

**Investigating the chloroplast-to-nucleus signaling that regulates plasmodesmata-
mediated intercellular trafficking in plants**

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Doctor of Philosophy

Degree

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DEDICATION

I would like to dedicate my dissertation to my father, Abdul Awal and my mother,

Taslima Akter

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First and foremost, I want to express my heartfelt thanks to my wife, Tasnim Rahman. Your unwavering love, patience, and support have been my anchor throughout this journey. Even in my toughest moments, you believed in me and reminded me of my purpose on this path.

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ABSTRACT

Intercellular communication via plasmodesmata (PD) is important for plant growth, development, and defense, yet its regulation remains poorly understood. Chloroplasts communicate information about the environment and the physiological state of the plant cell to the nucleus. Chloroplast-generated signals may change the expression of PD-related nuclear genes to regulate the trafficking of photosynthetic products and metabolites along with other molecules that act non-cell autonomously. We aim to identify the chloroplast retrograde signals that regulate intercellular trafficking via PD. Here, we show that tetrapyrroles, likely heme, regulate some aspects of PD-mediated intercellular trafficking. This regulation is not dependent on light. In addition to revealing the potential role of tetrapyrroles in regulating intercellular trafficking, we also revealed that the glucosinolates, a group of specialized metabolites best known for their roles in antimicrobial defense in select plant families, can act as signals to regulate PD-mediated intercellular communication. In contrast to previous reports, we did not identify any correlation between PD-mediated intercellular trafficking and the level of reactive oxygen species (ROS), the redox status of the chloroplast, or plastoquinone redox status. In a related study, we performed differential gene expression analyses and Gene Ontology (GO) analyses and then created protein-protein-interaction networks using differentially expressed genes (DEGs) encoding proteins. Understanding how tetrapyrroles, and in the broader sense chloroplast-to-nucleus retrograde signaling, control trafficking through PD could enable the engineering of plants to optimize carbon partitioning to various parts of the plant for higher yield or to limit pathogen spread. *In*

toto, the studies presented in this thesis have advanced our understanding of the cellular players that regulate the movement of molecules and signals between plant cells, an indispensable portion of the cell-to-cell communication that the coordinated growth, development and environmental responses that are characteristic of and necessary for multicellularity.

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CHAPTER 1

INTRODUCTION TO THE CHLOROPLAST-TO-NUCLEUS RETROGRADE SIGNALING INVOLVED IN REGULATING INTERCELLULAR TRAFFICKING VIA PLASMODESMATA IN PLANTS

I. Organelles-Nucleus-Plasmodesmata Signaling (ONPS): an update on its roles in plant physiology, metabolism and stress responses

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Mohammad was the first author of the paper and contributed to compiling the literature and preparing the manuscript.

ABSTRACT

Plasmodesmata allow movement of metabolites and signaling molecules between plant cells and are therefore critical players in plant development and physiology, and in responding to environmental signals and stresses. There is emerging evidence that plasmodesmata are controlled by signaling originating from other organelles, primarily the chloroplasts and mitochondria. These signals act in the nucleus to alter expression of genetic pathways that control both trafficking via plasmodesmata and the plasmodesmatal pores themselves. This control circuit was dubbed organelle-nucleus-plasmodesmata signaling (ONPS). Here we discuss how ONPS arose during plant evolution and highlight the discovery of an ONPS-like module for regulating stomata. We also consider recent findings that illuminate details of the ONPS circuit and its roles in plant physiology, metabolism and defense.

INTRODUCTION

Plasmodesmata (PD) are intercellular membrane-lined cytoplasmic pores that are essential for plant growth, development, and defense (1–3). PD, while being distinct cellular structures subject to their own regulatory processes, are actually comprised of an amalgam of other cellular components: the plasma membrane (PM) delimits the outer boundaries of the pore, the central desmotubule is formed by an appressed endoplasmic reticulum (ER) strand that is continuous between the connected cells, and the cytoplasmic continuum that is the main route for the trafficking of soluble molecules between cells (3). Subtending all this are the cell walls of the connected cells that surround each pore.

Identification of plasmodesmal proteins (4,5) and characterization of the lipid components of PD membranes (6) have contributed significantly to advancing our understanding of PD and how they function to not only traffic solutes like sugars and signaling molecules including hormones, RNA and proteins, but also to participate in signaling modules that mediate responses to the environment including during pathogen defense (3).

Despite their importance to plant survival and their myriad functions, PD remain among the least understood structures in plants. PD that form at cell division when strands of the ER are sheathed in the nascent cell wall are primary PD. PD may also form across existing walls in the absence of cell division, and these are called secondary PD. Reflecting their importance to plant function, PD structure and function vary temporally and spatially during plant development, as exemplified by changes to PD during the sink-to-source transition and in response to tissue differentiation (7). There is accumulating

evidence that specialization of PD structure is requisite for specialization of PD function. One recent example comes from the PD in the root pole pericycle that adopt a ‘funnel’ structure that facilitates phloem unloading (8) .

The best characterized mechanism for regulating PD trafficking in response to developmental and environmental signals is the controlled deposition and/or removal of callose, a β -1, 3 glucan, in the cell wall surrounding the pore (9). Callose accumulation effectively constricts the pore, inhibiting intercellular trafficking. During normal development callose is often deposited at PD when it is necessary to sequester a subset of cells from their neighbors to allow the execution of a specialized development program (10–12). Several proteins responsible for callose accumulation, callose synthases/glucan synthases (Cals/GSLs), have been identified and CalS1, 8 and 10 specifically deposit callose at PD (13). Removal of callose is mediated by β -1,3-glucanases, and several localize to PD(14). Perhaps the best studied of these is β -1,3-glucanase putative plasmodesmal associated protein (BG-ppap) that constitutively localizes to PD and mediates callose dynamics during various stresses including virus infection (15). There are other mechanisms for regulating PD function besides callose. The plant cytoskeleton components actin and myosin can exert influence over PD permeability (16–18). Phosphorylation of viral proteins and other non-cell-autonomous proteins can also regulate their transit through PD (19), raising the possibility that PD-resident kinases may also modulate intercellular flux (20,21). Finally, we and others have discovered a new role for other organelles, particularly chloroplasts and mitochondria, as critical regulators of PD biogenesis and function (22–24). Here we discuss the model we have developed

for organelle regulation of PD, dubbed organelle-nucleus-PD signaling (ONPS). We frame our discussion in the context of how such a system could have evolved, and then consider new evidence that supports the existence of the ONPS module. We also highlight areas for future research expenditure that would not only help test the ONPS hypothesis but could also greatly advance our understanding of the essential PD.

Developing the ONPS Hypothesis

Analyses of mutants with defects in intercellular trafficking led to the hypothesis that chloroplasts control PD formation and function. Early work in maize (*Zea mays*) identified the *sxd1* mutant which showed defects in exporting sucrose from the leaf. In this mutant, PD at the interface of bundle sheath (BS) and vascular parenchyma (VP) cells are blocked by callose deposition (25,26). *SXD1* encodes a chloroplast-localized tocopherol cyclase (*VTE1* homolog) (27), and expression of photosynthesis associated nuclear genes (PhANGs) was disrupted in the *sxd1* mutant (26). Thus, studies with *sxd1* were the first hint that PD development and function were tied to chloroplast signaling pathways.

Mutations that cause defects in PD and more general intercellular trafficking are expected to result in lethality, an assumption borne out by a dearth of such mutants. A genetic screen of embryonic lethal *Arabidopsis thaliana* mutants in the Zambryski lab identified 14 mutants with defects in intercellular trafficking of a 10-kDa fluorescently labeled dextran (28). Subsequent mapping and cloning identified two of these mutants, *ise1* and *ise2*, as encoding mitochondrial and chloroplast RNA helicases, respectively (22,29,30). A separate screen for altered intercellular trafficking in seedling-lethal

Arabidopsis mutants by the Jackson lab identified the *gat* series of mutants (23). *GAT1* encodes a chloroplast thioredoxin m3, and loss of GAT1 led to changes in chloroplast redox status and decreased intercellular trafficking. Other *gat* mutants also show defects in chloroplast oxidative states (31) .

Study of gene expression in the mitochondrial *ise1* and chloroplast *ise2* mutants revealed profound alterations in the patterns of expression of chloroplast-related genes, leading to the hypothesis that the observed changes in PD function and intercellular trafficking was caused by defective chloroplast function (22). We interpreted these findings from *ise1* to mean that defective mitochondria led to either altered mitochondrial-to-nucleus retrograde signaling that impinged on PhANG expression, or that defective mitochondria directly or indirectly perturbed chloroplast development and thence chloroplast-to-nucleus retrograde signaling to disrupt expression of PD associated nuclear genes (PDANGs). Indeed, crosstalk between chloroplast and mitochondrial retrograde signaling is well described (32). These findings from *ise1* and *ise2*, supported by the independent identification of the maize *sxd1* and Arabidopsis *gat* mutants, led to the hypothesis articulated as organelle-nucleus-PD signaling (ONPS, (11,22)).

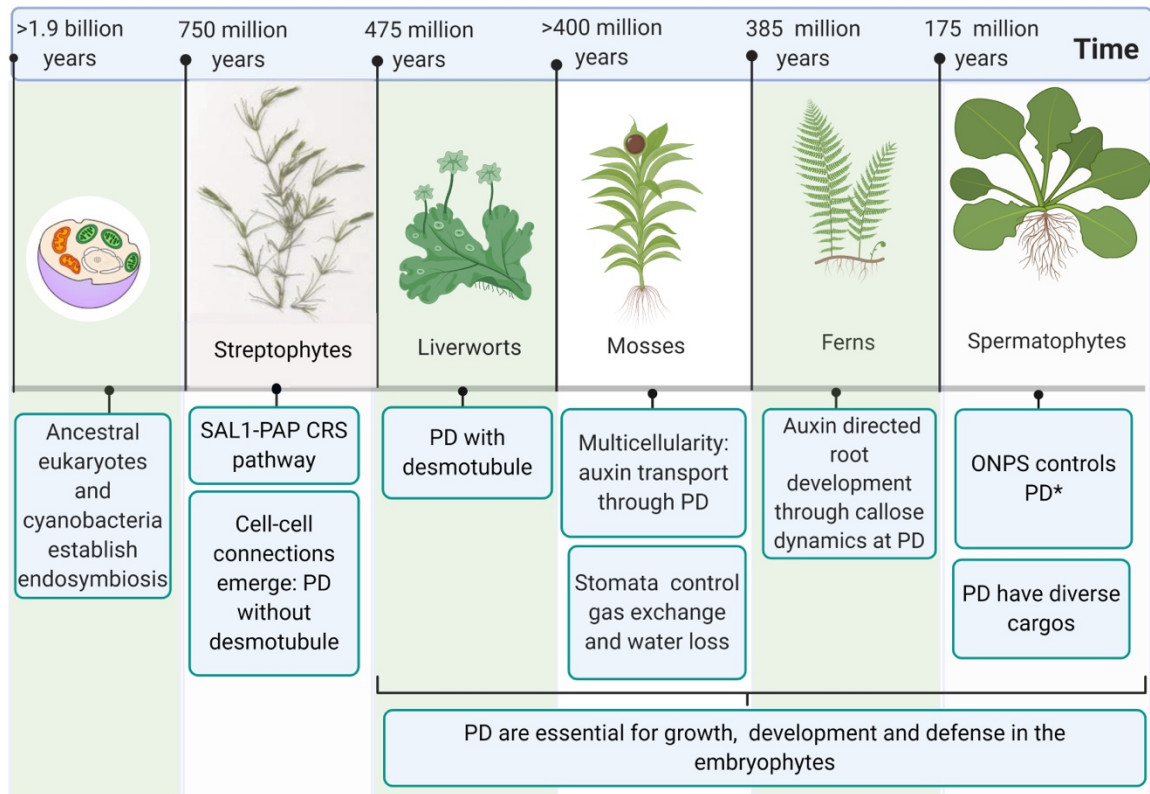
Plant evolution and intercellular trafficking

The establishment of the symbiotic relationship between cyanobacteria and the eukaryote host that would give rise to the plant lineage occurred at least 1.9 billion years ago, according to recent estimates (33), (**Fig. 1**). These early photosynthetic eukaryotes then gave rise to several algal lineages including the ancestors of land plants. How multicellular plants evolved from unicellular ancestors continues to be an important

question in understanding the processes of plant evolution and development. Recently, the role of PD in the evolution of multicellularity has been evaluated and we refer readers to these excellent discussions (34,35). Of note is the work of Newman, Niklas and colleagues who have conceived of dynamical patterning modules that they posit were critical to the evolution of multicellularity (36,37). Dynamical patterning modules theory positions intercellular trafficking and PD as central to the emergence of complex plant body plans through their contributions to cell, tissue and organ polarity and differentiation (35). They argue that the presence of intercellular connections is necessary for cell-type specification, a likely prerequisite for multicellularity. Auxin, like PD, is universally deployed in land plants for development and has its origins in the ancestors of land plants (38). Benitez and coworkers speculate that PD may have been crucial for establishing auxin as a central player in regulating plant development (35). While the transport of auxin by specialized protein carriers is well documented and extensively experimentally supported, there is strong emerging evidence that PD are also critical for establishing auxin gradients. First, in moss, auxin is transported exclusively through PD (39). Second, the importance of callose dynamics at PD is now known to be indispensable for auxin-directed root development (40). Third, mathematical modeling of auxin distribution in both the root tip and in leaves reveals that diffusive movement through PD is required (41,42). Another important finding was that auxin and the highly evolutionarily conserved metabolite/ chloroplast retrograde signal methylerythritol cyclodiphosphate (MEcPP) interact with each other, such that MEcPP modulates auxin

Figure 1. Evolution of PD and intercellular trafficking during land plant evolution.

More than 1.9 billion years ago an ancestral eukaryote established an endosymbiotic association with a photosynthetic cyanobacterium that would evolve into the chloroplast. The SAL1-PAP CRS module evolved in the streptophytes, a group that evolved around 750 million years ago (Mya) and includes the closest algal relatives of extant land plants. Intercellular pores lacking ER but resembling PD evolve in some streptophyte algal lineages. Between 500 and 450 Mya plants moved onto land and these ancestral land plants contained PD that incorporated encased ER, or the desmotubule. PD are found in all embryophytes where they are essential for plant survival. In the mosses, about 400 Mya, the body plan became more complex as auxin gradients were established via PD-mediated trafficking. Also in the mosses, stomata emerged, and they may use SAL1-PAP CRS as a second messenger for regulating these pores and water loss in a dry environment. In the ferns, callose deposition and removal at PD establish auxin gradients for root development. By the time spermatophytes arose, about 175 Mya, PD had evolved for trafficking of numerous types of cargo that allow coordinated responses to myriad intrinsic and extrinsic stimuli. While ONPS operates in spermatophytes the involvement of ONPS in other embryophytes has not yet been investigated.



homeostasis (43). These exciting discoveries linking auxin to PD and chloroplast retrograde signaling (CRS) support the coevolution of ancient systems to mediate major events in plant development, and support the concept that regulation of intercellular trafficking could indeed be controlled by other ancient plant components like the chloroplasts and mitochondria. Further support for the coupling of critical plant features in the evolution and establishment of land plants comes from recent discoveries regarding stomatal regulation. Stomata opening and closure are regulated by abscisic acid and involves the nucleotidase/phosphatase (SAL1)- phosphoadenosine-5-phosphate (PAP) CRS pathway as a second messenger pathway (44). Stomata evolved more than 400 million years ago as plants moved onto land, allowing them a means to control gas exchange and water loss (**Fig. 1**). Recent evolutionary analysis coupled with functional characterization revealed the ancient origins of the SAL1-PAP CRS pathway in streptophyte algae, a sister clade to modern land plants (45), (**Fig. 1**). The authors of this work speculated that the relationship between chloroplasts and stomata is ancient and essential for adaptation to land. We therefore posit that a similar relationship between chloroplasts and mitochondria and PD evolved during the development of multicellularity in plants. The validity of this hypothesis will only come from identifying the retrograde signaling pathways and PDANGs of ONPS.

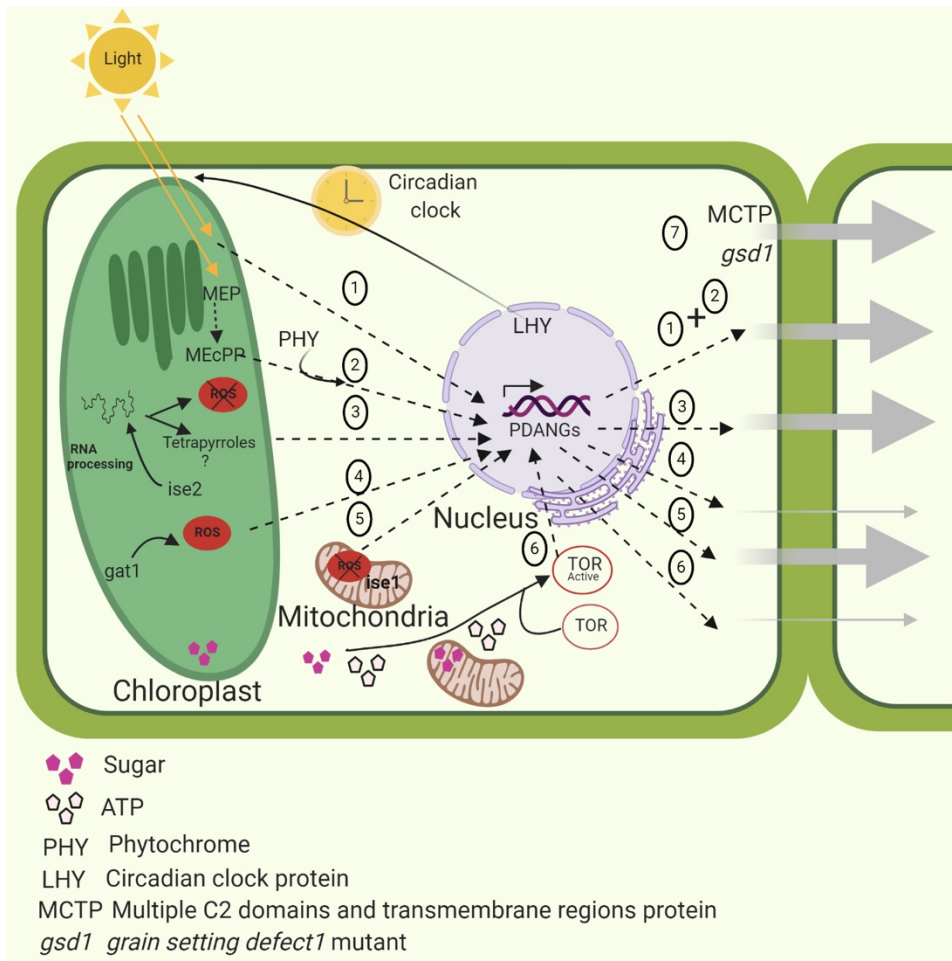
Chloroplast retrograde signaling in ONPS

Recent efforts have focused on elucidating the mechanism of ONPS. Knockdown of the gene encoding the chloroplast ribosomal protein uL15c led to defective chloroplast translation and increased PD-mediated trafficking (46), supporting previous observations

that chloroplast translation impinged on PD function (47). In a more comprehensive approach to understanding the relationship between chloroplast signaling and PD function, several nuclear genes encoding chloroplast RNA-processing proteins (*RH39*, *RH22*, *RH3*, *ISE2*, *IPII*, *RNaseJ*, *PNPase*) were silenced in *Nicotiana benthamiana* (**Fig. 2**). Reduced expression of *RH22* and one of the two *N. benthamiana* *PNPase* homologs, *PNPaseA*, resulted in increased intercellular trafficking of GFP(48). In contrast, knockdown of *RNaseJ*, *IPII*, *RH3* or both *PNPase* homeologs led to decreased intercellular trafficking. One important finding from this study is that there is no correlation between leaf chlorosis and intercellular trafficking, as plants with similar levels of chlorosis can display dramatic differences in PD flux, as illustrated by silencing different *PNPase* homeologs (48). Expression analysis of PhANGs revealed unique patterns of altered gene expression in each plant, suggesting that there may be unique signaling signatures generated by distinct defects in chloroplast RNA processing. This collection of silenced plants can be utilized for further studies to identify and characterize CRS pathways in ONPS. Another important observation made in this study was the positive correlation between PD and the extent of intercellular trafficking (48,49). This simple relationship seems intuitive and had been suggested by earlier observations (49). However, other experimental evidence argues against this positive correlation. One prominent example is the reduced trafficking in source tissues with elaborately branched PD compared with the extensive trafficking in sink tissues with simple, single pores (50). It is possible that PD structure per se is not the sole determinant of PD permeability, and that other factors like whether PD are primary (formed during cytokinesis) or secondary

Figure 2. ONPS in plant physiology and metabolism.

(1) Intercellular trafficking is higher in the day than night, and this is under circadian control (LHY) but likely does not involve callose metabolism. (2) Light regulation of PD may also involve of the MEcPP CRS pathway and the phytochrome (PHY) photoreceptors. (3) Defects in chloroplast processing, exemplified by the *ise2* mutation, leads to CRS possibly via tetrapyrroles and other pathways and increases PD formation and intercellular trafficking. Other RNA processing mutants can decrease intercellular trafficking. (4) The *gat1* mutation oxidizes the chloroplasts by increasing ROS production, and these ROS may themselves act as CRS or trigger other CRS pathways to decrease intercellular trafficking. (5) The *ise1* mutation leads to more reduced mitochondria and generates signals that trigger CRS or MRR that change PDANG expression to increase intercellular trafficking. (6) The balance of ATP and sugars from the chloroplasts and mitochondria activate the TOR kinase, and under conditions of abundance this leads to decreased intercellular trafficking. (7) An MCTP protein localized to PD is important for PD formation, and controlling intercellular trafficking. GSD1 also localizes to PD and the *gsd1* mutant shows increased intercellular trafficking.



(post-cytokinetic) also determine trafficking capacity.

Chloroplast and mitochondrial redox status correlate with PD function. Decreased *ISE2* expression led to reduced chloroplasts, and decreased *ISE1* expression resulted in oxidized mitochondria and reduced chloroplasts compared to wild-type chloroplasts (51), (**Fig. 2**). Similar effects on PD function were found upon treatment with chemicals inducing production of reactive oxygen species (ROS) in either chloroplasts or mitochondria. Several *gat* mutants also have altered chloroplast redox states (52). Somewhat discordant with these findings, the barley (*Hordeus vulgare*) and *Arabidopsis chlorina* mutants lacking *chlorophyllide-a-oxygenase (cao)* and chlorophyll b have increased chloroplast ROS levels, increased intercellular trafficking and enhanced PD formation with more PD connecting leaf mesophyll cells or the L1 layer of the shoot apical meristem(53) Thus, different ROS in varying quantities and combinations could act as signals to control PD formation and function (54) , via retrograde signaling or as direct signals to the PD, as may happen with the callose-modulating protein, PDL5 (24). However, the situation with chloroplast oxidation may not be straightforward as ROS signaling since changes in NADPH and ATP production are associated with ROS production. Silencing expression of a subunit of the chloroplast ATPase, *AtpC*, by VIGS resulted in increased cell-to-cell movement of GFP (55) and also plant viruses (56). Further critical examination of ATP and NADPH metabolism in mutants already known to have defects in PD will help clarify the importance of ROS in ONPS and may identify new components of ONPS.

ONPS in plant physiology and metabolism

At the core of ONPS is the idea that the balance between carbon supply from photosynthesis and PD development/function is regulated by chloroplasts. For carbon partitioning in a plant to be effective carbon fixation and transport must be coordinated (57,58). The cell-to-cell trafficking of this fixed carbon is largely via PD, and when PD function is perturbed or insufficient carbohydrates may accumulate in leaves, leading to feed-back inhibition of photosynthesis, as described in ‘**Developing the ONPS Hypothesis**’ for the *sxd1* mutant. On the other hand, if too great a proportion of a plant’s carbon resources is allocated to transport structures, carbon use efficiency is compromised. Both scenarios can lead to adverse outcomes in plant growth, development and yield (59,60). Besides *sxd1*, four other maize mutants with defects in carbohydrate partitioning have been identified. In the *tie-dyed(tdy)1* (61) and *tdy2* mutants (61), and the *carbohydrate partitioning defective(cpd)1* (62) and *cpd33* (63) mutants sucrose export to the phloem is reduced. The carbohydrate export defect was due to callose accumulation at PD at the mesophyll-phloem interface(64) or at sieve pores in the phloem (*cpd1*), or by defects in PD formation (*cpd33*) (63), although no obvious defects in PD were observed in the *tdy* mutants (61).

Callose dynamics at the PD are not the only means for controlling carbohydrate partitioning. *CPD33* encodes a Multiple C2 domains and transmembrane region (MCTP) protein (63). MCTPs are broadly distributed across eukaryotes and tether the ER to the PM. In Arabidopsis, MCTPs are also found to localize to PD and to control intercellular trafficking (65). PM proteins like the MCTPs may therefore be important for PD

formation, as revealed by the reduced numbers of PD in *cpd33* (61), (**Fig. 2**). Phloem loading was defective in the rice (*Oryza sativa*) *grain setting defect1-Dominant* (*gsd1-D*) mutant (66). *GSD1* encodes a putative Remorin protein that localizes to the plasma membrane and PD of phloem companion cells, and correct localization depends on S-acylation (67). GSD1 interacts with actin (OsACT1) at the PD to negatively regulate PD permeability (**Fig. 2**). Another rice mutant, *sugar accumulation 1* (*ossac1*) mutant has impaired sugar partitioning (68). *OsSAC1* was expressed at the bottoms of young leaves and in the developing leaf sheath and OsSAC1 localizes to the ER. Interestingly, OsGSD1 was detected in young leaves and developing leaf sheaths of the *ossac1* mutant. This suggests that OsSAC1 and GSD1 may act together to regulate sugar partitioning in young leaves and developing leaf sheaths. These examples highlight the interplay between PD and photosynthesis in carbon partitioning, and these mutants may be used for further examination of the mechanisms of ONPS.

Exciting recent data regarding the TARGET OF RAPAMYCIN (TOR) kinase signaling in plants further support that ONPS contributes to carbon partitioning. The TOR kinase complex acts by regulating mRNA transcription and translation in response to external signals and nutrient availability to promote development under favorable growth conditions, and its role extends throughout a plant's life (69–71). The same genetic screen that identified *ise1* and *ise2* (64), also identified *ise3* and *ise4*, later cloned and determined to be alleles of *At2g25570* and *At5g67630*, respectively (72). *At5g67630* encodes Reptin, a chaperone that is a component of the R2TP ATPase complex that contributes to TOR activation (73,74). Through a comprehensive approach involving

forward and reverse genetics as well as drug treatments, Brunkard and coworkers demonstrate that active TOR signaling negatively regulates intercellular trafficking (73). In addition, through use of chemical inhibitors, TOR signaling was revealed to promote the sink-to-source transition in leaves. TOR is significantly more active in mature leaves that produce excess sugars than in young growing leaves that must import sugars, and this increase in TOR activity in mature leaves correlates with decreased rates of PD transport (**Fig. 2**). Another interesting finding from this study was that the mitochondrial SEL1-like repeat containing (SLR) protein encoded by *At2g25570/ise3*, putatively involved in ATP synthesis during oxidative phosphorylation, is necessary for TOR activity. This supports the involvement of mitochondria in regulating PD-mediated intercellular signaling, previously described for *ise1* (30), (**Fig. 2**). Comparative gene expression analysis in the *ise3*, *ise4* and characterized *tor* mutants confirmed that defects in TOR signaling pathways were the primary defect in all the mutants. But an intriguing observation was that while changes in transcript levels of PDANGs were observed, the patterns of expression could not always be correlated with observed changes in intercellular trafficking, particularly as related to callose metabolism. This observation highlights not only the probable involvement of mechanisms besides callose in regulating PD during development, but there likely remain many undiscovered PDANGs.

Light has a profound effect on plant growth and development, and it is therefore not unexpected that light can modulate PD function and symplastic transport. Surprisingly, there are few studies that directly examine the effects of light on PD. Early work reported that white-light irradiation inhibited lateral transport from the stele into the

cortex in etiolated maize mesocotyls, suggesting that as chloroplast biogenesis initiated, PD trafficking was decreased (75). In contrast, at high light intensities, several species of C4 grasses showed higher photosynthetic efficiency along with higher PD frequency at Kranz mesophyll/bundle sheath interface in leaves(76). An important recent study directly addressed the effects of light on PD trafficking in plants. Using differing photoperiods for growth and a minimally invasive GFP movement assay in *N. benthamiana* leaves, Brunkard and Zambryski found that PD trafficking is higher in the day than night (55). This increased daytime trafficking was not only dependent on the light but also involved the circadian clock as revealed by knockdown of the clock component *LHY* (55), (**Fig. 2**). An interesting observation of this study was that differences in PD trafficking between day and night were not mediated by callose dynamics, suggesting that the situation is not always as clear cut as described in the literature, where callose deposition limits PD permeability (77). These findings are consistent with unexpected new findings about the roles of callose in modulating the properties of the cell wall. Addition of callose to cellulose hydrogels *in vitro* increased the elasticity of cellulose, a change that would not be expected to constrict PD. Future studies examining callose-cellulose interactions and their interactions with other cell wall components as well as their effects on plant cells should bring novel insight into PD function and how callose regulates PD. Also noteworthy are recent studies demonstrating that chloroplasts use the MEcPP CRS pathway to influence light detection by the phytochrome photoreceptors (78,79), (**Fig. 2**), and light regulates expression of

genes encoding the MEP biosynthesis pathway (80). The role of the MEcPP CRS pathway in mediating PD flux in response to light remains to be examined.

ONPS in response to biotic and abiotic stress

PD have important roles in mediating plant responses to biotic and abiotic stresses. Plants encounter multiple biotic stresses and have acquired adaptive tools of defense to respond to them. Chloroplasts are critical players in plant responses to biotic challenges, and include production of both defense molecules and chemicals that attract beneficial microbes and animals as reviewed (81–83). Salicylic acid (SA), jasmonic acid (JA) and ABA are phytohormones that are partially synthesized in the chloroplast and act as defense hormones. Further, CRS components are important for defense responses to pathogens. An *Arabidopsis cehl* mutant that accumulates MEcPP constitutively produces SA and constitutively expresses JA-responsive genes in an MEcPP-dependent manner (84). In contrast, in *sal1* mutants, SA- and JA-mediated defense responses were defective, leading to increased susceptibility to pathogen infection (85).

Besides launching local defense programs in the cells encountering an invading microbe, JA and SA are also important for systemic defense responses. The role of SA in systemic acquired resistance (SAR) has been controversial, but recent results provide very strong evidence supporting its importance to SAR (86). In addition to SA, chloroplasts also produce the signaling molecules azelaic acid (AzA) and glycerol-3-phosphate (G3P) in response to pathogenic attack to mediate SAR (87). AzA functions upstream of G3P and AzA-G3P branch functions in parallel to SA-derived signaling during SAR (88). AzA and G3P move cell-cell via PD through the action of the lipid

transfer proteins, AZI1 and DIR1, while SA moves via the apoplast in the cuticle under the control of transpiration (86,87) (**Fig. 3**). Interestingly, SA stimulates callose deposition at PD through the action of PD-LOCATED PROTEINs PDL1 and PDL5 to negatively regulate PD trafficking during pathogen infection (89), (**Fig. 3**). PDL1 and PDL5 are essential for SAR as well as the stability of AZI1, which is required for AzA- and G3P-induced SAR (87) Loss of either PDL1 or PDL5 increases chloroplast localization of AZI1, similar to pathogen infection (90), suggesting that plastidial localization or partitioning of AZI1 between the plastid and cytoplasm may be important for SAR. However, in a seeming paradox, treatment of Arabidopsis leaves with exogenous SA induced PD formation. In those leaves, the rate of complex PD formation is increased, although the final number of PD formed was the same as in control leaves (91). These studies highlight the interplay between chloroplasts and PD in mediating defense responses to biotic stresses, and further experiments could clarify the role of CRS and ONPS in these responses. While PD are important for systemic signaling in defense, studies with the PD-localized LYM2 chitin receptor reveal that PD can also act locally to respond to pathogens (92) (**Fig. 3**).

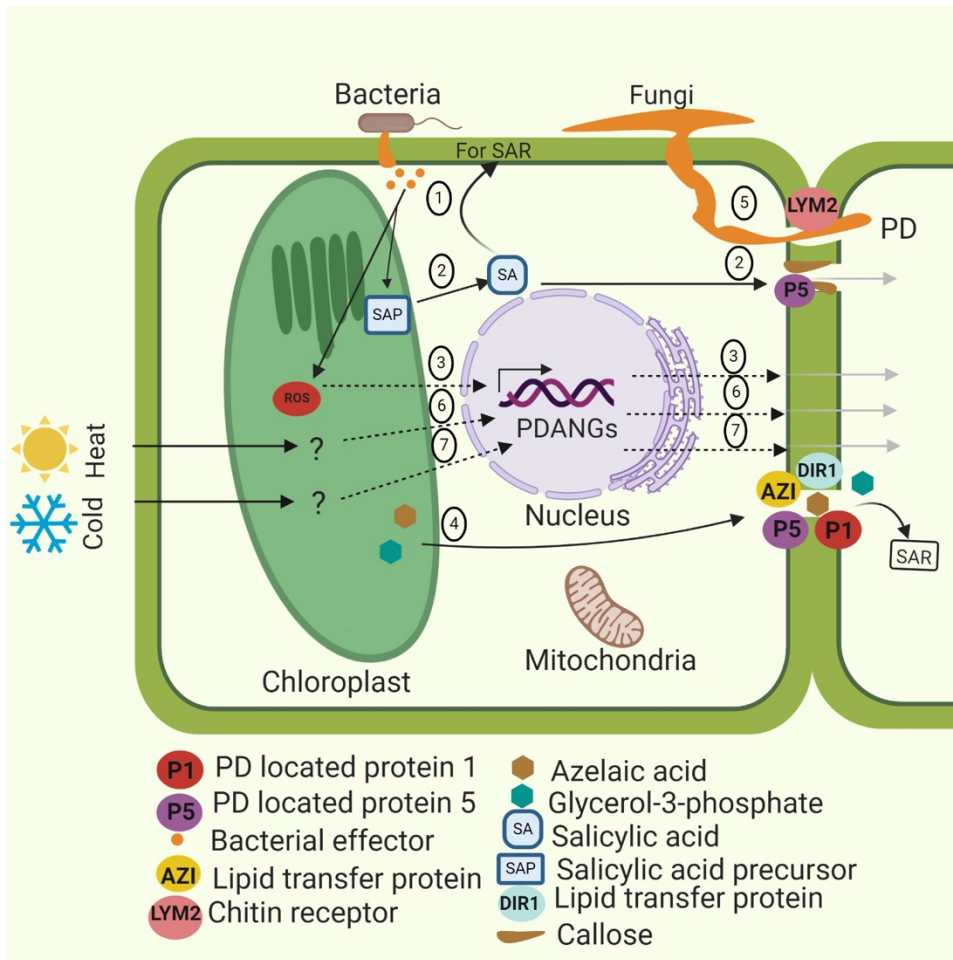
Compared to knowledge of ONPS in biotic stress, much less is known about ONPS in abiotic stress. In rice varieties tolerant or sensitive to heat stress, 40°C for 12 days, kernel weight was reduced due to the inhibition of the assimilate distribution (93). The authors reported that heat stress caused callose deposition at PD at the mesophyll-phloem interfaces in both the leaf and the sheath with more callose accumulating in the heat sensitive variety (**Fig. 3**). PD structure and function also respond to low

temperatures. Chilling maize plants at 14°C inhibited photosynthesis and restricted photoassimilate export to the phloem (94). In a chilling-sensitive line, changes in PD ultrastructure were observed particularly at the bundle sheath and Kranz mesophyll, and the bundle sheath and vascular parenchyma interfaces. These studies implicated calreticulin as responsible for the short-term restriction of PD trafficking and implied that callose was only later involved in modifying PD trafficking (94). Low temperatures also induced expression of PDANGs in maize, including *GRMZM2G081949* encoding a putative Remorin gene (95). While studies on PD structure and function during stress are uncommon, it should be noted that the hormone ABA mediates plant responses to drought and numerous other abiotic stresses (96). ABA also regulates PD function during the breaking of bud dormancy (97). Thus, it is possible that ABA regulates PD in response to abiotic stresses, although this remains to be experimentally verified.

Interestingly, ER stress has also been found to involve PD-mediated systemic signaling. In *Arabidopsis* plants the transcription factor bZIP60 was found to move systemically from the root to the shoot during the unfolded protein response (UPR) induced by tunicamycin (98). The cell-to-cell trafficking of bZIP60 was inhibited by callose accumulation at PD, suggesting that the movement of this transcription factor is via the symplast. In the future it will be interesting to determine whether the bZIP60 transcription factor regulates PDANGs to control PD and the extent of ER stress-associated systemic signaling. Regardless, these results demonstrate that other organelles besides chloroplasts and mitochondria mediate their effects via PD.

Figure 3. ONPS and PD in stress responses.

(1) Infection by pathogens including bacteria leads to the production of defense hormones which ultimately causes a defense response. For example, SA production is induced in the chloroplast and then SA enters the apoplast for SAR. (2) SA also triggers accumulation of callose at PD via PDL5 and reduces intercellular trafficking. (3) Chloroplast ROS are also produced during defense to reduce intercellular trafficking. (4) Aza and G-3-P are induced in the chloroplast by pathogen infection and they move systemically via the symplast to mediate SAR together with SA. PD proteins PDL1 and PDL5 together with the AZI1-DIR1 heterodimer coordinate the symplastic trafficking of Aza and G-3-P. (5) Some PD-mediated defense responses, e.g. those mediated by LYM2 in response to fungi and chitin, are local and the role of ONPS in those remains unknown. (6) Heat stress inhibits PD-mediated intercellular trafficking, reducing carbohydrate export from leaves and decreasing photosynthesis rates. (7) Cold stress causes changes to PD ultrastructure and reduces intercellular trafficking but the role of ONPS remains to be determined.



CONCLUSIONS

In recent years we have gained significant insight into the mechanisms and importance of organelle retrograde signaling for plant development, physiology and responses to stress (98). These findings along with studies into the evolutionary history of plants have made new insights into how ONPS may act to regulate intercellular trafficking. Beyond what we have covered here, PD have essential roles in development, allowing cell-to-cell movement of hormones, small signaling RNAs and transcription factors (9,99). Further investigations into how PD are regulated in these developmental processes as well as a better understanding of how PD are involved in stress responses, especially in non-photosynthetic tissues, will illuminate ONPS. The identification of signature PDANGs that are markers for organelle retrograde signaling and the identification of the retrograde signals themselves would go a long way towards fully characterizing ONPS and the interplay of PD with all other cellular processes that involve intercellular movement and communication. Identifying CRS that mediate ONPS is a focus of this thesis.

II. AIMS OF THE DISSERTATION

The research presented in this dissertation addresses three primary questions.

- 1. Do tetrapyrroles act as chloroplast retrograde signals to regulate trafficking through PD?**
- 2. Do reactive oxygen species act as chloroplast retrograde signals to regulate PD-mediated trafficking?**
- 3. What are candidate genes that contribute to the regulation of intercellular trafficking via PD?**

Chapter 2 focuses on investigating the potential role of tetrapyrrole as chloroplast retrograde signals that regulate trafficking via PD. The findings of this chapter are in preparation for submission to *PNAS*. Chapter 3 explores the potential role of reactive oxygen species and the redox states of chloroplasts and plastoquinone in regulating PD-mediated trafficking. Chapter 4 aims to perform differential gene expression analysis of known PD-related genes and identify new candidate PD proteins using the *in silico* pipeline PIP1. Chapter 5 focuses on the results from a primary research article that determines the role of glucosinolate, a specialized defense compound found in the mustard family and other Brassicales, in regulating trafficking through PD. The findings of these studies are integrated into a Discussion presented in Chapter 6.

CHAPTER 2

TETRAPYRROLES REGULATE INTERCELLULAR TRAFFICKING VIA

PLASMODESMATA

ABSTRACT

Regulating PD-mediated trafficking is essential for plant growth and defense.

Chloroplasts are sites of photosynthesis and fixed carbon from source to sink tissues move through PD. Thus, it is plausible to suggest that chloroplasts regulate PD-mediated trafficking for carbon distribution. Studies on the chloroplast RNA helicase ISE2 underpin the Organelle-nucleus-PD signaling (ONPS) hypothesis to explain how chloroplast gene expression led to signaling to the nucleus (chloroplast-to-nucleus retrograde signaling, CRS) to tune the expression of nucleus-encoded genes for plasmodesmal regulation. ISE2 has multiple roles in chloroplast RNA processing, and silencing ISE2 increases intercellular trafficking via PD. Studies on Arabidopsis *genomes uncoupled (gun)4* and *gun5* mutants have revealed the importance of tetrapyrrole intermediates in CRS. Heme positively regulates photosynthesis associated nuclear genes (PhANGs) and may act as a signaling molecule exported from chloroplasts. Here, we provide evidence that chloroplast-generated heme serves as a retrograde signal for controlling PD-mediated intracellular trafficking.

INTRODUCTION

Plasmodesmata (PD) are membrane-lined cytoplasmic pores that allow the movement of metabolites and signaling molecules between neighboring plant cells (100). A central membranous structure within the pore, derived from the endoplasmic reticulum (ER) of the connected cells, is called the desmotubule. The space between the desmotubule and plasma membrane is called the cytoplasmic sleeve or annulus, which is the primary route for the trafficking of soluble molecules between cells (101). PD that form during cell division, when strands of the ER become trapped in the cell wall, are designated as primary PD. PD may also form across existing cell walls when cells are not dividing, and such PD are called secondary PD (101).

Although surrounded by the cell wall, PD can affect their shape and structure in response to physiological and environmental cues (102). This is exemplified by PD converting from simple to branched forms during the sink-to-source transition in leaves (103). In vascular plants, plasmodesmal frequency is not a fixed cellular parameter, and the number of PD in a cell wall changes during cell wall expansion and in response to developmental and environmental signals, presumably to maintain a necessary minimal level of intercellular communication. The signals pathways that direct these changes in PD remain poorly understood. The organelle-nucleus-PD signaling (ONPS) hypothesis attempts to explain at least some of the regulation of PD. According to ONPS, signals originating in the mitochondria and chloroplast regulate the expression of cell wall- and PD-related nuclear genes to control PD-mediated trafficking(22,104). Since chloroplasts are the sites of photosynthesis and fixed carbon moves from source to sink tissues (105),

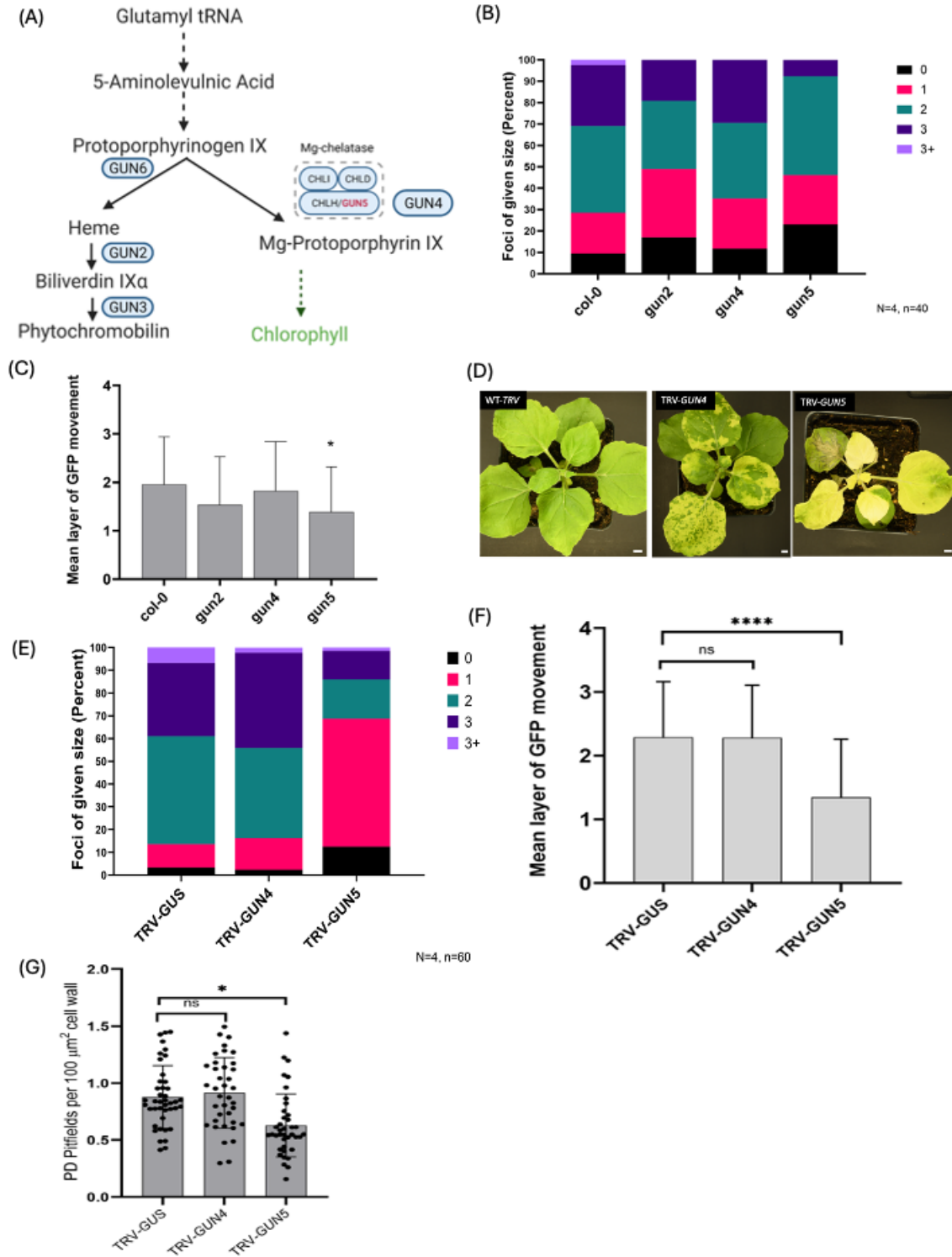
it is reasonable to postulate that chloroplasts would regulate PD-mediated trafficking to control the partitioning of fixed carbon. Studies of the chloroplast RNA helicase *ISE2* underpin the ONPS hypothesis and connect chloroplast gene expression with regulation of PD trafficking. Silencing *ISE2* increased intercellular trafficking via PD and stimulated the biogenesis of new PD (100). *ISE2* has multiple roles in plastid RNA processing including promoting splicing of group II introns, cytidine to uridine (C-to-U) post-transcriptional RNA editing, ribosomal RNA processing and accumulation (106). However, the signaling mechanism that *ISE2* uses to regulate PD trafficking remains unknown.

