Contrasting Roles of the Apoplastic Aspartyl Protease APOPLASTIC, *ENHANCED DISEASE SUSCEPTIBILITY1*-DEPENDENT1 and LEGUME LECTIN-LIKE PROTEIN1 in Arabidopsis Systemic Acquired Resistance[1,2][W]

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Systemic acquired resistance (SAR) is an inducible immune response that depends on *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*). Here, we show that Arabidopsis (*Arabidopsis thaliana*) *EDS1* is required for both SAR signal generation in primary infected leaves and SAR signal perception in systemic uninfected tissues. In contrast to SAR signal generation, local resistance remains intact in *eds1* mutant plants in response to *Pseudomonas syringae* delivering the effector protein AvrRpm1. We utilized the SAR-specific phenotype of the *eds1* mutant to identify new SAR regulatory proteins in plants conditionally expressing *AvrRpm1*. Comparative proteomic analysis of apoplast-enriched extracts from *AvrRpm1*-expressing wild-type and *eds1* mutant plants led to the identification of 12 APOPLASTIC, *EDS1*-DEPENDENT (AED) proteins. The genes encoding AED1, a predicted aspartyl protease, and another AED, LEGUME LECTIN-LIKE PROTEIN1 (LLP1), were induced locally and systemically during SAR signaling and locally by salicylic acid (SA) or its functional analog, benzo 1,2,3-thiadiazole-7-carbothioic acid *S*-methyl ester. Because conditional overaccumulation of AED1-hemagglutinin inhibited SA-induced resistance and SAR but not local resistance, the data suggest that AED1 is part of a homeostatic feedback mechanism regulating systemic immunity. In *llp1* mutant plants, SAR was compromised, whereas the local resistance that is normally associated with *EDS1* and SA as well as responses to exogenous SA appeared largely unaffected. Together, these data indicate that LLP1 promotes systemic rather than local immunity, possibly in parallel with SA. Our analysis reveals new positive and negative components of SAR and reinforces the notion that SAR represents a distinct phase of plant immunity beyond local resistance.

Systemic acquired resistance (SAR) is a form of induced resistance that is triggered in the systemic uninfected parts of plants after a local pathogen infection (Fu and Dong, 2013). The establishment of the systemic defense response is dependent on salicylic acid (SA), although SA itself is not the mobile signal responsible for SAR (Vernooij et al., 1994; Vlot et al., 2009). Over the past 10 years, a number of long-distance signals have been proposed to mediate systemic immune signaling. These signals include methyl salicylate, azelaic acid, glycerol-3-phosphate, dehydroabietinal, and pipecolic acid (Park et al., 2007; Jung et al., 2009; Chanda et al., 2011; Chaturvedi et al., 2012; Návarová et al., 2012). SAR signal generation has also been associated with actions of an extracellular aspartyl protease (Xia et al., 2004; Prasad et al., 2009), whereas SAR signal perception requires an intact cuticle in the systemic uninfected tissue (Xia et al., 2009, 2010). Together with the effects of environmental cues, such as light, on SAR (Zeier et al., 2004; Griebel and Zeier, 2008; Liu et al., 2011a), it is becoming clear that multiple signaling pathways converge to regulate SAR.

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[2] This article is dedicated to the memory of Chris Lamb (1950–2009).

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Recent findings provide further insight into defense-associated long-distance signaling, including the cooperation between signaling molecules mediating SAR (for review, see Dempsey and Klessig, 2012; Spoel and Dong, 2012; Kachroo and Robin, 2013; Shah and Zeier, 2013). Upon infection, SA is induced and converted to methyl salicylate (MeSA), which is believed to move to the systemic tissue, where its conversion back to SA is essential for SAR (Park et al., 2007). The putative lipid transfer protein DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) promotes SAR, possibly in part by directing the MeSA-SA equilibrium toward SA in systemic uninfected tissues (Maldonado et al., 2002; Liu et al., 2011b). Additionally, DIR1 contributes to a positive feedback loop promoting SAR together with another lipid transfer protein, AZELAIC ACID INDUCED1 (AZI1), and glycerol-3-phosphate (Yu et al., 2013). Azelaic acid acts upstream of DIR1/AZI1/glycerol-3-phosphate to activate SAR (Yu et al., 2013) and acts partly redundantly with dehydroabietinal, a mobile SAR signal that is active at picomolar concentrations (Chaturvedi et al., 2012). The SAR defects of Arabidopsis (*Arabidopsis thaliana*) mutants, in which the accumulation of MeSA or glycerol-3-phosphate is compromised, appear to be conditional and dependent on the length of light exposure of the plants after a primary SAR-inducing infection (Liu et al., 2011a). A similar effect of light was observed on the SAR-defective phenotype of *dir1* mutant plants, suggesting that MeSA, glycerol-3-phosphate, and DIR1 are essential for SAR only if plants receive a limited light exposure after infection (Liu et al., 2011a). Alternatively, SAR in the *dir1* mutant can be supported by the *DIR1* homolog *DIR1-like* (Champigny et al., 2013). Finally, accumulation of the nonprotein amino acid pipecolic acid in systemic uninfected tissues is essential for SAR (Návarová et al., 2012). Pipecolic acid potentiates systemic SA-mediated defenses via FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1), which is also required for signaling downstream of azelaic acid and dehydroabietinal and essential for SAR irrespective of the length of light exposure of the plants after infection (Mishina and Zeier, 2006; Liu et al., 2011a; Chaturvedi et al., 2012; Shah and Zeier, 2013).

SAR is induced in response to local infections that trigger SA signaling and is effective in systemic tissues at protecting against attack by a broad range of pathogens that are normally sensitive to SA-mediated defense (Vlot et al., 2009; Boatwright and Pajerowska-Mukhtar, 2013; Fu and Dong, 2013; Henry et al., 2013; Kachroo and Robin, 2013). A central regulator of SA signaling is encoded by *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*; Falk et al., 1999; Wiermer et al., 2005; Straus et al., 2010). EDS1 contains a noncatalytic N-terminal lipase-like domain with a classical α/β-hydrolase fold connected to an α-helical bundle C-terminal domain, and both domains are critical for EDS1 immune signaling functions (Wagner et al., 2013). These functions include promoting SA-mediated basal resistance, a low-level postinfection response that impedes the growth of virulent pathogens, and SAR (Falk et al., 1999; Wiermer et al., 2005; Truman et al., 2007; Vlot et al., 2009; Rietz et al., 2011). Additionally, EDS1 regulates SA-independent defense signaling in one or more pathways acting redundantly with SA (Bartsch et al., 2006; Venugopal et al., 2009; Roberts et al., 2013). *FMO1*, for example, acts upstream of SA in systemic uninfected tissues (Mishina and Zeier, 2006; Shah and Zeier, 2013) and is associated with *EDS1*-dependent local responses that are genetically separable from SA (Bartsch et al., 2006). Thus, EDS1 directs both the SA-dependent and SA-independent branches of basal resistance and SAR.

EDS1 also promotes effector-triggered immunity (ETI) mediated by a subclass of plant nucleotide-binding/leucine-rich repeat (NLR) receptors that have N-terminal Toll-Interleukin1 Receptor-like homology (referred to as TIR-NLRs) and at least two NLRs that contain an N-terminal coiled-coil domain (referred to as CC-NLRs; Aarts et al., 1998; Zhu et al., 2011; Roberts et al., 2013). ETI is induced by the direct or indirect recognition of pathogen effectors by NLR receptors and is a robust defense response often culminating in localized programmed cell death at infection foci known as a hypersensitive response (HR; Jones and Dangl, 2006; Maekawa et al., 2011; Bonardi and Dangl, 2012; Spoel and Dong, 2012). Arabidopsis EDS1 signals with two sequence-related proteins, PHYTOALEXIN DEFICIENT4 (PAD4) and SENESCENCE-ASSOCIATED GENE101 (SAG101), by forming separate nucleocytoplasmic and nuclear complexes, respectively, with each partner (Feys et al., 2001, 2005; Rietz et al., 2011; Wagner et al., 2013). Also, Arabidopsis EDS1 was found to reside in nuclear complexes with the TIR-NLR receptors RESISTANT TO PSEUDOMONAS SYRINGAE4 (RSP4), RPS6, and VARIATION IN COMPOUND TRIGGERED ROOT growth response, the RPS4- and RPS6-recognized effectors AvrRps4 and HopA1, as well as the CC-NLR HYPERSENSITIVE RESPONSE TO Turnip crinkle virus (HRT), recognizing turnip crinkle virus coat protein (Bhattacharjee et al., 2011; Heidrich et al., 2011; Zhu et al., 2011; Kim et al., 2012). EDS1 shuttles between the cytoplasm and nucleus through the nuclear pore machinery, and the accumulation of EDS1 inside nuclei is necessary for both TIR-NLR-mediated ETI and basal resistance and their associated transcriptional reprogramming of defense pathways (García et al., 2010; Heidrich et al., 2011). Therefore, direct or indirect interactions between EDS1 and NLR receptors have been proposed to connect effector recognition to nuclear transcriptional outputs and pathogen resistance (Bhattacharjee et al., 2011; Heidrich et al., 2011; Sohn et al., 2012).

In Arabidopsis, ETI mediated by the CC-NLR RESISTANCE TO *PSEUDOMONAS SYRINGAE* pathovar MACULICOLA1 (RPM1) is triggered by *Pseudomonas syringae* delivering the effector protein AvrRpm1 (Dangl et al., 1992). Although ETI conferred by RPM1 and another CC-NLR (RPS2) does not genetically require *EDS1* (Aarts et al., 1998), SAR induced by both CC-NLRs was abolished in the *eds1* mutant (Truman et al., 2007;

Rietz et al., 2011). Similarly, *pad4* mutant plants were SAR defective in response to a local activation of RPM1 or RPS2 (Jing et al., 2011; Rietz et al., 2011). In this work, we show that *EDS1* contributes to Arabidopsis SAR signaling in both the primary infected and systemic uninfected tissues. We utilize the SAR defect in *eds1* mutant plants in response to the *P. syringae* effector *AvrRpm1* to investigate systemic rather than local defense responses, focusing on *EDS1*-dependent changes in the leaf apoplast. Previous studies have shown that Arabidopsis protein secretion is highly regulated during *P. syringae*-induced defense responses, including SAR, with effectors such as AvrRpm1 likely directly affecting protein secretion (Hauck et al., 2003; Wang et al., 2005; Kaffarnik et al., 2009). Arabidopsis is an apoplastic phloem loader (Gottwald et al., 2000), and the phloem is believed to be the main conduit for SAR signal transmission (Guedes et al., 1980; Gaupels and Vlot, 2013). Therefore, we relate SAR to proteins differentially accumulating in the apoplast of wild-type and *eds1* leaves in response to AvrRpm1. Among 12 SAR-associated apoplastic proteins, we identify contrasting roles of a predicted aspartyl protease that appears to suppress SAR and a legume lectin-like protein that promotes SAR without affecting local resistance to virulent or avirulent *P. syringae*. In this work, we show that there are mechanistically distinct phases in local and systemic immunity.

RESULTS

EDS1 Is Necessary for SAR Signal Generation and Perception

The SAR-specific defect in *Arabidopsis eds1* null mutant plants in response to a primary infection with *P. syringae* pv *tomato* strain DC3000 (*Pst*) expressing *AvrRpm1* (*Pst/AvrRpm1*; Aarts et al., 1998; Truman et al., 2007) may be due to compromised responses in the locally infected tissue, the systemic untreated tissue, or both. To distinguish between these possibilities, we tested for the presence of defense-inducing signals in petiole exudates from leaves infected with *Pst/AvrRpm1*. In accordance with the terminology of previous studies (Chaturvedi et al., 2008), these petiole exudates will be referred to as AvrPEX. AvrPEX were collected from Wassilewskija-0 wild-type, *eds1-1* and *pad4-5* single mutant, and *eds1-1pad4-5* double mutant plants. As a control, AvrPEX from the *dir1* mutant were included (Maldonado et al., 2002). The presence of defense-inducing signals in the AvrPEX was investigated upon infiltration of the AvrPEX into the leaves of previously untreated plants using the induction of the SAR marker gene *PATHOGENESIS RELATED1* (*PR1*) as a marker for defense induction (Fig. 1).

