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

Running Title: Arabidopsis SAR Phloem Proteome

One Sentence Summary: Label-free quantitative proteomics analysis of Arabidopsis phloem exudates collected during the induction of systemic acquired resistance (SAR) identifies novel components of the SAR response.

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Author Contributions: Designed the research RKC, ACV, PC. Coordinated the research PC, SD. Performed experiments PC, DCW, RKC. Quantitative proteomics performed by JMP, SMH. Analysis of proteomes JMP, PC. Contributed research materials and equipment ACV, SMH, RKC. Wrote the manuscript PC, RKC, with significant contributions by DCW, ACV, and SD.

Figures: 7
Colour Figures: 2
Tables: 2
Supplementary Tables: 7
Supplementary Figures: 8
Supplementary Data: 1

**Word Count per Section**

- Abstract - 227
- Introduction – 1,180
- Methods – 675
- Results – 2,538
- Discussion – 2,715
- Figure Legends - 847
- References - 3,221
- Total (whole doc; from abstract down) - 11,709

**Abstract (250 words max - 236)**

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 thaliana*, local infection with virulent or avirulent strains of *Pseudomonas syringae pv. tomato* (Pst) 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 Col-0 plants were subjected to label-free quantitative LC-MS/MS proteomics (liquid chromatography, tandem mass spectrometry). 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 TRXh3, ACBP6, and PR1 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, T-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, UVR8 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.

**Introduction**

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 micro/macro-nutrients, small molecules, phytohormones, lipids, peptides/proteins, and coding/non-coding RNA (reviewed in Lucas et al. 2013). These molecules are involved in a number of inter-organ signaling responses, ranging from processes governing growth and development to stress-related responses to abiotic and biotic stimuli. Not surprisingly, some pathogens have co-opted 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 (Yadetta and Thomma 2013), and phloem-feeding herbivores (Kaloshian and Walling 2005). In response, plants have developed sophisticated inter-organ 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 (RNAi) (Yoo et al. 2004), induced systemic resistance (ISR) caused by beneficial microbes (Pieterse et al. 2014), and systemic acquired resistance (Cameron and Champigny 2009).

Systemic acquired resistance (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 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 PAMPs (pathogen-associated molecular patterns) to induce PAMP-triggered immunity (PTI). However, virulence effector proteins secreted into plant cells by P. syringae suppress this response and promote susceptibility in locally infected tissue (reviewed in Xin and He 2013). Incompatible or “avirulent” P. syringae strains carry effector proteins that are recognized in plant cells by cognate R (resistance) receptors to induce a robust local defense response termed effector-triggered immunity (ETI), which is usually associated with programmed cell death in the form of the hypersensitive response (reviewed in Cui et al. 2015). Classic SAR studies suggested that a necrotizing infection was important for SAR induction (reviewed in Sticher et al. 1997); however, recent studies demonstrate that the induction of PTI 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 determined that SAR signals travelled from induced rootstocks to distant scions to induce SAR (Jenns and Kuc, 1979). A specific role for the phloem in 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 demonstrated that the removal of stem sheath also resulted in a loss of systemic immunity (Tuzun and Kuc 1985), further supporting a role for 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 radiolabelled 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 recently supported 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 inter-organ communication.

The identification of long-distance SAR signals remains an active area of research, as they may represent novel bio-protective 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 (Navarova 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 (DA)-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 proteinacious 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 LC-MS/MS (liquid chromatography, tandem mass spectrometry) 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 P. 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 T-DNA mutants. This analysis identified a role in SAR for m-type thioredoxins, a putative major latex protein, and the UV-B photoreceptor UVR8 (UV-B RESISTANCE8). Further investigation of the UVR8 UV-B signaling pathway revealed a role for the E3 ubiquitin ligase COP1 (CONSTITUTIVE PHOTOMORPHOGENESIS1) and the bZIP transcription factor HY5 (ELONGATED HYPOCOTYL5) in the development of SAR. The Arabidopsis SAR phloem proteome provides new insights into the dynamic nature of the phloem during biotic stress and revealed that a number of previously unknown proteins accumulate in the phloem during SAR.

