Salicylic Acid–Independent ENHANCED DISEASE SUSCEPTIBILITY1 Signaling in Arabidopsis Immunity and Cell Death Is Regulated by the Monoxygenase FMO1 and the Nudix Hydrolase NUDT7

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Arabidopsis thaliana ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) controls defense activation and programmed cell death conditioned by intracellular Toll-related immune receptors that recognize specific pathogen effectors. EDS1 is also needed for basal resistance to invasive pathogens by restricting the progression of disease. In both responses, EDS1, assisted by its interacting partner, PHYTOALEXIN-DEFICIENT4 (PAD4), regulates accumulation of the phenolic defense molecule salicylic acid (SA) and other as yet unidentified signal intermediates. An Arabidopsis whole genome microarray experiment was designed to identify genes whose expression depends on EDS1 and PAD4, irrespective of local SA accumulation, and potential candidates of an SA-independent branch of EDS1 defense were found. We define two new immune regulators through analysis of corresponding Arabidopsis loss-of-function insertion mutants. FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1) positively regulates the EDS1 pathway, and one member (NUDT7) of a family of cytosolic Nudix hydrolases exerts negative control of EDS1 signaling. Analysis of fmo1 and nudt7 mutants alone or in combination with sid2-1, a mutation that severely depletes pathogen-induced SA production, points to SA-independent functions of FMO1 and NUDT7 in EDS1-conditioned disease resistance and cell death. We find instead that SA antagonizes initiation of cell death and stunting of growth in nudt7 mutants.

INTRODUCTION

Plants have evolved multiple layers of cellular innate immunity for protection against pathogen infection (Nürnberger et al., 2004; Lipka et al., 2005). An important defense layer (known as basal resistance) is expressed in response to invasive pathogens and serves to restrict their growth and the progression of disease (Glazebrook, 2005). Basal resistance depends on the timely activation of defense pathways and can be potentiated by receptors recognizing conserved pathogen-associated molecular patterns or specific pathogen effectors (Belkhadir et al., 2004; Nürnberger et al., 2004; Glazebrook, 2005).

Pathogen effector recognition by specialized immune receptors (known as Resistance [R] proteins) is normally associated with a localized burst of reactive oxygen species (ROS) and programmed plant cell death (the hypersensitive response [HR]) at infection sites. It also causes stimulation of basal defenses involving the phenolic molecule salicylic acid (SA) (Glazebrook et al., 2003; Eulgem et al., 2004). The processes by which R protein recognition connects to basal defense activation are unknown but are influenced by the particular receptor type. Thus, many R proteins of the nucleotide binding–leucine-rich repeat (NB-LRR) class have N-terminal coiled-coil (CC) domains requiring the membrane-attached protein NONSPECIFIC DISEASE RESISTANCE1 for resistance (Aarts et al., 1998; Coppinger et al., 2004). Plant NB-LRR proteins that instead have an N-terminal Toll Interleukin1 Receptor (TIR) domain depend on the intracellular protein EDS1 and its interacting partners, PAD4 and SENESCENCE-ASSOCIATED GENE101 (SAG101) (Aarts et al., 1998; Feyes et al., 2001, 2005). Significantly, the Arabidopsis thaliana CC-NB-LRR protein RESISTANCE TO PSEUDOMONAS SYRINGAE PV MACULICOLA1 (RPM1) conditions local programmed cell death, SA accumulation, and resistance independently of EDS1 or PAD4 but needs these regulators to transduce defense and death-promoting signals in cells surrounding pathogen infection foci (Rustérucci et al., 2001; Wiemer et al., 2005). Therefore, RPM1, and probably other structurally related CC-NB-LRR immune receptors, engages the ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1)/PHYTOALEXIN-DEFICIENT4 (PAD4) pathway even though it is not required for local resistance or cell death.

Arabidopsis EDS1 complexes are essential for basal resistance to invasive biotrophic and hemibiotrophic pathogens in the absence of R protein recognition (Zhou et al., 1998; Jirage et al., 1999; Feyes et al., 2001, 2005). In both the basal and TIR-NB-LRR conditioned responses, EDS1 and its partners control production...
of SA and other as yet undefined molecules to amplify defenses (Rustérucci et al., 2001; Eulgem et al., 2004; Song et al., 2004). Some genes that function in SA biosynthesis and signal relay have been isolated (Wildermuth et al., 2001; Nawrath et al., 2002; Dong, 2004). However, little is known about processes regulating the predicted SA-independent branch of EDS1 defense. Recent data reveal that EDS1 and PAD4 transduce ROS-derived signals in biotic and abiotic stress signaling (Rustérucci et al., 2001; Mateo et al., 2004), and this may be central to their activities beyond controlling SA (Wiermer et al., 2005).

In this study, we used Arabidopsis whole genome microarrays to examine the EDS1 regulatory node in disease resistance. In particular, we aimed to identify components of EDS1 signaling that are not within the SA pathway. We reasoned that the absence of effects of eds1 or pad4 null mutants on RPM1-triggered local resistance, cell death, and SA production (Feys et al., 2001; Rustérucci et al., 2001) would allow us to extract candidate genes that, beyond these events, are EDS1 and PAD4 dependent. Characterization of Arabidopsis insertion mutants in a discrete group of pathogen-responsive genes whose expression depends robustly on EDS1 and PAD4 resulted in identification of FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1) as a positive regulator and one member (NUDT7) of a cytosolic Nudix hydrolase family as a negative regulator of EDS1-conditioned resistance and cell death. Measurements of SA in these mutants combined with genetic analyses establish that FMO1 and NUDT7 are important for signaling through an EDS1-dependent but SA-independent branch of plant defense.

