
Functional and structural analysis of a fungal effector and its host target at plasmodesmata

Emma K. Turley

A thesis submitted for the degree of Doctor of Philosophy

University of East Anglia



John Innes Centre

September 2025

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with the author and that use of any information derived therefrom must be in accordance with current UK Copyright Law. In addition, any quotation or extract must include full attribution.

Abstract

To establish infection, phytopathogens deploy effector proteins to modify the plant intracellular environment and compromise host defences. Across diverse pathosystems, host proteins with heavy metal-associated (HMA) domains have been repeatedly identified as binding partners of effectors, as well as determinants of infection success. However, the endogenous functions of HMA proteins, including their roles in plant defence, are not well understood.

In this work, I show that ChEC108, a putative secreted effector from the anthracnose fungus, *Colletotrichum higginsianum*, binds a tandem HMA domain-containing protein from *Arabidopsis thaliana* called HIP6. To better understand why HMA proteins are frequently targeted by pathogens, I sought to structurally and functionally characterise the ChEC108-HIP6 complex.

Using X-ray crystallography, I discovered that ChEC108 is capable of binding either of the HIP6 HMA domains via a near-identical mechanism, involving coordination of a metal ion at the binding interface. In planta, both ChEC108 and HIP6 localise to plasmodesmata, and ChEC108 exploits these membrane-lined channels to move between cells. Although I initially hypothesised that ChEC108 might function as a virulence factor, exploiting HIP6 to promote its intercellular movement, I observed that the effector was dispensable for fungal virulence and remained cell-to-cell mobile even in the absence of HIP6, suggesting other host factors must facilitate its trafficking. Despite numerous HMA proteins being previously implicated as susceptibility factors in an infection context, my data suggest HIP6 is more likely to positively regulate defensive processes upon ChEC108 binding. Metal-binding residues of the HIP6 HMA domains are critical for both plasmodesmal localisation and complex formation with ChEC108, yet the functional implications of HIP6-metal association are currently unknown.

These findings have uncovered potential new roles for HMA proteins in plasmodesmata-localised signalling during fungal infection, while raising questions surrounding the function of metal-binding proteins at plasmodesmata and in plant immunity more generally.

Access Condition and Agreement

Each deposit in UEA Digital Repository is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the Data Collections is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form. You must obtain permission from the copyright holder, usually the author, for any other use. Exceptions only apply where a deposit may be explicitly provided under a stated licence, such as a Creative Commons licence or Open Government licence.

Electronic or print copies may not be offered, whether for sale or otherwise to anyone, unless explicitly stated under a Creative Commons or Open Government license. Unauthorised reproduction, editing or reformatting for resale purposes is explicitly prohibited (except where approved by the copyright holder themselves) and UEA reserves the right to take immediate 'take down' action on behalf of the copyright and/or rights holder if this Access condition of the UEA Digital Repository is breached. Any material in this database has been supplied on the understanding that it is copyright material and that no quotation from the material may be published without proper acknowledgement.

Contents

Abstract	3
List of figures	11
List of tables	15
Acknowledgements	17
1 Introduction	19
1.1 The plant immune system is multi-layered	19
1.1.1 Extracellular immunity	19
1.1.2 Secreted effector proteins modify host cells	21
1.1.3 Intracellular immunity	22
1.2 Heavy metal-associated (HMA) domain-containing proteins are commonly targeted by pathogens	25
1.2.1 What makes a HMA domain?	28
1.2.2 HMA proteins function in stress tolerance	32
1.2.3 HMA domains as effector host targets	33
1.2.4 HMA domains as bait for effectors	33
1.2.5 HMA proteins as susceptibility factors	34
1.2.6 HMA proteins as positive regulators of defence	35
1.2.7 Some HMA proteins localise to plasmodesmata	36
1.3 Non cell-autonomous immunity and the role of plasmodesmata	37
1.3.1 Plants send defence signals via the apoplast and symplast	37
1.3.2 Plasmodesmata establish symplastic continuity	38
1.3.3 Plasmodesmal function is altered in response to pathogens	41
1.3.4 Viruses employ diverse strategies to manipulate plasmodesmata	43
1.3.5 Some pathogen effectors are cell-to-cell mobile	44
1.4 <i>Colletotrichum higginsianum</i> (Ch) as a model hemibiotrophic fungal pathogen	45
1.4.1 Introducing ChEC108 - a hypermobile, PD-localised putative effector	47
1.5 Project aims	49

2	Materials and Methods	50
2.1	Cloning and bacterial methods	50
2.1.1	Golden Gate cloning	50
2.1.2	Gateway® cloning	51
2.1.3	In-Fusion® cloning	52
2.1.4	Transformation of chemically competent <i>Escherichia coli</i>	52
2.1.5	Bacterial screening and DNA extraction	52
2.1.6	Restriction digest	54
2.1.7	Preparation of electrocompetant <i>Agrobacterium tumefaciens</i>	55
2.1.8	Transformation of electrocompetant <i>A. tumefaciens</i>	55
2.2	Plant growth	55
2.2.1	Growth of <i>Nicotiana benthamiana</i>	55
2.2.2	Growth of Arabidopsis on soil	56
2.2.3	Growth of Arabidopsis on sterile media	56
2.2.4	Arabidopsis seed collection	57
2.3	Plant transformation	57
2.3.1	Agroinfiltration of <i>N. benthamiana</i> for transient expression	57
2.3.2	Particle bombardment for transient expression	58
2.3.3	Floral dipping of Arabidopsis for stable expression	59
2.4	Plant material and genotyping	60
2.4.1	Fluorescent seed selection	60
2.4.2	Seed selection by chemical resistance	60
2.4.3	Arabidopsis gDNA extraction	61
2.4.4	Arabidopsis genotyping	61
2.4.5	Mutation of Arabidopsis genes using CRISPR-Cas9	62
2.5	Confocal microscopy	62
2.5.1	Leaf staining and protein localisation	63
2.5.2	Quantification of fluorescent protein accumulation	63
2.5.3	Quantification of protein cell-to-cell mobility	64
2.5.4	Quantification of pitfield density	64
2.6	<i>Ch</i> work	66
2.6.1	<i>Ch</i> strains and propagation	66
2.6.2	<i>Ch</i> transformation	67
2.6.3	gDNA extraction from <i>Ch</i> mycelia	68
2.6.4	Protein extraction from <i>Ch</i> mycelia	68
2.6.5	<i>Ch</i> infection assays on Arabidopsis	68

2.6.6	<i>Ch</i> infection of Arabidopsis for protein extraction	69
2.6.7	<i>Ch</i> infection of Arabidopsis for microscopy	70
2.7	<i>Magnaporthe oryzae</i> work	70
2.7.1	<i>M. oryzae</i> transformation and culturing	70
2.7.2	Imaging <i>M. oryzae</i> infection of <i>Oryza sativa</i>	71
2.8	Co-immunoprecipitation (co-IP) and Western blotting	72
2.8.1	Protein extraction from plant material	72
2.8.2	Co-IP	72
2.8.3	SDS-PAGE for Western blotting	73
2.8.4	Western blotting	73
2.9	RNA work	75
2.9.1	RNA extraction from Arabidopsis	75
2.9.2	Reverse Transcription quantitative (RT-q)PCR	75
2.10	In vitro protein work	76
2.10.1	Protein production in <i>E. coli</i>	76
2.10.2	Protein purification from <i>E. coli</i> cell lysate	77
2.10.3	SDS-PAGE for protein purification	78
2.10.4	Intact mass spectrometry	78
2.10.5	Protein crystallisation	78
2.10.6	X-ray diffraction data collection and processing	79
2.10.7	Structure prediction and visualisation	80
2.10.8	Analytical gel filtration	80
2.10.9	Isothermal titration calorimetry (ITC)	81
2.11	Statistics and data presentation	81
3	Discovery and structural characterisation of the ChEC108-HIPP6 complex	82
3.1	Introduction	82
3.2	Results	85
3.2.1	ChEC108 associates with HIPP6 in <i>N. benthamiana</i>	85
3.2.2	The ChEC108-HIPP6 interface is not confidently predicted in silico . .	89
3.2.3	ChEC108 and HIPP6 HMA domains can be expressed in <i>E. coli</i>	95
3.2.4	The ChEC108-tHMA complex is unsuitable for crystallisation	98
3.2.5	A ChEC108-HMA1-HMA2 tripartite complex was not observed in vitro	104
3.2.6	Crystallisation of the ChEC108-HMA1 and ChEC108-HMA2 complexes	105
3.2.7	Processing ChEC108-HMA1 and ChEC108-HMA2 X-ray diffraction data	109
3.2.8	ChEC108 binds HMA1 and HMA2 via the same interface	113
3.2.9	HMA1-HMA2 association was not detected in vivo	119

3.2.10	Metal-binding residues are critical for ChEC108-HMA interactions . . .	122
3.3	Discussion	130
3.3.1	ChEC108 exhibits a novel mechanism of HMA targeting	130
3.3.2	What does the ChEC108-HIPP6 complex look like?	132
3.3.3	Can HIPP6 bind metal ions in the absence of ChEC108?	133
3.3.4	Conclusion	135
4	Investigating the function of ChEC108 and HIPP6 during infection	136
4.1	Introduction	136
4.1.1	The <i>Ch</i> infection cycle	136
4.1.2	<i>Ch</i> effectors are expressed in waves	139
4.2	Results	140
4.2.1	ChEC108 is dispensable for <i>Ch</i> virulence on Arabidopsis	140
4.2.2	<i>hipp6</i> mutants are marginally more susceptible to <i>Ch</i>	145
4.2.3	Efforts to complement <i>hipp6-3</i> mutants	150
4.2.4	Exploring trypan blue staining as an alternative method to quantify <i>Ch</i> virulence	154
4.2.5	Progress towards understanding the downstream consequences of ChEC108-HIPP6 association	157
4.3	Discussion	158
4.3.1	The challenges of studying effector function using reverse genetics . . .	159
4.3.2	Why has <i>Ch</i> retained ChEC108?	162
4.3.3	How specific is the ChEC108-HIPP6 interaction?	163
4.3.4	Conclusion	164
5	Understanding the subcellular localisation of ChEC108 and HIPP6	165
5.1	Introduction	165
5.2	Results	167
5.2.1	ChEC108 localises to PD	167
5.2.2	HIPP6 localises to PD	167
5.2.3	HIPP6 co-localises with ChEC108 at PD	176
5.2.4	ChEC108 enhances HIPP6 stability	180
5.2.5	ChEC108-mediated enhancement of intercellular trafficking was observed in <i>N. benthamiana</i> , but not Arabidopsis	182
5.2.6	PD localisation of ChEC108 does not depend on HIPP6	186
5.2.7	HIPP6 inhibits ChEC108 cell-to-cell mobility	192
5.2.8	ChEC108 overexpression shuts down intercellular trafficking	194

5.3	Discussion	195
5.3.1	How are ChEC108 and HIP6 targeted to PD?	196
5.3.2	How does ChEC108 manipulate PD in <i>N. benthamiana</i> ?	197
5.3.3	Why was ChEC108-mediated manipulation of PD not observed in Arabidopsis?	199
5.3.4	Conclusion	202
6	Seeking evidence of ChEC108 translocation to host cells	203
6.1	Introduction	203
6.1.1	<i>M. oryzae</i> effectors captured in the host symplast	203
6.1.2	<i>Colletotrichum</i> effectors captured at the host-pathogen interface	205
6.1.3	Proximity labelling for effector detection in planta	206
6.1.4	Towards indirect effector detection: using NLRs to engineer gain-of-resistance	207
6.2	Results	209
6.2.1	<i>Ch</i> -derived ChEC108-eGFP could not be detected in Arabidopsis	209
6.2.2	Exploring a TurboID-based strategy to detect <i>Ch</i> -derived ChEC108 in Arabidopsis	211
6.2.3	Attempts to engineer ChEC108 recognition by Pik NLRs were unsuccessful	225
6.2.4	ChEC108 localisation in <i>M. oryzae</i> and <i>O. sativa</i> host cells resembles that of a cytoplasmic effector	235
6.3	Discussion	238
6.3.1	Effector translocation from <i>Ch</i> is challenging to detect	238
6.3.2	Do Pik NLRs still have potential for engineering ChEC108 perception?	240
6.3.3	Alternative strategies to capture ChEC108 translocation	241
6.3.4	Conclusion	243
7	Discussion	244
7.1	How might HIP6 positively regulate plant defence?	245
7.1.1	HIP6 is unlikely to directly regulate transcription	247
7.1.2	HIP6 might signal via phytohormones	247
7.1.3	HIP6 might signal via ROS	250
7.1.4	HIP6 might regulate cell-to-cell trafficking	252
7.2	Is heavy metal binding important for HIP6 function?	254
7.2.1	Why would a metal-binding protein localise to PD?	256
7.2.2	Could HIP6 metal-binding and defensive functions be interlinked?	258
7.3	Conclusion	259

8	Appendix	261
8.1	Supplementary tables	261
8.2	Abbreviations	283
8.3	Publication	293
	References	311

List of figures

1.1	A simplified cell-autonomous model of the plant immune system	20
1.2	HMA domain structure and topology	28
1.3	HMA protein families in plants	30
1.4	Callose-dependent regulation of PD aperture	40
2.1	Post-bombardment set-up for <i>N. benthamiana</i> leaves.	59
2.2	Image analysis pipeline for quantification of pitfield density.	65
2.3	Cleavable N-terminal 6×His tag for IMAC.	76
3.1	Representative members of effector structural families from plant pathogens .	83
3.2	ChEC108 binds HIP6 in vivo.	87
3.3	HIP6 HMA domains are sufficient for ChEC108 binding in vivo	88
3.4	ChEC108 and HIP6 predicted structures.	90
3.5	ChEC108 bares some structural resemblance to GNAT enzymes.	91
3.6	Predicted structure of the ChEC108-HIP6 complex.	93
3.7	Predicted structures of ChEC108 in complex with HMA1 or HMA2.	94
3.8	Predicted structures of ChEC108 and HIP6 HMA domain truncations used for in vitro purification.	96
3.9	ChEC108, HMA1, HMA2 and tHMA can be expressed in <i>E. coli</i>	97
3.10	Co-purification of ChEC108 and tHMA from <i>E. coli</i>	99
3.11	Purification of ChEC108 from <i>E. coli</i>	101
3.12	Purification of tHMA from <i>E. coli</i>	102
3.13	ChEC108 and tHMA are highly susceptible to trypsin digestion.	103
3.14	HMA2 does not bind the ChEC108-HMA1 complex in vitro	104
3.15	Co-purification of ChEC108 with HMA1 or HMA2 from <i>E. coli</i>	105
3.16	Crystallisation of the ChEC108-HMA1 complex	106
3.17	Initial crystallisation of the ChEC108-HMA2 complex	107
3.18	Optimised crystallisation of the ChEC108-HMA2 complex	108

3.19	Protein crystals mounted prior to X-ray diffraction	109
3.20	ChEC108 binds HMA1 or HMA2 via a common binding interface with a coordinated metal ion	115
3.21	Intermolecular bonds at the ChEC108-HMA1/2 binding interfaces	116
3.22	Charge complementarity strengthens ChEC108-HMA binding interfaces	117
3.23	Zn was detected in ChEC108-HMA2 protein crystals	118
3.24	Predicted dimerisation of HIPP6 HMA domains.	120
3.25	HMA1-HMA2 association was not detected in vivo.	121
3.26	HIPP6 self-association was not detected in vivo	122
3.27	Mutation of metal-binding residues compromises ChEC108-HIPP6 association	124
3.28	Purification of HMA1 from <i>E. coli</i>	125
3.29	Purification of HMA2 from <i>E. coli</i>	126
3.30	ChEC108-HMA1 association was not detected in preliminary analytical gel filtration trials	127
3.31	Preliminary thermodynamic characterisation of ChEC108-HMA1 binding by ITC	129
3.32	Structurally characterised effectors in complex with the HMA or HMA-like domains of host target proteins	131
3.33	Predicted structure of HIPP6 in complex with Zn ²⁺	134
4.1	<i>Ch</i> infection progression at the microscopic scale	138
4.2	Progression of symptoms induced by <i>Ch</i> on a detached Arabidopsis leaf	138
4.3	Generation of $\Delta ChEC108$ by homologous recombination	141
4.4	Loss of <i>ChEC108</i> increases <i>Ch</i> virulence on Arabidopsis.	142
4.5	$\Delta ChEC108$ complementation strains generated by non-targeted insertion . . .	144
4.6	The enhanced virulence phenotype of $\Delta ChEC108$ could not be reproduced in subsequent assays with complemented strains	145
4.7	<i>hipp6</i> CRISPR mutant genotypes	146
4.8	Loss of <i>HIPP6</i> enhances Arabidopsis susceptibility to <i>Ch</i> expressing ChEC108.	148
4.9	<i>HIPP6</i> is required for the enhanced virulence of $\Delta ChEC108$	149
4.10	Constitutive <i>HIPP6</i> expression inhibits Arabidopsis growth.	151
4.11	Detection of GFP-HIPP6 and GFP-HIPP6 ^{MM} in Arabidopsis	152
4.12	The enhanced susceptibility phenotype of <i>hipp6-3</i> could not be reproduced in subsequent assays with complemented lines	153
4.13	Trypan blue-stained germinated <i>Ch</i> spores visible on Arabidopsis leaves	155
4.14	Preliminary data suggest early $\Delta ChEC108$ infection progression is faster than WT	156
4.15	Constitutive <i>ChEC108</i> expression severely inhibits Arabidopsis growth	158
4.16	Speculative model to illustrate the role of HIPP6 during <i>Ch</i> infection	159

4.17	ChEC32 is predicted to fold similarly to ChEC108	161
5.1	ChEC108 localises to PD	168
5.2	Heterogeneous distribution of ChEC108 in nuclei	169
5.3	HIPP6 localises to PD	170
5.4	Targeting of HIPP6 to PD does not require conserved C-terminal motifs	171
5.5	HIPP6 HMA domains are sufficient for PD localisation	172
5.6	Mutation of HIPP6 HMA domains reduces enrichment at PD in <i>N. benthamiana</i>	174
5.7	Mutation of HIPP6 HMA domains reduces enrichment at PD in Arabidopsis .	175
5.8	ChEC108 co-localises with HIPP6 in <i>N. benthamiana</i>	177
5.9	ChEC108 and HIPP6 co-localise at PD	178
5.10	HIPP6 localisation in Arabidopsis subtly changes in the presence of ChEC108	179
5.11	HIPP6 localisation is unchanged in the presence of ChEC108 when binding is compromised by mutation	180
5.12	HIPP6 is stabilised by ChEC108 in <i>N. benthamiana</i>	181
5.13	HIPP6, HMA2 and tHMA are stabilised by ChEC108 in <i>N. benthamiana</i>	182
5.14	ChEC108 enhances intercellular flux in <i>N. benthamiana</i>	184
5.15	ChEC108 does not increase pitfield density in <i>N. benthamiana</i>	185
5.16	ChEC108-mediated enhancement of intercellular transport was not detected in Arabidopsis	187
5.17	ChEC108 enhances intercellular flux in <i>N. benthamiana</i> when expressed via bombardment	188
5.18	ChEC108 is cell-to-cell mobile in Arabidopsis	189
5.19	ChEC108 PD localisation in <i>N. benthamiana</i> is not abolished by D164R or CCAA mutation.	190
5.20	ChEC108 PD localisation in Arabidopsis does not depend on HIPP6	191
5.21	Cell-to-cell mobility of ChEC108 in Arabidopsis does not depend on HIPP6 . .	193
5.22	<i>HIPP6</i> overexpression reduces ChEC108 mobility in <i>N. benthamiana</i>	194
5.23	Stable <i>ChEC108</i> expression dramatically reduces cell-to-cell trafficking in Arabidopsis	195
5.24	NbHIPP6 localises to PD	202
6.1	Structures exhibiting accumulation of effector proteins in <i>M. oryzae</i> and <i>Ch</i> . .	206
6.2	SP-ChEC108-eGFP can be constitutively expressed in <i>Ch</i>	210
6.3	<i>Ch</i> -derived ChEC108-eGFP could not be detected in Arabidopsis.	212
6.4	Illustration of the split-GFP TurboID approach to detect <i>Ch</i> -derived ChEC108 in Arabidopsis cells	213

6.5	Biotinylated <i>Ch</i> -derived ChEC108-Myc-GFP11 could not be detected in Arabidopsis.	215
6.6	Biotinylated ChEC108 was detected following transient co-expression with TurboID variants in <i>N. benthamiana</i>	216
6.7	<i>SP-ChEC108-4×Myc</i> and <i>ChEC108-4×Myc</i> can be constitutively expressed in <i>Ch</i>	217
6.8	Illustration of the TurboID-HMA1-based approach to detect <i>Ch</i> -derived ChEC108 in Arabidopsis cells	218
6.9	Biotinylated <i>Ch</i> -derived ChEC108-4×Myc was not detected in mature Arabidopsis leaves.	219
6.10	<i>Ch</i> -derived ChEC108-4×Myc was not detected in mature Arabidopsis leaves expressing <i>TurboID-HIPP6</i>	220
6.11	<i>Ch</i> -derived (SP-)ChEC108-4×Myc was detected in infected Arabidopsis seedlings.	222
6.12	Biotinylated <i>Ch</i> -derived ChEC108-4×Myc could not be detected in Arabidopsis seedlings.	224
6.13	Chimeric NLRs designed to engineer ChEC108 recognition	226
6.14	Sequence and structural alignment of the Pikm-1 HMA domain and HIPP6 HMA domains.	227
6.15	Integration of HMA1 or HMA2 into Pikm-1 triggers Pikp-2-dependent autoactivity.	228
6.16	Autoactivity of Pikm-1 ^{HMA1} and Pikm-1 ^{HMA2} could not be alleviated by extension of the HMA domain boundaries or by co-expression with Pikm-2. .	229
6.17	The metal-binding loop (mb1) does not underpin autoactivity of Pikm-1 ^{HMA1} .	231
6.18	The metal-binding loop (mb1) does not underpin autoactivity of Pikm-1 ^{HMA2} .	232
6.19	Autoactivity of Pikm-1 ^{HMA1} and Pikm-1 ^{HMA2} could not be alleviated by subtle modification of HMA domain boundaries, and resurfacing of the Pikm-1 HMA domain was insufficient to confer ChEC108 recognition.	234
6.20	Arg214 from HMA2 might help stabilise the ChEC108-HMA2 metal-binding site	235
6.21	The ChEC108 signal peptide is functional in the <i>M. oryzae</i> heterologous system.	237
7.1	Speculative model for ChEC108-HIPP6-mediated regulation of defence via cytokinin signalling	250
7.2	Speculative model for ChEC108-HIPP6-mediated enhancement of defensive ROS signalling	252
7.3	Speculative model for ChEC108-HIPP6-mediated stress signalling via PD closure	254

List of tables

1.1	HMA domain-containing proteins implicated in plant-pathogen interactions.	25
1.2	Number of <i>HIPP</i> and <i>HPP</i> genes identified in vascular plant genomes to date.	31
1.3	Examples of plant resistance proteins with HMA-like domains	34
2.1	Phusion PCR reaction mix.	51
2.2	Golden Gate reaction mixture.	51
2.3	Golden Gate thermocycler protocol.	51
2.4	Working concentrations of antibiotics.	53
2.5	Colony PCR reaction mix.	54
2.6	Colony PCR thermocycler protocol.	54
2.7	Restriction digest reaction mixture.	54
2.8	OD of bacterial suspensions used for <i>N. benthamiana</i> agroinfiltration experiments.	58
2.9	Excitation and detection wavelengths for visualising fluorescent proteins/stains.	63
2.10	<i>Ch</i> strains.	66
2.11	Composition of SDS-PAGE gels.	73
2.12	Antibodies used in this work.	74
2.13	RT-qPCR thermocycler protocol	76
2.14	Broad range crystallisation screens.	79
2.15	ITC experimental conditions.	81
3.1	Candidate ChEC108-associated proteins identified by IP-MS.	86
3.2	Mass spectrometry analysis of purified proteins.	98
3.3	X-ray data collection statistics.	111
3.4	Structure refinement and model statistics.	113
3.5	ChEC108-HMA1/2 binding parameters calculated by ITC	130
6.1	Attempts to optimise the TurboID-HMA1-based approach to detect <i>Ch</i> -derived ChEC108 in Arabidopsis cells	221

S1	DNA cloning primers	262
S2	Composition of buffers	266
S3	Golden Gate plasmids.	268
S4	Gateway [®] plasmids	272
S5	In-Fusion [®] plasmids	272
S6	Composition of media	273
S7	DNA sequencing primers	275
S8	Arabidopsis lines.	277
S9	DNA genotyping primers	278
S10	DNA primers for RT-qPCR.	280
S11	Top 25 proteins with structural similarity to ChEC108.	280
S12	Virulence assay experimental details.	281
S13	Percentage sequence identity matrix of clade I HIPPI HMA domains	282

Acknowledgements

I would like to thank so many people for their contributions to this work and my training over the past four years. Firstly, thank you to the **John Innes Foundation Rotation Programme** for financially supporting my studies. As part of a collaborative project, I am extremely fortunate to have been adopted by two research groups, where I have benefitted from the support of two passionate supervisors. I am incredibly grateful to my primary supervisor, **Christine**, for steering and shaping this project from the get-go and being patient with it (and me) even when progress was difficult. Next, a big thank you to **Mark** for his valuable contributions to project discussions and encouragement throughout. I suspect few supervisors would go to such lengths to make their secondary students feel so welcome in lab meetings and events, and I really appreciate it. Moving forward, the impressive presentations, written work and creative research ideas of both my supervisors will continue to inspire me to become a better scientist.

While finding my feet in the lab, I learned a lot from **Faulkner and Banfield lab members**, especially **Andy**, who taught me to use the gene gun and confocal microscope with such enthusiasm, and **Raf**, who patiently supervised my first rotation project and helped me appreciate the art of protein purification (not to mention pouring plates in a pyramid). I owe **Estee** and **Basti** for teaching me co-IP and Western blotting techniques, and **Sally** for her careful introduction to *Colletotrichum*. I am grateful also to **Barkha** and **Daniel** for joining the ChEC108/HIPP6 team as postdocs, and expanding the scope of this project with their unique skillsets. I extend my gratitude to all members of both labs for their feedback during lab meetings, for the lunchtime chats, generous sharing of snacks (especially by the Muffin Man himself, **Qi Yang**) and for providing motivational pep talks when I needed them most.

I am grateful to members of the **Talbot lab** for their guidance and collaboration. Firstly, to **Lauren** for agreeing to be part of my supervisory panel, and applying her expert fungal knowledge to help advance my experiments. In planta imaging of *Magnaporthe* infection would not have been possible without the generous help of **Weibin**, who carried out fungal transformations, and **Jave**, who supervised rice inoculations.

Thank you to **Phil** and the **Carella lab** members for hosting me during my third rotation, giving me a glimpse into the world of *Marchantia-Pseudomonas* interactions. During my third year, I had the valuable opportunity to supervise an undergraduate student, **Michael**, who contributed greatly to the NLR engineering efforts presented in Chapter 6 of this thesis. Many thanks also go to **Madi**, the certified TurboID guru of TSL, for sharing her hard-fought wisdom with me on numerous occasions.

As scientists at the JIC, we are lucky to work with excellent platform scientists and support staff, whose advice and expertise have made my lab life considerably easier. In particular, I would like to thank: **Dave** and **Julia** (Structural Biology); **Carlo** and **Gerhard** (Proteomics); **Phil** (Photography); the **Lab Support team** and **Horticultural Services**. A special mention goes to **John** from Hort Services for his friendly greetings and chats whenever I visited the B51 CERs.

Next, I would like to thank those who kept me (mostly) sane outside of work. To **Angela**, **Raf** and **Neil** for being awesome HIIT coaches, and **Angela again** for leading the hugely beneficial AWP interval training sessions every Wednesday. I wish I had joined both these groups from the start of my PhD as they never fail to give me a boost. Another big thank you goes to the **NRP parkrunning community** for giving me something to look forward to every weekend. I must mention **Christine** and **Mark** again here for motivating me to push myself and get fitter. In my final year, even with the thesis deadline looming, working with Mark to improve my running turned what probably should have been the most difficult summer of my PhD into the best one by far, so thank you for that!

I will forever be grateful to **Josh Joyce** for being a fantastic supervisor during my first ever research project back in 2020. His enthusiasm for aphids, amazing videos of calcium reporters and mega-organised, ambitious experimental schedule are what inspired me to do a PhD in the first place. Catching up with Josh and **Fran** periodically throughout my time in Norwich has been a much-appreciated a source of encouragement and good food.

Lastly, I would like to thank **my family** for their unconditional support and willingness to listen throughout the entire PhD process, despite my terrible lack of communication at times. I hope I have done you proud with this thesis.

Chapter 1

Introduction

1.1 The plant immune system is multi-layered

In their natural environment, plants are constantly threatened by a diverse array of pathogenic microbes. However, the majority of microbes are unable to establish themselves on any given plant species. Among other environmental and developmental factors, this non-host resistance is a testament to the highly sophisticated, multi-layered plant immune system. Like animals, plants deploy physical barriers as a first line of defence against infection, including rigid cell walls, waxy cuticles for their leaves, and cork for woody structures. Nevertheless, adapted pathogens can gain entry via a number of methods including via wounds, stomatal openings, the piercing mouthparts of hemipteran insects, or by actively puncturing cell walls. Once inside, a pathogen must somehow circumvent, inhibit or tolerate the interconnected extracellular and intracellular immune responses of the host (Figure 1.1).

1.1.1 Extracellular immunity

Integrated into the plasma membrane of plant cells are receptors for detection of pathogen signatures in the extracellular space. These include receptor-like kinases (RLKs), which contain an extracellular ligand-binding domain, transmembrane domain and cytosolic kinase domain. Receptor-like proteins (RLPs) function in complex with receptor kinases, but lack kinase domains for downstream signalling. These receptors enable perception of both self- and non-self-associated ligands in the apoplast, including damage-associated molecular patterns (DAMPs) and conserved microbe-associated molecular patterns (MAMPs) (for reviews highlighting the diversity of RLK/RLPs, their ligands and mechanisms of activation, see [DeFalco and Zipfel, 2021](#); [Dievart et al., 2020](#); [Tang et al., 2017](#)). Three of the most well-characterised RLKs are FLAGELLIN SENSITIVE 2 (FLS2), which recognises the bacterial

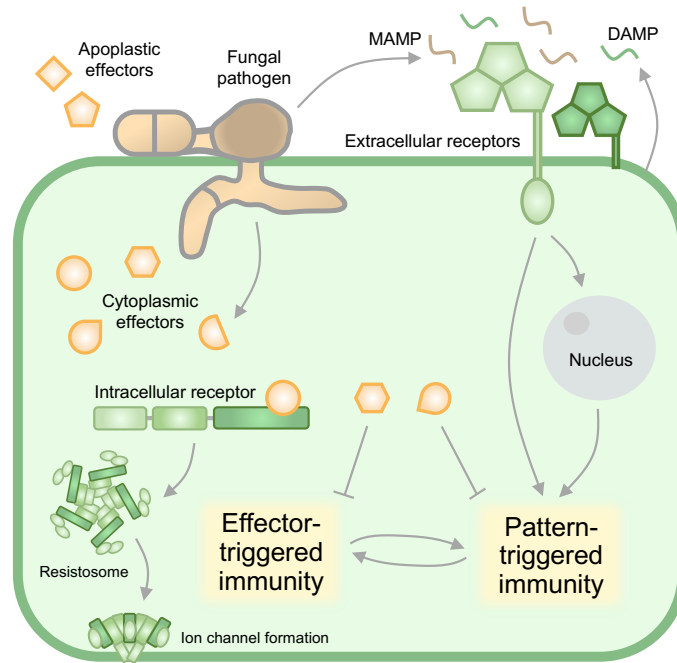


Figure 1.1 – A simplified cell-autonomous model of the plant immune system. Pathogen-derived microbe-associated molecular patterns (MAMPs) and plant-derived damage-associated molecular patterns (DAMPs) are perceived in the apoplast by extracellular receptors. This triggers signalling cascades and transcriptional reprogramming via the nucleus, giving rise to pattern-triggered immunity. Effectors are secreted by pathogens to manipulate host processes in the cytoplasm and apoplast. Some may be recognised by intracellular immune receptors, resulting in resistosome formation. The insertion of resistosomes into the plasma membrane or organelle membranes causes ion flux and cell death, resulting in effector-triggered immunity. Pattern- and effector-triggered immune responses can reinforce one another, although both are vulnerable to inhibition by effectors.

flagellin epitope flg22 (Chinchilla et al., 2006); ELONGATION FACTOR-TU RECEPTOR (EFR), which recognises elf18, a peptide derived from the bacterial elongation factor Tu (EF-Tu) (Zipfel et al., 2006); and LYSIN MOTIF (LysM)-CONTAINING CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1), which recognises chitin, an oligosaccharide component of fungal cell walls (Miya et al., 2007). Binding of ligands to RLK/RLPs typically results in recruitment of co-receptors and initiation of downstream signalling via phosphorylation cascades. Alongside, second messengers such as cytosolic calcium ions (Ca^{2+}), reactive oxygen species (ROS), nitric oxide and phosphatidic acid are elevated, amplifying the response (reviewed by Bigeard et al., 2015). Ultimately, signalling via extracellular receptors culminates in major transcriptional reprogramming (Bjornson et al., 2021) and activation of a variety of defences including cell wall remodelling and stomatal closure to reinforce physical barriers, as well as the synthesis of anti-microbial proteins and secondary metabolites. Together, these defences confer pattern-triggered immunity (PTI).

1.1.2 Secreted effector proteins modify host cells

Avoiding extracellular detection is hypothesised to be extremely difficult for pathogens given the important and conserved nature of proteins from which MAMPs are derived, and the inevitable release of DAMPs when plant tissues are damaged. To combat PTI, among other functions, pathogens have evolved arsenals of secreted proteins called effectors, which are released into the apoplast or cytoplasm of invaded cells. Effector-mediated antagonism of defence responses is known to be widespread (for an in-depth review, see [Zhang et al., 2022b](#)). For example, the *Fusarium oxysporum* effector Avr2 is described as a PTI inhibitor, given that flg22-induced ROS production, growth inhibition and callose deposition were reduced in Avr2-expressing tomato leaves ([Di et al., 2017](#)). Two effectors from *Pseudomonas syringae* pv. *tomato* (*Pto*), AvrPto and AvrPtoB are known to inhibit FLS2 signalling by perturbing complex formation between receptors and co-receptors ([Shan et al., 2008](#)). Also, apoplastic chitin-binding proteins have been identified in fungal effector repertoires, which may help conceal pathogens from extracellular receptors ([Martínez-Cruz et al., 2022](#); [Takahara et al., 2016](#)). As well as suppressing defence, effectors can modify the host cellular environment in favour of pathogen growth. An aqueous 'water-soaked' extracellular space, for example, is favourable for movement and reproduction of bacterial pathogens, and the *Pto* DC3000 effectors HopM1 and AvrE induce water-soaking of the *Arabidopsis thaliana* (hereon *Arabidopsis*) apoplast ([Xin et al., 2016](#)). Therefore, despite the evolution of host PTI, effector proteins can enable successful pathogen establishment via their diverse modes of action.

The route taken by effector proteins from pathogen to host cells varies depending on the pathosystem in question. Effector translocation from bacterial pathogens to plants is known to take place via the well-characterised needle-like type III secretion system (reviewed by [Deng et al., 2017](#)). In oomycetes, conserved motifs with consensus RXLR (Arg-X-Leu-Arg) and s/dEER (single/double Glu-Glu-Arg) were identified and shown to be required for host uptake ([Whisson et al., 2007](#)). In particular, the RXLR motif directs pre-secretion cleavage and N-acetylation of the *Phytophthora infestans* effector Avr3a ([Wawra et al., 2017](#)), likely facilitating its sorting and stabilisation. In contrast, translocation motifs have yet to be identified in fungal effectors and their mechanism of secretion and translocation is a topic of ongoing research, with mechanisms likely differing depending on the morphology of the host-pathogen interface and timing of effector expression relative to infection progression ([Giraldo et al., 2013](#); [Kleemann et al., 2012](#); [Stuer et al., 2023](#)). Imaging-based studies of the blast fungus *Magnaporthe oryzae* revealed that effectors destined for the host cytoplasm are first secreted and concentrated in a membranous structure known as the biotrophic interfacial complex (BIC) ([Khang et al., 2010](#)), before being packaged into vesicle-like compartments and translocated

via endocytosis (Oliveira-Garcia and Hamilton, 2024; Oliveira-Garcia et al., 2023). *P. infestans* effectors were similarly shown to rely on endocytosis for their translocation to *Nicotiana benthamiana* cells, following their secretion from haustoria (Wang et al., 2023a). In contrast, capturing the translocation of effectors from other filamentous pathogens, such as those in the genus *Colletotrichum*, has proved challenging (Kleemann et al., 2012) and many questions surrounding the mechanisms and selectivity of effector delivery remain unanswered. For more detail on fungal effector translocation, see 6.1 in Chapter 6 of this thesis.

1.1.3 Intracellular immunity

Adding an additional layer to their immune systems, plants deploy intracellular receptors to detect pathogen-derived effectors and mount localised defence responses with high specificity. Such effector-triggered immunity (ETI) is typically mediated by nucleotide-binding leucine-rich repeat (NLR) proteins, which exhibit a modular structure. Canonically, NLRs possess a C-terminal leucine-rich repeat (LRR) domain, central nucleotide-binding adapter shared by Apaf-1, resistance (R) proteins and CED-4 (NB-ARC) domain, and a variable N-terminal signalling domain. In plants, common N-terminal domains for NLRs are coiled-coils (CC), which can be further divided into several classes (Bentham et al., 2018) and toll/interleukin-1 receptor-type (TIR) domains, although recent studies have uncovered additional signalling domains in putative bryophyte NLRs, namely $\alpha\beta$ -hydrolase and protein kinase domains (Chia et al., 2024). So far, effector detection by NLRs is known to occur via three primary mechanisms (reviewed by Baggs et al., 2017; Bentham et al., 2020). Firstly, effectors can be bound directly via the LRR domain, as exemplified by Sr35 recognition of Avr35 from the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici* (Förderer et al., 2022). Secondly, indirect recognition can occur upon perception of an effector-modified host target or decoy protein (the latter being a non-functional host target mimic) (van der Hoorn and Kamoun, 2008). A classic example of an NLR-guarded host target is RPM1-INTERACTING PROTEIN 4 (RIN4), which is post-translationally modified by *Pto* DC3000 effectors AvrRpm1 and AvrB, triggering an ETI response via the Arabidopsis CC-NLR, RESISTANCE TO *Pseudomonas syringae* pv. *maculicola* 1 (RPM1) (Mackey et al., 2002). Finally, NLRs have been characterised with diverse integrated domains, which are hypothesised to structurally mimic host targets of cognate effectors, serving as bait to enable robust effector-triggered responses (Cesari et al., 2014, 2013; Maqbool et al., 2015; Mukhi et al., 2021; Sarris et al., 2016).

Downstream of effector perception, NLR protein conformation is adjusted such that nucleotide exchange (ADP for ATP) occurs in the NB-ARC domain, and signalling via the N-terminal domain is initiated. Depending on the NLR in question, the events proceeding effector perception can vary, although the end result is remarkably consistent: localised cell death

(termed the hypersensitive response, or HR), which restricts pathogen spread. In landmark studies by Wang et al. (2019b) and Förderer et al. (2022), the active conformations of the Arabidopsis CC-NLR, HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) and wheat (*Triticum monococcum*) CC-NLR, Sr35 were uncovered using cryogenic electron microscopy (cryo-EM). Upon pathogen perception, these NLRs assembled into pentameric wheel-like oligomers termed resistosomes, which included a funnel-like structure composed of the α 1-helices of CC domains. Further experiments suggested these pentamers are capable of inserting into cell membranes via their N-terminal funnels, forming inorganic ion channels (Bi et al., 2021; Förderer et al., 2022) and presumably inducing cell death as a consequence of ion flux and membrane depolarisation. Recent evidence also suggests NLRs to be capable disrupting organelle membranes, including the tonoplast, endoplasmic reticulum (ER) and the envelopes of chloroplasts and mitochondria (Ibrahim et al., 2024; Sunil et al., 2024).

In contrast to CC-NLRs, TIR-NLRs have been reported to form tetrameric oligomers with enzymatic activity (Ma et al., 2020). For instance, the nicotinamide adenine dinucleotide hydrolases (NADases) activity of oligomerised RECOGNITION OF *Peronospora parasitica* 1 (RPP1, from Arabidopsis) and RECOGNITION OF XopQ1 (ROQ1, from *N. benthamiana*) produce small, nucleotide-based metabolites capable of initiating immune responses via the widely-studied lipase-like proteins, ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE-ASSOCIATED GENE 101 (SAG101) (Huang et al., 2022). This in turn activates RWP8-like CC (CC^R)-NLRs in the subfamilies ACTIVATED DISEASE RESISTANCE 1 (ADR1) or N REQUIREMENT GENE 1 (NRG1), culminating once again in the formation of membrane-associated resistosomes and cell death (Feehan et al., 2023; Sun et al., 2021; Wang et al., 2023c).

As diverse NLRs continue to be discovered and structurally characterised, mechanisms of receptor activation and signalling, as well as the relationships between NLR proteins themselves, remain focusses of current research. With the exception of singleton NLRs, which are sufficient for both effector perception and downstream signalling (for example, ZAR1 Wang et al., 2019b), many NLRs are known to operate in pairs or networks, with 'sensors' required for effector perception, and 'helpers' required for signalling execution (Adachi et al., 2019; Wu et al., 2017). Pik-1 and Pik-2, for example, are a genetically linked CC-NLR pair from rice (*Oryza sativa*). Pik-1 contains a heavy metal-associated (HMA) integrated domain between its CC and NB-ARC domains, which directly binds AVR-Pik effectors from *M. oryzae* (Maqbool et al., 2015), while Pik-2 (a canonical CC-NLR with no integrated domain) is required to initiate AVR-Pik-dependent cell death (Zdrzałek et al., 2020). In contrast to this cooperative pair, the *O. sativa* sensor NLR, RESISTANCE GENE ANALOG 5 (RGA5), negatively regulates the activity of its helper RGA4, preventing constitutive cell death in the absence of corresponding

M. oryzae effectors (Césari et al., 2014). Further insights into helper activation mechanisms were gained from recent biochemical and cryo-EM analyses of purified NLR-REQUIRED FOR CELL DEATH 2 (NRC2), a CC-NLR from *N. benthamiana* that operates downstream of potato virus X (PVX) coat protein perception by the sensor, Rx (Contreras et al., 2023). In its resting state, NRC2 forms homodimers, which undergo major rearrangements upon activation by Rx, forming a hexameric resistosomes with the capacity to puncture membranes (Madhuprakash et al., 2025; Selvaraj et al., 2024). In the case of the Pik pair, the precise mechanism of sensor activation, and the composition of putative resistosomes have yet to be revealed in atomic level detail, although evidence from co-IP assays suggest an oligomer comprising Pik-1, Pik-2 and AVR-Pik effectors (or perhaps just Pik-2 alone, if sensor-helper association is transient) could be responsible for cell death initiation (Zdrzałek et al., 2020).

In recent years, it has become increasingly apparent that NLRs are not the only plant proteins to serve as intracellular immune receptors. Kinase fusion proteins (KFPs) are an emerging class of resistance proteins that commonly contain integrated domains alongside one or more kinase/pseudokinase domains (He et al., 2024; Reveguk et al., 2025; Wang et al., 2023b). Reminiscent of sensor NLRs, KFPs can directly bind pathogen effectors via their kinase domains (Chen et al., 2025), or alternatively exploit their integrated domains as bait (Bernasconi et al., 2025; Sung et al., 2025). Although the signalling events underlying KFP-mediated resistance is a topic of ongoing research and likely varies from protein to protein, two recent studies in wheat (*Triticum aestivum*) and its relatives demonstrated that KFP-NLR cooperation is possible, with the *Aegilops sharonensis* NLR, WHEAT TANDEM NBD 1 (WTN1, also referred to as Sr65^{NLR}) being required for signalling downstream of effector perception by KFPs WHEAT TANDEM KINASE 3 (WTK3) (Lu et al., 2025) and tandem kinase (TK) protein, Sr65^{TK} (Chen et al., 2025).

The evolution of plant intracellular immunity imposes a strong selection pressure onto pathogens, locking them in a continuous co-evolutionary arms race with their hosts. Accordingly, genes encoding putative effectors are known to evolve rapidly with respect to wider pathogen genomes (Huang et al., 2014; Sánchez-Vallet et al., 2018). Some effectors even inhibit the activation of intracellular immunity. For example, SPRYSEC 15 (SS15), a small secreted protein of the cyst nematode *Globodera rostochiensis*, is an established negative regulator of NRC activity in solanaceous plants (Derevnina et al., 2021).

Despite their spatial and temporal separation and distinct mechanisms of initiation, the immune responses that accompany PTI and ETI are interlinked. For instance, it was shown that Arabidopsis NLR activity increases the abundance of PTI signalling components, including cytosolic kinases and membrane-localised receptors, while PTI signalling enhances or is

required for NLR-induced cell death (Feehan et al., 2023; Ngou et al., 2021; Yuan et al., 2021). Thus, despite lacking the complex suite of mobile immune cells and adaptive systems characterised in animals, plants are well-equipped to fight infections using both extracellular and intercellular machinery. However, continual innovation in the form of effector gain, loss and diversification means some co-evolved pathogens remain capable of overwhelming the plant immune system to establish disease.

1.2 Heavy metal-associated (HMA) domain-containing proteins are commonly targeted by pathogens

To better understand the intracellular processes manipulated by pathogens during infection, it can be beneficial to study the binding partners of pathogen effectors using targeted or unbiased surveys of protein-protein interactions. Such investigations have led to the discovery of 'effector hub' host proteins, which are frequently targeted as a result of their integral roles in immunity (Carella et al., 2018). Examples include the TEOSINTE BRANCHED1 / CYCLOIDEA / PROLIFERATING CELL FACTOR (TCP) and JASMONATE ZIM-DOMAIN (JAZ) transcription factor families, which are key players in defensive hormone signalling (Ceulemans et al., 2021), receptor-like cytoplasmic kinases (RLCKs), which contribute to PTI signalling (Ngou et al., 2022), and lipase-like proteins such as EDS1, which function downstream of TIR-NLR activation (Wu and Derevnina, 2023). In the last decade, it has become increasingly apparent that host proteins with HMA domains are frequently targeted by pathogen-derived proteins (summarised in Table 1.1), hinting that these proteins might have widespread roles in plant immunity that evolutionarily distant pathogens have convergently evolved to exploit. However, the biological relevance of these interactions are not well understood in the majority of cases. In the following sections 1.2.1 to 1.2.6, I aim to introduce the families of HMA domain-containing proteins in plants and highlight their involvement in host-microbe interactions.

Table 1.1 – HMA domain-containing proteins implicated in plant-pathogen interactions. For each entry, gene identifiers and known pathogen-derived interactors are listed, as are the number of HMA/HMA-like domains (#HMA) and the sequence equivalent to the metal-binding motif (M/LXCXXC) in their $\beta 1$ - $\alpha 1$ loop region(s). Whether or not the HMA protein has been identified as a susceptibility factor (SF) in the pathosystem of interest is indicated by Y (yes), N (no), or - (not tested).

Host	HMA protein	#HMA	M/LXCXXC region	Pathogen	Pathogen protein	SF	Reference
Arabidopsis	CCS AT1G12520	1	MTCEGC	<i>Golovinomyces orontii</i>	OEC45	-	Weβling et al., 2014

Continued on next page

(Continued)

Host	HMA protein	#HMA	M/LXCXXC region	Pathogen	Pathogen protein	SF	Reference
Arabidopsis	HIPP13 AT5G52750	1	- ^a	<i>Hyaloperonospora arabidopsidis</i>	ATR13	-	Mukhtar et al., 2011
Arabidopsis	HIPP19 AT3G21490	1	MHCNDC	<i>Hyaloperonospora arabidopsidis</i>	HaRxL44	-	Mukhtar et al., 2011
				<i>Golovinomyces orontii</i>	OEC56 OEC115	-	Weßling et al., 2014
Arabidopsis	HIPP26 AT4G38580	1	MDCEGC	<i>Xanthomonas campestris</i>	XopK	-	González-Fuente et al., 2020
Arabidopsis	HIPP27 AT5G66110	1	MDCEGC	<i>Heterodera schachtii</i>	-	Y	Radakovic et al., 2018
				<i>Meloidogyne incognita</i>	-	Y	Dutta et al., 2023
Arabidopsis	AtHMP35 AT4G16380	1	LDCAKC	<i>Hyaloperonospora arabidopsidis</i>	HaRxL44	-	Mukhtar et al., 2011
Arabidopsis	CCP (HPP5) AT4G05030	1	MRCDKC	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	-	N	Chai et al., 2020
Arabidopsis	AthMAD1 (AtHMP9) AT1G51090	1	LNCSKC	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	-	Y	Imran et al., 2016
<i>Citrus sinensis</i>	CsHIPP03 Cs_ont_2g000480	2	MHCEAC MHCEAC	<i>Candidatus Liberibacter asiaticus</i>	SDE34	- ^b	Hu et al., 2023
<i>Eragrostis curvula</i>	EcHIPP43 EJB05_15595	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl2	-	Were et al., 2025
<i>Haynaldia villosa</i>	HIPP1-V KM216991	1	MDCEGC	<i>Blumeria graminis</i> f. sp. <i>tritici</i>	-	N ^c	Wang et al., 2022
				<i>Magnaporthe oryzae</i>	Pwl2	-	Were et al., 2025
<i>Hordeum vulgare</i>	HvHIPP43 HORVU.MOREX. r3.2HG0164110.1 or HORVU.MOREX .r3.3HG0267040.1 d	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl1 Pwl2 PWL2-3 Pwl3 Pwl4	-	Were et al., 2025
<i>Nicotiana benthamiana</i>	NbHIPP26 Niben101Scf 07109g05004	1	MDCEGC	Potato mop-top virus	TGB1	Y	Cowan et al., 2018
<i>Oryza sativa</i>	OsHIPP05 LOC_Os04g32850	1	LQCCRC	<i>Magnaporthe oryzae</i>	-	Y	Fukuoka et al., 2009 Nakao et al., 2011
<i>Oryza sativa</i>	OsHIPP19 LOC_Os04g39350	1	MPCEKS	<i>Magnaporthe oryzae</i>	AVR-PikA AVR-PikC AVR-PikD AVR-PikE AVR-PikF	N	Maidment et al., 2021 Oikawa et al., 2024
<i>Oryza sativa</i>	OsHIPP20 LOC_Os04g39010	1	MPCEKS	<i>Magnaporthe oryzae</i>	AVR-PikD	Y	Oikawa et al., 2024

Continued on next page

(Continued)

Host	HMA protein	#HMA	M/LXCXXC region	Pathogen	Pathogen protein	SF	Reference
<i>Oryza sativa</i>	OsHIPP21 LOC_Os09g09830	1	MTDERK	<i>Magnaporthe oryzae</i>	AVR-Pia	-	Maidment et al., 2025
<i>Oryza sativa</i>	OsHIPP28 LOC_Os10g14870	2	MHCEGC MHCDAC	<i>Rhizoctonia solani</i>	RsMf8HN	-	Niu et al., 2023
<i>Oryza sativa</i>	OsHIPP43 LOC_Os01g32330	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl1 Pwl2 Pwl2-2 Pwl2-3 Pwl3 Pwl4	Y	Zdrzałek et al., 2024
<i>Oryza sativa</i>	OsHPP02 LOC_Os02g37280	1	MASDKC	<i>Magnaporthe oryzae</i>	AVR-Mgk1	-	Stone, 2024
<i>Oryza sativa</i>	OsHPP03 LOC_Os02g37290	1	MASDKC	<i>Magnaporthe oryzae</i>	AVR-PikD AVR-Mgk1	-	Oikawa et al., 2024 Stone, 2024
<i>Oryza sativa</i>	OsHPP04 LOC_Os02g37300	1	MSCDKC	<i>Magnaporthe oryzae</i>	AVR-PikD AVR-Mgk1	N	Oikawa et al., 2024 Stone, 2024
				<i>Meloidogyne graminicola</i>	MgMO298	Y	Song et al., 2021 Huang et al., 2023b
<i>Oryza sativa</i>	OsHPP09 LOC_Os03g02070	1	MTDEKT	<i>Magnaporthe oryzae</i>	AVR-Pia	-	Maidment et al., 2025
<i>Oryza sativa</i>	OsHPP10 LOC_Os10g36200.2	1	MTDDKI	<i>Magnaporthe oryzae</i>	AVR-Pia	-	Maidment et al., 2025
<i>Oryza sativa</i>	OsHPP11 LOC_Os04g45130	1	MSDTKM	<i>Magnaporthe oryzae</i>	AVR-Pia	-	Maidment et al., 2025
<i>Setaria italica</i>	sHMA94 SETIT_012363mg	1	MPSPKN	<i>Magnaporthe oryzae</i>	AVR-PikD APikL2F	-	Bentham et al., 2021a ^e
<i>Setaria italica</i>	sHMA25 SETIT_011333mg	1	MSNAKS	<i>Magnaporthe oryzae</i>	AVR-PikD APikL2F APikLA	-	Bentham et al., 2021a ^e
<i>Setaria italica</i>	SitiHIPP43 SETIT_003232mg	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl2	-	Were et al., 2025
<i>Triticum aestivum</i>	TaHIPP1 TraesCS2A 01G064900	1	IDCEGC	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	-	Y	Zhang et al., 2015
<i>Triticum aestivum</i>	TaesHIPP43 TraesCS3D 02G209500	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl2	-	Were et al., 2025

^a The HIPP13 sequence included no conserved residues of the M/LXCXXC motif in the $\beta 1$ - $\alpha 1$ region.

^b Although not investigated in *C. sinensis*, the function of CsHIPP03 as a susceptibility factor was proposed, as independent silencing of three homologous transcripts (*NbHIPP3.1*, *NbHIPP3.2* and *NbHIPP3.3*) enhanced *N. benthamiana* resistance to *Pto* DC3000 Δ hopQ1-1.

^c Resistance to *Bgt* was assessed in *T. aestivum* HIPP1-V overexpression lines.

^d In yeast two-hybrid, Pwl2 bound two HvHIPP43 paralogs, both of which possess an M/LXCXXC motif reading MDCEGC.

^e 56, 19 and nine candidate sHMA binding partners were identified for AVR-PikD, APikL2A and APikL2F, respectively, originating from *O. sativa*, *S. italica* or *T. aestivum*. Only the two structurally characterised effector-HMA pairs from this study are included here.

1.2.1 What makes a HMA domain?

In 1994, Bull and Cox coined the term HMA to describe a collection of sequences from humans and bacteria, each possessing a conserved CXXC motif to facilitate metal ion coordination. These sequences included the Menkes and Wilson disease proteins from humans (ATPase copper transporting alpha (ATP7A) and beta (ATP7B), respectively), which are P_{1B}-type ATPases required to shuttle copper (Cu) across intracellular membranes within the secretory pathway (Tanzi et al., 1993; Vulpe et al., 1993). The characteristic 3D structure of a HMA domain was first described by Eriksson and Sahlman (1993), when the nuclear magnetic resonance (NMR) structure of MerP, a periplasmic mercury (Hg)-binding protein from *Pseudomonas aeruginosa*, revealed a $\beta\alpha\beta\beta\alpha\beta$ secondary structure, comprising an antiparallel β -sheet with two α -helices folded against it. A subsequent NMR structure of the fourth metal-binding domain of ATP7A (Gitschier et al., 1998), and crystal structure of the yeast (*Saccharomyces cerevisiae*) metallochaperone, antioxidant protein 1 (ATX1) (Rosenzweig et al., 1999, Fig. 1.2) each displayed homologous $\alpha\beta$ -sandwich folds, now recognised (alongside the CXXC motif) as a defining feature of HMA domains (InterPro: IPR006121).

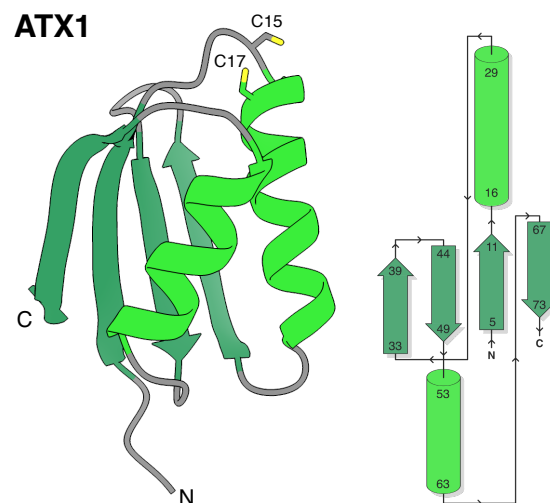


Figure 1.2 – HMA domain structure and topology. Crystal structure of ATX1 from *Saccharomyces cerevisiae* (1CC8) (Rosenzweig et al., 1999), demonstrating a typical $\beta\alpha\beta\beta\alpha\beta$ topology. Cys¹⁵ and Cys¹⁷ of the metal-binding motif (MTCSGC) are annotated. A topology diagram was drawn with the aid of PDBsum (Laskowski et al., 2018).

True to their name, in vitro studies indicated prototypical HMA domains were capable of directly binding heavy metals. Further structural characterisation of MerP revealed a Hg²⁺ ion coordinated between the two sulphur-containing Cys residues of the CXXC motif, forming approximately linear coordinate bonds (Steele and Opella, 1997). On the other hand, ATP7A, ATX1 and human ATX1 homologue 1 (HAH1, later named ATOX1) bound Cu⁺ in vitro (Hung

et al., 1998; Lutsenko et al., 1997; Pufahl et al., 1997). Across kingdoms, HMA proteins were initially characterised as metallochaperones: proteins that shuttle metal ions intracellularly to other metalloproteins. For instance, homologous Cu transport pathways in yeast, humans and Arabidopsis each employ a cytosolic HMA domain (belonging to ATX1, ATOX1 or AtATX1, respectively) to deliver imported Cu^+ to a HMA domain-containing ATPase embedded within post-Golgi vesicles (Klomp et al., 1997; Muller and Klomp, 2009; Pufahl et al., 1997; Puig et al., 2007; Yuan et al., 1995). Two more Cu chaperones with HMA domains have been characterised in Arabidopsis: COPPER CHAPERONE (CCH) and COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE (CCS) (Abdel-Ghany et al., 2005; Chu et al., 2005; Mira et al., 2001). Beyond this handful of Cu-binding examples, there is a growing body of evidence to support roles for plant HMA domains regulating heavy metal tolerance (see 1.2.2). However, numerous domains annotated as 'HMA' in plants lack the defining CXXC motif (and are thus referred to hereon as HMA-like domains), suggesting metals cannot be bound unless via an alternative mechanism. Considered alongside the diversity and expansion of HMA domain-containing families (see below and Table 1.2), this suggests the possibility of additional functions transcending metal homeostasis.

Plant proteins containing HMA or HMA-like domains (hereon HMA proteins) can be divided into three broad families: HMA PLANT PROTEINS (HPPs), HMA ISOPRENYLATED PLANT PROTEINS (HIPPs) and $\text{P}_{1\text{B}}$ -type ATPases (Figure 1.3). On rare occasions, HMA-like domains can also be found integrated within plant immune receptors (see 1.1.3 and 1.2.4), or fused to a SUPEROXIDE DISMUTASE (SOD)-like domain (InterPro: IPR001424), as is the case for Arabidopsis CCS (Figure 1.3). HIPPs, initially called *Arabidopsis thaliana* FARNESYLATED PROTEINS (ATFPs), or *Glycine max* FARNESYLATED PROTEINS (GMFPs), were first described by Dykema et al. (1999), following their identification in an in vitro screen for cDNAs encoding isoprenylated proteins (Crowell et al., 1996). These first HIPPs (ATFP2, 3, 6 and GMFP5, 7) were noted to possess both metal-binding (M/LXCXXC) and isoprenylation (CaaX, with 'a' indicating an aliphatic amino acid) motifs. Later BLAST-based searches uncovered a 45-member Arabidopsis HIPP family organised into five clades, the members of which vary in their number of HMA domains (one or two) and length of their C-terminal tails (de Abreu-Neto et al., 2013; Tehseen et al., 2010). Alongside, a 22-member Arabidopsis HPP family was described, including metallochaperones ATX1, CCS and CCH, the members of which lack an isoprenylation motif (de Abreu-Neto et al., 2013; Tehseen et al., 2010). Subsequently, using a hidden Markov model, Li et al. (2020) identified a further two HIPPs named HMA PROTEIN 9 (HMP09/HIPP40) and HMP35, and two HPPs named HMP18 and HMP28, bring the total number of family members to 47 and 24, respectively.

HMA domain-containing $\text{P}_{1\text{B}}$ -type ATPases constitute a smaller family of just eight proteins

in Arabidopsis, named HEAVY METAL ATPASE (HMA) 1-8 (not to be confused with the acronym for heavy metal-associated), only four of which possess at least one canonical HMA domain: HMA5, HMA6, HMA7 and HMA8 (Williams and Mills, 2005). These pumps utilise energy from ATP hydrolysis to shuttle cations between subcellular compartments, including from the cytosol into secretory vesicles or chloroplasts (reviewed by Hanikenne and Baurain, 2014). In contrast to HIPPs and HPPs, P_{1B}-type ATPases and HMA-SOD-like domain fusions have yet to emerge as host targets of pathogen effectors (Table 1.1). Hence, HIPPs and HPPs will be the focus of section 1.2 hereon.

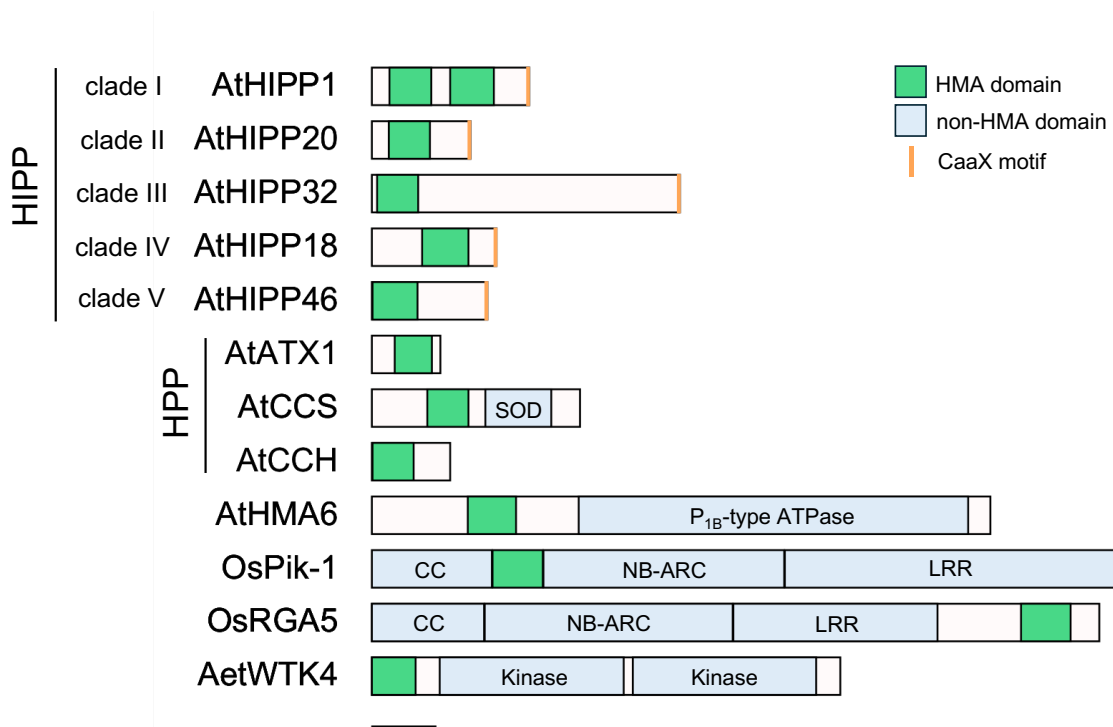


Figure 1.3 – HMA protein families in plants. Domain architectures of representative members of Arabidopsis HIPP, HPP and P_{1B}-type ATPase families. Assignment of HIPP proteins to clades I-V was based on phylogenetic work by de Abreu-Neto et al. (2013). Pik-1 and RGA5 from *Oryza sativa* represent sensor NLRs with integrated HMA domains. WTK4 from *Aegilops tauschii* represents KFPs with integrated HMA domains. Arabidopsis proteins were drawn based on domain annotations on the Uniprot website. Others were drawn based on domain annotations by De la Concepcion et al. (2018) (OsPik-1), Ortiz-Vallejo (2016) (OsRGA5) and Bernasconi et al. (2025) (AetWTK4). Scale bar = 100 amino acids.

Beyond Arabidopsis, catalogues of *HIPP* and *HPP* genes have been assembled from numerous plant species, including lycophytes, monocotyledonous and dicotyledonous flowering plants (Table 1.2). So far, HIPPs have been identified only in tracheophytes, meaning they likely arose after the divide of vascular and non-vascular plants. Tandem and segmental duplications have contributed to the major expansion of select gene families (Khan et al., 2019; Li et al., 2020; Ye et al., 2022), causing some species to accumulate over 50 *HIPP* genes (Table 1.2). Although, a recent survey of 13 plant genomes (including angiosperms, lycophytes and

bryophytes) suggested the number of HMA domain-containing proteins encoded is not always proportional to genome size [Sánchez-Pérez et al. \(2025\)](#). In this study, some exceptional proteins were uncovered with three or four HMA domains, and others were identified with additional domains such as zinc finger (zf)-BED, myotubularin (Myotub)-like phosphatase, pyrroloquinoline quinone 1 (PQQ1), PQQ2 and PQQ3, or domain of unknown function 659 (DUF659), suggesting the HMA domain may have been repeatedly repurposed to carry out diverse functions throughout plant evolution.

Table 1.2 – Number of *HIPP* and *HPP* genes identified in vascular plant genomes to date.
nr = not reported.

Species	<i>HIPP</i> genes	<i>HPP</i> genes	Reference
<i>Aegilops tauschii</i>	40	nr	Zhang et al., 2020b
<i>Arabidopsis thaliana</i>	47	24	de Abreu-Neto et al., 2013 ; Li et al., 2020
<i>Brachypodium distachyon</i>	40	nr	Zhang et al., 2020b
<i>Camellia sinensis</i>	38	9	Wei et al., 2023 ^a
<i>Chenopodium quinoa</i>	34	nr	Sun et al., 2022
<i>Cinnamomum micranthum</i>	42	9	Sánchez-Pérez et al., 2025
<i>Citrus sinensis</i>	26	nr	Huang et al., 2024
<i>Fagopyrum tataricum</i>	13	36	Ye et al., 2022 ^b
<i>Haynaldia villosa</i>	13	nr	Zhang et al., 2020b
<i>Hordeum vulgare</i>	33	nr	Zhang et al., 2020b
<i>Medicago sativa</i>	23	nr	Xia et al., 2025
<i>Oryza sativa</i>	59	7	de Abreu-Neto et al., 2013
<i>Persea americana</i>	27	8	Sánchez-Pérez et al., 2025
<i>Populus trichocarpa</i>	74	10	de Abreu-Neto et al., 2013
<i>Setaria italica</i>	52	3	de Abreu-Neto et al., 2013
<i>Selaginella moellendorffii</i>	5	4	de Abreu-Neto et al., 2013
<i>Solanum lycopersicum</i>	28	6	Xu et al., 2024 ^c
<i>Solanum tuberosum</i>	32	2	He et al., 2020
<i>Sorghum bicolor</i>	45	nr	Zhang et al., 2025
<i>Triticum aestivum</i>	114	nr	Zhang et al., 2020b
<i>Triticum dicoccoides</i>	58	nr	Zhang et al., 2020b
<i>Triticum urartu</i>	33	nr	Zhang et al., 2020b
<i>Zea mays</i>	66	nr	Gao et al., 2025

^a 56 genes were assigned the name *HIPP*. However, this list included nine genes encoding HPPs and nine genes encoding putative ATPases.

^b *HPP* genes were assigned the name *FtATX1*.

^c 34 genes were assigned the name *HIPP*. However, this list included six genes encoding HPPs.

1.2.2 HMA proteins function in stress tolerance

To date, various functions have been proposed for HIPP and HPP proteins within the fields of plant development and stress responses, with a range of spatial and stress-responsive expression profiles being reported for their corresponding genes. For instance, publicly available transcriptomic data or targeted reverse transcription quantitative PCR (RT-qPCR) experiments suggest that *HIPP* genes from *Arabidopsis*, *O. sativa*, *T. aestivum* and *H. vulgare* are responsive to abiotic stresses such as drought, cold, heat and salt (Barth et al., 2009, 2004; de Abreu-Neto et al., 2013; Zhang et al., 2020b, 2015; Zschiesche et al., 2015). In particular, *Arabidopsis HIPP26* (previously named *ATFP6*) was upregulated by cold, drought and salt stress, and is believed to positively regulate drought tolerance via the zinc finger transcription factor *Arabidopsis thaliana* HOMEBOX 29 (*ATHB29*) (Barth et al., 2009).

Unsurprisingly, HIPPs and HPPs have been widely implicated in regulating plant heavy metal tolerance. In *Arabidopsis*, *HIPP6*, *HIPP20*, *HIPP21*, *HIPP22* and *HIPP26* are induced by cadmium (Cd) exposure and positively regulate Cd tolerance (Gao et al., 2009; Suzuki et al., 2002; Tehseen et al., 2010). Following observation of higher Cd levels in overexpressing plants, *HIPP44* was also implicated in regulating Cd uptake (Zhang et al., 2019). Meanwhile, 11 *O. sativa* *HIPP/HPP* genes and three tea plant (*Camellia sinensis*) *HIPP* genes were upregulated under Cd stress (Khan et al., 2019; Wei et al., 2023), and *oshipp42* mutant growth was marginally reduced under Cu, zinc (Zn), Cd and manganese (Mn) stress (Khan et al., 2019). Furthermore, *OsHIPP29* overexpression enhanced Cd tolerance in *O. sativa* (Zhang et al., 2020a), and overexpression of *HIPP1-V* from *Haynaldia villosa* increased the tolerance of an elite bread wheat cultivar (*T. aestivum* Yangmai158) to select concentrations of Cd (Zhang et al., 2020b). Currently, the molecular mechanisms underlying these altered metal tolerance phenotypes (especially the contribution of direct heavy metal binding by HPP/HIPPs in this context) are poorly understood. For further discussion of this topic, see 7.2 in Chapter 7 of this thesis.

In the context of biotic stress, targeted RT-qPCR experiments frequently highlight *HPP/HIPP* genes as being upregulated in an infection context (Chai et al., 2020; Cowan et al., 2018; Dutta et al., 2023; Radakovic et al., 2018; Song et al., 2021; Wang et al., 2022; Were et al., 2025; Zhang et al., 2015; Zschiesche et al., 2015), yet a conserved defence-related function for these proteins has remained elusive. Instead, molecular and genetic studies suggest HMA proteins perform diverse (sometimes pathosystem-specific) roles, as discussed below.

1.2.3 HMA domains as effector host targets

As mentioned above, HPP/HIPPs from diverse plant species have been identified as direct targets of pathogen-derived molecules, including secreted effector proteins and a viral movement protein (MP) (Table 1.1). The most well-studied examples of effector-HMA interactions originate from *M. oryzae* pathosystems. OsHIPP19 is targeted by multiple AVR-Pik allelic effector variants (Maidment et al., 2021), and OsHIPP20 by the AVR-PikD variant (Oikawa et al., 2024). In vitro work revealed that OsHIPP43 is tightly bound by pathogenicity to weeping lovegrass (Pwl) effectors (Zdrzałek et al., 2024), and HvHIPP43, a barley (*Hordeum vulgare*) ortholog of OsHIPP43, was identified as a Pwl2 target using immunoprecipitation-mass spectrometry (IP-MS) (Were et al., 2025). Alongside, yeast two-hybrid screening efforts uncovered a plethora of small HMA (sHMA) proteins from *O. sativa*, foxtail millet (*Setaria italica*) and *T. aestivum* as potential targets of AVR-Pik and AVR-Pik like (APikL) effectors (Bentham et al., 2021a). Importantly, some *M. oryzae* effectors listed above are known to directly bind the HMA/HMA-like domains of their respective targets.

Beyond *M. oryzae*, targeting of host HMA proteins takes place across phytopathogen kingdoms. OsHIPP04 is a target of the root knot nematode (*Meloidogyne graminicola*) effector MgMO298 (Song et al., 2021), and was independently identified as a candidate binding partner of AVR-PikD from *M. oryzae* (Oikawa et al., 2024). Meanwhile, NbHIPP26 from *N. benthamiana* is targeted by the potato mop-top virus (PMTV) MP, triple gene block 1 (TGB1), although in this case, the isoprenylation motif of NbHIPP26 is known to be critical for TGB1 association, rather than the HMA domain (Cowan et al., 2018). In Arabidopsis studies, extensive yeast two-hybrid screens identified candidate HMA domain-containing targets of effectors from the powdery mildew fungus *Golovinomyces orontii*, downy mildew oomycete *Hyaloperonospora arabidopsidis* and bacterium *Xanthomonas campestris* (González-Fuente et al., 2020; Mukhtar et al., 2011; Weßling et al., 2014). Considered together, the repeated appearance of HPP and HIPP proteins in non-biased searches for host targets across diverse pathosystems hints at widespread roles for HMA proteins in plant immunity.

1.2.4 HMA domains as bait for effectors

As introduced in (1.1.3), HMA-like domains have been identified within plant NLR proteins (Figure 1.3), which function as part of a plant's intracellular defence signalling system. A variety of integrated domains have been reported in plant NLRs, including WRKY, BED, WD40 and NO₃-induced (NOI)/RIN4 domains (Grund et al., 2019; Sarris et al., 2016), however, HMAs remain one of the most well-studied (Table 1.3). Similarly, HMA domains have been reported within KFPs (Reveguk et al., 2025, Table 1.3), and a recent study demonstrated that

the WTK4 receptor from the wild wheat relative *Aegilops tauschii* directly binds AvrWTK4 from wheat powdery mildew (*Blumeria graminis* f. sp. *tritici*, or *Bgt*) via its N-terminal HMA domain (Bernasconi et al., 2025), highlighting another context in which plants exploit the HMA structure for effector recognition. Among NLRs with integrated domains, the recognition profiles of Pik-1 allelic variants from *O. sativa* have been studied in depth, and effector-binding specificity is known to be determined by their polymorphic HMA-like domains (De la Concepcion et al., 2019, 2018, 2021; Maqbool et al., 2015). Furthermore, Mla3 from *H. vulgare* was recently demonstrated to recognise Pw12 from *M. oryzae* via a HMA-like fold at the terminus of the receptor's LRR domain (Gómez De La Cruz et al., 2024). These discoveries support the hypothesis that pathogens frequently exploit HMA domains as host targets during infection.

Table 1.3 – Examples of plant resistance proteins with HMA-like domains and their corresponding helper NLRs and effectors, where known. The sequence equivalent to the metal-binding motif (M/LXCXXC) in the $\beta 1$ - $\alpha 1$ loop of the HMA-like domain is given. CC = coiled coil, NB = nucleotide-binding, LRR = leucine-rich repeat.

Plant	Sensor	Domain architecture	M/LXCXXC region	Helper	Pathogen	Effector(s)	References
<i>Aegilops tauschii</i>	WTK4	Pseudokinase-kinase-HMA	- ^a	Unknown	<i>Blumeria graminis</i> f. sp. <i>tritici</i>	AvrWTK4	Bernasconi et al., 2025
<i>Hordeum vulgare</i>	RPG1	HMA-pseudokinase-kinase	- ^a	Unknown	<i>Puccinia graminis</i> f. sp. <i>tritici</i>	Unknown	Brueggeman et al., 2002 Nirmala et al., 2006 Reveguk et al., 2025
<i>Hordeum vulgare</i>	MLA3	CC-NB-LRR	- ^b	-	<i>Magnaporthe oryzae</i>	Pw12	Gómez De La Cruz et al., 2024
<i>Oryza sativa</i>	Pikm-1	CC-HMA-NB-LRR	MVDDKS	Pikm-2	<i>Magnaporthe oryzae</i>	AVR-PikA AVR-PikD AVR-PikE AVR-Mgk1	De la Concepcion et al., 2018 Sugihara et al., 2023
<i>Oryza sativa</i>	Pikp-1	CC-HMA-NB-LRR	MEGNNC	Pikp-2	<i>Magnaporthe oryzae</i>	AVR-PikD AVR-Mgk1	De la Concepcion et al., 2018 Sugihara et al., 2023
<i>Oryza sativa</i>	RGA5	CC-NB-LRR-HMA	MPCGKS	RGA4	<i>Magnaporthe oryzae</i>	AVR-Pia AVR1-CO39	Cesari et al., 2013 Ortiz et al., 2017

^a The WTK4 and RPG1 sequences included no conserved residues of the M/LXCXXC motif in the $\beta 1$ - $\alpha 1$ loop.

^b Although MLA3 does not possess a full HMA-like fold, the final three repeats of the LRR structurally mimic the Pw12-binding surface of the OsHIPP43 HMA domain. This region does not include a $\beta 1$ - $\alpha 1$ loop.

1.2.5 HMA proteins as susceptibility factors

Multiple *HIPP* and *HPP* genes have been labelled as host susceptibility factors based on the observation that their loss of function results in enhanced resistance to a particular pathogen (Table 1.1). For example, the recessive *pi21* locus in *O. sativa* encodes a loss-of-function

OsHIPP05 mutation that enhanced resistance to ten races of *M. oryzae* (Fukuoka et al., 2009). Remarkably, heterologous expression of *OsHIPP05* in *Arabidopsis* (a non-host) enabled growth of *M. oryzae* hyphae, supporting the possibility that this protein functions as a negative regulator of host basal resistance (Nakao et al., 2011). While Oikawa et al. (2024) showed that *OsHIPP19* and *OsHIPP20* were each stabilised in complex with the effector AVR-PikD, deletion of *OsHIPP19* did not significantly impact *O. sativa* susceptibility to *M. oryzae*. However, *OsHIPP20* deletion enhanced resistance, suggesting AVR-PikD binding of this host target might ordinarily favour blast disease progression (Oikawa et al., 2024). *HvHIPP43* was also implicated as a susceptibility factor, as *H. vulgare* plants overexpressing this *HIPP* were more susceptible to *M. oryzae* (Were et al., 2025).

OsHIPP04 might likewise function as a susceptibility factor but in a pathogen-specific manner. Although *oshpp04* knockout mutants in *O. sativa* showed unaltered resistance to *M. oryzae* (Oikawa et al., 2024), an alternative mutant line (defective in the same gene) generated by Huang et al. (2023b) supported significantly fewer root knot nematodes (*M. graminicola*) than the wild-type background. In *Arabidopsis*, *HIPP27* function promoted susceptibility to two different nematode species: *Heterodera schachtii* (Radakovic et al., 2018) and *Meloidogyne incognita* (Dutta et al., 2023), while *HMA DOMAIN-CONTAINING 1 AtHMAD1* (*HMP09*) promoted susceptibility to *Pto* DC3000 (Imran et al., 2016). Whether or not the HMA domains of *HIPP27* and *AtHMAD1* are directly targeted by effectors remains to be determined.

Products of susceptibility genes can promote pathogen establishment via different mechanisms, including facilitation of host recognition and entry, pathogen nutrient acquisition and movement, or suppression of host immune responses (Garcia-Ruiz et al., 2021). Although not the case for all susceptibility factors described above (for example, *HIPP27*, Radakovic et al., 2018), genetic evidence suggests some HMA proteins may negatively regulate pathogen elicitor-induced ROS bursts and/or defence gene expression during infection (Fukuoka et al., 2009; Huang et al., 2023b; Were et al., 2025). Evidence for HPP/HIPP-mediated negative regulation of basal plant immune responses is thus building, however, the intracellular mechanisms underlying this suppression are unclear.

1.2.6 HMA proteins as positive regulators of defence

In contrast to the susceptibility factors described above, a select few HMA proteins have been shown to promote host resistance to pathogens. *HIPP1-V* is a single HMA domain-containing *HIPP* from the diploid wheat relative *H. villosa*, whose heterologous overexpression in hexaploid *T. aestivum* resulted in enhanced resistance to *Bgt* (Wang et al., 2022). To investigate the underpinning mechanism, Wang et al. (2022) identified *CMPG1-V* (so named for its

conserved Cys, Met, Pro and Gly residues) as a HIP1-V binding partner using a yeast two-hybrid approach. *CMPG1-V* was previously characterised as a resistance gene (Zhu et al., 2015), suggesting HIP1-V and *CMPG1-V* might cooperate to promote disease resistance. Another positive regulator of plant immunity is HPP5 from Arabidopsis, also named COPPER CHAPERONE INDUCED BY PATHOGENS (CCP). CCP overexpression enhanced Arabidopsis resistance to *Pto* DC3000, while its deletion enhanced susceptibility (Chai et al., 2020).

In summary, to date, both negative and positive regulators of defence have been identified amongst HMA proteins, making it especially challenging to assign a consensus immune function. As the number of HMA domains (Table 1.1) or pathogen-induced changes in expression patterns do not appear to be informative predictors, HPP/HIP influence on disease outcomes may depend on (1) protein subcellular localisation (see 1.2.7); (2) if/how the protein is targeted by effectors (which so far remains to be determined for HIP1-V and CCP); (3) the consequences of this (for instance, stabilisation versus degradation of the HMA protein) and/or (4) the distinct protein-protein interaction network of each family member.

1.2.7 Some HMA proteins localise to plasmodesmata

To better understand the diversity of HPPs and HIPs catalogued in genomic surveys, in silico localisation prediction tools have been applied on numerous occasions. The results suggest that HMA proteins could occupy almost any subcellular compartment, with the most commonly predicted locations being the cytoplasm, nuclei, chloroplasts, mitochondria and plasma membrane (Gao et al., 2025; He et al., 2020; Li et al., 2020; Sánchez-Pérez et al., 2025; Xia et al., 2025; Zhang et al., 2025), although a select few HIPs were predicted to be extracellular (de Abreu-Neto et al., 2013). Consistent with this, imaging based studies of individual HMA proteins have revealed a variety of subcellular localisations.

CCP and a selection of clade II Arabidopsis HIPs (numbered 20-27) and their *H. vulgare* relative, HvFP1, carry functional nuclear localisation signals, meanwhile mutation of the isoprenylation motifs of GFP-HIPP26 and GFP-HvFP1 modified the nuclear distribution of these HIPs in onion (*Allium cepa*) epidermal cells (Barth et al., 2009, 2004; Chai et al., 2020). Similarly, in *A. cepa* cells, GFP-HIPP3, TaHIP1-GFP and GFP-OsHIP05 were independently shown to localise to nuclei, with the latter also visible in the cytoplasm (Fukuoka et al., 2009; Zhang et al., 2015; Zschiesche et al., 2015). Meanwhile, OsHPP04-GFP (Song et al., 2021), CsHIP3-GFP (Huang et al., 2024) and GFP-HIP-V fusions from *H. villosa* were mainly nucleocytoplasmic when transiently expressed in *N. benthamiana*, although some co-localised with the plasma membrane marker mCherry-SYNTAXIN OF PLANTS 122 (SYP122) (Zhang et al., 2020b). Plasma membrane localisation has been reported for other HIP fusions in *N.*

benthamiana, including GFP-HIPP26 (Gao et al., 2009), GFP-NbHIPP26 (Cowan et al., 2018) and OsHIPP29-GFP, although the latter was also observed in nuclei (Zhang et al., 2020a). In contrast, GFP-HIPP32 from *Arabidopsis* co-localised with the cis-Golgi marker COMPLEX GLYCAN 1 (CGL1)-OFP (Leonte, 2020), while GFP-HIPP7 labelled the cytosolic surface of the ER (Guo et al., 2021), adding more potential sites of function to the list.

Of particular relevance for this thesis, HMA proteins have been identified as residents at plasmodesmata (PD) - the membrane-lined channels that connect adjacent cells. Specifically, 11 HIPP orthologs have emerged in PD proteomes (reviewed by Barr et al., 2023), and the subcellular localisations of four of these (HIPP1, HIPP7, HIPP26 and HIPP34) were confirmed in their respective native systems by confocal microscopy (Cowan et al., 2018; Guo et al., 2021; Leonte, 2020). In addition, HIPP33 and HIPP34 from *Arabidopsis*, as well as OsHIPP17, OsHIPP19 and OsHIPP20 from *O. sativa*, and HvHIPP43 from *H. vulgare* localised to PD when transiently expressed in *N. benthamiana* (Leonte, 2020; Oikawa et al., 2024; Were et al., 2025). HIPPs were even highlighted as one of 27 conserved protein orthogroups at PD, based on proteomic analysis of evolutionarily divergent species: *Arabidopsis*, *Populus trichocarpa* and *Physcomitrium patens* (Johnston et al., 2023). Alongside, HPP relatives of HIPPs were also detected in PD proteomes from *Arabidopsis* (Fernandez-Calvino et al., 2011), *N. benthamiana* (Park et al., 2017) and *P. patens* (Gombos et al., 2023). Despite the growing list of PD-located HMA proteins, exactly *why* they accumulate here remains a mystery. As the regulation of cell-to-cell communication via PD is known to be integral for determining outcomes of plant-pathogen interactions (discussed in the following section 1.3), it is possible that HIPP/HPP function at PD could explain their repeated appearance in studies of plant immunity.

1.3 Non cell-autonomous immunity and the role of plasmodesmata

1.3.1 Plants send defence signals via the apoplast and symplast

While the PTI and ETI responses described in 1.1 are cell-autonomous, pathogen colonisation of plants does not take place on a single-cell scale. As pathogens enter and spread throughout plant tissues, it remains advantageous for infected and/or damaged cells to signal to their neighbours, enabling pre-emptive enhancement of defences. Following a wound, amino acids such as Glu are released into the apoplast and diffuse outwards from stressed cells, activating ion-permeable channels on the surface of neighbours and triggering influxes of cytosolic Ca^{2+} second messengers as they spread (Bellandi et al., 2022). Also, immune-modulating endogenous secreted peptides (aptly named phytocytokines) are released into the apoplast by cells exposed to biotic stress (Huo et al., 2022). Here, these peptides are proteolytically processed

to yield an active form capable of triggering PTI-like responses via membrane-localised receptors (Rhodes et al., 2022, 2021). Given that secreted peptides are known to function non-cell autonomously in developmental contexts (for example, to maintain stem cell populations within the shoot apical meristem and vascular cambium Fletcher et al., 1999; Hirakawa et al., 2008), it is feasible that phyto cytokines could be perceived by and activate defence signalling in cells adjacent to those that perceived the stress.

At the scale of inter-organ communication, plants utilise mobile phytohormones, including salicylic acid (SA), jasmonic acid (JA) and ethylene to transcriptionally reprogramme cells via their interconnected signalling pathways (Verhage et al., 2010). Ethylene is a small gaseous molecule and can permeate cell membranes, while jasmonates (or JA precursors) are proposed to move both locally via the symplast, and systemically via vascular bundles (Gasparini et al., 2015; Lim et al., 2016). SA was shown to accumulate in apoplastic fluid upon exposure to pathogens (Lim et al., 2016), and its perception is required for the phenomenon known as systemic acquired resistance (SAR), which is activated in distal leaves upon local pathogen perception (Zhang and Li, 2019). The mobile molecule likely responsible for SAR is N-hydroxypipecolic acid (NHP), which accumulates in the phloem (reviewed by Sun and Zhang, 2021). Two other mobile signalling molecules, azelaic acid (AZA) and glycerol-3-phosphate (G3P) also contribute to SAR and were induced in petiole extracts (but not in apoplastic fluid) upon bacterial infection, suggesting they are similarly transported through the symplast (Lim et al., 2016). One might therefore hypothesise that distribution of defence signals via PD would be beneficial for hosts during infection. However, pathogens can exploit the same routes to facilitate their own spread and distribute virulence factors. As a result, the regulation of cell-to-cell connectivity is a critical yet often overlooked component of plant immunity.

1.3.2 Plasmodesmata establish symplastic continuity

As key components of a plant's internal communication system, PD directly connect the cytoplasm, plasma membrane and ER of adjacent cells, enabling transport of proteins, small RNAs, phytohormones and nutrients. The ultrastructure of an individual plasmodesma is typically represented as a plasma membrane-lined pore containing a cytoplasmic sleeve and appressed strand of ER called the desmotubule (Figure 1.4A). Building on this, electron microscopy studies have uncovered a diversity of PD structures including 'simple' (consisting of a single channel), twinned (two channels in close proximity, which may be centrally connected), and branched arrangements (with multiple openings and a central cavity) (Faulkner et al., 2008; Oparka et al., 1999). At specialised cell junctions such as those between phloem sieve elements and the phloem pole pericycle cells in root tips, funnel-like PD have

been described, which facilitate phloem unloading ([Ostermeyer et al., 2022](#)).

It has long been understood that primary PD arise during cytokinesis following mitosis, when strands of ER become trapped in the membranous cell plate as it expands and eventually fuses with parental membranes to divide daughter cells ([Hepler, 1982](#)). Recent electron microscopy and modelling experiments demonstrated that the presence of ER makes the closure of gaps (fenestrae) in expanding cell plates energetically unfavourable, and the resulting pores are stabilised following accumulation of MULTIPLE C2 DOMAINS AND TRANSMEMBRANE DOMAIN PROTEIN (MCTP) tethers, which bridge the ER and plasma membrane ([Li et al., 2024](#)). In mature cell walls, however, as opposed to being evenly or randomly distributed, PD are observed in clusters known as pitfields, which arise as a consequence of secondary PD formation in close proximity to existing channels ([Faulkner et al., 2008](#)).

Pitfield-associated cell walls are especially pectin-rich, and show variable accumulation of the β -1,3-glucan polymer, callose (reviewed by [Knox and Benitez-Alfonso, 2014](#)). Meanwhile, pitfield-associated plasma membrane domains are rich in sterols and sphingolipids with long-chained fatty acids ([Grison et al., 2015](#)). Distinct from the wider plasma membrane, PD harbour unique communities of proteins. Previously, meticulous fractionation and mass spectrometry were used to enrich for cell wall and PD proteins in plants and algae (for example, see [Bayer et al., 2006](#); [Faulkner et al., 2005](#)). This led to the identification of key regulators of PD function such as β -1,3-glucanases, which degrade PD-associated callose to enhance intercellular trafficking ([Benitez-Alfonso et al., 2013](#); [Levy et al., 2007](#)), and PLASMODESMATA-LOCATED PROTEINS (PDLs) that function in localised signalling cascades ([Tee et al., 2023](#); [Thomas et al., 2008](#)). Subsequent proteomic surveys uncovered PD resident proteins belonging to multiple families, including cytoskeletal components, peroxidases, C2 lipid-binding proteins, SKEWED 5 (SKU5) family proteins, tetraspanins and RLKs, to name a few ([Fernandez-Calvino et al., 2011](#); [Johnston et al., 2023](#)). Overall, their distinct molecular composition makes PD a unique form of membrane contact site, and one of many specialised signalling domains within the plasma membrane.

The maximum size of molecules permitted to move through PD (known as the size exclusion limit) can be altered by plants in response to internal and external signals. The best-understood mechanism by which this occurs is via callose metabolism (reviewed by [Wu et al., 2018](#)). Within the PD signalling domain, CALLOSE SYNTHASE (CALS) enzymes (also known as GSLs for GLUCAN SYNTHASE-LIKE) deposit callose at the neck regions of PD, thereby constricting the openings at either end and lowering the size exclusion limit ([Dickmanns et al., 2025](#), Figure 1.4B). Conversely, opening of PD is made possible by resident β -1,3-glucanase enzymes, which degrade callose. Frequently, a negative correlation is observed between the

extent of intercellular flux and PD-localised callose accumulation (Cheval et al., 2020; Lee et al., 2011; Li et al., 2021; Tee et al., 2023), and the identification of β -1,3-glucanase enzymes in angiosperm, moss and green algal PD proteomes suggests callose-mediated regulation of PD is evolutionary ancient (Faulkner et al., 2005; Johnston et al., 2023).

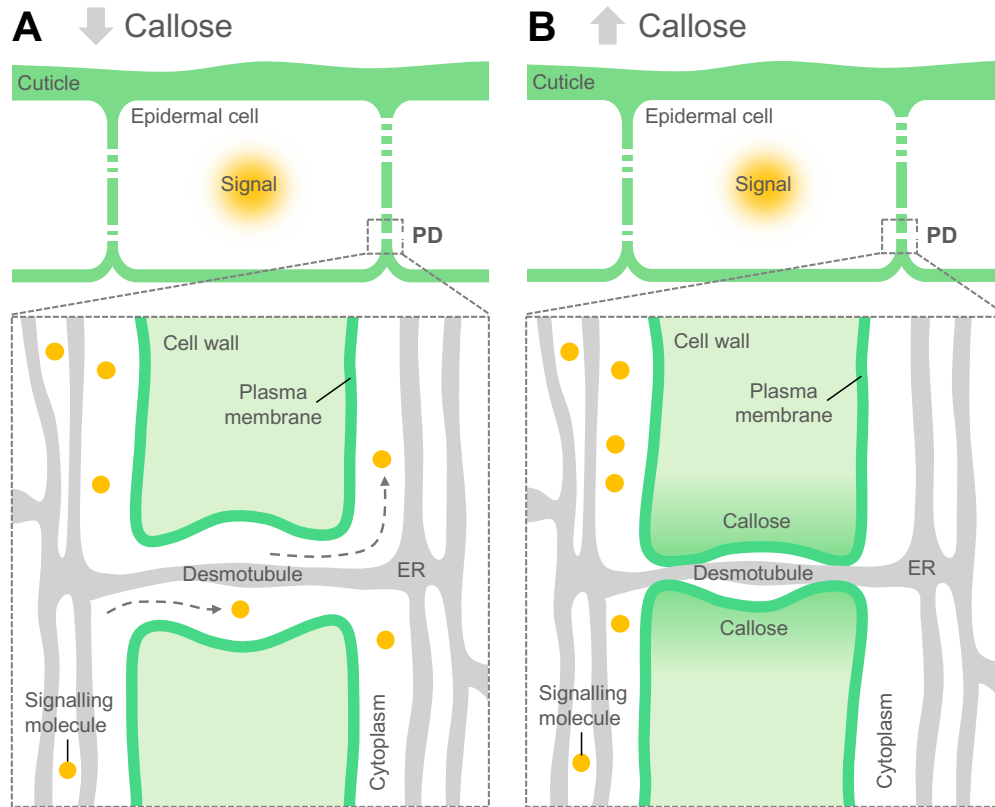


Figure 1.4 – Callose-dependent regulation of PD aperture. A-B. Schematic representation of a plant epidermal cell producing a signalling molecule (yellow), connected to its neighbours by PD. The structure of a single plasmodesma is represented in the lower panels. When callose accumulation is low (A) soluble signalling molecules move freely through the cytoplasmic sleeve. When callose accumulation is high (B), the PD neck regions become constricted, blocking cell-to-cell movement of the signal.

Interestingly, callose-independent enhancement and reduction of intercellular transport has been reported on multiple occasions (Blekemolen et al., 2022; Liu et al., 2022; Oparka et al., 1999), hinting at the existence of other more enigmatic mechanisms of PD regulation. Consistent with this, Pérez-Sancho et al. (2025) uncovered MCTP3, MCTP4 and MCTP6, as regulators of intercellular flux in *Arabidopsis* roots. At PD, MCTPs act as protein-lipid tethering complexes, being anchored to the desmotubule via their C-terminal transmembrane domain, while binding phosphatidylinositol 4-phosphate (PI4P) lipids within the plasma membrane. In the absence of these tethers, or when PI4P synthesis was inhibited, contacts between the ER and plasma membrane at PD were visibly compromised and cell-to-cell movement was enhanced (Pérez-Sancho et al., 2025).

Alteration of PD structure represents another callose-independent strategy for regulation of intercellular flux, given that branched PD were shown to have a lower size exclusion limit than simple PD in *Nicotiana tabacum* leaves (Oparka et al., 1999). In addition, plants may adjust the number or density of channels available for trafficking. While the molecular events that govern this process are still being elucidated, secondary PD formation has been reported to accompany cell expansion (Faulkner et al., 2008), developmental transitions (Complainville et al., 2003; Ormenese et al., 2006), C₄ photosynthetic induction (Schreier et al., 2024) and intraspecific graft formation (Chambaud et al., 2022). Remarkably, interspecific secondary PD have also been observed at graft junctions (Kollmann and Glockmann, 1991), and between parasitic plants and their hosts (Lee, 2009). Thus, through dynamic regulation of PD aperture, structure and development, plants are able to tightly control the connectivity of their cells.

1.3.3 Plasmodesmal function is altered in response to pathogens

Regulation of PD function is an integral part of basal plant immune responses. Following perception of apoplastic MAMPs flg22 and chitin, PD-associated callose deposition increases after just 30 minutes, reducing cell-to-cell connectivity in Arabidopsis leaves (Cheval et al., 2020; Faulkner et al., 2013; Xu et al., 2017). Chitin-triggered PD closure requires the membrane-localised receptor proteins LYSIN MOTIF DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEIN 2 (LYM2) and LYSIN MOTIF RECEPTOR-LIKE KINASE 4 (LYK4), both of which become enriched at PD in the presence of chitin (Cheval et al., 2020; Faulkner et al., 2013, Dr Sebastian Samwald, unpublished data). A third receptor, LYK5, is also required and is thought regulate LYK4 function as part of LYK4-LYK5 bipartite or LYM2-LYK4-LYK5 tripartite complexes outside PD signalling platforms (Cheval et al., 2020). How these receptors are sorted between membrane nanodomains within the PD and the wider plasma membrane is a topic of ongoing research. Notably, the canonical chitin receptor CERK1 is not required for chitin-induced PD closure, highlighting the existence of multiple independent PTI signalling cascades (Faulkner et al., 2013).

Downstream of chitin perception via LYM2, CALCIUM-DEPENDENT PROTEIN KINASES (CPKs), namely CPK6 and CPK11, phosphorylate specific residues of RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), triggering apoplastic ROS accumulation and eventual deposition of callose at PD neck regions via CALS1 (Cheval et al., 2020; Tee et al., 2023). Similarly, despite the requirement of different receptors for elicitor perception, flg22-induced PD closure also depends on CALS1 and RBOHD (Tee et al., 2023). Recently, chitin-, flg22- and SA-triggered responses were shown to converge at a signalling nexus comprising NON-RACE SPECIFIC DISEASE RESISTANCE/HIN1 HAIRPIN-INDUCED-LIKE 3 (NHL3) and PDL1/5 (Tee et al., 2023), which act upstream of CALS1 (Cui and Lee, 2016).

Evidence also suggests PD closure is part of anti-viral defence responses, as treatment of *N. benthamiana* with a synthetic double-stranded RNA analogue, polyinosinic-polycytidilic acid [poly(I:C)] triggered PD-localised callose deposition via a signalling cascade involving SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), BOTRYTIS INDUCED KINASE 1/AvrPphB SUSCEPTIBLE1-LIKE KINASE 1 (BIK1/PBL1) and PDL1/2/3 (Huang et al., 2023a). This cascade appears distinct from those mediating chitin- and flg22-induced closure, as enhanced callose deposition was evident in poly(I:C)-treated plants lacking *PDL5*. Although it remains unclear as to how viral RNA would ordinarily be transported to the apoplast, the reduced number and area of infection sites established by GFP-tagged tobacco mosaic virus (TMV) in *N. benthamiana* leaves treated with poly(I:C) suggests perception of this elicitor is beneficial for perturbing viral infection (Huang et al., 2023a).

Further supporting the hypothesis that a reduction in intercellular trafficking promotes pathogen resistance, *lym2* loss-of-function mutants, which fail to close their PD in response to chitin, were more susceptible to the necrotrophic fungus, *Botrytis cinerea* (Faulkner et al., 2013), and sustained closure of PD in *icals3m* plants, made possible by estradiol-inducible expression of an overactive variant of *CALS3*, enhanced resistance to the bacterial pathogen *Pto* DC3000 (Tee et al., 2025). However, Tee et al. (2025) discovered that induced *icals3m* plants displayed enhanced susceptibility to *B. cinerea*. This suggests the duration of host PD closure and accompanying changes in carbon distribution could be important factors determining the outcome of necrotrophic fungal infections, and that low connectivity does not translate to increased resistance in all scenarios.

While the signalling cascades underpinning PD closure were dissected using isolated elicitors, live imaging and protein mobility assays have enabled the study of PD function during pathogen colonisation. For instance, Tee et al. (2025) observed increased PD-associated callose deposition in *Arabidopsis* leaves at 48 and 72 hours post-inoculation (hpi) with *Pto* DC3000 *hrcC* mutants, suggesting closure is likely sustained in this system (that is, in the absence of secreted effectors) as a result of continuous exposure to elicitors. In contrast, during the early stages of *M. oryzae* infection of *O. sativa* (24-27 hpi), mCherry (transiently expressed via bombardment) was observed to move cell-to-cell, and enhanced callose accumulation (visualised using aniline blue stain) was *not* observed at the pitfields of primary infected cells, suggesting PD remained open (Sakulkoo et al., 2018). At later stages (30-34 hpi), callose deposition increased around hyphae at crossing points corresponding to pitfields, and at PD along the remainder of the cell wall (Sakulkoo et al., 2018). Given this delay in callose deposition, it is hypothesised that *M. oryzae* initially circumvents chitin-triggered PD closure to facilitate its invasion of neighbouring cells. The mechanisms and exact consequences of PD modification by pathogens remain themes of current research.

1.3.4 Viruses employ diverse strategies to manipulate plasmodesmata

Pathogen-mediated manipulation of PD function has been widely studied in the context of viral infection. As obligate intracellular pathogens, viruses must exploit the PD network to enable their local and subsequent systemic spread within plant hosts. To achieve this, viral MPs directly target and manipulate PD in a variety of ways, depending on the virus in question (for reviews, see [Alazem et al., 2025](#); [Lucas, 2006](#); [Taliensky et al., 2008](#)).

A key function of many MPs is their delivery of viral components to PD to facilitate their cell-to-cell transport. One of the earliest MPs to be characterised was the TMV 30 kDa MP (MP30) ([Ohno et al., 1983](#)). As a peripheral ER membrane protein, MP30 is capable of trafficking viral RNA as a ribonucleoprotein complex (RNP) along the ER/actin network towards PD ([Sambade et al., 2008](#)). Meanwhile, the MP of turnip mosaic virus (TuMV), named P3-N-terminal-pretty interesting potyviral open reading frame (P3N-PIPO), targets PD via its interaction with PLASMA MEMBRANE-ASSOCIATED CATION-BINDING PROTEIN 1 (PCaP1) in *Arabidopsis* ([Vijayapalani et al., 2012](#)) and acts as an anchor for viral cylindrical inclusion (CI) proteins ([Wei et al., 2010](#)). Here, CI proteins form cone-shaped structures, which funnel viral RNA and coat proteins through PD to neighbouring cells ([Roberts et al., 1998](#); [Rodríguez-Cerezo et al., 1997](#)). Interestingly, despite originating from a single-stranded DNA virus, the multifunctional MP of tomato yellow leaf curl virus (TYLCV), named C5, can partially rescue the reduced cell-to-cell trafficking phenotype of a TuMV mutant (a single-stranded RNA virus) lacking functional P3N-PIPO, by serving as an alternative anchor at PD ([Zhao et al., 2023](#)). Ordinarily, C5 promotes nuclear export and intracellular trafficking of a second TYLCV protein (V2), to PD along actin microfilaments, ultimately facilitating intercellular transport of the TYLCV genome ([Zhao et al., 2023](#)).

Once at PD, modification of host membranes and cytoskeletal components by viral proteins is often important for trafficking. This is exemplified by potex-like viruses and hordei-like viruses (including PVX and PMTV, respectively), which require a triple gene block system comprising three TGB proteins for their intercellular movement. In the case of PVX, viral movement is accomplished via 'coreplicative insertion', involving the establishment of localised replication sites at PD ([Tilsner et al., 2013](#)). For this to occur, TGB2 and TGB3 integrate into the ER membrane, forming a protein raft responsible for directing a complex of TGB1 and viral RNA to PD. Here, cortical ER is remodelled and membranous loops form, where replication complexes are assembled ([Tilsner et al., 2013](#)), and TGB1 gates PD via its reorganisation of actin and the ER network ([Howard et al., 2004](#); [Tilsner et al., 2012](#)). Constriction of ER tubules by TGB2 is also thought to contribute to PD gating ([Lazareva et al., 2021](#)), facilitating the loading of viral RNA for transport. On the other hand, the cucumber mosaic virus (CMV) MP, 3a, was discovered to

sever actin filaments and inhibit their polymerisation (Su et al., 2010). This activity is thought to increase PD aperture by removing obstructing material from the neck region (Chen et al., 2010), supported by the fact that stabilisation of actin following phalloidin treatment inhibited CMV-mediated PD gating (Su et al., 2010). Certain viruses are also proposed to promote callose catabolism to enhance the aperture of PD channels, for example via delivery of host β -1,3-glucanases within ER-derived vesicles (Epel, 2009; Guenoune-Gelbart et al., 2008).

Rather than manipulating endogenous PD regulation pathways, some viruses utilise surprisingly destructive strategies to shuttle their genomes. Tubule-forming viruses such as cowpea mosaic virus (CPMV), grapevine fanleaf virus (GFLV) and citrus psorosis virus (CPsV) manufacture molecular bridges to transport their RNPs or virions across the cell wall. These bridges are constructed from MP and coat protein oligomers and can be observed using transmission electron microscopical (TEM) study of pitfields, or seen protruding from the surface of protoplasts expressing viral genes (den Hollander et al., 2016; Luna et al., 2018; Ritzenthaler et al., 1995; van Lent et al., 1991). Xie et al. (2016) captured this phenomenon when studying PD ultrastructure in quinoa (*Chenopodium quinoa*) leaves inoculated with the broad bean wilt virus 2 (BBWV2). Here, TEM and electron tomography revealed that BBWV2-derived tubules (composed of the MP, VP37) displaced the desmotubule and expanded PD diameter, allowing packaging and movement of whole virions.

1.3.5 Some pathogen effectors are cell-to-cell mobile

While viruses are established masters of PD manipulation, it is becoming increasingly apparent that phytopathogens in other kingdoms similarly exploit PD to establish infections. For instance, secreted effectors from diverse pathogens are known to move cell-to-cell within their hosts. One of the first reports of effector mobility came from Khang et al. (2010), who demonstrated movement of fluorescently-tagged *M. oryzae* effectors Pwl2 and biotrophy-associated secreted 1 (Bas1) ahead of the infection front in *O. sativa* leaf sheath cells. Similarly, the *Ustilago maydis* effector chorismate mutase 1 (Cmu1) moved between cells when transiently expressed in maize (*Zea mays*) as a YFP fusion (Djamei et al., 2011). More recently, screens of putative effectors from two *Arabidopsis* pathogens, *Hyaloperonospora arabidopsidis* (an oomycete) and *Colletotrichum higginsianum* (*Ch*, an ascomycete fungus) identified 16 and 15 proteins, respectively, that were cell-to-cell mobile in the *N. benthamiana* transient system (Liu et al., 2022; Ohtsu et al., 2023). Li et al. (2021) also identified 16 effectors from *Pto* DC3000 that were cell-to-cell mobile as YFP fusions in *N. benthamiana*, and demonstrated inhibition of movement upon flg22 treatment or PDL5/7 overexpression. Thus, it is hypothesised that PD closure is induced upon pathogen perception (see 1.3.3) to protect adjacent cells from mobile effectors that may suppress their immune responses.

As a countermeasure, pathogens are hypothesised to secrete effectors capable of re-opening PD channels or preventing their initial closure. Indeed, a subset of hypermobile effectors were identified in both *H. arabidopsidis* and *Ch* effectomes (defined as moving further than expected, given their molecular weight), representing candidate PD manipulators (Liu et al., 2022; Ohtsu et al., 2023). Using agroinfiltration-based assays in *N. benthamiana* or bombardment-based assays in transgenic Arabidopsis, multiple putative effector proteins have been shown to enhance the movement of co-expressed fluorescent proteins relative to controls, namely HaRxL77 from *H. arabidopsidis* (Liu et al., 2022), *Ch* effector candidate 8 (ChEC8), ChEC127 and ChEC132 (Ohtsu et al., 2023), HopO1-1 from *Pto* DC3000 (Li et al., 2021) and the cooperating effector pair, secreted in xylem 5 (Six5) and Avr2 (also known as Six3) from *F. oxysporum* (Blekemolen et al., 2022; Cao et al., 2018). Although examples of PD-manipulating and/or PD-localised effectors continue to accumulate (see also Germain et al., 2018; Mosquera et al., 2009), exactly how these proteins modify PD remains poorly understood.

1.4 *Colletotrichum higginsianum* (*Ch*) as a model hemibiotrophic fungal pathogen

For those seeking an improved understanding of the mechanisms by which fungi might manipulate host cell-to-cell connectivity, the hemibiotrophic anthracnose pathogen *Ch* represents an attractive model system. In natural and agricultural settings, *Ch* infects the aerial tissues of a range of brassicaceous plants, including widely consumed crops such as cabbage, pak choi, mustard, turnip, swede (*Brassica spp.*) and radish (*Raphanus sativus*) (Damm et al., 2014; O'Connell et al., 2004). In a laboratory context, *Ch* is compatible with multiple ecotypes of Arabidopsis (Narusaka et al., 2004; O'Connell et al., 2004), offering a wealth of genetic resources and protocols to be utilised when investigating host-derived factors governing infection success. *Ch* itself is also genetically tractable and, in contrast to obligate biotrophic fungi, can be cultured axenically. Consequently, efficient protocols have been established to facilitate the stable expression of transgenes in *Ch* via *A. tumefaciens*-mediated transformation of spores (O'Connell et al., 2004) or polyethylene glycol (PEG)-mediated transformation of protoplasts (Dallery et al., 2017), as well as targeted gene knockout via homologous recombination (Korn et al., 2015). Recently, CRISPR-Cas9 (short for clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9) has also been applied to direct double-strand breaks, increasing the efficiency of *Ch* gene knockout by recombination approaches (Bhadauria et al., 2024; Yonehara et al., 2023).

Paving the way for gene discovery and functional analysis, genomic and accompanying stage-specific transcriptomic data were obtained for popular *Ch* laboratory strains. Following

its initial isolation from *Brassica campestris* subsp. *chinensis* (O'Connell et al., 2004), the IMI 349063 genome was first sequenced by O'Connell et al. (2012) using short-read sequencing, then again by Zampounis et al. (2016) using long-read PacBio and optical mapping methods. The closely related strain MAFF 305635, first isolated from *Brassica rapa* var. *perviridis* (Komatsuna), was engineered to express mRFP (referred to as MAFF 305635-RFP) (Hiruma et al., 2010) and had its genome sequenced by Tsushima et al. (2019), revealing strain-specific large-scale rearrangements of the genome attributed to transposon activity, and variation in candidate effector gene sets when compared to IMI 349063. Over the last two decades, equipped with these genetic resources and cell biological observation of infection structures, an improved understanding of the *Ch* infection cycle has been gained.

As a hemibiotroph, *Ch* exhibits a multi-stage infection cycle, first described in depth by O'Connell et al. (2004). First, the fungus penetrates host cells using a melanised appressorium to generate immense turgor pressure, and subsequently enters a biotrophic phase during which bulbous invasive hyphae are established within living host cells. Shortly after, fungal development undergoes a recognisable switch and the necrotrophic phase begins, involving death and degradation of host tissues as thin filamentous hyphae radiate from initially penetrated cells. For more detail on this topic, see 4.1.1 in Chapter 4. Early exploration of the factors underlying *Ch* pathogenicity involved high throughput forward genetic screens. Taking a random insertional mutagenesis approach using *A. tumefaciens*-mediated transformation, Huser et al. (2009), Liu et al. (2013) and Korn et al. (2015) generated a plethora of mutants with phenotypic defects at various stages of infection, from appressorium development, to necrotrophic growth. Subsequent amplification of genomic regions at T-DNA borders facilitated the discovery of multiple pathogenicity-related genes, including those encoding a putative β -1,3(4)-glucanase (required for host penetration), an importin- β 2 subunit (required for the biotrophic to necrotrophic switch) (Huser et al., 2009), a copper amine oxidase (required for full virulence) (Liu et al., 2013) and a plasma membrane H^+ -ATPase (required for invasive hyphal growth) (Korn et al., 2015), among many hypothetical proteins with yet unknown functions.

Reverse genetic approaches have also been valuable for discerning the functions of select fungal genes during *Ch* infection. For instance, melanin biosynthesis genes *scytalone dehydratase 1* (*SCD1*) and *polyketide synthase 1* (*PKS1*) were removed from *Ch* by targeted gene disruption, resulting in appressorial melanin deficiency and an inability of mutants to establish infection on *Arabidopsis* hosts (Yonehara et al., 2023). Elsewhere, deletion of *budding uninhibited by benzimidazole 2* (*BUB2*), a conserved regulator of G₁-S phase transitions in eukaryotes, was found to trigger premature nuclear division in *Ch* spores, leading to the formation of multinucleate appressoria and loss of virulence, demonstrating that regulation of cell cycle

phase transitions is crucial for development of functional infection structures in this pathogen (Fukada et al., 2019).

Following host penetration and the establishment of invasive hyphae, secreted effectors are hypothesised to promote *Ch* virulence via their manipulation of host processes, including defensive signalling. The *Ch* genome (strain IMI 349063) was initially predicted to encode a repertoire of 365 candidate secreted effectors, defined as possessing an N-terminal signal peptide, yet displaying no other significant homology to proteins outside the *Colletotrichum* genus (O'Connell et al., 2012). An additional analysis of expressed sequence tags by Kleemann et al. (2012) indicated that 198 effector candidate (*ChEC*) genes are transcribed during in planta infection stages, and 102 of these were preferentially expressed during biotrophy as opposed to necrotrophy. As is typical for putative phytopathogen effectors, most *ChEC* genes encode small proteins (67 amino acids, on average) that lack recognisable domains. Many are also specific to *Ch*, suggesting they are likely subject to diversifying selection pressures (Kleemann et al., 2012). For more detail on *ChEC* expression patterns and discussion of attempts to discern effector function using reverse genetics, see 4.1.2 in Chapter 4 of this thesis. Meanwhile, knowledge surrounding *ChEC* localisation and secretion is discussed in 5.1 in Chapter 5 and 6.1.2 in Chapter 6, respectively. Overall, considering the genetic tractability of both partners, availability of genomic and transcriptomic data, and long list of yet-uncharacterised *ChEC* genes, the *Ch*-Arabidopsis pathosystem represents an ideal model in which explore effector function during plant-fungus interactions.

1.4.1 Introducing ChEC108 - a hypermobile, PD-localised putative effector

Previously in the laboratory of Prof. Christine Faulkner, research effort was directed towards the characterisation of *Ch* effector subcellular localisation and cell-to-cell movement. Specifically, Dr Joanna Jennings and Assist. Prof. Mina Ohtsu screened 38 *ChEC* proteins, first establishing their localisation in *N. benthamiana* as C-terminal GFP fusions, then assessing their ability to exploit PD for symplastic movement. The latter objective was achieved using a quantitative assay in *N. benthamiana*, involving transient expression of an effector-GFP fusion at discrete single-cell transformation sites, and subsequent measurement of the number of adjacent (untransformed) cells into which the fusion moved. For more information on the outcomes of these screening efforts, see 5.1 in Chapter 5. Of particular relevance to this work, four effector candidates were discovered to be PD-localised: ChEC36, ChEC108, ChEC142 and ChEC155, and a fifth (ChEC147) was sighted at Arabidopsis PD (but not those of *N. benthamiana*) (Jennings, 2021). These *ChECs* represented prime candidate manipulators of cell-to-cell communication during infection.

Preliminary mobility assessments conducted by Dr Joanna Jennings suggested that, despite localising to intercellular channels, ChEC36-GFP and ChEC155-GFP were likely immobile, being confined to single transformed cells in *N. benthamiana*, while ChEC142-GFP was seen entering only cells immediately adjacent to those transformed. In contrast, ChEC108-GFP was discovered to be highly cell-to-cell mobile, marking it as the most promising candidate manipulator of PD (Jennings, 2021). A more thorough investigation of ChEC108 mobility was subsequently carried out, revealing that ChEC108-GFP moved significantly further than GFP alone, despite the former being larger in molecular weight. In addition, tandem dimer Tomato fused to a nuclear localisation signal (NLS-tdTomato, initially exploited as a transformation marker due to its low mobility) was observed to move significantly further upon co-expression with ChEC108-GFP, versus a GFP control (Jennings, 2021). Together, these experiments suggest ChEC108 can actively promote its own mobility *and* enhance general molecular flux through PD - a very intriguing behaviour for a fungus-derived protein.

In search of the mechanism by which ChEC108 might manipulate PD, an IP-MS experiment was carried out in *N. benthamiana* to identify candidate *in vivo* interactors in an unbiased manner (Jennings, 2021, see 3.2.1 in Chapter 3 for more detail). Arabidopsis homologs were identified for the top six most likely ChEC108-associated proteins, providing a list of candidate interactors from the *Ch* host. Based on the number of corresponding peptides identified in control versus experimental samples, the most likely binding partner to emerge from this analysis was the HMA domain-containing protein, HIP6 - a key finding that served as a foundation for the work presented in this thesis.

1.5 Project aims

From the outset of this project, I sought to better understand the mechanisms and consequences of fungal manipulation of plant intercellular communication by studying the PD-localised, hypermobile *Ch* effector, ChEC108. Additionally, with HIP6 emerging as a likely host target, the study of ChEC108 presented an opportunity to investigate the poorly understood roles of pathogen-targeted HMA proteins in plant immunity. Equipped with two genetically amenable model organisms in *Arabidopsis* and *Ch*, effort was directed towards the following aims:

1. **To determine the structural basis of ChEC108-HIP6 association.** Which protein domains and residues are required to form a complex? How does the mechanism of binding compare to other known effector-HMA protein interactions? These questions are explored in Chapter 3.
2. **To investigate the function of ChEC108 and HIP6 in an infection context.** How does genetic loss of either *ChEC108* or *HIP6* alter *Ch* virulence on *Arabidopsis*? This question is explored in Chapter 4.
3. **To study ChEC108 and HIP6 function at the subcellular level.** Where do ChEC108 and HIP6 localise in plant cells? Does ChEC108 influence PD function in *Arabidopsis* (a host of *Ch*) as well as in *N. benthamiana* (non-host)? These questions are explored in Chapter 5.
4. **To verify ChEC108 as a bona fide *Ch* effector.** Can *Ch*-derived ChEC108 be detected in *Arabidopsis* host cells during infection? This question is explored in Chapter 6.

Chapter 2

Materials and Methods

2.1 Cloning and bacterial methods

2.1.1 Golden Gate cloning

Single-stranded DNA primers were synthesised by [Integrated DNA Technologies \(IDT\)](#). For a full list of DNA primers used for cloning, see Table [S1](#). Target sequences were amplified from genomic DNA (gDNA) or plasmid templates by Bounce PCR ([Mugford and Hogenhout, 2018](#)) using Phusion[®] High Fidelity (HF) DNA polymerase (Table [2.1](#)). PCR products were loaded on 1% agarose gels stained with ethidium bromide, and electrophoresis was carried out in 0.5x Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer. DNA was extracted from agarose gels using a QIAquick[®] Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. As exceptions to the above, DNA sequences encoding candidate ChEC108-associated proteins were synthesised by [Genewiz](#), and select HMA domain fragments for integration into the Pikm-1 NLR chassis were obtained by annealing long DNA primers. To form a primer duplex, 1 µl of each single-stranded primer (100 µM) was diluted in 98 µl annealing buffer (Table [S2](#)). Dilutions were incubated at 95 °C for 4 min, then subject to 70 cycles of 1 min incubations, decreasing the temperature by 1 °C each cycle.

Target sequences were added to digestion-ligation reactions with BbsI (Thermo Scientific) or BsaI-HF[®] v2 enzymes (NEB) for incorporation into Golden Gate level 0 or level 1 backbones, respectively ([Engler et al., 2008](#), Tables [2.2](#) and [2.3](#)). Multi-gene constructs were assembled into level M or level P backbones using BbsI and BsaI-HF[®] v2 enzymes, respectively. Unless otherwise specified, all plasmid backbones and standard parts were obtained from [TSL SynBio](#). Plasmids assembled using Golden Gate cloning are listed in Table [S3](#).

Table 2.1 – Phusion PCR reaction mix.

Component	Volume (μl)
Template DNA (~10 ng)	1.0
Forward primer (10 μM)	2.0
Reverse primer (10 μM)	2.0
dNTP mix (10 mM)	0.8
Phusion [®] HF DNA polymerase (NEB)	0.4
5x Phusion [®] HF reaction buffer (NEB)	8.0
Deionised H ₂ O	25.8
Reaction volume	40.0

Table 2.2 – Golden Gate reaction mixture.

Component	Volume (μl)
T4 ligase buffer (NEB)	1.5
T4 DNA ligase (NEB)	1.0
10x Bovine Serum Albumin (NEB)	1.5
BsaI or BbsI	1.0
Acceptor plasmid (100 ng/μl)	1.0
Insert(s)	sufficient for 2:1 molar ratio of insert:vector
Deionised H ₂ O	up to 20.0
Reaction volume	20.0

Table 2.3 – Golden Gate thermocycler protocol.

Step	Temperature (°C)	Duration (min)
Initial digestion	37	0.3
Digestion	37	3.0
Ligation	16	4.0
Final digestion	50	15.0
Enzyme inactivation	80	5.0

35-50 cycles

2.1.2 Gateway[®] cloning

Historically in the laboratory of Christine Faulkner, high expression of fluorescent proteins in particle-bombarded cells (see 2.3.2) has been achieved using the Gateway[®] destination backbone, pB7WG2.0. To generate new constructs for bombardment, amplification and purification of gene fragments as blunt-ended PCR products was performed as described in 2.1.1. The pENTR[™]/D-TOPO[®] Cloning Kit (Invitrogen) was used to assemble PCR

products into entry clones with the following reaction composition: pENTRTM/D-TOPO[®] (~17 ng), salt solution (1 µl), PCR product (sufficient for 2:1 molar ratio of insert:vector), H₂O (up to 6 µl reaction volume). Reactions were incubated at room temperature for 3 h or overnight. Expression cassettes were generated using recombination reactions with the following composition: 5x Gateway[®] LR ClonaseTM II Enzyme mix (Invitrogen) (4 µl), destination vector pB7WG2.0 (300 ng), entry clone (300 ng), Tris-EDTA (TE) buffer (6 µl). Reactions were incubated at 25 °C for 4.5 h or overnight. Plasmids assembled using Gateway[®] cloning are listed in Table [S4](#).

2.1.3 In-Fusion[®] cloning

To assemble plasmids for transformation of *M. oryzae*, amplification and purification of gene fragments as blunt-ended PCR products was performed as described in [2.1.1](#). In-Fusion[®] cloning reactions were carried out with the following composition: 5x In-Fusion[®] Snap Assembly Master Mix (2 µl), linearised vector backbone (50-100 ng, provided by Jave Bautista), PCR product(s) (sufficient for 2:1 molar ratio of each insert:vector) and H₂O (up to 10 µl reaction volume). Reactions were incubated at 50 °C for 15 min. Plasmids assembled using In-Fusion[®] cloning are listed in Table [S5](#).

2.1.4 Transformation of chemically competent *Escherichia coli*

Golden Gate plasmids were transformed into DH5α *E. coli* cells by heat shock. 100 ng of plasmid or 2.5 µl of digestion-ligation reaction mixture were added to 60 µl cells and left on ice for 30 min. Cells were shocked in a water bath at 42 °C for 90 s, then left on ice for 2 min. 940 µl SOC was added and cells were recovered at 37 °C, shaking at 200 rpm for 1 h. 120 µl of culture was spread onto lysogeny broth (LB) agar plates (Table [S6](#)), supplemented with antibiotics (for concentrations, see Table [2.4](#)). Gateway[®] TOPO[®] and LR cloning reaction mixtures were transformed into One ShotTM TOP10 Chemically Competent *E. coli* (Invitrogen) according to the manufacturer's instructions. In-Fusion[®] cloning reaction mixtures were transformed into Stellar Competent Cells (Takara Bio) according to the manufacturer's instructions. *E. coli* plates were incubated at 37 °C for 12-16 h.

2.1.5 Bacterial screening and DNA extraction

Colony PCR was used to screen bacterial colonies for plasmids of interest. Single colonies were suspended in 15 µl liquid LB (Table [S6](#)), and 1 µl of this suspension was used as template in a PCR with GoTaq[®] G2 Green Master Mix (Tables [2.5](#) and [2.6](#)). PCR products were loaded on 1% agarose gels stained with ethidium bromide, and electrophoresis was carried out in 0.5x TBE buffer.

To extract plasmids from positive colonies, 10 ml liquid LB cultures were inoculated with 9 µl of colony suspension and appropriate antibiotics (Table 2.4), then incubated at 37 °C, shaking at 200 rpm for 12-16 h. To prepare glycerol stocks, 700 µl of culture was mixed with 700 µl of 40% glycerol and stored at -70 °C. Plasmids were purified from the remainder of the culture using a QIAprep® Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. The sequences of level 0 plasmid inserts were verified by Sanger sequencing, performed by Genewiz using primers listed in Table S7.

For production of plasmids stocks at high concentrations for particle bombardment (see 2.3.2), 10 ml liquid LB cultures were inoculated with a single bacterial colony and appropriate antibiotics (Table 2.4), then incubated at 37 °C, shaking at 200 rpm for 12-16 h. 2 ml of the resulting starter culture was added to 500 ml of liquid LB, and incubation at 37 °C and 200 rpm continued for 16-24 h. Plasmids were purified using a Plasmid Maxi Kit (QIAGEN) according to the manufacturer's procedure for high copy plasmids.

Table 2.4 – Working concentrations of antibiotics and other chemicals for selection. IPTG = isopropyl β-D-1-thiogalactopyranoside, PPT = L-phosphinothricin, X-gal = 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, DMSO = dimethyl sulfoxide, DMF = dimethylformamide.

Chemical	Dissolved in	Concentration (µg/ml)
Carbenicillin	H ₂ O	100
Cefotaxime	H ₂ O	100
Chloramphenicol	100% ethanol	34
Gentamycin	H ₂ O	10
Hygromycin	H ₂ O	100
IPTG	H ₂ O	238
Kanamycin	H ₂ O	50
PPT	H ₂ O	10 or 400 ^a
Rifampicin	DMSO	50
Spectinomycin	H ₂ O	50
X-gal	DMF	20

^a PPT was used at 10 µg/ml for selection of *Arabidopsis*, or 400 µg/ml for selection of *Ch*.

Table 2.5 – Colony PCR reaction mix. For primers used, see Table S7.

Component	Volume (µl)
Bacterial colony	1.0
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
GoTaq [®] G2 Green Master Mix (Promega)	5.0
Deionised H ₂ O	3.0
Reaction volume	10.0

Table 2.6 – Colony PCR thermocycler protocol. Annealing temperature was varied according to the melting temperature of primers used.

Step	Temperature (°C)	Duration (min)	
Initial denaturation	95	3.0	
Denaturation	95	0.5	
Annealing	variable	0.5	30 cycles
Elongation	72	~ 1.0 per kb	
Final elongation	72	5.0	

2.1.6 Restriction digest

To test whether mutli-gene constructs were assembled correctly without the need for multiple PCR or sequencing reactions, restriction digests were carried out. Reaction mixtures (Table 2.7) were prepared and incubated at 37 °C for 2 h, then enzymes were heat-inactivated (where possible) at 65 °C for 20 min. Restriction enzymes included DraIII-HF[®] (NEB), EcoN1 (NEB), BamH1-HF[®] (NEB) or HindIII-HF[®] (NEB), selected based on their ability to deferentially digest plasmids of interest and empty backbone controls. Digestion products were examined on 1% agarose gels stained with ethidium bromide, and electrophoresis was carried out in 0.5x TBE buffer.

Table 2.7 – Restriction digest reaction mixture.

Component	Volume (µl)
Plasmid DNA (500 ng – 1 µg)	10
Restriction enzyme	1
10x rCutSmart [™] buffer (NEB)	2
Deionised H ₂ O	7
Reaction volume	20.0

2.1.7 Preparation of electrocompetant *Agrobacterium tumefaciens*

Glycerol stocks of GV3101 or AGL1 *A. tumefaciens* strains were streaked onto LB agar plates, supplemented with appropriate antibiotics: rifampicin and gentamycin for GV3101, or rifampicin and carbenicillin for AGL1 (for concentrations, see Table 2.4). Plates were incubated at 27 °C for 2 days. 10 ml liquid LB cultures, supplemented with antibiotics as above, were inoculated with a single colony each, then incubated at 28 °C, shaking at 200 rpm for 24 h. 0.5 ml or 1 ml of this starter culture was used to inoculate 500 ml of liquid LB with antibiotics as above, and incubation continued at 28 °C and 200 rpm overnight, until optical density (OD) was within the range 0.5-1. OD was measured using a BioPhotometer spectrophotometer (Eppendorf) at 600 nm. Bacteria were pelleted by centrifuging at 4000 xg for 15 min at 4 °C, then resuspended in 500 ml of ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (1 mM, pH7). A series of centrifugation and resuspension steps were carried out as above, with the ice-cold resuspension solution varying as follows: 250 ml of HEPES buffer (1 mM, pH7), 200 ml of 10% glycerol, then 1.5 ml of 10% glycerol. Resuspended cells were divided into 20 µl aliquots, snap-frozen in liquid nitrogen and stored at -70 °C.

