

**Regulation of plasmodesmata by specialized metabolites
glucosinolates in *Arabidopsis thaliana***

**A Dissertation Presented for the
Doctor of Philosophy
Degree**

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DEDICATION

I dedicate this to all the kids from East LA. I was you and, you too, can do it. ¡Sí,
se puede!

And to the late Dr. Garcia from CSULA.

You touched the lives of so many first-generation college students and you
pushed us to move forward.

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ABSTRACT

Communication is an essential component to all living organisms. In plants, the additional cell wall surrounding each cell adds a layer of complexity not observed in animals. To overcome the literal wall separating cells, plants have evolved specialized pores to connect adjacent cells. Plasmodesmata (PD) allow plants to have a continuous cytoplasm between cells. Although plasmodesmata may appear simple and lack regulation, their structural components and their regulatory machinery is complex and not well understood. Organelle-to-nucleus-to-plasmodesmata signaling (ONPS) have been worked as a leading model for a possible regulatory mechanism. Many of the details of organelle-to-nucleus retrograde signaling pathways have been elucidated in yeast, mammalian and plant model systems. Understanding mechanisms of chloroplast-to-nucleus signaling will help elucidate the functions of retrograde signaling in all organisms including bacteria and apicomplexans. Our previous work with mutants lacking the chloroplast RNA helicase ISE2 indicates that chloroplasts are important regulators of plant intercellular communication and trafficking mediated by pores in the plant cell walls called plasmodesmata. Loss of ISE2 has suggested defects in glucosinolates and in this dissertation we show how glucosinolates can regulate intercellular trafficking via plasmodesmata. I uncover the potential pathway of how the changes in the chloroplast by the loss of ISE2 modulate nuclear signaling and ultimately disrupts the biosynthesis of glucosinolates. I find that plants overexpressing ISE2 results in global physiological defects that can partially be described as auxin defects. However, they are a more complex phenomenon. Further, I reveal that the addition of glucosinolates alone to plants results in an increase in intercellular trafficking in a dose-dependent manner.

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CHAPTER 1 INTRODUCTION

Intercellular communication across all multicellular organisms is a critical component for growth and development. In animals, most intercellular communication relies on cell surface receptors that detect and respond to the presence of cognate ligands on the surface of neighboring cells or small signaling molecules that are secreted by distant cells and are transported in the aqueous environment of the extracellular fluid or circulatory system. Animals also rely on specialized connections between cells, gap junctions, to relay information from cell to cell. In plants, these structures do not exist due to the complex cell-walls that provide the plant structure. These walls preclude the direct connection of plasma membranes of adjacent cells and also limit the diffusion of mobile signaling molecules like hormones. In order to compensate for the segregated cells, plants have developed specialized cell wall pores called plasmodesmata. Plasmodesmata (PD) are membrane-lined pores that provide cytoplasmic connectivity between adjacent plant cells.

Plasmodesmata Structure

Although plasmodesmata can simply be described as pores, they are structurally complex as depicted in Figure 1. The cell membranes of adjacent cells designate the outermost layer of plasmodesmata. A structure called the desmotubule, consisting of tightly coiled endoplasmic reticulum (ER) strand, forms a central rod and directly connects the two adjacent cells' ER (Tilney, 1991, Tilsner et al., 2011). The small space that remains between the desmotubule and the cell

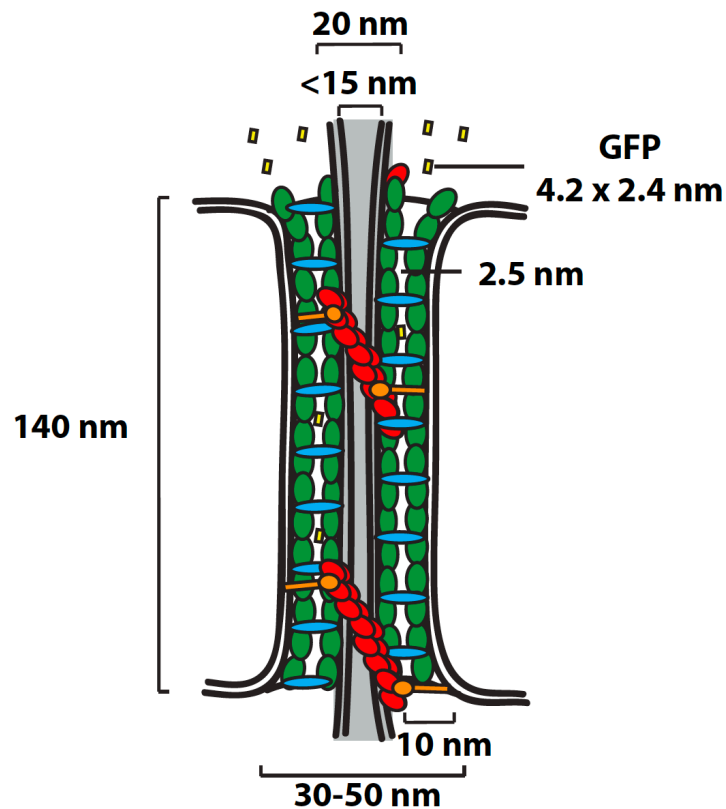


Figure 1 Plasmodesmata structure

Plasmodesmata structure consists of a continuous plasma membrane, a desmotubule (gray) that passes through the center of the pore and plasmodesmata associated and located proteins (green, orange, blue and red). GFP molecules can pass through the cytoplasmic sleeve (space between desmotubule and cell wall). Figure is drawn to scale.

membrane is occupied by cytoplasm and is called the cytoplasmic sleeve or annulus. Within the cytoplasmic sleeve, plasmodesmata localized proteins add further structural complexity and proteins called spokes tether the desmotubule to the cell membrane. Many other proteins located at plasmodesmata have been identified, yet their functions in the plasmodesmata have not been fully resolved (Fernandez-Calvino et al., 2011, Leijon et al., 2018). Contributing to further complexity, lipids found in the cell membrane within plasmodesmata are distinct from the rest of the cell membrane (Grison et al., 2015), indicating that the composition of lipids within plasmodesmata is important for function and structure.

In addition to the complexity found within PD, PD are further characterized based on their origin. Plasmodesmata originating from a cell division event are termed primary plasmodesmata. When the cell plate is formed, strands of ER are trapped within the cell plate and form the desmotubule of plasmodesmata. Plasmodesmata formed in an existing cell wall are secondary plasmodesmata. In this case, at the site of insertion, the part of the existing cell wall must be degraded or weakened, and the turgor pressure will allow the ER to insert through the plasma membrane (Burch-Smith et al., 2011b). The mechanisms of how secondary plasmodesmata form is still unclear.

Plasmodesmata are also structurally complex. Plasmodesmata exists as single pores (simple plasmodesmata) (Figure 2A), twinned plasmodesmata (two simple

less than 100 nm apart) (Figure 2B), and complex plasmodesmata (plasmodesmata with “Y” “X”, or “H” shapes) (Figure 2C) with different numbers of openings on either end. It is unclear if the shape of plasmodesmata play any role in their function. One evidence of function exists during the sink-to-source transition in plant leaves. Chloroplast make sugars that are transported from source leaves (mature leaves) to sinks (newer and not expanded leaves). These sugars depend on plasmodesmata for the local cell-to-cell movement (Liesche et al., 2011). Interestingly, as leaves transition from sink to source leaves, the plasmodesmata structure also changes from simple to complex suggesting that the structure of plasmodesmata may be dependent on its structure.

Intercellular trafficking via plasmodesmata

In addition to being complex structurally and in their composition, plasmodesmata are also complicated in what they traffic. When things move between cells via plasmodesmata, it is referred to as symplastic trafficking. Apoplastic trafficking is movement between cells via cell walls. Plasmodesmata are capable of trafficking small molecules, including ions, water, and small metabolites, and also large molecules like proteins, RNA, and viruses (Sager & Lee, 2018, Ruiz-Medrano et al., 2004). One probe routinely used as a tool to measure cell to cell trafficking is green fluorescent protein (GFP). GFP expressed

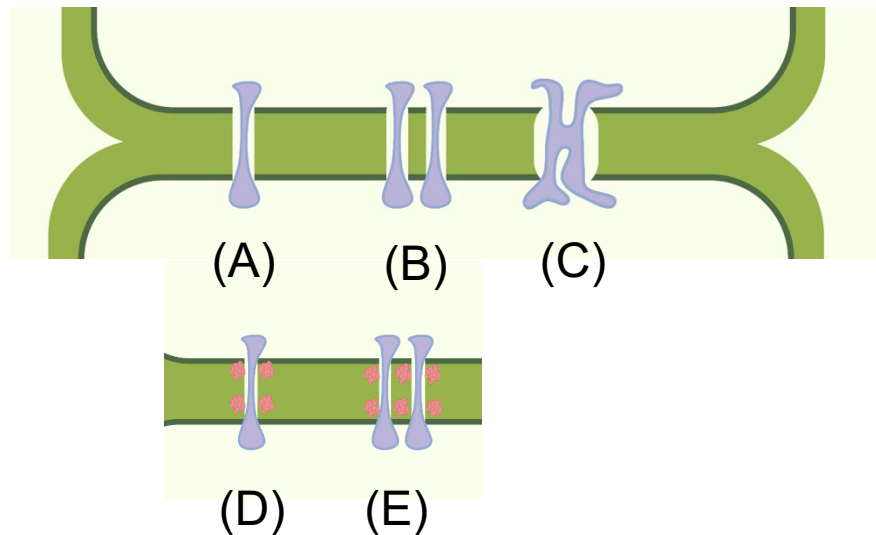


Figure 2 Different plasmodesmata structures

Plasmodesmata can exist in various shapes. Simple plasmodesmata as a single pore with one opening on either side (A). Twinned Plasmodesmata, two simple within 100 nm of each other (B). Branched Plasmodesmata contain central cavity and multiple openings (C). The deposition of callose (Red) on the “necks” of plasmodesmata (D,E).

in plant cells, as a non-native protein, moves freely within the cytoplasm of a cell and accumulates in the nucleus. An interesting feature of this protein is that it will only move from one cell to another via plasmodesmata via diffusion (Schonknecht et al., 2008).

The permeability of plasmodesmata varies and often is dependent on the size of the molecule being trafficked. For example, small molecules such as ions, sugars, and small proteins (such as GFP), can move from cell to cell by diffusion (Schonknecht et al., 2008, Dashevskaya et al., 2008). Regulating these molecules would depend greatly on whether or not the plasmodesmata are “opened or closed”. Open plasmodesmata allow molecules to pass through, closed plasmodesmata do not allow molecules to pass through (Crawford & Zambryski, 2000). This mechanism is believed to occur by the deposition of a β -1,3-glucan (callose).

Regulating Plasmodesmata through Callose

It is widely accepted that a major component physically regulating plasmodesmata is the deposition of a β -1,3-glucan or callose (Radford et al., 1998, Cui & Lee, 2016, Li et al., 2012). During cell division, plant cells deposit the cell plate made up of primarily callose between the cells and once the cell membrane expands and becomes continuous, the cell wall changes to have similar composition to existing cell walls (Samuels et al., 1995). Further, when

plants are wounded, callose is deposited between the plasma membrane and the cell wall. The general mechanism of how plasmodesmata are modified therefore changing intercellular trafficking, is through the deposition of callose at the neck of the plasmodesmata (Radford et al., 1998) (FIGURE 2 D,E). This addition of callose is thought to add rigidity and volume therefore constricting the flow through the cytoplasmic sleeve.

Callose is synthesized by the activity of callose synthase enzymes (glucan synthase-like GSL) and can be removed by callose β -1,3-glucanases (BGs) (Zavaliev et al., 2011, Chen & Kim, 2009, Zavaliev et al., 2013). *Arabidopsis thaliana* has 12 known callose synthase proteins (CalS or GSL). Three of the 12 GSLs associate to plasmodesmata; GSL8, GSL7, GSL12. The loss of these GSLs have been associated with defects in intercellular trafficking. For example, the loss of GSL8 has increased epidermal cell-to-cell trafficking and the protein SPEECHLESS (SPCH) that normally does not traffic from cell to cell is able to move to neighboring cells (Guseman et al., 2010). Another study found that GSL8 is important for establishing auxin gradients in *Arabidopsis* hypocotyls and therefore, mutants of GSL8 do not have phototropic responses (Han et al., 2014). The role of callose in the cell wall is generally thought to bring rigidity and in the case of plasmodesmata, a more rigid and constricted pore. Recent in vitro studies have revealed that a cellulose and callose mix results in varying rigidity. The more callose present, the less rigid the matrix was (Abou-Saleh et al., 2018),

calling in to question the mechanism by which callose restricts plasmodesmal permeability. This indicates that the role of callose at plasmodesmata may not be as simple as providing rigidity to regulate trafficking.

In *Arabidopsis thaliana*, the modulation of plasmodesmata localized callose is performed by β -1,3-glucanases and PdBGs. The hydrolysis of callose by the β -1,3-glucanases results in single β -1,3-glucan molecules (De Storme & Geelen, 2014). *Arabidopsis thaliana* has 50 β -1,3-glucanases but only a handful of proteins have been associated with PD and have been linked with regulating plasmodesmata. Three proteins PdBG1, PdBG2, and AtBG_ppap were found to localize at plasmodesmata (Benitez-Alfonso et al., 2013). The two proteins, PdBG1 and PdGB2 are functionally redundant proteins as single mutants do not have phenotypes and the double mutant does. Overexpressing PdGB1 resulted in an increase in intercellular trafficking, the opposite phenotype observed in double pdgb1pdgb2 mutants.

Plasmodesmata Located Proteins -PDLP

In *Arabidopsis*, PLASMODESMATA-LOCATED PROTEIN or PDLP, are a family of 8 plasmodesmata localized proteins. PDLP1, for example, has two domains of unknown function 26 (DUF26) and a single transmembrane domain followed by a short C-terminal tail (Thomas et al., 2008). Plants overexpressing *PDLP1* have a drastic decrease in intercellular trafficking however, the loss of PDLP1 did not result in an increase in trafficking. This could be due to

redundancy from the other PDLPS found in *Arabidopsis* (Thomas et al., 2008). Another protein, PDLP5, also has been shown to modulate intercellular trafficking. *Arabidopsis pdlp5* mutants have increased trafficking and the increased expression of *PDLP5* has decreased intercellular trafficking (Lee et al., 2011). PDLP5 also plays a major role in plant defense by aiding innate immunity during pathogen attack in a salicylic dependent manner (Lee et al., 2011) and through the deposition of callose from the GSL6 and GSL1 (Cui & Lee, 2016). In addition to local cell-to-cell signaling and trafficking, PDLP5 and PDLP1 play important roles in the propagation of systemic signaling. Grafting experiments have shown that PDLP5 and PDLP1 are required for the long-distance systemic response to high light stress (Fichman et al., 2021).

Molecules affecting PD trafficking

Salicylic Acid (SA) is an important hormone for plants to have a systemic acquired resistance (SAR). It is produced in response to bacterial or fungal infections and the exogenous application to plants induces pathogenesis-related gene expression, induces a hypersensitive response cell death and can establish systemic acquired resistance (Malamy et al., 1990, Mur et al., 2008, Shah et al., 2014, Shah, 2003). Salicylic acid also modulates intercellular trafficking via plasmodesmata. Exogenous application of SA results in a reduction intercellular trafficking via plasmodesmata (Wang et al., 2013) as a result of callose deposition by GSL6 and GSL1 (Cui & Lee, 2016). As pointed earlier, SA induces

the expression of *PDLP5* and it was found that plants overexpressing *PDLP5* had decreased plasmodesmata intercellular trafficking.

Auxin is a fundamental plant hormone and is able to regulate plasmodesmata by the deposition of GSL8 (Han et al., 2014). This mechanism occurs in a positive feedback loop in which a decrease in plasmodesmata trafficking results in an accumulation of auxin and therefore activating the transcription factor ARF7 which then induces expression of the GSL8. The deposition of callose by the GSL8 therefore reduces plasmodesmata trafficking ultimately feeding back to the same loop (Han et al., 2014).

The chloroplast is central to the synthesis and regulation of essential hormones that have important roles in biotic and abiotic interactions (Fernandez & Burch-Smith, 2019). The deposition of callose is enhanced by abscisic acid (ABA) by transcriptionally repressing *PATHOGEN-RELATED PROTEIN 2 (PR2)* (Oide et al., 2013). Further, ABA was also shown to reduce viral trafficking by the deposition of callose at plasmodesmata (as reviewed in Alazem & Lin, 2017).

Another chloroplast hormone, jasmonic acid (JA) is a lipid hormone that is one of the best-known hormones in plant responses to microbial pathogens (Fernandez & Burch-Smith, 2019). Wounding stress results in vascular pressure changes in plants (Farmer et al., 2014) and other studies found that bacterial flagellin (flg22) triggered both SA and JA and therefore callose deposition. This increase in

callose deposition therefore results in a decrease in intercellular trafficking (Yi et al., 2014).

There are a number of other metabolites that pass through plasmodesmata including sucrose, and glucosinolates (Stitt, 1996, Madsen et al., 2014, Andersen et al., 2013) however, their potential roles in plasmodesmata mediated trafficking have not yet been fully elucidated. One focus of this dissertation is to investigate if glucosinolates mediate plasmodesmata trafficking.

Chloroplasts and plasmodesmata

Although there have been numerous studies of PD, many questions about how they are regulated persist. Only a handful of proteins that influence PD structure or function have been identified. One of these is the chloroplast RNA helicase, ISE2 (Kobayashi et al., 2007). *Arabidopsis thaliana* *ise2* mutants are embryonically lethal (Kim et al., 2005, Kobayashi & Zambryski, 2007). *ise2* embryos or ISE2-silenced *Nicotiana benthamiana* plants had increased numbers of branched PD as well as increased intercellular trafficking (Burch-Smith et al., 2011a, Kobayashi et al., 2007). A long-standing question regarding plasmodesmata is 'What is the signal that modulates plasmodesmata structure and function?'.

A model described as organelle-nucleus-plasmodesmata-signaling (ONPS) describes how signals originating from the chloroplast or mitochondria modulate nuclear gene expression to turn induce or suppress genes involved

with plasmodesmata regulation. Given the close association of sugar transport and plasmodesmata, one can predict that the chloroplast and PD are directly linked to each other to transport sugar (or other metabolites) to the rest of the plant (Burch-Smith et al.). The loss of ISE2 or the other protein ISE1 from the mitochondria disrupt chloroplast gene expression and changes in plasmodesmata mediated trafficking is observed (Burch-Smith et al., 2011a, Stonebloom et al., 2009). Signals originating from the chloroplast are defined as Chloroplast Retrograde Signals (CRS) (Azim & Burch-Smith, 2020) and have an extremely complex network. These signals are sometimes directly sent from the chloroplast to the nucleus to alter nuclear gene expression. Examples of CRS signals include nucleotide 3'-phosphoadenosine 5'-phosphate (PAP) from the SAL-1-PAP signaling pathway originating from drought stress or methylerythritol phosphate (MEP) from stress responses, as reviewed in (Chi et al., 2015).

Chloroplast RNA metabolism

Chloroplast RNA metabolism is very complex and unique as it has retained many of the ancestral components as well as those acquired through evolution. The chloroplast RNA metabolism consists of intron removal, RNA editing and processing of polycistronic precursors such as those for ribosomal RNA. As chloroplast mRNA matures, proteins such as PNPase (Carpousis et al., 1999), RNaseJ and RNaseE (Stern et al., 2010) process the 5' and 3' end and those proteins are also responsible for the removal of intergenic regions. Later,

proteins such as pentatricopeptide proteins play roles in editing and further intron removal (Okuda et al., 2009). Overall, an arsenal of exonucleases and endoribonucleases ensure the maturation of mRNA's from the chloroplast. An interesting evolutionary perspective of the complexity of RNA maturation and metabolism is their potential functions. For example, in the chloroplast there are many proteins, such as the pentatricopeptide proteins (PPR) proteins with over 450 PPR encoded in *Arabidopsis thaliana* and rice genome (Nakamura et al., 2012). It is possible that the highly diverse roles that the many RNA processing proteins have in the chloroplast can modulate gene expression and therefore signals to the nucleus and structures such as plasmodesmata.

Chloroplast RNA metabolism linked to plasmodesmata regulation

Chloroplast RNA metabolism is a unique phenomenon in plants as chloroplast contain and regulate their own separate genome. However, through evolution, chloroplast have lost the majority of their genome and rely on nuclear encoded protein import to carry the major functions such as photosynthesis and RNA metabolism. The chloroplast genome is responsible for less than 100 proteins versus the 3000 nucleus-encoded proteins (Pesaresi et al., 2007, Jarvis & Lopez-Juez, 2013). The chloroplast genome is controlled primarily by post-transcriptional and translational regulation (Barkan, 2011b).

Fine-tuning chloroplast translation requires many nuclear encoded proteins including RNA helicases. RNA helicases are RNA binding proteins that through are able to rearrange RNA molecules for their expression or regulation. In the chloroplast, many of these RNA helicases play critical roles in the regulation of ribosomal RNA processing steps. The chloroplast RNA helicases RH3, RH22, RH39, and ISE2 have all been shown to play roles in rRNA chloroplast (Asakura et al., 2012, Chi et al., 2012, Nishimura et al., 2010, Bobik et al., 2017).

ISE2 has numerous roles in chloroplast RNA metabolism. ISE2 is responsible for the C-to-U RNA editing of six chloroplast transcripts (Bobik et al., 2017) and it is required for group II intron splicing (Carlotto et al., 2016, Bobik et al., 2017). In addition, ISE2 is responsible for the proper processing steps for chloroplast rRNA metabolism. In the chloroplast, ribosomal RNA must go through a number of processing steps before becoming the mature functional form. For example, the varying processed rRNA's from chloroplast, 16S, 23S, 4.5S and 5S are all transcribed from a single *rrn* operon (Figure 3). Further, the 23S is processed into three mature forms. Loss of ISE2 results in the accumulation of non-processed rRNA cistron (Bobik et al., 2017).

The loss of ISE2 also results in the reduction of the Lhcb (light-harvesting chlorophyll a/b) protein, which is associated with defective chloroplast-to-nucleus retrograde signaling. Disrupting chloroplast function is associated with changes

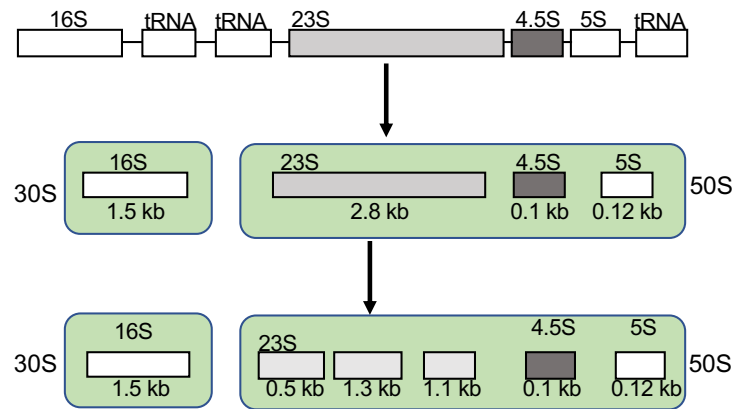


Figure 3 Processing schematic of the chloroplast *rrn* operon.

Single cistron *rrn* undergoes cleavage and trimming by RNases. Post-maturation processing and the introduction of the “hidden breaks” in the 23S.

to nuclear genes and often associated with defective expression of photosynthesis associated nuclear genes (*PhANGs*) and in the case of ISE2 and other chloroplast RNA helicases, has been associated with altered plasmodesmata trafficking (Ganusova et al., 2020). For example, silencing RH22, RH39, ISE2, PNPase, RNaseJ, IPI1 and RH3, all resulted in defective plasmodesmata function. As an RNA helicase, ISE2 likely interacts with other proteins as part of a larger functional complex. Therefore, a pull-down assay was used to identify potential protein partners of ISE2. One of these proteins was the chloroplast ribosomal protein uL15C (Bobik et al., 2019).

The ribosomal protein uL15C is a nuclear encoded chloroplast ribosomal protein. *RPL15* gene is an essential *Arabidopsis thaliana* gene and *rpl15* mutants are embryonically lethal. Reduced amounts of the uL15C protein results in defective ribosomal rRNA processing and defective chloroplast translation and protein accumulation. Plants with reduced uL15C also have increased plasmodesmata-mediated trafficking-similar to what was observed with plants with reduced ISE2.

Metabolic retrograde plasmodesmata signaling

Chloroplast signaling is not all the same. There are two distinct types of chloroplast retrograde control: biogenic control and operational control (Pogson et al., 2008). Biogenic control involves developmental control of the plastid

biogenesis, whereas operational control occurs in already matured chloroplast and regulates immediate changes when under developmental or environmental stress (Pogson et al., 2008). Together, the signals from the chloroplast to the nucleus are retrograde signals, however, it is important to differentiate the level of regulation these signals may originate from.

Although the model for plasmodesmata regulation by ONPS is well accepted, the signals that originate from the chloroplast to change nuclear gene expression have not been fully identified. The chloroplast is able to generate redox signals such as $^1\text{O}_2$ or H_2O_2 or metabolite signals such as PAP (3'-phosphoadenosine 5'-phosphate or MEcPP (methylerythritol cyclodiphosphate) that are capable of changing nuclear gene expression. For example, the metabolite MEcPP is transported into the nucleus and inducing the expression of the stressed-inducible *HPL* gene (Xiao et al., 2012). From the pathogen associated SAL1-PAP signaling pathway, PAP accumulates in the chloroplast and is transported into the nucleus to inhibit the activity of exoribonucleases (XNR) and therefore induce the nuclear gene expression of stress-responsive genes (Estavillo et al., 2011). The relationship between metabolic retrograde signaling and plasmodesmata regulation has not been fully elucidated. What is clear is that perturbing the chloroplast ultimately disrupts nuclear expression (Ganusova et al., 2020) and this may therefore be the cause for changes in plasmodesmata-mediated trafficking. Many of the metabolic signals originating from chloroplast have been

associated with stress including herbivore defense. For example, the metabolite MEcPP is linked with an induction of genes involved in glucosinolate biosynthesis (Wang et al., 2017). *Sal1-PAP Arabidopsis* mutants are incapable of accumulating methionine derived glucosinolates. Although the secondary metabolites, glucosinolates, have not been associated with PD, various glucosinolate related genes were misregulated in plants *ise2* mutant embryos Burch-Smith et al. (2011a).

