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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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

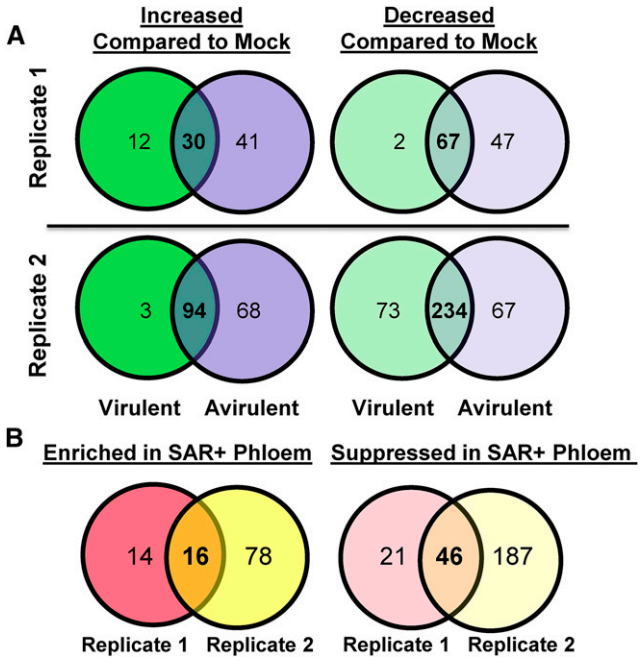

Figure 1. Comparative proteomics analysis of phloem exudates collected during the induction of SAR. Quantitative proteomics data of phloem exudates were collected from mock-inoculated (10 mM $MgCl_2$) and SAR-induced (virulent, *Pst* DC3000; and avirulent, *Pst* DC3000/avrRpt2) leaves of two experimental 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.

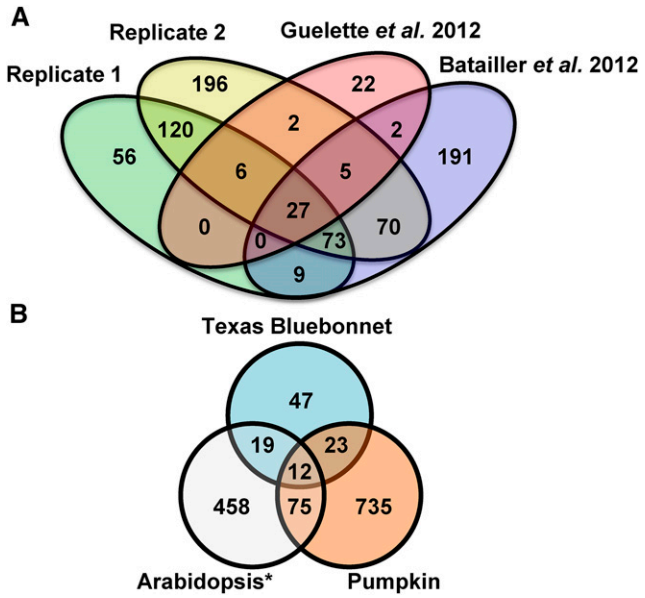

Figure 2. Comparing phloem exudate proteomes. A, Venn diagram comparing all proteins identified in replicates 1 and 2 of this study with the Arabidopsis phloem exudate proteomes described by Guelette et al. (2012) and Batailler et al. (2012). B, Comparison of all Arabidopsis proteins identified in this 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