AvrPEX from wild-type plants induced *PR1* expression in wild-type recipient plants (Fig. 1). As observed previously (Maldonado et al., 2002), AvrPEX from *dir1* failed to induce *PR1* expression in wild-type recipient plants (Fig. 1A). Similarly, AvrPEX from *eds1-1*, *pad4-5*,

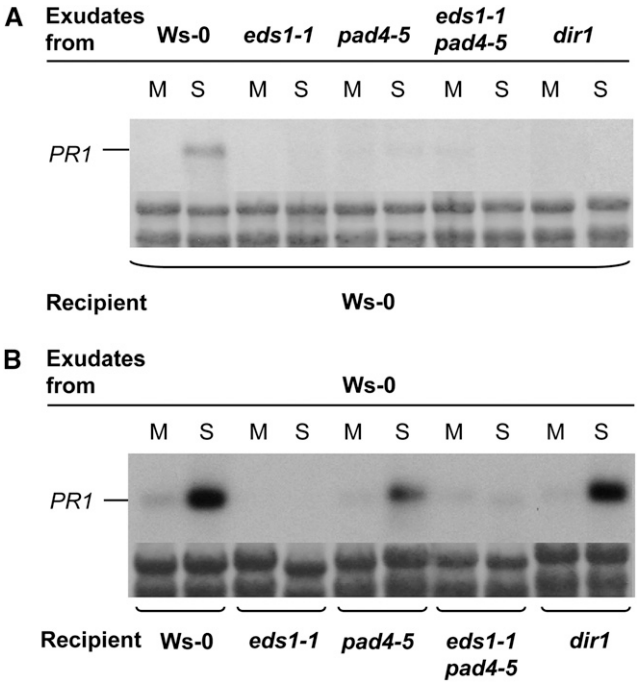

Figure 1. *EDS1* is required for SAR signal generation (A) and perception (B). *PR1* transcript accumulation is shown 24 h after infiltration of leaves with petiole exudates collected from mock-treated (M; 10 mM $MgCl_2$) or *Pst/AvrRpm1*-infected (S) leaves. The exudates were collected from the genotypes indicated above each RNA blot and infiltrated into the recipient plants indicated below each RNA blot. *PR1* transcript accumulation was analyzed on northern blots, and equal loading was controlled by on-blot Methylene Blue staining of ribosomal RNA (shown below each RNA blot). This experiment was repeated three times with similar results.

or *eds1-1pad4-5* mutants did not induce *PR1* expression in wild-type recipients. Thus, similar to *DIR1*, *EDS1* and its signaling partner *PAD4* are required for SAR signal generation in primary infected leaves. Reciprocally, AvrPEX from wild-type plants induced *PR1* expression in the *pad4-5* mutant and, as expected, the *dir1* mutant control (Fig. 1B; Maldonado et al., 2002). In contrast, wild-type AvrPEX did not induce *PR1* expression in *eds1-1* recipient plants or in the *eds1-1pad4-5* double mutant (Fig. 1B). Thus, unlike *DIR1*, whose activity is required only in the primary infected tissue, *EDS1* acts in both the primary infected and systemic uninfected tissues during SAR signal propagation.

Conditional Expression of *AvrRpm1* Triggers *EDS1*-Dependent Systemic Immunity

In screening for plant-derived SAR components, we sought to synchronize SAR induction as much as possible and to limit the presence of pathogen-derived proteins in the plants. Therefore, we used transgenic plants carrying the dexamethasone (DEX)-inducible transgene *pDEX:AvrRpm1-HA* that encodes C-terminally hemagglutinin (HA)-tagged AvrRpm1 (Mackey et al.,

2002). Before employing these transgenic plants for the identification of SAR-associated proteins, we assessed whether local DEX application, which induces the expression of *AvrRpm1-HA*, also effectively triggers SAR. The application of 30 μM DEX with a paintbrush to the first two true leaves of *pDEX:AvrRpm1-HA* plants resulted in the appearance of HR lesions on the systemic untreated leaves, presumably caused by DEX traveling from the treated tissues. Therefore, we monitored the expression of the transgene in local treated and systemic untreated leaves after the application of a much lower concentration of 1 μM DEX in 0.01% (v/v) Tween 20 using 0.01% (v/v) Tween 20 as a negative control. The Tween 20-treated leaves reproducibly displayed very low *AvrRpm1-HA* expression, as measured by quantitative reverse transcription (qRT)-PCR (Fig. 2A; Supplemental Fig. S1A). In contrast, the DEX application induced local *AvrRpm1-HA* expression to more than 100-fold over 48 to 72 h (Fig. 2A; Supplemental Fig. S1A), which is 2- to 8-fold higher than bacterial *AvrRpm1* transcript accumulation in Columbia-0 (Col-0) leaves 3 d after infiltration with 10^6 colony-forming units (cfu) mL^{-1} *Pst/AvrRpm1* (Supplemental Fig. S1B). Induced *AvrRpm1-HA* expression was not observed in systemic untreated leaves of the DEX-treated *pDEX:AvrRpm1-HA* plants (Fig. 2B; Supplemental Fig. S1C), suggesting that the 1 μM DEX application remained local.

We subsequently tested if the local application of 1 μM DEX in 0.01% (v/v) Tween 20 triggers systemic resistance in Col-0 *pDEX:AvrRpm1-HA* or *eds1-2 pDEX:AvrRpm1-HA* plants. The first two true leaves per *pDEX:AvrRpm1-HA* plant were treated with DEX or Tween 20, and the systemic untreated leaves were inoculated 3 d later with virulent *Pst*. The *Pst* titers in the secondary infected leaves were determined 4 d later, showing that the primary DEX treatment of Col-0 *pDEX:AvrRpm1-HA* but not *eds1-2 pDEX:AvrRpm1-HA* plants induced systemic resistance, resulting in reduced *Pst* growth in DEX-treated compared with Tween-treated plants (Fig. 2C). Because local DEX treatment of non-transgenic Col-0 plants did not trigger a systemic resistance response to *Pst* (Supplemental Fig. S2), the systemic resistance in the DEX-treated *pDEX:AvrRpm1-HA* plants was a consequence of local *AvrRpm1-HA* expression. Together, these data show that *AvrRpm1-HA* expression triggers *EDS1*-dependent systemic immunity.

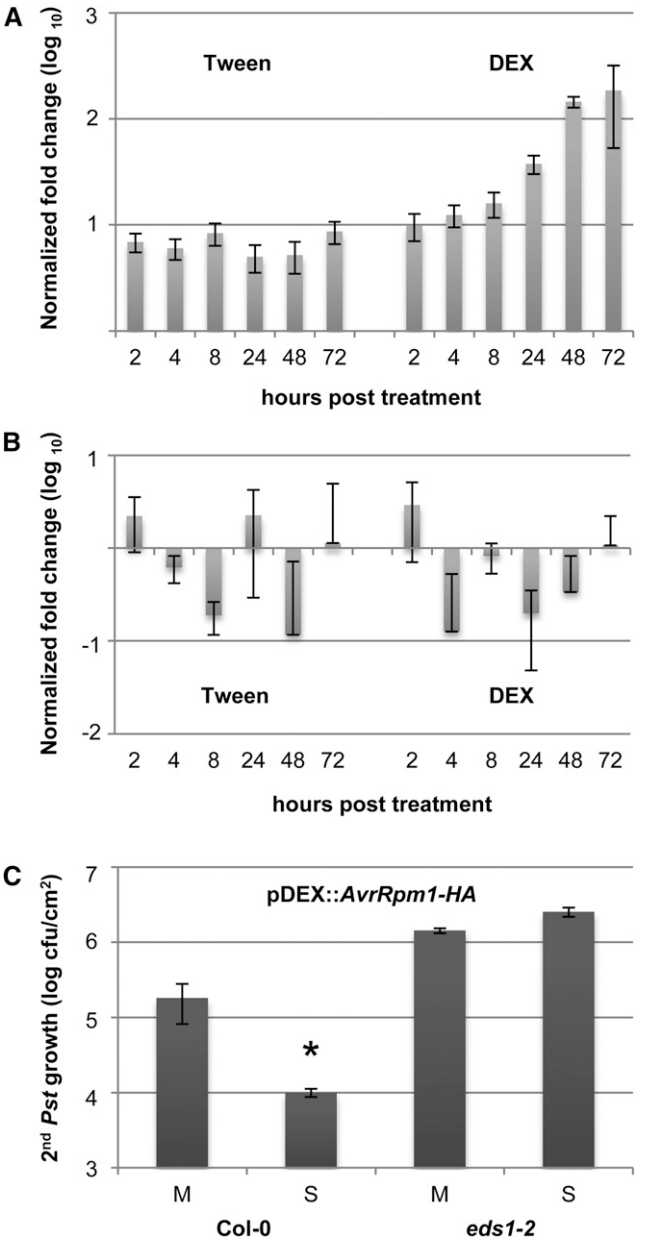

Figure 2. Pathogen-free, effector-triggered systemic immunity. A and B, *AvrRpm1-HA* transcript accumulation was normalized to *TUBULIN* in Col-0 *pDEX:AvrRpm1-HA* plants treated with 0.01% (v/v) Tween 20 or 1 μM DEX. Transcript accumulation is shown in the treated (A) and systemic untreated (B) leaves relative to the transcript levels in the same leaves from untreated plants. C, *Pst* titers are shown 4 d after a secondary infection of *pDEX:AvrRpm1-HA* plants that was systemic to primary treatments with 0.01% (v/v) Tween 20 (mock; M) or 1 μM DEX (SAR; S). The asterisk indicates a significant difference from the mock-treated plants ($P < 0.05$, Student's *t* test). This experiment was repeated three times with similar results.

Identification of Potential SAR Regulatory Proteins

Because *EDS1* is required for SAR signal generation (Fig. 1) but not local resistance in response to *Pst/AvrRpm1* (Aarts et al., 1998), we hypothesized that any proteins accumulating in an *EDS1*-dependent manner during *AvrRpm1-HA*-triggered responses may be related specifically to SAR. Proteomics experiments are often disturbed by highly abundant cellular proteins, such as Rubisco, that mask low-abundance proteins of interest. Therefore, we enriched the plant extracts for apoplastic proteins prior to proteomic analysis to both reduce the Rubisco content of the extracts and focus on the secreted proteins potentially related to SAR (see introduction; Wang et al., 2005). For protein isolation, we sprayed lawns of 3- to 4-week-old *pDEX:AvrRpm1-HA*

plants with 30 μM DEX in 0.01% (v/v) Tween 20, which did not confine the expression of the transgene to a specific location within the plant but instead maximized the leaf response to *AvrRpm1-HA*. Pilot experiments showed that the leaves treated with 1 μM DEX did not wilt and needed to remain on the plant for 4 to 6 h before removal without loss of systemic resistance to virulent *Pst* bacteria (Supplemental Fig. S3). Because the leaves treated with 30 μM DEX became severely wilted at 6 h after treatment, we harvested the aboveground tissue for protein isolation 4.5 to 5 h after spraying the plants with 30 μM DEX.

The protein accumulation in the apoplast-enriched extracts from *AvrRpm1-HA*-expressing wild-type and *eds1* mutant plants was compared using two-dimensional (2D) PAGE (Fig. 3; Supplemental Fig. S4A). Infection of Arabidopsis cultured cells with *Pst/AvrRpm1* induces the secretion of host proteins that do not carry an N-terminal secretion signal peptide (Kaffarnik et al., 2009). Nevertheless, proteins were selected as apoplastic only if they possessed a predicted signal peptide (determined by SignalP 4.1; Petersen et al., 2011), because the apoplast-enriched extracts from Arabidopsis contained a high proportion of cytosolic proteins due to cell membrane leakage during the extraction (Supplemental Table S1). Of the *EDS1*-dependent protein spots that were detected on the 2D gels, eight apoplastic proteins containing N-terminal signal peptides were identified after in-gel trypsin digestion of the spots followed by matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (MS; Fig. 3; Table I; Supplemental Fig. S4). This experiment was repeated four times with four biologically independent sample sets. Because not all of the differentially accumulating protein spots were trypsin digested and sequenced by MALDI-MS in every repetition, the reproducibility with which AED proteins were found is summarized in Supplemental Figure S4B. Four of the AED proteins were previously characterized as ASPARTIC PROTEASE IN GUARD CELL1 (ASPG1; Yao et al., 2012), β-D-XYLOSIDASE4 (XYL4; Minic et al., 2004), and PR2 and PR5 (van Loon et al., 2006). Similar but not identical to ASPG1, AED1 and AED3 are predicted aspartyl proteases (Simões and Faro, 2004; Faro and Gal, 2005). AED4 and AED5 are GDSL-motif lipases (Akoh et al., 2004).

