THE ROLE OF PHLOEM-MOBILE PROTEINS DURING SAR

EXPLORING THE ROLE OF DIR1 AND OTHER PHLOEM-MOBILE PROTEINS DURING SYSTEMIC ACQUIRED RESISTANCE IN *ARABIDOPSIS THALIANA*

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TITLE: Exploring the role of DIR1 and other phloem-mobile proteins during systemic acquired resistance in *Arabidopsis thaliana*

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Abstract

Systemic acquired resistance (SAR) is a defense response induced by an initial localized infection that leads to the generation of long-distance immune signals that travel to distant leaves to provide enhanced resistance to subsequent infections. The lipid transfer protein (LTP) DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) travels via the phloem from induced to distant leaves during SAR and may chaperone several long-distance signal candidates. In this thesis, the role of DIR1 during SAR is explored by examining the route of DIR1 movement, investigating the conservation of DIR1 structure and function, and by identifying DIR1-interacting proteins. I demonstrate that *Arabidopsis* plant lines with restricted cell-to-cell movement through plasmodesmata are negatively impacted in long-distance DIR1 movement, suggesting that cell-to-cell movement is important for DIR1 to access distant leaves. To elucidate the molecular function of DIR1, orthology analysis was performed with putative DIR1 orthologs. Structurally important amino acid residues that contribute to the hydrophobicity of the LTP cavity were identified, supporting the idea that DIR1 binds a hydrophobic ligand during SAR. RNAi-mediated knockdown of the *DIR1* paralog *DIR1-like* did not impact the SAR response, supporting the idea that DIR1-like plays a lesser role in SAR. In addition, targeted protein-protein interaction assays determined that LTP1 and LTP2 interact with DIR1, and SAR phenotypic analysis of an *ltp2-1* mutant supported a role for LTP2 in SAR. Lastly, a comparative proteomics approach identified several proteins with differential abundance in phloem exudates collected during the induction of SAR. Of these proteins, m-type thioredoxins, a major latex protein-like protein, and the UV-B photoreceptor UVR8 were essential for the manifestation of SAR. Together, these data provide insight into DIR1 function by identifying the importance of cell-to-cell movement through plasmodesmata, the DIR1 hydrophobic cavity, and DIR1-interacting proteins for DIR1-mediated SAR. In addition, this work identifies new phloem-localized proteins that contribute to the SAR response, providing fundamental knowledge on protein composition within the phloem during biotic stress.

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List of Abbreviations

μg	microgram
μL	microliter
5FCL	5-Formyltetrahydrofolate cycloligase
ABA	Abscisic acid
ACBP	Acyl-CoA Binding Protein
ACT	Actin
ALD1	AGD2-like defense response protein
ARR	Age-related resistance
Avr	Avirulence gene
AzA	Azelaic acid
AZI1	*Azelaic acid induced 1*
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSMT1	Benzoic acid and SA methyl transferase
CC	Companion cell
cDNA	Complementary DNA
CDR	constitutive disease resistance
cfu	Colony forming units
co-IP	co-immunoprecipitation
Col-0	Columbia
COP1	Constitutive photomorphogenesis
Cub	C-terminal half of ubiquitin
cYFP	C-terminal half of YFP
DA	Dehydroabietinal
DAMP	Damage associated molecular pattern
DHAP	Dihydroxyacetone Phosphate
DIR1	*Defective in Induced Resistance 1*
dpi	Days post inoculation
EARLI1	Early *Arabidopsis* aluminum induced gene
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
EYFP	Enhanced Yellow Fluorescent Protein
FAD	*Fatty Acid Desturase*
FLS2	*Flagellin sensing2*
FMO1	Flavin-Dependent Monooxygenase1
FP	Fluorescent Protein
FT	Flowering locus T
FTIP1	FT-interacting protein 1
GC-MS	gas chromatography – mass spectrometry
G3P	Glycerol-3-Phosphate
GFP	Green Fluorescent Protein
GO	Gene ontology
GPI	glycophosphatidylinositol
Gus	β-glucuronidase
Hpi	Hours post inoculation
Hpa	*Hyalopernospora arabidopsidis*
HR	Hypersensitive Response
HY5	Elongated Hypocotyl 5

ISR	Induced systemic resistance
JA	Jasmonic acid
kDa	Kilo Dalton
LC-MS/MS	Liquid chromatography, tandem mass spectrometry
ld	Leaf disc
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
LTP	Lipid Transfer Protein
LRR	Leucine rich repeats
MAMP	Microbe-Associated Molecular Pattern
MGDG1	*Monogalactosyldiacylglycerol Synthase1*
MES	Methyl esterase
MeSA	Methyl Salicylate
$MgCl_2$	Magnesium Chloride
ml	Milliliter
MLP	Major latex protein-like protein
mM	Millimolar
MMLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MPK	Mitogen activated protein kinase
MS	Murashige and Skoog
MS/MS	Tandem mass spectrometry
MTI	MAMP-triggered Immunity
NCBI	National Center for Biotechnology Information
NO	Nitric Oxide
NPR	*Nonexpresser of PR genes*
Nub	N-terminal half of ubiquitin
nYFP	N-terminal half of YFP
OD	Optical density
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PDLP	Plasmodesmata-located protein
Pip	Pipecolic acid
PLS	Plasmodesmata localization signal
PP	Phloem parenchyma
PR	Pathogenesis-Related Proteins
PRR	Pattern recognition receptor
Psm	*Pseudomonas syringae pv. maculicola*
Pss	*Pseudomonas syringae pv. syringae*
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-Triggered Immunity
qRT-PCR	Quantitative Real Time PCR
R	Resistance
RIN4	*RPM1-interacting protein 4*
RNAi	RNA interference
ROS	Reactive oxygen species
RPS2	Resistance to P. syringae 2
RT-PCR	Reverse transcription PCR
SA	Salicylic Acid
SABP2	SA Binding Protein 2
SAMT	SA methyl transferase
SAR	Systemic Acquired Resistance
SD	Synthetic defined media
SE	Sieve element

SFD	*Suppressor of Fatty Acid Desaturase Deficiency*
siRNA	small interfering RNA
SS	Signal Sequence
T3SS	Type III Secretion System
TAIR	The *Arabidopsis* Information Resource
T-DNA	Transfer DNA
TMV	Tobacco Mosaic Virus
TNS	6,P-toluidinylnaphthalene-2-sulfonate
Tris	Tris(hydroxymethyl) aminomethane
TRX	Thioredoxin
UVR8	Ultraviolet B resistance 8
wpg	Weeks post germination
Ws-2	Wassilewskija
XVE	Estrogen inducible promoter
YPD	Yeast Extract, Peptone, Dextrose

Chapter 1
Introduction

1.1 Plant Responses to the Environment

As sessile organisms, plants are unable to relocate during times of stress. Instead, they must tolerate adverse environmental conditions in order to survive long enough to reproduce. To accomplish this, plants have evolved a number of sophisticated strategies to quickly detect changes in environmental conditions and adjust their metabolism and development accordingly. This capacity for change is evident in numerous plant species and is exemplified by the so-called phenotypic plasticity of plants; where morphological phenotypes such as height, biomass, and color are adjusted to favor survival and reproduction under the current environmental conditions. As a result, genetically identical plants growing in different environments can often look strikingly different.

In natural environments there are a myriad of abiotic and biotic factors that serve as sources of stress that threaten plant survival. Examples of abiotic factors include light quality/quantity, water and nutrient availability, as well as temperature. Unfavorable levels of these factors can have serious impacts on plants and, as such, routinely threaten crop yields worldwide (Bray *et al.* 2000). Moreover, abiotic stress is becoming increasingly concerning as the severity of climate change-associated effects such as extreme weather events (storms, floods, etc.), temperature, CO_2 concentration and air pollution further threaten crop production (Tubiello *et al.* 2007; Lobell *et al.* 2008). Biotic stress, in comparison, is caused by living factors that negatively impact plant growth and development. Examples of biotic stressors include viral and microbial pathogens (bacteria, fungi, oomycetes, etc.) in addition to pests and herbivores (insects,

mammals, etc.). Pathogens, parasites, and pests are serious threats to the host plant's wellbeing, as they draw important metabolic resources away from the host and usually cause structural damage to plant tissues. The impact of plant pathogens on human history is best illustrated by the Irish potato famine (1845-1850), in which a highly virulent strain of *Phytophtora infestans* overtook an entire country's potato crop, resulting in mass human migration. Since then, efforts to mitigate the effects of plant pathogens on crops have prevented the recurrence of such a catastrophe. However, a number of economically important crops remain threatened by serious diseases to this day, as demonstrated by the vulnerability of citrus tress to Huanglongbing disease (da Graca *et al.* 2015) and bananas to Panama disease (Ordonez *et al.* 2015).

1.2 Plant-Microbe Interactions

Plants interact with a number of microbial species in their natural environment. These include beneficial as well as detrimental (pathogenic) varieties. Unlike mammals that possess both adaptive and innate immune responses, plants possess only innate immunity. Thus, they lack specialized mobile immune cells and as such each plant cell is thought to contain sophisticated immune machinery to allow for the rapid detection of invading microbes (Spoel and Dong 2012). An important distinction between these immune strategies is that adaptive responses result in a highly specific immune response that is effective against the initial infecting pathogen, while innate immunity involves non-specific, broad-spectrum resistance mechanisms. Plants detect and respond to the presence of microbes using a number of partially overlapping pathways (Jones and Dangl 2006). These responses can act locally at the site of perception as well as systemically throughout the plant, which allows for a coordinated plant-wide response to protect against pathogen ingress.

1.2.1 Local Responses to Pathogens

Plant pathogenic microbes form close associations with host cells in order to manipulate the host for the acquisition of metabolic resources. Molecular plant pathological research has focused on four groups of plant pathogens: pathogenic bacteria (Mansfield *et al.* 2012), fungi (Dean *et al.* 2012), fungus-like oomycetous microbes (Kamoun *et al.* 2015), and viruses (Scholthof *et al.* 2011). The strategies employed by these pathogens are sometimes similar, however each pathogen class employs unique tactics for host manipulation based on fundamental differences in life-style, mode of reproduction, or metabolic requirements. Immunity in plants is achieved through the use of “innate” immune machinery, which is composed of sensory receptors that monitor for the presence of common microbial signatures. Upon detection of such signatures, plants employ defense responses aimed at limiting pathogen proliferation. While these responses can be specific to the invading pathogen, many overlapping themes are evident during plant-pathogen interactions. Below, the major defense responses and outcomes that occur during plant-microbe interactions in locally infected tissue are outlined.

1.2.1.1 Basal Defense Responses

Most microbes lack the ability to form a pathogenic relationship with most plant hosts. This is largely due to the presence of preformed physical and chemical barriers that restrict pathogens from entering and/or colonizing plant tissues. Such physical barriers include the plant cell wall and the cuticle. The plant cell wall contains a number of complex, high molecular-weight polysaccharides that reinforce and rigidify plant cells, providing resistance to turgor and a barrier to pathogen infection (Somerville *et al.* 2004). The plant cuticle is a waxy

hydrophobic layer that coats and protects aerial plant structures such as leaves (Yeats and Rose 2013). This layer is resistant to most pests and pathogens, however certain phytopathogenic fungi can degrade and/or rupture the surface using specialized enzymes and infection structures (Serrano *et al.* 2014; Lo Presti *et al.* 2015). These penetrating fungal pathogens, in addition to bacterial pathogens that bypass the cuticle by swimming through natural openings such as stomata (Melotto *et al.* 2008), subsequently encounter the chemical barriers of the plant innate immune system. Such chemical barriers to pathogen proliferation include classes of specialized molecules such as phytoalexins, glucosinolates, and saponins. For example, constitutively produced glucosinolates stored in vacuoles are quickly translocated to the site of pathogen detection (or damage) and are metabolized by myrosinase enzymes to release toxic byproducts that include isothiocyanates and nitriles (Halkier and Gershenzon 2006). As such, these compounds are thought to act as antibiotics *in planta,* but may also be involved in regulating plant defense responses (reviewed in Piasecka *et al.* 2015). In addition to constitutive defenses, inducible responses such as those activated by the detection of foreign microbial signatures also contribute to basal defense. Plant pattern-recognition receptors (PRR) recognize conserved microbial motifs, termed MAMPs or PAMPS (microbial/pathogen-associated molecular patterns), which are specific to bacteria or fungi (e.g., flagellin, chitin, elongation factor). MAMP perception by PRRs initiate MAPK (mitogen-activated protein kinase) signalling cascades, resulting in changes in gene expression, the accumulation of the defense hormone salicylic acid (SA), antimicrobial pathogenesis-related (PR) proteins and chemicals (phytoalexins), as well as extensive cell wall modifications (e.g., callose deposition) to prevent pathogen ingress (reviewed in Tena *et al.* 2011; Meng and Zhang 2013; Bigeard *et al.* 2015). A classic example of MAMP perception in plants is the recognition of bacterial flagellin by the FLS2 (FLAGELLIN SENSING2) receptor, which binds a core flg22-sequence (RINSAKDD) using an extracellular leucine-rich repeat

(LRR) receptor domain (reviewed in Robatzek and Wirthmueller 2013). Receptor-ligand binding induces a signalling cascade that is transduced with the help of the BAK1 (BRI1-Associated Kinase1) co-receptor (Chinchilla *et al.* 2007), resulting in the activation of MAMP-triggered immunity (MTI; also termed PAMP-triggered immunity, or PTI). In addition, the perception of damage-associated molecular patterns (DAMPs) produced by the degradation or "damage" of plant-derived macromolecules by invading pathogens also initiates innate immune responses. DAMPs such as extracellular ATP (Tanaka *et al.* 2014), cutin-degradation products (Chassot *et al.* 2008), as well as endogenous peptides (Albert 2013) activate innate defense responses similar to MAMP-induced signaling (Yamaguchi and Huffaker 2011); however, our understanding of DAMP perception is less clear (Serrano *et al.* 2014).

1.2.1.2 Effector-Triggered Susceptibility

Plant pathogens must overcome basal defense to successfully infect a potential host. To accomplish this, phytopathogens utilize a repertoire of effector proteins to manipulate host cells. The function of these "virulence" effector proteins appears to be specific to the lifestyle of the invading pathogen, which can be necrotrophic, hemi-biotrophic, or biotrophic. Necrotrophic pathogens such as the bacterial pathogen *Pectobacterium carotovorum* (formerly *Erwinia carotovorum*) degrade plant tissues to obtain nutrients, causing the death or "rot" of infected tissue. Effectors employed by nectroptrophs such as *P. carotovorum* typically promote plant cell death, which act together with secreted extracellular cell wall degrading enzymes such as pectinases, cellulases, and glucanases to release nutrients from plant cells (discussed in Davidsson *et al.* 2013). In contrast, biotrophic pathogens must keep their hosts alive in order to benefit from the interaction. This is true for hemibiotrophs as well, however these pathogens can

shift to a necrotrophic phase in later stages of the infection (Dou and Zhou 2012). Both biotrophic and hemibiotrophic pathogens such as *Hyaloperonospora arabidopsidis* (Hpa) and *P. syringae* pv. *tomato* (Pst), respectively, rely on intracellular effector proteins to manipulate host plants. For many plant pathogenic bacteria, these effectors are delivered into host cells using type III or IV secretion systems, which are often described as molecular "hypodermic needles" (Costa *et al.* 2015). Plant pathogenic fungi and fungi-like oomycetes also require intracellular effector proteins to successfully invade and colonize host tissues, although how these effectors are delivered into host cells is not yet clear. In some cases the effectors appear to enter cells through specialized infection structures such as the "appressorium", while in other instances effectors appear to be translocated into cells from the apoplast (discussed in Giraldo and Valent 2013). In any case, intracellular effectors of biotrophic and hemibiotrophic pathogens play diverse roles in manipulating host plant cells. To date, effectors have been implicated in the direct transcriptional manipulation of host genes, blocking the protein secretory pathway, direct repression of MTI signaling responses, as well as manipulating hormone signalling by modifying or degrading hormones such as salicylic acid (reviewed in Dou and Zhou 2012; Giraldo and Valent 2013; Lee *et al.* 2013). The action of these effectors renders the host susceptible to infection, resulting in what is often described as effector-triggered susceptibility (ETS). The *Arabidopsis-Pseudomonas syringae* model pathosystem has been instrumental in the effort to identify and characterize the *P. syringae* effector repertoire, allowing for the discovery of several virulence effector proteins and their corresponding host targets (discussed in Xin and He 2013).

1.2.1.3 Effector-Triggered Immunity

Just as pathogens have evolved sophisticated virulence mechanisms to suppress host defenses and colonize plant tissues, host plants too have evolved sophisticated mechanisms to overcome or prevent manipulation by infecting microbes. A key strategy to accomplish this involves the detection of virulence effector proteins by plant resistance or "R" receptors. This detection can be direct, through physical interaction between the R receptor and effector proteins (termed "avirulence protein" when detected), or indirect, by interaction with other host proteins that are in some way modified by the effector proteins (Jones and Dangl 2006). This interaction leads to the establishment of a robust immune response termed effector-triggered immunity (ETI) or R gene-mediated resistance. The activation of ETI is associated with rapid defense gene activation, the accumulation of SA and pathogenesis-related (PR) proteins, increased levels of reactive oxygen species (ROS), and is often associated with a form of programmed cell death known as the hypersensitive response (reviewed in Cui *et al.* 2015). It is thought that the activation of ETI reestablishes and intensifies basal defense responses that would have otherwise been suppressed by the pathogen (Jones and Dangl 2006; Cui *et al.* 2015).

R receptor structure is conserved among different plant species, usually consisting of a TIR (toll-like interleukin) or CC (coiled coil) domain at the N terminus followed by the NBS-LRR (nucleotide-binding site, leucine-rich repeat) domains, and sometimes an additional (variable) domain (Sanseverino and Ercolano 2012). Although R receptors sometimes detect effector proteins through direct protein-protein interactions, as is the case for the *Arabidopsis* RPP1-WsB R receptor and the Atr1 effector from the oomycete *H. arabidopsidis* (Krasileva *et al.* 2010), most interactions between R receptors and cognate effector proteins are indirect. A classic example of this paradigm is the interaction of the

Arabidopsis basal defense regulator RIN4 (RPM1-interacting 4), with the R receptor proteins RPM1 and RPS2 (Kim *et al.* 2005). The RPS2 receptor detects proteolytic degradation of RIN4 caused by the *P. syringae* avrRpt2 effector protease (Axtell and Staskawicz 2003; Mackey *et al.* 2003). In contrast, RPM1 detects the phosphorylation of RIN4 caused by the *P. syringae* avrRpm1 and avrB effector proteins (Mackey *et al.* 2002; Chung *et al.* 2011). These observations, among others, helped to establish the "guard" hypothesis, which posits that plant R receptors monitor and protect host proteins targeted by pathogen effectors. Recent work has expanded our understanding of this hypothesis, identifying so-called "decoy" proteins that act as non-functional traps to facilitate the detection of pathogen effectors while protecting the functional targets (discussed in van der Hoorn and Kamoun 2008). An example of this strategy is described in Lewis *et al.* (2013), where the *P. syringae* HopZ1a effector acetylates the decoy ZED1 (hopZ-eti-deficient1) pseudokinase that is monitored by the R receptor ZAR1. Decoys can also be integrated into the R receptor itself, as demonstrated by the WRKY transcription factor domain present in the *Arabidopsis* RRS1 R receptor that acts as a decoy for the PopP2 effector of *Ralstonia solanacearum* that normally targets WRKY transcription factors to suppress basal defense (Le Roux *et al.* 2015). Biotechnological strategies to exploit this decoy system were recently explored by Kim *et al.* (2016), who demonstrated that engineered versions of the PBS1 decoy are able to detect an expanded repertoire of effector proteins to induce ETI.

1.2.1.4 Plant-Pathogen Co-Evolution: An Ongoing Arms Race

Local immune responses in plants are often described as having evolved in a linear fashion, from broad non-specific basal defense, to pathogen adaptation and host manipulation via effector proteins, followed by the emergence of R

receptor-mediated resistance. This paradigm is often described as an evolutionary arms race, where both plant and pathogen are continually evolving mechanisms to escape the restraints imposed on one another. This is most easily described by the "Zig-Zag" model described by Jones and Dangl (2006). In this model, the outcome of plant-pathogen interactions zig and zag from resistance to susceptibility (Figure 1.1). The logical next step in the evolutionary arms race is for pathogens to develop mechanisms to inhibit ETI signalling to re-establish a susceptible interaction. Recent evidence demonstrating the ETI-suppressing activity of the *P. syringae* effector HopD1 in *Arabidopsis* supports this idea (Block *et al.* 2014). A less obvious extrapolation of the Zig-Zag model was recently discussed by Keller *et al.* (2016), who postulate that pathogens with a necrotrophic lifestyle may induce ETI to the detriment of the host, causing effector-triggered immune pathologies (ETIPS, first described in animal immune systems; Stuart *et al.* 2013) that benefit the infecting pathogen. Previous observations of necrotrophic fungi that require R receptors for virulence support this idea (Lorang *et al.* 2007, 2012; Faris *et al.* 2010), which further demonstrates the usefulness of the zig-zag model in guiding plant-pathogen research.

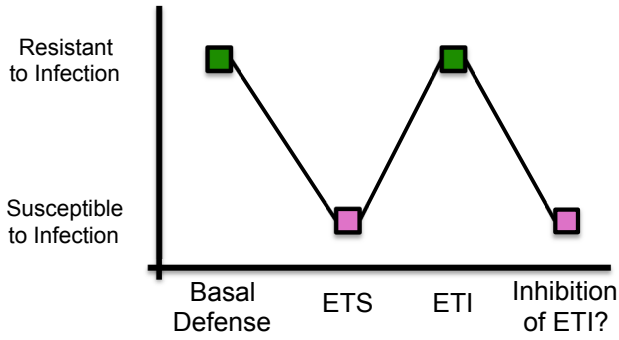

Figure 1.1 - The Zig-Zag Model of Plant-Pathogen Co-Evolution. A model of the various outcomes of plant-pathogen interactions adapted from Jones and Dangl (2006). The Y-axis represents the spectrum of the outcome of plant-pathogen interactions, with resistance to pathogen infection at the top and susceptibility to pathogen infection closer to the origin. The X-axis contains different plant-pathogen responses, including basal defense, effector-triggered susceptibility (ETS), effector-triggered immunity (ETI), and a hypothetical next step in the co-evolutionary arms race.

1.2.2 Systemic Immune Responses: Long-Distance Signalling During Plant-Microbe Interactions

Local detection of microbes also leads to the induction of systemic signalling responses in plants. Our current understanding of these plant-wide responses has expanded over the past 20 years, uncovering a diverse set of mechanisms that serve to warn and protect distal uninfected tissues from current and/or future pathogen attack. Systemic immune responses rely heavily on vasculature-mediated inter-organ communication. Classic examples of long-distance immune signalling in plants include virus-induced systemic small RNA signalling, induced systemic resistance (ISR), and systemic acquired resistance (SAR). Local plant-virus interactions lead to the DICER (RnaseIII)-dependent generation of virus-derived small interfering (si)RNAs that are loaded into RISC (RNA-induced silencing complex) for the detection and destruction of viral genomes (reviewed in

Ding and Voinnet 2007). These siRNAs are also translocated from the site of infection to distant uninfected tissues via the phloem, which is thought to contribute to systemic antiviral immunity (Yoo *et al.* 2004; Molnar *et al.* 2010; Brosnan and Voinnet 2011; Melnyk *et al.* 2011). Interactions with beneficial microbes can also induce systemic immune responses in plants. This occurs during the ISR response, where associations between plant roots and growth-promoting bacteria and/or mycorrhiza induce long-distance signalling to provide aerial tissues with enhanced resistance to pathogens (Pieterse *et al.* 2014). In addition, local interactions with pathogens (bacterial, fungal, viral) can elicit systemic immunity conferred by the SAR response.

1.2.2.1 Systemic Acquired Resistance

SAR is classically described as a systemic immune response that provides enhanced pathogen resistance to distal uninfected leaves following an initial localized infection. The term “systemic acquired resistance” was coined by Ross (1961), who observed enhanced immunity in the distal, uninfected leaves of tobacco plants following initial localized infections with tobacco mosaic virus (TMV). The SAR response can be divided into four distinct phases (Figure 1.2); (i) induction, (ii) signal propagation & mobilization, (iii) signal perception & defense priming, and (iv) the manifestation of SAR (reviewed in Champigny and Cameron 2009; Fu and Dong 2013). An overview of each phase is provided in the subsections below.

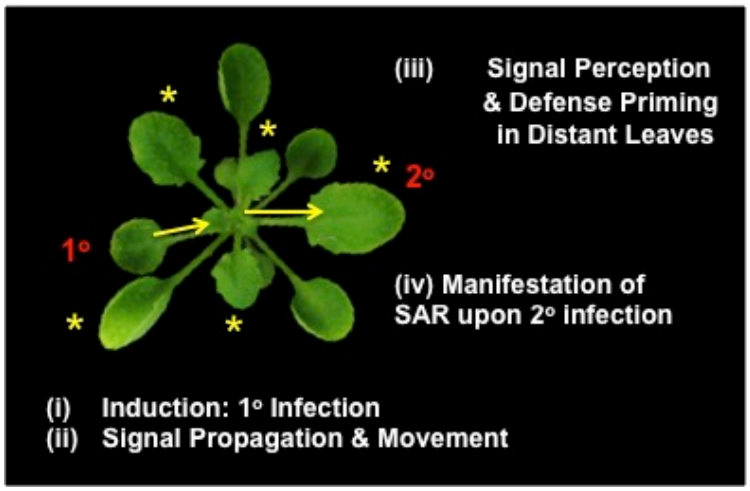

Figure 1.2 - The Four Phases of the SAR Response. A graphical representation of the four phases that occur during the SAR response in *Arabidopsis thaliana*. (i) The induction of SAR and (ii) the propagation and movement (yellow arrows) of mobile SAR signals from a lower leaf (1°) to distant, upper leaves (2°, yellow asterisks). (iii) Signal perception & defense priming, as well as the manifestation of SAR, occur in upper leaves.

1.2.2.1.1 (i) The Induction of SAR in Local Leaves

Since the early descriptions of TMV-induced SAR in tobacco (McKinney 1929; Ross 1961), several studies have described SAR responses in a number of plants, including tomato, soybean, cucumber, and the genetic model *Arabidopsis* (reviewed in Sticher *et al.* 1997; Champigny and Cameron 2009; Fu and Dong 2013). In *Arabidopsis*, SAR is induced by local infections with virulent or avirulent strains of *P. syringae* (Cameron *et al.* 1994; Mishina and Zeier 2007). Earlier SAR studies suggested that an initial necrotizing infection and the occurrence of an HR was essential for the induction of SAR (Kuc 1982), however recent studies demonstrate that the induction of MTI is sufficient to induce SAR in *Arabidopsis* (Mishina and Zeier 2007; Cecchini *et al.* 2015; Ross *et al.* 2015). Although this suggests that SAR induction is not associated with macroscopic cell death in

Arabidopsis, cell-death associated events such as the accumulation of ROS appear to be essential for the generation of SAR signals (Wang *et al.* 2014). In other plants, local interactions with a variety of bacterial, fungal, and viral pathogens have been shown to induce SAR, demonstrating the versatility and importance of this response. These interactions trigger local plant immune responses (MTI/ETI) that also lead to the generation and mobilization of long-distance SAR signals.

1.2.2.1.2 (ii) Signal Propagation & Mobilization

SAR-induced local leaves generate mobile immune signals that travel from induced to distant leaves to establish SAR. Classic SAR studies first described the timing and nature of signal movement rather than the identity of the SAR signal due to the technological limitations of the time. In tobacco, the SAR-induced leaf must remain on the plant for at least 60 hours after infection in order for SAR to be established (Ross 1961). Subsequent experiments in cucumber and *Arabidopsis* determined that SAR signal movement in these plants is substantially faster. Induced cucumber leaves must be attached to the plant for 4 hours post infection (hpi) to establish SAR in distant leaves at 24 hpi (Rasmussen *et al.* 1991). In *Arabidopsis*, SAR is established at 36-48 hpi, with the induced leaf requiring at least 4 hours to mobilize SAR signals (Cameron *et al.* 1994; Truman *et al.* 2007). Grafting experiments in tobacco were the first to suggest that a mobile signal generated in infected tissues travels through the vasculature to access distant leaves for the establishment of SAR (Jenns and Kuc 1979). Further evidence for vasculature-mediated SAR signalling was obtained in cucumber, where a girdling technique was used to isolate the vasculature of the induced leaf from the rest of the plant, resulting in complete loss of the SAR response (Guedes *et al.* 1980). Removal of the stem sheath in tobacco plants

resulted in a similar effect (Tuzun and Kuc 1985), demonstrating that signals require intact vascular connections for long-distance movement during SAR. A specific role for the phloem in long-distance SAR signalling was determined using elegant experiments that compared the pattern of SAR competence and SAR marker-gene expression with the translocation pattern of radiolabelled photosynthate ([^{14}C] Sucrose) following an initial SAR-inducing infection in a single leaf of *Arabidopsis* (Kiefer and Slusarenko 2003). SAR competence and marker gene expression was observed in upper leaves irrespective of the induced leaf orthostichy (vascular bundles that connect vertically arranged leaves), suggesting that SAR signals move via the phloem and perhaps also cell-to-cell (Kiefer and Slusarenko 2003). Since cell-to-cell movement alone is insufficient for rapid long-distance movement (Lucas *et al.* 2013), it is hypothesized that SAR signals move cell-to-cell to access different orthostichies to better disseminate SAR signals throughout the plant. In addition, several studies demonstrate the SAR-inducing activity of phloem exudates collected from SAR-induced but not mock-inoculated plants (Maldonado *et al.* 2002; Chaturvedi *et al.* 2008, 2012; Chanda *et al.* 2011; Xia *et al.* 2012), which further supports a role for the phloem in long-distance SAR signalling.

To date, several candidate SAR signals have been identified. The number and diversity of these putative signals is puzzling, and suggests that long-distance SAR signalling is more complex than expected (Dempsey and Klessig 2013). The defense hormone SA was initially hypothesized to act as a mobile SAR signal due to its accumulation in phloem exudates collected from SAR-induced leaves (Malamy *et al.* 1990; Metraux *et al.* 1990), but well-designed grafting experiments demonstrating that SA-deficient rootstocks (bottom) could generate and transmit SAR signals to wild-type scions (top) argued against this idea (Vernooj *et al.* 1994). Moreover, detailed analysis of SA accumulation and the timing of SAR establishment in cucumber demonstrated that mobile SAR signals exit the

induced leaf prior to the accumulation of SA in the phloem (Rasmussen *et al.* 1991). Further experimentation identified a role for the SA conjugate methyl salicylate (MeSA) during SAR in tobacco and *Arabidopsis*, leading to a model where SA produced in locally infected leaves is converted to MeSA by a methyltransferase (NtSAMT1 in tobacco, AtBSMT1 in *Arabidopsis*). MeSA then travels through the phloem to distant leaves where it is converted back to SA by a methylesterase (NtSABP2 in tobacco, AtMES1/7/9 in *Arabidopsis*) (Park *et al.* 2007; Vlot *et al.* 2008; Liu *et al.* 2010). A conflicting report disputed the importance of MeSA for SAR (Attaran *et al.* 2009), however further studies determined that the requirement of MeSA during SAR depends on whether plants are induced for SAR in the evening (MeSA required) or the morning (MeSA not required) (Liu *et al.* 2011a). The involvement of SA in SAR is further complicated by the SAR-defective phenotypes of mutants impaired in the accumulation of sulphonated-SA (Baek *et al.* 2012) or the production of amino acid-conjugates of SA-precursors (Nobuta *et al.* 2007; Lee *et al.* 2007; Jing *et al.* 2011). Whether these molecules act as *bona fide* long distance signals, or during local resistance responses (e.g., during SAR manifestation) by impacting total SA levels remains to be determined.

SAR studies from the early 2000's were the first to suggest the existence of a lipid-based SAR long-distance signal(s). A classic forward genetic screen for SAR-defective mutants identified the *dir1-1* mutant (*defective in induced resistance1*), which is defective in the generation or translocation of a long-distance SAR signal (Maldonado *et al.* 2002). Since *DIR1* encodes a putative lipid transfer protein (LTP), it was hypothesized that DIR1 translocates a lipidic signal to distant leaves during SAR (Maldonado *et al.* 2002). During the induction of SAR, DIR1 protein accesses the phloem for movement from locally infected to distant leaves, supporting the idea that DIR1, and to a lesser extent the DIR1 paralog DIR1-like, chaperones a signal(s) through the phloem (Champigny *et al.*

2013). Further studies identified roles for plastid-localized lipid biosynthetic enzymes such as SFD1 (SUPRESSOR OF FATTY ACID DESATURASE DEFICIENT1), SFD2, and FAD7 (FATTY ACID DESATURASE7) in SAR (Nandi *et al.* 2004; Chaturvedi *et al.* 2008). Phloem exudates collected from SAR-induced leaves of *dir1-1, sfd1,* and *fad7* mutants lack the ability to induce SAR when exogenously applied to naïve wild-type plants, however mixing exudates of *dir1-1* and *sfd1* (or *dir1-1* and *fad7*) restores SAR-inducing activity, suggesting that the DIR1 protein requires an SFD1/FAD7-derived lipidic signal in order to induce SAR (Chaturvedi *et al.* 2008). Subsequent studies have cast doubt on the role of FAD7 in SAR (Xia *et al.* 2010), however SFD1-produced glycerol-3-phosphate has emerged as an important precursor for a lipidic SAR signal that is dependent on DIR1 for SAR induction in *Arabidopsis* (Chanda *et al.* 2011; Yu *et al.* 2013). Moreover, recent studies have also determined that SAR requires Acyl-CoA binding proteins (Xia *et al.* 2012), wild-type cuticle development (Xia *et al.* 2010, 2012), and galactolipid biosynthesis (Chaturvedi *et al.* 2008; Gao *et al.* 2014), further supporting the idea that SAR long-distance signalling involves lipids.

Candidate SAR signals have also been identified through analytical biochemical analysis of phloem exudates collected from SAR-induced and mock-inoculated leaves. This approach has led to the identification of the candidate SAR signals azelaic acid (AzA; Jung *et al.* 2009), dehydroabietinal (DA; Chaturvedi *et al.* 2012), and pipecolic acid (Pip; Navarova *et al.* 2012). Comparative GC-MS (gas chromatography, mass spectrometry) analysis of phloem exudates collected from SAR-induced and mock-inoculated *Arabidopsis* leaves found that the nine-carbon dicarboxylic acid AzA accumulates in phloem exudates collected from SAR-induced, but not mock-inoculated, leaves (Jung *et al.* 2009). Exogenous application of AzA in naïve plants induces the accumulation of SA and *PR1* gene expression, resulting in the activation of local and systemic immune responses.

Moreover, the AzA-induced gene product *AZI1* (*AZELAIC ACID INDUCED1*) is required to generate SAR-activating phloem sap (Jung *et al.* 2009), demonstrating that AzA and AZI1 are required for the generation of long-distance SAR signals and are perhaps signals themselves. A comprehensive activity guided, mass spectrometry-based analytical screen was employed by Chaturvedi *et al.* (2012) to discover the SAR-activating diterpenoid dehydroabietinal (DA) in phloem exudates of SAR-induced wild-type *Arabidopsis* plants. DA and AzA appear to enhance each other's SAR-inducing capabilities, leading to the speculation that these molecules act in concert to induce SAR (Chaturvedi *et al.* 2012). Lastly, analysis of amino acid levels in phloem sap collected from SAR-induced *Arabidopsis* led to the identification of the SAR-inducing lysine catabolite, pipecolic acid (Navarova *et al.* 2012). Pip accumulates to high levels in the phloem following a localized SAR-inducing infection and leads to a primed or enhanced immune system in distal tissues (Navarova *et al.* 2012). Interestingly, Pip accumulation is dependent on key SAR genes such as NPR1 (NONEXPRESSOR OF PR1; Cao *et al.* 1997), FMO1 (FLAVIN MONOOXYGENASE1; Mishina and Zeier 2006) and ALD1 (AGD2-like DEFENSE RESPONSE PROTEIN1; Song *et al.* 2004), of which ALD1 appears to be involved in the Pip biosynthesis pathway (Navarova *et al.* 2012; Bernsdorff *et al.* 2016).

Comparatively less is known about protein composition in the phloem during SAR, with DIR1 being the only documented SAR mobile protein to date (Champigny *et al.* 2013). The DIR1-like protein, which shares 88% amino acid sequence similarity to DIR1 at the mature protein level (lacking signal peptide), may also be mobilized into the phloem during SAR; however, DIR1-like is hypothesized to have a reduced role in SAR compared to DIR1 (Champigny *et al.* 2013). Interestingly, DIR1 interacts with the SAR-related lipid transfer proteins AZI1 and EARLI1 (EARLY ARABIDOPSIS ALUMINUM INDUCED GENE1) in

tobacco epidermal cells (Yu *et al.* 2013; Cecchini *et al.* 2015) and co-fractionates with the SAR-activator DA in a proteinacious high-molecular-weight complex found in SAR-activated *Arabidopsis* phloem sap (Shah *et al.* 2014). Together, these results suggest that DIR1 and other proteins chaperone SAR signals through the phloem during SAR. Moreover, total protein levels are higher in phloem sap collected from SAR-induced relative to mock-inoculated leaves (Champigny *et al.* 2013), suggesting that protein mobilization into the phloem is important during SAR. Further evidence has implicated peptides as potential mobile SAR signals. A genetic screen of activation-tagged *Arabidopsis* lines identified the *CDR1-D* mutant, which overexpresses the apoplastic aspartyl protease CONSTITUTIVE DISEASE RESISTANCE1. The authors hypothesize that CDR1 generates a small peptide SAR signal, as *CDR1* overexpression induces SA accumulation, *PR1* expression, and micro-oxidative bursts (Xia *et al.* 2004). Together, these data demonstrate that proteins contribute to SAR long-distance signalling.

1.2.2.1.3 (iii) Signal Perception & Defense Priming

Our current understanding of signal perception in distant leaves lacks mechanistic detail, as receptors that interact with *bona fide* long-distance signals have not been identified. To date, the conversion of MeSA back to SA by the action of methyl esterases is the best-characterized mechanism of signal perception in distant leaves (Park *et al.* 2007; Vlot *et al.* 2008). Indeed, SA accumulation in distant leaves is required for the full manifestation of SAR, as demonstrated by early grafting experiments in tobacco where SA-deficient NahG (expressing SA hydroxylase) scions were incapable of manifesting SAR despite having received mobile signals from wild-type rootstocks (Vernooj *et al.* 1994). Transduction of the SAR signal(s) in distant leaves requires the transcriptional

co-regulator NPR1, as *npr1* mutants are defective in biologically- and SA-induced SAR-marker gene expression (Cao et al 1997). Further characterization of NPR1 function in *Arabidopsis* determined that NPR1 exists as an oligomer pool in the cytosol of naïve plants, from which active NPR1 monomers are released during SAR induction through the action of cytosolic thioredoxins (TRXh3, TRXh5), allowing for NPR1 accumulation and signalling in the nucleus (Tada *et al.* 2008). Recent evidence has expanded our understanding of NPR1 subcellular localization dynamics during SAR, identifying a mechanism whereby the SnRK2.8 kinase phosphorylates cytosolic NPR1 in distant leaves of SAR-induced plants to promote the nuclear accumulation of NPR1 (Lee *et al.* 2015). In the nucleus, NPR1 interacts with TGA transcription factors that are also important for SAR (Zhang *et al.* 2003). The mediator complex subunit MED16 is also essential for NPR1-mediated SAR, suggesting a direct link between the transcriptional co-activator NPR1 and the RNA polymerase machinery required to carry out SAR transcriptional reprogramming (Zhang *et al.* 2012). Specific gene targets of NPR1 were discovered by Wang *et al.* (2005), who identified the NPR1-dependent upregulation of defense-associated PR genes and components of the secretory pathway, which likely facilitates the secretion of these proteins to the apoplast where they function to prevent pathogen ingress. In addition, NPR1 targets the heat shock factor *HsfB1*, which is an important transcriptional regulator that initiates the transition from growth-to-defense signalling upon pathogen infection (Pajerowska-Mukhtar *et al.* 2012). Mutants in *HSFB1* are defective in MTI and SAR, demonstrating the importance of this transcriptional reprogramming for local and systemic immune responses (Pick *et al.* 2012; Pajerowska-Mukhtar *et al.* 2012). Whether NPR1 itself is the receptor that transduces the SA hormone signal in local and distant leaves has been the subject of recent debate (Kuai *et al.* 2015). One line of evidence suggests that the NPR1 paralogs NPR3 and NPR4 bind SA with different affinities to regulate the proteasomal degradation of NPR1 (Fu *et al.* 2012). In this model, background levels of SA favor the NPR1-

NPR4 interaction, which leads to NPR1 degradation via the proteasome. As SA levels rise, NPR4 binds SA, releasing NPR1 from targeted degradation and allowing for NPR1-mediated SA signalling. At very high levels of SA, NPR3 binds SA allowing for the NPR1-NPR3 interaction to occur, which again targets NPR1 for proteasomal degradation (Fu *et al.* 2012; Moreau *et al.* 2012; Attaran and He 2012). A separate line of evidence suggested that NPR1 acts as an SA receptor by directly binding to SA using key cysteine residues and a copper cofactor (Wu *et al.* 2012). NPR1-SA binding was shown to induce a conformational change that releases the auto-inhibition of NPR1's transactivation domain, which is thought to allow for NPR1-mediated SA signalling (Wu *et al.* 2012). The NPR1-SA interaction was recently recapitulated using a number of protein-SA interaction assays, lending further support to the idea that NPR1 itself is an SA receptor (Manohar *et al.* 2015). In any case, the true nature of the SA-receptor paradigm likely involves both models to some extent, as NPR1, NPR3 and NPR4 all play functional roles during SA-mediated defense responses such as SAR (Fu *et al.* 2012; Wu *et al.* 2012).

Recent work to elucidate the mechanism of Pip-induced SAR demonstrated the existence of Pip and SA amplification loops that are activated in distant leaves following the induction of SAR. In this model, the perception of SAR signals in distant leaves causes the ALD1-depedent accumulation of Pip, which then induces an FMO1-based signalling loop that primes SA-dependent and -independent signalling responses (Navarova *et al.* 2012; Gruner *et al.* 2013; Bernsdorff *et al.* 2016). This leads to significant reprogramming of the distant leaf transcriptome, suppressing genes associated with growth and development while minimally activating defense-related gene expression (Gruner *et al.* 2013; Bernsdorff *et al.* 2016).

The perception of SAR signals in distant leaves does not lead to the constitutive induction of local defense programs, as this would impose a high metabolic cost detrimental to growth and reproductive development (van Hulten *et al.* 2006). Rather, SAR in distant leaves relies on defense priming, a phenomenon that allows for the faster and stronger activation of defense upon secondary pathogen attack. This strategy effectively "prepares" the host for future encounters with pathogens and has reduced metabolic costs compared to the constitutive activation of defense programs (Heidel and Dong 2006; van Hulten *et al.* 2006; Conrath 2011). Priming responses that occur during SAR include the accumulation of inactive map kinases MPK3/MPK6 in the cytosol as well as epigenetic modifications at the promoters of defense-related genes (Conrath *et al.* 2015). Both of these modifications are thought to contribute to faster and enhanced defense responses during secondary pathogen infection. Data from two fundamental studies support the idea that the accumulation of large pools of inactive MPKs are quickly phosphorylated to better induce defense programs (Beckers *et al.* 2009) and that chromatin modifications that impart a relaxed chromatin state at defense gene promoters facilitate high levels of expression when activated (Jaskiewicz *et al.* 2011). Additional support for chromatin modification as a mechanism for SAR priming in distant leaves comes from the observation that the *Arabidopsis* histone-modifying protein FLD (FLOWERING LOCUS D) is required for the perception, but not the generation, of SAR signals (Singh *et al.* 2013). Moreover, exogenous application of a histone demethylase inhibitor 2-PCPA (trans-2-phenylcyclopropylamine) suppresses the SAR response in a manner that mimics *fld* loss-of-function mutants (Singh *et al.* 2014). Chromatin modifications are also associated with the inheritance of defense priming, whereby descendants of SAR-induced (by pathogen or chemical) parents exhibit enhanced defense responses (Luna *et al.* 2012; Slaughter *et al.* 2012). Such responses in primed descendants include enhanced activation of the *PR1, WRKY6,* and *WRKY53* genes, which is thought to be imparted by histone

modifications at the promoters of these genes (Luna *et al.* 2012). These findings demonstrate that the biological induction of SAR, as well as treatment with chemical inducers, is an attractive target for the development of agricultural strategies aimed at providing long-lasting, trans-generational disease resistance to vulnerable crops (Conrath *et al.* 2015).

1.2.2.1.4 (iv) Manifestation of SAR

The establishment of the "primed state" affords enhanced resistance to subsequent pathogen infection in distant leaves. The molecular machinery utilized during this secondary infection is thought to largely overlap with basal defense machinery, with the key difference being the faster and stronger activation of these responses during a secondary infection (Truman *et al.* 2007; Conrath 2011). In *Arabidopsis*, SAR provides enhanced resistance to virulent strains of *P. syringae* and the oomycete *H. arabidopsidis* (Cameron *et al.* 1994; Jing *et al.* 2011). In other plants, SAR has been demonstrated to afford enhanced resistance to a suite of bacterial, fungal, and viral pathogens that are normally virulent on naïve plants. Examples of SAR in agriculturally relevant plants include resistance to *P. infestans* in potato plants (*Solanum tuberosum*) induced for SAR using *P. syringae* (Kombrink *et al.* 1996) or *Phytophthora cryptogea (*Stromburg and Brishammar 1991). More recently SAR has been described in wheat (*Triticum aestivum*) in a study that demonstrated enhanced resistance to *Fusarium culmorum* in plants induced for SAR by an initial infection with *Bacillus mycoides* (Moya-Elizonda and Jacobsen 2016)

1.3 Current Understanding of DIR1 Function in *Arabidopsis*

As stated above, the lipid transfer protein DIR1 is the only protein that has been shown to move from locally infected to distant naïve tissues during SAR. The discovery of DIR1 in the early 2000's led to the hypothesis that SAR requires the long-distance movement of lipidic or lipid-derived mobile signals that are chaperoned through the phloem by DIR1 during SAR. Further characterization of DIR1 in *Arabidopsis* determined that *DIR1* is expressed in all green tissues of the plant and is downregulated during local *P. syringae* infection in a T3SS-dependent manner (Champigny *et al.* 2011). Importantly, *DIR1* is expressed in phloem companion cells, situating DIR1 in the right place for phloem-mediated long-distance movement (Champigny *et al.* 2011). Subcellular localization studies of fluorescent protein (FP) tagged DIR1 in *N. benthamiana* epidermal cells demonstrate that DIR1 is localized to the cell wall, apoplast, perinuclear endoplasmic reticulum (ER), and plasmodesmata (Champigny *et al.* 2011; Chanda *et al.* 2011). Localization of DIR1 to the cell wall/apoplast requires an N-terminal ER signal sequence (SS; amino acids 1-25). Intriguingly, the DIR1 signal sequence is not required for SAR, as demonstrated by the SAR competent phenotype of 35S:DIR1^{-SS}/*dir1-1* plants (Champigny *et al.* 2011). Moreover, DIR1^{-SS} is detected in phloem exudates collected from SAR-induced 35S:DIR1^{-SS}/*dir1-1* plants, suggesting that a cytosolic pool of DIR1 may be important during SAR (Champigny *et al.* 2011). Earlier structural analyses demonstrated that DIR1 is an atypically acidic LTP that contains a central hydrophobic cavity (242 Å^3) capable of accommodating mono-acylated fatty acids *in vitro* (Lascombe *et al.* 2008). An *in vivo* DIR1-ligand has not been identified, despite numerous studies demonstrating that DIR1 is required for the SAR-inducing activity of signal candidates such as AzA, G3P, and DA (Jung *et al.* 2009, Chanda *et al.* 2011; Chaturvedi *et al.* 2012). Moreover, DIR1 co-purifies with DA in phloem exudates collected from SAR-induced *Arabidopsis* (Shah *et al.* 2014), however a direct

interaction between DIR1 and DA has not been observed. In addition, the long-distance movement of the SAR-activator G3P is thought to be co-dependent on DIR1, such that both DIR1 and G3P are required for either molecule to travel in the phloem during SAR (Chanda *et al.* 2011). However, these experiments were based on the exogenous application of recombinant DIR1 protein, which may have induced cell death (discussed in Champigny *et al.* 2013). This idea is especially relevant when considering the authors observation that excess DIR1 protein activates SAR without pathogen-inoculation (Chanda *et al.* 2011), which is not observed in transgenic plant lines that overexpress *DIR1* (Maldonado *et al.* 2002; Champigny *et al.* 2011, 2013). Therefore, less intrusive *in vivo* experimentation is required to better explore the co-dependency of DIR1-G3P movement. Since DIR1 does not bind G3P directly, it was hypothesized that a G3P-derived SAR activator interacts with DIR1 during SAR (Chanda *et al.* 2011; Yu *et al.* 2013). Lastly, altered MeSA levels caused by increased *BSMT1* expression observed in *dir1-1* mutants during SAR-induction has also linked DIR1 to MeSA-mediated SAR (Liu *et al.* 2011b). Together, these studies demonstrate that DIR1 is a central component of the SAR response and is likely involved in regulating the activity (G3P, AzA) or abundance (MeSA) of SAR-related small molecules.

The *Arabidopsis* genome encodes a single DIR1 paralog, DIR1-like (AT5G48490), which shares 88% sequence similarity with DIR1 at the mature protein level (Champigny *et al.* 2013). The *dir1-1* mutant occasionally exhibits a SAR-competent phenotype and DIR1-antibody signals are sometimes observed in phloem exudates collected from SAR-induced *dir1-1* (Champigny *et al.* 2013). Since the polyclonal DIR1-antibody detects recombinant DIR1 and DIR1-like protein with similar efficiency, we hypothesized that DIR1-like is responsible for the antibody signals in SAR-induced *dir1-1* phloem exudates and the occasional SAR-competent phenotype (Champigny *et al.* 2013). This would imply that DIR1-

like is similarly mobilized to the phloem during the induction of SAR in *Arabidopsis*. Aside from this speculation, our understanding of DIR1-like's role during SAR is not well understood. Similar to DIR1, DIR1-like has an acidic isoelectric point and *DIR1-like* expression is also suppressed during local interactions with *P. syringae* (Champigny *et al.* 2011, 2013). Efforts to create a *DIR1-like* antisense line in the *dir1-1* background were unsuccessful, leading to the idea that a double *DIR1/DIR1-like* knockdown line may be lethal in *Arabidopsis*. Conditions that promote DIR1-like's participation in SAR are currently unknown. However, circumstantial evidence suggests that conditions at McMaster favor DIR1-like's participation during SAR, as relocation of the Cameron lab from Toronto to Hamilton (Ontario, Canada) led to the emergence of the "DIR1-like" phenotype.

1.4 Research Questions, Hypotheses, and Objectives

The overall goal of this thesis is to further explore the role of DIR1 and DIR1-like during SAR, and to identify novel phloem-mobile proteins that contribute to the SAR response in *Arabidopsis thaliana*.

Chapter 3: Plasmodesmata-located protein overexpression negatively impacts the manifestation of systemic acquired resistance and the long-distance movement of Defective in Induced Resistance1 in Arabidopsis

Hypothesis 1: DIR1 moves cell-to-cell to access the phloem for long-distance movement during SAR in *Arabidopsis thaliana*.

Objective 1: Characterize DIR1 movement and SAR phenotypes in *Arabidopsis* plant lines with reduced cell-to-cell movement through plasmodesmata caused by

the overexpression of PLASMODESMATA-LOCATED PROTEINs (35S:PDLP1, 35S:PDLP5).

Chapter 4: Orthology analysis and in vivo complementation studies to elucidate the role of DIR1 during Systemic Acquired Resistance in Arabidopsis thaliana and Cucumis sativus

Hypothesis 2: Conserved amino acid residues in putative DIR1 orthologs from *Nicotiana tabacum, Glycine max, Cucumis sativus,* and *Solanum lycopersicum* are important for DIR1 function.

Objective 2: Identify and test the importance of conserved amino acid residues in DIR1 orthologs through *in vivo* complementation studies and *in vitro* biochemical assays.

Chapter 5: Exploring the role of DIR1, DIR1-like and other lipid transfer proteins during systemic immunity in Arabidopsis

Hypothesis 3: DIR1-like is not essential for the SAR response in *Arabidopsis*.

Objective 3: Generate and characterize the SAR phenotypes of *DIR1-like*$_{RNAi}$ knockdown lines.

Hypothesis 4: Proteins that interact with DIR1 are important for SAR in *Arabidopsis*.

Objective 4: Identify DIR1 and DIR1-like interacting proteins using yeast- and plant-based interaction assays and explore the SAR phenotypes of T-DNA mutants corresponding to DIR1/DIR1like-interactors.

Chapter 6: Comparative Proteomics Analysis of Phloem Exudates Collected During the Induction of Systemic Acquired Resistance

Hypothesis 5: Proteins with differential abundance in the phloem during SAR play a functional role in the SAR response in *Arabidopsis*.

Objective 5: Use comparative proteomics techniques to identify proteins with differential abundance in phloem exudates collected from mock-inoculated and SAR-induced leaves. Identify phloem proteins with a functional role in SAR by testing the SAR phenotypes of corresponding T-DNA mutants.

1.5 Contributions Not Discussed in This Thesis

Long-Distance Movement of DIR1 During SAR

Our work to establish the long-distance movement of DIR1 during SAR in *Arabidopsis* (Champigny *et al.* 2013) was briefly described in the introduction. I contributed to this project by: (a) performing antibody specificity experiments to establish that the polyclonal DIR1 antibody recognizes both DIR1 and DIR1-like, (b) RT-PCR analysis to ensure that estrogen-induced *DIR1-EGFP* expression occurs only in the estrogen-treated leaf, (c) SAR assays using the *XVE:DIR1-EGFP/dir1-1* lines, and (c) phloem exudation collection experiments using the *XVE:DIR1-EGFP/dir1-1* lines.

Investigating the Age-Related Resistance (ARR) Response

Plants respond to pathogen infection in several ways. The Age-Related Resistance (ARR) response is an example of how resistance responses change throughout development to provide enhanced resistance to normally virulent pathogens (Develey-Riviere and Galiana 2007). In *Arabidopsis*, ARR is characterized by an increase in resistance to virulent strains of *P. syringae* (Kus *et al.* 2002) and *H. arabidopsidis* (Rusterucci *et al.* 2005) as plants age. The transition to flowering (Rusterucci *et al.* 2005) and a capacity to accumulate intra- and extracellular SA are associated with ARR in *Arabidopsis* (Cameron and Zaton 2005).

During my time in the Cameron lab, I participated in a number of ARR-related studies. Along with fellow co-first authors Dan Wilson and Marisa Isaacs, we determined that ARR in *Arabidopsis* is not triggered by the transition to flowering by assessing the ARR phenotypes of a number of early- and late-flowering mutant lines (Wilson *et al.* 2013). Interestingly, the MADS domain transcription factor SVP (SHORT VEGETATIVE PHASE) was ARR defective, unlike other early-flowering lines (Wilson *et al.* 2013). The investigation of SVP's role in ARR is ongoing.