Materials and Methods

Plant material and growth conditions

Wild-type Arabidopsis thaliana (ecotype Col-0) and homozygous T-DNA mutant seeds (Supplementary Figure S1) were surface sterilized and stratified at 4 °C in the dark for 2 days. Sterile seeds were plated on Murashige and Skoog (MS) plates and germinated for 5-7 days under continuous light. Seedlings were transplanted onto soil hydrated with 1 g/L 20-20-20 fertilizer and grown under short-day photoperiod conditions (9 hr light; 150 μE m⁻² s⁻¹) at 22 °C with 65-85% relative humidity. UV-B levels in growth chambers were undetectable (UV-X radiometer, UVP). Confirmed (homozygous) plant lines were obtained from ABRC or independent research labs (Fasano et al. 2014; Konopka-Postupolska et al. 2009; Tsuchiya et al. 2010). Homozygous mlp mutants (NASC; GK-089B08) were confirmed from heterozygous seed stock by germination on MS media containing sulfadiazine (5 μg ml⁻¹) followed by molecular characterization of mRNA levels using RT-PCR (Supplementary Figure S1).

Bacterial growth, inoculation, and quantitation

Standard SAR experiments and local resistance assays were performed as described in Carella et al. (2015) with Pseudomonas syringae pv. tomato (Pst) 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 MgCl₂ (mock-inoculation) or 10⁶ colony forming units (cfu) ml⁻¹ of virulent...
PstDC3000 (pVSP1) or avirulent PstDC3000/avrRpt2 (pVSP1 + avrRpt2). In planta Pst levels were quantified by dilution plating as described in Cameron et al. (1999) and Carella et al. (2015). Statistically significant differences in Pst levels were identified by ANOVA (Tukeys HSD, p < 0.05) analysis using R.

**Phloem exudation collection**

Phloem exudates were collected as described in Carella et al. (2015). At 24 hours post inoculation (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, 0.0006% bleach in 1 mM EDTA) and immediately placed into eppendorf tubes containing 1 mM EDTA for 1 hour. 12 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 hours (representing exudation from 25-48 hpi). For proteomics analysis, pooled exudates from >90 plants per treatment were concentrated using centrifugal concentrators with a 3 kDa cut-off (Vivaspin 20, GE Healthcare) according to manufacturer’s instructions until a final volume of ~ 7 ml. Concentrated exudates were equally subdivided into 4 tubes and protein levels were quantified using the BioRad protein reagent with bovine serum albumin (BSA) as a standard. Samples were then frozen in liquid nitrogen, lyophilized, and stored at -80 °C until further use. Phloem exudates used for immunoblots were collected as previously described (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 minutes at 4 °C. Each ~0.5 µg per sample were measured on a LTQ OrbitrapXL (Thermo Fisher Scientific) coupled to a Ultimate3000 nano-RSLC (Dionex) as described previously (Hauck et al., 2010; Molin et al., 2015).

Raw files of each dataset were analyzed separately with the 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 MS/MS features with charges +2 to +7 were exported for protein identification using the Mascot search engine (Matrix Science, version 2.5.0) in the TAIR 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 the Progenesis QI software and normalized abundances of unique peptides were summed up for every protein. These values were used for calculation of abundance ratios between groups and for statistical evaluation by Student’s t-tests (p < 0.05).
See Supplementary Data Online For Supplementary Methods: Protein isolation and immunoblotting. Sample preparation for mass spectrometry. RNA isolation and RT-PCR analysis.

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 hours 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 MgCl₂) or induced for SAR by inoculation with *Pst* strains that are virulent (*Pst*DC3000) or avirulent (*Pst*DC3000/avrRpt2) on Col-0 plants. SAR assays performed alongside exudate collection experiments confirmed that SAR was induced by both strains (Supplementary Figure S2A). 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 >90 plants per treatment were collected and concentrated using centrifugal concentrators (3 kDa cut-off) 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 (Supplementary Figure S2B). Concentrated phloem exudates from two independent experimental replicates were subjected to quantitative LC-MS/MS (see Supplementary Data for raw data). 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 (Supplementary Tables S1, S2). Since infection with either strain induces SAR to the same extent in Col-0 (Supplementary Figure 2A), 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 abundant in phloem exudates collected from leaves induced for SAR by both *Pst* strains relative to mock-inoculated phloem exudates (Figure 1B). A total of 16 proteins were enriched in phloem exudates collected from SAR-induced (virulent- and avirulent *Pst*) leaves compared to mock-inoculated controls (Table 1). In contrast, 46 proteins displayed decreased abundance in exudates collected from SAR-induced versus mock-inoculated leaves (Table 2, Supplementary Table S3).