RESULTS

Experimental Design and Microarray Data Analysis

We used Affymetrix ATH1 GeneChips representing 22,734 Arabidopsis genes (Redman et al., 2004) to examine transcriptional profiles of wild-type leaves of accession Wassilewskija (Ws-0) and the Ws-0 null mutants eds1-1 and pad4-5 before treatment and at 3 and 6 h after bacterial infiltration. A previous study revealed that avrRpm1-specific cellular changes are registered in cells ~3 h after bacterial infiltration (de Torres et al., 2003). Plants were either mock-inoculated with buffer (10 mM MgCl₂) or challenged with a high titer (10⁷ colony-forming units (cfu/mL) of Pseudomonas syringae pv tomato (Pst) DC3000 strains expressing either avrRpm1 (triggering EDS1/PAD4-independent RPM1 resistance) or avrRps4 (EDS1/PAD4-dependent RESISTANT TO PSEUDOMONAS SYRINGAE4 [RPS4] resistance) (Table 1). We reasoned that selection of pathogen-responsive genes whose expression was affected by both eds1 and pad4 null mutants would increase the likelihood of identifying genes that are robustly under transcriptional control of EDS1 and its interacting and cofunctioning partner, PAD4. A further criterion exploited the dispensability of EDS1 and PAD4 in RPM1 local resistance, SA production, and programmed cell death to extract candidate genes preferentially expressed in an SA-independent branch of EDS1 signaling. Untreated wild-type and mutant material was incorporated to the experiment (Table 1) to investigate whether defects in eds1 or pad4 plants could be detected at the transcriptional level in pathogen-unchallenged leaves.

To ensure uniformity of plant responses and reduce experimental noise, we recorded the timing of plant cell death after bacterial treatment in each experiment, and this was found to be consistent with previous analyses (Table 1) (Rustérucci et al., 2001). Equal amounts of total RNA from three biological replicates were pooled before cRNA labeling reactions, and the cRNA probes hybridized to individual Affymetrix (ATH1) GeneChips, producing 21 data sets (Table 1). Details of computational methods to process gene expression data are described in Methods.

By comparing transcriptional profiles of pathogen- and mock-inoculated wild-type samples, we identified probe sets whose expression changed (induced or repressed) at least twofold at 3 or 6 h in at least two pathogen treatments. This defined a group of 4522 Arabidopsis genes as pathogen responsive (see Supplemental Table 1 online).

Definition of EDS1/PAD4-Regulated Genes

In this article, we focus on analysis of pathogen-inducible genes whose expression was dependent on both EDS1 and PAD4 in RPM1-conditioned responses (referred to as Group I genes). Searching for genes that were at least twofold upregulated in the wild type but had a twofold lower expression in eds1-1 and pad4-5 revealed a group of seven genes, including PAD4, as shown in Table 2. Since the microarray analysis was not replicated, we considered these genes as candidates for further analysis. The identities of Group II genes (whose expression was strongly dependent on EDS1 and PAD4 in RPS4-triggered but not in RPM1-triggered responses) and Group III genes (that require both EDS1 and PAD4 for maximal expression in untreated tissues) are shown in Supplemental Tables 2 and 3 online.

**Table 1. Expression Microarray Sampling**

<table>
<thead>
<tr>
<th>Plant</th>
<th>Treatment</th>
<th>Harvest (h)</th>
<th>HR (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Ws-0)</td>
<td>Nontreated</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td>3, 6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Pst avrRpm1</td>
<td>3, 6</td>
<td>24</td>
</tr>
<tr>
<td>eds1-1</td>
<td>Nontreated</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td>3, 6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Pst avrRpm1</td>
<td>3, 6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Pst avrRps4</td>
<td>3, 6</td>
<td>6</td>
</tr>
<tr>
<td>pad4-5</td>
<td>Nontreated</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td>3, 6</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Pst avrRpm1</td>
<td>3, 6</td>
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<tr>
<td></td>
<td>Pst avrRps4</td>
<td>3, 6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Pst avrRps4</td>
<td>3, 6</td>
<td>24</td>
</tr>
</tbody>
</table>

*a* Pst DC3000 expressing avrRpm1 or avrRps4 was infiltrated at 10⁷ cfu/mL into leaves.

*b* Time of appearance of confluent HR in hours, measured by trypan blue staining. –, HR not detected within 24 h.

To investigate whether defects in eds1 or pad4 plants could be detected at the transcriptional level in pathogen-unchallenged leaves.

FMO1 Contributes to EDS1 Defense

Uregulation of five of the genes in Group I and their dependence on EDS1 and PAD4 in RPM1-triggered resistance was verified by semiquantitative or quantitative RT-PCR analysis of total RNA in
two independent experiments (Figure 1). We investigated whether any of the Group I genes are necessary for plant R gene conditioned resistance by isolating corresponding T-DNA insertion mutants in Arabidopsis accession Columbia-0 (Col-0) and performing pathology assays (see Methods for a list of insertion lines). In all cases except one (NUDT6), we identified insertion lines with an interruption of the open reading frame (Figures 2A and 2B; see Supplemental Figure 1A online). Lines homozygous for each T-DNA insertion were inoculated with an isolate of the oomycete pathogen Hyaloperonospora parasitica (Cala2) that is recognized by the TIR-NB-LRR gene RPP2. A T-DNA insertion in FMO1 (denoted fmo1-1, Figure 2A) encoding a predicted flavin-dependent monoxygenase partially disabled RPP2 resistance (Figure 2C). All other mutants produced discrete HR lesions at pathogen infection sites as seen in wild-type leaves (Figure 2C; see Supplemental Figure 1B online). Loss of resistance in fmo1-1 was manifested as trailing plant cell necrosis that was more severe than in inoculated leaves of sid2-1 (a mutant with a defect in the SA-biosynthetic enzyme isochorismate synthase; Wildermuth et al., 2001) but not as severe as a null mutant (Figure 2D). SA accumulation in fmo1-1 was not significantly different from that of the wild type in RPM1 and RPS4 responses (Figure 4A). We concluded that FMO1 is not essential for local SA production in either the CC- or TIR-NB-LRR-triggered resistance.

