The Genetic Network Controlling the *Arabidopsis* Transcriptional Response to *Pseudomonas syringae* pv. *maculicola*: Roles of Major Regulators and the Phytotoxin Coronatine

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Expression profiling of wild-type plants and mutants with defects in key components of the defense signaling network was used to model the *Arabidopsis* network 24 h after infection by *Pseudomonas syringae* pv. *maculicola* ES4326. Results using the Affymetrix ATH1 array revealed that expression levels of most pathogen-responsive genes were affected by mutations in *coi1*, *ein2*, *npr1*, *pad4*, or *sid2*. These five mutations defined a small number of different expression patterns displayed by the majority of pathogen-responsive genes. *P. syringae* pv. *tomato* strain DC3000 elicited a much weaker salicylic acid (SA) response than ES4326. Additional mutants were profiled using a custom array. Profiles of *pbs3* and *ndr1* revealed major effects of these mutations and allowed *PBS3* and *NDR1* to be placed between the *EDS1/PAD4* node and the SA synthesis node in the defense network. Comparison of *coi1*, *dde2*, and *jar1* profiles showed that many genes were affected by *coi1* but very few were affected by *dde2* or *jar1*. Profiles of *coi1* plants infected with ES4326 were very similar to those of wild-type plants infected with bacteria unable to produce the phytotoxin coronatine, indicating that, essentially, all COI1-dependent gene expression changes in this system are caused by coronatine.

The current consensus model of plant–biotroph interactions may be most easily understood in an evolutionary framework. Originally, plant defense responses were activated as a consequence of recognition of molecules characteristic of microbes (pathogen-associated molecular patterns [PAMPs]) by pattern recognition receptors (PRR), resulting in PAMP-triggered immunity (PTI). Subsequently, pathogens acquired effectors,

which are proteins or small molecules transported into host cells that promote virulence. Many of these effectors function to suppress PTI. Ultimately, plants acquired resistance (*R*) genes that recognize effectors, or host proteins modified by effectors, and trigger an alternative signaling cascade that activates plant defense responses. This response is known as effector-triggered immunity (ETI). The outcome of an interaction between a well-adapted pathogen and its host depends on the balance between the ability of pathogen effectors to turn off host defenses and the ability of the host to recognize the pathogen and activate defense responses (Jones and Dangl 2006).

The regulatory network that translates signals from PRR and *R* genes into activation of plant defense responses can be thought of as several interconnected sectors. The PTI sector includes a mitogen-activated protein kinase cascade, WRKY-class transcription factors, and AtRbohD, an NADPH oxidase required for a membrane-associated oxidative burst and callose deposition (Asai et al. 2002; Chinchilla et al. 2007; Gomez-Gomez and Boller 2000; Heese et al. 2007; Zhang et al. 2007; Zipfel et al. 2004). The salicylic acid (SA) sector is activated by ETI and by virulent pathogens that do not appear to trigger *R* gene recognition. SA activation by virulent pathogens may be due to weak recognition by *R* genes. PAMP recognition also activates SA signaling, and the SA sector is important for PTI (Tsuda et al. 2008). The jasmonic acid (JA) and ethylene (ET) sectors also have major effects on signaling during defense responses. JA and ET signaling tend to be important for resistance to necrotrophic pathogens and contribute little to resistance to biotrophs, although this is clearly an oversimplification (Glazebrook 2005). There is mutual negative cross-talk between JA and SA signaling but there is also evidence that JA, ET, and SA signaling may sometimes act synergistically (Mur et al. 2006; Schenk et al. 2000). Other plant hormones once thought of as mainly affecting development also impact defense signaling pathways (Robert-Seilanianantz et al. 2007). Absciscic acid (ABA) inhibits defense against *Pseudomonas syringae*, auxin inhibits SA signaling, and gibberellic acid (GA) may inhibit JA/ET signaling (de Torres-Zabala et al. 2007; Navarro et al. 2006; Robert-Seilanianantz et al. 2007).

Several major components of the SA sector are known. EDS1 and PAD4 are lipase-like proteins, but lipase activity does not seem to play a role in their functions. They interact physically and contribute to activation of SA synthesis in

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response to many, but not all, SA-inducing pathogens (Aarts et al. 1998; Falk et al. 1999; Feys et al. 2001, 2005; Jirage et al. 1999; Wiermer et al. 2005; Zhou et al. 1998). PAD4 is also required for pathogen-induced expression of many SA-independent genes (Glazebrook et al. 2003). SID2 and EDS5 are required for SA synthesis. SID2, also known as EDS16, is isochorismate synthase, an enzyme in the SA biosynthetic pathway, and EDS5 is a MATE transporter whose role in SA synthesis is not understood (Nawrath and Metraux 1999; Nawrath et al. 2002; Volko et al. 1998; Wildermuth et al. 2001). PBS3 is a member of the GH3-like family, which includes JAR1, a protein known to produce JA-Ile, the active form of JA. There are conflicting reports regarding SA levels in *pbs3* mutants after infection by various *P. syringae* strains, but it seems clear that SA levels are generally lower than in wild-type plants and that the enhanced susceptibility to *P. syringae* and reduced expression of the SA-responsive gene *PR-1* can be rescued by exogenous SA (Jagadeeswaran et al. 2007; Lee et al. 2007; Nobuta et al. 2007). NPR1 transmits the SA signal to the nucleus. At low SA levels, it exists as a cytoplasmic oligomer (Kinkema et al. 2000; Mou et al. 2003). In response to the altered redox state caused by rising SA levels, it becomes a monomer, translocates to the nucleus, and mediates changes in gene expression through interaction with TGA-family transcription factors (Cao et al. 1997; Despres et al. 2000; Dong 2004; Mou et al. 2003; Zhang et al. 1999). WRKY-family transcription factors are also involved in NPR1-dependent changes in gene expression (Li et al. 2004, 2006; Wang et al. 2006).

Within the JA sector, most of the components involved in JA synthesis are known (Wasternack 2007). The active form of JA is JA-Ile, produced by the GH3-like enzyme JAR1 (Staswick and Tiryaki 2004; Thines et al. 2007). JA-Ile binds to the E3 ubiquitin ligase F-box protein COI1, thereby promoting ubiquitination and degradation of a small family of JAZ proteins (Katsir et al. 2008). JIN1 is an MYC2 transcription factor that is induced by JA and which controls expression of some JA-responsive genes (Lorenzo et al. 2004). *P. syringae* produces coronatine, a toxin that mimics JA-Ile by binding to COI1 (Thines et al. 2007). This is thought to promote virulence of *P. syringae* by interfering with SA signaling through the negative effect of JA on the SA sector, and by easing bacterial entry through inhibition of stomatal closure (Brooks et al. 2005; Melotto et al. 2006).

