

The role of plasmodesmata in the growth-defence trade-off

A thesis submitted by

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Abstract

Plasmodesmata are plasma membrane-lined channels that connect adjacent plant cells to form a symplasmic network. These channels play a key role in both defence against pathogens and the phloem unloading of sugars. In mature leaf tissue, plasmodesmal closure is triggered upon detection of pathogen-associated molecular patterns (PAMPs), reducing cell-to-cell movement of molecules. In this thesis, I find PAMPs do not induce plasmodesmal closure in young *Arabidopsis* sink leaves. Other stress-related elicitors, such as hydrogen peroxide, salicylic acid, and abscisic acid also do not induce plasmodesmal closure in young sink leaves. Additionally, I find inducing the sink-source transition in young leaves changes plasmodesmal PAMP response. Young source leaves show PAMP-induced plasmodesmal closure, whilst young sink leaves do not. This indicates the change in PAMP response between young sink and mature source leaves is related to the sink-source status of the leaf rather than leaf age. I hypothesise the importance of plasmodesmata in symplastic phloem-unloading underpins this change in plasmodesmal stress response across the sink-source transition. Using lines with inducible plasmodesmal closure, I show that inducing plasmodesmal closure in young leaves and roots reduces phloem unloading and growth. Thus the regulation of plasmodesmal permeability in young sink tissue may be dependent on the balancing of a trade-off between growth and defence. Additionally, I find structure does not change across the sink-to-source transition meaning structure cannot account for changes to plasmodesmal response across the sink-source transition. Overall, these results suggest plasmodesmal response to PAMPs is dependent on the sink-source transition, highlighting the importance of maintaining symplastic connectivity in sink tissues.

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Chapter 1

Introduction

1.1 Introduction

Plasmodesmata are membrane-lined channels that form bridges across the plant cell wall connecting the cytoplasm of neighbouring cells. These cytoplasmic bridges facilitate cell-to-cell movement of molecules at a local scale between neighbouring cells, and on a much larger scale, enabling the transport of molecules across whole tissues and between organs. Plasmodesmal connections thus create a network of interconnected cells called the symplasm.

A large range of molecules are transported through plasmodesmata including hormones, nucleic acids, metabolites, and proteins. The functions of these molecules range from cellular patterning to phloem unloading, making plasmodesmata important to a wide range of plant processes. As a result of the various roles in plant biology, symplasmic connectivity through plasmodesmata is tightly controlled through both structural features and dynamic regulation of plasmodesmal aperture.

1.2 Formation and structure of plasmodesmata

1.2.1 Plasmodesmal ultrastructure

Plasmodesmata are plasma membrane-lined channels that connect the cytoplasm of neighbouring cells. Plasmodesmata typically consist of a continuous plasma membrane and cytoplasm spanned by a tube of endoplasmic reticulum, called the desmotubule (Figure 1.1). The cytoplasm-filled space between the plasma membrane and the desmotubule is called the cytoplasmic sleeve. Molecular trafficking of soluble proteins through plasmodesmata predominantly occurs through the cytoplasmic sleeve.

The desmotubule and plasma membrane are closely apposed at plasmodesmata, with distances of less than 10 nm observed between the desmotubule and plasma membrane (Nicolas et al., 2017). This close membrane apposition suggests plasmodesmata are specialised membrane contact sites (Tilsner et al., 2016). In general, membrane contact sites can be defined as cellular structures in close membrane apposition where there is no membrane fusion and proteins act as tethers between the membranes and (Scorrano et al., 2019). The closeness of the membranes is thought to allow rapid molecular crosstalk between the two structures. At plasmodesmata, the lack of membrane fusion and the presence of spoke-like tethers between the desmotubule and plasma membrane indicate plasmodesmata can be considered specialised membrane contact sites (Ding, Turgeon, & Parthasarathy, 1992; Tilsner et al., 2016).

MULTIPLE C2 DOMAINS AND TRANSMEMBRANE REGION PROTEINS (MCTPs) and synaptotagmins act as endoplasmic reticulum-plasma membrane tethers (Brault et al., 2019; Levy et al., 2015). These tethers are suggested to facilitate membrane curvature at plasmodesmata (Nicolas et al., 2017; Tilsner et al., 2016). The tethers are also critical for the formation of plasmodesmata, acting to stabilise the endoplasmic reticulum during plasmodesmal formation (Li et al., 2023). These tethers may also act in the regulation of plasmodesmal permeability by controlling the distance between the plasma membrane and desmotubule (Nicolas et al., 2017; Pérez-Sancho et al., 2023; Tilsner et al., 2016). Although further questions remain about the role of tethers in the control of plasmodesmal permeability, the membrane apposition of the desmotubule and plasma membrane is a key component of plasmodesmal structure.

1.2. Formation and structure of plasmodesmata

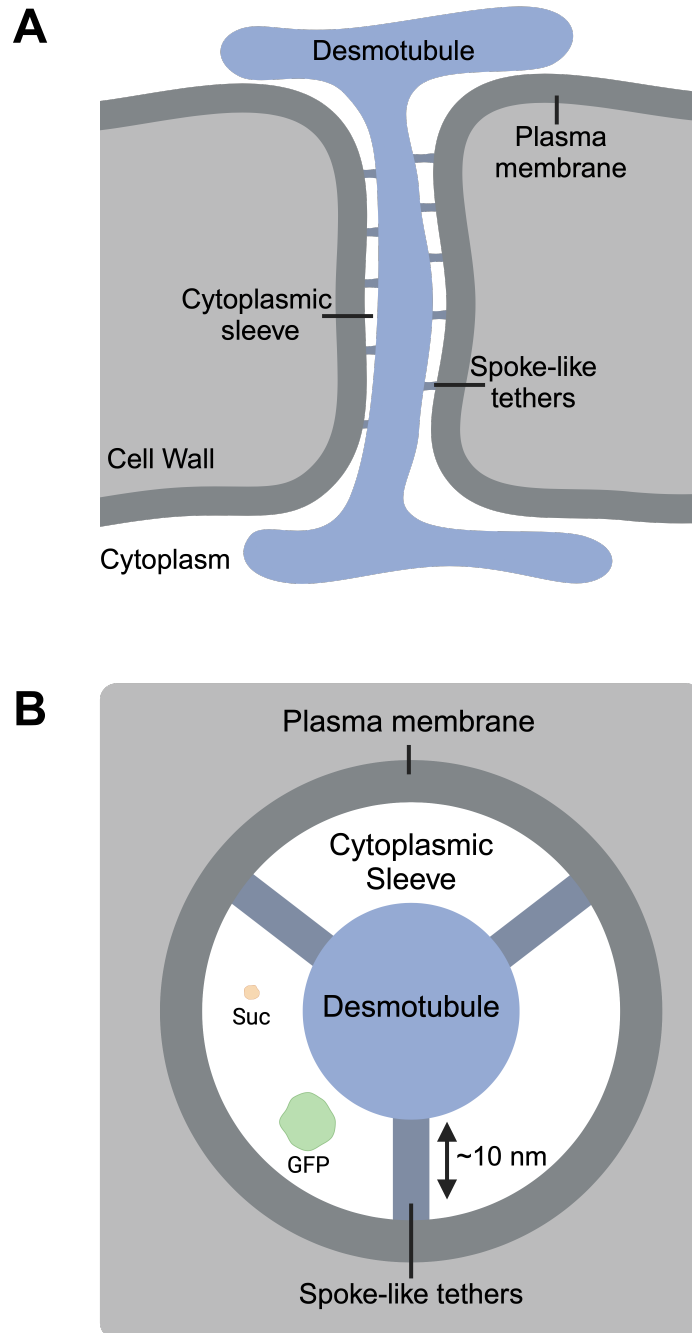


Figure 1.1: **Plasmodesmal ultrastructure** (A) Labelled plasmodesmal ultrastructure. (B) Cross-section of plasmodesmata showing the relative sizes of Green Fluorescent Protein (GFP) and Sucrose (Suc) in the cytoplasmic sleeve approximately drawn to scale. Redrawn with permission from Tee and Faulkner, 2024, created using BioRender.com

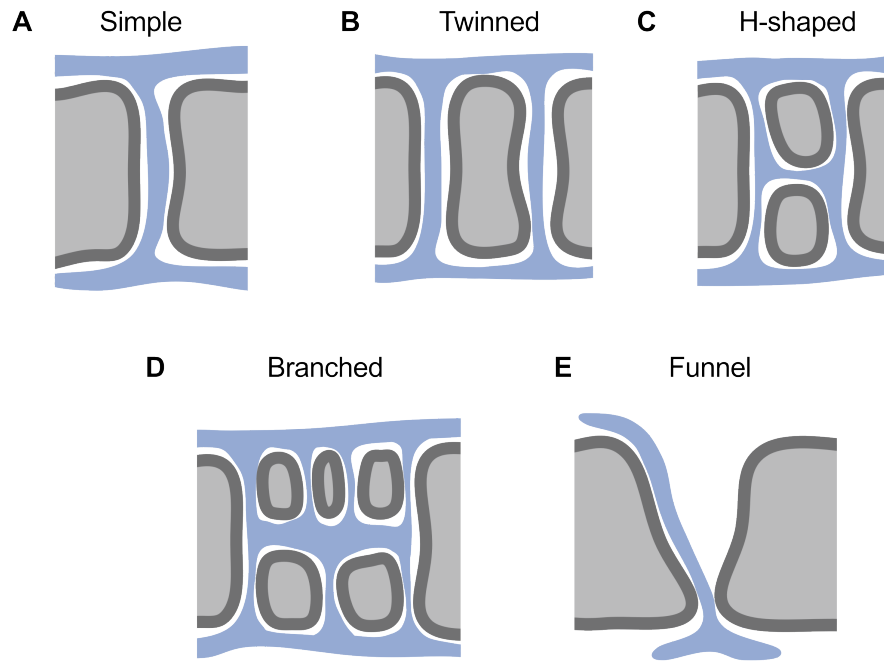


Figure 1.2: **Diversity of plasmodesmal structures** – (A) Simple plasmodesma, (B) Twinned plasmodesmata, (C) H-shaped plasmodesma, (D) Branched plasmodesma, (E) Funnel plasmodesma. Created using BioRender.com

1.2.2 Diversity of plasmodesmal structures

A diverse array of plasmodesmal structures can be found across different cell types and developmental stages. Simple single-channelled plasmodesmata (Figure 1.2 A) are predominantly found in developing tissues, such as embryos, roots, young leaves and shoot meristems (Burch-Smith & Zambryski, 2010; Nicolas et al., 2017; Oparka et al., 1999; Ormenese et al., 2000). Branched multi-channelled plasmodesmata (Figure 1.2 D), with several openings at the cell wall and a central cavity are mainly found in mature tissues (Brecknock et al., 2011; Faulkner et al., 2008; Oparka et al., 1999; Roberts et al., 2001; Wegner & Ehlers, 2024). Other plasmodesmal structures can be found in specialised cell types; for instance, in a diverse array of angiosperms, root phloem parenchyma cells have funnel-shaped plasmodesmata (Figure 1.2 E) (Ostermeyer et al., 2022; Ross-Elliott et al., 2017). The structure of plasmodesmata can vary with a cell at interfaces with different cell types. For instance, in protophloem sieve elements funnel-shaped plasmodesmata are found at the cell wall interface with phloem pole parenchyma cells, whilst simple plasmodesmata are found at the interface with metaphloem sieve elements (Ross-Elliott et al., 2017).

1.2. Formation and structure of plasmodesmata

This diversity of plasmodesmal structure between different cell types suggests structure is related closely to plasmodesmal function. For instance, simple plasmodesmata in developing tissue have increased trafficking capacity relative to branched plasmodesmata, meaning larger molecules can move through simple plasmodesmata (Kobayashi et al., 2005; Nicolas et al., 2017; Oparka et al., 1999). This greater trafficking through simple plasmodesmata in sink tissues could be important to growth and development in developing tissues (Oparka et al., 1999). Other plasmodesmal structures have been shown to maximise transport capacity. The funnel shape of plasmodesmata in root phloem parenchyma cells reduces hydraulic resistance, with the wide opening of the funnel into the sieve element cells and narrowing on the companion cell side (Esau & Thorsch, 1985; Ostermeyer et al., 2022). This asymmetric structure enables uni-directional phloem-unloading into the phloem parenchyma cells from the protophloem sieve elements (Ostermeyer et al., 2022). Similarly, asymmetric structures have been observed in mature leaves between sieve elements and companion cells. Branched plasmodesmata have more openings into companion cells than into the phloem sieve element lumen, which could facilitate one-directional phloem loading (Oparka & Turgeon, 1999). The structure of plasmodesmata is thus closely linked to plasmodesmal function by determining the direction and volume of molecular trafficking between cells.

1.2.3 Size limits on molecular movement through plasmodesmata

Electron micrographs suggest the cytoplasmic sleeve between the plasma membrane and desmotubule is between 10 to 50 nm wide, whilst the desmotubule is between 5 to 16 nm wide (Ehlers & Kollmann, 2001; Grison et al., 2015). The dimensions of the cytoplasmic sleeve were originally considered to determine passage through plasmodesmata, with the term “size exclusion limit” defining the upper size limit of molecules which can pass through a plasmodesma (Goodwin, 1983). Studies injecting dyes of different sizes initially placed this size exclusion limit at less than 1 kD (Goodwin, 1983). Since then, further studies have shown larger proteins can travel through plasmodesmata, with a 54 kD GREEN FLUORESCENT PROTEIN (GFP) moving symplasmically in *Nicotiana tabacum* (Figure 1.2 B) (Crawford & Zambryski, 2001). Additionally, larger messenger RNA (mRNA) molecules such as *AtLEAFY* mRNA (435 kD) and *zCas9* mRNA (1415 kD) have been suggested to move via plasmodesmata (Sessions et al., 2000; Wu et al., 2003; Yang et al., 2023). These molecules are much larger than the calculated dimensions of

plasmodesmata. This raises questions about how such large molecules could pass through plasmodesmata within our current understanding of size exclusion limits (Peters et al., 2021; Tee & Faulkner, 2024).

Further uncertainty around the size exclusion limit comes from questions about how molecules move through plasmodesmata with no cytoplasmic sleeves. Nicolas et al. (2017) identified plasmodesmata with no apparent cytoplasmic sleeves in young root cells due to very close apposition between the desmotubule and plasma membrane. These plasmodesmata, which lack visible cytoplasmic sleeves, are termed Type I, whilst plasmodesmata with cytoplasmic sleeves are termed Type II. Despite the absence of a cytoplasmic sleeve, GFP still can diffuse through Type I plasmodesmata. Liesche et al. (2019) placed further doubt on the relationship between structure and molecular traffic by finding structure did not correlate with trafficking capacity, indicating electron micrographs and the physical appearance of plasmodesmata are not always useful in determining plasmodesmal transport

Other factors beyond cytoplasmic sleeve size also need to be considered in the control of plasmodesmal conductivity. For instance, the electrical charge of a molecule could determine movement. Howell et al. (2024) present evidence suggesting electrical charge of a molecule controls plasmodesma conductivity by tracking the movement of fluorescent dyes through stamen hairs of *Tradescantia*. Howell et al. (2024) suggest tethering proteins between the desmotubule and the plasma membrane could lead to charge selective movement and limit the movement of positively charged molecules through the cytoplasmic sleeve. This leads to further questions about the role of tethering proteins in controlling cell-to-cell flux and raises questions about selectivity in the movement of molecules from cell to cell.

Additionally, plasmodesmal density can impact molecular trafficking. For instance, plasmodesmal density is important in species which carry out C_4 photosynthesis. C_4 photosynthesis involves the concentration of CO_2 in bundle sheath cells to reduce photorespiration (reviewed in Schlüter and Weber (2020)). In several C_4 species, CO_2 concentration relies on plasmodesmal transport of the carbon compounds malate and aspartate between mesophyll and bundle sheath cells. C_4 species have much higher densities of plasmodesmata between bundle sheath and mesophyll cells than C_3 species which facilitates malate and aspartate transport (Danila et al., 2016; Schreier et al., 2024). In the C_4 dicot *Gynandropsis gynandra*, exposure to higher light intensity induces a higher photosynthetic rate which correlates with an increase in plasmodesmal density, illustrating how plasmodesmal

1.2. Formation and structure of plasmodesmata

density can link to increased requirements for cell to cell trafficking (Danila et al., 2016; Schreier et al., 2024).

Plasmodesmal frequency also correlates with changes to transport in other developmental contexts. Across the sink-to-source transition plasmodesmal density decreases which correlates with a reduction in transport capacity (Roberts et al., 2001). In the root tip, transverse cell walls have a higher density of plasmodesmata than longitudinal cell walls (Zhu et al., 1998). Later experiments on cell-to-cell mobility in roots showed transverse cell layers with a higher density of plasmodesmata have higher cell-to-cell mobility (Gerlitz et al., 2018). Despite these examples showing increased plasmodesmal density correlates with increased molecular trafficking, a recent study by Liesche et al. (2019) suggested this is not always true. In studying phloem loading through plasmodesmata across different tree species, Liesche et al. (2019) found through quantifying plasmodesmata density using electron micrographs that density could not always predict levels of plasmodesmal permeability.

Although we can track movement through plasmodesmata using light microscopy and measure the size, structure and density of plasmodesmata using electron microscopy, many questions remain about the relationship between size, density and movement. Although mathematical modelling has helped to model the relationship between structure and movement i.e. Ostermeyer et al. (2022), current technological limitations limit our ability to simultaneously measure both size and structure and movement through plasmodesmata. Future development of nanoscale techniques to live image plasmodesmata could enable us to unlock a better understanding of how size, shape and structure determine molecular flux.

1.2.4 Plasmodesmal formation

The formation of plasmodesmata occurs both during and after cytokinesis. Ehlers and Kollmann (2001) categorise plasmodesmata as either primary or secondary depending on the developmental stage at which they are formed. Primary plasmodesmata form during Golgi-mediated formation of the cell wall in cytokinesis when the endoplasmic reticulum becomes trapped in the cell plate (Hepler, 1982). The endoplasmic reticulum-associated tethers MCTP 3, 4 and 6 are necessary to stabilise the endoplasmic reticulum during plasmodesma formation (Li et al., 2023). MCTPs shape the endoplasmic reticulum and prevent the complete abscission of the cell wall during cytokinesis, and as a result, the formation of plasmodes-

mal cytoplasmic bridges can occur during cytokinesis. These primary structures are initially simple single-channelled plasmodesmata. Secondary plasmodesmata form post-cytokinesis, and once formed, there is no way of distinguishing primary plasmodesmata from secondary plasmodesmata (Seagull, 1983).

The mechanism of secondary plasmodesmata formation remains unresolved. During cell wall expansion, there is an observed increase in the frequency of twinned plasmodesmata, defined as plasmodesmata less than 100nm apart, as well as an increase in the frequency of larger aggregates of plasmodesmata, termed pit fields (Barnett, 1987; Faulkner et al., 2008; Seagull, 1983; Wegner & Ehlers, 2024). In cell walls which have not undergone expansion, the distribution of plasmodesmata is less clustered and more regular, indicating numbers of plasmodesmata increase through the formation of new plasmodesmata adjacent to an existing plasmodesma during cell wall expansion (Barnett, 1987; Seagull, 1983).

One mechanism to explain secondary plasmodesmata formation during cell wall expansion has been suggested by Faulkner et al. (2008). Faulkner et al. (2008) propose the expansion of the cell wall surrounding a primary plasmodesma causes its cytoplasmic channel to widen. This widening enables the “active” expansion of the endoplasmic reticulum through the channel to form a second desmotubule (Faulkner et al., 2008). Subsequent cell wall growth between the split desmotubule divides the original channel in two, resulting in the formation of a secondary adjacent plasmodesma (Faulkner et al., 2008). This can explain the clustered appearance of plasmodesmata in pit fields after cell wall expansion.

Alternatively, Ehlers and van Bel (2009) propose a model of secondary plasmodesmata formation with “passive” endoplasmic reticulum. First deposition of new cell wall by Golgi vesicles causes plasmodesmata to form an ‘H’ shape structure (Figure 1.2 C), and then the plasmodesma separates into two adjacent single-channels (Ehlers & van Bel, 2009). Both the Ehlers and van Bel (2009) model and the Faulkner et al. (2008) model can explain the appearance of twinned plasmodesmata (Figure 1.2 B) in the cell wall and the aggregation of plasmodesmata into pit fields; however, without time course tracking of individual plasmodesma the exact mechanism of secondary plasmodesmata formation remain unresolved.

Once primary and secondary plasmodesmata have formed, further questions remain about how plasmodesmal structures change to form more complex structures such as branched or funnel plasmodesmata. Faulkner et al. (2008) suggest branched plasmodesmata form during twinned plasmodesmata formation through

1.2. Formation and structure of plasmodesmata

the presence of a central cavity connecting the neighbouring channels. Others suggest branched plasmodesmata form through the fusion of existing twinned plasmodesmata (Ehlers & Kollmann, 2001). Burch-Smith, Stonebloom, et al. (2011), however, argue against this “fusion” model, suggesting the contraction of the cell wall required to make space for plasmodesmal fusion is unlikely. Instead, Burch-Smith, Stonebloom, et al. (2011) suggest deposition of new cell material at plasmodesmata is key to plasmodesmata formation, with a “H” shaped structure being an intermediate to the formation of more complex plasmodesmata.

The signalling pathway controlling secondary plasmodesmata formation is not fully understood, but cytokinins are suggested to be involved. Cytokinins are hormones important in a wide range of processes related to growth and development. Exogenous application of cytokinins increases the frequency of secondary plasmodesmata in the shoot apical meristem of *Sinapis alba* (Ormenese et al., 2006). This cytokin-induced increase in the frequency of plasmodesmata may be linked to increased transport capacity through plasmodesmata. In *Nicotiana benthamiana* leaves, exogenous application of cytokinins increased plasmodesmal transport capacity, whilst silencing of cytokinin receptors reduced transport (Horner & Brunkard, 2021). Many questions remain regarding the importance of cytokinins in secondary plasmodesmal formation and the mechanisms by which cytokinins can indirectly control transport capacity through plasmodesmata.

1.2.5 Dynamic formation of plasmodesmata

Although no clear mechanism has been elucidated for the mechanism of secondary plasmodesmal formation, this process must be dynamically regulated in response to environmental cues. For instance, the frequency of branched plasmodesmata increases within an hour of leaf detachment as the sink leaf begins to transition to source (Fitzgibbon et al., 2013). Additionally, in C_4 dicots and monocots increased light intensity induces an increase in the numbers of plasmodesmata between mesophyll and bundle sheath cells within 24 hours (Schreier et al., 2024). The signalling pathway for the rapid formation of plasmodesmata in response to light and other environmental cues is unclear. Inhibited photosynthesis limits plasmodesmal formation whilst the application of exogenous sucrose is sufficient to restore plasmodesmal frequency in plants with inhibited photosynthesis (Schreier et al., 2024). This indicates sucrose or other photosynthates could act as a signal for plasmodesmal formation (Schreier et al., 2024). Other research has suggested

chloroplast-nucleus-plasmodesmata signalling plays a role in plasmodesmal formation, where the chloroplast redox state regulates plasmodesmata formation via retrograde signalling (Burch-Smith, Brunkard, et al., 2011; Ganusova et al., 2020).

Overall, the structure and frequency of plasmodesmata change dynamically in response to environmental and developmental cues. This indicates plasmodesmata are not simply fixed structures once cell expansion has finished. Instead, they are highly dynamic and responsive. Further questions remain as to what the signalling mechanism connecting these dynamic changes to external and internal cues. Additionally, it is unclear how dynamic changes in plasmodesmal frequency and structure impact molecular trafficking through plasmodesmata.

1.2.6 Callose-mediated plasmodesmal regulation

In addition to changes in plasmodesmal structure and frequency, molecular flux through plasmodesmata can be controlled through the deposition of the β -1,3-glucan callose in the cell wall. The deposition of the callose in the neck regions of plasmodesmata in the apoplast constricts the cytoplasmic sleeve, restricting movement through plasmodesmata (Figure 1.3)(Zavaliev et al., 2011). It is unclear how this model for callose restricting cell-to-cell mobility by constricting the cytoplasmic sleeve relates to the finding that Type I plasmodesmata in roots with very narrow cytoplasmic sleeves can facilitate cell-to-cell movement (Nicolas et al., 2017). Despite the uncertainty in how cell-to-cell movement is restricted by callose, it is a well-established mechanism to control plasmodesmal permeability.

CALLOSE SYNTHASES (CALSs) synthesise callose in the apoplast. CALSs use UDP-glucose as a substrate to build a callose polymer of β -1,3-glucose residues with few β -1,6-branches (Verma & Hong, 2001). In total there are twelve identified callose synthase genes in *Arabidopsis* with varying roles in controlling plasmodesmal permeability during stress signalling, floral development, and pollination, and in response to various developmental cues (reviewed in Chen and Kim (2009)).

Additionally, callose degradation by plasmodesmal localised β -1,3-glucases is important to the regulation of plasmodesmal permeability. β -1,3-glucases degrade callose to increase plasmodesmal permeability and also re-open plasmodesmata after callose-induced closure (Benitez-Alfonso et al., 2013; Levy et al., 2007; Zavaliev et al., 2013). The coordination of callose synthases and β -1,3-glucanases is thus important to the regulation of plasmodesmal permeability.

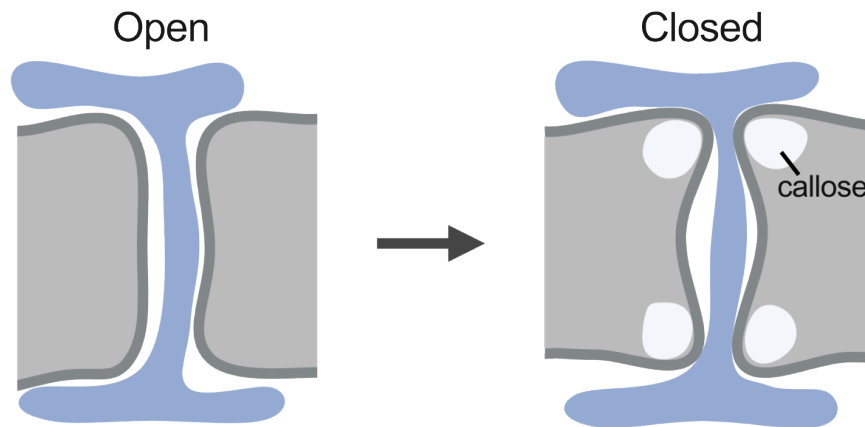


Figure 1.3: **Callose-induced plasmodesmal closure** – Plasmodesmal closure can be induced via callose deposition in the neck regions of plasmodesmata to restrict cell-to-cell movement. This can be triggered by various developmental and environmental cues. Created using BioRender.com

1.3 Regulation of plasmodesmata in defence

Regulation of cell-to-cell connectivity is important to the immune responses to pathogens and other potential threats in plants. The symplasmic network created by plasmodesmata is a potential weakness to plants, as pathogens can hijack this connectivity to facilitate their spread through the plant. As a result, plasmodesmata are a battleground in plant-pathogen interactions and form part of wider defensive responses.

1.3.1 Pattern-triggered immunity

Pattern-triggered immunity is one of the first lines of defence against pathogens. This immune response is induced upon the detection of pathogens through the recognition of conserved molecular signatures, called pathogen-associated molecular patterns (PAMPs), from different classes of pathogens (Dodds & Rathjen, 2010). Specific pattern recognition receptors (PRRs) at the plant cell surface recognise PAMPs to trigger immune signalling to fight pathogen infection in pattern-triggered immunity (Figure 1.4) (Jones & Dangl, 2006) .

Examples of PAMPs which trigger pattern-triggered immunity can be found

within different groups of pathogens. Recognition of fungal pathogens by PRRs can occur through the detection of chitin, a 1,4- β -linked polymer of *N-acetyl*-d-glucosamine, which is a highly conserved component of fungal cell walls. During infection, chitin is released as a by-product of fungal chitinase activity during cell wall growth or released by chitinases secreted by the plant (Langner & Göhre, 2016). Several PRRs and their co-receptors detect chitin to activate downstream immune signalling (Ngou et al., 2022). CHITIN ELICITOR RECEPTOR KINASE 1 (CERK1) is an essential co-receptor that forms a complex with other PRRs to mediate in general pattern-triggered immunity in response to chitin (Cao et al., 2014; Miya et al., 2007; Wan et al., 2008). Other PRRs such as LYSIN MOTIF DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYL-INOSITOL-ANCHORED PROTEIN 2 (LYM2) trigger more specific immune responses independently of CERK1. Perception of chitin by LYM2 leads to a reduction in molecular trafficking through plasmodesmata, which enhances defence against pathogens (Cheval et al., 2020; Faulkner et al., 2013) (discussed further in section 1.3.5 below).

Bacterial pathogens also trigger pattern-triggered immunity in plants through the detection of bacterial molecular signatures by a different set of PRRs to those involved in fungal PAMP detection. For instance, FLAGELLIN-SENSING2 (FLS2) is a well-characterised PRR which perceives bacterial flagellin (Gómez-Gómez & Boller, 2000). More specifically, FLS2 binds to the conserved 22 amino acid N-terminus domain of the flagellin protein, flg22 (Chinchilla et al., 2006; Felix et al., 1999; Gómez-Gómez & Boller, 2000). The binding of FLS2 and its co-receptor BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) to flg22 triggers an immune signalling response (Robatzek et al., 2006; Sun et al., 2013). FLS2 is the only known flagellin receptor, making it the canonical receptor with all flagellin-triggered responses induced through FLS2 binding. FLS2 is also required for flg22-triggered plasmodesmal closure (Xu et al., 2017). FLS2 activates the plasmodesmata-specific CALMODULIN-LIKE PROTEIN 41 (CML41) to trigger plasmodesmal closure through a pathway independent of other FLS2 triggered immune responses (Xu et al., 2017).

A wide range of downstream signalling and physiological defence responses are triggered upon PAMP recognition by PRRs. For instance, PRRs can trigger downstream phosphorylation of NADPH oxidases and RESPIRATORY BURST OXIDASE HOMOLOGUES (RBOHs) to trigger rapid apoplastic bursts of reactive oxygen species (ROS) (Kadota et al., 2014; Torres et al., 2002). ROS accumulation in the apoplast induces cell wall cross-linking to strengthen the cell wall against

1.3. Regulation of plasmodesmata in defence

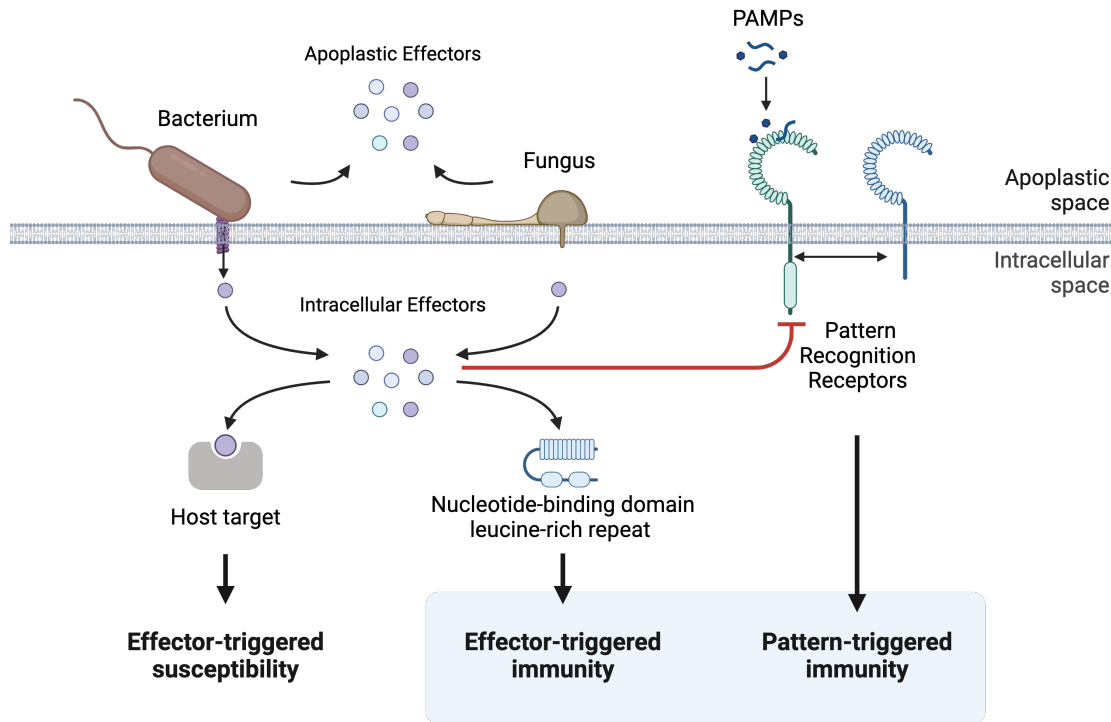


Figure 1.4: **Pattern-Triggered Immunity and Effector-Triggered Immunity** - Pattern recognition receptors (PRRs) bind to pathogen-associated molecular patterns (PAMPs) in the apoplastic space. This induces pattern-triggered immunity. Pathogens release effectors which target host processes to induce effector-triggered susceptibility. Plant nucleotide-binding domain leucine-rich repeat (NLRs) recognise effectors or effector activity to induce effector-triggered immunity. Redrawn with permission from Mukhi et al. (2020) and created using a template from BioRender.com

invading pathogens (Bradley et al., 1992; Voigt, 2014). ROS also acts as a signalling molecule to trigger further downstream immune responses such as stomatal closure to prevent further entry of microbial pathogens (Wang & Gou, 2021), plasmodesmal closure to trigger symplasmic isolation (Tee et al., 2023), and in some cases, the induction of hypersensitive response, resulting in cell death to limit the spread of pathogens (Yin et al., 2022).

PAMP recognition also triggers changes to gene expression. One of the most comprehensive collections of transcriptional responses to PAMPs was collected by Bjornson et al. (2021) in *Arabidopsis* seedlings. Bjornson et al. (2021) compared transcriptional responses across multiple PAMPs, finding different PAMP treatments trigger the up- or down-regulation of a shared set of core immune response genes. Many of these core immune response genes are also differentially expressed in response to abiotic stress responses. This indicates PAMP recognition triggers

the differential expression of general stress response genes as part of a defensive response.

Overall, the binding of PRRs to PAMPs is key to triggering the first line of defence upon detection of the presence of pathogens. PAMP-triggered plasmodesmal closure forms part of this defence.

1.3.2 Effector-triggered susceptibility and immunity

Pathogens have an arsenal of tools to limit pattern-triggered immune responses in plants. Pathogens release effector molecules to suppress or avoid plant defence responses in effector-triggered susceptibility (Figure 1.4). These effectors can directly target PRR recognition of PAMPs by blocking PRR binding to PAMPs e.g. de Jonge et al. (2010) and Irieda et al. (2019). Effectors can also target other plant processes to promote infection. For instance, effectors target plant defence-related gene expression to reduce the transcriptional changes triggered by PAMP detection (Arbibe et al., 2007; Doehlemann et al., 2009). Additionally, effectors can target plasmodesmata to increase plasmodesmal permeability by binding to plasmodesmal localised proteins Aung et al. (2020) and Cao et al. (2018). Effectors can also increase nutrient availability for the pathogen through the upregulation of sugar transports to increase apoplastic sugar concentration to fuel pathogen growth and development (Chen et al., 2010; Yan et al., 2021).

Although pathogens have evolved effectors to bypass and suppress pattern-triggered immunity, plants have also evolved defensive mechanisms to detect effectors. Plant genomes encode large repertoires of NUCLEOTIDE-BINDING LEUCINE RICH REPEAT RECEPTORS (NLRs) to recognise effectors to trigger effector-triggered immunity (reviewed in Cui, Tsuda, and Parker (2015) and Ngou et al. (2022)). The evolution of immunity and infection machinery is frequently viewed as a complex arms race between plants and pathogens. These complex interactions, and their evolution, lead to a multi-layered network of interactions that include pattern-triggered immunity, effector-triggered susceptibility, and effector-triggered immunity resulting in several layers of defence in plants to facilitate resistance against pathogens.

1.3.3 The role of plasmodesmal closure during defence

The regulation of plasmodesmal permeability is important in plant-pathogen interactions. Plasmodesmal closure is triggered by the binding of PAMPs to PRRs, which induces a signalling cascade resulting in reduced plasmodesmal permeability (Cheval et al., 2020; Faulkner et al., 2013; Xu et al., 2017).

Disrupting plasmodesmal closure during infection has been shown to increase susceptibility in a range of different infection contexts. Impairing flg22-triggered plasmodesmal closure, for example, increases susceptibility to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Xu et al., 2017). Disrupting chitin-triggered plasmodesmal closure by mutating the LYM2 chitin-receptor increases susceptibility to the fungal pathogen *Botrytis cinerea* (Faulkner et al., 2013). Additionally, impairing the activity of PLASMODESMAL LOCALISED PROTEIN 5 (PDLP5), involved in the PAMP-triggered plasmodesmal closure signalling pathway, increases susceptibility *P. syringae* pv *maculicola* (*Pma*) (Lee et al., 2011). These examples illustrate how disrupting PAMP-triggered plasmodesmal closure can result in increased susceptibility to pathogens.

There are many hypotheses explaining why plasmodesmal closure is beneficial to plant defence. Cheval and Faulkner (2018) suggest that temporary symplasmic isolation caused by plasmodesmal closure could enhance defence by enabling the build-up of defensive molecules to reach a threshold required to trigger a signalling response. Recent evidence supports this, showing inducing plasmodesmal closure in the absence of pathogens still activates stress signalling and results in the accumulation of the defence hormone salicylic acid (Tee et al., 2024). Plants with induced plasmodesmal closure before infection also have enhanced resistance of plants with induced plasmodesmal closure to the bacterial pathogen *Pst* DC3000 (Tee et al., 2024).

In addition to enabling the build-up of defensive molecules, plasmodesmal closure may promote defence against pathogens by creating a barrier to the symplasmic spread of pathogens and/or their associated molecules. Viruses, for instance, target plasmodesmata to increase plasmodesmal trafficking and facilitate cell-to-cell movement of viral RNA (Reagan & Burch-Smith, 2020; van Lent et al., 1991; Wolf et al., 1989). The fungal pathogen *Colletotrichum higginsianum* also capitalises on symplasmic connectivity, releasing several hypermobile effector proteins which spread from cell-to-cell through plasmodesmata, potentially priming

cells for infection ahead of the infection front (Ohtsu et al., 2024). Similarly, the bacterial pathogen *Pst* DC3000 expresses at least 16 mobile effectors during infection which are cell-to-cell mobile in *Nicotiana benthamiana*, which could enhance infection (Li et al., 2021). Plasmodesmal closure thus could be beneficial in preventing pathogens from harnessing symplasmic connectivity during infection.

1.3.4 Callose-mediated plasmodesmal closure during immune responses

The best-understood mechanism for PAMP-induced plasmodesmal closure is through the deposition of the β -1,3-glucan callose in the cell wall around the plasmodesmata. As discussed in Section 1.2.6, Callose deposition dynamically modifies plasmodesmal permeability through modifications to plasmodesmal aperture in response to PAMPs. (Zavaliev et al., 2011). Callose deposition is induced within 30 minutes of flg22 treatment (Xu et al., 2017), and then returns to baseline levels several hours later (Ohtsu, unpublished).

Coordination of callose synthases and β -1,3-glucases is important to regulating dynamic callose deposition at plasmodesmata in pattern-triggered immunity. The callose synthase CALS1 is activated in immune signalling triggered by flg22 and chitin to increase callose deposition and induce plasmodesmal closure (Tee et al., 2023). Whilst β -1,3-glucases play a role in the re-opening of plasmodesmata after PAMP-triggered closure through the degradation of callose (Benitez-Alfonso et al., 2013; Levy et al., 2007).

Callose-independent mechanisms of plasmodesmal closure have also been recently discovered. The endoplasmic reticulum - plasma membrane tether proteins MCTPs are suggested to pull the desmotubule and plasma membrane in closer apposition to reduce movement through the cytoplasmic sleeve (Pérez-Sancho et al., 2023). Although the role of MCTPs in plasmodesmal closure has, so far, only been shown in roots in response to brassinosteroid hormone and salt stress, a similar mechanism might also contribute to plasmodesmal closure in an infection context (Pérez-Sancho et al., 2023).

1.3.5 Mechanisms of PAMP-triggered plasmodesmal closure

Callose synthase activity is activated by PAMP-induced immune signalling to trigger plasmodesmal closure. The PRR and cell surface receptor LYM2 mediates plasmodesmal closure in response to the fungal PAMP chitin (Figure 1.5)(Cheval et al., 2020; Faulkner et al., 2013). LYM2 forms a complex with LYSM-CONTAINING RECEPTOR-LIKE KINASE 4 (LYK4) at plasmodesmata that can bind to chitin. This triggers the phosphorylation of RBOHD, which feeds into a signalling node that contains a complex of PDL1, PDL5, and NON-RACE-SPECIFIC DISEASE RESISTANCE GENE/HAIRPIN INDUCED GENE1 (NDR1/HIN1)-like protein 3 (NHL3). The NHL3-PDL complex activates CALS1, resulting in plasmodesmal callose deposition (Figure 1.5 , (Tee et al., 2023)).

The LYM2-mediated chitin-triggered plasmodesmal closure occurs independently of the CERK1-mediated chitin responses (Faulkner et al., 2013). Chitin-triggered plasmodesmal closure occurs in *cerk1* mutants, indicating LYM2 triggers a separate pathway (Faulkner et al., 2013). This indicates separate immune signalling pathways are triggered by the same PAMP binding to different PRRs. Also, RBOHD is involved in both CERK1 and LYM2 chitin responses, but the ROS burst in the CERK1-mediated pathway and the plasmodesmal closure pathway activated by LYM2 via RBOHD involved independent phosphorylation signatures of RBOHD (Cheval et al., 2020).

The bacterial PAMP flg22 also triggers plasmodesmal closure in a signalling pathway which converges with chitin-triggered signalling. Plasmodesmal closure is triggered through binding of flg22 to the cell surface receptor FLS2 (Faulkner et al., 2013; Xu et al., 2017). Downstream of FLS2, the signalling cascade is mediated by plasmodesmal specific CML41 proteins and converges with the chitin-induced LYM2 signalling pathway via RBOHD to activate the NHL3-PDL signalling complex, resulting in CALS1 activation (Tee et al., 2023). Thus, the PAMPs flg22 and chitin both trigger plasmodesmal closure via CALS1 to increase callose deposition.

PAMP-triggered plasmodesmal closure also converges with the salicylic-induced plasmodesmal closure signalling pathway. Salicylic acid is a defence hormone involved in a range of stress responses including hypersensitive response and eliciting systemic acquired resistance (Ding & Ding, 2020). Salicylic acid was

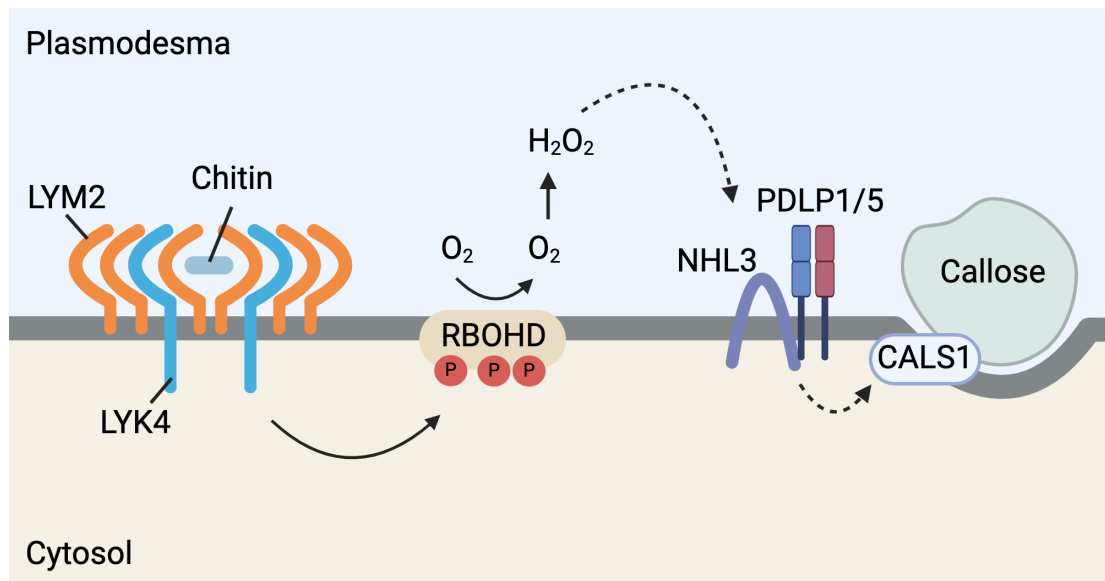


Figure 1.5: **Signalling pathway of chitin-triggered plasmodesmal closure** – Chitin binds to the LYM2-LYK4 receptor complex at plasmodesma triggering phosphorylation of RBOHD causing the production of ROS, signalling via NHL3-PDLP1/5 complex activates CALS1 resulting in callose deposition and plasmodesmal closure. Redrawn with permission from Tee and Faulkner (2024) and created with BioRender.com.

originally suggested to be a component of the PAMP-triggered plasmodesmal closure signalling pathway (Cui & Lee, 2016). Recent research, however, suggests salicylic acid does not form part of the flg22 and chitin pathway (Tee et al., 2023). Instead, salicylic acid signalling is suggested to converge with the chitin and flg22-signalling pathway downstream of RBOHD, with PDLP1, PDLP5 and CALS1 required for salicylic acid-triggered plasmodesmal closure (Tee et al., 2023; Wang et al., 2013). This indicates salicylic acid could induce plasmodesmal closure via the NHL3-PDLP complex. Thus, the PAMPs flg22 and chitin, and the phytohormone salicylic acid all trigger plasmodesmal closure via CalS1 activation increasing callose deposition.

Several pathogens target components of the PAMP-triggered plasmodesmal closure signalling pathway to facilitate infection. The pathogen *Phytophthora brassica* inhibits callose synthase activity to prevent callose deposition, whilst *Pst* interacts with plasmodesmal localised proteins (PDLPs) to increase plasmodesmal permeability (Aung et al., 2020; Tomczynska et al., 2020). Additionally, as mentioned in Section 1.3.1 above, loss of function of LYM2 and FLS2 receptors increases susceptibility to the fungal pathogen *Botrytis cinerea* and bacterial pathogen *Pst* DC3000 respectively (Faulkner et al., 2013). Thus PAMP-triggered plasmodesmal closure forms an important part of resistance against pathogens.

1.3.6 Changes to pathogen resistance across development

The regulation of plasmodesmal response in pattern-triggered immunity has mainly been studied in mature leaves, leading to questions about the induction of plasmodesmal closure across different stages of plant development. Although questions remain regarding changes to plasmodesmal response, studies have been carried out on general changes to pathogen resistance across development.

Plants can show varying levels of pathogen resistance across ages and developmental stages. These differences can be considered in terms of the changes in resistance of a whole plant across developmental stages, such as plants in the juvenile versus adult stage, or adult versus reproductive stage. Arabidopsis plants, for instance, have increased resistance to the bacterial pathogen *Pseudomonas syringae* pv. *Tomato* (*Pst*) upon the emergence of inflorescence shoots after the transition to flowering (Rusterucci et al., 2005). The increased resistance of more mature plants and tissues vs young plants and tissues is termed age-related resistance.

Within a plant, leaves at various developmental stages and ages can exhibit differences in resistance. This is termed leaf stage-related resistance. Whether young leaves are more or less resistant to pathogens than adult leaves depends on the plant species and pathosystem. In adult Arabidopsis plants, young leaves are more susceptible than adult leaves to the fungal pathogen *Sclerotinia sclerotiorum* and the bacterium *Pst* DC3000, but less susceptible than adult leaves to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Xu et al., 2018). Leaf stage-related resistance also occurs in other species. In potato and tomato plants, young leaves are more resistant than mature leaves to Early Blight (*Alternaria solani*) and Late Blight (*Phytophthora infestans*) respectively, whilst in grapevine young leaves are less resistant than mature leaves to downy mildew (*Plasmopara viticola*) (Dita Rodriguez et al., 2006; Nelson, 2006; Reuveni, 1998). These examples illustrate the variability between pathosystems and species in terms of resistance.

Leaf stage-related resistance may also vary within different environmental and developmental contexts. For instance, young onion leaves are less susceptible than mature leaves to Purple blotch (*Alternaria porri*) during bulb development; however, upon bulb maturation young leaves become more susceptible than mature leaves (Miller, 1983). Thus, leaf stage-related resistance can be affected by whole plant age. There is also weak evidence suggesting the strength of leaf stage-related

resistance depends on circadian rhythm, with differences in leaf stage-related resistance exhibited depending on the time of pathogen inoculation (Yamauchi et al., 2017). Further studies are needed to understand the relationship between circadian rhythms and leaf stage-related resistance.

The molecular mechanisms underpinning leaf stage- and age-related resistance are unclear and could be dependent on the pathogen. Where mature leaves/plants are less susceptible than younger leaves/plants, accumulation of phytohormones such as salicylic acid and jasmonic acid appears to play an important role in mediating resistance (Kus et al., 2002; McDowell et al., 2005; Xu et al., 2018). Differences in resistance could also be due to differential expression of defence-related genes in young and mature leaves, such as genes involved in ROS, cell wall strengthening, and defence signalling (Hu et al., 2023; Hugot et al., 2004; Xu et al., 2018). Additionally, changes in transcription factor activity might mediate changes to resistance. Recent work has revealed the association of regulators of development and stress to changes in pathogen resistance across developmental stages (Hu et al., 2023; Maeda et al., 2023). This includes changes in the activity of HOMEODOMAIN-LEUCINE ZIPPER SUBFAMILY I (HDZip) proteins, and the interactions of the microRNA156s family with SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors which could mediate changes in resistance (Hu et al., 2023; Maeda et al., 2023). These mechanistic studies have begun to reveal the complex interactions underpinning age and leaf stage-related pathogen resistance. Despite this progress, it remains unclear why in some pathosystems young leaves are more susceptible and in others less susceptible. It could be that infection mechanisms as well as the strength of basal resistance against a pathogen play a role. Additionally, given we know plasmodesmata play a role in defence - are there changes in plasmodesmal regulation across development that could relate to changes in resistance against pathogens?

1.4 Role of plasmodesmata in growth and development

In addition to a role in defence, plasmodesmata also play a role in growth and development. Symplasmic connectivity is important to the intercellular movement of sugars, proteins, nucleic acids and hormones at several stages of development. Equally, symplasmic isolation of individual cells or domains of cells is observed

1.4. Role of plasmodesmata in growth and development

across different developmental contexts to facilitate processes such as cellular differentiation and patterning. Structure and regulation underpin the varying functions of plasmodesmata across plant growth and development.

1.4.1 Symplasmic connectivity in phloem loading

Plasmodesmata play a role in the movement of sugars from the site of production in mature photosynthetic “source” tissue through the phloem to “sink” tissue such as young leaves and roots. This export of carbon from source tissue is important to fuelling growth and development in sink tissue. In *Arabidopsis*, for instance, carbon is transported to sink tissues overnight, at the end of the night, leaves have around 10% of overall carbon gained during the day (Usadel et al., 2008).

The main mechanism for long-distance sugar transport in the plant is through the phloem (Figure 1.6). Phloem tissue in *Arabidopsis* and other angiosperms consists of three main cell types: sieve elements, companion cells, and phloem parenchyma cells. Phloem parenchyma cells surround the sieve element and companion cells. Sieve elements are enucleated cells connected by heavily pored sieve plates to form structures called sieve tubes. Sieve tubes enable the long-distance movement of molecules across the plant. Sieve elements are connected via plasmodesmata to adjacent companion cells, which support sieve element function (van Bel & Knoblauch, 2000). Blocking plasmodesmal connections between sieve elements and companion cells prevents the export of sucrose through the phloem, highlighting the importance of symplasmic connectivity between these two cell types (Vilaine et al., 2013).

The phloem facilitates the long-range transport of RNA, proteins and sugars in phloem sap (Atkins et al., 2011; Kehr, 2006). In most plant species, sugars are predominantly transported in phloem sap in the form of sucrose. The Münch hypothesis suggests phloem sap moves by bulk flow driven by differences in hydrostatic pressures between sink and source tissues (Knoblauch et al., 2016; Münch, 1930). During phloem loading, sugars build up in the sieve elements creating osmotic pressure which drives the uptake of water and raises hydrostatic pressure. The unloading of sugars from the phloem creates osmotic withdrawal, with relatively low hydrostatic pressure forming in the sieve elements. The hydrostatic pressure difference between unloading and loading tissue is then suggested to drive the bulk flow of phloem sap into sink tissue (Knoblauch & Peters, 2010; Knoblauch et al., 2016; Münch, 1930).

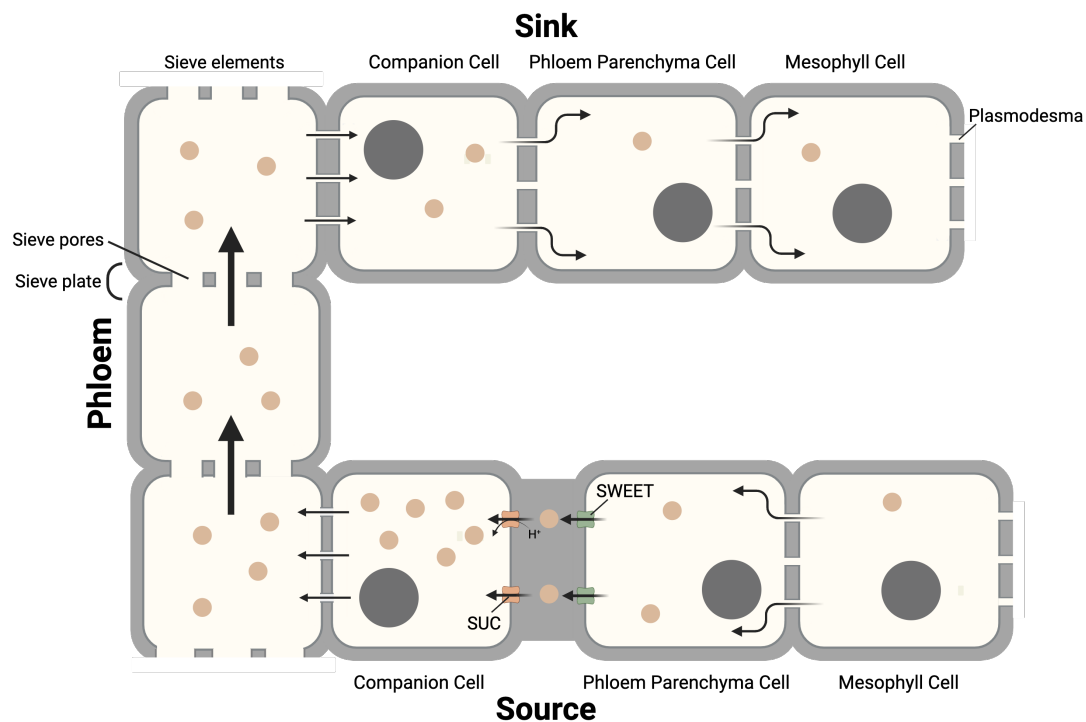


Figure 1.6: **Phloem loading and unloading in Arabidopsis** - Sucrose (indicated by orange circles) is synthesised in source tissue and transported symplasmically to phloem parenchyma cells. SUCROSE WILL EVENTUALLY BE TRANSPORTED (SWEETs) load sucrose into the apoplastic space from phloem parenchyma cells. The proton-sucrose symporters SUCROSE UPTAKE CARRIERS (SUCs) then import sucrose into companion cells. Sucrose is then loaded into the phloem through plasmodesmata. The phloem consists of sieve elements connected by sieve plates containing sieve pores to allow the passage of sucrose and other phloem-mobile molecules. In sink tissues, sugars are unloaded from the phloem symplasmically through plasmodesmata.

1.4. Role of plasmodesmata in growth and development

The pathway of sugar movement into the phloem can be split into two parts: pre-phloem loading and phloem loading. In pre-phloem loading, sugars diffuse through plasmodesmata from photosynthetic cells, e.g. mesophyll cells, down concentration gradients to phloem parenchyma cells (Haritatos et al., 2000; Schulz, 2015). The regular spacing of minor veins in *Arabidopsis* means mesophyll cells are generally between three to five cells away from the phloem parenchyma cells (Haritatos et al., 2000; Schulz, 2015). This results in a relatively short path from photoassimilate production to the phloem tissue. Additionally, tracking cell-to-cell movement in *N. tabacum* source leaves suggests there are high relative rates of molecular trafficking between mesophyll cells and bundle sheath cells, supporting the model that the pre-phloem loading pathway of sugar movement occurs symplasmically (Liesche & Schulz, 2012).

Once in the phloem parenchyma cells, sugars can be loaded into the phloem actively or passively via the apoplast or symplasm depending on the species, environment, and/or developmental context. *Arabidopsis* and most other herbaceous plants predominantly use active apoplastic phloem loading to build up sucrose concentrations in companion cells and sieve elements (Miras et al., 2022). In apoplastic loading, sucrose is passively exported into the apoplast from phloem parenchyma cells by the Sugars Will Eventually Be Exported Transporters (SWEET) family of transporters (Chen et al., 2012). Sucrose Uptake Carriers (SUCs) then actively uptake sucrose from the apoplast into companion cells via proton-sucrose symport (Boorer et al., 1996; Carpaneto et al., 2005). This builds up sucrose concentrations in the companion cell-sieve element complex. In apoplastic phloem loaders, there are reduced numbers of plasmodesmal connections between companion cells and phloem parenchyma cells, but some symplasmic movement still occurs, potentially enabling the symplasmic movement of other molecules into companion cells (Liesche & Schulz, 2012; van Bel et al., 1992).

In addition to apoplastic loading strategies, the phloem can also be loaded via the symplasm. The polymer trap mechanism, for instance, is suggested to be a method for active symplasmic loading. Sucrose diffuses down the concentration gradient into specialised “intermediate” cells, which could be considered specialised phloem parenchyma cells (Turgeon & Gowan, 1990). In these intermediate cells, sucrose (342 Da) is converted into larger raffinose oligosaccharides (594 Da) (Turgeon & Gowan, 1990). It is suggested that the larger size of raffinose means it is unable to diffuse back through plasmodesmata due to the size exclusion limit, raising questions about how plasmodesmata can be selective based on small size

differences between sucrose and raffinose (Peters et al., 2021, Botha and Murugan, 2021).

In addition to active symplasmic loading, passive symplasmic phloem loading mechanisms have also been characterised. First observed in willow (*Salix babylonica*) trees by Turgeon and Medville (1998), passive loading occurs due to high sugar concentrations in mesophyll cells that diffuse passively into the companion cell-sieve element complex during phloem loading (Rennie & Turgeon, 2009). Many tree species exhibit passive symplasmic loading whilst most herbaceous species exhibit active loading. This could be explained by differences in whole-plant hydraulic conductance, which measures the ease of water transport from soil to shoot (Fu et al., 2011). Trees have low hydraulic conductance due to their height (Fu et al., 2011). To maintain turgor pressure, trees produce high solute concentrations including high sugar contents. As a result of these high sugar contents, passive symplasmic phloem loading can occur in many tree species. This passive symplasmic loading mechanism is also seen in some herbaceous species with equally low hydraulic conductance and so these species also accumulate high sugar contents (Fu et al., 2011).

Overall plasmodesmata are important to facilitating and controlling symplasmic movement in pre-phloem loading and in both symplasmic and apoplastic phloem unloading. Questions remain about how the induction of plasmodesmal closure affects pre-phloem loading which usually occurs symplasmically. It is unknown whether an apoplastic pathway can be activated to ensure the pre-phloem transport of sugars to phloem parenchyma cells is maintained upon the induction of plasmodesmal closure in response to an environmental or development cue.

1.4.2 Symplasmic connectivity in phloem unloading

Phloem unloading into sink tissues can occur via the symplasm or apoplast. symplasmic unloading has been observed across sink tissues including roots and leaves in multiple species (Imlau et al., 1999; Oparka et al., 1994; Roberts et al., 1997; Wright & Oparka, 1996). Plasmodesmata are key to symplasmic unloading, forming connections both within phloem tissue and between the phloem and surrounding sink tissue cells.

Studies understanding the mechanisms of phloem unloading have mainly focused on roots. Unloading in roots occurs through the protophloem, which connects the

1.4. Role of plasmodesmata in growth and development

main conductive phloem to sink tissue (Oparka et al., 1994). Smaller molecules such as sucrose and GFP are unloaded into surrounding sink tissues through a convection flow system, involving by mass flow and diffusion (Ross-Elliott et al., 2017). Larger molecules such as nucleic acids and proteins greater than 100 kD are suggested to be unloaded in pulses, termed batch unloading, into a specific post-phloem domain (Ross-Elliott et al., 2017; Stadler et al., 2005).

Symplasmic unloading in sink tissues is facilitated by plasmodesmata with specific structures as discussed in section 1.2.2 above. The funnel-shaped plasmodesmata between the protophloem parenchyma cells and protophloem sieve elements minimise hydraulic drag and increase phloem unloading into root protophloem parenchyma cells, (Ostermeyer et al., 2022; Ross-Elliott et al., 2017). Intercellular trafficking is also increased in surrounding tissues as sink leaves have predominantly simple plasmodesmata (Oparka et al., 1999; Roberts et al., 2001). Simple plasmodesmata are thought to have increased transport capacity relative to the branched plasmodesmata found in source tissue (Oparka et al., 1999; Roberts et al., 2001). Plasmodesma structure thus may be important to maximising the efficiency of phloem unloading into sink tissues.

In addition to symplasmic unloading of sugars, apoplastic unloading of sugars is found in several developmental contexts. Apoplastic unloading has been observed into symplasmically isolated sink cells in fruits such as grape (Zhang et al., 2006), apple (Zhang et al., 2004), and walnut (Wu et al., 2004). Arabidopsis embryo development also involves apoplastic sugar unloading where SWEET sucrose transporters are upregulated to facilitate the transfer of stored sugars in the seed coat to the embryo (Chen et al., 2015). The switch between symplasmic and apoplastic unloading mechanisms occurs dynamically in certain cell types. Potato plants, for instance, switch from apoplastic to symplasmic unloading during tuberisation, which is linked to the increased sink strength in tubers being able to drive symplasmic unloading (Viola et al., 2001). This shift between mechanisms has also been associated with changes to plasmodesmal permeability. In grapes, for example, the shift to apoplastic transport is accompanied by a potential blocking of symplasmic plasmodesmal connections (Zhang et al., 2006). The dynamic changes between apoplastic and symplasmic loading allow plants to optimise unloading in different developmental contexts, especially where sink tissues are symplasmically isolated.

1.4.3 Symplasmic connectivity in other contexts

In addition to facilitating the movement of sugars from sink to source, symplasmic movement is important to defining tissue patterning and cellular differentiation through the intercellular movement of mobile signals. Several transcription factors act non-cell autonomously and move symplasmically. The mobile transcription factor *KNOTTED1*, for example, moves between cell layers in the shoot apical meristem to coordinate leaf initiation (Jackson et al., 1994). Similarly, *LEAFY* (*LFY*) acts non-cell autonomously to activate transcription in other cell layers beyond cells where *LFY* is expressed during flower development (Sessions et al., 2000).

In addition to proteins, mRNA is suggested to move symplasmically. Mobile mRNA has also been shown to move from cell to cell across a plant to promote translation in other tissues. mRNA has also been suggested to travel across in graft junctions, further supporting the existence of mobile mRNA (Yang et al., 2023). This mRNA mobility leads to questions of whether the mobile transcription factors act as the primary signal or whether mRNA is the key mobile signalling molecule if the mRNA is moving alongside the protein. Despite questions about the relative roles of mobile mRNA and proteins, studies suggest these processes are involved in the coordination of development, illustrating the importance of symplasmic movement during development.

1.4.4 Symplasmic isolation during development

Symplasmic isolation, where molecular trafficking no longer occurs through plasmodesmata into cells or regions of cells, is also important to development. During different developmental processes, symplasmic isolation occurs in several cell types and tissues. Apical bud dormancy in birch (*Betula pubescence*), for instance, involves symplasmic isolation of the shoot apical meristem (Rinne & van der Schoot, 1998). symplasmic isolation triggered by shortening photoperiods occurs through the formation of callose sphincters around the plasmodesmal neck region (Rinne & van der Schoot, 1998; Tylewicz et al., 2018). This symplasmic isolation is only transient, and prolonged cold temperatures release buds from dormancy by the activity of β -1,3-glucanases that degrade callose. This callose degradation enables the symplasmic movement of flowering and developmental signals such as *FLOWERING LOCUS T* to trigger bud growth (Rinne et al., 2011; Tylewicz

et al., 2018). Lateral root initiation also requires regulation of callose deposition for transient symplasmic isolation during development (Benitez-Alfonso et al., 2013). Disruption of callose regulation, through loss of β -1,3 glucanases results in perturbed lateral root hair patterning (Benitez-Alfonso et al., 2013).