2.1.8 Transformation of electrocompetant *A. tumefaciens*

Plasmids were transformed into GV3101 or AGL1 electrocompetent *A. tumefaciens* cells (prepared as described in 2.1.7) by electroporation. 20 µl of cells were incubated with 100 ng of plasmid on ice for 5-10 min. Cells were transferred to ice-cold 2 mm electroporation cuvettes (Cell Projects) and shocked with a 1800 V pulse, supplied by a Gene PulserTM electroporation system (BIO-RAD). Immediately after, cells were suspended in 500 µl of liquid LB and incubated at 28 °C, shaking at 220 rpm for 2-3 h. 120 µl of each culture were plated on LB agar plates with rifampicin, gentamycin and other appropriate antibiotics (Table 2.4), then incubated at 28 °C for 2 days. To prepare glycerol stocks, one colony was selected and incubated in 10 ml of liquid LB, supplemented with antibiotics as above, for 12-16 h. 800 µl of culture was mixed with 800 µl of 40% glycerol and stored at -70 °C.

2.2 Plant growth

2.2.1 Growth of *Nicotiana benthamiana*

N. benthamiana plants were grown in a controlled environment room under long-day conditions: 22 °C and 80% relative humidity, with a 16 h light/8 h dark photoperiod. Sowing, transplanting and watering were carried out by the John Innes Centre Horticultural Services team.

2.2.2 Growth of Arabidopsis on soil

Dry Arabidopsis seeds were incubated at 4 °C for at least 2 days before being placed on compost (Levington F2 Seed & Modular) in a 24-cell seed tray (1 seed per cell) or 9 cm pot (6-9 seeds per pot). For leaf assays, plants in 24-cell trays were grown in cabinets (Panasonic Versatile Environmental Test Chamber MLR-352-PE), equipped with Six Newlec LED T8 colour temperature 4000K and Nine Newlec LED T8 colour temperature 6500K lights. Short-day conditions were used to encourage vegetative growth: 22 °C with a 10 h light/14 h dark photoperiod. Tap water (~200 ml) was administered twice per week. Leaves were detached for experimentation 5-6 weeks post-sowing. Maximum rosette diameters (that is, the longest distance between the tips of two opposite leaves) of plants grown under short-day conditions were measured using Digital ABS Caliper CoolantProof IP67 calipers (Mitutoyo). To record growth phenotypes of mutants and transgenic lines under short-day growth conditions, photography was performed with the help of Phil Robinson.

For seed collection (see 2.2.4), plants were grown in 24-cell trays or 9 cm pots in a controlled environment room under long-day conditions to encourage reproductive growth: 22 °C with a 16 h light/8 h dark photoperiod. Plants destined for floral dipping (see 2.3.3) were grown in 9 cm pots under long-day conditions, as described above. Primary inflorescences were removed to promote growth of secondary inflorescences, thus increasing the number of buds available for dipping 1 week later.

2.2.3 Growth of Arabidopsis on sterile media

Before sowing on sterile plates for selection or infection, small amounts (< 50 µl volume) of dry seed were placed in open eppendorf tubes and sealed in a desiccator with chlorine gas to sterilise overnight (13-14 h). Before closing the lid of the desiccator, chlorine gas was released by adding 3 ml of concentrated HCl to 100 ml of commercial sodium hypochlorite solution (Honeywell). For selection of chemical-resistant T1 seedlings (see 2.4.2), or growth of seedling lawns for infection (see 2.6.6), seeds were sprinkled onto growth media without added glucose (GM-GLU) agar plates (Table S6), specifically chosen to reduce the risk of microbial contamination. For selection of chemical-resistant seedlings in T2 and subsequent generations and for assessment of segregation ratios, seeds were sown on Murashige and Skoog (MS) media with added sucrose (MS+S) agar plates (Table S6). Plates were sealed with two layers of micropore tape and left in the dark at 5 °C for 2-3 days, before being transferred to long-day growth conditions in a walk-in growth chamber: 20 °C, with a 16 h light/8 h dark photoperiod.

2.2.4 Arabidopsis seed collection

Arabidopsis inflorescences were tied to wooden stakes to reduce cross-contamination within and between trays. When siliques began to brown, inflorescences were contained within glassine bags, and the provision of water stopped. Approximately 2 weeks later (or when plants were fully dry), seeds were manually threshed, then stored in glassine bags at room temperature in the dark.

2.3 Plant transformation

2.3.1 Agroinfiltration of *N. benthamiana* for transient expression

Frozen *A. tumefaciens* glycerol stocks were streaked onto LB agar plates, supplemented with appropriate antibiotics (Table 2.4), then incubated at 28 °C for 2 days. 10 ml LB cultures, also supplemented with antibiotics, were inoculated using a single colony and incubated at 28 °C, shaking at 200 rpm for 12-24 h. Bacteria were pelleted by centrifuging at 3000 xg for 10 min at room temperature. Pellets were resuspended in infiltration buffer (Table S2) and the OD was measured using a BioPhotometer spectrophotometer (Eppendorf) at 600 nm. Bacterial suspensions were diluted in infiltration buffer to achieve a desired OD (Table 2.8). All infiltration mixtures were prepared with bacteria carrying the RNA silencing suppressor p19 at OD = 0.1. Mature leaves of 3-4-week-old *N. benthamiana* plants were infiltrated using needleless 1 ml syringes after the leaf surface was pricked in up to six locations with a 25G bevelled needle. Plants were transferred back to prior growth conditions until infiltrated leaves were collected for experimentation 2-3 days post-infiltration (see 2.5 and 2.8).

To test for activity of transiently-expressed TurboID fusion proteins in *N. benthamiana*, leaves were infiltrated with *A. tumefaciens* as above, then re-infiltrated with 50 µM biotin after ~17 h. After a further 21 h, leaves were detached and washed four times in 4 °C H₂O to remove excess biotin from the leaf surface, then dabbed dry using 2-ply blue paper. Leaf discs (8 mm diameter) were harvested from a minimum of three leaves, 2 days post-infiltration and frozen at -70 °C.

To visualise macroscopic cell death, bacterial suspensions were infiltrated in discrete spots on *N. benthamiana* leaves. 5 days post-infiltration, autofluorescence was visualised using ultraviolet (UV) photography of the abaxial surface of detached leaves. Images were taken with the help of Phil Robinson, using a Nikon D4 camera with a 60 mm macro lens. UV light was supplied with Blak-Ray® longwave (365 nm) B-100AP spotlight lamps, actively moved over the leaves to ensure even illumination. Post acquisition, raw images were converted to .tiff files by Phil Robinson, involving sharpness and contrast enhancements.

Table 2.8 – OD of bacterial suspensions used for *N. benthamiana* agroinfiltration experiments.

Experiment		Desired OD
Silencing suppression (p19)		0.1
Protein localisation		0.3-0.4 ^a
Protein extraction		0.3-0.4 ^b
Quantification of protein mobility	Mobile component	0.0001-0.0002
	Background component	0.4
Quantification of pitfield density	PD marker	0.5
	Background component	0.4
Cell death	NLR component	0.4
	Effector component	0.6

^a As an exception, to avoid excessive cell death, constructs encoding ChEC108-eGFP were infiltrated at ODs ranging from = 0.0001-0.1. In an attempt to boost protein accumulation, constructs encoding PDL5-mCherry (FP05207) and some mCherry-HIPP6 variants (FP16001, FP16118) were infiltrated at OD = 0.5.

^b As an exception, to avoid excessive protein accumulation, LTi6b-mCherry (FP09048) was infiltrated at OD = 0.1.

2.3.2 Particle bombardment for transient expression

To enable transient expression of fluorescent proteins at discrete locations across a leaf epidermis, solid gold particles were coated with plasmid DNA and fired at mature *Arabidopsis* leaves in a partial vacuum. First, 50 mg of 1.0 µm Gold Microcarriers (1652263, BIO-RAD) were prepared in 1 ml of H₂O and vortexed for 1-2 min. Particles were centrifuged at 13,000 xg for 1 min, then washed three times in 100% ethanol by vortexing, following by centrifugation as above. A final wash in H₂O was carried out, then particles were suspended in 1 ml of H₂O, distributed into 25 µl aliquots and stored at 4 °C.

Gold particle aliquots were prepared for bombardment by mixing with 5-10 µg of the required plasmid(s). 25 µl of 2.5 M CaCl₂ was added to precipitate the DNA onto the gold. To protect the DNA from DNases, 10 µl of 0.1 M spermidine was added, and the mixtures were left on ice for 30 min. Gold particles were pelleted using a short centrifuge spin, and the supernatant discarded. Particles were washed in 100% ethanol by pipetting, followed by a short centrifugation step, then finally resuspended in 100 µl of 100% ethanol. Immediately before bombardment, 5 µl of this gold particle preparation was applied to the centre of a circular microparticle carrier disc (1652335, BIO-RAD), loaded into a microcarrier holder (1652322, BIO-RAD).

Arabidopsis plants for bombardment were grown as described in 2.2.2. Immediately prior to bombardment, leaves were detached and placed on MS+S plates with 0.6% or 0.8% agar. 2-3 leaves were placed in the centre of each plate with their abaxial sides facing upwards. *N. benthamiana* plants for bombardment were grown as described in 2.2.1. Immediately

prior to bombardment, mature leaves were detached and placed on individual MS+S plates with their abaxial sides facing upwards. Bombardment was carried out using a Biolistic PDS-1000/He particle delivery system (BIO-RAD), fitted with 1,100 or 1,500 psi rupture disks (1652329 or 1652331, BIO-RAD) and stopping screens (1652336, BIO-RAD). Post-bombardment, *Arabidopsis* leaves were contained within their MS+S dishes to maintain humidity. However, given their thinner cuticle and tendency to lose turgor pressure when detached for long periods of time, *N. benthamiana* leaves were sealed in empty petri dishes containing a small reservoir of H₂O, such that when upright, the base of the freshly-trimmed petiole would be submerged (Figure 2.1). Leaves were left in daylight at room temperature for 18-24 h before bombarded sites were imaged using confocal microscopy (see 2.5.3).

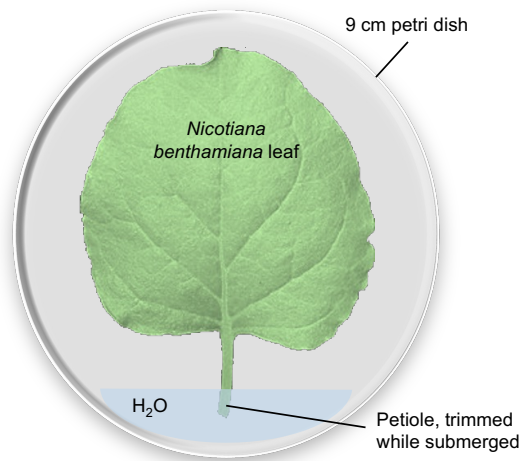


Figure 2.1 – Post-bombardment set-up for *N. benthamiana* leaves. Schematic representation of a leaf enclosed in a petri dish with a small reservoir of H₂O to prevent excessive loss of turgor pressure. Plates were sealed with parafilm.

2.3.3 Floral dipping of *Arabidopsis* for stable expression

Frozen *A. tumefaciens* glycerol stocks were streaked onto LB agar plates, supplemented with appropriate antibiotics (Table 2.4), then incubated at 28 °C for 2 days. 10 ml liquid LB starter cultures, also supplemented with antibiotics, were inoculated using a single colony and incubated at 28 °C, shaking at 200 rpm for 12-24 h. 200 ml of liquid LB with appropriate antibiotics was inoculated with 2 ml of this starter culture and incubated at 28 °C, shaking at 200 rpm for 20-24 h. Bacteria were collected by centrifugation at 5800 xg for 15 min at room temperature, then resuspended in 200 ml of dipping buffer (Table S2). 5 µl of Silwet L-77 was added and gently mixed.

Plants for dipping were grown as described in 2.2.2. Prior to dipping, visible siliques and open flowers were removed in an attempt to increase transformation efficiency. Inflorescences

were dipped in bacterial suspensions for 1 min with gently agitation, then covered with a black plastic bag to maintain humidity and exclude light. 24 h later, the cover was removed and plants were returned to prior growth conditions. In some cases, the dipping process was repeated 1 week later in an attempt to increase transformation efficiency further. T1 seeds were harvested as described in 2.2.4 and screened as described in 2.4.1.

2.4 Plant material and genotyping

Details of Arabidopsis lines generated and used in this work are given in Table S8.

2.4.1 Fluorescent seed selection

T1 seed expressing the FastRed fluorescent marker (Shimada et al., 2010) was selected using a Zeiss Lumar.V12 stereoscope, equipped with a 1.2x objective, 10x eyepiece lens, BP545/25 excitation filter and BP605/70 emission filter. Individual fluorescent seeds were selected using a toothpick, and sown on soil (2.2.2). Seeds were harvested from the resulting T2 plants (2.2.4). Non-fluorescent T2 seeds were selected as above, to ensure any resulting mutant lines were free from the FastRed selection marker and accompanying Cas9 enzyme. Selected T2 seeds were sown on soil and genotyped by PCR (see 2.4.3 and 2.4.4).

2.4.2 Seed selection by chemical resistance

Seedlings were grown on sterile media for selection as described in 2.2.3. T1 or T2 seeds carrying a selection marker for kanamycin resistance were sown on GM-GLU plates supplemented with kanamycin (Table 2.4). After 2-3 weeks of growth, surviving seedlings were transferred to 24-cell trays of soil (Levington F2 Seed & Modular) and moved to a controlled environment room for seed collection (2.2.2). When established on soil, plants were screened for transgene insertion by PCR (see 2.4.3 and 2.4.4).

T1 seeds carrying a selection marker for L-phosphinothricin (PPT) resistance were germinated on soil (Levington F2 Seed & Modular with added grit), under glasshouse conditions. When the first true leaves emerged, seedlings were sprayed three times with a PPT formulation (Finale, 2.5 ml/l), leaving 4-day intervals between sprays. Surviving seedlings were transplanted to 24-cell trays, left to grow under glasshouse conditions and screened for transgene insertion by PCR (see 2.4.3 and 2.4.4) before seed was harvested (2.2.4). Alternatively, PPT-resistant T1 and T2 seeds were selected on sterile GM-GLU plates supplemented with PPT (Table 2.4), as described above for kanamycin selection.

2.4.3 Arabidopsis gDNA extraction

For genotyping, high-throughput Arabidopsis gDNA extractions were carried out using the LiCl precipitation method. Leaf samples (~6-7 mm², consisting of a cotyledon, young leaf tip or cauline leaf) were collected in 96-well plates with a single glass bead (2 mm diameter, Z273627, Sigma-Aldrich). Samples were frozen by gently submerging in liquid nitrogen or storing at -70 °C before grinding with a 2010 Geno/Grinder (SPEX SamplePrep) took place, shaking at 1200 rpm for 1 min. After the addition of 100 µl of gDNA extraction buffer (Table S2) to each well, samples were vortexed vigorously, then centrifuged at 3200 xg for 10 min at 4 °C. 75 µl of each supernatant was transferred to a fresh well containing 75 µl of ice-cold 100% isopropanol, then mixed by pipetting to precipitate DNA. Centrifugation at 3200 xg for 15 min at 4 °C was carried out to pellet DNA. The pellet was washed briefly with 200 µl of 70% ethanol, air dried, and resuspended in 60 µl of H₂O. Concentration and purity of DNA extracts were assessed using a NanoDropTM One/One^C Microvolume UV-Vis Spectrophotometer.

2.4.4 Arabidopsis genotyping

gDNA fragments were amplified by PCR (Tables 2.5 and 2.6), with 1-5 µl of gDNA extract (2.4.3) as a template, using primers listed in Table S9. To genotype transfer DNA (T-DNA) insertion lines (specifically, ET007), PCR was used to test for presence of the wild-type gene fragment, and a fragment spanning the right border of the T-DNA insert and flanking genomic region, as recommended by SALK.

To genotype the *hipp6-2* mutation in lines ET006 and ET007, an internal PCR product was amplified as above, then digested with EcoN1 enzyme (2.1.6). *hipp6-2* mutant DNA fragments were resistant to digestion, as a Cas9-induced 1 bp insertion in the *HIPP6* coding sequence disrupted an EcoN1 recognition site. To genotype *hipp6-3* mutants (ET003), primers were designed to bind either side of the outermost Cas9 target sites. Wild-type or *hipp6-3* gDNA fragments were amplified by Phusion PCR (modified from the procedure described in 2.1 to allow for 10 µl reactions), with the latter product being smaller in size due to deletion of the *HIPP6* coding sequence (see Figure 4.7).

T1 or T2 lines expressing GFP-tagged proteins (ET013, ET020, ET022, ET026, ET028, ET030) were screened by a combination of PCR and confocal microscopy (see 2.5). T1 lines expressing non-fluorescent epitope-tagged proteins (ET015 and ET016) were screened via protein extraction and Western blotting (see 2.8).

2.4.5 Mutation of Arabidopsis genes using CRISPR-Cas9

Cas9 target sites within the *HIPP6* and *HIPP3* gDNA sequences were identified using an online tool, [CHOPCHOP](#) ([Labun et al., 2019](#)). These sites consisted of a 20 bp sequence, followed by a protospacer adjacent motif (PAM), with sequence NGG. Site selection criteria included low self-complementarity, high predicted efficiency of cutting and the identification of no off-target matches elsewhere in the genome. For each gene, two target sites were selected both upstream and downstream of the coding sequence. Constructs for expression of Cas9 and small guide RNAs (sgRNAs) were designed based on the optimised CRISPR-Cas9 protocol described by [Castel et al. \(2019\)](#), and assembled via Golden Gate cloning ([2.1](#)).

First, using custom primers encoding Cas9 target sites of choice (Table [S1](#)), sgRNAs were amplified from the template plasmid BCJJ434A, with a U6-26 terminator, BsaI recognition sites and Golden Gate-compatible overhangs. These sgRNAs were incorporated into level 1 plasmids with a U6-26 promoter, and then into level 2 or level M plasmids (Table [S3](#)). Level 2 plasmids enabled simultaneous expression of up to four sgRNAs, and were thus suitable for generating *hipp6* or *hipp3* single mutants. In an attempt to generate a double mutant, a level P plasmid was constructed, enabling expression of eight sgRNAs simultaneously. Also incorporated were *pYAO::Cas9-3(intron)::tE9* cassettes, encoded by the level 1 plasmid BCJJ345B, and FastRed selection cassettes ([Shimada et al., 2010](#)). As recommended by [Castel et al. \(2019\)](#), plasmids were constructed such that Cas9 was transcribed in the opposite direction to sgRNAs. BCJJ434A and BCJJ345B plasmids were acquired from the laboratory of Prof. Johnathan Jones, The Sainsbury Laboratory, UK.

Arabidopsis plants were transformed by floral dipping ([2.3.3](#)) and T1 seeds exhibiting red fluorescence were selected and grown for seed collection ([2.4.1](#)). T2 seeds were harvested and the resulting T2 plants genotyped by PCR ([2.4.3](#) and [2.4.4](#)) to identify individuals homozygous for mutations. Any mutant plants used for experimentation originated from T3 seeds or subsequent generations.

2.5 Confocal microscopy

Whole *Arabidopsis* leaves or *N. benthamiana* leaf discs (18 mm diameter) were mounted onto VWR[®] Microscope Slides (631-1552, avantor) with double-sided tape. Images were acquired using the Zeiss LSM800 confocal microscope and Zen software (Blue edition v2.6, Zeiss). Excitation wavelengths of solid state lasers and ranges of detection wavelengths used for visualisation of fluorescent proteins and stains are given in Table [2.9](#).

Table 2.9 – Excitation and detection wavelengths for visualising fluorescent proteins or stains. DAPI = 4',6-diamidino-2-phenylindole.

Fluorescent protein/stain	Excitation wavelength (nm)	Detection wavelength (nm)	
		Minimum	Maximum
Aniline blue	405	410	470
DAPI	405	410	547
GFP / eGFP / mEGFP	488	410	546
mCherry	561	562	617
mRFP	561	546	617
mScarlet	561	563	617
tdTomato	561	560	617

2.5.1 Leaf staining and protein localisation

To visualise callose deposition at PD, immediately prior to imaging, leaves were infiltrated with 0.01%, 0.1% or 1% aniline blue, dissolved in 1x Phosphate-Buffered Saline (PBS) using a blunt 1 ml syringe, then rinsed in H₂O, gently dabbed dry and mounted. Leaves were imaged under a VWR® cover slip (631-0120, avantor) using a 63× (C-Apochromat 63×/1.2 W Corr M27, Zeiss) water-immersion objective.

To visualise nuclei in *O. sativa* leaf sheath epidermal cells (see 2.7), hand-trimmed leaf sheaths were vacuum infiltrated in 4',6-diamidino-2-phenylindole (DAPI) staining solution (10 µg/ml, dissolved in PBS with 0.1% Triton100) using a 5 ml syringe. Samples were left submerged in staining solution for a further 30 min, before being mounted in PBS under a VWR® cover slip (631-0120, avantor), and imaged using a 63× (C-Apochromat 63×/1.2 W Corr M27, Zeiss) water-immersion objective.

Localisation of fluorescent proteins in unstained, non-infected leaves was studied using a 63× (W Plan-Apochromat 63×/1.0 M27, Zeiss), 40× (W Plan-Apochromat 40×/1.0 DIC M27, Zeiss) or 20× (W N-Achroplan 20×/0.5 M27, Zeiss) water-dipping objective len. Image acquisition settings varied depending on the nature and intensity of the signal in question. Post-acquisition addition of scale bars, stacking of Z-slices and adjustment of brightness, contrast and colouring was carried out in Fiji (Schindelin et al., 2012).

2.5.2 Quantification of fluorescent protein accumulation

N. benthamiana leaves were agroinfiltrated (2.3.1) and leaf discs (18 mm diameter) were harvested 2 days post-infiltration. Z-stacks were acquired to cover the entire region of visible fluorescent signal at five locations across a leaf, using consistent acquisition settings throughout

the experiment. Before analysis, images were assigned random numbers using an R script written by Dr Annalisa Bellandi, thereby hiding treatments from the experimenter to eliminate unconscious bias. Maximum intensity Z-projections and measurement of mean grey pixel values across the entire field of view were performed using a custom-built macro in Fiji.

2.5.3 Quantification of protein cell-to-cell mobility

N. benthamiana leaves were agroinfiltrated (2.3.1) and leaf discs (18 mm diameter) were harvested 2 days post-infiltration. Alternatively, detached *Arabidopsis* or *N. benthamiana* leaves were bombarded (2.3.2), and imaging was carried out 1 day post-bombardment, unless otherwise specified. Leaves were scanned and imaged using a 20× (W N-Achroplan 20×/0.5 M27, Zeiss) water-dipping objective. Z-stacks were acquired to cover the entire region of visible fluorescent signal at each transformation site, which consisted of single transformed cell and any surrounding cells into which fluorescent proteins had moved. When scanning leaf discs, all transformation sites encountered were recorded, up to a maximum of 15 or 20 sites per disc. However, sites were rejected if severe damage of fluorescent cells was evident. Laser power, detector gain and interval Z-distance were varied depending on the intensity of the signal in question, but kept consistent within an experiment.

Protein movement was quantified by counting fluorescent cell nuclei. However, due to variation in nuclear signal intensity between treatments, movement of ChEC108-GFP in Figure 5.22 was quantified by counting fluorescent cell outlines. Before analysis, images were assigned random numbers as described in 2.5.2. Counts were performed manually in Fiji using the [Cell Counter plugin](#).

2.5.4 Quantification of pitfield density

Pitfield density was measured using PDLF5-mCherry as a PD marker. *N. benthamiana* leaves were agroinfiltrated (2.3.1) and leaf discs (18 mm diameter) were harvested 2 days post-infiltration. Images were acquired using a 63× (W Plan-Apochromat 63×/1.0 M27, Zeiss) water-dipping objective at arbitrary locations where both PDLF5-mCherry and eGFP signal (from either free eGFP or ChEC108-eGFP) were observed. Z-stacks were acquired to cover the entire region of mCherry signal at a given location, using consistent acquisition settings throughout the experiment.

Image processing and analysis was carried out in Fiji, using a pipeline adapted from [Ganusova et al. \(2020\)](#). First, maximum intensity projections were generated for each Z-stack (Figure 2.2A). The length of visible cell wall was determined by manual tracing (Figure 2.2B), then cell wall area calculated for each image by multiplying the total length by the thickness of the Z-stack

(interval distance $\times$ number of slices-1). Background fluorescence was removed using the background subtraction tool, with a rolling ball radius of 50 pixels. Brightness and contrast for each image were manually adjusted to maximise the intensity of PDLP5-labelled pitfields and minimise background fluorescence. Images were converted to an 8-bit format, and a binary image was generated using the thresholding tool, using intensity limits 93 and 255 (Figure 2.2C). The watershed function was used to separate discrete foci in close proximity. A Fiji macro written by Dr Matthew Johnston, utilising the Analyze Particles tool, was used to quantify the number of foci in each binary image. To assess the efficacy of the script for recognising pitfields, a masked image showing the counted foci (Figure 2.2D) was directly compared to the corresponding maximum projection. Finally, pitfield density for each image was calculated by dividing the number of detected pitfields by the cell wall area.

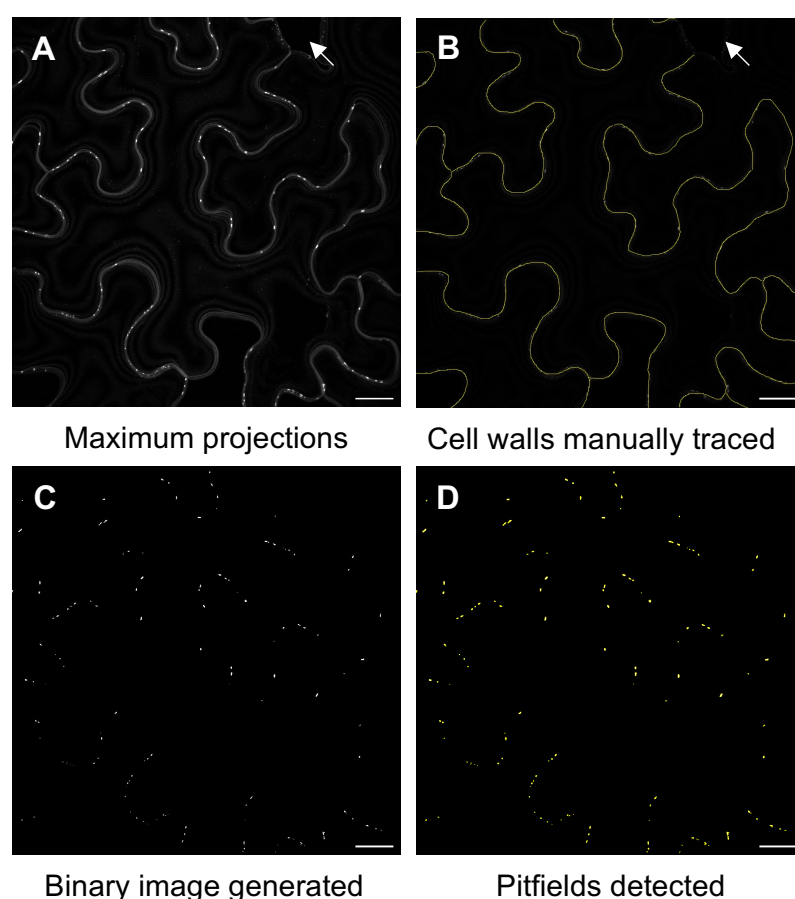


Figure 2.2 – Image analysis pipeline for quantification of pitfield density. A. Z-stacks were acquired and maximum projections generated. B. Cell walls were manually traced and their length measured. C. After manually adjusting brightness and contrast to maximise signal corresponding to pitfields, a binary 8-bit image was generated. D. Pitfields were automatically counted using the Analyze Particles tool in Fiji. Arrow in A and B indicates a guard cell, the cell wall of which was excluded from analysis. Scale bar = 20 μm .

2.6 *Ch* work

2.6.1 *Ch* strains and propagation

For a list of *Ch* strains used and generated in this work, see Table 2.10. The $\Delta Chku80$ strain, here referred to as wild-type (WT), was developed by Korn et al. (2015) and originally derived from the strain CY5535. This mutant lacks ChKu80 function due to replacement of the coding sequence with a nourseothricin resistance cassette. ChKu80 operates as part of the non-homologous end-joining pathway, and its loss-of-function decreases the likelihood of off-target transgene integration. The $\Delta ChEC108$ strain was generated by Dr Andrew Breakspear in the $\Delta Chku80$ background via targeted homologous recombination to replace the coding sequence of *ChEC108* (gene CH63R_09563) with a hygromycin resistance cassette (*HygR*). *Ch* strain IMI 349061 (here referred to as WT²) was acquired from Faulkner laboratory stocks. All other strains were generated via *A. tumefaciens*-mediated transformation of *Ch* as part of this work (see 2.6.2).

Table 2.10 – *Ch* strains. SP = signal peptide, Hyg = hygromycin, NTC = nourseothricin, PPT = L-phosphinothricin. If no source is given, the strain was generated as part of this work.

Strain	Background	Details	Selection	Source
WT	$\Delta Chku80$	-	NTC	Korn, 2016
WT ²	IMI 349061	-	-	Faulkner laboratory stock
$\Delta ChEC108$	WT	-	NTC, Hyg	Dr Andrew Breakspear
$\Delta ChEC108.ChEC108$	WT	<i>pChEC108::SP-ChEC108::tChEC108</i>	NTC, Hyg, PPT	
$\Delta ChEC108.ChEC108^{D164R}$	WT	<i>pChEC108::SP-ChEC108^{D164R}::tChEC108</i>	NTC, Hyg, PPT	
<i>Ch^{SP-ChEC108-eGFP}</i>	WT ²	<i>pcos1gpdA::SP-ChEC108-eGFP::ttrpC</i>	Hyg	
<i>Ch^{SP-ChEC108-Myc-GFP11}</i>	WT ²	<i>pgpdA::SP-ChEC108-Myc-GFP11::ttrpC</i>	Hyg	
<i>Ch^{SP-ChEC108-Myc}</i>	WT ²	<i>pcos1gpdA::SP-ChEC108-4×Myc::ttrpC</i>	Hyg	
<i>Ch^{ChEC108-Myc}</i>	WT ²	<i>pcos1gpdA::ChEC108-4×Myc::ttrpC</i>	Hyg	

Strains were cultured on Mathur's media (MM) (Table S6), a non-selective rich medium to promote sporulation. Plates were sealed with one layer of micropore tape and incubated at room temperature in the dark. Colonies were subcultured by transferring a small plug of mycelia-coated media onto the centre of a fresh MM plate. Occasionally, MM was supplemented with cefotaxime and spectinomycin to select against bacteria (see Table 2.4 for concentrations). To prepare glycerol stocks, spores were harvested from plate-grown colonies

by flooding the plates with H₂O and scraping the surface of the mycelia with a sterile cotton bud. The resulting spore suspensions were filtered using a 5 ml syringe stuffed with sterile cotton wool. Stocks were prepared using a 1:1 ratio (v/v) of spore suspension and 40% glycerol. To revive a strain stored in glycerol, ~10 µl of the stock was dropped onto the centre of an MM plate.

2.6.2 *Ch* transformation

A. tumefaciens (strain AGL1) carrying the plasmids FP06245, FP08082, FP16170, FP16171, FP16245 or FP16253 (see Table S3 for details) were streaked from a frozen glycerol stock onto LB agar plates, supplemented with rifampicin, carbenicillin and kanamycin (Table 2.4). Plates were incubated at 28 °C for 2 days, then 10 ml of liquid LB, supplemented with antibiotics as above, was inoculated using a single colony and incubated at 28 °C, shaking at 200 rpm for 16-24 h. Bacteria were pelleted by centrifuging at 3000 xg for 10 min at room temperature, then resuspended in glycerol induction (GI) broth (Table S6), and diluted to an OD of 0.4.

WT, WT² or $\Delta ChEC108$ strains (Table 2.10) were revived from glycerol stocks and cultured for 2-3 weeks before spore harvest, as described in 2.6.1 with the exception that GI broth was used to suspend the spores in place of H₂O. Spores and bacteria were mixed in 1:1 ratio (v/v) and 100 µl aliquots were spread onto sterile WhatmanTM mixed cellulose ester membrane filter discs (47 mm diameter, 7141-114, Cytiva), atop GI agar (Table S6). On these plates, spores and bacteria were co-cultivated at room temperature in the dark for 2 days, then the filter discs were inverted and laid onto fresh potato dextrose agar (PDA) plates (Table S6), supplemented with hygromycin or PPT to select for the plasmid of interest, and cefotaxime and spectinomycin to select against bacteria (see Table 2.4 for concentrations). After 2 days of dark incubation at room temperature, filter discs were removed and incubation continued for up to 2 weeks, or until fungal colonies emerged. Emerging colonies were sub-cultured onto fresh PDA plates, supplemented with antibiotics as above, and incubated for a further 2 weeks.

Given that colonies selected using the method above could potentially originate from a cluster of *Ch* spores, it was necessary to obtain colonies derived from single spores only. To achieve this, spores were harvested from transformants (2.6.1). 10-fold dilutions were prepared (10⁻¹, 10⁻², 10⁻³ and 10⁻⁴) and 100 µl of each dilution was spread onto PDA plates supplemented with hygromycin or PPT (Table 2.4). After 3 days of dark incubation at room temperature, the first emerging single colonies were transferred to fresh PDA plates, again supplemented with hygromycin or PPT. Incubation of these single-spored transformants continued for up to 2 weeks, then spores were harvested and glycerol stocks prepared (2.6.1).

2.6.3 gDNA extraction from *Ch* mycelia

To obtain material for gDNA extraction, 100 ml of potato dextrose broth (PDB) (Table S6) was inoculated with two ~25 mm² mycelial plugs, removed from the leading edge of a plate-grown *Ch* colony. Cultures were incubated at 27 °C, shaking at 100 rpm for 7 days. Mycelia were harvested by filtering cultures through two layers of miracloth (475855, Millipore), and rinsing with 100 ml of sterile H₂O. Mycelia were dabbed dry and flattened using layers of 2-ply blue paper and WhatmanTM filter paper (3030-917, Cytiva), before being frozen in liquid nitrogen and ground using a pestle and mortar. The resulting fine powder was divided into ~100 mg aliquots and stored at -70 °C. gDNA was extracted from aliquots using a DNeasy Plant Mini Kit (QIAGEN), according to the manufacturer's instructions.

2.6.4 Protein extraction from *Ch* mycelia

To obtain material for protein extraction, mycelia were grown in liquid culture and harvested as described in 2.6.3. Before grinding took place, prior to protein extraction, mycelial tissue was lyophilised at -55 °C overnight, using a LyotrapTM bench-top freeze dryer (LTE Scientific). When dry, mycelia were ground to a fine powder using a pestle and mortar, then resuspended in 5 ml protein extraction buffer (Table S2) and vortexed thoroughly. Each sample was divided into three 2 ml eppendorfs each containing one glass bead (3 mm, Z143928, Sigma-Aldrich), and rotated end-over-end at 4 °C for 30 min. Extracts were centrifuged at 21,130 xg for 10 min at 4 °C and the supernatant retained in a fresh eppendorf. This centrifugation step was repeated a further one or two times to maximise the removal of debris. Extracts could then be used for immunoprecipitation (IP) and/or Western blotting, as described in 2.8 for plant material.

2.6.5 *Ch* infection assays on Arabidopsis

Ch strains were revived from glycerol stocks and cultured for 2-3 weeks before spore harvest (2.6.1). Spore concentration was calculated using a haemocytometer with Neubauer rulings (Bright-LineTM Hemocytometer, Z359629, Sigma-Aldrich), and dilutions were carried out to ensure an equal concentration of spores for each strain within an experiment, ranging from 8.6x10⁵ - 2.0x10⁶ spores/ml. Concentrations of spores used for individual experiments and replicates can be found in Table S12.

Plant material for virulence assays was grown under short-day conditions as described in 2.2.2. Mature Arabidopsis rosette leaves were detached and secured on water agar (WA) plates (Table S6), with up to 10 leaves secured per plate. Avoiding the midrib, 3 µl droplets of spore suspension were applied to each leaf. Plates were sealed with at least two layers of parafilm to maintain humidity and incubated at 22 °C with a 10 h light/14 h dark photoperiod. To

prevent excessive light-induced stress of the fungus, plates were covered with one layer of 2-ply blue paper. Leaves were monitored for up to 7 days post-inoculation (dpi), and lesions were photographed using a digital camera in the region of 4-7 dpi, depending on rate of infection progress. Necrotic lesion areas were measured manually using Fiji. Lesions were excluded from analysis if smaller than 5 mm² (suggesting infection was not established) or if curling of the leaf impaired measurement.

2.6.6 *Ch* infection of *Arabidopsis* for protein extraction

To test for effector uptake in *Ch*-infected *Arabidopsis*, plant lines constitutively expressing *GFP1-10-TurboID* (ET011), *3×FLAG-TurboID-HIPP6* (ET015) or *3×FLAG-TurboID-HMA1* (ET016) or Col-0 plants as a control (Table S8), were grown on soil under short-day conditions to yield mature rosettes, or as lawns of seedlings on sterile plates (2.2.2 and 2.2.3). *Ch* colonies expressing *SP-ChEC108-Myc-GFP11* (where SP indicates inclusion of the native signal peptide), *SP-ChEC108-4×Myc*, *ChEC108-4×Myc*, or WT² *Ch* as a control (see Table 2.10), were cultured for 2-3 weeks before spore harvest (2.6.1). Spore concentration was calculated using a haemocytometer with Neubauer rulings (Bright-Line™ Hemocytometer, Z359629, Sigma-Aldrich). 3-25 ml dilutions of spores were prepared, depending on the inoculation method and scale of the experiment. Spore concentration varied between experiments, ranging from 2.73-6.55×10⁶ spores/ml.

To inoculate detached rosettes leaves or seedlings, spores were applied evenly across the adaxial surface using a 30 ml spray bottle or an airbrush (Silverline 380158). Spore-covered mature leaves were arranged on solid WA plates sealed with at least two layers of parafilm, whereas seedlings were sprayed and re-sealed in their original GM-GLU agar plates using two layers of parafilm or micropore tape. Plates were transferred to a growth cabinet at 22 or 24 °C with a 10 h light/14 h dark photoperiod and covered with one layer of 2-ply blue paper. 24-97 h post-infection, leaves/seedlings were removed from plates and vacuum infiltrated with 50 or 100 µM biotin solutions, or sterile H₂O as a control, then left submerged on an orbital shaker for 3-24 h. Leaves/seedlings were washed 1-4 times in 4 °C H₂O to remove excess biotin from the leaf surface, then dabbed dry using 2-ply blue paper, or air-dried on a bed of Whatman™ filter paper (3030-917, Cytiva). Samples were frozen in liquid nitrogen and stored at -70 °C, before proceeding with protein extraction, IP and Western blotting (see 2.8). For details on the plant age, genotype combinations, spore concentrations, time points and biotin treatment durations used in individual experiments, see Table 6.1.

2.6.7 *Ch* infection of *Arabidopsis* for microscopy

Col-0 *Arabidopsis* plants were grown on soil under short-day conditions (2.2.2). *Ch* colonies expressing *SP-ChEC108-eGFP* (Table 2.10) were cultured for 2-3 weeks before spore harvest (2.6.1). Spore concentration was calculated using a haemocytometer with Neubauer rulings (Bright-Line™ Hemocytometer, Z359629, Sigma-Aldrich), then dilutions were prepared, ranging from 1×10^5 to 3×10^6 spores/ml. Rosette leaves were detached and secured on WA plates. Avoiding the midrib, 1-3 μ l droplets of spore suspension were applied to cover the leaf adaxial surface. Alternatively, spores were applied evenly across the adaxial surface of leaves destined for trypan blue staining using an airbrush (Silverline 380158). Plates were sealed with at least two layers of parafilm, covered with one layer of 2-ply blue paper, and incubated at 22 or 24 °C, with a 10 h light/14 h dark photoperiod.

To image fluorescent strains in planta 1, 2, 3 and 4 dpi, leaves were mounted adaxial side-up in H₂O and imaged under a VWR® cover slip (631-0120, avantor) using a Zeiss LSM800 confocal microscope equipped with a 63 $\times$ (C-Apochromat 63 $\times$ /1.2 W Corr M27, Zeiss) water-immersion objective (2.5).

To stain infection structures for brightfield microscopy 1, 2, 3, 4 and 5 dpi, leaf discs (6 mm diameter) were harvested and boiled with trypan blue staining solution (Table S2) at 95 °C for 2.5 min, while shaking at 500 rpm. Leaves were submerged in staining solution for 30 min at room temperature, then rinsed with H₂O and destained in a 5:2 ratio (w/v) chloral hydrate:water solution with gentle agitation on an orbital shaker. After 1.5 h, the chloral hydrate solution was replaced, and destaining continued for at least 2 days. When sufficiently destained, leaf discs were mounted in 40% glycerol under a VWR® cover slip (631-0120, avantor) and imaged using a Zeiss Axio Imager Z2 with Zen software (Blue edition v2.6, Zeiss), equipped with a 40 $\times$ (EC Plan-Neofluar 40 $\times$ /0.75, Zeiss) air-immersion objective and AxioCam 712 color camera (Zeiss). To compensate for differing levels of background staining, pre-image acquisition, white balance was adjusted using the 'pick' function to select a white point for each leaf disc. Brightfield images were acquired as Z-stacks, and post-acquisition adjustment of brightness, contrast, scale bars and generation of minimum intensity Z-projections were carried out in Fiji.

2.7 *Magnaporthe oryzae* work

2.7.1 *M. oryzae* transformation and culturing

Protoplast-mediated transformation of *M. oryzae* (as described in Talbot et al., 1993) (strain Guy11) with plasmids FP16257, FP16258 or FP16261 (see Table S5) was conducted by Weibin

Ma and Jave Bautista in the laboratory of Prof. Nick Talbot, The Sainsbury Laboratory, UK. Monitoring and selection of the resulting PPT-resistant, or sulfonylurea-resistant transformants was performed by Jave Bautista.

For inoculation of *O. sativa*, *M. oryzae* strains were sub-cultured onto complete media (CM) agar plates (Table S6) and incubated for 6-8 days at 26 °C with a 12 h light/12 h dark photoperiod. To obtain filter stocks, colonies were subcultured onto CM agar plates scattered with sterile ~ 2 mm² WhatmanTM filter paper pieces (3030-917, Cytiva) and incubated for ~2 weeks at 26 °C with a 12 h light/12 h dark photoperiod, allowing time for the colony to fully cover the plate. Filter paper pieces were placed in sterile glassine bags and left to dehydrate in an airtight container with silica beads for at least 2 weeks at room temperature, before being transferred to -20 °C for long-term storage.

2.7.2 Imaging *M. oryzae* infection of *Oryza sativa*

Spores were harvested from 6-8-day-old *M. oryzae* colonies and suspended in 1 ml H₂O by gently scraping the surface of the colony with a blunted 1 ml pipette tip. To remove mycelia, the suspension was filtered through one layer of miracloth (475855, Millipore). As a wash step, spores were pelleted by centrifugation at 1200 xg for 5 min at room temperature, then resuspended in 1-2 ml H₂O, depending on the density of the pellet. Spore concentration was assessed using a haemocytometer with Neubauer rulings (Bright-LineTM Hemocytometer, Z359629, Sigma-Aldrich), and dilutions carried out to ensure a final concentration of < 1.25 x10⁵ spores/ml. Leaf sheaths were isolated by carefully peeling leaves off 3-5-week-old *O. sativa* cv. CO39 seedlings, then were trimmed to remove roots. The resulting hollow sheaths were filled with ~200 µl spore suspension. Up to ten inoculated leaf sheaths were rested on empty 0.2 ml tube strips within a square petri dish, lined with damp 2-ply blue paper to increase humidity, and incubated at 28 °C in the dark for 26-27 h.

In preparation for confocal imaging, with the help of Jave Bautista, inoculated leaf sheaths were trimmed by hand to remove green tissue, leaving only a thin layer of cells from the inner (inoculated) surface of the leaf sheath. Samples were mounted epidermis-side-up in water and imaged under a VWR[®] cover slip (631-0120, avantor) using a 63× (C-Apochromat 63×/1.2 W Corr M27, Zeiss) water-immersion objective (2.5). To visualise host nuclei, samples were DAPI-stained prior to imaging (2.5.1).

2.8 Co-immunoprecipitation (co-IP) and Western blotting

2.8.1 Protein extraction from plant material

To extract protein from *N. benthamiana*, six leaf discs (8 mm diameter) were harvested from a minimum of three leaves, 2-3 days post-infiltration. To extract protein from *Arabidopsis* for genotyping (2.4.4), nine leaf discs (6 mm diameter) were harvested from a minimum of three rosette leaves. Samples were frozen in liquid nitrogen with two glass beads (3 mm, Z143928, Sigma-Aldrich) and ground to a fine powder using a 2010 Geno/Grinder (SPEX SamplePrep), shaking at 1200 rpm for 1 min. Samples were vigorously vortexed with 500 μ l of protein extraction buffer (Table S2), then rotated end-over-end at 4 °C for 30 min. Extracts were centrifuged at 21,130 xg for 10 min at 4 °C and the supernatant was transferred to a fresh eppendorf. This centrifugation step was repeated to ensure complete removal of cell wall fragments.

To extract protein from biotin-treated, *Ch*-infected *Arabidopsis* leaves or seedlings (2.6.6), tissue was ground manually using a pestle and mortar. The resulting powder was vigorously vortexed with 1-24 ml of protein extraction buffer, then rotated end-over-end or placed on a rocking shaker at 4 °C for 0.5-1.5 h. Extracts were centrifuged at 30,000 xg for 30 min at 4 °C to pellet debris and the supernatant was transferred to a fresh vessel. For samples of large volume, an additional 'soft' spin at 7,082 xg for 30 min at 4 °C was performed before the spin at 30,000 xg took place.

2.8.2 Co-IP

32 μ l of protein extract (2.8.1) was set aside as the input fraction, and the remainder of the extract carried forward for IP. To enrich for GFP-tagged proteins, 20 μ l of GFP-Trap[®] magnetic agarose beads (Chromotek) were prepared per sample by washing four times in 500 μ l of protein extraction buffer. Magnetic beads were drawn to one side using a magnetic rack, allowing removal of the buffer by gentle inversion of the tubes after each wash step. To enrich for Myc-tagged proteins, 80-200 μ l of Pierce[™] anti-c-Myc agarose beads (Thermo Scientific, corresponding to 20-50 μ l of settled resin) were prepared per sample by washing four times in 200 μ l of protein extraction buffer. Between wash steps, resin was pelleted by centrifugation at 12,000 xg for 10 s at 4 °C, and the supernatant was discarded by pipetting. Pre-prepared beads and protein extracts were combined and rotated end-over-end at 5 °C for 2-5 h, or overnight. After, the beads were washed five times in 500 μ l of protein extraction buffer. Each wash involved rotating the tubes end-over-end at 5 °C for 5 min. As above for magnetic beads, a magnetic rack was used to enable removal of buffer after each wash step, while agarose beads

were pelleted by centrifugation at 4 °C: 12,000 xg for 10 s, or 1,500 xg for 5 min initially, if IP was carried out in 50 ml falcon tubes. Finally, beads were resuspended in 30 µl of protein extraction buffer.

2.8.3 SDS-PAGE for Western blotting

8-10 µl of SDS-PAGE loading buffer-1 (Table S2) was added to protein extracts (2.8.1 and 2.8.2), then mixtures were heated at 95 °C for 15 min with agitation (1000 rpm). 10 µl of heated samples were loaded into 10% or 15% stacking acrylamide gels with 1 mm thickness (Table 2.11) alongside PageRuler™ Plus pre-stained protein ladders (Thermo Scientific). Gels were cast using Mini-PROTEAN® Tetra Handcast Systems (BIO-RAD) and electrophoresis was carried out in 4-gel vertical electrophoresis tanks (165-8001, BIO-RAD). Gels were submerged in running buffer (Table S2) and run at 70 V for 15 min, then at 100-110 V until the loading dye front reached the bottom of the resolving gel.

Table 2.11 – Composition of SDS-PAGE gels. Gel components were dissolved in H₂O. Percentages are defined as volume / volume of solution. APS = ammonium persulfate. TEMED = tetramethylethylenediamine.

Component	Final concentration		
	10% gel (base)	15% gel (base)	Stacking layer
Acrylamide	10.00%	15.00%	5.00%
Bisacrylamide	0.27%	0.40%	0.13%
Tris-HCl (pH 8.8)	0.39 M	0.39 M	-
Tris-HCl (pH 6.8)	-	-	0.13 M
SDS	0.10%	0.10%	0.10%
APS	0.10%	0.10%	0.10%
TEMED	0.10%	0.10%	0.10%

2.8.4 Western blotting

Proteins were transferred onto activated polyvinylidene difluoride (PVDF) membranes using the Mini Trans-Blot® Cell transfer system (BIO-RAD), which required the SDS-PAGE gel and membrane to be sandwiched between four Whatman™ filter paper pieces (3030-917, Cytiva) and two outer sponge layers. Before assembling cassettes, PVDF membrane pieces were submerged briefly in 100% methanol. Sponges and filter paper pieces were pre-soaked in ice-cold transfer buffer (Table S2), and SDS-PAGE gels were washed briefly in transfer buffer to remove excess SDS. Assembled cassettes were submerged in transfer buffer, and the transfer took place at 5 °C, 30 V overnight, or at 100 V for 1.5-2 h.

Following a transfer, membranes were incubated in blocking buffer (Table S2) for 1-3 h at room temperature, followed by primary antibody solution at 5 °C. Antibody dilutions and incubation durations are detailed in Table 2.12. Membranes were washed three times in blocking buffer (5-10 min duration for each, at room temperature), then, if applicable, incubated in a secondary antibody at room temperature. As an exception to the above, when using streptavidin-horseradish peroxidase (Strep-HRP) conjugates (S911, Invitrogen) for detection of biotinylated proteins, membranes were blocked and antibodies diluted in 2% bovine serum albumin (BSA), thus avoiding excessive antibody depletion as a result of biotin presence in the blocking buffer.

Prior to blot development, membranes were washed three times (5-10 min duration for each, at room temperature) in Tris-buffered saline with Tween-20 (TBST) (Table S2). HRP-conjugated antibodies were visualised using a SuperSignal™ West Femto Trial Kit (Thermo Scientific). Luminol and stable peroxide chemiluminescent detection reagents were mixed in 1:1 ratio (v/v) immediately before imaging. 300 µl of the reagent mix was applied per membrane, and chemiluminescence visualised using an Amersham™ ImageQuant 800 CCD Imager. Total protein loading was observed using Coomassie Blue (CB) staining solution, followed by a 5-15 min incubation in CB de-staining solution (Table S2).

Table 2.12 – Antibodies used in this work. Details of antibody-conjugated magnetic or agarose beads for IP, and primary (1°) and secondary (2°) antibodies used for Western blotting are given.

	Antibody	Source	Catalogue number	Dilution	Incubation duration (h)
-	GFP-Trap® Magnetic beads	ChromoTek	gtma-20	-	2
-	Pierce™ Anti-c-Myc Agarose beads	Thermo Scientific	20168	-	2-5
1°	α-FLAG-HRP	abcam	ab49763	1/5000	12-24
1°	α-GFP (Mouse)	Roche	11814460001	1/1000	12-24
1°	α-Myc-HRP	abcam	ab62928	1/5000	12-24
1°	α-RFP (Rabbit)	abcam	ab34771	1/2000	12-24
1°	Strep-HRP	Invitrogen	S911	1/3000	12-24
2°	α-Mouse-HRP	Sigma-Aldrich	A0168	1/10,000	6
2°	α-Rabbit-HRP	Sigma-Aldrich	A0545	1/20,000	6

2.9 RNA work

2.9.1 RNA extraction from Arabidopsis

2-4 rosette leaves were harvested from one plant per sample (biological replicate), equating to 50-100 mg of fresh weight. Samples were frozen in liquid nitrogen with one glass bead (3 mm, Z143928, Sigma-Aldrich) and ground to a fine powder using a 2010 Geno/Grinder (SPEX SamplePrep), shaking at 1200 rpm for 1 min. RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN), using buffer RLT according to the manufacturer's instructions with the following modification: after tissue homogenisation using the QIAshredder spin column, 2-3 centrifugation steps were performed to maximise removal of debris. RNA was eluted from RNeasy spin columns in 30 µl of H₂O, and concentration and purity were assessed using a NanoDropTM One/One^C Microvolume UV-Vis Spectrophotometer. To remove contaminating DNA, RNA extracts were DNase-treated using the TURBO DNA-freeTM Kit (Invitrogen) according to the manufacturer's 'rigorous' procedure. Concentration and purity were assessed as above, before extracts were aliquoted and stored at -70 °C.

2.9.2 Reverse Transcription quantitative (RT-q)PCR

cDNA synthesis reactions using RNA extracts of equal concentration (2.9.1) were carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random primers according to the manufacturer's instructions. cDNA samples were diluted 1/10 in H₂O before 2 µl were added to 10 µl qPCR reactions with the following composition: LightCycler[®] 480 SYBR Green I Master Mix (5 µl, Roche), forward primer (1.5 µl, 10 µM), reverse primer (1.5 µl, 10 µM). Primers used for RT-qPCR are listed in Table S10. For each sample (genotype), three biological replicates (2.9.1) were included, and reactions were carried out in triplicate (technical replicates) in white LightCycler[®] Multiwell 384-well plates (Roche), using a LightCycler[®] 480 II Instrument (Roche). Cycle conditions are detailed in Table 2.13. Analysis of the resulting melting curves indicated that each primer pair generated a single product. Crossing point (Cp) values were calculated by the LightCycler[®] 480 Instrument software version 1.5, using the second derivative maximum method.

Table 2.13 – RT-qPCR thermocycler protocol.

Step		Temperature (°C)	Duration (min:s)	
Pre-incubation		95	05:00	
Amplification	Denaturation	95	00:10	45 cycles
	Annealing	60	00:10	
	Elongation	72	00:10	
Melting	Denaturation	95	00:05	^a
	Melting	65-97	04:51	
	Cooling	40	00:30	

^a Steadily increasing temperature by 0.11 °C/s to enable analysis of PCR product melt curves.

Cp values were processed to obtain relative expression levels according to the model outlined by Pfaffl (2001). Mean Cp values were calculated for each biological replicate, and Δ Cp was calculated by subtracting an arbitrary calibrator value, that is, the minimum Cp value obtained across all means for a given gene. Δ Cp values were transformed into relative quantities (RQs) using the exponential function with PCR efficiency (assumed to be 2, indicating 100% efficiency) as the base. RQs for the gene of interest (*HIPP6*) were normalised for each biological replicate by dividing by the corresponding RQ for the reference gene (*UBQ10*).

2.10 In vitro protein work

2.10.1 Protein production in *E. coli*

Proteins were expressed recombinantly in *E. coli* with cleavable N-terminal hexahistidine (6×His) affinity tags (Figure 2.3) to facilitate subsequent purification by (IMAC, 2.10.2). To achieve this, protein sequences of interest were cloned into pPGN-C, a Golden Gate level 1 backbone derived from pOPIN-F (Bentham et al., 2021b). When conducting protein expression tests, a cleavable solubility tag consisting of the B1 domain of Streptococcal protein G (GB1) preceded the 6×His sequence. For protein co-purification, one component of the complex of interest was expressed without an affinity tag. To achieve this, the coding sequence was cloned into pPGC-K, a Golden Gate level 1 backbone derived from pOPIN-A (Bentham et al., 2021b). For a full list of plasmids used for protein production, see Table S3.

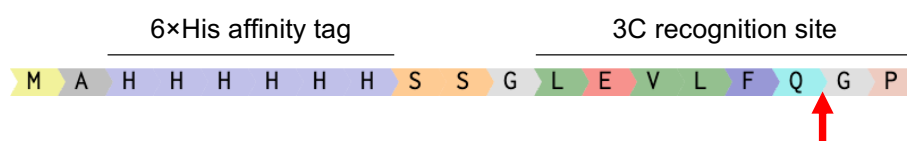


Figure 2.3 – Cleavable N-terminal 6×His tag for IMAC. The tag is preceded by a 3C protease cleavage site. Red arrow indicates the site of cleavage, leaving a Gly-Pro scar on the recombinant protein.

Plasmids were transformed into BL21- AITM One Shot[®] *E. coli* (Invitrogen, hereafter BL21-AI) or SHuffle (Lobstein et al., 2012) *E. coli* expression strains (2.1.4). 50 ml of liquid LB starter cultures, supplemented with appropriate antibiotics to select for plasmids of interest, plus chloramphenicol to select for BL21-AI cells if necessary (Table 2.4), were inoculated using a scrape from a frozen glycerol stocks and incubated overnight at 37 °C, shaking at 200 rpm. Liquid LB, supplemented with antibiotics and divided into 1 L aliquots in 2 L flasks, was inoculated with starter culture (5 ml/L), then incubated at 37 °C or 30 °C for BL21-AI or SHuffle cells, respectively, shaking at 200 rpm. When the OD was in range of 0.6-0.8, the incubation temperature was decreased to 18 °C and protein expression was induced by adding 0.2% arabinose or 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for BL21-AI or SHuffle cells, respectively. Induction was left to proceed for 17-24 h.

2.10.2 Protein purification from *E. coli* cell lysate

E. coli cells were harvested by centrifugation at 7500 xg for 10 min at 4 °C. Pellets were resuspended in 200 ml of A1 buffer (Table S2), supplemented with cOmpleteTM EDTA-free protease inhibitor cocktail tablets (Sigma-Aldrich). Cells were lysed by sonication (50% amplitude, 1s on/3 s off, 8 min pulse duration) using a Vibra CellTM VC505, equipped with a 13 mm probe (SONICS). Lysate was centrifuged at 45,000 xg for 30 min at 4 °C to obtain soluble protein, then the supernatant was loaded onto ÄKTAexpress for IMAC using 1 ml or 5 ml HisTrapTM HP NTA columns (Cytiva), equilibrated in A1 buffer. After elution with B1 buffer (Table S2), proteins were separated by gel filtration using a SuperdexTM 75 HiLoadTM 26/600 column (Cytiva), equilibrated in A4 buffer (Table S2). Fractions containing desired proteins were identified using SDS-PAGE (see 2.10.3), pooled and incubated with 6 $\times$ His-tagged human rhinovirus 3C protease (10 ug/mg of desired protein) for 12-16 h.

3C protease and cleaved affinity tags were removed by IMAC using 5 ml HisTrapTM columns, equilibrated in A4 buffer. Fractions containing desired proteins were identified using SDS-PAGE, pooled, then concentrated to 5 ml using VivaSpin[®] 20 ml concentrators (Sartorius) with a 3 kDa, 10 kDa, or 30 kDa molecular weight cut-off, depending on the size of the proteins of interest. Samples were loaded onto ÄKTAexpress for a second gel filtration step using SuperdexTM 75 HiLoadTM 16/600 columns (Cytiva), equilibrated in A4 buffer. Fractions containing desired proteins and minimal contamination were identified using SDS-PAGE, pooled and concentrated as above, then frozen in liquid nitrogen as 50 μ l aliquots and stored at -70 °C. Purification details for individual proteins can be found in Chapter 3.

2.10.3 SDS-PAGE for protein purification

2.5 µl of 4x SDS-PAGE loading buffer-2 (Table S2) was added to up to 7.5 µl of each protein sample, and mixtures were heated at 95 °C for 10 min. Up to 10 µl of heated samples were loaded into 15% stacking acrylamide gels with 1 mm thickness (Table 2.11) alongside Prestained Protein Ladders (ab116029, abcam). Gels were cast using Mini-PROTEAN® Tetra Handcast Systems (BIO-RAD) and electrophoresis was carried out in 4-gel vertical electrophoresis tank (165-8001, BIO-RAD). Gels were submerged in Tris/Glycine buffer (1610734, BIO-RAD) and run at 100 V for approximately 2 h, until the loading dye front reached the bottom edge of the resolving gel. Gels were stained in ReadyBlue™ Protein Gel Stain (RSB-1L, Sigma-Aldrich) while gently shaking at room temperature for 12-16 h, then destained by gently shaking in water for ~24 h. Stained gels were photographed using a GeneSys G:Box (Syngene).

2.10.4 Intact mass spectrometry

Intact mass spectrometry analysis was carried out by Dr Carlo Martins and Dr Gerhard Saalbach as part of the John Innes Centre Proteomics Platform. Spectra were obtained using a Synapt G2-Si mass spectrometer, coupled to an Acquity UPLC system for electrospray ionisation (Waters).

2.10.5 Protein crystallisation

Crystallisation experiments were conducted using the sitting drop vapour diffusion method, which involves mixing 0.3 µl of purified protein with 0.3 µl of precipitant solution, and sealing the resulting drop alongside a 40 µl reservoir of concentrated precipitant solution. Over time, evaporation of water from the drop concentrates the protein and precipitant within, encouraging the formation of nucleation points from which crystals can develop. Initial crystallisation trials were conducted using a variety of commercially available screens (Table 2.14), pre-dispensed into the reservoirs of Swissci 96-Well 2-Drop Plates (Molecular Dimensions) by the John Innes Centre Structural Biology Platform.

Using a two-drop setup enabled two protein concentrations to be tested simultaneously (for details, see Figures 3.16 to 3.18). Before loading onto crystal trays, proteins were defrosted on ice and centrifuged at 21,000 xg for 10 min at 4 °C to pellet any precipitants. Proteins were mixed with reservoir solutions and dispensed in sitting drops using the Oryx8 Protein Crystallization Robot (Douglas Instruments). Plates were immediately sealed using a ClearVue (UV light-permeable) adhesive seal (Molecular Dimensions) and stored at 20 °C. Trays were monitored periodically by imaging under visible and UV light, using the extended focus capabilities of a Rock Imager® (Formulatrix).

Crystals estimated to have a suitable morphology for X-ray diffraction were cryoprotected using 20% ethylene glycol or a solution of 10 % glycerol and 10% ethylene glycol, dissolved in precipitant solution from the relevant reservoir, then mounted onto X-ray-transparent loops and immediately frozen in liquid nitrogen by Julia Mundy. As an exception, crystals harvested from the Morpheus screen or custom optimisation plates were sufficiently cryoprotected by their precipitant solution, so additional cryoprotectants were not added.

Table 2.14 – Broad range crystallisation screens used in this study.

Screen	Source
Keep it Simple (KISS)	Dr Clare Stephenson, John Innes Centre
Morpheus	Molecular Dimensions
PEGs Suite	QIAGEN
ProPlex TM	Molecular Dimensions
ShotGun TM (SG1)	Molecular Dimensions
MIDASplus TM	Molecular Dimensions
JCSG-plus TM	Molecular Dimensions

2.10.6 X-ray diffraction data collection and processing

X-ray diffraction data were collected from crystals at beamline i04 and i24 of the Diamond Light Source synchrotron facility (Oxford, UK), driven remotely by Prof. David Lawson and Julia Mundy. Data were auto-processed via the *xia2* dials pipeline ([Winter, 2010](#)), accessed via the [Diamond ISPyB](#) website. Scaled, unmerged data were imported into ccp4i2 v.8.0 ([Agirre et al., 2023](#)) and processed using *AIMLESS* ([Evans and Murshudov, 2013](#)). Molecular replacement was carried out in *Phaser* ([McCoy et al., 2007](#)), followed by automatic model building with *Bucaneer* ([Cowtan, 2006](#)). Further manual building and refinement were carried out in the Crystallographic Object-Oriented Toolkit (*Coot*) ([Emsley et al., 2010](#)). Then, cycles of manual refinement and automatic refinement in *REFMAC5* ([Murshudov et al., 2011](#)) were carried out. For more details on data collection and processing steps for individual crystal structures, see Chapter 3.

2.10.7 Structure prediction and visualisation

Protein structure predictions were carried out using AlphaFold2 ([Jumper et al., 2021](#)), AlphaFold2 Multimer ([Evans et al., 2022](#)) or AlphaFold3 ([Abramson et al., 2024](#)). AlphaFold2 was accessed via the Google Colaboratory and ColabFold ([Mirdita et al., 2022](#)), and AlphaFold3 via the [AlphaFold Server](#). Predicted Aligned Error (PAE) data were visualised using an interactive web tool, [PAE Viewer](#) ([Elfmann and Stülke, 2023](#)). Predicted models and crystal structures were analysed and annotated using ChimeraX versions 1.5 through 1.9 ([Pettersen et al., 2021](#)).

2.10.8 Analytical gel filtration

To test for complex formation in vitro, proteins of interest were purified from *E. coli* ([2.10.1](#) and [2.10.2](#)) and diluted to generate 2x concentrated stocks at equimolar concentrations. For initial trials on the ÄKTA purifier system, equipped with a SuperdexTM 75 Increase 10/300 GL column (Cytiva, equilibrated in A4 buffer), proteins (120 µM each) were incubated on ice for 1.5 h, either in the presence or absence of their binding partner. 125 µl samples were loaded via a Hamilton glass syringe and 200 µl loop, and gel filtration carried out at 4 °C. For subsequent trials on the ÄKTA pure system, equipped with a SuperdexTM 75 Increase 5/150 GL column (Cytiva, equilibrated in A4 buffer), proteins (250 µM each) were incubated on ice for 1 h, either in the presence or absence of their binding partner. 90 µl samples were loaded via a Hamilton glass syringe and 50 µl loop, and gel filtration carried out at 4 °C. Column eluate was monitored by measuring absorbance at 280 nm and fraction contents was examined via SDS-PAGE ([2.10.3](#)).

2.10.9 Isothermal titration calorimetry (ITC)

To study the thermodynamics of in vitro interactions, proteins of interest were purified from *E. coli* (2.10.1 and 2.10.2) and loaded into Slide-A-Lyzer[®] MINI dialysis devices (Thermo Scientific) filled with A4 buffer, and left to dialyse on a gentle rocking shaker overnight. Proteins were diluted to desired concentrations before loading into the experimental cell (250 µl) or syringe (50 µl) of a MicroCal PEAQ-ITC machine (Malvern, UK). All experiments were conducted under the conditions listed in Table 2.15. Raw titration data were uploaded to AFFINIMeter (Piñeiro et al., 2019), where datasets were integrated, noise was removed, baselines were corrected and binding parameters calculated.

Table 2.15 – ITC experimental conditions.

Condition	Value
Temperature (°C)	25
Reference power (µcal/s)	10
Feedback	High
Stir speed (rpm)	750
Initial delay (s)	60
Injection volume (injection 1) (µl)	0.4
Injection volume (injections 2-18) (µl)	2
Injection spacing (s)	150
Injection duration (s)	4

2.11 Statistics and data presentation

Statistical analysis was carried out in R (R core team). Details of statistical tests conducted for individual experiments can be found in relevant figure legends. Data were visualised using the R package, ggplot2 (Wickham, 2011). Tables in this thesis were prepared with the aid of LaTeX Tables Editor.

Chapter 3

Discovery and structural characterisation of the ChEC108-HIPP6 complex

3.1 Introduction

Work in this chapter builds on results obtained by Dr Joanna Jennings, introduced in section 1.4.1. Initially, I was motivated to understand the mechanism by which the putative *Ch* effector ChEC108 might manipulate cell-to-cell communication in plants. A BLAST-based search for homologous proteins in the NCBI database yielded a short list of 'hypothetical' or 'uncharacterised' proteins, all from *Colletotrichum* species. Besides *Ch*, candidate orthologs were identified in *C. destructivum*, *C. shioi* and *C. tanacetii* - all fungal anthracnose pathogens with distinct host ranges. After scanning the primary sequence for known domains, [InterPro](#) (ver. 106.0) ([Blum et al., 2025](#)) was unable to assign ChEC108 to an existing protein family. Thus, based on sequence data alone, it was difficult to generate hypotheses regarding ChEC108 function.

Genes encoding putative effectors are known to evolve rapidly with respect to the wider genome ([Huang et al., 2014](#); [Sánchez-Vallet et al., 2018](#)). As a result, effector families are generally large and sequence diverse. However, recent advances in experimental and predictive structural biology have uncovered a number of conserved folds within phytopathogen effectomes, defining effector families with sequence-diverse members, perhaps sharing similar host targets and virulence mechanisms (for reviews, see [Le Naour-Vernet et al., 2025](#); [Outram et al., 2022](#)). A diverse array of experimentally-determined effector structures are displayed in Figure 3.1A-H, exemplifying some of these conserved folds.

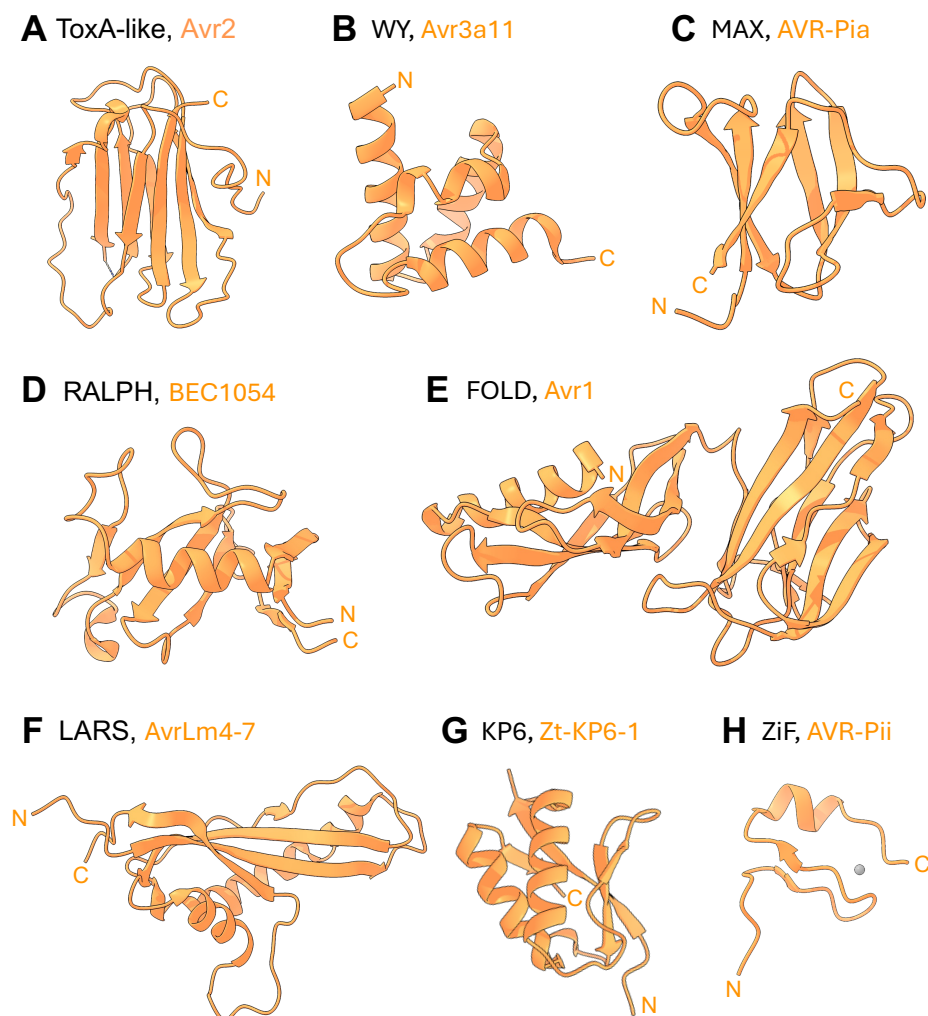


Figure 3.1 – Representative members of effector structural families from plant pathogens. **A.** ToxA-like effector, Avr2 from *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (5OD4) (Di et al., 2017). **B.** WY effector, Avr3a11 from *Phytophthora capsici* (3ZR8) (Boutemy et al., 2011). **C.** *Magnaporthe* AVR and ToxB-like (MAX) effector, AVR-Pia from *M. oryzae* (2MYW) (de Guillen et al., 2015). **D.** RNase-like proteins expressed in haustoria (RALPH) effector, BEC1054 from *Blumeria graminis* f. sp. *hordei* (6FMB) (Pennington et al., 2019). **E.** *Fol* dual-domain (FOLD) effector, Avr1 from *Fol* (7T6A) (Yu et al., 2024). **F.** *Leptosphaeria* avirulence-suppressing (LARS) effector, AvrLm4-7 from *Leptosphaeria maculans* (7AD5) (Lazar et al., 2022). **G.** Killer protein 6 (KP6) effector, Zt-KP6-1 from *Zymoseptoria tritici* (6QPK) (de Guillen et al., 2024). **H.** Zinc-finger (ZiF) effector, AVR-Pii from *M. oryzae* (7PP2) (De la Concepcion et al., 2022).

Understanding the 3D structure of proteins can be beneficial for discerning in planta functions. For example, using both InterProScan and the IntFOLD server, Pedersen et al. (2012) uncovered a suite of putative effectors from barley powdery mildew, *Blumeria graminis* f.sp. *hordei* (Bgh), with structural homology to ribonuclease enzymes, now named 'RNase-like proteins expressed in haustoria' (RALPH) effectors (Spanu, 2015, Figure 3.1D). X-ray crystallography confirmed that the RALPH family member BEC1054 displayed high structural similarity to fungal RNases, but lacked key catalytic residues for RNA cleavage (Pennington et al., 2019). This prompted

investigation of BEC1054 nucleic acid-binding capacity, ultimately leading to characterisation of the effector as a pseudoenzyme, positively regulating *B. graminis* virulence by inhibiting degradation of host rRNA (Pennington et al., 2019). More recently, the crystal structure of a killer protein 6 (KP6) effector from *Zymoseptoria tritici*, Zt-KP6-1, was reported (de Guillen et al., 2024, Figure 3.1G), confirming its predicted structural homology to virally encoded KP6 α killer toxins secreted by *Ustilago maydis* (Li et al., 1999). It was later confirmed that Zt-KP6-1 indeed possessed antimicrobial activity, being capable of inhibiting *Botrytis cinerea* growth and (to a lesser extent) *Z. tritici* growth, with no obvious toxic effects observed upon infiltration of *T. aestivum* leaves (de Guillen et al., 2024). This suggests that, rather than directly manipulating hosts, KP6 effectors may have evolved to modulate intermicrobial interactions during fungal colonisation of plants. These examples demonstrate that structure determination (or at least prediction) has been valuable for understanding phytopathogen effector biology.

Beginning with the open source release of Google DeepMind's groundbreaking AI systems AlphaFold2 (Jumper et al., 2021) and AlphaFold2 Multimer for protein-protein interactions (Evans et al., 2022), in silico protein structure prediction pipelines have become increasingly accurate. Despite the high computing power required to generate an AlphaFold2 model, user-friendly initiatives such as ColabFold (Mirdita et al., 2022) have made structure prediction accessible to researchers globally. Although not publicly available at the outset of this work, AlphaFold3 represents another leap in predictive power, being capable of modelling proteins in complex with DNA, RNA, small molecules and ions (Abramson et al., 2024). Even in research scenarios requiring experimentally derived structures, predictive models can be valuable starting points for hypothesis generation, guiding construct design for recombinant protein expression (Perrakis and Sixma, 2021), and as templates for molecular replacement during X-ray crystallography experiments (Flower and Hurley, 2021; Yu et al., 2024).

Despite its huge impact across numerous fields, in the face of all structure prediction problems, AlphaFold is not a silver bullet (reviewed by Agarwal and McShan, 2024). Being trained on patterns extracted from known structures in the Protein Data Bank (PDB), AlphaFold2 can struggle to accurately predict the structures of 'orphan' proteins with few close relatives in the PDB. In cases where the backbone of a structure is modelled correctly, fine details such as side chain rotamer positions could be inaccurate, given that AlphaFold2 is not primarily trained to consider the physical and chemical interactions that restrain protein folding. Modelling protein complexes in silico remains especially challenging, with AlphaFold2 Multimer reported to successfully predict dimer interfaces in 70-72% of cases, yet only 26-36% of predictions were considered 'highly accurate' (Evans et al., 2022). In agreement, a study by Zhu et al. (2023) found that the strengths of AlphaFold2 Multimer lie in predicting homodimers, while heteromers, especially those with multiple chain components, were less likely to be modelled

correctly. To validate *in silico* structure predictions, and for those seeking to characterise precise binding or enzymatic mechanisms, it is thus necessary to experimentally determine protein structures using techniques such as X-ray crystallography, cryo-EM or NMR, with the method of choice depending on the size and properties of the protein(s) in question.

As a first step towards characterising ChEC108 function, I continued the initial efforts of Dr Joanna Jennings to identify *in planta* host targets using a non-biased approach. Subsequently, to better understand the regions and residues of each binding partner required for interaction, I conducted co-IP experiments and sought to structurally characterise ChEC108 in complex with its newly-identified host target, HIPP6.

3.2 Results

3.2.1 ChEC108 associates with HIPP6 in *N. benthamiana*

To identify candidate *in vivo* interactors of ChEC108, an IP-MS experiment was carried out by Dr Joanna Jennings. ChEC108-eGFP (or eGFP as a control) was transiently expressed and immunoprecipitated from *N. benthamiana* leaves, and associated proteins were identified. Arabidopsis homologs were identified for the top six most likely ChEC108-associated proteins, providing a list of candidate interactors from the *Ch* host (Table 3.1). Five of these candidates were selected by Dr Jennings for further study: HIPP6, HIPP3, DYNAMIN RELATED PROTEIN 2B (DRP2B) and two REMORIN 1.4 (REM1.4) isoforms, denoted REM1.4.1 and REM1.4.3, and constructs to drive their expression as C-terminal mCherry fusion proteins were synthesised by Genewiz. To test for protein-protein associations *in vivo*, I used a targeted co-IP approach, expressing ChEC108-eGFP fusions alongside Dr Jennings' five candidates in *N. benthamiana* leaves. As a highly-expressed integral membrane protein that was not significantly enriched following ChEC108-eGFP IP-MS, LOW TEMPERATURE INDUCED PROTEIN 6B (AtLTI6b) was included as a negative control. After α -GFP IP, co-enrichment of HIPP6-mCherry was observed (Figure 3.2), a finding that was consistent across three independent repeats. While faint bands could indicate the presence of HIPP3-mCherry and DRP2B-mCherry in IP fractions, this result was not reproduced. Thus, among the five candidates screened, HIPP6 was identified as a likely host target of ChEC108.

Table 3.1 – Candidate ChEC108-associated proteins identified by IP-MS. Details of the *N. benthamiana* peptides and Arabidopsis orthologs identified as being the top six most likely ChEC108 interactors. Significance Analysis of INteractome (SAINT) scores were derived by SAINTq analysis (Teo et al., 2016). BFDR = bayesian false discovery rate.

Peptide (Niben101Scf)	SAINT score	BFDR	Arabidopsis ortholog	Full Name	Synonyms
09282g00004	0.88	0.008	AT5G03380.1	HEAVY METAL-ASSOCIATED ISOPRENYLATED PLANT PROTEIN 6	HIPP6 HMP43 CdI19
00504g02001	0.667	0.064	AT5G60800.2	HEAVY METAL-ASSOCIATED ISOPRENYLATED PLANT PROTEIN 3	HIPP3 HMP53
09472g02009	0.667	0.154	AT1G59610	DYNAMIN RELATED PROTEIN 2B	DRP2B DL3 ADL3
10476g05007	0.667	0.154	AT3G60190	DYNAMIN RELATED PROTEIN 1E	DRP1E DL1E ADL4 EDR3 ADLP2 ADL1E
08690g01009	0.667	0.226	AT1G53140	DYNAMIN RELATED PROTEIN 5A	DRP5A
02910g01040	0.667	0.244	AT5G23750.1 AT5G23750.3	REMORIN 1.4.1 REMORIN 1.4.3	REM1.4

As a member of the extensive HIPP family in Arabidopsis (introduced in 1.2.1), HIPP6 possesses two HMA domains towards the N-terminus (referred to throughout this work as HMA1 and HMA2), and a C-terminal isoprenylation motif (Figure 3.3A). Given that HMA domains have been previously identified as direct targets of pathogen-derived proteins, especially *Magnaporthe* AVR_s and ToxB-like (MAX) effectors (see 1.2.3), I hypothesised that ChEC108 may associate with one or both of the HMA domains of HIPP6. To test this, alongside ChEC108-eGFP, HMA1 and HMA2 were expressed in *N. benthamiana* as C-terminal mCherry fusions, both individually and in tandem (referred to hereon as tHMA), connected by their native linker. Full length HIPP6-mCherry and REM1.4.1-mCherry were included as positive and negative controls, respectively. HMA1, HMA2 and tHMA were all visible in the α -GFP IP fraction, despite HMA2 being barely visible in the input (Figure 3.3B). Curiously, across three repetitions, there was a noticeable reduction in ChEC108-eGFP signal in presence of HMA2, suggesting association with this domain may destabilise the effector. An additional higher molecular weight band was also observed for ChEC108-eGFP in the presence of tHMA, which

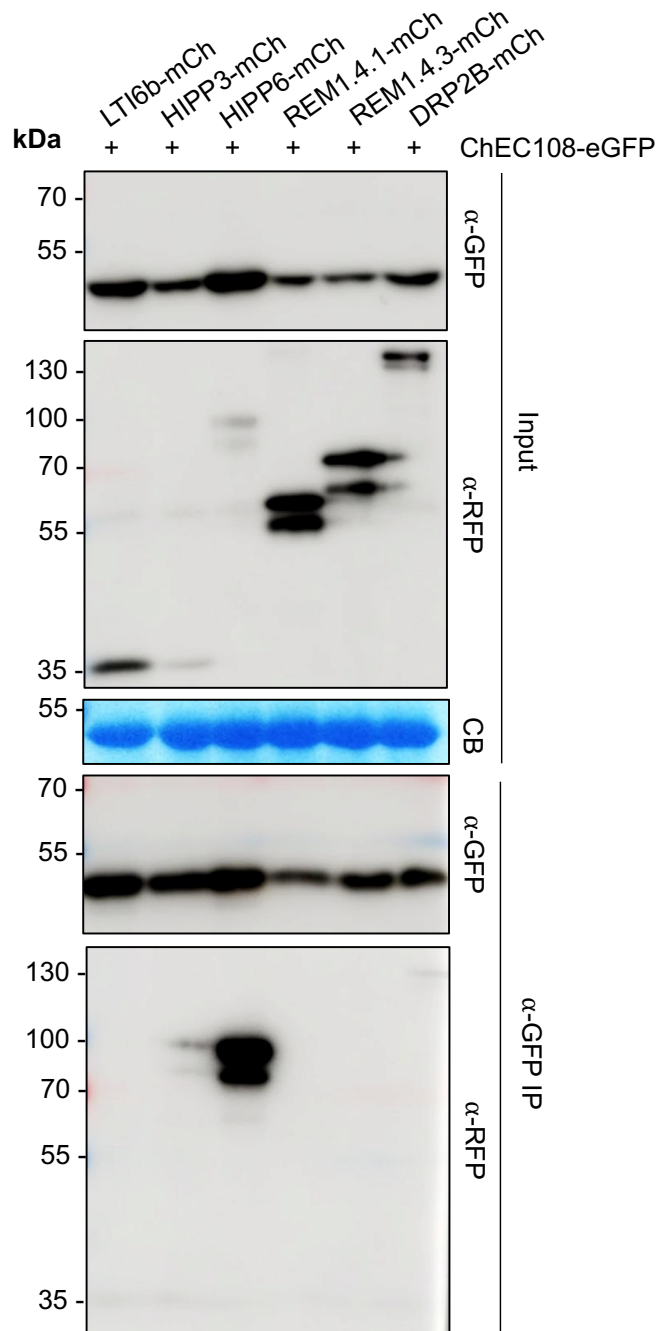


Figure 3.2 – ChEC108 binds HIPP6 in vivo. A. Co-IP demonstrating association of eGFP-tagged ChEC108 (47.0 kDa) with mCherry (mCh)-tagged HIPP6 (69.1 kDa), as observed in three independent repeats. LTI6b-mCh (33.0 kDa) was included as a negative control. Other mCh-tagged candidate interactors - HIPP3 (60.0 kDa), REM1.4.1 (49.3 kDa), REM1.4.3 (52.2 kDa) and DRP2B (127.1 kDa) - were co-expressed with ChEC108-eGFP, however reproducible association was not observed. Proteins were transiently expressed in *N. benthamiana* leaves. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit.

could indicate post-translational modification. While the significance of these phenomena is presently unclear, it seems that ChEC108 indeed targets HIPP6 via its HMA domains, and that a single domain (either HMA1 or HMA2) is sufficient for this association.

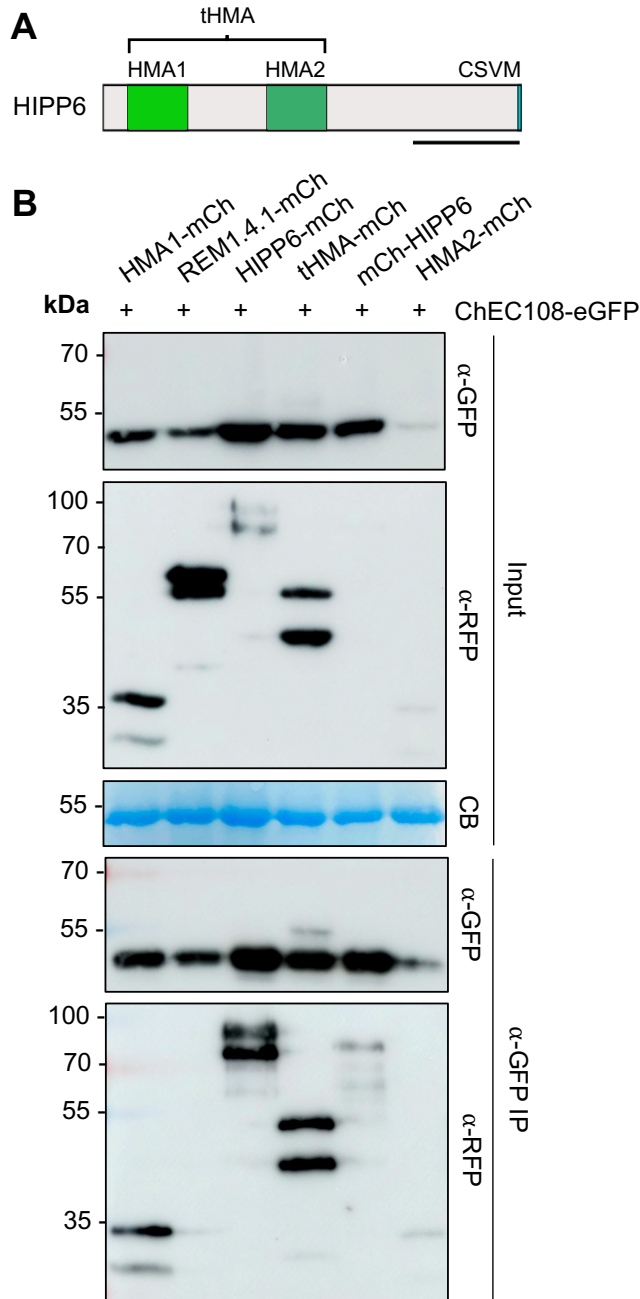


Figure 3.3 – HIPP6 HMA domains are sufficient for ChEC108 binding in vivo. **A.** Schematic representation of HIPP6 domain architecture, with HMA domains in green and isoprenylation motif (CSVM) in blue. Scale bar = 100 amino acids. **B.** Co-IP demonstrating association of eGFP-tagged ChEC108 (47.0 kDa) with mCherry (mCh)-tagged HMA1 (35.7 kDa), HMA2 (35.0 kDa) and tHMA (49.2 kDa). REM1.4.1-mCh (49.3 kDa) was included as a negative control, and full-length HIPP6 was included as a positive control with both N- and C-terminal mCh tags (69.8 and 69.1 kDa, respectively). Proteins were transiently expressed in *N. benthamiana* leaves. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. Representative of three independent repeats.

3.2.2 The ChEC108-HIPP6 interface is not confidently predicted in silico

Considering the evidence from co-IP presented above, during infection of *Arabidopsis*, it is conceivable that ChEC108 could bind either of the HIPP6 HMA domains independently. Alternatively, both HMA domains could be bound simultaneously via distinct sites. To better understand the mechanism of interaction and guide future protein-level manipulation, I sought to structurally characterise the ChEC108-HIPP6 complex. For initial exploration and hypothesis generation, I used AlphaFold2 to generate predictive models of each protein. When fed the 213-amino acid sequence of ChEC108, AlphaFold2 modelled a globular protein composed of an eight-stranded β -sheet and four α -helices (Figure 3.4A). An N-terminal α -helical extension corresponds to a putative signal peptide, as predicted by SignalP 6.0 (Teufel et al., 2022). The per-residue confidence estimates, as indicated by predicted Local Distance Difference Test (pLDDT) scores, are globally high, and the errors associated with relative positions of residues, as indicated by predicted aligned error (PAE) scores, are low with the exception of residues scored within the signal peptide (Figure 3.4A,B). Overall, this suggests that the ChEC108 structure alone can be predicted with high confidence.

To search for proteins with potentially similar structures (and by extension, functions) to ChEC108, I queried a representative subset of the PDB consisting of structures with < 25% similarity (known as PDB25) using the Distance matrix ALIgnment (DALI) server (Holm et al., 2023). Studying the descriptions of the top 25 ranked proteins that emerged, it seemed ChEC108 could be structurally similar to acetyltransferases, particularly those of the Gcn5-related N-acetyltransferase (GNAT) family (Table S11). GNATs are a superfamily of enzymes widely distributed in all kingdoms of life, which transfer acetyl groups from acetyl-CoA molecules to the N-terminus or Lys side chains of their bound substrates. The GNAT fold is highly conserved, consisting of an N-terminal β -strand followed by two α -helices, three antiparallel β -strands, then a central α -helix, fifth β -strand, fourth α -helix and a final β -strand (for reviews, see Dyda et al., 2002; Vetting et al., 2005). Other characteristic features of GNATs are (1) their V-shaped region for acetyl-CoA binding, resulting from a β -bulge (that is, disruption of antiparallel interactions within a β -sheet), and (2) a P-loop motif with consensus Q/RXXGXG/A, involved in binding the pyrophosphate group of acetyl-CoA ligands (Vetting et al., 2005). This core GNAT structure is exemplified by crystal structures deposited in the PDB: 3BLN from *Bacillus cereus* (Figure 3.5A), and 2PC1 from *Streptococcus agalactiae* (Figure 3.5B), both of which were predicted to be structurally similar to ChEC108 by the DALI server (Table S11).

The ChEC108 AlphaFold2 model bares some resemblance to 3BLN. For instance, it has an eight-stranded β -sheet and two central α -helices oriented similarly (Figure 3.5A). However, the ' β -bulge' of ChEC108 is predicted to be less pronounced than that of 3BLN, and the N-

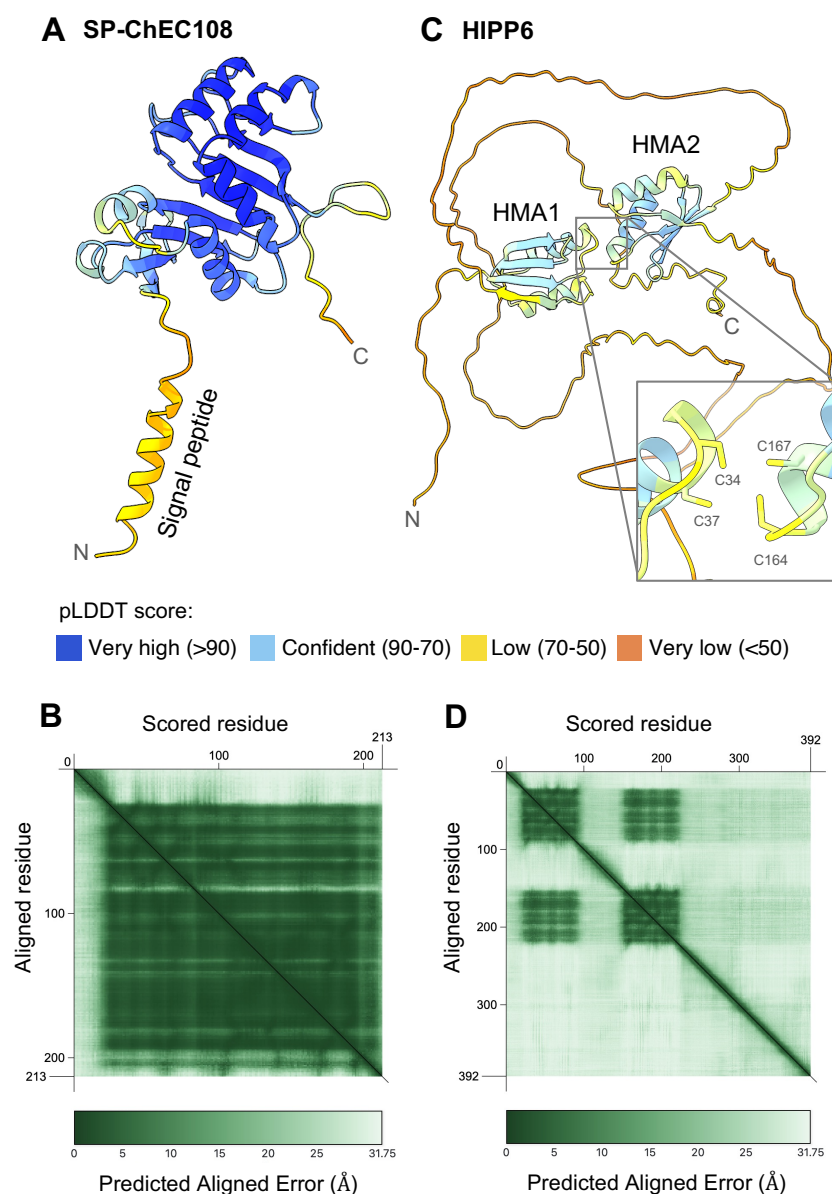


Figure 3.4 – ChEC108 and HIPP6 predicted structures. AlphaFold2 models of ChEC108 with its native signal peptide (SP) (A-B) and HIPP6 (C-D), coloured according to pLDDT confidence score. AlphaFold was accessed via ColabFold versions 1.5.2 and 1.3.0, respectively. Grey box in C highlights a potential inter-HMA interface, involving the CXXC motifs of each HMA domain. Predicted aligned error (PAE) plots in B and D show the predicted error associated with relative distances of residues.

and C-termini of both models are dissimilar (Figure 3.5A). An attempt to align the ChEC108 predicted structure to 2PC1 revealed even less similarity (Figure 3.5B).

GNAT enzymes commonly catalyse acetyl transfer using a general acid/base mechanism, utilising a Tyr or Cys residue as a general acid, and a His, Glu, or Asp residue as a general base (Baumgartner et al., 2021; Kotani and Takagi, 2008). The general acid is positioned within active site of the enzyme, formed by the β -bulge and surrounding loops (Krtenic et al., 2020). At the equivalent position in the ChEC108 AlphaFold2 structure, rendering of the protein

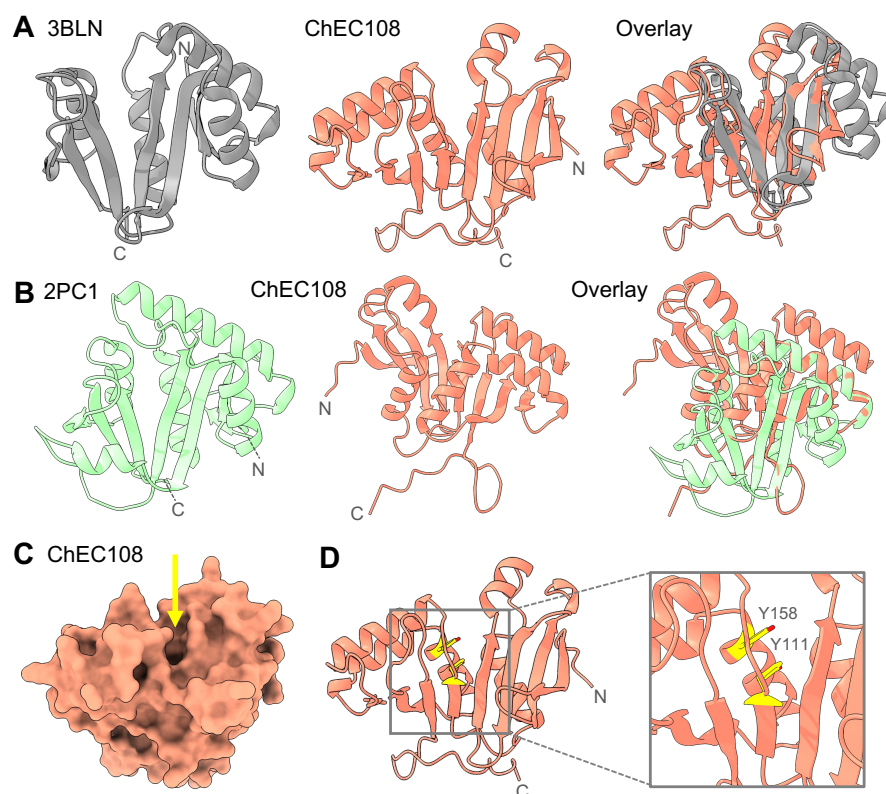


Figure 3.5 – ChEC108 bares some structural resemblance to GNAT enzymes. A-B. Comparison of the ChEC108 AlphaFold2 model (shown in Figure 3.4A with signal peptide removed) to GNAT structures from *B. cereus* (3BLN) and *S. agalactiae* (2PC1). Overlays shows structural alignments performed by ChimeraX using the matchmaker command. C. Surface rendering of the ChEC108 AlphaFold2 model, with a potential substrate binding pocket indicated by a yellow arrow. D. Ribbon model underlying surface model in C, with potential catalytic Tyr residues coloured yellow.

surface reveals a potential substrate binding pocket (Figure 3.5C). Within this pocket lie Tyr111 and Tyr158 (Figure 3.5D), which could potentially function as catalytic residues. However, the conserved P-loop motif implicated in GNAT substrate binding (with sequence RRRGYA and QFRGRG in 3BLN and 2PC1, respectively), is absent from ChEC108. Overall, structural comparisons conducted above have uncovered evidence both supporting and opposing the hypothesis that ChEC108 could function as an acetyltransferase.

When fed the 392-amino acid sequence of HIPP6 from *Arabidopsis*, AlphaFold2 identified two HMA domains with typical ferredoxin-like folds, each including two α -helices and a four-stranded antiparallel β -sheet in an α - β sandwich. However, the remainder of the protein, including the N- and C-termini and inter-HMA linker, were predicted to form no secondary structures (Figure 3.4C). Nevertheless, PAE values suggested the relative positions of HMA1 and HMA2 were predicted with high confidence, with the CXXC motifs of the HMA domains positioned directly opposite one another (Figure 3.4C,D).

Given that relatively confident predictions were obtained for both ChEC108 and HIPP6 HMA domains, I reasoned that AlphaFold2 Multimer might accurately predict the ChEC108-HIPP6 binding interface. To focus only on ChEC108 structures presumably available to HIPP6 in planta, the putative signal peptide was removed (residues 1-20 inclusive). When a single copy of ChEC108 and HIPP6 were fed to AlphaFold2 Multimer, the resulting model suggested ChEC108 could contact both HMA1 and HMA2 simultaneously, with the linker region and (presumably unstructured) C-terminus of HIPP6 forming no additional contacts (Figure 3.6A-B). However, predicted Template Modelling scores for the global structure (pTM, 0.38) and interfaces (ipTM, 0.22) suggest confidence in this prediction is low. In fact, ipTM scores lower than 0.6 generally indicate failed predictions of protein-protein interfaces. As disordered regions and low pLDDT scores can negatively impact ipTM metrics, I attempted to model ChEC108 in complex with tHMA only. Again, AlphaFold2 Multimer predicted that HMA1 and HMA2 could simultaneously contact ChEC108, yet confidence in the model and interface predictions remained low (pTM = 0.48 and ipTM = 0.22) (Figure 3.6C-D). As the ChEC108 chain is equivalently oriented in Figure 3.6A and C, it is clear that the predicted HMA binding surfaces of the ChEC108-tHMA complex differ from those predicted in the context of full length HIPP6.

When ChEC108 was modelled with either HMA1 or HMA2 in isolation, a similar binding interface was predicted for each complex, involving the CXXC motifs of the HMA domain, and a second CXXC motif at the C-terminus of ChEC108 (Figure 3.7). While removal of disordered regions increased the confidence of AlphaFold2 Multimer in the structures globally (pTM = 0.73 and 0.78, respectively), ipTM scores remained low (0.42 and 0.53, respectively), raising doubts about the accuracy of the predicted interaction mechanism. Overall, these data suggest AlphaFold2 was unable to confidently predict the binding interface of ChEC108 and HIPP6, justifying my subsequent pursuit of an experimentally-derived structure.

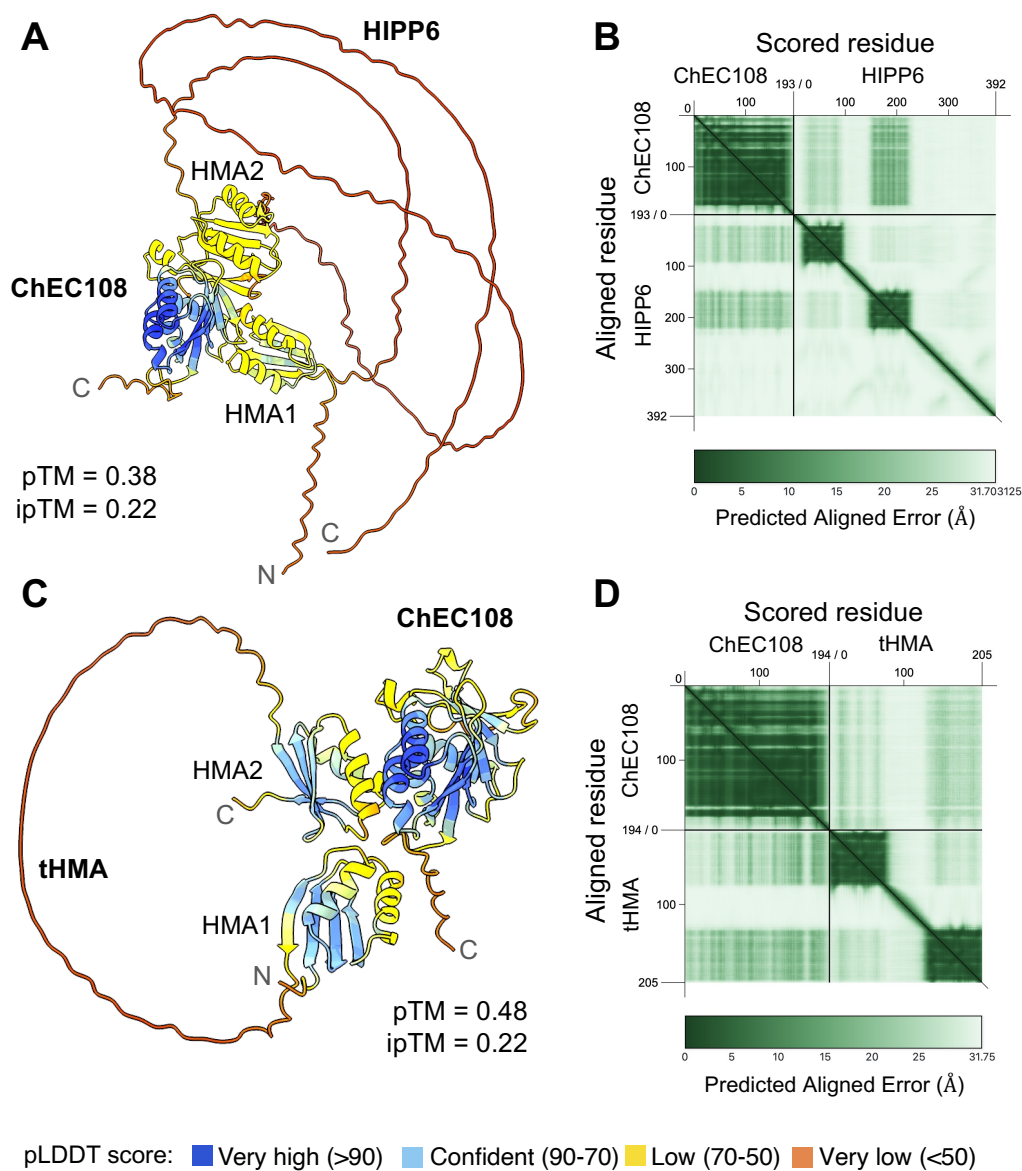


Figure 3.6 – Predicted structure of the ChEC108-HIPP6 complex. AlphaFold2 Multimer (accessed via ColabFold v.1.5.5) model of ChEC108 in complex with HIPP6 (**A-B**) or tHMA (**C-D**), coloured according to pLDDT confidence scores. Predicted Template Modelling scores for the overall structure (pTM) and interfaces (ipTM) are shown. Predicted aligned error (PAE) plots in B and D show the predicted error associated with relative distances of residues.

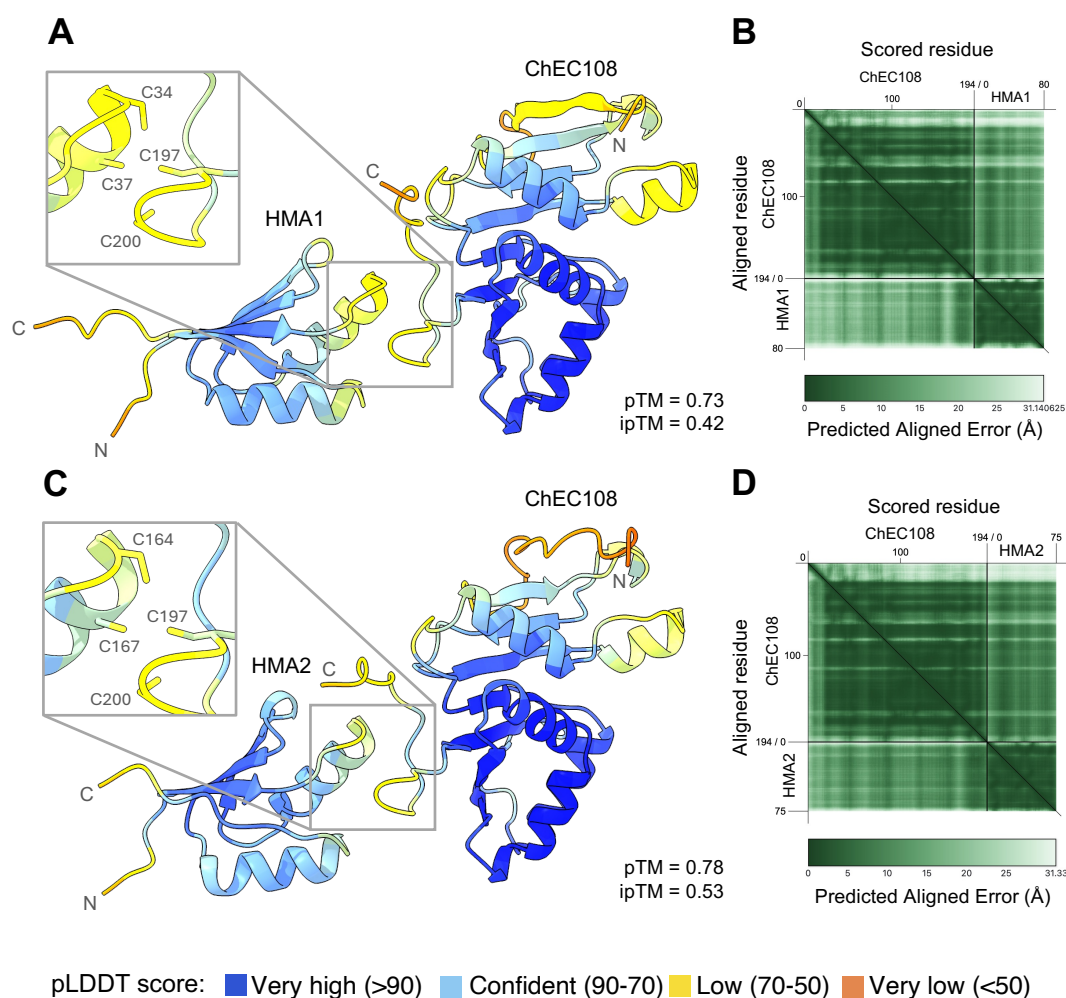


Figure 3.7 – Predicted structure of ChEC108 in complex with HMA1 or HMA2. AlphaFold2 Multimer (accessed via ColabFold v.1.5.5) models of ChEC108 in complex with HMA1 (**A-B**) or HMA2 (**C-D**) from HIPP6, coloured according to pLDDT confidence scores. Predicted Template Modelling scores for the overall structure (pTM) and interfaces (ipTM) are shown. Predicted aligned error (PAE) plots in B and D show the predicted error associated with relative distances of residues. Grey boxes in A and C show potential interfaces formed by the CXXC motifs of the HMA domains, and two Cys residues near the ChEC108 C-terminus.

3.2.3 ChEC108 and HIPP6 HMA domains can be expressed in *E. coli*

With the full-length HIPP6 protein predicted to be intrinsically disordered in all regions outside the HMA domains (68% of residues, according to [MobiDB](#)) ([Piovesan et al., 2025](#)), I anticipated that in vitro purification of a stable ChEC108-HIPP6 complex in sufficient quantities for X-ray crystallography would be highly challenging. Therefore, guided by co-IP experiments and AlphaFold2 models described above, I sought to structurally characterise ChEC108 in complex with HMA1, HMA2 or tHMA. First, sequences of these four proteins were cloned into pPGN vectors ([Bentham et al., 2021b](#)), flanked by bacteriophage T7 promoters and terminators to facilitate expression in *E. coli* as N-terminally tagged fusion proteins. Again, to reflect the ChEC108 structures likely accessible to HIPP6 during infection, the putative signal peptide was removed. AlphaFold2 models of all recombinant proteins produced in this work exhibited minimal disordered or flexible regions, with the exception of the inter-HMA linker of tHMA (Figure 3.8), suggesting these truncated variants could be soluble and amenable to crystallisation.

Before attempting large-scale purification of ChEC108 with HIPP6 domains, small-scale expression tests were carried out for each individual protein using two different N-terminal tags: (1) 6×His or (2) 6×His-GB1, in two different *E. coli* expression strains: (1) BL21-AI or (2) SHuffle ([Lobstein et al., 2012](#)). 6×His tags were chosen to enable binding to IMAC columns with immobilised nickel (Ni) ions, while GB1 was selected to promote solubility ([Cheng and Patel, 2004](#)). *E. coli* were grown in 1 l cultures, and the soluble fraction of the resulting cell lysate harvested for IMAC using 1 ml HisTrap™ columns (see 2.10.1 and 2.10.2 for details). Encouragingly, all affinity-tagged proteins of interest could be detected in lysate samples, and were significantly enriched following IMAC, as demonstrated by peaks in UV absorbance and observation of high protein abundance on SDS-PAGE gels (Figure 3.9). The use of a 6×His or 6×His-GB1 tag made little difference to protein accumulation levels. To minimally impact the structure and function of expressed proteins, and potentially enable greater purification yields, constructs for recombinant expression of ChEC108, HMA1, HMA2 and tHMA as 6×His-fusions were carried forward for larger scale preparations.

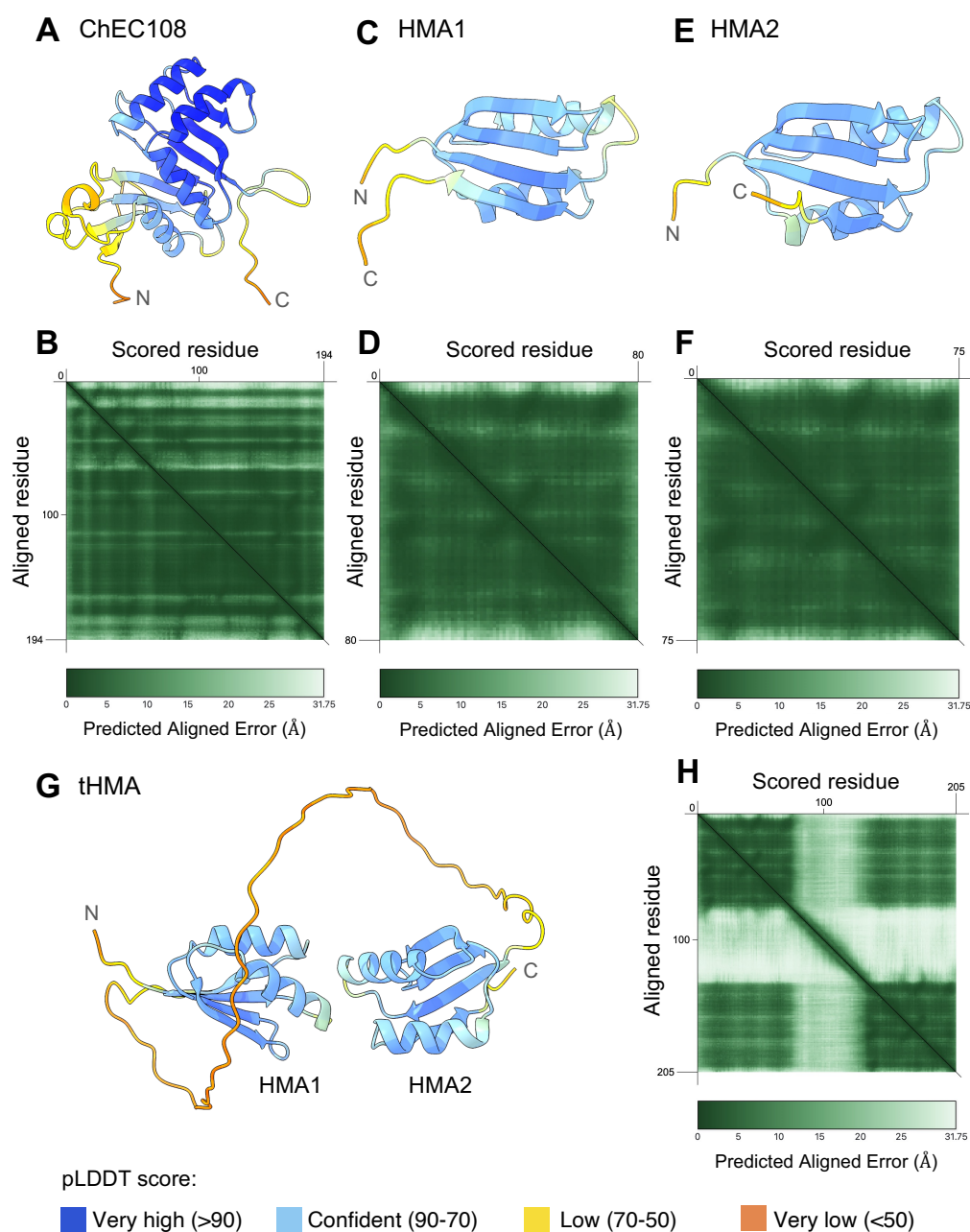


Figure 3.8 – Predicted structures of ChEC108 and HIPP6 HMA domain truncations used for in vitro purification. AlphaFold2 (accessed via ColabFold v.1.5.2) models of ChEC108 (A-B), HMA1 (C-D), HMA2 (E-F) and tHMA (G-H), coloured according to pLDDT confidence scores. Models were predicted based on sequences used for recombinant protein expression and co-purification, including N-terminal Gly-Pro scars where necessary. Predicted aligned error (PAE) plots in B, D, F and H show the predicted error associated with relative distances of residues.

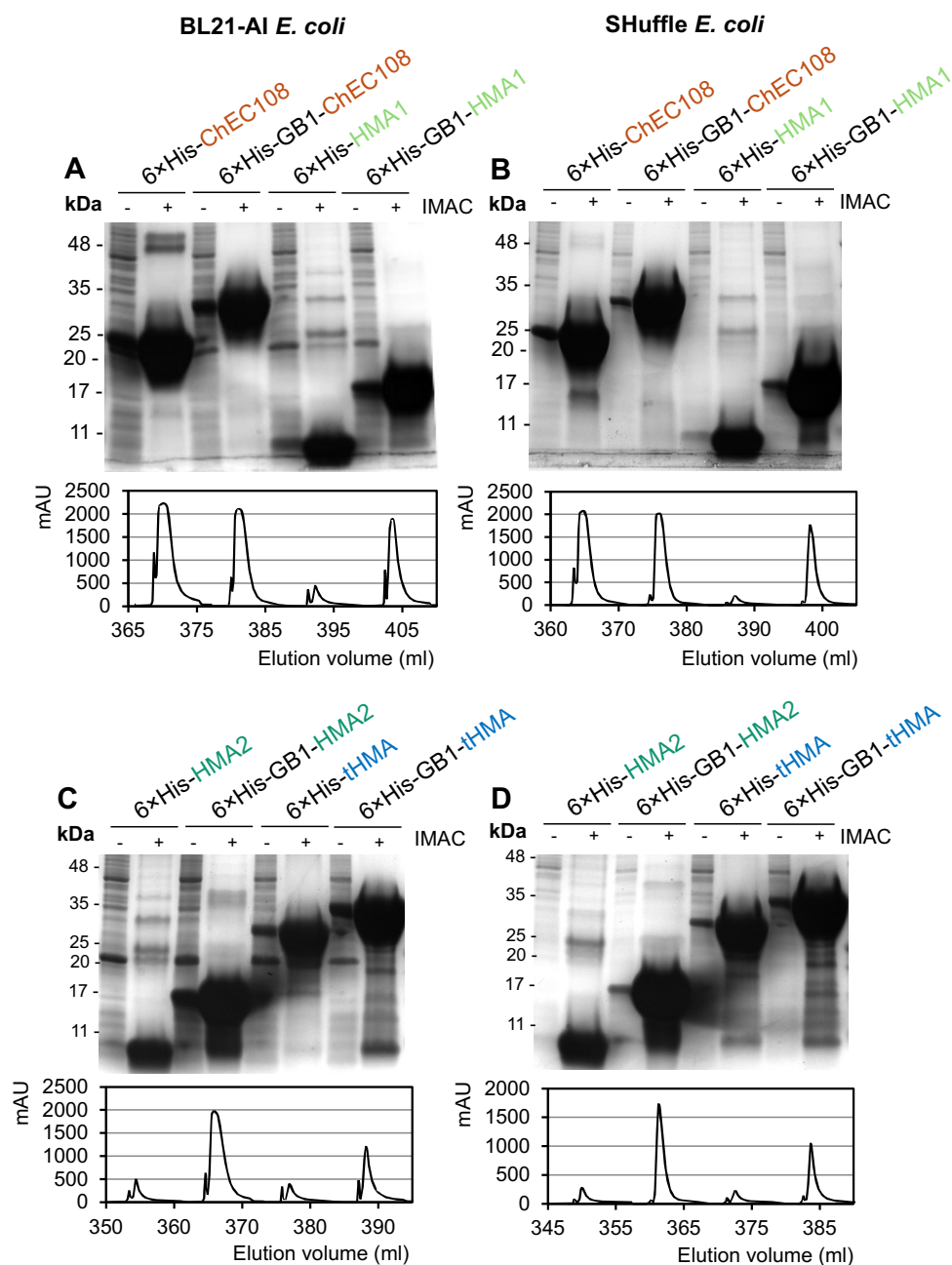


Figure 3.9 – ChEC108, HMA1, HMA2 and tHMA can be expressed in *E. coli*. Enrichment of affinity-tagged ChEC108 (A-B), HMA1 (A-B), HMA2 (C-D) and tHMA (C-D) from *E. coli* cell lysates by IMAC. Proteins were expressed with either a 6×His or 6×His-GB1 affinity tag, in either BL21-AI (A,C) or Shuffle (B,D) stains. Lysates were loaded onto separate 1 ml HisTrap™ columns and eluted in sequence. Upper panels are SDS-PAGE gels showing proteins of interest in cell lysates before the IMAC step (-) and in elution fractions within the peaks of the corresponding absorbance spectra (lower panels) at 280 nm (+). mAU = milli-absorbance units at 280 nm. Expected molecular weights (kDa): 6×His-ChEC108, 24.8; 6×His-GB1-ChEC108, 31.7; 6×His-HMA1, 10.9; 6×His-GB1-HMA1, 17.8; 6×His-HMA2, 10.3; 6×His-GB1-HMA2, 17.2; 6×His-tHMA, 24.5; 6×His-GB1-tHMA, 31.4.

3.2.4 The ChEC108-tHMA complex is unsuitable for crystallisation

Being the closest in sequence to the full-length HIPP6 protein, I first attempted to purify and crystallise tHMA in complex with ChEC108. Towards this goal, I generated a SHuffle *E. coli* strain to co-express untagged ChEC108 with 6×His-tHMA, and harvested soluble proteins from 8 l of the resulting culture. Subsequent IMAC and gel filtration steps enabled successful enrichment of a complex containing both proteins (Figure 3.10A). 6×His affinity tags were cleaved from tHMA by 3C protease and isolated from the sample using a second IMAC step (Figure 3.10B). Finally, a second gel filtration step was used to enhance purity of the sample (Figure 3.10C). After mass spectrometry analysis confirmed the presence of both ChEC108 and tHMA (Table 3.2), I attempted to crystallise the complex using protein concentrations of 20 mg/ml or 10 mg/ml in a variety of commercially available two-drop screens: Morpheus, PEG Suite, ProPlex, MIDASplus and SG1 (see 2.10.5). Unfortunately, no crystalline material was observed.

Table 3.2 – Mass spectrometry analysis of purified proteins. Expected and observed masses detected by mass spectrometry are given for each purified protein. Note that tag cleavage leaves a Gly-Pro scar (154.17 Da) on the N-terminus.

Figure	Protein	Post-tag cleavage?	Expected size (Da)	Observed size (Da)	Expected – observed size (Da)	
3.10	ChEC108	Y	22792.9	22790.4	2.5	^a
	tHMA	N	22323.4	22191.4	131.9	^b
3.15	ChEC108	N	22638.7	22638.3	0.4	
	HMA1	Y	8933.7	8932.8	0.8	
3.15	ChEC108	N	22638.7	22636.0	2.7	^a
	HMA2	Y	8321.0	8320.6	0.4	
3.11	ChEC108	Y	22792.9	22790.4	2.5	^a
3.28	HMA1	Y	8933.7	8931.5	2.2	^a
3.29	HMA2	Y	8321.0	8320.0	1.0	

^a Loss of approximately 2 Da is consistent with disulphide bridge formation.

^b Loss of approximately 131.19 Da is consistent with loss of N-terminal Met.

Despite being subject to one IMAC and two gel filtration steps, an abundance of low molecular weight contaminants were observed in the ‘pure’ ChEC108-tHMA sample (Figure 3.10C). I hypothesised that these might be a result of tHMA instability, owing to the presence of the presumably flexible linker between the two HMA domains (Figure 3.8G). Loops and other flexible regions, which compromise a protein’s conformational stability, are known to impede crystallisation by preventing tight assembly into lattices (Deller et al., 2016). To overcome such hurdles, in situ limited proteolysis has previously been applied to support crystallisation

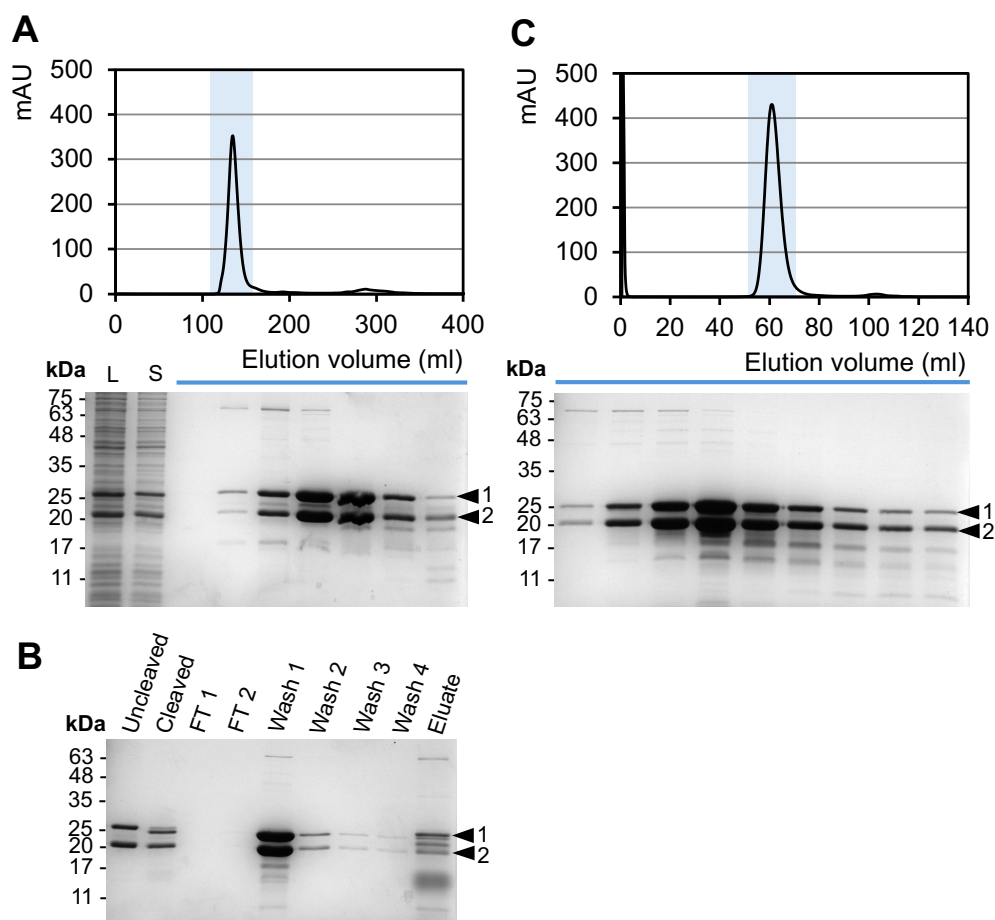


Figure 3.10 – Co-purification of ChEC108 and tHMA from *E. coli*. **A.** Enrichment of 6×His-tHMA (24.5 kDa, arrow 1) and ChEC108 (untagged, 22.6 kDa, arrow 2) from 8 l of SHuffle *E. coli* by IMAC and gel filtration. Both proteins were visible in cell lysate (L) and soluble fractions (S). Gel filtration fractions 110-162 ml were examined by SDS-PAGE. Fractions 118-162 ml were pooled and carried forward. **B.** Isolation of tHMA (22.5 kDa, arrow 1) and ChEC108 (22.6 kDa, arrow 2) by IMAC following cleavage of 6×His tags by 3C protease. The complex has natural affinity for the resin of the IMAC column and was absent from flowthrough (FT) fractions. Elution was achieved by washing with A1 buffer (Wash 1-4). His-tagged 3C protease and other contaminants were eluted with B1 buffer (Eluate). Wash 1-4 were pooled and carried forward. **C.** Final co-purification of tHMA (22.5 kDa, arrow 1) and ChEC108 (22.6 kDa, arrow 2) by gel filtration. Fractions 53-73 ml were examined by SDS-PAGE. Fractions 61-73 ml were pooled and concentrated. mAU = milli-absorbance units at 280 nm.

of a range of recalcitrant bacterial, fungal and human-derived proteins (Dong et al., 2007; Wernimont and Edwards, 2009; Zdrzałek et al., 2024). This approach involves incubation of proteins with a low concentration of protease (usually trypsin or chymotrypsin), encouraging cleavage of exposed loops and other flexible regions to leave behind a soluble, structured protein.

To assess the potential of limited proteolysis for improving the ChEC108-tHMA sample, I carried out small-scale trypsin digestion reactions using a dilution series to monitor the effect of increasing concentrations of trypsin on a fixed amount of protein (4 μg). As controls, identical experiments were conducted with ChEC108 and tHMA protein samples, purified in isolation from one another (for details, see Figures 3.11 and 3.12) and in the absence of a protein substrate for trypsin (Figure 3.13A). At high concentrations of trypsin (5 μg), ChEC108, tHMA and ChEC108-tHMA were fully digested, leaving no trace of the respective proteins when the reaction mixture was examined by SDS-PAGE (Figure 3.13B-D). ChEC108 appeared to resist digestion only at very low concentrations of trypsin (0.01 μg) (Figure 3.13B), while tHMA was digested even at this low concentration (Figure 3.13C). Interestingly, examination of control reactions in the absence of trypsin revealed stabilisation of tHMA in complex with ChEC108 (Figure 3.13C vs D). Nevertheless, even as part of a complex, ChEC108 and tHMA remained highly susceptible to digestion at low protease concentrations, making limited proteolysis an ineffective approach for sample optimisation in this case. Combined with the observed intrinsic instability of tHMA, this led me to conclude that the ChEC108-tHMA complex is unsuitable for crystallisation.