Glucosinolates

As sessile organism's incapable of fleeing predators or unfavorable environmental conditions, plants have evolved a series of self-defense mechanisms involving the production of toxic chemicals to deter herbivores and serve as mediators of stress responses (Figure 4). In Brassica plants, one such important mechanism is the breakdown of the secondary metabolites collectively called glucosinolates. This process has evolved from the cyanogenic glucosides that unlike glucosinolates, exists throughout the plant kingdom. Chemically, glucosinolates are nitrogen- and sulfur-containing moieties conjugated to glucose, that have highly diverse structures. Indeed, about 130-200 different types of glucosinolates have been cataloged in plants (Agerbirk & Olsen, 2012, Yi et al., 2015). Glucosinolates are made from amino acid precursors, including methionine (aliphatic glucosinolates), phenylalanine (aromatic glucosinolates), and tryptophan (indole glucosinolates). The overall synthesis of glucosinolates

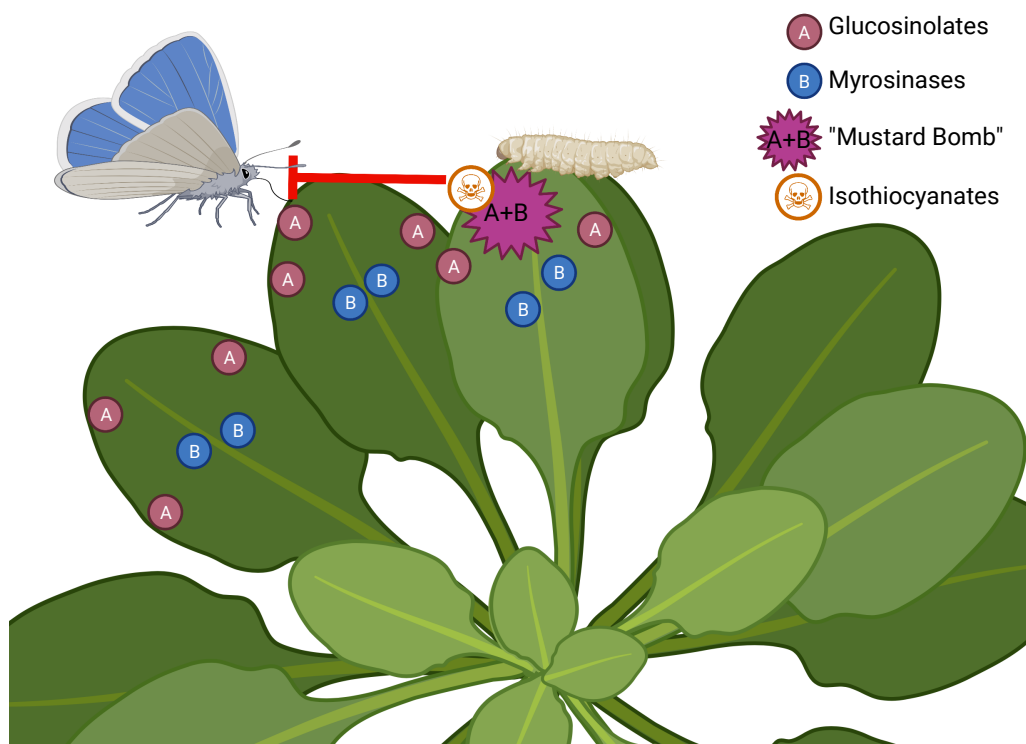


Figure 4 The role of glucosinolates in plant defense

Glucosinolates (A) accumulate at leaf margins and myrosinases (B) along the leaf margins. The “mustard bomb” occurs when glucosinolates and myrosinases are mixed by a feeding herbivore. The resulting isothiocyanates are toxic to the herbivore feeding and deters other approaching insects. Created with BioRender.com

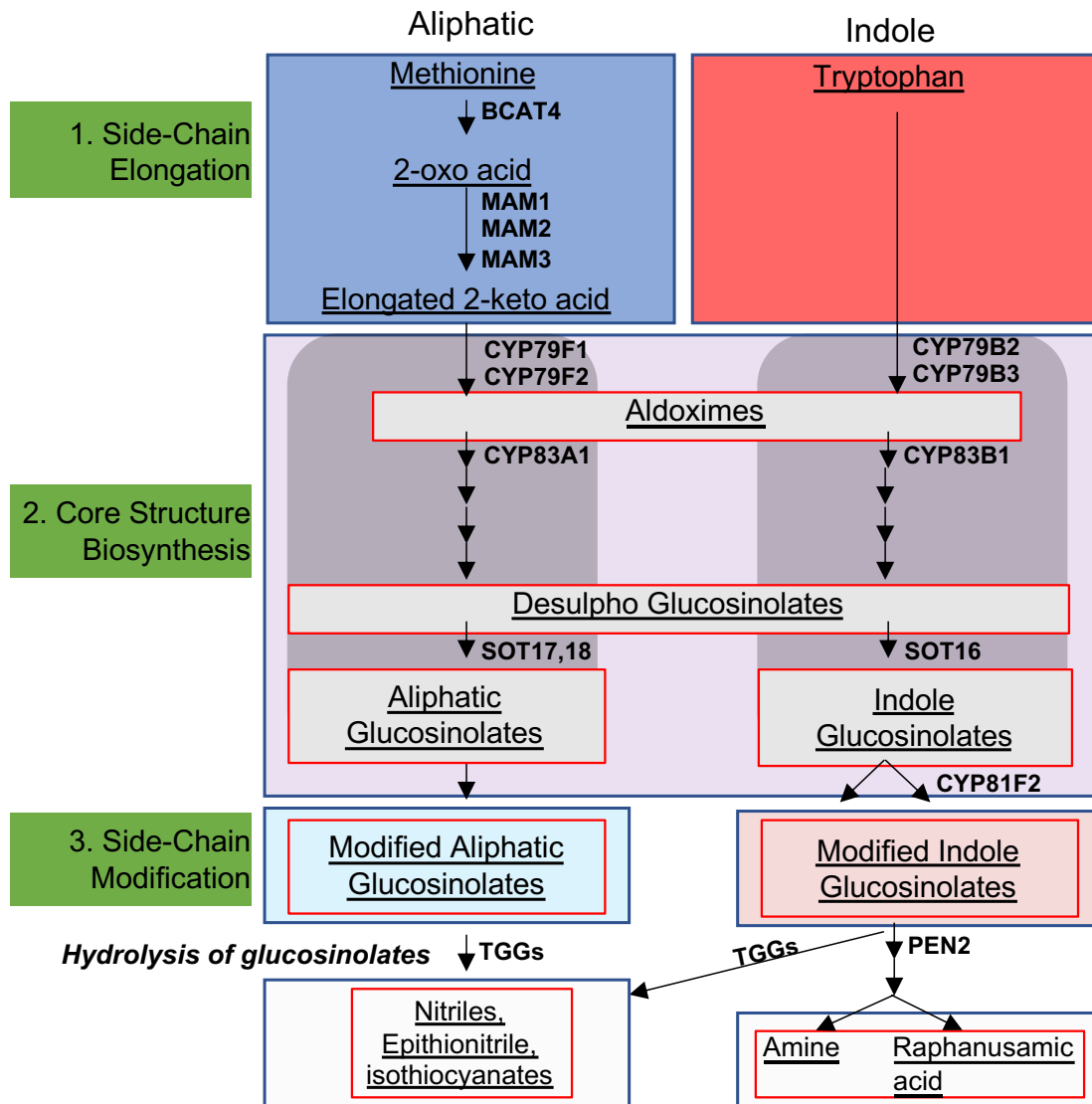
can be divided into three major steps: 1. chain elongation, 2. biosynthesis of core glucosinolate structure, and 3. secondary modifications (Figure 5).

Glucosinolates are unique to plants in the Brassicaceae family that includes cabbage, broccoli, mustard, and others. Commonly known as the “mustard family” the glucosinolates found in the Brassica plants are the compounds responsible for their pungent flavors. This flavor is a direct result of the mix between glucosinolates and the myrosinases (the enzymes that hydrolyze them) to make breakdown products including isothiocyanates. Because the hydrolysis reaction occurs quickly, the glucosinolates and myrosinases are stored spatially separated (Halkier & Gershenzon, 2006, Chhajed et al., 2020). This separation makes the myrosinase-glucosinolate system a “two-component” system and because of the pungent hydrolysis products it was then termed the “mustard oil bomb” (Bones & Rossiter, 1996). In plant defense, this reaction occurs during feeding of plant specialists that disrupt the spatially separated compartments harboring glucosinolates and myrosinases.

Glucosinolates themselves are not toxic. However, their breakdown by the myrosinase enzymes (for example, THIOGLUCOSIDE GLUCOHYDROLASE [TGG]), and other myrosinases generates intermediate molecules that may then be re-arranged through the action of other modifier enzymes to produce toxic by-products (Figure 6) (Wittstock & Burow, 2010). In Arabidopsis under normal developmental conditions, glucosinolates accumulate in glucosinolate storage

Figure 5 Aliphatic and Indole glucosinolate biosynthesis and breakdown.

The overall glucosinolate biosynthesis can be broken down into three major steps: 1. Side-Chain Elongation for methionine derived glucosinolates (Indole glucosinolates does not undergo this first step), 2. Core structure biosynthesis and 3. Side chain modifications. The hydrolysis of glucosinolates occurs by the myrosinases TGGs and by PEN2. Hydrolysis by TGGs produces Nitriles, Epithionitrile, or Isothiocyanates. Hydrolysis by PEN2 can produce Amines or Raphanusamic acid.



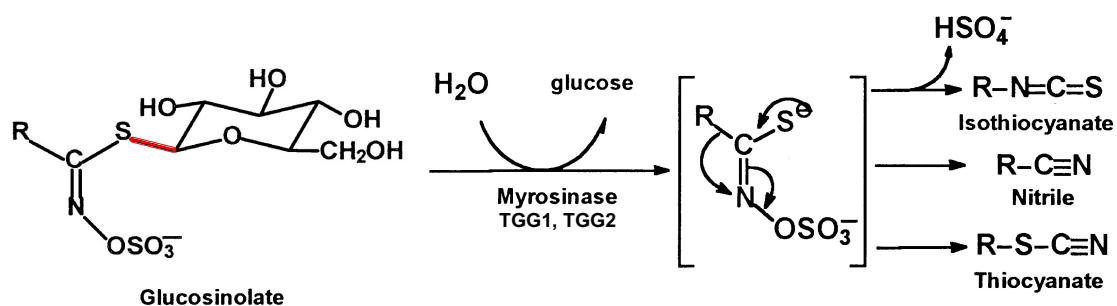


Figure 6 Breakdown of glucosinolates by myrosinases

Myrosinases hydrolyze the thioglucoside linkage (red bond) and form an unstable aglycone. Depending on many factors (side chain, specifier proteins) this can rearrange to isothiocyanates, nitriles or thiocyanates. Adapted with permission from Elsevier.

S-cells and myrosinases accumulate in myrosin cells (Koroleva et al., 2000, Rask et al., 2000, Shirakawa & Hara-Nishimura, 2018). This spatial separation is crucial, preventing the "mustard bomb" from occurring (Figure 4 "A+B").

Glucosinolates are synthesized in the vasculature however, they are found in high levels in epidermal cells (Nintemann et al., 2018). Further, glucosinolates are trafficked via plasmodesmata to S-cells by glucosinolate transporters GTR1 and GTR2 (Nour-Eldin et al., 2012). Recent data has shown that S-cells are rich in plasmodesmata and are not symplastically isolated. This was further observed by the diffusion of a photoactivatable dye from a phloem cell to S-cells (Hunziker et al., 2019). Although the roles of bulk glucosinolates in plant defense are well studied, other roles have not been fully discovered. For example, Arabidopsis seeds have large amounts of glucosinolates but upon germination, the glucosinolate content drops dramatically (Brown et al., 2003). This is significant because it is similar to other biological processes that depend on the concentrations of hormones to carry out cell differentiation or stress responses successfully i.e., lateral root formation or ABA-induced responses in roots (Benitez-Alfonso et al., 2013, Manzi et al., 2017).

Glucosinolate biosynthesis

Glucosinolates are metabolites derived from amino acids. The amino acids glutamate, leucine, isoleucine, methionine, phenylalanine, tryptophan, tyrosine, alanine, and valine can be used as precursors of glucosinolates.

Glucosinolates derived from tryptophan are classified as indole glucosinolates. Aliphatic glucosinolates use the amino acids methionine, leucine, isoleucine, valine, alanine and glutamate. Benzenic glucosinolates use phenylalanine and tyrosine. Each one of these glucosinolate types, indole, methionine, and benzenic, have unique glucosinolate biosynthetic pathways.

Aliphatic glucosinolate biosynthesis

General biosynthesis of glucosinolates consists of three major steps: 1. The Chain elongation step, 2. The biosynthesis of the core structure, and 3. Secondary modifications (Sonderby et al., 2010, Halkier & Gershenzon, 2006, Chhajed et al., 2020, Grubb & Abel, 2006) (Figure 5). The first major step occurs in those glucosinolates that undergo side-chain elongation and consists of three major steps that are repeated, depending on the glucosinolate being synthesized. For simplicity, the three steps in the first major step will be broken down as 1a-1c. For methionine derived aliphatic glucosinolates, prior to entering step 1a, the amino acid is deaminated to form a 2-Oxo acid and step by the enzyme branched-chain amino acid aminotransferase 4 (BCAT4) and transported to the chloroplast by the bile acid transporter 5 (BAT5). Step 1a is the condensation reaction of the 2-Oxo acid with an acetyl-CoA by a methylthioalkylmalate synthase 1 (MAM1), MAM2, and MAM3 and forms 2-malate derivatives (Kumar et al., 2019). In the following step 1b, the 2-malate derivatives isomerize through an isopropylmalate isomerase large subunit 1

(IPMI) and then undergoes oxidation decarboxylation by isopropylmalate dehydrogenase 1 (IPMDH1) (step 1c) (Sonderby et al., 2010). These three steps 1a-1c complete a chain elongation cycle, which can be repeated up to nine times in plants (Halkier & Gershenzon, 2006, Chhajer et al., 2020).

After the chain elongation steps, the elongated-amino acid derivative undergoes the second major step, 2. biosynthesis of the core structure. At this step, all amino acid derivatives are converted to aldoximes by cytochromes P450 enzymes (Halkier & Gershenzon, 2006, Chhajer et al., 2020) and in the case of aliphatic glucosinolates, this occurs by the enzymes CYP79F1 and CYP79F2. The next step involves a different CYP83A1, and an unidentified intermediate is formed that is then conjugated with glutathione by glutathione S-transferase F11. The next steps produce thiohydroximate and then desulpho glucosinolates and sulfotransferase 17 and 18 (SOT17, SOT18) (Hirschmann et al., 2017, Halkier & Gershenzon, 2006) form aliphatic glucosinolates ready to enter the third and final major step; 3. Side-chain modification.

In general, aliphatic glucosinolates can have side chain modifications of oxygenation, hydroxylation, alkenylation, benzylation, and methoxylation (Ishida et al., 2014). These steps are done by a number of varying enzymes including flavin-monooxygenases (FMOs), 2-oxoglutarate-dependent dioxygenases (AOP2, AOP2) (Neal et al., 2010), and 2-oxoglutarate-dependent dioxygenase (GS-OH).

Indole glucosinolate biosynthesis

Unlike aliphatic glucosinolates, Indole glucosinolates do not have the side-elongation step, therefore, the first major step in the synthesis of indole glucosinolates is the conversion of tryptophan to indole-3-acetaldoxime by the enzymes CYP79B2 and CYP79B3. The aldoxime is converted to an unspecified intermediate by the enzyme CYP83B1 and further to that glutathione conjugate by GST F9, 10. The glutathione conjugate is then converted to a thiohydroximate and finally a desulpho glucosinolate that is sulfonated by SOT16 to form indole glucosinolates. (Halkier & Gershenzon, 2006) (Figure 7).

Sulfur metabolism and glucosinolates

Every glucosinolate contains two sulfur atoms, one in the thioglucosidic bond and another that is used when modifying the core structure. Aliphatic glucosinolates often times contain a third sulfur and in *Arabidopsis thaliana*, aliphatic glucosinolates make up the majority of glucosinolates found (Sonderby et al., 2010). Glucosinolates are also tightly coordinated to sulfur availability to the plant, for example if the sulfur availability is low, glucosinolate accumulation is decreased. Sulfur integration in plants follows a diurnal cycle and this is also true with the accumulation of glucosinolates (Huseby et al., 2013). These trends



can also be observed when the plants are placed in prolonged darkness where there is a decrease in sulfur integration as well as glucosinolate accumulation (Huseby et al., 2013). Further, reduced sulfur availability in *Brassica napus* also led to reduced glucosinolate accumulation (Blake-Kalff et al., 1998).

Glucosinolates consists of two sulfur atoms (three for aliphatic glucosinolates) and therefore, radiolabeled ^{35}S showed that roughly 2% of the integrated sulfur is incorporated into glucosinolates (Mugford et al., 2011).

One pathway for sulphur assimilation in plants is to be converted into adenosine phosphosulfate (APS), by ATP sulfurylases (ATPSs) and then converted into 3'-phosphoadenosine 5'-phosphosulfate (PAPS). This conversion into PAPS is done by the enzymatic activity of adenosine 5'-phosphosulfate kinases (APK) (reviewed in (Chan et al., 2019).

The addition of sulfur to desulfo-glucosinolates is performed by sulfotransferases (SOT) enzymes where the sulfur from PAPS is transferred resulting in a 3'-phosphoadenosine 5'-phosphate (PAP). This reaction is part of the last step of glucosinolate biosynthesis and is carried out by SOT16, SOT17, and SOT18 (Chhajed et al., 2020, Chan et al., 2019). PAP, the by-product of PAPS, can act as a chloroplast-to-nucleus retrograde signal during oxidative stress and activate stress-responsive genes such as *ASCORBATE PEROXIDASE 2 APX2* in high light (Chan et al., 2019, Chi et al., 2015). *Sal1* encodes a gene for a phosphatase, and it regulates the accumulation of PAP,

and *sal1* mutants were susceptible to pathogens and also accumulation aliphatic glucosinolates (Fernandez & Burch-Smith, 2019). It remains unclear what the physiological significance between sulfur assimilation, PAPS, PAP, and glucosinolates.

Glucosinolate diversity

The complex biosynthesis of glucosinolates give rise to many and diverse amounts of glucosinolates. To date, there are about 137 predicted glucosinolates and a total of 88 identified structures (Blazevic et al., 2020) and *A. thaliana* is predicted to have only 40 with the majority of them derived from methionine (Sonderby et al., 2010). The quantification and/or identification of glucosinolates is usually performed in laboratories by mass spectrometry, high performance liquid chromatography (HPLC), or nuclear magnetic resonance and most of these techniques rely on the use of authentic standards (Blazevic et al., 2020). The glucosinolate profiles for 58 *Arabidopsis thaliana* ecotypes revealed that the ecotypes accumulated a diverse range of glucosinolates (Panthee et al., 2011). For example, the total glucosinolate concentration found in shoots of the Columbia ecotype was 27.85 $\mu\text{mol/g}$ dry weight vs 2.0 $\mu\text{mol/g}$ dry weight of the CS925 (Litva ecotype) (Panthee et al., 2011).

In addition to glucosinolate profiles being diverse among *Arabidopsis thaliana* ecotypes, glucosinolates within a single ecotype in different tissues varies among organs and different developmental stages (Brown et al., 2003).

For example, total glucosinolate accumulation in *Arabidopsis thaliana* seeds is 63.4 μmol per gram dry weight and in rosette leaves the concentration is 17.9 μmol per gram dry weight (Brown et al., 2003). This variation is further complicated when the individual types of glucosinolates are identified in these tissues. The glucosinolate glucoraphanin (4MSOB) concentrations range greatly, for example: rosette leaves are about 10.63 μmol per gram dry weight, roots have 7.48 μmol per gram dry weight, and seeds have 2.43 μmol per gram dry weight (Brown et al., 2003).

Glucosinolate roles in signaling

Glucosinolates serve as essential components of plant responses to both biotic and abiotic environmental stresses and potential roles in signaling (Ishiga et al., 2017, Urbancsok et al., 2017, Wang et al., 2017). Although glucosinolates are not known to be signaling molecules, their actions on the plant definitely resemble those of signaling molecules.

Aliphatic glucosinolates are regulated by auxin pathways and during drought stress, plants are able to regulate glucosinolate levels and change the drought response (Salehin, 2019). Further, auxin mutants, *iaa5*, 6, 19 have a suppression of genes in the aliphatic glucosinolate biosynthesis pathway. Further, the amount of glucosinolates (4MSO) was also decreased in the auxin mutants. When *iaa5*, 6, 19 was challenged in drought conditions, it was not as resistant as the wild-type but the plants increased their drought resistance with the exogenous

application of glucosinolates (Salehin, 2019). Further, the defects in drought response were due to the stomata regulation and interestingly, was linked to be in response to aliphatic glucosinolates and not indole glucosinolates. The myrosinase TGG1 is highly abundant in guard cells (Zhao et al., 2008) where single-cell type proteomic of guard cells identified this protein in high abundance. Further, *tgg1* mutants were not responsive to ABA inhibition of stomatal opening. Another example of glucosinolate concentrations in plants affecting physiological processes is demonstrated in plants overexpressing the myrosinase TGG1. Plants overexpressing TGG1 have an enhanced defense against *Pseudomonas syringae* infection and have delayed flowering due to the induction of FLOWERING LOCUS C (FLC) that makes a transcription factor that suppresses flowering (Zhang et al., 2019).

The biosynthesis and accumulation of glucosinolates in plants is tightly regulated. Comprising of up to 3% of the dry weight in *Arabidopsis thaliana* (Brown et al., 2003). It is also energetically costly for plants to generate such large quantities of glucosinolates where their production could result in an increase of photosynthetic output by 15% (Bekaert et al., 2012). Therefore, the biosynthesis of glucosinolate is coordinated with the nutrient conditions. Glucosinolates are themselves sulfur and nitrogen rich compounds. In intact plants, the availability of sulfur is directly correlated with methionine derived glucosinolates while the nitrogen availability is correlated with indole glucosinolates (Jeschke et al., 2019).

However, when a carbon source was given to plants, the total glucosinolates were increased (Jeschke et al., 2019). This further supports that glucosinolates of intact and not damaged leaves are responsive to their external stimuli.

Glucosinolate hydrolysis-myrosinases

The hydrolysis of the thioglucosidase bond in glucosinolates results in a glucose and an unstable aglycone (Halkier & Gershenzon, 2006). The aglycone can change to various compounds either spontaneously through changes in pH or epithiospecifier proteins (ESP) (Andreasson et al., 2001). The myrosinases, also known as beta-glucosidases, consists of a family of 47 genes (Xu et al., 2004). These consist of two types of myrosinases, classical and atypical myrosinases. In Arabidopsis there are six classical myrosinases, myrosinases TGG1-TGG6 (Figure 8) and there are 16 genes that have predicted coding sequences for atypical myrosinases (Sugiyama & Hirai, 2019). Interestingly, the hydrolysis of glucosinolates by classical myrosinases, specifically glucosinolates TGG1 and TGG2 does not seem to occur in intact plants (Barth & Jander). The major differences between these two types of myrosinases is that typical myrosinases have a glutamine and glutamic acid (QE) that are predicted to be required for their activity of the hydrolysis of glucosinolates. The predicted catalytic site in atypical myrosinases has replaced the glutamine with a glutamic acid (EE) (Rask et al., 2000). Two enzymes, PENETRATION 2 (PEN2) and PYK10 (beta-

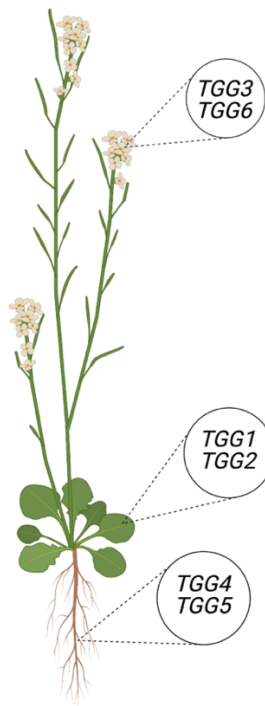


Figure 8 Schematic of myrosinase expression patterns in *Arabidopsis thaliana*.

Thioglucoside glucohydrolase commonly known as myrosinases are expressed in different tissues of *Arabidopsis*. *TGG1* and *TGG2* are both expressed in phloem-associated cells and *TGG1* is also expressed in stomata (Barth & Jander, 2006). *TGG4* and *TGG5* are expressed in roots (Carlotto et al., 2016). *TGG3* and *TGG6* are expressed mainly in pollen (Wang et al., 2009). glucosidase23, BGLU23) continue to have myrosinase activity although lacking the QE residues (Nakano et al., 2017).

The localization within the tissues of atypical myrosinases greatly varies and further, their localization can change depending on the environmental stress (Sugiyama & Hirai, 2019). For example, expression of BGLU18 under wounding stress can be found in both roots and shoots, whereas under drought stress it is only found in the shoots (Sugiyama & Hirai, 2019). Further, the glucosinolate specificity of atypical myrosinases also varies with BGLU18 targeting the glucosinolate 4MI3G (4-methoxyindol-3-ylmethyl glucosinolate) (Nakazaki et al., 2019) and BGLU23 targets specifically I3G (indol-3-ylmethyl glucosinolate) (Schmid et al., 2005).

The hydrolysis of glucosinolates is also as complex as their synthesis. For example, the myrosinases that exist in *Arabidopsis thaliana* do not all exist in all tissues nor do they hydrolyze all glucosinolates. For example, the TGG1 myrosinases do not hydrolyze specific glucosinolates, whereas the PYK10 glucosinolate hydrolyzes indole glucosinolates (Nakano et al., 2017).

PEN2 was found to be important for the pre-invasive resistance to powdery mildew. Flg22 resulted in a decrease in indole glucosinolates and further, a secondary modification by the enzyme CYP81F2 is necessary for PEN2 immune response (Lipka et al., 2005). This specific modification and enzymatic activity beg the question of whether these biological compounds themselves serve unique and specific roles in plants that ultimately may go beyond their simple defense response.

Aims of the dissertation

Based on the prior information, the specific aims of this dissertation are to address the following questions:

What is the role of the ISE2 interacting protein uL15C in chloroplast RNA metabolism and intercellular trafficking via plasmodesmata?

Chapter 2 focuses specifically on the ISE2 interacting protein uL15C and the data from this chapter was published in New Phytologist (Bobik et al., 2019). Similar to ISE2, loss of uL15C results in increased plasmodesmata permeability and disrupts ribosomal RNA processing and accumulation.

Is there a relationship to glucosinolate biosynthesis or glucosinolate imbalances to plasmodesmata mediated trafficking? What is the role of ISE2 and glucosinolate biosynthesis?

Chapter 3 focuses on the model Organelle-Nucleus-Plasmodesmata signaling and specifically focuses on the chloroplast changes induced by the loss of ISE2. The chapter focuses on changes on nuclear gene expression and plasmodesmata-mediated trafficking in the perspective of glucosinolate imbalances.

