Exploring the movement of DIR1 into the phloem during SAR and identification of genes upregulated during SAR induction

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Title: Exploring the movement of DIR1 into the phloem during SAR and identification of genes upregulated during SAR induction

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Abstract

Plants respond to pathogens both locally at the site of infection, as well as systemically in distant leaves. Systemic Acquired Resistance (SAR) is an immune response that involves the long-distance transport of SAR signal via the phloem from the site of infection to distant, uninfected leaves to establish long-lasting resistance. The *Arabidopsis thaliana* Defective in Induced Resistance 1 (DIR1) protein, which is required for SAR, accesses the phloem during SAR for long-distance travel to systemic leaves, and is thought to be part of a SAR signal complex. However, many questions remain about the long-distance movement of DIR1 during SAR – including the cellular route travelled to reach the phloem and whether other proteins are required for DIR1 movement. Fluorescent fusion lines of DIR1 and the related protein DIR1-like were previously created were investigated as potential tools to trace the movement of DIR1/DIR1-like during SAR. Immunoblot analysis of leaf extracts from these DIR1/DIR1-like fluorescent fusion lines revealed no signal, indicating that no fusion protein was present in these lines and therefore, they were likely not useful as a tool for assessing the movement of DIR1/DIR1-like during SAR. Lipid Transfer Protein 2 (LTP2) is required for SAR and interacted with DIR1 in a yeast-two-hybrid assay. To investigate if LTP2 is required for DIR1 movement into the phloem and long-distance, DIR1 signal was investigated by immunoblotting of phloem exudates from SAR induced *ltp2-1* mutant plants. The presence of DIR1

signal in phloem exudates of local *ltp2-1* leaves but not distant *ltp2-1* leaves suggested that LTP2 may be required for the long-distance movement of DIR1 during SAR, but not for DIR1 to enter the phloem in induced leaves.

Gene expression changes in the systemic, uninfected leaves are associated with the establishment of SAR, however, it remains less clear if there is a core set of genes important for SAR induction upregulated at the initial site of infection. To investigate this question, SAR was induced through differing treatments that first activated the PAMP-triggered immunity (PTI) pathway or Effector-triggered immunity (ETI) pathway. Common genes upregulated between all three SAR-inducing treatments were identified, revealing genes previously and currently under investigation by the Cameron lab, as well as genes that represent candidates for possible future studies.

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List of Abbreviations

ALD1 – AGD2-like defense response protein 1

AZA – Azelaic Acid

AZI1 – Azelaic Acid Induced 1

AZI3 – Azelaic Acid Induced 3

BAK1 -Brassinosteroid insensitive1-associated receptor kinase 1

BKK1 – BAK1- like 1

BiFC – Bimolecular Fluorescence Complementation

CaM – Calmodulin

CERK1 – Chitin Elicitor Receptor Kinase 1

CBP60g – CaM Binding Protein 60 g

DA - Dehydroabietinal

DIR1 – Defective in Induced Resistance 1

EARLI1 – Early Arabidopsis Aluminum Induced 1

EDS5 – Enhanced Disease Susceptibility 5

EFR –Elongation Factor Tu Receptor

EGFP – Enhanced Green Fluorescent Protein

EPS1 -Enhanced Pseudomonas Susceptibility 1

ER - Endoplasmic Reticulum

ETI – Effector-Triggered Immunity

ETS – Effector Triggered Susceptibility

Flg22 – Flagellin 22

FLS2 – Flagellin-Sensing 2

FMO1 – Flavin-dependent Monooxygenase 1

G3P – Glycerol-3-Phosphate

HA – Hemagluttanin

hpi – Hours post-inocultion

HR – Hypersensitive response

ICS1 – Isochorismate Synthase 1

LTP – Lipid Transfer Protein

LTP2 – Lipid Transfer Protein 2

LTPG5 – Lipid Transfer Protein GPI-anchored 5

MATE – Multidrug and Toxin extrusion

MAPK – Mitogen activated protein kinase

MCTP – Multiple C2 domain and transmembrane region protein

MeSA – Methyl Salicylate

NAD – Nicotinamide adenine dinucleotide

NADP – Nicotinamide adenine dinucleotide phosphate

NHP – N-hydroxypipecolic Acid

NLR – Nucleotide binding Leucine rich repeat Receptors

NPR1 – Nonexpressor of Pathogenesis Related 1

NPR3/4 – Nonexpressor of pathogenesis related 3/4

PBS3 – avrPphB Susceptible 3

P2C – 1-piperideine-2-carboxylic acid

PAMP – Pathogen Associated Molecular Pattern

PDLP – Plasmodesmata-Located Protein

PP2 – Phloem Protein 2

PR1 – Pathogenesis-Related 1

PRR – Pattern Recognition Receptor

PTI – PAMP-Triggered Immunity

Psm – Pseudomonas syringae pv. *maculicola*

Pst – Pseudomonas syringae pv. *tomato*

RIN4 – RPM1 Interacting protein 4

ROS – Reactive Oxygen Species

RPS2 – Resistance to *P. syringae* 2

SA – Salicylic Acid

SABP2 – Salicylic Acid Binding Protein 2

SAR – Systemic Acquired Resistance

SARD1 – Systemic Acquired Resistance Deficient 1

SARD4 – Systemic Acquired Resistance Deficient 4

SYP – Syntaxin of Plants

TMV – Tobacco Mosaic Virus

SID2 – Salicylic acid Induction Deficient 2

wpg – weeks post-germination

YFP – Yellow Fluorescent Protein

Declaration of Academic Achievement

I declare this thesis to be an original report of my research, except where indicated by referencing. No part of this work has been submitted, in whole or in part, in any previous applications or publications for a degree at another institution.

Chapter 1: Introduction

1.1 Plant immunity and the *Arabidopsis thaliana– Pseudomonas syringae* pathosystem

Every year, we lose a significant proportion of crops to pests and disease, with current mean estimates of crop losses ranging from 21.5 % in wheat to 30.3% in rice (Savary et al., 2019). This is especially concerning as the world population is growing, and food production will need to increase to meet this demand – all the while climate change is predicted to increase the frequency and severity of emerging plant diseases (Ristaino et al., 2021). Recent work has also highlighted that plant immunity can become compromised at higher temperatures (Huot et al., 2017). Together, this highlights the need for creating disease-resistant crops, and the study of the plant immune system can contribute to this goal.

There are numerous plant-pathogen model systems used to study the plant immune response, including the well-studied *Arabidopsis thaliana – Pseudomonas syringae* model system. While not a crop plant, there are many advantages to using Arabidopsis as a model plant, including its sequenced genome (The Arabidopsis Genome Initiative, 2000), small size, short generation time and ability to produce many seeds. The gram negative bacterial phytopathogen *Pseudomonas syringae* has numerous pathovars, with many of them infecting economically important crops (Xin et al., 2018). Certain strains of *P. syringae* can infect Arabidopsis - with the

discovery of the strains *Pseudomonas syringae* pv. *maculicola* (*Psm*) ES4326 (Dong et al., 1991) and *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Whalen et al., 1991) leading to the establishment of Arabidopsis and *P. syringae* as a model system for the study of plant immunity.

Infection normally occurs when *P. syringae* bacteria on the leaf surface enter the plant via wounds or open stomata (Katagiri et al., 2002). Once inside the plant, *P. syringae* bacteria can multiply within the leaf intercellular spaces and establish an aqueous environment – resulting in the appearance of water-soaked lesions on leaf tissue (Katagiri et al, 2002, Xin et al., 2018). It has been suggested that drawing liquid into the apoplast to create an aqueous environment may increase nutrient availability or dilute plant-produced antimicrobial compounds, ultimately benefiting bacterial multiplication (Xin et al., 2018).

1.2 Local Immune Responses

1.2.1 PAMP-triggered Immunity (PTI)

To effectively respond to biotic threats, plant cells must perceive these biotic threats. Plants can recognize pathogens via conserved molecules known as pathogen-associated molecular patterns (PAMPs) (Couto and Zipfel, 2016). These PAMPs are molecules that make up essential components of a pathogen's structure – for example, the peptide epitope flg22, derived from bacterial flagella (Felix et al., 1999). PAMPs are recognized by the plant through an array of cell surface receptors

known as pattern recognition receptors (PRRs) (Couto and Zipfel, 2016). For example, the PAMP flg22 is recognized by the cell surface receptor FLAGELLIN SENSING 2 (FLS2) in the model plant *Arabidopsis thaliana* (Gomez-Gomez & Boller, 2000; Chinchilla et al., 2005). The recognition of PAMPs by the plant activates pattern-triggered immunity (PTI) – resulting in calcium fluxes, the production of reactive oxygen species (ROS), hormone signaling, and defense-related gene expression (Couto and Zipfel, 2016; Yuan et al., 2021a).

1.2.2 Effector-triggered Immunity (ETI)

Since plants can detect pathogen attack via perception of conserved PAMPs, successful virulent pathogens are able to evade PTI. Pathogens such as *P. syringae* can inject effector proteins into plant cells to disrupt or shut down plant defense responses and promote infection (Cui et al., 2015). If the plant is unable to recognize and respond to these effector proteins, it can lead to Effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). However, plants have a second class of intracellular immune receptors containing nucleotide binding domains and leucine rich repeats (NLRs) (Cui et al., 2015). These receptors allow plants to respond to the actions of effector proteins and initiate Effector-triggered immunity (ETI) (Jones and Dangl, 2006; Cui et al., 2015). NLRs sometimes recognize effector proteins directly through interaction with them, but this appears to be less common than indirect recognition of effectors via their activities (Cui et al., 2015). The activities of effectors on plant

proteins can result in the creation of a 'modified self', that when recognized by NLRs, can trigger ETI (Cui et al., 2015). For example, the Arabidopsis protein RPM1-INTERACTING PROTEIN 4 (RIN4) is the target of several *P. syringae* effectors – including AvrRpt2 (Axtell and Staskawicz, 2003; Kim et al., 2005). AvrRpt2 is a protease that cleaves RIN4 (Kim et al., 2005), resulting in the production of two fragments designated ACP2 and ACP3 – with evidence suggesting that ACP2 and ACP3 function in suppressing PTI (Afzal et al., 2011). The Arabidopsis NLR RPS2 'guards' RIN4 (Cui et al., 2015), with the cleavage and subsequent absence of RIN4 resulting in RPS2 activation, which triggers the ETI response (Axtell and Staskawicz, 2003).

1.2.3 Connections between PTI and ETI

It was previously thought that PTI and ETI were different, distinct branches of the plant immune system, but recent work has revealed they are more interconnected than originally thought. Ngou et al. (2021) created an Arabidopsis line that expresses the *P. syringae* pathovar *tomato (Pst)* effector AvrRps4 in response to estrogen treatment, allowing initiation of ETI in the absence of PTI. Treatment of these plants with the flg22 peptide to initiate PTI alone or estrogen to initiate ETI alone resulted in less ROS production, callose deposition, and expression of PTI-responsive genes compared to plants treated with both flg22 and estrogen to activate PTI and ETI together. This suggests that the PTI response is enhanced by ETI. In addition, Yuan et

al. (2021b) investigated ETI responses in the Arabidopsis PTI PRR receptor mutant *fls2 efr cerk1* and the PTI PRR co-receptor mutant *bak1 bkk1 cerk1*. These mutants were then infected with *Pst* carrying the AvrRpt2 effector (*Pst AvrRpt2*), which activates ETI via the RPS2 receptor in wild-type plants (Axtell and Staskawicz, 2003, Cui et al., 2015). However, the PTI PRR and PTI co-receptor triple mutants failed to display an ETI response after infection with *Pst AvrRpt2*, suggesting that PTI signaling components are required for an efficient ETI response. Together, Ngou et al. (2021) and Yuan et al. (2021b) have revealed that PTI and ETI enhance one another and that ETI depends on PTI, changing our understanding of these local defense responses.

1.3 Systemic Immunity

1.3.1 Systemic Acquired Resistance (SAR)

It has been observed for over a century that after infection, plants often become more resistant to subsequent infections, with many of these early observations being compiled into a publication by Chester (1933). In 1961, Ross observed that *Nicotiana tabacum* plants previously infected with tobacco mosaic virus (TMV) were more resistant to subsequent TMV infection in distant, uninfected leaves – and he named this phenomenon Systemic Acquired Resistance (SAR). SAR occurs when an initial infection results in a plant developing resistance to a variety of other pathogens, in distant, uninfected leaves (Champigny and Cameron, 2009). SAR is also long-lasting, with evidence to suggest that it can even be transgenerational

(Luna et al., 2012, Luna and Ton, 2012). SAR can be described in four stages (Figure 1.1, Champigny and Cameron, 2009): induction, propagation, establishment, and manifestation.

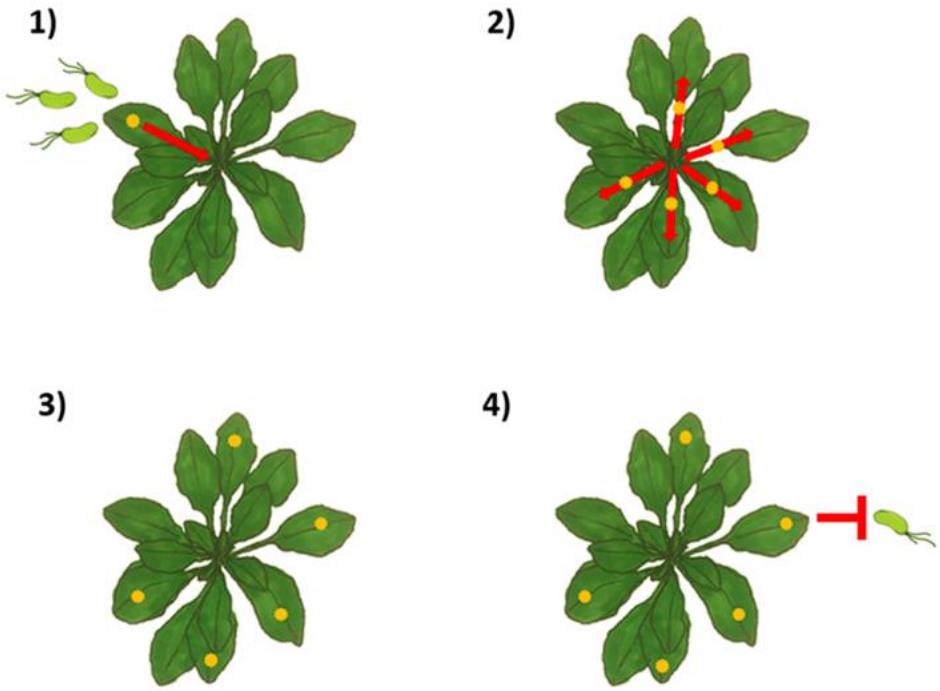


Figure 1.1: The four phases of Systemic Acquired Resistance. Systemic Acquired Resistance (SAR) can be divided into four phases. In the first phase – induction- a local infection results in local immune responses as well as the generation of mobile SAR signals (represented by the yellow dot). The second phase of SAR is propagation of mobile signals (again represented by the yellow dots) – where SAR signals travel via the phloem from the original site of infection to distant, uninfected leaves. Establishment, the third phase of SAR, occurs when the SAR signals reach distant leaves and are perceived. This results in defense priming, a series of responses that puts the distant leaf into an immune-ready state. Subsequent infection of primed leaves results in manifestation of SAR – the fourth phase – where a faster and stronger immune response by the plant ultimately results in resistance against the invading pathogen.

1.3.2 Salicylic Acid (SA)

The phytohormone salicylic acid (SA) is essential for local resistance responses (Nawrath and Metraux, 1999), but also plays a key role in SAR. SA accumulates in infected leaves during the first stage of SAR and accumulates modestly in distant leaves during the establishment phase of SAR (Champigny and Cameron, 2009). Early evidence highlighting the requirement of SA for SAR was provided by expressing the bacterial salicylate hydroxylase NahG, which converts SA to catechol (Yamamoto et al., 1965), in tobacco plants (Gaffney et al., 1993). Not only did the NahG-expressing plants accumulate lower levels of SA compared to wild-type plants, but they also displayed a defective SAR response (Gaffney et al., 1993). Further work revealed that Arabidopsis *sid2-1* mutants, which display reduced SA accumulation upon infection, were also SAR-defective (Nawrath and Metraux, 1999). It was later discovered that the *sid2-1* mutant corresponded to a mutation in the gene for the enzyme isochorismate synthase 1 (ICS1) (Wildermuth et al., 2001), an enzyme that catalyzes the first step in the synthesis of salicylic acid from chorismite in the chloroplast (Wildermuth et al., 2001, Huang et al., 2020). After the conversion of chorismite to isochorismate, it has been proposed that isochorismate is transported to the cytosol via the Multidrug and Toxin Extrusion (MATE) transporter ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) (Serrano et al., 2013; Rekhter et al., 2019a), which is localized to the chloroplast envelope (Serrano et al., 2013). The cytosolically localized avrPphB SUSCEPTIBLE 3 (PBS3) joins isochorismate to

glutamate, generating isochorismate-9-glutamate (Rekhter et al., 2019a, Torrens-Spence et al., 2019). ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (EPS1) then promotes the conversion of the resulting isochorismate-9-glutamate to SA (Torrens-Spence et al., 2019) in the cytosol. The regulation of SA biosynthetic gene expression occurs through the two redundant master immune transcription factors CBP60g (CaM-Binding Protein 60 g) and SARD1 (SAR- deficient 1) (Wang et al., 2009, Wang et al., 2011, Zhang et al., 2010).

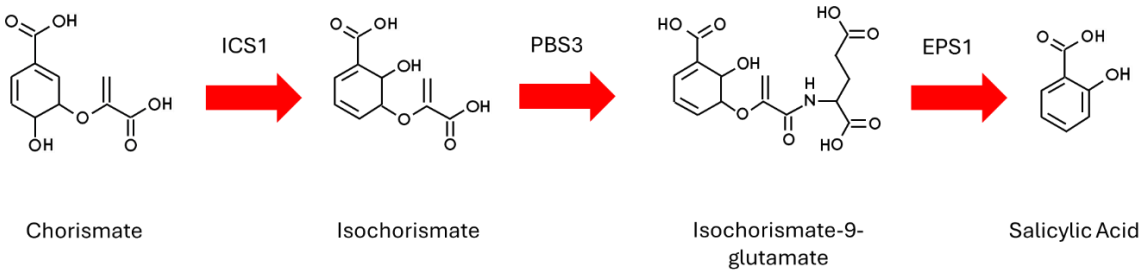


Figure 1.2: Biosynthesis of salicylic acid (SA). SA biosynthesis begins in the chloroplast, where ICS1 converts chorismite to isochorismate. The isochorismate is then transported to the cytosol by the MATE transporter EDS5. Once in the cytosol, PBS3 joins isochorismate and glutamate, resulting in isochorismate-9-glutamate. The conversion of isochorismate-9-glutamate can occur spontaneously but is promoted by EPS1. Modified from Huang et al. (2020).

1.3.3 SAR Induction

Pathogen infection not only results in local immune responses but can also result in the production of mobile SAR signals at the site of infection (Champigny and Cameron, 2009). The hypersensitive response (HR) or disease-caused necrosis is often associated with SAR induction (Champigny and Cameron, 2009). When SAR is

induced by a pathogen such as *PstAvrRpt2*, both PTI and ETI responses will be activated in the induced leaf. However, there is evidence that suggests SAR can be induced through the activation of PTI or ETI alone. Mishina and Zeier (2007) treated lower leaves of wild-type Arabidopsis plants with the PAMP flg22 before infecting untreated systemic leaves with *Psm* ES4326. The flg22-treated plants showed significant reductions in *Psm* ES4326 levels in systemic leaves compared to mock-treated plants, indicating that the flg22 treatment induced resistance in the systemic leaves. However, Jelenska et al. (2017) observed that the flg22 peptide moved from its initial site of infiltration in one leaf to distant leaves and accumulated in distant leaf vasculature. This raises questions if flg22 treatment is truly inducing SAR, or flg22 is moving through the plants and inducing PTI in systemic leaves. In addition, recent work in the Cameron lab by Natalie Belu with plants that express the effector AvrRpt2, in response to estrogen treatment, also suggests that SAR can be induced by ETI alone, however it has been shown that some effectors can also move via the phloem in plants (Zhang et al., 2024).

1.3.4 SAR signals and signal propagation

In the second stage of SAR, mobile SAR signals generated at the site of infection travel from the induced leaf to distant leaves via the phloem (Champigny and Cameron, 2009). Numerous candidate SAR mobile signals have been proposed including azelaic acid (AZA) (Jung et al., 2009), glycerol-3-phosphate (G3P) (Chanda

et al., 2011), dehydroabietinal (DA) (Chaturvedi et al., 2012), methyl salicylate (MeSA) (Park et al., 2007), N-hydroxypipecolic acid (NHP) (Chen et al., 2018), the protein Defective in Induced Resistance 1 (DIR1) (Champigny and Cameron, 2009), and nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate (NAD/NADP) (Wang et al., 2019). It was previously proposed that SA itself was the mobile SAR signal, but a series of experiments provided evidence that this is not the case. Rasmussen et al. (1991) demonstrated that inoculation of a cucumber leaf with *P. syringae* pv. *syringae* D20, followed by detaching that leaf resulted in detectable SA in systemic leaves at 4 hours post-inoculation, despite SA only appearing in phloem exudates at 8 hours post-inoculation. This led to the idea that another signal was translocating through the phloem to promote SA accumulation in distant leaves. This study was followed by grafting experiments conducted by Vernooij et al. (1994) in which the shoots of wild-type tobacco plants were grafted onto the rootstocks of plants expressing the SA-degrading salicylate hydroxylase NahG (Gaffney et al., 1993). Infection of these SA-deficient rootstocks still resulted in SAR-competent shoots, strongly suggesting that SA was not the mobile signal involved in SAR (Vernooij et al., 2014).