To quantify the relative differences in protein accumulation between the *AvrRpm1-HA*-induced wild-type and *eds1* mutant plants, proteins in the different extracts were analyzed by isotope-coded protein labeling (ICPL). To this end, the proteins were chemically labeled with mass tags containing different isotopic substitutions and mixed prior to liquid chromatography-tandem mass spectrometry (MS/MS)-based protein identification, thus allowing the immediate and relative quantification of the proteins in extracts from wild-type and *eds1* plants. Differentially labeled standards were added as internal controls (the quantitation results are shown in Supplemental Fig. S5A). Across three biologically independent replicates, 758 proteins were identified in the apoplast-enriched extracts, 609 of which could be quantified (Supplemental Table S1). As expected, the log normal distribution of quantitation followed a Gaussian distribution (Supplemental Fig. S5B). In the ICPL experiment, ASPG1 and AED4 each displayed trends toward an approximately 1.4-fold enhanced accumulation in the extracts from the wild type compared with those from *eds1* mutant plants (Table I; Supplemental Table S1). The accumulation of AED3 and XYL4 was not different between wild-type and *eds1* extracts (Supplemental Table S1). However, each of these proteins was found in multiple spots on the 2D gels. Notably, only a single spot per protein (i.e. one isoform

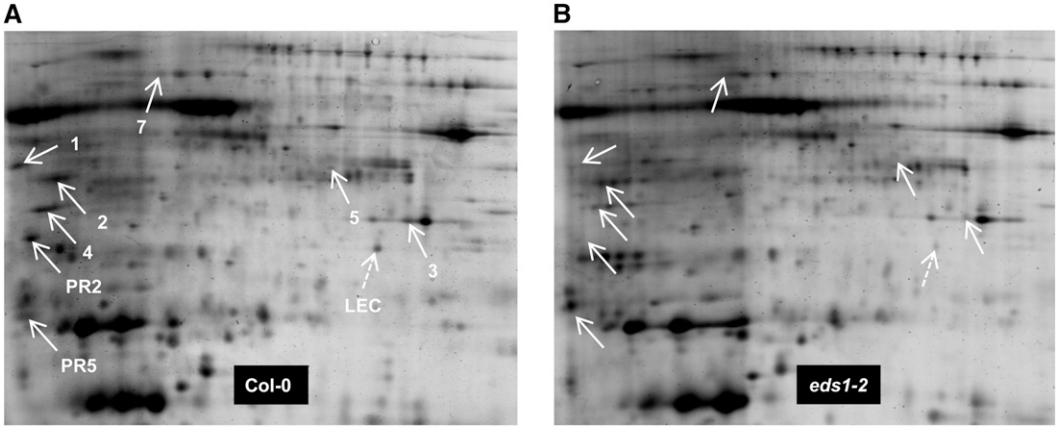

Figure 3. 2D gel analysis of apoplast-enriched extracts from *AvrRpm1-HA*-expressing Col-0 (A) and *eds1-2* mutant (B) plants. Isoelectric focusing was performed along the horizontal axis across a pI range from 4 to 7, and proteins were resolved according to their mass along the vertical axis on 12% (w/v) polyacrylamide gels. White arrows indicate the positions of AED proteins. A putative lectin encoded by At3g16530 was identified on this gel set only and is marked with the dashed arrows (LEC). Numbers correspond to the number of each AED protein in Table I. This experiment was repeated multiple times (for reproducibility per spot, see Supplemental Fig. S4).

Table I. *Summary of AED proteins identified by 2D gel or ICPL analyses with their ICPL quantitation and significance as well as possible biological functions (Gene Ontology analysis; www.arabidopsis.org)*
ND, Not determined.

Gene	Locus	Protein (Predicted Size)	ICPL Ratio, Wild Type to *eds1-2*	*P* (ICPL)[a]	Biological Process
AED1	At5g10760	Eukaryotic aspartyl protease family protein (49.4 kD)	ND	ND	Response to stress/response to abiotic or biotic stimulus/protein metabolism/signal transduction/other cellular processes
ASPG1, AED2	At3g18490	Aspartic protease in guard cell1 (53.2 kD)	1.39	0.20	Response to stress/response to abiotic or biotic stimulus/protein metabolism
AED3	At1g09750	Eukaryotic aspartyl protease family protein (47.6 kD)	1.22	0.45	Protein metabolism
AED4	At1g29660	GDSL-motif lipase (40.1 kD)	1.45	0.15	Response to stress/response to abiotic or biotic stimulus/transport
AED5	At3g05180	GDSL-motif lipase (42.3 kD)	ND	ND	Other metabolic processes
XYL4, AED7	At5g64570	β-Xylosidase (84.3 kD)	1.15	0.61	Other metabolic and cellular processes
PR2	At3g57260	Pathogenesis-related protein2 (37.3 kD)	5.62	1.5E-11	Response to stress/response to abiotic or biotic stimulus/signal transduction/other cellular and metabolic processes
PR5	At1g75040	Pathogenesis-related protein5 (25.2 kD)	2.9	3.2E-05	Response to stress/response to abiotic or biotic stimulus/signal transduction/other cellular and metabolic processes
LLP1, AED9	At5g03350	Legume lectin family protein (30.1 kD)	2.0	0.007	Unknown
PNP-A, AED14	At2g18660	Plant natriuretic peptide A (14.5 kD)	2.65	0.0001	Response to stress/response to abiotic or biotic stimulus/electron transport or energy pathways/other metabolic and cellular processes
AED15	At2g43570	Putative chitinase (29.7 kD)	2.34	0.0009	Other metabolic and cellular processes
EP1, AED19	At4g23170	Cys-rich receptor-like kinase (29.7 kD)	1.96	0.009	Other cellular processes

[a]According to the Perseus statistical tool (www.perseus-framework.org; Cox and Mann, 2011).

or posttranslationally modified variant) displayed differential accumulation between the extracts from the wild-type and *eds1* mutant plants (Supplemental Fig. S4). Finally, AED1 and AED5 were not identified in the ICPL analyses. For AED5, this result might be due to its relatively low abundance (Fig. 3A). AED1, however, appears to be a quite abundant protein in the wild-type extracts (Fig. 3A). The amino acid sequence and the theoretical peptide masses after an in silico trypsin digestion of AED1 did not indicate why this protein was not identified by liquid chromatography-MS/MS.

In the ICPL experiment, six AED proteins displaying a statistically significant *EDS1*-dependent accumulation were detected in all three independent biological replicates and contained predicted N-terminal secretion signal peptides (Table I; Supplemental Table S1). These AEDs included PR2, PR5, the putative PR protein PLANT NATRIURETIC PEPTIDE A (PNP-A; Meier et al., 2008), the predicted chitinase AED15, a receptor-like kinase that is encoded by the SA-responsive *EP1* gene (Blanco et al., 2005), and the SA-inducible LEGUME LECTIN-LIKE PROTEIN SAi-LLP1 (LLP1; Krinke et al., 2007; Blanco et al., 2009; Armijo et al., 2013). LLP1 is 63% identical to a protein that is encoded by locus At3g16530 and was detected on one 2D gel set (Fig. 3). The LLP1-like protein showed a slight trend of 1.3-fold enhanced accumulation in the extracts from the wild type compared with those from *eds1* mutant plants (Supplemental Table S1). Another sequence-related protein, *At*LEC (for *Arabidopsis thaliana* LECTIN; Lyou et al., 2009), which is 60% identical to LLP1 and 89% identical to the LLP1-like protein, did not differentially accumulate between the wild-type and *eds1* extracts (Supplemental Table S1).

In summary, we assigned 12 AED proteins that (besides PR2, PR5, and PNP-A) have not previously been associated with SAR. A Gene Ontology term analysis (www.arabidopsis.org) linked six AED proteins to responses to biotic and/or abiotic stresses (Table I).

Infection Induces Local and Systemic *AED* Gene Expression Changes

To gain further insight into the regulation of the *AED* genes in locally infected and systemic uninfected leaves during SAR establishment, we analyzed *AED* transcript accumulation using qRT-PCR in the local infected and systemic uninfected leaves of *Pst/AvrRpm1*-inoculated plants. The transcript accumulation of *AED1*, *LLP1*, *PNP-A*, and *PR5* was induced by *Pst/AvrRpm1* relative to the mock-treated controls both locally and

systemically (Fig. 4, A and B). Their local induction was independent of *EDS1* (Fig. 4A). Similarly, *AED4* transcript accumulation was locally repressed by *Pst/AvrRpm1* compared with the mock-treated controls, and this repression was independent of *EDS1* (Fig. 4A). Systemically, a local mock treatment led to a slight induction of *AED1* transcript accumulation and an apparent repression in these tissues of the other genes tested, shown in Figure 4B. Although local *Pst/AvrRpm1* infection enhanced systemic *AED1* transcript accumulation approximately 2-fold compared with the mock-treated controls in both the wild-type and *eds1* mutant plants, the relative level of *AED1* transcripts remained much lower in *eds1* compared with the wild type (Fig. 4B). The systemic *LLP1* transcript accumulation was 4-fold higher in the locally *Pst/AvrRpm1*-infected compared with the mock-treated plants, and this induction was dependent on *EDS1* (Fig. 4B). Similarly, the transcript accumulation of the putative *PR* gene *PNP-A* and *PR5* was induced systemically in an *EDS1*-dependent manner by *Pst/AvrRpm1*. In contrast, the systemic regulation of *AED4* was not different between the local mock and *Pst/AvrRpm1* treatments and did not require *EDS1*. Taken together, we established that the systemic but not local transcript accumulation of *AED1* and the transcriptional regulation of *LLP1*, *PNP-A*, and *PR5* in response to *Pst/AvrRpm1* are dependent on *EDS1* and thus likely related to SAR.