Chloroplast biogenesis from proplastids or etioplasts in response to light requires massive changes in the expression of nuclear genes, which are not limited to PhANGs (107) but includes genes that affect cell wall synthesis and/or modification, sugar transport, stress response, and responses to reactive oxygen species (ROS) (108). Signals from chloroplasts to the nucleus during chloroplast biogenesis are called biogenic chloroplast retrograde signals (CRS) (109). We hypothesize that the biogenic developmental changes of the chloroplasts are expected to be coordinated with intercellular trafficking as the need to modulate the trafficking of photosynthetic products arises (104). Biogenic CRS pathways have been identified using mutants that lack normal chloroplast development. For instance, if chloroplast development is blocked using herbicides (e.g., norflurazon) or by blocking plastid translation (e.g., lincomycin), the nucleus responds by reducing the expression of PhANGs. A forward genetic screen identified mutants that still retain PhANG expression even when chloroplast development

is blocked in this manner and were termed *genomes uncoupled* (*gun*) mutants. These mutants were identified originally as having elevated expression of the PhANG *LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN1.2* (*LHCBI.2*) when chloroplast development was blocked, a response that has become known as a *gun* phenotype. The original genetic screens for mutants with defects in chloroplast-to-nucleus retrograde signaling identified six loci (*gun1-6*) that are important in retrograde signaling. Five of these (*gun2-6*) encode components of the tetrapyrrole biosynthesis pathway (110–112), while the sixth, *gun1*, encodes a pentatricopeptide repeat protein (113). *GUN2*, *GUN3*, and *GUN5* encode heme oxygenase, phytylchromobilin synthase, and the H subunit of Mg-chelatase (CHLH), respectively (110), while *GUN4* encodes a regulator of Mg-chelatase activity (111). The branchpoint of the tetrapyrrole biosynthetic pathway – where protoporphyrin (ProtoIX) can be a substrate for either Mg-chelatase or Fe-chelatase - is highly regulated to prevent the accumulation of the photosensitizing and singlet oxygen-producing intermediates (114). It was originally proposed that Mg-protoporphyrin IX (Mg-proto IX) accumulated in undeveloped chloroplasts and acts as a CRS to negatively regulate nuclear gene expression (115). This hypothesis was challenged by additional biochemical and genetic analyses that demonstrated that bulk Mg-Proto levels did not correlate with PhANG expression (116,117). Instead, the identification of the dominant *gun6* mutation (*gun6-D*) that results in elevated ferrochelatase (FC)1 activity led to the hypothesis that synthesis of the FC1 product (and developed chloroplasts), heme was a positive regulator of PhANGs (112). Heme has been established as a signaling molecule in many systems and it has been shown to be

Figure 4. *GENOME UNCOUPLED (GUN)* genes have distinct role in PD-mediated trafficking.

(A) Tetrapyrrole pathway featuring *GUN*-encoding enzymes is illustrated. The four subunits (CHLI, CHLD, CHLH/*GUN5*, and *GUN4*) of magnesium chelatase are highlighted. (B-C) PD-mediated trafficking was measured using a movement assay by tracking the expression of GFP from a single cell into adjacent cells (called layers). Stacked bar graph (B) presents the percentage of foci with different layers (0,1,2,3 or >3) of GFP movement in *gun* mutants. Mean layer of GFP movement in *gun* mutants are shown in (C). Silencing *GUN4* (TRV-*GUN4*) shows variegated phenotype (D) while silencing *GUN5* (TRV-*GUN5*) results in severe chlorosis in *Nicotiana benthamiana*. (E-F) GFP movement in these plants are shown as the percentage of foci with different layer movement in stacked bar charts showing all data (E) and in (F) as mean layer of GFP movement. Statistical significance compared to non-silenced control (TRV-*GUS*) was performed by Mann-Whitney U-test with significance at $p < 0.0001$ (indicated by **** asterisks). Data shown here are from at least three biological replicates with 15-20 z-sections per replicate. Clusters of PD were labeled with Arabidopsis PD marker PDL1 fused with GFP, and quantification is shown in (G). Student's t-test was performed for determining statistical significant difference compared to non-silenced control (TRV-*GUS*), asterisks indicate $p < 0.05$. ns= $p > 0.05$. Error bars indicate +/- standard deviation.



exported from chloroplasts (118). In this study, we investigated the role of these tetrapyrroles in regulating intercellular trafficking via PD through their actions as CRSs. Here, we showed that tetrapyrroles, likely heme, regulate some aspects of PD-mediated intercellular trafficking.

RESULTS

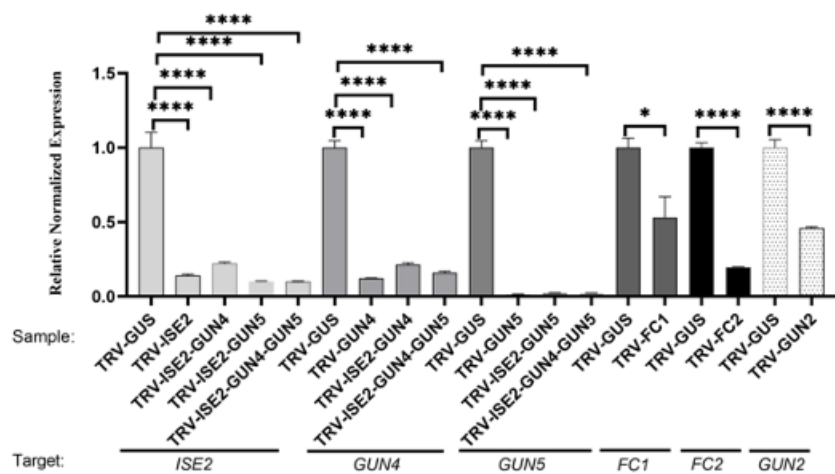
GUN5 has a conserved role in regulating intercellular trafficking in Arabidopsis and N. benthamiana.

Given that the tetrapyrrole biosynthesis pathway provides retrograde signals for chloroplast biogenesis (119), we hypothesized that this pathway influences intercellular trafficking. In the tetrapyrrole biosynthetic pathway, GUN2 belongs to the Fe branch, while GUN4 and GUN5 are part of the Mg branch (**Fig. 4A**). *GUN2* and *GUN4* encode HEME OXYGENASE and a regulator of Mg-CHELATASE activity (120), respectively. *GUN5* encodes the catalytic subunit of Mg-CHELATASE (CHLH). Arabidopsis *gun* mutants showed impaired chloroplast-to-nucleus signaling (121). A GFP movement assay using low-pressure particle bombardment of the *GFP*-expression construct was used to investigate whether the CRS in *gun* mutants impacts PD-mediated trafficking. While *gun2* and *gun4* mutants did not demonstrate significant differences in GFP movement, *gun5* mutants displayed decreased PD-mediated intercellular trafficking revealed by a 20% reduction (compared to the non-silence controls) in foci where GFP had moved into the three surrounding rings of cells and no foci larger than three surrounding layers (**Fig. 4B**).

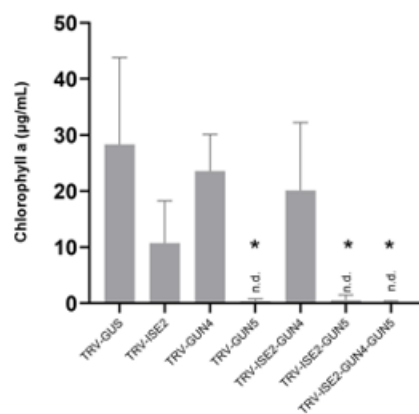
Figure 5. Silencing confirmation, chlorophyll, and carotenoid content in *ISE2-GUNs* silenced plant.

(A) qPCR confirmed silencing of *ISE2*, *GUN4*, *GUN5*, *FC1*, *FC2* and *GUN2*. . All are normalized to the housekeeping genes *EF1- α* and then to the non-silenced control TRV-*GUS*. (B) Chlorophyll a, b and carotenoid level in *ISE2*- only (TRV-*ISE2*) or with *GUN4*- (TRV-*ISE2-GUN4*) and *GUN5*- (TRV-*ISE2-GUN5*) and *GUN4-GUN5*- (TRV-*ISE2-GUN4-GUN5*) -silenced plant.

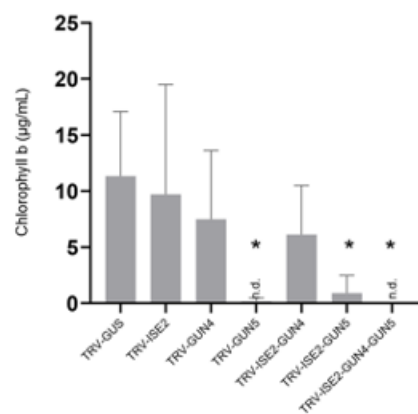
(A)



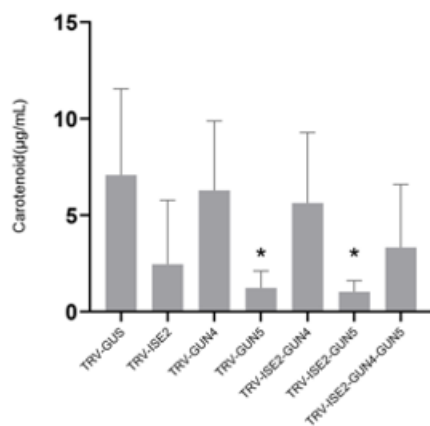
(B)



(C)



(D)



GUN4 and *GUN5* were silenced in *Nicotiana benthamiana* by tobacco rattle virus (TRV)-mediated virus-induced gene silencing (VIGS) to further explore their functions in PD-mediated trafficking. Unlike typical genetic mutants, VIGS enables us to examine the acute effects of reduced gene expression, likely resulting in fewer compensatory responses associated with the loss of gene function (e.g. delayed chloroplast development) (48). Silencing of *GUN4* and *GUN5* was confirmed by quantitative reverse transcription followed by PCR (qRT-PCR) and up to 90% reduction of the expression of target genes when compared to a non-silenced control plant (TRV-*GUS*) was measured (**Fig. 5A**). Whether *N. benthamiana* *GUN4* and *GUN5* are involved in CRS was assessed by measuring the expression of PHANGs *LHCB1.2* and *LHCB2.1* by qRT-PCR (**Fig. 8A, B**). The increased expression of those PHANGs in the *GUN4*- and *GUN5*- silenced leaves compared to non-silenced controls confirms dysregulation of CRS and that *GUN4* and *GUN5* contribute to CRS in *N. benthamiana* as they do in *Arabidopsis*. The reduced levels of chlorophyll *a*, chlorophyll *b*, and carotenoids measured in the leaves of silenced plants (**Fig. 5B-D**) were supported by the observed leaf chlorosis, which ranged from a variegated phenotype in *GUN4*-silenced plants to severe yellowing in *GUN5*-silenced plants (**Fig. 4D**). GFP movement assays revealed that silencing *GUN4* did not perturb intracellular trafficking, while *GUN5*-silenced plants had a drastic decrease in PD-mediated trafficking (**Fig. 4D-E**). This suggests that *GUN5* positively regulates intercellular trafficking. Interestingly, *GUN4* and *GUN5* exhibited distinct roles in regulating intercellular trafficking even though both participate in the same step of tetrapyrrole biosynthesis (**Fig. 4A**).

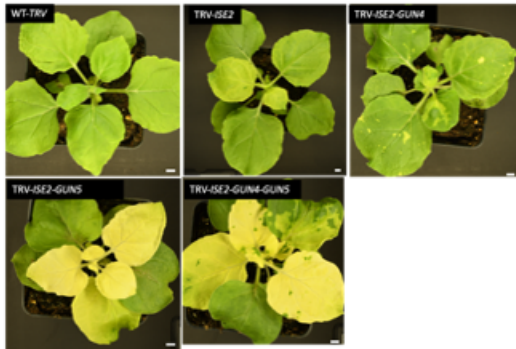
Defects in intercellular trafficking are associated with changes in PD density. Arabidopsis PLASMODESMATA-LOCATED PROTEIN1, a general marker for PD (122), tagged with GFP (PDL1-GFP) was expressed in the silenced leaves to measure PD distribution. PD are nanopores with an average diameter of 30 nm, so the GFP foci in the cell wall observed by confocal microscopy are likely clusters of PD since individual PD are beyond the resolution limits of confocal microscopy. Therefore, we tested if changes in the number of clusters of PD accompanied changes to intercellular trafficking observed in the silenced plants. The average number of pitfields (per 100 μm^2 cell wall) was significantly lower in *GUN5*-silenced plants but did not change in *GUN4*-silenced plants compared to the non-silenced control (TRV-*GUS*) plants (**Fig. 4F**). This suggests that changes in PD distribution may be responsible for the changes in intercellular trafficking observed in the *GUN5*-silenced tissues. ISE2 may regulate PD-mediated trafficking via GUN5-dependent signaling pathway.

ISE2 has important roles in chloroplast RNA metabolism (123,124) and regulates PD-mediated trafficking (125,126). Given that ISE2, GUN4, and GUN5 have distinct roles in chloroplast development, we investigated whether ISE2, GUN4, and GUN5 regulate PD via the same signaling pathway. For this, we co-silenced *ISE2* with *GUN4* (TRV-*ISE2-GUN4*) and *ISE2* with *GUN5* (TRV-*ISE2-GUN5*). Leaves on *ISE2*-silenced plants showed mild chlorosis (**Fig. 6A**). When *ISE2* is co-silenced with *GUN4* (TRV-*ISE2-GUN4*), leaves showed a mild chlorotic phenotype like that of *ISE2*-silenced plants. Slightly reduced levels of chlorophyll a, chlorophyll b, and carotenoid content (**Fig. 7**) support mild chlorosis in these silenced tissues. In contrast, a severe chlorotic phenotype

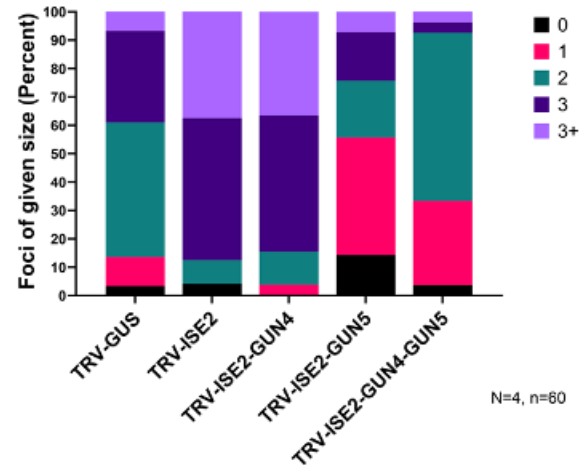
Figure 6. GUN5 has an epistatic relation with ISE2 in regulating trafficking through plasmodesmata.

(A) Silencing *ISE2*- only (TRV-*ISE2*) or with *GUN4*- (TRV-*ISE2-GUN4*) and *GUN5*- (TRV-*ISE2-GUN5*) results in different levels of chlorosis. (A) *ISE2*- and *ISE2-GUN4*-silenced plants have mild chlorosis, while *GUN5*- and *ISE2-GUN5*- and *ISE2-GUN4-GUN5*- (TRV-*ISE2-GUN4-GUN5*) silenced plants have severe chlorosis. **(B-C)** GFP movement in these plants are shown in stacked bar charts showing all data **(B)** and in **(C)** as mean layer of GFP movement. Statistical significance compared to the non-silenced control (TRV-*GUS*) was performed by the Mann-Whitney U-test. **(D)** Clusters of PD were labeled with the general PD marker PDL1-GFP, and data for three replicates of PD clusters are used for the statistical analyses using Student's t-test ($p < 0.05$).

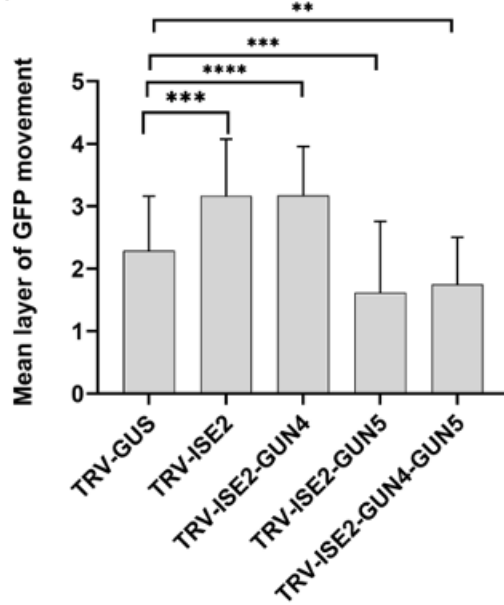
(A)



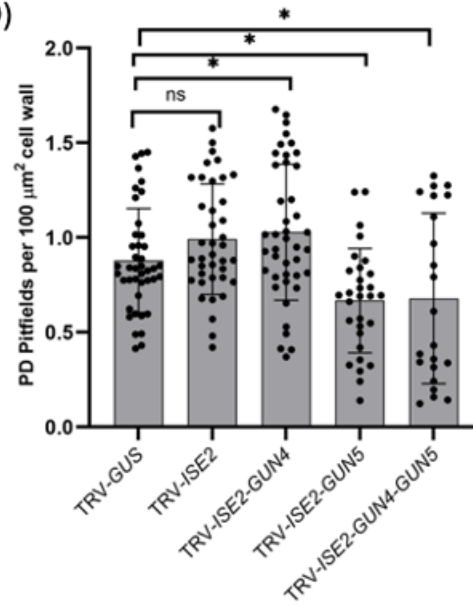
(B)



(C)



(D)



was observed when *ISE2* is co-silenced with *GUN5* in TRV-*ISE2-GUN5*-silenced plants (**Fig. 6A**), similar to that of plants silenced for *GUN5* alone (**Fig. 4C**). Furthermore, when *ISE2* was co-silenced with *GUN4* and *GUN5* together (TRV-*ISE2-GUN4-GUN5*), a severe chlorotic phenotype was observed, similar to the *GUN5*-silenced plants (**Fig. 6A**). The very low chlorophyll a, b, and carotenoid levels in these plants support severe chlorosis (**Fig. 5B-D**). Despite severe chlorosis, the silenced plants grew and eventually matured to set seed.

GFP movement assays were performed to measure the effects of silencing various combinations of genes on intercellular trafficking. As expected, in *ISE2*-silenced leaves, the mean layers of GFP movement increased compared to the non-silenced control (TRV-*GUS*), indicating increased PD-mediated trafficking (**Fig. 6B and 6C**). When *ISE2* was co-silenced with *GUN4* in TRV-*ISE2-GUN4* plants, increased trafficking was observed (**Fig. 6B**). In contrast, co-silencing of *ISE2* with *GUN5* in TRV-*ISE2-GUN5* showed decreased trafficking (**Fig. 6B**), indicating that *GUN5* is epistatic to *ISE2*. This suggests an epistatic relation of *ISE2* with *GUN4* and *GUN5* in regulating PD trafficking, in which *ISE2* is epistatic to *GUN4* while *GUN5* is epistatic to *ISE2*. This relationship is supported by the intercellular trafficking measured in plants where *ISE2* was co-silenced with both *GUN4* and *GUN5* (TRV-*ISE2-GUN4-GUN5*). In those plants, trafficking was similar to that measured in *GUN5* or *ISE2/GUN5*-silenced plants (**Fig. 6B-C**). Together, these results suggest that *ISE2* regulates PD-mediated trafficking via a *GUN5*-dependent signaling pathway.

The relationship between PD-mediated trafficking and PD density was assessed in

Figure 7. Callose staining using aniline blue in *ISE2-GUs*-silenced plants.

Clusters of PD did not correlate with intercellular trafficking in *ISE2*- only (TRV-*ISE2*) or with *GUN4*- (TRV-*ISE2-GUN4*) and *GUN5*- (TRV-*ISE2-GUN5*)- silenced plant. Aniline blue staining was used to detect callose at PD. The cluster of PD punctae stained by aniline blue were counted, and outliers were removed by interquartile range (IQR) analysis. Each dot represents an independent region of interest. Statistical significance was measured by Student's *t*-test , * $p < 0.05$.

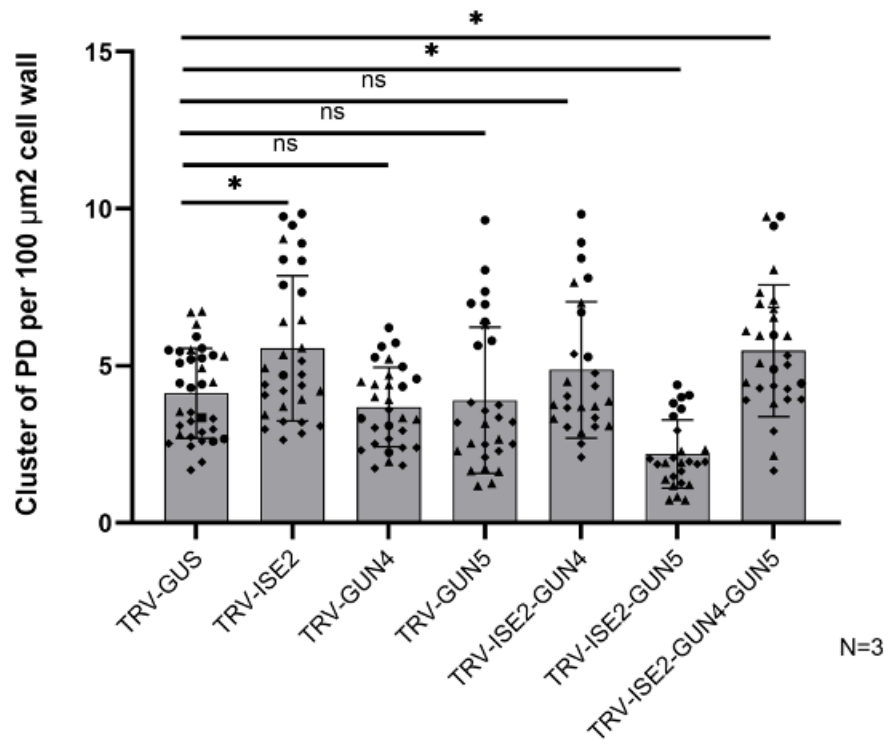
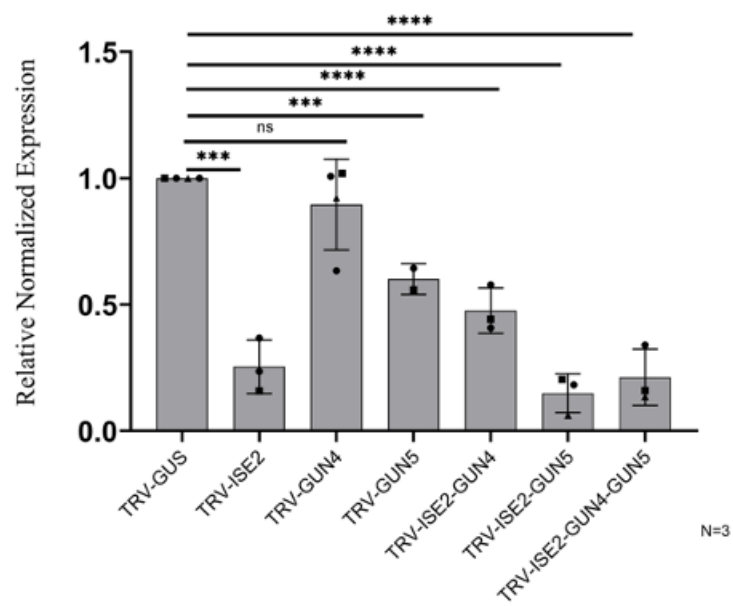


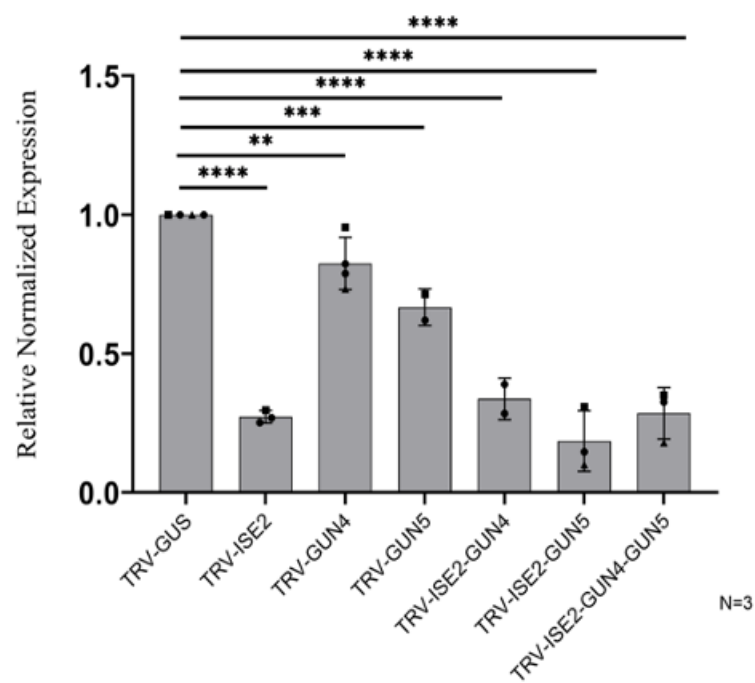
Figure 8. qPCR quantifying the expression *LHCB1.2* and *LHCB2.1*.

Expression of *LHCBs* after silencing *ISE2*- only (TRV-*ISE2*) or with *GUN4*- (TRV-*ISE2-GUN4*) and *GUN5*- (TRV-*ISE2-GUN5*) and *GUN4-GUN5*- (TRV-*ISE2-GUN4-GUN5*). All are normalized to the housekeeping genes *EF1- α* and then to the non-silenced control TRV-*GUS*. TRV-*ISE2*-, TRV-*GUN4*-, TRV-*GUN5*-, TRV-*ISE2-GUN4*-, TRV-*GUN5*-, TRV-*ISE2-GUN4-GUN5* results in different levels of reduction of the expression of *LHCB1.2* (A) and *LHCB2.1* (B).

(A)



(B)



plants where *ISE2* was co-silenced with *GUN4* and/or *GUN5*. PD density, measured as PDL1-GFP clusters per 100 μm^2 cell wall, was increased in *ISE2* and *ISE2-GUN4* - silenced leaves (**Fig. 6D**). In contrast, in both *ISE2-GUN5*- and *ISE2-GUN4-GUN5*- silenced plants PD density was significantly decreased (**Fig. 6D**). Thus, as we found previously, there is a positive correlation between intercellular trafficking and PD density.

Callose is a beta-1,3-glucan polymer that can accumulate in the cell wall surrounding PD and regulate PD-mediated intercellular trafficking. Increased callose accumulation is associated with decreased intercellular trafficking (127). To investigate the relationship between callose accumulation at PD and intercellular trafficking, we measured callose levels in silenced plants. In our study, TRV-*ISE2*-silenced plants showed increased callose accumulation, whereas *GUN4*- and *GUN5*-silenced plants showed no changes in callose accumulation compared to the control (TRV-*GUS*) (**Fig. 7**). For co-silenced plants, *ISE2-GUN4*-silenced plants also showed no change in callose accumulation, but *ISE2/GUN5*- and *ISE2/GUN4/GUN5*-silenced plants exhibited large decreases in callose accumulation (Fig. 7). In summary, the distribution of callose matches the PD density measured, and suggests decreased numbers of PD when there is decreased trafficking.

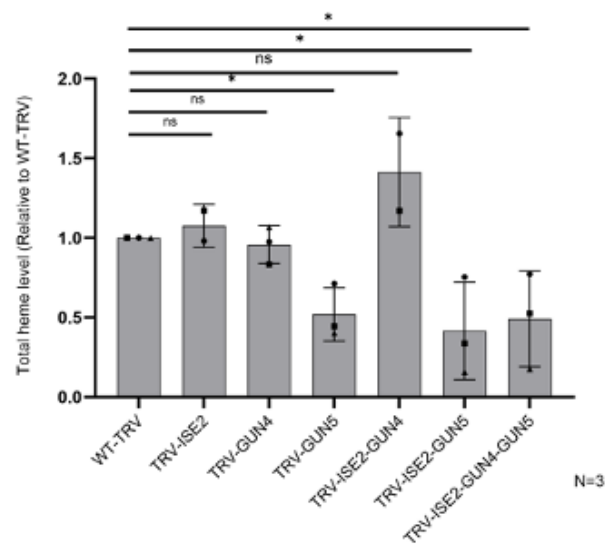
Retrograde signaling is initiated in ISE2-GUNs-silenced plants

To test if retrograde signaling is initiated in the silenced plants, we have measured the expression of the photosynthesis-associated nuclear genes *LHCB1.2* and *LHCB2.1* encoding the antennae proteins of the photosystem-associated light- harvesting

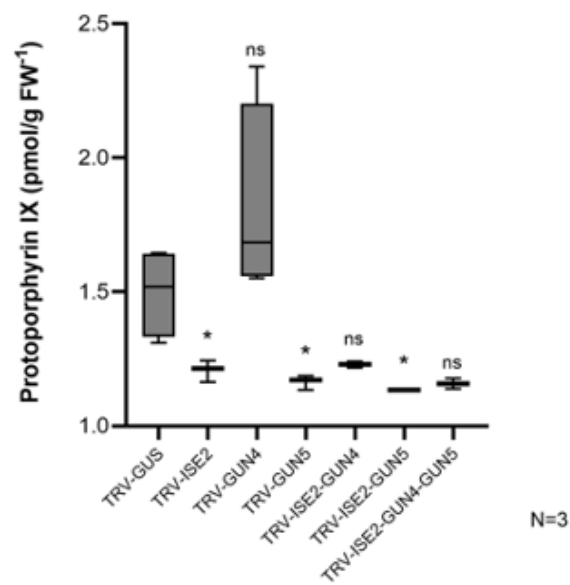
Figure 9. Plasmodesmata-mediated trafficking is affected by the level of heme.

The steady-state level of non-covalently bound total heme (A) and protoporphyrin IX (B) are measured from *N. benthamiana* in non-silenced control (TRV-GUS) versus TRV-*ISE2*, TRV-*GUN4*, TRV-*GUN5*, TRV-*ISE2-GUN4*, TRV-*ISE2-GUN5*, and TRV-*ISE2-GUN4-GUN5*. Total heme levels were measured per mg of fresh weight (FW) and are shown relative to non-silenced control (TRV-*GUS*). (C) Total heme levels were also measured after silencing previously known genes (TRV-*RH22*, TRV-*RH39*, TRV-*ISE2*, TRV-*PNPaseA*, TRV-*PNPaseA/B* (silenced both homologs of *PNPases*: *PNPaseA* and *PNPaseB*), TRV-*RNaseJ*, TRV-*IPII*, TRV-*RH3*) that regulate PD-mediated trafficking. Three biological replicates (each replicate contained tissue pooled from three plants) were used to quantify total heme and protoporphyrin IX. Student's t-test was performed to determine the statistical significance ($p < 0.05$).

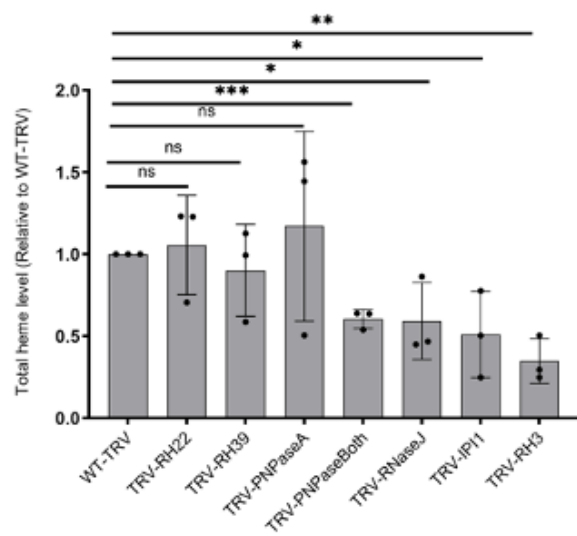
(A)



(B)



(C)



complexes. We used EF1 α as a reference gene; expression normalized to the housekeeping gene EF1- α and then to the non-silenced control TRV-*GUS*.

Expression of *LHCB1.2* and *LHCB2.1* was suppressed in *ISE2*-silenced plants (**Fig. 8A, B**). This suggests that disrupting chloroplast RNA processing by silencing *ISE2* triggered retrograde signals. Silencing *GUN4* significantly reduced the expression of only *LHCB2.1* (**Fig. 8B**); it did not affect the expression of *LHCB1.2* (**Fig. 8A**). In contrast, silencing *GUN5* reduced the expression of both *LHCB1.2* and *LHCB2.1* (**Fig. 8A,B**). Co-silencing *ISE2* with *GUN4* (TRV-*ISE2-GUN4*-), with *GUN5* (TRV-*ISE2-GUN5*-), and with *GUN4-GUN5*- (TRV-*ISE2-GUN4-GUN5*-) suppressed the expression of *LHCB1.2* and *LHCB2.1* (**Fig. 8A,B**). This suggests key genes (*GUN4* and *GUN5*) in the tetrapyrrole pathway with/without chloroplast metabolism genes *ISE2* initiate retrograde signals.

Heme may contribute to regulating PD-mediated trafficking

In plant cells heme is bound covalently to c-type cytochromes, and non-covalently to b-type cytochromes and the vast majority of other hemoproteins, including cytochromes P450, nitrate reductase, NADPH oxidases, peroxidases, and catalases (128). It is generally assumed that there is a pool of free heme that may be involved in signaling, and this pool is likely to be small compared with total cellular heme. Heme signal may act in the nucleus directly or via interaction with cytoplasmic signaling (129). We measured the total heme content of leaves from plants where *ISE2* alone or *ISE2* combined with *GUN4* and/or *GUN5* were silenced. Total heme levels in *ISE2*, *GUN4*, and *ISE2-GUN4*-silenced plants were not significantly reduced compared to those in the non-silenced control (TRV-*GUS*) plants (**Fig. 9A**). In contrast, *GUN5*-, *ISE2-GUN5*, and *ISE2-GUN4*-

GUN5-silenced plants showed a significantly lower total heme than non-silenced control plants (**Fig. 9A**). This suggests a correlation between the reduced level of total heme and decreased PD-mediated trafficking.

We next tested the apparent correlation between total heme and PD-mediated trafficking in plants where chloroplast RNA metabolism genes which were shown to regulate PD-mediated trafficking were silenced. We measured the total heme level after silencing these genes, including *RH3*, *RH22*, *RH39*, *IP11*, *RnaseJ*, and two homologs of *PNPases* (*PNPaseA* and *PNPaseB*), as done previously(48).

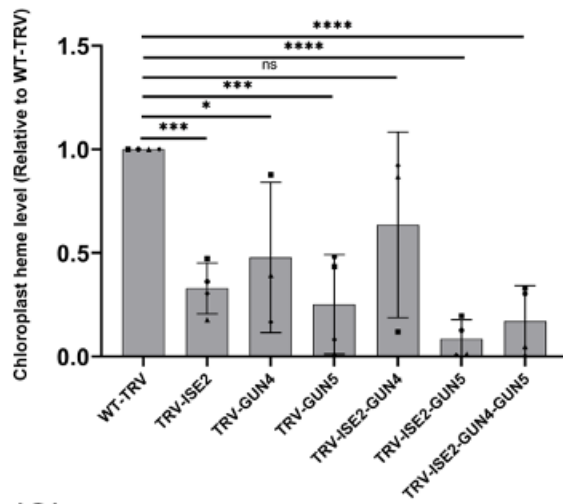
The total heme levels of leaves from *RH22*-, *RH39*-, *ISE2*-, and *PNPaseA*-silenced plants previously reported to increase PD-mediated trafficking were not different from those of non-silenced control plants (TRV-*GUS*) (**Fig. 9C**). In contrast, leaves from plants directly or via interaction with cytoplasmic signaling (129). We measured the total heme content of leaves from plants where *ISE2* alone or *ISE2* combined with *GUN4* and/or *GUN5* were silenced. Total heme levels in *ISE2*, *GUN4*, and *ISE2-GUN4*-silenced plants where both *PNPases*, *RNaseJ*, *IP11*, or *RH3* were silenced had reduced levels of total heme (**Fig. 9A**). These plants with decreased total heme content have reduced intercellular PD-mediated trafficking. They also suggest that a certain minimum level of heme is necessary to be able to increase intercellular trafficking.

To examine whether a specific pool of heme plays a role in regulating PD-mediated trafficking, we measured heme extracted from isolated chloroplasts, and the heme level was normalized to the total number of chloroplasts (**Fig. 10A**). Silencing

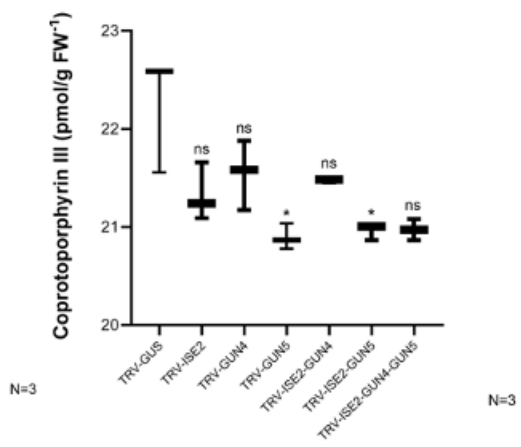
Figure 10. Quantification of heme levels in chloroplast and Coproporphyrinogen III levels in *ISE2-GUNs*- and *FC1*-, *FC2*- and *GUN2*- silenced plants.

(A) Non-covalently bound hemes were quantified from isolated chloroplasts and normalized by the number of chloroplasts and the level of non-silenced control (TRV-*GUS*) plant. (B-C) Coproporphyrinogen III levels are quantified in TRV-*ISE2*, TRV-*GUN4*, TRV-*GUN5*, TRV-*ISE2-GUN4*, TRV-*ISE2-GUN5*, and TRV-*ISE2-GUN4-GUN5* plants (B) and in silenced plants with heme biosynthetic (*FC1*- and *FC2*- silenced) and degrading (*GUN2*-silenced) genes. Coproporphyrinogen III level are measured per fresh weight (FW). Student's t-test was performed to determine the statistical significance ($p < 0.05$).

(A)



(B)



(C)

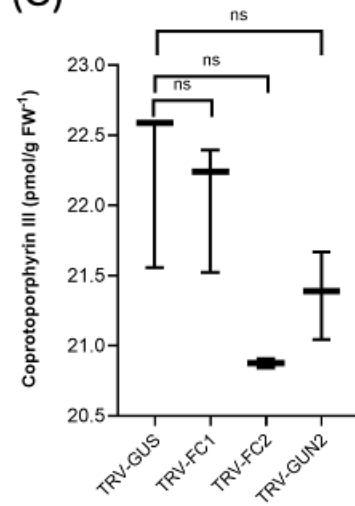
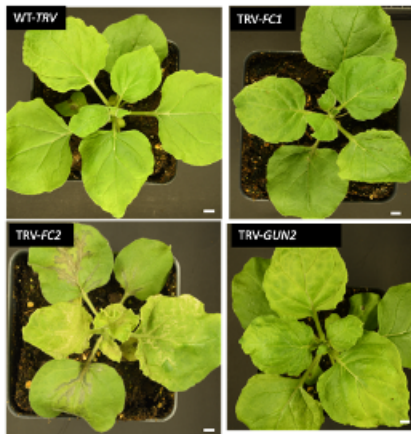


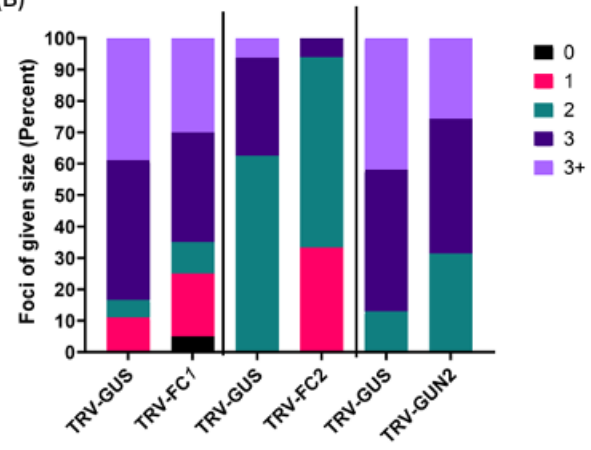
Figure 11. Heme metabolism genes have distinct role in changing intercellular trafficking through PD

(A) Silencing heme biosynthetic genes *FC1* (TRV-*FC1*) and *FC2*- (TRV-*FC2*) and heme-degrading enzyme encoding gene *GUN2* (TRV-*GUN2*) results in different levels of chlorosis. (A) *FC1*-silenced plants exhibit no chlorosis, while *FC2*-silenced plants show severe chlorosis and *GUN2*-silenced plants show mild chlorosis. (B-C) GFP movement in these plants are shown in stacked bar charts showing all data (B) and in (C) as mean layer of GFP movement. Statistical significance was compared to the non-silenced control (TRV-*GUS*) performed by the Mann-Whitney U-test. (D) Heme level was measured from isolated chloroplasts and normalized by the number of chloroplasts. Chloroplast heme level (D) and total heme (E) were shown as relative to non-silenced control plant (TRV-*GUS*). Statistical analyses were performed by Student's t-test ($p < 0.05$). Total heme levels (E) and protoporphyrin IX (F) were measured per mg of fresh weight (FW) and are shown as relative to non-silenced control (TRV-*GUS*). Three biological replicates (each replicate contained tissue pooled from three plants) were used to quantify total heme and protoporphyrin IX.