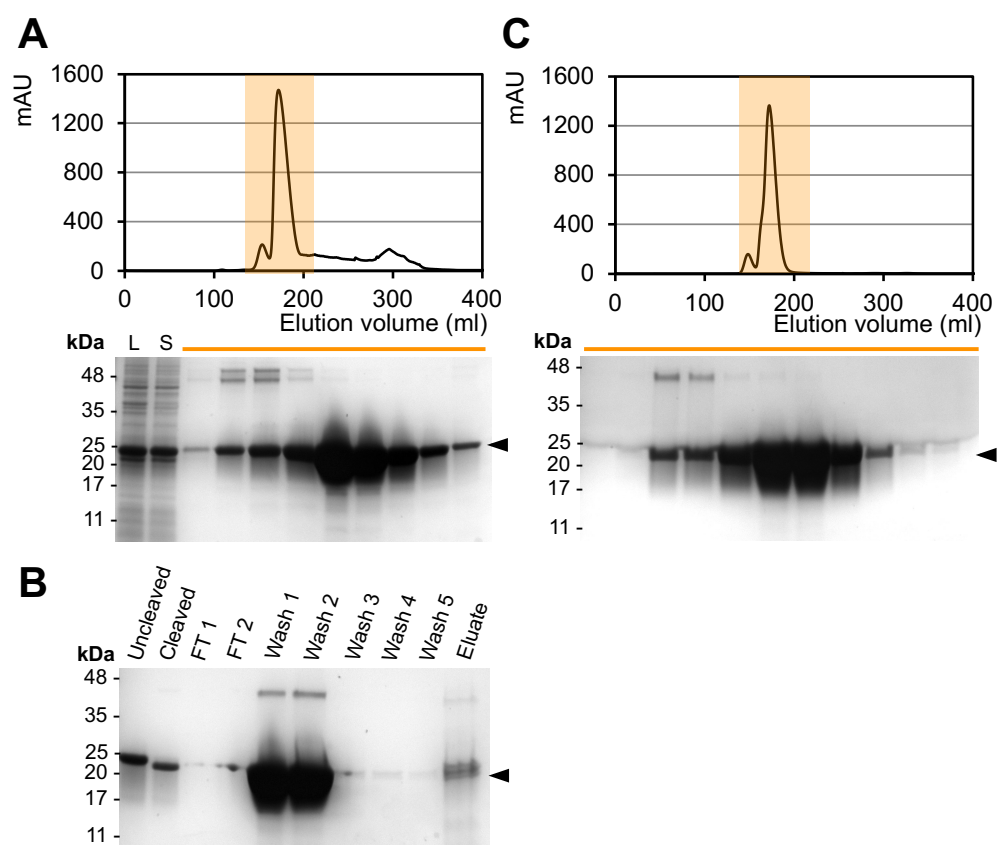


Figure 3.11 – Purification of ChEC108 from *E. coli*. **A.** Enrichment of 6×His-ChEC108 from 8 l of BL21-AI *E. coli* by IMAC and gel filtration. 6×His-ChEC108 (24.8 kDa, arrow) was visible in cell lysate (L) and soluble fractions (S). Gel filtration fractions 142-208 ml were examined by SDS-PAGE, pooled and carried forward. **B.** Isolation of ChEC108 (22.8 kDa, arrow) by IMAC following cleavage of 6×His tags by 3C protease. ChEC108 has natural affinity for the resin of the IMAC column and was absent from flowthrough (FT) fractions. Elution was achieved by washing with A1 buffer (Wash 1-4). His-tagged 3C protease and other contaminants were eluted with B1 buffer (Eluate). Wash 1-2 were pooled and carried forward. **C.** Final co-purification of ChEC108 (22.8 kDa, arrow) by gel filtration. Fractions 125-212 ml were examined by SDS-PAGE. Fractions 157-212 ml were pooled and concentrated. mAU = milli-absorbance units at 280 nm.

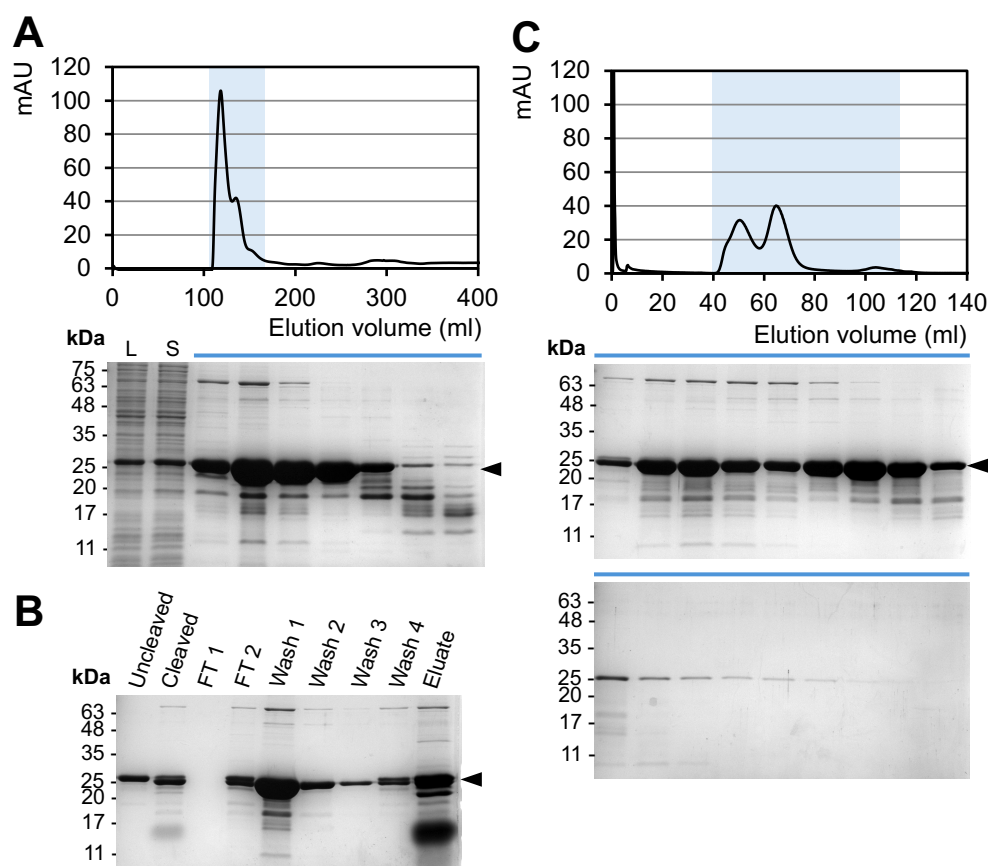


Figure 3.12 – Purification of tHMA from *E. coli*. **A.** Enrichment of 6×His-tHMA from 8 l of SHuffle *E. coli* by IMAC and gel filtration. 6×His-tHMA (24.5 kDa, arrow) was visible in cell lysate (L) and soluble fractions (S). Gel filtration fractions 110-167 ml were examined by SDS-PAGE. Fractions 110-142 ml were pooled and carried forward. **B.** Isolation of tHMA (22.5 kDa, arrow) by IMAC following cleavage of 6×His tags by 3C protease. tHMA has a natural (weak) affinity for the resin of the IMAC column and was not fully eluted in flowthrough (FT) fractions. Further elution was achieved by washing with A1 buffer (Wash 1-4). His-tagged 3C protease and other contaminants were eluted with B1 buffer (Eluate). Wash 1-3 were pooled and carried forward. **C.** Final co-purification of tHMA (22.5 kDa, arrow) by gel filtration. Fractions 44-118 ml were examined by SDS-PAGE. Fractions 42-82 ml were pooled and concentrated. mAU = milli-absorbance units at 280 nm.

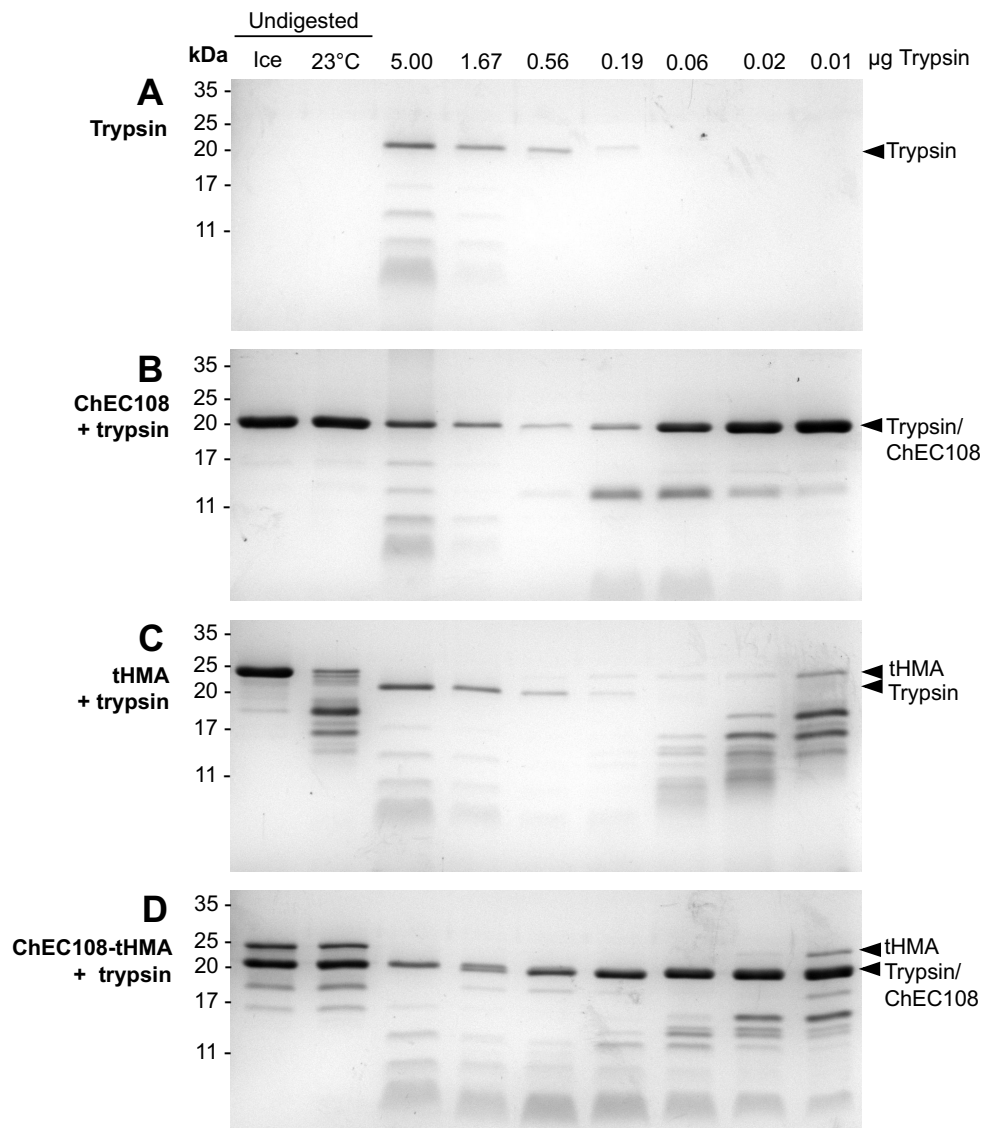


Figure 3.13 – ChEC108 and tHMA are highly susceptible to trypsin digestion. SDS-PAGE gels showing in vitro digestion of purified proteins (4 µg per 25 µl reaction) with decreasing concentrations of trypsin. Reactions were carried out at 23 °C for 30 min. Undigested control reactions did not contain trypsin. **A.** Control dilution series showing bands corresponding to trypsin and accompanying impurities. **B-D.** Digestion of ChEC108 (B), tHMA (C) and the ChEC108-tHMA complex (D).

3.2.5 A ChEC108-HMA1-HMA2 tripartite complex was not observed in vitro

In contrast to tHMA, HMA1 and HMA2 are not predicted to possess large flexible or unstructured regions (Figure 3.8B-C), making them more likely to crystallise. I therefore devised a co-purification strategy to obtain a ChEC108-HMA1-HMA2 tripartite complex, lacking the potentially problematic inter-HMA linker. First, a complex comprising 6×His-HMA1 and untagged ChEC108 was expressed in SHuffle *E. coli* (Figure 3.14 lanes 1 and 3). Independently, untagged HMA2 was expressed (Figure 3.14 lanes 2 and 4). Cell lysates from these two strains were mixed for 1 h at 4 °C, then carried forward for IMAC and gel filtration. While ChEC108 and HMA1 were successfully co-eluted, HMA2 was absent from the IMAC column eluate (Figure 3.14), suggesting it did not have sufficiently strong affinity for the 6×His-HMA1-ChEC108 complex to be retained during the column washes. Given that a ChEC108-HMA1-HMA2 tripartite complex has yet to be observed, it is possible that ChEC108 may be incapable of binding HMA1 and HMA2 simultaneously.

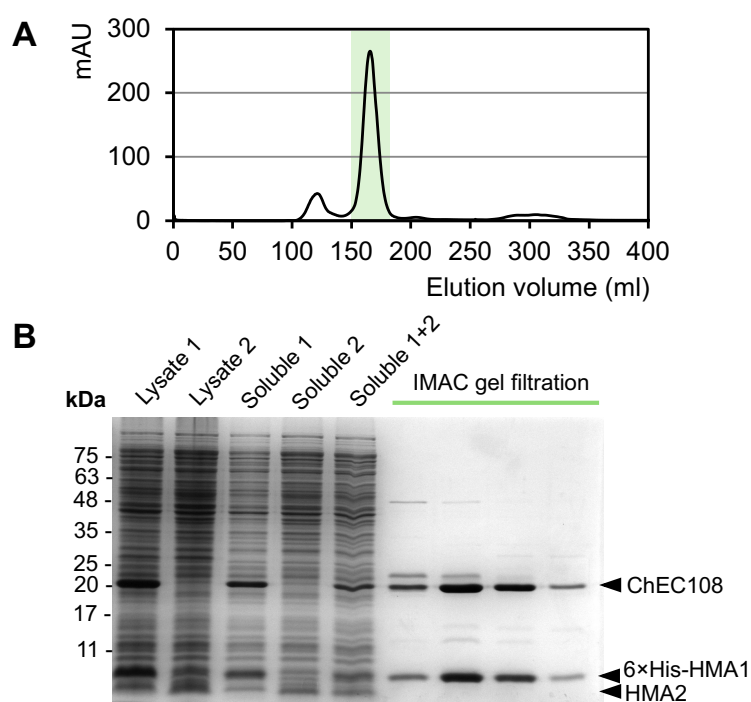


Figure 3.14 – HMA2 does not bind the ChEC108-HMA1 complex in vitro. ChEC108 (untagged, 22.6 kDa) and 6×His-HMA1 (10.9 kDa) were co-expressed in 2 l of SHuffle *E. coli* (1). Independently, HMA2 (untagged, 8.2 kDa) was expressed in 2 l of SHuffle *E. coli* (2). **A.** The soluble fractions from cells harvested from (1) and (2) were combined, incubated at 4 °C for 1 h, and loaded onto an IMAC column, followed by gel filtration of the column eluate. mAU = milli-absorbance units at 280 nm. **B.** Examination of cell lysate, soluble fractions and gel filtration fractions 150-180 ml by SDS-PAGE.

3.2.6 Crystallisation of the ChEC108-HMA1 and ChEC108-HMA2 complexes

In the absence of a ChEC108-HMA1-HMA2 tripartite complex, I sought to crystallise ChEC108-HMA1 and ChEC108-HMA2 independently. A SHuffle *E. coli* strain was generated to co-express untagged ChEC108 with 6×His-HMA1, enabling purification of both proteins in complex (Figure 3.15A) using the same strategy described in section 3.2.4 for ChEC108-tHMA. In parallel, a ChEC108-HMA2 complex was purified from SHuffle *E. coli* expressing untagged ChEC108 with 6×His-HMA2 (Figure 3.15B). Encouragingly, few low molecular weight contaminating bands were observed during the final gel filtration steps, suggesting these bipartite complexes might be more stable than ChEC108-tHMA.

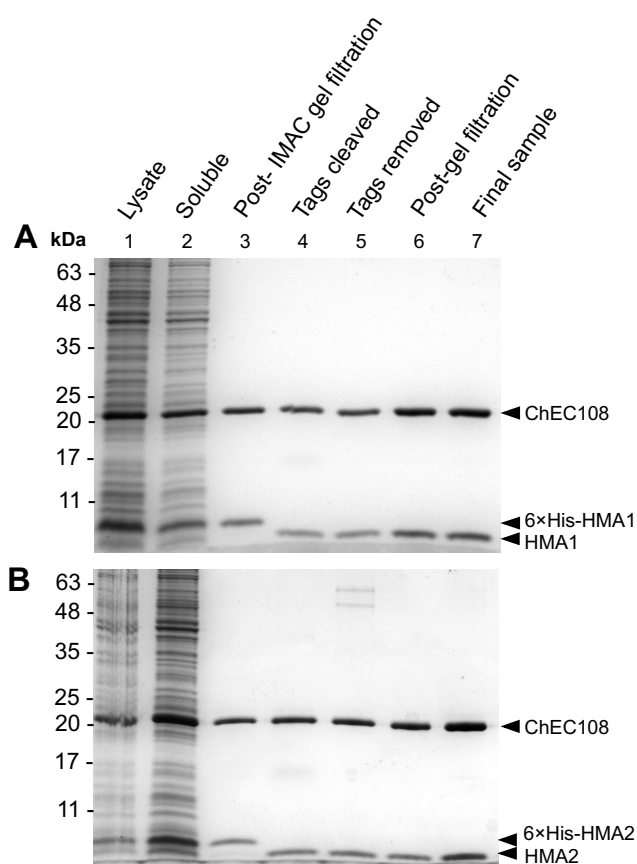


Figure 3.15 – Co-purification of ChEC108 with HMA1 or HMA2 from *E. coli*. **A.** ChEC108 (untagged, 22.6 kDa) was co-expressed with 6×His-HMA1 (10.9 kDa, **A**) or 6×His-HMA2 (10.1 kDa, **B**). Proteins of interest were visible in the lysate and soluble fraction of harvested cells (lanes 1-2). IMAC and gel filtration were used to enrich for His-tagged proteins and their binding partners (lane 3). 6×His tags were cleaved using 3C protease (lane 4). Free 6×His tags and 6×His-3C protease were removed using IMAC (lane 5). Gel filtration and concentration steps were carried out to increase purity of the final product (lanes 6-7).

I attempted to crystallise ChEC108-HMA1 using protein concentrations of 20 mg/ml or 10 mg/ml in four different two-drop screens: PEGs Suite, ProPlex, ShotGun™ (SG1™), and one custom screen provided by the John Innes Centre Structural Biology Platform, called

Keep it Simple (KISS). Crystalline material was observed in the SG1 plate under the following conditions: 20% (w/v) PEG 3350 with 0.2 M CaCl_2 dihydrate, and fluorescence under UV light suggested this material contained protein as opposed to purely salt from the precipitant solution (Figure 3.16A, E). As the small size and excessive clustering of these crystals made them unsuitable for X-ray diffraction, optimisation of crystal morphology was attempted using a range of PEG 3350 concentrations (10-30%). However, all emerging crystals had a similar small, clustered shape (Figure 3.16B, E) and no further optimisation was attempted with this precipitant.

In addition to the SG1 hit, ChEC108-HMA1 crystals were later observed in single drops sharing the same well conditions in both the ProPlexTM and PEGs Suite screens: 25% (w/v) PEG MME2K with 0.1 M HEPES (7.5 pH). These crystals resembled thin plates, growing from a cluster of precipitated protein (Figure 3.16C-E). In an attempt to reproduce this result and grow larger, thicker crystals, I set up an optimisation screen with varying PEG MME2K concentrations (22-28%). However, no crystalline material was observed. Nevertheless, with the help of Julia Mundy, the crystals pictured in Figure 3.16C and D were harvested with cryoprotectant (20% ethylene glycol) and submitted for X-ray diffraction.

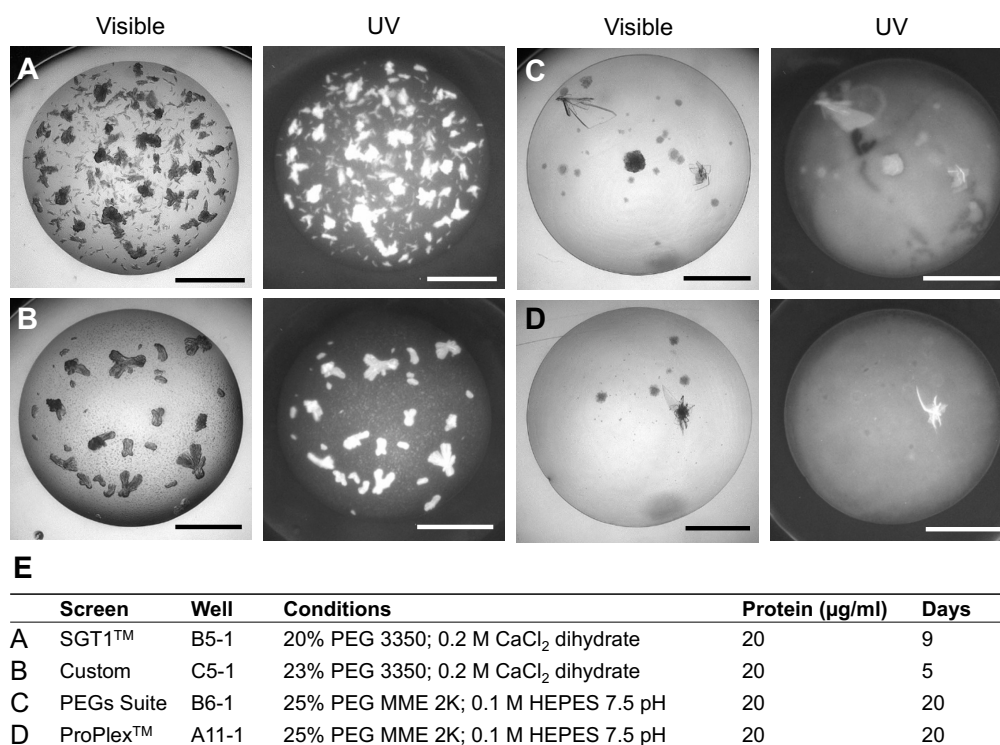


Figure 3.16 – Crystallisation of the ChEC108-HMA1 complex. A-D. Protein crystals photographed under visible and UV light. Scale bar = 500 μm . E. Conditions that yielded crystals pictured in A-D.

In parallel, using protein concentrations of 20 mg/ml or 10 mg/ml, I set up four two-drop screens with the aim of crystallising ChEC108-HMA2: Morpheus, PEG Suite, ProPlex™ and SG1™. Surprisingly, crystals were observed in a plethora of drops containing PEG precipitant, buffered between pH 6.5-8.5. Crystal morphology consisted of thin shards or plates of varying thickness and area, often clustered or arising from a protein aggregate. A selection of plate-like crystals (Figure 3.17A-G) were harvested by Julia Mundy with cryoprotectant (20% ethylene glycol, where necessary) and submitted for X-ray diffraction. Unfortunately, all samples diffracted weakly and warping of certain plate-like crystals resulted in distorted diffraction patterns, meaning high resolution data for structure determination could not be acquired.

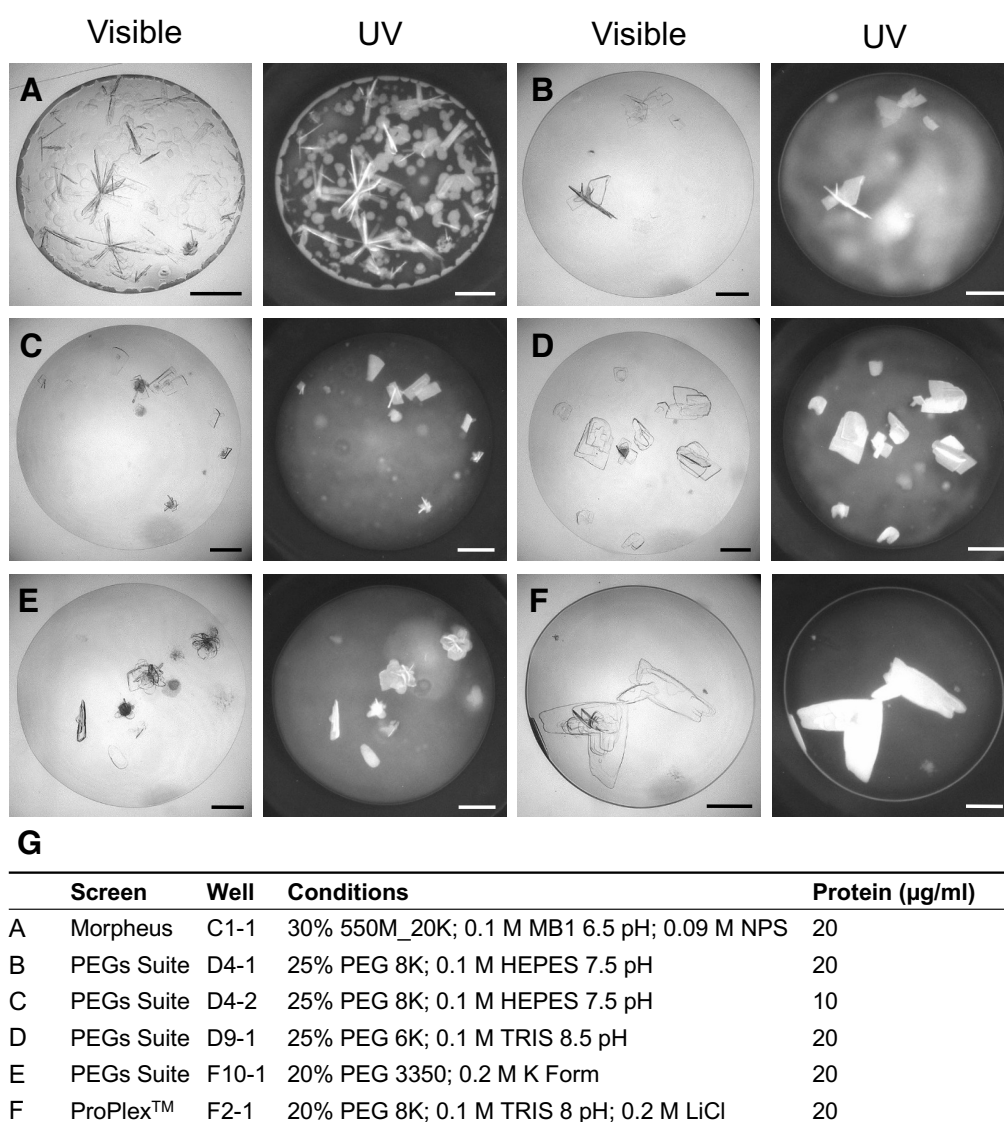


Figure 3.17 – Initial crystallisation of the ChEC108-HMA2 complex. A-F. Protein crystals photographed under visible and UV light 17 days post set-up and subsequently selected for X-ray diffraction. Scale bar = 250 µm. G. Conditions that yielded crystals pictured in A-F.

To encourage the formation of thicker ChEC108-HMA2 crystals with less tendency to cluster, I set up two optimisation screens to explore a range of PEG concentrations at pH 7.5 and pH 8.5. PEG 6K was chosen as a precipitant, given that an average weight of 6000 g/mol is within the range of PEG weights (3350 - 8000 g/mol) used to form the crystals displayed in Figure 3.17A-F (see Figure 3.17G for details). A range of shard- and plate-like crystals arose, including some that appeared thicker than those previously observed (Figure 3.18). Multiple of these more promising crystals were harvested with the aid of Julia Mundy, mixed with cryoprotectant (10 % glycerol, 10% ethylene glycol), and submitted for X-ray diffraction.

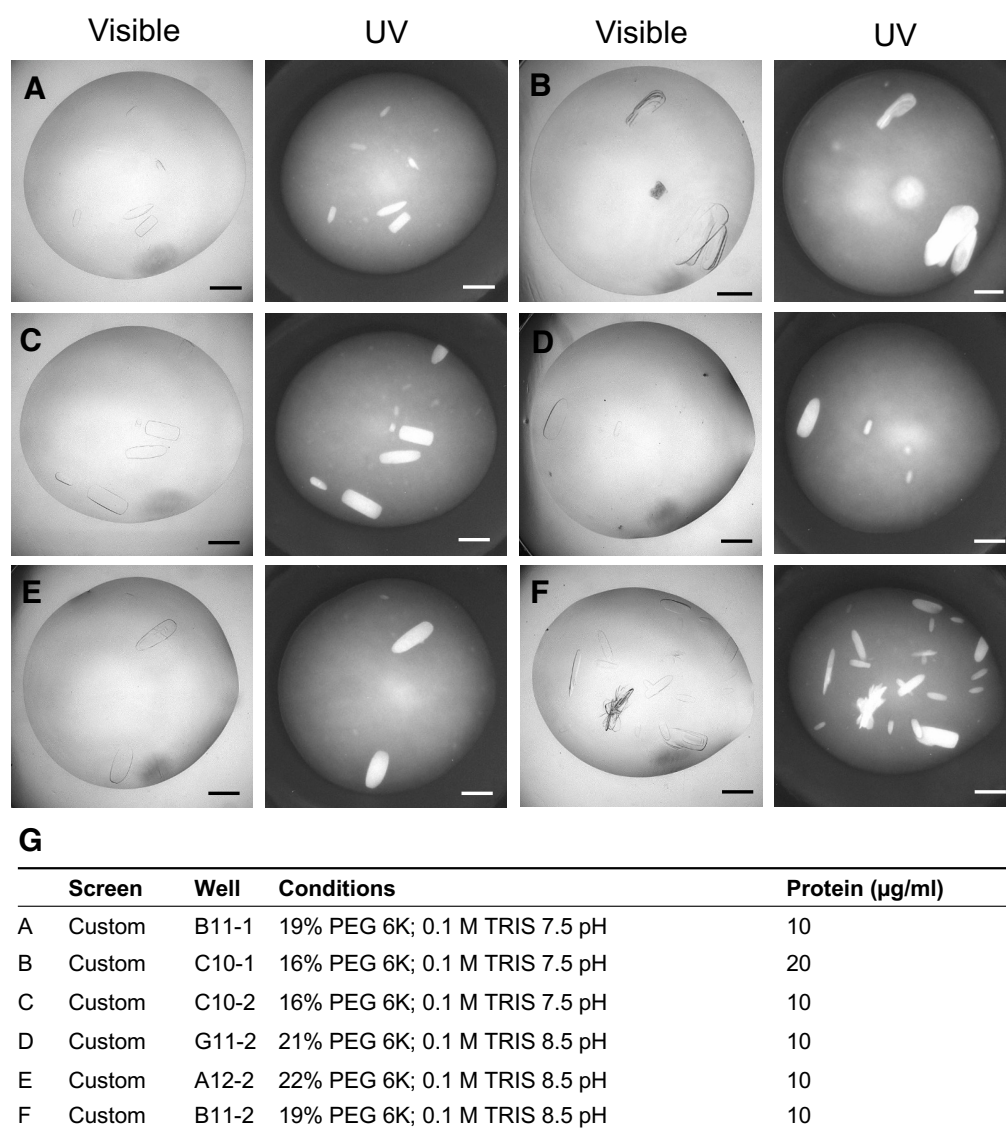


Figure 3.18 – Optimised crystallisation of the ChEC108-HMA2 complex. A-F. Protein crystals photographed under visible and UV light 7 days post set-up and subsequently selected for X-ray diffraction. Scale bar = 250 μm. G. Conditions that yielded crystals pictured in A-F.

3.2.7 Processing ChEC108-HMA1 and ChEC108-HMA2 X-ray diffraction data

X-ray diffraction data were collected from ChEC108-HMA1 crystals at beamline i24 of the Diamond Light Source synchrotron, operated remotely by Prof. David Lawson and Julia Mundy. Weak diffraction was observed, likely owing to the thin, plate-like nature of the crystals. Nevertheless, a diffraction dataset (comprising 3600 images, representing a 360° rotation of the crystal in the beamline) was collected from the crystal pictured in 3.16A (shown mounted in Figure 3.19A) at a wavelength of 0.619 Å and oscillation angle of 0.10°. An expected resolution of 2.5 Å was assigned, based on examination of three prior test images.

Meanwhile, X-ray diffraction data were collected from ChEC108-HMA2 crystals at beamline i04. Across ten submitted samples, the strongest diffracting crystal was that pictured in Figure 3.18B (lower righthand corner of the drop), shown mounted in Figure 3.19B. A diffraction dataset (comprising 3600 images) was collected at a wavelength of 0.954 Å and oscillation angle of 0.10°. An expected resolution of 2.0 Å was assigned, based on examination of three prior test images. A selection of auto processing pipelines accessed via [Diamond ISPyB](#) indicated that the symmetry of the ChEC108-HMA2 crystal lattice could be described by the space group *P* 1, a triclinic group with the lowest possible symmetry. With this space group, due to a lack of symmetry-related atoms in each repeating unit of the lattice, it is typically difficult to obtain diffraction data with high multiplicity (that is, the number of measurements collected for each unique reflection), potentially lowering the accuracy of final (averaged) intensity measurements. In light of this, and considering collection of the first dataset caused minimal radiation damage to the crystal, a second dataset (comprising a further 3600 images) was collected and merged with the first.

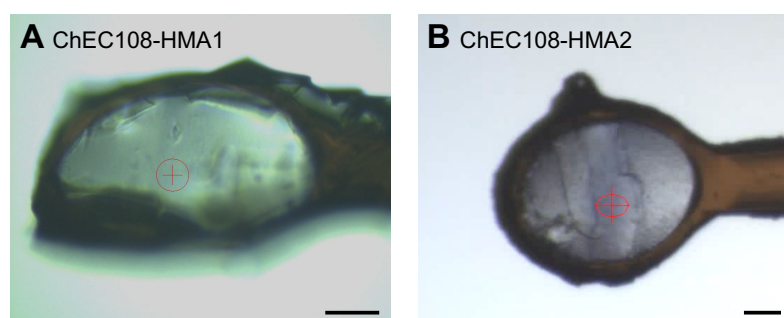


Figure 3.19 – Protein crystals mounted prior to X-ray diffraction. A. ChEC108-HMA1 crystal, as pictured in Figure 3.16A. B. ChEC108-HMA2 crystal, as pictured in Figure 3.18B. Scale bar = 50 µm.

With the aid of Dr Adam Bentham and Prof. Mark Banfield, I first processed the data collected from ChEC108-HMA2 crystals, given their stronger diffraction versus ChEC108-HMA1 crystals. Auto-processed data obtained via the *xia2* dials pipeline ([Winter, 2010](#)), were exported from [Diamond ISPyB](#). Scaled, unmerged data were imported into ccp4i2 v.8.0 ([Agirre et al.,](#)

2023) and processed using *AIMLESS* (Evans and Murshudov, 2013). Initially, all available data were processed, and the data collection statistics calculated by *AIMLESS* used to guide exclusion of data points (images) in subsequent processing steps. In an attempt to increase the overall consistency of the data, variation in the half-dataset correlation coefficient [CC(1/2)] was studied to decide on an appropriate maximum resolution cut-off of 2.1 Å. A space group of *P* 1 was confirmed by *AIMLESS*, and the overall R_{merge} of processed data was 13.9%, indicating reasonable precision of measurements (internal consistency) within the dataset.

Phases were solved by molecular replacement, using *Phaser* (McCoy et al., 2007) in expert mode to allow the use of two search models (one for ChEC108, and another for HMA2). AlphaFold2 predicted structures were used as search models (Figure 3.8A,E), and a unique solution was found with a translation function (TF) Z-score of 6.8, indicative of sub-optimal phasing (considering a score of > 8 generally indicates success). Cell contents analysis conducted by *Phaser* revealed a high probability (0.57) that the asymmetric unit of ChEC108-HMA2 crystals contained two copies of the complex, whereas the probability that it contained one copy was calculated to be 0. Thus, molecular replacement was repeated using two copies each of the ChEC108 and HMA2 search models. A more promising solution was reached with a TF Z-score of 12.29. Automatic model building was subsequently carried out using *Bucaneer* (Cowtan, 2006) to re-build the molecular replacement model into the electron density map. Further manual building (including correction of errors made by *Bucaneer*), refinement and addition of water and ligand molecules were carried out in *Coot* (Emsley et al., 2010), followed by refinement in *REFMAC5* (Murshudov et al., 2011), all the while using the R factor (R_{work}) and free R factor (R_{free}) calculated by *REFMAC5* as an indicator of model quality.

R factors represent the discrepancy between the observed X-ray data and the manually-refined atomic model, such that a low R factor (typically between 0.1-0.35) suggests a model is of high quality, fitting the observed data well. R_{free} is calculated for cross-validation using a random portion of the data excluded from refinement (5% in this case). My initial refinement attempts yielded an atomic model with R_{work} and R_{free} statistics of 0.19 and 0.24, respectively. This model was reviewed and improved further by excluding poorer quality data (images 10201-10500 and 12001-12300) and assigning a more modest resolution cut-off (2.2 Å) during the data reduction step (for final data processing statistics, see Table 3.3). Following additional manual adjustments, R_{work} and R_{free} were lowered to 0.18 and 0.23, respectively, indicating the model was of sufficient quality to proceed with further analysis.

X-ray diffraction data for the ChEC108-HMA1 complex were auto-processed via the *xia2* pipeline and processed using *AIMLESS*, as above for ChEC108-HMA2. After an initial *AIMLESS* run, I studied per-image variation in R_{merge} to decide whether to exclude portions of

Table 3.3 – X-ray data collection statistics for the ChEC108-HMA1 and ChEC108-HMA2 complexes. Statistics were calculated by *AIMLESS*, implemented in *ccp4i2*.

Statistic		ChEC108-HMA1	ChEC108-HMA2
Wavelength (Å)		0.6199	0.9537
Space group		<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 1
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)		34.91, 76.64, 99.21	37.70 60.11, 77.12
Resolution (Å)	Overall	35.74 - 2.75	72.61 – 2.20
	Outer shell	2.90 – 2.75	2.27 – 2.20
<i>R</i> _{merge} (%)	Overall	18.9	11.4
	Outer shell	91.7	85.2
<i>I</i> / σ <i>I</i>	Overall	7.9	9.0
	Outer shell	2.1	1.3
Completeness (%)	Overall	100	99.4
	Outer shell	100	96.7
Unique reflections	Overall	7405	32007
	Outer shell	1060	2659
Redundancy	Overall	8.7	6.1
	Outer shell	9.1	4.4
CC(1/2) (%)	Overall	99.3	99.6
	Outer shell	85.6	74.8

data, given that significant increases in this statistic could indicate misalignment of the crystal in the X-ray beam, or radiation damage towards the end of a collection cycle. For these reasons, images 1-100 and 3000-3600 were excluded. A maximum resolution cut-off of 2.75 Å was applied to improve data consistency. The space group *P* 2₁ 2₁ 2₁ was assigned by *AIMLESS*, and the overall *R*_{merge} of processed data was 20.5%, indicating an acceptable level of internal data consistency.

Phases were solved by molecular replacement using expert *Phaser*, as above for ChEC108-HMA2. Initially, I used AlphaFold2 predicted structures of ChEC108 and HMA1 (Figure 3.8A, C) as search models, and a unique solution was found with a TF Z-score of 6.8, indicative of sub-optimal phasing. Considering the low resolution of this data, further model building and refinement would have been extremely challenging. As an alternative strategy, due to the high similarity of predicted HMA1 and HMA2 structures (Figure 3.8C vs E), the newly-solved ChEC108 and HMA2 crystal structures were exploited as templates for molecular replacement. Encouragingly, a unique solution was found with a TF score of 19.8, indicative of successful phasing. Omitting the automatic model building step, I instead used *Coot* to

manually mutate the HMA1 molecular replacement model, replacing its primary sequence with that of HMA2. Further manual adjustments, including addition of a limited number of water molecules, were carried out in *Coot*, followed by refinement with *REFMAC5*.

Due to the lower resolution of this dataset relative to that collected from the ChEC108-HMA2 crystal, positioning of side chains and water molecules was considerably more challenging. My initial refinement attempts yielded an atomic model with R_{work} and R_{free} statistics of 0.19 and 0.31, respectively. Such divergence of R_{work} and R_{free} (typically a > 0.05 difference) indicates overfitting, that is, when a model fails to describe unseen data effectively due to the influence of noise or errors made during the refinement process. In an attempt to remedy this, additional images judged to be of poorer quality (601-1050) were excluded and the data reduction step repeated (for final data processing statistics, see Table 3.3). Further manual adjustments were made to some of the challenging regions of the model, bringing R_{work} and R_{free} to 0.20 and 0.29, respectively. Considering the sub-optimal nature of the original diffraction data, further convergence of the R statistics was likely impossible, and so this final model was deemed ready for further analysis. Final refinement and model-building statistics for both the ChEC108-HMA2 and ChEC108-HMA1 structures are listed in Table 3.4.

Table 3.4 – Structure refinement and model statistics. Values were calculated by *REFMAC*, implemented in *ccp4i2*, or by MolProbity (Davis et al., 2007) (*). RMSD = root mean square deviation.

Statistic		ChEC108-HMA1	ChEC108-HMA2
Resolution range (Å)		35.77 - 2.75	72.60 - 2.20
Reflections (all / free)		7368 / 379	31998 / 1663
R _{work} / R _{free}		0.204 / 0.295	0.176 / 0.226
Atoms	Amino acids	2010	4069
	Ions	1	4
	Ligands	0	32
	Water	19	292
B-factors	Amino acids	36.29	45.44
	Ions	35.62	56.02
	Ligands	0	52.21
	Water	39.32	44.55
RMSD	Bond lengths (Å)	0.0081	0.0139
	Bond angles (°)	1.61	2.25
Ramachandran *	Favoured	218 (91.21%)	477 (96.75%)
	Allowed	20 (8.37%)	16 (3.25%)
	Outliers	1 (0.42%)	0 (0.00%)
Clash score *		2.44	0.85
MolProbity score *		6.72	1.29

3.2.8 ChEC108 binds HMA1 and HMA2 via the same interface

Considering a tripartite complex comprising ChEC108, HMA1 and HMA2 could not be purified (3.2.5), I hypothesised that both HMA domains may compete for the same binding site on ChEC108. Crystal structures of ChEC108-HMA1 and ChEC108-HMA2 support this, revealing 1:1 effector-HMA complexes of remarkable similarity (Figure 3.20A-C). As anticipated, despite sharing only 45% sequence identity, HMA1 and HMA2 displayed similar HMA folds, and could be superimposed with root mean square deviation (RMSD) = 1.084 Å across all 66 C α pairs, suggesting their peptide backbones are closely aligned. When the ChEC108 atomic models from both crystal structures were superimposed, RMSD = 0.758 Å across all 178 C α pairs, suggesting the effector adopts the same conformation when bound to either HMA domain. This conformation is highly similar to that predicted by AlphaFold2 (Figure 3.8A), which is unsurprising given this model was utilised successfully as a search model during molecular replacement. While it seems AlphaFold2 was able to predict the individual structures of ChEC108, HMA1 and HMA2 with high accuracy, were the effector-HMA interfaces predicted by AlphaFold2 Multimer (Figure 3.7) also correct?

Surprisingly, crystal structures revealed that HMA1 and HMA2 occupied a binding site on ChEC108 involving three α -helices (α -1, α -2 and α -5), that was not observed in any predicted models involving HIPP6, tHMA, or HMA domains in isolation (Figures 3.6 and 3.7). On the HMA side, it is predominantly α -1 that interacts with the effector, with additional contacts provided by the loop connecting β -strand-1 to α -1, and that connecting α -2 to β -4. A metal ion was identified at equivalent positions within both the ChEC108-HMA1 and ChEC108-HMA2 binding interfaces, coordinated by three HMA-derived residues (His33, Cys34 and Cys37 from HMA1, or His163, Cys164 and Cys167 from HMA2), and one ChEC108-derived residue (Asp164 in both structures) (Figure 3.20A-B). Notably, these four Cys residues belong to the putative metal-binding motifs (M/LXCXXC) of HIPP6 (Figure 3.20C).

To study ChEC108-HMA binding in more detail, I used QtPISA (for QuickTime Protein Interfaces, Surfaces and Assemblies) (Krissinel and Henrick, 2007, implemented in ccp4i2), a software widely used to calculate interface properties such as binding energy and hydrophobicity, and determine ranks for each parameter based on statistical analysis of all interfaces in the PDB. However, as QtPISA does not factor metal-ligand coordinate bonds into its calculations, which likely contribute significantly to ChEC108-HMA binding affinity, I deemed these measures unsuitable for describing the interfaces in their entirety. Nevertheless, QtPISA was used to identify additional intermolecular bonds likely important for enhancing stability of the complexes and conferring specificity. For ease of presentation, the ChEC108-HMA1/2 interfaces can be divided into three regions (Figure 3.21A). As displayed in Figure 3.21B, the first involves negatively charged ChEC108 residues Asp22 and Glu90, which each form salt bridges with a conserved Arg residue of HMA1 or HMA2 (Arg43 or Arg173, respectively). Here, hydrogen bonds are also established between carbonyl oxygens along the peptide backbone of ChEC108, and side chains of Lys, Arg and (in the case of HMA2) His residues of the HMA domains. In this region, the side chain of ChEC108 Trp78 exhibits a pi-pi interaction, stacking favourably against the hydrophobic region of Lys39 (HMA1) or His169 (HMA2) side chains. An additional salt bridge is conserved within the ChEC108-HMA1 and ChEC108-HMA2 interfaces, where Asp89 of ChEC108 contacts Lys40 (HMA1) or Lys170 (HMA2) (Figure 3.21C). Closer inspection of the metal-binding sites revealed two ChEC108 residues (Arg157 and Glu160) which likely support the position of metal coordinating Cys residues (Cys34 in HMA1 or Cys164 in HMA2), given their close proximity (Figure 3.21D).

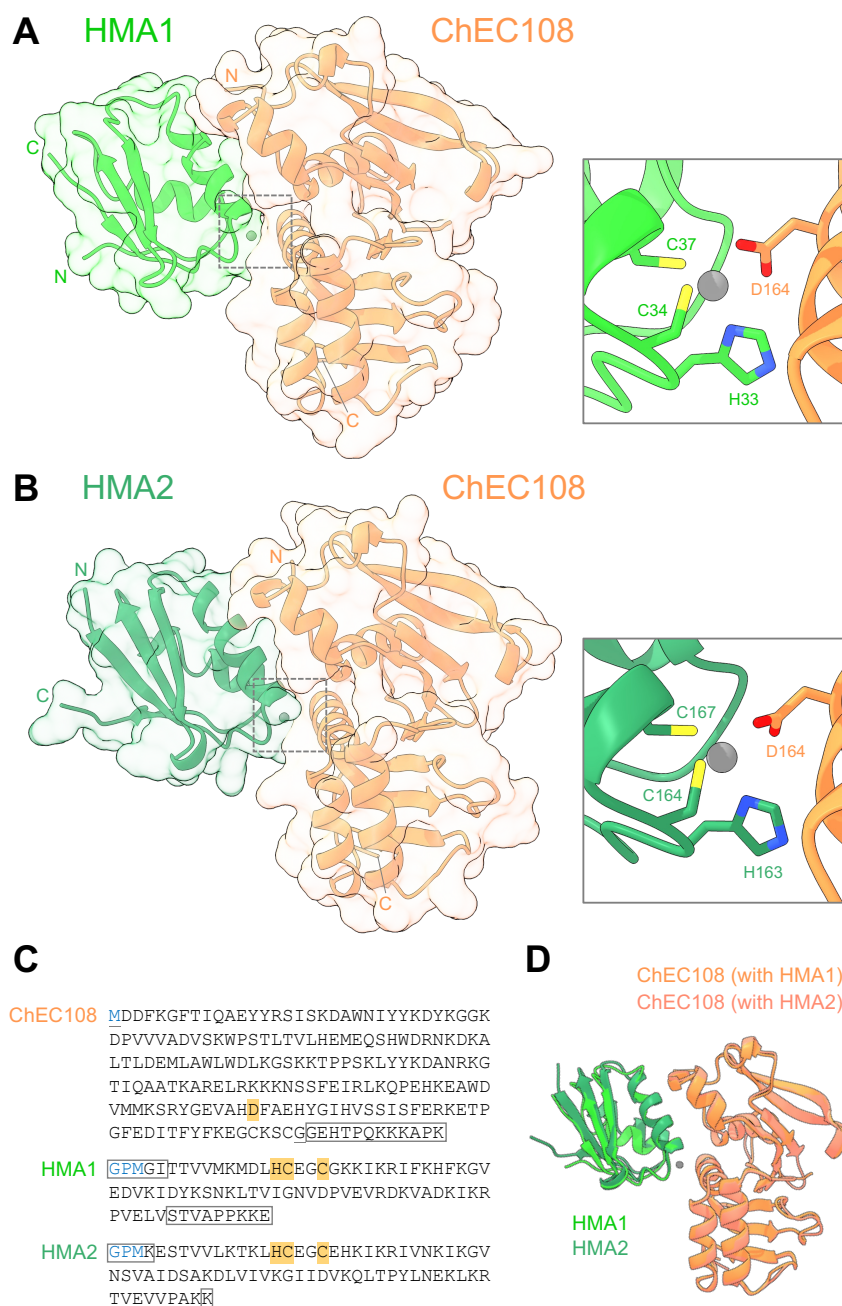


Figure 3.20 – ChEC108 binds HMA1 or HMA2 via a common binding interface with a coordinated metal ion. A-B. Crystal structures of the ChEC108-HMA1 (A) and ChEC108-HMA2 (B) complexes. Grey boxes show close-up views of metal-binding sites for each complex. C. Primary sequences of chains displayed in A-B. Residues involved in metal coordination are highlighted in yellow. Residues in blue indicate non-native N-terminal scars necessary for recombinant protein production. Boxed residues could not be included in atomic models due to a lack of electron density to inform their positioning during refinement. Underlined residues at the N- and C-terminus of ChEC108 were absent from the ChEC108-HMA2 and ChEC108-HMA1 models, respectively, for the same reason. D. Overlay of crystal structures from A and B, performed by ChimeraX using the matchmaker command.

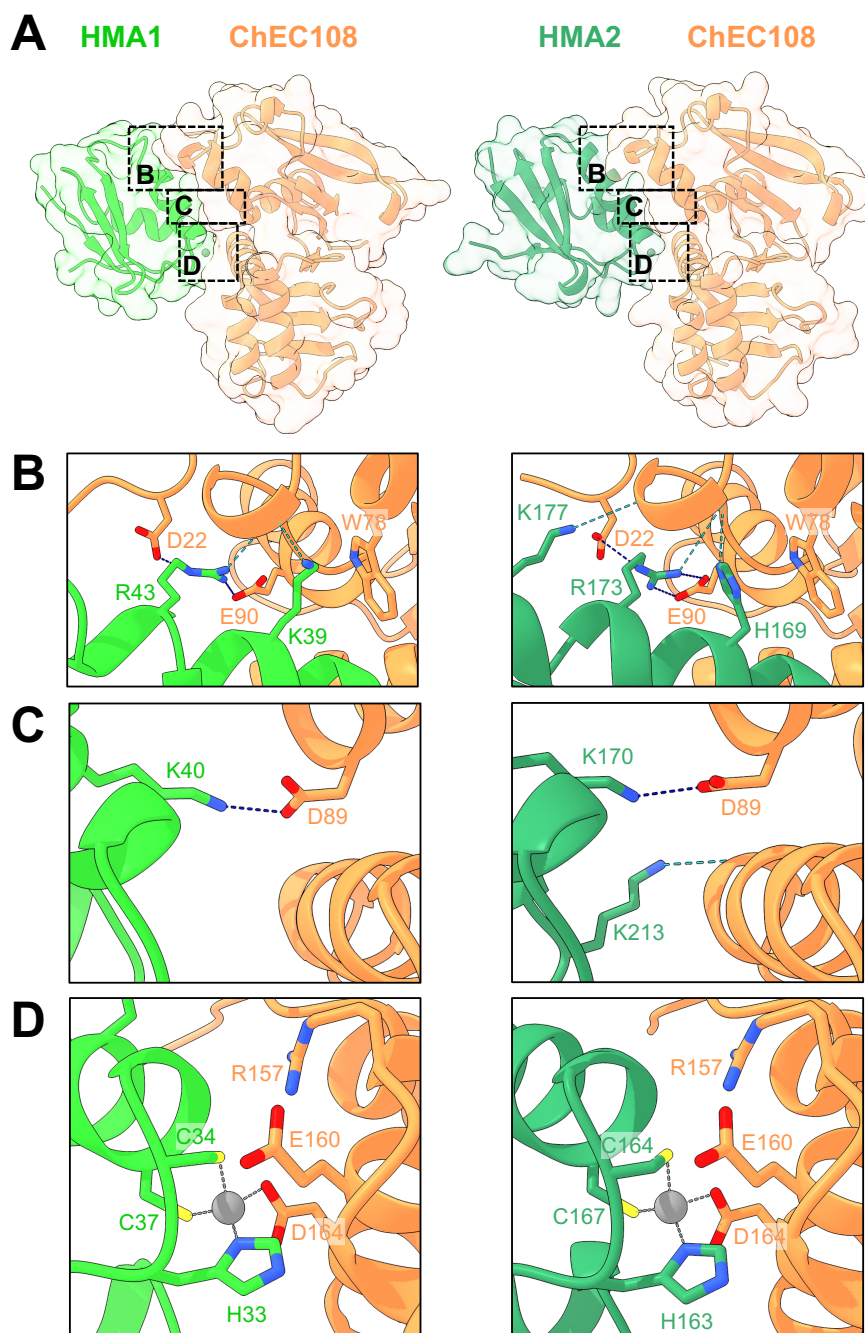


Figure 3.21 – Intermolecular bonds at the ChEC108-HMA1/2 binding interfaces. **A.** Black boxes highlight regions of the ChEC108-HMA1 (left) and ChEC108-HMA2 (right) binding interfaces, shown in more detail in corresponding panels **B-D** below. Intermolecular bonds are represented as dashed lines, with hydrogen bonds coloured cyan, salt bridges coloured blue and coordinate bonds between amino acid side chains and metal ions (spheres in panel **D**) coloured grey.

Examination of electrostatic potential revealed highly charged surface patches at ChEC108-HMA binding interfaces. Specifically, a positively charged patch formed by four residues of HMA1 (Lys39, Lys40, Arg43 and Lys83) or HMA2 (His169, Lys170, Arg173 and Lys213) contacts a negatively charged patch formed by six residues of ChEC108 (Asp22, Asp89, Glu90, Glu160, Asp164 and Glu167) (Figure 3.22). In combination with the strong coordinate bonds and stabilising salt bridges and hydrogen bonds described above, such charge complementarity could make ChEC108-HMA1/2 interactions especially favourable.

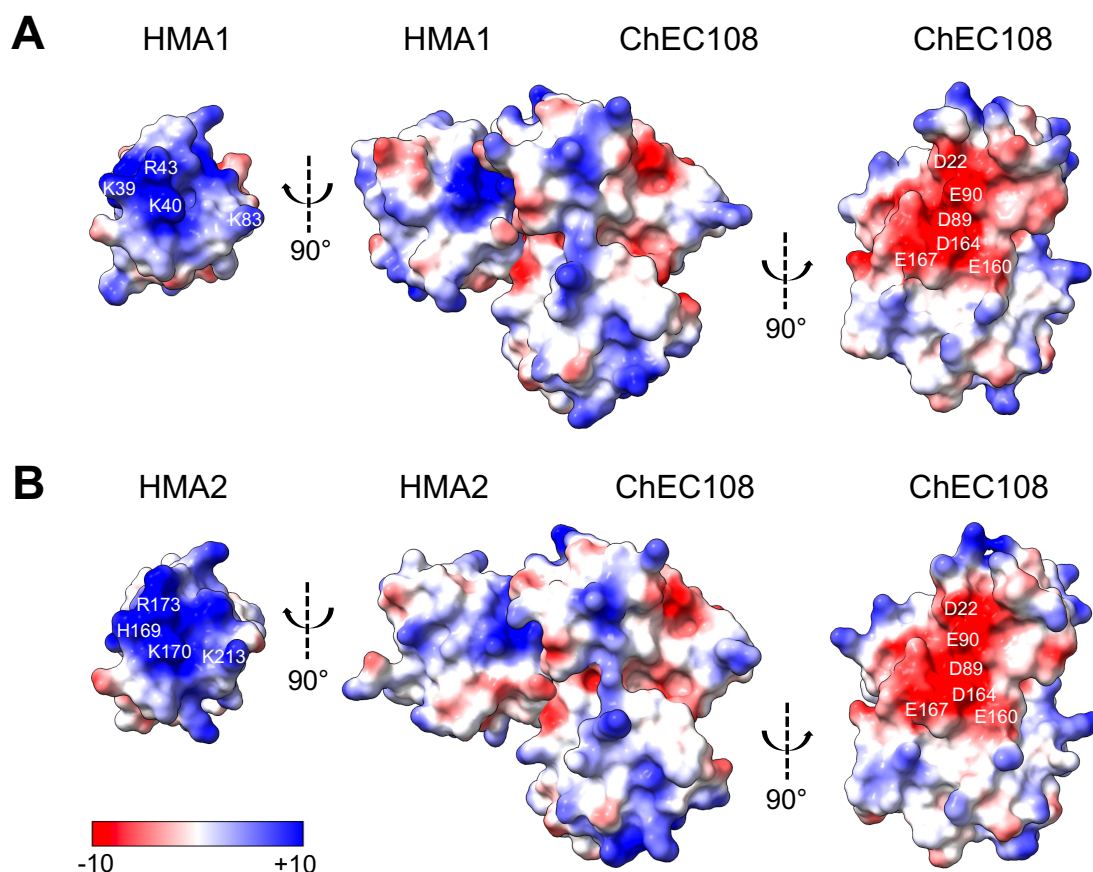


Figure 3.22 – Charge complementarity strengthens ChEC108-HMA binding interfaces. A-B. Surface rendering of ChEC108-HMA1 (A) and ChEC108-HMA2 (2) crystal structures, coloured according to electrostatic potential. Monomers of ChEC108 and HMA1/2 have been rotated to view charged surfaces otherwise buried in the binding interfaces.

To identify the metal ion captured at the ChEC108-HMA2 interface, I submitted further crystals for X-ray fluorescence scanning at Diamond Light Source. Such crystals were obtained in a custom optimisation screen and were similar in morphology to those presented in Figure 3.18. At beamline i04, operated remotely by Prof. David Lawson and Julia Mundy, ChEC108-HMA2 crystals were exposed to high energy (13,000 electron volts, or eV) incident X-rays, and the energy of emitted X-rays quantified to indicate the presence of heavy elements. The

resulting spectrum exhibited two peaks in energy at ~8600 eV and ~9600 eV, suggestive of Zn presence (Figure 3.23), which, given the biological context of the element, must be in the Zn^{2+} state. While the identity of the metal ion associated with ChEC108-HMA1 was not experimentally determined due lack of available crystalline material, Zn^{2+} is likely present, considering (1) the striking similarity of the coordination site with that in the ChEC108-HMA2 complex (Figure 3.20A-B), (2) the fact that similar procedures and buffers were used to purify both samples from *E. coli*, and (3) conditions that enabled the growth of crystals for structure determination were not supplemented with alternative heavy metals (Figures 3.16 and 3.18).

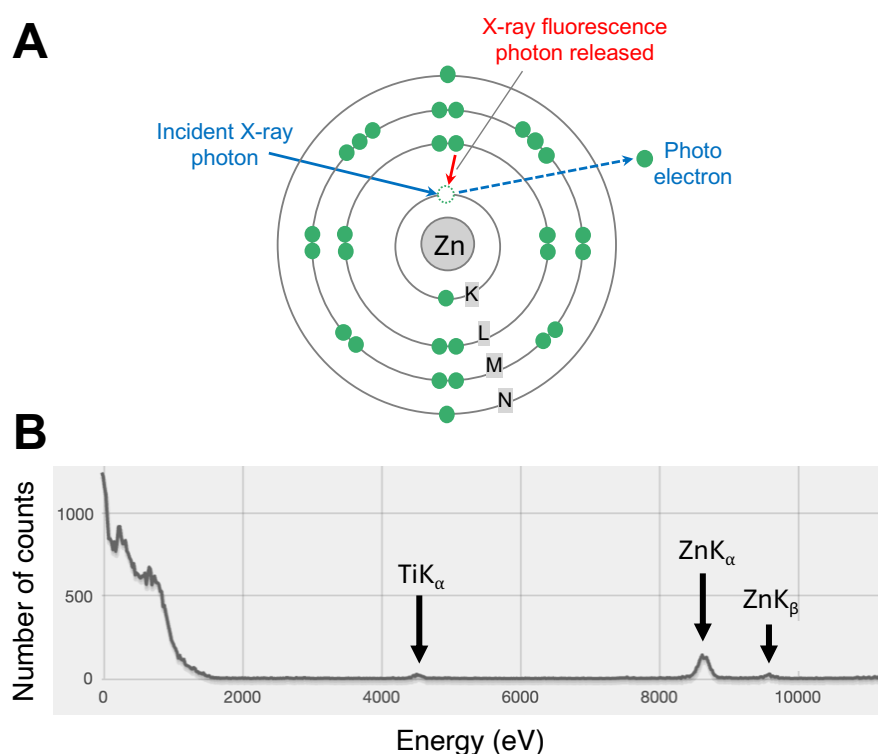


Figure 3.23 – Zn was detected in ChEC108-HMA2 protein crystals. **A.** Schematic of a Zn atom to illustrate the principle of X-ray fluorescence, with electrons represented as green circles. High energy incident X-ray photons eject electrons from the inner (K) shell of the atom. When electrons from outer shells fall to the K shell, excess energy is released in the form of X-ray fluorescence. The energy of the emitted X-rays is characteristic of the element from which they originate. **B.** X-ray fluorescence spectrum collected from protein crystals of the ChEC108-HMA2 complex. Arrows highlight peaks in spectral counts at ~8600 eV and ~9600 eV, indicative of ZnK_{α} and ZnK_{β} , respectively. The peak at 4500 eV can be attributed to Ti, a contaminant introduced during the scan. K_{α} represents X-rays emitted as electrons fall from the L-shell to the K-shell. K_{β} indicates X-rays emitted as electrons fall from the M-shell to the K-shell.

3.2.9 HMA1-HMA2 association was not detected in vivo

The appearance of a metal ion in both the ChEC108-HMA1 and ChEC108-HMA2 crystal structures raises the possibility that, true to its name, HIPP6 might ordinarily bind metals in the absence of ChEC108. In support of this, AlphaFold2 predicted that the two HMA domains of HIPP6 are oriented with their metal-binding loops in close proximity, forming a putative metal-binding site (Figure 3.4C). Additionally, AlphaFold2 Multimer predicted that, when hypothetically isolated from the full length HIPP6 protein, HMA1 and HMA2 might homo- and heterodimerise (Figure 3.24A-B). Despite the low confidence of AlphaFold2 in these models, as reflected by the low ipTM scores (Figure 3.24A), at the interface of all predicted dimers, the HMA CXXC motifs are oriented in close proximity (Figure 3.24C), much like those in the full-length HIPP6 model. These predictions led me to hypothesise that HIPP6 might ordinarily coordinate a metal ion via the CXXC motifs of both HMA1 and HMA2, and that ChEC108 must therefore displace one of the domains upon HIPP6 binding, contributing its own Asp164 to keep the metal bound.

To test for HMA1-HMA2 association in vivo, I designed a co-IP experiment in *N. benthamiana* that involved fusing the HMA domains with two different epitope tags. Initial attempts using eGFP, mCherry and 3×FLAG tags were unsuccessful due to instability of the HMA fusions in *N. benthamiana*, especially those containing HMA2. To remedy this, bait domains were expressed as 4×Myc-TurboID fusions, while prey domains were expressed as 3×FLAG-TurboID fusions. As a relatively large (35.7 kDa), stable and soluble protein, at least when expressed in Arabidopsis or *N. benthamiana*, TurboID was chosen to act as an in planta stability tag, much like in vitro solubility tags commonly employed for recombinant protein expression in *E. coli*. 3×FLAG-TurboID and 3×FLAG-TurboID-ChEC108 were included as negative and positive controls, respectively. Evidence of HMA1-HMA1 homomer formation were reproducibly detected, while HMA1-HMA2 heteromers and HMA2-HMA2 homomers were not (Figure 3.25). Contrary to AlphaFold2 Multimer models described above, it seems that HMA1-HMA2 association is unlikely, or perhaps too weak to be detected in isolation from the remainder of the HIPP6 protein.

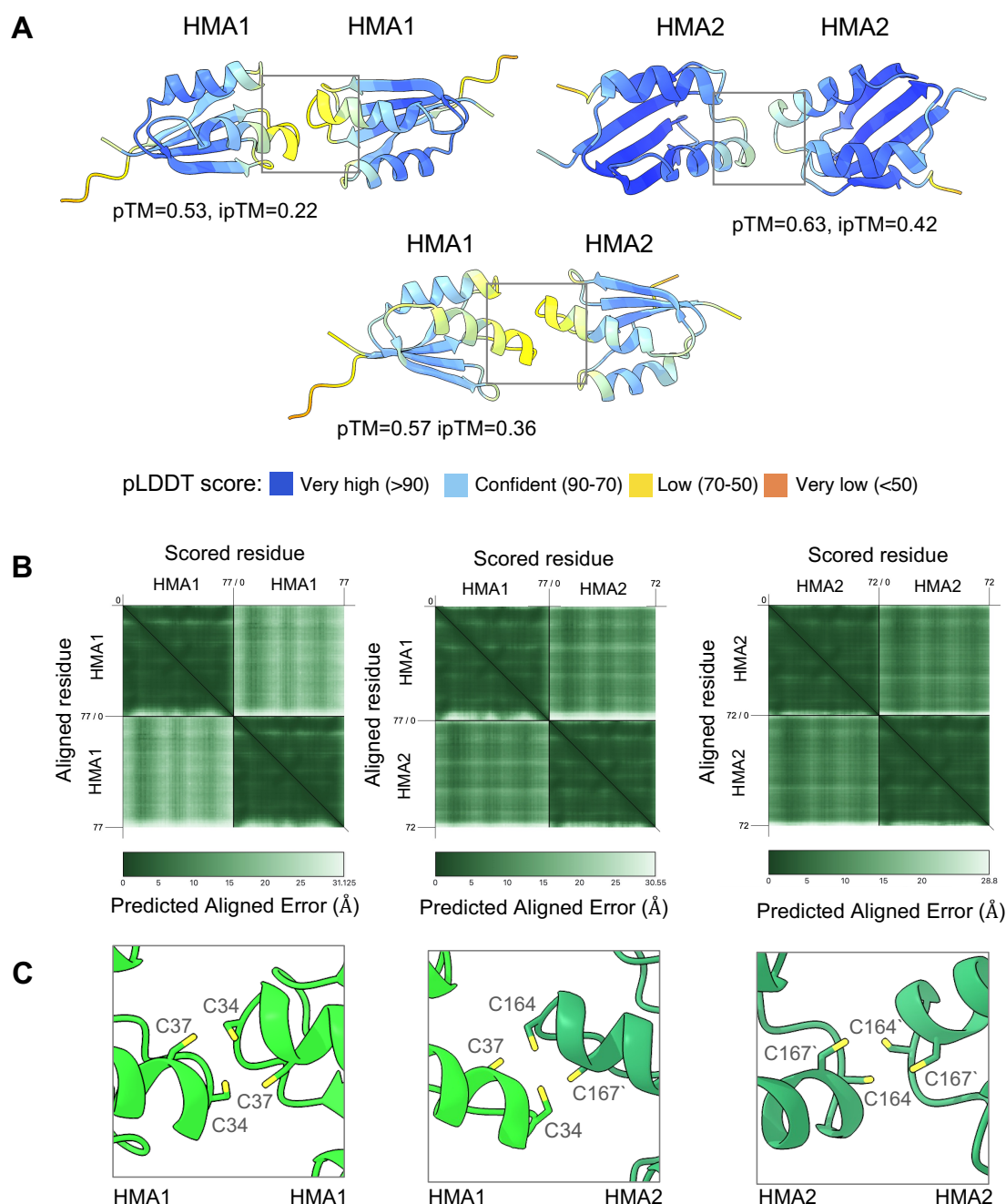


Figure 3.24 – Predicted dimerisation of HIPP6 HMA domains. **A.** AlphaFold2 Multimer (accessed via ColabFold v1.5.5) models of a HMA1 homodimers, HMA2 homodimer and HMA1-HMA2 heterodimer, coloured according to pLDDT confidence scores. Predicted Template Modelling scores for the overall structure (pTM) and interfaces (ipTM) are shown. **B.** Predicted aligned error (PAE) plots show the predicted error associated with relative distances of residues in dimer models shown in **A**. **C.** Zoomed in view of grey boxes in **A**, showing Cys residues of CXXC motifs, hypothesised to facilitate metal binding.

Nevertheless, the observation that HMA1 can self-associate raised the intriguing possibility of HIPP6 dimerisation *in vivo*, mediated by HMA1-HMA1 contacts. To investigate this, I performed a co-IP experiment in *N. benthamiana* using 4×Myc-TurboID-HIPP6 as bait, and probed for co-expressed 3×FLAG-TurboID-HIPP6 in the IP fraction. Again, 3×FLAG-TurboID and 3×FLAG-TurboID-ChEC108 served as negative and positive controls, respectively. Although accumulation of 3×FLAG-TurboID-HIPP6 was admittedly low in comparison to control fusions, across three independent repeats, HIPP6 self-association was not detected (Figure 3.26). In summary, at least in the *N. benthamiana* heterologous system, I was unable to detect evidence of metal-facilitated intra- or inter-HIPP6 interactions.

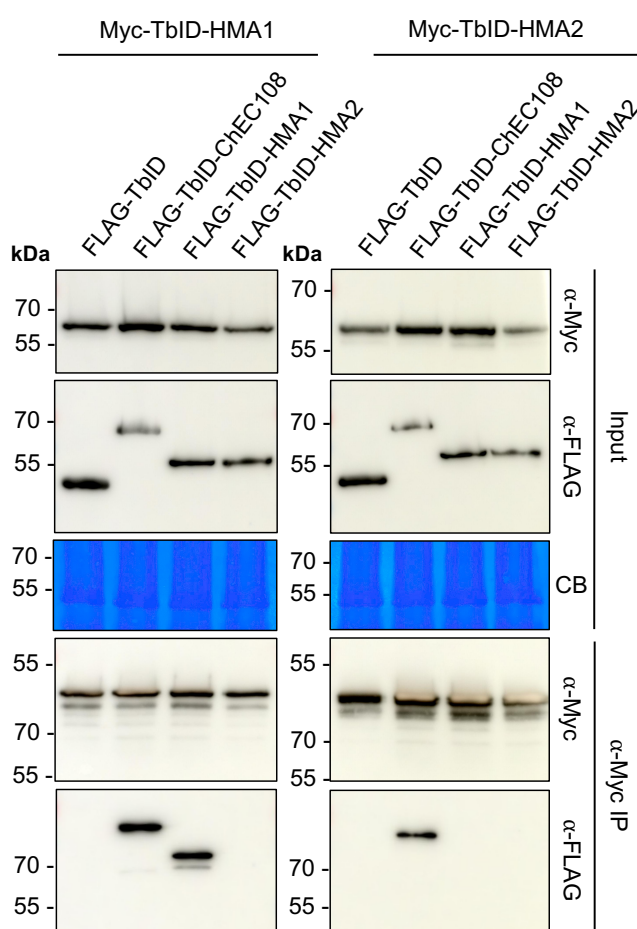


Figure 3.25 – HMA1-HMA2 association was not detected *in vivo*. Co-IP demonstrating HMA1 self-association, but not HMA1-HMA2 or HMA2 self-association. Bait and prey proteins were tagged with 4×Myc-TurboID (Myc-TbID) or 3×FLAG-TurboID (FLAG-TbID), respectively, to promote stability and enable immunodetection. FLAG-TbID and FLAG-TbID-ChEC108 were included as negative and positive controls, respectively. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. Representative of two independent experiments. Expected molecular weights (kDa): Myc-TbID-HMA1, 50.9; Myc-TbID-HMA2, 50.3; FLAG-TbID, 40.6; FLAG-TbID-ChEC108, 62.8; FLAG-TbID-HMA1, 49.2; FLAG-TbID-HMA2, 48.6.

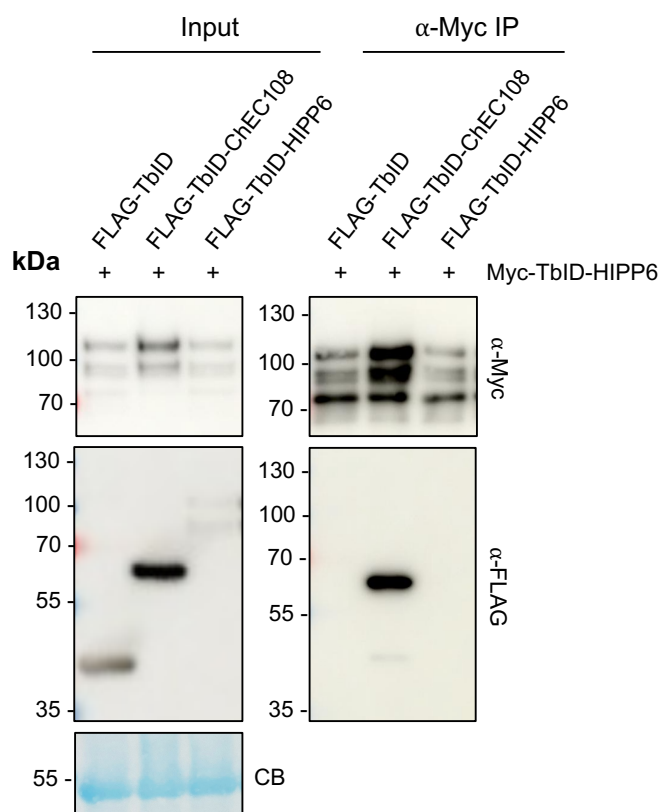


Figure 3.26 – HIPP6 self-association was not detected *in vivo*. Co-IP demonstrating no detectable HIPP6 self-association. Bait and prey proteins were tagged with 4×Myc-TurboID (Myc-TbID) or 3×FLAG-TurboID (FLAG-TbID), respectively, to promote stability and enable immunodetection. FLAG-TbID and FLAG-TbID-ChEC108 were included as negative and positive controls, respectively. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. Representative of three independent experiments. Expected molecular weights (kDa): Myc-TbID-HIPP6, 84.4; FLAG-TbID, 40.6; FLAG-TbID-ChEC108, 62.8; FLAG-TbID-HIPP6, 82.7.

3.2.10 Metal-binding residues are critical for ChEC108-HMA interactions

To validate the crystal structures presented in 3.2.8 and generate useful variants for probing the biological relevance of ChEC108-HIPP6 association in planta, binding interface mutations were designed with the goal of introducing steric clashes to compromise binding. As coordinate bonds identified in the ChEC108-HMA crystal structures are expected to strengthen the binding interfaces significantly, I hypothesised that the four metal-coordinating residues - Cys, Cys, His and Asp in both structures - would be critical for complex formation. I therefore designed three HIPP6 mutants with compromised metal-binding motifs: CXXC substituted for GxxG in HMA1 only (HIPP6^{M1}), HMA2 only (HIPP6^{M2}) or both HMA1 and HMA2 (HIPP6^{MM}) (Figure 3.27A). In parallel, a ChEC108 mutant was designed with a single amino acid substitution: Asp164 to Arg (ChEC108^{D164R}). In this case, exchanging a negatively charged Asp for a positive Arg is predicted to prevent coordinate bond formation with metals, while introducing a steric clash at the binding interface.

With these structure-guided mutants, I conducted a co-IP experiment in *N. benthamiana* and discovered that HIPP6^{M1} and HIPP6^{M2} retained their ability to associate with ChEC108 (Figure 3.27B). This suggests the loss of one metal-binding motif is insufficient to compromise complex formation. However, when HMA1 and HMA2 CXXC motifs were simultaneously mutated in HIPP6^{MM}, ChEC108 association was not detected (Figure 3.27C). Similarly, association of ChEC108^{D164R} with wild-type HIPP6 was not observed (Figure 3.27B). This result supports the hypothesis that metal coordination makes a significant contribution to ChEC108-HMA interfaces, and is essential for interaction.

After solving the structures of ChEC108 in complex with HMA1 and HMA2, multiple questions were raised: does ChEC108 have a higher affinity for one of the HMA domains over the other? How does the binding affinity compare to other effector-HMA interactions reported to date? Can alternative metal species substitute for Zn²⁺ at the binding interface? To help answer these questions, I sought to establish in vitro binding assays using purified ChEC108 and HIPP6 HMA domains, allowing the impact of buffer conditions and amino acid substitutions on binding affinity to be assessed quantitatively. As demonstrated in Figure 3.11, ChEC108 can be purified from BL21-AI *E. coli* in high quantities, making use of a 6×His affinity tag for IMAC. I initially attempted to purify HMA1 using the same strategy, beginning by collecting the soluble fraction of BL21-AI cell lysate, harvested from 6 l of culture expressing 6×His-HMA1. Subsequent IMAC and gel filtration resulted in an abundance of protein being eluted from the column, and inspection of the fractions via SDS-PAGE confirmed the presence of 6×His-HMA1 (Figure 3.28). However, the volume at which the protein eluted raised concern. Such early elution (within the void volume of the column) occurs when proteins are too large to interact with the gel matrix. Considering the small size of 6×His-HMA1 monomers (10.9 kDa), it is likely that the first peak fractions displayed in Figure 3.28A contained aggregates, which (assuming they cannot be disassembled without compromising protein integrity) would make the sample unsuitable for sensitive in vitro assays.

It is possible that the cellular environment offered by BL21-AI cells was sub-optimal for 6×His-HMA1 folding. I next attempted to purify 6×His-HMA1 from 6 l of SHuffle cells. As before, during gel filtration post-IMAC, a large peak in UV absorption was observed around the column's void volume, indicating potential aggregation of the protein (Figure 3.28B). However, an additional peak was observed around 200 ml, which likely represented aggregates of a lower molecular weight. Inspection of the fractions via SDS-PAGE revealed that both peaks contained 6×His-HMA1, but only the later-eluting peak fractions were carried forward for further purification, to avoid contamination of the final sample with aggregates. After cleavage of affinity tags and a second round of gel filtration, I obtained a sufficient yield of pure HMA1 protein to proceed with in vitro binding assays (Figure 3.28C-D). HMA2 was subsequently

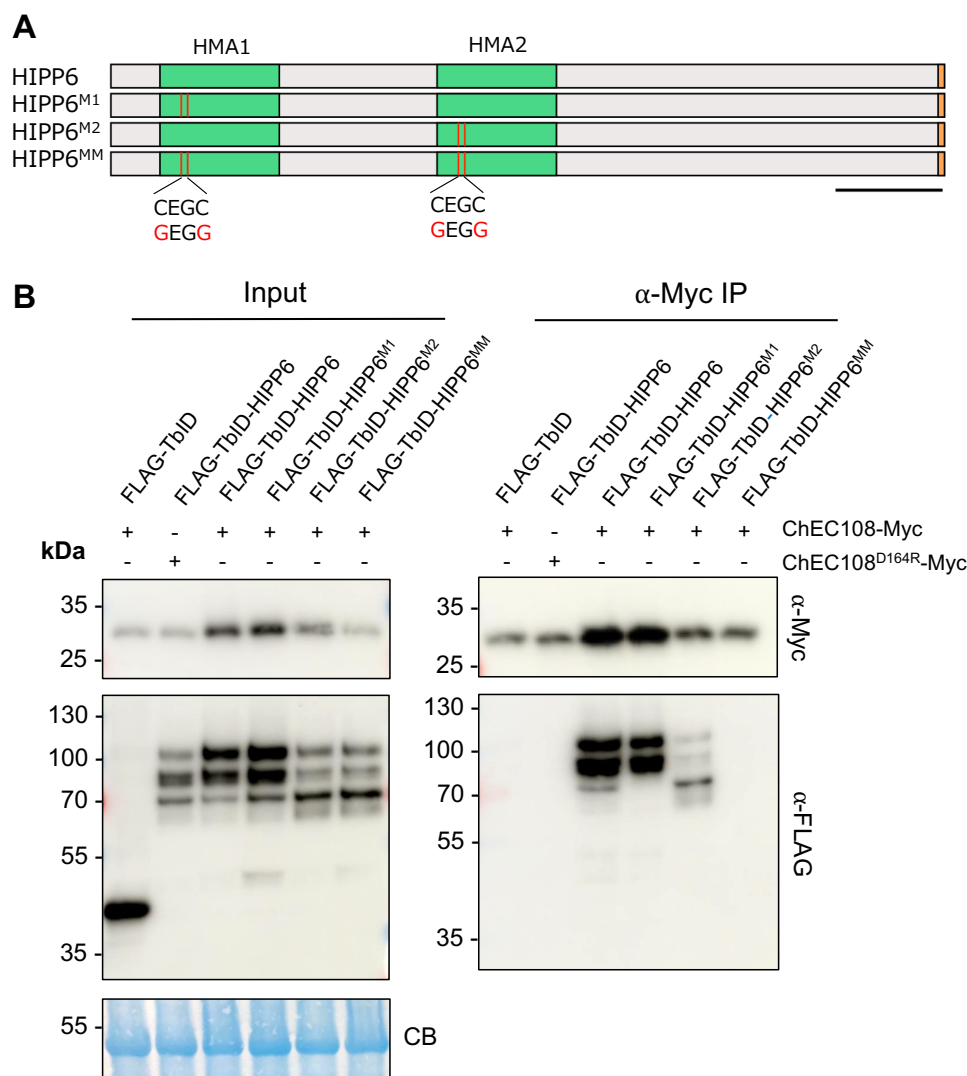


Figure 3.27 – Mutation of metal-binding residues compromises ChEC108-HIPP6 association in vivo. **A.** Domain structure of wild-type HIPP6 and metal-binding site mutants lacking a CXXC motif in HMA1 (M1), HMA2 (M2) or both (MM). Scale bar = 50 amino acids. **B.** Co-IP demonstrating loss of ChEC108-HIPP6 association in *N. benthamiana* upon mutation of ChEC108 Asp164 to Arg (ChEC108^{D164R}), or both HIPP6 metal-binding motifs from CXXC to GXXG (HIPP6^{MM}). Bait proteins were tagged with 4×Myc (Myc). Prey proteins were tagged with 3×FLAG-TurboID (FLAG-TbID) to promote stability and enable immunodetection. FLAG-TbID alone and FLAG-TbID-HIPP6 were included as negative and positive controls, respectively. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. Representative of three independent repeats. Expected molecular weights (kDa): ChEC108-Myc, 27.6; ChEC108^{D164R}-Myc, 27.6; FLAG-TbID-HIPP6, 82.7; FLAG-TbID-HIPP6^{M1}, 82.6; FLAG-TbID-HIPP6^{M2}, 82.6; FLAG-TbID-HIPP6^{MM}, 82.5.

purified from 8 l SHuffle cells using a similar strategy, with the exception that early-eluting peak fractions from the initial gel filtration step were carried forward for further purification in an attempt to increase yield (Figure 3.29A-B). Promisingly, HMA2 elution resulted in a single peak in UV absorption during the second gel filtration step (Figure 3.29C), at a similar volume to HMA1 (Figure 3.28D). This suggests the sample was not dominated by aggregates, which may have disassembled upon cleavage and removal of 6×His tags.

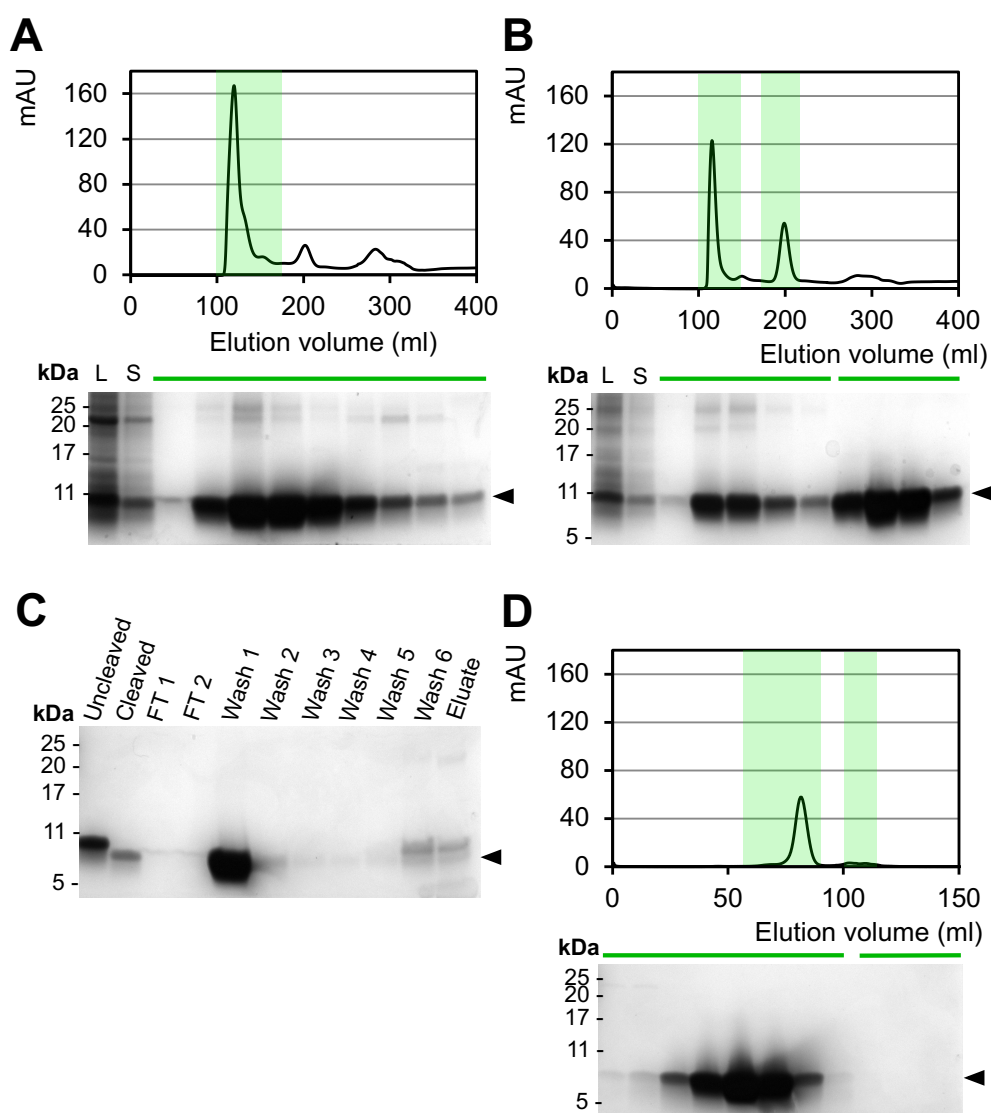
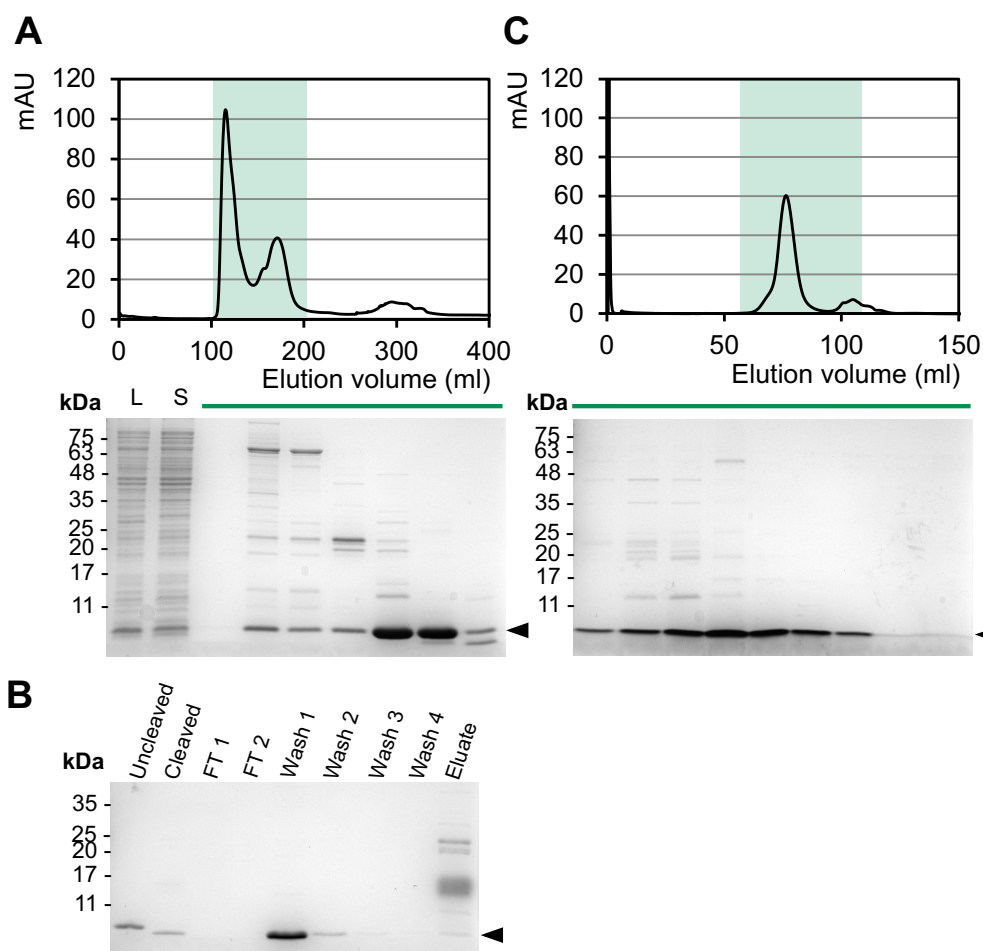


Figure 3.28 – Purification of HMA1 from *E. coli*. A-B. Enrichment of 6×His-HMA1 from 8 l of BL21-AI *E. coli* (A) or 6 l of SHuffle *E. coli* (B) by IMAC and gel filtration. 6×His-HMA1 (10.9 kDa, arrow) was visible in cell lysate (L) and soluble fractions (S). In A, gel filtration fractions 102-173 were examined by SDS-PAGE. The premature absorption peak at 115 ml indicates aggregates of large molecular weight, while the smaller peak at 200 ml likely indicates non-aggregated 6×His-HMA1. These proteins were not purified further. In B, fractions 109-141 ml and 189-215 were examined by SDS-PAGE, and fractions 181-223 ml were pooled and carried forward. C. Isolation of HMA1 (8.9 kDa, arrow) by IMAC following cleavage of 6×His tags by 3C protease. HMA1 has natural affinity for the resin of the IMAC column and was absent from flowthrough (FT) fractions. Elution was achieved by washing with A1 buffer (Wash 1-3), then A1 buffer supplemented with additional imidazole (30 mM, 50 mM and 100 mM in Wash 4-6, respectively). His-tagged 3C protease and other contaminants were eluted with B1 buffer (Eluate). Wash 1-3 were pooled and carried forward. D. Final purification of HMA1 (8.9 kDa, arrow) by gel filtration. Fractions 66-93 ml and 101-111 ml were examined by SDS-PAGE. Fractions 64-91 ml were pooled and concentrated. mAU = milli-absorbance units at 280 nm.



was observed at 13.2 ml, showing a very minor leftward shift in comparison to that observed with ChEC108 alone (Figure 3.30A). As this trial did not provide definitive evidence of in vitro interaction, I repeated the experiment using an alternative system, ÄKTA pure, equipped with a higher resolution chromatography column for use with small sample volumes. From this column, HMA1 alone eluted at 1.648 ml, ChEC108 eluted at 1.508 ml, and an equimolar mixture of ChEC108 and HMA1 eluted at 1.501 ml (Figure 3.30B). Inspection of the eluted fractions by SDS-PAGE revealed that peaks in ChEC108 and HMA1 abundance did not coincide (Figure 3.30C). Together, these analytical gel filtration attempts suggested a ChEC108-HMA1 complex could not be reconstituted in vitro, at least using these specific protein samples.

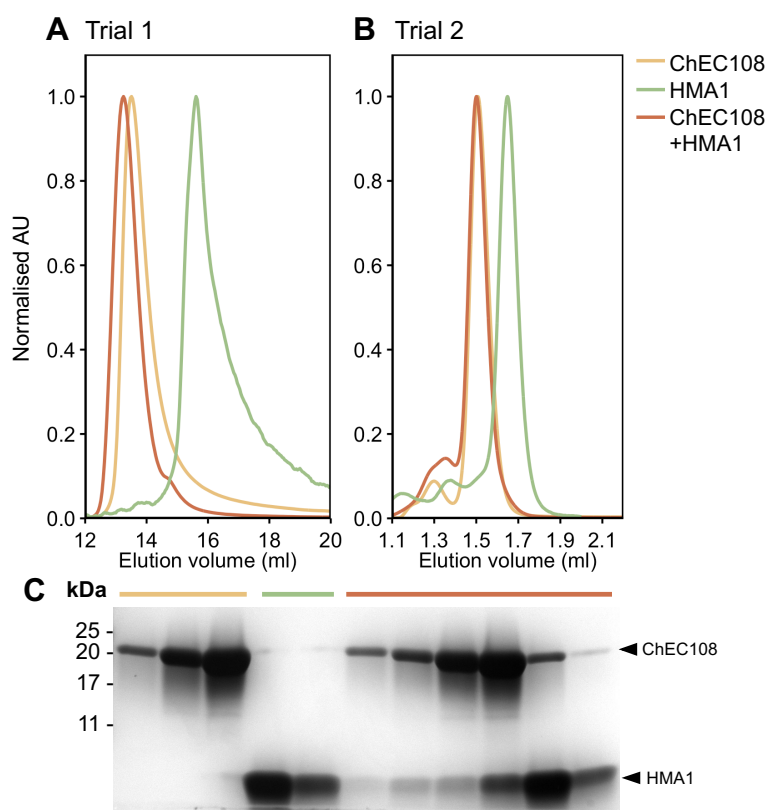


Figure 3.30 – ChEC108-HMA1 association was not detected in preliminary analytical gel filtration trials. Normalised absorption units (AU) of elution fractions at 280 nm following analytical gel filtration. **A.** An initial trial on the ÄKTA purifier system (Trial 1) revealed a very minor leftward shift of the ChEC108+HMA1 peak, compared to peaks representing ChEC108 or HMA1 alone. **B.** A second trial on the ÄKTA pure system (Trial 2) revealed no leftward shift of the ChEC108+HMA1 peak. **C.** Examination of peak fractions from B via SDS-PAGE, showing the presence of ChEC108 (22.8 kDa) and HMA1 (8.9 kDa). In the ChEC108+HMA1 sample, peaks of ChEC108 and HMA1 abundance do not coincide, indicative of no complex formation.

Alongside analytical gel filtration, I sought to study the thermodynamics of ChEC108-HMA binding via ITC, a highly sensitive quantitative technique for profiling protein-protein interactions in solution. As heat is exchanged when one protein is sequentially injected into a solution containing a binding partner (within an experimental cell), thermograms can be

obtained, integrated with respect to time and normalized for protein concentration to generate a titration curve. When these curves are fitted to a binding model, the dissociation constant (K_d), which indicates binding affinity, and enthalpy change (ΔH) can be calculated. Based on the crystal structures presented in Figure 3.20, I anticipated a 1:1 stoichiometry for ChEC108-HMA1 interactions. Given that the experimental cell volume of the ITC instrument is ~5x the volume of the syringe, and the ligand should ideally be in excess within the cell by the end of the experiment, I sequentially injected ChEC108 at 200 μ M into a cell containing HMA1 at 20 μ M. However, the resulting thermograms indicated negligible heat exchange with each injection, indicative of little/no binding events taking place. In subsequent runs, after increasing the concentration of HMA1 in the experimental cell to 120 μ M, exothermic binding events could be observed (Figure 3.31A, upper panel). However, given the huge excess of HMA1 molecules required to see such events, the estimated stoichiometry of ChEC108-HMA1 interactions was roughly 1:5 (Figure 3.31A, central panel), and any binding parameters calculated based on a 1:1 binding model would not be meaningful. Being contradictory to evidence gathered via co-IP and X-ray crystallography, such negative results were initially puzzling, especially as ChEC108 and HMA1 samples showed no signs of precipitation or degradation pre- or post-ITC runs (Figure 3.31B).

Crucially, following subsequent re-purification and analysis of isolated HIPP6 HMA domains, Dr Daniel Yu recognised that disulphide bonds could be perturbing complex formation with ChEC108. Indeed, mass spectrometry analysis of purified proteins used for analytical gel filtration and ITC above indicated that a disulphide bond was present in HMA1, as the observed mass was 2 Da lower than anticipated (Table 3.2), a difference attributed to the loss of one hydrogen atom each from two oxidised Cys side chains. HMA1 contains only two Cys residues, Cys34 and Cys37 within the CXXC motif - a region demonstrated to be critical for ChEC108-binding (Figure 3.27). It is therefore reasonable to assume that metal-binding, and thus ChEC108-binding, would be greatly perturbed by a disulphide bond in this region. For this reason, while I was unfortunately unable to investigate the thermodynamics of 1:1 protein interactions in vitro within the timeframe of this project, I unwittingly gathered further support for the hypothesis that metal-binding residues are essential for ChEC108 interaction with HIPP6 HMA domains.

While my research efforts going forward became increasingly focussed on in planta experiments (see Chapters 4 to 6), Dr Daniel Yu made additional progress towards understanding the structural basis of the ChEC108-HIPP6 complex. After independently re-purifying ChEC108, HMA1 and HMA2 from *E. coli*, and confirming the presence of disulphides in all three samples by mass spectrometry, analytical gel filtration was conducted in the presence of 1 mM dithiothreitol (DTT), a reducing agent to maintain Cys residues in a reduced form, and

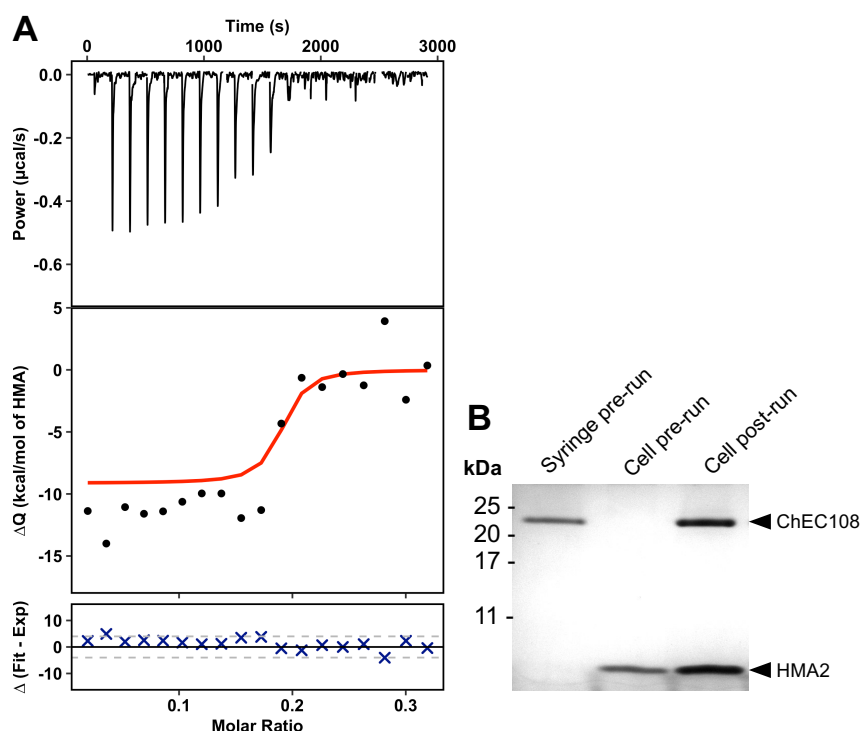


Figure 3.31 – Preliminary thermodynamic characterisation of ChEC108-HMA1 binding by ITC. **A.** Purified ChEC108 (200 μM) was injected step-wise into a cell containing HMA1 (120 μM). Upper panel shows raw processed thermogram after baseline correction and noise removal. Central panel shows experimental binding isotherm with a global fitted curve (red), obtained from 4 repeats. Lower panel indicates the difference between the actual and fitted estimates of heat transfer (ΔQ). **B.** Integrity of pure ChEC108 (22.8 kDa) and HMA1 (8.9 kDa) proteins was confirmed pre- and post-run via SDS-PAGE.

50 μM ZnCl_2 as a source of Zn^{2+} ions. Encouragingly, mixing ChEC108 with the reduced form of HMA1 or HMA2 triggered a pronounced leftward peak shift of the resulting UV spectrum relative to peaks representing ChEC108 or the HMA domains alone (Dr Daniel Yu, personal communication). Furthermore, using ITC in the presence of 1 mM DTT and 50 μM ZnCl_2 , ChEC108 was found to have high affinity for both reduced HMA1 and HMA2, with K_d for each complex being calculated at 7.2 nM and 18.3 nM, respectively (Table 3.5). However, mutation of ChEC108 Asp164 to Arg abolished interaction with HMA1 and HMA2 entirely in ITC experiments, such that binding parameters could not be calculated (Table 3.5). These results validate the ChEC108-HMA binding models obtained via X-ray crystallography (Figure 3.20), and complement evidence from *in vivo* co-IP (Figure 3.27), suggesting the metal-coordinating ChEC108 residue, Arg164, is critical for interaction.

Table 3.5 – ChEC108-HMA1/2 binding parameters calculated by ITC. Samples in both the syringe and cell were supplemented with 1 mM DTT and 50 μ M ZnCl₂. nd = not determined due to negligible binding. Data were provided by Dr Daniel Yu.

Cell		Syringe		K_d (nM)	ΔH (kcal/mol)
Protein	μ M	Protein	μ M		
HMA1	20	ChEC108	200	7.2	-13.3
HMA2	30	ChEC108	300	18.3	-14.3
HMA1	20	ChEC108 ^{D164R}	200	nd	nd
HMA2	30	ChEC108 ^{D164R}	300	nd	nd

3.3 Discussion

In this chapter, I described a novel protein complex between the putative *Ch* effector, ChEC108, and its host target from *Arabidopsis*, HIPP6. Using co-IP and in vitro purification, I discovered that the HIPP6 HMA domains were sufficient for ChEC108 binding, and proceeded to obtain X-ray crystallographic structures of the ChEC108-HMA1 and ChEC108-HMA2 complexes. At least in vitro, these structures suggest ChEC108 targets HMA1 and HMA2 via the same mechanism, involving coordination of a divalent metal ion with tetrahedral geometry (Zn²⁺, in the case of HMA2). Despite the binding interfaces of both complexes being relatively small (involving < 20 residues from each partner, according to analysis conducted with QtPISA), strong coordinate bonds formed between amino acid side chains and this metal ion likely provide a strong bridge to facilitate interaction. Using co-IP, I confirmed the metal-binding residues contributed by both ChEC108 and HIPP6 to be critical for complex formation.

3.3.1 ChEC108 exhibits a novel mechanism of HMA targeting

To date, multiple *M. oryzae* effectors belonging to the MAX effector family have been structurally characterised in complex with HMA domains (De la Concepcion et al., 2018, 2021; Guo et al., 2018; Maidment et al., 2021; Maqbool et al., 2015; Stone, 2024; Varden et al., 2019; Zdrzałek et al., 2024). MAX folds consist of two three-stranded antiparallel β -sheets arranged face-to-face (de Guillen et al., 2015) - a fold not observed in ChEC108. In Figure 3.32A-D, four MAX effector-HMA complexes from *M. oryzae* pathosystems are displayed side-by-side, highlighting their distinct binding interfaces. Despite this diversity in positioning of the effector relative to the HMA fold, in all four cases, non-covalent interactions between β -strands of the MAX effectors and HMA partners contribute significantly to the interactions, while the metal-binding loops (β 1- α 1) of the HMA domains (coloured blue) do not participate. In contrast, the ChEC108-HMA interfaces described in this work (exemplified by ChEC108-HMA1 in Figure 3.32E) are formed entirely from α -helices and loop regions, including the metal-binding

loop of the HMA domains. Notably, three example host targets displayed in Figure 3.32 (OsHIPP19, sHMA25 and OsHPP02) lack a conserved M/LXCXXC motif in their $\beta 1$ - $\alpha 1$ loop, suggesting they have no capacity for metal-binding. This motif is similarly absent from the integrated HMA domains of *O. sativa* NLRs Pik-1 and RGA5 (Table 1.3), suggesting metal-binding is not necessary for baiting effectors in these systems. While OsHIPP43 *does* possess a metal-binding motif (MDCEGC), in the Pwl2-OsHIPP43^{HMA} complex, the Cys side chains of this motif are engaged in a disulphide bond (Zdrzałek et al., 2024). It thus appears that ChEC108 from *Ch* and MAX effectors from *M. oryzae* have convergently evolved to target HMAs via very different mechanisms.

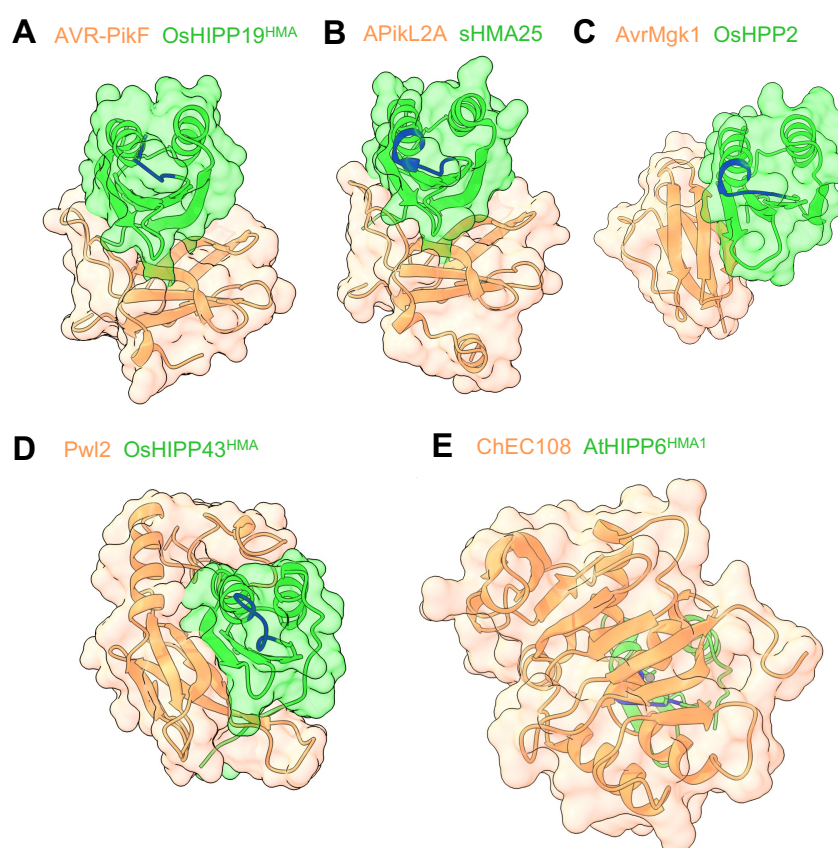


Figure 3.32 – Structurally characterised effectors in complex with the HMA or HMA-like domains of host target proteins. Crystal structures of A. AVR-PikF with the HMA-like domain of OsHIPP19 from *O. sativa* (7B1I) (Maidment et al., 2021); B. APikL2A with the HMA-like domain of sHMA25 from *S. italica* (7NLJ) (Bentham et al., 2021a); C. AvrMgk1 with the HMA-like domain of OsHPP2 from *O. sativa* (Stone, 2024). D. Pwl2 with the HMA domain of OsHIPP43 from *O. sativa* (8R7D) (Zdrzałek et al., 2024); E. ChEC108 with the HMA1 domain of HIPP6 from Arabidopsis. Complexes are displayed such that the HMA/HMA-like domains (coloured green) are in equivalent orientations. Effectors are coloured orange. Regions of ribbon models equivalent to the metal-binding motif (M/LXCXXC) in the $\beta 1$ - $\alpha 1$ loop region of HMA/HMA-like domains are coloured blue.

3.3.2 What does the ChEC108-HIPP6 complex look like?

In the absence of ChEC108-tHMA crystals, the structural basis of ChEC108 interaction with full length HIPP6 remains an open question. As the ChEC108-HMA co-structures described in this chapter were remarkably similar, and ITC did not reveal a stark difference in ChEC108 binding affinity for each HMA domain, one could hypothesise that ChEC108 contacts either HMA1 or HMA2 in planta with no obvious preference, leaving the unbound HMA domain free-floating. Alternatively, could there be a secondary HMA-binding site on ChEC108, potentially strengthening any ChEC108-HIPP6 interactions? While co-IP data in Figure 3.27 suggested contact with both HMA domains (at least via their CXXC motifs) is not necessary for ChEC108 to target HIPP6, whether or not interaction with both domains is required to alter HIPP6 *function* remains unknown.

In the ChEC108 crystal structure, a disulphide bond is formed between Cys197 and Cys200, forming a closed loop which, given its C-terminal location, is unlikely to be essential for supporting the effector's structure, and could even be an artefact introduced by vitro purification. Noticing this disulphide, Dr Daniel Yu hypothesised that a metal ion could be coordinated here, with additional ligands provided by the CXXC motif of a HMA domain, reminiscent of AlphaFold2 Multimer predictions of ChEC108-HMA interactions presented in Figure 3.7. Although HMA2 seemingly failed to bind the ChEC108-HMA1 complex in vitro, potentially contradicting this hypothesis (Figure 3.14), disulphide bonds in both ChEC108 and HMA2 likely blocked any metal coordination at this putative second site. Repetition of this in vitro co-purification attempt under reducing conditions, leaving Cys thiol groups exposed, would be beneficial when continuing the search for a ChEC108-HMA1-HMA2 tripartite complex.

If HMA domains *can* associate with the C-terminus of ChEC108, one might expect the ChEC108^{D164R} mutant to retain HMA1/2 binding in vitro, contradicting ITC results described in Table 3.5. However, perhaps interaction with one HMA domain at the primary interface reported in this work is first necessary to anchor HIPP6 to ChEC108, enabling an otherwise weaker or transient interaction with the second HMA domain. This might also explain why 1:2 ChEC108:HMA stoichiometry was not observed using X-ray crystallography following co-expression of the effector with HMA domains isolated from their native linker. To test these ideas, it would be valuable to study ChEC108-tHMA interaction in greater detail, although attempts to crystallise this complex were unfortunately unsuccessful. This could be due to a number of reasons: (1) the precise chemical environment required to crystallise this complex was not encountered as part of the five commercially available screens used; (2) as discussed in 3.2.4, instability of the inter-HMA linker resulted in contamination of the sample with excessive

breakdown products; (3) the flexible nature of the linker inhibited the close, repeated packing of proteins required to form a crystal, even if both HMA domains were bound; (4) if only one HMA domain was bound, other free-floating tHMA regions likely impeded protein packing, and lastly, (5) a heterogeneous mixture of ChEC108-HMA1 and ChEC108-HMA2 interfaces within the sample made it recalcitrant to crystallisation. In summary, ChEC108-tHMA remains a challenging sample to structurally characterise, although it is possible that optimisation of purification conditions to maximise stability, and further exploration of crystallisation conditions could lead to future breakthroughs.

3.3.3 Can HIPP6 bind metal ions in the absence of ChEC108?

Confirmation of HMA1 and HMA2 metal-binding capacity in complex with ChEC108 raises questions as to the relevance of metal association for HIPP6 function, both in the presence and absence of effector binding. My working hypothesis is that HIPP6 is ordinarily capable of coordinating a metal ion via four Cys residues, acting as a bridge between HMA1 and HMA2, potentially helping to stabilise full-length HIPP6 in planta. Consistent with the initial AlphaFold2 prediction displayed in Figure 3.4C, when asked to model HIPP6 with Zn^{2+} , AlphaFold3 predicts a tetrahedral Zn^{2+} coordination site with high confidence (ipTM = 0.95) (Figure 3.33A-B). A study by Suzuki et al. (2002) provides additional support for this hypothesis, as circular dichroism (CD) spectra suggested HIPP6 (here named CADMIUM-INDUCED 19 (CdI19) and purified from *E. coli* as an N-terminal fragment encompassing the HMA domains) bound Zn, Cu and Hg ions in vitro, but not when the CXXC motifs of the HMA domains were mutated to GXXG.

Contrary to this hypothesis, HMA1-HMA2 association was not observed in vivo (Figure 3.25). This result was surprising, as dimerisation of HMA domains, facilitated by metal-binding, has been described on multiple occasions. For instance, purified human and yeast ATX1 orthologs dimerise in the presence of Cu, as demonstrated by surface plasmon resonance (SPR) and analytical gel filtration, respectively (Miras et al., 2008; Tanchou et al., 2004). X-ray crystallography revealed a distorted tetrahedral geometry involving four Cys residues at the Cu^+ -binding sites of these dimers, stabilised by hydrogen bonds (Lee et al., 2017; Wernimont et al., 2000). Alongside, ATX1 from the cyanobacterium *Synechocystis* PCC6803 was crystallised as a dimer, loaded with two or four Cu^+ ions (Badarau et al., 2010). In plants, bimolecular fluorescence complementation (BiFC) assays suggested HPP5/CCP from *Arabidopsis* self-associates before entering *N. benthamiana* nuclei, but not in the presence of the Cu chelator, bathocuproine sulfonate (BCS), and not when the CXXC motif is mutated to GXXG (Chai et al., 2020). Self-association of HIPP7, a tandem HMA protein in the same clade as HIPP6, was observed via co-IP, and yeast two-hybrid assays suggested these interactions could be abolished

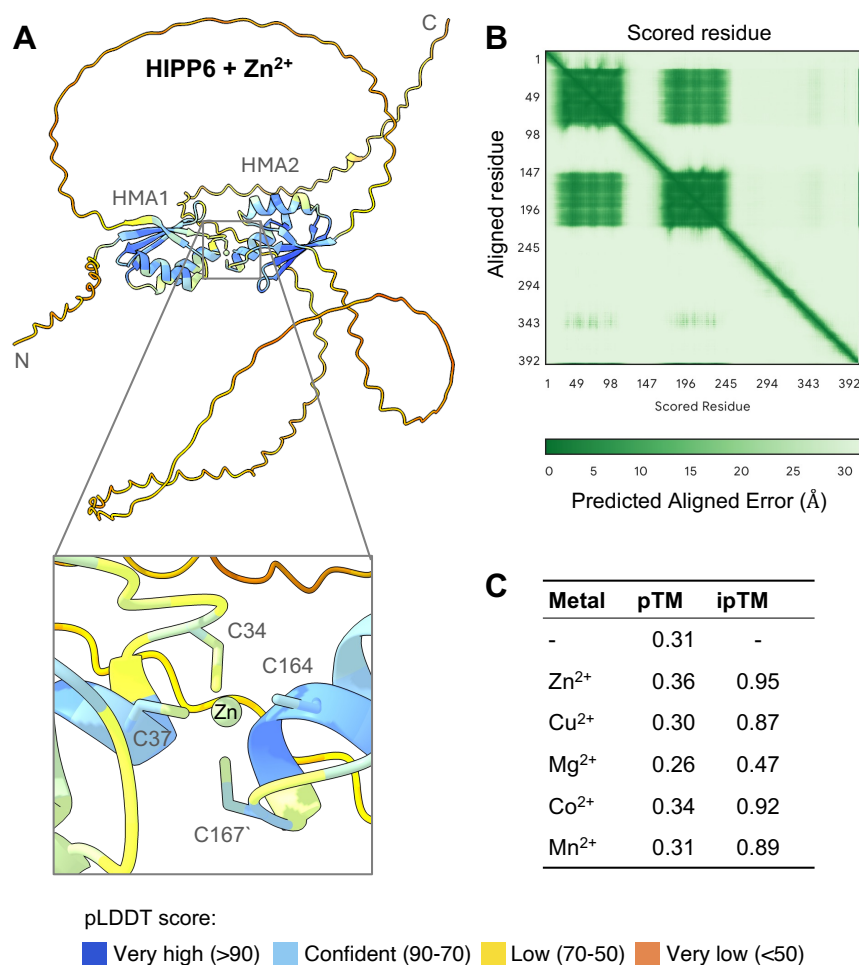


Figure 3.33 – Predicted structure of HIPP6 in complex with Zn²⁺. AlphaFold3 (accessed via the AlphaFold Server in July 2024) model of HIPP6 in complex with a single Zn²⁺ ion, coloured according to pLDDT confidence scores. Grey box shows the site of predicted Zn²⁺ coordination, formed by the CXXC motifs of the HIPP6 HMA domains. **B.** Predicted aligned error (PAE) plot shows the predicted error associated with relative distances of residues in the model shown in A. **C.** Summary of AlphaFold3 outputs when asked to model HIPP6 in complex with different metal species. Predicted Template Modelling scores for the overall structures (pTM) and HIPP6-metal interfaces (ipTM) are given for each model.

by Cys to Gly mutation in the CXXC motifs (Guo et al., 2021). Given that HIPP6 HMA1-HMA1 homomers were detected (Figure 3.25), it is possible that instability of HMA2 impeded detection of HMA1-HMA2 and HMA2-HMA2 association in co-IP assays reported here.

Assuming HIPP6 *can* bind metals alone, which metal might it prefer? And, importantly, which metal(s) could be most relevant for HIPP6 function? While Zn²⁺ was identified in the ChEC108-HMA2 structure, it is possible that alternative ions could be bound via the same coordination site, or indeed via a putative Cys₄ tetrahedral site in HIPP6 monomers. In the subcellular compartment to which HIPP6 is localised in planta, availability of individual metal species likely differs from the environment in which recombinant ChEC108-HMA complexes

were expressed and purified, making it challenging to be confident in the biological relevance of HIPP6-Zn binding. In fact, [CheckMyMetal \(CMM\)](#), an online server for metal-binding site validation ([Gucwa et al., 2023](#)) ranked Zn, iron (Fe) and Cu as being equally likely to occupy the ChEC108-HMA2 interface. At the ChEC108-HMA1 interface, Zn was given the same ranking as Co, behind Fe and Cu. However, this could be attributed to the overall poorer quality of data informing the geometry of metal-binding residues in this particular structure. Interestingly, AlphaFold3 suggested HIPP6-Cu²⁺, HIPP6-Co²⁺ and HIPP6-Mn²⁺ complexes could be feasible, alongside HIPP6-Zn²⁺ (Figure 3.33C). Furthermore, bicinehoninic acid (BCA) protein assays conducted in the laboratory of Prof. Christopher Dennison, Newcastle University, UK, confirmed that HMA1 and HMA2 have high affinity for Cu⁺ ions when their Cys side chains are reduced (personal communication). Further exploration of HIPP6 metal binding specificity, perhaps exploiting purified tHMA, could be attempted using the colorimetric 4-(2-pyridylazo)resorcinol (PAR) assay, which has been previously applied to quantify Co²⁺, Ni²⁺, Cu²⁺, and Zn²⁺ in protein samples ([McCall and Fierke, 2000](#); [Säbel et al., 2009](#)). ITC could also be useful to study metal-binding kinetics, assuming tHMA can be purified in a reduced state and stripped of any pre-bound metals before experimentation. Ultimately, genetic experiments would be beneficial to investigate the relevance of metal-binding for potential HIPP6 functions in heavy metal homeostasis and/or plant immunity (for further discussion of this topic, see 7.2).

3.3.4 Conclusion

While the structural basis of ChEC108 interaction with the full length HIPP6 protein has yet to be elucidated in its entirety, in vitro protein work presented in this chapter has shed light on the molecular mechanism by which ChEC108 binds isolated HMA domains, enabling the design of structure-guided variants with compromised binding abilities. Equipped with these insights and tools, I next sought to investigate the biological relevance of ChEC108-HIPP6 binding events in the context of *Ch* infection.

Chapter 4

Investigating the function of ChEC108 and HIP6 during infection

4.1 Introduction

Confirmation of ChEC108 association with the HMA domains of HIP6 using both in vitro and in vivo techniques (see Chapter 3) served as a robust foundational discovery, opening research avenues for functional exploration. The *Ch*-*Arabidopsis* pathosystem represents an ideal model for such investigations, given the wealth of genetic tools available and relatively short lifecycles of both pathogen and host, among other reasons discussed previously (1.4).

4.1.1 The *Ch* infection cycle

As a hemibiotroph, *Ch* exhibits a dual-stage life cycle involving an initial biotrophic phase, when the fungus establishes itself within living cells, and a subsequent necrotrophic phase, involving death and degradation of host cells to fuel fungal growth. The invasion structures associated with each phase differ in their morphology, schematically illustrated in Figure 4.1.

Before infection can commence, *Ch* must first penetrate the waxy cuticle and cell wall of the leaf epidermis. This is achieved using mechanical force generated by specialised cells called appressoria, which develop following the germination of asexual spores (conidia) - a strategy that *Ch* shares with other phytopathogenic fungi in the genus *Colletotrichum*, and the blast fungus, *M. oryzae* (Chethana et al., 2021). Appressorium differentiation is induced by contact with the solid leaf surface, with studies in *M. oryzae* highlighting surface hydrophobicity as a key determining factor (Lee and Dean, 1994). Based on studies of *Colletotrichum gloeosporioides*, physical contact with hard surfaces triggers touch-responsive signalling cascades, which may also prime fungi to respond to other appressoria-inducing host signals such as ethylene and

wax (Yeon-Ki et al., 1998). In *M. oryzae* appressoria, high turgor is attributed to the accumulation of glycerol and other polyols, which encourage water entry via osmosis. Meanwhile, the highly melanised appressorial cell wall prevents leakage of these osmotic compounds, facilitating the generation of up to 8 MPa of penetrative force (reviewed by Cruz-Mireles et al., 2021; Foster et al., 2017). The *Ch* appressorium is thought to function similarly, directing mechanical pressure to its base and enabling emergence of a penetration peg (O'Connell et al., 2004). Studies of gene expression during the appressorial phase of *Ch* and *Colletotrichum lindemuthianum* infections suggested hydrolytic enzymes might also contribute to penetration by degrading cuticle and cell wall components such as cutin, pectin, cellulose and hemicellulose (Bernard et al., 1999; O'Connell et al., 2012). After breaching the cell wall, a bulbous multiseptate biotrophic hypha (4.5-8.5 µm in diameter) develops, enveloped by the extra-invasive hyphal membrane (EIHM). These hyphae-adjacent host membranes are invaginated, enriched in phosphoinositides and remain intact during biotrophy (O'Connell et al., 2004; Shimada et al., 2019).

Ch exhibits 'localised biotrophy', meaning primary invasive hyphae are restricted to one host cell only (Latunde-Dada and Lucas, 2007). This is in contrast to certain other hemibiotrophic pathogens such as *C. lindemuthianum* and *Colletotrichum graminicola*, which first establish a multicellular network of biotrophic hyphae before necrotrophy ensues at the lesion centre (O'Connell et al., 1985, 2012). Microscopic studies of *Ch* infection on *Arabidopsis* rosette leaves suggest the biotrophic phase is short-lived, lasting only 24 h before necrotrophic growth is initiated (O'Connell et al., 2004). Upon the switch to necrotrophy, the host tonoplast and plasma membranes become fragmented (Regmi et al., 2023), and filamentous secondary hyphae (2.2-4.0 µm in diameter) radiate from biotrophic hyphal tips into adjacent cells (O'Connell et al., 2004). These hyphae macerate host tissues, producing dark grey lesions visible at the macroscopic level, sometimes with chlorotic halos (Figure 4.2). Although the molecular events signifying the end of biotrophy are not fully understood, the necrotrophic phase is characterised by enhanced expression of genes encoding hydrolytic enzymes and plasma membrane transporters to facilitate fungal nutrient acquisition (O'Connell et al., 2012). Closing the loop on the asexual lifecycle of *Ch*, fruiting bodies (acervuli) develop on the surface of dead host tissues. Here, conidia are manufactured within specialised hyphae (conidiophores), from which they are dispersed via water splash (O'Connell et al., 2004).

How *Ch* necrotrophic hyphae spread to adjacent host cells is not fully understood. During *M. oryzae* infection of *O. sativa*, as part of the sequential biotrophic invasion strategy of this fungus, invasive hyphae were seen 'scanning' the periphery of primary invaded cells, before temporarily swelling and puncturing the cell wall using a tightly constricted penetration peg (Kankanala et al., 2007; Sakulkoo et al., 2018). Given their resemblance to appressoria, these specialised structures were named transpressoria, yet it remains unclear as to whether

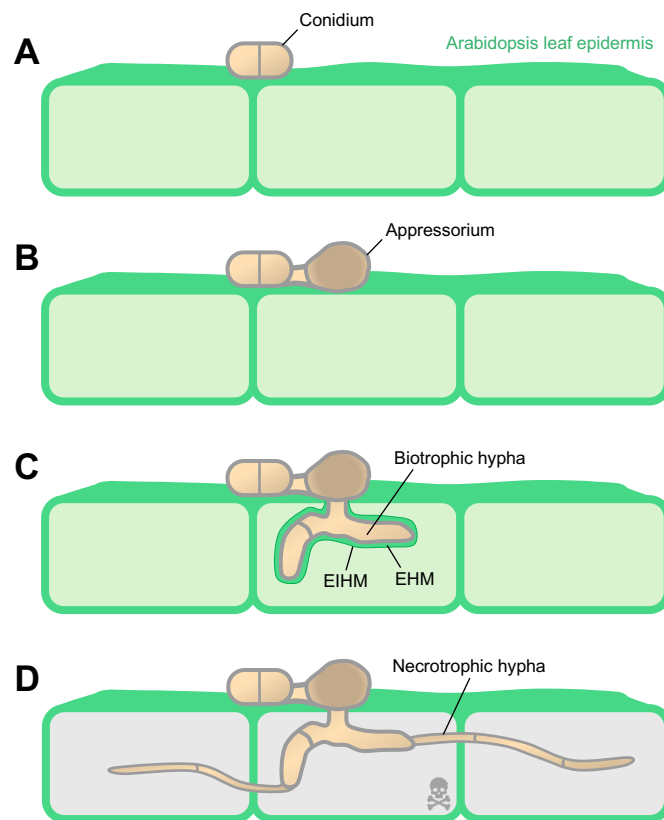


Figure 4.1 – *Ch* infection progression at the microscopic scale. Schematic illustration of *Ch* infection stage transitions in Arabidopsis epidermal cells. **A-B.** A conidium lands on the cuticle and germinates, forming a melanised appressorium to facilitate penetration of the host cuticle and cell wall. **C.** A penetration peg develops at the base of the appressoria, which grows into a bulbous, often branched, biotrophic hypha. At this stage, the host plasma membrane remains intact, and hyphae are isolated from the host cytoplasm by the extra-invasive hyphal membrane (EIHM). The extra-invasive hyphal matrix (EHM) is continuous with the apoplast and surrounds hyphae within the EIHM. **D.** Thin necrotrophic hyphae emerge from the tips of biotrophic hyphae and invade neighbouring cells. As membrane integrity is compromised, host cells die and their contents are degraded.



Figure 4.2 – Progression of symptoms induced by *Ch* on a detached Arabidopsis leaf. Arrowhead indicates the site of application of *Ch* spore suspension.

mechanical force and/or enzymatic digestion are necessary for cell wall traversal (reviewed by [Cruz-Mireles et al., 2021](#)). Interestingly, detailed ultrastructural analysis using TEM revealed *M. oryzae* hyphal constriction coincided with pitfields, where PD are clustered in the host cell wall, suggesting a preference of the fungus for these sites as intercellular crossing points ([Kankanala et al., 2007](#)). In contrast, necrotrophic *Ch* hyphae have not been observed to develop transpressoria or exhibit constriction as they exit primary invaded cells, at least not to the same extent as *M. oryzae* hyphae ([O'Connell et al., 2004, 2012](#)). Furthermore, *Ch* necrotrophic hyphae are not restricted to the symplast, as recent TEM data revealed their presence in the extracellular space surrounding invaded cells ([Regmi et al., 2023](#)). Thus, unlike *M. oryzae*, it seems unlikely that *Ch* specifically target pitfields to enable hyphal spread during infection, and may instead rely heavily on enzymatic degradation of host membranes and cell walls.

4.1.2 *Ch* effectors are expressed in waves

Stage-specific transcriptomic studies have offered key insights into the molecular events that occur as *Ch* infection of *Arabidopsis* progresses, especially concerning the secretion of effector proteins. Notably, the majority of genes encoding candidate effectors were found to be upregulated during biotrophy, suggesting effector-mediated manipulation of host processes is particularly prevalent here, and that biotrophic hyphae are likely major platforms for effector delivery ([O'Connell et al., 2012](#)). Since this initial study, expression profiling of putative effector genes with finer temporal resolution has uncovered waves of effector gene expression, coinciding with different stages of infection. In particular, based on analysis of 17 *ChEC* genes, [Kleemann et al. \(2012\)](#) defined four distinct patterns of effector induction: (1) prior to host penetration, (2) prior to host penetration, followed by persistently high expression as the biotrophic phase commences, (3) during biotrophy and (4) during the transition to necrotrophy. In multiple independent studies, *ChEC108* expression has been shown to peak during the biotrophic phase of infection ([Dallery et al., 2017](#); [O'Connell et al., 2012](#); [Robin et al., 2018](#)), reminiscent of 'wave three' *ChEC* genes ([Kleemann et al., 2012](#)), making *ChEC108* a likely manipulator of live host cells. For further discussion of effector delivery mechanisms in *Ch* and other pathogens, see 6.1 in Chapter 6.

Although many have been characterised as biotrophy-associated, the in planta functions of only a few putative *Ch* effectors have been studied in depth. For example, *Ch* extracellular LysM motif protein 1 (ChELP1) and ChELP2 are thought to suppress host PTI by binding chitin oligomers, concealing this elicitor from plant immune receptors in the apoplast ([Takahara et al., 2016](#)). When delivered directly into the cytoplasm of *Arabidopsis* cells via the bacterial type III secretion system, *ChEC3* and *ChEC89* enhanced *Pto* DC3000 virulence, presumably as a result of host defence suppression by these effectors ([Kleemann et al., 2012](#)). Additionally,

when transiently expressed in *N. benthamiana* alongside the toxin ChNLP1 (for *Ch* necrosis- and ethylene-inducing peptide 1), wave two- and three-associated effectors, ChEC3, ChEC3a, ChEC5, ChEC6 and ChEC34, inhibited ChNLP1-induced cell death, implicating these proteins in biotrophy maintenance (Kleemann et al., 2012). In contrast, the highly conserved core effector of *Colletotrichum* 3 (CEC3) is thought to support the switch of *Ch* to necrotrophy, as its transient expression induced cell death in *N. benthamiana* (Tsushima et al., 2021).