1.3.5 SAR establishment and priming

The third stage of SAR, establishment, occurs when the SAR signal arrives in the distant leaves and is perceived. This stage is associated with modest accumulation

of the phytohormone salicylic acid (Champigny and Cameron, 2009) and the establishment of defense priming. Conrath (2006) describes this priming as a form of 'plant memory'. Priming is thought to create an immune-ready state through various means, such as histone modifications associated with chromatin openness occurring at the promoters of defense-related genes (Jaskiewicz et al., 2011). Building on the work of Jaskiewicz et al. (2011), Baum et al. (2019) were able to use FAIRE-seq to identify large-scale chromatin accessibility changes during the priming phase of SAR – noting that chromatin became more open upstream of the transcription start sites of numerous genes, while others displayed reductions in chromatin accessibility. This was followed up by an RNA-seq analysis. Plants were mock-inoculated or SAR-induced with *Psm* bacteria, followed by challenging systemic leaves with water and collecting the challenged leaf tissue for RNA-seq (Baum et al., 2019). This led to the observation that, many of the genes identified as having more open chromatin in the primed state displayed greater levels of transcription during challenge with water (Baum et al., 2019). In particular, Baum et al. (2019) identified that the defense related genes *WRKY6*, *WRKY29*, and *WRKY53,* as well as *FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1)* – a gene required for SAR (Mishina and Zeier, 2006) and involved in the synthesis of the metabolite N-hydroxypipecolic acid (Chen et al., 2018, Hartmann et al., 2018) also followed this pattern. Together, the FAIRE-seq and RNA-seq analyses suggest that increased expression of SAR-related genes at the manifestation of SAR is preceded by open

chromatin at the promoters of those genes during the priming stage of SAR (Baum et al., 2019).

Transcriptional changes are also a hallmark of SAR establishment (Gruner et al., 2013), with the transcriptional upregulation of genes such as *PATHOGENESIS RELATED-1* (*PR1*) in systemic leaves becoming a marker for the establishment and/or manifestation of SAR (Klessig and Malamy, 1994). It was also suggested that priming could be associated with the accumulation of inactive immune signal transduction components. Conrath (2006) observed that priming was associated with increases in enzymatically inactive Mitogen Activated Protein Kinase (MAPK) proteins in systemic leaves. Beckers et al. (2009) then went on to show that priming was associated with greater expression of MAPK3 in systemic leaves in plants that had been induced for SAR, as well as greater levels of MAPK3 and MAPK6 protein, although this protein did not show phosphorylation of the activation loop, indicating that primed leaves accumulated greater levels of these inactive MAPKs. Together, this suggests that after SAR induction and movement of SAR signals to distant leave, leaves become primed to accumulate transcripts and proteins related to immune signaling and the immune response. This is thought to contribute to an immune-ready state, allowing the plant to respond faster and stronger to a subsequent infection (Conrath, 2006, Beckers et al., 2009).

1.3.6 SAR manifestation

The final stage of SAR – manifestation - occurs when a pathogen attempts to infect a primed distant leaf and the plant responds more quickly and successfully, resulting in resistance to the infection (Champigny & Cameron, 2009). Manifestation of SAR is associated with increases in SA levels and defense gene expression (Champigny and Cameron, 2009), with SA being a key regulator of the defense response in SAR. Early work demonstrated that Arabidopsis *npr1* mutants were unresponsive to SA treatments as shown by the loss of the expression of SAR marker gene PR1 or in response to infection (Cao et al., 1994, Delaney et al., 1995, Glazebrook et al., 1996, Shah et al., 1997), highlighting the important role of NPR1 in SAR and the response to SA. Further studies revealed that NPR1 localized to the nucleus (Kinkema et al., 2000) and was able to interact with TGA family transcription factors (Zhang et al., 1999, Despres et al., 2000, Zhou et al., 2000; Fan and Dong, 2002), with these interactions being strengthened in the presence of SA (Fan and Dong, 2002). The current model proposes that NPR1 acts as a receptor for SA. (Manohar et al., 2015, Wu et al., 2012, Ding et al., 2018). The binding of SA by NPR1 results in a conformational change (Wu et al., 2012) after which NPR1 moves to the nucleus and interacts with TGA transcription factors TGA2, TGA5 and TGA6 (Zhou et al., 2000, Fan and Dong, 2002, Zhang et al., 2003) to promote defense-related gene expression.

NPR1 is not the only NPR gene involved in the regulation of SA-responsive genes, as demonstrated by enhanced *PR* gene expression and pathogen resistance of plants lacking functional NPR3 and NPR4 compared to wild-type Arabidopsis (Zhang et al.

2006). The ability of NPR3 and NPR4 to interact with TGA transcription factors suggests they may also play a regulatory role in immunity (Zhang et al., 2006). Like NPR1, NPR3 and NPR4 also bind SA (Wu et al., 2012) and Ding et al. (2018) demonstrated that NPR3/NPR4 act as transcriptional repressors of defense-related genes, and their repressor activities become inactivated after binding SA. Therefore, NPR1 and NPR3/NPR4 play opposing roles in the regulation of SA responsive genes during SAR (Ding et al., 2018, Zhang and Li, 2019).

1.4 Candidate SAR signals

1.4.1 Glycerol-3-Phosphate (G3P)

Glycerol-3-Phosphate (G3P) plays a role in immunity, as demonstrated in *Arabidopsis* with mutants in the glycerol-3-phosphate dehydrogenase GLY1, that were SAR defective compared to wild-type plants (Nandi et al., 2004). In addition, plants with mutations in another enzyme involved in G3P synthesis, GLI1, demonstrated susceptibility to strains of *P. syringae* normally unable to successfully infect Arabidopsis (Lu et al., 2001; Kang et al., 2003) and led Chanda et al. (2011) to investigate the role of GLI1 in SAR. Chanda et al. (2011) demonstrated that SAR-induced *gli1* plants supported high bacterial levels in systemic leaves just like mock-inoculated plants, indicating that *gli1* plants were SAR defective. The application of exogenous G3P to *gly1* and *gli1* mutants concurrently with a SAR-inducing *Pst* AvrRpt2 inoculation resulted in restoration of the SAR response, though not to wild-

type levels, which suggests that G3P is a contributor to the SAR response. In addition to this, Chanda et al. (2011) detected G3P accumulation in phloem exudates of SAR-induced plants at 6 hours-post-inoculation. However, radiolabelled G3P alone was not able to translocate to distant tissues, but infiltrating leaves with radiolabelled G3P and recombinant DEFECTIVE IN INDUCED RESISTANCE 1(DIR1) protein resulted in translocation of the radiolabelled G3P to distant leaves. Chanda et al. (2011) suggest that DIR1 promotes the movement of G3P in the phloem, however the recombinant DIR1 used in this experiment was produced in *E. coli*. Previous work in the Cameron lab used Rosetta-gami *E. coli* to produce recombinant DIR1 (Isaacs et al., 2016), as this strain of *E. coli* promotes disulfide bond formation in the bacterial cytosol (Rosano and Ceccarelli, 2014). The structure of DIR1 contains 4 disulfide bonds (Lascombe et al., 2008), therefore the recombinant DIR1 used by Chanda et al. (2011) was not correctly folded. Finally, DIR1 and G3P did not associate during *in vitro* binding assays (Chanda et al., 2011), making it unclear whether DIR1 truly promotes G3P movement in the phloem during SAR.

1.4.2 Dehydroabietinal (DA)

Another potential SAR signal is dehydroabietinal (DA), which Chaturvedi et al. (2012) detected in a fraction isolated from phloem exudates of SAR-induced Arabidopsis leaves. Infiltrating lower leaves with concentrations of DA 1 pM or greater was able to significantly reduce bacterial levels in distant leaves challenged with *Psm*. In

addition, DA treatment of lower leaves was able to induce expression of the SAR marker gene PR1 and SA biosynthetic gene ICS1 in distant leaves (Chaturvedi et al., 2012). Together, this suggests that DA treatment of a local leaf induces systemic resistance in distant leaves. Chaturvedi et al. (2012) also investigated whether DA was able to move systemically through plants. Application of deuterated DA to lower leaves resulted in its detection in systemic leaves within 15 minutes, providing evidence that it can move systemically within plants (Chaturvedi et al., 2012). All together, this suggests DA may be a mobile SAR signal.

1.4.3 Methyl Salicylate (MeSA)

Methyl Salicylate (MeSA) has emerged as another potential SAR signal (Park et al., 2007). The tobacco SABP2 (Salicylic acid binding protein 2) converts MeSA to SA (Forouhar et al., 2005) and is needed for the SAR response (Kumar and Klessig, 2003). This led Park et al. (2007) to investigate the role of SABP2 and MeSA in long-distance movement of SAR signals. To investigate whether SABP2 is involved in the production of SAR signals at the site of infection or perception of SAR signals in distant tissues, grafting experiments were performed in *Nicotiana tabacum*. Grafting SABP2-silenced shoots onto wild type rootstocks resulted in SAR defects. However, grafting wild-type shoots onto SABP2-silenced rootstocks resulted in SAR competent plants, suggesting that SABP2 is required for SAR in distant leaves, but not for the production of SAR signals in induced leaves (Park et al., 2007). When

mutant versions of SABP2 were created and expressed in systemic leaves via the XVE estrogen-inducible promoter system, versions with defects in methyl esterase activity showed SAR defects indicating the methyl esterase activity of SABP2 was important for SAR in systemic tissues (Park et al., 2007). Gas-chromatography mass-spectrometry analysis of the phloem exudates from locally SAR-induced and systemic leaves revealed that MeSA levels peaked from 48-72 hours post-inoculation, suggesting that MeSA enters the phloem during SAR (Park et al., 2007). Building on the work of Park et al., (2007), Vlot et al. (2008) identified orthologs of SABP2 in Arabidopsis and demonstrated that underexpression of these genes also resulted in SAR defects. Together, this indicates that SABP2/SABP2 orthologs and MeSA play a role in SAR.

1.4.4 Nicotinamide Adenine Dinucleotide (NAD) / Nicotinamide Adenine Dinucleotide Phosphate (NADP)

There is evidence to suggest that nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate (NAD/NADP) could be potential SAR signals. Zhang and Mou (2012) overexpressed the NADP-metabolizing human enzyme CD38 in *Arabidopsis* and observed significantly reduced levels of NADP in leaf intercellular washing fluids after *Pst avrRpt2* infection in the CD38-overexpressing plants when compared to wild-type plants. When local leaves were induced for SAR with *Pst avrRpt2*, the CD38-overexpressing plants displayed

reduced PR1 expression in distant leaves compared to wild-type plants. Additionally, these SAR-induced CD38-overexpressing plants were defective for SAR compared to mock-treated plants, suggesting that NADP accumulation is required for SAR. Wang et al. (2019) proposed that accumulation of NADP in the intercellular spaces in response to a SAR-inducing pathogen may contribute to the induction stage of SAR. To investigate this, leaves were mock-induced or SAR-induced with *Psm* bacteria followed by cutting discs from induced leaves ten minutes later. These leaf discs were placed into water and NAD/NADP leakage was measured over time, revealing that *Psm*-treated leaf discs leaked more NAD/NADP into the surrounding water than mock-treated leaf discs. This provided evidence that *Psm* infection does result in the leakage of NAD/NADP into the intercellular space. The ability of NADP or NAD+ to move systemically to distant leaves was also investigated by Wang et al (2019) and Li et al. (2023). Radiolabelling NADP (Wang et al., 2019) or NAD+ (Li et al., 2023), followed by infiltration into the lower leaves of Arabidopsis and *Nicotiana benthamiana,* resulted in radioactive signal being detected in systemic leaves 24 hours later, suggesting that NADP and NAD+ can move systemically within plants. However, there are limitations to radiolabelling experiments as it is possible that radiolabelled molecules can be modified or cleaved (Cuyckens et al., 2024). This could lead to the loss of the radiolabel from the compound of interest, resulting in the radiolabel no longer representing the molecule of interest being traced (Cuyckens et al., 2024).

1.4.5 Azelaic Acid (AZA)

To investigate signals involved in the priming stage of SAR, Jung et al. (2009) subjected mock-treated and SAR-induced phloem exudates collected from wild-type Arabidopsis Col-0 plants to gas chromatography – mass spectrometry (GC-MS). This revealed that SAR-induced phloem exudates contained on average 6.2-fold higher levels of the compound azelaic acid (AZA) compared to the phloem exudates from mock-treated plants. Jung et al. (2009) then investigated whether AZA spray treatment induced resistance to *Psm* strain Dg3. Plants were sprayed with a mock solution or 1, 10, 100 or 1000 µM AZA, then inoculated with *Psm* Dg3 at 48 hours post treatment. Bacterial levels were reduced in the plants treated with 100 or 1000 µM AZA compared to mock-treated plants. Additionally, the application of 1 mM AZA to local leaves was able to significantly reduce bacterial levels in systemic leaves. These two experiments suggest AZA treatment can induce both local and systemic resistance. Finally, Jung et al. (2009) applied 1 mM of radiolabelled AZA to local leaves to investigate whether AZA was able to move systemically throughout the plant. Detection of radiolabelled AZA in both the phloem and systemic leaves at various timepoints after application suggests that AZA can move systemically through the plant and within the phloem (Jung et al., 2009). Despite the limitations of radiolabelling infiltration experiments (Cuyckens et al., 2024), the evidence indicates that AZA plays a role in both local and systemic resistance, and could be a SAR signal (Jung et al., 2009).

1.4.6 Pipecolic acid and N-Hydroxypipecolic acid (NHP)

SA is not the only signal necessary for local and systemic resistance – the metabolites pipecolic acid (Navarova et al., 2012) and N-hydroxypipecolic acid (NHP) (Chen et al., 2018, Hartmann et al., 2018) are also important. Navarova et al. (2012) uncovered a role for the amino acid pipecolic acid in SAR and defense priming. Pipecolic acid accumulated in systemic leaves after inoculation of lower leaves with *Psm ES4326*, with this accumulation preceding SA accumulation in these leaves, suggesting Pipecolic acid acts upstream of SA in the establishment of SAR in systemic leaves (Navarova et al., 2012). Aminotransferase AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1) (Song et al., 2004a) was thought to be involved in the synthesis of pipecolic acid, therefore Navarova et al. (2012) measured pipecolic acid levels in phloem exudates, induced leaves, and systemic leaves of *Psm*-inoculated wild-type and *ald1* mutant Arabidopsis. The *ald1* mutants showed significantly reduced levels of pipecolic acid compared to wild-type plants in exudates and induced and systemic leaves, providing strong evidence that the synthesis of pipecolic acid required ALD1. Additionally, these *ald1* plants were SAR defective (Song et al., 2004b, Navarova et al. 2012) demonstrating that pipecolic acid is an important metabolite for SAR.

Building on the work of Navarova et al. (2012), investigation by Hartmann et al. (2018) revealed that pipecolic acid undergoes N-hydroxylation by FMO1 to produce

the metabolite N-hydroxypipecolic acid (NHP). The *fmo1* mutant is unable to accumulate NHP (Chen et al., 2018) and is SAR defective (Mishina and Zeier 2006), which provides evidence that NHP plays a role in SAR. When Chen et al. (2018) treated local leaves of both *fmo1*-mutant and Col-0 control plants with NHP, accumulation of SAR-marker gene transcripts such as PR1 was detected in distant, untreated leaves. Distant leaves of NHP-treated Col-0 and *fmo1* plants supported lower levels of *Psm* ES4326 compared to mock-inoculated plants (Chen et al., 2018). This indicates that NHP treatment induces resistance and perhaps SAR in Arabidopsis.

The current model of NHP synthesis proposes that like SA, the synthesis of NHP starts off in the plastid, where ALD1 converts lysine to ε-amino-α-keto caproic acid, which spontaneously converts to Δ1-piperideine-2-carboxylic acid (P2C) (Ding et al., 2016, Hartmann et al., 2017). P2C is converted to pipecolic acid by SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 4 (SARD4) (Ding et al., 2016, Hartmann et al., 2017). Followed by pipecolic acid transport to the cytosol via the transporter EDS5 (Rekhter et al., 2019b, Huang et al., 2020), where FMO1 N-hydroxylates pipecolic acid to produce NHP (Chen et al., 2018, Hartmann et al., 2018).

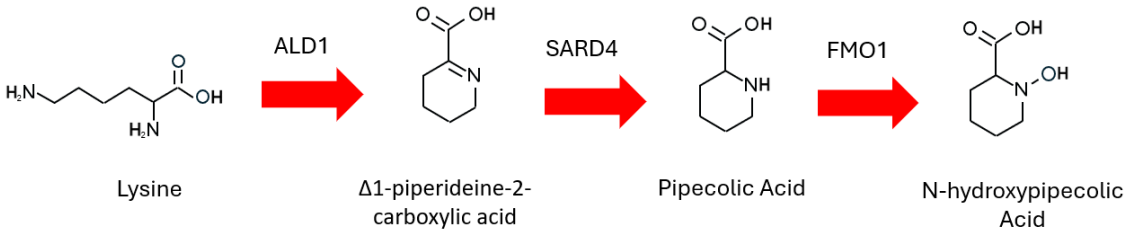


Figure 1.3: Biosynthesis of N-hydroxypipecolic acid (NHP). L-lysine is converted to Δ-1-piperideine-2-carboxylic acid (P2C) by ALD1 in the chloroplast. P2C is converted to pipecolic acid by SARD4, before transport out of the chloroplast by EDS5. In the cytosol, FMO1 n-hydroxylates pipecolic acid to produce NHP. Modified from Huang et al. (2020).

1.5 Defective in Induced Resistance 1 (DIR1)

Defective in Induced Resistance 1 (DIR1) is an important protein needed for SAR and was discovered using a SAR mutant screen (Maldonado et al., 2002). Arabidopsis *dir1-1* mutants show SAR defects, such as reduced transcript levels of SAR marker genes like *PR1* in distant leaves but show no impairment in local defense responses (Maldonado et al., 2002). DIR1 is part of the Lipid Transfer Protein (LTP) family, a large protein family with diverse functions and the ability to bind hydrophobic ligands *in vitro* (Edqvist et al., 2018). The structure of DIR1 was examined by x-ray crystallography to reveal that DIR1 has four disulfide bridges and a hydrophobic binding pocket typical of LTPs, and DIR1 binds 2 lysostearoylphosphatidylcholine molecules *in vitro* (Lascombe et al. 2008).

1.5.2 Localization and Movement of DIR1

DIR1 contains an endoplasmic reticulum (ER) signal sequence (SS) and is localized to the apoplast and ER (Champigny et al., 2011), as well as plasmodesmata (Chanda et al., 2011). At the tissue level, DIR1is found in all living cell types, including companion cells and sieve elements of the phloem (Champigny et al., 2011). DIR1 is detected in phloem exudates upon induction of SAR, suggesting that it accesses the phloem for movement to distant tissues (Champigny et al., 2013). Immunoblotting of phloem exudates with DIR1 antibody revealed that DIR1 accumulates in phloem exudates from 28 to 33 hours post SAR induction in wild-type Ws plants, and 9 to 23 or 23 to 28 hours post SAR induction in DIR1-GUS expressing plants (Champigny et al., 2013). Using an estrogen-inducible DIR1-EGFP construct expressed in the Arabidopsis *dir1-1* mutant background, DIR1 expression was induced in one leaf by estrogen treatment, followed by SAR induction of the same leaf. Collection of petiole exudates from distant leaves followed by DIR1 immunoblotting revealed a DIR1-EGFP signal, providing strong evidence that DIR1 travels through the phloem to distant leaves during SAR (Champigny et al., 2013). DIR1 has also been identified as part of a high-molecular weight protein-containing fraction that forms in phloem exudates after SAR induction, suggesting that it interacts with other proteins once it arrives in the phloem (Shah et al., 2014). This, along with the ability of DIR1 to bind lipids *in vitro*, suggests that DIR1 binds

lipophilic SAR signals as part of a SAR signal complex and moves to distant leaves during SAR. (Carella, Wilson and Cameron, 2015; Cameron et al., 2016).

As DIR1 is expressed in leaves including companion cells and sieve elements (Champigny et al., 2011), it is possible that DIR1 enters the phloem via plasmodesmata joining sieve elements and companion cells. To investigate this, Arabidopsis lines with reduced plasmodesmatal apertures due to overexpression of PLASMODESMATA-LOCATED PROTEINs (PDLP) 1 and 5 (Thomas et al., 2008; Lee et al., 2011) were used (Carella et al., 2015). PDLP1- and PDLP5-overexpressing plants supported high bacterial levels when mock-inoculated or SAR-induced with *PstAvrRpt2*, indicating that SAR is negatively affected by the overexpression of PDLP proteins (Carella et al., 2015). Additionally, immunoblots with DIR1 antibody of phloem exudates of SAR-induced PDLP-overexpressing plants revealed a reduction in DIR1 signals in distant leaves compared to SAR-induced exudates of wild-type plants (Carella et al., 2015). Together, this suggests that DIR1 requires open plasmodesmata to access distant leaves during SAR.