Similar to *Pst/AvrRpm1*, two other *Pst* strains, *Pst* expressing the effector *AvrRps4* (*Pst/AvrRps4*) and virulent *Pst*, locally repressed *AED4* transcript accumulation (Fig. 4, C and D). This repression occurred independently of *EDS1* or was enhanced in the infected *eds1-2* compared with wild-type plants (Fig. 4C). In contrast

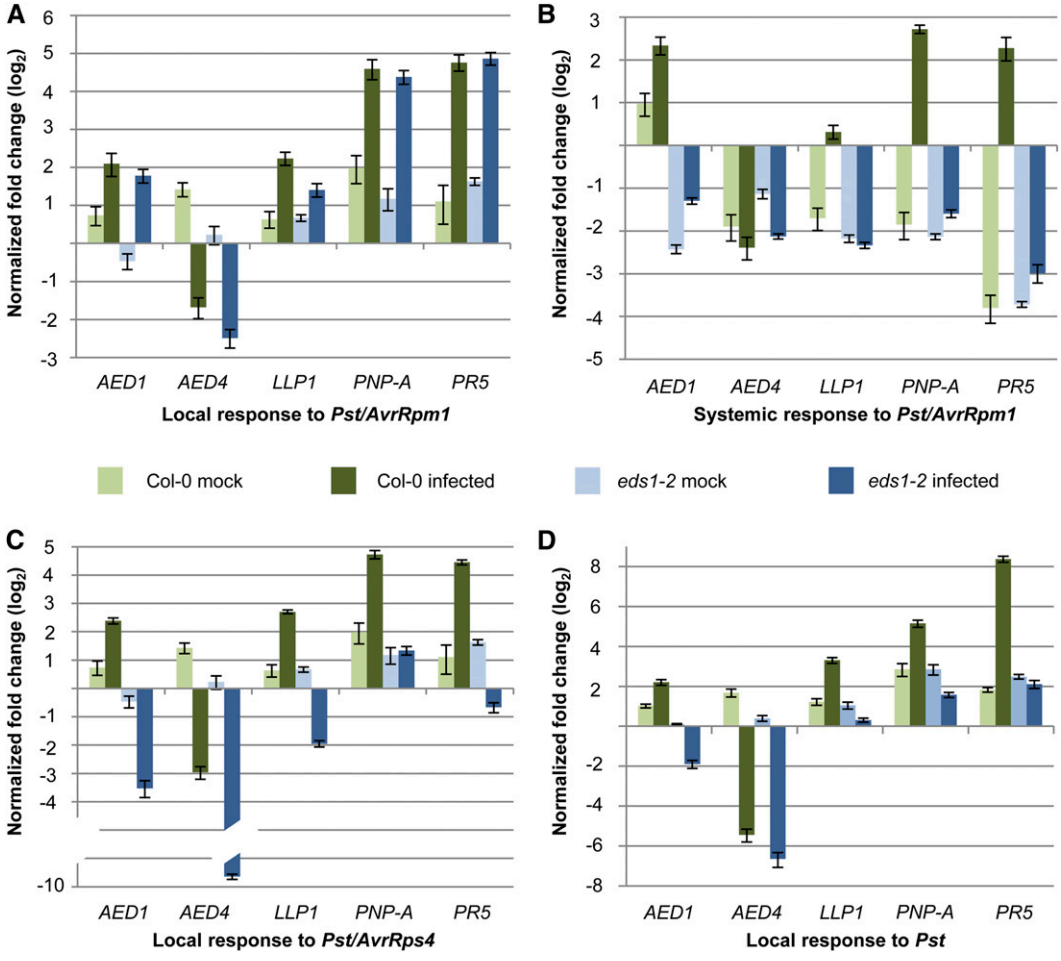

Figure 4. *AED* transcript accumulation in response to avirulent and virulent *Pst*. Col-0 wild-type (green bars) and *eds1-2* mutant (blue bars) plants were treated with 10 mM $MgCl_2$ (mock; light colored bars) or infected (dark colored bars). Three days later, the transcript accumulation of *AED1*, *AED4*, *LLP1*, *PNP-A*, and *PR5* was analyzed by qRT-PCR and normalized to *UBIQUITIN* in leaves infected with *Pst/AvrRpm1* (A), in systemic untreated leaves of *Pst/AvrRpm1*-infected plants (B), in leaves infected with *Pst/AvrRps4* (C), and in leaves infected with *Pst* (D). The transcript accumulation is shown relative to the corresponding transcript levels in leaves of untreated plants of the same age. These experiments were repeated three (*AED4*, *LLP1*, and *PNP-A*) or more (*AED1* and *PR5*) times with similar results.

to an *EDS1*-independent induction of *AED1*, *LLP1*, *PNP-A*, and *PR5* in leaves that were infected with *Pst/AvrRpm1*, these genes were locally induced by *Pst/AvrRps4* or *Pst* in an *EDS1*-dependent manner (Fig. 4, C and D). Because ETI triggered by *Pst/AvrRpm1* is *EDS1* independent but ETI triggered by *Pst/AvrRps4* and basal resistance to *Pst* genetically require *EDS1* (Aarts et al., 1998; Falk et al., 1999; Rietz et al., 2011), the transcriptional up-regulation of the *AED1*, *LLP1*, *PNP-A*, and *PR5* genes in infected leaves appears to correlate with the local resistance response rather than SAR.

AED1 Likely Suppresses SAR

CONSTITUTIVE DISEASE RESISTANCE1 (*CDR1*) encodes an apoplastic aspartyl protease that is 30% identical to AED1 at the amino acid level and is reported to contribute to SAR (Xia et al., 2004). Here, we studied SAR in two transfer DNA (T-DNA) insertion mutants of *AED1*. Whereas *AED1* transcript accumulation was virtually undetectable in the *aed1-1* knockout mutant, *AED1* expression was reduced to approximately 15% compared with wild-type levels in the *aed1-2* knockdown mutant, which carried a T-DNA insertion in the promoter region of *AED1* (Fig. 5A). SAR was induced in these plants by infection of the first two true leaves per plant with *Pst/AvrRpm1*. Three days later, the next two systemic leaves were infected with virulent *Pst*, and the bacterial titers were determined at 4 d post infection (dpi). Wild-type plants and both *aed1-1* and *aed1-2* mutants preinfected with *Pst/AvrRpm1* supported significantly lower *Pst* growth than did the mock-pretreated plants, which was indicative of an SAR response (Fig. 5B). Because the transcripts of the neighboring locus *At5g10770*, which is 70% homologous at the nucleotide level with *AED1*, were elevated in the *aed1-1* and *aed1-2* mutant plants compared with the wild-type (Fig. 5A), we cannot exclude overcompensation for the loss of functional *AED1* in, for example, *aed1-1* by *At5g10770*.

To exclude any effects of possible functional redundancy between *AED1* and its neighboring locus, we attempted to silence the expression of both *AED1* and *At5g10770* using double-stranded RNA interference (RNAi). The RNAi construct included two consecutive 400-nucleotide stretches with full complementarity to the respective transcripts and was used to transform Col-0 plants. T1 plants were cultivated on sterile medium for 2 weeks as described in "Materials and Methods" and subsequently transferred to soil. Compared with similarly treated Col-0 plants, the RNAi plants were severely stunted (Fig. 5C). With time, these plants produced a few flowers and seeds, but we were unable to retrieve viable T2 transgenic plants. Therefore, we pooled the above-ground tissue of three 5-week-old T1 plants to produce sufficient material to allow for gene expression analysis. In two biologically independent samples, the *AED1* transcript accumulation was 3-fold lower in *RNAi:AED1/At5g10770* plants compared with wild-type plants, while *At5g10770* transcript accumulation was not reduced. In contrast to the *aed1-1* and *aed1-2* mutants, the RNAi plants accumulated wild-type but not elevated levels of *At5g10770* transcripts (Fig. 5A). Therefore, the stunted phenotype was most likely related to the reduced *AED1* expression. Notably, the transcript accumulation of the SAR marker gene *PR1* was elevated in both of the RNAi samples (Fig. 5A), suggesting the hyperactivation of SAR in these plants. These data show that AED1 likely restricts SA or SAR signaling, although it is possible that the stunted phenotype of the *RNAi:AED1/At5g10770* plants is due to another function of *AED1*.

To gain further insight into the role of AED1 during SAR, we constructed transgenic lines that conditionally overaccumulate AED1 with a C-terminal HA tag. The expression of the transgene was controlled by the β-estradiol-inducible *XVE* promoter, containing the DNA-binding domain of the bacterial repressor LexA (*X*), the acidic transactivating domain of VP16 (*V*) and the regulatory region of the human estrogen receptor (*E*; Zuo et al., 2000). In the absence of β-estradiol, two independent homozygous *XVE:AED1-HA* transgenic lines did not display AED1-HA protein accumulation, as detected by immunoblot analysis using anti-HA antibodies (Fig. 5D). After spraying the same plants with 100 μM β-estradiol in 0.01% Tween 20 (v/v), we detected the accumulation of a protein band corresponding to the predicted size of AED1-HA (49 kD; Fig. 5D). Compared with untreated Col-0 plants, *AED1* transcript accumulation was induced 3- to 16-fold in *XVE:AED1-HA* plants that were treated with 30 μM β-estradiol (Fig. 5A), the concentration used to induce AED1-HA accumulation in subsequent experiments. Thus, compared with the approximately 4-fold induction of *AED1* expression locally or systemically by infection of wild-type plants (Fig. 4), the treatment of the *XVE:AED1-HA* plants with 30 μM β-estradiol caused either a comparable (transgenic line 108-194) or an approximately 4-fold higher (transgenic line 154-47) induction of *AED1* transcript accumulation, suggesting that the overaccumulation of AED1-HA likely remains within or close to physiologically relevant levels.

Without β-estradiol, both of the *XVE:AED1-HA* transgenic lines mounted normal SAR responses, resulting in the reduced growth of virulent *Pst* in the systemic leaves of preinfected compared with mock-pretreated plants (Fig. 5E). β-Estradiol is normally dissolved in ethanol. Because we observed that the spray treatment of wild-type plants with a low concentration of ethanol repressed SAR (Supplemental Fig. S6), we dissolved β-estradiol in methanol for the following experiments. A control spray treatment of plants with a low concentration of methanol did not affect SAR (Supplemental Fig. S6). Spraying the *XVE:AED1-HA* plants with 30 μM β-estradiol 1 d prior to the start of the experiment suppressed *Pst/AvrRpm1*-induced SAR against *Pst* (Fig. 5F; Supplemental Fig. S6), although local resistance to *Pst/AvrRpm1* and *Pst* did not change (Supplemental Fig. S7). Because the same β-estradiol treatment did not alter SAR in Col-0 wild-type plants (Fig. 5F; Supplemental Fig. S6), we concluded