As introduced in 1.4.1, ChEC108 was found to target PD and exhibited extensive cell-to-cell mobility when transiently expressed in *N. benthamiana*. Maintenance of symplastic connectivity is proposed to benefit a range of pathogens, including fungi ((reviewed by Cheval and Faulkner, 2018)). Although the underlying reasons for this are not fully elucidated, it is proposed that fungi exploit PD to enable the spread of virulence factors and suppression of defence responses ahead of their primary invasion front (see also 1.3). In support, evidence from agroinfiltration-based protein mobility assays in *N. benthamiana* implicated ChEC8, ChEC127 and ChEC132 as indirect manipulators of PD function, enhancing general intercellular trafficking without being localised to PD themselves (Ohtsu et al., 2023). Although not the case for ChEC132, Arabidopsis leaves overexpressing ChEC127 displayed enhanced susceptibility to *Ch* relative to Col-0, suggesting the function of this gene favours *Ch* establishment (Ohtsu et al., 2023). With this in mind, I hypothesised that ChEC108 might similarly function as a virulence factor, enhancing cell-to-cell trafficking at and around infection sites, thereby facilitating the spread of other *Ch*-derived effectors and metabolites. Accordingly, I reasoned HIP6 could be targeted by ChEC108 to promote pathogenesis via as yet unknown mechanisms, aligning with numerous reports of HMA proteins as susceptibility factors in diverse pathosystems, as discussed in 1.2.5. In the following section, I document my efforts to test these hypotheses, primarily using reverse genetics and quantitative pathogenicity assays.

4.2 Results

4.2.1 ChEC108 is dispensable for *Ch* virulence on Arabidopsis

To first investigate the contribution of ChEC108 to *Ch* virulence, I made use of genetic resources and protocols established in the laboratory of Prof. Christine Faulkner. Specifically, a loss-of-function ChEC108 *Ch* strain (referred to as Δ ChEC108) was generated Dr Andrew Breakspear in the Δ Chku80 genetic background, referred to as WT in this work. To generate this strain, the native ChEC108 coding sequence was exchanged for a *HygR* cassette via homologous recombination (Figure 4.3A). In a background lacking ChKu80 (a component of the non-homologous end joining pathway) the efficiency of site-specific integration of the *HygR* cassette is enhanced (Korn et al., 2015). In vitro growth on non-selective sterile media

was comparable between the $\Delta ChEC108$ and WT background strain (Figure 4.3B). This is unsurprising considering *ChEC108* expression was previously shown to be induced during the biotrophic phase of infection, not during axenic growth (Dallery et al., 2017). Before experimentation, I confirmed deletion of the *ChEC108* coding sequence and site-specific integration of the *HygR* cassette by PCR from *Ch* genomic DNA (gDNA), extracted from liquid-grown mycelia (Figure 4.3C).

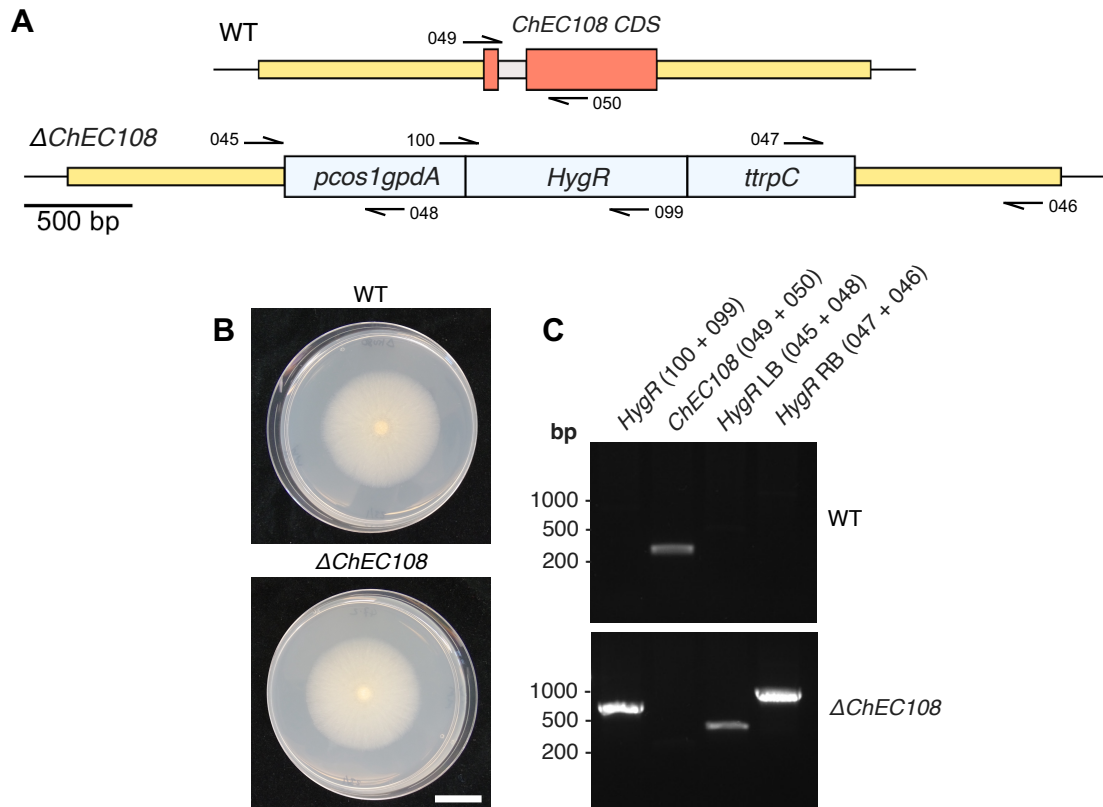


Figure 4.3 – Generation of $\Delta ChEC108$ by homologous recombination. **A.** Schematic of wild-type and $\Delta ChEC108$ genomic loci in the $\Delta Chku80$ (WT) background. In the $\Delta ChEC108$ strain, the *ChEC108* coding sequence (CDS) is replaced by a hygromycin resistance gene (*HygR*), under control of an *Aspergillus nidulans* *cos1gpdA* promoter and *trpC* terminator. Homologous recombination to integrate the *HygR* cassette was guided by the 5' and 3' flanking regions of the *ChEC108* locus (yellow). The binding sites of primers (not to scale) used for genotyping in C are shown. **B.** Comparable growth of WT and $\Delta ChEC108$ strains on MM plates. Photographs were provided by Dr Andrew Breakspear. Scale bar = 2 cm. **C.** Genotyping of $\Delta ChEC108$ by PCR from gDNA. Numbers represent primers used, the sequences of which are listed in Table S9.

To study the virulence of $\Delta ChEC108$ in relation to the WT background, detached Arabidopsis leaves (accession Col-0) were drop-inoculated with spore suspensions from each strain. Lesion area was quantified 6 dpi, when infection of WT-treated samples was judged to be well-established, but not to the extent that leaves were fully consumed by the pathogen. Using a detached leaf experimental setup, upon infection of a compatible host, *Ch* induces grey necrotic lesions where host tissue is macerated, surrounded by symptomatic chlorotic

tissue not yet invaded by the fungus (Figure 4.2). As chlorotic symptoms were shown to be exacerbated by senescence in *Ch*-infected detached leaves (Liu et al., 2007), and not typically observed to the same severity in attached leaf setups (Hiruma and Saijo, 2016; Narusaka et al., 2004; Olivé and Campo, 2021), I judged necrotic lesion area to be the most informative proxy for *Ch* infection success. Unexpectedly, Δ *ChEC108* formed marginally (yet significantly) larger necrotic lesions than WT *Ch* (Figure 4.4A), a trend consistent across three independent experiments (Figure 4.4B). Contrary to the hypothesis outlined in 4.1.2, this finding suggests ChEC108 negatively impacts infection progression of *Ch* on Arabidopsis, with the effector being dispensable for virulence.

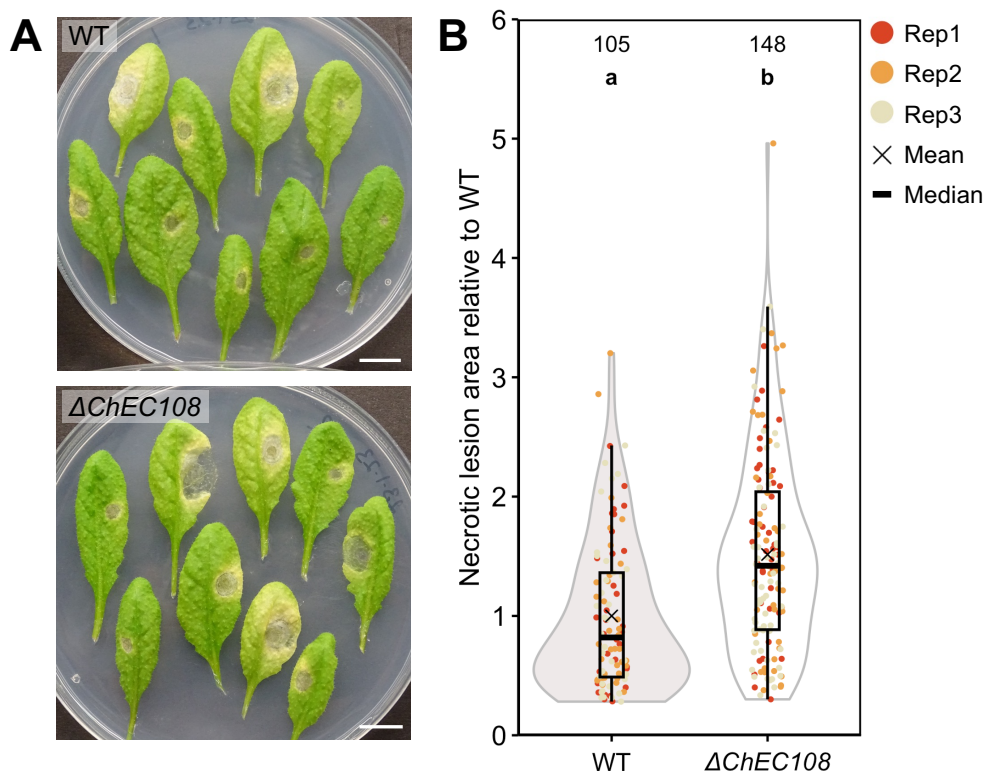


Figure 4.4 – Loss of *ChEC108* increases *Ch* virulence on Arabidopsis. **A.** Representative photographs of disease lesions induced by WT or Δ *ChEC108* on Col-0 Arabidopsis leaves. Photographs were taken 6 dpi. Scale bar = 1 cm. **B.** Quantification of 2D area of necrotic lesions. Three independent replicates were carried out (Rep1-3), and data were normalised by dividing all measurements by the mean area of WT lesions for a given replicate. Numeric annotations indicate sample size (leaves). Letters indicate significant differences between genotypes ($p < 0.05$), as determined using a linear mixed-effects model, with replicate as a random effect and genotype as a fixed effect, followed by an ANOVA (Satterthwaite's Method): $F = 29.2$, $df = 1$, $p < 0.001$. For experimental details of each replicate, see Table S12.

Although the Δ *Chku80* genetic background was specifically chosen to promote targeted genomic insertion of constructs into the *Ch* genome, there remains a possibility that off-target insertion events disrupted the expression of genes besides *ChEC108* in the Δ *ChEC108* strain. To confirm that the enhanced virulence phenotype of Δ *ChEC108* was underpinned by deletion of *ChEC108* only, I sought to complement the mutant by re-inserting a wild-type gene copy.

Towards this goal, Dr Andrew Breakspear constructed a novel plasmid backbone for *Ch* transformation (FP08077), including a PPT resistance cassette (*Bar*) to facilitate selection of transformants in genetic backgrounds already including a *HygR* gene. PPT (a glutamate analogue, and the active ingredient in Basta[®] herbicide) is an inhibitor of glutamine synthetases, which are critical for nitrogen metabolism in plants and fungi alike (Albrecht and Kortekamp, 2009; D'Halluin et al., 1992; Vallejo et al., 2017). *Bar* encodes a PPT acetyltransferase, enabling detoxification of PPT via acetylation, and has been previously employed as a selectable transformation marker for *Neurospora crassa* (Avalos et al., 1989) and *Aspergillus niger* (Ahuja and Puneekar, 2008). As observed for many fungal species (reviewed by Ahuja and Puneekar, 2008) *Ch* proved only moderately susceptible to PPT, with mycelial growth still visible on solid media containing 2000 µg/ml. Nevertheless, via the optimisation efforts of myself and Dr Andrew Breakspear, we discovered that a PPT concentration of 400 µg/ml could be used to distinguish faster-growing transformants carrying the *Bar* gene from other marginally PPT-resistant, non-transformed colonies. With a new selection system established, Dr Breakspear cloned the *ChEC108* coding sequence into the FP08077 backbone, flanked by the native *ChEC108* promoter (1000 bp upstream of the start codon) and terminator (950 bp downstream of the stop codon).

The pathogenicity phenotype described above for $\Delta ChEC108$ raises the intriguing possibility that ChEC108 effectors secreted by wild-type *Ch* might be perceived by Arabidopsis, leading to signalling events that ordinarily culminate in a marginal reduction of *Ch* growth. To test whether the Arabidopsis protein HIPP6 - a newly identified binding partner of ChEC108 - could be involved in these events, I sought to complement $\Delta ChEC108$ with a mutant gene copy, encoding ChEC108^{D164R}. This effector variant failed to bind wild-type HIPP6 in vitro and in vivo (see 3.2.10), and so allows specific investigation of the consequences of ChEC108-HIPP6 binding in an infection context. I cloned the *ChEC108*^{D164R} coding sequence into the FP08077 backbone, again flanked by the native *ChEC108* promoter and terminator, and proceeded to transform spores of $\Delta ChEC108$ with both this plasmid and that encoding the wild-type *ChEC108* sequence described earlier.

As the complementation strategy outlined above requires insertion of the *Bar* gene to enable selection of transformants, targeted insertion at the original *ChEC108* genomic locus would be inappropriate, and I instead took a random insertion approach. Despite the supposedly reduced efficiency of non-site-specific integrations in the $\Delta Chku80$ background (Korn et al., 2015), I obtained multiple PPT-resistant *Ch* transformants. After screening via PCR from gDNA, I confirmed the presence of both *HygR* and *Bar* genes, as well as the *ChEC108* coding sequence, in at least one transformant for each desired strain (Figure 4.5A, lanes1-3). Using a primer targeted to the terminator of the *HygR* cassette and another targeting the native *ChEC108*

terminator (as used in Figure 4.3), I confirmed the $\Delta ChEC108$ genetic background of these transformants (Figure 4.5A, lane 4). In vitro appearance and growth of $\Delta ChEC108.ChEC108$ and $\Delta ChEC108.ChEC108^{D164R}$ colonies were indistinguishable from WT and $\Delta ChEC108$ controls (Figure 4.5B), suggesting random insertion of constructs did not perturb the function of key genes for fungal development.

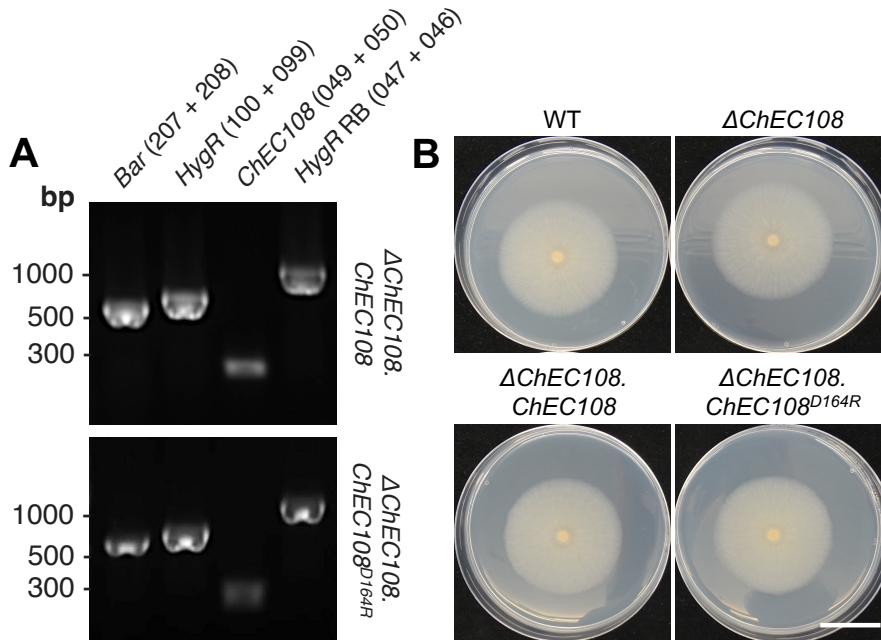


Figure 4.5 – $\Delta ChEC108$ complementation strains generated by non-targeted insertion. **A.** Genotyping of the $\Delta ChEC108.ChEC108$ and $\Delta ChEC108.ChEC108^{D164R}$ strains by PCR from gDNA. Numbers in brackets represent primers used, the sequences of which are listed in Table S9. **B.** Comparable growth of strains in vitro. Plates were photographed 8 days post-application of 5 μ l droplets of spore suspension (2×10^6 spores/ml) to the centre of MM plates. Scale bar = 2 cm.

Following drop-inoculation of Col-0 Arabidopsis leaves, I anticipated that $\Delta ChEC108.ChEC108$ and WT would induce lesions of a similar size, while $\Delta ChEC108$ controls would induce larger lesions, as observed in Figure 4.4. Meanwhile, if a lack of ChEC108-HIPP6 binding events *does* underpin the $\Delta ChEC108$ phenotype, $\Delta ChEC108.ChEC108^{D164R}$ mutants (being unable to target HIPP6 in planta) would be expected to induce lesions of a similar size to $\Delta ChEC108$. I was therefore surprised to observe that lesions induced by all four *Ch* strains - WT, $\Delta ChEC108$, $\Delta ChEC108.ChEC108$ and $\Delta ChEC108.ChEC108^{D164R}$ - were similar in size (Figure 4.6A), a finding consistent across four independent repeats (Figure 4.6B). Although no major adjustments to infection assay protocols were made during the 26-month interval between data collection for Figure 4.4 and Figure 4.6, it is possible that minor environmental changes (for instance, light intensity and humidity in growth cabinets, or room temperature during *Ch* cultivation) could influence fungal performance. This suggests that the $\Delta ChEC108$ phenotype originally observed in Figure 4.4 could be more subtle and context-dependent than initially thought.

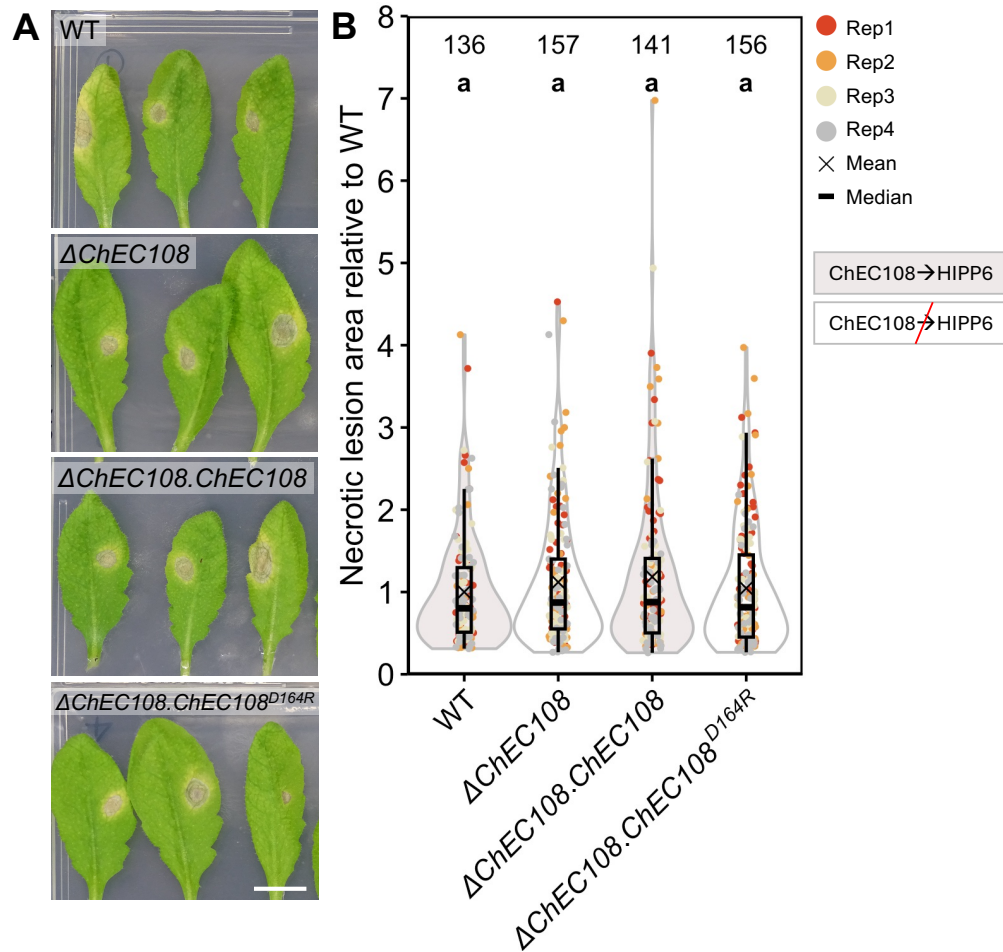


Figure 4.6 – The enhanced virulence phenotype of Δ ChEC108 could not be reproduced in subsequent assays with complemented strains. **A.** Representative photographs of disease lesions induced by WT, Δ ChEC108, Δ ChEC108.ChEC108 and Δ ChEC108.ChEC108^{D164R} on Col-0 Arabidopsis leaves. Photographs were taken 6 dpi. Scale bar = 1 cm. **B.** Quantification of 2D area of necrotic lesions. Four independent replicates were carried out (Rep1-4), and data were normalised by dividing all measurements by the mean area of WT lesions for a given replicate. Grey and white violins represent scenarios where the ChEC108-HIPP6 interaction could and should not occur in planta, respectively. Numeric annotations indicate sample size (leaves). Letters indicate no significant differences between genotypes ($p < 0.05$), as determined using a linear mixed-effects model, with replicate as a random effect and genotype as a fixed effect, followed by an ANOVA (Satterthwaite's Method): $F = 1.4$, $df = 3$, $p = 0.249$. For experimental details of each replicate, see Table S12.

4.2.2 *hipp6* mutants are marginally more susceptible to *Ch*

Independent of ChEC108, to investigate the function of HIPP6 in plant immunity, I acquired Arabidopsis lines with *HIPP6* loss-of-function. *hipp6-2* seeds were generously provided by Prof. Tomáš Werner, University of Graz, Austria. In this mutant, a 1 bp insertion in the third exon of *HIPP6* (introduced via CRISPR-Cas9), results in frame shift and a premature stop codon, preventing translation of HMA2 or the C-terminal tail of HIPP6 (Figure 4.7A-B). To obtain an additional loss-of-function allele, I generated *hipp6-3* mutants using CRISPR-Cas9, eliminating the entire coding sequence of *HIPP6* by Cas9-induced cutting within both the promoter region

and 3' UTR (Figure 4.7A). Homozygous deletion of a 2367 bp fragment from the *HIPP6* locus was confirmed by PCR and Sanger sequencing, using primers either side of the Cas9-targeted sites (Figure 4.7C).

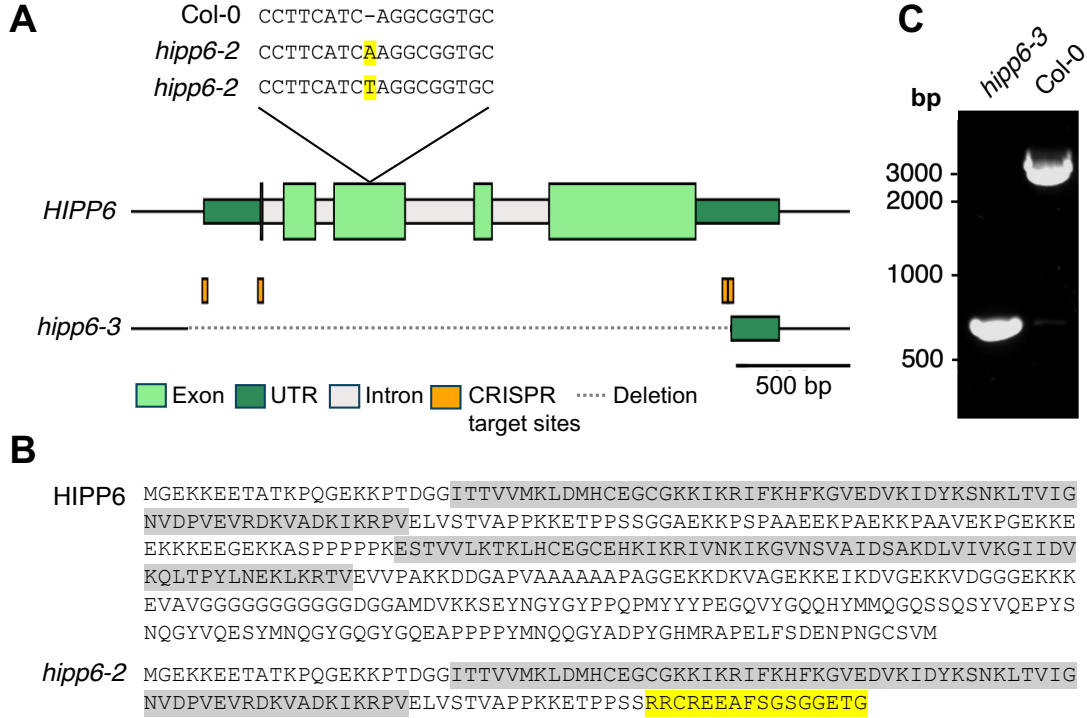


Figure 4.7 – *hipp6* CRISPR mutant genotypes. **A.** Schematic showing mutations present in *hipp6-2* and *hipp6-3* lines. *hipp6-2* is heterozygous for a 1 bp insertion (either A or T) in the third exon, resulting in frame shift and a premature stop codon. *hipp6-3* was generated by simultaneously targeting Cas9 to the promoter and 3' UTR, resulting in a 2367 bp deletion encompassing the 5' UTR and all exons and introns. **B.** Primary sequence of wild-type HIPP6, and the truncated protein encoded in the *hipp6-2* genomic background. HMA domains are coloured grey. Non-native amino acids introduced due to a frame shift in *hipp6-2* are coloured yellow. **C.** Genotyping of *hipp6-3* by PCR using primers either side of the CRISPR target sites (085 and 086, see Table S9 for primer sequences), confirming deletion of the *HIPP6* coding sequence.

As functional redundancy is predicted to be common within the HIPP family (de Abreu-Neto et al., 2013; Guo et al., 2021), I anticipated that higher order mutants could be required to study the impact of *HIPP* loss-of-function during *Ch* infection. HIPP6 belongs to clade I of the Arabidopsis HIPP family (de Abreu-Neto et al., 2013), the members of which (named HIPP1-9, excluding HIPP2) all share a tandem HMA domain architecture. With this in mind, and considering HIPP3 as another candidate host target of ChEC108 (albeit an unconfirmed target in vivo) (Figure 3.2 and table 3.1), attempts were made to generate a *hipp6-3 hipp3* double mutant alongside *hipp6-3*. However, during PCR screening efforts, I was unable to identify large Cas9-induced deletions within *HIPP3* genomic loci, suggesting Cas9 did not cut efficiently at the target sites selected. Elsewhere, a study by Guo et al. (2021) identified

HIPP5, *HIPP6* and *HIPP7* to be early cytokinin-responsive genes, implicated in controlling Arabidopsis root development via regulation of cytokinin signalling. It is therefore plausible that *HIPP5*, *HIPP6* and *HIPP7* share redundant or overlapping functions. To test this idea in the context of *Ch* infection, a *hipp5 hipp6-2 hipp7* triple mutant was provided by Prof. Tomáš Werner. This line carries the *hipp6-2* CRISPR mutation, as well as T-DNA insertions in *HIPP5* and *HIPP7* (see Table S8 for details).

If *HIPP6* is targeted by ChEC108 to promote *Ch* virulence, one would expect *HIPP6* loss-of-function to enhance Arabidopsis resistance to this pathogen. Conversely, *HIPP6*-mediated perception of ChEC108 could hinder *Ch* infection progress, which might explain the enhanced virulence of Δ ChEC108 reported in Figure 4.4. When drop-inoculated with WT *Ch* spores, *hipp6-2*, *hipp6-3* and *hipp5,6,7* mutant leaves displayed enhanced susceptibility relative to Col-0, reflected by their marginally larger necrotic lesions (Figure 4.8A). Also, the enhanced susceptibility phenotype of *hipp6-3* and *hipp5,6,7* was dependent on the presence of ChEC108, as Δ ChEC108 induced lesions of a similar size on Col-0, *hipp6-3* and *hipp5,6,7* leaves (Figure 4.8B). Curiously, *hipp6-2* remained marginally more susceptible to Δ ChEC108, despite sharing an identical *HIPP6* mutation with *hipp5,6,7*. The reasons for this remain unclear, but there are at least three possible explanations: (1) constitutive expression of *pUBQ10:3×mCherry-NLS* in the *hipp6-2* background (which is absent from *hipp5,6,7*) promotes susceptibility, perhaps due to gene disruption at the insertion site of the transgene; (2) off-target mutations induced by Cas9 during the generation of this line somehow promote susceptibility. Such background mutations may not have been crossed into *hipp5,6,7* alongside the *hipp6-2* mutation; (3) simultaneous loss of *HIPP5* and *HIPP7* function may somehow dampen the more severe susceptibility phenotype caused by *hipp6-2* mutation. Nevertheless, the ChEC108-dependent enhanced susceptibility of *hipp6-3* implicates *HIPP6* as a positive regulator of Arabidopsis immunity, with a role that perhaps involves ChEC108 perception. To challenge this hypothesis further, I infected *hipp6-2* and *hipp6-3* leaves with both WT and Δ ChEC108 within the same experiment. On each plant genotype, lesions induced by WT and Δ ChEC108 were of a comparable size, suggesting Δ ChEC108 no-longer exhibits enhanced virulence in the absence of *HIPP6* (Figure 4.9).

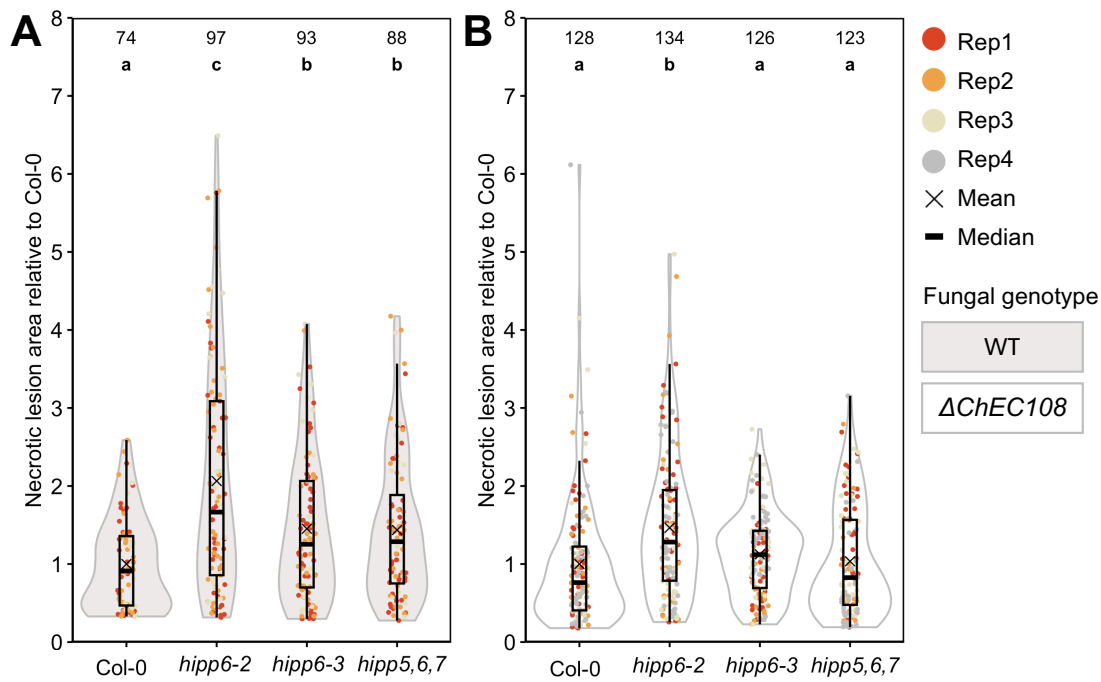


Figure 4.8 – Loss of *HIPP6* enhances Arabidopsis susceptibility to *Ch* expressing ChEC108. A-B. Quantification of 2D area of necrotic lesions induced by WT (A) or Δ ChEC108 (B) on leaves of four Arabidopsis genotypes: Col-0, *hipp6-2*, *hipp6-3* and *hipp5,6,7*. Three (A) or four (B) independent replicates were carried out (Rep1-4), and data were normalised by dividing all measurements by the mean area of Col-0 lesions for a given replicate. Numeric annotations indicate sample size (leaves). Letters indicate significant differences between genotypes ($p < 0.05$), as determined using a linear mixed-effects model, with replicate as a random effect and genotype as a fixed effect, followed by an ANOVA (Satterthwaite's Method). For panel A: $F = 14.4$, $df = 3$, $p > 0.001$. For panel B: $F = 3.21$, $df = 3$, $p = 0.023$. For experimental details of each replicate, see Table S12.

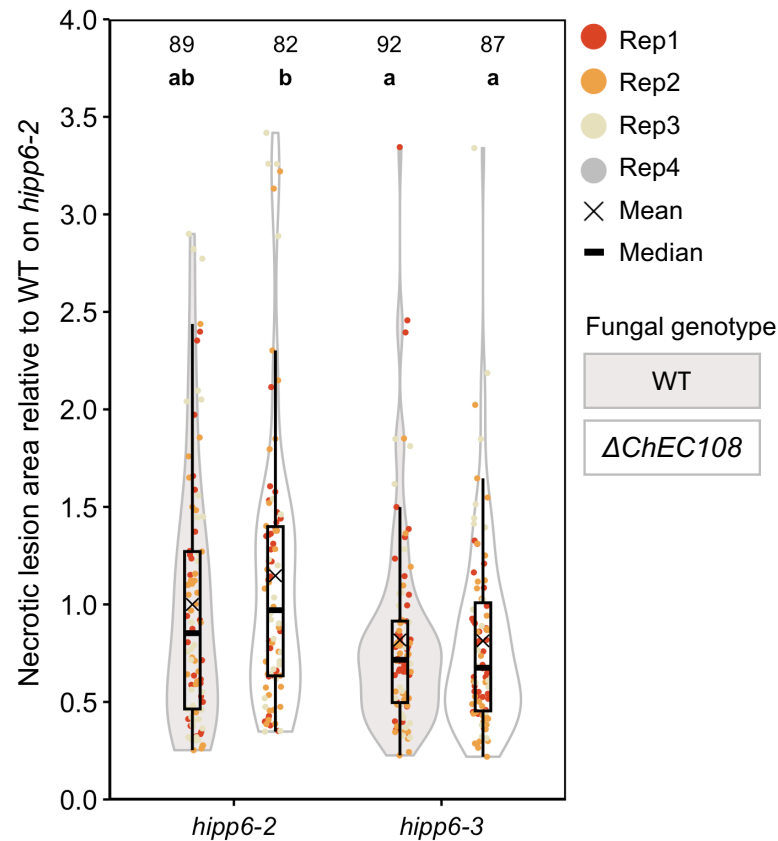


Figure 4.9 – *HIPP6* is required for the enhanced virulence of $\Delta ChEC108$. Quantification of 2D area of necrotic lesions induced by WT or $\Delta ChEC108$ on leaves of *hipp6-2* and *hipp6-3* Arabidopsis. Four independent replicates were carried out (Rep1-4), and data were normalised by dividing all measurements by the mean area of lesions induced by WT on *hipp6-2* for a given replicate. Numeric annotations indicate sample size (leaves). Letters indicate significant differences between genotypes ($p < 0.05$), as determined using a linear mixed-effects model, with replicate as a random effect and genotype as a fixed effect, followed by an ANOVA (Satterthwaite's Method): $F = 6.0$, $df = 3$, $p > 0.001$. For experimental details of each replicate, see Table S12.

4.2.3 Efforts to complement *hipp6-3* mutants

Given the possibility that off-target Cas9 activity could have disrupted the function of genes beyond *HIPP6* in *hipp6-3* plants, I sought to complement this mutant with a functional gene copy. I thus generated Arabidopsis lines expressing HIP6 as an N-terminal mGFP (from hereon GFP) fusion protein under the control of an Arabidopsis *UBIQUITIN 10* (*AtUBQ10*) promoter and cauliflower mosaic virus (CaMV) 35S terminator in the *hipp6-3* mutant background. When screening transformants via confocal microscopy and RT-qPCR, I observed high variation in GFP-HIP6 expression between individuals, likely underpinned by random integration of the expression cassette into the Arabidopsis genome (Figure 4.10). Notably, transformant #18 (named *hipp6-3.GFP-HIP6-18*) displayed high levels of GFP-HIP6 expression, stunted rosette growth in comparison to *hipp6-3* controls, and failed to set seed in a homozygous state (Figure 4.10A-B, E-F). Although my screening efforts did not uncover a second transformant with similar levels of GFP-HIP6 accumulation in the *hipp6-3* genetic background, *GFP-HIP6-15* (in the Col-0 background), exhibited a similar phenotype (Figure 4.10C, E-F), suggesting stunted rosette growth is likely a result of *HIPP6* overexpression and not random disruption of a key developmental regulator. Alongside, transformants *hipp6-3.GFP-HIP6-32* and *GFP-HIP6-16* were identified with markedly lower GFP-HIP6 expression and rosette growth comparable to that of *hipp6-3* control plants (Figure 4.10A-C, E-F). To qualitatively compare GFP-HIP6 protein levels in the aforementioned transgenic lines, I conducted a Western blot using proteins extracted from mature rosette leaves, including Col-0 and *hipp6-3* leaves as negative controls. As expected, protein abundance positively correlated with expression levels measured via RT-qPCR (Figure 4.11 versus Figure 4.10F).

As a negative control for upcoming infection assays, I sought to complement *hipp6-3* mutants with the HIP6^{MM} variant, which contains CXXC to GXXG mutations in both HMA domains and cannot be bound by ChEC108 according to in vivo co-IP data (Figure 3.27). Interestingly, despite exhibiting high levels of GFP-HIP6^{MM} expression (Figure 4.10F), the rosette growth of *hipp6-3.GFP-HIP6^{MM}-11* plants was comparable that of *hipp6-3* controls (Figure 4.10A, D-E). Alongside, I identified a further two transformants (#8 and #13) with high levels of GFP-HIP6^{MM} accumulation, later confirmed by Dr Barkha Ravi to be lacking obvious developmental phenotypes (personal communication). Overall, phenotypic analysis suggests constitutive *HIPP6* expression inhibits Arabidopsis vegetative growth in a dose-dependent manner, via a mechanism that requires functional metal-binding motifs in the HMA domains.

Before proceeding with further experiments, I recognised that pleiotropic developmental phenotypes observed in *hipp6-3.GFP-HIP6-18* plants could confound the results of infection

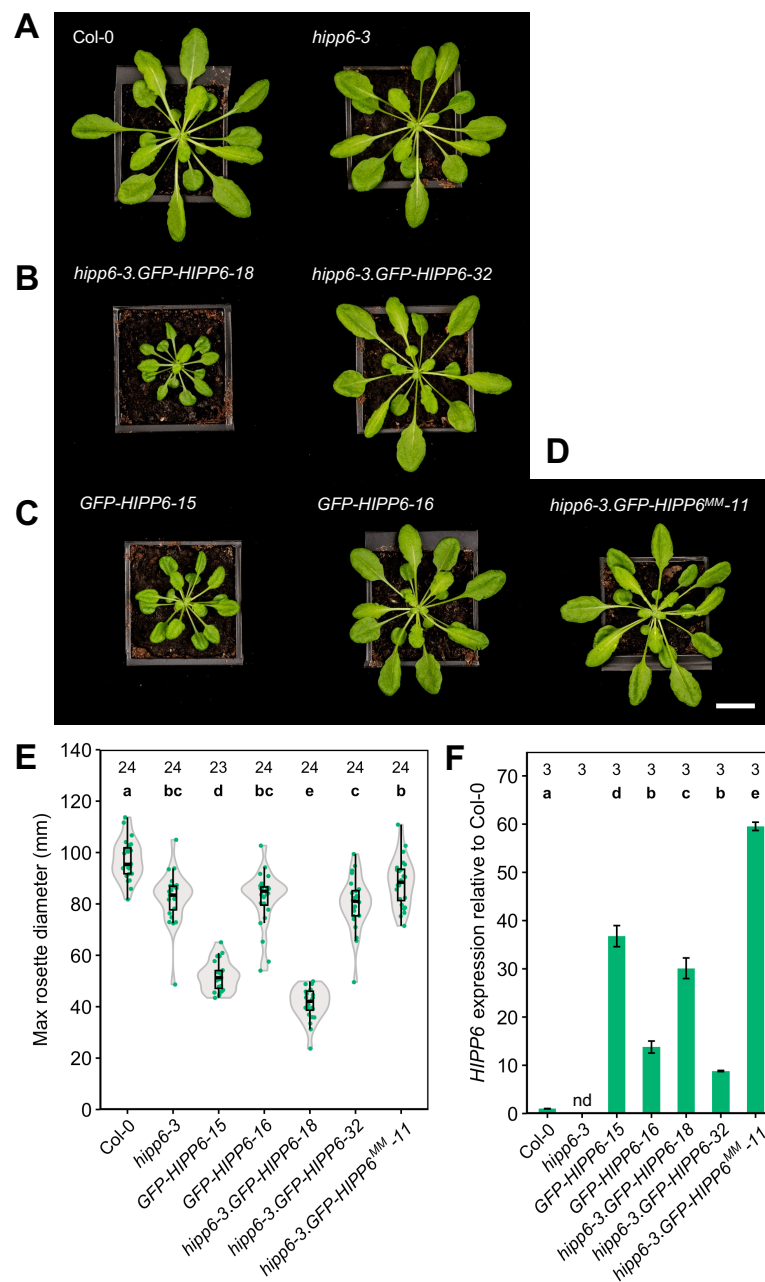


Figure 4.10 – Constitutive *HIPP6* expression inhibits *Arabidopsis* growth. A-D. 6-week-old *Arabidopsis* rosettes of indicated genotypes: Col-0 and *hipp6-3* (A), or independent lines constitutively expressing *GFP-HIPP6* in the *hipp6-3* (B) or Col-0 (C) background, or constitutively expressing *GFP-HIPP6^{MM}* in the *hipp6-3* background (D). Scale bar = 2 cm. E. Maximum rosette diameter of genotypes represented in A-D. Numeric annotations indicate sample size (plants, distributed across seven 24-cell trays). Letters indicate significant differences between genotypes ($p < 0.05$), as determined using a linear mixed-effects model, with tray as a random effect and genotype as a fixed effect, followed by an ANOVA (Satterthwaite's Method): $F = 125.6$, $df = 6$, $p < 0.001$. F. RT-qPCR analysis of *HIPP6* transcript levels in leaves of genotypes represented in A-D. Expression was normalised to that of *AtUBQ10* and plotted as a mean relative to Col-0. Numeric annotations indicate sample size (plants). Bars show standard error of the mean. Letters indicate significant differences between genotypes ($p < 0.05$), as determined using a one-way ANOVA followed by Tukey post-hoc tests: $F = 236.6$, $df = 5$, $p < 0.001$. *HIPP6* transcripts were not detected (nd) in *hipp6-3*, so this genotype was excluded from statistical analyses.

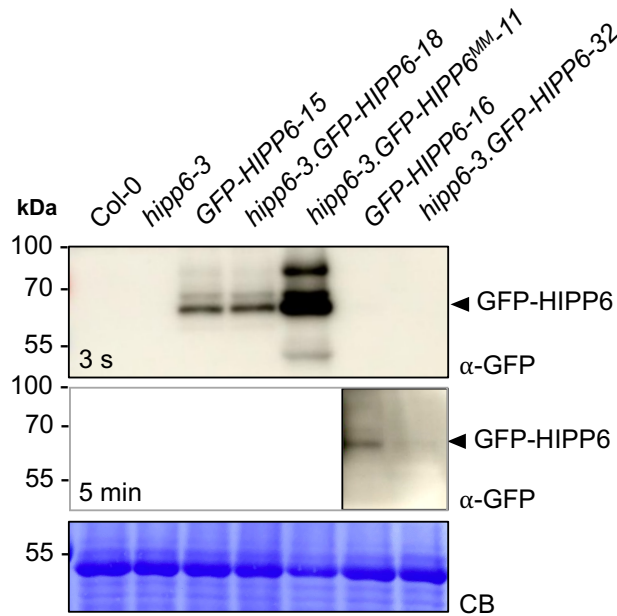


Figure 4.11 – Detection of GFP-HIPP6 and GFP-HIPP6^{MM} in Arabidopsis. Western blot confirming accumulation of GFP-HIPP6 (69.3 kDa) or GFP-HIPP6^{MM} (69.1 kDa) in mature rosette leaves of Arabidopsis transgenic lines detailed in Figure 4.10. Col-0 and *hipp6-3* protein extracts were included as negative controls. Upper and central panels show the same membrane, developed using different exposure times (3 s or 5 min, respectively), with over-exposed signal cropped out of the lower panel for clarity. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit.

assays, while also presenting a technical challenge for lesion area quantification. On the other hand, *hipp6-3.GFP-HIPP6-32* plants exhibited *HIPP6* expression levels greater than Col-0 (Figure 4.10F), yet their rosette size and morphology were similar to those of Col-0 and *hipp6-3* controls (Figure 4.10A, B, E). Thus, to test for complementation of *hipp6-3* phenotypes, I carried forward the *hipp6-3.GFP-HIPP6-32* line, with *hipp6-3.GFP-HIPP6^{MM}-11* serving as a negative control.

Considering the aforementioned enhanced susceptibility of *hipp6-3* mutants (Figure 4.8), I hypothesised that complemented *hipp6-3.GFP-HIPP6-32* plants should display a wild-type (lower) level of *Ch* susceptibility. Meanwhile, if the comparatively poorer performance of *Ch* on Col-0 plants was underpinned by ChEC108-HIPP6 binding events, *hipp6-3.GFP-HIPP6^{MM}-11* should remain highly susceptible. I was therefore surprised to find that Col-0, *hipp6-3*, *hipp6-3.GFP-HIPP6-32* and *hipp6-3.GFP-HIPP6^{MM}-11* plants were all equally susceptible to WT *Ch* in pathogenicity assays (Figure 4.12A), a trend consistent across two independent repeats (Figure 4.12B). As discussed in 4.2.1 in relation to assays involving Δ ChEC108, it is likely that unknown environmental fluctuations could be responsible for the variation in *hipp6-3* phenotypes observed here (Figure 4.12 versus Figure 4.8), as experiments were conducted 24 months apart. This highlights the subtle nature of the previously-described *hipp6-3*

susceptibility phenotype, as well as the challenges associated with discerning the biological relevance of ChEC108-HIPP6 interaction at the level of macroscopic lesion formation.

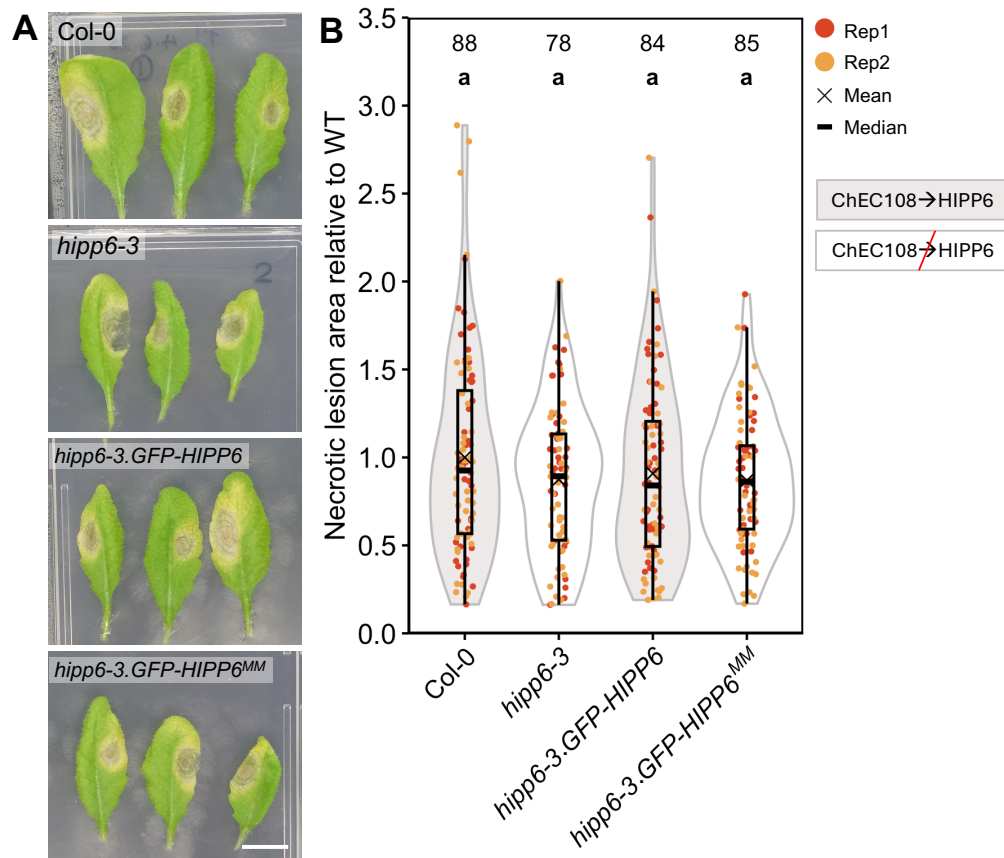


Figure 4.12 – The enhanced susceptibility phenotype of *hipp6-3* could not be reproduced in subsequent assays with complemented lines. **A.** Representative photographs of disease lesions induced by WT *Ch* on Col-0, *hipp6-3* and *hipp6-3.GFP-HIPP6*-32 and *hipp6-3.GFP-HIPP6^{MM}*-11 Arabidopsis leaves. Photographs were taken 6 dpi. Scale bar = 1 cm. **B.** Quantification of 2D area of necrotic lesions. Two independent replicates were carried out (Rep1-2), and data were normalised by dividing all measurements by the mean area of Col-0 lesions for a given replicate. Grey and white violins represent scenarios where the ChEC108-HIPP6 interaction could and should not occur in planta, respectively. Numeric annotations indicate sample size (leaves). Letters indicate no significant differences between genotypes, as determined using a linear mixed-effects model, with replicate as a random effect and genotype as a fixed effect, followed by an ANOVA (Satterthwaite's Method): $F = 1.4$, $df = 3$, $p = 0.253$. For experimental details of each replicate, see Table S12.

4.2.4 Exploring trypan blue staining as an alternative method to quantify *Ch* virulence

According to infection assay data described thus far, it seems that neither ChEC108 nor HIP6 are critical determinants of *Ch* symptom development on *Arabidopsis*. Instead, it is possible that the functions of these two proteins and the outcomes of the interaction between them are only relevant (or at least, can only be detected) at specific stages within the *Ch* infection cycle. For example, loss of the ChEC108 effector could result in an altered frequency of host penetration events, abnormal hyphal development, or an altered rate of transition between the biotrophic and necrotrophic stages of infection. Such perturbations might not influence the eventual size of necrotic lesions at later stages of infection, but could contribute to overall fungal fitness. With this in mind, as an alternative to macroscopic lesion quantification, I established a trypan blue staining protocol to study infection structures at the microscopic level.

With the aim of comparing intracellular structures at discrete infection sites, I spray-inoculated mature Col-0 *Arabidopsis* leaves with spores of WT or Δ ChEC108, and harvested leaf discs 1, 2, 3, 4 and 5 dpi. 1-3 dpi, appressoria were seen peppering the leaf surfaces, indicative of spore germination and incipient host penetration events (Figure 4.13). 4-5 dpi, invasive hyphae of various shapes and sizes were identified in both WT- and Δ ChEC108-infected leaves, signifying a range of infection stages from biotrophy (Figure 4.14A-C) to necrotrophy (Figure 4.14D-E). In the case of both fungal genotypes, hyphae at multiple developmental stages were observed at single time points and even on individual leaves, emphasising the somewhat stochastic nature of *Ch* infection progression.

At each stage, hyphae with similar morphologies were observed in both WT- and Δ ChEC108-infected leaves, suggesting loss of ChEC108 does not severely alter *Ch* development in planta. Nevertheless, closer examination revealed that invasive hyphae (relative to the number of appressoria captured) were more abundant in Δ ChEC108-infected samples versus those infected with WT, a trend consistent for both biotrophic and necrotrophic hyphae, at both 4 and 5 dpi (Figure 4.14F-G). Although acquired as part of a preliminary experiment with a small sample size (only 4 leaves per genotype per time point), this data provides a hint that Δ ChEC108 might progress from appressorium development to invasive hyphal growth more rapidly than WT, which could underlie the marginally enhanced virulence phenotype reported in Figure 4.4. Given the inherently high variation associated with this biological system, future large-scale trypan blue staining efforts would be required to increase confidence in this conclusion.

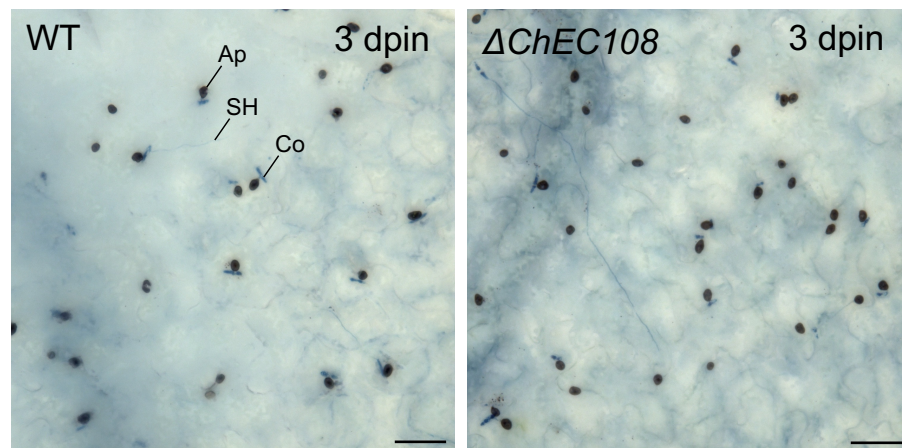


Figure 4.13 – Trypan blue-stained germinated *Ch* spores visible on *Arabidopsis* leaves. Brightfield image of *Arabidopsis* epidermal cells inoculated with WT (left) or $\Delta ChEC108$ (right) conidia (Co), stained with trypan blue. Brown ovate structures are appressoria (Ap). Occasionally, especially in densely inoculated areas, thin surface hyphae (SH) were seen extending from conidia, with or without associated appressoria. Images were acquired 3 dpi, but are representative of those acquired 1-3 dpi. At least 5 images were taken of inoculated sites on each leaf, with 4 leaves imaged per genotype per time point. Scale bar = 30 μ m.

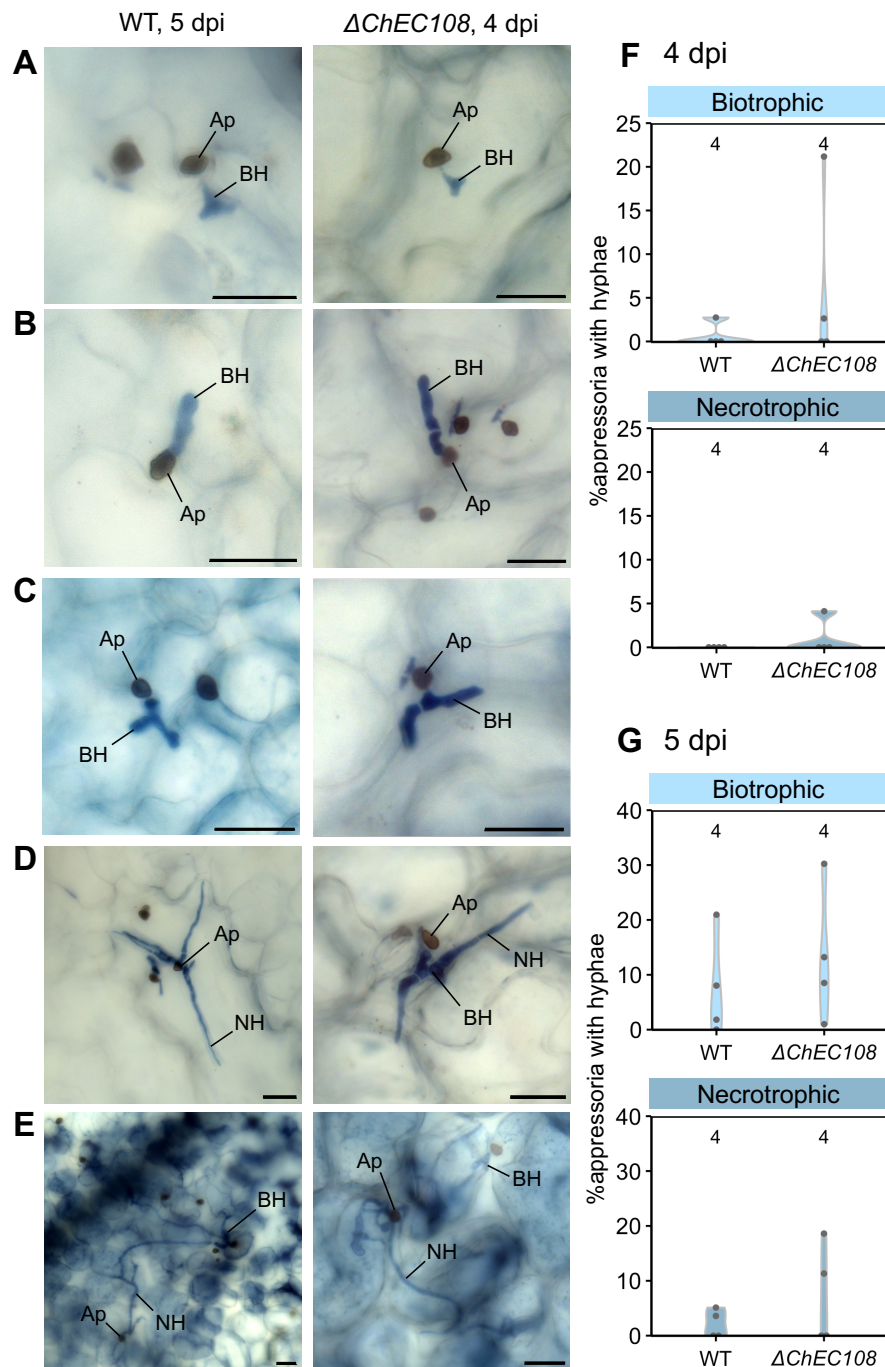


Figure 4.14 – Preliminary data suggest early $\Delta ChEC108$ infection progression is faster than WT. A-E. Trypan-blue stained hyphae of WT or $\Delta ChEC108$ within *Arabidopsis* leaf epidermal cells, acquired 5 and 4 dpi, respectively. Images were selected to represent different stages of infection, including the development of appressoria (Ap) from germinated conidia, and formation of early invasive biotrophic hyphae (BH) (A), growth (and often branching) of biotrophic hyphae (B-C), formation of necrotrophic hyphae (NH) (D), and finally, extensive necrotrophic hyphal growth, accompanied by host cell death (E). Scale bar = 20 μ m. **F-G.** Percentage of appressoria associated with biotrophic and necrotrophic hyphae observed 4 dpi (F) or 5 dpi (G). Numerical annotations indicate sample size (leaves), with number of appressoria counted per leaf ranging from 39-170 (F) and 43-167 (G).

4.2.5 Progress towards understanding the downstream consequences of ChEC108-HIPP6 association

Although often viewed as a cruder approach to understand gene function in comparison to reverse genetics, overexpression of genes of interest can provide functional insights when loss-of-function mutants are particularly challenging to generate, or prove uninformative. Alongside the generation of fungal and plant genetic resources described thus far, I continued the efforts of Dr Joanna Jennings to generate transgenic *ChEC108*-expressing *Arabidopsis* lines. Previously, Dr Jennings attempted to drive *ChEC108-eGFP* expression via the constitutive CaMV 35S promoter, but was unable to obtain positive transformants in the T1 generation while screening for PPT-resistant seedlings on soil (Jennings, 2021). When repeating this, I was able to identify only one transformant with confirmed integration of the *ChEC108* coding sequence via PCR. However, this individual displayed heavily stunted growth and did not survive to set seed. On the premise that ChEC108 production is toxic to *Arabidopsis*, Dr Jennings employed both estradiol-inducible and Cre-Lox recombination expression systems to restrict *ChEC108* expression during early developmental phases and enable activation only when desired by an experimenter. However, in all cases, positive transformants were not identified (Jennings, 2021). Unexpectedly, for reasons unknown, using a selection system that involved screening for kanamycin-resistant T1 seedlings on sterile media, I was able to identify *Arabidopsis* lines expressing *ChEC108-eGFP* via the constitutive *AtUBQ10* promoter. Alongside, as a negative control for HIPP6-binding, I generated lines expressing *ChEC108^{D164R}-eGFP* via the same approach.

Plants constitutively expressing *ChEC108-eGFP* displayed severe developmental phenotypes, including stunted rosettes with small, curled leaves (Figure 4.15). In contrast, plants constitutively expressing *ChEC108^{D164R}-eGFP* developed larger rosettes that resembled those of Col-0 plants (Figure 4.15). Although comparisons of expression level and protein accumulation have yet to be carried out, evidence from confocal microscopy suggested the accumulation of effector-GFP fusions was similarly low in each of the four transgenic lines pictured in Figure 4.15 (for confocal images, see Figure 5.20 in Chapter 5). I am therefore doubtful that variation in expression levels are solely responsible for the phenotypic differences of *ChEC108-eGFP* versus *ChEC108^{D164R}-eGFP* lines. Overall, these preliminary observations suggest ChEC108 might induce stress in *Arabidopsis*, heavily stunting growth. Moreover, this activity seems dependent on Asp164 - a residue that contributes to metal ion coordination and facilitates interaction with the HIPP6 HMA domains (Section 3.2.10). This raises the possibility that HIPP6 could participate in ChEC108-mediated growth inhibition.



Figure 4.15 – Constitutive *ChEC108* expression severely inhibits *Arabidopsis* growth. 6-week-old rosettes of Col-0 *Arabidopsis*, and two independent lines constitutively expressing *ChEC108-eGFP* or *ChEC108^{D164R}-eGFP*. Scale bar = 2 cm.

4.3 Discussion

In this chapter, I described genetic experiments designed to probe the functions of ChEC108 and its host target HIP6 in the context of *Ch* infection. Initially, data collected from infection assays utilising $\Delta ChEC108$ and *hipp6-3* loss-of-function mutants supported a model whereby HIP6 positively regulates *Arabidopsis* resistance to *Ch* in a ChEC108-dependent manner, as summarised in Figure 4.16. In this scenario, as Col-0 plants (expressing functional HIP6) remain susceptible to WT *Ch* (putatively secreting functional ChEC108), it seems that HIP6-mediated defence must be quantitative as opposed to the qualitative ‘all-or-nothing’ resistance responses typically associated with resistance (R) gene / avirulence (Avr) gene pairings.

Considering evidence gathered from transgenic lines generated as part of this work, it is plausible that HIP6 ordinarily modulates *Arabidopsis* stress responses, such that its overexpression triggers overfiring of protective signalling pathways, culminating in growth inhibition (Figure 4.10). In the presence of ChEC108, stabilisation of HIP6 (as observed in co-IP experiments displayed in Figures 3.2, 3.3, 3.26 and 3.27) could have a similar effect and at least partially underpin the stunted growth of *ChEC108-eGFP*-expressing plants (Figure 4.15). In an infection context, ChEC108-dependent promotion of HIP6 signalling could marginally enhance *Arabidopsis* resistance to *Ch*, but not to the extent that fungal growth is halted. Gathering further support for this hypothesis has proved challenging, as I was unable to reproduce both the $\Delta ChEC108$ (enhanced virulence) and *hipp6-3* (enhanced susceptibility)

mutant phenotypes when testing for complementation in subsequent infection assays. This implies that the influence of ChEC108 and HIP6 on *Ch* infection outcomes is minor and perhaps context-dependent.

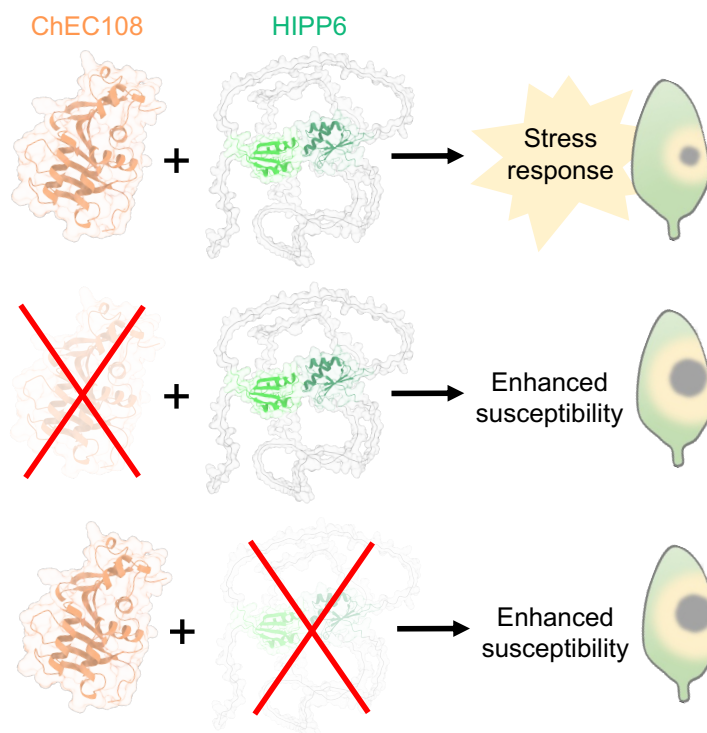


Figure 4.16 – Speculative model to illustrate the role of HIP6 during *Ch* infection of Arabidopsis. ChEC108-HIP6 binding events might stabilise HIP6, triggering a stress response in Arabidopsis that marginally enhances resistance to *Ch*, as represented by a small macroscopic lesion area on a mature infected leaf. However, in the absence of either ChEC108 or HIP6, a stress response is not triggered, leading to enhanced susceptibility and formation of larger disease lesions.

4.3.1 The challenges of studying effector function using reverse genetics

Filamentous pathogens are renowned for producing extensive arsenals of secreted effector proteins (Guyon et al., 2014; Haas et al., 2009; Yan et al., 2023), which is perhaps a testament to their complex infection stage transitions, necessity for ETI suppression and broad host ranges (Thordal-Christensen et al., 2018). *Ch* is no exception. Initially, the *Ch* genome was predicted to encode a repertoire of 365 candidate secreted effectors, defined as having no significant homology to proteins outside the *Colletotrichum* genus (O’Connell et al., 2012). Utilising an alternative bioinformatic pipeline, a later search by Lu et al. (2022) boosted this estimate to 417 candidates. However, analysis of expressed sequence tags by Kleemann et al. (2012) indicated that a more modest number of effector candidates (198 proteins, assigned the name ChEC) are transcribed during in planta infection stages, with 102 of these being preferentially expressed during biotrophy as opposed to necrotrophy. Regardless of the exact number, it is clear that

ChEC108 remains one small protein in a hypothetical sea of secreted effectors.

From this perspective, it is perhaps unsurprising that *ChEC108* loss-of-function resulted in only a subtle modification (Figure 4.4) or no detectable modification (Figure 4.6) of *Ch* virulence. Such findings are not uncommon in plant pathology, especially when only a single stage of infection is examined. For instance, following a screen of 50 effector candidates from *Bgh* for virulence functions in *H. vulgare* leaves using host-induced gene silencing (HIGS), only eight were found to have a significant impact on haustoria formation (Pliego et al., 2013). Similarly, after individually disrupting 78 candidate effector genes from *M. oryzae* using transposon arrayed gene knockout (TAG-KO), Saitoh et al. (2012) identified only one gene with a measurable impact on *O. sativa* and *H. vulgare* susceptibility to blast. Even loss of the highly conserved, putatively cell-death inducing CEC3 effector did not detectably influence *Ch* virulence on Arabidopsis (Tsushima et al., 2021). These observations could be underpinned by (1) the virulence contributions of most effectors being too small to detect at the macroscopic level, or (2) functional redundancy among effectors, perhaps involving compensation for loss of individual effectors at the level of transcription or translation.

To search for proteins that could potentially function redundantly with ChEC108, I used FoldSeek (van Kempen et al., 2024) to query the AlphaFold Database (AFDB50), and discovered four ChEC108-like candidates from *Ch* among the resulting list of proteins with similar predicted structures, each annotated as a 'hypothetical protein'. Three of these (encoded by genes CH63R_12089, CH63R_00260 and CH63R_00332) were predicted by SignalP-6.0 (Teufel et al., 2022) to be secreted proteins, yet had not been previously classified as effectors. However, the fourth, encoded by the gene CH63R_05615, was previously named ChEC32 (Kleemann et al., 2012). In a stage-specific transcriptomic study, expression of *ChEC32* was found to peak during the biotrophic phase of *Ch* infection in a similar manner to *ChEC108* (Dallery et al., 2017). With its putative signal peptide removed (amino acids 1-21 inclusive), the AlphaFold3 predicted structure of ChEC32 closely aligns with that of ChEC108 (Figure 4.17), suggesting these two proteins might function similarly, despite their primary sequences sharing only 24.5% identity. While further genetic experiments, mutational analysis and cell biological studies would be required to test this hypothesis, the identification of other candidate ChEC108-like folds within the *Ch* secretome highlights the potential for functional redundancy.

It is becoming increasingly apparent that plant-associated microbes utilise complex effector networks to manipulate their hosts, rife with epistatic interactions and redundancy, such that global effectome function does not equate to a simple sum of individual effector functions (reviewed by Arroyo-Velez et al., 2020). Significant progress towards a more holistic understanding of effectome function has been made using the bacterium *Pto* DC3000,

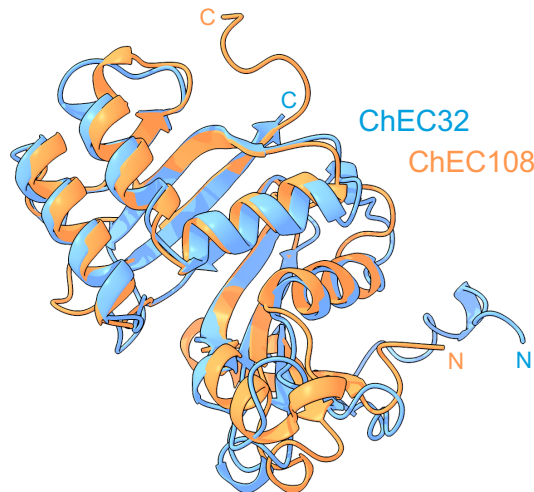


Figure 4.17 – ChEC32 is predicted to fold similarly to ChEC108. ChEC32 AlphaFold3 predicted structure (blue) with its putative signal peptide removed, overlaid with the ChEC108 crystal structure (orange), extracted from the ChEC108-HMA2 complex (see 3.2.8). Structural alignment was performed in ChimeraX using the matchmaker command. RMSD across all 165 C α pairs = 3.339.

specifically by systematic restoration of effector genes in the polymutant $\Delta 36E$ background, which lacks all 36 type III secreted effectors encoded by the wild-type genome (Wei et al., 2015). Considering the vast (and often uncertain) number of candidate effectors encoded by filamentous pathogen genomes, as well as the requirement of efficient tools for gene deletion and high-throughput pathogenicity assays, deciphering effector networks remains a current challenge for fungal effector biologists.

Meanwhile, to gain further insight into ChEC108 function, it would be beneficial to compare the virulence of WT and $\Delta ChEC108$ at the microscopic scale using trypan blue staining of fungal structures, as discussed in 4.2.4. This method has been previously used alongside images of macroscopic disease symptoms to qualitatively illustrate differences in infection progression rate between *Ch* genotypes, and/or to quantify host penetration events (biotrophic hyphal counts, relative to appressoria) and necrotrophic switches (necrotrophic hyphal counts, relative to biotrophic hyphal counts) (for examples, see Liu et al., 2007; Plaumann et al., 2018; Schmidpeter et al., 2017). Alternative methods could also be explored, such as the assessment of absolute fungal biomass via quantitative PCR (qPCR)-amplification of a *Ch* gDNA fragment within infected leaf samples, or RT-qPCR-based assessment of *Ch* housekeeping gene expression, relative to a host housekeeper (Narusaka et al., 2009; Olivé and Campo, 2021). Building on this, the doctoral thesis of Dr Sally Jones (Jones, 2023) proposed a ‘kompetitive’ allele-specific PCR (KASP)-based assay as a novel approach to study the competitiveness of *Ch* genotypes in planta. Using differentially fluorescently-labelled primer pairs carefully designed to amplify strain-specific gDNA fragments, the proportion of DNA

in infected leaves corresponding to individual strains of interest could be assessed via qPCR. Assuming leaves are initially inoculated with a 1:1 spore mixture, any deviation from a 1:1 ratio of fluorescent signals following qPCR could indicate enhanced fitness of one strain over another. If reproducible insights into $\Delta ChEC108$ virulence versus WT can be gained, employing such methods might help to reinforce any conclusions drawn from lesion area measurements reported in this work.

Alongside assessments of *Ch* virulence, it would be interesting to explore the potential downstream implications of ChEC108-HIP6 binding at the transcriptomic level. Towards this goal, RNA-sequencing experiments are currently underway, being driven by myself and Dr Barkha Ravi, exploiting genetic resources described in Figures 4.3, 4.7 and 4.15.

4.3.2 Why has *Ch* retained ChEC108?

According to the speculative model presented in Figure 4.16, perception of ChEC108 by Arabidopsis (tentatively via HIP6), could lead to enhanced resistance of Arabidopsis to *Ch*, begging the question: why has this fungus not deleted *ChEC108* from its genome? With wild-type *Ch* remaining highly virulent on Col-0 Arabidopsis, it seems that possessing ChEC108 does not confer a major disadvantage, meaning evolutionary selection pressures might not be sufficient to drive deletion of this effector. Alternatively, the virulence functions of ChEC108 could be host-specific. Consistent with this, ChEC108 was annotated as a '*Ch*-specific' effector by O'Connell et al. (2012), meaning, rather than being part of a core battery of effectors with likely conserved and essential functions across *Colletotrichum* species, this protein could serve a comparatively niche function. *Ch* infects a broad range of hosts besides Arabidopsis, including Brassicaceous plants: cabbage, pak choi, mustard, turnip and swede (all *Brassica* spp.) and radish (*Raphanus sativus*) (Damm et al., 2014; O'Connell et al., 2004), as well as bellflowers (*Campanula* spp.) of the family Campanulaceae (Khodaei and Arzanlou, 2019), sorrel (*Rumex acetosa*) of the family Polygonaceae (Zhang et al., 2018) and brown lentil (*Lens culinaris*) of the family Leguminosae (O'Connell et al., 2004). It is therefore plausible that ChEC108 could make a significant positive contribution to *Ch* virulence during colonisation of these alternative hosts, meanwhile other *Ch*-specific effectors evolved to promote colonisation of Arabidopsis. To challenge this hypothesis in future and gauge the potential prevalence of ChEC108 'perception' across the *Ch* host range, it could be interesting to search for HIP6 orthologs within the genomes of alternative *Ch* hosts and determine whether their encoded proteins can bind ChEC108.

4.3.3 How specific is the ChEC108-HIP6 interaction?

As for $\Delta\text{ChEC108}$, genetic redundancy could be underpinning the subtle (Figure 4.8) or absent (Figure 4.12) *hipp6-3* susceptibility phenotypes reported in this work. Although *hipp5,6,7* triple mutants did not display enhanced susceptibility to *Ch* relative to *hipp6-3* single mutants (Figure 4.8), the Arabidopsis genome hosts a plethora of HMA proteins (de Abreu-Neto et al., 2013; Li et al., 2020), and the specificity of ChEC108-HIP6 interaction in planta has yet to be fully explored. HIP3 was identified as a potential binding partner for ChEC108 via IP-MS (Table 3.1), yet targeted co-IP assays failed to provide convincing evidence for ChEC108-HIP3 association (Figure 3.2). Another candidate interactor from Arabidopsis whose *N. benthamiana* ortholog was enriched following ChEC108 IP (albeit not significantly) is HPP1 (AT5G50740), a HMA domain-containing protein lacking an isoprenylation motif (Dr Joanna Jennings, unpublished data). Furthermore, work on in vitro purified proteins described in Chapter 3 revealed ChEC108 to be capable of binding either HMA1 or HMA2 from HIP6 with similarly high affinity. While these two domains do share a highly similar structure, their amino acid sequences are only 42% identical. Among clade I members of the HIP family, including HIP6 and HIP3, the sequence identities of HMA domains vary from 22-69% (Table S13), raising the possibility that ChEC108 could target multiple Arabidopsis HMA domains, even those with divergent sequences.

Support for this hypothesis has recently been gathered by Dr Daniel Yu, who demonstrated that ChEC108 can weakly bind the isolated HMA domains of HIP3 (HIP3^{HMA1} and HIP3^{HMA2}), and bind tightly to HIP5^{HMA1} in ITC experiments in the presence of 1 mM DTT and 50 μM ZnCl_2 (personal communication). As for the HIP6 HMA domains, binding to HIP5^{HMA1} could be abolished by Asp164Arg mutation of ChEC108, suggesting the effector could potentially target HIP6^{HMA1/2} and HIP5^{HMA1} via the same mechanism. However ChEC108 failed to bind HIP5^{HMA2} in ITC experiments, perhaps owing to the presence of a bulky Glu169 residue, which would hypothetically substitute for positively charged Lys39 (in HIP6^{HMA1}), or His169 in (HIP6^{HMA2}) at the ChEC108 binding interface, based on a primary sequence alignment (Dr Daniel Yu, personal communication). With further explorative tests and exploitation of the ChEC108-HMA1/2 crystal structures, one could predict the likelihood (and perhaps even affinity) of ChEC108-binding across the phylogeny of Arabidopsis HMA proteins. Additional candidate interactors could then be validated in planta, and their subcellular localisations compared to that of HIP6 (see work in Chapter 5), informing the generation of higher order mutants. With multiple ChEC108 host targets absent, it is possible that Arabidopsis susceptibility to *Ch* might be more severely impacted, and a more confident conclusion drawn regarding the role of HIPs in plant immunity.

4.3.4 Conclusion

Due to the complexity and inherent stochasticity of plant-fungus interactions, data obtained from infection assays in this context are variable, meaning extensive repetition and support from additional methods and/or time points are often required to confidently characterise the function of pathogen or host genes. Facing such challenges, efforts to study *ChEC108* and *HIP6* function during *Ch* infection of *Arabidopsis* using reverse genetics have given rise to a model in which HIP6, rather than functioning as a susceptibility factor, instead positively regulates *Arabidopsis* resistance to *Ch*, potentially via signalling pathways downstream of ChEC108 perception. Speculative hypotheses regarding the mechanism by which HIP6 could signal in this context are discussed in 7.1. Supporting this, rather than functioning as a virulence factor, ChEC108 appears to contribute to negative regulation of *Ch* virulence. Although further support for these ideas are undoubtedly required, I am hopeful that ongoing RNA-sequencing and microscopic observation of *Ch* infection structures will shed light on the consequences of ChEC108-HIP6 binding in planta. In the meantime, to better understand the intracellular functions of these two proteins, I continued my investigations at cell biology level.

Chapter 5

Understanding the subcellular localisation of ChEC108 and HIP6

5.1 Introduction

To explore the function of ChEC108 and HIP6 at the cellular level, work in this chapter builds on the cell biological studies of Dr Joanna Jennings and Assist. Prof. Mina Ohtsu. Previously, as part of a broad screen to explore *Ch* effector biology, the localisations of 38 candidate effectors (all designated ChEC proteins) were determined via transient expression in *N. benthamiana* as C-terminal GFP fusions ([Jennings, 2021](#)). These candidates localised to diverse compartments within cells, with nucleocytoplasmic signal being most commonly observed (23/38 proteins). Other candidates were reported in nuclei exclusively (ChEC111), in chloroplasts (ChEC153), and at PD (ChEC36, ChEC108, ChEC142, ChEC147 and ChEC155), prompting closer investigation into their molecular functions at these localities ([Jennings, 2021](#); [Jones, 2023](#), this work).

In an independent study, the localisation of 61 ChEC proteins were similarly assessed in *N. benthamiana*, but this time as N-terminal GFP fusions. This work uncovered additional subcellular structures that could be targeted by *Ch* effectors during biotrophy: peroxisomes (ChEC51a, ChEC89, and ChEC96), Golgi bodies (ChEC21) and microtubules (ChEC9, ChEC17 and ChEC113) ([Robin et al., 2018](#)). When expressed in *Ch* and visualised using immunogold labelling and confocal microscopy, ChEC6-mRFP and ChEC36-mRFP fusions were identified at the basal appressorium pore, suggesting they may be secreted early during infection and remain in the apoplast ([Kleemann et al., 2012](#)). In the same study, *Ch*-derived ChEC3-mRFP, ChEC13-mRFP, ChEC34-mRFP and ChEC89-mRFP labelled the surface of biotrophic hyphae, occupying the apoplastic space between the fungal cell wall and host plasma membrane.

Together, these localisation studies suggest the *Ch* secretome harbours potential manipulators of multiple compartments within plant tissues.

To investigate the capacity of *Ch* effectors to move cell-to-cell, Dr Jennings and Assist. Prof. Ohtsu conducted agroinfiltration-based assays in the *N. benthamiana* transient system. Of the 24 nuclear and/or cytoplasmic effectors screened, 15 exhibited mobility, while nine were immobile (Jennings, 2021). Alongside, by quantifying the mobility of fluorescent proteins of various sizes, a standard curve was constructed to enable the extent of an effector's mobility to be predicted given its molecular weight (Ohtsu et al., 2023). When screened for mobility, five ChEC proteins (ChEC111, ChEC127, ChEC129, ChEC130 and ChEC132) rose above this standard curve and were deemed 'hypermobile', suggesting they might exploit active mechanisms to enhance their cell-to-cell movement. Indeed, ChEC127 and ChEC132, alongside the non-hypermobile ChEC8, were subsequently shown to indirectly modify PD function, promoting the movement of co-expressed fluorescent proteins, despite not being enriched at PD themselves (Ohtsu et al., 2023). As pitfield-associated callose was found to be unchanged in transgenic Arabidopsis lines expressing *ChEC127* or *ChEC132*, such PD modification is thought to occur via callose-independent pathways. Overall, this study adds strength to the hypothesis that *Ch* employs effectors to manipulate cell-to-cell connectivity during infection, although the mechanisms by which this is achieved remain to be elucidated.

As mentioned previously (1.4.1), ChEC108 was identified as a PD-localised candidate effector and a likely *direct* manipulator of PD function by Dr Jennings. Interestingly, Robin et al. (2018) first described ChEC108 to be a nuclear protein, as N-terminal GFP fusions labelled the nucleoplasm, nucleolus and sub-nuclear structures known as Cajal bodies, but not PD. Consistent with this, Jennings (2021) independently verified that N-terminal tagging abolished PD localisation of ChEC108. Although excluded from the mobility screen described above, subsequent agroinfiltration-based assays in *N. benthamiana* revealed ChEC108 to be highly cell-to-cell mobile, while other PD-localised ChECs exhibited limited or no capacity for movement (Jennings, 2021). How does ChEC108 reach and become enriched at PD? And how does it manipulate PD to drive its own movement? Such questions inspired many of the live imaging experiments reported in this chapter.

As a host target of ChEC108, I hypothesised that HIPP6 could be responsible for recruiting ChEC108 to PD in Arabidopsis. As discussed in 1.2.7, HIPPs and their HPP relatives have emerged in the PD proteomes of various plant species (reviewed by Barr et al., 2023), and the subcellular localisations of multiple Arabidopsis HIPPs (HIPP1, HIPP7, HIPP32, HIPP33 and HIPP34) have been confirmed via live imaging (Guo et al., 2021; Leonte, 2020). Despite this, the functions of HMA proteins at this locality remain poorly understood.

On the other hand, some Arabidopsis HIPPs have been shown to accumulate in nuclei and even possess nuclear localisation signals (Barth et al., 2009; Zschiesche et al., 2015). In fact, the subcellular localisation prediction tool CELLO v.2.5 (Yu et al., 2006) predicted HIPP6 to be nuclear-localised in eukaryotes, based on its primary sequence. One might therefore hypothesise that ChEC108 and HIPP6 carry out as yet uncharacterised functions together in nuclei, potentially modulating gene expression during infection. Contradicting this, GFP-HIPP6 (also named GFP-CdI19) was reported to localise exclusively to the plasma membrane when transiently expressed in *N. tabacum* Bright Yellow-2 (BY2) cells (Suzuki et al., 2002). To gain further insights, after reproducing the previously described localisation of ChEC108, I sought to characterise the subcellular localisation of HIPP6 using a live imaging approach, first exploiting the convenient heterologous system of *N. benthamiana*, then subsequently seeking confirmation of key findings in the native system of Arabidopsis.

5.2 Results

5.2.1 ChEC108 localises to PD

To reproduce the results of Dr Joanna Jennings, I transiently expressed *ChEC108-eGFP* in *N. benthamiana* leaf epidermal cells via agroinfiltration and studied the distribution of eGFP signal via confocal microscopy. As anticipated, bright punctate signal was observed at cell peripheries, and diffuse signal was observed in cell nuclei (including the nucleolus) and cytoplasm (Figure 5.1A). Occasionally, ChEC108-eGFP signal in the nucleus appeared heterogeneous, in agreement with previous reports (Figure 5.2, Robin et al., 2018). To establish whether or not the plasma membrane-localised puncta corresponded to pitfields, I tested for co-localisation of ChEC108-eGFP with two PD markers: (1) aniline blue, containing sirofluor to stain callose deposits at PD neck regions (Conrath, 2024), and (2) PDL5-mCherry, an established resident and regulator of PD (Lee et al., 2011). In *N. benthamiana*, ChEC108-eGFP signal aligned closely with both aniline blue- and PDL5-mCherry-labelled puncta, confirming ChEC108 to be a PD-localised protein. When stably expressed in Arabidopsis, punctate ChEC108-eGFP signal was also observed (albeit faintly) at cell peripheries, reminiscent of pitfields, while cytoplasmic and nuclear localisation was not observed (Figure 5.1D). This suggests ChEC108 predominantly localises to PD in the *Ch* host.

5.2.2 HIPP6 localises to PD

Following the identification of HIPP6 as a candidate interactor of ChEC108 via IP-MS (3.1), Dr Joanna Jennings began initial investigations into HIPP6 subcellular localisation. When transiently expressed in *N. benthamiana* leaf epidermal cells, HIPP6-mCherry was found to be

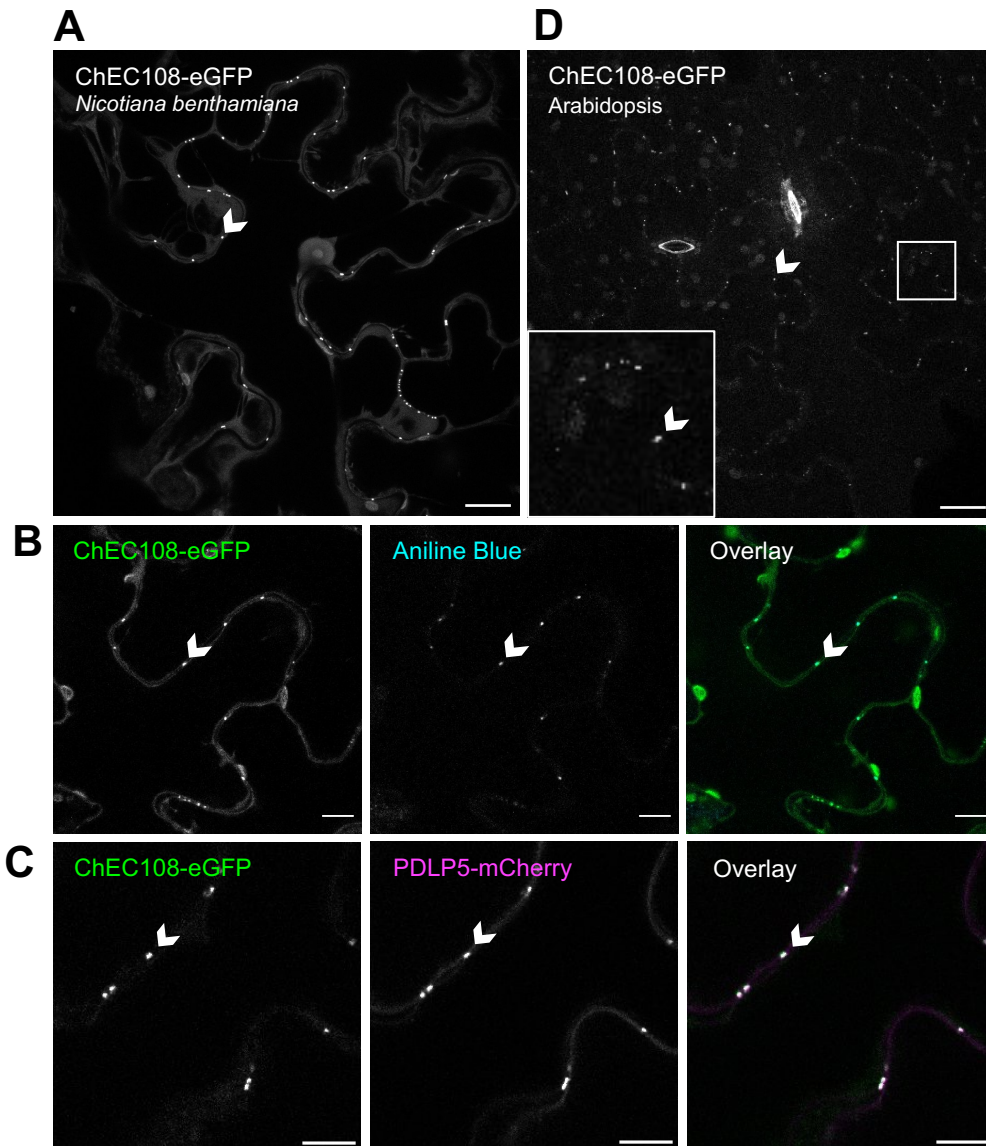


Figure 5.1 – ChEC108 localises to PD. **A.** *ChEC108-eGFP* transiently expressed in *N. benthamiana* leaf epidermal cells. Scale bar = 20 μm. **B-C.** Co-localisation of *ChEC108-eGFP* with pitfields in *N. benthamiana*, highlighted by aniline blue stain (B) or the PD marker, PDLP5-mCherry (C). Scale bar = 10 μm. **D.** *ChEC108-eGFP* stably expressed in *Arabidopsis* leaf epidermal cells. Insert panel shows a magnified view of the region indicated by a white box. Scale bar = 20 μm. Arrowheads indicate example pitfields.

predominantly nucleocytoplasmic, with small puncta and mobile aggregates visible (Jennings, 2021). HIPP6-mCherry-labelled puncta did not co-localise with aniline blue signal in this case, leading Dr Jennings to conclude that HIPP6 was not PD-localised (Jennings, 2021).

More recently, evidence has emerged to suggest the C-terminal isoprenylation motif of HIPPs can promote PD localisation, presumably by enabling association with the plasma membrane (Cowan et al., 2018; Guo et al., 2021; Were et al., 2025). Under the premise that a C-terminal mCherry might block isoprenylation, I aimed to study HIPP6 localisation using an N-terminal

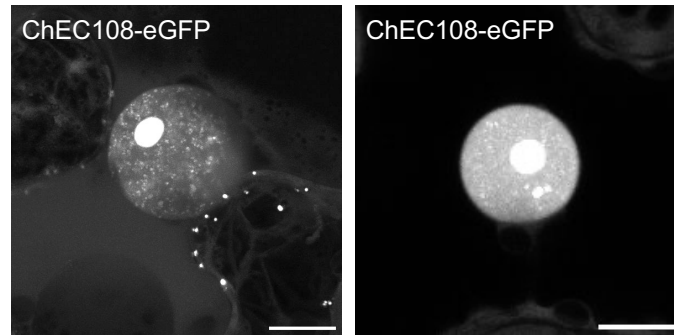


Figure 5.2 – Heterogeneous distribution of ChEC108 in nuclei. ChEC108-eGFP-labelled bodies of irregular sizes in the nuclei of *N. benthamiana* epidermal cells. Scale bar = 10 μ m.

tag. When transiently expressed in *N. benthamiana*, mCherry-HIPP6 was detected primarily at discrete puncta on the plasma membrane of epidermal cells, although weak nuclear signal was also observed (Figure 5.3A). When *meGFP-HIPP6* was stably expressed in Arabidopsis leaf epidermal cells (line *GFP-HIPP6-15*, introduced in 4.2.3), membrane-associated puncta were clearly observed, while nuclear signal was not (Figure 5.3B). In both *N. benthamiana* and Arabidopsis, HIPP6-labelled puncta aligned closely with aniline blue-stained pitfields (Figure 5.3C-D). Together, these images highlight pitfields as the subcellular compartment to which HIPP6 predominantly localises in the native system of Arabidopsis.

To better understand which regions of HIPP6 are responsible for its PD targeting, I generated variants with altered C-termini and studied their localisation in *N. benthamiana*. Consistent with the observations of Dr Joanna Jennings, HIPP6-mCherry appeared nucleocytoplasmic and did not accumulate at obvious puncta along the plasma membrane (Figure 5.4A). To determine if inhibition of isoprenylation could be responsible, I deleted the CaaX motif (CSVM) at the HIPP6 C-terminus (HIPP6 Δ C⁴) (Figure 5.4B) and fused this variant to an N-terminal mCherry tag. Unexpectedly, mCherry-HIPP6 Δ C⁴ accumulated at pitfields (Figure 5.4C, E), making its localisation indistinguishable from that of wild-type mCherry-HIPP6 (Figure 5.3A, C) and suggesting that targeting of HIPP6 to PD (at least in *N. benthamiana*) does not require isoprenylation.

I therefore hypothesised that other C-terminal features might be important for directing HIPP6 to PD. In two independent studies, Dykema et al. (1999) and Austin et al. (2007) discovered an eight-amino-acid sequence preceding the CaaX motif that was conserved among Arabidopsis and *O. sativa* HIPPs. However, this motif was absent from HMA proteins in yeast, that is, an organism lacking PD (Dykema et al., 1999). The consensus for this sequence is FSDENPNA, yet in HIPP6 it reads FSDENPNG. By deleting both the isoprenylation and FSDENPNG motifs, I generated the HIPP6 Δ C¹² variant (Figure 5.4B). Once again, mCherry-HIPP6 Δ C¹² fusions displayed a distinct punctate localisation along *N. benthamiana* plasma membranes (Figure 5.4D,

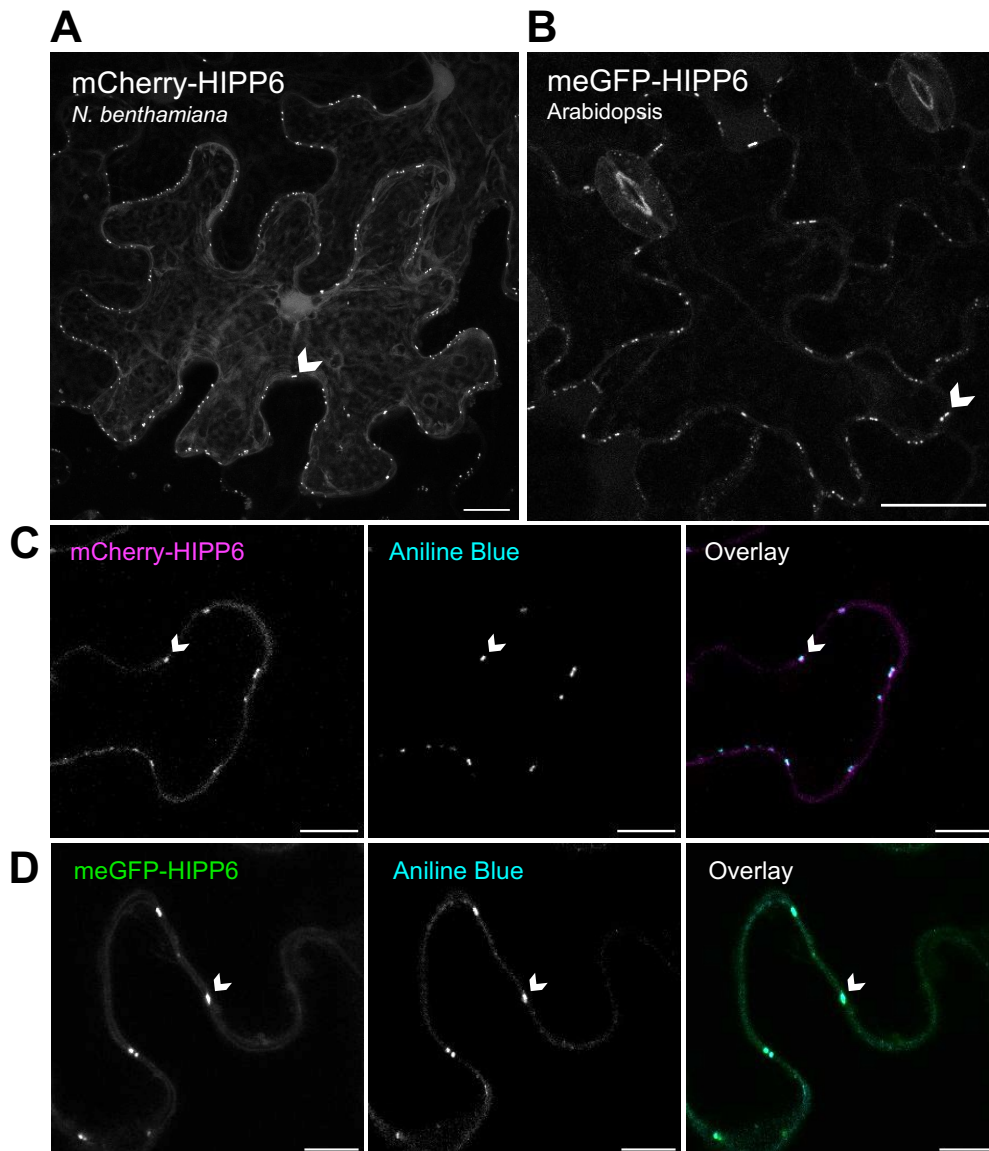


Figure 5.3 – HIPP6 localises to PD. **A.** *mCherry-HIPP6* transiently expressed in *N. benthamiana* leaf epidermal cells. Scale bar = 20 μm. **B.** *meGFP-HIPP6* stably expressed in Arabidopsis leaf epidermal cells. Scale bar = 20 μm. **C-D.** Co-localisation of *mCherry-HIPP6* (C) or *meGFP-HIPP6* (D) with pitfields in *N. benthamiana* and Arabidopsis, respectively, highlighted by aniline blue stain. Scale bar = 10 μm. Arrowheads indicate example pitfields.

F), similar to *mCherry-HIPP6* (Figure 5.3A, C) and *mCherry-HIPP6*^{ΔC4} (Figure 5.4C, E). It thus seems likely that other C-terminal features upstream of the FSDENPNG motif must be important for HIPP6 accumulation at PD, although such motifs have yet to be identified.

To further explore the regions responsible for PD targeting, the subcellular localisation of individual HIPP6 HMA domains were studied in *N. benthamiana*. HMA1-*mCherry*, HMA2-*mCherry* and tHMA-*mCherry* fusions localised primarily to the nucleus (including the nucleolus) and cytosol of *N. benthamiana* cells (Figure 5.5A). However, closer examination revealed subtle enrichment of HMA1-*mCherry* at puncta along the plasma membrane

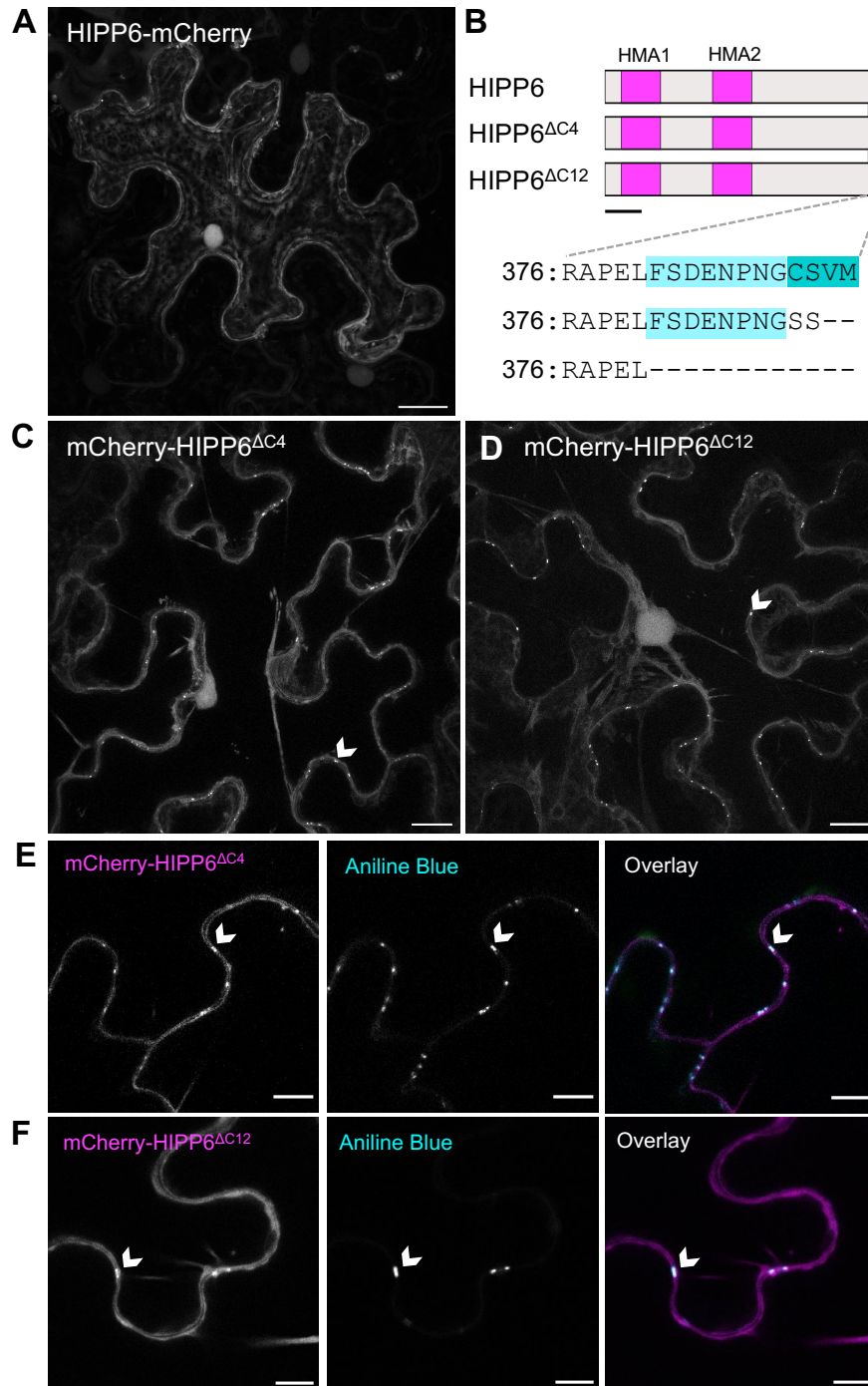


Figure 5.4 – Targeting of HIPP6 to PD does not require conserved C-terminal motifs. **A.** HIPP6-mCherry transiently expressed in *N. benthamiana* leaf epidermal cells, displaying nucleocytoplasmic localisation. Scale bar = 20 μ m. **B.** Schematic representation of wild-type HIPP6 and C-terminally truncated variants. Scale bar = 50 amino acids. In HIPP6 Δ C4, the CaaX motif has been replaced by an SerSer cloning scar. **C-D.** mCherry-HIPP6 Δ C4 (**C**) and mCherry-HIPP6 Δ C12 (**D**) transiently expressed in *N. benthamiana* leaf epidermal cells, displaying PD localisation. Scale bar = 20 μ m. **E-F.** Co-localisation of mCherry-HIPP6 Δ C4 (**E**) and mCherry-HIPP6 Δ C12 (**F**) with pitfields, highlighted by aniline blue staining. Scale bar = 10 μ m. Arrowheads indicate example pitfields.

(Figure 5.5B), which co-localised with aniline blue-stained callose deposits (Figure 5.5C). In contrast to HMA1-mCherry fusions, HMA2-mCherry and tHMA-mCherry fusions accumulated to very low levels in *N. benthamiana*, making confocal imaging challenging, especially after infiltration of aniline blue solution. However, on the rare occasions that mCherry signal was detectable, pitfield-like puncta were sometimes visible (Figure 5.5B). Together, these findings suggests the HIP6 HMA domains could be sufficient to direct HIP6 to PD, while isoprenylation is dispensable.

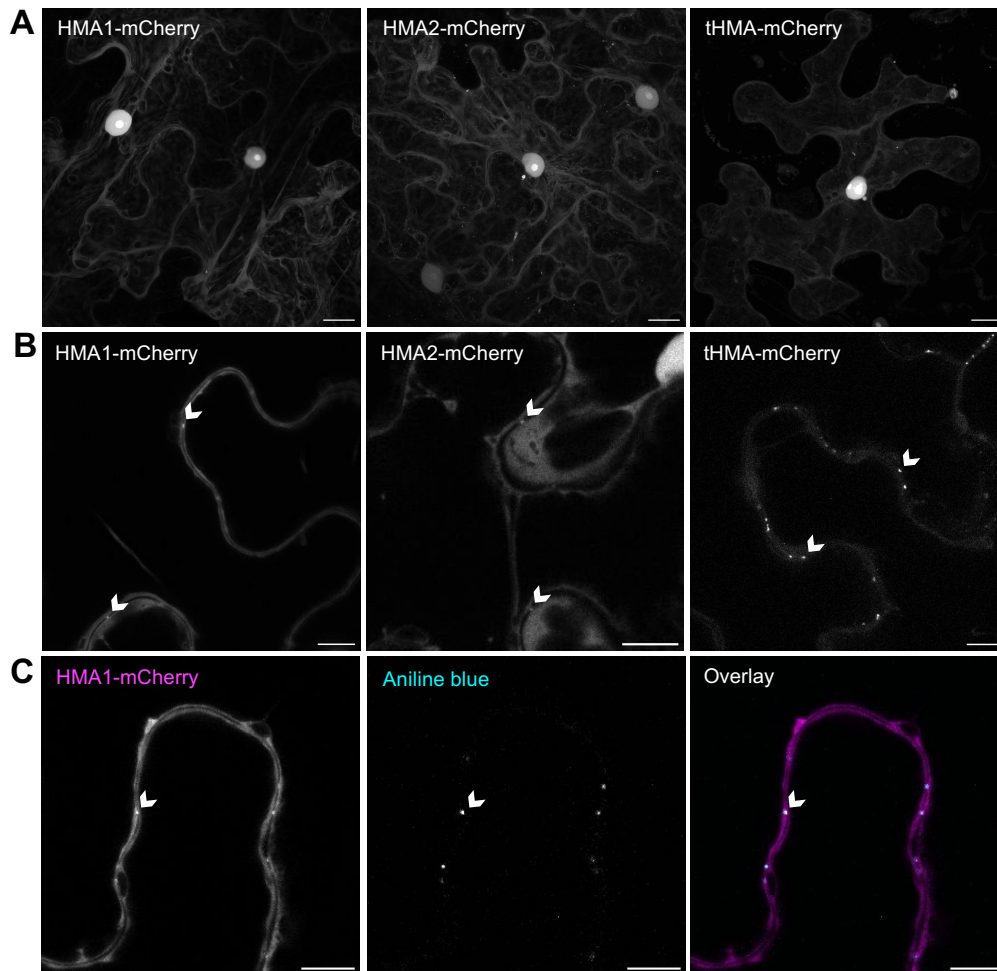


Figure 5.5 – HIP6 HMA domains are sufficient for PD localisation. **A.** HMA1-mCherry, HMA2-mCherry and tHMA-mCherry transiently expressed in *N. benthamiana* leaf epidermal cells, displaying nucleocytoplasmic localisation. Scale bar = 20 μm . **B.** Occasionally, HMA1-mCherry, HMA2-mCherry and tHMA-mCherry were observed at puncta along the plasma membrane. Scale bar = 10 μm . **C.** Co-localisation of HMA1-mCherry puncta with callose, highlighted by aniline blue stain. Scale bar = 10 μm . Arrowheads indicate example pitfields.

Examination of X-ray crystallographic structures presented in Chapter 3 gave rise to the hypothesis that HIP6 might bind metal ions via the CXXC motifs of its HMA domains (3.3.3). To establish whether these conserved Cys residues are also involved in establishing proper HIP6 localisation, I made use of variants designed in 3.2.10 with compromised metal-binding motifs: HIP6^{M1}, HIP6^{M2} and HIP6^{MM} (Figure 5.6A). I fused meGFP to the N-terminus of these variants, and transiently expressed them in *N. benthamiana* leaves. meGFP-HIP6 was included as a positive control for PD localisation (Figure 5.6B). With a functional CXXC motif in HMA2, but not HMA1, meGFP-HIP6^{M1} was observed to be enriched at pitfields, although the ratio of pitfield to non-pitfield-associated signal appeared lower in comparison to the meGFP-HIP6 control (Figure 5.6C versus B). With a functional CXXC motif in HMA1, but not HMA2, meGFP-HIP6^{M2} mutants accumulated poorly in *N. benthamiana* and were challenging to image. However, on rare occasions, pitfield-like puncta were observed (Figure 5.6D). On the other hand, meGFP-HIP6^{MM} mutants accumulated highly in the *N. benthamiana* cytoplasm, and PD were rarely observed (Figure 5.6E). Crucially, these findings were recapitulated in the Arabidopsis native system, using transgenic lines introduced in 4.2.3. In the *hipp6-3* mutant background, meGFP-HIP6 and meGFP-HIP6^{M1} localised to pitfields (Figure 5.7A-B), whereas meGFP-HIP6^{MM} predominantly localised to the cytoplasm (Figure 5.7C). This suggests the CXXC motifs of the HIP6 HMA domains (and potentially by extension, metal-binding), positively regulate PD targeting, such that loss of the CXXC motif in one domain can be partially compensated for by the other.

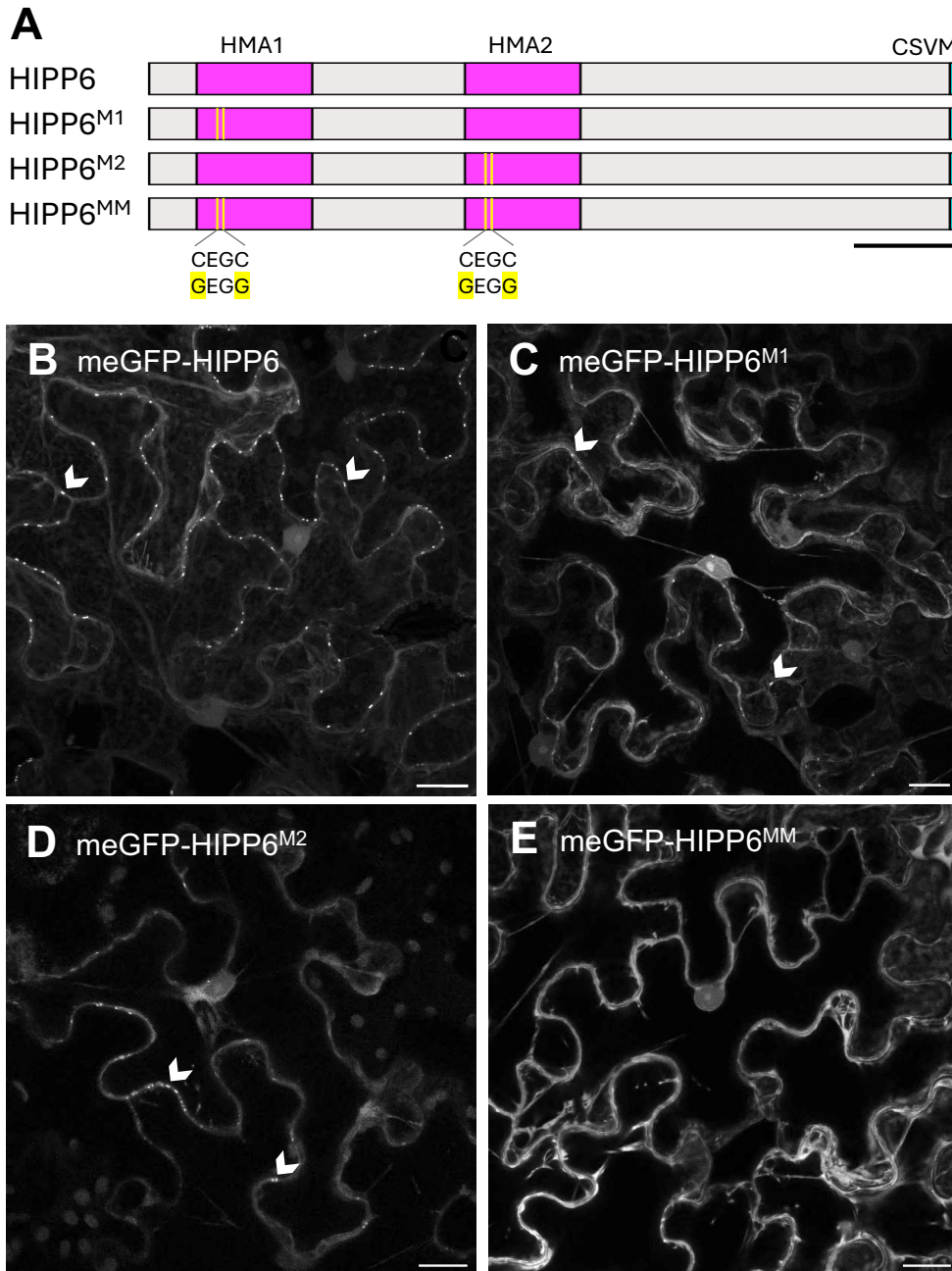


Figure 5.6 – Mutation of HIPP6 HMA domains reduces enrichment at PD in *N. benthamiana*. **A.** Schematic representation of wild-type HIPP6 and mutant variants with Cys to Gly mutations in their HMA domains. Scale bar = 50 amino acids. **B-D.** meGFP-HIPP6 (B), meGFP-HIPP6^{M1} (C) or meGFP-HIPP6^{M2} (D) transiently expressed in *N. benthamiana* leaf epidermal cells, displaying PD localisation. Arrowheads indicate example pitfields. Scale bar = 20 μ m. **D.** meGFP-HIPP6^{MM} transiently expressed in *N. benthamiana* leaf epidermal cells, with no visible enrichment at PD. Scale bar = 20 μ m.

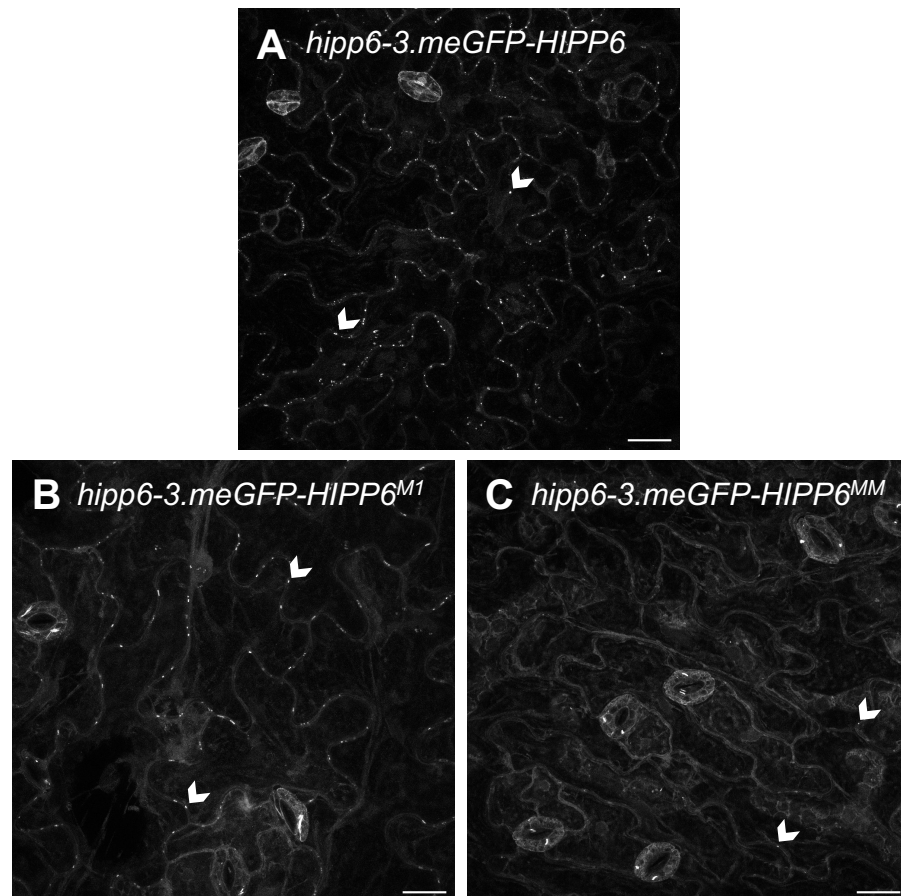


Figure 5.7 – Mutation of HIPP6 HMA domains reduces enrichment at PD in Arabidopsis. A-B. meGFP-HIPP6 (A) or meGFP-HIPP6^{M1} (B) stably expressed in Arabidopsis leaf epidermal cells, displaying PD localisation. Scale bar = 20 μm. C. meGFP-HIPP6^{MM} stably expressed in Arabidopsis leaf epidermal cells, displaying reduced enrichment at PD. Arrowheads indicate example pitfields. Scale bar = 20 μm.

5.2.3 HIP6 co-localises with ChEC108 at PD

Having established both ChEC108 and HIP6 to be PD-localised proteins, I next proceeded to test whether localisation of one binding partner was perturbed in the presence of the other. When transiently expressed in *N. benthamiana* leaves and viewed using a low magnification (20×) objective lens, ChEC108-eGFP and HIP6 (with either an N- or C-terminal mCherry tag) co-localised at the periphery of epidermal cells (Figure 5.8A-B). As nuclei were labelled intensely by ChEC108-eGFP, but not by HIP6 fusions in the same cells, this suggests nuclei are unlikely to be the main site of ChEC108-HIP6 interaction. Further supporting this conclusion, on occasion, ChEC108-eGFP nuclear accumulation seemed depleted in the presence of HIP6, accompanied by elevated signal surrounding the nuclear envelope, which is reminiscent of ER localisation (Figure 5.8C-D).

Although not immediately obvious (for example, see Figure 5.8C), occasionally, ChEC108-eGFP and mCherry-HIP6 exhibited co-localisation at *N. benthamiana* pitfields (Figure 5.9A). To test for co-localisation in the Arabidopsis native system, I used particle bombardment to transiently expressed *ChEC108-mScarlet* in Arabidopsis leaves stably expressing *meGFP-HIP6*. Where visible, ChEC108- and HIP6-labelled pitfields were found to closely align (Figure 5.9B), highlighting PD as a potential site of ChEC108-HIP6 association.

Initially, visualisation of whole cells expressing *ChEC108-mScarlet* in leaves expressing *meGFP-HIP6* gave an indication that ChEC108 might perturb HIP6 localisation. Specifically, I observed enhancement of nuclear meGFP-HIP6 signal in bombarded cells, relative to surrounding cells that lacked ChEC108-mScarlet signal (Figure 5.10A). This led me to hypothesise that (contrary to results obtained in *N. benthamiana*), ChEC108 ordinarily dislodges HIP6 from PD, driving its accumulation in the nucleus. However, bombardment of the same Arabidopsis lines with mScarlet as a control resulted in a similar phenomenon (Figure 5.10B), suggesting enrichment of nuclear meGFP-HIP6 signal in bombarded cells was an artefact, and not a consequence of ChEC108-HIP6 interaction. Nevertheless, close examination of meGFP-HIP6 signal sometimes revealed fine strands crisscrossing *ChEC108-mScarlet*-expressing cells (Figure 5.10A). Such strands were not observed in cells expressing *mScarlet* alone (Figure 5.10B). Given the low signal intensity and absence of appropriate markers for co-localisation, it is unclear whether these meGFP-labelled structures represent the ER, cytoskeletal elements, or simply cytoplasmic strands. Even so, the observation suggests ChEC108 might subtly alter HIP6 localisation in Arabidopsis.

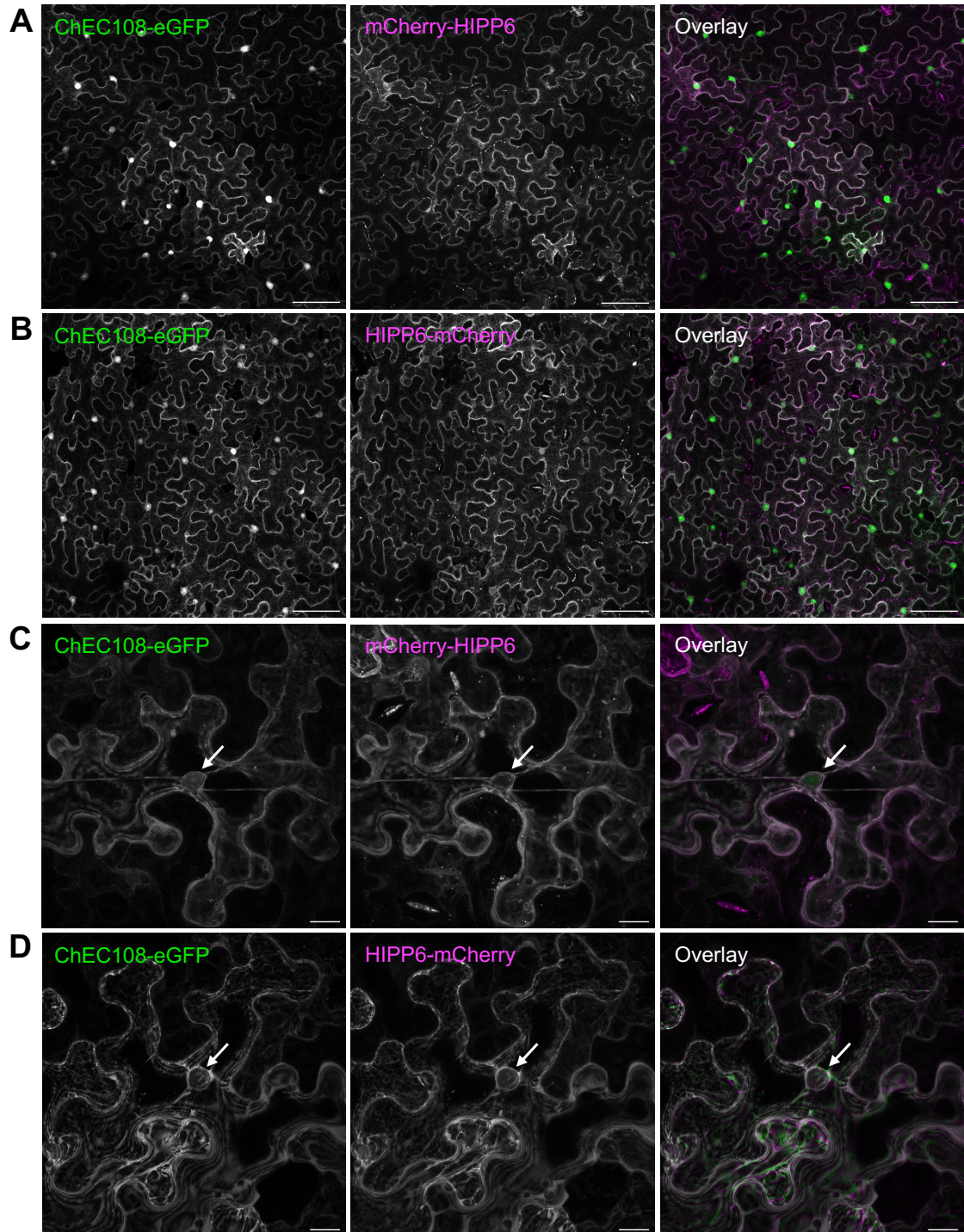


Figure 5.8 – ChEC108 co-localises with HIPP6 in *N. benthamiana*. A-D. ChEC108-eGFP and mCherry-HIPP6 (A,C) or HIPP6-mCherry (B,D) transiently co-expressed in *N. benthamiana* leaf epidermal cells. In both cases, co-localisation is visible (A-B, Scale bar = 100 µm). Occasionally, nuclear ChEC108-eGFP signal appeared reduced, and ChEC108-eGFP and mCherry-HIPP6 were observed surrounding the nucleus, indicated by arrows (C-D, Scale bar = 20 µm).

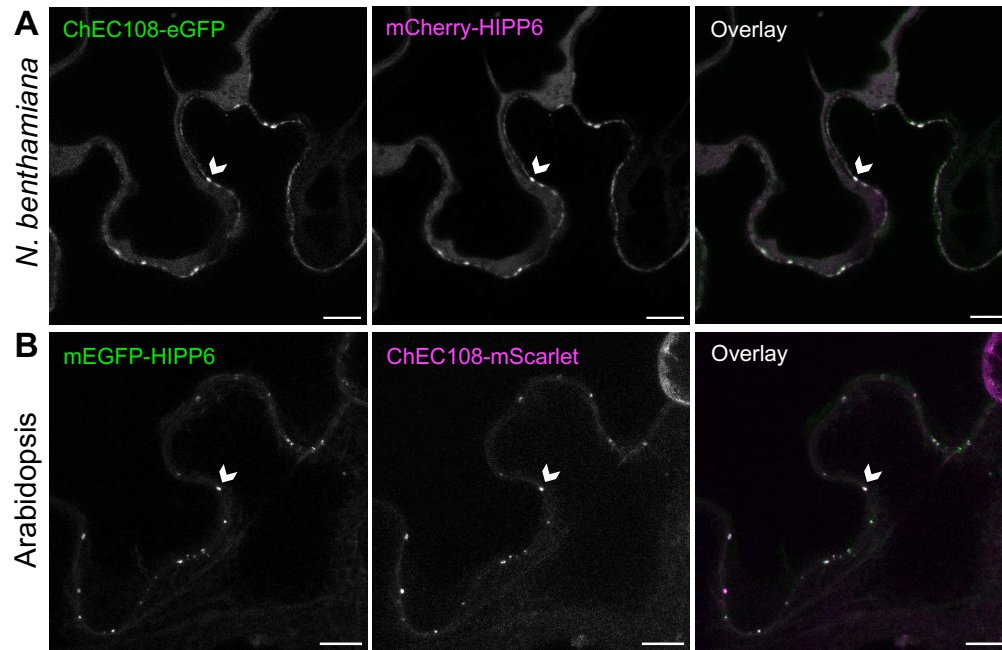


Figure 5.9 – ChEC108 and HIPP6 co-localise at PD. **A.** *ChEC108-eGFP* and *mCherry-HIPP6* transiently co-expressed in *N. benthamiana* leaf epidermal cells. **B.** *ChEC108-mScarlet* transiently expressed via bombardment in *Arabidopsis* leaf epidermal cells stably expressing *meGFP-HIPP6*. Arrowheads indicate co-localisation of eGFP and mCherry signal at example pitfields. Scale bar = 10 µm.

Further supporting this conclusion, perturbation of meGFP-HIPP6 localisation (beyond the aforementioned artefactual enhancement of nuclear signal) was not observed in cells bombarded with ChEC108^{D164R}-mScarlet (Figure 5.11A) - a variant of the effector that failed to bind HIPP6 (3.2.10). Similarly, meGFP-HIPP6^{MM} - a variant of HIPP6 that failed to bind ChEC108 (3.2.10) - did not display detectable shifts in its localisation following bombardment of ChEC108-mScarlet, or an mScarlet control (Figure 5.11B-C). Collectively, these data suggest that the recruitment of meGFP-HIPP6 to fine strands in Figure 5.10A depends on ChEC108-HIPP6 complex formation.