1.5.3 Defective in Induced Resistance 1– like (DIR1-like)

Although the *dir1-1* mutant was revealed to be SAR-defective, a SAR response was occasionally observed in *dir1-1* plants (Champigny et al., 2013). A search of the Arabidopsis genome revealed a gene located beside DIR1 (At5g48485) on chromosome 5 that displayed strong similarity to DIR1 at both the gene and protein

level – this gene (At5g48490) was named DIR1-like (Champigny et al., 2013). The DIR1-like protein is also similar enough to DIR1 that the DIR1 polyclonal antibody used in the Cameron Lab to immunoblot DIR1 also recognizes DIR1-like (Champigny et al., 2013). When DIR1-like was transiently expressed via *Agrobacterium* in *dir1-1* Arabidopsis plants, phloem exudates collected from SAR-induced leaves and immunoblotted using the DIR1 polyclonal antibody displayed a DIR1 signal, suggesting that DIR1-like also moves into the phloem upon SAR induction (Champigny et al., 2013).

1.6 Other Lipid Transfer Proteins (LTPs) and SAR

In addition to DIR1, the LTPs Azelaic Acid Induced 1 (AZI1) and Early Arabidopsis Aluminum Induced 1 (EARLI1) also play a role in SAR. The *azi1* and *earli1* mutants show SAR defects, with both mutants supporting high levels of bacterial growth in systemic leaves of mock-inoculated or SAR-induced plants(Cecchini et al., 2015). In addition, both AZI1 and EARLI1 interact with DIR1 in transient transformation experiments in *Nicotiana benthamiana* (Yu et al., 2013; Cecchini et al., 2015). Carella et al. (2017) demonstrated that Arabidopsis Lipid Transfer Protein 2 (LTP2) is also required for SAR as *ltp2-1* mutants were defective for SAR, indicating that LTP2 function is required for a successful SAR response (Carella et al., 2017). In addition, Carella at al. (2017) determined that LTP2 interacts with DIR1 in a split-ubiquitin yeast interaction assay, but a Bimolecular Fluorescence Complementation (BiFC)

assay performed in healthy uninoculated *N. benthamiana* plants indicated that DIR1 and LTP2 do not interact (Carella et al., 2017). It is possible that DIR1 and LTP2 only interact during SAR (Carella et al., 2017) as the BiFC assay was conducted in healthy plants not suring SAR. Moreover, fluorescently-tagged LTP2 and DIR1 did not co-localize when expressed transiently in healthy *Nicotiana benthamiana* leaves that were not induced for SAR (Carella et al., 2017).

1.7 DIR1 movement and the SAR signal complex

Because DIR1 interacts with other proteins necessary for SAR such as AZI1 (Yu et al., 2013; Cecchini et al., 2015) and is part of a high-molecular weight protein-containing fraction isolated from SAR-induced phloem exudates (Shah et al., 2014), it is possible that DIR1 travels to distant leaves via the phloem as part of a SAR signal complex (Cameron et al., 2016). This raises the possibility that proteins that interact with DIR1 or are necessary for SAR are also necessary for the movement of DIR1 into the phloem. The requirement of functional LTP2 for SAR and the possibility that LTP2 and DIR1 interact during SAR leads to the question, is functional LTP2 required for the movement of DIR1 into the phloem in induced leaves or to distant leaves?

In addition to investigating factors necessary for the movement of DIR1, tracing the cellular route travelled by DIR1 during SAR is also of interest. DIR1 is expressed in the companion cells and sieve elements (Champigny et al., 2011) as well as plasmodesmata (Chanda et al., 2011), and reduction of plasmodesmata aperture

both negatively affects SAR and reduces movement of DIR1 to distant leaves (Carella et al., 2015). This suggests that DIR1 accesses the phloem via plasmodesmata that connect companion cells and sieve elements. Visualizing DIR1 at companion cells and sieve element plasmodesmata during SAR would provide additional evidence for how DIR1 accesses the phloem for movement to distant leaves.

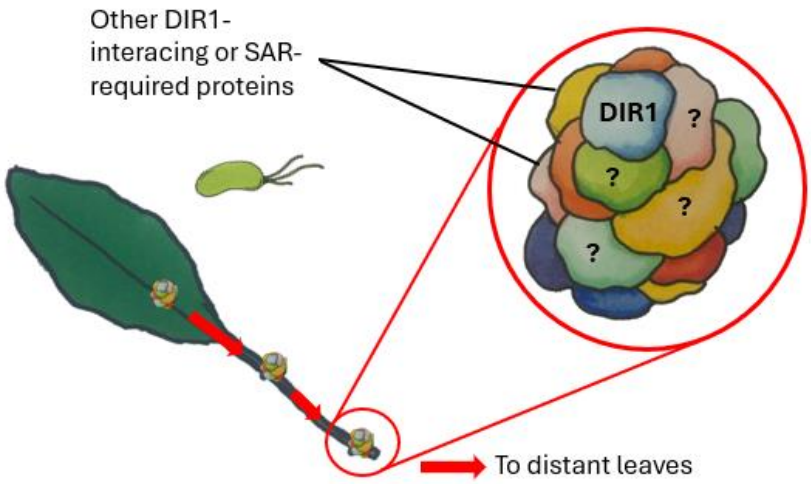


Figure 1.4: Model of SAR signaling complex in the phloem. Model of DIR1 movement in the phloem during SAR. After infection by a pathogen such as *Pst*, DIR1 is hypothesized to travel from infected leaves to distant tissues in the phloem as part of a multiprotein SAR signalling complex.

1.8 Biological Questions, Hypotheses and Objectives:

Chapter 3: Validation of fluorescently tagged DIR1 and DIR1-like fusion protein transgenic lines for use as tools for tracking DIR1/DIR1-like movement during SAR

Objectives:

1) Determine if DIR1 and DIR1-like are stably fused with the fluorescent tags (ILOV or PHILOV).
2) If these lines produce detectable HA-DIR1-ILOV and FLAG-DIR1-like-PHILOV, use these lines to investigate the cellular route travelled by DIR1 during SAR.

Chapter 4: Investigating LTP2's potential role in DIR1 movement during SAR

1) Is LTP2 required for the movement of DIR1 during SAR?

Objective: Assess SAR-induced *ltp2-1* phloem exudates from induced and distant leaves for the presence of DIR1.

2) Does LTP2 enter the phloem during SAR?

Objective: Assess SAR-induced 35S-LTP2-FLAG phloem exudates for the presence of FLAG-tagged LTP2.

Chapter 5: Bioinformatic analysis of SAR transcriptomes to understand the induction stage of SAR

1) **Hypothesis:** A core set of genes is expressed during the induction stage of SAR regardless of the SAR-inducing treatment.

Objective: Compare gene expression patterns in wild-type plants induced for SAR in response to PTI and ETI, to identify genes that contribute to the induction stage of SAR.

1.9 Contributions not presented in this thesis:

Work in the Cameron lab also focused on investigating the formation of *in planta Pst* biofilm-like aggregates and whether the polysaccharide alginate was required by *Pst* for biofilm-like aggregate formation. I assisted current and former Cameron lab members Noah Xiao, Garrett Nunn, Natalie Belu, and Evan Krysmanski in staining and imaging *in planta Pst* biofilm-like aggregates and with conducting bacterial quantification assays. This work was recently published in Molecular Plant Pathology (Xiao et al., 2024).

Chapter 2: Methods

2.1 Plant Growth Conditions and Plant Antibiotic Resistance Assay

Arabidopsis thaliana seeds (Col-0, *ltp2-1*, 35S-LTP2-FLAG, SS-HA-DIR1-iLOV, SS-FLAG-DIR1-like-phiLOV, *dir1-1*) were surface sterilized, washed with water, then resuspended in 0.1% Phytoblend (Cassion). Seeds were stratified for 2-3 days at 4° C before being plated onto Murashige and Skoog (MS) media solidified with 5.5-6.5% Phytoblend (Cassion) and germinated under continuous light (90 µmol m^{-2} s^{-1}). At about 1-week post-germination, plants were transplanted to soil watered with 1g/L 20-20-20 fertilizer. Plants were grown under short day conditions (9h of light) at 22°

C, 70-80% relative humidity, and light levels of 100-120 µmol m^{-2} s^{-1} (chamber 17). Plants were fertilized again with 1g/L 20-20-20 fertilizer between 2 and 3 weeks old.

To assess hygromycin resistance, *Arabidopsis thaliana* Col-0, DIR1-GUS, DIR1-EGFP, 35S-LTP2-FLAG, SS-HA-DIR1-iLOV, and SS-FLAG-DIR1-like-phiLOV seeds were surface sterilized, washed with water, then resuspended in 0.1% Phytoblend (Cassion). Seeds were stratified for 3 days at 4° C before being plated onto MS media solidified with 6% Phytoblend (Cassion) containing 15 ug/ml hygromycin B (Sigma) and germinated under continuous light (90 µmol m^{-2} s^{-1}).

2.2 Bacterial Growth, Inoculations, and SAR Assay

Overnight cultures of *P. syringae* pv. *tomato* DC3000 (*Pst*) without (virulent) or with the avirulence gene *avrRpt2* (Whalen et al., 1991) were grown with shaking (200rpm) at room temperature in King's B (KB) liquid media supplemented with 50 µg/ml kanamycin. Cultures were pelleted at 1000 x g, resuspended in 10 mM $MgCl_2$, followed by optical density (OD_{600}) measurements. Cultures with an OD_{600} between 0.2 and 0.6 were diluted with 10 mM $MgCl_2$ to be used for inoculations. Plants were inoculated on the abaxial side of the leaf using a needleless syringe.

For SAR assays, 3.5 to 4-week-old plants were mock inoculated with 10 mM $MgCl_2$ or SAR-induced using 10^6 colony forming units per ml (cfu/ml) avirulent *Pst avrRpt2* on 2 to 3 lower leaves. Two days later, upper leaves were challenged with 10^5 cfu/ml virulent *Pst*. Three days later, 24 challenged leaves per treatment were collected and

surface sterilized. Three replicates of 8 leaf discs each were cut using a cork borer and placed in a solution of 0.1% silwet in 10 mM $MgCl_2$, for an hour at 200 rpm to isolate bacteria from inside the leaf tissue, followed by serial dilution and plating on KB plates to quantify bacterial multiplication inside leaves (Cameron et al., 1999). Bacterial quantification plots were statistically assessed in R using analysis of variance (ANOVA) at $p < 0.05$, followed with Tukey's honestly significant differences (HSD) test.

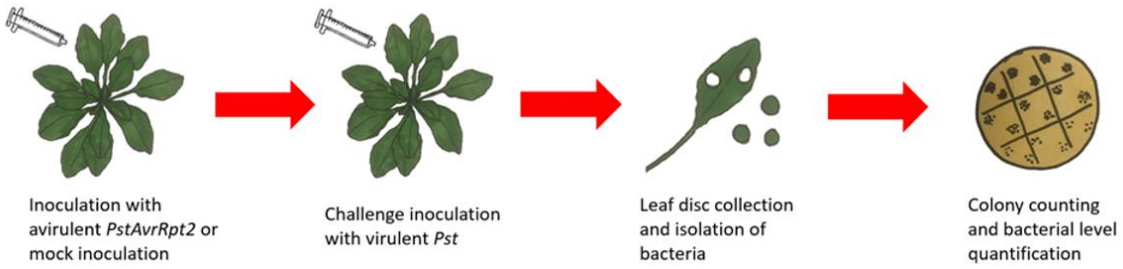


Figure 2.1: Conducting a SAR Assay

2.3 Phloem Exudate Collection

3.5 to 4-week-old plants were mock-inoculated with 10 mM $MgCl_2$, or SAR-induced with 10^6 cfu/ml *Pst avrRpt2*. Three replicates per treatment of around ten leaves each were cut at the petioles one at a time 24 hours later and surface sterilized, followed by washing with 1 mM EDTA. Petioles were submerged into 1.5 ml tubes containing 1 mM EDTA (to prevent sieve elements from closing; King and Zeevert, 1974) and 50 ug/ml ampicillin for 1 hour, and then moved to water and allowed to exude for ~24 hours in a humid flat. Exudates were frozen and stored at -20° C.

Protein levels were determined using a Bradford assay (Biorad protein assay kit). Exudates were lyophilized, resuspended in 20 µl nuclease free water, and all replicates were pooled together.

2.4 Protein Extraction from Leaf Tissue

About 100 mg of leaf tissue from SS-HA-DIR1-iLOV, *dir1-1*, SS-FLAG-DIR1-like-phiLOV, and 35S-LTP2-FLAG plants was collected, and flash frozen using liquid nitrogen. Leaf tissue was ground on dry ice using a drill, and 250 µl of BCA-compatible RIPA buffer (1 % SDS, 1% Nonidet P40 substitute, 2.5 % Tris HCl) was added after extracts thawed. Extracts were vortexed, and 3.3 µl of protease inhibitor cocktail (Sigma; contains AEBSF, 10-Phenanthroline, Pepstatin A, Leupeptin, Bestatin, and E-64) was added. Extracts were incubated at 4° C on an end-over-end shaker, followed by centrifugation at 4 °C at 20,0000 x g. Supernatants were collected and stored at -20° C. Protein levels were measured via BCA assay (Pierce).

2.5 SDS-PAGE and Immunoblotting

Phloem exudates (16.5 µl) or leaf extracts (diluted in nuclease-free water to 20-30 µg/ml protein) were mixed with 5 µl 5X SDS loading dye (350 mM Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 0.02% bromophenol blue, 700 mM DTT) before being boiled at 95° C. Samples were run on NuPAGE 4-12% Bis-Tris polyacrylamide gels (Invitrogen) in MES-SDS running buffer. Proteins were transferred to nitrocellulose membranes (Biorad) using a semi-dry transfer in Towbin buffer (25 mM Tris base, 192

mM glycine, 20% methanol). Membranes were blocked in 5% skim milk in TBST for 1 hour at room temperature. Membranes were incubated overnight at 4°C in blocking solution with primary antibody. 1:20,000 dilution was used for DIR1 antibody (Maldonado et al., 2002), 1:7000 dilution for FLAG antibody, and 1:5000 dilution for HA antibody. Antibody binding was detected using goat anti-rabbit (for DIR1 and HA antibodies) or goat anti-mouse (for FLAG antibody) horseradish peroxidase conjugated secondary antibody and the SuperSignal West Femto kit (Pierce).

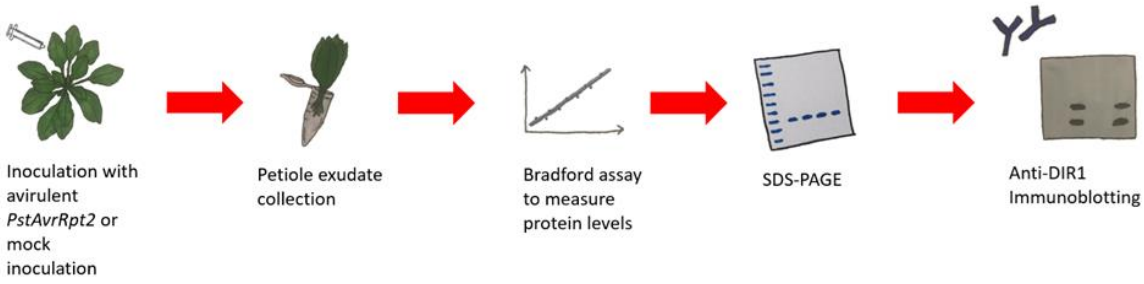


Figure 2.2: DIR1 antibody immunoblotting of phloem exudates

2.6 Bioinformatic Analysis

PTI RNA-seq data was generated by Garrett Nunn and Noah Xiao (manuscript in preparation). *Arabidopsis thaliana* Col-0 plants were left untreated, mock-inoculated with sterile water, or infiltrated with 1 µM flg22 peptide (PhytoTech Labs) in sterile water to induce PTI. Leaf tissue was collected at 0-, 6-, 12-, 18-, and 24-hours post-treatment (hpt) and flash frozen in liquid nitrogen. RNA extraction was performed using TriZol reagent (Invitrogen), and samples were sequenced on an Illumina HiSeq machine. This data was pre-processed by Garrett Nunn using the

pipeline shown in Figure 2.4. FastQC (Andrews, 2010) was used to assess data quality, while trimmomatic (Bolger et al., 2014) was used to trim adapter and low-quality sequences. The data was aligned to the *Arabidopsis thaliana* genome using STAR (Dobin et al., 2013), and reads were counted using Htseq-count (Anders et al., 2015). RSeQC (Wang et al., 2012) was used to perform additional quality assessments post-alignment.

The Cameron lab PTI dataset was compared to a dataset from Mine et al. (2018) (GEO accession GSE88798). The data was downloaded from the NCBI's SRA database to the Golding lab cluster (info@mcmaster.ca) using SRA Toolkit and miniconda. All pre-processing of the data was performed on the cluster, with scripts available on GitHub at https://github.com/rkbrookman/Biology-722-project.

The Mine et al. (2018) dataset was also pre-processed using the pipeline shown in Figure 2.4. Data was first quality assessed using FastQC (Andrews, 2010). Some samples consisted of multiple separate files, which were merged and assessed with FastQC again post-merging. The data was then trimmed using trimmomatic v. 0.36 (Bolger et al., 2014) to remove low quality bases and adapter sequence contamination. The data was quality assessed one last time with FastQC to ensure the trimming successfully removed low-quality bases and adapter contamination. The *Arabidopsis thaliana* TAIR10 genome assembly and corresponding annotations were downloaded from Ensembl and used to generate a genome index for alignment

using STAR aligner v. 2.7.7a (Dobin et al., 2013). STAR was then used to align the sequence data against the genome index. RSeQC (Wang et al., 2012) was used to perform additional quality assessments post-alignment, with SAMtools (Li et al., 2009) being used to convert between SAM and BAM file formats. Htseq-count v. 0.11.2 (Anders et al., 2015) was used to count how many transcripts corresponded to each gene in the genome. Before both sets of counts were imported into R for comparison, noncoding RNA genes and their counts were removed from the PTI count data on the Golding lab cluster to ensure that both sets of counts contained the same set of genes. Both sets of counts were then imported into R and the DESeq2 package (Love et al., 2014) was used for modelling and differential expression analyses. A total of three separate analyses were conducted to assess differential gene expression between mock and treated plants at 6-, 12-, and 24-hours post treatment. Genes from each analysis with $\log_2$ fold changes in expression of >2 and an adjusted p-value of <0.05 were then compiled and TAIR (Reiser et al., 2024) was used to investigate gene names and descriptions.

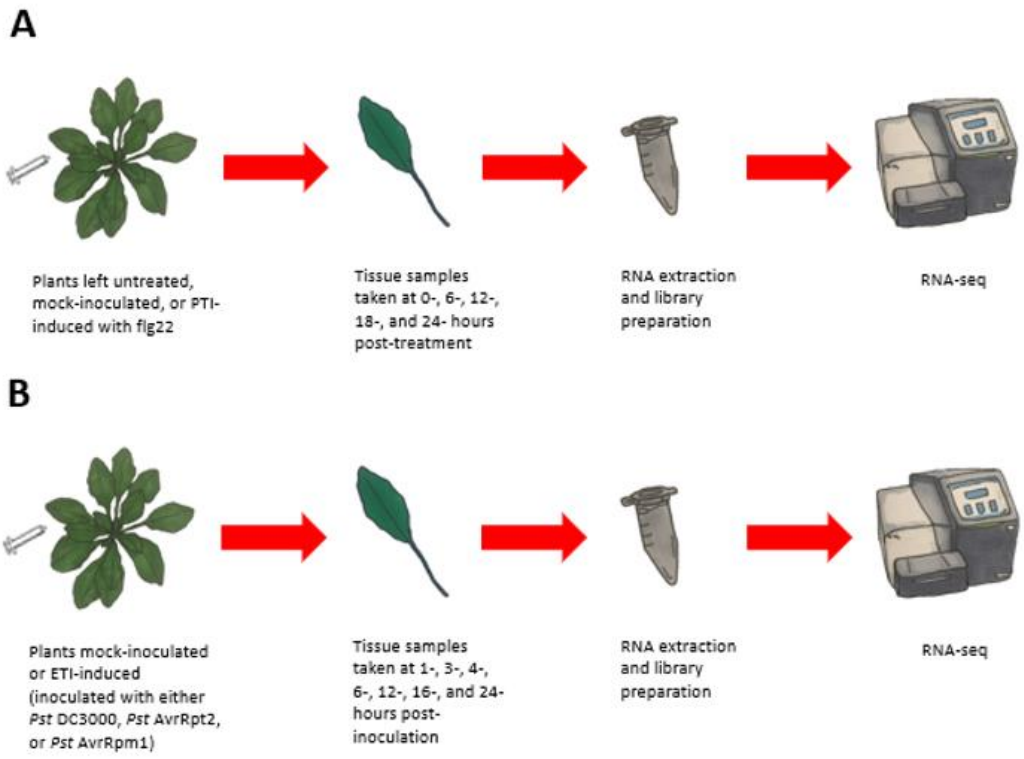


Figure 2.3: Sampling of plant tissue for RNA-seq analysis. Sampling of *Arabidopsis thaliana* leaf tissue in A) the Cameron lab PTI RNA-seq dataset and B) the Mine et al. (2018) dataset

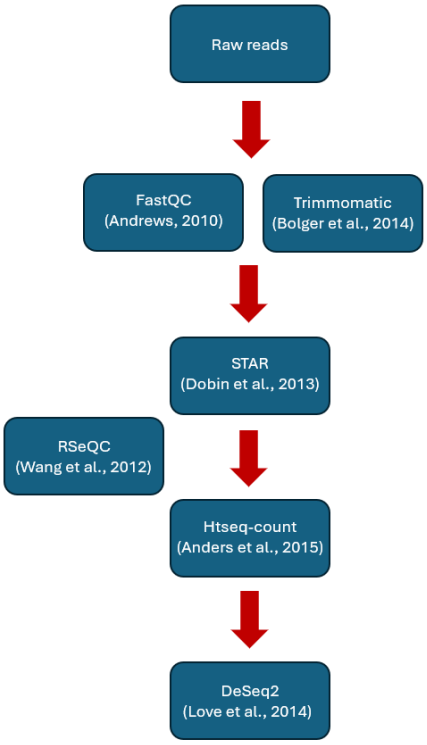


Figure 2.4: Bioinformatic pipeline used to pre-process both RNA-seq datasets.