Pathology assays with Pst DC3000 strains expressing avrRps4 or avrRpm1 established that the fmo1-1 and fmo1-2 mutations compromised RPS4 but did not affect RPM1 resistance, as shown for fmo1-1 (Figure 3A). The Ler-0 CC-NB-LRR gene RPP8 also conferred resistance to H. parasitica independently of FMO1 (see Supplemental Figure 2 online). Basal resistance in fmo1-1 (data not shown) and fmo1-2 (Figure 3B) to virulent isolates of H. parasitica was reduced. These data show that defects in FMO1 partially disable TIR-NB-LRR resistance and basal defense but can be overridden by CC-NB-LRR recognition. We concluded that FMO1 specifically affects the EDS1 pathway rather than promoting R protein–triggered resistance in general.

**Defects in Arabidopsis fmo1 Mutants Are Not Coupled to SA Accumulation**

We assessed whether the fmo1 mutant defects are associated with SA production. Levels of total SA were measured at 24 h in wild-type, pad4-1, and fmo1-1 leaves after infiltration of Pst DC3000 expressing either avrRpm1 or avrRps4. Leaves of sid2-1 plants were infiltrated alongside as an SA-deficient control. pad4-1 plants accumulated amounts of SA that were slightly reduced compared with the wild type after activation of RPM1 resistance and were strongly reduced in RPS4-triggered tissues (Figure 4C). SA accumulation in fmo1-1 was not significantly different from that of the wild type in RPM1 and RPS4 responses (Figure 4A). We concluded that FMO1 is not essential for local SA production in either the CC- or TIR-NB-LRR–triggered resistance.

To test further the hypothesis that FMO1 activity is independent of SA, the fmo1-1 mutation in Col-0 was crossed with sid2-1 and homozygous double mutant lines selected. Single and double mutant combinations were then inoculated with H. parasitica isolate Cala2 (recognized by RPP2). The fmo1-1 sid2-1 double mutant displayed loss of resistance that was significantly greater than either sid2-1 or fmo1-1 alone, measured by an increase in the frequency of trailing necrosis (Figure 4B) and pathogen spore production (Figure 4C). Therefore, the defects of fmo1-1 and sid2-1 in TIR-NB-LRR resistance are genetically additive and consistent with incremental activities of FMO1 and SID2 in defense signal relay. These data argue for a role of FMO1 in an SA-independent branch of the EDS1 pathway that, together with SA production, is required for full expression of resistance.

FMOs bind the cofactor flavin adenine nucleotide (FAD) and catalyze oxygenation of substrates containing nucleophilic nitrogen, phosphorous, sulfur, or selenium at the expense of NADPH. Extensively studied mammalian FMOs function in detoxification of xenobiotics (Lawton et al., 1994); the single yeast FMO (yFMO) acts as a redox regulator by oxidizing biological thiols (Suh et al., 1999), and an insect FMO (SNO) inactivates a plant toxin (Naumann et al., 2002). Little is known about FMO proteins in plants. One FMO clade containing Arabidopsis Yucca homologs and the Floozy gene from Petunia was shown to N-hydroxylate

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**Table 2.** Group I Candidate Genes Induced by Pst avrRpm1 in an EDS1- and PAD4-Dependent Manner

<table>
<thead>
<tr>
<th>Probe Set ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AGI Number&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Gene Description</th>
<th>Gene Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>257185_at</td>
<td>At3g13100</td>
<td>ABC transporter</td>
<td></td>
</tr>
<tr>
<td>260179_at</td>
<td>At1g70690</td>
<td>Kinase-related</td>
<td></td>
</tr>
<tr>
<td>248062_at&lt;sup&gt;c&lt;/sup&gt;</td>
<td>At5g55450</td>
<td>Lipid transfer protein</td>
<td></td>
</tr>
<tr>
<td>256012_at</td>
<td>At1g19250</td>
<td>Flavin-containing monoxygenase</td>
<td>FMO1</td>
</tr>
<tr>
<td>263852_at</td>
<td>At2g04450</td>
<td>MutT/Nudix family</td>
<td>NUDT6</td>
</tr>
<tr>
<td>249743_at</td>
<td>At5g24550/At5g24540</td>
<td>Glycosyl hydrolase</td>
<td></td>
</tr>
<tr>
<td>252060_at</td>
<td>At5g52430</td>
<td>Phytalexin-deficient 4 protein</td>
<td>PAD4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Probes sets met the following criteria. (1) A log<sub>2</sub> expression ratio of $\geq 1$ upon avrRpm1 versus MgCl<sub>2</sub> (at 3 and 6 h) in the wild type. (2) Suppression in both eds1 and pad4 versus the wild type ($\leq \log_2 -1$) at 3 and 6 h. Group I genes were also EDS1/PAD4 dependent in RPS4-triggered resistance, but expression values at 3 h were below the threshold to meet selection criteria for Group II. (3) A P value $\leq 0.05$ calculated by Wilcoxon rank sum test in MAS5.0. (4) An adjusted P value $\leq 0.05$ using false discovery rate (FDR) adjustment (see Methods).

<sup>b</sup>AGI, Arabidopsis Genome Initiative.