Most of what is known about the topology of the defense signaling network is based on comparing the effects of various defense signaling mutants on a few particular phenotypes. Considering the large number of genes induced or repressed in response to pathogen attack and the apparent complexity of the signaling network, a comparison of mutant phenotypes on a larger scale is desirable. One method for obtaining system-wide information about mutant phenotypes is expression profiling. Expression profiling has been used in studies of responses to pathogens; for example, to describe the response to PAMPs (Navarro et al. 2004), to discern the defense-suppressing activities of effectors (Truman et al. 2006), and to characterize particular defense-signaling mutants, such as *mpk4* (Petersen et al. 2000) and *pmr4* (Nishimura et al. 2003). Previously, we used expression profiling to model the defense network by comparing similarities among the profiles of a suite of defense signaling mutants (Glazebrook et al. 2003). This work was limited by the use of an early microarray representing only 8,000 *Arabidopsis* genes and by the lack of statistical power due to the absence of replicates. The data analysis considered only genes that were induced by infection.

Here, we report studies of the *Arabidopsis* response to infection by *P. syringae* pv. *maculicola* ES4326 at 24 h after infection. We used expression profiling of wild-type plants and mu-

tants with defects in key defense-signaling components (*coi1*, *ein2*, *pad4*, *npr1*, and *sid2*) to define the major patterns of regulation governing the response to this pathogen, thereby creating a model of the defense network at a single timepoint. The model describes the relationships among the regulators and defines groups of genes that are subject to similar regulation. We found that most induced genes fit one of a small number of regulatory patterns defined by the effects of the five mutations. The repressed genes fit similar patterns, demonstrating that these five genes comprise a regulatory network that controls activation of induced genes, as well as suppression of repressed genes. Comparison of our data obtained with strain ES4326 to public data obtained with other strains suggested that *P. syringae* pv. *tomato* DC3000 induces a much weaker SA response than ES4326. We found that this is true, because SA levels in plants infected with DC3000 are much lower than in plants infected with ES4326. We refined the network model by profiling additional mutants, revealing major effects of PBS3 and NDR1 on signaling, and allowing us to place these mutants between the node defined by PAD4 and EDS1 and the SA-synthesis node. Finally, we found that essentially all of the COI1-dependent gene expression changes in this system are due to the phytotoxin coronatine.

RESULTS

A major portion of the *Arabidopsis* genome responds to infection by strain ES4326.

To obtain a global view of gene expression changes triggered by *P. syringae* pv. *maculicola* ES4326 infection, we compared expression profiles of wild-type Col-0 plants infected with ES4326 to profiles of mock-infected plants. The experiment consisted of three independent biological replicates, each including samples collected 9, 24, and 32 h after infection. We obtained expression profiles from each of the resulting 18 samples using the Affymetrix ATH1 microarray (Redman et al. 2004). To determine which genes showed significantly different expression levels in corresponding mock- and ES4326-infected samples, we used a mixed-effect linear model with a *q* value cut-off of 0.01, as described below. The *q* value cut-off of 0.01 corresponds to a false discovery rate (FDR) of 1%, excluding most genes that are not differentially expressed. We identified 381, 4,076, and 3,340 ES4326-induced genes and 210, 4,541, and 3,983 ES4326-repressed genes at 9, 24, and 32 h after infection, respectively.

The sets of induced or repressed genes from different timepoints overlapped extensively (Fig. 1). These similarities were reflected in the correlations for pairwise comparisons of the log₂ fold-change values of the differentially expressed genes. For the set of 591 genes differentially expressed at 9 h postinoculation (hpi), the correlations between the 9 hpi values and the 24 and 32 hpi values were 0.81 and 0.88, respectively. For the 9,993 genes differentially expressed at 24 or 32 hpi, the correlation between the 24 and 32 hpi values was 0.89. The data for all the genes on the array are shown in Supplementary Table S1.

Most *P. syringae* pv. *maculicola* ES4326-responsive genes show altered expression in defense signaling mutants.

Sectors of the defense signaling network defined by the hormones SA, JA, and ET have major roles in regulating pathogen-responsive gene expression. To assess the contributions of these sectors on the gene expression changes induced by ES4326 infection, we compared expression profiles of wild-type Col-0 to those of various defense signaling mutants: *pad4* (blocks SA and other signals), *sid2* (blocks SA synthesis), *npr1* (blocks transmission of the SA signal), *ein2* (blocks ET signal-

ing), and *coi1* (blocks JA signaling). The experiment above revealed the largest number of expression changes at 24 h after infection; therefore, we chose this timepoint for mutant analysis. The experiment consisted of three independent biological replicates, each including wild-type plants that were mock in-

oculated or ES4326 infected and mutants that were ES4326 infected, all sampled after 24 h. We obtained expression profiles from each of these 21 samples using the ATH1 microarray.

We decided to focus on a set of genes that show robust expression changes in response to ES4326 infection. To define these, we analyzed the profiles from all six samples of mock-inoculated and ES4326-infected wild-type plants 24 hpi (three from the time course experiment and three from the signaling mutants experiment), using a mixed-effect linear model. Results for all the genes on the array are shown in Supplementary Table S2. We first selected genes that were differentially expressed with q values <0.01 . Then, to eliminate genes with small expression changes, we further selected genes that showed a greater than fourfold change in expression, resulting in a final set of 1,140 ES4326-induced and 1,397 ES4326-repressed genes, for a total of 2,537.

To determine which of the 2,537 genes induced or repressed by ES4326 showed altered expression levels in plant defense signaling mutants, we compared profiles from infected wild-type plants with those of infected mutants. Expression levels in infected wild-type plants were compared with those in infected mutant plants using a mixed-effect linear model. Supplementary Table S3 contains the data for all the genes on the array. For each mutant, we determined which of the 1,140 or 1,397 genes induced or repressed by ES4326 infection in wild-type plants showed significantly different expression levels in mutant plants, using a q value cut-off of $q < 0.01$. We did not include a fold-change criterion, because we wanted to include genes that were only modestly affected by one or more mutations. Although all mutations studied altered expression of some ES4326-responsive genes, the mutation with the strongest effect on both induced and repressed genes was *pad4*, which reduced expression of 710 of 1,140 induced genes and increased expression of 897 of 1,397 repressed genes (Table 1).

For the vast majority of genes differentially expressed between infected mutants and infected wild type, defense signaling mutations reduced ES4326-triggered expression changes. For ES4326-induced genes, nearly all effects of mutations were reductions of gene expression, indicating that the signaling factors act to induce expression of these genes. For ES4326-repressed genes, nearly all effects of mutations were increases of gene expression, indicating that the signaling factors act to repress expression of these genes.

The gene circuits controlling gene induction and gene repression are similar.

To define patterns of defense gene regulation and reveal relationships among network components, we sorted the induced genes into groups according to the defense signaling mutations that significantly ($q < 0.01$) affected their expression (Supplementary Table S4). Of the 1,140 induced genes, 189 were not affected by any mutation, indicating that they are controlled by network sectors other than those involving SA, JA, or ET or are controlled by these sectors in a redundant manner. Of the remaining 951 genes, 848 fit one of nine major

Fig. 1. Venn diagrams showing the number of genes whose expression levels are affected by *Pseudomonas syringae* pv. *maculicola* ES4326 infection. The total number of genes induced or repressed at each timepoint is shown outside the circles; hpi = hours postinoculation. **A**, Induced genes. **B**, Repressed genes.