A forward genetic screen identified the ARR-defective *iap1-1* mutant, which is defective in the ability to accumulate SA during ARR (Carviel *et al.* 2009). I contributed to the characterization of *iap1-1* by performing SA quantification experiments and helping with ion leakage assays (midnight shift-work), which together demonstrated that *iap1-1* is compromised in ETI and ETI-induced SA accumulation (Carviel *et al.* 2014). In addition, Dan Wilson and I co-wrote a perspective article focusing on SA accumulation and signalling during ARR for a research topic in *Frontiers in Plant Science* (Carella *et al.* 2015c).

Investigating Disease Resistance in Eutrema salsugineum

Eutrema salsugineum is an extremophile model plant that has an enhanced capacity to tolerate abiotic stressors such as cold temperature, drought, and salinity. To learn more about how plants deal with biotic as well as abiotic stress, we investigated disease resistance in two *Eutrema* accessions derived from the Yukon Territory (Canada) and Shandong Province (China). Co-first author May Yeo and I demonstrated that both *Eutrema* accessions are more resistant to *P. syringae* compared to *Arabidopsis* Col-0, with Shandong plants displaying an exceptionally strong resistance response reminiscent of defense-primed accessions of *Arabidopsis* (Yeo *et al.* 2015). This work also described ETI and ARR responses in *Eutrema,* in addition to investigating the highly resistant nature of Shandong *Eutrema* (Yeo *et al.* 2015). Our work demonstrates that a capacity to withstand abiotic stress does not impact disease resistance responses, and sets the framework for future experiments to investigate the effect of subsequent and/or concurrent stress responses in *Eutrema.*

Chapter 2
Materials & Methods

PREFACE:

This chapter contains the materials and methods for Chapter 5 and the data presented in the Appendix. Each of the published chapters (Chapter 3, 4, 6) have self-contained methods sections. Please note that there is significant overlap between the methods described below and the methods in Chapters 3, 4, and 6. References contained in Chapter 2 are included in the reference list for Chapter 5.

2.1 Plant Growth Conditions

Seeds of wild-type *Arabidopsis thaliana* (Col-0), *dir1-2* (GK403C01), *ltp2-1* (GK638E09), 35S:DIR1-like$_{RNAi}$ and 35S:LTP2-FLAG/*ltp2-1* were surface sterilized, followed by stratification in the dark for 48 hours at 4 °C. Seeds were then plated on Murashige and Skoog (MS) media plates solidified with 6% phytagar (Caisson Labs) and germinated under continuous light for 5-7 days. Seedlings were transplanted to soil hydrated with 1 g L^{-1} of 20-20-20 fertilizer and grown under short day photoperiod conditions (9 hr light; 150 µE m^{-2} s^{-1}) in 60-85% relative humidity at 22 °C. *Nicotiana benthamiana* seeds were germinated directly on soil hydrated with 1 g L^{-1} of 20-20-20 fertilizer and grown under long day photoperiod conditions (16 hr light; 150 µE m^{-2} s^{-1}) in 60-85% relative humidity at 22 °C. All lines used in this study were homozygous as determined by molecular characterization (*dir1-2* and *ltp2-1*) and/or germination on selective MS media supplemented with sulfadiazine (5 µg ml^{-1}) or hygromycin B (25 µg ml^{-1}). All plant lines used in this study are described in Table S1/2.1.

Table 2.1 – Plant Lines Used in This Study (Chapter 5, Table S1)

Plant Line	Mutation	Source
dir1-2 (GK403C01)	T-DNA in coding sequence	Generated by GABI-KAT, Obtained from NASC (N438617)
ltp2-1 (GK639E08)	T-DNA in coding sequence	Generated by GABI-KAT, Obtained from NASC (N461304)
35S:DIR1-like$_{RNAi}$	*DIR1-like* knockdown in Col-0	This Study
35S:LTP2-FLAG/*ltp2-1*	*LTP2-FLAG* OE in *ltp2-1*	This Study

2.2 Bacterial Growth and Inoculations

Pathogen inoculation experiments utilized virulent *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (pVSP1) and avirulent Pst DC3000/AvrRpt2 (pVSP1 + avrRpt2) (Whalen *et al.* 1991). *P. syringae* was cultured by shaking overnight (room temperature) in King's B media (King *et al.* 1954) supplemented with kanamycin (50 µg ml^{-1}). Local resistance assays were performed by inoculating leaves of 4 week-old plants with 10^6 colony forming units (cfu) mL^{-1} of virulent Pst DC3000 or avirulent Pst DC3000/AvrRpt2 via pressure infiltration using a needleless syringe. SAR assays were performed as described in Carella *et al.* (2016a). Two lower leaves of 3.5 to 4 week-old plants were mock-inoculated (10 mM $MgCl_2$) or induced for SAR with 10^6 cfu mL^{-1} avirulent Pst DC3000/AvrRpt2. Two days later, three upper leaves were challenged for SAR with 10^6 cfu mL^{-1} virulent Pst DC3000. Bacterial densities were quantified at 0 and 3 days post inoculation (dpi) for local resistance assays, and at 3 dpi for SAR assays, by serial dilution plating as described in Kus *et al.* (2002). *Agrobacterium tumefaciens* strains (GV3101 background) were cultured by shaking overnight at 28 °C in LB media supplemented with rifampicin (50 µg ml^{-1}) and gentamycin (25 µg ml^{-1}), as well as kanamycin (50 µg ml^{-1}) or spectinomycin (50 µg ml^{-1}) when appropriate.

2.3 Phloem Exudate Collection and Immunoblotting

Phloem exudates were collected from 3.5-4 week-old *Arabidopsis* plants that were mock-inoculated (10 mM $MgCl_2$) or induced for SAR with 10^6 cfu mL^{-1} of avirulent Pst DC3000/AvrRpt2 as described in Carella *et al.* (2015). Exudates were collected from 25-48 hours post inoculation (hpi) using the optimized EDTA exudation protocol described in Guelette *et al.* (2012) with modifications that are described in Carella *et al.* (2015). Exudates were lyophilized, resuspended in water, and loaded into NuPAGE Novex 4-12% Bis-Tris pre-cast protein gels (Life Technologies). Each lane was loaded with a single tube of exudate (10-12 leaves per tube), blotted to nitrocellulose, and probed with a polyclonal DIR1 antibody as previously described (Champigny *et al.* 2013).

2.4 Generation of Constructs and Transgenic Plants

**All primers used to generate the constructs described below can be found in Table 2.2. Lower case letters denote restriction sequences, adaptor sequences, or modifications to maintain proper open reading frames.

Constructs for Split Ubiquitin Assays

Prey constructs for split ubiquitin interaction assays were generated by homologous recombination of PCR amplified inserts into pNXgate32 (ABRC, CD3-935) in the *Saccharomyces cerevisiae* THY.AP5 strain (ABRC, CD3-809) as described in Obrdlik *et al.* (2004). Inserts were created by two rounds of PCR; an initial amplification using gene specific primers, followed by a subsequent reaction to generate sequences necessary for homologous recombination using the B1 and B2 adapter primers (Adpt-B1 + Adpt-B2). All sequences used for split ubiquitin construct generation lack signal peptides as determined by SignalP.

Homologous recombination was achieved by co-transformaing the THY.AP5 strain with purified gene specific inserts and linearized (EcoRI + SmaI) pNXgate32. Yeast transformation was performed using the LiAC-ssDNA carrier method as described in Gietz and Schiestl (2007). Transformants were selected on Synthetic Defined (SD; 6.7 g L^{-1} yeast nitrogen base without amino acids, 2% glucose, 20 g L^{-1} agar when needed) minimal media supplemented with amino acid dropout mix lacking tryptophan (See Clontech Yeast Protocols Handbook, or Cold Spring Harbor Recipe, doi:10.1101/pdb.rec085639). Transformants were tested for G418 sensitivity to confirm successful recombination of the insert. Bait constructs for split ubiquitin interaction assays were generated by restriction-ligation cloning in *Escherichia coli*. Gene specific inserts lacking signal peptides were cloned into the bait vector pDHB1 by directional cloning using the SfiI enzyme. Constructs were then purified and transformed into the *S. cerevisiae* THY.AP4 strain (ABRC, CD3-808) using the transformation protocol described in Gietz and Schiestl (2007). Transformants were selected on SD dropout media plates lacking leucine.

Table 2.2 – Primers for Construct Generation (Chapter 5, Table S2)

Primer Name	Sequence (5' - 3')	Construct
Adpt-B1	ACAAGTTTGTACAAAAAAGCAGGCTCTCCAACCACCATG	N/A
Adpt-B2	TCCGCCACCACCAACCACTTTGTACAAGAAAGCTGGGTA	N/A
B1-DIR1-ss	gcaggctctccaaccaccATGGCGATAGATCTCTGCGGCAT	DIR1-NubG
B2-DIR1	gtacaagaaagctgggtaACAAGTTGGGGCGTTGGCTAG	
B1-DLike-ss	gcaggctctccaaccaccATGGCGATTGACCTTTGTGGCATG	DIR1-like-NubG
B2-DLike	gtacaagaaagctgggtaACAAGTTGGGGCGTTGGTTAG	
B1-AZI1-ss	gcaggctctccaaccaccATGACAAATTGCAACTGCAAGCC	AZI1-NubG
B2-AZI1	gtacaagaaagctgggtaAGCACATTGGAAACCAGATG	
B1-NPR1	gcaggctctccaaccaccATGGACACCACCATTGATGG	NPR1-NubG
B2-NPR1	gtacaagaaagctgggtaCCGACGACGATGAGAGAGTT	
B1-MES9	GCAGGCTCTCCAACCACCATGAAGCATTATGTGCTAGT	MES9-NubG
B2-MES9	GTACAAGAAAGCTGGGTAGGGATATTTATCAGCAATCT	
B1-MES1	GCAGGCTCTCCAACCACCATGAGTGAGGAAAAGAGGAAAC	MES1-NubG
B2-MES1	GTACAAGAAAGCTGGGTAAACGAATTTGTCCGCGATTTTC	
B1-MES7	GCAGGCTCTCCAACCACCATGGATAAGAATAACCAGAAG	MES7-NubG
B2-MES7	GTACAAGAAAGCTGGGTAGGCGTATTTATCTGCAATCTC	
B1-LTP1-ss	GCAGGCTCTCCAACCACCATGGCGCTAAGCTGTGGCTCAGT	LTP1-NubG
B2-LTP1	GTACAAGAAAGCTGGGTACCTCACGGTTTTGCAGTTGG	
B1-LTP2-ss	GCAGGCTCTCCAACCACCATGCTTATGAGTTGTGGCACCGT	LTP2-NubG
B2-LTP2	GTACAAGAAAGCTGGGTACCTCACGGTGTTGCAGTTGG	
SfiI-DIR1-ss	ATTAACAAGGCCATTACGGCCGCGATAGATCTCTGCGGCATG	DHB1-DIR1-Cub
SfiI-DIR1-R	aactgattGGCCGAGGCGGCCccACAAGTTGGGGCGTTGGCTAG	
SfiI-DLike-ss	ATTAACAAGGCCATTACGGCCGCGATTGACCTTTGTGGCATG	DHB1-DIR1-like-Cub
SfiI-DLike-R	aactgattGGCCGAGGCGGCCccACAAGTTGGGGCGTTGGTTAG	
SacI-DIR1-F	GGAACAGAGCTCATGGCGAGCAAGAAAGCAGCTATGG	DIR1-nYFP, DIR1-cYFP
BamHI-DIR1-R	TGTCCAGGATCCcACAAGTTGGGGCGTTGGCTAGACC	
SacI-DLike-F	GGAACAGAGCTCATGACAAGCAAGAAGGTGGC	DIR1-like-nYFP, DIR1-like-cYFP
BamHI-DLike-R	TGTCCAGGATCCcACAAGTTGGGGCGTTGGTTAGGTC	
SacI-LTP1-F	GGAACAGAGCTCATGGCTGGAGTGATGAAGTTGGCATGCTTGC	LTP1-nYFP, LTP1-cYFP
BamHI-LTP1-R	TGTCCAGGATCCcCCTCACGGTTTTGCAGTTGGTGCTGG	
SacI-LTP2-F	GGAACAGAGCTCATGGCTGGAGTGATGAAGTTGGCATGCATGG	LTP2-nYFP, LTP2-cYFP
BamHI-LTP2-R	TGTCCAGGATCCcCCTCACGGTGTTGCAGTTGGTGC	
SpeI-DLike-F	GGAACAactagtATGACAAGCAAGAAGGTGGC	DIR1-like-GFP
KpnI-DLike-R	tcaaggGGTACCcccACAAGTTGGGGCGTTGGTTAGGTC	
SpeI-LTP1-F	GGAACAactagtATGGCTGGAGTGATGAAGTTGGC	LTP1-GFP
KpnI-LTP1-R	tcaaggGGTACCccCCTCACGGTTTTGCAGTTGGTGC	
SpeI-LTP2-F	GGAACAactagtATGGCTGGAGTGATGAAGTTGGC	LTP2-GFP
KpnI-LTP2-R	tcaaggGGTACCccCCTCACGGTGTTGCAGTTGGTGC	
AscI-LTP2-F	CACACGGGCGCGCCaccATGGCTGGAGTGATGAAGTTGG	LTP2-FLAG
LTP2-FLAG-R1	catcgtcatccttgtaatcCCTCACGGTGTTGCAGTTGG	
SacI-LTP2FLAG-R2	GGAACAGAGCTCTCActtgtcatcgtcatccttgtaatcCCT	
XhoI/AscI-SenseDLike-F	TCGCTCGAGGGCGCGCCATTGACCTTTGTGGCATGACTC	DIR1-Like-RNAi
KpnI-SenseDLike-R	TCGGGTACCTTAACAAGTTGGGGCGTTG	
ClaI-AntiDlike-F	CTGATCGATTTAACAAGTTGGGGCGTTG	
XbaI/SacI-AntiDLike-R	CTGTCTAGAGAGCTCATTGACCTTTGTGGCATGAC	

Constructs for BiFC and Subcellular localization

Constructs for bimolecular fluorescence complementation studies were generated by restriction-ligation cloning in *E. coli.* Full-length coding sequences lacking stop codons were directionally cloned (SacI + BamHI) into pSAT4A-nEYFP-N1 (ABRC, CD3-1083) and pSAT4A-cEYFP-N1 (ABRC, CD3-1079) to generate nYFP and cYFP fusion proteins respectively. Sequence confirmed vectors were then sub-cloned into the binary vector pPZP-RCS2-BAR-SmaI (ABRC, CD3-1059) using the ISce-I meganuclease to generate constructs suitable for transient *Agrobacterium*-mediated expression. GFP fusion constructs for subcellular localization studies were generated by restriction-ligation cloning in *E. coli.* Full-length coding sequences lacking stop codons were directionally cloned (SpeI + KpnI) into pMDC83 (ABRC, CD3-742) to generate GFP fusions. All vectors were sequenced and transformed into *A. tumefaciens* GV3101.

Constructs Used to Generate Transgenic Arabidopsis Lines

The LTP2-FLAG amplicon was generated by performing two rounds of PCR amplification (Forward + Reverse1 followed by Forward +Reverse2). Full-length LTP2-FLAG was directionally cloned into pMDC32 (ABRC, CD3-738) using AscI and SacI. Vectors to generate *DIR1-like* RNAi plants were generated in two steps. The *DIR1-like* RNAi cassette was first created by cloning sense and antisense *DIR1-like* sequence into the pHANNIBAL RNAi vector by directional cloning. Sense *DIR1-like* sequence was cloned using the XhoI and KpnI sites, while antisense *DIR1-like* was cloned using ClaI and XbaI. The RNAi cassette was then sub-cloned into the pMDC32 expression vector by directional cloning using AscI and SacI. All vectors were sequenced and transformed into *A. tumefaciens* GV3101.

Transformation of Arabidopsis

Transgenic *Arabidopsis* lines were created by *Agrobacterium*-mediated transformation using the *Arabidopsis* floral dip method (Clough and Bent 1998). Bulked seed from transformed plants was plated on MS media supplemented with timentin (300 µg ml^{-1}) to select against *A. tumefaciens* growth and Hygromycin B (25 µg ml^{-1}, 35S:LTP2-FLAG and 35S:DIR1-$like_{RNAi}$) to select for transformants. Homozygous plants with single T-DNA inserts were isolated through segregation analysis of the Hygromycin resistance marker.

2.5 Split Ubiquitin Yeast Two-Hybrid Interaction Assays

Split ubiquitin interaction assays were performed as described in Obrdlik *et al.* (2004). In brief, pairwise interactions were tested using diploid yeast generated by mating appropriate THY.AP4 (Bait, mating type "a") and THY.AP5 (Prey, mating type "α") strains on yeast extract/peptone/dextrose (YPD; 10 g L^{-1} yeast extract, 20 g L^{-1} bacto-peptone, 2% glucose, 15 g L^{-1} agar when needed) plates for 8 hours at 28 °C. Diploid cells were then selected by streaking onto SC dropout media plates lacking leucine, tryptophan and uracil, and incubated at 28 °C. 2-3 days later, diploid cells were collected and streaked onto interaction specific SC dropout media plates lacking adenine, tryptophan, histidine, leucine, uracil, and methionine (-ATHLUM). Plates were supplemented with 3 mM 3-aminotriazole (3-AT) to ensure stringent histidine auxotrophy. Growth (at 28 °C) was observed 3-7 days post plating on interaction specific media.

2.6 Transient *Agro*-mediated Expression and Confocal Microscopy

Overnight cultures of *A. tumefaciens* GV3101 containing split YFP constructs (DIR1-nYFP, DIR1-cYFP, DIR1-like-nYFP, DIR1-like-cYFP, LTP1-cYFP, LTP2-

cYFP) were centrifuged at 2000 x *g* for 5 minutes, resuspended in Agro-infiltration "induction" buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, 100 µM acetosyringone) and adjusted to OD_{600} = 1 for BiFC experiments, or OD_{600} = 0.5 for subcellular localization experiments. Agrobacteria were then incubated with shaking in the dark for 2 hours. Appropriate 1:1 mixtures of cYFP and nYFP containing constructs were mixed thoroughly and co-infiltrated into fully expanded *N. benthamiana* leaves using a needleless syringe. Confocal fluorescence microscopy was performed using the Leica TCS SP5 platform, with default settings for YFP or GFP detection.

2.7 RNA Isolation, cDNA Synthesis, and (q) RT-PCR Analyses

RNA was isolated from frozen leaf tissue using the Sigma TRI-reagent as described in Carella *et al.* (2015a). 2 µg of RNA was used for cDNA synthesis using Sigma M-MLV reverse transcriptase following the manufacturer's instructions. RT-PCR analysis was performed using Taq polymerase (BioBasic Inc, Canada), with an annealing temperature of 60 °C unless specified otherwise. Quantitative (q)-RT-PCR analysis was performed in a 10 µL reaction consisting of 2 µL of diluted cDNA (2.5-fold with water), 1x LuminoCT SYBR Green qPCR Ready Mix (Sigma), and 200 nM (*DIR1-Like*) or 400 nM (*5-FCL*) of each primer. Reaction mixes were loaded onto low profile 96-well plates and qRT-PCR was performed using the BioRad CFX96 Real-Time PCR Detection System (BioRad). Results were analyzed using BioRad CFX Manager software version 2.0. Primers used in this study (Chapter 5) can be found in Table S3/2.3.

Table 2.3 – Primers Used for (q) RT-PCR Analyses (Chapter 5, Table S3)

Primer Name	Sequence (5' - 3')	Amplicon
DIR1-RT-F	GATCGTGATAATGGCTATGTTGGTCGATACATC	DIR1
DIR1-RT-R	GCGTTGGCTAGACCACACTGTTTGGGGAGAGC	
TruncDIR1-RT-R	CCACAAAGACATGCAAAATCAGCGTGTTGCA	Truncated DIR1
LTP2-RT-F	CAATCACAGCGAACGCGCTTATG	LTP2
LTP2-RT-R	GTAGCTTCATTTGACCGTCGCTC	
ACTIN1-RT-F	GGCGATGAAGCTCAATCCAAACG	ACTIN1
ACTIN1-RT-R	GGTCACGACCAGCAAGATCAAGACG	
DIR1like-qRT-F	AATAAAGAGGATAAAATGACAAGC	DIR1-like
DIR1like-qRT-R	CTGGTAAGCATTCATTCAACTC	
5FCL-qRT-F	TGTCCGCAAATCCCTAAAAG	5-FCL
5FCL-qRT-R	CCAGGGAGCTTCAAGAACAG	

2.8 Methods for Appendix Material

Creating 35S:DIR1-FLAG Plant Lines

The DIR1-FLAG construct was generated by recombination-based gateway cloning. A C-terminal DIR1-FLAG fusion insert suitable for use in gateway cloning was initially synthesized by performing two rounds of PCR (Primers can be found in Table 2.4). The first reaction (F + R1) added half of the FLAG sequence to the C-terminal end of DIR1, while the second round (F + R2) completed the FLAG sequence and added the attB1 sequence necessary for gateway cloning. DIR1-FLAG was cloned into pDONR221 by an initial BP clonase reaction to generate the DIR1-FLAG entry clone, followed by an LR recombinase reaction to transfer DIR1-FLAG into pK7WG2D to generate the 35S:DIR1-FLAG construct. Enzymes for gateway cloning (LR and BP enzymes, Thermofisher) were used following the manufacturer's instructions. 35S:DIR1-FLAG was transformed into the XVE:avrRpt2 and XVE:avrRpt2;rps2-101 plant lines (Tsuda *et al.* 2012) by *Agrobacterium*-mediated floral dip transformation (Clough and Bent 1998). Transformants were isolated by germination on MS media supplemented with timentin (300 μg mL^{-1}) and kanamycin (50 μg mL^{-1}). Single insert, homozygous

transgenic lines were isolated by following the segregation of the kanamycin selectable marker, and were used to isolate lines with suitable DIR1-FLAG overexpression as determined by RT-PCR and immunoblot analyses. Primers for RT-PCR characterization can be found in Table 2.4. A monoclonal FLAG antibody was used to detect DIR1-FLAG in protein extracted from whole seedlings (~20-50 µg protein loaded per lane) using a mild protein extraction buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100).

Table 2.4 – Primers for Appendix Material

Primer Name	Sequence (5' - 3')	Construct/ Amplicon
attB1-DIR1FLAG-F	GGGGACAAGTTTgtacaaaaaagcaggctTCACCATGGCGAGCAAGAA AGCAGC	DIR1-FLAG
DIR1-FLAG-R1	cgtcatccttgtaatcACAAGTTGGGGCGTTGGCTAGACC	
attB2-DIR1FLAG-R2	GGGGACCACTTTGTACAAGaaagctgggtCTTActtgtcatcgtcatccttg	
DIR1-RT-F	GATCGTGATAATGGCTATGTTGGTCGATACATC	DIR1
DIR1-RT-R	GCGTTGGCTAGACCACACTGTTTGGGGAGAGC	
ACTIN1-RT-F	GGCGATGAAGCTCAATCCAAACG	ACTIN1
ACTIN1-RT-R	GGTCACGACCAGCAAGATCAAGACG	

Estrogen-activated SAR System: SAR assays and Phloem Exudate Collection

SAR assays and phloem exudate collection for experiments involving the 35S:DIR1-FLAG/XVE:avrRpt2 and 35S:DIR1-FLAG/XVE:avrRpt2;r*ps2* plant lines were performed essentially as described in the methods sections above, with the key exception being that plants were treated with estrogen (β –estradiol, Sigma) rather than pathogens to induce SAR. Plants were induced by pressure infiltrating 50 µM β-estradiol into lower leaves of 4 week-old plants. Exudate collection, immunoblotting, SAR challenge inoculations, and bacterial quantification were performed as described above.

Immunoprecipitation

Immunoprecipitation was performed on total protein extracts of 35S:DIR1-FLAG seedlings (1-2 weeks post germination) using the M2 FLAG affinity matrix kit (Sigma). Protein was extracted using the protein extraction buffer provided in the kit (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). Protein levels were quantified using the BCA protein quantification kit (Thermofisher) following the manufacturer's instructions. 1 mg of total protein was incubated with 20 µL of sepharose A beads (Roche) for 1 hour at 4 °C while turning end-over-end to pre-clear the lysate of proteins that non-specifically interact with beads. To immunoprecipitate DIR1-FLAG, pre-cleared lysates were incubated with 40 µL of the M2 FLAG affinity matrix at 4 °C while turning end-over-end for 8 hours. Beads were washed, and immunoprecipitated protein was eluted from the beads with 3x-FLAG peptide following the manufacturer's instructions.

Chapter 3

Plasmodesmata-located protein overexpression negatively impacts the manifestation of systemic acquired resistance and the long-distance movement of Defective in Induced Resistance1 in Arabidopsis

PREFACE:

This chapter consists of the published article entitled "***Plasmodesmata-located protein overexpression negatively impacts the manifestation of systemic acquired resistance and the long-distance movement of Defective in Induced Resistance1 in Arabidopsis***" by Carella P, Isaacs M, and Cameron RK (*Plant Biology* 17, 395-401, 2015). This publication has been reproduced in its original format with permission for use in a dissertation/thesis under a license provided by John Wiley & Sons to Philip Carella through RightsLink (License Number: 3802141488467, License Date: Feb 4, 2016). An addendum to this article is also included to provide an extended discussion of this work. The addendum article is entitled "***Mind the Gap: Signal movement though plasmodesmata is critical for the manifestation of SAR****"* by Carella P, Wilson DC, and Cameron RK (*Plant Signaling & Behavior,* e1075683, 2015). The addendum has been reproduced in its original format under the terms of the "Creative Commons Attribution-Non-Commercial License" which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited appropriately.

This work describes the SAR phenotypic analysis of transgenic *Arabidopsis* plant lines with restricted symplastic trafficking through plasmodesmata caused by the overexpression of plasmodesmata-located proteins PDLP1 and PDLP5. Both PDLP1 and PDLP5 overexpressing plant lines were defective in the manifestation of SAR. Moreover, the long-distance movement of DIR1 was suppressed in PDLP-overexpressing plants, such that DIR1 could not be detected in phloem exudates collected from distant leaves of SAR-induced plants. Together, the data suggest that DIR1 and other SAR signals require symplastic movement through plasmodesmata during SAR.

Contributions:

Philip Carella generated all of the data and figures contained in this article. Marisa Isaacs assisted with SAR assays and phloem exudate collection experiments. Philip Carella and Robin Cameron wrote the manuscript, Marisa Isaacs helped with data analysis and editing the manuscript. The transgenic plant lines used in this study were obtained from Dr. J-Y Lee (University of Delaware; 35S:PDLP5) and Dr. Y Benitez-Alfonso (University of Leeds; 35S:PDLP1). Philip Carella and Robin Cameron wrote the article addendum with help from Dan Wilson. Philip Carella created Figure 1 of the addendum with the help of Robin Cameron and Dan Wilson.

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RESEARCH PAPER

Plasmodesmata-located protein overexpression negatively impacts the manifestation of systemic acquired resistance and the long-distance movement of Defective in Induced Resistance1 in *Arabidopsis*

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Keywords
Cell-to-cell movement; DIR1; lipid transfer protein; long-distance signalling; PDLP; plasmodesmata; systemic acquired resistance.

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ABSTRACT

Systemic acquired resistance (SAR) is a plant defence response that provides immunity to distant uninfected leaves after an initial localised infection. The lipid transfer protein (LTP) Defective in Induced Resistance1 (DIR1) is an essential component of SAR that moves from induced to distant leaves following a SAR-inducing local infection. To understand how DIR1 is transported to distant leaves during SAR, we analysed DIR1 movement in transgenic *Arabidopsis* lines with reduced cell-to-cell movement caused by the overexpression of Plasmodesmata-Located Proteins PDLP1 and PDLP5. These PDLP-overexpressing lines were defective for SAR, and DIR1 antibody signals were not observed in phloem sap-enriched petiole exudates collected from distant leaves. Our data support the idea that cell-to-cell movement of DIR1 through plasmodesmata is important during long-distance SAR signalling in *Arabidopsis*.

INTRODUCTION

Systemic acquired resistance (SAR) is a plant disease resistance pathway in which an initial infection elicits movement of mobile signals that establish broad-spectrum pathogen resistance in distant uninfected leaves. SAR can be divided into four stages, the first being the induction stage. In *Arabidopsis*, local responses to virulent and avirulent strains of the bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst*) initiate the SAR response (Cameron *et al.* 1999; Mishina & Zeier 2007). The initial infection in local leaves leads to the second stage of SAR, signal propagation and movement. During this stage, mobile signals generated in the SAR-induced leaf move to distant, uninfected leaves. To understand SAR signal movement, Kiefer & Slusarenko (2003) investigated source–sink relationships (orthostichies) in the *Arabidopsis* rosette in relation to SAR competence. They observed that the movement of SAR signals from induced to distant leaves occurred within and outside the orthostichy of the induced leaf, suggesting that *Arabidopsis* long-distance SAR signals move *via* the phloem and other means, perhaps cell-to-cell (Kiefer & Slusarenko 2003). To date, several proteinaceous and chemical components have been identified as important for the generation and/or movement of long-distance SAR signals in *Arabidopsis* (Champigny & Cameron 2009; Dempsey & Klessig 2012; Fu & Dong 2013; Shah & Zeier 2013). These include lipid transfer proteins (LTPs) (Maldonado *et al.* 2002; Jung *et al.* 2009; Champigny *et al.* 2013), dehydroabietinal (Chaturvedi *et al.* 2012), azelaic acid (Jung *et al.* 2009), a glycerol-3-phosphate derivative (Chanda *et al.* 2011), methyl salicylate (Vlot *et al.* 2008), pipecolic acid (Navarova *et al.* 2013) and a CDR1 (CONSTITUTIVE DISEASE RESISTANCE1)-derived peptide (Xia *et al.* 2004). How these SAR signals are dispersed and perceived within distant leaf cells remains to be determined. However, the defence hormone salicylic acid (SA) and the transcriptional regulator NPR1 (NONEXPRESSOR OF PR GENES1), as well as the NPR3 and NPR4 proteins that regulate NPR1 accumulation, are required for the manifestation of SAR in distant leaves (Vernooij *et al.* 1994; Lawton *et al.* 1995; Cao *et al.* 1997; Fu *et al.* 2012). Recognition of SAR mobile signals leads to changes in gene expression in distant leaves (Gruner *et al.* 2013), and is associated with epigenetic modifications in defence-related genes to establish defence priming (Jaskiewicz *et al.* 2011). In the last stage, SAR is manifested during a secondary infection in the primed distant leaf, such that a fast and effective resistance response is initiated to virulent pathogens.

The LTP DIR1 (DEFECTIVE IN INDUCED RESISTANCE1) has emerged as a key component of the SAR response. The *dir1-1* mutant was identified in a classical genetic screen designed to identify genes essential for SAR (Maldonado *et al.* 2002). Further characterisation of DIR1 expression and localisation indicate that DIR1 is expressed in all living cell types of leaves, including companion cells and sieve elements. DIR1 has been localised to the cell wall as well as intracellularly to the endoplasmic reticulum (ER) (Champigny *et al.* 2011). In addition, transient co-expression studies in *Nicotiana benthamiana*

demonstrated co-localisation of DIR1-RFP (Red Fluorescent Protein) and the plasmodesmata-localised Movement Protein 30 (MP30)-GFP (Green Fluorescent Protein) of Tobacco Mosaic Virus suggesting that DIR1 may also be localised to plasmodesmata (Chanda *et al.* 2011; Yu *et al.* 2013). Expression of DIR1-YFP (Yellow Fluorescent Protein) lacking the wild-type DIR1 N-terminal ER signal sequence ($DIR1^{\Delta 1\text{-}25}$) resulted in the accumulation of DIR1 in the cytosol of *N. benthamiana* in transient *Agrobacterium*-mediated expression experiments, and reduced accumulation in *Arabidopsis* intercellular washing fluids (Champigny *et al.* 2011). Additionally, $DIR1^{\Delta 1\text{-}25}$ rescued the SAR-defect in *dir1-1* suggesting that non-secreted DIR1 can participate in SAR (Champigny *et al.* 2011). We further demonstrated that DIR1-EGFP (Enhanced GFP) moves into phloem sap-enriched petiole exudates collected from local and distant leaves of SAR-induced plants, providing compelling evidence that DIR1 moves to distant tissues during biologically-induced SAR in *Arabidopsis* (Champigny *et al.* 2013). While examining long-distance movement of DIR1 during SAR, we identified a highly similar DIR1 paralogue, DIR1-like. We hypothesise that DIR1-like protein sometimes compensates for the SAR defect in *dir1-1*, as we occasionally observed that *dir1-1* is SAR-competent and our polyclonal DIR1 antibody recognises recombinant DIR1-like protein. Furthermore, transient *Agrobacterium*-mediated expression of DIR1-like rescued the SAR defect in *dir1-1*, suggesting that DIR1-like is capable of participating in SAR (Champigny *et al.* 2013). The importance of DIR1 in SAR is highlighted by the observations that resistance induced by exogenous application of the SAR signals dehydroabietinal, azelaic acid and glycerol-3-phosphate all require functional DIR1 (Chaturvedi *et al.* 2012; Jung *et al.* 2009; Chanda *et al.* 2011). When considered with our observation that DIR1 moves from induced to distant leaves during SAR, this may suggest that DIR1 is a mediator of SAR signal movement. In support of this hypothesis, DIR1 is found to complex with other SAR-related proteins and signals. For example, DIR1 forms DIR1-DIR1 homo-dimers and hetero-dimers with the SAR-related LTP AZI1 (AZELAIC ACID INDUCED1) in co-immunoprecipitation and bimolecular fluorescence complementation experiments performed using agroinfiltration in *N. benthamiana* (Yu *et al.* 2013). DIR1 protein is also present in a high-molecular weight (>100 kDa), dehydroabietinal-containing fraction prepared using petiole exudates collected from SAR-induced leaves (Shah *et al.* 2014). Together, these data suggest that DIR1 is a member of a signal complex that translocates to distant leaves during SAR.

Transient expression experiments in tobacco epidermal cells localised DIR1 to the ER and apoplast (Champigny *et al.* 2011), and also to plasmodesmata (Chanda *et al.* 2011; Yu *et al.* 2013). Plasmodesmata are plant specific cell–cell junctions that connect adjacent cells *via* a cytosolic sleeve and contiguous ER, allowing for the cell-to-cell (symplastic) movement of signals and macromolecules. These channels regulate a number of developmental and stress-related processes in plants, and are major regulators of cell-to-cell movement of macromolecules in various cell types (Burch-Smith *et al.* 2011; Maule *et al.* 2012). In the phloem, companion cells are connected to sieve elements by plasmodesmata that facilitate the loading and unloading of materials into and out of the phloem, indicating that plasmodesmata also play an important role in the long-distance movement of macromolecules (Turgeon & Wolf 2009). While technological limitations and the nanoscale nature of plasmodesmata have limited our knowledge of their composition and regulation, some plasmodesmata-specific proteins have been identified (Maule *et al.* 2012). Among these is the Plasmodesmata-Located Protein (PDLP1-8) family of transmembrane receptor-like proteins. Recent studies on PDLP proteins demonstrated their location in the plasma membrane within plasmodesmatal channels, and characterised their function in regulating cell-to-cell movement of viruses and macromolecules in plants (Thomas *et al.* 2008; Amari *et al.* 2010; Lee *et al.* 2011). Reduction in cell-to-cell movement of fluorescent molecules (0.5–30.0 kDa) and viral movement proteins (>30 kDa) was observed in plant lines that overexpress PDLP1a and PDLP5 (Thomas *et al.* 2008; Lee *et al.* 2011). Lee and co-workers subsequently demonstrated that in response to virulent *P. syringae*, SA-dependent callose deposition at plasmodesmata is mediated by PDLP5 (Wang *et al.* 2013). In addition to their previous work using PDLP5 overexpression and knockout mutants, this suggests that the reduced cell-to-cell movement observed in PDPL5-overexpressing lines is due to increased callose deposition at plasmodesmata (Lee 2014). A number of studies suggest that SAR long-distance signals move *via* the phloem to distant leaves during SAR (reviewed in Champigny & Cameron 2009; Dempsey & Klessig 2012; Shah & Zeier 2013). Movement of SAR signals in the phloem, expression of DIR1 in companion cells and the fact that plant lines expressing non-secreted DIR1 rescue the SAR-defect in *dir1-1* (Champigny *et al.* 2011) support the idea that DIR1 gains access to the phloem *via* companion cell plasmodesmata for movement during SAR. PDLP1a- and PDLP5-overexpressing lines were used to examine the effects of reduced movement through plasmodesmata on the long-distance translocation of DIR1 and the manifestation of SAR.

MATERIAL AND METHODS

Plant and bacterial growth conditions

Seeds were surface sterilised and stratified for 2 days at 4 °C before plating on MS (Musharige and Skoog) media, where they germinated under constant light at 22 °C. Seedlings were transplanted to soil hydrated with 1 $g{\cdot}l^{-1}$ 20-20-20 fertiliser and grown at 22 °C under a short-day photoperiod (9 h light) with a light intensity of 150 $\mu E{\cdot}m^{-2}{\cdot}s^{-1}$ and 60–85% relative humidity. Plants were supplemented with 1 $g{\cdot}l^{-1}$ 20-20-20 fertiliser at 2 weeks post germination.

Avirulent *P. syringae* pv. *tomato* (*Pst-avrRpt2*) and virulent *Pst* (DC3000) were used in this study. Both *P. syringae* strains were cultured by shaking in King's B (KB) media supplemented with 50 $\mu g{\cdot}ml^{-1}$ kanamycin at room temperature. Bacteria were grown overnight until the exponential phase (OD_{600} 0.2–0.6) and were resuspended in 10 mM $MgCl_2$.

Pathogen inoculation assays

The SAR assays were performed as previously described (Champigny *et al.* 2013). SAR was induced by inoculating two lower leaves of 3.5-week-old plants with 10^6 colony forming units (cfu) ml^{-1} of *Pst-avrRpt2*, or 10 mM $MgCl_2$ (mock-treated). Two days later, upper leaves were challenged for SAR by inoculating with 10^5 cfu ml^{-1} virulent *Pst* (DC3000). *In*

planta bacterial density of upper leaves inoculated with *Pst* (DC3000) was performed 3 days post inoculation (dpi) as previously described (Champigny *et al.* 2013). Local disease resistance assays were performed by inoculating leaves of 3.5-week-old plants with 10^6 cfu ml^{-1} of *Pst* (DC3000) or *Pst-avrRpt2*, and quantifying *in planta* bacterial density at 0 and 3 dpi. *In planta* bacterial levels are expressed as cfu per leaf disc. Leaf discs were collected using a No. 2 cork borer (4-mm diameter).

Petiole exudate collection and analyses

Petiole exudates were collected from 3.5-week-old plants that were induced for SAR (*Pst-avrRpt2*) or mock-inoculated with 10 mM $MgCl_2$. At 24 h post inoculation (hpi), inoculated (local) or distant leaves were cut at the base of the petiole and placed in 1 mM EDTA for 1 h to prevent sieve element occlusion. A total of 12 leaves were used per tube of exudate. To prevent EDTA-facilitated cell leakage, leaves were transferred from the EDTA solution to sterile water (Guelette *et al.* 2012) and allowed to exude from 25–48 hpi. Where indicated, total protein levels of petiole exudates were determined by Bradford analysis prior to lyophilisation. Exudates were then lyophilised, resuspended in water, loaded into protein gels, blotted to nitrocellulose and probed with a DIR1-antibody as previously described (Champigny *et al.* 2013). Each lane was loaded with a single exudate.

Reverse transcription polymerase chain reaction (RT-PCR)

Leaf tissue from 3.5-week-old plants was harvested, frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated using the Sigma TRI Reagent (Sigma, St. Louis, MI, USA) according to the manufacturer's instructions. Prior to RNA quantification, residual DNA was degraded using Turbo DNA-free (Life Technologies, Carlsbad, CA, USA). A total of 2 µg RNA was used for cDNA synthesis with M-MLV Reverse Transcriptase (Sigma). PCR primers and conditions used in this study are included in Table S1.

RESULTS AND DISCUSSION

We determined if restricted cell-to-cell movement due to PDLP overexpression impacts the manifestation of SAR in distant leaves. To accomplish this, we compared the SAR phenotypes of two independent PDLP-overexpressing *Arabidopsis* lines, 35S:PDLP1a-HA (hereafter referred to as 35S:PDLP1) and 35S:PDLP5, to wild-type Col-0. If DIR1 and/or other SAR signals travel through plasmodesmata, we predict that 35S:PDLP-associated plasmodesmatal occlusion will impact signal movement, and ultimately, the manifestation of SAR. To assess the SAR response, two lower leaves of 3.5-week-old plants were inoculated with 10 mM $MgCl_2$ (Mock) or 10^6 cfu·ml^{-1} of *Pst-avrRpt2* to induce SAR. Two days post inoculation (dpi), upper/distant leaves were challenged with 10^5 cfu ml^{-1} virulent *Pst* (DC3000), and 3 days later *in planta* bacterial levels were determined. Wild-type Col-0 plants displayed a strong and statistically significant (ANOVA, Tukey HSD, $P < 0.05$) SAR response, as indicated by a 42-fold difference in bacterial levels in mock-inoculated [5.6×10^6 cfu ld (leaf disc)$^{-1}$] *versus* SAR-induced (1.3×10^5 cfu ld^{-1}) Col-0 plants (Fig. 1). In contrast to wild-type Col-0, both 35S:PDLP plant lines were impaired in the manifestation of SAR, as demonstrated by high levels of *Pst* in distant leaves of mock- and SAR-induced 35S:PDLP1/5 plants. The reduced capacity to manifest the SAR response in 35S:PDLP1 and 35S:PDLP5 demonstrates that PDLP overexpression has a negative impact on SAR (Fig. 1).

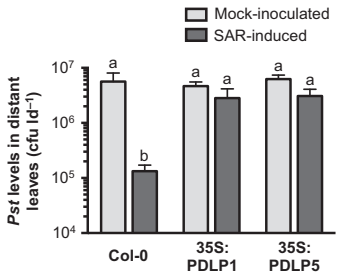

Fig. 1. SAR is negatively impacted in PDLP-overexpressing *Arabidopsis*. SAR assays were performed on 3.5-week-old Col-0, 35S:PDLP1 and 35S:PDLP5. Plants were induced for SAR (*Pst-avrRpt2*) or mock-inoculated with 10 mM $MgCl_2$. Distant leaves were inoculated with virulent *Pst* (DC3000) to determine SAR competence. Bacterial density was quantified in distant leaves 3 dpi. Values represent mean ± SD of three sample replicates. Different letters indicate significant differences (ANOVA, Tukey HSD, $P < 0.05$). This experiment was performed four times with similar results.

Since both PDLP-overexpressing plant lines were defective for the SAR response (Fig. 1), and PDLP overexpression reduces cell-to-cell movement (Thomas *et al.* 2008; Lee *et al.* 2011), we hypothesised that DIR1 movement to distant tissues would be reduced in these lines. We assayed DIR1 movement by protein gel blot analysis of phloem sap-enriched petiole exudates collected from local and distant leaves of Col-0, 35S:PDLP5 and 35S:PDLP1 plants that were mock or SAR induced with *Pst-avRpt2*. In Col-0, DIR1 antibody signals (~7 and ~14 kDa) were detected in exudates collected from inoculated (local) and distant leaves of SAR-induced, but not mock-inoculated, plants (Fig. 2A and B, Figure S1). This is consistent with previous reports of DIR1 movement from local to distant leaves during SAR in *Arabidopsis* (Champigny *et al.* 2013). DIR1 antibody signals were observed in petiole exudates collected from 35S:PDLP1 and 35S:PDLP5 leaves induced for SAR, but not mock-inoculated leaves (Fig. 2A, Figure S1). Weaker DIR1 antibody signals were observed in petiole exudates collected from local 35S:PDLP1/5 leaves induced for SAR as compared to wild-type Col-0 (Fig. 2A). However in a replicate experiment, similar DIR1 antibody signals were observed in 35S:PDLP1/5 and Col-0 (Figure S1). It is difficult to quantify and compare DIR1 antibody signals in petiole exudates due to the variable nature of protein exudation from the phloem of SAR-induced leaves. This is demonstrated in Fig. 2C, in which total protein levels in petiole exudates varied among biological replicates. Therefore we suggest that PDLP overexpression minimally impacts DIR1 movement into the phloem in local leaves induced for SAR. DIR1 antibody signals were not observed in distant leaf petiole exudates of mock-inoculated or SAR-induced 35S:PDLP1 and 35S:PDLP5 plants, while DIR1 antibody signals were observed in distant leaf exudates of SAR-induced Col-0 (Fig. 2B, Figure S1). The DIR1 antibody detects

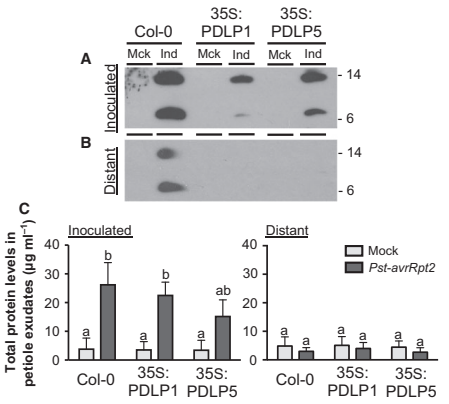

Fig. 2. DIR1 movement is reduced in PDLP-overexpressing *Arabidopsis*. Petiole exudates were collected from 3.5-week-old Col-0, 35S:PDLP1 and 35S:PDLP5 plants induced for SAR with 10^6 cfu ml^{-1} *Pst-avrRpt2* (Ind) or mock-inoculated with 10 mM $MgCl_2$ (Mck). Exudates were collected from (A) inoculated and (B) distant leaves from 25–48 hpi. Exudates were lyophilised and subjected to protein gel blot analyses using a DIR1 antibody. Each lane was loaded with a single phloem exudate. Protein molecular weight markers are indicated (6 and 14 kDa). Similar results were obtained for (A) induced leaf exudates in three independent experiments, and (B) distant leaf exudates in two independent experiments. (C) Average protein levels in petiole exudates collected from inoculated and distant leaves that were mock-inoculated or inoculated with 10^6 cfu ml^{-1} *Pst-avrRpt2* from 25–48 hpi. Values represent mean ± SD of three experimental replicates for inoculated leaf exudates (n = 14), and two experimental replicates for distant leaf exudates (n = 8). Different letters indicate significant differences (ANOVA, Tukey HSD, $P < 0.05$). Statistical analyses for inoculated and distant leaf exudates were performed separately.

both DIR1 and DIR1-like, therefore both may be present in protein blot signals observed using the DIR1 antibody (Champigny *et al.* 2013). These data suggest that while DIR1/DIR1-like can access the phloem in SAR-induced leaves in PDLP-overexpressing plants, movement to or within distant leaves is impeded. We hypothesise that the impaired movement of DIR1 is responsible for the SAR defect in 35S:PDLP1/5 *Arabidopsis*.

Plasmodesmata-located protein overexpression is hypothesised to restrict molecular traffic through plasmodesmata in a non-specific manner, which may have a broad impact on the loading of protein into the phloem. To examine the impact of PDLP overexpression on protein accumulation in the phloem during SAR, we determined total protein levels of petiole exudates collected from inoculated and distant leaves that were mock-inoculated or induced for SAR (*Pst-avrRpt2*) (Fig. 2C). Mock-inoculated local (inoculated) and distant leaf exudates contained similar levels of total protein (~4 µg·ml^{-1}) in Col-0, 35S:PDLP1 and 35S:PDLP5 plants. Petiole exudates collected from SAR-induced Col-0, PDLP1- or PDLP5-overexpressing leaves contained statistically similar protein levels (ANOVA, Tukey HSD, $P < 0.05$). However, protein levels in exudates collected from SAR-induced 35S:PDLP5 leaves were also statistically similar to levels in mock-induced petiole exudates, indicating that phloem protein accumulation may be modestly impacted in this line. The average total protein and variation observed among biological replicates is similar to previous reports (Maldonado *et al.* 2002; Champigny *et al.* 2013). Distant leaf exudates collected from SAR-induced plants contained total protein levels similar to mock-inoculated local and distant leaf exudates regardless of genotype (~3 µg·ml^{-1}). Although total protein in petiole exudates was not significantly affected by PDLP1/5 overexpression, the accumulation of DIR1/DIR1-like in distant leaf exudates was highly reduced, suggesting that the defect in long-distance DIR1/DIR1-like movement in these lines is not associated with a general defect in protein loading into the phloem.

We reason that reduced symplastic movement caused by plasmodesmatal occlusion is responsible for the reduction of DIR1 antibody signals in petiole exudates collected from distant leaves of SAR-induced 35S:PDLP1/5 plants. However, *PDLP1/5* overexpression may indirectly affect *DIR1* and *DIR1-like* expression levels, leading to reduced DIR1/DIR1-like signals in petiole exudates collected from SAR-induced 35S:PDLP1/5 plants. To examine this possibility, *DIR1*, *DIR1-like*, *PDLP1* and *PDLP5* expression was monitored in untreated leaf tissue of 3.5-week-old Col-0, 35S:PDLP1 and 35S:PDLP5 plants (Fig. 3A). As expected, *PDLP1* and *PDLP5* were highly expressed in the respective overexpressing line compared to wild-type plants. The expression of *DIR1* and *DIR1-like* was similar in untreated Col-0, 35S:PDLP1 and 35S:PDLP5 leaves (Fig. 3A, Figure S2), demonstrating that *PDLP1/5* overexpression does not affect *DIR1* or *DIR1-like* expression. In this

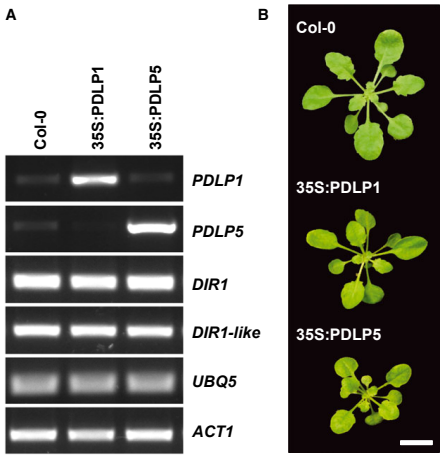

Fig. 3. PDLP overexpression does not affect *DIR1* or *DIR1-like* expression. (A) *PDLP1*, *PDLP5*, *DIR1*, *DIR1-Like* expression monitored in leaf tissue collected from 3.5-week-old Col-0, 35S:PDLP1 and 35S:PDLP5 plants. *ACT1* and *UBQ5* were monitored as internal controls. This experiment was performed three times with similar results. (B) Morphological phenotypes of 3.5-week-old Col-0, 35S:PDLP1 and 35S:PDLP5 plants grown under short-day conditions. Scale bar = 1 cm.

study, 35S:PDLP1 and 35S:PDLP5 plants grown under a short-day photoperiod (9 h light) were smaller than Col-0 (Fig. 3B), but the affect on plant growth was less severe compared to growth in long days (Thomas *et al.* 2008; Lee *et al.* 2011). Taken together, these data suggest that reduced DIR1/DIR1-like accumulation in distant exudates collected from SAR-induced 35S:PDLP1/5 plants is not due to pleiotropic effects of PDLP overexpression on gene expression or plant size.

The overexpression of PDLP5 impacts not only cell-to-cell movement, but also SA-mediated defence signalling, as demonstrated by constitutive *PR1* (*PATHOGENESIS RELATED1*) expression, SA and callose accumulation and enhanced resistance to *P. syringae* pv. *maculicola* (*Psm*) in 35S:PDLP5 compared to wild-type and *pdlp5* mutant plants (Lee *et al.* 2011; Wang *et al.* 2013). In contrast, we observed that 35S:PDLP5 plants do not display enhanced resistance to *Pst* (DC3000) compared to wild-type Col-0 in our SAR assays. Differences in the pathogen and plant growth conditions may contribute to this disparity. In our experiments, plants were grown under a 9-h photoperiod, challenged with 10^5 cfu ml^{-1} *Pst*, and assayed for *in planta* bacterial levels at 3 days post inoculation (dpi). In contrast, a 16-h photoperiod, inoculation with 10^6 cfu ml^{-1} *Psm*, and bacterial quantification at 2 dpi were used by Lee *et al.* (2011). Because PDLP5 is involved in defence, the effect of *PDLP5* overexpression on SAR could be due to altered SA signalling rather than reduced cell-to-cell movement. Therefore we included 35S:PDLP1a-HA, which is similarly impaired in the cell-to-cell movement of fluorescent dyes (Thomas *et al.* 2008), and importantly, is not believed to be involved in SA-mediated defence responses. Moreover, *PDLP1a* is not up-regulated in response to *Psm* or *Pst*, as determined by querying publicly available microarray data (bar.utoronto.ca – data not shown; Winter *et al.* 2007). Nevertheless, the SAR defects observed in 35S:PDLP1 and 35S:PDLP5 plants may be due to PDLP overexpression-associated effects in local leaves induced for SAR with *Pst-avrRpt2*. To examine this possibility, we assessed local responses to *Pst-avrRpt2* in 3.5-week-old PDLP-overexpressing and wild-type plants. Expression of the defence marker genes *PR1* and *PR2* (*PATHOGENESIS RELATED1* and *2*) was monitored in untreated, mock-inoculated and *Pst-avrRpt2*-inoculated (SAR-induced) leaves at 2 dpi using RT-PCR (Fig. 4A). In response to *Pst-avrRpt2*, *PR1* and *PR2* were up-regulated compared to untreated or mock-inoculated leaves in a similar manner in Col-0 and 35S:PDLP1 plants. 35S:PDLP5 plants displayed *PR1* and *PR2* expression in untreated, mock-inoculated and *Pst-avrRpt2*-treated leaves, which is consistent with previous reports (Lee *et al.* 2011; Wang *et al.* 2013). To complement these expression data, we assessed the local resistance phenotypes of 35S:PDLP1 and 35S:PDLP5 to virulent *Pst* (DC3000) and the SAR-inducing avirulent *Pst-avrRpt2* strain. PDLP-overexpressing and wild-type Col-0 plants were inoculated with 10^6 cfu ml^{-1} *Pst-avrRpt2* or *Pst* (DC3000) at 3.5 weeks post germination, and *in planta* bacterial density was measured at 0 and 3 dpi (Fig. 4B). At 0 dpi, all plant lines supported similar levels of virulent and avirulent *Pst*. By 3 dpi, virulent *Pst* (DC3000) density was high in Col-0 (3.3×10^7 cfu ld^{-1}), 35S:PDLP1 (3.5×10^7 cfu ld^{-1}) and 35S:PDLP5 (3.3×10^7 cfu ld^{-1}) plants. In contrast, avirulent *Pst-avrRpt2* levels were ten-fold lower in Col-0 (3.3×10^6 cfu ld^{-1}), 11.8-fold lower in 35S:PDLP1 (2.9×10^6 cfu ld^{-1}) and 11.7-fold lower in 35S:PDLP5 (2.8×10^6 cfu ld^{-1}) plants, indicative of an R gene-mediated resistance response. No statistically significant differences in *Pst* numbers were observed between different plant lines inoculated with the same strain of *Pst* when compared at the same time point (ANOVA, Tukey HSD, $P < 0.05$). In this study, constitutive *PR1/PR2* expression in 35S:PDLP5 was not associated with enhanced pathogen resistance, which is unusual but has been observed in other *Arabidopsis* mutants (Greenberg *et al.* 2000; Lu *et al.* 2009). Based on these studies, we conclude that local leaves of PDLP-overexpressing plants respond to avirulent *Pst-avrRpt2* like wild-type Col-0. This suggests that PDLP overexpression negatively impacts SAR downstream of the initial SAR induction stage in local leaves.