CHAPTER 2 CHLOROPLAST RNA PROCESSING AND PLASMODESMATA MEDIATED TRAFFICKING

Most of the research from this chapter was originally published by Krzysztof Bobik, Jessica C. Fernandez, Sara R. Hardin, Ben Ernest, Elena E. Ganusova, Margaret E. Staton, and Tessa M. Burch-Smith. The article citation is as follows:

Bobik, K., Fernandez, J.C., Hardin, S.R., Ernest, B., Ganusova, E.E., Staton, M.E., Burch-Smith, T.M. (2019) The essential chloroplast ribosomal protein uL15c interacts with the chloroplast RNA helicase ISE2 and affects intercellular trafficking through plasmodesmata. *New Phytologist*, 221(2), 850-865.

Fernandez completed the experiments and made five of the figures. She also was part of the written revisions and resubmission after reviews. Bobik and Burch-Smith conceived and designed the research. Bobik, Hardin, Ganusova and Burch-Smith performed experiments. Bobik, Fernandez, Staton, and Burch-Smith analyzed data.

Abstract

The chloroplast RNA helicase INCREASED SIZE EXCLUSION LIMIT 2 an essential protein for plant development. Loss of ISE2 expression results in abnormal chloroplast development. Beyond splicing of group II introns, ISE2 plays important roles in multiple chloroplast RNA processing including C-to-U RNA editing and defective processing of chloroplast ribosomal RNAs. RNA helicase function in large protein complexes and therefore, previous efforts were

in place to identify interacting proteins. Plastid ribosomal protein L 15 (uL15c previously RPL15) was previously found to interact with ISE2 in both a yeast two-hybrid and in a pull-down assay. uL15c was found to also play a role in chloroplast ribosomal RNA processing and the assembly of ribosomal subunits were disrupted in plants with reduced expression of *RPL15*. Loss of *rp15* also resulted in chloroplast translational defects.

Introduction

The translation of mRNA into protein is a universal process and the machinery that facilitates this is highly conserved across all domains of life. Chloroplasts, descendants of prokaryotes, have retained part of their genomes while the remainder of the gene has been transferred to the host nucleus (Jensen & Leister, 2014). Chloroplast genomes typically encode fewer than 100 proteins, considerably fewer than the estimated 3,000 nucleus-encoded proteins found in chloroplasts (Pesaresi et al., 2007, Jarvis & Lopez-Juez, 2013). Thus, the function of a plant cell as a successful unit depends on the coordinated expression of the nuclear and chloroplast genomes. Chloroplast gene expression is mainly controlled at the post-transcriptional level, and translation is one critical point for such control (Barkan, 2011a, Sun & Zerges, 2015).

Given their origins as cyanobacteria, it is not surprising that the chloroplast ribosomes (chloro-ribosomes) are 70S bacterial type. However, there are several unique features of chloro-ribosomes. One of the most striking is their increased

protein content compared to bacterial ribosomes. Chloro-ribosomes contain a 2:3 protein:RNA ratio, while bacterial ribosomes have a 1:3 protein ratio (Tiller & Bock, 2014). Much of this difference can be attributed to the larger sizes of chloro-ribosome proteins because of N and C-terminal extensions (Yamaguchi et al., 2002, Yamaguchi & Subramanian, 2003, Manuell et al., 2007). In addition, two bacterial-type proteins, bL25 and uL30, are absent from chloro-ribosomes, but five plastid-specific ribosomal proteins (PSRPs) have been acquired (Yamaguchi & Subramanian, 2000, Yamaguchi et al., 2000). [The new nomenclature for ribosomal proteins has been adopted herein (Ban et al., 2014)]. A distinguishing characteristic of chloroplast rRNA of some green algae and all land plants is the presence of a 4.5S rRNA that is similar to the 3' end of the bacterial 23S rRNA and other RNA species (Harris et al., 1994). Despite this difference, the nascent 23S rRNAs of the bacterial and chloro-ribosomes are still of similar size, approximately 4,500 nucleotides. Recent structural analyses of chloro-ribosomes have begun to reveal their unique features and spatial arrangements (Sharma et al., 2007, Manuell et al., 2007, Ahmed et al., 2016, Bieri et al., 2017).

The genes encoding chloro-ribosomal proteins are split between the chloroplast and nuclear genomes. Regardless of the genome encoding them, some chloro-ribosomal proteins are essential while others are not. The general pattern is that proteins essential in *Escherichia coli* are also essential in chloroplasts (Tiller &

Bock, 2014). Mutational analysis by transplastomics revealed that the chloroplast-encoded bL33 (RPL33), bL36 (RPL36) and uS15 (RPS15) are dispensable for plant survival (Rogalski et al., 2008, Fleischmann et al., 2011), whereas bL32 (RPL32) is indispensable (Fleischmann et al., 2011). Similarly, some nucleus-encoded chloro-ribosomal proteins are essential while others are not, and the correspondence between essentiality in *E. coli* and chloroplasts appears to hold. However, the importance of some ribosomal proteins for ribosome assembly and function remains unclear even in *E. coli*. Conflicting evidence for the essential nature of uL15, bL21, uL24, bL27, bL28, uL29, uL30 and bL34 has been reported (reviewed in (Tiller & Bock, 2014)).

Besides ribosomal proteins, other nucleus-encoded proteins are imported into chloroplasts for expression of the organellar genome. Among these are several RNA helicases. RNA helicases are a large group of RNA-binding proteins with ATPase activity that regulate gene expression through the rearrangement of ribonucleoprotein structures. Three chloroplast DEAD-box helicases, RH3 (Asakura et al., 2012) RH22 (Chi et al., 2012), and RH39 (Nishimura et al., 2010), and the DEAH-type RNA helicase ISE2 (Bobik et al., 2017) are required for rRNA processing in chloroplasts. In addition to its role in rRNA processing, ISE2 is required for group II intron splicing (Carlotto et al., 2016, Bobik et al., 2017) chloroplast transcript accumulation, and is involved in C-to-U RNA editing of six chloroplast transcripts (Bobik et al., 2017).

The *Arabidopsis* increased size exclusion limit (ise) mutants, including ise2, were identified by a genetic screen for mutants with defects in intercellular trafficking via plasmodesmata (PD) (Kim et al., 2002). A collection of embryonically lethal *Arabidopsis* mutants were tested for increased transport of 10-kDa fluorescent dextrans (Kim et al., 2002, Burch-Smith & Zambryski, 2012). In addition to increased PD trafficking, loss of ISE2 also leads to increased formation of PD. At every stage of embryogenesis examined, ise2 embryos contained a larger proportion of twinned or branched PD than did wild-type embryos (Burch-Smith & Zambryski, 2010). Consistent with the changes observed in *Arabidopsis* ise2 embryos, formation of twinned and branched PD was also increased in *Nicotiana benthamiana* leaves where ISE2 expression was reduced by virus-induced gene silencing (VIGS) (Burch-Smith et al.). Indeed, loss of ISE2 in *N. benthamiana* leaves resulted in defective chloroplasts and leaf chlorosis (Burch-Smith & Zambryski, 2010). Thus, ISE2 impacts two distinct subcellular locations: chloroplasts and PD.

We identified plastid ribosomal protein L15, uL15c (formerly RPL15), as interacting with ISE2. We also found that RPL15 is an essential gene in *Arabidopsis*, and *rpl15* null mutants are embryonically lethal. Loss of uL15c led to aberrant chloroplast rRNA processing as well as defective chloroplast translation. Together, the data provide further evidence for a connection between the functional status of chloroplasts and PD biogenesis and function.

Results

Localization of uL15c and characterization of *Arabidopsis* Line

Arabidopsis thaliana line expressing 35S:*RPL-YFP* has overexpression of the *RPL15* gene and similar to *ISE2-OE* lines, co-suppression was observed and measured by qPCR (Figure 9e). Plants co-suppressing the *RPL15* gene have bleached leaves and those overexpressing the gene have no phenotype. Confocal microscopy confirmed uL15c-YFP's localization to chloroplast (Figure 9 a-d).

uL15c is required for accumulation of RNA of the 30S and 50S ribosomal subunits

rRNAs do not accumulate in the absence of assembled ribosomes. Therefore, the abundance of ribosomal subunits can be extrapolated from the abundance of various rRNAs. This relationship has been used to characterize several chloroplast ribosome biogenesis mutants including *ise2* (Bobik et al., 2017), *rbf1* (Fristedt et al., 2014), *psrp2*, 3, 4, 5 and 6 (Tiller et al., 2012), *rps15* (Fleischmann et al., 2011), and *rne* (Walter et al., 2010). Levels of the various rRNAs of the cytosolic and chloroplast ribosomes were measured by microfluidics for two independent uL15c-SUP lines and compared to those of the corresponding uL15c-OE lines and WT Col-0 controls. We then calculated the ratios of chloroplast to cytosolic rRNAs and the ratios of rRNAs of small to large

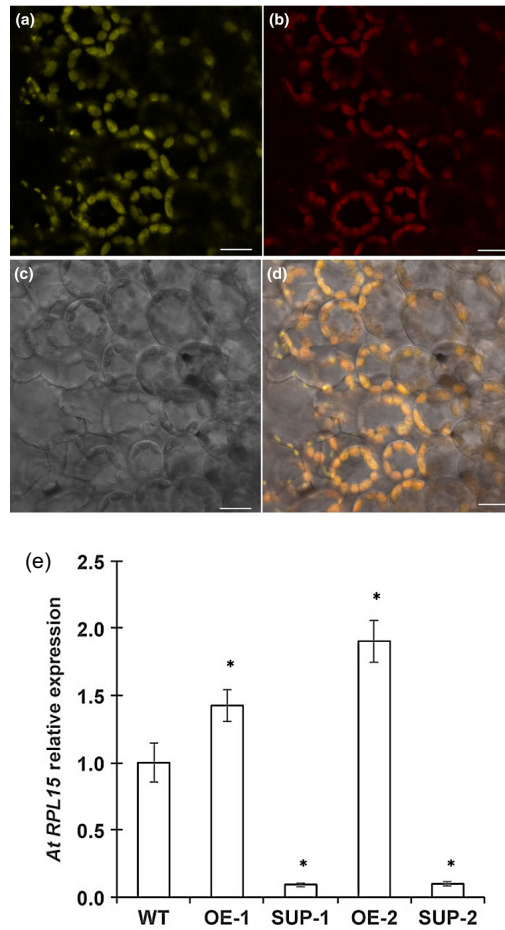


Figure 9 Localization of uL15C-YFP and expression of RPL15.

Localization of uL15c-YFP was determined by confocal fluorescence microscopy.

(a) uL15c-YFP signal, (b) Chloroplast autofluorescence, (c) DIC image, (d)

Merge. (e) expression of *RPL15* in Wild-type, uL15c-OE and uL15c-SUP plants

by qPCR.

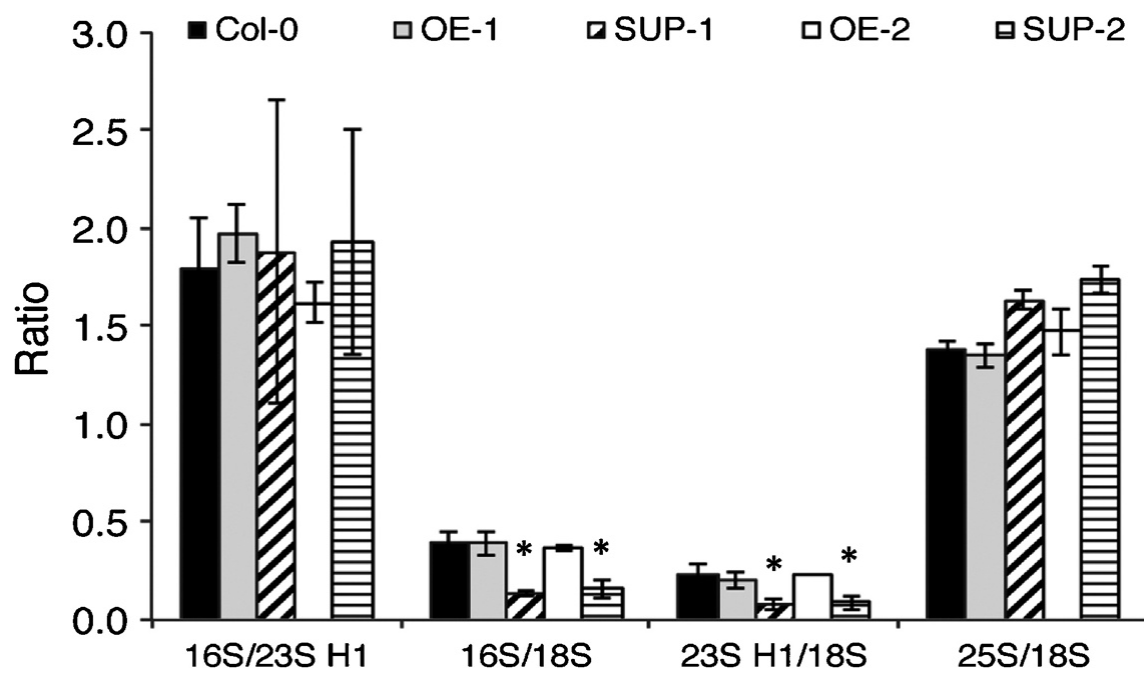
subunits of the chloro-ribosome (Figure 10). Both sets of uL15c-SUP plants showed a decrease in the 23S H1 : 18S ratio, suggesting reduced amounts of 23S rRNA and 50S ribosomal subunit RNA (Figure 10). In addition, these plants contained reduced amounts of 30S ribosomal subunit RNA that led to decreased 16S : 18S rRNA ratios. The rRNA compositions of the uL15c-OE plants were not different from the WT Col-0 plants. Thus, uL15c is required for the correct assembly of 50S ribosomal subunits as well as for assembly of 30S ribosomal subunits.

rRNA processing is impaired in plants with reduced uL15c levels

We next examined the processing of each chloroplast rRNA in leaves of Arabidopsis plants with co-suppressed RPL15 (uL15c-SUP) by Northern blot with probes specific to each species. Chloro-ribosome RNAs are transcribed as a single cistron that is then systematically processed by endo- and exonucleolytic cleavages to eventually produce the mature RNA species (Figure 3; (Nishimura et al., 2010). Reductions in the amounts of correctly processed 16S and 23S rRNAs in uL15c-SUP samples were readily observed on denaturing agarose gels (Figure 11a). Northern blotting with specific digoxigenin (DIG)-labeled probes

Figure 10 Ribosomal subunit assembly is reduced in Arabidopsis uL15c-SUP plants.

Ribosomal subunit abundance is represented through the accumulation of rRNAs. Total RNA was isolated from leaves of 4-wk-old plants grown in soil, and the abundance of rRNAs was measured by a microfluidics-based technique. 16S, the 16S rRNA of the 30S subunit of the chloro-ribosome; 23S H1, the 1.1-kb fragment of the 23S rRNA of the 50S subunit of the chloro-ribosome; 18S, the 18S rRNA of the 40S subunit of the cytosolic ribosome; 25S, the 25S rRNA of the 60S subunit of the cytosolic ribosome. RNA was quantified for at least three biological replicates for each sample. Error bars indicate ± 1 SD from the mean and asterisks indicate statistical significance as calculated by t-test, $P < 0.05$.



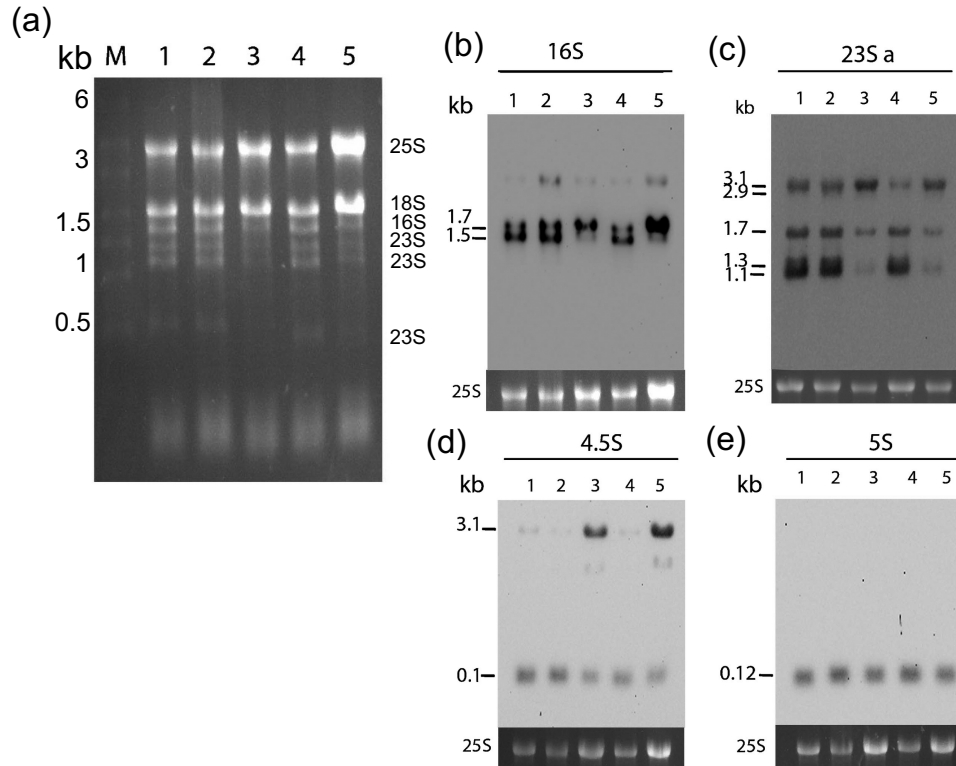


Figure 11 Processing of Arabidopsis chloroplast rRNAs is affected by reduced uL15c.

(a) Total RNA isolated from leaves of 4-wk-old Arabidopsis wild type (WT) Col-0, uL15c-OE and uL15c-SUP plants grown in soil was run on a denaturing formaldehyde gel. The gel was stained with ethidium bromide. Lane 1, WT Col-0; lane 2, uL15c-OE-1; lane 3, uL15c-SUP-1; lane 4, uL15c-OE-2; lane 5, uL15c-SUP-2. (b-e) RNA samples from Col-0, uL15c-OE and uL15c-SUP leaves were analyzed by Northern blot analyses for (b) 16S, (c) full-length 23S, (d) 4.5S and (e) 5S rRNAs using specific DIG-labeled probes. Lanes are as described in (a).

revealed that the pre-16S rRNA accumulated in uL15c-SUP tissues compared to the uL15c-OE and Col-0 WT tissues (Figure 11b). There were also significant changes in the levels of various processing intermediates of the 23S rRNA in the uL15c-SUP samples (Figure 11c). There was a marked accumulation of the full-length 23S+4.5S transcript (3.1 kb). This was concordant with a decrease in accumulation of the 1.7 kb intermediate (0.5 + 1.3 kb) and the 1.3- and 1.1-kb fragments that are produced by cleavage of the RNA after incorporation into chloro-ribosomal subunits in the uL15c-SUP samples. The 4.5S rRNA levels were reduced in uL15c-SUP leaves, consistent with the drastic accumulation of the 23S+4.5S intermediate (Figure 11d). In contrast to the other RNA species, the 5S rRNA levels showed a slight reduction in leaves with reduced amounts of uL15c (Figure 11e). Together, these results revealed that uL15c is necessary for correct processing of chloroplast rRNA species.

Chloroplast translational defects in plants with reduced uL15c levels

The translational efficiency in plants with reduced levels of uL15c was then directly measured. For this we generated polysome profiles of plants from the two independent uL15c-SUP lines and compared them to profiles from WT Col-0 plants. The polysomes containing mRNAs and translating ribosomes were

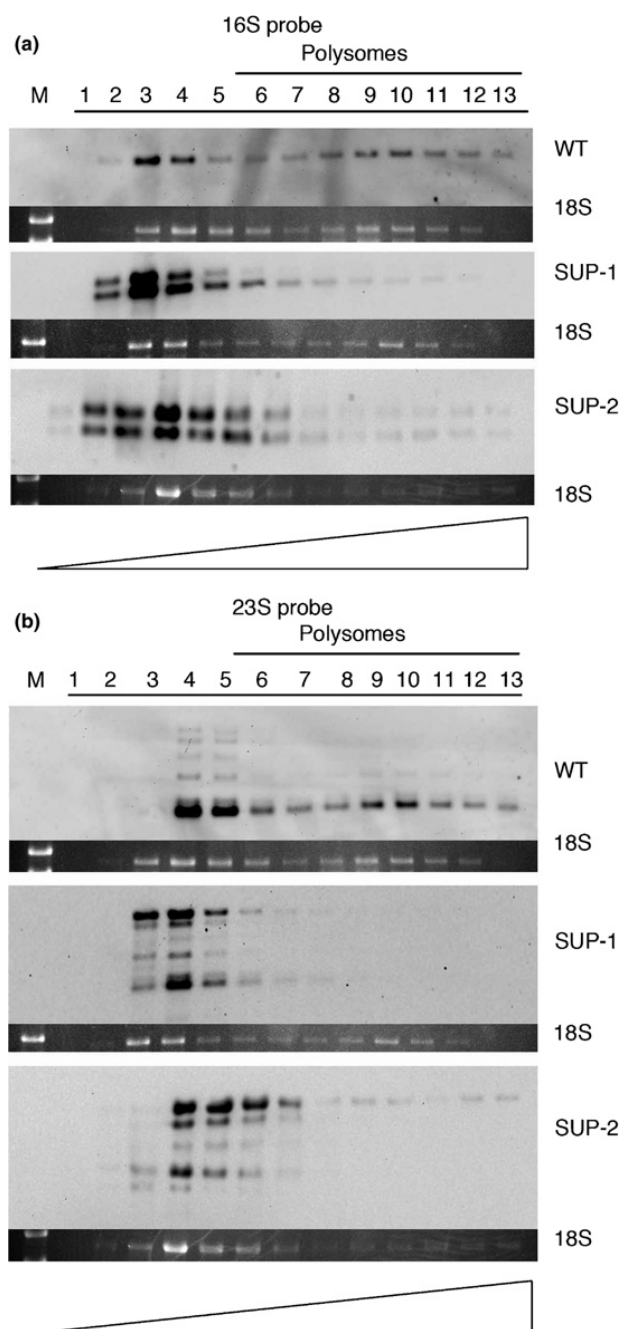
collected by centrifugation on a continuous sucrose gradient. Highly translated mRNAs are associated with more ribosomes and collect toward the bottom of the gradient. Poorly translated or non-translated mRNAs can be found at the top of the gradient. The distribution of the 16S and 23S rRNAs along the gradient was first examined by hybridization to specific probes in gradient fractions of equal proportions. In both sets of uL15c-SUP samples, the 16S rRNA was more likely to be found toward the top of the gradient than it was in WT samples (Figure 12a). As has been previously reported (Fristedt et al., 2014), incompletely processed 16S rRNAs were also found in polysomes, albeit at relatively low levels. There were similar results with the 23S rRNA, with a reduction in 23S rRNA present in polysomes in the uL15c-SUP samples (Figure 1b). We also examined the ribosome loading of chloroplast transcripts. While the *rbcl* transcript was associated with polysomes and in the heavier fractions in the WT sample, the transcript was found in the lighter fractions in the uL15c-SUP samples (Figure 13). These results are consistent with reduced translation efficiency in plants with reduced RPL15 expression.

Discussion

In the last few years, intense investigation has revealed the importance of various nucleus-encoded factors for plastid gene expression

Figure 12 rRNAs are redistributed between free ribosomes and polysomes in *Arabidopsis* uL15c-SUP leaves

Sucrose density gradient centrifugation was used to separate free ribosomes from polyribosomes. Fractions of equal proportions were collected from the top of the gradient, so Fraction 1 is the lightest fraction and wedges indicate increasing gradient density. Free ribosomes are enriched in the light fractions; the polysomes are enriched in the heavy fractions (6–13). 18S rRNA is shown as a control. (a) The distribution of chloroplast 16S rRNA and pre-rRNA between fractions in wild-type (WT) (top samples) or uL15-c-SUP samples (bottom two samples) was determined by Northern blot with a specific DIG-labeled probe. (b) The distribution of chloroplast 23S rRNA and processing intermediates between fractions was determined by probing with a specific DIG-labeled probe.



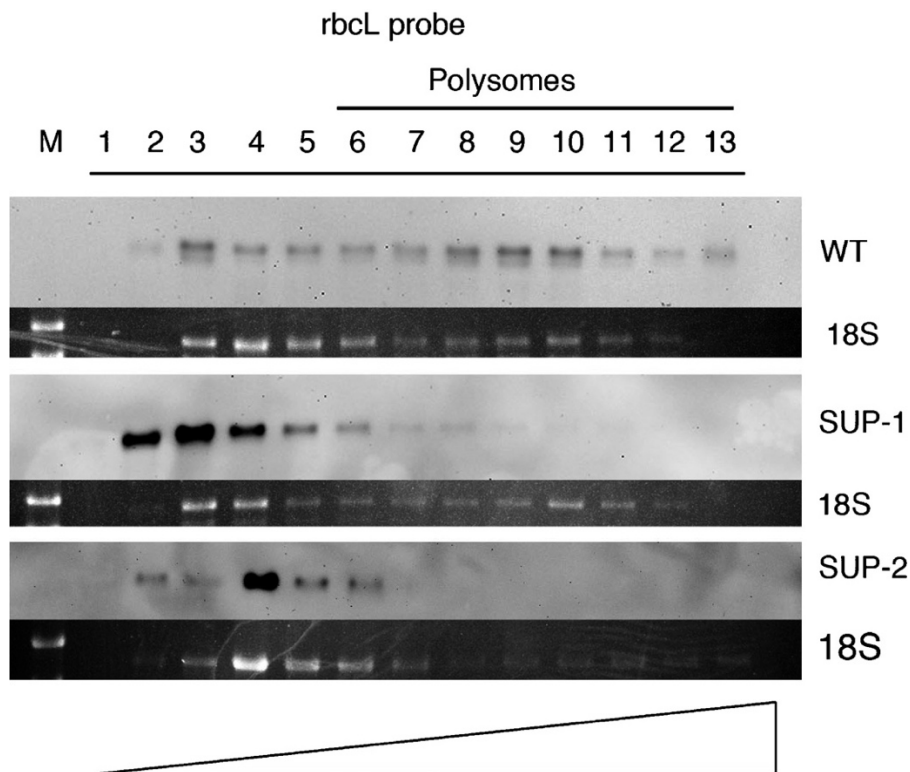


Figure 13 Distribution of rbcL mRNA between free ribosomes and polyribosomes in Arabidopsis uL15c-SUP leaves.