Table 1. Genes differentially expressed in mutant plants^a

Expression (total no. of genes)	Level ^b	Genes				
		<i>pad4</i>	<i>sid2</i>	<i>npr1</i>	<i>ein2</i>	<i>coi1</i>
Induced by infection (1,140)	L	710	153	220	177	349
	H	14	28	24	2	4
Repressed by infection (1,397)	L	0	3	1	0	7
	H	897	111	317	167	239

^a For each mutant, the number of genes expressed at significantly different levels in infected mutant plants than in infected wild-type plants is shown. Data were obtained from the ATH1 microarray.

^b L and H indicate genes expressed at lower and higher levels, respectively, in mutant than in wild-type plants ($q < 0.01$).

patterns in which one or more mutations reduced expression (Fig. 2A). Three sets of genes were affected by only one mutation: *pad4*, *coi1*, or *ein2*. In the remaining six patterns, multiple mutations affected gene expression. Three sets of genes were affected by pairwise combinations of *pad4*, *coi1* and *ein2*; one set of genes was affected by all three mutations; one set of genes was affected by *npr1* and *pad4*; and the final set was affected by *sid2*, *npr1*, and *pad4*. Thus, genes affected by the SA-related mutations *npr1* and *sid2* were distinct from genes affected by the JA- and ET-related mutations *coi1* and *ein2*. The mutation in *pad4* affected all the *NPR1*- or *SID2*-dependent genes and a subset of the *COI1*- or *EIN2*-dependent genes. The 103 genes that did not fit one of the major patterns represented 31 different minor patterns. The largest of these minor patterns were a set of 11 genes that were affected only by *pad4* and *sid2* and a set of 11 genes that were affected only by *npr1* and *sid2*. Fewer than 10 genes fit each of the other minor patterns. Some minor patterns included genes that were expressed at higher levels in mutant plants than in wild-type plants. We conclude that the five signaling mutations studied affect expression of most of the genes induced by ES4326 infection and that the regulation of most genes can be described by one of nine major patterns.

To determine the extent of the similarity between the gene circuits controlling gene induction and gene repression, we carried out a similar analysis using the ES4326-repressed genes (Supplementary Table S5). Of the 1,397 repressed genes, 420 were not affected by any mutation (Fig. 2B). Of the remaining 977 genes, 891 fit one of nine major patterns. Six of these nine major patterns for repressed genes were also found as major patterns controlling the induced genes. The remaining three major patterns with the smallest number of induced genes (*ein2* only, *pad4* and *ein2* only, and *coi1* and *ein2* only) were minor patterns among the repressed genes. The repressed genes showed three additional major patterns that were minor or nonexistent among the induced genes: genes affected by all mutations ($n = 17$), genes affected by all mutations except *sid2* ($n = 35$), and genes affected by *ein2*, *pad4*, and *npr1* ($n = 30$). Among the major patterns of repressed genes, these were the three with the smallest numbers of genes. The 86 repressed genes that did not fit in one of the major patterns represented 19 different minor patterns. These included 14 genes affected by *coi1*, *pad4*, and *npr1* only; 13 affected by all mutations except *coi1*; and 13 affected by *ein2* and *pad4* only. The other patterns included some in which genes were repressed even further than in wild-type plants, and comprised fewer than 10 genes each. Overall, the gene circuitry controlling gene repression was very similar to the one controlling induction, with a few differences: more repressed genes were unaffected by any mutation (420 versus 189), more repressed genes were affected only by *pad4* (467 versus 337), and more repressed genes were affected by combinations of SA-related *npr1* or *sid2* mutations and JA- and ET-related mutations *coi1* and *ein2* (82 versus 21, all in minor classes).

***P. syringae* pv. *maculicola* ES4326 is a stronger inducer of the SA network sector than is *P. syringae* pv. *tomato* DC3000.**

P. syringae pv. *maculicola* ES4326 infection induces strong expression of the canonical SA-regulated gene *PR-1* whereas *P. syringae* pv. *tomato* DC3000 induces very weak expression (Glazebrook et al. 1996; Ham et al. 2007). We tested the possibility that this is a reflection of a general difference in the activity of the SA network sector during infection by these two strains. Defense responses to different bacterial strains were compared using expression profiling data in the NASC-120 data set. This data includes expression profiles of wild-type

Col-0 plants 24 h after mock inoculation or inoculation with strains DC3000, DC3000 *avrRpm1* (a strain that triggers ETI due to recognition of AvrRpm1 by the *R* gene *RPM1*), DC3000 *hrcC* (the *hrcC* mutation blocks secretion of effectors; therefore, this strain acts as an inducer of the PAMP response), and *P. syringae* pv. *phaseolicola* race 6 (a bean pathogen that fails to cause disease on *Arabidopsis*). The data were analyzed using a mixed-effect linear model, as described below. The $\log_2$ expression values, the $\log_2$ fold-change values, and the q values for each pathogen compared with mock at 24 h are shown in Table S6. Any gene for which the q value for the comparison of mock and pathogen inoculation was less than 0.01 was considered responsive to that pathogen, regardless of the fold change. We chose this less-stringent criterion to minimize false negatives when testing for ES4326-responsive genes that also respond to the other strains. DC3000 affected the most genes ($n = 3,369$) while DC3000 *hrcC* affected the fewest ($n = 1,660$) (Table 2). This is the expected result, because PAMP responses are rapid and are waning by 24 h after treatment (Navarro et al. 2004; Truman et al. 2006). DC3000 *hrcC* fails to deliver effectors and grows much less than DC3000, which may also contribute to differences in gene expression.

SA-dependent responses are critical for resistance to *P. syringae* (Glazebrook 2005). Consequently, we examined induction of SA-dependent genes, defined as the 107 genes that are induced by ES4326 in a *PAD4*-, *NPR1*-, and *SID2*-dependent manner, by the other pathogen strains. We found that DC3000 induced the fewest of these genes ($n = 30$) (Table 2). Further-

Fig. 2. *Pseudomonas syringae* pv. *maculicola* ES4326-responsive genes that are controlled by major defense signaling genes. Numbers in ovals indicate the number of genes whose induction or repression is compromised by mutation in certain regulators. For example, A shows that the induction of 337 genes was compromised in *pad4* but not in any other mutants tested. The induction of 74 additional genes was compromised in only *pad4* and *npr1* mutants. A, Induced genes. B, Repressed genes.

more, the average DC3000-induced $\log_2$ fold-change of the 107 genes was very low compared with the averages induced by the other strains (0.6 compared with > 1 for all the other strains) (Table 2). To test whether this effect was specific to SA-dependent genes, we carried out a similar analysis on the entire set of 1,140 genes strongly induced by ES4326. In marked contrast to the results with the 107 SA-induced genes, DC3000 and DC3000 *avrRpm1* induced the largest number of these genes (831 and 835, respectively), with fewer genes induced by *P. syringae* pv. *phaseolicola* or *P. syringae* pv. *tomato* DC3000 *hrcC*. Similarly, the average $\log_2$ fold-change values for these 1,140 genes were highest for these two strains (1.90 for DC3000 and 1.86 for DC3000 *avrRpm1*) (Table 2). The difference in induction of SA-dependent genes in DC3000- versus ES4326-infected plants is unlikely to be due solely to differences in infection conditions for two reasons. First, DC3000 was a weaker inducer of SA-dependent genes when compared with other strains within the NASC-120 data set, not just when compared with ES4326. Second, the difference in expression of SA-dependent genes between DC3000 and ES4326 was much more pronounced than the difference for the complete set of ES4326-induced genes.