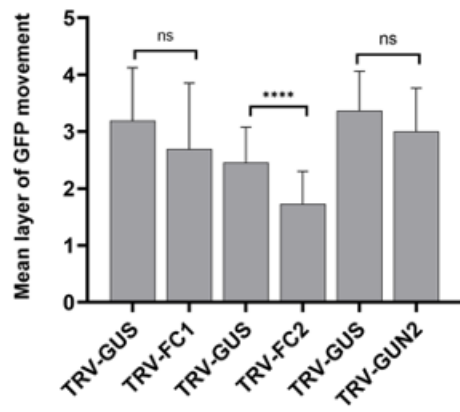
(A)



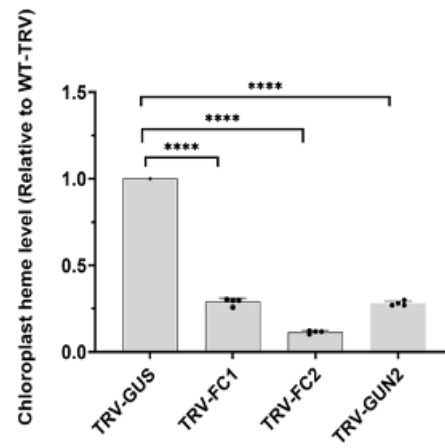
(B)



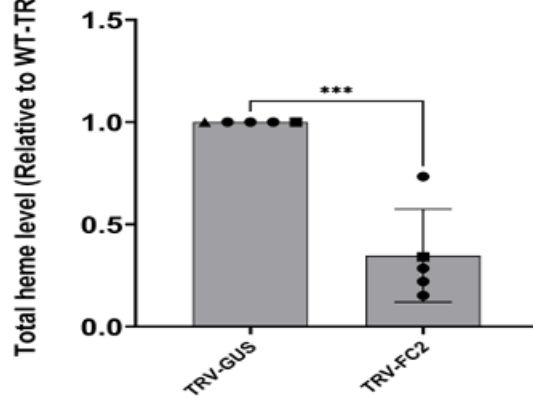
(C)



(D)



(E)



ISE2, *GUN5* or *ISE2/GUN5* reduced chloroplast heme levels compared to the non-silenced control (TRV- *GUS*) plants (**Fig. 10A**). Notably, silencing *GUN4* showed no significant changes in heme level in the chloroplast. This result demonstrates no correlation between the chloroplast heme level and PD-mediated trafficking (**Fig. 10A**).

Quantification of co-pro III and proto-IX

The involvement of other tetrapyrrole intermediates in addition to or besides heme in regulating PD-mediated trafficking was also investigated. We quantified the coproporphyrinogen III (copro III) and protoporphyrin IX (proto-IX) levels in leaves of plants where *GUN4* and *GUN5* were silenced by themselves or together with *ISE2*. Proto IX is at the branching point, where it can either be converted into heme via ferrochelatase or into other tetrapyrroles in the Mg-branch, while co-pro III is upstream of the branching point. Measuring proto-IX and co-pro III will help determine whether changes in heme levels are due to branch point or upstream metabolic regulation. The proto IX content was reduced in *ISE2*-, *GUN5*-, and *ISE2-GUN5*-silenced plants but did not change significantly in other silenced plants (**Fig. 9B**). We found that only *GUN5*-silenced and *ISE2-GUN5*-silenced plants exhibited reduced levels of copro III compared to the non-silenced control (TRV-*GUS*) plants (**Fig. 10B**). Thus, there appears to be no simple correlation between the amount of these intermediates and intercellular trafficking.

Distinct pools of heme uniquely affect PD-mediated trafficking

A specific pool of heme generated from the TPB pathway acts as a positive signal that promotes the expression of genes required for chloroplast development (112). The chloroplast-localized enzymes Fe-CHELATASE 1 (FC1) and FC2 produce two different

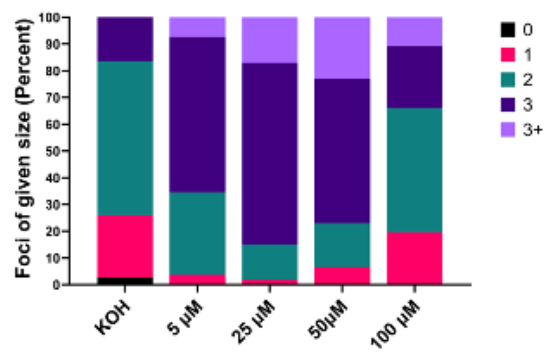
pools of heme (130). While FC2-synthesized heme remains in the chloroplast where it associates with pETC proteins, FC1 synthesizes heme for the rest of the cell (131). We reasoned that silencing *FC1*, *FC2*, and *GUN2* genes could explain how heme may play a role in regulating PD. *FC1* and *FC2* were silenced to disrupt the different pools of heme. We found that *FC1*-silenced plants showed no visible phenotype and were indistinguishable from non-silenced control plants. Silencing *FC1* exhibited reduced level of the chloroplast heme compared to the control (**Fig. 11D**). Intercellular trafficking was not affected in FC1 compared to non-silenced controls (**Fig. 11C**). This suggests that FC1-generated chloroplast heme pool does not regulate PD permeability. In contrast, *FC2*-silenced plants showed severe chlorosis (**Fig. 11A**). Leaves of *FC2*-silenced plants had decreased PD permeability (**Fig. 11C**) along with reduced levels of both chloroplast and total heme (**Fig. 11D**), suggesting that a heme pool produced by FC2 is critical for maintaining PD-mediated intercellular trafficking.

GUN2 encodes HEME OXYGENASE that degrades heme (132), and silencing *GUN2* may lead to accumulation of heme (131). *GUN2*-silenced plants showed mild chlorosis (**Fig. 11A**), and it did not accumulate heme in the chloroplast (**Fig. 11D**). *GUN2*-silenced plants did not show a statistically significant difference in PD permeability compared to control plants (**Fig. 11C**). Silencing *GUN2* did not elevate heme level in chloroplast (**Fig. 11C**) may be due to feedback inhibition of 5-aminolevulinic acid (ALA) biosynthesis (**Fig. 4A**). We also measured the copro III content in *FC1*-, *FC2*-, and *GUN2*-silenced plants and found no statistically significant difference compared to the non-silenced controls (**Fig. 10C**). This supports our

Figure 12. Exogenous application of heme and ALA increases intercellular trafficking.

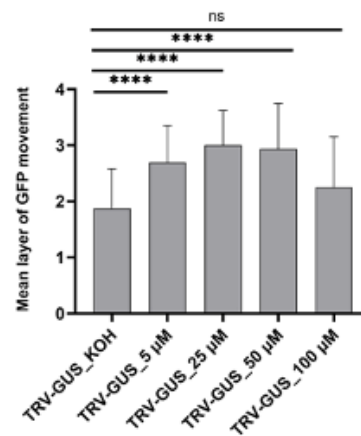
(A-B) Intercellular trafficking was measured after infiltrating with 5 μ M to 100 μ M heme. (C-D) Application of the ALA also caused a significant increase in intercellular trafficking over a range of concentration from 0.2 to 5 mM. (E-F) Infiltration of 50 μ M of heme, not 1 mM ALA, can partially rescue the PD-mediated trafficking in *GUN5*-silenced plants. Statistical significance was measured by the Mann-Whitney U-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ (asterisks). ns = $p > 0.05$. Error bars indicate $\pm$ standard deviation.

(A)

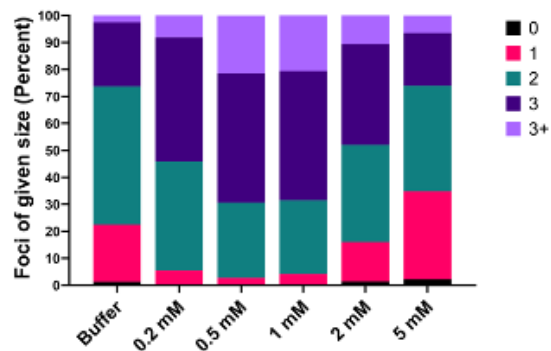


N=3, n=55

(B)

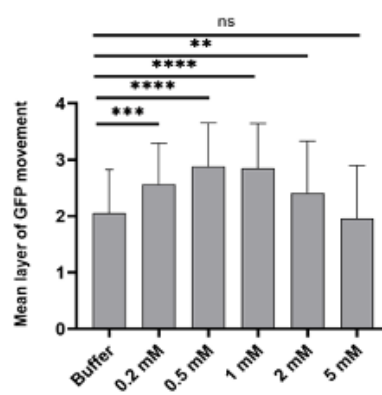


(C)

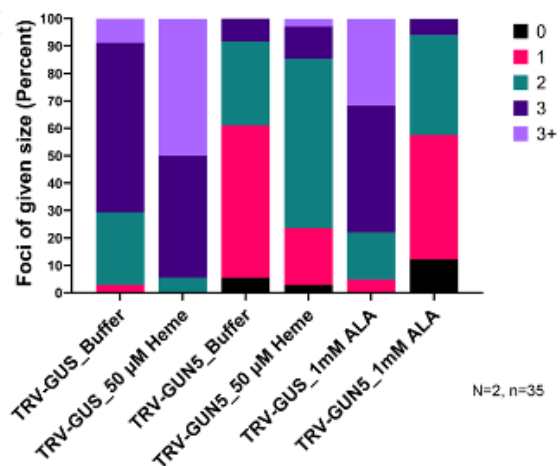


N=3, n=50

(D)

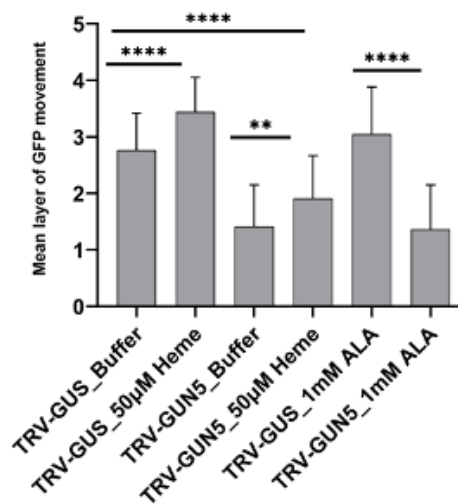


(E)



N=2, n=35

(F)



hypothesis that copro III and proto-IX levels in these silenced plants do not correlate with intercellular trafficking through PD.

Application of exogenous heme increased the PD-mediated trafficking

Our data suggested that heme may act in signaling to regulate intercellular trafficking. The endogenous level of heme is tightly controlled through feedback inhibition of the synthesis of 5- 5-aminolevulinic acid (ALA), a key precursor in the tetrapyrrole pathway (**Fig. 4**). This feedback loop creates a bottleneck that limits heme accumulation. To investigate whether heme was sufficient to regulate intercellular trafficking, we infiltrated plants with heme over a range of concentrations (0, 5, 25, 50, and 100 μ M) and performed GFP movement assays in the infiltrated leaves. A low concentration of heme (5 μ M) increased intercellular trafficking compared to the mock treatment (KOH only) (**Fig. 12A, B**). Higher heme concentrations of 25 μ M and 50 μ M resulted even larger increases in intercellular trafficking compared to the mock treatment (**Fig. 12A, B**). The highest concentration of heme tested, 100 μ M, did not change intercellular trafficking compared to the mock-treated control (**Fig. 12A, B**). The observed effects of heme on intercellular trafficking were somewhat unexpected as heme is expected to not to have biological activity when introduced exogenously. The simplest interpretation of these observations is that, at least in *N. benthamiana*, the infiltration of heme probably increased total cellular heme levels and this led to increased intercellular trafficking.

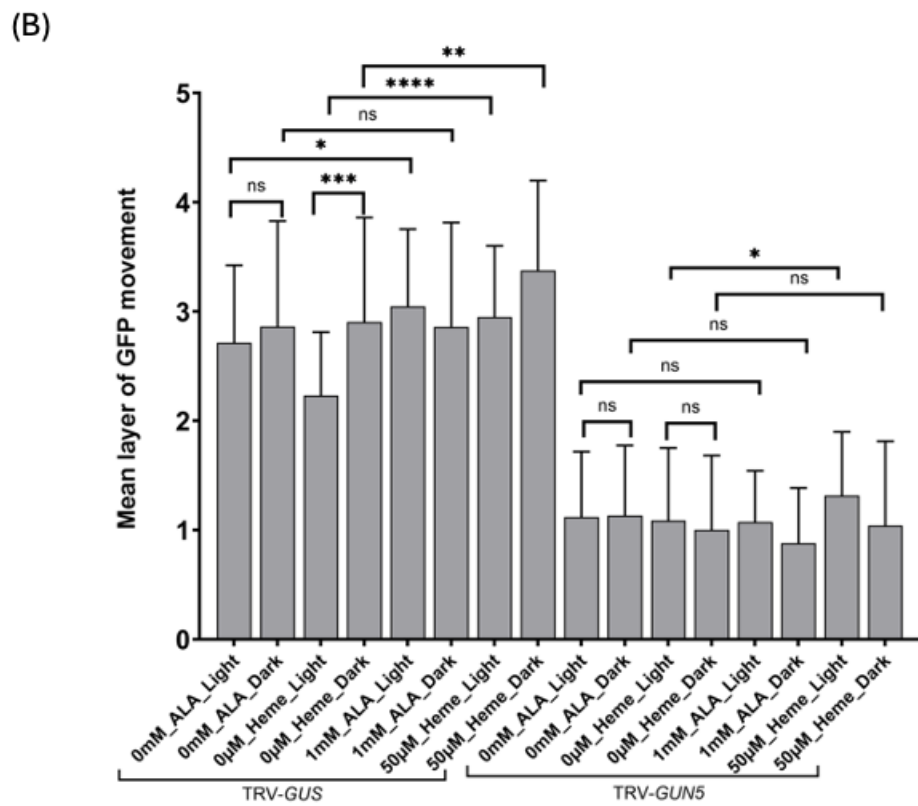
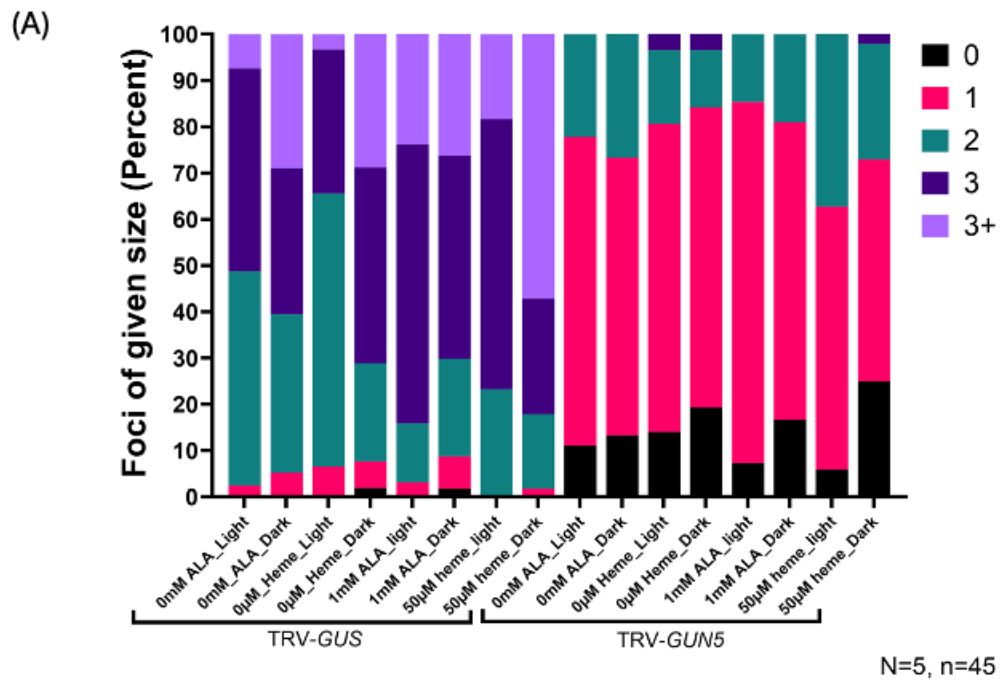
To overcome the bottleneck of heme accumulation due to the negative feedback to ALA, we treated plants with ALA to try to increase heme levels. This approach was demonstrated to bypass feedback inhibition and promote increased heme biosynthesis

(133). We hypothesized that if heme could indeed regulate intercellular trafficking, this approach would increase intercellular trafficking by increasing the heme levels in the treated plant. To investigate the effect of exogenous ALA on intercellular trafficking, we infiltrated different concentrations (0, 0.2, 0.5, 1, 2, and 5 mM) of ALA into plants and then measured intercellular trafficking in ALA-treated leaves. At a very low concentration of ALA of 0.2 mM, ALA increased intercellular trafficking compared to the mock treatment (phosphate buffer) (**Fig. 12C, D**). Increasing ALA concentration to 0.5, 1 or 2 mM significantly increased intercellular trafficking (**Fig. 12C-D**). In contrast, 5 mM ALA, had no effect on intercellular trafficking compared to the mock-treated control. The observed increased trafficking with increased ALA concentrations supports the observation that increased heme levels, up to a point, can increase intercellular trafficking.

Finally, we predicted that application of heme could rescue the intercellular trafficking defect in silenced plants with reduced heme levels. To test this, we infiltrated *GUN5*-silenced plants with heme or ALA. Exogenous application of heme at 50 μ M increased intracellular trafficking statistically significantly compared to the mock treatment (KOH only) in *GUN5*-silenced plants (**Fig. 11E-F**). However, it did not completely rescue the intercellular trafficking to the level observed in non-silenced plants. Interestingly, exogenous ALA (1 mM) did not increase intercellular trafficking in the *GUN5*-silenced plants compared to mock treatment (**Fig. 11E-F**). This suggests that ALA feeding in *GUN5*-silenced plants might not increase the heme level, potentially due

Figure 13. Light dependence of exogenous heme and ALA regulating PD-mediated trafficking.

A. Stacked bar graph showing the percentage of foci with different layers of GFP movement after treatment. Mock (0 mM ALA/0 μ M heme, buffer only) and 1 mM ALA/ 50 μ M heme were infiltrated into non-silenced control (TRV-*GUS*) and *GUN5*- (TRV-*GUN5*) silenced plant in standard light conditions or dark conditions (with infiltrated leaves covered in aluminum foil) for 24 hours. GFP movement assay was performed using confocal microscopy 48 hours post-infiltration of Agrobacterium containing the GFP construct. **B.** Mean layer of GFP movement following mock, 1 mM ALA, and 50 μ M heme infiltration. Statistical significance was measured by the Mann-Whitney U-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$ (asterisks). ns = $p > 0.05$. Error bars indicate +/- standard deviation.



to the defect in heme synthesis. Taken together, these experiments suggest that heme can act as the CRS to regulate intercellular trafficking via PD.

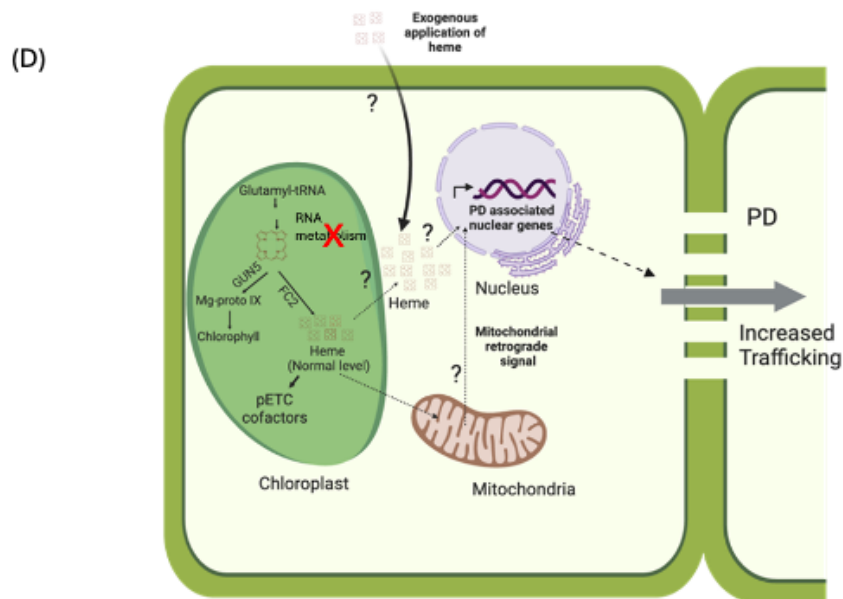
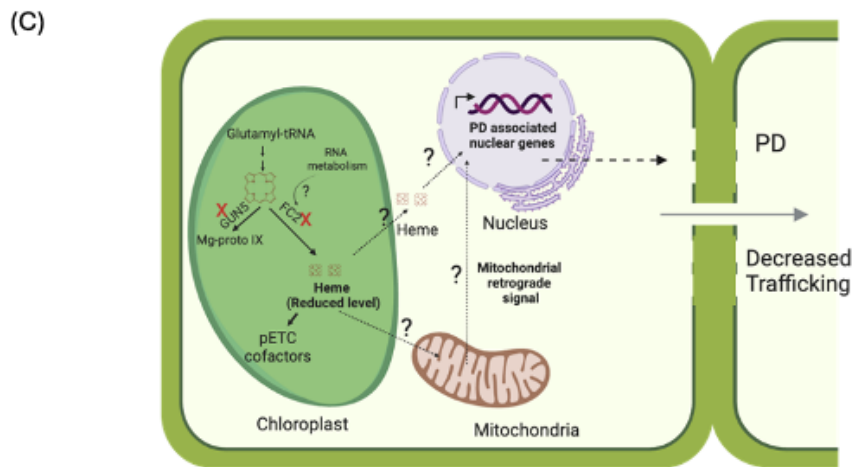
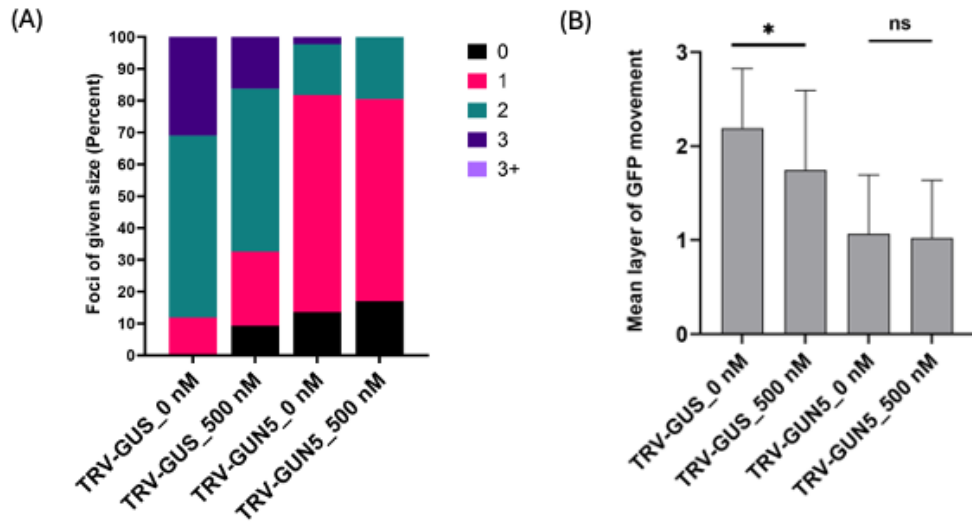
Heme regulates PD-mediated trafficking in a light-independent manner

To investigate if exogenous heme/ALA regulates PD-mediated trafficking in a light-dependent manner, we treated leaves with 50 μ M heme, either exposing them to standard light or covering them with aluminum foil to create dark conditions. In non-silenced control (TRV-*GUS*), exogenous heme significantly increased trafficking through PD, in both light and dark conditions, suggesting that light is not required for heme function in the non-silenced control plants (**Fig. 13 A, B**). However, in *GUN5*-silenced plants, exogenous heme significantly increased trafficking through PD only in light, not in the dark, suggesting that light is required for heme function in these plants (**Fig. 13 A, B**).

To compare this with heme synthesis driven by precursors, we infiltrated 1 mM ALA into non-silenced and *GUN5*-silenced plants. In non-silenced control plants, ALA treatment increased trafficking only under light conditions, not in darkness. In contrast, *GUN5*-silenced plants did not respond to ALA treatment in either light or dark conditions, suggesting the defects in heme synthesis for ALA precursor in these plants. Interestingly, dark conditions without any treatment resulted in increased PD-mediated trafficking in non-silenced control plants compared to light conditions. However, only the mock treatment (0 μ M heme, buffer only) for heme showed a statistically significant increase in PD-mediated trafficking, while the mock treatment (0 mM ALA, buffer only) for ALA showed a similar trend toward increased trafficking, although it was not statistically significant (**Fig. 13 A, B**). However, this increase in trafficking was not

Figure 14. Flg22 induced PTI does not decrease intercellular trafficking at reduced heme levels in *GUN5*-silenced plants.

(A-B) Infiltrating with 500 nM flg22 significantly decreases intercellular trafficking in non-silenced control plants but did not after trafficking through PD in *GUN5*-silenced plants. Statistical significance was measured by the Mann-Whitney U-test. * $p < 0.05$, ns = no statistically significant difference. Error bars indicate standard deviation. (C-D) Model of heme regulating intercellular trafficking. (C) Reduced heme levels may change the cell wall- and/or PD-related gene expression to decrease intercellular trafficking. (D) Increased/Control level of heme level, along with other unknown signal/s, may change nuclear gene expression to increased trafficking.



observed in
65

GUN5-silenced plants, where PD-mediated trafficking showed no change in both light and dark environments.

Flg22 depends on heme level to regulate PD-mediated trafficking

The biological role of ONPS and its potential CRSs like heme have not been clearly demonstrated. We therefore assessed the importance of heme-mediated ONPS in response to a pathogen. For this we turned to the model bacterial elicitor, flagellin, and its flg22 peptide that is a potent inducer of pattern triggered immunity (PTI) (134). Flg22-induced PTI results in decreased PD-mediated intercellular trafficking (135). It has also been reported to induce heme production (131). To determine whether flg22 regulates PD-mediated trafficking in a heme-dependent manner, we infiltrated 500 nM flg22 into *GUN5*-silenced *N. benthamiana* plants, which had reduced heme levels. We found that treating a non-silenced control plant (TRV-*GUS*) with 500 nM flg22 significantly decreased intercellular trafficking (**Fig. 12A,B**). The flg22 treatment (500 nM) did not decrease intercellular trafficking in *GUN5*-silenced plants compared to the mock treatment (**Fig. 14A, B**). This lack of response to flg22 in *GUN5*-silenced plants suggests that there was insufficient heme to regulate intercellular trafficking in the silence plants. These findings point to a critical role for heme in facilitating the flg22-mediated regulation of intercellular trafficking, suggesting that this signaling pathway may have a wider role in plant-pathogen interactions.

DISCUSSION

Previous studies revealed that *genome uncoupled* (*gun*) mutants have defective CRS,

resulting in the deregulation of expression of PHANGs gene expression (136,137). We show that at least one of these mutants, *gun5*, also has deregulated intercellular trafficking via PD (**Fig. 4B**). Silencing the *N. benthamiana* *GUN5* homolog also perturbed intercellular trafficking (**Fig. 4D and 4E**). While silencing or knockout of *GUN5* disrupted intercellular trafficking, similar changes in *GUN4* expression had no effect on PD-mediated intercellular trafficking (**Fig. 4D and 4E**), pointing to the enzymatic activity of the Mg-CHELATASE as a critical control point for generating signals that regulate PD permeability. The connection between PD and Mg-CHELATASE is supported by the observed correlation between the cluster of PD and PD-mediated trafficking, where decreased cluster of PD was measured in plants with decreased intercellular trafficking and vice versa (**Fig. 4D and 4F**).

Chloroplast RNA metabolism gene *ISE2* was found to regulate PD-mediated trafficking in *GUN5* dependent pathway. Reducing heme level to regulate intercellular trafficking is a common mechanism by other known chloroplast RNA metabolism genes (*RH39*, *RH22*, *RH3*, *PNPaseBoth* (*PNPaseA* and *PNPaseB*), *PNPaseA*, *IP11*), with evidence showing that silencing those genes showed a correlation between decreased trafficking and reduced heme levels. Exogenous heme and ALA can increase PD-mediated trafficking. However, while exogenous heme partially rescued intercellular trafficking in *GUN5*-silenced plants, exogenous ALA did not exhibit the same effect. Moreover, bacterial elicitor flg22 requires basal level heme to regulate PD-mediated trafficking.

ISE2 regulates intercellular trafficking via PD in a GUN5-dependent manner

ISE2 and *GUN5* encode chloroplast-targeted proteins, with *ISE2* involved in chloroplast RNA metabolism and *GUN5* encoding biosynthetic enzymes for the tetrapyrrole pathway. Both processes involve chloroplast-to-nucleus signaling (119,126). Silencing *GUN5* decreases PD-mediated trafficking, possibly affecting the key signal in the chloroplast-to-nucleus signaling pathway. Silencing *ISE2* and *GUN5* genes may uncover how chloroplast signaling interacts to regulate intercellular trafficking. We found that co-silencing *ISE2*-*GUN5* decreases trafficking, suggesting that the loss of *ISE2*'s negative role in regulating intercellular trafficking depends on *GUN5*'s role in trafficking. Therefore, we conclude that *ISE2* regulates intercellular trafficking in a *GUN5*-dependent manner. The previous study supports this and showed that silencing *ISE2* increased the expression of *GUN5* while decreasing *GUN4* expression (48). The possibility of the physical interaction of *ISE2* and *GUN5* and its role in regulating intercellular trafficking was not ruled out.

Threshold of heme levels in may regulate intercellular trafficking

Heme is reported to translocate from the chloroplast to the nucleus, where it regulates transcription, RNA metabolism, and chromatin remodeling (138). Heme-related signals are proposed to be the primary signal during chloroplast biogenesis (139). We expect that the expression of PD-related and cell wall metabolism genes will be co-regulated with genes responsive to heme signals, including RNA helicase DBRHs, histone deacetylase (HAD) SRT1, and HDA3 complex subunit *CMLL100C* (138). The DBRH family participates in broad aspects of RNA metabolism, such as transcription, translation, RNA

decay, and miRNA processing. HAD removes an acetyl group from Lys residues of histone, resulting in the histone wrapping DNA more tightly to repress the gene expression from the removed chromatin region (138).

For the extraction and determination of total heme (including non-covalently bound and free heme), the standard method involves sequential acetone extractions (140–145). Initially, basic acetone mixed with ammonia removes major pigments like chlorophyll and carotenoids from plant tissues. After washing these precipitates with 80% acetone, non-covalently bound heme is extracted using an acidic acetone solution containing HCl, typically 80% acetone and 20% 0.6–2.1 M HCl (140,146,147). Importantly, free heme levels are maintained at low concentrations to avoid oxidative stress from uncontrolled heme iron oxidation (148). Heme's low solubility in water suggests it predominantly exists either dissolved in membranes or loosely attached to proteins (147). Thus, free heme is defined as heme that isn't attached to hemoproteins functioning as prosthetic groups; rather, it is loosely associated with membranes or proteins and plays a role as a metabolic intermediate or second messenger in vital biological processes. Studies indicate that loosely associated free heme can be extracted with basic acetone without causing hemoprotein denaturation in isolated chloroplasts and healthy seedlings (147,149). However, unbound free heme concentration is very low in both plant and mammalian cells (148). The neutral acetone-extracted free heme corresponds to 3% of total heme in these plants (150). Moreover, when chloroplast structure is disrupted by norflurazon, an inhibitor of carotenoid biosynthesis, exhibited significant increase in endogenous free heme levels occurs (150). However, free heme

levels do not correlate with the *gun* phenotype, suggesting that if heme acts as an organelle-derived signal, its transfer likely occurs through specific trafficking rather than remaining unbound. Neutral acetone-extractable heme levels were higher in *gun2* and lower in *gun4* and *gun5* compared to wild type, suggesting that unbound free heme levels don't correlate with the *gun* phenotype. In NF-treated *gun2*, higher accumulation of total heme was noted (119), indicating decreased heme catabolism may lead to higher total and unbound heme accumulation. In contrast, mutations in *gun4* and *gun5* might redirect protoporphyrin IX into heme biosynthesis, yet free heme levels decreased, suggesting feedback inhibition regulates heme levels in plant cells to maintain homeostasis.

We found that *GUN5*-silenced plants had decreased heme content, possibly by regulating the expression of *FC1* and/or *FC2* or their activity and reducing the heme level and affecting the chloroplast to nucleus signaling, which eventually may affect the expression of PD-related and/or cell-wall remodeling genes (**discussed in Chapter 4**) to decrease the intercellular trafficking via PD (**Fig. 14C**). Silencing *ISE2* or *GUN4* alone, and the combination *ISE2-GUN4* does not significantly impact heme levels compared to the non-silenced control. These silenced plants either increase or do not affect PD-mediated trafficking (**Fig. 14D**). In contrast, *GUN5*, *ISE2-GUN5*, and *ISE2-GUN4-GUN5* exhibit reduced levels of heme and decreased PD-mediated trafficking. This suggests that reduced heme levels correlate with decreased intercellular trafficking, indicating that heme is essential for normal trafficking. Intercellular trafficking may depend on maintaining a certain heme threshold. If heme levels fall below this threshold (e.g., in *GUN5* or *ISE2-GUN5* co-silenced plants), trafficking was reduced. At or above this

threshold, trafficking remains stable or increases. However, heme alone may not be sufficient to increase intercellular trafficking. The reduced heme levels in *GUN5* and other silenced plants may influence the demand for heme in cytochrome b6f, affecting electron transfer in the photosynthetic machinery and generating reactive oxygen species. We explored this possibility by measuring the levels of reactive oxygen species, examining the chloroplast's redox status, and assessing the plastoquinone redox state in the silenced leaves. We found no correlation between the levels of reactive oxygen species, the chloroplasts' redox status, and the plastoquinone redox state concerning the regulation of intercellular trafficking in our silenced plants.

Proto-IX and copro III levels do not correlate with PD-mediated trafficking

Silencing key genes in the tetrapyrrole pathway (*GUN4* and *GUN5*) and chloroplast RNA metabolism (*ISE2*) differentially impacts the accumulation of copro III and proto-IX. However, the levels of copro III and proto IX did not correlate with intercellular trafficking via PD.

None of the silenced plants showed increased accumulation of co-pro II and proto IX; therefore, we did not observe any cell death or necrosis under our growth conditions (light intensity: $120 \mu\text{mol m}^{-2}\text{s}^{-1}$). ProtoIX levels did not differ significantly in the leaves of two FC1 antisense lines relative to control seedlings (151). However, tobacco lines with decreased FC2 expression were characterized by elevated steady-state levels of ProtoIX in leaves (152). This may be due to differences in light intensity and plant species studied. Our plants were grown under light intensity 2.5 times lower than the light intensity ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$) used in the (152).

Exogenous heme can partially rescue the intercellular trafficking via PD

Exogenous heme and its precursor, ALA, increase intercellular trafficking in a dose-dependent manner (**Fig. 11E,F**). However, exogenous heme application could only partially restore intercellular trafficking to the levels observed in non-silenced control leaves. This partial rescue suggests that the *GUN5*-silenced plant retains the capacity to utilize exogenous heme to regulate PD-mediated trafficking but might not be able to rescue completely. This indicates that other signal(s) may be necessary for complete rescue. Although the exact mechanism of penetration and mode of action remains unclear, we hypothesize that heme may act directly as a signaling molecule in the cytoplasm and nucleus and binds to proteins or transcription factors to alter their activity. Thus, it may alter the expression of PD- and/or cell-wall remodeling genes, thereby positively regulating intercellular trafficking. An alternative hypothesis is that exogenously applied heme accumulates in the apoplast and acts locally (on PD) to affect intercellular trafficking. Importantly, the signaling role of heme is not limited to higher plants. In *Chlamydomonas reinhardtii*, exogenous heme treatment resulted in differential expression of hundreds of genes - most of which were unrelated to photosynthesis (153) - highlighting a broader signaling potential. This supports our finding that exogenous heme increases PD-mediated trafficking, likely through regulatory pathways beyond its classic metabolic roles.

ALA is a small, water-soluble molecule that plant tissues can absorb through passive diffusion or specific transport proteins (119), although the exact transporters have yet to be identified. Once inside the cell, ALA can participate in the tetrapyrrole

biosynthesis pathway (133). For ALA to contribute to heme biosynthesis, it must be imported into the chloroplast. Exogenous application of ALA did not increase the intercellular trafficking in *GUN5*-silenced plants treatment, suggesting the silenced plants could not utilize the exogenous precursor ALA to synthesize heme.

Heme regulation of plasmodesmata is not light dependent

Exogenous heme exhibited regulation of PD-mediated trafficking independently of light in non-silenced plants, while exogenous ALA infiltration only increased PD-mediated trafficking in light (**Fig. 13 A, B**). This suggests that heme biosynthesis from ALA depends on chloroplast activity, which is light dependent. This aligns with known regulatory mechanisms of enzymes in the tetrapyrrole pathway (154). In *GUN5*-silenced plants, ALA treatment failed to increase trafficking through PD in light or dark, indicating *GUN5* is necessary for heme synthesis and underlining the defect in heme biosynthesis in these plants. In contrast, heme treatment showed increased trafficking only in light, which suggests that the introduced heme can modulate PD function, possibly through redox signaling, callose regulation, or interactions with PD-associated proteins.

In the absence of heme/ALA treatment, we observed increased PD-mediated trafficking in non-silenced control plants, indicating that dark condition alone can modulate PD-mediated trafficking independently of any treatments (**Figure 13 A, B**). In the dark condition, reduction of ATP levels due to reduced photosynthesis would open the PD for the export of the assimilated carbon from the leaves ((155). *GUN5*-silenced plants did not show increased trafficking in dark conditions, indicating that endogenous

heme or tetrapyrrole signaling is necessary for this regulation. Additionally, GUN5 may play a crucial role in integrating light cues into PD function, potentially through a retrograde signaling pathway.

Distinct role of two different pools of heme in regulating PD-mediated trafficking

FC2 produces heme which is utilized by photosynthetic machinery (156) and this pool of heme was found to be more critical for intercellular trafficking than the heme produced FC1. We found reduced levels of total heme and chloroplast heme in *FC2*-silenced plants. Disrupting heme production in chloroplasts can also affect the supply to other organelles, such as mitochondria and peroxisomes. In mitochondria, heme is essential for cytochrome c in the mitochondrial electron transport chain to transfer electrons critical for ATP production. In peroxisomes, heme acts as a co-factor for peroxidase and catalase in redox signaling. We did not rule out the possibility of heme acting through the other organelles to regulate the PD-mediated trafficking. Heme might be transported to mitochondria/other cellular organelles or directly to the nucleus to regulate the expression of PD/cell-wall-associated nuclear genes to regulate PD-mediated trafficking.

Mitochondrial localized protein ISE1 is shown to regulate the trafficking through PD (157). Since chloroplast heme is required for the mitochondrial electron transport system (mETC), reduced total heme level may affect the mitochondrial function, which can regulate the trafficking through PD.

MATERIAL AND METHODS

Plant Materials and Growth Conditions

N. benthamiana seeds were sown in pots filled with hydrated soil (Berger 35). After 10 days, germinated seedlings were transplanted into individual pots and allowed to grow for 10-12 days in a 16-hour photoperiod before performing VIGS. **Appendix Table 3** lists the *Arabidopsis thaliana* ecotype Columbia (Col-0) and *gun* mutant lines used in this study. These mutant lines are in the Col-0 background and have previously been described (121). Arabidopsis seeds were also sown directly in pots and stratified for 3 days at 4°C before moving to a growth chamber with a 16-8-hour photoperiod of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 23°C until further experimentation.

RNA isolation, cDNA synthesis and quantitative polymerase chain reaction (qPCR)

The ninth or tenth leaf (counted from the bottom) of silenced or non-silenced *N. benthamiana* plants was collected and flash-frozen in liquid nitrogen. RNA was isolated from three independent samples using the RNA isolation kit (Qiagen), following the manufacturer's instructions. To remove DNA, the RNA was treated with DNase using the DNA-free DNA removal kit (Invitrogen, CA) for subsequent qPCR or RNA sequencing analysis. Complementary DNA (cDNA) was synthesized with oligo-dT primers and M-MLV reverse transcriptase (Promega, WI), following the manufacturer's instructions. cDNA was then used for gene cloning or quantitative polymerase chain reaction (qPCR). Diluted cDNA (1:5) with nuclease-free water is used for qPCR to quantify the expression of PHANGs and to confirm the silencing in VIGS-infiltrated plants. qPCR was performed using the LightCycler 480 SYBR Green I Master Mix and a LightCycler 480 Real-Time PCR system (Roche, Indianapolis, IN, USA) with the following thermal cycling program: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 59°C for 10 seconds,

and 72°C for 20 seconds. The expressions of PHANGs and silencing genes are normalized to the expression of *EF1-α*.

Gene cloning to generate Virus-Induced Gene Silencing (VIGS) constructs

The *Arabidopsis thaliana* genome uncoupled (GUN) loci were obtained from The Arabidopsis Information Resource database (TAIR, <https://www.arabidopsis.org/index.jsp>). These gene loci are AtGUN2 (AT2G26670), AtGUN4 (AT3G59400), and AtGUN5 (AT5G13630). The protein sequences of each gene loci were used to obtain *N. benthamiana* gene sequences (http://solgenomics.net/organism/Nicotiana_benthamiana/genome). The gene loci in *N. benthamiana* are *NbGUN2* (Niben101Scf12135g00014.1, Niben101Scf06347g04017.1), *NbGUN4* (Niben101Scf03113g03014.1, Niben101Scf00078g02007.1), *NbGUN5* (Niben101Scf07214g00015.1, Niben101Scf04388g00011.1), *NbFC1* (Niben101Scf04539g00003.1, Niben101Scf02338g00006.1), *NbFC2* (Niben101Scf03375g02028.1). Gene fragments, approximately 400 bp in length, were amplified from cDNA from wildtype *N. benthamiana* plants using Phusion DNA polymerase (New England Biolabs, Ipswich, MA) and primers and plasmids are listed in **Appendix Table 2-3**. The amplified silencing fragment was cloned into the pYL156 vector (containing TRV RNA2) after digestion with restriction enzymes (New England Biolabs). To generate a co-silencing construct with *ISE2*, the *GUN4* and/or *GUN5* silencing fragments were digested and ligated to pTBS136, which already carries a fragment for silencing *ISE2*. Ligations were carried out with T4 DNA ligase (New England Biolabs). The resulting ligated plasmids were transformed into chemically

competent DH5a *Escherichia coli* cells and cultured on LB media containing kanamycin for selection. Positive clones were screened through colony PCR. Plasmids were isolated from overnight-cultured positive clones using a mini-prep kit (QIAGEN), and the nucleotide sequences of silencing constructs in plasmid were confirmed through Sanger sequencing. These positive plasmids containing the silencing constructs were transformed into *Agrobacterium tumefaciens* GV3101 competent cells by chemical transformation (freeze/thaw) and used for VIGS experiments.

Virus Induced Gene Silencing

We performed VIGS as previously described (158). Briefly, pYL192 (encoding TRV RNA1) and pYL156 (which encodes TRV RNA2 carrying the silencing construct) were cultured overnight, centrifuged, and resuspended in an infiltration buffer (10 mM MgCl₂ and 2-(N-morpholino) ethane sulfonic acid (MES, pH 5.5, adjusted with KOH), along with 200 µM acetosyringone) to an OD of 0.1 at 600 nm. An equal volume of pYL192 and pYL156 derivatives were mixed before infiltrating into the leaves of three-week-old plants. TRV2 vector pYC1 containing pYL156:GUS was a negative control for all VIGS experiments. Movement assays were conducted 10-12 days after infiltration.

GFP Movement Assay

Intercellular trafficking in *N. benthamiana* was measured by a GFP movement assay, as described previously (158). In brief, *Agrobacteria* carrying a binary construct for GFP expression (*p35S: GFP*) were cultured overnight in LB medium with antibiotics at 28°C. The cultures were centrifuged at 3000 rpm for 15 minutes, washed, and then resuspended to an optical density of 0.0001 at 600 nm in infiltration buffer consisting of 10 mM

MgCl₂ and 2-(N-morpholino) ethane sulfonic acid (MES, pH 5.5, adjusted with KOH), along with 200 μ M acetosyringone (Sigma, St Louis, MO, USA). To induce virulence, the *Agrobacteria* were incubated at room temperature for 3 hours with gentle shaking before infiltrating them into silenced leaves using a needleless syringe. Imaging was performed 48 hours post infiltration using a Leica SP8 confocal microscope (Leica Microsystems, Buffalo Grove, IL) with excitation and emission wavelengths of 488 nm and 500-530 nm, respectively. Images were collected using 20X objective with the pinhole aperture was set to 1 Airy unit. Z-stacks of GFP foci were maximum projected and analyzed using ImageJ (48). The number of cell layers in the epidermis surrounding the original GFP-expressing cell was determined. At least three biological replicates, each including at least 20 foci, were performed.

To measure intercellular trafficking in *gun* mutant lines, the fifth and sixth leaves from five three-week-old plants were collected and low-pressure (60 psi) bombarded with plasmid pRTNL2rsGFP for the GFP movement assay as described(159). After bombardment, the leaves were incubated abaxial side down on half-strength MS media at 23°C in a growth chamber. After 24 hours of bombardment, confocal microscopy was performed as described above to measure the GFP movement. At least 15 foci per sample were collected to analyze the GFP movement, and experiments were performed for at least three biological replicates. For statistical analyses, the Mann-Whitney U test and the bootstrap method (160) were performed to compare the GFP movement between samples.