Comparison to Published Phloem Exudate Proteomes
To assess the quality of our proteomes, we compared our dataset (all proteins, regardless of treatment) to 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 in all three proteomes (Figure 2A; Supplementary Table S4). Our combined phloem proteome (replicate 1 + replicate 2) overlapped with 49% of the proteins identified by Batailler et al. (2012) and 63% of those described in Guelette et al. (2012). By comparison, the Batailler et al. (2012) dataset overlapped with 47% of proteins identified in Guelette et al. (2012). Further, 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) (Figure 2B). Only 12 proteins were present in the proteomes of all three species (Supplementary 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 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 vs -suppressed Phloem Proteins**

To gain insight into the nature of SAR-enriched and SAR-suppressed phloem proteins, comparative GO (gene ontology) Slim analysis was performed (Supplementary Figure S3). 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 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 were also more frequent in SAR-enriched compared to 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” (Figure 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, extracellular) and thylakoid localization, while SAR-suppressed phloem proteins were associated with intracellular terms (ribosome, endoplasmic reticulum, vacuole, nucleus, plastid, cytosol, intracellular) (Figure 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 TRXh3 and ACBP6 (AcylCoA Binding
Protein6) were significantly enriched in phloem exudates collected from SAR-
induced compared to mock-inoculated leaves (Table 1). TRX-h3 regulates the
oligomeric status of the master SAR signaling protein NPR1 along with TRX-h5
to control the induction of SAR (Tada et al. 2008). Single mutants of trx-h3 and
trx-h5 are modestly impacted in SAR, however loss of the NTRA (NADPH-
dependent Thioredoxin ReductaseA) 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 have also 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 protein 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 PR1 (PATHOGENESIS-RELATED1) 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 MgCl2) and SAR-induced (PstDC3000/avrRpt2) Col-0 leaves
were collected from 25-48 hpi, concentrated, and probed with a polyclonal PR1
antibody. As a positive control, exudates were also 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 PstDC3000/avrRpt2-
inoculated leaf tissue (48 hpi) were assayed for PR1 and DIR1 accumulation. As
expected, DIR1 antibody signals (7 and 14 kDa) were detected in phloem
exudates collected from SAR-induced but not mock-inoculated leaves, and were
undetectable in leaf extracts (Figure 4). In comparison, PR1 was detected in total
protein extracts of PstDC3000/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 dataset 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. TRX-h3 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 to 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 to 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 vs mock-inoculated plants (Figure 5A). The SAR phenotype of the trxm2 mutant ranged from partially SAR defective to fully competent in three independent experiments (Supplementary Figure 4). 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 (>74%) to one another (Supplementary Table 6). 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 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 wild-type Col-0 at both 0 and 3 dpi (Figure 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 (GPI)-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 (AT4G23670, MLP) accumulated in phloem exudates collected from PstDC3000/avrRpt2-induced leaves (Table 1, Supplementary Table 1). The SAR phenotypes of ltpg6 and mlp mutants were compared to 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 (Supplementary Figure 4). In contrast, an mlp T-DNA mutant (Supplementary Figure S1) displayed a 2-fold reduction in Pst levels in
distant leaves of SAR-induced compared to mock-inoculated plants, whereas a
22-fold reduction was observed in Col-0 (Figure 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 to Col-0
(Figure 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 *TRXm1-4* 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* 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 (1-4), *MLP*, or *ACT1* after
*Pst* inoculation. In contrast, the defense marker *PR1* was highly induced at 24
and 48 hpi (Supplementary Figure 5). The data indicate that the *TRXm1-4* 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
genomes 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 (Supplementary Figure 4). In contrast, the 35S:UVR8
overexpression line and the *uvr8*-6 mutant were defective for SAR compared to
wild-type Col-0 (Figure 6). However, the severity of the defect varied between
experiments, such that partial (Figure 6A) or full (Figure 6B) defects in the SAR
response of *uvr8*-6 and 35S:UVR8 were observed in 3 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 (Figure 6C), indicating
that these lines are specifically impaired in SAR. The 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
were also 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 (reviewed in 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 (Figure 7A). Since subtle changes in gene expression cannot be quantified using RT-PCR, we queried publically 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 to mock- or untreated controls (Supplementary Figures S6-S8). Levels of UVR8 and COP1 decreased <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 may also 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 to mock-inoculated plants, while hy5 displayed a partial defect in SAR (3.3-fold decrease) and 35S:GUS-COP1 was fully defective in SAR (Figure 7B). The cop1-4 mutant was similarly defective in SAR, as Pst levels were similar in both mock- and SAR-induced plants (Figure 7C). These data demonstrate that HY5 and COP1 are required for SAR.