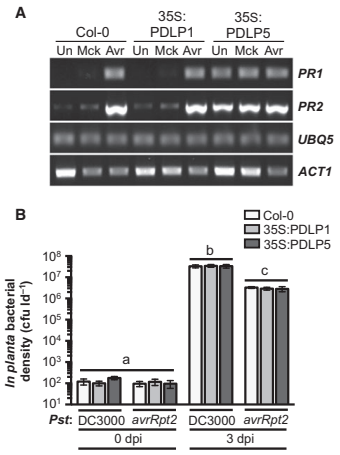

Fig. 4. Local resistance to *Pst-avrRpt2* is not affected by PDLP overexpression. (A) *PR1* and *PR2* expression monitored in leaf tissue collected from 3.5-week-old Col-0, 35S:PDLP1 and 35S:PDLP5 plants that were left untreated (Un), mock-inoculated with 10 mM $MgCl_2$ (Mck) or inoculated with 10^6 cfu ml^{-1} *Pst-avrRpt2* (Avr). Expression was analysed 2 days after inoculation. *UBQ5* and *ACT1* were monitored as internal controls. This experiment was performed twice with similar results. (B) 3.5-week-old Col-0, 35S:PDLP1 and 35S:PDLP5 plants inoculated with 10^6 cfu ml^{-1} *Pst* (DC3000) or *Pst-avrRpt2*. *In planta* bacterial densities were quantified at 0 and 3 dpi. Values represent mean ± SD of three biological replicates. Different letters represent significant differences (ANOVA, Tukey HSD, $P < 0.05$). This experiment was performed twice with similar results for *Pst* (DC3000) and three times with similar results for *Pst-avrRpt2*.

The movement of DIR1/DIR1-like to distant leaf petioles and the manifestation of SAR were negatively impacted in plant lines overexpressing PDLP proteins, suggesting that cell-to-cell movement of DIR1 is important during SAR. Similar total protein and DIR1 antibody signals in petiole exudates collected from SAR-induced Col-0 and 35S:PDLP1/5 plants indicate that reduction in cell-to-cell movement did not prevent DIR1/DIR1-like from accessing the phloem in local leaves. These data lead us to suggest that DIR1/DIR1-like may enter the phloem from the apoplast rather than the symplast, although this is unlikely as loading of proteins into the phloem is thought to occur through companion cell to sieve element

plasmodesmata (Imlau *et al.* 1999; Stadler *et al.* 2005; Turgeon & Wolf 2009). Alternatively, symplastic loading of DIR1/DIR1-like protein into the phloem of SAR-induced leaves may occur, since PDLP overexpression reduces but does not fully prevent cell-cell movement of fluorescent markers like carboxyfluorescein diacetate (CFDA, ~0.5 kDa) and GFP (~27 kDa) (Thomas *et al.* 2008; Lee *et al.* 2011). Moreover, DIR1 protein is expressed in companion cells (Champigny *et al.* 2011), which have numerous plasmodesmata with larger pores in comparison to other cell types (Stadler *et al.* 2005; Turgeon & Wolf 2009). Taken together, these observations support the idea that during SAR induction in the PDLP-overexpressing lines, DIR1, a small protein (~7 kDa) expressed in companion cells, moves through partially occluded plasmodesmata connecting companion cells with sieve elements to access the translocation stream, leading to its detection in petiole exudates collected from SAR-induced local leaves.

Arabidopsis is an apoplastic loader of sugars into the phloem of source leaves, however proteins are thought to access the phloem symplastically using companion cell plasmodesmata. Plant viruses access the phloem symplastically *via* plasmodesmata (reviewed in Hipper *et al.* 2013), and soluble GFP fusions expressed in companion cells access the phloem *via* companion cell–sieve element plasmodesmata (Imlau *et al.* 1999; Stadler *et al.* 2005), providing further support for the symplastic loading of proteins into the phloem. In sink tissue, the unloading of both proteins and sugars is symplastic, and requires open plasmodesmata between sieve elements and companion cells (Turgeon & Wolf 2009). Reports indicate that sink plasmodesmata generally have larger pores than source plasmodesmata, facilitating the symplastic unloading of phloem content into sink tissues (Imlau *et al.* 1999; Baluska *et al.* 2001; Turgeon & Wolf 2009; Burch-Smith *et al.* 2011). PDLP overexpression in *Arabidopsis* restricts the cell-to-cell movement of fluorescent markers such as the 27 kDa GFP protein, and may impact symplastic loading and unloading of DIR1 and/or other SAR signals in companion cells of local and distant leaves. In PDLP-overexpressing plants, DIR1/DIR1-like moved out of induced leaves, while movement to or within distant leaves was severely reduced. Furthermore, it is possible that mesophyll cells are more sensitive to PDLP over-accumulation because these cells are connected to each other by plasmodesmata with smaller pores compared to companion cells and sieve elements (Stadler *et al.* 2005; Turgeon & Wolf 2009). This may explain why DIR1/DIR1-like was not observed in distant leaf exudates. In addition, DIR1 and other SAR signals may participate in long-distance signalling as members of a large signal complex. This idea is supported by detection of protein complexes containing DIR1 (>100 kDa) in high-molecular weight fractions collected from SAR-induced petiole exudates (Shah *et al.* 2014). Taken together, the absence of DIR1 antibody signals in distant leaves of SAR-induced 35S:PDLP1/5 plants leads us to propose that PDLP-associated plasmodesmata occlusion restricts the unloading of SAR signal complexes from the phloem in distant petioles and leaves.

In *Arabidopsis*, the movement of SAR signals is not restricted to the orthostitchy of the SAR-induced leaf, indicating that signals can move between vascular bundles to establish SAR in upper leaves (Kiefer & Slusarenko 2003). How the signal moves between orthostitchies is unknown, although cell-to-cell movement between vascular bundles is plausible and would require plasmodesmata. The defect in SAR manifestation and DIR1/DIR1-like movement in 35S:PDLP1/5 plants may be associated with a defect in signal distribution to leaves outside of the induced leaf orthostitchy. This would prevent DIR1 and other signals from accessing distant leaves outside of the orthostitchy of the induced leaf, causing fewer leaves on the plant to be induced for SAR. The proposed defects in SAR signal movement and orthostitchy distribution suggest that the restriction of cell-to-cell movement caused by PDLP overexpression has a broad impact on SAR, and that the regulation of plasmodesmatal pore size is important for SAR at multiple sites in the plant.

The SAR-defective response observed in both 35S:PDLP5 and 35S:PDLP1 suggests that plasmodesmatal occlusion impairs SAR signal movement. Our results indicate that DIR1/DIR1-like movement to distant leaf petioles is abolished in these lines, and when considered in combination with multiple reports on the requirement of DIR1 for SAR (Maldonado *et al.* 2002; Jung *et al.* 2009; Chanda *et al.* 2011; Chaturvedi *et al.* 2012; Champigny *et al.* 2013), suggests that this defect is responsible for the reduced manifestation of SAR in PDLP-overexpressing *Arabidopsis* lines. Our data provide additional evidence of the importance of DIR1 during SAR in *Arabidopsis* and identifies plasmodesmata as an important avenue for SAR signal movement. We hypothesise that the regulation of plasmodesmatal aperture is important for long-distance signalling during SAR.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. DIR1 is present in inoculated but not distant leaf exudates in SAR-induced PDLP-overexpressing *Arabidopsis*.

Figure S2. *DIR1* and *DIR1-like* expression is not altered by PDLP1 or PDLP5 overexpression.

Table S1. PCR Primers and conditions used in this study.

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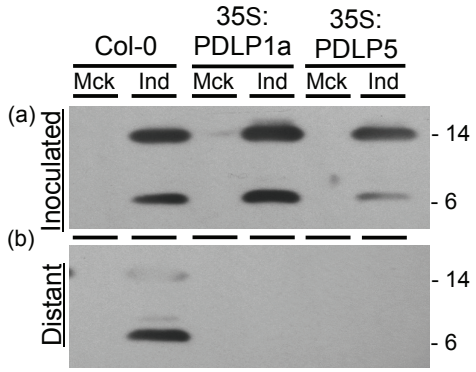
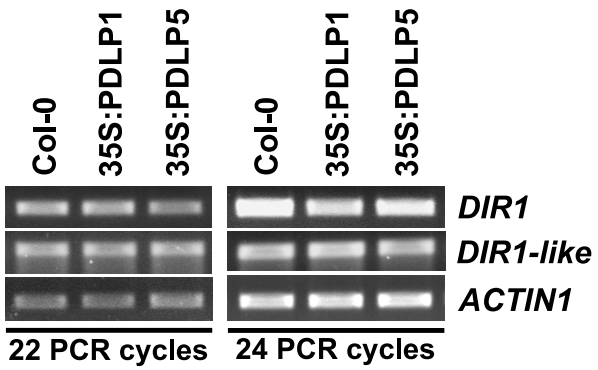
3.8 – Online Supplementary Figures and Tables

Supporting figures are shown in their original, published format.



Supporting Figure S1. DIR1 is present in inoculated but not distant leaf exudates in SAR-induced PDLP-overexpressing Arabidopsis. Petiole exudates were collected from 3.5-week-old Col-0, 35S:PDLP1, and 35S:PDLP5 plants induced for SAR with 10^6 cfu/ml *Pst-avrRpt2* (Ind) or mock-inoculated with 10 mM $MgCl_2$ (Mck). Exudates were collected from (a) inoculated and (b) distant leaves from 24-48 hpi. Exudates were lyophilized and subjected to protein gel blot analyses using a DIR1 antibody. Protein molecular weight markers are indicated (6 and 14 kDa). This is an experimental replicate for Figure 2.



Supporting Figure S2. *DIR1* and *DIR1-like* expression is not altered by PDLP1 or PDLP5 Overexpression. RT-PCR of untreated, 3.5 week-old Col-0, 35S:PDLP1, and 35S:PDLP5 leaf tissue. *DIR1*, *DIR1-like*, and *ACTIN1* were monitored at 22 and 24 PCR cycles to avoid oversaturating PCR products. This experiment was performed 3 times with similar results and is in support of the data presented in Figure 3A.

Target	Primer Sequence (5'-3')	Annealing Temp (° C)	PCR Cycle #	Reference
ACT1	F: GGCGATGAAGCTCAATCCAAACG	61	22/24/26	Champigny *et al.* 2013
	R: GGTCACGACCAGCAAGATCAAGACG			
DIR1	F: GATCGTGATAATGGCTATGTTGGTCGATACATC	61	22/24/26	Champigny *et al.* 2013
	R: GCGTTGGCTAGACCACACTGTTTGGGGAGAGC			
DIR1-like	F: AATGGTGATGGCTAGTTTAGTCGTTGAGAGG	61	22/24/26	Champigny *et al.* 2013
	R: TAAACAAACAAAGGAAAACACCATAATGC			
PDLP1	F: TTCTAACGATCCTGGGTTTGTCG	59	28	This study
	R: TCTCTCCACCTGATAATGGCTCT			
PDLP5	F: TTCCTTCGTCACCTCAACCG	61	26	This study
	R: GATTTGAGCCGTCCGATTGC			
PR1	F: GCAATGGAGTTTGTGGTCAC	61	26	Kim *et al.* 2010
	R: GTTCACATAATTCCCACGAGG			
PR2	F: ATGCTACGGGATGCTAGGCG	61	26	Kim *et al.* 2010
	R: TCTCCGACACCACGATTTCC			
UBQ5	F: AGCTTACAAAATTCCCAAATAGAAATGCAG	61	26	Champigny *et al.* 2013
	R: ACCTACGTTTACCAGAAAGAAGGAGTTGAA			

Supporting Table S1. PCR Primers and conditions used in this study. Primer sequences for *ACTIN1* (AT2G37620), *DIR1* (AT5G48485), *DIR1-like* (AT5G48490), *PDLP1* (AT5G43980), *PDLP5* (AT1G70690), *PR1* (AT2G14610), *PR2* (AT3G57260), and *UBIQUITIN5* (AT3G62250). PCR cycle number and primer annealing temperatures (°C) are indicated.

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ARTICLE ADDENDUM

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Mind the gap: Signal movement through plasmodesmata is critical for the manifestation of SAR

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Systemic acquired resistance (SAR) is a plant defense response in which an initial localized infection affords enhanced pathogen resistance to distant, uninfected leaves. SAR requires efficient long-distance signaling between the infected leaf, where SAR signals are generated, and the distant uninfected leaves that receive them. A growing body of evidence indicates that the lipid transfer protein DIR1 (Defective in Induced Resistance) is an important mediator of long-distance SAR signaling. In a recent publication, we investigated if cell-to-cell movement through plasmodesmata is required for long-distance movement of DIR1 during SAR. We determined that overexpression of Plasmodesmata-Located Proteins (PDLP1 and 5) negatively impacted long-distance DIR1 movement and SAR competence, suggesting that movement through plasmodesmata contributes to long-distance signal movement during SAR.

Keywords: *Arabidopsis*, cell-to-cell movement, DIR1, phloem, plasmodesmata, *Pseudomonas syringae*, systemic acquired resistance

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In Arabidopsis, mobile SAR signals are hypothesized to travel from induced to distant leaves via the phloem, as the establishment of SAR competence in distant leaves is predominately confined to the orthostichy (a collection of vascular bundles connecting vertically aligned leaves) of the induced leaf. However, other routes of transport cannot be ruled out, as upper leaves outside of the induced leaf orthostichy also become SAR competent.[1] This suggests that SAR signals move cell-to-cell to access other orthostichies. While much of the SAR research to date has focused on screening for SAR-defective mutants and identifying mobile SAR signals, little is known about the physiological mechanisms and routes of signal movement. In plants, the cell-to-cell (symplastic) movement of macromolecules occurs via membrane lined cell-to-cell plasmodesmatal junctions, which cytosolically connect neighboring cells.[2] In the phloem, plasmodesmata provide an important conduit for cell-to-cell movement between companion cells and sieve elements, implicating plasmodesmata as important regulators of long-distance movement of macromolecules.

In healthy plants, the lipid transfer protein DIR1 is targeted to the cell wall[3] and plasmodesmata.[4] During the induction phase of SAR, DIR1 is activated to move from induced to distant leaves via the phloem to establish SAR competence.[5] Other putative SAR mobile signals can be detected in phloem sap and some of these signals (azelaic acid,[6] a glycerol-3-phosphate-derived molecule,[4] dehydroabietinal[7]) require functional DIR1 to contribute to SAR. Our knowledge of phloem loading and the detection of SAR mobile signals in the phloem led us to speculate that SAR mobile signals move through plasmodesmata to access the phloem for long-distance movement during SAR. To investigate if movement through plasmodesmata is important for SAR mobile signaling, we examined the impact of restricting plasmodesmatal pore size on long-distance movement of DIR1 during SAR.

We took advantage of transgenic lines that overexpress members of the Plasmodesmata-Located Protein (PDLP) family, which have been shown to accumulate at plasmodesmata and reduce the cell-to-cell movement of fluorescent dyes (35S:PDLP5[8]) and proteins (35S:PDLP1[9]). Given that cell-to-cell movement through plasmodesmata is important for loading

and unloading macromolecules in the phloem, we hypothesized that overexpression of PDLP1 or 5 would negatively impact the long-distance movement of DIR1, leading to a defect in SAR. As predicted, both PDLP-overexpressing lines were defective in the manifestation of SAR.[10] DIR1 antibody signals were observed in phloem exudates collected from local and distant leaves of SAR-induced but not mock-inoculated wild-type plants. In contrast, DIR1 antibody signals were not detected in phloem exudates collected from distant leaves of SAR-induced PDLP-overexpressing lines.[10] Our results suggest that PDLP overexpression suppresses the long-distance movement of DIR1 during SAR.

Our findings suggest that the regulation of plasmodesmatal pore size is important for long-distance SAR signaling. We hypothesize that movement of SAR signals into phloem cells is particularly sensitive to plasmodesmatal occlusion since proteins are thought to move into and out of the phloem symplastically through plasmodesmata.[11-14] In locally infected leaves, *Pseudomonas* bacteria have been observed to form clusters in the apoplast near mesophyll cells,[15] however it is not known if phloem parenchyma or companion cells are similarly infected. It is conceivable that mesophyll cells produce intra- and/or extracellular signals in response to SAR-inducing pathogens that move symplastically (via plasmodesmata) or apoplastically to access the vasculature. Our model in **Figure 1** illustrates symplastic and apoplastic routes of SAR mobile signal movement starting from phloem parenchyma cells. Using a symplastic route, intracellular SAR signals in phloem parenchyma (PP) cells access companion cells (CC) via plasmodesmata, and then move from companion cells to sieve elements (SE) via plasmodesmata for long-distance transport. Alternatively, an apoplastic route could be used to access the phloem, where SAR signals would be loaded into companion cells or sieve elements from the apoplast by membrane-localized transporters. However, the evidence to date indicates that proteins and larger macromolecules such as RNA and viral genome complexes access the phloem symplastically from companion cells.[14,16] Given the number of putative SAR signals,[17] it is possible that some of these signals access the phloem via the symplastic route, while others may use an apoplastic route. Upon entering the phloem, mobile SAR signals move from locally infected to distant leaves. Currently, the mechanism of signal dissemination within distant leaves is unknown. We hypothesize that mobile SAR signals that arrive in distant leaves are symplastically unloaded from sieve elements to companion cells, and then from companion cells to phloem parenchyma, similar to other phloem mobile macromolecules.[11,12] Once inside phloem parenchyma, long-distance SAR signals may move to other leaf cell types to induce SAR. Alternatively, the unloading of SAR signals in distant leaf vasculature may lead to the generation of secondary signals that communicate with leaf mesophyll cells, either through plasmodesmatal cell-to-cell movement or via the apoplast.

In conclusion, our work suggests that the regulation of plasmodesmatal pore size is essential for movement of SAR signals to distant leaves. Whether plasmodesmata are actively dilated to facilitate SAR signal movement is currently unknown and should be addressed in future investigations. Moreover, investigating the relative contributions of apoplastic and symplastic phloem loading will provide deeper insight into the regulation of SAR and long-distance transport processes in general.

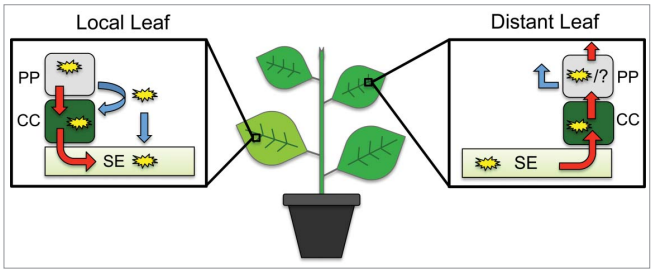

Figure 1. Potential routes of signal movement during SAR. Signals (yellow graphic) generated in the local/SAR-induced leaf are present inside phloem parenchyma (PP) cells and/or the apoplast. The signal(s) may move from PP into companion cells (CC) symplastically through plasmodesmata (red arrows) or directly into CCs or sieve elements (SE) from the apoplast (blue arrows). Once inside CCs, signals move symplastically into SEs for movement to distant leaves. In distant leaves, SAR signals are unloaded from the phloem symplastically through plasmodesmata connecting SEs and CCs, then from CCs to PP cells. In PP cells, mobile SAR signals (yellow graphic) or unknown secondary signals (?) move symplastically (red arrow) or apoplastically (blue arrow) to mesophyll cells (not shown).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Chapter 4

Orthology analysis and in vivo complementation studies to elucidate the role of DIR1 during Systemic Acquired Resistance in Arabidopsis thaliana and Cucumis sativus

PREFACE:

This chapter consists of the published article entitled "***Orthology analysis and in vivo complementation studies to elucidate the role of DIR1 during Systemic Acquired Resistance in Arabidopsis thaliana and Cucumis sativus***" by Isaacs M, Carella P, Faubert J, Rose JKC, and Cameron RK (*Frontiers in Plant Science* 7, 566. 2016, doi: 10.3389/fpls.2016.00566).

This work describes orthology analysis of DIR1 for the identification of conserved protein motifs to better understand the role of DIR1 in SAR. Bioinformatic analysis of several DIR1 orthologs from agriculturally relevant plants such as cucumber (*Cucumis sativus*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), and tomato (*Solanum lycopersicum*) revealed conserved amino acid residues within the central hydrophobic cavity of DIR1. The importance of these motifs was tested by subjecting recombinant DIR1 variant proteins lacking these residues to an *in vitro* lipid-binding assay. This analysis revealed a role for aspartic acid 39 and leucine 43 in maintaining the size of the hydrophobic cavity. Further, the conservation of DIR1 function during SAR in cucumber was determined.

Contributions:

Philip Carella and Marisa Isaacs contributed equally as first authors. Marisa Isaacs performed the orthology analysis and homology modeling depicted in Figures 1, 2, 3A, and 8C. Philip Carella generated and analyzed data for Figures 3B, 4, 5, 6, 7, S1, S2, and S3. Jennifer Faubert & Robin Cameron generated Figure 8AB. Marisa Isaacs and Philip Carella performed the experiments described in Figures 4, 5, 6, 7, and S2 together. The TNS-binding assays (Figures 4, 5, 6, S1, S2) were performed by Philip Carella at the Mac Biophotonics center (McMaster University) or by Philip Carella and Marisa Isaacs at Cornell University (Ithaca, New York, U.S.A) with the assistance of Dr. Jocelyn KC Rose and his lab. Philip Carella and Robin Cameron wrote the manuscript, with significant contributions by Marisa Isaacs and Jocelyn KC Rose.

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Orthology Analysis and *In Vivo* Complementation Studies to Elucidate the Role of DIR1 during Systemic Acquired Resistance in *Arabidopsis thaliana* and *Cucumis sativus*

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AtDIR1 (Defective in Induced Resistance1) is an acidic lipid transfer protein essential for systemic acquired resistance (SAR) in *Arabidopsis thaliana*. Upon SAR induction, DIR1 moves from locally infected to distant uninfected leaves to activate defense priming; however, a molecular function for DIR1 has not been elucidated. Bioinformatic analysis and *in silico* homology modeling identified putative AtDIR1 orthologs in crop species, revealing conserved protein motifs within and outside of DIR1's central hydrophobic cavity. *In vitro* assays to compare the capacity of recombinant AtDIR1 and targeted AtDIR1-variant proteins to bind the lipophilic probe TNS (6,*P*-toluidinylnaphthalene-2-sulfonate) provided evidence that conserved leucine 43 and aspartic acid 39 contribute to the size of the DIR1 hydrophobic cavity and possibly hydrophobic ligand binding. An *Arabidopsis*–cucumber SAR model was developed to investigate the conservation of DIR1 function in cucumber (*Cucumis sativus*), and we demonstrated that phloem exudates from SAR-induced cucumber rescued the SAR defect in the *Arabidopsis dir1-1* mutant. Additionally, an AtDIR1 antibody detected a protein of the same size as AtDIR1 in SAR-induced cucumber phloem exudates, providing evidence that DIR1 function during SAR is conserved in *Arabidopsis* and cucumber. *In vitro* TNS displacement assays demonstrated that recombinant AtDIR1 did not bind the SAR signals azelaic acid (AzA), glycerol-3-phosphate or pipecolic acid. However, recombinant CsDIR1 and CsDIR2 interacted weakly with AzA and pipecolic acid. Bioinformatic and functional analyses using the *Arabidopsis*–cucumber SAR model provide evidence that DIR1 orthologs exist in tobacco, tomato, cucumber, and soybean, and that DIR1-mediated SAR signaling is conserved in *Arabidopsis* and cucumber.

Keywords: cucumber, DIR1, hydrophobic cavity, lipid transfer protein, long-distance signaling, systemic acquired resistance

INTRODUCTION

Systemic acquired resistance (SAR) is a plant defense response during which an initial infection leads to resistance to a broad spectrum of normally virulent pathogens in distant naive tissues. Pathogen-induced mobile SAR signals produced in locally infected leaves travel to distant leaves, resulting in signal perception and the manifestation of SAR. A number of physiological experiments demonstrated that mobile SAR signals travel from induced to distant tissues, predominantly via the phloem (reviewed in Guedes et al., 1980; Tuzun and Kuc, 1985; Champigny and Cameron, 2009). More recently this was also shown in *Arabidopsis* (Kiefer and Slusarenko, 2003). To date, a number of potential SAR mobile signals have been identified (reviewed in Dempsey and Klessig, 2012; Shah and Zeier, 2013; Shah et al., 2014), including lipid transfer proteins (LTPs; Maldonado et al., 2002; Jung et al., 2009; Xia et al., 2012; Champigny et al., 2013; Li et al., 2014; Cecchini et al., 2015), methyl salicylate (MeSA; Park et al., 2007; Vlot et al., 2008), azelaic acid (AzA; Jung et al., 2009; Wittek et al., 2014; Cecchini et al., 2015), a glycerol-3-phosphate (G3P)-derived molecule (Chanda et al., 2011), pipecolic acid (Pip; Navarova et al., 2012; Vogel-Adghough et al., 2013), and the abietane diterpenoid dehydroabietinal (DA; Chaturvedi et al., 2012). The existence of numerous putative SAR signals illustrates the complexity of the SAR signaling pathway and highlights the need to better understand the roles of these signals during SAR.

Since plants cannot predict which leaf will become infected, each leaf must have the capacity to produce SAR long-distance signals. Additionally, long-distance SAR signals must move from SAR-induced to distant leaves to establish SAR. The LTP DIR1 (Defective in Induced Resistance 1) possesses these characteristics as it is expressed in all living cells of leaves (Champigny et al., 2011) and experiments using an estrogen-inducible DIR1–GFP line provide compelling evidence that DIR1 is a mobile signal or chaperone that becomes activated in locally infected leaves to access the phloem and move to establish SAR in distant leaves (Champigny et al., 2013). Moreover, the resistance-promoting activity of G3P, AzA, and DA all require functional DIR1 (Jung et al., 2009; Chanda et al., 2011; Chaturvedi et al., 2012) and the SAR-related LTPs AzA Induced 1 (AZI1) and Early Arabidopsis Aluminum Induced 1 (EARLI1) have been shown to interact with DIR1 in transient expression experiments in *Nicotiana benthamiana* (Yu et al., 2013; Cecchini et al., 2015). These findings suggest that DIR1 participates as a member of a SAR signal complex. In support of this idea, a high molecular weight protein complex was identified in petiole exudates collected from SAR-induced leaves (Chaturvedi et al., 2012) and immunoblot analysis provided evidence that DIR1 is present in this complex (Shah et al., 2014). Taken together, these studies support the idea that DIR1 is an integral component of long-distance signaling during SAR.

Analysis of the DIR1 crystal structure revealed that DIR1 is a unique non-specific (ns)-LTP, most similar to members of the LTP2 family (Lascombe et al., 2008). Like other nsLTPs, DIR1 has eight cysteine residues that participate in four disulfide bonds to form a central hydrophobic cavity or pocket. Unlike other LTP2 proteins, DIR1 has an acidic isoelectric point (pI), it binds two monoacylated lipids within its hydrophobic pocket *in vitro* and it possesses a putative protein interaction PxxP motif (where P is proline and x is any amino acid; Lascombe et al., 2008). Given the characteristics of DIR1, it is possible that it interacts with lipids or other hydrophobic molecules, acting as a chaperone and/or as part of a larger protein complex that translocates from induced to distant tissues during SAR.

The importance of DIR1 in the SAR response is further supported by studies of DIR1 orthologs in other plant species. A putative DIR1 ortholog was identified in tomato and immunoblot analysis confirmed its presence in petiole exudates collected from healthy tomato plants (Mitton et al., 2009); however, its role during SAR was not investigated. Transgenic *Arabidopsis* plants expressing two putative DIR1 orthologs from *Nicotiana tabacum* rescued the SAR defect in the *Arabidopsis dir1-1* mutant and RNAi-mediated knockdown of these orthologs in *N. tabacum* impaired SAR (Liu et al., 2011). These studies suggest that DIR1 and DIR1-mediated SAR are conserved in other plants. Additionally, a DIR1-like protein with high sequence similarity to DIR1 (88% of the mature protein at the amino acid level) is present in *Arabidopsis*. Phylogenetic analysis, and the fact that *DIR1* and *DIR1-like* are adjacent to one another on chromosome 5, suggests they arose from a duplication event (Champigny et al., 2013). *DIR1* and *DIR1-like* are similarly expressed in naïve and pathogen-treated plants, and transiently expressed DIR1-like complements the *dir1-1* SAR defect (Champigny et al., 2013). Moreover, the *dir1-1* mutant occasionally displays a partially SAR-competent phenotype, suggesting that in some circumstances DIR1-like acts redundantly to DIR1 (Champigny et al., 2013).

To further understand the role of DIR1 during SAR, we used bioinformatic analyses and *in silico* homology modeling to identify and characterize orthologous DIR1 proteins from a number of agriculturally relevant plants. Conserved motifs in areas important for LTP structure were identified. Mutations were introduced into these motifs and their effect on the formation of the DIR1 hydrophobic cavity was examined. Further, we combined the cucumber and *Arabidopsis* SAR model systems to provide evidence that cucumber DIR1 orthologs are functionally equivalent to AtDIR1.

MATERIALS AND METHODS

Plant Growth Conditions

Wild-type *Arabidopsis thaliana* Wassilewskija (Ws-2), *dir1-1,* and *npr1-2* seeds were surface sterilized and stratified at 4°C for 2 days in the dark. Sterilized seeds were plated on Murashige and Skoog (MS) plates and germinated under continuous light for 5–7 days. Seedlings were transplanted to soil hydrated with 1 g L^{-1} 20–20–20 fertilizer. *Arabidopsis* plants were grown in short day photoperiod conditions (9 h light; 150 μE m^{-2} s^{-1}) in 65–85% relative humidity at 22°C. Cucumber Wisconsin S.M.R 58 146B seeds (Stokes Seeds LTD., St. Catharines, ON, Canada) were

sown directly onto soil hydrated with 1 g L^{-1} 20–20–20 fertilizer and grown in 65–85% relative humidity at 22°C in a long day photoperiod (16 h light: 150 μE m^{-2} s^{-1}).

Pathogen Culture and Inoculation

Systemic acquired resistance experiments with *Arabidopsis* employed virulent *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (pVSP1) and avirulent *Pst* (DC3000 containing pVSP1 + avrRpt2) described in Whalen et al. (1991). SAR experiments with cucumber employed *P. syringae* pv. *syringae* D20 (Rasmussen et al., 1991). *Pseudomonas* strains were cultured overnight with shaking at room temperature in sterile King's B (KB) medium (King et al., 1954). *Pst* cultures were supplemented with 100 μg ml^{-1} rifampicin and 50 μg ml^{-1} kanamycin. SAR assays in *Arabidopsis* were performed as described in Champigny et al. (2013). In cucumber, SAR was induced in 3–4 week-old plants by resuspending *Pss* D20 in 10 mM $MgCl_2$ and infiltrating leaves with 10^8 colony forming units (cfu) ml^{-1}. *In planta* bacterial levels were quantified by dilution plating as described by Cameron et al. (1999).

Cucumber Petiole Exudate Collection

Cucumber exudates were collected according to Rasmussen et al. (1991) by cutting the petiole on an angle 3–5 cm below the leaf blade with a razor blade. Exudate droplets (30–40 μl) were collected from the petiole cut ends using capillary pipettes and immediately added to 300 μl of cucumber exudate buffer (0.05 M Tris-HCl, pH 7.5 with 0.1% β-mercaptoethanol). Cucumber exudates contained between 5 and 15 μg μl^{-1} total protein (Biorad Protein Assay Kit). Samples were used immediately in cucumber–*Arabidopsis* SAR-rescue experiments or frozen at −20°C for later concentration by lyophilization and protein gel blot analysis.

Agro-SAR and Petiole Exudate-Swapping SAR Assays

Agro-SAR assays were performed as described in Champigny et al. (2013) using *Agrobacterium tumefaciens* GV3101 (pMP90) expressing EYFP, DIR1-YFP, CsDIR1, or CsDIR2. The EYFP and DIR1-YFP constructs are described in Champigny et al. (2013). Cucumber DIR1 orthologs were cloned into pMDC32 (Curtis and Grossniklaus, 2003) to create the 35S:CsDIR1 and 35S:CsDIR2 constructs. Constructs were verified by sequencing. Cloning primers are described in Supplementary Table S1.

Cucumber petiole exudates were collected from leaves that were mock-inoculated or induced for SAR with 10^7 cfu ml^{-1} *Pseudomonas syringae* pv *syringae* D20 at 8 and 22 h post inoculation (hpi). Cucumber exudates were filter sterilized (0.45 μm, EMD Millipore) and samples were stored at −20°C for future lyophilization and protein gel blot analysis or used immediately in the cucumber–*Arabidopsis* SAR assay. Cucumber exudates from mock-inoculated and SAR-induced leaves were diluted 10-fold in sterile distilled water then pressure-infiltrated into two lower *Arabidopsis* leaves using a needleless syringe. Two days later, distant upper leaves were challenge-inoculated with virulent *Pst* at 10^5 cfu ml^{-1}, followed by *in planta Pst* quantitation 3 days post-inoculation (dpi).

Phylogenetic and Bioinformatic Analyses

A rooted phylogenetic Maximum Likelihood tree was created for AtDIR1 (AT5G48485), Brassicaceae ortholog family members and crop plant DIR1 orthologs. The phylogeny used protein sequences lacking the divergent ER signal sequence. Signal P 4.0 was used to determine the location of the signal sequence cleavage site (Perterson et al., 2011). The sequences were aligned in MEGA 5 using Muscle (Tamura et al., 2011). The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter (Kimura, 1980) model with discrete Gamma distribution using MEGA 5 (Tamura et al., 2011). A total of 10,000 bootstrap replicates were conducted and percent bootstrap values were placed on the branches (Felsenstein, 1985). Branches were drawn to scale, measured in number of substitutions per site and were labeled by species name followed by The Arabidopsis Information Resource (TAIR[1]) gene number or Phytozome 8.0 accession (Goodstein et al., 2012). For the Brassicaceae plus crop plant DIR1 ortholog phylogeny, branches with <50% bootstrap values were collapsed using Archaeopteryx software (Han and Zmasek, 2009). The phylogenetic tree was viewed in FigTree v1.4 (Drummond et al., 2012).

The coding sequences and amino acid sequences of AtDIR1 (AT5G48485) and AtDIR1-like (AT5G48490), AtLTP2.12 (AT5G38170), were retrieved from TAIR. Tobacco DIR1 ortholog sequences were retrieved from the National Center for Biotechnology Information (NCBI[2]) website. Cucumber, tomato, and soybean DIR1 ortholog sequences were retrieved using Phytozome[3]. Sequences were compared using the EMBOSS Needleman–Wunsch pairwise alignment algorithm[4]. Signal peptides were deduced using the SignalP 3.0 prediction server[5] (Perterson et al., 2011). SWISS-MODEL homology models of AtDIR1-like, CsDIR1 and CsDIR2 were produced using the AtDIR1-phospholipid crystal structure (Lascombe et al., 2008) as a template (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006; Kiefer et al., 2009). The Swiss-pdf viewer 4.0.1 and ICM browser were used to compare the AtDIR1 structure and the AtDIR1-like, CsDIR1, and CsDIR2 protein models[6] (Guex and Peitsch, 1997). A Sequence Logo plot was created by submitting the Muscle aligned mature DIR1 ortholog protein sequences in FASTA format to the Web logo program[7].

DIR1 Variant Cloning and Recombinant Protein Purification

DIR1 variants were synthesized by BioBasic Inc (Markham, ON, Canada). Full sequence information for each variant can

[1]http://www.arabidopsis.org
[2]http://www.ncbi.nlm.nih.gov/
[3]http://phytozome.net
[4]http://www.ebi.ac.uk/emboss/align
[5]http://www.cbs.dtu.dk/services/SignalP
[6]http://www.expasy.org/spdbv/
[7]http://weblogo.berkeley.edu

be found in Supplementary Document 1. Variants lacking the N-terminal ER signal sequence were subcloned into the pET29b expression vector (Novagen) using the primers in Supplementary Table S1 and were verified by sequencing. The constructs were transformed into competent Rosetta Gami *E. coli* cells (Novagen). For protein expression, 250 and 500 ml Rosetta Gami *E. coli* cultures were grown overnight in liquid LB (Luria–Bertani) at 30°C with shaking at 200 rpm. At an OD_{600} of 0.6, 100 ml of each culture was poured into a new flask and 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, BioShop) was added to induce protein expression. Cultures were shaken for another 4 h at 30°C. Cells were harvested by centrifugation in two sterile 50 ml tubes at 4000 × *g* for 20 min at 4°C. One milliliter of cells was also collected for crude protein extraction. The pellets were dried and kept −80°C prior to either a crude total protein extraction or S-Tag thrombin protein purification (Novagen).

For crude extraction of total protein, pellets from 1 mL of IPTG-induced *E. coli* cells were resuspended in 100 μl of lysis buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM KH_2PO_4, Phosphate Buffered Saline pH 7.3, 1 mM phenylmethylsulfonyl fluoride, 0.1% TritonX-100). This mixture was sonicated three times for 10 s each (60% amplitude, cooling on ice in between. After lysis the cells were centrifuged for 5 min at 20,000 × *g* (4°C) to separate soluble and insoluble fractions.

An S-Tag thrombin purification kit (Novagen) was used to purify the recombinant Rosetta Gami expressed proteins. Proteins were purified according to manufacturer's instructions (Novagen) and quantified (Biorad Protein Assay Kit) using bovine serum albumin (BSA) as a standard. Samples were stored at −80°C until further use.

Immunoblot Analysis

Protein samples (crude protein extract, purified protein or petiole exudates concentrated by lyophilization) were mixed with 5x SDS loading buffer (350 mM Tris-HCl pH 6.8, 30% glycerol, 10% SDS, 0.01% bromophenol blue and 200 mM dithiothreitol) followed by boiling for 5 min. Samples were loaded onto 4–12% NuPAGE Bis-Tris polyacrylamide gels (Life Science Technologies) and subjected to electrophoresis in denaturing MES running buffer (9.7 g L^{-1} MES, 6.0 g L^{-1} Tris, 1 g L^{-1} SDS, 0.37 g L^{-1} EDTA). Proteins were transferred to nitrocellulose membranes in Towbin transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol, pH 8.3). Membranes were probed with anti-DIR1 antisera (Maldonado et al., 2002) at a 1:20,000 dilution, or anti-6-His (Covance) at a 1:3,000 dilution in 5% non-fat milk in 1X Tris Buffered Saline with Tween 20 (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Tween 20). Antibody binding was detected with a goat anti-rabbit (AtDIR1-antibody) or goat anti-mouse (His-antibody, Sigma–Aldrich) horseradish peroxidase conjugate and WestFemto reagents (Pierce) as described by the manufacturer.

TNS Binding Assay and TNS Displacement Experiments

Proteins were engineered to resemble mature protein lacking the ER signal sequence and were expressed in Rosetta Gami *E. coli*. For TNS binding assays, increasing concentrations (0–30 μM) of 6,*P*-toluidinylnaphthalene-2-sulfonate (TNS, Sigma–Aldrich) were added to 1 μg of each Rosetta Gami *E. coli* (Novagen) purified protein in measurement buffer (0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.175 M mannitol, 5 mM MES, pH 7) and TNS concentrations were increased in 5 μM increments from a 0.3 mM TNS stock (dissolved in DMSO). As a control, proteins were denatured by boiling in a solution of 6 M urea for 10 min and analyzed. Samples were loaded into microflor two black bottom 96 well microtiter plates (Thermo Fisher) and analyzed using the Gen5 Synergy 4 plate reader (BioTek Instruments). Experiments using the $DIR1^{\Delta Cys}$ variant were performed using an Infinite M1000 (TECAN) plate reader. TNS-binding curves displayed similar trends irrespective of the instrument used. Samples were excited at 320 nm and emission was collected at 437 nm. The change in fluorescence (*x* μM TNS – 0 μM TNS) was calculated for three technical replicates.

6,*P*-Toluidinylnaphthalene-2-sulfonate displacement/ligand binding assays were performed using the same plates and instrument. Fluorescence was first measured when TNS (5 μm) and putative ligands (16 μm AzA, pipecolic acid, or G3P; Sigma–Aldrich) were co-incubated for 3 min in a 96 well plate, then a second reading was recorded after purified proteins were added. Plates were loaded with 1 μg of protein in measurement buffer (0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.175 M mannitol, 5 mM MES, pH 7) and 5 μM TNS. AzA was prepared in 5 mM MES (pH 5.6), while all other putative ligands were prepared in water. Fluorescence was measured at an excitation wavelength of 320 nm and emission was collected at 437 nm in technical triplicate. Ligand binding was represented by the percent of TNS fluorescence that was quenched by the addition of the purified proteins.

RNA Isolation and Reverse Transcription (RT)-PCR

Total RNA was isolated from frozen leaf tissue using the Sigma TRI-Reagent as previously described (Carella et al., 2015). Primers for RT-PCR analysis can be found in Supplementary Table S1. Twenty-eight PCR cycles were used.

RESULTS

Identification of Putative DIR1 Orthologs

To gain insight into the phylogenetic relationships among DIR1 orthologs, we identified and examined putative DIR1 sequences in model and crop plants. Putative DIR1 orthologs from members of the Brassica family (*Arabidopsis thaliana*, *Arabidopsis lyrata*, *Brassica rapa*, *Eutrema salsugineum*), *Nicotiana tabacum* (tobacco), *Solanum lycopersicum* (tomato), *Cucumis sativus* (cucumber), and *Glycine max* (soybean) were identified as having >51% amino acid sequence similarity with mature AtDIR1 (**Table 1**). The evolutionary relationships between AtDIR1 and putative orthologs were examined by constructing a phylogenetic tree (**Figure 1**). We selected the *Arabidopsis*

TABLE 1 | Amino acid sequence similarity and identity of putative DIR1 orthologs.

Organism	Protein	Amino Acid Sequence	
		Similarity[a]	Identity[a]
Arabidopsis thaliana	AtDIR1-like	88%	80%
	AtLTP2.12[b]	38%	27%
Arabidopsis lyrata	AlDIR1	96%	93%
	AlDIR1-like	87%	80%
Brassica rapa	BrDIR1	83%	72%
Eutrema salsugineum	EsDIR1	86%	77%
Nicotiana tabacum	NtDIR1	62%	52%
	NtDIR2	57%	44%
	NtDIR3	67%	51%
Solanum lycopersicum	SlDIR1	63%	50%
	SlDIR2	63%	47%
	SlDIR3	58%	46%
Cucumis sativus	CsDIR1	61%	48%
	CsDIR2	65%	47%
Glycine max	GmDIR1	59%	47%
	GmDIR2	57%	46%
	GmDIR3	61%	49%

[a]*Mature protein lacking signal peptide.* [b]*Outgroup for phylogenetic analysis.*

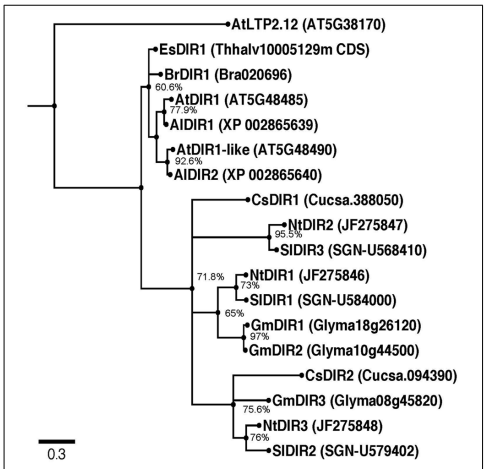


FIGURE 1 | Rooted Phylogenetic Maximum Likelihood tree of putative DIR1 orthologs. DNA sequences lacking the divergent ER signal sequence were aligned using MUSCLE. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model with discrete Gamma distribution using MEGA 5. Ten thousand bootstrap replicates were conducted and percent bootstrap values were placed on corresponding branches. Nodes with bootstrap values below 50% were collapsed using Archaeopteryx software. Phylogeny was viewed in FigTree v1.4. Branches were drawn to scale, measured in number of substitutions per site and branches were labeled by species name followed by TAIR gene number or Phytozome 8.0 accession. Al: *Arabidopsis lyrata*, At: *Arabidopsis thaliana*, Br: *Brassica rapa*, Cs: *Cucumis sativus*, Es: *Eutrema salsugineum*, Gm: *Glycine max*, Nt: *Nicotiana tabacum*, Sl: *Solanum lycopersicum*.

ortholog of wheat LTP2, AtLTP2.12 (At5g38170; Edstam et al., 2011), as an outgroup because it has only 38% amino acid sequence similarity to AtDIR1. As with the *DIR1 Brassica* phylogeny described previously (Champigny et al., 2013), this expanded phylogeny (**Figure 1**) predicted that AtDIR1 and AtDIR1-like were the result of a tandem duplication event in an ancestor of *A. lyrata* and *A. thaliana*. Independent DIR1 duplications in tomato, cucumber, tobacco, and soybean are also predicted. A lineage-specific duplication event was predicted in soybean, resulting in *GmDIR1* and *GmDIR2*, while duplication events in cucumber, tomato, and tobacco occurred in a common ancestor. Moreover, the *Arabidopsis DIR1/DIR1-like* and cucumber *DIR1/DIR2* duplications occurred independently. This DIR1 phylogeny provided evidence that DIR1 is conserved in agriculturally relevant plant species. Additionally, the identification of several DIR1 orthologs facilitates the discovery of conserved motifs and residues that can be used to learn more about DIR1 structure and function.

Homology Modeling Supports the Existence of DIR1 Orthologs in *Cucumis sativus*

The cucumber SAR model system was used in seminal SAR studies to investigate the nature of long-distance signaling (Guedes et al., 1980; Rasmussen et al., 1991; Smith-Becker et al., 1998), largely due to the ability to directly collect phloem sap from cut cucumber petioles. Therefore, we chose to investigate DIR1 orthologs in this well-developed SAR model system. Homology modeling was used to compare the protein structure of AtDIR1 and the putative cucumber DIR1 orthologs CsDIR1 and CsDIR2. Using the AtDIR1-phospholipid crystal structure (Lascombe et al., 2006, 2008) as a template, a homology model of each cucumber ortholog was generated using the SWISS-MODEL server (**Figure 2**) (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006; Kiefer et al., 2009). AtDIR1, CsDIR1, and CsDIR2 all share a similar hydrophobic cavity into which hydrophobic phospholipid tails may extend (**Figure 2A**). The AtDIR1 PxxPxxP motif is a proposed protein–protein interaction site (Lascombe et al., 2008). CsDIR1 (PxPxxxPP) and CsDIR2 (PPxPxPP) contain a proline-rich region, rather than the canonical PxxPxxP motif (**Figure 2B**). Another region considered to be important for *in vitro* phospholipid docking is the entrance of the hydrophobic cavity, which contains hydrophilic residues. Lascombe et al. (2008) postulated that these charged residues interact with the hydrophilic region of putative lipid head groups, while the hydrophobic acyl tails are bound within the hydrophobic cavity. AtDIR1 possesses three hydrophilic residues within 5 Å of the phospholipid head groups (GLN9, ASN13, LYS16), while CsDIR1 and CsDIR2 both possess two residues (TYR7, ARG10 and GLU8, THR12, respectively; **Figure 2C**). Homology models of the putative

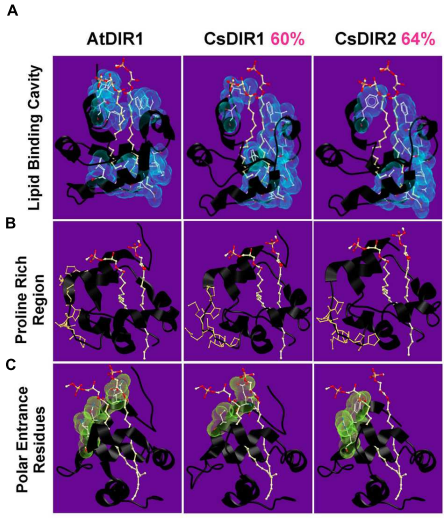


FIGURE 2 | Homology models of CsDIR1 and CsDIR2. Proteins were modeled with SWISS-MODEL 4.0.1 server using AtDIR1-phospholipid crystal structure as a template and modeled with SWISS-MODEL 4.0.1 server and viewed using Molsoft ICM browser. Percent sequence similarity compared to AtDIR1 is identified in pink. **(A–C)** phospholipids in orange (phosphate), red (oxygen), and white (carbon). **(A)** The hydrophobic residues (blue) that are within 5 Å of the phospholipids highlight the inner hydrophobic cavity. **(B)** The proline residues of the proline rich regions are highlighted in orange. **(C)** The polar residues at the cavity entrance are highlighted in green.

DIR1 orthologs from tobacco, tomato, and soybean were generated and all were structurally similar to AtDIR1 (data not shown). Homology modeling of AtDIR1 and the putative DIR1 orthologs identified common structural motifs, providing additional support for the importance of these motifs for DIR1 function. Moreover, the high degree of structural similarity between cucumber, tobacco, and soybean DIR1 proteins with AtDIR1 validates our bioinformatics analyses and provides support for the list of putative DIR1 orthologs (**Table 1**; **Figure 1**).

Sequence Alignment of DIR1 Orthologs Identifies Conserved Motifs

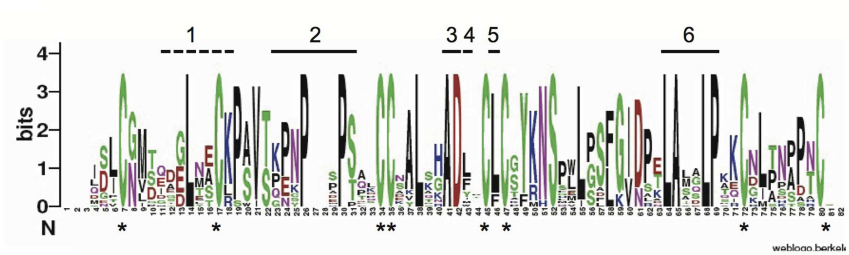
Given that the DIR1 phylogeny presented in **Figure 1** supports the existence of DIR1 orthologs, we used these DIR1 sequences to identify additional conserved protein motifs that may be important for DIR1 function during SAR. To identify conserved protein motifs a Sequence Logo was created using the online Web Logo algorithm (Schneider and Stephens, 1990; Crooks et al., 2004). This provides a visual output of multiple sequence alignments, where residues are stacked on top of one another and their frequency determines the height of the residue letter, allowing identification of conserved regions. Alignment of AtDIR1 and predicted DIR1 orthologs (**Table 1**) was performed using MEGA and submitted to the Sequence Logo online application. The Sequence Logo output (**Figure 3A**) predicted several conserved regions (**Figure 3B**), including the eight-cysteine motif that forms the four intramolecular disulfide bonds essential for LTP protein structure. The proline rich PxxP-like regions, which represent possible sites of protein–protein interaction, were common to all DIR1 proteins. Moreover, two previously unidentified motifs, AD and LAxxLP, were identified. Both motifs are located at the bottom of the hydrophobic cavity. In the AD motif, alanine faces inward toward the hydrophobic cavity and aspartic acid reaches outward toward the solution, while the LAxxLP motif is exposed to the solution. The polar tyrosine residue inside the hydrophobic cavity of AtDIR1-like (Y40 of mature polypeptide) was only present in AtDIR1-like and its corresponding putative ortholog in *A. lyrata*, while the predicted DIR1 orthologs had various non-polar residues at this location.

Modification of AtDIR1 to Investigate Conserved Motif Function

The importance of the motifs identified in the Sequence Logo was investigated by creating several DIR1 variants with amino acid substitutions in these regions (**Figure 3B**). The PxxPxxP motif in AtDIR1 was selected for modification because of its high conservation among putative DIR1 orthologs and because it could be a site of protein–protein interactions (Lascombe et al., 2008). Multiple PxxP motifs are thought to strengthen such interactions (Williamson, 1994), therefore each proline residue was changed to an alanine (PxxPxxP to AxxAxxA). Although not identified in the Sequence Logo alignment, the polar amino acids at the DIR1 hydrophobic cavity entrance (Gln9, Asn13, Lys16) may be essential for stabilizing ligand binding (Lascombe et al., 2008). These residues were investigated using a variant in which the polar head group residues are changed to alanine to create the "No Polar Head group" (NPH) DIR1 variant (Gln9Ala, Asn13Ala, Lys16Ala).

The hydrophobic pocket of DIR1 was investigated using a DIR1 variant in which non-polar phenylalanine 40 is modified to tyrosine to resemble AtDIR1-like, which has a polar tyrosine at this position. This difference is hypothesized to affect the ability of AtDIR1-like to participate in SAR by disrupting the integrity of the pocket and/or reducing ligand interactions (Champigny et al., 2013). This idea is supported by the Sequence Logo alignment in which only AtDIR1-like and AlDIR1-like possessed a polar residue at this location. Leucine 43 lies between two cysteine residues in the hydrophobic cavity of many nsLTP2 proteins and has been shown to be important for protein folding, therefore a DIR1 variant with Cys-Asp43-Cys (L43D) was created (Samuel et al., 2002; Cheng et al., 2008). As an additional control, a DIR1 variant that lacks all 8 of the conserved cysteine residues required for disulfide bond formation was created (DIR1$^{\Delta Cys}$, eight alanine residues substituted for eight cysteine residues) to generate an unfolded protein lacking the hydrophobic pocket entirely.

A



B

	Motif	Variant Name	Function/Description
*	8 cysteine motif	DIR1$^{\Delta Cys}$	Essential to generate central hydrophobic cavity
1	Polar Head Groups	No Polar Head Groups (NPH)	Polar head groups at cavity entrance hypothesized to stabilize ligand interaction
2	PxxP	AxxAxxA	Proline-rich region, hypothesized involvement in protein-protein interactions
3	AD	D39Q	Unknown function
4	F40	F40Y	Hypothesized function in ligand stabilization.
5	CLC	L43D	Important for proper cysteine-pairing during hydrophobic cavity formation
6	LAxxLP	-	Unknown function

FIGURE 3 | Identification of conserved amino acid residues and motifs among DIR1 orthologs. (A) Sequence Logo plot of Muscle aligned mature DIR1 orthologs protein sequences. Residues of the orthologs are stacked on top of one another and their frequency determines the height of the residue letter in the plot. Bits is a measure of the information content at that position in the sequence. Conserved cysteine residues are indicated by an asterisk (*) that appears under the *x*-axis. Other conserved areas are highlighted with numbers above the corresponding letter. **(B)** Summary of identified motifs. Motif name, DIR1 variant protein generated, and a short description are included.