Chapter 3: Validation of fluorescently tagged DIR1 and DIR1-like fusion protein transgenic lines for use as tools for tracking DIR1/DIR1-like movement during SAR

3.1 Previous work conducted on the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines

While there is good evidence indicating that DIR1 travels to distant leaves via the phloem during SAR (Champigny et al., 2013), it is unknown how DIR1 moves at the cellular level to enter the phloem in induced leaves or what happens to it once it reaches distant leaves. As DIR1 is expressed in all living cell types including companion cells and sieve elements (Champigny et al., 2011) and localizes to plasmodesmata (Chanda et al., 2011), this led to the idea that DIR1 may access the phloem through plasmodesmata that join sieve elements to companion cells (Carella et al., 2015). Since less DIR1 signal was detected in distant leaf phloem exudates when plasmodesmata were partially occluded due to overexpression of PLASMODESMATA-LOCATED PROTEINS (PDLP) 1 or 5 (Carella et al., 2015), this suggested that DIR1 moves from companion cells to sieve elements through plasmodesmatal connections.

To microscopically examine the route travelled by DIR1 during SAR, DIR1 and DIR1-like fluorescent protein fusions were created. Previous attempts to attach tags to DIR1 such as GUS or EGFP resulted in the cleavage of the tag from the C terminus of

DIR1 (Champigny et al., 2013). To avoid this, Isaacs (2013) created double 35S-promoter driven fluorescent protein fusions of DIR1 and DIR1-like in the *dir1-1* mutant background using the iLOV (Chapman et al., 2008) and phiLOV (Christie et al., 2012) fluorescent protein tags. As the iLOV and phiLOV tags are around 10 kDa in size, much smaller than GUS or EGFP, it was proposed that these protein tags may interfere less with the stability and/or function of DIR1/DIR1-like (Isaacs, 2013). Transient *Agrobacterium*-mediated transformation of *Nicotiana benthamiana* plants with the fusion protein constructs revealed fluorescent signal in leaves, however the stability of the DIR1/DIR1-like-fluorescent tag fusion proteins was not investigated (Isaacs, 2013). The constructs were also stably transformed into Arabidopsis and fluorescence was investigated in both 2-week-old and 4.5-week-old plants by previous lab member Abdul Halim. While Halim saw faint fluorescence in some 2-week-old plants, no fluorescence was observed in the 4.5 week-old plants. An additional investigation of 4 to 4.5-week-old *dir1-1*, HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV plants using fluorescence microscopy performed by myself confirmed these findings, with no fluorescent signal being observed in any plants (data not shown).

3.2 DIR1/DIR1-like signal is absent in HA-DIR1-ILOV and FLAG-DIR1-like-phiLOV lines

The faintness or absence of fluorescent signal in both HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV leaves may indicate that the fluorescent tags were cleaved from the C terminus of DIR1 and/or DIR1-like. To investigate if the fluorescent tags remain fused to DIR1 and/or DIR1-like, immunoblotting with a DIR1/DIR1-like antibody (Champigny et al., 2013) of HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV leaf extracts was conducted. If the fluorescent tags were cleaved from the C termini of DIR1 and or DIR1-like, DIR1/DIR1-like signals should be observed at the usual size of 7 kDa (Lascombe et al., 2008) or at 14 kDa, due to the ability of DIR1and/or DIR1-like to form dimers even after sample denaturation (Champigny et al., 2013). Initial immunoblots with *dir1-1*, HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV leaf extracts revealed no DIR1 signal (data not shown, see lab notebooks 1 and 2). DIR1 signal appeared in the positive control lane containing leaf extracts from plants overexpressing DIR1 (dD5e; Maldonado et al., 2002) indicating there were no issues with the immunoblotting procedure. Despite repeating leaf protein extractions on dry ice to reduce protein degradation and observing high protein concentrations in leaf extracts (Figure 3.1), DIR1/DIR1-like signal was still not observed (Figure 3.2). The lack of DIR1/DIR1-like signal at the expected size of the fusion (17-34 kDa) or the expected size of DIR1 or DIR1-like suggests that the fusion proteins were cleaved and/or degraded.

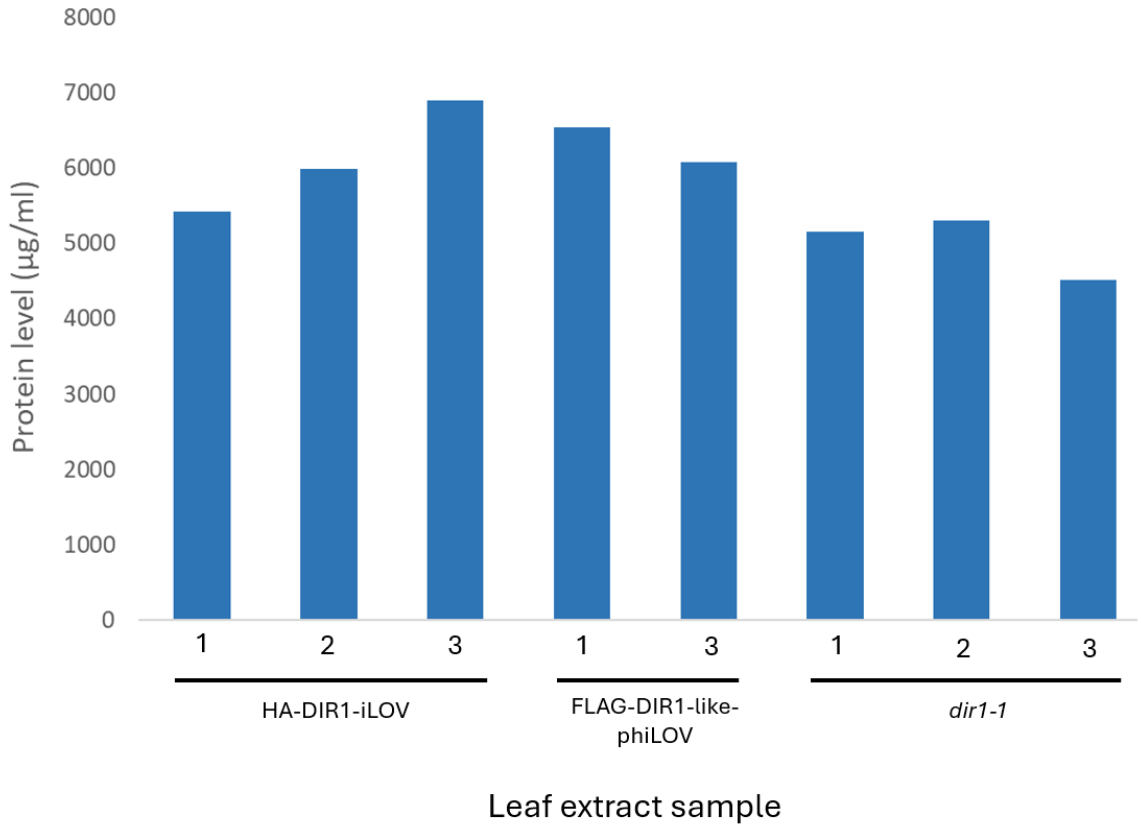


Figure 3.1: Protein concentration in *dir1-1*, HA-DIR1-iLOV, and FLAG-DIR1-like-phiLOV leaf extracts. The leaves of 4 to 4.5-week-old *dir1-1*, HA-DIR1-ILOV, and FLAG-DIR1-like-PHILOV plants were collected, frozen, ground on dry ice and subjected to protein extraction. Protein levels measured in µg/ml were determined by BCA assay (Pierce).

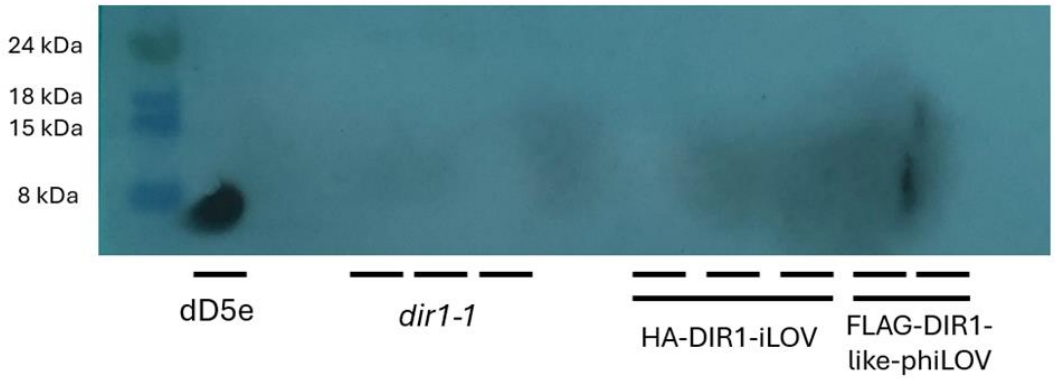


Figure 3.2: DIR1 immunoblot of *dir1-1*, HA-DIR1-iLOV, and FLAG-DIR1-like-phiLOV leaf extracts. *dir1-1*, HA-DIR1-iLOV, and FLAG-DIR1-like-phiLOV leaf extracts were subjected to SDS-PAGE followed by immunoblotting with a polyclonal DIR1 antibody that recognizes DIR1 and DIR1-like. The leftmost lane represents the protein ladder, with the next lane representing leaf extracts from DIR1-overexpressing plants (dD5e; Maldonado et al., 2002) as a positive control. The *dir1-1* leaf extracts represent a negative control.

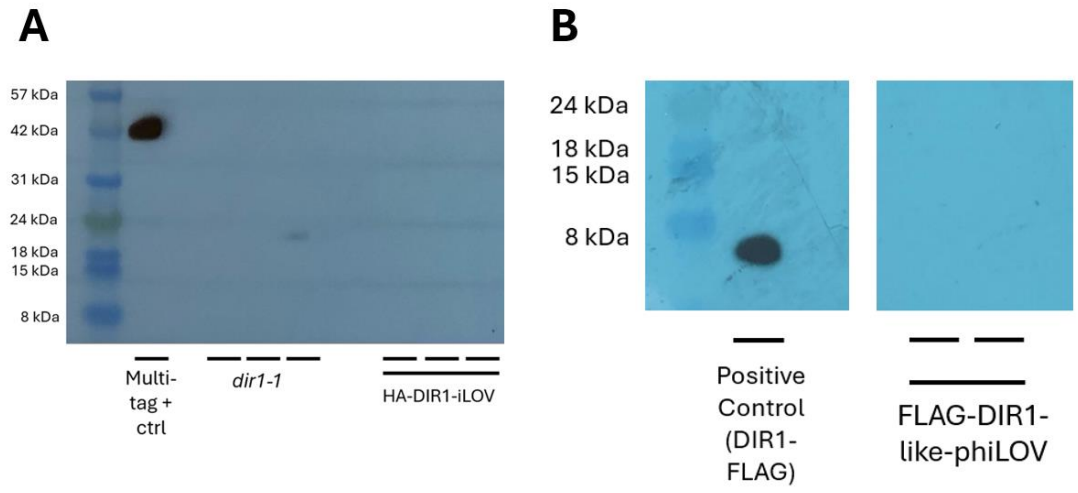


Figure 3.3: HA and FLAG immunoblots of HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV leaf extracts. In **A,** *dir1-1* and HA-DIR1-iLOV leaf extracts were subjected to SDS-PAGE followed by immunoblotting with an HA antibody. The leftmost lane represents the protein ladder, while the lane to the right of it represents a positive control (Abcam Recombinant *E. coli* Multi Tag protein). In **B**, FLAG-DIR1-like-phiLOV leaf extracts were subjected to SDS-PAGE followed by immunoblotting with FLAG antibodies. The leftmost lane represents the protein ladder, followed by the positive control next to it (DIR1-FLAG leaf extract; Carella 2016). The middle of this blot is not shown as it contains unrelated samples (35S-LTP2-FLAG leaf extracts; these samples are shown in Figure 4.3).

3.3 HA and FLAG signal is absent in DIR1-ILOV and DIR1-LIKE-PHILOV leaf extracts

The HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines also have HA or FLAG peptide tags fused at the N termini of DIR1 or DIR1-like, which should be detectable by immunoblotting of leaf extracts with HA or FLAG antibodies. However, if the fusion proteins are not present, then HA or FLAG signals will not be observed when immunoblotted with the peptide antibodies. HA antibody immunoblots were conducted with *dir1-1* and HA-DIR1-iLOV leaf extracts (Figure 3.3 A), but HA signals were not observed in lanes loaded with *dir1-1* leaf extract as expected. HA signals were not observed in lanes loaded with the HA-DIR1-iLOV leaf extracts. HA signal of the expected size of 45 kDa was observed in the positive control lane, indicating the HA immunoblotting procedure was successful. The lack of HA signals indicate that the fusion protein was not present in HA-DIR1-iLOV leaf extracts. Similarly, FLAG signal was not observed when FLAG-DIR1-like-phiLOV leaf extracts were immunoblotted using a FLAG antibody. FLAG signal was observed in the positive control lane (DIR1-FLAG leaf extract) indicating the FLAG immunoblotting procedure was successful, (Figure 3.3 B). The absence of FLAG signals indicates that the fusion protein was not present in the FLAG-DIR1-like-phiLOV leaf extracts.

3.4 Growth phenotypes of HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV seedlings on hygromycin-containing media

The HA-DIR1-ILOV and FLAG-DIR1-like-PHILOV lines also contain a hygromycin resistance gene that acted as a selection marker when making these transgenic lines. To assess whether the T-DNAs in these transgenic lines contain the hygromycin resistance, the ability of seedlings to grow on hygromycin was examined. Inability to grow on hygromycin would indicate an issue with the transgenes or expression of the transgenes in these lines. HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV seeds were plated onto media containing 15 µg/ml hygromycin alongside Col-0 as a negative control and the DIR1-GUS and DIR1-EGFP lines (Champigny et al., 2013) as positive controls. Col-0 seedlings germinated on the hygromycin containing plates, but over time these seedlings appeared small and discoloured as expected (Figure 3.4). The DIR1-GUS seedlings displayed healthy growth on the hygromycin containing media, however, most (122/168, 104/154) of the DIR1-EGFP seedlings failed to germinate, though of the seedlings that grew. most displayed a wild-type phenotype (37/50 and 32/46) (Figure 3.4). The majority (42/63 and 40/59) of the HA-DIR1-iLOV seedlings displayed a wild-type growth phenotype on the hygromycin containing media, however, very few of the FLAG-DIR1-like-phiLOV seedlings displayed wild-type growth (4/63, 11/61), while most were small or stunted (59/63, 44/61) (Figure 3.4). This suggests that in the HA-DIR1-iLOV line, the transgenic constructs are likely intact and expressed. However, the

FLAG-DIR1-like-phiLOV line appears to contain few wild-type appearing plants and a majority of stunted plants. This suggests that transgene expression may not be intact in this line, but it also could result from contamination of this seed stock, as these plants were grown for seed alongside plants of other genotypes, including Col-0.

3.5 The HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines cannot be used to trace DIR1/DIR1-like movement during SAR

Despite the transgenic constructs likely being intact in the HA-DIR1-iLOV line, DIR1/DIR1-like fusion proteins were absent in both the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines. Additionally, the lack of wild-type growth of FLAG-DIR1-like-phiLOV seedlings on the hygromycin-containing media suggests that the transgenic constructs in this line are either not intact or expressed, or the seed line is contaminated. Overall, this means that these lines cannot be used as tools to trace DIR1/DIR1-like movement during SAR.

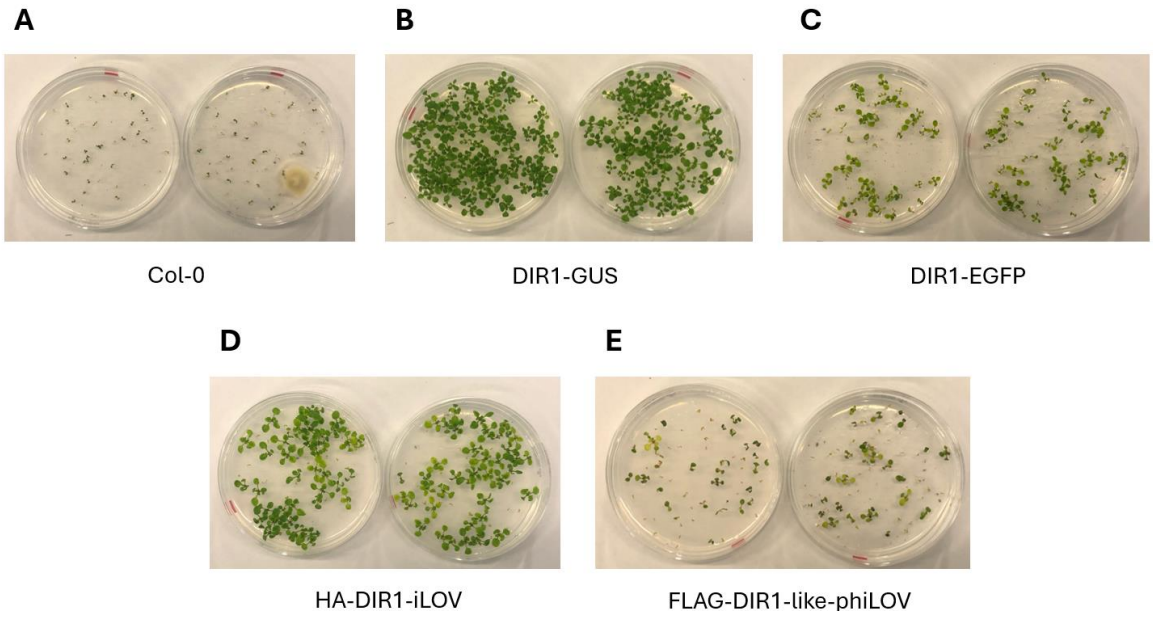


Figure 3.4: Growth of HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV seedlings on hygromycin-containing media. Seeds were sterilized and stratified followed by plating onto MS media containing 15 µg/ml hygromycin. **A** Col-0 represents the negative control, while **B** DIR1-GUS and **C** DIR1-EGFP represent positive controls. These controls were grown alongside **D** HA-DIR1-iLOV and **E** FLAG-DIR1-like-phiLOV.

Chapter 4: Investigating LTP2's potential role in DIR1 movement during SAR

4.1 Is LTP2 required for DIR1 to access the phloem during SAR?

There is good evidence that DIR1 travels through the phloem to distant leaves during SAR (Champigny et al., 2013), and may be part of a SAR signaling complex (Cameron et al., 2016). The lipid transfer protein LTP2 is required for SAR and interacts with DIR1 in yeast-2-hybrid experiments (Carella et al., 2017). This leads to the question of whether LTP2 is required for the movement of DIR1 into the phloem during SAR. If DIR1movement during SAR does require LTP2, DIR1 will not be observed in phloem

exudates collected from SAR-induced *ltp2-1* mutant plants. To investigate this, phloem exudates were collected from wild-type (Col-0) and *ltp2-1* mutant local leaves either mock-induced with 10 mM $MgCl_2$ or SAR-induced with 10^6 cfu/ml *Pst AvrRpt2,* followed by immunoblotting with the DIR1 antibody. DIR1 signal was not observed in mock-induced Col-0 or *ltp2-1* local leaf exudates, as expected (Figure 4.1 B, D). DIR1 signal was observed in SAR-induced exudates from local Col-0 leaves at the expected sizes of 7 kDa (Lascombe et al., 2008) and/or 14 kDa (Champigny et al., 2013). Phloem exudates collected from SAR-induced *ltp2-1* leaves also had DIR1 signal at the expected size of 7 and/or 14 kDa, suggesting that DIR1 is present in the phloem of SAR-induced *ltp2-1* mutant plants. This experiment was replicated numerous times, with eight out of nine experiments giving this result despite the presence of various stresses including high levels of fungal contamination during the growth of the plants (See Figure S1A, C, E for some additional replicates).

The presence of DIR1 signal in *ltp2-1* SAR-induced phloem exudates suggests that DIR1 accesses the phloem in *ltp2*-1 mutants and leads to the next question – is LTP2 required for long-distance movement of DIR1 to systemic leaves during SAR. Phloem exudates collected from the distant leaves of Col-0 and *ltp2-1* plants mock-induced with 10 mM $MgCl_2$ had no DIR1 signal, while DIR1 signals were present in the distant leaf phloem exudates collected from SAR-induced (Pst AvrRpt2) Col-0 plants (Figure 4.2 A). While *ltp2-1* SAR-induced exudates collected from local leaves almost always had DIR1 signal (Figure 4.1 B, D, Figure S1 A, C, E), DIR1 signal at 7 or 14 kDa

was not observed in SAR-induced exudates collected from distant *ltp2-1* leaves (Figure 4.2 A), in multiple experiments (Figure S1 A, C, E). In the replicates displayed in Figure 4.2 and Figure S1 A, there is a faint band present in the lane with SAR-induced phloem exudates from distant *ltp2-1* leaves, however, this band only appeared after very long exposure times and has a higher molecular weight than DIR1 (7 or 14 kDa), making it unlikely to be DIR1. The absence of signal at the expected size for DIR1 in phloem exudates collected from distant leaves of SAR-induced *ltp2-1* plants suggests that DIR1 is unable to move to distant leaves in the *ltp2-1* mutant. These data suggest that LTP2 is not required for DIR1 to access the phloem but may be required for DIR1 to travel to distant leaves during SAR.