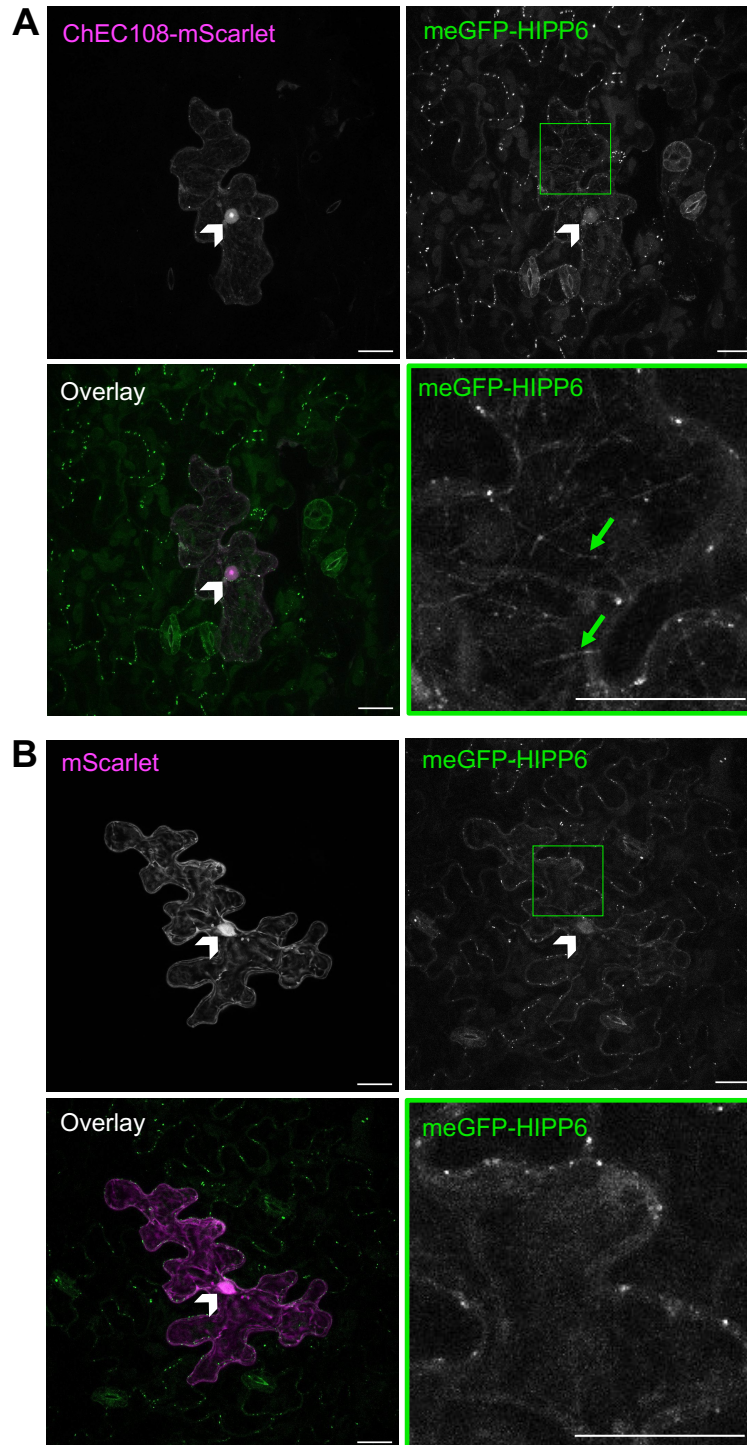


Figure 5.10 – HIPP6 localisation in Arabidopsis subtly changes in the presence of ChEC108. A-B. *ChEC108-mScarlet* (A) or *mScarlet* (B) transiently expressed via bombardment in Arabidopsis leaf epidermal cells stably expressing *meGFP-HIPP6*. Arrowheads indicate the nucleus of a bombarded cell. Enhancement of *meGFP* nuclear signal is an artefact caused by bombardment. Bottom right panels display a magnified view of bombarded cells in top right panels, indicated by a green box. Green arrows indicate thin strands observed in the presence of *ChEC108-mScarlet*. Scale bar = 20 μ m.

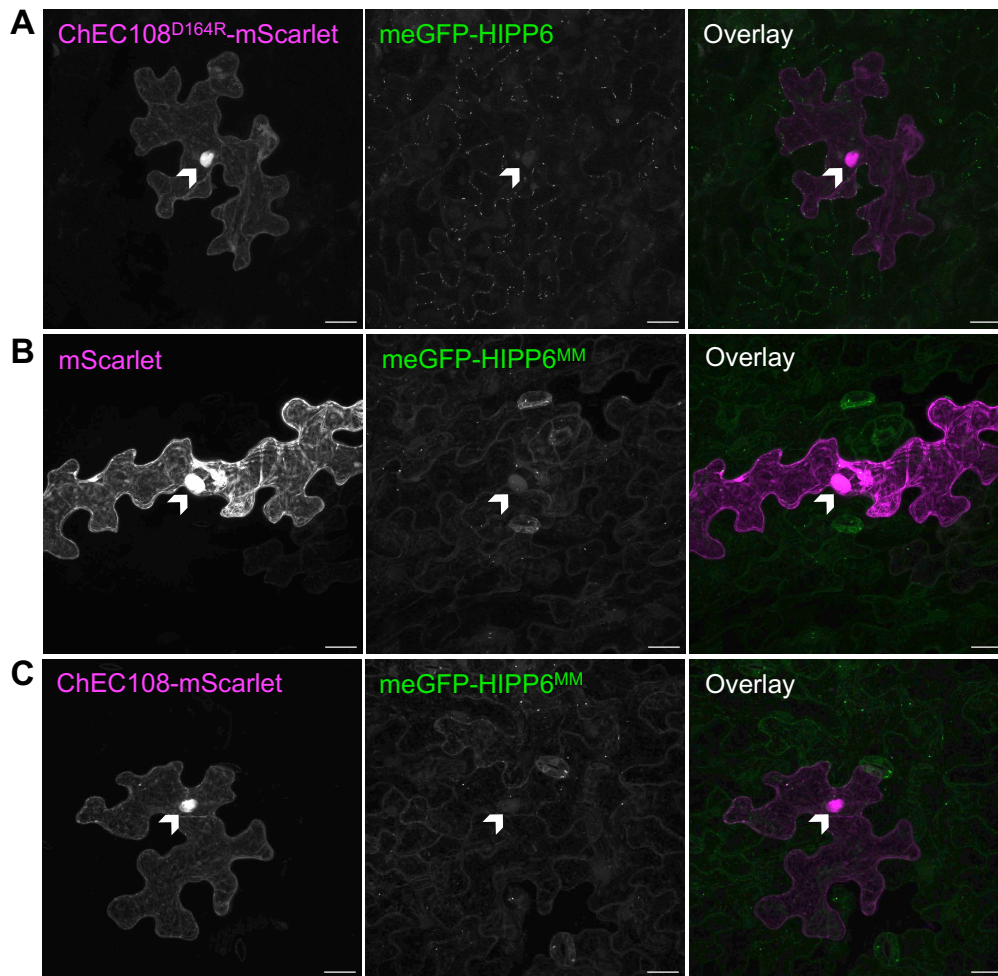


Figure 5.11 – HIPP6 localisation is unchanged in the presence of ChEC108 when binding is compromised by mutation. **A.** *ChEC108^{D164R}-mScarlet* transiently expressed via bombardment in Arabidopsis leaf epidermal cells stably expressing *meGFP-HIPP6*. **B-C.** *mScarlet* (B) or *ChEC108-mScarlet* (C) transiently expressed via bombardment in Arabidopsis leaf epidermal cells stably expressing *meGFP-HIPP6^{MM}*. Arrowheads indicate the nucleus of a bombarded cell, in which meGFP signal appears unchanged in comparison to surrounding cells. Enhancement of meGFP nuclear signal is an artefact caused by bombardment. Scale bar = 20 μ m.

5.2.4 ChEC108 enhances HIPP6 stability

Previously, co-IP experiments presented in Figures 3.2, 3.3, 3.26 and 3.27 revealed an enhancement of ChEC108 accumulation in the presence of wild-type HIPP6. The repeatability of this observation lends support to the hypothesis that the ChEC108-HIPP6 interaction is stabilising. To confirm this using a more quantitative method, I used confocal microscopy to capture mCherry-HIPP6 signal in the presence of ChEC108-eGFP or free eGFP as a negative control, following transient co-expression of each construct pair in *N. benthamiana* leaves. When expressed alongside eGFP, mCherry-HIPP6 signal was extremely faint (Figure 5.12A). However, in the presence of ChEC108-eGFP, mCherry-HIPP6 was more easily visualised and, consistent with observations in Figure 5.8, co-localisation of the two proteins was evident

(Figure 5.12B). In agreement, average signal intensity measurements collected from three leaves per construct pair revealed significantly higher accumulation of mCherry-HIPP6 upon co-expression with ChEC108-eGFP versus eGFP (Figure 5.12C).

While investigating the localisation of HIPP6 HMA domains in *N. benthamiana* (5.2.2), I noted that the accumulation of HMA2-mCherry and tHMA-mCherry fusions was especially poor, such that fluorescent signal was not detectable in the vast majority of cells. To test whether ChEC108 could enhance the accumulation of these isolated domains, I performed a Western blotting experiment, involving expression of HMA1-mCherry, HMA2-mCherry and tHMA-mCherry in *N. benthamiana* leaves in the presence or absence of ChEC108-eGFP. HIPP6-mCherry was included as a positive control. When expressed alone, HMA1-mCherry was the only mCherry fusion to be detected on a Western blot, suggesting the presence of HMA2 in the other three fusions might confer instability via unknown mechanisms (Figure 5.13). Upon co-expression with ChEC108, all four mCherry fusions were detected (although bands corresponding to HMA2-mCherry were faint) (Figure 5.13), confirming that effector-binding helps overcome the inherent instability of HIPP6 and its isolated domains.

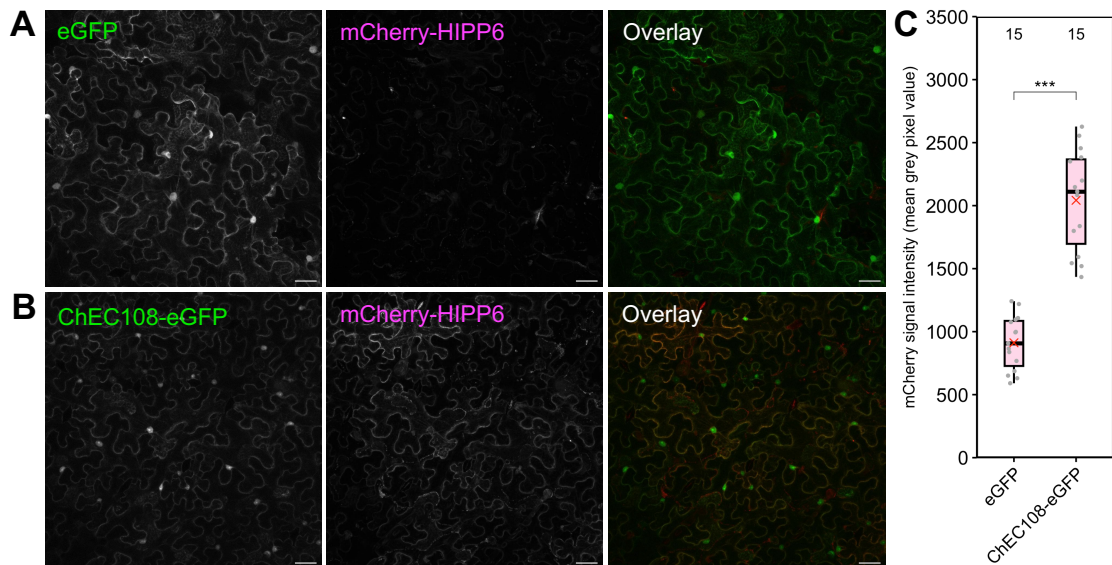


Figure 5.12 – HIPP6 is stabilised by ChEC108 in *N. benthamiana*. A-C. mCherry-HIPP6 transiently expressed in *N. benthamiana* leaf epidermal cells alongside eGFP (A) or ChEC108-eGFP (B). Protein accumulation 2 days post-infiltration was assessed via confocal microscopy. A and B are representative images from datasets collected using identical parameters across 3 leaves, with 5 images acquired per leaf. Scale bar = 50 μ m. Quantification of mCherry signal intensity (mean grey pixel value across a maximum projection of each image) is shown in C. $\times$ indicates the mean, and numerical annotations indicate sample size (images). *** indicates $p < 0.001$, as determined by a Welch two sample t-test ($t = -9.67$, $df = 21.51$).

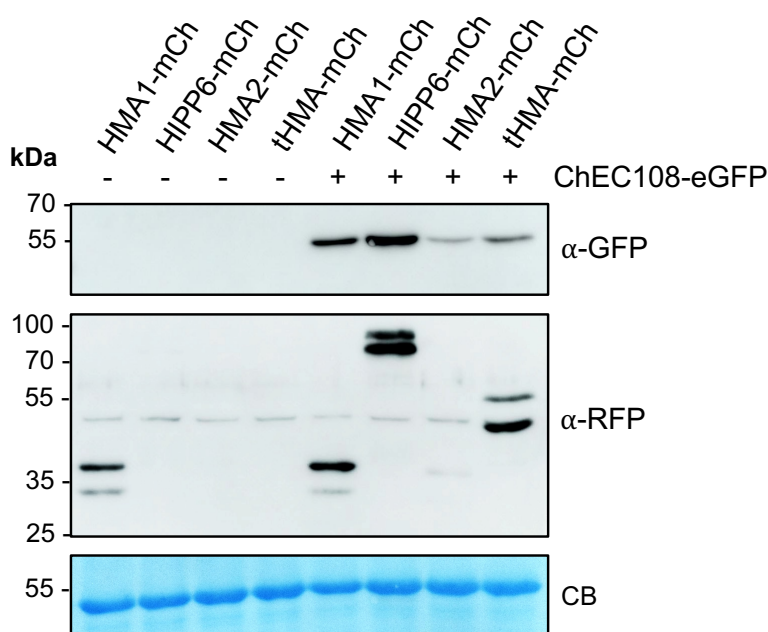


Figure 5.13 – Stabilisation of HIPP6, HMA2 and tHMA upon co-expression with ChEC108 in *N. benthamiana*. HIPP6, HMA1, HMA2 or tHMA were transiently expressed in *N. benthamiana* leaves with C-terminal mCherry tags (mCh) in the presence and absence of ChEC108-eGFP. Protein accumulation 3 days post-infiltration was assessed by Western blotting. Representative of two independent repeats.

5.2.5 ChEC108-mediated enhancement of intercellular trafficking was observed in *N. benthamiana*, but not *Arabidopsis*

Having established the localisation of both ChEC108 and HIPP6, I next sought to investigate the influence of these two proteins on cell-to-cell trafficking via PD. When transiently expressed in *N. benthamiana*, large fluorescent proteins such as 2×GFP (55 kDa) and NLS-tdTomato (62 kDa) typically exhibit limited (if any) cell-to-cell movement, and have thus been employed as markers for single cell transformation events (Jennings, 2021; Ohtsu et al., 2023). Yet surprisingly, a mobility assay conducted by Dr Joanna Jennings suggested the presence of ChEC108 (23 kDa) with either a single or double GFP C-terminal tag (enlarging the fusion to 50 or 78 kDa, respectively) could markedly enhance the intercellular movement of NLS-tdTomato (Jennings, 2021). To reproduce this result, I transiently co-expressed *ChEC108-2×GFP* or *2×GFP* as a negative control, alongside *NLS-tdTomato* in *N. benthamiana* leaves using a low density of *A. tumefaciens* (OD = 0.0001) to enable visualisation of discrete transformation events. As anticipated, ChEC108-2×GFP moved significantly further than 2×GFP alone, as reflected by the average number of nuclei displaying GFP signal at transformation sites (Figure 5.14A, D-E). Also consistent with Dr Jennings' results, NLS-tdTomato in the presence of 2×GFP exhibited little/no movement beyond transformed cells (Figure 5.14B, D). On the other hand, when co-expressed with ChEC108-2×GFP, movement of this nuclear marker was significantly

enhanced (Figure 5.14B, E). Overall, a positive correlation was observed between the number of ChEC108-2×GFP- and NLS-tdTomato-labelled nuclei at transformation sites, supporting the idea that ChEC108 was responsible for enhancing NLS-tdTomato movement (Figure 5.14C). In summary, as well as promoting its own intercellular mobility, ChEC108 is capable of enhancing general intercellular flux via PD in *N. benthamiana*.

Leading on from the identification of ChEC108 as a PD-localised protein and manipulator of *N. benthamiana* intercellular transport, I was curious to understand the mechanism by which this protein might function. With HIPP6 established as host target, it is possible that ChEC108 might enhance cytokinin signalling within plant cells, inducing secondary PD formation. This idea stemmed from (1) the observation that HIPP6, alongside HIPP1 and HIPP7, was shown to regulate ER-associated degradation (ERAD) of cytokinin-degrading enzymes (Guo et al., 2021), and (2) exogenous cytokinin application enhanced cell-to-cell movement of fluorescent proteins in *N. benthamiana* leaves (Horner and Brunkard, 2021), speculatively via rapid de novo formation of secondary (post-cytokinesis) plasmodesmal pores - a phenomenon previously demonstrated in the shoot apical meristem of white mustard (*Sinapis alba*) (Ormenese et al., 2006). To test this hypothesis, I quantified the density of pitfields in leaves expressing *ChEC108-eGFP* or *eGFP* as a control, alongside *PDL5-mCherry* as a PD marker, revealing no significant difference in pitfield density between the two treatments (Figure 5.15A-C). Although further repetitions of this experiment would be desirable, this preliminary evidence suggests the induction of secondary PD formation is an unlikely mechanism by which ChEC108 manipulates *N. benthamiana* cell-to-cell connectivity.

Next, I sought to confirm the biological relevance of putative ChEC108 function by testing for modification of intercellular trafficking in the *Ch* host, Arabidopsis. As a more efficient alternative to agroinfiltration in this system, I used particle bombardment to express *NLS-tdTomato* in single Arabidopsis epidermal cells alongside *ChEC108-2×GFP* or *2×GFP* as a control. 1 day post-bombardment, cell-to-cell flux was quantified by counting the number of GFP- and NLS-tdTomato-labelled nuclei at bombarded sites. Surprisingly, the presence of ChEC108-2×GFP had no significant impact on NLS-tdTomato mobility, and ChEC108-2×GFP itself was rarely observed to move beyond transformed cells (Figure 5.16A-D).

Given that the PD of mature Arabidopsis and *N. benthamiana* leaves may vary in their structure and/or aperture, it is possible that fusion of such a large (55 kDa) 2×GFP tag inhibited the ability of ChEC108 to manipulate Arabidopsis PD. With this in mind, I quantified NLS-tdTomato movement in the presence of ChEC108-eGFP or eGFP as a control, this time leaving 2 days between bombardment and image acquisition to allow ample time for fluorescent proteins to be expressed and for ChEC108 to carry out its putative function.

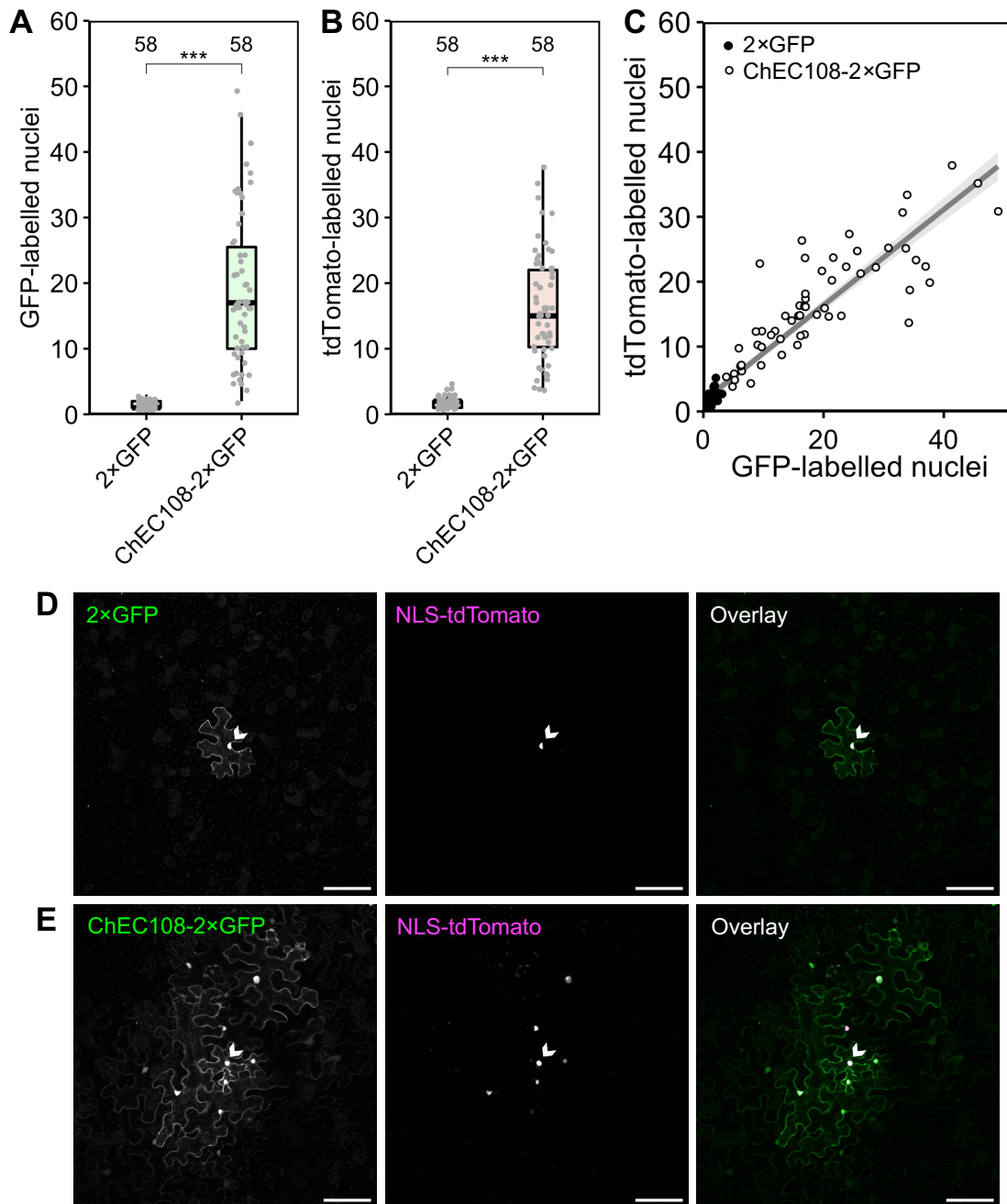


Figure 5.14 – ChEC108 enhances intercellular flux in *N. benthamiana*. **A-B.** NLS-tdTomato was transiently expressed in single *N. benthamiana* leaf epidermal cells via low-OD agroinfiltration alongside ChEC108-2xGFP or 2xGFP as a control. Mobility of GFP (A) or NLS-tdTomato signal (B) was quantified 3 days post-infiltration by counting the number of fluorescent nuclei at transformation sites. Numeric annotations indicate sample size (sites), collected across 3 leaves per treatment, with a maximum of 20 sites per leaf. *** indicates $p < 0.001$, determined by pairwise bootstrapping analysis with 5000 iterations. **C.** Correlation of GFP and NLS-tdTomato mobility at transformation sites shown in A and B. A linear regression line and 95% confidence intervals are included in dark and light grey, respectively. **D-E.** Representative transformation sites from datasets shown in A-C. Arrowheads indicate the nuclei of single transformed cells. Scale bar = 100 µm.

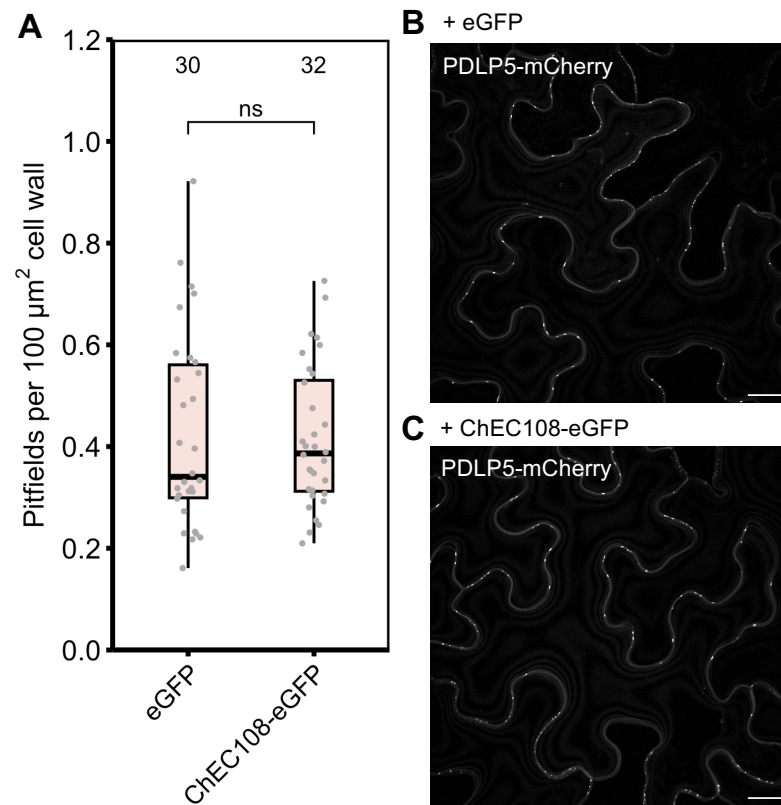


Figure 5.15 – ChEC108 does not increase pitfield density in *N. benthamiana*. **A.** *PDLP5-mCherry* was transiently expressed in *N. benthamiana* leaf epidermal cells alongside *ChEC108-eGFP* or *eGFP* as a control, and *PDLP5*-labelled pitfield density was quantified 2 days post-infiltration. Data were collected using identical imaging parameters across 8 leaves, with 3-4 images acquired per leaf. Numerical annotations indicate sample size (images). ns = not significant, determined by a Mann-Whitney U test ($W = 456$, $p = 0.7424$). **B-C.** Representative images of *PDLP5*-labelled pitfields from datasets shown in A. Scale bar = 20 μm .

However, NLS-tdTomato movement was not significantly altered at *ChEC108-eGFP*-expressing sites versus *eGFP*-expressing control sites (Figure 5.16E). Similarly, cytosolic GFP did not exhibit enhanced cell-to-cell movement in the presence of *ChEC108-mCherry* versus mRFP (Figure 5.16F). In summary, despite multiple efforts, *ChEC108*-mediated manipulation of PD has yet to be recapitulated in *Arabidopsis*.

Leading on from this negative result, I sought to investigate whether the transformation technique used (agroinfiltration vs particle bombardment) could be underlying the disparate results obtained from mobility assays in *N. benthamiana* and *Arabidopsis*. I thus bombarded *N. benthamiana* leaves with the same constructs employed for bombardment of *Arabidopsis* in Figure 5.16E. In agreement with result obtained via agroinfiltration, NLS-tdTomato exhibited significantly enhanced cell-to-cell mobility in the presence of *ChEC108-eGFP*, relative to *eGFP* (Figure 5.17A-D). However, in the same experiment, hypermobility of *ChEC108-eGFP* versus *eGFP* was not detected (Figure 5.17A). This suggests agroinfiltration ordinarily drives

greater accumulation of ChEC108 in comparison to particle bombardment, perhaps due to the influence of bacterial factors. Alternatively, differing levels of ChEC108 accumulation could be underpinned by the putatively different mechanisms of foreign DNA integration and/or expression associated with each technique. Regardless, data depicted in Figure 5.17A-B suggest that ChEC108-mediated enhancement of intercellular trafficking is measurable in *N. benthamiana* even when ChEC108 itself is difficult to detect. In turn, this implies the negative results obtained from mobility assays in Arabidopsis (Figure 5.16B, E-F) might not be entirely underpinned by low accumulation of ChEC108 in transformed cells. Instead, host-specific factors could be 'protecting' Arabidopsis PD from ChEC108-mediated manipulation.

5.2.6 PD localisation of ChEC108 does not depend on HIP6

As a PD-associated binding partner of ChEC108 with a potentially positive impact on Arabidopsis resistance to *Ch* (see Section 4.2.2), HIP6 seemed like a promising candidate 'guardian' of Arabidopsis PD, which might be responsible for inhibiting ChEC108 activity in the *Ch* host. Before testing this hypothesis, I sought to establish whether or not ChEC108 was capable of moving cell-to-cell in Arabidopsis, as images acquired using a 20× objective for bombardment experiments failed to provide convincing evidence of this phenomenon (Figure 5.16A, D). I therefore bombarded ChEC108-eGFP into Arabidopsis leaves and examined the resulting transformation sites using a 63× objective. At this higher magnification, ChEC108-eGFP was observed in cells adjacent to bombarded cells, exclusively at PD and occasionally reaching the boundaries of the field of view. However, this was often visible only when image brightness was enhanced post-acquisition (Figure 5.18). This confirms that ChEC108 is cell-to-cell mobile in Arabidopsis, but due to the faint, PD-exclusive nature of the fluorescent signal, this could not be quantified using the methods described in 5.2.5.

If HIP6 has defence-promoting functions in Arabidopsis, one might expect this protein to meet ChEC108 at PD, as opposed to facilitating proper effector localisation as a susceptibility factor. To test this idea, I studied the localisation of ChEC108^{D164R}-eGFP in *N. benthamiana*. Despite being putatively unable to bind HIP6, ChEC108^{D164R}-eGFP retained its PD localisation, although enrichment of this fusion at puncta seemed marginally reduced in comparison to wild-type ChEC108-eGFP (Figure 5.19A-B). As discussed in 3.3.2, there is a possibility that ChEC108 possess a second HMA-binding site at its C-terminus, involving Cys197 and Cys200. Despite the relevance of this in the context of a full-length ChEC108-HIP6 complex being presently unclear, I generated constructs to express ChEC108^{CCAA}-eGFP and ChEC108^{D164R-CCAA}-eGFP fusions, in which Cys197 and Cys200 are mutated to Ala, allowing me to test whether ChEC108^{D164R}-eGFP PD localisation could be facilitated by this second binding

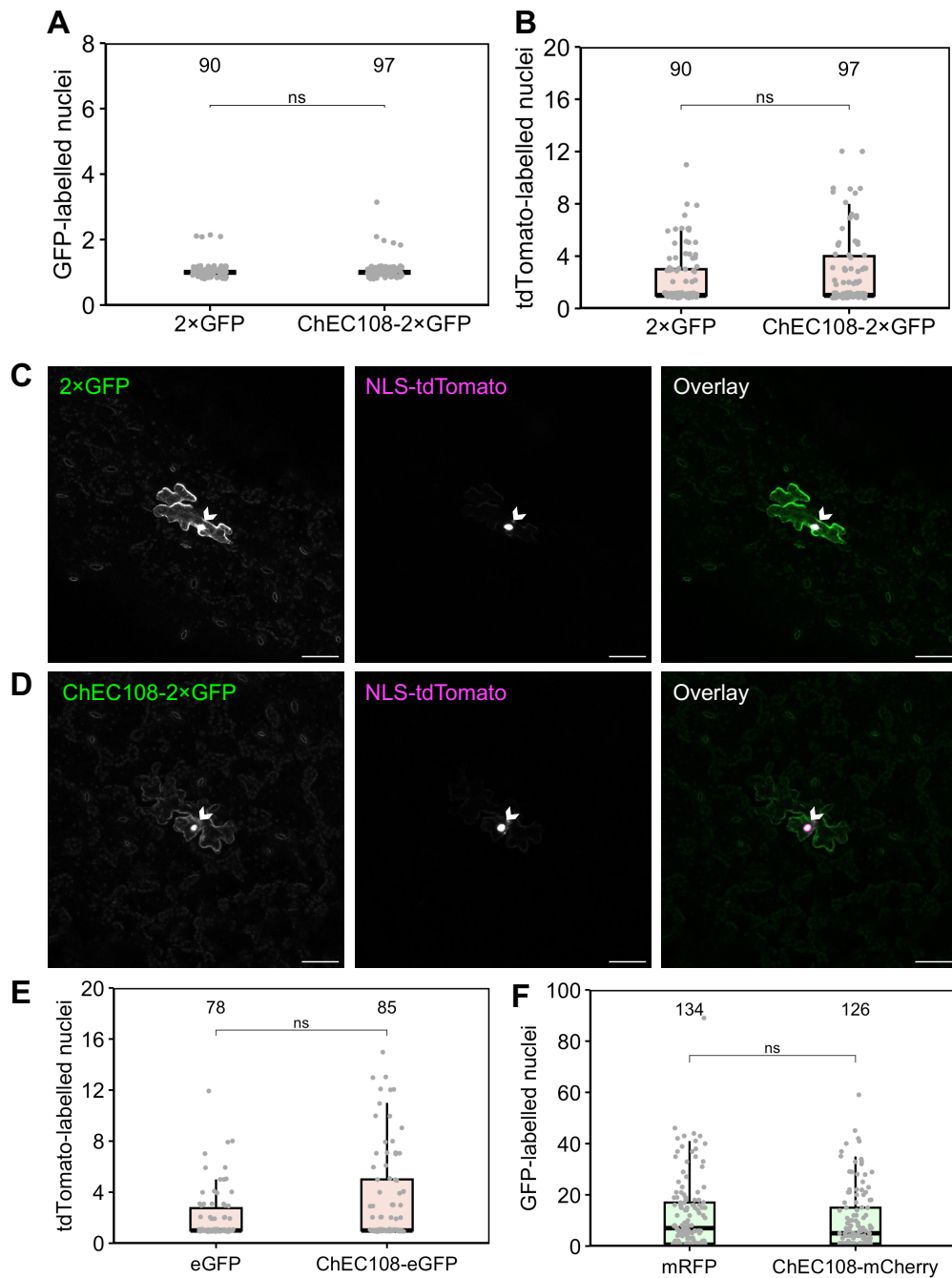


Figure 5.16 – ChEC108-mediated enhancement of intercellular transport was not detected in Arabidopsis. **A-B.** *NLS-tdTomato* was transiently expressed in Arabidopsis leaf epidermal cells via bombardment alongside *ChEC108-2×GFP* or *2×GFP* as a control. Mobility of GFP (A) or NLS-tdTomato signal (B) was quantified 1 day post-bombardment by counting the number of fluorescent nuclei at transformation sites across 5 leaves, with a maximum of 20 sites per leaf. **C-D.** Representative transformation sites from datasets in A-B. Arrowheads indicate the nuclei of single transformed cells. Scale bar = 50 μ m. **E.** *NLS-tdTomato* was transiently expressed via bombardment alongside *ChEC108-eGFP* or *eGFP* as a control. Mobility of NLS-tdTomato signal was quantified 2 days post bombardment at transformation sites across 4-5 leaves, with a maximum of 20 sites per leaf. **F.** *eGFP* was transiently expressed via bombardment alongside *ChEC108-mCherry* or *mRFP* as a control. Mobility of eGFP signal was quantified 1 day post-bombardment at transformation sites across 18-20 leaves, with a maximum of 10 sites per leaf. In panels A-B and E-F, numeric annotations indicate sample size (sites). ns = not significant, determined by pairwise bootstrapping analysis with 5000 iterations.

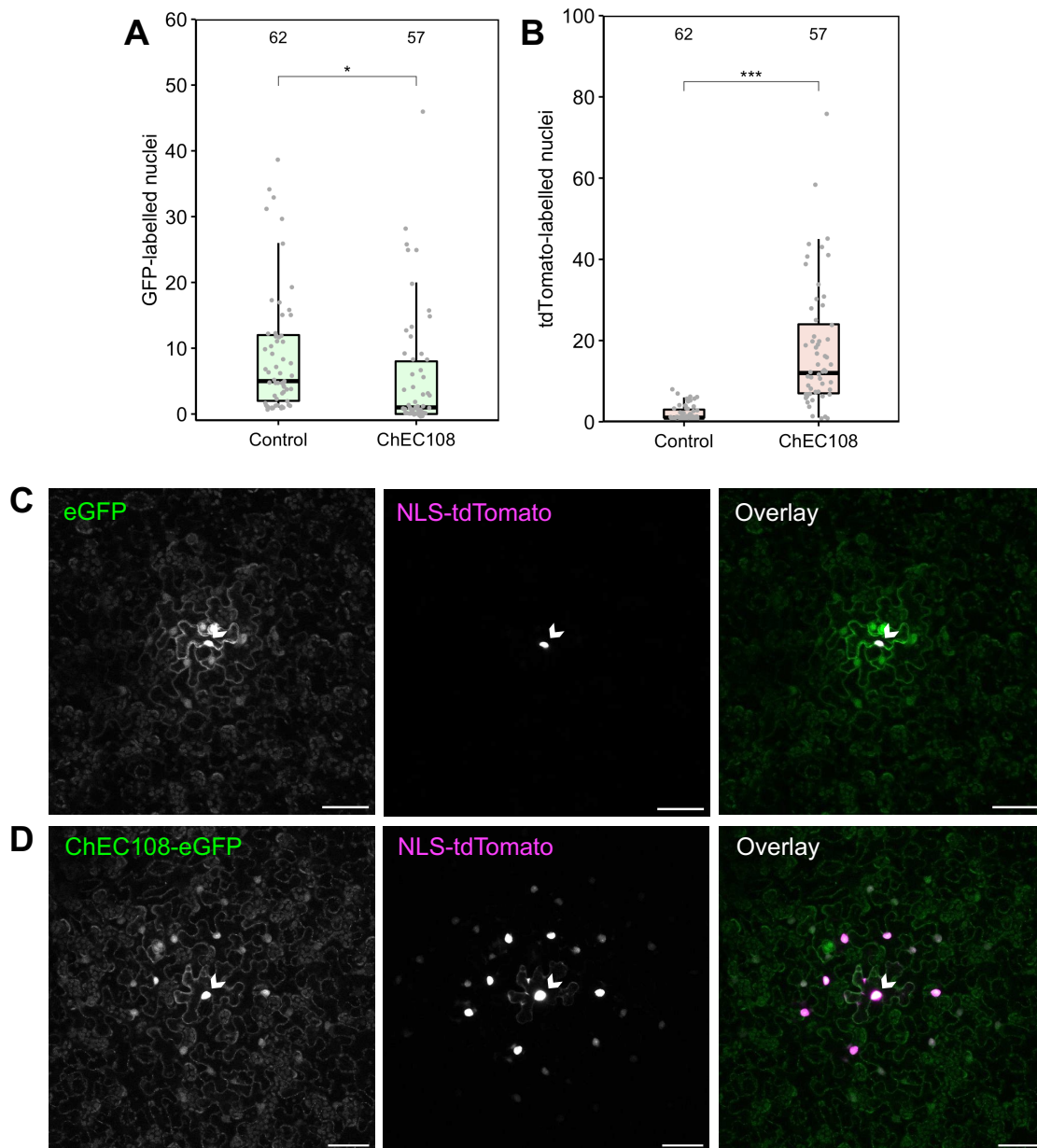


Figure 5.17 – ChEC108 enhances intercellular flux in *N. benthamiana* when expressed via bombardment. **A-B.** *NLS-tdTomato* was transiently expressed in *N. benthamiana* leaf epidermal cells via bombardment alongside *ChEC108-eGFP* or *eGFP* as a control. Mobility of *eGFP* (A) or *NLS-tdTomato* signal (B) was quantified 1 day post-bombardment by counting the number of fluorescent nuclei at transformation sites. Numeric annotations indicate sample size (sites), collected across 4 leaves per treatment, with a maximum of 20 sites imaged per leaf. * indicates $p < 0.05$, *** indicates $p < 0.001$, determined by pairwise bootstrapping analysis with 5000 iterations. **C-D.** Representative transformation sites from datasets shown in A-B. Arrowheads indicate the nuclei of single transformed cells. Scale bar = 50 μm .

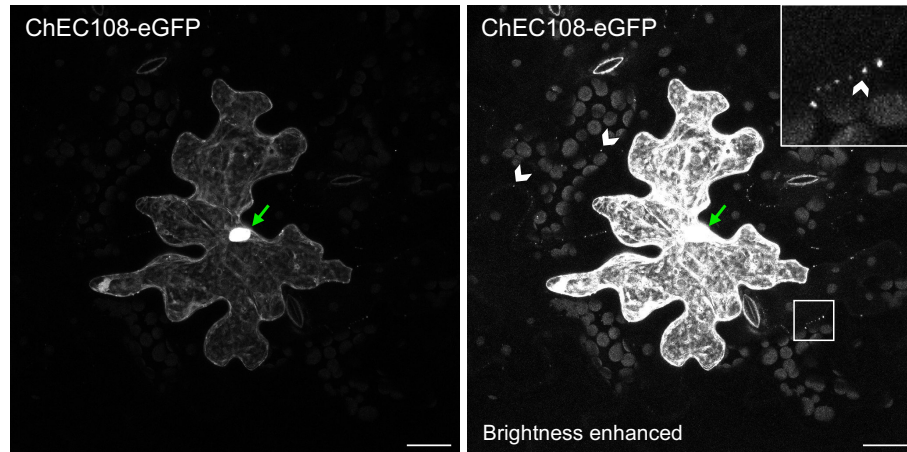


Figure 5.18 – ChEC108 is cell-to-cell mobile in Arabidopsis. *ChEC108-eGFP* transiently expressed via bombardment in a single Arabidopsis leaf epidermal cell, the nucleus of which is marked by a green arrow. When brightness is enhanced post-acquisition (right), eGFP signal is visible at PD in adjacent cells, alongside chlorophyll autofluorescence. Insert panel shows a magnified view of the region indicated by a white box. Arrowheads indicate example pitfields. Scale bar = 20 μm .

site. In *N. benthamiana*, both *ChEC108^{CCAA}-eGFP* and *ChEC108^{D164R-CCAA}-eGFP* were observed at PD, suggesting Cys197 and Cys200 are not important for guiding ChEC108 localisation (Figure 5.19C-D). This is perhaps unsurprising, as evidence from ITC and co-IP experiments implied Asp164Arg mutation was sufficient to prevent ChEC108 complex formation with isolated HMA domains and full-length HIP6, respectively (Table 3.5 and fig. 3.27). Together, these mutational analyses support the hypothesis that PD localisation of ChEC108 does not require HIP6.

To challenge this idea further, I turned to the native system of Arabidopsis, in which ChEC108-eGFP was detected exclusively at PD when its stable expression was driven by an *AtUBQ10* promoter (Figure 5.1D and Figure 5.20A). When stably expressed in Arabidopsis using the same approach, *ChEC108^{D164R}-eGFP* was also identified exclusively at PD in the Col-0 background (Figure 5.20B) and wild-type ChEC108-eGFP exhibited the same localisation in the *hipp6-3* mutant background (Figure 5.20C). Consistent with data obtained from *N. benthamiana*, it seems HIP6-binding is not required for ChEC108 to target Arabidopsis PD.

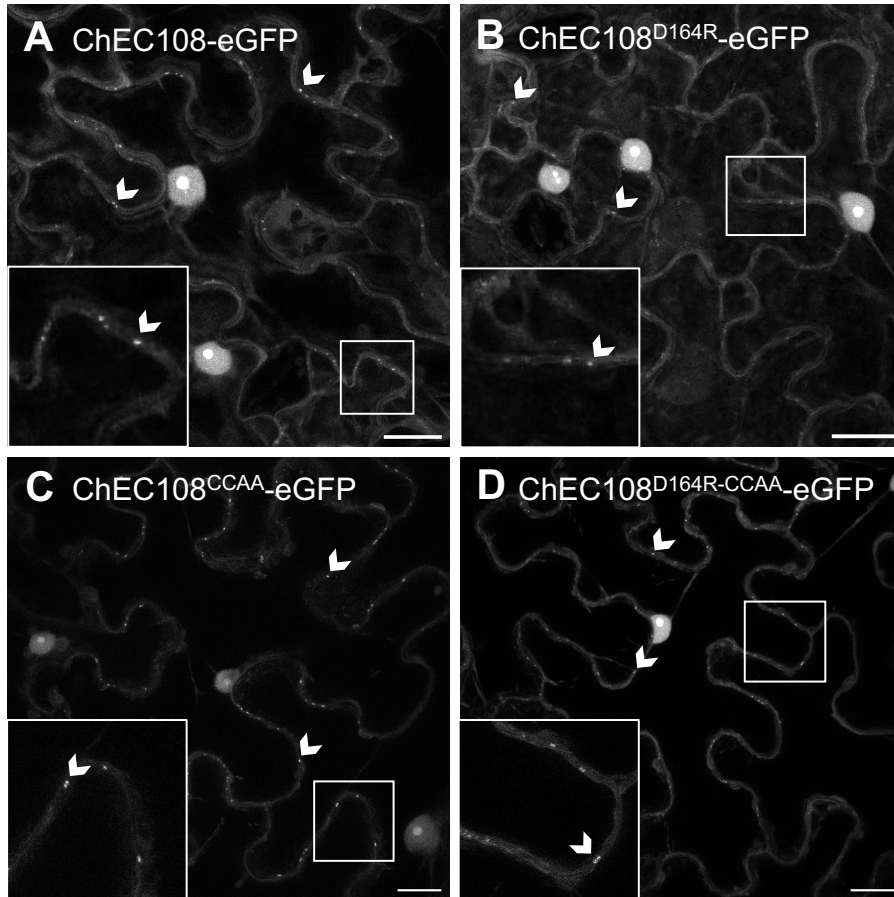


Figure 5.19 – ChEC108 PD localisation is not abolished by D164R or CCAA mutation. A-D. ChEC108-eGFP (A), ChEC108^{D164R}-eGFP (B), ChEC108^{CCAA}-eGFP (C) or ChEC108^{D164R-CCAA}-eGFP (D) transiently expressed in *N. benthamiana* leaf epidermal cells, displaying nuclear and PD localisation. Insert panels show a magnified view of regions indicated by white boxes. Arrowheads indicate example pitfields. Scale bar = 20 μm.

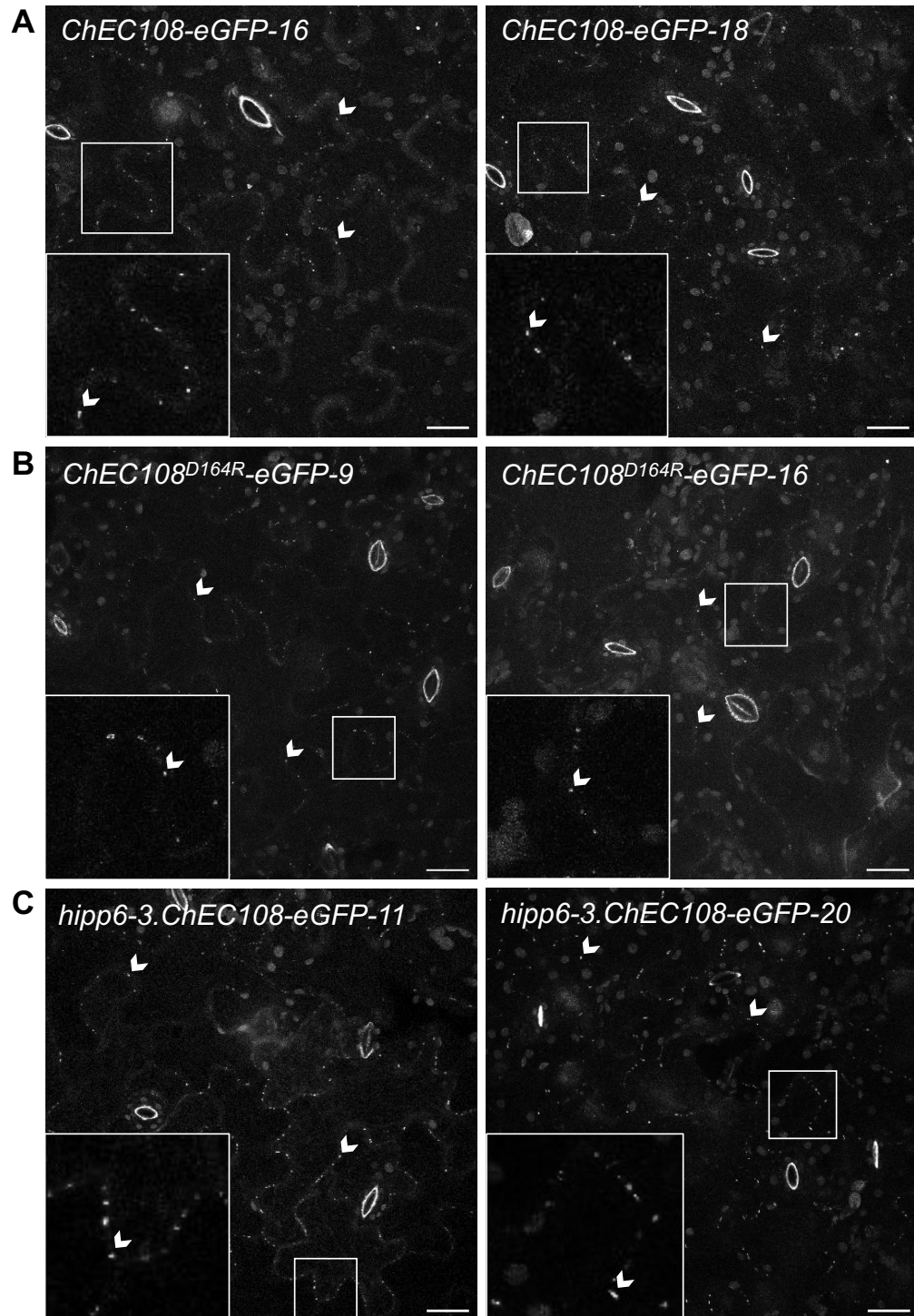


Figure 5.20 – ChEC108 PD localisation in Arabidopsis does not depend on HIPP6. A-C. PD-localised eGFP signal in leaf epidermal cells of two independent transgenic Arabidopsis lines expressing *ChEC108-eGFP* in the Col-0 background (A), *ChEC108^{D164R}-eGFP* in the Col-0 background (B) or *ChEC108-eGFP* in the *hipp6-3* mutant background (C). Insert panels show a magnified view of regions indicated by white boxes. Arrowheads indicate example pitfields. Scale bar = 20 μ m.

5.2.7 HIP6 inhibits ChEC108 cell-to-cell mobility

To further explore the idea that HIP6 helps guard Arabidopsis PD against putative manipulation by ChEC108, I sought to determine whether loss of HIP6-binding capacity could influence ChEC108 mobility. I thus bombarded ChEC108-eGFP into *hipp6-3* mutant leaves and studied transformed cells using confocal microscopy at high magnification, as described in 5.2.6. Similar to results obtained in the Col-0 background (Figure 5.18), in *hipp6-3* leaves, ChEC108-eGFP was identified at the PD of cells adjacent to those bombarded, suggesting cell-to-cell movement of the effector persists in the absence of HIP6 (Figure 5.21A). In agreement, mutant variants ChEC108^{D164R}-eGFP, ChEC108^{CCAA}-eGFP and ChEC108^{D164R-CCAA}-eGFP all exhibited cell-to-cell movement when delivered to Col-0 Arabidopsis via bombardment (Figure 5.21B-D). Unexpectedly, contrary to observations made in *N. benthamiana* using the same construct (Figure 5.19D), ChEC108^{D164R-CCAA}-eGFP was not identified at pitfields in Arabidopsis (Figure 5.21D). However, it is difficult to be confident in this negative result due to the extremely low accumulation of this variant in bombarded cells. Unfortunately, it remains impossible to meaningfully quantify ChEC108 movement in this system due to the low intensity of fluorescent signal, preventing assessment of potentially subtle changes to the *extent* of ChEC108 mobility in different scenarios. Nevertheless, these data confirm that HIP6 is not required for ChEC108 to move through PD.

As an alternative approach to study the influence of HIP6, I quantified ChEC108-eGFP movement in *N. benthamiana* leaves transiently overexpressing *mCherry-HIP6*, or *mCherry* as a control. This was achieved using a high density (OD = 0.4) of *A. tumefaciens* to drive expression of mCherry fusions in the majority of cells. Meanwhile, a low density of *A. tumefaciens* (OD = 0.0001) was used drive expression of *ChEC108-eGFP*, enabling single cell transformation events to be captured. On average, ChEC108-eGFP was restricted to a smaller number of cells at transformation sites when expressed alongside *mCherry-HIP6*, implying ChEC108 mobility was inhibited by overabundance of its binding partner (Figure 5.22A). Although the number of ChEC108-eGFP-labelled cells displayed a high degree of variance, this is likely explained by heterogeneous accumulation of mCherry-HIP6 in this transient system. As previously observed during Western blotting and confocal microscopy experiments presented in 5.2.4, unlike mCherry alone, mCherry-HIP6 is inherently unstable when transiently expressed, such that patchy fluorescent signal could only be observed using a high laser power and after enhancing the brightness of images post-acquisition (Figure 5.22B-C). It is therefore surprising that such a significant decrease in ChEC108-eGFP movement was detected in the presence of mCherry-HIP6 (Figure 5.22A). Overall, this suggests HIP6 could be an inhibitor of ChEC108 cell-to-cell movement.

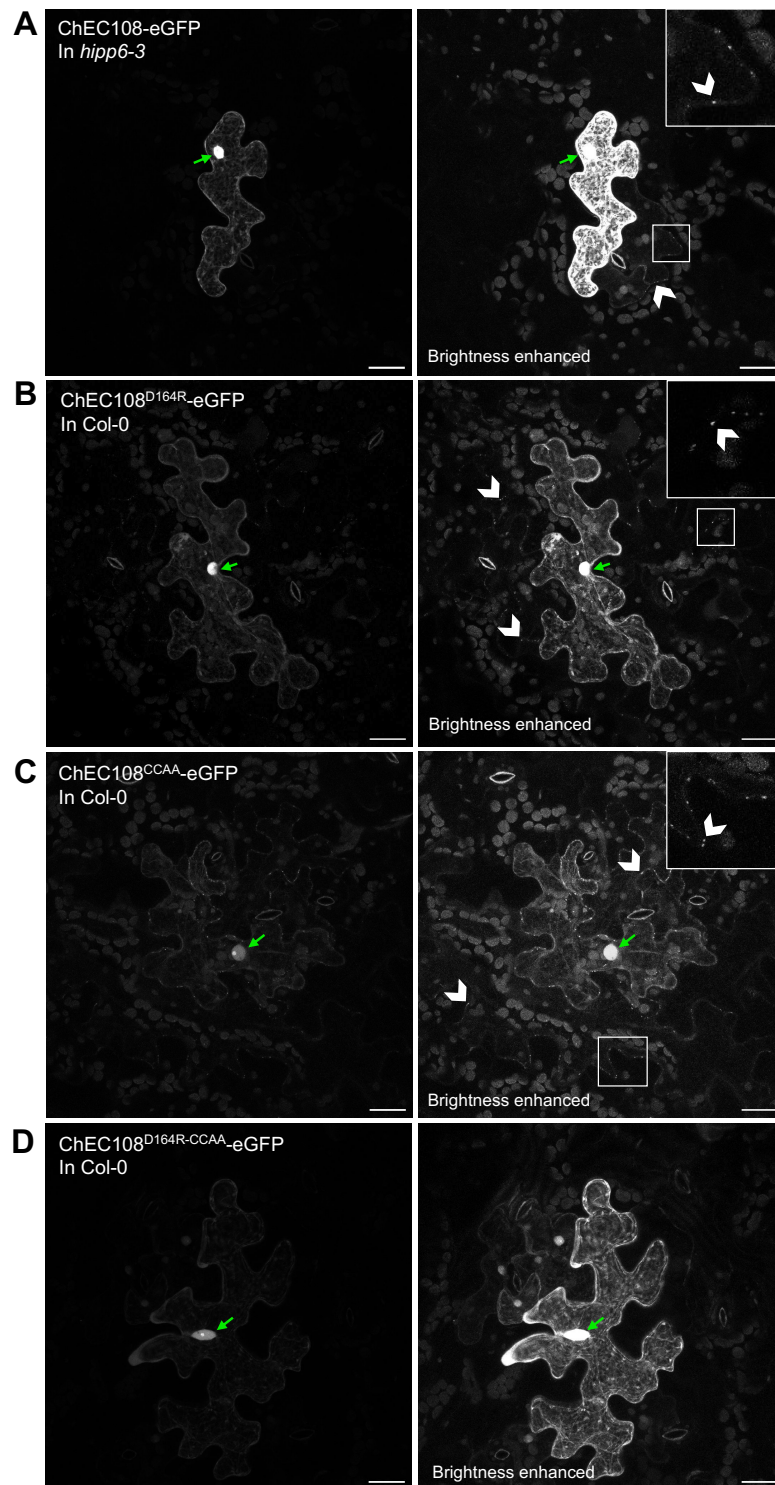


Figure 5.21 – Cell-to-cell mobility of ChEC108 in Arabidopsis does not depend on HIPP6. **A.** *ChEC108-eGFP* transiently expressed via bombardment in a single *hipp6-3* Arabidopsis leaf epidermal cell. **B-D.** *ChEC108^{D164R}-eGFP* (B), *ChEC108^{CCAA}-eGFP* (C) or *ChEC108^{D164R-CCAA}-eGFP* (D) transiently expressed via bombardment in single Col-0 Arabidopsis leaf epidermal cells. In panels A-D, when brightness is enhanced post-acquisition (right), eGFP signal is visible at PD and/or in the nuclei of adjacent cells, alongside chlorophyll autofluorescence. Insert panels show a magnified view of regions indicated by white boxes. Green arrows indicate the nuclei of bombarded cells. Arrowheads indicate example pitfields. Scale bar = 20 μ m.

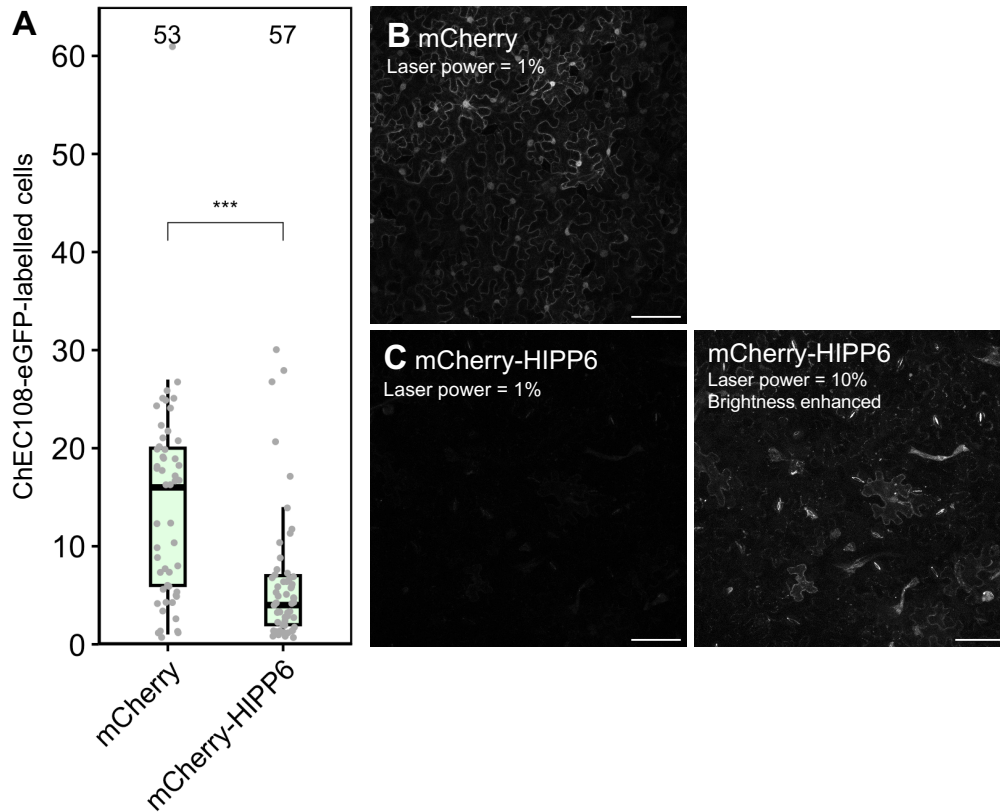


Figure 5.22 – HIPP6 overexpression reduces ChEC108 mobility in *N. benthamiana*. **A.** *ChEC108-eGFP* was transiently expressed in single *N. benthamiana* epidermal cells via low-OD agroinfiltration of leaves transiently expressing *mCherry* or *mCherry-HIPP6* via high-OD agroinfiltration. Mobility of *ChEC108-eGFP* was quantified 2 days post-infiltration by counting the number of fluorescent cell outlines at transformation sites. Numeric annotations indicate sample size (sites), collected across 4 leaves per treatment, with a maximum of 20 sites per leaf. *** indicates $p < 0.001$, determined by pairwise bootstrapping analysis with 5000 iterations. **B.** Representative image of *mCherry* signal, acquired using 1% laser power. **C.** Representative image of *mCherry-HIPP6* signal, acquired using 1% laser power (left), or 10% laser power with post-acquisition brightness enhancement (right). Scale bar = 100 μm .

5.2.8 ChEC108 overexpression shuts down intercellular trafficking

To further explore the influence of *ChEC108-HIPP6* association on PD function, I exploited genetic resources first introduced in 4.2.5. Specifically, Col-0 and *ChEC108-eGFP*-expressing plants (*ChEC108-eGFP-18*) were bombarded with mScarlet - a bright, cell-to-cell mobile cytosolic fluorophore, the mobility of which can be quantified as a proxy for baseline PD aperture. *ChEC108^{D164R}-eGFP-16* plants were included as negative controls for HIPP6 binding. If *ChEC108-HIPP6* binding events positively regulate intercellular trafficking, one would expect enhanced mScarlet movement at bombardment sites on *ChEC108-eGFP-18* leaves. On the other hand, if *ChEC108-HIPP6* binding shuts down trafficking, one would expect mScarlet movement to be greater in Col-0 and *ChEC108^{D164R}-eGFP-16* leaves. The data obtained support the latter hypothesis, as mScarlet movement was significantly lower in *ChEC108-eGFP-18* leaves, relative to Col-0 (Figure 5.23A-B). In fact, at the majority of sites

in this line (44/51), mScarlet was restricted to a single cell only. Unexpectedly, mScarlet movement in *ChEC108-eGFP-18* versus *ChEC108^{D164R}-eGFP-16* was not significantly different (Figure 5.23A), which could be explained by the high variance of mScarlet movement in the latter genotype, or the lower number of sites imaged on the former. Nevertheless, the extent of mScarlet movement in Col-0 and *ChEC108^{D164R}-eGFP-16* was found to be statistically similar. Considered alongside the severe growth phenotypes of *ChEC108-eGFP*-expressing plants that were absent in lines expressing *ChEC108^{D164R}-eGFP* (Figure 4.15), these data suggest ChEC108 and HIP6 may together induce growth inhibition and PD closure.

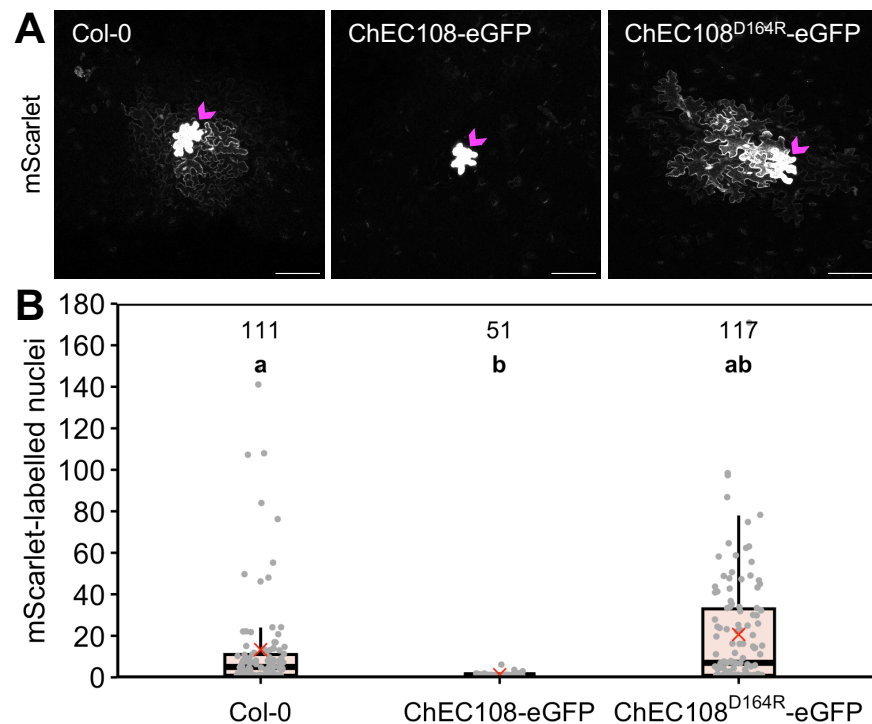


Figure 5.23 – Stable *ChEC108* expression dramatically reduces cell-to-cell trafficking in Arabidopsis. **A.** *mScarlet* transiently expressed in Arabidopsis leaf epidermal cells via bombardment. Leaves were taken from Col-0 plants (left) or those stably expressing *ChEC108-eGFP* (middle) or *ChEC108^{D164R}-eGFP* (right). Representative of image datasets collected across 8-10 leaves per genotype, with a maximum of 20 sites per leaf. Magenta arrowheads indicate the nuclei of bombarded cells. Scale bar = 100 μ m. **B.** Mobility of mScarlet was quantified by counting the number of fluorescent nuclei at transformation sites. $\times$ indicates the mean, and numerical annotations indicate sample size (sites). Letters indicate significant differences ($p < 0.001$), determined by pairwise bootstrapping analyses with 5000 iterations.

5.3 Discussion

In this chapter, I characterised the subcellular localisation of ChEC108 and HIP6 proteins, exploiting both *N. benthamiana* and Arabidopsis expression systems for live imaging experiments. I confirmed ChEC108 and HIP6 to be PD-localised proteins, and demonstrated that their interaction has a stabilising effect on HIP6. Outside PD compartments,

co-localisation data suggested ChEC108-HIPP6 complexes might associate with the ER.

While investigating the putative plasmodesmal functions of ChEC108 - a protein hypothesised to open up PD by [Jennings \(2021\)](#) - I made a series of unexpected observations: (1) ChEC108-mediated enhancement of intercellular flux was not detected in Arabidopsis; (2) *HIPP6* overexpression appeared to inhibit ChEC108 movement; (3) together, ChEC108 and HIPP6 might suppress intercellular trafficking in Arabidopsis. These findings open up many mechanistic questions, discussed below.

5.3.1 How are ChEC108 and HIPP6 targeted to PD?

In both *N. benthamiana* and Arabidopsis, ChEC108 and HIPP6 are enriched at pitfields, as demonstrated by their co-localisation with aniline blue-stained callose deposits (Figures 5.1 and 5.3). However, the mechanism by which these proteins reach PD remains unclear. While a universal PD targeting motif has yet to be identified, various divergent signals have been discovered in different classes of PD resident proteins. For instance, two regions of PDLTP proteins were identified to act as PD targeting signals, falling at the N- and C-terminal sides of the 'juxta membrane' region on the extracellular portion of these integral membrane proteins ([Luna et al., 2023](#)). In contrast, the MP of TMV possesses a targeting signal within its N-terminal 50 amino acids ([Yuan et al., 2016](#)), and association of this region with Arabidopsis SYNAPTOTAGMIN A (SYTA) is thought to anchor the protein at PD ([Yuan et al., 2018](#)). The fig mosaic virus (FMV) MP possesses a signal peptide comprising 19 N-terminal amino acids (a rare feature among viral MPs) ([Ishikawa et al., 2013](#)) with seemingly low translocation efficiency, resulting in dual targeting of the protein to the ER and plasma membrane microdomains. It is proposed that, as part of ER-plasma membrane contact sites, ER-translocated MP_{FMV} recruits plasma membrane-localised MP_{FMV} to PD via an unknown mechanism, making both pools of MP_{FMV} necessary for PD targeting ([Ishikawa et al., 2017](#)).

Recognising the remarkable variety of motifs and modifications reported to underpin the PD localisation of proteins, [Barr and Tilsner \(2025\)](#) recently proposed a retention-focussed perspective: rather than asking 'how does a protein reach PD?', a more appropriate question would be 'how is a protein *retained* at PD after arriving there randomly?'. Similar to the viral MP examples given above, I expect the signal(s) responsible for retaining ChEC108 at PD to reside at the N-terminus of the protein, given that GFP-ChEC108 fusions were not observed at pitfields ([Jennings, 2021](#); [Robin et al., 2018](#)). Future mutational studies could be used to test this hypothesis and to identify key underlying residues.

In addition to endogenous sequences, post-translational modifications are essential for the PD localisation of select proteins. For example, the *Pto* effector HopO1-1 requires

N-myristoylation for plasma membrane association, as well as its ADP-ribosyltransferase domain for enrichment at PD (Aung et al., 2019). Meanwhile, SUMOylation promoted enrichment of the carrot mottle virus (CMoV) MP, open reading frame 4 (ORF4), at PD (Jiang et al., 2021). Glycosylphosphatidylinositol (GPI) anchors for membrane association are present in a range of PD-localised proteins, including the β -1,3-glucanase 'putative PD-associated protein' (BG_pap), PD-associated callose-binding (PDCB) proteins and the LYM2 chitin receptor, where they are thought to act as PD sorting signals (Faulkner et al., 2013; Levy et al., 2007; Zavaliev et al., 2016).

Adding to this growing list of modifications, HIPP proteins putatively undergo C-terminal isoprenylation. Previously, Cys to Gly mutation at the CaaX motif was shown to compromise PD-enrichment of OsHIPP19 (Dr Josephine Maidment, unpublished data) and HIPP7 from *Arabidopsis* (Guo et al., 2021). HvHIPP43 localisation to PD was also reported to be abolished upon deletion of the isoprenylation motif (Were et al., 2025). Such mutations are expected to prevent prenylation (that is, the conjugation of farnesyl or geranylgeranyl lipids to Cys), which is proceeded by proteolytic removal of -aaX and methylation of the lipid-modified Cys (Crowell, 2000; Wang and Casey, 2016). It is hypothesised that prenylation provides proteins with a hydrophobic tail, thereby facilitating their association with membranes. The apparent irrelevance of the CaaX for regulation of HIPP6 localisation is therefore surprising (Figure 5.4). In fact, it seems likely that the HMA domains of HIPP6 (particularly their CXXC motifs) are responsible for anchoring HIPP6 to PD (Figures 5.5 to 5.7). This finding suggests PD retention signals are not necessarily conserved among HIPPs.

Although work described in this chapter did not uncover a detailed mechanism to explain ChEC108 or HIPP6 subcellular localisation, the data presented support a model whereby soluble ChEC108 becomes enriched at PD via an unknown mechanism, where it encounters HIPP6. Upon ChEC108-binding, the native conformation of HIPP6 is altered and a stable complex forms, which possibly dissociates from PD. This model assumes ChEC108 has a higher affinity for the HIPP6 HMA domains than other putative PD-localised binding partners. As HIPP6 is clearly not the 'anchor' exploited by ChEC108 for its enrichment at PD, it would be interesting to identify additional host targets of this effector.

5.3.2 How does ChEC108 manipulate PD in *N. benthamiana*?

In some ways, the behaviour of ChEC108 in *N. benthamiana* is reminiscent of a viral MP, in that it targets PD and increases their trafficking capacity (Figure 5.14). As discussed in 1.3.4, MPs actively manipulate PD via diverse mechanisms to facilitate the spread of their genetic material. Such mechanisms include (but are not limited to) severing of the actin cytoskeleton

(Su et al., 2010), trafficking along and modification of cortical ER membranes (Ham et al., 2023; Tilsner et al., 2013) and the formation of destructive tubules that span the cell wall (Xie et al., 2016) (for a recent in-depth review on this topic, see Alazem et al., 2025).

As ChEC108 localises to both PD and the cytoplasm of *N. benthamiana* cells (Figure 5.1), it is conceivable that this protein moves cell-to-cell via the cytoplasmic sleeve, and that its extensive mobility could be a consequence of dilation of existing PD channels. Currently, it remains uncertain as to whether ChEC108 manipulates PD-associated callose metabolism (either directly or indirectly) to achieve this goal. Given that deposition of callose at PD neck regions typically negatively correlates with trafficking capacity (Cheval et al., 2020; Vatén et al., 2011; Xu et al., 2017), I would expect callose levels to be reduced in *ChEC108*-expressing *N. benthamiana* leaves - a hypothesis that could be tested in future by quantifying the intensity of aniline blue-stained puncta as a proxy for callose accumulation (Johnston et al., 2022).

Alternatively, as demonstrated by studies of viral MPs above and, more recently, MCTP tethering proteins in *Arabidopsis* roots (Pérez-Sancho et al., 2025), callose-independent mechanisms of PD regulation remain a possibility for ChEC108. Tubule formation represents a common strategy employed by diverse plant viruses to effectively shuttle material between cells (reviewed by Schoelz et al., 2011). Even in the absence of cell walls, virus-induced tubules are known to assemble on the surface of host protoplasts, enabling closer examination of their structure and investigation into the viral or host components required for their induction (den Hollander et al., 2016; Kasteel et al., 1996; van Lent et al., 1991). However, ChEC108-induced tubule formation at PD is considered unlikely, as protoplasts generated from *ChEC108-eGFP*-expressing *N. benthamiana* leaves exhibited no tubule-like growths (Prof. Christine Faulkner, personal communication).

A preview of recent work by Reagan et al. (2023, currently under review) hints that a surprising additional strategy could be employed by viruses to enhance intercellular flux: de novo formation of secondary PD. Alongside the dilation of existing channels, it is expected that an increase in the number of pitfields (or the number of individual channels within), could greatly increase the available opportunities for molecules to exit cells. Indeed, secondary PD were shown to be rapidly induced during de-etiolation at the mesophyll-bundle sheath interface within the C_4 plant, *Gynandropsis gynandra*, crucially enabling more efficient exchange of metabolites as C_4 photosynthesis is induced (Schreier et al., 2024). Secondary PD formation has also been captured during the expansion of *N. tabacum* leaf trichome cells (Faulkner et al., 2008) and during xylem and phloem differentiation in the cambial zone of tomato (*Lycopersicon esculentum*) (Ehlers and van Bel, 2010). Initially, it was hypothesised that ChEC108 could exploit endogenous developmental pathways, rapidly forging new channels to facilitate the

movement of itself and other proteins (see 5.2.5). However, I have yet to uncover evidence to support this hypothesis, as ChEC108-mediated enhancement of pitfield density was not observed in *N. benthamiana* (Figure 5.15). Nevertheless, this conclusion is based on a single experiment, and one cannot eliminate the possibility that ChEC108 enhances the density of PD *within* individual pitfields - a phenomenon that could not be detected by quantifying PDL5-labelled puncta.

Instead of driving the formation of bona fide secondary PD, could ChEC108 simply ‘punch’ additional holes in the cell wall? Again, this seems unlikely, as ChEC108-labelled puncta closely co-localised with the PD marker PDL5 (Figure 5.1), indicating ChEC108-associated channels retain at least one member of the canonical PD proteome. However, it is possible that ChEC108 presence induces shifts in the composition of protein populations at PD, which could alter trafficking capacity. This could be investigated by studying the localisation of other known PD resident proteins alongside ChEC108, or via proteomic analysis of PD-enriched subcellular fractions in the presence and absence of the effector.

Further investigation is required to determine which (if any) viral MP-like strategies are adopted by ChEC108 to manipulate PD in *N. benthamiana*. In particular, although technically challenging, TEM techniques (for example, see Danila et al., 2016; Ostermeyer et al., 2022; Xie et al., 2016) could be beneficial to measure the density of PD in ChEC108-expressing leaves, and/or study their ultrastructure in finer detail. Although work in this chapter suggests ChEC108 plasmodesmal functions inferred from experiments in *N. benthamiana* might not translate directly to the more biologically relevant system of Arabidopsis, an improved understanding of ChEC108-mediated enhancement of intercellular trafficking could lead to exciting fundamental discoveries surrounding PD regulation.

5.3.3 Why was ChEC108-mediated manipulation of PD not observed in Arabidopsis?

Considering the extensive mobility of ChEC108 in *N. benthamiana* (Figure 5.14), the apparent inability of this protein to enhance general cell-to-cell flux following bombardment of Arabidopsis leaves (Figure 5.16B-F) came as a surprise. However, I managed to capture (but not quantify) ChEC108 mobility in the *Ch* host using high-magnification confocal imaging, as PD-localised signal was observed in cells adjacent to those initially transformed (Figure 5.18). At present, whether or not ChEC108 is *hypermobile* in Arabidopsis remains unclear. As traditional bombardment assays can not be used to quantify the movement of PD-exclusive signal, gaining a detailed understanding of ChEC108 mobility in host cells will be challenging. Potentially, increasingly large fluorescent tags could be utilised to discern the limits of ChEC108

mobility, asking the question: how large of a tag can be tolerated before fusions becomes restricted to single cells only? In combination with a standard curve constructed based on the mobility of standard fluorescent proteins (for an example from the *N. benthamiana* system, see [Ohtsu et al., 2023](#)), this approach could be used to determine whether ChEC108 fusions remain cell-to-cell mobile at larger molecular weights than expected, which could indicate an ability to manipulate PD.

There remains a possibility that the rapid radial spread of ChEC108 from bombarded cells resulted in dilution of fluorescent signal, giving a false impression of limited/no mobility. Such behaviour was previously encountered during development of the small fluorescent protein iLOV as a tool for monitoring viral spread ([Chapman et al., 2008](#), Prof. Christine Faulkner, personal communication). However, in this scenario, assuming ChEC108 alters general flux through PD, I would still expect to capture co-expressed fluorophores moving further alongside the effector - a phenomenon that was not observed in Arabidopsis (Figure 5.16). Currently, I favour the hypothesis that host-specific factors have evolved to oppose ChEC108 activity in Arabidopsis, either degrading the protein (which could explain the low ChEC108-eGFP signal intensity in bombarded cells) or blocking its manipulation of PD. As a direct interactor of ChEC108 and fellow PD-localised protein, could HIP6 be this host-specific factor?

Considering the reduced mobility of ChEC108 observed upon overexpression of HIP6 in *N. benthamiana* (Figure 5.22), one could imagine that HIP6 'captures' ChEC108, inhibiting cell-to-cell movement of the effector by increasing its effective molecular weight, and perhaps preventing interaction with other host targets that would otherwise be exploited to alter PD. Although ChEC108 and HIP6 were both found to predominantly localise to PD when stably expressed in Arabidopsis (Figures 5.1 and 5.3), co-localisation images presented in Figure 5.8 and Figure 5.10 raise the possibility that ChEC108-HIP6 complexes could be stabilised around the ER, rather than at PD exclusively. Effector- or MP-mediated mislocalisation of HIP6 proteins has been reported previously. For example, when transiently expressed in *N. benthamiana* in the presence of the *M. oryzae* effector AVR-PikD, OsHIP19 and OsHIP20 showed highly compromised PD localisation, instead accumulating in the cytoplasm ([Oikawa et al., 2024](#)). PD localisation of HvHIP43 was similarly perturbed in the presence of Pw12 from *M. oryzae* ([Were et al., 2025](#)), and NbHIP26 accumulated in cell nuclei and along microtubules when co-expressed with TGB1 from PMTV ([Cowan et al., 2018](#)). Even so, given the subtlety of ChEC108-mediated mislocalisation of HIP6 presented in Figure 5.10, the idea that ChEC108 pulls HIP6 away from PD requires further experimental support.

Although the precise subcellular location of ChEC108-HIP6 complexes remains to be confirmed, my data support a model whereby the interaction between these two proteins

initiates downstream signalling events, bringing about growth inhibition (Figure 4.15), accompanied by suppression of cell-to-cell trafficking (Figure 5.23). Currently, it remains unclear as to whether PD closure in the *ChEC108-eGFP-18* line could be a *cause* or *consequence* of stress signalling. In support of the 'consequence' hypothesis, callose-dependent PD closure is known to occur following perception of pathogen-associated elicitors by membrane-localised receptor complexes, initiating a common signalling pathway via RBOHD, a complex of PDL1/PDL5/NHL3, and ultimately, CALS1 (Tee et al., 2023). However, whether off-shoots of such PD-localised signalling events could also trigger major transcriptional reprogramming and growth stunting downstream of ChEC108-HIPP6 interaction is uncertain. Alternatively, in support of the 'cause' hypothesis, multiple transgenic lines exhibiting PD closure also exhibit stunted growth, including those overexpressing PD regulators, *PDL1* (Thomas et al., 2008), *PDL5* (Lee et al., 2011) and *PDL6* (Li et al., 2024). Recently, RNA-sequencing experiments utilising two independent tools to enable estradiol-inducible PD closure in *Arabidopsis* were conducted by Tee et al. (2025), demonstrating that closure itself triggers a stress response in leaves, accompanied by accumulation of the defence hormone SA, which potentially reinforces closure via a positive feedback loop. Furthermore, the homogenous, sustained PD closure achieved by induction of the *LexA::icals3m* line led to a significant reduction in leaf growth (Tee et al., 2025). It is therefore possible that suppression of intercellular trafficking downstream of ChEC108 perception by HIPP6 could initiate similar responses, restricting the optimal flow of sugars and other resources between source and sink rosette leaves. In future, it could be interesting to continue the efforts of Dr Joanna Jennings to generate estradiol-inducible ChEC108 lines (Jennings, 2021). This would enable closer examination of the consequences of effector function on mature leaves, disconnected from the pleiotropic phenotypes resulting from constitutive, ectopic expression throughout development.

Opposing both the hypotheses outlined above is the fact that ChEC108 was found to bind the *N. benthamiana* ortholog of HIPP6 in an IP-MS experiment (Table 3.1), while the effector remains highly cell-to-cell mobile in this species. Perhaps, although similarly bound by ChEC108, HIPP6 and NbHIPP6 functions are not entirely conserved, owing to differing protein-protein interaction networks or expression levels, despite both proteins being localised to PD (Figures 5.3 and 5.24). It is also plausible that other *Arabidopsis*-specific factors might inhibit ChEC108 function alongside HIPP6. While the disparate results collected in host and non-host systems in this work are presently difficult to explain, future functional comparisons of HIPP6 and NbHIPP6 could shed light on this.

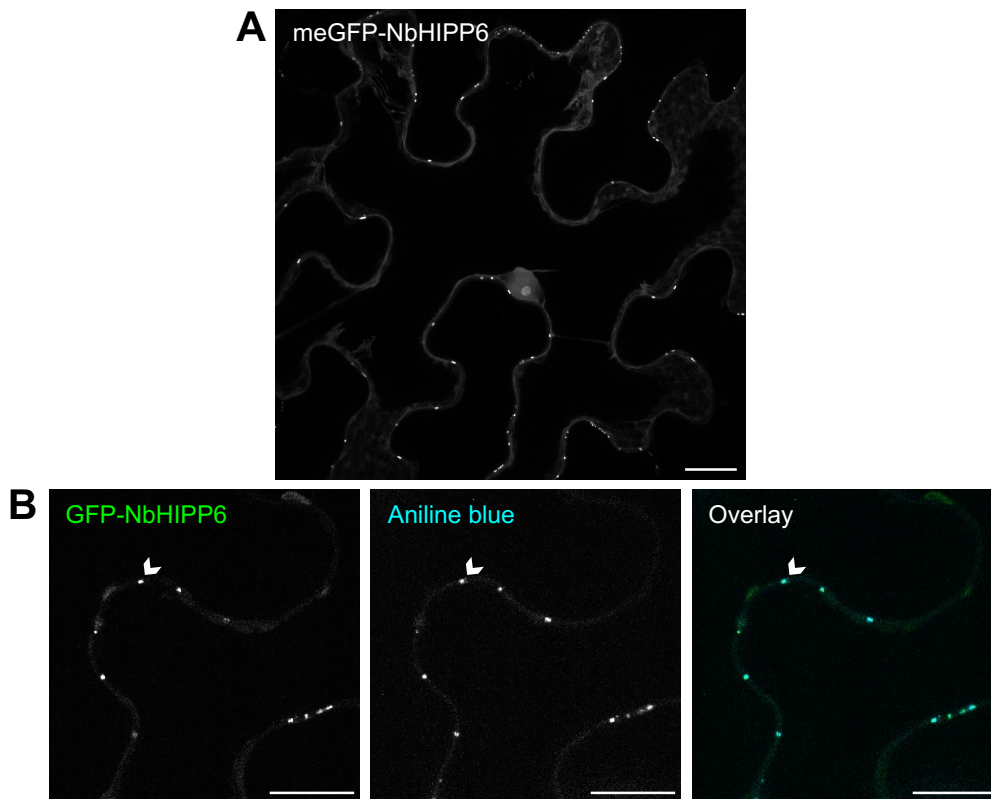


Figure 5.24 – NbHIPP6 localises to PD. **A.** *meGFP-NbHIPP6* transiently expressed in *N. benthamiana* leaf epidermal cells. Scale bar = 20 µm. **B.** Co-localisation of *meGFP-NbHIPP6* with pitfields in *N. benthamiana*, highlighted by aniline blue stain. Scale bar = 20 µm. Arrowheads indicate example pitfields.

5.3.4 Conclusion

Although the function of ChEC108 at PD in *Arabidopsis* has yet to be clarified, it seems unlikely for a fungal protein to target such a specific subcellular structure (and a plant-specific structure, a that) purely by chance. My working hypothesis is that ChEC108 evolved as a virulence factor, enabling *Ch* to target and manipulate host PD and help maintain symplastic continuity during infection. However, at least in *Arabidopsis*, the resident protein HIPP6 might guard PD against these virulence functions, restricting ChEC108 mobility and activating a stress signal, albeit a signal that is insufficient to halt *Ch* colonisation in its entirety. Further speculations and discussion surrounding ChEC108 and HIPP6 function at PD can be found in Chapter 7. Meanwhile, my efforts to study these two proteins in planta have so far left a key question unexplored: is ChEC108 a genuine secreted effector with access to the host symplast (and by extension, HIPP6) during infection? In the final results chapter of this thesis, I document my efforts to experimentally demonstrate ChEC108 secretion from *Ch* and its subsequent translocation to *Arabidopsis* cells.

Chapter 6

Seeking evidence of ChEC108 translocation to host cells

6.1 Introduction

A notable limitation of work described in Chapters 3 to 5 is that ChEC108 remains a *candidate* effector until secretion from *Ch* and translocation to Arabidopsis cells can be experimentally demonstrated. This protein is predicted by the [SignalP 6.0](#) protein language model to include a 20-amino-acid signal peptide with high probability (0.98) ([Teufel et al., 2022](#)). Similarly, machine learning models implemented by [EffectorP 3.0](#) predicted ChEC108 to be a cytoplasmic secreted effector with high confidence (0.82) ([Sperschneider and Dodds, 2022](#)). Supporting this further is evidence from independent transcriptomic studies, suggesting *ChEC108* is expressed preferentially during the biotrophic phase of infection ([Dallery et al., 2017](#); [O'Connell et al., 2012](#); [Robin et al., 2018](#)). Considered alongside the observed localisation of ChEC108 to PD (Figure 5.1), a plant-specific subcellular structure, these data point towards a function for ChEC108 as a bona fide effector.

6.1.1 *M. oryzae* effectors captured in the host symplast

Among filamentous phytopathogens, *M. oryzae* is established as an amenable system in which to study the translocation of effectors. Using confocal microscopy, mRFP-tagged Pwl2 and Bas1, each expressed in transgenic *M. oryzae*, were detected in the cytoplasm of *O. sativa* leaf sheath cells, and their cell-to-cell movement inferred based on the observation of fluorescent signal in cells ahead of the invasion front ([Khang et al., 2010](#)). Conversely, the apoplastic effector Bas4 exclusively outlined invasive *M. oryzae* hyphae when expressed with a GFP tag, and these fusions only spilled into the host cytoplasm upon collapse of the EIHM ([Khang](#)

et al., 2010; Sakulkoo et al., 2018). Besides these examples, live imaging of effector-fluorophore fusions during *M. oryzae* colonisation of *O. sativa* has been applied to demonstrate effector secretion in multiple studies, as well as to determine whether a given effector resides in the apoplast or enters the host symplast (Irieda et al., 2014; Mosquera et al., 2009; Quime et al., 2025; Yan et al., 2023).

As introduced in 4.1.1, the hemibiotrophic lifecycles of *M. oryzae* and *Ch* share some similarities. For instance, both pathogens deploy appressoria to generate turgor pressure for host penetration, underneath which emerge primary invasive hyphae. These primary hyphae develop into multiseptate, bulbous biotrophic hyphae, but remain isolated from the host symplast by the plant-derived EIHM (Giraldo and Valent, 2013). However, the *M. oryzae* BIC - a membrane-rich structure that functions as a localised point of effector delivery (Khang et al., 2010), has yet to be observed during *Ch* infection (Figure 6.1A). The BIC initially develops at the tip of an *M. oryzae* primary hypha as it extends from the penetration pore, but later becomes a 'side BIC', being displaced from its position at the tip as the hypha swells and branches (Shipman et al., 2017). As the BIC is associated with a concentration of *host* plasma membrane and ER, and lies outside the fungal plasma membrane, accumulation of fluorescently-tagged effectors at this locality serves as an indicator of their secretion (Giraldo et al., 2013).

Advances in *M. oryzae* cell biology have uncovered layers of complexity surrounding the regulation of blast effector secretion. While pharmacological studies suggest apoplastic effectors are trafficked via a conventional secretory pathway dependent on the ER and Golgi, cytoplasmic effectors are concentrated at the BIC, into which they are delivered via an unconventional pathway requiring members of the exocyst complex (Giraldo et al., 2013). Delving further into this mechanism, Li et al. (2023) discovered that tRNA modification (specifically 2-thiolation of base U₃₄ in the anticodon loop of tRNAs) helps fine-tune cytoplasmic effector secretion by reducing ribosome pausing at mRNA codons ending in '-AA'. Such codons are enriched in cytoplasmic versus apoplastic effectors, and are thought to be important for preventing effector overproduction and BIC instability (Li et al., 2023). Genetic and pharmacological experiments suggest that, following their arrival at the BIC, cytoplasmic effectors are packaged into dynamic vesicle-like compartments and taken up by host cells via clathrin-mediated endocytosis (Oliveira-Garcia et al., 2023). At present, given the structural diversity of host-pathogen interfaces observed (Giraldo and Valent, 2013), it is unclear whether other fungal phytopathogens employ the same mechanisms of effector delivery as *M. oryzae*.

6.1.2 *Colletotrichum* effectors captured at the host-pathogen interface

In contrast to *M. oryzae* studies, the localisation of few *Ch* effectors have been reported in an infection context. Expressed as C-terminal mRFP fusions under control of their native promoters, ChEC36 and ChEC6 labelled the *Ch* penetration pore, but not subsequently-emerging biotrophic hyphae, suggesting the base of appressorium could serve as a platform for effector delivery prior to host penetration (Kleemann et al., 2012). However, in the same study, immunogold labelling revealed ChEC36 to be absent from the host cytoplasm, and instead associated with the 'pore wall overlay', a layer continuous with the wall of an incipient penetration hypha. Meanwhile, three mCherry-tagged *Colletotrichum obiculare* effectors, DN3, MC69 and NIS1 were observed to localise to membranous ring-shaped compartments surrounding the necks of biotrophic hyphae underneath appressoria on cucumber leaves (*Cucumis sativus*), termed the 'ring signal interface' (Irieda et al., 2014). A similar pattern was observed following expression of DN3-mCherry in *Colletotrichum trifolii* and *Ch* during infection their respective hosts, alfalfa (*Medicago sativa*) and Komatsuna (*Brassica rapa*) (Ogawa et al., 2021), and in *C. obiculare* during infection of melon (*Cucumis melo*) and *N. benthamiana* (Irieda et al., 2016). Directed by their signal peptides, evidence from genetic experiments and confocal microscopy suggests effectors are delivered to this compartment via the ER-Golgi secretion (Sec) pathway, requiring exocytosis-related proteins Sec4 and Sec22 (Irieda et al., 2014). Beyond their targeting to the ring signal interface, if and how these *C. obiculare* effectors cross the surrounding plant plasma membrane to enter host cells remains unknown.

Later during infection, some effectors are known to associate with *Ch* biotrophic hyphae. Namely, ChEC3-mRFP, ChEC13-mRFP, ChEC34-mRFP and ChEC89-mRFP were observed at the *Ch*-host interface, occupying the apoplastic space between the fungal cell wall and plant plasma membrane (Kleemann et al., 2012). Here, ChEC34 and ChEC89 were observed at foci decorating the hyphal surface, revealed by TEM to correspond to electron-opaque structures termed 'interfacial bodies' (Figure 6.1B). Although these structures are proposed to function in effector delivery, none of the four effector-fluorophore fusions examined were observed in the host cytoplasm. The putative effector ChELP2 (for *Ch* extracellular LysM protein) similarly did not appear to translocate to host cells, and instead localised to the cell walls of biotrophic hyphae, where it is proposed to bind chitin for suppression of PTI (Takahara et al., 2016). Overall, at least three sites for *Colletotrichum* effector delivery have been proposed based on in planta studies: the penetration pore, the ring signal interface and interfacial bodies. However, explicit demonstration of effector translocation to the host cytoplasm remains a challenge for the field, and the mechanisms involved in effector uptake are poorly understood.

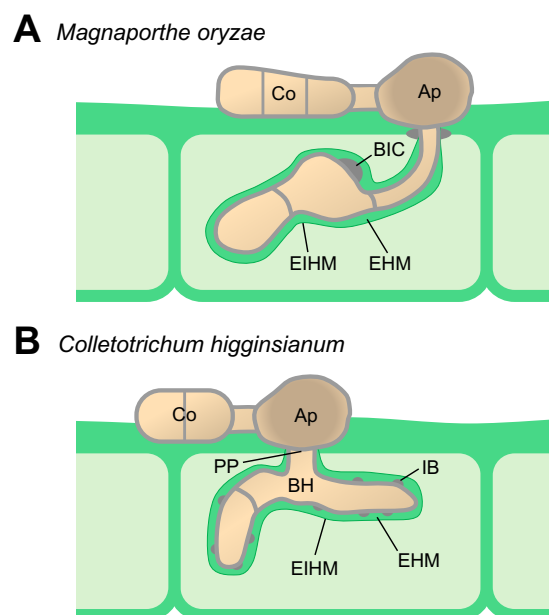


Figure 6.1 – Structures exhibiting accumulation of effector proteins in *M. oryzae* and *Ch*. A-B. Schematic representation of primary invasive hyphae of *M. oryzae* (A) and *Ch* (B) during the biotrophic phase of infection. Co = conidium, Ap = appressorium, BH = biotrophic hypha, EIH = extra-invasive hypha, EHM = extra-invasive hyphal matrix, BIC = biotrophic interfacial complex, IB = interfacial bodies, PP = penetration pore.

6.1.3 Proximity labelling for effector detection in planta

Alongside live imaging of pathogen invasion sites, enzyme-catalysed proximity labelling constitutes a sensitive biochemical technique that has been utilised to detect effectors in planta. These approaches typically involve fusion of a bait protein to an enzyme such as bifunctional ligase/repressor A (BirA), a biotin ligase derived from *E. coli*, enabling post-translational modification of proteins in the immediate vicinity of the fusion. In the case of BirA and its derivatives, biotinyl-5'-AMP formation is catalysed in the presence of ATP and biotin substrates, which becomes covalently conjugated to Lys residues or the N-termini of proteins within a 10-35 nm radius, depending on the exact enzyme and experimental conditions used (Kim et al., 2014; May et al., 2020). Biotinylated proteins can be subsequently identified following streptavidin-based enrichment and mass spectrometry for unbiased analysis, and/or Western blotting for targeted analysis.

Applying proximity labelling techniques to study *U. maydis* infection of *Z. mays*, Presti et al. (2017) were able to demonstrate translocation of effectors chorismate mutase 1 (Cmu1) and tumour inducing 3 (Tin3) to the plant cytoplasm. Here, *U. maydis* strains were engineered to express each effector fused with hemagglutinin (HA) for immunodetection, in addition to a 15-amino-acid biotinylation tag (Avitag). The Avitags served as effective substrates for a codon-optimised Myc-tagged variant of BirA (expressed in transgenic *Z. mays*), removing

the need for a BirA-fused bait protein. In such experiments, appropriate negative controls for background biotinylation events and potential biotinylation of proteins following cell lysis are necessary to distinguish genuine translocation events from experimental noise.

TurboID is biotin ligase derived from BirA, engineered via directed evolution to improve its catalytic efficiency and thermostability (Branon et al., 2018). This makes it a sensitive tool for mapping the interactomes of proteins, including those of pathogen effectors (Shi et al., 2023). Recently, TurboID-dependent biotinylation has been successfully applied in native infection contexts as a marker to indicate effector uptake by host cells. For instance, as bait to confirm translocation of the *P. infestans* avirulence proteins AVRamr1 and AVRamr3, TurboID was fused to corresponding CC-NLRs, Resistance to *Phytophthora infestans* from *Solanum americanum* 1 (Rpi-amr1) or Rpi-amr3, respectively (Szymansky, 2025). In *N. benthamiana* plants transiently expressing these TurboID fusions and subsequently infected with *P. infestans*, biotinylated AVRamr1 and AVRamr3 were detected in the presence of their corresponding NLR via streptavidin pull-down coupled with mass spectrometry, but were not detected in control leaves expressing *mCherry-TurboID*.

As well as confirming uptake of known effectors under native conditions, Szymansky (2025) utilised TurboID for the discovery of novel avirulence factors in the *Albugo candida*-*Arabidopsis* pathosystem. In this case, TurboID was fused to the TIR-NLR WHITE RUST RESISTANCE 4 (WRR4) and stably expressed in *Arabidopsis*. Following infection with *A. candida* and isolation of biotinylated proteins, mass spectrometry uncovered two CCG proteins (so-named for their C₂C₅G motifs) in the WRR4 putative interactome: CCG14 and CCG41. Despite not being listed among the eight CCG proteins already known to be recognised by this TIR-NLR, cell death assays in *N. tabacum* suggested CCG14 and CCG41 were capable of triggering WRR4-dependent immune responses (Szymansky, 2025). Overall, this work demonstrates that when materials and protocols are sufficiently optimised, TurboID-based approaches have the potential to capture pathogen-derived effectors (even those of low abundance, expressed under their native promoters) in host cells during infection.

6.1.4 Towards indirect effector detection: using NLRs to engineer gain-of-resistance

As an alternative to the direct detection methods described thus far, it is possible for the presence of effectors in planta to be monitored *indirectly* via their activation of intracellular immune receptors. Col-0 *Arabidopsis* remains highly susceptible to *Ch* strains carrying the *ChEC108* gene, suggesting a ChEC108-responsive NLR is absent in this system. However, with advances in structural and synthetic biology, as well as protein structure prediction and de novo design (Dauparas et al., 2022; Watson et al., 2023), the generation of novel NLRs with

alternative or expanded effector recognition specificities represents a promising solution to this problem (for reviews, see [Cadiou et al., 2023](#); [Zdrzałek et al., 2023](#)). Consistent with the fact that some effectors are perceived via direct binding to NLR LRR domains, LRR resurfacing has been successfully applied to alter NLR recognition specificities ([Förderer et al., 2022](#); [Tamborski et al., 2023](#)). Alongside, the effector-binding integrated domains of paired NLRs represent attractive targets for engineering. For example, in a study of the *O. sativa*-*M. oryzae* pathosystem, [De la Concepcion et al. \(2019\)](#) used structure-guided engineering of the Pikp-1 HMA domain to expand the recognition profile of this sensor to include AVR-PikA and AVR-PikE, alongside the cognate effector, AVR-PikD. Similarly guided by effector structures, [Liu et al. \(2021\)](#) designed a mutant HMA domain for RGA5, enabling it to recognise AVR-Pib and confer resistance to *M. oryzae* carrying this non-corresponding effector. Also, recognition of AVR-PikD was achieved by mutation of the RGA5 HMA domain on two separate occasions ([Cesari et al., 2022](#); [Zhang et al., 2024b](#)), and one of these strategies (involving mutation of the Lys-rich region adjacent to the HMA) translated to resistance in transgenic *O. sativa* ([Zhang et al., 2024b](#)).

In addition to strategic point mutation, it is possible to alter the recognition profiles of NLRs by replacement of their entire integrated domains. This strategy is exemplified by recent work on 'pikobodies', a term given to chimeric NLRs comprising Pikm-1 with a camelid antibody fragment (nanobody) in place of the native HMA domain. By integrating nanobodies specifically raised to recognise fluorescent proteins, [Kourelis et al. \(2023\)](#) engineered pikobodies capable of activating immune responses following GFP or mCherry perception in *N. benthamiana*, and conferring resistance to GFP- and mCherry-expressing PVX. As well as nanobodies, integration of domains from other NLRs ([Bentham et al., 2023](#)) or effector host targets has proven successful for adapting the effector recognition profiles of NLRs. For example, exchange of the Pikm-1 HMA domain for that of OsHIP43 enabled Pikp-2-dependent recognition of *M. oryzae* Pwl family effectors ([Zdrzałek et al., 2024](#)). By replacing the HMA domain of Pikp-1 with that of OsHIP19 and introducing further mutations to its loop regions, [Maidment et al. \(2023\)](#) generated transgenic *O. sativa* with enhanced resistance to *M. oryzae* isolates expressing previously unrecognised effectors, AVR-PikC and AVR-PikF, guided by structural information gained from effector-host target complexes in vitro ([Maidment et al., 2021](#)).

In light of these advances, the discovery of HIP6 as a HMA domain-containing target for ChEC108, coupled with insights from crystal structures described in Chapter 3, makes NLR engineering a promising research avenue to explore. While *Arabidopsis* is known to encode NLRs with diverse integrated domains, comprehensive resistance gene enrichment sequencing (RenSeq) analysis of 64 accessions did not uncover a HMA domain-containing sensor within the pan-NLRome ([Van de Weyer et al., 2019](#)). Nevertheless, given the widespread conservation

of NLR signalling mechanisms across plants families (Chia et al., 2024; Maekawa et al., 2012), it is possible for NLRs expressed in transgenic plants to retain their signalling capabilities downstream of effector perception, even when transferred from other species (Du et al., 2025). While also representing an ambitious novel approach to enhance the resistance of host plants to *Ch*, generation of a ChEC108-responsive *O. sativa* NLR pair could be beneficial to provide a detectable readout of effector presence in host cells during native infection.

In summary, so far I have described three unique approaches to obtain evidence of effector translocation during fungal infection: (1) confocal imaging to assess protein subcellular localisation, (2) proximity labelling to serve as a marker for effector presence in host cells, and (3) indirect detection of effectors via activation of synthetic NLRs. In the following chapter, I report on my efforts to implement all three strategies, seeking evidence that ChEC108 is a bona fide secreted effector with access to the host symplast.

6.2 Results

6.2.1 *Ch*-derived ChEC108-eGFP could not be detected in Arabidopsis

With the aim of establishing the subcellular localisation of ChEC108 during infection of Arabidopsis, I generated *Ch* strains to produce ChEC108-eGFP, fused to its native N-terminal signal peptide (SP). I cloned the *SP-ChEC108-eGFP* coding sequence into a custom-made backbone for fungal transformation, called pCH-HPH (Jennings, 2021). To drive constitutive expression of the transgene, I flanked the coding sequence with the *cos1*-glyceraldehyde-3-phosphate dehydrogenase (*cos1gpdA*) promoter and *tryptophan biosynthesis* (*trpC*) terminator from *A. nidulans*, both originally amplified from the vector pBin-GFP-hph (O'Connell et al., 2004). Admittedly, the relevance of the 387 bp *cos1* sequence is unclear, as its origin is not explained by O'Connell et al. (2004) and a BLAST-based search suggested the region is derived from *E. coli*. Nevertheless, the *gpdA* promoter has been widely used to drive high-level expression of transgenes in filamentous fungi (Punt et al., 1991), and the specific 405 bp fragment contained within the *cos1gpdA* promoter can be traced back to Herbert et al. (2002), who exploited this region to drive fluorescent protein expression in *C. lindemuthianum*. Using a non-targeted *A. tumefaciens*-mediated transformation approach, I transformed the plasmid encoding SP-ChEC108-eGFP into wild-type IMI 349061 *Ch* (here referred to as WT²) and identified multiple hygromycin-resistant transformants.

To increase the likelihood of ChEC108-eGFP detection during infection, I sought to identify a *Ch* transformant with high levels of transgene expression. Reasoning that rapid growth on hygromycin-containing media could indicate high expression of *HygR* and the accompanying

ChEC108-eGFP transgene, I carried forward four of the fastest-growing transformants: #4, #7, #12 and #15. Unfortunately, obvious eGFP signal was not observed upon examination of spores collected from these transformants via confocal microscopy. As an alternative screening method, I extracted protein from liquid-grown mycelia and probed for eGFP signal via Western blotting, using WT² mycelia as a control. In these initial extracts, only a low molecular weight band (~24 kDa) could be detected, likely corresponding to free eGFP (Figure 6.2 upper panel for #7, #12 and #15). However, when an IP step was conducted to enrich for GFP-tagged proteins, full-length SP-ChEC108-eGFP was detected in #12 (Figure 6.2). Although this suggested expression and/or accumulation of SP-ChEC108-eGFP was low, it was unclear whether cleavage of the C-terminal tags occurred in the *Ch* mycelia or during extraction, and I thus carried forward #12 (referred to as *Ch*^{SP-ChEC108-eGFP}) for experimentation.

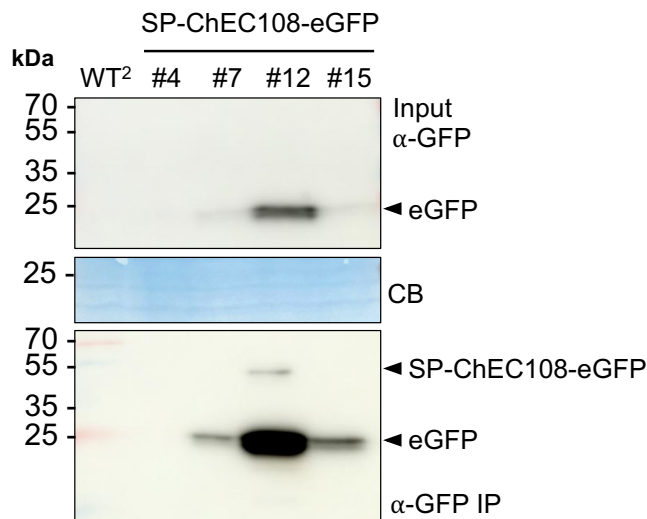


Figure 6.2 – SP-ChEC108-eGFP can be constitutively expressed in *Ch*. Protein extracts from liquid-grown *Ch* mycelia were probed with α-GFP antibodies. In input fractions, free (cleaved) eGFP (~24 kDa) was observed in transformants #7, #12 and #15, but not in #4 or the WT² control. After enrichment via α-GFP IP, intact SP-ChEC108-GFP (52 kDa) was detected in #12. Total protein loading is shown by Coomassie Blue (CB) staining.

Following drop-inoculation of Col-0 Arabidopsis leaves with *Ch*^{SP-ChEC108-eGFP} spores, I searched for signs of invasive fungal growth via confocal microscopy 2 and 3 dpi. As anticipated given the low levels of transgene expression reported above, fluorescent signal was difficult to locate. Nevertheless, I was able to identify conidia and appressoria on the leaf surface (Figure 6.3A), accompanied by hyphae at a select few invasion sites. On one occasion, puncta resembling pitfields were seen in close proximity to a cell containing a small biotrophic hypha (Figure 6.3B), however, given the lack of repeatability of this observation and extremely low signal intensity, I cannot confidently conclude that these represent ChEC108-labelled PD. In the majority of biotrophic hyphae observed, faint fluorescent signal seemed diffuse,

with septa being especially evident in Figure 6.3C, while concentration of signal at a BIC-like structure was not observed (Figure 6.3B-D). Given the extremely faint nature of fluorescent signal associated with *Ch*^{SP-ChEC108-eGFP} hyphae, I deemed the experiment to be inconclusive regarding the translocation status of ChEC108.

6.2.2 Exploring a TurboID-based strategy to detect *Ch*-derived ChEC108 in Arabidopsis

Making use of a smaller epitope tag, a proximity labelling-based strategy combined with a split-GFP system for in planta visualisation (Avilov and Aleksandrova, 2019) was devised to test for ChEC108 translocation. Previously, an Arabidopsis line was developed by Dr Joanna Jennings to constitutively express β -strands 1-10 of GFP (GFP1-10) fused to TurboID, under regulation of the CaMV 35S promoter and Arabidopsis *HEAT SHOCK PROTEIN 18* (*HSP*) terminator. Upon infection of these transgenic Arabidopsis with *Ch* expressing SP-ChEC108 fused to β -strand 11 of GFP (GFP11), one would hypothesise that the effector fusion would be secreted and taken up by host cells, and that GFP fluorescence could be visualised following association of the fungus-derived GFP11 with plant-derived GFP1-10. Meanwhile cells infected with a WT² control strain would not be expected to fluoresce (Figure 6.4A-B). Similar approaches exploiting split-GFP have been employed to demonstrate delivery of bacterial effectors via the type III or IV secretion system (reviewed by Fritsch and Hensel, 2025). In preparation to test this system in the context of *Ch* infection, I generated a *Ch* strain in the WT² background to constitutively produce SP-ChEC108, C-terminally fused to Myc for immunodetection and GFP11, under regulation of the *cos1gdpA* promoter and *trpC* terminator. To confirm expression of the transgene in *Ch* transformants recovered on selective media, I extracted protein from liquid-grown mycelia and performed an α -Myc IP. When examined via Western blotting, bands corresponding to SP-ChEC108-Myc-GFP11 were detected in IP fractions corresponding to transformants #8 and #15 (with signal intensity being marginally higher for the former), although a nearby non-specific band impeded detection of the fusion protein in input fractions (Figure 6.4C).

Carrying forward transformant #8 for experimentation, I conducted a trial infection of GFP1-10-TurboID-expressing plants involving spray-inoculation of detached mature leaves. However, when leaves were examined by confocal microscopy 25 hpi, GFP signal was not observed in cells beneath appressoria sighted on the leaf surface. Verifying that GFP-1-10-TurboID was expressed and functioning as expected, I bombarded leaves of this transgenic line with a plasmid encoding β -glucuronidase (GUS)-GFP11 and was able to detect fluorescence in transformed cells, suggesting functional GFP could be assembled and visualised (Figure 6.4D). I therefore concluded the abundance of ChEC108-Myc-GFP11

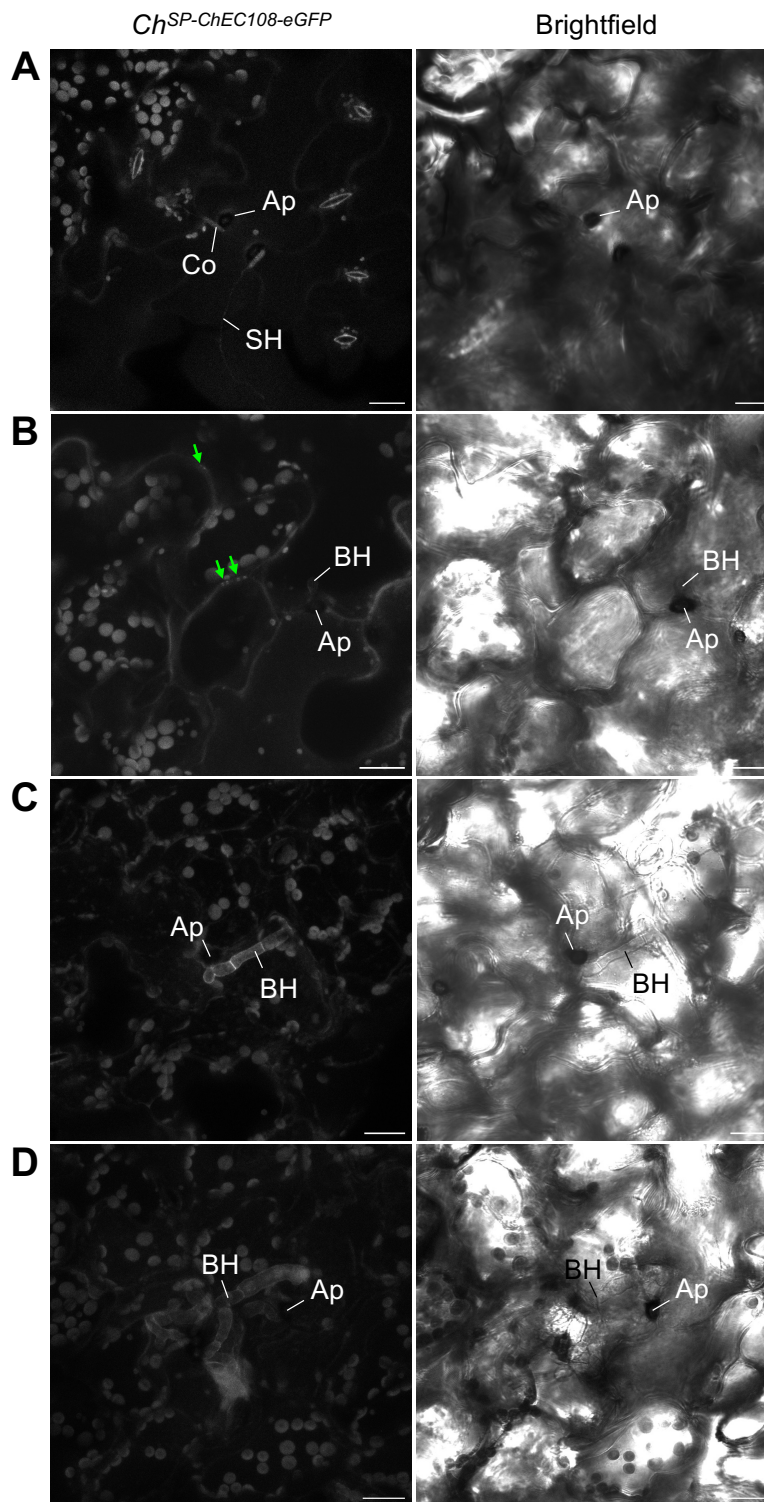


Figure 6.3 – *Ch*-derived ChEC108-eGFP could not be detected in Arabidopsis. A-D. eGFP signal and autofluorescent background of Arabidopsis leaves infected with *Ch*^{SP-ChEC108-eGFP}. Images were acquired 3 dpi, and represent the pre-penetration phase (A), early biotrophic phase (B-C) and mid biotrophic phase of infection (D). Co = conidium, Ap = appressorium, SH = surface hyphae, BH = biotrophic hyphae, NH = necrotrophic hyphae. Green arrows in B indicate puncta resembling PD in cells adjacent to an invaded cell, observed only once. Scale bar = 20 μm.

(and therefore fully assembled GFP) must have been extremely low during the trial infection. Although optimisation of time points and infected tissue type could have been beneficial to better assess the efficacy of this imaging-based strategy, with *Ch* proving difficult to image in planta using confocal microscopy (Figure 6.3), I decided to focus my efforts on an alternative biochemical approach to capture *Ch* effector translocation, utilising the same biological materials.

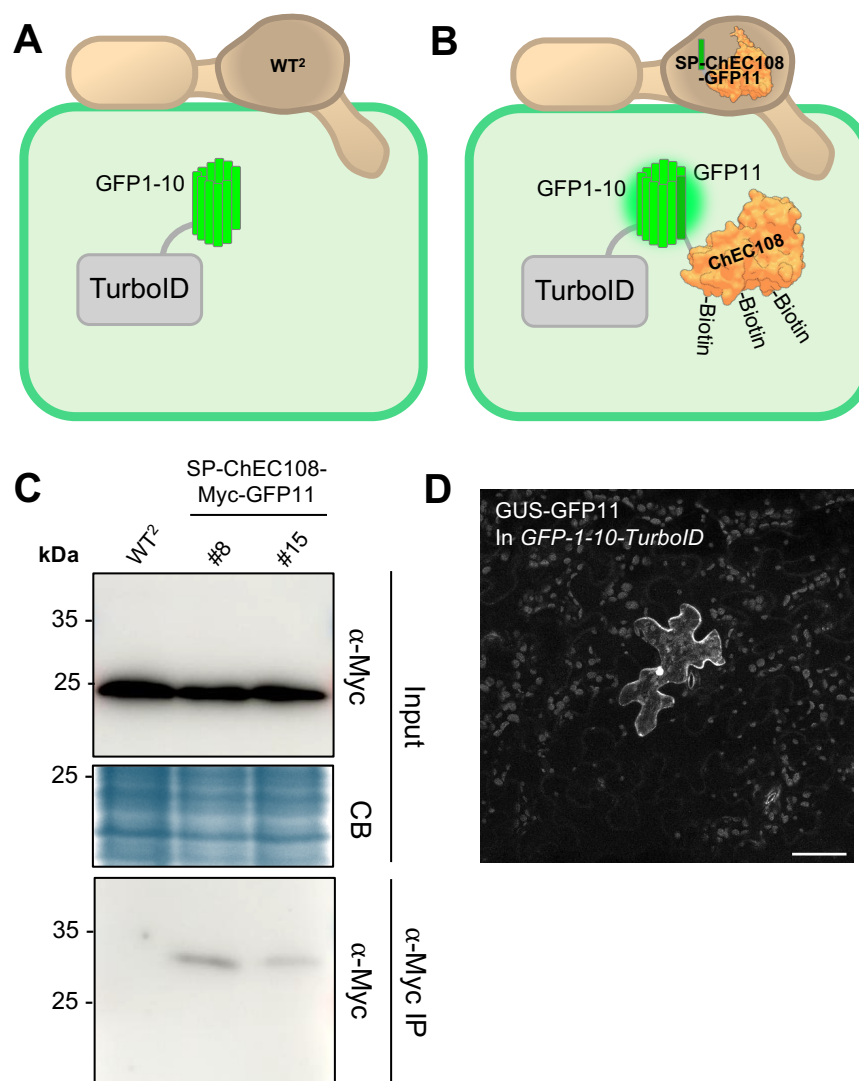


Figure 6.4 – Illustration of the split-GFP TurboID approach to detect *Ch*-derived ChEC108 in *Arabidopsis* cells. **A-B.** If GFP1-10-TurboID were constitutively expressed in *Arabidopsis* during infection with WT² *Ch*, fluorescence should not be observed (A). However, during infection with *Ch* expressing SP-ChEC108-Myc-GFP11, assembly of GFP in the cytosol via interaction of GFP11 with GFP1-10 should enable fluorescence (B). Alternatively, biotinylation of ChEC108 (a consequence of close proximity to TurboID) could be detected via Western blotting. **C.** Protein extracts from liquid-grown *Ch* mycelia were probed with α-Myc antibodies. In input fractions, a non-specific band was observed in all extracts, including the WT² control. After enrichment via α-Myc IP, intact SP-ChEC108-Myc-GFP11 (31 kDa) was detected in transformants #8 and #15. Total protein loading is shown by Coomassie Blue (CB) staining. **D.** GFP assembly visualised when GUS-GFP11 was transiently expressed in a single *Arabidopsis* epidermal cell via bombardment, in a leaf background constitutively expressing GFP-1-10-TurboID. Scale bar = 50 μm.

Besides assembling a functional GFP monomer, the association of GFP1-10-TurboID and ChEC108-Myc-GFP11 in the split-GFP system described above is expected to promote biotinylation of ChEC108 by the now-adjacent TurboID enzyme (Figure 6.4B). Hypothetically, an α -Myc IP followed by Western blotting with a streptavidin conjugate could be used to detect these labelled effector proteins, serving as evidence of the effector's secretion and entry into the host cytoplasm. As experimental evidence suggests TurboID to be capable of biotinylating proteins within a ~10 nm radius (Kim et al., 2014), one would not expect an intermediate GFP tag, comprising a cylinder with length 4.2 nm and diameter 2.4 nm (Hink et al., 2000), to prevent labelling of ChEC108 in this scenario. However, an initial trial experiment conducted using spray-inoculated mature *Arabidopsis* leaves proved inconclusive, as SP-ChEC108-Myc-GFP11 could not be detected in the protein extracts of samples harvested 24 hpi, even following α -Myc IP (Figure 6.5). Despite this, autobiotinylated GFP1-10-TurboID was detected in input fractions alongside background biotinylated proteins only when leaves were infiltrated with 50 μ M biotin, and not in the Col-0 control samples (Figure 6.5). This demonstrated that the plant material and sample preparation procedures were suitable for induction and detection of *Arabidopsis* protein biotinylation by TurboID.

Even when expressed via the constitutive *cos1gpdA* promoter, it seems that SP-ChEC108-Myc-GFP11 levels were insufficient to be detected by Western blotting in an infection context. With the goal of increasing ChEC108 stability and ease of immunodetection, I devised a modified TurboID-based strategy (Figure 6.8). In place of GFP1-10, I fused TurboID to the host target of ChEC108, HIPP6, or its individual HMA domains, along with an N-terminal 3 $\times$ FLAG tag. As in vitro work suggested ChEC108 affinity for HIPP6^{HMA1} and HIPP6^{HMA2} to be strong (Table 3.5), I hypothesised that these fusions might serve as effective baits to bring ChEC108 into close proximity with TurboID. Additionally, I exchanged the epitope tag on ChEC108 for 4 $\times$ Myc (4.8 kDa), which is marginally smaller than Myc-GFP11 (5.4 kDa) and could potentially enhance IP and/or detection of this protein by α -Myc antibodies.

To demonstrate a proof of concept for this approach (and the split-GFP alternative), TurboID and ChEC108 variants described above were transiently expressed in *N. benthamiana*. Following biotin treatment of the leaves, I used an α -Myc IP to enrich for Myc-tagged ChEC108 variants and probed for their biotinylation with streptavidin conjugates. Encouragingly, biotinylated ChEC108-4 $\times$ Myc was detected upon co-expression with 3 $\times$ FLAG-TurboID fused to either HMA1, HMA2 or full-length HIPP6, but not in the absence of TurboID, or when co-expressed with GFP1-10-TurboID as a negative control (Figure 6.6). Alongside, biotinylated ChEC108-Myc-GFP11 was detected upon co-expression with GFP1-10-TurboID, but not in the absence of TurboID (Figure 6.6). This suggests both the split-GFP and HIPP6-mediated bait systems have the potential to facilitate ChEC108 biotinylation.

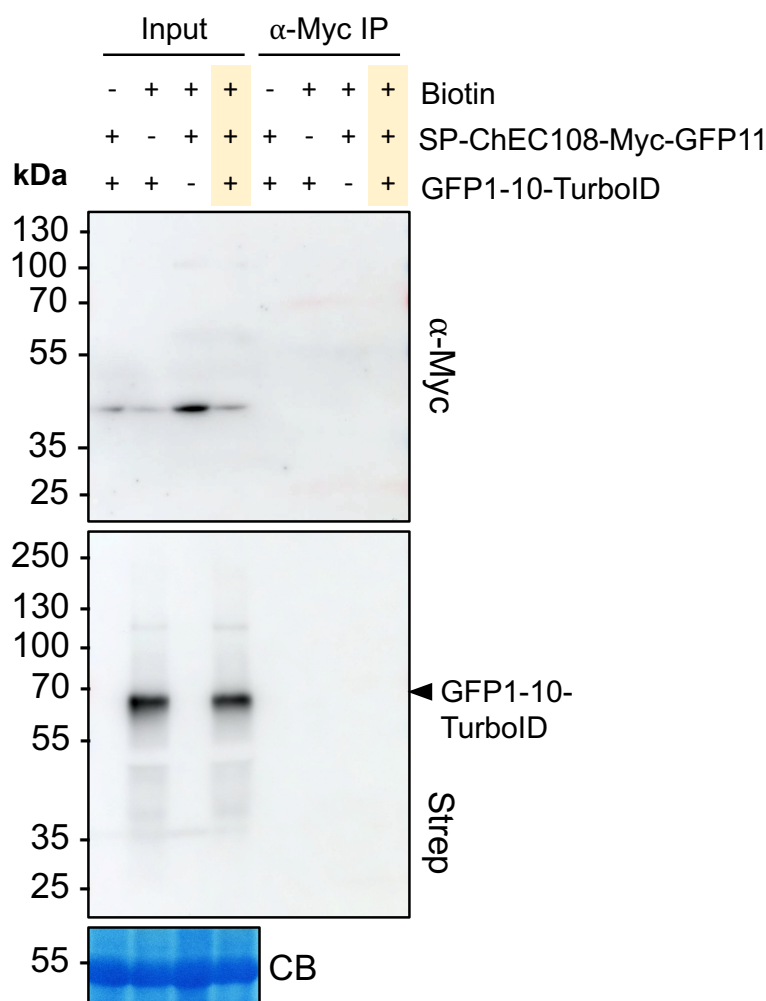


Figure 6.5 – Biotinylated *Ch*-derived ChEC108-Myc-GFP11 could not be detected in Arabidopsis. Proteins were extracted from mature Arabidopsis leaves expressing *GFP1-10-TurboID*, infected with *Ch* expressing *SP-ChEC108-Myc-GFP*, after 3 h biotin (50 μ M) treatment (yellow lanes). Autobiotinylated GFP1-10-TurboID (63 kDa), alongside other biotinylated proteins (smears), were observed when input fractions were probed with a streptavidin (strep)-HRP conjugate. Considering background levels of biotinylation, mock treatment (H_2O in place of biotin), or Col-0 leaves were used as negative controls. Infection with WT² *Ch* was performed as a negative control for specific Myc bands. When probed with α -Myc antibodies, a non-specific band was observed in all input fractions. Despite enriching for Myc-tagged proteins via α -Myc IP, ChEC108-Myc-GFP (28 kDa) or SP-ChEC108-Myc-GFP11 (31 kDa) could not be detected. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit.

Moving forward, I generated Arabidopsis lines to constitutively express 3 $\times$ FLAG-TurboID-HIPP6, 3 $\times$ FLAG-TurboID-HMA1 or 3 $\times$ FLAG-TurboID-HMA2 under regulation of a 35S promoter and HSP terminator. For the majority of subsequent experiments, I chose 3 $\times$ FLAG-TurboID-HMA1 as the preferred bait construct for three reasons: (1) ChEC108-4 $\times$ Myc was stabilised, and its biotinylation easily detectable in the presence of this fusion (Figure 6.6), (2) ChEC108 has a marginally higher affinity for HMA1 over HMA2, according to K_d values calculated via ITC (Table 3.5), and (3) in contrast to those expressing 3 $\times$ FLAG-TurboID-HIPP6, Arabidopsis plants

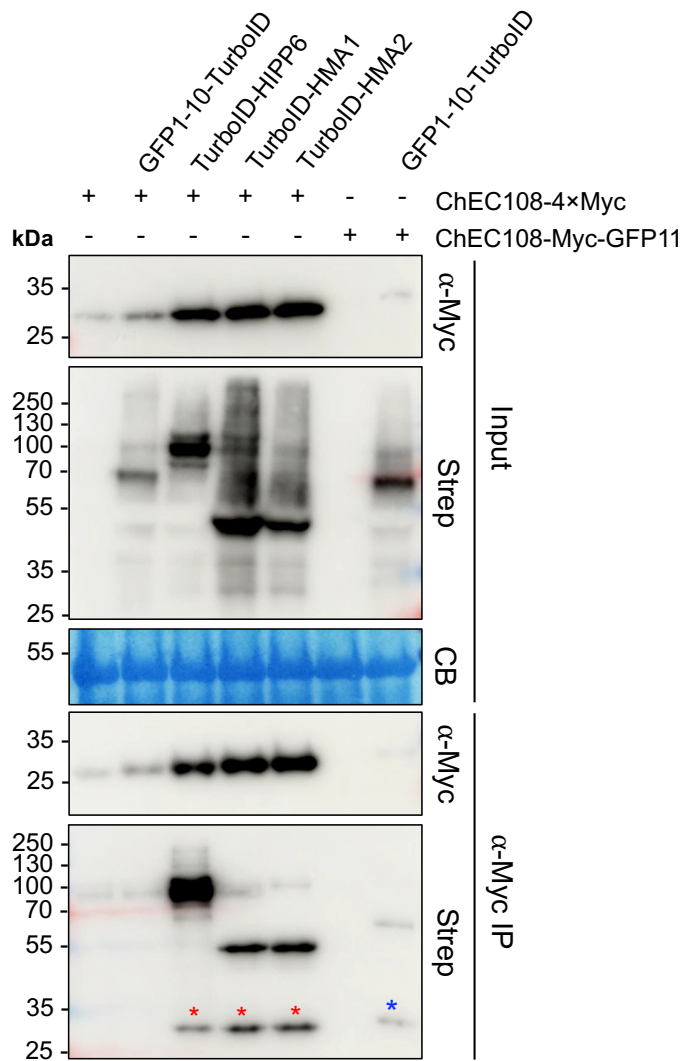


Figure 6.6 – Biotinylated ChEC108 was detected following transient co-expression with TurboID variants in *N. benthamiana*. *ChEC108-4×Myc* (28 kDa) or *ChEC108-Myc-GFP11* (28 kDa) were co-expressed in *N. benthamiana* leaves alongside TurboID fusions: *GFP1-10-TurboID* (63 kDa), *3×FLAG-TurboID-HIPP6* (83 kDa), *3×FLAG-TurboID-HMA1* (49 kDa) or *3×FLAG-TurboID-HMA2* (49 kDa). As a negative control, TurboID was excluded. Leaves were harvested 2 days post-infiltration and incubated with 50 μ M biotin for 21 h. Autobiotinylated TurboID fusions, alongside other biotinylated proteins (smears), were observed when protein extracts were probed with a streptavidin (strep)-HRP conjugate. After enriching for Myc-tagged proteins via α -Myc IP, biotinylated *ChEC108-4×Myc* (*) and *ChEC108-Myc-GFP11* (*) were observed. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit.

expressing *3×FLAG-TurboID-HMA1* did not exhibit stunted growth in a homozygous state, making it easier to cultivate material for infection in large quantities.

To accompany these transgenic *Arabidopsis* lines, I generated a *Ch* strain expressing *SP-ChEC108-4×Myc* under control of the *cos1gpdA* promoter and *trpC* terminator in the WT² background, referred to as *Ch*^{*SP-ChEC108-Myc*}. Alongside, I generated *Ch*^{*ChEC108-Myc*} as negative control, in which *ChEC108-4×Myc* is produced without an N-terminal signal peptide.

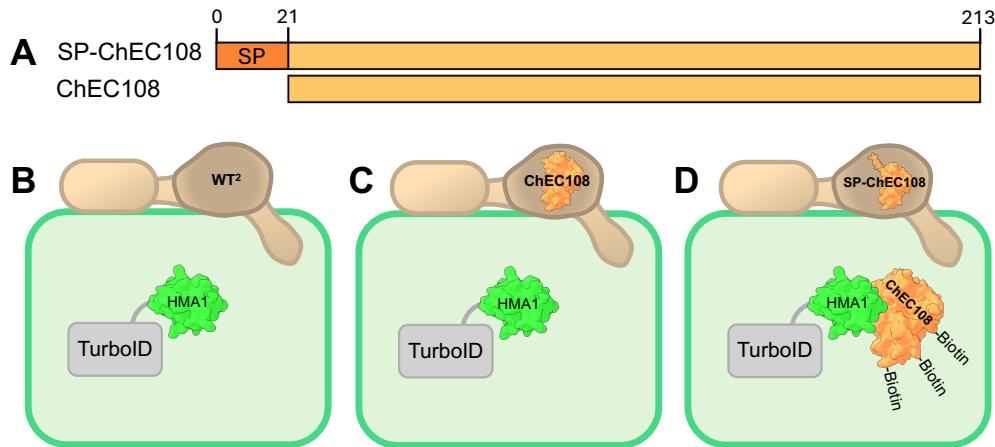


Figure 6.8 – Illustration of the TurboID-HMA1-based approach to detect *Ch*-derived ChEC108 in Arabidopsis cells. **A.** Schematic representation of ChEC108 with and without an N-terminal signal peptide (SP). **B-D.** If 3×FLAG-TurboID-HMA1 were constitutively expressed in Arabidopsis during infection with WT² (A) or *Ch* expressing ChEC108-4×Myc (B), biotinylated ChEC108 should not be detected. However, SP-ChEC108-4×Myc is expected to be secreted from *Ch* and translocate to the Arabidopsis cytosol following SP cleavage. In this scenario (C), ChEC108-HMA1 interaction would facilitate biotinylation of ChEC108 by TurboID, which could be detected via Western blotting.