Other developmental processes involve permeant symplasmic isolation. Guard cells become permanently symplasmically isolated during development (Ding et al., 1997; Palevitz & Hepler, 1985; Willmer & Sexton, 1979). The symplasmic isolation is suggested to regulate stomatal patterning by segregating epidermal versus stomatal cell fate signalling. CALS10 mediates symplasmic isolation in guard cells through the deposition of callose at plasmodesmata (Guseman et al., 2010). Disrupting CALS10 activity prevents this symplasmic isolation meaning the hypothesised stomatal cell-fate determinant *SPEECHLESS* (*SPCH*) moves symplasmically into neighbouring epidermal pavement cells, resulting in defective cell patterning (Guseman et al., 2010). This highlights how symplasmic isolation is key to guard cell differentiation.

Overall, symplasmically isolated cells and domains form in different developmental contexts. This contrasts with the role of plasmodesmata in mediating symplasmic connectivity for certain phloem loading and unloading mechanisms and in the movement of mobile signals for other cellular differentiation processes. Symplasmic connectivity must thus be tightly regulated in response to metabolic, developmental, and environmental cues to optimise where symplasmic connectivity or isolation is triggered to ensure correct developmental patterning occurs along with maintaining the transport of nutrients and other signalling molecules through plasmodesmata where needed.

1.5 Trade-offs in plants

Trade-offs occur in plants to balance the demands of growth and defence. As plasmodesmata play a role in both growth and defence, I hypothesise plasmodesmal regulation may result in a trade-off. In the following section, I discuss the current understanding of growth-defence trade-offs and mechanisms underlying the balance of this trade-off.

1.5.1 Growth-defence trade-offs

The growth defence trade-off is based on the assumption a plant has limited resources that can be used in either growth or defence. Defensive responses have high fitness costs in terms of energy diverted away from growth, development, and reproduction (Huot et al., 2014). For instance, constitutive activation of pattern-triggered immunity inhibits growth (Gómez-Gómez & Boller, 2000; Zipfel et al., 2006). Similarly, activating salicylic acid-mediated resistance in the absence of pathogens results in loss of fitness with reduced seed production and biomass (Heil et al., 2000). When activated during defence, induced defensive responses increase overall fitness by reducing losses to the pathogen. For instance, inducing salicylic acid-mediated defences during infection with the oomycete downy mildew increases resistance to pathogens (Görlach et al., 1996). Studies have also shown loss of inducible defence responses reduces fitness due to increased susceptibility to pathogens (Heidel et al., 2004). These examples indicate inducible defence is a key strategy to ensure resources are only invested into defence when pathogens are present to limit costs to growth, and these defensive responses are important to optimising fitness.

To fuel inducible defensive responses, resources are likely to be reallocated within a plant away from growth and towards prioritising defence. The presence of pathogens triggers transcriptional reprogramming and changes to the proteome to increase the activity of defensive proteins and the production of defensive compounds (Zou et al., 2005). As part of this reprogramming, several photosynthesis-related genes are also downregulated, whilst carbohydrate catabolism is increased (Bilgin et al., 2010; Chen et al., 2011; Denoux et al., 2008; Zou et al., 2005). Additionally, carbon and nitrogen are allocated towards defence-related compounds to fuel defence. For example in *Nicotiana attenuata*, during a defence response fixed nitrogen is allocated into defensive compounds such as nicotine and phenolamide rather than into nitrogen compounds involved in photosynthesis such as RuBisCo (Ullmann-Zeunert et al., 2013).

Many studies into the growth-defence relationship explore it as an antagonistic trade-off of growth versus defence. More recent studies have begun to reframe this trade-off in line with more recent evidence on limitations and the regulatory pathways coordinating growth and defence. Kliebenstein (2016), for instance, suggest growth and defence should be considered less antagonistic and more cooperative, where fitness is optimised based on the coordination of defence and

growth. Plants must optimise their response in highly complex environments involving multiple stimuli, meaning the optimal balance of growth and defence is likely to vary across different contexts. Additionally, Kliebenstein (2016) challenges the general assumption that resources are diverted towards growth or defence, arguing instead that the impact of resource limitation on growth and defence is likely to be more nuanced, especially given different resources are limiting in different environments. Overall, considering the growth-defence trade-off as a balanced coordinated response to maximise fitness allows for a more dynamic interpretation of how the growth-defence trade-off is regulated in response to internal and external cues.

1.5.2 Mechanisms to regulate trade-offs

Hormone crosstalk tightly regulates the relationship between growth and defence to optimise response to complex biological stimuli. An important group of hormones that regulate the growth-defence trade-off are jasmonates (JAs). Growth is inhibited by the accumulation of JA during defence responses, whilst active growth inhibits defence response by inhibiting the accumulation of JAs to facilitate growth (Campos et al., 2014; Zhang & Turner, 2008). JA signalling regulates defence responses via JASMONATE-ZIM DOMAIN (JAZ) repressor proteins which bind to MYC transcription factors under non-stressed conditions to repress MYC activity (Fernández-Calvo et al., 2011). Increases in the accumulation of JAs during stress result in the degradation of JAZ proteins to release MYC transcription factors, which then trigger the expression of defence-related genes (Liu et al., 2021; Schweizer et al., 2013; Wasternack & Song, 2017).

Additionally, JA signalling results in growth inhibition. One suggested mechanism of JA-induced growth inhibition is that JAZ degradation interacts with the signalling cascade of the growth hormone gibberellin to inhibit growth (Yang et al., 2012). Thus, the gibberellin growth signalling and JA defence signalling pathways exhibit crosstalk to regulate growth during defensive responses. Additionally, JA-independent growth inhibition mediated by the red-light receptor phytochrome B (phyB) (Campos et al., 2014, 2016; Cerrudo et al., 2012; Chico et al., 2014; Major et al., 2020). PhyB regulates growth based on light conditions and is important in shade avoidance. Shade inactivates phyB, unleashing downstream signalling which promotes growth. This pathway is independent of JA-mediated signalling; thus, shading can promote growth even during a defence response to respond to light

conditions (Campos et al., 2016; Major et al., 2020). The crosstalk between JA and growth signalling pathways and light environments is thus key to optimising the balance of growth and defence where, in certain cases, both growth and defence occur.

Plant immune signalling receptors and growth receptors have shared signalling components which could facilitate the balance between growth and defence. FLS2 activates immune responses upon detection of flagellin, and BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptor acts as a steroid-mediated growth receptor (Lin et al., 2013; Lu et al., 2010). Both FLS2 and BRI1 signal via a common receptor-like cytoplasmic kinase Botrytis-Induced Kinase 1 (BIK1) (Lin et al., 2013; Lu et al., 2010). BIK1 thus has a dual function as a positive regulator of pattern-triggered immunity and a negative regulator of brassinosteroid-mediated growth and development. Bücherl et al. (2017) suggest the discrete functions of BIK1 are facilitated by the FLS2 and BRI1 receptors localising to separate nanodomains within the plasma membrane. The shared components between these growth and defence signalling pathways suggest the growth-defence trade-off is finely balanced and compartmentalised within cells.

In addition, research indicates trade-offs can be regulated in an age-dependent manner through hormone crosstalk. Berens et al. (2019) found that there are leaf age-specific responses to simultaneous abiotic stress and biotic stress. In mature leaves, abscisic acid suppresses salicylic acid biosynthesis to reduce biotic immune responses and boost abiotic stress responses, whereas in young leaves, increased salicylic acid signalling prevents abscisic acid from suppressing this immune response. This results in young leaves having increased biotic stress responses compared to abiotic responses Berens et al. (2019). The balance of this stress for optimising plant fitness is evident as disrupting the balance of abiotic and biotic stress response in young leaves results in a growth and reproductive penalty (Berens et al., 2019).

1.5.3 Questions on the role of plasmodesmata in trade-offs in growth and defence

The regulation of symplasmic connectivity is important in both growth and defence. As discussed in Section 1.4 above, the pre-phloem loading and phloem unloading pathway in *Arabidopsis* leaves occur through plasmodesmata, meaning maintaining

connectivity through plasmodesmata is likely to be important for both the optimal transport of sugars into phloem parenchyma cells and for unloading into sink leaves. On the other hand, as discussed in Section 1.3.3, PAMP-triggered plasmodesmal closure to reduce molecular traffic is important for resistance against pathogens.

To date, research has either focused on the role of plasmodesmata in development or the role of plasmodesmata in defence. As far as I am aware, no studies have explored the function and regulation of plasmodesmata across development during defensive responses. The contrasting regulation of plasmodesmata in different contexts raises multiple questions: is there a trade-off between growth and defence in the regulation of plasmodesmal permeability during an immune response? How is plasmodesmal PAMP response regulated in a developmental context where maintaining symplasmic connectivity is important? And how are plasmodesmata regulated in response to potentially conflicting signals? All of these questions focus on whether the regulation of plasmodesmal permeability balances a trade-off between the role of plasmodesmata in growth and defence.

1.6 Questions and aims of this work

To date, research has focused independently on plasmodesmal function in defence and growth, with little exploration of how the regulation of plasmodesmata in defence and growth is coordinated. In this work, I aim to investigate how the regulation of plasmodesmata impacts growth and defence, and how these two processes are intertwined with plasmodesmal permeability.

Many studies focus on the role of plasmodesmata in defence but only focus on specific tissue types such as mature leaves. This raises questions about whether tissues with different plasmodesmal structures and functions have differential regulation of plasmodesmata in response to stress. The initial objective of this work is to explore plasmodesmal response to PAMPs across different leaf ages and in roots to investigate whether there are changes in plasmodesmal defence response across different developmental stages.

Additionally, symplasmic phloem unloading occurs through plasmodesmata in leaf sink tissue. Given plasmodesmal structure in sink leaves is suggested to maximise unloading, it is not clear whether apoplastic pathways would be able to compensate for reduced symplasmic transport upon plasmodesmal closure. In this work, I aimed to investigate how plasmodesmal closure impacts the movement of sugars into sink tissues. Furthermore, I aimed to explore how the role of plasmodesmata in sink tissues is coordinated with plasmodesmal regulation in response to PAMPs.

Overall, in this work, I aimed to explore how plasmodesmata are regulated in response to potentially competing demands on plasmodesmal connectivity. I wanted to bring together new findings with existing understandings of the separate roles of plasmodesmata in growth and defence, to build a picture of how these roles are combined to optimise plant growth and defence.

Chapter 2

Materials and Methods

The chapter contains general materials and methods used within this thesis. Specific materials and methods are included within each results chapter.

2.1 Plant Material

2.1.1 Transgenic and mutant lines

Arabidopsis thaliana (Arabidopsis) lines in the Columbia ecotype background (Col-0) were used in this study. Table 2.1 lists the sources for lines used in this work.

Line	Source
Col-0	na
<i>lym2/LYM2pro::Citrine-LYM2</i>	C. Faulkner (John Innes Centre)
<i>LexA::PLUG</i>	Tee et al. (2024)
<i>LexA::icals3m</i>	Bellandi et al. (2022)
<i>cher1</i>	Kraner et al. (2017)
<i>p35S::MP17-GFP</i>	Vogel et al. (2007)
<i>p35S::PAGFP</i>	C. Faulkner (John Innes Centre)
<i>pAtSUC2::GFP</i>	Imlau et al. (1999)
<i>p35S::PA-GFP</i> $\times$ <i>LexA::icals3m</i>	This work

Table 2.1: **Arabidopsis mutant and transgenic lines** – Names and references for lines used in this work.

2.1.2 Plant material

Plate-grown *Arabidopsis* seedlings were grown in a long-day controlled environment room (CER) at 20°C and 80% relative humidity with a 16-hour light and 8-hour dark period. *Arabidopsis* seeds were stratified for a minimum of 48 hours and then sterilised by either a wet or dry sterilisation method. For wet sterilisation, seeds were first incubated in 70% ethanol for 1 minute, and then in a sterilization solution (2% Sodium Hypochlorite, 0.02% Triton X-100, sterile water) for 10 minutes, before washing five times with sterile distilled water. For dry sterilisation, seeds were sterilised in chlorine gas (3% hydrochloric acid, 10% Sodium Hypochlorite) for 4 to 6 hours.

Unless otherwise specified, seedlings were grown on 0.5× Murashige & Skoog media without sucrose (0.5× MS-S) which consisted of 2.205 g of MS including vitamins (Duchefa Biochemie) and 8 g of Agar (Formedium).

2.1.3 Soil-grown plants

All *Arabidopsis* seed was stratified for a minimum of 48 hours in the dark at 4°C before being sown onto John Innes F2 starter soil (100% Peat, 4Kg/m³ Dolomitic limestone, 1.2Kg/m³ Osmocote Start). Soil-grown plants for experimental assays were grown in growth cabinets (Panasonic Versatile Environmental Test Chamber MLR-352-PE) with a short-day 10-hour light and 14-hour dark cycle. Plants grown for bulking and crossing were grown in long-day CERs with 16-hour light and 8-hour dark periods at 22°C and 80% relative humidity.

2.2 Leaf numbering

In assays comparing young and mature leaves, young leaves were defined as the three youngest emerged leaves on an *Arabidopsis* rosette (Figure 2.1) and mature leaves were defined as the 7th, 8th, 9th or 10th oldest leaf on the rosette. Leaves were numbered relative to the youngest emerged leaf to allow for comparison of young leaves at similar developmental stages: e.g. the youngest emerged leaf was marked as “1”, the 2nd youngest emerged leaf was marked as “2”, and 3rd youngest emerged leaf was marked as ”3” and so on (Figure 2.1).

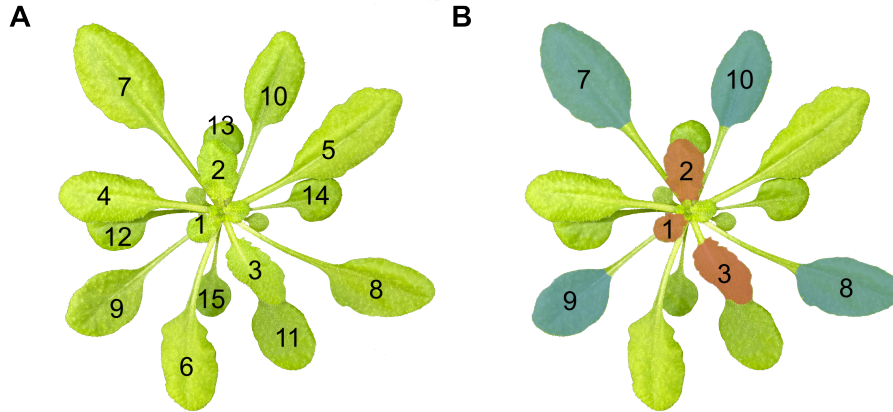


Figure 2.1: **Leaf numbering of Arabidopsis** - (A) A 5-week-old Col-0 rosette with leaves numbered from youngest to oldest, where the youngest emerged leaf is labelled "1". (B) Highlighted in blue are the leaves defined as "Mature" leaves and highlighted in red are leaves defined as "young" leaves.

2.3 Microprojectile bombardments

Microprojectile bombardments were carried out to determine cell-to-cell movement of GFP in young and mature leaves. Leaves were harvested from five-week-old plants and placed on 1% sucrose 0.8% agar MS plates and bombarded abaxial side up within one hour of detachment. As described in Tee et al. (2022), cells were transformed with a plasmid encoding the expression of free GFP (pB7WG2.0.GFP) and a plasmid encoding immobile endoplasmic reticulum (ER)-targeted RFP (pB7WG2.0.RFP_{ER}). The ER-targeted RFP acts as a transformation marker as it localises to the ER of the transformed cell (Figure 2.2 B). The expression of GFP acts as a marker for cell-to-cell mobility, as GFP can move cell-to-cell depending on the permeability of plasmodesmata (Figure 2.2 C).

To transform epidermal cells, a Bio-Rad Biolistic PDS-1000/He particle delivery system was used (Bio-Rad, 1652257) as described in Tee et al. (2022). To prepare the plasmids for bombardment, a 1 nM aliquot of gold particles (Bio-Rad, 1652263) was coated with 8 µg of GFP and 8 µg of ER-RFP Plasmid DNA. The DNA was co-precipitated onto gold by adding 2.5 M CaCl₂ and protected from DNases with 0.1 M spermidine. Gold particles were washed in ethanol and resuspended with 100% ethanol using sonication. A 7 µL aliquot of gold particles was fired at the abaxial side of leaves at 1100 PSI to transform lower epidermal cells. Successful transformations occurred when a plasmid-coated gold particle hit the nuclei of an epidermal cell resulting in the expression of plasmid-encoded genes.

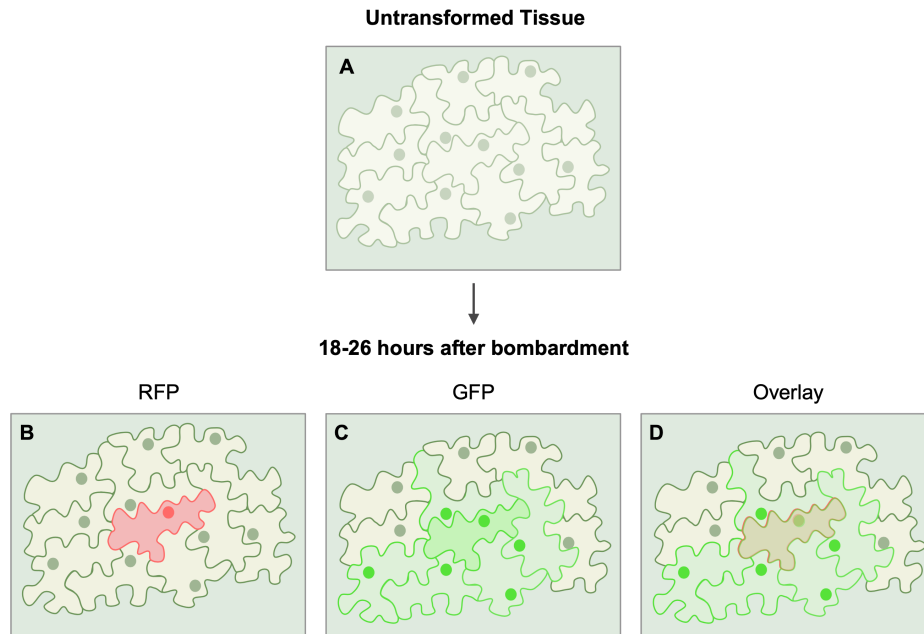


Figure 2.2: **Overview of GFP mobility assay** - Pavement cells in the lower epidermis (A) were transformed using microprojectile bombardments with free GFP and ER-targeted RFP. Successful transformation sites were identified 18-26 hours later via the ER-targeted RFP marker (B), which does not diffuse out of the transformed cell. GFP moves out of the transformed cell through plasmodesmata into neighbouring cells (C) and so cell-to-cell mobility was quantified by counting the number of cells showing GFP. In (C) the number of cells showing GFP would be counted as six. (D) shows an overlay of RFP and GFP channels.

Leaves were imaged 18 to 26 hours after carrying out microprojectile bombardments. Bombardment sites were identified in the lower epidermal layer by selecting cells expressing the ER-RFP transformation markers (Figure 2.2 B). Z-stack projections of sites were acquired using a Zeiss LSM800 confocal microscope with a 20 $\times$ objective (Apochromat 20 $\times$ /0.8 water immersion). GFP was excited at 488 nm and collected at 500-546 nm, RFP was excited at 587 nm and collected at 562-617 nm. The upper and lower limits of the z-stacks were defined by the limits of GFP fluorescence.

After image acquisition, cell-to-cell movement of GFP was quantified. The number of nuclei showing GFP fluorescence was counted, where a count of one nucleus indicates GFP has not spread from the transformed cell (Figure 2.2). Where specified, GFP movement was shown normalised to the mean of mock treatments within each genotype and/or tissue age. Bombardment data was analysed using a median bootstrap approach to account for the non-normal distribution and unequal

variance of data Johnston and Faulkner (2021). For comparisons of more than two treatments, a multiple comparisons test was incorporated into the median bootstrapping using analysis from Tee et al., 2023.

2.4 Leaf plasmodesmal callose staining

Aniline blue was used to quantify plasmodesmal callose deposition in leaves. Callose in mature and young leaves was stained by syringe-infiltrating leaves with 1% Aniline Blue (Sigma-Aldrich, 415049) in 1× Phosphate-buffered saline solution, pH 7.4. Immediately after staining, the abaxial side of leaves was imaged with a Zeiss LSM800 confocal microscope using a water immersion 63× objective (C-Apochromat 63×/1.20 W Korr UV VIS IR-water). Aniline blue was excited using the 405 nm laser and collected at 410-546 nm. Z-stacks were acquired, with the upper and lower limits defined to capture aniline blue staining in the epidermal cell wall.

Automated image quantification of aniline blue-stained plasmodesmal callose deposits was performed in FIJI (Schindelin et al., 2012) using a macro developed by Johnston (2021) and Bellandi (2021). Prior to quantification, stomata were manually removed from images, as the high callose content in guard cell walls disrupted the automated image quantification. As described in Johnston (2021) and Bellandi (2021), the automated script quantifies aniline blue staining intensity at plasmodesma puncta. For each slice of the z-stack, the script creates a binary image of pixels above a user-defined intensity threshold. Plasmodesmal callose deposits are detected where the number of pixels above the threshold is between the minimum and maximum size. The integrated density of these callose deposits is calculated from the total intensity of all pixels within the deposit. An R (RCoreTeam, 2023) script developed by Johnston (2021) was adapted to analyse the result outputs. This analysis calculated mean particle area, mean particle number, and mean and total integrated density per callose deposit for a given z-stack. These metrics could then be used to compare the intensity, area, and number of plasmodesmal callose deposits between treatments and genotypes.

2.5 Transcriptomics

Three different transcriptomic datasets were collected and analysed in this thesis. Details on the experimental set-up for each dataset can be found within the methods section of each chapter.

RNA extraction

To extract RNA, tissue was flash frozen in liquid nitrogen immediately upon harvesting. RNA was extracted using a Qiagen RNeasy micro kit following the manufacturer's instructions with buffer RLT. Prior to sending for sequencing, RNA samples were treated to remove DNA using TURBO-DNase (ThermoFisher).

RNA sequencing

Depending on the dataset, libraries were constructed and sequenced by Genewiz (Azenta Life Sciences) or Novogene. Reads for all datasets were checked for quality using FastQC (v0.11.8) (Andrews, 2010). Adaptors were trimmed and low quality reads with a phred of less than 20 were removed using TrimGalore (v0.5.0) and Cutadapt (v1.7) (Martin, 2011)(DOI 10.5281/zenodo.7598955 Felix Kruger). Reads were then mapped against the TAIR10 reference Arabidopsis genome using Hisat2 (v2.1.0) (Kim et al., 2019). SAMtools was used to convert files for input into StringTie, which was used calculate read counts and create a matrix of counts (Li et al., 2009; Pertea et al., 2016).

Data analysis

Raw read counts were used in differential gene expression analysis using DESeq2 (v1.38.3, (Love et al., 2014) in R studio (version 4.3.3 (2024-02-29))). Additional R packages were used to carry out data analysis: GO-term enrichment analysis was carried out using topGO (v.2.54.0, Alexa and Rahnenfuhrer (2023)); Heatmaps were built using ComplexHeatmap (v.2.18.0, Gu et al. (2016)); Volcano plots were plotted using EnhancedVolcano (v1.20.0, Blighe et al. (2023)); clusters in likelihood ratio tests were identified and plotted using DEGreport (v1.38.5, Pantano (2023)); shared differentially expressed genes were identified using RVenN (v.1.1.0, Akyol

(2019)).

2.6 Data visualisation and statistics

Data was analysed using R Studio (version 4.3.3; RCoreTeam (2023)). Data was visualised using R base or the ggplot2 package (Wickham, 2016). Analysis of variance (ANOVA) statistical tests were performed using the R base package and Tukey Honestly Significance Difference (HSD) tests were performed using the emmeans R package (v1.10.2, Lenth (2024)). All plotted boxplots showed the default R ggplot2 package settings, where the line in the centre of the box represents the median 50th percentile (Q2), whilst the upper boundary of the box represents the 75th percentile (Q3) and the lower boundary represents the 25th percentile (Q1). The boundaries of the box thus represent the interquartile range (IQR), which is equal to $Q3 - Q1$. The upper whisker extends up to the highest value datapoint or to $1.5 \times \text{IQR} + Q3$, depending on whichever is smaller. The same calculation is carried out for the lower whisker with the whisker extending to the lowest value datapoint or $1.5 \times \text{IQR} + Q1$, whichever is greater. Where notches are included in box plots, the notches indicate the confidence interval around the median which is the median $\pm 1.57 \times \text{IQR} / \sqrt{n}$.

Figures were prepared using Inkscape (v1.3.2, inkscape.org), BioRender.com, and FIJI (Schindelin et al., 2012).

Chapter 3

Characterising plasmodesmal stress response across leaf age

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3.1 Introduction

Symplastic transport through plasmodesmata is dynamically regulated in response to a wide range of biotic and abiotic stresses. Understanding this regulation under different developmental contexts is key to expanding our knowledge of the function of plasmodesmata. Much of the current work on the role of plasmodesmata in immunity focuses on responses seen in mature leaves. Plasmodesmal regulation across different leaf ages has not been previously studied in the context of immunity. This raises questions about how the regulation of plasmodesmal permeability and other aspects of pattern-triggered immunity change during development.

3.1.1 PAMP-induced plasmodesmal closure

Plasmodesmata are dynamically regulated in response to pathogen-associated molecular patterns (PAMPs). As outlined in Section 1.3.5, PAMPs act as molecular signatures for the presence of pathogens and trigger signalling cascades leading to plasmodesmal closure. The pattern recognition receptor LYSIN MOTIF DOMAIN-CONTAINING GLYCOSYLPHOSPHATIDYL-INOSITOL-ANCHORED PROTEIN 2 (LYM2) forms a complex with LYSM-CONTAINING RECEPTOR-LIKE KINASE 4 (LYK4) to bind to the fungal PAMP chitin at the cell surface and triggers an immune signalling cascade via calcium-dependent protein kinases (CPK) mediated phosphorylation of RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) (Cheval et al., 2020; Faulkner et al., 2013. RBOHD then activates

the signalling complex PLASMODESMAL-LOCALISED PROTEIN 1 (PDLP1) or 5 and NON-RACE-SPECIFIC DISEASE RESISTANCE GENE/HAIRPIN INDUCED GENE1-like protein 3 (NHL3) resulting in plasmodesmal closure via CALLOSE SYNTHASE 1 (CALS1) activation (Cheval et al., 2020; Faulkner et al., 2013; Tee et al., 2023). In a convergent pathway, the bacterial PAMP flg22, induces plasmodesmal closure by triggering an immune signalling cascade upon binding to the cell surface receptor FLAGELLIN-SENSING2 (FLS2) which activates CALMODULIN-LIKE 41 (CML41) (Tee et al., 2023; Xu et al., 2017). The chitin and flg22 immune signalling cascades are suggested to converge via RBOHD to activate CALLOSE SYNTHASE 1 (CALS1) via the PDLP-NHL3 complex (Figure 3.1).

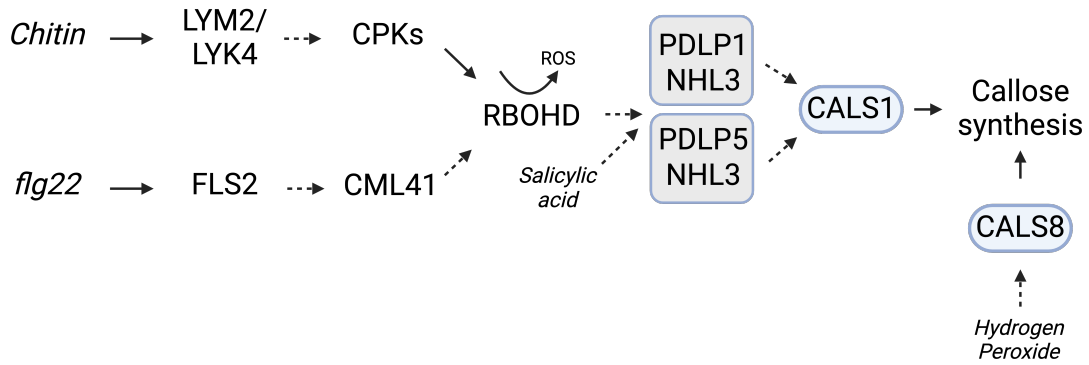


Figure 3.1: **Covergent immune signalling pathways trigger plasmodesmal closure** - Chitin and flg22 trigger a signalling cascade through separate receptor complexes that converges through RBOHD signalling. RBOHD triggered signals converge with salicylic acid-triggered signalling at either a PDLP1-NHL3 or PDLP5-NHL3 complex. Downstream of the PDLP-NHL3 complex, CALS1 is activated to promote callose synthesis at plasmodesmata. Hydrogen peroxide triggers callose synthesis through an independent pathway via CALS8. Solid arrows indicated established pathways and dashed arrows indicate yet to be established pathways. Reproduced with permission from Tee et al. (2023) using Biorender.com

The immune signalling cascades leading to PAMP-triggered plasmodesmal closure have been predominantly studied in mature leaves. Whether plasmodesmata are regulated differently in response to PAMPs in leaves at other developmental stages remains an outstanding question.

3.1.2 Phytohormone-induced plasmodesmal closure

Plant hormones regulate a variety of developmental and stress responses, and many have been found to both travel through plasmodesmata and regulate their function. For example, abscisic acid and salicylic acid are important signalling molecules known to impact plasmodesmal permeability but also have wider roles in coordinating responses to pathogen invasion, abiotic stress, and developmental cues.

The defence hormone salicylic acid is involved in a range of stress responses including hypersensitive response, eliciting systemic acquired resistance (reviewed in Ding and Ding (2020)), and regulating plasmodesmal permeability. Inoculation of plants with fungal and bacterial pathogens induces the accumulation of salicylic acid in both local and systemic tissues (Malamy et al., 1990; Wildermuth et al., 2001). Salicylic acid binds to salicylic acid-binding proteins and activates signalling pathways to trigger downstream defensive responses such as defence gene activation and hypersensitive response (Moreau et al., 2013; Rate & Greenberg, 2001; Tada et al., 2008; Tian et al., 2012). The salicylic acid biosynthesis protein ISOCHORISMATE SYNTHASE 1 (ICS1) is important to salicylic-acid mediated defensive responses, with *ics1* mutants showing reduced pathogen-triggered salicylic acid synthesis (Wildermuth et al., 2001).

Salicylic acid can also regulate plasmodesmal aperture. Treatment with exogenous salicylic induces callose deposition resulting in plasmodesmal closure (Wang et al., 2013). Salicylic acid was originally suggested by Cui and Lee (2016) to be a component of the PAMP-triggered plasmodesmal closure signalling pathway. However, PAMP-triggered plasmodesmal closure does not require functional ICS1, indicating that PAMP-induced production of salicylic acid is not required for PAMP-triggered plasmodesmal closure (Tee et al., 2023). Instead, salicylic acid feeds into the PAMP-triggered plasmodesmal closure pathway downstream of RBOHD, with PDL1, PDL5, and CALS1 required for salicylic-acid triggered plasmodesmal closure (Figure 3.1) (Tee et al., 2023; Wang et al., 2013). Thus, in addition to its role in wider defence responses, salicylic acid regulates cell-to-cell connectivity.

The phytohormone abscisic acid also regulates plasmodesmal permeability in response to different developmental and environmental cues. For instance, bud dormancy in aspen trees during short photoperiods is maintained by abscisic

acid-mediated plasmodesmal closure to prevent the symplasmic movement of growth signals (Tylewicz et al., 2018). This is suggested to be maintained by the formation of callose dormancy sphincters at plasmodesmata which could constrict cell-to-cell movement (Tylewicz et al., 2018). In addition to a role in dormancy, abscisic acid plays a role in Arabidopsis in regulating plasmodesmal permeability in root xerobranching, a process where the formation of lateral roots is repressed in the absence of water (Mehra et al., 2022). Abscisic acid represses lateral root development by blocking the symplasmic movement of auxin through the up-regulation of PDLs (Mehra et al., 2022). The up-regulation of PDLs then triggers plasmodesmal closure via callose deposition (Mehra et al., 2022). Additionally, abscisic acid also plays a role in the dynamic regulation of symplasmic connectivity in non-vascular plants. In the moss *Physcomitrella patens*, abscisic acid treatment reduced cell-to-cell movement in a callose-independent mechanism involving short-term changes in plasmodesmal structure (Kitagawa et al., 2019). Overall abscisic acid signalling mediates reductions in plasmodesmal permeability in various developmental and environmental contexts.

The role of abscisic acid has also been explored in an infection context, but it is less clear how this links to the control of plasmodesmal permeability. For instance, abscisic acid has previously been shown during infection to transcriptionally repress pathogenesis-related protein 2 (PR2), resulting in the decreased activity of β -1,3-glucanases and increased callose deposition (Oide et al., 2013; Rezzonico et al., 1998). However, these studies focused on larger macroscopic callose deposits and plasmodesmal callose deposition was not explored (Oide et al., 2013; Rezzonico et al., 1998). In a recent commentary, Benitez-Alfonso (2019) suggested the impact of abscisic acid on virus resistance in plants correlates with callose synthesis and reduced plasmodesmal permeability; however, there is currently limited evidence to back this up. Additionally, abscisic acid-mediated viral resistance to Bamboo Mosaic Virus is independent of plasmodesmal permeability, instead requiring the activity of ARGONAUTE proteins to trigger defensive transcriptional changes (Alazem et al., 2017). Further work is needed to determine how abscisic acid impacts plasmodesmal permeability in response to PAMPs and during viral infections and whether this leads to increased resistance.

Overall phytohormones have been shown to induce plasmodesmal closure in response to various developmental and environmental cues. Whilst the role of abscisic acid in regulating plasmodesmal permeability in defence is less well defined, salicylic acid is characterised to mediate plasmodesmal permeability in response

to PAMPs. Additional questions remain as to whether the roles of abscisic acid and salicylic acid in defence vary across leaf developmental contexts.

3.1.3 Reactive Oxygen Species as signalling molecules

Modifications to cell-to-cell movement through plasmodesmata often occur as part of wider cellular changes in response to stress or developmental cues. Understanding how plasmodesmal permeability is regulated alongside these wider responses is important to understanding the spatial and temporal roles of plasmodesmata. Reactive Oxygen Species (ROS) are signalling molecules that trigger plasmodesmal closure, but also play a wider role in PAMP-triggered immunity. If and how these signalling events interact is not known, but previous studies suggest that they might be executed independently.

ROS act as signalling molecules during development and in response to abiotic stress (Foreman et al., 2003; Miller et al., 2009). ROS is particularly important in plant defence responses, where it is produced upon detection of a pathogen infection (Bestwick et al., 1997; Doke, 1983), and also in response to wounding (Orozco-Cardenas & Ryan, 1999). There are multiple sources of intracellular ROS including photorespiration in peroxisomes and electron transport chains in chloroplasts; however, the rapid oxidative bursts of ROS released in response to pathogens are mainly extracellular events (Wrzaczek et al., 2013). These extracellular oxidative bursts are generated by NADPH oxidases and peroxidases. The NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGUES (RBOH) produces ROS by oxidising cytosolic NADPH to generate an apoplastic O_2^- superoxide ion (Torres et al., 1998, 2002). This superoxide ion is then dismutated by a superoxide dismutase to produce hydrogen peroxide (H_2O_2) (Auh & Murphy, 1995). In addition to RBOHs, Class III cell wall peroxidases also produce ROS, and account for half of the apoplastic ROS produced in response to pathogens in Arabidopsis cell cultures (O'Brien et al., 2012). These peroxidases oxidise plant hormones such as salicylic acid and indole-3-acetic acid to form H_2O_2 in the apoplast in response to pathogens (Bindschedler et al., 2006). RBOHs and peroxidases thus play an important role in generating apoplastic ROS bursts during pathogen infection.

The perception of increased apoplastic ROS concentrations is key to triggering immune signalling in response to stress. Several intracellular mechanisms for sensing ROS have been suggested, including oxidation of amino acid residues such as cysteines as well as lipid oxidation to produce oxylipins (Jeong et al.,

2011; Montillet et al., 2004; Wrzaczek et al., 2013). Other suggested mechanisms involve perception and response to changes in cellular redox balance, with redox-regulated proteins such as NON-EXPRESSER OF PR GENE 1 (NPR1) undergoing cytoplasmic redox-induced conformational changes to trigger downstream signalling (Brosché & Kangasjärvi, 2012; Després et al., 2003; Tada et al., 2008). Further evidence is needed to explain how apoplastic ROS enters the cytosol to trigger these intracellular sensors. Tian et al. (2016) suggest apoplastic H_2O_2 diffuses into cells via aquaporins. Tian et al. (2016) find disrupting H_2O_2 transport through the aquaporin AtPIP results in increased apoplastic H_2O_2 accumulation, reduced intracellular signalling, and increased susceptibility to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Thus, the diffusion of H_2O_2 into cells from the apoplast may be key to activating intracellular immune signalling. Overall, the apoplastic production of ROS and intracellular perception is key to triggering defensive immune signalling, with H_2O_2 playing an important role as a signalling molecule in pathogen-triggered immunity.

Questions remain about the role and specificity of oxidative bursts in plasmodesmal regulation. The NADPH oxidase RBOHD plays a role both in the regulation of reactive oxygen species production (Torres et al., 2002), and in PAMP-triggered plasmodesmal closure (Cheval et al., 2020; Tee et al., 2023). RBOHD forms part of the converging flg22 and chitin signalling pathway, with *rbohD* mutants showing no plasmodesmal closure in response to flg22 (Tee et al., 2023) and chitin (Cheval et al., 2020). Interestingly, *lym2* mutants with no LYM2 chitin receptor cannot execute chitin-triggered plasmodesmal closure, but produce an apoplastic ROS burst in response to chitin. Conversely, mutants lacking the *chitin elicitor receptor kinase 1* (*cerk1*) chitin receptor, can execute chitin-triggered plasmodesmal closure, but not the apoplastic ROS burst in response to chitin (Faulkner et al., 2013). This illustrates apoplastic ROS bursts are not required for chitin-triggered plasmodesmal closure, with CERK1 activating RBOHD-mediated oxidative ROS bursts and LYM2 inducing RBOHD-mediated plasmodesmal closure. This could indicate ROS signalling is localised, i.e. that ROS produced in the apoplastic burst does not migrate to the apoplast surrounding plasmodesmata to induce plasmodesmal closure (Cheval et al., 2020; Faulkner et al., 2013).

H_2O_2 induces plasmodesmal closure independently of the convergent salicylic acid, chitin, and flg22 signalling pathways (Figure 3.1). H_2O_2 -triggered reductions in plasmodesmal permeability do not require PDL5, NHL3 and CALS1 (Cui & Lee, 2016; Tee et al., 2023). Instead, H_2O_2 -mediated plasmodesmal closure

involves the activation of CALLOSE SYNTHASE 8 (CALS8) (Cui & Lee, 2016).

ROS bursts play an important role in pathogen-triggered immunity and plasmodesmal closure; however, there is little study of ROS signalling at different stages of leaf development. Questions remain about how development overlays with differences in ROS signalling at plasmodesmata and the surrounding apoplast.

3.1.4 Callose deposition

In addition to plasmodesmal callose deposits, larger macroscopic callose deposits are also observed in response to PAMPs. These are irregular deposits seen across the treated leaf tissue that are much larger than plasmodesmal callose deposits. Macroscopic callose deposits are not thought to be directly related to callose deposition at plasmodesmata. Instead, macroscopic callose deposition reinforces larger areas of the cell wall with extra callose (Luna et al., 2011). The presence of macroscopic callose deposits is often used as an indicator for the induction of pattern-triggered immunity (Luna et al., 2011); however, the function of PAMP-triggered macroscopic callose deposition is not clear.

Specific macroscopic callose structures are seen in papillae formation during leaf infection with fungal pathogens such as powdery mildew. Papillae are cell wall appositions formed at sites of fungal pathogen penetration where the cell wall is thickened by additional callose deposition (Aist, 1976; Brown et al., 1998; Eggert et al., 2014). However, unlike PAMP-triggered macroscopic callose which forms irregularly-sized deposits, papillae form specific ring-like shapes around the fungal penetration peg (Eggert et al., 2014). The same callose synthase protein, CALS12, is linked to both callose formation at papillae and in PAMP-triggered macroscopic deposits (Luna et al., 2011; Nishimura et al., 2003; Vogel & Somerville, 2000). Despite the shared callose synthase, it is unclear whether the ring-like macroscopic callose structures in papillae form through a different signalling pathway to the irregular PAMP-triggered macroscopic callose deposits.

The benefits of papillae formation in pathogen defence are unclear. Despite *cals12* mutants having almost no macroscopic callose deposition or papillae formation, these mutants have increased resistance to various powdery mildews (Jacobs et al., 2003; Vogel & Somerville, 2000). This increased resistance is suggested to be due to hyperactivation of the salicylic acid pathway in these mutants (Nishimura et al., 2003). Conversely, overexpression of *CALS12* leads to enhanced resistance

to the powdery mildew *Golovinomyces cichoracearum* due to increased callose deposition (Ellinger et al., 2013). In these *CALS12* overexpression lines, even spraying leaves with water induces callose formation, indicating a high sensitivity to any form of pressure. Ellinger et al. (2013) suggest the pathogen's infection strategy determines the impact of *CALS12* activity on susceptibility, suggesting callose deposition plays a more important role in defence against necrotrophs. Characterisation of macroscopic callose in response to other biotrophic pathogens would help to ascertain the benefits of induced deposition and papillae formation in different contexts. Although the role of PAMP-triggered macroscopic callose deposition in immunity is not well understood, it is still considered an indicator of pattern-triggered immune responses.

3.1.5 Aims

PAMP-triggered plasmodesmal closure and its accompanying immune signalling pathway has been well characterised in mature leaf tissue, but not in other leaf developmental stages. Given plasmodesmata have different roles across development, in this chapter I aimed to determine whether plasmodesmal regulation in response to PAMPs changes across different leaf developmental stages. Also, given key components of signalling pathways leading to plasmodesmal closure have been well characterised in mature leaves, I aimed to explore how these signalling pathways are expressed in young leaves and whether this links to any potential changes in plasmodesmal regulation.

Additionally, limited studies of changes in other pattern-triggered immune responses have been carried out in leaves at different developmental stages. I aimed to test whether young and mature leaves have different pattern-triggered immune responses by exploring ROS burst production, macroscopic callose deposition, and transcriptional responses to chitin in young and mature leaf tissue. I further aimed to bring these findings together to address gaps in our understanding of how plasmodesmal regulation and wider pattern-triggered immune responses shift across development.

3.2 Materials and Methods

3.2.1 Microprojectile bombardments

Microprojectile bombardments were performed as described in Section 2.3. Young and mature leaves were sampled based on leaf number where the leaves 1 to 3 were defined as young leaves and leaves 7 to 10 were defined as mature leaves as described in Figure 2.1 and Section 2.2. Leaves were bombarded and abaxially syringe-infiltrated with a mock solution or elicitor two hours later. Treatments always included a water mock (distilled water) compared to chitin (500 $\mu\text{g}/\text{ml}$, distilled water), flg22 (100 nM, distilled water), salicylic acid (100 μM , distilled water), hydrogen peroxide (10 mM, distilled water) or abscisic acid (50 μM , distilled water).

3.2.2 Measuring Reactive Oxygen Species Bursts

The production of reactive oxygen species (ROS) was measured in five-week-old *Arabidopsis* plants. Circular leaf discs were harvested using a 5 mm corer. Young leaf discs were sampled from the three youngest leaves and mature leaf discs were sampled from leaves 7, 8, and 9 as defined in Figure 2.1.

After harvesting, leaf discs were immediately placed adaxial side face-up in a 96-well plate with water. Six hours later, the water was replaced with a working solution of 20 mg/ml peroxidase, 20 mM L-012, and sterile distilled water. Immediately before measurement, leaf discs were treated with either sterile distilled water (mock), flg22 (100 nM final concentration), or chitin (500 ng/ml chitin final concentration). Emitted luminescence was measured for 90 minutes after the addition of elicitors by a Varioskan Flash Plate Reader (Thermo Fisher Scientific). The intensity of the first ROS peak was quantified by integrating the relative light unit (RLU) in the first 45 minutes of luminescence measurements to calculate the cumulative RLU.

A total of twelve plants were sampled across three experimental replicates for each leaf age and treatment. From each plant, three leaf discs were harvested and the cumulative RLU for each plant was determined by taking the mean cumulative RLU of three leaf discs. Chitin, flg22, and mock treatments were

performed within the same experiment. However, due to large differences in scales of magnitude of ROS response between flg22 and chitin, the chitin and flg22 treatments were analysed as separate experiments. Cumulative RLU was compared between leaf ages and treatments in a mean bootstrap analysis due to the non-normal distribution and uneven variance of the data. This analysis was adapted from the median bootstrap approach (Johnston & Faulkner, 2021) by comparing means instead of medians due to the cumulative RLU being continuous data. Additionally, a script from Tee et al. (2023) was used to adjust for multiple comparisons within the mean bootstrap analysis.

3.2.3 Quantifying Macroscopic Callose

Macroscopic callose staining was performed on young and mature leaves of five-week-old Col-0 plants. Leaves were syringe-infiltrated with either sterile water, 100nM of flg22, or 1 μ M of flg22. After 22-30 hours, leaves were stained with aniline blue to visualise callose deposition.

Leaves were handled in two ways: either leaves were detached before flg22/water infiltration and placed on 0.5 $\times$ MS-S plates for 22-30 hours, or leaves were infiltrated and left attached to the plant until immediately before aniline blue staining. Aniline blue staining was performed by syringe-infiltrating leaves with 1% Aniline Blue in 1 $\times$ PBS pH 7.4 solution. Immediately after infiltration with aniline blue, images were acquired on a Zeiss LSM 800 confocal microscope using a 10 $\times$ objective (EC Plan-Neofluar 10 $\times$ /0.30 M27-Air). Aniline blue was excited using a 405 nm laser and collected at 410-500 nm. The bright-field filter was used to select sites for imaging based on where aniline blue had been successfully infiltrated into the leaf. This prevented bias in selecting imaging sites as aniline blue staining of callose deposits was not visible in the bright-field filter. Z-stacks from five sites were acquired per leaf, and maximum projections of these z-stacks were used in image quantification.

Macroscopic callose was quantified in FIJI (Schindelin et al., 2012). The plasmodesmal callose detection FIJI macro described in section 2.4 was modified to detect the intensity of macroscopic callose deposits. As macroscopic callose deposits are larger and have more intense aniline blue staining than plasmodesmal callose deposits, the threshold intensity of particle detection was increased along with the minimum and maximum area of detection. Trial and error was used to optimise particle detection parameters within each experiment. For each imaged

leaf site, the total and average intensity and the total and average area of each callose deposit were calculated along with the number of callose deposits per image. R studio (R Core Team, 2023) was used to calculate the mean and total size, intensity, and number of callose deposits for each leaf. In the attached leaves, the size and intensity of callose deposits per leaf between water and flg22 treatments were compared using a multiple comparisons mean bootstrap approach described in Section 3.2.2.

For experiments comparing 1 μM of flg22 to water treatment, a minimum of six leaves were imaged for leaf age and treatment combination. For experiments comparing 100 nM of flg22 to water treatment, at least 16 leaves were imaged per leaf age and treatment combination across four experimental replicates. In a preliminary assay on macroscopic callose deposition in detached leaves, two attached mature leaves treated with flg22 were compared to three detached water-treated leaves and six detached flg22-treated leaves

3.2.4 Young vs mature leaf transcriptomic analysis

Mature leaves from five-week-old Col-0 and *lym2-1* mutant lines were syringe infiltrated with mock (water and 0.00001% Silwet L-77) or chitin (500 $\mu\text{g}/\text{ml}$ chitin, water and 0.00001% Silwet L-77) and harvested 30 minutes later. Mature leaves were defined as leaf eight (sampled according to the numbering system outlined in Figure 2.1), and three mature leaves were harvested from three plants pooled together into one mature leaf sample. Young leaves were defined as leaves two and three and six young leaves from three plants were pooled into one young leaf sample. Each pooled sample was considered to be a single biological replicate, with three biological replicates sampled per leaf age and elicitor combination.

As described in more detail in section 2.5, I extracted RNA for sequencing by Novogene, calculated gene read counts, and used DESeq2 to perform gene expression analysis in R studio (Love et al., 2014; RCoreTeam, 2023).

3.2.5 Imaging of *Citrine-LYM2* in young leaves

Young leaves of *lym2/LYM2pro::Citrine-LYM2* plants were detached from 5-week-old plants and immediately imaged to confirm the expression of Citrine-LYM2 in young leaves. Images were acquired using a Zeiss 800 confocal microscope with a

3.2. Materials and Methods

C-Apochromat 63x/1.20 W Korr UV VIS IR. LYM2-Citrine was excited using a 488nm laser and collected at 500-617 nm. Citrine-LYM2 expression was screened across more than 10 young leaves on three separate days to determine there was no leaf-to-leaf variation in localisation.

3.3 Results

3.3.1 Plasmodesmata do not close in response to chitin or flg22 in young leaves

To investigate plasmodesmal PAMP response in young *Arabidopsis* leaves and compare these responses to those in mature leaves, I quantified changes in cell-to-cell mobility using microprojectile bombardment assays in response to the fungal PAMP chitin and bacterial PAMP flg22. The number of cells showing GFP was counted in each image as a measure for cell-to-cell mobility. Mature leaves showed a significant reduction in the number of cells showing GFP when treated with chitin compared in comparison to mock, similar to previous research (Figure 3.2 A, D-E)(Faulkner et al., 2013). In contrast, young leaves showed no significant difference in cell-to-cell GFP movement between mock and chitin treatments (Figure 3.2 A-C). This indicates plasmodesmal permeability in young leaves is not reduced in response to chitin, which differs from the response found in mature leaves. Similarly, treatment with flg22 reduces cell-to-cell movement of GFP in mature leaves but has no effect on cell-to-cell movement in young leaves (Figure 3.2 F, G-J, data collected with Catherine Walker). The lack of change in cell-to-cell mobility in response to flg22 and chitin in young leaves indicates there is a change in plasmodesmal response to PAMPs across leaf development.

3.3.2 Plasmodesmata do not close in response to common stress elicitors in young leaves

To investigate whether the lack of plasmodesmal closure in young leaves is PAMP-specific I explored plasmodesmal responses to other stress elicitors. Both salicylic acid and hydrogen peroxide (H_2O_2) induce plasmodesmal closure in mature leaves through independent signalling cascades that trigger callose deposition (Tee et al., 2023). As previously found, treatment with salicylic acid and H_2O_2 reduces cell-to-cell mobility of GFP compared to a mock (distilled water) treatment in mature leaves (Figure 3.3 A)(Cui & Lee, 2016; Wang et al., 2013). Young leaves, however, show no significant difference in cell-to-cell GFP mobility when leaves are treated with either salicylic acid or H_2O_2 compared with a mock treatment.

Abscisic acid treatment also induces a reduction in the spread of GFP between

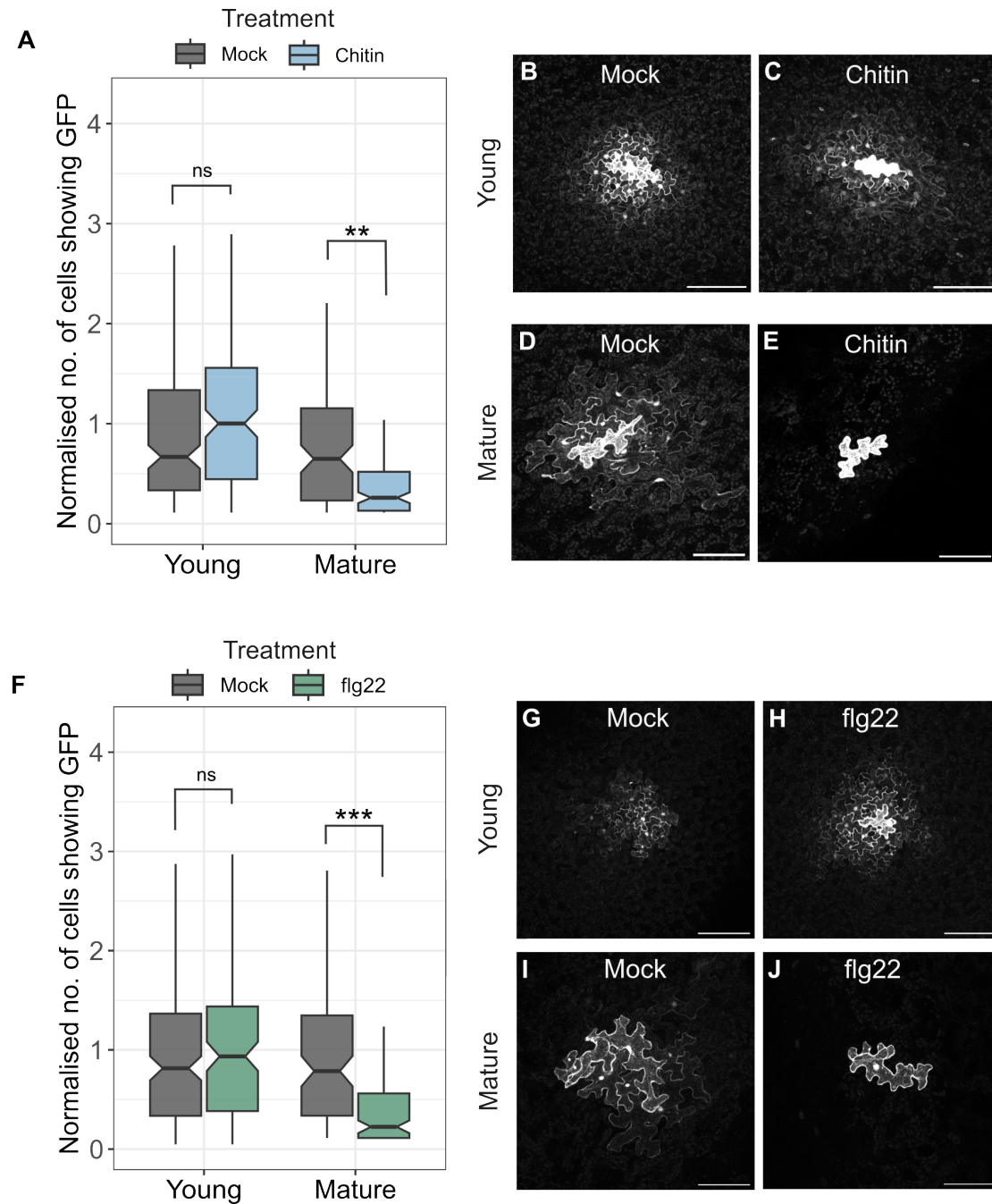


Figure 3.2: **Cell-to-cell mobility assays on young and mature leaves treated with PAMPs** – (A) GFP movement after treatment with mock (grey) and chitin (blue) normalised to mean movement of mock within each tissue age. (B-E). Confocal images of single bombardment sites from leaves treated with mock (B, D) or chitin (C, E) in young (B, C) and mature (D, E) leaves. (F) Normalised GFP movement after treatment with mock (grey) and flg22 (green) (G-J). Confocal images of single bombardment sites treated with mock (G, I) or flg22 (H, J) in young (G, H) and mature (I, J) leaves. The scale bar indicates 100 μ m. Significance values: ‘ns’, no significance; ‘*’ = $p < 0.05$; ‘**’ = $p < 0.01$, ‘***’ = $p < 0.001$. $N > 110$ per treatment and age combination. GFP movement is normalised to mean movement of mock within each tissue age.

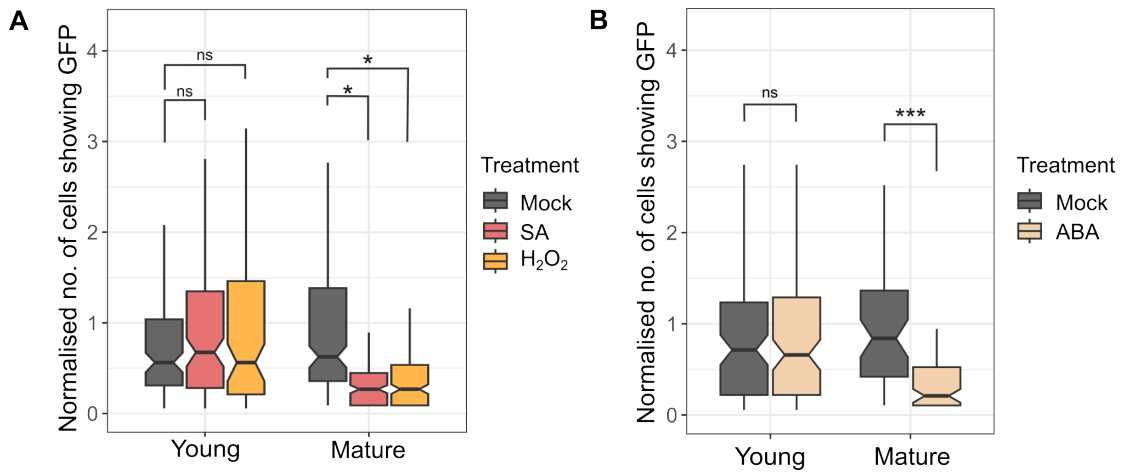


Figure 3.3: Cell-to-cell mobility assay on young and mature leaves in response to stress elicitors - (A) GFP movement in response to mock (grey), salicylic acid (SA, red), and hydrogen peroxide (H₂O₂, orange) in young and mature leaves, where (B) GFP movement assays on young and mature leaves in response to abscisic acid (ABA, beige). Significance values: ‘ns’ = no significance. ‘*’ = $p < 0.05$, ‘***’ = $p < 0.00$. $N > 38$ for each treatment and age combination in mature leaves, and $N > 98$ in young leaves. GFP movement is normalised to mean movement of mock within each tissue age.

cells in mature leaves, indicating a reduction in plasmodesmal permeability. However, similarly to all other elicitors tested, abscisic acid treatment in young leaves did not induce a change in plasmodesmal permeability (Figure 3.3 B). Overall, the lack of changes in cell-to-cell mobility in response to salicylic acid, H₂O₂, and abscisic acid suggests that similar to PAMPs, plasmodesmal closure is not triggered in response to stress in young leaves.

3.3.3 Apoplastic Reactive Oxygen Species Bursts are triggered in response to PAMPs in young leaves

To investigate whether the differences in PAMP responses between young and mature leaves are plasmodesmal-specific or reflective of wider differences in immune responses across leaf ages, I first explored how the PAMP-triggered apoplastic ROS burst varied across leaves of different ages. I sampled leaf discs from young and mature leaf tissue, and then used a luminol-based assay to track apoplastic ROS production in young and mature leaf tissue after the addition of either water (mock), chitin, or flg22 elicitors. ROS production in leaf discs was measured over 3000 seconds after the addition of elicitors (Figure 3.4 A and C). The relative light units (RLU) was averaged across the leaf discs within each plant, with the average area under the curve for each plant calculated as cumulative RLU. Both young

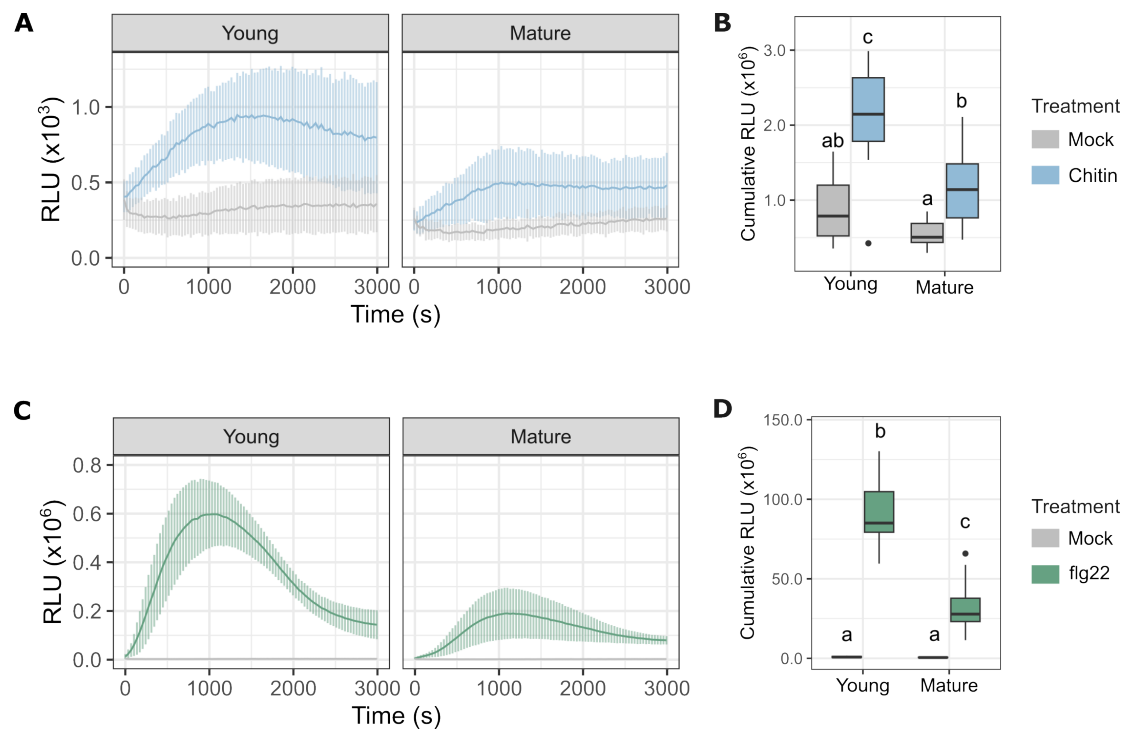


Figure 3.4: **Reactive Oxygen Species bursts in response to PAMPs young and mature leaves** – (A) Relative light units (RLU) released from young and mature leaves in response to mock (grey) and chitin (blue) over time. (B) Cumulative RLU in young and mature leaves in response to chitin. (C) RLU over time in response to treatment with mock (grey) and flg22 (green). (D) Cumulative RLU in young and mature leaves in response to flg22. Different letters represent significant differences of $p < 0.05$

and mature leaf tissue produced significantly higher cumulative RLU in response to flg22 and chitin compared to mock (Figure 3.4 A-D). Additionally, young leaves show a significantly higher cumulative RLU compared to mature leaves in response to both flg22 and chitin.

3.3.4 Macroscopic callose deposition is triggered in response to flg22 in young leaves

Another previously characterised PAMP response in mature leaves is the deposition of macroscopic callose. These deposits are thought to act to strengthen the cell wall against pathogen attack (Brown et al., 1998; Eggert et al., 2014). Macroscopic callose deposition is regulated by a different set of enzymes to plasmodesmal callose deposition, and so could be triggered in the absence of plasmodesmal callose deposition. Thus, I investigated how these independent callose responses are executed in young leaves.

Established methods for visualising macroscopic callose typically involve overnight staining in aniline blue and/or tissue clearing (Xu et al., 2017); however, I found infiltrating with aniline blue and immediately imaging more effective for visualising callose and maintaining intact leaves. Thus, I stained macroscopic callose deposits with aniline blue 24 hours after treatment with either water (mock), flg22 100nM, or flg22 1 mM. I observed substantial variation in callose deposition in the macroscopic callose response in each leaf, with Figure 3.5 A-B showing a selection of images from the same leaves. At higher magnification, the macroscopic callose deposits can be seen to be centred around stomata in young leaves (Figure 3.5 C and D).