Quantification of PD density and staining callose with aniline blue

Clusters of PD in *N. benthamiana* plants were quantified after transiently expressing Arabidopsis PDL1 fused to GFP (p35S: PDL1-GFP) as described previously(161). Briefly, *Agrobacterium* containing PDL1-GFP was infiltrated at OD_{600 nm}= 0.001. After 72 hours post infiltration, 15 μ m z-stacks of epidermal cell images at a step size of 1 μ m were collected with a Leica SP8 confocal microscope with a White Light Laser (Leica Microsystem, IL, USA) using a 25x water emersion objective. Three biological replicates, each consisting of sixty foci, were collected for each sample. Maximum intensity projections were generated for each of the z-stacks using ImageJ. Cell wall lengths were measured by tracing the cell wall in the field of view. Images were then converted to 8-bit images and inverted. After adjusting the intensity threshold, the particle analyze function was used to count the total number of punctate in the image. PD pitfields are measured per 100 μ m² cell wall area (cell length was multiplied by z-stacks size to measure the cell wall area). Mann-Whitney U-test was performed to measure the statistical significance comparing the PD pitfields between silencing and non-silenced control plants.

Callose at PD was stained using aniline blue and quantified using Method 1, as described previously (162). Images were obtained by exciting the aniline blue with a 405 nm laser, and emission was captured from 415 to 525 nm using a 40X water immersion objective. Z-stacks were acquired using a system-optimized step size of less than 1 μ m. In total, 60 foci/regions of interest were collected for each sample across three biological replicates.

Flg22 treatment, ALA feeding, and heme feeding

The stock solutions of flg22 (GenScript, Piscataway, NJ, USA, Cat. No. RP19986) and ALA (Sigma-Aldrich Inc., Cat. No. A3785) were freshly prepared in sterile water. Flg22 was further diluted to the desired concentration (0 nM, 100 nM, and 500 nM) using water. ALA was diluted to the desired concentrations (0 mM, 0.2 mM, 0.5 mM, 1 mM, 2 mM, 5 mM) using 10 mM phosphate buffer and 5 mM MgCl₂. The stock solution of heme (100 mM) was prepared in DMSO and further diluted freshly to different concentrations in 100 mM KOH.

For the flg22 treatment, freshly prepared flg22 at desired concentrations were syringe-infiltrated into the leaves of *GUN5*- and non-silenced plants seven hours before confocal microscopy. For ALA and heme feeding, the desired concentrations of ALA or heme are infiltrated into *GUN5*- and non-silenced control plants 24 hours before confocal microscopy. Plants were kept in the dark overnight and moved to light for another 12 hours. Infiltrated leaves were covered with aluminum foil after heme or ALA treatment for 24 hours for dark conditions experiments. For the GFP movement assay, these leaves were infiltrated with *Agrobacterium* carrying 35S: GFP 48 hours before confocal microscopy. Movement assays were performed as described previously.

Chlorophyll and carotenoid determination

Chlorophyll and carotenoid were extracted following the method described previously(48). Briefly, ten leaf discs were weighed and homogenized in 3 mL of 100% acetone using a mortar and pestle. Two mL of the resulting extracts were transferred to a reaction tube and centrifuged at 500 x g for 5 minutes. The supernatants were used to

measure absorption at 470 nm, 646.8 nm, and 661.6 nm. Quantification of total carotenoids (x+c, xanthophylls, and carotenes) and chlorophyll a and b were performed using the equation described in reference (163).

Heme measurements

Non-covalently bound heme was measured in plants 10-12 days post VIGS infiltration. Ten leaf discs were collected from the eighth or ninth leaf of a plant and weighed to determine fresh weight. 100 mg of plant tissue was ground in liquid nitrogen using a mortar and pestle and the powder was dissolved in 1 mL of ice-cold extraction solution 1 (90% (v/v) acetone and 10% (v/v) 0.1 M NH₄OH) and centrifuge at 10,000 x g for 10 minutes and the supernatant was discarded. The pigment extraction steps were repeated four times. To extract heme from the pellet, 500 µL of extraction solution 2 (80% (v/v) acetone, 16% (v/v) DMSO, and 4% (v/v) concentrated HCl) was added and mixed thoroughly. The solution was incubated for 10 minutes at room temperature and centrifuged at 10,000 x g for 10 minutes. The supernatants were diluted 100 to 1,000-fold with 10 mM KOH, and 10 µL of diluted samples were used for heme quantification.

Heme extracts were diluted in 10 mM KOH before being applied to the Horse radish peroxidase (HRP) enzyme assay. The HRP apoenzyme spontaneously reconstitutes with heme, resulting in an active enzyme (164). The peroxidase activity of HRP (Biozyme) is utilized to oxidize luminol in the presence of H₂O₂, and the resulting luminescence signal is proportional to the amount of heme present in the sample. To prepare the master mix for each reaction, 50 µL of 2x reaction buffer, 30 µL of H₂O, and HRP are added to achieve a final concentration of 2.5 nM. Eighty µL of the master mix

was incubated with 20 μ L of the sample or standards for 30 minutes at room temperature, followed by the addition of the western blot detection reagent (Thermo Scientific™ Pierce™ ECL, Cat No. PI32106), prepared according to the manufacturer's instructions (50 μ L of luminol and 50 μ L of peroxide solution), and incubated for 12-15 minutes. Finally, the luminescence was measured at 430 nm using a 96-well plate reader in luminescence mode with an integration time of 0.5 seconds. We made two standard curves of 250-4000 nM and 50-400 nM to quantify total and chloroplast heme.

Porphyrin measurements

Protoporphyrin IX (Proto) and Uroporphyrinogen III (Uro III) were measured by HPLC analysis as described in (145). Briefly, ten leaf discs from each VIGS plants were weighed to measure the fresh weight and then frozen in liquid nitrogen. The discs were ground using mortar and pestle, and homogenized were resuspended with 250 μ L of 50 mM potassium phosphate buffer, pH 7.8, and centrifuged at 10 000 x g for 10 minutes at 4°C. The supernatant was transferred to a dark tube and kept on ice. To extract tetrapyrrole from the pellet, an additional 500 μ L 90% (v/v) methanol and 10% (v/v) 0.1 M NH₄OH were added, followed by centrifugation at 10, 000 x g for 10 minutes at 4°C. The supernatants were combined, and tetrapyrroles were extracted from the pellet again with 500 μ L 90% (v/v) acetone and 10% (v/v) 0.1 M NH₄OH. This mixture was incubated at 20°C for 20 minutes and then centrifuged at 16,000 x g for 10 minutes at 4°C. All the extracts were combined and subjected to final centrifugation at 16,000 x g for 10 minutes at 4°C. To convert Uro III to fluorescent (detectable) porphyrins, the 400

μL extracts were mixed with 10 μL 1M acetic acid and 10 μL of 2-butanone for oxidation prior to injection into HPLC.

Tetrapyrroles were separated using a Waters Symmetry C18 column (5 μm, 4.6 x 150 mm) with the following HPLC method: 7 min at 100% Solvent A (10% (v/v) methanol and 10% (v/v) 1M ammonium acetate, pH 5.2), 13 min 100% Solvent B (90% (v/v) methanol and 10% (v/v) 1 M ammonium acetate, pH 5.2), 9 min 100% Solvent A. Fluorescence detection was performed at an excitation/emission wavelength of 405/625 nm for Uro II and Proto IX. Standard curves were generated using authentic tetrapyrroles (Frontier Scientific, Logan, UT).

APPENDIX

Table 1 Primers used in this study

Gene	Forward sequence	Reverse sequence	Purpose
<i>GUN2</i>	gattctagaATGGCTTCAATTACACC CTTATC	gatctcgagTCAAGACAATATTAATC GGAGG	VIGS construct for silencing
<i>GUN2</i>	TTCTCAGGGGAAATCCTGCG	TGAGCTCCAATGTACAACCTACC	qPCR for confirma tion of silencing
<i>FC1</i>	ATCggatccGCTAAAAATGGCTCTA GAAAC	TGAgaattcAGAACCAGTTGTAGAG ATAG	VIGS construct for silencing
<i>FC1</i>	CGATTTCTCCAACGCCATT	TTGCAGCGTACCCTTCCTTA	qPCR for confirma tion of silencing
<i>FC2</i>	ATCgaattcTGGGAAAGGAATGTGC CTGC	TGAggatccCCACACATTCCTCCAT TTCA	VIGS construct for silencing
<i>FC2</i>	GCTCGTCAGTCATTGGTTCC	GCCAACATAGCAGCTCTTCC	qPCR for confirma tion of silencing
<i>GUN4</i>	TAAgaattcGGAGAAGGCATTCTAT TGATTGCCC	AGCggatccCACTTTGTTCATATT TTCTTTTG	VIGS construct for silencing
<i>GUN4</i>	ACAAGCAAGTTTTTCTGGGGA	ACTCCTCAAACCACCCAACA	qPCR for confirma tion of silencing
<i>GUN5</i>	ATGgaattcGCTCAGCGAGCTCATT TCCTC	ATCggatccGGTTAGCTTGTCATTG ACATTAC	VIGS construct

Table 1 continued

<i>GUN5</i>	CCGAGCAGTCAAGATGGTTG	GCTTGTCTAGTGCATGTTTCC	qPCR for confirma tion of silencing
<i>NbLHCB 1.2</i>	CAGATGGGCTATGCTTGGTG	GCCTTGAACCATAACAGCCTC	qPCR d primer
<i>NbLHCB 1.2</i>	AGGCCCACTTGGTGAAGG	TCGGGATCATCAGCGAAC	qPCR d primer
<i>EF1α</i>	CAAGAGGCCCTCAGACAAAC	CACAACCATAACCAGGCTTGA	qPCR d primer
<i>RH22*</i>	CTAtctagaGAGCAAGTGGTTCGAA TG	CTAggatccGATAACTGCTTCTCAT C	VIGS construct for silencing
<i>RH39*</i>	TATtctagaCACCTGAACAATAGGC AACAT	TATggatccAACAGGAAGAGCACT GCTAAA	VIGS construct for silencing
<i>ISE2*</i>	GACctcgagGGGGAAGGTTGATAC TTTGA	GATggatccTCAAACGGGAGACCT TATTC	VIGS construct for silencing
<i>PNPase A*</i>	CGTgaattcATGCCTTGGCAGTTAC GGC	CGAtctagaCGACTAGCTTATACAG CTC	VIGS construct for silencing
<i>PNPase A/B*</i>	CGTgaattcGATGCATCAGGAGATA TGG	CGAtctagaCGTCCAGGCGCAATCT CCA	VIGS construct for silencing
<i>RNaseJ*</i>	GTAtctagaGGCCTTGAGGAACTGT C	CATggatccCAGAGTTGATGGATCT ATTG	VIGS construct for silencing
<i>IP11*</i>	CACgaattcCCTGTGTCCTTGTGAT ATC	CATtctagaCATAAGAAGCCCAAAG GTTA	VIGS construct for silencing
<i>RH3*</i>	AAATTTggatccTTTTGTTAGGGCA TTCGGATG	CTTAAtctagaAAAATTCACGTTCA GGGTGCT	VIGS construct for silencing

Table 1 continued

<i>PDS</i> *	CGAggatccCTGACGAGCTTTCGAT GCAGTGCAT	TCGctcgagATATATGGACATTTAT CACAGGAAC	VIGS construct for silencing
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*From Ganusova et al., 2020

Table 2 Plasmids used in this study

Plasmid	Description
pTBS13*	<i>ISE2</i> silencing constructs in pYL156 for VIGS
pLBG269	<i>GUN4</i> silencing constructs in pYL156 for VIGS
pLBG270	<i>GUN5</i> silencing constructs in pYL156 for VIGS
pMFA337	<i>ISE2</i> and <i>GUN4</i> silencing constructs in pTBS136 for VIGS
pMFA338	<i>ISE2</i> and <i>GUN5</i> silencing constructs in pTBS136 for VIGS
pMFA340	<i>ISE2</i> and <i>GUN4</i> and <i>GUN5</i> silencing constructs in pTBS136 for VIGS
pMFA342	<i>FC1</i> silencing constructs in pYL156 for VIGS
pMFA343	<i>FC2</i> silencing constructs in pYL156 for VIGS
pMFA345	<i>GUN2</i> silencing constructs in pYL156 for VIGS

*From Ganusova et al., 2020

Table 3 Arabidopsis mutants used in this study

Mutant	Background	Reference
<i>gun2</i>	Col-o	Susek et al., 1993
<i>gun4-1</i>	Col-0	Koussevitzky et al., 2007
<i>gun5-1</i>	Col-0	Koussevitzky et al., 2007

CHAPTER 3

**INVESTIGATING THE ROLE OF REACTIVE OXYGEN SPECIES AND THE
REDOX STATUS OF CHLOROPLASTS IN REGULATING TRAFFICKING
THROUGH PLASMODESMATA**

ABSTRACT

Reactive oxygen species (ROS) have been proposed as chloroplast retrograde signals that potentially regulate intercellular trafficking through plasmodesmata (PD). In this chapter, we investigate whether ROS, the chloroplast's redox status, and the plastoquinone (PQ) redox status can regulate PD-mediated trafficking. We measured ROS levels, chloroplast redox status, and PQ redox status following the silencing of the chloroplast RNA metabolism gene (*ISE2*) and the tetrapyrrole biosynthetic genes (*GUN4*, *GUN5*), either alone or in combination with *ISE2*. In Chapter 2, we showed that these genes regulate PD-mediated trafficking. We also measured ROS, chloroplast redox status, and PQ redox status after silencing other chloroplast RNA metabolism genes, including *RH3*, *RH22*, *RH39*, *IP11*, *RNaseJ*, PNPaseA, and *PNPaseA/B*, which are known to regulate trafficking via PD. We measured ROS levels using DAB staining, while the chloroplast redox status was measured using chloroplast-targeted redox-sensitive GFP (ro-GFP2). PQ redox status was determined from chlorophyll fluorescence parameters using the LI-COR 6800. Our results show no consistent correlation between ROS levels, chloroplast redox state, or PQ redox status and PD-mediated trafficking. These findings indicate that PD regulation in these silenced plants may occur independently of generalized redox signaling, highlighting the need to further investigate specific redox signaling mechanisms.

INTRODUCTION

In addition to tetrapyrrole (discussed in **Chapter 2**), reactive oxygen species (ROS) represent another potential class of chloroplast-to-nucleus retrograde signals (165–167). ROS including superoxide anion (O_2^-), singlet oxygen (1O_2), hydroxyl radical ($OH^\bullet$), and hydrogen peroxide (H_2O_2), are produced during growth and in response to environmental stresses like light, cold, heat, pathogens (168). H_2O_2 and O_2^- are mainly generated at Photosystem I (PSI), while 1O_2 is primarily produced at Photosystem II (PSII)(169), potentially altering the stromal redox state and regulating plastid redox-associated nuclear genes (PRANGs). Plastid-derived H_2O_2 induces unique nuclear transcriptional changes, unlike peroxisomal H_2O_2 (170). Redox buffers, such as ascorbic acid/dehydroascorbic acid (AA/DHA) and glutathione/glutathione disulfide (GSH/GSSG), help regulate cellular responses to environmental shifts (171).

Reduced chloroplast heme content may reduce the amount of heme incorporated in the cytochrome b_6f complex of the photosynthetic electron transport chain (130). Defective cytochrome b_6f complexes may generate reactive oxygen species (ROS). We found that silencing the genes that encode enzymes of the TBS, *GUN4*, and *GUN5*, did not result in heme accumulation (**Chapter 2**). This suggested the possibility of a higher accumulation level of the tetrapyrrole intermediate Protoporphyrin IX (Proto). Proto is an extremely photosensitive molecule that produces singlet oxygen (1O_2) (172). Since 1O_2 has an extremely short half-life (~ 0.5 - $1.0 \mu\text{sec}$) (173), secondary messengers may be involved in propagating the chloroplast 1O_2 signal to change nuclear gene expression through retrograde signaling (174). Silencing *GUN5* also led to significantly decreased

intercellular trafficking via PD, further supporting the possibility that chloroplast ROS may have a role in determining plasmodesmal flux.

Organellar redox states in plant cells influences intercellular trafficking through PD. Reduced trafficking was correlated with higher ROS accumulation in the *gat1* mutant, a plastid-localized thioredoxin *m3* mutant (175). (In contrast, oxidative stress in mitochondria has been shown to enhance intercellular trafficking (176). Reducing the expression of mitochondrial RNA helicase *ISE1* or chloroplast RNA helicase *ISE2* led to a reductive shift in chloroplast redox states and consequently increased trafficking through PD. This finding aligns with Benitez-Alfonso et al. (2009), which noted that an oxidative shift in chloroplasts diminishes PD transport. Silencing *ISE1* or *ISE2* increased plasmodesmal trafficking; however, *ISE1*-silenced plants exhibited a more oxidized state in mitochondria, whereas *ISE2*-silenced plants had a reduced state in chloroplasts (157).

In this study, we investigated the role of ROS and the chloroplasts' redox status in regulating intercellular communication via PD. We used a variety of cell and molecular biology approaches in a set of chloroplast RNA metabolism genes that have previously been reported to regulate PD-mediated trafficking. Together, our results suggest that these chloroplast-generated signals may not act as retrograde signals to control intercellular trafficking, at least under our experimental conditions and protocols.

RESULTS

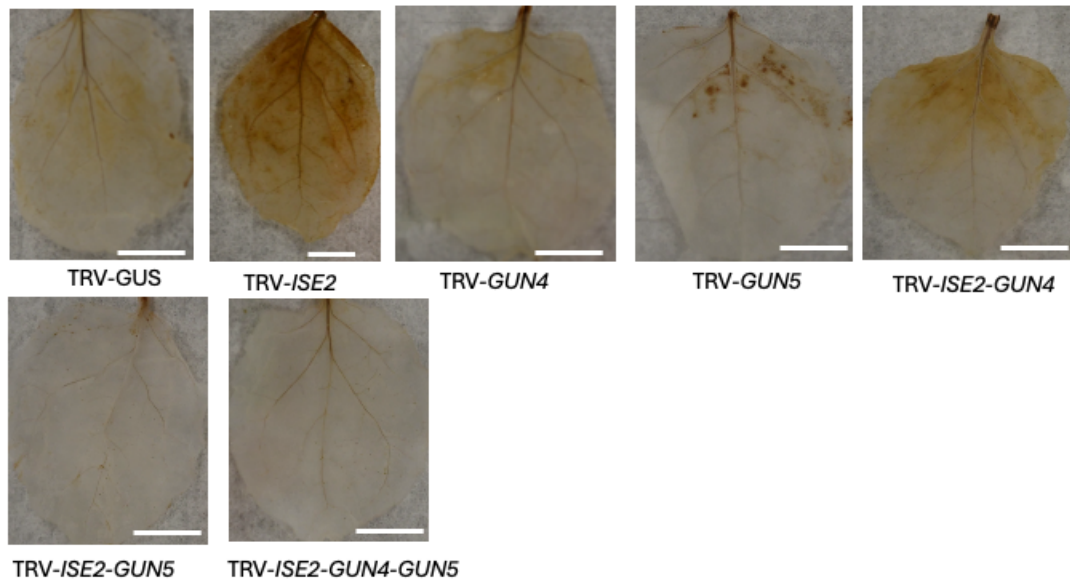
Leaf H₂O₂ levels do not correlate with trafficking through plasmodesmata

To understand the role of ROS in regulating trafficking through plasmodesmata (PD), we measured ROS production, chloroplast redox status, and the reduced status of

Figure 15. Measurement of reactive oxygen species (ROS) levels in *ISE2-GUNs*-silenced plants.

3,3'-Diaminobenzidine (DAB) stains H₂O₂ levels, with representative images (A) from non-silenced control (TRV-*GUS*) and TRV-*ISE2*-, TRV-*GUN4*-, TRV-*GUN5*-, TRV-*ISE2-GUN4*-, TRV-*ISE2-GUN5*-, and TRV-*ISE2-GUN4-GUN5*-silenced plants. The staining intensity was quantified (B) using ImageJ software, with DAB staining performed for two biological replicates. At least three plants per biological replicate were used. A Student's t-test was performed to determine statistical significance ($p < 0.05$).

(A)



(B)

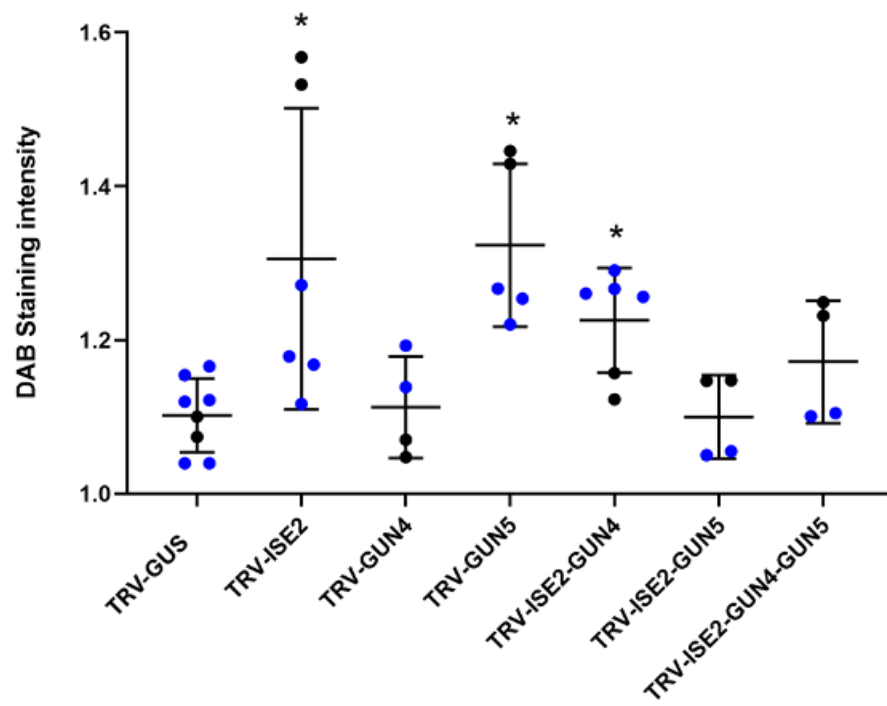
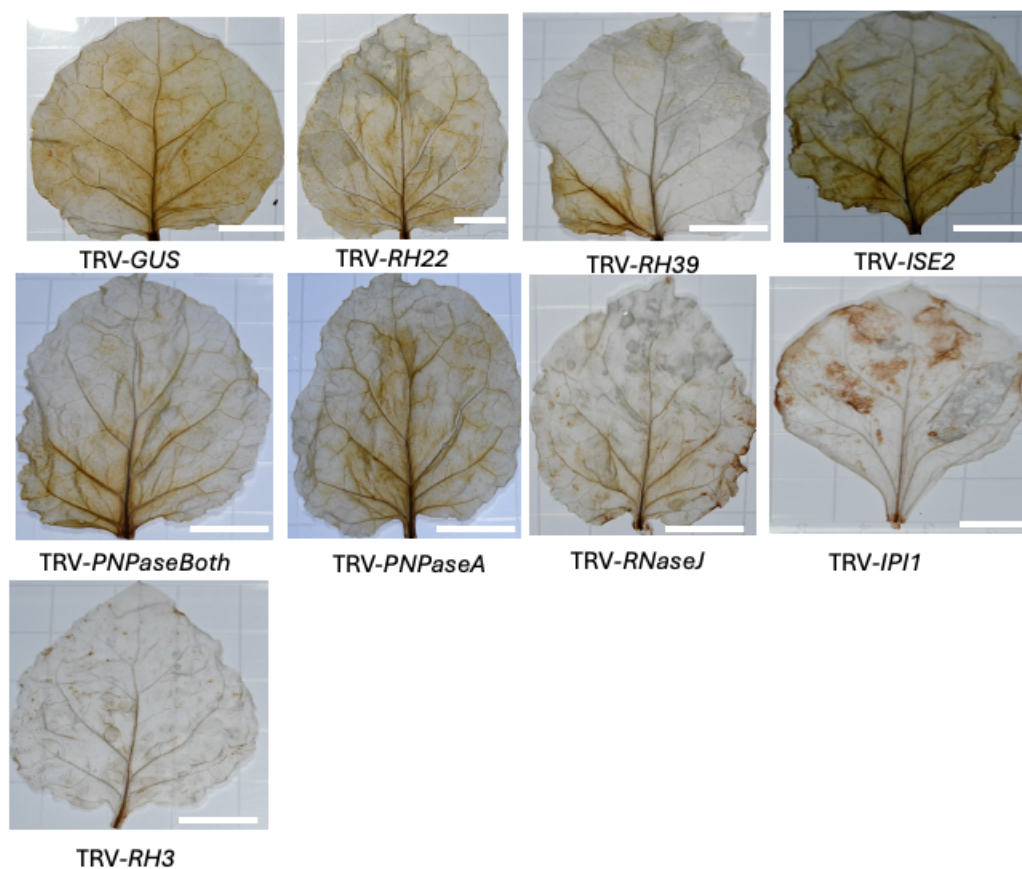


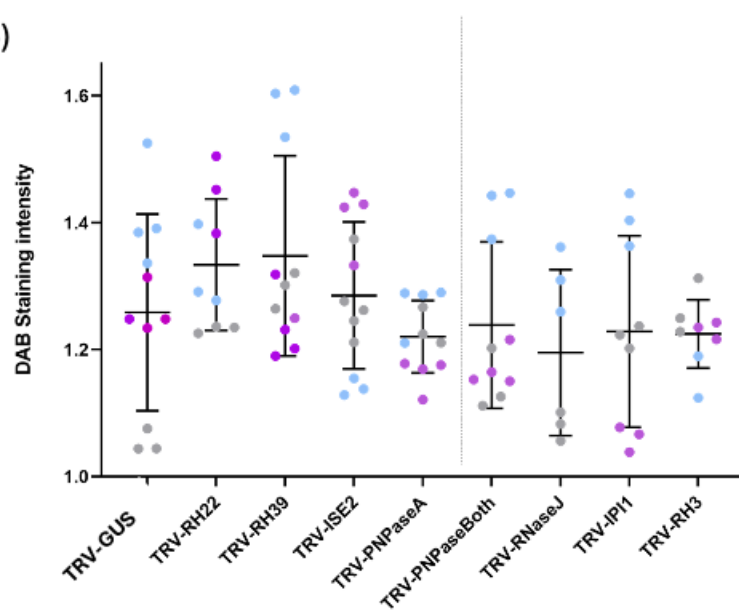
Figure 16. Reactive oxygen species (ROS) levels are measured after silencing previously known genes that regulate PD-mediated trafficking.

Measurement of reactive oxygen species (ROS) levels after silencing previously known genes (TRV-*RH22*, TRV-*RH39*, TRV-*ISE2*, TRV-*PNPaseA*, TRV-*PNPaseBoth* (silenced both homologs of *PNPases*: *PNPaseA* and *PNPaseB*), TRV-*RNaseJ*, TRV-*IP11*, TRV-*RH3*) that regulate PD-mediated trafficking. 3,3'-Diaminobenzidine (DAB) stains H₂O₂ levels, with representative images from non-silenced control (TRV-*GUS*) and TRV-*RH22*, TRV-*RH39*, TRV-*ISE2*, TRV-*PNPaseA*, TRV-*PNPaseA/B* (silenced both homologs of *PNPases*: *PNPaseA* and *PNPaseB*), TRV-*RNaseJ*, TRV-*IP11*, TRV-*RH3*-silenced plants. The staining intensity was quantified using ImageJ software, with DAB staining performed for two biological replicates. At least three plants per biological replicate were used. A Student's t-test was performed to determine statistical significance ($p < 0.05$).

(A)



(B)



plastoquinone. To assess the level of H₂O₂ in *ISE2* and/or *GUNs*-silenced plants, we used 3,3'-diaminobenzidine (DAB) staining of whole leaves. *ISE2*, *GUN5*, and *ISE2-GUN4*-silenced plants produced significantly higher ROS levels than the non-silenced control (*TRV-GUS*) plants (**Fig. 15 A,B**). In contrast, *GUN4*- and *ISE2-GUN5*- and *ISE2-GUN4-GUN5*-silenced plants exhibited lower levels of ROS production than the non-silenced control plants (**Fig. 15 A,B**). The silencing of *ISE2* led to increased trafficking, whereas silencing *GUN5* caused decreased trafficking (**Chapter 2**).

We next measured the H₂O₂ levels after silencing known chloroplast RNA metabolism genes that regulate PD-mediated trafficking, including *RH3*, *RH22*, *RH39*, *IP11*, *RNaseJ*, and two homologs of *PNPases* (*PNPaseA* and *PNPaseB*) (48). We found no statistically significant difference in H₂O₂ accumulation in silenced plants compared to non-silenced plants (**Fig 16 A,B**). Together, these results suggest that there was no correlation between H₂O₂ levels (as determined by DAB staining) and trafficking through PD.

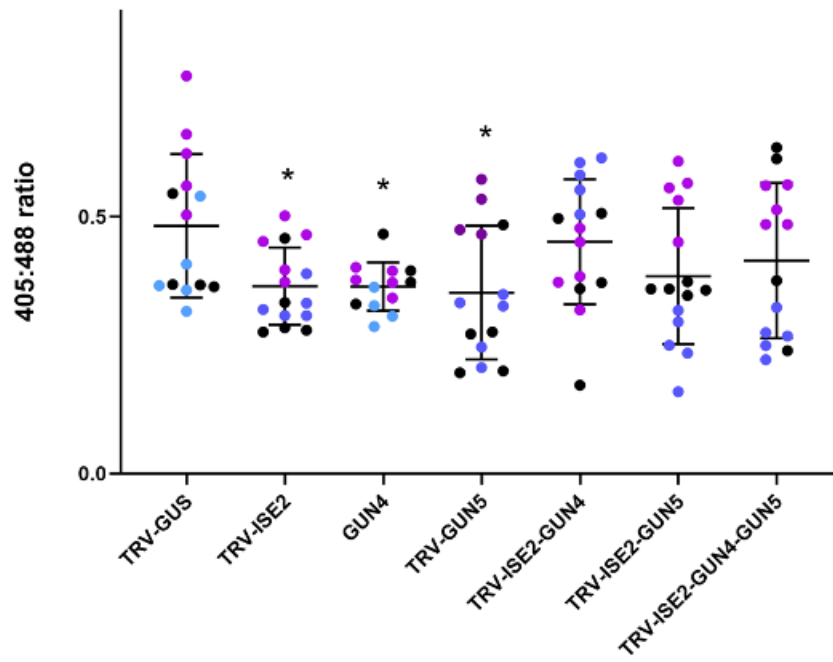
Chloroplast redox status does not correlate with trafficking through plasmodesmata

We assessed the chloroplast redox status in *ISE2-GUNs* plants using redox-sensitive fluorescent probes (chloroplast-targeted ro-GFP), performing ratiometric measurements at 405 nm and 488 nm (51). These GFP variants contain two cysteine residues that modify the amino acid side chains associated with the chromophore, and this modification promotes the formation of a neutral chromophore instead of an anionic one(177). The neutral and anionic versions show different fluorescence excitation peaks

Figure 17. Measurement of the redox status of chloroplast and plastoquinone redox status in the *ISE2-GUNs* -silenced plants.

(A). Relative redox state of the chloroplast in the non-silenced control (TRV-GUS) and TRV-*ISE2*-, TRV-*GUN4*-, TRV-*GUN5*-, TRV-*ISE2-GUN4*-, TRV-*ISE2-GUN5*-, TRV-*ISE2-GUN4-GUN5*- silenced plants were measured using the redox state sensor ro-GFP2 targeted to the chloroplast. The emission was detected in the window between 505 and 520 nm using confocal microscopy. The graph shows the ratio of fluorescence intensities 405:488 analyzed by ImageJ. **(B)** Plastoquinone redox status (QA) was measured using the LI-6800 instrument. Light response curve shows light intensities at 200, 400, 800, and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Different light intensities are shown in different color shades. The redox status of chloroplast **(A)** was performed for three biological replicates, while plastoquinone redox status **(B)** was performed for two biological replicates. At least three plants per biological replicates were used.

(A)



(B)

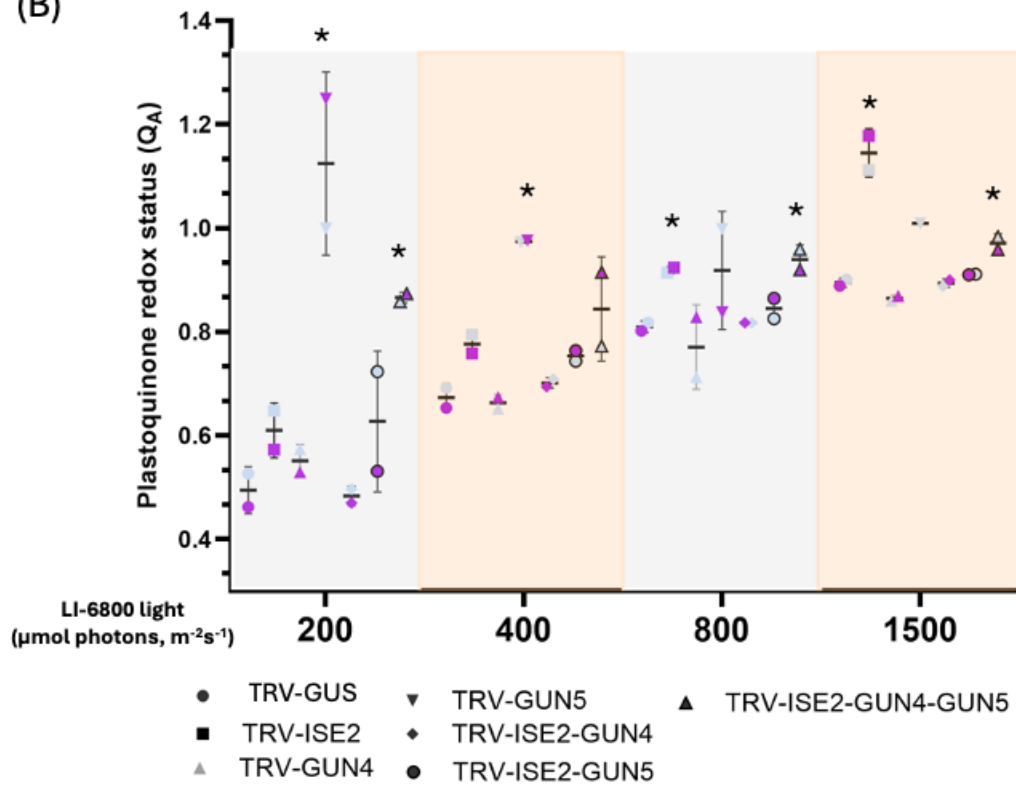
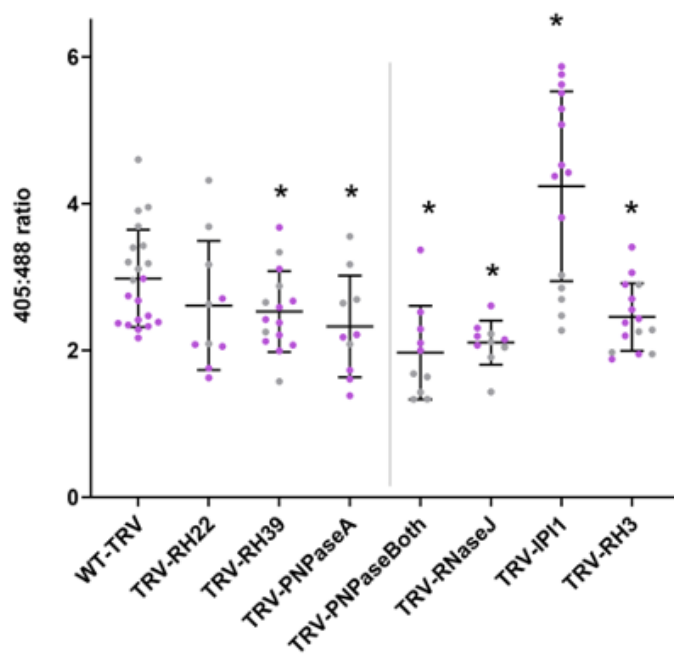


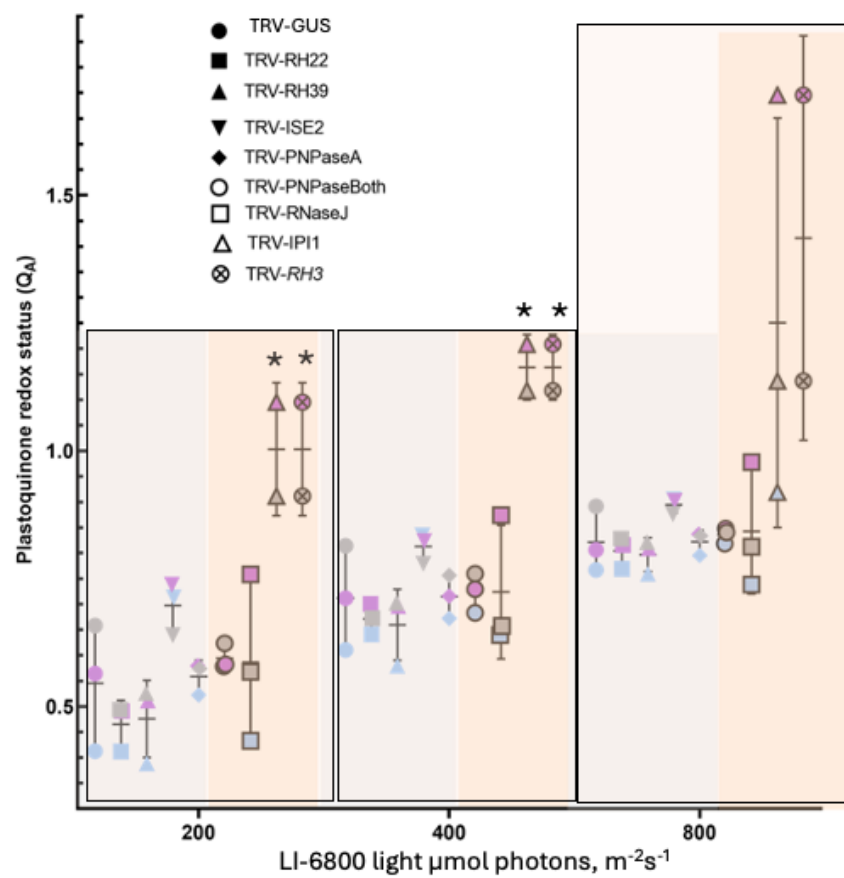
Figure 18. Redox status of chloroplast and plastoquinone redox status after silencing previously known genes that regulate PD-mediated trafficking.

(A). Relative redox state of the chloroplast in the non-silenced control (TRV-GUS) and TRV-*RH22*, TRV-*RH39*, TRV-*ISE2*, TRV-*PNPaseA*, TRV-*PNPaseBoth* (silenced both homologs of *PNPases*: *PNPaseA* and *PNPaseB*), TRV-*RNaseJ*, TRV-*IP11*, TRV-*RH3*-silenced plants were measured using the redox state sensor ro-GFP2 targeted to the chloroplast. The emission was detected in the window between 505 and 520 nm using confocal microscopy. The graph shows the ratio of fluorescence intensities 405:488 analyzed the ImageJ. **(B)** Plastoquinone redox status (QA) was measured using the LI-6800 instrument. Light response curve shows light intensities at 200, 400, 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The redox status of chloroplast (A) was performed for one biological replicate, while plastoquinone redox status (B) was performed for two biological replicates. At least three plants per biological replicates were used. Student's t-test was performed to determine statistical significance ($p < 0.05$).

(A)



(B)



at 405 and 495 nm, respectively. We determined its reduced or oxidized state by analyzing roGFP fluorescence intensity at these wavelengths. Notably, roGFP equilibrates with the redox potential of the glutathione (GSH) pool (178). Changes in oxidized roGFP levels reflect GSH to GSSG ratio changes within the specific subcellular compartment.

Chloroplast-targeted roGFP2 was utilized to evaluate the induced changes in redox potential following the silencing of *ISE2*, *GUN4*, or *GUN5* and the co-silencing of *ISE2-GUN4*, *ISE2-GUN5*, and *ISE2-GUN4-GUN5* (**Fig. 17 A**). The *ISE2*-, *GUN4*-, and *GUN5*-silenced plants exhibited more reduced chloroplasts than the non-silenced control plants (TRV-*GUS*). Conversely, *ISE2-GUN4*-, *ISE2-GUN5*, and *ISE2-GUN4-GUN5*-silenced plants did not display any changes in the redox state of the chloroplast glutathione pool. Thus, no consistent correlation pattern was found between the chloroplast's redox state and PD-mediated trafficking.

Next, we measured the chloroplast redox status in *RH3*-, *RH22*-, *RH39*-, *IPI1*-, *RNaseJ*-, *PNPaseA*, and *PNPaseA/B* (two homologs of *PNPases*: *PNPaseA* and *PNPaseB*)(48) (**Fig. 18A**). We found that only *RNaseJ*- and *IPI1*-silenced plants exhibited changes in redox status, while the other silenced plants did not show statistically significant changes in chloroplast redox status. Silencing *RNaseJ* reduced the chloroplast's state, whereas silencing *IPI1* resulted in an oxidized state. However, silencing these genes decreased PD-mediated trafficking. Overall, like with the TBS pathway genes and/or *ISE2* we observed no correlation between the chloroplast's redox status and PD-mediated trafficking.

No correlation between plastoquinone redox status and PD-mediated trafficking

To investigate the relationship between the redox state of plastoquinone (PQ) and PD-mediated trafficking, we measured QA—the redox state of the primary quinone electron acceptor of PSII—as an indicator of plastoquinone redox dynamics at different light intensities (200, 400, 800, and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) in *Nicotiana benthamiana* plants silenced for *ISE2*, *GUN4*, or *GUN5*, as well as co-silenced plants *ISE2-GUN4*, *ISE2-GUN5*, and *ISE2-GUN4-GUN5*, (**Fig 17B**) alongside several chloroplast RNA metabolism genes (*RH3*, *RH22*, *RH39*, *IPI1*, *RNaseJ*, *PNPaseA*, and *PNPaseA/B*) (**Fig 18B**). These genes were previously reported to regulate trafficking through PD. A higher PQ value indicates a more reduced PQ pool, while a lower QA value indicates a more oxidized state.

The PQ pool is crucial in photosynthetic electron transport within chloroplasts, serving as an electron shuttle between Photosystem II (PSII) and the cytochrome b_6f complex (179). In light reactions, PSII transfers electrons to PQ, transforming it into plastoquinol (PQH₂). The cytochrome b_6f complex then re-oxidizes PQH₂, enabling electron flow towards Photosystem I and eventually to NADP⁺. The redox status of the PQ pool, the ratio of reduced PQH₂ to oxidized PQ, acts as a sensitive indicator of electron transport balance and the chloroplast's redox state (180). When light absorption surpasses the ability of downstream electron acceptors- such as during high light stress or metabolic disruptions- electrons build up, resulting in a more reduced PQ pool. In contrast, an oxidized PQ pool indicates effective electron transport and balanced photosynthesis. Since the redox state of the PQ pool affects the production of reactive

oxygen species (ROS) and the initiation of photoprotective responses, it provides insight into the physiological status of the chloroplast.

Silencing *ISE2* showed a statistically significant increase in QA at 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and above, indicating a reduced PQ pool under high light conditions. Silencing *GUN5* also led to a significant increase in QA at both 200 and 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$. In contrast, silencing *GUN4* did not have a significant impact on QA at any light intensity. In the *ISE2-GUN4-GUN5*-silenced plants, we observed significant increase in QA across a broader range of light intensities (200, 800, and 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) indicating reduced PQ pool.

We also investigated the plastoquinone redox status in *RH3*-, *RH22*-, *RH39*-, *IP11*-, *RNaseJ*-, *PNPaseA*-, and *PNPaseB*—(the two homologs of PNPases). Only the *IP11*- and *RH3*- silenced plant significantly increased QA, indicating a more oxidized PQ pool. Despite these changes, we found no consistent correlation between PQ redox status and PD-mediated trafficking across the investigated silenced plants. For example, although silencing *ISE2* and *GUN5* both leads to reduced PQ, silencing *ISE2* increases trafficking while silencing *GUN5* decreases trafficking via PD. These findings suggest that PQ redox status alone may not be a direct or sufficient regulator of PD function and that other signals or pathways likely play a role in chloroplast retrograde signaling to regulate intercellular communication through PD.

DISCUSSION

Differential effect of silencing GUN4, GUN5 and ISE2 on ROS accumulation

Silencing *ISE2*, *GUN4*, and *GUN5* in *N. benthamiana* led to a reduced chloroplast state,

but only silencing *GUN5* and *ISE2* resulted in ROS accumulation. In contrast, silencing *GUN4* did not cause ROS accumulation despite a reduced chloroplast state. Since *GUN4* serves a photoprotective role in Arabidopsis, it helps to channel protoporphyrin IX and minimize ROS formation (181). The lack of ROS accumulation in *N. benthamiana*, a *GUN4*-silenced plant, may reflect species-specific regulation. *GUN5*, encoding the catalytic H subunit of Mg-chelatase, likely causes significant disruption in chlorophyll biosynthesis (**Chapter 2**), resulting in a tetrapyrrole imbalance that aligns with ROS accumulation. *ISE2*, which plays a role in chloroplast RNA metabolism, also led to increased ROS production, indicating that disturbances in chloroplast gene expression may indirectly disrupt redox balance.