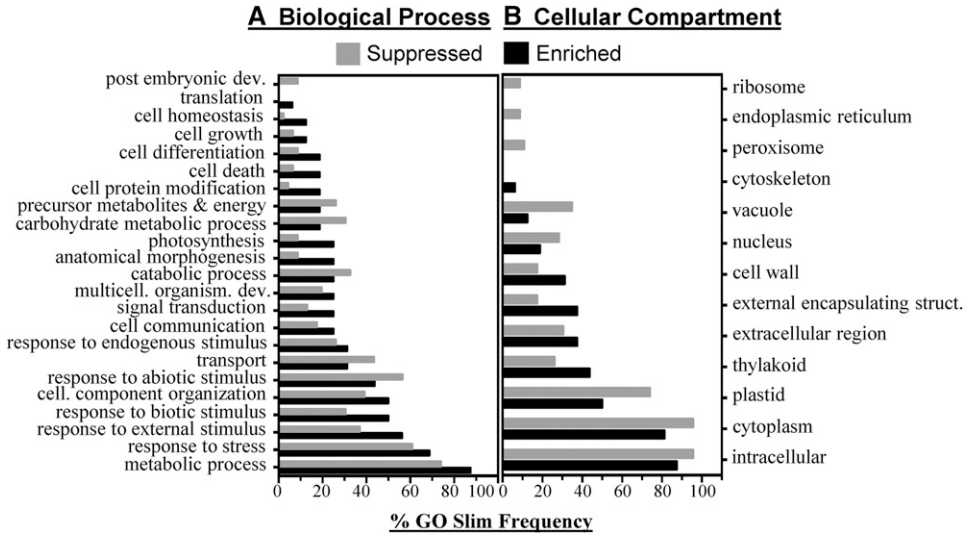

Figure 3. GO Slim analysis of proteins enriched or suppressed in SAR-induced phloem exudates. GO Slim terms are given pertaining to biological process (A) and cellular compartment (B) of SAR-enriched (Enriched; $n = 16$) compared with SAR-suppressed (Suppressed; $n = 46$) proteins. Only GO Slim terms 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

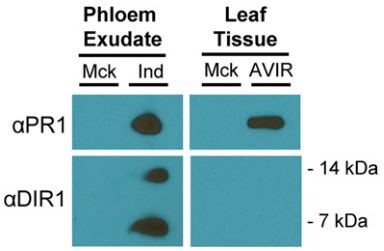

Figure 4. PR1 accumulates in phloem exudates of SAR-induced leaves. Immunoblots are from phloem exudates and leaf tissue collected from 4-week-old Col-0 plants that were mock inoculated (Mck; 10 mM $MgCl_2$) or induced (Ind) for SAR (10^6 colony-forming units [cfu] mL^{-1} *Pst* DC3000/avrRpt2). Phloem 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.

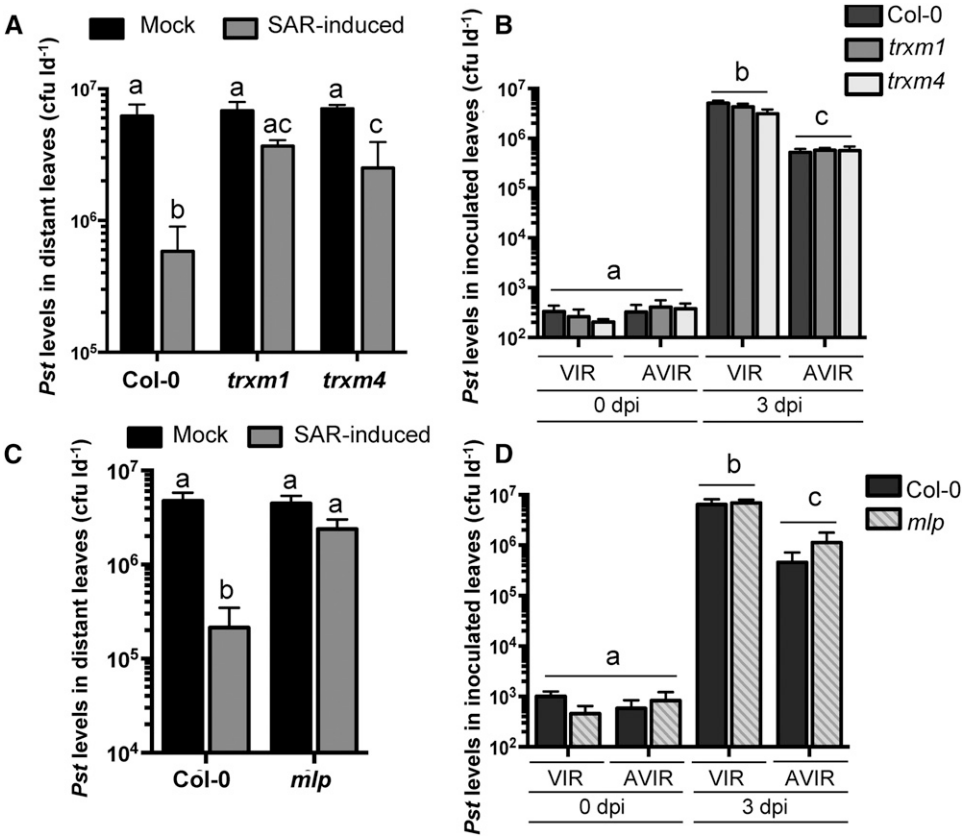

Figure 5. The SAR-enriched phloem proteins TRXm1, TRXm4, and MLP are involved in SAR. A and D, Standard SAR assays comparing wild-type Col-0 with *trxm1* and *trxm4* (A) or *mlp* (D). Leaves of 4-week-old plants were mock inoculated (10 mM $MgCl_2$) or induced for SAR by pressure infiltration with 10^6 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