Fractions containing free ribosomes and polyribosomes were prepared by sucrose density gradient centrifugation. Fractions of equal proportions were collected from the top of the gradient, so Fraction 1 is the lightest fraction and wedges indicate increasing gradient density. Free ribosomes are enriched in the light fractions; the polysomes are enriched in the heavy fractions (6–13). RNA distribution between fractions was determined by Northern blot with a specific DIG-labeled probe. 18S RNA is shown as a control.

(Tiller & Bock, 2014). One of the nucleus-encoded plastid ribosomal proteins whose importance for plant survival remained to be clarified was uL15c. Here we have demonstrated that uL15c interacts with ISE2 and is indispensable for proper chloroplast development under autotrophic conditions.

We found that reduced expression of RPL15 led to defects in rRNA processing and chloro-ribosome assembly. In uL15c-SUP samples the levels of the processed 16S rRNA were reduced and those of correctly processed 23S rRNA were drastically reduced compared to controls (Figure 11). In total, these changes are congruent with the reduced accumulation of 50S chloro-ribosome subunits (Figure 10). These changes in rRNA processing are grossly similar to changes observed with reduced expression of ISE2 (Bobik et al., 2017). When ISE2 expression was reduced there was widespread accumulation of rRNA intermediates, suggesting general defects in rRNA processing. When the amount of uL15c was reduced, there was less accumulation of the mature 4.5S rRNA (Figure 11d). However, uL15c has not been reported to interact with the 4.5S rRNA. In the spinach 70S chloro-ribosome the 4.5S rRNA interacts with uL3c, uL13c, b17c, bL19c and bL32c ribosomal proteins, with plastid-specific extensions bL19c and uL3c being important for stabilizing the interactions (Bieri et al., 2017). These interactions are distal from uL15c, supporting the notion that uL15c plays a role in the proper incorporation/positioning of the 23S–4.5S rRNA

precursor into the 50S subunit, a hypothesis supported by our analysis of the incorporation of 23S rRNA into the ribosomes (Figure 12b). Our results (Figure 12b) and those of others (Nishimura et al., 2010, Fristedt et al., 2014) reveal the presence of the 3.1 kb 23S–4.5S precursor in ribosomes, consistent with processing of this molecule occurring in the assembled ribosome.

The chloroplast translational states influence intercellular trafficking and

PD

Plasmodesmata are pores that connect neighboring plant cells, allowing the trafficking of solutes, metabolites and signaling molecules between cells (Burch-Smith & Zambryski, 2012). Characterization of the few identified mutants with altered intercellular trafficking has increased our understanding of PD biogenesis and regulation (Brunkard et al., 2015b). Among these mutants, *Arabidopsis* *ise1* and *ise2* are the only ones demonstrated to have increased intercellular transport and increased frequency of PD (Stonebloom et al., 2009, Burch-Smith & Zambryski, 2010). Surprisingly, ISE1 and ISE2 encode RNA helicases that localize to mitochondria and chloroplasts, respectively. These discoveries shed new light on the possible roles of organelles in governing cell-to-cell communication, but how organelle-localized RNA helicases regulate and govern cell-wall-associated processes remains an intriguing question.

Importantly, similar to the reduced expression of ISE2, reduced expression of RPL15 also resulted in increased intercellular trafficking of GFP. Loss of the mitochondrial RNA helicase ISE1 has also been shown to cause defective chloroplast development and lead to increased intercellular trafficking and increased formation of PD (Stonebloom et al., 2009). Thus, uL15c joins a small group of proteins that have been shown to function in chloroplasts and to modulate PD function. The data presented here support the hypothesis that the functional status of chloroplasts can influence intercellular trafficking flux via PD. Also lending support to this hypothesis is the finding that the *clpr2* mutant has increased intercellular trafficking (Carlotto et al., 2016).

Signaling between the chloroplast and cell wall to control PD

If the chloroplast functional state regulates intercellular trafficking via PD, an intriguing question emerges how are changes in chloroplast function transmitted to the cell wall and PD. One proposed signaling pathway depends on the redox state of chloroplasts. It has been shown that treatment with varying concentrations of reactive oxygen species can affect PD-mediated intercellular flux. Application of low concentrations (0.6 mM) of H₂O₂ doubled plasmodesma flux while higher concentrations (6 mM) greatly restricted PD-mediated trafficking (Rutschow et al., 2011). There is also evidence that the redox state of various

organelles has differing effects on PD. Treatment with chemicals that specifically oxidized mitochondria increased plasmodesmal flux, while those that oxidized chloroplasts had the opposite effect and reduced plasmodesmal flux (Stonebloom et al., 2012). These results were supported by the additional finding that silencing ISE1 expression led to oxidized mitochondria, and silencing ISE1 or ISE2 resulted in more reduced chloroplasts. Arabidopsis GAT1 encodes a chloroplast-localized m-type thioredoxin whose reduced expression led to increased generation of reactive oxygen species, increased deposition of callose at PD and decreased intercellular trafficking (Benitez-Alfonso et al., 2009). Similar to *ise2* mutants, the *gat1* mutant also displays a lethal phenotype, with mutants dying as seedlings. Overexpression of GAT1 results in the opposite phenotype of increased intercellular transport.

We propose three additional mechanisms by which chloroplast-to-PD communication could be accomplished. The first is through modulating the expression of cell-wall-related genes through chloroplast-to-nucleus retrograde signaling. It is well established that reduced chloroplast translation rates trigger the production of retrograde signals that modulate expression of photosynthesis-related and other nuclear genes (Koussevitzky et al., 2007, Cottage et al., 2008, Yu et al., 2012). Chloroplast-to-nucleus retrograde signaling regulates the expression of *Lhcb* genes (Jasper et al., 1991). Concordantly, there was an

increase in Lhcb levels when the amount of uL15c decreased, suggesting that the chloroplast-retrograde signaling pathway regulating Lhcb expression was initiated in plants with decreased uL15c, where translation was reduced but not completely inhibited as occurs with chemical translation inhibitors. This contrasts with the drastic reduction in Lhcb levels observed in ISE2-silenced tissues (Bobik et al., 2017), indicating that reductions in uL15c or ISE2 amounts differentially impact chloroplast-to-nucleus retrograde signaling. Because reduced expression of either leads to a similar phenotype including increased PD frequency followed by increased intercellular trafficking, one could suggest that multiple chloroplast-derived signals may act to regulate nuclear gene expression to regulate PD and intercellular trafficking.

A second mechanism could be through hormonal signaling. Chloroplasts are the source of several plant hormones including salicylic, abscisic and jasmonic acids, and these hormones are known to influence intercellular trafficking (reviewed by (Sager & Lee, 2014, Han & Kim, 2015)). It is possible that altered hormone levels in mutants with defective chloroplasts could also impact intercellular trafficking and PD development. We have found that increased ISE2 expression is associated with elevated jasmonic acid accumulation and signaling in response to pathogen infection (Ganusova et al., 2017).

A third mechanism for chloroplast to PD communication could involve the metabolic coupling between chloroplasts and endoplasmic reticulum. This coupling has been demonstrated for lipid biosynthetic pathways that require the activity of enzymes partitioned between the two organelles (Mehrshahi et al., 2014, Mehrshahi et al., 2013). However, further experimentation is necessary to elucidate which of these proposed mechanisms is involved in communicating the translational and functional state of chloroplasts to the cell wall.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana Columbia (Col-0) ecotype or *N. benthamiana* plants used in all experiments were grown as previously described (Bobik et al., 2017). The 35S:RPL15-YFP was previously made by following Clough & Bent (1998) was used to transform *A. thaliana* Columbia (Col-0) ecotype

Fluorescence microscopy

Live cell imaging of uL15c-YFP used a laser confocal scanning microscope (Zeiss LSM 710; Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). Tissue samples were cut from *Arabidopsis* leaves and infiltrated with water. The 514-nm

and 561-nm laser lines with appropriate emission filters were used to image enhanced yellow fluorescent protein (EYFP) and chloroplast autofluorescence, respectively. Images were collected with a Plan-Apochromat ×63 oil immersion lens. For measuring intercellular trafficking, images were collected on a Leica SP2 or SP8 with White Light Laser (Leica Microsystems Inc., Buffalo Grove, IL, USA). The 488-nm laser line was used to excite green fluorescent protein (GFP). A ×25 or ×25 water immersion lens was used to collect images.

cDNA synthesis and quantitative RT-PCR

cDNA synthesis was performed according to the manufacturer's instructions (Promega). At the end of the reaction the mixture including the cDNA was mixed with 25 µl of nuclease-free water and 1 µl was used as template for subsequent DNA amplification (PCR). Two and a half microliters of cDNA diluted five-fold, 0.5 µl of each gene-specific primer (Table 1) and 5 µl of Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) were used in 10 µl reactions for quantitative reverse transcriptase PCR (qRT-PCR) analysis with an iQ5 Multicolor RT PCR Detection System (Bio-Rad). RPL15 transcript levels were normalized relative to ACTIN mRNA levels and a relative quantification method (Pfaffl, 2001).

Northern blotting

Two micrograms of total RNA were separated on 1% or 1.25% agarose denaturing gels and then transferred to positively charged nylon membranes (Roche, Basal, Switzerland) according to Church & Gilbert (1984). The hybridization was performed at 57°C for 12–16 h using PCR-generated DIG-labeled probes (PCR DIG Probe Synthesis Kit, Roche Diagnostics) using primers indicated in Table 1. Full-length probes were generated for 23S rRNA, 5S rRNA and 4.5S rRNA that correspond to 2811 bp, 121 bp and 103 bp, respectively. A probe specific for the 0.5-kb fragment of the 23S rRNA was designed as described previously (Nishimura et al., 2010). A 770 bp probe was used to detect 16S rRNA. Hybridization and detection were performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics).

Polysome fractionation

Leaves of 4-wk-old WT and uL15c-OE or -SUP plants were collected and pulverized in liquid nitrogen. Polysome fractionation on 15–55% sucrose gradients were performed according to Barkan (1998).

Table 1 Primers used for this study

Name	Forward Sequence	Reverse Sequence	Gene	Aim	Source
KB 634/635	TGAAGGTGGTCAAACCTGCTC	TCATCTCCCTCTTGAAACCC	<i>AtRPL15</i>	qRT-PCR	Bobik, 2019
JCF 908/873	CGGTGGTTCCATTCTTGC	GGACCTGCCTCATCATACTC	<i>AtACTIN</i>	qRT-PCR	Bobik, 2019
TBS 210/211	ACGGGTGAGTAACGCGTAA G	TGAGTTTCATTCTTGCGAAC GTACTC	<i>rrn 16S</i>	DIG probe	Bobik, 2017
TBS 424/425	TTCAAACGAGGAAAGGCTTA CGGTG	AGGAGAGCACTCATCTTGGG GTGG	<i>rrn 23S</i>	DIG probe	Bobik, 2017
TBS 214/215	GAAGGTCACGGCGAGACGA GC	GTTCAAGTCTACCGGTCTGT TAGGATG	<i>rrn 4.5S</i>	DIG probe	Bobik, 2017
TBS 216/217	TATTCTGGTGTCTAGGCGT AGAGG	ATCCTGGCGTCGAGCTATTT TT	<i>rrn 5S</i>	DIG probe	Bobik, 2017
JCF 1451/1452	CGAGGAAAGGCTTACGGTG GATAC	AAGATGTTTCAGTTCGCCAG GTTG	<i>Rrn 23S</i> <i>0.5kb</i>	DIG probe	Nishimura <i>et al.</i> , 2010

CHAPTER 3 GLUCOSINOLATES MODULATE INTERCELLULAR TRAFFICKING VIA PLASMODESMATA

Introduction

Plasmodesmata (PD) allow for the direct cell-to-cell communication between two adjacent cells across the cellulosic cell wall. This provides direct cytoplasmic connectivity, and the movement of various sugars, signals, proteins, nucleic acids, and metabolites are essential for plant growth and development (Sager & Lee, 2018, Reagan et al., 2018). Despite the importance of plasmodesmata in growth and development, how plasmodesmata are regulated has not been fully investigated. Only a few proteins have been identified to play a role in the regulation of plasmodesmata. The chloroplast RNA helicase ISE2 has major functions in chloroplast RNA metabolism and processing (Bobik et al., 2019, Bobik et al., 2017, Carlotto et al., 2016), and photosynthesis associated nuclear genes are misregulated (Ganusova et al., 2020, Burch-Smith et al., 2011a), such as the expression of LHB1.2, and LHCB2.1. Additionally, plants with reduced ISE2 expression display increased plasmodesmata-mediated trafficking and increased numbers of plasmodesmata (Ganusova et al., 2020, Burch-Smith & Zambryski, Kobayashi et al., 2007).

Plants have evolved a series of self-defense responses involving toxic chemicals. In Brassica plants, one such important mechanism is the breakdown of the secondary metabolites collectively called glucosinolates. Glucosinolates are well known for their roles in defense and in Arabidopsis they are predominantly formed from tryptophan (indole glucosinolates) and methionine (aliphatic

glucosinolates) (Sonderby et al., 2010). The Brassicaceae (mustard family) includes plants such as cabbage, broccoli, mustard, and *Arabidopsis*. Glucosinolates serve as essential components of plant responses to both biotic and abiotic environmental stresses and potential roles in signaling (Ishiga et al., 2017, Urbancsok et al., 2017, Wang et al., 2017). In *Arabidopsis* ecotypes the chemical composition of glucosinolates vary vastly from one ecotype to another and even between tissues, as observed with the Columbia-0 ecotype (Panthee et al., Brown et al.). Further, the overall quantity of glucosinolates as well as their overall diversity of types of glucosinolates vary drastically throughout development, in *Arabidopsis thaliana* (Petersen et al., 2002, Brown et al., 2003). Given the changes of glucosinolate composition in development, one can assume additional roles besides defense. In *Arabidopsis*, glucosinolate metabolism has been linked to stomatal control, drought resistance, auxin signaling, root growth, and biomass, and bolting (Zhao et al., 2008, Ahuja et al., 2021, Salehin, 2019, Katz et al., 2020, Zhang et al., 2019, Bak et al., 2001). Glucosinolates are synthesized in three steps involving different subcellular compartments: 1. chain elongation in the chloroplasts, 2. biosynthesis of core glucosinolate structure in the chloroplast and cytoplasm, and 3. secondary modifications in the cytoplasm (Figure 5). Amino acids are precursors to glucosinolates. The specific enzymes responsible for the many steps of synthesis tend to be unique to the amino acid precursors, meaning that methionine derived

glucosinolates have unique enzymes responsible for their synthesis (as reviewed in (Chhajed et al., 2020)).

Due to the overall defects that the loss of ISE2 has in RNA metabolism and intercellular trafficking, we decided to investigate the transcriptional network in order to identify the potential signal responsible for these changes. In mutant embryos, the loss of ISE2 had an increased representation of genes involved in glucosinolate metabolism (Burch-Smith et al., 2011a). Therefore, we investigated if the loss of ISE2 induced changes in glucosinolate metabolism, ultimately changing Plasmodesmata Associated Nuclear Genes (PDANGS) and ultimately altering intercellular communication via plasmodesmata. Intercellular trafficking via plasmodesmata is reduced in mutant *ise2* embryos and in silenced plants (Burch-Smith & Zambryski, 2010, Kobayashi et al., 2007). In order to investigate the relationship between the chloroplast RNA helicase ISE2 and plasmodesmata, we studied gene expression in *Arabidopsis* where ISE2 function was limited. For this, we used two approaches. 1. Tobacco rattle virus (TRV)-based virus induced gene silencing (VIGS) to acutely silence ISE2 in leaves and analyzed gene expression by tiling microarrays (Burch-Smith et al., 2011a) and 2. Using a stable rescue line where co-suppression is observed (allowing for a system of plants over expressing the gene ISE2 (ISE-OE plants) and suppression of ISE2 (ISE2-SUP)), we analyzed gene expression via RNA seq. Here we present data demonstrating that changing the expression of the chloroplast RNA helicase

ISE2, results in potential chloroplast-to-nucleus signaling ultimately altering the gene expression of genes involved in glucosinolate metabolism. The change in glucosinolate profiles thus results in physiological changes including flowering, root development, stress response. Further, we show that glucosinolates act as signaling molecules that alter intercellular trafficking via plasmodesmata.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana Colombia (Col-0) ecotype plants and various lines and mutant lines were all from this ecotype. Seeds that were germinated on soil were directly planted onto soil and stratified at 4 °C for three days and then transferred to a growth chamber under standard growth and conditions (22 °C, 16 h Light, 8 h Dark). Seeds germinated on plates were germinated 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. First, seeds were 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 stratified on plates at 4 °C for 3 days and were then transferred to a growth chamber under standard growth and conditions (22 °C, 16 h light, 8 h dark, 60% humidity).

Measuring effects of drought

Plants were grown in soil for 20 days with normal watering in 4 cm x 4 cm x 4 cm pots with two plants per pot. Plants were grown under long day conditions (16h/8h light/dark) in a chamber at 21 °C day and 18 °C night with a light intensity of 100-110 $\mu\text{mol m}^{-2} \text{s}^{-1}$. After 20 days, the pots were saturated with water, drained and weighed. Water was withheld for 12 days to reduce the weight of the pot to 50% of its original weight and then rewatered to 75% original weight by injecting water with a needle into the middle of the pot. The pots were then subjected to re-drought for 10 days to reduce the pot weights to 50-60%. Fresh weight and dry weights of each rosette were measured. For rosette dry weights, the plants were incubated for two days at 65 °C in an oven.

Measuring Osmotic Stress

Seeds were surface sterilized and sowed on $\frac{1}{2}$ X MS media (recipe above) and stratified at 4 °C for three days. Plates were then transferred to a growth chamber under long-day conditions for 5 days. Prior to seedling transfer, 35 ml MS plates were soaked with 50 ml 30% filtered-sterilized PEG-8000 overnight. The excess PEG was drained the following day and the 5-day-old seedlings were transferred to MS plates or PEG-treated plates. The root length and plant fresh weight were measured 10 days post transfer.

Stomata Analysis

Stomatal impressions were made using clear nail polish on the abaxial side of 3–4-week-old *Arabidopsis* leaves. A thin coat of nail polish was applied to the abaxial side of the leaves and allowed to dry for 30–40 minutes. The leaf was then carefully placed on clear transparent tape (Scotch transparent tape-glossy finish #MMM144) nail polish-side down on tape and the leaf was peeled off leaving behind the nail polish. The tape was then placed on a glass slide and observed under 25x objective.

Stomata were counted per area 0.1 mm² and their lengths and widths were measured using Image J (Schindelin et al., 2012) To reduce overcounting, partial stomata were counted from the top and right boundaries of the image but not from the left and bottom boundaries. For the two biological replicates, the leaves were collected at the same circadian time.

Glucosinolate Quantification

Glucosinolate extraction and quantification was followed as (Charron & Sams, 2004). Glucosinolates were desulphated using a DEAE Sephadex A-25 column (Raney & McGregor, 1993). 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 µm) with a UV detector at 230 nm. The identification and quantification of the

desulfoglucosinolates were made using authentic standards (Charron & Sams, 2004).

RNA Isolation

RNA was isolated from tissue samples following the TRIZOL RNA isolation protocol according to manufacturer's protocol (Invitrogen, 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 μ l 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 μ l of isopropanol was added then the samples were incubated at room temperature for 10 minutes. The RNA was centrifuged for 10 minutes at 12,000 x g at 4 °C. The RNA precipitate was washed 2 times by adding cold 75% ethanol and centrifuging at 7,500 x g at 4 °C. The RNA pellet was resuspended in 40 μ l DEPC-treated water and the concentration was determined by nanodrop analysis.

cDNA synthesis

In order to remove genomic DNA from the sample, the total RNA was DNase treated using the DNA-free kit TM 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.

Quantitative Real-Time PCR (RT-qPCR)

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 ACTIN (Table 3) and the reference gene At4g26410 (Wang et al., 2014).

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 final concentration of 100 µM. Working stocks (10 µM) were made for all experiments. They are presented in Table 2.

qPCR primer optimization plasmid

Full length targets were amplified with specific primers (Table 2) 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 a standard curve was generated by the amplification efficiency of five dilution points according to (Krefft et al.).

Cloning of *AtTGG1* under 35S promoter through gateway cloning

The full-length TGG1 gene from *A. thaliana* was amplified from cDNA using Phusion High-Fidelity PCR Master Mix (Thermo Scientific) with GATEWAY compatible sites. Briefly, 50 µl reactions were made with 2.0 µl of 1:5 diluted cDNA and each primer at 0.5 µM final concentration. attB-PCR products were cleaned up by using the GATEWAY cloning PEG purification protocol and analyzed by agarose gel electrophoresis for purity. 1 µl of the purified PCR product was used for the BP reaction into the donor vector pDONR/Zeo to create the entry vector.

LR reactions were done using pGWB405 and 406 destination vectors from the Nakagawa Lab to generate TGG1 tagged with GFP at the N- or C- terminus, respectively.

Table 2. Cloning and PCR primers

<u>Gene</u>	<u>Forward</u>	<u>Reverse</u>
<i>CYP83A1</i>	ATGGAAGATATCATCATCGGCGTGG	CATACTTGTTCACTTTCTCTGGAAC
<i>CYP83B1</i>	ATGGATCTCTTATTGATTATAGCCGG	GCAGATGTGTTTCGTTGGTG
<i>BCAT</i>	GTATGGCCAGGGTTTATATGAAG	CTGCCAATGGTATCCAAACAG
<i>CYP79B2</i>	ATGAACACTTTTACCTCAAAC	TCACTTCACCGTCGG
<i>CYP79B3</i>	ATGGATACTTTAGCTTCAAAC	TCACTTCACCATTTGGG
<i>MAM1</i>	CCGCATTGTTCATCTCATGTTGC	CACGGCCATCTCAATCACTTC
<i>TGG1*</i>	GGGGACAAGTTTGTACAAAAAAGCAGGC TTTATGAAGCTTCTTATGCTCGCC	ACCACTTTGTACAAGAAAGCTGGGTCTGCA TCTGCAAGACTCTTCCG
<i>TGG1**</i>	CTTCTTATGCTCGCCTTTGTTTTTC	GAGCGGAGTAGATCTTGGTTCGTAG
<i>TGG2**</i>	CTTCTTGGGTTCGCCTTAGC	TATCTTGGTTCTTGGTGGTATCTC
<i>UBQ10**</i>	GATCTTTGCCGGAAAACAATTGGAGGAT GGT	CGACTTGTCATTAGAAAGAAAGAGATAACAG G
*Gateway Cloning attb sites **Genotyping (Jander, 2006)		

Table 3 qPCR primers used in this study

<u>Gene</u>	<u>Forward</u>	<u>Reverse</u>
<i>BCAT</i>	ACCAGACCAAAACGGTCTCC	TGTTTGATGGCGGAGACGAA
<i>MAM1</i>	CGCCATAGCTCGATGCAAAC	CGAGTATCCTTGGCCTCTTCG
<i>CYP83B1</i>	CAAGAACTTCTAGACGAGAC	GGAGAAAGGTTGGTCTTTG
<i>CYP83A1</i>	GATTCCTCTCCTTATCCCTC	TAAACTCGTAGTCCGTGCCT
<i>CYP81F2*</i>	GTCACAGGGAGACGCTACTAC	CACCACTGTTGTCATTGATGTC
<i>CYP79B2</i>	GCACTTTCTGACACAACCGT	AAACTTTTGGGTTACGGCCC
<i>CYP79B3</i>	AGCCTTTGCTTACCGCTGAT	TATCATCTCCGCAATGGCCC
<i>TGG1</i>	AGACCTCAAAGCATCTGGCA	CTCCTTATCTATGGAGCAAG
<i>TGG2</i>	CTTATGGTACCAGTCGTTCTTG	GTGAGGCTCTTCCTATCCCC
<i>AT4g26410**</i>	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC
* Wang, 2017		
** Wang, 2014		

Intercellular trafficking via plasmodesmata

Intercellular trafficking was done using two separate approaches. To measure intercellular trafficking in mutant plants whole plants were placed in the bombardment chamber and bombarded. For the treatments of glucosinolates, isothiocyanates and the double mutant *tgg1tgg2* leaves were removed and placed on plates prior to bombardment. The same age leaves were used for all analysis.

The pRTL2 plasmid for expressing free GFP using a modified vacuum desiccator as the bombardment “gun” apparatus for low-pressure bombardment (Fernandez, submitted). The pressure was generated with a vacuum and helium gas was used to produce the blast at 60 psi.

Confocal microscopy for movement assays

Plants were analyzed 24 hours post bombardment. Individual transformed cells expressing GFP were identified, and a z-stack was collected with the Leica SP8 confocal with White Light Laser (excitation 488 nm, collecting emission 500-530 nm) (Leica Microsystems, Buffalo Grove, IL). 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 were collected however due to the undulating nature of the leaves, the thickness varied. Z-stacks were reconstructed with FIJI (Schindelin et al., 2012). The number of cell

layers in which the GFP spread was tabulated and analyzed. Significance was determined by Mann-Whitney test with a significance at p-value <0.05.

RNA analysis for RNAseq

RNA from plants grown as above (RNA isolation) was used for analysis. Approximately 25-500 ng/ul of purified RNA was subjected to microfluidics analysis on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) in which integrity of the RNA and the quantification of rRNA was determined. The Plant RNA Pico Assay chip was used according to manufacturer's instructions. RIN values were determined, and the RNA used for RNA seq analysis.

RNA sequence analysis

The nine libraries (three samples and three biological replicates were multiplexed and sequenced Illumina NovaSeq (Illumina Corp., San Diego, CA, USA) with 150-bp pair-end reads. Quality assessment of sequenced reads was performed using fastq. The remaining high-quality reads were mapped to the latest TAIR Arabidopsis genome using HISAT2 version 2.1.0. DESeq2 (version 1.18.1) was subsequently used to determine which genes were differentially expressed between pairs of samples. The parameters used to call a gene differentially expressed (DE) between conditions were p-value < 0.05.

Results

Plants with altered ISE2 expression have altered glucosinolate-related genes.