Discussion

Phloem Proteomics

Proteomic analysis provides a snap shot 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 50-63% 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 to 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, non-phloem cell types. While this may be indicative of unavoidable contamination caused by cellular leakage from non-phloem 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, nuclear-encoded proteins with predicted plastid-localization peptides may localize to non-plastid sub-cellular sites in the phloem. Comparisons to exudate proteomes derived from different plant species yielded fewer similarities, which suggests that protein composition within the phloem is specialized. This may also be due to differences in exudate collection techniques and/or fundamental differences in phloem architecture. This is especially important in comparisons to the Cucurbit family, where phloem exudates collected directly from the cut ends of petioles are largely comprised 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 have recently conducted complex comparative proteomics studies of phloem exudates collected during stress. These include comparative phloem proteomes derived from poplar and pumpkin upon wounding stress (Dafoe et al. 2009; Gaupels et al. 2012), rice exposed to plant-hopper insects (Du et al. 2015), salt-stressed cucumber (Fan et al. 2015), melon 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 inter-organ communication. A number of studies have focused on the identification of SAR-activating small molecules that accumulate in the phloem during SAR (reviewed in Dempsey and Klessig, 2012), yet little attention has been given to proteins. This gap in knowledge was addressed by performing comparative proteomics 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 to 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; Wang et al. 2013; Faulkner 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 previously associated 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. 2010; 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 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 inter-organellar lipid transport (Chen et al. 2008), while DIR1 binds mono-acylated 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-disulphide conversion of redox-sensitive cysteine residues (Tada et al. 2008). How TRXh3 functions in the phloem during the induction of SAR is unknown, but it may function in thiol-disulphide conversion of NPR1 or other cysteine-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 PrxII (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 cysteine-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. 2012). Nevertheless, TRXm protein (this study; Guelette et al. 2012) and mRNA (Deeken et al. 2008) accumulates 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 Nicotiana tabacum suggests that TRXm proteins act as molecular chaperones that protect target proteins during stress (Sanz-Barrio et al. 2011). 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 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 PR-10 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

Accumulation of a number of proteins was suppressed in phloem exudates collected from SAR-induced leaves, some of which were previously associated with plant defense and include TGG1 myrosinase (Barth and Jander 2006), the JA-responsive mannose-binding lectin JR1 (León et al. 1998), calreticulin-2 (Qui et al. 2012), plastidial chaperonin CPN60B (Ishikawa et al. 2003), fasciclin-like arabinogalactan-protein FLA8 (Gruner et al. 2014), and glycine-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. 2014; Bernsdorff et al. 2015/6) 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, was also suppressed in melon phloem during viral infection (Serra-Soriano et al. 2012), 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 to wild-type plants. The UVR8 photoreceptor is a seven-bladed β-propeller protein that perceives UV-B wavelengths using intrinsic tryptophan residues (Christie et al. 2012). Upon UV-B photo-activation, UVR8 homodimers monomerize and translocate from the cytosol to the nucleus (Kaiserli and Jenkins 2007). In the nucleus, UVR8 interacts with COP1 to induce 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 the present study, we observed reduced levels of UVR8 in phloem exudates of SAR-induced compared to 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 suppression of UVR8 involves proteasomal degradation and/or post-transcriptional regulation.

In addition to its well-established role in the UV-B stress response (reviewed in 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 cinera (Demkura and Ballare 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 is probably not contributing 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 indicates 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 the 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 are also 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 (reviewed in 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 et al. 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 to 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 (ROS), and programmed cell death.
(PCD) resulting in a SAR-like response (Muhlenbock et al. 2008). Recent evidence demonstrated that HY5 is required for light-induced PCD 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 (Zhang et al. 2007). Further, 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.

Conclusions

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 dataset 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 inter-organ communication during stress.

Tables:

Table 1. Proteins Enriched in the Phloem During SAR (SAR-Enriched)

Table 2. Selected Proteins Suppressed in the Phloem During SAR (SAR-Suppressed)

Figure Legends:

Figure 1. Comparative Proteomics Analysis of Phloem Exudates Collected During the Induction of SAR. Quantitative proteomics of phloem exudates collected from mock (10 mM MgCl₂) and SAR-induced (Virulent – PstDC3000; or Avirulent – PstDC3000/avrRpt2) leaves of two experimental replicates. Values inside Venn diagrams represent the number of unique proteins (at least 2 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 to 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 to mock-inoculated leaves. Venn diagrams generated in
were remade using Microsoft Office Powerpoint.