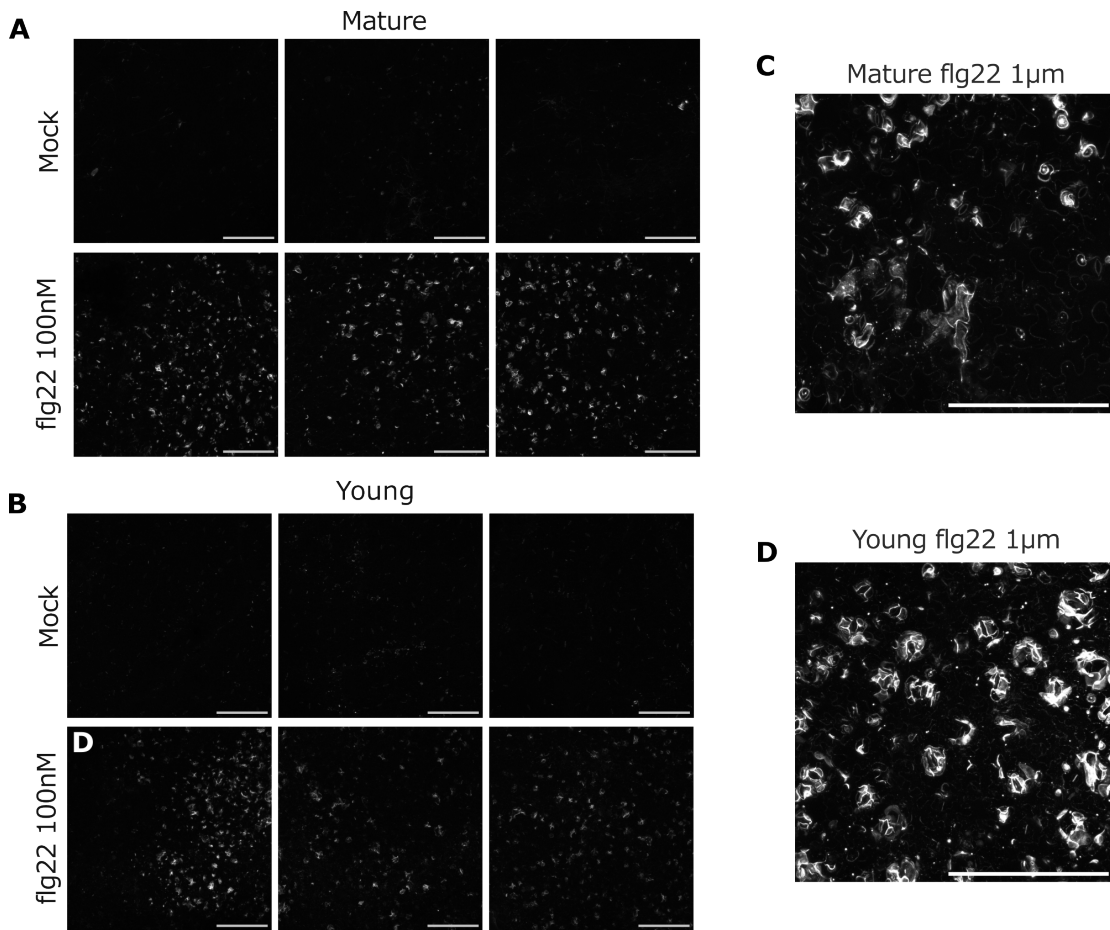


Figure 3.5: **Confocal Images of aniline blue staining of macroscopic callose deposits** – Rows show three sites from the same leaf from mature (A) and Young (B) leaves stained with aniline blue 24 hours after treatment with water (mock) or 100 nM flg22. Aniline-blue stained mature leaves (C) and young leaves (D) treated with 1 μM flg22. Scale bars indicate 200 μm.

To determine whether my infiltration method is effective for quantifying the macroscopic callose deposition response, I first replicated the experiments in Xu et al. (2017) with 1 μM flg22 compared to water (mock). For this, I quantified the

size and intensity of macroscopic callose deposits using an automated detection script (Figure 3.6 A, detailed method can be found in Section 3.2.3). In both young and mature leaves, macroscopic callose deposition was significantly increased in response to 1 μ M flg22, with mock-treated showing no or low levels of macroscopic callose (Figure 3.6 B and C). This indicates my method of callose staining is effective at detecting PAMP-triggered macroscopic callose deposition.

To directly compare the plasmodesmal PAMP response to macroscopic callose deposition, I repeated this experiment using 100 nM flg22 treatment as my cell-to-cell mobility assays in Section 3.3.1 were performed with a 10-fold lower flg22 concentration. I found 100 nM of flg22 induces a significant increase in macroscopic callose area and intensity compared to water treatment in both young and mature leaves (Figure 3.7 E and F). This suggests despite the lack of flg22-triggered plasmodesmal closure in young leaves, macroscopic callose deposition is still induced by flg22 treatment.

These results suggest there are differences in the mechanisms of plasmodesmal response and macroscopic callose deposition. However, it could be that these differences are related to the experimental setup. Plasmodesmal permeability was quantified by measuring GFP movement in microprojectile bombardment assays in images acquired 18-26 hours after leaf detachment, whereas leaves used in macroscopic callose deposition were treated with flg22, left 24 hours and imaged immediately after detachment. To recreate the conditions of the plasmodesmal permeability assay, I carried out macroscopic callose staining on detached leaves. I detached mature leaves, infiltrated them with 100 nM flg22 and stained them with aniline blue 18-26 hours later. Despite two preliminary experimental repetitions, I found very low levels of macroscopic callose in mature detached leaves compared to attached leaves in response to 100 nM flg22 (Figure 3.7). Detaching the leaf thus appears to disrupt the process of macroscopic callose deposition in mature leaves indicating it is not an effective method to compare plasmodesmal callose to macroscopic callose.

Overall, my results indicate that flg22 triggers macroscopic callose deposition but not plasmodesmal closure in young leaves. This correlates with the differences in machinery involved in each response and suggests that the lack of plasmodesmal response in young leaves is not due to an inability of these tissues to synthesise callose.

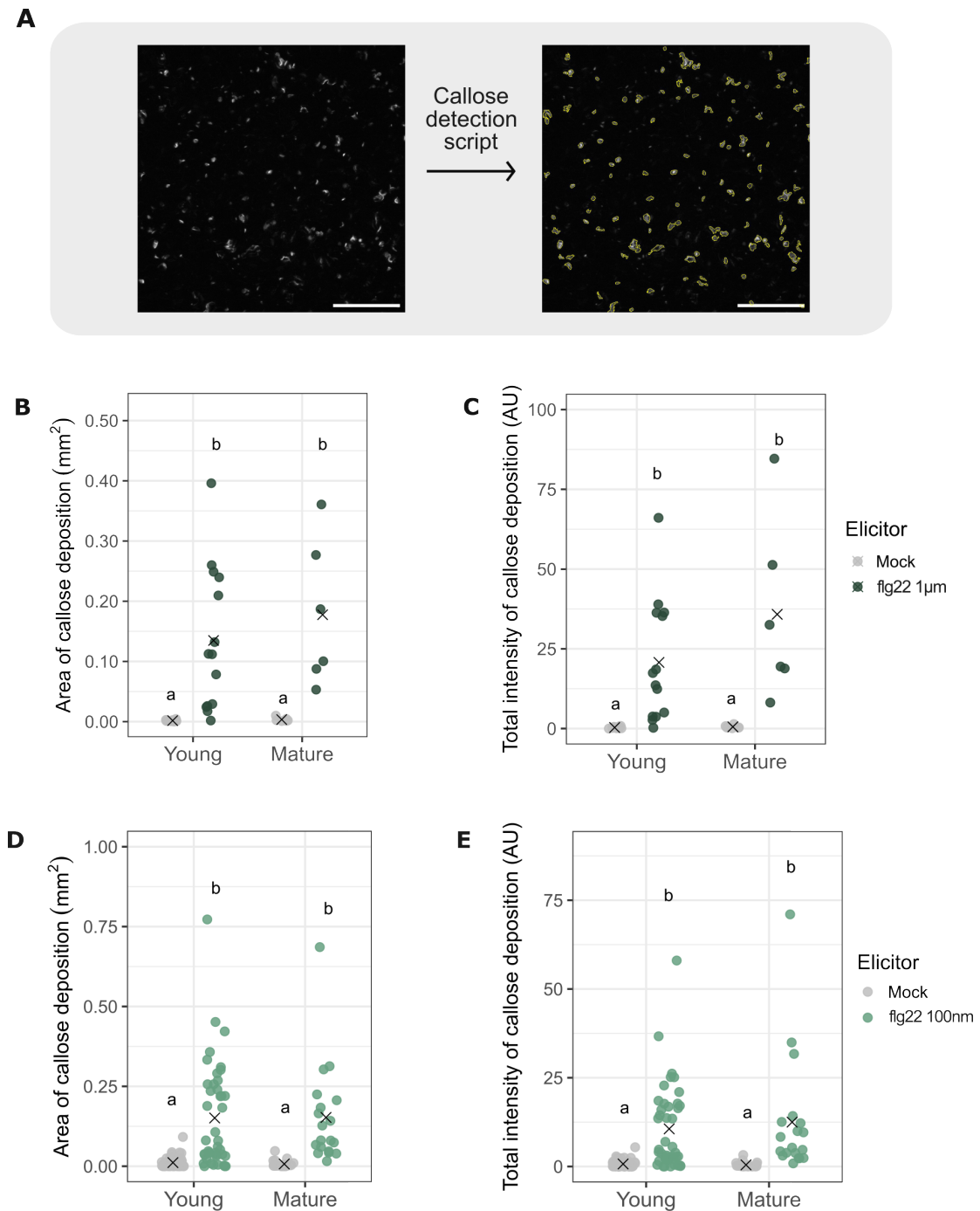


Figure 3.6: **Quantification of macroscopic callose deposits in response to flg22**
 – (A) Quantification of callose deposits through drawing a mask around callose deposits stained with aniline blue. (B) Area and (C) intensity of callose deposits in young and mature tissue treated with $1\mu\text{M}$ flg22 (dark green) or a mock water (grey) treatment. (D) Area and (E) Intensity of callose deposits in young and mature tissue treated with 100 nM flg22 (light green) or mock (grey). Scale bars indicate $200\mu\text{m}$. Different letters represent significant differences of $p < 0.05$ between samples. ‘X’ indicates the mean value.

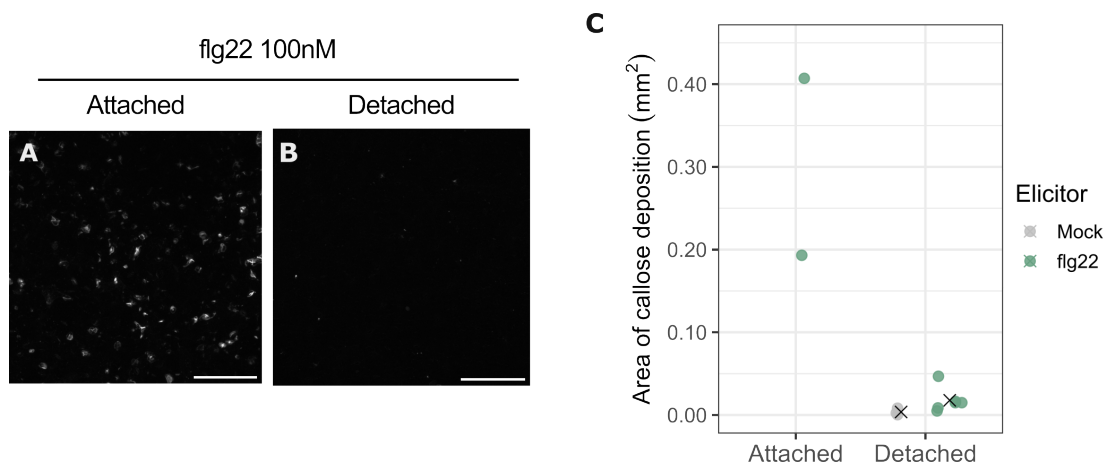


Figure 3.7: **Quantification of macroscopic callose deposits in detached leaves** – Staining of callose deposits in attached (A) compared to detached (B) leaves. Quantification of Area (C) of callose deposits in young and mature tissue treated with 100 nM flg22 (dark green) or mock (grey). Scale bars indicate 200 μ m.

3.3.5 Young leaf tissue has a reduced transcriptional response to chitin

ROS bursts and macroscopic callose deposition are triggered in young leaves, indicating several immune responses are induced during leaf development even when plasmodesmal closure is not. To explore this phenomenon further, I performed an analysis of chitin-induced transcriptional responses in young and mature leaf tissue. For this, I infiltrated young and mature leaves with a chitin (500 μ g/ml) or mock (water) solution and harvested 30 minutes post infiltration. Two genotypes were investigated: Col-0 wildtype; and *lym2-1*, which cannot close its plasmodesmata in response to chitin due to a lack of a functional LYM2 receptor. To identify differentially expressed genes I used a Wald test, including all young and mature samples from Col-0 and *lym2* treated with water or chitin. Differentially expressed genes between leaf ages and treatments were identified in a single model with an adjusted p-value cut-off of 0.05 and Log₂ fold change cut-off of greater than 0.5 or less than -0.5.

Young leaf tissue showed an overall reduced transcriptional response to chitin compared to mature leaf tissue. There were fewer significantly differentially expressed genes in response to chitin in young leaves compared to mature leaves, with 569 up-regulated genes mature leaves but only 94 in young leaves in response to chitin (Figure 3.8). There were 143 genes down-regulated in response to chitin mature leaves and only 9 genes in young leaves. Overall, this suggests chitin elicits a smaller, or weaker, transcriptional response in the first 30 minutes after chitin

treatment in young leaves than mature tissue.

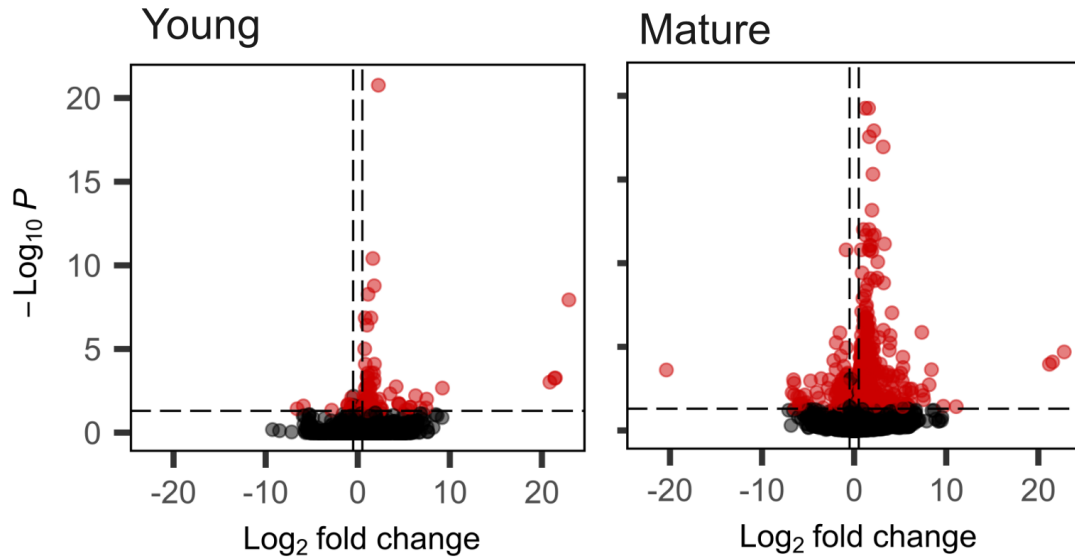


Figure 3.8: **Volcano plots of differentially expressed genes in young and mature leaves** – Differentially expressed genes in response to chitin compared to mock water treatment. Red points are significantly differentially expressed genes with an adjusted p-value of < 0.05 and $\log_2$ fold change $> |0.5|$, black points are genes which are not significantly differentially expressed. Vertical dashed lines mark the thresholds for significance and $\log_2$ fold change.

3.3.6 Young and mature leaf tissue share a common transcriptional defensive response

To determine if young leaves share similar differentially expressed genes to mature leaves, and therefore elicit the same response, I compared the differentially expressed genes in mature and young leaves (Figure 3.9 A and B). I identified 64 up-regulated genes shared between young and mature leaves (Figure 3.9 B). A Gene Ontology (GO)-term analysis of these overlapping genes suggests involvement in defence and stress signalling with enriched terms including “Defence response to bacterium” and “Regulation of jasmonic acid-mediated signalling” (Figure 3.9 C). This indicates despite differences in the magnitude of the responses, chitin induces shared stress and defence transcriptional responses between young and mature leaf tissue

Despite the shared transcriptional response between young and mature tissue, there are many defence-related genes only up-regulated in mature leaf tissue. GO-terms such as “Plant-hypersensitive response” and “Protein-phosphorylation” are only found in mature leaf tissue, indicating there a subset of defence genes are only differentially expressed in mature leaves (Figure 3.9 D).

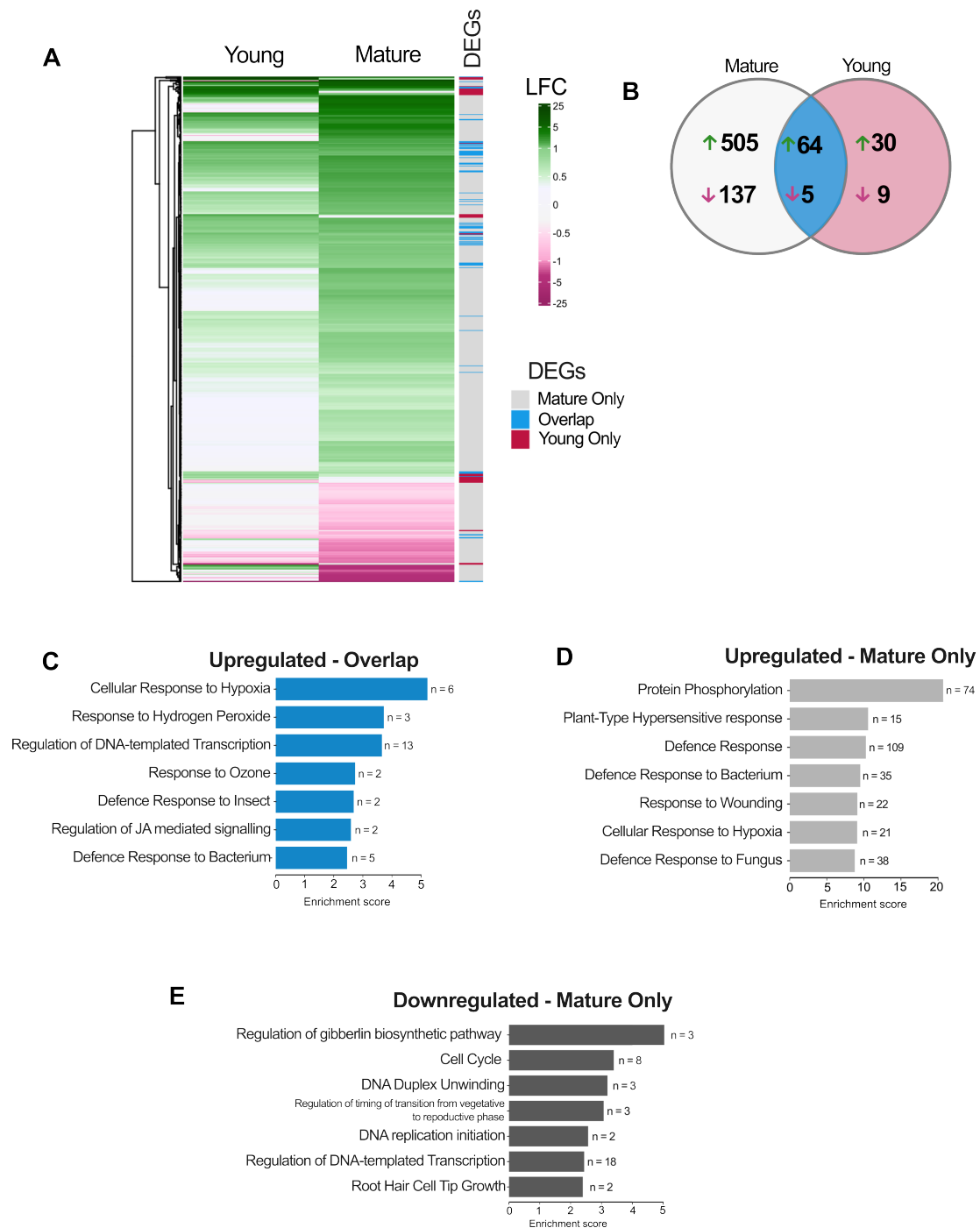


Figure 3.9: Differences in chitin-responsive genes in Mature and Young Tissue – (A) Heatmap of differentially expressed genes (DEGs) in response to chitin in both mature and young leaves. Heatmap annotations indicate where genes are significantly differentially expressed in mature only (grey), young only (Red) or in both young and mature (blue). The scale indicates $\log_2$ fold change (LFC) of gene expression. (B) A Venn diagram showing genes categorised into up-regulated (green up arrow) and down-regulated (red arrow) in mature (grey circle) and young (red circle), where the overlap indicates shared significantly differentially expressed genes. GO-term analysis of parts of the Venn diagram: (C) up-regulated-overlapping, (D) up-regulated-Mature Only, (E) down-regulated-Mature Only, where n indicates the number of DEGs in that category.

To identify processes specifically up-regulated in young leaf tissue, I explored the 30 genes only up-regulated in young leaves in response to chitin. On such a small number of genes, the GO-term analysis is ineffective as one gene annotated with multiple GO-terms can bias the analysis, or enriched GO-terms appear with the presence of just one gene. Therefore, I carried out a manual search for potentially interesting defence-, age- or signalling-related genes that were up-regulated only in young tissue. Out of the 30 genes, there are several genes of interest including *Ethylene Response Factor 1* (*EFR1*), involved in the ethylene signalling cascade; *Forked 1* (*FKD1*) and *ATP-binding cassette subfamily B4* (*ABCB4*), involved in auxin transport; and *WRKY DNA-binding protein 75* (*WRKY75*), a transcription factor found to regulate jasmonic acid defence in response to fungal pathogens (Chen et al., 2020). It is possible that some of the genes only up-regulated in young make-up an age-specific response.

The genes down-regulated in response to chitin in mature and young leaves share a similar trend to the up-regulated gene pattern, with fewer down-regulated genes in young leaves than mature (Figure 3.8, Figure 3.9 A-B). A GO-term analysis on the genes only down-regulated in response to chitin in mature leaves finds GO-terms such as “Cell Cycle” and “Regulation of DNA-templated transcription” down-regulated in response to chitin (Figure 3.9 E). This suggests downregulation of the cell cycle and gene expression-related processes occurs as part of a response to chitin in mature leaves. Only five genes were down-regulated in response to chitin in both mature and young tissue. A manual search revealed there were two uncharacterised genes: AT3G19680 and AT1G22570, as well as three known genes: *brz insensitive long hypocotyl 9* (*BIL9*), which promotes growth during drought stress through brassinosteroid signalling (Surina et al., 2024), *Trehalose-6-phosphatase synthase 8* (*TPS8*), involved in trehalose-6-phosphate synthesis (Nunes et al., 2013); *acyl activating enzyme* (*AAE2*), involved in the catalysis of CoA ester formation (Srividya et al., 2020). Given the small number of overlapping down-regulated genes, it is difficult to draw conclusions around this shared response in young and mature leave.

Several of the genes only down-regulated in young leaves have known stress response functions. Of the ten down-regulated genes specific to young tissue, previously characterised genes are: *Vacuolar Iron Transporter-like 1* (*VTL1*), a regulator of iron accumulation (Gollhofer et al., 2014); *Enhancer of RNA-dependent RNA polymerase 1* (*ENOR3*), proposed to be a magnesium transporter involved in antiviral defence in RNAi-based immunity (Gao et al., 2018; Zhu

et al., 2017); *Protein Phosphatase 2C 74*, which interacts with SnRK1 (Tsugama et al., 2012); *Serine carboxypeptidase-like 35 (SCPL35)* is an acyltransferase from a wider protein family suggested to be involved in stress responses (Liu et al., 2008; Milkowski & Strack, 2004); *Early-responsive to dehydration 4, ERD4*, which responds to drought and is involved in salt tolerance (Liu et al., 2009); *Suppressor of overexpression of CO 1 (SOC1)*, a key flowering transcription factor that coordinates flowering, but is not related to stress response; The other four genes down-regulated in young remain uncharacterised: AT5G08260, AT4G13505, AT4G04410, and AT5G09670. Although several of these genes have not been characterised in a defence-specific context, these genes may play a role in defence against pathogens. Further characterisation of these genes could help to determine their function in chitin-response young leaves.

Overall, the transcriptional differences in mature and young leaves in response to chitin suggest that young leaves have a smaller transcriptional response to chitin. Despite this, there are still several shared up-regulated defensive genes that are in both leaf ages. The large number of genes up-regulated only in mature leaves (505 genes) allows many more processes and genes to be identified as tissue-specific defence responses in mature leaves. It is possible some of these could be related to plasmodesmal closure, as they are only seen in mature tissue.

3.3.7 Identifying chitin response genes linked to plasmodesmal closure

I hypothesised several of the genes only up-regulated in response to chitin in mature Col-0 leaves and not in young Col-0 are related to plasmodesmal-closure. However, the differences in chitin response between Col-0 mature and young tissue are not just related to differences in plasmodesmal response. There could also be chitin-response genes that are tissue age-specific. To uncouple age effects from plasmodesmal closure effects, I performed a Likelihood Ratio Test comparing the chitin-treated tissue from Col-0 mature, Col-0 young, *lym2* mature, and *lym2* young leaves, assigning each sample a plasmodesmal variable of “open” or “closed” (Figure 3.10 A). Callose deposition occurs within 15 minutes of chitin infiltration and so at the 30-minute timepoint plasmodesmata are closed, or at least have reduced permeability, and so chitin-infiltrated Col-0 mature leaf samples were grouped under a plasmodesmata “closed” variable (Ohtsu, unpublished). Chitin-infiltrated *lym2* mature leaf, chitin-infiltrated Col-0 young leaf, and chitin-

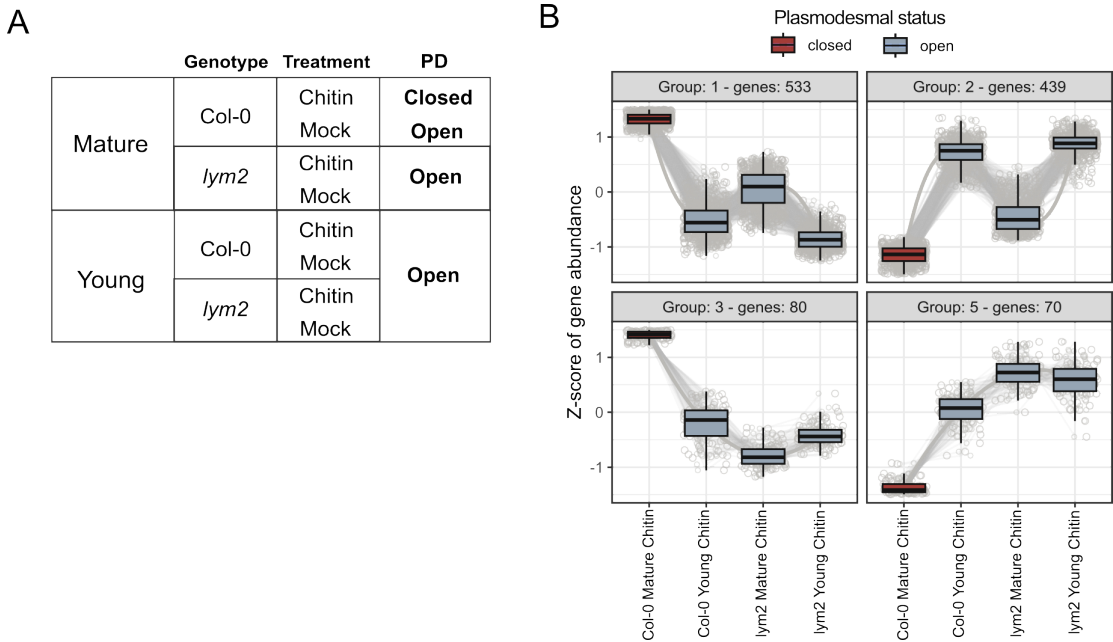


Figure 3.10: **Likelihood Ratio Test on Col-0 and *lym2* young and mature leaves** – (A) Table showing genotypes sampled and the corresponding plasmodesmal (PD) status in these treatments. (B) Boxplots of cluster groups identified in a Likelihood Ratio Test comparing gene abundance in samples with plasmodesmal closure (red) to samples where plasmodesmata are open (blue). Individual points represent the abundance of individual genes.

infiltrated *lym2* young leaf samples were grouped under the same plasmodesmal “open” variable, allowing me to compare responses when plasmodesmata are open with responses when plasmodesmata are open. Any age-specific chitin responses would be shared between Col-0 and *lym2* mature leaves, and so not picked up as differentially expressed between the plasmodesmal “open” and “closed” variables.

The Likelihood Ratio Test identified four cluster groups of interest (Figure 3.10 B). Groups 1 and 3 contained genes with higher abundance when plasmodesmata are closed in response to chitin compared to when plasmodesmata are open in response to chitin. Groups 2 and 5 contained genes with a lower abundance where plasmodesmata are closed in response to chitin.

To further explore these gene clusters, I combined Groups 1 and 3 to analyse genes up-regulated with chitin-induced plasmodesmal closure. Plotting the relative gene counts of these 613 genes illustrates their higher abundance in Col-0 chitin-treated mature leaf samples (Figure 3.11 A). A GO-term analysis reveals multiple enriched terms related to jasmonic acid signalling and synthesis (Figure 3.11 B). Other secondary metabolic pathways are also enriched with multiple GO-terms connected to alpha-amino acid metabolic processes such as “sulphate assimilation”

and “cysteine biosynthetic process”. Other interesting enriched terms include “starch catabolic process” and glucosinolate biosynthesis-related terms. Overall, the GO-term analysis reveals interesting chitin responses mainly related to secondary metabolism that are of a higher abundance where plasmodesmal closure is induced.

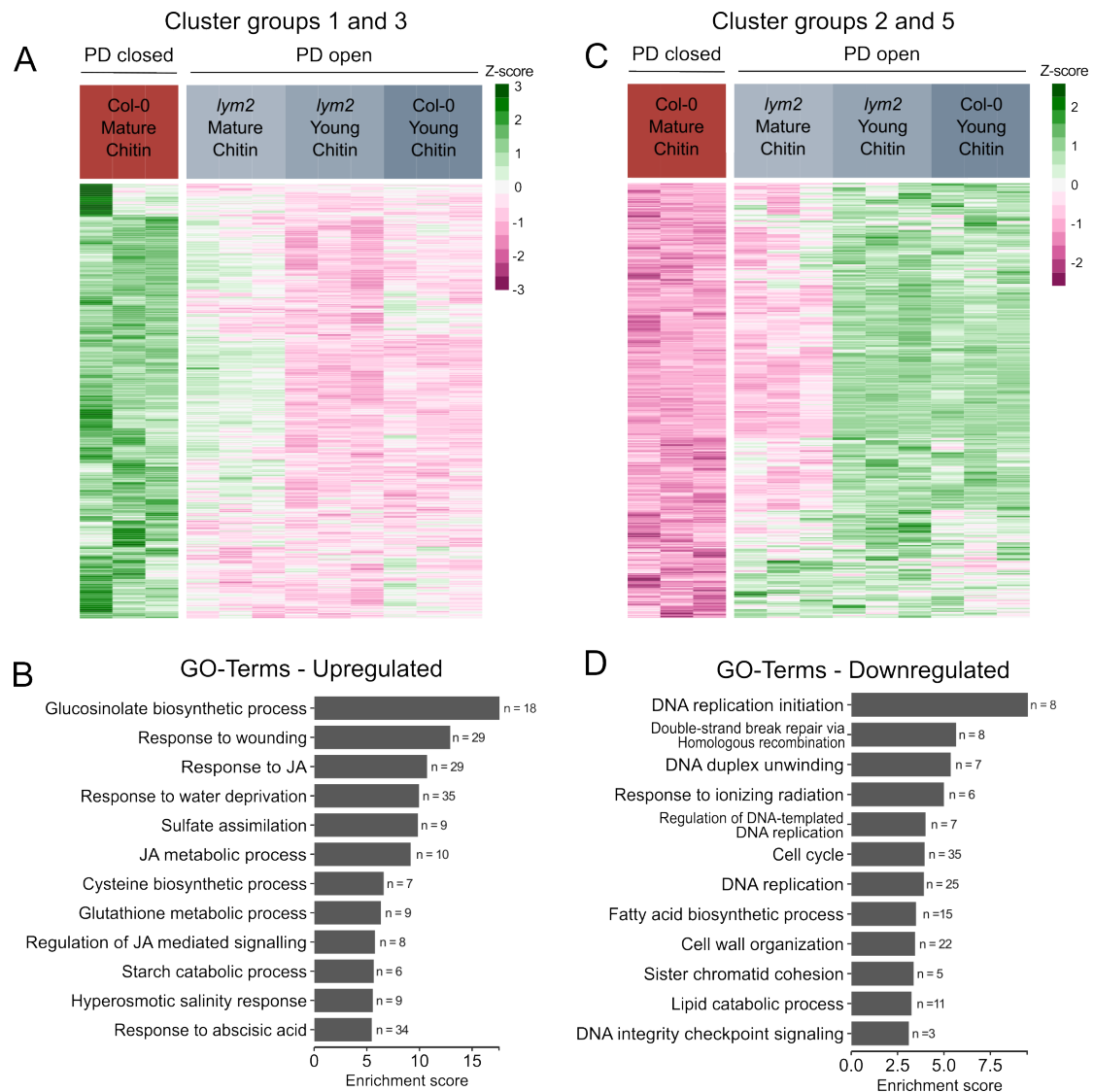


Figure 3.11: GO-term analysis on gene clusters in Col-0 and *lym2* – (A) Heatmap showing normalised gene counts from genes within cluster groups 1 and 3, where genes have a significantly higher abundance in chitin-treated samples where plasmodesmata are closed. Each column indicates a biological replicate, with three biological replicate per sample. (B) GO-terms enriched in cluster groups 1 and 3. (C) Heatmap showing normalised gene counts from genes within groups 2 and 5, where genes have a significantly lower abundance in chitin-treated samples where plasmodesmata are open. (D) GO-terms enriched in cluster groups 2 and 5.

I also explored the genes that exhibit lower abundance in Groups 2 and 5 to identify genes down-regulated in a plasmodesmal-closed chitin response. Combining Groups 2 and 5 reveals 509 genes of lower abundance in these clusters (Figure 3.11

C). Although the heatmap showed some similarities in abundance between Col-0 Mature and *lym2* mature samples in Group 2; I decided to continue grouping Groups 2 and 5 together, as this cluster was identified in the likelihood ratio test to show significant overall differences between plasmodesmal “open” and “closed” samples. The enriched GO-terms are similar to the genes revealed by a Wald test conducted earlier for differentially expressed genes found only in chitin-treated tissue from Col-0 Mature leaves (Figure 3.9 E), with common enriched GO-terms related to cell cycle and DNA replication. Other GO-terms found enriched in chitin-induced plasmodesmal closure are fatty acid and lipid related GO-terms such as “cell wall organisation” and “lipid catabolic process”. Overall, this suggests the downregulation of cell cycle processes as well as other fatty acid/cell wall-related processes in chitin-induced plasmodesmal closure responses.

3.3.8 Expression of plasmodesma-related genes

Young leaves have a chitin-induced transcriptional response as indicated by the upregulation of several defence-related genes. To investigate whether this transcriptional data might reveal mechanistic insights into why young leaves lack plasmodesmal closure, I compared the expression of key plasmodesmal-related genes in young and mature tissue. I collated a list of genes associated with the PAMP-triggered plasmodesmal signalling pathway. To investigate if any differences in baseline transcription could explain why plasmodesmata do not close in young leaves, I compared gene expression of these candidate genes between mock-treated mature leaves and mock-treated young leaves (Figure 3.12 A). This analysis showed the plasmodesmal-related genes are similarly expressed in young leaves and mature leaves, except for *CML41* and *NHL3*. *NHL3* showed a 0.6 log₂ fold increase in expression in mature tissue compared to young leaves, whilst *CML41* showed a 3.5 log₂ fold increase in expression in mature leaves compared to young leaves. The baseline expression of *NHL3* is still high in young leaves, suggesting the protein is likely to still be present in young leaves. Other plasmodesmal-related genes do not show differences in baseline expression between young and mature leaves. This indicates despite a lack of plasmodesmal closure in young leaves, key plasmodesmal-related genes are expressed in both mature and young tissue.

Along with this transcriptional analysis of gene expression, I also verified the expression of *LYM2* in young leaves using *pAtLYM2::LYM2-citrine* lines. As previously found in mature leaves, *LYM2-citrine* localises to the plasma mem-

brane in young leaves (Faulkner et al., 2013) (Figure 3.12 B). This supports the transcriptional data suggesting that *LYM2* is expressed in young leaves.

I also investigated differences in chitin-induced gene expression between young and mature tissues to determine if there were differences in chitin-induced differential expression of these defensive genes. I compared $\log_2$ fold changes in gene expression in response to chitin in mature leaf tissue to $\log_2$ fold changes in gene expression in response to chitin in young leaf tissue (Figure 3.12 C). I found that four genes are significantly up-regulated in mature leaves but not in young leaves: *CML41*, *LYM2*, *CERK1*, and *NHL3*. Many of these genes show positive log fold changes in both young and mature leaves but do not show significant (adjusted p-value < 0.05) increases in gene expression. Interestingly both chitin receptors *LYM2* and *CERK1* show increases in gene expression in response to chitin in mature leaves, but not in young leaves. Other genes such as the *Plasmodesmal localised protein 1 (PDLP1)* and *PDLP5* show no difference in gene expression after chitin induction. Overall, the changes in receptor expression could be involved in the differences in chitin response but given the baseline expression and plasma membrane localisation of the receptor *LYM2* in young leaf tissue (Figure 3.12 A and B) it is unclear whether this increased expression of the receptor in mature leaves in response to chitin can explain why plasmodesmal closure occurs.

As there are few differences between young and mature leaves in terms of plasmodesmal immune signalling related proteins I also examined genes associated with plasmodesmal callose synthesis and hydrolysis, i.e., callose synthases (CALs) and β ,1-3, glucanases (PDBGs), to determine if callose synthesis or degradation is up-regulated in young and mature leaves. *CALS8*, *PDBG1* and *PDBG4* have a significantly higher expression in young leaves compared to mature leaves (Figure 3.13), but none of these genes showed differential expression in response to chitin. The differences in the baseline turnover of callose could relate to differences in plasmodesmal permeability between young and mature leaves in response to PAMPs if these proteins were activated post-translationally after chitin treatment. It is unclear if these transcriptional differences are related to the lack of plasmodesmal response to PAMPs.

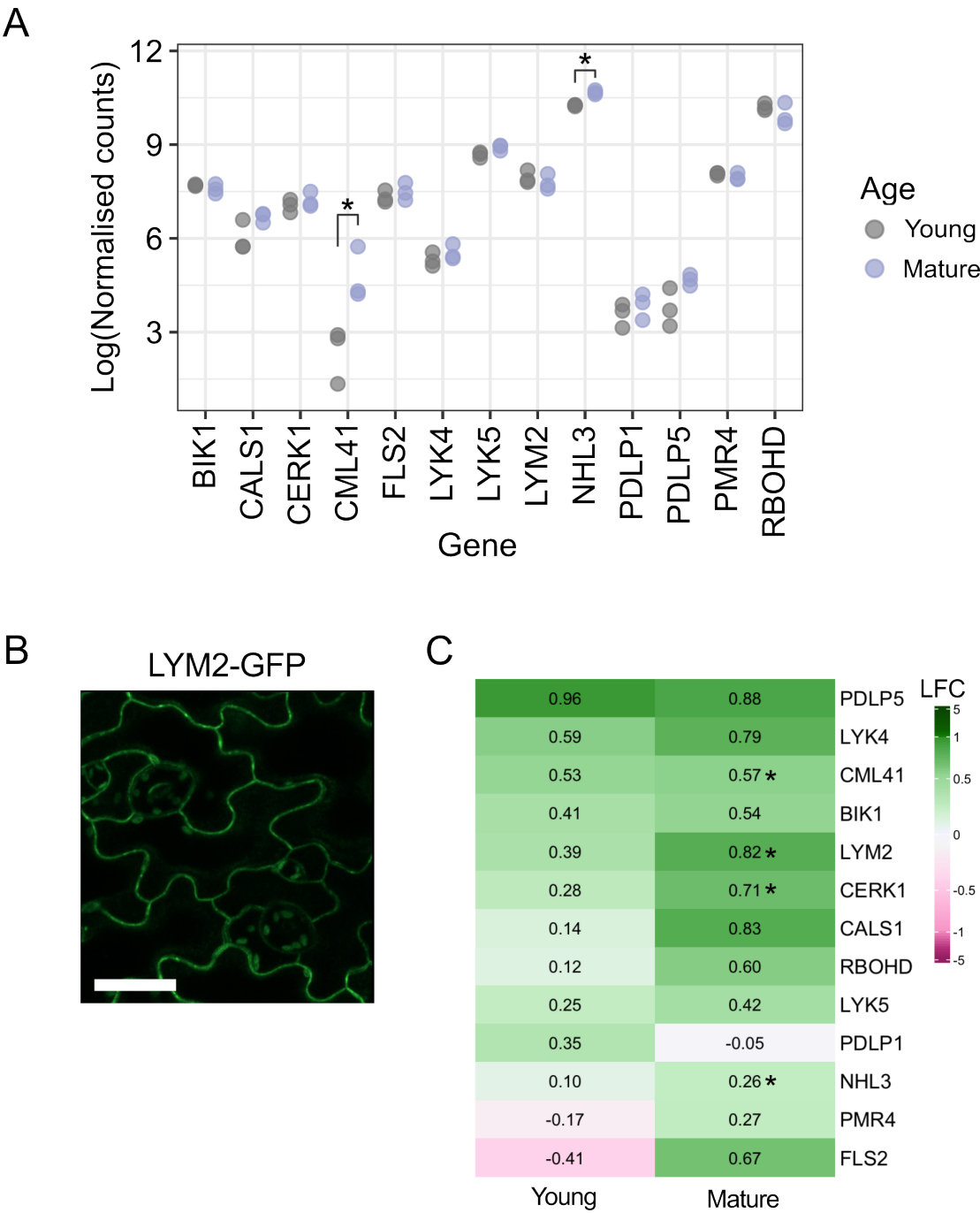


Figure 3.12: **Plasmodesmal and Defence-related gene expression** - (A) Normalised gene counts of defence-related genes expressed in Young and Mature samples in mock-treated samples, where “*” indicates an adjusted p-value < 0.05 for significantly differently expressed genes between young and mature mock-treated tissue. (B) *pAtLYM2::LYM2-citrine* expression in young leaves where the scale bar indicates 200 μ m. (C) Log₂ fold change (LFC) of differentially expressed genes in response to chitin in young and mature leaves, where “*” indicates an adjusted p-value of < 0.05 for significantly differentially expressed genes in response to chitin

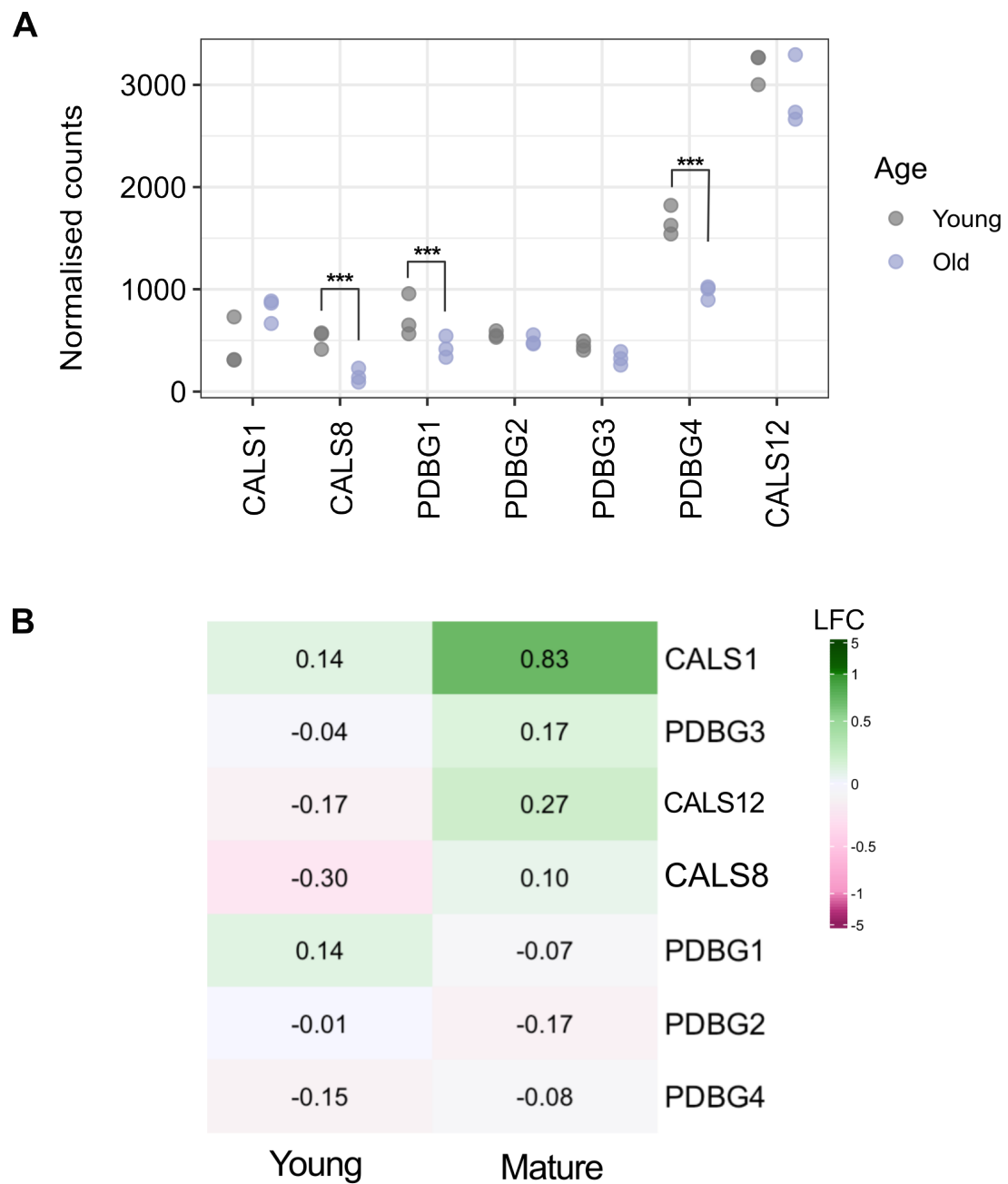


Figure 3.13: **Expression of Callose synthases and β ,1-3, glucanases** – (A) Expression of genes in young and mature leaves treated with mock. Each point represents the normalised counts from a single sample, $n=3$ for each age. Where the adjusted p -value $< 0.001 = “***”$, no label = no significant difference. (B) Differential expression of callose synthases and β ,1-3, glucanases in response to chitin, where the scalebar indicates $\log_2$ fold change

3.4 Discussion

3.4.1 Plasmodesmal response to stress in young leaves

In mature leaves, plasmodesmal permeability is reduced upon recognition of pathogen associated molecular patterns (PAMPs) (Cheval et al., 2020; Faulkner et al., 2013; Xu et al., 2017). Here, I characterised the plasmodesmal response to the fungal and bacterial PAMPs chitin and flg22, respectively, to show neither induces plasmodesmal closure in younger tissues (Figure 3.2). Other elicitors that trigger plasmodesmal closure in mature leaves, such as salicylic acid and hydrogen peroxide (Cui & Lee, 2016; Tee et al., 2023), also do not induce plasmodesmal closure in young leaves (Figure 3.3). This suggests the lack of plasmodesmal closure in response to stress is a general feature of plasmodesmal regulation in young leaves.

The reduced plasmodesmal permeability in mature leaves in response to PAMPs is due to increased callose depositions in the neck regions of plasmodesmata. I was not able to quantify plasmodesmal callose in young leaves due to the high callose contents in the cell walls of young leaves causing background noise meaning it was not possible to detect plasmodesmal callose deposits. In young leaves, I assumed the lack of reduction in plasmodesmal permeability means there is not a significant increase in callose deposition at plasmodesmata. This raises questions about why the presence of PAMPs does not trigger increased callose synthesis to reduce plasmodesmal permeability in young leaves.

Chitin and flg22 trigger plasmodesmal closure by binding to LYM2 and FLS2 receptors respectively (Faulkner et al., 2013; Xu et al., 2017). These receptors then trigger signalling cascades that converge into the same downstream pathway involving RBOHD which feeds into a PDLP-NHL3 complex (Figure 3.1) (Tee et al., 2023). Salicylic acid-induced plasmodesmal signalling then converges with FLS2- and LYM2-triggered signalling downstream of RBOHD (Tee et al., 2023). My results indicates that treating young leaves with elicitors of both signalling pathways is not sufficient to induce plasmodesmal closure in young leaves. It is possible the PDLP-NHL3 complex is not active in young leaves or that other unknown signalling components downstream of PDLP1/5-NHL3 are not active in young leaves to trigger PAMP-induced plasmodesmal closure.

The lack of hydrogen peroxide-induced plasmodesmal closure suggests multiple

plasmodesmal closure pathways are not active in young leaves. Hydrogen peroxide triggers plasmodesmal closure through an independent signalling pathway via a different callose synthase, CALS8, in mature leaves and does not require PDLP5, NHL3, or CALS1 (Cui & Lee, 2016; Tee et al., 2023). Given the independence of hydrogen peroxide-induced plasmodesmal closure from salicylic acid and PAMP-induced closure, one explanation is that an inhibitor of plasmodesmal responses acts on both pathways. For instance, it could be CALS1 and CALS8 activity are regulated by a shared factor blocking callose deposition at plasmodesmata in young leaves, but that does not block CALS12 induced macroscopic callose deposition. Further work on these pathways is required to determine if there are any shared regulators of these pathways in young leaves.

I also determined there is abscisic acid-triggered plasmodesmal closure in mature leaves but not young leaves. Previously abscisic acid had been linked to cell-to-cell mobility (Benitez-Alfonso, 2019). Here I show that abscisic acid treatment directly induces plasmodesmal closure in mature leaves, indicating that the accumulation of abscisic acid during pathogen response could trigger plasmodesmal closure.

3.4.2 Plasmodesmal-closure related gene expression in young leaves

To determine if the lack of plasmodesmal closure could be explained by changes to transcriptional regulation of the plasmodesmal signalling pathway I explored expression of specific genes in the chitin-signalling pathway. Overall, the data suggests key parts of the chitin signalling pathway are expressed in young leaves. *LYM2*, *RBHOD*, *CALS1*, *PDLP1*, and *PDLP5* are all similarly expressed in young and mature mock-treated leaf samples. Likewise, the *LYM2* receptor localises to the plasma membrane when tagged with mCitrine in young leaves. The expression of these genes raises questions as to why there is a lack of plasmodesmal closure despite all the components being present. One hypothesis is there could be post-transcriptional differences in protein synthesis or modification that abrogate function. For instance, the localisation of proteins such as *PDLP1* and *PDLP5* could be different in young leaves; if they were not localised to plasmodesmata potentially they do not induce plasmodesmal closure. Another alternative, as mentioned above, is that the machinery is present, but an additional factor is inhibiting a part of the signalling pathway to prevent plasmodesmal closure.

NHL3 was more highly expressed in mature leaf tissue than young leaf tissue, and more significantly up-regulated in response to chitin (LFC of 0.26) in mature leaves, with no significant change in response to chitin in young leaves (Figure 3.12). Given that *NHL3* is still expressed in young leaves, it is unclear whether this 0.26 LFC difference in expression would be enough to impact plasmodesmal closure signalling. Additionally, *CML41*, an important component of flg22 signalling has a lower expression in young leaves than mature, with low gene counts. This is interesting as it could link to the absence of plasmodesmal closure in young leaves in response to flg22. However, the lack of downstream plasmodesmal closure triggered by salicylic acid, which acts downstream of CML41 indicates that reduced CML41 is not enough to explain the lack of plasmodesmal closure in response to flg22. Overall transcriptional differences in plasmodesmal immune signalling components does not sufficiently explain the lack of plasmodesmal closure in young leaves.

Another hypothesis to explain differences in plasmodesmal regulation is that callose synthesis and degradation are differentially regulated in young leaves compared to mature leaves. I questioned whether the availability of the substrate for callose synthesis could limit callose deposition at plasmodesmata; however, the presence of large macroscopic callose deposits in young leaves indicates the glucose substrate is available for callose synthesis (Figure 3.6). Transcriptional data also suggests the difference in plasmodesmal response does not arise from the downregulation of callose synthases. *CALS1* is not differentially expressed between young and mature leaves, nor in response to chitin, and *CALS8* expression is higher in young leaves compared to mature leaves (Figure 3.13). I also hypothesised callose degradation is higher in young leaves. Plasmodesmal localised β -1,3-glucanases are associated with callose degradation at plasmodesmata (Benitez-Alfonso et al., 2013; Levy et al., 2007). The expression of the plasmodesmal localised β -1,3-glucanases *PDBG1* and *PDBG4* is higher in young leaves compared to mature leaves, but there is no significant differential expression in response to chitin. This indicates it is unlikely to be due to a mechanism where callose deposition is induced by callose deposition and then rapidly degraded at plasmodesmata through transcriptional up-regulation of the PDBGs. Overall, the data suggests regulation of expression of callose synthases and β -1,3-glucanases cannot account for the lack of plasmodesmal closure in young leaves. Further assays on callose synthase and β -1,3-glucanase activity could yield more conclusive results about the regulation of callose synthesis and degradation post-transcriptionally. Many questions remain about the mechanism controlling the lack of plasmodesmal closure in young leaves, although there is no clear indication that the transcriptional components of the

immune signalling pathway play a role

3.4.3 Common defence responses in mature and young leaves

My transcriptional data revealed that there are differences beyond plasmodesmal-related genes between young and mature leaves. Firstly, I found a much smaller transcriptional response to chitin in young leaves, with a 64 gene overlap in the differentially expressed genes between mature and young leaves. A GO-term analysis of this shared set of genes reveals a set of shared defence-related genes between young and mature leaves. This suggests there is a core set of defensive response genes between leaves of different ages. However, despite the overlap, there is a much larger number of genes only up-regulated in mature leaves, with many of these genes also involved in defensive processes. This suggests there are a range of defensive genes not up-regulated in young leaves. One hypothesis to explain this could be that the timings of defensive gene induction are different in mature and young tissue. Bjornson et al. (2021) find clear temporal patterns in gene induction post-PAMP treatment. Also, temporal transcriptomic analyses of chitin-treated mature leaves revealed different transcriptional responses at 30 minutes and 1 hour post-infiltration (Thomas, unpublished). If young leaves had a slower transcriptional activation than mature leaves it would mean the datasets may be out of phase with each other and could explain the smaller transcriptional response in young leaves at the time point harvested.

Another hypothesis for the dampened response is that plasmodesmal closure is critical to the activation of a strong transcriptional response. However, here I also collected data from *lym2* mutants and observed that mature *lym2* leaf tissue has a stronger transcriptional response to chitin than young leaf tissue. This indicates the lack of plasmodesmal closure in young leaves is likely not causing the dampened young leaf transcriptional response. Instead, there could be wider factors in young leaves causing different differential gene expressions in response to chitin.

Although the differences in plasmodesmal closure do not appear to be the cause of the smaller transcriptional response in young leaves, I used this dataset to pull out interesting gene expression trends associated with chitin-induced plasmodesmal closure. To uncouple the impact of age and plasmodesmal closure

on chitin response, I carried out a likelihood ratio test comparing gene abundance in young and mature Col-0 and *lym2* leaf samples treated with chitin. I find in plasmodesmal-closure specific response there are key secondary metabolism-associated genes with metabolic signalling such as jasmonic acid-related pathways and amino acid synthesis. This indicates these secondary metabolic processes may be related to plasmodesmal closure, either upstream or downstream of the closure response. Likewise, the downregulation of DNA replication and cell cycle genes perhaps indicates a diversion away from core cellular processes and a shift towards defensive processes upon plasmodesmal closure. Thus chitin-induced plasmodesmal closure may trigger downstream defensive responses.

Previously, Bjornson et al. (2021) suggested there are 39 Core Immune Response (CIR) genes that are differentially expressed in response to multiple PAMPs in mature tissues. The CIRs are suggested to be fundamental components of transcriptional pattern-triggered immunity response (Bjornson et al., 2021). I was interested to see if these CIRs were also up-regulated in mature and young leaves from my transcriptomic dataset. I first compared genes up-regulated in response to chitin in mature leaves to find ten of these CIR genes are also differentially expressed in the mature leaves. Only one of these CIR genes is up-regulated in young leaves (uncharacterised *Nac Domain Containing Protein 61*). The overlaps in mature leaves despite different experimental conditions support the idea of Bjornson et al. (2021) for a core transcriptional immune response, indicating it is a robust transcriptional module. However, the lack of CIR genes up-regulated in young leaves in response to PAMPs suggests these CIR genes may be specific to mature tissue types. This indicates that core transcriptional responses to PAMPs vary across leaf ages, and further research is needed to determine if there is a separate core immune response in young leaves.

3.4.4 General immune responses are triggered in young leaves

Given the absence of plasmodesmal closure triggered in young leaves and the differences in transcriptional responses between mature and young leaves, it is interesting I still find ROS bursts and macroscopic callose deposition are triggered by PAMPs in young leaf tissue. The presence of ROS bursts in young leaves supports the evidence that ROS bursts can occur in the absence of plasmodesmal closure. As discussed in the introduction to this chapter, ROS bursts have

previously been found to occur in the absence of plasmodesmal closure in *lym2* and *cml41* mutants, (Faulkner et al., 2013; Xu et al., 2017). In young leaves, I observed that ROS bursts are triggered by both flg22 and chitin in the absence of plasmodesmal closure. This supports previous findings on the independence of plasmodesmal responses and ROS bursts. It also indicates other general stress responses are activated in young leaves.

Interestingly, I found young leaves had a stronger ROS response than mature leaves. It is unclear whether this greater ROS response is due to a stronger immune response in young leaves, or an increased cell density with smaller cell size resulting in more cells producing ROS. Alternatively, the cell walls in young tissues might also take up a greater volume of reagents, leading to greater reactivity of the assay. Further assays quantifying cell density and size and ROS response across additional leaf ages could help to determine whether there is a correlation between measured ROS response and cell density.

In addition to ROS, macroscopic callose deposition was also induced in young leaves. Both macroscopic and plasmodesmal callose are synthesised by CALSs using UDP-glucose as a substrate, with CALS12 mediating macroscopic callose deposition and CALS1 mediating PAMP-triggered plasmodesmal callose deposition (Cui & Lee, 2016; Tee et al., 2023; Vogel & Somerville, 2000). The flg22-triggered macroscopic callose deposition in young leaves indicates the lack of plasmodesmal callose deposition in young leaves is not due to an inability of young leaves to supply the substrates required for callose synthesis.

Also this data supports the hypothesis that macroscopic and plasmodesmal callose deposition is regulated by different pathways. Instead, there may be other factors causing the lack of plasmodesmal closure in young leaves. This could occur via the activity of separate callose synthases being differentially regulated in the same developmental and environmental contexts. It is unclear why detached leaves show no macroscopic callose deposition in mature leaves, especially as detached mature leaves can deposit plasmodesmal callose as evidenced by microprojectile bombardment assays. Further experiments could be carried out to determine if changing the conditions in which detached leaves are kept before callose staining could impact the deposition of macroscopic callose.

3.4.5 Plasmodesmal regulation and a growth defence trade-off.

The main question following on from this chapter is why young leaves have no stress-triggered plasmodesmal closure whilst still inducing other pattern-triggered immune responses and a core set of transcriptional defensive genes. One reason could be the young leaves are balancing a trade-off in growth and defence. Plasmodesmata are important in the process of phloem unloading into young leaf tissue to provide sugars for growth and development. Inducing plasmodesmal closure may benefit defence against pathogens but could hinder growth. Macroscopic callose deposition has not been related to changes in phloem unloading, so the growth costs of macroscopic callose may not be as high as the costs of inducing plasmodesmal closure. In terms of the activation of ROS bursts in young leaves, it could be that ROS bursts activate downstream defensive responses that are beneficial in young leaf defence, although the growth costs of initiating ROS bursts to facilitate growth are undefined. Thus, the possibility exists that plasmodesmata play a more important role in the growth-defence trade-off than other pattern-triggered immune responses.

Chapter 4

Exploring the role of plasmodesmata in the trade-off between growth and defence in young leaves

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4.1 Introduction

Symplasmic connectivity is important to various plant processes such as the movement of photoassimilates from source to sink tissues and the cell-to-cell diffusion of mobile signals during tissue patterning. Conversely, symplasmic isolation also plays an important role in defence and differentiation. For instance, plasmodesmal closure enhances resistance against infection in pattern-triggered immunity, whilst some cell types require symplasmic isolation to facilitate cellular differentiation. Existing research has mainly focused on the role of plasmodesmata in either symplasmic connectivity or defence. This results in many unanswered questions regarding how plasmodesmal permeability is regulated in complex situations where there may be benefits to maintaining symplasmic connectivity for growth or development but also benefits to inducing plasmodesmal closure in terms of defence.

I hypothesise the lack of PAMP-triggered plasmodesmal closure in young leaves determined in Chapter 3 is due to the importance of maintaining symplasmic connectivity in young leaves. Phloem unloading in *Arabidopsis* sink leaves occurs symplasmically, with sugars moving out of the phloem and into surrounding tissues through plasmodesmata via bulk flow and diffusion as discussed in more detail in 1.4.2. Thus symplasmic phloem unloading relies on cell-to-cell connectivity and inducing plasmodesmal closure could restrict the symplasmic movement of sugars

and potentially hinder growth and development if apoplastic sugar transport was unable to compensate.

The apoplastic movement of sugars relies on the activity of cytoplasmic-apoplastic sugar transporters. For instance, the SUCROSE WILL EVENTUALLY BE TRANSPORTED (SWEET) family of proteins are sugar efflux proteins which transport sugars passively down the concentration gradient into the apoplast (Chen et al., 2010, 2012). For active transport, Sucrose Transporters/Sucrose Uptake Carriers (SUT/SUCs) actively transport sugars against the concentration gradient to build up sugars in the cytoplasm or apoplast (Sauer & Stolz, 1994). SUC2 is important in the transport of sucrose from the apoplast into companion cells to facilitate phloem loading in *Arabidopsis* source tissues. Other sugars in the form of the hexoses glucose, fructose and/or galactose can be transported in and out of the apoplast via SUGAR TRANSPORTER PROTEINS (STPs). Increased activity of these STPs is usually accompanied by increased activity of cell wall invertases (CWINs) and cell wall β -galactosidases that break down sucrose into hexoses (Büttner & Sauer, 2000; Fotopoulos et al., 2003; Poschet et al., 2010; Sturm & Chrispeels, 1990). Overall, the expression of different sugar transports can facilitate the apoplastic import and export of sugars into the cytosol as an alternative to symplasmic movement.

Currently, little is known about the flexibility of switching phloem unloading from symplasmic to apoplastic unloading in young leaves. In several plant species and tissue types, a switch between apoplastic and symplasmic unloading is observed at specific, pre-programmed points in development. For instance in fruits, sugar unloading switches from symplasmic to apoplastic at specific points during ripening (Manning et al., 2001; Zhang et al., 2004). This switch in loading strategies is mediated by an upregulation in apoplastic sugar transporters including SWEETs, SUCs and cell wall invertases (Guo et al., 2021; Manning et al., 2001; Vignault et al., 2005; Zhang et al., 2006). Beyond the context of sugar unloading, other switches to increase apoplastic transport can be mediated by pathogens. For instance, the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and the fungal pathogen *Golovinomyces cichoracearum* can induce the upregulation of SWEETs to increase the concentrations of sugars in the apoplast (Chen et al., 2010). Thus, sugar transport into the apoplast can be dynamically increased through increased expression of apoplastic sugar transporters. The question remains as to whether apoplastic phloem unloading could occur in sink leaves if symplasmic unloading is blocked. The answer to this question could offer insight into whether the lack

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of plasmodesmal response to stress in young leaves is related to an inability of the apoplastic unloading pathway to compensate for a reduction in symplasmic unloading.

The lack of plasmodesmal closure in young leaves determined in Chapter 3 also raises questions given the previously characterised potential defensive benefits of inducing plasmodesmal closure in mature leaves. As discussed in Section 1.3.3, impairing pathogen-associated molecular pattern (PAMP)-triggered plasmodesmal closure in mature leaves increases susceptibility to pathogens including the bacterial pathogens *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and *P. syringae* pv. *maculicola* (*Pma*), and the fungal pathogen *Botrytis cinerea* (Faulkner et al., 2013; Lee et al., 2011; Xu et al., 2017). The proposed reasons for the benefits of PAMP-triggered reductions in plasmodesmal permeability include preventing cell-to-cell spread of hypermobile effector proteins, limiting symplasmic movement of viral RNA, and enabling the accumulation of defensive signalling molecules such as salicylic acid (Cheval & Faulkner, 2018; Ohtsu et al., 2024; Tee et al., 2024; van Lent et al., 1991). Questions remain as to whether the defensive benefits of plasmodesmal closure in mature leaves also extend to young leaves.