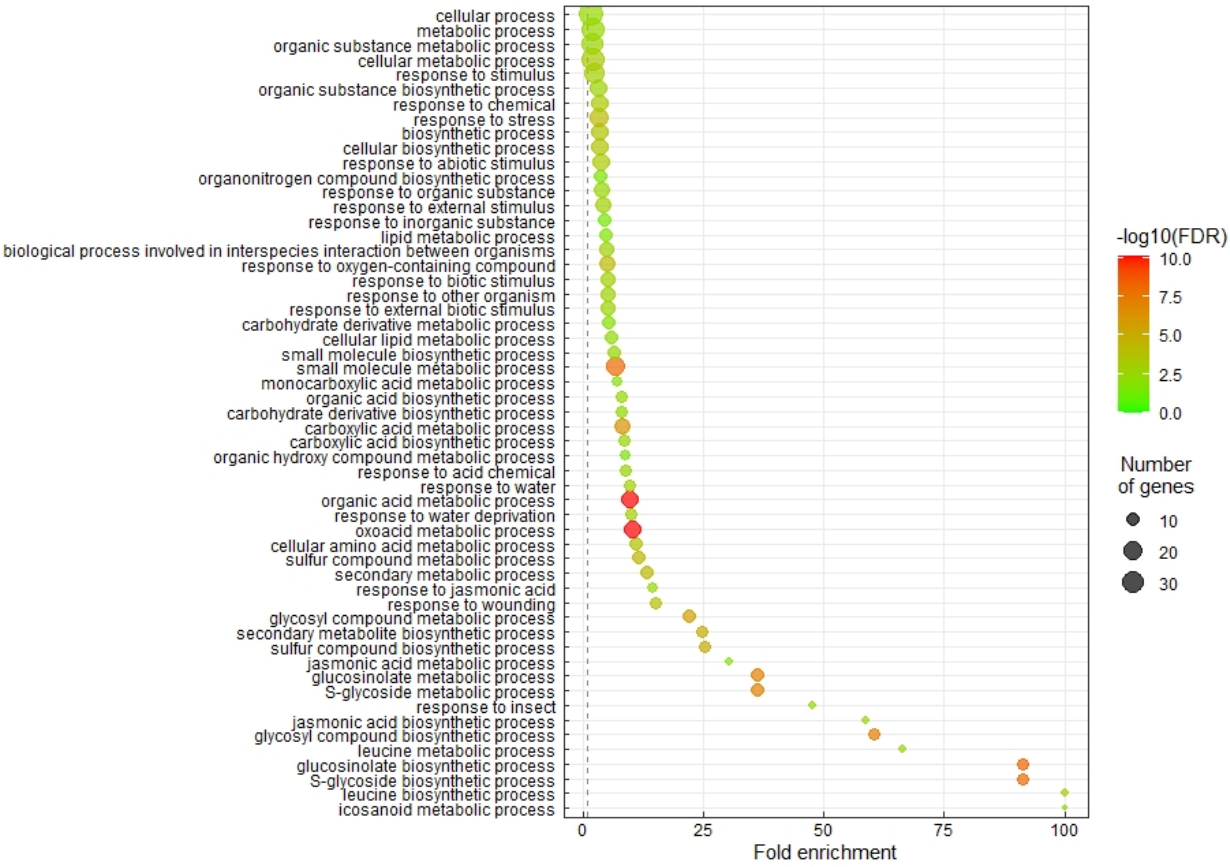
A small group of 64 genes showed abnormal expression in those silenced leaves and this group included genes required for the biosynthesis and metabolism of glucosinolates (Figure 14). Indeed, glucosinolate-related genes were overrepresented among ISE2-affected genes.

As a second approach we investigated gene expression in a system where expression of ISE2 was stably disrupted. For this, we analyzed gene expression in leaves of 3–4-week-old plants overexpressing *ISE2-GFP* via a rescue transgene (ISE2-OE) and plants suppressing the *ISE2-GFP* transgene, effectively producing very low ISE2 levels (ISE2-SUP) as previously described (Bobik et al., 2017) (Figure 15). Consistent with the gene expression analysis in the *ISE2*-silenced leaves (microarray data), several glucosinolate-related genes were also identified in the RNA seq analysis for the ISE2-SUP samples as well as in the ISE2-OE samples (Figure 16). We identified that in addition to the aliphatic glucosinolate pathway, genes involved in indole glucosinolate synthesis and the genes involved in the secondary modifications of glucosinolates upregulated in ISE2-SUP plants (Figure 16).

Figure 14 Gene ontology and heat map for the microarray expression data

(a) Gene ontology analysis for ISE2 TRV-silenced microarray data highlighting glucosinolate metabolic process and glucosinolate biosynthetic process (blue-lined yellow box). (b) heat map of the microarray gene expression data of glucosinolate related genes.

a)



(b)

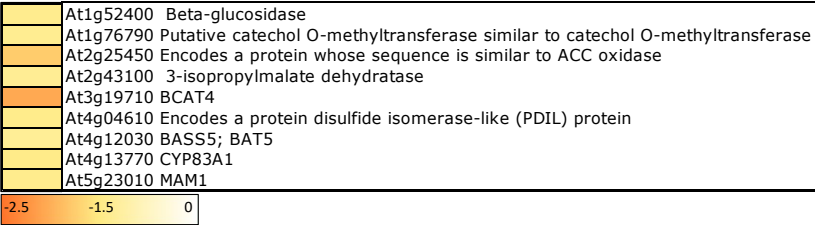




Figure 15 Co-suppression of ISE2 results in chlorotic leaves

Wild-type plants (left) were grown alongside rescue *ise2* mutants stably expressing 35S:*ISE2-GFP* (right). ISE2-OE overexpress *ISE2*, and plants have green leaves. ISE2-SUP plants are silencing *ISE2* and have yellow leaves.

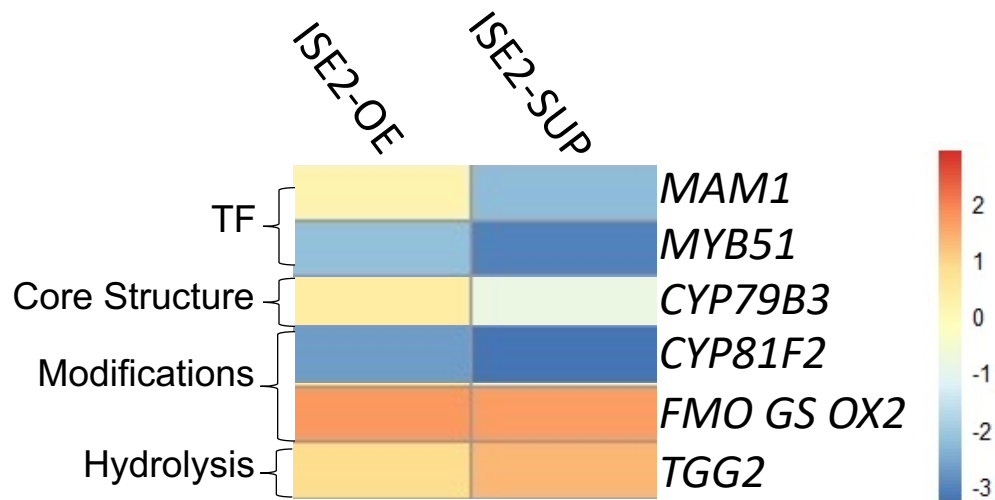


Figure 16 ISE2 mediates genes associated with glucosinolate biosynthesis.

Heatmap of genes involved in glucosinolate biosynthesis. (Transcription factors, Core structure synthesis, modification and hydrolysis). Genes representing various key genes involved in glucosinolate biosynthesis including *MAM1* (for elongation) and a master transcription factor *MYB51*.

qPCR analysis to confirm changes in glucosinolate gene expression

The relative abundance of transcripts of glucosinolate-related genes in leaves of Col-0, ISE2-OE and ISE2-SUP plants was measured by real-time qPCR analysis. (Figure 17). The glucosinolate biosynthetic and breakdown pathways are shown (Figure 5). The first gene whose expression was measured was *BCAT4* which encodes the enzyme responsible for the first step in the synthesis of aliphatic glucosinolates. In ISE2-OE plants, *BCAT4* expression was induced while in *ISE2-SUP plants* expression was suppressed. The same pattern of altered expression was observed for *MAM1*, the enzyme that is responsible for the condensation of the 2-oxo acid with acetyl-CoA, and *CYP83A1* which converts the aldoximes to other intermediates (Figure 5). There was increased expression of *CYP79B2*, *CYP79B3* (the enzymes that convert tryptophan to indole-3-acetaldoxime) and *CYP83B1* (catalyzes aldoxime to produce a glucosinolate intermediate), only in ISE2-OE. The only gene suppressed in only ISE2-SUP was *CYP81F2*, a p450 enzyme responsible for the production of 4-hydroxyindole glucosinolate. Interestingly, the genes involved in the breakdown of glucosinolates, myrosinases *TGG1* and *TGG2* were induced in OE ISE2. Suppressing *ISE2* resulted in an even greater gene expression of these genes. Thus, when ISE2 levels are perturbed in Arabidopsis leaves, expression of glucosinolate-related genes is perturbed, suggesting that glucosinolate metabolism changes in response to ISE2 function.

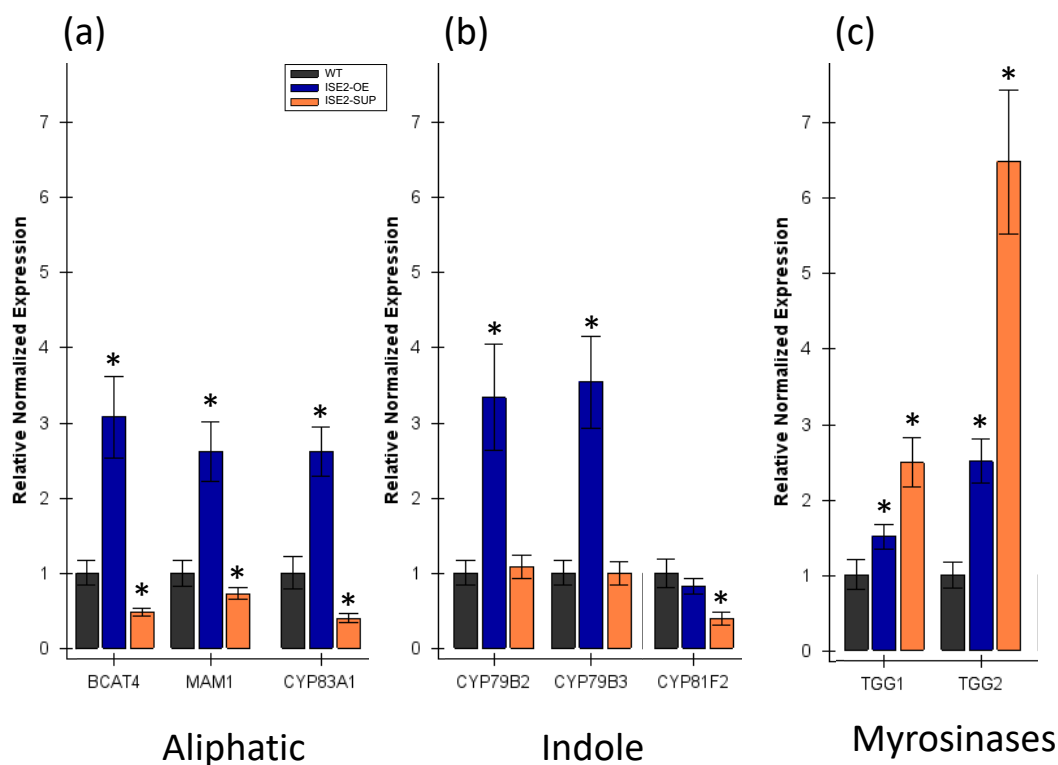


Figure 17 qPCR analysis of glucosinolate related genes.

Gene expression of aliphatic biosynthesis, indole, and myrosinases was analyzed in Wild-type, ISE2-OE, and ISE2-SUP. (a) Expression of methionine-derived aliphatic glucosinolate biosynthetic genes. (b) Expression of Tryptophan-derived indole glucosinolate biosynthetic genes. (c) Expression of classical myrosinases *TGG1* and *TGG2*. Data is from three biological replicates and each experiment had four technical replicates. Data was normalized to Actin and to reference gene AT4g26410. Error bars represent standard deviation and differences were significant when $p < 0.05$ (*).

Varying levels of ISE2 expression phenocopy glucosinolate mutants

Plants with defects in glucosinolate metabolism have been reported to have defects in responses to water availability. Deficiency in aliphatic glucosinolates is linked to decreased drought tolerance (Salehin, 2019). Due to the increased expression of *BCAT4* and *CYP83A1*, we hypothesized that ISE2-OE plants may contain increased levels of aliphatic glucosinolates.

When water was withheld from soil-grown ISE2-OE plants, they were able to tolerate drought better than wild-type counterparts (Figure. 18a), displaying higher fresh weights under water restriction (Figure. 18b) although there was no difference in dry weights for the two groups (Figure 18c). Interestingly, ISE2-OE plants had significantly more adventitious roots than did wild-type, with an average of 2.5 adventitious roots per plant versus the wild-type average of 1 (Figure 18d-f).

To determine if the increase in drought tolerance was linked with reduced transpiration, stomatal apertures were measured. ISE2-OE plants had increased numbers of stomata relative to wild-type plants (Figure 19), however, the lengths and widths were smaller making the total leaf area occupied by stomata smaller than that of wild-type leaves wild-type counterparts (Figure 19b). Further, the increased number of stomata are also due to the fact that epidermal cells in ISE2-OE plants are smaller. Arabidopsis plants with increased *TGG1* expression have delayed bolting (Zhang et al., 2019)..

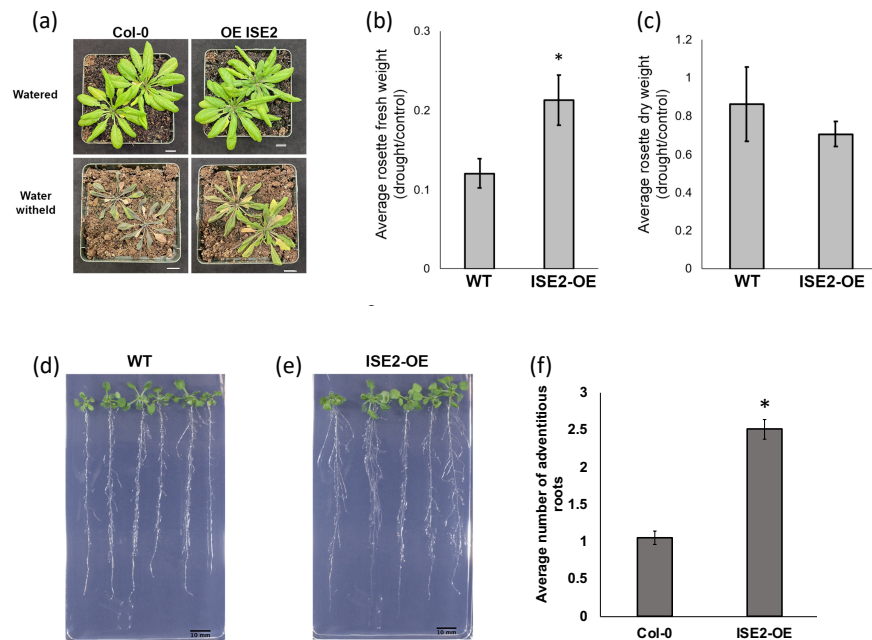


Figure 18 Drought tolerance and adventitious roots in ISE2-OE plants

Representative images for drought experiment. (a-c). Wild-type Col-0 plants were grown alongside ISE2-OE plants. ISE2-OE plants are more drought tolerant than WT Col-0. (d-e) ISE2-OE plants have an increase in number of adventitious roots. Drought experiment is from one independent experiment, $n=8$ for each condition. Error bars represent standard error. Differences between ISE2-OE and Col-0 are significant at $p<0.05$ (*). Results for (a) are from one biological replicate.

Results for (f) are from three biological experiments, Col-0 $n=69$, ISE2-OE $n=72$.

Error bars represent standard error and are significant at $p<0.05$ (*). (a) Scale bar 10 mm.

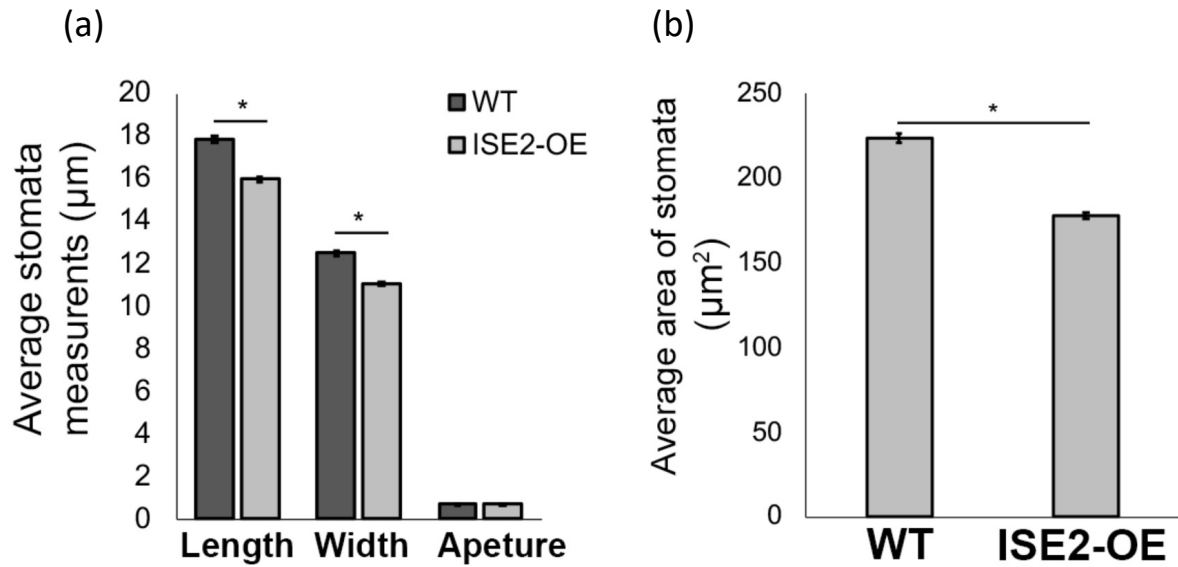


Figure 19 ISE2-OE plants have smaller than wild-type stomata.

(a) Average stomata measurements. lengths, widths, were measured with ImageJ and aperture was calculated by W/L. The average stomata area was calculated by W*L. Data represents two biological replicates. WT n=457 ISE2-OE n=740. Error bars represent standard error and significance $p < 0.05$ (*).

In order to determine if ISE2-OE plants display this same phenotype (plants have increased *TGG1* expression) we determined the days to bolting for WT and ISE2-OE. In ISE2-OE, the average day of bolting was 25 days vs 23 days for WT. Overall, the phenotypic data suggests that altering the level of ISE2 in the chloroplast results in phenotypes previously described in glucosinolate-related mutants (Zhang et al., 2019, Salehin et al., 2019, Bak et al., 2001). *TGG1* expression have delayed bolting (Zhang et al., 2019)

Quantification of glucosinolates in plants with varying ISE2 expression

To determine if the increased expression of genes involved in glucosinolate biosynthesis altered the actual biosynthesis of glucosinolates, we measured the specialized metabolites by high performance liquid chromatography. Aliphatic glucosinolates were increased in ISE2-SUP plants and indole glucosinolates are increased in ISE2-OE plants and decreased in ISE2-SUP plants (Figure 20a). Aliphatic glucosinolates 4MSOB and 4MTB were individually quantified and indole I3M and IMO-I3M glucosinolates were individually quantified. I3M is an indole glucosinolate that has not undergone secondary modification. ISE2-OE plants had an accumulation of I3M and a reduced accumulation in ISE2-SUP (Figure 20b). Overall, these data suggest that the balance of glucosinolates in ISE2-OE plants and in ISE2-SUP plants is altered. This imbalance correlates with the altered gene expression suggesting that there could be a potential signal

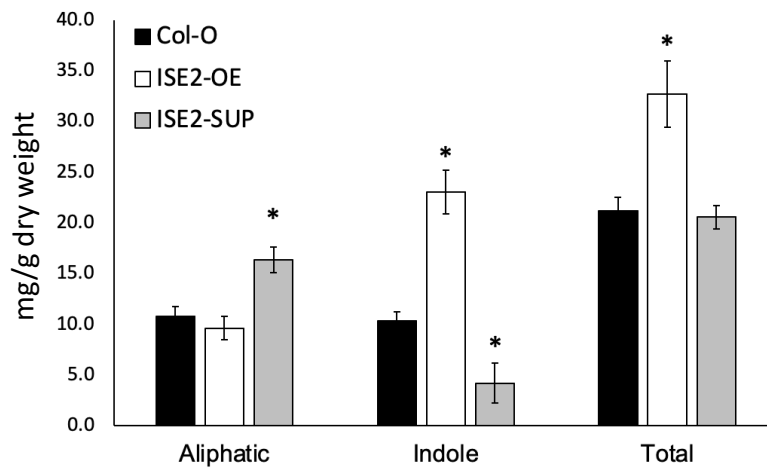
Figure 20 Glucosinolate accumulation imbalance in ISE2-OE and ISE2-SUP plants.

(a) Aliphatic and indole glucosinolates used as a proxy for total. These two glucosinolates make up the majority of glucosinolates in *Arabidopsis thaliana* Col-0. ISE2-OE plants have increased total glucosinolates.

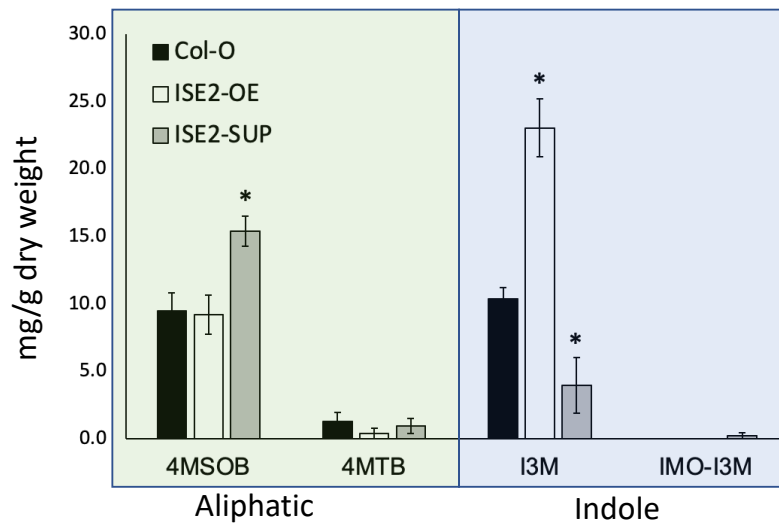
(b) Aliphatic glucosinolates 4MSOB and 4MTB were measured in 21-day old plants and Indole glucosinolates I3M and IMO-I3M were measured in 21-day old plants.

Col-0 N=3, ISE2-OE N=3, ISE2-SUP N=4. Error bars represent standard error and significance $p < 0.05$ (*).

a)



(b)



from the chloroplast altering expression of glucosinolate biosynthesis genes in the nucleus.

Glucosinolate-related mutants have defects in intercellular assays

In order to determine if intercellular trafficking was defective in glucosinolate mutants, we measured intercellular trafficking via plasmodesmata by implementing a movement assay by particle bombardment (Fernandez, submitted) (Figure 21). Representative images and a cartoon representation of a focus with two-layers of intercellular trafficking using the same color schematic from the data graphs are found in Figure 21. Wild-type plants have approximately 45% zero layers of intercellular trafficking, ISE2-OE plants have decreased intercellular trafficking, relative to wild-type with 55% of the foci having zero layers and an overall decrease in foci with 1 and 2 layers and a lack of foci with 3 layers. ISE2-SUP plants had increased intercellular trafficking with a decrease in zero layers and an increased number of foci with 2, 3, and greater than 3 layers (Figure 22a). The double mutant *cyp79b2, b3* nor does that line overexpressing CYP79B2 have changes in intercellular trafficking (Figure 22b). Single mutants *tgg1* and *tgg2* also had a slight increase in intercellular trafficking. Two of the three plants with increased *TGG1* expression (TGG1-OEL1, TGG1-OEL2, TGG1-OEL3) had increased trafficking (TGG1-OEL1 and TGG1-OEL3) (Figure 22c).

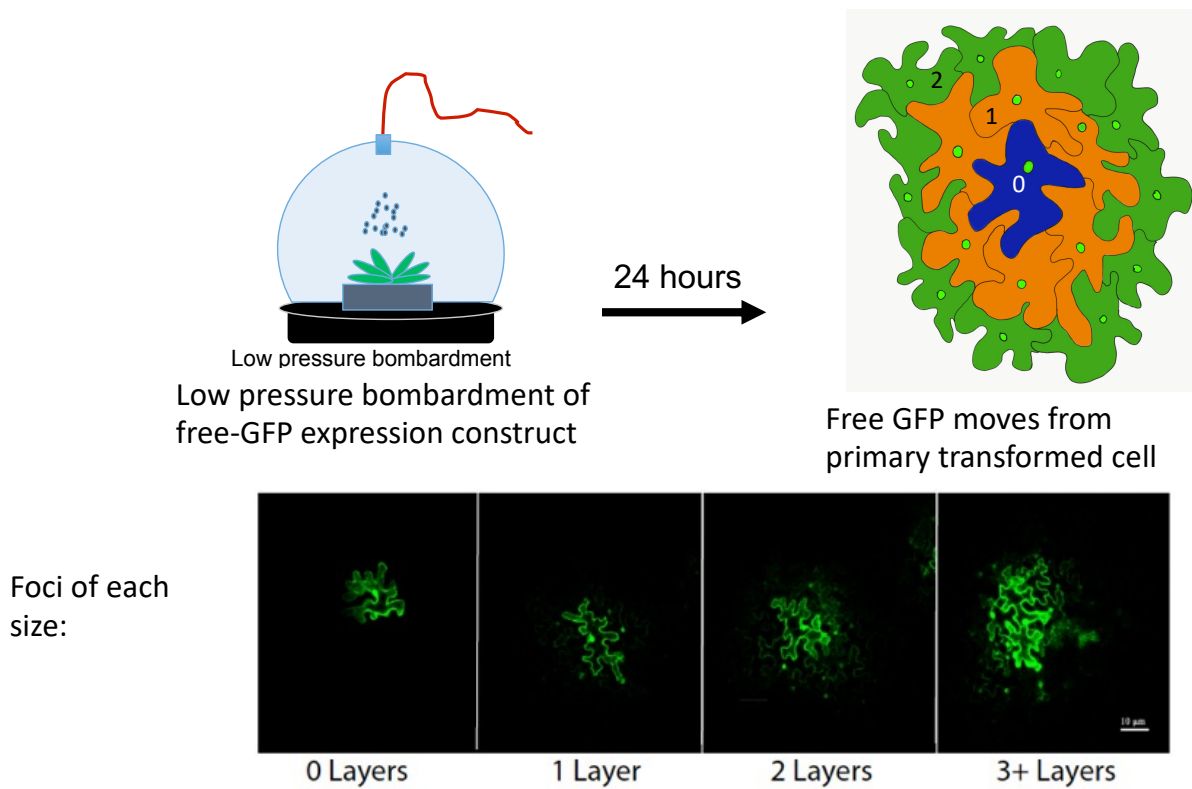
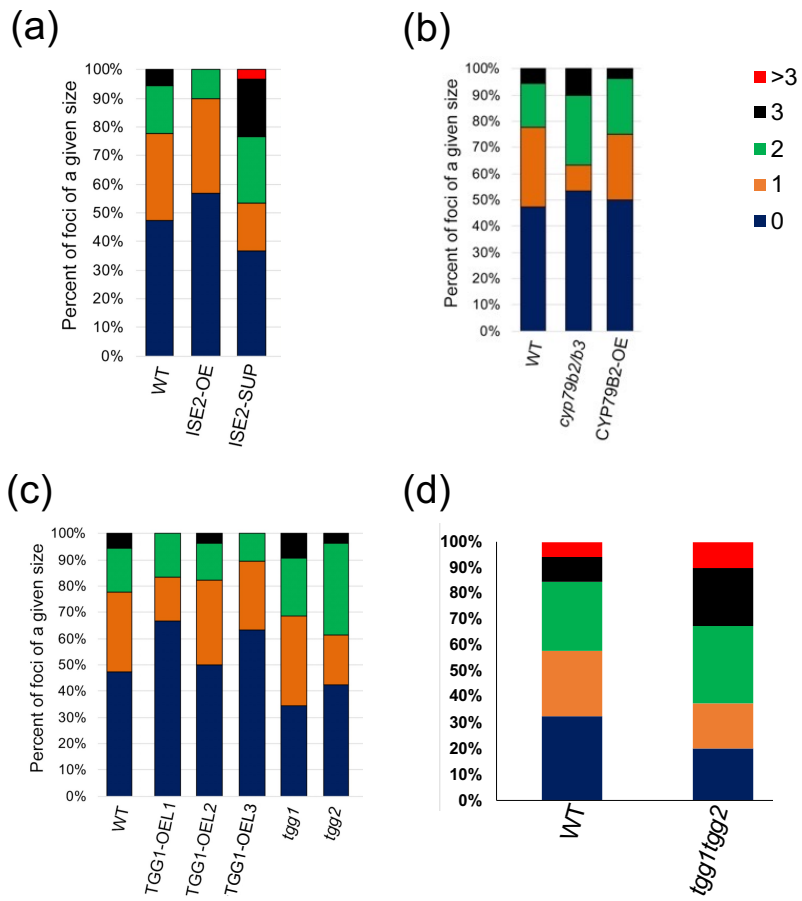


Figure 21 Schematic of the Movement Assay.