**Figure 2. Comparing Phloem Exudate Proteomes.** (A) Venn diagram
comparing all proteins identified in Replicate 1 and Replicate 2 of this study to the
Arabidopsis phloem exudate proteomes described in Guelette et al. (2012) and
Batailler et al. (2012). (B) Comparison of all Arabidopsis proteins identified in this
study (Arabidopsis*) to phloem exudate proteomes of pumpkin (*Cucurbita
maxima* – Lin et al. 2009) and Texas Bluebonnet (*Lupinus texensis* – Lattanzio et
al. 2013). Venn diagrams obtained from Venny 2.0 (Olivieros 2015,
http://bioinfogp.cnb.csic.es/tools/venny/index.html) were remade in Microsoft
Office Powerpoint.

**Figure 3. GO Slim Analysis of Proteins Enriched or Suppressed in SAR-
induced Phloem exudates.** Gene ontology (GO) slim terms pertaining to (A)
“Biological Process” and (B) “Cellular Compartment” of SAR-enriched (Enriched,
n=16) compared to 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 the
supporting information online.

**Figure 4. PR1 accumulates in Phloem Exudates of SAR-induced Leaves.**
Immunoblots of phloem exudates and leaf tissue collected from 4 week-old Col-0
plants that were mock-inoculated (10 mM MgCl$_2$) or induced for SAR ($10^6$ colony
forming units ml$^{-1}$ of PstDC3000/avrRpt2). Phloem exudates were collected from
24-48 hours post inoculation (hpi) and leaf tissue was harvested at 48 hpi.
Immunoblots were performed using PR1 (1:3000) and DIR1 (1:10,000)
antibodies. Similar results were obtained in 3 independent experiments.

**Figure 5. The SAR-enriched Phloem Proteins TRXm1, TRXm4, and MLP are
Involved in SAR.** Standard SAR assays comparing wild-type Col-0 to *trx1* and
*trx4* (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 $10^6$ colony forming units
ml$^{-1}$ PstDC3000/avrRpt2. Two days later, distant leaves were challenged with $10^6$
PstDC3000 and Pst levels in these leaves were quantified 3 days post
inoculation (dpi). Experiments were repeated at least 3 times with similar results.
Local resistance assays comparing wild-type Col-0 to *trx1* and *trx2* (B) or *mlp*
(C). Local resistance to virulent (PstDC3000) and avirulent (PstDC3000/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 (A, B, C, D) represent the mean +/- standard deviation of three sample
replicates. Different letters indicate statistically significant differences (ANOVA, Tukey HSD, P < 0.05).

**Figure 6. The UV-B Photoreceptor UVR8 is required for SAR.** (A, B) Standard SAR assays of 4 week-old Col-0, *uvr8-6*, and 35S:UVR8 plants. Leaves were mock-inoculated (10 mM MgCl₂) or induced for SAR by pressure infiltration with 10⁶ colony forming units ml⁻¹ PstDC3000/avrRpt2. Two days later, distant leaves were challenged with 10⁶ PstDC3000 and Pst levels in these leaves were quantified 3 days post inoculation (dpi). This experiment was performed 6 times, with similar results in (A) and (B) observed 3 times each. (C) Local resistance assays of Col-0, *uvr8-6* and 35S:UVR8 to virulent (VIR - PstDC3000) and avirulent (AVIR - PstDC3000/avrRpt2) strains of Pst. Leaves of 4 week-old plants were inoculated with 10⁶ cfu ml⁻¹ of either strain, and *in planta* bacterial density was calculated at 0 and 3 days post inoculation. This experiment was performed 3 times with similar results. All values (A, B, C) represent the mean +/- standard deviation of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey HSD, P < 0.05).