Overall the role of plasmodesmata in growth and defence raises questions about whether there is a trade-off occurring in the regulation of plasmodesmal permeability in mature and young leaves. Trade-offs between growth and defence have previously been studied in many different developmental contexts and tissue types, with various hypotheses developed to explain the costs and benefits of defence versus growth. A wider overview of growth-defence trade-offs can be found in Section 1.5.1. Here I explore how previous research on the growth-defence trade-off relates to differences between young and mature leaves.

One theory – the optimal defence theory – suggests young tissue is more valuable than mature tissue, and thus more resources are invested in prioritising defence of young tissue (McKey, 1974). This higher value placed on young leaves is due to a higher concentration of carbon assimilates and nutrients invested in young leaves during growth, as well as their higher photosynthetic capacity (Berens et al., 2019; Harper, 1989). A meta-analysis by McCall and Fordyce (2010) supports the optimal defence theory by showing, in general, young leaves accumulate higher levels of energy-expensive defensive chemicals. Other evidence in *Arabidopsis* found young leaves invest more in resources in a stronger inducible defence response than mature leaves (Zeier, 2005). These findings suggest within the rosette there is variation in the defence response, which depends on balancing investment in

growth and defence to optimise fitness in young leaves.

Despite the optimal defence theory supporting the idea of resource investment in young leaves in a growth-defence trade-off, studies on leaf stage-related resistance raise questions about how applicable these theories are in the outcomes of plant-pathogen interactions. As introduced in Section 1.3.6, leaf stage-related resistance is a term used to describe when leaves of different ages within a plant are more or less resistant to pathogens. For instance, adult *Nicotiana Benthamiana* plants young leaves are more susceptible than adult leaves to the fungal pathogen *Sclerotinia sclerotiorum* and the bacterium *Pst* DC3000, but less susceptible than adult leaves to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* Xu et al. (2018). The concept of leaf stage-related resistance supports the idea of differences in defensive responses in young and mature leaves in the optimal defence theory; however, questions remain as to how the balance between growth and defence leads to real-world changes in leaf stage-related resistance.

Overall, as plasmodesmata play a role in both growth and defence, the regulation of plasmodesmata in response to different cues might also be tightly regulated through development. Currently, the majority of plasmodesmal research has focused on mature leaves, roots, and seedlings, with less focus on age-specific differences in plasmodesmal regulation across leaf ages. Despite the known roles of plasmodesmata in growth and defence, there has been little focus on plasmodesmal regulation where both defence and growth are occurring. Bringing together existing knowledge of trade-offs, plasmodesmal regulation, and findings from Chapter Three raises many questions on the role of plasmodesmata in the growth and defence trade-off.

Aims

In mature leaves, PAMP-triggered plasmodesmal closure is known to play an important role in defence, whilst in young leaves plasmodesmal connectivity is suggested to be important to symplasmic phloem unloading. I hypothesised that the age-specific plasmodesmal response to PAMPs discovered in Chapter Three is underpinned by different roles of plasmodesmata in growth and defence in young leaves. To test this hypothesis, I aim to explore the susceptibility to the biotrophic pathogen *Pst* DC3000 of young and mature tissue and determine how inducing plasmodesmal closure in young leaves influences this. I also used inducible plasmodesmal closure lines, *LexA::icals3m*, to manipulate plasmodesmal

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permeability in young leaves, to understand how inducing plasmodesmal closure influences phloem unloading and growth in young leaves. Additionally, I aim to complement these experimental approaches with an accompanying transcriptomics experiment to explore whether transcriptional processes are also altered with the induction of plasmodesmal closure in young leaves.

4.2 Materials and Methods

4.2.1 Inducing transgene expression with estradiol in leaves

Transgene expression in *LexA::icals3m* and *LexA::PLUG* lines was induced using estradiol treatment. To prepare the estradiol treatment, β -estradiol 17-acetate (Sigma-Aldrich) was first dissolved in dimethyl sulfoxide (DMSO) and then diluted with distilled water containing 0.0001% Silwet L-77 (De Sangosse) to achieve a final concentration of 20 μ M estradiol. A corresponding mock solution was prepared by diluting DMSO to a final concentration of 0.001% in water with 0.0001% Silwet L-77. Both abaxial and adaxial surfaces of young and mature leaves were painted with either the 20 μ M estradiol solution or the 0.001% DMSO mock solution. Following treatment, plants were incubated for 24 hours, as previous transcriptomic studies in *LexA::icals3m* and *LexA::PLUG* indicated that the toxicity response to estradiol diminishes within this time frame (Tee et al., 2024).

4.2.2 Leaf area measurements

The four youngest leaves (leaf one to four, in Figure 2.1) of five-week-old Col-0 and *LexA::icals3m* plants were painted abaxially and adaxially with 20 μ M estradiol or 0.001% DMSO (mock). Four days post-treatment, leaf area was measured. The curling of leaves after estradiol treatment made measuring leaf area whilst attached challenging, and so leaves were detached and flattened, so the whole leaf area could be photographed. Leaf area was quantified from images using FIJI (Scheidler 2012). A total of four plants were quantified per genotype, with four young leaves measured per plant. A three-way ANOVA was conducted to determine the effect of treatment (estradiol vs DMSO), genotype (Col-0 vs *LexA::icals3m*), and leaf number (one to four) on leaf area with an interaction term included to determine whether the effect of treatment on leaf area varied across different genotypes and leaf numbers. Following the three-way ANOVA, a Tukey HSD test was carried out to determine significant differences between treatments, genotypes, and leaf numbers.

4.2.3 Phloem-unloading of 5(6)-Carboxyfluorescein diacetate

Dye unloading assays were carried out using 5(6)-Carboxyfluorescein diacetate (CFDA) on five-week-old *Arabidopsis* plants. As outlined in Bellandi et al. (2022), 10 to 20 μ L droplets of 1 mM CFDA in water (Sigma Aldrich, C4916) were placed on mature source leaves. Mature source leaves were then wrapped in clingfilm to prevent the CFDA solution from evaporating. Loading dye onto all mature leaves increased the consistency of dye unloading in young leaves because, as outlined in Dengler (2007), certain leaves have closer vascular connections depending on their positioning in the vasculature. The abaxial side of unwrapped leaves was imaged 4 to 6 hours later using an Axio ZoomV16 stereomicroscope with a Zeiss Plan Z1.5 $\times$ /0.25, monochrome camera ORCA FLASH4 (C13440, Hamamatsu), and SPECTRA Light Engine (Lumencor). Leaves were illuminated with 463 nm to 487 nm light and fluorescence was collected with a 525/50 nm band-pass filter. Z-stack projections were generated using the Zeiss Blue v2.6 built-in software for the extended depth of focus wavelets method.

To investigate phloem unloading in young sink leaves with induced plasmodesmal closure, a dye-unloading assay was performed in *LexA::icals3m* lines. The second youngest leaf (leaf two in Figure 2.1) of five-week-old *LexA::icals3m* was painted with 20 μ M estradiol or 0.001% DMSO (mock). After 24 hours, CFDA dye-loading was carried out as described above in section 4.2.3. An image acquired was acquired of the left and right side of each leaf, and a minimum of seven different plants were imaged per leaf age per treatment.

To quantify phloem unloading of 5(6)-Carboxyfluorescein (CF), an image analysis pipeline was developed with Annalisa Bellandi as described in Bellandi et al. (2022). To analyse phloem unloading, 50 x 50 gridlines were scanned across each image, with each gridline creating a profile of intensity that was analysed using R (RCoreTeam, 2023) (Figure 4.13 A). A curve was fitted to the intensity data of each gridline, and the peaks and troughs of each curve were detected (Figure 4.13 B). For each peak, the width and maximum intensity were calculated and represents the detected movement of carboxyfluorescein out of the phloem and into the surrounding tissue. Code for this analysis can be found here: doi.org/10.5281/zenodo.6585010. This quantification method was developed in collaboration with Annalisa Bellandi. The script writing and execution in FIJI and R was carried out by Annalisa Bellandi. The mean peak widths between estradiol

and DMSO-treated leaves were compared using a Wilcoxon rank-sum test.

4.2.4 Pathoassay with *Pseudomonas syringae* DC3000

Pathoassays with *Pseudomonas syringae* DC3000 were carried out on five-week-old Arabidopsis Col-0 and *LexA::icals3m*. If required for the experiment, the three youngest leaves (leaves one to three) of *LexA::icals3m* and Col-0 plants were painted with 20 μ M estradiol or 0.001% DMSO 24 hours before inoculation.

To prepare the bacterial suspension for inoculation, *P. syringae* DC3000 was first grown at 28 °C on Lysogeny broth (LB) agar with 50 μ g/mL Rifamycin. After 48 hours, a single colony was picked and cultured overnight at 28°C in liquid LB with 50 μ g/mL Rifamycin. The overnight culture was pelleted, washed with 10 mM MgCl₂, and diluted to a concentration of 5×10^4 CFU/mL. The young leaves treated with estradiol or DMSO were syringe-infiltrated with the 5×10^4 CFU/mL bacterial suspension. Four days after inoculation, leaf discs were harvested using a 5 mm corer and placed into 300 μ L of water. Six leaf discs from three plants were pooled during harvesting to make up one biological replicate. Leaf samples were lysed in water at 1100 rpm for 2 minutes using a Geno/Grinder (SPEX SamplePrep2010). A dilution series was then carried out and each dilution step was plated out with three technical replicates for each biological replicate. Plates were incubated for 48 hours at 27°C before colonies were counted and the concentrations of bacterial colonies were calculated in Colony Forming Units (CFU).

In the assay comparing Col-0 and *LexA::icals3m* susceptibility after estradiol vs DMSO treatments, data from three separate experimental replicates was pooled to make a total of 12 biological replicates for each treatment in Col-0 plants and 20 biological replicates for each treatment in *LexA::icals3m* plants. Data was analysed using a linear mixed-effects model comparing the impact of genotype and treatment on the log of CFU, with the experimental replicate included as a nested random variable using the lmerTest R package (Kuznetsova et al., 2017). Significant differences between genotypes and treatments were determined in a post-hoc Tukey pairs comparison.

In the assay comparing Col-0 young and mature leaf susceptibility, the experiment was replicated three times, with four biological replicate samples per experiment for a total of twelve samples. Data was analysed using a linear

mixed-effects model comparing the impact of age on the log of CFU, with the experimental replicate included as a nested random variable using the lmerTest R package (Kuznetsova et al., 2017).

4.2.5 Transcriptional response to plasmodesmal closure and chitin treatment

The transcriptional response to inducing plasmodesmal closure and chitin treatment was compared in Col-0 and *LexA::icals3m* lines. Young leaves of Col-0 and *LexA::icals3m* lines were painted with 20 μ M estradiol or 0.001% DMSO (mock). 24 hours later leaves were syringe-infiltrated with a chitin (500 μ g/mL chitin in distilled water) or mock (distilled water) solution. Leaf tissue samples were harvested 1 hour after elicitor treatment. Six young leaves from three plants were pooled per biological replicate, and five biological replicates were collected for each treatment-elicitor-genotype combination.

As described in more detail in Section 2.5, RNA was extracted and sequenced by Genewiz (Azenta Life Sciences). Gene expression analysis was performed on gene read counts using DESeq2 in R studio (Love et al., 2014; RCoreTeam, 2023).

4.3 Results

4.3.1 Young leaves are more susceptible to *Pst* DC3000 than mature leaves

Previously young leaves have been found to show different levels of resistance to pathogens than mature leaves in leaf stage-related resistance. I performed a pathoassay to investigate whether young leaves are more susceptible than mature leaves to *Pst* DC3000 under my experimental condition. Four days after inoculation, young leaves appeared less healthy than mature leaves with visible areas of chlorosis, as well as the appearance of lesions suggesting cell death (Figure 4.1 A and B). Assays of bacterial growth showed that four days post-inoculation young leaves had significantly higher numbers of bacterial CFU than mature leaves (Figure 4.1 C). This suggests young leaves are more susceptible to *Pst* DC3000 than mature leaves.

4.3.2 Estradiol treatment of *LexA::icals3m* lines induces plasmodesmal closure in young leaves

To investigate how plasmodesmal closure impacts susceptibility to *Pst* DC3000 in young leaves, I first tested whether I could manipulate plasmodesmal permeability in young leaves using *LexA::icals3m* lines. *LexA::icals3m* lines have inducible plasmodesmal closure through the expression of an overactive *callose synthase 3* (*Cals3*) allele under the estradiol-inducible *XVE* chimeric promoter and *LexA* operator (Bellandi et al., 2022; Tee et al., 2024; Vatén et al., 2011). Estradiol-induced plasmodesmal closure in *LexA::icals3m* lines has previously been characterised in mature leaves and seedlings, and so I performed a cell-to-cell mobility assay to determine if estradiol also induces plasmodesmal closure in young leaves of *LexA::icals3m* lines (Bellandi et al., 2022; Tee et al., 2024). Young leaves from *LexA::icals3m* plants were treated with estradiol or DMSO (mock) 24 hours before microprojectile bombardments. A minimum of 39 bombardment sites were imaged per treatment and age combination. Estradiol treatment resulted in a significant reduction in the normalised number of cells showing GFP signal compared to DMSO treatment in both young and mature tissue (Figure 4.2 A and B). Thus, the *LexA::icals3m* expression system induces reduced plasmodesmal permeability

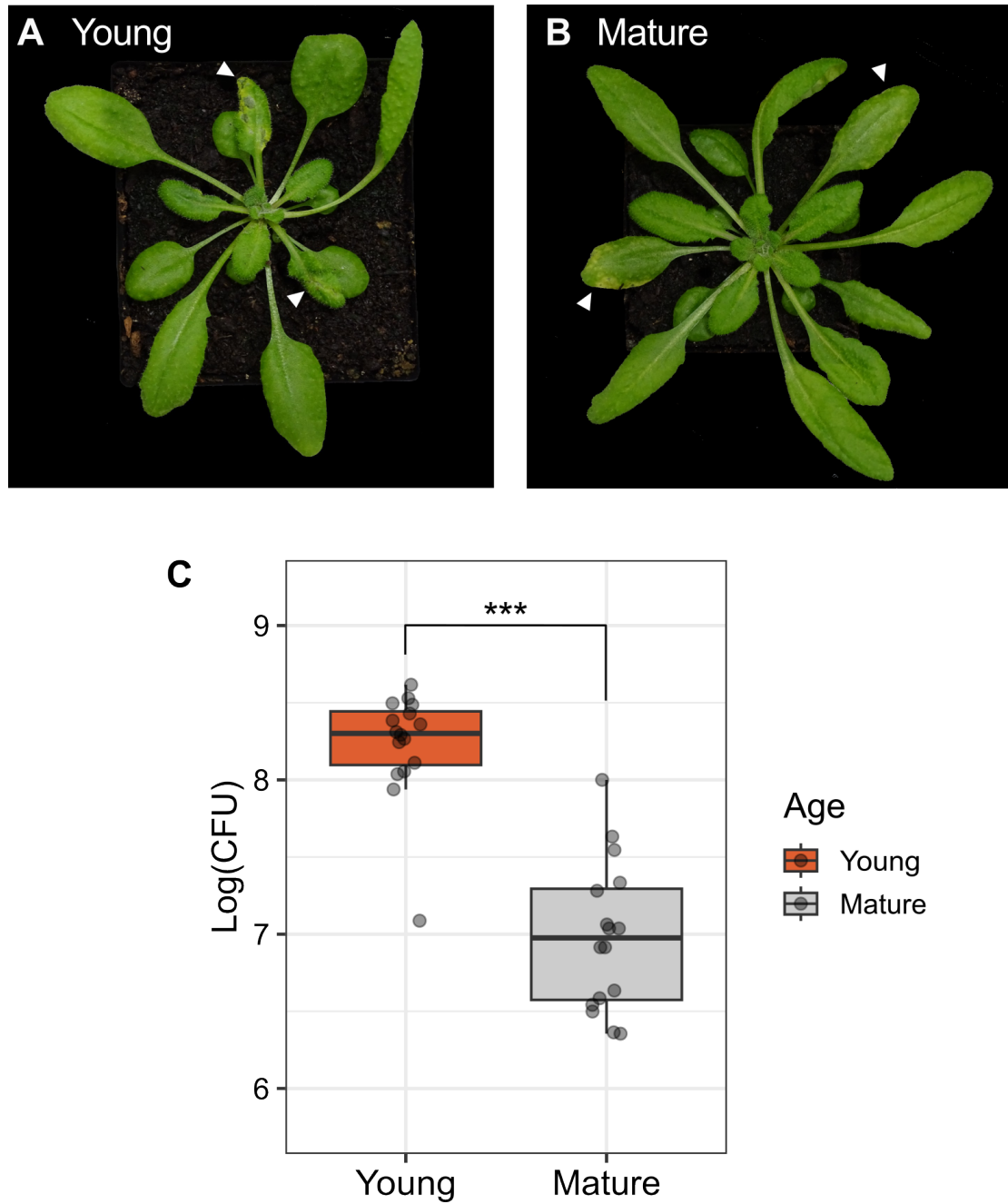


Figure 4.1: **Susceptibility of young and mature leaves to *Pst* DC3000** – Images of young (A) and mature (B) Col-0 leaves four days post inoculation with *Pst* DC3000, where the white arrows indicate infiltrated leaves on each plant. (C) Colony Forming Units (CFU) of *Pst* DC3000 in young (orange) and mature (grey) Col-0 leaves four days post-inoculation. Points show individual biological replicates and leaf ages were compared in a linear mixed-effects model. $p < 0.001 = \text{“***”}$.

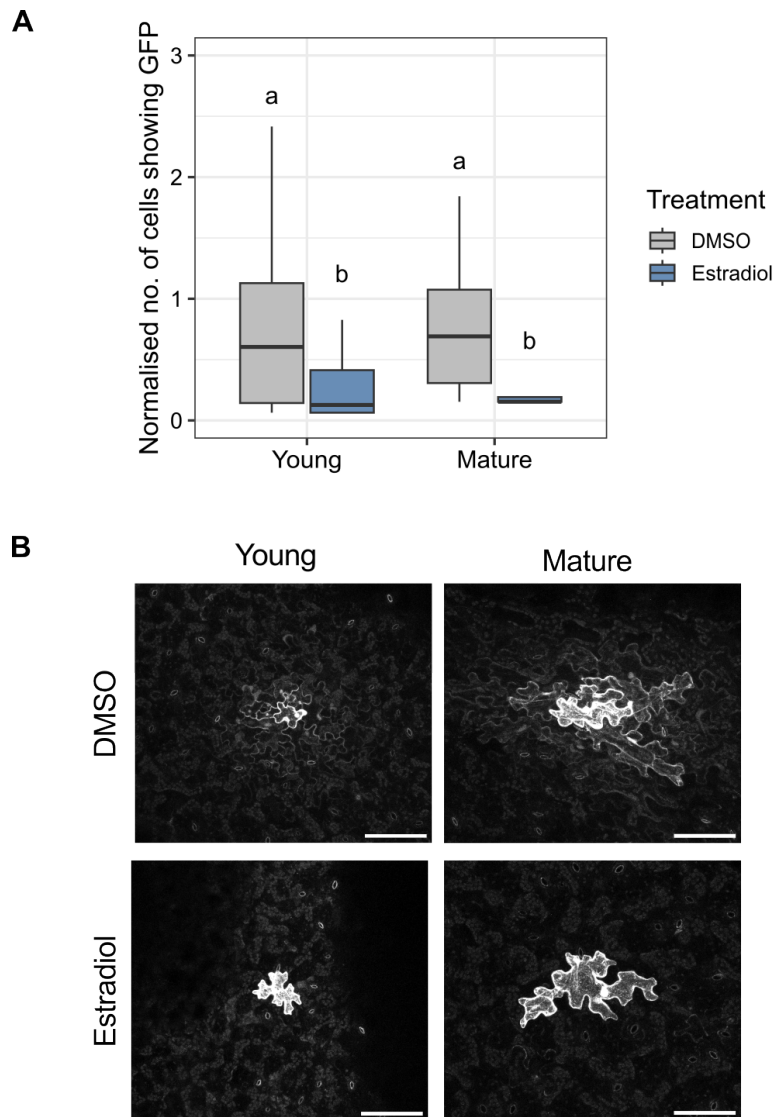


Figure 4.2: **GFP mobility assay on *LexA::icals3m* leaves** – (A) Cell-to-cell movement of GFP in *LexA::icals3m* leaves treated with either DMSO (grey) or estradiol (blue). Different letters indicate significant differences in a bootstrapping analysis of the medians. Confocal z-stack projections of GFP movement in DMSO (B and C) and estradiol (D and E) treated young (B and D) and mature (C and E) leaves. Scale bars indicate 100 μ m.

in young leaves.

4.3.3 Inducing plasmodesmal closure increases resistance to *Pst* DC3000 in young leaves

To determine whether inducing plasmodesmal closure in young leaves impacts susceptibility to *Pst* DC3000, I performed a pathoassay on young leaves with

induced plasmodesmal closure. I treated young leaves from Col-0 and *LexA::icals3m* plants with DMSO (mock) or estradiol and inoculated with *Pst* DC3000 24 hours later.

Four days post-inoculation, estradiol-treated *LexA::icals3m* young leaves had significantly less bacterial growth than DMSO-treated young leaves (Figure 4.2). This suggested inducing plasmodesmal closure in *LexA::icals3m* reduces susceptibility to *Pst* DC3000 in young leaves. Additionally, I observed no differences in susceptibility between Col-0 estradiol-treated leaves compared to Col-0 DMSO-treated leaves, indicating estradiol treatment itself has no impact on susceptibility to *Pst* DC3000. There were also basal differences in susceptibility between Col-0 and *LexA::icals3m* leaves, with DMSO-treated *LexA::icals3m* having significantly lower bacterial growth than DMSO-treated Col-0 (Figure 4.2). This indicates *LexA::icals3m* has lower baseline susceptibility to *Pst* DC3000 relative to Col-0.

Overall, the reduced susceptibility in *LexA::icals3m* young leaves with induced plasmodesmal closure compared to *LexA::icals3m* young leaves without plasmodesmal closure raises an important question about why young leaves do not show PAMP-triggered plasmodesmal closure given the potential defensive benefits.

4.3.4 Estradiol treatment induces *LexA::icals3m* expression in young leaves

To further understand the impact of inducing plasmodesmal closure on young leaves, I transcriptionally profiled the response of young leaves to the induction of plasmodesmal closure. I painted young leaves of Col-0 and *LexA::icals3m* lines with estradiol or DMSO, and 24 hours later infiltrated leaves with a chitin or water solution, and harvested 1 hour after elicitor treatment (Table 4.1). I used a Wald test to extract differences in gene expression between genotype, treatment, and elicitor, incorporating all Col-0 and *LexA::icals3m* samples into the analysis. Differentially expressed genes were identified based on a threshold of $\log_2$ fold change of greater than 0.5 or less than -0.5 and an adjusted p-value of less than 0.05.

To first verify the effectiveness of my treatments and controls on *icals3m* expression, I investigated gene expression levels in estradiol- and DMSO-treated plants. Since reads of the native *CALS3* allele and the *icals3m* allele are indistinguishable, normalised counts of *CALS3* were used to represent the expression of both alle-

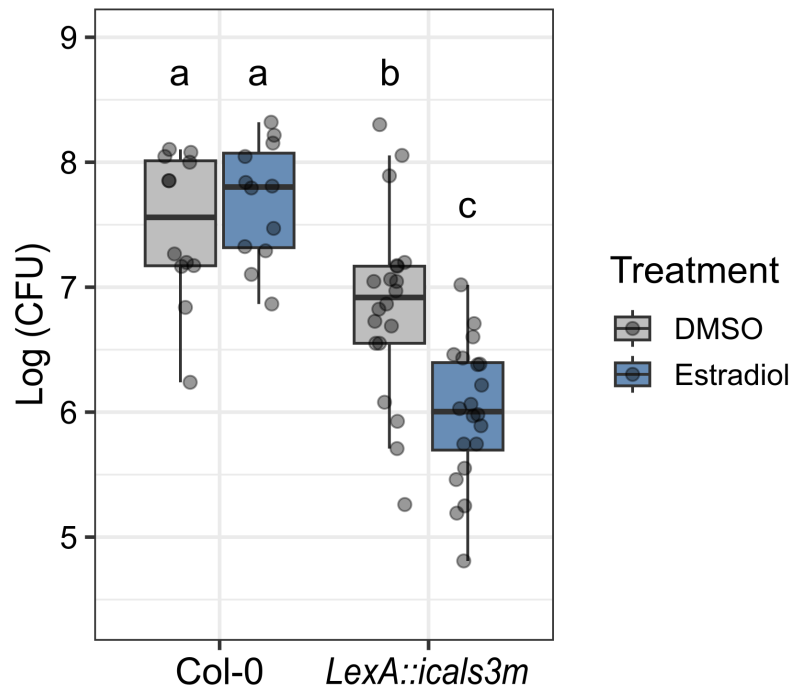


Figure 4.3: **Pathoassay on Col-0 and *LexA::icals3m* young leaves** – young leaves from Col-0 and *LexA::icals3m* plants were treated with either DMSO (grey) or estradiol (blue) and infiltrated with *Pst* DC3000. Different letters indicate significant differences ($p < 0.05$)

Genotype	Treatment	Elicitor	Plasmodesmata
Col-0	Estradiol	Chitin	Open
		Water	
	DMSO	Chitin	
		Water	
icals3m	Estradiol	Chitin	Induced closure
		Water	
	DMSO	Chitin	Open
		Water	

Table 4.1: **Transcriptomic treatment, genotype, and elicitor combinations** - Leaves from Col-0 and *LexA::icals3m* genotypes were painted with estradiol or DMSO and 24 hours later infiltrated with either chitin or water (mock). Leaves were harvested one hour after infiltration with chitin or water. The “Plasmodesmata” column indicates where plasmodesmal closure is induced with estradiol treatment in *LexA::icals3m* lines.

les. In Col-0, *CALS3* was expressed at low background levels in samples treated with either DMSO or estradiol (Figure 4.4 A). As Col-0 lacks the *LexA::icals3m* construct, this measured *CALS3* expression in Col-0 should only represent the expression of the native *CALS3* construct.

In *LexA::icals3m* lines, estradiol treatment induces a large increase in the expression of *CALS3/icals3m*, with a 45-fold increase in *CALS3* normalised counts compared to DMSO treatment. This indicated estradiol treatment was effective at activating the *LexA::icals3m* expression in young leaves, supporting the reduction in plasmodesmal permeability observed after estradiol treatment 4.2. Interestingly, the background expression of *CALS3/icals3m* was 3.4-fold higher in *LexA::icals3m* lines treated with DMSO compared to Col-0 lines (Figure 4.4). It is unclear whether this was due to higher background expression of the native *CALS3* allele or leaky expression of *icals3m* from the *LexA::icals3m* construct. Nevertheless, previous work from Tee et al. (2024) showed increased baseline levels *CALS3/icals3m* expression does not significantly impact plasmodesmal permeability, as no differences in cell-to-cell mobility of GFP were observed between *LexA::icals3m* and Col-0 in mature leaves. Overall, *LexA::icals3m* has a higher baseline of *CALS3/icals3m* expression than Col-0, but estradiol induces increased expression of *CALS3/icals3m* many folds higher than DMSO in *LexA::icals3m*. This supports the evidence for estradiol-induced reduction of plasmodesmal permeability in young leaves of *LexA::icals3m* treated with estradiol compared to DMSO as seen in Figure 4.2.

4.3.5 Impact of estradiol treatment on gene expression in Col-0

To determine if there were any background effects of estradiol treatment unrelated to plasmodesmal closure, I explored the impact of estradiol on gene expression in Col-0. Previously, in mature leaves, Tee et al. (2024) found a small estradiol effect on gene expression 24 hours after application, identifying 14 genes associated with estradiol treatment. To determine whether the estradiol effect is also small in young leaves, I compared gene expression between the DMSO- and estradiol-treated Col-0 samples that had been infiltrated with water. This revealed 7 genes were up-regulated, and 11 genes were down-regulated in response to estradiol in Col-0 (Figure 4.5). None of these genes overlapped with the previously identified differentially expressed genes in response to estradiol from Tee et al. (2024),

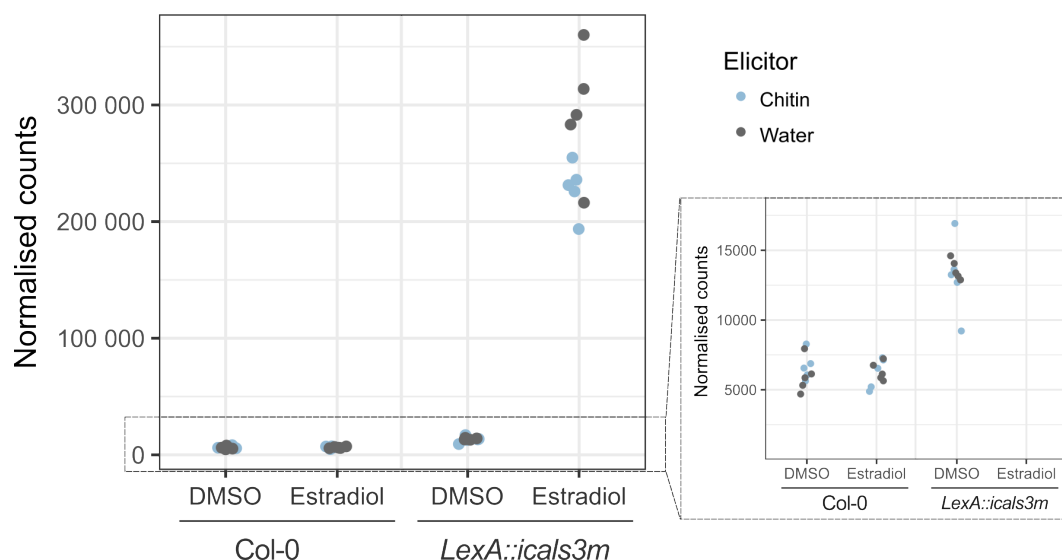


Figure 4.4: **Transcriptional response to estradiol treatment in leaves of Col-0 and *LexA::icals3m*** – Normalised counts of *Callose Synthase 3* (*CALS3*) across samples treated with DMSO or Estradiol with both chitin (blue) and water (mock, grey) infiltrated samples

indicating the impact of estradiol on gene expression in Col-0 young leaves is equally small but potentially different to in mature leaves.

Interestingly, there appeared to be an interactive effect between chitin treatment and estradiol treatment in Col-0. There were 166 differentially expressed genes in response to chitin in estradiol-treated tissue, but only 18 differentially expressed genes in response to chitin in DMSO-treated tissue (Figure 4.5). A Gene Ontology (GO)-term analysis of up-regulated genes in response to chitin within estradiol-treated tissue showed the top three enriched GO-terms were response to abscisic acid, response to jasmonic acid, and response to toxic substance. This indicates estradiol and chitin treatment potentially have an interactive effect, resulting in a stress-related transcriptional response that is not seen in DMSO-treated Col-0 infiltrated with chitin.

4.3.6 Inducing plasmodesmal closure triggers stress responses in young leaves

To first explore how induced plasmodesmal closure affects gene expression in young leaves, I identified genes that were significantly differentially expressed after the induction of plasmodesmal closure. I performed a likelihood ratio test comparing all samples that were infiltrated with water to focus on plasmodesmal closure in

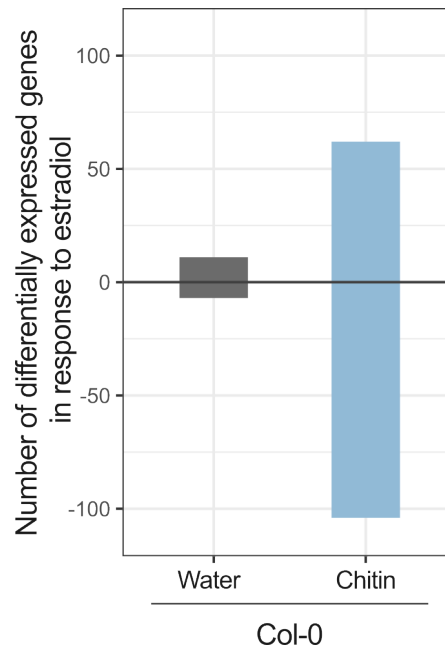


Figure 4.5: **Number of differentially expressed genes in response to estradiol** - up-regulated and down-regulated genes in response to estradiol in Col-0 samples infiltrated with mock (grey) or chitin (blue)

the absence of the chitin elicitor. I split these water-infiltrated samples into two groups; where plasmodesmata are closed (estradiol-treated *LexA::icals3m*), and where plasmodesmata are open (DMSO-treated *LexA::icals3m*; DMSO-treated Col-0; and estradiol-treated Col-0) (Table 4.1). Including estradiol-treated Col-0 samples allowed me to factor in any estradiol toxicity effects into the analysis. The cluster analysis revealed three gene expression clusters: Group 1, Group 2 and Group 3 (Figure 4.6 A). Group 1 contains genes that are more abundant in estradiol-treated *LexA::icals3m* where plasmodesmata are closed, while Groups 2 and 3 contain genes that are less abundant in estradiol-treated *LexA::icals3m* where plasmodesmata are closed.

An analysis of enriched GO-terms in Group 1, containing genes that are more abundant in response to plasmodesmal closure, revealed genes associated with defence signalling processes such as response to jasmonic acid, glucosinolate biosynthesis, and response to bacterium are up-regulated with plasmodesmal closure (Figure 4.6 B). A GO-term enrichment analysis on Groups 2 and 3, containing genes that are less abundant when plasmodesmata are closed, suggests a downregulation of translational processes (Figure 4.6 C). Enriched GO-terms include rRNA processing, translational elongation and protein folding, as well as several chloroplast-related terms with terms for chloroplast organisation and RNA processing enriched. Supporting this, I performed an additional KEGG

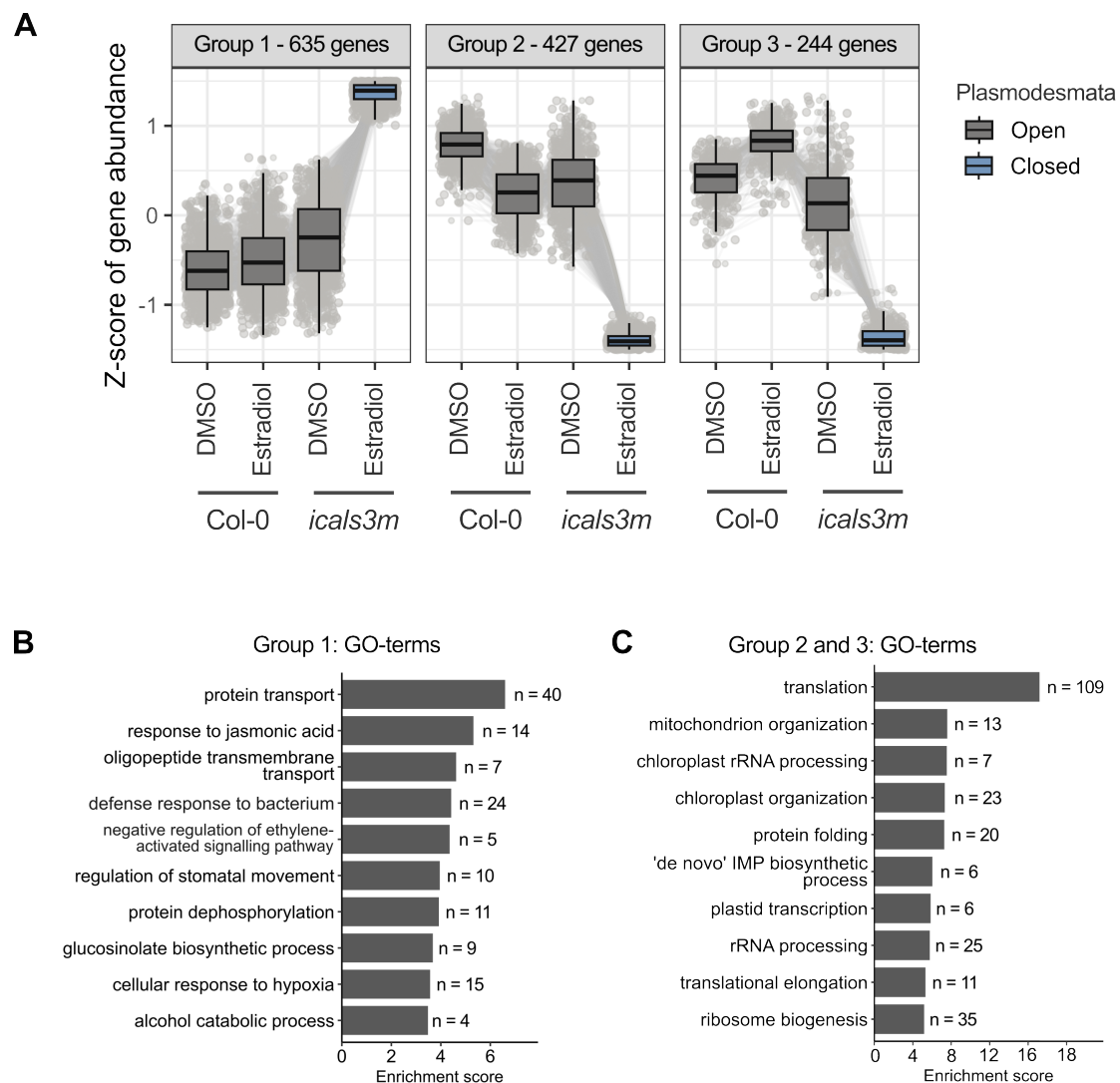


Figure 4.6: **Inducing plasmodesmal closure triggers stress response in young leaves** – (A) Cluster groups identified in the Likelihood Ratio Test, comparing gene abundance in samples with plasmodesmal closure (open) to samples where plasmodesmata are closed (blue). Individual points represent the abundance of individual genes. GO-term enrichment analysis on Group 1 (B) and Groups 2 and 3 combined (C), where n = the number of genes associated with that GO-term.

(Kyoto Encyclopaedia of Genes and Genomes) profile analysis on the genes in Groups 2 and 3 to determine enriched pathways. There is significant enrichment of 4 pathways: ribosome (ath03010), purine metabolism (ath00230), pyrimidine metabolism (ath00240), and nucleotide metabolism (ath01232). The GO-terms and KEGG analysis suggest a downregulation of translation and RNA metabolism in response to plasmodesmal closure.

4.3.7 Inducing plasmodesmal closure increases the transcriptional response to chitin in young leaves

In chapter 3, I showed plasmodesmal closure is not induced in response to PAMPs in young leaves. In this chapter, however, my results suggest inducing plasmodesmal closure in young leaves reduces resistance to *Pst* DC3000 (Figure 4.3). To further understand how plasmodesmal closure affects PAMP response, I explored the effect of inducing plasmodesmal closure on the transcriptional response to chitin. I performed a Wald test to compare genes that are differentially expressed in response to chitin when plasmodesmata are closed (*LexA::icals3m* estradiol treatment, chitin vs water) and when plasmodesmata are open (*LexA::icals3m* DMSO treatment, chitin vs water). Thresholds for differential gene expression were set as a log₂ change of greater than 0.5 or less than -0.5 and an adjusted p-value of less than 0.05.

The differential expression analysis indicated there was a stronger transcriptional response to chitin treatment in young leaves 24 hours after the induction of plasmodesmal closure compared to samples with no plasmodesmal closure. There were 503 up-regulated genes in response to chitin when plasmodesmata were closed (Figure 4.7 A). In contrast, there are only 47 up-regulated genes in response to chitin when plasmodesmata are open. There was a subset of 38 shared up-regulated genes between samples where plasmodesmata are open and closed. These shared genes could be core chitin response genes in young leaves that are not impacted by changes in symplasmic connectivity. Overall this analysis identified three subsets of differentially expressed genes in response to chitin: genes up-regulated when plasmodesmata are closed, genes up-regulated when plasmodesmata are open, and genes up-regulated regardless of plasmodesmal permeability.

A GO-term analysis was performed to identify enriched GO-terms in response to chitin across the three subsets of differentially expressed genes. All three subsets have enrichment of defence-related GO-terms such as response to chitin and response to fungus (Figure 4.7). However, a much larger range of defensive genes are up-regulated when plasmodesmata are closed.

A similar trend is seen amongst the down-regulated genes, with 134 differentially expressed genes specific to when plasmodesmata are closed, and 53 differentially expressed genes specific to when plasmodesmata are open (Figure 4.7 A). A GO-term analysis showed the enrichment of a range of down-regulated processes in

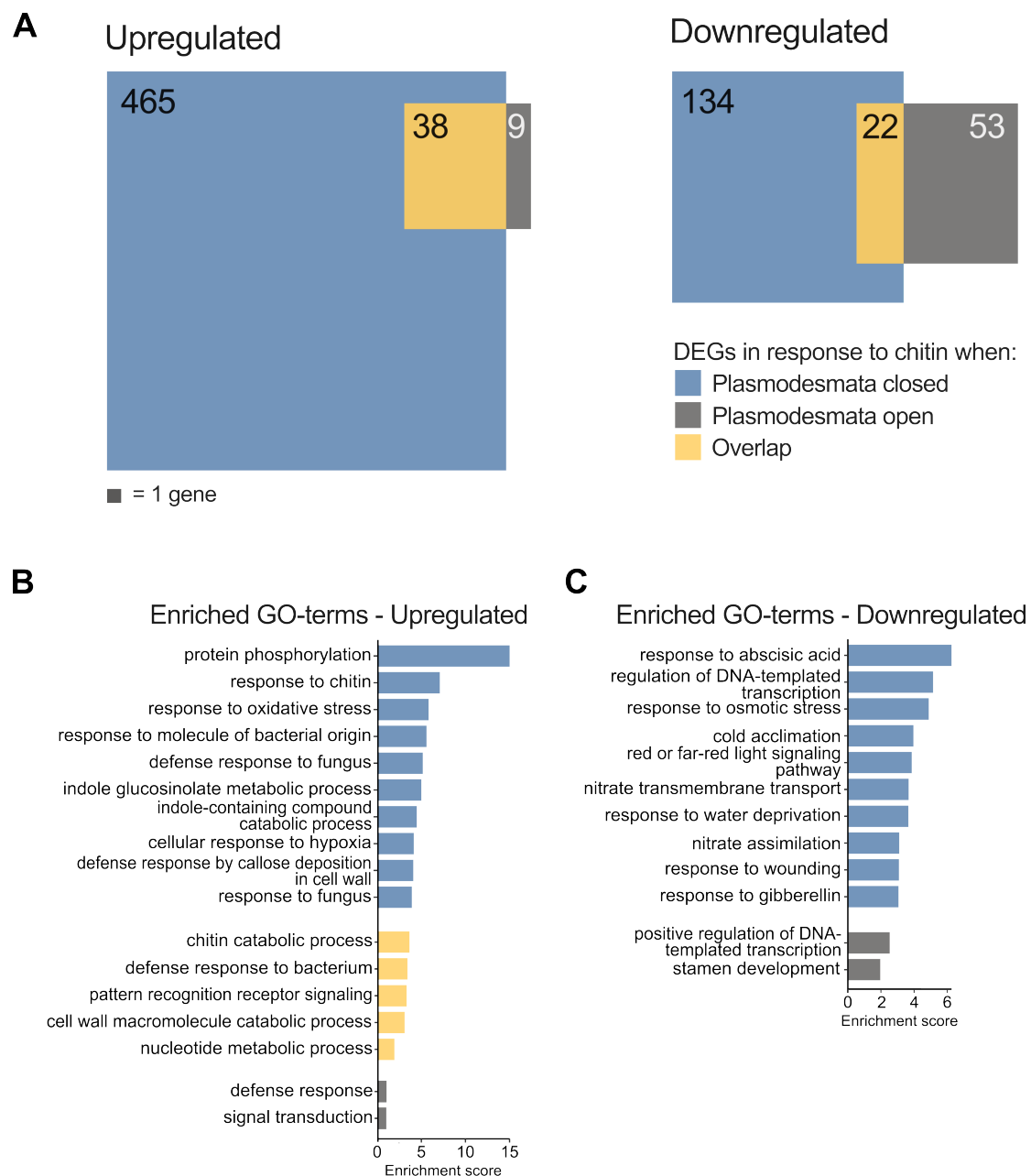


Figure 4.7: Differentially Expressed Genes in response to chitin after plasmodesmal closure – (A) Venn diagrams showing differentially expressed genes (DEGs) genes in response to chitin in *LexA::icals3m* when plasmodesmal closure is induced (blue), when plasmodesmata are open (grey), and genes differentially expressed in both sets (yellow) ($\log_2$ fold change $> |0.5|$, adjusted p-value < 0.05). Enriched GO-terms for genes up-regulated (B) and down-regulated (C) in response to chitin, with colours representing the Venn diagram sections in (A).

response to chitin when plasmodesmata are closed such as response to abscisic acid, response to osmotic stress and wounding (Figure 4.7 C). The downregulation of DNA-templated transcriptional processes was seen in response to chitin both when plasmodesmata are both open and closed. The subset of genes down-regulated regardless of plasmodesmal aperture was small at only 22 genes, and showed no enriched GO-terms.

Overall, young leaves from *LexA::icals3m* with induced plasmodesmal closure show an increased transcriptional response to chitin compared to young leaves with no induced plasmodesmal closure. This suggests a stronger PAMP-triggered transcriptional response is induced when plasmodesmata are closed.

I investigated if the large transcriptional response to chitin in *LexA::icals3m* lines with estradiol-induced plasmodesmal closure was related to the potential chitin $\times$ estradiol treatment interaction effect I identified in section 4.3.5 above (Figure 4.5). I compared the lists of differentially expressed genes identified in response to chitin in estradiol-treated Col-0 and estradiol-treated *LexA::icals3m* and found only 6 genes were differentially expressed in response to chitin in both estradiol-treated genotypes. This indicates the vast majority of the chitin-induced genes in *LexA::icals3m* are not the same genes as those linked to the interaction between estradiol and chitin in Col-0. It is unclear, however, if plasmodesmal closure, estradiol treatment, and chitin treatment have some sort of interactive effect that would result in a different set of genes being up-regulated in estradiol-treated chitin-infiltrated *LexA::icals3m* leaves compared to Col-0. Further questions remain on a potential interactive effect on gene expression between chitin and estradiol treatments.

4.3.8 Chitin-induced plasmodesmal closure-associated genes in young leaves differ from mature leaves

In section 3.3.7 above, I identified differentially expressed genes that I proposed were specifically induced upon chitin treatment when plasmodesmata were closed. These genes were identified using a likelihood ratio test comparing chitin response across young and mature leaves in Col-0 and *lym2* mutants (which lack chitin-induced plasmodesmal closure in mature leaves), in the following text I refer to this group of differentially expressed genes as the "Col-0 *lym2*" dataset (see table 4.2). I suggested the differentially expressed genes in chitin-treated Col-0 mature

Dataset name	Treatment description	Analysis method
Col-0 <i>lym2</i>	Young and mature leaves from Col-0 and <i>lym2</i> genotypes were infiltrated with chitin solution and harvested 30 minutes later.	A likelihood ratio test in Section 3.3.7 to identify groups of differentially expressed genes in Col-0 mature plants with chitin-induced plasmodesmal closure compared to <i>lym2</i> mature and young leaves and Col-0 young leaves with no chitin-induced plasmodesmal closure
<i>LexA::icals3m</i>	Young leaves from <i>LexA::icals3m</i> were treated with estradiol to induce plasmodesmal closure and 24-hours later infiltrated with chitin or water and harvested 1 hour later.	A Walds test in Section 4.3.7 to identify differentially expressed genes between water and chitin treatments in <i>LexA::icals3m</i> young leaves with induced plasmodesmal closure
Thomas and Walker	Mature leaves from Col-0 were infiltrated with chitin or water and harvested 1 hour later.	A Walds test performed by Thomas and Walker (unpublished) to identify differentially expressed genes between chitin and water treatments in mature Col-0 leaves. The same thresholds of $LFC > 0.5 $ and adjusted p-value < 0.05 were applied.

Table 4.2: **Datasets used to identify shared up- and down-regulated genes in plasmodesmal closure-related chitin responses**

leaves, but not in Col-0 young leaves and *lym2* young and mature leaves, were chitin-response genes related to plasmodesmal closure. I hypothesised that the chitin-response genes related to plasmodesmal closure identified in Section 3.3.7 could be similar to the chitin-response genes after the induction of plasmodesmal closure I identified in young *LexA::icals3m* leaves in the previous section above 4.3.7, I refer to this group of differentially expressed genes as the “*LexA::icals3m*” subset, see table 4.2.

Despite my hypothesis, I found very few shared differentially expressed genes between the Col-0 *lym2* and *LexA::icals3m* datasets. I compared the list of differentially expressed genes between the two datasets to find only a very small overlap of shared differentially expressed genes (figure 4.8). This indicates that the induced chitin response after plasmodesma closure in young leaves is very different to that of mature leaves.

The lack of shared transcriptional response between the Col-0 *lym2* and *LexA::icals3m* datasets could be due to differences in the time points of collection. In the *LexA::icals3m* dataset, I collected samples 1-hour post-chitin infiltration, whereas in the Col-0 *lym2* dataset I collected samples 30-minutes post-chitin infiltration. Differences in time points could result in different transcriptional

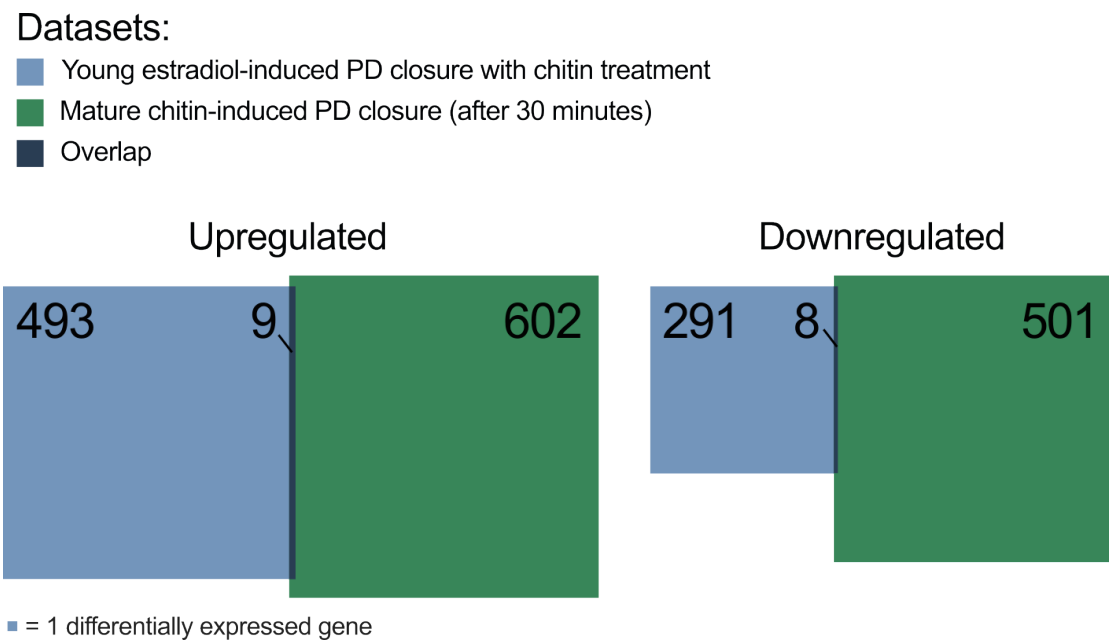


Figure 4.8: **Comparison of differentially expressed genes between young inducible plasmodesmal closure and mature chitin-induced closure** – Venn diagrams showing the number of up-regulated and down-regulated genes in the *LexA::icals3m* young leaf dataset, where differential gene expression in response to chitin was identified in leaves with induced plasmodesmal closure (blue), and the mature and young leaf dataset (from Chapter 3) where differentially expressed genes in response to a 30-minute chitin treatment were identified (green). Common differentially expressed genes are also identified as the overlap between the two Venn squares (dark blue)

responses as Bjornson et al. (2021) find distinct transcriptional responses at 30 minutes and 1-hour post-treatment with a range of PAMPs.

I therefore identified a third unpublished transcriptomic dataset with more similar time points to my *LexA::icals3m* dataset that was collected and analysed by Thomas and Walker (from the Faulkner group, John Innes Centre, U.K.). Thomas and Walker infiltrated leaves of mature Col-0 plants with chitin (500 µg/ml) or water (mock), and harvested leaves 1 hour later, RNA was then extracted, sequenced and analysed using the same methods as described in 2.5. Thomas and Walker identified differentially expressed genes in response to chitin in mature Col-0 plants 1-hour post-infiltration. I thus extracted a list of differentially expressed genes induced by chitin treatment in mature leaves from their dataset. I refer to this dataset as the “Thomas and Walker” dataset (see Table 4.2). In Figure 4.9, I compared the Thomas and Walker dataset to the differentially expressed genes identified in the *LexA::icals3m* dataset. I found a shared 127 genes up-regulated in both datasets (Figure 4.8). A GO-term analysis of these shared genes shows upregulation of protein phosphorylation, defence response to fungus and chitin, as well as other defensive processes. These genes may be core up-regulated defensive genes in response to both chitin treatment and plasmodesmal closure. Additionally, there are only 13 overlapping down-regulated genes, which is in part proportional to the small number of genes down-regulated after chitin treatment in mature tissue.

Overall, the transcriptomic response to chitin in *LexA::icals3m* young leaves with induced plasmodesmal closure shows overlap with the transcriptomic response to chitin in mature leaves with PAMP-triggered plasmodesmal closure identified by Thomas and Walker (unpublished). The lack of shared genes between the *LexA::icals3m* dataset and Col-0 *lym2* dataset could thus be due to the different timepoints, and suggests time is an important factor when comparing transcriptional responses to PAMPs.

4.3.9 Inducing plasmodesmal closure in young leaves results in growth defects

The transcriptional defence activation and increased *Pst* DC3000 resistance observed after plasmodesmal closure (Figures 4.7 and 4.3), raises the question of why young leaves do not execute PAMP-induced plasmodesmal closure if there

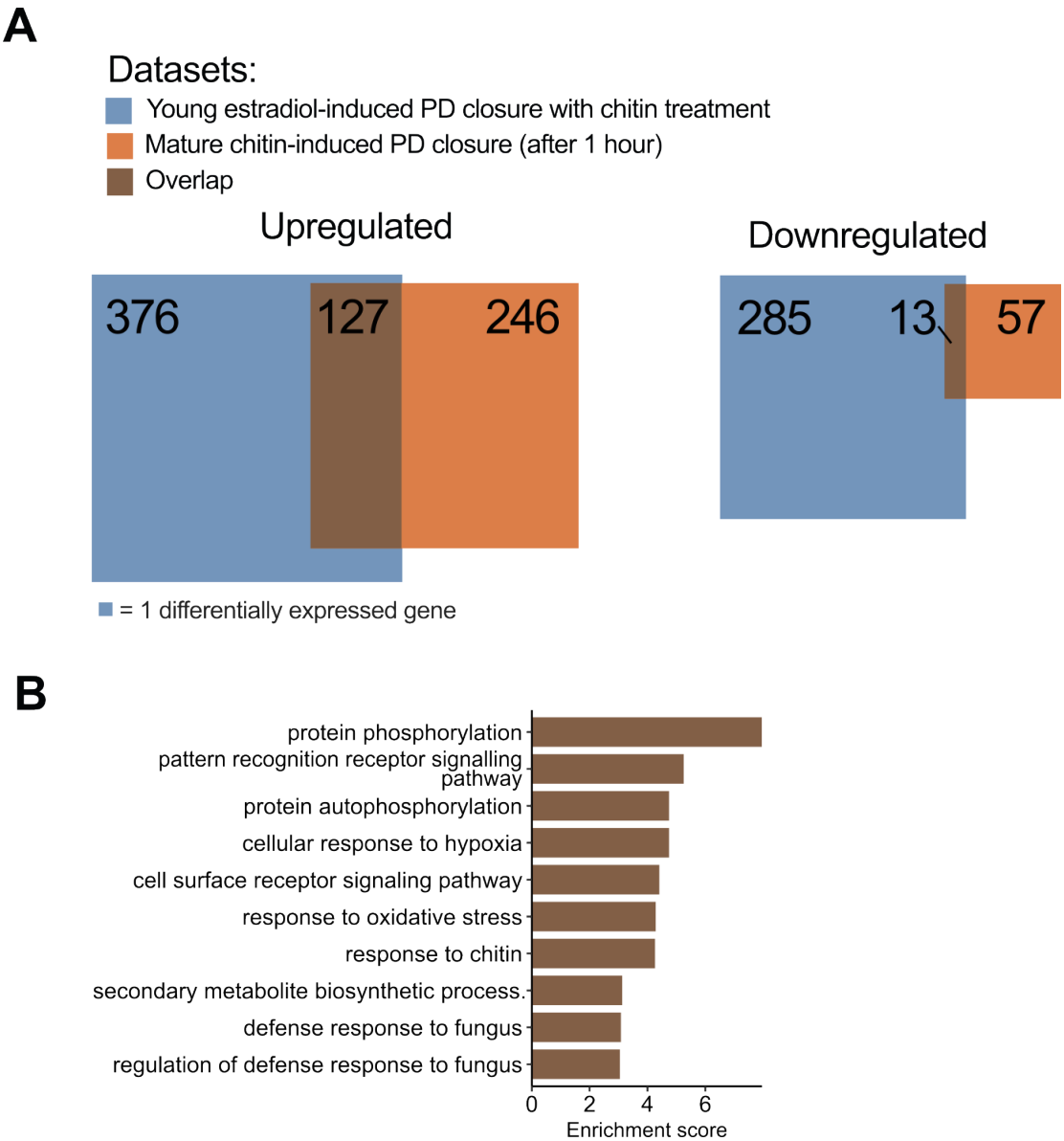


Figure 4.9: **Comparison of differentially expressed genes in response to chitin between young and mature leaf tissue 1 hour after chitin treatment** – (A) Venn diagrams showing the number of up-regulated and down-regulated genes in the *LexA::icals3m* young leaf dataset, where differential gene expression in response to chitin was identified in leaves with induced plasmodesmal closure (blue), compared to differentially expressed genes from Thomas and Walker (unpublished) 1 hour after chitin treatment in mature leaves (orange). Shared differentially expressed genes between the two datasets are also shown.

could be potentially enhanced defensive responses. I hypothesised this is due to the importance of plasmodesmata in phloem unloading, which provides a source of carbon to support growth. To investigate this hypothesis and determine if inducing plasmodesmal closure impacts growth, I measured leaf area in Col-0 and *LexA::icals3m* lines four days after treating the four youngest emerged leaves with estradiol or DMSO. Col-0 leaves showed no significant difference in area between estradiol and DMSO-treatments at any leaf age (Figure 4.8 A). In *LexA::icals3m* leaves, estradiol-treatment reduced leaf growth across leaves two, three, and four, but not in the youngest leaf. The youngest leaves do show a trend towards reduced growth, but it is not statistically significant. *LexA::icals3m* leaves also show a leaf curling phenotype, where the leaf edges curl upwards (Figure 4.10 B). The reduced leaf area in *LexA::icals3m* leaves treated with estradiol indicates inducing plasmodesmal closure reduces overall leaf growth.

4.3.10 Phloem unloading is reduced upon plasmodesmal closure

Sugars are unloaded from the phloem through the symplasm in Arabidopsis, meaning plasmodesmata are important for the supply of sugars. To determine whether the reduced growth in young leaves correlates with reduced phloem unloading I developed a 5(6)-carboxyfluorescein diacetate (CFDA) dye loading assay. CFDA is a membrane-permeable and non-fluorescent dye which is rapidly uptaken into cells due to its structure containing acetyl groups (Cui & Lee, 2016). Once inside a cell, CFDA is converted by cellular esterases into carboxyfluorescein (CF). CF is a fluorescent, non-membrane-permeable molecule which moves cell-to-cell through plasmodesmata. The molecular weight of CF is 460 Da, making it similar in size to sucrose, which is 342 Da, and so it can act as a tracer for cell-to-cell movement of sugars (Cui & Lee, 2016).

To investigate phloem-unloading patterns, I tracked dye movement across a single plant in which I only loaded half the mature leaves with CFDA. In mature leaves, the CF signal remains within the vasculature and does not unload from the phloem (Figure 4.11 A to C). In young leaves the CF signal is visible in the mesophyll layers as the CF unloads from the phloem and into the surrounding tissue, indicating these leaves are sink leaves (Figure 4.11 D-E). I imaged CF movement in additional leaves from the same plant to show the CF pattern in each leaf was variable across leaf ages (Figure 4.12). Leaves 11 and 13 have a

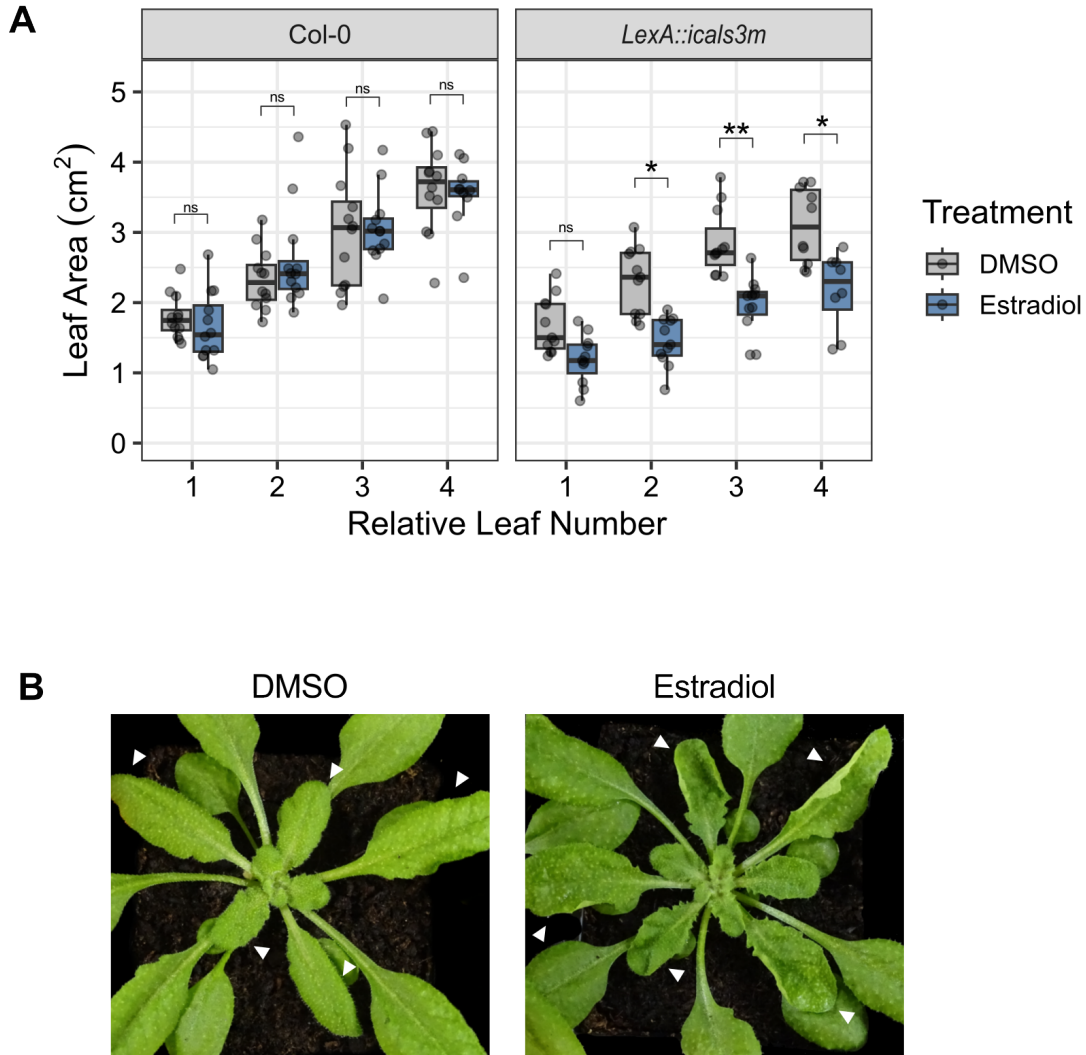


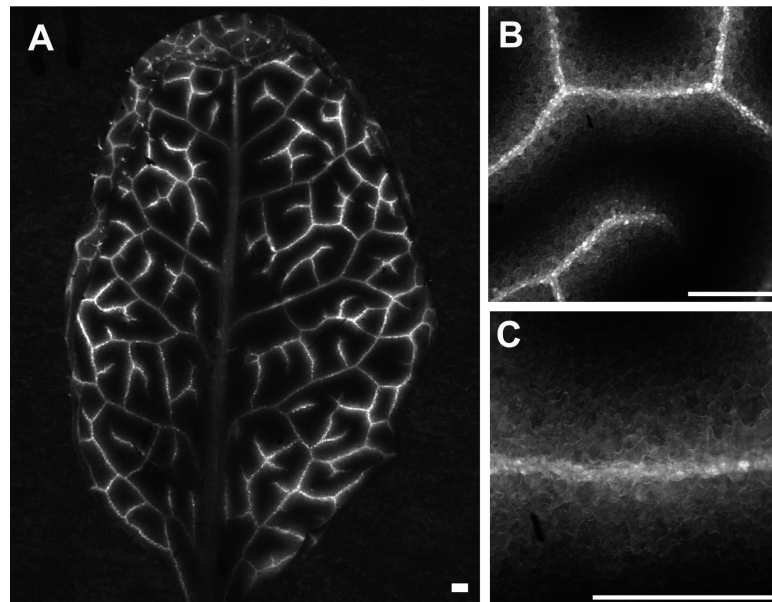
Figure 4.10: **Leaf growth in *LexA::icals3m* young leaves after plasmodesmal closure** – (A) Leaf area of Col-0 and *LexA::icals3m* after treatment with DMSO (grey) or estradiol (blue) in relative leaf numbers 1 to 4, where 1 is the youngest. All combinations were tested following an ANOVA in a posthoc Tukey HSD test with only specific combinations within each relative leaf number shown here (B) Images of young leaves from *LexA::icals3m* treated with estradiol or DMSO, treated leaves are indicated with a white arrow, ns = no significance, “*” = $p < 0.05$, “**” = $p < 0.01$.

more discrete CF signal within the vasculature (Figure 4.12 I-L), while leaves 17, 18 and 19 show a more diffuse CF signal indicating CF is unloaded into the surrounding cells (Figure 4.12 A-F). Although I was able to image CF unloading in leaves across the same plants in Figures 4.11 and 4.12, obtaining CFDA unloading patterns across multiple leaf ages of the same plant was challenging. For instance, different leaves show highly variable signal strength depending on the amount of CF present in the vasculature, so in Figures 4.11 and 4.12 the images of leaves are set at different brightnesses and laser strengths due to very high unloading variability. Additionally, in some plants, some leaves showed weak or absent CF unloading despite CFDA being loaded onto mature leaves. As a result of these issues the plant in Figure 4.11 and 4.12 was one of the few for which I managed to obtain a whole sequence of images of different leaf ages. Instead of tracking CF movement across the whole rosette, I developed a method to quantify phloem unloading in young leaves which addresses the challenges listed here.