As a first attempt, I spray-inoculated mature Arabidopsis leaves expressing 3×FLAG-TurboID-HMA1 with *Ch*^{ChEC108-Myc}, *Ch*^{SP-ChEC108-Myc} or H₂O as a negative control, and harvested samples at three time points post-infection: 24, 48 and 93 h, with the aim of capturing the biotrophic phase of *Ch* growth. Total protein was extracted and probed for Myc-tagged and biotinylated proteins, and an α-Myc IP carried out to enrich for ChEC108 fusions. Although autobiotinylated 3×FLAG-TurboID-HMA1 was detected in high abundance, ChEC108 fusions were not detected in input fractions, likely due to the low abundance of fungal material relative to plant material in this setup (Figure 6.9). In the IP fractions, a faint band corresponding to ChEC108-4×Myc was detected at all three time points, whereas (SP-)ChEC108-4×Myc was detected only at 93 hpi (Figure 6.9). Consistent with data presented in Figure 6.7, due to the resolution limits of Western blotting, it was not possible to deduce whether bands observed in *Ch*^{SP-ChEC108-Myc}-infected samples represented ChEC108-4×Myc in the plant apoplast or symplast (post-cleavage of the signal peptide), or full length SP-ChEC108-4×Myc proteins within *Ch* cells. In this scenario at 93 hpi, biotinylation of *Ch*^{SP-ChEC108-Myc}-derived ChEC108-4×Myc (but not *Ch*^{ChEC108-Myc}-derived ChEC108-4×Myc) would support the hypothesis that ChEC108 translocates to Arabidopsis cells during infection. However, when the IP fractions were probed with streptavidin conjugates, only non-specific bands were visible, perhaps because the putative pool of biotinylated ChEC108 was too small to detect.

Following this, I conducted three more independent experiments on mature leaves, using higher concentrations of *Ch* spores in an attempt to increase the proportion of infected cells at the point of harvest. However, in all cases, Myc-tagged ChEC108 fusions could not be

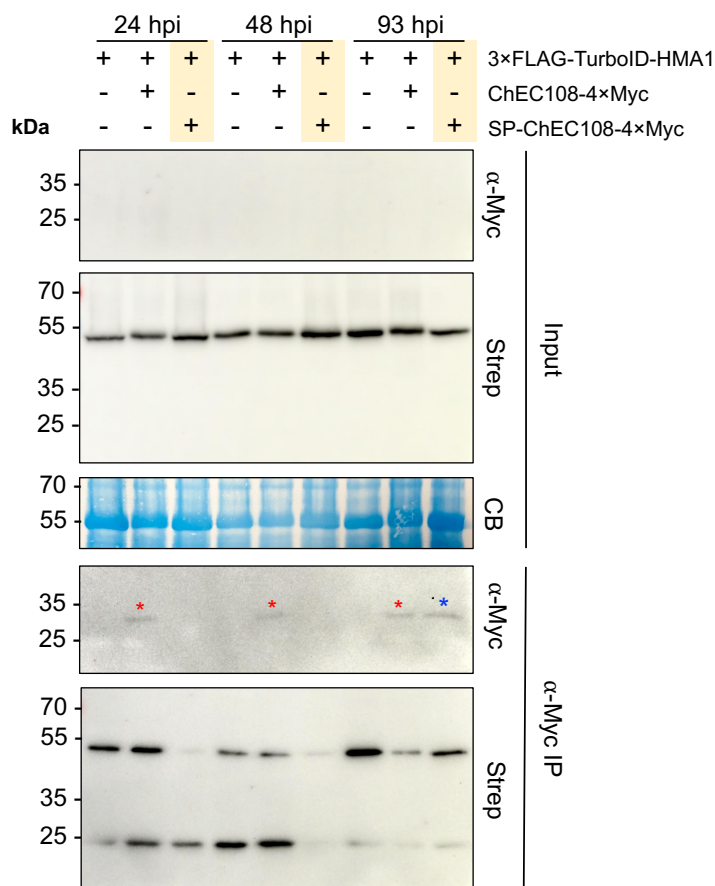


Figure 6.9 – Biotinylated *Ch*-derived ChEC108-4×Myc was not detected in mature *Arabidopsis* leaves. Proteins were extracted from leaves expressing 3×FLAG-TurboID-HMA1 (49 kDa), infected with *Ch* expressing SP-ChEC108-4×Myc (30 kDa), after a 3 h biotin (50 μM) treatment, carried out 24, 48 or 93 hpi (yellow lanes). Autobiotinylated 3×FLAG-TurboID-HMA1 was observed when input fractions were probed with a streptavidin (strep)-HRP conjugate. A mock infection (H₂O in place of *Ch* spores) was performed as a negative control for specific Myc bands, and infection with *Ch* expressing ChEC108-4×Myc (28 kDa) was performed as a negative control for effector secretion. When protein extracts were probed with α-Myc antibodies, ChEC108-4×Myc (*) and (SP-)ChEC108-4×Myc (*) were detected, but only after enriching for Myc-tagged proteins via α-Myc IP. Biotinylation of neither protein could be detected by probing the IP fraction with strep-HRP. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. For experimental details, see Table 6.1 #1.

detected, even in IP fractions. For each attempt, specific modifications to the experimental design and protocols are detailed in Table 6.1. Notably, ChEC108 did not co-immunoprecipitate alongside 3×FLAG-TurboID-HMA1 fusions when α-FLAG IP was conducted in place of α-Myc IP (Table 6.1 #3), suggesting once again that any ChEC108 molecules entering the host cytoplasm must be very low in abundance. As live imaging suggested ChEC108 and HIPP6 predominantly localise to PD in *Arabidopsis* when stably expressed (Figure 5.1D and Figure 5.3B), I tested whether 3×FLAG-TurboID-HIPP6 could serve as a more effective bait construct, as HIPP6-directed PD localisation of TurboID might increase its frequency of encounter with ChEC108 molecules (Table 6.1 #4). However, this experiment was similarly inconclusive due to insufficient accumulation of ChEC108 fusions (Figure 6.10).

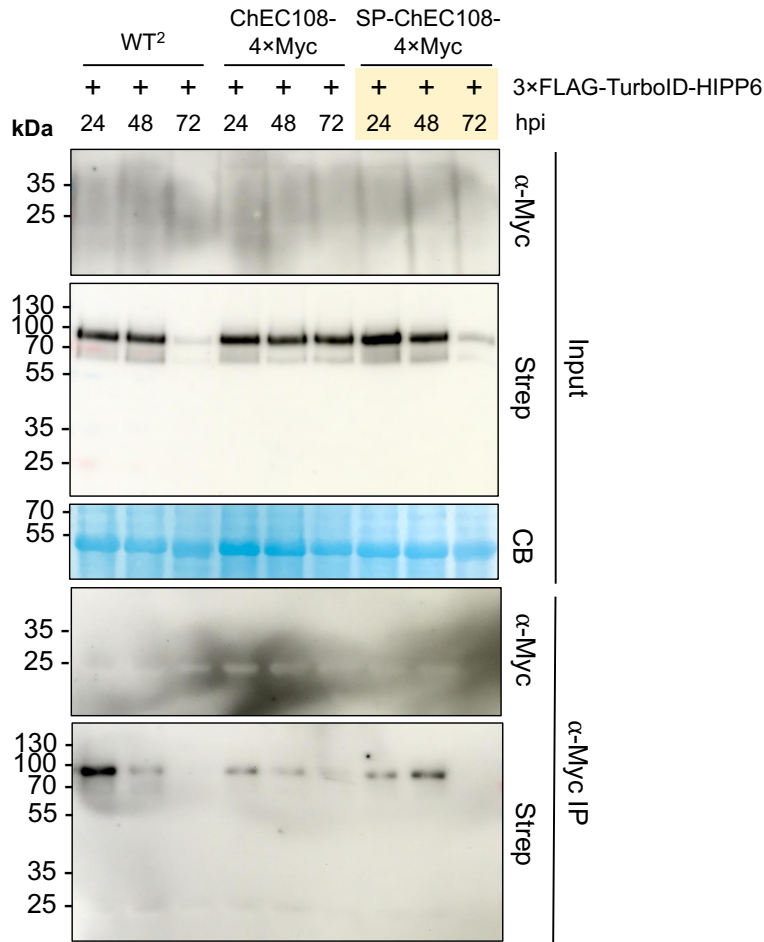


Figure 6.10 – *Ch*-derived ChEC108-4xMyc was not detected in mature *Arabidopsis* leaves expressing *TurboID-HIPP6*. Proteins were extracted from leaves expressing 3xFLAG-TurboID-HIPP6 (83 kDa), infected with *Ch* expressing SP-ChEC108-4xMyc (30 kDa), after ~16 h biotin (50 μM) treatment, carried out 24, 48 or 72 hpi (yellow lanes). Auto-biotinylated 3xFLAG-TurboID-HIPP6 was observed when input fractions were probed with a strep-HRP conjugate. Infection with WT² was performed as a negative control for specific Myc bands, and infection with *Ch* expressing ChEC108-4xMyc (28 kDa) was performed as a negative control for effector secretion. When protein extracts were probed with α-Myc antibodies, neither ChEC108-4xMyc nor (SP-)ChEC108-4xMyc were detected, even after enriching for Myc-tagged proteins via α-Myc IP. Biotinylation of neither protein could be detected by probing the IP fraction with a strep-HRP conjugate. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. For experimental details, see Table 6.1 #4.

Table 6.1 – Attempts to optimise the TurboID-HMA1-based approach to detect *Ch*-derived ChEC108 in Arabidopsis cells. T-HMA1 and T-HIPP6 refer to plants expressing 3×FLAG-TurboID-HMA1 and 3×FLAG-TurboID-HIPP6, respectively. -SP and +SP refer to strains *Ch*^{ChEC108-Myc} and *Ch*^{SP-ChEC108-Myc}, respectively. Plant age is given in days post sowing. hpi = hours post infection, Y = yes, N = no, - = not tested. Notes are listed overleaf.

#	Plant tissue	Plates /sample	Leaves /plate	Plant age (days)	Time (hpi)	Plant lines	<i>Ch</i> strains	Spores/ml	Biotin treatment (h)	ChEC108 detected? (Y/N)	Biotinylated ChEC108 detected? (Y/N)	Notes
1	Mature	1	16-17	45	25, 49, 93	T-HMA1	Mock, -SP, +SP	2.00 x10 ⁶	3	Y	N	a, b
2	Mature	4	16-17	50	72	Col-0, T-HMA1	WT ² , -SP, +SP	3.00 x10 ⁶	24	N	N	b, c
3	Mature	2	16-17	49	50, 72, 92	T-HMA1	-SP, +SP	6.55x10 ⁶	-	N	-	b, d, e
4	Mature	1	16-17	46	24, 48, 72	T-HIPP6	WT ² , -SP, +SP	3.00 x10 ⁶	16	N	N	b, c
5	Seedlings	1	-	11	24, 48, 72	Col-0	WT ² , +SP	3.00 x10 ⁶	-	Y	-	f
6	Seedlings	2	-	15	97	Col-0, T-HMA1	WT ² , -SP, +SP	3.00 x10 ⁶	22	N	N	c
7	Seedlings	4	-	15	50	Col-0, T-HMA1	WT ² , -SP, +SP	3.00 x10 ⁶	17	Y	N	g
8	Seedlings	4 or 8	-	17	50	T-HMA1	WT ² , -SP, +SP	3.00 x10 ⁶	21	Y	N	g, h
9	Seedlings	4	-	19	48	T-HMA1	WT ² , -SP, +SP	4.50 x10 ⁶	16	N	N	d, g
10	Seedlings	20	-	15	49	T-HIPP6	WT ² , +SP	2.73 x10 ⁶	15	N	N	g, i

- ^a Mock indicates leaves were sprayed with H₂O in place of *Ch* spores.
^b Plants were germinated and grown on sterile media, then transplanted to soil as seedlings after 15-18 days.
^c Samples were likely compromised due to severe infection progression.
^d α -FLAG IP was used in place of α -Myc IP to test a co-IP approach for detection of ChEC108-4×Myc.
^e Samples were likely compromised due to an incident with the freeze-dryer.
^f Represents an initial trial to test growth conditions, time points and detectability of ChEC108-4×Myc in infected seedlings. Seedlings were grown on sterile media with 0.8% agar. In attempt #6 onwards, a switch to 2% agar was made to facilitate easier harvesting of seedlings.
^g In contrast to previous attempts, plates of infected seedlings were sealed with micropore tape, which seemed more secure than parafilm, despite being breathable.
^h In an attempt to compensate for differing expression levels of *ChEC108-4×Myc* and *SP-ChEC108-4×Myc* in the *Ch* strains used, 4 plates of seedlings were infected with WT² and -SP strains, while 8 plates were infected with the +SP strain.
ⁱ IP with α -Myc beads took place overnight (~16 h).

With detection of *Ch*-derived ChEC108 in mature leaves proving challenging, I adapted the experimental setup to instead utilise seedling-stage plants (aged 11-19 days post-sowing on sterile media). This switch came with two benefits. Firstly, protein extraction from seedlings is expected to be more efficient in comparison to mature leaves, as reduced lignification of cell walls at this early stage makes them easier to break down. Secondly, a shorter growth period for plant material was preferable when multiple rounds of optimisation were anticipated. Before scaling up to include multiple control treatments, I first tested whether (SP-)ChEC108-4×Myc could be detected on spray-inoculated Col-0 seedlings (Table 6.1 #5). Alongside, WT² inoculations were carried out as negative controls. Although absent from input fractions, (SP-)ChEC108-4×Myc was detected in samples collected 48 and 72 hpi (Figure 6.11), helping inform the timing of seedling harvest in subsequent experiments.

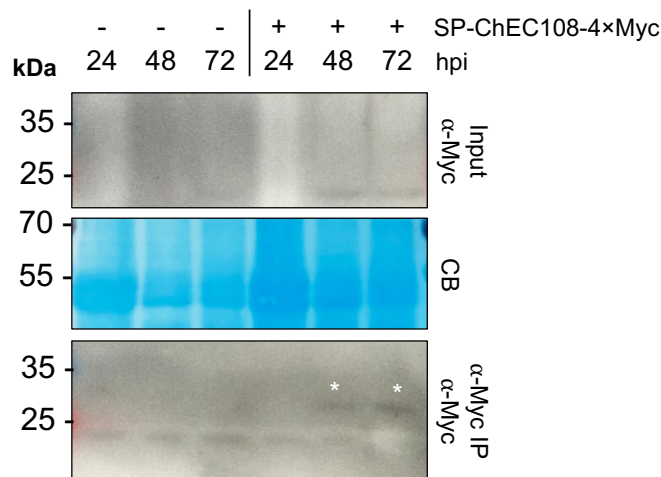


Figure 6.11 – *Ch*-derived (SP-)ChEC108-4×Myc was detected in infected *Arabidopsis* seedlings. Proteins were extracted from Col-0 seedlings, infected with *Ch* expressing *SP-ChEC108-4×Myc* (30 kDa). Seedlings were harvested 24, 48 or 72 hpi. Infection with WT² *Ch* was performed as a negative control for specific Myc bands. When protein extracts were probed with α -Myc antibodies, (SP-)ChEC108-4×Myc was detected at 48 and 72 hpi (*), but only after enriching for Myc-tagged proteins via α -Myc IP. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. For experimental details, see Table 6.1 #5.

Unfortunately, across a further five independent experiments conducted on infected seedlings, biotinylated ChEC108-4×Myc remained elusive. As summarised in Table 6.1 #6-10, modifications to the experimental setup included the use of progressively larger amounts of tissue as input for protein extraction, elevated spore concentrations for inoculation, and lastly, the use of 3×FLAG-TurboID-HIPP6 as an alternative bait construct. On one occasion, ChEC108-4×Myc and (SP-)ChEC108-4×Myc were both detected following α -Myc IP from infected seedlings harvested 50 hpi (Table 6.1 #8). However, when the same fractions were probed with streptavidin conjugates, non-specific smears obscured the detection of any biotinylated proteins of an equivalent molecular weight (Figure 6.12). Ultimately, despite multiple attempts, my efforts to experimentally demonstrate ChEC108 translocation to Arabidopsis using proximity labelling were inconclusive, with variability in the rate of infection progression making it difficult to identify the optimal time point at which to harvest samples.

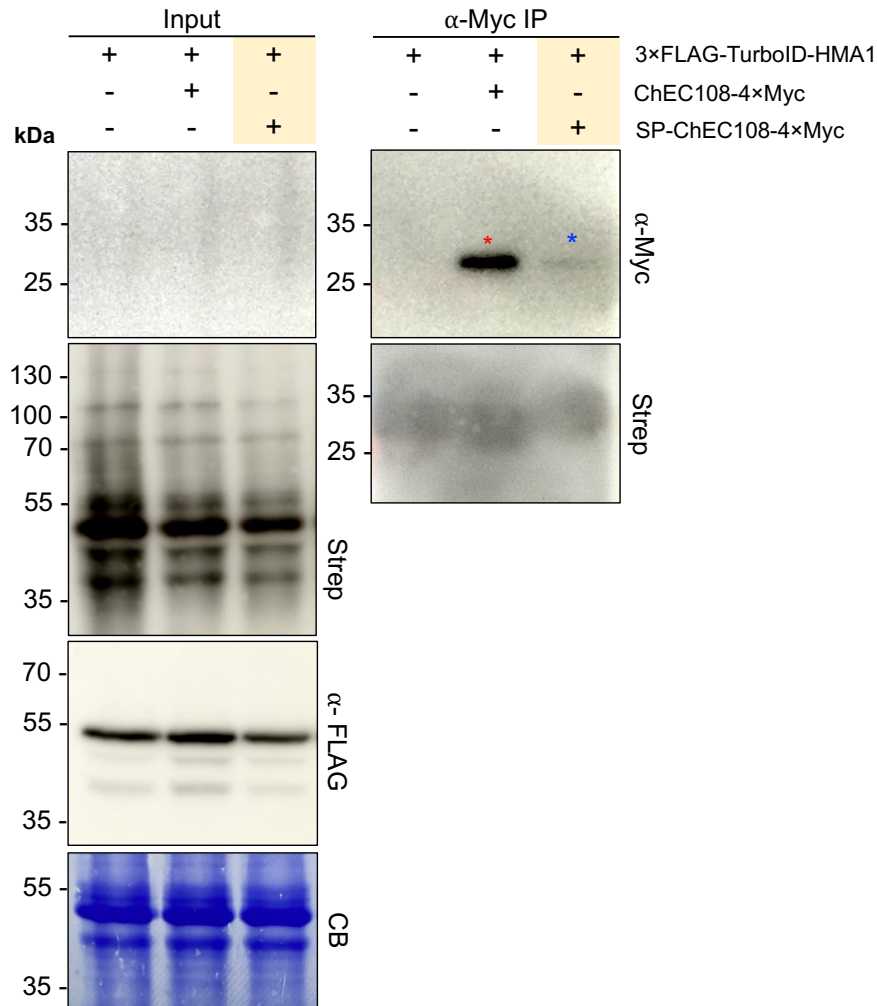


Figure 6.12 – Biotinylated *Ch*-derived ChEC108-4xMyc could not be detected in *Arabidopsis* seedlings. Proteins were extracted from seedlings expressing 3xFLAG-TurboID-HMA1 (49 kDa), infected with *Ch* expressing SP-ChEC108-4xMyc (30 kDa), after a 21 h biotin (100 μ M) treatment carried out 50 hpi (yellow lanes). Autobiotinylated 3xFLAG-TurboID-HMA1 was observed when input fractions were probed with a streptavidin (strep)-HRP conjugate or α -FLAG antibodies. Infection with WT² was performed as a negative control for specific Myc bands, and infection with *Ch* expressing ChEC108-4xMyc (28 kDa) was performed as a negative control for effector secretion. When protein extracts were probed with α -Myc antibodies, ChEC108-4xMyc (*) and (SP-)ChEC108-4xMyc (*) were detected, but only after enriching for Myc-tagged proteins via α -Myc IP. Biotinylation of neither protein could be detected by probing the IP fraction with strep-HRP. Total protein loading is shown by Coomassie Blue (CB) staining of the Rubisco large subunit. For experimental details, see Table 6.1 #8.

6.2.3 Attempts to engineer ChEC108 recognition by Pik NLRs were unsuccessful

In search of an alternative strategy to verify ChEC108 effector function, I turned to NLR biology. As introduced in 6.1.4, the Pik-1 NLR chassis has been previously employed to engineer recognition of non-cognate effectors via mutation or full exchange of the integrated HMA domain. Expressed in *O. sativa*, either natively or via a transgene, the Pik-1/Pik-2 pair confer robust resistance to pathogens carrying corresponding effectors (Kanzaki et al., 2012; Maidment et al., 2023; Varden et al., 2019). In the context of Arabidopsis infection, I thus hypothesised that a strong resistance phenotype resulting from effector recognition by Pik NLRs could serve as a robust readout to indicate the presence of ChEC108 in planta. This idea is founded upon two major assumptions: (1) a variant of Pik-1 can be designed to elicit ChEC108-dependent immune signalling, and (2) when stably expressed, this engineered Pik-1 sensor can cooperate with Pik-2 to protect transgenic Arabidopsis against *Ch*. To address the first assumption, I sought to engineer ChEC108 recognition by Pik-1 using a domain swapping approach.

Successful engineering of Pik-1 to confer novel or expanded recognition capabilities often requires circumventing receptor autoactivity, that is, constitutive immune activation in the absence of cognate effectors, which frequently occurs following modification of NLR architectures (Kourelis et al., 2023; Maidment et al., 2023). However, extensive testing by Bentham et al. (2023) demonstrated that autoactivity can sometimes be attenuated by pairing a modified Pikm-1 allelic variant with Pikp-2, without compromising effector recognition. Demonstrating the utility of this helper mis-matching strategy, Zdrzałek et al. (2024) successfully engineered Pwl2 recognition by Pikm-1^{OsHIPP43} in combination with Pikp-2, despite Pikm-1^{OsHIPP43} exhibiting autoactivity in the presence of the corresponding Pikm-2 helper. Thus, to avoid triggering immune responses in the absence of ChEC108, which would be detrimental to any future transgenic Arabidopsis lines expressing these NLRs, I decided to similarly mis-match a modified 'm' variant of Pik-1 with the 'p' variant of Pik-2.

Exploiting the discovery of HIPP6 as a HMA domain-containing host target of ChEC108, I integrated HMA1 (Gly22-Glu98 inclusive) and HMA2 (Lys152-Lys223 inclusive) into the Pikm-1 chassis in place of the native HMA domain. This was made possible using a custom level 0 Golden Gate backbone, designed by Dr Adam Bentham and Mark Youles at TSLSynBio. The resulting chimeric NLR coding sequences, referred to hereafter as *Pikm-1*^{HMA1} or *Pikm-1*^{HMA2}, were assembled into cassettes for their independent expression or co-expression with *Pikp-2* (Figure 6.13). Multi-gene constructs were assembled to express *Pikm-1* variants and *Pikp-2* under the regulation of different promoters and terminators: 35S for *Pikm-1* and *A. tumefaciens mannopine synthase (mas)* for *Pikp-2*, with C-terminal epitope tags: 6×His-3×FLAG

(Hellfire, or HF) for Pikm-1 and 6×hemagglutinin (HA) for Pikip-2. Importantly, these tags did not impede Pik NLR signalling in previous engineering schemes (Bialas et al., 2021; Maidment et al., 2023; Zdrzałek et al., 2024). Despite being dissimilar in primary sequence (Figure 6.14A), HMA1 and HMA2 share a highly similar structure to the native HMA domain of Pikm-1 (Figure 6.14B-C), decreasing the likelihood that their integration will compromise NLR function. As I previously showed that HMA1 or HMA2 are alone sufficient for ChEC108 binding (Chapter 3), I hypothesised that ChEC108 would be capable of activating both Pikm-1^{HMA1} and Pikm-1^{HMA2} sensors, triggering a hypersensitive response in the presence of Pikip-2.

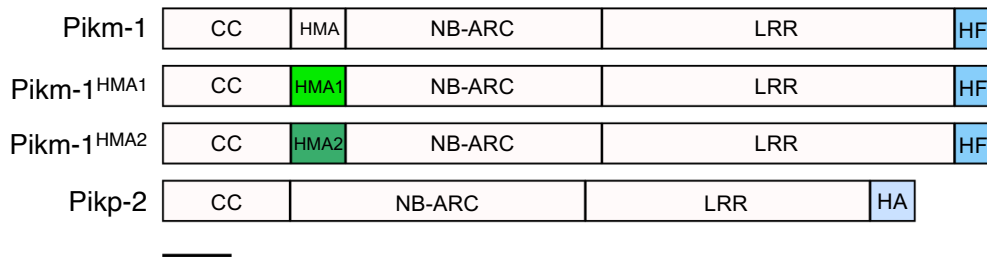


Figure 6.13 – Chimeric NLRs designed to engineer ChEC108 recognition. Native Pik domains are shown in white. C-terminal epitope tags are shown in blue. Integrated HIPP6 HMA domains are shown in green. HF = Hellfire, HA = 6×hemagglutinin. Scale bar = 100 amino acids.

Before proceeding to generate transgenic Arabidopsis lines, I assessed the ability of Pikm-1^{HMA1} and Pikm-1^{HMA2} to specifically recognise and respond to ChEC108 when transiently expressed in *N. benthamiana* - a system widely employed to observe and quantify NLR-mediated cell death responses in a high throughput manner (De la Concepcion et al., 2019, 2018, 2021; Maqbool et al., 2015; Varden et al., 2019; Xi et al., 2021). The two chimeric sensors were expressed alongside Pikip-2 and ChEC108 as a test for activity, or alongside an empty vector (EV) to test for autoactivity. Pikm-1^{OsHIPP43} was expressed alongside Pikip-2 and Pwl2 as a positive control (Zdrzałek et al., 2024). Alongside ChEC108 or another non-recognised *M. oryzae* effector, AVR-PikD, the Pikm-1^{OsHIPP43}/Pikip-2 pair served as a negative control. Consistent with previous tests for Pik NLR activation in this system, all effectors were expressed via an *AtUBQ10* promoter and with C-terminal 4×Myc tags. Surprisingly, despite using the mis-matched Pikm1/Pikip-2 pair, both Pikm-1^{HMA1} and Pikm-1^{HMA2} initiated a strong cell death response in the absence of ChEC108, as evidenced by autofluorescence within leaf zones infiltrated with these construct combinations (Figure 6.15A-B). A lack of fluorescence in leaf zones lacking Pikip-2 suggests the aforementioned responses are a result of autoactivation of the Pik pair, and not due to unconventional initiation of cell death by engineered Pikm-1 operating as a singleton (Figure 6.15C). With the end goal of achieving specific ChEC108-dependent cell death activation, methods to eliminate such autoactivity were trialled with the help of Michael Henderson, an undergraduate student at the University of East Anglia.

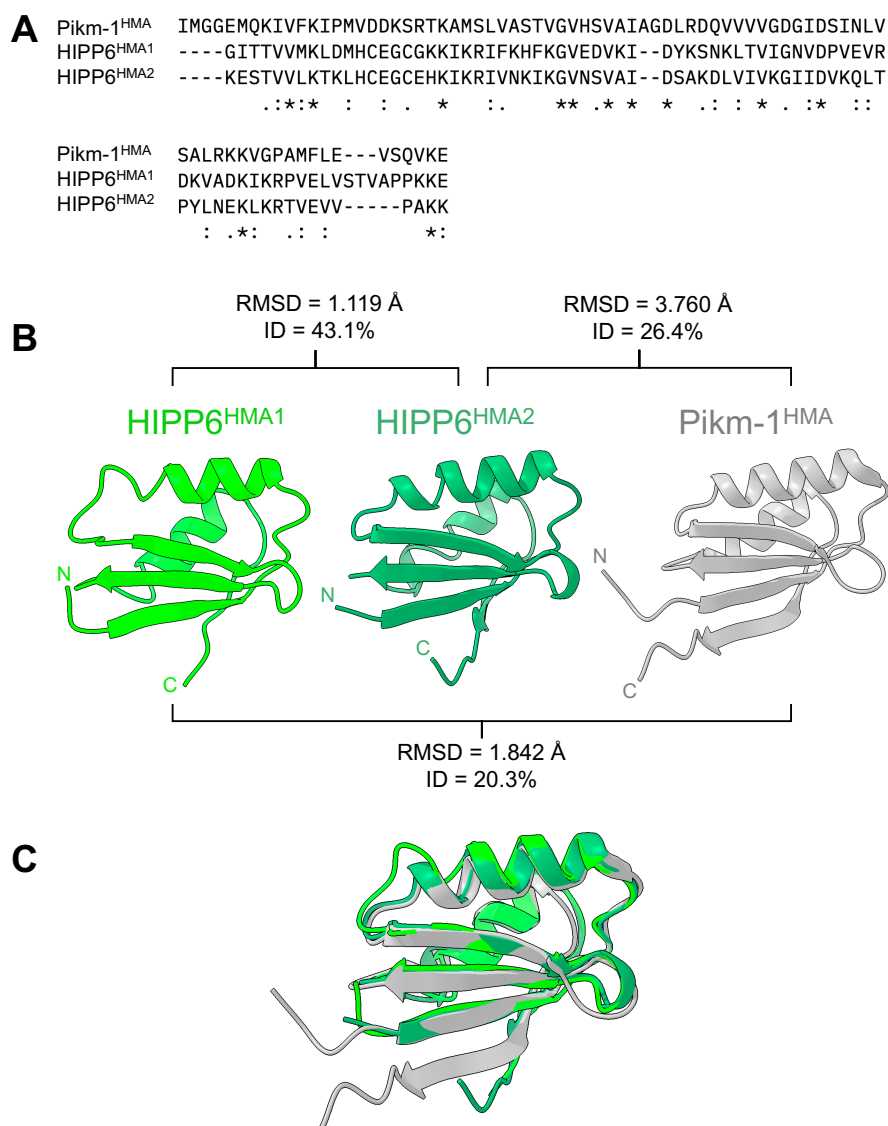


Figure 6.14 – Sequence and structural alignment of the Pikm-1 HMA domain and HIPP6 HMA domains. **A.** Primary sequence alignment generated and annotated by [MUSCLE v.3.8](#) ([Edgar, 2004](#)). Asterisks (*) indicate fully conserved residues; colons (:) indicate residues of strongly similar properties; full stop (.) indicates residues of weakly similar properties. **B.** Crystal structures of HMA1, HMA2 (see [3.2.8](#)) and the Pikm-1 HMA domain (6FU9) ([De la Concepcion et al., 2018](#)). Similarity between the three domains are indicated by root mean square deviation (RMSD) (Å, considering all C α pairs) and percentage sequence identity (ID). **C.** Overlay of the three structures shown in B, performed by ChimeraX using the matchmaker command.

In the chimeras tested above, it is possible that integration of HMA1 or HMA2 introduced steric clashes and/or disrupted Pikm-1 confirmation to such an extent that the protein became locked in an active conformation. Careful positioning of non-native integrated domains relative to the adjacent CC and NR-ARC domains may therefore be necessary to retain proper Pikm-1 function. In an attempt to alleviate Pikm-1^{HMA1} and Pikm-1^{HMA2} autoactivity, HMA1 and HMA2 were cloned into a modified Pikm-1 chassis with extended (ex) integrated domain

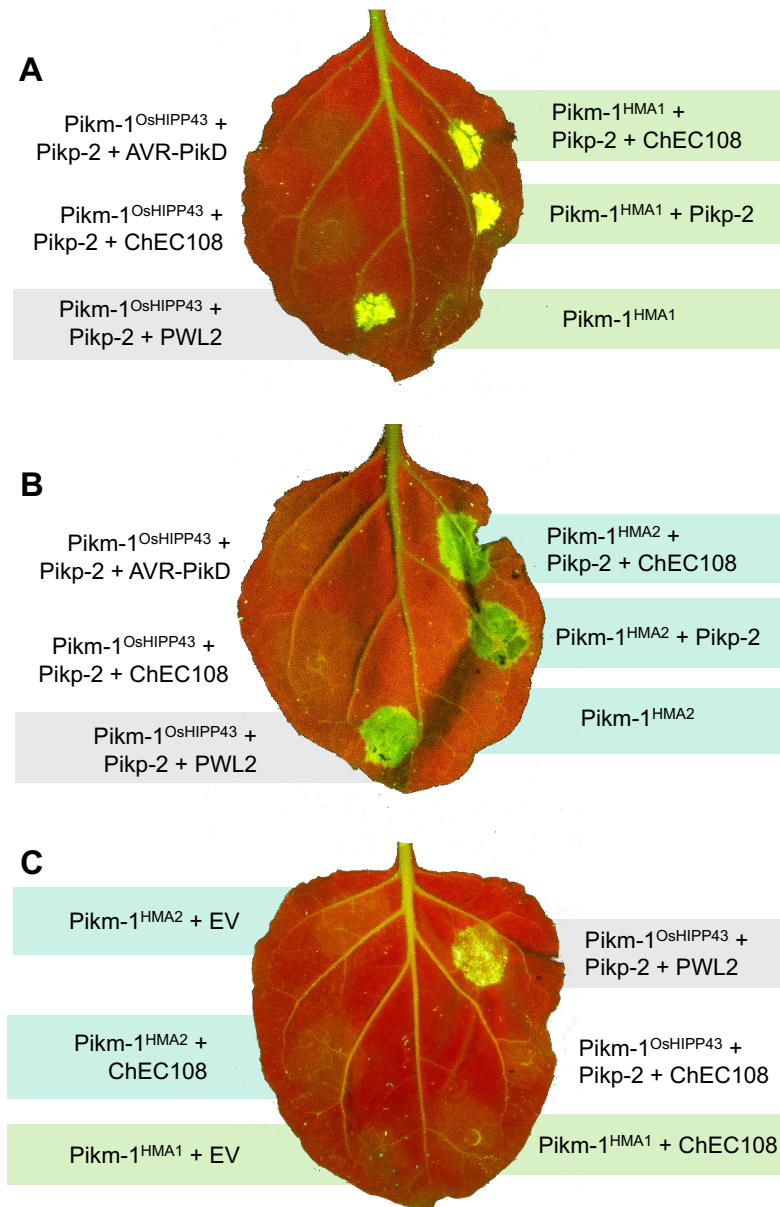


Figure 6.15 – Integration of HMA1 or HMA2 into Pikm-1 triggers Pikp-2-dependent autoactivity. A-C. Indicated proteins were transiently expressed in discrete regions of *N. benthamiana* leaves via agroinfiltration. Each leaf is representative of $n = 6$, taken from 3 plants, photographed 5 days post-infiltration under UV to reveal autofluorescence as a consequence of cell death. Zones relating to HMA1 and HMA2 are labelled in green and teal, respectively. Positive and negative control zones are labelled in grey and white, respectively. Conducted with help from Michael Henderson.

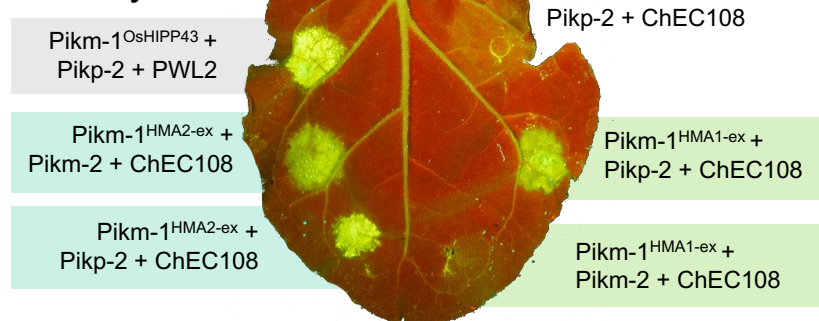
boundaries, assembled by Mark Youles, giving rise to $\text{Pikm-1}^{\text{HMA1-ex}}$ and $\text{Pikm-1}^{\text{HMA2-ex}}$. In this chassis, the addition of six native HMA-derived amino acids either side of the integrated domain (Figure 6.16A) are hypothesised to confer enhanced flexibility to the NLR structure. Also, to investigate the unlikely but non-zero possibility that allelic mis-matching underpinned the autoactivity observed in Figure 6.15, $\text{Pikm-1}^{\text{HMA1-ex}}$ and $\text{Pikm-1}^{\text{HMA2-ex}}$ were co-expressed alongside their corresponding helper, *Pikp-2*. Unfortunately, neither the extension of HMA

domain boundaries nor allelic matching were sufficient to alleviate autoactivity of the chimeras. Constitutive cell death was observed upon co-expression of *Pikm-1*^{HMA1-ex} or *Pikm-1*^{HMA2-ex} with *Pikp-2*, and upon *Pikm-1*^{HMA2-ex} co-expression with *Pikm-2* (Figure 6.16B-C). Although *Pikm-1*^{HMA1-ex} did not display autoactivity in the presence of *Pikm-2* (Figure 6.16C), the similar lack of cell death observed in the presence of ChEC108 suggests this NLR pair is unable to perceive the effector (Figure 6.16B).

A *Pikm-1* HMA domain boundaries

Original	174	RKRKPHVPLR-----	HMA	-----DVKEITAMLA
Extended (ex)	174	RKRKPHVPLRIMGGEM	HMA	ASQVKE DVKEITAMLA

B Activity tests



Auto-activity tests

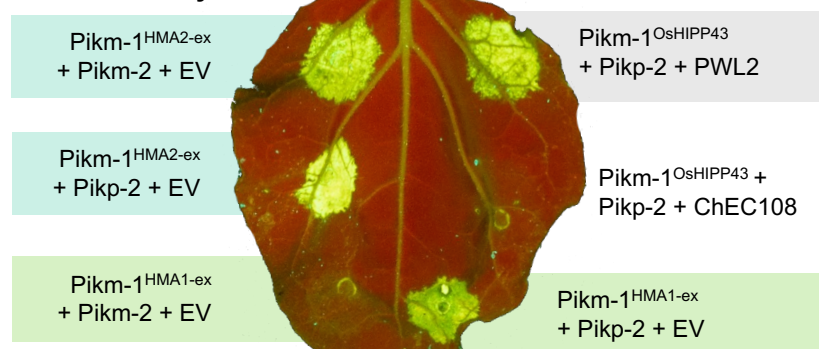


Figure 6.16 – Autoactivity of *Pikm-1*^{HMA1} and *Pikm-1*^{HMA2} could not be alleviated by extension of the HMA domain boundaries or by co-expression with *Pikm-2*. A. Domain boundaries either side of the HMA domain in the original and extended (ex) *Pikm-1* chassis. B. Indicated proteins were transiently expressed in discrete regions of *N. benthamiana* leaves via agroinfiltration. Tests for activity in the presence and absence of ChEC108 are displayed on the upper and lower leaf, respectively. Each leaf is representative of n = 6, taken from 3 plants, photographed 5 days post-infiltration under UV to reveal autofluorescence as a consequence of cell death. Zones relating to HMA1 and HMA2 are labelled in green and teal, respectively. Positive and negative control zones are labelled in grey and white, respectively. Conducted with help from Michael Henderson.

Given that the integrated domain of the Pik system is responsible for determining both effector recognition specificity and sensor-helper compatibility (Bentham et al., 2023), mutation within the HMA domain itself could be a promising strategy to remove autoactivity from chimeras. As part of an evolutionary study of Pik NLRs, Białas et al. (2021) generated an autoactive sensor with an integrated ancestral HMA domain (ancHMA), referred to as Pikp-1:ancHMA. After substituting residues of ancHMA for equivalent residues in the native Pikp-1 HMA domain, a seven-amino-acid region corresponding to the metal-binding loop (mbl) was found to underpin autoactivity: PMASDK (residues 196-201, inclusive) and His251. When these residues were exchanged for the equivalent Pikp-1 mbl sequence (AMEGNN and D), Pikp-1:ancHMA autoactivity in the presence of Pikp-2 was removed (Białas et al., 2021). Also, Maidment et al. (2023) successfully abolished Pikp-2-dependent autoactivity of the Pikp-1^{OsHIPP19} chimera by exchanging seven adjacent residues in the mbl of the integrated HMA domain (SMPCEKS) for those of Pikp-1 (AMEGNNC, residues 196-202, inclusive). Mimicking this strategy, I sought to test whether replacement of Pikm-1^{HMA1} and Pikm-1^{HMA2} mbl regions with the AMEGNNC sequence might similarly remove autoactivity in the presence of Pikp-2.

Due to concern that mutation of residues involved in metal ion coordination could compromise ChEC108 binding (see 3.2.8 and 3.2.10), additional mbl variants were designed, as summarised in Figure 6.17A and Figure 6.18A. Firstly, mbl-C variants included mbl amino acids from Pikp-1, while retaining the CXXC metal-binding motif of HMA1 and HMA2. Secondly, mbl-43 variants were designed to introduce mbl amino acids from the OsHIPP43 HMA domain, which harbours a CXXC motif yet did not trigger autoactivity when integrated into the Pikm-1 chassis in a previous study (Zdrzałek et al., 2024). Despite these efforts, all tested *Pikm-1*^{HMA-mbl} mutants triggered cell death when expressed in *N. benthamiana* alongside *Pikp-2* and either *ChEC108* or an EV control, although the response induced by *Pikm-1*^{HMA2-mbl-C} was notably weaker (Figure 6.17B-C and Figure 6.18B-C). This suggests that, unlike the examples cited above, the mbl region does not underpin autoactivity in *Pikm-1*^{HMA1} and *Pikm-1*^{HMA2} chimeras.

Seeking alternative strategies to alleviate autoactivity, I turned again to HMA domain boundary modification in the *Pikm-1* chassis. Instead of introducing excessive flexibility by extending the domain boundaries on both sides (Figure 6.16A), I sought to closely mimic the native *Pikm-1* integrated domain. Primary sequence alignment revealed four N-terminal amino acids in the *Pikm-1* HMA domain (IMGG) that were missing equivalents in the HMA1 and HMA2 integrated sequences (Figure 6.14A). While examination of a published crystal structure suggested this IMGG region is not part of secondary structures making up the *Pikm-1* HMA fold (De la Concepcion et al., 2018, Figure 6.14B), it could be crucial for correctly positioning the domain within the wider NLR structure. *Pikm-1*^{HMA1-IMGG} and *Pikm-1*^{HMA2-IMGG} mutants were thus designed and tested for ChEC108-dependent activity in *N. benthamiana* (Figure 6.19A,

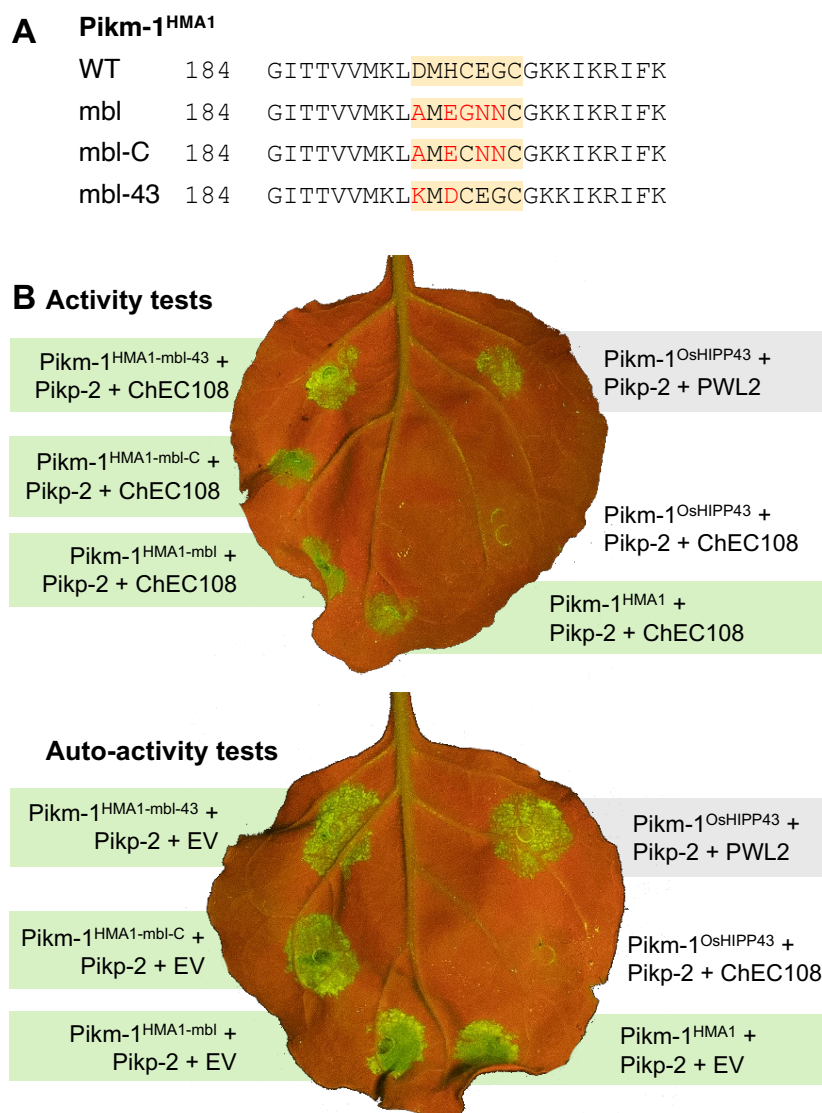


Figure 6.17 – The metal-binding loop (mb1) sequence does not underpin autoactivity of Pikm-1^{HMA1}. **A.** Substitutions within the mb1 region (yellow) tested in the Pikm-1^{HMA1} chimera. Mutated residues are coloured red. WT indicates the wild-type sequence of integrated HMA1. **B.** Indicated proteins were transiently expressed in discrete regions of *N. benthamiana* leaves via agroinfiltration. Tests for activity in the presence and absence of ChEC108 are displayed on the upper and lower leaf, respectively. Each leaf is representative of $n = 6$, taken from 3 plants, photographed 5 days post-infiltration under UV to reveal autofluorescence as a consequence of cell death. Positive and negative control zones are labelled grey and white, respectively. Conducted with help from Michael Henderson.

C, D). Although both chimeras exhibited autoactivity when co-expressed with *Pikp-2* and the EV control (Figure 6.19D), only Pikm-1^{HMA1}-IMGG triggered cell death in the presence of ChEC108 (Figure 6.19C), hinting at potential effector-mediated inhibition of Pikm-1^{HMA2}-IMGG activation. Regardless, the addition of Pikm-1-derived IMGG at the N-terminus of the integrated HMA boundary was insufficient to alleviate Pikm-1^{HMA1} and Pikm-1^{HMA2} autoactivity.

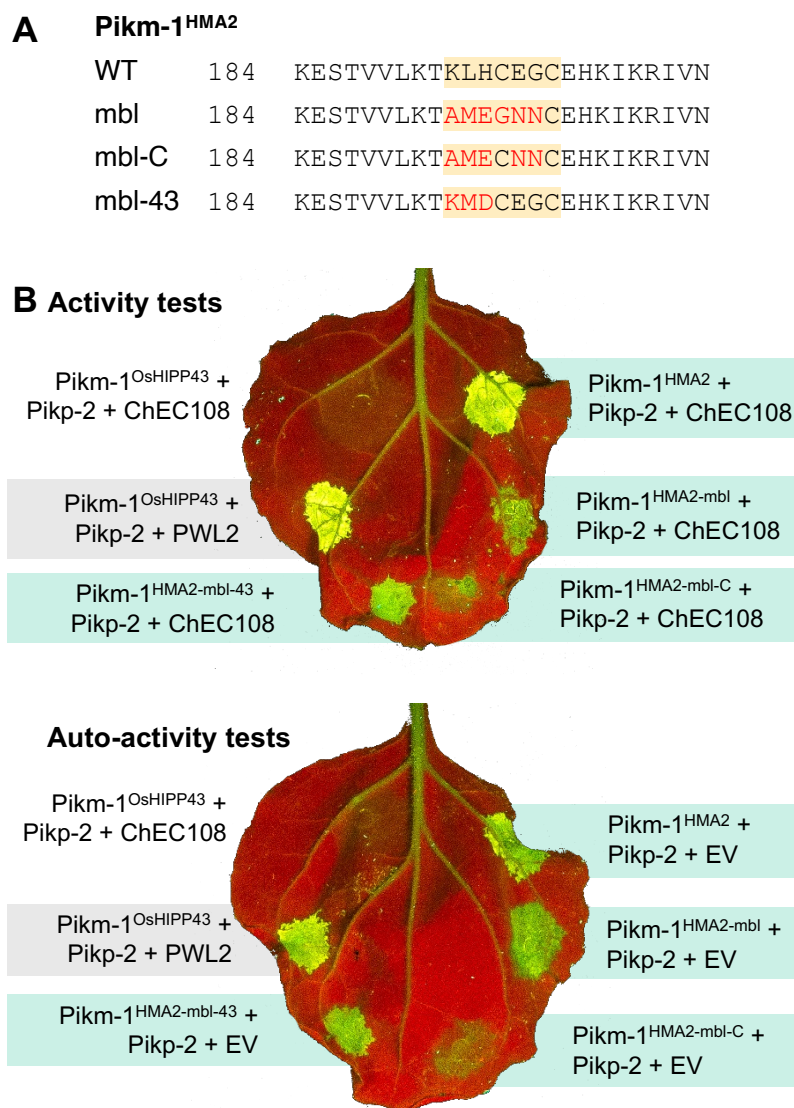


Figure 6.18 – The metal-binding loop (mb1) sequence does not underpin autoactivity of Pikm-1^{HMA2}. A. Substitutions within the mbl region (yellow) tested in the Pikm-1^{HMA2} chimera. Mutated residues are coloured red. WT indicates the wild-type sequence of integrated HMA2. B. Indicated proteins were transiently expressed in discrete regions of *N. benthamiana* leaves via agroinfiltration. Tests for activity in the presence and absence of ChEC108 are displayed on the upper and lower leaf, respectively. Each leaf is representative of $n = 12$, taken from 3 plants, photographed 5 days post-infiltration under UV to reveal autofluorescence as a consequence of cell death. Positive and negative control zones are labelled grey and white, respectively. Conducted with help from Michael Henderson.

Despite the high similarity of HMA1, HMA2 and Pikm-1^{HMA} folds (Figure 6.14B-C), it remains possible that subtle structural dissimilarities or unfavourable side chain positions underpin the autoactivity of all Pikm-1 chimeras tested thus far. As an alternative to domain swapping, I hypothesised that ChEC108 recognition could be engineered by strategic mutation of the already non-autoactive Pikm-1 integrated domain, using knowledge of ChEC108-HMA binding interfaces presented in Chapter 3. A similar strategy involving six amino acid substitutions, guided by the structure of AVR-PikF with the OshIPP19 HMA domain

(Maidment et al., 2021), was used to engineer recognition of AVR-Pik effectors by the RGA5 HMA domain as part of a Pikm-1 chimera (Bentham et al., 2023). Given that metal-binding residues are critical for ChEC108-HMA interaction, I generated the Pikm-1^{mb1-HMA2} mutant, in which seven amino acids at the mbl region of Pikm-1 (PMVDDKS) were mutated to equivalent residues in HMA2 (KLHCEGC) (Figure 6.19B), including residues His163, Cys164 and Cys167 required for Zn²⁺ coordination in the ChEC108-HMA2 crystal structure (Figure 3.20B). Alongside, I resurfaced (RS) the Pikm-1 HMA domain to incorporate residues from HMA2 that form contacts with ChEC108 when in complex. In total, ten substitutions were made, giving rise to the Pikm-1^{HMA-RS} mutant (Figure 6.19B).

Firstly, native Pikm-1 residues Val199 to Ser203 (inclusive, reading VDDKS) were replaced with residues His163 to Cys167 from HIP6 (inclusive, reading HCEGC) in the hopes of generating a functional metal-binding loop capable of coordinating a metal ion alongside Asp164 from ChEC108 (Figure 3.21D). Secondly, Thr205 was substituted for His169 from HIP6 as the latter was shown to form pi-pi interactions with the side chain of ChEC108 residue Trp78, and also form a hydrogen bond with the effector's peptide backbone (Figure 3.21B). Thirdly, Ser209 and Ser213 were substituted for Arg173 and Lys177 from HIP6, respectively, with the goal of establishing more hydrogen bonds with the ChEC108 peptide backbone. Arg173 was also shown to form salt bridges with ChEC108 residues Asp22 and Glu90, so its inclusion could strengthen the hypothetical ChEC108-HMA^{RS} interface (Figure 3.21B). Finally, Gly251 and Pro252 were substituted for Lys213 and Arg214 from HIP6. Lys213 forms a hydrogen bond with the ChEC108 peptide backbone (Figure 3.21C), while Arg214 is oriented towards the metal-binding site, suggesting it could be important for positioning nearby residues in this region (Figure 6.20).

Despite these efforts, neither Pikm-1^{mb1-HMA2} or Pikm-1^{HMA-RS} triggered ChEC108-dependent cell death in *N. benthamiana*, suggesting ChEC108 binding (or at least, binding of sufficiently high affinity) was not achieved (Figure 6.19C-D). It appears that further Pikm-1 resurfacing would be required to engineer an NLR pair capable of inducing ChEC108-dependent cell death. In the absence of a functioning synthetic NLR to confer recognition, my ultimate goal of testing for ChEC108-induced resistance during *Ch* infection of Arabidopsis was unfortunately unachievable.

A Pikm-1 HMA domain boundaries

Original	174	RKRKPHVPLR----	HMA	DVKEITAMLA
IMGG	174	RKRKPHVPLRIMGG	HMA	DVKEITAMLA

B Pikm-1 HMA domain

Original	184	IMGGEMQKIVFKIPMVDDKSRTKAMSLVASTVGVHSAIA
	224	GDLRDQVVVVG DGIDSINLV SALRKKVGPAMFLEVSQVKE
mb1-HMA2	184	IMGGEMQKIVFKIKLHCEGCR TKAMSLVASTVGVHSAIA
	224	GDLRDQVVVVG DGIDSINLV SALRKKVGPAMFLEVSQVKE
HMA-RS	184	IMGGEMQKIVFKIPMHCEGCRHKAMRLVAKTVGVHSAIA
	224	GDLRDQVVVVG DGIDSINLV SALRKKVKRAMFLEVSQVKE

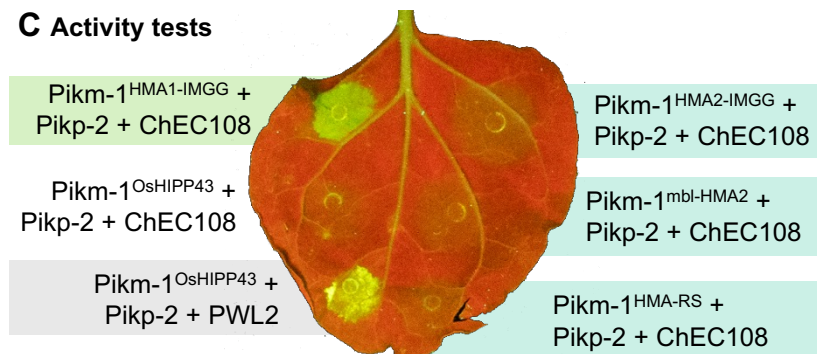
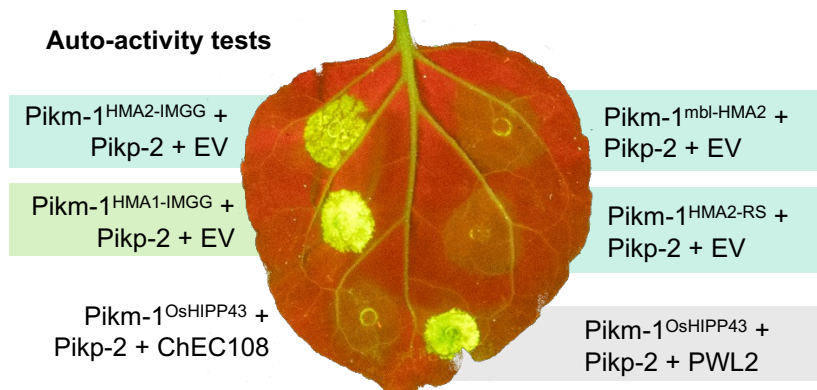
C Activity tests**Auto-activity tests**

Figure 6.19 – Autoactivity of Pikm-1^{HMA1} and Pikm-1^{HMA2} could not be alleviated by subtle modification of HMA domain boundaries, and resurfacing of the Pikm-1 HMA domain was insufficient to confer ChEC108 recognition. **A.** Addition of IMGG to N-terminal HMA domain boundaries in Pikm-1^{HMA1} and Pikm-1^{HMA2}. **B.** Mutation of native Pikm-1 HMA to resemble HMA2 specifically at the metal-binding loop (mb1), or broadly across regions in contact with ChEC108, indicated by RS for ‘resurfaced’. **C.** Indicated proteins were transiently expressed in discrete regions of *N. benthamiana* leaves via agroinfiltration. Tests for activity in the presence and absence of ChEC108 are displayed on the upper and lower leaf, respectively. Each leaf is representative of $n = 6$, taken from 3 plants, photographed 5 days post-infiltration under UV to reveal autofluorescence as a consequence of cell death. Zones relating to HMA1 and HMA2 are labelled in green and teal, respectively. Positive and negative control zones are labelled grey and white, respectively. Conducted with help from Michael Henderson.

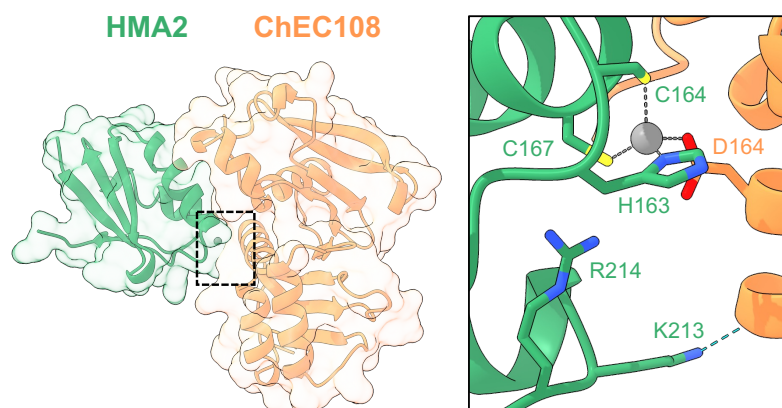


Figure 6.20 – Arg214 from HMA2 might help stabilise the ChEC108-HMA2 metal-binding site. In a region of the ChEC108-HMA2 crystal structure (indicated by a black box), Lys213 from HMA2 forms a hydrogen bond with the ChEC108 main chain, and Arg214 is oriented towards the metal-binding site involving ChEC108 Asp164 and HMA2 residues: His163, Cys164 and Cys167. Intermolecular bonds are represented as dashed lines, with hydrogen bonds coloured cyan and dative covalent bonds between amino acid side chains and the metal ion (sphere) coloured grey.

6.2.4 ChEC108 localisation in *M. oryzae* and *O. sativa* host cells resembles that of a cytoplasmic effector

In the absence of a functioning assay to directly demonstrate ChEC108 translocation in the native *Ch*-*Arabidopsis* pathosystem (6.2.1 and 6.2.2), and with indirect detection via NLR activation unfeasible (6.2.3), I set out to corroborate the annotation of ChEC108 as a secreted protein using an alternative expression system. Previously, tests for signal peptide function in yeast (*S. cerevisiae*) invertase assays have been used to instill confidence in the annotation of many phytopathogen effectors (Chen et al., 2022; Wang et al., 2019a; Zhang et al., 2024a). Inspired by live imaging studies of effectors described in 6.1.1, I reasoned that *M. oryzae* - an appressorium-forming phytopathogen with a lifestyle more closely resembling that of *Ch* than *S. cerevisiae* - could serve as a preferable heterologous system in which to test for ChEC108 secretion.

To generate transgenic *M. oryzae* strains expressing fluorescently-tagged ChEC108, I collaborated with Jave Bautista and Weibin Ma from the laboratory of Prof. Nick Talbot, The Sainsbury Laboratory, UK. I began by cloning the *SP-ChEC108-eGFP* and *ChEC108-eGFP* coding sequences into a custom vector for *M. oryzae* transformation, pscBAR. Considering the possibility that the native *ChEC108* promoter might not function identically in *Ch* and *M. oryzae*, I used alternative regulatory sequences to drive transgene expression in this system. Namely, I chose the promoter of the *M. oryzae* biotrophy-associated *AVR-PikD* effector gene (Yan et al., 2023), which has been previously employed to drive high levels of transgene expression in combination with the constitutive *A. nidulans trpC* terminator

(Jave Bautista, personal communication). I flanked the *SP-ChEC108-eGFP* and *ChEC108-eGFP* coding sequences with these two regulatory elements, and provided the resulting plasmids to Weibin Ma, who performed *M. oryzae* protoplast transformations and monitored the resulting transformants.

To observe the subcellular localisation of ChEC108 fusions during the early stages of *M. oryzae* infection, I inoculated *O. sativa* leaf sheaths with transgenic strains described above and performed confocal imaging on trimmed sheath samples, prepared with the aid of Jave Bautista. 26-30 hpi, appressoria and accompanying invasive hyphae were abundant, with the majority of colonisation events being restricted to one host cell. SP-ChEC108-eGFP signal was concentrated at a single punctum associated with invasive hyphae, either at the tip of filamentous primary (undivided) hyphae, or adjacent to a branch point if hyphal development was further advanced (Figure 6.21A). This localisation pattern is reminiscent of the BIC, where cytoplasmic *M. oryzae* effectors such as Pwl2, Bas1, Bas2 and AVR-Pia are known to concentrate before translocating to the host (Khang et al., 2010; Mosquera et al., 2009; Sornkom et al., 2017). On the other hand, ChEC108-eGFP appeared diffuse within all appressoria and invasive hyphae observed, with signal being concentrated within hyphal nuclei, but excluded from vacuoles (Figure 6.21B). This suggests the native signal peptide directs ChEC108 via the secretory pathway to single hyphae-associated foci at *M. oryzae* invasion sites, which likely represent the BIC.

As fluorescent tags do not appear to impede effector uptake by *O. sativa*, cytoplasmic *M. oryzae* effectors can be distinguished from apoplastic effectors via examination of their subcellular localisation. In this system, translocation of *M. oryzae*-derived ChEC108 to the *O. sativa* symplast would lend support to the hypothesis that the protein functions as a cytoplasmic effector. On the other hand, exclusive localisation of ChEC108 to the extra-invasive hyphal matrix (EHM) of *M. oryzae* would favour the idea that the effector is apoplastic. As the pool of fluorescent effectors entering the host cytoplasm is expected to be low, non-native NLS sequences can be added to concentrate effector fusions in nuclei, facilitating easier detection via confocal microscopy (Khang et al., 2010). In collaboration with the Talbot laboratory once again, an *M. oryzae* strain was generated to express *SP-ChEC108-GFP-3×NLS*, driven by an *AVR-PikD* promoter and *trpC* terminator. Similar to the localisation of SP-ChEC108-eGFP reported in Figure 6.21A, during infection of *O. sativa* leaf sheaths, SP-ChEC108-GFP-3×NLS was observed at BIC-like puncta (Figure 6.21C-D). Host nuclei were also clearly labelled, suggesting ChEC108 fusions were secreted from *M. oryzae* and effectively translocated to the *O. sativa* symplast.

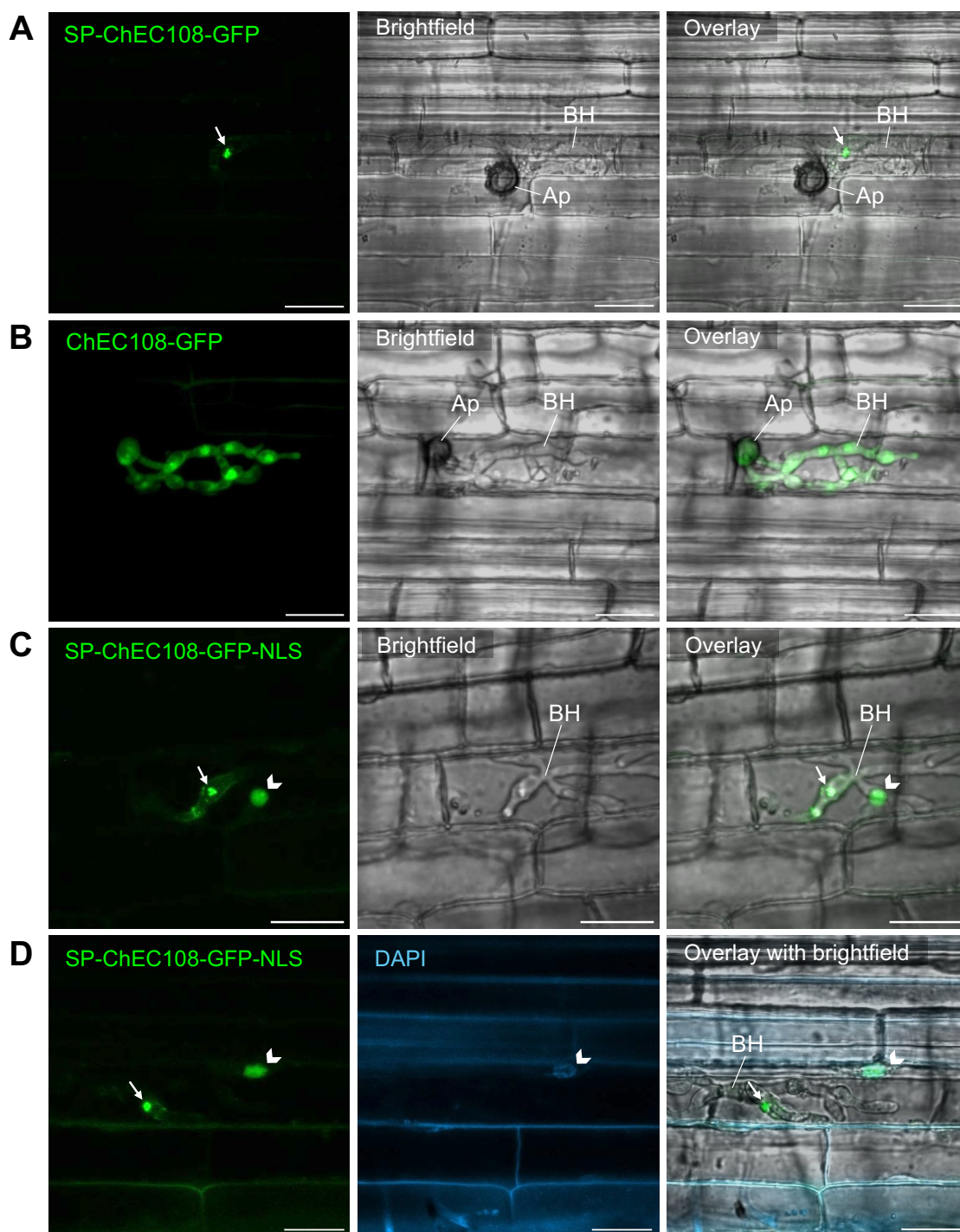


Figure 6.21 – The ChEC108 signal peptide is functional in the *M. oryzae* heterologous system. A-B. *O. sativa* leaf sheath epidermal cells were inoculated with *M. oryzae* strains expressing SP-ChEC108-eGFP with the native N-terminal signal peptide included (A) or ChEC108-eGFP with the signal peptide absent (B). Concentration of eGFP signal at the BIC can be seen in A only. **C-D.** SP-ChEC108-GFP-3×NLS localises to the BIC and host cell nuclei, highlighted with DAPI staining in D. Images were acquired 26-30 hpi. Arrows indicate the BIC. Arrowheads indicate host nuclei. Images are representative of at least two independent *M. oryzae* strains generated to express each fusion protein. Scale bar = 20 μm.

6.3 Discussion

Using confocal imaging or immunodetection following proximity labelling, I have been unable to explicitly demonstrate ChEC108 presence in Arabidopsis cells during *Ch* colonisation. Meanwhile, efforts to generate a ChEC108-responsive NLR using the Pik-1 chassis were unsuccessful, meaning detection of effector uptake via Arabidopsis gain-of-resistance to *Ch* could not be achieved. Nevertheless, I demonstrated signal peptide-dependent secretion of ChEC108 from an alternative fungal pathogen more amenable to confocal imaging, *M. oryzae*, and observed translocation of the effector to *O. sativa* cells during infection, providing support for the hypothesis that ChEC108 is a secreted protein. Below, I discuss some of the wider difficulties associated with demonstrating effector translocation, and suggest methodological improvements and alternatives that could be applied to build upon the efforts documented in this chapter.

6.3.1 Effector translocation from *Ch* is challenging to detect

The lack of evidence for ChEC108-eGFP translocation reported in 6.2.1 is likely a consequence of low effector abundance in the transgenic *Ch* strain used, such that any fluorescence was below the detection threshold. In studies of other pathosystems, adding an NLS to drive nuclear accumulation of secreted effector proteins after their entry into the host symplast has proven effective for concentration of fluorescent signal (Irieda et al., 2014; Khang et al., 2010; Yan et al., 2023). Similarly, the native NLS of the *C. graminicola* effector protein 1 (CgEP1) likely enhanced the detectability of fungus-derived CgEP1-mCherry fusions in *Z. mays* leaf sheath cells (Vargas et al., 2016). With ChEC108 being enriched at PD when stably expressed in Arabidopsis host plants (Figure 5.1), I hypothesised that fluorescently-tagged ChEC108 might be sufficiently concentrated at puncta, increasing the likelihood of its detection. As this proved not to be the case (Figure 6.3), nuclear targeting could facilitate easier detection of ChEC108 in future.

ChEC108-eGFP is by no means the first pathogen-expressed effector-fluorophore fusion not to be detected in planta. As discussed in 6.1.2, the retention of effector fusions within pathogen tissues, or at the host-pathogen interface adjacent to the host plasma membrane has been commonly reported (Djamei et al., 2011; Irieda et al., 2014; Kleemann et al., 2012; Tanaka et al., 2015). Although seemingly unimportant in the *M. oryzae*-*O. sativa* pathosystem, there remains a possibility that, regardless of protein accumulation levels, large fluorescent tags might impede translocation across the plasma membrane. For instance, if translocation requires unfolding and refolding of effectors, the rigid folds of fluorescent proteins might pose a restriction. To better understand the mechanisms putatively inhibiting the delivery of effector fusions from *Colletotrichum*, a deeper understanding of the sequence of molecular events governing

effector translocation would be beneficial, especially those localised to previously-reported sites of effector accumulation such as interfacial bodies and the basal appressorium pore (Figure 6.1).

Exemplified by the doctoral thesis of Dr Camille-Madeleine Szymansky ([Szymansky, 2025](#)), proximity labelling in combination with sensitive mass spectrometry can be used to identify even low abundance effectors under native infection conditions, presenting an alternative to confocal imaging or Western blotting for effector detection. However, such feats required extensive optimisation of protocols, including selection of appropriate time points to ensure sufficient pathogen proliferation, as well as the generation of plant materials with optimal accumulation of TurboID fusions, improving the signal to noise ratio within resulting datasets. Using IP to enrich for Myc-tagged ChEC108 in biotin-treated infected material, I had hoped to reduce the likelihood of biotinylated ChEC108 being obscured within a large pool of endogenous biotinylated proteins, meaning plant lines with high expression of TurboID fusions and long biotin incubation times could be used without major concern for background biotinylation levels. Despite this, ChEC108 (regardless of its biotinylation status) remained challenging to detect via Western blotting. Although the sensitivity of detection methods can vary depending on the protein in question, as well as sample purity and the exact instruments or antibodies used (Dr Carlo Martins, personal communication), it might be beneficial to explore the possibility of ChEC108 detection via mass spectrometry in this context.

It is also worth noting that proximity labelling approaches for effector detection in planta are best suited for the study of biotrophic pathogens, which proliferate within live host cells where TurboID remains functional. *A. candida*, for example, is an obligate biotroph on *Arabidopsis*, while *P. infestans* is a hemibiotroph on *N. benthamiana* ([Szymansky, 2025](#)). *Ch* likewise exhibits an early biotrophic phase during infection, however this is short-lived and takes place in single host cells, lasting only 24 h before necrotrophy ensues ([O'Connell et al., 2004](#)). Combined with inherent stochasticity and the influence of environmental fluctuations on infection progression rate, this made the selection of an appropriate post-infection time point for biotin treatment and sample harvest very difficult. A too-early harvest would likely result in sub-optimal levels of pathogen proliferation and thus a low abundance of secreted effectors, while a too-late harvest would result in excessive host cell death and degradation of TurboID (alongside any biotinylated proteins) at the onset of necrotrophy.

Moving forward, to boost the likelihood of translocated ChEC108 being detected via confocal imaging or proximity labelling approaches reported in 6.2.1 and 6.2.2, I expect that enhancement of effector abundance within transgenic *Ch* strains would be necessary. Towards this goal, more extensive screening of initial *Ch* transformants could be carried out to identify those with

higher levels of transgene expression. In addition, alternative constitutive promoters such as the full-length *A. nidulans gpdA* promoter (Punt et al., 1991), *Aureobasidium pullulans translation elongation factor (TEF)* promoter (Wymelenberg et al., 1997), or the *M. grisea ribosomal protein 27 RP27* promoter (Bourett et al., 2002) (as used by Vijai et al., 2013; Were et al., 2025) could be trialled to drive *ChEC108* expression. On the other hand, inspired by experiments reported in 6.2.4, it might be beneficial to select the promoter of a *Ch* gene exhibiting high levels of expression during biotrophy, such as those upstream of *ChEC6* (CH63R_13188) or *ChEC116* (CH63R_14384), as time course RNA-sequencing data suggests native expression levels of *ChEC108* to be low in comparison (Dallery et al., 2017).

6.3.2 Do Pik NLRs still have potential for engineering ChEC108 perception?

Cell death assays described in 6.2.3 suggest that simply replacing the HMA domain of Pikm-1 with HMA1 or HMA2 from HIP6 is (on its own) an unsuitable strategy to synthetically engineer ChEC108 recognition, given that chimeras Pikm-1^{HMA1} and Pikm-1^{HMA2} triggered Pikp-2-dependent cell death in the absence of ChEC108. Multiple strategies were implemented to remove this autoactivity, including moderate and subtle extension of the HMA domain boundaries, and mutation of the mbl region - all to no avail. While some NLR variants were engineered with apparently weaker activity such as Pikm-1^{HMA2-mbl-C}, cell death was similarly elicited in the absence of ChEC108. Undesired autoactivity among synthetic NLRs is not uncommon, and has been frequently observed upon modification of NLR integrated domains, including within the Pik system (Bentham et al., 2023; Białas et al., 2021; Kourelis et al., 2023; Maidment et al., 2023; Stone, 2024; Zdrzałek et al., 2024). Even allelic mis-matching of Pikm-1 with Pikp-2, which proved successful in the case of Pikm-1^{OsHIP643} engineering (Zdrzałek et al., 2024), was not sufficient to remove autoactivity from *all* Pikm-1-nanobody fusions tested by Bentham et al. (2023). At present, in the absence of high-resolution structural information regarding the active and resting states of Pik NLRs, the precise molecular mechanisms underlying effector-independent activation remain unknown.

A previous study suggested careful matching of HMA size to be key for generating functional Pik-1 chimeras, based on the finding that deletion of a single amino acid in wild-type Pikm-1 (Gly186) resulted in strong autoactivity (Bentham et al., 2023). Although structural alignments suggest the native Pikm-1^{HMA} and HMA1/HMA2 from HIP6 to be similar (Figure 6.14), it is possible that minor gaps and insertions may be sufficient to nudge the overall NLR conformation out of its usual resting state. Although addition of IMG to the N-terminal HMA boundary did not alleviate autoactivity of Pik-1 chimeras in this work, it is possible that further minor adjustment of HMA1 and HMA2 structures to more closely match that of Pikm-1^{HMA} could give rise to a functional NLR. As suggested by Zhang et al. (2024b), mutation

of HMA-adjacent regions could also be beneficial for improving chimeric receptor function, although rational design of such mutations is challenging.

An alternative method trialled to engineer ChEC108 recognition while avoiding autoactivity was that of structure-guided resurfacing of wild-type Pikm-1^{HMA} to more closely resemble HMA2 from HIPP6. However, neither Pikm-1^{mb1-HMA2} (containing seven HMA2-derived residues in the mbl region) or Pikm-1^{HMA-RS} (containing ten HMA2-derived residues contributing to the ChEC108 binding interface) were able to elicit ChEC108-dependent cell death in *N. benthamiana*. To better understand this negative result, first, it would be necessary to test for ChEC108 association with these resurfaced chimeras. If binding is not observed, further amino acid substitutions could be integrated. If binding is detected, the lack of ChEC108-triggered cell death could be a consequence of low binding affinity. Through a combination of cell death and surface plasmon resonance (SPR) experiments, [Bentham et al. \(2023\)](#) demonstrated that high affinity interfaces were key for initiating a cell death response via engineered Pik NLRs. A correlation between strength of in vitro binding and cell death severity was similarly observed when comparing Pikp-1 perception of AVR-PikD and AVR-Pia ([Varden et al., 2019](#)). Therefore, in future, more extensive resurfacing of Pikm-1^{HMA} to enhance the affinity of this domain for ChEC108 could be beneficial.

Ultimately, if the strategies suggested above yield no functional Pik-1 chimeras with the ability to recognise ChEC108, alternative NLR chassis could be trialled. For example, sensor NLRs RGA5 from *O. sativa* (paired with its helper, RGA4) and resistance gene homolog 2 (RGH2) from *H. vulgare* (paired with its helper, RGH3) possess C-terminal integrated HMA and Exo70 domains, respectively ([Brabham et al., 2023](#); [Ortiz et al., 2017](#)). Exchange of these domains for HMA1 or HMA2 may be less likely to disrupt the wider NLR structure, which presumably underlies Pikm-1^{HMA1} and Pikm-1^{HMA2} autoactivity.

6.3.3 Alternative strategies to capture ChEC108 translocation

Beyond the techniques applied in this work, effector biologists have developed a plethora of strategies to investigate the translocation of proteins of interest, many of which could be optimised and applied to study ChEC108. For example, immunogold labelling in combination with TEM has been used to visualise the delivery of effectors from a range of organisms to the cytosol or specific subcellular organelles of host plants, including those derived from aphids ([Mugford et al., 2016](#)), oomycetes ([Djamei et al., 2011](#); [Presti et al., 2017](#)) and fungi ([Liu et al., 2025](#); [Rafiqi et al., 2010](#)). Taking this approach would require the development of α -ChEC108 antibodies or fusion of the effector to a sufficiently small epitope tag that does not impede secretion or uptake by the host. Ultimately, as suggested in [6.3.1](#), increasing the expression

of *ChEC108* in transgenic *Ch* strains would be highly desirable alongside implementation of this challenging technique, as genuine translocation events could be difficult to distinguish from artefacts if the number of visible gold particles is low.

In pursuit of evidence that the *U. maydis* effector, stop penetration 1 (Stp1) is translocated to *Z. mays* hosts, [Tanaka et al. \(2015\)](#) trialled a site-directed recombination-based assay. This involved transformation of *Z. mays* with a construct encoding a *GUS* reporter gene with an upstream *ZmUBQ1* promoter. Blocking constitutive transcription of *GUS* was a spacer sequence flanked by flippase recombination target (FRT) sites, which are recognised by flippase (FLP) recombinase enzymes. Alongside, a *U. maydis* strain was generated to produce Stp1-FLP, under the premise that delivery of this fusion to host cells would trigger excision of the spacer sequence and expression of *GUS* via the now-adjacent *ZmUBQ1* promoter. Although Stp1 delivery could not be detected using this method ([Tanaka et al., 2015](#)), similar recombination-based assays with detectable readouts could be applied to the *Ch*-Arabidopsis pathosystem.

Although I gathered evidence to suggest ChEC108 possesses a functional signal peptide by demonstrating its secretion in a heterologous system, it remains to be determined whether ChEC108 can be trafficked across the Arabidopsis plasma membrane. Previous studies of oomycete and fungal effectors suggest this process could be pathogen-independent ([Rafiqi et al., 2010](#)), relying on lipid raft-mediated endocytosis ([Kale et al., 2010](#)). If this similarly applies to *Ch*, effector translocation could be investigated following direct treatment of Arabidopsis tissue with purified ChEC108 protein, followed by live imaging (for example, using purified ChEC108-GFP11 and plant-derived GFP1-10) or proximity labelling-based approaches (for example, using purified ChEC108-4×Myc and plant-derived TurboID-HIPP6) to monitor effector internalisation. Even so, observation of *Ch*-derived effector translocation in an infection context would naturally be considered the gold standard method.

6.3.4 Conclusion

Effector translocation remains a poorly understood phenomenon in the field of plant-microbial interactions, especially in the context of fungal phytopathogens. For effectors lacking corresponding R proteins in nature, delivery to host plants can be challenging to explicitly demonstrate, especially in cases where the temporal window for effector expression and secretion is narrow, and/or the abundance of the effector protein is low. Consequently, due to technical concerns, it can be difficult to confidently conclude that an effector is *not* secreted following negative results obtained from effector translocation assays. Thus, although I was unable to detect fluorescently-tagged or biotinylated ChEC108 in planta following its expression in *Ch* (6.2.1 and 6.2.2), I am confident in the annotation of ChEC108 as a secreted protein following experiments described in 6.2.4, and am hopeful that future experiments, including pathogen virulence assessments (discussed in 4.3) will shed light on the in planta functions of this protein.

Chapter 7

Discussion

At the outset of this project, with the goal of better understanding how fungal pathogens manipulate cell-to-cell communication within their hosts, I sought to functionally characterise the *Ch* protein, ChEC108 - a hypermobile putative effector that localises to PD and enhances intercellular transport in *N. benthamiana*. Following the identification of HIPP6 - a PD-localised Arabidopsis protein - as a likely host target of ChEC108, I used a combination of co-IP experiments, in vitro protein purification and X-ray crystallography to study the mechanism by which ChEC108 targets the HMA domains of HIPP6, and identified key metal-binding residues making a critical contribution to the binding interface (Chapter 3). However, following a series of unexpected results obtained when investigating ChEC108 function at the cellular level and in an infection context, the overall focus of the project pivoted towards understanding the consequences of ChEC108-HIPP6 association.

As initially discussed in 5.3, I hypothesise that ChEC108 evolved as a *Colletotrichum*-specific virulence factor, enabling *Ch* to manipulate host PD and help maintain symplastic continuity. This would be expected to favour fungal establishment by promoting the spread of other effectors (for example, those shown to be cell-to-cell mobile by Ohtsu et al., 2023), some of which might have immunosuppressive functions. As a virulence factor, ChEC108 could also allow *Ch* to circumvent chitin-triggered PD closure within the host, and thus avoid triggering stress responses that might otherwise antagonise pathogen growth during the biotrophic phase (Tee et al., 2025). However, experiments presented in Chapters 4 and 5 support a model whereby host plants (at least, in the case of Col-0 Arabidopsis) have evolved to combat these putative ChEC108 virulence functions, perhaps promoting degradation of the effector or employing PD-localised defences to slow its intercellular movement. This idea is supported by the following observations: (1) bombardment-based mobility assays in Arabidopsis failed to recapitulate the ChEC108-mediated enhancement of intercellular flux previously observed in

N. benthamiana; (2) although I captured cell-to-cell movement of ChEC108-eGFP in Arabidopsis using live imaging, fluorescent signal was extremely faint, suggesting the effector could be unstable or actively degraded; (3) *ChEC108* was dispensable for *Ch* virulence, and possession of a functional copy of this gene even hindered macroscopic symptom development in some contexts; (4) rather than opening up PD, stable overexpression of *ChEC108* in Arabidopsis led to a shutdown of intercellular trafficking and heavily stunted rosette growth. Overall, these findings suggest that host-specific factors could be blocking or masking the outcome of suspected ChEC108 virulence functions at PD.

Building on the above hypothesis, I propose that HIP6 contributes to host defence against ChEC108 by binding the effector when it reaches PD. This idea is supported by the following: (1) targeting of ChEC108 to PD did not depend on HIP6, suggesting the two proteins are capable of reaching this location independently; (2) an agroinfiltration-based mobility assay conducted in *N. benthamiana* suggested that the formation of stable ChEC108-HIP6 complexes could hinder trafficking of the effector via PD; (3) in some contexts, *hip6* loss-of-function mutants displayed enhanced susceptibility to *Ch*, in a manner that depended on the presence of ChEC108; (4) in contrast to those expressing wild-type *ChEC108*, Arabidopsis plants stably overexpressing a non-interacting effector variant, *ChEC108*^{D164R}, did not exhibit severe growth inhibition and PD closure. Together, these results suggest HIP6 positively regulates host resistance to *Ch*, and could be responsible for initiating signalling events downstream of ChEC108 perception, culminating in a minor (and likely context-dependent) increase in Arabidopsis resistance.

In the following discussion, I highlight outstanding questions that accompany the model proposed above, and draw on observations made in studies of other plant HMA proteins to speculate in more detail about the intracellular functions of the ChEC108-HIP6 complex.

7.1 How might HIP6 positively regulate plant defence?

Considering HMA proteins have been frequently identified as susceptibility factors in a diverse array of pathosystems (see Table 1.1 and 1.2.5), it is surprising to consider that HIP6 may have an opposite role - one involved in defence activation. Currently, literature on plant recognition of pathogens is dominated by the study of extracellular receptors (such as RLKs, RLPs and their co-receptors), intracellular NLRs, and more recently KFPs (see 1.1). As HIP6 belongs to none of these families, one might hypothesise that it operates as a guard or decoy protein, enabling indirect recognition of ChEC108 activity via an unidentified intracellular receptor. However, considering the fact that wild-type *Ch* is highly virulent on Arabidopsis, any defence

signals elicited by ChEC108-HIPP6 binding are unlikely to lead to a canonical ETI response, as effector recognition by NLRs typically results in strong (qualitative) resistance (Zhang et al., 2022a), as opposed to a minor adjustment of disease symptom severity.

Beyond the classic gene-for-gene model of qualitative resistance, quantitative resistance (sometimes referred to as partial or broad-spectrum resistance) is a polygenic trait, associated with a continuous distribution of disease symptoms (Corwin and Kliebenstein, 2017; French et al., 2016). Being somewhat of a grey area within the field of plant immunity, there are several hypotheses that could explain quantitative resistance, only some of which involve the recognition of effectors (Poland et al., 2009). For instance, Meile et al. (2023) reported that recognition of different isoforms of the *Zymoseptoria tritici* effector Avr3D1 by an unknown R protein underpinned a continuous distribution of resistance magnitudes on *T. aestivum* as opposed to triggering either complete resistance or susceptibility, blurring the lines between quantitative resistance and ETI (for a commentary, see Rudd, 2023). Alternatively, genes affecting plant morphology and development could have pleiotropic effects on resistance (Zhu et al., 1999), and genes governing the production of antimicrobial compounds (Parisy et al., 2007), detoxification of pathogen-derived compounds (Wang et al., 2021) or defence signal transduction (Qiu et al., 2007) could all influence disease severity. In their review of this topic, Poland et al. (2009) also recognised that ‘unique sets of unidentified genes’ could explain quantitative disease resistance traits, citing *OsHIPP05* (encoded by the *Pi21* locus) as an example of a susceptibility gene not previously recognised to function in immunity (Fukuoka et al., 2009). I therefore hypothesise that *HIPP6* acts as a quantitative resistance locus in the context of *Ch* infection, speculatively with a role in defence signalling downstream of ChEC108 perception.

To better understand HIPP6 function, one could identify protein binding partners using a non-targeted IP-MS approach, making use of the *hipp6-3.GFP-HIPP6-18* and *hipp6-3.GFP-HIPP6^{MM}-11* resources described in Chapter 4. Alongside, RNA-sequencing experiments could be used to study host transcriptomic changes during infection in the presence and absence of ChEC108. Such experiments might aid in the identification of immune signalling modules that are differentially regulated in the event of ChEC108-HIPP6 binding, but not when this interaction is compromised. In the meantime, informed by studies of other HMA proteins in plant immunity and beyond, I continue to speculate about the intracellular functions of HIPP6.

7.1.1 HIPP6 is unlikely to directly regulate transcription

Several HMA proteins have been characterised as nuclear-localised (see 1.2.7), and are known to interact directly with transcription factors to modulate stress responses. For example, CCP/HIPP5 was imported into the nuclei of Arabidopsis protoplasts, requiring a functional CXXC motif and nuclear localisation signal (KKVGF) to do so (Chai et al., 2020). CCP was also shown to bind the transcription factor TGACG-BINDING 2 (TGA2), activating transcription of the defence gene *PATHOGENESIS-RELATED PROTEIN 1* (PR1) (Chai et al., 2020), which encodes a widely-studied antimicrobial signalling protein (Han et al., 2023). In *N. benthamiana*, NbHIPP26 is proposed to shuttle from the plasma membrane to nuclei in the presence of the PMTV movement protein TGB1, promoting drought tolerance and long-distance PMTV transport via an unknown mechanism (Cowan et al., 2018). An ortholog of this HIPP, ATFP6/HIPP26, similarly localised to the nuclei of *A. cepa* epidermal cells, and is thought to manipulate drought-responsive gene expression in the Arabidopsis native system via its zinc finger transcription factor binding partner, ATHB29 (Barth et al., 2009). In fact, several HIPPs were suggested to bind ATHB29 in a yeast two-hybrid experiment, namely HIPP20, 21, 23, 24, 27 and 30, with HIPP30 showing additional interactions with ATHB21 and ATHB30 (Barth et al., 2009). Elsewhere, a BiFC experiment in *N. benthamiana* suggested CqHIPP34 from *C. quinoa* bound the transcription factor ZINC FINGER HOMEODOMAIN 14 (CqZF-HD14), another positive regulator of drought tolerance (Sun et al., 2022). It is thus conceivable that other HMA proteins (including those in complex with effectors or viral movement proteins), might target transcription factors in nuclei to reprogramme host cells during infection.

In contrast to the examples of nuclear HMA proteins given above, I observed HIPP6 to be primarily PD-localised in the native system of Arabidopsis. Although nuclear signal was observed upon transient overexpression in *N. benthamiana*, the intensity of this signal was not enhanced upon co-expression with ChEC108, despite the effector being abundant in nuclei. Instead, co-localisation data suggest ChEC108 and HIPP6 likely interact at PD or on the outer surface of the ER. Thus, it seems unlikely that HIPP6 or the ChEC108-HIPP6 complex act as direct manipulators of transcription factors within nuclei.

7.1.2 HIPP6 might signal via phytohormones

Given its potential association with the ER, HIPP6 could regulate phytohormone signalling, specifically in the context of cytokinin. This idea stems from the findings of Guo et al. (2021), who characterised Arabidopsis *HIPP5*, *HIPP6* and *HIPP7* as early cytokinin-responsive genes (specifically, their expression was downregulated 30 min post-cytokinin treatment), and implicated their encoded proteins in the regulation of intracellular cytokinin levels via

ER-associated degradation of CYTOKININ OXIDASE/DEHYDROGENASE (CKX) enzymes, which degrade cytokinin. In this study, overexpression of *GFP-HIPP6* or *GFP-HIPP7* via a *35S* promoter, or *GFP-HIPP1* via a *UBQ10* promoter, gave rise to stunted rosettes with curled leaves - a phenotype indicative of cytokinin hypersensitivity and attributed to enhanced proliferation and delayed differentiation of leaf cells in *GFP-HIPP7* lines. *GFP-HIPP1* overexpression triggered a reduction in CKX1-Myc protein levels in transgenic Arabidopsis, yet *GFP-HIPP6* or *GFP-HIPP7* overexpression led to increased accumulation of this protein. Although, in the latter case, this pool of excess CKX enzyme is hypothesised to be non-functional, given the consistent cytokinin hypersensitivity phenotypes of all three lines. Alongside, the doctoral thesis of Dr Georgeta Leonte (Leonte, 2020) revealed CKX1-binding capacity for three other Arabidopsis proteins: HIPP32, HIPP33 and HIPP34, suggesting regulation of cytokinin signalling may not be exclusive to clade I of the HIPP family.

In agreement with the work described above, as reported in 4.2.3, I observed stunted rosette growth of Arabidopsis lines expressing *meGFP-HIPP6*, yet serrated and crinkly leaf margins were not observed. This could be attributed to variation in growth conditions (that is, a long-day photoperiod in a greenhouse, used by Guo et al. (2021), versus a short-day photoperiod in a growth cabinet), or variation in transgene expression levels between lines, perhaps owing to the use of different promoters (*35S* versus *AtUBQ10*). Nevertheless, could regulation of cytokinin signalling explain the proposed function of HIPP6 in defence?

While cytokinin is not a hormone family classically associated with plant immunity, its influence on plant-microbe interactions has been explored. For example, cytokinins were shown to positively regulate resistance of tomato (*Solanum lycopersicum*) plants to the necrotrophic fungus, *B. cinerea* and biotrophic fungus, *Oidium neolycopersici*, in a manner dependent on SA and ethylene signalling (Gupta et al., 2020). Cytokinins were also found to promote Arabidopsis resistance to bacterial pathogens, again involving crosstalk with SA (Choi et al., 2010). In this study, a complex of the SA response factor TGA3 and cytokinin-activated transcription factor ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2) was shown to drive expression of the defence gene *PR1*, with SA signalling enhancing binding of the complex to the *PR1* promoter. Supporting this model, overexpression of CKX genes (leading to reduced endogenous cytokinin levels), or mutation of ARABIDOPSIS HISTIDINE KINASE cytokinin receptor genes *AHK2* and *AHK3*, triggered enhanced susceptibility to *Pto* DC3000 (Choi et al., 2010). In an independent study, when treated with high concentrations of exogenous cytokinin (benzyl adenine), Arabidopsis displayed enhanced resistance to the oomycete *H. arabidopsidis*, accompanied by enhanced SA-responsive defence gene expression (Argueso et al., 2012). These phenotypes were not observed in *eds16* mutant plants deficient in SA biosynthesis (Argueso et al., 2012). Together, these studies highlight a relationship between cytokinin and

SA signalling that mediates defence gene expression in multiple pathosystems.

In addition to cytokinins, previous studies have hinted towards SA-related functions for HMA proteins. For example, transcripts related to SA signalling, as well as accumulation of SA itself, were increased in *T. aestivum* plants overexpressing *HIPP1-V* (Wang et al., 2022), suggesting this HIPP might regulate SA-responsive pathways. Likewise, CCP may promote SA responses via its interaction with TGA2, a known regulator of SA-dependent gene expression (Boyle et al., 2009; Chai et al., 2020). Both *HIPP1-V* and CCP were identified as positive regulators of immunity in their respective systems (see 1.2.6). In contrast, *HIPP3* was implicated as a negative regulator of SA responses, based on co-expression analysis of Arabidopsis plants constitutively expressing this *HIPP* (Zschiesche et al., 2015). AtHMAD1/HMP09 is also thought to negatively regulate SA signalling, as *athmad1* mutants displayed increased expression of SAR marker genes post-inoculation with *Pto* DC3000 (Imran et al., 2016), emphasising that not all HMA proteins interact with SA signalling in the same way.

Bringing together concepts described above, I speculate that *HIPP6* could positively regulate Arabidopsis defence against *Ch* via inhibition of CKX enzyme function and enhancement of cytokinin signalling (summarised in Figure 7.1). In the presence of ChEC108, stabilisation of *HIPP6* on the outer surface of the ER might enhance this activity further, leading to elevated cytokinin levels and downstream defence signalling. To test this, it would be beneficial to study the ER-localised pool of *HIPP6* in more depth, perhaps testing for co-localisation with established ER markers in the presence and absence of ChEC108. It would also be interesting to quantify the expression of cytokinin- and SA-responsive genes during the biotrophic phase of *Ch* infection, especially in scenarios where the ChEC108-*HIPP6* complex can and cannot form.

Admittedly, the model described in Figure 7.1 does not take into account the PD localisation of *HIPP6*, and whether or not metal-binding would be required for functions in hormonal regulation is unclear. Interestingly, Guo et al. (2021) observed both GFP-*HIPP1* and GFP-*HIPP7* at PD in Arabidopsis, yet the functional relevance of this was not discussed. This raises the possibility that HIPP proteins could be multifunctional, with both ER- and PD-localised roles.

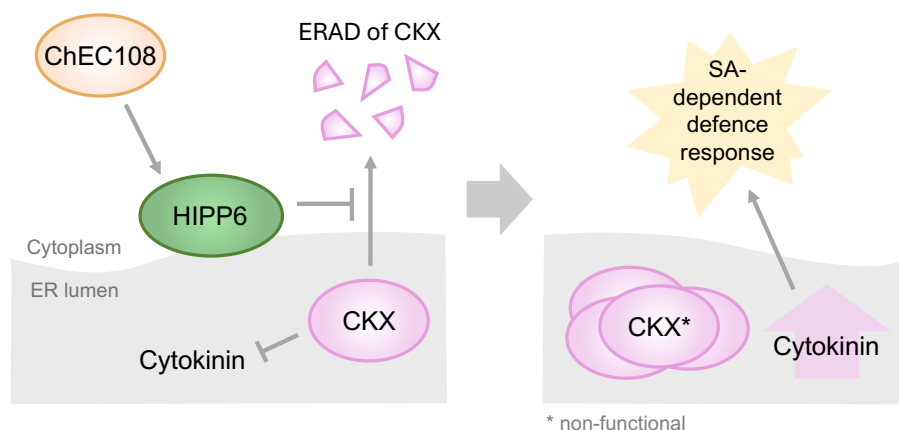


Figure 7.1 – Speculative model for ChEC108-HIPP6-mediated regulation of defence via cytokinin signalling. Based on the findings of [Guo et al. \(2021\)](#), on the outer surface of the ER, HIPP6 inhibits ER-associated degradation (ERAD) of CKX enzymes, leading to accumulation of a non-functional pool of enzyme in the ER (CKX*). This activity could be enhanced by ChEC108-binding, leading to elevated cytokinin levels, activation of SA-responsive gene expression and enhanced resistance to pathogens.

7.1.3 HIPP6 might signal via ROS

As an alternative to phytohormone regulation, HIPP6 could function at the level of second messenger signalling. ROS are established second messengers in plants, and their accumulation is associated with both abiotic and biotic stress (for reviews, see [Castro et al., 2021](#); [Mittler et al., 2022](#)). On perception of stressful stimuli, ROS build-up can occur in chloroplasts and mitochondria as a consequence of electron transport chain malfunction. ROS may also be actively produced via oxidases in peroxisomes, and in the apoplast via cell wall peroxidases and RBOH enzymes. To prevent excessive oxidative damage to cellular machinery during stress, plants utilise ROS scavenging and transport proteins, as well as non-enzymatic antioxidants. To explain its involvement in *Ch*-*Arabidopsis* interactions, could HIPP6 also regulate these broad-spectrum stress signals? Supporting this idea are multiple studies implicating HMA proteins in ROS detoxification.

The prototypical HMA protein ATX1 was first identified in a genetic screen for antioxidant factors capable of functionally substituting for *S. cerevisiae* Cu/Zn-SOD 1 (SOD1) ([Lin and Culotta, 1995](#)), a metalloenzyme that catalyses the dismutation of damaging superoxide, forming hydrogen peroxide and oxygen ([Bermingham-McDonogh et al., 1988](#)). Although not responsible for direct delivery of metal cofactors to SOD1 ([Culotta et al., 1997](#)), ATX1 is thought to potentially dampen oxidative stress using its own (albeit inefficient compared to SOD1) superoxide dismutase activity ([Portnoy et al., 1999](#)). In plants, participation in ROS scavenging has similarly been suggested for HPPs. Through the use of alternative translation start sites, CCS transcripts were found to encode both cytosolic and chloroplast-targeted protein isoforms, enabling activation of three *Arabidopsis* Cu/Zn-SOD (CSD) enzymes: CSD1 (cytoplasmic),

CSD2 (stromal) and CSD3 (peroxisomal) (Abdel-Ghany et al., 2005; Chu et al., 2005). Later, a study in the *M. graminicola*-*O. sativa* system suggested OsHPP04 delivers Cu cofactors to cytosolic Cu/Zn-SOD 2, and upon nematode infection, binding of the MgMO298 effector to OsHPP04 is thought to promote ROS scavenging, thereby dampening host immune responses and increasing susceptibility to nematodes (Song et al., 2021). Recent work by Were et al. (2025) uncovered a potential link between HIPPs and ROS homeostasis, as *H. vulgare* plants overexpressing *HvHIPP43* exhibited reduced ROS bursts in response to pathogen elicitors flg22 and chitin. It is not yet established whether these plants scavenged ROS more efficiently, or were compromised in ROS production. Nevertheless, it is proposed that binding of the *M. oryzae* effector Pwl2 might somehow enhance the ability of *HvHIPP43* to negatively regulate ROS signalling, as similar phenotypes were observed in *HvHIPP43*- and *Pwl2*-overexpressing lines (Were et al., 2025).

Considering these findings, one could hypothesise that ChEC108 similarly exploits HIPP6 to suppress ROS signalling and thus compromise Arabidopsis immunity, perhaps by promoting SOD-dependent ROS scavenging. However, as my data do not suggest HIPP6 functions as a susceptibility factor, this scenario seems unlikely. Instead, by forming a stable complex and occupying the metal-binding loop(s) of HIPP6 HMA domains, ChEC108 might inhibit HIPP6-mediated delivery of metal ion co-factors, resulting in reduced ROS scavenging via SODs and an elevated immune response (Figure 7.2). Consistent with this, HIPP1-V was suggested to promote ROS accumulation in *T. aestivum*, as levels of oxidative response-related transcripts, NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) oxidase enzymes and hydrogen peroxide were increased in plants overexpressing this *HIPP*, which may have contributed to their increased *Bgt* resistance (Wang et al., 2022).

Again, how the PD localisation of HIPP6 fits into this model is unclear. It is possible that HIPP6 promotes highly localised ROS scavenging, recruiting cytosolic enzymes or supporting the function of PD-resident enzymes to protect other PD components from oxidative damage. HIPPs may also help fine-tune PD-localised signalling cascades involving ROS as a second messenger. For example, extracellular flg22 or chitin perception culminates in RBOHD activation via independent pathways, triggering apoplastic ROS accumulation and closure of PD via CALS1-mediated callose deposition (Cheval et al., 2020; Tee et al., 2023; Xu et al., 2017). However, it is puzzling to consider how intracellular HIPP6 proteins (which lack a transmembrane and extracellular domain), could contribute to PD regulation via apoplastic ROS.

Alternatively, it is possible that HIPPs influence ROS signalling in other cellular compartments. mCherry-*HvHIPP43* (which dampens ROS responses) was shown to be enriched at N.

benthamiana PD by Were et al. (2025), yet this localisation was compromised in the presence of YFP-Pw12, with both proteins co-localising in the cytosol. In contrast, I observed ChEC108 and HIPP6 co-localisation at PD in Arabidopsis. These findings could suggest that HIPP-mediated detoxification of ROS occurs primarily in the cytoplasm, such that stabilisation of HIPP6 by ChEC108 at PD might negatively regulates this process. In future, it would be interesting to assess the ability of HIPP6 to bind and provide metals to SOD enzymes, especially to Arabidopsis CSD1, which resides in the cytoplasm (Kliebenstein et al., 1998).

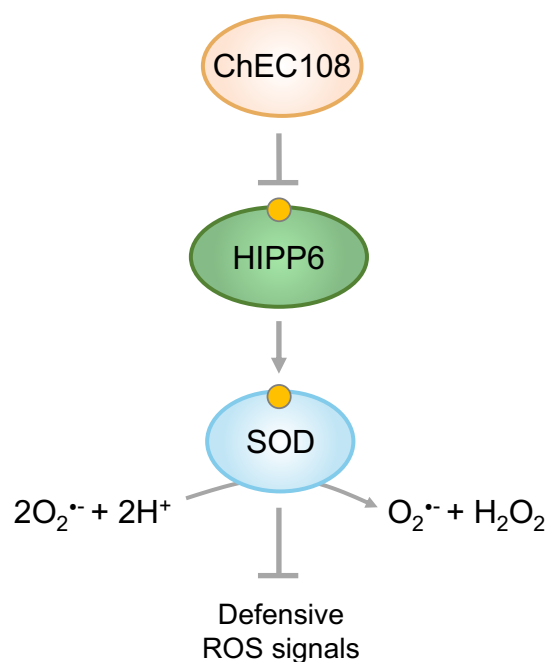


Figure 7.2 – Speculative model for ChEC108-HIPP6-mediated enhancement of defensive ROS signalling. By stabilising HIPP6 at PD or occupying the metal-binding loop of its HMA domains, ChEC108 inhibits the delivery of metal ion cofactors (represented in yellow) to cytoplasmic SOD enzymes via HIPP6. SODs catalyse the dismutation of superoxide radicals, meaning inhibition of SOD activity might enhance defensive ROS signalling.

7.1.4 HIPP6 might regulate cell-to-cell trafficking

Besides HIPP6, other pathogen-targeted PD-located HIPPs are known susceptibility factors (Cowan et al., 2018; Oikawa et al., 2024; Were et al., 2025), hinting that HIPP functions in immunity could be linked to PD regulation. As discussed in 1.3.3, control of cell-to-cell trafficking is key during infection, as PD enable the distribution of plant signalling molecules, nutrients and defensive compounds, while also offering routes for the intercellular spread of pathogens and their effectors. To maintain symplastic connectivity and overcome elicitor-induced channel closure, pathogen-mediated manipulation of PD function is hypothesised to be widespread, with ChEC108 representing a promising case study for this phenomenon in a fungal pathosystem.

I initially hypothesised that HIP6 might be an endogenous regulator of PD aperture, targeted by pathogens to maintain cell-to-cell connectivity during infection. Contrary to these ideas, bombardment experiments described in Chapter 5 suggest ChEC108 does not require HIP6 to target PD or move cell-to-cell. In fact, overaccumulation of HIP6 reduced ChEC108 movement in *N. benthamiana*, and constitutive expression of *ChEC108* left Arabidopsis plants with reduced cell-to-cell trafficking capacity. These results imply that, together, ChEC108 and HIP6 might trigger PD closure, speculatively enforcing a state of cellular stress associated with the accumulation of SA and sugars (see 5.3.3, Tee et al., 2025), which could help explain the stunted growth of *ChEC108*-expressing Arabidopsis lines.

The mechanism by which the ChEC108-HIP6 complex might regulate PD is up for debate. As discussed above (7.1.3), HIP6-mediated modulation of PD-localised ROS signalling could be responsible, yet the involvement of other protein players and precise molecular mechanism remain unknown. Data collected by Dr Barkha Ravi suggests callose metabolism could be involved. When assayed via bombardment, *hipp6-3* mutants displayed reduced cell-to-cell trafficking capacity in comparison to Col-0, accompanied by enhanced callose deposition at pitfields (personal communication). This would suggest HIP6 ordinarily functions in maintaining or promoting intercellular flux, rather than shutting it down (Figure 7.3A). In light of this, perhaps ChEC108 binding inhibits endogenous HIP6 function at PD, removing a negative regulator of channel closure and perturbing its own intercellular movement in the process (Figure 7.3B). Once again, characterisation of HIP6 binding partners would be beneficial to shed light on this topic, as would investigation into the contribution of HIP6 to known PD-localised signalling cascades.

Opposing the model proposed above is the concept of genetic redundancy. The Arabidopsis HIP family is expansive (de Abreu-Neto et al., 2013), and HIP6 is not the only HMA protein to be targeted to PD in this species (Guo et al., 2021; Leonte, 2020), begging the question: would inhibition of HIP6 function by ChEC108 not be compensated for by other HIPs? Beyond PD regulation, it is thus likely that other signalling events might underly the severe growth phenotypes of *HIP6*- and *ChEC108*-expressing plants, such as those discussed in 7.1.2. Alternatively, as proposed by Guo et al. (2021) in the context of CKX degradation in the ER, it is possible that HIPs possess overlapping yet non-identical functions at PD. Indeed, Arabidopsis *HIP33* and *HIP34* were previously reported to *negatively* regulate PD permeability in roots, as independent or simultaneous mutation of these genes enhanced phloem unloading of GFP, expressed under control of the companion cell-specific *SUCROSE SYMPORTER 2* (*SUC2*) promoter. Clearly there remains much to learn about the biological significance of HIP localisation to PD, as well as the extent of genetic redundancy in this context.

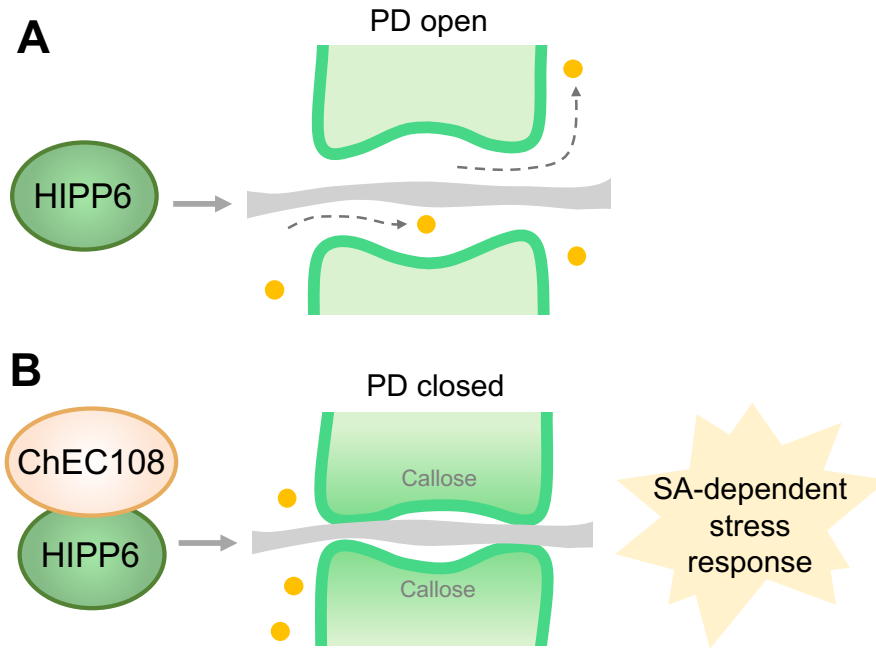


Figure 7.3 – Speculative model for ChEC108-HIPP6-mediated stress signalling via PD closure. A. HIPP6 ordinarily promotes cell-to-cell trafficking by maintaining PD channels in an open state. B. ChEC108 binding inhibits the endogenous functions of HIPP6, leading to channel closure (likely involving enhanced callose deposition) and induction of a stress response, which could be a consequence of SA accumulation.

7.2 Is heavy metal binding important for HIPP6 function?

In addition to being implicated in immunity, data collected as part of this work suggest HIPP6 could function as a metalloprotein. As discussed in 3.3.3, following the discovery of a metal ion positioned at the binding interface of both ChEC108-HMA1 and ChEC108-HMA2 crystal structures (identified as Zn^{2+} in the latter), I hypothesise that with its tandem HMA domains, HIPP6 might associate with metals in the absence of ChEC108. This idea is supported by in silico structural modelling and the work of Suzuki et al. (2002), who suggested HIPP6 (CdI19) HMA domains are capable of binding Cd, Cu and Hg in vitro. Alternatively, as metal ions are known to serve as ‘matchmakers’ for proteins, acting as bridges by enabling the formation of strong intermolecular bonds (Lu, 2010), one cannot rule out the possibility that metal coordination by HIPP6 merely facilitates protein-protein interaction and occurs only in the presence of ChEC108. However, given three out of four residues at the metal-binding site of ChEC108-HMA complexes originate from the HMA, and free heavy metals are not expected to be highly accessible in the plant cytosol due to their inherent toxicity, it seems likely that HIPP6 would provide the metal in the context of in vivo ChEC108-HIPP6 interaction.

Looking beyond HIPP6, HMA proteins are frequently described as metallochaperones, and have been reported to function in transport and detoxification of heavy metals in

both prokaryotes and eukaryotes (Harrison et al., 2000, also see 1.2.1 and 1.2.2). When the plant-specific HIPP family was first described by Dykema et al. (1999), ATPF3 (HIPP7) was discovered to bind Cu^{2+} , Ni^{2+} or Zn^{2+} in a reversible manner within IMAC columns. Subsequently, using in vitro gel-binding assays, ATPF6 (HIPP26) was shown to bind Pb^{2+} , Cd^{2+} and Cu^{2+} ions (Gao et al., 2009), and HIPP3 was shown to bind Zn^{2+} by inductively coupled plasma mass spectrometry (Zschiesche et al., 2015). OsHIPP04 from *O. sativa* and TaHIPP1 from *T. aestivum* were also suggested function as a Cu chaperones, as expression of these *HIPP* genes favoured yeast growth on media with excess Cu^{2+} (Song et al., 2021; Zhang et al., 2015). Although metal-binding capacity seems common among in vitro-purified HMA domains, evidence for direct binding in planta is notably lacking, and the specificity and fate of associated metals is unclear.

Despite a lack of mechanistic insights, HIPPs are becoming increasingly recognised as regulators of plant heavy metal homeostasis. Namely, HIPP6, HIPP20, HIPP21, HIPP22 and HIPP26 are thought to promote Cd detoxification in Arabidopsis (Gao et al., 2009; Suzuki et al., 2002; Tehseen et al., 2010), and studies in crops continue to identify numerous metal stress-responsive *HIPP* genes as positive regulators of metal tolerance (Khan et al., 2019; Wei et al., 2023; Zhang et al., 2020a, 2025, 2020b). Admittedly, it is puzzling to consider why vascular plants would require expansive HIPP and HPP families to fulfil such niche roles in heavy metal homeostasis. Also, the CXXC metal-binding motif is not conserved throughout the entire HIPP family, suggesting metals must not be critical for the function of *all* HIPPs. Considering this, one cannot rule out the possibility that HPP/HIPP regulation of heavy metal tolerance is indirect, for example, via regulation of broad stress-responsive signals such as ROS (see 7.1.3). Nevertheless, the appearance of Zn^{2+} in the ChEC108-HMA2 crystal structure raises the possibility that HIPP6 could contribute to heavy metal tolerance by directly binding toxic ions.

It is worth noting that metals identified in complex with recombinant proteins do not necessarily match the biologically-relevant metal species, as sample preparation procedures, metal contamination and experimental conditions such as pH and temperature can affect the occupancy of metal-binding sites (Handing et al., 2018). Zn is the most common metal to appear in protein structures deposited on the PDB, followed by Fe, Ca, and Mg (Permyakov, 2021), and I recognise that the presence of Zn^{2+} at the ChEC108-HMA2 interface could be an artefact of in vitro purification. To explore the biological relevance of HIPP6-Zn binding, Dr Barkha Ravi measured the primary root growth of Col-0 and *hipp6-3* seedlings on sterile media with or without 350 μM ZnSO_4 supplementation, yet root growth was similarly inhibited by ZnSO_4 across both genotypes (personal communication). In contrast, *hipp6-3* seedlings were found to be hypersusceptible to Cu stress, as suggested by their more severe root growth inhibition (versus Col-0) in the presence of 10, 25 or 50 μM CuSO_4 (Dr Barkha Ravi, personal

communication). These phenotypes implicate HIP6 as a positive regulator of Cu tolerance, suggesting this protein likely binds Cu in planta, as opposed to Zn.

As previously discussed (3.3.3), analysis of crystal structures using the CMM online tool suggests Cu could feasibly occupy the position of Zn at the ChEC108-HMA interfaces, and AlphaFold3 modelling suggests full-length HIP6 could bind Cu^{2+} using both its HMA domains. Meanwhile, in vitro data from the laboratory of Prof. Christopher Dennison, Newcastle University, UK suggested HMA1 and HMA2 from HIP6 can each bind Cu^+ ions with 1:1 stoichiometry (personal communication). I can therefore imagine two possible scenarios concerning HIP6 metal-binding in planta: (1) HMA1 and HMA2 ordinarily sequester a single Cu ion, with the four Cys residues from their two CXXC motifs forming a tetrahedral coordination site (Cys_4). In the presence of ChEC108, one of the HMA domains is displaced, yet Cu would be retained in the complex, coordinated at a $\text{Cys}_2\text{HisAsp}$ site. Alternatively, (2) HMA1 and HMA2 might each bind Cu via their respective CXXC motifs, either of which could be incorporated into a ChEC108-HMA interface.

In future, deciphering the precise geometry and specificity of metal-binding could be valuable for providing insight into HIP6 function, and indeed the consequences of ChEC108 binding. For example, high-coordinate (involving 4-5 ligands bonded to the metal) high affinity metal environments are often utilised by metalloenzymes, allowing them to retain their co-factors during catalysis (for a review of Cu coordination chemistry, see Rubino and Franz, 2012). Conversely, metal trafficking proteins might contain high-coordinate low affinity, or low-coordinate (2-3 ligands) environments, facilitating transfer of associated metals when necessary. Cys-rich sites, such as those offered by HIP6, often favour Cu^+ over Cu^{2+} , and are commonly found in Cu trafficking proteins, allowing them to avoid redox cycling of Cu^{2+} to Cu^+ and visa versa, which could result in the production of toxic hydroxyl radicals (Rubino and Franz, 2012). One could therefore speculate that alteration of the redox potential of HIP6-associated metal environments upon ChEC108 binding could influence local ROS levels. Whether or not ChEC108 (or *Ch* infection more generally) influences the function of HIP6 in heavy metal tolerance has yet to be investigated.

7.2.1 Why would a metal-binding protein localise to PD?

To date, very few studies have investigated the relationship between heavy metal homeostasis and PD function, making it a challenge to understand the functional relevance of HIP6 localisation to PD. A possible clue comes from a study by O'Leary et al. (2018), in which heavy metal species were reported to have distinct effects on PD function in roots. Specifically, Fe stress reduced channel permeability in a CALS12-dependent manner, while Cu stress enhanced

permeability in a β -1,3-glucanase-dependent manner. While the components involved in metal sensing and signalling in this context remain unknown, it is tempting to speculate that HPPs could be involved; ‘capturing’ heavy metals and perhaps transferring them to metalloenzymes, thereby triggering signalling cascades that feedback to modify PD function.

If HPPs and HPPs function as metallochaperones at PD, which proteins could be the recipients of their associated metals? As discussed above (7.1.3) HPP/HIPP function has been previously linked to Cu/Zn SOD activity, although SODs are not known to be enriched at PD. On the other hand, CATALASE enzymes (CAT2 and CAT3), which utilise Fe cofactors to degrade hydrogen peroxide (Mhamdi et al., 2010), appeared in an Arabidopsis PD proteome (Fernandez-Calvino et al., 2011). Whether or not HIPP6 is capable of trafficking Fe to promote ROS scavenging remains to be determined.

Looking beyond, proteomic analysis suggests Arabidopsis PD could house numerous Zn- and Cu-associated proteins representing candidate metal recipients (Fernandez-Calvino et al., 2011; Kraner et al., 2017), although their localisations have not been confirmed. These include SKU5 and multiple SKU5-SIMILAR (SKS) proteins, which were further highlighted as core PD components by comparative proteomic work (Johnston et al., 2023). SKU5 and SKS1 are GPI-anchored proteins that direct cell wall formation in roots by controlling apoplastic Fe accumulation upstream of ROS production via RBOH enzymes (Chen et al., 2023). However, despite exhibiting structural similarity to multicopper oxidases enzymes, which employ multiple Cu cofactors organised into mononuclear and trinuclear centres for electron transfer (InterPro: IPR045087), SKU5 and SKS proteins lack highly conserved His residues for Cu-binding, making their enzymatic function unclear (Sedbrook et al., 2002).

UCLACYANIN 2 (UCC2), a putative Cu-containing phytocyanin family member, was also identified in the Arabidopsis PD proteome (Fernandez-Calvino et al., 2011). Spectroscopic analysis of the purified Cu-binding domain of a related protein, UCC1, suggests phytocyanins likely associates with Cu in planta, where they are proposed to take part in redox reactions with small molecular weight compounds (Nersissian et al., 1998). Delving deeper into phytocyanin function, Reyt et al. (2020) discovered that UCC1 localises to a central nanodomain of the Casparian strip of Arabidopsis vascular tissue and together with UCC2, promotes lignification of the cell wall in this area. However, the significance of Cu-binding was not discussed, and mCherry-UCC1 fusions were not observed at PD (Reyt et al., 2020). Other Cu-containing proteins uncovered in PD proteomes include a COPPER AMINE OXIDASE (CuAO) and L-ASCORBATE OXIDASE (AO) (Fernandez-Calvino et al., 2011), each involved in redox processes, yet are reported elsewhere to be extracellular proteins (Mellidou and Kanellis, 2024; Planas-Portell et al., 2013). Furthermore (unlike HIPP6), SKU5, SKS proteins, UCC1

and UCC2 possess GPI anchors, suggesting they function in the apoplast (Borner et al., 2003). Therefore, hypothetically, any HIPP6-associated heavy metals must somehow traverse the plasma membrane in order to be delivered to these proteins, assuming their cofactors were not already acquired en route to the extracellular space.

Rather than passing cofactors to enzymes, HIPP6 could shuttle metals to transporters to facilitate their export from cells, or even accept them upon import. COPPER TRANSPORTER 5 (COPT5) and NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3 (NRAMP3) were detected in PD fractions (Fernandez-Calvino et al., 2011), despite being functionally characterised as metal exporters on the tonoplast membrane (Klaumann et al., 2011; Thomine et al., 2003). In the *Populus trichocarpa* PD proteome, an ortholog of ZINC TRANSPORTER PRECURSOR 11 (ZIP11) was identified (Leijon et al., 2018), which is proposed to import Zn based on experiments conducted in *S. cerevisiae* (Milner et al., 2013). For now, it remains unclear as to why metal transporters would be enriched at intercellular channels. Further cell biological experiments would be required to confirm the presence of metal transporters at PD, and investigate the influence of HIPPs on metal influx and efflux. In summary, there is no obvious protein candidate to receive putative HIPP6-associated metals, and the functions of various metal-bound proteins at PD could benefit from further study.

7.2.2 Could HIPP6 metal-binding and defensive functions be interlinked?

Even outside the context of PD, it is challenging to connect the concept of HIPP6 metal-binding to plant immunity. In one study, Zhang et al. (2015) identified TaHIPP1 as a negative regulator of *T. aestivum* resistance to *Puccinia striiformis* f. sp. *tritici*, and also observed enhanced tolerance of yeast (*Schizosaccharomyces pombe*) to Cu toxicity when TaHIPP1 was heterologously expressed. However, a mechanistic link between these two findings has yet to be established. Elsewhere, CCP was characterised as a positive regulator of Arabidopsis resistance to *Pto* DC3000, supposedly via recruitment of TGA2 to the promoters of defence genes (Chai et al., 2020). Although Cu-binding by CCP was not explicitly demonstrated, mutation of the CXXC motif or treatment with the Cu chelator BCS inhibited CPP self-association and nuclear import (Chai et al., 2020), suggesting metal-binding is critical for proper localisation of this HPP. Metal-binding also appears to be critical for HIPP6 localisation, as I observed HIPP6^{MM} mutants (lacking functional CXXC motifs) to be compromised in their ability to target PD. In addition, as discussed above (7.1.3) HPP/HIPP-mediated delivery of metal cofactors to enzymes involved in ROS metabolism could influence plant immune responses (Song et al., 2021), yet a metallochaperone function for HIPP6 has yet to be confirmed.

A speculative hypothesis is that heavy metals carried by HIPP6 domains could be released during stress and employed as anti-microbial weapons. Evidence from studies of hyperaccumulator plants suggest that high concentrations of heavy metals might deter or kill herbivores and pathogens directly, referred to as the ‘elemental defence hypothesis’ (reviewed by De et al., 2024; Höerger et al., 2013). For example, hyperaccumulation of Zn, Ni, or Cd by *Thlaspi caerulescens* enhanced resistance to the apoplastic bacterium *P. syringae* pv. *maculicola* (Fones et al., 2010). Also, local accumulation of Zn around invasion sites was associated with increased resistance of *Arabidopsis* (a non-accumulator plant), to the fungal necrotroph *Plectosphaerella cucumerina* (Escudero et al., 2022). One could imagine that HIPP6 might sequester metals within PD, ready for delivery or release as part of a strategy to enhance stress tolerance. However, evidence for elemental defence against intracellular pathogens is limited, and it is difficult to comprehend how the storage of metals at such small subcellular compartments at the cell periphery could make a meaningful defensive contribution.

Beyond the examples cited above, there is evidence to suggest metal-binding is dispensable for HMA protein function in plant immunity. While the majority of host HMA proteins implicated in plant microbe interactions contain a conserved metal-binding motif, there are notable exceptions: OsHIPP19, OsHIPP20, OsHIPP02 and OsHIPP03 from *O. sativa*, and sHMA94 and sHMA25 from *S. italica* (Table 1.1). Also, regions corresponding to the metal-binding loop (between $\beta 1$ and $\alpha 1$) of HMA-like integrated domains within NLRs Pik-1 and RGA5 do not include two Cys residues (Table 1.3), suggesting metal-binding is not required for baiting effectors in these systems. Consistent with this, crystal structures of MAX effectors in complex with HMA domains from their corresponding host targets have revealed that the $\beta 1$ - $\alpha 1$ loop rarely constitutes part of the binding interface (see 3.3.1). It thus seems that HMA protein function is diverse even amongst those targeted by pathogens, with some (including HIPP6) likely associating with metals, and others not.

7.3 Conclusion

Are we any closer to understanding why HMA proteins are frequently targeted by pathogens?

In summary of the work presented in this thesis, I have identified an *Arabidopsis* HIPP protein that is bound directly by the PD-localised, cell-to-cell mobile candidate *Ch* effector, ChEC108. With a novel binding mechanism involving coordination of a heavy metal ion between ChEC108 and the HIPP6 HMA domains, this adds support to the idea that phytopathogens have convergently evolved to target host HMA proteins. Although the intracellular consequences of ChEC108-HIPP6 binding were not fully established, I have shown

that HIP6 is more likely to promote Arabidopsis resistance to *Ch* than act as a susceptibility factor, and that ChEC108-HIP6 association might initiate signalling events accompanied by a decrease in intercellular trafficking. These findings raise questions surrounding the fundamental role of metal-binding proteins at PD, as well as the mechanisms by which plants might defend their PD from effector-mediated manipulation.

A key takeaway from this thesis (based on the experimental work and accompanying literature review) is that HIPs and HPPs, despite sharing common domains, are functionally diverse. Given the contrasting subcellular localisations, domain architectures and binding partners of these proteins, I conclude that there is unlikely to be a common mechanism that could simultaneously explain their involvement in evolutionarily distinct pathosystems *and* their regulation of plant tolerance to various abiotic stresses. I still favour the hypothesis that HPP/HIPs play important roles in plant immunity, acting as both susceptibility factors or positive regulators of resistance, depending on the protein and pathosystem in question, and am confident that mechanistic insights could be gained by in-depth study of individual cases in future.

Chapter 8

Appendix

8.1 Supplementary tables

To accompany Chapter 2, the following Tables S1 to S10 contain details of the plasmids, primers and plant materials used in this work, as well as the composition of buffers and media. Table S11 contains supplementary data relevant to Chapter 3. Table S12 and Table S13 contain supplementary data relevant to Chapter 4.

Table S1 – DNA cloning primers. GG = Golden Gate, GW = Gateway[®], IF = In-Fusion[®]. Unless otherwise specified, primers were designed as part of this work.