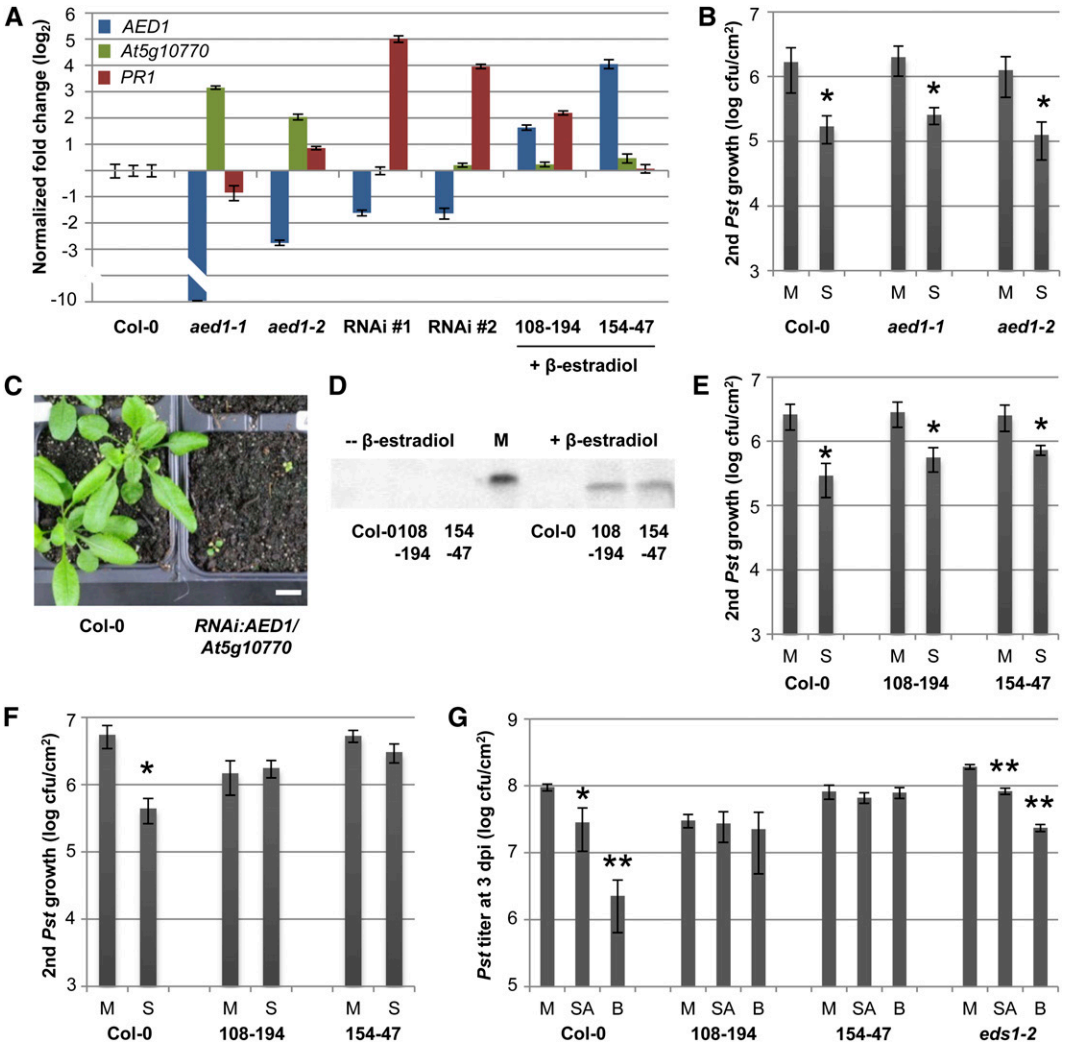

Figure 5. AED1 likely suppresses SAR. A, Transcript levels of *AED1* (blue bars), its neighboring locus *At5g10770* (green bars), and *PR1* (red bars) were normalized to *UBIQUITIN* in the *aed1-1* and *aed1-2* mutant plants, in two aerial tissue pools that each contained three *RNAi:AED1/At5g10770* transgenic plants (T1), and in *XVE:AED1-HA* lines 108-194 and 154-47 at 24 h after treatment with 30 μM β-estradiol. For each set of mutant or transgenic plants, the transcript levels are shown relative to those in Col-0 plants of the same age (one representative Col-0 sample is shown). B, SAR in *aed1-1* and *aed1-2*. *Pst* titers are shown 4 d after a secondary infection that was systemic to primary treatments with 10 mM $MgCl_2$ (mock; M) or *Pst/AvrRpm1* (SAR; S). C, Five-week-old *RNAi:AED1/At5g10770* transgenic plants (T1) compared with Col-0. Both genotypes were grown for the first 2 weeks on Murashige and Skoog medium without (Col-0) or with (transgenic plants) antibiotics, transferred to soil, and propagated for another 3 weeks. D, Anti-HA western blot of whole protein extracts before (left) or 24 h after (right) treatment of Col-0 plants and *XVE:AED1-HA* lines 108-194 and 154-47 with 30 μM β-estradiol. M, Molecular size marker; the visible band corresponds to 50 kD. E and F, SAR in *XVE:AED1-HA* plants. The plants were either untreated (E) or sprayed with 30 μM β-estradiol 24 h before SAR induction (F). SAR was analyzed as in B. G, SA-induced resistance in *XVE:AED1-HA* plants. At 24 h after the treatment of Col-0, *XVE:AED1-HA* lines 108-194 and 154-47, and *eds1-2* plants with 30 μM β-estradiol, all of the plants were sprayed with 0.01% (v/v) Tween 20 (mock; M) or with 1 mM SA or 1 mM BTH (B) in 0.01% (v/v) Tween 20. After another 24 h, the treated leaves were infected with *Pst*. The in planta *Pst* titers are shown at 4 dpi. Asterisks (B and E–G) indicate significant differences compared with the mock-treated controls (*$P < 0.05$, **$P < 0.005$, Student's *t* test). These experiments were repeated two (*aed1-2* [A and B] and RNAi lines [B]) to at least three times with similar results.

that the overaccumulation of the AED1 protein leads to a suppression of SAR.

Because SAR is highly correlated with SA, we investigated the SA-induced resistance response in plants overaccumulating AED1-HA. For this, we treated plants first with 30 μM β-estradiol and 24 h later with SA or the SA functional analog benzo 1,2,3-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) in 0.01% (v/v) Tween 20 or with 0.01% (v/v) Tween 20 as a mock control. Twenty-four hours later, the treated leaves were inoculated with *Pst*, and the in planta *Pst* titers were determined at 4 dpi. Both SA and BTH locally enhanced

resistance in Col-0 plants, resulting in reduced *Pst* growth in treated plants compared with the mock-treated controls (Fig. 5G). As expected, the *eds1-2* plants responded to both SA and BTH with enhanced resistance to *Pst* (Fig. 5G; Falk et al., 1999). Notably, overaccumulation of AED1-HA rendered the *XVE:AED1-HA* plants nonresponsive to SA and BTH, with both independent transgenic lines supporting *Pst* growth to similar levels in the mock-treated, SA-treated, and BTH-treated plants (Fig. 5G). Taken together, these data suggest that AED1 acts downstream of SA to suppress systemic immunity.

LLP1 Is Essential for SAR

To investigate a role of the *LLP1* gene in SAR, we examined the local and systemic defense responses of two Col-0 mutant lines carrying T-DNA insertions upstream or in the vicinity of the start codon of *LLP1* (Fig. 6A). Both insertions strongly reduced *LLP1* transcript accumulation. Because *LLP1* transcript accumulation is enhanced by SA (Krinke et al., 2007; Blanco et al., 2009) and BTH (Fig. 6A), we measured *LLP1* expression in BTH-treated leaves of the *llp1* insertion lines. In *llp1-1* and *llp1-3* mutant plants, BTH elevated the *LLP1* transcript accumulation to 1% and 2%, respectively, of the BTH-induced levels in wild-type plants, confirming that both are knockdown mutants (Fig. 6A). To study local resistance responses, we monitored the growth of three *Pst* strains in the *llp1* mutants compared with the wild-type and *eds1-2* control plants. The *llp1-1* and *llp1-3* mutants supported the growth of *Pst/AvrRpm1* to comparable levels as the Col-0 and *eds1-2* plants (Fig. 6B), indicating that local resistance to *Pst/AvrRpm1* was not affected by mutations in *LLP1*. Supporting this conclusion, free SA was induced to comparable levels in the *Pst/AvrRpm1*-infected leaves of wild-type and *llp1-1*, *llp1-3*, and *eds1-2* mutant plants (Supplemental Fig. S8). In contrast to *eds1-2*, which displayed enhanced susceptibility to *Pst/AvrRps4* and virulent *Pst*, both *llp1-1* and *llp1-3* supported growth of these *Pst* strains that was comparable to that in the Col-0 wild type (Fig. 6, C and D), indicating that local *EDS1*-dependent immunity was not altered in the *llp1* mutants. However, SAR triggered by *Pst/AvrRpm1* was abolished in both the *llp1-1* and *llp1-3* mutant plants (Fig. 6E). Because plants overexpressing *LLP1* showed marginally higher resistance than wild-type plants against *Pst/AvrRpm1* (Armijo et al., 2013), we reasoned that the *llp1* SAR-defective phenotype might arise from weak local effects of *llp1* mutations specifically to *Pst/AvrRpm1*. Therefore, we used *Pst/AvrRps4*, whose growth is not affected in plants overexpressing *LLP1* (Armijo et al., 2013), as a primary inoculum to trigger SAR. The pretreatment of Col-0 plants with *Pst/AvrRps4* triggered SAR, leading to reduced *Pst* growth in systemic leaves compared with the mock pretreatment (Fig. 6E). Similar to *Pst/AvrRpm1*, *Pst/AvrRps4* did not trigger SAR in the *llp1-1* and *llp1-3* mutant plants (Fig. 6E). Together, these data suggest an SAR-specific function for *LLP1* in plant immunity.

SAR induced by *Pst/AvrRpm1* was accompanied by enhanced *PR1* transcript accumulation in the systemic uninfected leaves of the preinfected compared with the mock-pretreated wild-type plants (Fig. 6F). In contrast to the SAR-defective *eds1-2* mutant (Truman et al., 2007; Rietz et al., 2011), which did not support the systemic induction of *PR1* (Fig. 6F), the *llp1* mutant plants responded to the local infection with modestly induced *PR1* expression in the systemic uninfected tissue that was 8- to 20-fold lower compared with that in the wild-type plants (Fig. 6F). These data indicate that immune signaling was compromised but not abolished in the systemic uninfected tissue of locally infected *llp1* mutant plants. To gain further insight into a possible role of LLP1 in SA signaling, we investigated the responses of the *llp1* mutants to the application of exogenous SA or BTH. In the wild-type and *eds1-2* plants, both SA and BTH induced resistance against *Pst* (Fig. 6G), which was accompanied by enhanced *PR1* transcript accumulation in the treated leaves (Fig. 6H). Similarly, endogenous *AED1* transcript accumulation was induced by both SA and BTH in the wild-type and *eds1-2* plants to a comparable degree as by infection of the wild-type plants (Figs. 4 and 6H). These results confirmed the role of *EDS1* upstream of SA in SA-induced immunity (Falk et al., 1999). Additionally, *LLP1* transcript accumulation was induced in BTH-treated wild-type and *eds1-2* plants (Fig. 6A), indicating that BTH induced *LLP1* expression independently of *EDS1*. Both the *llp1-1* and *llp1-3* mutants responded to SA and BTH with enhanced resistance to *Pst*, which was not significantly different from that induced in the wild-type plants (Fig. 6G). Additionally, SA and BTH induced comparable levels of *AED1* and *PR1* transcript accumulation in the *llp1-1* and *llp1-3* mutants compared with those in the wild-type and *eds1-2* plants (Fig. 6H). These data show that LLP1 is not necessary for immune signaling downstream of SA. Altogether, the data suggest that LLP1 is an important promoter of systemic resistance, possibly acting in parallel with SA.

DISCUSSION

Here, we have utilized the SAR-specific defect of *eds1* mutant plants in response to the *Pst* effector AvrRpm1 to identify new SAR components using a comparative proteomics approach to identify potential proteinaceous signaling components in the apoplast. We showed that *EDS1* is required for both SAR signal generation in the primary infected leaves and SAR signal perception in the systemic tissues. Therefore, the identified proteins might operate during one or more phases of SAR. Of the 12 identified AED proteins, nine have not previously been associated with SAR. Of these, the predicted aspartyl protease AED1 appears to suppress systemic immunity as part of a homeostatic control mechanism regulating SAR (Figs. 5 and 7), whereas the legume lectin-like protein LLP1