Previously CF movement has been used to quantify plasmodesmal permeability by tracking movement in mature *Arabidopsis* leaves in Drop-And-See assays (Cui & Lee, 2016) and to track phloem unloading in various tissue-types including sink leaves and roots (Oparka et al., 1994; Roberts et al., 1997). I adapted and expanded on these methods to quantify phloem unloading of CF across young leaves. As described in more detail in the methods section 4.2.3, CF unloading was imaged and quantified using a pipeline I developed in collaboration with Annalisa Bellandi and published in (Belliandi et al., 2022). Briefly, the pipeline tracked fluorescence intensity along 50 x 50 gridlines across each image and the profile of intensity was analysed to detect the peaks and troughs of each profile (Figure 4.13 A and B). The width of the peaks from each profile was taken to represent the distance CF moves out of the phloem into the surrounding tissue and therefore used as a metric for phloem unloading.

Quantification of phloem unloading of CF in young leaves of *LexA::icals3m* treated with estradiol or DMSO revealed estradiol-treated leaves had significantly smaller peak widths than DMSO-treated leaves (Figure 4.13 C and D, and published in Bellandi et al. (2022)). This indicated inducing plasmodesmal closure in *LexA::icals3m* reduces the movement of CF out of the phloem. As CF has a similar molecular weight to sucrose and moves symplasmically, reduced CF unloading suggests there would also be reduced symplasmic phloem unloading of sucrose when plasmodesmal closure is induced in young leaves.

Mature



Young

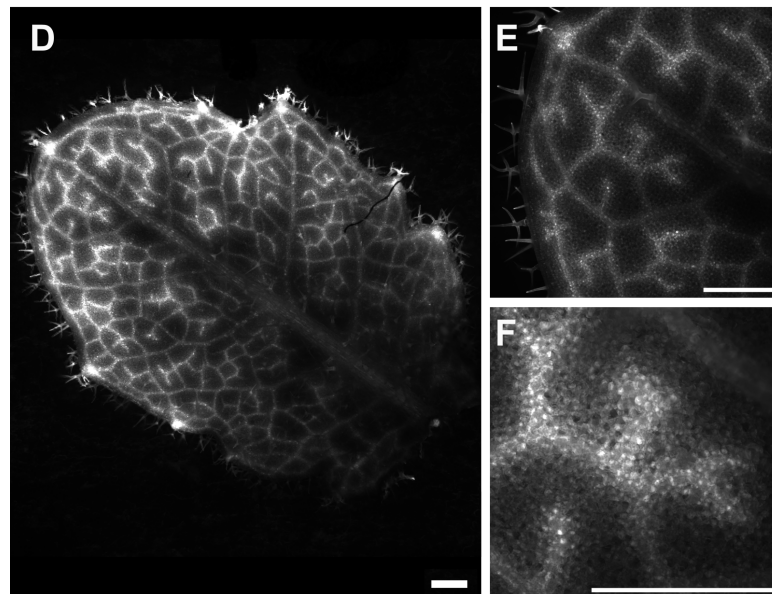


Figure 4.11: **Carboxyfluorescein phloem unloading in young and mature leaves** – (A - C) Carboxyfluorescein (CF) pattern within the same mature leaf shown at different scales. (B-D) CF unloading pattern within the same young leaf shown at different scales. Scale bar indicates 500 μm

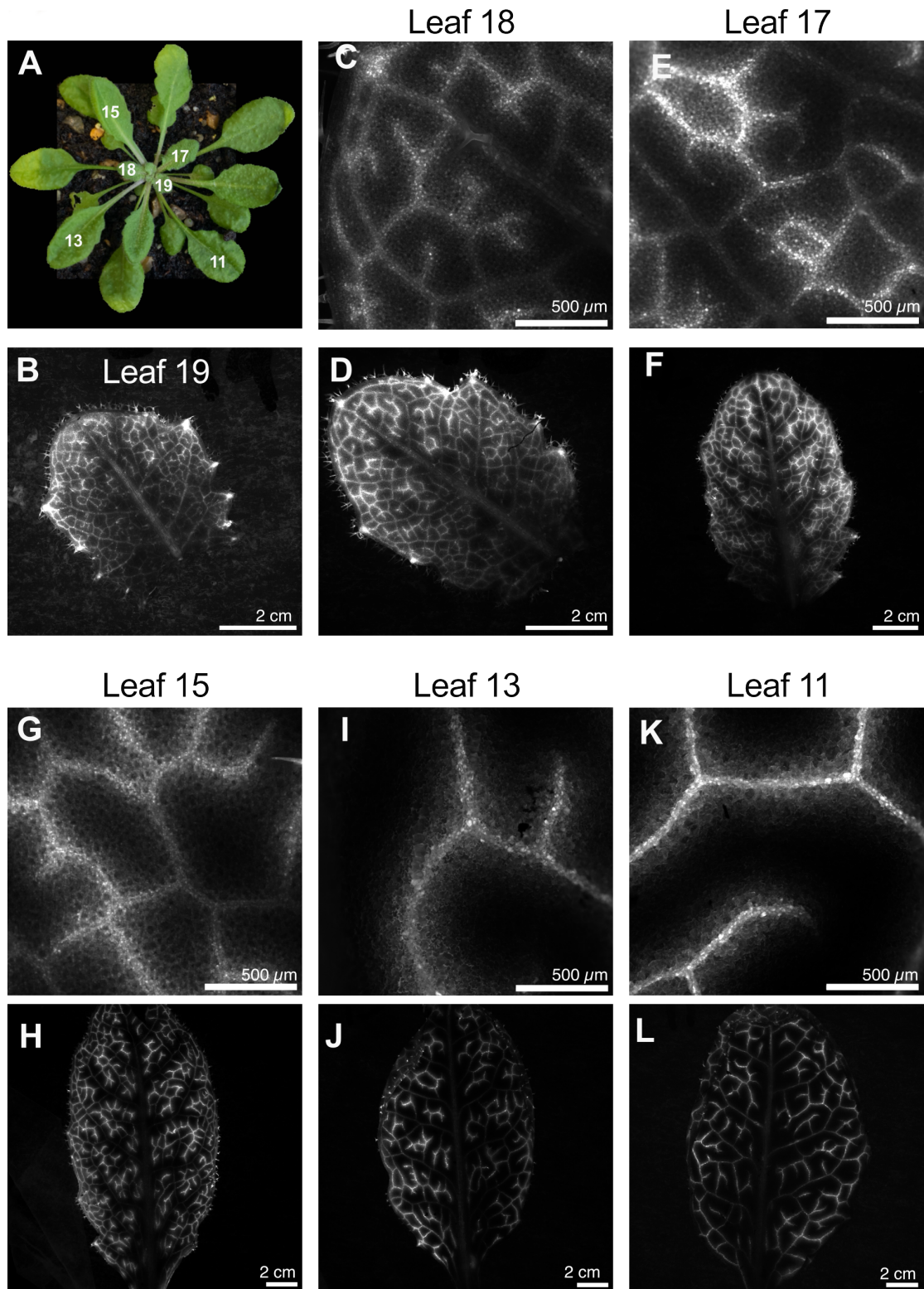


Figure 4.12: **Carboxyfluorescein movement across multiple leaf ages** – (A) Rosette with leaf numbers according to the leaves shown in this figure: Leaf 19 (B), Leaf 18 (C,D), Leaf 17 (E,F), Leaf 15 (G,H), Leaf 13 (I,J), and Leaf 11 (K,L). Scale bars on C,E,G,I,K indicate 500 µm, and scale bars on B,D,F,H,J, and L indicate 2 cm.

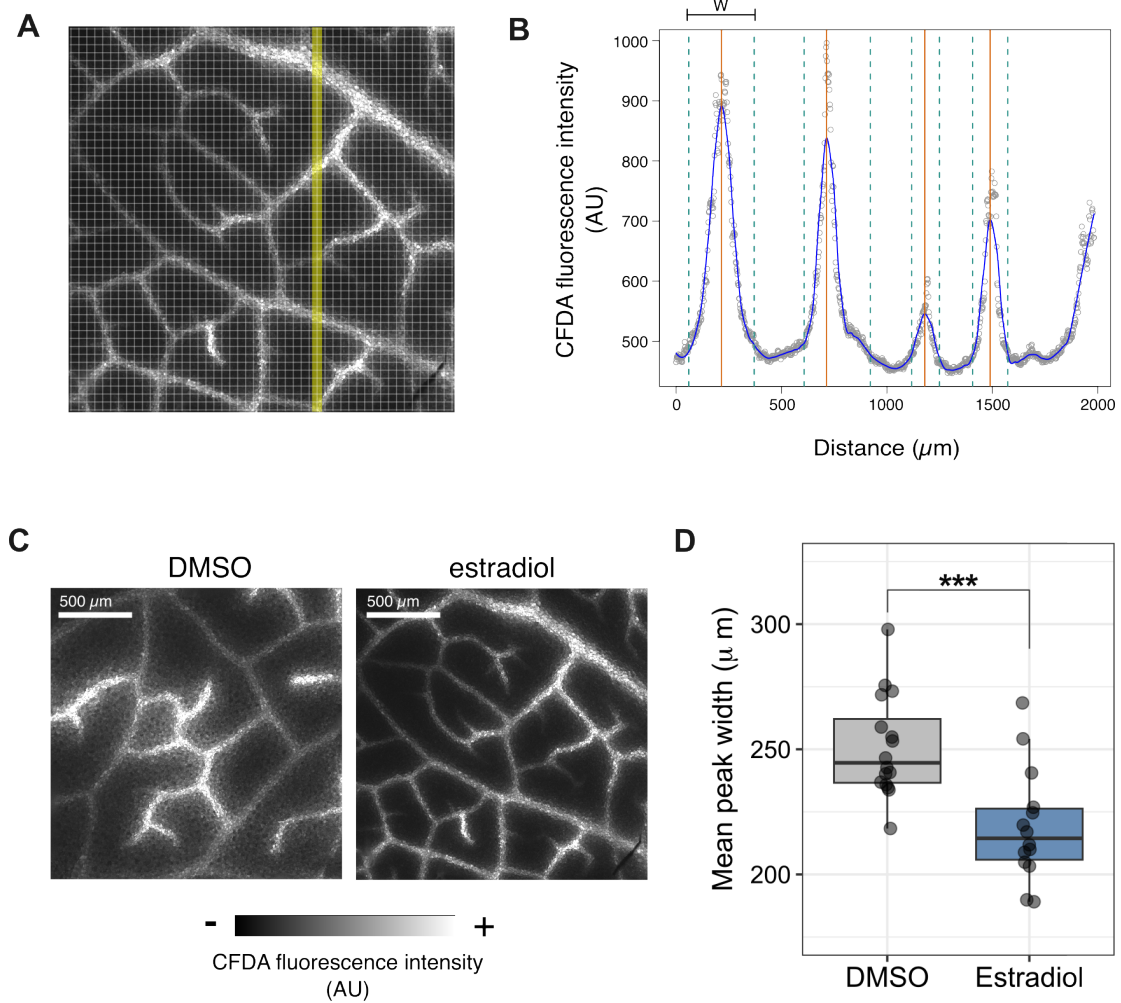


Figure 4.13: **Quantifying carboxyfluorescein unloading after inducing plasmodial closure** - (A) Each image of CF being unloaded from the phloem was divided up into 50 x 50 grid. (B) CF fluorescence intensity was measured across each grid transect, with the width of each peak (W) calculated. (C) Images of CF unloading in DMSO vs. estradiol treated *LexA::icals3m* plants. (D) Box-plot of the mean peak width in DMSO and estradiol treatments. Each point represents the mean peak width for a single image. $P < 0.001 = \text{'***'}$. Figure from Bellandi et al. (2022))

SWEET Proteins		Sucrose Transporter Proteins		Sucrose Transporters		Cell Wall Invertases and Cell Wall β -galactosidases	
Gene ID	Name	Gene ID	Name	Gene ID	Name	Gene ID	Name
AT1G21460	SWEET1	AT1G11260	STP1	AT1G71880	SUC1	AT3G13790	CWINV1
AT3G14770	SWEET2	AT1G07340	STP2	AT1G22710	SUC2	AT3G52600	CWINV2
AT5G53190	SWEET3	AT3G19930	STP4 *	AT2G02860	SUC3	AT2G36190	CWINV4
AT3G28007	SWEET4	AT3G05960	STP6	AT1G09960	SUC4	AT3G13784	CWINV5
AT5G62850	SWEET5	AT4G02050	STP7	AT1G71890	SUC5	AT5G63800	MUM2
AT1G66770	SWEET6	AT5G26250	STP8	AT5G43610	SUC6	AT3G52840	GAL2
AT4G10850	SWEET7	AT1G50310	STP9	AT1G66570	SUC7		
AT5G40260	SWEET8	AT3G19940	STP10	AT2G14670	SUC8		
AT2G39060	SWEET9	AT5G23270	STP11	AT5G06170	SUC9		
AT5G50790	SWEET10	AT4G21480	STP12				
AT3G48740	SWEET11	AT5G26340	STP13				
AT5G23660	SWEET12	AT1G77210	STP14 *				
AT5G50800	SWEET13						
AT4G25010	SWEET14						
AT5G13170	SWEET15						
AT3G16690	SWEET16						
AT4G15920	SWEET17						

Table 4.3: **List of known sugar transporters in Arabidopsis** - List of sugar transporters in Arabidopsis. A Wald test was performed to identify differentially expressed genes in response to estradiol in *LexA::icals3m* leaves. Significantly up-regulated genes are indicated by “*”, where the adjusted p-value < 0.05. None of these genes were significantly up-regulated in Col-0 in response to estradiol.

4.3.11 Exploring upregulation of sugar transporters after plasmodesmal closure

The induction of plasmodesmal closure in *LexA::icals3m* resulted in reduced phloem unloading. In several plant processes where plasmodesmata are closed and cells are symplasmically isolated, such as fruiting and seedling development, plants supply sugars to symplasmically isolated cells using apoplastic transport (Chen et al., 2015; Manning et al., 2001; Zhang et al., 2006). I mined my transcriptomic dataset in Section 4.3.4 to determine whether there is an upregulation of apoplastic sugar transporters to compensate for the reduction in symplasmic transport in response to plasmodesmal closure. I use the Wald test performed in Section 4.3.4 to identify differentially expressed genes between water-treated *LexA::icals3m* DMSO and water-treated *LexA::icals3m* estradiol. I explored the expression of major sugar transports (listed in Table 4.3) and found none of the *SWEET* transporter genes or *SUC* transporter genes are up-regulated. Similarly, there is no upregulation of the *Cell Wall Invertases* (*CWIN*), which have previously been found to breakdown sucrose in the apoplast to facilitate hexose transport between the apoplast and cytoplasm (Büttner & Sauer, 2000; Sturm & Chrispeels, 1990). Only *Sugar*

Transporter Protein 4 (*STP4*) and *STP14* are up-regulated after plasmodesmal closure (Figure 4.14 A and B). *STP4* had a 0.81 LFC in expression and *STP14* had a 1.58 LFC in expression after plasmodesmal closure induction, and both are significantly differentially expressed. The normalised gene counts have a large within-sample variation for *STP4* in particular, with two estradiol-treated samples in *LexA::icals3m* showing higher expression than the other three samples. Although the difference in expression overall is significant it is important to consider that the response is not uniform across all three samples.

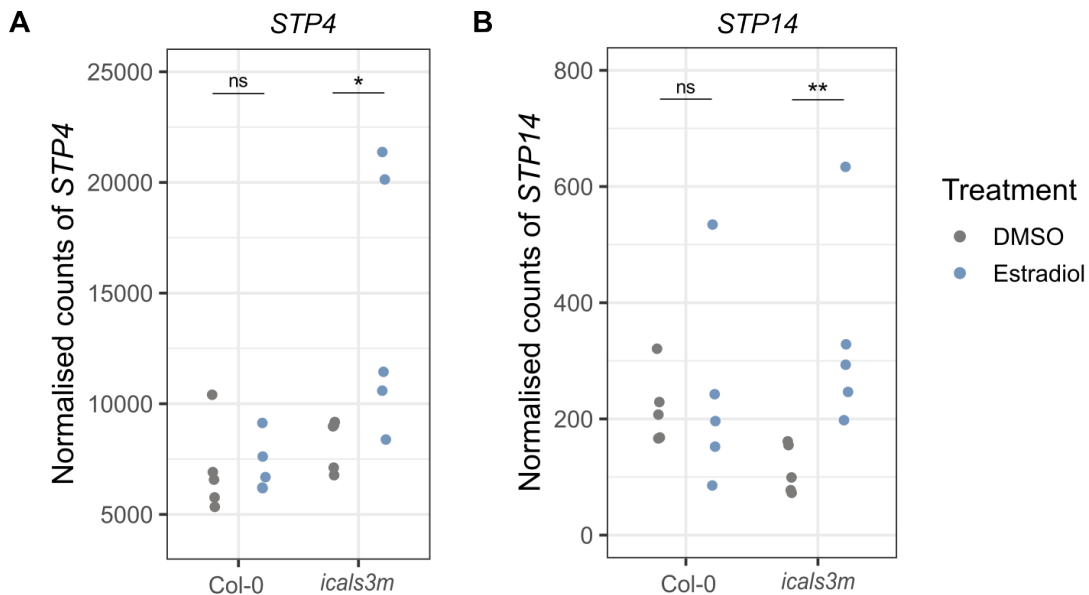


Figure 4.14: – **Differential expression of sugar transporters** – Normalised counts of Sugar transporter (*STP*) 4 and *STP* 14 comparing DMSO treatment and estradiol treatment in Col-0 and *LexA::icals3m(icals3m)*. Differential gene expression in response to estradiol in both genotypes was determined in a Wald test, and adjusted p-values were calculated. “*” $p < 0.05$, “***” $p < 0.001$)

4.4 Discussion

In this chapter, I investigated the role of plasmodesmal permeability in mediating defence responses and growth. I found inducing plasmodesmal closure in young leaves reduces phloem unloading and growth; however, it also increases resistance to *Pst* DC3000 and induces a transcriptional stress response. The antagonism between reduced growth and increased defence after induced plasmodesmal closure suggests plasmodesmal permeability could be regulated to optimise the balance between growth and defence in young leaves.

4.4.1 Plasmodesmal closure increases defence response in young leaves

Previously, in Section 3.3.1, I found young leaves do not execute PAMP-triggered plasmodesmal closure. To determine whether this correlates with increased susceptibility to pathogens, I performed a pathoassay on Col-0 plants. I found young leaves were more susceptible to *Pst* DC3000 than mature leaves in Col-0 (Figure 4.1). This is consistent with previous findings from (Xu et al., 2017), who found younger Col-0 Arabidopsis leaves are more susceptible than older leaves to *Pst* DC3000 in 8-week-old Arabidopsis. Kus et al. (2002), however, found Arabidopsis Col-0 young and mature leaves have similar levels of resistance to *Pst* DC3000. The discrepancies between the results of my pathoassay and that of Kus et al. (2002) could be due to differences in pre-inoculation environmental stress exposure. Kus et al. (2002) noted anecdotally that their plants had been under stress from pests during development, and that this environmental stress altered the development of leaf-stage related resistance. In my experiments, there was limited known exposure to pests due to the use of growth cabinets. Therefore, the differences between my findings and those of Kus et al. (2002) could result from their increased pre-inoculation stress exposure. This indicates in future experiments studying leaf stage-related resistance it is key to take note of the presence of any potential stresses pre-inoculation as it is likely to be a source of variation in leaf stage-related resistance in plants.

Questions remain about the differences in susceptibility between Arabidopsis in young and mature leaves to other pathogens, as I only explored susceptibility to *Pst* DC3000. Xu et al. (2018), for instance, show young Arabidopsis leaves

are less susceptible than adult leaves to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*, whilst (Zeier, 2005) show young leaves exhibit no differences in susceptibility relative to mature leaves to the avirulent *P. syringae* strain *P. syringae* pv. *maculicola* (*avrRPM1*). Furthermore, there may even be differences in leaf stage-related resistance to *Pst* DC3000 between Arabidopsis ecotypes. For instance, Zipfel et al. (2004) find in Arabidopsis plants of the Landsburg erecta (Ler-0) ecotype young leaves are less susceptible than mature leaves to *Pst* DC3000. Only a direct comparison of Arabidopsis ecotypes under the same experimental conditions could determine whether this is due to differences in ecotypes or experimental conditions. Overall these examples paint a complex landscape of leaf stage-related resistance in Arabidopsis, with limited understanding of why young leaves are more or less susceptible to infection than mature leaves. Environmental differences between experimental set-ups could underpin changes in the balance of leaf stage-related resistance, as suggested above to explain the discrepancies between my results and those from Kus et al. (2002). Alternatively, Xu et al. (2018) suggest differences in defensive gene expression and phytohormone signalling between young and mature leaves underpin the variation in leaf stage-related resistance. Pathogens could be more successful in young leaves if they were better able to overcome the defensive responses in young leaves compared to the defensive response in mature leaves. My transcriptional data from 3 support this, where I saw differences in PAMP-triggered genes in young and mature leaves and potentially larger ROS bursts in young leaves. Overall, studying differences in the susceptibility of mature and young leaves to various pathogens under my experimental conditions could help to limit the impact of environmental variation on resistance and allow me to explore further how differences in defensive signalling could underpin leaf stage-related resistance.

The role of plasmodesmal closure in leaf stage-related resistance is unclear. A lack of PAMP-induced plasmodesmal closure in young leaves is correlated with increased susceptibility to *Pst* DC3000, whilst inducing plasmodesmal closure itself in young leaves increased resistance to *Pst* DC3000 (Figure 4.3). Previous findings in mature leaves support the role of plasmodesmata in resistance, with plasmodesmal closure in *LexA::icals3m* mature leaves also increasing resistance to *Pst* DC3000 (Tee et al., 2024). Similarly, mutants with impaired PAMP-triggered plasmodesmal closure have increased susceptibility (Faulkner et al., 2013; Lee et al., 2011; Xu et al., 2017). The question remains as to why inducing plasmodesmal closure increases resistance in young leaves to *Pst* DC3000, and leads to the wider question of why plasmodesmal closure is not triggered by the presence of PAMPs

in young leaves if it has the potential to increase resistance.

The increased resistance to *Pst* DC3000 in young leaves with plasmodesmal closure could be due to defensive priming induced by plasmodesmal closure. Young *LexA::icals3m* leaves show a transcriptional upregulation of stress-related processes such as response to jasmonic acid and response to bacterium 24 hours after plasmodesmal closure (Figure 4.6). This results in cells being primed for stress and so upon infection with *Pst* DC3000, defence is already activated making leaves less susceptible to *Pst* DC3000. Furthermore, a defensive priming effect could cause increased chitin-triggered transcriptional responses in young leaves with estradiol-induced plasmodesmal closure compared to those with no induced closure (Figure 4.7). This hypothesis is supported by previous findings from Tee et al. (2024) showing the induction of plasmodesmal closure in mature leaves causes stress activation and results in an accumulation of salicylic acid 72 hours later. Although I did not quantify the accumulation of defence hormones, it is possible plasmodesmal closure in young leaves also induces hormone accumulation leading to priming. There is transcriptional evidence for this, with response to jasmonic acid identified as an enriched GO-term associated with plasmodesmal closure (Figure 4.6). Further quantification of the accumulation of jasmonates as well as salicylic acid could help to address this defensive priming hypothesis.

In my transcriptional analysis of the impact of inducing plasmodesmal closure in young leaves of *LexA::icals3m* lines, I identified a subset of chitin-response genes specific to leaf tissue with induced plasmodesmal closure in young leaves (Figure 4.7). I compared this *LexA::icals3m* subset to previously identified plasmodesmal-closure associated chitin response genes from my Col-0 *lym2* dataset from Chapter 3. I found a lack of shared differentially expressed genes between these datasets. I attributed this lack of overlapping genes to the 30-minute difference in the time points of tissue collection between the two datasets, rather than due to either dataset not being representative of the chitin-induced plasmodesmal closure response. After comparing my *LexA::icals3m* list of differentially expressed genes to those from Thomas and Walker’s unpublished dataset, I identified 127 shared chitin-response genes which may be related to a plasmodesmal-closure specific response (Figure 4.9). The GO-term analysis of these shared genes revealed enriched terms such as “response to fungus” and several signalling and protein phosphorylation-related terms. These genes could be a subset of age-independent chitin-response genes associated with plasmodesmal closure and defensive signalling related to plasmodesmal closure.

Overall, evidence presented here suggests plasmodesmata have a role in defence in young leaves. I specifically explored this in terms of resistance to *Pst* DC3000. Further investigation of the impact of plasmodesmal closure on susceptibility to other pathogens in young leaves could determine whether this is shared across pathosystems. Whether the increased resistance is due to priming triggered by the induction of plasmodesmal closure, or due to other indirect impacts of plasmodesmal closure remains unclear. Additionally, chitin treatment after the induction of plasmodesmal closure triggers the activation of a separate subset of chitin-response genes. Further exploration of the accumulation of phytohormones in young leaves when plasmodesmata are open or closed could help to clarify the role of plasmodesmata in leaf stage-related resistance.

4.4.2 Plasmodesmal closure reduces phloem unloading and growth

In young leaves of *LexA::icals3m*, the induction of plasmodesmal closure resulted in reductions in phloem unloading and growth. A CF dye-unloading assay showed phloem unloading is reduced in young leaves 24 hours after estradiol treatment (Figure 4.13). Additionally, cell-to-cell movement of GFP is reduced after estradiol treatment (Figure 4.2). The restriction of symplasmic transport indicates the supply of sugars to young leaves through the symplasm is reduced. This may explain the reduced growth I observed four days after induced plasmodesmal closure in young leaves (Figure 4.6). The transcriptional data also points towards fewer nutrients reaching the young leaves, as there is a downregulation of translational machinery-associated genes both in my KEGG and GO-term analyses. Down-regulation of translation and ribosomes has been previously linked to nutrient availability through TARGET OF RAPAMYCIN (TOR) signalling, with purine and pyrimidine biosynthesis found to promote TOR activity (Busche et al., 2021). In young leaves with reduced phloem unloading the lack of nutrients could be triggering a similar nutrient deficiency response limiting translation and growth.

The reduced symplasmic transport after plasmodesmal closure in young leaves also coincides with an upregulation of the transmembrane sugar transporter *STP4* (Figure 4.14). *STP4* is a monosaccharide H⁺ symporter expressed in multiple tissue types including symplasmically isolated guard cells and sink tissue such as root tips and anthers (Flütsch et al., 2020; Truernit et al., 1996). Additionally, *STP4* is up-regulated in response to wounding and pathogen attack to increase

monosaccharide transport in temporary sinks, such as wounded or infected tissue with enhanced carbohydrate requirement (Truernit et al., 1996). The up-regulation of *STP4* in response to plasmodesmal closure could be a direct response to the lack of symplasmic sugar transport if there was a mechanism for sensing intracellular sugar availability. Alternatively, the upregulation of *STP4* could be up-regulated as part of a general stress response induced by plasmodesmal closure and unrelated to intracellular sugar availability. I also explored if there was an up-regulation of apoplastic processes that could increase glucose availability for *STP4* to transport. Increased *CWIN* expression has previously been linked to pathogen-mediated increases in cell wall invertase activity to break down sucrose and increase the availability of glucose for *STP4* transport (Fotopoulos et al., 2003). While in my dataset I see no differential expression of any *CWINs*, assaying for *CWIN* activity would enable me to determine if activity is increased post-translationally.

In addition to *STP4*, another sugar transporter gene, *STP14*, is up-regulated twenty-four hours after plasmodesmal closure, with a $\log_2$ fold change of 1.58. *STP14* is a galactose transporter expressed in both mature source leaves and in sink tissues (Poschet et al., 2010). Galactose forms part of cell wall polysaccharides and is used as a source of sugars in cell wall recycling. During sugar starvation induced by extended darkness, expression of *STP14* and β -galactosidases is increased to induce polysaccharide breakdown and is suggested to have a role in increased galactose import into the cell during cell wall recycling (Poschet et al., 2010). The upregulation of *STP14* could be part of a sugar starvation response induced by plasmodesmal closure which triggers cell wall recycling. However, the mechanism for increased galactose production is unclear as plasmodesmal closure is not accompanied by an upregulation of β -galactosidases. It is possible β -galactosidase activity is increased without an increase in expression, or that there are other mechanisms to increase apoplastic galactose concentrations to match increased *STP14*. More evidence of apoplastic galactose levels and β -galactosidase activity in response to plasmodesmal closure is needed to determine whether *STP14* upregulation leads to increased cell wall recycling as a sugar starvation response.

Overall, the upregulation of *STP4* and *STP14* after plasmodesmal closure could increase sugar transport from the apoplast into the cell, but there is little transcriptional evidence to suggest widespread apoplastic phloem unloading is occurring. For the switch from symplasmic to apoplastic unloading to occur I would expect a wider transcriptional reprogramming. For instance, one would expect upregulation of the apoplastic loading *SUCs*, or the sugar importing *SWEETs*,

or upregulation of *CWINs*. Overall, the transcriptional data from the induction of plasmodesmal closure in young leaves indicates there is an up-regulation of two sugar transporters, but other apoplastic transporters and enzymes are not up-regulated and there is a reduction in growth, indicating that young leaves are not able to completely compensate for a reduction in symplasmic isolation through an increase in apoplastic phloem unloading.

In addition to reducing phloem unloading, plasmodesmal closure in young leaves reduced leaf growth and induced a leaf curling phenotype (Figure 4.6), where the abaxial surface curls upwards. The reason for this is unclear. One explanation is that when painting both sides of the leaf, the estradiol treatment could drip off faster from the abaxial side, whilst the droplets may stay longer on the adaxial side. Thus, the estradiol effect may be more enhanced on the adaxial side leading to a more strongly reduced growth phenotype and so the faster-growing abaxial side curls upwards. A flaw in this argument, however, is that estradiol treatment was still effective at reducing cell-to-cell movement of GFP after painting on the abaxial side in microprojectile bombardment assays (Figure 4.2). Alternatively, the curled phenotype could be due to the reduced movement of symplasmic signals across young leaves. Maybe these symplasmic signals are required to coordinate abaxial and adaxial cell division and expansion. Other developmental processes not quantified here could also be disrupted by plasmodesmal closure. For instance, stomatal development requires the symplasmic isolation of guard cells, and if plasmodesmata are closed in all cells it could disrupt development and explain the reason for the enrichment of GO-terms such as “Regulation of stomatal movement”. Further quantification of developmental changes with plasmodesmal closure such as cell expansion, division, and stomatal structure could help to address this hypothesis.

Although I have suggested inducing plasmodesmal closure could reduce growth by reduced phloem unloading, growth could be indirectly reduced upon plasmodesmal closure by increased jasmonic acid signalling. Jasmonic acid leads to growth inhibition (reviewed in Havko et al. (2016)). My results show plasmodesmal closure in young *LexA::icals3m* leaves results in the upregulation of a subset of genes associated with the GO-term “response to jasmonic acid”, with jasmonate responsive genes including *MYC3* and *JASMONATE RESPONSIVE 1 (JR1)* up-regulated. Although I have not determined whether concentrations of jasmonic acid increase in young leaves, if they do this could lead to growth inhibition. Further quantification of phytohormone levels could help to determine if jasmonic acid accumulation

is linked to the observed reduced growth. In any case, if plasmodesmal closure indirectly triggers reduced growth through plasmodesmal signalling, the result is still a reduction in growth and thus could still fit into a trade-off between growth and defence.

4.4.3 A growth-defence trade-off

Considering my results in this chapter along with the lack of PAMP-triggered plasmodesmal closure in young leaves, I suggest the regulation of plasmodesmal closure is linked to a trade-off between growth and defence processes. Young leaves could prioritise growth by maintaining symplasmic connectivity for phloem unloading, even at a potential defensive cost. This is supported by the lack of solid evidence that young leaves can compensate for plasmodesmal closure via transcriptional mechanisms to boost apoplastic phloem unloading. The reduced growth but increased resistance to *Pst* DC3000 indicates in some cases plasmodesmal closure comes at a cost to growth but benefits defence.

The optimal defence theory suggests young tissue is more valuable, and so more resources are invested in prioritising defence (McKey, 1974). How the regulation of plasmodesmata fits into fitness costs and benefits is less clear. Inducing plasmodesmal closure in young leaves reduces growth which could be a fitness cost, but also keeping plasmodesmata open with increased susceptibility to pathogens could also be a fitness cost. Maybe keeping plasmodesmata open allows growth and a smaller defence response which is more beneficial in the long term, whilst inducing plasmodesmal closure prevents growth with a larger defence response. In practice, as young leaves do not close plasmodesmata, it suggests growth is prioritised over defence.

It is important to note that *LexA::icals3m* induces a very strong and uniform plasmodesmal closure response, while physiological PAMP-triggered plasmodesmal closure is more heterogeneous (Tee and Faulkner, 2024). It would be interesting to explore the impact of inducing plasmodesmal closure in a more heterogeneous way, to more closely resemble PAMP-triggered plasmodesmal closure in mature leaves. The *LexA::PD-PLUG* line induces callose deposition following estradiol treatment in a more heterogeneous way using a fusion of the *PLASMODESMAL LOCALISED PROTEIN 1* (*PDLP1*) signal peptide, transmembrane domain and cytoplasmic tail to mCherry (Caillaud et al., 2014; Tee et al., 2024). Using this line may provide insight into whether a less uniform plasmodesmal closure response in

Chapter Four

young leaves impacts growth or defence responses.

How plasmodesmata are regulated to mediate this defence-growth trade-off across development is unclear. In the search to understand what underlies the trade-off between growth and defence, I explore the role of the sink-to-source transition on plasmodesmal regulation in Chapter 5.

Chapter 5

Plasmodesmal response to stress changes across the sink-source transition

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5.1 Introduction

Plasmodesmata play an important role in the long-range movement of molecules through a plant, particularly in the unloading of sugars from the phloem. Excess sugars synthesised in photosynthetic “source” tissues are loaded into the phloem and unloaded into “sink” tissues, which require sugars for growth and development. During phloem unloading sugars travel passively through plasmodesmata out of the sieve elements and into surrounding cells (Ross-Elliott et al., 2017). I hypothesise maintaining symplasmic connectivity during phloem unloading is important for normal leaf growth and development, and thus plasmodesmal closure is not induced in response to pathogen-associated molecular patterns (PAMPs) in young leaves. This hypothesis is based on evidence from previous chapters showing there is a lack of stress-triggered plasmodesmal closure in young sink leaves (Section 3.3.1) and that inducing plasmodesmal closure disrupts symplasmic phloem unloading and reduces growth (Sections 4.3.9 and 4.3.10). If this hypothesis is correct, and the importance of symplasmic connectivity in phloem unloading underpins plasmodesmal PAMP response, one would predict a shift in plasmodesmal response to stress across the sink-to-source transition.

The sink-to-source transition in leaves can be defined as when net carbon flux becomes positive, meaning the leaf is exporting more carbon than it is importing. Sink leaves import carbon from source leaves to fuel the high levels of respiration needed to drive growth and development (Marchi et al., 2005a, 2005b). As sink leaves develop, rates of carbon assimilation increase until eventually the net carbon gain exceeds the loss of carbon through respiration, at which point leaves transition from sink to source (Marchi et al., 2005a, 2005b). This transition point occurs at different stages depending on leaf position and plant species. In dicotyledonous plants, for instance, leaves typically become net exporters when the leaf is between 30-60% expanded, varying across different species and leaf positions on the stem (Marchi et al., 2005b; Turgeon, 1989). The transition is not uniform across a leaf, instead it occurs basipetally in dicotyledonous plants with leaf tissue at the tip of the leaf beginning to export sugars whilst tissue at the base continues to import sugars (Jones & Eagles, 1962; Turgeon & Webb, 1973).

The transition from sink-to-source in *Arabidopsis* involves a shift in sugar transport from phloem unloading via the symplasmic pathway to phloem loading via the apoplastic pathway. As discussed in more detail in Chapter 1 Section 1.4.1, apoplastic loading in *Arabidopsis* requires the activity of SUCROSE WILL

EVENTUALLY BE TRANSPORTED (SWEET) efflux proteins. SWEET11 and 12 transport sugar passively down the concentration gradient from the cytosol and into the apoplast for apoplastic phloem loading (Chen et al., 2012). Sucrose Uptake Carrier 2 (SUC2) then actively transports sucrose out of the apoplast and into companion cells (Gottwald et al., 2000). Although the specific transporters involved in apoplastic loading are well-characterised, the mechanism by which this apoplastic loading system is initiated during the sink-to-source transition is unknown. Previously, Fellows and Geiger (1974) and Turgeon (1989) suggested that maturation of phloem tissue occurs well before the carbon export is actually initiated, and hypothesise an unknown factor then drives the loading of sugars into the mature phloem tissue. Since the proposition of this hypothesis, there has been little progress in determining what triggers the initiation of loading. Questions remain as to whether there are physiological or developmental cues that trigger the expression of key sugar transporters to initiate phloem loading.

In addition to the initiation of apoplastic loading, structural changes to plasmodesmata have been associated with the sink-to-source transition. Studies in *Nicotiana tabacum* (tobacco) have shown 90% of plasmodesmata in sink leaves have a simple single-channel structure, whilst in source leaves 80% of plasmodesmata predominantly show a more complex branched plasmodesmal structure (Oparka et al., 1999; Roberts et al., 2001). Similarly, dye-unloading assays in *Arabidopsis* show detaching mature leaves accelerates the sink-to-source transition and increases the frequency of branched plasmodesmata (Fitzgibbon et al., 2013). This change is rapid, with an increase in branching seen within the first hour of detachment (Fitzgibbon et al., 2013). Thus, dynamic changes in plasmodesmal structure correlate with the sink-to-source transition.

The correlation between structure and sink/source leaf status has been suggested to link to changes in the transport capacity of different plasmodesmal structures. Simple plasmodesmata have been shown to have a higher transport capacity than branched plasmodesmata and enable rapid unloading of sugars in sink tissues, whilst the branched structure in source tissues could allow for increased control and regulation of sugar export (Brunkard et al., 2020; Kobayashi et al., 2005; Oparka et al., 1999). The TOR (TARGET OF RAPAMYCIN) signalling network is suggested to play a regulatory role in controlling this restriction of flow. Increased glucose signalling in source tissue is suggested to increase TOR activity, which is then associated with reduced plasmodesmal transport in source tissue (Brunkard et al., 2020). Thus TOR signalling is hypothesised to link carbohydrate availability

and plasmodesmal permeability (Brunkard et al., 2020). Despite the hypothesised relationship between the sink-to-source transition and plasmodesmal structure, it is also possible that plasmodesmal structure is only correlated with sink/source leaf status. More evidence is needed to determine if other developmental-, or age-related processes could be driving the shift in structure in different leaf ages.

Although the mechanisms and signalling driving the formation of branched and secondary plasmodesmata¹ remain unclear, genetic screens have identified proteins involved in the process. Kraner et al. (2017) carried out a forwards genetic screen in *Arabidopsis* using plants constitutively expressing the GFP-tagged 17 kDa movement protein (MP17-GFP) from *Potato Leaf Roll Virus*. MP17-GFP only localises to branched plasmodesmata and not simple plasmodesmata (Kronberg et al., 2007). To identify mutants lacking branched plasmodesmata, Kraner et al. (2017) identified mutants lacking MP17-GFP labelled plasmodesmata. Kraner et al. (2017) were able to identify the *Choline transporter-like 1* (*cher1*) mutants which lack MP17-GFP labelled branched plasmodesmata and have no formation of secondary plasmodesmata. The *cher1* gene encodes a protein involved in the subcellular transport of choline, a precursor in phospholipid synthesis, and is linked to lipid remodelling during cell wall expansion (Li-Beisson et al., 2013). Thus subcellular transport of choline may be important to the formation of branched and secondary plasmodesmata. Additionally, *cher1* mutants have excess starch accumulation in the photosynthetic cells of mature rosette leaves (Kraner et al., 2017; Li et al., 2021). This indicates the formation of branched and secondary plasmodesmata could have a role in sugar export out of photosynthetic cells.

In addition to the identification of *cher1*, other genetic screens have identified proteins with altered secondary plasmodesmal formation. The *increased size exclusion limit* (*ise*) mutants *ise1* and *2* were identified in *Arabidopsis* embryos with increased cell-to-cell dye movement (Kim et al., 2002). Both ISE1 and ISE2 are RNA helicases found in the mitochondria and chloroplasts, respectively, which mediate the formation of secondary plasmodesmata (Burch-Smith & Zambryski, 2010; Kobayashi et al., 2007). Burch-Smith, Brunkard, et al. (2011) suggested that defective chloroplast transcription and translation in *ise2* influences plasmodesmal formation through organelle-nucleus-plasmodesmal signalling. More recent evidence supports this, with Ganusova et al. (2020) finding that chloroplast-retrograde

¹As defined in Ding, Haudenschild, et al. (1992), the term "secondary" refers to any plasmodesmata which has formed post-cytokinesis and is a description of ontology, and the term "branched" refers the structure of the plasmodesma and is a description of morphology. All branched plasmodesmata could be considered secondary, but not all secondary plasmodesmata are branched.

Chapter Five

signalling regulates trafficking through plasmodesmata via the tetrapyrrole biosynthesis pathway. Thus plasmodesmal formation can be regulated in response to chloroplast signals.

Many questions remain about changes to plasmodesmal signalling and formation, as well as the role of plasmodesmata in sink tissue. Recent research raises questions about plasmodesmal regulation in roots. Arabidopsis roots are true sink tissue as they lack photosynthetic capacity. To study plasmodesmal permeability in roots, Gerlitz et al. (2018) developed a method to track cell-to-cell movement in roots using the DRONPA-S protein. DRONPA-S is a protein that can be photoconverted between a high and non-fluorescent state using a two-photon laser microscope (Ando et al., 2004; Lummer et al., 2011). Using this DRONPA-S system Gerlitz et al. (2018) and Pérez-Sancho et al. (2023) show reduced cell-to-cell mobility in root cortex cells 10 minutes after treatment with flg22 and NaCl. This result indicates plasmodesmata close in response to stress in root sink tissue, unlike in young sink leaves. These root experiments, however, were performed on sucrose-supplemented plates, and adding sucrose to media has previously been shown to alter plant-microbe interactions (Cabrera et al., 2022). Plasmodesmal response to flg22 and other stresses may also be altered in these sucrose-supplemented plates and not necessarily reflective of root responses in soil. Additionally, given previously I studied cell-to-cell mobility over several hours in microprojectile bombardment assays in leaves it is not clear whether the same plasmodesmal responses are captured by the different temporal scales, i.e. whether the flg22-triggered plasmodesmal closure in roots sustained for several hours. Several questions remain about whether PAMP-triggered plasmodesmal closure in roots is comparable to plasmodesmal closure in leaves, and additionally, how this plasmodesmal closure could affect the supply of nutrients to sink tissue in roots.

Overall, many questions remain regarding how plasmodesmal structure and permeability are dynamically regulated in response to developmental, physiological, and environmental cues. Here, I explore these questions by investigating how plasmodesmal regulation shifts between sink and source tissues in young leaves and roots, and additionally investigate the link between plasmodesmal regulation and structure.

Aims

In this chapter, I aimed to investigate my hypothesis that the sink-to-source

transition in leaves underpins the shift in plasmodesmal regulation between young and mature leaf tissue. I induced the sink-to-source transition in young leaves to uncouple the effect of leaf age (mature vs young) from the effect of sink vs source status on plasmodesmal function and structure. This enabled me to separate potential factors that underpin how plasmodesmata are regulated in response to stress.

Additionally, previous research found a correlation between plasmodesmal structure and leaf sink-source status. I questioned whether this correlation may also link to a change in plasmodesmal regulation across the sink-to-source transition. I aimed to investigate the role of plasmodesmal structure in plasmodesmal regulation across leaf age and the sink-to-source transition using existing transgenic *Arabidopsis* lines to image fluorescently labelled branched plasmodesmata and characterising plasmodesmal response in mutants with no branched plasmodesmata. Further to using transgenic and mutant lines, I aimed to quantify plasmodesmal structure across the sink-to-source transition in wild-type Col-0 lines using Serial Block Face-Scanning Electron Microscopy (SBF-SEM).

Finally, I aimed to explore another sink tissue type – root tissue. I tested the impact of inducing plasmodesmal closure in roots and aimed to develop a method to investigate how roots are regulated in response to PAMPs. This allowed me to determine whether my hypothesis around the sink-to-source transition in leaves is also applicable to other sink tissue types.

5.2 Methods

5.2.1 Inducing the sink-to-source transition

The sink-source transition was induced in young leaves of five-week-old Col-0 plants by covering mature leaves in a layer of foil with a circle cut in the middle for the three youngest leaves to access light (Figure 5.1). Young leaves harvested from covered plants were termed “young covered” leaves, and mature leaves that had been covered were termed “mature covered” leaves. Additionally, I harvested young and mature leaves from uncovered plants, termed “young uncovered” and “mature uncovered” leaves respectively.

5.2.2 Phloem unloading of GFP in leaves

Changes in GFP unloading in young leaves were quantified using *pAtSUC2::GFP* lines expressing GFP under the promoter of SUC2, a sucrose H⁺ symporter specifically expressed in phloem companion cells (Imlau et al., 1999). Five-week-old *pAtSUC2::GFP* plants were covered in foil or left uncovered as described in section 5.2.1 for either 24-30 or 48-52 hours. Young and mature leaves from uncovered plants were imaged alongside young leaves from covered plants. Leaves one to three (as defined in Figure 2.1) were imaged per plant to represent young leaves, and leaves eight and nine were imaged to represent mature leaves. Both the left and right abaxial side of each leaf were imaged. Images of GFP unloading in young leaves from covered and uncovered plants were acquired using an Axio ZoomV16 stereomicroscope with a Zeiss Plan Z1.5×/0.25, monochrome camera ORCA FLASH4 (C13440, Hamamatsu), and SPECTRA Light Engine (Lumencor). Leaves were illuminated with 463 nm to 487 nm light and fluorescence was collected with a 525/50 nm band-pass filter. Z-stack projections were generated using the Zeiss Blue v2.6 built-in software for the extended depth of focus wavelets method.

Images of GFP unloading were analysed using FIJI and R scripts originally developed in collaboration with Annalisa Bellandi for quantifying CFDA unloading (as described in section 4.2.3, Bellandi et al. (2022)). The images taken on either side of a leaf were considered technical replicates and averaged, resulting in one mean peak width value per leaf. Each leaf was considered a single biological replicate. A minimum of 13 mature leaves and 20 young leaves were quantified for

each tissue type and time point. A Wilcoxon rank sum test was used to compare the mean widths of peaks for each leaf between different tissue types. p-values were adjusted for multiple comparisons using the Holm correction in the ggpvr R package ((Holm, 1979); v0.0.6 Kassambara (2023)).

An additional analysis was carried out for images taken at the 24-30 hour timepoint. GFP unloading in young covered and young uncovered tissue was compared across different young leaf ages. A two-way ANOVA was conducted to determine the effect of treatment (young leaves from covered plants vs young leaves from uncovered plants) and leaf age (relative leaf age 1, 2 and 3) on mean peak width, with an interaction term included to determine whether the effect of treatment on mean peak width varied across different leaf ages. Following the two-way ANOVA, a Tukey HSD test was carried out to determine significant differences in treatments within and between different relative ages.

5.2.3 Quantifying starch and soluble sugars

Young and mature leaves were harvested from covered and uncovered plants at the end of the day, either 24 or 48 hours after wrapping treatment (Figure 5.4 A). Leaf tissues were harvested at the end of day as in *Arabidopsis* starch accumulates over the day, and so is at its highest level at the end of day, before the start of night (reviewed in (Smith & Zeeman, 2020)). Immediately after harvesting, leaves were weighed and flash-frozen in liquid nitrogen. Each young sample consisted of six harvested young leaves pooled from three different plants. Each mature sample consisted of three mature leaves pooled from three separate plants. Each sample was considered a biological replicate. For the 24-hour timepoint, a total of twelve samples were pooled across two experimental replicates for each tissue age and wrapping treatment. For the 48-hour timepoint, a single experimental replicate was carried out with six samples per tissue type.

To extract starch and soluble sugars, leaf material was first homogenised in 0.7 M perchloric acid using a mix mill (Retsch). Homogenised samples were pelleted by centrifugation with the insoluble pellet used to quantify starch and the supernatant used to quantify soluble sugars.

Starch content quantification

The insoluble pellet was washed in 80% ethanol, resuspended in water, and gelatinised for 15 minutes at 95°C as described in Smith and Zeeman (2006). The starch was then digested to glucose by adding α -amylase (Roche) and amyloglucosidase (Roche) in 0.22 M Na-Acetate Buffer (pH 4.8) and incubating at 37°C for two hours. As a control, samples were incubated with 0.22 M Na-Acetate Buffer (pH 4.8) without the addition of enzymes. These controls should remain as non-digested samples. Starch content was quantified by measuring the digested glucose content. NADH production was monitored in an Omega FLUOstar microplate reader in a hexokinase/glucose-6-phosphate dehydrogenase assay (Roche), measuring the absorbance of samples at 340 nm. Three technical replicates were included per digested sample, with one technical replicate for the non-digested sample. The control non-digested samples were checked to ensure they had no absorbance at 340 nm. The mean absorbance of the three digested technical replicates was used to calculate the digested starch content of the original sample in mg per gram of fresh weight. Starch contents were then analysed using an ANOVA, with significant differences between tissue types determined in a post-hoc Tukey HSD test.

Soluble sugar quantification

Soluble sugars were quantified by taking the supernatant from homogenized leaf tissue samples. The supernatant was neutralised to a pH of 6-7 using neutralisation buffer (2 M KOH, 400 mM MES) and quantified in a solution of 1M HEPES.KOH (pH 7.6), 1 mM ATP, 1 mM NAD, 100 mM MgCl_2 , 1.4U Hexokinase and H_2O . An Omega FLUOstar microplate reader was used to quantify soluble sugars in an enzymatic assay. Glucose, fructose, and sucrose contents were determined by measuring sample absorbance after adding glucose-6-phosphate dehydrogenase, phosphoglucose isomerase and invertase, respectively. The mean absorbance of three technical replicates was used to calculate soluble sugar contents in mg per g of fresh weight. Soluble sugar contents were then analysed using an ANOVA, with significant differences between individual tissue types determined in a post-hoc Tukey HSD test.

5.2.4 Transcriptional analysis

Five-week-old Col-0 plants were either covered or left uncovered as described in 5.2.1. 24 hours later, mature leaves from uncovered plants and young leaves from uncovered and covered plants were syringe-infiltrated with water or chitin (500 µg/ml) and harvested 30 minutes later (see Table 5.1 for all tissue-type and elicitor combinations). Six young leaves from three plants were pooled per young biological replicate, and four mature leaves were pooled from two plants per mature biological replicate. Five biological replicates in total were collected for each tissue type-elicitor combination. As described in more detail in section 2.5, I extracted RNA for sequencing by Genewiz (Azenta Life Sciences), calculated gene read counts, and used DESeq2 (v.1.38.3) to perform gene expression analysis in R studio (v2022.12.0+353) (Love et al., 2014; RCoreTeam, 2023).

5.2.5 Quantification of PSII efficiency

The operating efficiency of PSII (ΦPSII) can be calculated using chlorophyll fluorescence emission. As defined by Genty et al. (1989), ΦPSII can be calculated as

$$\Phi(\text{PSII}) = \frac{(F_{m'} - F')}{F_{m'}} \quad (5.1)$$

where F is defined as steady-state chlorophyll fluorescence under actinic light conditions and $F_{m'}$ is the maximum chlorophyll fluorescence emission in light-adapted tissues when reaction centres are closed. F' can be determined from chlorophyll fluorescence in actinic light conditions, whilst $F_{m'}$ can be determined by exposing light-adapted leaves to a saturating light pulse.

To determine ΦPSII in the growth conditions used here, chlorophyll fluorescence was measured using an IMAGING-PAM Maxi Chlorophyll Fluorometer (Heinz Walz GmbH). First, Col-0 plants were dark-adapted for a minimum of 45 minutes, and then young and mature leaves were detached and placed in the IMAGING-PAM. Leaves were first exposed to a saturating light pulse of 845 µmol m⁻²s⁻¹ and then actinic light was set to match growth cabinet conditions at 189 µmol m⁻²s⁻¹. Every 60 seconds a saturating light pulse was used to measure $F_{m'}$. After approximately 20 mins of acclimation to actinic light, the value of $F_{m'}$ reaches its maximum, at

which point F_m' and F' can be used to calculate $\Phi PSII$ under the actinic light of $189 \mu\text{mol m}^{-2}\text{s}^{-1}$ using equation 5.1.

$\Phi PSII$ was quantified in both young and mature uncovered and covered leaves and from 5-week-old Col-0 plants. Chlorophyll fluorescence in the three youngest leaves (leaves one to three) and mature leaves (leaves eight and nine) were measured. In total a minimum of twenty-eight young leaves of each leaf number and twelve mature leaves were measured across two experimental replicates. A linear mixed model analysis was performed with experimental replicate included as a nested variable and a Tukey HSD multiple comparisons test was used to compare $\Phi PSII$ efficiency between all tissue types and leaf ages.

5.2.6 Estradiol treatment in roots

Transgene expression in the roots of *LexA::icals3m* and *PAGFP*×*LexA::icals3m* seedlings was induced using estradiol-containing media. Seeds were first stratified and then sterilised by the wet sterilisation method described in Section 2.1.2. Seedlings were sown onto $0.5\times$ MS-S plates and grown vertically. After 9 or 10 days of growth seedlings were transplanted onto $0.5\times$ MS-S plates with estradiol added to the media. To prepare the estradiol media, β -estradiol 17-acetate (Sigma-Aldrich) in DMSO was diluted in $0.5\times$ MS-S to a final concentration of $20 \mu\text{M}$ estradiol. A corresponding mock solution was prepared by diluting DMSO to a final concentration of 0.001% DMSO in $0.5\times$ MS-S.

5.2.7 Quantification of callose deposition in roots

LexA::icals3m seedlings were stratified, sterilised by wet sterilisation and grown vertically on $0.5\times$ MS-S plates for 9 days and transplanted onto $0.5\times$ MS-S plates containing $20 \mu\text{M}$ estradiol or 0.001% DMSO. 24 hours after transplanting, callose deposition was quantified by aniline blue staining. Roots were dipped in 1% aniline blue $1\times$ PBS and immediately imaged on a Zeiss LSM 800 confocal microscope. I imaged two root regions: the root tip and the upper edge of the elongation zone, just before cells begin to differentiate into root hair cells, referred to here as the elongation zone (Figure 5.22). Images were acquired using a $20\times$ objective (Plan-Apochromat 20x/0.8 M27). Aniline blue was excited using the 405 nm laser and collected at 410-500 nm.

To quantify the general intensity of callose staining in the elongation zone and root tip using FIJI (Schindelin et al., 2012). In the elongation zone, a circle of set area in each root z-stack was drawn and the mean intensity of aniline blue within the circles was measured. In the root tip, I repeated the same quantification method as in the elongation zone, drawing a circle at the same distance from the root cap in all root tips to measure the mean intensity of aniline blue staining. 11 roots were imaged for each treatment, and the mean intensities of callose staining compared in a Wilcoxon rank-sum test.

5.2.8 Quantification of root growth

Root growth was quantified in Col-0 and *LexA::icals3m* lines. Col-0 and *LexA::icals3m* seeds were transplanted from 0.5× MS-S vertical plates at nine days post germination onto 0.5× MS-S vertical plates containing 20 µM estradiol or 0.001% DMSO. Images were taken of seedlings two and four days after transplanting onto estradiol plates. Root length was measured using FIJI (Schindelin et al., 2012). Change in root length between days was calculated and compared between treatments (DMSO vs estradiol) and time points (day two vs day four) using a two-way ANOVA, and individual comparisons between treatments within individual time points were made using a Tukey HSD test.

5.2.9 Phloem unloading of 5(6)-Carboxyfluorescein in roots

To measure CF unloading in estradiol-treated roots, I transplanted *LexA::icals3m* seedlings from 0.5× MS-S vertical plates at nine days post germination onto 0.5× MS-S plates containing 20 µM estradiol or 0.001% DMSO. Dye loading was carried out 24 hours after transplanting onto estradiol or DMSO plates. As described in Kalmbach et al. (2023), 0.3 µL droplets of 1 mM CFDA solution, with added 0.5% Adigor (Syngenta) to enable CFDA to pass more easily through the cuticle, were placed onto cotyledons. CF unloading in the root was imaged 45 minutes after dye-loading, and additional exploratory images were taken 3 hours after dye-loading. Images were acquired using a Zeiss LSM 800 confocal microscope with a water immersion 20× objective (Plan-Apochromat 20× /0.8 M27). A minimum of nine roots were imaged for each treatment.

5.2.10 Photoactivatable GFP movement in roots.

Seedlings expressing *Photoactivatable GFP* (*PAGFP*) under the 35S promoter were grown for nine or ten days on 0.5× MS-S plates before treatment and photoactivation. Using a confocal microscope, a 70 µm × 30 µm region located 175 µm from the root tip was activated using a 405 nm laser and 75% laser power. Images of the photoactivated root were acquired immediately after photoactivation and 3.5 hours later.

The change in the area of PAGFP between images taken at the two time points was used as a metric for cell-to-cell movement as shown in Figure 5.28 A. To quantify the area of PAGFP, I changed the parameters of the particle detection FIJI macro previously described in section 2.4 for callose deposit detection. I used trial and error to optimise the threshold and maximum and minimum area for the detection of PAGFP fluorescence. I set the pixel threshold through trial and error, ensuring it was low enough to detect the diffusion of small amounts of PAGFP but high enough to avoid picking up background noise. The same intensity threshold was used across timepoints and in the control and treatment.

For NaCl treatment, I treated PAGFP seedlings with NaCl or water for 15 minutes before photoactivation. I used the same NaCl concentration of 20 µM previously described to induce plasmodesmal closure as Pérez-Sancho et al. (2023). Photoactivation and image acquisition were acquired using a Zeiss LSM 880 confocal microscope with a 25× objective (LD LCI Plan-Apochromat 25x/0.8 Imm Korr DIC M27). Photoactivation was performed using the in-built Zen Blue (Zeiss) bleaching program, photoactivating with a 405 nm laser at a laser strength of 10 with 3 cycles at scanning speed 1. Image acquisition was carried out using the same objective, with PAGFP excited by the 488 nm laser and emission collected at 500-600 nm. A total of six roots were imaged per treatment and the change in movement of GFP was compared between water and NaCl treatments using a student's t-test comparing the means of the two treatments.

For *LexA::icals3m* x PAGFP lines, seedlings from 0.5× MS-S plates were transplanted onto 0.5× MS-S plates containing 20 µM estradiol or 0.001% DMSO. 24 hours after transplanting, PAGFP movement was assayed for in roots. The same image acquisition and photoactivation settings on a Zeiss 880 confocal microscope were used as described for the NaCl treatment above. I carried out three experimental replicates with a total of twelve seedlings per treatment. Each

seedling was considered a biological replicate. Change in the movement of GFP was compared between DMSO and estradiol treatments using a one-way ANOVA for each experimental replicate.

For flg22 treatment, PAGFP seedlings were treated with 100 μ M flg22 or water 30 minutes before photoactivation. Photoactivation and image acquisition were carried out using a Zeiss 800 confocal microscope with a 20 $\times$ objective (Plan-Apochromat 20x/0.8 M27). Photoactivation was carried out using the in-built Zen Blue (Zeiss) bleaching program with parameters set to match the bleaching settings used for NaCl and *LexA::icals3m* x PAGFP line assays. I quantified movement in 12 roots per treatment and carried out a student's t-test comparing the means of water versus flg22 treatment.

5.2.11 PA-GFP x *LexA::icals3m* crosses movement in roots

In this chapter, *LexA::icals3m* x PA-GFP lines were generated. *LexA::icals3m* was selected as the female line and *p35S::PAGFP* lines were selected as the male line. The female line was prepared by emasculinising partially developed flowers by removing the stamens to leave only the gynoecium. The following day stamens from the male line were rubbed on gynaecium from the female line and wrapped in clingfilm. Several days later, siliques were bagged once they began to develop. F1 seeds were bulked up and self-crossed.

Segregating F2 populations were phenotyped to identify offspring homozygous for *PAGFP* and *LexA::icals3m* alleles. The presence of homozygous *LexA::icals3m* alleles was determined by growing F2 seed on estradiol plates alongside *LexA::icals3m* and Col-0 lines in a short-day 10-hour light and 14-hour dark cycle (Panasonic Versatile Environmental Test Chamber MLR-352-PE). F2 seedlings with homozygous *LexA::icals3m* alleles exhibit a chlorotic phenotype in leaves touching the estradiol media and show retarded growth relative to Col-0 and heterozygous plants. F2 seedlings with homozygous *PAGFP* alleles were screened for using a Zeiss 800 confocal microscope. The expression of PAGFP was tested by photoactivating leaves using the same photoactivation parameters as section 5.2.10. It was difficult to determine whether PAGFP lines were homozygous or heterozygous based on the strength of photoactivation, so seedlings showing both PAGFP activation and slowed growth and chlorosis were taken to the F3 generation. Multiple offspring from each of the suspected F2 homozygous segregating lines were sown, and any F2 lines with offspring not showing growth defects on estradiol or not showing

fluorescent were deemed to be heterozygous. F3 offspring from F2 lines suspected to be homozygous were taken to the next generation and again homozygosity of F3 lines was checked to determine if F4 seedlings showed homozygous phenotypes for *PAGFP* and *LexA::icals3m* alleles. Two homozygous F3 lines were confirmed using this method and used for PAGFP assays.