Although mutants like fluorescent (*flu*) and *fc2* in Arabidopsis accumulate protochlorophyllide or protoporphyrin IX, generating singlet oxygen (119), our silenced lines showed no accumulation of ProtoIX or coproporphyrinogen III (Copro III) (Chapter 2). This indicates that the ROS accumulation observed during *GUN5* and *ISE2* silencing could arise from different sources or signaling mechanisms not related to these photoreactive intermediates.

PD-mediated trafficking does not correlate with ROS levels, the chloroplast redox state (glutathione pool), or the plastoquinone redox state.

We did not find any significant correlation between PD-mediated trafficking and the overall levels of ROS, chloroplast redox state, or plastoquinone redox state. ROS levels were evaluated using DAB staining to specifically detect H₂O₂ in the presence of peroxidase. Although DAB staining is commonly used for a rough approximation of ROS

accumulation, it solely reflects H₂O₂ levels. It does not identify other reactive oxygen species such as superoxide, hydroxyl radicals, or singlet oxygen.

Studies show that stressors like pathogens or heavy metals trigger callose and reactive oxygen species (ROS) production, regulating plasmodesmata (PD) trafficking (182,183). Also, ROS stress has been shown to impede the intercellular movement of pSUC2-GFP and hinder phloem unloading in roots (184). However, ROS alone does not lead to a specific effect on PD trafficking. For instance, *ise1* mutants and chloroplast thioredoxin *gat1* mutants both produce elevated levels of ROS, but they have opposing effects on PD-mediated intercellular trafficking: *ise1* mutants lead to an increase in PD-mediated trafficking, while *gat1* mutants decrease it (157,175,185). This indicates distinct redox-responsive signaling factors regulate PD trafficking differently in each mutant. There may be a correlation between ROS production and its effect on PD trafficking. Lower hydrogen peroxide levels increase PD-mediated trafficking, while ten times higher concentrations decrease it (54).

We also measured the relative reduced and oxidized states of chloroplast by measuring roGFP fluorescence at two different excitation maxima. Adding the RuBisCO chloroplast targeting peptide sequence allows roGFP2 import into chloroplasts and subsequent measurement of changes in the chloroplast redox state (176). Stonebloom et al. (2011) noted a correlation between a reduced state of the chloroplast and decreased PD-mediated trafficking. Consistent with this finding, we observed a reduced chloroplast redox state in *ISE2*-silenced plants, which showed increased trafficking through PD. However, when we measured the redox states following the silencing of other chloroplast

RNA metabolism genes (*RH3*, *RH22*, *RH39*, *PNPases*, *IPII*, *RNaseJ*) and tetrapyrrole metabolic genes (*GUN4*, *GUN5*), we did not find an overall correlation between PD-mediated trafficking and the chloroplast redox state. It is important to note that roGFP2 only indicates the redox potential of the glutathione pool (GSH/GSSG) and does not respond to other redox-active pools like plastoquinone, thioredoxin, and ascorbic acid/dehydroascorbic acid (171).

None of these global measurements showed a correlation with PD-mediated trafficking, indicating that global redox changes do not directly affect PD-mediated trafficking. However, we cannot rule out the possibility of localized redox changes occurring near PDs or specific ROS species that our methods did not detect. Moreover, this study did not investigate other redox systems, such as thioredoxin or ascorbate. Future research will be crucial to determine whether local or specialized redox changes are responsible for regulating PD function.

MATERIALS AND METHODS

Plant materials and growth conditions

N. benthamiana seeds were sown in pots filled with hydrated soil (Berger 35). After 10 days, germinated seedlings were transplanted into individual pots and allowed to grow for 10-12 days in a 16-hours photoperiod before performing VIGS.

Virus-Induced Gene Silencing (VIGS)

We performed VIGS as previously described in previous **chapter 2**.

DAB staining

DAB staining of the silenced leaves was performed as described by (186). First, we harvested the ninth or tenth leaf from the silenced plant after 12 to 14 days of virus-induced gene silencing (VIGS) and transferred it to a 50 ml centrifuge tube. Next, we added DAB solution (1 mg/ml 3,3-diaminobenzidine-HCl, pH 3.8) to the leaves and incubated them in the dark for at least 8 hours at room temperature with gentle agitation. After incubation, we discarded the DAB solution and rinsed the leaves with water to remove excess stains. Subsequently, we added absolute ethanol to cover the leaves and incubated them in a 65°C water bath for 2 hours to clear the chlorophyll. We then replaced the absolute ethanol with 75% ethanol and incubated for 1 hour. This was followed by replacing it with 50% ethanol until the leaves completely lost their green color. Finally, we transferred the leaves to petri dishes filled with water for dehydration and photographed them using a Nikon camera. The images were then analyzed using ImageJ. Fluorescence intensity was measured from 2-3 regions of interest (ROI) on each leaf and normalized by dividing the background intensity. The analysis was performed using two biological replicates.

Measurement of Plastoquinone redox status

Plastoquinone redox status was measured using LI-6800 as previously described (187). Briefly, eight/night leaves after 12-14 days of silencing were used to measure plastoquinone redox status. First, plants were dark-adapted for 20 min in a Li-6800 chamber under constant CO₂ partial pressure of 400 ppm in the sample cell, leaf temperature 25°C, leaf VPD 1.5 kPa, fan speed 10,000 RPM, and flow rate 500 μ mol-s⁻¹.

To measure the light response curves, LI-6800 light intensities of 200, 400, and 800 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ were used to measure the light response curves.

Confocal microscopy

In the redox state detection experiment, we modified the confocal imaging as described in (188). We observed roGFP2 emission using 405 nm (oxidized) and 488 nm (reduced) lasers, capturing emissions from 505 to 520 nm. Chlorophyll autofluorescence was recorded from 690 to 750 nm using a 488 nm laser. We used a 20x objective and 3x zoom and averaged 2 frames for imaging the leaves. Regions were scanned unidirectionally at 400 Hz. Fluorescence emissions were collected across three channels with a z-stack of 10 steps covering at least 30 μm . Images were analyzed by importing TIFF files, converting them to grayscale, filtering out low-intensity pixels, and applying spatial adaptive thresholding for binary conversion. We calculated the fluorescence intensity ratio (405:488) per pixel within the chloroplast masks.

Statistical Analysis

Analyses were performed using the *t*-tests for statistical significance. Unless otherwise indicated, $P < 0.05$ was considered to be significant.

CHAPTER 4

IDENTIFICATION OF PLASMODESMATA-RELATED GENES BY

DIFFERENTIAL GENE EXPRESSION ANALYSES

ABSTRACT

While the protein and lipid composition at the plasmodesmata (PD) are extensively studied, the transcriptional expression networks related to plasmodesmal dynamics are less explored. To understand the regulation of trafficking through PD, we investigated the interactions of PD-associated genes at the transcriptomic level and identified key hub genes that regulate PD-mediated trafficking. We performed RNA sequencing after silencing the chloroplast RNA metabolism gene *ISE2* (TRV-*ISE2*-) alone and/or in combination with the tetrapyrrole biosynthetic genes *GUN4* (TRV-*ISE2*-*GUN4*) and *GUN5* (TRV-*ISE2*-*GUN5*), or both (TRV-*ISE2*-*GUN4*-*GUN5*). In Chapter 2, we showed that silencing *ISE2*, *GUN4*, and *GUN5* alone or in combination resulted in the differential regulation of PD-mediated trafficking. In this chapter, we analyzed these RNA-seq data using differential gene expression (DEG) analyses and identified four clusters of DEGs from the analysis that whose functions may be significantly related to PD as determined by Gene Ontology (GO) analysis. Additionally, we utilized a prediction pipeline tool called plasmodesmata in silico proteome 1 (PIP1), to identify the candidate PD genes that are enriched in PD-related GO terms. The enrichment of PD-related GO terms suggest biological relevance and could help uncover unknown regulatory mechanisms associated with PD regulation.

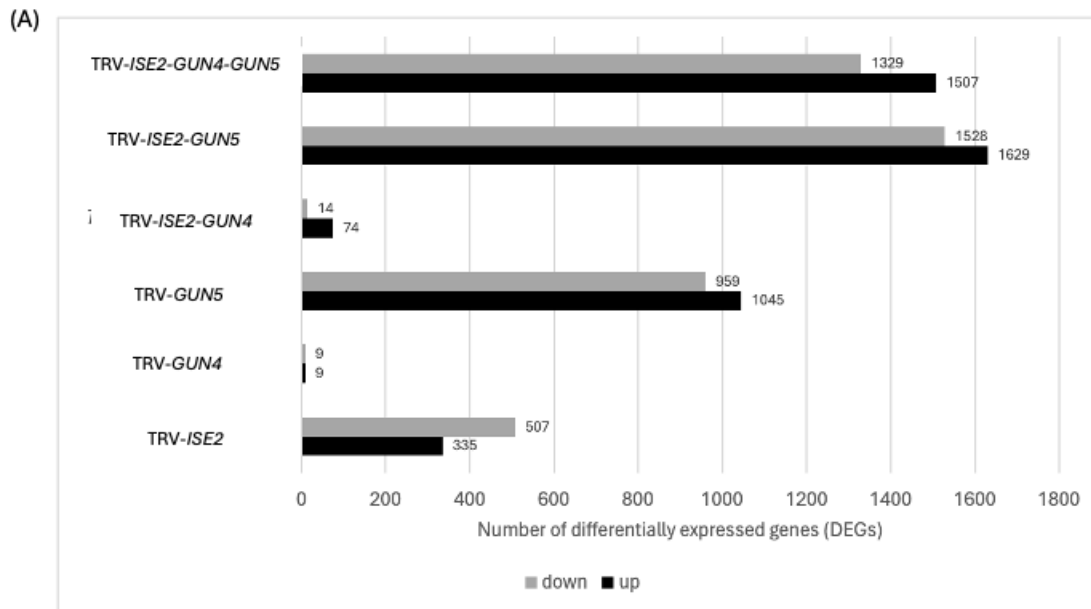
INTRODUCTION

The studies of the protein and lipid composition of plasmodesmata (PD) aim to enhance our understanding of their roles. Structural proteins such as actin and myosin as well as scaffolding proteins like remorin and tetraspanins are found at PD. MCTPs and SYNAPTOTAGMIN (SYT) connect the endoplasmic reticulum (ER) to the plasma membrane (PM) (65,189). Tetraspanins are also localized at PD (190,191) Signaling proteins at PD include receptor proteins that respond to various plant growth and defense mechanisms against pathogens (192). FLAGELLIN SENSING2 (FLS2) and LYSIN MOTIF DOMAIN-CONTAINING PROTEIN2 (LYM2) act as receptors for bacterial and fungal pathogens (193) CALMODULIN-LIKE41 (CAML41) operates downstream of FLS2, while PLASMODESMATA-LOCATED PROTEIN 5 (PDL5) is crucial for bacterial signaling immunity.

Callose, a polysaccharide of glucose synthesized by CALLOSE SYNTHASES (CalSs), restricts cell-to-cell communication by accumulating near the necks of plasmodesmata, restricting transit through the pore (194). Six of twelve CalS are reportedly located at the plasmodesmata (195–199). Beta-1,3-glucanases from family GH17 are involved in callose degradation; four beta-1,3-glucanases, including AtBG_PPAP and PLASMODESMATA LOCALIZED BETA-1,3-GLUCANASES (PDBG1-3), associate with PD (200). Furthermore, PLASMODESMATA CALLOSE-BINDING PROTEINS (PDCBs) bind to callose at the PD (201). The developmental signaling proteins CLAVATA 1 (CLV1) and ARABIDOPSIS CRINKLY 4 (ACR4) are also located at the PD (202).

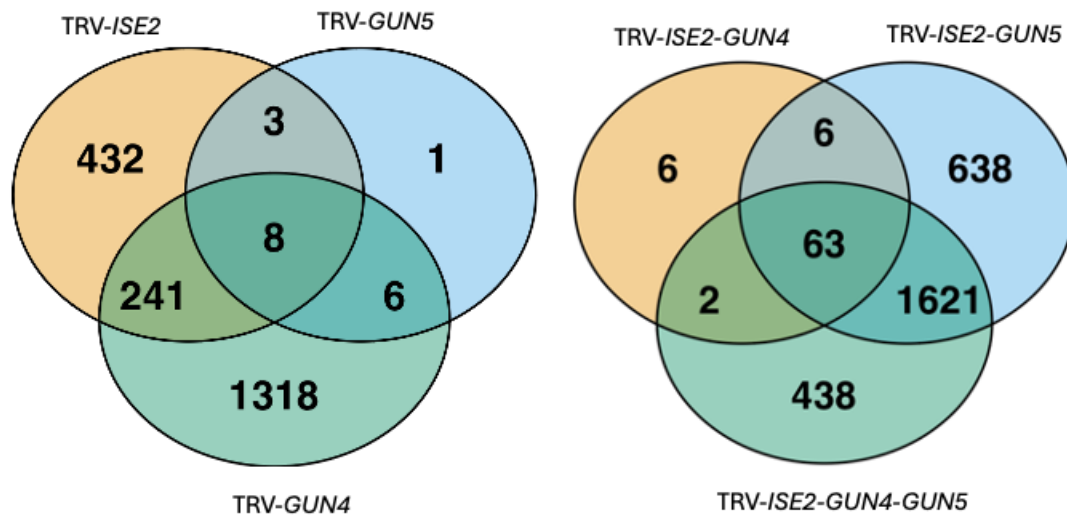
Figure 19. RNA sequencing analyses of *ISE2-GUNs*- silenced plants.

(A) Number of DEGs in *ISE2-GUNs* silenced plants. (B) Venn diagram showing the overlap of the DEGs in *ISE2*-, *GUN4*- and *GUN5*- silenced plants. (C) Venn diagram showing the overlap of the DEGs in *ISE2-GUN4*-, *ISE2-GUN5*- and *ISE2-GUN4-GUN5*- silenced plants. Only DEGs with adjusted p values below 0.05 are shown, and values on the heat map show $\log_2$ fold change in expression.



(B)

(C)



To enhance our understanding of the structure and regulation of PD, it is crucial to explore transcriptional expression networks related to plasmodesmal dynamics. In this study, we will investigate the transcriptional change of PD- and cell-wall- associated genes, aim to identify the protein-protein interaction (PPI) network of proteins encoded by PD-related genes.

RESULTS

Differential gene expression analysis in ISE2, either alone or co-silenced with GUN4 and/or GUN5 plants

We aimed to identify the shared differential expression genes that regulate PD-mediated trafficking by integrating the RNA-seq data from plants with different defective RNA processing and defective retrograde signaling that is correlated with increased or decreased intercellular trafficking. To understand which genes are involved in regulating PD-mediated trafficking by *ISE2* and *GUN5* and whether *ISE2* and *GUN5* utilize the same pathway for this regulation, we conducted a transcriptomic analysis. In the *ISE2*-silenced plant, 842 genes were significantly differentially expressed, with a cutoff for adjusted p-values of <0.05. This analysis revealed 335 genes whose expression was induced and 507 whose expression was repressed (**Fig 19A**).

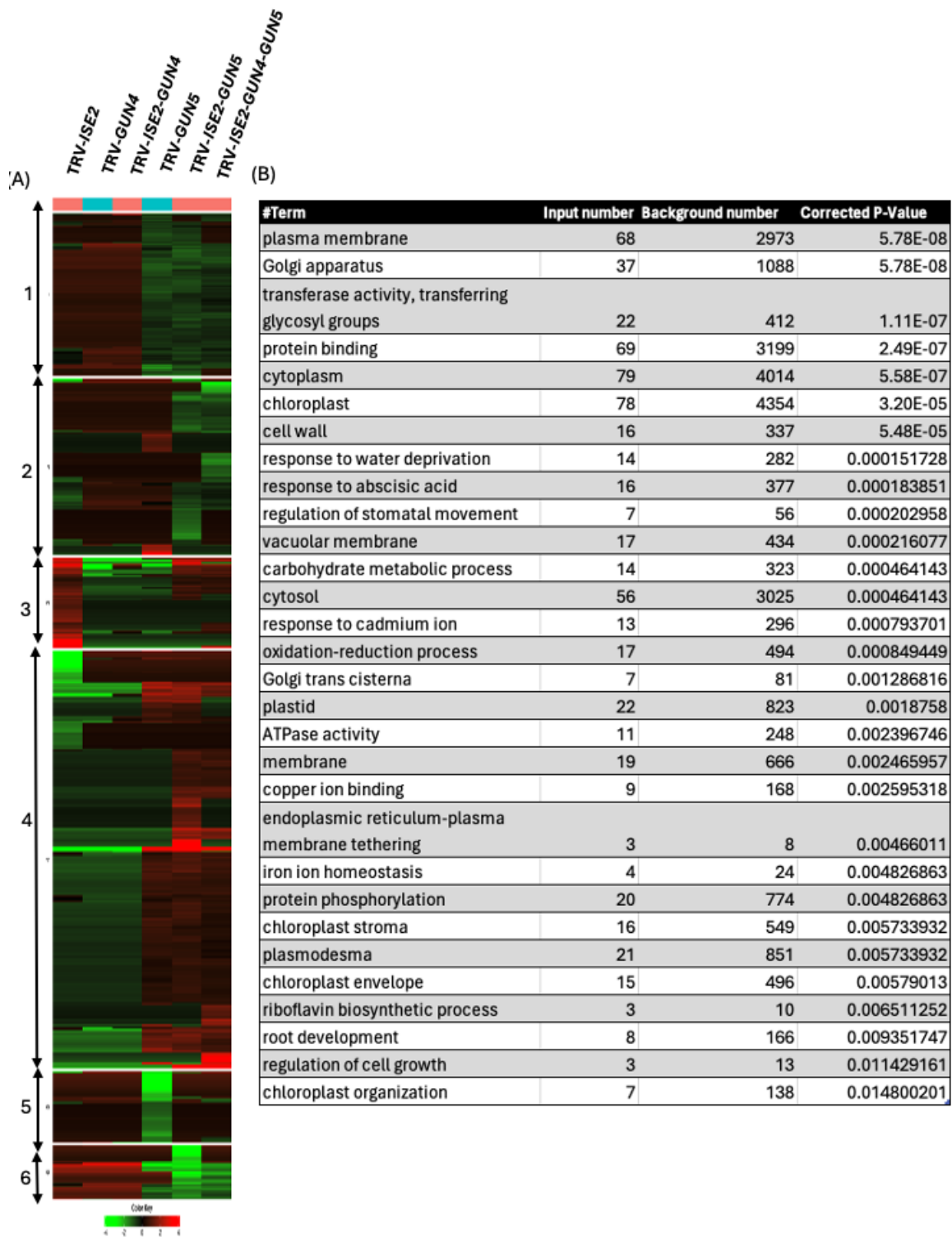
In the *GUN4*-silenced plants, only 18 genes were significantly differentially expressed, with nine genes induced and 9 suppressed (**Fig 19**). In striking contrast, *GUN5*-silenced plants showed that 2,004 genes were significantly differentially expressed, where 1,045 genes were induced, and 959 genes were suppressed. For co-silenced plants, *ISE2*-

GUN4-silenced plants exhibit only 88 genes (74 induced and 14 suppressed genes) that are differentially expressed. In contrast, *ISE2-GUN5* and *ISE2-GUN4-GUN5* silenced plants show 3,155 genes (1,629 induced and 1,528 suppressed genes) and 2,836 genes (1,507 induced and 1,329 suppressed genes). Silencing *GUN5*- alone showed the highest number of DEGs genes compared to *ISE2*- and *GUN4*- alone silenced plants. For co-silenced plants, *ISE2-GUN5*- exhibited the highest number of DEGs for co-silenced plants compared to *ISE2-GUN4*- and *ISE2-GUN4-GUN5*- silenced plants.

The Venn diagram analysis of differentially expressed genes (DEGs) revealed only 8 DEGs shared among *ISE2*, *GUN4*, and *GUN5*-silenced plants. A total of 241 genes were shared between *ISE2* and *GUN5*- silenced plants, indicating a common regulatory mechanism (**Fig. 19B**). In terms of unique DEGs, *ISE2* silencing displayed 432 uniquely expressed genes, *GUN4* silencing presented only 1 unique gene, and *GUN5* silencing showed 1,318 unique genes, suggesting that each silencing has distinct gene-specific effects. Regarding pairwise overlaps, the highest overlap was detected between *ISE2*- and *GUN5*-silenced plants, with 241 genes shared, while only 6 genes were common between *GUN4* and *GUN5*, and another 6 genes were shared between *ISE2*- and *GUN4*-silenced plants. The substantial overlap of shared genes between *ISE2* and *GUN5* suggests a potential functional link where *ISE2*-mediated RNA processing intersects with *GUN5*'s roles in plastid signaling. This implies that gene expression and tetrapyrrole biosynthesis in plastids are connected.

For co-silenced plants, *ISE2-GUN4*-silenced plants showed 8 uniquely expressed genes, while *ISE2-GUN5*- and *ISE2-GUN4-GUN5*-silenced plants showed 838 and

Figure 20. Heatmap of k-means-clustered DEGs and GO term enrichment analysis.
(A) Differentially expressed genes of *ISE2-GUNs* silenced plant are clustered into six groups using k-means. (B) GO-term enrichment (top 30) in cluster 1. Only DEGs with adjusted p values below 0.05 are shown, and values on the heat map show log₂ fold change in expression.



438 expressed genes, respectively (**Fig. 19C**). For shared DEGs, *ISE2-GUN4*- and *ISE2-GUN5*- silenced plants shared only 6 genes, whereas *ISE2-GUN5*- and *ISE2-GUN4-GUN5*- silenced plants shared 1621 DEGs. This suggests that *GUN5*-silencing has a higher impact on differential gene expression in these co-silenced plants compared to silencing *ISE2*- or *GUN4*.

Clustering analysis of DEGs in heatmap following silencing *ISE2*-, *GUN4*-, *GUN5*- alone and their co-silencing *ISE2-GUN4*-, *ISE2-GUN5*-, *ISE2-GUN4-GUN5*- reveals six clusters (**Fig. 20 A**). A striking observation was the opposing regulation of these clusters following the silencing of different genes alone or in combination. When *ISE2*, *GUN4*, and *ISE2-GUN4* were silenced, DEGs in cluster 1 were upregulated while downregulated after silencing *GUN5*, *ISE2-GUN5*, and *ISE2-GUN4-GUN5*.

Enrichment of gene ontology (GO) terms in cluster 1 and analysis of protein-protein networks for PD-related proteins

To gain a broad understanding of the functions of DEGs of cluster 1, GO term and KEGG pathway (**Appendix Fig. 25**) enrichment were performed. GO enrichment analysis revealed that DEGs in cluster 1 enriched several cellular compartments, including plasma membrane, cell wall, chloroplast, plasmodesma, and endoplasmic reticulum-plasma membrane tethering (**Fig. 20B**). This suggests a link between chloroplast functionality and intercellular communication through PD. In addition, GO terms, including response to abscisic acid, iron ion homeostasis, and oxidation-reduction process, were also enriched in cluster 1.

Protein-protein interaction (PPI) networks were constructed using 21 genes

enriched for PD-related GO terms, revealing distinct interactions like QKY-SYT1-F4F15.170 (**Fig. 21A,B**). QKY (QUIRKY) is a C2-domain protein found at PD (203), while SYT1 (SYNAPTOTAGMIN-1) facilitates membrane tethering and lipid transfer between the plasma membrane and ER (204). The protein F4F15.170 is a beta-1,6-N-acetylglucosaminyl transferase-like enzyme (AtGnTL) located at PD (205) and may link SYT1-dependent vesicle trafficking to QKY-regulated lipid remodeling. Another network, PIP2.1-AHA1-AKT2, includes PIP2.1 (aquaporin), AHA (proton gradient control) and AKT2 (potassium channel) (206), suggesting a role in ion and water trafficking through PD.

In the SPP2-BIP2 network, SPP2 (PROBABLE SUCROSE PHOSPHATASE) relates to sucrose metabolism (207), accompanied by BIP2, a chaperone protein for proper folding (208). This interaction may regulate carbon partitioning and sugar transport via PD. The PER42-TMK2 network features PER42 (PEROXIDASE 42) in oxidative stress response (209) and TMK2 (TRANSMEMBRANE KINASE 2 (210), is a transmembrane kinase activates RHO-RELATED GTPASES FROM PLANTS (ROPs) GTPase are part of auxin signaling system, indicating a potential redox-dependent and hormonal regulatory role at PD. However, in our silenced plants, we found no correlation between redox signaling and differential regulation of trafficking through PD (**Chapter 3**). On the other hand, auxin diffusion through PD is essential for distributing auxin, which influences organ initiation, tropic responses, and growth (211,212). The potential role of TMK2 in regulating PD-mediated trafficking could be further investigated.

Some proteins in the network lack direct interactions but may still influence PD regulation. MS1 (5-METHYLTETRAHYDROPTEROYTRIGLUTAMATE-HOMOCYSTEINE METHYLTRANSFERASE 1) is involved in methionine biosynthesis (213), potentially modulating protein activity that affects trafficking. SKS5, a pectinesterase-like protein, may assist in cell wall remodeling (214) at PD. PDCB4 (PLASMODESMATA CALLOSE-BINDING PROTEIN 4) binds callose and its metabolism involve in regulating PD-mediated trafficking (215). RPL13B (60S ribosomal protein L13-1) and GLN1-1 (GLUTAMATE SYNTHETASE1-1) likely contribute to protein synthesis and nitrogen assimilation (216), respectively, and may be affecting amino acid trafficking. NRAMP3 regulates iron homeostasis under deficiency (217) may affect plasmodesmal permeability through callose metabolism, as iron homeostasis has been previously reported to regulate callose deposition (218). RAN3, a small GTPase (219), may facilitate vesicle trafficking for plasmodesmal protein delivery or membrane restructuring. NAT (Nucleobase-Ascorbate transporter)1 likely assists in ascorbate transport (220) and ROS scavenging, may influence trafficking. CYP19-2 (CYCLOPHILIN 19-2) regulates PD function, directing mRNA transport and communication(221). Finally, T4L20.60 (GLUCAN ENDO-BETA-1,3-GLUCOSIDASE) hydrolyzes beta-1,3-glucan, critical for callose degradation(222), suggesting modulation of plasmodesmal function.

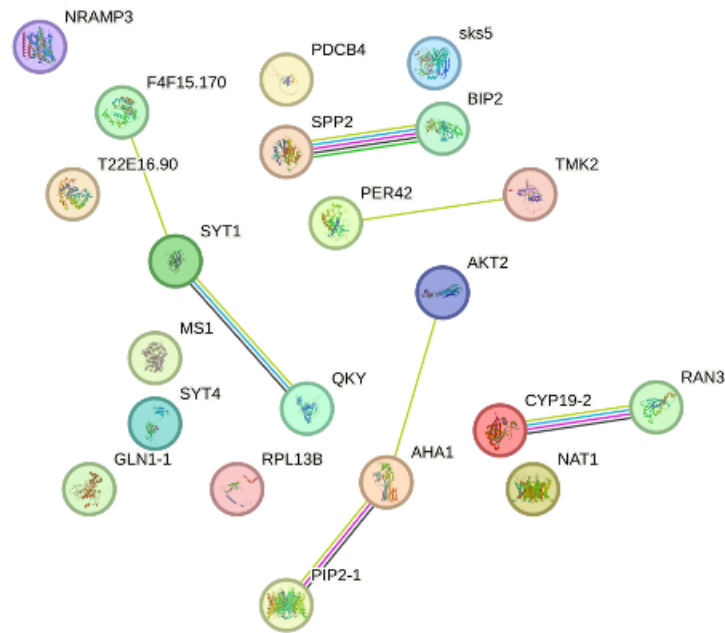
Enrichment of gene ontology (GO) terms in cluster 2 and analysis of protein-protein networks for PD-related proteins

GO enrichment analysis of DEGs in cluster 2 identified cellular compartments, including

Figure 21. Protein-protein network showing the interaction and description of plasmodesmata-related protein enriched in GO term analysis of cluster 1.

(A) Protein-protein networks are made using the STRING database (<https://string-db.org/>) and description of these protein are shown in (B). Network nodes represent proteins and the edge represents protein-protein association. Association between proteins means they jointly contribute to shared function and does not necessarily mean they are physically binding to each other. Cyan and purple edges represent known interactions from curated databases or experimentally determined. Green, red, and blue edges represent predicted interactions. Light green, black, and sky blue edges represent other interactions from textmining, co-expression, and protein homology, respectively.

(A)



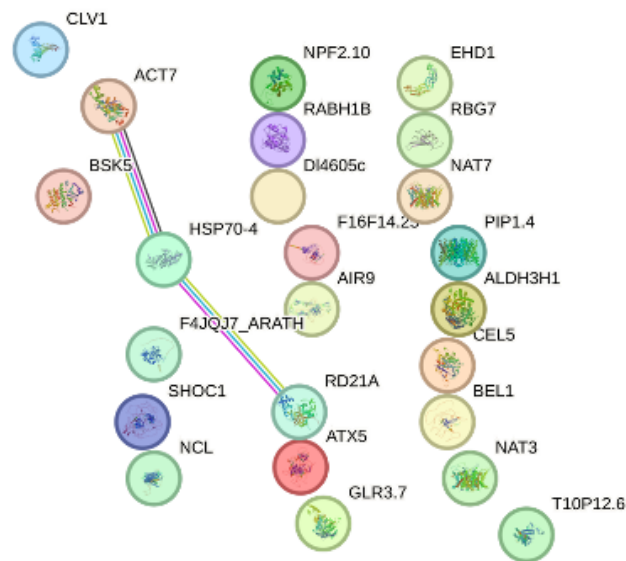
(B)

Gene Symbol	Description
CYP19-2	Peptidyl-prolyl cis-trans isomerase CYP19-2
AHA1	ATPase 1, plasma membrane-type
NAT1	Nucleobase-ascorbate transporter 1
PER42	Peroxidase 42
SYT1	Synaptotagmin-1
QKY	Protein QUIRKY
SYT4	Synaptotagmin-4
sks5	AT1G76160 protein. (541 aa)
AKT2	Potassium channel AKT2/3
NRAMP3	Metal transporter Nramp3
RPL13B	60S ribosomal protein L13-1. (206 aa)
TMK2	Receptor-like kinase TMK2
SPP2	Probable sucrose-phosphatase 2
T22E16.90	O-Glycosyl hydrolases family 17 protein
PDCB4	PLASMODESMATA CALLOSE-BINDING PROTEIN 4. (222 aa)
PIP2-1	Aquaporin PIP2-1
MS1	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase 1
GLN1-1	Glutamine synthetase cytosolic isozyme 1-1
RAN3	GTP-binding nuclear protein Ran-3
F4F15.170	Core-2/1-branching beta-1,6-N-acetylglucosaminyltransferase family protein. (346 aa)
BIP2	Heat shock 70 kDa protein BIP2

Figure 22. Protein-protein network showing the interaction and description of plasmodesmata-related protein enriched in the GO term analysis of cluster 2.

Protein-protein networks (A) were made using the STRING database (<https://string-db.org/>). The description of these proteins are shown in (B). Network nodes represent proteins, and the edge represents protein-protein association. Association between proteins means they jointly contribute to shared function and does not necessarily mean they are physically binding to each other. Cyan and purple edges represent known interactions from curated databases or experimentally determined. Green, red, and blue edges represent predicted interactions. Light green, black, and sky blue edges represent other interactions from textmining, co-expression, and protein homology, respectively.

(A)



(B)

Gene Symbol	Description
ATX5	Histone-lysine N-methyltransferase ATX5
CEL5	Endoglucanase 3
ALDH3H1	Aldehyde dehydrogenase family 3 member H1
GLR3.7	Glutamate receptor 3.7
NPF2.10	Protein NRT1/PTR FAMILY 2.10
HSP70-4	Heat shock 70 kDa protein 4
PIP1.4	Probable aquaporin PIP1-4
CLV1	Receptor protein kinase CLAVATA1
SHOC1	Protein SHORTAGE IN CHIASMATA 1
RABH1B	Ras-related protein RABH1b
F16F14.25	Probable LRR receptor-like serine/threonine-protein kinase At2g16250
BSK5	Serine/threonine-protein kinase BSK5
ACT7	Actin-7
NAT7	Nucleobase-ascorbate transporter 7
DI4605c	Pleckstrin homology (PH) domain-containing protein. (4219 aa)
BEL1	Homeobox protein BEL1 homolog
AIR9	187-kDa microtubule-associated protein AIR9
EHD1	EH domain-containing protein 1
RBG7	Glycine-rich RNA-binding protein 7
NAT3	Nucleobase-ascorbate transporter 3
T10P12.6	Sphingomyelin synthetase family protein. (421 aa)
NCL	Sodium/calcium exchanger NCL
F4JQJ7_ARATH	PLC-like phosphodiesterases superfamily protein. (408 aa)
RD21A	Cysteine proteinase RD21A

the plasma membrane and chloroplast (envelope, stroma, thylakoid membrane), linking chloroplast and intercellular trafficking via PD (**Appendix Fig. 26**). The PPI network of 23 DEGs enriched in the PD related GO term showed that only RD21A, HSP70-4, and ACT7 interacted, while the others showed no known interaction. RD21A facilitates protein turnover (223), HSP70-4 aids protein folding (224), and ACT7 supports the cytoskeleton(225) (**Fig 22A, B**). Their interaction suggests a regulatory mechanism at PD, integrating actin remodeling, protein turnover, and folding to maintain function. Twenty genes in cluster 2 exhibited no known direct interactions in the PPI network. CEL5-Endoglucanase 3 can alter cell walls (226) and may regulate PD. Membrane transporters such as PIP1.4 (aquaporin) (227), NAT3 (nucleobase-ascorbate transporter)(228), and NCL (sodium/calcium exchanger) (228) may also play a role. Signaling proteins and kinases, including BSK5 (BRASSINOSTEROID-SIGNALING KINASE 5), is a serine/threonine kinase that is localized to the cell periphery (229) and may be involved in regulating PD function, while CLV1 (receptor kinase) is known to regulate PD trafficking (230). RNA binding proteins like RBG7 (glycine-rich RNA-binding protein 7) (231) may facilitate selective mRNA movement through PD.

Enrichment of gene ontology (GO) terms in cluster 3

Cluster 3 showed significant enrichment in functional categories related to protein processing, stress response, transcriptional regulation, and cellular signaling (**Appendix Fig. 27**). GO terms such as "response to heat," "protein folding," and "chaperone-mediated protein complex assembly" highlight Cluster 3's role in maintaining protein homeostasis and adapting to stress. Moreover, GO terms like "response to reactive

oxygen species" and "L-ascorbate peroxidase activity" underscore Cluster 4's function in redox homeostasis. The GO term "positive regulation of transcription" indicates Cluster 3's participation in gene expression. Additionally, GO terms such as "phosphatidylinositol binding" suggest a link to membrane trafficking and signaling, which are essential for endosomal sorting.

Enrichment of gene ontology (GO) terms in cluster 4 and analysis of protein-protein networks for PD-related proteins

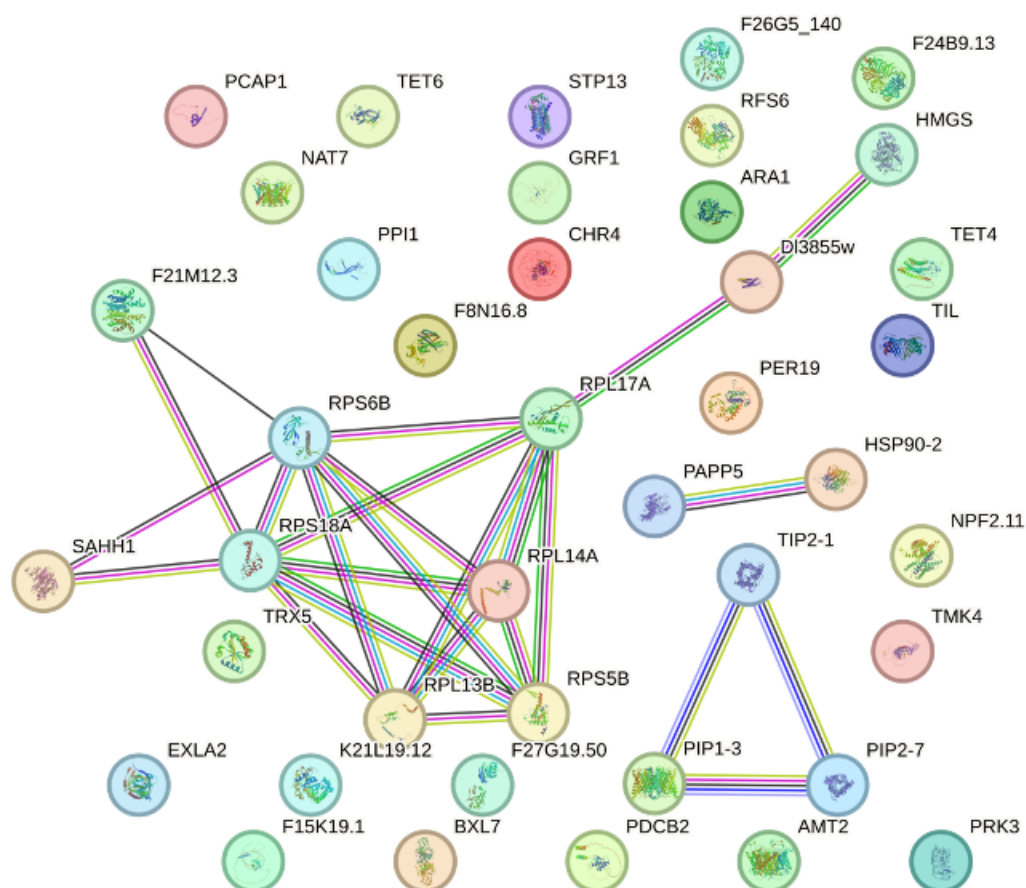
Cluster 4 shows GO term enrichment in biological processes, including responses to water deprivation, oxidative stress, wounding, and salicylic acid (**Appendix Fig. 28**). Plastid transcription indicates genes that regulate chloroplast gene expression. The regulation of jasmonic acid transport and ATP transmembrane transport suggests roles in hormone signaling and metabolic transport. GO terms like plastid and PD highlight involvement in chloroplast functions and intercellular communication. The analysis indicates a strong representation of genes in plastid function and photosynthesis regulation, suggesting nuclear-encoded factors significantly influence chloroplast gene expression. Overall, this GO enrichment analysis emphasizes functional categories related to chloroplast function, transcriptional regulation, stress responses, and intercellular communication.

The PPI network analysis of PD-enriched genes in cluster 4 reveals interactions among genes and proteins related to PD. RPL17A, RPS18A, RPL13B, RPL14A, and RPS5B cluster together, highlighting the importance of protein synthesis for PD-mediated trafficking (**Fig 23A,B**). Aquaporins PIP1-3, PIP2-7, and TIP2-1 transport

Figure 23. Protein-protein network showing the interaction and description of plasmodesmata-related protein enriched in GO term analysis of cluster 4.

(A) Protein-protein networks are made using the STRING database (<https://string-db.org/>). The descriptions of these proteins are shown in (B). Network nodes represent proteins and the edge represents protein-protein association. Association between proteins means they jointly contribute to shared function and does not necessarily mean they are physically binding to each other. Cyan and purple edges represent known interactions from curated databases or experimentally determined. Green, red, and blue edges represent predicted interactions. Light green, black, and sky blue edges represent other interactions from textmining, co-expression, and protein homology, respectively.

(A)



(B)

Gene Symbol	Description
CHR4	Protein CHROMATIN REMODELING 4
PER19	Peroxidase 19
F8N16.8	Pathogenesis-related thaumatin superfamily protein. (274 aa)
PDCB2	PLASMODESMATA CALLOSE-BINDING PROTEIN 2
ARA1	L-arabinokinase
F15K19.1	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein. (291 aa)
PRK3	Pollen receptor-like kinase 3
PIP2-7	Aquaporin PIP2-7
TIL	Temperature-induced lipocalin-1
STP13	Sugar transport protein 13
PCAP1	Plasma membrane-associated cation-binding protein 1
TMK4	Receptor-like kinase TMK4
RPL14A	60S ribosomal protein L14-1
DI3855w	CASP-like protein 1E1. (190 aa)
HSP90-2	Heat shock protein 90-2
BXL7	Probable beta-D-xylosidase 7. (767 aa)
SAHH1	Adenosylhomocysteinase 1
RPL13B	60S ribosomal protein L13-1. (206 aa)
RPS5B	40S ribosomal protein S5-2
NPF2.11	Protein NRT1/ PTR FAMILY 2.11
RFS6	Probable galactinol-sucrose galactosyltransferase 6
TET6	Tetraspanin-6
NAT7	Nucleobase-ascorbate transporter 7
PIP1-3	Aquaporin PIP1-3
TRX5	Thioredoxin H5
GRF1	Growth-regulating factor 1
F24B9.13	RmlC-like cupins superfamily protein. (356 aa)
AMT2	Ammonium transporter 2
TET4	Tetraspanin-4
RPL17A	60S ribosomal protein L17-1
F21M12.3	Probable elongation factor 1-gamma 1
HMG5	Hydroxymethylglutaryl-CoA synthase
F27G19.50	Aluminum induced protein with YGL and LRDR motifs. (250 aa)
F26G5_140	FAD/NAD(P)-binding oxidoreductase family protein. (367 aa)
RPS18A	40S ribosomal protein S18
K21L19.12	Glucan endo-1,3-beta-glucosidase 6. (477 aa)
PPI1	Proton pump-interactor 1
RPS6B	40S ribosomal protein S6-2
EXLA2	Expansin-like A2. (265 aa)
TIP2-1	Aquaporin TIP2-1
PAPP5	Serine/threonine-protein phosphatase 5

water and solutes (232), while NPF2.10 (nitrate transporter) facilitate nitrate transport (233) and AMT2 (ammonium transporter) facilitate nutrient exchange (234). HSP90-2 (heat shock protein) (235), PER19 (ascorbate peroxidase) contribute to stress responses (236). SAHH1 (Adenosylhomocysteinase 1) is linked to methylation(237), while CHR4 (Chromatin Remodeling 4) (238) may regulate the translation of plasmodesmal proteins.

GRF1 (Growth-Regulating Factor 1) (239) and TRX1 (Thioredoxin 1) are involved in redox regulation and growth and may impact PD function. ARA1 (L-arabinokinase) and BXL7 (beta-D-xylosidase 7) modify the cell wall(240,241), which may be important for the PD function. TET6 and TET4 belong to the tetraspanins family of proteins that have been found to localize at PD (5,190), suggesting their role in the recruitment or activity of signaling components at PD. PRK3 (Pollen Receptor-Like Kinase 3) plays a role in pollen tube (242), while TMK4 is a receptor kinase on the plasma membrane that plays a vital role in cell growth(243). F26G5_140 (FAD/NAD(P)-binding oxidoreductase family protein) may catalyze the redox reaction ((244). PRK3, TMK4 and F26G5_140 may play a role, by a yet unknown mechanism, as a signaling protein to regulate PD function. PCAP1 (Plasma Membrane-Associated Cation Binding Protein 1) is critical for calcium signaling which plays role in modulating PD by affecting the function of myosin (245). EXLA2 (Expansin-Like A2) may contribute to cell wall loosening (246), which is necessary for plasmodesmal permeability.

Enrichment of gene ontology (GO) terms in cluster 5 and 6 and analysis of protein-protein networks for PD-related proteins

DEGs in cluster 5 demonstrate opposite regulation in *GUN5* alone when compared to the

other single silencing of *ISE2*, *GUN4*, and co-silencing of *ISE2-GUN4*, *ISE2-GUN5*, and *ISE2-GUN4-GUN5*. GO term enrichment analysis of Cluster 5 reveals that its genes are involved in cytoskeletal regulation, intercellular communication, stress responses, and developmental processes (**Appendix Fig. 29 and Appendix Fig. 30**). The strong enrichment related to microtubules indicates that these genes contribute to intracellular trafficking, vesicle transport, and structural organization. The connection between microtubule-based movement and the plasma membrane suggests active vesicular transport and interactions with PD. PD-related genes highlight a role in cell communication, regulating signaling molecules and nutrient exchange.

Transmembrane and sugar transporters indicate involvement in solute exchange, contributing to vascular tissue formation. Phospholipid catabolic processes and phospholipase C activity highlight involvement in lipid signaling, which is crucial for membrane remodeling. Finally, the cellular response to potassium and phosphate starvation indicates nutrient sensing, while thioredoxin peroxidase and hydrogen peroxide processes strengthen redox signaling and the regulation of oxidative stress.