The results from microarray analysis demonstrated that SA-dependent gene expression is less active in DC3000-infected plants than in ES4326-infected plants, suggesting that SA levels might be lower in DC3000-infected plants. To test this idea, we assayed SA in wild-type, *pad4*, and *sid2* plants 9 and 24 hpi. SA levels were dramatically lower in DC3000-infected plants than in ES4326-infected plants (Fig. 3). We conclude that our inference from microarray data is correct. DC3000 either actively represses or avoids inducing the strong SA response induced by ES4326.

Exploration of the SA and JA network sectors using the miniarray.

The SA and JA network sectors are known to play major roles in disease resistance (Glazebrook 2005). To define the roles of additional signaling components in these sectors, we selected mutants known, or which we thought likely, to have defects in SA or JA signaling. We then profiled these mutants 24 h after infection by strain ES4326, using a custom microarray that monitors expression of 464 pathogen-responsive genes, which we call a “miniarray” (Sato et al. 2007). For SA signaling, we chose *eds1* because the amino acid sequence of EDS1 is similar to PAD4, EDS1 and PAD4 physically interact, and *eds1* mutants have reduced SA levels (Falk et al. 1999; Feys et al. 2001); *eds5* because *eds5* mutants have SA levels as low as those in *sid2* and EDS5 is thought to be required for SA synthesis (Nawrath and Metraux 1999; Nawrath et al. 2002); *pbs3* because some groups have reported that SA levels are low in *pbs3* plants (Jagadeeswaran et al. 2007; Lee et al. 2007); *fmo1* because pathogen-induced *FMO1* expression is under EDS1 and PAD4 control and *fmo1* mutants are defective in systemic acquired resistance, an SA-requiring process (Bartsch et al. 2006; Mishina and Zeier 2006); and *ndr1* be-

cause we recently found that an *ndr1* mutation has a major effect on gene expression changes triggered by infection with DC3000 *avrRpt2*, raising the possibility of an SA signaling defect (Sato et al. 2007). We also profiled *pad4*, *npr1*, and *sid2*. For JA signaling, we chose *dde2*, a mutation in allene oxide synthase that blocks JA synthesis (von Malek et al. 2002); *jar1*, a mutation in a JA-conjugating enzyme that blocks production of JA-isoleucine (Staswick and Tiryaki 2004); *jin1*, a mutation in the MYB2 transcription factor required for a subset of responses to JA (Lorenzo et al. 2004); and *coi1*, a mutation in an E3-ubiquitin ligase required for JA signaling (Xie et al. 1998). As in our experiments using the ATH1 microarray, wild-type plants were mock inoculated or inoculated with ES4326 and mutant plants were inoculated with ES4326. Samples for profiling were collected after 24 h. Each experiment included three independent biological replicates and data were analyzed using mixed-effect linear models as described below.

We defined genes responding to ES4326 infection in wild-type plants using data from two experiments, each consisting of three replicates each of mock- and ES4326-inoculated wild-type plants. The $\log_2$ expression values, the $\log_2$ fold-change values, and the *q* values for all the genes on the array are

Fig. 3. Salicylic acid (SA) levels in plants infected with *Pseudomonas syringae* pv. *tomato* DC3000 or *P. syringae* pv. *maculicola* ES4326. Plants were mock inoculated (M) or inoculated with DC3000 (Pst) or ES4326 (Psm), and sampled 9 or 24 h postinoculation (hpi). Uninoculated plants were sampled at the beginning of the experiment (0 hpi). Each bar represents the mean and standard error of SA levels from two independent experiments, calculated by analysis of variance. Note the $\log_{10}$ scale on the y-axis. At both 9 and 24 h, SA levels in wild-type (Col-0) plants were much lower after DC3000 inoculation than after ES4326-inoculation ($P < 0.01$).

Table 2. Induction of *Pseudomonas syringae* pv. *maculicola* ES4326-induced genes by other *P. syringae* strains

Strains	Total no. of genes induced ($q < 0.01$)	No. of 107 salicylic acid (SA)-dependent genes induced ($q < 0.01$)	Average $\log_2$ fold-change of 107 SA-dependent genes (infected vs. mock)	No. of 1,140 ES4326-induced genes induced ($q < 0.01$)	Average $\log_2$ fold-change of 1140 ES4326-induced genes (infected vs. mock)
<i>P. syringae</i> pv. <i>maculicola</i> ES4326	4,076	107	2.91	1,140	3.12
<i>P. syringae</i> pv. <i>tomato</i> DC3000	3,369	31	0.60	831	1.90
<i>P. syringae</i> pv. <i>tomato</i> DC3000 <i>avrRpm1</i>	2,438	50	1.14	835	1.86
<i>P. syringae</i> pv. <i>tomato</i> DC3000 <i>hrcC</i>	1,660	55	1.10	443	0.86
<i>P. syringae</i> pv. <i>phaseolicola</i>	2,170	81	1.70	637	1.27

shown in Supplementary Table S7. ES4326-responsive genes were selected by requiring a $\log_2$ fold-change of <-1 or >1 (twofold) and $q < 0.05$, resulting in 223 genes. These criteria are less stringent than the criteria we used to define ES4326-responsive genes on the ATH1 array. They were chosen because the miniarray is less sensitive than the ATH1 array (Sato et al. 2007) and the smaller number of genes monitored reduces the number of false positives ($q < 0.05$ with 223 genes will detect an average of 11 false positives). To detect the effects of mutations on gene expression, we compared expression levels in mutant and wild-type plants, as shown in Supplementary Table S8. Then, among the 223 ES4326-responsive genes, we selected those whose expression levels in ES4326-infected plants were significantly altered by at least one mutation ($q < 0.05$), resulting in 205 genes. The $\log_2$ fold-change and q values for these 205 genes are shown in Supplementary Table S9. The number of genes affected by each mutation is shown in Table 3. To explore the relationships among the mutations, the $\log_2$ fold-change values for each mutation versus wild type were subjected to complete linkage hierarchical clustering using Cluster, and the results were visualized using Treeview (Fig. 4) (Eisen et al. 1998). Correlations between the $\log_2$ fold-change values for each pair of mutants are shown in Table 4. Beginning with the SA signaling mutants, we used the results to define the functions of signaling components relative to those of *PAD4*, *NPR1*, *SID2*, and *COI1*.

EDS1: The profile of *eds1* was more similar to that of *pad4* than to any other mutation in our set (correlation 0.84) (Table 4). The hierarchical clustering does not show any large groups of genes that are affected differently by these two mutations. Growth of ES4326 in *eds1* was similar to *pad4*, with bacterial titers approximately 300-fold higher than in wild-type plants (Fig. 5A). We conclude that, within the limits of resolution of our analysis, EDS1 and PAD4 affect the same function.