In Vitro TNS Binding Assays to Compare Hydrophobic Cavities of Recombinant DIR1 and DIR1 Variant Proteins

Recombinant protein of AtDIR1, AtDIR1-like, AtLTP2.12, CsDIR1, CsDIR2, and the DIR1 variants discussed above were expressed in Rosetta Gami *E. coli*. Unlike many *E. coli* strains used to isolate recombinant proteins, Rosetta Gami *E. coli* promotes disulfide bond formation of proteins in the bacterial cytosol (Rosano and Ceccarelli, 2014). Disulfide bond formation contributes to formation of the LTP hydrophobic cavity (Desormeaux et al., 1992; Douliez et al., 2000; Yeats and Rose, 2008). To confirm that the proteins produced in Rosetta Gami *E. coli* were folded, and to begin to investigate the structural effects of modifying the conserved DIR1 motifs identified above (**Figure 3**), a protein-folding assay, based on the lipophilic synthetic probe TNS, was employed. TNS fluoresces in hydrophobic environments (McClure and Edelman, 1966) and is routinely used to investigate the hydrophobic cavities of LTPs (Mikes et al., 1998; Girault et al., 2008; DeBono et al., 2009; Guo et al., 2013). The formation of a hydrophobic cavity was examined by incubating each Rosetta Gami-generated recombinant protein with increasing concentrations of TNS and measuring the change in fluorescence. As a control, TNS binding curves of recombinant protein were compared to curves generated using protein samples that were denatured by boiling in urea. The TNS binding curves of each DIR1 variant is shown alongside folded and denatured AtDIR1 to illustrate the TNS binding capacity of each protein relative to AtDIR1 (**Figure 4**). Individual TNS binding curves for each protein (folded and denatured) are shown in **Supplementary Figure S1** and batch-to-batch repeatability of TNS assays for selected proteins is shown

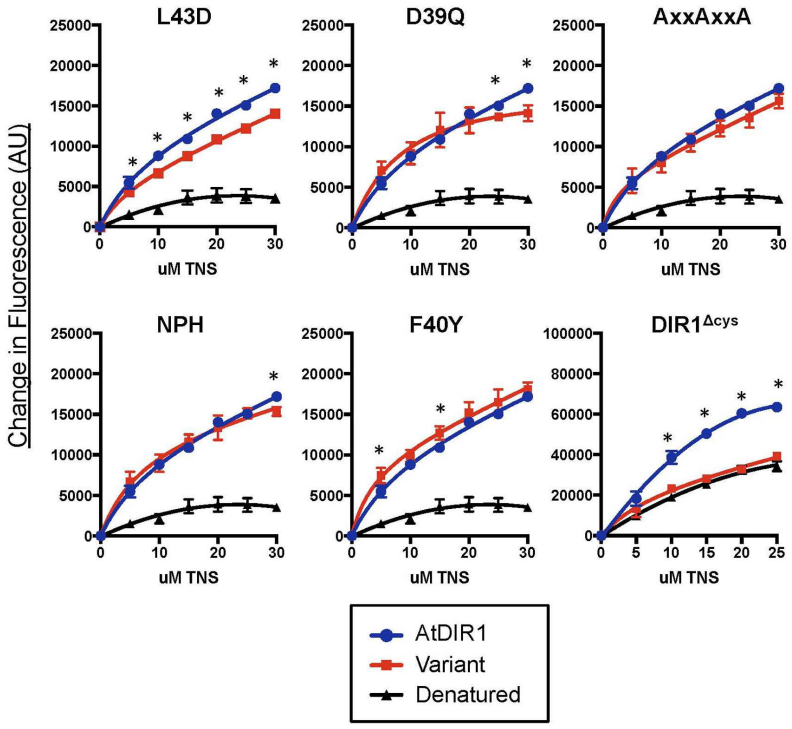


FIGURE 4 | Comparative *in vitro* TNS (6,*P*-toluidinylnaphthalene-2-sulfonate) binding assays of recombinant DIR1 variant proteins and AtDIR1. DIR1 variants (L43D, D39Q, AxxAxxA, NPH, F40Y, and DIR1$^{\Delta cys}$: red lines, squares) are compared to natured (blue lines, circles) and denatured (black lines, triangles) AtDIR1 protein. Increasing concentrations of TNS were added to each Rosetta Gami *E. coli* purified protein lacking the ER signal sequence. TNS binding curves were generated in PRISM6 by non-linear curve fitting for one site saturation binding. Proteins were denatured by boiling in 6 M Urea. Samples were excited at 320 nm and emission at 437 nm and the change in fluorescence was calculated for three replicates. Error bars represent the standard deviation. Significant differences between AtDIR1 and each variant at a given concentration of TNS were determined by Student's *t*-test ($p < 0.05$) and are indicated by an asterisk (*). The DIR1$^{\Delta cys}$ data was obtained using a different instrument (see Section "Materials and methods").

in **Supplementary Figure S2**. Denatured recombinant proteins displayed little change in fluorescence with increasing TNS, and were used as negative controls for TNS binding (**Figures 4** and **5**; **Supplementary Figures S1** and **S2**). In assays containing native AtDIR1 and DIR1 variant proteins, fluorescence increased with increasing TNS concentrations in comparison to denatured protein negative controls, providing evidence that Rosetta Gami *E. coli* produced properly folded LTP proteins (**Figure 4**). The AxxAxxA, F40Y, and NPH variants displayed statistically similar TNS binding profiles in comparison to AtDIR1, which suggests that these residues are not essential for the formation or maintenance of the hydrophobic cavity. As expected, the TNS binding curve of the DIR1$^{\Delta Cys}$ variant was similar to that of denatured AtDIR1, confirming the importance of the disulfide bonds of DIR1 for protein folding. Interestingly, TNS fluorescence was reduced in assays containing the L43D and D39Q variants compared to native AtDIR1 (**Figure 5**), indicating that replacing these hydrophobic cavity residues with charged or polar residues reduced the TNS-DIR1 interaction.

TNS Displacement Assays to Determine whether *Arabidopsis* or Cucumber DIR1 Proteins Interact with Putative SAR Signals

Fluorescence displacement assays can be used to assess *in vitro* binding of lipids to LTP proteins (Buhot et al., 2004; Krasikov et al., 2011; Lei et al., 2014). To determine if AtDIR1 and/or DIR1 homologs from *Arabidopsis* or cucumber interact with known SAR-activating small molecules, we

purified recombinant AtDIR1, AtDIR1-like, CsDIR1, CsDIR2, and the unrelated AtLTP2.12 protein from Rosetta Gami *E. coli* for use in fluorescence displacement assays. Before performing ligand-interaction assays, the folding status of each recombinant protein was assessed using *in vitro* TNS binding assays. In assays comparing native AtDIR1, AtDIR1-like, CsDIR1, CsDIR2, and AtLTP2.12, fluorescence increased with increasing TNS concentrations in comparison to denatured protein controls, providing evidence that these LTP proteins are properly folded (**Figure 5**). Interestingly, the unrelated AtLTP2.12 protein displayed significantly higher TNS fluorescence compared to AtDIR1 with 10, 20, and 30 μM TNS (Student's *t*-test, $p < 0.05$). This result was expected, as AtLTP2.12 is a putative ortholog of wheat LTP2 that has two hydrophobic cavities with volumes of 300 and 103 $Å^3$ (Hoh et al., 2005) compared to AtDIR1, which has a single hydrophobic cavity with a volume of 242 $Å^3$ (Lascombe et al., 2008).

To investigate whether the AtDIR1 hydrophobic cavity interacts with known SAR signal molecules, TNS displacement assays were performed with AtDIR1, AtDIR1-like, CsDIR1, CsDIR2, and AtLTP2.12 proteins and commercially available SAR signal molecules, AzA, pipecolic acid (Pip), and G3P, as well as a buffer control (MES). Fluorescence was measured before and after the addition of each SAR molecule to a mixture of recombinant protein and TNS. If a putative ligand enters the LTP hydrophobic cavity, TNS molecules are displaced and fluorescence decreases. No significant differences in TNS fluorescence were observed for AtLTP2.12, AtDIR1 and AtDIR1-like, regardless of whether MES or SAR signal molecules were added (**Figure 6**). Interestingly, the addition of AzA lead to reduced TNS fluorescence in both CsDIR1 (∼25% lower than the MES control) and CsDIR2 (∼30% lower), while the addition of Pip to CsDIR1 resulted in a similar reduction in TNS fluorescence (∼25% lower). This suggests that features of the CsDIR1 and CsDIR2 hydrophobic cavities allow for modest binding of AzA or Pip *in vitro*. Alternatively, Pip and AzA may interact outside of the cavity, resulting in a conformational change that modestly impacts the TNS binding of CsDIR1/2. In contrast, interaction of AtDIR1 with these SAR signals was not observed, perhaps because AtDIR1 requires *in vivo* factors to facilitate signal binding.

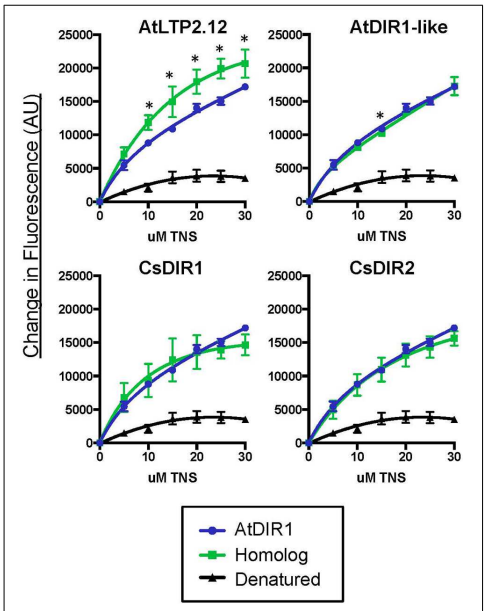


FIGURE 5 | Comparative *in vitro* TNS binding assays of recombinant AtDIR1, AtDIR1-like, AtLTP2.12, and the CsDIR1/CsDIR2 orthologs. DIR1 homologs and the AtLTP2.12 control (green lines, squares) are compared to natured (blue lines, circles) and denatured (black lines, triangles) AtDIR1 protein. Increasing concentrations of TNS were added to each Rosetta Gami *E. coli* purified protein lacking the ER signal sequence. TNS binding curves were generated in PRISM6 by non-linear curve fitting for one site saturation binding. Proteins were denatured by boiling in 6 M Urea. Samples were excited at 320 nm and emission at 437 nm and the change in fluorescence was calculated for three replicates. Error bars represent the standard deviation of the technical variation. Significant differences between AtDIR1 and each homolog at a given concentration of TNS were determined by Student's *t*-test ($p < 0.05$) and are indicated by an asterisk (*).

Validation of *In Silico* Orthology Analysis Using the Cucumber and *Arabidopsis* Model Systems

To validate our *in silico* orthology data (**Figures 1** and **2**; **Table 1**) we investigated the role of DIR1 during SAR in cucumber by combining the *Arabidopsis* and cucumber SAR model systems (Rasmussen et al., 1991; Cameron et al., 1994, 1999) in two ways. First, we reasoned that if cucumber and *Arabidopsis* DIR1 proteins are functionally equivalent, transiently expressed CsDIR1/2 proteins should rescue the SAR-defective *dir1-1 Arabidopsis* mutant. The *Agrobacterium*-mediated transient-SAR (Agro-SAR) assay previously developed in our lab (Champigny et al., 2013) was used to transiently express *CsDIR1* or *CsDIR2* in one leaf of *dir1-1*. *Agrobacterium*-mediated transient expression of AtDIR1 and EYFP was included as a positive and negative control, respectively. RT-PCR analysis confirmed successful Agro-mediated transient expression of each transgene in *dir1-1* leaves 4 days post agro-infiltration (**Supplementary Figure S3**). Four days after *Agrobacterium* inoculation, leaves were induced for SAR using 10^6 cfu ml^{-1} *Pst(avrRpt2)*. The SAR response was measured in distant leaves at 3 dpi with 10^5 cfu ml^{-1} virulent *Pst*. The positive control gave the expected result in that transient expression of AtDIR1 complemented the *dir1-1* mutation, as indicated by a significant ∼fivefold reduction (Student's *t*-test, $p < 0.05$) in bacterial density in distant leaves of SAR-induced plants compared to mock-inoculated control plants (**Figure 7A**). Expression of EYFP did not complement the

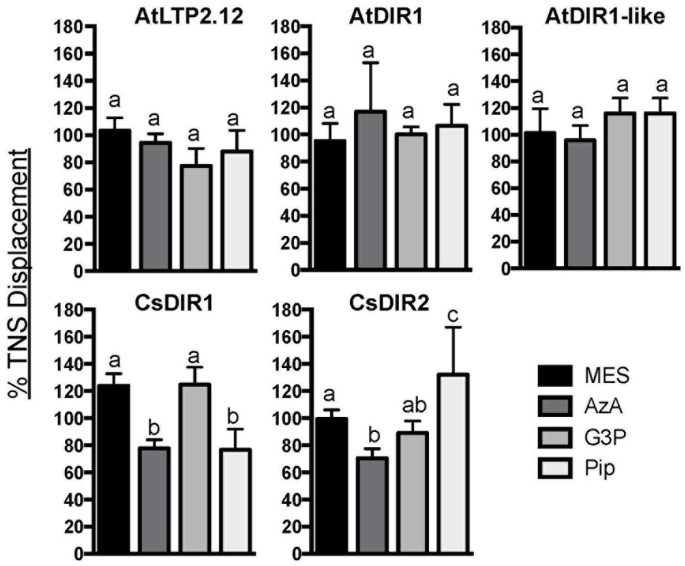


FIGURE 6 | *In vitro* ligand binding assays. TNS displacement assays using AtDIR1, AtLTP2.12, AtDIR1-like, CsDIR1, and CsDIR2 to test the binding of azelaic acid (AzA), glycerol-3-phosphate (G3P), pipecolic acid (Pip), and MES buffer control. Fluorescence was measured when TNS (3 μm) and putative ligands (16 μm) were incubated together for 3 min, then again after purified proteins were added. Fluorescence was measured at excitation wavelength of 320 nm and emission at 437 nm in triplicate. The data is represented as the percent TNS Displacement, which indicates the fluorescence level of protein-ligand-TNS compared to TNS-protein alone (100%). Error bars indicate standard deviation of three replicate measurements. Different letters indicate significant differences (ANOVA, Tukey's HSD, $p < 0.05$).

SAR defect in *dir1-1*, confirming that *Agrobacterium* infection did not induce the SAR response (**Figure 7A**). Expression of *CsDIR1* in one leaf of the *dir1-1* mutant resulted in a significant ∼12-fold reduction in *Pst* levels in SAR-induced versus mock-inoculated plants (**Figure 7A**), while expression of *CsDIR2* in a separate experiment resulted in a significant ∼sixfold reduction (**Figure 7B**). We concluded that transient expression of CsDIR1 and CsDIR2 complemented the *dir1-1* SAR defect, providing evidence that both cucumber orthologs are functionally similar to AtDIR1.

We further combined the cucumber and *Arabidopsis* SAR models to examine the importance of CsDIR1/2 during SAR and to determine if cucumber DIR1 proteins act as SAR long distance signals in cucumber. Phloem exudates collected from mock- and SAR-induced cucumber leaves were collected and infiltrated into the *dir1-1* and *npr1-2 Arabidopsis* SAR mutants to determine whether SAR-induced cucumber exudates contain SAR signals capable of rescuing these mutants. In cucumber, SAR signals move out of induced leaves between 4 and 8 hpi (Rasmussen et al., 1991). Therefore, cucumber phloem exudates were collected at two time-points after inoculation with 10^8 cfu ml^{-1} *P. syringae* pv *syringae* D20 (*Pss*): at 8 hpi when SAR signals are accumulating and at 22 hpi when SAR signals are no longer present in the phloem. Cucumber exudates containing 5–15 mg ml^{-1} protein were diluted 15-fold and infiltrated into two lower leaves of *dir1-1* or *npr1-2* (negative control). Two days later, distant *Arabidopsis* leaves were inoculated with 10^5 cfu ml^{-1} virulent *Pst DC3000* and bacterial levels were measured 3 dpi to assay for SAR competence. The *npr1-2* mutant is defective in acting on mobile SAR signals in systemic leaves (Cao et al., 1997; Fu and Dong, 2013). Therefore, as expected, SAR was not established in *npr1-2* distant leaves after infiltration of SAR-induced (8 hpi) cucumber exudates, as demonstrated by similar *Pst* levels in plants that received exudates collected from either mock-inoculated or SAR-induced leaves (**Figure 8A**). SAR was established in *dir1-1* distant leaves after infiltration of SAR-induced cucumber exudates (8 hpi), as demonstrated by a sixfold reduction in *Pst* levels compared to plants that were infiltrated with exudates collected from mock-inoculated leaves or inactive (22 hpi) exudates (**Figure 8A**). Similar to previous reports (Rasmussen et al., 1991; Smith-Becker et al., 1998), SA was not detected in the 8 hpi cucumber exudates (data not shown), ruling out the possibility that SA present in the exudates induced the observed resistance. These experiments suggest that cucumber petiole exudates collected at 8 hpi, but not at 22 hpi contain

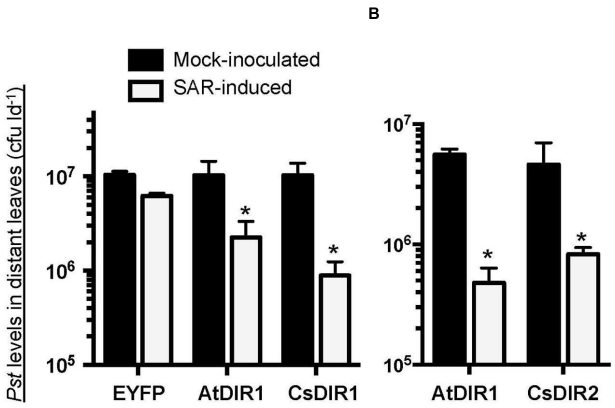


FIGURE 7 | *In vivo* complementation of *dir1-1* by *Agrobacterium*-mediated transient expression of (A) *CsDIR1* and (B) *CsDIR2*. At 3 weeks post germination (wpg), two leaves of *dir1-1* were inoculated with *Agrobacterium* expressing EYFP, AtDIR1(-EYFP), CsDIR1, or CsDIR2. Four days later, the same leaves were mock-inoculated (10 mM MgCl2) or induced for SAR with 10^6 cfu ml^{-1} *Pst* (*avrRpt2*). Distant leaves were challenged for SAR 2 days post mock/SAR-induction with virulent *Pst* (10^5 cfu ml^{-1}) and *in planta* bacterial levels were determined in distant leaves 3 days post challenge. Statistically significant differences between mock-induced and SAR-induced plants (Student's *t*-test $p < 0.05$) are indicated with asterisks (*). **(A)** was performed four times with similar results and **(B)** was performed three times with similar results.

SAR long-distance signals that rescue the *dir1-1* SAR defect, and that mobile SAR signals are conserved between cucumber and *Arabidopsis*. Moreover, this provides additional evidence that the cucumber genome contains DIR1 orthologs that can compensate for the absence of AtDIR1 protein in the *dir1-1* mutant.

Rescue of the *dir1-1* SAR defect by transient expression of CsDIR1/2 or by infiltration of SAR-induced cucumber exudates suggested that cucumber DIR1 orthologs function like AtDIR1 during SAR. To obtain additional support for this idea, we investigated whether the AtDIR1 antibody detected orthologous CsDIR1/2 proteins in cucumber petiole exudates. Petiole exudates collected from mock- (10 mM $MgCl_2$) and SAR-induced (*Pss* D20) cucumber leaves at 8 hpi and 22 hpi were subjected to immunoblot analysis with the AtDIR1 antibody. A ~15 kDa band was detected in untreated, mock-inoculated and SAR-induced petiole exudates, suggesting that a protein related to AtDIR1 is constitutively present in cucumber phloem (**Figure 8B**). To confirm that the AtDIR1 antibody detects CsDIR1 and/or CsDIR2, these proteins, along with AtDIR1 and AtDIR1like controls, were ectopically expressed in Rosetta Gami *E. coli*. Immunoblots revealed that, as with AtDIR1 and AtDIR1like, the AtDIR1 antibody detected both CsDIR1 and CsDIR2 proteins (**Figure 8C**). This suggests that the proteins detected by the AtDIR1 antibody in the cucumber phloem samples (**Figure 8B**) represent CsDIR1 and/or CsDIR2, providing further support for the hypothesis that CsDIR1/2 act as long-distance signals during SAR.

DISCUSSION

Orthology Analysis Identifies Conserved Residues in DIR1

DIR1 is hypothesized to bind and translocate a lipidic signal during SAR. In support of this hypothesis, *in vitro* experiments have demonstrated the non-specific loading of fatty acids into the hydrophobic pocket of DIR1 (Lascombe et al., 2008). However, *in vivo* evidence supporting this or any biochemical function during SAR has not been reported. In this study, we identified and compared DIR1 orthologs to reveal conserved residues or motifs. Many conserved residues among DIR1 orthologs were involved in the formation of the central hydrophobic cavity. This result is not surprising, as LTPs are small proteins whose defining feature is a central cavity that can accommodate fatty acids. We generated DIR1 variant proteins to determine the structural importance of each conserved residue/motif. This was achieved using an *in vitro* binding assay based on the fluorescent lipophilic probe TNS. As described previously, the TNS probe fluoresces only in hydrophobic environments (McClure and Edelman, 1966). Since LTPs are small proteins with a central hydrophobic cavity, TNS fluorescence in the presence of an LTP is attributed to loading of TNS into the cavity. As such, this assay is often used as an indicator of LTP folding status and lipid-binding capacity *in vitro* (Mikes et al., 1998; Girault et al., 2008; DeBono et al., 2009; Choi et al., 2012; Guo et al., 2013). Among the DIR1 variants tested, a significant reduction in TNS-binding was observed in the D39Q, L43D, and $DIR1^{\Delta Cys}$ proteins compared to the wild-type AtDIR1 protein. The largest reduction in TNS-binding was

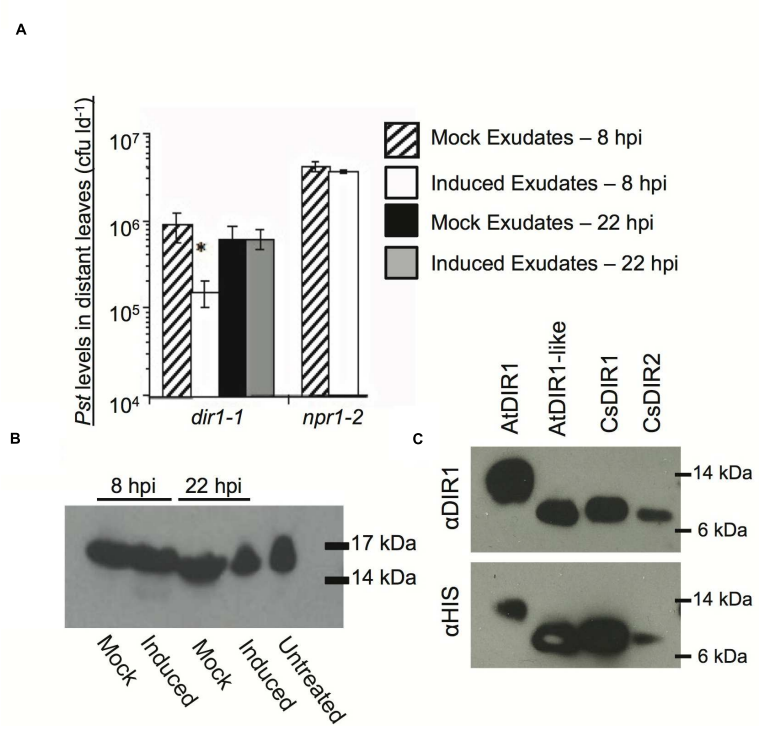


FIGURE 8 | Characterization of DIR1-mediated SAR in *Cucumis sativus*. (A) SAR-induced cucumber exudates rescue the SAR defect in *dir1-1*. Petiole exudates were collected from cut cucumber ends at 8 or 22 hpi with 10 mM $MgCl_2$ (mock) or SAR-inducing (induced) *Pseudomonas syringae* pv. *syringae* D20. Exudates from Mock (8 or 22 hpi) or SAR-induced (8 or 22 hpi) were infiltrated into 2 *dir1-1* or *npr1-2* leaves. Two days later distant leaves were inoculated with virulent *Pst* (10^5 cfu ml-*1*) and 3 dpi *Pst* levels were measured. An asterisk (*) indicates a significant difference between plants infiltrated with mock and induced exudates. **(B)** A DIR1-sized band is present in cucumber phloem before and after SAR induction Exudates were lyophilized and subjected to immunoblot analysis using the DIR1 antibody. 17 and 14 kDa protein molecular weight markers are indicated. **(C)** Recombinant CsDIR1 and CsDIR2 protein is detected by the polyclonal DIR1 antibody. Immunoblots of crude extracts from IPTG-induced Rosetta-gami strains expressing His-tagged AtDIR1, AtDIR1-like, CsDIR1, and CsDIR2 proteins using anti-DIR1 or anti-HIS antibodies. **(A)** Was repeated two additional times, **(B,C)** were repeated once, all with similar results.

observed in the $DIR1^{\Delta Cys}$ protein, which lacks the eight cysteine residues responsible for the generation of four disulphide bonds that establish the hydrophobic cavity. This variant displayed reduced TNS-fluorescence levels that were comparable to those observed with denatured AtDIR1, indicating that the $DIR1^{\Delta Cys}$ protein does not contain the hydrophobic cavity. Both the L43D and D39Q variants also displayed reduced TNS-binding compared to the wild-type AtDIR1. Leucine 43 is located between two cysteine residues, a position that is hypothesized to be important for cysteine bond pairing (Samuel et al., 2002). In the L43D DIR1 variant, the conserved non-polar leucine residue was modified to a polar aspartic acid residue. This modification resulted in reduced TNS-fluorescence compared to that observed with AtDIR1, suggesting that this residue is important for hydrophobic ligand interactions, or that it contributes to the size or shape of the hydrophobic cavity. A similar effect was reported in mutagenesis studies of the rice (*Oryza sativa*) OsLTP2 protein, where modifying the same position from phenylalanine to alanine (F36A) disrupted the formation of the hydrophobic cavity, as determined by circular dichroism, NMR spectroscopy, and a fluorescence-based assay using the ANS (1-anilino-8-naphthalene sulfonate) probe (Cheng et al., 2008). Using the comparable TNS binding assay, we observed similar binding defects for the L43D AtDIR1 variant as was observed for the OsLTP2 F36A variant (Cheng et al., 2008). The conserved D39 (aspartic acid) residue was modified to determine the importance of the DIR1-specific AD motif present at the bottom of the DIR1 cavity. In this variant, the charged aspartic acid residue

was modified to a similarly shaped but non-charged glutamine residue. TNS binding was affected to a small degree in the D39Q variant compared to the wild-type protein, suggesting that this residue contributes to ligand binding. Alternatively, the AD motif could be the site of an adduct formation with potential SAR signals/ligands, as a similarly exposed aspartic acid residue in the barley LTP1 protein is the site of allene oxide adduct formation (Bakan et al., 2006).

The other DIR1 variants (AxxAxxA, F40Y, and NPH) displayed similar TNS-binding profiles to that of wild-type AtDIR1. The NPH variant lacks three polar residues (Gln9, Asn13, Lys16) at the entrance of the hydrophobic cavity. It is not surprising that that TNS-binding was unaffected in this variant as these exterior residues are thought to stabilize a ligand possessing a hydrophilic moiety that remains outside of the hydrophobic cavity. In contrast, the F40 phenylalanine residue is located within the central hydrophobic cavity. AtDIR1-like, which is hypothesized to have a reduced capacity to participate in SAR (Champigny et al., 2013), contains a polar tyrosine residue at position 40, while AtDIR1 and other orthologs contain a non-polar phenylalanine residue. In this study, we demonstrated that the F40Y and AtDIR1-like proteins have similar TNS-binding curves to that of AtDIR1, indicating that the F40 residue does not affect the ability of the cavity to bind TNS molecules. As outlined previously (Champigny et al., 2013), we hypothesize that the F40Y substitution decreases the affinity of DIR1-like for a specific ligand, thereby compromising its ability to participate in SAR.

The conserved PxxPxxP and LAxxLP motifs are located on the exterior of AtDIR1 and are thought to contribute little to cavity shape, size or ligand interaction. The PxxPxxP motif is predicted to mediate protein–protein interactions (Lascombe et al., 2008), however PxxP motifs are thought to interact with SH3 domain-containing proteins (Li, 2005). It is possible that DIR1 interacts with one of the three predicted SH3 domain-containing proteins (AtSH3P1-3) in *Arabidopsis* (Lam et al., 2001), or that the mechanism of PxxP-based protein–protein interaction in *Arabidopsis* is distinct from the classical PxxP–SH3 interaction. Lastly, the LAxxLP motif is located on the surface of DIR1 and may also be involved in mediating, or maintaining, protein–protein interactions.

DIR1 Is Conserved in Tobacco, Tomato, Cucumber, and Soybean

DIR1 is known to play a significant role during SAR in *Arabidopsis*; however, its importance in other plants has not been well defined. Mitton et al. (2009) identified a putative DIR1 ortholog in tomato, SlDIR1 (SGN-U584000, formerly LeDIR1; SGN-327306). SlDIR1 is 63% similar to mature AtDIR1 at the amino acid level and has a similarly low pI of 4.03. Interestingly, SlDIR1 is present in petiole exudates of untreated tomato leaves (Mitton et al., 2009), whereas AtDIR1 only accumulates to detectable levels in petiole exudates collected from local and distant leaves of SAR-induced plants (Champigny et al., 2013; Shah et al., 2014; Carella et al., 2015). Nevertheless, the presence of SlDIR1 in petiole exudates suggests that it participates in long-distance SAR signaling, and thus may function similarly to AtDIR1. A previous report hinted at the functional redundancy of SAR signals between *Arabidopsis* and tomato, as infiltration of petiole exudates collected from SAR-induced leaves of *Arabidopsis* induced SAR in tomato (Chaturvedi et al., 2008). We hypothesize that SlDIR1 (and/or SlDIR2/3) participates in tomato long-distance SAR signaling.

A more comprehensive study demonstrated that DIR1-mediated SAR signaling is conserved in tobacco. Of the three *N. tabacum* DIR1 orthologs, NtDIR2 and NtDIR3 complemented the SAR-defect in *Arabidopsis dir1-1,* demonstrating that these two orthologs (which have 57 and 67% protein sequence similarity, respectively, to AtDIR1) have a similar function to AtDIR1 (Liu et al., 2011). Knockdown of *NtDIR2/3* expression in tobacco RNAi lines resulted in a loss of SAR to TMV (tobacco mosaic virus), which was associated with heightened *SAMT1* (Salicylic Acid Methyl-transferase) expression and increased MeSA levels compared to wild-type plants. This was consistent with the observation that *dir1-1* mutants have increased MeSA levels and higher *BSMT1* (Benzoic acid/Salicylic acid Carboxyl Methyl-transferase) expression after pathogen infection relative to wild-type plants (Liu et al., 2011). Interestingly, NtDIR1, which shares 62% amino acid similarity with AtDIR1, was unable to complement *Arabidopsis dir1-1* and NtDIR1-RNAi tobacco lines with reduced NtDIR1 expression (but wild-type NtDIR2/3 expression levels), were SAR-competent. Although NtDIR2/3 movement into petiole exudates during SAR was not determined, the authors suggested that NtDIR2/3 are translocated through the phloem to activate SAR in distant tobacco leaves (Liu et al., 2011). Taken together, the existence of DIR1 orthologs in several crop plants, and the conservation of DIR1 function in tobacco and cucumber, suggests that DIR1-mediated SAR is important in a number of plant species.

DIR1-Mediated SAR in *Cucumis sativus*

Two putative DIR1 orthologs are encoded in the *C. sativus* genome, CsDIR1 and CsDIR2, which share 61 and 65% amino acid sequence similarity, respectively, with mature AtDIR1. Homology models of the CsDIR1/2 proteins identified structural similarities with AtDIR1. TNS-binding experiments demonstrated that CsDIR1, CsDIR2, and AtDIR1 have statistically similar affinities for the lipophilic TNS probe, confirming that these proteins are indeed structurally similar. Phloem exudate rescue and Agro-SAR rescue experiments demonstrated that DIR1-mediated SAR is conserved in cucumber. Interestingly, proteins that cross-reacted with the AtDIR1-antibody were detected in phloem exudates collected from both mock- and SAR-induced cucumber leaves at 8 and 22 hpi. Accumulation of the LeDIR1 protein in petiole exudates collected from untreated tomato plants was also observed (Mitton et al., 2009). However, DIR1-antibody signals were only detected in *Arabidopsis* exudates after SAR induction (Champigny et al., 2013; Shah et al., 2014; Carella et al., 2015). Importantly, only exudates from SAR-induced cucumber leaves collected at 8 hpi, when SAR signals are present, were able to induce SAR in the *Arabidopsis dir1-1* mutant, even though DIR1-antibody cross-reacting proteins were present in all exudates.

This result supports the idea that both cucumber and *Arabidopsis* DIR1 are activated during SAR-induction, perhaps by binding a ligand(s) and/or becoming part of a mobile signal complex.

Searching for DIR1 Ligands

We used an *in vitro* TNS-based fluorescence displacement assay to determine whether AtDIR1 can interact with known inducers of SAR (G3P, AzA, Pip). Although these molecules do not resemble typical fatty acid ligands of LTPs *in vitro*, we examined their ability to displace TNS from the hydrophobic cavity of DIR1 because these signals are present in the phloem during SAR induction, along with DIR1. Moreover, G3P and AzA both require DIR1 for their resistance-inducing activity (Jung et al., 2009; Chanda et al., 2011). AtDIR1 and AtDIR1like failed to interact with any of the SAR inducers, suggesting that these molecules are not DIR1 ligands. However, it also possible that SAR induction causes DIR1 or AzA/G3P/Pip modification and/or the formation of a SAR signal complex *in planta* that is required for DIR1–ligand interaction. Alternatively, DIR1 may bind a different SAR-inducing molecule or an unknown ligand. Given that DIR1-containing high molecular weight complexes co-fractionate with the diterpenoid SAR-inducer dehydroabietinal in phloem exudates collected from SAR-induced *Arabidopsis* leaves (Chaturvedi et al., 2012; Shah et al., 2014), we speculate that DIR1 may interact with dehydroabietinal. Moreover, DIR1's association with a high molecular weight complex may indicate that other proteins are required for DIR1-ligand binding, which could explain why purified recombinant AtDIR1 did not interact with the tested SAR signaling molecules.

Intriguingly, the TNS displacement assays suggest that AzA and Pip displace TNS from the hydrophobic cavity of CsDIR1, while AzA displaces TNS from the CsDIR2 cavity. This leads us to speculate that AzA and Pip may contribute to DIR1 function in cucumber, as ligands that enter the DIR1 hydrophobic cavity. Alternatively, AzA and Pip may act outside of the hydrophobic cavity to cause allosteric effects that alter the shape or size of the cavity. It is currently unknown whether AzA or Pip accumulate in phloem exudates during SAR induction in cucumber, therefore further experimentation is needed to characterize the role of these SAR signal molecules during the cucumber SAR response.

CONCLUSION

DIR1 orthology analysis identified amino acid motifs (L43, AD, and the eight cysteine motif) that are important for TNS binding, supporting the hypothesis that they contribute to the size, shape, or lipid binding ability of the hydrophobic cavity, an essential feature for hydrophobic ligand–LTP interaction. In addition, we developed an *Arabidopsis*–cucumber SAR model to further explore the role of DIR1 during SAR. Using this model, we discovered two cucumber orthologs that function similarly to AtDIR1 during SAR. Although DIR1-antibody signals are constitutively present in cucumber phloem sap, only SAR-induced cucumber phloem exudates rescued the SAR defect in *dir1-1*. Together, these data suggest that DIR1-mediated SAR signaling is conserved in cucumber, further demonstrating the importance of DIR1 in long-distance systemic immune signaling in plants.

AUTHOR CONTRIBUTIONS

MI and PC contributed equally as first authors. Designed experiments: MI, PC, JR, and RC. Performed experiments: MI, PC, and JF. Analyzed data: MI, PC, JF, and RC. Provided reagents and equipment: JR and RC. PC and RC wrote the bulk of the manuscript, with significant contributions by MI and JR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00566

FIGURE S1 | Individual *in vitro* TNS (6,*P*-toluidinylnaphthalene-2-sulfonate) binding assays of recombinant AtDIR1, AtDIR1-like, AtLTP2.12, CsDIR1, CsDIR2, and DIR1 variant proteins. Natured protein (black lines, circles) is compared to denatured protein for sample (gray lines, squares). Increasing concentrations of TNS were added to each Rosetta Gami *E. coli* purified protein lacking the ER signal sequence. TNS binding curves were generated in PRISM6 by non-linear curve fitting for one site saturation binding. Proteins were denatured by boiling in 6 M Urea. Samples were excited at 320 nm and emission at 437 nm and the change in fluorescence was calculated for three replicates. Error bars represent the standard deviation. An asterisk (*) indicates proteins that were analyzed using a TECAN M1000 rather than the Gen5 BioTek fluorometer.

FIGURE S2 | Batch Reproducibility of the TNS binding assay. TNS binding curves of identical proteins purified from different batches was performed for AtDIR1 **(A)** and AtDIR1- like **(B)**. Student's *t*-tests ($p < 0.05$) of natured proteins from batch 1 and batch 2 did not identify statistically significant differences in TNS binding capacity (means of denatured proteins were not compared). Values represent the mean ± standard deviation of 3 technical replicates. These assays were performed using a Gen5 Synergy 4 (BioTek) plate reader.

FIGURE S3 | Confirmation of successful *Agrobacterium*-mediated expression in *dir1-1*. RT-PCR analysis was used to monitor the expression of *EYFP, AtDIR1-EYFP, CsDIR1,* and *CsDIR2* four days post agro-infiltration of *dir1-1* leaves (3.5 week-old plants). Expression of the endogenous *ACTIN1* housekeeping gene was monitored as a loading control. Primers for RT-PCR analysis can be found in Table S1. This was performed twice with similar results.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

4.12 – Online Supplementary Figures and Tables

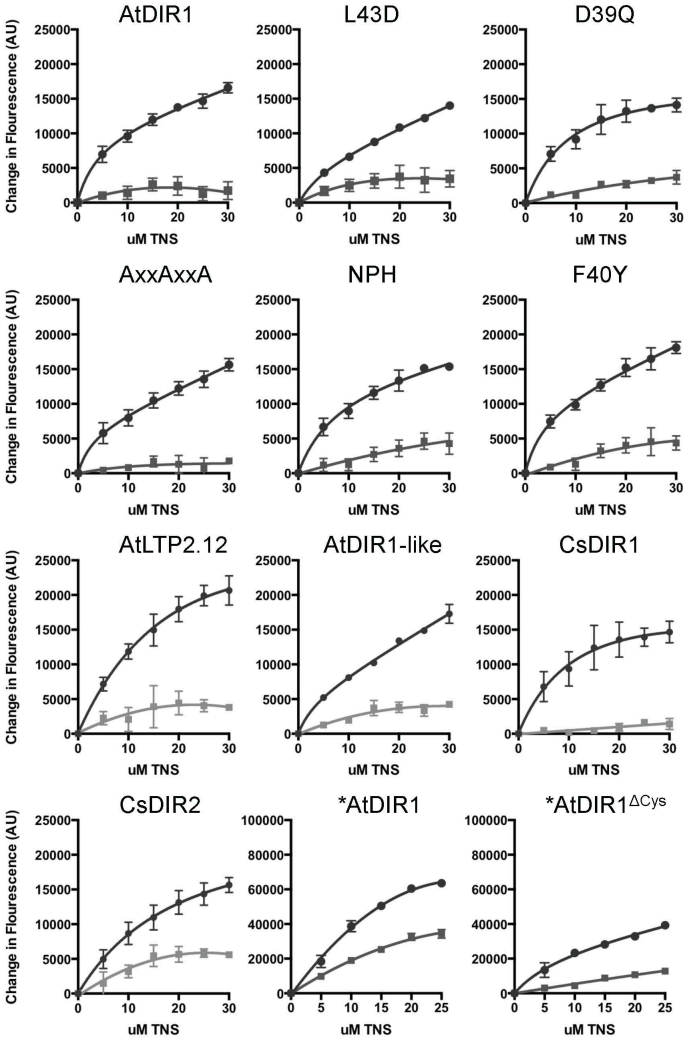

Figure 4.9/S1 - Individual *In vitro* TNS (6,P-toluidinylnaphthalene-2-sulfonate) binding assays of recombinant AtDIR1, AtDIR1-like, AtLTP2.12, CsDIR1, CsDIR2, and DIR1 variant proteins. Natured protein (black lines, circles) is compared to denatured protein for sample (grey lines, squares). Increasing concentrations of TNS were added to each Rosetta Gami *E. coli* purified protein lacking the ER signal sequence. TNS binding curves were generated in PRISM6 by non-linear curve fitting for one site saturation binding. Proteins were denatured by boiling in 6 M Urea. Samples were excited at 320 nm and emission at 437 nm and the change in fluorescence was calculated for three replicates. Error bars represent the standard deviation. An asterisk (*) indicates proteins that were analyzed using a TECAN M1000 rather than the Gen5 BioTek fluorometer.

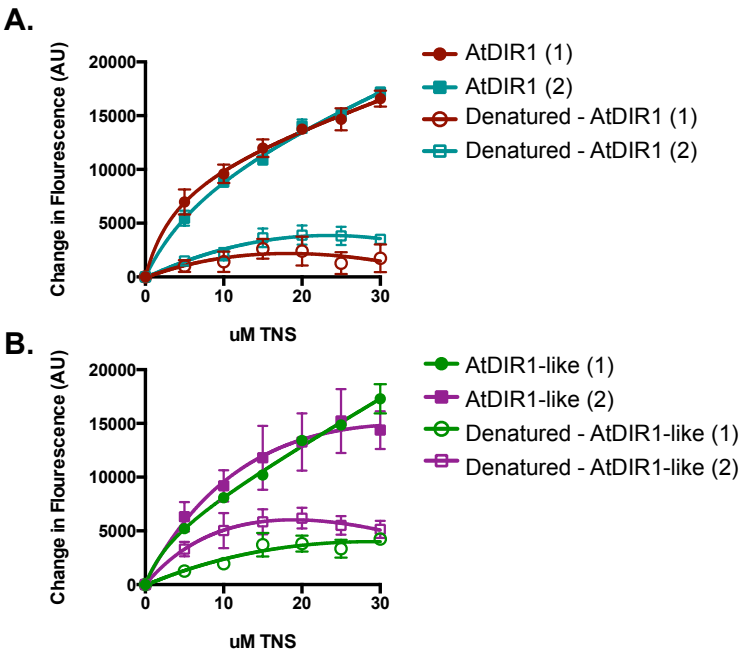

Fig. S2 – Batch Reproducibility of the TNS binding assay. TNS binding curves of identical proteins purified from different batches was performed for AtDIR1 (A) and AtDIR1-like (B). Student's t-tests ($p < 0.05$) of natured proteins from batch 1 and batch 2 did not identify statistically significant differences in TNS binding capacity (means of denatured proteins were not compared). Values represent the mean +/- standard deviation of 3 technical replicates. These assays were performed using a Gen5 Synergy 4 (BioTek) plate reader.

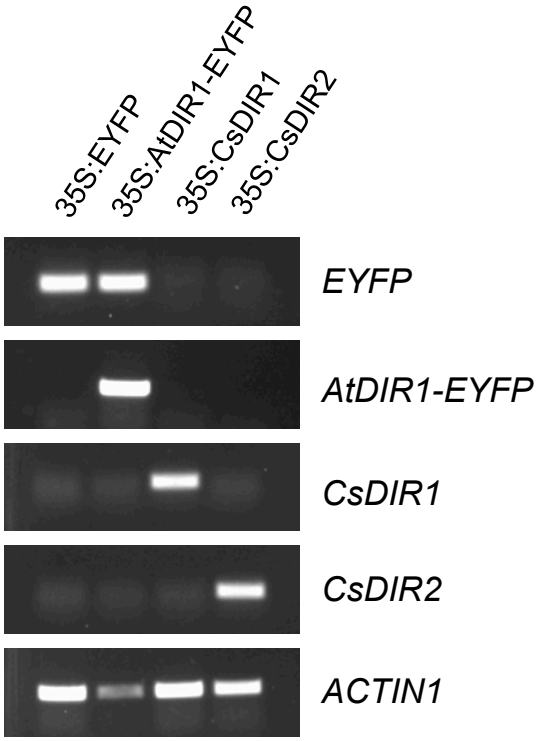

Figure S3 Confirmation of successful Agrobacterium-mediated expression in *dir1-1*. RT-PCR analysis was used to monitor the expression of *EYFP*, *AtDIR1-EYFP*, *CsDIR1*, and *CsDIR2* four days post agro-infiltration of *dir1-1* leaves (3.5 week-old plants). Expression of the endogenous *ACTIN1* housekeeping gene was monitored as a loading control. Primers for RT-PCR analysis can be found in Table S1. This was performed twice with similar results.

Table S1. Primers used in this study

Primer	Sequence (5' - 3')	Construct/ Amplicon
BamHI-AtDIR1like-F	TCGGGATCCGGCGATTGACCTTTGTGGCATG	pET29b-DIR1like
XhoI-AtDIR1like-R	AGCCTCGAGACAAGTTGGGGCGTTGGTTAGG	
BamHI-AtLTP2.12-F	TCGGGATCCGACTGAGGTCAAACTTTCTGGAGG	pET29b-AtLTP2.12
XhoI-AtLTP2.12-R	AGCCTCGAGACAAGTAGGATAAGGAACACCAC	
BamHI-CsDIR1-F	TCGGGATCCGATGGAAGTTTGCGGCGTCGACG	pET29b-CsDIR1
XhoI-CsDIR1-R	GCCTCGAGAGCAGAGCAAGTGGGAGTGTTAGG	
BamHI-CsDIR2-F	TCGGGATCCGCAATCCATTTGCAACATGCC	pET29b-CsDIR2
XhoI-CsDIR2-R	AGCCTCGAGGCAATTTGGAGACTTAGAAATG	
BamHI-DIR1$^{\Delta Cys}$-F	TCGGGATCCGGCGATAGATCTCGCTGGCATG	pET29b-DIR1$^{\Delta Cys}$
XhoI-DIR1$^{\Delta Cys}$-R	AGCCTCGAGAGCAGTTGGGGCGTTGGCTAGA	
BamHI-Variants-F*	CGGGATCCGGCGATAGATCTCTGCGGCATGAGC	pET29b-Variants
XhoI-Variants-R*	AGCCTCGAGACAAGTTGGGGCGTTGGCTAGACC	
AscI-CsDIR1-F	CACACGGGCGCGCCACCATGGAGATGGCTCAAAAGGTG	35S:CsDIR1 (pMDC32)
SacI-CsDIR1-R	GGAACAGAGCTCTTAAAGGTTTAAGCAGAGCAAG	
KpnI-CsDIR2-F	AGCGGTACCTTAGCAGTTGGGAGGATGAGG	35S:CsDIR2 (pMDC32)
SpeI-CsDIR2-R	AGCACTAGTCTAGCAATTTGGAGACTTAGAAATG	
RT-CsDIR1-F	GGTGACGGTGATGGTGGTGCTG	*CsDIR1*
RT-CsDIR1-R	CCAAATGAGGATAGCAACATTG	
RT-CsDIR2-F	GCTATGAAAGTTGTGGCTTTAGC	*CsDIR2*
RT-CsDIR2-R	GCCAAAAGAAGAAAGAGCTCCCG	
RT-AtDIR1-F	GATCGTGATAATGGCTATGTTGGTCGATA	*AtDIR1-EYFP*
RT-nEYFP-R	TCGCCGGACACGCTGAACTTGTGG	
RT-EYFP-F	TGCAGTGCTTCGCCCGCTAC	*EYFP*
RT-EYFP-R	CGGTTCACCAGGGTGTCGCC	
RT-AtACT1-F	GGCGATGAAGCTCAATCCAAACG	*ACTIN1*
RT-AtACT1-R	GGTCACGACCAGCAAGATCAAGACG	

*These primers are suitable for the amplification of the L49D, D39Q, NPH, F40Y, and AxxAxxA variants.

Supporting Document 1. Nucleotide and amino acid sequences of DIR1 variants used in this study. Modified amino acids are highlighted.

>L49D_nt
ATGGCGAGCAAGAAAGCAGCTATGGTTATGATGGCGATGATCGTGATAATG
GCTATGTTGGTCGATACATCAGTAGCGATAGATCTCTGCGGCATGAGCCAG
GATGAGTTGAATGAGTGCAAACCAGCGGTTAGCAAGGAGAATCCGACGAGC
CCATCACAGCCTTGCTGCACCGCTCTGCAACACGCTGATTTTGCATGTGATT
GTGGTTACAAGAACTCTCCATGGCTCGGTTCTTTCGGTGTTGATCCTGAACT
CGCTTCTGCTCTCCCCAAACAGTGTGGTCTAGCCAACGCCCCAACTTGTTAA

>L49D_aa
MASKKAAMVMMAMIVIMAMLVDTSVAIDLCGMSQDELNECKPAVSKENPTSPS
QPCCTALQHADFAC**D**CGYKNSPWLGSFGVDPELASALPKQCGLANAPTC

>D39Q_nt
ATGGCGAGCAAGAAAGCAGCTATGGTTATGATGGCGATGATCGTGATAATG
GCTATGTTGGTCGATACATCAGTAGCGATAGATCTCTGCGGCATGAGCCAG
GATGAGTTGAATGAGTGCAAACCAGCGGTTAGCAAGGAGAATCCGACGAGC
CCATCACAGCCTTGCTGCACCGCTCTGCAACACGCTCAATTTGCATGTCTTT
GTGGTTACAAGAACTCTCCATGGCTCGGTTCTTTCGGTGTTGATCCTGAACT
CGCTTCTGCTCTCCCCAAACAGTGTGGTCTAGCCAACGCCCCAACTTGTTAA

>D39Q_aa
MASKKAAMVMMAMIVIMAMLVDTSVAIDLCGMSQDELNECKPAVSKENPTSPS
QPCCTALQHA**Q**FACLCGYKNSPWLGSFGVDPELASALPKQCGLANAPTC

>F40Y_nt
ATGGCGAGCAAGAAAGCAGCTATGGTTATGATGGCGATGATCGTGATAATG
GCTATGTTGGTCGATACATCAGTAGCGATAGATCTCTGCGGCATGAGCCAG
GATGAGTTGAATGAGTGCAAACCAGCGGTTAGCAAGGAGAATCCGACGAGC
CCATCACAGCCTTGCTGCACCGCTCTGCAACACGCTGATTACGCATGTCTTT
GTGGTTACAAGAACTCTCCATGGCTCGGTTCTTTCGGTGTTGATCCTGAACT
CGCTTCTGCTCTCCCCAAACAGTGTGGTCTAGCCAACGCCCCAACTTGTTAA

>F40Y_aa
MASKKAAMVMMAMIVIMAMLVDTSVAIDLCGMSQDELNECKPAVSKENPTSPS
QPCCTALQHAD**Y**ACLCGYKNSPWLGSFGVDPELASALPKQCGLANAPTC

>AxxAxxA_nt
ATGGCGAGCAAGAAAGCAGCTATGGTTATGATGGCGATGATCGTGATAATG
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GATGAGTTGAATGAGTGCAAACCAGCGGTTAGCAAGGAGAATGCTACGAGC
GCTTCACAGGCTTGCTGCACCGCTCTGCAACACGCTGATTTTGCATGTCTTT
GTGGTTACAAGAACTCTCCATGGCTCGGTTCTTTCGGTGTTGATCCTGAACT
CGCTTCTGCTCTCCCCAAACAGTGTGGTCTAGCCAACGCCCCAACTTGTTAA

>AxxAxxA_aa
MASKKAAMVMMAMIVIMAMLVDTSVAIDLCGMSQDELNECKPAVSKEN**A**TS**A**S
Q**A**CCTALQHADFACLCGYKNSPWLGSFGVDPELASALPKQCGLANAPTC

>NPH_nt
ATGGCGAGCAAGAAAGCAGCTATGGTTATGATGGCGATGATCGTGATAATG
GCTATGTTGGTCGATACATCAGTAGCGATAGATCTCTGCGGCATGAGCGCT
GATGAGTTGGCTGAGTGCGCTCCAGCGGTTAGCAAGGAGAATCCGACGAG
CCCATCACAGCCTTGCTGCACCGCTCTGCAACACGCTGATTTTGCATGTCTT
TGTGGTTACAAGAACTCTCCATGGCTCGGTTCTTTCGGTGTTGATCCTGAAC
TCGCTTCTGCTCTCCCCAAACAGTGTGGTCTAGCCAACGCCCCAACTTGTTA
A

>NPH_aa
MASKKAAMVMMAMIVIMAMLVDTSVAIDLCGMS**A**DEL**A**EC**A**PAVSKENPTSPS
QPCCTALQHADFACLCGYKNSPWLGSFGVDPELASALPKQCGLANAPTC

>DIR1$^{\Delta Cys}$_nt
ATGGCGAGCAAGAAAGCAGCTATGGTTATGATGGCGATGATCGTGATAATG
GCTATGTTGGTCGATACATCAGTAGCGATAGATCTCGCTGGCATGAGCCAG
GATGAGTTGAATGAGGCTAAACCAGCGGTTAGCAAGGAGAATCCGACGAGC
CCATCACAGCCTGCTGCTACCGCTCTGCAACACGCTGATTTTGCAGCTCTT
GCTGGTTACAAGAACTCTCCATGGCTCGGTTCTTTCGGTGTTGATCCTGAAC
TCGCTTCTGCTCTCCCCAAACAGGCTGGTCTAGCCAACGCCCCAACTGCTT
AA

> DIR1$^{\Delta Cys}$_aa
MASKKAAMVMMAMIVIMAMLVDTSVAIDL**A**GMSQDELNE**A**KPAVSKENPTSPS
QP**AA**TALQHADFA**A**L**A**GYKNSPWLGSFGVDPELASALPKQ**A**GLANAPT**A**

Chapter 5

Exploring the role of DIR1, DIR1-like and other lipid transfer proteins during systemic immunity in Arabidopsis

PREFACE:

This chapter consists of a manuscript in preparation entitled "***Exploring the role of DIR1, DIR1-like and other lipid transfer proteins during systemic immunity in Arabidopsis***" by Carella P, Kempthorne CJ, Wilson DC, Isaacs M, and Cameron RK. This manuscript is being prepared for submission to PLoS ONE, which publishes OPEN ACCESS articles under the "Creative Commons Attribution-Non-Commercial License".

This work describes the SAR phenotypic analysis of *DIR1-like* knockdown lines and a *dir1-2* mutant allele in the *Arabidopsis* accession Col-0. The results support the idea that DIR1-like has a reduced role in SAR long-distance signalling compared to DIR1. Further characterization of DIR1-like revealed similar subcellular localization of DIR1-like-GFP and DIR1-YFP when transiently expressed in *Nicotiana benthamiana* epidermal cells, which is different compared to other LTPs (LTP1-GFP, LTP2-GFP). Lastly, targeted yeast- and plant-based protein-protein interaction assays were employed to identify DIR1 and DIR1-like interacting proteins. Through this analysis, it was determined that DIR1 interacts with DIR1-like, and perhaps with LTP1 and LTP2 as well. The SAR-defective phenotype of an *ltp2-1* knockout mutant revealed a role for LTP2 in SAR.