GO term enrichment analysis of cluster 6 showed significant enrichment for iron ion and heme binding terms (**Appendix Fig. 31**), indicating potential disruption of heme hemostasis. This observation aligns with our data showing reduced heme levels, supporting the hypothesis that altered expression of cluster 6 genes reflects the cellular response to heme deficiency.

Predicting candidate PDANGs from different clusters of DEGs

Having obtained several candidate clusters of PDANGS, we performed an *in silico* analysis to test the PD association of these candidate using the established Plasmodesmata In silico Proteome 1 (PIP1) (247). We limited our analysis to gene enriched in PD-related GO terms across clusters 1, 2, 4, and 5. This pipeline compares four published PD proteomes, which significantly overlap in terms of protein family and subfamily composition (248–251). The pipeline output classifies whether the subfamily appears in one or multiple PD proteomes and is based on predicted distinctive features associated with PD-verified genes (247). The pipeline generates four lists of genes/proteins. Candidate list A includes genes encoding proteins from subfamilies found in more than one PD proteome with predicted signal peptide (SP), glycoposphatidylinositol anchor (GPI), or transmembrane domain (TM), which are the features enriched in PD-verified proteins. Proteins in Candidate list B, while also appearing in multiple proteomes, do not have membrane-localizing features. Candidate lists C and D comprise proteins from subfamilies identified in a single proteome, with candidate list C including proteins with predicted targeting features and candidate list D including those without (235). Since experimentally verified PD proteins typically exhibit membrane-targeting features, candidate lists A and C are the most promising candidates. Clusters 1, 2, 4, and 5 contain a total of 97 genes, 37 of which fall under candidate list A or C (**Table 4**). From cluster 1, the genes AHA1, NAT1, OKY, SKS5, T22E16.90, and BIP1 are included in candidate list A. In cluster 2, NAT7, NAT3, NCL, and RD21A are part of candidate list A, whereas GFR3.7, NPF2.10, and AIR9 are in candidate list C.

Table 4. Predicted candidate PD genes from PD-enriched GO terms in clusters 1, 2, 4, and 5 using the Plasmodesmata In silico Proteome 1 (PIP1) pipeline.

Arabidopsis orthologs were used for the analyses. The output of the candidate list is classified into A/B/C/D, and or if they encode known *A. thaliana* PD protein. Genes not in the candidate list or known PD proteins are denoted by ‘NOT’. Candidate A are genes encoding proteins from subfamilies present in more than one PD proteome with predicted signal peptide (SP), glycosphosphatidylinositol anchor (GPI) or transmembrane domain (TM) domains. Candidate B is also present in multiple proteomes but lacks the membrane-localizing features. Candidate C and D are proteins in subfamilies found in a single proteome, either with predicted targeting features (C) or without them (D).

Nicotiana benthamiana Gene Symbol	TAIR ID (Arabidopsis Ortholog)	Cluster	Candidate_list or Known PD gene	Nicotiana benthamiana Gene Symbol	TAIR_ID (Arabidopsis Ortholog)	#Cluster	Candidate_list or Known PD_gene
CYP19-2	AT2G16600	1	D	BSK5	AT5G59010	2	D
AHA1	AT2G18960	1	A	ACT7	AT5G09810	2	D
NAT1	AT2G05760	1	A	NAT7	AT1G60030	2	A
PER42	AT4G21960	1	NOT	DI4605c	AT1G77730	2	NOT
SYT1	AT2G20990	1	NOT	BEL1	AT5G41410	2	D
QKY	AT1G74720	1	A	AIR9	AT2G34680	2	C
SYT4	AT5G11100	1	NOT	EHD1	AT3G20290	2	B
sks5	AT1G76160	1	A	RBG7	AT5G61030	2	D
AKT2	AT4G22200	1	NOT	NAT3	AT2G26510	2	A
NRAMP3	AT2G23150	1	NOT	T10P12.6	AT1G43580	2	C
RPL13B	At3g49010	1	NOT	NCL	AT1G53210	2	A
TMK2	AT1G24650	1	NOT	F4JQJ7_ARATH	AT4G36945	2	NOT
SPP2	AT3G52340	1	NOT	RD21A	AT1G47128	2	A
T22E16.90	AT1G64760	1	A	CHR4	AT5G44800	4	D
PDCB4	AT1G69295	1	NOT	PER19	AT5G17550	4	NOT
PIP2-1	AT3G53420	1	NOT	F8N16.8	AT2G28790	4	D
MS1	AT5G22260	1	NOT	PDCB2	AT5G08000	4	NOT
GLN1-1	AT5G37600	1	B	ARA1	AT4G16130	4	D
RAN3	AT5G55190	1	D	F15K19.1	AT2G10940	4	C
F4F15.170	AT3G52060	1	NOT	PRK3	AT3G42880	4	C
BIP2	AT5G42020	1	A	PIP2-7	AT4G35100	4	A
ATX5	AT5G53430	2	D	TIL	AT5G58070	4	D
CEL5	AT1G22880	2	NOT	STP13	AT5G26340	4	A
ALDH3H1	AT1G44170	2	D	PCAP1	AT4G20260	4	D
GLR3.7	AT2G32400	2	C	TMK4	AT3G23750	4	A
NPF2.10	AT3G47960	2	C	RPL14A	ATCG00780	4	D
HSP70-4	AT3G12580	2	NOT	DI3855w	AT4G15630	4	C
PIP1.4	AT4G00430	2	NOT	HSP90-2	AT5G52640	4	NOT
CLV1	AT1G75820	2	NOT	BXL7	AT5G49360	4	C
SHOC1	AT5G52290	2	D	SAHH1	AT4G13940	4	B
RABH1B	AT1G79830	2	NOT	RPL13B	At3g49010	4	D
F16F14.25	AT2G16250	2	NOT	RPS5B	AT2G37270	4	B

Table 4. continued.

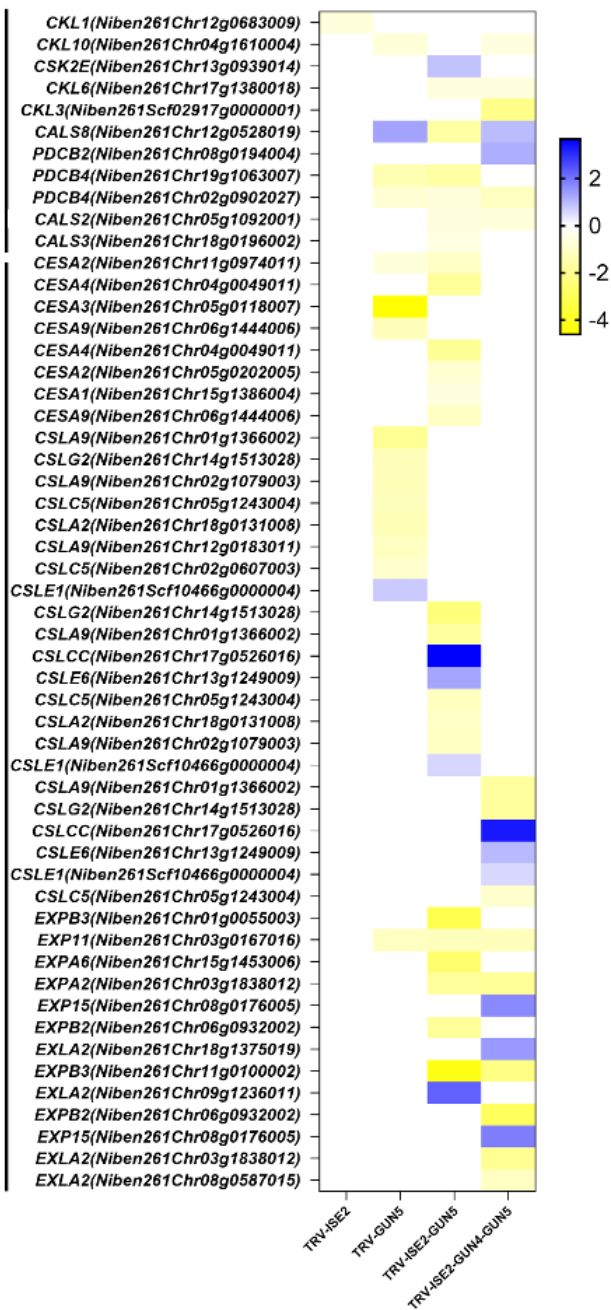
Nicotiana benthamiana Gene Symbol	TAIR_ID (Arabidopsis Ortholog)	#Cluster	Candidate_list or Known PD_gene	Nicotiana benthamiana Gene Symbol	TAIR ID (Arabidopsis Ortholog)	Cluster	Candidate_list or Known PD gene
RFS6	AT5G46470	4	NOT	RPS6B	AT5G10360	4	D
TET6	AT3G12090	4	NOT	EXLA2	AT4G38400	4	A
NAT7	AT1G60030	4	A	TIP2-1	AT3G16240	4	C
PIP1-3	AT1G01620	4	C	PAPP5	AT2G42810	4	D
TRX5	AT1G45145	4	D	NPF2.11	AT5G62680	4	C
GRF1	AT4G09000	4	C	MJL12_25	At3g25290	5	A
F24B9.13	AT1G07750	4	D	APA1	AT1G11910	5	A
AMT2	AT2G38290	4	A	HVA22C	AT1G69700	5	A
TET4	AT5G60220	4	NOT	CSLC12	AT4G07960	5	C
RPL17A	AT1G27400	4	D	RLK902	AT3G17840	5	C
F21M12.3	AT1G09970	4	NOT	EXPB3	AT4G28250	5	C
HMGS	AT4G11820	4	D	PIN7	AT1G23080	5	C
F27G19.50	AT4G27450	4	D	EXPA6	AT2G28950	5	C
F26G5_140	AT3G44190	4	NOT	VIL2	AT4G30200	5	D
RPS18A	ATCG00650	4	D	PIP2-2	AT2G37170	5	C
K21L19.12	AT5G58090	4	A	ACO1-2	AT2G19590	5	NOT
PPI1	AT1G02280	4	NOT				

Figure 24. Heatmap showing expression of callose, PD- and cell wall-related genes. Callose, PD-related DEGs (A), and cell wall—and plasma membrane-associated and ER-membrane tethering protein encoding DEGs (B) are determined by RNA-seq analysis. Only DEGs with adjusted p values below 0.05 are shown, and values on the heat map show log₂ fold change in expression.

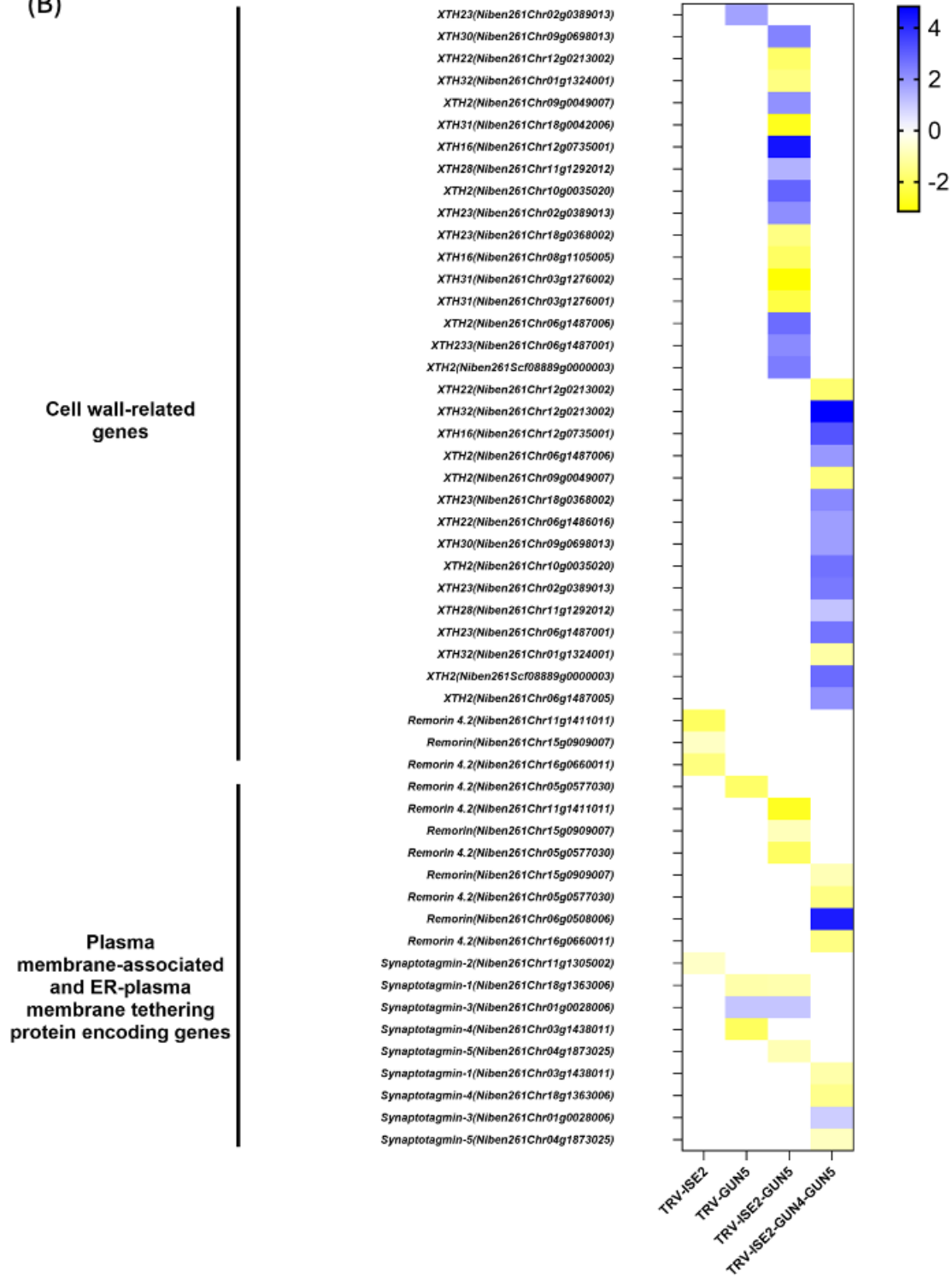
(A)

Callose and
PD-related
genes

Cell wall-related
genes



(B)



Cluster 4 includes F15K19.1, PRK3, DI3855W, BXL7, PIP1-3, GFR1, TIP2-1, and NPF2.11 in candidate list C, while PIP2-7, STP13, TMK4, NAT7, AMT2, K21L19.12, and EXLA2 are in candidate list A. Lastly, in cluster 5, MJL12_25, APA1, and HVA22C are found in candidate list A, with CSLC12, RLK902, EXPB3, PIN7, EXPA6, and PIP2-2 in candidate list C. Thus, the proteins belonging to candidate lists A and C in **Table 4** are potentially representative of newly identified *N. benthamiana* PDANGs.

Differential expression of known callose-, PD- and cell wall-related genes

To further understand the link between intercellular trafficking and chloroplast retrograde signaling, we examine the differential expression of callose and cell wall-related genes (**Fig. 24A, B**). Most of these cell wall- and PD-related genes are differentially expressed following silencing *GUN5* alone or co-silencing with *ISE2* in *ISE2-GUN5* and *ISE2-GUN4-GUN5*. In contrast, *ISE2* silencing only showed differential expression for remorin, synaptotagmin-2, and CKL1, while none of these genes were differentially expressed in *ISE2-GUN4*-silenced plants.

Among the callose and PD-related DEGs were several *CASEIN KINASE I-LIKE* (*CKL*) proteins, specifically *CKL1*, *CKL2*, *CK36*, *CKL6*, and *CKL10*. The Arabidopsis genome contains 14 *CKL* genes, with *CKL1* found to be differentially expressed in the *ise2* mutant. *CKL6* associates with cortical microtubules and phosphorylates tubulins in vitro, suggesting a role in cytoskeletal dynamics and potentially in PD function. The PD-associated gene PLASMODESMATA CALLOSE BINDING (PDCB) 4 is also included among the DEGs (**Fig. 24A**). This set of DEGs includes *CELLULOSE SYNTHASES* and other genes involved in cell wall modification, featuring several *EXPANSINS* and

XYLOGLUCAN ENDOTRANSGLYCOSYLASE/HYDROLASES (XTHs). REMORIN is a plant-specific lipid raft and PD protein, and SYNAPTOTAGMIN is a plasma membrane protein that regulates the cell-to-cell movement of viruses (189,252). This indicates that the cell wall and PD-related genes respond differentially to chloroplast retrograde signals and may be involved in regulating trafficking through PD.

DISCUSSION

Differential gene expression analyses of known PD-related genes and predicting novel PD proteins using an in silico pipeline PIP1

To find the genes associated with PD-mediated trafficking, we used different clusters of DEGs identified via heatmap analysis to conduct a GO term enrichment analysis in the cellular component category. Several clusters showed enrichment for the GO term plasmodesma (GO:0009506), indicating their potential crucial roles in intercellular trafficking through PD. We further investigated the functional connections among these genes using the STRING database to create protein-protein interaction networks, which helped us identify potential co-regulatory or co-functional partners within the PD-enriched groups. The enrichment in plasmodesmal function highlights meaningful biological relevance, suggesting that intercellular communication via PD is dynamically regulated.

Although Gene Ontology offers a useful framework for functional interference, novel genes related to PD through GO enrichment might not be true PD-localized proteins. Given the current annotation limitations, caution is necessary when interpreting the enrichment of PD GO terms. The PD GO term (GO:0009506) includes approximately

3,111(249). While AtCells represents one of the most extensive datasets for plasmodesmal proteomics, containing 39 validated plasmodesmal proteins, it remains unfiltered and likely contains contaminants and specific extraction artifacts. For example, about 12% of the dataset's proteins are classified as cell wall proteins, which may not accurately reflect true plasmodesmal localization but could instead result from proximity or overlap during the isolation process of cell walls and membranes. Identifying components in PD proteomes does not guarantee their localization to PD, as these preparations are often contaminated with non-PD structures, such as the plasma membrane (PM), cell walls, and the endoplasmic reticulum (ER) (248). To eliminate contamination, PD-enriched fractions were created, and their proteomes were compared to other membrane proteomes (248,250). However, this solution is not ideal, especially given the finding that PM proteins can temporarily localize in PD regions(253,254). Additionally, a significant limitation is that all current PD proteomes are derived from cell cultures and likely represent the proteomes of secondary PD which are forming as cells contact each other in the absence of cytokinesis. To further narrow down our list of potential PDANGs, we utilized an *in silico* pipeline, PIP1, to predict likely PD localization of candidate PD proteins encoded by genes enriched from our GO analysis. The pipeline provided four lists of candidate PD genes, where candidate lists A and C contain secreted proteins and are the most likely PD components. The candidate B list contains mobile and peripheral membrane proteins, whereas the candidate list D is likely comprised of other membrane or cell wall proteins not related to PD proteins. Our *in silico* analysis identified a set of candidate lists A (20) and C (17) that are most likely localized to PD and may have

direct roles in regulating PD function. Functional characterization along with co-localization with known plasmodesmal markers or biochemical fractionation of these candidate PD proteins will be necessary to experimentally validate the roles of these candidate genes in PD function.

Potential for discovering cis-elements in plasmodesmata-enriched DEGs

In addition to functional enrichment and interaction analyses, identifying PD-related genes from our DEGs cluster and hub genes offers a chance to investigate the regulatory mechanisms governing plasmodesmal dynamics. Genes enriched in the PD GO term form clusters or are identified through *in silico* analyses, making them potential candidates for cis-regulatory element analysis. Examining the promoter regions of these genes could reveal common transcription factor binding motifs that influence their expression in response to developmental cues. One challenge with our analysis is that our DEGs were identified in *N. benthamiana*. Current genome of this genome limits our ability to identify *cis* regulatory elements.

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type *Nicotiana benthamiana* seeds were directly planted onto soil and then transferred to a growth chamber under standard growth conditions (25 °C, 16 h light, 8 h dark). VIGS was performed in around two-and-a-half to three-week-old plant.

RNA isolation for RNA sequencing, quality control, and reads mapping to the reference genome

RNA was isolated from plant tissue from eighth/ninth leaves after 12-14 days of VIGS

using Qiagen RNeasy® Plant Mini Kit and according to manufacturer protocol (Qiagen, Cat No. 74904) and digested with DNase I (Roche). RNA, approximately 25 to 500 ng/μl, was used for microfluidics analysis using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) to assess RNA integrity using the Plant RNA Pico Assay chip following the manufacturer's instructions. RNA Integrity Number (RIN) values were calculated to evaluate the quality of RNA samples and the RNA used for RNA seq analysis. RNA sequencing was performed by Novogene. Raw reads were analyzed for quality by FASTQC, and reads were mapped to the reference genome using HISAT2. The reads were mapped to the *N. benthamiana* V0.4.4 reference genome. The reference genome and gene model annotation files were all downloaded from the genome website (http://solgenomics.net/organism/Nicotiana_benthamiana/genome).

Differential Expression Analysis and Functional Enrichment

Gene counts were analyzed for gene-level differential expression using DESeq2 (255). Sample distances were calculated using the normalized log transformation of gene counts from the DESeq2 package. Significantly differentially expressed genes (DEGs) were identified with a fold change greater than 0 and an adjusted P-value of less than 0.05. Gene ontology (GO) and KEGG pathway enrichment analyses of DEGs were conducted using the STRING database (<https://string-db.org/>) (255,256) with Arabidopsis genomes. GO enrichment analyses utilized the hypergeometric statistical model, and p-values were adjusted using the Benjamini-Hochberg method to control the false discovery rate (FDR). GO terms with FDR < 0.05 were considered significantly enriched.

Predicting candidate PD genes from DEGs genes

Arabidopsis orthologs of DEGs were obtained from The Arabidopsis Information Resource (TAIR) database. TAIR IDs were used for in silico prediction of PD genes using the PIP1 pipeline (247). PIP1 is a R-based tool that classifies candidate genes encoding PD proteins using predictions from proteomics and structural features, such as the presence or absence of GPI, SP, and TM. Genes are annotated based on their identification as known PD genes or the subfamily frequency observed in the proteomes analyzed from four prior studies (248–251).

APPENDIX

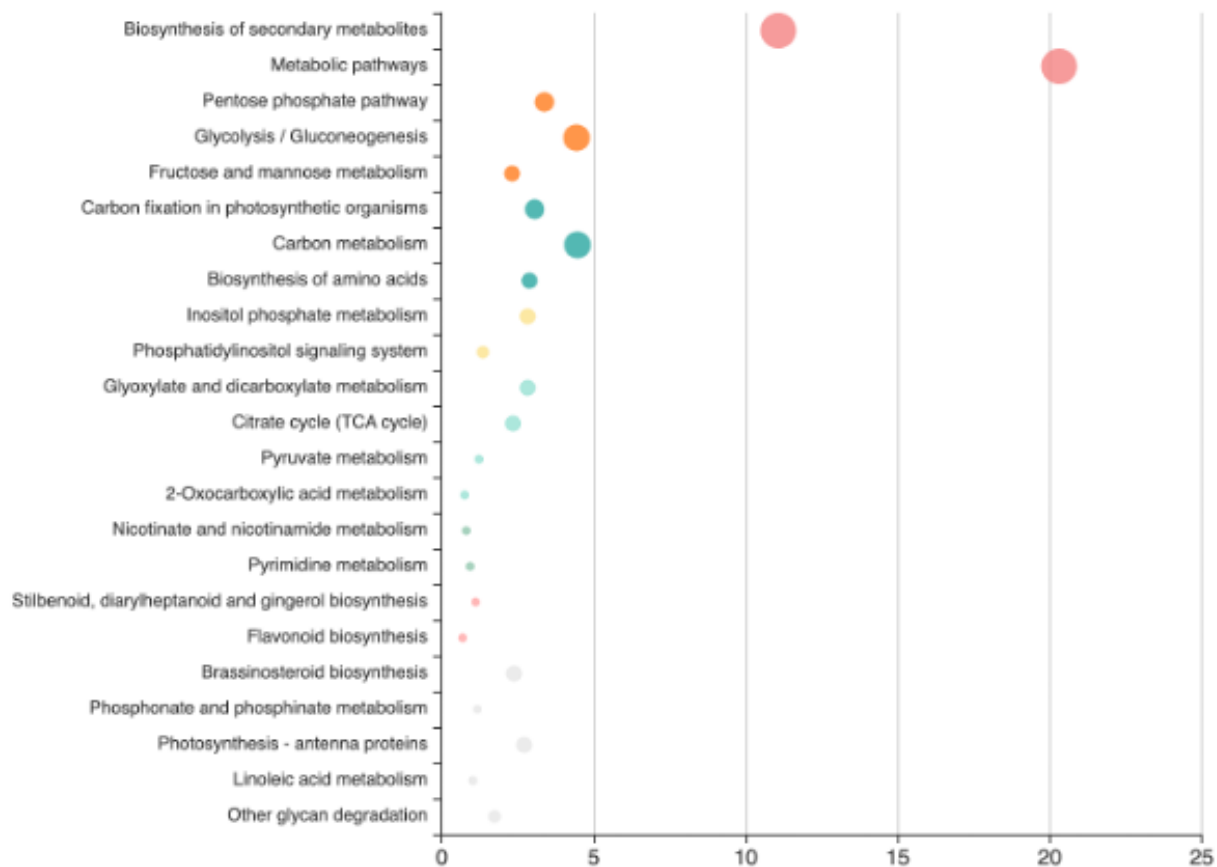


Figure 25. KEGG pathway enrichment analysis of cluster 1 from the heatmap shown in Fig.19

KEGG pathway enrichment analysis was performed in KOBAS website (<http://kobas.cbi.pku.edu.cn>). KEGG pathways only with corrected p-values below 0.05 are shown. Same color dots represent KEGG pathways that belong to the same cluster.

#Term	Input number	Background number	Corrected P-Value
protein binding	95	3199	5.95E-16
plasma membrane	86	2973	1.03E-13
chloroplast	103	4354	1.23E-11
chloroplast envelope	30	496	4.54E-11
chloroplast stroma	29	549	2.19E-09
response to cold	20	310	1.20E-07
chloroplast thylakoid membrane	18	291	1.36E-06
chlorophyll binding	7	21	1.62E-06
cytoplasm	79	4014	2.81E-05
photosynthesis, light harvesting in photosystem I	6	23	4.47E-05
response to light stimulus	13	199	4.72E-05
apoplast	15	275	5.45E-05
thylakoid	12	181	9.55E-05
single-stranded DNA binding	8	73	0.000165581
DNA-binding transcription factor activity	37	1513	0.000446876
response to cadmium ion	14	296	0.000467687
response to salt stress	15	340	0.000467687
plastoglobule	7	62	0.000467687
MCM complex	4	10	0.000537122
chloroplast thylakoid	11	190	0.000601831
plastid	24	823	0.000889838
response to wounding	11	202	0.000889838
response to water deprivation	13	282	0.000889838
cellular response to phosphate starvation	7	72	0.000889838
cytosol	58	3025	0.001149265
plasmodesma	24	851	0.001363392
mRNA binding	24	855	0.001415156
transcription regulatory region sequence-specific DNA binding	16	460	0.002505127
DNA replication origin binding	4	18	0.002517498
ammonia assimilation cycle	3	6	0.002867809

Figure 26. GO term enrichment analysis of cluster 2 from the heatmap shown in Fig.19.

GO term enrichment analysis was performed in KOBAS website (<http://kobas.cbi.pku.edu.cn>). Go term only with corrected p-values below 0.05 are shown.

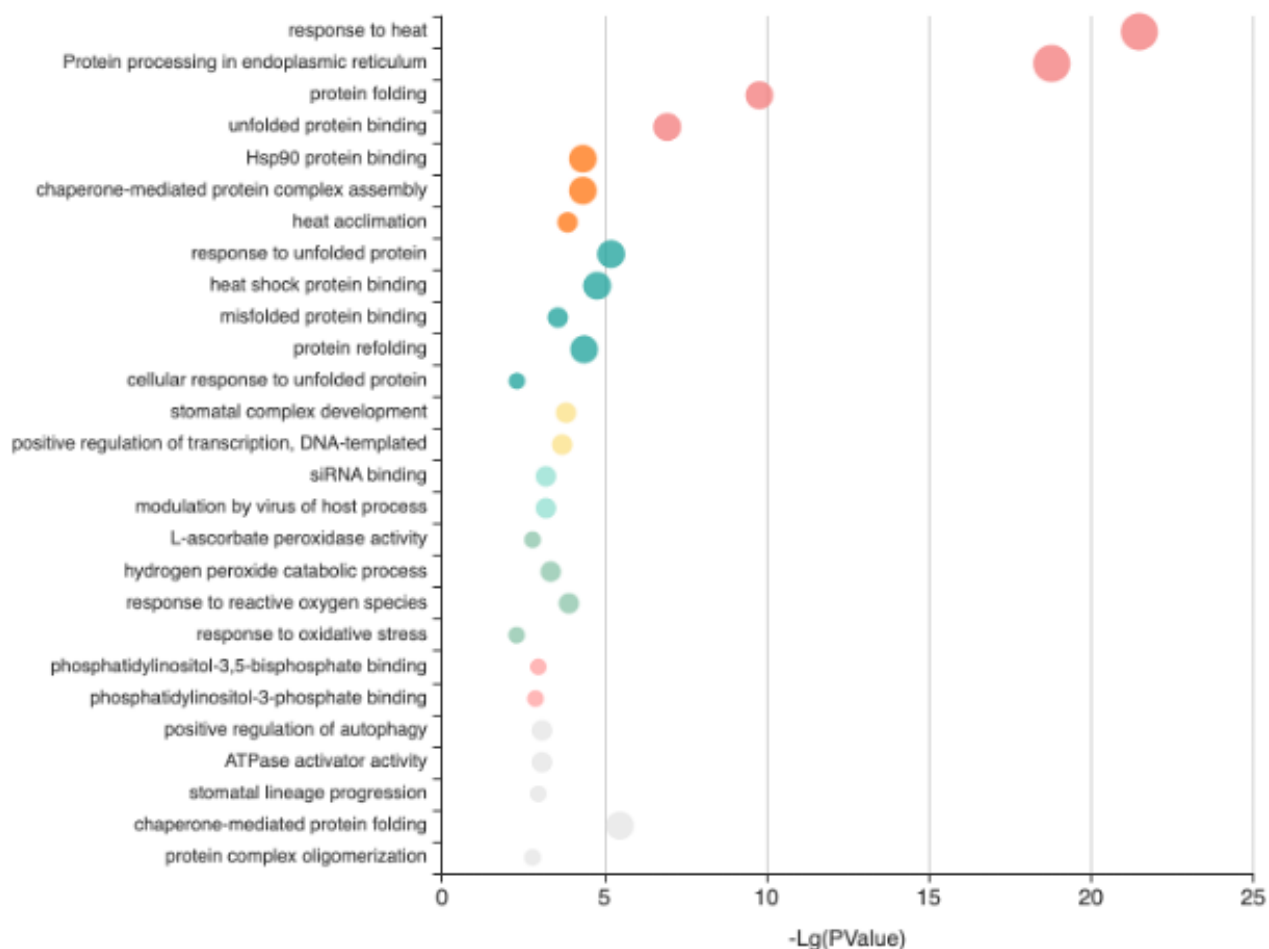


Figure 27. GO term and KEGG pathway enrichment analysis of cluster 3 from the heatmap shown in Fig.19

GO term and KEGG pathway enrichment analysis were performed in KOBAS website (<http://kobas.cbi.pku.edu.cn>). GO terms and KEGG pathways only with corrected p-values below 0.05 are shown. Same color dots represent GO terms or KEGG pathways that belong to the same cluster.

Figure 28. GO term enrichment analysis of cluster 4 from the heatmap shown in Fig.19.

GO term enrichment analysis were performed in KOBAS website (<http://kobas.cbi.pku.edu.cn>). GO terms only with corrected p-values below 0.05 are shown.

#Term	Input number	Background number	Corrected P-Value
cytosol	159	3025	4.01E-14
protein binding	165	3199	4.01E-14
plasma membrane	146	2973	1.03E-10
vacuole	47	598	3.48E-08
chloroplast	179	4354	1.34E-07
mRNA binding	53	855	4.02E-06
cytoplasm	160	4014	7.86E-06
RNA binding	33	423	1.29E-05
vacuolar membrane	33	434	2.02E-05
response to water deprivation	24	282	0.000121
cellular response to hypoxia	20	204	0.000121
plastid transcription	6	11	0.000219
cytosolic ribosome	18	178	0.000238
cytosolic large ribosomal subunit	14	126	0.001057
response to bacterium	13	110	0.001108
DNA-binding transcription factor activity	68	1513	0.001172
secretory vesicle	15	148	0.001219
transcription regulatory region sequence-specific DNA binding	29	460	0.001492
leaf senescence	12	99	0.001492
response to oxidative stress	21	276	0.001492
response to salicylic acid	12	100	0.001492
regulation of defense response	9	54	0.001492
defense response to Gram-negative bacterium	6	19	0.00152
defense response to bacterium	23	324	0.00152
hydrolase activity, hydrolyzing O-glycosyl compounds	13	124	0.002176
response to wounding	17	202	0.002176
photomorphogenesis	9	61	0.002917
response to oomycetes	6	23	0.00314
ATP transmembrane transporter activity	7	35	0.003304
regulation of jasmonic acid mediated signaling pathway	7	36	0.003744
plastid	41	823	0.004182
identical protein binding	15	178	0.004854
transmembrane transport	24	389	0.006003
nucleolus	24	391	0.006276
plasmodesma	41	851	0.007299
plastid chromosome	5	18	0.007845
plastid-encoded plastid RNA polymerase complex	4	9	0.007845
defense response to bacterium, incompatible interaction	7	43	0.007845
calmodulin binding	14	169	0.007845
extracellular region	102	2749	0.007845

#Term	Input number	Background number	Corrected P-Value
microtubule	7	65	1.41E-05
microtubule binding	7	117	0.000281589
plasma membrane	32	2973	0.000281589
phospholipid catabolic process	3	19	0.008827161
microtubule motor activity	4	54	0.008827161
microtubule-based movement	4	56	0.008827161
kinesin complex	3	21	0.008827161
phospholipase C activity	2	7	0.029219938
protein binding	27	3199	0.029219938
cellular response to potassium ion starvation	2	8	0.029219938
thioredoxin peroxidase activity	2	8	0.029219938
plasmodesma	11	851	0.048776268
protein homodimerization activity	5	191	0.053341965
protein heterodimerization activity	4	121	0.058141139
syncytium formation	2	15	0.058141139
transferase activity, transferring glycosyl groups	7	412	0.058141139
phragmoplast	3	60	0.058141139
response to sucrose	3	61	0.058141139
protein homooligomerization	2	17	0.061157218
sugar transmembrane transporter activity	2	19	0.065141407
DNA unwinding involved in DNA replication	2	19	0.065141407
carbohydrate transport	2	19	0.065141407
cellular response to phosphate starvation	3	72	0.070944988
sexual reproduction	2	21	0.070944988
hydrogen peroxide catabolic process	2	24	0.087343508
nucleoside metabolic process	2	26	0.095934217
root development	4	166	0.095934217
transmembrane transport	6	389	0.106242626
xylem and phloem pattern formation	2	29	0.106242626

Figure 29. GO term pathway enrichment analysis of cluster 5 from the heatmap shown in Fig.19.

GO term enrichment analysis was performed in KOBAS website (<http://kobas.cbi.pku.edu.cn>). GO terms only with corrected p-values below 0.05 are shown.

Figure 30. GO term and protein-protein network analysis of cluster 5 from the heatmap shown in Fig. 19.

Description (A) of plasmodesmata-related protein, and protein-protein network (B) showing interaction of protein enriched in GO term analysis of cluster 5. Protein-protein networks are made using the STRING database (<https://string-db.org/>). Network nodes represent proteins and the edge represents protein-protein association. Association between proteins means they jointly contribute to shared function and does not necessarily mean they are physically binding to each other. Cyan and purple edges represent known interactions from curated databases or experimentally determined. Green, red, and blue edges represent predicted interactions. Light green, black, and sky blue edges represent other interactions from textmining, co-expression, and protein homology, respectively.

(A)

Gene Symbol	Description
MJL12_25	Cytochrome b561 and DOMON domain-containing protein At3g25290
APA1	Aspartic proteinase A1
HVA22C	HVA22-like protein c
CSLC12	Probable xyloglucan glycosyltransferase 12
RLK902	Probable inactive receptor kinase RLK902
EXPB3	Expansin-B3
PIN7	Auxin efflux carrier component 7
EXPA6	Expansin-A6
VIL2	VIN3-like protein 2
PIP2-2	Aquaporin PIP2-2
ACO1-2	1-aminocyclopropane-1-carboxylate oxidase 1

(B)

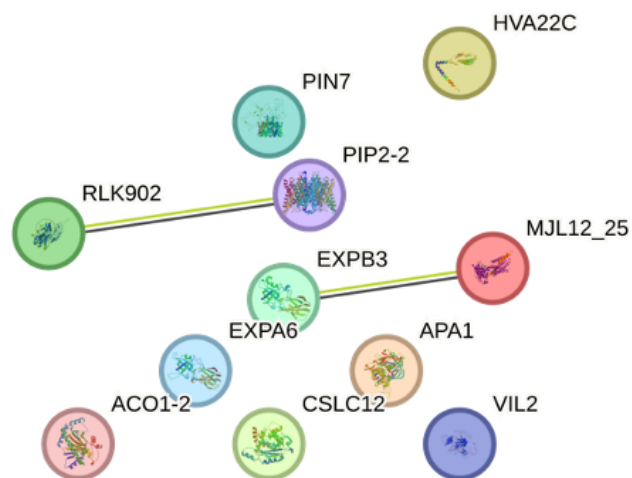


Figure 31. GO term and KEGG pathway enrichment analysis of cluster 6 from the heatmap shown in Fig.19.

GO term and KEGG pathway enrichment analysis was performed in KOBAS website (<http://kobas.cbi.pku.edu.cn>). GO terms and KEGG pathways only with corrected p-values below 0.05 are shown.

#Term	Input number	Background number	Corrected P-Value
oxidation-reduction process	11	494	0.000305178
integral component of plasma membrane	6	102	0.000305178
carbohydrate transport	3	19	0.003770931
sugar transmembrane transporter activity	3	19	0.003770931
iron ion binding	7	306	0.005219274
heme binding	7	357	0.01021201
abscisic acid catabolic process	2	5	0.01021201
phosphatase activity	3	42	0.016434604
chlorophyll biosynthetic process	3	42	0.016434604
dioxygenase activity	3	52	0.026965124
sucrose transmembrane transporter activity	2	13	0.033453962
sucrose transport	2	16	0.043209462
protein homooligomerization	2	17	0.043209462
sulfate transmembrane transporter activity	2	17	0.043209462
anthocyanin-containing compound biosynthetic process	2	18	0.04491246
oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	4	161	0.047695655
defense response to insect	2	20	0.047695655
response to nematode	3	78	0.047695655
plasma membrane	19	2973	0.072529945
sterol metabolic process	2	41	0.110601121
transmembrane transport	5	389	0.110601121
gravitropism	2	44	0.110601121
dephosphorylation	2	45	0.110601121
ubiquitin protein ligase activity	3	151	0.110601121
integral component of membrane	3	152	0.110601121
chloroplast	23	4354	0.110601121
negative regulation of response to water deprivation	1	5	0.110601121
dUTP phosphohydrolase activity	1	5	0.110601121
GTP phosphohydrolase activity	1	5	0.110601121

CHAPTER 5

GLUCOSINOLATES CAN ACT AS SIGNALS TO MODULATE INTERCELLULAR TRAFFICKING VIA PLASMODESMATA

**Glucosinolates can act as signals to modulate intercellular trafficking via
plasmodesmata**

Disclosure: A version of this chapter was previously published in the journal of New Phytologist by Jessica C. Fernandez, Mohammad F. Azim, Morgan Strong, Jacob O. Brunkard, Min Xu, Sarbottam Piya, Tarek A. F. Hewezi, Carl E. Sams and Tessa M. Burch-Smith.

Jessica C. Fernandez, Mohammad F. Azim, Morgan Strong, Jacob O. Brunkard, Min Xu, Sarbottam Piya, Tarek A. F. Hewezi, Carl E. Sams and Tessa M. Burch-Smith (2025).

Glucosinolates can act as signals to modulate intercellular trafficking via plasmodesmata. New Phytologist.

Jessica Fernandez and Mohammad Azim share the first authorship of this manuscript. I contributed to RNA sequencing and qPCR analyses, and performed the GFP movement assay and callose staining following the exogenous application of sinigrin and glucobrassicin. I also prepared all the figures used in this manuscript.

ABSTRACT

Plasmodesmata (PD) allow direct communication across the cellulosic plant cell wall, facilitating the intercellular movement of metabolites and signaling molecules within the symplast. This connectivity allows coordinated responses to developmental and environmental cues. In plants with reduced levels of the chloroplast RNA helicase *ISE2*, intercellular trafficking and the number of branched PD were increased. Gene expression analyses in *Arabidopsis thaliana* tissues where *ISE2* expression was perturbed identified genes associated with the biosynthesis and metabolism of glucosinolates (GLSs) as highly affected by changes in *ISE2* expression. Concomitant with changes in expression of GLS-related genes, plants with increased or reduced *ISE2* expression contained altered GLS metabolic profiles compared to wildtype counterparts. Indeed, changes in expression of GLS-associated genes led to altered intercellular trafficking in *Arabidopsis* leaves. Exogenous application of GLSs but not their breakdown products also resulted in altered intercellular trafficking in a dose-dependent manner. These changes in trafficking may be mediated by callose levels at PD as exogenous GLS treatment was sufficient to change callose in wildtype plants. Further, auxin levels were perturbed in plants with increased indole type GLS levels and the plants also displayed auxin-related phenotypes. These findings suggest that GLSs, which are themselves transported between cells via PD, can act on PD to regulate their trafficking capacity.

INTRODUCTION

Plants have evolved pores called plasmodesmata (PD) that allow direct communication between cells across the cell wall. The direct cytoplasmic connectivity provided by PD allows the intercellular distribution of various metabolites like sugars, and signaling molecules including proteins and nucleic acids, that are essential for plant growth and development (9,99). Besides the cytoplasm other cellular compartments are associated with and continuous between cells via PD. The external boundaries of PD are the plasma membranes of the cells they connect while the PD of land plants often contain central appressed strands of the endoplasmic reticulum, called the desmotubule. Both the PD plasma membrane and desmotubule each have a unique lipid and protein composition that distinguish them from each other and from other cellular membranes (257,258). An updated view of PD is that they are specialized membrane contact sites (259,260). Despite this improved understanding of the structural composition of PD, PD regulation is not fully understood. One of the best studied mechanisms of regulating PD is the controlled accumulation of the β -1,3-glucan, callose in the cell wall surrounding PD. Accumulation of callose restricts PD-mediated trafficking, while removal of callose allows the resumption of, or increased, trafficking (261,262). Other potential mechanisms for regulating PD include signals and molecules originating from the chloroplast (263,264).

Previous studies with mutants in the chloroplast RNA helicase ISE2 suggested the chloroplast and mitochondria can generate signals that act on the nucleus to influence PD function and formation(51,263,265,266). *Arabidopsis thaliana* *ise2* mutant embryos

displayed increased PD-mediated trafficking (267) concomitant with increased numbers of PD pores in the form of increased branching (49). Similarly, *Nicotiana benthamiana* leaves with reduced *ISE2* expression had increased numbers of PD and intercellular trafficking(48). *ISE2* has major functions in chloroplast RNA metabolism and processing (47,268,269) and plants with reduced *ISE2* levels also mis-expressed photosynthesis associated nuclear genes (PhANGs), like *LHCB1.2* and *LHCB2.1*, indicating that chloroplast-to-nucleus retrograde signaling (CRS) was perturbed (22,48). The molecule responsible for CRS in *ISE2*-defective plants has yet to be identified but it is now known that CRS associated with defects in PD formation and function is common in plants where chloroplast gene expression is perturbed (46,48,55,155,269).

Abiotic stress is a potent trigger of CRS. The chloroplast metabolite and retrograde signal 3'-phosphoadenosine 5'-phosphate (PAP) accumulates in response to drought, high light and other chloroplast stresses (270,271) while another chloroplast metabolite methylerythritol cyclodiphosphate (MEcPP) accumulates and acts a retrograde signal to control expression of genes that regulate both local and systemic stress responses, (272,273). Stress-induced CRS can also influence secondary metabolism and in *Arabidopsis* this is pronounced in the case of GLS metabolism (274). For example, the *ceh1* mutant (*constitutively expressing HPL*) accumulates MEcPP, leading to disrupted indole glucosinolate synthesis and altering the formation of ER bodies which store enzymes that hydrolyze GLSs (275).