EDS5 and NPR1: The profiles of *eds5*, *npr1*, and *sid2* are all very similar to each other (correlations *eds5* to *npr1*, *eds5* to *sid2*, and *npr1* to *sid2* = 0.84, 0.81, and 0.78, respectively) (Table 4). These mutations also appear to define the same function, which is presumably the activity of SA. In the ATH1 data, we observed a group of 74 genes whose induction required *NPR1* but not *SID2*, seemingly contradicting the results from the miniarray. Only three of these genes are represented on the miniarray and only one of them met the criteria for significant induction in wild-type plants. Thus, the miniarray cannot detect the group of genes whose induction requires *NPR1* but not *SID2*.

FMO1: In *fmo1*, expression of only one gene, *FMO1* itself (At1g19250), was significantly altered. Hierarchical clustering did not detect a close similarity between *fmo1* and any of other mutants studied (Fig. 4). We conclude that *fmo1* does not have a major effect on gene expression 24 h after ES4326 infection. Mishina and Zeier reported that *fmo1* is not more susceptible to infection by ES4326 (Mishina and Zeier 2006) but we found a modest increase in susceptibility to ES4326 (Fig. 5A). This

discrepancy could be due to differences in growth conditions between laboratories.

PBS3: The expression profile of *pbs3* was similar to those of *eds1* (correlation 0.59), *pad4* (0.68), *npr1* (0.71), *eds5* (0.71), and *sid2* (0.51) (Table 4). The number of genes affected by *pbs3* was greater than the number affected by *npr1*, *eds5*, or *sid2* but smaller than the number affected by *eds1* or *pad4* (Table 3). Cluster grouped *pbs3* with *pad4* and *eds1*, in a separate group from *npr1*, *eds5*, and *sid2* (Fig. 4). Inspection of Figure 4 explains these results. There is a group of genes that is affected similarly by *pbs3*, *eds1*, and *pad4* but not by *npr1*, *eds5*, or *sid2* (Fig. 4, group A). There are other groups of genes that are affected by *pad4* and *eds1* but not by *pbs3*, *npr1*, *eds5*, or *sid2* (Fig. 4, groups B and C). We found a major increase in susceptibility to ES4326 in *pbs3* (Fig. 5B), consistent with the results of Nobuta and associates (2007). Assuming that signal flow through the network is divergent, we conclude that *PBS3* acts at a point intermediate between the node defined by *pad4* and *eds1* and the node defined by *npr1*, *eds5*, and *sid2*.

NDR1: The expression profile of *ndr1* is very similar to those of *eds1* and *pad4* (correlations of 0.79 and 0.75, respectively), and slightly less similar to *pbs3* (correlation of 0.69). Correlations between *ndr1* and *eds5* or *sid2* are low, less than 0.40. Hierarchical clustering places *ndr1* in the group with *pbs3*, *eds1*, and *pad4*, closer to *eds1* and *pad4* than to *pbs3*. Inspection of Figure 4 reveals a small group of genes (group C) affected by *pad4* and *eds1* but not by *ndr1* or *pbs3*. Another group of genes (group B) is affected by *pad4*, *eds1*, and *ndr1* but not by *pbs3*. There are no groups of genes affected by *pbs3* but not *ndr1*; therefore, we conclude that *NDR1* likely acts at a point between the node defined by *PAD4* and *EDS1* and the node defined by *PBS3*.

COI1-dependent gene expression changes are a response to coronatine.

We studied three additional mutations affecting the JA sector. Two of these, *dde2* and *jar1*, block synthesis of the active form of JA, JA-Ile. *COI1* is required for responses to JA. Consequently, if endogenous JA-Ile is responsible for activation of the *COI1*-dependent genes, the profiles of *dde2*, *jar1*, and *coi1* should all be quite similar. Our results from the miniarray are strikingly inconsistent with this idea, because very few genes were significantly affected by *dde2* ($n = 16$) or *jar1* ($n = 9$), whereas many were affected by *coi1* ($n = 96$). Furthermore, there was almost no correlation between the profile of *coi1* and those of *dde2* and *jar1* (-0.17 and 0.15 , respectively). This raised the possibility that coronatine, a toxin produced by ES4326 and DC3000 that mimics the effects of JA-Ile, might be responsible for induction of the *COI1*-dependent genes.

To study the role of coronatine in ES4326-induced gene expression, we profiled wild-type and *coi1* plants after mock inoculation, inoculation with ES4326, or inoculation with ES4326 *cor*, a coronatine-deficient mutant. The experiment consisted of three biological replicates and data were analyzed using

Table 3. Genes differentially expressed in mutant plants^a

Genes (no.) ^b	Level ^c	Mutants											
		<i>coi1</i>	<i>dde2</i>	<i>eds1</i>	<i>eds5</i>	<i>fmo1</i>	<i>jir1</i>	<i>jar1</i>	<i>ndr1</i>	<i>npr1</i>	<i>pad4</i>	<i>pbs3</i>	<i>sid2</i>
Induced in WT (177)	L	74	9	88	22	1	8	7	57	25	103	56	22
	H	11	4	8	3	0	0	2	11	3	10	19	4
Repressed in WT (28)	L	0	3	0	0	0	0	0	4	0	0	0	0
	H	11	0	16	0	0	1	0	5	3	10	16	1

^a For each mutant, the number of genes expressed at significantly different levels in infected mutant plants than in infected wild-type (WT) plants is shown. Data were obtained from the miniarray.

^b Number in parentheses = total number of genes expressed.

^c L and H indicate genes expressed at lower and higher levels, respectively, in mutant than in wild-type plants ($q < 0.05$).

mixed-effect linear models, as described below. Using this data, we compared two sets of $\log_2$ fold-change data for all the genes on the array; *coi1* infected with ES4326 versus wild type infected with ES4326 and wild type infected with ES4326 *cor* versus wild type infected with ES4326 (Supplementary Table S10). These values are almost identical, with a correlation of 0.96 (Fig. 6). We conclude that essentially all of the *COI1*-dependent gene expression changes triggered by ES4326 at

24 h after infection are a response to coronatine produced by the pathogen.

DDE2, JAR1, and JIN1 have small effects on coronatine responses.

Although *dde2* and *jar1* affect only a small number of genes, there is a reasonable correlation between their profiles (0.58) (Table 4). Among the 22 genes significantly affected by at least one of these mutants, the correlation is 0.75, suggesting that endogenous JA-Ile contributes to changes in expression of a small group of genes. Curiously, for the 16 genes significantly affected by *dde2*, the correlation between *coi1* and *dde2* is -0.72 , suggesting that endogenous JA-Ile is inhibiting *COI1*. Nine genes were significantly affected by *jin1*. Of these nine genes, five are similarly affected by *coi1* and four are not. This suggests that *JIN1* mediates a small fraction of the gene expression changes triggered by the action of *COI1*. The four genes that are not affected by *coi1* may indicate a *COI1*-independent function of *JIN1* but this data set is too small to draw a firm conclusion.