Please note that experiments pertaining to Figure 4 are ongoing. The SAR phenotype of 35S:LTP2-FLAG/*ltp2-1* lines will be further tested after the final submission of this thesis. Analysis of LTP2-FLAG accumulation in phloem

exudates during SAR will also be assessed during this time. In addition, 7 independent homozygous LTP1-RNAi lines (obtained from NASC – Agrikola project) are currently being screened to identify *LTP1* knockdown lines suitable to assess the importance of LTP1 during SAR. Preliminary data suggests that the expression of both *LTP1* and *LTP2* are reduced in 5 of these lines.

Contributions:

DIR1-like RNAi lines (T_0) were generated by Marisa Isaacs, and homozygous lines with reduced *DIR1-like* expression were isolated and characterized by Philip Carella. Data presented in Figures 1, 2, 3, 4 was generated by Philip Carella. CJ Kempthorne assisted with SAR experiments described in Figure 4 and RT-PCR analysis presented in the supporting information**.** DC Wilson and CJ Kempthorne assisted with the isolation and characterization of 35S:LTP2-FLAG/*ltp2-1* and LTP1-RNAi plant lines. Philip Carella wrote the manuscript, with significant contributions by Robin Cameron and DC Wilson.

5.1 ABSTRACT

The lipid transfer protein (LTP) DIR1 (DEFECTIVE IN INDUCED RESISTANCE1) is a key component of systemic acquired resistance (SAR), a defense response in which an initial localized infection induces long-distance signaling that provides distant tissues with enhanced resistance to future pathogen attack. While DIR1 is an important mobile component that moves from local to distant leaves during SAR, evidence suggests that the DIR1-like paralog may compensate for the lack of DIR1 in the *dir1-1* mutant in the Ws-2 accession background. In this study, a *dir1-2* mutant was isolated and *DIR1-like* knockdown lines were created using RNAi to investigate the roles of DIR1 and DIR1-like during SAR in the commonly used *Arabidopsis* accession Col-0. SAR phenotype analysis demonstrated that the *dir1-2*, but not 35S:DIR1-like$_{RNAi}$, plants were defective in SAR. Further characterization of DIR1-like demonstrated that both DIR1 and DIR1-like exhibit similar subcellular localization patterns when heterologously expressed in *Nicotiana benthamiana*, which were different compared to LTP1 and LTP2. Targeted yeast- and plant-based interaction assays were employed to determine if DIR1 and DIR1-like interact with SAR-related proteins such as AZI1, NPR1, and methyl esterase enzymes MES1/7/9. This analysis demonstrated that DIR1 and DIR1-like form hetero- and homo-dimers with one another and with the LTP1 and LTP2 controls. Further investigation of an *ltp2-1* knockout mutant and 35S:LTP2-FLAG/*ltp2-1* plant lines suggests a functional role for LTP2 in long-distance SAR signaling. Together, this work begins to clarify the roles of DIR1 and DIR1-like during SAR and further highlights the importance of lipid transfer proteins for the SAR response.

5.2 INTRODUCTION

Systemic acquired resistance (SAR) is a defense response in which an initial localized infection leads to the dissemination of mobile immune signals that travel to distant naïve leaves to protect against future infection. The SAR response is broadly described by four key stages - induction, signal mobilization, signal perception in distant leaves, and the manifestation of SAR upon subsequent pathogen attack (reviewed in Champigny and Cameron 2009; Fu and Dong 2013). In *Arabidopsis*, SAR is induced by local infection with virulent and avirulent strains of the hemibiotrophic phytopathogen *Pseudomonas syringae* pv. *tomato* (Pst) (Cameron *et al.* 1999; Mishina and Zeier 2007). This interaction leads to the generation and movement of long-distance SAR signals that travel via the phloem to access distant leaves (Kiefer and Slusarenko 2003). To date, several candidate mobile SAR signals have been discovered in *Arabidopsis* and tobacco, including azelaic acid (Jung *et al.* 2009; Wittek *et al.* 2014), dehydroabietinal (Chaturvedi *et al.* 2012), a glycerol-3-phosphate-derived compound (Chanda *et al.* 2011), methyl-salicylate (Park *et al.* 2007; Vlot *et al.* 2008), and pipecolic acid (Navarova *et al.* 2012; Vogel-Adgough *et al.* 2013). Our understanding of signal perception in distant leaves is limited, however the salicylic acid (SA) and SAR master regulator NONEXPRESSER of PATHOGENESIS-RELATED GENES1 (NPR1) is required to activate defense following the perception of mobile SAR signals (Cao *et al.* 1997). The activation of SAR in distant leaves is associated with significant transcriptional reprogramming that prioritizes defense over metabolism and photosynthesis (Gruner *et al.* 2013; Bernsdorff *et al.* 2016). This ultimately results in a phenomenon known as defense priming, where SAR-activated plants contain a sensitized immune system that is more responsive and effective during subsequent interactions with pathogens (Conrath *et al.* 2015). This sensitization, known as the “primed state”, is responsible for the manifestation of SAR during a secondary infection, where SAR-induced plants

display enhanced resistance to normally virulent pathogens compared to mock-inoculated or naïve plants.

DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) is a non-specific lipid transfer protein (ns-LTP) that is required for the generation and/or translocation of mobile SAR signals (Maldonado *et al.* 2002). Upon the induction of SAR, DIR1 accesses the phloem for long-distance transport from locally infected to distant, uninfected leaves (Champigny *et al.* 2013; Carella *et al.* 2015). The crystal structure of DIR1 demonstrates the presence of a single, central hydrophobic pocket that accommodates fatty acids *in vitro*, leading to the speculation that DIR1 chaperones a lipidic (or hydrophobic) signal to distant leaves during SAR. The identity of the DIR1 ligand remains unknown, however functional DIR1 is required for the SAR-inducing activity of the candidate SAR signals azelaic acid (Jung *et al.* 2009), dehydroabietinal (Chaturvedi *et al.* 2012), and glycerol-3-phosphate (Chanda *et al.* 2011). In addition, SAR-regulatory MeSA accumulation and signalling is perturbed in *dir1-1* mutants during SAR induction, suggesting overlap between DIR1 function and MeSA (Liu *et al.* 2011). DIR1 protein co-fractionates with a high molecular weight, dehydroabietinal-containing complex in phloem exudates collected from SAR-induced leaves (Shah *et al.* 2014) and interacts with the SAR-related LTPs, AZELAIC ACID INDUCED1 (AZI1) and EARLY *ARABIDOPSIS* ALUMINUM INDUCED1 (EARLI1) via interaction assays in tobacco epidermal cells (Yu *et al.* 2013; Cecchini *et al.* 2015). These studies demonstrate that DIR1 interacts with other proteins during SAR and suggest that DIR1 plays a central role in the mobilization of long-distance SAR signals.

The *Arabidopsis* genome encodes the *DIR1* paralog *DIR1-like*, which is 88% similar to DIR1 at the amino acid level (Champigny *et al.* 2013; Isaacs *et al.* 2016). A polyclonal DIR1 antibody recognizes recombinant DIR1-like protein, and *dir1-1* mutant plants occasionally display DIR1 antibody signals in immunoblots of

SAR-induced phloem exudates suggesting that DIR1-like is mobilized to the phloem during SAR. Moreover, the *dir1-1* mutant sometimes displays a partially SAR-competent phenotype and transient *Agrobacterium*-mediated expression of *DIR1-like* rescues the *dir1-1* SAR-defect (Champigny *et al.* 2013). Together, these studies suggest that DIR1-like may also play a role in SAR.

The *Arabidopsis* ecotype Col-0 is more commonly used in defense studies; therefore we examined the SAR phenotypes of a *dir1-2* mutant and *DIR1-like*^{RNAi} (RNA interference) knockdown lines in Col-0. We also performed protein-protein interaction assays in yeast and *in planta* to determine whether DIR1 and DIR1-like interact with each other and/or with SAR-related proteins. Our results demonstrate that DIR1-like participates in homo- and heterodimer formation with DIR1, and that DIR1 is required for SAR in Col-0. In contrast to Ws-2, evidence is presented indicating that DIR1-like is not a major contributor to the SAR response in Col-0. In addition, we provide evidence that both LTP1 and LTP2 are putative DIR1-interacting proteins that are likely involved in long-distance SAR signaling.

5.3 RESULTS

Examination of DIR1 and DIR1-like during SAR in Arabidopsis ecotype Col-0

To better understand the role of DIR1-like during SAR, we created *DIR1-like* RNAi knockdown lines in the *Arabidopsis* Col-0 ecotype and assayed them for SAR competence. This ecotype was chosen because the majority of SAR mutant studies are performed using Col-0. A *DIR1-like* RNAi cassette was created by cloning sense and antisense *DIR1-like* sequence into the pHANNIBAL RNAi vector. This cassette was subcloned into the pMDC32 expression vector to create 35S:DIR1-like^{RNAi} and used to transform wild-type Col-0 (see methods).

Initial screening of 35S:DIR1-like^{RNAi} plants using quantitative reverse transcription PCR (qRT-PCR) identified two independent lines with consistently reduced *DIR1-like* levels compared to wild-type Col-0 plants (Figure 5.1A). Normalized relative expression of *DIR1-like* was reduced to ~50% in the 35S:DIR1-like^{RNAi}/8n line compared to Col-0, while *DIR1-like* transcripts were essentially undetectable in 35S:DIR1like^{RNAi}/5i (0.002% of wild-type). *DIR1* transcript levels in 35S:DIR1like^{RNAi}/5i and 8n were similar to wild-type Col-0 (Figure S1/5.5). To determine if reduced *DIR1-like* expression impacts SAR, the SAR phenotypes of 35S:DIR1like^{RNAi}/8n and 35S:DIR1like^{RNAi}/5i were compared to Col-0. Wild-type Col-0 plants that received an initial SAR-inducing inoculation (*Pst-DC3000/avrRpt2*) were 17-fold more resistant to a second infection with virulent *Pst-DC3000* in upper leaves compared to mock-inoculated controls, indicative of a strong SAR response. The 35S:DIR1-like^{RNAi}/8n and 35S:DIR1-like^{RNAi}/5i lines were similarly competent for SAR, with 26-fold and 30-fold less bacterial growth in distant leaves of SAR-induced plants compared to mock-inoculated controls (Figure 5.1B). Local resistance assays demonstrated that both 35S:DIR1-like^{RNAi} lines were competent in effector-triggered immunity (ETI) to avirulent *Pst-DC3000/avrRpt2* and were no more susceptible to virulent *Pst-DC3000* than wild-type plants (Figure 5.1C). Together, these data demonstrate that reduced *DIR1-like* expression has no effect on local or systemic immune responses in *Arabidopsis*.

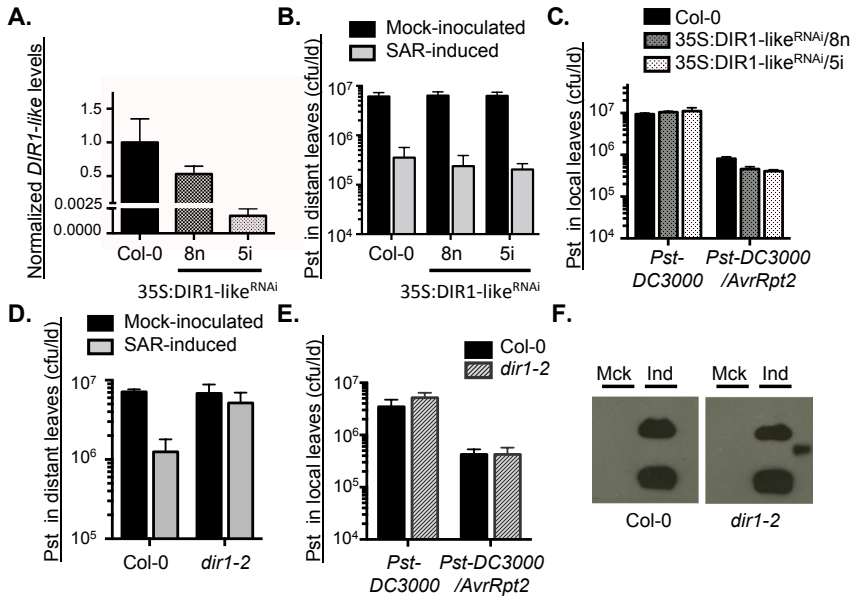

Figure 5.1 – DIR1, but not DIR1-like, is required for SAR in *Arabidopsis* Col-0. Analysis of *DIR1-like* mRNA levels in leaf tissue of 4 week-old Col-0 and 35S:DIR1-like^{RNAi} plants was performed using quantitative reverse transcription (q-RT)-PCR **(A)**. *DIR1-like* levels were normalized to *5FCL* (AT5G13050; 5-Formyltetrahydrofolate cycloligase). This was performed 3 times with similar results. Standard SAR **(B)** and local resistance **(C)** assays of 4 week-old Col-0 and *DIR1-like^{RNAi}* expressing plants. Standard SAR **(D)** and local resistance **(E)** assays of 4 week-old Col-0 and *dir1-2* plants. Local resistance assays were performed by quantifying bacterial density (colony forming units per leaf disc) 3 days post inoculation (dpi) with 10^6 cfu/ml of virulent Pst-DC3000 or avirulent Pst-DC3000/AvrRpt2. Standard SAR assays were performed by quantifying bacterial density of virulent Pst-DC3000 (3dpi) in plants that received an initial mock-inoculation (10 mM $MgCl_2$) or were induced for SAR with 10^6 cfu/ml Pst-DC3000/AvrRpt2 (SAR-induced). Values represent the mean +/- standard deviation of three sample replicates (A,B,C,D,E). DIR1-antibody signals in phloem exudates collected from leaves of Col-0 or *dir1-2* plants that were mock-inoculated (Mck) or induced for SAR (Ind) with 10^6 cfu/ml avirulent Pst-DC3000/AvrRpt2 **(F)**. Exudates were collected from 25-48 hours post inoculation. All experiments were performed at least 3 times with similar results.

The majority of SAR studies utilize mutants generated in the Col-0 ecotype, which is likely due to the greater number of genetic resources available in the Col-0 background compared to other ecotypes. To better understand the role of DIR1 during SAR, we characterized a *dir1-2* mutant allele generated in the Col-0 background by the GABI-KAT initiative (Kleinboelting *et al.* 2012). The *dir1-2* mutant (GK-403C01) harbors a T-DNA insertion at the 3' end of the *DIR1* (AT5G48485) coding region that disrupts the last 14 amino acids of the mature protein (Figure 5.6/S2A), resulting in a loss of native *DIR1* transcript expression compared to wild-type Col-0 (Figure 5.6/S2B). Importantly, this region contains 2 of the 8 cysteine residues that participate in the four disulphide bonds that are important for the generation of properly folded DIR1 protein. As such, this results in a DIR1 protein variant that lacks the C32-C69 and C44-C77 disulphide bonds that establish the bottom of the hydrophobic cavity (Figure 5.6/S2C). To determine if the *dir1-2* mutation impacts SAR, we performed standard SAR assays comparing *dir1-2* to Col-0. As expected, wild-type Col-0 plants displayed SAR, such that SAR-induced plants supported ~6-fold less virulent *Pst* in distant leaves compared to mock-inoculated controls (Figure 5.1D). In comparison, *dir1-2* mutants displayed a SAR-defective phenotype similar to that of the previously described *dir1-1* mutant in Ws-2 (Maldonado *et al.* 2002). Since the *dir1-1* mutant is occasionally SAR competent (Champigny *et al.* 2013) multiple SAR assays performed with the *dir1-2* mutant are presented in Figure 5.7/S3. These data, collected over a 4-year period, demonstrate that the *dir1-2* mutant is SAR defective in most experiments, however a partially SAR-defective response was observed. In addition, local disease resistance assays demonstrated that the *dir1-2* mutant is not affected in local immune responses to virulent and avirulent *Pst* strains (Figure 5.1E), similar to what has been observed for *dir1-1* (Maldonado *et al.* 2002).

To further characterize the *dir1-2* allele we assayed DIR1 movement into the phloem during SAR. Phloem exudates collected from SAR-induced (*Pst-DC3000/avrRpt2*) and mock-inoculated (10 mM $MgCl_2$) Col-0 and *dir1-2* were subjected to immunoblot analysis using the polyclonal DIR1 antibody. As expected, DIR1 antibody signals were not detected in exudates collected from mock-inoculated plants (Figure 5.1F). Phloem exudates collected from SAR-induced Col-0 and *dir1-2* leaves displayed DIR1-antibody signals. Since the polyclonal DIR1 antibody recognizes both DIR1 and DIR1-like protein (Champigny *et al.* 2013), we speculate that the antibody signals observed in exudates of SAR-induced *dir1-2* represent the detection of DIR1-like. However, it is possible that a mutated version of DIR1 may be produced and detected in *dir1-2*. To explore this possibility, *dir1-2* mutants were assayed for the expression of a *DIR1-T-DNA* read-through mRNA using a forward DIR1-specific primer and a T-DNA specific reverse primer. A T-DNA read-through product was detected in *dir1-2* but not wild-type Col-0 (Figure 5.6/S2B). Using the ExPASy translate program (web.expasy.org/translate/), the amino acid sequence of this product was predicted to result in a protein of 13.8 kDa. Given that DIR1 antibody signals in SAR-induced exudates of *dir1-2* appear at the DIR1/DIR1-like monomer and dimer sizes (~7 and 14 kDa), which coincidentally is similar to the predicted DIR1-2 read-through product (13.8 kDa), it is possible that the movement of a DIR1-2 read-through product and/or DIR1-like into the phloem during SAR contributes to the signals observed in exudates collected from SAR-induced *dir1-2* plants.

Subcellular localization of DIR1-like in N. benthamiana

To gain further insight into the nature of DIR1-like, subcellular localization studies of DIR1-like-GFP were performed in *N. benthamiana* using transient *Agrobacterium*-mediated expression. The localization pattern of DIR1-like-GFP

was compared to DIR1-YFP and two unrelated LTPs from the PR-14 family (LTP1-GFP and LTP2-GFP). The subcellular localization pattern of DIR1-YFP and DIR1-like-GFP were similar, with fluorescence detected at the cell periphery peri-nuclear ER, and at punctate structures reminiscent of plasmodesmata (Figure 5.2; Figure 5.8/S4). This localization pattern is consistent with previous reports for DIR1 (Champigny *et al.* 2011; Chanda *et al.* 2011). In comparison, both LTP1-GFP and LTP2-GFP were uniformly distributed at the cell periphery, with the strongest fluorescence signals detected at the cell wall/apoplast (Figure 5.2; Figure 5.8/S4). Previous immunocytochemical studies have also identified LTP1 epitopes in the cell wall of *Arabidopsis* explants during somatic embryogenesis (Potocka *et al.* 2012). Together, the data demonstrate that DIR1-like and DIR1 are similarly distributed in plant cells.

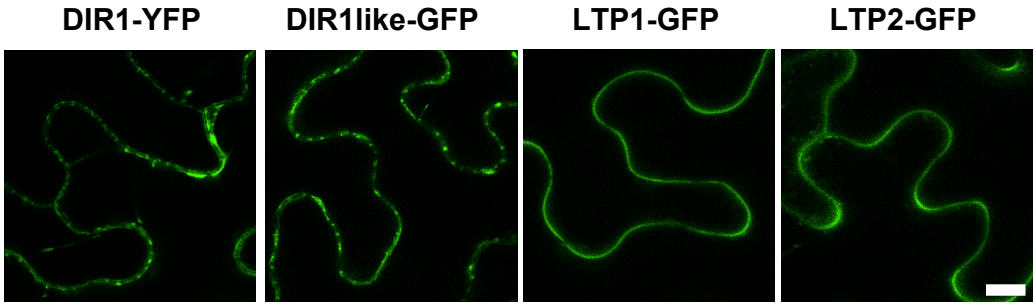

Figure 5.2 - Subcellular localization of DIR1, DIR1-like, LTP1, and LTP2. DIR1-YFP, DIR1-like-GFP, LTP1-GFP, and LTP2-GFP fusions proteins were expressed in expanded leaves of *Nicotiana benthamiana* via *Agrobacterium*-mediated transient expression. Fluorescent fusion proteins were visualized in epidermal cells 2 days after inoculation with *Agrobacteria* using confocal microscopy. Scale bar = 10 µm. Fluorescence is false colored green. This experiment was performed twice with similar results

Identification of DIR1 and DIR1-like Interacting Proteins

Previous studies demonstrated that DIR1 interacts with itself and the SAR-related LTPs AZI1 and EARLI1 in *N. benthamiana*-based protein-protein interaction

assays (Yu *et al.* 2013; Cecchini *et al.* 2015). Moreover, DIR1 co-fractionates with a high molecular weight complex in phloem exudates collected from SAR-induced wild-type plants (Shah *et al.* 2014). Together, these data suggest that DIR1 interacts with other proteins during SAR, perhaps during the long-distance movement of a signal complex. To identify additional DIR1-interacting proteins, we performed targeted protein-protein interaction assays in yeast (*Saccharomyces cerevisiae*) using the mating-based split ubiquitin protein-protein interaction system (Obrdlik *et al.* 2004). DIR1 and DIR1-like bait strains containing mature DIR1/DIR1-like (lacking signal peptides) fused to the C-terminal half of ubiquitin (Cub) and a synthetic transcriptional activator were mated with various prey strains expressing SAR-related proteins fused to a mutated version of the N-terminal half of ubiquitin (NubG). In this system, protein-protein interaction brings the two halves of ubiquitin together, resulting in the cleavage of the synthetic transcriptional activator, which travels to the nucleus to activate auxotrophic reporter genes that allow for growth on minimal media lacking histidine. Key to the effectiveness of this system, the NubG mutant protein prevents the spontaneous recombination of the two halves of ubiquitin, reducing the number of false positive interactions (Obrdlik *et al.* 2004).

Evidence demonstrates that DIR1 is transported to distant leaves (Champigny *et al.* 2013; Carella *et al.* 2015), however very little is known in regard to its function in these tissues. Since NPR1 is a central component of SAR establishment in distant leaves and DIR1 has been associated with MeSA signalling (Cao *et al.* 1997; Liu *et al.* 2011), we performed targeted interaction assays to determine if DIR1, DIR1-like, or the unrelated KAT1 (potassium ion channel) baits interact with NPR1 or the SAR-related SA methyl esterase enzymes MES1 (METHYL ESTERASE1), MES7, or MES9. In addition, interactions with SAR-related (DIR1, DIR1-like, AZI1) and randomly selected LTPs (LTP1 and LTP2) were also tested. Baits were also screened against prey expressing KAT1 as a positive control for

KAT1-KAT1 interaction. All three baits interacted successfully with the Nub^{WT} positive control and failed to interact with mutated Nub^{G}, indicating that the bait strains do not auto-activate (false positive) and are suitable for targeted interaction assays (Figure 5.3A). Moreover, KAT1-Cub, but not DIR1/DIR1-like-Cub, interacted with KAT1-Nub^{G}, demonstrating the specificity of the system. DIR1 bait failed to interact with MES1/7/9 or NPR1 prey (Figure 5.9/S5). Interestingly, DIR1 interacted with itself (DIR1), DIR1-like, AZI1, LTP1, and LTP2 prey, while DIR1-like bait interacted with DIR1, DIR1-like, and LTP2 prey (Figure 5.3A). Minimal growth on interaction specific media was observed for the DIR1-like/LTP1 interaction compared to DIR1/LTP1 suggesting a weaker interaction between DIR1-like and LTP1.

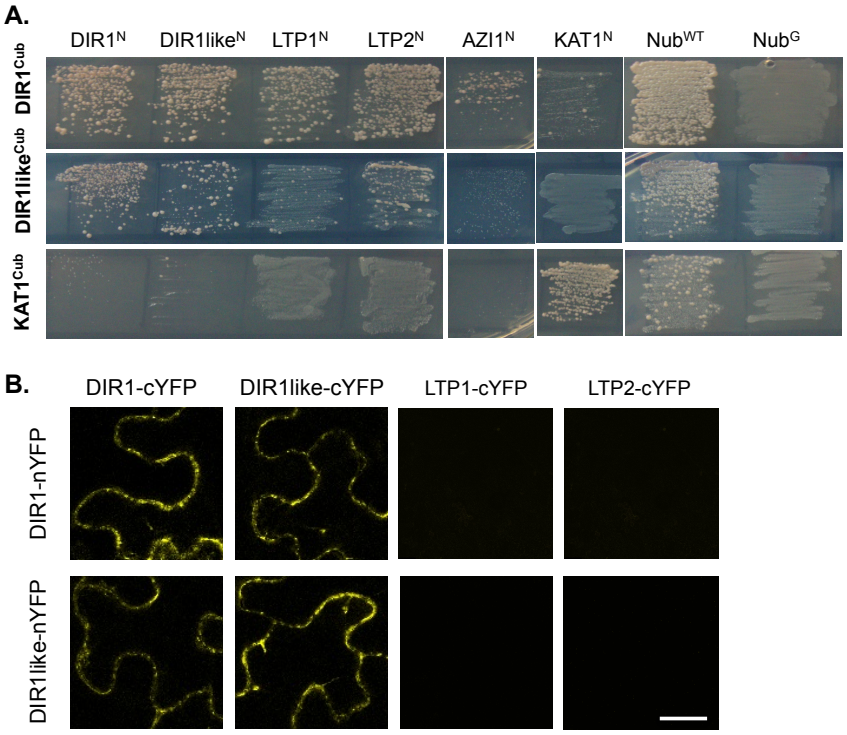

Figure 5.3 - Yeast- and plant-based interaction assays identify DIR1- and DIR1-like-interacting proteins. Split-ubiquitin interaction assays in *Saccharomyces cerevisiae* **(A)**. Growth on interaction specific, minimal media indicates interactions of prey (N terminal half of ubiquitin = Nub) with DIR1, DIR1-like, or KAT1 bait fusions (Cub = C terminal portion of ubiquitin). Baits were screened against self-activating Nub^{WT} and interaction specific Nub^{G} as controls. Prey constructs encode proteins of interest fused to Nub^{G} and are indicated by the superscript ^{N}. This experiment was performed three times with similar results. Bimolecular fluorescence complementation (BiFC) interaction assays in *Nicotiana benthamiana* **(B)**. Fusion proteins containing the N-terminal or C-terminal halves of Yellow Fluorescent Protein (nYFP, cYFP) were co-expressed in 4-5 week-old *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression. Fluorescence complementation was monitored in epidermal cells two days after inoculation with *Agrobacteria* using confocal microscopy. Scale bar = 20 µm. Each interaction was performed at least 2 times with similar results.

To confirm the DIR1 and DIR1-like interactions identified in yeast, bimolecular fluorescence complementation (BiFC) experiments were performed in *N. benthamiana*. Co-infiltration of *N. benthamiana* leaves with *Agrobacterium* strains expressing proteins fused to the N- or C-terminal halves of YFP were monitored for fluorescence as an indicator of protein-protein interactions that reconstitute the YFP protein (Citovsky *et al.* 2004). Using this assay, the DIR1/DIR1, DIR1/DIR1-like, and the DIR1-like/DIR1-like interactions were confirmed *in planta*, as co-infiltration of *N. benthamiana* leaves with these combinations of the corresponding nYFP/cYFP-fusion constructs resulted in fluorescence complementation (Figure 5.3B). Interactions between DIR1 or DIR1-like with LTP1 or LTP2 were not detected in this assay. These results demonstrate that DIR1 and DIR1-like participate in homo- and heterodimer formation with one another, in both yeast and *N. benthamiana*, while DIR1/LTP interactions were only observed in yeast. A number of factors may prevent the detection of DIR1/LTP interactions in this assay, such as different subcellular localizations *in planta* as well as cell-type and/or context specificity of the interaction. In support of this idea, DIR1 and DIR1-like displayed a different subcellular localization pattern in *N. benthamiana* compared to LTP1 and LTP2 (Figure 5.2).

Is LTP2 Required for SAR?

The interaction of DIR1 with LTP1 and LTP2 suggests that these LTPs are involved in DIR1-mediated SAR. To test this hypothesis, we isolated the *ltp2-1* knockout mutant and created a 35S:LTP2-FLAG/*ltp2-1* overexpression line (Figure 5.10/S6) for use in SAR assays. The importance of LTP1 in SAR could not be determined because *ltp1* mutant lines are not available. A strong SAR response was observed in wild-type Col-0, where SAR-induced plants supported 23-fold less virulent *Pst* in distant leaves compared to mock-inoculated controls.

In contrast, high levels of *Pst* were observed in mock- and SAR-induced *ltp2-1* plants, indicative of a SAR-defective phenotype (Figure 5.4A). No differences in the growth of virulent or avirulent *Pst* between the *ltp2-1* mutant and wild-type Col-0 were observed (Figure 5.4B), demonstrating that the *ltp2-1* mutant exhibits wild-type local resistance responses to *Pst.* Importantly, 35S:LTP2-FLAG overexpression rescued the *ltp2-1* SAR-defect, as two independent 35S:LTP2-FLAG/*ltp2-1* lines (10M and 31C) exhibited a SAR competent phenotype (Figure 5.4C) Taken together, these data demonstrate that LTP2 is required for systemic immune responses in *Arabidopsis*.

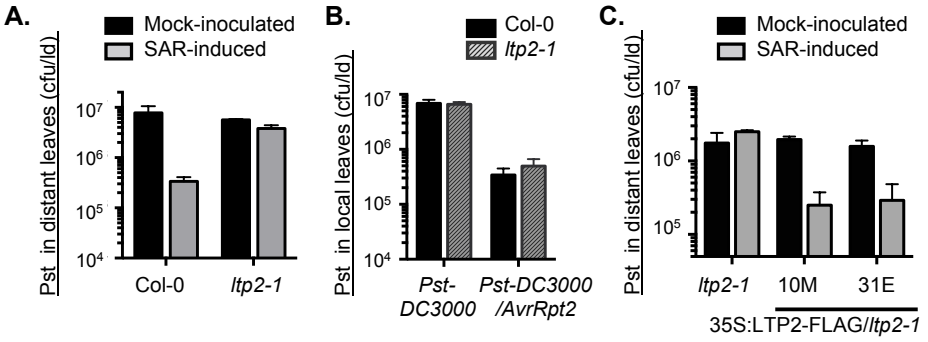

Figure 5.4 - LTP2 is required for SAR in *Arabidopsis*. Standard SAR assays of wild-type Col-0 and *ltp2-1* **(A)** or 35S:LTP2-FLAG/*ltp2-1* plants **(C)**. Plants were induced for SAR by inoculation with 10^6 cfu per ml avirulent Pst-DC3000/AvrRpt2 (SAR-induced) or were mock-inoculated with 10 mM $MgCl^2$ (mock-inoculated). Two days later, upper leaves were challenged by inoculation with 10^6 cfu per ml virulent Pst-DC3000. Bacterial densities in upper, challenged leaves were determined three days post inoculation (dpi). Local resistance assays of 4 week-old Col-0 and *ltp2-1* plants **(B)**. Bacterial densities were determined three dpi with avirulent (Pst-DC3000/AvrRpt2) or virulent Pst-DC3000. SAR assays were performed at least 3 times with similar results (A,C). Local resistance assays were performed twice with similar results (B). All values represent the mean +/- standard deviation of three sample replicates.

5.4 DISCUSSION

The accumulated evidence indicates that unlike DIR1, DIR1-like is not essential for SAR in *Arabidopsis* (Champigny *et al.* 2013, Isaacs *et al.* 2016). The reason for this disparity may be due to an amino acid substitution within the hydrophobic cavity of DIR1-like (Champigny *et al.* 2013; Isaacs *et al.* 2016). A nonpolar phenylalanine residue is present at position 40 across all DIR1 orthologs (mature protein lacking signal peptide), while a polar tyrosine residue is present at this position only in DIR1-like orthologs (Isaacs *et al.* 2016). This amino acid substitution is hypothesized to destabilize interactions with hydrophobic ligands allowing DIR1-like to contribute occasionally to SAR in the *dir1-1* mutant (Champigny *et al.* 2013). Analysis of the *dir1-2* mutant in Col-0 yielded similar results, however *dir1-2* appears to be SAR-defective more often than *dir1-1* in Ws-2. Given that the SAR phenotype of *dir1-1* has been tested for over a decade (in multiple labs) and *dir1-2* has been tested for only 4 years, we cannot definitively claim that *dir1-2* is SAR-defective more often than *dir1-1*. Moreover, DIR1 antibody signals were observed in immunoblots of phloem exudates collected from SAR-induced *dir1-2* leaves, similar to studies using *dir1-1*. Since the DIR1 antibody recognizes both DIR1 and DIR1-like to a similar degree (Champigny *et al.* 2013), we speculate that these signals are caused by the detection of DIR1-like, but we cannot rule out the possibility of a DIR1-2 read-through product. The generation of antibodies specific to DIR1 and DIR1-like are needed to accurately identify and compare each protein's ability to move into the phloem during SAR.

Defense and SAR research is often conducted using mutants generated in the Col-0 background, while our work with the *dir1-1* mutant was conducted using Ws-2. Important differences between these ecotypes exist which led us to investigate the SAR phenotype of *dir1-2* in Col-0. While both ecotypes are SAR competent, accession-specific differences in immune mechanisms have been

observed. For instance, Ws-2 plants lack a functional *FLS2* allele and cannot induce SAR upon flg22 treatment (Mishina and Zeier 2007). The induction of *PR1* expression is less responsive during SA treatment in Ws-2 and these plants are more susceptible to *Pst* infection than Col-0 (Ahmad *et al.* 2011). Basal transcription levels of priming-associated genes are also lower in Ws-2 compared to Col-0. Moreover, steady state levels of abscisic acid (ABA), a hormone that can suppress defense responses including SAR (Yasuda *et al.* 2008) are higher in Ws-2 compared to Col-0 (North *et al.* 2007). Together, these observations suggest that the Col-0 ecotype is better equipped for the SAR response than Ws-2. How DIR1 and DIR1-like are affected by these differences is not yet clear. However, our data suggest that DIR1-like accumulates in the phloem during SAR in both ecotypes and yet DIR1-like does not significantly contribute to SAR in Ws-2 (Champigny *et al.* 2013) or Col-0 (this work).

The creation of a *dir1/dir1-like* double mutant is needed to understand why *dir1* mutants are occasionally SAR competent. Previous attempts to generate a *dir1-1/dir1-like* double mutant using antisense technology failed to identify viable *DIR1-like* knockdown lines (Champigny *et al.* 2013). Further attempts to generate a *dir1/dir1-like* double mutant by crossing 35S:DIR1-like^{RNAi}/5i with *dir1-2* were similarly unsuccessful (data not shown). This may suggest that a *dir1/dir1-like* mutant is lethal in *Arabidopsis*, although more detailed genetic analyses are required. In any case, these observations suggest that DIR1 and DIR1-like play a role in development. Future efforts to create inducible *DIR1-like* RNAi lines in the *dir1-2* and *dir1-1* backgrounds will contribute to determining the roles of DIR1 and DIR1-like during development and SAR.

DIR1 and DIR1-like participate in hetero- and homo-dimers with one another in yeast and *in planta* (this study). Additionally we confirmed that DIR1 interacts with AZI1 in yeast (Yu *et al.* 2013; Cecchini *et al.* 2015), while DIR1-like does not. This

finding provides support for the hypothesis that DIR1-like does not significantly contribute to SAR. Unexpectedly, the targeted interaction assays in yeast demonstrated that DIR1 interacts with the randomly selected LTP1 and LTP2 proteins. However, these interactions were not observed using BiFC analysis in *N. benthamiana*. Subcellular localization studies using GFP fusions in *N. benthamiana* determined that DIR1 and DIR1-like display a different localization pattern compared to LTP1 and LTP2. Interactions in yeast were likely identified because the signal peptide-lacking proteins co-localize in the cytosol. However, in BiFC experiments performed in healthy plants, these proteins would display pre-SAR localization patterns that may prevent interaction with DIR1. Therefore, we speculate that interactions between DIR1 and LTP1/2 are context specific, such that these interactions only occur during SAR. Co-immunoprecipitation studies using the LTP2-FLAG/ltp2-1 line may determine whether DIR1 and LTP2 interact before or after the induction of SAR *in planta.*

To explore the role of DIR1 in distant leaves during SAR, we performed targeted interaction assays with known regulators of SAR establishment. Interactions between DIR1 and NPR1 were not observed in yeast, suggesting that DIR1 does not physically associate with NPR1 to induce SAR in distant leaves. Recent evidence demonstrated physical interactions between the SnRK2.8 kinase and NPR1 in distant leaves during SAR, which leads to the phosphorylation and subsequent activation of NPR1 that is required for SAR establishment (Lee *et al.* 2015). A role for DIR1 upstream of this step may therefore be possible, or perhaps DIR1 acts to establish SAR by other means. DIR1 also failed to interact with SA methyl esterase enzymes (MES1/7/9) required for the generation of SA from MeSA in distant leaves. This possibility was explored since *dir1-1* mutants have increased MeSA accumulation during infection with *P. syringae* (Liu *et al.* 2011), suggesting a potential regulatory role for DIR1 in MeSA signalling. Our

data suggest that such a role does not involve direct interactions with MES1/7/9 enzymes.

The putative DIR1/LTP2 interaction suggests that LTP2 is involved in the SAR response. In support of this idea, the *ltp2-1* T-DNA insertion mutant was specifically defective in systemic and not local immune responses. Both LTP1 and LTP2 are members of the PR-14 family of LTPs, which are hypothesized to act as pathogen-inducible antimicrobial proteins based on sequence similarity to barley LTPs with demonstrated antimicrobial activity against *Clavibacter michiganensis* (Molina *et al.* 1993; Garcia-Olmedo *et al.* 1995; Sels *et al.* 2008). However, a recent study demonstrated that LTP3 and LTP4 of the PR-14 family are negative regulators of plant immunity such that overexpression of *LTP3* enhances susceptibility to *Pst* and *ltp3/ltp4* double mutants are more resistant to *Pst* compared to wild-type plants (Gao *et al.* 2015). Moreover, Gao *et al.* (2015) demonstrated that of the 15 PR-14 family members, only *LTP3, LTP4, LTP6, LTP9, LTP10,* and *LTP12* are upregulated in response to *Pst* within the first 24 hours of infection. *LTP2* transcripts were not upregulated in response to *Pst* (Gao *et al.* 2015), which is consistent with our observations that LTP2 is not required for local immune responses. Whether LTP1 is involved in local and/or systemic immune responses in *Arabidopsis* remains to be determined. Future SAR studies involving *LTP1* knockdown and overexpression lines are needed to clarify the role of LTP1 in SAR.

Several studies demonstrate an important role for LTPs and other lipid-binding proteins in the SAR response. Currently, the lipid-binding proteins DIR1, DIR1-like, AZI1, EARLI1, ACBP3/4/8 (Acyl-CoA Binding Proteins), DHyPRP1 (Double Hybrid Proline-rich Protein1) and MLP (Major Latex Protein-like Protein) have all been implicated in SAR (Maldonado *et al.* 2002; Jung *et al.* 2009; Xia *et al.* 2012; Champigny *et al.* 2013; Li *et al.* 2014; Cecchini *et al.* 2015; Carella *et al.* 2016). In

this study, we identified a role for LTP2 and provide evidence that LTP1 may also play a role in SAR. The importance of lipid binding proteins for SAR is not surprising given that plastid-derived lipids, membrane lipids, and lipid precursors (G3P) are all important for SAR (Nandi *et al.* 2004; Chaturvedi *et al.* 2008; Chanda *et al.* 2011; Gao *et al.* 2014). Given this association between lipids and SAR, it is therefore likely that a number of lipid binding proteins are required for the generation, translocation, and perception of lipidic signals during SAR. Where LTP1 and LTP2 fit into lipid-based SAR signalling is not yet clear, however interactions with DIR1 may suggest a role in long-distance signalling. The idea that lipid-binding proteins are essential for the long-distance transport of lipids through the phloem is emerging as an important component of abiotic and biotic stress signalling (Benning *et al.* 2012; Barbaglia *et al.* 2016). Further characterizing the roles of LTP1 and LTP2 in SAR will clarify whether these proteins are involved in long-distance signalling or other stages of the SAR response.

In this study, we demonstrated that DIR1 is required for SAR in the Col-0 accession of *Arabidopsis*. Moreover, we generated *DIR1-like* RNAi lines and determined that DIR1-like does not significantly contribute to SAR in Col-0 plants that carry functional DIR1. Further characterization of DIR1 and DIR1-like determined similar subcellular localization patterns in healthy tobacco epidermal cells as well as homo- and heterodimer formation with one another in yeast and *in planta.* Targeted protein-protein interaction assays also determined that DIR1 interacts with LTP1 and LTP2, and that DIR1-like interacts with LTP2. Although DIR1 failed to interact with LTP1 or LTP2 *in planta*, phenotypic analysis of an *ltp2-1* mutant identified a role for LTP2 in SAR. This suggests that DIR1-LTP2 may interact during SAR in *Arabidopsis*. Together, the data demonstrate once again that DIR1 and other LTPs are an important component of the SAR response.

5.5 ACKNOWLEDGMENTS

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5.7 SUPPORTING MATERIAL

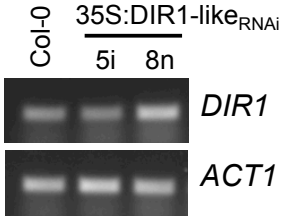

Figure 5.5/S1 – *DIR1* expression in 35S:DIR1-like$_{RNAi}$ lines. RT-PCR analysis of *DIR1* and *ACT1* expression in 4 week-old Col-0 and 35S:DIR1-like$_{RNAi}$ lines. This experiment was performed 3 times with similar results.

A.

MASKKAAMVMMAMIVIMAMLVDTSVAIDLCGMSQDELNECKPAVSKENPTSP
SQPCCTALQHADFACLCGYKNSPWLGSFGVDPELAS**ALPKQCGLANAPTC**

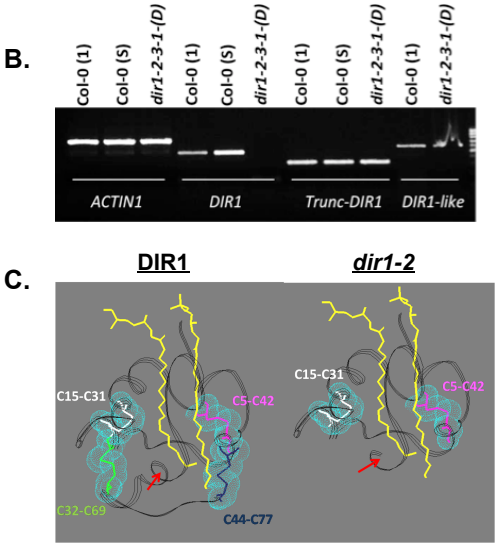

Figure 5.6/S2 – Characterization of the *dir1-2* mutant. (A) Location of the T-DNA insertion in the last 14 amino acids (bolded and underlined) of *dir1-2* mutant. The affected cysteine residues are displayed in red. **(B)** Expression of *DIR1*, a DIR1-2-read-through product *(Trunc DIR1), DIR1-like*, and the *ACTIN1* housekeeping control in 4 week-old Col-0 and the *dir1-2* mutant. **(C)** Homology model depicting the site of disruption in DIR1-2. Red arrow indicates the site of T-DNA insertion that disrupts cysteine bond pairing (C32-C69 and C44-C77) in DIR1-2.

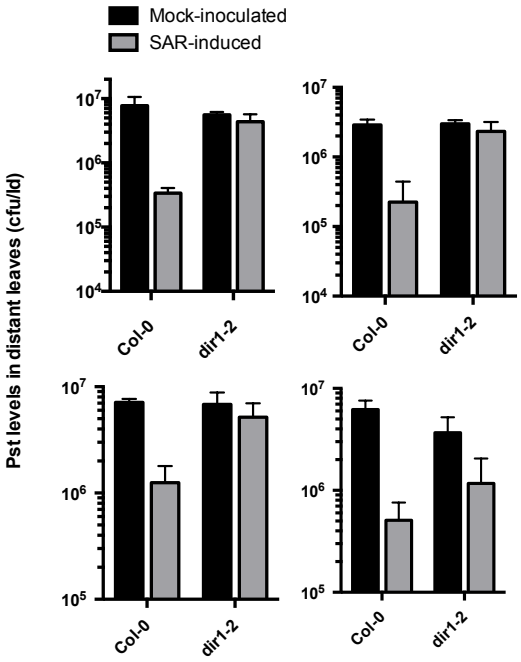

Figure 5.7/S3 – Experimental replicates of *dir1-2* SAR assays. Replicates of tandard SAR assays performed on 3.5-4 week-old Col-0 and *dir1-2* plants. Plants were either Mock-inoculated (10 mM $MgCl_2$) or SAR-induced with PstDC3000/avrRPt2. Two days later, these plants were challenged for SAR by inoculation with virulent PstDC3000. 3 days post SAR challenge, *in planta* bacterial densities were quantified (colony forming units [cfu] per leaf disc [ld]). Results are presented as the mean +/- standard deviation of three sample replicates.

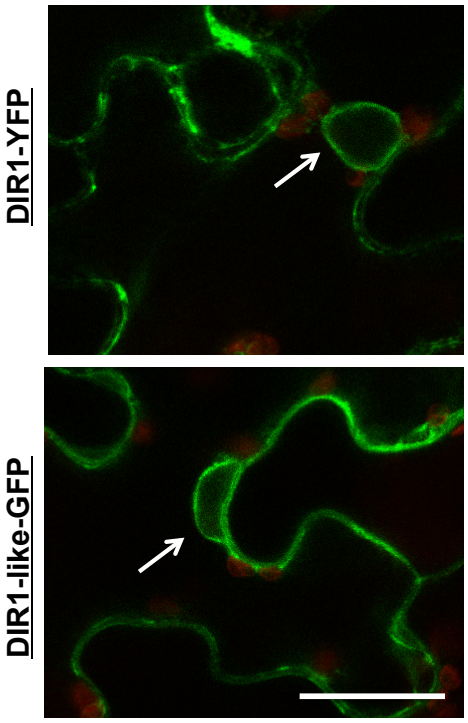

Figure 5.8/S4 – Expanded subcellular localization of DIR1 and DIR1-like. DIR1-YFP and DIR1-like-GFP fusions proteins were expressed in expanded leaves of *Nicotiana benthamiana* via *Agrobacterium*-mediated transient expression. Fluorescent fusion proteins were visualized in epidermal cells 2 days after inoculation with *Agrobacteria* using confocal microscopy. Scale bar = 25 µm. Fluorescence of YFP and GFP is false colored green and overlayed with chlorophyll autofluorescence (red). White arrows indicate localization at peri-nuclear ER. This experiment was performed twice with similar results.

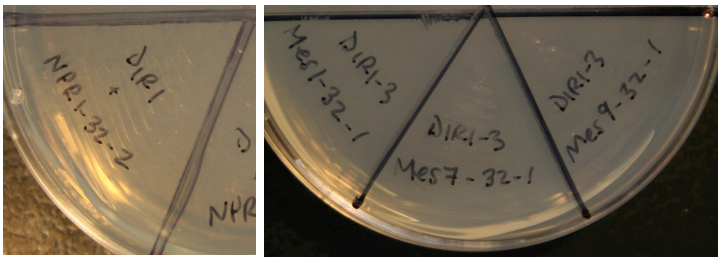

Figure 5.9/S5 – DIR1 does not interact with NPR1 or MES1/7/9 in yeast. Split ubiquitin interaction assays demonstrate that DIR1-Cub baits do not interact with NPR1-NubG, MES1-NubG, MES7-NubG, or MES9-NubG. This experiment was performed twice with similar results.

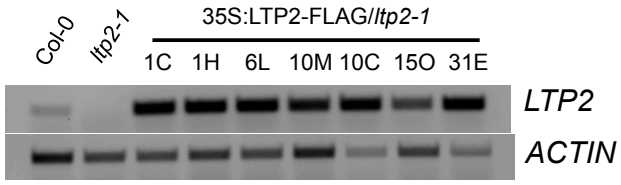

Figure 5.10/S6 – Characterization of *ltp2-1* and 35S:LTP2-FLAG/*ltp2-1.* RT-PCR analysis of *LTP2* and *ACTIN1* expression in 4 week-old Col-0, *ltp2, and* several independent *35S:LTP2-FLAG/ltp2-1* lines. This experiment was performed twice with similar results.

Chapter 6

Comparative Proteomics Analysis of Phloem Exudates Collected During the Induction of Systemic Acquired Resistance

PREFACE:

This chapter consists of the published article entitled "***Comparative Proteomics Analysis of Phloem Exudates Collected During the Induction of Systemic Acquired Resistance***" by Carella P, Merl-Pham J, Wilson DC, Dey S, Hauck SM, Vlot AC, and Cameron RK (*Plant Physiology, 172, 1495-1510. 2016,* doi: 10.1104/pp.16.00269). This publication has been reproduced in its original format with permission from The American Society of Plant Biologists, who allows authors to reproduce their own articles in full for non-commercial purposes. This reproduction requires a full citation in addition to the information below.

Citation: Carella P, Merl-Pham J, Wilson DC, Dey S, Hauck SM, Vlot AC, Cameron RK. 2016. Comparative proteomics analysis of phloem exudates collected during the induction of systemic acquired resistance. *Plant Physiology* **172:** 1495-1510.

Journal URL: www.plantphysiol.org

This work describes comparative proteomics analysis of phloem exudates collected from wild-type *Arabidopsis* leaves during the induction of SAR. This analysis identified several proteins whose abundance increased or decreased in the phloem during SAR, leading to the identification of novel components of the SAR response. In this manuscript, a role for m-type thioredoxins, a major latex

protein-like protein, and the UVR8-COP1-HY5 light-signalling pathway is described.

Contributions:

Philip Carella, Robin Cameron, and Corina Vlot designed the research. Philip Carella and Sanjukta Dey coordinated the research in the Cameron and Vlot labs, respectively. Philip Carella, Dan Wilson, and Robin Cameron collected phloem exudates for proteomics analysis, with Philip Carella being responsible for sample preparation (concentration, protein quantification, etc). Juliane Merl-Pham and Stephanie Hauck were responsible for label-free proteomics analysis of phloem exudates. Juliane Merl-Pham performed statistical analyses on proteomes to identify differentially abundant proteins. Philip Carella analyzed the proteomes for similarity to published phloem sap proteomes and analyzed statistical tests performed by Juliane Merl-Pham to generate lists of SAR-enriched and SAR-suppressed proteins. Philip Carella and Dan Wilson performed SAR assays on T-DNA insertion and transgenic overexpression lines. Philip Carella generated figures 1-7 and all supplementary materials, with the exception of the raw proteomics data. Philip Carella and Robin Cameron wrote the manuscript, with significant contributions by Dan Wilson, Sanjukta Dey, and Corina Vlot.

Comparative Proteomics Analysis of Phloem Exudates Collected during the Induction of Systemic Acquired Resistance[1][OPEN]

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Systemic acquired resistance (SAR) is a plant defense response that provides long-lasting, broad-spectrum pathogen resistance to uninfected systemic leaves following an initial localized infection. In Arabidopsis (*Arabidopsis thaliana*), local infection with virulent or avirulent strains of *Pseudomonas syringae* pv *tomato* generates long-distance SAR signals that travel from locally infected to distant leaves through the phloem to establish SAR. In this study, a proteomics approach was used to identify proteins that accumulate in phloem exudates in response to the induction of SAR. To accomplish this, phloem exudates collected from mock-inoculated or SAR-induced leaves of wild-type Columbia-0 plants were subjected to label-free quantitative liquid chromatography-tandem mass spectrometry proteomics. Comparing mock- and SAR-induced phloem exudate proteomes, 16 proteins were enriched in phloem exudates collected from SAR-induced plants, while 46 proteins were suppressed. SAR-related proteins THIOREDOXIN h3, ACYL-COENZYME A-BINDING PROTEIN6, and PATHOGENESIS-RELATED1 were enriched in phloem exudates of SAR-induced plants, demonstrating the strength of this approach and suggesting a role for these proteins in the phloem during SAR. To identify novel components of SAR, transfer DNA mutants of differentially abundant phloem proteins were assayed for SAR competence. This analysis identified a number of new proteins (m-type thioredoxins, major latex protein-like protein, ULTRAVIOLET-B RESISTANCE8 photoreceptor) that contribute to the SAR response. The Arabidopsis SAR phloem proteome is a valuable resource for understanding SAR long-distance signaling and the dynamic nature of the phloem during plant-pathogen interactions.

Plants responding to their environment must communicate over short and long distances to optimize growth and development. At short distances, growth- and stress-related signals move cell to cell through plasmodesmata (symplastically) or diffuse through the apoplast for communication with neighboring cells. At greater distances, macromolecules must access the plant vasculature for long-distance movement from one organ to another. A large body of evidence demonstrates the importance of the xylem and phloem as conduits for the long-distance movement of a diverse set of signals/macromolecules, such as micronutrients/macronutrients, small molecules, phytohormones, lipids, peptides/proteins, and coding/noncoding RNA (for review, see Lucas et al., 2013). These molecules are involved in a number of interorgan signaling responses, ranging from processes governing growth and development to stress-related responses to abiotic and biotic stimuli. Not surprisingly, some pathogens have coopted the plant vasculature to better exploit their hosts. Classic examples of this strategy include the systemic movement of plant viruses through the phloem (Hipper et al., 2013), vasculature-infecting microbes (Yadeta and Thomma, 2013), and phloem-feeding herbivores (Kaloshian and Walling, 2005; Howe and Jander, 2008). In response, plants have developed sophisticated interorgan resistance responses to limit the spread of infecting pathogens as well as to prevent and/or limit the effectiveness of future infection(s). Such responses include virus-induced RNA interference (Yoo et al.,

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R.K.C., A.C.V., and P.C. designed the research; P.C. and S.D. coordinated the research; P.C., D.C.W., and R.K.C. performed experiments; J.M.-P. and S.M.H. performed quantitative proteomics; J.M.-P. and P.C. performed analysis of proteomes; A.C.V., S.M.H., and R.K.C. contributed research materials and equipment; P.C. and R.K.C. wrote the article with significant contributions by D.C.W., A.C.V., and S.D.

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2004), induced systemic resistance caused by beneficial microbes (Pieterse et al., 2014), and systemic acquired resistance (SAR; Champigny and Cameron, 2009).

SAR is classically described as a plant defense response that provides long-lasting, broad-spectrum pathogen resistance to uninfected systemic leaves following an initial localized infection. In Arabidopsis (*Arabidopsis thaliana*), SAR is induced after a localized infection with compatible or incompatible strains of the hemibiotrophic bacterial phytopathogen *Pseudomonas syringae* (Cameron et al., 1994). During the compatible interaction with virulent *P. syringae*, Arabidopsis pattern recognition receptors recognize conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) to induce PAMP-triggered immunity. However, virulence effector proteins secreted into plant cells by *P. syringae* suppress this response and promote susceptibility in locally infected tissue (for review, see Xin and He, 2013). Incompatible or avirulent *P. syringae* strains carry effector proteins that are recognized in plant cells by cognate resistance receptors to induce a robust local defense response termed effector-triggered immunity, which is usually associated with programmed cell death in the form of the hypersensitive response (for review, see Cui et al., 2015). Classic SAR studies suggested that a necrotizing infection was important for SAR induction (for review, see Sticher et al., 1997); however, recent studies demonstrate that the induction of PAMP-triggered immunity is sufficient to induce SAR in Arabidopsis (Mishina and Zeier, 2007). Nevertheless, local infection with virulent or avirulent *P. syringae* strains leads to the generation of mobile SAR signals that travel from locally infected to distant leaves to initiate SAR.