<sup>c</sup>248062_at displayed a consistent EDS1/PAD4 dependency and was therefore included in Group I but did not meet the criteria log<sub>2</sub> $\geq 1$ in the ratio wild-type avrRpm1/wild-type MgCl<sub>2</sub> at 6 h as the absolute expression level in wild-type MgCl<sub>2</sub> at 6 h was already high.
tryptamine in a proposed step of auxin biosynthesis (Zhao et al., 2001; Tobena-Santamaria et al., 2002). Sequencing of FMO1 cDNA derived from Col-0 revealed that the published The Arabidopsis Information Resource (TAIR) coding sequence (CDS) for At1g19250 lacks a stretch of 45 nucleotides in exon 4 (http://www.arabidopsis.org/). The coding sequence (1593 bp) was translated to a 530–amino acid sequence that is identical to protein AAF82235 from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/), and this was used in further analyses. Multiple sequence alignments of FMO1 with functionally characterized FMOs from Arabidopsis, bacteria, yeast, insect, and humans showed that FMO1 possesses an FAD binding site, an FMO-identifying motif, and an NADPH binding domain that are characteristic of functional FMO enzymes (Figure 5A). Lack of conservation of the second Gly of the NADPH binding domain in FMO1 occurs also in a catalytically functional bacterial FMO (Figure 5A; Choi et al., 2003). Previous phylogenetic analyses show that Arabidopsis FMO-like proteins form three distinct clusters (Fraaije et al., 2002; Naumann et al., 2002). FMO1 and another gene, At5g45180, are the sole representatives of one of the three clusters. Microarray data from this study and from the Affymetrix database of the Nottingham Arabidopsis Stock Centre (NASC; http://arabidopsis.info/) reveal that At5g45180 (ATH1 array element 248987_at) is not expressed and therefore likely to be a pseudogene. A BLASTP database search identified an FMO-like protein in rice (Oryza sativa) of unknown function with 56% amino acid identity (Os FMO, Figure 5A).

Other analyses have demonstrated the importance of the conserved Gly residues in the FAD and NADPH binding sites for cofactor binding and enzymatic activity (Rescigno and Perham, 1994; Kubo et al., 1997). We therefore generated by site-directed mutagenesis variants of FMO1 in which the conserved Gly residues of these motifs were exchanged to Ala residues (Figure 5A).
Wild-type FMO1 and the fmo1 mutants under the control of the cauliflower mosaic virus 35S promoter and fused to a C-terminal Strept affinity purification tag were stably transformed into fmo1-1, and several independent single insertion homozygous lines were selected for each construct. Although constitutively expressed wild-type FMO1 protein was hardly detectable in transgenic leaf extracts (Figure 5B), it complemented the fmo1-1 defect in basal resistance to virulent *H. parasitica* (Figure 5C). Moreover, constitutive expression of FMO1 caused significantly enhanced resistance in two out of three independent transgenic lines (Figure 5C). By contrast, accumulation of the predicted catalytically inactive fmo1 variant proteins was much higher than wild-type FMO1 (Figure 5B), but these failed to complement the fmo1-1 defect in basal resistance (Figure 5C). We concluded that intact FAD and NADPH binding are required for FMO1 defense function.

Mutations in NUDT7 Encoding a Cytosolic Nudix Hydrolase Deregulate Resistance

Expression of NUDT6 (At2g04450), encoding a putative cytosolic Nudix hydrolase, was also strongly EDS1 and PAD4 dependent after triggering of RPM1 resistance (Table 2). However, a loss-of-function mutant of this gene was not found. A T-DNA insertion was identified in the promoter of NUDT6, but this did not deplete its mRNA (see Supplemental Figure 1 online). We noted that a second predicted cytosolic Nudix hydrolase, NUDT7 (At4g12720), that is highly sequence related to NUDT6 (61% amino acid identity; Figure 6) was also strongly induced in RPM1 and RPS4 responses and was registered as EDS1 and PAD4 dependent in the RPS4-triggered tissues using our selection criteria (see Supplemental Table 2 online). In another study, NUDT7 was identified among seven PAD4 coregulated genes (including EDS1) in SA-independent resistance conditioned by RPP4 (a TIR-NB-LRR–type gene) (Eulgem et al., 2004). Nudix (for nucleoside diphosphates linked to moiety X) hydrolases that are characterized by a conserved motif, G_E_E_X_E_GU (where U is Ile, Leu, or Val), are found in a wide range of species from viruses to humans (McLennan, 1999). They catalyze hydrolysis of nucleoside diphosphate derivatives and have been shown to regulate levels of toxic molecules and signal intermediates in the cell (Bessman et al., 1996; Perraud et al., 2005).

Disruption of NUDT7 in two independent T-DNA insertion lines, denoted nudt7-1 and nudt7-2 (Figures 2A and 2B), caused visible growth retardation (Figures 7A and 7B) and strongly enhanced basal resistance to virulent *H. parasitica* (Figure 7C) suggestive of deregulated defense in these mutants. Consistent with a constitutive resistance phenotype, SA levels were approximately
mutants, such as *acd11* (Lorrain et al., 2003). Some of these mutants have been identified that deregulate plant cell death (Lorrain et al., 2003). These dead cells can be detected by staining with lactophenol trypan blue.