**Figure 7. The UV-B Signaling Components COP1 and HY5 are required for the Manifestation of SAR.** (A) RT-PCR of cDNA generated from leaves of 4 week-old Col-0 plants that were untreated (Un) or inoculated with 10⁶ cfu ml⁻¹ PstDC3000 at the indicated time points (hours post inoculation – hpi). *UVR8, HY5, COP1* expression was compared to the ACTIN1 and PR1 controls. This experiment was performed 3 times with similar results. Standard SAR assays comparing wild-type Col-0 to *hy5* and 35S:GUS-COP1 (B) or *cop1-4* (C). Leaves were mock-inoculated (10 mM MgCl₂) or induced for SAR by pressure infiltration with 10⁶ colony forming units ml⁻¹ PstDC3000/avrRpt2. Two days later, distant leaves were challenged with 10⁶ PstDC3000 and Pst levels in these leaves were quantified 3 days post inoculation (dpi). Values (B, C) represent the mean +/- standard deviation of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey HSD, P < 0.05). These experiments were performed 3 times with similar results.

**Supplementary Data:**

**Supplementary Methods**

**Figure S1.** Plant Lines Used in This Study

**Figure S2.** SAR Assay and Phloem Exudate Collection Controls

**Figure S3.** Complete GO Slim Analysis of Proteins Enriched or Suppressed in SAR-induced Phloem Exudates

**Figure S4.** Supporting SAR Assays
Figure S5. TRXm and MLP expression Analysis

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

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

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

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

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

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

Table S4. Common Arabidopsis Phloem Proteins

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

Table S6. TRXm-family Similarity Matrix

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

Data S1. Raw Proteomics Data

Acknowledgments:
The authors wish to thank Dr. Xinnian Dong (Duke University, USA) for the PR1 antibody, Dr. Antonella Leone (University of Salerno, Italy) for the uvr8-6 and 35S:UVR8 plant lines, Dr. Jacek Hennig (Polish Academy of Sciences, Poland) for the annat1 and 35S:AnnAt1 plant lines, Dr. Peter McCourt (University of Toronto, Canada) for the hy5 (SALK_056405), cop1-4, and 35S:GUS-COP1 plants lines, May T.S. Yeo for help with phloem exudate collection, as well as the ABRC (Ohio State, USA) and NASC (Nottingham, UK) for TDNA insertion lines. This work was supported by NSERC Discovery, RTI and CFI Leadership grants to RKC, an Ontario Graduate Scholarship to PC, and an NSERC Scholarship to DCW.

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<td>CCoAOMT1</td>
<td>SAM-methyltransferase</td>
<td>4.3</td>
<td>2.4</td>
<td>3.3</td>
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<tr>
<td>AT2G19760</td>
<td>PFN1</td>
<td>Profilin</td>
<td>5.6</td>
<td>1.5</td>
<td>21.6</td>
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<tr>
<td>AT4G02450</td>
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<td>HSP20-like</td>
<td>7.5</td>
<td>4.7</td>
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<tr>
<td>AT2G29450</td>
<td>GSTU5</td>
<td>Glutathione S-transferase</td>
<td>3.3</td>
<td>1.8</td>
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<td>AT1G55260</td>
<td>LTPG6</td>
<td>Lipid transfer protein</td>
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<td>4.8</td>
<td>9.2</td>
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<td>AT1G31812</td>
<td>ACBP6</td>
<td>AcylCoA Binding Protein</td>
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<td>4.4</td>
<td>111.1</td>
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<td>AT3G15360</td>
<td>TRXm4</td>
<td>Thioredoxin</td>
<td>5.5</td>
<td>0.6</td>
<td>9.3</td>
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<tr>
<td>AT4G23670</td>
<td></td>
<td>Polyketide cyclase</td>
<td>5.7</td>
<td>1.1§</td>
<td>30.2</td>
</tr>
</tbody>
</table>