SAR studies in non-Arabidopsis model systems first suggested that SAR signals move via the phloem. Early grafting experiments in cucumber (*Cucumis sativus*) determined that SAR signals traveled from induced rootstocks to distant scions to induce SAR (Jenns and Kuc, 1979). A specific role for the phloem in the long-distance transport of SAR signals was identified in cucumber, where restricting vascular connections of induced leaf petioles using a wool/hot-water girdling technique prevented the manifestation of SAR in distant leaves (Guedes et al., 1980). Experiments performed in tobacco (*Nicotiana tabacum*) demonstrated that the removal of stem sheath also resulted in a loss of systemic immunity (Tuzun and Kuc, 1985), further supporting a role for the plant vasculature in long-distance immune signaling. In Arabidopsis, the transport of SAR signals from locally infected to distant leaves also occurs via the phloem, as demonstrated by overlapping translocation patterns for radiolabeled photosynthate and SAR signals (Kiefer and Slusarenko, 2003). Interestingly, the results did not preclude additional mechanisms of transport, as SAR signal movement was not strictly limited to the orthostichy (vascular bundle) of the induced leaf, suggesting that SAR signals move cell to cell from one orthostichy to another to better disseminate the signal. This idea was supported recently by the observation that plant lines with reduced cell-to-cell movement through plasmodesmata are defective in SAR and the long-distance movement of DEFECTIVE IN INDUCED RESISTANCE1 (DIR1; Carella et al., 2015). Taken together, these studies demonstrate that long-distance SAR signaling is dependent on the phloem for efficient interorgan communication.

The identification of long-distance SAR signals remains an active area of research, as they may represent novel bioprotective agents suitable for use in agriculture (Conrath et al., 2015). Both genetic and analytical biochemical screens have been performed to isolate genes and metabolites important for SAR. A common approach for identifying SAR-activating small molecules is to perform biochemical screens with phloem exudates collected from SAR-induced Arabidopsis leaves. Activity-guided analytical screening of SAR-induced phloem exudates was used to identify the SAR activators azelaic acid and dehydroabietinal (Jung et al., 2009; Chaturvedi et al., 2012) and to analyze amino acid levels during SAR, leading to the identification of pipecolic acid (Návarová et al., 2012). Together, these studies demonstrate that phloem exudates are a rich source of SAR-activating small molecules that may work in concert to induce SAR in distant tissues.

In comparison, our knowledge of protein composition within the phloem during SAR is extremely limited. The lipid transfer protein (LTP) DIR1 is currently the only protein demonstrated to move from SAR-induced to distant tissues via the phloem (Champigny et al., 2013). Recent studies demonstrate that DIR1 interacts with other SAR-related LTPs in untreated tobacco leaves (Yu et al., 2013; Cecchini et al., 2015) and is associated with a dehydroabietinal-containing, trypsin-sensitive, high-molecular-weight fraction of phloem exudates collected from SAR-induced leaves (Shah et al., 2014). This suggests that DIR1 is a member of a large proteinaceous complex that travels to distant leaves in the phloem during SAR. Additionally, total protein levels are typically higher in phloem exudates collected from SAR-induced versus mock-inoculated leaves (Champigny et al., 2013; Carella et al., 2015), supporting the notion that numerous proteins are loaded into the phloem during SAR.

In this study, a proteomics approach was taken to identify proteins that accumulate in phloem exudates during the induction of SAR and, therefore, could be involved in the long-distance signaling stage of SAR. Label-free quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics was used to identify and quantify proteins present in phloem exudates collected from leaves that were mock inoculated or induced for SAR with virulent or avirulent *Pseudomonas syringae* pv *tomato* (Pst). By comparing mock- and SAR-induced exudate proteomes, 16 proteins accumulated and 46 proteins decreased in abundance in phloem exudates during SAR. The functional relevance of these proteins to SAR was explored by performing SAR assays on the corresponding transfer

DNA (T-DNA) mutants. This analysis identified a role in SAR for m-type thioredoxins, a putative major latex protein, and the UV-B photoreceptor ULTRAVIOLET-B RESISTANCE8 (UVR8). Further investigation of the UVR8 UV-B signaling pathway revealed a role for the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1) and the bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) in the development of SAR. The Arabidopsis SAR phloem proteome provides new insights into the dynamic nature of the phloem during biotic stress and reveals that a number of previously unknown proteins accumulate in the phloem during SAR.

RESULTS

Quantitative Proteomics of Phloem Exudates during SAR

To identify proteins that accumulate in the phloem in response to the induction of SAR, we subjected phloem exudates collected from mock- and SAR-induced Arabidopsis leaves to quantitative label-free LC-MS/MS. Phloem exudates were collected from 24 to 48 h post inoculation (hpi) because the SAR-mobile DIR1 protein accumulates to high levels during this interval (Champigny et al., 2013). Phloem exudates were collected from leaves that were mock inoculated (10 mM $MgCl_2$) or induced for SAR by inoculation with *Pst* strains that are virulent (*Pst* DC3000) or avirulent (*Pst* DC3000/avrRpt2) on Columbia-0 (Col-0) plants. SAR assays performed alongside exudate collection experiments confirmed that SAR was induced by both strains (Supplemental Fig. S1A). This was further supported by observing DIR1 antibody signals in immunoblots of phloem exudates collected from SAR-induced, but not mock-inoculated, leaves (data not shown). To obtain protein levels suitable for LC-MS/MS, exudates from more than 90 plants per treatment were collected and concentrated using centrifugal concentrators (3-kD cutoff) followed by lyophilization. Similar to previous reports (Champigny et al., 2013; Carella et al., 2015), phloem exudates collected from SAR-induced leaves contained higher total protein levels than exudates collected from mock-induced leaves (Supplemental Fig S1B). Concentrated phloem exudates from two independent experimental replicates were subjected to quantitative LC-MS/MS (Supplemental Data S1). Venn diagrams in Figure 1A show the number of proteins that were significantly enriched or suppressed in SAR-induced exudates relative to mock-inoculated controls. Not surprisingly, the exudate proteomes of leaves treated with virulent or avirulent *Pst* were not identical, as several proteins displayed strain-specific differences in abundance (Supplemental Tables S1 and S2). Since infection with either strain induces SAR to the same extent in Col-0 (Supplemental Fig. S1A), we reasoned that key proteins involved in SAR should accumulate to a similar degree after either treatment. Therefore, we compiled a list of proteins that were differentially

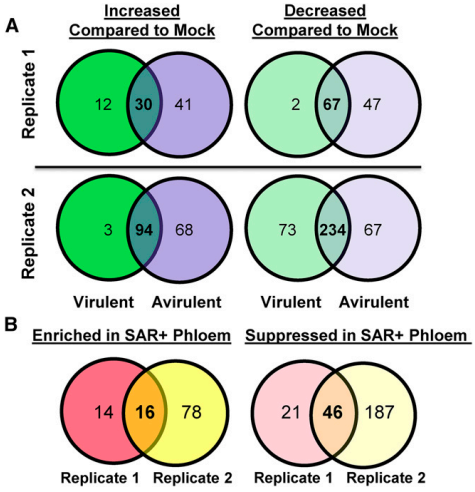

Figure 1. Comparative proteomics analysis of phloem exudates collected during the induction of SAR. Quantitative proteomics data of phloem exudates were collected from mock-inoculated (10 mM $MgCl_2$) and SAR-induced (virulent, *Pst* DC3000; and avirulent, *Pst* DC3000/avrRpt2) leaves of two experimental replicates. Values inside Venn diagrams represent the number of unique proteins (at least two peptides) that were differentially abundant (Student's *t* test, $P < 0.05$) between treatments. A, Proteins with increased or decreased abundance in phloem exudates of SAR-induced (virulent or avirulent) leaves compared with mock-inoculated controls in each experimental replicate. B, Proteins that are similarly enriched or suppressed in phloem exudates collected from SAR-induced (virulent and avirulent) compared with mock-inoculated leaves. Venn diagrams generated in Venny 2.0 (Oliveros, 2015; http://bioinfogp.cnb.csic.es/tools/venny/index.html) were remade using Microsoft Office Powerpoint.

abundant in phloem exudates collected from leaves induced for SAR by both *Pst* strains relative to mock-inoculated phloem exudates (Fig. 1B). A total of 16 proteins were enriched in phloem exudates collected from SAR-induced (virulent and avirulent *Pst*) leaves compared with mock-inoculated controls (Table I). In contrast, 46 proteins displayed decreased abundance in exudates collected from SAR-induced versus mock-inoculated leaves (Table II; Supplemental Table S3).

Comparison with Published Phloem Exudate Proteomes

To assess the quality of our proteomes, we compared our data set (all proteins, regardless of treatment) with previously published phloem exudate proteomes. Comparisons were performed with two studies that used LC-MS/MS-based proteomics to identify proteins in phloem exudates collected from untreated Arabidopsis leaves (Batailler et al., 2012; Guelette et al., 2012). A total of 27 common phloem proteins were identified

Table I. *Proteins enriched in the phloem during SAR*

Locus	Gene Symbol	Description	Relative Abundance (Virulent/Mock)		Relative Abundance (Avirulent/Mock)		Peptides Used for Quantitation	
			Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
AT3G52960	PrxIIE	Peroxiredoxin	8.7	8.6	12.6	19.70	8	10
AT1G03680	TRXm1	Thioredoxin	7.5	2	174.3	9.5	6	8
AT1G06680	PsbP1	PSII subunit	2.9	5.3	10.7	15.1	6	6
AT5G42980	TRXh3	Thioredoxin	4.2	4.3	3.5	7.2	5	4
AT4G03520	TRXm2	Thioredoxin	16.5	3.7	79.7	14.9	4	5
AT2G43570	CHI/AED15	Chitinase	3.9	1.5	2.8	1.7	4	3
AT2G44920	–	Tetratricopeptide-like	13	13.9	18.6	41.4	3	4
AT1G20340	PETE2	Plastocyanin	2.9	15.6	16.7	23.9	3	8
AT5G40370	GRXC2	Glutaredoxin	10.9	11.1	8.4	17.6	3	4
AT3G50820	PsbO2	PSII subunit	13.7	6.8	37.4	57.3	3	3
AT2G14610	PR1	Pathogenesis-related	4.8	3.8	12.2	14.6	3	2
AT4G34050	CCoAOMT1	*S*-Adenosyl-L-Met methyltransferase	4.3	2.4	3.3	1.8	2	2
AT2G19760	PFN1	Profilin	5.6	1.5	21.6	1.8	2	4
AT4G02450	–	HEAT SHOCK PROTEIN20 (HSP20)-like	7.5	4.7	7.2	8.2	2	3
AT2G29450	GSTU5	Glutathione *S*-transferase	3.3	1.8	6.2	3.5	2	2
AT1G55260	LTPG6	Lipid transfer protein	4.3	4.8	9.2	9	2	2
AT1G31812	ACBP6	Acyl-CoA-binding protein	119.4	4.4	111.1	7.9	1[a]	4
AT3G15360[b]	TRXm4	Thioredoxin	5.5	0.6	9.3	1.9	4	5
AT4G23670[b]	MLP	Major latex protein-like	5.7	1.1[c]	30.2	6	2	4

[a]Only one peptide was available for quantitation. [b]Peptides with significant enrichment in SAR plus phloem in one of two replicates. [c]Not statistically significant.

in all three proteomes (Fig. 2A; Supplemental Table S4). Our combined phloem proteome (replicates 1 and 2) overlapped with 49% of the proteins identified by Batailler et al. (2012) and 63% of those described by Guelette et al. (2012). By comparison, the Batailler et al. (2012) data set overlapped with 47% of proteins identified by Guelette et al. (2012). Furthermore, we compared our proteome with phloem proteomes obtained from pumpkin (*Cucurbita maxima*; Lin et al., 2009) and Texas bluebonnet (*Lupinus texensis*; Lattanzio et al., 2013; Fig. 2B). Only 12 proteins were present in the proteomes of all three species (Supplemental Table S5). Our Arabidopsis phloem proteome overlapped with 10% of proteins identified in pumpkin exudates and 31% of proteins identified in Texas bluebonnet exudates. In comparison, the Batailler et al. (2012) proteome

Table II. *Selected proteins suppressed in the phloem during SAR*

Locus	Gene Symbol	Description	Relative Abundance (Virulent/Mock)		Relative Abundance (Avirulent/Mock)		Peptides Used for Quantitation	
			Replicate 1	Replicate 2	Replicate 1	Replicate 2	Replicate 1	Replicate 2
AT3G05900	–	Neurofilament protein-related	0.07	0.03	0.03	0.03	10	15
AT5G66190	FNR1	Ferrodoxin oxidoreductase	0.37	0.14	0.20	0.21	4	17
AT2G04030	HSP90.5	Chaperone protein	0.51	0.13	0.26	0.18	6	6
AT5G26000	TGG1	Thioglucoside glucohydrolase	0.29	0.40	0.28	0.75	6	10
AT3G16470	JR1	Man-binding lectin	0.20	0.26	0.04	0.20	5	8
AT1G55490	CPN60B	Chaperonin	0.24	0.15	0.14	0.19	5	7
AT3G16400	NSP1	Nitrile specifier protein	0.16	0.11	0.03	0.08	4	5
AT1G09210	CRT1b	Calreticulin	0.09	0.08	0.03	0.08	4	2
AT1G56340	CRT1a	Calreticulin	0.15	0.09	0.04	0.09	4	4
AT5G54770	THI1	Thiazole biosynthetic enzyme	0.08	0.09	0.02	0.03	3	2
AT5G28540	BiP1	HSP70	0.38	0.35	0.36	0.47	3	7
AT2G28000	CPN60A	Chaperonin	0.20	0.29	0.12	0.27	3	4
AT1G72150	PATL1	Patellin	0.06	0.09	0.06	0.10	2	3
AT1G76180	ERD14	Dehydrin	0.06	0.05	0.03	0.04	2	6
AT1G35720	ANNAT1	Annexin	0.17	0.08	0.06	0.06	2	6
AT4G22670	HIP1	HSP70-interacting	0.03	0.01	0.00	0.04	2	2
AT2G21660	GRP7	Gly-rich protein	0.19	0.10	0.06	0.06	2	5
AT5G63860	UVR8	UVB photoreceptor	0.27	0.05	0.18	0.06	2	1[a]

[a]Only one peptide was used for quantitation.

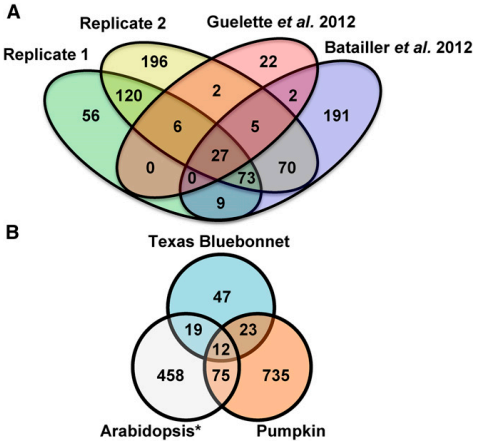

Figure 2. Comparing phloem exudate proteomes. A, Venn diagram comparing all proteins identified in replicates 1 and 2 of this study with the Arabidopsis phloem exudate proteomes described by Guelette et al. (2012) and Batailler et al. (2012). B, Comparison of all Arabidopsis proteins identified in this study (Arabidopsis*) with phloem exudate proteomes of pumpkin (Lin et al., 2009) and Texas bluebonnet (Lattanzio et al., 2013). Venn diagrams obtained from Venny 2.0 (Oliveros, 2015; http://bioinfogp.cnb.csic.es/tools/venny/index.html) were remade in Microsoft Office Powerpoint.

overlapped with 8% of pumpkin and 22% of Texas bluebonnet phloem proteins. This demonstrates that although there is variation in the protein profiles of phloem exudates within and between species, the phloem proteome generated in this study shares similarity with previously published phloem proteomes.

GO Slim Analysis of SAR-Enriched Versus SAR-Suppressed Phloem Proteins

To gain insight into the nature of SAR-enriched and SAR-suppressed phloem proteins, comparative GO Slim analysis was performed (Supplemental Fig. S2). GO Slim terms with a difference of 5% or greater between SAR-enriched and SAR-suppressed phloem proteins were included in Figure 3. SAR-enriched phloem proteins were associated with the Gene Ontology (GO) terms response to stress, response to biotic stimulus, cell death, and response to external stimulus; however, the metabolic process, anatomical morphology, and photosynthesis terms also were more frequent in SAR-enriched compared with SAR-suppressed phloem proteins. In contrast, SAR-suppressed phloem proteins were associated with the GO terms response to abiotic stress, transport, catabolic process, carbohydrate metabolic process, and metabolite precursor and energy (Fig. 3A). In comparing cellular compartment GO terms, it was evident that SAR-enriched phloem proteins were frequently associated with terms representing extracellular (cell wall, external encapsulating structure, and extracellular) and thylakoid localization, while SAR-suppressed phloem proteins were associated with intracellular terms (ribosome, endoplasmic reticulum, vacuole, nucleus, plastid, cytosol, and intracellular; Fig. 3B). The molecular function GO terms catalytic activity, nucleotide binding, RNA binding, transferase activity, and enzyme regulator activity were more frequent in SAR-enriched phloem proteins, whereas binding, protein binding, transporter, carbohydrate binding, and hydrolase were more frequent in suppressed phloem proteins. Although qualitative, the GO Slim analysis demonstrates that the induction of SAR leads to the accumulation and suppression of two distinct sets of proteins.

SAR Phloem Proteome Validation

Among the 16 SAR-enriched phloem proteins, two known regulators of SAR were present. The cytosolic THIOREDOXIN h3 (TRXh3) and ACYL-COENZYME A-BINDING PROTEIN6 (ACBP6) were significantly enriched in phloem exudates collected from SAR-induced compared with mock-inoculated leaves (Table I). TRXh3 regulates the oligomeric status of the master SAR signaling protein NPR1 along with TRXh5 to control the induction of SAR (Tada et al., 2008). Single mutants *trxh3* and *trxh5* are modestly impacted in SAR; however, loss of the NADPH-DEPENDENT THIOREDOXIN REDUCTASE A protein that regulates their activity results in a full loss of SAR, suggesting that TRXs are important components of the SAR response (Tada et al., 2008). ACBPs including ACBP6 also have been implicated in SAR, such that *acbp6* mutants are defective in the generation and/or translocation of SAR signals (Xia et al., 2012). Unexpectedly, DIR1 was not identified in our proteomes despite being readily observed via immunoblot analysis (Champigny et al., 2013). This may be explained by the demonstrated resistance of LTPs to proteolytic degradation (Lindorff-Larsen and Winther, 2001; Scheurer et al., 2004), preventing DIR1 detection during quantitative proteomics analysis of phloem exudates. In support of this idea, recombinant DIR1 protein was not detected using LC-MS/MS. Lastly, the accumulation of the SAR molecular marker PATHOGENESIS-RELATED1 (PR1) was detected in SAR-induced phloem exudates, which together with finding TRXh3 and ACBP6 indicates that the phloem proteomes from pathogen-inoculated leaves represent SAR-activated phloem sap.

To further assess the validity of our SAR proteome, immunoblot experiments were performed to confirm PR1 protein accumulation in phloem exudates during SAR. PR1 was selected because it is an important SAR molecular marker and a reliable antibody was available (Wang et al., 2005). Phloem exudates from mock-inoculated (10 mM $MgCl_2$) and SAR-induced (*Pst* DC3000/avrRpt2) Col-0 leaves were collected from 25

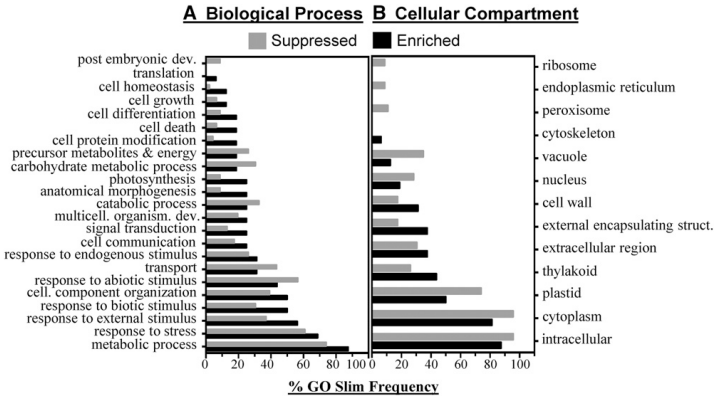

Figure 3. GO Slim analysis of proteins enriched or suppressed in SAR-induced phloem exudates. GO Slim terms are given pertaining to biological process (A) and cellular compartment (B) of SAR-enriched (Enriched; $n = 16$) compared with SAR-suppressed (Suppressed; $n = 46$) proteins. Only GO Slim terms with a difference in frequency of at least 5% between the enriched and suppressed groups are shown. The full GO analysis can be found in Supplemental Fig. S2.

to 48 hpi, concentrated, and probed with a polyclonal PR1 antibody. As a positive control, exudates also were probed for DIR1, a protein with demonstrated phloem accumulation during SAR (Champigny et al., 2013). As an additional control, total protein extracts from mock- and *Pst* DC3000/avrRpt2-inoculated leaf tissue (48 hpi) were assayed for PR1 and DIR1 accumulation. As expected, DIR1 antibody signals (7 and 14 kD) were detected in phloem exudates collected from SAR-induced but not mock-inoculated leaves and were undetectable in leaf extracts (Fig. 4). In comparison, PR1 was detected in total protein extracts of *Pst* DC3000/avrRpt2- but not mock-inoculated leaves. Importantly, PR1 was detected in phloem exudates collected from SAR-induced but not mock-inoculated leaves, confirming that PR1 protein accumulates in the phloem during SAR. This observation further validates the proteomics data set and identifies PR1 as a marker for SAR-activated phloem sap.

Functional Characterization of SAR-Enriched Phloem Proteins

SAR assays were performed on a number of T-DNA insertion mutants corresponding to SAR-enriched phloem exudate proteins to determine if they contribute to SAR. TRXh3 and ACBP6 mutants were not tested because these proteins have been shown to be required for SAR (Tada et al., 2008; Xia et al., 2012). Three members of the TRXm family (TRXm1, TRXm2, and TRXm4) were identified in the proteomics analysis. Both TRXm1 and TRXm2 were enriched in exudates from SAR-induced leaves, while TRXm4 was enriched in exudates collected from leaves induced with avirulent *Pst*. To determine if this protein family is important for SAR, we compared the SAR phenotypes of the *trxm1*, *trxm2*, and *trxm4* mutants with that of wild-type Col-0. Distant leaves of SAR-induced Col-0 plants supported 29-fold less bacterial growth than mock-inoculated controls, indicative of a strong SAR response. In comparison, both *trxm1* and *trxm4* displayed partial defects in the SAR response compared with wild-type Col-0, such that *trxm1* and *trxm4* plants were 2.5- and 3.5-fold more resistant to *Pst* in distant leaves of induced versus mock-inoculated plants (Fig. 5A). The SAR phenotype of the *trxm2* mutant ranged from partially SAR defective to fully competent in three independent experiments (Supplemental Fig. S3). The partial SAR-defective phenotypes of *trxm1* and *trxm4* and the variable phenotype of *trxm2* may be due to genetic redundancy in the TRXm family. This idea is supported by the observation that TRXm1, TRXm2, and TRXm4 all share high amino acid sequence similarity (greater than 74%) to one another (Supplemental Table S6). To ensure that the partial SAR defects observed in the *trxm1* and *trxm4* mutants were not caused by a defect in local immune responses, we performed

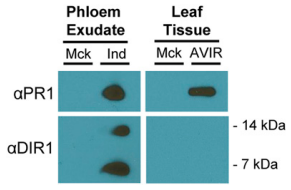

Figure 4. PR1 accumulates in phloem exudates of SAR-induced leaves. Immunoblots are from phloem exudates and leaf tissue collected from 4-week-old Col-0 plants that were mock inoculated (Mck; 10 mM $MgCl_2$) or induced (Ind) for SAR (10^6 colony-forming units [cfu] mL^{-1} *Pst* DC3000/avrRpt2). Phloem exudates were collected from 24 to 48 hpi, and leaf tissue was harvested at 48 hpi. Immunoblotting was performed using PR1 (1:3,000) and DIR1 (1:10,000) antibodies. Similar results were obtained in three independent experiments. AVIR, Avirulent.

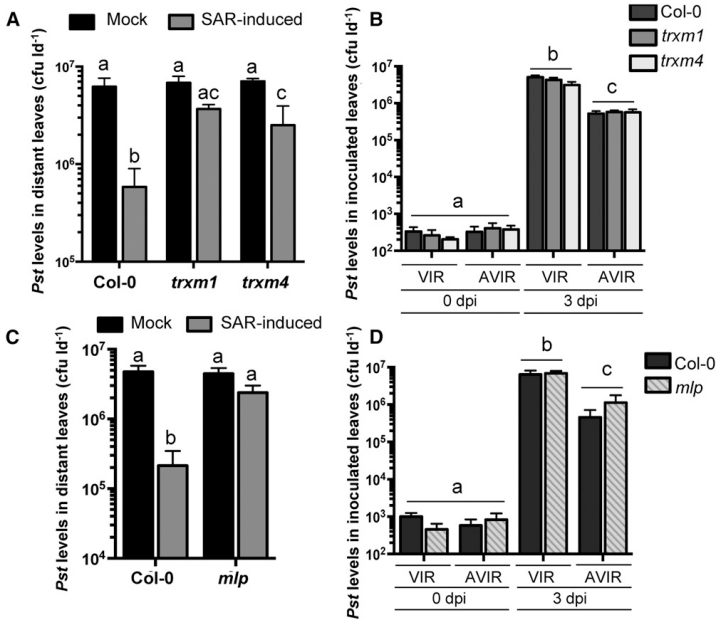

Figure 5. The SAR-enriched phloem proteins TRXm1, TRXm4, and MLP are involved in SAR. A and D, Standard SAR assays comparing wild-type Col-0 with *trxm1* and *trxm4* (A) or *mlp* (D). Leaves of 4-week-old plants were mock inoculated (10 mM $MgCl_2$) or induced for SAR by pressure infiltration with 10^6 cfu mL^{-1} *Pst* DC3000/avrRpt2. Two days later, distant leaves were challenged with 10^6 cfu mL^{-1} *Pst* DC3000, and *Pst* levels in these leaves were quantified 3 d post inoculation (dpi). Experiments were repeated at least three times with similar results. B and C, Local resistance assays comparing wild-type Col-0 with *trxm1* and *trxm2* (B) or *mlp* (C). Local resistance to virulent (VIR; *Pst* DC3000) and avirulent (AVIR; *Pst* DC3000/avrRpt2) strains of *Pst* was assessed by inoculating leaves of 4-week-old plants with 10^6 cfu mL^{-1} of either strain. Bacterial densities were determined at 0 and 3 dpi. All values represent means ± SD of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey's honestly significant difference [HSD], $P < 0.05$).

disease resistance assays to assess local responses to virulent and avirulent *Pst*. In planta bacterial levels of virulent and avirulent *Pst* in *trxm1* and *trxm4* were similar to those in wild-type Col-0 at both 0 and 3 dpi (Fig. 5B), demonstrating that *trxm1* and *trxm4* are not impaired in local immune responses to *Pst*.

Several lipid transfer/binding proteins contribute to the SAR response (Jung et al., 2009; Xia et al., 2012; Champigny et al., 2013; Cecchini et al., 2015). Two lipid-binding proteins were identified in our SAR phloem proteome. Glycosylphosphatidylinositol-anchored LIPID TRANSFER PROTEIN6 (LTPG6) accumulated in phloem exudates collected from leaves induced with virulent and avirulent *Pst*, and a putative lipid-binding major latex protein (MLP; AT4G23670) accumulated in phloem exudates collected from *Pst* DC3000/avrRpt2-induced leaves (Table I; Supplemental Table S1). The SAR phenotypes of *ltpg6* and *mlp* mutants were compared with that of wild-type Col-0 to determine if these lipid-binding proteins are involved in SAR. In two independent experiments, the *ltpg6-2* mutant displayed a strong SAR response similar to that of Col-0, indicating that LTPG6 is not required for SAR (Supplemental Fig. S3). In contrast, an *mlp* T-DNA mutant (Supplemental Fig. S4) displayed a 2-fold reduction in *Pst* levels in distant leaves of SAR-induced compared with mock-inoculated plants, whereas a 22-fold reduction was observed in Col-0 (Fig. 5C), providing evidence that MLP is involved in SAR. Local resistance assays demonstrated that the *mlp* mutant supports similar levels of virulent and avirulent *Pst* compared with Col-0 (Fig. 5D), ruling out the possibility that a defect in local resistance is responsible for the SAR-defective phenotype of the *mlp* mutant. The data support a role for MLP in long-distance SAR signaling.

Expression levels of *TRXm1* to *TRXm4* and *MLP* were monitored in wild-type Col-0 plants during local infection with virulent *Pst* to determine if increases in gene expression explain why these proteins accumulated in phloem exudates during SAR. *ACTIN1* (*ACT1*) and *PR1* were monitored as controls for equal loading

and defense activation, respectively. No appreciable changes in gene expression were observed for any of the *TRXm* family members (*TRXm1–TRXm4*), *MLP*, or *ACT1* after *Pst* inoculation. In contrast, the defense marker *PR1* was highly induced at 24 and 48 hpi (Supplemental Fig. S5). These data indicate that the *TRXm1* to *TRXm4* and *MLP* genes are not induced during the induction of SAR, suggesting that the increase in protein abundance in phloem exudates may be due to mobilization into the phloem during SAR.

Functional Characterization of SAR-Suppressed Phloem Proteins

A potential function of proteins whose abundance is reduced in the phloem during SAR may be to act as negative regulators of SAR. To explore this possibility, SAR assays were conducted with mutant and overexpression lines of genes corresponding to two SAR-suppressed phloem exudate proteins. Of the 46 proteins with decreased abundance in SAR phloem exudates, we obtained and tested plant lines with altered expression levels of ANNEXIN1 and UVR8. The ANNEXIN1 overexpression line (35S:AnnAt1) and mutant (*annat1-1*) were fully SAR competent (Supplemental Fig. S3). In contrast, the 35S:UVR8 overexpression line and the *uvr8-6* mutant were defective for SAR compared with wild-type Col-0 (Fig. 6). However, the severity of the defect varied between experiments, such that partial (Fig. 6A) or full (Fig. 6B) defects in the SAR response of *uvr8-6* and 35S:UVR8 were observed in three separate experiments. It is possible that environmental conditions, such as variable UV-B radiation, may have impacted the involvement of UVR8 in SAR; however, UV-B radiation was undetectable in our growth chambers. Local resistance responses to virulent and avirulent *Pst* were unaffected in 35S:UVR8 and *uvr8-6* (Fig. 6C), indicating that these lines are specifically impaired in SAR. These data suggest that UVR8 may function as both a positive and negative regulator of SAR.

To determine if reduced UVR8 protein in phloem exudates of SAR-induced plants is associated with a decrease in *UVR8* mRNA, we monitored *UVR8* gene expression during local infection with virulent *Pst*. The *COP1* and *HY5* genes also were monitored to determine if the UV-B signaling module is perturbed during infection. In Arabidopsis, COP1 and HY5 are important positive regulators of the UVR8 signaling module (for review, see Tilbrook et al., 2013). *ACT1* and *PR1* were monitored as loading and defense-activation controls, respectively. As expected, *PR1* levels were high at 24 and 48 hpi. Subtle changes in gene expression were observed for *UVR8*, *COP1*, and *HY5* (Fig. 7A). Since subtle changes

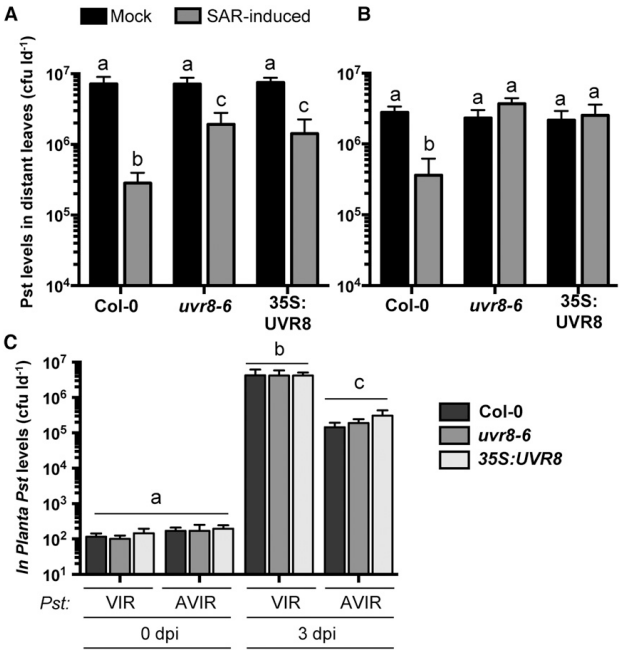

Figure 6. The UV-B photoreceptor UVR8 is required for SAR. A and B, Standard SAR assays of 4-week-old Col-0, *uvr8-6*, and 35S:UVR8 plants. Leaves were mock inoculated (10 mM $MgCl_2$) or induced for SAR by pressure infiltration with 10^6 cfu mL^{-1} *Pst* DC3000/avrRpt2. Two days later, distant leaves were challenged with 10^6 cfu mL^{-1} *Pst* DC3000, and *Pst* levels in these leaves were quantified 3 dpi. This experiment was performed six times, with similar results observed three times each. C, Local resistance assays of Col-0, *uvr8-6*, and 35S:UVR8 to virulent (VIR; *Pst* DC3000) and avirulent (AVIR; *Pst* DC3000/avrRpt2) strains of *Pst*. Leaves of 4-week-old plants were inoculated with 10^6 cfu mL^{-1} of either strain, and in planta bacterial density was calculated at 0 and 3 dpi. This experiment was performed three times with similar results. All values represent means ± SD of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey's HSD, $P < 0.05$).

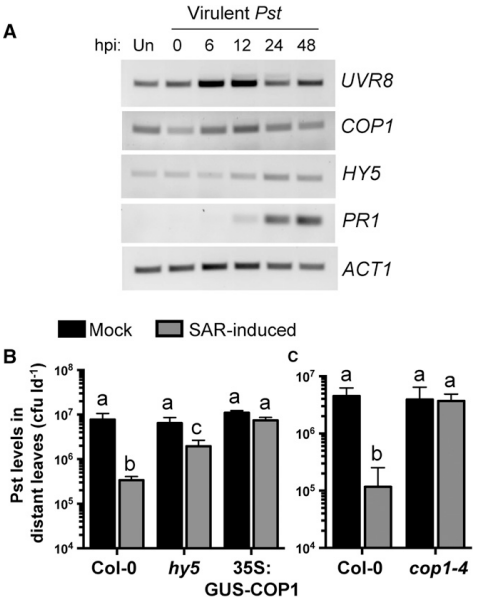

Figure 7. The UV-B signaling components COP1 and HY5 are required for the manifestation of SAR. A, Reverse transcription (RT)-PCR of complementary DNA generated from leaves of 4-week-old Col-0 plants that were untreated (Un) or inoculated with 10^6 cfu mL^{-1} *Pst* DC3000 at the indicated time points (hpi). *UVR8*, *HY5*, and *COP1* expression was compared with that of the *ACT1* and *PR1* controls. This experiment was performed three times with similar results. B and C, Standard SAR assays comparing wild-type Col-0 with *hy5* and 35S:GUS-COP1 (B) or *cop1-4* (C). Leaves were mock inoculated (10 mM $MgCl_2$) or induced for SAR by pressure infiltration with 10^6 cfu mL^{-1} *Pst* DC3000/avrRpt2. Two days later, distant leaves were challenged with 10^6 cfu mL^{-1} *Pst* DC3000, and *Pst* levels in these leaves were quantified 3 dpi. Values represent means ± SD of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey's HSD, $P < 0.05$). These experiments were performed three times with similar results.

in gene expression cannot be quantified using RT-PCR, we queried publicly available gene expression databases (Genevestigator, the Arabidopsis Gene Expression Browser, and the Arabidopsis eFP Browser) for *UVR8*, *COP1*, and *HY5* expression during local interactions with *P. syringae* (Winter et al., 2007; Hruz et al., 2008; Zhang et al., 2010). Several studies identified enhanced *HY5* expression (4.5-fold maximally) in pathogen-treated compared with mock-treated or untreated controls (Supplemental Figs. S6–S8). Levels of *UVR8* and *COP1* decreased less than 2-fold during pathogen infection or did not change at all. Altogether, the data suggest that reduced levels of UVR8 in SAR-induced phloem exudates are not associated with reduced *UVR8* mRNA levels and that the expression of *HY5* is enhanced during local inoculation with virulent *Pst*.

Given that reduced or elevated levels of UVR8 impair SAR, we hypothesized that altered levels of COP1 and HY5 also may impact systemic immunity. Alternatively, UVR8 function during SAR may be independent of COP1 or HY5. To test these hypotheses, SAR assays were performed with a COP1 mutant and overexpression line as well as a T-DNA insertion mutant of HY5. Wild-type Col-0 displayed a 23-fold decrease in distant leaf *Pst* levels in SAR-induced compared with mock-inoculated plants, while *hy5* displayed a partial defect in SAR (3.3-fold decrease) and 35S:GUS-COP1 was fully defective in SAR (Fig. 7B). The *cop1-4* mutant was similarly defective in SAR, as *Pst* levels were similar in both mock- and SAR-induced plants (Fig. 7C). These data demonstrate that HY5 and COP1 are required for SAR.

DISCUSSION

Phloem Proteomics

Proteomic analysis provides a snapshot of the proteins present in a particular tissue at a given stage of development under a particular set of environmental conditions. The phloem proteome described in this study shared 50% to 63% similarity with two previously published Arabidopsis phloem exudate proteomes. Plants used in this study were short-day grown and young (4 weeks post germination) compared with the older, long-day-grown plants used by Batailler et al. (2012) and Guelette et al. (2012). Despite these differences, 27 common phloem proteins were identified. These included known phloem proteins as well as plastid-targeted proteins that are normally associated with photosynthetic, nonphloem cell types. While this may be indicative of unavoidable contamination caused by cellular leakage from nonphloem cells during exudation, companion cells and sieve elements do contain plastids that could harbor these proteins (Froelich et al., 2011; Cayla et al., 2015). In support of this idea, live imaging of Arabidopsis phloem recently determined that Rubisco-containing plastids occupy a large volume of the companion cell cytoplasm (Cayla et al., 2015). Alternatively, nucleus-encoded proteins with predicted plastid-localization peptides may localize to nonplastid subcellular sites in the phloem. Comparisons with exudate proteomes derived from different plant species yielded fewer similarities, which suggests that protein composition within the phloem is specialized. This also may be due to differences in exudate collection techniques and/or fundamental differences in phloem architecture. This is especially important in comparisons with the cucurbit family, where phloem exudates collected directly from the cut ends of petioles are largely composed of apoplastic fluid mixed with the contents of a specialized extrafascicular phloem system that is not present in other plants (Zhang et al., 2012).

Several groups recently conducted complex comparative proteomics studies of phloem exudates collected during stress. These include comparative phloem proteomes derived from poplar (*Populus* spp.) and pumpkin upon wounding stress (Dafoe et al., 2009; Gaupels et al., 2012), rice (*Oryza sativa*) exposed to plant-hopper insects (Du et al., 2015), salt-stressed cucumber (Fan et al., 2015), melon (*Cucumis melo*) responding to viral infection (Serra-Soriano et al., 2015), and iron-limited *Brassica napus* (Gutierrez-Carbonell et al., 2015). A common theme among these proteomes, including this study, is the accumulation of redox-related proteins during stress. The presence of a sieve element antioxidant system is well described and is hypothesized to be important for phloem protein regeneration/protection, as enucleate sieve elements cannot easily replace damaged proteins (Walz et al., 2002). Therefore, the accumulation and maintenance of redox-associated proteins is likely essential to maintain phloem function during stress.

The SAR-Induced Phloem Proteome

Inducible, systemic responses such as SAR often rely on the phloem as an avenue for efficient interorgan communication. A number of studies have focused on the identification of SAR-activating small molecules that accumulate in the phloem during SAR (for review, see Dempsey and Klessig, 2012), yet little attention has been given to proteins. This gap in knowledge was addressed by performing comparative proteomics studies to determine the protein profiles of phloem exudates collected from mock-inoculated and SAR-induced plants. To identify SAR-specific phloem proteins, plants were induced for SAR using both virulent and avirulent *Pst*. These strains induce SAR to the same extent in Arabidopsis Col-0 (Mishina and Zeier; 2007; this study), allowing us to differentiate SAR phloem proteins from those specifically associated with susceptible or resistant interactions. Label-free quantitative LC-MS/MS proteomics of two experimental replicates identified a total of 564 phloem proteins, from which we identified 16 proteins that accumulate and 46 proteins that decrease in abundance in the phloem during SAR induced by both virulent and avirulent *Pst*. Comparative GO analyses revealed that SAR-enriched proteins were associated with stress-related extracellular terms, while SAR-suppressed proteins were associated with metabolism-related intracellular terms. This result is not surprising, as previous studies demonstrated that pathogen infection modifies host metabolism (Ward et al., 2010) and induces protein secretion to the apoplast (Wang et al., 2005).

Consistent with previous reports, total protein levels were higher in phloem exudates collected from SAR-induced compared with mock-inoculated leaves (Champigny et al., 2013; Carella et al., 2015), which may suggest that the induction of SAR leads to the mass translocation of a number of proteins through the phloem. If this is indeed true, then significant modifications to companion cell plasmodesmatal pore size are likely required to facilitate increased protein loading into the phloem. This idea is consistent with current hypotheses linking plasmodesmata to local and systemic immunity (Lee et al., 2011; Faulkner et al., 2013; Wang et al., 2013; Carella et al., 2015), although the impact of biotic stress on plasmodesmatal permeability in the phloem has yet to be studied. Alternatively, increased protein levels in SAR-induced phloem exudates may result from contamination caused by the deterioration of plant tissues that occurs during infection with pathogens. Indeed, proteins classified as extracellular were enriched in phloem exudates collected during SAR, which may support that cellular contamination is more likely to occur during infection. However, petiole damage was not detected in mock- or SAR-induced leaves in this study. Moreover, extracellular PR proteins are routinely identified in phloem exudate proteomes of healthy plants, including this study (Rodriguez-Celma et al., 2016), suggesting that extracellular proteins access the phloem translocation stream.

Proteins Enriched in SAR-Induced Phloem That Contribute to the SAR Response

We identified 16 proteins that accumulate in phloem exudates during the induction of SAR. Of these, PR1, the putative chitinase AED15, TRXh3, and ACBP6 were associated previously with SAR, demonstrating that SAR-related proteins are present in our SAR phloem proteome. The AED15 and PR1 proteins are known to accumulate in the apoplast during SAR (Moreno et al., 2012; Breitenbach et al., 2014). The localization of these proteins in the phloem suggests that plants produce these antimicrobial and antiherbivory proteins to protect against phloem sap-feeding insects and/or phloem-restricted microbial pathogens.

The SAR-enriched phloem proteins ACBP6 and TRXh3 are required for the manifestation of SAR in Arabidopsis (Tada et al., 2008; Xia et al., 2012). Phloem exudate-swapping experiments with the *acbp6* mutant suggest that ACBP6 is required for the production or movement of SAR signals (Xia et al., 2012), similar to the lipid transfer protein DIR1 (Maldonado et al., 2002). In vitro studies indicate that ACBP6 binds acyl-CoA and phosphatidylcholine (Engeseth et al., 1996; Chen et al., 2008) and may be involved in interorganellar lipid transport (Chen et al., 2008), while DIR1 binds monoacylated phospholipids (Lascombe et al., 2008). Accumulation of the ACBP6 (this study) and DIR1 lipid-binding proteins in the phloem during SAR supports the idea that lipid-based long-distance signaling is important for systemic immunity.

TRXh3 contributes to SAR in concert with TRXh5 by regulating the oligomer-to-monomer transition of cytosolic NPR1 via the thiol-disulfide conversion of redox-sensitive Cys residues (Tada et al., 2008). How TRXh3 functions in the phloem during the induction of

SAR is unknown, but it may function in the thiol-disulfide conversion of NPR1 or other Cys-containing SAR proteins such as DIR1. Recent evidence demonstrating the effectiveness of phloem-specific *AtNPR1* expression in protecting citrus trees against Huanglongbing disease hints that NPR1 function may be important in the phloem (Dutt et al., 2015).

Several redox-related proteins accumulated in the phloem during SAR, including PrxIIE (peroxiredoxin), GRXC2 (glutaredoxin), GSTU5 (glutathione *S*-transferase), and the m-type thioredoxins TRXm1/2/4. Given that thioredoxins are associated with SAR (Tada et al., 2008), the importance of TRXm1/2/4 function during SAR was investigated. T-DNA mutants in TRXm1 and TRXm4 were partially SAR defective, providing evidence that these thioredoxins are involved in SAR. TRXm1 and TRXm4 belong to the m-type family of plastid-targeted thioredoxins, which also includes TRXm2 and TRXm3 (Collin et al., 2003). Aside from TRXm3, which is involved in mediating intercellular transport during meristem development (Benitez-Alfonso et al., 2009), m-type thioredoxins are thought to play a redundant role in the redox regulation of plastidial enzymes associated with carbon metabolism (Collin et al., 2003). Given their localization in plastids and accumulation in phloem exudates, the function of TRXm1/4 during SAR may involve the redox regulation of target proteins in companion cell and/or sieve element plastids, which is intriguing given that lipidic SAR signals and some Cys-containing SAR proteins (AZI1 and EARLI1) are produced or located in plastids (Chaturvedi et al., 2008; Cecchini et al., 2015).

It is conceivable that TRXm proteins localize to other subcellular compartments in phloem cells during SAR, which would allow for their accumulation in phloem exudates. This idea is supported by observations of dual cytosolic and plastidial localization of TRXm2 (Holscher et al., 2014). Nevertheless, TRXm protein (Guelette et al., 2012; this study) and mRNA (Deeken et al., 2008) accumulate in phloem exudates, and TRXm1 and TRXm4 contribute to SAR (this work). How these proteins contribute to SAR remains to be determined, but recent evidence demonstrating the molecular holdase/foldase activity of NtTRXm in tobacco suggests that TRXm proteins act as molecular chaperones that protect target proteins during stress (Sanz-Barrio et al., 2012). As such, TRXm proteins may protect redox-sensitive proteins important for SAR in the phloem. In addition, TRXm1 was recently shown to bind the defense hormone salicylic acid (SA) using a number of protein-ligand-binding techniques (Manohar et al., 2015). Whether TRXm1 function in the phloem during SAR requires SA remains to be determined.

The putative lipid-binding protein MLP joins a number of lipid-associated proteins important for SAR. Analysis of an *mlp* T-DNA insertion mutant demonstrated a role for MLP in the SAR response. MLP belongs to a largely uncharacterized family of proteins that contain a BetvI (major birch [*Betula* spp.] pollen allergen) fold, which produces a forked hydrophobic cavity capable of binding large hydrophobic molecules (Gajhede et al., 1996; Radauer et al., 2008). This protein family includes the defense-associated intracellular PR10 protein, whose molecular function is unknown (Osmark et al., 1998). Since the main feature of MLP appears to be the BetvI fold, we speculate that MLP may bind a hydrophobic SAR signal. The diterpenoid SAR signal dehydroabietinal is a potential MLP ligand, as dehydroabietinal accumulates in the phloem during SAR (Chaturvedi et al., 2012). Future studies to examine if MLP binds dehydroabietinal or other hydrophobic defense activators will shed light on its role during SAR.

Proteins Suppressed in the SAR Phloem Proteome

The accumulation of a number of proteins was suppressed in phloem exudates collected from SAR-induced leaves, some of which were associated previously with plant defense and include TGG1 myrosinase (Barth and Jander, 2006), the jasmonic acid-responsive Man-binding lectin JR1 (León et al., 1998), CALRETICULIN2 (Qiu et al., 2012), the plastidial chaperonin CPN60B (Ishikawa et al., 2003), the fasciclin-like arabinogalactan-protein FLA8 (Gruner et al., 2013), and the Gly-rich RNA-binding protein GRP7 (Fu et al., 2007). Of these proteins, *JR1* and *FLA8* are down-regulated in distant leaves of SAR-induced plants (Gruner et al., 2013; Bernsdorff et al., 2016), and analysis of *cpn60B* knockout mutants demonstrated a constitutive SAR-like response to *P. syringae* pv *maculicola* (Ishikawa et al., 2003). Interestingly, CPN60, a chloroplastic chaperon protein, also was suppressed in melon phloem during viral infection (Serra-Soriano et al., 2015), hinting that CPN60 may act as a negative regulator of disease resistance responses in the phloem.

The UVR8-Signaling Module Is Important for SAR

Phenotypic analysis of the SAR response in mutant and overexpression lines of a number of SAR-suppressed proteins identified a role for UVR8 in SAR, as both *uvr8-6* and 35S:UVR8 plant lines were SAR defective compared with wild-type plants. The UVR8 photoreceptor is a seven-bladed β-propeller protein that perceives UV-B wavelengths using intrinsic Trp residues (Christie et al., 2012). Upon UV-B photoactivation, UVR8 homodimers monomerize and translocate from the cytosol to the nucleus (Kaiserli and Jenkins, 2007). In the nucleus, UVR8 interacts with COP1 to induce the expression of the bZIP transcription factor HY5, which in turn activates UV-B-responsive gene expression (Favory et al., 2009; Rizzini et al., 2011). In this study, we observed reduced levels of UVR8 in phloem exudates of SAR-induced compared with mock-induced plants. It is tempting to speculate

that SAR induction causes the accumulation of UVR8 in the nucleus, leading to decreased levels of cytosolic UVR8 available for movement into the phloem translocation stream. Alternatively, UVR8 may be negatively regulated during the induction of SAR. Given that *UVR8* gene expression is not affected by inoculation with virulent *Pst*, we speculate that the suppression of UVR8 involves proteasomal degradation and/or posttranscriptional regulation.

In addition to its well-established role in the UV-B stress response (for review, see Tilbrook et al., 2013), recent evidence demonstrated a positive role for UVR8 in abiotic stress responses (Fasano et al., 2014) as well as UV-B-induced resistance to the necrotrophic fungus *Botrytis cinerea* (Demkura and Ballaré, 2012). Our analysis of the *uvr8-6* mutant and a UVR8 overexpression line suggests that UVR8 plays both a positive and negative role during SAR, which may indicate that UVR8 regulates distinct processes during the SAR response, perhaps in different tissues. Overexpression of wild-type UVR8 protein does not activate UV-B-response gene expression in the absence of UV-B (Heijde et al., 2013). Since UV-B radiation is not detectable in our growth chambers, UVR8 signaling activated by UV-B light probably does not contribute to the SAR defect observed in the UVR8 overexpression line. Rather, increased pools of inactive UVR8 protein in the UVR8 overexpression line may have a dominant-negative effect. In any case, the SAR phenotypes of the UVR8 overexpression and mutant lines indicate that UVR8 is required for SAR, perhaps by regulating core light signaling or UV-response genes.

SAR Utilizes Core Components of Light Signaling Pathways

We further investigated the importance of UVR8 in SAR by assessing the SAR phenotypes of *hy5*, *cop1-4*, and 35S:GUS-COP1. Both COP1 and HY5 positively regulate UV-B responses downstream of UVR8 (Tilbrook et al., 2013). SAR was negatively impacted in each of these plant lines, demonstrating that the core members of the UV-B signaling pathway are important for SAR. In addition to their involvement in UV-B signaling, COP1 and HY5 also are central regulators of other light-signaling responses (Jiao et al., 2007), suggesting that core light-signaling machinery is required for SAR. Indeed, several studies indicate an association of light signaling with local and systemic pathogen defense responses (for review, see Roden and Ingle, 2009). The accumulation of SA, *PR* gene expression, and the manifestation of SAR all require exposure to light (Zeier et al., 2004). Moreover, light signaling components are important for this response, as the red light photoreceptor double mutant *phyA/phyB* is defective in SAR under typical growth conditions (Griebel and Zeier, 2008) and the blue light photoreceptor CRY1 is required for SAR in continuous light (Wu and Yang, 2010). The duration of light perceived following pathogen infection also impacts SAR, such that plants induced for SAR in the morning are less dependent on methyl salicylate-mediated responses compared with plants induced in the evening (Liu et al., 2011). In addition, exposure to high light intensities induces SA accumulation, the generation of reactive oxygen species, and programmed cell death, resulting in a SAR-like response (Mühlenbock et al., 2008). Recent evidence demonstrated that HY5 is required for light-induced programmed cell death and SA accumulation through the positive regulation of the immune regulator EDS1 (Chai et al., 2015), which itself is required for the generation and perception of mobile SAR signals (Breitenbach et al., 2014). This may suggest that HY5 is a positive regulator of EDS1 and other defense-related genes during the induction of SAR, which is supported by the identification of *NPR1*, *NIMIN2*, *ADR1*, *PAD4*, and *TRXm4* as putative HY5-binding targets (Lee et al., 2007). Furthermore, a recent study identified COP1 as a putative binding target of the SAR transcription factor SARD1 (Sun et al., 2015). Together, these results argue for a central role of light signaling in the establishment of local and systemic immune responses.

CONCLUSION

A comparative proteomics analysis of Arabidopsis phloem exudates collected from mock- and SAR-induced plants identified several proteins with differential abundance. Of these proteins, m-type thioredoxins, a major latex protein-like protein, and UVR8 were discovered to play a role in the SAR response. Further exploration of the UV-B signaling pathway identified COP1 and HY5 as additional regulators of SAR, which is in agreement with several studies that associate light signaling and systemic immunity. Importantly, the proteomics data set obtained in this study bridges fundamental gaps in knowledge by significantly adding to the limited understanding of protein composition in Arabidopsis phloem exudates while providing an in-depth look at phloem proteins associated with SAR long-distance signaling. This study contributes to the emerging field of comparative proteomic analysis of plant vascular sap that will provide insights into interorgan communication during stress.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Col-0) and homozygous T-DNA mutant seeds (Supplemental Fig. S4) were surface sterilized and stratified at 4°C in the dark for 2 d. Sterile seeds were plated on Murashige and Skoog plates and germinated for 5 to 7 d under continuous light. Seedlings were transplanted onto soil hydrated with 1 g L^{-1} 20-20-20 fertilizer and grown under short-day photoperiod conditions (9 h of light; 150 $\mu E\ m^{-2}\ s^{-1}$) at 22°C with 65% to 85% relative humidity. UV-B levels in growth chambers were undetectable (UV-X radiometer; UVP). Confirmed (homozygous) plant lines

were obtained from the Arabidopsis Biological Resource Center or independent research laboratories (Konopka-Postupolska et al., 2009; Tsuchiya et al., 2010; Fasano et al., 2014). Homozygous *mlp* mutants (Nottingham Arabidopsis Stock Centre; GK-089B08) were confirmed from heterozygous seed stock by germination on Murashige and Skoog medium containing sulfadiazine (5 μg mL^{-1}) followed by molecular characterization of mRNA levels using RT-PCR (Supplemental Fig. S4).