(Figure 7E) In pathogen assays, we observed an increase in the number of dead cells in *nudt7-1* compared with the wild type (Figure 7D). The marked difference in phenotypes between *nudt7-1* and *sid2-1* mutants was intermediate between that exhibited by *sid2-1* and *nudt7-1*. We confirmed that the marked difference in phenotypes between *nudt7-1* and *sid2-1* was due to differences in SA accumulation since both lines were strongly depleted in total SA (Figure 7D). These data show that growth retardation and cell death initiation in *nudt7-1* require *EDS1* but are antagonized by SA. By contrast, both SA-dependent and SA-unrelated processes contribute to enhanced resistance of *nudt7* mutants to *H. parasitica*.

**DISCUSSION**

*EDS1* regulatory complexes are needed for expression of plant basal resistance against invasive pathogens and connect specific pathogen recognition by TIR-NB-LRR–type immune receptors to activation of basal defenses (Feyes et al., 2005; Lipka et al., 2005). Here, we used Arabidopsis gene expression microarrays to identify potential new components of the Arabidopsis *EDS1* pathway. Phenotypic analysis of insertion mutants in a discrete set of *RPM1*-induced *EDS1*/*PAD4*-regulated Arabidopsis genes (Group I; Table 2) uncovered one positive regulator of *EDS1* signaling, *FMO1*. By cross-referencing Group I and Group II (*RPS4*-induced *EDS1*/*PAD4*-regulated; see Supplemental Table 2 online) genes, we discovered one member of a family of cytosolic Nudix hydrolases (*NUDT7*) that negatively regulates *EDS1*-conditioned plant defense and programmed cell death. Insertion mutants corresponding to the remaining Group I genes did not display altered defense in *RPP2* or basal resistance (see Supplemental Figure 1 online). It is possible that functional redundancy might have hindered identification of an altered defense phenotype. For example, the lipid transfer protein-like gene *At5g55450* has three sequence-related genes. Two of these (*At5g55410* and *At5g55460*) are pathogen inducible as measured by RT-PCR (M. Bartsch and J.E. Parker, unpublished data). The third gene, *DESTRUCTIVE IN INDUCED RESISTANCE1* (*At5g48485*), is not pathogen responsive but was shown to contribute to establishment of plant systemic resistance (Maldonado et al., 2002).

*RPM1* is required for full expression of TIR-NB-LRR–conditioned resistance to avirulent pathogens and for basal resistance to invasive virulent pathogens (Figures 2 and 3). As in plants with defects in *EDS1* or its interacting partners *PAD4* and *SAG101* (Feyes et al., 2005), the deficiency of an *fmo1-1* null mutant is overridden by *RPM1* and *RPP8* recognition. All of these features are consistent with a role of *FMO1* in the *EDS1* pathway.
Uncoupling fmo1-1 defects from SA accumulation (Figure 4) further supports a function of FMO1 in an EDS1-regulated but SA-independent mechanism that promotes resistance and cell death at pathogen infection sites.

EDS1 and PAD4 drive defense signal amplification involving positive feedback on expression of multiple genes, including themselves (Wiermer et al., 2005). We assessed whether fmo1-1 affects PAD4 or EDS1 mRNA accumulation. We found that EDS1 and PAD4 transcripts were marginally reduced in fmo1-1 compared with the wild type in healthy tissues and were not significantly affected in pathogen-treated tissues (M. Bartsch and J.E. Parker, unpublished data), inconsistent with a primary role of FMO1 in stimulating expression of these regulators. Instead, we favor the idea that FMO1 acts posttranslationally on one or more EDS1 pathway components. Like EDS1 and PAD4 (Feys et al., 2001), preexisting FMO1 protein inside the cell may exert a major activity once the plant defense pathway is triggered and together with other positive and negative components be subject to rapid upregulation, as demonstrated here. Pathology tests of fmo1-1 stable transgenic lines constitutively expressing wild-type FMO1 showed that they were complemented for pathogen resistance, whereas transgenics expressing fmo1 amino acid exchange variants in the conserved Gly residues of the FAD and NADPH binding motifs were not (Figure 5C). Thus, flavin-dependent monooxygenase activity is required for FMO1 defense function.

The marked difference in accumulation of wild-type FMO1 and the catalytically inactive fmo1 variants coupled with enhanced basal resistance only of fmo1-1 plants expressing wild-type FMO1 suggests that active FMO1 strongly influences the degree of resistance. FMO1 levels may therefore be strictly regulated inside the cell. Uncovering the precise biochemical activity of FMO1 should provide insight into processes involved in EDS1 signaling. One possibility is that FMO1 regulates oxidative metabolism, as was found for the single yeast FMO protein (Suh et al., 1999). Brodersen et al. (2002) reported that FMO1 (referred to as FMO) mRNA accumulates to high levels in the acd11 mutant that triggers EDS1-dependent cell death. Recent studies reveal that FMO1 was also transcriptionally upregulated in response to superoxide generation but not by hydrogen peroxide or ozone (Olszak et al., 2006), suggesting a close link between provision of certain ROS and FMO1 expression. In the same study, FMO1 mRNA was elevated in the lsd1 mutant but not in the constitutive defense mutants ctr1, cec1, cpr6-1, or mpk4, indicating that FMO1 is not activated simply by deregulating SA, jasmonic acid, or ethylene signaling (Olszak et al., 2006). An intrinsic activity of EDS1 and PAD4 is to process ROS-derived signals in biotic and abiotic stress responses (Rustèrucci et al., 2001; Mateo et al., 2004). FMO1 may alter the redox state of a signal intermediate or components of the EDS1 system to promote signal relay. Alternatively, FMO1 could N- or S-oxygenate a molecule that contributes additively with SA to resistance.