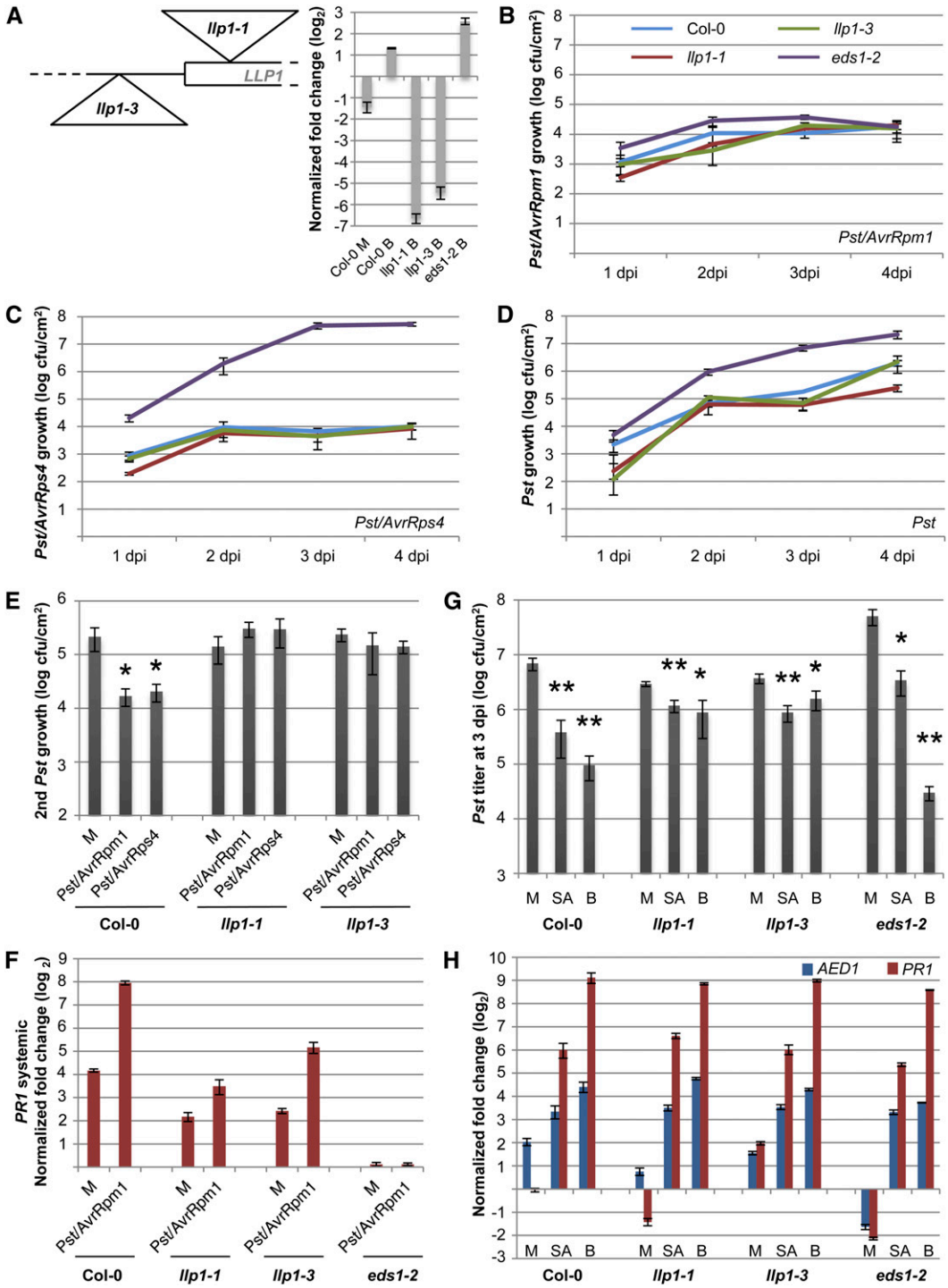

Figure 6. *LLP1* promotes SAR. A, Schematic drawing of the positions of the *llp1-1* and *llp1-3* T-DNA insertions near the start codon of *LLP1* and transcript levels of *LLP1* normalized to *UBIQUITIN* in the Col-0, *llp1-1*, *llp1-3*, and *eds1-2* plants 24 h after infiltration of the leaves with 10 mM $MgCl_2$ (mock; M) or with 100 μM BTH (B; inset). Transcript accumulation is shown relative to that in untreated Col-0 plants. B to D, Growth curves of *Pst/AvrRpm1* (B), *Pst/AvrRps4* (C), and *Pst* (D) in the wild-type (blue), *llp1-1* (red), *llp1-3* (green), and *eds1-2* (purple) plants. The bacterial titers in the infected leaves were determined at 1, 2, 3, and 4 dpi. E, SAR in Col-0, *llp1-1*, and *llp1-3* plants. *Pst* titers are shown 4 d after a secondary infection that was systemic to primary treatments with 10 mM $MgCl_2$ (M) or with *Pst/AvrRpm1* or *Pst/AvrRps4*. F, Systemic *PR1* induction. *PR1* transcript levels were normalized to *UBIQUITIN* in the systemic untreated leaves 3 d after a primary treatment of Col-0, *llp1-1*, *llp1-3*, and *eds1-2* plants with 10 mM $MgCl_2$ (M) or *Pst/AvrRpm1*. G and H, SA-induced resistance in the *llp1* mutants. Col-0 and *llp1-1*, *llp1-3*, and *eds1-2* mutant plants were sprayed with 0.01% (v/v) Tween 20 (mock; M) or with 1 mM SA or 1 mM BTH (B) in 0.01% (v/v)

is essential for promoting SAR, possibly in parallel with SA (Figs. 6 and 7). Systemic but not local *AED1* and *LLP1* transcript accumulation was correlated with *EDS1*-dependent *Pst/AvrRpm1*-induced SAR (Fig. 4). Moreover, both AED1 and LLP1 have pronounced effects on SAR but only mild or no influence on local defenses (Figs. 5 and 6; Supplemental Fig. S7; Armijo et al., 2013). Thus, together with documented differences between local and systemic transcriptional and metabolic profiles (Gruner et al., 2013), our data support a mechanistic separation between the local and systemic phases of SAR. Both AED1 and LLP1 predominantly operate in the systemic phase.

Similar to mitogen-activated protein kinases 3 and 6 (Tsuda et al., 2013), EDS1/PAD4 signaling can compensate for the loss of SA signaling during local resistance responses activated via CC-NLRs similar to RPM1 responding to AvrRpm1 (Venugopal et al., 2009). For SAR, however, EDS1 and SA do not appear to act redundantly (Fig. 1; Truman et al., 2007; Rietz et al., 2011). Although we cannot rule out that EDS1 and PAD4, as a nucleocytoplasmic complex functioning in basal resistance (Feys et al., 2005; García et al., 2010; Rietz et al., 2011), function in the synthesis of one or more SAR signaling molecules, a more upstream transcriptional reprogramming role seems more likely between AvrRpm1 recognition and SAR signal generation (Bartsch et al., 2006; García et al., 2010). Whereas both EDS1 and PAD4 are necessary for SAR signal generation, EDS1 but not PAD4 is essential for SAR signal perception (Fig. 1). Because the *eds1* mutant responds normally to SA (Figs. 5 and 6; Falk et al., 1999), the data place EDS1 upstream of SA, also during SAR signal perception. Arabidopsis *EDS1*, *PAD4*, and *SAG101* have separable functions in TIR-NLR and basal resistance responses (Rietz et al., 2011; Wagner et al., 2013) and in viral resistance mediated by the CC-NLR HRT (Chandra-Shekara et al., 2004; Zhu et al., 2011). Also, it is known that *PAD4* has a unique action, without *EDS1* or *SAG101*, in phloem-based defenses against green peach aphid infestation (Pegadaraju et al., 2007). Similarly, EDS1 might individually contribute to SAR signal perception without its signaling partner PAD4 (Figs. 1 and 7). Because the EDS1/PAD4 complex is essential for basal resistance and the full extent of SAR (Rietz et al., 2011), loss of *PAD4* is likely partially compensated for by *SAG101* for at least a subset of SAR defense outputs (Feys et al., 2005; Wagner et al., 2013; Fig. 7). Thus, EDS1, together with its signaling partner PAD4 or SAG101, might support SAR signal perception by fortifying SA-mediated defenses, including SA-associated transcriptional reprogramming (Fig. 7; Feys et al., 2005; Wiermer et al., 2005; Vlot et al., 2009; García et al., 2010; Rietz et al., 2011).

AvrRpm1 transcript accumulation upon treatment of *pDEX:AvrRpm1-HA* plants with 1 μM DEX reaches higher levels than during an SAR-inducing infection of Col-0 plants with *Pst/AvrRpm1* (Supplemental Fig. S1). Because it is unclear how bacterial translation and subsequent protein transfer into plant cells compares with the translation of the foreign transcript by the Arabidopsis translational machinery, it is difficult to draw conclusions on the relative potency of each AvrRpm1 delivery. However, the macroscopic cell death symptoms that are induced locally by both treatments are comparable (Supplemental Fig. S1, D and E). Prior studies have shown that ETI-inducing pathogens trigger SAR independently of HR cell death (Cameron et al., 1994; Mishina and Zeier, 2007; Liu et al., 2010). Altogether, the systemic resistance induced by the local expression of *AvrRpm1-HA* (Fig. 2) is likely caused by an *AvrRpm1*-dependent signaling event and not, for example, by nonspecific processes following tissue damage in DEX-treated *pDEX:AvrRpm1-HA* tissues. In the absence of effectors, systemic immunity can be induced by the local application of purified pathogen-associated molecular patterns (PAMPs), such as flagellin or lipopolysaccharides (Mishina and Zeier, 2007), eliciting basal PAMP-triggered immunity (PTI), which is not normally associated with HR cell death (Jones and Dangl, 2006; Henry et al., 2013). The fact that either PAMPs (Mishina and Zeier, 2007) or the effector AvrRpm1 (Fig. 2) can trigger systemic immunity indicates that local defense signaling in the form of PTI or ETI is possibly sufficient for SAR.

Using the *eds1* mutant with an SAR-specific phenotype in response to *Pst/AvrRpm1* (Aarts et al., 1998; Truman et al., 2007), we expected to identify proteins that are more predominantly associated with systemic than local defenses. In support of this hypothesis, there was limited overlap between the AED proteins and proteins that were identified in proteomic studies of local responses of Arabidopsis plants or cultured cells to either SA or virulent and avirulent *Pst* (Oh et al., 2005; Jones et al., 2006; Kaffarnik et al., 2009). However, these studies identified proteins with predicted enzymatic activities that are related to some of the AEDs found here. For example, GDSL LIPASE-LIKE1 shares 25% identity with AED4 at the amino acid level, is secreted upon the SA treatment of Arabidopsis cultured cells (Oh et al., 2005), and is involved in the local and systemic defense responses that are associated

Figure 6. (*Continued.*)
Tween 20. After 24 h, leaves of the treated plants were either infected with *Pst* (G) or harvested for qRT-PCR analysis (H). In G, the in planta *Pst* titers are shown at 4 dpi; in H, the transcript levels of *AED1* and *PR1* were normalized to *UBIQUITIN* and are shown relative to those in untreated Col-0 plants of the same age. Asterisks indicate significant differences from the mock-treated controls (*$P < 0.05$, **$P < 0.005$, Student's *t* test). These experiments were repeated two times (E [*Pst/AvrRps4*], F, and H) to at least three times (A–D, E [*Pst/AvrRpm1*], and G) with similar results.

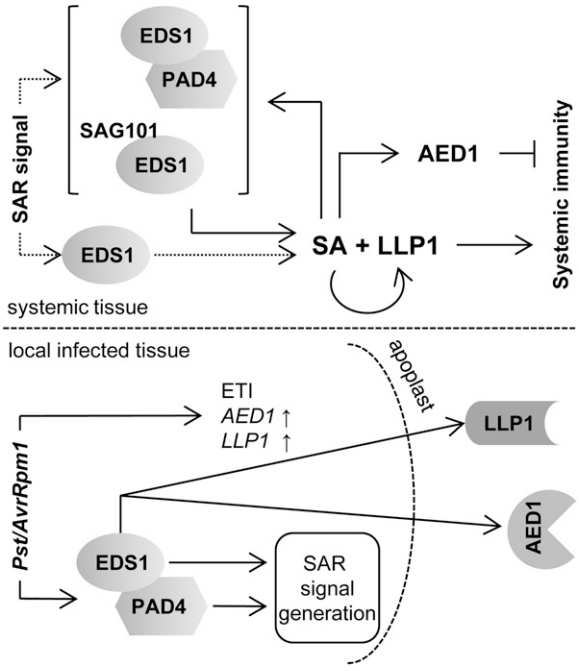

Figure 7. Model integrating EDS1, PAD4, AED1, and LLP1 in SAR signaling. Both EDS1 and PAD4 are essential for SAR signal generation in *Pst/AvrRpm1*-infected tissue. In addition, signaling downstream of EDS1 leads to the accumulation of AED1 and LLP1 in the apoplast. Systemically, EDS1 either alone or together with PAD4 or SAG101 (both options are shown in dotted lines) mediates SAR signal perception upstream of SA. SA signaling is most likely fortified by the positive feedback loop of SA with EDS1/PAD4/SAG101. The local accumulation of *AED1* and *LLP1* transcripts in response to *Pst/AvrRpm1* is *EDS1* independent and appears to be related to ETI, whereas systemic *AED1* and *LLP1* expression is regulated by *EDS1* and SA and may be associated with SAR. The predicted aspartyl protease AED1 likely suppresses systemic immunity, and the legume lectin-like protein LLP1 appears to promote systemic immunity via actions in parallel with SA.

with ethylene but not SA (Oh et al., 2005; Kwon et al., 2009). Our data implicate the GDSL-motif lipase AED4 in *EDS1*-dependent SAR (Fig. 3). The protein was found in petiole exudates from 6-week-old, long-day-grown Arabidopsis plants, indicating that AED4 may be mobile through the phloem (Benning et al., 2012; Guelette et al., 2012). Because the expression of *AED4* is repressed by the infection of plants with virulent or avirulent *Pst* (Fig. 4) or by drought (Huang et al., 2008), it is tempting to speculate that AED4 is involved in phloem-mediated long-distance signaling regulating responses to biotic and abiotic stress.