Bacterial Growth, Inoculation, and Quantitation

Standard SAR experiments and local resistance assays were performed as described by Carella et al. (2015) with *Pseudomonas syringae* pv *tomato* strains cultured overnight with shaking in King's B medium (King et al., 1954) supplemented with 50 μg mL^{-1} kanamycin. For large-scale phloem exudate collection experiments, leaves of 4-week-old Col-0 were pressure infiltrated with 10 mM $MgCl_2$ (mock inoculation) or 10^6 cfu mL^{-1} virulent *Pst* DC3000 (pVSP1) or avirulent *Pst* DC3000/avrRpt2 (pVSP1 + avrRpt2). In planta *Pst* levels were quantified by dilution plating as described by Cameron et al. (1999) and Carella et al. (2015). Statistically significant differences in *Pst* levels were identified by ANOVA (Tukey's HSD, $P < 0.05$) using R.

Phloem Exudate Collection

Phloem exudates were collected as described by Carella et al. (2015). At 24 hpi, leaves of mock-inoculated or SAR-induced plants (4-week-old Col-0) were cut at the base of the petiole, surface sterilized quickly (50% ethanol and 0.0006% bleach in 1 mM EDTA), and immediately placed into Eppendorf tubes containing 1 mM EDTA for 1 h. Twelve leaves were placed into each Eppendorf tube. Leaves were then transferred to tubes containing sterile water and allowed to exude in a humidity chamber for 23 h (representing exudation from 25 to 48 hpi). For proteomics analysis, pooled exudates from more than 90 plants per treatment were concentrated using centrifugal concentrators with a 3-kD cutoff (Vivaspin 20; GE Healthcare) according to the manufacturer's instructions to a final volume of approximately 7 mL. Concentrated exudates were equally subdivided into four tubes, and protein levels were quantified using the Bio-Rad protein reagent with bovine serum albumin as a standard. Samples were then frozen in liquid nitrogen, lyophilized, and stored at −80°C until further use. Phloem exudates used for immunoblotting were collected as described previously (Carella et al., 2015).

LC-MS/MS Measurement, Label-Free Quantitative Analysis, and Database Search

Prior to LC-MS/MS analysis, the samples were centrifuged for 5 min at 4°C. Each approximately 0.5-μg sample was measured on an LTQ OrbitrapXL (Thermo Fisher Scientific) coupled to an Ultimate3000 nano-RSLC device (Dionex) as described previously (Hauck et al., 2010; Molin et al., 2015).

Raw files of each data set were analyzed separately with Progenesis QI software for proteomics as described previously (Hauck et al., 2010; Merl et al., 2012). Briefly, peptide features in the individual runs were aligned to reach a maximum overlay of at least 80%. The samples were assigned to the three individual groups, and all tandem mass spectrometry features with charges +2 to +7 were exported for protein identification using the Mascot search engine (version 2.5.0; Matrix Science) in The Arabidopsis Information Resource database (version 10). Search results were filtered for $P < 0.05$ and Mascot percolator score ≥ 15 to reach a false discovery rate of 1% (Brosch et al., 2009). Protein identifications were reimported in Progenesis QI software, and normalized abundances of unique peptides were summed for every protein. These values were used for the calculation of abundance ratios between groups and for statistical evaluation by Student's *t* test ($P < 0.05$).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Information Resource under accession numbers AT1G31812 (ACBP6), AT1G35720 (ANNAT1), AT2G32950 (COP1), AT5G48485 (DIR1), AT5G11260 (HY5), AT1G55260 (LTPG6), AT4G23670 (MLP), AT2G14610 (PR1), AT5G42980 (TRXh3), AT1G03680 (TRXm1), AT4G03520 (TRXm2), AT3G15360 (TRXm4), AT5G63860 (UVR8).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. SAR assay and phloem exudate collection controls.

Supplemental Figure S2. Complete GO Slim analysis of proteins enriched or suppressed in SAR-induced phloem exudates.

Supplemental Figure S3. Supporting SAR assays.

Supplemental Figure S4. Plant lines used in this study.

Supplemental Figure S5. *TRXm* and *MLP* expression analysis.

Supplemental Figure S6. Exploring *UVR8/COP1/HY5* expression dynamics in publicly available data obtained from Genevestigator.

Supplemental Figure S7. Exploring *UVR8/COP1/HY5* expression dynamics in publicly available data obtained from the Arabidopsis Gene Expression Browser.

Supplemental Figure S8. Exploring *UVR8/COP1/HY5* expression dynamics in publicly available data obtained from the Arabidopsis eFP Expression Browser.

Supplemental Table S1. Differentially abundant phloem proteins specific to avirulent *Pst* treatment.

Supplemental Table S2. Differentially abundant phloem proteins specific to virulent *Pst* treatment.

Supplemental Table S3. Complete list of proteins suppressed in the phloem during SAR.

Supplemental Table S4. Common Arabidopsis phloem proteins.

Supplemental Table S5. Common phloem proteins in pumpkin, Texas bluebonnet, and Arabidopsis.

Supplemental Table S6. TRXm family similarity matrix.

Supplemental Data S1. Raw proteomics data.

Supplemental Methods S1. Protein isolation and immunoblotting, sample preparation for mass spectrometry, RNA isolation, PCR primers and RT-PCR analysis.

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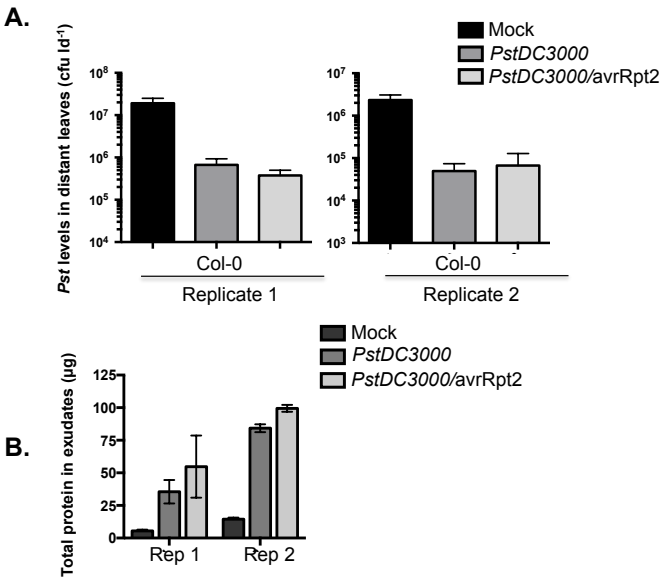
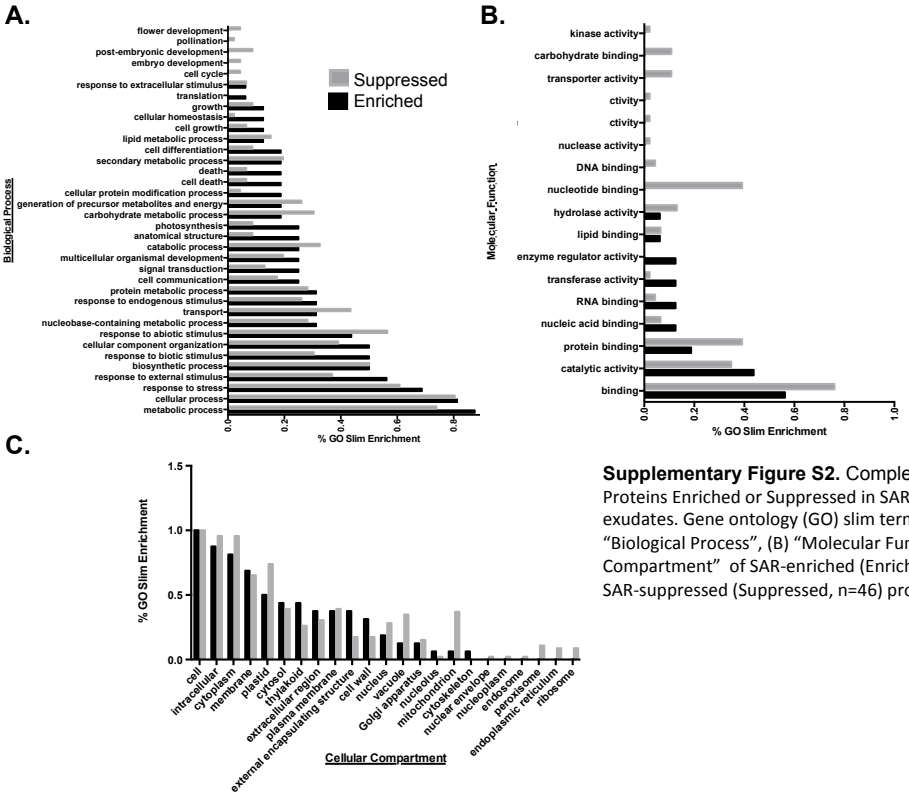
6.9 – Online Supplementary Figures, Tables, and Methods

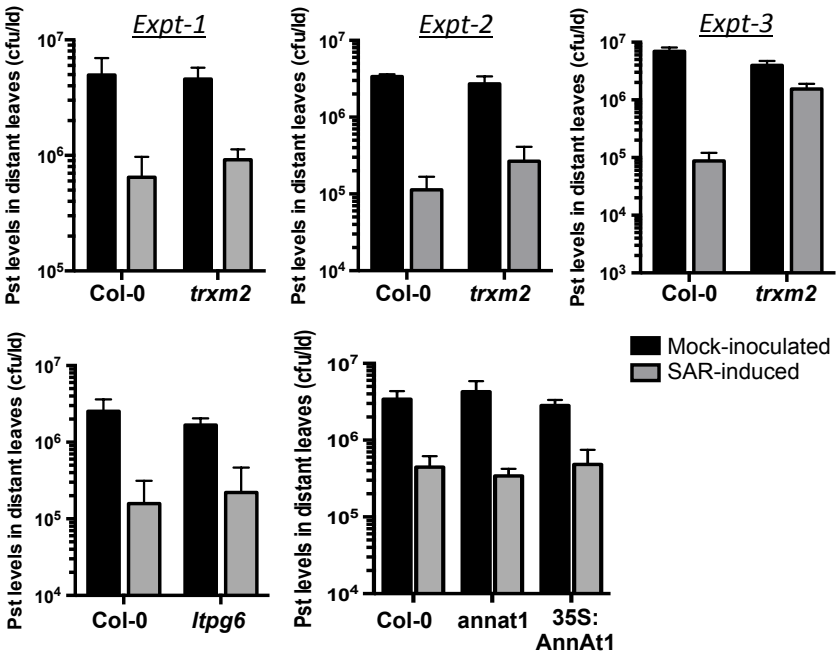
All supplementary material appears as it does online.



Supplementary Figure S1. SAR assay and phloem exudate collection controls. 4 week-old Col-0 plants were mock-inoculated (Mock, 10 mM $MgCl_2$) or induced for SAR by pressure infiltration of 10^6 colony forming units (cfu) ml^{-1} of virulent (*PstDC3000*) or avirulent (*PstDC3000/avrRpt2*) strains of *Pst.* (A) SAR was assessed 2 days after induction by challenging distant leaves with 10^5 cfu ml^{-1} *PstDC3000*, and quantifying *Pst* levels in these leaves 3 days later. Values represent the mean +/- standard deviation of 3 biological replicates. (B) Phloem exudate collected from mock and SAR-induced plants were subjected to Bradford analysis. Values represent mean total protein levels (µg) +/- standard deviation of 4 biological replicates.



Supplementary Figure S2. Complete GO Slim Analysis of Proteins Enriched or Suppressed in SAR-induced Phloem exudates. Gene ontology (GO) slim terms pertaining to (A) "Biological Process", (B) "Molecular Function", and (C) "Cellular Compartment" of SAR-enriched (Enriched, n=16) compared to SAR-suppressed (Suppressed, n=46) proteins

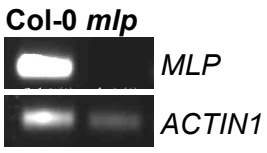


Supplementary Figure S3. Supporting SAR Assays. Standard SAR assays comparing wild-type Col-0 to *trxm2*, *ltpg6, annat1-1* and 35S:AnnAt1. Leaves of 4 week-old plants were mock-inoculated (10 mM $MgCl_2$) or induced for SAR by pressure infiltration with 10^6 colony forming units ml^{-1} *PstDC3000/avrRpt2* (SAR-induced). Two days later, distant leaves were challenged with 10^6 *Pst/DC3000* and *Pst* levels in these leaves were quantified 3 days post inoculation (dpi). Experiments with *ltpg6, annat1,* and 35S:AnnAt1 were repeated twice with similar results.

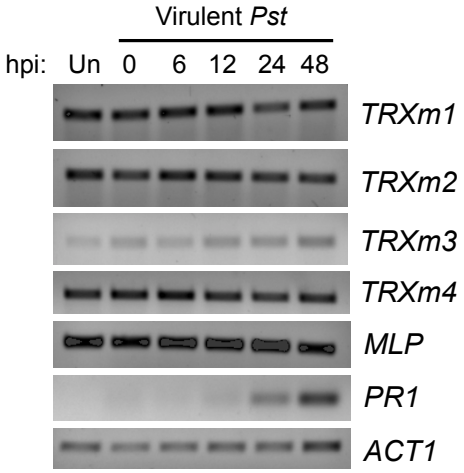
Supplementary Figure S4. Plant Lines Used in This Study

Name	Mutation/Line	Obtained From	Described in
trxm1	T-DNA (SALK_ 087118C)	ABRC	Laugier et al. 2013
trxm4	T-DNA (SALK_023810C)	ABRC	Laugier et al. 2013
trxm2	T-DNA (SALK_ 123570C)	ABRC	Okegawa & Motohashi 2015
mlp	T-DNA (GK-089B08)	NASC	This Study
ltpg6-2	T-DNA (SAIL_793_B12)	ABRC	Edstam & Edqvist 2014
hy5	T-DNA (SALK_056405)	McCourt Lab	Tsuchiya et al. 2010
cop1-4	Substitution (nonsense)	McCourt Lab	McNellis et al. 1994
35S:GUS-COP1	Overexpression line	McCourt Lab	Tsuchiya et al. 2010
uvr8-6	T-DNA (SALK_033468)	Leone Lab	Favory et al. 2009
35S:UVR8	Overexpression line	Leone Lab	Fasano et al. 2014
annat1-1	T-DNA (SALK_015426)	Hennig Lab	Konopka-Postupolska et al. 2009
35S:AnnAt1	Overexpression line	Hennig Lab	Konopka-Postupolska et al. 2009

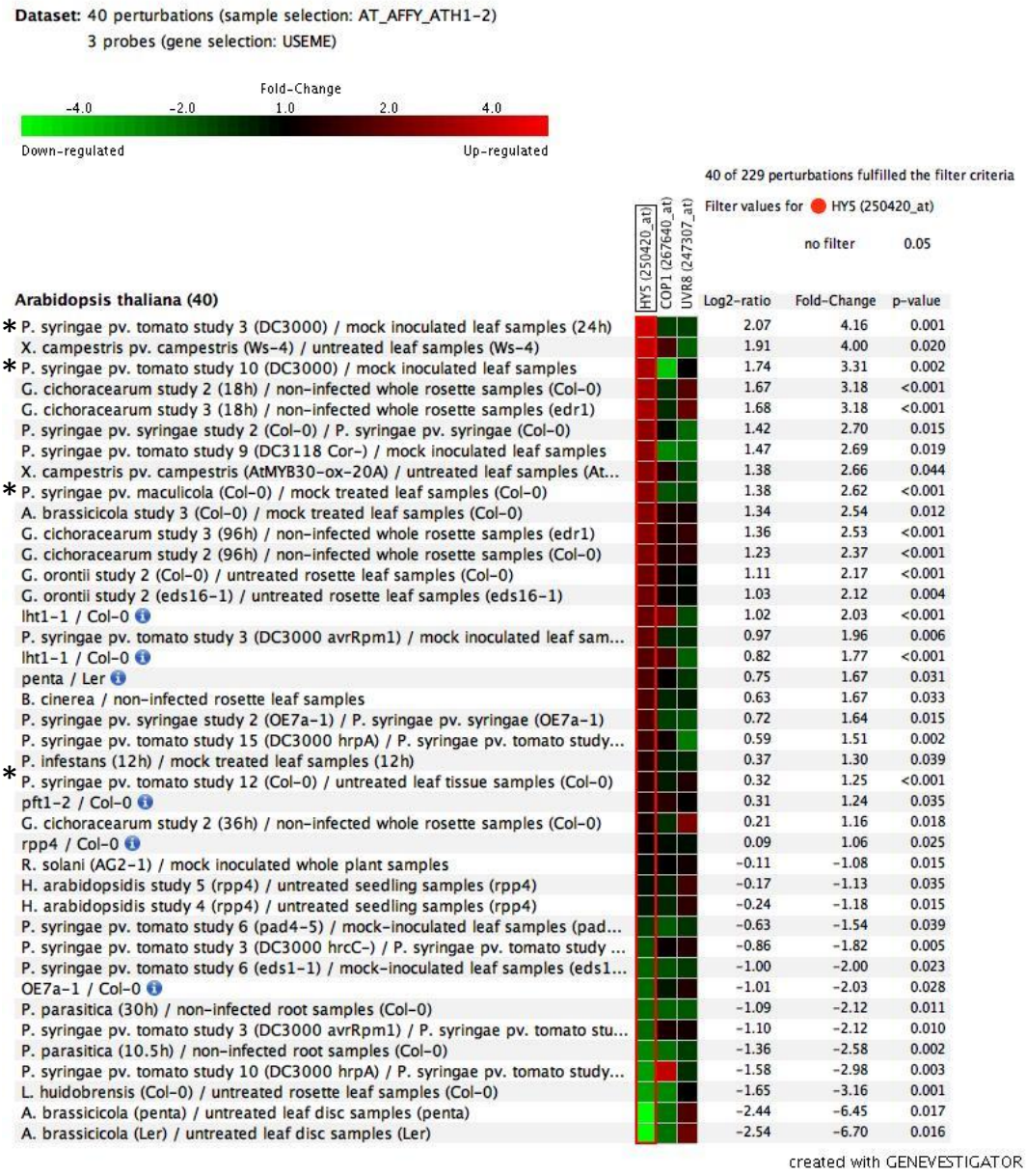
ABRC – Arabidopsis Biological Resource Centre, NASC – Nottingham Arabidopsis Stock Centre



RT-PCR of cDNA generated from leaves of 4 week-old Col-0 and *mlp* plants. *MLP* expression was compared to the ACTIN1 control. This experiment was performed twice with similar results.



Supplementary Figure S5. *TRXm* and *MLP* expression analysis. RT-PCR of cDNA generated from leaves of 4 week-old Col-0 plants that were untreated (Un) or inoculated with 10^6 cfu ml^{-1} *PstDC3000* at the indicated time points (hours post inoculation – hpi). *TRXm1, TRXm2, TRXm3, TRXm4, and MLP* expression was compared to the ACTIN1 and *PR1* controls. This experiment was performed 3 times with similar results.



Supplementary Figure S6. Exploring *UVR8/COP1/HY5* expression dynamics in publically available data obtained from Genevestigator (Hruz et al. 2008). Experiments with statistically significant differences in *HY5* regulation during pathogen infection were obtained using the filter setting (p-value <0.05). Expression of *HY5* is compared to *UVR8* and *COP1* in the heat map. Experiments comparing Col-0 plants infected with virulent *Pseudomonas syringae* to mock- or untreated controls are indicated by an asterisk *.

Supporting Figure S7 - Exploring *UVR8/COP1/HY5* expression dynamics in publically available data obtained from the "Arabidopsis Gene Expression Browser". The data below was obtained from www.expressionbrowser.com (Zhang et al. 2010) by querying all expression studies for "Pseudomonas syringae".

Pseudomonas syringae pv tomato DC3000 infiltration for 2 hr: infiltrated with 1x108 cfu/ml Pseudomonas syringae pv tomato DC3000, 2 leaves per plant, 8 plants pooled, harvested after 2h

Gene	Treatment	Control	Fold-Change	p-value
UVR8	808	763	1.05	0.645
COP1	205	212	-1.03	0.792
HY5	64	49	1.3	0.1879

Pseudomonas syringae pv tomato DC3000 infiltration for 6 hr: infiltrated with 1x108 cfu/ml Pseudomonas syringae pv tomato DC3000, 2 leaves per plant, 8 plants pooled, harvested after 6h

Gene	Treatment	Control	Fold-Change	p-value
UVR8	979	1128	-1.15	0.2776
COP1	254	276	-1.08	0.5707
HY5	75	60	1.24	0.1051

Pseudomonas syringae pv tomato DC3000 infiltration for 24 hr: infiltrated with 1x108 cfu/ml Pseudomonas syringae pv tomato DC3000, 2 leaves per plant, 8 plants pooled, harvested after 24hr

Gene	Treatment	Control	Fold-Change	p-value
UVR8	643	968	-1.5	0.0272
COP1	253	382	-1.5	0.0094
HY5	212	47	4.45	1.8E-4

Pst DC3000 infection 24 hr: Plants were inoculated by vacuum infiltration with Pseudomonas syringae pv. tomato strain DC3000 bacteria at a concentration of 10e6 cfu/ml. Inoculated leaf tissue from at least 15 plants was collected for RNA isolation

Gene	Treatment	Control	Fold-Change	p-value
UVR8	618	878	-1.41	0.1576
COP1	138	144	-1.03	0.9219
HY5	119	63	1.88	0.1919

Pst DC3000 infection 7 hr: Plants were inoculated by vacuum infiltration with a suspension of Pseudomonas syringae pv. tomato DC3000 bacteria at 10e8 cfu/ml. Inoculated leaf tissue from at least 15 plants was collected 7 hours after inoculation for RNA isolation

Gene	Treatment	Control	Fold-Change	p-value
UVR8	594	715	-1.2	0.641
COP1	67	244	-3.59	0.1106
HY5	187	67	2.78	0.0218

PsmES4326 infection for 9 hr: col-0 plant infected by PsmES4326 (Pseudomonas syringae ES4326) for 9 hr.

Gene	Treatment	Control	Fold-Change	p-value
UVR8	620	896	-1.44	0.1658
COP1	179	214	-1.19	0.5544
HY5	20	21	-1.02	0.4669

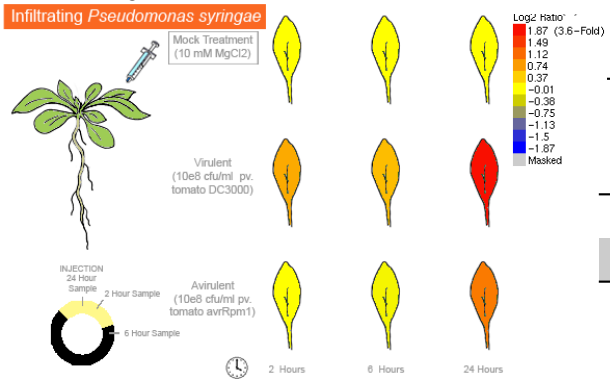
PsmES4326 infection for 24 hr: col-0 plant infected by PsmES4326 (Pseudomonas syringae ES4326) for 24 hr.

Gene	Treatment	Control	Fold-Change	p-value
UVR8	160	318	-1.98	0.0168
COP1	102	358	-3.48	0.0069
HY5	152	172	-1.12	0.2929

PsmES4326 infection for 32 hr: col-0 plant infected by PsmES4326 (Pseudomonas syringae ES4326) for 32 hr.

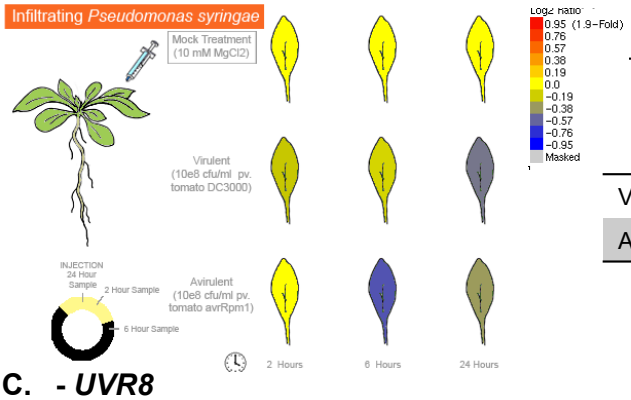
Gene	Treatment	Control	Fold-Change	p-value
UVR8	335	796	-2.37	0.0098
COP1	111	234	-2.1	0.0231
HY5	76	18	4.09	0.0529

A. - *HY5*



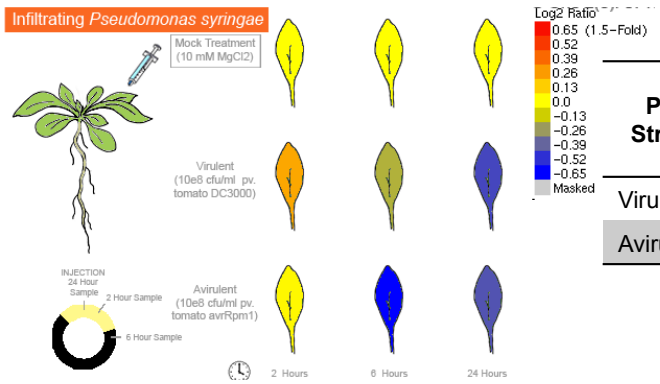
Pst Strain	Fold-Change (treatment vs. mock)		
	2 hours	6 hours	24 hours
Virulent	1.55	1.41	3.67
Avirulent	1	0.94	1.99

B. - *COP1*



Pst Strain	Fold-Change (treatment vs. mock)		
	2 hours	6 hours	24 hours
Virulent	0.86	0.89	0.70
Avirulent	1	0.64	0.77

C. - *UVR8*



Pst Strain	Fold-Change (treatment vs. mock)		
	2 hours	6 hours	24 hours
Virulent	1.17	0.87	0.72
Avirulent	1.01	0.63	0.73

Supplementary Figure S8. Exploring *UVR8/COP1/HY5* expression dynamics in publically available data obtained from the Arabidopsis eFP Expression Browser (Winter et al. 2007). Expression data for *HY5 (A), COP1 (B), and UVR8 (C)* are provided in graphical and tabular formats. Data was obtained from a microarray experiment performed on Arabidopsis Col-0 plants infected with virulent and avirulent (AvrRpt2) strains of *Pseudomonas syringae* pv. *tomato* (Pst). Times given in table represent the time of sample collection post infection with Pst. Further details on the experiment can be found at (http://www.bar.utoronto.ca/NASCArrays/index.php?ExpID=120).

Table S1. Differentially Abundant Phloem Proteins Specific to Avirulent *Pst*-treatment

Locus	Gene Symbol	Description	Relative Abundance (Virulent/Mock)		Relative Abundance (Avirulent/Mock)		Peptides Used for Quantitation	
			Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
Increased Abundance:								
AT1G08830/ AT5G18100	CSD1	Superoxide dismutase	0.52	1.64	3.09	3.41	2	2
AT1G09310/ AT1G56580	-	Unknown function	1.91	1.17	1.73	2.51	4	5
AT1G18210	-	Calcium binding EF hand	1.92	2.06	6.79	5.88	5	7
AT1G22400	UGT85A1	UDP-Glucosyl transferase	1.73	1.06	28.12	2.93	2	3
AT1G32470	-	Single hybrid motif family	3.88	1.01	2.09	3.57	6	2
AT1G55480	ZKT	PDZ, K-box, TPR motifs	2.29	1.99	11.84	5.39	2	3
AT1G66410/ AT2G27030	CAM4/CAM5	Calmodulin	1.02	1.37	2.76	4.68	4	4
AT1G70890	MLP43	Major latex protein-like	1.25	1.46	1.85	1.82	3	2
AT1G73260	KTI1	Kunitz trypsin inhibitor	2.86	1.46	12.31	4.28	4	7
AT1G76100	PETE1	Plastocyanin	2.24	12.02	10.19	21.55	2	2
AT1G77490	TAPX	Thylakoidal ascorbate peroxidase	74.06	1.25	14.05	2.51	2	3
AT1G80380	-	P-loop containing	36.05	1.59	3.95	2.15	3	4
AT2G02930/ AT4G02520	GSTF2/GSTF3	Glutathione S-transferase	0.05	0.24	3.02	1.16	2	8
AT2G20270	-	Thioredoxin	1.79	4.50	9.80	10.74	3	4
AT2G28190	CSD2	Superoxide dismutase	0.47	2.42	8.66	5.35	2	2
AT2G38870	-	Serine proteinase inhibitor	0.75	2.58	11.90	5.12	3	3
AT2G42590	GRF9	General regulatory factor	2.25	0.77	2.70	1.43	2	3
AT2G43510	ATTI1	Trypsin inhibitor	2.18	1.19	16.77	2.20	2	3
AT2G44060	-	LEA family	1.81	0.86	3.99	1.68	3	2
AT2G44790	UCC2	Uclacyanin	0.63	0.56	1.86	1.89	2	3
AT2G47470	PDI2	Protein disulphide isomerase	0.76	1.12	3.88	2.59	2	2
AT3G01500	CA1	Carbonic anhydrase	14.27	0.76	24.74	2.19	2	4
AT3G10060	-	FKBP-like	2.41	0.85	2.39	4.34	2	5
AT3G15360	TRXm4	Thioredoxin	5.50	0.58	9.29	1.94	4	5
AT3G48870/ AT5G50920	CLPC/CLPC2	Similar to ATP-dependent Clp protease ATP-binding subunit	0.96	0.63	2.53	2.54	5	10
AT4G02530	-	Thylakoid lumen protein	0.80	52.69	13.94	166.06	2	2
AT4G03280	PETC	Photosynth. electron transfer	1.34	3.38	81.99	20.02	2	2
AT4G10300	RmlC-like	Cupin superfamily	9.50	7.29	52.42	16.34	2	3
AT4G23670	-	Polyketide cyclase	5.72	1.10	30.23	5.96	2	4
AT4G37530	-	Peroxidase	0.68	1.40	3.07	1.61	3	6
AT5G51070	ERD1	Clp ATPase	1.64	0.65	2.83	1.96	2	4
AT5G53490	-	Tetratricopeptide-like	1.84	4.36	3.38	11.02	3	5
AT5G66570	PSBO-1	PSII Subunit	0.86	1.42	6.91	3.20	2	4
Decreased Abundance:								
AT1G67090	RBCS1A	RuBisCO small subunit	1.13	0.69	0.36	0.69	3	4
AT1G03475	LIN2	Coproporphyrinogen III oxidase	0.99	0.50	0.47	0.84	3	5
AT1G11840	GLX1	Glyoxalase	0.69	0.28	0.12	0.35	2	4
AT1G20020	FNR2	Ferredoxin oxidoreductase	0.70	0.17	0.60	0.32	5	14
AT1G20440	COR47	Dehydrin	0.46	0.23	0.14	0.23	2	6
AT1G49630	PREP2	Presequence protease	0.57	0.11	0.14	0.19	7	4

Table S1. Differentially Abundant Phloem Proteins Specific to Avirulent *Pst*-treatment

AT1G67280	-	Glyoxalase	1.27	0.22	0.33	0.35	2	5
AT2G05990	MOD1	NADP-binding rossmann-fold superfamily	0.69	0.26	0.20	0.49	2	4
AT2G34790	EDA28	FAD-binding Berberine family protein	0.77	0.10	0.06	0.06	3	3
AT2G44160/ AT3G59970	MTHFR1/ MTHFR2	Methylenetetrahydrofolate reductase	0.26	0.11	0.05	0.15	2	4
AT3G01480	CYP38	Cyclophilin	1.09	0.22	0.58	0.43	3	7
AT3G03250	UGP1	UDP-Glucose pyrophosphorylase	0.54	0.14	0.16	0.11	2	3
AT3G62410	CP12	CP12 domain-containing	0.87	0.07	0.26	0.08	2	2
AT4G04640	ATPC1	ATPase	0.59	0.32	0.22	0.20	4	5
AT4G20260	PCAP1	Cation-binding protein	0.15	0.02	0.04	0.04	4	9
AT4G23600	JR2	Tyrosine transmaminase	4.71	0.50	0.49	0.37	3	2
AT5G04140	GLU1	Glutamate synthase	1.01	0.10	0.40	0.14	4	14
AT5G09650	PPa6	Pyrophosphorylase	1.14	0.30	0.39	0.47	6	8
AT5G12040	-	Nitriliase	1.75	0.23	0.21	0.40	3	2
AT5G17310	UGP2	UDP-Glucose pyrophosphorylase	0.60	0.26	0.09	0.24	2	2

Table S2. Differentially Abundant Phloem Proteins Specific to Virulent *Pst*-treatment

Locus	Gene Symbol	Description	Relative Abundance (Virulent/Mock)		Relative Abundance (Avirulent/Mock)		Peptides Used for Quantitation	
			Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
Increased Abundance:								
AT2G21170	TIM	Triosphosphate isomerase	2.21	2.06	1.05	2.31	3	5
AT2G37660	-	NADP-binding Rossmann-fold superfamily	2.63	1.58	0.70	3.14	5	8
AT3G14067	-	Subtilase	1.94	2.07	1.19	4.97	3	4
Decreased Abundance:								
AT1G12080	-	Vacuolar calcium-binding	0.19	0.33	0.91	0.26	3	4
AT1G78320/ AT1G78380	GSTU23/GSTU19	Glutathione S-transferase	0.62	0.39	0.50	1.09	3	5
AT4G37930/ AT5G26780	SHM1/SHM2	Serine trans-hydroxymethyltransferase	0.32	0.28	0.54	0.87	4	5
AT2G36460	-	Aldolase	0.19	0.63	0.07	1.03	2	5

Table S3. Complete List of Proteins Suppressed in the Phloem During SAR (SAR-Suppressed)

Locus	Gene Symbol	Description	Relative Abundance (Virulent/Mock)		Relative Abundance (Avirulent/Mock)		Peptides Used for Quantitation	
			Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
AT3G05900	-	Neurofilament protein-related	0.07	0.03	0.03	0.03	10	15
ATCG00480	ATPB	ATP Synthase	0.42	0.22	0.21	0.14	10	9
AT5G40450	-	Unknown Protein	0.03	0.03	0.01	0.04	7	12
ATCG00120	ATPA	ATP Synthase	0.31	0.34	0.15	0.17	6	7
AT1G16080	-	Unknown Protein	0.41	0.16	0.17	0.23	6	5
AT5G66190	FNR1	Ferrodoxin oxidoreductase	0.37	0.14	0.20	0.21	4	17
AT2G04030	HSP90.5	Chaperone Protein	0.51	0.13	0.26	0.18	6	6
AT5G26000	TGG1	Thioglucoside glucohydrolase	0.29	0.40	0.28	0.75	6	10
AT3G16470	JR1	Mannose-binding Lectin	0.20	0.26	0.04	0.20	5	8
AT1G65930	cICDH	Cytosolic NADP-dependent isocitrate dehdrogenase	0.29	0.13	0.08	0.20	5	8
AT4G09000	GRF1	General Regulatory Factor	0.39	0.19	0.24	0.28	5	4
AT5G38480	GRF3	General Regulatory Factor	0.50	0.27	0.38	0.41	5	3
AT1G55490	CPN60B	Chaperonin	0.24	0.15	0.14	0.19	5	7
AT3G16400	NSP1	Nitrile Specifier Protein	0.16	0.11	0.03	0.08	4	5
AT1G42970	GAPB	Glyceraldehyde-3-phosphate dehydrogenase	0.28	0.23	0.55	0.56	4	5
AT1G09210	CRT1b	Calreticulin	0.09	0.08	0.03	0.08	4	2
AT1G56340	CRT1a	Calreticulin	0.15	0.09	0.04	0.09	4	4
AT5G54770	THI1	Thiazole Biosynthetic Enzyme	0.08	0.09	0.02	0.03	3	2
AT3G53420	PIP2	Plasmamembrane instrisic protein	0.06	0.02	0.07	0.01	3	2
AT4G02510	TOC159	Chloroplast Outer Membrane Translocon	0.11	0.08	0.03	0.11	3	8
AT1G78300	GRF2	General Regulatory Factor	0.23	0.09	0.11	0.08	3	3
AT1G62660	-	Glycosyl transferase	0.16	0.04	0.05	0.04	3	4
AT5G07440	GDH2	Glutamate Dehydrogenase	0.29	0.08	0.19	0.21	3	5
AT2G32240	-	Unknown Function	0.18	0.29	0.21	0.36	3	8
AT5G28540	BiP1	Heat Shock Protein 70	0.38	0.35	0.36	0.47	3	7
AT2G28000	CPN60A	Chaperonin	0.20	0.29	0.12	0.27	3	4
AT3G20390	-	Endoribonuclease	0.58	0.39	0.39	0.56	3	7
AT1G22300	GRF10	General Regulatory Factor	0.34	0.30	0.29	0.38	3	4
AT1G72150	PATL1	Patellin	0.06	0.09	0.06	0.10	2	3
AT1G76180	ERD14	Dehydrin	0.06	0.05	0.03	0.04	2	6
AT1G22530	PATL2	Patellin	0.04	0.15	0.10	0.17	2	3
AT1G35720	ANNAT1	Annexin	0.17	0.08	0.06	0.06	2	6
AT4G22670	HIP1	HSP70-interacting	0.03	0.01	0.00	0.04	2	2
AT2G21660	GRP7	Glycine-rich Protein	0.19	0.10	0.06	0.06	2	5
AT2G01140	-	Aldolase	0.18	0.04	0.07	0.03	2	3
AT3G12390	-	Nascent polypeptide-associated complex subunit	0.30	0.02	0.04	0.05	2	2
AT1G13440	GAPC2	Glyceraldehyde-3-phosphate dehydrogenase	0.06	0.36	0.28	0.29	2	2
AT2G22795	-	Unknown Protein	0.00	0.01	0.00	0.00	2	3
AT2G45470	FLA8	Fasciclin-like Arabinogalactin Protein	0.36	0.30	0.36	0.38	2	3
AT1G52410	TSA1	TSK-associating Protein	0.01	0.01	0.01	0.00	2	3
AT5G63310	NDPK2	Nucelotide Diphosphate Kinase	0.38	0.12	0.19	0.27	2	8

Table S4. Common Arabidopsis Phloem Proteins

Locus	Gene Symbol	Description
AT1G08830	SOD1	Superoxide dismutase
AT1G11840	GLX1	Glyoxalase
AT1G13440	GAPC2	Glycerol-3-phosphate dehydrogenase
AT1G22300	GRF10	General regulatory factor
AT1G23740	AOR	Oxidoreductase
AT1G29660	-	GDSL-motif lipase
AT1G35720	ANNAT1	Annexin
AT1G67090	RBCS1A	RuBiSCO small chain
AT1G70890	MLP43	Major latex protein
AT1G75040	PR-5	Pathogenesis-related
AT1G78380	GST8	Glutathione transferase
AT2G21660	GRP7	Glycine-rich RNA binding protein
AT2G28190	SOD2	Superoxide dismutase
AT2G37660	-	NAD(P)-binding
AT3G01500	CA1	Carbonic anhydrase
AT3G03250	UGP1	UDP-glucose pyrophosphorylase
AT3G16470	JR1	Jacalin-related lectin
AT3G20390	RIDA	Reactive intermediate deaminase
AT3G55440	TPI	Triosephosphate isomerase
AT3G55800	SBPASE	Sedoheptulose-bisphosphatase
AT3G62030	ROC4	Cyclophilin
AT4G38970	FBA2	Fructose-bisphosphate aldolase
AT4G39730	PLAT1	Lipase/lipooxygenase
AT5G04140	GLU1	Glutamate synthase
AT5G26000	TGG1	Thioglucoside glucohydrolase
AT5G42980	TRX-h3	Thioredoxin
ATCG00490	RBCL	RuBiSCO large subunit

Table S5. Common Phloem Proteins in Pumpkin, Texas bluebonnet, and Arabidopsis*

Locus	Gene Symbol	Description
AT1G56070	LOS1	Translation elongation factor
AT1G78900	VHA-A	ATP synthase
AT2G21660	GRP7	Glycine rich, RNA binding
AT2G36460	FBA6	Fructose-bisphosphate aldolase
AT2G36530	ENO2	Enolase
AT3G52880	MDAR1	Monodehydroascorbate reductase
AT3G55440	TPI	Triosephosphate isomerase
AT4G09320	NDK1	Nucleoside diphosphate kinase
AT5G02500	HSC70	Heat shock cognate protein
AT5G03340	CDC48C	ATPase
AT5G42020	BiP2	Luminal binding protein
AT5G57330	-	Galactose mutarotase-like

Table S6. TRXm-family Similarity Matrix (Displaying % Identity/Similarity)*

	TRXm1	**TRXm2**	**TRXm3**	**TRXm4**
TRXm1	-	-	-	-
TRXm2	75/82%	-	-	-
TRXm3	37/65%	35/65%	-	-
TRXm4	55/74%	52/74%	42/59%	-

*As determined by pairwise BLASTP analysis

Table S7. PCR Primers and Conditions Used in This Study.

Target	Primer Sequence (5'-3')	Annealing Temp (° C)	PCR Cycle #	Reference
ACT1	F: GGCGATGAAGCTCAATCCAAACG	61	26	Champigny *et al.* 2013
	R: GGTCACGACCAGCAAGATCAAGACG			
PR-1	F: GCAATGGAGTTTGTGGTCAC	61	26	Kim *et al.* 2010
	R: GTTCACATAATTCCCACGAGG			
UVR8	F: CTACTGGTGGTGAGAAAATGTC	61	26	Kliebenstein *et al.* 2002?
	R: TGTTGTCCACTTGCTCCATCG			
HY5	F: GCTGCAAGCTCTTTACCATC	59	26	Brown & Jenkins 2008
	R: AGCATCTGGTTCTCGTTCTG			
COP1	F: AGTTATAGCAGAAATCCGGCATGG	61	26	This study
	R: TTAACCTTGCAGTCGTCACTACC			
TRXm1	F: CGACAGATGTTCTCTGTGTTGC	61	26	This study
	R: GATAGTTGGGATGCTTCTAACACC			
TRXm2	F: CGTTGACCTCGATTCATCAACC	61	26	This study
	R: CTATGATTGTATCCTTCTTCTCGC			
TRXm3	F: TCATCTCCGTCGCGTCTCTTCC	61	26	This study
	R: GTGGACCATCCGACATGGACC			
TRXm4	F: AGCTGCTCCGTCGGTTTCACG	61	26	This study
	R: TAGGCACAGCTCCAATGATGC			
MLP	F: TACAAGAGCTGGAAGAGCGAGAACC	61	26	This study
	R: GCCTTCAATGTCAACAACCATTTGC			

Primer sequences for *ACTIN1* (AT2G37620), *PR1* (AT2G14610), *UVR8* (AT5G63860), *HY5* (AT5G11260), *COP1* (AT2G32950), *TRXm1* (AT1G03680), *TRXm2* (AT4G03520), *TRXm3* (AT2G15570), *TRXm4* (AT3G15360), and *MLP* (AT4G23670). PCR cycle number and primer annealing temperatures (°C) are indicated.

References:

Brown B.A., Jenkins G.I. (2008) UV-B signaling pathways with different fluence-rate response profiles are distinguished in mature arabidopsis leaf tissue by requirement for UVR8, HY5, and HYH. *Plant Physiology,* **146(2)**: 576-588. doi: http://dx.doi.org/10.1104/pp.107.108456

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Kim S.H., Gao F., Bhattacharjee S., Adiasor J.A., Nam J.C., Gassmann W. (2010) The Arabidopsis resistance-like gene SNC1 is activated by mutations in SRFR1 and contributes to resistance to the bacterial effector AvrRps4. *PLoS Pathogens,* **6(11)**: doi:10.1371/journal.ppat.1001172.

Kliebenstein D.J., Lim J.E., Landry L.G., Last, R.L. (2002) Arabidopsis UVR8 regulates ultraviolet-B signal transduction and tolerance and contains sequence similarity to human regulator of chromatin condensation 1. *Plant Physiology,* **130(1)**: 234-243. doi: http://dx.doi.org/10.1104/pp.005041

Supplementary Methods

Protein Isolation, quantitation, and immunoblotting

Total protein extracts of leaves were obtained from 4 week-old Col-0 plants 48 hours post inoculation with 10 mM $MgCl_2$ or 10^6 cfu ml^{-1} *PstDC3000/avrRpt2.* Leaves were frozen in liquid nitrogen, ground into a powder, suspended in extraction buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% TRITON X-100), and incubated at 4 °C on an end-over-end shaker for 30 minutes. Prior to quantitation, extracts were cleared of debris by centrifugation at 13000x g for 10 minutes at 4 °C. Total protein levels of leaf extracts were quantified using the BCA protein assay (Thermofisher) while protein levels of concentrated phloem exudates were quantified using the BioRad reagent. Both assays were performed according to the manufacturer's instructions using BSA as a standard. Immunoblots were performed as previously described (Carella et al. 2015) using 50 µg of protein from leaf extracts per lane or 1 tube of phloem exudate per lane (Champigny et al. 2013; Carella et al. 2015).

Sample preparation for mass spectrometry

Dried phloem exudates were reconstituted and each 10 µg of protein were subjected to tryptic digest using a modified filter-aided sample preparation (FASP) protocol (Wiśniewski et al., 2009; Heim et al., 2014; Molin et al., 2015). Briefly, the samples were diluted in 50 mM ammoniumbicarbonate to 100 µl and then reduced with DTT at 60°C for 30 minutes, followed by cysteine alkylation with iodoacetamide for 30 minutes at room temperature. After dilution with 8 M urea in 0.1 M Tris/HCl, pH 8.5, samples were centrifuged on a 30 kDa cut-off filter (Pall corporation). After washing with 8 M urea in 0.1 M Tris/HCl, pH 8.5, and with 50 mM ammoniumbicarbonate, the immobilized proteins were pre-digested with 1 µg Lys-C (Wako Chemicals GmbH) for 2 hours at room temperature followed by an overnight digest at 37°C with 2 µg trypsin (Promega). Peptides were collected by centrifugation, acidified with trifluoroacetic acid and stored at -20 °C.

Extraction of RNA and RT-PCR analysis

Leaf tissue from untreated and pathogen-treated 4 week-old Col-0 plants were harvested, frozen in liquid nitrogen, and stored at -80 °C. Total RNA was isolated using the Sigma TRI reagent (Sigma, St. Louis, USA) following the manufacturer's instructions. Residual DNA was degraded using the Turbo DNA-free kit (Life Technologies, Carlsbad, USA) prior to RNA quantification. cDNA was synthesized from 2 µg of total RNA with M-MLV reverse transcriptase

(Sigma, St. Louis, USA). All PCR primers and reaction conditions used in this study are included in below.

PCR primers and conditions used in this study:

Target	Primer Sequence (5'-3')	Annealing Temp (° C)	PCR Cycle #	Reference
ACT1	F: GGCGATGAAGCTCAATCCAAACG	61	26	Champigny *et al.* 2013
	R: GGTCACGACCAGCAAGATCAAGACG			
PR-1	F: GCAATGGAGTTTGTGGTCAC	61	26	Kim *et al.* 2010
	R: GTTCACATAATTCCCACGAGG			
UVR8	F: CTACTGGTGGTGAGAAAATGTC	61	26	Kliebenstein *et al.* 2002
	R: TGTTGTCCACTTGCTCCATCG			
HY5	F: GCTGCAAGCTCTTTACCATC	59	26	Brown & Jenkins 2008
	R: AGCATCTGGTTCTCGTTCTG			
COP1	F: AGTTATAGCAGAAATCCGGCATGG	61	26	This study
	R: TTAACCTTGCAGTCGTCACTACC			
TRXm1	F: CGACAGATGTTCTCTGTGTTGC	61	26	This study
	R: GATAGTTGGGATGCTTCTAACACC			
TRXm2	F: CGTTGACCTCGATTCATCAACC	61	26	This study
	R: CTATGATTGTATCCTTCTTCTCGC			
TRXm3	F: TCATCTCCGTCGCGTCTCTTCC	61	26	This study
	R: GTGGACCATCCGACATGGACC			
TRXm4	F: AGCTGCTCCGTCGGTTTCACG	61	26	This study
	R: TAGGCACAGCTCCAATGATGC			
MLP	F: TACAAGAGCTGGAAGAGCGAGAACC	61	26	This study
	R: GCCTTCAATGTCAACAACCATTTGC			

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Chapter 7

Discussion

Since much of my work has focused on the lipid transfer protein DIR1, a simplified model outlining the role of DIR1 during SAR is presented (Figure 7.1). The model depicts DIR1 at various stages of SAR, beginning in local leaves induced for SAR with *P. syringae*. SAR induction leads to the generation of putative SAR signals (DA, dehydroabietinal; G3P, glycerol-3-phosphate; AzA, azelaic acid; Pip, pipecolic acid; MeSA, methyl-salicylate) and prompts DIR1 to access the phloem, likely via symplastic plasmodesmatal connections (red arrow), for long-distance transport to distant leaves. DIR1 and other phloem-mobile proteins (in blue), as well as putative SAR signals (in black), accumulate in the phloem during SAR. DIR1 is unloaded from the phloem via plasmodesmata (red arrow) and enters distant leaf cells. Here, DIR1 is thought to contribute to the initiation of SAR priming, likely through the accumulation of pipecolic acid and salicylic acid. This leads to the activation of the SnRK2.8 kinase, which subsequently activates NPR1-mediated SAR priming. The experimental evidence relevant to this model is discussed below.

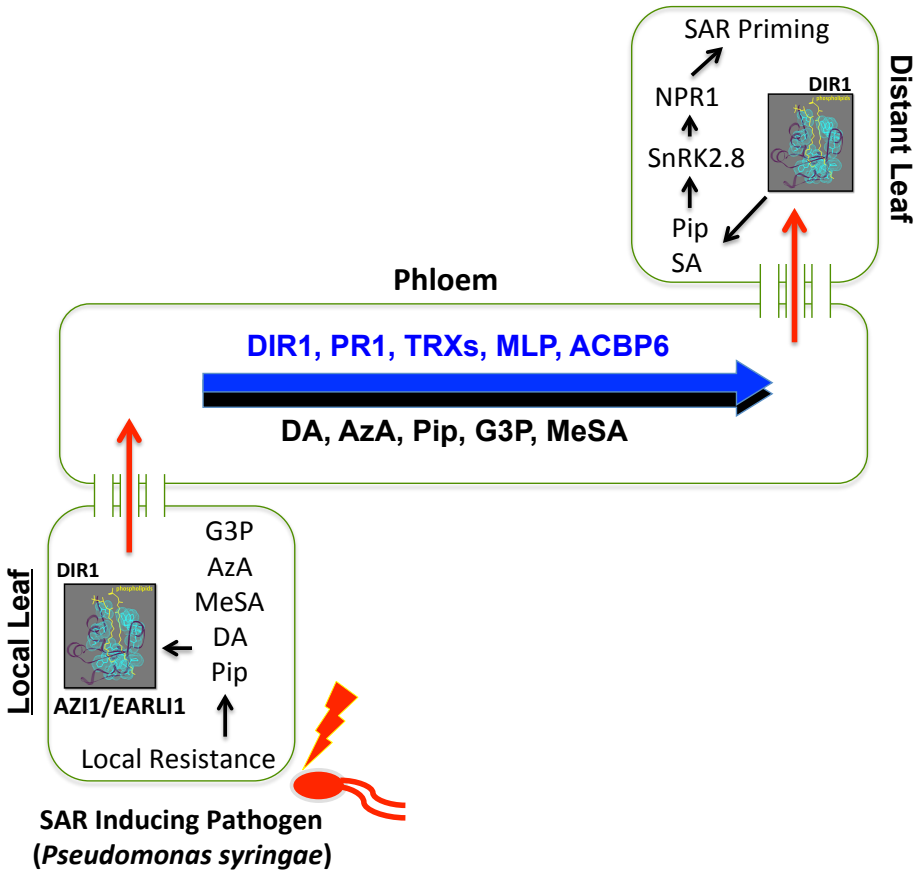

Figure 7.1 – Simplified Model of DIR1-mediated SAR in *Arabidopsis*. Local resistance responses to a SAR-inducing pathogen such as *Pseudomonas syringae* lead to the generation/activation of SAR-activating small molecules, including pipecolic acid (Pip), dehydroabietinal (DA), methyl salicylate (MeSA), azelaic acid (AzA), and glycerol-3-phosphate (G3P). The SAR-inducing activity of these molecules requires DIR1 and perhaps AZI1/EARLI1. SAR-activated DIR1 accesses the phloem symplastically through plasmodesmata (red arrow) for long-distance movement. Several proteins (blue arrow) and SAR-activating small molecules (black arrow) accumulate in the phloem and are likely transported to distant tissues. DIR1 is symplastically unloaded from the phloem via distant leaf plasmodesmata (red arrow). Here, DIR1 and other SAR signals activate downstream SAR signalling cascades that include the accumulation of Pip and salicylic acid (SA), the activation of SnRK2.8 and NPR1, and ultimately the establishment of SAR priming.

7.1. The Role of DIR1 and DIR1-like During SAR

Early experiments demonstrated that DIR1 is required for the generation and/or translocation of a long-distance SAR signal in *Arabidopsis* (Maldonado *et al.* 2002). Since this discovery, several groups have demonstrated that functional DIR1 is essential for the resistance-inducing activity of putative SAR signals (Jung *et al.* 2009; Chanda *et al.* 2011; Chaturvedi *et al.* 2012). This thesis further advances our understanding of DIR1-mediated SAR, identifying plasmodesmata as important regulators of DIR1 movement (Carella *et al.* 2015a), characterizing DIR1 orthologs and identifying conserved motifs important for DIR1 structure (Isaacs *et al.* 2016), and identifying LTP1 and LTP2 as putative DIR1-interacting proteins important for SAR. Despite these advancements, we still know little about the biochemical function of DIR1 during SAR. Moreover, our understanding of DIR1-mediated SAR is further complicated by the recent discovery of the highly similar DIR1-like protein. Below, I discuss our current understanding of DIR1 and DIR1-like function during the various stages of SAR.