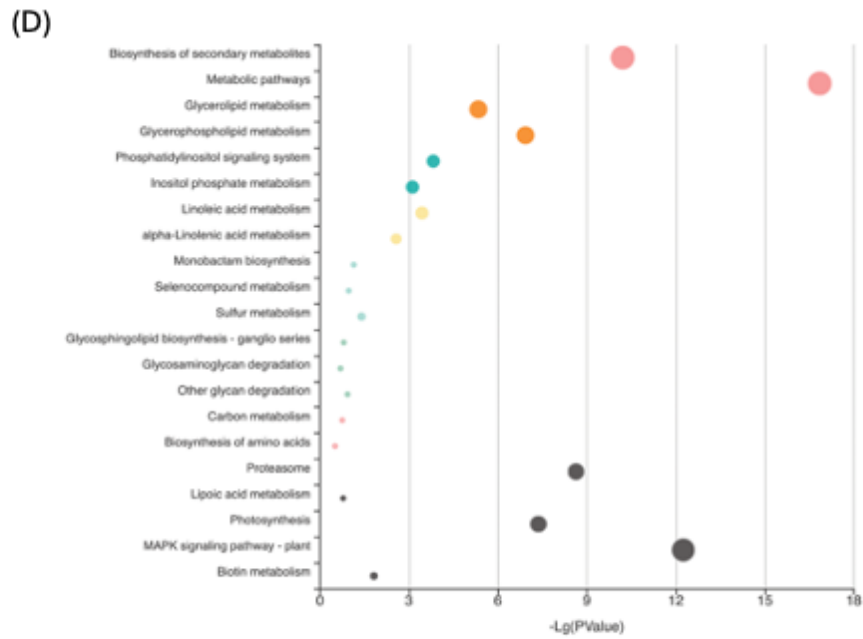
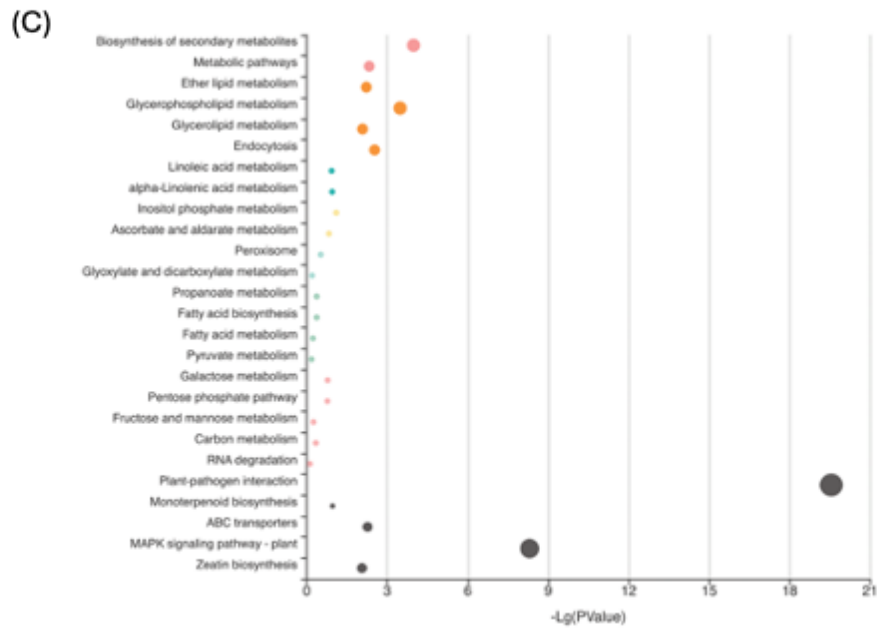
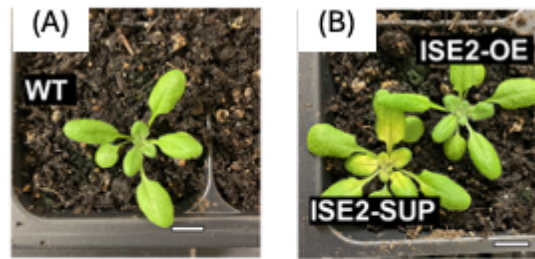
Glucosinolates (GLSs) are a class of specialized amino acid-derived, sulfur-containing metabolites that are synthesized by plants in the order *Brassicales*, including

in the model dicot *Arabidopsis thaliana*. GLSs have been intensely studied for their roles in plant defense and human health(276,277). When plant tissues are damaged, GLSs are hydrolyzed by enzymes called myrosinases to produce reactive molecules like nitriles, epithionitriles, and thiocyanates that are toxic to insects, herbivores and other pathogens (276). In *Arabidopsis* GLSs are predominantly formed from tryptophan (indole GLSs) and methionine (aliphatic GLSs) (278). They are synthesized in three steps involving different subcellular compartments: 1. amino acid chain elongation in the chloroplasts, 2. biosynthesis of core GLS structure in the chloroplast and cytoplasm, and 3. secondary modifications in the cytoplasm. The structural diversity of GLSs is astounding, with more than 200 identified in the *Brassicaceae* and more than 30 in *A. thaliana*. In *A. thaliana* the repertoire of GLSs varies between ecotypes, and may also vary even between tissues, as observed with the Columbia-0 ecotype(279,280). Further, the overall quantity of GLSs as well as their diversity of types of GLSs vary drastically throughout *Arabidopsis* development (281,282). Recent discoveries have revealed that, not only do GLSs serve as essential components of plant responses to biotic stress, but that they also have roles in abiotic stress responses, acting as potential signaling molecules (85,275,283). In *Arabidopsis*, GLS metabolism has been linked to stomatal control, drought resistance, auxin signaling, root growth and biomass, and bolting (284–289).

Due to the extensive defects in chloroplast RNA metabolism and gene expression, together with the complex PD-related phenotypes that result from perturbation of normal ISE2 function, we examined the nuclear transcriptional landscape in plants with loss or

Figure 32. Changes in ISE2 expression induced large changes in the transcriptomes of ISE2-OE and ISE2-SUP plants, with DEGs related to secondary metabolism showing strong effects.

(A-B) Wild-type Col-0 plants were grown alongside rescued *ise2* mutants stably expressing 35S:*ISE2-GFP* (ISE2-OE) and rescued plants suppressing the *ISE2-GFP* transgene (ISE2-SUP) plants have yellow leaves. (C) Analysis of KEGG pathways using KOBAS identified pathways affected by DEGs in ISE2-OE plants, and pathways are represented as clusters with similar levels of significance (p-values). Larger circles indicate more DEGs affected in a category. (D) Analysis of KEGG pathways using KOBAS identified pathways affected by DEGs in ISE2-SUP plants.



overexpression of ISE2 to identify the potential signal(s) and gene expression responses that were responsible for the observed changes in PD function. In a previous analysis of gene expression in *ise2* mutant embryos, several genes involved in GLS metabolism showed very large changes in expression (263). Here, we report that in mature *Arabidopsis* tissues genes associated with both the biosynthesis and breakdown of GLSs were differentially regulated when *ISE2* was overexpressed or its expression was reduced by silencing. Changes in expression of GLS-related genes were reflected in changes in the composition and size of the GLS pools. Further, we show that exogenously applied GLSs are sufficient to modulate intercellular trafficking via PD and that changes in PD-mediated trafficking are associated with modified callose levels at PD. We interpret these data to mean that GLSs can act as signaling molecules that regulate PD function through callose metabolism at PD. We have incorporated these findings into a model where chloroplast derived signals (CRS) regulate nuclear gene expression to modulate GLSs that in turn act to regulate callose metabolism and thence PD.

RESULTS

Expression of genes related to secondary metabolism is affected by altered ISE2 expression

Plants with reduced *ISE2* expression have revealed the role of CRS in regulating PD formation and function(48,263). To further investigate the relationship between CRS initiated in response to defective chloroplast gene expression and altered PD permeability, we measured gene expression in *Arabidopsis* with varied levels of *ISE2* expression. We used a previously described experimental system consisting of Col-0

ecotype plants as the wild-type controls (**Fig. 32A**), *ise2* mutants that were rescued by constitutively expressing *ISE2-GFP* and were visually indistinguishable from the wild type (ISE2-OE), and rescued plants that spontaneously co-suppressed the *ISE2-GFP* transgene resulting in mature plants with chlorotic leaves and severely reduced *ISE2* expression [(ISE2-SUP), (**Fig. 32B**), (47)].

We performed transcriptomic analysis on leaves of plants at 28 days old and three biological replicates for each group of plants were used to prepare a total of 9 RNA-seq libraries. Principal component analysis (PCA) revealed that the biological samples were highly similar between the biological replicates for each sample, with some replicates being almost identical to each other. A false-discovery rate (FDR) of 0.1 and a log fold2 ≥ 1.5 identified a total of 6028 differentially expressed genes (DEGs) in the ISE2-OE plants relative to the wild-type controls, where expression of 2820 genes was reduced, and expression of 3208 genes was induced. In the ISE2-SUP plants, a total of 7019 DEGs were identified with 3724 genes down-regulated, and 3295 genes up-regulated. Significantly DEGs were assigned to significantly affected bioprocesses using Gene Ontology (GO) analysis and the Generally Applicable Gene-set Enrichment (GAGE) method in iDEP (290). In both ISE2-OE and ISE2-SUP datasets, genes related to abiotic stress like hypoxia and decreased oxygen levels, and biotic stress including responses to bacteria and immunity were significantly repressed. In the ISE2-OE dataset, genes related to secondary metabolism were significantly enriched among up-regulated genes. In the ISE2-SUP dataset, expression of genes related to RNA metabolism was significantly increased.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg>) and GAGE method in iDEP were used to assign DEGs to their relevant metabolic pathways. In the ISE2-OE plants, the most significantly enriched pathway for genes showing increased expression comprised genes involved in biosynthesis of various compounds including cutin, suberin and waxes, phenylpropanoids. Flavonoids and glucosinolates (GLSs) (**Fig. 32C**). For down-regulated genes in the same plants, the most significantly enriched pathway were associated with plant-pathogen interactions and endocytosis (**Fig. 32C**). The most enriched pathways associated with upregulated genes in the ISE2-SUP plants were related to the ribosome and other RNA metabolism machinery including the spliceosome, consistent with ISE2's functions as an RNA helicase (46,47). Other pathways with increased gene expression included flavonoid biosynthesis and cutin, suberin, and wax biosynthesis, the converse of changes to these pathways in ISE2-OE plants (**Fig. 32D**). In the ISE2-SUP dataset, expression of genes associated with plant-pathogen interaction, photosynthesis and endocytosis was significantly decreased (**Fig. 32D**).

Further analysis revealed that many of the genes associated with secondary metabolism were involved in GLS production and metabolism. Genes associated with the aliphatic GLS (AG) pathway were mostly induced in the ISE2-OE plants, including genes involved in core AG biosynthesis (e.g., *BAT5*, *BCAT4*, *CYP79F1*, *CYP79F2*, *MAM1*, *SOT17*, *SOT18*) and modification (*FMO GS-OX1-4*). Consistent with the increased expression of AG biosynthesis genes, the expression of *MYB28* and *MYB29*, encoding transcriptional regulators involved in GLS synthesis, was also increased.

Figure 33. Genes involved in AG biosynthesis are affected by changes in ISE2 expression.

(A) Illustration of the biosynthetic pathway for production of glucoraphanin, the most abundant AG in Arabidopsis Col-0. Enzymes involved in each step are presented. (B) Representative expression of select AG biosynthesis genes was measured by qPCR in ISE2-OE and ISE2-SUP samples and compared to levels in wildtype Col-0. Statistical significance was calculated by Student's t-test, asterisks indicate $p < 0.05$.

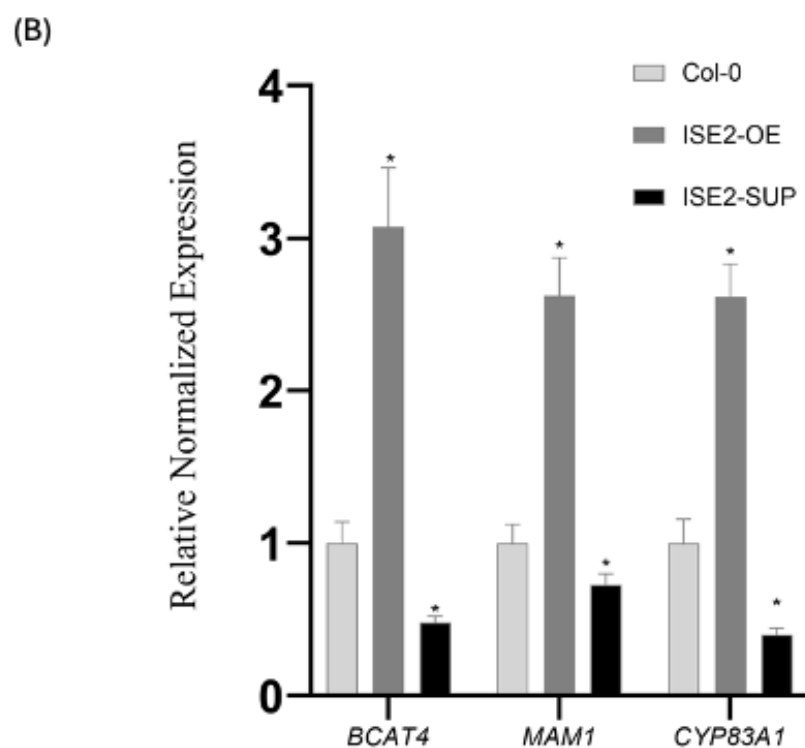
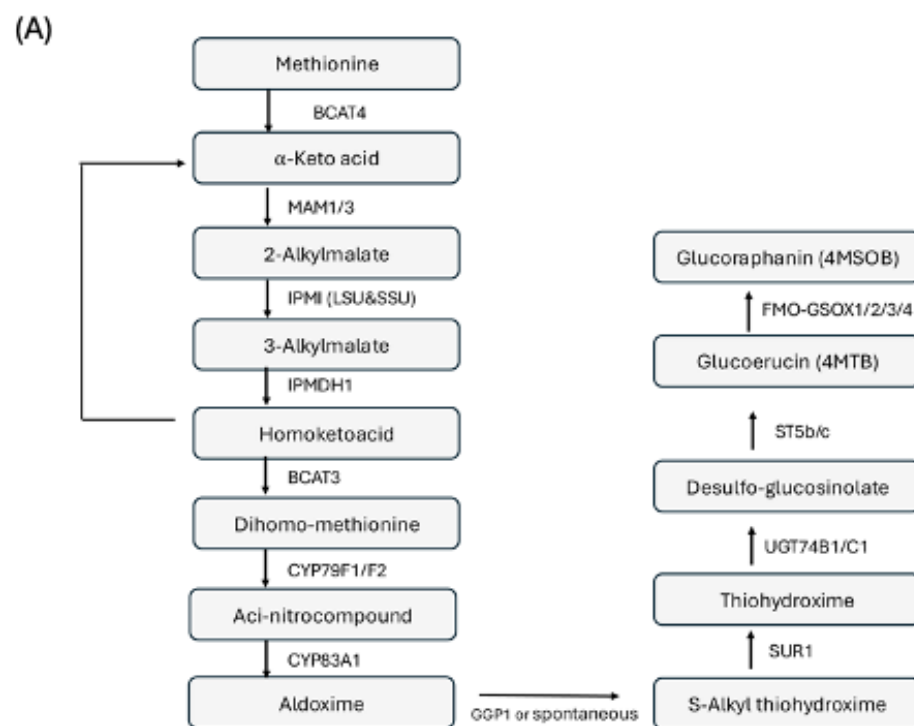
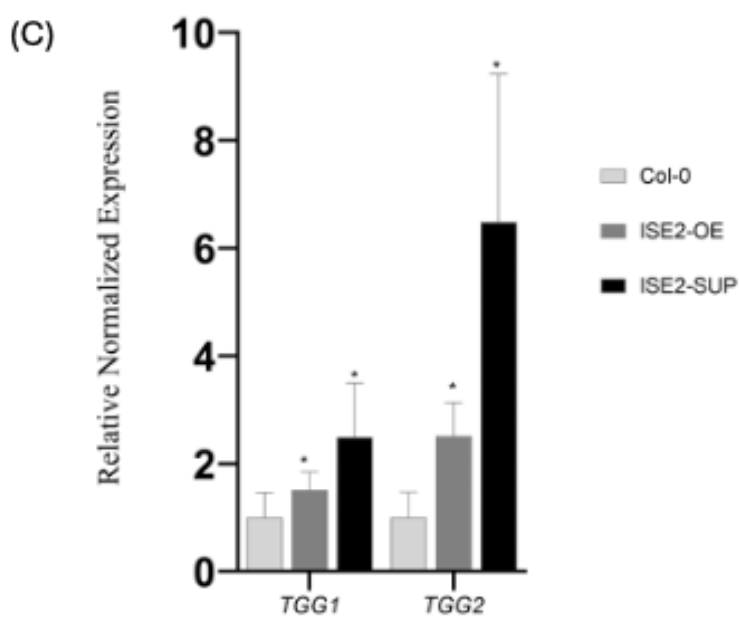
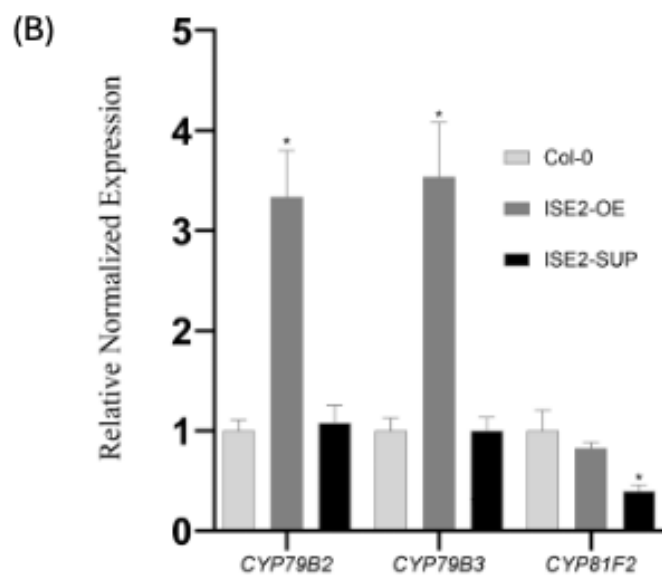
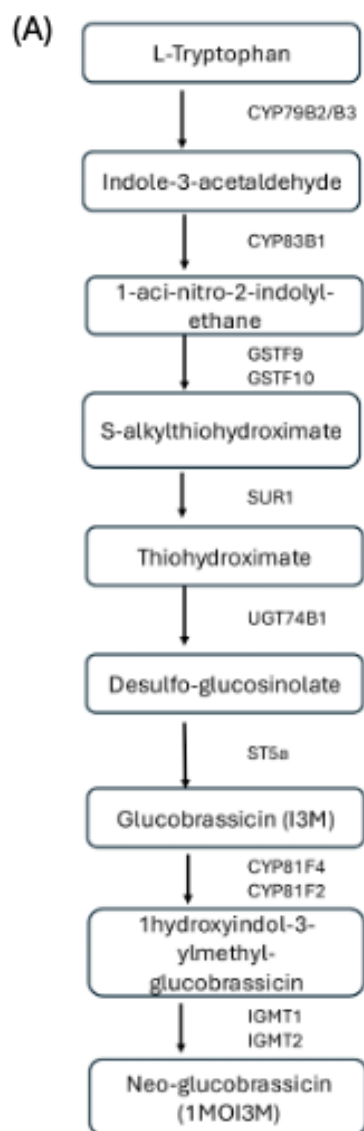


Figure 34. Genes involved in IG biosynthesis and GLS hydrolysis are affected by changes in *ISE2* expression.

(A) Illustration of the biosynthetic pathway for production of glucobrassicin and its derivative, the most abundant IGs in Arabidopsis Col-0. Enzymes involved in each step are presented. (B) Representative graph showing expression of select IG biosynthesis genes was measured by qPCR in ISE2-OE and ISE2-SUP samples and compared to levels in wildtype Col-0. (C) Expression of leaf myrosinase genes was measured by qPCR in ISE2-OE and ISE2-SUP samples and compared to levels in wildtype Col-0. Statistical significance was calculated by Student's t-test, asterisks indicate $p < 0.05$.



Expression of *NSP4*, and *NSP1* that encode specifier proteins that promote rearrangement of the products of myrosinase hydrolysis of GLSs toward simple nitriles, epithionitriles and organic thiocyanates, was also induced in the ISE2-OE plants. With the exception of *PKYK10*, encoding a myrosinase, and *NSP4*, encoding a specifier protein, changes in expression were relatively small although statistically significant. In contrast, most GLS-related genes were not differentially expressed in the leaves of ISE2-SUP plants. The exceptions included the repression of *MYB51* that regulates the expression of indole GLSs (291) and the increased expression of the myrosinase gene *PYK10*. This DEG analysis suggested that GLS metabolism was altered in plants with perturbed *ISE2* expression.

Expression of genes encoding functions related to GLS metabolism changes in response to ISE2 levels

In a parallel study to determine the acute effects of reduced ISE2 expression, Tobacco rattle virus (TRV)-based virus induced gene silencing (VIGS,(292)) was used to silence *ISE2* in Arabidopsis and changes in gene expression were determined by tiling microarrays. Here, a small group of 64 genes showed differential expression in the *ISE2*-silenced leaves. Genes related to GLS metabolism were overrepresented among the DEGs. This finding prompted us to re-examine previous gene expression analysis of *ise2* mutant embryos(293). Several genes related to GLS metabolism were among those differentially expressed in *ise2* mutant embryos.

Reverse transcription followed by quantitative PCR (RT-qPCR) was used to quantify changes in expression of GLS-related genes in ISE2-OE and ISE2-SUP plants.

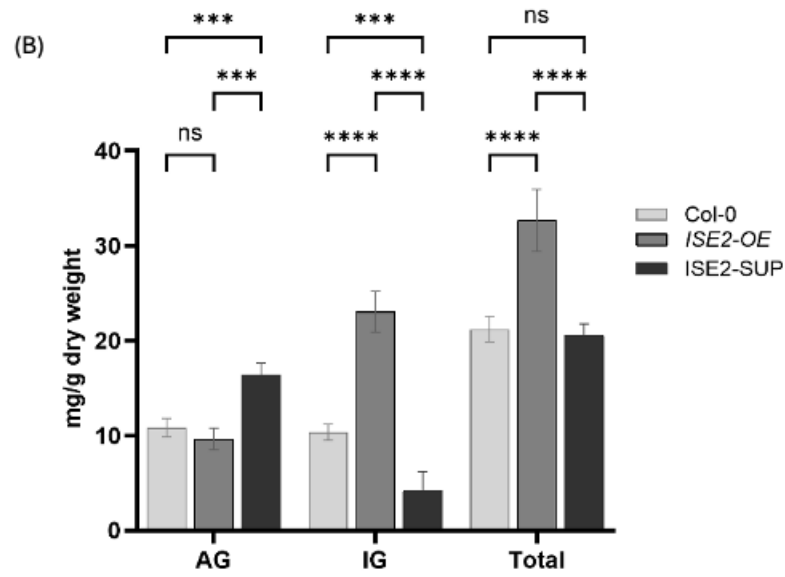
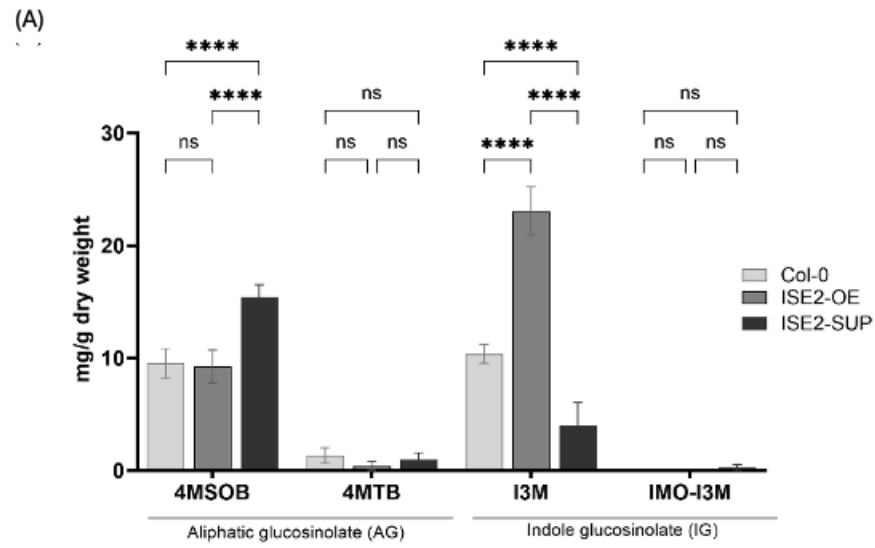
The biosynthetic pathways for the major Arabidopsis Col-0 AGs are shown (**Fig. 33A**). In ISE2-OE plants, expression of *BCAT4*, which encodes the enzyme responsible for the first step in the synthesis of AG, was induced while expression was suppressed in *ISE2-SUP* plants (**Fig. 38b**). The same pattern of increased expression in ISE2-OE plants but decreased expression in ISE2-SUP plants was observed for *MAMI*, the enzyme responsible for the condensation of the 2-oxo acid with acetyl-CoA, and for *CYP83A1* which converts the aldoximes to other intermediates (**Fig. 33B**). These results suggested that AG biosynthesis may be increased in ISE2-OE plants but decreased in ISE2-SUP plants.

For indole GLS biosynthesis (**Fig. 34A**), there was increased expression of the *CYP79B2* and *CYP79B3* enzymes that convert tryptophan to indole-3-acetaldoxime, the first step in IG biosynthesis, in ISE2-OE plants while expression of these genes was not different from the WT controls in ISE2-SUP plants (**Fig. 34B**). This suggests that there is increased flux into the indole GLS biosynthetic pathway in ISE2-OE plants. Expression of *CYP81F2*, encoding a p450 enzyme responsible for production of 4-hydroxyindole glucosinolate, was suppressed in ISE2-SUP plants but not affected in ISE2-OE plants relative to controls (**Fig. 34B**).

Interestingly, expression of the genes involved in GLS breakdown in leaves, myrosinases *TGG1* and *TGG2*, were induced in ISE2-OE plants relative to WT controls, and there was even higher expression of those genes in ISE2-SUP plants compared to controls (**Fig. 34C**). We confirmed the changes in expression of GLS-related genes in two independent lines that had been rescued with the same transgenes (47)).

Figure 35. GLS content is altered in ISE2-OE plants and in ISE2-SUP plants.

(A) ISE2-OE plants accumulated glucobrassicin (I3M) while ISE2-SUP plants accumulated glucoraphanin (4MSOB) but had decreased glucobrassicin (I3M) levels compared to the Col-0 controls. (B) ISE2-SUP plants had higher AG and lower IG levels than ISE2-OE or Col-0 plants while ISE2-OE plants accumulated higher levels of IG. The total GLS content was increased in ISE2-OE plants. GLS were measured in leaves of 22-day old plants. For Col-0 N=3, ISE2-OE N=3 and ISE2-SUP N=4. Error bars represent standard error. Statistically significant differences between means were determined using ANOVA followed by Tukey's HSD.



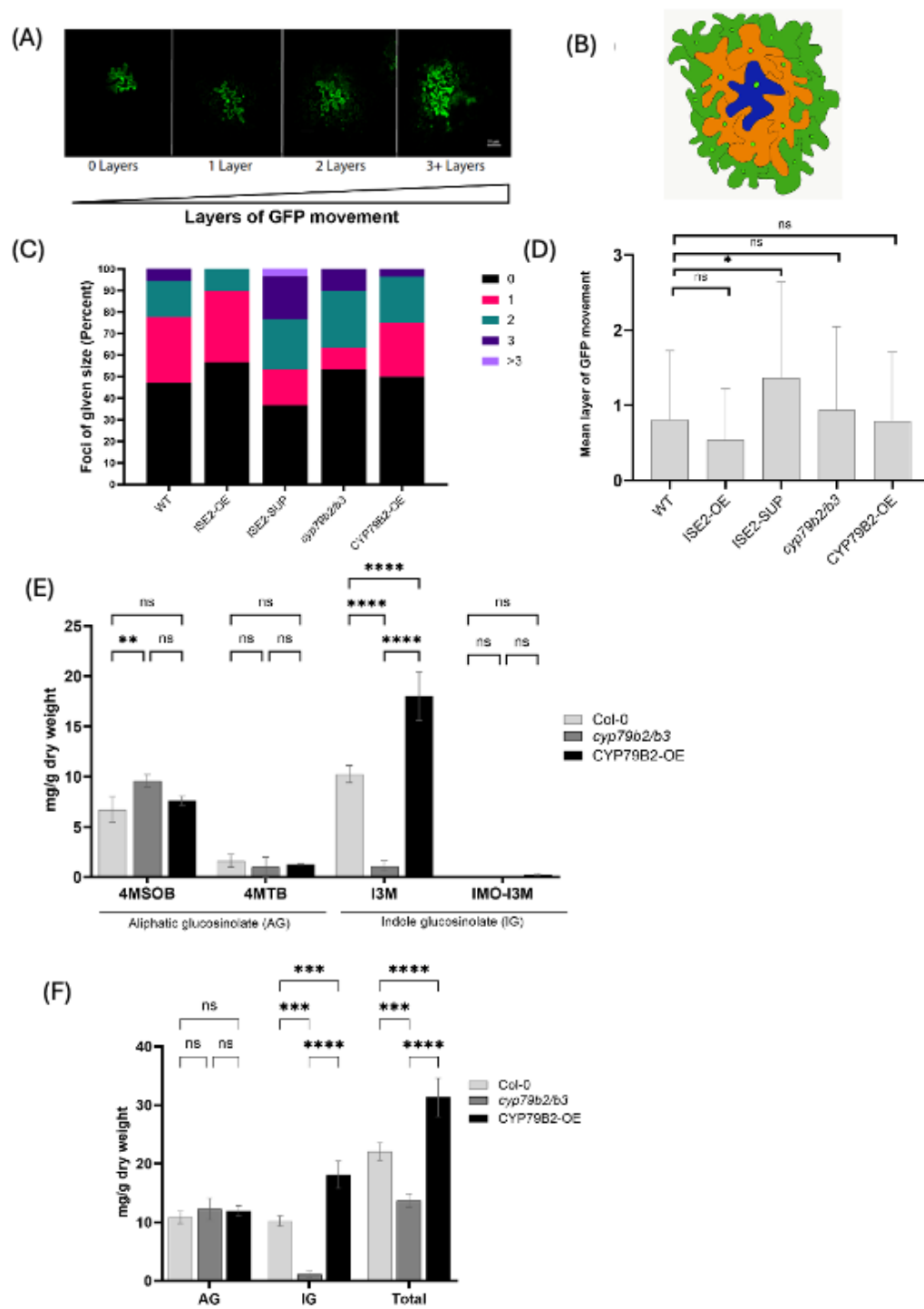
Thus, when ISE2 levels were perturbed in Arabidopsis leaves, expression of GLS-related genes was changed.

Quantification of GLSs in plants with varying ISE2 expression

To determine how the abnormal expression of GLS-related genes altered GLS levels in plants with varying levels of *ISE2* expression, we measured GLSs by high performance liquid chromatography(294). In total, the levels of 13 GLSs reported to occur in Arabidopsis were measured (See Materials and Methods). As expected, several of those GLSs were not detected in our samples, and there were very low levels of aromatic GLSs. Indeed, AGs and IGs dominated the GLS pools in the WT, ISE2-OE and ISE2-SUP. Focusing on the most abundant GLSs, ISE2-OE plants had significantly increased accumulation of the IG glucobrassicin (also referred to as indol-3-ylmethyl, I3M) (**Fig. 35A**). This was consistent with the changes in IG biosynthesis genes observed (**Fig. 34**). ISE2-SUP plants had a statistically significant increase in the AG glucoraphanin (or 4-methylsulfinylbutyl, 4MSOB) (**Fig. 35A**). ISE2-SUP plants also had significantly decreased glucobrassicin IG content relative to the wild-type controls (**Fig. 35A**). While the decreased IG content in ISE2-SUP plants was expected from the gene expression analysis (**Fig. 34**), the increased AG content was unexpected, suggesting that other genes in the pathway may be responsible for this increase or that gene expression levels do not reflect enzyme level or activities. Levels of the other minor GLSs, 4-methylthio-3-butenyl (4MTB) AG and 4-methylglucobrassicin (IMO-I3M) IG, were not significantly changed in ISE-OE or ISE2-SUP plants (**Fig. 35A**). The changes in these few GLSs may explain the observed differences in the total GLS levels in plants with differing levels of

Figure 36. Intercellular trafficking is affected by GLS content.

(A) PD-mediated trafficking was measured by a movement assay where the expression of GFP is tracked from a single cell into neighboring cells (called layers). (B) A cartoon representation of a GFP-expressing cells (blue shape labeled with 0) to the surrounding concentric layers (orange cells labeled as layer 1 and green cells labeled as layer 2). (CID) Movement assays in *ISE2-OE* and *ISE2-SUP* plants and known GLS mutants, *cyp79b2 cyp79b3* and a line overexpressing *CYP79B2*. Results are presented as stacked bar charts showing all data (C) or as the mean number of layers moved (D). Data shown are from at least three biological replicates, with 10-15 z-sections collected per genotype per replicate. Z-stacks were reconstructed using FIJI (Scheindelin *et al.*, 2012) and significance was determined by Mann-Whitney test with significance at $p < 0.05$ (indicated by asterisk). ns = no statistically significant difference. Error-bars indicate standard deviation. (E) *cyp79b2 cyp79b3* plants accumulated glucoraphanin (4MSOB) while glucobrassicin (I3M) levels were significantly lower. The *CYP79B3* plants accumulated glucobrassicin but wildtype levels of glucoraphanin. (F) Total AG levels were unaffected in both *cyp79* mutants while significant changes to IG levels were found for each mutant, with the total GLS content significantly decreased in *cyp79b2 cyp79b3* plants but significantly increased in *CYP79B3OE* plants. GLS were measured in leaves of 22-day old plants. For all genotypes, $N=2$, with each replicate containing a pool of leaves from at least 6 plants. Error bars represent standard error. Statistically significant differences between means were determined using ANOVA followed by Tukey's HSD.



ISE2. While AG levels were unchanged in ISE2-OE plants the large increase in IG levels likely contributed to a significant increase in total GLS levels (**Fig. 35B**). Total GLS levels were unchanged in ISE2-SUP plants due to the increased AG content being balanced by decreased IG content (**Fig. 35B**). These data suggest different imbalances of IG and AG in ISE2-OE and in ISE2-SUP plants compared to wild-type control plants. Thus, the differential expression of GLS-associated genes in response to *ISE2* expression resulted in altered GLS content.

GLS-related mutants have defects in intercellular trafficking

Intercellular trafficking via PD was measured in plants with altered GLS levels by implementing a movement assay by particle bombardment to introduce a plasmid encoding soluble green fluorescent protein (sGFP) into individual leaf epidermal cells(295). The movement of GFP from the primary transformed (GFP-producing) cell into contiguous layers of surrounding cells was then scored. Representative images of cell clusters with increased intercellular trafficking GFP are depicted (**Fig. 36A**, from left to right). **Fig. 36B** shows a cartoon illustration of a GFP focus with two layers of intercellular trafficking . Data collected from 3 biological replicates each containing a minimum of 20 events were presented in a stacked bar chart (**Fig. 36C**). The average size of GFP-containing cell cluster was determined and represented as mean layers of movement (**Fig. 36D**). In wildtype plants 48% of clusters had no intercellular trafficking (**Fig. 36C**) and the mean number of layers of GFP spread was 0.7 (**Fig. 36D**). Consistent with previous findings in *N. benthamiana* leaves, ISE2-OE plants have decreased intercellular trafficking while intercellular trafficking increased in ISE2-SUP leaves

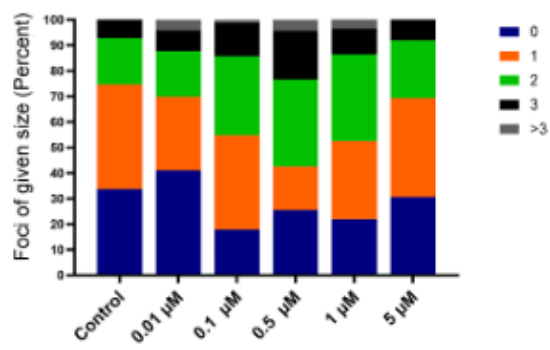
(48,126), although the change in ISE2-OE plants was not statistically significant. For ISE2-OE leaves, ~57% of the foci had no GFP trafficking, and the average cluster size decreased to 0.6 layers (**Fig. 36C,D**). For ISE2-SUP leaves only ~36% of clusters showed no movement and there were increased numbers of clusters with 2, 3, and more layers (**Fig. 36C**), and the mean cluster size increased to ~1.5 layers (**Fig. 36D**). Thus, ISE2-OE plants with increased IG content had somewhat decreased intercellular trafficking whereas ISE2-SUP plants with increased AG content had increased intercellular trafficking.

We then tested whether intercellular trafficking was defective in other plants with altered GLS levels. For this, we selected the *cyp79b2 cyp79b3* double mutant, which was reported to have a drastic reduction in IG biosynthesis (296), and an established transgenic line overexpressing *CYP79B2* (CYP79B2-OE) that was expected to have increased glucobrassicin (I3M) IG levels (296). We confirmed that IG levels were significantly reduced in the *cyp79b2/b3* mutant and observed a small but statistically significant increase in the AG glucoraphanin in the mutant (**Fig. 36E**). The CYP79B2-OE line had significantly elevated levels of IG, specifically of glucobrassicin (I3M), like the ISE2-OE plants (**Fig. 36E,F**). The AG content in the CYP79B2-OE plants were not different from those in wild-type control plants (**Fig. 36E**). While the GLS pools of the mutants were altered in patterns similar to those of the *ISE2* mutants, the measured amounts of the GLSs differed with higher GLS content in the *ISE2* plants (compare **Fig. 35C** and **Fig. 36C**). Cell-to-cell movement of GFP in *cyp79b2 cyp79b3b3* mutant plants and the CYP79B2-OE plants was not significantly different from the wild-type controls,

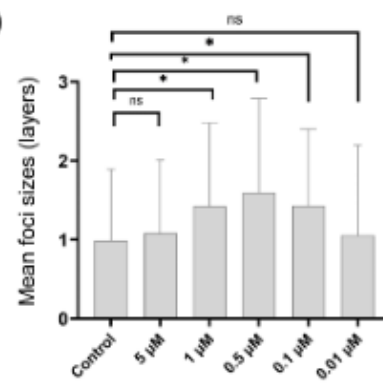
Figure 37. Exogenous application of GLS was sufficient to modify intercellular trafficking.

(A,B) Intercellular trafficking was significantly increased in plants treated with 0.1 μ M, to 1 μ M sinigrin AG. (C-D) Application of the IG glucobrassicin caused a significant decrease in intercellular trafficking over a range of concentrations from 0.1 to 5 μ M. (E-F) Treatment with a high dose of glucobrassicin, near levels found in ISE2-OE plants, reduced intercellular trafficking. (G-H) Exogenous application of ITC did not alter intercellular trafficking. For each treatment, N=3, n=10-20 z-stacks per replicate and significance was determined by Mann-Whitney test, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ (asterisks). ns = no statistically significant difference. Error bars indicate standard deviation.

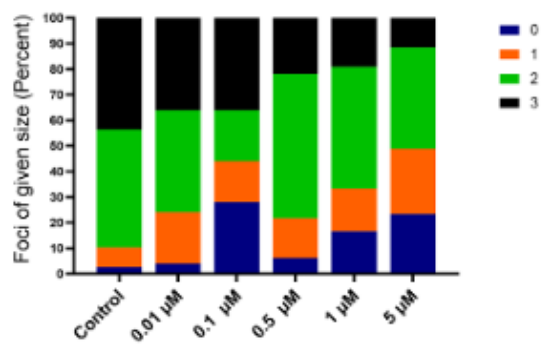
(A) Sinigrin application



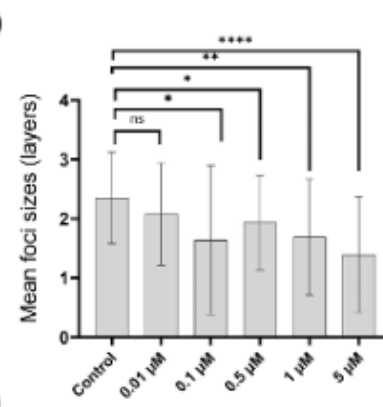
(B)



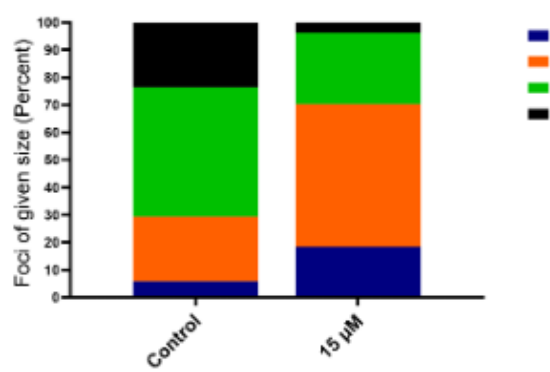
(C) Glucobrassicin application



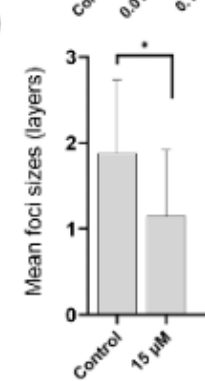
(D)



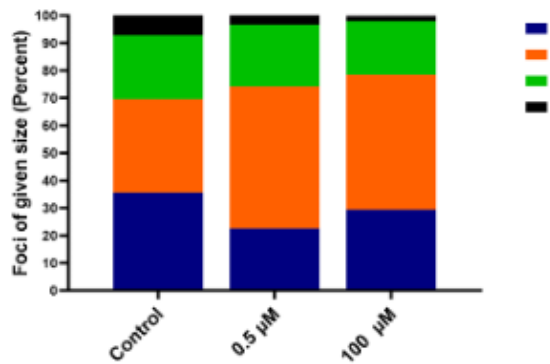
(E)



(F)



(G) ITC application



(H)

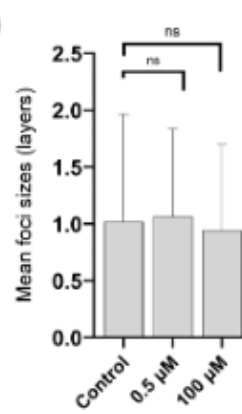


Figure 38. GLSs can modulate callose levels at PD.

(A) Aniline blue staining was used to detect callose at plasmodesmata in Col-0 that were treated with GLSs at indicated concentrations. Representative images are shown, with bright dots indicating PD callose in a more diffusely stained cell wall. Scale bar represents 10 μm . (B) Leaves from wildtype Col-0 plants were treated with indicated GLSs as described for after bombardment. The number of aniline-blue stained punctae were counted and outliers removed by IQR analysis. Each dot represents an independent region of interest, and data was collected from at least 10 leaves per treatment. Statistical significance at $p < 0.05$ was calculated by Student's t -test (asterisks). Ns = no statistically significant difference. (C) DEGs related to callose metabolism as determined by RNAseq analysis. Only DEGs with adjusted p values below 0.05 are shown, and values on the heat map show $\log_2$ fold change in expression.