*Peptides with significant enrichment in SAR+ phloem in 1 of 2 replicates.
# - Only one peptide available for Quantitation, § = Not statistically significant
Table 2. Selected Proteins Suppressed in the Phloem During SAR (SAR-Suppressed)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>Relative Abundance (Virulent/Mock)</th>
<th>Relative Abundance (Avirulent/Mock)</th>
<th>Peptides Used for Quantitation</th>
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<tbody>
<tr>
<td>AT3G05900</td>
<td>-</td>
<td>Neurofilament protein-related</td>
<td>Rep 1: 0.07, Rep 2: 0.03</td>
<td>Rep 1: 0.03, Rep 2: 0.03</td>
<td>10, 15</td>
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<tr>
<td>AT5G66190</td>
<td>FNR1</td>
<td>Ferredoxin oxidoreductase</td>
<td>Rep 1: 0.37, Rep 2: 0.14</td>
<td>Rep 1: 0.20, Rep 2: 0.21</td>
<td>4, 17</td>
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<tr>
<td>AT2G04030</td>
<td>HSP90.5</td>
<td>Chaperone Protein</td>
<td>Rep 1: 0.51, Rep 2: 0.13</td>
<td>Rep 1: 0.26, Rep 2: 0.18</td>
<td>6, 6</td>
</tr>
<tr>
<td>AT5G26000</td>
<td>TGG1</td>
<td>Thioglucoside glucohydrolase</td>
<td>Rep 1: 0.29, Rep 2: 0.40</td>
<td>Rep 1: 0.28, Rep 2: 0.75</td>
<td>6, 10</td>
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<tr>
<td>AT3G16470</td>
<td>JR1</td>
<td>Mannose-binding Lectin</td>
<td>Rep 1: 0.20, Rep 2: 0.26</td>
<td>Rep 1: 0.04, Rep 2: 0.20</td>
<td>5, 8</td>
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<td>AT1G55490</td>
<td>CPN60B</td>
<td>Chaperonin</td>
<td>Rep 1: 0.24, Rep 2: 0.15</td>
<td>Rep 1: 0.14, Rep 2: 0.19</td>
<td>5, 7</td>
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<tr>
<td>AT3G16400</td>
<td>NSP1</td>
<td>Nitrile Specifier Protein</td>
<td>Rep 1: 0.16, Rep 2: 0.11</td>
<td>Rep 1: 0.03, Rep 2: 0.08</td>
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<td>AT1G09210</td>
<td>CRT1b</td>
<td>Calreticulin</td>
<td>Rep 1: 0.09, Rep 2: 0.08</td>
<td>Rep 1: 0.03, Rep 2: 0.08</td>
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<td>AT1G56340</td>
<td>CRT1a</td>
<td>Calreticulin</td>
<td>Rep 1: 0.15, Rep 2: 0.09</td>
<td>Rep 1: 0.04, Rep 2: 0.09</td>
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<tr>
<td>AT5G54770</td>
<td>THI1</td>
<td>Thiazole Biosynthetic Enzyme</td>
<td>Rep 1: 0.08, Rep 2: 0.09</td>
<td>Rep 1: 0.02, Rep 2: 0.03</td>
<td>3, 2</td>
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<tr>
<td>AT5G28540</td>
<td>BiP1</td>
<td>Heat Shock Protein 70</td>
<td>Rep 1: 0.38, Rep 2: 0.35</td>
<td>Rep 1: 0.36, Rep 2: 0.47</td>
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<tr>
<td>AT2G28000</td>
<td>CPN60A</td>
<td>Chaperonin</td>
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<td>Rep 1: 0.12, Rep 2: 0.27</td>
<td>3, 4</td>
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<td>AT1G72150</td>
<td>PATL1</td>
<td>Patellin</td>
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<td>Rep 1: 0.06, Rep 2: 0.10</td>
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<tr>
<td>AT1G76180</td>
<td>ERD14</td>
<td>Dehydrin</td>
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<td>ANNAT1</td>
<td>Annexin</td>
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<tr>
<td>AT4G22670</td>
<td>HIP1</td>
<td>HSP70-interacting</td>
<td>Rep 1: 0.03, Rep 2: 0.01</td>
<td>Rep 1: 0.00, Rep 2: 0.04</td>
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<td>AT2G21660</td>
<td>GRP7</td>
<td>Glycine-rich Protein</td>
<td>Rep 1: 0.19, Rep 2: 0.10</td>
<td>Rep 1: 0.06, Rep 2: 0.06</td>
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<tr>
<td>AT5G63860</td>
<td>UVR8</td>
<td>UVB Photoreceptor</td>
<td>Rep 1: 0.27, Rep 2: 0.05</td>
<td>Rep 1: 0.18, Rep 2: 0.06</td>
<td>2, 1*</td>
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*Only 1 peptide used for quantitation.
Figure 1. Comparative Proteomics Analysis of Phloem Exudates Collected During the Induction of SAR. Quantitative proteomics of phloem exudates collected from mock (10 mM MgCl$_2$) and SAR-induced (Virulent – PstDC3000; or Avirulent – PstDC3000/avrRpt2) leaves of two experimental replicates. Values inside Venn diagrams represent the number of unique proteins (at least 2 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 to 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 to mock-inoculated leaves. Venn diagrams generated in Venny 2.0 (Olivieros 2015, http://bioinfogp.cnb.csic.es/tools/venny/index.html) were remade using Microsoft Office Powerpoint.