7.1.1. DIR1 Localization and Function Prior to SAR-induction

Both DIR1 (AT5G48485) and DIR1-like (AT5G48490) are members of the non-specific lipid transfer protein (nsLTP) family. The hallmark characteristic of LTPs is the presence of 8 conserved cysteine residues that participate in 4 intramolecular disulphide bonds, which form a central hydrophobic cavity that accommodates lipids *in vitro* (Yeats and Rose 2008). DIR1 is thought to be an atypical member of the LTP2 family of nsLTPs, with an acidic isoelectric point (pI: 4.5) and putative protein-protein interaction motifs (PxxPxxP) (Lascombe *et al.* 2008). DIR1-like appears similarly atypical, with a predicted acidic isoelectric point (pI: 5) and a single PxxP motif (Champigny *et al.* 2013). Analysis of

DIR1pro:DIR1-GUS/*dir1-1* plant lines demonstrated that *DIR1* is expressed in all living tissues, including roots (Champigny *et al.* 2011), which is corroborated by multiple microarray experiments available via the online BAR (Bio-Analytic Resource for Plant Biology) expression analysis platform "*Arabidopsis* eFP Browser" (Winter *et al.* 2007). Expression levels of *DIR1-like* are similar to *DIR1* in *Arabidopsis* leaves, as indicated by RT-PCR analysis (Champigny *et al.* 2013; Carella *et al.* 2015) and the BAR resource (Winter *et al.* 2007). Both DIR1 and DIR1-like contain an N-terminal ER signal peptide for localization to the ER, where the establishment of the 4 intramolecular disulphide bonds is thought to occur (Frand *et al.* 2000; Onda 2013). Several subcellular localization studies have visualized DIR1-FP (fluorescent protein) fusions in plant cells. Transient *Agrobacterium*-mediated expression of DIR1-YFP fusions in healthy *N. benthamiana* epidermal cells initially demonstrated that DIR1 is targeted to the cell wall, apoplast, and perinuclear ER (Champigny *et al.* 2011). The apoplastic localization of DIR1-YFP was supported by co-localization with propidium iodide, a marker that is selectively excluded from living cells. Moreover, these localization patterns were dependent on the N-terminal ER signal sequence (SS; amino acids 1-25), as demonstrated by the cytosolic localization of a DIR1$^{\Delta 1\text{-}25}$-YFP fusion. Subsequent DIR1 localization studies visualized DIR1-RFP co-localization with GFP-tagged TMV movement protein (MP), which is often used as a molecular marker to demarcate plasmodesmata (Chanda *et al.* 2011). More recently, DIR1-RFP was observed to co-localize with the SAR-related LTPs AZI1-GFP and EARLI-GFP at plasmodesmata, perinuclear ER, and potentially at ER-chloroplast contact sites (Cecchini *et al.* 2015). These LTPs have also been demonstrated to interact with DIR1 using protein-protein interaction assays (co-immunoprecitiation and BiFC) in healthy *N. benthamiana* plants (Yu *et al.* 2013; Cecchini *et al.* 2015). In this thesis, targeted yeast-based split-ubiquitin assays using DIR1 and DIR1-like as bait demonstrated that both DIR1 and DIR1-like participate in homo- and hetero-dimer formation with one another, and that DIR1

interacts with AZI1, LTP1 and LTP2 while DIR1-like interacts with LTP2 and perhaps LTP1. Whether these proteins form complexes in *Arabidopsis*, before or during the induction of SAR, remains to be determined. Subcellular localization of DIR1-like-GFP in healthy *N. benthamiana* epidermal cells is highly similar to that of DIR1-YFP, with localization at plasmodesmata, perinuclear ER, and the cell wall/apoplast (Chapter 5). This pattern was not observed for other LTPs (LTP1-GFP and LTP2-GFP), which displayed a more uniform localization pattern at the cell periphery. This is consistent with previous reports in which LTP1 was visualized in *Arabidopsis* explants undergoing somatic embryogenesis during callus formation (reprogramming of somatic cells to embryos) (Potocka *et al.* 2012).

7.1.1.1. How are DIR1/DIR1-like Targeted to Plasmodesmata?

The localization of DIR1 and DIR1-like in the perinuclear ER and the cell wall/apoplast is in agreement with conventional thinking, given that these proteins contain N-terminal ER signal peptides. In comparison, the localization of DIR1/DIR1-like (and proteins in general) to plasmodesmata is not well understood. To date, the Plasmodesmata-Located Protein (PDLP) family of type I membrane receptor-like proteins are among the best-characterized proteins located at plasmodesmata, requiring a transmembrane domain for localization in the plasma membrane of plasmodesmata (Thomas *et al.* 2008; Amari *et al.* 2010). A number of additional proteins and protein families have been associated with plasmodesmata (Fernandez-Calvino *et al.* 2011: Salmon and Bayer 2013), including callose synthases, β-1,3-glucanases, and callose binding proteins that appear to regulate plasmodesmatal aperture by the selective deposition of callose at the neck (opening) region (Lee 2015). Interestingly, many of these proteins contain GPI (glycophosphatidylinositol) anchors that are required for

their targeting to plasmodesmata, suggesting that lipid-binding/anchoring at plasmodesmata is an important component of plasmodesmata-biology.

Consistent with this idea, lipid membranes at plasmodesmata are enriched in sterols and sphingolipids, and modifications to total sterol levels alters the GPI anchoring of PDCB1 (PLASMODESMATA CALLOSE BINDING PROTEIN1) and PdBG2 (PLASMODESMATAL β-1,3-GLUCANASE2) at plasmodesmata (Grison *et al.* 2015). Whether the localization of DIR1/DIR1-like to plasmodesmata is regulated through interactions with plasmodesmata-enriched lipid species remains to be determined. Additional plasmodesmata-located proteins include multiple C2 domain-containing proteins such as SYT1 (SYNAPTOTAGMIN), QKY (QUIRKY), and SRC2.2 (SOYBEAN RESPONSE TO COLD2.2) (discussed in Tilsner *et al.* 2016). C2 domains of plant and mammalian proteins are involved in lipid binding (Schulz and Cruetz 2004; Stahelin 2009; Tilsner *et al.* 2016) and appear to accumulate at membrane contact sites such as those between plasmodesmata and plasma membranes (Tilsner *et al.* 2016). Moreover, a recent study identified a so-called plasmodesmata localization signal (PLS) in the first 50 amino acids at the N-terminus of the TMV cell-cell Movement Protein30 (MP30) (Yuan *et al.* 2016). Currently, the identification of canonical PLS sequences in plant proteins have yet to be discovered. It is possible that such PLS sequences are important for the localization of non-transmembrane, GPI-lacking plasmodesmata-located proteins. Future studies to identify motifs important for the plasmodesmatal-localization of DIR1/DIR1-like may shed light on a novel aspect of plasmodesmata biology.

The targeting of DIR1/DIR1-like to the ER is important for localization at plasmodesmata, as a DIR1$^{\Delta 1\text{-}25}$-YFP variant lacking the ER signal peptide is cytosolically located *in planta* (Champginy *et al.* 2011). This suggests that DIR1/DIR1-like could be located inside constricted ER strands (desmotubules)

contained within plasmodesmata. Alternatively, the lack of plasmodesmatal localization of YFP-tagged DIR1$^{\Delta 1\text{-}25}$ may be due to defects in lipid-binding, since ER-signal sequence containing proteins like DIR1 are co-translated and secreted into the ER where intramolecular disulphide bonds form the lipid-binding hydrophobic cavity. Therefore, assessing the subcellular localization of GFP-tagged DIR1 variants that contain the ER signal peptide but are impacted in lipid binding (DIR1$^{\Delta CYS}$-GFP or DIR1^{L43D}-GFP) will determine whether a capacity for lipid binding is important for DIR1 localization at plasmodesmata. If lipid binding is an important aspect of DIR1 localization to plasmodesmata, it may implicate lipid species enriched at plasmodesmata such as long-chain sterol and sphingolipid fatty acids (Grison *et al.* 2015) as potential DIR1 ligands.

Additionally, DIR1 targeting to plasmodesmata may involve protein-protein interactions with *bona fide* plasmodesmatal proteins such as PDLPs or C2 domain-containing proteins. Whether DIR1/DIR1-like interact with these types of proteins is unknown, however previous research has identified a mechanism by which the C2 domain-containing FTIP1 (FT-INTERACTING PROTEIN1) protein, located in the ER and at plasmodesmata, regulates the long-distance movement of the floral regulator FT (FLOWERING LOCUS T) (Liu *et al.* 2012). Whether such a mechanism is important for DIR1 function during SAR will be discussed below in subsection 7.1.2.1.

7.1.1.2. Post-transcriptional Dynamics of DIR1

DIR1 is present at low levels before the induction of SAR and is only detected by immunoblotting of concentrated phloem exudates collected from SAR-induced wild-type plants or leaf extracts of transgenic DIR1-overexpressing plant lines. This may be explained by low levels of *DIR1* transcripts in wild-type plants (Champigny *et al.* 2011; Yu *et al.* 2013), however post-

transcriptional/translational modifications may also impact DIR1 protein accumulation. One study has recently suggested an association between G3P and DIR1 levels, such that *DIR1* transcript and protein levels are reduced in the G3P-deficient *gly1* and *gli1* mutants. Moreover, exogenous G3P application increased DIR1-GFP (but not *DIR1-GFP* mRNA*)* levels in locally treated and systemic leaves of *DIR1pro:DIR1-GFP* Col-0 plants (Yu *et al.* 2013). This phenomenon was also observed for AZI1, leading the authors to speculate that G3P accumulation stabilizes *DIR1* and *AZI1* transcripts allowing for increased translation (Yu *et al.* 2013). However, the mechanism by which G3P stabilizes *DIR1* is unclear. High G3P levels are associated with the accumulation of DIR1 protein without increased *DIR1* expression suggesting that G3P either increases the translatability of *DIR1* transcripts or perhaps prevents targeted degradation of DIR1 protein. On the other hand, decreased *DIR1* transcript levels in G3P-deficient mutants suggest that G3P is required to prevent the degradation of *DIR1* transcripts. While the authors suggest that G3P is required only to stabilize *DIR1* transcripts, the possibility of G3P-mediated protection of DIR1 protein from proteasomal degradation remains a possibility. The results described in this study are in direct contrast to data pertaining to *DIR1* mRNA and protein levels during local interactions with *P. syringae* in the Ws-2 ecotype of *Arabidopsis* (Champigny *et al.* 2011). This will be discussed in more detail in section 7.1.2.

7.1.1.3. Importance of DIR1/DIR1-like Before SAR-induction

Efforts to create a *dir1/dir1like* double mutant have been unsuccessful thus far. Marisa Isaacs previously attempted to knockdown *DIR1-like* levels in the *dir1-1* mutant background using antisense technology (35S:antisense*DIR1-like*/*dir1-1),* but failed to identify viable lines in the T3 generation (discussed in Champigny *et al.* 2013). To determine if this result was due to ineffective suppression of *DIR1-*

like expression in the antisense lines, Marisa created *35S:DIR1-like*^{RNAi} constructs and transformed *dir1-1,* Ws-2, and Col-0 plants. I took over this project when Marisa graduated and isolated *DIR1-like* knockdown lines in the Col-0 accession of *Arabidopsis*. Col-0 exhibits more robust growth (in our conditions) and is used by *Arabidopsis* researchers more often than the Ws-2 ecotype. Of the two *DIR1-like* knockdown lines obtained, the *35S:DIR1-like*^{RNAi} line with the lowest *DIR1-like* levels (Line 5i) was crossed with *dir1-2* (Col-0 background) to generate a *dir1/dir1like* double mutant. Although F1 plants containing both the *dir1-2* and *35S:DIR1-like*^{RNAi} loci were recovered, molecular (PCR) analysis of >70 F2 progeny failed to identify homozygous *dir1-2* plants that also carry the *35S:DIR1-like*^{RNAi} locus. Given that both of our attempts to create a *dir1/dir1like* line have failed, we hypothesize that the combined loss of both *DIR1* and *DIR1-like* is lethal.

The potential lethality of *dir1/dir1like* double mutants suggests that DIR1 and DIR1-like have an essential, non SAR-related function in development. LTPs are implicated in several biological processes in plants, including the regulation of stress responses and the control of growth and development (reviewed in Kader 1996; Yeats and Rose 2008). Our research with DIR1 and DIR1-like suggest that these LTPs carry out multiple functions as signalling agents and potential regulators of an essential, yet to be determined developmental process. Circumstantial evidence from our lab suggests that this function may be related to seed development/longevity, as *dir1-1* mutant seed exhibits reduced longevity and germination efficiency on soil compared to MS media (unpublished). Though speculative, this idea is supported by the accumulation of LTPs in the seeds of several plants (Kader 1996; Yeats and Rose 2008) and the observation that *DIR1* and *DIR1-like* are expressed during seed development in *Arabidopsis* (BAR eFP Browser; Winter *et al.* 2007). Moreover, LTPs share similarity with seed storage 2s albumin proteins that act as an important nutrient source in seeds (reviewed in

Moreno and Clemente 2008). Moving forward, the lethality of the *dir1-2/35S:DIR1-like*$_{RNAi}$ plant line should be examined by appropriate genetics analysis. If supported, a conditional *DIR1-like*^{RNAi} knockdown line using estrogen- or dexamethasone-inducible expression systems (Aoyama and Chua 1997; Zuo *et al.* 2000) should be generated in the *dir1-2* (Col-0) mutant to explore the redundancy of DIR1 and DIR1-like in SAR and development.

7.1.2. Activation of DIR1 for Movement During SAR

The idea that DIR1 is mobilized to the phloem for long-distance transport to distant leaves during SAR is supported by numerous observations of DIR1 accumulation in phloem exudates collected from SAR-induced but not mock-inoculated leaves of wild-type plants (Champigny *et al.* 2013; Shah *et al.* 2014; Carella *et al.* 2015a, 2016). Importantly, estradiol-induced *DIR1-GFP* expression in SAR-induced local leaves of XVE:DIR1-GFP/*dir1-1* plants rescues the *dir1-1* SAR defect and DIR1-GFP is detected in phloem exudates collected from local and distant leaves of SAR-induced plants (Champigny *et al.* 2013). This type of analysis was important to demonstrate that DIR1 moves from induced to distant leaves during SAR, given that DIR1 is expressed in all aerial tissues in *Arabidopsis* (Champigny *et al.* 2011). DIR1 accumulation in SAR-induced phloem exudates occurs despite T3SS-dependent suppression of *DIR1* transcripts in Ws-2 plants inoculated with virulent or avirulent strains of Pst (Champigny *et al.* 2011, 2013). Recently, a conflicting report demonstrated the absence of T3SS-dependent *DIR1* suppression in Col-0 plants infected with avirulent Pst (Yu *et al.* 2013), which suggests that genetic differences between the two ecotypes, or perhaps differences in the growth conditions used in these studies, impacts *DIR1* transcript levels. In any case, both studies support the idea that DIR1 protein accumulates without the upregulation of *DIR1* transcripts. Exactly how DIR1

accumulates in phloem exudates is currently unknown, however it may involve a combination of increased protein/mRNA stability and mobilization of DIR1 from mesophyll to phloem cells. In support of this idea, Yu *et al.* (2013) demonstrated that local infections with avirulent Pst(DC3000/avrRpt2) or exogenous application of the SAR-inducing small molecule G3P leads to increased DIR1-GFP protein but not mRNA levels in local and distant leaves of DIR1pro:DIR1-GFP plants via fluorescence microscopy and immunoblot analysis, suggesting that posttranscriptional dynamics impact DIR1 accumulation during SAR. This finding is somewhat controversial, as our lab has failed to observe DIR1 accumulation in leaf extracts of local or distant leaves responding to SAR-induction by avirulent Pst and DIR1-fusion proteins (-YFP, -EGFP, -GUS) are often cleaved during SAR (Champigny *et al.* 2011, 2013). Differences in growth conditions such as photoperiod length (9 hr short days for Ws-2 work; 12 hr photoperiod for Col-0), in addition to genetic differences between the Ws-2 and Col-0 accessions used in these studies may influence the stability of DIR1 (-fusion) proteins. Future studies to examine DIR1 fusion stability during SAR must examine both accessions grown in similar conditions to clarify this issue.

7.1.2.1. How Does DIR1 Access the Phloem?

Subcellular localization studies place wild-type DIR1 in the perinuclear ER, apoplast, cell wall, and at plasmodesmata in *N. benthamiana* and *Arabidopsis* (Champigny et al 2011; Chanda *et al.* 2011; Yu *et al.* 2013; this thesis). Constitutive expression of non-secreted DIR1$^{\Delta 1\text{-}25}$ rescues the *dir1-1* SAR-defect, suggesting that a cytosolic pool of DIR1 may be important during SAR (Champigny *et al.* 2011). The cytosolic localization of DIR1 is consistent with the current understanding of protein mobilization into the phloem, in which proteins synthesized in companion cells move symplastically (cell-to-cell) through

plasmodesmata to access sieve elements for long-distance transport (Imlau *et al.* 1999; Stadler *et al.* 2005; Turgeon and Wolfe 2009; Lucas *et al.* 2013). Based on this knowledge, I hypothesized that cell-to-cell symplastic movement of DIR1 is important during long-distance SAR signalling. Analysis of DIR1 movement and SAR in *Arabidopsis* lines with restricted molecular traffic through plasmodesmata caused by PDLP1/5 overexpression supported the idea that symplastic movement through plasmodesmata is required for DIR1 to access distant leaves during SAR (Carella *et al.* 2015a). However, the impact of altered signalling at plasmodesmata caused by PDLP1/5 overexpression cannot be ruled out, as PDLP5 and PDLP1 are receptor-like proteins that have been associated with local defense responses to *P. syringae* and *H. arabidopsidis*, respectively (Lee *et al.* 2011; Wang *et al.* 2013; Caillaude *et al.* 2014). In any case, the physical restrictions placed on plasmodesmatal pores by PDLP-overexpression (Thomas *et al.* 2008; Lee *et al.* 2011; Wang *et al.* 2013) is likely the main factor contributing to suppressed DIR1 movement during SAR, especially if DIR1 moves as part of a larger proteinacious complex (Shah *et al.* 2014).

How SAR induction affects DIR1 subcellular localization is unclear. While DIR1 is normally targeted to the ER, apoplast, and plasmodesmata, it is detected in phloem exudates collected from SAR-induced plants, which suggests that cytosolic localization occurs at some point. How DIR1 accesses the phloem during SAR is currently unknown; however, recent research on the long-distance movement of the floral regulator FT may shed light on this issue. Upon the perception of long-day photoperiods, FT protein accesses the phloem for long distance movement to the shoot apical meristem (SAM) to induce the transition from vegetative to reproductive development (Corbesier *et al.* 2007; Jaeger and Wigge 2007). FTIP1, an ER and plasmodesmata-located C2 domain-containing protein, interacts with FT in phloem cells and is essential for the long-distance movement of FT during the floral transition (Liu *et al.* 2012). The authors suggest

that FT and FTIP1 interact inside the ER, with FT moving cell-to-cell through the plasmodesmata desmotubule (contiguous ER) and FTIP1 facilitating the accumulation of FT in the cytosol of sieve elements by some unknown mechanism (Liu *et al.* 2013). However, the authors could not rule out that cytosolic FT in companion cells interacts with FTIP1 for cell-to-cell movement through the cytosolic "sleeve" of plasmodesmata (discussed in Liu *et al.* 2012, 2013). This research underscores the complexity of subcellular localization dynamics in the phloem and implicates C2 domain-containing proteins as important regulators of symplastic movement in the phloem.

Whether DIR1 interacts with a C2 domain-containing protein like FTIP1 remains to be determined. Given that other C2 domain-containing proteins like SYTA (SYNAPTOTAGMIN) and QKY (QUIRKY) have been implicated as positive regulators of the symplastic movement of viruses and developmental signals in the phloem (Levy *et al.* 2010; Vaddepalli *et al.* 2014), it is tempting to speculate that DIR1 interacts with a C2 domain-containing protein for cell-to-cell movement during SAR. Future research to understand the mechanisms by which C2 domain-containing proteins facilitate the movement of proteins into the phloem may identify fundamental processes in cell biology important for protein mobilization into the phloem. Such mechanisms may rely on interactions with lipids, as C2 domain-containing proteins are thought to interact with lipids (Schapire *et al.* 2008; Stahelin 2009; Giordano *et al.* 2013; Perez-Sancho *et al.* 2015; Tilsner *et al.* 2106) and the mobile proteins FT and DIR1 both interact with lipids *in vitro* (Lascombe *et al.* 2008; Namamura *et al.* 2014).

7.1.2.2. Interactions With Other SAR Regulators

Several studies support the idea that DIR1 is a central component of the long-distance signalling phase of SAR. The candidate SAR signals azelaic acid, G3P, and dehydroabietinal all require functional DIR1 for their ability to induce SAR (Jung *et al.* 2009; Chanda *et al.* 2011; Chaturvedi *et al.* 2012). Moreover, *BSMT1* expression and MeSA levels are elevated during the induction of SAR in *dir1-1* compared to wild-type controls (Liu *et al.* 2011). This phenotype may be associated with the inability of *dir1-1* mutants to accumulate pathogen-triggered G3P, as the application of exogenous G3P suppresses *BSMT1* expression in wild-type plants (Chanda *et al.* 2011). The absence of G3P accumulation in *dir1-1* may therefore be responsible for altered MeSA levels in *dir1-1.* This idea could be examined by analyzing MeSA and *BSMT1* levels in Pst-inoculated *dir1-1* plants supplied with exogenous G3P.

How DIR1 is involved in G3P accumulation is unknown. G3P is synthesized in the cytosol by the phosphorylation of glycerol via the GLI1 glycerol kinase, or in plastids by G3P dehydrogenase enzymes that convert dihydroxyacetonephosphate (DHAP) to G3P (Venugopal *et al.* 2009; Chanda *et al.* 2011). Recently, it has been suggested that G3P accumulation also requires membrane lipid peroxidation via interactions with reactive oxygen species (ROS). The authors hypothesize that pathogen-triggered ROS interact with MGDG (monogalactosyldiacylglycerol) membrane lipids to generate AzA, which in turn is converted to (or induces the synthesis of) G3P (Gao *et al.* 2014; Wang *et al.* 2014). How DIR1 fits into this model is not clear, however DIR1 may bind released membrane lipids given that DIR1 is an LTP. DIR1 may also be involved in the AzA-induced accumulation of G3P (Yu *et al.* 2013; Gao *et al.* 2014; Wang *et al.* 2014), which could explain why DIR1 is required for the SAR-inducing ability of AzA (Jung *et al.* 2009). Given that *azi1* mutants are also defective in

pathogen-triggered G3P accumulation and DIR1 interacts with AZI1/EARLI1 it is possible that the combined action of DIR1, AZI1, and perhaps EARLI1 is required for signal generation or translocation during SAR. Whether DIR1-like, LTP1, or LTP2 are also involved in this aspect of DIR1-mediated SAR should be assessed in the future, perhaps by analyzing pathogen-induced G3P accumulation in the corresponding mutant and/or overexpressing lines. Examining long-distance DIR1-like movement upon exogenous G3P application is also important. Given that DIR1-like sometimes compensates for DIR1, it can be hypothesized that DIR1-like movement during SAR occurs irrespective of G3P levels. If this were the case, it would explain why DIR1-like is capable of moving through the phloem in *dir1-1* mutants that are defective in pathogen-induced G3P accumulation, providing clarity on how DIR1-like compensates for DIR1 in *dir1-1* mutants. However, it is likely that additional environmental factors are also contributing since DIR1-like only compensates for *dir1-1* on some occasions (Champigny *et al.* 2013).

DIR1 co-purifies with the SAR-activator DA (dehydroabietinal) from SAR-activated phloem exudates (Shah *et al.* 2014) and is required for its ability to induce SAR (Chaturvedi *et al.* 2012). Moreover, DIR1 is present in a high molecular weight complex in phloem exudates collected from SAR-induced plants suggesting that DIR1 and other proteins are required for the transport or activation of DA. It is possible that one or more of the SAR-enriched phloem proteins identified in our proteomics analysis are additional members of this complex. DIR1 may bind DA directly or may be required for the formation of the protein complex, with other phloem-mobile lipid-binding proteins (ACBP6, MLP) acting as signal chaperones. In any case, it appears that DIR1 is a central component of the hypothesized long-distance signal complex. Future studies to determine how the SAR-enriched phloem proteins ACBP6, MLP, TRXm1/4, and PR1 contribute to DIR1-mediated SAR may improve our understanding of this

complex. Targeted interaction assays with DIR1 in yeast and *N. benthamiana* could be performed to explore this idea.

An understudied aspect of DIR1-mediated SAR is the role of pipecolic acid (Pip) in the activation and/or translocation of DIR1. Analysis of DIR1 movement in the Pip-deficient mutant *ald1,* in addition to determining whether exogenous Pip application induces DIR1 movement in wild-type plants, would begin to clarify this issue. Moreover, how Pip interacts with other candidate SAR signals such as G3P, AzA, and DA is also unknown (discussed in Gao *et al.* 2015). Pipecolic acid appears to be an essential regulator of the induction of SAR priming in distant leaves (Navarova *et al.* 2012; Bernsdorff *et al.* 2015), therefore any overlap between Pip and DIR1/G3P/AzA/DA function may occur in distant leaves during signal perception and defense priming.

7.1.3. What is the Role of DIR1 in Distant Leaves?

Studies to explore DIR1's function during SAR have focused on DIR1 mobilization into the phloem for long-distance transport. Thus, our current understanding of DIR1 function in distant leaves is severely limited. An early hypothesis suggested that DIR1 is required for the generation and/or translocation of SAR signals (Maldonado *et al.* 2002). Several lines of evidence support this idea; DIR1 travels to distant leaves during SAR (Champigny *et al.* 2013; Carella *et al.* 2015a), *dir1* mutants are defective in the generation of pathogen-triggered G3P (Chanda *et al.* 2011), and functional DIR1 is required for the activity of several SAR-inducing small molecules (Jung *et al.* 2009; Chanda *et al.* 2011; Chaturvedi *et al.* 2012). These lines of evidence suggest that DIR1 links signal generation with SAR priming in distant leaves. In general, proteins and other phloem contents are symplastically unloaded from the phloem in sink

leaves, moving through sieve element plasmodesmata to access companion cells, and then from companion cells to phloem parenchyma (discussed in Turgeon and Wolf 2009). Based on this current thinking, I hypothesize that DIR1 is symplastically unloaded in distant leaf phloem, moving symplastically from sieve elements to companion cells, then into phloem parenchyma. From there, DIR1 may move symplastically into mesophyll cells to deliver SAR signals and activate defense priming (discussed in Carella *et al.* 2015b). This idea is supported by the SAR-defective phenotype of PDLP-overexpressing plants that fail to accumulate DIR1 in distant leaf petioles during SAR (Carella *et al.* 2015a). However, the plasmodesmata of all cell-types are restricted in these lines, preventing identification of the precise cell type(s) that DIR1 is moving through during SAR. Therefore, a more controlled approach that would allow for the conditional occlusion of plasmodesmata specifically in phloem versus mesophyll cells is required to obtained a more detailed understanding of DIR1 movement during SAR. It is also possible that SAR-activated DIR1 is directly detected in distant leaf phloem cells (companion cells or phloem parenchyma), leading to the generation of secondary SAR signals that travel throughout the distant leaf to activate defense priming. Pipecolic acid is currently the best candidate for such a signal, as its accumulation in distant leaves is required to activate defense priming (Navarova *et al.* 2012). Interestingly, *in vitro* TNS displacement assays demonstrated that Pip weakly interacted with the cucumber DIR1 ortholog CsDIR1, which led us to speculate that Pip may induce allosteric changes in DIR1 cavity conformation (Isaacs *et al.* 2016). However, this weak interaction was not observed for AtDIR1 and the *in vitro* TNS displacement assay alone is not sufficient to detect subtle conformational changes (discussed in Isaacs *et al.* 2016).

DIR1 in distant leaves may interact with a SAR-receptor protein to activate defense priming. Alternatively the DIR1-containing mobile SAR complex may

dissociate in distant leaves, releasing mobile signals that activate downstream components of SAR. Currently, DIR1 has been shown to interact with SAR-related LTPs such as AZI1, EARLI1, DIR1-like, and LTP1/LTP2 in targeted yeast 2-hybrid and plant-based interaction assays (Yu *et al.* 2013; Cecchini *et al.* 2015; this work). It is intriguing to speculate that an interaction between DIR1 and any of these proteins contributes to SAR signal perception in distant leaves. It is likely that DIR1/AZI1/EARLI1 interactions are required only in local leaves given that *AZI1*/*EARLI1* expression is only required during the induction phase of SAR (Cecchini *et al.* 2015). Although little is known regarding LTP1/LTP2 function during SAR it is tempting to hypothesize that DIR1 interacts with these proteins in distant leaves to induce SAR. Further studies with LTP1/LTP2 knockdown and overexpression lines should be aimed at determining whether LTP1/LTP2 interact with DIR1 *in vivo* and if these proteins are required for the perception of SAR signals. If LTP1/LTP2 are not involved in the signal perception stage of SAR, other DIR1-interacting proteins may be responsible for detecting DIR1 in distant leaves. Given that DIR1 contains a tandem PxxP motif, which is associated with protein-protein interactions with SH3 domain-containing proteins (Li 2005), it is possible that DIR1 interacts with one of three *Arabidopsis* SH3-like proteins (Lam *et al.* 2003). T-DNA knockout mutants in these lines should be tested for SAR competence to determine if they are required for DIR1-mediated SAR.

Lastly, the activation of SAR priming may not require an interaction between DIR1 and a dedicated receptor in distant leaves, but instead may involve changes in DIR1-ligand binding affinity. In distant leaves there may be an exchange of lipids in the DIR1 hydrophobic cavity such that a compound with higher affinity replaces the bound SAR signal from the DIR1 cavity, allowing for its detection and the induction of SAR. It is also possible that protein-protein interactions or post-translational modifications induce a conformational change in DIR1 structure that releases the DIR1 ligand/SAR signal for detection by an

unknown SAR receptor protein. However, it is important to note that these ideas are entirely speculative, as *in vivo* DIR1 ligands have not been identified.

7.1.4. Future Studies to Identify DIR1-interactors

The identification of DIR1-interacting proteins and/or small molecules is important to provide insight into DIR1 function during SAR. As discussed above, putative DIR1-interacting proteins may be involved in the activation of DIR1 during the induction of SAR in local leaves, the transport of a DIR1-containing signal complex through the phloem, or the perception of DIR1 and/or it's ligand(s) in distant leaves. Moreover, the hypothesized DIR1 ligand may be a novel SAR-inducing compound with commercial potential in agriculture, making its identification of significant academic and industrial value. This thesis described two methods to identify SAR-relevant proteins and small molecules that interact with DIR1. Yeast and plant-based interaction assays identified DIR1-like, LTP1, and LTP2 as putative DIR1-interacting proteins, which was not entirely surprising as DIR1 was demonstrated to interact with LTP(-like) proteins in other studies (Yu *et al.* 2013; Cecchini *et al.* 2015). TNS-based fluorescence displacement assays failed to identify interactions between AtDIR1 and known SAR-activators (AzA, Pip, and G3P), indicating that DIR1 either binds a different/novel SAR-activator or perhaps requires additional *in vivo* components, like the hypothesized high molecular weight complex, in order for binding to occur. While these studies further our knowledge of DIR1-mediated SAR, more comprehensive approaches are required to identify DIR1-interactors. Potential approaches for future investigations include a saturated split-ubiquitin screen using a DIR1 bait strain and an *Arabidopsis*-ORF prey library, biochemical screening of recombinant DIR1 protein against chemical libraries using techniques such as surface plasmon resonance (SPR), and/or MS-based identification of proteins/molecules

that co-immunoprecipitate with DIR1. Since yeast-based interaction screens have limitations that include the promiscuous binding of LTPs (see Chapter 5 discussion) and it is challenging to collect sufficient amounts of folded recombinant DIR1 for chemical screening, co-immunoprecipitation (co-IP) of epitope-tagged DIR1 is the most viable option.

To this end, 35S:DIR1-FLAG overexpression lines were generated in the Col-0 background for future use in co-IP-based interactor screens (Figure A1). Characterization of these lines demonstrates that the DIR1-FLAG fusion is stable *in planta,* which may indicate that our previous issues with fusion cleavage were due to the addition of large tags (YFP/GUS/GFP). Alternatively, this result may support the idea that DIR1 fusion proteins are more stable in Col-0 compared to Ws-2. The DIR1-FLAG overexpression line also conditionally expresses the *P. syringae* effector AvrRpt2 in the wild-type (Col-0) and *rps2* mutant backgrounds using the estrogen-inducible expression system (Tsuda *et al.* 2012). Since the detection of AvrRpt2 by the plant RPS2 resistance receptor leads to the induction of SAR, this system allows for the chemical (estrogen) induction of SAR in Col-0, but not in the *rps2* mutant background. As such, DIR1-FLAG can be isolated from plants that are competent (XVE:AvrRpt2/Col-0) or incompetent (XVE:AvrRpt2/rps2) for SAR upon the application of estrogen (Figure A1). A key advantage of this system is that SAR can be efficiently and easily induced in a large number of leaves by spraying plants with estrogen. This feature aids in the collection of large volumes of phloem exudates, which are needed to obtain high levels of DIR1-FLAG suitable for immunoprecipitation. Preliminary co-IP experiments using these lines demonstrated that DIR1-FLAG could be isolated from total protein extracts of estrogen-treated seedlings using a commercial FLAG-tag affinity matrix (Figure A2). However, high levels of background (non-specific binding of proteins to the affinity matrix) detected during LC-MS/MS analysis prevented the identification of DIR1-interacting proteins, demonstrating

the need for further optimization. Therefore, immunoprecipitating DIR1-FLAG from SAR-activated phloem exudates rather than leaf tissue may be more suitable for MS-based interactor identification, as the protein profiles of phloem exudates are less complex in comparison to total protein extracts derived from leaves.

An additional concern is that DIR1 itself appears to be recalcitrant to MS-based protein detection, as purified recombinant DIR1 protein is not detected by LC-MS/MS analysis (Carella *et al.* 2016a). One hypothesis to explain this observation is that DIR1 may be resistant to the action of proteases, similar to other LTPs (Lindorff-Larsen and Winther 2001; Scheurer *et al.* 2004). This would effectively prevent DIR1 protein from being digested into small tryptic fragments, which is required for protein detection using LC-MS/MS. An additional challenge for MS analysis of co-IPs is the optimization of protein extraction buffers. Strong detergents and denaturing buffers disrupt non-covalent protein-protein and protein-ligand interactions, while weak buffers may not fully solubilize leaf tissue, preventing efficient immunoprecipitation. Therefore, it may be difficult to balance co-IP conditions for the successful identification of DIR1-interacting proteins, especially if DIR1 interacts with membrane localized receptor proteins. An alternative approach to identify DIR1-interacting proteins should be considered. The birA* biotinylation system is as an excellent candidate for future DIR1-interactor screens. This system is based on a promiscuous biotin ligase enzyme from *E.coli* (birA*) that, when fused to a protein of interest, biotinylates proximally located proteins (Roux *et al.* 2012). An advantage of this technique over co-immunoprecipitation is that strong detergents/denaturants can be used to extract total proteins since the birA* technique does not rely on keeping protein-protein interactions intact, but rather on the purification of biotinylated proteins using streptavidin-based biotin purification matrixes, followed by LC-MS/MS-based protein identification. DIR1 proximal proteins identified by this approach may be

direct DIR1-interactors or members of the SAR long-distance signal complex. The vector required to create the DIR1-birA* fusion is publicly available on Addgene (Plasmid #36047) and should be considered as a viable alternative to co-IP-based approaches for the identification of DIR1-interactors.

7.2. Exploring the SAR Phloem Proteome

With the exception of DIR1, very little is known in regard to protein movement through the phloem during SAR. We therefore collaborated with Drs. Corina Vlot, Stefanie Hauck and Juliane Merl-Pham of the Helmholtz Zentrum in Munich (Germany) to perform comparative proteomics on phloem exudates collected during SAR (Chapter 6, Carella *et al.* 2016a). Through this analysis, 16 proteins that consistently accumulated in phloem exudates during SAR-induction were identified, as well as 46 proteins that decreased in abundance. Of these proteins, putative roles in SAR were determined for m-type thioredoxins (TRXm1, TRXm4), a major latex protein-like protein (MLP), the UV-B photoreceptor UVR8 and its downstream components HY5 and COP1. Moreover, proteins previously associated with SAR such as ACBP6, PR1, and TRXh3 were also identified, hinting at novel roles for these proteins in the phloem during SAR. How each of these proteins contribute to long-distance SAR signaling is not yet clear, but several hypotheses are explored to explain these results.

It is tempting to hypothesize a role for these proteins in the long-distance movement of hydrophobic SAR signals, as is predicted for DIR1. Given that DIR1 and the SAR-activator DA co-fractionate with a proposed high-molecular weight signal complex, it is likely that some of the proteins identified in the SAR phloem exudate proteome are additional members of this complex. Two plausible candidates include the lipid-binding protein ACBP6 and the BetvI-fold containing

protein MLP. As discussed above, *acbp6* and *mlp* mutants are defective in SAR, suggesting a functional role for these proteins in the phloem during SAR. Such a role may involve the interactions with hydrophobic SAR signals like DA, since ACBP6 has demonstrated lipid-binding (acyl CoA and phosphatidylcholine) activity *in vivo* (Engeseth *et al.* 1998; Chen *et al.* 2008) and MLP contains a BetvI fold that forms a large hydrophobic cavity capable of binding hydrophobic ligands (Gajhede *et al.* 1996; Radauer *et al.* 2008). In addition, ACBP6 and MLP may interact with DIR1, as DIR1 appears to have an affinity for protein-protein interactions with other lipid-binding proteins (AZI1, EARLI1, DIR1-like, LTP1/2) (Yu *et al.* 2013; Cecchini *et al.* 2015; this thesis). Together, these observations suggest a role for ACBP6 and MLP in SAR signal movement via a large proteinacious complex.

Given the importance of the phloem for long-distance communication, it is likely that several housekeeping/maintenance proteins are required to prevent oxidative damage to structural and/or signalling proteins during stress. This idea appears to be especially important for phloem sieve elements (Walz *et al.* 2002), which cannot easily replace damaged proteins due to a lack of nuclei and protein synthesis machinery (Turgeon and Wolf 2009). Several proteins identified in the SAR phloem proteome had molecular functions associated with oxidative stress tolerance, and as such, may protect the proteinacious components of the mobile signal complex. The TRXm1, TRXm4, and TRXh3 thioredoxins may be especially important since the corresponding mutants are negatively impacted in SAR (Tada *et al.* 2008; Carella *et al.* 2016a). Thioredoxins are involved in the redox regulation of cysteine-containing proteins, often acting as important components in the protection against oxidative stress (Dos Santos and Rey, 2006). Specifically, these proteins can act to repair proteins with oxidative damage as well as prevent oxidative damage by providing reducing power to detoxifying enzymes such as peroxiredoxins that limit the accumulation of peroxides

(discussed in Dos Santos and Rey 2006; Meyer *et al.* 2008; Collett and Messens 2010). In non-phloem cells, the cytosolic thioredoxins TRXh3 and TRXh5 are thought to contribute to SAR by regulating the oligomer-to-monomer transition of NPR1 via the reduction of inter-molecular disulphide bonds (Tada *et al.* 2008). Whether TRXm1/4 and TRXh3 regulate NPR1 function in phloem cells is currently unknown, but remains a possibility. It is likely that phloem thioredoxins protect signalling proteins from oxidative damage, as both proteins and free radicals co-accumulate in the phloem (Wang *et al.* 2014; Carella *et al.* 2016a; Gaupels *et al.* 2016).

Many of the phloem proteins that decreased in abundance during SAR were associated with metabolism, which is consistent with previous reports demonstrating that responses to pathogen-infection (including SAR-induction) prioritize defense over normal growth and development (Ward *et al.* 2010; Gruner *et al.* 2013; Bersndorff *et al.* 2016). In addition, several proteins associated with biotic and abiotic stress were observed, including Annexin1, CPN60B, JR1, GRP7, TGG1, ERD14, and UVR8. A role for UVR8 during SAR was identified, as both the *uvr8-6* mutant and a 35S:UVR8 overexpression line were compromised in the manifestation of SAR (Carella *et al.* 2016a). Further, two downstream signalling components in the UV-B response pathway, COP1 and HY5, were similarly required for the full manifestation of the SAR response. Previous studies also implicated light signalling machinery as an important component of SAR, identifying SAR defects in red and blue light photoreceptor mutants *phyB* and *cry1*, respectively (Griebel and Zeier 2008; Wu and Yang 2010). Moreover, the duration of light experienced by SAR-induced plants has been shown to impact SAR, such that plants induced for SAR in the evening (less, or no light after infection) require the SAR activator MeSA, while plants induced in the morning (receives more light after infection) do not (Liu *et al.* 2011). Why this occurs is not clear; however, water flow in the vasculature is reduced during the night, which

may dictate a need for volatile SAR signals like MeSA. Therefore, it is likely that light perception as well as physiological factors influence long-distance SAR signal movement. Whether light signalling machinery is required for SAR in phloem cells is an interesting idea, and is supported by the fact that we identified UVR8 in phloem exudates. However, it is likely that light signalling impacts all tissues (local leaf, phloem, distant leaf) throughout the SAR response given the fact that several photoreceptors are required for SAR. Alternatively, the lack of SAR in these mutants may implicate common downstream light signalling machinery such as COP1 or HY5 as central regulators of the SAR response. Future experimentation to determine whether COP1 and HY5 are required for the generation, translocation, or perception of SAR signals will clarify the role of light signalling during SAR.

7.3. An Emerging Role for Lipid-binding Proteins in the SAR Response

Several studies have identified roles for lipid-binding proteins in SAR, including DIR1 and DIR1-like (Maldonado *et al.* 2002; Champigny *et al.* 2013), AZI1 (Jung *et al.* 2009), EARLI1 (Cecchini *et al.* 2015), ACBPs (Xia *et al.* 2012), and perhaps DOUBLE HYBRID PROLINE-RICH PROTEIN1 (DHyPRP1, Li *et al.* 2014). Joining this list are the MLP and LTP2 lipid-binding proteins described in this thesis. Several groups postulate the importance of lipid-derived or lipid-like SAR long-distance signals, which is suggested by the requirement of plastidial lipid biosynthesis enzymes (Nandi *et al.* 2004; Chaturvedi *et al.* 2004), lipid precursors (G3P, Chanda *et al.* 2011), lipid fragmentation products (AzA, Jung *et al.* 2009), cuticular lipids (Xia *et al.* 2010, 2012), and membrane lipids (Gao *et al.* 2014) for the full establishment of SAR. SAR-related lipid-binding proteins may therefore be involved in the generation, translocation, and perception of lipidic SAR signals.

Lipid-binding proteins associated with SAR signal generation include AZI1, EARLI1, ACBPs, and perhaps DIR1. The ACBP proteins ACBP3, ACBP4, and ACBP6 have been implicated in SAR signal generation, as mutants lacking these proteins fail to generate long-distance SAR signals (Xia *et al.* 2012). In addition, AZI1 and DIR1 are thought to contribute to the generation of the SAR activator G3P through an undetermined mechanism (Yu *et al.* 2013). A recent study further demonstrated that AZI1/EARLI1 are required for SAR signal mobilization to distant tissues, and that these proteins localize to ER-plasmodesmata and ER-plastid contact sites during SAR (Cecchini *et al.* 2015). Moreover, the authors identified co-localization of AZI1/EARLI1 with DIR1 at ER-plastid contact sites (Cecchini *et al.* 2015). An intriguing hypothesis to explain this data is that lipid-binding proteins are required to shuttle lipidic signals through different subcellular compartments during SAR. In such a scenario, plastid-derived lipid signals could be transferred from AZI1/EARLI1 to DIR1 at plastid-ER contact sites, followed by the symplastic movement of liganded DIR1 into the phloem for long-distance transport to distant leaves. How ACBPs participate in such a mechanism is unknown. ACBP3 is localized in the apoplast, while both ACBP4 and ACBP6 are present in the cytosol (Xiao and Chye 2009). It may be that ACBP3 is important to shuttle membrane or extracellular (cuticular) lipids into the cytosol using ACBP4/ACBP6, with ACBP6 being especially important in the phloem. The idea of lipid-binding proteins acting as shuttles for intracellular transport through different subcellular compartments has been discussed as a possibility (Hurlock *et al.* 2014; Li *et al.* 2016) and may contribute to SAR.

The translocation of lipid-derived or lipid-like SAR signals through the phloem likely requires the action of lipid-binding proteins, given that the phloem translocation stream is an aqueous environment (discussed in Benning *et al.* 2012; Barbaglia *et al.* 2016). Consistent with this idea, lipid-binding proteins have been identified in phloem exudates collected from healthy plants (Guelette *et al.*

2012; Barbaglia *et al.* 2016; Carella *et al.* 2016a,b) as well as plants induced for SAR (Champigny *et al.* 2013; Carella *et al.* 2016a). DIR1, ACBP6, and MLP have been identified in phloem exudates collected from SAR-induced plants and were shown to be important for SAR by mutant phenotype analysis (Champigny *et al.* 2013; Xia *et al.* 2012; Carella *et al.* 2016a). This may suggest a functional role for these proteins as chaperones for the long-distance movement of lipid signals through the phloem during SAR. However, convincing data for long-distance transport to distant leaves (petioles) has only been demonstrated for DIR1, underscoring a need to further explore the importance of ACBP6 and MLP in phloem exudates. Moreover, the MLP protein is only hypothesized to bind lipids based on homology to BetvI-containing proteins that accommodate large hydrophobic molecules within a large, forked hydrophobic cavity (Gajhede *et al.* 1996; Radauer *et al.* 2008). Further experimentation is needed to determine whether MLP is indeed a lipid-binding protein.

Significantly less is known about the perception of lipidic SAR signals in distant leaf cells, which likely requires a receptor with lipid-binding ability. Current candidates may include lipid-binding proteins whose function during SAR is not yet clear. For example, both LTP2 and DHyPRP1 are important for SAR but little is known about how these proteins participate in SAR. While it may be unlikely that these proteins are *bona fide* signal receptors due to the lack of typical receptor transducing domains, future experimentation should be undertaken to determine whether these mutants are responsive to exogenous application of individual signals (AzA, G3P, DA, Pip) or SAR-activated phloem exudates.

Examples of lipid or lipid-like receptor proteins in plants include the jasmonic acid (JA) receptor COI1 (CORONATINE INSENSITIVE1), the abcisic acid receptor family of PYL/PYR/RCAR proteins, and the newly identified LPS (lipopolysaccharide) receptor LORE. The perception of bioative jasmonic acid

[(+)-7-iso-JA-Ile)] or the *P. syringae* JA-mimicking toxin coronatine (COR) occurs within a binding pocket in the LRR domain of COI1 (Yan *et al.* 2009; Sheard *et al.* 2010), an F-box protein that forms an SCF (Skip-Cullen-F-box)-E3 ubiquitin ligase complex. JA/COR perception leads to the ubiquitination and subsequent proteasome-mediated degradation of JAZ repressor proteins that negatively regulate the JA signalling pathway, allowing for de-repression of the MYC2 transcription factor to initiate JA-responsive gene expression (reviewed in Pieterse *et al.* 2012). Interestingly, the novel SAR-regulator COP1 (Carella *et al.* 2016a) is an E3 ubiquitin ligase involved in proteasomal degradation of light signalling machinery (Osterlund *et al.* 2000; Seo *et al.* 2004; Jang *et al.* 2008). Based on this I hypothesize that COP1 mediates SAR signal perception through associations with an unknown receptor that, upon ligand binding, leads to the selected degradation and/or de-repression of SAR signalling machinery in distant leaves.

In contrast, the abiotic stress hormone ABA is perceived by a family of proteins that contain the START/BetvI domain also present in major latex proteins such as MLP. In the absence of ABA, these receptors form homo-dimers with their hydrophobic cavities open to the solvent to facilitate future interactions with ABA. In this scenario, free PP2C (Protein Phosphatase 2C) negatively regulates the activity of SnRK kinases to suppress the ABA pathway. Upon the binding of ABA within the large hydrophobic cavity of the PYL/PYR/RCAR receptors, these dimers dissociate leading to subsequent interactions between the receptor and PP2C. This prevents the negative regulation of SnRK kinases, which then phosphorylate transcriptional activators to induce ABA-responsive gene expression (pathway reviewed in Klingler *et al.* 2010). The MLP protein identified in SAR-activated phloem exudates is important for SAR and may also function as a potential signal receptor, as it too contains the BetvI-fold. Interestingly, a SnRK kinase was recently implicated in the activation of NPR1 function in distant leaves

of SAR-induced plants, which may hint at a similar BetvI-PP2C-SnRK signalling mechanism for SAR signal perception.

Lastly, a lipidic SAR signal receptor may have similarity to the newly identified LORE (Lipooligosaccharide-specific Reduced Elicitation) receptor that recognizes bacterial LPS to induce basal defenses against bacterial pathogens. LORE is a plasma membrane-localized SD lectin RLK (Receptor-like Kinase) protein that is hypothesized to interact with the lipid A moeity of LPS via the extracellular lectin domain, leading to activation of the cytosolic kinase domain and subsequent phosphorylation/activation of downstream MTI signalling machinery (Ranf *et al.* 2015). Given that the perception of LPS via LORE is thought to occur in the extracellular space, an analogous mechanism important for SAR long-distance signal perception would require that mobile SAR signals are unloaded into the apoplast in distant leaves, which is unlikely given the number of studies that suggest symplastic unloading of phloem contents (Imlau *et al.* 1999; Baluska *et al.* 2001; Turgeon and Wolf 2009; Burch-Smith *et al.* 2011). Rather, a putative lectin-RLK SAR receptor could perhaps be located to different subcellular compartments, such as the ER. However, the nature (and existence) of the SAR signal receptor is entirely speculative at this time. Future research aimed at clarifying the roles of mobile SAR activating small molecules and identifying putative receptor proteins that detect these (or other) signals in distant leaves should be undertaken. Analysis of proteins that associate with the SnRK2.8 kinase is an intriguing starting point for the identification of upstream signalling components involved in SAR signal perception, as SnRK2.8 is currently the only component in distant leaves that appears to integrate SA-dependent and –independent signals to activate NPR1-mediated SAR signalling (Lee *et al.* 2015).

7.4. Conclusion

This thesis significantly contributes to our understanding of DIR1/protein movement in the phloem during SAR. In this thesis, I provided evidence of symplastic DIR1 movement through plasmodesmata during SAR (Carella *et al.* 2015a), demonstrated that DIR1 structure and function are conserved in agriculturally relevant plants such as cucumber (Isaacs *et al.* 2016), and determined that DIR1 interacts with LTPs important for SAR (Chapter 5, unpublished). In addition, comparative proteomics analysis of phloem exudates collected during SAR led to the identification of novel phloem proteins important for SAR (Carella *et al.* 2016a). Together, this work argues that DIR1, phloem proteins, and lipid-binding proteins are important components of the SAR response. Most of the data in this thesis suggests a role for these proteins in phloem-mediated long-distance signalling during SAR. As a single body of work, this thesis sheds light on multiple aspects of DIR1. This work lays the foundation for future studies aimed at understanding how DIR1 and other phloem proteins participate in long-distance SAR signalling. Moreover, this work provides new insights on the protein composition of the phloem and how it is affected during interaction with pathogens.

Appendix

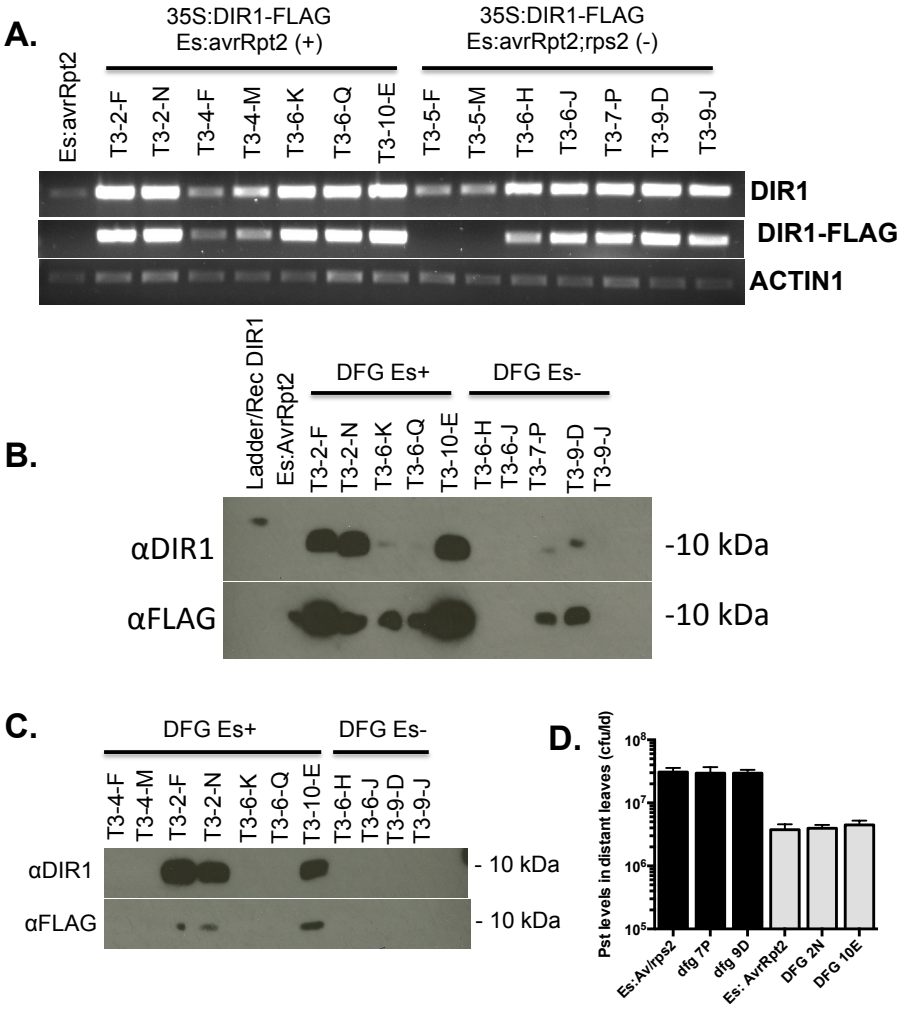

Figure A1 – Characterization of 35S:DIR1-FLAG lines. Molecular characterization of 35S:DIR1-FLAG (DFG) lines in the Es:AvrRpt2 (+/Es+) and Es:AvrRpt2;rps2 (-/Es-)backgrounds. **(A)** RT-PCR analysis of untreated 3-4 week-old leaf tissue. **(B)** Immunoblot analysis of total protein extracted from untreated 1-2 week old seedlings using a polyclonal DIR1 antibody or a monoclonal FLAG antibody. **(C)** Immunoblot analysis of phloem exudates collected from 4 week-old plants 24 hours after estrogen treatment. Note that all plants were sprayed with estrogen and that Es+/- refers only to the Es:AvrRpt2 (Es+) or Es:AvrRpt2;rps2 (Es-) backgrounds. **(D)** SAR analysis of DFG lines that received an initial inoculation with estrogen, followed by SAR challenge in upper leaves.

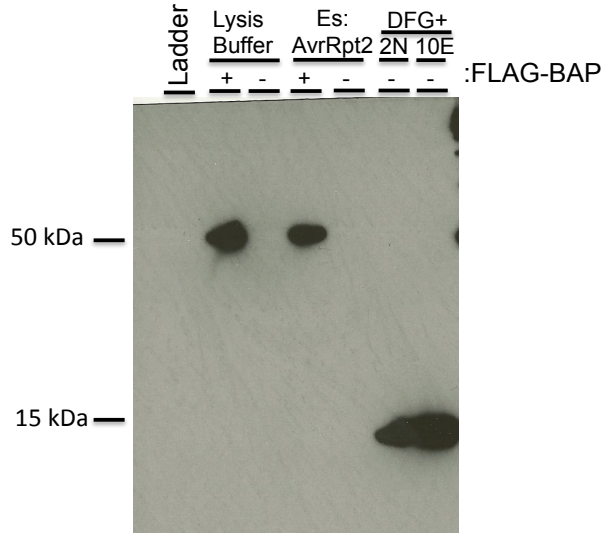

Figure A2 – Immunoprecipitation of DIR1-FLAG. Immunoblot depicting immunoprecipitation of DIR1-FLAG from 35S:DIR1-FLAG/Es:AvrRpt2 (DFG+) or parental Es:AvrRpt2 or Lysis Buffer controls. The recombinant FLAG-BAP control protein (~50 kDa) was spiked into certain samples as an internal control to ensure successful immunoprecipitation. Total protein from extracts was not run on this gel, only proteins immunoprecipitated using anti-FLAG M2 beads is depicted.

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