Our genetic analyses show that growth inhibition, enhanced basal resistance, and lesioning of nudt7 plants also depend on functional EDS1 (Figure 7). Strikingly, the sporadic death of individual cells in the epidermis and palisade and spongy mesophyll layers of nudt7 leaves (Figure 7E) did not progress to confluent lesioning, suggesting that NUDT7 normally restricts the initiation rather than propagation of cell death. Also, lesion formation in nudt7 did not depend on isochorismate synthase encoded by SID2 (Figures 7E and 7F) that provides the major route for pathogen-induced SA accumulation (Wildermuth et al., 2001). Instead, SA depletion exacerbated nudt7-1 conditioned cell death (Figures 7E and 7F) and growth retardation (Figures 7A and 7B). This contrasts markedly with deregulated cell death in acd11 or lsd1 that require functional SID2 (Brodersen et al., 2005; Torres et al., 2005). In lsd1 leaves, SA appears to act as a
Figure 5. FMO1 Catalytic Domains Are Required for Defense Signaling.

(A) Alignment of amino acid sequences from *Arabidopsis* (At FMO1 and YUCCA; Zhao et al., 2001), rice (Os FMO), *Methylophaga* sp Strain SK1 (bFMO; Choi et al., 2003), yeast (yFMO; Zhang and Robertus, 2002), and human (hFMO1; Lawton et al., 1994) was performed, and the N-terminal sequences are shown here. FMO-defining motifs and the conserved Gly residues exchanged by site-directed mutagenesis are indicated above the top line: I, FAD binding motif GXGXXG; II, FMO identifying sequence motif FXGXXXHXXX(Y/F); and III, NADPH binding domain GXGX(G/A). Multiple alignments were visualized using GeneDoc (Nicholas et al., 1997) with conserved residue shading mode set to level 4 using default settings and enabled similarity groups function. Amino acids with 100, 80, and 60% conservation are presented as white letters on black background, white letters on dark-gray background, and black letters on light-gray background, respectively.

(B) Expression of wild-type and mutant forms of FMO1-StrepII in independent *fmo1-1* transformants. Protein gel blot analysis was performed after StrepII affinity purification. Equal amounts of the input fraction are shown by Coomassie blue staining.

(C) Intact FAD and NADPH binding sites in FMO1 are required for basal resistance to virulent strain *H. parasitica*. The plant lines correspond to those tested in (B). Numbers of pathogen conidiospores were measured on leaves 6 d after inoculation. Values are the average of four replicate samples (±SD).
promotive signal in propagation of cell death that is inhibited by NADPH-oxidase generated ROS (Torres et al., 2005). These and our data imply differences in signal relay between cell death initiation and maintenance programs. Although sid2-2 mutants suppressed acd11-triggered cell death and SA accumulation, application of SA to acd11 sid2-2 leaves failed to restore lesioning indicative of isochorismate synthase-derived compounds other than SA as mediators of programmed cell death in acd11 (Brodersen et al., 2005). While aspects of acd11 and nudt7 phenotypes are clearly distinct, both deregulated resistance pathways generate EDS1-dependent signals that are not SA itself. Recent studies showed that NUDT6 and NUDT7 hydrolyze preferentially ADP-ribose and NADH in vitro (Ogawa et al., 2005; Olejnik and Kraszewska, 2005). In mammalian cells, ADP-ribose has been implicated as an intracellular second messenger in oxidative stress-induced ion channel activation and apoptosis (Perraud et al., 2001, 2005; Kolisek et al., 2005). Levels of free ADP-ribose increase under oxidative stress conditions due to NAD⁺ decay catalyzed by the enzymes PARP/PARG (poly-ADP-ribose polymerase/poly-ADP-ribose glycohydrolase) in the nucleus or by mitochondrial damage and subsequent ADP-ribose leakage (Richter and Schlegel, 1993; Kolisek et al., 2005). A role for free ADP-ribose in plant cells is unknown but as a potentially toxic intermediate that can modify and inactivate proteins by mono-ADP-ribosylation (McDonald et al., 1992; Bektas et al., 2005), and its levels are likely to be strictly controlled. Downregulation of PARP enzymes that could potentially produce ADP-ribose rendered plants more tolerant to a broad range of stresses (De Block et al., 2005). A requirement for EDS1 and PAD4 in pathogen-stimulated accumulation of NUDT6 and NUDT7 enzymes that would eliminate ADP-ribose suggests that ADP-ribose might be generated through EDS1/PAD4 activity as a proapoptotic and resistance promoting signal. Therefore, these Nudix hydrolases could serve to restrict the damaging effects of the plant defense response. Supporting this idea, several pathogen-derived Nudix enzymes were shown to contribute to infectivity of mammalian cells and may protect the pathogen against damaging oxidative stresses.

**Figure 6. Phylogeny of Arabidopsis Nudix Hydrolase Family Members.**

A phylogenetic tree drawn from neighbor-joining analysis using Mega 3.0 software (Kumar et al., 2004). Bootstrap values (1000 replicates) are shown. Annotations of Nudix hydrolase–like proteins were taken from Ogawa et al. (2005).
Figure 7. Developmental and Basal Resistance Phenotypes of nudt7 Single and Double Mutants.

(A) Attenuated growth of nudt7-1 is suppressed by eds1-2 but exacerbated by sid2-1. Four-week-old soil-grown plants representative of single or double mutants are shown. Bar = 1 cm.

(B) Average fresh weight (FW) of 4-week-old plants (± SD) calculated from the aerial tissue weight of six plants per genotype.

(C) Enhanced basal resistance to H. parasitica isolate Noco2 in nudt7 mutants is dependent on EDS1 and partially requires SID2. Pathogen spores were counted as in Figure 3B.

(D) Levels of total SA in leaves of healthy 4-week-old plants. Data points are the average of three replicate samples (± SD).