Primer	System	Sequence (5'-3')	For amplification of
001_HIPP6_Lv0_FWD	GG	TGAAGACGAAATGGGTGAGAAAAAGGAAGAAACGG	HIPP6
004_HIPP6_Lv0_no_CaaX_REV	GG	TGAAGACGACGAACTCCCATTTTGGATTCTCATCAC	HIPP6 ^{ΔC4}
011_Lv0_ChEC108_CDS1_Rev	GG	TGAAGACGAAAGCCCTACTTAGGCGCCCTTTTCTTTTG	ChEC108
012_Lv0_ChEC108_CDS1_Fw	GG	TGAAGACGAAATGGACGACTTCAAAAGGATTC	ChEC108
013_Lv0_HIPP6_HMA1_Fw	GG	TGAAGACGAAATGGGAATCACCCCGTC	HMA1
014_Lv0_HIPP6_HMA1_Rev	GG	TGAAGACGAAAGCCCTACTCTTTCTTCGGCGCG	HMA1
015_Lv0_HIPP6_HMA2_Fw	GG	TGAAGACGAAATGAAAGAGAGTACTGTGGTTTIG	HMA2
016_Lv0_HIPP6_HMA2_Rev	GG	TGAAGACGAAAGCCCTACTTTTGGCCGGAACAACCTTC	HMA2
021_sgRNA_Rev	GG	TGTGCTCTCAAGCGACCCAGAAATTGAAC	sgRNA templates ^a
023_HIPP6_C1_B	GG	TGTGCTCTCAATTGTACTACTACGATGGGTGTTAAGAGCTATGCTGGAA	HIPP6 sgRNA templates
024_HIPP6_C2_A	GG	TGTGCTCTCAATTGGTTAGAACCGGTTTGTGAGGTTTAAAGAGCTATGCTGGAA	HIPP6 sgRNA templates
025_HIPP6_C2_B	GG	TGTGCTCTCAATTGTAATCTAAACCGGCAAGATGTTTAAAGAGCTATGCTGGAA	HIPP6 sgRNA templates
026_HIPP3_C1_A	GG	TGTGCTCTCAATTGTACCTCGCCCATGATGAATGTTTAAAGAGCTATGCTGGAA	HIPP3 sgRNA templates
027_HIPP3_C1_B	GG	TGTGCTCTCAATTGTATACGCAACAACATAGGTGTTTAAAGAGCTATGCTGGAA	HIPP3 sgRNA templates
028_HIPP3_C2_A	GG	TGTGCTCTCAATTGAATGTCAACATTCACITTAGGTTTAAAGAGCTATGCTGGAA	HIPP3 sgRNA templates
029_HIPP3_C2_B	GG	TGTGCTCTCAATTGTATTTGGACGAATTAGTTTAAAGAGCTATGCTGGAA	HIPP3 sgRNA templates
030_HIPP6_C1_C	GG	TGTGCTCTCAATTGACGAGGTCAGGATTAGTATGGTTTAAAGAGCTATGCTGGAA	HIPP6 sgRNA templates
053_Lv0_ChEC108_Rev2	GG	TGAAGACGACGAACTCTTAGGGCCCTTTTCTTTTG	ChEC108
095_HMA1_forPikm-1_Fw	GG	TGAAGACGACAGAGGAATCACCAACCGTCGTTATG	HMA1 for integration into Pikm-1
096_HMA1_forPikm-1_Rev	GG	TGAAGACGACATCCTCTTTCTTCGGCGG	HMA1 for integration into Pikm-1
097_HMA2_forPikm-1_Fw	GG	TGAAGACGACAGAAAAAGAGACTACTGTGGTTTGTGAAAC	HMA2 for integration into Pikm-1
098_HMA2_forPikm-1_Rev	GG	TGAAGACGACATCCTTTTGGCCGGAAC	HMA2 for integration into Pikm-1

Continued on next page

(Continued)

Primer	System	Sequence (5'-3')	For amplification of
108_HIPP6_Lv0_trunc_Rev	GG	AGAAGACCTAAGCTTACAACTCAGGCGCACGC	HIPP6 ^{ΔC12}
118_Lv0_HIPP6_CDS1_Rev	GG	AGAAGACCTAAGCTTACATCACAGAACACCCATTGTGGATTG	HIPP6
121_Lv0_HMA1_GxxG_Fw	GG	TGAAGACGAGCATGGTGAAGGTGGCGGG	HMA1 ^{GXXG}
122_Lv0_HMA1_GxxG_Rev	GG	TGAAGACGAATGCATATCAAGCTTCATAACGACGGT	HMA1 ^{GXXG}
123_Lv0_HMA2_GxxG_Fw	GG	TGAAGACGAGTTACATGGTGAAGGTGGCGAAC	HMA2 ^{GXXG}
124_Lv0_HMA2_GxxG_Rev	GG	TGAAGACGATAACTTGGTTTTCAAAAACACAGTACTCTCTTTAG	HMA2 ^{GXXG}
125_Lv0_ChEC108_D164R_Fw	GG	TGAAGACGACGTTTGTGCTGAACATATATGGCATAACACGTTTC	ChEC108 ^{D164R}
126_Lv0_ChEC108_D164R_Rev	GG	TGAAGACGAAACGATGGGCAACCTCCCCA	ChEC108 ^{D164R}
127_Lv0_mScarlet_NT_Fw	GG	TGAAGACGACCATGGTGAAGCAAGGCGGAGG	mScarlet
128_Lv0_mScarlet_NT_Rev	GG	TGAAGACGACATTCACCCCTTGACAGCTCGTCCATG	mScarlet
129_TurboID_NT1_frag2_Fw	GG	TGGTCTCAITCTGGCGGAGGTTACAGG	4xFLAG-TurboID
130_TurboID_NT1_frag2_Rev	GG	TGGTCTCACATTGAACCTCTCCAGATCC	4xFLAG-TurboID
131_4xMyc_NT1_frag1_Fw	GG	TGGTCTCACCATGGAACAAAAAGTTGATCTCTGAAGAG	4xMyc
132_4xMyc_NT1_frag1_Rev	GG	TGGTCTCAAGAAAGGTCTCTTCAGAAATAAGTTTITG	4xMyc
136_Lv1_ChEC108_CDS1ns_Fw	GG	TGGTCTCACCATGGACGACTTCAAAGGATTC	ChEC108 for <i>Ch</i>
137_Lv1_ChEC108_CDS1ns_Rev	GG	TGGTCTCACGAACTCTTAGG	ChEC108 for <i>Ch</i>
144_HMA1_Pikm-1_Fw_2	GG	TGAAGACGAAAAAAAATCAAAACGCATC	HMA1 mutants for integration into Pikm-1
145_HMA2_Pikm-1_Fw_2	GG	TGAAGACGACATAAAATCAAAAGAATAGTC	HMA2 mutants for integration into Pikm-1
146_HMA1_Pikm-1_mbl_F	GG	TGAAGACGACAGAGGAATCACCACCGTCGTATGAAGCTTGCTAT GGAAGGAAATAATTGCGGCAAAATCGTCTTCA	HMA1 mutants for integration into Pikm-1

Continued on next page

(Continued)

Primer	System	Sequence (5'-3')	For amplification of
147_HMA1_Pikm-1_mbl_R	GG	TGAAGACGATTTTCCGCAATTATTTCTTCCATAGCAAGCTTCA TAACGACGGTGGTGATTCCTCTCTGTCGCTTCA	HMA1 mutants for integration into Pikm-1
148_HMA1_Pikm-1_mbl-C_F	GG	TGAAGACGACAGAGGAATCACCAACCGTCGTATGAAAGCTTGCTAT GGAATGCAATAATTGGGCAAAATCGTCTTCA	HMA1 mutants for integration into Pikm-1
149_HMA1_Pikm-1_mbl-C_R	GG	TGAAGACGATTTTCCGCAATTATTCATTCATAGCAAGCTTCA TAACGACGGTGGTGATTCCTCTCTGTCGCTTCA	HMA1 mutants for integration into Pikm-1
150_HMA1_Pikm-1_mbl-43_F	GG	TGAAGACGACAGAGGAATCACCAACCGTCGTATGAAAGCTTAAAGAT GGATTGTGAAGGTTCGGCAAAATCGTCTTCA	HMA1 mutants for integration into Pikm-1
151_HMA1_Pikm-1_mbl-43_R	GG	TGAAGACGATTTTCCGCAACCTTCACATCCATCTTAAGCTTCA TAACGACGGTGGTGATTCCTCTCTGTCGCTTCA	HMA1 mutants for integration into Pikm-1
152_HMA2_Pikm-1_mbl_F	GG	TGAAGACGACAGAAAAGAGAGTACTGTGTTTGA AAAACCGCTAT GGAAGGAAAATAATTGGCAACATATCGTCTTCA	HMA2 mutants for integration into Pikm-1
153_HMA2_Pikm-1_mbl_R	GG	TGAAGACGATGTTCGCAATTATTCCTTCCATAGCGGTTTCA AAACCAAGTACTCTCTTTTCTGTCGCTTCA	HMA2 mutants for integration into Pikm-1
154_HMA2_Pikm-1_mbl-C_F	GG	TGAAGACGACAGAAAAGAGAGTACTGTGTTTGA AAAACCGCTAT GGAATGCAATAATTGGCAACATATCGTCTTCA	HMA2 mutants for integration into Pikm-1
155_HMA2_Pikm-1_mbl-C_R	GG	TGAAGACGATGTTCGCAATTATTCATTCATAGCGGTTTCA AAACCAAGTACTCTCTTTTCTGTCGCTTCA	HMA2 mutants for integration into Pikm-1
156_HMA2_Pikm-1_mbl-43_F	GG	TGAAGACGACAGAAAAGAGAGTACTGTGTTTGA AAAACCAAGAT GGATTGTGAAGGTTCGCAACATATCGTCTTCA	HMA2 mutants for integration into Pikm-1
157_HMA2_Pikm-1_mbl-43_R	GG	TGAAGACGATGTTCGCAACCTTCACATCCATCTTGGTTTCA AAACCAAGTACTCTTTTCTGTCGCTTCA	HMA2 mutants for integration into Pikm-1
158_HMA1_forPikm-1_Lv2_Rev	GG	TGGTCTCAAAGCCTCTTCTTCGGCGGGCG	HMA1 for integration into extended (ex) Pikm-1

Continued on next page

(Continued)

Primer	System	Sequence (5'-3')	For amplification of
159_HMA2_forPikm-1_Lv2_Rev	GG	TGGTCTCAAAGCCTTTTGGCCGGAACAACTTC	HMA2 for integration into extended (ex) Pikm-1
162_ChEC108_Gateway_Fw	GW	CACCATGGACGACTTCAAAGGATTCA	ChEC108
163_eGFP_mScarlet_Gateway_Rev	GW	TTACTTGTACAGCTCGTCCATG	eGFP or mScarlet
164_HMA1_IMGG_forPikm-1_Fw	GG	TGAAGACGACAGATAATGGAGGGGGAATCACACCCTG	HMA1 ^{IMGG} for integration into Pikm-1
165_HMA2_IMGG_forPikm-1_Fw	GG	TGAAGACGACAGATAATGGAGGGAAAGAGAGTACTGTGG	HMA2 ^{IMGG} for integration into Pikm-1
171_Lv1_Sig-ChEC108_CDS1_Rev	GG	TGGTCTCATACCCCTACTTAGGGCCCTTTTCTTTTG	ChEC108 ^{D164R} for <i>Ch</i>
180_IF_AvrPikD_pro_Fw	IF	GCGGCCGCTCTAGAACTAGTAATAAAATCTTGCAGCCGT	AVR-PikD promoter for <i>M. oryzae</i>
181_IF_AvrPikD_pro_Rev	IF	GATGAGAAAGAGCCCTCATTTATTGACTTTTCAAAATAATGTC	AVR-PikD promoter for <i>M. oryzae</i>
182_IF_ChEC108_CDS1ns_Fw	IF	AAAGTCAATAATGAGGCTTCTTCTCATCTTTGCC	SP-ChEC108 for <i>M. oryzae</i>
183_IF_ChEC108_CDS1ns_Rev	IF	CTTGCTCACCATGAATCCGAACTCTTAGGGCCCTTT	SP-ChEC108 for <i>M. oryzae</i>
184_IF_AvrPikD_pro_Fw2	IF	TGCAGCCCAATGTGGAATTCATAATAAATCTTGCAGCCGT	AVR-PikD promoter for <i>M. oryzae</i>
185_IF_ChEC108_CDS1ns_Rev2	IF	CCTTGCTCACCATCGAACTCTTAGGGCCCTTTTTC	SP-ChEC108 for <i>M. oryzae</i>
195_IF_AvrPikD_pro_Rev2	IF	TCCTTTGAAGTCGTCCATTATTGACTTTTCAAAATAATGTC	AVR-PikD promoter for <i>M. oryzae</i>
196_IF_ChEC108_CDS-SP_Fw2	IF	AAAGTCAATAATGGACGACTTCAAGGATTCACT	ChEC108 for <i>M. oryzae</i>
209_tTrpC_Rev	IF	TCGACGGTATCGATAAGCTCTCGAGTGGAGATGTGGAGTGGG	<i>ChEC108-GFP::tTrpC</i> for <i>M. oryzae</i> ^b

^a Sourced from Dr Sebastian Samwald^b Sourced from Jave Bautista

Table S2 – Composition of buffers. All buffers are dissolved in water, unless otherwise specified. DTT = dithiothreitol, EDTA = ethylenediaminetetraacetic acid, HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, MES = 2-(N-morpholino)ethanesulfonic acid, PMSF = phenylmethylsulfonyl fluoride.

Name	Composition
A1	150 mM Tris-HCl, pH 8.0 500 mM NaCl, 5% (v/v) Glycerol 50 mM Glycine 20 mM Imidazole
A4	20 mM HEPES, pH 7.5 150 mM NaCl
B1	See recipe for A1 + 500 mM Imidazole
Blocking buffer	20 mM Tris-HCl, pH 7.5 150 mM NaCl 0.1% (v/v) Tween20 5% (w/v) Skimmed milk powder
Coomassie blue (CB) staining solution	3.8 mM Coomassie blue 10% (v/v) Acetic acid 40% (v/v) Methanol
CB de-staining solution	5.2% (v/v) Acetic acid 42% (v/v) Methanol
Dipping buffer	0.5x Murashige Skoog Medium, including vitamins (Duchefa Biochemie) 5% Sucrose (w/v) 0.0025% (v/v) Silwet L-77
gDNA extraction buffer	0.2 M Tris-HCl, pH 8 0.4 M LiCl 25 mM EDTA 1% (w/v) SDS
Infiltration buffer	10 mM MES, pH 5.7 10 mM MgCl ₂ 100 µM Acetosyringone

Continued on next page

(Continued)

Name	Composition
Protein extraction buffer	50mM Tris-HCl, pH 7.5 150mM NaCl 1mM EDTA, pH 8 10% (v/v) Glycerol 0.50% (v/v) NP40 / IGEPAL CA-630 cOmplete™ ULTRA EDTA-free glass vials protease inhibitor tablets (1 per 50 mL) (Roche) PhosSTOP EASYpack phosphatase inhibitor cocktail tablets (1 tablet per 50 mL) (Roche) 1 mM Na ₂ MoO ₄ ·2H ₂ O 1 mM NaF 5 mM DTT 1 mM PMSF
Running buffer	25 mM Tris 200 mM Glycine 0.1% (w/v) SDS
SDS-PAGE loading buffer-1	100 mM Tris, pH 6.8 42% (v/v) Glycerol 2.5% (w/v) SDS 0.04% (w/v) Bromophenol blue 10% (v/v) β-mercaptoethanol
SDS-PAGE loading buffer-2	0.25 M Tris, pH 6.75 40% (v/v) Glycerol 8% SDS (w/v) 0.02% (w/v) Bromophenol blue
Tris-buffered saline with Tween-20 (TBST)	20 mM Tris-HCl, pH 7.5 150 mM NaCl 0.1% (v/v) Tween20
Transfer buffer	25 mM Tris 200 mM Glycine
Trypan blue staining solution	25% (v/v) Glycerol 25% (v/v) Lactic acid 25% (v/v) Phenol 0.025% (w/v) Trypan blue

Table S3 – Golden Gate plasmids. If no source is given, the plasmid was generated as part of this work.

Code	Level	Contents	Source
FP16001	Lv1	<i>p35S::mCherry-HIPP6::t35S</i>	
FP06281	Lv1	<i>p35S::REM1.4-mCherry::t35S</i>	Dr Joanna Jennings
FP06282	Lv1	<i>p35S::REMX-mCherry::t35S</i>	Dr Joanna Jennings
FP06283	Lv1	<i>p35S::DRP2B-mCherry::t35S</i>	Dr Joanna Jennings
FP06285	Lv1	<i>p35S::HIPP3-mCherry::t35S</i>	Dr Joanna Jennings
FP06284	Lv1	<i>p35S::HIPP6-mCherry::t35S</i>	Dr Joanna Jennings
FP09048	Lv2	<i>p35S::LTI6b-mCherry::tHSP</i>	Dr Sebastian Samwald
FP16005	Lv1	<i>p35S::mCherry-HIPP6^{ΔC4}::t35S</i>	
FP08055	Lv1	<i>p35S::HMA1-mCherry::t35S</i>	Dr Andrew Breakspear
FP08056	Lv1	<i>p35S::HMA2-mCherry::t35S</i>	Dr Andrew Breakspear
FP08057	Lv1	<i>p35S::tHMA-mCherry::t35S</i>	Dr Andrew Breakspear
FP08063	Lv1	<i>p35S::mCherry::t35S</i>	Dr Andrew Breakspear
FP16012	Lv1	<i>pT7::6×His-ChEC108::tT7</i>	
FP16013	Lv1	<i>pT7::6×His-GB1-ChEC108::tT7</i>	
FP16014	Lv1	<i>pT7::6×His-MBP-ChEC108::tT7</i>	
FP16015	Lv1	<i>pT7::6×His-HMA1::tT7</i>	
FP16016	Lv1	<i>pT7::6×His-GB1-HMA1::tT7</i>	
FP16017	Lv1	<i>pT7::6×His-MBP-HMA1::tT7</i>	
FP16018	Lv1	<i>pT7::6×His-HMA2::tT7</i>	
FP16019	Lv1	<i>pT7::6×His-GB1-HMA2::tT7</i>	
FP16020	Lv1	<i>pT7::6×His-MBP-HMA2::tT7</i>	
FP16021	Lv1	<i>pT7::6×His-tHMA::tT7</i>	
FP16022	Lv1	<i>pT7::6×His-GB1-tHMA::tT7</i>	
FP16023	Lv1	<i>pT7::6×His-MBP-tHMA::tT7</i>	
FP16036	Lv2	<i>pOLE1::FastRed::tOLE1</i> <i>.pYAO::Cas9::tE9</i> <i>.4xHIPP6 sgRNAs</i>	
FP16037	Lv2	<i>pOLE1::FastRed::tOLE1</i> <i>.pYAO::Cas9::tE9</i> <i>.4xHIPP3 sgRNAs</i>	

Continued on next page

(Continued)

Code	Level	Contents	Source
FP16040	LvP	<i>pOLE1::FastRed::tOLE1</i> <i>.pYAO::Cas9::tE9</i> <i>.4xHIPP6 sgRNAs</i> <i>4xHIPP3 sgRNAs</i>	
FP16046	Lv1	<i>pT7::ChEC108::tT7</i>	
FP16047	Lv1	<i>pT7::HMA1::tT7</i>	
FP16048	Lv1	<i>pT7::HMA2::tT7</i>	
FP16049	Lv1	<i>pT7::tHMA::tT7</i>	
FP16054	Lv1	<i>p35S::eGFP::t35S</i>	
FP16055	Lv1	<i>p35S::mCherry::t35S</i>	
FP16057	Lv1	<i>p35S::ChEC108-eGFP::t35S</i>	
FP16072	Lv1	<i>p35S::ChEC108-mCherry::t35S</i>	
FP05207	Lv2	<i>p35S::PDLP5-mCherry</i>	Dr Matthew Johnston
FP03017	Lv1	<i>p35S::NLS-dTomato::t35S</i>	Dr Mina Ohtsu
FP16093	Lv1	<i>p35S::Pikm-1HMA1-HF::t35S</i>	
FP16094	Lv1	<i>p35S::Pikm-1HMA2-HF::t35S</i>	
FP16099	Lv1	<i>pmas::Pikp-2-6xHA::tmas</i>	
FP16103	Lv2	<i>p35S::Pikm-1^{HMA1}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16107	Lv2	<i>p35S::Pikm-1^{HMA2}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP06245	Lv1	<i>pgpdA::SP-ChEC108-Myc-GFP11::tTrpC</i>	
FP16118	Lv1	<i>p35S::mCherry-HIPP6^{ΔC12}::t35S</i>	
FP16121	Lv1	<i>p35S::mCherry-HIPP6::t35S</i>	
FP06151	Lv1	<i>p35S::2xGFP::t35S</i> <i>.pmas::NLS-tdTomato::tmas</i>	Dr Joanna Jennings
FP06272	Lv1	<i>p35S::ChEC108-2xGFP::t35S</i> <i>.pmas::NLS-tdTomato::tmas</i>	Dr Joanna Jennings
-	Lv2	<i>p35S::Pikm-1^{WT}-HF</i> <i>.p35S::Pikp-2-HA</i>	Dr Rafał Zdrzałek
-	Lv1	<i>pAtUBQ10::Myc-AVR-PikD</i>	Dr Rafał Zdrzałek
FP16125	Lv1	<i>pAtUBQ10::ChEC108-4×Myc::t35S</i>	

Continued on next page

(Continued)

Code	Level	Contents	Source
FP16126	Lv2	<i>pAtUBQ10::meGFP-HIPP6::t35S</i>	Dr Joanna Jennings
FP16136	Lv1	<i>p35S::3×FLAG-TurboID-HIPP6::tHSP</i>	
FP16137	Lv1	<i>p35S::3×FLAG-TurboID-HMA1::tHSP</i>	
FP16138	Lv1	<i>p35S::3×FLAG-TurboID-HMA2::tHSP</i>	
FP16140	Lv1	<i>p35S::ChEC108-Myc-GFP11::t35S</i>	
FP06293	Lv2	<i>p35S::GFP1-10-TurboID::tHSP</i>	
FP16147	Lv1	<i>p35S::ChEC108^{D164R}-eGFP::t35S</i>	
FP16149	Lv1	<i>p35S::ChEC108^{D164R}-4×Myc::t35S</i>	
FP16154	Lv1	<i>p35S::ChEC108-mScarlet::t35S</i>	
FP16155	Lv1	<i>pT7::6×His-HMA1^{GxxG}::tT7</i>	
FP16156	Lv1	<i>pT7::6×His-HMA2^{GxxG}::tT7</i>	
FP16157	Lv1	<i>pT7::HMA1^{GxxG}::tT7</i>	
FP16158	Lv1	<i>pT7::HMA2^{GxxG}::tT7</i>	
FP16159	Lv1	<i>pT7::6×His-ChEC108^{D164R}::tT7</i>	
FP16160	Lv1	<i>pT7::ChEC108^{D164R}::tT7</i>	
FP16161	Lv1	<i>pAtUBQ10::meGFP-HIPP6^{M1}::t35S</i>	
FP16162	Lv1	<i>pAtUBQ10::meGFP-HIPP6^{M2}::t35S</i>	
FP16163	Lv1	<i>pAtUBQ10::meGFP-HIPP6^{MM}::t35S</i>	
FP16165	Lv1	<i>p35S::4×Myc-TurboID-HMA1::tHSP</i>	
FP16166	Lv1	<i>p35S::4×Myc-TurboID-HMA2::tHSP</i>	
FP16167	Lv1	<i>p35S::3×FLAG-TurboID::tHSP</i>	
FP16168	Lv1	<i>p35S::3×FLAG-TurboID-ChEC108::tHSP</i>	
FP16170	Lv1	<i>pcos1gpda::SP-ChEC108-4×Myc::tTrpC</i>	
FP16171	Lv1	<i>pcos1gpda::ChEC108-4×Myc::tTrpC</i>	
FP16181	Lv1	<i>p35S::Pikm-1^{HMA1-mbl}-HF::t35S</i>	
FP16182	Lv1	<i>p35S::Pikm-1^{HMA1-mbl-C}-HF::t35S</i>	
FP16183	Lv1	<i>p35S::Pikm-1^{HMA1-mbl-43}-HF::t35S</i>	
FP16184	Lv1	<i>p35S::Pikm-1^{HMA2-mbl}-HF::t35S</i>	
FP16185	Lv1	<i>p35S::Pikm-1^{HMA2-mbl-C}-HF::t35S</i>	
FP16186	Lv1	<i>p35S::Pikm-1^{HMA2-mbl-43}-HF::t35S</i>	
FP16187	Lv2	<i>p35S::Pikm-1^{HMA1}-HF::t35S</i>	
		<i>.pmas::Pikp-2-6xHA::tmas</i>	

Continued on next page

(Continued)

Code	Level	Contents	Source
FP16188	Lv2	<i>p35S::Pikm-1^{HMA1}-HF::t35S</i> <i>.pmas::Pikm-2-6xHA::tmas</i>	
FP16189	Lv2	<i>p35S::Pikm-1^{HMA2}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16190	Lv2	<i>p35S::Pikm-1^{HMA2}-HF::t35S</i> <i>.pmas::Pikm-2-6xHA::tmas</i>	
FP16191	Lv2	<i>p35S::Pikm-1^{HMA1-mbl}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16192	Lv2	<i>p35S::Pikm-1^{HMA1-mbl-C}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16193	Lv2	<i>p35S::Pikm-1^{HMA1-mbl-43}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16194	Lv2	<i>p35S::Pikm-1^{HMA2-mbl}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16195	Lv2	<i>p35S::Pikm-1^{HMA2-mbl-C}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16196	Lv2	<i>p35S::Pikm-1^{HMA2-mbl-43}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
-	Lv1	<i>pAtUBQ10::PWL2-Myc</i>	Dr Rafał Zdrzałek
-	Lv2	<i>p35S::Pikm-1^{OsHIPP43}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA</i>	Dr Rafał Zdrzałek
FP16216	Lv1	<i>pAtUBQ10::ChEC108^{D164R}-eGFP::t35S</i>	
FP16221	Lv1	<i>pAtUBQ10::ChEC108-eGFP::t35S</i>	
FP16222	Lv1	<i>p35S::3×FLAG-TurboID-HIPP6^{M1}::tHSP</i>	
FP16223	Lv1	<i>p35S::3×FLAG-TurboID-HIPP6^{M2}::tHSP</i>	
FP16224	Lv1	<i>p35S::3×FLAG-TurboID-HIPP6^{MM}::tHSP</i>	
FP16239	Lv2	<i>p35S::Pikm-1^{HMA1-IMGG}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16240	Lv2	<i>p35S::Pikm-1^{HMA2-IMGG}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16241	Lv2	<i>p35S::Pikm-1^{mbl-HMA2}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	

Continued on next page

(Continued)

Code	Level	Contents	Source
FP16242	Lv2	<i>p35S::Pikm-1^{HMA-RS}-HF::t35S</i> <i>.pmas::Pikp-2-6xHA::tmas</i>	
FP16243	Lv1	<i>p35S::ChEC108^{D164R}-2xGFP::t35S</i> <i>.pmas::NLS-tdTomato::tmas</i>	
FP16244	Lv1	<i>p35S::ChEC108^{D164R}-GFP::t35S</i> <i>.pmas::NLS-tdTomato::tmas</i>	
FP16245	Lv1	<i>pChEC108::SP-ChEC108^{D164R}::tChEC108</i>	
FP08082	Lv1	<i>pChEC108::SP-ChEC108::tChEC108</i>	
FP16252	Lv1	<i>p35S::4×Myc-TurboID-HIPP6::tHSP</i>	
FP16253	Lv1	<i>pcos1gpda::SP-ChEC108-eGFP::tTrpC</i>	
FP16255	Lv1	<i>p35S::ChEC108^{CCAA}-eGFP::t35S</i>	
FP16256	Lv1	<i>p35S::ChEC108^{D164R-CCAA}-eGFP::t35S</i>	

Table S4 – Gateway® plasmids used in this work.

Code	Contents	Source
-	<i>p35S::eGFP::t35S</i>	Prof. Christine Faulkner
-	<i>p35S::SP^{BIP2}-mRFP-KDEL::p35S</i>	Prof. Christine Faulkner
-	<i>p35S::mRFP::t35S</i>	Prof. Christine Faulkner
-	<i>p35S::mScarlet::t35S</i>	Dr Andrew Breakspear
FP16212	<i>p35S::ChEC108-eGFP::t35S</i>	This work
FP16213	<i>p35S::ChEC108-mScarlet::t35S</i>	This work
FP16214	<i>p35S::ChEC108^{D164R}-eGFP::t35S</i>	This work

Table S5 – In-Fusion® plasmids generated as part of this work.

Code	Contents
FP16257	<i>pAvrPikD::SP-ChEC108-eGFP-3xNLS::tTrpC</i>
FP16258	<i>pAvrPikD::SP-ChEC108-eGFP::tTrpC</i>
FP16261	<i>pAvrPikD::ChEC108-eGFP::tTrpC</i>

Table S6 – Composition of media used in this work. MES = 2-(N-morpholino)ethanesulfonic acid.

Name	Composition
Complete Medium (CM) agar	1 g/l Casamino acids 10 g/l Glucose 2 g/l Peptone 1 g/l Yeast extract 0.1% (v/v) Vitamin supplement: [0.001 g/l Biotin 0.001 g/l Pyridoxine 0.001 g/l Thiamine 0.001 g/l Riboflavin 0.001 g/l Nicotinic acid] 6 g/l NaNO ₃ 0.5 g/l KCl 0.5 g/l MgSO ₄ 1.5 g/l KH ₂ PO ₄ , pH 6.5 ~0.1% (v/v) Trace elements: [22 mg/l ZnSO ₄ ·7H ₂ O 11 mg/l H ₃ BO ₃ 5 mg/l MnCl ₂ ·4H ₂ O 5 mg/l FeSO ₄ ·7H ₂ O 1.7 mg/l CoCl ₂ ·6H ₂ O 1.6 mg/l CuSO ₄ ·5H ₂ O 1.5 mg/l Na ₂ MoO ₄ ·2H ₂ O 50 mg/l EDTA] 15 g/l Agar (Sigma-Aldrich A1296) After autoclaving and cooling to 50 °C, media was supplemented with penicillin-streptomycin (50 units/ml, Fisher Scientific 15140122)
GI agar	1 g/l NH ₄ Cl 0.3 g/l MgSO ₄ ·7H ₂ O 0.15 g/l KCl 0.01 g/l CaCl ₂ ·2H ₂ O 0.0025 g/l FeSO ₄ ·7H ₂ O 0.69 g/l NaH ₂ PO ₄ ·H ₂ O 1.8 g/l Glucose 5 g/l Glycerol 9.75 g/l MES/NaOH 30 g/l Agar (Formedium AGA03) 200 µM Acetosyringone

Continued on next page

(Continued)

Name	Composition
GI broth	1 g/l NH ₄ Cl 0.3 g/l MgSO ₄ ·7H ₂ O 0.15 g/l KCl 0.01 g/l CaCl ₂ ·2H ₂ O 0.0025 g/l FeSO ₄ ·7H ₂ O 1.8 g/l Glucose 5 g/l Glycerol 9.75 g/l MES/NaOH 200 µM Acetosyringone
GM-GLU agar	4.4 g/l Murashige Skoog Medium, including vitamins (Duchefa M0222) 0.50 g/l MES hydrate, pH6 (Sigma M8250) 20 g/l Agar (Difco 214030)
Lysogeny Broth (LB)	25 g/l LB Broth Miller (Formedium LMM5L)
LB agar	36 g/l LB Agar Miller John Innes (Formedium LMM9901)
Mathur's medium (MM) agar	2.8 g/l Oxoid mycological peptone 3.6 g/l Glucose 1.2 g/l MgSO ₄ 2.7 g/l KH ₂ PO ₄ 20 g/l Agar (Formedium AGA03)
Murashige Skoog with added sucrose (MS+S) agar	4.4 g/l Murashige Skoog Medium, including vitamins (Duchefa M0222) 1.6 g/l Agar (Formedium AGA03)
Potato Dextrose Agar (PDA)	41 g/l PDA (Formedium PDA0102)
Potato Dextrose Broth (PDB)	12 g/l PDB (Formedium PDB0102)
Super Optimal broth with Catabolite repression (SOC)	20 g/l Tryptone 5 g/l Yeast extract 0.58 g/l NaCl 0.186 g/l KCl 2.03 g/l MgCl ₂ 2.46 g/l MgSO ₄ 3.6 g/l Glucose
Water Agar (WA)	15 g/l Agar (Formedium AGA03)

Table S7 – DNA sequencing primers. GG = Golden Gate, GW = Gateway[®], IF = In-Fusion[®].
If no source is given, the primer was designed as part of this work.

Primer	Sequence (5'-3')	Target	Source
006_Lv0_Fw	TCACATGTTCTTTCCTGCG	GG Lv0 backbone	TSL SynBio
007_Lv0_Rev	GTCTCATGAGCGGATACATATTGAAATG	GG Lv0 backbone	TSL SynBio
008_Lv1_Rev	GAACCCCTGTGTTGGCATGCACATAC	GG Lv1 backbone	TSL SynBio
009_Lv1_Fw	CTGGTGGCAGGATATATTGTGGTG	GG Lv1 backbone	TSL SynBio
010_p35S_Fw	AGGTGGCTCCTACAAATGCC	35S promoter	Dr Estee Tee
017_T7_Promoter_Fw	TAATACGACTCACTATAGGG	T7 promoter	Dr Rafał Zdrzałek
018_T7_Terminator_Rev	CACCACCTTCTGTATAGGCAG	T7 terminator	Dr Rafał Zdrzałek
019_MBP_Fw	GGTGAAATCATGCCGAACATC	MBP	Dr Rafał Zdrzałek
040_SENSE_PRLM_Rev	TCGGTCCACATGTGCATCCTC	GG Lv2 backbone	Dr Sebastian Samwald
069_mCherry_Fw	CCACTACGACGCTGAGGTC	mCherry	
070_mCherry_Rev	CTTCAGCTTGGCGGTCTG	mCherry	
071_GFP_Fw	CCGACCACTACCAGCAGAA	GFP	
072_GFP_Rev	AAGTCGTGCTGCTTCAATGTG	GFP	
110_Pikm-1_CC_Fw	TTGGAGCTCAATTTCCGAGC	Pimk-1 CC domain	
111_Pikm-1_NB_REV	GAGATTGATGCCGAAAACCC	Pimk-1 NB-ARC domain	
112_Ayb133_pgdpA_Fw	CGAGCTTTCCCACTTCATCG	gpdA promoter	
113_Ayb134_fTrpC_Rev	CACCTTTGCTGCTTGGACA	TrpC terminator	
114_AyB90_mas_ter_Rev	TATGCAGGAGCGGATCAITC	mas terminator	Dr Andrew Breakspear
115_mas_pro_Fw	CAAAATTACCAATACATTACACTAGCATCTG	mas promoter	
116_Pikp-2_Fw1	CCCTCACCTCAGCGCTAAG	Pikp-2	
117_Pikp-2_Fw2	ACATCATGTCCAAATCAATCGAAG	Pikp-2	

Continued on next page

(Continued)

Primer	Sequence (5'-3')	Target	Source
133_pAtUBQ10_Fw	GCTTCACCGCCTTAGC	AtUBQ10 promoter	
166_M13_F	GTAAAAACGACGGCCAGT	GW entry vector	Dr Andrew Breakspear
167_M13_R	AACAGCTATGACCATG	GW entry vector	Dr Andrew Breakspear
174_ChEC108_Fw_seq	CTGAAACAGCCCGGAACACAA	ChEC108	
186_PSC_Fw	GGCTGCAGCCCAATGTGG	IF PSC backbone	
187_PSC_Rev	TGTGTGGAATTGTGAGCGG	IF PSC backbone	

Table S8 – Arabidopsis lines. NASC = Nottingham Arabidopsis Stock Centre. For details of plasmids used, see Table S3. For details of CRISPR mutations in ET003 and ET006, see Figure 4.7. If no source is given, the line was generated as part of this work.

ID	Genotype	Gene(s) mutated	Plasmid used	Source	Notes
ET003	<i>hipp6-3</i>	AT5G03380	FP16038		CRISPR mutant
ET006	<i>hipp6-2</i>	AT5G03380	-	Prof. Tomáš Werner	CRISPR mutant
ET007	<i>hipp5 hipp6-2 hipp7</i>	AT2G36950 AT5G03380 AT5G63530	-	Prof. Tomáš Werner	T-DNA insertions, SALK_069207 (<i>hipp5</i>) and SALK_091924C (<i>hipp7</i>) CRISPR mutant (<i>hipp6-2</i>)
ET010	Columbia-0 (Col-0)	-	-	Faulkner laboratory stocks	
ET011	<i>p35S::GFP1-10-TurboID::tHSP</i>	-	FP06213	Dr Joanna Jennings	
ET013	<i>pAtUBQ10::GFP-HIPP6::t35S</i>	AT5G03380	FP16126		
ET016	<i>p35S::3×FLAG-TurboID-HMA1::HSP</i>	-	FP16137		
ET020	<i>hipp6-3 pAtUBQ10::meGFP-HIPP6::t35S</i>	AT5G03380	FP16126		
ET022	<i>hipp6-3 pAtUBQ10::meGFP-HIPP6^{M1}::t35S</i>	AT5G03380	FP16161		
ET026	<i>hipp6-3 pAtUBQ10::meGFP-HIPP6^{MM}::t35S</i>	AT5G03380	FP16163		
ET028	<i>pAtUBQ10::ChEC108-eGFP::t35S</i>	-	FP16221		
ET030	<i>pAtUBQ10::ChEC108^{D164R}-eGFP::t35S</i>	-	FP16216		

Table S9 – DNA genotyping primers. If no source is given, the primer was designed as part of this work. *Ch* = *Colletotrichum higginsianum*.

Primer	Sequence (5'-3')	For genotyping	Line/strain	Source
010_p35S_Fw	AGGTGGCTCTCTACAAATGCC	Arabidopsis	ET015, ET016, ET017, ET018	Dr Estee Tee
032_LBb1.3	ATTTTGCCGATTTTCGGAAC	Arabidopsis	SALK T-DNA insertion lines	SIGnAL
034_hipp6-1_RP	AGGAGGTCTCTCTTTCTTCG	Arabidopsis	ET020	SIGnAL
045_ChEC108_5'Flank_Fw	TTCGCTCAACCCCTAGTACC	<i>Ch</i>	Δ ChEC108	
046_ChEC108_3'Flank_Rev	GACCCAGACATCCAGAACCT	<i>Ch</i>	Δ ChEC108	
047_Trpc_ter_Fw	GTGAATGCTCCGTAAACACC	<i>Ch</i>	Δ ChEC108	
048_Cos1_Rev	TCGATCTGTTCCTGCCAG	<i>Ch</i>	Δ ChEC108	
049_ChEC108_Fw	TGCGACGTAATGTTTCCTTCC	<i>Ch</i>	Δ ChEC108	
050_ChEC108_Rev	GTCGGCCACTACTACTGGATC	<i>Ch</i>	Δ ChEC108	
072_GFP_Rev	AAGTCGTGCTGCTTCATGTG	Arabidopsis	ET028, ET030	
085_CRISPR_HIPP6_Fw3	GCACCAAGTCCCGGTTAAAAAC	Arabidopsis	<i>hipp6-3</i>	
086_CRISPR_HIPP6_Rev3	ACACCAATTGGAACCAAGACC	Arabidopsis	<i>hipp6-3</i>	
089_hipp5_LP	ATGGGAGAGGTCCAAGAAAG	Arabidopsis	<i>hipp5</i> T-DNA insertion	Guo et al., 2021
090_hipp5_RP	TTACATAACAGAGCAACCAATTG	Arabidopsis	<i>hipp5</i> T-DNA insertion	Guo et al., 2021
091_hipp7_LP	GTTGTCTCAAAAGGCTTCG	Arabidopsis	<i>hipp7</i> T-DNA insertion	Guo et al., 2021
092_hipp7_RP	CCTACCAATACACCCCATG	Arabidopsis	<i>hipp7</i> T-DNA insertion	Guo et al., 2021
093_TW_HIPP6_CRISPR_F3	ACGATGGGTGAGGTTAGTCA	Arabidopsis	<i>hipp6-2</i>	Prof. Tomás Werner
094_TW_HIPP6_CRISPR_R3	CCGTAAAGTTTCGAAAGTGACA	Arabidopsis	<i>hipp6-2</i>	Prof. Tomás Werner
099_Sjp040_Hyg_Rev	ATGTTGGCGACCTCGTATTG	<i>Ch</i>	Δ ChEC108	Dr Sally Jones
100_Sjp046_Hyg_Fw	CTGATCGAAAAGTTCGACAGC	<i>Ch</i>	Δ ChEC108	Dr Sally Jones
102_CK2903_ChKU80_Fw	ATGGAGGGAGTTGAGTCGGTGTC	<i>Ch</i>	Δ Chku80	Korn, 2016
103_CK2904_ChKU80_Rev	CAGTCCATGGAATCGACGTAGT	<i>Ch</i>	Δ Chku80	Korn, 2016

Continued on next page

(Continued)

Primer	Sequence (5'-3')	For genotyping	Line/strain	Source
104_CK2912_5'HB_Fw	CGTACCGGGCCCTTCATGACGGT	<i>Ch</i>	$\Delta Chku80$	Korn, 2016
105_CK2913_5'HB_Rev	GGAAATGTTCTGAATGGAGACACTAT	<i>Ch</i>	$\Delta Chku80$	Korn, 2016
106_CK2914_3'HB_Fw	TCATCGCGGCTGCTGGTGAGTTTCC	<i>Ch</i>	$\Delta Chku80$	Korn, 2016
107_CK2915_3'HB_Rev	CCAAAGAGCAGCAAGAAGAAGCAG	<i>Ch</i>	$\Delta Chku80$	Korn, 2016
133_pAtUBQ10_Fw	GCTTCACCGCCTTAGC	Arabidopsis	ET020, ET028, ET030	
160_HMA1_Rev	TACGTTTCCGATCACCGTCA	Arabidopsis	ET015, ET016, ET018	
161_HSP_Rev	TATAGGTCCTCGCCTCGTT	Arabidopsis	ET017	
207_AyB269_Bar_Fw	TGAAGACGAAATGAGCCCAGAACGACGC	<i>Ch</i>	ETCh01, ETCh02	Dr Andrew Breakspear
208_AyB270_Bar_Rev	TGAAGACGAAAGCCTAAATCTCGGTGACGGG	<i>Ch</i>	ETCh01, ETCh02	Dr Andrew Breakspear

Table S10 – DNA primers for RT-qPCR.

Primer	Sequence (5'-3')	Target	Source
201_HIPP6_qPCR_Fw3	CCTCCACCACCACCTAAAGA	<i>HIPP6</i>	This work
202_HIPP6_qPCR_Rev3	AGCAACCGAATTAACCCCTT	<i>HIPP6</i>	This work
210_AyB34_UBQ10_Fw	AGTCTACTCTTCACTTGGTCCTGC	<i>AtUBQ10</i>	Dr Andrew Breakspear
211_AyB35_UBQ10_Rev	GCCCCAAAACACAAACCACCAAAG	<i>AtUBQ10</i>	Dr Andrew Breakspear

Table S11 – Top 25 proteins with structural similarity to ChEC108, identified by searching PDB25 via the DALI server using the ChEC108 AlphaFold2 model as query. Z-score (Z) = statistical significance of an alignment. Root Mean Square Deviation (RMSD) = the average distance between aligned C α atoms. Number of equivalent residues (LALI) = number of aligned C α atoms. NRES = total number of residues in the target structure. ID = sequence identity. GNAT = Gcn5-related N-acetyltransferase.

Rank	Model-Chain	Z	RMSD	LALI	NRES	ID (%)	Description
1	4ZSV-A	7.6	3.6	121	294	9	Uncharacterized protein
2	2K5T-A	6.6	3	99	128	7	Putative N-Acetyltransferase
3	3DNS-A	6.4	3.3	104	131	10	Ribosomal-protein-alanine acetyltransferase
4	6SJY-B	6.2	4.4	128	175	9	L-2,4-diaminobutyric acid acetyltransferase
5	1Y9K-B	6.1	3.7	111	154	5	IAA acetyltransferase
6	3EXN-A	5.7	3.4	102	154	10	Probable acetyltransferase
7	2FIA-B	5.6	3.3	108	159	6	Acetyltransferase
8	2REF-A	5.6	3.9	128	203	4	GNATL domain
9	3BLN-A	5.4	3.5	101	142	6	Acetyltransferase GNAT family
10	3F5B-A	5.4	3.4	101	172	11	Aminoglycoside N(6')acetyltransferase
11	1YRE-B	5.4	3.5	113	187	5	Hypothetical protein
12	5YGE-A	5.4	3.4	102	170	8	Amino-acid acetyltransferase
13	5C82-A	5.3	3.4	103	170	10	Nourseothricin acetyltransferase
14	3EFA-A	5.3	3.4	103	146	4	Putative acetyltransferase
15	6R45-A	5.3	2.9	103	208	6	O-glcnacase
16	3SHP-A	5.2	4.1	111	168	12	Putative acetyltransferase
17	2ZW6-A	5.2	3.4	110	297	12	Bleomycin acetyltransferase
18	1Z4E-A	5.2	3	99	150	10	Transcriptional regulator
19	1S7F-A	5.2	3.3	107	181	7	Acetyltransferase
20	2Q7B-A	5.1	3.5	103	164	9	Acetyltransferase, GNAT family
21	3OWC-A	5.1	3.3	102	172	5	Probable acetyltransferase
22	2VZZ-A	5	3.3	105	210	10	Succinyltransferase with the GNAT Fold.
23	3EO4-D	5	3.8	103	162	8	Uncharacterized protein
24	2FIW-A	5	3.4	107	160	7	GNAT, aminotransferase
25	2PC1-A	5	3	100	173	12	Acetyltransferase, GNAT family

Table S12 – Virulence assay experimental details. For each replicate, the time of lesion image acquisition is given in days post-infection (dpi), along with the concentration of spores applied.

Figure	Panel	Replicate	Images acquired (dpi)	Spore concentration (/ml)
4.4	B	1	6	9.0×10^5
	B	2	6	2.0×10^6
	B	3	6	2.0×10^6
4.6	B	1	6	2.0×10^6
	B	2	6	2.0×10^6
	B	3	6	2.0×10^6
	B	4	6	2.0×10^6
4.8	A-B	1	6	2.0×10^6
	A-B	2	7	1.9×10^6
	A-B	3	8	9.1×10^5
	C-D	1	6	1.5×10^6
	C-D	2	6	8.6×10^5
	C-D	3	6	1.2×10^6
	C-D	4	7	2.0×10^6
	C-D	4	7	2.0×10^6
4.9	A-B	1	5	1.2×10^6
	A-B	2	6	1.8×10^6
	A-B	3	7	2.0×10^6
4.12	B	1	6	2.0×10^6
	B	2	6	2.0×10^6

Table S13 – Percentage sequence identity matrix of clade I HIPP HMA domains, generated using MUSCLE. Gene IDs: HIPPI, AT2G28090; HIPPI3, AT5G60800; HIPPI4, AT1G29000; HIPPI5, AT2G36950; HIPPI6, AT5G03380; HIPPI7, AT5G63530; HIPPI8, AT3G02960; HIPPI9, AT5G24580.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	HIPP4_HMA2	100	35	27	29	35	37	29	27	32	30	28	25	28	28	27
2	HIPP4_HMA1	35	100	28	26	37	31	34	33	28	34	28	36	31	39	41
3	HIPP9_HMA1	27	28	100	30	36	39	41	34	31	31	38	36	38	34	39
4	HIPP9_HMA2	29	26	30	100	45	34	32	28	32	33	36	31	22	27	41
5	HIPP7_HMA2	35	37	36	45	100	48	37	34	31	34	38	34	36	42	34
6	HIPP8_HMA2	37	31	39	34	48	100	40	41	34	38	30	30	33	33	33
7	HIPP5_HMA2	29	34	41	32	37	40	100	69	41	50	34	27	39	42	39
8	HIPP6_HMA2	27	33	34	28	34	41	69	100	44	52	36	25	33	42	41
9	HIPP1_HMA2	32	28	31	32	31	34	41	44	100	56	31	23	36	39	41
10	HIPP3_HMA2	30	34	31	33	34	38	50	52	56	100	30	25	38	39	41
11	HIPP7_HMA1	30	28	38	36	38	30	34	36	31	30	100	55	37	47	45
12	HIPP8_HMA1	28	27	30	31	34	30	27	25	23	25	55	100	32	33	41
13	HIPP1_HMA1	25	36	36	22	34	30	39	33	36	38	37	32	100	45	53
14	HIPP6_HMA1	28	31	38	27	36	33	42	42	39	39	47	33	45	100	48
15	HIPP3_HMA1	28	39	34	41	42	33	39	41	41	41	45	41	53	48	100
16	HIPP5_HMA1	27	41	39	27	34	33	38	44	33	44	45	38	47	55	56
																100

8.2 Abbreviations

Throughout this thesis, standard three-letter abbreviations are used to refer to amino acids in the main text. In figures and tables or as part of sequences, standard one-letter amino acid codes are used. X indicates any amino acid. Metal elements are denoted by their standard atomic symbols, defined at first use in the main text. Other abbreviations are listed below:

ADP	adenosine diphosphate
ADR1	ACTIVATED DISEASE RESISTANCE 1
AHK	ARABIDOPSIS HISTIDINE KINASE
anchHMA	ancestral HMA
AO	L-ASCORBATE OXIDASE
APikL	AVR-Pik like
APS	ammonium persulfate
ARR	ARABIDOPSIS RESPONSE REGULATOR
ATFP	<i>Arabidopsis thaliana</i> FARNESYLATED PROTEIN
ATHB	<i>Arabidopsis thaliana</i> HOMEODOMAIN 29
AtHMAD1	<i>Arabidopsis thaliana</i> HMA DOMAIN-CONTAINING 1
ATOX1	antioxidant protein 1 (human-specific)
ATP	adenosine triphosphate
ATP7A	ATPase copper transporting alpha
ATP7B	ATPase copper transporting beta
ATX1	antioxidant protein 1
AU	absorption units
Avr / AVR	avirulence
AzA	azelaic acid
Bas1	biotrophy-associated secreted 1
BBWV2	broad bean wilt virus 2
BCA	bicinchoninic acid
BCS	bathocuproine sulfonate
BFDR	bayesian false discovery rate

Bgh	<i>Blumeria graminis</i> f. sp. <i>hordei</i>
BG_pap	β -1,3-glucanase putative plasmodesmal-associated protein
Bgt	<i>Blumeria graminis</i> f. sp. <i>tritici</i>
BIC	biotrophic interfacial complex
BiFC	bimolecular fluorescence complementation
BIK1	BOTRYTIS INDUCED KINASE 1
BirA	bifunctional ligase/repressor A
BLAST	basic local alignment search tool
BSA	bovine serum albumin
BUB2	budding uninhibited by benzimidazole 2
BY-2	Bright Yellow-2
CALS	CALLOSE SYNTHASE
CaMV	cauliflower mosaic virus
Cas9	CRISPR-associated protein 9
CAT	CATALASE
CB	coomassie blue
CC	coiled-coil
CC(1/2)	half-dataset correlation coefficient
CC^R	RWP8-like coiled coil
CCAA	indicates simultaneous mutation of Cys197Ala and Cys200Ala in ChEC108
CCH	COPPER CHAPERONE
CCP	COPPER CHAPERONE INDUCED BY PATHOGENS
CCS	COPPER CHAPERONE FOR SUPEROXIDE DISMUTASE
cCu/Zn-SOD	cytosolic Cu/Zn-SUPEROXIDE DISMUTASE
CD	circular dichroism
CdI19	CADMIUM-INDUCED 19
cDNA	complementary DNA
CDS	coding sequence
CEC3	conserved core effector of <i>Colletotrichum</i> 3

CERK1	LYSIN MOTIF-CONTAINING CHITIN ELICITOR RECEPTOR KINASE 1
CGL1	COMPLEX GLYCAN 1
CgEP1	<i>Colletotrichum graminicola</i> effector protein 1
Ch	<i>Colletotrichum higginsianum</i>
ChEC	<i>Colletotrichum higginsianum</i> effector candidate
ChELP	<i>Colletotrichum higginsianum</i> extracellular lysin motif protein
ChNLP1	<i>Colletotrichum higginsianum</i> necrosis- and ethylene-inducing peptide 1
CI	cylindrical inclusion
CM	complete media
CMoV	carrot mottle virus
Cmu1	chorismate mutase 1
CMV	cucumber mosaic virus
co-IP	co-immunoprecipitation
Col-0	Columbia-0
Coot	Crystallographic Object-Oriented Toolkit
COPT5	COPPER TRANSPORTER 5
Cp	crossing point
CPK	CALCIUM-DEPENDENT PROTEIN KINASE
CPMV	cowpea mosaic virus
CPsV	citrus psorosis virus
CRISPR	clustered regularly interspaced short palindromic repeats
CSD	Cu/Zn-SUPEROXIDE DISMUTASE
CuAO	COPPER AMINE OXIDASE
cryo-EM	cryogenic electron microscopy
DALI	Distance matrix ALIgnment
DAMP	damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DMF	dimethylformamide
DMSO	dimethyl sulfoxide

dpi	days post-inoculation
DRP	DYNAMIN RELATED PROTEIN
DTT	dithiothreitol
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
EDTA	ethylenediaminetetraacetic acid
EFR	ELONGATION FACTOR-TU RECEPTOR
eGFP	enhanced green fluorescent protein
EHM	extrahaustorial matrix
EIHM	extra-invasive hyphal membrane
EM	electron microscopy
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ETI	effector-triggered immunity
eV	electron volts
ex	extended
Exo	exocyst complex [subunit]
FLP	flippase
FLS2	FLAGELLIN SENSITIVE 2
FMV	fig mosaic virus
FOLD	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i> dual-domain
FP	FARNESYLATED PROTEIN
FRT	flippase recombination target
G3P	glycerol-3-phosphate
GB1	Streptococcal protein G B1 domain
gDNA	genomic DNA
GFLV	grapevine fanleaf virus
GFP	green fluorescent protein
GI	glycerol induction
GM	growth media
GM-GLU	growth media without added glucose

GMFP	<i>Glycine max</i> FARNESYLATED PROTEINS
GNAT	Gcn5-related N-acetyltransferase
gpdA	glyceraldehyde-3-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GSL	GLUCAN SYNTHASE-LIKE
GUS	β -glucuronidase
HA	hemagglutinin
HAH1	human ATX1 homologue 1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HF	high fidelity (in relation to enzymes) or Hellfire (6 $\times$ His-3 $\times$ FLAG, in relation to epitope tags)
HIGS	host-induced gene silencing
HIPP	HEAVY METAL-ASSOCIATED ISOPRENYLATED PLANT PROTEIN
HMA	heavy metal-associated or HEAVY METAL ATPASE (the latter applies to gene names only)
HMP	HEAVY METAL-ASSOCIATED PROTEIN
hpi	hours post-inoculation
HPP	HEAVY METAL-ASSOCIATED PLANT PROTEIN
HR	hypersensitive response
HRP	horseradish peroxidase
HSP	HEAT SHOCK PROTEIN 18
HygR	hygromycin resistance gene
IDT	Integrated DNA Technologies
IMAC	immobilised metal affinity chromatography
IP-MS	immunoprecipitation-mass spectrometry
IPTG	isopropyl β -D-1-thiogalactopyranoside
ipTM	interface predicted Template Modelling
ITC	isothermal titration calorimetry
JA	jasmolic acid
JAZ	JASMONATE ZIM-DOMAIN

KASP	kompetitive allele-specific PCR
KFP	kinase fusion protein
KP	killer protein
LARS	<i>Leptosphaeria</i> avirulence and suppressing
LB	lysogeny broth
LRR	leucine-rich repeat
LTi6b	LOW TEMPERATURE INDUCED PROTEIN 6B
LYK	LYSIN MOTIF RECEPTOR-LIKE KINASE
LYM2	LYSIN MOTIF DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED PROTEIN 2
M1	indicates metal-binding loop mutation (CXXC to GXXG) in HMA1 of HIP6
M2	indicates metal-binding loop mutation (CXXC to GXXG) in HMA2 of HIP6
MAMP	microbe-associated molecular pattern
mas	mannopine synthase
mAU	milli-absorbance units
MAX	<i>Magnaporthe</i> AVR _s and ToxB-like
mb1	metal-binding loop
mCherry	monomeric Cherry
MCTP	MULTIPLE C2 DOMAINS AND TRANSMEMBRANE DOMAIN PROTEIN
meGFP	monomeric enhanced green fluorescent protein
MES	2-(N-morpholino)ethanesulfonic acid
MM	Mathur's medium or indicates double metal-binding loop mutations (CXXC to GXXG) in the HMA domains of HIP6
MP	movement protein
MP30	30 kDa movement protein
mRFP	monomeric red fluorescent protein
MS	Murashige and Skoog

MS+S	Murashige Skoog media with added sucrose
Myotub	myotubularin
NADase	nicotinamide adenine dinucleotide hydrolases
NADPH	nicotinamide adenine dinucleotide phosphate
NASC	Nottingham Arabidopsis stock centre
NB-ARC	nucleotide-binding adapter shared by Apaf-1, resistance (R) proteins and CED-4
NCBI	national center for biotechnology information
NHL3	NON-RACE SPECIFIC DISEASE RESISTANCE /HIN1 HAIRPIN-INDUCED-LIKE 3
NHP	N-hydroxypipicolinic acid
NLR	nucleotide-binding leucine-rich repeat
NLS	nuclear localisation signal
NMR	nuclear magnetic resonance
NOI	NO ₃ -induced
NPH	N-hydroxypipicolinic acid
NRAMP3	NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3
NRC2	NLR-REQUIRED FOR CELL DEATH 2
NRG1	N REQUIREMENT GENE 1
NTC	nourseothricin
OD	optical density
OFP	orange fluorescent protein
ORF	open reading frame
P3N-PIPO	P3-N-terminal-pretty interesting potyviral open reading frame
PAD4	PHYTOALEXIN DEFICIENT 4
PAE	predicted aligned error
PAM	protospacer adjacent motif
PAR	4-(2-pyridylazo)resorcinol
PBL1	AVRPPHB SUSCEPTIBLE1-LIKE KINASE 1
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PD	plasmodesmata
PDA	potato dextrose agar
PDB	Protein Data Bank or potato dextrose broth
PDCB	plasmodesmata-associated callose-binding
PDLP	PLASMODESMATA-LOCATED PROTEIN
PEG	polyethylene glycol
PI4P	phosphatidylinositol 4-phosphate
PKS1	polyketide synthase 1
pLDDT	predicted Local Distance Difference Test
PMSF	phenylmethylsulfonyl fluoride
PMTV	potato mop top virus
poly(I:C)	polyinosinic-polycytidilic acid
PPQ	pyrroloquinoline quinone
PPT	L-phosphinothricin
PR1	PATHOGENESIS-RELATED PROTEIN 1
PSbMV	pea seed-borne mosaic potyvirus
PTI	pattern-triggered immunity
pTM	predicted Template Modelling
<i>Pto</i> DC3000	<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000
PVDF	polyvinylidene difluoride
PVX	potato virus X
Pwl	pathogenicity to weeping lovegrass
qPCR	quantitative PCR
QtPISA	QuickTime Protein Interfaces, Surfaces and Assemblies
R	resistance [gene/protein]
RALPH	RNase-like proteins expressed in haustoria
RBOH	RESPIRATORY BURST OXIDASE HOMOLOG
REM	REMORIN
RFP	red fluorescent protein

RGA	resistance gene analog
RGH	resistance gene homolog
RIN4	RPM1-INTERACTING PROTEIN 4
RLCK	receptor-like cytoplasmic kinase
RLK	receptor-like kinase
RLP	receptor-like protein
RMSD	root mean square deviation
RNP	ribonucleoprotein complex
ROQ1	RECOGNITION OF XOPQ1
ROS	reactive oxygen species
RP27	ribosomal protein 27
Rpi-amr	Resistance to <i>Phytophthora infestans</i> from <i>Solanum americanum</i>
RPM1	RESISTANCE TO <i>Pseudomonas syringae</i> pv. <i>maculicola</i> 1
RPP1	RECOGNITION OF <i>Peronospora parasitica</i> 1
RQ	relative quantities
rRNA	ribosomal RNA
RS	resurfaced
RT-qPCR	reverse transcription quantitative PCR
RXLR	Arg-X-Leu-Arg
s/dEER	single/double Glu-Glu-Arg
SA	salicylic acid
SAG101	SENESCENCE-ASSOCIATED GENE 101
SAINT	Significance Analysis of INteractome
SAR	systemic acquired resistance
SCD1	scytalone dehydratase 1
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec	secretory [pathway gene]
SERK1	SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1
sgRNA	small guide RNA

sHMA	small HMA
Six	secreted in xylem
SKS	SKU5-SIMILAR
SKU5	SKEWED 5
SOC	super optimal broth with catabolite repression
SOD	SUPEROXIDE DISMUTASE
SP	signal peptide
SPR	surface plasmon resonance
SS15	SPRYSEC 15
Stp1	Stop penetration 1
Strep	streptavidin
SYP122	SYNTAXIN OF PLANTS 122
SYTA	SYNAPTOTAGMIN A
T-DNA	transfer DNA
TAG-KO	transposon arrayed gene knockout
TBE	Tris-borate-EDTA
TBST	Tris-buffered saline with Tween-20
TCP	TEOSINTE BRANCHED1 / CYCLOIDEA / PROLIFERATING CELL FACTOR
tdTomato	tandem dimer tomato
TE	Tris-EDTA
TEF	translation elongation factor
TEM	transmission electron microscopy
TEMED	tetramethylethylenediamine
TF	translation function
TGA2	TGACG-BINDING 2
TGB	triple gene block
tHMA	tandem HMA
Tin3	Tumour inducing 3
TIR	toll/interleukin-1 receptor

TMV	tobacco mosaic virus
tRNA	transfer RNA
trpC	tryptophan biosynthesis C
TuMV	turnip mosaic virus
TYLCV	tomato yellow leaf curl virus
UBQ10	UBIQUITIN 10
UCC	UCLACYANIN
UV	ultraviolet
WA	water agar
WRR4	WHITE RUST RESISTANCE 4
WT	wild-type <i>Colletotrichum higginsianum</i> strain $\Delta Chku80$
WT ²	wild-type <i>Colletotrichum higginsianum</i> strain IMI 349063
WTN1	WHEAT TANDEM NUCLEOTIDE-BINDING DOMAIN 1
X-gal	5-bromo-4-chloro-3-indolyl β -D-galactopyranoside
YFP	yellow fluorescent protein
ZAR1	HOPZ-ACTIVATED RESISTANCE 1
zf-BED	zinc-finger-BED
ZF-HD	ZINC FINGER HOMEODOMAIN
ZiF	zinc-finger
ZIP11	ZINC TRANSPORTER PRECURSOR 11

8.3 Publication

Text/ideas, tables and figures from Chapter 1, as well as text/ideas from Chapter 7 of this thesis contributed to the following publication, written in collaboration with Prof. Christine Faulkner. A copy of the article is included below.

Turley EK and Faulkner C (2025). HIPPs of all trades: insights from pathogens on the function of HMA proteins at plasmodesmata. *Journal of Experimental Botany*, eraf234.



Journal of Experimental Botany, Vol. 00, No. 0 pp. 1–17, 2025
<https://doi.org/10.1093/jxb/eraf234> Advance Access Publication 28 May 2025



Downloaded from <https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/eraf234/8152035> by Norwich Bioscience Institutes Library (INACTIVE) user on 23 September 2025

REVIEW PAPER

HIPPs of all trades: insights from pathogens on the function of HMA proteins at plasmodesmata

Emma K. Turley¹ and Christine Faulkner^{1*}

Cell and Developmental Biology, John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK

* Correspondence: christine.faulkner@jic.ac.uk

Received 11 February 2025; Editorial decision 23 May 2025; Accepted 27 May 2025

Editor: Jacob Brunkard, University of Wisconsin, Madison, USA

Abstract

Within plant cell walls, plasmodesmal channels harbour unique collections of proteins to maintain their structure and facilitate dynamic regulation of cell-to-cell connectivity. Proteomic surveys, combined with evidence from confocal microscopy, have identified heavy metal-associated (HMA) domain-containing proteins as residents at plasmodesmata; however, the functional relevance of this localization is currently unknown. Although HMA domains themselves are present in all kingdoms of life, in plants they can be found in three main families: HPPs, HIPPs, and P_{1B}-type ATPases. Within the last decade, HPPs and HIPPs have emerged as frequent host targets of pathogen-derived molecules, including secreted effectors from bacteria, fungi, and oomycetes, and a viral movement protein. The seemingly conserved targeting of HMA domains throughout distantly related pathosystems suggests that these proteins could play integral roles in plant immunity. This is corroborated by observations of HMA-like domains being integrated into plant immune receptors, enabling direct binding of effectors to activate downstream signalling, as well as genetic evidence highlighting the influence of HPPs and HIPPs on disease susceptibility. Drawing especially from plant pathology studies, we speculate about the potential metallochaperone and signalling functions of these enigmatic plasmodesmal components.

Keywords: Effector, heavy metal, HIPP, HMA, HPP, metallochaperone, NLR, pathogen, plasmodesmata.

Introduction

As key components of the internal communication system of plants, plasmodesmata directly connect the cytoplasm, plasma membrane, and endoplasmic reticulum (ER) of adjacent cells, enabling transport of proteins, small RNAs, phytohormones, and nutrients. The ultrastructure of an individual plasmodesma is typically represented as a plasma membrane-lined pore containing a cytoplasmic sleeve and appressed strand of ER called the desmotubule (Sager and Lee, 2018).

In comparison with the wider plasma membrane, plasmodesmata harbour unique communities of proteins. Previously, meticulous fractionation and MS were used to

enrich for cell wall and plasmodesmal proteins in plants and algae (e.g. see Faulkner *et al.*, 2005; Bayer *et al.*, 2006). This led to the identification of key regulators of plasmodesmal function such as β -1,3-glucanases, which degrade plasmodesmata-associated callose to enhance intercellular trafficking (Levy *et al.*, 2007; Benitez-Alfonso *et al.*, 2013), and PLASMODESMATA-LOCATED PROTEINS (PDLPs) that function in localized signalling cascades (Thomas *et al.*, 2008; Tee *et al.*, 2023).

More recently, armed with novel and previously published proteomes from divergent species, namely *Arabidopsis thaliana*

© The Author(s) 2025. Published by Oxford University Press on behalf of the Society for Experimental Biology.
 This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

2 | Turley and Faulkner

(from hereon *Arabidopsis*), *Populus trichocarpa*, and *Physcomitrium patens*, Johnston *et al.* (2023) identified conserved protein orthogroups represented in plasmodesmata. One such orthogroup consisted of HEAVY METAL-ASSOCIATED ISOPRENYLATED PLANT PROTEINs (HIPPs), which are prevalent throughout vascular plant genomes and defined as having one or two heavy metal-associated (HMA) domains, and a C-terminal isoprenylation motif (Barth *et al.*, 2009; de Abreu-Neto *et al.*, 2013).

So far, 11 HIPP orthologues have emerged in plasmodesmal proteomes (reviewed by Barr *et al.*, 2023), and the subcellular localizations of three of these (AtHIPP01 and AtHIPP07 from *Arabidopsis*, and NbHIPP26 from *Nicotiana benthamiana*) were confirmed (Cowan *et al.*, 2018; Guo *et al.*, 2021). Additionally, OsHIPP17, OsHIPP19, and OsHIPP20 from rice (*Oryza sativa*) and HvHIPP43 from barley (*Hordeum vulgare*) were identified in different contexts and subsequently observed to localize to plasmodesmata (Oikawa *et al.*, 2024; Were *et al.*, 2025). Although lacking an isoprenylation motif, HMA PLANT PROTEIN (HPP) relatives of HIPPs were also detected in plasmodesmal proteomes from *Arabidopsis* (Fernandez-Calvino *et al.*, 2011), *N. benthamiana* (Park *et al.*, 2017), and *P. patens* (Gombos *et al.*, 2023). Despite the growing list of plasmodesmal HMA domain-containing proteins, exactly why they accumulate here remains a mystery.

Independent of their appearance in plasmodesmata, HIPPs and HPPs are common players in plant-pathogen interactions, being frequently targeted by pathogen-derived proteins and identified as regulators of infection success. To explain this phenomenon, we hypothesize that these proteins might play important and potentially diverse roles in plant immunity that evolutionarily distant pathogens have convergently evolved to manipulate. In this review, we explore the known functions of HMA domain-containing proteins, drawing especially from plant pathology studies to provide clues as to the endogenous functions of these enigmatic proteins at plasmodesmata.

A historical perspective: what makes a HMA domain?

Perhaps the most obvious hint towards HMA domain function can be found in the name. Bull and Cox (1994) coined the term HMA to describe a collection of sequences from humans and bacteria, each possessing a conserved CXXC motif to facilitate metal ion co-ordination. These sequences included the Menkes and Wilson disease proteins from humans (ATP7A and ATP7B, respectively), which are P_{1B}-type ATPases required to shuttle copper (Cu) across intracellular membranes within the secretory pathway (Tanzi *et al.*, 1993; Vulpe *et al.*, 1993). The characteristic 3D structure of a HMA domain was first described by Eriksson and Sahlman (1993), when the NMR structure of MerP, a periplasmic mercury

(Hg)-binding protein from *Pseudomonas aeruginosa*, revealed a βαβαβ secondary structure, comprising an antiparallel β-sheet with two α-helices folded against it. A subsequent NMR structure of the fourth metal-binding domain of ATP7A (Gitschier *et al.*, 1998) and the crystal structure of the yeast (*Saccharomyces cerevisiae*) metallochaperone, antioxidant protein 1 (ATX1) (Rosenzweig *et al.*, 1999, Fig. 1), each displayed homologous αβ-sandwich folds, now recognized (alongside the CXXC motif) as a defining feature of HMA domains (InterPro: IPR006121).

In vitro studies indicated that prototypical HMA domains were capable of direct heavy metal binding. Further structural characterization of MerP revealed a Hg(II) ion co-ordinated between the two Cys residues of the CXXC motif, forming approximately linear co-ordinate bonds (Steele and Opella, 1997). On the other hand, ATP7A, ATX1, and its human homologue HAH1 (later named ATOX1) bound Cu(I) *in vitro* (Lutsenko *et al.*, 1997; Pufahl *et al.*, 1997; Hung *et al.*, 1998).

Across kingdoms, HMA domain-containing proteins were initially characterized as metallochaperones: proteins that shuttle metal ions intracellularly to other metalloproteins. For instance, homologous Cu transport pathways in yeast, humans, and *Arabidopsis* each employ a cytosolic HMA domain (ATX1, ATOX1, or AtATX1, respectively) to deliver imported Cu(I) to a HMA domain-containing ATPase embedded within post-Golgi vesicles (CCC2, ATP7A/B, or RAN1, respectively) (Yuan *et al.*, 1995; Klomp *et al.*, 1997; Pufahl *et al.*, 1997; Puig *et al.*, 2007; Muller and Klomp, 2009). Two more Cu chaperones with HMA domains have been characterized in *Arabidopsis*: AtCCH (Cu chaperone) and AtCCS, a Cu chaperone for Cu-zinc (Zn) superoxide dismutase (Cu/Zn-SOD) (Mira *et al.*, 2001; Abdel-Ghany *et al.*, 2005; Chu *et al.*, 2005). Beyond this handful of Cu-binding examples, there is a growing body of evidence to support roles for plant HMA domains in regulation of heavy metal tolerance (see ‘What microbes aren’t telling us: HPP/HIPP function in heavy metal tolerance’). However, numerous domains annotated as ‘HMA’ in plants lack the defining CXXC motif (and are thus referred to from hereon as ‘HMA-like’ domains), suggesting that metals cannot be bound unless via an alternative mechanism. Considered alongside the diversity and expansion of HMA domain-containing families in plants (see below and Table 1), this suggests the possibility of additional functions transcending metal homeostasis.

Plant proteins sporting HMA or HMA-like domains (from hereon HMA proteins) can be divided into three broad families: HPPs, HIPPs, and P_{1B}-type ATPases. On rare occasions, HMA-like domains can also be found integrated within plant immune receptors (see ‘HMA domains as bait for effectors’). HIPPs, initially called *Arabidopsis thaliana* farnesylated proteins (ATFPs), or *Glycine max* farnesylated proteins (GMFPs), were first described by Dykema *et al.* (1999), following their identification in an *in vitro* screen for cDNAs encoding isoprenylated proteins (Crowell *et al.*, 1996). These

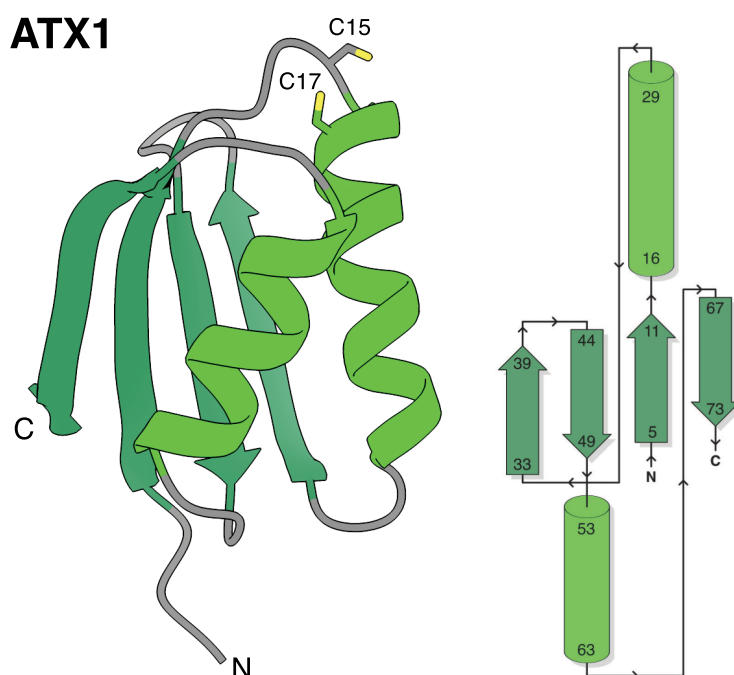


Fig. 1. HMA domain structure and topology. Crystal structure of ATX1 from *S. cerevisiae* (1CC8) (Rosenzweig *et al.*, 1999), demonstrating a typical $\beta\alpha\beta\alpha\beta$ topology. Cys15 and Cys17 of the metal-binding motif (MTCSGC) are annotated. A topology diagram was drawn with the aid of PDBsum (Laskowski *et al.*, 2018).

first HPPs (ATFP2, 3, 6, and GMFP5, 7) were noted to possess both metal-binding (M/LXCXXC) and isoprenylation (CaaX) motifs. Later BLAST-based searches uncovered a 45 member Arabidopsis HPP family, organized into five clades (Barth *et al.*, 2009; Tehseen *et al.*, 2010; de Abreu-Neto *et al.*, 2013). In addition, a 22 member Arabidopsis HPP family was described, including metallochaperones AtATX1, AtCCS, and AtCCH (Tehseen *et al.*, 2010; de Abreu-Neto *et al.*, 2013), the members of which lack an isoprenylation motif. Subsequently, using a hidden Markov model, Li *et al.* (2020) identified a further two HPPs (AtHMP09 and AtHMP35, for heavy metal-associated protein) and two HPPs (AtHMP18 and AtHMP28), bring the total number of family members to 47 and 24, respectively.

HMA domain-containing P_{1B} -type ATPases constitute a smaller family of just eight proteins in Arabidopsis, named HMA1–HMA8 (for heavy metal ATPase, not to be confused with heavy metal-associated), only four of which possess at least one canonical HMA domain: HMA5, HMA6/PAA1, HMA7/RAN1, and HMA8/PAA2 (Williams and Mills, 2005). These pumps utilize energy from ATP hydrolysis to shuttle cations

between subcellular compartments, including from the cytosol into secretory vesicles or chloroplasts (reviewed by Hanikenne and Baurain, 2014). Only HMA4 has appeared in plasmodesmal proteomes to date (Fernandez-Calvino *et al.*, 2011), and this localization has yet to be confirmed, suggesting that P_{1B} -type ATPases are unlikely to be core regulators of plasmodesmal function.

Beyond Arabidopsis, catalogues of HIPP and HPP genes have been assembled from numerous plant species, including lycophytes, and monocotyledonous and dicotyledonous flowering plants (Table 1). Tandem and segmental duplications have contributed to the major expansion of select families (Khan *et al.*, 2019; Li *et al.*, 2020; Ye *et al.*, 2022), causing some species to accumulate >50 HIPP genes. Notably, HPPs appear to be exclusive to tracheophytes. Thus, HPPs, like PDLs (Lee, 2014), seem to have arisen after the division of vascular and non-vascular plants. As plasmodesmata are present in both lineages, this suggests that HPPs may be regulators of plasmodesmal function, rather than essential structural components or governors of plasmodesmal morphogenesis.

Table 1. Number of *HIPP* and *HPP* genes identified in surveys of vascular plant genomes to date^a

Species	HIPP genes	HPP genes	Reference
<i>Aegilops tauschii</i>	40	nr	H. Zhang <i>et al.</i> (2020)
<i>Arabidopsis thaliana</i>	47	21	de Abreu-Neto <i>et al.</i> (2013); Li <i>et al.</i> (2020)
<i>Brachypodium distachyon</i>	40	nr	H. Zhang <i>et al.</i> (2020)
<i>Camellia sinensis</i>	38	9	Wei <i>et al.</i> (2023) ^b
<i>Chenopodium quinoa</i>	34	nr	Sun <i>et al.</i> (2022)
<i>Citrus sinensis</i>	26	nr	Huang <i>et al.</i> (2024)
<i>Fagopyrum tataricum</i>	13	36	Ye <i>et al.</i> (2022) ^c
<i>Haynaldia villosa</i>	13	nr	H. Zhang <i>et al.</i> (2020)
<i>Hordeum vulgare</i>	33	nr	H. Zhang <i>et al.</i> (2020)
<i>Oryza sativa</i>	59	7	de Abreu-Neto <i>et al.</i> (2013)
<i>Populus trichocarpa</i>	74	10	de Abreu-Neto <i>et al.</i> (2013)
<i>Setaria italica</i>	52	3	de Abreu-Neto <i>et al.</i> (2013)
<i>Selaginella moellendorffii</i>	5	4	de Abreu-Neto <i>et al.</i> (2013)
<i>Solanum lycopersicum</i>	28	6	Xu <i>et al.</i> (2024) ^d
<i>Triticum aestivum</i>	114	nr	H. Zhang <i>et al.</i> (2020)
<i>Triticum dicoccoides</i>	58	nr	H. Zhang <i>et al.</i> (2020)
<i>Triticum urartu</i>	33	nr	H. Zhang <i>et al.</i> (2020)

^anr, not reported. ^bA total of 56 genes were assigned the name *HIPP*. However, this list included nine genes encoding HPPs and nine genes encoding putative ATPases. ^c*HPP* genes were assigned the name *FIATX1*. ^dA total of 34 genes were assigned the name *HIPP*. However, this list included six genes encoding HPPs.

A protein perspective: HMA domains are frequently targeted by pathogens

HMA domains as effector host targets

While there remains much to learn about HMA proteins in the context of plasmodesmata, evidence is continually emerging for their involvement in plant–pathogen interactions. HPP/HIPPs from diverse plant species have been identified as direct targets of pathogen–derived molecules, including secreted effector proteins and a viral movement protein (Table 2).

The most well-studied examples of effector–HMA interactions originate from *Magnaporthe oryzae* (rice/wheat blast) pathosystems. OsHIPP19 is targeted by multiple AVR–Pik allelic effector variants (Maidment *et al.*, 2021), and OsHIPP20 by the AVR–PikD variant (Oikawa *et al.*, 2024). *In vitro* work revealed that OsHIPP43 is tightly bound by Pwl effectors (Zdrzałek *et al.*, 2024), and HvHIPP43, a barley orthologue of OsHIPP43, was identified as a Pwl2 target using immunoprecipitation–MS (IP–MS) (Were *et al.*, 2025). Furthermore, yeast two–hybrid screening efforts uncovered a plethora of small HMA (sHMA) proteins from rice, foxtail millet (*Setaria italica*), and wheat (*Triticum aestivum*) as potential

targets of AVR–Pik and AVR–Pik like (APikL) effectors (Bentham *et al.*, 2021). Importantly, some *M. oryzae* effectors listed above are known to directly bind the HMA/HMA–like domains of their respective targets (Fig. 2A–C).

In addition to *M. oryzae*, targeting of host HMA proteins takes place across phytopathogen kingdoms. OsHPP04 is a target of the root knot nematode (*Meloidogyne graminicola*) effector MgMO298 (Song *et al.*, 2021), and was also identified as a candidate binding partner of AVR–PikD (Oikawa *et al.*, 2024). Meanwhile, NbHIPP26 from *N. benthamiana* was identified as a target of the potato mop–top virus (PMTV) movement protein TGB1, although in this case, the isoprenylation motif of NbHIPP26 was found to be critical for TGB1 association, rather than the HMA domain (Cowan *et al.*, 2018). In Arabidopsis studies, extensive yeast two–hybrid screens identified candidate HMA domain–containing targets of effectors from the powdery mildew fungus, *Golovinomyces orontii*, downy mildew oomycete, *Hyaloperonospora arabidopsidis*, and the bacterium, *Xanthomonas campestris* (Table 2). Considered together, the repeated appearance of HPP and HIPP proteins in non–biased searches for host targets across diverse pathosystems hints at widespread roles for HMA proteins in plant immunity.

HMA domains as bait for effectors

HMA–like domains have been identified within plant resistance proteins, including nucleotide–binding leucine–rich repeat (NLR) receptors, and tandem kinase proteins (TKPs), which form part of the intracellular defence signalling system of plants. Typically, NLRs are composed of a C–terminal leucine–rich repeat (LRR) domain, central NB–ARC (nucleotide–binding adaptor shared by APAF–1, R proteins, and CED–4) domain, and a variable N–terminal signalling domain, capable of triggering localized cell death when activated, for example via direct or indirect recognition of effectors (for a recent review, see Locci and Parker, 2024). Additionally, some NLRs contain integrated domains (IDs), including WRKY, WD–40, and, of relevance here, HMA–like domains (Sarris *et al.*, 2016; Grund *et al.*, 2019; Table 3). It is hypothesized that NLR IDs structurally mimic host targets of their cognate effectors, serving as bait to enable robust effector–triggered responses (Cesari *et al.*, 2013, 2014; Maqbool *et al.*, 2015; Ortiz *et al.*, 2017; Mukhi *et al.*, 2021). Beyond NLRs, TKPs are an emerging class of resistance proteins that commonly contain non–kinase IDs (Reveguk *et al.*, 2025; Table 3), hinting at another context in which plants might exploit the HMA structure for effector recognition.

Among ID–NLRs, the recognition profiles of Pik–1 allelic variants from rice have been studied in depth, and effector binding specificity is known to be determined by their polymorphic HMA–like IDs (Maqbool *et al.*, 2015; De la Concepcion *et al.*, 2018, 2019, 2021). Structural studies suggest that HMA binding might have evolved convergently among *M. oryzae* effectors, as some form distinct interfaces with their

Insights from pathogens on HMA protein function | 5

Table 2. HMA proteins implicated in plant-pathogen interactions^a

Host	HMA protein	#HMA	M/LXCXXC region	Pathogen	Pathogen protein	SF	Reference
Arabidopsis	AtCCS AT1G12520	1	MTCEGC	<i>Golovinomyces orontii</i>	OEC45	–	Wefling <i>et al.</i> (2014)
Arabidopsis	AthIPP13 AT5G52750	1	– ^b	<i>Hyaloperonospora arabidopsidis</i>	ATR13	–	Mukhtar <i>et al.</i> (2011)
Arabidopsis	AthIPP19 AT3G21490	1	MHCNDC	<i>Hyaloperonospora arabidopsidis</i> <i>Golovinomyces orontii</i>	HaRxL44 OEC56 OEC115	–	Mukhtar <i>et al.</i> (2011) Wefling <i>et al.</i> (2014)
Arabidopsis	AthIPP26 AT4G38580	1	MDCEGC	<i>Xanthomonas campestris</i>	XopK	–	González-Fuente <i>et al.</i> (2020)
Arabidopsis	AthIPP27 AT5G66110	1	MDCEGC	<i>Heterodera schachtii</i>	–	Y	Radakovic <i>et al.</i> (2018)
Arabidopsis	AthHMP35 AT4G16380	1	LDCAKC	<i>Meloidogyne incognita</i> <i>Hyaloperonospora arabidopsidis</i>	– HaRxL44	Y –	Dutta <i>et al.</i> (2023) Mukhtar <i>et al.</i> (2011)
Arabidopsis	CCP (AthIPP05) AT4G05030	1	MRCDKC	<i>Pseudomonas syringae</i> pv. tomato DC3000	–	N	Chai <i>et al.</i> (2020)
Arabidopsis	AthMAD1 (AthHMP09) AT1G51090	1	LNCSKC	<i>Pseudomonas syringae</i> pv. tomato DC3000	–	Y	Imran <i>et al.</i> (2016)
<i>Citrus sinensis</i>	CsHIPP03 Cs_ont_2g000480	2	MHCEAC MHCEAC	<i>Candidatus Liberibacter asiaticus</i>	SDE34	– ^c	Hu <i>et al.</i> (2023)
<i>Eragrostis curvula</i>	EJB05_15595	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl2	–	Were <i>et al.</i> (2025)
<i>Haynaldia villosa</i>	HIPP1-V KM216991	1	MDCEGC	<i>Blumeria graminis</i> f. sp. <i>tritici</i> <i>Magnaporthe oryzae</i>	– Pwl2 Pwl1	N ^d – –	Wang <i>et al.</i> (2023) Were <i>et al.</i> (2025) Were <i>et al.</i> (2025)
<i>Hordeum vulgare</i>	HvHIPP43 HORVU.MOREX.r3.2HG0164110.1 or HORVU.MOREX.r3.3HG0267040.1 ^e	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl2 PWL2-3 Pwl3 Pwl4	–	
<i>Nicotiana benthamiana</i>	NbHIPP26	1	MDCEGC	Potato mop-top virus	TGB1	Y	Cowan <i>et al.</i> (2018)
<i>Oryza sativa</i>	Niben101Scf 07109g05004 OshIPP05 LOC_Os04g32850	1	LQCCRC	<i>Magnaporthe oryzae</i>	–	Y	Fukuoka <i>et al.</i> (2009); Nakao <i>et al.</i> (2011)
<i>Oryza sativa</i>	OshIPP19 LOC_Os04g39350	1	MPCEKS	<i>Magnaporthe oryzae</i>	AVR-PikA AVR-PikC AVR-PikD AVR-PikE AVR-PikF	N	Maidment <i>et al.</i> (2021); Oikawa <i>et al.</i> (2024)
<i>Oryza sativa</i>	OshIPP20 LOC_Os04g39010	1	MPCEKS	<i>Magnaporthe oryzae</i>	AVR-PikD	Y	Oikawa <i>et al.</i> (2024)
<i>Oryza sativa</i>	OshIPP28 LOC_Os10g14870	2	MHCEGC MHCDAC	<i>Rhizoctonia solani</i>	RsMf8HN	–	Niu <i>et al.</i> (2023)
<i>Oryza sativa</i>	OshIPP43 LOC_Os01g32330	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pwl1 Pwl2 Pwl2-2 Pwl2-3 Pwl3 Pwl4	Y	Zdrzalek <i>et al.</i> (2024)
<i>Oryza sativa</i>	OshPP03 LOC_Os02g37290	1	MASDKC	<i>Magnaporthe oryzae</i>	AVR-PikD	–	Oikawa <i>et al.</i> (2024)

(continued)

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erz234/8152035 by Norwich Bioscience Institutes Library (INACTIVE) user on 23 September 2025

Table 2. Continued

Host	HMA protein	#HMA	M/LXCXXC region	Pathogen	Pathogen protein	SF	Reference
<i>Oryza sativa</i>	OsHPP04	1	MSCDKC	<i>Magnaporthe oryzae</i>	AVR-PikD	N	Oikawa et al. (2024)
	LOC_Os02g37300			<i>Meloidogyne graminicola</i>	MgMO298	Y	Song et al. (2021); Huang et al. (2023)
<i>Setaria italica</i>	sHMA94	1	MPSPKN	<i>Magnaporthe oryzae</i>	AVR-PikD	–	Bentham et al. (2021) ^f
<i>Setaria italica</i>	SETIT_012363mg	1	MSNAKS	<i>Magnaporthe oryzae</i>	APIkL2F	–	Bentham et al. (2021) ^f
	sHMA25				AVR-PikD		
<i>Setaria italica</i>	SETIT_011333mg	1	MDCEGC	<i>Magnaporthe oryzae</i>	APIkL2F	–	Were et al. (2025)
	SitiHIPP43				APIkLA		
<i>Triticum aestivum</i>	SETIT_003232mg	1	IDCEGC	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	Pw12	Y	Zhang et al. (2015)
<i>Triticum aestivum</i>	TaHIPP1	1	IDCEGC	<i>Puccinia striiformis</i> f. sp. <i>tritici</i>	–	–	–
<i>Triticum aestivum</i>	TraesCS2A_01G064900	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pw12	–	Were et al. (2025)
<i>Triticum aestivum</i>	TaHIPP43	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pw12	–	Were et al. (2025)
<i>Triticum aestivum</i>	TraesCS3D_02G209500	1	MDCEGC	<i>Magnaporthe oryzae</i>	Pw12	–	Were et al. (2025)

^aFor each entry, gene identifiers and known pathogen-derived interactors are listed, as are the number of HMA/HMA-like domains (#HMA) and the sequence equivalent to the metal-binding motif (M/LXCXXC) in their $\beta 1$ - $\alpha 1$ loop region(s). Whether or not the HMA protein has been identified as a susceptibility factor (SF) in the pathosystem of interest is indicated by Y (yes), N (no), or – (not tested). ^bThe AtHIPP13 sequence included no conserved residues of the M/LXCXXC motif in the $\beta 1$ - $\alpha 1$ region. ^cAlthough not investigated in *C. sinensis*, the function of CsHIPP03 as a susceptibility factor was proposed, as independent silencing of three homologous transcripts (*NbHIPP3.1*, *NbHIPP3.2*, and *NbHIPP3.3*) enhanced *N. benthamiana* resistance to *Pto* DC3000 *dhopQ1-1*. ^dResistance to *Bgt* was assessed in *T. aestivum* HIPP1-V overexpression lines. ^eIn a yeast two-hybrid assay, Pw12 bound two HvHIPP43 paralogues, both of which possess an M/LXCXXC region reading MDCEGC. ^fA total of 56, 19, and nine candidate sHMA-binding partners were identified for AVR-PikD, APIkL2A, and APIkL2F, respectively, originating from *O. sativa*, *S. italica*, and *T. aestivum*. Only the two structurally characterized effector-HMA pairs from this study are included here.

respective targets, despite sharing conserved folds (Fig. 2). Overall, the repeated integration of HMA-like structures into plant resistance proteins corroborates the hypothesis that HPPs and HIPPs are frequently targeted by pathogens as a result of important, but poorly understood, roles in plant immunity.

A genetic perspective: HMA proteins contribute to plant disease outcomes

RNA-sequencing and microarray studies have revealed a range of expression profiles for HPP/HIPP genes under various abiotic and biotic stresses, suggesting a degree of functional divergence within the families (de Abreu-Neto et al., 2013). However, targeted quantitative reverse transcription-PCR (RT-qPCR) experiments frequently highlight genes encoding pathogen-targeted HPP/HIPPs as being up-regulated in an infection context: *NbHIPP26* in response to PMTV (Cowan et al., 2018); *OsHIPP43* in response to *M. oryzae* (Were et al., 2025); and *OsHPP04* in response to *Meloidogyne graminicola* (Song et al., 2021), among others whose products are not yet characterized as pathogen host targets (Zhang et al., 2015; Zschiesche et al., 2015; Radakovic et al., 2018; Chai et al., 2020; Dutta et al., 2023; Wang et al., 2023). To investigate the potential consequences of these expression changes within a given pathosystem, genetic studies exploiting mutants and

overexpression lines have been key. To date, both negative and positive regulators of defence have been identified amongst HMA proteins, making it especially challenging to assign a consensus immune function.

HMA proteins as susceptibility factors

Multiple HIPP and HPP genes have been labelled as host susceptibility factors based on the observation that their loss of function results in enhanced resistance to a particular pathogen (Table 2). For example, the recessive *pi21* locus in rice encodes a loss-of-function *OsHIPP05* mutation that enhanced resistance to 10 races of *M. oryzae* (Fukuoka et al., 2009). Remarkably, heterologous expression of *OsHIPP05* in Arabidopsis (a non-host) enabled growth of *M. oryzae* hyphae, supporting the possibility that the encoded protein functions as a negative regulator of host basal resistance (Nakao et al., 2011).

While Oikawa et al. (2024) showed that *OsHIPP19* and *OsHIPP20* were each stabilized in complex with the effector AVR-PikD, deletion of *OsHIPP19* did not significantly impact rice susceptibility to *M. oryzae*. However, *OsHIPP20* deletion enhanced resistance, suggesting that AVR-PikD binding of this host target might ordinarily favour blast disease progression (Oikawa et al., 2024). HvHIPP43 was also implicated as a susceptibility factor, as barley plants overexpressing this HIPP were more susceptible to *M. oryzae* (Were et al., 2025).

Insights from pathogens on HMA protein function | 7

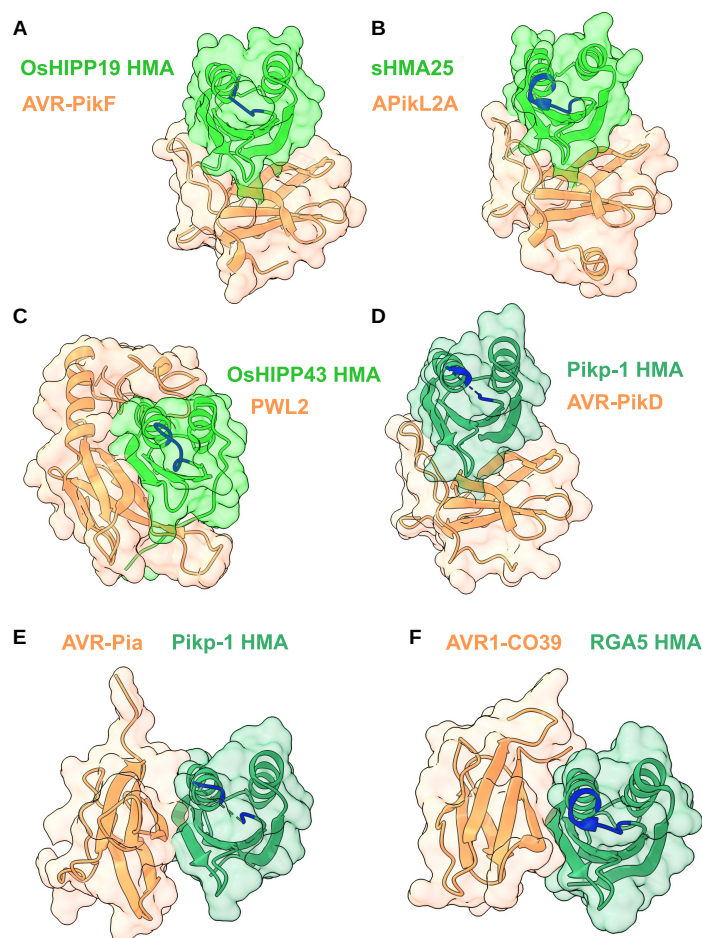


Fig. 2. Structurally characterized *M. oryzae* effectors in complex with the HMA or HMA-like domains of host target proteins and ID-NLRs. Crystal structures of (A) AVR-PikF with the HMA-like domain of OsHIPP19 from rice (7B1I) (Maidment *et al.*, 2021); (B) APikL2A with the HMA-like domain of sHMA25 from foxtail millet (7NLJ) (Bentham *et al.*, 2021); (C) Pwl2 with the HMA domain of OsHIPP43 from rice (8R7D) (Zdrzalek *et al.*, 2024); (D) AVR-PikD with the HMA-like ID of Pikp-1 from rice (5A6V) (Maqbool *et al.*, 2015); (E) AVR-Pia with the HMA-like ID of Pikp-1 (6Q76) (Varden *et al.*, 2019); (F) AVR1-CO39 with the HMA-like ID of RGA5 from rice (5ZNG) (Guo *et al.*, 2018). Complexes are displayed such that the HMA/HMA-like domains are in equivalent orientations. Domains from HPP/HIPP host targets are coloured light green. Domains from ID-NLRs are coloured dark green. Regions of ribbon models equivalent to the metal-binding motif (M/LXCXXC) in the $\beta 1$ - $\alpha 1$ loop region of HMA/HMA-like domains are coloured blue. Effectors are coloured orange.

OsHPP04 might likewise function as a susceptibility factor but in a pathogen-specific manner. Although a knockout mutant of *OsHPP04* in rice showed unaltered resistance to *M. oryzae* (Oikawa *et al.*, 2024), an alternative mutant allele of the same gene generated by Huang *et al.* (2023) supported significantly fewer root knot nematodes (*M. graminicola*) than wild-type rice. In Arabidopsis, *AtHIPP27* function promoted susceptibility to two different nematode species: *Heterodera*

schachtii (Radakovic *et al.*, 2018) and *Meloidogyne incognita* (Dutta *et al.*, 2023), while *AtHMAD1* (*AtHMP09*) promoted susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 (Imran *et al.*, 2016). Whether or not the HMA domains of *AtHIPP27* and *AtHMAD1* are directly targeted by effectors remains to be determined.

Products of susceptibility genes can promote pathogen establishment via different mechanisms, including facilitation of

Table 3. Examples of plant resistance proteins with HMA-like domains and their corresponding helper NLRs and effectors, where known^a

Plant	Sensor	Domain architecture	M/LXCXXC region	Helper	Pathogen	Effector(s)	References
<i>Hordeum vulgare</i>	RPG1	HMA-Pseudokinase-kinase	— ^a	Unknown	<i>Puccinia graminis</i> f. sp. <i>tritici</i>	Unknown	Brueggeman <i>et al.</i> (2002); Nimala <i>et al.</i> (2006); Reveguk <i>et al.</i> (2025)
<i>Hordeum vulgare</i>	MLA3	CC-NB-LRR	— ^b	—	<i>Magnaporthe oryzae</i>	Pwl2	Gómez De la Cruz <i>et al.</i> (2024, Preprint)
<i>Oryza sativa</i>	Pikm-1	CC-HMA-NB-LRR	MVDDKS	Pikm-2	<i>Magnaporthe oryzae</i>	AVR-PikA AVR-PikD AVR-PikE AVR-Mgk1	De la Concepcion <i>et al.</i> (2018); Sugihara <i>et al.</i> (2023)
<i>Oryza sativa</i>	Pikp-1	CC-HMA-NB-LRR	MEGNNC	Pikp-2	<i>Magnaporthe oryzae</i>	AVR-PikD AVR-Mgk1	De la Concepcion <i>et al.</i> (2018); Sugihara <i>et al.</i> (2023)
<i>Oryza sativa</i>	RGA5	CC-NB-LRR-HMA	MPCGKS	RGA4	<i>Magnaporthe oryzae</i>	AVR-Pia AVR1-CO39	Cesari <i>et al.</i> (2013); Ortiz <i>et al.</i> (2017)

The sequence equivalent to the metal-binding motif (M/LXCXXC) in the $\beta 1$ - $\alpha 1$ loop region of the HMA-like domain is given. CC, coiled coil; NB, nucleotide-binding; LRR, leucine-rich repeat. ^aThe RPG1 sequence included no conserved residues of the M/LXCXXC motif in the $\beta 1$ - $\alpha 1$ loop. ^bAlthough MLA3 does not possess a full HMA-like fold, the final three repeats of the LRR structurally mimic the Pwl2-binding surface of the OsHIPP43 HMA domain. This region does not include a $\beta 1$ - $\alpha 1$ loop.

host recognition and entry, pathogen nutrient acquisition and movement, or via suppression of host immune responses (Garcia-Ruiz *et al.*, 2021). Although not the case for all susceptibility factors described above (e.g. AtHIPP27, Radakovic *et al.*, 2018), genetic evidence suggests that some HMA proteins may negatively regulate pathogen elicitor-induced reactive oxygen species (ROS) bursts and/or defence gene expression during infection (Fukuoka *et al.*, 2009; Huang *et al.*, 2023; Were *et al.*, 2025). Evidence for HPP/HIPP-mediated negative regulation of basal plant immune responses is thus building; however, the intracellular mechanisms underlying this suppression are unclear.

HMA proteins as positive regulators of defence

In contrast to the susceptibility factors described above, a select few HMA proteins have been shown to promote host resistance to pathogens. HIPP1-V is a single HMA domain-containing HIPP from the diploid wheat relative, *Haynaldia villosa*, whose heterologous overexpression in hexaploid wheat resulted in enhanced resistance to the powdery mildew fungus, *Blumeria graminis* f. sp. *tritici* (Bgt) (Wang *et al.*, 2023). To investigate the underpinning mechanism, Wang *et al.* (2023) identified CMPG1-V as a HIPP1-V binding partner using a yeast two-hybrid approach. CMPG1-V was previously characterized as a resistance gene (Zhu *et al.*, 2015), suggesting that HIPP1-V and CMPG1-V might co-operate to promote disease resistance. Specifically, HIPP1-V is proposed to recruit CMPG1-V to the plasma membrane following its isoprenylation, thereby initiating defence signalling (Wang *et al.*, 2023).

Another positive regulator of plant immunity is AtHPP05, also named CCP (copper chaperone induced by pathogens) (Chai *et al.*, 2020). CCP overexpression enhanced Arabidopsis resistance to *Pto* DC3000, while its deletion enhanced susceptibility. Although heavy metal binding was not

explicitly demonstrated, CCP is homologous to human ATOX1, and is proposed to bind Cu via its HMA domain, facilitating its homodimerization and nuclear import. There, CCP was found to bind the transcription factor TGA2, activating transcription of the defence gene *PR1* (Chai *et al.*, 2020), which encodes a widely studied antimicrobial signalling protein (Han *et al.*, 2023).

Considering the examples above, whether HMA proteins are positive or negative regulators of immunity does not seem to correlate with their number of HMA domains (Table 2), pathogen-induced changes in expression patterns, or subcellular localization. Instead, the influence of HPP/HIPPs on disease outcomes may depend on (i) if/how the protein is targeted by effectors (which so far remains to be determined for HIPP1-V and CCP), (ii) the consequences of this (for instance, stabilization versus degradation of the HMA protein), and (iii) the distinct protein–protein interaction networks of HPP/HIPP family members. To better understand the variety of immune-related functions reported for HMA proteins, more data surrounding these three aspects are required.

Lessons from microbes and beyond

As HMA proteins have emerged as host targets of diverse pathogen-derived proteins and/or determinants of host susceptibility, we draw on observations made in plant pathology studies, as well as those focused on plant development and abiotic stress tolerance, to generate hypotheses about their intracellular functions, including at plasmodesmata.

HPP/HIPPs regulate phytohormone signalling

HPP and HIPP proteins have been implicated in phytohormone regulation, including in developmental and defensive contexts. Firstly, a handful of ER-associated Arabidopsis

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/eraf234/8152035 by Norwich Bioscience Institutes Library (INACTIVE) user on 23 September 2025

HIPPs were characterized as regulators of cytokinin signalling by Guo *et al.* (2021). Overexpression of *AtHIPP01* led to enhanced degradation of the cytokinin-degrading enzyme CKX1 (cytokinin oxidase/dehydrogenase), increasing cytokinin sensitivity and severely stunting rosette growth. In this work, both *AtHIPP01* and *AtHIPP07* were observed at plasmodesmata, yet the functional relevance of this was not discussed. Exogenous cytokinin application was recently shown to enhance cell-to-cell movement of fluorescent proteins in *N. benthamiana* leaves (Horner and Brunkard, 2021), speculatively via rapid *de novo* formation of secondary (post-cytokinesis) plasmodesmal pores—a phenomenon previously demonstrated in the shoot apical meristem of *Sinapis alba* (Ormenese *et al.*, 2006). Whether or not HIPP-mediated modulation of cytokinin responses ordinarily governs secondary plasmodesmata formation remains to be studied.

Secondly, pathology studies hint at a role for HMA proteins in salicylic acid (SA) signalling. For example, *AtHIPP03* was implicated as a negative regulator of SA responses, based on co-expression analysis of Arabidopsis plants constitutively expressing this HIPP (Zschiesche *et al.*, 2015). *AtHMAD1* is also thought to negatively regulate SA signalling, as *athmad1* mutants displayed increased expression of systemic acquired resistance (SAR) marker genes post-inoculation with *Pto* DC3000 (Imran *et al.*, 2016). In contrast, transcripts related to SA signalling, as well as accumulation of SA itself, were increased in wheat plants overexpressing *HIPP1-V* (Wang *et al.*, 2023), suggesting that this HIPP might positively regulate SA-responsive pathways. Likewise, CCP may promote SA responses via its interaction with TGA2, a known regulator of SA-dependent gene expression (Boyle *et al.*, 2009; Chai *et al.*, 2020). As SA is an established elicitor of plasmodesmal closure via callose deposition (Wang *et al.*, 2013; Huang *et al.*, 2019; Tee *et al.*, 2023), it is tempting to speculate that HMA proteins could regulate plasmodesmal aperture indirectly via SA. However, why these proteins should require plasmodesmal localization to do so remains unclear.

HPP/HIPPs regulate oxidative stress tolerance

ROS are established second messengers in plant signalling, and their accumulation is associated with both abiotic and biotic stress (for reviews, see Castro *et al.*, 2021; Mittler *et al.*, 2022). On perception of stressful stimuli, ROS build-up can occur in chloroplasts and mitochondria as a consequence of electron transport chain malfunction. ROS may also be actively produced via oxidases in peroxisomes, and in the apoplast via cell wall peroxidases and respiratory burst oxidase homologue (RBOH) enzymes. To prevent excessive oxidative damage to the cellular machinery during stress, plants utilize ROS-scavenging and transport proteins, as well as non-enzymatic antioxidants. To explain their apparent involvement in so many plant processes and pathosystems (de Abreu-Neto *et al.*, 2013; Table 2), could HMA proteins somehow regulate these broad-spectrum stress signals?

The prototypical HMA protein ATX1 was first identified in a genetic screen for antioxidant factors capable of functionally substituting for *S. cerevisiae* Cu/Zn-SOD 1 (SOD1) (Lin and Culotta, 1995), a metalloenzyme that catalyses the dismutation of damaging superoxide, forming hydrogen peroxide and oxygen (Bermingham-McDonogh *et al.*, 1988). Although not responsible for direct delivery of metal cofactors to SOD1 (Culotta *et al.*, 1997), ATX1 is thought to potentially dampen oxidative stress using its own (albeit inefficient compared with SOD1) superoxide dismutase activity (Portnoy *et al.*, 1999).

In plants, participation in ROS scavenging has similarly been suggested for HPPs. Through the use of alternative translation start sites, *AtCCS* transcripts were found to encode both cytosolic- and chloroplast-targeted protein isoforms, enabling activation of three Arabidopsis Cu/Zn-SOD (CSD) enzymes: CSD1 (cytoplasmic), CSD2 (stromal), and CSD3 (peroxisomal) (Abdel-Ghany *et al.*, 2005; Chu *et al.*, 2005). Later, a study in the *M. graminicola*-rice system suggested that OsHPP04 may deliver Cu cofactors to cytosolic Cu/Zn-SOD 2 (cCu/Zn-SOD2) and, upon nematode infection, binding of the MgMO298 effector to OsHPP04 is thought to promote ROS scavenging, thereby dampening host immune responses (Song *et al.*, 2021). Besides nematodes, it is conceivable that other phytopathogens might have evolved to exploit HPP-mediated delivery of metal ion cofactors to enhance enzymatic ROS scavenging.

Recent work by Were *et al.* (2025) uncovered a potential link between a plasmodesmata-localized HIPP and ROS homeostasis, as barley plants overexpressing *HvHIPP43* exhibited reduced ROS bursts in response to pathogen elicitors flg22 and chitin. It is not yet established whether these plants scavenged ROS more efficiently, or were compromised in ROS production. Nevertheless, it is proposed that binding of the *M. oryzae* effector Pw12 might somehow enhance the ability of *HvHIPP43* to negatively regulate ROS signalling, as similar phenotypes were observed in *HvHIPP43*- and *Pw12*-overexpressing plants (Were *et al.*, 2025). Currently, it is unclear how these phenotypes relate to the plasmodesmal localization of *HvHIPP43*, which live cell imaging suggests to be compromised by Pw12 (Were *et al.*, 2025).

In contrast, *HIPP1-V* was suggested to promote ROS accumulation, as levels of oxidative response-related transcripts, NADPH oxidase enzymes, and hydrogen peroxide were increased in wheat plants overexpressing this HIPP, which may have contributed to their increased *Bgt* resistance (Wang *et al.*, 2023). Unlike the mechanisms described for *AtCCS* and OsHPP04 above, exactly how *HvHIPP43* and *HIPP1-V* might modulate ROS signalling remains unknown.

As an alternative to metallochaperone-based models, one could hypothesize that HMA domains regulate oxidative stress tolerance by participating in redox reactions. ROS accumulation generates local oxidative environments within cellular compartments, and is thus tightly linked to redox homeostasis. To combat oxidative stress, plants maintain large pools of

10 | Turley and Faulkner

antioxidants, including low molecular weight compounds such as glutathione, a tripeptide of Glu–Cys–Gly, which possess a solvent-accessible thiol (–SH) group (Jozefczak *et al.*, 2012).

With exposed thiol groups as part of their CXXC metal-binding motif(s), it is possible that HPPs and HIPPs might also fluctuate between oxidized and reduced forms. Indeed, ATOX1 from humans is oxidized by glutathione, resulting in intramolecular disulfide bond formation that blocks Cu co-ordination in the HMA domain (Hatori *et al.*, 2012; Brose *et al.*, 2014). As redox environments are predicted to vary between cell types and subcellular compartments, it is proposed that ATOX1 couples Cu distribution with cellular redox status to regulate mammalian cell viability and differentiation (reviewed in depth by Hatori and Lutsenko, 2016). Whether or not HMA proteins function as key redox regulators in plants, as well as the consequences of this for ROS detoxification, remains to be studied.

In summary, multiple lines of evidence point towards roles for HMA proteins in ROS homeostasis, which has the potential to underpin their involvement in both abiotic and biotic stress tolerance. However, much of this evidence comes from the study of HPPs or mammalian HMA domains, leaving the question of how HIPPs alter plant ROS metabolism unanswered.

HPP/HIPPs might be translocated intracellularly to regulate transcription

Live-cell imaging has uncovered HMA proteins in multiple subcellular compartments, from the cytoplasm and nucleus, to the plasma membrane and plasmodesmata (Fukuoka *et al.*, 2009; Zhang *et al.*, 2015; Cowan *et al.*, 2018; Chai *et al.*, 2020; Song *et al.*, 2021; Wang *et al.*, 2023; Huang *et al.*, 2024; Oikawa *et al.*, 2024; Were *et al.*, 2025). However, the motifs and/or structural features that promote and maintain these localizations remain largely unknown. Nevertheless, studies on the *N. benthamiana* and Arabidopsis HIP26 homologues have shed light on this.

Visualized with an N-terminal fluorescent tag, NbHIP26 was found to localize to *N. benthamiana* nuclei (including the nucleoplasm and nucleoli) and to the plasma membrane, with enrichment at plasmodesmata (Cowan *et al.*, 2018). However, when co-expressed with the PMTV movement protein TGB1 in a bimolecular fluorescence complementation (BiFC) experiment, NbHIP26 predominantly localized to nucleoli and microtubules. These observations prompted Cowan *et al.* (2018) to propose a model whereby TGB1 binds and shuttles NbHIP26 to the nucleus of vascular parenchyma cells (where *NbHIP26* promoter activity is concentrated), promoting drought tolerance and long-distance PMTV movement via an unknown mechanism. *AtHIP26* promoter activity was similarly enriched in Arabidopsis vascular tissue, and the *AtHIP26* protein localized to the nuclei of onion (*Allium cepa*) epidermal cells (Barth *et al.*, 2009). In nuclei, *AtHIP26* is

thought to manipulate drought-responsive gene expression via its binding partner, zinc finger homeobox protein ATHB29 (Barth *et al.*, 2009). It is conceivable that the TGB1–NbHIP26 complex might also manipulate transcription factor function to reprogramme *N. benthamiana* vascular cells.

AtHIP26 is not the only HMA protein to bind transcription factors. Indeed, several HIPPs were suggested to bind ATHB29 in a yeast two-hybrid experiment, namely *AtHIP20*, 21, 23, 24, 27, and 30, with *AtHIP30* showing additional interactions with ATHB21 and ATHB30 (Barth *et al.*, 2009). Also, a BiFC experiment in *N. benthamiana* suggested that CqHIP34 from quinoa (*Chenopodium quinoa*) bound CqZF-HD14, another positive regulator of drought tolerance (Sun *et al.*, 2022). As mentioned earlier, CCP binds the bZIP transcription factor TGA2, recruiting it to the promoter of defence gene *PR1* (Chai *et al.*, 2020).

As multiple HMA proteins appear to operate in cell nuclei, this raises mechanistic questions. Live imaging suggests that the TGB1–NbHIP26 complex could be transported from plasmodesmata to nuclei via microtubules (Cowan *et al.*, 2018). In this case, a combination of C-terminal isoprenylation and S-acetylation of a central Cys residue promoted membrane association of NbHIP26, whereas mutation of the CXXC motif in the HMA domain had no noticeable effect on localization (Cowan *et al.*, 2018). Consistent with this, mutation of the isoprenylation motif compromised plasmodesmal localization of other HIPPs: *AtHIP07* and *HvHIP43* (Guo *et al.*, 2021; Were *et al.*, 2025). Lipid modifications might also regulate the nuclear distribution of HIPPs. *AtHIP26* formed speckles within the nucleoplasm of onion cells, but when its isoprenylation motif was mutated, nuclear distribution was more homogenous (Barth *et al.*, 2009). As isoprenylation has been documented to influence both protein–protein and protein–lipid interactions (Crowell, 2000; Wang *et al.*, 2020), this phenomenon could speculatively be explained by facilitation or inhibition of *AtHIP26* binding to other nuclear proteins.

In contrast, CCP lacks an isoprenylation motif, and its nuclear import in Arabidopsis protoplasts required a functional CXXC motif and nuclear localization signal (KKVGF) near its C-terminus (Chai *et al.*, 2020). According to Barth *et al.* (2009), *AtHIP26* likewise possesses a nuclear localization signal (DCSHGHKIKKKR), but this is found near the N-terminus of the protein. Thus, although it seems that multiple HMA proteins carry out signalling functions in nuclei, the routes they take to get there are not necessarily conserved.

HPP/HIPPs might regulate plasmodesmal aperture

Some pathogen-targeted plasmodesmata-located HIPPs are known susceptibility factors (Cowan *et al.*, 2018; Oikawa *et al.*, 2024; Were *et al.*, 2025), hinting that putative Hipp functions in immunity and plasmodesmal regulation could be linked. Plasmodesmata are critical during infection, as they

offer routes for the intercellular spread of pathogens and their secreted effectors. Indeed, imaging-based screens have revealed cell-to-cell mobility to be common among nucleocytoplasmic effectors from the fungal pathogen *Colletotrichum higginsianum* (Ohtsu *et al.*, 2024), the oomycete *H. arabidopsidis* (Liu *et al.*, 2022, Preprint), and the bacterium *Pto* DC3000 (Li *et al.*, 2021). Such mobile effectors could feasibly allow pathogens to suppress host defences ahead of their primary invasion front.

To enhance intercellular trafficking and overcome elicitor-induced closure of plasmodesmata, pathogen-mediated manipulation of plasmodesmal function is hypothesized to be widespread (Cheval and Faulkner, 2018). Viral movement proteins are known to target and open plasmodesmal channels in a variety of ways, including via alteration of callose metabolism, cytoskeleton dynamics, and tubule formation (for reviews, see Lucas, 2006; Taliany *et al.*, 2008; Reagan and Burch-Smith, 2020). Elsewhere, plasmodesmata-located effectors from bacteria, fungi, and oomycetes have been identified, some of which are modifiers of plasmodesmal function. For instance, HaRxL77 from *H. arabidopsidis* (Liu *et al.*, 2022, Preprint), ChEC8, ChEC127, and ChEC132 from *C. higginsianum* (Ohtsu *et al.*, 2024), and the co-operating effector pair, secreted in xylem 5 (Six5) and Avr2 (Six3) from *Fusarium oxysporum* (Cao *et al.*, 2018) can enhance intercellular trafficking via unknown mechanisms. While Six5 does not modify plasmodesmal callose (Blekemolen *et al.*, 2022), RxRL3 from the oomycete *Phytophthora brassicae* and HopO1 from *Pto* DC3000 both suppress callose accumulation to enhance plasmodesmal trafficking capacity (Aung *et al.*, 2020; Tomczynska *et al.*, 2020). Although empirical evidence is currently lacking, one could hypothesize that plasmodesmal HMA proteins might be endogenous regulators of plasmodesmal aperture, and are thus targeted by pathogens to maintain cell-to-cell connectivity during infection.

If HPPs and/or HIPPps do regulate plasmodesmal function, could ROS be involved? A handful of plant HMA proteins have been implicated in ROS homeostasis (discussed above) and, as part of elicitor-induced signalling cascades, ROS are known regulators of plasmodesmal dynamics. For example, extracellular flg22 or chitin perception culminates in RBOHD activation via independent pathways, triggering apoplastic ROS accumulation and closure of plasmodesmata via CALLOSE SYNTHASE 1 (CALS1)-mediated callose deposition (Xu *et al.*, 2017; Cheval *et al.*, 2020; Tee *et al.*, 2023). With this in mind, it is puzzling to consider how intracellular HMA proteins, which lack transmembrane and extracellular domains, could contribute to plasmodesmal regulation via apoplastic ROS. A greater understanding of HPP/HIPP binding partners at plasmodesmata would help address this.

Alternatively, as some HMA proteins have been identified as positive regulators of defence, it is possible that a subset may reside at plasmodesmata as part of a stress perception system. Reminiscent of IDs in immune receptors (see ‘HMA domains as bait for effectors’), perhaps some defence-promoting HPP/

HIPPps employ their HMA domains as bait, perturbing cell-to-cell movement and/or virulence functions of effectors, thus slowing infection spread. Building on the established role of plasmodesmata as signalling hubs within the plasma membrane, and the potential nuclear functions of HPPps and HIPPps discussed above, it is possible that effector–HMA binding events at plasmodesmata might trigger downstream immune responses, including transcriptional reprogramming to fortify host defences. Further characterization of the intracellular functions of defence-promoting HMA proteins is required to test these hypotheses.

What microbes aren’t telling us: HPP/HIPP function in heavy metal tolerance

Perhaps one of the biggest challenges in HMA protein research is uncovering empirical links between three major themes discussed in this review: plasmodesmata, immunity, and heavy metal binding. Some of the first described HMA domains belonged to metallochaperones participating in ion transport within cells, including widely conserved Cu chaperones (see ‘A historical perspective: what makes a HMA domain?’). Unsurprisingly, in plants, HIPPps and HPPps have been implicated in regulating heavy metal tolerance, especially regarding the toxic non-nutrient metal, cadmium (Cd). AtHIPP06, 20, 21, 22, and 26 are induced by Cd exposure and positively regulate Cd tolerance in Arabidopsis (Suzuki *et al.*, 2002; Gao *et al.*, 2009; Tehseen *et al.*, 2010). Following observations of higher Cd levels in overexpressing plants, AtHIPP44 was also implicated in regulating Cd uptake (Zhang *et al.*, 2019). Meanwhile, 11 rice HPP/HIPP genes and three tea plant (*Camellia sinensis*) HIPP genes were up-regulated under Cd stress, and the growth of *oshipp42* rice mutants was marginally reduced under Cu, Zn, Cd, and manganese (Mn) stress (Khan *et al.*, 2019; Wei *et al.*, 2023). *OsHIPP29* and *HIPP1-V* overexpression enhanced Cd tolerance in rice and bread wheat, respectively (B.Q. Zhang *et al.*, 2020; H. Zhang *et al.*, 2020). Despite the growing number of studies reporting HPP/HIPP involvement in metal homeostasis, many questions regarding the underlying mechanisms remain.

Firstly, do all HPPps and HIPPps bind metal ions directly? Dykema *et al.* (1999) demonstrated that AtHIPP07 (originally ATPF3) bound Cu(II), Ni(II), or Zn(II) in a reversible manner within immobilized metal affinity chromatography columns. Subsequently, using different *in vitro* methods, AtHIPP26 (originally ATPF6) was discovered to bind Pb(II), Cd(II), and Cu(II) ions (Gao *et al.*, 2009). AtHIPP03 was shown to bind Zn(II) (Zschiesche *et al.*, 2015), and AtHIPP06 (originally Cdi19 for Cd-induced 19) was shown to bind Cd(II), Cu(II), and Hg(II) (Suzuki *et al.*, 2002). Despite this, evidence for *in planta* metal binding of HIPPps and HPPps is notably lacking, and the specificity and fate of associated metals are unclear. Also, one cannot rule out the possibility that HPP/HIPP

12 | Turley and Faulkner

regulation of heavy metal tolerance could be indirect, for example via regulation of ROS (see earlier), given that ROS production is induced by metal stress (Shahid *et al.*, 2014). Alternatively, Zhou *et al.* (2023) proposed a transcription-based tolerance mechanism, as overexpression of *ApHIPP26* from *Arabidopsis paniculata* enhanced *Arabidopsis* growth under Cd stress, accompanied by increased expression of genes involved in metal uptake and translocation.

Secondly, what can the number of HMA domains tell us about putative metallochaperone function? So far, the biological significance of a HPP or HIPP possessing one versus two HMA domains has not been directly studied. Structural characterization of HMA domains from yeast, bacteria, and humans suggests that a single HMA fold, complete with a CXXC motif, is sufficient for metal binding (Steele and Opella, 1997; Gitschier *et al.*, 1998; Hung *et al.*, 1998; Rosenzweig *et al.*, 1999); however, metals have yet to appear in structures of plant HMA or HMA-like domains (Maqbool *et al.*, 2015; De la Concepcion *et al.*, 2018, 2021; Guo *et al.*, 2018; Varden *et al.*, 2019; Bentham *et al.*, 2021; Maidment *et al.*, 2021; Zdrzałek *et al.*, 2024, Fig. 2), and the structures of double-HMA HPP/HIPP proteins have not been characterized.

It is plausible that a double-HMA architecture might increase the versatility of a given protein by facilitating association with more/different metal species. Interestingly, ATX1 from the cyanobacterium *Synechocystis* PCC6803 has been crystallized as a dimer, loaded with two or four Cu(I) ions (Badarau *et al.*, 2010). The human and yeast ATX1 orthologues also dimerize in the presence of Cu (Tanchou *et al.*, 2004; Miras *et al.*, 2008), and X-ray crystallography revealed a distorted tetrahedral geometry involving four Cys residues at the Cu(I)-binding site of these dimers, stabilized by hydrogen bonds (Wernimont *et al.*, 2000; Lee *et al.*, 2017). Speculatively, depending on the relative orientation of their domains, a double-HMA protein might also be capable of binding Cu(I) or divalent cations such as Zn(II) in a tetrahedral environment, akin to the 'structural' metal-binding sites of some Zn finger proteins (Permyakov, 2021). Indeed, crystal structures suggest that ATOX1 from humans can bind Zn(II), Cd(II), and platinum [Pt(II)] as a homodimer (Wernimont *et al.*, 2000; Belviso *et al.*, 2016; Mangini *et al.*, 2022). Such tetrahedral co-ordination sites might be better suited for metal sequestration and/or enhancing protein stability, as opposed to metal trafficking. Hinting at the possibility of metal-dependent HIPP–HIPP interactions, Guo *et al.* (2021) observed homodimerization of AtHIPP7 in yeast, which was abolished by Cys to Gly mutation in the CXXC motifs. Clearly, there remains much to learn about the chemistry and specificity of metal-binding sites in plant HMA domains.

Thirdly, why might proteins involved in metal homeostasis be localized to plasmodesmata? A possible clue comes from a study by O'Lexy *et al.* (2018) (also summarized by Barr *et al.*, 2023) in which heavy metal species were reported to have

distinct effects on plasmodesmal function in roots. Specifically, iron (Fe) stress reduced channel permeability in a CALS12-dependent manner, while Cu stress enhanced permeability in a β -1,3-glucanase-dependent manner. While the components involved in metal sensing and signalling in this context remain unknown, it is tempting to speculate that HIPPs could be involved; 'capturing' heavy metals and perhaps transferring them to metalloenzymes, thereby triggering signalling cascades that feed back to modify plasmodesmal function.

Finally, is metal binding relevant for HMA protein function in plant immunity? While the majority of host HMA proteins implicated in plant–microbe interactions sport a conserved metal-binding motif, there are notable exceptions: OsHIPP19, OsHIPP20, and OsHIPP03 from rice, and sHMA94 and sHMA25 from foxtail millet (Table 2). Also, regions corresponding to the 'metal-binding loop' (between β 1 and α 1) of HMA-like IDs within rice NLRs Pik-1 and RGA5 do not include two Cys residues (Table 3), suggesting that metal binding is not required for baiting effectors. Consistent with this, in effector–HMA crystal structures, the β 1– α 1 loop rarely constitutes part of the binding interface (Fig. 2). Nevertheless, indirect evidence from a handful of studies suggests functional links between HPP/HIPP metallochaperone functions and plant immunity (Zhang *et al.*, 2015; Chai *et al.*, 2020; Song *et al.*, 2021).

A speculative hypothesis is that heavy metals carried by HMA domains could be released during stress and employed as anti-microbial weapons, or delivered to intracellular defensive enzymes such as SODs and metalloproteases (Moreau *et al.*, 2013). Curiously, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1), a transcriptional co-regulator of SA-responsive gene expression, was shown to bind SA via a co-ordinated transition metal ion (probably Cu, according to *in vitro* analyses) (Wu *et al.*, 2012). Should HMA proteins be implicated in provision or removal of NPR1-associated metals, this would corroborate observations of HPP/HIPP influence on SA signalling.

Alternatively, evidence from studies of hyperaccumulator plants suggests that high concentrations of heavy metals might deter or kill herbivores and pathogens directly, referred to as the 'elemental defence hypothesis' (reviewed by Hörger *et al.*, 2013; De *et al.*, 2025). For example, hyperaccumulation of Zn, nickel (Ni), or Cd by *Thlaspi caerulescens* enhanced resistance to the apoplastic bacterium *P. syringae* pv. *maculicola* (Fones *et al.*, 2010). Also, local accumulation of Zn around invasion sites was associated with increased resistance of *Arabidopsis* (a non-accumulator plant) to the fungal necrotroph *Plectosphaerella cucumerina* BMM (Escudero *et al.*, 2022). Although evidence for elemental defence against intracellular pathogens is limited, one could imagine that some HMA proteins might sequester metals within vulnerable tissues such as the vasculature, where HPP/HIPP expression is often enriched (Mira *et al.*, 2001; Barth *et al.*, 2009; Tehseen *et al.*, 2010; Cowan *et al.*, 2018) or in subcellular structures such as

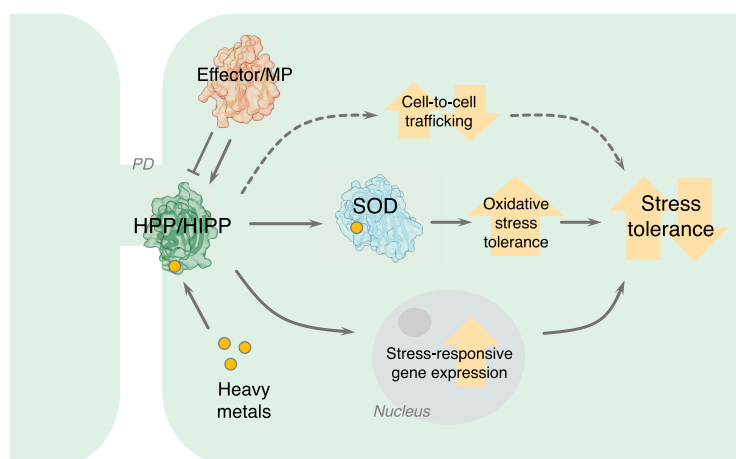


Fig. 3. Speculative functions for plasmodesmal HMA proteins. HPP/HIPPs may be endogenous regulators of cell-to-cell trafficking and/or ROS metabolism, perhaps via provision of cofactors to metalloenzymes such as superoxide dismutase (SOD). Alternatively, they may enter the nucleus in times of stress, altering gene expression via their interaction with transcription factors. Binding of pathogen effectors or viral movement proteins (MPs) may promote or inhibit HPP/HIPP function. Dashed lines indicate hypotheses currently unsupported by empirical evidence. PD, plasmodesma.

plasmodesmata, ready for delivery or release as part of a strategy to enhance stress tolerance.

Conclusion

HMA domains can be found in multiple protein families in plants, including HPPs, HIPPs, P_{1B}-type ATPases, and (occasionally, as HMA-like structures) immune receptors. While the functions of the latter two families are established, many questions regarding HPP and HIPP function remain unanswered. Molecular and genetic evidence highlighted in this review suggests that HPP/HIPPs might play important roles in plant immunity, acting as both susceptibility factors and positive regulators of resistance, depending on the protein and pathosystem in question. HPPs and HIPPs have also been implicated in heavy metal homeostasis and tolerance to other abiotic stresses. Connecting these three themes could be an underlying ability for HMA proteins to directly or indirectly regulate ROS accumulation and/or modulate stress-responsive gene expression via transcription factors. A greater understanding of plant HMA biochemistry and cell biology is needed to explain their proposed roles in heavy metal tolerance. At present, it is challenging to mechanistically link observations of ion binding by HMA domains *in vitro* to *in planta* metal tolerance phenotypes.

Further study is also required to test the biological significance of HPP/HIPP plasmodesmal localization, although some speculative hypotheses are outlined in Fig. 3. Are these proteins lying in wait for effectors, viral movement proteins, or heavy metals, ready to shuttle to cell nuclei in times of stress to modify gene expression? Are they endogenous regulators of plasmodesmal

aperture, targeted by pathogens to adjust cell-to-cell connectivity during infection? Do they regulate plasmodesmata-localized ROS responses via unknown interactors? In-depth studies of pathogen-targeted HPP/HIPP cell biology, including surveys of their binding partners and localization during stresses, may deepen our understanding in the future.

Acknowledgements

We would like to thank Mark Banfield for his advice when brainstorming the contents and structure of this review, and Estee Tee and Barkha Ravi for their feedback on the manuscript.

Author contributions

ET and CF: conceptualization and funding acquisition; ET: investigation and writing (first draft); CF: supervision and writing (review and editing).

Conflict of interest

The authors declare that there are no conflicts of interest.

Funding

Work in the Faulkner lab is supported by Biotechnology and Biological Science Research Council grants BB/X010996/1, BB/X007685/1, BB/X016056/1, and BB/Y008782/1. ET is funded by a John Innes Foundation Rotation PhD.

Data availability

No new data was generated for this review.