5.2.12 MP17-GFP lines

Lines expressing *MP17-GFP* under the 35S promoter were used in this work to visualise plasmodesmal branching. As MP17-GFP and Col-0 lines grow at different rates, leaves from seven-week-old MP17-GFP plants were compared to leaves from five-week-old Col-0 plants, as that is when both lines typically show 14-17 true leaves. Microprojectile bombardments were carried out on mature leaves from MP17-GFP plants as described in section 2.3. Callose staining of MP17-GFP lines was carried out as described in section 2.4 with adjusted image acquisition settings to capture both GFP and aniline blue fluorescence. The GFP fluorescence was excited using the 488 nm laser and collected at 500 - 617 nm, aniline blue was excited using the 405 nm laser and collected at 410 - 480 nm.

5.2.13 *cher1* lines

Choline transporter-like1 (*cher1*) mutant lines which have a T-DNA insertion in the *cher1* gene were used to study changes in plasmodesmal structure (Kraner et al., 2017). Microprojectile bombardments were carried out on 5-week-old *cher1* lines as described in section 2.3 with leaves infiltrated leaves with water (distilled) or chitin (500 µg/mL) two hours after bombardment. Aniline blue staining was carried out on mature leaves from five-week-old Col-0 and *cher1* lines as described in section 2.4.

5.2.14 Imaging plasmodesmal structure using Serial Block-face Scanning Electron Microscopy

Young leaves from 5-week-old Col-0 uncovered and covered plants were sampled 24 hours after covering. Three samples were imaged per treatment. The fixation was carried out by Rhea Stringer as outlined in Atkinson et al. (2024). Briefly,

samples were fixed in 2.5% (v/v) glutaraldehyde (GA), 2% (v/v) paraformaldehyde (PFA), and 2 mM CaCl_2 in 0.1 M sodium cacodylate buffer (NaCaC), pH 7.4 and inserted into a Pelco BioWave Pro+ (Agar Scientific) for irradiation under vacuum. Samples were embedded into a 13-mm flat TAAB cavity mould containing fresh 100% Durcupan resin (Merk Life Sciences) at 60 °C for 48 hours. A razor blade was utilized to trim the samples to the appropriate size. The trimmed samples were then attached to a Gatan 3View specimen pin, standard 1.4 mm flat type (Agar Scientific), using conductive epoxy glue. To further refine the geometry of the resin block, a Leica ARTOS 3D Ultramicrotome (Leica Microsystems) and a DiATOME trim 45 diamond trimming tool (Leica Microsystems) were employed. The sample pins were then sputter-coated with a 15 nm layer of platinum using a Leica ACE 600 sputter coater (Leica Microsystems). Subsequently, the samples were placed in a Zeiss Gemini 300 SEM (Carl Zeiss Microscopy) with focal charge compensation, integrated with a Gatan 3View® 2XP in situ ultramicrotome and an OnPoint detector (AMETEK). Gatan DigitalMicrograph DMS3 software (AMETEK) was used to capture images in high vacuum conditions at 1.5kV. Sections were taken at a thickness of 50 nm, with a pixel size of 30 nm, pixel dwell time of 8 μs , and image size of $2,048 \times 2,048$. Datasets were aligned and segmented by Rhea Stringer on Microscopy Image Browser (MIB) (<https://mib.helsinki.fi>, Microscopy Image Browser), and visualized using Amira software (Thermo Fisher Scientific).

5.3 Results

5.3.1 Method to cover mature leaves to induce the sink-to-source transition

To determine if plasmodesmal regulation in young leaves is underpinned by the importance of plasmodesmata for phloem unloading in sink tissues, I manipulated the sink-source status young leaves by shading mature leaves. I aimed to decouple the impact of leaf age from the impact of sink-source status on plasmodesmal regulation. As young leaves are defined as sink due to carbon imports from mature source leaves, I hypothesised covering mature leaves limits the light-reactions of photosynthesis, resulting in reduced production and export of photosynthates to sink tissues. Starved of their carbon supply, I then predicted the remaining uncovered young sink leaves would no longer be sink tissue, and would, by definition, become source tissue as there is little or no import of carbon from mature leaves. Thus, I hypothesised plasmodesmata would be “free” from their role in phloem unloading and regulated differently.

I covered mature leaves with foil to block out light and left three young sink leaves uncovered (Figure 5.1). Young leaves assayed from covered plants were termed “young covered” leaves, and mature leaves that had been covered were termed “mature covered” leaves (Figure 5.1). Additionally, I assayed young and mature leaves from uncovered plants, termed “young uncovered” and “mature uncovered” leaves respectively. During the foil-wrapping process, I made a note of which leaves in young uncovered plants were of equivalent age to the leaves in young covered plants. I then performed assays with these specific young leaves the next day. This was to ensure young covered and young uncovered leaves were at the same initial developmental timepoint before being left uncovered or covered.

5.3.2 Covering mature leaves reduces GFP unloading in young leaves

I predicted phloem unloading would be reduced in young leaves from covered plants. To test this prediction, I investigated phloem unloading patterns in young leaves from covered and uncovered plants using *pAtSUC2::GFP* lines. These Arabidopsis lines express *GFP* under the promoter of *SUC2*, a sucrose H⁺ symporter specifically



Figure 5.1: **Labeled images of uncovered and foil-covered Arabidopsis - Col-0** with young (dark orange) and mature (light orange) leaves from uncovered plants and young (dark green) and mature (light green) leaves from plants with foil-covered mature leaves

expressed in phloem companion cells (Imlau et al., 1999). In mature source leaves GFP is synthesised in companion cells and loaded into the phloem, and then unloaded from the phloem in sink leaves (Imlau et al., 1999; Wright et al., 2003). I covered *pAtSUC2::GFP* plants in foil or left them uncovered for 24-30 or 48-52 hours before imaging leaves. To quantify GFP unloading I used the same image quantification method as in the carboxyfluorescein unloading assay outlined in Chapter 4 Section 4.2.3. The image analysis generates a 50 x 50 grid across each image, with the fluorescence intensity measured across each transect and the mean width of the intensity peaks calculated for each leaf (Figure 5.2 A-C). I used the mean width of GFP signal as a measure of GFP movement out of the phloem.

The width of GFP signal around the vein was significantly greater in young uncovered leaves compared to mature uncovered leaves (Figure 5.2 D). This indicates GFP moved further out of the phloem in young sink leaves than mature source leaves. 24-30 hours after foil covering, young leaves from plants with covered mature leaves showed no significant difference in the width of GFP movement compared to mature leaves and a significantly reduced width compared to young sink leaves. The same trend was seen 48-52 hours after covering, suggesting young leaves from covered plants have reduced phloem unloading of GFP. This supports my hypothesis that covering mature leaves could reduce phloem unloading into young leaves and induce the sink-to-source transition.

Additionally, I questioned whether the age of young leaves impacts the change

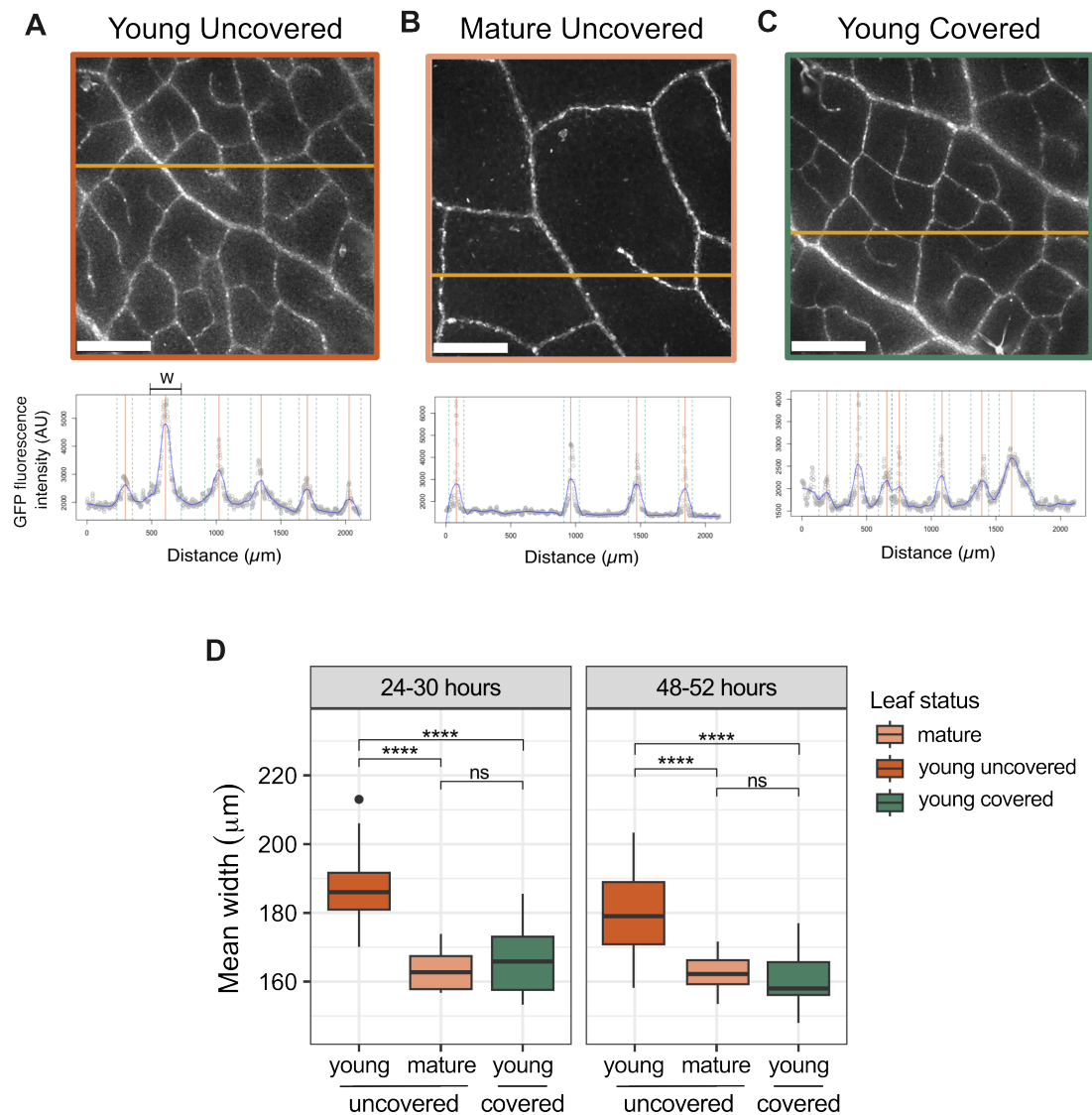


Figure 5.2: **Phloem unloading of GFP in covered and uncovered plants** – Extended Depth of Focus images of (A) mature, (B) young sink, and (C) young source plants in foil covered and uncovered plants, the orange line indicates a transect with the intensity of this transect plotted below each image, where “W” indicates the width of the peak and scale bars indicate 500 μm . (D) Boxplots of mean widths in young uncovered (dark orange), mature uncovered (light orange) and young covered (dark green) leaves. “ns” indicates no significant difference and “****” indicates $p < 0.001$.

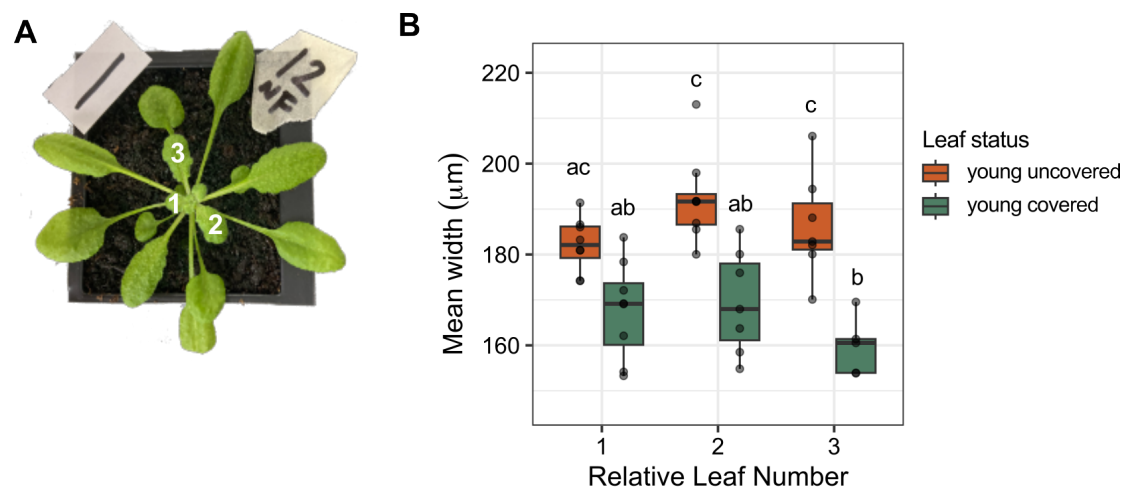


Figure 5.3: **GFP unloading across different young leaf ages** – (A) Arabidopsis rosette labelled with relative young leaf numbers where “1” is the youngest and “3” is the oldest. (B) Boxplots showing mean width of GFP unloading across young relative leaf numbers, different letters indicate significant differences where $p < 0.05$. Boxplots show median $\pm$ interquartile range, with each individual point representing an individual leaf.

in phloem unloading. I quantified phloem unloading in the three youngest leaves (Figure 5.3 A). Relative leaf numbers 2 and 3 showed significantly increased GFP unloading in young leaves from covered plants compared to young uncovered leaves. Although there is a similar trend in the youngest leaf, relative leaf number 1, there is no significant difference in the mean width of GFP unloading between young covered and uncovered leaves (Figure 5.3 B).

5.3.3 Starch content, but not fructose or sucrose content, increases in young leaves from foil-covered plants

The reduction in phloem unloading of GFP into young leaves from covered plants indicates there could also be reduced unloading of sugars. I hypothesised a reduction in phloem unloading of sugars could result in changes to leaf physiology. To determine if there are changes to carbon partitioning and storage, I quantified starch and soluble sugar contents in covered and uncovered leaves. Twenty-four hours after mature leaves were covered or left uncovered, leaf tissue from young and mature leaves was harvested at the end of day for starch and soluble sugar extractions.

Young leaves from covered plants showed a significantly higher accumulation of starch than in both uncovered young and uncovered mature leaves (Figure 5.4 B).

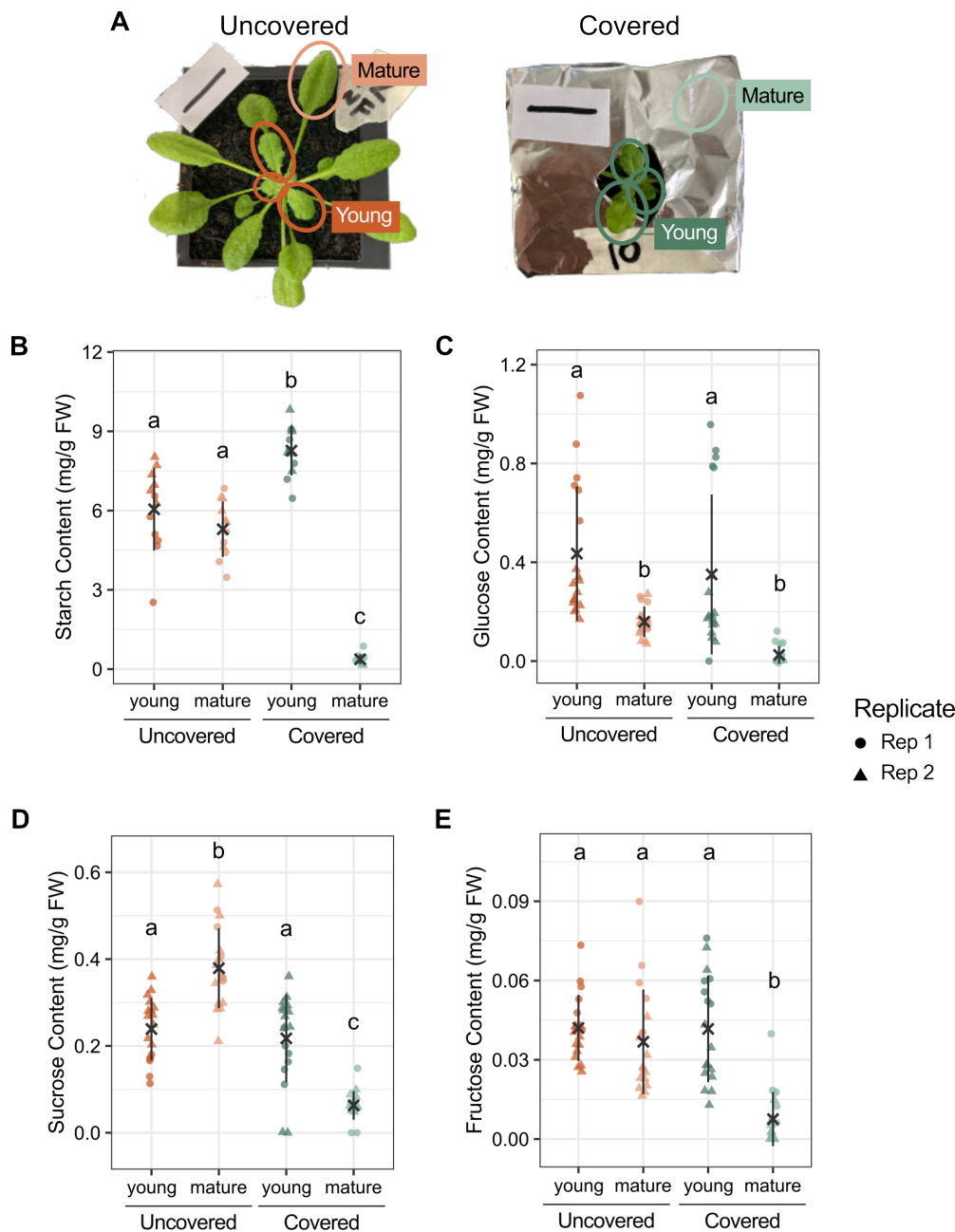


Figure 5.4: Starch and soluble sugar quantification in plants with covered mature leaves after 24 hours – (A) Images of uncovered and foil-covered *Arabidopsis* Col-0 with labelled young (dark orange) and mature (light orange) leaves from uncovered plants and labelled young (dark green) and mature (light green) leaves from plants with foil-covered mature leaves. Plots of starch content (B), glucose content (C), sucrose content (D), and fructose content (E) showing mg/g of fresh weight (FW). Different letters indicate significant differences between tissue types where $p < 0.05$, crosses indicate the mean value, and lines indicate $\pm$ standard deviation. Circles represent individual samples from the first replicate, and triangles indicate individual samples from the second replicate.

Additionally, covering mature leaves with foil significantly depletes starch stores after 24 hours, with covered mature leaves showing very low starch contents. This indicated the covering of mature leaves resulted in increased starch accumulation in young leaves from covered plants and starch depletion in the covered mature leaves.

Quantification of soluble sugars revealed no significant differences in glucose, sucrose, and fructose contents between young leaves from uncovered and covered plants (Figure 5.4 C to E). There was substantial between-replicate variation in glucose contents in young leaves, with replicate 1 showing a trend of higher glucose contents compared to replicate 2 (Figure 5.4 C). The variability between replicates makes it more difficult to conclude whether there are differences in glucose accumulation between young uncovered and young covered due to the high standard deviation when both replicates are pooled.

Mature and young leaves showed age-specific differences in soluble sugar contents. There was significantly higher sucrose and lower glucose contents in mature uncovered leaves compared to both young uncovered and young covered leaves. Additionally, covered mature leaves had very low levels of glucose, sucrose and fructose, indicating soluble sugars are depleted after light is blocked. Overall, twenty-four hours after mature leaves were covered there were significant changes in starch contents between young covered and uncovered leaves, but not soluble sugars.

To determine if a longer period of covering resulted in additional trends in starch and soluble sugar contents, I harvested samples 48 hours after mature leaves were covered. As this was an exploratory assay, I carried out a single experimental replicate with six samples per tissue type. In this single replicate, trends in starch content at a 48-hour timepoint are the same as at the 24-hour timepoint, with starch accumulating at higher levels at the end of day in young covered leaves (Figure 5.5 A). Trends in glucose content did shift, with glucose significantly reduced in young leaves from covered plants compared to young uncovered leaves after 48 hours (Figure 5.5 B). The trends for sucrose and fructose were the same at 24 and 48 hours (Figure 5.5 C and D).

Overall, covering mature leaves resulted in increased accumulation of end-of-day starch in young leaves at both 24- and 48-hour time-points. This increased starch accumulation may link to the decreased glucose levels after 24 hours, with more carbon being diverted to starch storage and less to glucose production, or

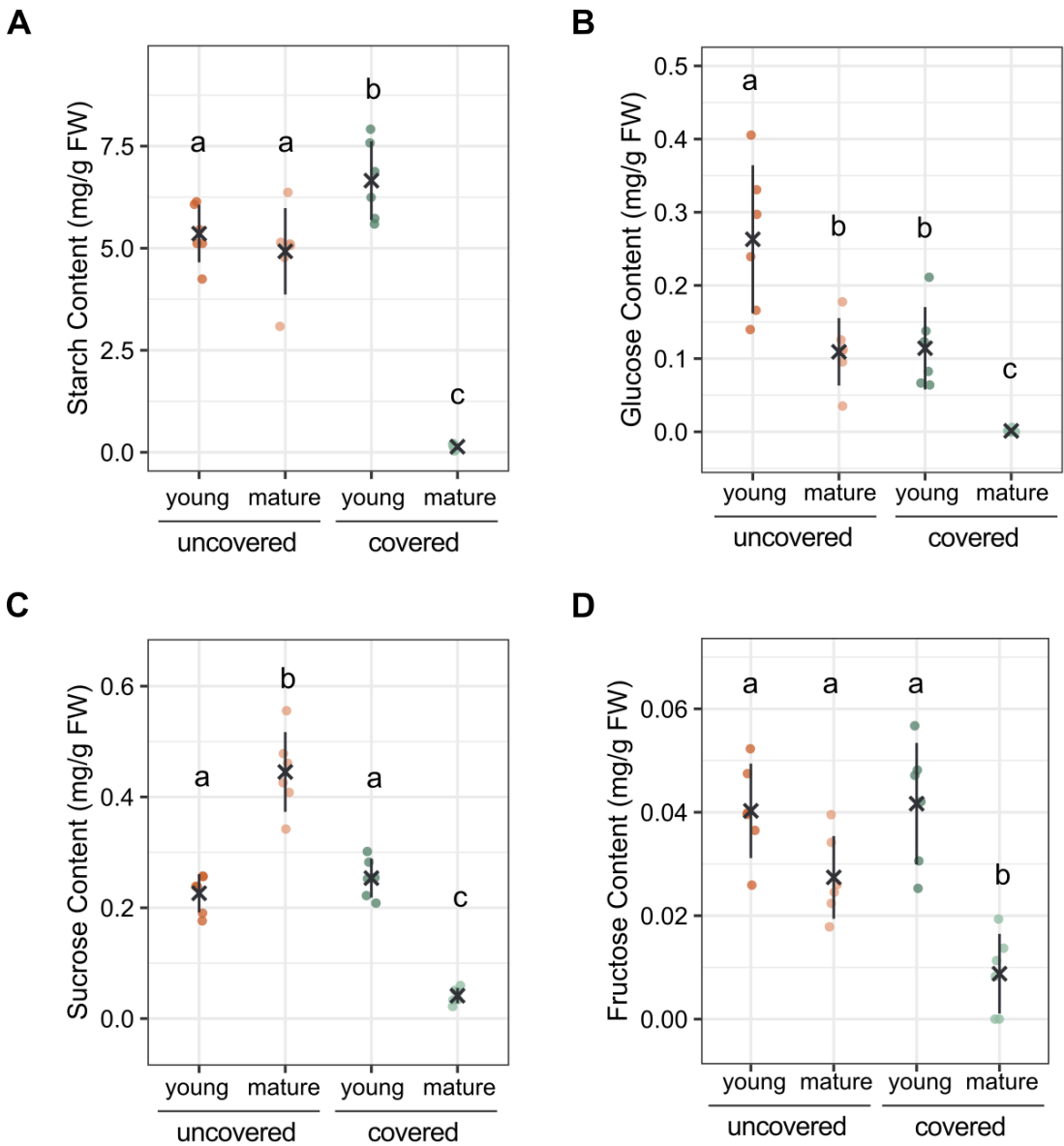


Figure 5.5: **Starch and sugar quantification 48 hours after foil wrapping** – Starch (A), glucose (B), sucrose (C), and fructose (D) contents in mg/g of fresh weight (FW) in young (dark orange) and mature (light orange) leaves from uncovered plants and young (dark green) and mature (light green) leaves from plants with mature leaves covered. Different letters indicate significant differences between tissue types where $p < 0.05$, crosses indicate the mean value, and lines indicate $\pm$ standard deviation. Circles represent individual samples

alternatively, there could be increased carbon fixation resulting in more carbon availability for storage.

5.3.4 PSII operating efficiency increases in young leaves from covered plants

The increased starch accumulation in young leaves from covered plants indicates there is a shift in carbon partitioning. Sugars could be diverted from existing pools of carbon towards starch storage or there could be an increase in carbon assimilation. This increase in carbon assimilation may involve changes to the activity of the photosynthetic machinery. To explore this, I used chlorophyll fluorescence to measure the light use efficiency of Photosystem II (Φ PSII). Twenty-four hours after mature leaves were covered or left uncovered, I measured operating Φ PSII at an ambient photosynthetic active radiation (PAR) of $189 \mu\text{mol m}^{-2} \text{s}^{-1}$, which was a similar PAR to the growth cabinet conditions. To determine if there were leaf-age specific differences between young leaves I tracked leaf number, with the youngest leaf numbered “1”, the second youngest numbered “2” and third youngest numbered “3” (Figure 5.6 A). The youngest young leaf, leaf 1, showed no significant difference in operating Φ PSII at ambient PAR between young covered and young uncovered leaves (Figure 5.6 B). Leaves 2 and 3 both showed significant increases in operating Φ PSII in young covered compared to young uncovered leaves. This suggests a leaf-age dependent increase in operating Φ PSII in young leaves from covered plants relative to young leaves from uncovered plants.

In addition to differences in young leaves, I found mature leaves from covered plants had a significant decreased operating Φ PSII at ambient PAR compared to mature leaves from uncovered plants (Figure 5.6 B). This indicates covering mature leaves results in reduced operating Φ PSII, which could indicate there is a degradation of photosynthetic machinery or alternations to the electron transport chain (Baker, 2008). Additionally Φ PSII was lower in mature leaves compared to young leaves across all leaf ages from both covered and uncovered plants. This indicates there are baseline differences in Φ PSII efficiency between young and mature leaves, and that young leaves from covered plants do not have a similar operating Φ PSII to mature source leaves.

Overall, the decreased operating quantum PSII efficiency and reduced accumulation of sugars in mature covered leaves indicated they have reduced ability to produce and export sugars to young leaves. This suggests young leaves from covered plants are unlikely to receive a supply of sugars from covered mature leaves. Additionally the reduced phloem unloading, increased starch content, and increased PSII operating efficiency in young leaves “2” and “3” indicates

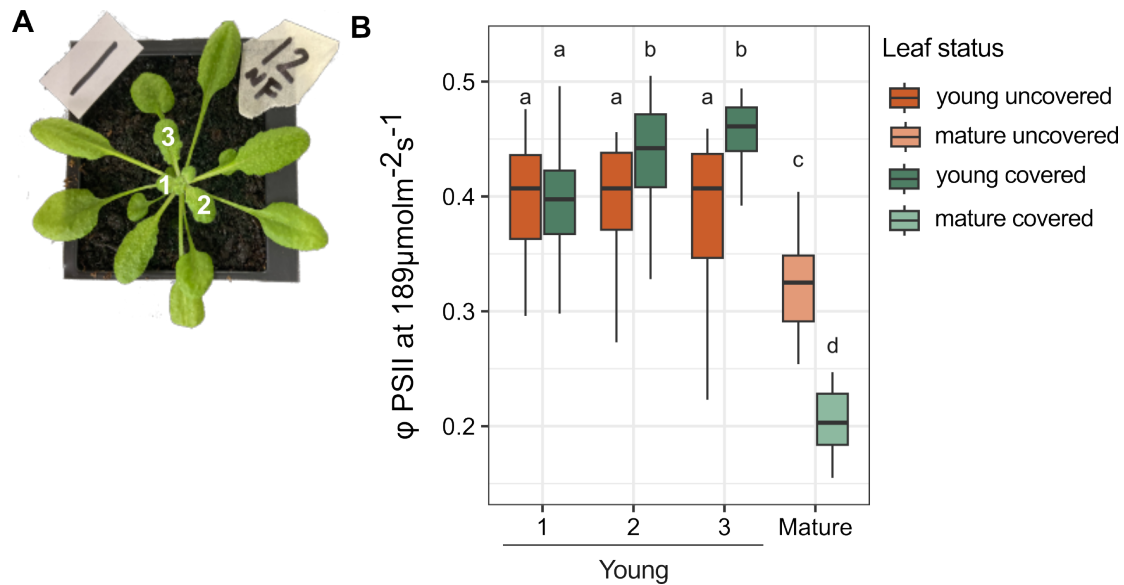


Figure 5.6: **Operating quantum efficiency of Photosystem II at ambient light** – (A) Labelled young leaf numbers where “1” is the youngest of leaves sampled and “3” is the oldest sampled young leaf. (B) Boxplot showing the operating quantum efficiency of Photosystem II (Φ_{PSII}) at ambient light across three ages of young leaf and mature leaves comparing young uncovered (dark orange) to young covered (dark green), and comparing mature uncovered (light orange) to mature covered (light green). Different letters indicate significant differences between tissue types where $p < 0.05$.

physiological changes occurring in these young leaves. As a result of these data, I suggest young leaves “2” and “3” are undergoing a sink-to-source transition. From here onwards I refer to young leaves “2” and “3” from covered plants as young “source” leaves and young leaves from uncovered plants as young “sink” leaves.

5.3.5 Inducing the sink-to-source transition changes plasmodesmal PAMP response.

To determine whether inducing the sink-to-source transition in young leaves changes plasmodesmal regulation in response to pathogen associated molecular patterns (PAMPs), I carried out GFP cell-to-cell movement assays. Mature leaves were either covered with foil or left uncovered and sampled for microprojectile bombardments twenty-four hours later. Leaves were infiltrated with mock (distilled H_2O), chitin ($500 \mu\text{g/mL}$) or flg22 (100 nM) two hours after microprojectile bombardments. GFP movement in a minimum of 130 bombardment sites for each combination of elicitor and tissue type and results were pooled across several days of experiments.

As previously found in chapter three, chitin treatment in young sink leaves did

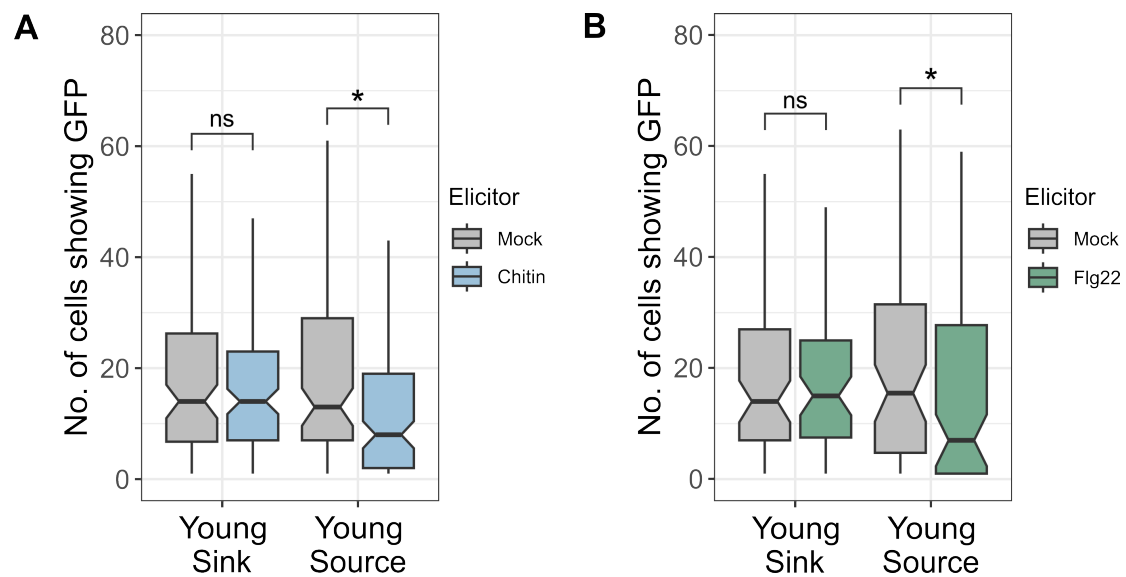


Figure 5.7: Cell-to-cell mobility in response to chitin and flg22 in young sink and young source leaves – (A) Boxplot of cell-to-cell mobility assay after treatment with mock (water, grey) vs chitin (blue). (B) Boxplot of cell-to-cell mobility assay after treatment with mock (water, grey) vs flg22 (green). “ns” = non-significant, “*” = $p < 0.05$.

not induce any significant change in cell-to-cell mobility of GFP (Figure 5.7 A). In young source leaves, however, where the sink-to-source transition had been induced, cell-to-cell mobility was significantly reduced in response to chitin. Similarly, young sink leaves show no significant differences in cell-to-cell mobility in response to flg22 whilst young source leaves show a significant reduction in cell-to-cell mobility (Figure 5.7 B). These results suggest inducing a premature sink-to-source transition in young leaves changes plasmodesmal regulation in response to PAMPs. This indicates sink vs source status is important in determining PAMP response in young leaves.

5.3.6 Transcriptional changes across the sink-to-source transition

To explore whether transcriptional changes across the sink-to-source transition underpin the shift in plasmodesmal response, I transcriptionally profiled the sink-to-source transition and chitin responses across the sink-to-source transition. Mature leaves from Col-0 plants were covered in foil or left uncovered for 24 hours and then young and mature leaves were infiltrated with chitin (500 $\mu\text{g}/\text{ml}$) or water (distilled water) and harvested 30 minutes later (note: chitin responses across the sink-to-source transition are discussed in the next section, section 5.3.7). I harvested

Genotype	Tissue Type	Elicitor	Plasmodesmata
Col-0	Young Sink	Water	Open
		Chitin	
	Young Source	Water	Open
		Chitin	Closed
	Mature Source	Water	Open
		Chitin	Closed

Table 5.1: **Transcriptomic treatment, genotype and elicitor combination** - Mature source tissue and young sink tissue were harvested from Col-0 plants, whilst young source tissue was harvested from Col-0 plants 24 hours after mature leaves were covered. Leaves were infiltrated with chitin or water and harvested 30 minutes later. The “Plasmodesmata” column indicates where plasmodesmal closure is induced by elicitor treatment.

young source leaves from covered plants, young sink leaves from uncovered plants, and mature source leaves from uncovered plants with five biological replicates collected for each tissue type-elicitor combination (Table 5.1).

To determine if there were transcriptional changes associated with the sink-to-source transition, I first focused on the water-infiltrated samples. Using a Wald test, I identified differentially expressed genes between tissue types using a threshold of an adjusted p-value of less than 0.05 and a $\log_2$ fold change (LFC) of greater than 0.5 or less than -0.5. I found a total of 130 differentially expressed genes between young sink and young source tissue, with 72 up-regulated and 58 down-regulated in young source tissue. These differentially expressed genes could be potential candidates for genes associated with the sink/source tissue status (Figure 5.8 B).

I reasoned that genes associated with the sink/source tissue status would also be differentially expressed between young sink and mature source tissue. I found over 9,000 differentially expressed genes between young sink and mature source tissue (Figure 5.8 C). From these 9,000 differentially expressed genes it is unclear which genes are related to sink/source status and which genes may be related to tissue age and development.

To further narrow down genes expressed in source leaves regardless of leaf age, I compared two sets of differentially expressed genes: genes differentially

expressed between young sink and young source (referred to as Set A), and genes differentially expressed between young sink and mature source (referred to as Set B) (Figure 5.8 A). I hypothesised the common genes between Set A and Set B would be genes specific to source leaves regardless of leaf age. A subset of 51 genes were up-regulated and 38 genes were down-regulated in both mature source and young source leaves relative to young sink leaves (Figure 5.8 D). These shared genes could be important indicators of sink-source status. I explored these shared genes further and identified several genes of interest which are up-regulated and down-regulated in both mature source and young source tissue compared to young sink tissue (Figure 5.9 A). The identities of these genes are discussed further in the discussion.

The genes shared between mature source and young source are potential candidates for genes important in source leaves regardless of tissue age. There could also be genes only differentially expressed during or immediately after the sink-to-source transition in young source leaves, that are not up-regulated in mature source leaves. To identify these genes, I also explore the list of genes only up- or down-regulated in young source tissue relative to young sink tissue (Figure 5.10 A and B).

Overall, the differentially expressed genes between mature source and young source leaves relative to young sink leaves have identified several future candidate genes for exploration involved in the regulation of nutrient transport, chloroplast function, and oxidative stress response are altered across the sink to source transition.

5.3.7 Transcriptional response to chitin is small across all tissue types

To determine whether transcriptional changes to chitin response underpin changes in plasmodesmal response between sink and source tissue I explored the transcriptional differences in chitin response. I carried out a Wald test within each tissue type to identify the number of differentially expressed genes in response to chitin, where the adjusted p-value was greater than 0.05 and the $\log_2$ fold change is greater or less than 0.5. All three tissue types have a small number of differentially expressed genes in response to chitin, with less than 100 genes up or down-regulated (Figure 5.11 A). Previously in mature leaves I found 569 up-regulated genes in

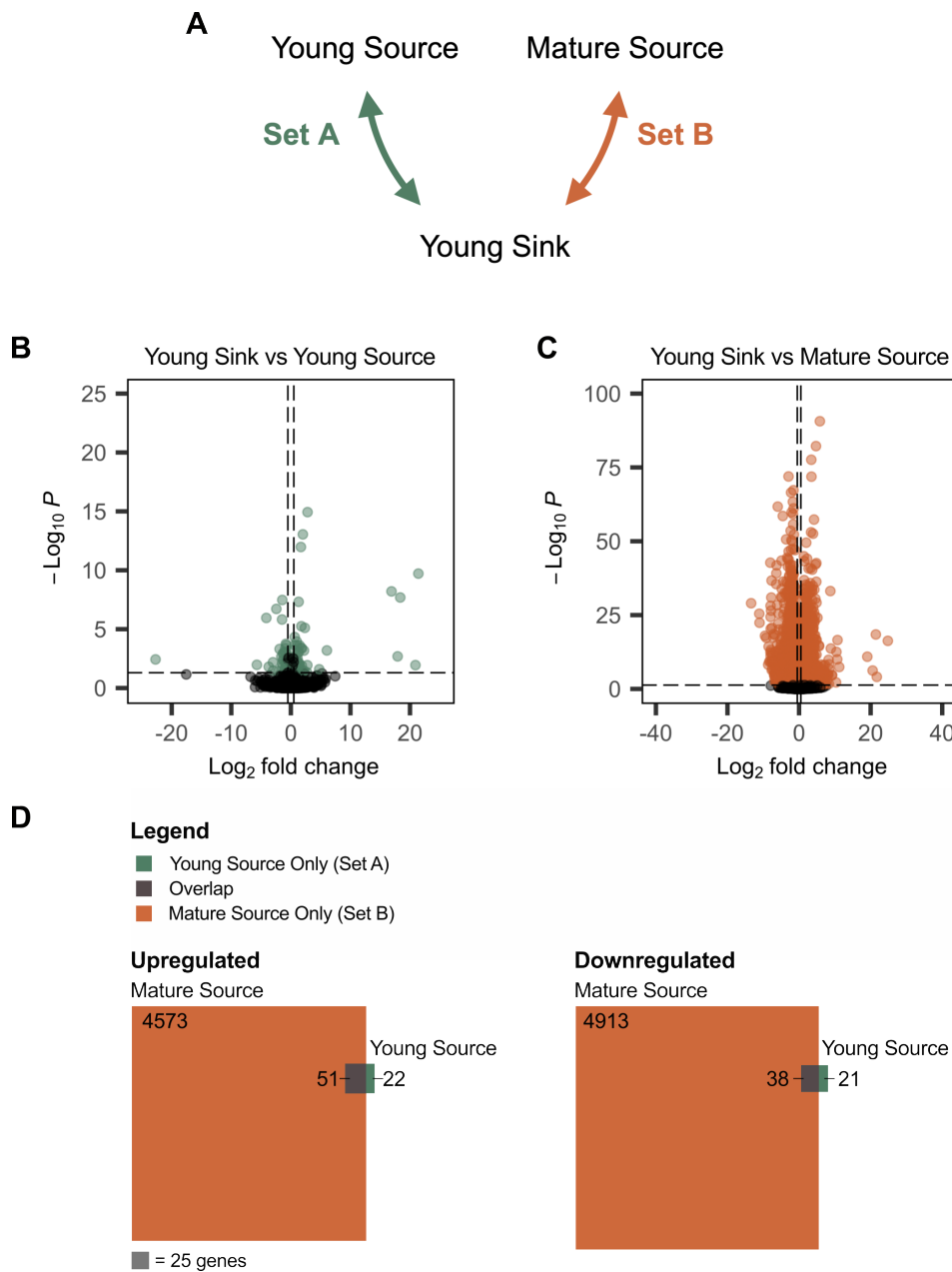


Figure 5.8: **Differentially expressed genes between Young Source, Young sink and Mature Source** – (A) Schematic of differentially expressed gene sets identified between Young Sink and Young Source (Set A) and Young Sink and Mature Source (Set B) (B) Volcano plot showing differentially expressed genes (green points, adjusted p-value < 0.05, log₂ fold change > |0.5|) in young sink vs young source tissue, where a positive log₂ fold change indicates upregulation in young source relative to young sink. (C) Volcano plot showing differentially expressed genes (orange, adjusted p-value < 0.05, log₂ fold change > |0.5|) in young sink vs mature source tissue, where a positive log fold change indicates upregulation in mature source relative to young sink. Dashed lines indicate a Log fold change of +/- 0.5 and adjusted p-value = 0.05, and points represent individual genes (D) Square Venn diagrams to scale showing differentially expressed genes only in mature source relative to young sink tissue (Orange), only in young source relative to young sink tissue (Green) and shared differentially expressed genes (brown). Numbers indicate the number of genes in each Venn section.

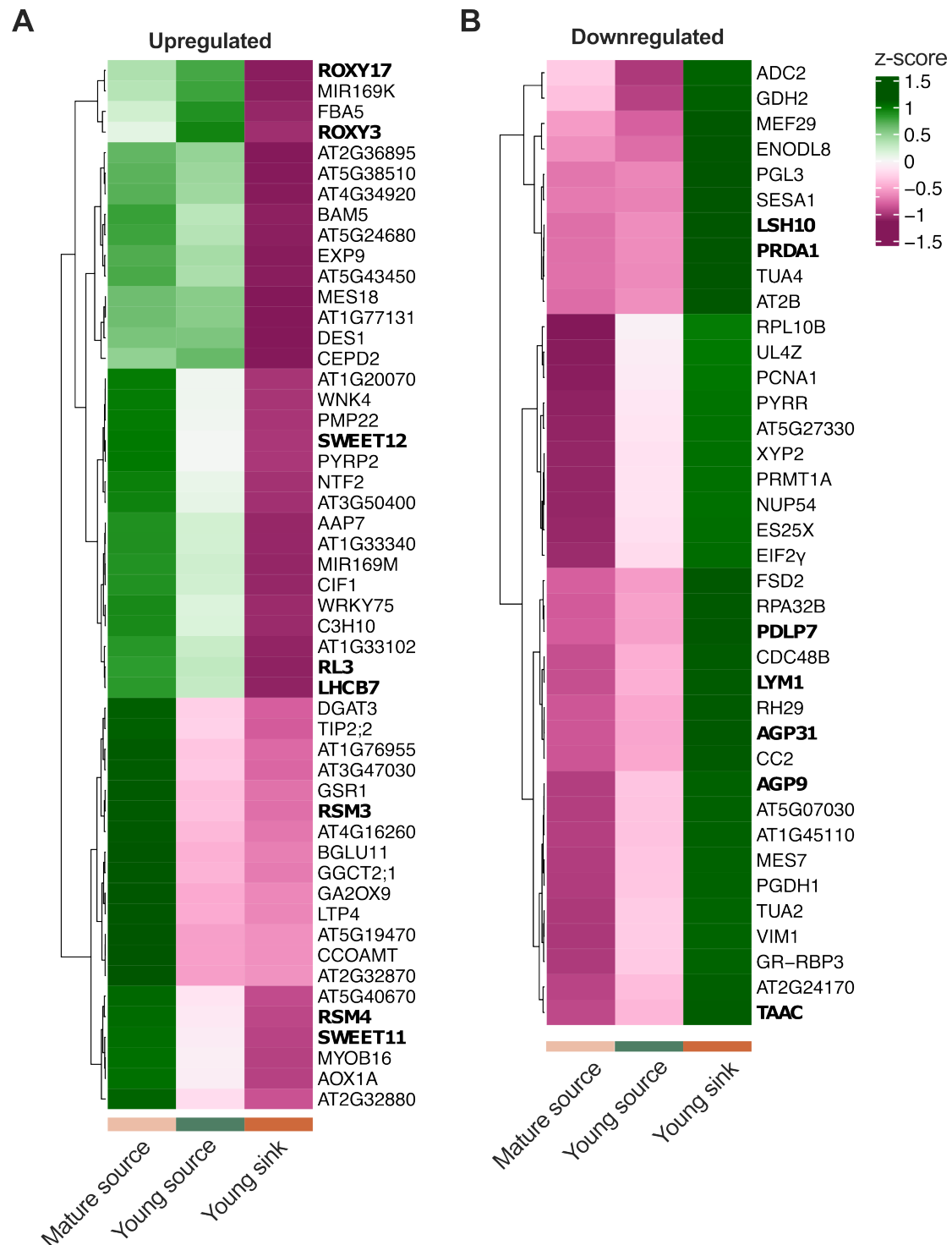


Figure 5.9: **Heatmap of shared differentially expressed genes in young and mature source tissue** – Heatmaps showing scaled normalised counts of: (A) shared up-regulated genes and (B) shared down-regulated genes in Mature source and Young source tissue relative to Young sink tissue. Each row represents scaled normalised counts (z-score) in a specific differentially expressed gene. Genes of interest discussed in the discussion are highlighted in bold. Each column represents a different tissue type: Mature source (light orange), Young source (dark green), and Young sink (dark orange). Z-score colour scales are the same in (A) and (B).

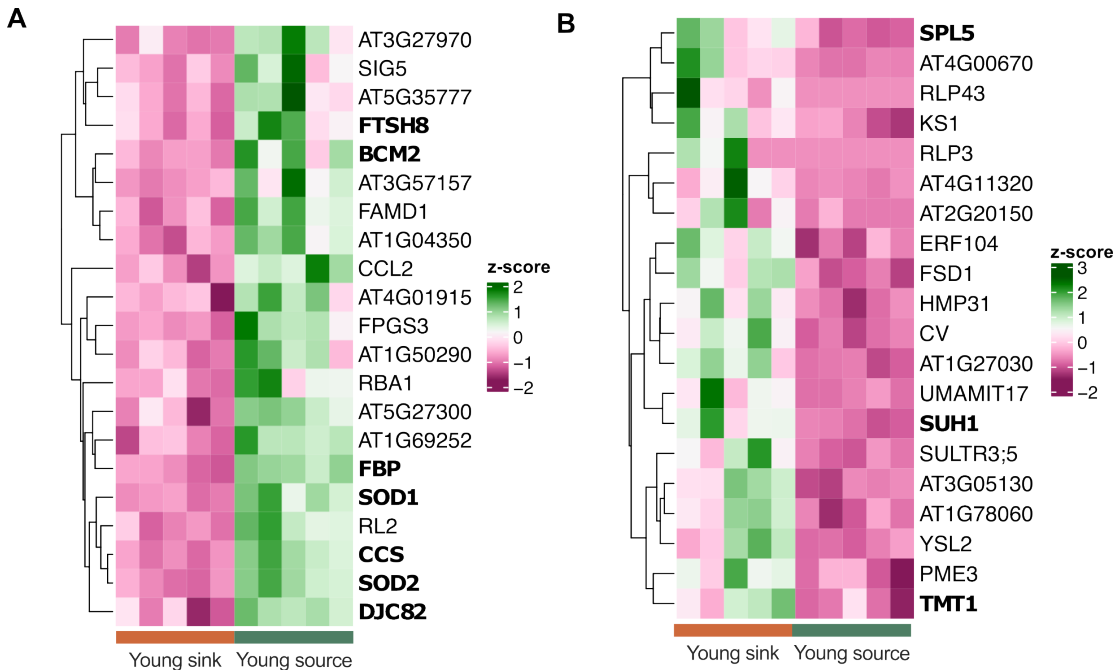


Figure 5.10: **Heatmap of differentially expressed genes only in young source tissue** – Scaled normalised counts of (A) up-regulated genes and (B) down-regulated genes only differentially expressed in young source compared to young sink, and not differentially expressed in mature source compared to young sink. Each row represents scaled normalised counts (z-score) in a specific differentially expressed gene. Genes of interest discussed in the discussion are highlighted in bold. Each column represents a sample from: Young sink (dark orange) or Young source (dark green)

response to chitin in mature source leaves, and 104 in young sink tissue (Chapter 3, Figure 3.8). These numbers are higher than the 72 up-regulated in mature and 4 up-regulated in young sink tissue found here. The transcriptional response induced by chitin in these leaves appears to be reduced compared to previous transcriptomic experiments carried out under similar conditions.

To determine if the chitin treatment elicited a defensive response, I carried out a GO-term analysis on the up-regulated genes in response to chitin in young source and mature source leaves. In young source leaves general terms such as “defence response” and “protein phosphorylation” are enriched, indicating the chitin treatment is eliciting some kind of defence response (Figure 5.11 B). Similarly in mature source leaves GO-terms such as “jasmonic acid metabolic process” and “response to wounding” are enriched, but even so few genes are associated with these GO-terms (Figure 5.11 C). The typical “defence response” GO-terms are not up-regulated, indicating a weak transcriptional defence response to chitin. Overall, this small transcriptional response in young and mature leaves makes it challenging to identify changes in chitin response across the sink to source transition as it is not clear if the chitin elicitor was effective given differences to my previous chitin-response transcriptomic datasets.

5.3.8 Lower epidermal cells of young sink leaves lack MP17-GFP-labelled branched plasmodesmata

The change in plasmodesmal regulation across the sink-to-source transition leads to questions regarding the underlying mechanisms controlling this change. Plasmodesmal structure has been previously found to shift from simple to branched across the sink to source transition (Fitzgibbon et al., 2013; Oparka et al., 1999; Roberts et al., 2001). I hypothesise changes in plasmodesmal structure could be related to the change in regulation.

To visualise changes in plasmodesmal structure between young and mature leaves I used Col-0 lines constitutively expressing a GFP-tagged 17kDa movement protein (MP17-GFP) from Potato Leaf Roll Virus (Kronberg et al., 2007). MP17-GFP localises to branched but not simple plasmodesmata, allowing me to investigate where branching occurs under my experimental conditions (Fitzgibbon et al., 2013; Kronberg et al., 2007).

MP17-GFP and wild-type Col-0 lines have many phenotypic differences, in-

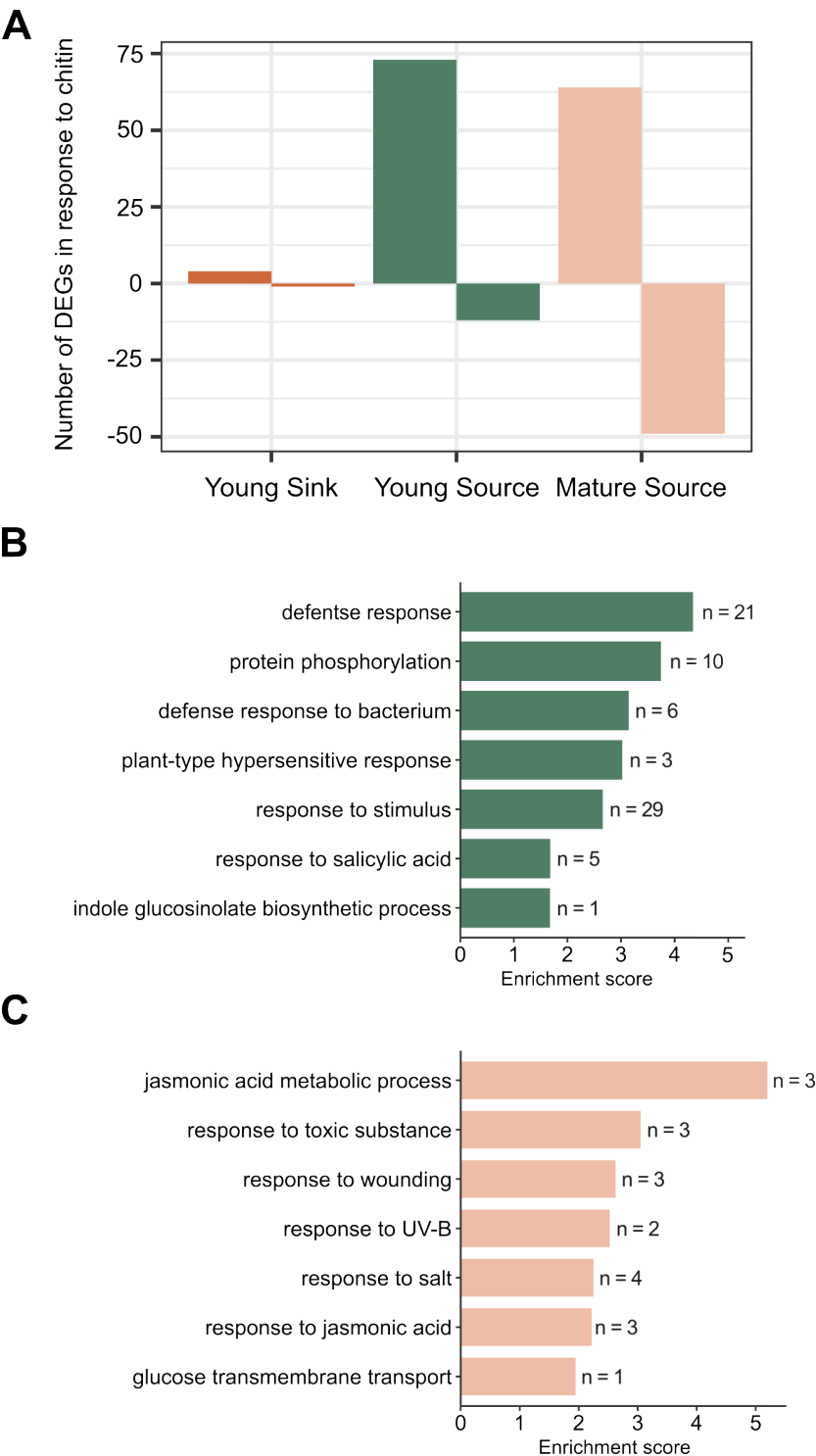


Figure 5.11: **Chitin response in sink and source tissue** – (A) Number of Differentially Expressed Genes (DEGs) in response to chitin treatment in Young sink (dark orange), Young source (green) and Mature source (light orange) leaves. (B and C) Bar plot showing results of GO-term analysis on genes up-regulated in response to chitin in (B) young source leaves and (C) mature source leaves. “n” indicates the number of genes enriched in that GO-term category.

cluding slower growth as characterised in Kronberg et al. (2007). I observed plant size is visually smaller in MP17-GFP, and growth is slower (Figure 5.12 A). To compare Col-0 and MP17-GFP leaves at similar developmental stages I sampled leaves from 7-week-old MP17-GFP plants and leaves from 5-week-old Col-0 plants, as that is when MP17-GFP lines show similar numbers of true leaves to 5-week-old Col-0. In addition to slower growth, MP17-GFP leaves showed chlorotic tissue with the largest areas of chlorosis in the oldest leaves (Figure 5.12 B). Tissue chlorosis in MP17-GFP leaves started at the tip and spread down through the leaf basipetally, with areas surrounding the vasculature and mid-rib remaining green the longest. This chlorosis has previously been linked to disruption of sugar transport in MP17-GFP lines and supports previous suggestions that constitutive expression of MP17-GFP has wide ranging phenotypic effects (Kronberg et al., 2007).

Despite these phenotypic effects, I continued to explore the potential of MP17-GFP as a tool to label branched plasmodesmata. To determine at what leaf stage plasmodesmal branching occurs I stained leaves with aniline blue to identify where MP17-GFP co-localises with plasmodesmal callose deposits. The abaxial side of young and mature leaves from three plants were stained with aniline blue and imaged under a confocal microscope (Figure 5.13 A). Based on previous research I assumed all plasmodesmal callose deposits co-localising with MP17-GFP were callose deposits at branched plasmodesmata, whilst plasmodesmal callose deposits with no MP17-GFP fluorescence were callose deposits at simple plasmodesmata (Fitzgibbon et al., 2013).

In mature leaves, MP17-GFP co-localises with plasmodesmal callose deposits at epidermal-epidermal cell connections, indicating the presence of branched plasmodesmata (Figure 5.13 B, indicated by white arrow). In young leaves, localisation of MP17-GFP was cell layer-specific. To visualise this, I split the z-stack into two sections to capture mainly mesophyll-mesophyll plasmodesmata in the lower slices and mainly epidermal-epidermal plasmodesmata in the upper slices. In the upper portion of the z-stack, MP17-GFP is not present at callose deposits in epidermal-epidermal cell wall connections, indicating only simple plasmodesmata are present (see white arrow in Figure 5.12 C). MP17-GFP can also be seen co-localising with plasmodesmal callose deposits at mesophyll-mesophyll junctions in the upper z-stack (see red arrows in Figure 5.12 C). These mesophyll-mesophyll junctions are distinguishable from epidermal-epidermal junctions by the more globular mesophyll cell shape faintly visible from callose staining and

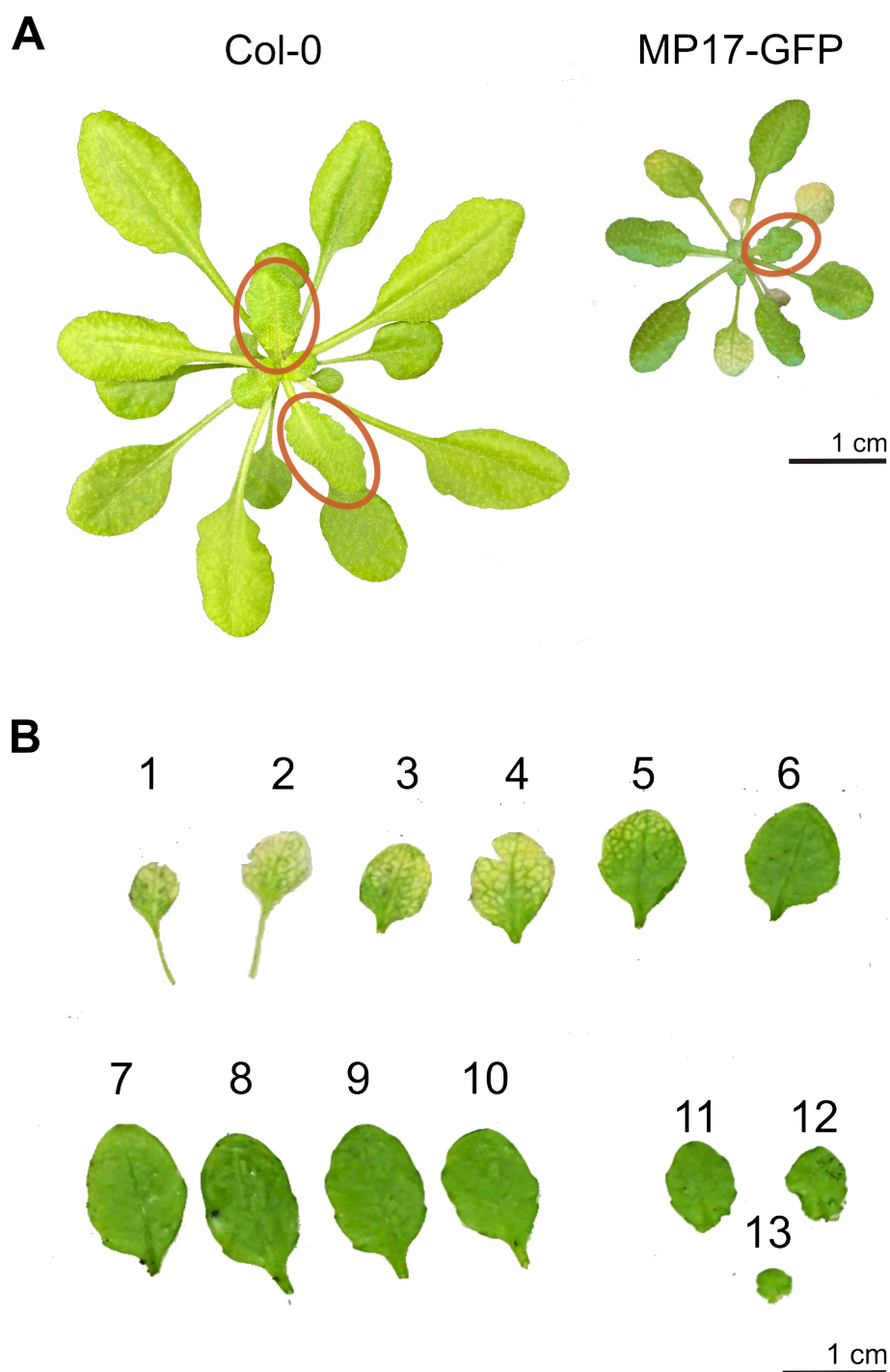


Figure 5.12: **Col-0 and MP17-GFP rosettes** – (A) Images comparing 5-week-old Col-0 and 7-week-old MP17-GFP Arabidopsis lines at similar developmental stage of having 14-16 true leaves at the same scale. (B) Individual adaxial side of detached leaves of MP17-GFP from leaf number 1 (oldest) to leaf number 13 (youngest).

the shorter walls at cell-to-cell connections due to the airspaces between cells. In the lower z-stack showing mesophyll-mesophyll cell wall connections, MP17-GFP colocalises with plasmodesmal callose deposits (see white arrows in Figure 5.13 D). This suggested young leaves had simple plasmodesmata between epidermal cells, and MP17-GFP-labelled branched plasmodesmata in between mesophyll cells, indicating initiation of branching occurs at different times in different cell types. Overall, MP17-GFP labelling of branched plasmodesmata is found in the mesophyll layers before labelling in epidermal cell layers. This contrasts to findings in *Nicotiana tabacum* from Roberts et al. (2001) where branching is first initiated in the upper and lower epidermal cells and later in the mesophyll cells layer using labelling by a Tobacco Mosaic Virus movement protein.

5.3.9 *MP17-GFP* expression blocks cell-to-cell movement in mature leaves

As I hypothesised plasmodesmal structure plays a role in plasmodesmal regulation, I initially aimed to quantify plasmodesmal response to PAMPs in cell-to-cell mobility assays before and after the appearance of MP17-GFP labelled branched plasmodesmata in epidermal-epidermal cell connections. To test whether this would be a valid approach I compared baseline plasmodesmal permeability in MP17-GFP transgenic plants and Col-0. Mature leaves from Col-0 and MP17-GFP leaves were bombarded and imaged 18-26 hours later with a minimum of 80 sites imaged per genotype. As MP17-GFP fluorescence localises to plasmodesmata, it was possible to count nuclei showing GFP with limited interference from MP17-GFP fluorescence. Cell-to-cell movement of GFP was significantly lower in MP17-GFP leaves compared to Col-0 leaves, with many cells showing no GFP movement in MP17-GFP (Figure 5.14 A-C). This is consistent with findings in Kronberg et al. (2007), where cell-to-cell movement of carboxyfluorescein dye is reduced in lines constitutively expressing MP17-GFP. The reduced baseline plasmodesmal permeability, even in the absence of any elicitor, means MP17-GFP lines are not a suitable model to understand how plasmodesmal structure relates to plasmodesmal regulation.