DISCUSSION

In this work, we used similarities among expression profiles from plants with loss-of-function mutations in key components of the defense signaling network to study the network at a fixed time after infection with a particular pathogen, ES4326. We found that most ES4326-responsive genes were affected by at least one mutation in the study set and that most affected genes fit one of a few simple patterns of regulation. Comparison of the ES4326 profiling data with profiles from DC3000-infected plants indicated that the SA sector is much less active after DC3000 infection. Expansion of the set of mutants studied using a custom miniarray allowed *EDS1*, *NDR1*, *PBS3*, and *EDS5* to be positioned in the SA sector. The lack of correlation between profiles from *coi1* and those of *dde2* and *jar1* led to an experiment that showed that essentially all *COI1*-dependent gene expression changes are attributable to the effects of coronatine.

Profiling showed that expression levels of most (948 of 1,140) ES4326-induced genes were reduced by one or more of the mutations *pad4*, *npr1*, *sid2*, *ein2*, or *coi1*. Most ES4326-induced genes (1,037 of 1,140) (Fig. 2A) fit 1 of 10 major expression patterns (including the pattern unaffected by any mutation), far fewer than the 32 possible patterns formed by all combinations of unaffected or reduced expression in each mutant. A few genes ($n = 103$) constituted 31 minor patterns, including some in which genes were induced at even higher levels in mutants than in wild-type plants. Some of the minor patterns are likely not biologically meaningful, because many of the genes constituting them were excluded from one of the major patterns by q values close to the cut-off. Interestingly,

Fig. 4. Clustering of $\log_2$ fold-change values of mutants versus the wild type. Complete linkage hierarchical clustering was performed. Vertical dendrograms describe similarities among genes and the horizontal dendrogram describes similarities among mutants. Columns labeled by mutant names contain $\log_2$ fold-change values of mutants versus the wild type. The column labeled “Col-0” contains the $\log_2$ fold-change values of *Pseudomonas syringae* pv. *maculicola* ES4326-inoculated versus mock-inoculated wild-type plants. These values were not used in the clustering. Negative values shown in blue indicate lower expression in a mutant than in wild-type plants or repression in wild-type plants. Positive values shown in yellow indicate higher expression in a mutant than in wild-type plants or induction in wild-type plants. A color scale is provided at the bottom of the figure. Vertical bars at the right labeled “A”, “B”, or “C” mark clusters of genes that are discussed in the text.

repression of many (976 of 1,397) of the ES4326-induced genes was attenuated by the same set of mutants, and the major expression patterns of repressed genes were quite similar to those of the induced genes. It is tempting to think of repressed genes as being targets of pathogen effectors whose repression favors the pathogen. However, for the many genes whose repression is attenuated by *pad4*, *npr1*, or *sid2*, this seems unlikely to be true. Rather, it is likely that the SA-dependent defense response includes increased expression of some genes and decreased expression of others.

We found that the SA network sector is much less active following DC3000 infection than following ES4326 infection. This could be due to better recognition of ES4326 by the plant or better suppression of SA signaling by DC3000 effectors. These possibilities might be distinguished by introducing individual effectors from each strain into the other, and screening for DC3000 effectors that weaken the SA response to ES4326 and for ES4326 effectors that strengthen the SA response to DC3000.

Using profiles from a custom miniarray, we found that *pad4* and *eds1* have very similar effects on network function that we could not differentiate, as did *eds5*, *npr1*, and *sid2*. We did differentiate *npr1* and *sid2* using the ATH1 array but very few of the differentiating genes were present on the miniarray. We identified PBS3 and NDR1 as network components acting between the *pad4/eds1* node and the *eds5/npr1/sid2* node, as shown in Figure 7. NDR1 was placed above PBS3 in the network due to the group of genes affected by *pad4*, *eds1*, and *ndr1* but not by *pbs3* (Fig. 4, group B). PBS3 was placed above NPR1 due to the group of genes affected by *pad4*, *eds1*, *ndr1*, and *pbs3* but not by *npr1* (Fig. 5, group A). NPR1 was placed above EDS5 and SID2 due to the group of genes identified on the ATH1 array that were affected by *npr1* but not by *sid2*. This placement may seem strange because NPR1 is well known to transmit the SA signal to the nucleus. Our model rests on the assumption of signal divergence through the network. This assumption may not be correct. Alternatively, NPR1 may react to a signal in addition to SA. This was suggested as an explanation for why an induced systemic resistance response required NPR1 but not SA (Pieterse et al. 1998). To explain our data, such an additional signal would have to require PAD4, EDS1, NDR1, and PBS3. We did not attempt to place *fmo1* in the network, because we did not detect any significant changes in gene expression in this mutant.

PBS3 encodes a GH3-like protein. Because other family members, such as JAR1, catalyze formation of hormone-amino acid conjugates, it is tempting to speculate that PBS3 produces an SA conjugate. However, this is unlikely because the

enhanced susceptibility and reduced *PR-1* expression phenotypes of *pbs3* can be rescued by exogenous SA. There is conflicting data regarding levels of free (not glucoside conjugates) SA levels in infected *pbs3* plants. Nobuta and associates (2007) reported increased SA 24 h after infection by DC3000 *avrRpt2*. Lee and associates (2007) reported reduced SA 24 h after infection by *P. syringae* pv. *maculicola* DG3 (very similar to ES4326), 6 h after infection by DG6 (a DG3 derivative carrying *avrRpt2*) and 24 h after infection by *P. syringae* pv. *maculicola* DG34 (a DG3 derivative carrying *avrRpm1*). Jagadeeswaran and associates (2007) found reduced levels 12 and 24 h after infection by DC3000 carrying *avrRpm1*. Our profiling data strongly suggests reduced SA signaling 24 h after infection by ES4326, consistent with the reduced SA levels observed by Lee and associates (2007). It is entirely possible that all of the reported SA levels are correct and that the contribution of PBS3 to SA levels varies according to the inducing strain or with environmental conditions. This would be reminiscent of *pad4*, which shows reduced SA levels after ES4326 infection but not after ES4326 *avrRpt2* infection (Zhou et al. 1998). Also like *PAD4*, *PBS3* affects expression of many more genes than canonical SA signaling mutants. This suggests that the effect of *PBS3* on SA levels is regulatory rather than biosynthetic.

NDR1 was first identified in a screen for mutations that interfere with resistance due to recognition of the AvrB effector by the R protein RPM1 (Century et al. 1995). Subsequently, it was found to be required for resistance mediated by many other, but not all, *R* genes (Aarts et al. 1998; Century et al. 1995; McDowell et al. 2000). Based on these results, NDR1 has long been considered as a protein required for *R*-gene-mediated resistance. Indeed, expression profiling of responses to DC3000 *avrRpt2* in wild-type, *ndr1*, and *rps2* plants showed that essentially all of the gene expression changes that required the cognate *R* gene *RPS2* also required *NDR1* (Sato et al. 2007). However, this study also showed that there were additional genes whose expression required *NDR1* but not *RPS2*. Many of these genes were also PAMP responsive, suggesting a role for NDR1 in the PAMP response. This idea was supported by the subsequent finding that DC3000 *hrcC* grows to higher titers in *ndr1* than in wild-type plants (Katagiri and Sato 2007). Here, we found that essentially all SA-dependent genes were also *NDR1*-dependent, as were many SA-independent genes. This may reflect a role for *R* genes in activating responses to ES4326 or it may reflect a general role for NDR1 in SA signaling and a branch of *PAD4*- and *EDS1*-dependent signaling. Profiling of *ndr1* plants after treatment with exogenous SA might help to resolve these possibilities.