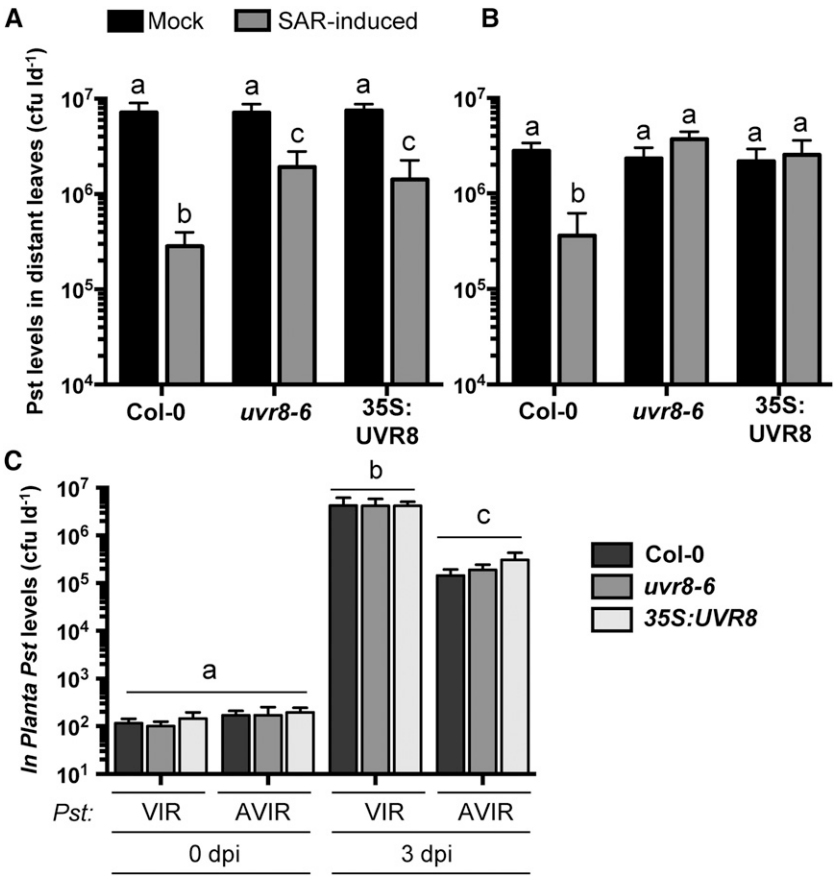

Figure 6. The UV-B photoreceptor UVR8 is required for SAR. A and B, Standard SAR assays of 4-week-old Col-0, *uvr8-6*, and 35S:UVR8 plants. Leaves were mock inoculated (10 mM $MgCl_2$) or induced for SAR by pressure infiltration with 10^6 cfu mL^{-1} *Pst* DC3000/avrRpt2. Two days later, distant leaves 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, $P < 0.05$).

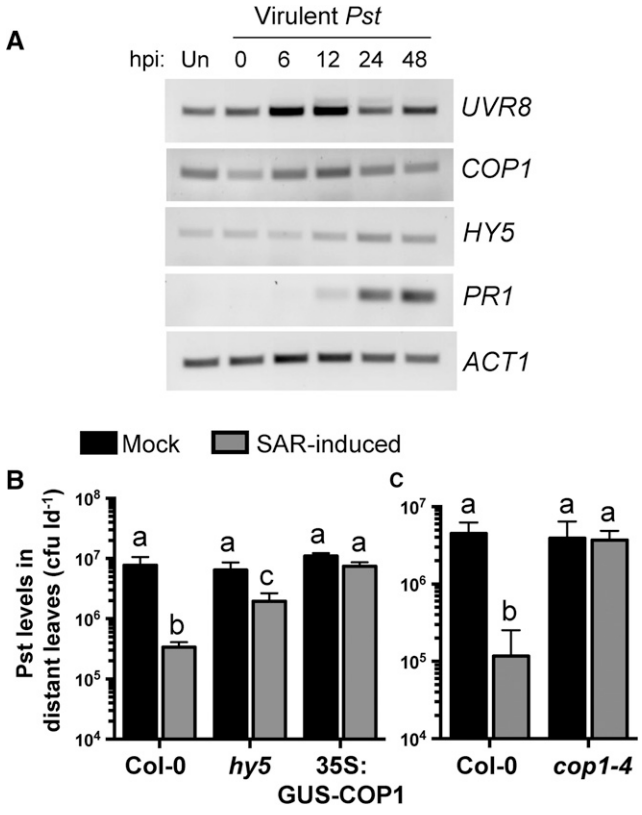

Figure 7. The UV-B signaling components COP1 and HY5 are required for the manifestation of SAR. A, Reverse transcription (RT)-PCR of complementary DNA generated from leaves of 4-week-old Col-0 plants that were untreated (Un) or inoculated with 10^6 cfu mL^{-1} *Pst* DC3000 at the indicated time points (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 μE 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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