Figure 2. Comparing Phloem Exudate Proteomes. (A) Venn diagram comparing all proteins identified in Replicate 1 and Replicate 2 of this study to the Arabidopsis phloem exudate proteomes described in Guelette et al. (2012) and Batailler et al. (2012). (B) Comparison of all Arabidopsis proteins identified in this study (Arabidopsis*) to phloem exudate proteomes of pumpkin (*Cucurbita maxima* – Lin et al. 2009) and Texas Bluebonnet (*Lupinus texensis* – Lattanzio et al. 2013). Venn diagrams obtained from Venny 2.0 (Oliviers 2015, http://bioinfogp.cnb.csic.es/tools/venny/index.html) were remade in Microsoft Office Powerpoint.
Figure 3. GO Slim Analysis of Proteins Enriched or Suppressed in SAR-induced Phloem exudates. Gene ontology (GO) slim terms pertaining to (A) “Biological Process” and (B) “Cellular Compartment” of SAR-enriched (Enriched, n=16) compared to 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 the supporting information online.
Figure 4. PR1 accumulates in Phloem Exudates of SAR-induced Leaves. Immunoblots of phloem exudates and leaf tissue collected from 4 week-old Col-0 plants that were mock-inoculated (10 mM MgCl₂) or induced for SAR (10⁶ colony forming units ml⁻¹ of PstDC3000/avrRpt2). Phloem exudates were collected from 24-48 hours post inoculation (hpi) and leaf tissue was harvested at 48 hpi. Immunoblots were performed using PR1 (1:3000) and DIR1 (1:10,000) antibodies. Similar results were obtained in 3 independent experiments.
Figure 5. The SAR-enriched Phloem Proteins TRXm1, TRXm4, and MLP are Involved in SAR. Standard SAR assays comparing wild-type Col-0 to trxm1 and trxm4 (A) or mlp (D). Leaves of 4 week-old plants were mock-inoculated (10 mM MgCl₂) or induced for SAR by pressure infiltration with 10⁶ colony forming units ml⁻¹ PstDC3000/avrRpt2. Two days later, distant leaves were challenged with 10⁶ PstDC3000 and Pst levels in these leaves were quantified 3 days post inoculation (dpi). Experiments were repeated at least 3 times with similar results. Local resistance assays comparing wild-type Col-0 to trxm1 and trxm2 (B) or mlp (C). Local resistance to virulent (PstDC3000) and avirulent (PstDC3000/avrRpt2) strains of Pst was assessed by inoculating leaves of 4 week-old plants with 10⁶ cfu ml⁻¹ of either strain. Bacterial densities were determined at 0 and 3 dpi. All values (A, B, C, D) represent the mean +/- standard deviation of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey HSD, P < 0.05).
Figure 6. The UV-B Photoreceptor UVR8 is required for SAR. (A, B) Standard SAR assays of 4 week-old Col-0, *uvr8-6*, and 35S:UVR8 plants. Leaves were mock-inoculated (10 mM MgCl2) or induced for SAR by pressure infiltration with 10^6 colony forming units ml\(^{-1}\) PstDC3000/avrRpt2. Two days later, distant leaves were challenged with 10^6 PstDC3000 and Pst levels in these leaves were quantified 3 days post inoculation (dpi). This experiment was performed 6 times, with similar results in (A) and (B) observed 3 times each. (C) Local resistance assays of Col-0, *uvr8-6* and 35S:UVR8 to virulent (VIR - PstDC3000) and avirulent (AVIR - PstDC3000/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 days post inoculation. This experiment was performed 3 times with similar results. All values (A, B, C) represent the mean +/- standard deviation of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey HSD, P < 0.05).
Figure 7. The UV-B Signaling Components COP1 and HY5 are required for the Manifestation of SAR. (A) 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). UVR8, HY5, COP1 expression was compared to the ACTIN1 and PR1 controls. This experiment was performed 3 times with similar results. Standard SAR assays comparing wild-type Col-0 to 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 $10^6$ colony forming units ml$^{-1}$ PstDC3000/avrRpt2. Two days later, distant leaves were challenged with $10^6$ PstDC3000 and Pst levels in these leaves were quantified 3 days post inoculation (dpi). Values (B, C) represent the mean +/- standard deviation of three sample replicates. Different letters indicate statistically significant differences (ANOVA, Tukey HSD, $P < 0.05$). These experiments were performed 3 times with similar results.