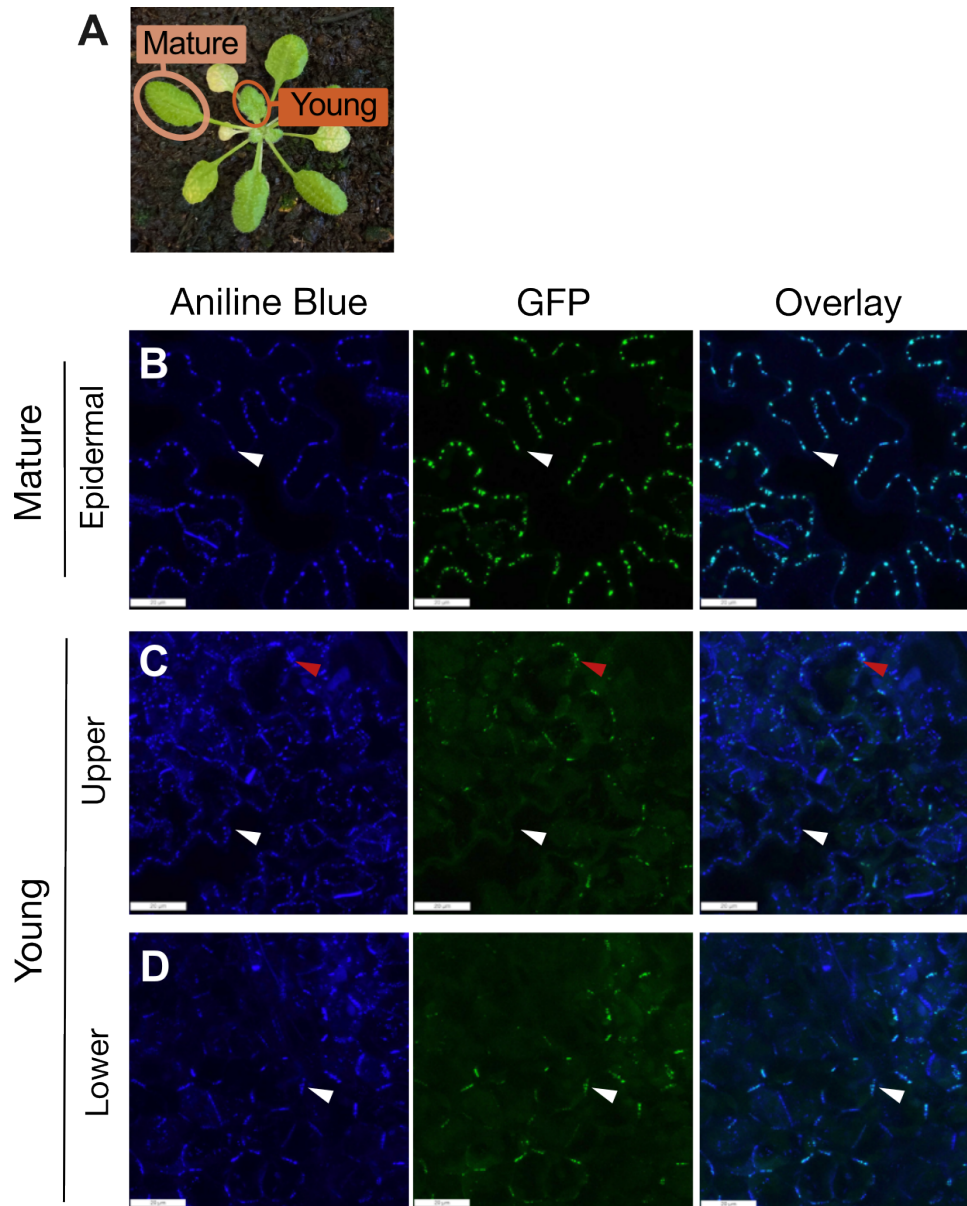


Figure 5.13: **Aniline blue staining of MP17-GFP lines** – (A) Image of MP17-GFP rosette with circled mature and young leaves of ages that were stained with aniline blue. (B) Confocal image of mature MP17-GFP leaf tissue stained with aniline blue where the white arrow indicates a plasmodesmal callose deposit of an epidermal cell (C) Upper z-stack of young leaf tissue stained with aniline blue, where the white arrow indicates plasmodesmata that are stained in an epidermal cell not colocalising with MP17-GFP and the red arrow indicates plasmodesmal callose deposits colocalising with MP17-GFP at mesophyll-mesophyll connections. (D) Lower z-stack of young leaf where stained plasmodesmal callose deposits in the mesophyll are indicated with a white arrow. Scale bar indicates 20 µm.

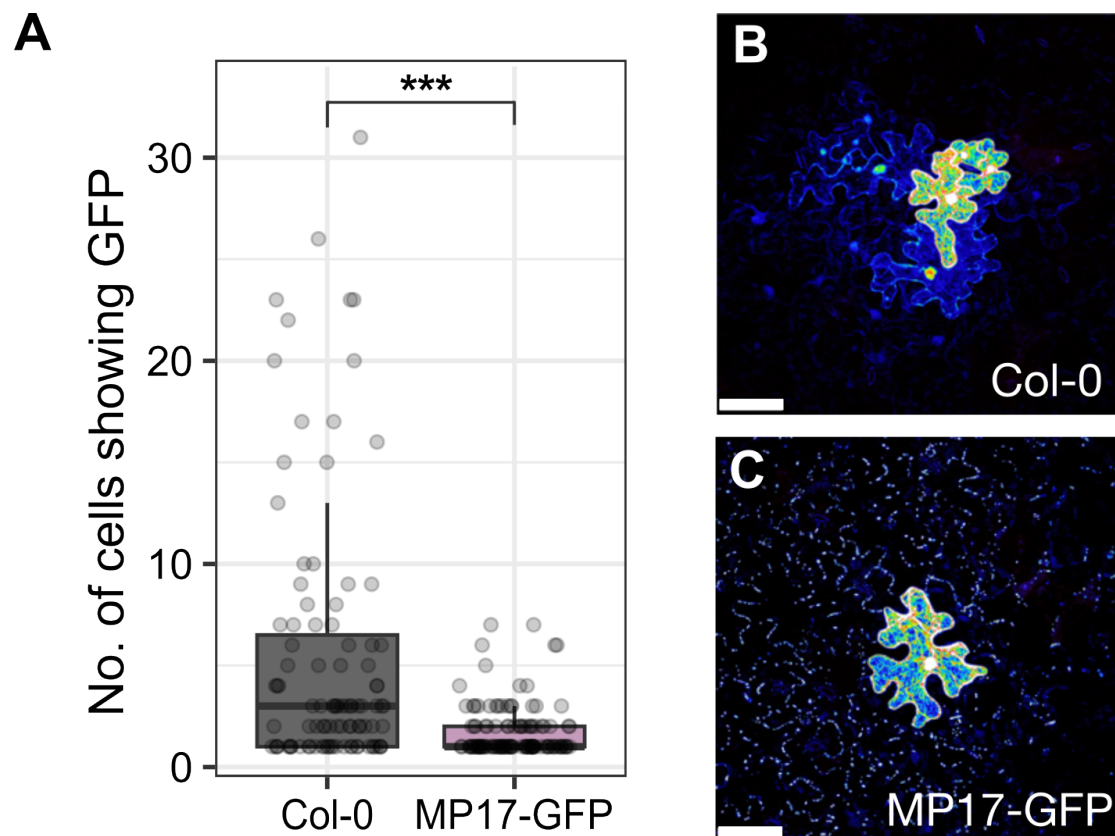


Figure 5.14: **Quantification of cell-to-cell mobility in Col-0 and MP17-GFP** – (A) Boxplots of cell-to-cell mobility in Col-0 and MP17-GFP. “***” indicates $p < 0.001$, individual points represent cells showing GFP in individual bombardment sites. Confocal images of bombardment sites in (B) Col-0 and (C) MP17-GFP showing localisation of MP17-GFP at plasmodesmata and cell-to-cell movement of free GFP. Scale bars indicate 50 μm .

5.3.10 MP17-GFP expression results in reduced callose deposition.

The reduction in plasmodesmal permeability caused by the constitutive expression of movement proteins has previously been linked to increased callose deposition. Rinne et al. (2005) suggest constitutive expression of a Tobacco topsovirus NS_M movement protein triggers a defensive response leading to increased callose deposition at plasmodesmata, which results in reduced plasmodesmal permeability. To determine if the lack of cell-to-cell movement in MP17-GFP is due to increased callose deposition I carried out an aniline blue staining assay. I stained plasmodesmal callose using aniline blue in Col-0 and MP17-GFP (Figure 5.15 A and B). The number of plasmodesmata detected per image was not significantly different between Col-0 and MP17-GFP lines (Figure 5.15 B). Both the mean size and mean intensity of the callose deposits were significantly reduced in MP17-GFP compared to Col-0 (Figure 5.14 C and D). This indicates, that despite decreases in cell-to-cell mobility, MP17-GFP has fewer plasmodesmal callose deposits and smaller callose deposits at plasmodesmata. This indicates constitutive expression of MP17-GFP does not induce callose deposition and other factors may be responsible for the reduced plasmodesmal permeability.

Overall, the phenotypic effects of constitutive MP17-GFP expression made it an unsuitable for use in studying the relationship between plasmodesmal structure and regulation. Instead, I explored other experimental systems to investigate the role of plasmodesmal structure in the change in plasmodesmal regulation across the sink-to-source transition.

5.3.11 *cher1* mutants have chitin-triggered plasmodesmal closure in mature leaves

I hypothesised changes to plasmodesmal structure across the sink to source transition could directly link to plasmodesmal response to PAMPs. It is possible structure determines plasmodesmal response to stress. This led me to predict that simple plasmodesmata do not respond to PAMPs regardless of whether leaves are sink or source. To test this prediction, I used *Choline transporter-like1* (*cher1*) mutant lines which have a T-DNA insertion in the *cher1* gene, and as a result only have simple plasmodesmata in mature leaves (Kraner et al., 2017). Loss of

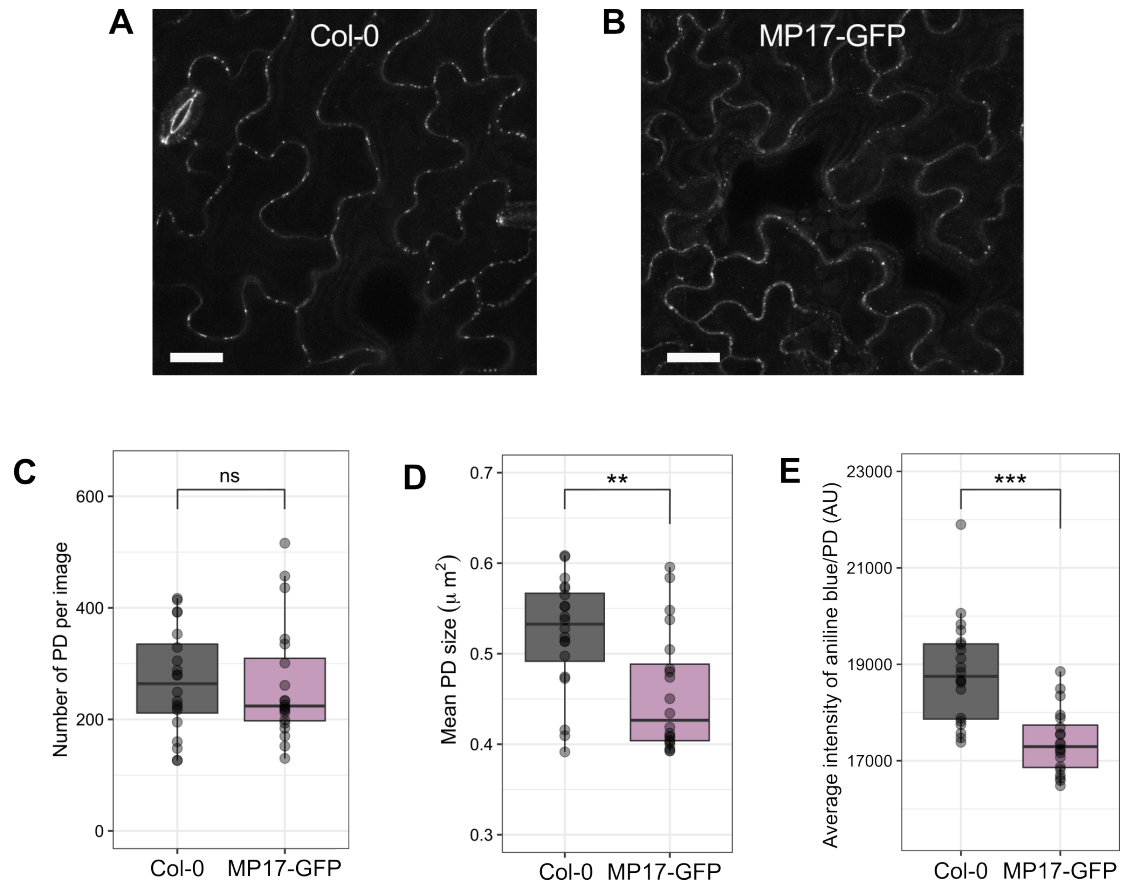


Figure 5.15: **Quantification of callose deposition in MP17-GFP lines** – (A) Confocal images of aniline-blue stained plasmodesmal callose deposits in Col-0 (B) Confocal images of aniline blue stained callose in MP17-GFP. (C) Boxplot of the number of plasmodesmata (PD) per image in Col-0 and MP17-GFP lines. (D) Mean PD size in Col-0 and MP17-GFP. (E) Average intensity of aniline blue per PD in Col-0 and MP17-GFP. Each point represents an individual staining site, and “ns” = no significant difference, “***” = $p < 0.01$, “****” = $p < 0.001$, and boxplots show the median +/- the interquartile range. 10 μm scalebar

function in *cher1* prevents the formation of secondary and branched plasmodesmata (Kraner et al., 2017) Limiting secondary plasmodesmata formation prevents the formation of branched plasmodesmata (Kraner et al., 2017). I used these *cher1* lines to decouple the sink-to-source transition from plasmodesmal structure, testing whether mature *cher1* source leaves with only simple plasmodesmata have PAMP-triggered plasmodesmal closure.

I performed a GFP mobility assay on *cher1* mature leaves to determine plasmodesmal regulation in response to chitin. I infiltrated leaves with a mock (distilled H₂O) or chitin (500 µg/mL) solution two hours after bombarding, and imaged leaves 18-26 hours later, with a minimum of 40 sites quantified per treatment. Chitin triggered a significant reduction in the number of cells showing GFP in *cher1* mutant lines (Figure 5.16). This indicated *cher1* mature leaves with only simple plasmodesmata exhibit chitin-triggered plasmodesmal closure, suggesting plasmodesmal structure is not directly related to regulation. The relationship between structure and regulation could be dependent on developmental context, or simply a correlation across the sink-to-source transition, or there could also effects of mutating *cher1* that interfere with plasmodesmal PAMP response.

5.3.12 *cher1* mutants have increased cell-to-cell mobility

Previously, several phenotypic differences have been found between *cher1* and wild-type Arabidopsis lines. These include decreased numbers of plasmodesmata, increased starch accumulation in mature leaves, and reduced growth (Kraner et al., 2017). I also observed under my growth conditions *cher1* rosettes have a smaller size than Col-0 (Figure 5.17 A). As *cher1* lines have previously been characterised to have fewer plasmodesmata than Col-0, I used a GFP mobility assay to explore if this could result in different baseline plasmodesmal permeabilities. Mature leaves from Col-0 and *cher1* were bombarded and imaged under a confocal microscope 18-26 hours later with a minimum of 45 sites imaged per treatment. *cher1* had significantly increased cell-to-cell GFP movement compared to Col-0 (Figure 5.17 B). This indicates *cher1* has increased plasmodesmal permeability, which is interesting as previous studies show *cher1* as having fewer plasmodesmata than Col-0 (Kraner et al., 2017).

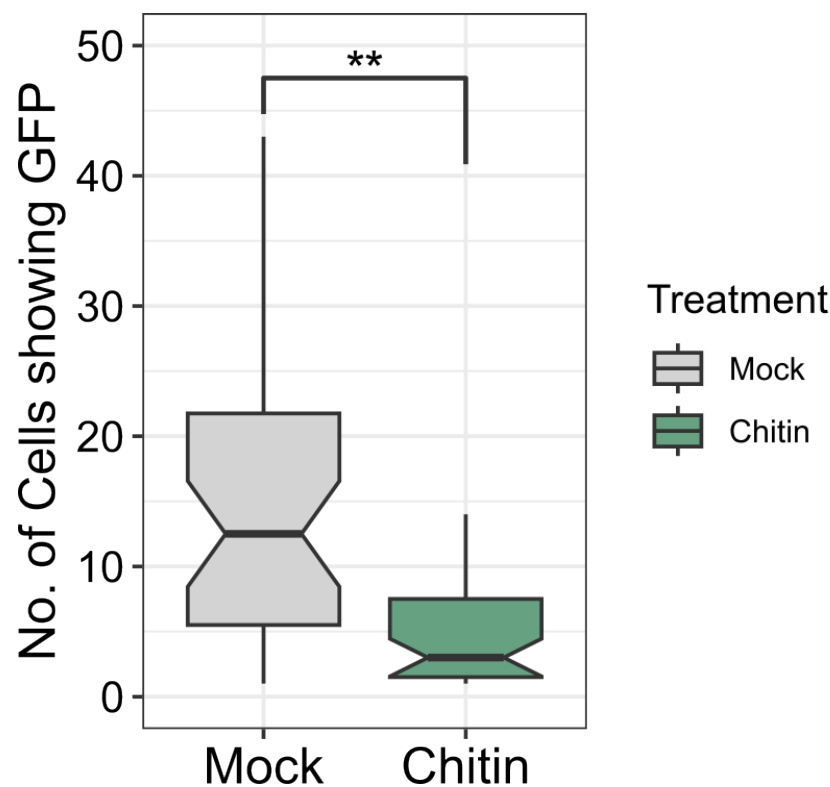


Figure 5.16: **Quantification of cell-to-cell mobility in *cher1* in response to chitin** – Boxplots of microprojectile bombardments in *cher1* after mock (water) or chitin treatment.. “***” indicates $p < 0.01$. Boxplots indicate the median +/- the interquartile range

5.3.13 *cher1* mutants have less callose accumulation at plasmodesmata

To determine whether the increased cell-to-cell mobility in *cher1* is related to reduced callose deposition I stained plasmodesmal callose deposits using aniline blue. Col-0 aniline blue staining shows distinct sites of callose deposits spread relatively evenly across the cell wall (Figure 5.18 A). *cher1* callose deposits, however, seemed to be absent for large parts of the cell wall and then clustered in certain parts of the cell wall (Figure 5.18 B). I quantified the size, mean intensity and callose deposits in *cher1* and Col-0 using the plasmodesmal callose detection script from Johnston (2021) as described in section 2.4. As this script depends on detecting puncta of increased intensity, automation of callose deposit detection was challenging in *cher1* due to an increase in background callose deposition noise. As a result, I manually removed background fluorescence from the images. The source of this increased background callose deposition is unclear, other than that the mutation in *cher1* may impact lipid composition and may could impact callose distribution.

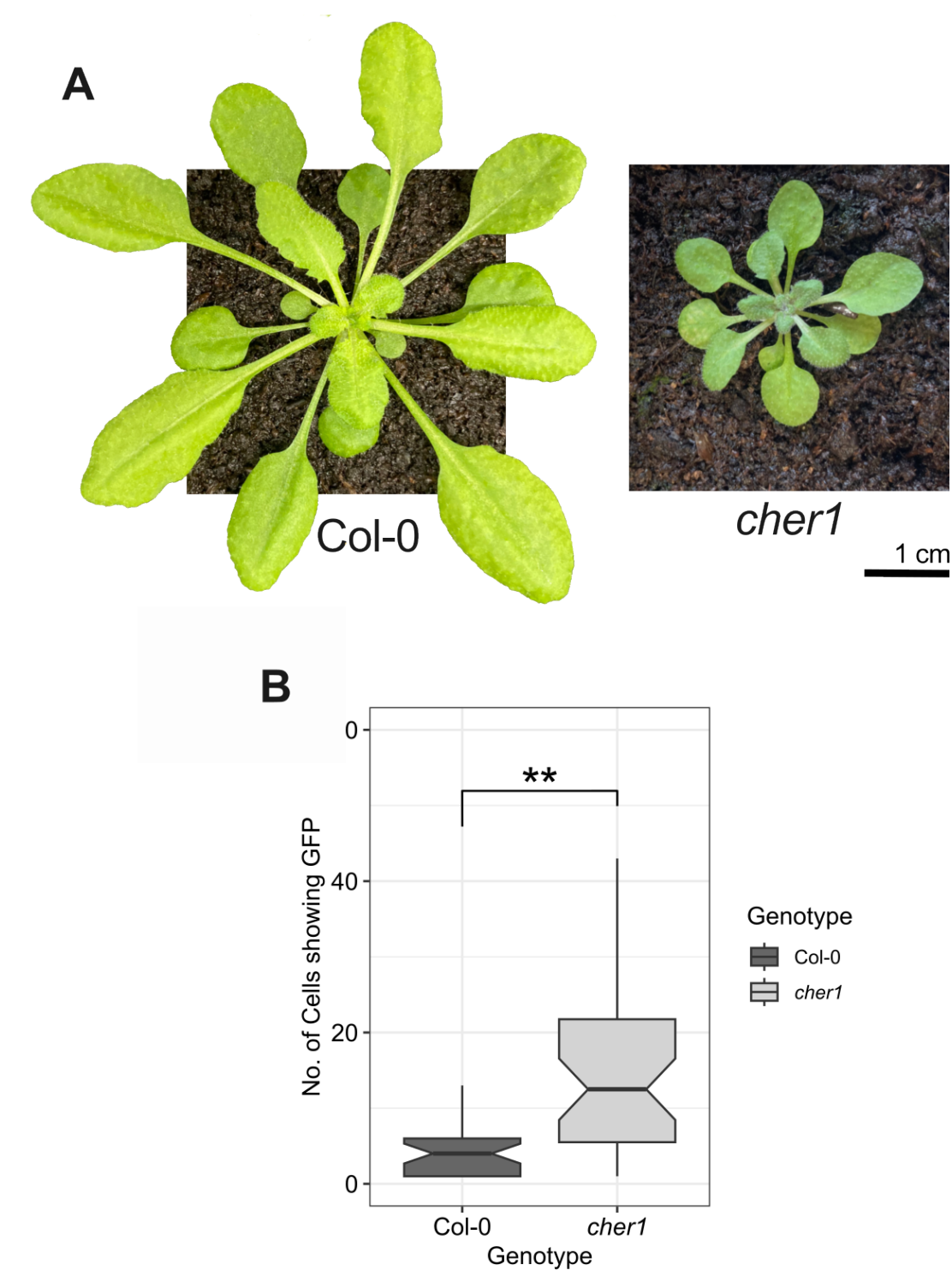


Figure 5.17: **Quantification of cell-to-cell mobility in Col-0 and *cher1*** – (A) Images of Col-0 and *cher1* rosettes, scale bar = 1cm. (B) Cell to cell GFP mobility assay comparing Col-0 (dark grey) and *cher1* (light grey), where ‘**’ indicates $p < 0.01$ and boxplots show the median +/- the interquartile range.

I found the number of plasmodesmal callose deposits per image was not significantly different between Col-0 and *cher1* lines (Figure 5.18 C). This contrasts with previous findings from TEM images showing the number of plasmodesmata are increased in *cher1* compared to wild-type plants (Kraner et al., 2017). The mean size and intensity of plasmodesmata was significantly reduced in *cher1* compared to Col-0 indicating lower amounts of callose accumulated at *cher1* plasmodesmata compared to Col-0 (Figure 5.18 D and E). The differences in callose deposition indicates lower amounts of callose at plasmodesmata, which could explain the increased cell to cell movement in *cher1*.

Overall, despite showing *cher1* mature leaves with simple plasmodesmata can close in response to chitin, there are large phenotypic differences between Col-0 and *cher1*. This makes it difficult to conclude from results in *cher1* whether shifts in plasmodesmal structure across the sink-source transition link to changes in plasmodesmal PAMP response in Col-0.

5.3.14 Plasmodesmal structure does not change upon inducing the sink-to-source transition

As both the constitutive expression of MP17-GFP and mutations in *cher1* mutant had secondary effects on plasmodesmal permeability and plant development, it is difficult to draw direct comparisons to how plasmodesmal structure could impact the change in regulation of plasmodesmata across the sink-to-source transition in wildtype Col-0 plants.

To explore whether there are structural changes to plasmodesmata across the sink-to-source transition, I used Serial Block Face Scanning Electron Microscopy (SBF-SEM). I sampled three young sink and three young source leaves from either uncovered or covered plants 24 hours after covering. Rhea Stringer then fixed, sectioned, and imaged the tissue as outlined in the section 5.2.14. Plasmodesmata with only one opening on either side of the cell wall were defined as simple. Plasmodesmata with more than one opening on either side of the cell wall were defined as branched (Figure 5.19).

To quantify the changes in plasmodesmal structure across the sink-source transition, Rhea Stringer segmented the plasmodesmata from each of the three sections and categorised the structure as simple or branched (Figure 5.20 A and B). Although there was a trend towards an increased proportion of plasmodes-

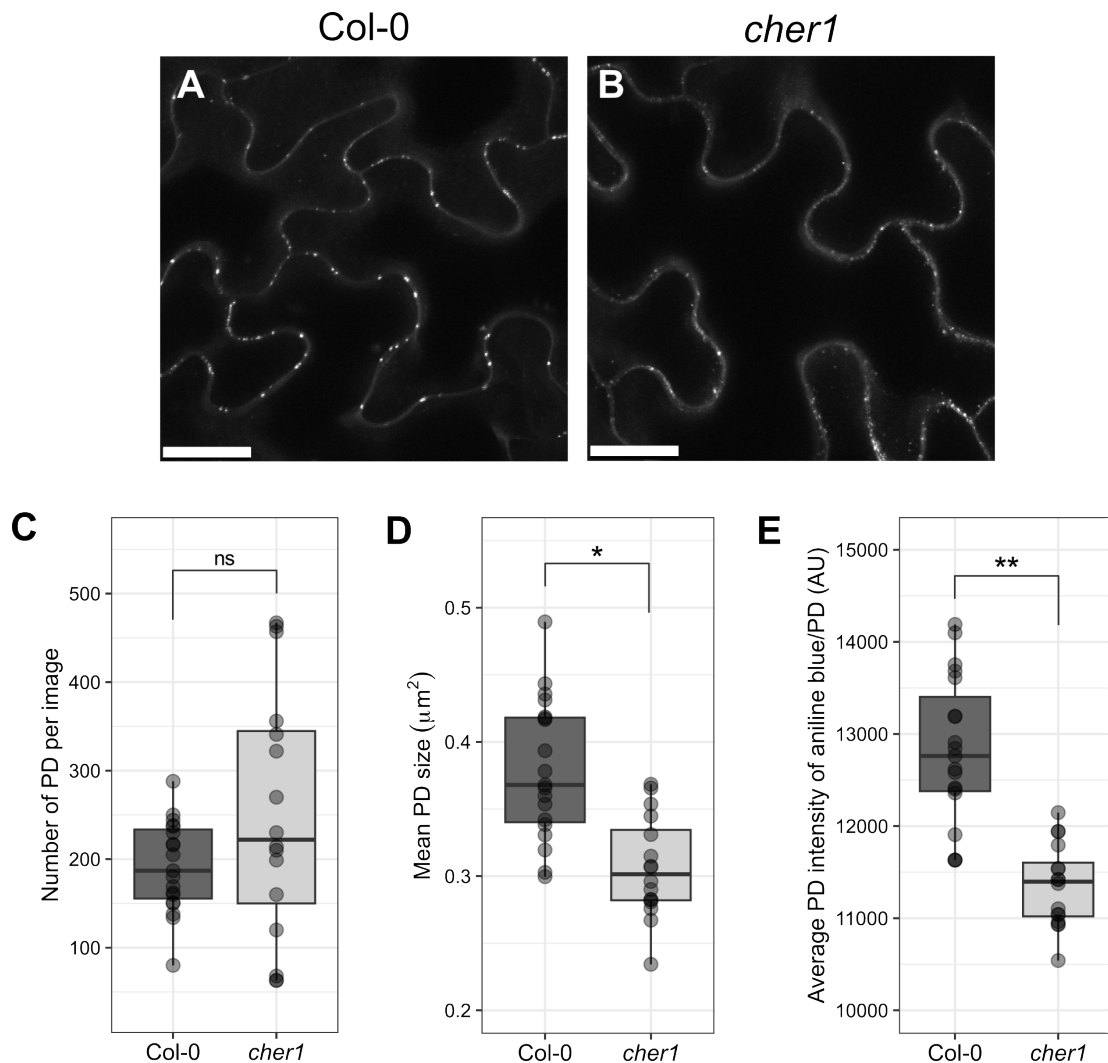


Figure 5.18: **Quantification of callose deposition *cher1***– (A) Confocal images of aniline blue staining of plasmodesmal callose deposition in Col-0 lines and (B) Aniline blue staining of callose deposition in *cher1* lines, scale bar indicates 20 μm . (C) Boxplots of the number of plasmodesmata (PD) per image in Col-0 (dark grey) and *cher1* (light grey) lines. (D) Mean size of plasmodesmata in Col-0 and *cher1*. (E) Average plasmodesmal intensity of aniline blue per plasmodesmata in Col-0 and *cher1*. Boxplots show the median +/- the standard deviation, with individual points representing individual images.

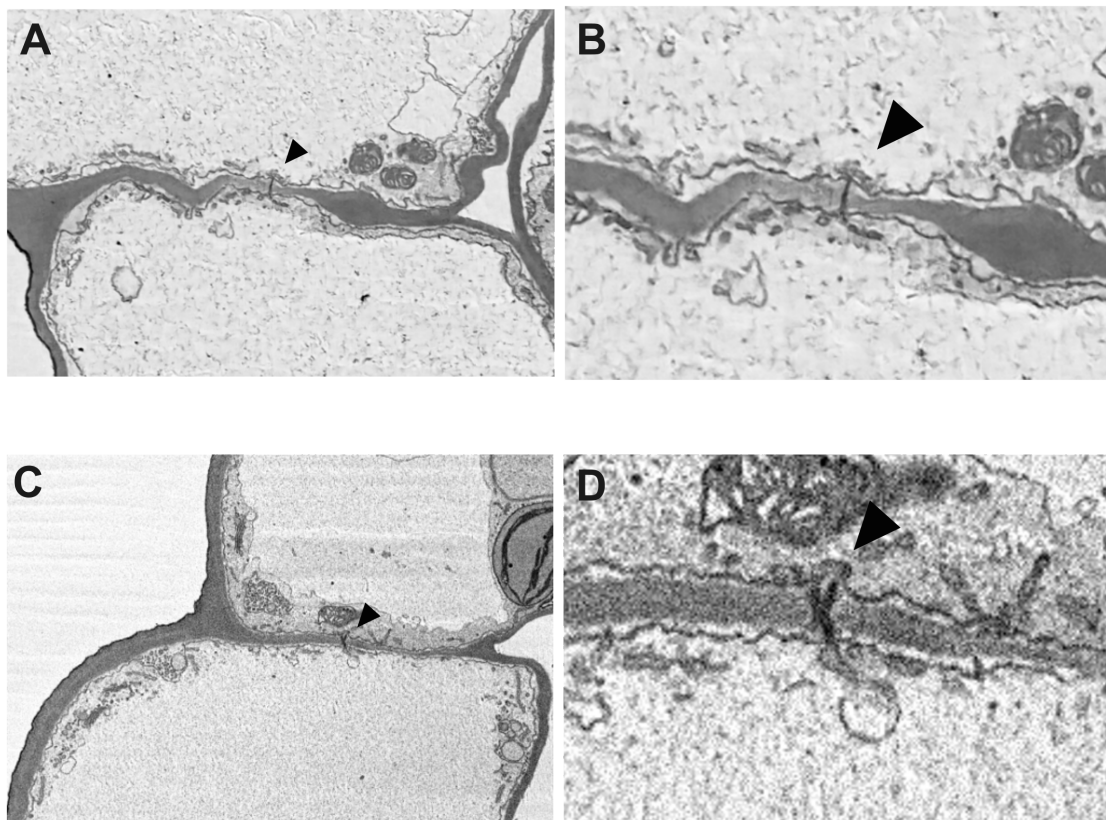


Figure 5.19: **SBF-SEM images of simple and branched plasmodesmata** – Images from SEM-SBF microscopy where (A) shows an example of a simple plasmodesma, with an enlarged version in (B); and (C) shows an example of a branched plasmodesma with an enlarged version in (D). Black arrows indicate the location of the plasmodesma of interest.

mata being branched in sink and source tissue, overall adding up the sum of all plasmodesmal structures in each sample resulted in a mean of 36% branched in young sink leaves and 42% branched in young source leaves. This contrasts with previous findings showing a much larger shift in plasmodesmal structure across the sink-to-source transition in *N. tabaccum* leaves, where 90% of plasmodesmata in sink leaves have a simple single-channel structure, whilst in source leaves 80% of plasmodesmata have a more complex branched plasmodesmal structure (Oparka et al., 1999; Roberts et al., 2001).

Additionally, I determined if the number of plasmodesmata changes across the sink-to-source transition. Comparing the cumulative number of plasmodesmata across all samples shows young sink leaves have higher numbers of plasmodesmata than young source leaves. Young sink leaves have just over 320 plasmodesmata whereas young source leaves have 147 plasmodesmata. This increase in the total number of plasmodesmata was not due to more image slices being taken in young leaves, as young sink leaves and young source leaves had similar numbers of total image slices at 737 and 720 slices respectively. Despite similar numbers of slices taken for both treatments, it is possible more cell wall was imaged in young sink leaves. Further quantification of the number of plasmodesmata per μm of cell wall is needed to determine if the density of plasmodesmata changes between young sink and young source.

5.3.15 Inducing *LexA::icals3m* expression increases callose deposition in roots

Roots are another important sink tissue in plants where phloem unloading occurs through plasmodesmata and maintaining plasmodesmal connectivity is likely to be critical for root growth and development. I questioned whether inducing plasmodesmal closure across whole root tissues reduces phloem unloading and growth, as previously seen sink leaves (Chapter 4). To induce plasmodesmal closure in roots I used *LexA::icals3m* lines. These lines have estradiol inducible expression of an allele of *callose synthase 3* using *LexA* promoter Bellandi et al., 2022. As these lines have previously been characterised in leaves (Bellandi et al., 2022; Tee et al., 2024) and Chapter 4, I first tested whether estradiol treatment induces callose deposition in roots of seedlings. Seedlings were imaged 24 hours after estradiol or DMSO (mock) treatment. I imaged two root regions: the root tip and the upper edge of the elongation zone, just before cells begin to differentiate

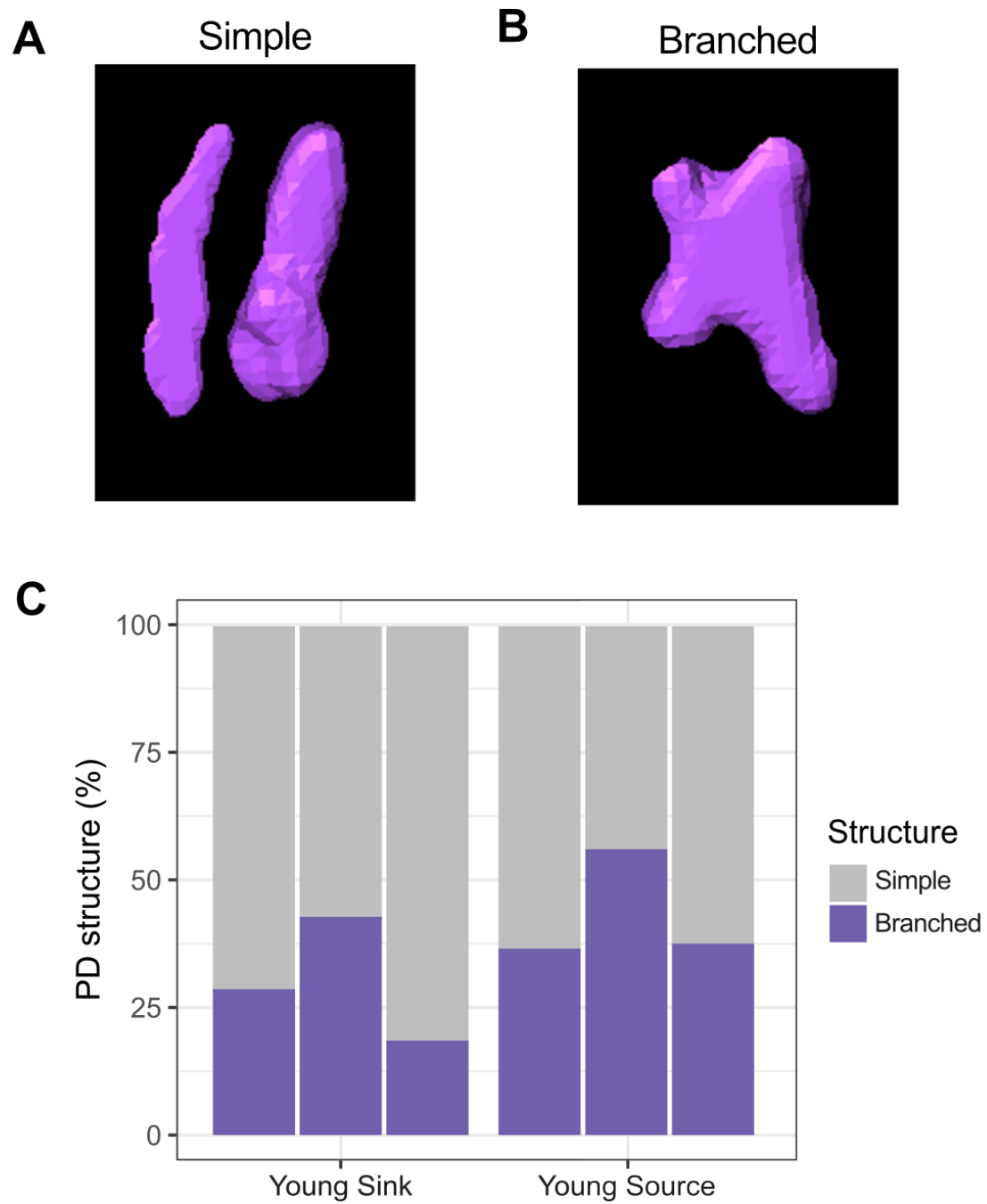


Figure 5.20: **SBF-SEM images of simple and branched plasmodesmata** – Example segmentations of a simple (A) and branched (B) plasmodesma. (C) Barplot showing percentage of plasmodesmata which are simple or branched across three young sink and young source samples

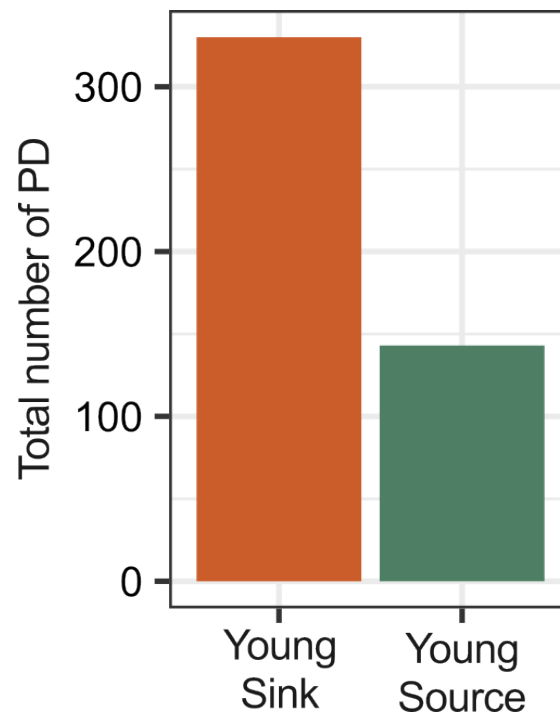


Figure 5.21: **SBF-SEM quantification of the number of plasmodesmata in Young sink and Young source tissue** – Total number of plasmodesmata (PD) across all three samples in young sink (orange) and young source (green) tissue.

into root hair cells, referred to here as the elongation zone (Figure 5.22).

In the elongation zone, aniline blue staining in DMSO-treated roots showed general callose deposition in the cell wall with plasmodesmal puncta visible in the cell wall (indicated by the white arrow in Figure 5.23 A). In estradiol-treated roots, aniline blue staining reveals more pronounced callose deposition, with defined staining in both horizontal and vertical cell walls, and clearer plasmodesmal puncta visible connecting cell layers (indicated by the white arrow in Figure 5.23 B).

In leaves, callose staining at plasmodesmal puncta is much more intense than in the surrounding cell wall, making it easier to quantify callose deposition at individual puncta. In roots, the increased intensity of callose staining of the cell wall makes it more difficult to distinguish plasmodesmal callose puncta from the cell wall. Instead of quantifying changes in plasmodesmal callose deposition, I quantified the general intensity of callose staining in the root. I drew a circle of set area in each root z-stack and compared the mean intensity of aniline blue within the circles between estradiol and mock DMSO treatments. In the elongation/differentiation zone of the root, total intensity of aniline blue was significantly higher in roots treated with estradiol than those treated with the mock DMSO solution (Figure 5.23 C).

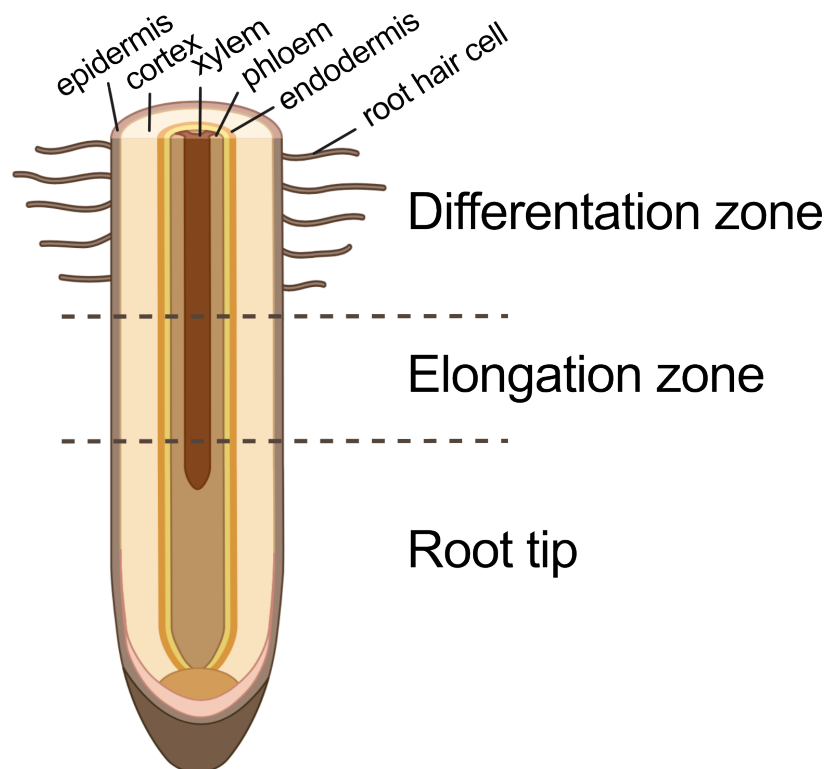


Figure 5.22: **Diagram of root zones** – Diagram of root divided up into three zones: Differentiation zone, elongation zone and root tip, and annotated tissue types with the epidermis, cortex, endodermic, phloem, xylem and root hair cells. Created using Biorender.com.

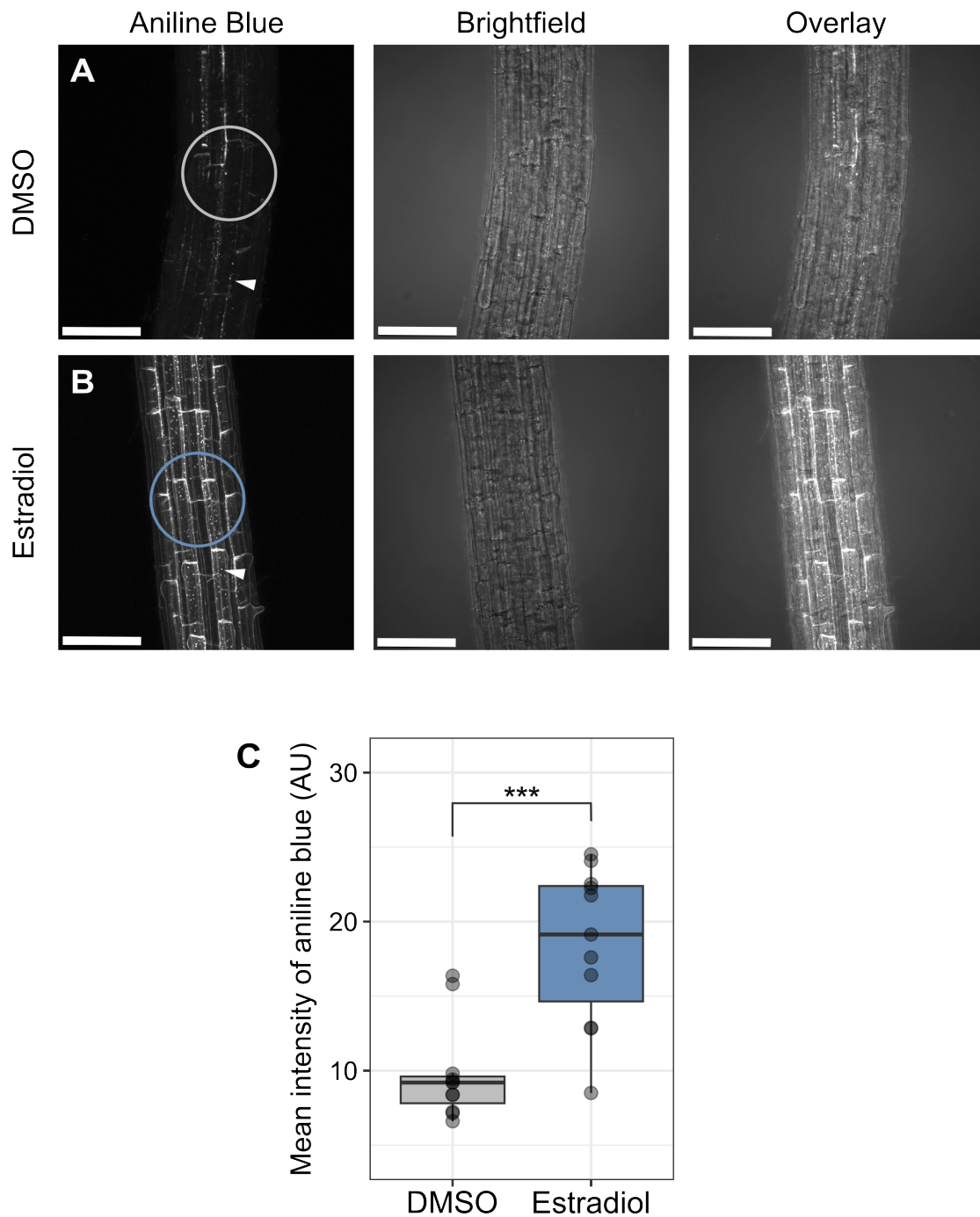


Figure 5.23: **Callose staining in LexA::icals3m roots** – Confocal images of Aniline blue staining of callose in the root cell elongation zone in roots transplanted onto mock DMSO (A) or Estradiol (B) plates, with a circle draw to represent the area of aniline blue intensity quantified where the white arrows indicate plasmodemata. (C) Boxplot quantification of mean intensity of aniline blue staining in root elongation zone after DMSO (grey) or Estradiol (blue) treatment, where each point represents intensity of a single root. Scale bars indicate 100 μ m and “***” indicates $p < 0.001$.

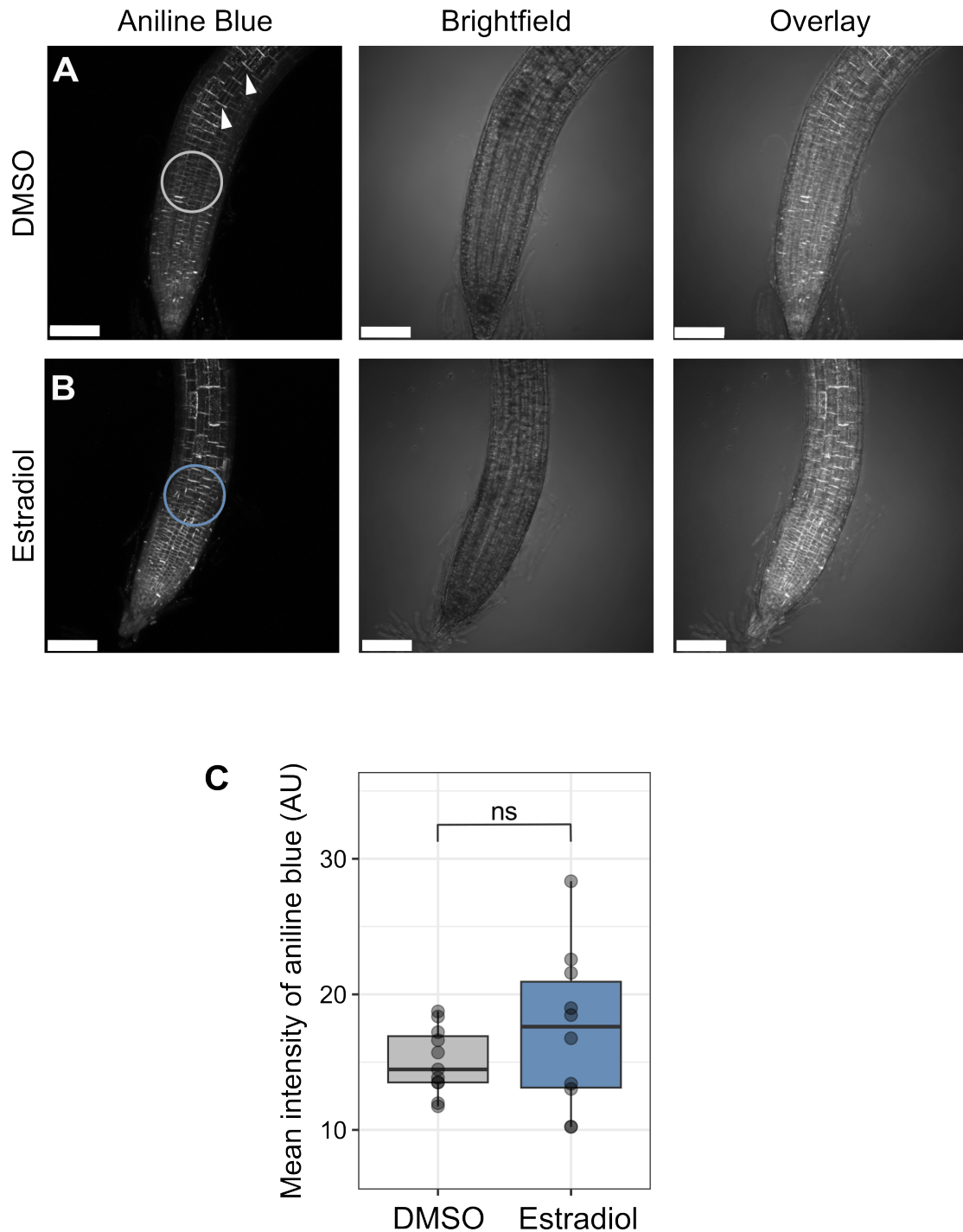


Figure 5.24: **Callose staining in *LexA::icals3m* root tips** – (A) Confocal image of aniline blue staining of callose in the root tip 24 hours after being transplanted onto mock DMSO or Estradiol plates, with a circle drawn to represent the area of aniline blue intensity quantified and arrows indicating horizontal cell wall. (B) Quantification of mean intensity of aniline blue staining after DMSO (grey) or Estradiol (blue) treatment, where each point represents intensity of a single root. Scale bars indicate 100 μ m and “***” indicates $p < 0.001$

I also quantified aniline blue staining in the root tip 24 hours after transplanting onto mock DMSO or estradiol plates. I repeated the same quantification method as in the elongation zone, drawing a circle at the same distance from the root cap in all root tips to measure the mean intensity of aniline blue staining. Compared to the elongation zone, the aniline blue staining was noisier at the root tip, with DMSO-treated roots showing callose staining of horizontal cell walls (Figure 5.24 A, indicated by white arrows). Estradiol-treated root tips were also noisy, with callose deposition seen in cell walls throughout the tip (Figure 5.24 B). There were no significant differences in the intensity of callose staining between DMSO and estradiol-treated roots in root tips (Figure 5.24 B and C).

The lack of estradiol-induced increase in callose deposition in the root tip led me to question whether estradiol is effective at inducing the expression of *icals3m* in the root tip. To test this, I explored the activity of the LexA promoter. I visualised the expression of *LexA::PD-Plug* lines, which express an internal fusion of *mCherry* to the *Plasmodesmal Localised Protein 1* (*PDLP1*) signal peptide at the N-terminus, and the transmembrane domain and cytoplasmic tail (Caillaud et al., 2014) at the C-terminus. This allowed me to visualise where the LexA promoter is active. I transplanted 9-day-old *LexA::PD-Plug* seedlings onto estradiol or DMSO media and imaged after 24 hours. Figure 5.25 shows strong PD-Plug intensity in the vasculature and root epidermal cells, but weaker intensity closer to the root cap where cell division is occurring. The reduction of PD-Plug intensity close to the root cap where high levels of cell division occur suggests callose deposition may not be induced in that region of the root tip. However, the area of callose staining I quantified in *LexA::icals3m* root tips was in a zone where PD-Plug expression can be seen in epidermal cells and the vasculature. This raises the possibility that the lack of estradiol-triggered increase in callose deposition in the tip is not related to reduced gene induction. Instead, it could be that the background callose deposition is higher in roots and so increases in callose deposition triggered by *LexA::icals3m* may not have a significant impact on overall callose staining in my method of quantification.

Overall, estradiol treatment induces callose deposition in the root elongation zone but not the root tip. As there is a strong increase in callose deposition in the root elongation zone I continued to use *LexA::icals3m* to understand the impact of inducing callose deposition on growth and plasmodesmal permeability in roots.

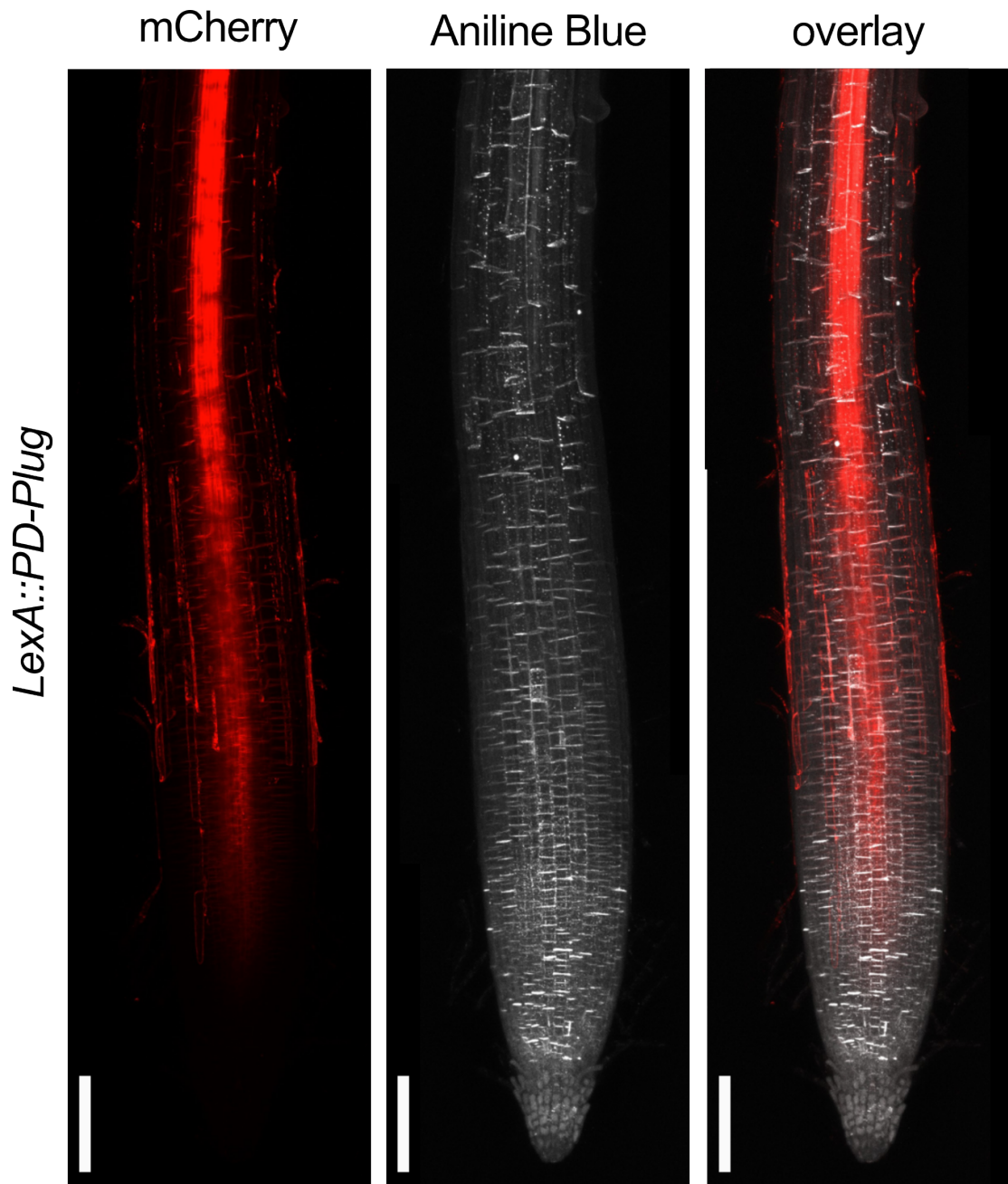


Figure 5.25: **PD-Plug mCherry expression in roots** – Confocal image of *LexA::PD-Plug* lines in roots of 10-day-old seedlings after 24 hours of treatment on estradiol plates. Scale bars indicate 100 μm .

5.3.16 Inducing *icals3m* expression in roots decreases growth and phloem transport

To explore how inducing callose deposition impacts root growth, *LexA::icals3m* roots were transplanted onto estradiol or mock-DMSO plates and root length was quantified two and four days after transplanting. Root growth is significantly reduced in roots transplanted on estradiol plates compared to roots transplanted onto mock DMSO plates (Figure 5.26 A-C). The change in root length of DMSO-treated roots also increased from a mean of 0.8 cm between day 0 and 2 to a mean of 1.2 cm between day 2 to 4, whilst estradiol-treated roots had similar mean values of around 0.6 cm per day across the two time periods. These results show callose deposition reduces the growth rate of roots within the first 48 hours of treatment. This could be due to the callose deposition at plasmodesmata reducing cell-to-cell mobility and phloem unloading.

To explore whether the reduced growth is correlated with reduced phloem unloading I carried out a carboxy fluorescein dye-loading assay. 5(6)-carboxyfluorescein diacetate (CFDA) is a membrane permeable and non-fluorescent molecule which is rapidly up-taken into cells due to its acetyl groups (Cui, Wang, & Lee, 2015). Once inside cells, CFDA is converted by cellular esterases into carboxyfluorescein (CF). CF is a fluorescent, membrane impermeable molecule which moves cell-to-cell through plasmodesmata. The molecular weight of CF is 460 Da, making it similar in size to sucrose, which is 342 Da, and so it can act as a tracer for cell-to-cell movement of sugars (Cui, Wang, & Lee, 2015). I used the method from Kalmbach et al. (2023) to load CF onto the leaves of nine-day old seedlings, placing droplets of CFDA on leaves, and imaging roots 45 minutes later. Preliminary results suggested estradiol treatment slows phloem unloading of CF. In DMSO-treated roots, CF is unloaded in the root tip 45 minutes after dye-loading, with the dye moving out of the phloem and into the root tip tissue (Figure 5.27 A). In contrast, no fluorescence was seen in estradiol-treated root tip tissue (Figure 5.27 B). The lack of CF in the vascular bundle of estradiol-treated root tips indicated the dye did not reach the root tip through the phloem. Three hours after dye loading CF can be seen in the phloem of the elongation zone (Figure 5.27 C, indicated by white arrows), and fluorescence can be seen in epidermal cells, but no fluorescence is seen in root tips yet. This suggests inducing callose deposition in the root blocks movement through the phloem and thus makes it difficult to determine what effect estradiol treatment has on cell-to-cell plasmodesmal permeability in

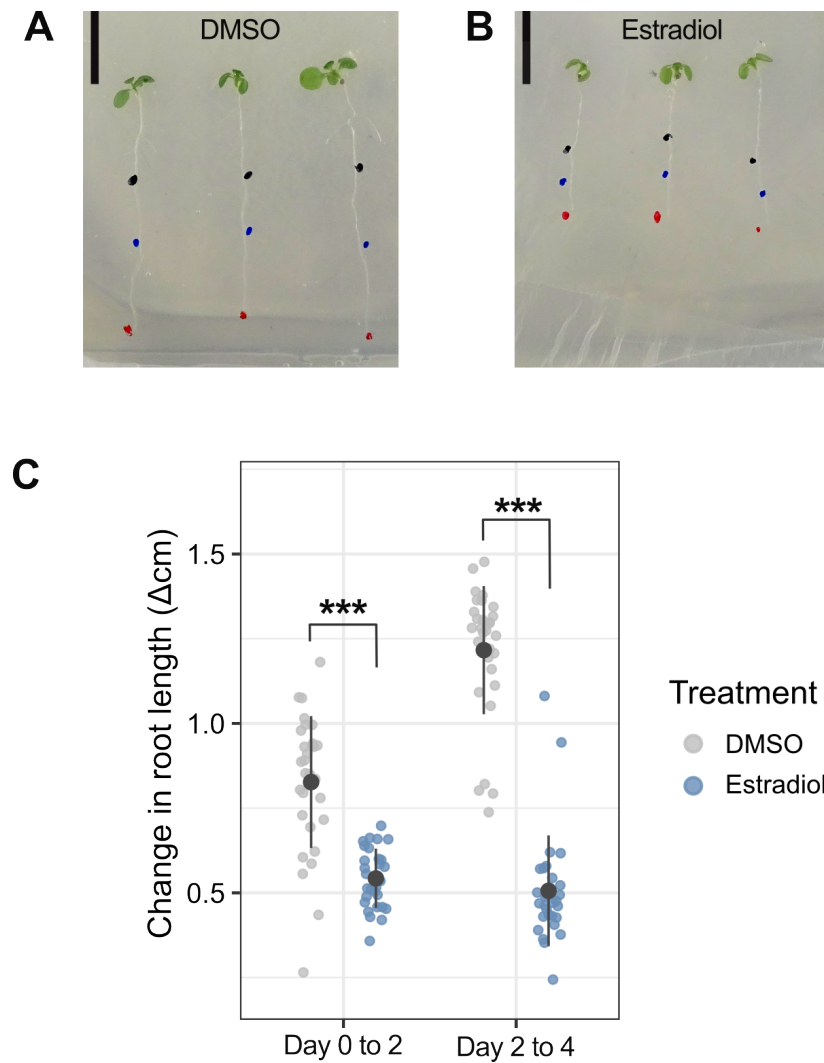


Figure 5.26: *LexA::icals3m* root growth after estradiol treatment – Images of (A) Mock DMSO treated roots and (B) Estradiol treated roots, where black dots indicate root length on the day of transplanting, the blue dot indicates root length after 2 days, and the red dot indicates root length after 4 days. (C) Boxplot of change in root length from day 0 to day 2, and from day 2 to day 4 in mock DMSO (grey) treated roots compared to estradiol treated roots (blue), individual small circles represent individual seedlings, and the large dark grey circles indicate the mean $\pm$ standard deviation, $p < 0.001$ is indicated by “***”. Scale bars indicate 1 cm.