Arabidopsis plants are bombarded on a low-pressure bombardment system and observed after 24 hours with confocal microscopy. Individual transformed cells (blue cell label 0) are identified, and number of cell layers are tabulated (orange cell 1, green cell 2). The images below are examples of image stacks representing a focus of a given size.

Figure 22 Intercellular trafficking of 1x GFP via plasmodesmata

(a) As proof of concept, ISE2-OE plants have decrease intercellular trafficking and reduced *ISE2* in ISE2-SUP plants have increased intercellular trafficking. (b) Indole glucosinolate double *cyp79b2cyp79b3* mutant and CYP79B2-OE plants do not have major changes in intercellular trafficking. (c) Two independent lines overexpressing the myrosinase *TGG1* have a slight decrease in intercellular trafficking (L1, L3). (C) Single mutant *tgg1* and *tgg2* have increased intercellular trafficking. (d) Double mutant had a significant increase in intercellular trafficking, relative to WT plants. Significance of the increase in intercellular trafficking in *tgg1tgg2* mutants was determined by Mann-Whitney test and significance was at a $p < 0.05$.



The double mutant *tgg1tgg2* has been documented to have increased glucosinolate accumulation (Barth & Jander, 2006) and therefore, this mutant was used to determine if increased accumulation of glucosinolates correlates with changes in intercellular trafficking. Approximately 30% of the foci counted in wild-type had zero layers of movement, vs. 18% in the double mutant. The double *tgg1tgg2* mutant also had an increase in the number of foci counted with 3 layers and greater than 3 layers counted (Figure 22d). Overall, the data suggest that when plants have altered glucosinolate accumulation, plasmodesmata-mediated trafficking is disrupted.

Exogenous application of glucosinolates increase plasmodesmata-mediated trafficking

In order to determine if glucosinolates alone could change plasmodesmata flux, we treated *Arabidopsis* with exogenous sinigrin. Columbia-0 is naïve to allyl glucosinolates, such as sinigrin as it lacks 2-oxoglutarate-dependent dioxygenases2 (AOP2), the enzyme required for the biosynthesis of sinigrin (Neal et al., 2010). At very low concentrations, 0.01 μ M sinigrin concentrations, intercellular trafficking slightly increased (Figure 23). At 0.01 μ M sinigrin concentration we observed a phenotype often observed during these assays. There was a slight increase in the number of foci with zero layers and there was an increase in the number of foci with -3 and >3 layers, However, when the

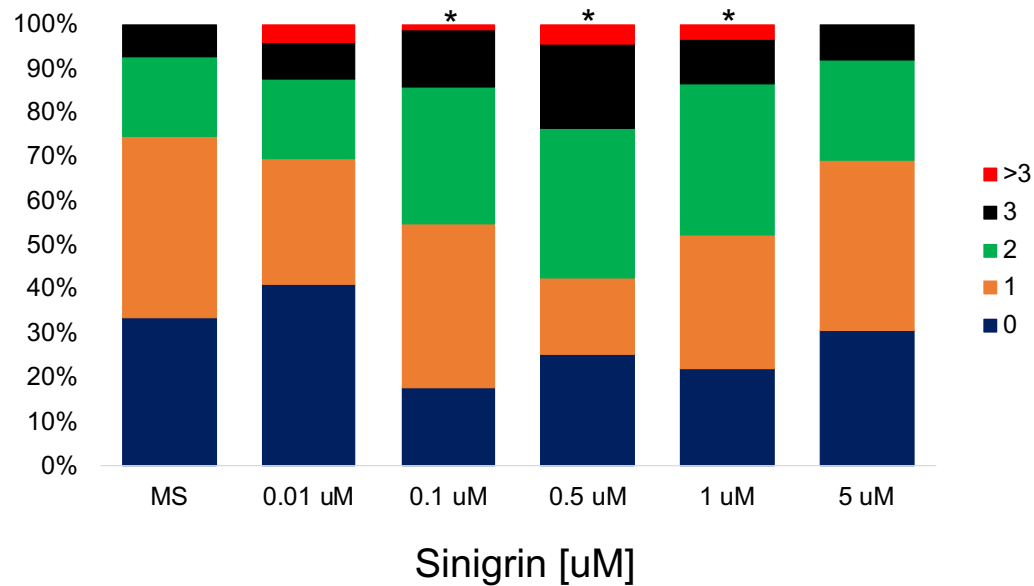


Figure 23 Exogenous application of the glucosinolate sinigrin alters plasmodesmata mediated trafficking.

Exogenous application of Sinigrin at the following concentrations: 0.01 μ M, 0.1 μ M, 0.5 μ M, 1 μ M, and 5 μ M. All conditions N=3, MS n=83, 0.01 μ M n=73, 0.1 μ M n=84, 0.5 μ M n=47, 1 μ M n=59, and 5 μ M n=62.

concentration of sinigrin was increased to 0.1 μM , 0.5 μM , and 1 μM the total number of foci with zero layers counted decreased to 15%, 22%, and 20% respectively. These two treatments also resulted in an increase in the number of foci with 3 or >3 layers of trafficking. Interestingly, when the concentration was increased to 5 μM concentration sinigrin, the intercellular trafficking looked very similar to that of WT (MS) control. Thus, intercellular trafficking changed in a dose-dependent manner.

Exogenous application of 4-hydroxybenzyl changes plasmodesmata mediated trafficking

In order to determine if other types of glucosinolates also mediated intercellular trafficking via plasmodesmata, we implemented the same assay with an aromatic glucosinolate (4-hydroxybenzyl). We used 0.5 μM and 5 μM concentrations, as these concentrations resulted in altered trafficking and wild-type levels in trafficking with Sinigrin. At the lower concentration of 0.5 μM concentration, intercellular trafficking was increased as the percentage of foci with zero layers of trafficking decreased from 33% in the control to 18% in the 0.5 μM 4-hydroxybenzyl treated leaves (Figure 24). Interestingly, the same restoration of WT (control) trafficking with 5 μM was not observed. The number of foci with zero layers were 19% however there was an increase in the numbers of foci with 1 layer and a presence of foci counted with more than three layers of trafficking.

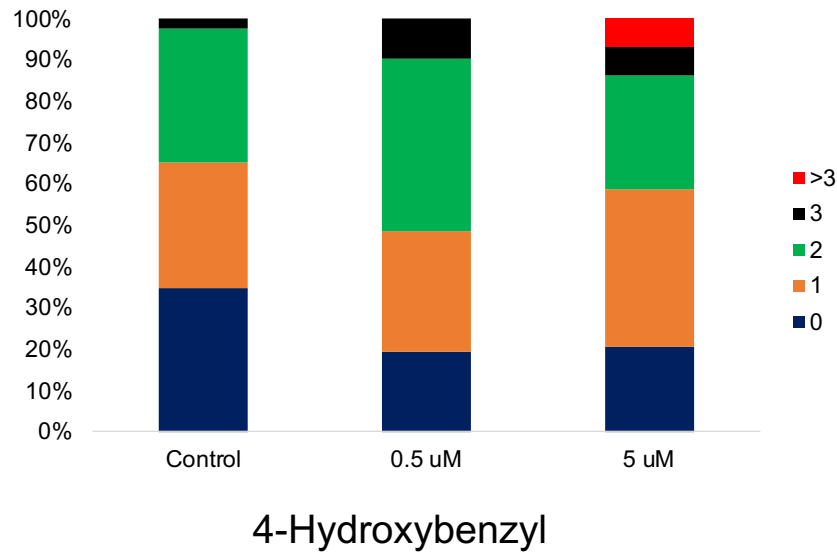


Figure 24 Exogenous application of the glucosinolate 4-hydroxybenzyl

Exogenous application of 0.5 μM and 5 μM 4-hydroxybenzyl. Control N=4, n=53.

0.5 μM N=2, n=31. 100 μM N=4, n=51.

**Exogenous application of Allyl isothiocyanate does not mediate
intercellular trafficking via plasmodesmata.**

In order to determine if the hydrolysis products of glucosinolates were responsible for the changes in intercellular trafficking we treated leaves with allyl isothiocyanate (ITC). The concentrations 0.5 μ M and 100 μ M were selected to represent one at the lower concentrations we observed with the glucosinolates and the higher 100 μ M to represent those concentrations that may be observed during leaf disruption. Although for both, 0.5 μ M and 100 μ M had reduced number of foci with zero layers of movement, the overall intercellular trafficking was similar to the WT DMSO control Figure 25. This suggest that the chemical compound affecting intercellular trafficking is not the breakdown product from sinigrin-allyl isothiocyanates-but the glucosinolates themselves.

Expression of sulfur metabolic pathway, MEcPP, and Auxin.

In an effort to identify if existing pathways are at play in regulating the nuclear gene expression of glucosinolates, we identified genes involved in the response of MEcPP, those involved in sulfonating glucosinolates (SOT16, SOT17, and SOT 18), any of the sulfur transferases involved in hormonal signaling. We also looked into genes involved in auxin biosynthesis and those in response to Salicylic acid and Jasmonic acid. *HPL*, the gene that is responsive to MEcPP

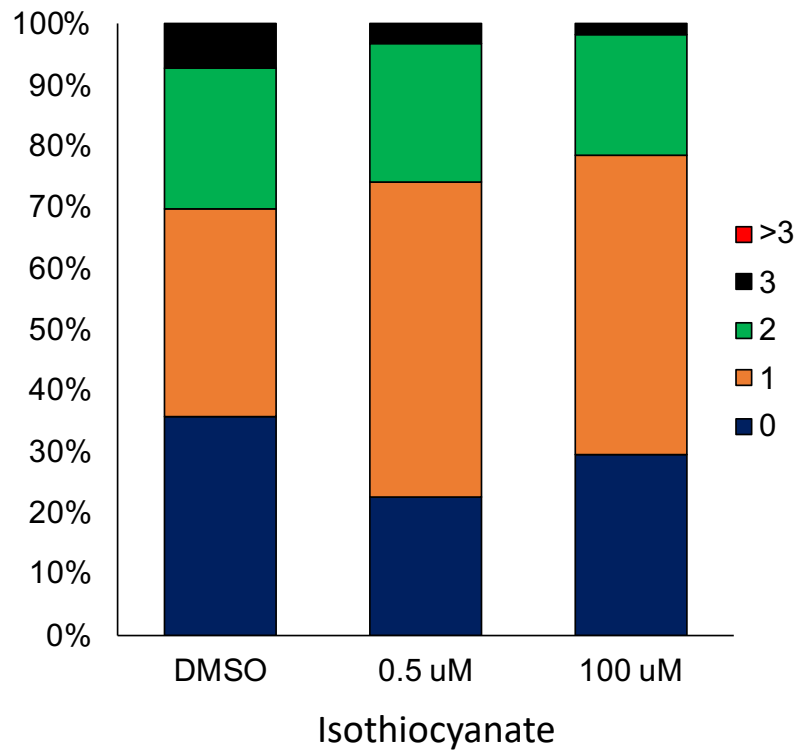


Figure 25 Exogenous application of the sinigrin derived isothiocyanate does not affect intercellular trafficking.

Exogenous application of the sinigrin derived isothiocyanate at the two concentrations, 0.5 μ M and 100 μ M. DMSO N=4, n=56, 0.5 μ M N=2, n=31. 100 μ M N=4, n=51.

was not misregulated, indicating that there is no accumulation of the metabolite.

To assure of this, we looked at the expression of other biosynthetic genes involved in the metabolism of MEcPP and found similar trends.

Utilizing the RNAseq data and microarray data, we do not find any differentially expressed genes involved in sulfur metabolism related to glucosinolate sulfation nor those in SAL1-PAP. Interestingly, we did find that the sulfotransferase involved in the sulfating brassinosteroids, SA, and flavonoids (SOT12) was suppressed, although the suppression was low (Log2FC -1 and -0.97 in ISE2-OE and ISE2-SUP, respectively). These results suggest an alternate pathway to activating glucosinolate biosynthesis.

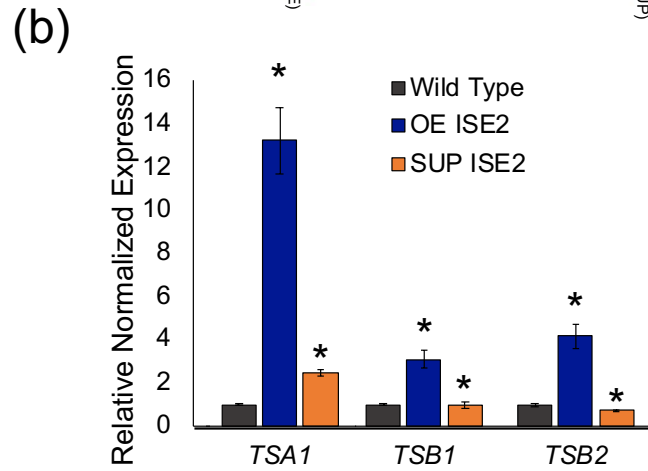
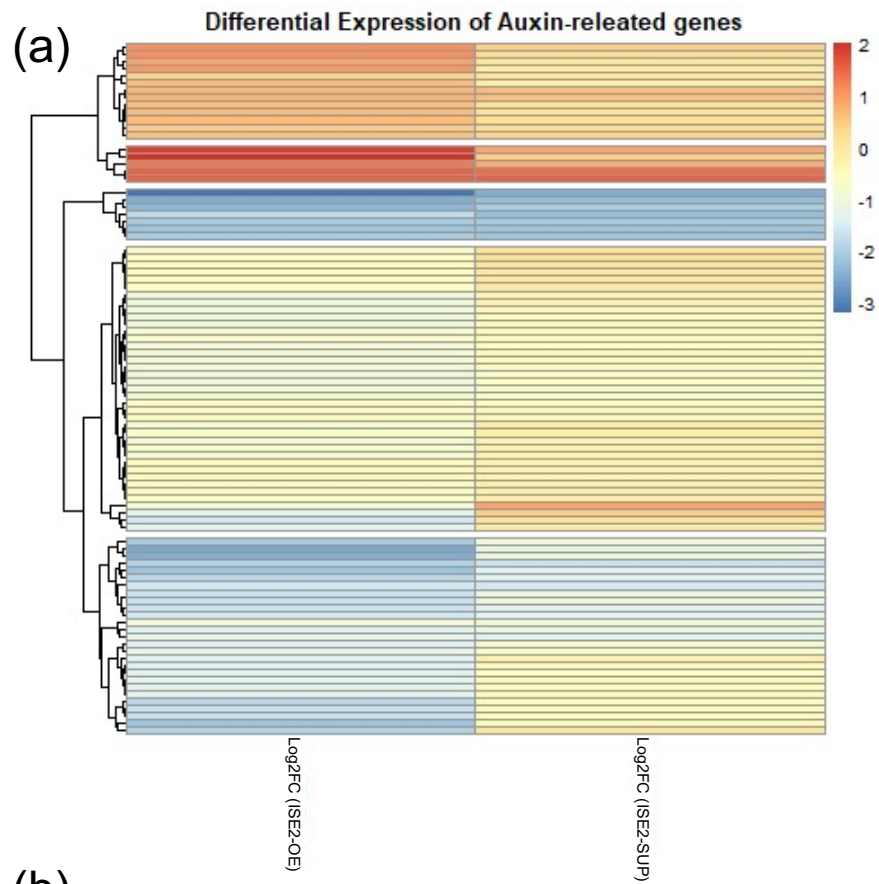
Genes related to Auxin-biosynthesis or response- were differentially expressed in ISE2-OE plants and in ISE2-SUP plants (Figure 26a). Upon further investigation, we observed that ISE2-OE plants have an induction in the genes responsible for Tryptophan synthesis, *TSA1*, *TSB1*, and *TSB2* (Figure 26b) the precursor to both Auxin and Indole glucosinolates. This suggests that there is an increased flux into tryptophan synthesis and potentially the indole glucosinolate pathway.

Discussion

When chloroplast perceive stress, this results in changes in secondary metabolism. For example, the *ceh1* mutant (constitutively expressing HPL) results in the accumulation of the metabolite and retrograde signal

Figure 26 Differential expression of Auxin biosynthesis related genes

(a) Heat map of differentially expressed genes in ISE2-OE and ISE2-SUP plants relative to Wild-type control from RNA seq analysis. (b) qPCR of genes responsible for the synthesis of tryptophan, precursor amino acid to both auxin and indole glucosinolates.



methylethylerythritol cyclodiphosphate (MEcPP) ultimately disrupting indole glucosinolate synthesis (Wang et al., 2017). Changes in photosynthesis associated nuclear genes (PhANGs) are misregulated in plants with decreased expression of ISE2, however, the signal responsible for these changes has not yet been identified. Multiple retrograde signaling pathways have implicated the roles of glucosinolate metabolism (Chi et al., 2015).

Here we have demonstrated that disrupting *ISE2* has resulted in changes in genes involved in glucosinolate synthesis and breakdown. However, many of the phenotypes observed, are oftentimes controlled by other hormones such as auxin. Interestingly, indole glucosinolates are closely linked to auxin since the first step in the synthesis of both auxin and indole glucosinolates utilizes a tryptophan and further, indole glucosinolates can be hydrolyzed by myrosinases to indole-3-acetonitrile (IAN) which then through the enzymatic activity of nitrilase be converted to indole-3-acetic acid (IAA) (Fu et al., 2016). The ISE2-OE line and the ISE2-SUP line has a number of auxin related phenotypes that have been reported in other glucosinolate mutants. However, due to imbalance of glucosinolate found in these lines, it is possible that two independent pathways have crossed ways. For example, the gain of function *CYP83B1* mutants have decreased apical dominance (Bak et al., 2001). Null mutants of *CYP83B1*, called *mt1-1* had a “runt” phenotype and also had delayed bolting, shorter plants and an increase number of rosette leaves. Further, complementing the *mt1* mutant with

the wild-type *CYP83B1* gene, resulted in more dramatic phenotypes. The complemented lines had an increase number of inflorescences, a reduced height, more bushy appearance and reduced seed set (Bak et al., 2001).

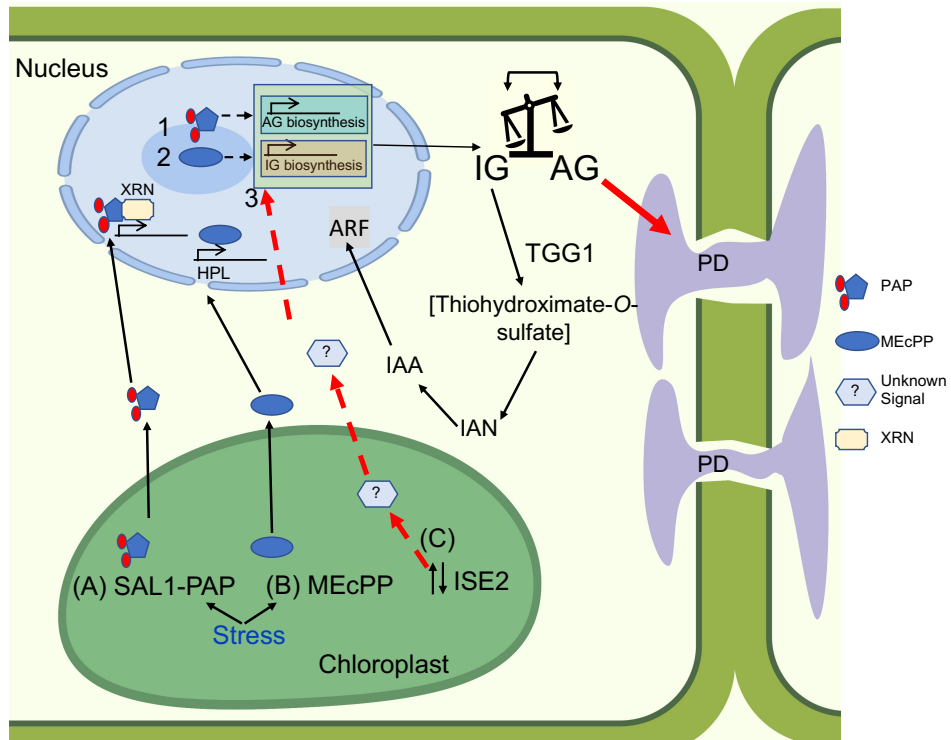
The relationships between glucosinolates and plasmodesmata remains unclear however, we have established that indeed a connection exists. ISE2-SUP plants also have an increased aliphatic glucosinolates and these plants have increased intercellular trafficking. Further we have demonstrated that the aliphatic glucosinolate sinigrin is capable in increasing intercellular trafficking. In parallel, ISE2-OE plants have increased indole glucosinolates and ISE2-SUP plants have decreased indole glucosinolates this is in similar pattern to their intercellular trafficking as well. ISE2-OE plants have decreased intercellular trafficking and ISE2-SUP have increased intercellular trafficking. Interestingly, we did not see such relationship in the indole glucosinolate mutants *cyp79b2cyp79b3* mutants indicating additional layers of regulation of Plasmodesmata.

What we have accomplished is establishing glucosinolates and its metabolism to plasmodesmata mediated trafficking. The chloroplast disruption by the overexpression of ISE2 has disrupted how plasmodesmata function and we predict it is through a signal or metabolite originating from chloroplast (Figure 27). Metabolic retrograde signaling is shown to alter nuclear gene expression and two pathways have links with glucosinolate biosynthesis. The two pathways are

Figure 27 ISE2 expression modulates the nuclear gene expression of glucosinolate biosynthesis and its relationship to PD.

(A) Inhibition of Sal1 under stress results in the accumulation of PAP. Transfer of PAP to the nucleus binds to exoribonuclease (XRN) and allows the gene expression of stress responsive genes (Chan et al., 2019). 1. SAL1-PAP where mutants accumulate aliphatic glucosinolates (AG) (B) The metabolite methylerythritol cyclodiphosphate (MEcPP) is accumulated in chloroplast and can act as a retrograde signaling molecule inducing stress responsive genes, including *hydroperoxide lyase (HPL)* (Xiao et al., 2012). 2. MEcPP induces biosynthesis of indole glucosinolates (IG). (C) ISE2-induced chloroplast changes signal to the nucleus by an unidentified signal. 3. Novel proposed pathway misregulating glucosinolate biosynthesis by the unknown signal originating from chloroplast.

The imbalance of glucosinolates results in aliphatic glucosinolates mediating plasmodesmata (PD) intercellular trafficking and indole glucosinolate metabolism shifting to the IAA/AUX pathway (Bak et al., 2001). IAN, indole-3-acetonitrile; IAA, indole-3-acetic acid; ARF, Auxin Response Factors. Red arrows signify findings from this dissertation.



SAL1-PAP, and 4. MEcPP (Chi et al., 2015) have been implicated in glucosinolate metabolism. However, the lines overexpressing ISE2 do not appear to have any defects in the genes involved in both SAL1-PAP nor in the MEcPP pathway. This suggest that there could exist a third mechanism of chloroplast retrograde signaling by an unknown signal that modulates glucosinolate biosynthesis and which could therefore modulate intercellular trafficking.

CHAPTER 4 DISCUSSION

Glucosinolates and Plasmodesmata

The research from this dissertation has shown a new and unique relationship of glucosinolates and their regulation on plasmodesmata. As mobile molecules, glucosinolates can move from cell-to-cell through plasmodesmata and can be found to accumulate at leaf margins. Here, we show for the first time that the exogenous application of an aliphatic glucosinolate, and not its degradation product, is able to regulate plasmodesmata. It is still unknown if the regulation is direct or indirect.

Chloroplast Retrograde Signaling

This dissertation explores the important role chloroplast retrograde signaling has on plasmodesmata mediated trafficking. At a basic fundamental level, general chloroplast RNA metabolism is found to alter plasmodesmata mediated trafficking (Bobik et al., Bobik et al., 2019, Ganusova et al., 2020). Ganusova, 2020 shows that silencing those proteins involved in RNA metabolism including INCREASED SIZE EXCLUSION LIMIT2 (ISE2), ISE2 PROTEIN INTERACTOR1 (IP1), polynucleotide phosphorylase (PNPase), RNA helicase22 (RH22) have unique intercellular trafficking and more striking have unique chloroplast retrograde signaling. Retrograde signaling in plants can arise from various processes including signals arising from photosynthesis, ROS production, or metabolic retrograde signaling from stress (Chi et al., 2015) their unique

pathways and their potential connection to plasmodesmata regulation has not been fully elucidated and this dissertation aims at identifying a unique role for ISE2.