(E) Visualization of dead cells in leaves of 4-week-old nudt7 single and double mutants after staining with lactophenol trypan blue. The scale bar unit is in micrometers.

(F) Quantification of leaf cell death. Numbers of dead cells were determined in leaves of 3-week-old plants after staining with lactophenol trypan blue. Data represent samplings from 10 leaves from at least five plants per genotype (± SD). Single cell death did not occur in Col eds1-2 or sid2-1 (data not shown).
RT-PCR was performed using gene-specific primers: T-DNA Express homepage. Primer sequences are available on request.

METHODS

Plant Material and Mutant Characterization

Arabidopsis thaliana wild-type Ws-0, Ler-0, Col-0, eds1-1 (Falk et al., 1999), pad4-5, pad4-1 (Jirage et al., 1999), and sid2-1 (Wildermuth et al., 2001) mutants have been described. The null eds1-2 mutation in the Col-0 background (referred to as Col eds1-2) was derived from a cross between Col-0 and eds1-2 (Ler-0) followed by eight backcrosses to Col-0. Insertion mutants of EDS1/PAD4-dependent genes were identified using the SIGnAL T-DNA Express Arabidopsis gene mapping tool (http://signal.salk.edu/). SALK lines SALK_026163 (Falk et al., 2001), SALK_120950 (Ler-0) followed by eight backcrosses to Col-0. Inser-
tion mutants of eds1-1, eds1-2, sid2-1, and eds1-2 dependent genes were identified using the T-DNA Express homepage. Primer sequences are available on request.

To evaluate if the isolated mutants were mRNA nulls, semiquantitative RT-PCR was performed using gene-specific primers: FM01-forward (5’-GGAAGCGGAATAGGATGAC-3’), FM01-reverse (5’-CTACCTGGTGATTCCTGCTG-3’), NU07-forward (5’-TTTAGGTTTACTTCTCCTGTCG-3’), NU07-reverse (5’-GGGATGCATTTAAGGCGCTTG-3’), ACTIN-forward (5’-TTCTGACAATGAGTGGAGTGC-3’), and ACTIN-reverse (5’-CGCTCCTGAGTTCCTGCTCG-3’). Double mutants were generated by crossing individual mutants and identifying homozygous double mutant combinations by PCR or sequencing of PCR-amplified DNA fragments. T-DNA insertion lines of genes in Group I that exhibited no defects in RPP2 resistance are SALK_120950 (At3g13100), SAIL_46_E06 (At1g070690), SALK_105557 (At5g55540), and SALK_038957 (At5g24540).

Plant Growth and Pathology Assays

Plants were grown in controlled environment chambers under a regime of a 10-h light period at 150 to 200 μE m⁻² s⁻¹, 23°C, and 65% relative humidity. Four-week-old plants were used for microarray experiments. 2-week-old plants for infection assays with Hyaloperonospora parasitica, and 4- to 5-week-old plants for infections with Pseudomonas syringae strains. H. parasitica was spray inoculated at 4 × 10⁶ cfu ml⁻¹ except otherwise stated. Detailed procedures for pathogen inoculations and determination of pathogen growth have been described (Feyts et al., 2005). P. syringae inoculations of leaves were done with a needleless syringe for microarray sampling or by vacuum infiltration for bacterial growth assays. Plant cell death and H. parasitica infection structures were visualized under a light microscope after staining of leaves with lactophenol trypan blue (Aarts et al., 1998).

Microarray Sampling and Data Collection

Leaves were harvested at the indicated time points (Table 1) and total RNA extracted using RNAwiz reagent (Ambion). cRNA was prepared following the manufacturer’s instructions (www.affymetrix.com/support/technical/manual/expression_manual.affx). Labeled cRNA transcripts were purified using the Sample Cleanup Module (Affymetrix). Fragmentation of cRNA transcripts, hybridization, and scanning of the high-density oligonucleotide microarrays (Arabidopsis ATH1 genome array; Affymetrix) were performed according to the manufacturer’s GeneChip Expression Analysis Technical Manual.

Microarray Data Analysis

For data collection and assessment, we used Affymetrix Microarray Analysis Suite Version 5.0 (MAS5.0) and R language (bioconductor project). Following standard protocols for data analysis using MAS5.0, the fluorescence intensity of each array was scaled to an overall intensity of 100 to enable comparison of all arrays. Signal log ratios and corresponding P values were calculated using standard MAS5.0 algorithms (statistical algorithms description document, technical report; Affymetrix, 2002). As a second approach for background adjustment, normalization, and collation, we used the RMA (robust multichip average) model in R language (Irizarry et al., 2003). Fold changes were calculated based on signal values derived by the RMA method. To assign a level of confidence for expression differences between the different experimental conditions, P values were calculated for each probe set individually by conducting a paired Wilcoxon rank sum test based on 11 individual values (mismatch signal subtracted from the perfect match signal) of a respective probe set. FDR calculations were performed according to the Benjamini and Hochberg definition of FDR (Benjamini and Hochberg, 1995) in R language (multitest package). Hierarchical cluster analysis was performed in R language (hclust package). Annotations of Arabidopsis genes based on the probe set identifiers were obtained from TAIR (www.arabidopsis.org).