The expression of *AED1*, *AED4*, *LLP1*, *PNP-A*, and *PR5* in leaves infected with virulent or avirulent *Pst* was predominantly associated with local defenses (Figs. 4 and 7). In contrast, the accumulation of the corresponding proteins in apoplast-enriched extracts from the *AvrRpm1-HA*-expressing plants was *EDS1* dependent and thus was correlated with SAR (Figs. 3 and 7; Supplemental Table S1). The accumulation or modification of AED proteins is possibly regulated posttranscriptionally or posttranslationally independently of or prior to transcriptional changes. Such a scenario was previously suggested to explain early changes in intracellular protein accumulation in Arabidopsis plants infected with different *Pst* strains, including *Pst/AvrRpm1* (Jones et al., 2006), and is supported by the identification of two AED proteins in multiple 2D gel spots (Supplemental Fig. S4). Also, for differentially apoplast-enriched protein spots, we could not distinguish between differences in overall protein accumulation or their secretion. In the systemic uninfected leaves of *Pst/AvrRpm1*-infected plants, the transcript accumulation of *AED1*, *LLP1*, *PNP-A*, and *PR5* was tightly correlated with the extent of SAR (Figs. 4 and 7). For *PNP-A*, the data provide further experimental support for a previous bioinformatics study linking *PNP-A* to SA-mediated defense signaling and SAR (Meier et al., 2008). Natriuretic peptides occur in animals and plants and have similarities with cell wall-loosening expansins (Gehring and Irving, 2003). PNP-A localizes to the apoplast and is associated with plant homeostasis regulating ion fluxes and cellular water uptake (Gehring and Irving, 2003; Wang et al., 2011). Because PNP-A was suggested to regulate dark respiration via long-distance, possibly phloem-mediated signaling (Ruzvidzo et al., 2011), we will investigate whether the PNP-A-triggered local and/or systemic changes in plant homeostasis affect SAR.

The aspartyl protease CDR1 accumulates in the apoplast upon infection of Arabidopsis plants with *Pst/AvrRpm1* (Xia et al., 2004). Similar to its rice (*Oryza sativa*) homolog *Os*CDR1 (Prasad et al., 2009), *At*CDR1 is believed to release a peptide between 3 and 10 kD in size that triggers *PR2* transcription both locally and systemically (Xia et al., 2004). Similar to CDR1, AED1 is an atypical aspartyl protease lacking a plant-specific insertion that is found in canonical plant aspartyl proteases (Faro and Gal, 2005). However, in contrast to CDR1, AED1 appears to repress SAR by restricting systemic immunity downstream of SA (Fig. 5). Previous studies have firmly linked the expression of *AED1* in Arabidopsis with *EDS1* or SA-mediated defense responses (Chini et al., 2004; Eulgem et al., 2004; Mosher et al., 2006; van Damme et al., 2008), but its biological role has remained elusive. We showed that *AED1* transcript accumulation is induced locally and systemically by infection and locally by SA (Figs. 4 and 6). Because the conditional overexpression of *AED1-HA* repressed both SAR and SA-induced resistance without affecting the growth of *Pst* in healthy plants (Fig. 5; Supplemental Fig. S7), AED1 might be part of a homeostatic mechanism to limit SAR signaling (Fig. 7) and thus regulate the resource allocation in the trade-off between defense and plant growth (Heidel and Dong, 2006; van Hulten et al., 2006; Traw et al., 2007; Pajerowska-Mukhtar et al., 2012). In support of this, we found that reduced *AED1* transcript levels in Arabidopsis *RNAi:AED1/At5g10770* plants caused severe stunting, a phenotype that is often observed in constitutive defense mutants (Shirano et al., 2002;

Yoshioka et al., 2006; Bi et al., 2010; Zhu et al., 2010). The stunted growth of the *RNAi:AED1/At5g10770* plants was accompanied by an elevated transcript accumulation of the SAR marker gene *PR1*, underscoring a negative regulatory role of AED1 during SAR.

The primary structure of AED1 resembles that of its tobacco (*Nicotiana tabacum*) homolog, CHLOROPLASTIC NUCLEOID DNA-BINDING41 (CND41), which degrades the chloroplast protein Rubisco during the initial steps of senescence (Nakano et al., 1997; Kato et al., 2004, 2005). Consequently, *CND41* antisense tobacco displayed delayed senescence in older leaves, whereas the plants were dwarfed, similar to the *RNAi:AED1/At5g10770* Arabidopsis plants (Fig. 5; Nakano et al., 2003; Kato et al., 2004). Therefore, we speculate that *CND41* may also play a role in the defense against pathogens, given the proposed connections between senescence and innate immunity, with autophagy-associated genes antagonizing SA-mediated immune signaling (for review, see Dickman and Fluhr, 2013). Elucidating the exact mode of AED1 action during SAR, including its apparent recruitment in the systemic rather than the local phase of SA-mediated immunity, requires further investigation. Similar to tobacco and tomato (*Solanum lycopersicum*) apoplastic aspartyl proteases that degrade PR proteins (Rodrigo et al., 1989, 1991), AED1 may suppress SAR by degrading one or more proteins in the apoplast of SAR-induced leaves.

LLP1 is one of 226 lectin genes encoding carbohydrate-binding proteins in the Arabidopsis genome (Peumans and Van Damme, 1995; Sharon and Lis, 2004; Armijo et al., 2013). The plant lectins are divided into 12 families, and LLP1, along with its two closest homologs At3g16530 and AtLEC, belongs to the lectin-legB family of legume lectin-like proteins (Jiang et al., 2010; Armijo et al., 2013). In contrast to LLP1, most Arabidopsis legume lectin-like proteins contain kinase domains (Armijo et al., 2013). Notably, several lectin receptor kinases with other types of lectin domains promote SA-associated immunity, including PTI (for review, see Singh and Zimmerli, 2013). In contrast to the *LLP1* homolog *AtLEC*, whose expression is associated with jasmonic acid- and ethylene-mediated signaling (Lyou et al., 2009), the expression of *LLP1* is induced early after the SA treatment of Arabidopsis plants in a *NONEXPRESSOR OF PR GENES1* (*NPR1*)-dependent manner (Krinke et al., 2007; Blanco et al., 2009) and by virulent and avirulent *Pst* in an *EDS1*-dependent manner (Fig. 4). The *LLP1* expression that was induced by BTH was independent of *EDS1* (Fig. 6), suggesting that the induction of *LLP1* by SA occurs downstream of *EDS1* (Fig. 7). Similar to EDS1 (Falk et al., 1999; Truman et al., 2007; Heidrich et al., 2011), LLP1 is necessary for systemic immunity triggered by the local inoculation of plants with *Pst/AvrRpm1* or *Pst/AvrRps4* but not for the local immunity triggered by the application of exogenous SA (Fig. 6). The latter result indicates that LLP1 probably does not act downstream of SA. However, a function of LLP1 upstream of SA seems equally unlikely, because LLP1 does not appear to affect the local resistance responses to virulent and avirulent *Pst* that are normally associated with *EDS1* and SA (Fig. 6; Armijo et al., 2013). Additionally, LLP1 did not affect *EDS1*-independent free SA accumulation in Arabidopsis in response to *Pst/AvrRpm1*. Altogether, the data link LLP1 most closely with systemic rather than local immunity and suggest that LLP1 may act in parallel with SA. Because the constitutive overaccumulation of LLP1 did not trigger a significant resistance response to, for example, *Pst* (Armijo et al., 2013), LLP1 likely cooperates with one or more additional components, possibly including SA. Further investigation is required to clarify how LLP1 acts and which signaling components, in addition to SA, may cooperate with LLP1 to promote the systemic phase of SAR.

Mammalian lectins perform essential functions in animal innate immunity (summarized in Rabinovich et al., 2012). These lectins are believed to operate in the recognition of self, nonself, or altered-self molecules or associated entities (e.g. tumor cells) and to mediate cellular trafficking, cell-cell communication, and immune signaling, among other activities (Sharon and Lis, 2004; Rabinovich et al., 2012). Similar to plant lectin receptor kinases (Singh and Zimmerli, 2013), a subset of lectins functions as PAMP receptors in mammalian immunity (Rabinovich et al., 2012). Analogous to animal systems, LLP1 may regulate SAR in plants by mediating the recognition of, for example, altered-self glycan structures accumulating in locally infected or systemically responding tissues. Interestingly, LLP1 is localized to the plasma membrane facing the apoplast (Armijo et al., 2013) and might sense changes to the glycan composition of the cell wall, possibly caused by another AED, XYL4, which functions as an extracellular β-D-xylosidase (Fig. 3; Table I; Minic et al., 2004). Alternatively, LLP1 might recognize components of the cuticle, which contributes to SAR signal perception or propagation in systemic uninfected tissues via a currently unknown mechanism (Xia et al., 2009, 2010).

In conclusion, we uncovered two new components that regulate different aspects of SAR. An aspartyl protease, AED1, represses SAR likely as part of a feedback regulatory mechanism. In contrast, the legume lectin-like protein LLP1 promotes SAR, possibly in parallel with SA. Future research will be directed at elucidating whether LLP1-mediated extracellular glycan sensing acts on systemic immunity.

MATERIALS AND METHODS

Plants and Cultivation Conditions

The Arabidopsis (*Arabidopsis thaliana*) cv Wassilewskija-0 (petiole exudate analyses) and cv Col-0 (all other experiments) were used. The mutants *eds1-1*, *pad4-5*, *eds1-1pad4-5*, *dir1*, and *eds1-2* have been described previously (Falk et al., 1999; Feys et al., 2001; Maldonado et al., 2002; Bartsch et al., 2006). Col-0 *pDEX:AvrRpm1-HA* has been described previously (Mackey et al., 2002) and was crossed with *eds1-2* to yield *eds1-2 pDEX:AvrRpm1-HA*. The T-DNA insertion lines SALK_111104 (*aed1-1*), SALK_091655 (*aed1-2*), SALK_036814 (*llp1-1*), and SALK_074760 (*llp1-3*) were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al., 2000). Homozygous plants were

selected for seed stocks and experiments. The plants were grown on normal potting soil mixed with silica sand at a ratio of 5:1 and kept at 20°C/22°C (night/day), 70% relative humidity, and 100 μE m^{-2} s^{-1} light for 10-h days.

DNA Constructs and Plant Transformation

The RNAi construct targeting *AED1* and *At5g10770* was made in pHANNIBAL (Wesley et al., 2001). From each target gene, a 400-nucleotide fragment of the coding sequence was amplified by reverse transcription-PCR with the primer sets pRNAi-1-5′cl/pRNAi-1-3′cl and pRNAi-70-5′cl/pRNAi-70-3′cl (Supplemental Table S2), respectively, using RNA that was isolated from *Pseudomonas syringae* pv *tomato* strain DC3000 expressing *AvrRpm1*-infected Col-0 plants as a template. Subsequently, the two fragments were annealed and amplified by PCR using the primers pRNAi-1-5′cl and pRNAi-70-3′cl. The resulting 800-nucleotide fragment was cloned into pENTR/dTOPO (Invitrogen), sequenced, and cloned into pHANNIBAL in the sense and antisense orientations using *Eco*RI/*Xho*I and *Hin*dIII/*Xba*I, respectively. Finally, the *AED1/At5g10770* RNAi cassette from pHANNIBAL was transferred to the binary vector pART27 using *Not*I.

The *AED1* overexpression construct was made in pER8-GW-C-term-3XHA-Strep. To generate pER8-GW-C-term-3XHA-StrepII, the 3XHA-StrepII fragment from pXCSG-HAStrep (Stuttmann et al., 2009) was cut with *Xho*I and *Xba*I and transferred to Gateway-compatible pER8 (Curtis and Grossniklaus, 2003) that was cut with *Xho*I and *Spe*I. The coding sequence of *AED1* (At5g10760) lacking the stop codon was amplified by reverse transcription-PCR with the primers pAED1-5′cl and pAED1-3′cl (Supplemental Table S2) using RNA isolated from Col-0 plants infected with *Pst/AvrRpm1* as a template. The resulting DNA fragment was cloned into pENTR/dTOPO (Invitrogen) and sequenced. Subsequently, *AED1* complementary DNA was transferred into the destination vector pER8-GW-C-term-3XHA-StrepII by standard Gateway technology (Invitrogen).