ISE2 plays many roles in the chloroplast ranging from rRNA processing, cytosine to uracil editing, splicing, and the regulation of plasmodesmata mediated trafficking. Further, in evolutionary studies, ISE2 was one of the few non-photosynthesis associated proteins to be identified in the GreenCut2 study, where they identified essential “green” proteins across all organisms (Karpowicz et al., 2011). Therefore, we must look at the system used in this dissertation with a unique perspective. There is no situation in which the loss of ISE2 can exist in plants, however, using adult tissues with a loss of *ISE2* has provided us with a tool to tease apart these complex pathways that are absolutely necessary for chloroplast function.

Metabolic Retrograde Signaling

In this dissertation we have further identified a novel pathway linking *ISE2* with metabolic retrograde signaling. In our model, there are two pathways originating from the chloroplast that use a retrograde signaling metabolite for changing glucosinolate biosynthesis. The SAL-1-PAP pathway is tightly coordinated and linked with sulfur metabolism. Sulfur has many links with hormonal signaling, specialized metabolite formation, and defense (Chan et al., 2019). One of the final steps in the synthesis of the specialized metabolite glucosinolate is the

addition of sulfur from PAPS by sulfur transferases. Further, SAL-1-PAP mutants have a decreased accumulation in aliphatic glucosinolates (Ishiga et al., 2017) further an increase accumulation of aliphatic glucosinolates has been correlated with a high tolerance to salt stress (Martínez-Ballesta et al., 2015). Through an unknown mechanism, PAP, presumably in the nucleus, is able to modulate the expression of aliphatic glucosinolate synthesis. We hypothesized then that the increase in aliphatic glucosinolates could maybe be linked to sulfur metabolism and/or the SAL-1-PAP pathway but the data from this research shows that the increase in *aliphatic* glucosinolates that we observe in ISE2-SUP plants is not due to the SAL1-PAP pathway.

The chloroplast MEP pathway produces a retrograde signaling metabolite, methylerythritol cyclodiphosphate (MEcPP). MEcPP induces the expression of *HYDROPEROXIDE LYASE (HPL)*, a stress induced gene and generally used as a marker gene for stress response (Xiao et al., 2012). The accumulation of the metabolite MEcPP in chloroplast in the *ceh1* mutant (*constitutively expressing HPL*) induced the expression of indole glucosinolate biosynthesis (Wang et al., 2017). Similar to the above, ISE2-OE plants have *decreased* indole glucosinolates. We then hypothesized that maybe the MEcPP could be the signal in the ISE2 lines. However, qPCR analysis and RNA seq results suggest that indeed, the signaling altering glucosinolate synthesis in the chloroplast is through an unknown pathway (Figure 27 (c) and 3).

Auxin signaling linked to glucosinolates

When indole glucosinolates derived from tryptophan are hydrolyzed by myrosinases (TGG1 or TGG2), the IAA precursor IAN (indole-3-acetonitrile) is made. Through the action of two nitrilases, NIT1 and NIT2, IAA is formed. To enter this particular pathway, tryptophan is catalyzed by two Cytochrome P450 enzymes CYP79B2, and CYP79B3 to form Indole-3-acetaldoxime which through varying steps make IAA or enter the glucosinolate synthesis pathway. The Indole-3-acetaldoxime is catalyzed by a CYP83B1, considered a marker gene for this pathway. Mutants for this CYP83B1 have increased accumulation of IAA and have many phenotypes associated with auxin since the aldoxime has only one pathway to enter, the direct IAA pathway. It is interesting to note that in ISE2-OE, the expression of the CYP79B2 and CYP79B3 were significantly increased and further, the expression of the myrosinases was increased. These plants also had an increased in indole glucosinolates. We, therefore, suggest that the phenotypes observed in these plants, such “auxin” phenotypes, are actually phenotypes associated with an imbalance of indole glucosinolates. It is possible that the delay in bolting, increased adventitious roots and drought tolerance are auxin phenotypes but the origin cause is in fact the glucosinolates. A comprehensive study on the metabolites and hormones for ISE2-OE and ISE2-SUP is needed to tease apart the complex network at play.

Special Considerations and Care

In light of this information, the breakdown of glucosinolates should therefore always be considered when describing auxin phenotypes downstream of IAN. The complexity and close-knit association of the glucosinolate pathway and auxin IAA production is highlighted in (Bak & Feyereisen, 2001) where the crosstalk between the aliphatic glucosinolates and indole glucosinolates was closely investigated. Overexpressing the aliphatic CYP83A1 (producing aliphatic aldoximes) reduced the “auxin” phenotype of *cyp83b1* mutants, however, these two enzymes are not considered redundant as they have different affinities to substrates. There is enough overlap in auxin mutants and glucosinolate mutants that the Superroot2 (*sur2*) mutant was previously described in 1998 as an auxin mutant and it had increased levels of free IAA (Delarue et al., 1998). Later, it was found that the “auxin” mutant gene *SUR2* was allelic to the gene that encodes *CYP83B1*, the CYP responsible for the conversion of indole glucosinolates to aldoxime (Bak et al., 2001). How can we ensure phenotypes we are observing are strictly phenotypes associated to auxin? Due to Brassicaceae having glucosinolate pathways, it would be beneficial for future studies regarding free IAA or IAN to include an additional model system to ensure that the phenotypes are not accidental crosstalk from the specialized glucosinolate metabolism pathway.

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APPENDIX 1

A version of this chapter was originally submitted for publication by Jessica Fernandez and Tessa Burch-Smith:

Jessica C Fernandez, Tessa Burch-Smith. "Investigating plasmodesmata function in *Arabidopsis thaliana* using a low-pressure bombardment system and GFP movement assay". Methods in Molecular Biology, Plasmodesmata. (Not yet published chapter in book).

Summary

Plasmodesmata are nanopores in the plant cell wall that allow direct cell-cell communication, and they are key for plant growth, development and defense. However, studying these pores is challenging due to their small size, with diameters of 30 to 50 nm, and lengths that match cell wall thickness. One particular challenge is measuring how much cell-cell trafficking is facilitated by the plasmodesmata in a tissue or between particular cells. Here, we present an approach for studying plasmodesmata-mediated trafficking in the model plant *Arabidopsis thaliana* by using an easy to build and affordable low-pressure particle bombardment apparatus. Using low-pressure particle bombardment at around 60 psi, we are able to transform individual cells in the leaf epidermis and then study by confocal fluorescence microscopy the subsequent cell-to-cell trafficking of the diffusible molecule green fluorescent protein (GFP). This technique and this equipment could be used by any plant biologist to measure

intercellular trafficking through plasmodesmata under varying growth conditions including under stress, different light conditions, chemical treatments, or in various mutant backgrounds.

Key Words: Plasmodesmata, Green fluorescent protein, particle bombardment, gene gun, movement assay, intercellular trafficking, Arabidopsis, Agroinfiltration

Introduction

Plasmodesmata (PD) are cell wall pores that connect adjacent plant cells, thus allowing direct cell-cell communication and intercellular trafficking of molecules including metabolites, signaling molecules and ions. Many small molecules, such as metabolites and small ions move through plasmodesmata via simple diffusion. Some small proteins, like the green fluorescent protein (GFP), also traverse plasmodesmata by diffusion (Schonknecht et al., 2008, Liarzi & Epel, 2005). However, other proteins, exemplified by the WUSHEL (WUS) transcription factor and its mRNA, move in a directional manner via plasmodesmata from the “organizing center” to the outer layer of the meristem (Layer 1) indicating a more controlled and targeted movement (Daum et al., 2014).

Determining plasmodesmal flux is challenging because of the need to introduce a labeled molecule whose movement can be easily tracked between

cells without disrupting normal tissue and plasmodesmata function (reviewed in (Stadler et al., 2005)). One of the earliest techniques used to measure intercellular trafficking was microinjection of fluorescent small molecules and dyes into plant cells (Kragler, 2015, Lucas, 1995, Storms et al., 1998). Microinjection presented a number of problems including requiring a great deal of skill to master the technique. Further, the large vacuoles of plant cells rendered the technique extremely difficult since the dye needed to be delivered to the cytoplasm as this is the path for cell-to-cell diffusion, but often the dye was delivered instead to the large central vacuole. A recently developed technique, the “Drop-ANd-See” or DANS assay, utilizes carboxyfluorescein diacetate (CFDA) as a proxy to measure plasmodesmata permeability by measuring the spread of the dye (Li et al., 2016). CFDA is colorless until it enters cells where esterases cleave the diacetate from the carboxyfluorescein (CF) moiety, yielding a CF molecule that fluoresces after excitation by ultraviolet light (Wright & Oparka, 1996). The dye is applied to the abaxial side of the leaf and the size of the cell cluster containing CF on the adaxial side of the leaf is measured a short time later. An overall assessment of plasmodesmata mediated trafficking could be quantified using this assay. In the DANS assay, the overall diameter of the CF-containing cell cluster is quantified and, therefore, cell-to-cell spread quantification is not possible. CFDA can also be used to measure the import of the dye into the vascular tissue and measuring the movement and unloading into

sink tissues although this gives estimates of systemic spread and is more challenging for determining cell-to-cell trafficking (Ross-Elliott et al., 2017, Péron et al., 2017)

Other plasmodesmal flux studies have utilized free cytoplasmic GFP as a proxy for cell-to-cell spread. In *Arabidopsis* roots, GFP expressed from the *SUC2* promoter in vascular cells is commonly used to study unloading from the phloem into companion cells via plasmodesmata (Stadler et al., 2005, Fitzgibbon et al., 2013). While this model for measuring intercellular trafficking in roots is robust, studying plasmodesmata in *Arabidopsis* leaves remains challenging. Therefore, measuring the diffusion of GFP between cells in leaves would provide a good understanding of plasmodesmal function in leaf tissues. Monitoring intercellular trafficking by measuring the cell-to-cell spread of fluorescent proteins has been used with much success in the *Nicotiana benthamiana* model system (Burch-Smith & Zambryski, 2010, Bobik et al., 2019, Brunkard et al., 2020, Ganusova et al., 2020). Here, *Agrobacterium* carrying a *35S:GFP* construct can be infiltrated into leaves at very low concentrations, resulting in transformation of individual cells (Brunkard et al., 2015a). This approach has been coupled with virus-induced gene silencing (VIGS), drug treatments and varying growth conditions to understand how genetic and environmental perturbations impact intercellular trafficking (Ganusova et al., 2020, Brunkard et al., 2020, Brunkard & Zambryski). Unfortunately, the *N. benthamiana* genome is not yet well annotated and genetic

manipulation outside VIGS through the generation of stable transgenic lines is fairly uncommon.

Here we present a method to measure plasmodesmata-mediated trafficking using an easily constructed low-pressure bombardment system in the well-studied *Arabidopsis* model system. In this system, small metal particles are coated with plasmid DNA encoding GFP expressed from the 35S promoter and then shot into plant tissues under low pressure. Transformed cells express GFP that then diffuses into neighboring cells allowing quantification of intercellular trafficking, similar to the method used for trafficking assays in *N. benthamiana*. The large number of mutant lines available allow any researcher to study how a mutation of interest may affect intercellular trafficking. Commercial particle bombardment systems are expensive and the typical bombardment for plant transformation is performed around 1,000 psi. However, these high pressures can inhibit plasmodesmata-mediated trafficking by forcing closure of the pores (Oparka & Prior, 1992). Low-pressure bombardment ensures that plasmodesmata are not shut down (Crawford & Zambryski, 2000), and therefore is an excellent method to quantifying cell-to-cell trafficking in systems like *Arabidopsis* that are resistant to transient expression by *Agrobacterium* (Stonebloom et al., 2012). Another advantage is that the low-pressure bombardment system uses common lab materials and supplies readily available from university machine shops or facilities departments. Using this low-pressure

bombardment system, a variety of mutants, treatments and growth conditions can also be easily studied for their effects on plasmodesmata and intercellular trafficking. For example, the effects of various metabolites or hormones on plasmodesmata mediated trafficking could be measured. Here we offer methods for a single experiment with one wild-type *Arabidopsis* ecotype and a mutant of the same ecotype. The experiment should be replicated at least 3 times and should identify and count a minimum of 15 foci (individual transformed cells and surrounding cells into which GFP has diffused) per replicate.

Materials

PLANTS

Arabidopsis accessions Columbia (*Col-0*), Landsberg *erecta* (*Ler*), or Nossen (No-0) wild-type ecotypes.

Soil (any soil mix which provides adequate drainage and aeration)

Small 5 cm x 5 cm - inserts for flats

Cleaned and sanitized flats

Clear plastic humidity domes

Plant growth chambers

PLASMIDS FOR PARTICLE BOMBARDMENT:

E. coli competent cells for transformation.

Plasmid pRTL2rsGFP (Crawford & Zambryski, 2000) (see Note 1).

MEDIA

Luria-Bertani broth and plates containing 100 µg/ml carbenicillin for the growth of *E. coli* containing pRTL2rsGFP.

0.5x MS + 0.1% sucrose plant agar plates (see Note 2)

Low pressure particle bombardment

Modified plastic vacuum desiccator (see Note 3)

Aluminium disks (see Note 4)

Metal microcarriers for bombardment (tungsten is discontinued -1.0 µm gold microcarriers #1652263 from Bio-Rad are recommended)

MICROSCOPY

A confocal laser scanning microscope with a 25x lens. GFP excitation wavelength of 488 nm and emission between wavelengths 500 nm and 530 nm.

Glass microscope slides and cover slips

Single-edged razor blades

Image J image processing software

Methods

Construction of low-pressure bombardment system (Figure 28)

Modifying a vacuum desiccator by attaching a refrigerated vapor trap system and a vacuum pump creates the low-pressure

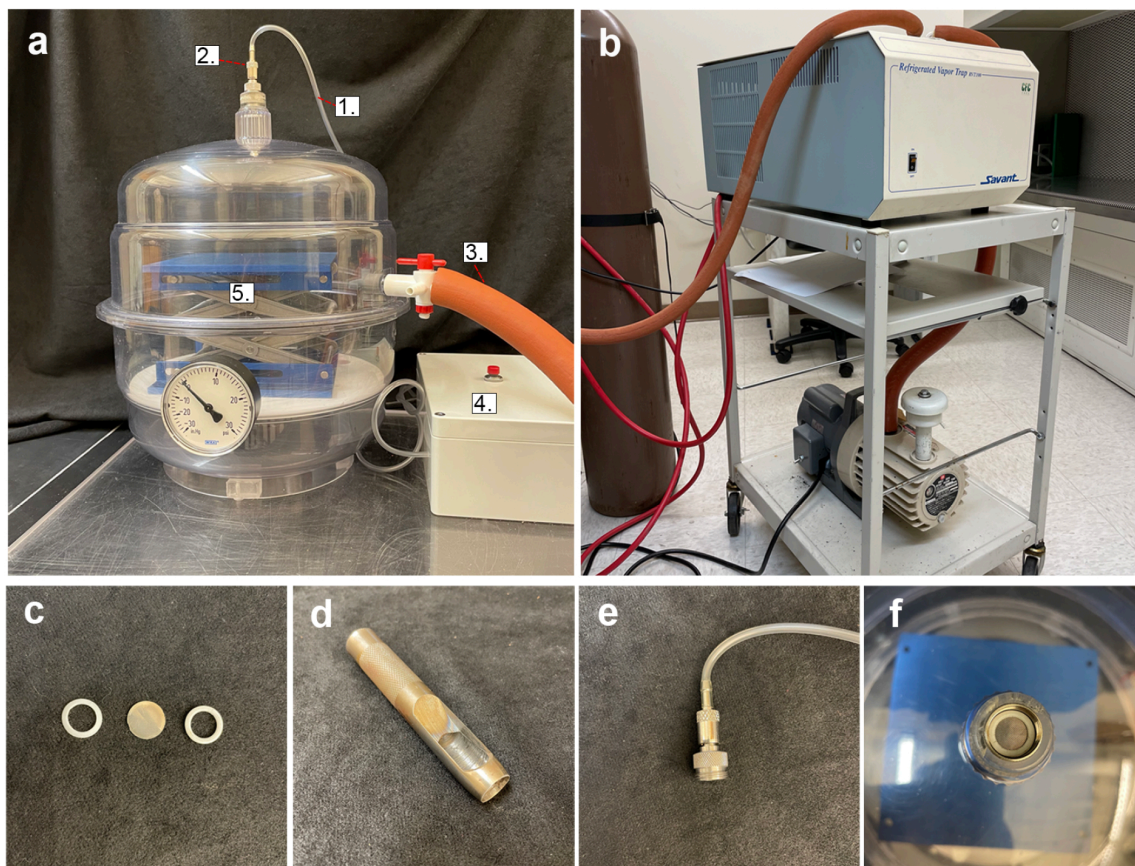


Figure 28 Bombardment apparatus.

(a) Modified vacuum desiccator with 1. 1/16 inch Tygon tubing, 2. Connection of Tygon tubing and a 1/16 inch x 10-32 hose barb, 1/8 inch npt 10-32 bushing secured with a pipe bulkhead, 3. Rubber vacuum rubber hose, 4. Gun switch (box), 5. Laboratory jack. (b) Refrigerated vapor trap and vacuum. (c) Flat silicone washer, mesh grid, and washer (from left to right). (d) Hole punch for bombardment aluminium disk. (e) Exit gun attachment. (f) Top down view of bombardment apparatus.

bombardment apparatus. Pressure is generated in the chamber with helium and the pressure is brought to 60 psi. Machine shops at universities can easily modify the vacuum desiccator.

The lid of the vacuum desiccator (Figure 28f) is re-tooled to include a DNA holder that consists of washers for holding the aluminium disks and mesh grids (Figure 28c). A hole is made on the top to fit a 1/8 inch npt 10-32 bushing secured with a pipe bulkhead. The gun connects to the bushing via 1/16 inch Tygon tubing and a 1/16 inch x 10-32 hose barb.

The gun can be assembled by the electronics facility in most departments. The gun consists of a timer circuit in which a momentary switch and attenuator that activates the pressured air release into the Tygon tubing for 1 second.

The vacuum (EW-06514-30, Bel-Art) is connected to a refrigerated vapor trap system (RVT400-115, ThermoFisher) via a vacuum rubber hose. A second hose from the vapor trap is then attached to the vacuum desiccator.

Plant Growth

Plant *Arabidopsis* seeds directly to soil then allow them to stratify at 4 °C for 2 days.

Move flats to a plant growth chamber under standard conditions of 16h day 22 °C, 8h night 20°C (light intensity of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Water from the bottom until soil is wet and then discard excess water.

Once plants are 3 weeks old, take leaves 5 and 6 from five plants (see Note 5) and carefully place them on 0.5x MS + 0.1% sucrose plate. Place 5-6 leaves per plate.

DNA Plasmid Preparation for particle bombardment and Tungsten preparation

Grow an overnight culture of *E. coli* containing the pRTL2rsGFP plasmid with 100 µg/ml carbenicillin. Isolate the plasmid using any plasmid isolation kit (example: QIAGEN cat: 27106).

Generate large amounts of plasmid and ensure a concentration greater than 300 ng/ul. Vacuum concentrate, if necessary, to achieve this concentration.

Wash 5 mg of gold or tungsten particles three times with 100 µl ddH₂O and centrifuge at 1,000 rpm for 1 minute. After the last wash resuspend in 80 µl ddH₂O and make 12.5 µl aliquots. 5 µg of plasmid pRTL2rsGFP DNA is added to the washed metal. Then add 12.5 µl of filter-sterilized 2.5 M CaCl₂, gently resuspend the mixture once, and then place onto a vortex shaker (level 2-3 on Fisher brand Vortex Genie). Quickly add 5 µl of 0.1 M spermidine (free base, freshly made and filter-sterilized), wait 30 seconds and finger vortex. Shake the suspension in the vortex shaker for 10 minutes. (see Note 6). After the ten minutes, remove the tubes and let them settle for 3-4 minutes. Centrifuge the tubes for 15 seconds at 5,000 rpm. Remove the supernatant and resuspend the metal in 100 µl 70% ethanol by finger vortexing (see Note 7). Allow the tubes to

settle (3-5 minutes) and centrifuge for 15 seconds at 5,000 rpm. Finally, remove the supernatant and resuspend by finger vortexing with 40 μ l 100% ethanol.

Macro-carrier assembly and low-pressure bombardment

Load each carrier with 3-5 μ l of DNA-coated tungsten and once the ethanol evaporates, it is placed DNA-side down onto the bombardment system.

Place the washers and grid in the DNA holder in the following order: flat washer, grid, and top washer (Figure 28c). The aluminium bombardment disk (with the DNA-coated microcarriers) rests on top with the DNA-coated side face down (Figure 29a inset).

Use a sharpened metal hole punch (Figure 28d) to make bombardment disks to a final diameter of 1.3 cm. Disks are made by layering aluminium foil and a thin paper towel to three layers total. The hole punches are done with a hammer and the disks are separated and then autoclaved in a dry cycle.

Place the plate containing the leaves 10.5 cm from the exit of gun (Fig. 30a) and align with the gun. The height can be adjusted and optimized using laboratory jack (Figure 29a). The vacuum hose is connected to the attachment (Figure 29a) and the vacuum is turned on until the gauge reaches 25 inches Hg. Once this pressure is reached, activate the particle gun twice (press button twice) per macro-carrier. Bombard each plate twice (with two macro-carriers) and shift the plate slightly from the starting position between bombardments to ensure transformation across different

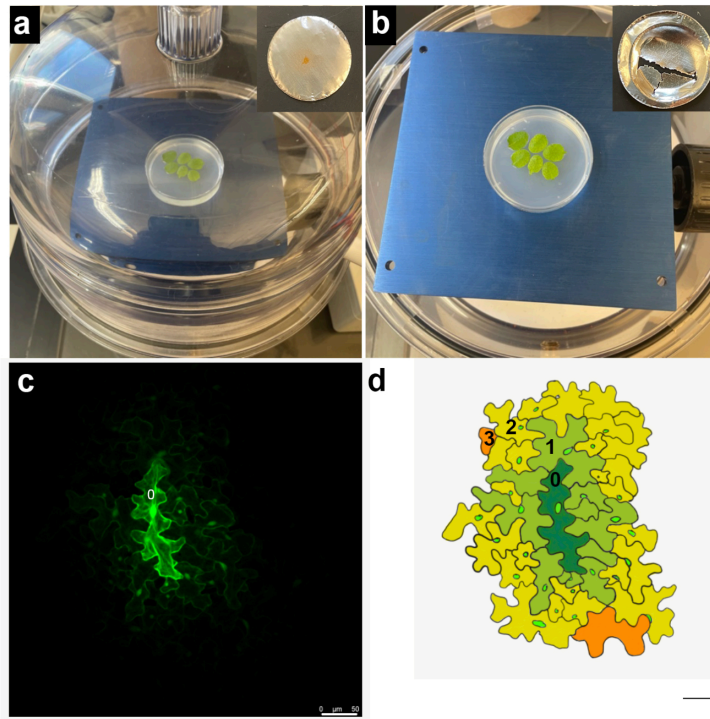


Figure 29 Bombardment of leaves on a plate and GFP movement assay

(a) MS plate with *Arabidopsis* leaves is aligned with the exit of the gun. Inset: Pre-bombardment aluminium disk with gold particles. (b) After bombardment, the plate should not appear much different than prior to bombardment. Inset: Disk ruptured by bombardment. (c) Primary transformed cell, labeled 0, has the brightest fluorescence in a focus as seen under the confocal fluorescence microscope. The GFP has trafficked into the neighboring cells with weaker fluorescence signal. (d) Cartoon depicting the three different cell layers surrounding the primary transformed cell in dark green (0). Layer 1 in lime green, layer 2 in yellow, and layer 3 in orange. Nuclei are in bright green. Scale bars are 50 μm .

leaves. The bombardment is performed under vacuum with 60 psi (helium). The leaves should not drastically differ from pre-bombardment appearance, with only faint marks from the microcarriers being visible (Figure 29b) while the bombardment aluminium macro-carrier is ruptured after the bombardment event (Figure 29b inset).

Allow the leaves to recover for 1 hour after bombardment on the same plate and then transfer to new plates as appropriate. The plates are placed into the growth chamber and imaged 24 hours post bombardment. The bombardment and the following imaging should be performed at the same point in the diurnal cycle for each replicate.

Measuring intercellular trafficking by confocal fluorescence microscopy

Take a leaf from the post-bombardment plate and remove the midrib with a single-edge razor blade. Place the remaining leaf tissue between a glass slide and coverslip and ensure the tissue is fully submerged in water. Image the fluorescent foci on the confocal fluorescence microscope with the 25X objective and with 488 nm excitation wavelength and collecting emission from 500-530 nm.

Single transformed epidermal cells expressing GFP are identified by their bright fluorescence (see Note 8) (Figure 29c, 29d). Once the primary GFP-expressing cell is identified, move up and down the z-plane and locate contiguous and neighboring cells containing GFP. Collect successive images in the z-plane to

generate a z-stack containing all the GFP-expressing cells in a cluster. The typical z-stack consists of 10-15 slices, however, due to the undulating nature of the leaf, the thickness of the z-stack will vary. This should be optimized for each focus to ensure enough detail in the images collected of the GFP spread.

Analysis of movement data

Using the Leica LasX software (<https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/>) or ImageJ (<https://imagej.nih.gov/ij/index.html>) (Schindelin et al., 2012), identify the primary transformed cell within a stack of images and count the layers of cells to which the GFP spread, taking caution to identify layers (Figure 29d) (see Note 8). Cell layers are counted as they represent discrete rounds of trafficking through PD. Besides cell layers, the total number of cells containing GFP from the primary transformed cell can also be counted.

Notes

Note 1: The plasmid concentration for bombardment needs to be above 350 ng/ μ l for adequate transformation efficiency. If the concentrations are below 350 ng/ μ l after the isolation procedure, the DNA can be concentrated by using a vacuum dryer to remove water.

Note 2: If the bombarded leaves are to be transferred to media with selective chemical or hormone treatments, fresh media should be made the day of bombardment, unless the hormone or chemical does not degrade.

Note 3: Bombardment system and conditions should be optimized prior to performing experiments.

Note 4: If the aluminium disks are made in house, use regular aluminium foil and not heavy duty aluminium foil. Heavy duty aluminium foil will not burst during the bombardment event and therefore not transform the leaves. The aluminium foil is purchased from a regular supermarket or local store.

Note 5: For reproducibility and consistency, leaves of similar age and size should always be used. This will ensure that any variability in movement is not due to developmental differences between leaves.

Note 6: During the ten-minute incubation, the refrigerated vapor trap should be turned on and allowed to run for about 30 minutes total prior to bombardment.

Note 7: The metal at this point should have a very silky appearance and there should be no clumping. If there are clumps, do not use the metal-this will

cause severe damage to bombarded tissue and will not give reliable movement data. Finger vortexing the sample ensures that the metal will not aggregate but instead fully mix.