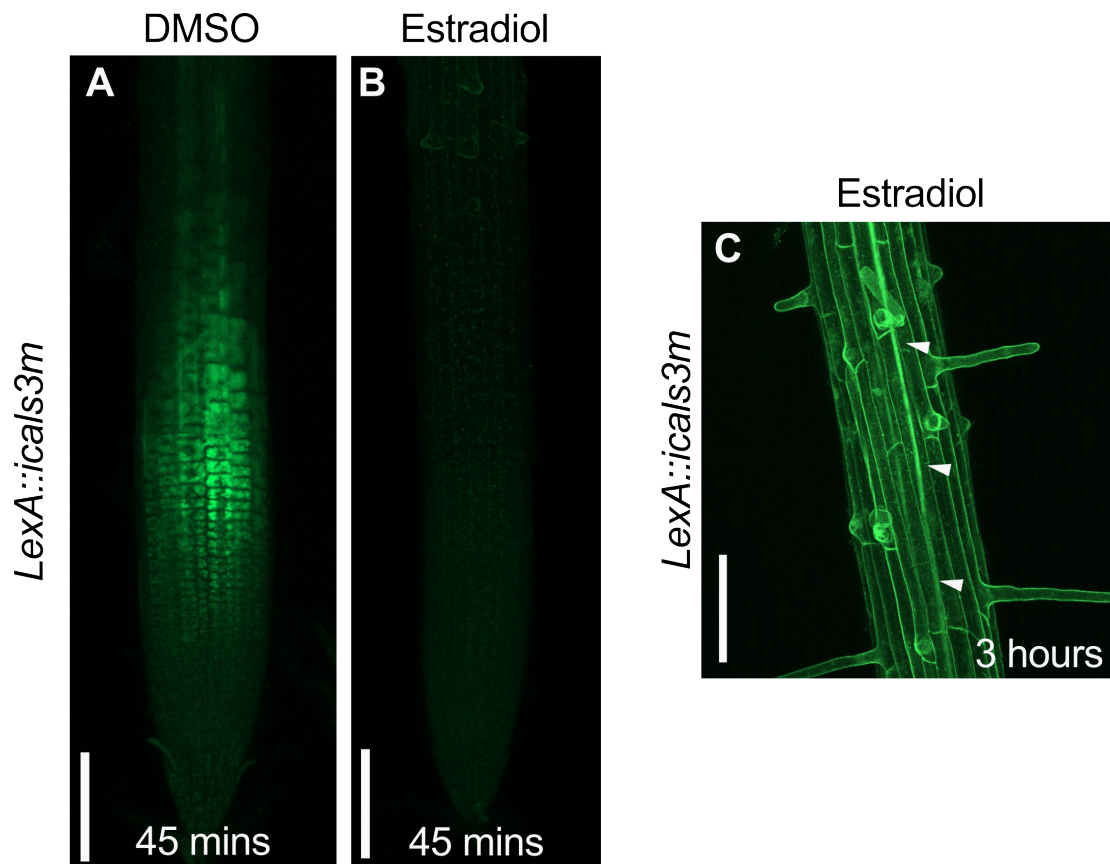


Figure 5.27: **Carboxyfluorescein unloading in *LexA::icals3m* roots** – Confocal images of (A) Mock DMSO-treated roots and (B) Estradiol-treated *LexA::icals3m* roots 45 minutes after loading carboxy fluorescein (CF) onto leaves. (C) The upper section Estradiol-treated *LexA::icals3m* roots 3 hours after CF-loading onto leaves, movement of carboxy fluorescein down the vasculature is indicated by white arrows. Scale bars indicate 100 μm.

the root tip. Instead of further quantifying CF unloading, I decided to take a different approach. I attempted to develop a new method to study cell-to-cell mobility in roots to determine if plasmodesmal closure is induced in *LexA::ical3m* roots tips and investigated plasmodesmal stress response in roots.

5.3.17 Root PAMP-induced plasmodesmal closure was not detected in a PAGFP mobility assay

To determine whether plasmodesmal closure is induced during response to stress in roots I attempted to develop an assay to detect plasmodesmal closure using Arabidopsis lines constitutively expressing photoactivatable GFP (PAGFP). Unlike normal GFP, PAGFP has very low fluorescent emission under 488 nm light. When photoactivated by 413 nm light, the fluorescence of PAGFP increases 100-fold

(Patterson & Lippincott-Schwartz, 2002). Using a confocal microscope, I activated PAGFP in a 30 x 70 μm region located 175 μm from the root tip. I imaged the photoactivation site immediately after photoactivation and 3.5 hours later (Figure 5.28 A and B). Over 3.5 hours the pool of activated PAGFP diffused from the initial activated region into neighbouring cells (Figure 5.28 B). I quantified the change in the area of root showing PAGFP fluorescence at 0 hours and 3.5 hours.

To determine whether this method is effective at detecting plasmodesmal closure I first tried to detect NaCl-triggered plasmodesmal closure, previously demonstrated in roots (Pérez-Sancho et al., 2023). I found no significant difference between the change in the area of PAGFP after NaCl treatment versus water treatment (Figure 5.28 C). This contrasts the NaCl-triggered reduction in plasmodesmal permeability found by Pérez-Sancho et al. (2023). Pérez-Sancho et al. (2023), however, use a DRONPA-S assay to quantify cell to cell movement, which is a much more precise tool to study plasmodesmal permeability over 10 minutes from a single cell. This means the spatial and temporal scales are different to my experiment. It could be under my experimental conditions there is no plasmodesmal closure in response to NaCl, or my experimental set-up may not have enough resolution to detect plasmodesmal closure.

To further test whether my experimental set-up can detect plasmodesmal closure I generated a cross between PAGFP x *LexA::icals3m* lines. Even though my previous results using *LexA::icals3m* in tips have been inconclusive, I photoactivated root tips in an area where the LexA promoter is active so callose deposition at plasmodesmata may be increased even if not detected by aniline blue staining. I transplanted the T4 homozygous PAGFP x *LexA::icals3m* seedlings onto estradiol or mock DMSO plates for 24 hours and then photoactivated PAGFP. I carried out three replicates with a minimum of 4 seedlings per treatment in each replicate. Within each replicate, I saw no difference in GFP movement 3.5 hours after photoactivation in PAGFP x *LexA::icals3m* (Figure 5.28 D). There was variation in trends between replicates, with replicates 1 and 2 showing a non-significant trend towards a reduction movement of PAGFP, whilst replicate 3 shows an increase in PAGFP movement. These preliminary data suggests the PAGFP method is variable and further replicates are needed to determine whether estradiol can induce plasmodesmal closure in PAGFP x *LexA::icals3m* seedlings.

As my previous positive controls were inconclusive, I used flg22 to attempt to induce plasmodesmal closure. flg22 has previously been found to induce plasmodesmal closure in the root cortex in seedlings grown on sucrose plates (Gerlitz et al.,

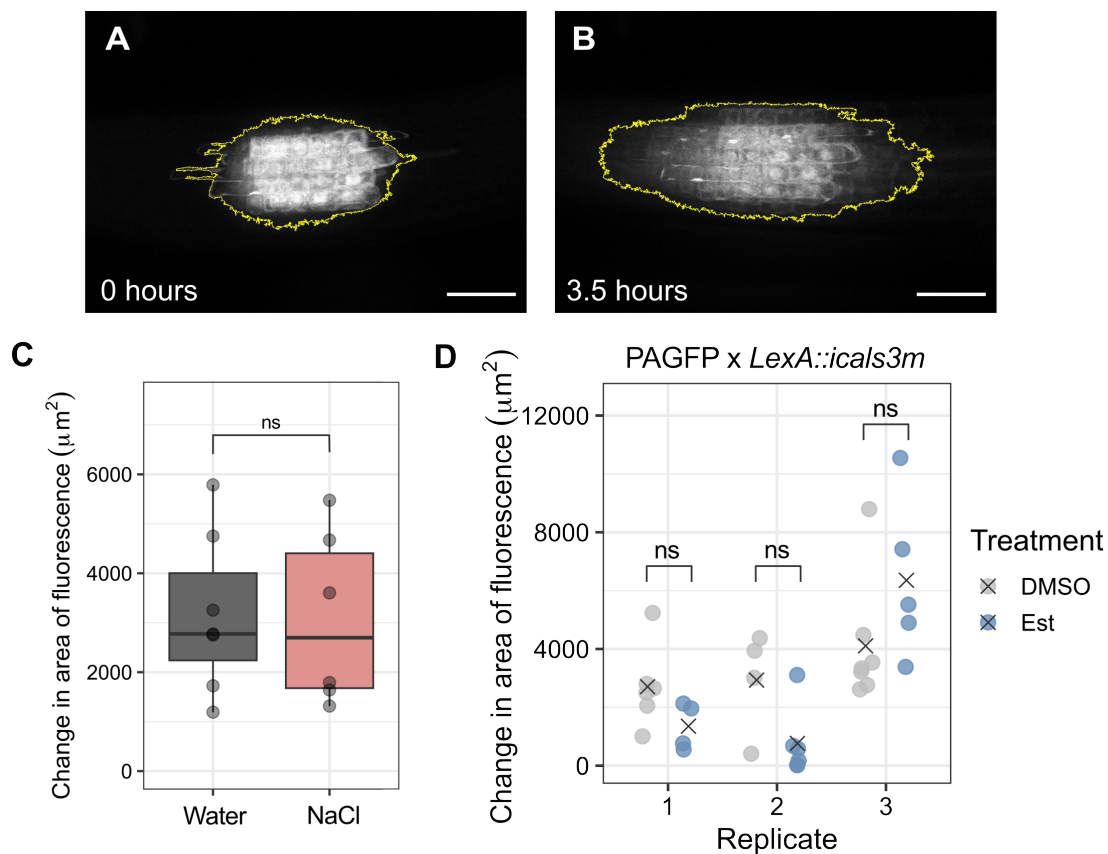


Figure 5.28: **Photoactivatable GFP movement assay in roots** – Confocal images of Photoactivated photoactivable GFP (PAGFP) immediately after activation (A, 0 hours) and 3.5 hours later where the yellow outline indicates the area of fluorescence intensity above the given threshold of 3000 Arbitrary unit (AU) and scale bars indicate 50 μm . (C) Box plot showing quantification of the change in area of fluorescence between 0 hours and 3.5 hours after photoactivation of PAGFP in comparing NaCl (coral) to mock water (grey) treatment. (D) Plot of change in area of fluorescence in PAGFP x *LexA::icals3m* lines after DMSO (grey) and estradiol (blue) treatment across three replicates. “ns” indicates where no statistically significant difference was found, and box plots show the median +/- the interquartile range.

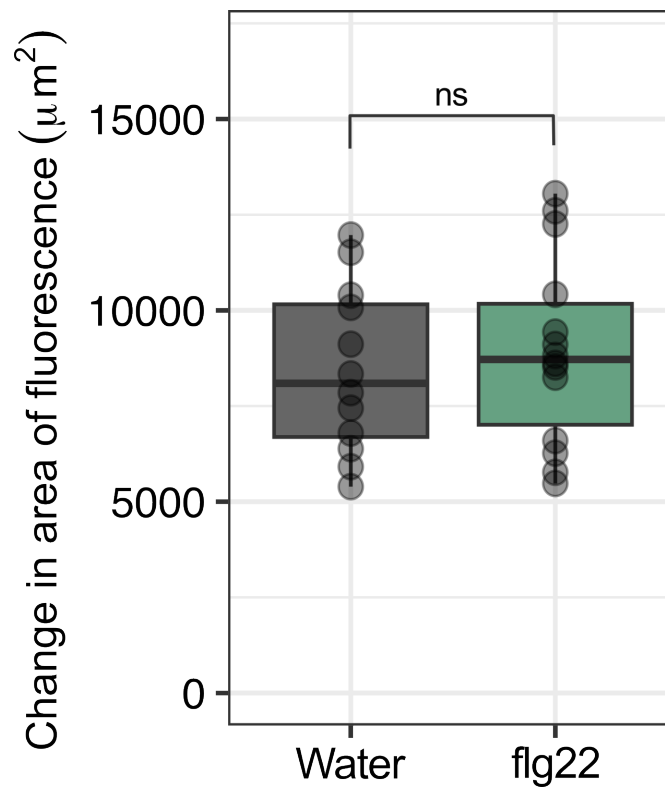


Figure 5.29: **Photoactivatable GFP movement assay in response to 100 μ M flg22**– Plot showing change in area in fluorescence of activated photoactivable GFP (PAGFP) comparing the area of PAGFP fluorescence in flg22 treatment (green) to water (grey) treatment, where individual points represent PAGFP fluorescence in a single root. “ns” indicates where no statistically significant difference was found, and box plots show the median +/- the interquartile range.

2018), I tested plasmodesmal closure in roots grown on plates without sucrose. I treated roots with 100 μ M flg22 30 minutes before photoactivation. I quantified movement in 12 roots per treatment and carried out a student’s t-test comparing means of the two treatments. I found no significant difference in PAGFP movement between water and flg22 treatment (Figure 5.29). Given the lack of positive control for this PAGFP experiment I cannot be sure whether this is due to there being no changes in plasmodesmal permeability, or if there are changes in plasmodesmal permeability but my assay lacks sensitivity to detect them. Overall, quantifying cell-to-cell movement in roots has proved challenging and I lack concrete answers on whether flg22 or NaCl induces plasmodesmal closure in roots in my experimental conditions.

5.4 Discussion

5.4.1 Covering mature leaves induces the sink-source transition in young leaves

In this chapter I found plasmodesmal responses to PAMPs change with the induction of the premature sink to source transition (Figure 5.7). I induced the sink to source transition in young leaves by covering mature leaf tissue with foil and leaving three young leaves exposed. Physiological changes in the covered mature leaves and young leaves from the covered plants strongly indicate the young leaves from covered plants have transitioned from sink to source. The mature covered leaves show a depletion of starch and soluble sugars along with a reduction in the quantum efficiency of photosystem II (Figure 5.4 and 5.6). Previous research showed covering individual leaves triggers a senescence response, with an exchange of nutrients between the darkened leaf and the plant as cellular components of the leaf are retrieved and recycled (Keech et al., 2007; Law et al., 2018). Covering whole rosettes leads to a “standby” response where key cellular machinery is maintained as long as possible (Keech et al., 2007; Law et al., 2018). In my experiment, it is unclear whether the covered mature leaves adopt a senescence or stand-by response. The decline in PSII efficiency does indicate some sort of senescence response, with chlorophyll and photosynthesis related proteins found to degrade during shading-induced senescence in *Arabidopsis* (Brouwer et al., 2012).

Even if the nutrient exchange between mature and young leaves occurs as part of a senescence response, sugars are unlikely to be transported to young leaves through-out my experimental timeframe. The complete depletion of starch and measured soluble sugars in mature leaves after 24 and 48 hours indicate supply of carbon in the form of sugars is fully depleted. If sugars are being transported to young leaves they would have to be from the breakdown of carbon stores such as the cell wall. Further evidence that the sugar transport to young leaves is reduced in covered plants is seen in the reduction in phloem unloading in young source leaves compared to young sink leaves. Young source leaves show similar patterns of GFP unloading out of the phloem to mature source leaves indicating that phloem unloading is reduced (Figure 5.2).

Another indication the sink-to-source transition has occurred in young leaves from covered plants is the higher accumulation of starch in young source leaves

compared to young sink leaves at both 24 and 48 hours (Figure 5.4 and 5.4). This increased starch content at the end of the day may be remobilised at night to supply remaining sink tissues such as roots or almost-emerged leaves with a carbon supply, or used to fuel growth in the leaf itself. Further experiments involving carbon tracing could enable me to track where this starch store is being utilised.

To facilitate the increase in carbon stored as starch, there could be a change in carbon partitioning. It could be that the same amount of carbon assimilation occurs in young sink and young source leaves but more carbon is diverted towards starch storage. Although glucose, sucrose and fructose contents are unchanged between the young sink and young source, there could be partitioning of carbon from other unmeasured carbon stores towards starch storage. Alternatively, the increased starch content could be due to increased carbon assimilation. Previously, characterisation of the sink-to-source transition in olive and peach trees showed carbon assimilation increased as leaves transitioned from sink to source. As I was not able to directly measure carbon assimilation due to technical and equipment limitations, I measured the operating efficiency of PSII at ambient light to gain insight into changes in photosynthetic processes. I found the operating efficiency is increased in PSII in young source leaves compared to young sink leaves (Figure 5.6). The relationship between PSII and CO₂ assimilation is not linear in C₃ plants, making it difficult to conclude CO₂ assimilation from PSII efficiency (Baker, 2008). Calculating CO₂ assimilation from PSII operating efficiency requires knowledge of how many electrons produced in the light reactions of photosynthesis are partitioned towards alternative electron sinks, such as photorespiration (Baker, 2008; Genty et al., 1989). Although I could assume the electrons are partitioned identically in young sink and young source leaves, this is unlikely due to developmental differences between sink and source leaves potentially impacting photorespiration or other electron sinks. As a result, I cannot directly link the higher quantum efficiency of PSII to increased carbon assimilation. The increase in PSII quantum efficiency still indicates there is increased photosynthetic conversion capacity in young source leaves, whether this is used in CO₂ assimilation or other electron sinks is unknown.

Transcriptomic data from young source leaves supports the experimental evidence that there is an increase in PSII efficiency, showing upregulation of chloroplast and oxidative stress related genes (Figure 5.10). The increased expression of FtsH8, a subunit of the FtsH protease, could be involved in increased PSII efficiency. FtsH functions in repair of PSII and previously FtsH8 has been found to be

up-regulated in high light, and so could be up-regulated to increase repair and photoprotection of PSII if it is working at increased efficiency (Kato & Sakamoto, 2018; Sinvany-Villalobo et al., 2004). The light-dependent chloroplast regulator SIG2 and chlorophyll homeostasis regulator BCM2 are up-regulated. These genes could be mediating an increase in PSII efficiency through altering chloroplast gene expression and chlorophyll ratios and content, with potentially wider chloroplast changes mediated by SIG2. Additionally, the upregulation of superoxide radical detoxification genes SOD1 and SOD2, along with the SOD1-associated chaperone CCS could be triggered by extra oxidising agents produced by an increased PSII efficiency. The upregulation of three genes could protect the cell from potential superoxide radicals generated by increased oxidation occurring at PSII. Overall, the transcriptional data supports my finding that PSII efficiency is increased in young source leaves, and suggests several transcriptional changes may facilitate this through increased expression of PSII photoprotection- and chlorophyll-related genes as well as increased expression of genes involved in protection against oxidative stress.

The sink-source transition appears to be occurring strongest in the “older” of the young leaves, termed relative leaves 2 and 3. Quantification of PSII efficiency and phloem unloading showed no differences between the youngest leaf (“leaf 1”) of the young sink and source tissue (Figure 5.6, 5.3). These leaves may continue to be supported by the older young leaves which are transitioning from sink to source. Alternatively, due to their small size and earlier developmental stage, there could be more variation between these young leaves in terms of operating PSII efficiency and phloem unloading. In relative leaf 1, the median values for operating PSII efficiency and phloem unloading are lower in the young source leaves compared to the young sink leaves but this trend is not significant, potentially due to higher variation in these leaves. Overall as the young relative leaves 2 and 3 show stronger physiological differences between young sink and young source leaves, and so I conclude that these leaves are undergoing the sink to source transition, but not relative leaf 1.

Further evidence for the induction of the sink-to-source transition in young source leaves can be found in transcriptional data (Figure 5.9). I used my dataset to identify genes up-regulated in both young source and mature source tissue compared to young sink leaves and identified these genes as candidate genes related to source tissue. I identified two genes of particular interest – the *SWEET* sugar efflux transports *SWEET11* and *SWEET12*. *SWEET11* and *12* are expressed in

a wide range of tissues and their expression is induced by biotrophic pathogens (Chen et al., 2010). *SWEET11* and *12* play an important role in phloem loading, they are expressed in phloem parenchyma and localise to the plasma membrane, loading sucrose into the apoplast from phloem parenchyma cells (Chen et al., 2012). Sucrose loaded into the apoplast is then transported by the sucrose H⁺ symporter *SUC2* into companion cells where sucrose then travels through the phloem (Chen et al., 2012). The upregulation of *SWEET11* and *12* in young source leaves could result in a build-up of sugars in the apoplast ready for phloem loading. The upregulation of *SWEET*s could be key to driving the initiation of sucrose export during the sink to source transition.

Previous research has suggested prior to the sucrose export being initiated, as leaves transition from sink to source, the phloem has reached maturity, with an unknown mechanism suggested to drive loading of sugars into companion cells (Fellows & Geiger, 1974; Turgeon, 1989). It could be that the expression of *SWEET 11* and *12* are the drivers of the export of sucrose, initiating the carbon concentration mechanism that establishes the osmotic pressure gradient that drives sugar flow in the phloem. Additionally, expression of *SWEET* genes is known to be dynamically regulated in response to environmental cues such as cold stress and drought stress, as well as expression is induced by biotrophic pathogens to supply sugars to the pathogen (Chen et al., 2010; Durand et al., 2016; Le Hir et al., 2015; Walerowski et al., 2018). This indicates expression of *SWEET11* and *12* can be dynamically induced, and so in the sink-to-source transition some internal cue could trigger this induction of gene expression.

If *SWEET 11* and *12* are up-regulated to increase sucrose transport into the apoplast for phloem loading, one could also predict that *SUC2*, which transports sucrose from the apoplast into companion cells, would also be up-regulated. However, I did not see the upregulation of *SUC2* in young or mature source leaves relative to young sink leaves. As there is no difference in transcription between young source, young sink, and mature source and *SUC2* is already expressed in young sink leaves, it could be that upregulation is not needed to facilitate phloem loading. Alternatively, there could be upregulation of *SUC2* within companion cells that is not detected by my whole leaf transcriptional approach. A single-cell approach could help to determine if there are transcriptional changes in *SUC2* companion cells. Overall, the lack of increase in *SUC2* expression raises questions about whether an increase in *SWEET11* and *12* expression alone could facilitate phloem loading in source leaves.

In addition to the *SWEET11* and *12* genes, my transcriptomic data suggested other potential candidate proteins that could be related to the sink to source transition. The transcription factors *RAD-LIKE 5/ RADIALIS-LIKE SANT/MYB* (*RL/RSM*) are up-regulated in both young source and mature source tissue, and have previously been associated with leaf morphogenesis and floral development (Baxter et al., 2007; Hamaguchi et al., 2008). One hypothesis could be that these transcription factors have an additional role in coordinating source-leaf specific transcription to ensure the vegetative phase is maintained. Additionally, the up-regulation of two CC-type glutaredoxins *ROXY3* and *ROXY17* could coordinate cellular and redox stress response in source tissue. Other CC-type glutaredoxins are known to bind to TGA transcription factors; however, the binding activity of *ROXY3* and *17* is uncharacterised despite sequence similarity and the roles of *ROXY3* and *ROXY17* have not been characterised in the sink-to-source context (Gutsche et al., 2015; Jung et al., 2018). Additionally in young source leaves the gene encoding chlorophyll a-b binding protein *LIGHT HARVESTING COMPLEX B 7 (LHCB7)* is also upregulated, which functions in the light harvesting complex and could be related to increased photosynthetic efficiency of PSII in young source leaves compared to young sink (Peterson & Schultes, 2014). Other down-regulated genes in source tissue such as the vascular cell wall development-related protein *AGP31*, or the cell expansion coordination protein *LSH10* could have an impact on source-specific tissue maintenance and development (Hijazi et al., 2014; Vo Phan et al., 2023). The transcriptomic data has revealed multiple potential candidate genes that could have roles alongside *SWEET11* and *12* in the sink-to-source transition and maintenance of source tissue. Further investigation is needed to confirm if the differential expression of these potential candidate genes plays a role in source tissue formation and maintenance.

In summary, my results in young source and sink leaves, as well as physiological changes in covered mature leaves indicate covering mature leaves reduces phloem transport of sugars to young leaves (Figure 5.30). This reduction in carbon supply to young leaves thus forces them to shift their physiology, with an increase in PSII efficiency and starch accumulation as well as expression of *SWEET11* and *SWEET12* indicating phloem loading could be induced in these young source leaves.

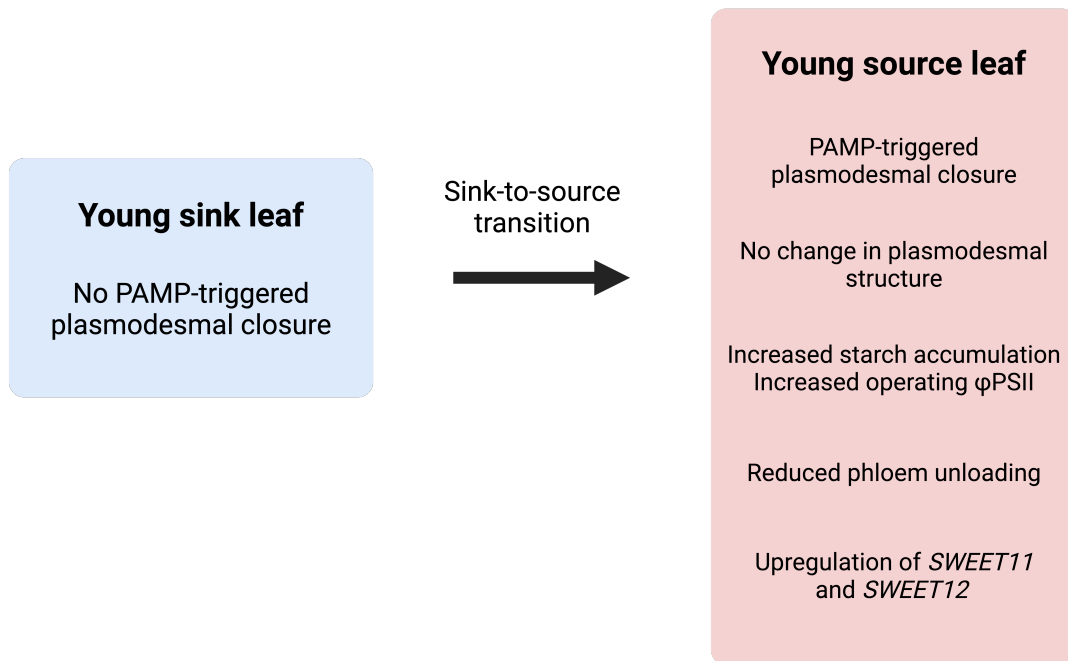


Figure 5.30: **Summary of changes associated with the sink-to-source transition** – Schematic showing the observed changes in young leaves upon the induction of the sink-to-source transition.

5.4.2 Inducing the sink-to-source transition changes plasmodesmal regulation in response to PAMPs

Inducing the sink-to-source transition in young leaves changes plasmodesmal responses to PAMPs in young leaves. Young source leaves show chitin-induced plasmodesmal closure whilst young sink leaves do not (Figure 5.7). This supports my hypothesis that the importance of maintaining plasmodesmal permeability in sink tissue underpins the lack of stress-triggered plasmodesmal closure. The induction of plasmodesmal closure in tissue of the same biological age in young sink and young source also shows that the differences between young and mature leaves are not dependent on tissue age. Instead, the sink or source status plays a more important role in determining plasmodesmal response.

Although my transcriptomic dataset did not provide insights on how chitin response shifts across the sink-to-source transition due to the small transcriptional response to chitin, the downregulation of *PDLP7* in both young source and mature source relative to young sink raises questions about whether this could impact plasmodesmal response to stress (Figure 5.9). *PDLP7* has been previously established to play a role in plasmodesmal callose deposition (Chen et al., 2024).

Transient expression of *PDLP7* in *Nicotiana benthamiana* previously increased callose deposition at plasmodesmata (Li et al., 2021), whilst destabilisation of *PDLP7* by the *P. syringae* HopO1-1 effector increased cell-to-cell mobility in Arabidopsis (Aung et al., 2020). These previous results would suggest that decreased *PDLP7* expression in source leaves could decrease callose deposition and increase cell-to-cell mobility. However, cell-to-cell mobility has been typically suggested to be reduced across the sink-to-source transition. Further exploration of *PDLP7* activity in sink vs source leaves is needed to understand how the decreased expression of *PDLP7* could be related to the regulation of plasmodesmal permeability in sink vs source leaves.

5.4.3 Constitutive expression of MP17-GFP reduces movement through a callose-independent mechanism

Previously, a shift in plasmodesmal structure has been associated with the sink-to-source transition in Arabidopsis (Fitzgibbon et al., 2013). I hypothesised the changes in PAMP response could relate to this change in structure. I initially attempted to use the Potato Leaf Roll Virus movement protein MP17-GFP as a label for branched plasmodesmata; however, I found constitutive expression of MP17-GFP induces plasmodesmal closure (Figure 5.14). This is interesting as movement proteins expressed during infection have been suggested to target plasmodesmata to facilitate viral infection, often by increasing plasmodesmal permeability (Talianky et al., 2008; Wolf et al., 1989). My results support previous findings that constitutive expression of MP17-GFP and other movement proteins has previously been associated with reduced cell-to-cell mobility (Kronberg et al., 2007; Rinne et al., 2005). The reason for the reduction in cell-to-cell mobility induced by constitutive movement protein expression is unclear. Kronberg et al. (2007) and Rinne et al. (2005) suggest viral movement protein expression induces a defensive response in leaves which results in increased callose deposition. Constitutive expression of MP17-GFP, however, did not increase callose deposition at plasmodesmata (Figure 5.15). Instead, the reduction in cell-to-cell mobility could be because the MP17-GFP protein itself directly blocks movement through plasmodesmata. Fitzgibbon et al. (2013) suggest the MP17-GFP protein accumulates in the central cavity of branched plasmodesmata. This could result in reduced cell-to-cell movement if the MP17-GFP protein accumulation in the central plasmodesmal cavity physically blocked movement through plasmodesmata. Overall, the reduction in cell-to-cell movement in MP17-GFP lines and other

phenotypic effects i.e. reduced growth, reduced callose deposition, and chlorosis phenotype, meant it was not a suitable tool for studying the relationship between plasmodesmal regulation and structure. The results still raise questions about why the constitutive expression of MP17-GFP has such a large impact on cell-to-cell mobility.

Overall, I faced several challenges in quantifying plasmodesmal structure, with mutants and transgenic lines having secondary phenotypic effects that limit comparisons to trends in Col-0.

5.4.4 Plasmodesmal structure does not shift across the sink to the source transition

As the MP17-GFP approach to quantify changes in plasmodesmal structure proved unsuccessful, I used SBF-SEM to quantify changes in plasmodesmal structure across the sink-to-source transition. I hypothesised one potential mechanism for the shift in plasmodesmal response across the sink-to-source transition could be a shift in the plasmodesmal structure; however, I found inducing the sink-to-source transition resulted in no major increase in the frequency of branched plasmodesmata compared to simple. Young sink and young source leaves showed similar ratios of simple to branched plasmodesmata. This suggests structure does not underpin regulation. This is further supported by the result that chitin can induce plasmodesmal closure in mature leaves of *cher1* mutants, which only have simple plasmodesmata. Thus, plasmodesmal structure alone is not a determinant of PAMP-response.

The lack of change in plasmodesmal structure across the sink-to-source transition contrasts with previous studies which correlated increased frequency of branched plasmodesmata across the sink-to-source transition. In *Nicotiana tabacum* leaves, there is an increase in the frequency of branched plasmodesmata in electron micrographs taken across where the authors predict the sink-to-source transition to be occurring (Oparka et al., 1999; Roberts et al., 2001). Other factors could have influenced the increase in plasmodesmal branching across the sink-to-source transition in these plants. For instance, in trichome hairs and cotton buds branching of plasmodesmata is seen at specific developmental time points independently of the sink-to-source transition (Faulkner et al., 2008; Ruan et al., 2001). It could be that another developmental or age-related cue is triggering this

shift in plasmodesmal structure. My experiment uncoupled the impact of leaf age from the sink-to-source transition which could explain the differences in results.

More evidence for the change in plasmodesmal structure across the sink-to-source transition was previously presented by Fitzgibbon et al. (2013). Fitzgibbon et al. (2013) induced the sink-to-source transition in Arabidopsis by detaching leaves and then quantified an increase in the frequency of branched plasmodesmata using MP17-GFP labelling as an indicator. My results, however, revealed that constitutive expression of MP17-GFP impacts plasmodesmal permeability and growth. Therefore the increased MP17-GFP labelling of branched plasmodesmal across the sink-to-source transition found by Fitzgibbon et al. (2013) may also be related to indirect impacts of MP17-GFP expression. Additionally, the detachment of young sink leaves to induce the sink-to-source transition may cause other stress responses which trigger branched plasmodesmata formation that is not induced upon the less mechanically damaging covering of mature leaves in my experimental set-up to induce the sink-to-source transition.

Although I did not find any change in plasmodesmal structure, I did see a trend towards increased numbers of plasmodesmata in young sink tissues. Further segmentation of the cell wall to measure the density of plasmodesmata per μm of the cell wall could help to determine if this is an artefact of differences in cell wall lengths captured in different samples. If there is an increase in plasmodesmal density in young sink leaves, it could indicate plasmodesmal formation is slowed in source leaves whilst the rate of plasmodesmal formation continues as usual in young sink leaves. This is also interesting as my microprojectile bombardment assays showed the medians of the cell-to-cell movement were the same between the mock-treated young sink leaves and mock-treated young source leaves. This indicates if there is increased plasmodesmal density it does impact the intercellular molecular trafficking of GFP. There are varying studies showing density does (Oparka et al., 1999; Roberts et al., 2001) or does not (Ganusova et al., 2020; Liesche et al., 2019) correlate with plasmodesmal permeability. Additional quantification of my data could help to reveal whether inducing the sink-to-source transition induces a relative reduction in plasmodesmal density.

Further questions about the relationship between plasmodesmal number, structure, and cell-to-cell mobility were raised in differences between baseline plasmodesmal permeability in *cher1*. *cher1* has an increased baseline cell-to-cell movement compared to Col-0 (Figure 5.17). I find no change in the number of plasmodesmal callose deposits, but the TEM data from Kraner et al. (2017) suggests there is a

reduction in the number of plasmodesmata (Figure 5.18). The counting of plasmodesmata is more precise and accurate from TEM images, as the detection of callose deposits does not identify individual plasmodesmata. As a result, the findings from Kraner et al. (2017) are more compelling in showing there are fewer plasmodesmata in young tissue. The decrease in plasmodesmal number but increased transport compared to Col-0 could be related to the lack of branched plasmodesmata in *cher1*. It could be that the fewer simple plasmodesmata have a larger transport capacity than the more branched plasmodesmata in Col-0. The reduction in callose deposition in *cher1* compared to Col-0 supports this. Thus, the increased baseline movement in *cher1* may be due to increased transport capacity through simple plasmodesmata.

Overall I found changes in PAMP response across the sink-to-source transition were not correlated with changes in plasmodesmal structure. Further questions remain about how plasmodesmal structure and density are related to cell-to-cell mobility. One of the main barriers to answering this is current technological limitations meaning it is not possible to simultaneously measure both structure, density and movement through plasmodesmata.

5.4.5 Uncertainty around root plasmodesmal response

I attempted to explore root plasmodesmal response to investigate whether plasmodesmal response in sink leaves was common across other sink tissues. Similarly to sink leaves, I find inducing increased callose deposition in *LexA::icals3m* roots reduces growth (Figure 5.26). This decrease in growth could be due to reduced transport through the phloem, with preliminary results suggesting carboxyfluorescein dye takes longer to move through the phloem in plants with induced callose deposition (Figure 5.27). Additionally, the decrease in root growth of *LexA::icals3m* lines could also be due to reduced phloem unloading due to induced callose deposition reducing plasmodesmal permeability, but I have limited evidence to confirm or reject this hypothesis. Callose staining in staining showed a non-significant trend towards increased callose deposition in root tips, potentially due to high background levels of callose deposition in young roots (Figure 5.24). I was also unable to quantify CF unloading in the root tip, as estradiol-treated roots had very low CF fluorescence and it was unclear if this was due to reduced unloading or reduced phloem transport. Other attempts to quantify cell-to-cell movement after inducing callose deposition in crossed PAGFP x *LexA::icals3m*

resulted in variability between replicates (Figure 5.28). This made it difficult to determine whether inducing callose deposition in *LexA::icasl3m* lines resulted in reduced plasmodesmal permeability and whether this was linked to the reduced growth in roots.

Further questions also remain surrounding my unsuccessful attempt to develop a novel cell-to-cell mobility assay in roots using PAGFP. I was unable to see changes in PAGFP movement in response to the elicitors flg22 and NaCl which have been previously been shown to induce plasmodesmal closure using the DRONPA-S system (Figure 5.28) (Gerlitz et al., 2018; Pérez-Sancho et al., 2023). The reason for the lack of plasmodesmal closure in my system could be because I am studying a different spatial and temporal scale of plasmodesmal responses. The DRONPA-S system quantifies plasmodesmal permeability for 10 minutes whereas my assay looks over the time course of 3.5 hours. In roots there could be a transient plasmodesmal closure response initiated in response to stimuli that is not detected by my assay. Additionally, the experiments are carried out on different spatial scales. The induced closure by NaCl and flg22 in DRONPA-S was studied in root cortex cells, whereas my PAGFP assay targets a larger area of root due to a lack of laser precision, and I activated PAGFP across multiple cell layers. The DRONPA-S system has shown different root cell layers have different plasmodesmal permeabilities and so these different cell types could respond to flg22 or NaCl differently. As I activated PAGFP across different cell layers I may be picking up a general signal from mixed plasmodesmal responses across cell layers.

Despite potential issues with my PAGFP assays, it could still be that I am picking up a genuine lack of plasmodesmal response not seen in previous studies. One major difference between my experimental setup and the DRONPA-S system is the difference in the media used. The DRONPA-S experiments were performed on sucrose-supplemented plates, and adding sucrose to media has previously been shown to alter plant-microbe interactions (Cabrera et al., 2022). Plasmodesmal response to flg22 and other stresses could also be altered in these sucrose-supplemented plates and as a result, roots on plates without sucrose could respond differently. Further experiments using my PAGFP assay on roots grown on sucrose plates could help test this hypothesis. Overall I was unable to detect any plasmodesmal closure in roots, although I am unsure if that is due to a lack of plasmodesmal closure in my experimental conditions or if my method is not able to detect plasmodesmal closure. Further exploration and method refinement is needed to determine whether plasmodesmal closure is induced in my experimental

conditions in roots.

Overall, this chapter shows inducing the sink-to-source transition in young leaves changes plasmodesmal response to PAMPs. I show this change in plasmodesmal regulation is not accompanied by a change in structure. The extent to which this relationship between structure and regulation is correlation or causation remains to be determined due to difficulties in studying plasmodesmal structure alongside plasmodesmal regulation. I also explored plasmodesmal response in root tissue but was unable to verify my method could detect plasmodesmal closure.

Chapter 6

General Discussion

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6.1 Introduction

In this chapter, I will discuss the results, hypotheses, and further questions that arise from this thesis. Across this thesis, I investigated the role of plasmodesmata in growth and defence in young and mature leaf tissue. In Chapter 3, I found plasmodesmal closure is not triggered by pathogen-associated molecular patterns (PAMPs) or stress elicitors in young leaves, despite the induction of other pattern-triggered immune responses. In Chapter 4, I explored how this lack of plasmodesmal closure could be related to the role of plasmodesmata in growth and defence to find

inducing plasmodesmal closure in young sink leaves reduced phloem unloading and growth, whilst also reducing susceptibility to the bacterial pathogen *Pseudomonas syringae* (*Pst*) DC3000. In Chapter 5, I found induction of the sink-to-source transition in young leaves changes the regulation of plasmodesmal permeability in response to PAMPs, linking the sink-source status of leaves to the plasmodesmal response. Overall, my findings suggest the regulation of plasmodesmal permeability is important in balancing the trade-off between growth and defence.

6.2 The role of plasmodesmata in growth

Phloem unloading has previously been shown to occur symplasmically in *Arabidopsis* sink leaves and roots (Imlau et al., 1999; Oparka et al., 1999; Ross-Elliott et al., 2017; Wright & Oparka, 1996). Phloem unloading supplies sugar to young leaves, and so I hypothesised phloem unloading through plasmodesmata was important to growth and development. My findings in Chapter 4 support this, showing the induction of plasmodesmal closure reduced both phloem unloading of 5,6-carboxyfluorescein (CF) and leaf growth in young sink tissue. Similarly, experiments on *LexA::icals3m* roots in Chapter 5, showed inducing plasmodesmal closure in roots reduced growth. My results are less clear on the effect of inducing plasmodesmal closure in *LexA::icals3m* root tips on phloem unloading and cell-to-cell mobility. Preliminary data in roots suggests phloem transport of CF is reduced after the induction of plasmodesmal closure, but I was unable to determine how this increased callose deposition impacts cell-to-cell mobility as the dye did not unload from the phloem. Although questions remain in roots, overall my results in leaves show maintaining symplasmic connectivity through plasmodesmata is important to symplasmic phloem unloading and growth.

In certain developmental contexts, many plant species can switch between symplasmic and apoplastic phloem unloading. For instance, during fruit development regions of cells become symplasmically isolated and apoplastic transport increases to facilitate sugar transport (Wu et al., 2004; Zhang et al., 2004; Zhang et al., 2006). The switch from symplasmic to apoplastic sugar import is also seen in *Arabidopsis* guard cells and root embryo cells, which become symplasmically isolated during development (Chen et al., 2015; Daloso et al., 2016; Stadler et al., 2003). If I assume the reduced growth in young leaves after plasmodesmal closure is directly linked to a reduction in sugar supply, it indicates young sink leaves cannot fully compensate for a reduction in symplasmic unloading by switching to apoplastic

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phloem unloading. To determine if there is any evidence of a switch to apoplastic loading I explored the expression of cytoplasmic-apoplastic sugar transporters upon plasmodesmal closure in young *LexA::icals3m* leaves (section 4.3.11). The only transcriptional changes to cytoplasmic-apoplastic sugar transporter expression were the up-regulation of genes encoding the monosaccharide H⁺ efflux protein *SUGAR TRANSPORTER PROTEIN (STP) 4* and the galactose transporter *STP14* after plasmodesmal closure. *STP4* and *STP14* were significantly upregulated with log₂ fold changes of 0.81 and 1.58 respectively. Increased expression of these genes could increase sugar transport from the apoplast into the cell if transport capacity is increased; however, the reduced growth in these young sink leaves indicates any potential increased sugar import as a result of *STP* upregulation is not enough to meet the needs for growth. Apoplastic transport could be increased through a post-translational mechanism e.g. increased protein activity of already present sugar transporters, but again any apoplastic transport mechanism does not seem to fully compensate for reduced symplasmic unloading as overall leaf growth is reduced. The lack of strong evidence for an increase in apoplastic sugar transport to compensate for reduced symplasmic connectivity highlights the importance of maintaining symplasmic connectivity in sink tissues for the movement of sugars in growth and development.

6.3 The role of plasmodesmata in defence

In mature leaves, PAMP-triggered reductions in flux through plasmodesmata have a well-established role in defence. Previous studies have used plants with mutations in genes encoding plasmodesmal-specific PAMP receptors, e.g. *lym2*, or components of the PAMP-triggered signalling pathway, e.g. *pdlp5*, to show disrupting PAMP-triggered plasmodesmal closure can lead to increased susceptibility (Cheval et al., 2020; Faulkner et al., 2013; Lee et al., 2011; Xu et al., 2017). These loss-of-function studies have been key in highlighting the importance of PAMP-triggered plasmodesmal closure in defence against pathogens in mature leaves. In this work, I explored the role of plasmodesmata in defence in young leaves. As PAMPs do not trigger reductions in plasmodesmal permeability in young sink leaves, I could only explore the potential defensive benefits that would arise if plasmodesmata were to close. In Chapter 5, I performed a pathoassay in young leaves of *LexA::icals3m* lines, finding that the induction plasmodesmal closure reduced susceptibility to *Pst* DC3000. These findings indicate there are defensive benefits to PAMP-triggered

plasmodesmal closure in young leaves. Performing additional pathogen assays could help to determine if the induction of plasmodesmal closure also reduces the susceptibility of young leaves to other pathogens such as the fungal pathogens *Botrytis cinera* and *Colletotrichum higginsianum*.

The reduction in susceptibility of young leaves with induced plasmodesmal closure to *Pst* DC3000 could be due to direct or indirect effects of reducing symplasmic connectivity on bacterial infection. Tee et al. (2024) suggested inducing plasmodesmal closure before infection in mature leaves of *LexA::icals3m* reduced susceptibility to certain pathogens due to the accumulation of salicylic acid in a potential priming effect. However, these assays in mature leaves compared susceptibility between plants with induced plasmodesmal closure (24 hours before inoculation) to plants with “normal” PAMP-triggered plasmodesmal closure at the point of inoculation. As young leaves have no baseline PAMP-triggered plasmodesmal closure, in my pathoassays I was comparing the susceptibility of plants with a complete lack of plasmodesmal closure to plants with induced plasmodesmal closure. Therefore, there could be additional benefits to inducing plasmodesmal closure in young leaves that lead to this reduced susceptibility compared to young leaves without induced plasmodesmal closure beyond a potential salicylic acid priming effect. These benefits could prevent the cell-to-cell spread of bacterial effectors through plasmodesmata or enable the accumulation of other defensive molecules. Assaying for the accumulation of salicylic acid in young leaves with and without induced plasmodesmal closure, whilst also studying effector mobility in young leaf tissue could help answer questions about reasons for reduced susceptibility to *Pst* DC3000 in young leaves with plasmodesmal closure.

To conclude, there are established defensive benefits to inducing plasmodesmal closure in mature leaves, and my results using *Pst* DC3000 suggest plasmodesmal closure can also enhance defence in young leaves. Further exploration is needed to fully understand the benefits of inducing plasmodesmal closure in young leaves, and whether these benefits apply to defence against different pathosystems.

6.4 A trade-off between growth and defence?

The concept of pattern-triggered immunity is based on the activation of defence in the presence of a pathogen (Dodds & Rathjen, 2010). This means potentially energy-expensive defensive responses are only induced in the presence of

6.4. A trade-off between growth and defence?

a threat (Huot et al., 2014). In young leaves, I found PAMP treatment induces pattern-triggered immune responses such as reactive oxygen species (ROS) bursts, macroscopic callose deposition, and differential expression of several defensive genes. Plasmodesmal closure, however, is not induced in response to PAMPs in young sink leaves. This leads to the question of why the plasmodesmal pattern-triggered immunity is not induced in young sink leaves.

As discussed above, my results suggest maintaining plasmodesmal permeability is key to growth and phloem unloading in young sink leaves, whilst inducing plasmodesmal closure has defensive benefits against pathogen infection. Thus I hypothesise plasmodesmal closure is not triggered in young sink leaves to prioritise maintaining a symplasmic supply of sugars despite the potential defensive benefits of triggering plasmodesmal closure. This hypothesis suggests plasmodesmal stress response is optimised to balance the trade-off between growth and defence.

One major piece of evidence to support this hypothesis is my finding in Chapter 5 that inducing the sink-to-source transition in young leaves changes plasmodesmal PAMP response. Young source leaves showed PAMP-triggered plasmodesmal closure, whilst young sink leaves of equivalent age showed no change in plasmodesmal response to PAMPs as found in 3. This experiment uncoupled the effect of tissue age from sink-source status, revealing differences in plasmodesmal regulation between young and mature leaves are correlated with sink-source status and not tissue age.

If my hypothesis about the role of plasmodesmata in the balance between growth and defence is correct, the changes in plasmodesmal PAMP response during the sink-to-source transition could be attributed to physiological changes occurring during this developmental shift. Covering the mature leaves of a plant induced the sink-to-source transition in young leaves. This was indicated by the depletion of starch in mature covered leaves indicating a reduced sugar supply to young leaves, as well as the reduced phloem unloading into young source leaves, and increased expression of *Sucrose will eventually be transported 11* (*SWEET11*) and *SWEET12* cytosol-apoplast sugar transporters in young source leaves. Young source leaves showed increased starch accumulation and increased operating efficiency of Photosystem II (Φ PSII) at ambient light compared to young sink leaves. This indicates young source leaves have either increased carbon fixation to increase starch stores or have reallocated carbon from other stores. In any case, this increased starch accumulation, despite reduced phloem unloading, points towards young source leaves becoming more self-sufficient in terms of carbon supply. I suggest during the

sink-to-source transition the symplasmic import of sugars is no longer integral to growth due to reduced import and increased self-sufficiency, and thus the PAMP-induced plasmodesmal closure is less detrimental to sugar supply. The change in plasmodesmal response to PAMPs across the sink-to-source transition could thus be explained as a shift in the balance between the importance of symplasmic connectivity for growth and the defensive benefits of plasmodesmal closure for defence.

Interestingly other pattern-triggered immune responses tested here are induced in young sink leaves, indicating that different pattern-triggered immune responses are regulated independently. This supports previous findings that apoplastic ROS bursts and macroscopic callose deposition are triggered independently of plasmodesmal closure (Faulkner et al., 2013; Xu et al., 2017). This independence may allow modulation of pattern-triggered immune responses depending on the resources available and tissue type to ensure the optimal level of defence is triggered, rather than an all-or-nothing response.

Additionally, a supply of sugars may be required to fuel pattern-triggered immune responses. For instance, UDP-glucose is used as a substrate by CALLOSE SYNTHASEs (CALs) for macroscopic callose synthesis, so inducing plasmodesmal closure could limit macroscopic callose synthesis although the responses appear to be triggered independently. Further characterisation of ROS bursts and macroscopic deposition in young leaves of *LexA::icals3m* lines with induced plasmodesmal closure could help to determine how inducing plasmodesmal closure impacts other pattern-triggered immune responses.

The hypothesised trade-off between growth and defence could be framed as antagonistic, with growth processes competing with defence signalling to maintain plasmodesmal permeability. Alternatively, taking the perspective as proposed in a review criticising the “false idolatry” of the growth-defence trade-off by Kliebenstein (2016), this growth-defence trade-off could be considered as more of a coordination of plasmodesmal response to optimise the balance between growth and defence. Under changing conditions where different resources are limited this trade-off could be balanced differently in young sink leaves. Results showing the change in the regulation of plasmodesmal permeability occurring within 24 hours of inducing the sink-to-source transition support this argument for dynamic and responsive balancing of growth and defence. Additional experiments exploring plasmodesmal response in young sink leaves in other abiotic conditions such as high light or nutrient-limited soils could help to determine the balance of this

6.4. A trade-off between growth and defence?

trade-off within young leaves.

The regulation of plasmodesmal permeability in defence in roots may differ from young sink leaves, despite both being sink tissues. As discussed in Section 6.2 above, maintaining plasmodesmal permeability is important to root growth and potentially also to symplasmic phloem unloading. Previous research, however, found plasmodesmal closure is induced in response to flg22 and NaCl in root cell cortex cells (Gerlitz et al., 2018; Pérez-Sancho et al., 2023). I attempted to repeat this experiment using photoactivatable GFP movement in section 5.3.17; however, was unable to develop the assay with a suitable positive control. There could be biological reasons for the lack of plasmodesmal closure I observed in roots. Unlike previous studies, I did not include sucrose in the media the roots were grown on, which could have impacted the regulation of plasmodesmal permeability. Maybe the lack of sucrose in the media means more dependence on phloem unloading through plasmodesmata. However, there could have also been technical reasons for the lack of plasmodesmal closure. For example, my photoactivatable (PAGFP) GFP movement assay may not have been sensitive enough to detect changes in cell-to-cell mobility in roots.

If we assume my results are due to technical limitations, previous results show root tip cells do close plasmodesmata in response to flg22 (Pérez-Sancho et al., 2023). This could indicate the growth-defence trade-off is optimised differently in roots compared to sink leaves. Maybe defence is more strongly prioritised over growth, even in contexts where plasmodesmal closure reduces sugar import. Additionally, sterile plate-grown conditions of root growth may further impact the trade-off between growth and defence if roots have no prior exposure to pathogens. Further research on PAMP-triggered plasmodesmal closure in roots under different conditions could help to establish plasmodesmal PAMP-response in roots, and additional studies developing methods to study the regulation of plasmodesmata in other sink tissues, e.g. flowers and seeds could provide more insight on how the trade-off is optimised in different contexts.

Additionally, a strength of this growth-defence trade-off hypothesis is that it can account for the potential benefits of maintaining symplasmic connectivity in defence in sink tissue. Although the potential benefits of maintaining symplasmic connectivity in defence were not studied here, previous research indicates some innate defences against herbivores rely on symplasmic connectivity. For instance, the secondary metabolites glucosinolates are transported symplasmically from mature leaves into sink leaf tissue and into leaf margins where they accumulate

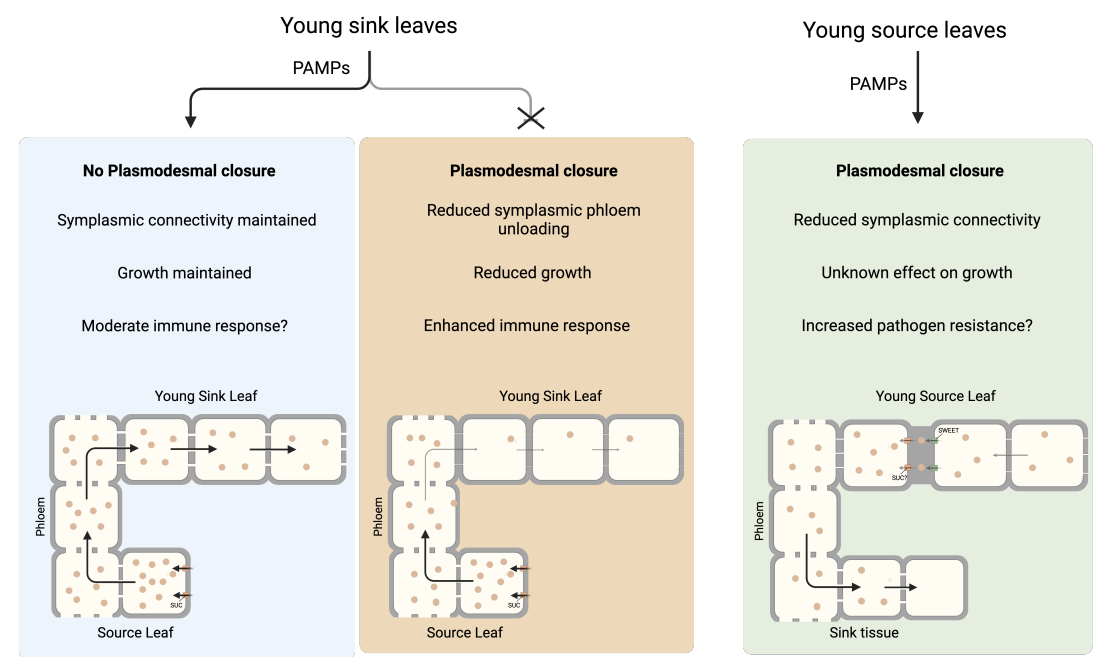


Figure 6.1: **Summary of plasmodesmal response to PAMPs in young leaves** – In young sink leaves, pathogen-associated molecular patterns (PAMPs) do not trigger plasmodesmal closure meaning symplasmic connectivity is maintained and sugars (indicated by the orange circles) can be unloaded from the phloem through the symplasm. Plasmodesmal closure is not induced by PAMPs in sink leaves as this reduces phloem unloading and growth, despite defensive benefits. In young source leaves plasmodesmal closure is triggered in response to PAMPs to reduce symplasmic connectivity. The impacts of PAMP-induced plasmodesmal closure on growth and pathogen resistance are unknown in young source leaves.

6.5. Mechanisms underpinning the change in plasmodesmal regulation

at high concentrations to defend young leaves against herbivores (Hunziker et al., 2021; Madsen et al., 2014). Herbivores then avoid consuming young leaves with high glucosinolate concentrations (Hunziker et al., 2021). It could be that similar symplastic transport of defensive compounds against fungal and bacterial pathogens in young leaves could provide defensive benefits in young leaves, especially if these compounds are energetically expensive to make. In summary, there could be both growth and defensive benefits of maintaining plasmodesmal permeability that outweighs the benefits of inducing plasmodesmal closure and sway the optimal balance of plasmodesmal regulation (Figure 6.1).

6.5 Mechanisms underpinning the change in plasmodesmal regulation

The differences in PAMP-triggered plasmodesmal response in sink and source tissues raise the question of the underlying mechanism driving this plasmodesmal response.

6.5.1 Plasmodesmal Structure

In Chapter 5, I investigated whether shifts in plasmodesmal structure across development could underpin changes to the plasmodesmal stress response. Previous studies have reported plasmodesmal structure shifts from simple to branched across the sink-to-source transition in *Arabidopsis* and various species from the *Nicotiana* genus (Crawford & Zambryski, 2001; Fitzgibbon et al., 2013; Oparka et al., 1999; Roberts et al., 2001). I hypothesised potential changes in plasmodesmal structure across the sink-source transition might link to changes in plasmodesmal PAMP response. My results, however, do not show a change in plasmodesmal structure after inducing the sink-to-source transition. This indicates the change in plasmodesmal regulation across the sink-to-source transition does not correlate with a change in plasmodesmal structure. The lack of correlation between PAMP response and structure is further supported by the finding that chitin-triggered plasmodesmal closure is induced in *cher1* mutant lines that only develop simple plasmodesmata. Overall, despite previous studies having found plasmodesmal structure changes with the induction of the sink-to-source transition in *Arabidopsis* (Fitzgibbon et al., 2013), I did not find evidence to support this and additionally

found no link between plasmodesmal structure and PAMP response.

6.5.2 Changes to PAMP-triggered signalling cascades

An alternative mechanism mediating differences in plasmodesmal regulation across the sink-to-source transition could be a change in the expression or activity of immune signalling machinery. The flg22, chitin, and salicylic acid-triggered plasmodesmal closure signalling pathways converge to trigger plasmodesmal closure through CALLOSE SYNTHASE 1 (CALS1) activity (Tee et al., 2023). A shared signalling component of these pathways may be differentially regulated in young and mature leaves. Analysis of the gene expression of known signalling components in young sink leaves compared to mature leaves in section 3.3.8 did not reveal any clear differentially expressed candidates. Differences in *NHL3* expression could be of potential interest, but baseline expression of *NHL3* is still high in young leaves so it is unclear if these expression differences have a significant impact on protein activity. The regulation of plasmodesmal permeability through *NHL3* expression is thus unconvincing and overall baseline expression of signalling machinery in young leaves appears similar to mature leaves and young source leaves.

In the absence of major transcriptional differences, post-translational differences in protein activity or localisation of components of the signalling pathway could lead to changes in plasmodesmal response. Callose synthase activity, for instance, could be repressed by an unknown sink-related regulator in young tissue. Callose deposition in mature leaves is mediated through CALS1 activity and is triggered by flg22, chitin and salicylic acid-mediated signalling, whilst H₂O₂-induced plasmodesmal closure requires CALS8 (Cui & Lee, 2016; Tee et al., 2023; Wang et al., 2013). Inhibition of the activity of these two callose synthases may prevent PAMP-triggered plasmodesmal closure in young leaves. This inhibition must be specific to CALS1 and CALS8 as plasmodesmal closure can be induced in young sink leaves through the induction of a *cals3* allele in *LexA::icals3m* lines and macroscopic callose deposition seen in young leaves is controlled by CALS12 activity (Luna et al., 2011; Nishimura et al., 2003; Vogel et al., 2007). Alternatively, instead of callose synthase activity being reduced, it could be that the activity of β -glucanases which are associated with callose degradation at plasmodesmata is increased in young leaves (Benitez-Alfonso et al., 2013; Levy et al., 2007). To further test my hypothesis on the role of callose synthases, I need to establish whether the known signalling components of PAMP-triggered plasmodesmal closure upstream

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of CALS1 or CALS8 are all functional. I could also establish whether subcellular localisations and activity of these callose synthases are the same in young and mature leaves, and search for potential regulators of callose synthase activity by screening for CALS1 interactors in young leaves using co-immunoprecipitation.

Although there is currently no clear candidate for an altered signalling mechanism between young and mature leaves, further experimental work could help to determine and characterise whether there is an altered regulation of components of the signalling pathway.

6.5.3 Changes to phytohormone crosstalk across the sink to source transition

Phytohormone crosstalk is already established as a mechanism for regulating growth and defence. It could be that this phytohormone crosstalk feeds into the regulation of plasmodesmal response in young and mature tissues. For instance, jasmonates (JA) are important in the coordination of growth and defence. JA accumulates during defence responses to inhibit growth, whilst active growth inhibits JA accumulation (Campos et al., 2014; Zhang & Turner, 2008). Across my three results chapters, I identified JA-related GO-terms enriched in response to chitin in mature leaves (Chapter 3); after the induction of plasmodesmal closure in young leaves of *LexA::icals3m* lines (Chapter 4); and again in mature leaves in response to chitin treatment (Chapter 5). Although this suggests jasmonic acid is upregulated as part of a plasmodesmal closure/chitin response, it is unclear whether jasmonic acid feeds into the signalling control of the initial induction of plasmodesmal closure. Additionally, no characterised jasmonic acid-related genes are differentially expressed in the young sink and young source tissue (Chapter 5), making it unclear how jasmonic acid signalling could mediate changes to plasmodesmal regulation unless there are undetected non-transcriptional changes.

Phytohormone crosstalk between salicylic acid and abscisic acid signalling has previously been found to regulate an age-specific trade-off between abiotic stress and growth (Berens et al., 2019). However, neither salicylic acid nor abscisic acid directly triggers plasmodesmal closure in young sink leaves, which suggests increasing phytohormone concentrations does not induce closure. Thus if salicylic acid or abscisic acid is involved in plasmodesmal regulation, it is unlikely to be related to increases in concentration across development.

Overall I found little evidence to support a role for phytohormones in regulating the change in plasmodesmal responses across the sink-to-source transition, despite their previous association with balancing growth and defence.

6.5.4 Changes to sugar availability and signalling across the sink-to-source transition

Physiological changes during the sink-to-source transition could regulate the shift in plasmodesmal PAMP response. One hypothesis is that changes in substrate availability for callose synthesis result in changes to plasmodesmal response. Callose synthases are suggested to form complexes with sucrose synthases to catalyse the formation of UDP-glucose, which is then supplied as a substrate to callose synthases (Amor et al., 1995; Brownfield et al., 2007; Verma & Hong, 2001). It could be that carbon is partitioned towards growth in young sink leaves, leaving a limited supply of sucrose or UDP-glucose for plasmodesmal callose synthesis. The main flaw in this hypothesis is that PAMP-triggered macroscopic callose deposition occurs in young leaves, indicating there is a supply of sugars for other callose synthesis processes in the cell wall, even if plasmodesmal callose is not synthesised. A possible explanation could be if the callose synthase enzymes are competitive for substrate and if CALS12 had a higher affinity for UDP-glucose or a more active sucrose synthase partner than CALS1 or CALS8, meaning that substrate supply was limited macroscopic callose deposition would be prioritised. There is, however, no evidence for differences in enzyme activity of different callose synthases or sucrose synthases. Additionally, glucose concentrations do not differ between young sink and source leaves, indicating pools of available glucose and sucrose which could be used if callose synthesis remains unchanged. A more precise method of calculating glucose and UDP-glucose, such as mass spectrometry could reveal more subtle differences in substrate accumulation not detected by my glucose extraction method. Overall, the hypothesis for changes in sugar availability in young leaves is undermined by the PAMP-induced increase in macroscopic callose deposition in young leaves.

Alternatively, there could be other signalling mechanisms influencing plasmodesmal PAMP response related to physiological changes during the sink-to-source transition. Young source leaves have an increase in the operating efficiency of Φ PSII relative to young sink leaves (Chapter 5). Although I am unable to directly relate this to increased carbon assimilation it does raise the question of whether

this increased efficiency of Φ PSII triggers other chloroplast-derived signals that could link to plasmodesmal regulation. One possibility is that chloroplast ATP and NADPH production are increased during the sink-to-source transition which could link to changes in plasmodesmal permeability. This hypothesis is based on evidence from Dmitrieva et al. (2021) showing that decreases in plasmodesmal permeability in several *Arabidopsis* chloroplast mutants could be related to decreased chloroplast ATP and NADPH production and other unidentified chloroplast-related signals. Brunkard et al. (2020) also suggest that ATP synthesis relates to plasmodesmal transport in the TARGET OF RAPAMYCIN - glucose signalling axis to regulate plasmodesmal permeability. If the sink-to-source transition altered the production of ATP or other chloroplast-related signalling molecules it could feed into regulation of plasmodesmal transport. Further exploration of plasmodesmal regulation in response to PAMPs in young and mature leaves in chloroplast and TOR signalling mutants could help to determine if these mutants have changes to plasmodesmal permeability in response to PAMPs.

Overall, I have limited evidence to suggest physiological changes associated with the sink-to-source transition could be leading to changes in plasmodesmal closure. However, given existing research linking plastid signalling and plasmodesmal permeability, it is possible that this signalling also extends to regulating a dynamic plasmodesmal response in young sink tissue. One major challenge with identifying these signalling components is that the data collected in this thesis has focused on downstream immune signalling or physiological outcomes (i.e plasmodesmal permeability, phloem unloading, starch accumulation, callose deposition, ROS bursts) or transcriptomic data. More research on the activity of PAMP-triggered signalling pathways and potential factors regulating these pathways may offer more insights into a mechanism for how plasmodesmal-PAMP response changes across the sink-to-source transition.

6.6 Conclusion

Overall, the results from my thesis suggest plasmodesmal permeability in response to PAMPs is regulated to optimise the balance between growth and defence in leaf tissue. These results highlight how the importance of maintaining symplasmic connectivity varies across development, whilst different defensive strategies might be implemented in different contexts. The mechanism underlying these changes in plasmodesmal PAMP responses remains unclear. Potential mechanisms include

the regulation of callose synthases, chloroplast signalling or changes in signalling machinery. Further research exploring the signalling changes which occur upon the induction of the sink-to-source transition could elucidate the mechanisms underlying this change in plasmodesmal regulation.

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