RT-PCR Analysis

Total RNA was extracted as described for the preparation of the microarray samples. RT reactions were performed with 1 μg of total RNA and 0.5 μg of oligo(dT)₁₆ primer at 42°C using reverse transcriptase and RNAase inhibitor RNasin (both from Promega) in a 20-μL reaction volume. Aliquots of 1 μL RT-reaction product were subsequently used for quantitative and semiquantitative RT-PCR analysis using the following gene-specific primers: NU07-forward (5’-CCTACCTGGACTGGTACTG-3’), NU07-reverse (5’-TAAATGGGACGGTGAGTCT-3’), At3g13100-forward (5’-GGGACATTTGGAATGAGTGC-3’), At3g13100-reverse (5’-CGTCCTGCAATGCTGCTG-3’), At5g55540-forward (5’-CGTCCTGCAATGCTGCTG-3’), and At5g55540-reverse (5’-CCTGGAGAGGCCGACATTCA-3’). For data collection and assessment, we used Affymetrix Microarray Analysis Suite Version 5.0 (MAS5.0) and R language (bioconductor project). Following standard protocols for data analysis using MAS5.0, the fluorescence intensity of each array was scaled to an overall intensity of 100 to enable comparison of all arrays. Signal log ratios and corresponding P values were calculated using standard MAS5.0 algorithms (statistical algorithms description document, technical report; Affymetrix, 2002). As a second approach for background adjustment, normalization, and collation, we used the RMA (robust multichip average) model in R language (Irizarry et al., 2003). Fold changes were calculated based on signal values derived by the RMA method. To assign a level of confidence for expression differences between the different experimental conditions, P values were calculated for each probe set individually by conducting a paired Wilcoxon rank sum test based on 11 individual values (mismatch signal subtracted from the perfect match signal) of a respective probe set. FDR calculations were performed according to the Benjamini and Hochberg definition of FDR (Benjamini and Hochberg, 1995) in R language (multitest package). Hierarchical cluster analysis was performed in R language (hclust package). Annotations of Arabidopsis genes based on the probe set identifiers were obtained from TAIR (www.arabidopsis.org).

SA Quantification

Leaf material (100 to 200 mg fresh weight) was extracted with aqueous methanol (Bednarek et al., 2005). Leaf extracts were hydrolyzed with β-glucosidase (EC 3.2.1.21; Sigma-Aldrich), and released SA was reextracted as described (Lee and Raskin, 1998). HPLC analyses were performed on an Agilent 1100 HPLC system.
Wild-Type or Mutant FMO1

The FMO1 coding region (without the stop codon) was amplified from Arabidopsis Col-0 cDNA using gene-specific primers (sequences available on request). Wild-type and mutant forms of FMO1 in pENTR/D-TOPO were recombined into the expression vector pXG50-Strep to generate an in-frame C-terminal fusion to the eight–amino acid StrepII affinity purification tag and transformed into fmo1-1 plants as described previously (Witte et al., 2004). Independent, homozygous, single insertion lines were identified and analyzed for FMO1-StrepII expression by StrepII previously (Witte et al., 2004). Independent, homozygous, single insertion fmo1-1 affinity purification tag and transformed into generate an in-frame C-terminal fusion to the eight–amino acid StrepII able on request). Wild-type and mutant forms of FMO1

Sequence Alignments and Phylogenetic Analysis

Alignment of amino acid sequences was performed using ClustalX (version 1.8) (Thompson et al., 1997) and edited for display with GeneDoc (version 2.6.002) (Nicholas et al., 1997). Phylogenetic analysis of Arabidopsis Nudix hydrolase amino acid sequences was performed using MEGA3 (Kumar et al., 2004). A neighbor-joining tree was constructed using a p-distance amino acid distance model and complete deletion. Bootstrap values from 1000 replications are shown at the tree nodes. The alignment of the Nudix hydrolase sequences is shown in Supplemental Figure 3 online.

Accession Numbers

The accession numbers for the genes discussed in this article are as follows: EDS1 (At1g348090), PAD4 (At3g52430), SID2 (At1g74710), FMO1 (At1g19250, corrected open reading frame has been reported to TAIR and will be updated for the next annotation release), NUDT6 (At2g04450), NUDT7 (At1g12720), ACTIN (At3g18780), and UBQ10 (At4g03240). Arabidopsis Genome Initiative numbers for candidate genes from Groups I, II, and III are given in Table 2 and in Supplemental Tables 2 and 3 online, respectively. Proteins in the alignment shown in Figure 5A are as follows (GenBank accession or Arabidopsis Genome Initiative numbers are indicated in parentheses): FMO1 (AAF82253), SID2 (AAC04789), and FMO1 (NP_001002108). Microarray data from this article have been deposited with the ArrayExpress data library (http://www.ebi.ac.uk/arrayexpress/) under accession number E-MEXP-546.

Supplemental Data

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

Supplemental Table 1. Expression Data of 4333 Pathogen-Responsive Probe Sets.

Supplemental Table 2. DC3000/avrRps4–Induced Genes in an EDS1- and PAD4-Dependent Manner (30 Probe Sets/33 Genes; Group II).

Supplemental Table 3. Genes Suppressed in eds1 and pad4 in Untreated Tissue (20 Probe Sets/21 Genes; Group III).

Supplemental Figure 1. Characterization of Insertion Lines Corresponding to Candidate Genes with EDS1/PAD4-Dependent Expression.

Supplemental Figure 2. Plant Resistance Phenotypes in Response to Inoculation with Avirulent H. parasitica Isolate Emrco5 (Recognized by RPP8) on Ler-0 Wild Type, eds1-2, pad4-2, and fmo1-2.

Supplemental Figure 3. Full Alignment of Arabidopsis Nudix Hydrolases.

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NOTE ADDED IN PROOF
Identification of NUDT7 (referred to as GFG1) was also reported by Jambunathan and Mahalingam (2005).

Salicylic Acid–Independent ENHANCED DISEASE SUSCEPTIBILITY1 Signaling in Arabidopsis Immunity and Cell Death Is Regulated by the Monooxygenase FMO1 and the Nudix Hydrolase NUDT7
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