Note 8: It is critical to find isolated transformed cells. One easy way to identify the transformed cell is to observe the fluorescence. Typically, a single transformed cell will consist of one cell brightly expressing GFP (Figure 29c labeled “0”) and the GFP fills the nucleus and cytoplasm. Neighboring cells into which the GFP has diffused will be dimmer with less fluorescence in the cytoplasm and nucleus. In some cases, the GFP has spread more to the mesophyll layers than the neighboring epidermal layers and this could be quantified as well, as it represents an increase in trafficking through secondary plasmodesmata (those plasmodesmata formed across established cell walls). Further, some cells extend further than others and counting layers needs to be done more cautiously (Figure 29d labels “2 and 3”). In addition to layers, one can quantify the numbers of cells in which the GFP diffused as a metric for quantification of spread.

APPENDIX 2

A version of this chapter was originally submitted for publication by Jessica Fernandez and Tessa Burch-Smith:

Fernandez JC, Burch-Smith TM: Chloroplasts as mediators of plant biotic interactions over short and long distances. *Current Opinion in Plant Biology* 2019, 50:148-155 <http://dx.doi.org/10.1016/j.pbi.2019.06.002>.

Abstract

In nature, plants interact with numerous other organisms. Some interactions benefit both the plant and the other organism(s), while others lead to disease or even death of the plant hosts. The traditional focus of research into plant biotic interactions has been on the negative effects on plants and the strategies plants use to mitigate or prevent these. Over the last several years there has been increasing appreciation for the diversity and importance of plant biotic interactions in plant success as well as the evolution and stabilization of ecosystems. With this new perspective, it is also becoming clear that the metabolic output of chloroplasts in plants is critical to establishing and maintaining these interactions. Here we highlight the roles of chloroplasts in diverse biotic interactions. Photosynthetic chloroplasts are the source of hormones, small molecules and a prodigious number of specialized metabolites, a significant portion of which influence plant biotic interactions. Importantly, the effects of chloroplasts on these interactions are not limited to sites of direct

association or contact but also act at a distance in systemic leaves and roots, in the rhizosphere, in the air surrounding a plant and in neighboring plants, and they can persist over several years.

Introduction

Plants are invaluable sources of food and shelter for microbes, nematodes, insects and various animals and even for other plants. Interactions with some bacteria, fungi and nematodes can disrupt plant growth and development, leading to disease. Other interactions with bacteria and fungi can benefit the plant, leading to enhanced growth and better resilience in challenging environmental conditions (Reviews in this special issue to be cited). Parasitic plants can deplete their host of nutrients leading to stunted growth and development. Plants are not passive participants in these interactions and they produce a wide array of signaling and bioactive molecules that can help determine the outcome of a given interaction. These compounds can act locally or can be released into the soil, transported to distal tissues to warn of the infection or even secreted into the air to influence insect feeding and behavior or to signal to neighboring plants. Most of these compounds are either synthesized in plastids or are made from precursors generated by plastids.

Plastids are metabolic hubs that generate numerous molecules essential for plant growth and responses to the environment. Chloroplasts are the specialized

plastids of leaves that carry out oxygenic photosynthesis, fixing inorganic carbon into carbohydrates with the release of oxygen into the atmosphere. Chloroplasts and other plastids also synthesize amino acids, many vitamins and a plethora of specialized metabolites (Maeda & Dudareva, 2012). Chloroplasts are also the source of numerous small signaling molecules including the plant hormones salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA), all crucial players in determining the outcomes of plant biotic interactions (Preece et al., 2018). The *de novo* synthesis of fatty acids also occurs in the chloroplast. The lipid hormone JA is among the best-known players in plant responses to microbial infection, and numerous other lipid-derived molecules also act as signals during plant biotic interactions (Lim et al., 2017).

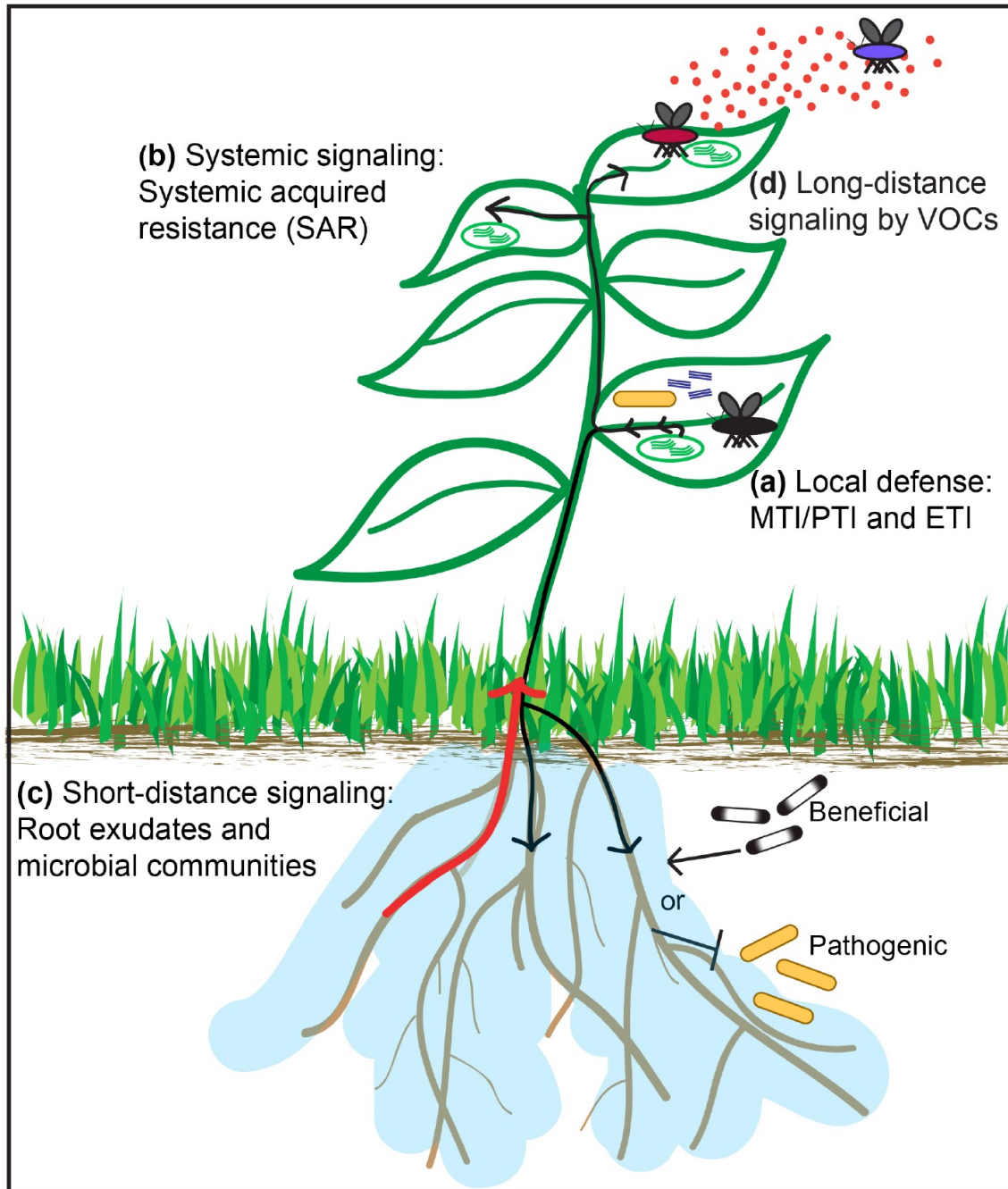
In this review, focusing on findings from the last two years, we highlight the roles of chloroplast-derived signals in shaping how plants defend from potentially harmful organisms and how other chloroplast metabolites and signals can produce and influence beneficial biotic interactions. Our discussion draws attention to the physical and temporal distances at which chloroplast signals act, further evidence of the importance of this organelle for plant survival. We also briefly discuss the impact a changing environment will have on chloroplast biology and its implications for plant biotic interactions.

Chloroplasts are a source of hormones and signaling molecules that act locally

Microbes first encounter the cuticle of leaves or bark of stems if interacting with the aerial parts of the plant. If associating with roots, then first contact would be with plant products in the form of root exudates before reaching the root epidermis. In both instances, the waxy cuticle and plant cell wall would serve as a barrier to microbial access to the protoplast contents (Wang et al., 2018, Ziv et al., 2018). Once the cell wall is breached, the microbe must then contend with diverse suites of cellular defense mechanisms that depend on both the plant host and on microbial surface molecules or secreted small molecules like effector proteins. One strategy for plant defense includes cell-surface-localized receptors that recognize conserved microbe or pathogen-associated molecular patterns (MAMPs or PAMPs) (Figure 30). These cell-surface receptors then initiate defense signaling leading to a suite of responses whose goal is to ultimately limit the overall infection. This plant recognition and response is called MAMP- or PAMP-triggered immunity (MTI or PTI). If a microbe overcomes MTI or PTI and gains entry into a cell, it will release effector proteins that specifically recognize and modify the behavior of target host proteins. These targets are often involved in triggering or mediating plant defense responses. Again, plants encode receptors (R proteins) that recognize these effectors and initiate defense responses, a process called effector triggered immunity (ETI). Both MTI and PTI

Figure 30 Chloroplast products in interactions with microbes across time and space.

(a) At sites of microbial ingress or herbivore feeding, chloroplast products including the phytohormones SA, JA and ABA, reactive oxygen species (ROS) and signals that modulate nuclear gene expression are produced in relatively short time frames of minutes to days and act locally. **(b)** Systemic signals induce defense at sites away from primary infections and result in systemic acquired resistance (SAR). The chloroplast products SA, G3P, DA, and AzA are systemic signals that are involved in SAR. SAR is activated within days of primary infections and can persist for several weeks. **(c)** Root exudates in the rhizosphere contain numerous chloroplast metabolites including sugars and amino acids. Root exudates help shape the microbial communities of the rhizosphere and these in turn initiate ISR in the plants, with effects that can persist for years. **(d)** Chloroplasts metabolism produces numerous VOCs including terpene and lipid derivatives. These can signal systemically to the plant of origin or can signal to neighboring plant up to 1 meter away to prime defense responses. This can give population-level resistance to infection. VOC production is induced by herbivory, and the VOCs can act to recruit predators that feed on the herbivores.



are very well studied and their general mechanisms have been described (Bigeard et al., 2015, Peng et al., 2018). Studies on MTI and ETI have been crucial in revealing the importance of chloroplasts to plant defense.

As part of general stress responses and during interactions with microbes chloroplasts produce numerous reactive oxygen species (ROS) that mediate redox flux leading to changes in nuclear gene expression and activation of downstream defense responses (Preece et al., 2018, Rossi et al., 2017). Other chloroplast metabolites act as retrograde signals during defense to regulate nuclear gene expression (Sowden et al., 2018). Methylerythritol cyclodiphosphate (MEcPP) is an intermediate of the chloroplast methylerythritol phosphate (MEP) pathway that generates isoprenoid precursors for terpenes, carotenoids and tocopherols. During stress MEcPP accumulates and acts as a retrograde signaling molecule. The Arabidopsis *ceh1* mutant that cannot convert MEcPP to hydroxymethylbutenyl diphosphate (HMBPP) accumulates MEcPP, eventually resulting in the constitutive production of salicylic acid (SA) (Xiao et al., 2012), but increased induction of JA-responsive genes (Lemos et al., 2016). Systems-level analyses have resolved this paradox of *ceh1* plants by revealing that JA induction is due to MEcPP signaling and does not involve SA (Bjornson et al., 2017). Another mediator of chloroplast retrograde signaling during stress is 3'-phosphoadenosine 5'-phosphate (PAP) (Estavillo et al., 2011). The *SAL1* gene encodes a phosphatase that regulates the accumulation of PAP. In Arabidopsis

sal1 mutants SA- and JA-mediated defense signaling was compromised, SA levels were lower than in wild type plants, and plants were hypersusceptible to multiple pathogens (Ishiga et al., 2017). The induction of the sulfur-containing glucosinolate defense molecules in response to *P. syringae* pv. *tomato* DC3000 infection was also compromised in *sal1* plants. Additional support for the notion that chloroplasts are critical for defense comes from the revelations that many microbe-derived effectors target chloroplasts or chloroplast-localized proteins (Bobik & Burch-Smith, 2015, Park et al., 2018). Intriguingly, proteins that seemingly have no obvious role in defense are also effector targets, including components of the photosynthetic electron transport chain (reviewed in (Preece et al., 2018, Bobik & Burch-Smith, 2015)). This indicates that even basal chloroplast functions make contributions to defense.

Chloroplast products often act systemically to regulate plant-microbe interactions

Beyond their roles in limiting microbe ingress at the primary infection site, ETI and PTI have systemic components. Systemic acquired resistance (SAR) is initiated by ETI and it renders parts of the plant distal from the site of infection resistant to secondary infections (Shah et al., 2014). SAR is mediated by several chloroplast-generated molecules including SA, the SA derivative methyl salicylate (MeSA), the oxylipin azelaic acid (AzA), glycerol-3-phosphate (G3P),

and dehydroabietinal (DA), an abietane diterpenoid (Shah et al., 2014). The lysine catabolite pipecolic acid (PIP) is also an important mediator of SAR (Navarova et al., 2012). The inducer of SAR has recently been identified as N-hydroxy-pipecolic acid (N-OH-Pip or NHP) that is generated from PIP by the action of FMO1 (flavin-dependent-monooxygenase 1) (Chen et al., 2018, Hartmann et al., 2018).

Importantly, N-OH-Pip and its derivatives were detected in systemic leaves after inoculation of local leaves of the *fmo1* mutant (Chen et al., 2018). Since *fmo1* plants cannot synthesize N-OH-Pip, its presence in systemic leaves suggested that N-OH-Pip and/or its derivatives are mobile. This is consistent with results for SA, Aza, G3P and DA that show that these molecules can move systemically from sites of infection to distal parts of the plant where they act non-cell-autonomously to confer SAR (Shah et al., 2014). SA is transported through the apoplast while Aza and G3P use the symplastic route provided by plasmodesmata (PD) for systemic spread (Lim et al., 2016). The transport route for N-OH-Pip or its derivatives has not yet been described (Chen et al., 2018). Interestingly, while Aza moves from infected to systemic leaves, it does not move systemically from Arabidopsis roots to shoots when applied exogenously although it induced systemic resistance (Cecchini et al., 2019). This finding

suggests that there may be important differences between root- and shoot-produced Aza in inducing SAR.

Control over production and distribution: plasmodesmata and chloroplasts

Organelle-nucleus-plasmodesmata signaling (ONPS) describes a model that places chloroplasts as regulators of plasmodesmata (PD), either via retrograde signaling to the nucleus to control expression of PD-related genes, or by direct signaling to PD (Burch-Smith et al., 2011a, Brunkard & Burch-Smith, 2018). This model is based on the observation that plants with defects in chloroplast function may exhibit defects in intercellular trafficking mediated by plasmodesmata (Bobik et al., 2019, Bobik et al., 2017, Carlotto et al., 2016, Benitez-Alfonso et al., 2009)

PD are plasma membrane-lined pores that connect the cytoplasms of neighboring cells. In a plant, most cells are connected to at least a few of its neighbors by PD. Thus, the PD can be thought of as a superhighway for the intercellular movement of water, small molecules, metabolites and signaling molecules (Sager & Lee, 2018). Not surprisingly then, PD and intercellular trafficking are regulated and are subject to control by developmental and environmental signals (Sager & Lee, 2018). PD are also crucial players in pathogen defense (Ganusova & Burch-Smith, 2019). Indeed, PD provide the symplastic route for the trafficking of SAR-related and other signaling molecules

(see above). The PD-localized proteins PDL1 and PDL5 are both required for SAR (Lim et al., 2016, Carella et al., 2015), albeit through differing mechanisms (Lim et al., 2016). Overexpression of *PDL5* reduced PD-mediated trafficking (Lee et al., 2011, Lim et al., 2016) and also induced SA-mediated defense responses (Lee et al., 2011). The link between PD and plant defense is supported by data showing that plants with defects in intercellular trafficking have compromised resistance to virus infection (Ganusova et al., 2017). Interestingly, the defects in intercellular trafficking reported by Ganusova et al. were due to changes in expression of a chloroplast RNA helicase, ISE2 (Ganusova et al., 2017). ISE2 is important for the processing of chloroplast RNAs. Numerous aspects of chloroplast gene expression are compromised when ISE2 expression is reduced including chloroplast ribosomal composition culminating in altered chloroplast protein accumulation (Bobik et al., 2017). These defects in gene expression trigger chloroplast-to-nucleus retrograde signaling (Chan et al., 2016). Results from work with ISE2 thus support the ONPS model where changes in chloroplast gene expression can modulate PD function and suggest that this may be accomplished through retrograde signaling to the nucleus to regulate expression of PD-related nuclear genes (Brunkard & Burch-Smith, 2018). Thus, chloroplasts control both the production and distribution of signaling molecules for systemic communication, giving chloroplasts exquisite control over responses to microbes.

Even longer range signaling: chloroplasts shape the rhizosphere

The interactions between plants and soil microbes and nematodes, especially those associated with roots, have been investigated for their potential use in pharmaceuticals (Lica et al., 2018), agriculture (Hu et al., 2018) and even rhizoremediation of contaminated soils (Kuiper et al., 2004). Plant roots secrete numerous metabolites into the surrounding soil (rhizosphere) to generate a complex mixture referred to as root exudate (Sasse et al., 2018). Many of the metabolites found in root exudates and those involved in plant defense, are either fully or partly synthesized in the chloroplast. Exudates are divided into two classes: low-molecular weight and high-molecular weight compounds. Low-molecular weight compounds include sugars like glucose and fructose (Hoysted et al., 2018), amino acids including tryptophan (Sasse et al., 2018), organic acids like malic acid (Sasse et al., 2018), and phytohormones including strigolactones (Kretzschmar et al., 2012). High-molecular weight compounds include polysaccharides and proteins (Bertin et al., 2003). Indeed, plants may contribute as much as 20% of their total fixed carbon to root exudate (Venturi & Keel, 2016). Those metabolites, when excreted into the soil, shape the type of bacteria or fungi that can grow in the vicinity of roots and also determine which microbes colonize the roots (Venturi & Keel, 2016, Hoysted et al., 2018). If a potential colonizer is a pathogen, the plant may secrete antimicrobial indoles, antifungals,

or nematicidal compounds (Lombardi et al., 2018, Martin-Mora et al., 2018, Li et al., 2018). When Welsh onion was grown with cucumber as a companion plant, the hatching of root-knot nematodes of cucumber plants was greatly reduced (Li et al., 2018). Exogenous application of 4-hydroxy-benzeneethanol, identified as the predominant component of onion exudate, reduced the hatching of the root-knot nematodes and was lethal to J2 nematodes. In a split-root system, tomato plants exposed to various biotic stresses secreted chemoattractants to recruit a beneficial fungus to their roots (Lombardi et al., 2018). Thus, plants respond to pathogen attack by creating a hostile environment and to mutualists and symbionts by creating a non-hostile environment. Defense compounds found in root exudates include isothiocyanates (ITC), the breakdown products of glucosinolates (Strehmel et al., 2014), and indole-derived metabolites collectively called benzoxazinoids (BXs) (Hu et al., 2018).

Induced systemic resistance (ISR) is another systemic plant response to microbial colonization. ISR is established when, in response to infections of the shoot, root exudates induce colonization by plant growth promoting rhizobia (PGPR) and there is subsequent activation of systemic defense against shoot pathogens (Pieterse et al., 2001, Yuan et al., 2018). For example, *Pseudomonas syringae* pathovar *tomato* (*Pst*) infection of *Arabidopsis* leaves resulted in soil-borne resistance or “soil memory” in the roots. *Arabidopsis* grown in soils where “memory” had been established by infecting five consecutive generations of

plants with *Pst* had higher resistance to *Pst* (Yuan et al., 2018). It was concluded that the five generations of *Pst*-infected plants produced root exudates that remodeled the root microbial community and led to ISR in the plants tested. Root exudates from the *Pst*-infected *Arabidopsis* were characterized and the exogenous application of the identified metabolites, specifically long-chained organic acids (LCOAs) and amino acids (AAs), was sufficient to induce similar soil-borne resistance to that observed from the *Pst* infection. Importantly, soil that was free of microbes but contained exudates did not confer resistance, indicating that resistance was due to the microbial community and not the exudates (Yuan et al., 2018). In another example, maize plants where ISR was activated by the beneficial root fungus *Trichoderma atroviride*, levels of defense phytohormones were found to be altered in silks infected by the fungal pathogen *Fusarium verticilloides* (Agostini et al., 2019). Most prominently SA levels were significantly reduced likely due to increased conversion of SA to MeSA as suggested by gene transcription analysis. The authors suggest that SA metabolism is central for signaling, both local and systemic, during *F. verticilloides* infection of the silks undergoing ISR (Agostini et al., 2019). Thus, by contributing metabolites and defense molecules to the rhizosphere in root exudates, chloroplasts are able to shape the long-distance signals important for plant-biotic interactions.

Volatiles-the perfume of chloroplast: acting at even greater distances

Chloroplasts also produce a variety of volatile organic compounds (VOCs). These VOCs are products of the MEP pathway like terpenoid and carotenoid derivatives, and the fatty acid derivatives like aldehydes and alcohols (Bobik & Burch-Smith, 2015). Recent analysis of VOCs from 12 different Brassicaceae vegetables identified volatiles generated from glucosinolate metabolism in addition to those from MEP and fatty acids (Liu et al., 2018). The metabolites and volatiles found among the 12 species varied greatly (Liu et al., 2018). Although glucosinolates are not volatile, some of their isothiocyanate products are (Dufour et al., 2015).

VOCs produced by a given plant can act to direct systemic signaling or they can signal to plants about a meter away (Benevenuto et al., 2018). VOC production is often induced by stress and infection or predation, and these VOCs can prime defense responses. This defense priming may protect not only the infected plant but also helps protect surrounding plants, yielding population-level resistance (Benevenuto et al., 2018). In addition to directly discouraging plant herbivores or pests, VOCs can also signal to the predators of those herbivores and pests to attract them to infected plants and to predate on the herbivores (Cortes et al., 2016). In one intriguing example of this phenomenon the deposition of sawfly

eggs on Scots pine needles induced production of the VOC (*E*)- β -farnesene and led to increased feeding by birds that usually feed on sawfly larvae and adults (Mantyla et al., 2018). This mechanism of “long distance” resistance has been adopted in agriculture in the practice of “crop training” (Llandres et al., 2018). Cotton crops that are mechanically damaged by trimming and topping, so-called “trained crops”, will export VOCs to attract enemies of pests and also prime neighboring crops for resistant to pathogens or insects (Renou et al., 2011).

Climate change impacts of chloroplasts and plant-microbe interactions

Aside from their fundamental contributions to plant metabolism and biotic interactions, defense, chloroplasts also sense environmental cues to adapt to the changing environment (Watson et al., 2018). As the sites of photosynthesis, chloroplasts must necessarily respond to environmental conditions in a manner that ensures minimal fluctuations in energy output and that the correct metabolic fluxes are maintained to support life (Watson et al., 2018, Suzuki et al., 2012). Therefore, the responses of plants and especially chloroplasts to current global climate changes have been the focus of much interest. Many studies suggest that increased atmospheric carbon may actually be advantageous to photosynthesis (Dusenge et al., 2019, Gutteridge, 2018). However, elevated CO₂ levels could have complex effects on plants. There is accumulating data suggesting that increased CO₂ induces elevated defense including those related

to SA and ROS and enhanced pathogen resistance (Cassia et al., 2018, Noctor & Mhamdi, 2017). But there are recent examples of herbivores being able to overcome plant defenses at high temperatures. *Phaseolus vulgaris* plants grown in elevated CO₂, had increased sugar and amino acid content but decreased activity of antioxidant enzymes like superoxide dismutase, peroxidase, and catalase (Qian et al., 2018). Feeding of thrips, *Frankliniella occidentalis*, initially induced plant antioxidant enzyme activity but over time the thrips were able to produce counter-defense enzymes of their own and overcome plant defense (Qian et al., 2018). These results suggest that in the long run those plants would have decreased defense against herbivore predation.

Chloroplast metabolism can deteriorate under stressful environmental conditions such as those resulting from the effects of climate change: increased global temperatures, extreme drought, heat waves or flooding, increased temperature. In general, heat stress leads to numerous changes in chloroplasts including increases chlorophyll degradation, deactivation of Rubisco activase (RCA) and increased turnover of photosystem proteins among others (Wang et al., 2018). *In toto* these changes can be expected to reduce chloroplast photosynthetic capacity. However, under heat stress, increases in antioxidant defenses in parallel with an increase in ROS production have also been observed (Soengas et al., 2018, Wang et al., 2018). It is likely that changes in plant biotic interactions

at elevated temperatures are specific to a given species or variety. Heat-sensitive potato plants subject to heat stress had decreased defense gene expression and increased susceptibility to potato virus Y, while a heat-tolerant cultivar showed no temperature-related changes in defense gene expression or virus susceptibility (Makarova et al., 2018). Engineering plants to have increased carbon fixation under emerging conditions resulting from climate change can be a way to address this issue. However, major challenges involve improving overall Rubisco biogenesis and CO₂ capacity (Sharwood, 2017). The changing climate can be expected to cause ecosystem-level changes in plant populations and distribution, and this in turn will impact the microbial and insect populations associated with plants (Van der Putten et al., 2010). Therefore, although the details of the effects of climate change on individual plant-biotic interactions remain murky, there will no doubt be considerable large-scale alterations to plant biotic interactions.

Conclusions

Deeper understanding of the mechanisms of plant biotic interactions has revealed that chloroplasts are the source of numerous molecules and signals that mediate those interactions. Some of these acts directly, like sugars, amino acids and antimicrobials in root exudates, and some act indirectly, like retrograde signals that regulate nuclear gene expression to modulate defense responses. Although it is unclear how these interactions will be affected in a climate where

'above-average' temperatures become more common and even the norm, we expect that these will be impacted as chloroplasts adjust to cope with environmental changes. Going forward, efforts to engineer chloroplasts should keep in mind the importance of chloroplast metabolism and homeostasis to establishing interactions with beneficial organisms to avoid their unintended disruption. Similarly changes to chloroplasts aimed for crop improvement should be careful to avoid enhancing susceptibility to pathogens.

VITA

Jessica Cisneros Fernandez was born in Los Angeles, California on June 24th in the '80s. She is the youngest daughter of two immigrant parents that came to this country illegally. Overcoming many obstacles in life, Jessica finished her High School Studies at Schurr High School where a teacher convinced her to apply for college. She completed her Bachelor of Science in Microbiology from California State University, Los Angeles. She then applied to the University of Tennessee, Knoxville, and began in the Biochemistry and Cellular & Molecular Biology Graduate Program in the fall of 2014. She was a PEER fellow, and she was the first in her department to apply for an NIH F31 fellowship and receive it. She will complete her doctorate in the Spring of 2021 after a year of living in an unbearable and frustrating pandemic.