To ensure that SAR was in fact induced and established during each experiment, SAR assays (Cameron et al., 1994) were performed alongside the phloem exudate collection experiments. Additional Col-0 and *ltp2-1* plants were SAR-induced or mock-induced, followed by challenge inoculation of distant, upper leaves with 10^5 cfu/ml virulent *Pst* 2 days later. Three days post-inoculation, leaf discs were taken for quantification of *in planta* bacterial levels. Bacterial levels and SAR responses varied across experiments (Figure 4.1 A,C, Figure S1 B, D, F). Statistically significant decreases in bacterial levels of 32-fold (Figure 4.1 A) and 16-fold (Figure 4.1 C) were observed between the mock-induced and SAR-induced Col-0 plants, indicating that SAR was successfully induced in these experiments. However, in the three replicates presented in Figure S1 (B, D, F), bacterial levels reached similar levels in

mock-induced versus SAR-induced Col-0 plants, indicating that SAR was not induced and established in these experiments. However, in these three experiments (Figure S1, A, C, E) phloem exudates collected from SAR-induced Col-0 leaves displayed DIR1 signals. The absence of SAR in mock- and SAR-induced Col-0 plants combined with the presence of DIR1 in phloem exudates in these experiments suggests that SAR induction did occur, as DIR1 was found in induced leaf exudates, but SAR was not established and/or manifested in the distant leaves.

Despite previous work by Carella et al. (2017) demonstrating that LTP2 was required for SAR in Arabidopsis, *ltp2-1* plants were not always SAR-defective as demonstrated by 5-fold (Figure 4.1 A) and 10-fold (Figure 4.1 B) reductions in *Pst* multiplication in SAR-induced compared to mock-induced *ltp2-1*. Additionally, the SAR competency of the *ltp2-1* mutants did not influence whether DIR1 signal was observed in SAR-induced *ltp2-1* local phloem exudates (Figure 4.1) as DIR1 signal was observed in experiments in which *ltp2-1* was SAR competent (Figure 4.1 C, D) and in other experiments in which *ltp2-1* was partially SAR defective (Figure 4.1 A, B).

Most replicate SAR assays (Figure 4.1 A, C, Figure S1 B, D) resulted in lower than expected levels of bacteria of 10^7 cfu/ld in the mock-induced Col-0 plants (Cameron et al., 1999, Carella et al., 2017). Low bacterial levels are often associated with stress, and many different stresses occurred during these experiments, including flooding of the growth chamber, power shutdowns, possible transgenerational

resistance in the seeds used, overwatering, fungal growth on the soil surface, and low light levels. Together, the variable SAR assays and the low bacterial levels in the control plants (mock-induced then challenged with virulent *Pst*) suggest that the stresses that occurred during the growth of the plants impacted the results of the experiments. Therefore, we cannot draw conclusions regarding the role of LTP2 in DIR1 movement from these experiments.

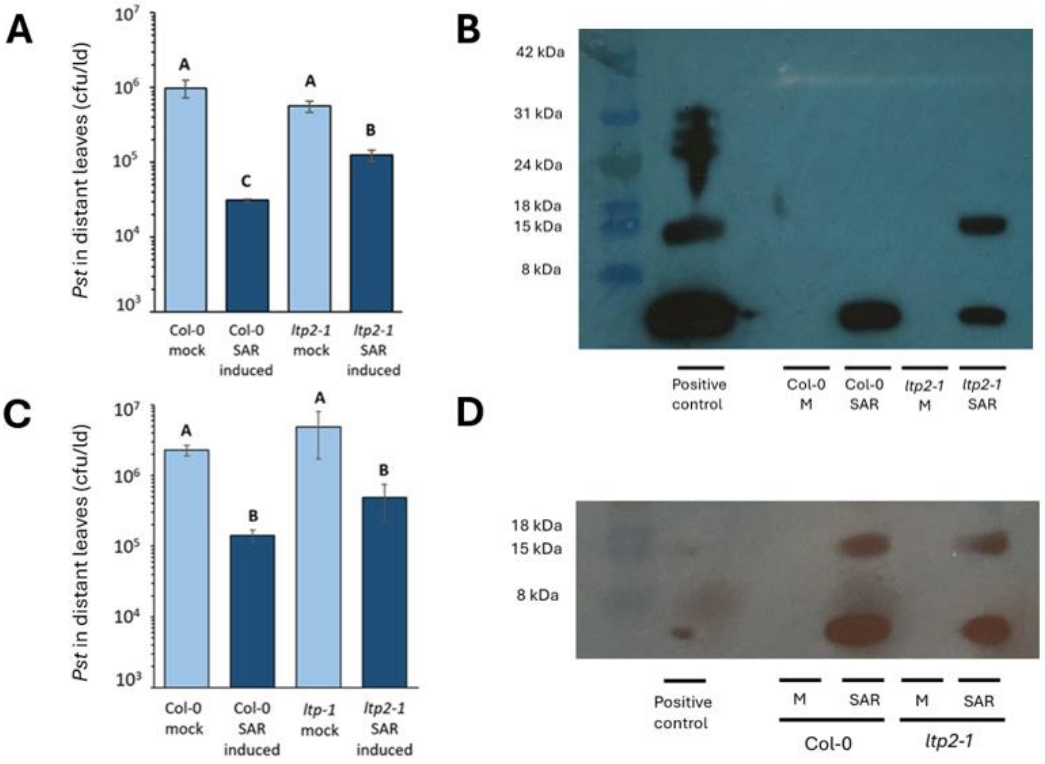


Figure 4.1: DIR1 is present in phloem exudates collected from SAR-induced (local) leaves of *ltp2-1* plants. A and **C**, bacterial levels in separate SAR assays conducted on Col-0 and *ltp2-1* plants. In each SAR assay, two lower leaves of Col-0 and *ltp2-1* plants were mock-induced with 10 mM $MgCl_2$ or SAR-induced with 10^6 colony forming units/ml (cfu/ml) *Pst AvrRpt2* bacteria. Two days later, plants were challenge-inoculated on upper leaves with 10^5 cfu/ml *Pst* bacteria. Bacteria were isolated from leaves three days later and plated for counting. Significant differences

are indicated by different letter groups (ANOVA with Tukey's honestly significant differences test, alpha = 0.05). **B** and **D** represent the corresponding DIR1 antibody immunoblots conducted on phloem exudates taken from local leaves of mock-induced (M) and SAR-induced (SAR) Col-0 and *ltp2-1* plants. The leftmost lane of each blot contains the protein ladder, while the positive controls are SAR-induced Col-0 phloem exudate from previous experiments where SAR was confirmed to be induced.

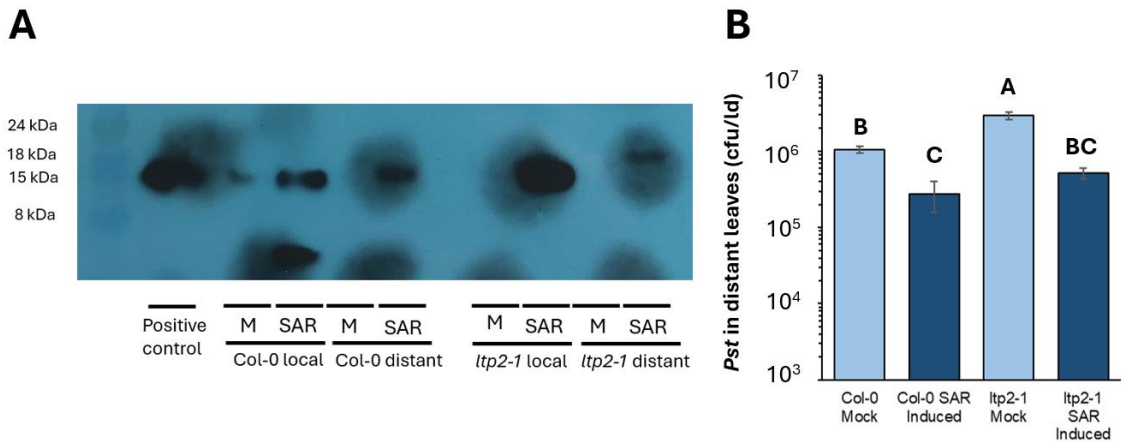


Figure 4.2: DIR1 signal is absent in phloem exudates of distant leaves from SAR-induced *ltp2-1* plants. A represents DIR1 antibody immunoblot of phloem exudates collected from local and distant leaves of mock-induced (M) and SAR-induced (SAR) Col-0 and *ltp2-1* plants. The leftmost lane contains the protein ladder, and the lane beside it contains leaf extracts from dD5e DIR1-overexpressing plants as a positive control (Maldonado et al., 2002). **B** Bacterial levels in a SAR assay on Col-0 and *ltp2-1* plants. Two lower leaves of 3.5 to 4-week-old Col-0 and *ltp2-1* plants were either mock-induced with 10 mM $MgCl_2$ or SAR-induced with 10^6 cfu/ml *Pst AvrRpt2* bacteria. Two days later, upper leaves were challenged with 10^5 cfu/ml *Pst* bacteria, followed by bacterial quantification three days later. Significant differences are indicated by different letter groups (ANOVA with Tukey's honestly significant differences test, alpha = 0.05).

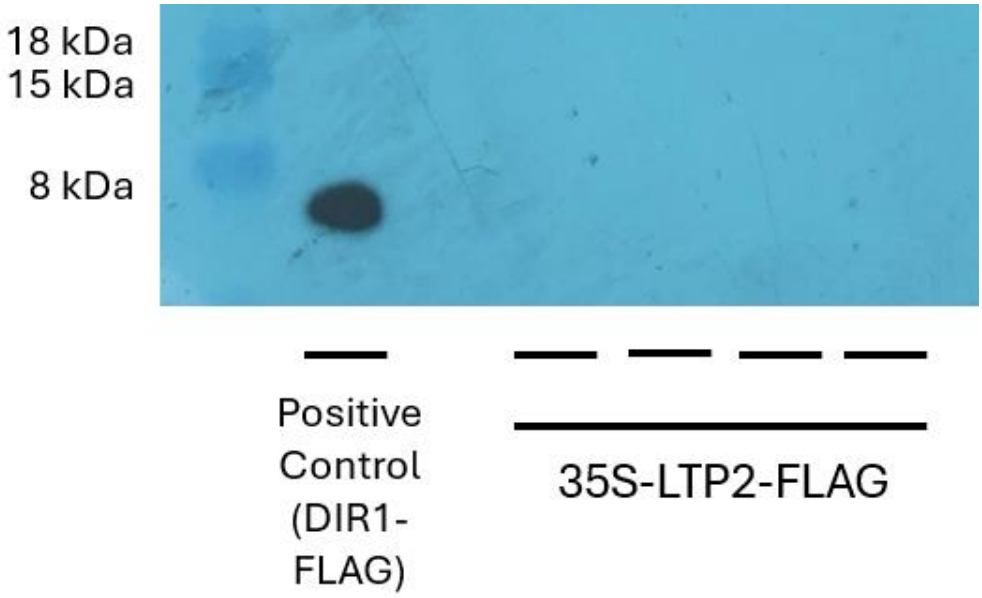


Figure 4.3: FLAG antibody immunoblot of 35S-LTP2-FLAG leaf extracts. 35S-LTP2-FLAG leaf extracts were subjected to SDS-PAGE followed by immunoblotting with a FLAG antibody. The protein ladder is shown in the leftmost lane, followed by the positive control (DIR1-FLAG leaf extracts; Carella 2016) in the lane next to it.

4.2 Examination of LTP2-FLAG stability in the 35S-LTP2-FLAG line

Given that DIR1 and LTP2 interacted with one another in a yeast-2-hybrid assay, it is possible that they also interact during SAR (Carella et al., 2017) and LTP2 may access the phloem during SAR like DIR1. To investigate whether LTP2 accesses the phloem during SAR, immunoblotting with FLAG antibodies of phloem exudates collected from SAR-induced 35S-LTP2-FLAG plants (Carella et al., 2017) was performed (Figure S2). Given the history of cleavage of DIR1 from protein tags (Champigny et al., 2013), and the fact that LTP2 is also a small lipid transfer protein, the stability of the LTP2-FLAG fusion was examined in the 35S-LTP2-FLAG line. The presence of FLAG signal in 35S-LTP2-FLAG leaf extracts would indicate the FLAG tag

was still attached, and therefore, that the line could be used to assess the presence of LTP2 in SAR-induced phloem exudates. Leaf extracts from 35S-LTP2-FLAG plants were subjected to SDS-PAGE followed by FLAG antibody immunoblotting. FLAG signal was not observed in any of the leaf extracts in a number of replicate experiments (data not shown, see lab notebook 2). Additional protein extractions were conducted on dry ice to reduce protein degradation, but FLAG signal was still not observed (Figure 4.3). Despite high protein levels in these extracts as measured by BCA assay (Figure 4.4), FLAG signal was only observed in the lane for the DIR1-FLAG positive control (Figure 4.3), or FLAG-BAP positive control (Figure S2). The absence of FLAG signal in these leaf extracts suggests that the FLAG tag does not remain attached to LTP2 in the 35S-LTP2-FLAG line. An experiment showed similar results where FLAG signals were not observed in phloem exudates collected from local leaves of mock-induced and SAR-induced 35S-LTP2-FLAG plants (Figure S2). The absence of FLAG signals in leaf extracts and phloem exudates provides strong evidence that the LTP2-FLAG fusion protein is not stable in the 35S-LTP2-FLAG transgenic line.

The ability of the 35S-LTP2-FLAG line to grow on hygromycin containing media was also investigated alongside the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines from chapter 3, to investigate if the transgenes were intact and expressed in these lines. The growth of 35S-LTP2-FLAG seedlings on hygromycin-containing media was investigated and compared to the growth of Col-0, DIR1-GUS, and DIR1-EGFP

seedlings as controls. The Col-0 seedlings demonstrated stunted growth, and all the DIR1-GUS seedlings appeared healthy as expected (Figure 4.5), however, most of the DIR1-EGFP seedlings did not all germinate (122/154, 104/168). Out of the DIR1-EGFP seeds that did germinate, the majority displayed a wild-type like growth phenotype (37/50, 32/46), This may be a result of the age of the DIR1-EGFP seeds (2019), which were older than the rest of the seeds used. 35S-LTP2-FLAG seedlings displayed a growth phenotype identical to the stunted growth of the Col-0 seedlings, with none displaying a healthy growth phenotype (Figure 4.5). This suggests that the 35S-LTP2-FLAG line is not expressing its transgenes and/or the 35S-LTP2-FLAG gene is not intact in the transgenic line.

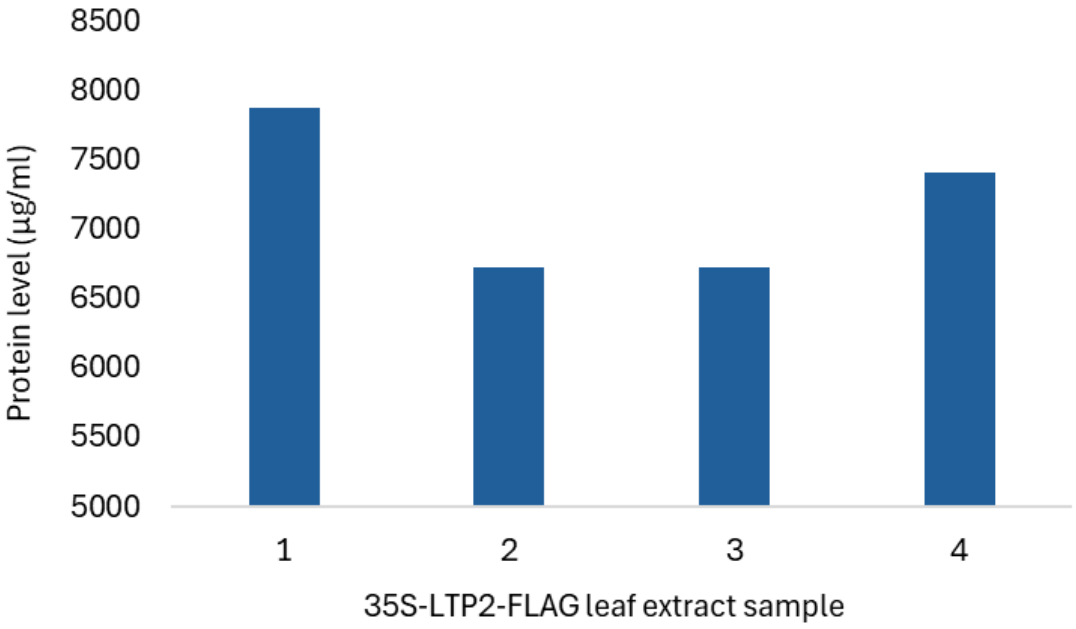


Figure 4.4: Protein levels in 35S-LTP2-FLAG leaf extracts. The leaves of 4 to 4.5-week-old 35S-LTP2-FLAG plants were collected, frozen, ground on dry ice and subjected to crude protein extraction. Leaf extract protein levels in µg/ml were measured using a BCA assay (Pierce).

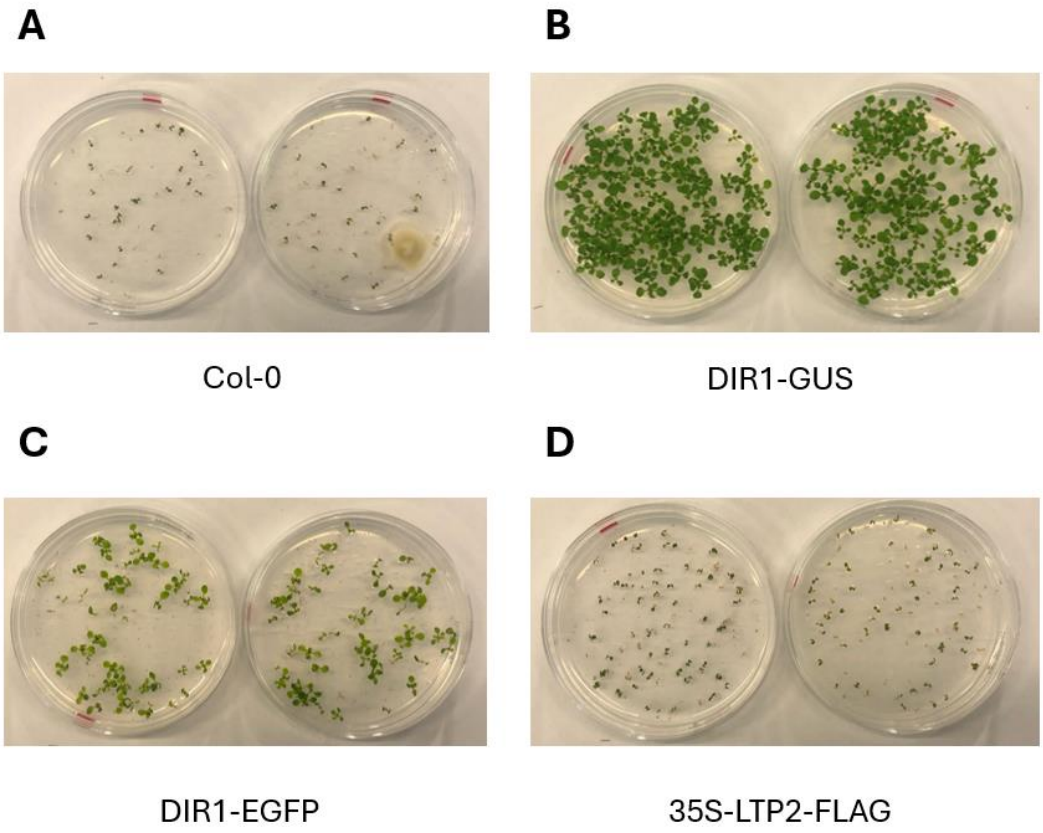


Figure 4.5: Growth of 35S-LTP2-FLAG seedlings on hygromycin-containing media. Seeds were sterilized and stratified followed by plating onto MS media containing 15 µg/ml hygromycin. **A** Col-0 represents the negative control, while **B** DIR1-GUS and **C** DIR1-EGFP represent positive controls. These controls were grown alongside **D** 35S-LTP2-FLAG.

Chapter 5: Exploring a core set of genes upregulated in the induction of SAR by varying treatments

5.1 Is there a core set of genes involved in the induction stage of SAR?

Large-scale gene expression changes occur in distant leaves during the establishment phase of SAR (Gruner et al., 2013), with the expression of genes such as PR1 being used as a marker for SAR establishment in distant tissues (Klessig and

Malamy, 1994). However, there is less information about the genes that are expressed and important for the induction stage of SAR. At the site of infection, local immune responses occur, making it difficult to disentangle which changes in gene expression are related to local immunity versus the SAR induction stage. To identify genes whose functions may contribute to the induction stage of SAR, a bioinformatic approach was taken to compare the genes expressed in leaves after different SAR-inducing treatments. A core set of genes was hypothesized to be expressed in all SAR-inducing treatments. These genes would be candidates for further investigation of their potential roles in SAR by the Cameron lab.