References

- Abdel-Ghany SE, Burkhead JL, Gogolin KA, Andrés-Colás N, Bodecker JR, Puig S, Peñarubia L, Pilon M. 2005. AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. *FEBS Letters* **579**, 2307-2312.
- Aung K, Kim P, Li Z, Joe A, Kvitko B, Alfano JR, He SY. 2020. Pathogenic bacteria target plant plasmodesmata to colonize and invade surrounding tissues. *The Plant Cell* **32**, 595-611.
- Badarau A, Firbank SJ, McCarthy AA, Banfield MJ, Dennison C. 2010. Visualizing the metal-binding versatility of copper trafficking sites. *Biochemistry* **49**, 7798-7810.
- Barr ZK, Werner T, Tilsner J. 2023. Heavy metal-associated isoprenylated plant proteins (HIPPs) at plasmodesmata: exploring the link between localization and function. *Plants (Basel, Switzerland)* **12**, 315.
- Barth O, Vogt S, Uhlemann R, Zschiesche W, Humbeck K. 2009. Stress induced and nuclear localized HIP26 from *Arabidopsis thaliana* interacts via its heavy metal associated domain with the drought stress related zinc finger transcription factor ATHB29. *Plant Molecular Biology* **69**, 213-226.
- Bayer EM, Bottrill AR, Walshaw J, Vigouroux M, Naldrett MJ, Thomas CL, Maule AJ. 2006. *Arabidopsis* cell wall proteome defined using multidimensional protein identification technology. *Proteomics* **6**, 301-311.
- Belviso BD, Galliani A, Lasorsa A, Mirabelli V, Caliendo R, Arnesano F, Natile G. 2016. Oxaliplatin binding to human copper chaperone Atox1 and protein dimerization. *Inorganic Chemistry* **55**, 6563-6573.
- Benítez-Alfonso Y, Faulkner C, Pendle A, Miyashima S, Helariutta Y, Maule A. 2013. Symplastic intercellular connectivity regulates lateral root patterning. *Developmental Cell* **26**, 136-147.
- Bentham AR, Petit-Houénou Y, Win J, Chuma I, Terauchi R, Banfield MJ, Kamoun S, Langner T. 2021. A single amino acid polymorphism in a conserved effector of the multihost blast fungus pathogen expands host-target binding spectrum. *PLoS Pathogens* **17**, e1009957.
- Bermingham-McDonogh O, Gralla EB, Valentine JS. 1988. The copper, zinc-superoxide dismutase gene of *Saccharomyces cerevisiae*: cloning, sequencing, and biological activity. *Proceedings of the National Academy of Sciences, USA* **85**, 4789-4793.
- Blekemolen MC, Cao L, Tintor N, de Groot T, Papp D, Faulkner C, Takken FLW. 2022. The primary function of Six5 of *Fusarium oxysporum* is to facilitate Avr2 activity by together manipulating the size exclusion limit of plasmodesmata. *Frontiers in Plant Science* **13**, 910594.
- Boyle P, Le Su E, Rochon A, Shearer HL, Murmu J, Chu JY, Fobert PR, Després C. 2009. The BTB/POZ domain of the *Arabidopsis* disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *The Plant Cell* **21**, 3700-3713.
- Brose J, La Fontaine S, Wedd AG, Xiao Z. 2014. Redox sulfur chemistry of the copper chaperone Atox1 is regulated by the enzyme glutaredoxin 1, the reduction potential of the glutathione couple GSSG/2GSH and the availability of Cu(I). *Metallomics* **6**, 793-808.
- Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B, Kleinhofs A. 2002. The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. *Proceedings of the National Academy of Sciences, USA* **99**, 9328-9333.
- Bull PC, Cox DW. 1994. Wilson disease and Menkes disease: new handles on heavy-metal transport. *Trends in Genetics* **10**, 246-252.
- Cao L, Blekemolen MC, Tintor N, Cornelissen BJC, Takken FLW. 2018. The *Fusarium oxysporum* Avr2-Six5 effector pair alters plasmodesmal exclusion selectivity to facilitate cell-to-cell movement of Avr2. *Molecular Plant* **11**, 691-705.
- Castro B, Citterico M, Kimura S, Stevens DM, Wrzaczek M, Coaker G. 2021. Stress-induced reactive oxygen species compartmentalization, perception and signalling. *Nature Plants* **7**, 403-412.
- Cesari S, Thilliez G, Ribot C, et al. 2013. The rice resistance protein pair RGA4/RGA5 recognizes the *Magnaporthe oryzae* effectors AVR-Pia and AVR1-CO39 by direct binding. *The Plant Cell* **25**, 1463-1481.
- Cesari S, Bernoux M, Moncuquet P, Kroj T, Dodds PN. 2014. A novel conserved mechanism for plant NLR protein pairs: the 'integrated decoy' hypothesis. *Frontiers in Plant Science* **5**, 606.
- Chai LX, Dong K, Liu SY, Zhang Z, Zhang XP, Tong X, Zhu FF, Zou JZ, Wang XB. 2020. A putative nuclear copper chaperone promotes plant immunity in *Arabidopsis*. *Journal of Experimental Botany* **71**, 6684-6696.
- Cheval C, Faulkner C. 2018. Plasmodesmal regulation during plant-pathogen interactions. *New Phytologist* **217**, 62-67.
- Cheval C, Samwald S, Johnston MG, de Keijzer J, Breakspear A, Liu X, Bellandi A, Kadota Y, Zipfel C, Faulkner C. 2020. Chitin perception in plasmodesmata characterizes submembrane immune-signaling specificity in plants. *Proceedings of the National Academy of Sciences, USA* **117**, 9621-9629.
- Chu CC, Lee WC, Guo WY, Pan SM, Chen LJ, Li HM, Jinn TL. 2005. A copper chaperone for superoxide dismutase that confers three types of copper/zinc superoxide dismutase activity in *Arabidopsis*. *Plant Physiology* **139**, 425-436.
- Cowan GH, Roberts AG, Jones S, Kumar P, Kalyandurg PB, Gil JF, Savenkov EI, Hemsley PA, Torrance L. 2018. Potato mop-top virus co-opts the stress sensor HIP26 for long-distance movement. *Plant Physiology* **176**, 2052-2070.
- Crowell DN, Biermann BJ, Randall SK. 1996. Identification of cDNAs encoding isoprenylated proteins. *Molecular Biotechnology* **5**, 253-258.
- Crowell DN. 2000. Functional implications of protein isoprenylation in plants. *Progress in Lipid Research* **39**, 393-408.
- Culotta VC, Klomp LWJ, Strain J, Casareno RLB, Krems B, Gitlin JD. 1997. The copper chaperone for superoxide dismutase. *Journal of Biological Chemistry* **272**, 23469-23472.
- De A, Hoang CV, Escudero V, Armas AM, Echavarri-Erasun C, González-Guerrero M, Jordá L. 2025. Combating plant diseases through transition metal allocation. *New Phytologist* **245**, 1833-1842.
- de Abreu-Neto JB, Turchetto-Zolet AC, Oliveira LFVD, Zanettini MHB, Margis-Pinheiro M. 2013. Heavy metal-associated isoprenylated plant protein (HIP): characterization of a family of proteins exclusive to plants. *The FEBS Journal* **280**, 1604-1616.
- De la Concepcion JC, Franceschetti M, MacLean D, Terauchi R, Kamoun S, Banfield MJ. 2019. Protein engineering expands the effector recognition profile of a rice NLR immune receptor. *eLife* **8**, e47713.
- De la Concepcion JC, Franceschetti M, Maqbool A, Saitoh H, Terauchi R, Kamoun S, Banfield MJ. 2018. Polymorphic residues in rice NLRs expand binding and response to effectors of the blast pathogen. *Nature Plants* **4**, 576-585.
- De la Concepcion JC, Maidment JHR, Longya A, Xiao G, Franceschetti M, Banfield MJ. 2021. The allelic rice immune receptor Pikh confers extended resistance to strains of the blast fungus through a single polymorphism in the effector binding interface. *PLoS Pathogens* **17**, e1009368.
- Dutta TK, Vashisth N, Ray S, Phani V, Chinnusamy V, Sirohi A. 2023. Functional analysis of a susceptibility gene (*HIPP27*) in the *Arabidopsis thaliana-Meloidogyne incognita* pathosystem by using a genome editing strategy. *BMC Plant Biology* **23**, 390.
- Dykema PE, Sipes PR, Marie A, Biermann BJ, Crowell DN, Randall SK. 1999. A new class of proteins capable of binding transition metals. *Plant Molecular Biology* **41**, 139-150.
- Eriksson PO, Sahiman L. 1993. ¹H NMR studies of the mercuric ion binding protein MerP: sequential assignment, secondary structure and global fold of oxidized MerP. *Journal of Biomolecular NMR* **3**, 613-626.
- Escudero V, Ferreira Sánchez D, Abreu I, Sopena-Torres S, Makarovskiy-Saavedra N, Bernal M, Krämer U, Grolimund D, González-Guerrero M, Jordá L. 2022. *Arabidopsis thaliana* Zn²⁺-efflux ATPases HMA2 and HMA4 are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina* BMM. *Journal of Experimental Botany* **73**, 339-350.

- Faulkner CR, Blackman LM, Cordwell SJ, Overall RL. 2005. Proteomic identification of putative plasmodesmal proteins from *Chara corallina*. *Proteomics* **5**, 2866-2875.
- Fernandez-Calvino L, Faulkner C, Walshaw J, Saalbach G, Bayer E, Benítez-Alfonso Y, Maule A. 2011. Arabidopsis plasmodesmal proteome. *PLoS One* **6**, e18880.
- Fones H, Davis CAR, Rico A, Fang F, Smith JAC, Preston GM. 2010. Metal hyperaccumulation armors plants against disease. *PLoS Pathogens* **6**, e1001093.
- Fukuoka S, Saka N, Koga H, et al. 2009. Loss of function of a proline-containing protein confers durable disease resistance in rice. *Science* **325**, 998-1001.
- Gao W, Xiao S, Li HY, Tsao SW, Chye ML. 2009. Arabidopsis thaliana acyl-CoA-binding protein ACP2 interacts with heavy-metal-binding farnesylated protein AtFP6. *New Phytologist* **181**, 89-102.
- García-Ruiz H, Szurek B, Van den Ackerveken G. 2021. Stop helping pathogens: engineering plant susceptibility genes for durable resistance. *Current Opinion in Biotechnology* **70**, 187-195.
- Gitschier J, Moffat B, Reilly D, Wood WI, Fairbrother WJ. 1998. Solution structure of the fourth metal-binding domain from the Menkes copper-transporting ATPase. *Nature Structural Biology* **5**, 47-54.
- Gombos S, Miras M, Howe V, et al. 2023. A high-confidence *Physcomitrium patens* plasmodesmata proteome by iterative scoring and validation reveals diversification of cell wall proteins during evolution. *New Phytologist* **238**, 637-653.
- Gómez De La Cruz D, Zdrzałek R, Banfield MJ, Talbot NJ, Moscou MJ. 2024. Molecular mimicry of a pathogen virulence target by a plant immune receptor. *bioRxiv*. doi: [10.1101/2024.07.26.605320](https://doi.org/10.1101/2024.07.26.605320). [Preprint].
- González-Fuente M, Carrère S, Monachello D, et al. 2020. Effectork, a comprehensive resource to mine for *Ralstonia*, *Xanthomonas*, and other published effector interactors in the Arabidopsis proteome. *Molecular Plant Pathology* **21**, 1257-1270.
- Grund E, Tremousaygue D, Deslandes L. 2019. Plant NLRs with integrated domains: unity makes strength. *Plant Physiology* **179**, 1227-1235.
- Guo L, Cesari S, de Guillen K, et al. 2018. Specific recognition of two MAX effectors by integrated HMA domains in plant immune receptors involves distinct binding surfaces. *Proceedings of the National Academy of Sciences, USA* **115**, 11637-11642.
- Guo T, Weber H, Niemann MCE, Theisl L, Leonte G, Novák O, Werner T. 2021. Arabidopsis HIPP proteins regulate endoplasmic reticulum-associated degradation of CKX proteins and cytokinin responses. *Molecular Plant* **14**, 1918-1934.
- Han Z, Xiong D, Schneider R, Tian C. 2023. The function of plant PR1 and other members of the CAP protein superfamily in plant-pathogen interactions. *Molecular Plant Pathology* **24**, 651-668.
- Hanikenne M, Baurain D. 2014. Origin and evolution of metal P-type ATPases in plantae (Archaeplastida). *Frontiers in Plant Science* **4**, 544.
- Hatori Y, Clasen S, Hasan NM, Barry AN, Lutsenko S. 2012. Functional partnership of the copper export machinery and glutathione balance in human cells. *Journal of Biological Chemistry* **287**, 26678-26687.
- Hatori Y, Lutsenko S. 2016. The role of copper chaperone Atx1 in coupling redox homeostasis to intracellular copper distribution. *Antioxidants (Basel, Switzerland)* **5**, 25.
- Hörger AC, Fones HN, Preston GM. 2013. The current status of the elemental defense hypothesis in relation to pathogens. *Frontiers in Plant Science* **4**, 395.
- Horner W, Brunkard JO. 2021. Cytokinin stimulate plasmodesmal transport in leaves. *Frontiers in Plant Science* **12**, 674128.
- Hu Y, Zeng L, Li Z, Li F, Huang Y, Li R, Huang G. 2023. Construction of yeast two-hybrid library and screening the host targets of the effector SDE34 from *Candidatus Liberibacter asiaticus*. *Journal of Agricultural Biotechnology* **31**, 2589-2598.
- Huang D, Sun Y, Ma Z, et al. 2019. Salicylic acid-mediated plasmodesmal closure via Remorin-dependent lipid organization. *Proceedings of the National Academy of Sciences, USA* **116**, 21274-21284.
- Huang G, Hu Y, Li F, Zuo X, Wang X, Li F, Li R. 2024. Genome-wide characterization of heavy metal-associated isoprenylated plant protein gene family from *Citrus sinensis* in response to huanglongbing. *Frontiers in Plant Science* **15**, 1369883.
- Huang Q, Lin B, Cao Y, Zhang Y, Song H, Huang C, Sun T, Long C, Liao J, Zhuo K. 2023. CRISPR/Cas9-mediated mutagenesis of the susceptibility gene OsHPP04 in rice confers enhanced resistance to rice root-knot nematode. *Frontiers in Plant Science* **14**, 1134653.
- Hung IH, Casareno RLB, Labesse G, Mathews FS, Gitlin JD. 1998. HAH1 is a copper-binding protein with distinct amino acid residues mediating copper homeostasis and antioxidant defense. *Journal of Biological Chemistry* **273**, 1749-1754.
- Imran QM, Falak N, Hussain A, Mun BG, Sharma A, Lee SU, Kim KM, Liao BW. 2016. Nitric oxide responsive heavy metal-associated gene *ATHMAD1* contributes to development and disease resistance in Arabidopsis thaliana. *Frontiers in Plant Science* **7**, 1712.
- Johnston MG, Breakspear A, Samwald S, Zhang D, Papp D, Faulkner C, de Keijzer J. 2023. Comparative phyloproteomics identifies conserved plasmodesmal proteins. *Journal of Experimental Botany* **74**, 1821-1835.
- Jozefczak M, Remans T, Vangronsveld J, Cuypers A. 2012. Glutathione is a key player in metal-induced oxidative stress defenses. *International Journal of Molecular Sciences* **13**, 3145-3175.
- Khan IU, Rono JK, Zhang BQ, Liu XS, Wang MQ, Wang LL, Wu XC, Chen X, Cao HW, Yang ZM. 2019. Identification of novel rice (*Oryza sativa*) HPP and HIPP genes tolerant to heavy metal toxicity. *Ecotoxicology and Environmental Safety* **175**, 8-18.
- Klomp LW, Lin SJ, Yuan DS, Klausner RD, Culotta VC, Gitlin JD. 1997. Identification and functional expression of HAH1, a novel human gene involved in copper homeostasis. *Journal of Biological Chemistry* **272**, 9221-9226.
- Laskowski RA, Jablonska J, Pravda L, Vařeková RS, Thornton JM. 2018. PDBsum: structural summaries of PDB entries. *Protein Science* **27**, 129-134.
- Lee JY. 2014. New and old roles of plasmodesmata in immunity and parallels to tunneling nanotubes. *Plant Science* **221-222**, 13-20.
- Lee M, Cooray NDG, Maher MJ. 2017. The crystal structures of a copper-bound metallochaperone from *Saccharomyces cerevisiae*. *Journal of Inorganic Biochemistry* **177**, 368-374.
- Levy A, Erlanger M, Rosenthal M, Epel BL. 2007. A plasmodesmata-associated β -1,3-glucanase in Arabidopsis. *The Plant Journal* **49**, 669-682.
- Li J, Zhang M, Sun J, Mao X, Wang J, Liu H, Zheng H, Li X, Zhao H, Zou D. 2020. Heavy metal stress-associated proteins in rice and Arabidopsis: genome-wide identification, phylogenetics, duplication, and expression profiles analysis. *Frontiers in Genetics* **11**, 477.
- Li Z, Variz H, Chen Y, Liu SL, Aung K. 2021. Plasmodesmata-dependent intercellular movement of bacterial effectors. *Frontiers in Plant Science* **12**, 640277.
- Lin SJ, Culotta VC. 1995. The *ATX1* gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proceedings of the National Academy of Sciences, USA* **92**, 3784-3788.
- Liu X, Bellandi A, Johnston MG, Faulkner C. 2022. The *Hyaloperonospora arabidopsidis* effector HaRxL77 is hypermobile between cells and manipulates host defence. *bioRxiv* doi: [10.1101/2022.01.24.477405](https://doi.org/10.1101/2022.01.24.477405). [Preprint].
- Locci F, Parker JE. 2024. Plant NLR immunity activation and execution: a biochemical perspective. *Open Biology* **14**, 230387.
- Lucas WJ. 2006. Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. *Virology* **344**, 169-184.
- Lutsenko S, Petrukhin K, Cooper MJ, Gilliam CT, Kaplan JH. 1997. N-terminal domains of human copper-transporting adenosine triphosphatases (the Wilson's and Menkes disease proteins) bind copper selectively *in vivo* and *in vitro* with stoichiometry of one copper per metal-binding repeat. *Journal of Biological Chemistry* **272**, 18939-18944.

16 | Turley and Faulkner

- Maidment JHR, Franceschetti M, Maqbool A, Saitoh H, Jantasuriyarat C, Kamoun S, Terauchi R, Banfield MJ. 2021. Multiple variants of the fungal effector AVR-Pik bind the HMA domain of the rice protein OshIPP19, providing a foundation to engineer plant defense. *Journal of Biological Chemistry* **296**, 100371.
- Mangini V, Belviso BD, Nardella MI, Natile G, Arnesano F, Caliendo R. 2022. Crystal structure of the human copper chaperone ATOX1 bound to zinc ion. *Biomolecules* **12**, 1494.
- Maqbool A, Saitoh H, Franceschetti M, Stevenson CE, Uemura A, Kanzaki H, Kamoun S, Terauchi R, Banfield MJ. 2015. Structural basis of pathogen recognition by an integrated HMA domain in a plant NLR immune receptor. *eLife* **4**, e08709.
- Mira H, Martínez-García F, Peñarrubia L. 2001. Evidence for the plant-specific intercellular transport of the Arabidopsis copper chaperone CCH. *The Plant Journal* **25**, 521-528.
- Miras R, Morin I, Jacquin O, Cuillel M, Guillaud F, Mintz E. 2008. Interplay between glutathione, Atx1 and copper. 1. Copper(I) glutathione induced dimerization of Atx1. *Journal of Biological Inorganic Chemistry* **13**, 195-205.
- Mittler R, Zandalinas SI, Fichman Y, Van Breusegem F. 2022. Reactive oxygen species signalling in plant stress responses. *Nature Reviews. Molecular Cell Biology* **23**, 663-679.
- Moreau M, Westlake T, Zampogna G, Popescu G, Tian M, Noutsos C, Popescu S. 2013. The Arabidopsis oligopeptidases TOP1 and TOP2 are salicylic acid targets that modulate SA-mediated signaling and the immune response. *The Plant Journal* **76**, 603-614.
- Mukhi N, Brown H, Gorenkin D, Ding P, Bentham AR, Stevenson CEM, Jones JDG, Banfield MJ. 2021. Perception of structurally distinct effectors by the integrated WRKY domain of a plant immune receptor. *Proceedings of the National Academy of Sciences, USA* **118**, e2113996118.
- Mukhtar MS, Carvunis AR, Dreze M, et al. 2011. Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* **333**, 596-601.
- Muller PAJ, Klomp LWJ. 2009. ATOX1: a novel copper-responsive transcription factor in mammals? *International Journal of Biochemistry & Cell Biology* **41**, 1233-1236.
- Nakao M, Nakamura R, Kita K, Inukai R, Ishikawa A. 2011. Non-host resistance to penetration and hyphal growth of *Magnaporthe oryzae* in *Arabidopsis*. *Scientific Reports* **1**, 171.
- Nirmala J, Brueggeman R, Maier C, Clay C, Rostoks N, Kannangara CG, von Wettstein D, Steffenson BJ, Kleinhofs A. 2006. Subcellular localization and functions of the barley stem rust resistance receptor-like serine/threonine-specific protein kinase Rpg1. *Proceedings of the National Academy of Sciences, USA* **103**, 7518-7523.
- Niu X, Yamamoto N, Yang G, Lin H, Jiang L, Liu Y, Zheng A. 2023. A small secreted protein, RsMf8HN, in *Rhizoctonia solani* triggers plant immune response, which interacts with rice OshIPP28. *Microbiological Research* **266**, 127219.
- Ohtsu M, Jennings J, Johnston M, Breakspear A, Liu X, Stark K, Morris RJ, de Keijzer J, Faulkner C. 2024. Assaying effector cell-to-cell mobility in plant tissues identifies hypermobility and indirect manipulation of plasmodesmata. *Molecular Plant-Microbe Interactions* **37**, 84-92.
- Oikawa K, Fujisaki K, Shimizu M, et al. 2024. The blast pathogen effector AVR-Pik binds and stabilizes rice heavy metal-associated (HMA) proteins to co-opt their function in immunity. *PLoS Pathogens* **20**, e1012647.
- O'Lexy R, Kasai K, Clark N, Fujiwara T, Sozzani R, Gallagher KL. 2018. Exposure to heavy metal stress triggers changes in plasmodesmal permeability via deposition and breakdown of callose. *Journal of Experimental Botany* **69**, 3715-3728.
- Ormenese S, Bernier G, Périlleux C. 2006. Cytokinin application to the shoot apical meristem of *Sinapis alba* enhances secondary plasmodesmata formation. *Planta* **224**, 1481-1484.
- Ortiz D, de Guillen K, Cesari S, Chalvon V, Gracy J, Padilla A, Kroj T. 2017. Recognition of the *Magnaporthe oryzae* effector AVR-Pia by the decoy domain of the rice NLR immune receptor RGA5. *The Plant Cell* **29**, 156-168.
- Park SH, Li F, Renaud J, Shen W, Li Y, Guo L, Cui H, Sumarah M, Wang A. 2017. NbEXPA1, an α -expansin, is plasmodesmata-specific and a novel host factor for potyviral infection. *The Plant Journal* **92**, 846-861.
- Permyakov EA. 2021. Metal binding proteins. *Encyclopedia* **1**, 261-292.
- Portnoy ME, Rosenzweig AC, Rae T, Huffman DL, O'Halloran TV, Culotta VC. 1999. Structure-function analyses of the ATX1 metallochaperone. *Journal of Biological Chemistry* **274**, 15041-15045.
- Pufahl RA, Singer CP, Peariso KL, Lin SJ, Schmidt PJ, Fahrni CJ, Culotta VC, Penner-Hahn JE, O'Halloran TV. 1997. Metal ion chaperone function of the soluble Cu(I) receptor Atx1. *Science* **278**, 853-856.
- Puig S, Mira H, Dorcey E, et al. 2007. Higher plants possess two different types of ATX1-like copper chaperones. *Biochemical and Biophysical Research Communications* **354**, 385-390.
- Radakovic ZS, Anjam MS, Escobar E, Chopra D, Cabrera J, Silva AC, Escobar C, Sobczak M, Grundler FMW, Siddique S. 2018. Arabidopsis HIPP27 is a host susceptibility gene for the beet cyst nematode *Heterodera schachtli*. *Molecular Plant Pathology* **19**, 1917-1928.
- Reagan BC, Burch-Smith TM. 2020. Viruses reveal the secrets of plasmodesmal cell biology. *Molecular Plant-Microbe Interactions* **33**, 26-39.
- Reveguk T, Fatiukha A, Potapenko E, et al. 2025. Tandem kinase proteins across the plant kingdom. *Nature Genetics* **57**, 254-262.
- Rosenzweig AC, Huffman DL, Hou MY, Wernimont AK, Pufahl RA, O'Halloran TV. 1999. Crystal structure of the Atx1 metallochaperone protein at 1.02 Å resolution. *Structure* **7**, 605-617.
- Sager RE, Lee JY. 2018. Plasmodesmata at a glance. *Journal of Cell Science* **131**, jcs209346.
- Sarris PF, Cevik V, Dagdas G, Jones JDG, Krasileva KV. 2016. Comparative analysis of plant immune receptor architectures uncovers host proteins likely targeted by pathogens. *BMC Biology* **14**, 8.
- Shahid M, Pourrut B, Dumat C, Nadeem M, Aslam M, Pinelli E. 2014. Heavy-metal-induced reactive oxygen species: phytotoxicity and physico-chemical changes in plants. In: Whitacre DM, ed. *Reviews of environmental contamination and toxicology*, Vol. **232**. Cham: Springer International Publishing, 1-44.
- Song H, Lin B, Huang Q, Sun L, Chen J, Hu L, Zhuo K, Liao J. 2021. The *Meloidogyne graminicola* effector MgMO289 targets a novel copper metallochaperone to suppress immunity in rice. *Journal of Experimental Botany* **72**, 5638-5655.
- Steele RA, Opella SJ. 1997. Structures of the reduced and mercury-bound forms of MerP, the periplasmic protein from the bacterial mercury detoxification system. *Biochemistry* **36**, 6885-6895.
- Sugihara Y, Abe Y, Takagi H, et al. 2023. Disentangling the complex gene interaction networks between rice and the blast fungus identifies a new pathogen effector. *PLoS Biology* **21**, e3001945.
- Sun W, Wei J, Wu G, et al. 2022. CqZF-HD14 enhances drought tolerance in quinoa seedlings through interaction with CqHIPP34 and CqNAC79. *Plant Science* **323**, 111406.
- Suzuki N, Yamaguchi Y, Koizumi N, Sano H. 2002. Functional characterization of a heavy metal binding protein Cdl19 from *Arabidopsis*. *The Plant Journal* **32**, 165-173.
- Taliansky M, Torrance L, Kalinina NO. 2008. Role of plant virus movement proteins. In: Foster GD, Johansen IE, Hong Y, Nagy PD, eds. *Plant virology protocols: from viral sequence to protein function*. Totowa, NJ: Humana Press, 33-54.
- Tanchou V, Gas F, Urvoas A, Cougoulouéne F, Ruat S, Averseng O, Quéméneur E. 2004. Copper-mediated homo-dimerisation for the HAH1 metallochaperone. *Biochemical and Biophysical Research Communications* **325**, 388-394.
- Tanzi RE, Petrukhin K, Chernov I, et al. 1993. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nature Genetics* **5**, 344-350.
- Tee EE, Johnston MG, Papp D, Faulkner C. 2023. A PDLNHL3 complex integrates plasmodesmal immune signaling cascades. *Proceedings of the National Academy of Sciences, USA* **120**, e2216397120.

- Tehseen M, Cairns N, Sherson S, Cobbett CS. 2010. Metallochaperone-like genes in *Arabidopsis thaliana*. *Metallomics* **2**, 556-564.
- Thomas CL, Bayer EM, Ritzenthaler C, Fernandez-Calvino L, Maule AJ. 2008. Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. *PLoS Biology* **6**, e7.
- Tomczynska I, Stumpe M, Doan TG, Mauch F. 2020. A phytophthora effector protein promotes symplastic cell-to-cell trafficking by physical interaction with plasmodesmata-localised callose synthases. *New Phytologist* **227**, 1467-1478.
- Varden FA, Saitoh H, Yoshino K, Franceschetti M, Kamoun S, Terauchi R, Banfield MJ. 2019. Cross-reactivity of a rice NLR immune receptor to distinct effectors from the rice blast pathogen *Magnaporthe oryzae* provides partial disease resistance. *Journal of Biological Chemistry* **294**, 13006-13016.
- Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J. 1993. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nature Genetics* **3**, 7-13.
- Wang Q, Liu M, Zang Y, Xiao W. 2020. The C-terminal extension of *Arabidopsis* Uev1A/B with putative prenylation site plays critical roles in protein interaction, subcellular distribution and membrane association. *Plant Science* **291**, 110324.
- Wang X, Sager R, Cui W, Zhang C, Lu H, Lee JY. 2013. Salicylic acid regulates plasmodesmata closure during innate immune responses in *Arabidopsis*. *The Plant Cell* **25**, 2315-2329.
- Wang Z, Zhang H, Li Y, et al. 2023. Isoprenylation modification is required for HIP1-mediated powdery mildew resistance in wheat. *Plant, Cell & Environment* **46**, 288-305.
- Weßling R, Eppe P, Altmann S, et al. 2014. Convergent targeting of a common host protein-network by pathogen effectors from three kingdoms of life. *Cell Host & Microbe* **16**, 364-375.
- Wei Y, Peng X, Wang X, Wang C. 2023. The heavy metal-associated isoprenylated plant protein (HIP) gene family plays a crucial role in cadmium resistance and accumulation in the tea plant (*Camellia sinensis* L.). *Ecotoxicology and Environmental Safety* **260**, 115077.
- Were VM, Yan X, Foster AJ, et al. 2025. The *Magnaporthe oryzae* effector Pwl2 alters HIP43 localization to suppress host immunity. *The Plant Cell*. doi:10.1093/plcell/koaf116
- Wernimont AK, Huffman DL, Lamb AL, O'Halloran TV, Rosenzweig AC. 2000. Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins. *Nature Structural Biology* **7**, 766-771.
- Williams LE, Mills RF. 2005. P_{1B}-ATPases—an ancient family of transition metal pumps with diverse functions in plants. *Trends in Plant Science* **10**, 491-502.
- Wu Y, Zhang D, Chu JY, Boyle P, Wang Y, Brindle ID, De Luca V, Després C. 2012. The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. *Cell Reports* **1**, 639-647.
- Xu B, Cheval C, Laohavisit A, Hocking B, Chiasson D, Olsson TSG, Shirasu K, Faulkner C, Gilliam M. 2017. A calmodulin-like protein regulates plasmodesmal closure during bacterial immune responses. *New Phytologist* **215**, 77-84.
- Xu J, Cui J, He Q, Liu Y, Lu X, Qi J, Xiong J, Yu W, Li C. 2024. Genome-wide identification of *HIPP* and mechanism of *SlHIPP4/7/9/21/26/32* mediated phytohormones response to Cd, osmotic, and salt stresses in tomato. *Plant Physiology and Biochemistry* **217**, 109220.
- Ye X, Liu C, Yan H, Wan Y, Wu Q, Wu X, Zhao G, Zou L, Xiang D. 2022. Genome-wide identification and transcriptome analysis of the heavy metal-associated (HMA) gene family in tartary buckwheat and their regulatory roles under cadmium stress. *Gene* **847**, 146884.
- Yuan DS, Stearman R, Dancis A, Dunn T, Beeler T, Klausner RD. 1995. The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proceedings of the National Academy of Sciences, USA* **92**, 2632-2636.
- Zdrzalek R, Xi Y, Langner T, et al. 2024. Bioengineering a plant NLR immune receptor with a robust binding interface toward a conserved fungal pathogen effector. *Proceedings of the National Academy of Sciences, USA* **121**, e2402872121.
- Zhang BQ, Liu XS, Feng SJ, Zhao YN, Wang LL, Rono JK, Li H, Yang ZM. 2020. Developing a cadmium resistant rice genotype with OsHIP29 locus for limiting cadmium accumulation in the paddy crop. *Chemosphere* **247**, 125958.
- Zhang H, Zhang X, Liu J, et al. 2020. Characterization of the heavy-metal-associated isoprenylated plant protein (*HIPP*) gene family from *Triticeae* species. *International Journal of Molecular Sciences* **21**, 6191.
- Zhang P, Wang R, Ju Q, Li W, Tran LSP, Xu J. 2019. The R2R3-MYB transcription factor MYB49 regulates cadmium accumulation. *Plant Physiology* **180**, 529-542.
- Zhang X, Feng H, Feng C, et al. 2015. Isolation and characterisation of cDNA encoding a wheat heavy metal-associated isoprenylated protein involved in stress responses. *Plant Biology* **17**, 1176-1186.
- Zhou L, Ye L, Pang B, Hou Y, Yu J, Du X, Gu L, Wang H, Zhu B. 2023. Overexpression of ApHIP26 from the hyperaccumulator *Arabidopsis paniculata* confers enhanced cadmium tolerance and accumulation to *Arabidopsis thaliana*. *International Journal of Molecular Sciences* **24**, 15052.
- Zhu Y, Li Y, Fei F, et al. 2015. E3 ubiquitin ligase gene *CMPG1-V* from *Haynaldia villosa* L. contributes to powdery mildew resistance in common wheat (*Triticum aestivum* L.). *The Plant Journal* **84**, 154-168.
- Zschiesche W, Barth O, Daniel K, Böhme S, Rausche J, Humbeck K. 2015. The zinc-binding nuclear protein HIP3 acts as an upstream regulator of the salicylate-dependent plant immunity pathway and of flowering time in *Arabidopsis thaliana*. *New Phytologist* **207**, 1084-1096.

References

- Abdel-Ghany SE, Burkhead JL, Gogolin KA, et al.** (2005). AtCCS is a functional homolog of the yeast copper chaperone Ccs1/Lys7. *FEBS Letters* **579**, 2307–2312.
- Abramson J, Adler J, Dunger J, et al.** (2024). Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**, 493–500.
- Adachi H, Derevnina L, and Kamoun S** (2019). NLR singletons, pairs, and networks: evolution, assembly, and regulation of the intracellular immunoreceptor circuitry of plants. *Current Opinion in Plant Biology* **50**, 121–131.
- Agarwal V and McShan AC** (2024). The power and pitfalls of AlphaFold2 for structure prediction beyond rigid globular proteins. *Nature Chemical Biology* **20**, 950–959.
- Agirre J, Atanasova M, Bagdonas H, et al.** (2023). The CCP4 suite: integrative software for macromolecular crystallography. *Acta Crystallographica Section D* **79**, 449–461.
- Ahuja M and Punekar NS** (2008). Phosphinothricin resistance in *Aspergillus niger* and its utility as a selectable transformation marker. *Fungal Genetics and Biology* **45**, 1103–1110.
- Alazem M, Nuzzi SN, and Burch-Smith TM** (2025). Regulation of cell-to-cell trafficking by viral movement proteins. *Journal of Experimental Botany*, eaf184.
- Albrecht M and Kortekamp A** (2009). The in vitro effect of the herbicide Basta® (glufosinate ammonium) on potential fungal grapevine pathogens. *European Journal of Horticultural Science* **74**, 112–117.
- Argueso CT, Ferreira FJ, Eppe P, et al.** (2012). Two-Component Elements Mediate Interactions between Cytokinin and Salicylic Acid in Plant Immunity. *PLOS Genetics* **8**, e1002448.
- Arroyo-Velez N, González-Fuente M, Peeters N, et al.** (2020). From effectors to effectomes: Are functional studies of individual effectors enough to decipher plant pathogen infectious strategies?. *PLOS Pathogens* **16**, e1009059.
- Aung K, Kim P, Li Z, et al.** (2019). Pathogenic Bacteria Target Plant Plasmodesmata to Colonize and Invade Surrounding Tissues. *The Plant Cell* **32**, 595–611.
- Austin RS, Provart NJ, and Cutler SR** (2007). C-terminal motif prediction in eukaryotic proteomes using comparative genomics and statistical over-representation across protein families. *BMC Genomics* **8**.
- Avalos J, Geever RE, and Case ME** (1989). Bialaphos resistance as a dominant selectable marker in *Neurospora crassa*. *Current Genetics* **16**, 369–372.
- Avilov SV and Aleksandrova N** (2019). Fluorescence protein complementation in microscopy: applications beyond detecting bi-molecular interactions. *Methods and Applications in Fluorescence* **7**, 012001.

- Badarau A, Firbank SJ, McCarthy AA, et al.** (2010). Visualizing the Metal-Binding Versatility of Copper Trafficking Sites. *Biochemistry* **49**, 7798–7810.
- Baggs E, Dagdas G, and Krasileva KV** (2017). NLR diversity, helpers and integrated domains: making sense of the NLR IDentity. *Current Opinion in Plant Biology* **38**, 59–67.
- Barr Z, Werner T, and Tilsner J** (2023). Heavy metal-associated isoprenylated plant proteins (HIPPs) at plasmodesmata: exploring the link between localization and function. *Plants* **12**, 3015.
- Barr ZK and Tilsner J** (2025). Should I stay or should I go – does protein localisation to plasmodesmata depend on targeting signals?. *Journal of Experimental Botany* **eraf278**.
- Barth O, Vogt S, Uhlemann R, et al.** (2009). Stress induced and nuclear localized HIP26 from *Arabidopsis thaliana* interacts via its heavy metal associated domain with the drought stress related zinc finger transcription factor ATHB29. *Plant Molecular Biology* **69**, 213–226.
- Barth O, Zschiesche W, Siersleben S, and Humbeck K** (2004). Isolation of a novel barley cDNA encoding a nuclear protein involved in stress response and leaf senescence. *Physiologia plantarum* **121**, 282–293.
- Baumgartner JT, Mohammad TSH, Czub MP, et al.** (2021). Gcn5-Related N-Acetyltransferases (GNATs) With a Catalytic Serine Residue Can Play Ping-Pong Too. *Frontiers in Molecular Biosciences* **8**, 154.
- Bayer EM, Bottrill AR, Walshaw J, et al.** (2006). *Arabidopsis* cell wall proteome defined using multidimensional protein identification technology. *Proteomics* **6**, 301–311.
- Bellandi A, Papp D, Breakspear A, et al.** (2022). Diffusion and bulk flow of amino acids mediate calcium waves in plants. *Science Advances* **8**, eabo6693.
- Benitez-Alfonso Y, Faulkner C, Pendle A, et al.** (2013). Symplastic Intercellular Connectivity Regulates Lateral Root Patterning. *Developmental Cell* **26**, 136–147.
- Bentham AR, De la Concepcion JC, Benjumea JV, et al.** (2023). Allelic compatibility in plant immune receptors facilitates engineering of new effector recognition specificities. *The Plant Cell* **35**, 3809–3827.
- Bentham AR, De la Concepcion JC, Mukhi N, et al.** (2020). A molecular roadmap to the plant immune system. *Journal of Biological Chemistry* **295**, 14916–14935.
- Bentham AR, Petit-Houdenot Y, Win J, et al.** (2021a). A single amino acid polymorphism in a conserved effector of the multihost blast fungus pathogen expands host-target binding spectrum. *PLOS Pathogens* **17**, e1009957.
- Bentham AR, Youles M, Mendel MN, et al.** (2021b). pOPIN-GG: A resource for modular assembly in protein expression vectors. *bioRxiv* 2021.08.10.455798.
- Bentham AR, Zdrzalek R, De la Concepcion JC, and Banfield MJ** (2018). Uncoiling CNLs: Structure/Function Approaches to Understanding CC Domain Function in Plant NLRs. *Plant and Cell Physiology* **59**, 2398–2408.
- Bermingham-McDonogh O, Gralla EB, and Valentine JS** (1988). The copper, zinc-superoxide dismutase gene of *Saccharomyces cerevisiae*: cloning, sequencing, and biological activity. *Proceedings of the National Academy of Sciences* **85**, 4789–4793.
- Bernard D, Sylvie C, Nathalie S, and Marie-Thérèse ET** (1999). Use of Green Fluorescent Protein To Detect Expression of an Endopolygalacturonase Gene of *Colletotrichum lindemuthianum* during Bean Infection. *Applied and Environmental Microbiology* **65**, 1769–1771.

- Bernasconi Z, Stirnemann U, Xu Y, et al. (2025). An HMA-like integrated domain in the wheat tandem kinase WTK4 recognises an RNase-like pathogen effector. *bioRxiv* 2025.08.26.672365.
- Bhadauria V, Han T, Li G, et al. (2024). A gln-tRNA-based CRISPR/Cas9 knockout system enables the functional characterization of genes in the genetically recalcitrant brassica anthracnose fungus *Colletotrichum higginsianum*. *International Journal of Biological Macromolecules* **254**, 127953.
- Bi G, Su M, Li N, et al. (2021). The ZAR1 resistosome is a calcium-permeable channel triggering plant immune signaling. *Cell* **184**, 3528–3541.e12.
- Bialas A, Langner T, Harant A, et al. (2021). Two NLR immune receptors acquired high-affinity binding to a fungal effector through convergent evolution of their integrated domain. *eLife* **10**, e66961.
- Bigeard J, Colcombet J, and Hirt H (2015). Signaling Mechanisms in Pattern-Triggered Immunity (PTI). *Molecular Plant* **8**, 521–539.
- Bjornson M, Pimprikar P, Nürnberger T, and Zipfel C (2021). The transcriptional landscape of *Arabidopsis thaliana* pattern-triggered immunity. *Nature Plants* **7**, 579–586.
- Blekemolen MC, Cao L, Tintor N, et al. (2022). The primary function of Six5 of *Fusarium oxysporum* is to facilitate Avr2 activity by together manipulating the size exclusion limit of plasmodesmata. *Frontiers in Plant Science* **13**.
- Blum M, Andreeva A, Florentino L, et al. (2025). InterPro: the protein sequence classification resource in 2025. *Nucleic Acids Research* **53**, D444–D456.
- Borner GHH, Lilley KS, Stevens TJ, and Dupree P (2003). Identification of Glycosylphosphatidylinositol-Anchored Proteins in Arabidopsis. A Proteomic and Genomic Analysis. *Plant Physiology* **132**, 568–577.
- Bourett TM, Sweigard JA, Czymmek KJ, et al. (2002). Reef coral fluorescent proteins for visualizing fungal pathogens. *Fungal Genetics and Biology* **37**, 211–220.
- Boutemy LS, King SRF, Win J, et al. (2011). Structures of *Phytophthora* RXLR Effector Proteins. *Journal of Biological Chemistry* **286**, 35834–35842.
- Boyle P, Su EL, Rochon A, et al. (2009). The BTB/POZ Domain of the *Arabidopsis* Disease Resistance Protein NPR1 Interacts with the Repression Domain of TGA2 to Negate Its Function. *The Plant Cell* **21**, 3700–3713.
- Brabham HJ, Gómez De La Cruz D, Were V, et al. (2023). Barley MLA3 recognizes the host-specificity effector Pw12 from *Magnaporthe oryzae*. *The Plant Cell* **36**, 447–470.
- Branon TC, Bosch JA, Sanchez AD, et al. (2018). Efficient proximity labeling in living cells and organisms with TurboID. *Nature Biotechnology* **36**, 880–887.
- Brueggeman R, Rostoks N, Kudrna D, et al. (2002). The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. *Proceedings of the National Academy of Sciences* **99**, 9328–9333.
- Bull PC and Cox DW (1994). Wilson disease and Menkes disease: new handles on heavy-metal transport. *Trends in Genetics* **10**, 246–252.
- Cadiou L, Brunisholz F, Cesari S, and Kroj T (2023). Molecular engineering of plant immune receptors for tailored crop disease resistance. *Current Opinion in Plant Biology* **74**, 102381.

- Cao L, Blekemolen MC, Tintor N, et al.** (2018). The *Fusarium oxysporum* Avr2-Six5 Effector Pair Alters Plasmodesmatal Exclusion Selectivity to Facilitate Cell-to-Cell Movement of Avr2. *Molecular Plant* **11**, 691–705.
- Carella P, Evangelisti E, and Schornack S** (2018). Sticking to it: phytopathogen effector molecules may converge on evolutionarily conserved host targets in green plants. *Current Opinion in Plant Biology* **44**, 175–180.
- Césari S, Kanzaki H, Fujiwara T, et al.** (2014). The NB-LRR proteins RGA4 and RGA5 interact functionally and physically to confer disease resistance. *The EMBO Journal* **33**, 1941–1959–1959.
- Castel B, Tomlinson L, Locci F, et al.** (2019). Optimization of T-DNA architecture for Cas9-mediated mutagenesis in Arabidopsis. *PLOS One* **14**, e0204778.
- Castro B, Citterico M, Kimura S, et al.** (2021). Stress-induced reactive oxygen species compartmentalization, perception and signalling. *Nature Plants* **7**, 403–412.
- Cesari S, Bernoux M, Moncuquet P, et al.** (2014). A novel conserved mechanism for plant NLR protein pairs: the ‘integrated decoy’ hypothesis. *Frontiers in Plant Science* **5**.
- Cesari S, Thilliez G, Ribot C, et al.** (2013). The Rice Resistance Protein Pair RGA4/RGA5 Recognizes the *Magnaporthe oryzae* Effectors AVR-Pia and AVR1-CO39 by Direct Binding. *The Plant Cell* **25**, 1463–1481.
- Cesari S, Xi Y, Declerck N, et al.** (2022). New recognition specificity in a plant immune receptor by molecular engineering of its integrated domain. *Nature Communications* **13**, 1524.
- Ceulemans E, Ibrahim HMM, Coninck BD, and Goossens A** (2021). Pathogen Effectors: Exploiting the Promiscuity of Plant Signaling Hubs. *Trends in Plant Science* **26**, 780–795.
- Chai LX, Dong K, Liu SY, et al.** (2020). A putative nuclear copper chaperone promotes plant immunity in Arabidopsis. *Journal of Experimental Botany* **71**, 6684–6696.
- Chambaud C, Cookson SJ, Ollat N, et al.** (2022). A correlative light electron microscopy approach reveals plasmodesmata ultrastructure at the graft interface. *Plant Physiology* **188**, 44–55.
- Chapman S, Faulkner C, Kaiserli E, et al.** (2008). The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection. *Proceedings of the National Academy of Sciences* **105**, 20038–20043.
- Chen C, Zhang Y, Cai J, et al.** (2023). Multi-copper oxidases SKU5 and SKS1 coordinate cell wall formation using apoplastic redox-based reactions in roots. *Plant Physiology* **192**, 2243–2260.
- Chen C, Zhang Y, Zhu L, and Yuan M** (2010). The actin cytoskeleton is involved in the regulation of the plasmodesmal size exclusion limit. *Plant Signaling & Behavior* **5**, 1663–1665.
- Chen R, Chen J, Powell OR, et al.** (2025). A wheat tandem kinase activates an NLR to trigger immunity. *Science* **387**, 1402–1408.
- Chen X, Duan Y, Qiao F, et al.** (2022). A secreted fungal effector suppresses rice immunity through host histone hypoacetylation. *New Phytologist* **235**, 1977–1994.
- Cheng Y and Patel DJ** (2004). An efficient system for small protein expression and refolding. *Biochemical and biophysical research communications* **317**, 401.

- Chethana KWT, Jayawardena RS, Chen YJ, et al.** (2021). Diversity and Function of Appressoria. *Pathogens* **10**, 746.
- Cheval C and Faulkner C** (2018). Plasmodesmal regulation during plant–pathogen interactions. *New Phytologist* **217**, 62–67.
- Cheval C, Samwald S, Johnston MG, et al.** (2020). Chitin perception in plasmodesmata characterizes submembrane immune-signaling specificity in plants. *Proceedings of the National Academy of Sciences* **117**, 9621–9629.
- Chia KS, Kourelis J, Teulet A, et al.** (2024). The N-terminal domains of NLR immune receptors exhibit structural and functional similarities across divergent plant lineages. *The Plant Cell* **36**, 2491–2511.
- Chinchilla D, Bauer Z, Regenass M, et al.** (2006). The Arabidopsis Receptor Kinase FLS2 Binds flg22 and Determines the Specificity of Flagellin Perception. *The Plant Cell* **18**, 465.
- Choi J, Huh SU, Kojima M, et al.** (2010). The Cytokinin-Activated Transcription Factor ARR2 Promotes Plant Immunity via TGA3/NPR1-Dependent Salicylic Acid Signaling in *Arabidopsis*. *Developmental Cell* **19**, 284–295.
- Chu CC, Lee WC, Guo WY, et al.** (2005). A Copper Chaperone for Superoxide Dismutase That Confers Three Types of Copper/Zinc Superoxide Dismutase Activity in *Arabidopsis*. *Plant Physiology* **139**, 425–436.
- Complainville A, Brocard L, Roberts I, et al.** (2003). Nodule Initiation Involves the Creation of a New Symplasmic Field in Specific Root Cells of *Medicago* Species. *The Plant Cell* **15**, 2778–2791.
- Conrath U** (2024). Unlocking Precision in Callose Staining: Unveiling the Role of Sirofluor. *Molecular Plant-Microbe Interactions* **37**, 595.
- Contreras MP, Pai H, Tumtas Y, et al.** (2023). Sensor NLR immune proteins activate oligomerization of their NRC helpers in response to plant pathogens. *The EMBO Journal* **42**, e111519.
- Corwin JA and Kliebenstein DJ** (2017). Quantitative Resistance: More Than Just Perception of a Pathogen. *The Plant Cell* **29**, 655–665.
- Cowan GH, Roberts AG, Jones S, et al.** (2018). Potato Mop-Top Virus Co-Opts the Stress Sensor HIPP26 for Long-Distance Movement.. *Plant Physiology* **176**, 2052–2070.
- Cowtan K** (2006). The *Buccaneer* software for automated model building. 1. Tracing protein chains. *Acta Crystallographica Section D* **62**, 1002–1011.
- Crowell DN** (2000). Functional implications of protein isoprenylation in plants. *Progress in Lipid Research* **39**, 393–408.
- Crowell DN, Biermann BJ, and Randall SK** (1996). Identification of cDNAs encoding isoprenylated proteins. *Molecular Biotechnology* **5**, 253–258.
- Cruz-Mireles N, Eseola AB, Osés-Ruiz M, et al.** (2021). From appressorium to transpressorium—Defining the morphogenetic basis of host cell invasion by the rice blast fungus. *PLOS Pathogens* **17**, e1009779.
- Cui W and Lee JY** (2016). Arabidopsis callose synthases CalS1/8 regulate plasmodesmal permeability during stress. *Nature Plants* **2**, 16034.

- Culotta VC, Klomp LWJ, Strain J, et al.** (1997). The Copper Chaperone for Superoxide Dismutase. *Journal of Biological Chemistry* **272**, 23469–23472.
- Dallery JF, Lapalu N, Zampounis A, et al.** (2017). Gapless genome assembly of *Colletotrichum higginsianum* reveals chromosome structure and association of transposable elements with secondary metabolite gene clusters. *BMC Genomics* **18**, 667.
- Damm U, O’Connell R, Groenewald J, and Crous P** (2014). The *Colletotrichum destructivum* species complex-hemibiotrophic pathogens of forage and field crops. *Studies in Mycology* **79**, 49–84.
- Danila FR, Quick WP, White RG, et al.** (2016). The Metabolite Pathway between Bundle Sheath and Mesophyll: Quantification of Plasmodesmata in Leaves of C3 and C4 Monocots. *The Plant Cell* **28**, 1461–1471.
- Dauparas J, Anishchenko I, Bennett N, et al.** (2022). Robust deep learning-based protein sequence design using ProteinMPNN. *Science* **378**, 49–56.
- Davis IW, Leaver-Fay A, Chen VB, et al.** (2007). MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Research* **35**, 375–383.
- De A, Hoang CV, Escudero V, et al.** (2024). Combating plant diseases through transition metal allocation. *New Phytologist* **245**, 1833–1842.
- de Abreu-Neto JB, Turchetto-Zolet AC, Oliveira LFVD, et al.** (2013). Heavy metal-associated isoprenylated plant protein (HIPP): characterization of a family of proteins exclusive to plants. *The FEBS Journal* **280**, 1604–1616.
- de Guillen K, Mammri L, Gracy J, et al.** (2024). *Zymoseptoria tritici* effectors structurally related to killer proteins UmV-KP4 and UmV-KP6 are toxic to fungi, and define extended protein families in fungi. *bioRxiv* 2024.10.14.618152.
- de Guillen K, Ortiz-Vallejo D, Gracy J, et al.** (2015). Structure Analysis Uncovers a Highly Diverse but Structurally Conserved Effector Family in Phytopathogenic Fungi. *PLOS Pathogens* **11**, e1005228.
- De la Concepcion JC, Franceschetti M, MacLean D, et al.** (2019). Protein engineering expands the effector recognition profile of a rice NLR immune receptor. *eLife* **8**, e47713.
- De la Concepcion JC, Franceschetti M, Maqbool A, et al.** (2018). Polymorphic residues in rice NLRs expand binding and response to effectors of the blast pathogen. *Nature Plants* **4**, 576–585.
- De la Concepcion JC, Fujisaki K, Benthall AR, et al.** (2022). A blast fungus zinc-finger fold effector binds to a hydrophobic pocket in host Exo70 proteins to modulate immune recognition in rice. *Proceedings of the National Academy of Sciences* **119**, e2210559119.
- De la Concepcion JC, Maidment JHR, Longya A, et al.** (2021). The allelic rice immune receptor Pikh confers extended resistance to strains of the blast fungus through a single polymorphism in the effector binding interface. *PLOS Pathogens* **17**, e1009368.
- DeFalco TA and Zipfel C** (2021). Molecular mechanisms of early plant pattern-triggered immune signaling. *Molecular Cell* **81**, 3449–3467.
- Deller MC, Kong L, and Rupp B** (2016). Protein stability: a crystallographer’s perspective. *Acta Crystallographica Section F* **72**, 72–95.

- den Hollander PW, Kieper SN, Borst JW, and van Lent JW (2016). The role of plasmodesma-located proteins in tubule-guided virus transport is limited to the plasmodesmata. *Archives of Virology* **161**, 2431–2440.
- Deng W, Marshall NC, Rowland JL, et al. (2017). Assembly, structure, function and regulation of type III secretion systems. *Nature Reviews Microbiology* 2017 15:6 **15**, 323–337.
- Derevnina L, Contreras MP, Adachi H, et al. (2021). Plant pathogens convergently evolved to counteract redundant nodes of an NLR immune receptor network. *PLOS Biology* **19**, e3001136.
- D'Halluin K, Block MD, Denecke J, et al. (1992). The *bar* gene has selectable and screenable marker in plant engineering. *Methods in Enzymology* **216**, 415–426.
- Di X, Cao L, Hughes RK, et al. (2017). Structure–function analysis of the *Fusarium oxysporum* Avr2 effector allows uncoupling of its immune-suppressing activity from recognition. *New Phytologist* **216**, 897–914.
- Dickmanns M, Pöge M, Xu P, et al. (2025). In situ architecture of plasmodesmata suggests mechanisms controlling intercellular exchange. *bioRxiv* 2025.07.24.666190.
- Dievart A, Gottin C, Peacuterin C, et al. (2020). Origin and Diversity of Plant Receptor-Like Kinases. *Annual Review of Plant Biology* **71**, 131–156.
- Djamei A, Schipper K, Rabe F, et al. (2011). Metabolic priming by a secreted fungal effector. *Nature* **478**, 395–398.
- Dong A, Xu X, Edwards AM, et al. (2007). In situ proteolysis for protein crystallization and structure determination. *Nature Methods* **4**, 1019–1021.
- Du X, Alam M, Witek K, et al. (2025). Interfamily co-transfer of sensor and helper NLRs extends immune receptor functionality between angiosperms. *Cell* **188**, 4505–4516.
- Dutta TK, Vashisth N, Ray S, et al. (2023). Functional analysis of a susceptibility gene (*HIPP27*) in the *Arabidopsis thaliana*-*Meloidogyne incognita* pathosystem by using a genome editing strategy. *BMC Plant Biology* **23**, 390.
- Dyda F, Klein DC, and Hickman AB (2002). GCN5-Related N-Acetyltransferases: A Structural Overview. *Annual Review of Biophysics and Biomolecular Structure* **29**, 81–103.
- Dykema PE, Sipes PR, Marie A, et al. (1999). A new class of proteins capable of binding transition metals. *Plant Molecular Biology* **41**, 139–150.
- Edgar RC (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.
- Ehlers K and van Bel AJE (2010). Dynamics of plasmodesmal connectivity in successive interfaces of the cambial zone. *Planta* **231**, 371–385.
- Elfmann C and Stülke J (2023). PAE Viewer: A webserver for the interactive visualization of the predicted aligned error for multimer structure predictions and crosslinks. *bioRxiv* 2023.03.06.531253.
- Emsley P, Lohkamp B, Scott WG, and Cowtan K (2010). Features and development of *Coot*. *Acta Crystallographica Section D: Biological Crystallography* **66**, 486.
- Engler C, Kandzia R, and Marillonnet S (2008). A one pot, one step, precision cloning method with high throughput capability. *PLOS One* **3**, e3647.

- Epel BL** (2009). Plant viruses spread by diffusion on ER-associated movement-protein-rafts through plasmodesmata gated by viral induced host β -1,3-glucanases. *Seminars in Cell & Developmental Biology* **20**, 1074–1081.
- Eriksson PO and Sahlman L** (1993). ^1H NMR studies of the mercuric ion binding protein MerP: Sequential assignment, secondary structure and global fold of oxidized MerP. *Journal of Biomolecular NMR* **3**, 613–626.
- Escudero V, Sánchez DE, Abreu I, et al.** (2022). *Arabidopsis thaliana* Zn^{2+} -efflux ATPases HMA2 and HMA4 are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina* BMM. *Journal of Experimental Botany* **73**, 339–350.
- Evans PR and Murshudov GN** (2013). How good are my data and what is the resolution?. *Acta Crystallographica Section D* **69**, 1204–1214.
- Evans R, O'Neill M, Pritzel A, et al.** (2022). Protein complex prediction with AlphaFold-Multimer. *bioRxiv* 2021.10.04.463034.
- Förderer A, Li E, Lawson AW, et al.** (2022). A wheat resistosome defines common principles of immune receptor channels. *Nature* 2022 610:7932 **610**, 532–539.
- Faulkner C, Akman OE, Bell K, et al.** (2008). Peeking into pit fields: A multiple twinning model of secondary plasmodesmata formation in tobacco. *Plant Cell* **20**, 1504–1518.
- Faulkner C, Petutschnig E, Benitez-Alfonso Y, et al.** (2013). LYM2-dependent chitin perception limits molecular flux via plasmodesmata. *Proceedings of the National Academy of Sciences* **110**, 9166–9170.
- Faulkner CR, Blackman LM, Cordwell SJ, and Overall RL** (2005). Proteomic identification of putative plasmodesmatal proteins from *Chara corallina*. *Proteomics* **5**, 2866–2875.
- Feehan JM, Wang J, Sun X, et al.** (2023). Oligomerization of a plant helper NLR requires cell-surface and intracellular immune receptor activation. *Proceedings of the National Academy of Sciences* **120**, e2210406120.
- Fernandez-Calvino L, Faulkner C, Walshaw J, et al.** (2011). Arabidopsis Plasmodesmal Proteome. *PLOS One* **6**, e18880.
- Fletcher JC, Brand U, Running MP, et al.** (1999). Signaling of Cell Fate Decisions by *CLAVATA3* in Arabidopsis Shoot Meristems. *Science* **283**, 1911–1914.
- Flower TG and Hurley JH** (2021). Crystallographic molecular replacement using an in silico-generated search model of SARS-CoV-2 ORF8. *Protein Science: A Publication of the Protein Society* **30**, 728.
- Fones H, Davis CAR, Rico A, et al.** (2010). Metal Hyperaccumulation Armors Plants against Disease. *PLOS Pathogens* **6**, e1001093.
- Foster AJ, Ryder LS, Kershaw MJ, and Talbot NJ** (2017). The role of glycerol in the pathogenic lifestyle of the rice blast fungus *Magnaporthe oryzae*. *Environmental Microbiology* **19**, 1008–1016.
- French E, Kim BS, and Iyer-Pascuzzi AS** (2016). Mechanisms of quantitative disease resistance in plants. *Seminars in Cell & Developmental Biology* **56**, 201–208.
- Fritsch VN and Hensel M** (2025). Experimental Approaches to Visualize Effector Protein Translocation During Host-Pathogen Interactions. *BioEssays* **47**, e202400188.

- Fukada F, Kodama S, Nishiuchi T, et al.** (2019). Plant pathogenic fungi *Colletotrichum* and *Magnaporthe* share a common G1 phase monitoring strategy for proper appressorium development. *New Phytologist* **222**, 1909–1923.
- Fukuoka S, Saka N, Koga H, et al.** (2009). Loss of Function of a Proline-Containing Protein Confers Durable Disease Resistance in Rice. *Science* **325**, 998–1001.
- Gómez De La Cruz D, Zdrzałek R, Banfield MJ, et al.** (2024). Molecular mimicry of a pathogen virulence target by a plant immune receptor. *bioRxiv* 2024.07.26.605320.
- Ganusova EE, Reagan BC, Fernandez JC, et al.** (2020). Chloroplast-to-nucleus retrograde signalling controls intercellular trafficking via plasmodesmata formation. *Philosophical Transactions of the Royal Society B: Biological Sciences* **375**, 20190408.
- Gao C, Zhang Z, Zhu Y, et al.** (2025). Genome-Wide Analysis of *HIPP* Gene Family in Maize Reveals Its Role in the Cadmium Stress Response. *Genes* **16**, 770.
- Gao W, Xiao S, Li HY, et al.** (2009). *Arabidopsis thaliana* acyl-CoA-binding protein ACBP2 interacts with heavy-metal-binding farnesylated protein AtFP6. *New Phytologist* **181**, 89–102.
- Garcia-Ruiz H, Szurek B, and den Ackerveken GV** (2021). Stop helping pathogens: engineering plant susceptibility genes for durable resistance. *Current Opinion in Biotechnology* **70**, 187–195.
- Gasperini D, Chauvin A, Acosta IF, et al.** (2015). Axial and Radial Oxylipin Transport. *Plant Physiology* **169**, 2244–2254.
- Germain H, Joly DL, Mireault C, et al.** (2018). Infection assays in *Arabidopsis* reveal candidate effectors from the poplar rust fungus that promote susceptibility to bacteria and oomycete pathogens. *Molecular Plant Pathology* **19**, 191–200.
- Giraldo MC, Dagdas YF, Gupta YK, et al.** (2013). Two distinct secretion systems facilitate tissue invasion by the rice blast fungus *Magnaporthe oryzae*. *Nature Communications* **4**, 1996.
- Giraldo MC and Valent B** (2013). Filamentous plant pathogen effectors in action. *Nature Reviews Microbiology* **11**, 800–814.
- Gitschier J, Moffat B, Reilly D, et al.** (1998). Solution structure of the fourth metal-binding domain from the Menkes copper-transporting ATPase. *Nature Structural Biology* **5**, 47–54.
- Gombos S, Miras M, Howe V, et al.** (2023). A high-confidence *Physcomitrium patens* plasmodesmata proteome by iterative scoring and validation reveals diversification of cell wall proteins during evolution. *New Phytologist* **238**, 637–653.
- González-Fuente M, Carrère S, Monachello D, et al.** (2020). EffectorK, a comprehensive resource to mine for *Ralstonia*, *Xanthomonas*, and other published effector interactors in the *Arabidopsis* proteome. *Molecular Plant Pathology* **21**, 1257–1270.
- Grisson MS, Brocard L, Fouillen L, et al.** (2015). Specific Membrane Lipid Composition Is Important for Plasmodesmata Function in *Arabidopsis*. *The Plant Cell* **27**, 1228–1250.
- Grund E, Tremousaygue D, and Deslandes L** (2019). Plant NLRs with Integrated Domains: Unity Makes Strength. *Plant Physiology* **179**, 1227–1235.
- Gucwa M, Lenkiewicz J, Zheng H, et al.** (2023). CMM—An enhanced platform for interactive validation of metal binding sites. *Protein Science* **32**, e4525.
- Guenoun-Gelbart D, Elbaum M, Sagi G, et al.** (2008). Tobacco mosaic virus (TMV) Replicase and Movement Protein Function Synergistically in Facilitating TMV Spread by

- Lateral Diffusion in the Plasmodesmal Desmotubule of *Nicotiana benthamiana*. *Molecular Plant-Microbe Interactions* **21**, 335–345.
- Guo L, Cesari S, de Guillen K, et al.** (2018). Specific recognition of two MAX effectors by integrated HMA domains in plant immune receptors involves distinct binding surfaces. *Proceedings of the National Academy of Sciences* **115**, 11637–11642.
- Guo T, Weber H, Niemann MCE, et al.** (2021). *Arabidopsis* HIPPs regulate endoplasmic reticulum-associated degradation of CKX proteins and cytokinin responses. *Molecular Plant* **14**, 1918–1934.
- Gupta R, Pizarro L, Leibman-Markus M, et al.** (2020). Cytokinin response induces immunity and fungal pathogen resistance, and modulates trafficking of the PRR LeEIX2 in tomato. *Molecular Plant Pathology* **21**, 1287–1306.
- Guyon K, Balagué C, Roby D, and Raffaele S** (2014). Secretome analysis reveals effector candidates associated with broad host range necrotrophy in the fungal plant pathogen *Sclerotinia sclerotiorum*. *BMC Genomics* **15**, 336.
- Haas BJ, Kamoun S, Zody MC, et al.** (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature* **461**, 393–398.
- Ham BK, Wang X, Toscano-Morales R, et al.** (2023). Plasmodesmal endoplasmic reticulum proteins regulate intercellular trafficking of Cucumber mosaic virus in *Arabidopsis*. *Journal of Experimental Botany*, erad190.
- Han Z, Xiong D, Schneider R, and Tian C** (2023). The function of plant PR1 and other members of the CAP protein superfamily in plant–pathogen interactions. *Molecular Plant Pathology* **24**, 651–668.
- Handing KB, Niedzialkowska E, Shabalin IG, et al.** (2018). Characterizing metal-binding sites in proteins with X-ray crystallography. *Nature Protocols* **13**, 1062–1090.
- Hanikenne M and Baurain D** (2014). Origin and evolution of metal P-type ATPases in Plantae (Archaeplastida). *Frontiers in Plant Science* **5**, 544.
- Harrison MD, Jones CE, Solioz M, and Dameron CT** (2000). Intracellular copper routing: the role of copper chaperones. *Trends in Biochemical Sciences* **25**, 29–32.
- He G, Qin L, Tian W, et al.** (2020). Heavy Metal Transporters-Associated Proteins in *Solanum tuberosum*: Genome-Wide Identification, Comprehensive Gene Feature, Evolution and Expression Analysis. *Genes* **11**, 1269.
- He H, Chen Z, Fan R, et al.** (2024). A kinase fusion protein from *Aegilops longissima* confers resistance to wheat powdery mildew. *Nature Communications* **15**, 6512.
- Hepler PK** (1982). Endoplasmic reticulum in the formation of the cell plate and plasmodesmata. *Protoplasma* **111**, 121–133.
- Herbert C, Jacquet C, Borel C, et al.** (2002). A cis-Acting Sequence Homologous to the Yeast Filamentation and Invasion Response Element Regulates Expression of a Pectinase Gene from the Bean Pathogen *Colletotrichum lindemuthianum*. *Journal of Biological Chemistry* **277**, 29125–29131.
- Hink MA, Griep RA, Borst JW, et al.** (2000). Structural Dynamics of Green Fluorescent Protein Alone and Fused with a Single Chain Fv Protein. *Journal of Biological Chemistry* **275**, 17556–17560.

- Hirakawa Y, Shinohara H, Kondo Y, et al. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proceedings of the National Academy of Sciences* **105**, 15208–15213.
- Hiruma K, Onozawa-Komori M, Takahashi F, et al. (2010). Entry Mode-Dependent Function of an Indole Glucosinolate Pathway in Arabidopsis for Nonhost Resistance against Anthracnose Pathogens. *The Plant Cell* **22**, 2429–2443.
- Hiruma K and Saijo Y (2016). Plant Inoculation with the Fungal Leaf Pathogen *Colletotrichum higginsianum*. In P. Duque, ed., *Environmental Responses in Plants: Methods and Protocols*. Springer New York. ISBN 978-1-4939-3356-3. pages 313–318.
- Höerger AC, Fones HN, and Preston G (2013). The current status of the elemental defense hypothesis in relation to pathogens. *Frontiers in Plant Science* **4**.
- Holm L, Laiho A, Törönen P, and Salgado M (2023). DALI shines a light on remote homologs: One hundred discoveries. *Protein Science* **32**, e4519.
- Horner W and Brunkard JO (2021). Cytokinins Stimulate Plasmodesmatal Transport in Leaves. *Frontiers in Plant Science* **12**.
- Howard AR, Heppler ML, Ju HJ, et al. (2004). Potato virus X TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves. *Virology* **328**, 185–197.
- Hu Y, Zeng L, Li Z, et al. (2023). Construction of yeast two-hybrid library and screening the host targets of the effector SDE34 from *Candidatus Liberibacter asiaticus*. *J. Agric. Biotechnol* **31**, 2589–2598.
- Huang C, Sede AR, Elvira-González L, et al. (2023a). DsRNA-induced immunity targets plasmodesmata and is suppressed by viral movement proteins. *The Plant Cell* **35**, 3845–3869.
- Huang G, Hu Y, Li F, et al. (2024). Genome-wide characterization of heavy metal-associated isoprenylated plant protein gene family from *Citrus sinensis* in response to huanglongbing. *Frontiers in Plant Science* **15**.
- Huang J, Si W, Deng Q, et al. (2014). Rapid evolution of avirulence genes in rice blast fungus *Magnaporthe oryzae*. *BMC Genetics* **15**, 45.
- Huang Q, Lin B, Cao Y, et al. (2023b). CRISPR/Cas9-mediated mutagenesis of the susceptibility gene *OsHPP04* in rice confers enhanced resistance to rice root-knot nematode. *Frontiers in Plant Science* **14**.
- Huang S, Jia A, Song W, et al. (2022). Identification and receptor mechanism of TIR-catalyzed small molecules in plant immunity. *Science* **377**, eabq3297.
- Hung IH, Casareno RLB, Labesse G, et al. (1998). HAH1 Is a Copper-binding Protein with Distinct Amino Acid Residues Mediating Copper Homeostasis and Antioxidant Defense. *Journal of Biological Chemistry* **273**, 1749–1754.
- Huo C, He L, Yu T, et al. (2022). The Superoxide Dismutase Gene Family in *Nicotiana tabacum*: Genome-Wide Identification, Characterization, Expression Profiling and Functional Analysis in Response to Heavy Metal Stress. *Frontiers in Plant Science* **13**.
- Huser A, Takahara H, Schmalenbach W, and O'Connell R (2009). Discovery of Pathogenicity Genes in the Crucifer Anthracnose Fungus *Colletotrichum higginsianum*, Using Random Insertional Mutagenesis. *Molecular Plant-Microbe Interactions* **22**, 143–156.

- Ibrahim T, Yuen ELH, Wang HY, et al.** (2024). A helper NLR targets organellar membranes to trigger immunity. *bioRxiv* 2024.09.19.613839.
- Imran QM, Falak N, Hussain A, et al.** (2016). Nitric Oxide Responsive Heavy Metal-Associated Gene *AtHMAD1* Contributes to Development and Disease Resistance in *Arabidopsis thaliana*. *Frontiers in Plant Science* **7**.
- Irieda H, Maeda H, Akiyama K, et al.** (2014). *Colletotrichum orbiculare* Secretes Virulence Effectors to a Biotrophic Interface at the Primary Hyphal Neck via Exocytosis Coupled with SEC22-Mediated Traffic. *The Plant Cell* **26**, 2265–2281.
- Irieda H, Ogawa S, and Takano Y** (2016). Focal effector accumulation in a biotrophic interface at the primary invasion sites of *Colletotrichum orbiculare* in multiple susceptible plants. *Plant Signaling & Behavior* **11**, e1137407.
- Ishikawa K, Hashimoto M, Yusa A, et al.** (2017). Dual targeting of a virus movement protein to ER and plasma membrane subdomains is essential for plasmodesmata localization. *PLOS Pathogens* **13**, e1006463.
- Ishikawa K, Maejima K, Komatsu K, et al.** (2013). Fig mosaic emaravirus p4 protein is involved in cell-to-cell movement. *Journal of General Virology* **94**, 682–686.
- Jennings J** (2021). *Colletotrichum higginsianum* exploitation of host cell-to-cell connectivity. Ph.D. thesis. University of East Anglia.
- Jiang J, Kuo YW, Salem N, et al.** (2021). Carrot mottle virus ORF4 movement protein targets plasmodesmata by interacting with the host cell SUMOylation system. *New Phytologist* **231**, 382–398.
- Johnston MG, Bellandi A, and Faulkner C** (2022). faulknerfalcons/PD_detection: Toolkit for quantification of callose at plasmodesmata. *Zenodo* 10.5281/zenodo.6583765.
- Johnston MG, Breakspear A, Samwald S, et al.** (2023). Comparative phyloproteomics identifies conserved plasmodesmal proteins. *Journal of Experimental Botany* **74**, 1821–1835.
- Jones SL** (2023). Investigating phytopathogen putative effector proteins that target starch granule initiation. Ph.D. thesis. University of East Anglia.
- Jumper J, Evans R, Pritzel A, et al.** (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* **596**, 583–589.
- Kale SD, Gu B, Capelluto DGS, et al.** (2010). External Lipid PI3P Mediates Entry of Eukaryotic Pathogen Effectors into Plant and Animal Host Cells. *Cell* **142**, 284–295.
- Kankanala P, Czymmek K, and Valent B** (2007). Roles for Rice Membrane Dynamics and Plasmodesmata during Biotrophic Invasion by the Blast Fungus. *The Plant Cell* **19**, 706–724.
- Kanzaki H, Yoshida K, Saitoh H, et al.** (2012). Arms race co-evolution of *Magnaporthe oryzae* AVR-Pik and rice Pik genes driven by their physical interactions. *The Plant Journal* **72**, 894–907.
- Kasteel DTJ, Perbal MC, Boyer JC, et al.** (1996). The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells. *Journal of General Virology* **77**, 2857–2864.
- Khan IU, Rono JK, Zhang BQ, et al.** (2019). Identification of novel rice (*Oryza sativa*) HPP and HIPP genes tolerant to heavy metal toxicity. *Ecotoxicology and Environmental Safety* **175**, 8–18.
- Khang CH, Berruyer R, Giraldo MC, et al.** (2010). Translocation of *Magnaporthe oryzae* Effectors into Rice Cells and Their Subsequent Cell-to-Cell Movement. *The Plant Cell* **22**, 1388–1403.

- Khodaei S and Arzanlou M** (2019). Novel hosts in the genus *Colletotrichum* and first report of *C. higginsianum* from Iran. *Nova Hedwigia* **108**, 449–463.
- Kim DI, KC B, Zhu W, et al.** (2014). Probing nuclear pore complex architecture with proximity-dependent biotinylation. *Proceedings of the National Academy of Sciences* **111**, 2453–2461.
- Klaumann S, Nickolaus SD, Fürst SH, et al.** (2011). The tonoplast copper transporter COPT5 acts as an exporter and is required for interorgan allocation of copper in *Arabidopsis thaliana*. *New Phytologist* **192**, 393–404.
- Kleemann J, Rincon-Rivera LJ, Takahara H, et al.** (2012). Sequential Delivery of Host-Induced Virulence Effectors by Appressoria and Intracellular Hyphae of the Phytopathogen *Colletotrichum higginsianum*. *PLOS Pathogens* **8**, e1002643.
- Kliebenstein DJ, Monde RA, and Last RL** (1998). Superoxide Dismutase in Arabidopsis: An Eclectic Enzyme Family with Disparate Regulation and Protein Localization. *Plant Physiology* **118**, 637–650.
- Klomp LWJ, Lin SJ, S.Yuan D, et al.** (1997). Identification and Functional Expression of *HAH1*, a Novel Human Gene Involved in Copper Homeostasis. *Journal of Biological Chemistry* **272**, 9221–9226.
- Knox JP and Benitez-Alfonso Y** (2014). Roles and regulation of plant cell walls surrounding plasmodesmata. *Current Opinion in Plant Biology* **22**, 93–100.
- Kollmann R and Glockmann C** (1991). Studies on graft unions - III. On the mechanism of secondary formation of plasmodesmata at the graft interface. *Protoplasma* **165**, 71–85.
- Korn M** (2016). Identifizierung und Analyse der Protonenpumpe ChPma2 als Pathogenitätsfaktor der Interaktion zwischen *Colletotrichum higginsianum* und *Arabidopsis thaliana*. Ph.D. thesis. Friedrich-Alexander Universität Erlangen-Nürnberg.
- Korn M, Schmidpeter J, Dahl M, et al.** (2015). A Genetic Screen for Pathogenicity Genes in the Hemibiotrophic Fungus *Colletotrichum higginsianum* Identifies the Plasma Membrane Proton Pump Pma2 Required for Host Penetration. *PLOS One* **10**, e0125960.
- Kotani T and Takagi H** (2008). Identification of amino acid residues essential for the yeast N-acetyltransferase Mpr1 activity by site-directed mutagenesis. *FEMS Yeast Research* **8**, 607–614.
- Kourelis J, Marchal C, Posbeyikian A, et al.** (2023). NLR immune receptor-nanobody fusions confer plant disease resistance. *Science* **379**, 934–939.
- Kraner ME, Müller C, and Sonnewald U** (2017). Comparative proteomic profiling of the choline transporter-like1 (CHER1) mutant provides insights into plasmodesmata composition of fully developed *Arabidopsis thaliana* leaves. *The Plant Journal* **92**, 696–709.
- Krissinel E and Henrick K** (2007). Inference of Macromolecular Assemblies from Crystalline State. *Journal of Molecular Biology* **372**, 774–797.
- Krtenic B, Drazic A, Arnesen T, and Reuter N** (2020). Classification and phylogeny for the annotation of novel eukaryotic GNAT acetyltransferases. *PLOS Computational Biology* **16**, e1007988.
- Labun K, Montague TG, Krause M, et al.** (2019). CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research* **47**, 171–174.

- Laskowski RA, Jabłońska J, Pravda L, et al. (2018). PDBsum: Structural summaries of PDB entries. *Protein Science* **27**, 129–134.
- Latunde-Dada AO and Lucas JA (2007). Localized hemibiotrophy in *Colletotrichum*: cytological and molecular taxonomic similarities among *C. destructivum*, *C. linicola* and *C. truncatum*. *Plant Pathology* **56**, 437–447.
- Lazar N, Mesarich CH, Petit-Houdenot Y, et al. (2022). A new family of structurally conserved fungal effectors displays epistatic interactions with plant resistance proteins. *PLOS Pathogens* **18**, e1010664.
- Lazareva EA, Lezzhov AA, Chergintsev DA, et al. (2021). Reticulon-like properties of a plant virus-encoded movement protein. *New Phytologist* **229**, 1052–1066.
- Le Naour-Vernet M, Lahfa M, Maidment JHR, et al. (2025). Structure-guided insights into the biology of fungal effectors. *New Phytologist* **246**, 1460–1477.
- Lee JY, Wang X, Cui W, et al. (2011). A Plasmodesmata-Localized Protein Mediates Crosstalk between Cell-to-Cell Communication and Innate Immunity in *Arabidopsis*. *The Plant Cell* **23**, 3353–3373.
- Lee KB (2009). Structure and development of the endophyte in the parasitic angiosperm *Cuscuta japonica*. *Journal of Plant Biology* **52**, 355–363.
- Lee M, Cooray NDG, and Maher MJ (2017). The crystal structures of a copper-bound metallochaperone from *Saccharomyces cerevisiae*. *Journal of Inorganic Biochemistry* **177**, 368–374.
- Lee YH and Dean RA (1994). Hydrophobicity of contact surface induces appressorium formation in *Magnaporthe grise*. *FEMS Microbiology Letters* **115**, 71–75.
- Leijon F, Melzer M, Zhou Q, et al. (2018). Proteomic Analysis of Plasmodesmata From *Populus* Cell Suspension Cultures in Relation With Callose Biosynthesis. *Frontiers in Plant Science* **9**.
- Leonte G (2020). Characterization of cluster-III HIPP proteins targeted to plasmodesmata in *Arabidopsis thaliana*. Ph.D. thesis. Freie Universität Berlin.
- Levy A, Erlanger M, Rosenthal M, and Epel BL (2007). A plasmodesmata-associated β -1,3-glucanase in *Arabidopsis*. *The Plant Journal* **49**, 669–682.
- Li G, Dulal N, Gong Z, and Wilson RA (2023). Unconventional secretion of *Magnaporthe oryzae* effectors in rice cells is regulated by tRNA modification and codon usage control. *Nature Microbiology* **8**, 1706–1716.
- Li J, Zhang M, Sun J, et al. (2020). Heavy Metal Stress-Associated Proteins in Rice and *Arabidopsis*: Genome-Wide Identification, Phylogenetics, Duplication, and Expression Profiles Analysis. *Frontiers in Genetics* **11**.
- Li N, Erman M, Pangborn W, et al. (1999). Structure of *Ustilago maydis* Killer Toxin KP6 α -Subunit. *Journal of Biological Chemistry* **274**, 20425–20431.
- Li Z, Variz H, Chen Y, et al. (2021). Plasmodesmata-Dependent Intercellular Movement of Bacterial Effectors. *Frontiers in Plant Science* **12**.
- Li ZP, Moreau H, Petit JD, et al. (2024). Plant plasmodesmata bridges form through ER-dependent incomplete cytokinesis. *Science* **386**, 538–545.
- Lim GH, Shine MB, de Lorenzo L, et al. (2016). Plasmodesmata Localizing Proteins Regulate Transport and Signaling during Systemic Acquired Immunity in Plants. *Cell Host & Microbe* **19**, 541–549.

- Lin SJ and Culotta VC** (1995). The *ATX1* gene of *Saccharomyces cerevisiae* encodes a small metal homeostasis factor that protects cells against reactive oxygen toxicity. *Proceedings of the National Academy of Sciences* **92**, 3784–3788.
- Liu G, Kennedy R, Greenshields DL, et al.** (2007). Detached and Attached Arabidopsis Leaf Assays Reveal Distinctive Defense Responses Against Hemibiotrophic *Colletotrichum* spp. *Molecular Plant-Microbe Interactions* **20**, 1308–1319.
- Liu L, Zhao D, Zheng L, et al.** (2013). Identification of virulence genes in the crucifer anthracnose fungus *Colletotrichum higginsianum* by insertional mutagenesis. *Microbial Pathogenesis* **64**, 6–17.
- Liu M, Guo Z, Hu J, et al.** (2025). The multifunctional ascorbate peroxidase MoApx1 secreted by *Magnaporthe oryzae* mediates the suppression of rice immunity. *The Plant Cell* **37**, 146.
- Liu X, Bellandi A, Johnston MG, and Faulkner C** (2022). The *Hyaloperanospora arabidopsidis* effector HaRxL77 is hypermobile between cells and manipulates host defence. *bioRxiv* 2022.01.24.477405.
- Liu Y, Zhang X, Yuan G, et al.** (2021). A designer rice NLR immune receptor confers resistance to the rice blast fungus carrying noncorresponding avirulence effectors. *Proceedings of the National Academy of Sciences* **118**.
- Lobstein J, Emrich CA, Jeans C, et al.** (2012). SHuffle, a novel *Escherichia coli* protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. *Microbial Cell Factories* **11**, 753.
- Lu P, Zhang G, Li J, et al.** (2025). A wheat tandem kinase and NLR pair confers resistance to multiple fungal pathogens. *Science* **387**, 1418–1424.
- Lu X, Miao J, Shen D, and Dou D** (2022). Proteinaceous Effector Discovery and Characterization in Plant Pathogenic *Colletotrichum* Fungi. *Frontiers in Microbiology* **13**.
- Lu Y** (2010). Metal ions as matchmakers for proteins. *Proceedings of the National Academy of Sciences* **107**, 1811–1812.
- Lucas WJ** (2006). Plant viral movement proteins: Agents for cell-to-cell trafficking of viral genomes. *Virology* **344**, 169–184.
- Luna GR, Li J, Wang X, et al.** (2023). Targeting of plasmodesmal proteins requires unconventional signals. *The Plant Cell* **35**, 3035–3052.
- Luna GR, Peña EJ, Borniego MB, et al.** (2018). Citrus Psorosis Virus Movement Protein Contains an Aspartic Protease Required for Autocleavage and the Formation of Tubule-Like Structures at Plasmodesmata. *Journal of Virology* **92**, e00355–18.
- Lutsenko S, Petrukhin K, Cooper MJ, et al.** (1997). N-terminal Domains of Human Copper-transporting Adenosine Triphosphatases (the Wilson's and Menkes Disease Proteins) Bind Copper Selectively *in Vivo* and *in Vitro* with Stoichiometry of One Copper Per Metal-binding Repeat. *Journal of Biological Chemistry* **272**, 18939–18944.
- Ma S, Lapin D, Liu L, et al.** (2020). Direct pathogen-induced assembly of an NLR immune receptor complex to form a holoenzyme. *Science* **370**, eabe3069.
- Mackey D, Holt BF, Wiig A, and Dangl JL** (2002). RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. *Cell* **108**, 743–754.

- Madhuprakash J, Toghani A, Contreras MP, et al.** (2025). A disease resistance protein triggers oligomerization of its NLR helper into a hexameric resistosome to mediate innate immunity. *Science Advances* **10**, eadr2594.
- Maekawa T, Kracher B, Vernaldi S, et al.** (2012). Conservation of NLR-triggered immunity across plant lineages. *Proceedings of the National Academy of Sciences* **109**, 20119–20123.
- Maidment JHR, Franceschetti M, Maqbool A, et al.** (2021). Multiple variants of the fungal effector AVR-Pik bind the HMA domain of the rice protein OsHIP19, providing a foundation to engineer plant defense. *Journal of Biological Chemistry* **296**, 100371.
- Maidment JHR, Saile SC, Bocquet A, et al.** (2025). The *Magnaporthe oryzae* MAX effector AVR-Pia binds a novel group of rice HMA domain-containing proteins. *bioRxiv* 2025.07.11.664054.
- Maidment JHR, Shimizu M, Bentham AR, et al.** (2023). Effector target-guided engineering of an integrated domain expands the disease resistance profile of a rice NLR immune receptor. *eLife* **12**, e81123.
- Maqbool A, Saitoh H, Franceschetti M, et al.** (2015). Structural basis of pathogen recognition by an integrated HMA domain in a plant NLR immune receptor. *eLife* **4**, e08709.
- Martínez-Cruz JM, Polonio Á, Ruiz-Jiménez L, et al.** (2022). Suppression of Chitin-Triggered Immunity by a New Fungal Chitin-Binding Effector Resulting from Alternative Splicing of a Chitin Deacetylase Gene. *Journal of Fungi* **8**, 1022.
- May DG, Scott KL, Campos AR, and Roux KJ** (2020). Comparative Application of BioID and TurboID for Protein-Proximity Biotinylation. *Cells* **9**, 1070.
- McCall KA and Fierke CA** (2000). Colorimetric and Fluorimetric Assays to Quantitate Micromolar Concentrations of Transition Metals. *Analytical Biochemistry* **284**, 307–315.
- McCoy AJ, Grosse-Kunstleve RW, Adams PD, et al.** (2007). Phaser crystallographic software. *Journal of Applied Crystallography* **40**, 658–674.
- Meile L, Garrido-Arandia M, Bernasconi Z, et al.** (2023). Natural variation in *Avr3D1* from *Zymoseptoria* sp. contributes to quantitative gene-for-gene resistance and to host specificity. *New Phytologist* **238**, 1562–1577.
- Mellidou I and Kanellis AK** (2024). Revisiting the role of ascorbate oxidase in plant systems. *Journal of Experimental Botany* **75**, 2740–2753.
- Mhamdi A, Queval G, Chaouch S, et al.** (2010). Catalase function in plants: a focus on *Arabidopsis* mutants as stress-mimic models. *Journal of Experimental Botany* **61**, 4197–4220.
- Milner MJ, Seamon J, Craft E, and Kochian LV** (2013). Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis. *Journal of Experimental Botany* **64**, 369–381.
- Mira H, Martínez-García F, and Peñarrubia L** (2001). Evidence for the plant-specific intercellular transport of the Arabidopsis copper chaperone CCH. *The Plant Journal* **25**, 521–528.
- Miras R, Morin I, Jacquin O, et al.** (2008). Interplay between glutathione, Atx1 and copper. 1. Copper(I) glutathionate induced dimerization of Atx1. *JBIC Journal of Biological Inorganic Chemistry* **13**, 195–205.
- Mirdita M, Schütze K, Moriwaki Y, et al.** (2022). ColabFold: making protein folding accessible to all. *Nature Methods* **19**, 679–682.

- Mittler R, Zandalinas SI, Fichman Y, and Breusegem FV** (2022). Reactive oxygen species signalling in plant stress responses. *Nature Reviews Molecular Cell Biology* **23**, 663–679.
- Miya A, Albert P, Shinya T, et al.** (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proceedings of the National Academy of Sciences* **104**, 19613–19618.
- Mosquera G, Giraldo MC, Khang CH, et al.** (2009). Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease. *Plant Cell* **21**, 1273–1290.
- Mugford ST, Barclay E, Drurey C, et al.** (2016). An Immuno-Suppressive Aphid Saliva Protein Is Delivered into the Cytosol of Plant Mesophyll Cells During Feeding. *Mol Plant Microbe Interact* **29**, 854–861.
- Mugford ST and Hogenhout S** (2018). Bounce PCR V.3. *protocols.io* doi.org/10.17504/protocols.io.vhge33w.
- Mukhi N, Brown H, Gorenkin D, et al.** (2021). Perception of structurally distinct effectors by the integrated WRKY domain of a plant immune receptor. *Proceedings of the National Academy of Sciences* **118**, e2113996118.
- Mukhtar MS, Carvunis AR, Dreze M, et al.** (2011). Independently Evolved Virulence Effectors Converge onto Hubs in a Plant Immune System Network. *Science* **333**, 596–601.
- Muller PAJ and Klomp LWJ** (2009). ATOX1: A novel copper-responsive transcription factor in mammals?. *The International Journal of Biochemistry & Cell Biology* **41**, 1233–1236.
- Murshudov GN, Skubák P, Lebedev AA, et al.** (2011). REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallographica Section D* **67**, 355–367.
- Nakao M, Nakamura R, Kita K, et al.** (2011). Non-host resistance to penetration and hyphal growth of *Magnaporthe oryzae* in *Arabidopsis*. *Scientific Reports* **1**, 171.
- Narusaka M, Shirasu K, Noutoshi Y, et al.** (2009). RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens. *The Plant Journal* **60**, 218–226.
- Narusaka Y, Narusaka M, Park P, et al.** (2004). RCH1, a Locus in *Arabidopsis* That Confers Resistance to the Hemibiotrophic Fungal Pathogen *Colletotrichum higginsianum*. *Molecular Plant-Microbe Interactions* **17**, 749–762.
- Nersissian AM, Valentine JS, Immoos C, et al.** (1998). Uclacyanins, stellacyanins, and plantacyanins are distinct subfamilies of phytoeyanins: Plant-specific mononuclear blue copper proteins. *Protein Science* **7**, 1915–1929.
- Ngou BPM, Ahn HK, Ding P, and Jones JDG** (2021). Mutual potentiation of plant immunity by cell-surface and intracellular receptors. *Nature* **592**, 110–115.
- Ngou BPM, Ding P, and Jones JDG** (2022). Thirty years of resistance: Zig-zag through the plant immune system. *The Plant Cell* **34**, 1447–1478.
- Nirmala J, Brueggeman R, Maier C, et al.** (2006). Subcellular localization and functions of the barley stem rust resistance receptor-like serine/threonine-specific protein kinase Rpg1. *Proceedings of the National Academy of Sciences* **103**, 7518–7523.
- Niu X, Yamamoto N, Yang G, et al.** (2023). A small secreted protein, RsMf8HN, in *Rhizoctonia solani* triggers plant immune response, which interacts with rice OsHIPP28. *Microbiological Research* **266**, 127219.

- O'Connell R, Herbert C, Sreenivasaprasad S, et al.** (2004). A Novel *Arabidopsis-Colletotrichum* Pathosystem for the Molecular Dissection of Plant-Fungal Interactions. *Molecular Plant-Microbe Interactions* **17**, 272–282.
- O'Connell RJ, Bailey JA, and Richmond DV** (1985). Cytology and physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. *Physiological Plant Pathology* **27**, 75–98.
- O'Connell RJ, Thon MR, Hacquard S, et al.** (2012). Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. *Nature Genetics* **44**, 1060–1065.
- Ogawa T, Chen J, Mise K, and Takano Y** (2021). Multiple *Colletotrichum* species commonly exhibit focal effector accumulation in a biotrophic interface at the primary invasion sites in their host plants. *Plant Signaling & Behavior* **16**, 1935604.
- Ohno T, Takamatsu N, Meshi T, et al.** (1983). Single amino acid substitution in 30K protein of TMV defective in virus transport function. *Virology* **131**, 255–258.
- Ohtsu M, Jennings J, Johnston M, et al.** (2023). Assaying Effector Cell-to-Cell Mobility in Plant Tissues Identifies Hypermobility and Indirect Manipulation of Plasmodesmata. *Molecular Plant-Microbe Interactions* **37**, 84–92.
- Oikawa K, Fujisaki K, Shimizu M, et al.** (2024). The blast pathogen effector AVR-Pik binds and stabilizes rice heavy metal-associated (HMA) proteins to co-opt their function in immunity. *PLOS Pathogens* **20**, e1012647.
- O'Lexy R, Kasai K, Clark N, et al.** (2018). Exposure to heavy metal stress triggers changes in plasmodesmatal permeability via deposition and breakdown of callose. *Journal of Experimental Botany* **69**, 3715–3728.
- Olivé M and Campo S** (2021). The dsRNA mycovirus ChNRV1 causes mild hypervirulence in the fungal phytopathogen *Colletotrichum higginsianum*. *Archives of Microbiology* **203**, 241–249.
- Oliveira-Garcia E and Hamilton AJ** (2024). A pharmacological approach to investigating effector translocation in rice-*Magnaporthe oryzae* interactions. *Plant Signaling & Behavior* **19**, 2350869.
- Oliveira-Garcia E, Tamang TM, Park J, et al.** (2023). Clathrin-mediated endocytosis facilitates the internalization of *Magnaporthe oryzae* effectors into rice cells. *The Plant Cell* **35**, 2527–2551.
- Oparka KJ, Roberts AG, Boevink P, et al.** (1999). Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. *Cell* **97**, 743–754.
- Ormenese S, Bernier G, and Périlleux C** (2006). Cytokinin application to the shoot apical meristem of *Sinapis alba* enhances secondary plasmodesmata formation. *Planta* **224**, 1481–1484.
- Ortiz D, de Guillen K, Cesari S, et al.** (2017). Recognition of the *Magnaporthe oryzae* Effector AVR-Pia by the Decoy Domain of the Rice NLR Immune Receptor RGA5. *The Plant Cell* **29**, 156–168.
- Ortiz-Vallejo D** (2016). Study of the molecular basis of recognition of the fungal effector AVR-Pia by the rice immune receptor RGA5. Ph.D. thesis. Montpellier SupAgro.
- Ostermeyer GP, Jensen KH, Franzen AR, et al.** (2022). Diversity of funnel plasmodesmata in angiosperms: the impact of geometry on plasmodesmal resistance. *The Plant Journal* **110**, 707–719.

- Outram MA, Figueroa M, Sperschneider J, et al.** (2022). Seeing is believing: Exploiting advances in structural biology to understand and engineer plant immunity. *Current Opinion in Plant Biology* **67**, 102210.
- Pérez-Sancho J, Smokvarska M, Dubois G, et al.** (2025). Plasmodesmata act as unconventional membrane contact sites regulating intercellular molecular exchange in plants. *Cell* **188**, 958–977.
- Parisy V, Poinssot B, Owsianowski L, et al.** (2007). Identification of *PAD2* as a γ -glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of *Arabidopsis*. *The Plant Journal* **49**, 159–172.
- Park SH, Li F, Renaud J, et al.** (2017). NbEXPA1, an α -expansin, is plasmodesmata-specific and a novel host factor for potyviral infection. *The Plant Journal* **92**, 846–861.
- Pedersen C, van Themaat EVL, McGuffin LJ, et al.** (2012). Structure and evolution of barley powdery mildew effector candidates. *BMC Genomics* **13**, 694.
- Pennington HG, Jones R, Kwon S, et al.** (2019). The fungal ribonuclease-like effector protein CSEP0064/BEC1054 represses plant immunity and interferes with degradation of host ribosomal RNA. *PLOS Pathogens* **15**, e1007620.
- Permyakov EA** (2021). Metal Binding Proteins. *Encyclopedia* **1**, 261–292.
- Perrakis A and Sixma TK** (2021). AI revolutions in biology. *EMBO reports* **22**, e54046.
- Pettersen EF, Goddard TD, Huang CC, et al.** (2021). UCSF ChimeraX: Structure visualization for researchers, educators, and developers. *Protein Science* **30**, 70–82.
- Pfaffl MW** (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, 45.
- Piñeiro A, Muñoz E, Sabín J, et al.** (2019). AFFINImeter: A software to analyze molecular recognition processes from experimental data. *Analytical Biochemistry* **577**, 117–134.
- Piovesan D, Del Conte A, Mehdiabadi M, et al.** (2025). MOBIDB in 2025: integrating ensemble properties and function annotations for intrinsically disordered proteins. *Nucleic Acids Research* **53**, 495–503.
- Planas-Portell J, Gallart M, Tiburcio AF, and Altabella T** (2013). Copper-containing amine oxidases contribute to terminal polyamine oxidation in peroxisomes and apoplast of *Arabidopsis thaliana*. *BMC Plant Biology* **13**, 109.
- Plaumann PL, Schmidpeter J, Dahl M, et al.** (2018). A Dispensable Chromosome Is Required for Virulence in the Hemibiotrophic Plant Pathogen *Colletotrichum higginsianum*. *Frontiers in Microbiology* **9**.
- Pliego C, Nowara D, Bonciani G, et al.** (2013). Host-Induced Gene Silencing in Barley Powdery Mildew Reveals a Class of Ribonuclease-Like Effectors. *Molecular Plant-Microbe Interactions* **26**, 633–642.
- Poland JA, Balint-Kurti PJ, Wissner RJ, et al.** (2009). Shades of gray: the world of quantitative disease resistance. *Trends in Plant Science* **14**, 21–29.
- Portnoy ME, Rosenzweig AC, Rae T, et al.** (1999). Structure-Function Analyses of the ATX1 Metallochaperone. *Journal of Biological Chemistry* **274**, 15041–15045.
- Presti LL, Zechmann B, Kumlehn J, et al.** (2017). An assay for entry of secreted fungal effectors into plant cells. *New Phytologist* **213**, 956–964.

- Pufahl RA, Singer CP, Peariso KL, et al.** (1997). Metal Ion Chaperone Function of the Soluble Cu(I) Receptor Atx1. *Science* **278**, 853–856.
- Puig S, Mira H, Dorcey E, et al.** (2007). Higher plants possess two different types of ATX1-like copper chaperones. *Biochemical and Biophysical Research Communications* **354**, 385–390.
- Punt PJ, Zegers ND, Busscher M, et al.** (1991). Intracellular and extracellular production of proteins in *Aspergillus* under the control of expression signals of the highly expressed *Aspergillus nidulans* *gpdA* gene. *Journal of Biotechnology* **17**, 19–33.
- Qiu D, Xiao J, Ding X, et al.** (2007). OsWRKY13 Mediates Rice Disease Resistance by Regulating Defense-Related Genes in Salicylate- and Jasmonate-Dependent Signaling. *Molecular Plant-Microbe Interactions* **20**, 492–499.
- Quime BG, Ryder LS, and Talbot NJ** (2025). Live cell imaging of plant infection provides new insight into the biology of pathogenesis by the rice blast fungus *Magnaporthe oryzae*. *Journal of Microscopy* **297**, 274–288.
- Radakovic ZS, Anjam MS, Escobar E, et al.** (2018). Arabidopsis HIPP27 is a host susceptibility gene for the beet cyst nematode *Heterodera schachtii*. *Molecular Plant Pathology* **19**, 1917–1928.
- Rafiqi M, Gan PHP, Ravensdale M, et al.** (2010). Internalization of Flax Rust Avirulence Proteins into Flax and Tobacco Cells Can Occur in the Absence of the Pathogen. *The Plant Cell* **22**, 2017–2032.
- Reagan B, Nuzzi S, Zanini A, and Burch-Smith T** (2023). Plant Viruses Induce Plasmodesmata Formation During Infection. *Current Biology* .
- Regmi KC, Ghosh S, Koch B, et al.** (2023). Three-Dimensional Ultrastructure of *Arabidopsis* Cotyledons Infected with *Colletotrichum higginsianum*. *Molecular Plant-Microbe Interactions* **37**, 396–406.
- Reveguk T, Fatiukha A, Potapenko E, et al.** (2025). Tandem kinase proteins across the plant kingdom. *Nature Genetics* **57**, 254–262.
- Reyt G, Chao Z, Flis P, et al.** (2020). Uclacyanin Proteins Are Required for Lignified Nanodomain Formation within Casparian Strips. *Current Biology* **30**, 4103–4111.
- Rhodes J, Roman AO, Bjornson M, et al.** (2022). Perception of a conserved family of plant signalling peptides by the receptor kinase HSL3. *eLife* **11**, e74687.
- Rhodes J, Yang H, Moussu S, et al.** (2021). Perception of a divergent family of phytocytokines by the Arabidopsis receptor kinase MIK2. *Nature Communications* **12**, 705.
- Ritzenthaler C, Schmit A, Michler P, et al.** (1995). Grapevine fanleaf nepovirus P38 putative movement protein is located on tubules in vivo. *Molecular Plant-Microbe Interactions* **8**, 379–387.
- Roberts IM, Wang D, Findlay K, and Maule AJ** (1998). Ultrastructural and Temporal Observations of the Potyvirus Cylindrical Inclusions (CIs) Show That the CI Protein Acts Transiently in Aiding Virus Movement. *Virology* **245**, 173–181.
- Robin GP, Kleemann J, Neumann U, et al.** (2018). Subcellular localization screening of *Colletotrichum higginsianum* effector candidates identifies fungal proteins targeted to plant peroxisomes, golgi bodies, and microtubules. *Frontiers in Plant Science* **9**.
- Rodríguez-Cerezo E, Findlay K, Shaw JG, et al.** (1997). The Coat and Cylindrical Inclusion Proteins of a Potyvirus Are Associated with Connections between Plant Cells. *Virology* **236**, 296–306.

- Rosenzweig AC, Huffman DL, Hou MY, et al. (1999). Crystal structure of the Atx1 metallochaperone protein at 1.02 Å resolution. *Structure* **7**, 605–617.
- Rubino JT and Franz KJ (2012). Coordination chemistry of copper proteins: How nature handles a toxic cargo for essential function. *Journal of Inorganic Biochemistry* **107**, 129–143.
- Rudd JJ (2023). Effector-mediated partial and nonhost disease resistance in wheat. *New Phytologist* **238**, 1340–1342.
- Säbel CE, Shepherd JL, and Siemann S (2009). A direct spectrophotometric method for the simultaneous determination of zinc and cobalt in metalloproteins using 4-(2-pyridylazo)resorcinol. *Analytical Biochemistry* **391**, 74–76.
- Saitoh H, Fujisawa S, Mitsuoka C, et al. (2012). Large-Scale Gene Disruption in *Magnaporthe oryzae* Identifies MC69, a Secreted Protein Required for Infection by Monocot and Dicot Fungal Pathogens. *PLOS Pathogens* **8**, e1002711.
- Sakulkoo W, Osés-Ruiz M, Garcia EO, et al. (2018). A single fungal MAP kinase controls plant cell-to-cell invasion by the rice blast fungus. *Science* **359**, 1399–1403.
- Sambade A, Brandner K, Hofmann C, et al. (2008). Transport of TMV Movement Protein Particles Associated with the Targeting of RNA to Plasmodesmata. *Traffic* **9**, 2073–2088.
- Sánchez-Pérez CR, Castaño E, Rodríguez-López CE, et al. (2025). Heavy Metal-Associated Proteins in Plants: Genome-Wide Identification and Functional Insights from Angiosperms and Ancestral Plants. *Journal of Molecular Evolution* **93**, 527–542.
- Sánchez-Vallet A, Fouché S, Fudal I, et al. (2018). The Genome Biology of Effector Gene Evolution in Filamentous Plant Pathogens. *Annual review of phytopathology* **56**, 21–40.
- Sarris PF, Cevik V, Dagdas G, et al. (2016). Comparative analysis of plant immune receptor architectures uncovers host proteins likely targeted by pathogens. *BMC Biology* **14**, 8.
- Schindelin J, Arganda-Carreras I, Frise E, et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**, 676–682.
- Schmidpeter J, Dahl M, Hofmann J, and Koch C (2017). ChMob2 binds to ChCbk1 and promotes virulence and conidiation of the fungal pathogen *Colletotrichum higginsianum*. *BMC Microbiology* **17**, 22.
- Schoelz JE, Harries PA, and Nelson RS (2011). Intracellular Transport of Plant Viruses: Finding the Door out of the Cell. *Molecular Plant* **4**, 813–831.
- Schreier TB, Müller KH, Eicke S, et al. (2024). Plasmodesmal connectivity in C4 *Gynandropsis gynandra* is induced by light and dependent on photosynthesis. *New Phytologist* **241**, 298–313.
- Sedbrook JC, Carroll KL, Hung KF, et al. (2002). The Arabidopsis *SKU5* Gene Encodes an Extracellular Glycosyl Phosphatidylinositol–Anchored Glycoprotein Involved in Directional Root Growth. *The Plant Cell* **14**, 1635–1648.
- Selvaraj M, Toghani A, Pai H, et al. (2024). Activation of plant immunity through conversion of a helper NLR homodimer into a resistosome. *PLOS Biology* **22**, e3002868.
- Shan L, He P, Li J, et al. (2008). Bacterial Effectors Target the Common Signaling Partner BAK1 to Disrupt Multiple MAMP Receptor-Signaling Complexes and Impede Plant Immunity. *Cell Host & Microbe* **4**, 17–27.

- Shi W, Stolze SC, Nakagami H, et al.** (2023). Combination of in vivo proximity labeling and co-immunoprecipitation identifies the host target network of a tumor-inducing effector in the fungal maize pathogen *Ustilago maydis*. *Journal of Experimental Botany* **74**, 4736–4750.
- Shimada TL, Betsuyaku S, Inada N, et al.** (2019). Enrichment of Phosphatidylinositol 4,5-Bisphosphate in the Extra-Invasive Hyphal Membrane Promotes *Colletotrichum* Infection of *Arabidopsis thaliana*. *Plant and Cell Physiology* **60**, 1514–1524.
- Shimada TL, Shimada T, and Hara-Nishimura I** (2010). A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of *Arabidopsis thaliana*. *Plant Journal* **61**, 519–528.
- Shipman EN, Jones K, Jenkinson CB, et al.** (2017). Nuclear and structural dynamics during the establishment of a specialized effector-secreting cell by *Magnaporthe oryzae* in living rice cells. *BMC Cell Biology* **18**, 11.
- Song H, Lin B, Huang Q, et al.** (2021). The *Meloidogyne graminicola* effector MgMO289 targets a novel copper metallochaperone to suppress immunity in rice. *Journal of Experimental Botany* **72**, 5638–5655.
- Sornkom W, Miki S, Takeuchi S, et al.** (2017). Fluorescent reporter analysis revealed the timing and localization of AVR-Pia expression, an avirulence effector of *Magnaporthe oryzae*. *Molecular Plant Pathology* **18**, 1138–1149.
- Spanu PD** (2015). RNA–protein interactions in plant disease: hackers at the dinner table. *New Phytologist* **207**, 991–995.
- Sperschneider J and Dodds PN** (2022). EffectorP 3.0: Prediction of Apoplastic and Cytoplasmic Effectors in Fungi and Oomycetes. *Molecular Plant-Microbe Interactions* **35**, 146–156.
- Steele RA and Opella SJ** (1997). Structures of the Reduced and Mercury-Bound Forms of MerP, the Periplasmic Protein from the Bacterial Mercury Detoxification System. *Biochemistry* **36**, 6885–6895.
- Stone CE** (2024). Interactions of blast fungus effectors with small HMAs and the paired rice NLR Pik. Ph.D. thesis. University of East Anglia.
- Stuer N, Damme PV, Goormachtig S, and Dingenen JV** (2023). Seeking the interspecies crosswalk for filamentous microbe effectors. *Trends in Plant Science* **28**, 1045–1059.
- Su S, Liu Z, Chen C, et al.** (2010). Cucumber Mosaic Virus Movement Protein Severs Actin Filaments to Increase the Plasmodesmal Size Exclusion Limit in Tobacco. *The Plant Cell* **22**, 1373–1387.
- Sugihara Y, Abe Y, Takagi H, et al.** (2023). Disentangling the complex gene interaction networks between rice and the blast fungus identifies a new pathogen effector. *PLOS Biology* **21**, e3001945.
- Sun T and Zhang Y** (2021). Short- and long-distance signaling in plant defense. *The Plant Journal* **105**, 505–517.
- Sun W, Wei J, Wu G, et al.** (2022). CqZF-HD14 enhances drought tolerance in quinoa seedlings through interaction with CqHIPP34 and CqNAC79. *Plant Science* **323**, 111406.
- Sun X, Lapin D, Feehan JM, et al.** (2021). Pathogen effector recognition-dependent association of NRG1 with EDS1 and SAG101 in TNL receptor immunity. *Nature Communications* **12**, 3335.

- Sung YC, Li Y, Bernasconi Z, et al. (2025). Wheat tandem kinase RWT4 directly binds a fungal effector to activate defense. *Nature Genetics* **57**, 1238–1249.
- Sunil S, Beeh S, Stöbbe E, et al. (2024). Activation of an atypical plant NLR with an N-terminal deletion initiates cell death at the vacuole. *EMBO reports* **25**, 4358–4386.
- Suzuki N, Yamaguchi Y, Koizumi N, and Sano H (2002). Functional characterization of a heavy metal binding protein CdI19 from *Arabidopsis*. *The Plant Journal* **32**, 165–173.
- Szymansky CM (2025). Discovery of pathogen virulence and plant defense mechanisms by proximity labelling. Ph.D. thesis. University of East Anglia.
- Takahara H, Hacquard S, Kombrink A, et al. (2016). *Colletotrichum higginsianum* extracellular LysM proteins play dual roles in appressorial function and suppression of chitin-triggered plant immunity. *New Phytologist* **211**, 1323–1337.
- Talbot NJ, Ebbole DJ, and Hamer JE (1993). Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *The Plant Cell* **5**, 1575–1590.
- Taliansky M, Torrance L, and Kalinina NO (2008). Role of Plant Virus Movement Proteins. *Plant Virology Protocols: From Viral Sequence to Protein Function* **451**, 33–54.
- Tamborski J, Seong K, Liu F, et al. (2023). Altering Specificity and Autoactivity of Plant Immune Receptors Sr33 and Sr50 Via a Rational Engineering Approach. *Molecular Plant-Microbe Interactions* **36**, 434–446.
- Tanaka S, Djamei A, Presti LL, et al. (2015). Experimental approaches to investigate effector translocation into host cells in the *Ustilago maydis*/maize pathosystem. *European Journal of Cell Biology* **94**, 349–358.
- Tanchou V, Gas F, Urvoas A, et al. (2004). Copper-mediated homo-dimerisation for the HAH1 metallochaperone. *Biochemical and Biophysical Research Communications* **325**, 388–394.
- Tang D, Wang G, and Zhou JM (2017). Receptor Kinases in Plant-Pathogen Interactions: More Than Pattern Recognition. *The Plant Cell* **29**, 618–637.
- Tanzi RE, Petrukhin K, Chernov I, et al. (1993). The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nature Genetics* **5**, 344–350.
- Tee EE, Breakspear A, Papp D, et al. (2025). Plasmodesmal closure elicits stress responses. *bioRxiv* 2024.05.08.593115.
- Tee EE, Johnston MG, Papp D, and Faulkner C (2023). A PDLN-NHL3 complex integrates plasmodesmal immune signaling cascades. *Proceedings of the National Academy of Sciences* **120**, e2216397120.
- Tehseen M, Cairns N, Sherson S, and Cobbett CS (2010). Metallochaperone-like genes in *Arabidopsis thaliana*. *Metallomics* **2**, 556–564.
- Teo G, Koh H, Fermin D, et al. (2016). SAINTq: Scoring protein-protein interactions in affinity purification – mass spectrometry experiments with fragment or peptide intensity data. *Proteomics* **16**, 2238–2245.
- Teufel F, Armenteros JJA, Johansen AR, et al. (2022). SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology* **40**, 1023–1025.
- Thomas CL, Bayer EM, Ritzenthaler C, et al. (2008). Specific Targeting of a Plasmodesmal Protein Affecting Cell-to-Cell Communication. *PLOS Biology* **6**, e7.

- Thomine S, Lelièvre F, Debarbieux E, et al.** (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *The Plant Journal* **34**, 685–695.
- Thordal-Christensen H, Birch PRJ, Spanu PD, and Panstruga R** (2018). Why did filamentous plant pathogens evolve the potential to secrete hundreds of effectors to enable disease?. *Molecular Plant Pathology* **19**, 781–785.
- Tilsner J, Linnik O, Louveaux M, et al.** (2013). Replication and trafficking of a plant virus are coupled at the entrances of plasmodesmata. *Journal of Cell Biology* **201**, 981–995.
- Tilsner J, Linnik O, Wright KM, et al.** (2012). The TGB1 Movement Protein of Potato virus X Reorganizes Actin and Endomembranes into the X-Body, a Viral Replication Factory. *Plant Physiology* **158**, 1359–1370.
- Tsushima A, Gan P, Kumakura N, et al.** (2019). Genomic plasticity mediated by transposable elements in the plant pathogenic fungus *Colletotrichum higginsianum*. *Genome Biology and Evolution* **11**, 1487–1500.
- Tsushima A, Narusaka M, Gan P, et al.** (2021). The Conserved *Colletotrichum* spp. Effector Candidate CEC3 Induces Nuclear Expansion and Cell Death in Plants. *Frontiers in Microbiology* **12**.
- Vallejo B, Picazo C, Orozco H, et al.** (2017). Herbicide glufosinate inhibits yeast growth and extends longevity during wine fermentation. *Scientific Reports* **7**, 12414.
- Van de Weyer AL, Monteiro F, Furzer OJ, et al.** (2019). A Species-Wide Inventory of NLR Genes and Alleles in *Arabidopsis thaliana*. *Cell* **178**, 1260–1272.
- van der Hoorn RAL and Kamoun S** (2008). From Guard to Decoy: A New Model for Perception of Plant Pathogen Effectors. *The Plant Cell* **20**, 2009–2017.
- van Kempen M, Kim SS, Tumescheit C, et al.** (2024). Fast and accurate protein structure search with Foldseek. *Nature Biotechnology* **42**, 243–246.
- van Lent J, Storms M, van der Meer F, et al.** (1991). Tubular structures involved in movement of cowpea mosaic virus are also formed in infected cowpea protoplasts. *Journal of General Virology* **72**, 2615–2623.
- Varden FA, Saitoh H, Yoshino K, et al.** (2019). Cross-reactivity of a rice NLR immune receptor to distinct effectors from the rice blast pathogen *Magnaporthe oryzae* provides partial disease resistance. *Journal of Biological Chemistry* **294**, 13006–13016.
- Vargas WA, Sanz-Martín JM, Rech GE, et al.** (2016). A Fungal effector with host nuclear localization and DNA-binding properties is required for maize anthracnose development. *Molecular Plant-Microbe Interactions* **29**, 83–95.
- Vatén A, Dettmer J, Wu S, et al.** (2011). Callose Biosynthesis Regulates Symplastic Trafficking during Root Development. *Developmental Cell* **21**, 1144–1155.
- Verhage A, van Wees SC, and Pieterse CM** (2010). Plant Immunity: It's the Hormones Talking, But What Do They Say?. *Plant Physiology* **154**, 536–540.
- Vetting MW, de Carvalho LPS, Yu M, et al.** (2005). Structure and functions of the GNAT superfamily of acetyltransferases. *Archives of Biochemistry and Biophysics* **433**, 212–226.
- Vijai B, Sabine B, Albert V, et al.** (2013). Overexpression of a Novel Biotrophy-Specific *Colletotrichum truncatum* Effector, CtNUDIX, in Hemibiotrophic Fungal Phytopathogens Causes Incompatibility with Their Host Plants. *Eukaryotic Cell* **12**, 2–11.

- Vijayapalani P, Maeshima M, Nagasaki-Takekuchi N, and Miller WA** (2012). Interaction of the Trans-Frame Potyvirus Protein P3N-PIPO with Host Protein PCaP1 Facilitates Potyvirus Movement. *PLOS Pathogens* **8**, e1002639.
- Vulpe C, Levinson B, Whitney S, et al.** (1993). Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. *Nature Genetics* **3**, 7–13.
- Wang A, Pan L, Niu X, et al.** (2019a). Comparative secretome analysis of different smut fungi and identification of plant cell death-inducing secreted proteins from *Tilletia horrida*. *BMC Plant Biology* **19**, 360.
- Wang F, Li X, Li Y, et al.** (2021). *Arabidopsis* P4 ATPase-mediated cell detoxification confers resistance to *Fusarium graminearum* and *Verticillium dahliae*. *Nature Communications* **12**, 6426.
- Wang H, Wang S, Wang W, et al.** (2023a). Uptake of oomycete RXLR effectors into host cells by clathrin-mediated endocytosis. *The Plant Cell* **35**, 2504–2526.
- Wang J, Hu M, Wang J, et al.** (2019b). Reconstitution and structure of a plant NLR resistosome conferring immunity. *Science* **364**, eaav5870.
- Wang M and Casey PJ** (2016). Protein prenylation: unique fats make their mark on biology. *Nature Reviews Molecular Cell Biology* **17**, 110–122.
- Wang Y, Abrouk M, Gourdoups S, et al.** (2023b). An unusual tandem kinase fusion protein confers leaf rust resistance in wheat. *Nature Genetics* **55**, 914–920.
- Wang Z, Liu X, Yu J, et al.** (2023c). Plasma membrane association and resistosome formation of plant helper immune receptors. *bioRxiv* 2023.01.23.525201.
- Wang Z, Zhang H, Li Y, et al.** (2022). Isoprenylation modification is required for HIP1-mediated powdery mildew resistance in wheat. *Plant, Cell & Environment* **46**, 288–305.
- Watson JL, Juergens D, Bennett NR, et al.** (2023). De novo design of protein structure and function with RFdiffusion. *Nature* **620**, 1089–1100.
- Wawra S, Trusch F, Matena A, et al.** (2017). The RxLR Motif of the Host Targeting Effector AVR3a of *Phytophthora infestans* Is Cleaved before Secretion. *The Plant Cell* **29**, 1184–1195.
- Wei HL, Chakravarthy S, Mathieu J, et al.** (2015). *Pseudomonas syringae* pv. *tomato* DC3000 Type III Secretion Effector Polymutants Reveal an Interplay between HopAD1 and AvrPtoB. *Cell Host & Microbe* **17**, 752–762.
- Wei T, Zhang C, Hong J, et al.** (2010). Formation of Complexes at Plasmodesmata for Potyvirus Intercellular Movement Is Mediated by the Viral Protein P3N-PIPO. *PLOS Pathogens* **6**, e1000962.
- Wei Y, Peng X, Wang X, and Wang C** (2023). The heavy metal-associated isoprenylated plant protein (HIPP) gene family plays a crucial role in cadmium resistance and accumulation in the tea plant (*Camellia sinensis* L.). *Ecotoxicology and Environmental Safety* **260**, 115077.
- Weßling R, Eppele P, Altmann S, et al.** (2014). Convergent Targeting of a Common Host Protein-Network by Pathogen Effectors from Three Kingdoms of Life. *Cell Host & Microbe* **16**, 364–375.
- Were VM, Yan X, Foster AJ, et al.** (2025). The *Magnaporthe oryzae* effector Pwl2 alters HIP43 localization to suppress host immunity. *The Plant Cell* **37**, 116.
- Wernimont A and Edwards A** (2009). In Situ proteolysis to generate crystals for structure determination: An update. *PLOS One* **4**, e5094.

- Wernimont AK, Huffman DL, Lamb AL, et al. (2000). Structural basis for copper transfer by the metallochaperone for the Menkes/Wilson disease proteins. *Nature Structural Biology* **7**, 766–771.
- Whisson SC, Boevink PC, Moleleki L, et al. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* **450**, 115–118.
- Wickham H (2011). ggplot2. *WIREs Computational Statistics* **3**, 180–185.
- Williams LE and Mills RF (2005). P_{1B}-ATPases - an ancient family of transition metal pumps with diverse functions in plants. *Trends in Plant Science* **10**, 491–502.
- Winter G (2010). xia2: an expert system for macromolecular crystallography data reduction. *Journal of Applied Crystallography* **43**, 186–190.
- Wu C and Derevnina L (2023). The battle within: How pathogen effectors suppress NLR-mediated immunity. *Current Opinion in Plant Biology* **74**, 102396.
- Wu CH, Abd-El-Hallem A, Bozkurt TO, et al. (2017). NLR network mediates immunity to diverse plant pathogens. *Proceedings of the National Academy of Sciences* **114**, 8113.
- Wu SW, Kumar R, Iswanto ABB, and Kim JY (2018). Callose balancing at plasmodesmata. *Journal of Experimental Botany* **69**, 5325–5339.
- Wymelenberg AJV, Cullen D, Spear RN, et al. (1997). Expression of Green Fluorescent Protein in *Aureobasidium pullulans* and Quantification of the Fungus on Leaf Surfaces. *BioTechniques* **23**, 686–690.
- Xi Y, Chochois V, Kroj T, and Cesari S (2021). A novel robust and high-throughput method to measure cell death in *Nicotiana benthamiana* leaves by fluorescence imaging. *Molecular Plant Pathology* **22**, 1688–1696.
- Xia H, Jing X, He H, et al. (2025). Genome-wide identification of the HIPPs gene family and functional validation of MsHIPP12 in enhancing cadmium tolerance in *Medicago sativa*. *Journal of Hazardous Materials* **491**, 137894.
- Xie L, Shang W, Liu C, et al. (2016). Mutual association of Broad bean wilt virus 2 VP37-derived tubules and plasmodesmata obtained from cytological observation. *Scientific Reports* **6**, 21552.
- Xin XF, Nomura K, Aung K, et al. (2016). Bacteria establish an aqueous living space in plants crucial for virulence. *Nature* **539**, 524.
- Xu B, Cheval C, Laohavisit A, et al. (2017). A calmodulin-like protein regulates plasmodesmal closure during bacterial immune responses. *New Phytologist* **215**, 77–84.
- Xu J, Cui J, He Q, et al. (2024). Genome-wide identification of HIPP and mechanism of SIHIPP4/7/9/21/26/32 mediated phytohormones response to Cd, osmotic, and salt stresses in tomato. *Plant Physiology and Biochemistry* **217**, 109220.
- Yan X, Tang B, Ryder LS, et al. (2023). The transcriptional landscape of plant infection by the rice blast fungus *Magnaporthe oryzae* reveals distinct families of temporally co-regulated and structurally conserved effectors. *The Plant Cell* **35**, 1360–1385.
- Ye X, Liu C, Yan H, et al. (2022). Genome-wide identification and transcriptome analysis of the heavy metal-associated (HMA) gene family in Tartary buckwheat and their regulatory roles under cadmium stress. *Gene* **847**, 146884.

- Yeon-Ki K, Daoxin L, and E KP (1998). Induction of Ca^{2+} -Calmodulin Signaling by Hard-Surface Contact Primes *Colletotrichum gloeosporioides* Conidia To Germinate and Form Appressoria. *Journal of Bacteriology* **180**, 5144–5150.
- Yonehara K, Kumakura N, Motoyama T, et al. (2023). Efficient multiple gene knockout in *Colletotrichum higginsianum* via CRISPR/Cas9 ribonucleoprotein and *URA3*-based marker recycling. *Molecular Plant Pathology* **24**, 1451–1464.
- Yu CS, Chen YC, Lu CH, and Hwang JK (2006). Prediction of protein subcellular localization. *Proteins: Structure, Function, and Bioinformatics* **64**, 643–651.
- Yu DS, Outram MA, Smith A, et al. (2024). The structural repertoire of *Fusarium oxysporum* f. sp. *lycopersici* effectors revealed by experimental and computational studies. *eLife* **12**, RP89280.
- Yuan C, Lazarowitz SG, and Citovsky V (2016). Identification of a functional plasmodesmal localization signal in a plant viral cell-to-cell-movement protein. *mBio* **7**, e02052–15.
- Yuan C, Lazarowitz SG, and Citovsky V (2018). The plasmodesmal localization signal of TMV MP is recognized by plant synaptotagmin SYTA. *mBio* **9**, e01314–18.
- Yuan DS, Stearman R, Dancis A, et al. (1995). The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proceedings of the National Academy of Sciences* **92**, 2632–2636.
- Yuan M, Jiang Z, Bi G, et al. (2021). Pattern-recognition receptors are required for NLR-mediated plant immunity. *Nature* **592**, 105–109.
- Zampounis A, Pigné S, Dallery JF, et al. (2016). Genome Sequence and Annotation of *Colletotrichum higginsianum*, a Causal Agent of Crucifer Anthracnose Disease. *Genome Announcements* **4**, e00821–16.
- Zavaliev R, Dong X, and Epel BL (2016). Glycosylphosphatidylinositol (GPI) Modification Serves as a Primary Plasmodesmal Sorting Signal. *Plant Physiology* **172**, 1061–1073.
- Zdrzałek R, Kamoun S, Terauchi R, et al. (2020). The rice NLR pair Pikp-1/Pikp-2 initiates cell death through receptor cooperation rather than negative regulation. *PLOS One* **15**, e0238616.
- Zdrzałek R, Stone C, De la Concepcion JC, et al. (2023). Pathways to engineering plant intracellular NLR immune receptors. *Current Opinion in Plant Biology* **74**, 102380.
- Zdrzałek R, Xi Y, Langner T, et al. (2024). Bioengineering a plant NLR immune receptor with a robust binding interface toward a conserved fungal pathogen effector. *Proceedings of the National Academy of Sciences* **121**, e2402872121.
- Zhang B, Liu M, Wang Y, et al. (2022a). Plant NLRs: Evolving with pathogen effectors and engineerable to improve resistance. *Frontiers in Microbiology* **13**.
- Zhang BQ, Liu XS, Feng SJ, et al. (2020a). Developing a cadmium resistant rice genotype with *OsHIPP29* locus for limiting cadmium accumulation in the paddy crop. *Chemosphere* **247**, 125958.
- Zhang H, hui Wen S, hang Li P, et al. (2024a). LysM protein BdLM1 of *Botryosphaeria dothidea* plays an important role in full virulence and inhibits plant immunity by binding chitin and protecting hyphae from hydrolysis. *Frontiers in Plant Science* **14**.
- Zhang H, Zhai G, Ni X, et al. (2025). Genome-wide identification of HIPP genes family in sorghum reveals the novel role of *SbHIPP40* in accumulation of cadmium. *Journal of Hazardous Materials* **494**, 138478.

- Zhang H, Zhang X, Liu J, et al.** (2020b). Characterization of the Heavy-Metal-Associated Isoprenylated Plant Protein (HIPP) Gene Family from *Triticeae* Species. *International Journal of Molecular Sciences* **21**, 6191.
- Zhang P, Wang R, Ju Q, et al.** (2019). The R2R3-MYB Transcription Factor MYB49 Regulates Cadmium Accumulation. *Plant Physiology* **180**, 529–542.
- Zhang S, Li C, Si J, et al.** (2022b). Action Mechanisms of Effectors in Plant-Pathogen Interaction. *International Journal of Molecular Sciences* **23**, 6758.
- Zhang X, Feng H, Feng C, et al.** (2015). Isolation and characterisation of cDNA encoding a wheat heavy metal-associated isoprenylated protein involved in stress responses. *Plant Biology* **17**, 1176–1186.
- Zhang X, Liu Y, Yuan G, et al.** (2024b). The synthetic NLR RGA5^{HMA5} requires multiple interfaces within and outside the integrated domain for effector recognition. *Nature Communications* **15**, 1104.
- Zhang Y and Li X** (2019). Salicylic acid: biosynthesis, perception, and contributions to plant immunity. *Current Opinion in Plant Biology* **50**, 29–36.
- Zhang YW, Xue LH, and Li CJ** (2018). First Report of Anthracnose Caused by *Colletotrichum higginsianum* on *Rumex acetosa* in China. *Plant Disease* **102**, 1174.
- Zhao S, Gong P, Liu J, et al.** (2023). Geminivirus C5 proteins mediate formation of virus complexes at plasmodesmata for viral intercellular movement. *Plant Physiology* **193**, 322–338.
- Zhu H, Gilchrist L, Hayes P, et al.** (1999). Does function follow form? Principal QTLs for Fusarium head blight (FHB) resistance are coincident with QTLs for inflorescence traits and plant height in a doubled-haploid population of barley. *Theoretical and Applied Genetics* **99**, 1221–1232.
- Zhu W, Shenoy A, Kundrotas P, and Elofsson A** (2023). Evaluation of AlphaFold-Multimer prediction on multi-chain protein complexes. *Bioinformatics* **39**, btad424.
- Zhu Y, Li Y, Fei F, et al.** (2015). E3 ubiquitin ligase gene *CMPG1-V* from *Haynaldia villosa* L. contributes to powdery mildew resistance in common wheat (*Triticum aestivum* L.). *The Plant Journal* **84**, 154–168.
- Zipfel C, Kunze G, Chinchilla D, et al.** (2006). Perception of the Bacterial PAMP EF-Tu by the Receptor EFR Restricts *Agrobacterium*-Mediated Transformation. *Cell* **125**, 749–760.
- Zschiesche W, Barth O, Daniel K, et al.** (2015). The zinc-binding nuclear protein HIPP3 acts as an upstream regulator of the salicylate-dependent plant immunity pathway and of flowering time in *Arabidopsis thaliana*. *New Phytologist* **207**, 1084–1096.