Table 4. Pairwise correlations between mutants^a

	<i>coi1</i>	<i>dde2</i>	<i>eds1</i>	<i>eds5</i>	<i>fmo1</i>	<i>jin1</i>	<i>jar1</i>	<i>ndr1</i>	<i>npr1</i>	<i>pad4</i>	<i>pbs3</i>	<i>sid2</i>
<i>coi1</i>	1	-0.17	0.06	-0.21	-0.06	0.28	0.15	-0.01	-0.16	0.13	-0.19	-0.19
<i>dde2</i>	-0.17	1	-0.04	0.04	0.08	0.23	0.58	-0.11	-0.06	-0.15	-0.14	0.04
<i>eds1</i>	0.06	-0.04	1	0.35	0.22	-0.09	0.11	0.79	0.45	0.84	0.59	0.23
<i>eds5</i>	-0.21	0.04	0.35	1	0.03	-0.12	0.06	0.29	0.84	0.55	0.71	0.81
<i>fmo1</i>	-0.06	0.08	0.22	0.03	1	-0.06	-0.04	0.27	0.02	0.16	0.11	-0.03
<i>jin1</i>	0.28	0.23	-0.09	-0.12	-0.06	1	0.20	-0.11	-0.06	-0.08	-0.10	-0.03
<i>jar1</i>	0.15	0.58	0.11	0.06	-0.04	0.20	1	-0.07	-0.08	-0.03	-0.08	0.05
<i>ndr1</i>	-0.01	-0.11	0.79	0.29	0.27	-0.11	-0.07	1	0.39	0.75	0.69	0.11
<i>npr1</i>	-0.16	-0.06	0.45	0.84	0.02	-0.06	-0.08	0.39	1	0.70	0.71	0.78
<i>pad4</i>	0.13	-0.15	0.84	0.55	0.16	-0.08	-0.03	0.75	0.70	1	0.68	0.45
<i>pbs3</i>	-0.19	-0.14	0.59	0.71	0.11	-0.10	-0.08	0.69	0.71	0.68	1	0.51
<i>sid2</i>	-0.19	0.04	0.23	0.81	-0.03	-0.03	0.05	0.11	0.78	0.45	0.51	1

^a Pairwise correlations for the values of log fold-change of 205 genes for the indicated mutants versus wild-type. Correlations greater than 0.5 are shown in bold type. The uncentered Pearson correlations between the log₂ fold-change values of the infected mutant versus infected wild type were calculated for the set of 205 genes significantly altered by a least twofold in infected wild type versus mock-infected wild type, and significantly different in at least one infected mutant relative to infected wild type.

The phytotoxin coronatine has been suspected to mimic the action of JA since the isolation of *coi1* plants, which are resistant to coronatine and insensitive to JA (Feys et al. 1994). It is now clear that both coronatine and JA-Ile, the active form of JA, act by binding to the F-box protein COI1, thereby promoting degradation of the JAZ repressor proteins (Chini et al. 2007; Katsir et al. 2008; Thines et al. 2007). Expression profiling studies have shown that coronatine has a major effect on host gene expression during *P. syringae* infection (Thilmony et al. 2006), that methyl-JA and coronatine have similar effects on gene expression in tomato (Uppalapati et al. 2005), and that the effects of JA on gene expression depend largely on *COI1* (Feng et al. 2003). In our work, data from the ATH1 array showed that many gene expression changes depend on *COI1*. Comparison of the

effects of *coi1* and coronatine deficiency in the pathogen using the miniarray showed that essentially all *coi1*-dependent gene expression changes are attributable to coronatine. Defects in synthesis of endogenous JA had little effect on gene expression.

Fig. 5. Growth of *Pseudomonas syringae* pv. *maculicola* ES4326 in various genotypes. Plants were inoculated with ES4326 and bacterial titers were determined immediately (0) or 3 days postinoculation (dpi). All four mutants supported significantly higher concentrations of bacteria than the wild type ($P < 0.001$ by Mann-Whitney U test). Similar results were obtained in at least three independent experiments. **A**, Growth in *fmo1*, *eds1*, and *pad4*. Bars represent means and standard deviations of 4 replicates at 0 dpi, and 24, 16, 16, or 8 replicates for the wild type (Col-0), *fmo1*, *eds1*, and *pad4*, respectively, at 3 dpi. **B**, Growth in *pbs3*. Bars represent means and standard deviations of 4 replicates at 0 dpi and 16 replicates at 3 dpi.

Fig. 6. Presence of *coi1* in the plant or coronatine deficiency in the pathogen have very similar effects. The $\log_2$ ratios of infected *coi1* versus infected wild type and wild type infected with *Pseudomonas syringae* pv. *maculicola* ES4326 *cor* versus wild type infected with ES4326 were plotted for all the genes on the miniarray.

Fig. 7. Model of the network sector including salicylic acid. The model is based mainly on the results from the miniarray, assuming signal divergence as signal proceeds through the network. Numbered ovals represent groups of similarly-regulated genes. Group 4 is based on the results from the ATH1 array. Because this group of genes is not present on the miniarray, the roles of EDS1, PBS3, and NDR1 in their regulation are not directly known.

Thus, our results show that, in this plant–pathogen interaction, there is very little role for endogenous JA but coronatine produced by the pathogen has a major effect. This result differs from those of Zhao and associates (2003), who did a similar analysis comparing *P. syringae* pv. *tomato* strains DC3000 and DC3000 *cor* on wild-type and *jail* tomato (tomato *JAIL* is likely orthologous to *Arabidopsis COI1*). Although there was substantial overlap between coronatine-dependent and *JAIL*-dependent genes, a substantial number of *jail*-dependent genes were not coronatine dependent (Zhao et al. 2003). There may be more effect of endogenous JA or other oxylipins in the DC3000–tomato system than in the ES4326–*Arabidopsis* system.

Curiously, among the few genes affected by loss of JA synthesis due to the presence of *dde2*, there was a negative correlation between the effect of *dde2* and the effect of *coi1*. This does not appear to be consistent with the fact that coronatine and JA-Ile act similarly in binding to COI1. If the COI1–coronatine complex is more active than the COI1–JA-Ile complex, then COI1 binding to JA-Ile might reduce COI1 activity relative to COI1 binding to coronatine, explaining our results.