Transcriptome data was available for three different SAR-inducing treatments (1 PTI and 2 ETI). To investigate gene expression changes in PTI-responding plants, an RNA-seq dataset was generated by Cameron lab members Noah Xiao and Garrett Nunn. Col-0 leaves were mock-treated or treated with 1 μM of the PAMP flg22 to induce PTI, and leaves were collected at 0-, 6-, 12-, 18-, and 24- hours post-treatment. Leaves were subjected to RNA extraction and sent for RNA sequencing analysis. PAMP treatment alone is thought to be sufficient to induce SAR (Mishina and Zeier, 2007), therefore the PTI RNA-seq dataset may provide information about the induction of SAR via PTI alone. The PTI RNA-seq data was compared to the ETI RNA-seq dataset generated by Mine et al. (2018), in which Col-0 plants were inoculated with 5x 10^5 cfu/ml *Pst AvrRpt2* or *Pst AvrRpm1* bacteria, followed by leaf collection at 3-, 6-, 12-, 16-, and 24-hours post-inoculation. As these strains of *P. syringae* carry the effector

proteins AvrRpt2 and AvrRpm1 respectively, they initiate ETI as Arabidopsis carries the corresponding NLR receptor genes, *RPS2* and *RPM1*. While these ETI datasets provide information about SAR induced via ETI, these *Pst* strains also carry PAMPs, therefore SAR was induced by both PTI and ETI.

5.2 Pre-processing and quality assessment of RNA-seq data

Before the datasets could be compared, the Mine et al. (2018) dataset was pre-processed using the same pipeline used to pre-process the Cameron Lab PTI RNA-seq data (Nunn, 2020). Initial quality assessment was performed using FastQC (Andrews, 2010), with the FastQC reports indicating some reads had lower quality scores at their ends (Figure S3 A, B), and were contaminated by adapter sequences used as part of the sequencing procedure (Figure S4 A, B). To remove the trailing low-quality bases and contaminating adapter sequences, the sequences were processed using Trimmomatic (Bolger et al., 2014). FastQC reports of samples after trimming revealed an increase in quality scores near the read ends, in addition to reductions in adapter contamination, suggesting that Trimmomatic was successful in removing low quality bases and adapter sequences (Figure S3 C, D; Figure S4 C, D). Once the reads were trimmed, they were aligned against the *Arabidopsis thaliana* TAIR 10 genome assembly using the STAR aligner (Dobin et al., 2013). Htseq-count (Anders et al., 2014) was used to count how many reads corresponded to each gene in the genome and to throw out any reads that mapped ambiguously

(to more than one gene). Counts were imported into R alongside the Cameron lab PTI count data. Since this comparison is being conducted on data collected in two different labs, it is possible that the datasets will have differences in expression that result from different lab conditions, even though the Mine et al. (2018) dataset was collected using similar plant growth conditions as the Cameron Lab.

To investigate if the data differs between labs, a PCA was performed using a model of all the data where 'dataset' was the only variable used to test for differences (Figure 5.1 A). The PCA demonstrated that the data clustered based on dataset – the Cameron lab data, labelled as 'PTI' clustered closer to the upper left corner of the PCA plot, whereas the Mine et al. (2018) data, labelled as 'ETI', clustered near the bottom right corner of the plot. This pattern of clustering does suggest that some of the differences in the data are due to between-lab differences. A second PCA plot conducted using 'timepoint' and 'treatment' as variables revealed that the mock-treated samples clustered close together with one another, while the samples for each treatment tended to cluster near one another as well (Figure 5.1 B). Samples also clustered further based on timepoint in addition to treatment – for example, the flg22 treated samples collected at 12 hours post-treatment cluster closely to one another and separately from the flg22 treated samples collected at 6- and 24-hours post-treatment. This suggests that the RNA-seq count data clusters based on treatment and timepoint in addition to clustering by dataset.

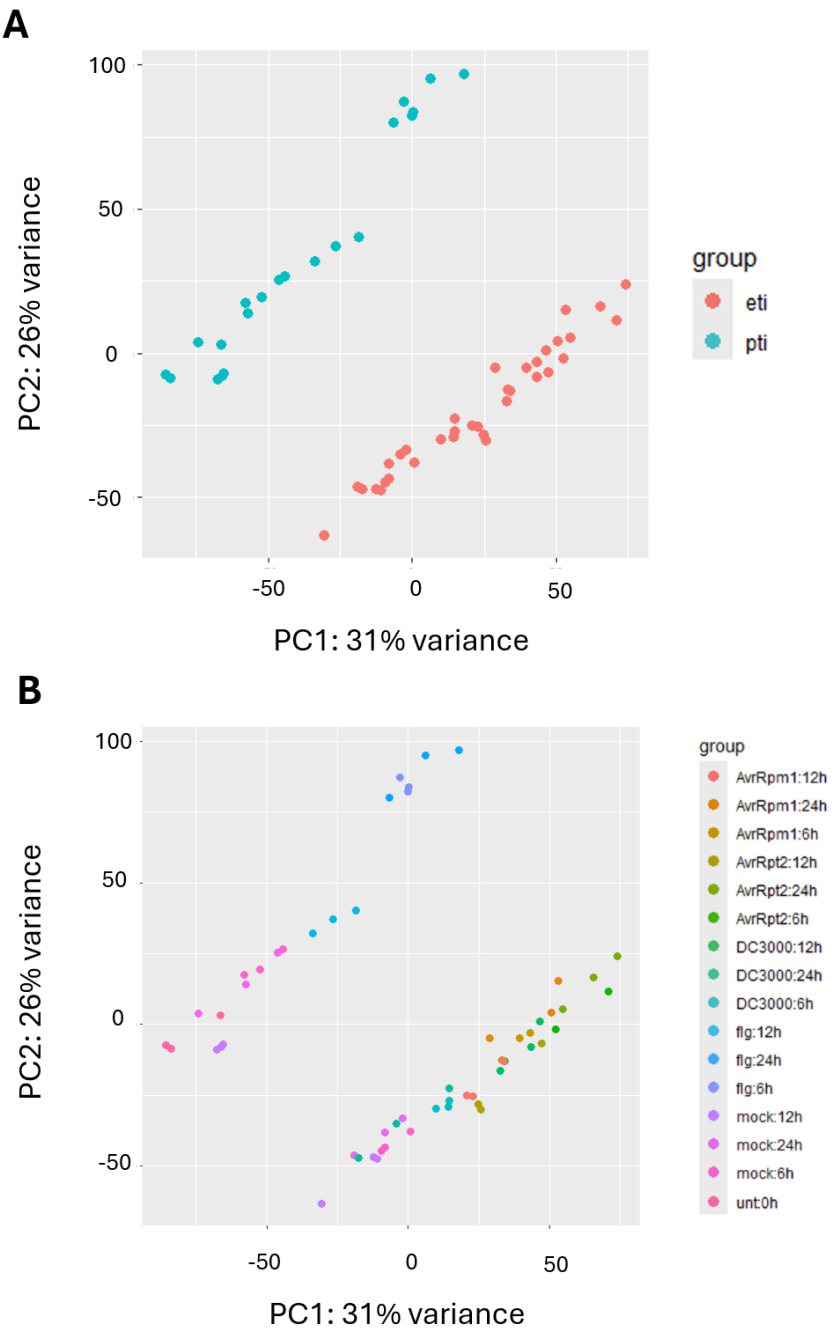


Figure 5.1: **Clustering of RNA-seq count data by principal component analysis (PCA).** *Arabidopsis* Col-0 leaf tissue was mock-treated or treated with 1 µM flg22 peptide, collected at 6-, 12-, or 24-hours post-treatment, and subjected to RNA extraction followed by RNA-seq analysis. This data was compared against RNA-seq data collected by Mine et al. (2018) consisting of Col-0 leaf tissue that was mock-inoculated, inoculated with 5 x 10^5 cfu/ml of either *Pst AvrRpt2* or *Pst AvrRpm1* bacteria and collected at 6-, 12-, or 24-hours post-treatment. **A** Clustering of the

count data via PCA using 'dataset' as the only variable, with the Cameron lab data ('PTI') shown in turquoise, and the Mine et al. (2018) data ('ETI') shown in red. **B** PCA plot with timepoint and treatment used as variables. The legend indicating timepoint/treatment group is shown at the right.

5.3 Comparison of datasets

To investigate which genes were upregulated post treatment between all three SAR-inducing treatments, the DESeq2 package (Love et al., 2014) for R was used to perform differential expression analyses on the 6-, 12-, and 24-hour post treatment data. Once differentially expressed genes were identified, they were filtered by adjusted p-value (cutoff of <0.05) and $\log_2$ fold change (>2). The filtered results of each differential expression analysis were compared to generate Venn diagrams of the genes upregulated by all three treatments at each timepoint (6-, 12-, and 24-hours post-treatment). This revealed that 352 genes were upregulated within 6 hours post treatment in all three treatments, while 102 were upregulated in all three treatments 12 hours post treatment (Figure 5.2). Only 41 genes were upregulated in all three treatments 24 hours post treatment, a much smaller number than seen at 6- or 12- hours post treatment (Figure 5.2). A summary table of SAR-related genes and genes of interest upregulated in all three timepoints is shown below (Table 5.1). Additionally, a summary table of uncharacterized genes/genes of unknown function upregulated by all three treatments at multiple timepoints is available in the supplementary information (Table S2). Out of these uncharacterized genes/genes with unknown functions, only four were upregulated at all three timepoints, while

fourteen were upregulated at 6- and 12-hours post treatment, three were upregulated at 6- and 24-hours post treatment, and only one was upregulated at both 12- and 24-hours post treatment.

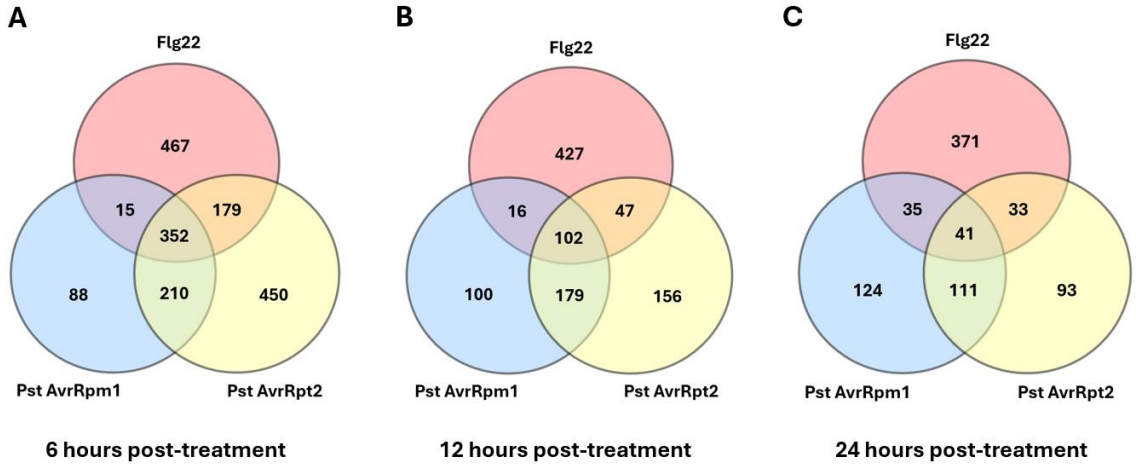


Figure 5.2: Common genes upregulated during SAR induction by differing methods at multiple timepoints. *Arabidopsis* Col-0 plants were mock-treated or treated with 1 µM flg22 followed by tissue collection 6-, 12-, or 24-hours post-treatment. Tissue was frozen and subjected to RNA extraction and RNA sequencing analysis. This data was compared to RNA-seq data collected by Mine et al. (2018) where Col-0 plants were mock-inoculated or SAR-induced using 5 x 10^5 cfu/ml of either *Pst AvrRpt2* or *Pst AvrRpm1* bacteria and leaf tissue was collected 6-, 12-, or 24-hours post treatment. Both datasets were subjected to differential expression analysis using the DESeq2 (Love et al., 2014) package for R and genes upregulated in response to each SAR-inducing treatment were compared via Venn diagram (adjusted p-value cutoff was <0.05; $\log_2$ fold change cutoff was >2). **A** Genes upregulated at 6 hours post treatment. **B** Genes upregulated at 12 hours post treatment. **C** Genes upregulated 24 hours post treatment.

Within 6 hours, many well-studied genes important for the PTI, ETI and SAR responses were upregulated in all treatments, including NHP biosynthesis genes, *FMO1* and *ALD1*, the salicylic acid biosynthesis genes *ICS1*, *PBS3*, and the SA/NHP

transporter *EDS5* (Table 5.1). This demonstrates that PTI, ETI and SAR were induced appropriately in these experiments. Other SAR-related genes were also upregulated, including SAR marker gene *PR1* and the glycosyltransferase *UGT76B1*, responsible for glycosylating NHP (Holmes et al., 2021). In addition to genes expected to be upregulated during the induction of SAR, numerous other genes of interest were upregulated in response to all three treatments (Table 5.1). This includes genes currently under investigation by the Cameron lab, such as *PLEIOTROPIC DRUG RESISTANCE 12* (*PDR12*), a putative SA transporter (G. Nunn), and *MULTIPLE C2 DOMAIN AND TRANSMEMBRANE PROTEIN 9* (*MCTP9*), which may play a role in DIR1 movement during SAR (N. Belu). The *SYNTAXIN OF PLANTS 122* (*SYP122*) gene was included as a gene of interest as it is closely related to *SYNTAXIN OF PLANTS 121* (*SYP121*) gene (Sanderfoot et al., 2000), which is also being investigated in the Cameron lab for a potential role in SAR.

Additionally, genes previously investigated by the Cameron lab also appeared, such as PDLP5, a plasmodesmata-located protein which when overexpressed reduces movement through plasmodesmata (Lee et al., 2011) and was shown to impede DIR1 movement to distant leaves (Carella et al., 2015). However, this list also included genes not previously investigated by the Cameron lab, such as the three genes encoding members of the Arabidopsis Phloem Protein 2 family (AtPP2), specifically members A6, A7, and B6. Finally, members of the lipid transfer protein

family were seen, including LTPG5, and AZI3 - a member of the same family of LTPs as the SAR-required protein AZI1 (Cecchini et al., 2015).

AGI ID	Gene Symbol	Description	Timepoint (hpt)
AT1G15520	PDR12	Pleiotropic Drug Resistance 12	6
AT1G19250	FMO1	Flavin-dependent Monooxygenase 1	6, 12
AT1G70690	PDLP5	Plasmodesmata Located Protein 5	6
AT1G74710	ICS1	Isochorismate Synthase 1	6
AT2G02310	ATPP2-B6	Phloem protein 2 – B6	12
AT2G13810	ALD1	AGD-like Defense Response Protein 1	12
AT2G14610	PR1	Pathogenesis-Related 1	6, 12, 24
AT3G11340	UGT76B1	UDP-glycosyltransferase 76B1	6
AT3G22600	LTPG5	Lipid Transfer Protein GPI-anchored 5	6
AT3G52400	SYP122	Syntaxin of Plants 122	6
AT4G00700	MCTP9	Multiple C2 domain and transmembrane region protein 9	6
AT4G12490	AZI3	Azelaic acid induced 3	24
AT4G39030	EDS5	Enhanced disease susceptibility 5	6
AT5G13320	PBS3	AvrPphB Susceptible 3	6
AT5G45080	ATPP2-A6	Phloem protein 2 – A6	6
AT5G45090	ATPP2-A7	Phloem protein 2 – A7	12, 24

Table 5.1: Genes upregulated during SAR induction by flg22, *Pst AvrRpt2*, and *Pst AvrRpm1*. *Arabidopsis* Col-0 leaf tissue was mock-treated or treated with 1 µM flg22 followed by tissue collection at 6- 12 and 24-hours post-treatment. Leaf tissue was subjected to RNA extraction followed by RNA-sequencing analysis. This data was compared to data collected by Mine et al. (2018), where leaf tissue was mock-inoculated, or SAR-induced using either *Pst AvrRpt2* or *Pst AvrRpm1* and tissue was collected at 6-, 12-, or 24-hours post-treatment. The DESeq2 package for R was used to compare the count data between datasets and identify differentially expressed genes upregulated during SAR induction (adjusted p-value cutoff <0.05; $\log_2$ fold change cutoff >2). The table above lists a selection of genes identified as being upregulated between all three SAR-inducing treatments, as well as the timepoint(s) in hours-post-treatment (hpt) they appeared to be upregulated at. The table lists both well-known genes involved in SAR (highlighted in blue) and genes of interest (highlighted in grey).

Chapter 6: Discussion, Conclusions, and Future Directions

6.1 The HA-DIR1-ILOV and FLAG-DIR1-PHILOV lines do not produce fusion protein

The growth of HA-DIR1-iLOV on hygromycin-containing media indicated that the hygromycin gene is present and expressed in this line, and the transgenic constructs are intact. The poor growth of some of the FLAG-DIR1-like-phiLOV seedlings may indicate that the transgenic constructs are not intact or expressed or may be due to contamination of the seed stock, as these plants were grown alongside plants of other genotypes such as Col-0. As the FLAG-DIR1-like-phiLOV seeds used in this experiment are relatively new (2023), the poor growth of some of the FLAG-DIR1-like-phiLOV seedlings is unlikely to be related to seed age.

While previous DIR1 fusion proteins using GUS, EGFP, or EYFP tags resulted in the cleavage of the tags from the C terminus of DIR1 leaving DIR1 intact (Champigny et al., 2013), the lack of DIR1 signal observed in leaf extracts of the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines suggests these tags are not being cleaved from DIR1 in a similar manner as observed in Champigny et al. (2013). Both the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV constructs are driven by a double 35S promoter, meaning if the tags were being cleaved from the fusion proteins leaving intact DIR1, we would expect to still see high levels of DIR1/DIR1-like protein in the leaf extracts, appearing as strong signal at 7 and/or 14 kDa on DIR1 immunoblots. The observation of no DIR1/DIR1-like, HA, or FLAG signal at all instead suggests that the fusion

protein is not present in the leaf extracts. It is possible that the addition of either the C-terminal fluorescent tags or N-terminal peptide tags results in instability that leads to degradation of the fusion proteins.

When creating these lines, Isaacs (2013) proposed that one of the reasons for the cleavage of EGFP and/or GUS tags from earlier DIR1 fusions was due to the tag location at the C terminus of DIR1 -as the C terminus of DIR1 participates in a disulfide bond (Lascombe et al., 2008). Additionally, it was observed that the cleavage of the C-terminal tags was associated with the movement of DIR1 into the phloem during the induction stage of SAR (Champigny et al., 2013) – which could suggest that the C terminus of DIR1 is important for the structure or function of DIR1 during SAR. As the fluorescent iLOV and phiLOV tags in the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines are also attached to the C-terminus of DIR1/DIR1-like, it is possible that they could be interfering with the proper structure or folding of DIR1. Additionally, these fusion proteins contain HA or FLAG peptides fused to the N terminus of DIR1/DIR1-like. DIR1 is secreted to the apoplast and requires an N-terminal signal peptide to first direct it to the endoplasmic reticulum (ER) (Champigny et al., 2011). Due to the presence of the signal peptide, the peptide tags had to be fused internally after the signal peptide, making it possible that the fusion of the peptide tags after the signal peptide resulted in improper folding of the protein. Improper folding of proteins in the endoplasmic reticulum (ER) can result in endoplasmic reticulum assisted degradation (ERAD) of the misfolded proteins, as

the accumulation of large amounts of misfolded protein can result in ER stress and the unfolded protein response (UPR) (Fanata et al., 2013). If the fusion proteins were being degraded because of improper folding, it could result in the observed absence of signal on the immunoblots of leaf extracts conducted with both the DIR1 and HA/FLAG antibodies. Since the fusion proteins are absent from leaf extracts and possibly being degraded, the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines cannot be used as a tool to trace the movement of DIR1 during SAR.

6.2 The role of LTP2 in DIR1 movement during SAR

Since multiple types of stress occurred during each SAR assay and phloem exudate collection experiment, it is difficult to draw concrete conclusions from the data. However, the presence of DIR1 signal in the phloem exudates of local but not distant *ltp2-1* leaves was relatively consistent when SAR was and was not successfully manifested, despite various stresses occurring during plant growth, and despite variations in SAR competency of the *ltp2-1* mutant. The consistency of this phenotype in *ltp2-1* plants despite these differing conditions between experiments does provide evidence to suggest that LTP2 may not be required for DIR1 to enter the phloem but may be required for the movement of DIR1 to distant tissues during SAR. If this is the case, it also provides support for the idea that DIR1 and LTP2 may interact in the phloem during SAR (Carella et al., 2017), potentially as part of a multiprotein SAR signaling complex. Still, the various stresses that occurred during

the growth of the plants, such as the fungal contamination of the soil, represent confounding factors. The experiments conducted with fungal growth present on the soil surface tended to display modest or nonexistent SAR responses and low bacterial levels in mock-treated Col-0 plants in the SAR assay portion of the experiment. This suggests that the fungus growing on the soil may induce resistance or impact the SAR response in Arabidopsis.