Both pART27 containing the *AED1/At5g10770* RNAi cassette and pER8-GW-C-term-3XHA-StrepII carrying *AED1* were transferred into *Agrobacterium tumefaciens* strain GV3101. Subsequently, Col-0 plants were transformed with each construct by floral dipping according to Logemann et al. (2006). Transgenic T1 plants were selected on Murashige and Skoog medium containing 50 μg mL^{-1} kanamycin (RNAi lines) or 50 μg mL^{-1} hygromycin (*AED1* overexpression lines). The surviving RNAi plants were transferred to soil 2 weeks after germination and used for experiments 3 weeks later. Homozygous *AED1* overexpression lines of the third (T3; line 108-194) or fourth (T4; line 154-47) generation were used for all of the experiments.

Pathogens, Infection Methods, and SAR Assays

Four- to 5-week-old plants were used for all of the infection experiments. Bacterial propagation and the infection of plants with virulent or avirulent *Pst* were carried out as described previously (Aarts et al., 1998; Vlot et al., 2008). For bacterial growth curves or gene expression analysis in infected tissues, rosette leaves 3 and 4 were infiltrated with 10^5 cfu mL^{-1} of the appropriate bacterium. SAR or systemic gene expression changes were induced by infiltrating the first two true leaves per plant with 10^6 cfu mL^{-1} *Pst/AvrRpm1* or *Pst/AvrRps4* or 10 mM $MgCl_2$ as a control. For DEX-induced SAR, the first two true leaves of the *pDEX:AvrRpm1-HA* plants were surface treated (painted) with 1 μM DEX (Sigma-Aldrich) in 0.01% (v/v) Tween 20 or 0.01% (v/v) Tween 20 as a control. For systemic gene expression or SAR analyses, two systemic leaves (leaves 3 and 4) were either harvested or infected with 10^5 cfu mL^{-1} *Pst* 3 d after the primary treatment. The in planta *Pst* titers were determined 4 d later as described previously (Vlot et al., 2008). For experiments with the *AED1-HA*-overexpressing plants, the *XVE:AED1-HA* plants were sprayed with 30 μM β-estradiol in 0.01% (v/v) Tween 20 at 24 h prior to the start of the experiment. β-Estradiol was freshly dissolved in methanol and diluted to the appropriate concentration prior to every experiment for the optimal induction of AED1-HA accumulation.

Chemically Induced Resistance

BTH (purchased commercially under the trade name Bion; Ciba-Geigy) and SA (Sigma-Aldrich) were each dissolved in water to produce stock solutions of 100 mM. The local induction of *LLP1* transcript accumulation was triggered via syringe infiltration of the first two true leaves of 4- to 5-week-old plants with 100 μM BTH. To induce local resistance with SA or BTH, 4- to 5-week-old plants were sprayed until dropoff with 1 mM of either compound in 0.01% (v/v) Tween 20 or with 0.01% (v/v) Tween 20 as a control. After 24 h, leaves 3 and 4 of the treated plants were either harvested for gene expression analysis or syringe infiltrated with 10^5 cfu mL^{-1} *Pst*. The in planta *Pst* titers were determined at 4 dpi as described above.

Petiole Exudates

The petiole exudates were collected as described previously (Maldonado et al., 2002), except that leaves were treated with 10^6 cfu mL^{-1} *Pst/AvrRpm1* or 10 mM $MgCl_2$. The exudates from five to 10 leaves in 1.5 mL of 1 mM EDTA were diluted 2-fold with water and infiltrated into untreated healthy plants. The exudate-infiltrated leaves were collected 24 h later, and *PR1* expression was analyzed on northern blots as described previously (Maldonado et al., 2002). In parallel, 100 μL of exudate per petiole was plated on sterile medium and cultivated for *Pst/AvrRpm1* growth as described (Aarts et al., 1998); experiments were evaluated and considered for this work only if the petiole exudates did not contain bacteria.

Isolation of Apoplast-Enriched Protein Extracts

The Col-0 *DEX::AvrRpm1-HA* and *eds1-2 DEX::AvrRpm1-HA* plants were sown as a lawn. Three- to 4-week-old plants were sprayed until dropoff with 30 μM DEX in 0.01% (v/v) Tween 20. Four to 5 h later, all of the aboveground tissue was harvested. The apoplast-enriched protein extracts were isolated in either APO buffer I (2.5 mM Tris, pH 7.4, 1 mM EDTA, and 30 mM mannitol; used for 2D gel analysis) or APO buffer II (2.5 mM HEPES, pH 7.4, 1 mM EDTA, and 30 mM $MgCl_2$; used for ICPL analysis). The plant tissue in the appropriate buffer was exposed to a mild vacuum for up to 10 min in a normal vacuum chamber. Afterward, the vacuum was slowly released. This procedure was repeated two to three times until the tissue was completely infiltrated. The infiltrated plants were transferred to 20- to 30-mL syringes that were hung in 50-mL tubes and centrifuged at 2,250 rpm at 4°C for 20 min. The flow through was collected as the apoplast-enriched protein extract.

Proteomics

2D Gel Analysis

A total of 200 μg of protein was loaded onto 17-cm (two biological replicates, including Fig. 3 and Supplemental Fig. S4A) or 7-cm (two additional replicates in Supplemental Fig. S4B) nonlinear isoelectric focusing strips, pI 4 to 7 (Bio-Rad), and run according to the manufacturer's instructions. The second dimension with the 17-cm strips was performed using 12% (w/v) polyacrylamide gels in the Laemmli buffer system and run in a Bio-Rad Protean II cell. The second dimension using the 7-cm strips was performed using NuPAGE precast 4% to 12% (w/v) polyacrylamide gradient gels (Invitrogen) in the MES buffer system. Both large gels and one small gel were then stained using SYPRO Ruby (Invitrogen); the other small gel was stained using Coomassie PageBlue (Fermentas). For the software-assisted comparison of 2D gels containing proteins from the wild-type and *eds1* mutant plants, gel images were overlapped, combined, and analyzed using the Delta2D gel-analysis system (DECODON). The spots selected for MALDI analysis were robotically excised, digested, and spotted using the Proteineer SP + DP systems (Bruker Daltonik), and the data were collected on an Ultraflex III (Bruker Daltonik) using two-stage data collection as described previously (March et al., 2012). The peptide mass fingerprints and peptide fragmentation fingerprints were searched against the National Center for Biotechnology Information nonredundant database using Mascot (Matrix Science; www.matrixscience.com) with the parameters described in Supplemental Protocol S1.

ICPL

The proteins in the apoplast-enriched extracts were concentrated on 3-kD size-exclusion columns (Amicon Ultra; Millipore) according to the manufacturer's instructions. Subsequently, 70 μg of protein per sample (at a concentration of 3.5 μg μL^{-1}) was labeled with d0-*N*-nicotinoyloxy-succinimide (ICPL-0 or light tag; wild-type extracts) or d4-*N*-nicotinoyloxy-succinimide (ICPL-4 or heavy tag; *eds1-2* extracts) according to the ICPL duplex kit instructions (Serva Electrophoresis). The differentially labeled wild-type and mutant extracts were then pooled per biologically independent repetition, and the proteins were separated on one-dimensional polyacrylamide gels (12% [w/v]). After Coomassie Blue staining and washing of the gels, three slices with visible protein bands were excised per sample and subjected to in-gel trypsin

digestion as described previously (Sarioglu et al., 2006). The digested peptides were analyzed according to Gaupels et al. (2012) by nano-HPLC using the Ultimate 3000 (Dionex) device coupled to a linear quadrupole ion-trap Orbitrap (LTQ Orbitrap XL) mass spectrometer (Thermo Fisher) equipped with a nano-electrospray ionization source. All of the MS/MS spectra were analyzed using Mascot (version 2.2.06; Matrix Science). Mascot was set up to search the National Center for Biotechnology Information Arabidopsis database (version of April 19, 2012 [64,961 sequences]), assuming the digestion enzyme trypsin and with a fragment ion mass tolerance (MS/MS) of 0.6 D and parent ion tolerance of 10 ppm (MS). The iodoacetamide derivatization of Cys was specified in Mascot as a stable modification. The oxidations of Met and ICPL-0 and ICPL-4 for Lys were specified as variable modifications. The data processing for the ICPL analysis was performed according to Gaupels et al. (2012) with one exception: proteins that were identified by at least one unique peptide but were present in all three biological replicates were considered, of which proteins displaying a significant differential accumulation between the extracts from the wild-type and *eds1* mutant plants were considered AEDs ($P < 0.05$, Perseus statistical tool; http://www.perseus-framework.org/; Cox and Mann, 2011).

SA Measurement

Free SA was isolated from pooled leaves from six individual plants per sample and measured essentially as described previously (von Saint Paul et al., 2011), except that the extraction was performed with a mixture of 98:2 (v/v) methanol:formic acid. For the solid phase extraction, the mixtures were adjusted to 30:69.4:0.6 (v/v) methanol:water:formic acid, and the quantification was based on SA fluorescence (excitation, 305 nm; emission, 438 nm), with 3,4-dihydroxy benzoic acid added as an internal control during the extraction.

RNA Isolation and qRT-PCR

Total RNA was isolated using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The complementary DNA was generated using oligo (dT) (20-mer) and SuperScript II Reverse Transcriptase (Invitrogen), and PCR was performed using the primers outlined in Supplemental Table S2. Quantitative PCR was performed with the SensiMix SYBR Low-ROX Kit (Bioline) on a 7500 real-time PCR system (Applied Biosystems). The transcript accumulation of target genes was normalized to *TUBULIN* or *UBIQUITIN* using relative quantification with the 7500 Fast System Software 1.3.1. The presented quantitative PCR results are averages of three technical repetitions per sample ± SD.

Protein Immunoblots

Four- to 5-week-old *XVE:AED1-HA* plants (or Col-0 as a control) were sprayed until dropoff with 100 μM β-estradiol in 0.01% (v/v) Tween 20. After 24 h, 100 mg of leaf tissue was ground in 200 μL of 2× Laemmli sample buffer and boiled for 5 min. Subsequently, 15 μL per sample was analyzed by western blotting (Towbin et al., 1979) using Hybond-P polyvinylidene difluoride membranes (GE Healthcare) and monoclonal anti-HA antibodies conjugated to horseradish peroxidase (HRP; Sigma-Aldrich). HRP was visualized using the chemiluminescence kit Immun-Star WesternC (Bio-Rad) and a Typhoon Trio+ (GE Healthcare). The protein size was compared with the Precision Plus Protein Size Marker (Bio-Rad) tagged on the blot with Precision Protein Strep Tactin-HRP conjugate (Bio-Rad) according to the manufacturer's instructions.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *AvrRpm1-HA* transcript accumulation in infected Col-0 plants and in *eds1-2 pDEX:AvrRpm1-HA* plants.

Supplemental Figure S2. DEX does not induce SAR in Col-0 plants.

Supplemental Figure S3. SAR signals are emitted from the DEX-treated leaves of *pDEX:AvrRpm1-HA* plants between 4 and 6 h after DEX treatment.

Supplemental Figure S4. Summary of 2D gel analyses of apoplast-enriched extracts from *AvrRpm1-HA*-expressing plants.

Supplemental Figure S5. ICPL controls.

Supplemental Figure S6. Effects of organic solvents on SAR.

Supplemental Figure S7. Pathogen growth in infected leaves of plants overaccumulating AED1-HA.

Supplemental Figure S8. Local accumulation of free SA before and after infection of *llp1* mutants.

Supplemental Table S1. Summary of ICPL data.

Supplemental Table S2. Primers used in this study.

Supplemental Protocol S1. MALDI-MS parameters for protein identification in 2D gel spots.

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