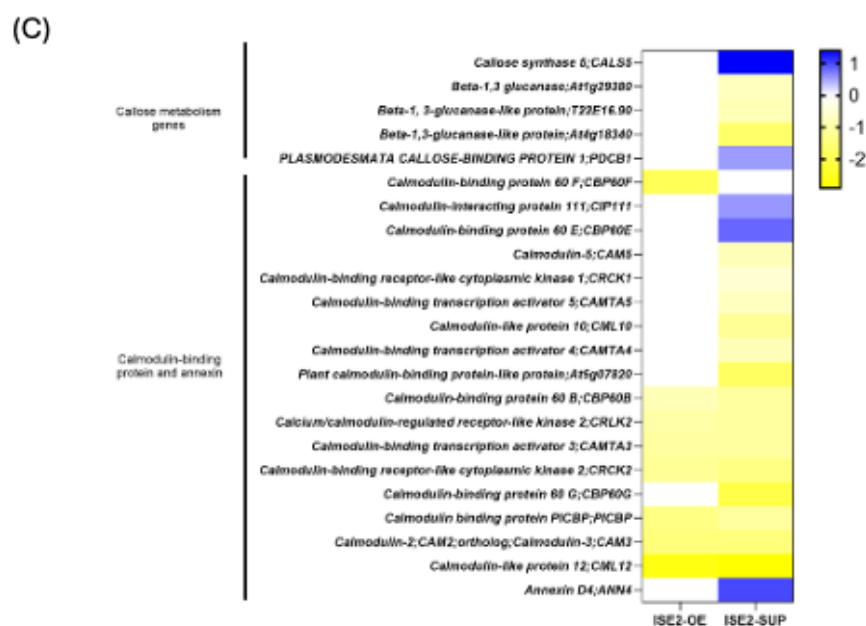
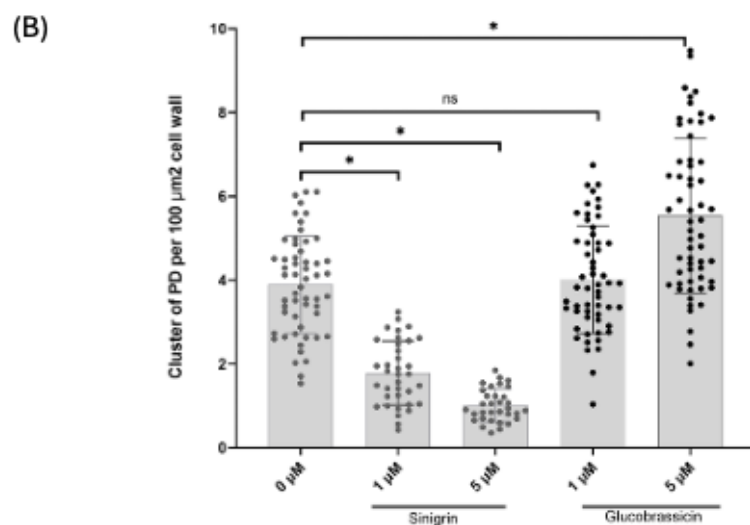
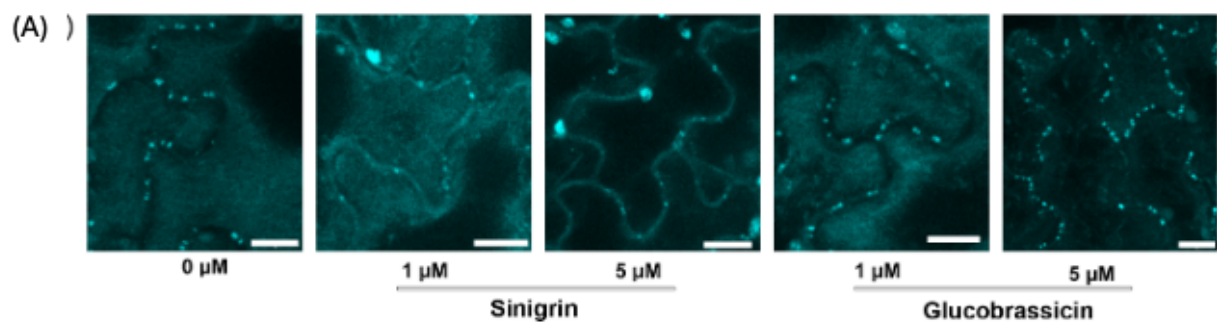
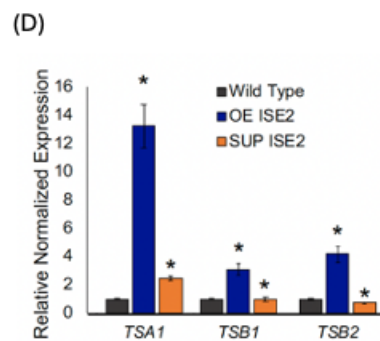
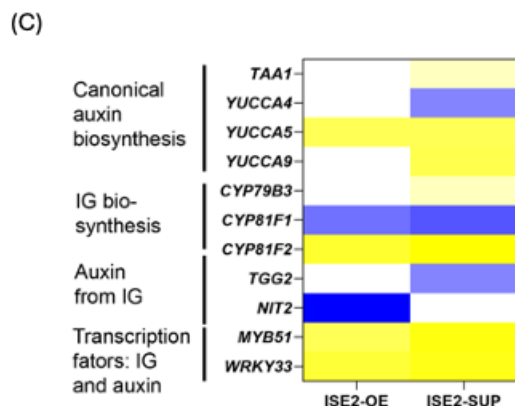
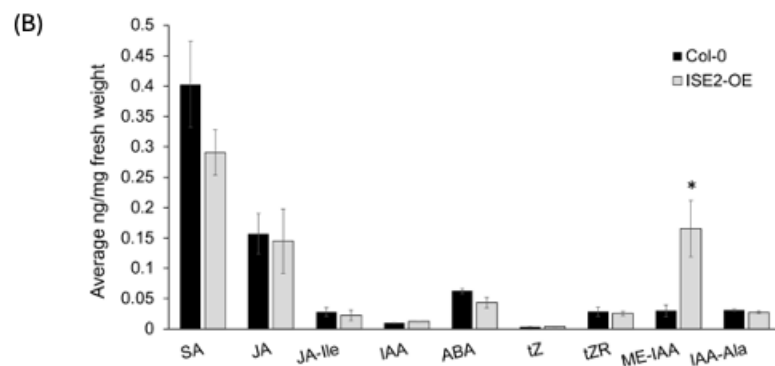
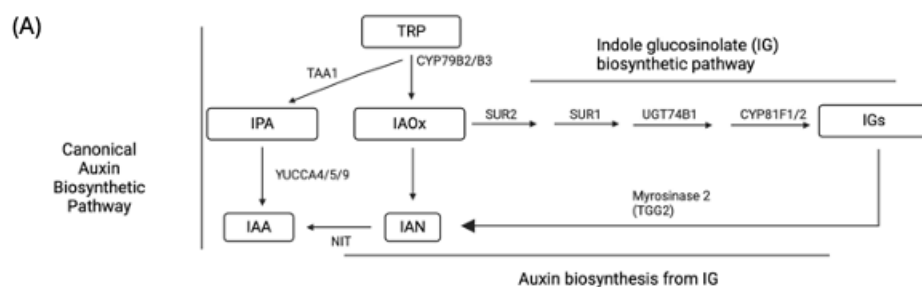


Figure 39. ISE2-OE plants with elevated IG levels have increased auxin metabolism.

(A) Scheme showing auxin biosynthesis from tryptophan via the canonical TAA/YUC pathway or IG. (B) ISE2-OE plants have significantly higher MeIAA levels compared to wild-type Col-0 plants. SA: salicylic acid, JA: jasmonic acid, JA-Ile: jasmonyl isoleucine, IAA: Indole-3-acetic acid, ABA: Trans abscisic acid, tZ: trans-Zeatin, tZR: trans-zeatin 9-riboside, ME-IAA: Methyl-indole-3-acetate, IAA-Ala: N-indole-3-acetyl-alanine. N=3, n=6. Significance at $p < 0.05$ (*) determined by Student's *t*-test. (C) Expression of auxin and IG-related genes compared to Col-0 control plants revealed by RNA-seq. Only DEGs with adjusted *p* values below 0.05 are shown, and values on the heat map show $\log_2$ fold change in expression. (D) Expression of TRP biosynthesis genes in ISE2-OE and ISE2-SUP plants compared to Col-0 control plants measured by RT-qPCR. Significance at $p < 0.05$ (*) determined by Student's *t*-test.



although a tendency towards increased trafficking (larger foci) could be observed in the *cyp79b2 cyp79b3* plants (**Fig. 36C,D**). This result suggested that the balance of GLS between the IG and AG pool and the amounts of GLS both contributed to regulating intercellular trafficking.

We next tested intercellular trafficking in mutants with defects in the TGG1 and TGG2 myrosinases. The *tgg1-2* mutant has a statistically significant increase in intercellular trafficking while the increase in trafficking in the *tgg2-1* mutant was not. *3, PDCB4, PLASMODESMATA LOCATED PROTEIN (PDLP)3 and PDLP6*, were also found among the DEGs (**Fig. 38C**). The activity of callose synthase enzymes is proposed to involve several regulatory factors including CALMODULIN (CAM) calcium sensors (297–299), and several genes related to CAMs were identified in the callose and cell wall-related DEG set. The DEGs set also included several *CELLULOSE SYNTHASES* and other genes involved in cell wall modification including several *EXPANSINS* and *XYLOGLUCAN ENDOTRANSGLYCOSYLASES/HYDROLASES (XTHs)*. Together, the measurement of PD callose in response to GLS and this set of DEGs suggest that GLS may modulate PD callose metabolism to modulate intercellular trafficking.

ISE2-OE plants have elevated flux through auxin

Auxin and IG biosynthesis both start with tryptophan as the initial compound in their synthesis (300,301). A schema for the biosynthesis of auxin is presented in **Fig. 39A**. We measured hormone levels in wild type Col-0 and ISE2-OE plants. There was a drastic, statistically significant increase in methyl-IAA (MeIAA) levels in the ISE2-OE plants compared to controls while levels of other hormones like the cytokinin trans-zeatin were

not different between the two groups of plants (**Fig. 39B**). Conversion of free IAA to the methylated form is likely used to control free IAA levels, and hence auxin levels and activity in plants (302), although the hydrophobic MeIAA may have distinct roles in plant development (303). However, the levels free IAA in both groups of plants were below the level of quantification for our method (**Fig. 39B**). Nevertheless, the increased levels of MeIAA suggest that auxin homeostasis is disturbed in ISE2-OE plants. Analysis of our RNA-seq data revealed that genes related to auxin biosynthesis were differentially expressed in ISE2-OE plants and in ISE2-SUP plants compared to Col-0 plants (**Fig. 39C**). Expression of the tryptophan biosynthesis genes, *TSA1*, *TSB1*, and *TSB2* was significantly induced in ISE2-OE plants as revealed by qPCR (**Fig. 39D**). Expression of both *TAA1* and *CYP79B2*, encoding the first enzyme in auxin and IG biosynthesis, respectively, was induced in the ISE2-OE plants (**Fig. 39C**). This points to a potential increase in tryptophan synthesis and is consistent with the increased IAA and IG levels in ISE2-OE plants. (**Fig. 39C**). Notably, expression of the *NITRILASE* gene that converts IAN to auxin was strongly induced in ISE2-OE plants but only mildly affected in ISE2-SUP plants (**Fig. 39C**). Expression of *TGG2*, encoding the myrosinase involved in IG conversion to IAA was also included in the DEG set (**Fig. 39C**). Further exploration of the effects of the observed accumulation of Me-IAA and other changes in auxin metabolism in the ISE2-OE and ISE2-SUP plants is certainly warranted.

DISCUSSION

Studies with the chloroplast RNA helicase, ISE2, have been important for revealing a potential role for chloroplast signals in regulating intercellular trafficking via

plasmodesmata(126). In this study we employed a system of plants with varying levels of ISE2 expression to further investigate ISE2's effects on intercellular trafficking. We found that disrupting *ISE2* expression resulted in changes in expression of genes involved in GLS synthesis and breakdown (**Fig. 33-34**). This unexpected observation was supported by the elevated IG levels in ISE2-OE plants and AG accumulation in ISE-SUP plants (**Fig. 35**). These observations were consistent with altered intercellular trafficking in known GLS mutants or plants with altered expression of GLS genes (**Fig. 36**). Indeed, changes in intercellular trafficking could be mediated by GLS themselves and do not require their hydrolysis (**Fig. 37**). Exogenous application of GLSs modulated callose levels at PD, suggesting that GLS could alter callose homeostasis at PD to control intercellular flux (Fig. 44). The observed correlation between PD callose levels is supported by studies in *ISE2*-silenced *N. benthamiana* plants where increased intercellular trafficking was accompanied by reduced PD callose (304). Together, these findings identify a previously unknown potential role for GLS in signaling to control intercellular trafficking, possibly by regulating callose levels at PD.

In leaves, GLSs are synthesized in the phloem parenchyma cells (305). GLSs are then transported to S cells near the vasculature and in the leaf epidermis for storage at the leaf margins (278,305,306). S cells are elongated cells in the phloem parenchyma with altered cellular contents which accumulate high levels of GLS and can be the progenitors of phloem fibers(307,308). PD and symplastic trafficking are also crucial for GLS transport across leaves (309). Consistent with this, numerous functional PD connect S-cells to the neighboring cells that synthesize GLSs (308). GLSs are transported long

distances in the plant via the vasculature following the source-sink route of sugars(305,310–312). Short-chained AG are synthesized in the shoot and transported to the roots via the phloem before entering the root xylem for transport back to the shoots (309). This transport was found to depend on the GLS transporters GTR1 and GTR2, suggesting that they may load into the phloem for symplastic transport. When viewed in this context, it is not surprising, then, that GLS would have effects on PD and their intercellular trafficking capacities. That GLSs use PD to move to storage cells from synthesis cells, would also explain why the GLSs themselves and not their hydrolysis products affect PD.

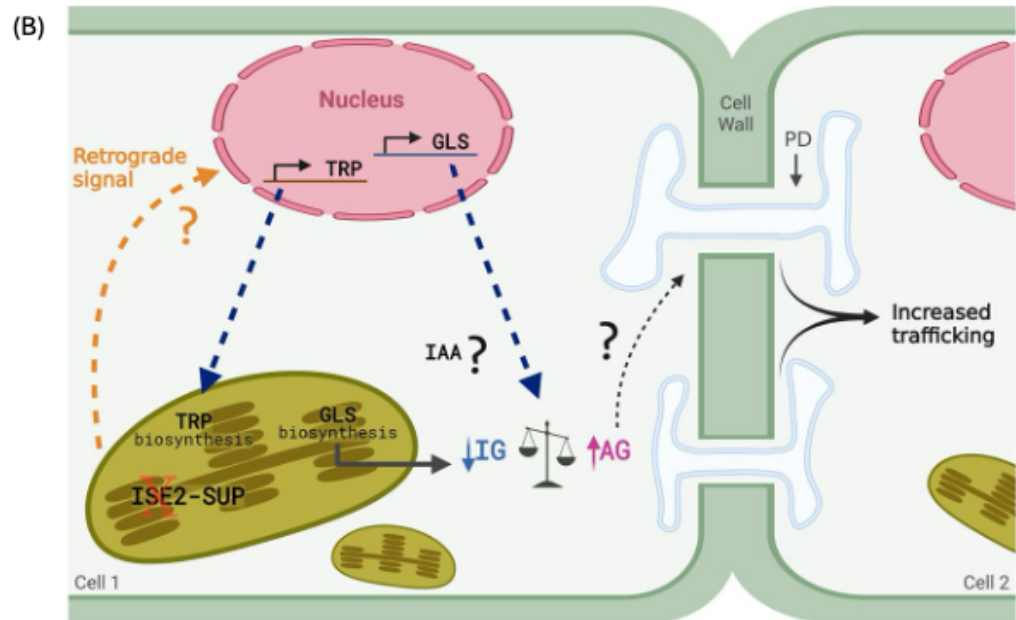
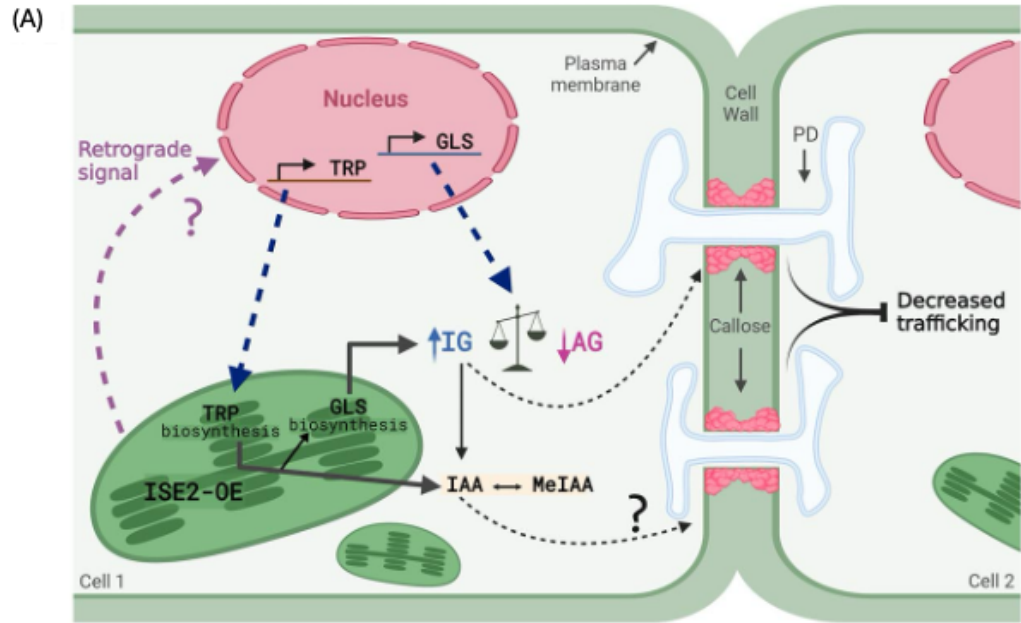
IGs are closely linked to auxin since both use tryptophan as a precursor. Indeed, there is enough overlap in auxin mutants and GLS mutants that the *superroot2* (*sur2*) mutant was previously described as an auxin mutant and it had increased levels of free IAA (313). Later, it was found that the “auxin” mutant gene *SUR2* is allelic to the gene that encodes CYP83B1, the CYP responsible for the conversion of aldoxime to IGs (314). When IGs are hydrolyzed by myrosinases (TGG1 or TGG2), the IAA precursor IAN (indole-3-acetonitrile) is made. In the first steps of IG biosynthesis, tryptophan is acted on by two cytochrome P450 enzymes, CYP79B2 and CYP79B3, to produce Indole-3-acetaldoxime (IAOx) which then enters the IG biosynthetic pathway that includes CYP83B1 (*SUR2*) as the first of a series of enzymes. Through the action of two nitrilases, NIT1 and NIT2, IAA is formed from IAN. *CYP83B1* is therefore considered a marker gene for the IG biosynthetic pathway. Note that in ISE2-OE plants, the expression of *CYP79B2* and *CYP79B3* was significantly increased as was expression of the

myrosinase genes *TGG1* and *TGG2* (**Fig. 32**). The auxin phenotypes of the ISE2-OE plants could be attributed to an imbalance in GLS and increased flux through the IG biosynthesis pathway leading to increased IA levels. While the delay in bolting, increased production of adventitious roots and drought tolerance are indeed auxin phenotypes, the original cause may, in fact, be the increased IG content. A comprehensive study on the metabolites and hormones for ISE2-OE and ISE2-SUP is needed to tease apart the complex network at play.

This investigation adds a new layer to our growing understanding of the connections between phytohormones and PD trafficking (315). For example, an investigation of root development revealed that auxin regulates PD transport, and, moreover, that rates of PD transport impact auxin accumulation and signaling (316). Similar to the discovery reported here that GLS can regulate their own trafficking by modulating symplastic transport, it was recently shown that brassinosteroids (BRs) regulate PD trafficking to coordinate the balance between the levels of BR precursors, which are synthesized in one cell type, and the active signaling forms of BR, which are synthesized in a different cell type (317). Transcriptional profiling of *ise2* embryos and other *ise* mutants, including *ise1* (30), *ise3*, and *ise4* (72), revealed disruption of cytokinin signaling, and subsequent experiments in *N. benthamiana* leaves confirmed that cytokinins can promote PD transport(318). Salicylic acid, a phytohormone associated with defense against pathogens, regulates PD trafficking during immune responses; although SA itself does not primarily move through PD, some parallel signaling molecules synthesized during infections, including glycerol-3-phosphate and azelaic acid,

Figure 40. Modeling ISE2 effects on intra- and intercellular trafficking.

(A) Altered ISE2 levels trigger retrograde signaling to impinge nuclear gene expression, perturbing GLS and TRP metabolism in the chloroplast and leading to higher IG and MeIAA levels. In those cells, intercellular trafficking is restricted, likely by callose accumulation at PD. (B) Different retrograde signals may be produced by chloroplasts with reduced ISE2 content, leading to distinct changes in TRP and GLS metabolism. Higher AG levels increase intercellular trafficking, likely by decreasing callose levels at PD.



preferentially travel through PD(86,87). This current study demonstrates the complexity of the connections between phytohormones and PD by showing that ISE2 regulates synthesis of GLS, which are another class of defense compounds, and that GLS can impact auxin accumulation and PD transport.

ISE2 is a chloroplast RNA helicase whose role in posttranscriptional processing of organellar RNAs has been characterized (269,304). Reduced ISE2 levels are associated with altered nuclear gene expression(22,48). Based on this knowledge and the findings presented here we propose a model for how ISE2 may impinge on GLS and auxin to regulate PD. Changes in ISE2 levels, leading to altered chloroplast physiology, trigger retrograde signals that alter expression of GLS- and TRP-related genes among others, with signal identity or strength depending on ISE2 levels (**Fig. 40**). It should be noted that both GLS and TRP metabolism involve chloroplast-localized enzymes.

In ISE2-OE tissues with increased ISE2 levels, this changes chloroplast metabolism, impacting TRP biosynthesis and therefore both GLS and auxin biosynthesis (**Fig. 39A**). The imbalance in GLS levels, with elevated IG, changes callose homeostasis in the cell walls surrounding PD leading to accumulation of callose and ultimately decreased intercellular trafficking. Changes in auxin metabolism, possibly the elevated MeIAA levels caused by more flux through IAA, may also contribute to altered PD function (**Fig. 39A**). When ISE2 levels are lower (ISE2-SUP), different retrograde signals are produced which have distinct effects on the expression genes of the GLS and other pathways. In this instance, the GLS imbalance favors AG accumulation (**Fig. 39B**). In these tissues, intercellular trafficking increases, possibly with decreased accumulation

of callose contributing to this change (304). Due to the technical limitations of our system, a role for auxin could not be examined because IAA levels were not measured but a role for auxin cannot be ruled out (**Fig. 39B**). Thus, GLS may act on the callose-metabolizing proteins at the PD to control permeability of the pores and increase or decrease intercellular trafficking. In such a scenario, the balance between IG and AG in a cell or tissue would therefore be important for determining intercellular trafficking as it pertains to the PD connecting those cells (**Fig. 40**). Several components of this model metabolites generated during stress, pest attack and/or pathogen infection, the role of chloroplasts in these responses, and the impacts of these events on PD transport and signaling events within and beyond affected tissues, will be needed to understand how these various signaling modules interact to confer resilience (295,319).

MATERIALS AND METHODS

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* Colombia-0 (Col-0) ecotype plants and derivative transgenic and mutant lines were used for all studies. Seeds were directly planted onto soil and stratified at 4 °C for three days and then transferred to a growth chamber under standard growth conditions (22 °C, 16 h light, 8 h dark, 60% humidity). Alternatively, seeds were germinated on plates on ½ X strength Murashige and Skoog Basal Salt Mixture (MS) media (Sigma M5524-1L), 0.7% plant agarose (PlantMedia, SKU# 40100072-1) pH 5.7. Seeds were first surface sterilized with 50% ethanol for 1 minute, followed by a 10% bleach and 0.01% Triton X-100 solution for 11 min, followed by six

5-minute washes in sterile water. Seeds were then stratified on plates at 4 °C for 3 days before transfer to a growth chamber under standard growth conditions.

RNA isolation for RNA-seq

RNA was isolated from plant tissue samples using TRIzol® Reagent and according to manufacturer's protocol (Invitrogen, Life Technologies, Carlsbad, CA). Briefly, tissues were ground in a mortar and pestle under liquid nitrogen. RNA was isolated from 100 mg of ground tissue by adding 1 ml of TRIzol and incubated at room temperature for 5 minutes. 200 µl of chloroform was added to the sample and shaken vigorously by hand for 10 seconds and then incubated at room temperature for 3 minutes. Samples were then centrifuged for 15 minutes at 12,000 x g at 4 °C. The aqueous phase was collected and 500 µl of isopropanol was added. Samples were incubated at room temperature for 10 minutes before the RNA was collected by centrifugation for 10 minutes at 12,000 x g at 4 °C. The RNA pellet was washed twice with cold 75% ethanol and centrifugation at 7,500 x g at 4 °C. Approximately 25-500 ng/µl of purified RNA was subjected to microfluidics analysis on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) to determine RNA integrity and quantification of rRNA. The Plant RNA Pico Assay chip was used according to manufacturer's instructions. RNA Integrity Number (RIN) values were determined to assess the quality of RNA samples and the RNA used for RNA seq analysis.

RNA sequencing analysis

Library creation and sequencing was performed at the Joint Genome Institute (JGI). About 60 million filtered reads were obtained for each sample, with four biological

replicates for each sample. Raw reads were processed at Oak Ridge National Lab (ORNL) using the JGI QC pipeline. Raw fastq file reads were filtered and trimmed using the JGI QC pipeline. Filtered reads from each library were aligned to the reference genome using HISAT2 version 2.1.0 (320) (BAMs/ directory). featureCounts (321) was used to generate the raw gene counts file using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts. Raw gene counts were used to evaluate the level of correlation between biological replicates using Pearson's correlation and determine which replicates would be used in the DGE analysis.

RNAseq Data Analysis

The read counts from three replicates for each sample group were processed through the integrated Differential Expression and Pathway analysis (iDEP 2.01, <https://bioinformatics.sdstate.edu/idep/>) web application(290), where the reads were converted and filtered then aligned to the *Arabidopsis thaliana* (TAIR10) genome. The method for differential expression was using the CountsDEGMethod (DESeq2), with an FDR cutoff of 0.1 and a minimum fold change of 1.5. Significantly DEGs were determined using an adjusted *P* value cutoff of 0.05. Gene Ontology (GO) and KEGG pathway analysis in iDEP used GAGE analysis and an FDR cutoff of 0.1.

Arabidopsis VIGS and Tiling Microarray

A 400-bp fragment from *AtISE2* was cloned into TRV RNA2 silencing vector (pYL156) (322). *Arabidopsis* VIGS was performed as described previously(292). Leaves were collected from *ISE2*-silenced or non-silenced, TRV infected control plants on the fourteenth day after infiltration for VIGS. RNA was extracted by Plant RNeasy Mini Kit

(QIAGEN) and prepared for tiling microarray hybridization as previously described (323). Samples were hybridized to *Arabidopsis* GeneChip 1.0R tiling arrays and hybridization, scanning and analysis were performed as described previously (323).

GLS Quantification

GLS extraction and quantification were conducted as previously reported (Charron & Sams, 2004). GLSs were desulfated using a DEAE Sephadex A-25 column.

Desulfoglucosinolates were separated with a Hewlett-Packard (Palo Alto, CA) high performance liquid chromatograph (HPLC) with a C-18 ODS reverse-phase column (250 x 4.6 mm ID, 5 mm) with a UV detector at 230 nm. The identification and quantification of the desulfoglucosinolates were made using authentic standards (Charron & Sams, 2004). The 13 GLSs measured were Glucoiberin (3MSOP), Glucoprogoin (2S)-2-hydroxy-3-butenyl glucosinolate), Glucoepiprogoitrin ((2R)-2-Hydroxybut-3-enyl), Glucoraphanin (4MSOB), Sinigrin (2-Propenyl), Glucosinalbin (4-Hydroxybenzyl), Gluconapin (3-butenyl), Glucobarbarin ((2R)-Hydroxy-2-phenylethyl), Glucoerucin (4MTB), Glucobrassicin (I3M), 4-methylglucobrassicin (4MOI3M), Gluconasturtiin (2PE), and Neo-glucobrassicin (1MOI3M).

Hormone Analysis

Hormone analysis was performed by Creative Proteomics (Shirley, NY) as follows. Plant samples were ground into powder and 50 mg of the sample were added into a test tube with 1 mL of a mixture of methanol: water: formic acid (v: v: v, 15:4:1). The sample was vortexed for 2 min and put in an ultrasonic bath for 20 min. Then the sample was stored in a fridge at -20°C for 16 h to avoid light. Samples were centrifuged at 10000 rpm and

4°C for 10 min and supernatant was transferred to another tube. The residue was added with 0.5 mL of a mixture of methanol: water: formic acid (v: v: v, 15:4:1) and extracted twice. The collected supernatant (three times) was pooled and dried by a gentle flow of nitrogen gas at room temperature. The dried residue was re-dissolved in 200 µL of methanol:water (50:50, v/v). The re-dissolved sample was filtered through a 0.22 µm membrane filter before analysis by UPLC-MS/MS using an AB SCIEX 4000 Qtrap mass spectrometer (AB Sciex LLC, Framingham, MA) connected with a Waters Acquity UPLC (Waters Corporation, Milford, MA). The standards were dissolved in methanol to achieve 1 mg/mL as stocking solutions, respectively. Seven standards (IAA, tZ, tZR, IAA-Asp, ME-IAA, IAA-Ala, and IAA-Trp) analyzed in positive mode on MS were mixed to get gradient concentrations from 0.1 ng/mL to 500 ng/mL. The other 4 standards (SA, JA, JA-Ile, and ABA) analyzed in negative mode on MS were mixed to get gradient concentrations from 0.1 mg/mL to 500 ng/mL.

cDNA synthesis

RNA was extracted with TRIzol® Reagent as described above. The RNA pellet was resuspended in 40 µl DEPC-treated water and the concentration was determined by spectrophotometric analysis on a Nanodrop. Genomic DNA was removed from the sample by treating total RNA with DNase using the DNA-free kit™ according to manufacturer's protocol (Ambion). RNA concentration and purity were again analyzed and 2 µg of RNA was used for cDNA synthesis using the M-MLV Reverse Transcriptase kit (Promega, Madison, WI) with oligo-dT primers.

Reverse transcription-quantitative PCR (RT-qPCR)

Full length targets were amplified with specific primers (Table S5) using ExTaq (Takara, Mountain View, CA) and cloned into pMiniT 2.0 vector using the NEB PCR Cloning Kit (New England Biolabs, Ipswich, MA). The isolated plasmid was used as template and amplification efficiency was calculated from a standard curve generated by the amplification of five dilution points. Gene expression was measured using qPCR on the CFX96 Touch Real-Time PCR Detection System (BIO-RAD Hercules, CA). cDNA was diluted 1:5 for analysis. The Master mix was made using the LightCycler 480 SYBR Green I Master Mix with the following thermocycling conditions: 40 cycles of 95 °C 10 s, 59 °C 10 s, 72 °C for 20 s. CT values were normalized to the reference gene *At4g26410* (324) .

Primers

All primers for cloning, qPCR and probe synthesis were designed with DNASTAR Lasergene Software (Madison, WI) and ordered from ThermoFisher (Invitrogen, Carlsbad, CA). The lyophilized primers are resuspended in water to a final concentration of 100 mM. Working stocks (10 mM) were made for all experiments. They are presented in Table S6.

Transgenic lines generation

The full-length *TGG1* gene from *A. thaliana* was amplified from cDNA using Phusion High-Fidelity PCR Master Mix (ThermoFisher Scientific) with GATEWAY compatible sites (Table S5). The PCR product was cloned into the pDONR-Zeo donor vector and subsequently into the binary destination vectors pGWB405 and 406 (325) following the

GATEWAY protocol (Invitrogen, Life Technologies, Carlsbad, CA). The resulting plasmids were transformed into wild-type Arabidopsis Col-0 plants by floral dip (326).

Intercellular trafficking via plasmodesmata

Intercellular trafficking was done using two separate approaches. First, to measure intercellular trafficking in mutant plants whole plants were placed in the bombardment chamber and bombardments were done to whole intact plants. The pRTL2rsGFP plasmid for expressing free GFP was bombarded into leaves under low pressure using a modified vacuum desiccator as the bombardment “gun” apparatus (295). The pressure was generated with a vacuum and helium gas was used to produce the blast at 60 psi. Second, to assay the effects of GLS on intercellular trafficking, fifth and sixth leaves (starting from the oldest leaf at the base of the rosette as the first leaf and counting upward toward the youngest leaf) from five three-week-old plants were collected, placed on agar plates, and low-pressure bombarded with GFP-containing plasmid pRTNL2rsGFP for a GFP movement assay as described above. After bombardment, the leaves were incubated abaxial side down on half-strength MS media containing different concentrations of GLS: sinigrin (Sigma-Aldrich, Cat. No. PHL89279) or glucobrassicin (Sigma-Aldrich, Cat. No. PHL80593) at 0.01, 0.1, 0.5, 1, 5 mM; or glucosinalbin (Sigma-Aldrich, Cat. No. PHL89793) or allyl isothiocyanate (ITC; Sigma-Aldrich, Cat. No.377430) at 0, 5 or 100 mM for 24 hours at 23°C in a growth chamber before imaging GFP movement by confocal microscopy. At least 15 foci per treatment were collected to analyze GFP movement. Experiments were performed for at least three biological replicates.

Confocal microscopy for movement assays

Cell-to-cell movement of GFP was analyzed 24 hours post bombardment. Individual transformed cells expressing GFP on the abaxial surface of the leaves were identified and clusters of cells containing GFP were imaged with the Leica SP8 confocal with White Light Laser (Leica Microsystems, Buffalo Grove, IL) with excitation and emission wavelengths of 488 nm and 500-530 nm, respectively. The z-stacks were collected so that all the layers of cells surrounding the transformed cell (especially if those cells had GFP fluorescence) were imaged. Typically, 10-15 slices for z-stacks were collected with a step size of 3 mm was used; however, due to the undulating nature of the leaves, the thickness of the z-stacks varied. Z-stacks were reconstructed with FIJI (327). The number of cell layers to which the GFP spread was counted(295).

Callose measurements

Leaves from wildtype Col-0 plants were treated with sinigrin or glucobrassicin at 1 or 5 mM as described above. Twenty-four hours after moving the leaves to treatment plates, callose at PD was visualized by aniline blue staining following Method 1 described in a previous publication and aniline blue-stained punctae were counted with the manual method as described (328).

Statistics

Statistical significance for qPCR gene expression analysis was calculated by Student's *t*-test. For movement assays, statistical significance of differences between treatments and the untreated control or between the Col-0 wild-type plants and *ISE2-OE* or *ISE2-SUP* plants were calculated using the previously described Bootstrap method (Johnston &

Faulkner) or with Mann-Whitney U test in GraphPad Prism (Dotmatics, Boston, MA). In all cases, p values less than 0.05 were taken as significant. For glucosinolate measurements statistically significant differences between pairs of means was calculated by AVOVA followed by Tukey's HSD post hoc analysis in GraphPad Prism.

Data Availability

RNA-seq data accession number is PRJNA886667.

CHAPTER 6
CONCLUSION

Conclusion

This dissertation reveals the multifaceted regulatory pathways that orchestrate chloroplast-mediated regulation of intercellular communication via PD. The results suggest that the primary and secondary metabolism in chloroplasts plays a vital role in maintaining intercellular communication via PD, along with maintaining cellular energy.

In Chapter 1, we reviewed the ONPS hypothesis and explored the organelle-initiated signaling pathway that regulates trafficking through PD in the context of plant physiology and development. The central concept of ONPS is the balance between carbon supply from photosynthesis and the formation and function of PD (57, 58). The intercellular trafficking of fixed carbon mainly occurs via PD, which is essential for plant growth, development, and yield. When the PD function is compromised, excess carbohydrates accumulate in the leaves, causing feedback inhibition of photosynthesis.

In chapter 2, we showed that heme can regulate PD-mediated intercellular trafficking. Heme is synthesized from the primary metabolite glutamate (Glu) in the chloroplast. Aminoacylation of tRNA (Glu) may ensure protein synthesis or heme synthesis. Glu-tRNA binds to elongation factor Tu for protein synthesis or is reduced by GluTR in the first step of tetrapyrrole biosynthesis in the chloroplast (329). Altering the availability of glutamate-tRNA impacts the protein translational efficiency and tetrapyrrole biosynthesis in the chloroplast (329). Heme binds ferrous iron (Fe^{2+}) through four nitrogen atoms in its protoporphyrin ring, functioning as a prosthetic group in various apoproteins. The main type is heme b, synthesized from protoporphyrin IX by ferrochelatase (FC)(330,331). Other heme forms, including a and c, derive from heme b.

Heme a and b attach non-covalently to hemoproteins, while heme c is covalently linked to cysteine residues, as in cytochrome C. Beyond a prosthetic group, heme also serves as a regulatory molecule, with free heme produced by FCs for metabolic processes (332). Heme has been found to play new roles in influencing transcription (333–337), translation (338), post-translational modification (339), and ion-channel function (340). In this dissertation, we showed that heme can modulate PD functions that reinforces the view that heme is not confined to organelle-localized activity, but instead acts as a multi-functional signal that integrates chloroplast and nuclear processes to modulate PD functions.

In chapter 3, we tested whether PD trafficking correlates with other well-characterized chloroplast-derived retrograde signals. Measurement of reactive oxygen species (ROS), chloroplast redox status, and plastoquinone (PQ) state revealed no consistent correlation between these redox metrics and changes in PD-mediated trafficking. This suggests heme may not act through redox signaling but may through heme-binding proteins or transcriptional regulators that affect PD structure and function. Notably, a proteomics study identified that nearly half of the heme-binding candidate proteins are localized to the nucleus, supporting the idea that heme is actively transported to the nucleus where it may directly affect transcriptional regulation or act as nuclear signal (341). While heme acts as signaling molecule, the mechanism of its intracellular trafficking remains poorly understood. In animals, ABC transporters and TSPO proteins mediate tetrapyrrole transport (342). In plants, *Arabidopsis* vacuolar ABC transporters (AtMRP1-3) and ABCG23, a plastid-envelop WBC subfamily member, are potential

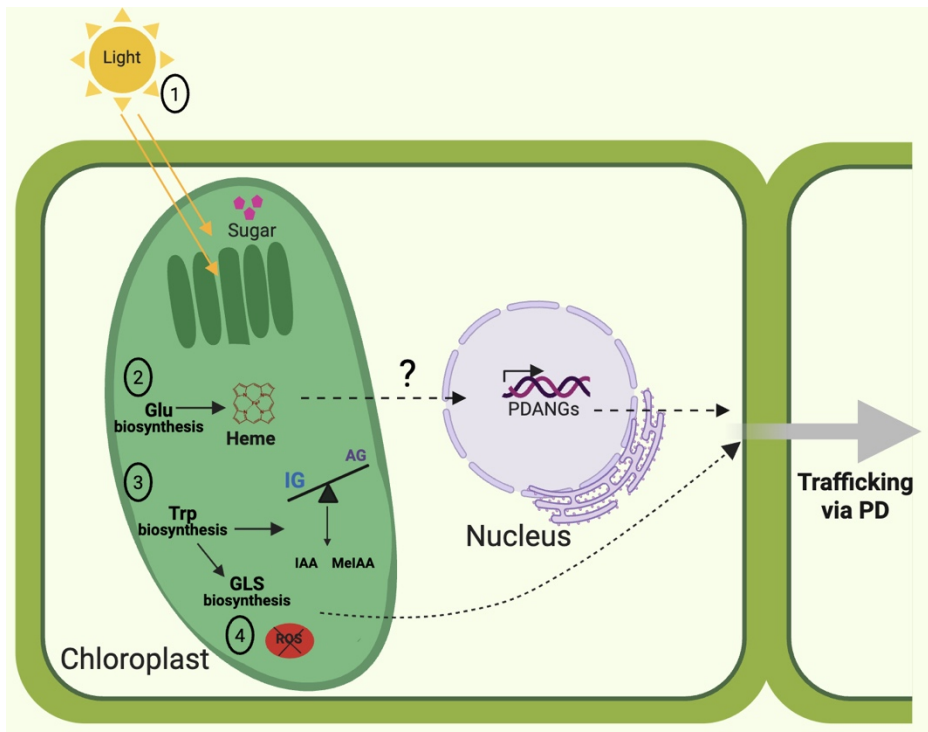
candidates for heme export and redistribution (342,343). Meanwhile, heme-binding TSPO homologs and SOUL proteins, including a cytosolic plant homolog with strong heme affinity, may also serve as carriers or buffer (344,345).

The known PD regulator ISE2 interacts with IPI1 (ISE2 Interacting Protein), which has a heme-binding motif and also can regulate PD-mediated trafficking (48). When heme levels are adequate, it may bind to IPI1, maintaining IPI1's role as a positive regulator of PD permeability. IPI1 is a pentatricopeptide repeat (PPR) protein, a category of RNA-binding proteins that can impact organellar RNA metabolic processes (48). This potential mechanism highlights how metabolic signals can link to RNA metabolism and influence intercellular communication. The *Arabidopsis* genome encodes over 450 PPR proteins; most are targeted to the chloroplast or mitochondria (346). PPR proteins that belong to the DYW subclass edit organelle RNA. This subclass of plant-specific PLS subfamily has a conserved C-terminal region with a predicted CXXXCH heme binding motif. (347). Synthetic PPR proteins offer RNA binding specificity without requiring guide RNA. As a result, these synthetic PPR proteins present significant potential as valuable tools in biotechnology for engineering the regulation of organelle transcripts (348). The potential to regulate heme binding to PPR proteins might enhance our ability to regulate chloroplast RNA editing and intercellular trafficking in plants.

In Chapter 4, we demonstrated the differential expression of cell wall and plasmodesmata-related genes in response to changes in chloroplast function caused by impaired RNA metabolism and/or tetrapyrrole biosynthesis. We showed differential

Figure 41. A proposed model for chloroplast as a regulatory hub, integrating metabolic pathways that regulate intercellular trafficking through PD

(1) Carbon assimilation via photosynthesis may modulate PD permeability to regulate the distribution of fixed carbon (e.g., sugar). (2) Heme, synthesized synthesized in the chloroplast from glutamyl-tRNA after aminoacylation with glutamate, can regulate PD-mediated trafficking by altering the expression of PD-associated nuclear genes (PDANGs). (3) Auxin (IAA, MeIAA) and indole glucosinolates (IG) are synthesized from the common precursor tryptophan (Trp) in the chloroplast. Aliphatic glucosinolates (AG) are synthesized from methionine. GLS has been shown to modulate PD permeability. (4) Reactive oxygen species (ROS) generated due to chloroplast dysfunction do not appear to correlated with changes in PD-mediated trafficking.



expression of known PD proteins and identified new candidate PD proteins that can be tested in future research.

In chapter 5, we demonstrated how glucosinolates, secondary metabolites derived from the primary metabolite tryptophan (278), modulate trafficking through PD. Notably, the *ise2* mutants displayed altered glucosinolate metabolism and disrupted auxin levels, linking chloroplast secondary metabolism with hormonal balance and PD permeability. This finding highlights a broader theme: primary and secondary metabolites produced in the chloroplast work together to regulate PDs, thereby integrating photosynthesis, amino acid biosynthesis, and metabolic signaling.

The proposed unifying model suggests that the chloroplast serves as a regulatory hub, translating metabolic outputs, such as fixed carbon, heme, and secondary metabolites such as glucosinolates, into signals that actively regulate PD function (**Fig. 41**). Additionally, these findings provide a compelling explanation for why many plant viruses target chloroplasts (349,350), potentially hijacking chloroplast-derived metabolic pathways to influence PD permeability, promoting their systemic movement throughout the plants.

One avenue for future research could be investigating how reduced heme levels affect mitochondrial function in plants, as heme is essential for cytochrome activity in the electron transport chain (351). Mitochondrial functionality play a role in regulating PD-mediated trafficking (157). Another avenue of investigation could focus on understanding the potential heme-regulated PD permeability during stress conditions like drought or pathogen attacks, as well as during key developmental stages such as the transition from

sink to source transition. Studying heme-mediated PD regulation in early land plants, such as mosses and liverworts, may reveal ancient mechanisms crucial for colonization on land.

A parallel mechanism could involve ISE1, a mitochondrial DEAD-box RNA helicase known to regulate PD permeability. It has been noted that heme can bind to DEAD-box RNA helicase in the nucleus remodeling (138), which raises the possibility that heme may also influence mitochondrial helicases such as ISE1. If so, this introduces a mitochondrial layer to the heme-PD regulatory axis, suggesting heme regulates PD function by affecting RNA helicase across organelles, supporting our ONPS hypothesis.

While we utilized tissue-level techniques for this study, it is important to consider cell-specific variations in chloroplast function, redox state, and PD structures. Different cell types, such as epidermal or mesophyll, exhibit distinct PD structures and trafficking regulations, indicating that future studies should adopt single-cell resolution methods to investigate how heme influences PD regulation in various cell types. Techniques such as single-cell RNA-seq and spatial transcriptomics are essential for uncovering localized signaling processes and regulatory networks. Simultaneously, high-resolution single-cell microscopy could be critical for directly observing trafficking behaviors at the cellular level in response to heme. These tools will play a crucial role in understanding the spatial and temporal mapping of heme-regulated intercellular communication in plants.

Finally, the insights derived from this dissertation could have significant applications in crop improvement. By engineering heme-responsive PD regulators or altering PD permeability may enhance nutrient transport, stress resilience, and defense

signaling, forming new strategies for improving agricultural productivity in changing environment.

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