6.3 Abiotic stresses can influence the plant response to biotic stress

In addition to the fungal contamination issues, some of the stresses present during plant growth were abiotic stresses such as overwatering or low light levels. Abiotic stresses such as drought and flooding have the potential to influence the plant response to biotic stress through crosstalk between abiotic and biotic stress signaling, with Atkinson and Urwin (2012) highlighting in a review that plants respond very differently to combinations of stresses compared to individual stresses. The phytohormone abscisic acid (ABA), involved in the response to abiotic stresses such as drought, has the potential to negatively or positively influence the plant response to biotic stresses (Atkinsion and Urwin, 2012). A recent review by Pandey et al. (2023) noted that exposure to drought stress preceding the exposure to *P. syringae* has the potential to reduce the severity of the infection. Pandey et al. (2023) use the term ‘eustress’ to describe this ability of one stress to result in a protection against another type of stress. Additionally, it has been demonstrated

that the ABA and SA pathways interact with and influence one another (Atkinson and Urwin, 2012, Mohr and Cahill, 2007, Yasuda et al. 2008). For example, the transcription factor MYB96 is activated in response to ABA and is proposed to promote cuticular wax biosynthesis as a way to compensate for drought stress (Seo et al., 2011). MYB96 is also promotes the biosynthesis of SA (Seo and Park, 2010), and MYB96-overexpressing plants express the SA biosynthetic gene *ICS1* to a greater degree and accumulate higher levels of SA compared to wild-type plants.

While a relationship between overwatering and the development of resistance has been observed in the Cameron Lab over many years, Hsu et al. (2013) reported that flooding was associated with resistance to *Pst* DC3000 and the expression of defense-related genes in Arabidopsis. Both *ltp2-1* and Col-0 plants grown in late 2022 for seed were both under- and over watered, followed by use of these seeds for experiments throughout the spring and summer of 2023. As overwatering has been associated with resistance, and it has been demonstrated that SAR can be transgenerational (Luna et al., 2011, Luna and Ton, 2012), it is possible that the plants used in these experimental replicates were displaying transgenerational SAR, resulting in the low bacterial levels in mock-inoculated Col-0 plants. Additionally, it is possible this transgenerational resistance could explain the SAR-competent phenotype of *ltp2-1* seen in some of these experiments which conflicts with the SAR-defective phenotype observed in *ltp2-1* described by Carella et al. (2017).

6.4 The LTP2-FLAG protein is not present in the 35S-LTP2-FLAG line

The 35S-LTP2-FLAG line was created in the *ltp2-1* mutant background and overexpression of LTP2-FLAG in these lines restored the wild-type, SAR-competent phenotype (Carella et al., 2017) suggesting that the 35S-LTP2-FLAG line expressed functional LTP2 protein. In my studies, the absence of FLAG signal in the 35S-LTP2-FLAG leaf extracts could suggest that the FLAG tag is being cleaved from LTP2 in this line as was seen in the DIR1-GUS, DIR1-EGFP and DIR1-EYFP lines (Champigny et al., 2013). The AlphaFold Protein Structure Database (Varadi et al., 2022) predicts LTP2 to contain four alpha helices, while UniProt (The UniProt Consortium, 2023) lists LTP2 as containing four disulfide bonds, with one of those bonds predicted to occur near the C terminus – this is similar to the structure of DIR1 described by Lascombe et al. (2008). Interestingly protein tags fused to DIR1 are thought to be cleaved from the C terminus of DIR1 after SAR induction during movement of DIR1 to distant leaves via the phloem (Champigny et al., 2013), which suggests the C-terminus of DIR1 could be important for its function during SAR. It is possible that like DIR1, the C-terminus of LTP2 is important for its structure or function either during SAR or in healthy tissues, resulting in the FLAG tag destabilizing LTP2 and ultimately being cleaved.

The inability of the 35S-LTP2-FLAG seedlings to grow on hygromycin-containing media also raises the possibility that the transgenes in this line have been silenced,

with numerous cases of 35S-promoter driven transgene silencing being reported in the literature (Meyer et al., 1992, Elmayan and Vaucheret, 1996, Mishiba et al., 2005). Silencing of transgenes can occur at the transcriptional level, preventing the transcription of the gene, or at the post-transcriptional level, preventing transcript accumulation (Rajeevkumar et al., 2015). At the transcriptional level, cytosine methylation occurring in the promoter (Meyer et al., 1992, Mishiba et a., 2005) or coding region (Mishiba et al., 2005) of the transgene can lead to silencing (Rajeevkumar et al., 2015). Rajeevkumar et al. (2015) highlight that this methylation may be a result of the site the transgene integrated into the genome, the copy number of the transgene, methylation status of homologous genes, or environmental changes (Meyer et al., 1992). Additionally, silencing of transgenes can occur at the post-transcriptional level, with Schubert et al. (2004) proposing a model where expression over a gene-specific threshold triggers silencing. Post-transcriptional silencing can be triggered by the formation of double-stranded RNA (dsRNA) due to the transcription of an inverted repeat of the transgene or a homologous gene (Rajeevkumar et al., 2015). Additionally, recent work by Butel et al. (2021) suggests that transgenes are epigenetically distinct from endogenous genes, resulting in an increased level of aberrant RNA transcription that leaves transgenes susceptible to post-transcriptional silencing.

If silencing occurred in the 35S-LTP2-FLAG line, little to no LTP2-FLAG protein would be produced, consistent with the absence of FLAG signal on immunoblots of 35S-

LTP2-FLAG leaf extracts. While it is interesting to speculate on the possible reasons for the absence of LTP2-FLAG protein in the 35S-LTP2-FLAG line, this absence means this line is not useful for investigating the presence of LTP2 in the phloem during SAR through FLAG antibody immunoblotting of LTP2-FLAG.

6.5 Genes previously and currently investigated by the Cameron lab are upregulated during SAR induction

Many of the genes upregulated during SAR induction in response to all three treatments were genes that were expected to be upregulated, such as the SA and NHP biosynthetic genes *ICS1*, *PBS3*, *FMO1* and *ALD1*. Some of the genes that were upregulated were genes currently or previously studied by the Cameron lab – such as the *PDLP5* gene (Carella et al., 2015). *PDLP5* is also known as *HOPW1-1 INDUCED GENE 1* as its expression is upregulated in response to infection by *P. syringae* strains carrying the HopW1-1 effector protein (Lee et al., 2008). PDLP5 is localized to plasmodesmata and plays a role in the deposition of callose during infection (Lee et al., 2011), with callose deposition being thought to fortify plant cell walls (Li et al., 2016) and narrow the plasmodesmatal aperture (Xu et al., 2017). Overexpression of PDLP5 was used by Carella et al. (2015) to reduce movement through plasmodesmatal apertures and investigate the movement of DIR1 to distant leaves during SAR. Work by Lee et al. (2011) revealed a role for PDLP5 in local resistance, as *pdlp5-1* mutant plants inoculated with *Psm* supported higher

bacterial levels than Col-0. Further study (Tee et al., 2023) identified that the protein NON-RACE SPECIFIC DISEASE RESISTANCE /HIN1 HAIRPIN-INDUCED-LIKE 3 (NHL3) interacted with PDLP5 at plasmodesmata and was required for PDLP5's function in plasmodesmatal callose deposition in response to infection (Tee et al., 2023). This led Tee et al. (2023) to propose a model where PDLP5 and NHL3 work together to regulate callose deposition at plasmodesmata during PTI. However, the fact that PDLP5 expression was upregulated at 6-hours post-treatment in all treatments suggests it is important during the early part of the SAR induction stage and warrants further investigation.

Another gene studied by the Cameron lab and upregulated in response to all three SAR-inducing treatments is MCTP9 (MULTIPLE C2 DOMAIN AND TRANSMEMBRANE REGION PROTEIN 9), a plasma-membrane localized protein that is part of the MCTP family of proteins, with 16 members in Arabidopsis (Liu et al., 2018). Liu et al. (2012) demonstrated that the MCTP protein FT-INTERACTING PROTEIN 1 (FTIP1) is involved in the trafficking of the mobile peptide signal FLOWERING LOCUS T (FT) through plasmodesmata from companion cells into sieve elements. As this is a similar to how DIR1 is thought to access sieve elements for the movement to distant leaves during SAR (Carella et al., 2015), ongoing work in the Cameron lab by Natalie Belu is focused on the role of MCTPs, including MCTP9, in SAR. The fact that expression of MCTP9 is upregulated in response to all three SAR-inducing treatments provides

additional evidence to suggest that MCTP9 may play a role in SAR and is a good candidate for continued investigation.

6.6 Phloem Protein 2 (PP2) genes A6, A7, and B6 represent candidates for investigation

At multiple timepoints, expression of the *ATPP2-A6*, *-A7* and *-B6* genes was upregulated in response to all SAR induction treatments. Dinant et al. (2003) described PP2 and PP2-like proteins in multiple plant species, including monocots and dicots, as well as the non-vascular plant *Physcomitrella patens*. These proteins are members of a 30-member protein family in Arabidopsis (Dinant et al., 2003). The PP2 family members *AgPP2-1* and *AgPP2-2* from celery were expressed in the phloem as shown by *in situ* hybridization, with strong signals observed in sieve elements and companion cells (Dinant et al., 2003) Similarly, expression of *AtPP2-A1* and *AtPP2-A2* from Arabidopsis was also observed in the phloem at sieve elements and companion cells using *in situ* hybridization (Dinant et al., 2003). Additionally, Golecki et al. (1999) observed the translocation of PP2 proteins in the phloem by grafting rootstocks of *Cucurbita maxima* to shoots of *Cucumis sativa*, followed by the collection of phloem exudates from the *Cucumis sativa* shoot 15 days after grafting. Immunoblotting of these phloem exudates revealed that *Cucurbita maxima* PP2 was present in the phloem of *Cucumis sativa* shoots, suggesting it can translocate in the phloem (Golecki et al., 1999). While it remains

unknown if Arabidopsis AtPP2-A6, A7, and B6 are expressed in sieve elements and companion cells of the phloem or translocate long-distance via the phloem, the fact that some PP2 proteins are expressed in this location and move through the phloem is of interest considering that DIR1 travels to distant tissues in the phloem via plasmodesmata joining companion cells and sieve elements (Carella et al., 2015).

The 30 PP2 Arabidopsis family members also contain various N-terminal domains, with A6 containing a TIR domain, and B6 containing an F-box domain. Dinant et al. (2003) proposed that the variety of domains associated with signaling and protein interaction mean that PP2 proteins are likely involved in signaling. Some members of this family in Arabidopsis may function in immunity, with work by Santamaria et al. (2019) revealing a role in immunity for AtPP2-A5, which appears to be structurally similar to the ATPP2-A6 protein that was expressed in response to all 3 SAR-inducing treatments (Dinant et al., 2003). The overexpression of *ATPP2-A5* in Arabidopsis resulted in increased resistance to spider mites, while *atpp2-5* mutants were more susceptible to spider mites (Santamaria et al., 2019). Overall, the expression patterns of PP2 family proteins (Dinant et al., 2003), their potential long-distance travel in the phloem (Golecki et al., 1999), and functions in immunity (Santamaria et al., 2019) suggest PP2s could be involved in SAR signal transport into the phloem or be associated with a SAR signaling complex in the phloem, and therefore PP2 A6, A7, and B6 are candidates for further investigation.

6.7 Two Lipid Transfer Proteins are upregulated during SAR induction

As DIR1 and LTP2 are lipid transfer proteins that play roles in SAR, it was also of interest to see if other LTP genes were upregulated by all three SAR-inducing treatments. Two LTP genes were identified as being commonly upregulated by all SAR-inducing treatments, with LTPG5 being upregulated at 6 hours post treatment, and AZI3 being upregulated 24 hours post treatment. AZI3 is a protein containing a Proline rich region followed by a l8-cysteine motif lipid transfer protein domain and is part of the same protein family as AZI1- which plays a role in SAR (Cecchini et al., 2015). Work by Maier et al. (2021) investigating genes upregulated in response to infection by 38 strains of bacteria from 16 families identified that AZI3 was part of 24 genes commonly upregulated in response to infection, with this group of genes being designated as part of a General Non-Self Response (GNSR). Additionally, work by Chassot et al. (2007) identified that the overexpression of AZI3 resulted in enhanced resistance to the fungal pathogen *Botrytis cinerea*. While the similarity of AZI3 to AZI1, including similar structure and localization (Cecchini et al., 2015), makes it a potential candidate for a role in SAR, it may also be involved in local defense responses rather than in SAR.

6.8 Limitations of comparing flg22-induced SAR to bacterial-induced SAR

As SAR was induced through PTI alone in the Cameron lab dataset, and by PTI and ETI together in the Mine et al. (2018) dataset, it is possible that many of the

upregulated genes commonly expressed in all three methods of inducing SAR, are related to the PTI response. As PTI was induced in all three treatments, and transcriptional changes occur in the infected leaf as part of the PTI response (Couto and Zipfel, 2016; Yuan et al., 2021a), this makes it difficult to distinguish which genes are acting in the induction of SAR rather than in the PTI response. And while some genes, such as the SA biosynthetic gene *ICS1* (Wildermuth et al., 2001) are important for both local and systemic immunity, the dual role of some genes in local and systemic immunity also makes it more difficult to distinguish which genes are acting specifically during SAR induction specifically. To more clearly discern which genes are involved in the induction of SAR, a better approach would be to compare SAR induced by PTI to SAR induced solely by ETI. The estrogen-inducible AvrRpt2-expressing Arabidopsis line, XVE:AvrRpt2/RPS2 could be used. In this line, the effector protein AvrRpt2 is expressed under the control of the estrogen-inducible XVE promoter, where estrogen treatment results in AvrRpt2 expression, and the recognition of AvrRpt2 by RPS2 receptor which initiates ETI (Axtell and Staskawicz, 2003).

6.9 Future Directions

1) As various stresses occurred during the experiments aimed at determining whether LTP2 was required for the movement of DIR1 into the phloem or to distant

leaves during SAR, these experiments will need to be repeated in the absence of stresses.

2) The stunted growth phenotype of the 35S-LTP2-FLAG seedlings grown on hygromycin-containing media suggests that the transgenic constructs in this line have been silenced. However, the seeds used to grow 35S-LTP2-FLAG plants in this thesis were from 2023. It may be of interest to grow older 35S-LTP2-FLAG seeds (from 2016) on hygromycin-containing media to investigate if the transgenic constructs are being expressed in these older seeds.

3) The investigation of a common set of genes involved in the induction stage of SAR revealed some interesting candidates for further investigation. AZI3, a lipid transfer protein in the same family as the SAR-required AZI1 (Cecchini et al., 2015), the phloem proteins PP2 A6, A7, and B6, and the previously investigated PDLP5 represent candidates for further investigation. To investigate whether these genes are required for SAR, it may be of interest to perform SAR assays on *azi3*, *pdlp5*, *pp2-a6*, *a7*, and *b6* mutants to identify if they are SAR defective.

4) It is possible that some of the genes of interest upregulated during SAR induction by all three treatments were related to the PTI response rather than SAR induction. To investigate their potential involvement in the PTI response, local resistance assays could be conducted on *azi3, pp2-a6, -a7*, and *-b6* mutants using *Pst* bacteria and/ or treatment with flg22 peptide.

5) Phloem proteins from the PP2 family have been previously localized to sieve-element companion cell complexes (Dinant et al., 2003), can translocate in the phloem (Golecki et al., 1999), and function in immunity (Santamaria et al., 2019) Additionally, PP2-A6, -A7, and -B6 are upregulated at the induction of SAR. Together, this leads to questions of whether PP2-A6, -A7, and/or -B6 interact with DIR1 in the phloem during SAR or are part of a SAR signal complex during SAR. To investigate this, the movement of DIR1 into the phloem during SAR induction by DIR1 immunoblotting could be performed on phloem exudates collected from mock-induced and SAR-induced *pp2-a6, -a7*, and *-b6* mutants.

6.10 Conclusions

The HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV fluorescent fusion lines could have been tools to provide us with greater clarity on the path taken by DIR1 to access the phloem during SAR at the cellular level. However, the absence of DIR1/DIR1-like and HA and FLAG signal in leaf extracts from the HA-DIR1-iLOV and FLAG-DIR1-like-phiLOV lines indicate that the fusion protein is not present in these lines, possibly as a result of degradation of the fusion protein. Additionally, the poor growth of the FLAG-DIR1-like-phiLOV seedlings on hygromycin-containing media suggests the transgenic constructs in this line may not be intact or expressed. Therefore, the HA-DIR1-iLOV and FLAG-DIR1-like phiLOV lines cannot be used as tools for monitoring the movement of DIR1 and DIR1-like during SAR.

Investigation of LTP2's role in the movement of DIR1 during SAR suggested that LTP2 may not be required for the movement of DIR1 into the phloem at the induction stage of SAR. The consistent absence of DIR1 from phloem exudates of distant SAR-induced *ltp2-1* mutant leaves suggests that LTP2 may be required for the long-distance movement of DIR1 to systemic leaves during SAR. Despite this, the presence of LTP2 in the phloem during SAR was unable to be assessed, as the absence of FLAG signal on immunoblots of 35S-LTP2-FLAG leaf extracts indicates that the LTP2-FLAG protein is not present in this line.

Investigation of a common set genes expressed at the induction stage of SAR identified numerous genes upregulated in all SAR-inducing treatments. Some of these genes are genes previously (PDLP5) or currently (MCTP9) under investigation by the Cameron lab, however, numerous new genes of interest were identified, such as AZI3, and the phloem proteins PP2-A6, A7, and B6. These genes of interest represent candidates for future studies to investigate their potential roles in SAR.

Supplementary Figures

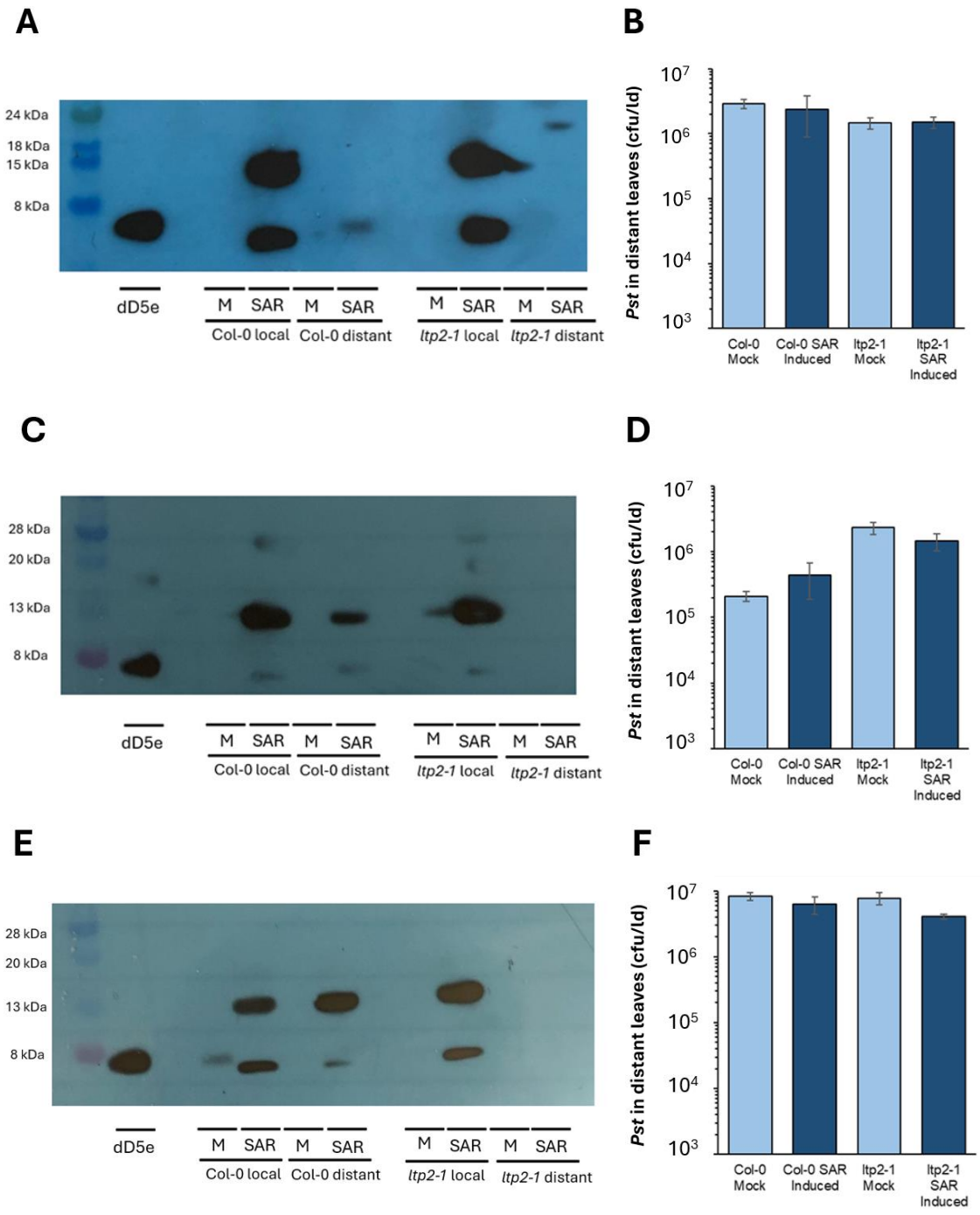


Figure S1: Additional replicates of DIR1 antibody immunoblotting of local and distant leaf phloem exudates and SAR assays conducted on Col-0 and *ltp2-1* plants. A, C and **E** represent DIR1 antibody immunoblots of phloem exudates

collected from local and distant leaves of mock-induced (M) and SAR-induced (SAR) Col-0 and *ltp2-1* plants. The leftmost lanes contain the protein ladders, while the lane beside the ladder contains leaf extract from dD5e DIR1-overexpessing plants (Maldonado et al., 2002) as a positive control. **B**, **D**, and **F** Bacterial levels in corresponding SAR assays conducted alongside phloem exudate collection. Col-0 and *ltp2-1* plants were mock-induced with $MgCl_2$ or SAR-induced with 10^6 cfu/ml *Pst AvrRpt2* followed by challenge of upper leaves with 10^5 cfu/ml virulent *Pst* 3 days later. Bacteria were isolated from leaves for quantification three days post-challenge and plated for counting.

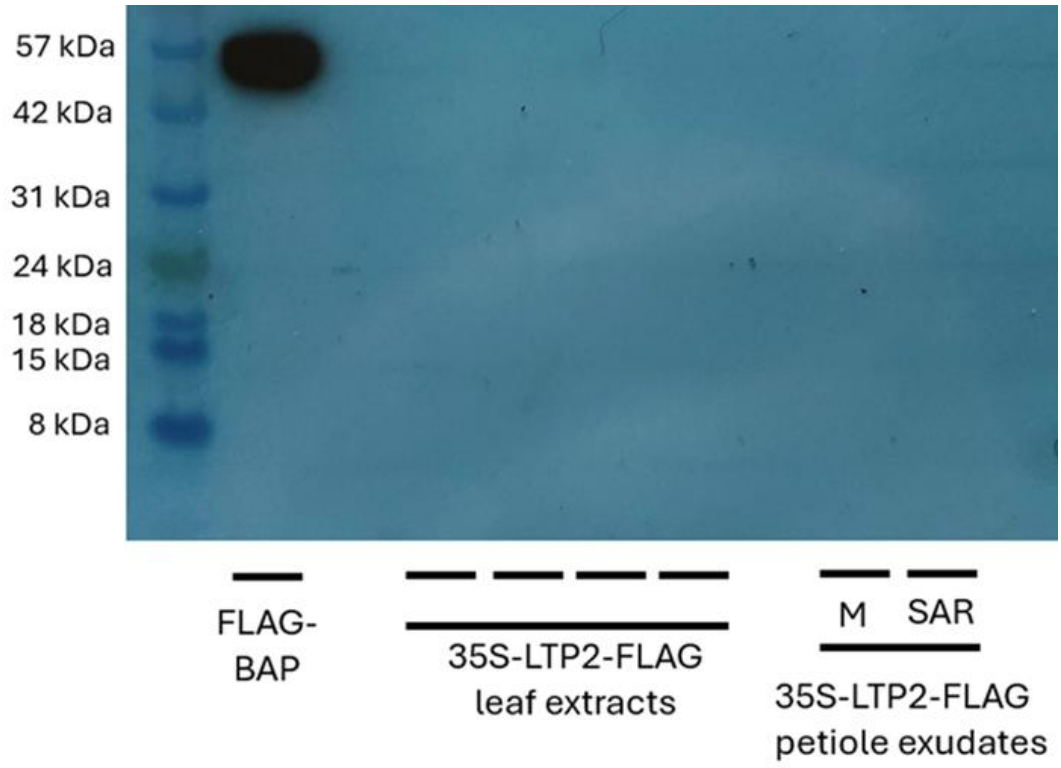
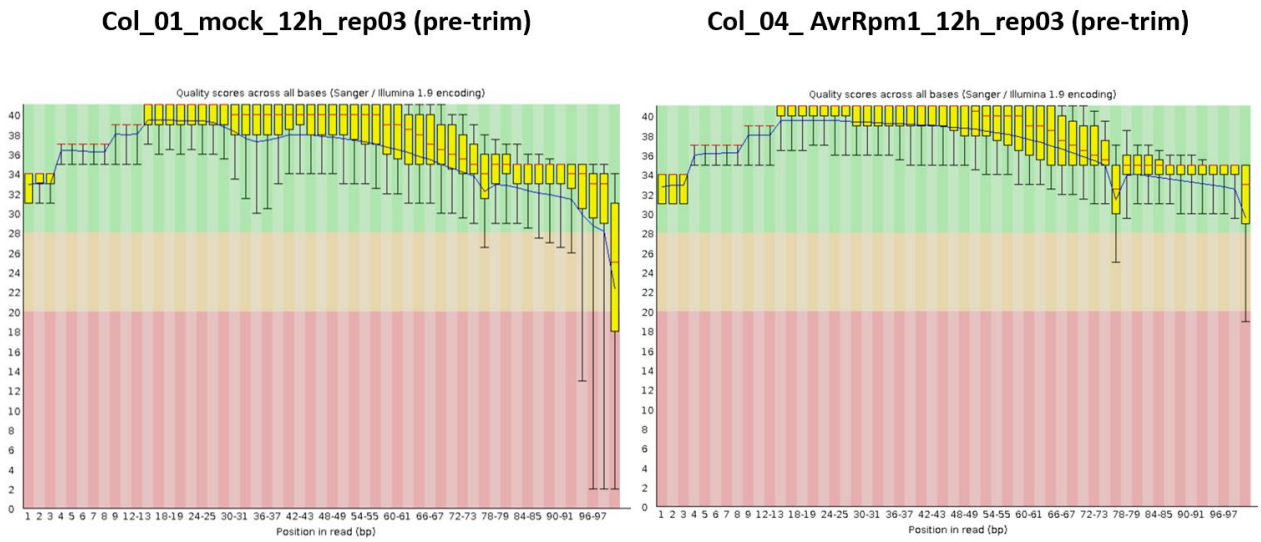


Figure S2: FLAG antibody immunoblot of 35S-LTP2-FLAG leaf extracts and phloem exudates. Leaf extracts and phloem exudates (mock-induced and SAR-induced) collected from 35S-LTP2-FLAG plants were subjected to SDS-PAGE followed by FLAG antibody immunoblotting. The leftmost lane contains the protein ladder, and the lane to the right of it contains the FLAG-BAP (Sigma) positive control.

A



B

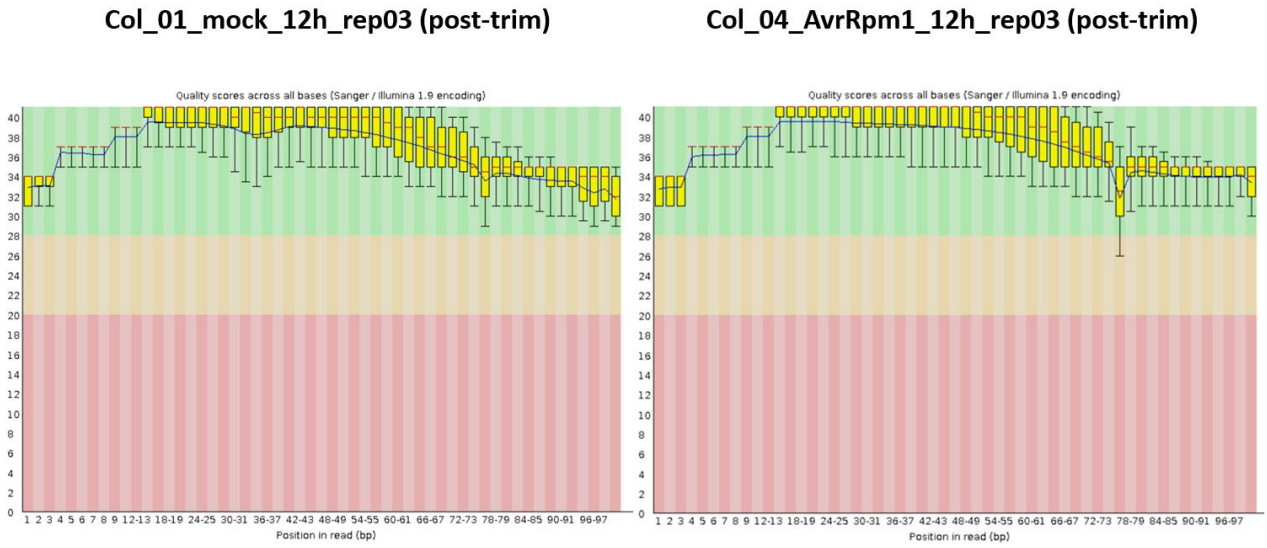
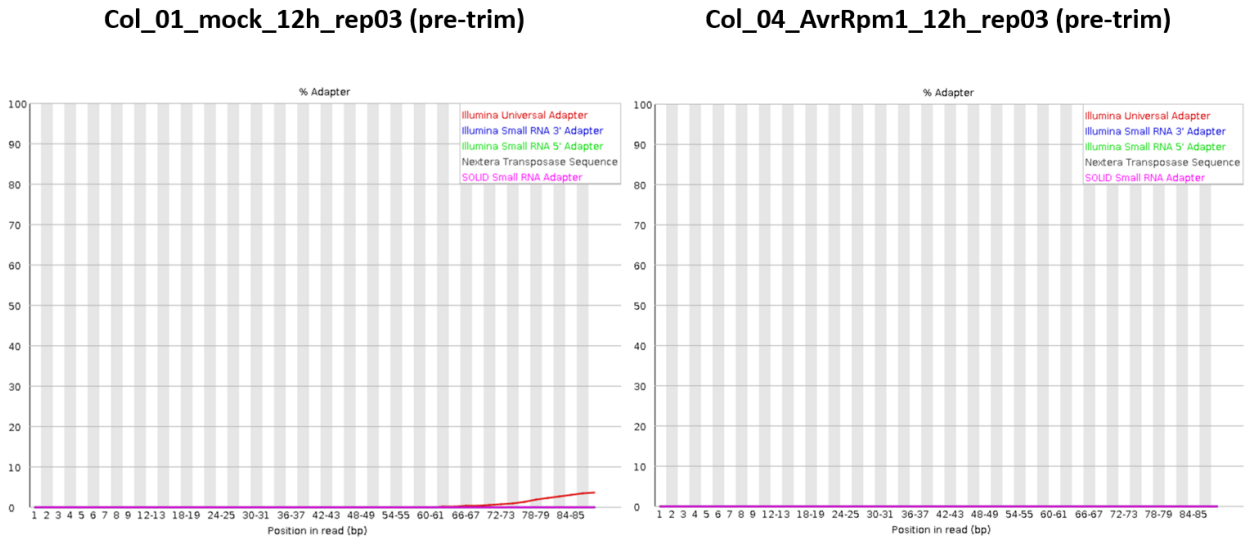


Figure S3: Quality scoring before and after trimming RNA-seq reads. Quality scores across the length of reads from two representative RNA-seq samples (Col-0 tissue mock-inoculated or inoculated with *Pst AvrRpm1* bacteria and collected at 12 hours post-treatment) from the Mine et al. (2018) dataset as measured by FastQC (Andrews, 2010) before and after trimming. **A** shows quality scores before trimming reads with trimmomatic (Bolger et al., 2014) and **B** shows quality scores after trimming

A



B

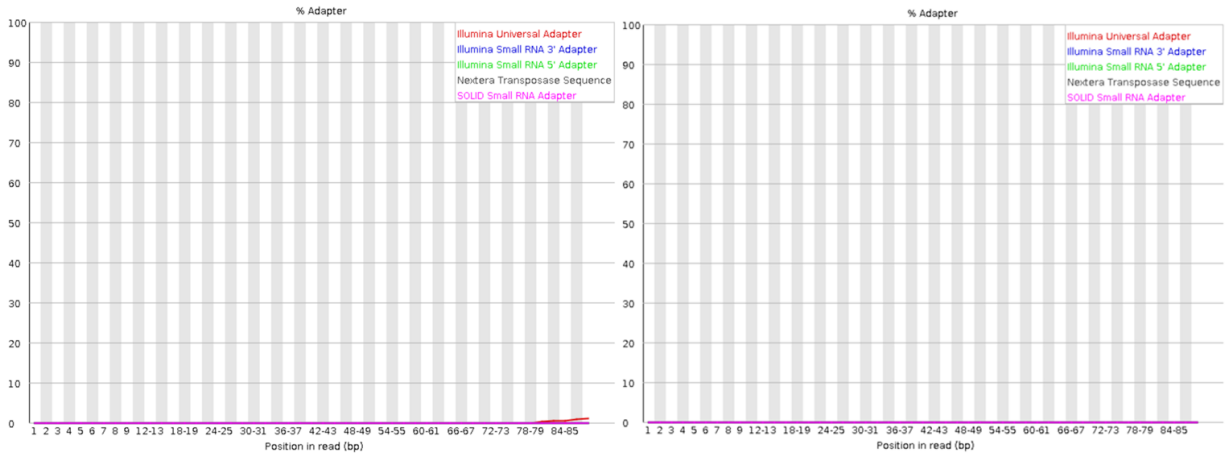


Figure S4: Adapter contamination before and after trimming RNA-seq reads. Adapter contamination in two representative RNA-seq samples (Col-0 tissue mock-inoculated or inoculated with *Pst AvrRpm1* bacteria and collected at 12 hours post-treatment) from the Mine et al. (2018) dataset as reported by FastQC (Andrews, 2010) before and after trimming samples. **A** represents the adapter contamination before trimming reads with trimmomatic (Bolger et al., 2014) while **B** represents the same two samples after trimming.

Dataset	Lab(s)	Data Type	Timepoints	Plant Age	Plant Growth Conditions	Information on Experimental Design/Samples Collected
Bjornson et al. 2021	Zipfel	RNA-seq	0, 5, 10, 30, 90, 180 min post treatment	14 days post-stratification*	Seedlings germinated on standard MS media under 16 h light/8 h darkness at 22°C and were then moved to 24-well plates containing 1 mL liquid MS media per well.	Used wild type *Arabidopsis thaliana* Col-0 plants, as well as the mutants *fls2, efr1-1, pepr1-1, pepr2-1, rlp23-1, lyk4, lyk5, sd1-29, bak1-5,* and *camta/dsc1/dsc2*. Treatments were conducted using various immune elicitors at different concentrations: 1 µm flg22, 1 µm elf18, 1 µm pep1, 1 µm nlp20, 100 µg/ml OGs, 1 µm CO8, 1 µm 3-OH-FA. Every well of each plate contained 2 seedlings, and 2 wells were pooled for each genotype/treatment/timepoint, resulting in each replicate containing a total of 4 seedlings.
Mine et al., 2018	Tsuda	RNA-seq	1, 3, 4, 6, 9, 12, 16, 24 hours post treatment	4-5 weeks post germination	Plants were grown under 10 h light/14 h darkness at 22 °C and 60% relative humidity for 3 weeks, then grown under 12 h light at 22 °C and 60% relative humidity for a week.	Used wild type *Arabidopsis thaliana* Col-0 plants, as well as the mutants *pad4, sid2, pad4 sid2, rpm1 rps2*, and *dde2 ein2 pad4 sid2*. Plants were inoculated with water (control) or syringe infiltrated with the *Pseudomonas syringae* strains *Pst* DC3000, *Pst* AvrRpt2, or *Pst* AvrRpm1 (OD_{600} = 0.001). The authors defined one biological replicate as 3 fully expanded leaves pooled from 3 separate plants. Three biological replicates were taken for each combination of genotype/treatment/time.
Gruner et al., 2013	Zeier	Microarray	48 hours post treatment	5-6 weeks post germination	Plants were grown under 9 h light at 21 °C/15 h darkness at 18 °C, with 70% relative humidity, and a light level (photon flux density) of 70 µmol m^{-2} s^{-1}	*Arabidopsis thaliana* wild type Col-0 and *fmo1* mutant plants were syringe infiltrated on 3 lower leaves with 10 mM $MgCl_2$ (control) or SAR induced with *P. syringae* strain *Psm* ES4326 (OD600=0.005). Uninfiltrated upper leaves were then collected 48 hours later. Three biological replicates were conducted for each treatment, with each replicate consisting of 2 leaves pooled from different plants of the same treatment group.
Baum et al., 2019	Conrath	RNA-seq	74.5 hours post treatment	4-5 weeks post germination	Plants were grown under 8 h light/16 h darkness at 20 °C, and a light level (photon flux density) of 100 µmol m^{-2} s^{-1}	Three lower leaves of wild-type *Arabidopsis thaliana* Col-0 plants were syringe infiltrated with 10 mM $MgCl_2$ (control) or with $3x10^8$ colony forming units (cfu)/ml *Psm* bacteria to induce SAR. 72 hours later, upper distant leaves were either left untreated or challenged with tap water infiltration before harvesting for RNA-Seq 2.5 hours later. Each replicate consists of 9 leaves (3 leaves/plant from 3 plants), and a total of 3 replicates were collected per treatment for a total of 12 samples.
Bernsdorff et al., 2016	Zeier	RNA-seq	48 hours post treatment	5-6 weeks post germination	Plants were grown under 10 h light at 21 °C/14 h darkness at 18 °C, with 70% relative humidity, and light levels (photon flux density) of 100 µmol m^{-2} s^{-1}	Three lower leaves of wild type *Arabidopsis thaliana* wild-type Col-0 or mutant *sid2* or *ald1* plants were syringe infiltrated with *Psm* bacteria (OD_{600} = 0.005) or 10 mM $MgCl_2$ (control). Upper, uninfiltrated leaves were harvested 48 hours later for RNA-Seq. For each treatment/genotype, 6 or more upper leaves from 6 plants were pooled as one biological replicate.
Golisz et al., 2021	Kufel & Jarmolowski	RNA-seq	48 hours post treatment	6 weeks post germination	Plants were grown under 8 h light at 22 °C /16 h darkness at 19 °C.	Wild type *Arabidopsis thaliana* Col-0 plants and *smd3b-1* mutants were spray inoculated with 10 mM $MgCl_2$ (control) or 10^6 cfu/ml of *Pst* DC3000 bacteria. Tissue was collected from 10 or more plants per treatment/timepoint, but it is unclear how much tissue/how many different plants make up each replicate specifically.
Salguero-Linares et al., 2022	Rengel & Coll	RNA-seq	0, 1, 2, 4, and 6 hours post treatment	4 weeks post germination	Plants were grown under 9 h light /15 h darkness, with 65% relative humidity.	*Arabidopsis thaliana* wild type Col-0 leaves were syringe infiltrated with 10 mM $MgCl_2$ (control) or $2.5x10^7$ cfu/ml *Pst AvrRpm1*. Each biological replicate consists of tissue from 6 leaves from 3 separate plants, and 3 biological replicates were collected per timepoint /treatment /leaf area. Total of 60 samples.
Roussin-Leveillee et al., 2022	He & Moffett	RNA-seq	36 hours post treatment	4-5 weeks post germination	Plants were grown under 12 h light/darkness at 21°C, with relative humidity of 60%.	*Arabidopsis thaliana* wild-type Col-0 leaves were syringe infiltrated with either a control, *Pst* DC3000, *Pst hopM1*$^-$, *Pst avrE1*$^-$, or *Pst hopM1 avrE1* at a dose of 10^6 cfu/ml. Three biological replicates were collected per treatment, but it is unclear how much tissue (and from how many plants) was collected per replicate.

Table S1: Choosing a gene expression dataset for comparison. Compilation of the various datasets considered for comparison to the Cameron lab PTI RNA-seq data. Datasets were assessed for type (RNA-seq, microarray etc.), plant growth conditions, treatments, plant age during the analysis, and experimental design. This allowed a dataset to be chosen with plant growth conditions as similar as possible to the Cameron lab plant growth conditions to minimize any differences in expression that may have arisen due to differences in experimental conditions between labs.

AGI ID	Description	Timepoint (hpt)
AT1G03660	Ankyrin-repeat containing protein	6, 12
AT1G51860	Leucine-rich repeat protein kinase family protein	6, 12
AT1G51890	Leucine-rich repeat protein kinase family protein	6, 24
AT2G37750	Hypothetical protein	6, 12
AT3G09960	Calcineurin-like metallo-phosphoesterase superfamily protein	6, 12, 24
AT3G18250	Putative membrane lipoprotein	6, 12, 24
AT3G28510	P-loop containing nucleoside triphosphate hydrolases superfamily protein	6, 24
AT3G46280	Kinase-like protein	6, 12
AT3G46690	UDP-Glycosyltransferase superfamily protein	6, 12, 24
AT3G47050	Glycosyl hydrolase family protein	6, 12, 24
AT3G49130	SWAP (Suppressor-of-White-Apricot)/surp RNA-binding domain-containing protein	6, 12
AT3G51360	Eukaryotic aspartyl protease family protein	12, 24
AT3G57380	Glycosyltransferase family 61 protein	6, 12
AT3G60470	Transmembrane protein, putative (DUF247)	6, 12
AT4G09770	TRAF-like family protein	6, 12
AT4G19970	Nucleotide-diphospho-sugar transferase family protein	6, 12
AT4G25070	Caldesmon-like protein	6, 12
AT4G40020	Myosin heavy chain-related protein	6, 24
AT5G42830	HXXXD-type acyl-transferase family protein	6, 12
AT5G44990	Glutathione S-transferase family protein	6, 12
AT5G59490	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein	6, 12
AT5G62150	Peptidoglycan-binding LysM domain-containing protein	6, 12

Table S2: Uncharacterized genes/genes of unknown function upregulated during SAR induction by flg22, *Pst AvrRpt2,* and *Pst AvrRpm1* at multiple timepoints. Arabidopsis Col-0 leaf tissue was mock-treated or treated with 1 µM flg22 before being collected at 6-, 12-, or 24-hours post treatment. Tissue was then subjected to RNA extraction and RNA-sequencing analysis. This data was compared to RNA-seq data from Mine et al. (2018), where Arabidopsis Col-0 plants were mock-inoculated or SAR-induced with either *Pst AvrRpt2* or *Pst AvrRpm1* bacteria, followed by tissue collection at 6-, 12-, or 24-hours post-treatment. The DESeq2 package for R was used to compare the count data between datasets and identify differentially expressed genes upregulated by all three SAR-inducing treatments (adjusted p-

value cutoff <0.05; $\log_2$ fold change cutoff >2). The table above lists uncharacterized genes/ genes with unknown functions upregulated by all three SAR-inducing treatments at multiple timepoints indicated in hours post-treatment (hpt). Genes upregulated in all three timepoints are highlighted in blue.

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