In summary, we have investigated the relationships among defense signaling network components defined by mutations using expression profiles as detailed descriptions of mutant phenotypes. It is worth emphasizing that, in this approach, we do not assume that the transcript level of a particular gene represents its activity. Rather, we use causal relationships between the presence of a mutation and changes in the expression profile. Although our analysis at this stage was mostly limited to qualitative comparisons of gene sets that are differentially regulated in single mutants, we succeeded in revealing complex relationships among network components that affect the SA sector. This approach has the potential to extract additional information about the network. It has been shown that even low-amplitude profiles can contain statistically significant information when whole profiles are used as phenotypes (Hughes et al. 2000). Therefore, quantitative analysis of these data could reveal more subtle relationships among network components. The data in this study were generated under a single experimental condition: 24 h after infection by ES4326. A different pathogen, such as a necrotrophic fungal pathogen, might induce different signal flow patterns in the network and reveal relationships that may have escaped detection using ES4326. Data from multiple timepoints would allow the direction of signal flow to be determined and might reveal relationships that are undetectable at the single timepoint we used.

MATERIALS AND METHODS

Plant genotypes and growth conditions.

All genotypes were derived from the Col-0 accession. They were *sid2-2* (Wildermuth et al. 2001), *eds5-1* (Nawrath et al. 2002), *pad4-1* (Jirage et al. 1999), *ein2-1* (Alonso et al. 1999), *coi1-1* (Xie et al. 1998), *ndr1-1* (Century et al. 1997), *dde2-2* (von Malek et al. 2002), *jar1-1* (Staswick et al. 2002), and *jin1-1* (Berger et al. 1996; Lorenzo et al. 2004). The *eds1* plants were derived by introgression of *eds1-2* into Col-0 (Bartsch et al. 2006), *fmo1-1* was SALK_026163 (Bartsch et al. 2006), and *pbs3-2* was SALK_018225 (Nobuta et al. 2007). All plants were grown in controlled-environment chambers at 22°C and 75% relative humidity with 12 h of light (100 $\mu\text{m}^{-2} \text{s}^{-1}$ fluorescent illumination) and 12 h of darkness.

P. syringae pv. *maculicola* ES4326 infection and SA assays.

Pseudomonas syringae pv. *maculicola* was cultured in King's B medium at room temperature (22 to 25°C) as described (Glazebrook and Ausubel 1994). For microarray experiments, overnight liquid cultures were washed and resuspended in 5

mM MgSO_4 . Bacteria were diluted to a concentration equivalent to an optical density at 600 nm (OD_{600}) = 0.002 and introduced into leaves of 30-day-old plants using a needleless syringe. Mock-infected plants were similarly treated with 5 mM MgSO_4 . For bacterial growth experiments, 4- to 5-week-old plants were inoculated at a concentration of OD_{600} = 0.0001. Two leaf discs were cut from each inoculated leaf with a no. 3 cork borer, ground in 5 mM MgSO_4 , serially diluted, and plated on King's B medium. For free (nonconjugated) SA assays, mature leaves of 4- to 5-week-old plants were inoculated with DC3000 or ES4326 suspensions of OD_{600} = 0.01. SA was determined by solid-phase extraction and gas chromatography–mass spectrometry as described previously (Tsuda et al. 2008).

Microarray experiments.

Microarrays were Affymetrix ATH1 (Redman et al. 2004) and the custom miniarray described previously (Sato et al. 2007). The ATH1 experiments included a sixth mutant, *pad2-1*. This mutation had relatively little effect on gene expression and it was not discussed here. The data are available from NASC-Arrays (accession numbers below). The NASC-120 data collected by Nürnberger and associates was obtained from NASC-Arrays (Craigon et al. 2004). Only the data from the 24-h timepoint was analyzed. Miniarray experiments were conducted in four batches of seven to eight samples each, with three biological replicates of each set. Each batch included wild-type plants inoculated with ES4326 and all sampling was done 24 hpi. Two batches included mock-inoculated wild-type plants. One batch included wild-type and *coi1* plants inoculated with ES4326 or ES4326 *cor* or mock inoculated. Another batch also included ES4326-inoculated *coi1* plants, making a total of six replicates for this genotype–treatment combination. Some of the genotypes included in the four batches are not relevant to the analysis reported here and, therefore, were not discussed. The complete data sets are available from GEO (accession numbers below). Preparation of RNA, labeling, hybridization to arrays, and scanning were performed as described previously (Sato et al. 2007). ATH1 data generated in this work, as well as NASC-120 data, were normalized using RMAExpress (Irizarry et al. 2003), and miniarray data were normalized using stable genes-based quantile normalization (SBQ) (Sato et al. 2007).

Microarray data analysis.

Generally, the normalized expression profile data sets were subjected to mixed-effect linear models to obtain the mean estimate and standard error values. The contrast of the second model in each case was set to obtain standard errors for particular comparisons. Student's *t* test (two tails) was used to obtain the *P* values for particular comparisons. The *P* values were corrected for multiple testing using the Benjamini-Hochberg FDR, and the corrected values were designated as the *q* values. All these analyses were done in R (ver. 2.5.1) using the lme4 package for the mixed models. The models used in each of the following comparisons are described below as cases A, B, and C.

i) Comparison of ES4326-inoculated and mock-inoculated wild-type plants at three timepoints using ATH1 data: case C with two levels of the inoculum effect *I*, ES4326 and mock inoculation, and three levels of the condition effect *Y*, 9, 24, and 32 h.

ii) Comparison of ES4326-inoculated and mock-inoculated wild-type plants at 24 h using ATH1 data from two experiments: case B with two levels of the sample effect *S*, ES4326 and mock inoculation.

iii) Comparison of wild-type and mutant plants inoculated with ES4326 using ATH1 data: case A with seven levels of the

sample effect S , Col, *coil*, *ein2*, *npr1*, *pad2*, *pad4*, and *sid2*. All the pairwise comparisons among the genotypes were made.

iv) Comparison of pathogen- and mock-inoculated plants in the NASC120 data set: case A with five levels of the sample effect S , DC3000, DC3000 *avrRpm1*, DC3000 *hrcC*, *P. syringae* pv. *phaseolicola*, and mock inoculation. Each strain was compared with mock inoculation and all the pairwise comparisons between strains were made.

v) Comparison of ES4326-inoculated and mock-inoculated wild-type plants using miniarray data: case B with two levels of the sample effect S , ES4326 and mock inoculation.

vi) Comparison of wild-type and mutant plants inoculated with ES4326 using miniarray data: case B with 22 levels of the sample effect S , Col, *eds5*, *npr1*, *pad4*, *sid2*, *coil*, *dde2*, *ein2*, *jnl1*, *jar1*, *eds1*, *fmo*, *ndr1*, *pbs3*, and 8 other genotypes profiled together but not used in this study. Note that this data set did not have a factorial design. Each mutant was compared with Col.

vii) Comparisons using the ES4326 *cor* data: case C with two levels of the condition effect, Col and *coil*, and three levels of the inoculum effect I , ES4326, ES4326 *cor*, and mock inoculation.

Case A: for data generated as three biological replicates within a single experimental set:

$$\log_2(y_{gsr}) = \mu + R_r + \gamma_{gsr}$$

And then, for each g th gene, the next model was fit separately:

$$\gamma_{gsr} = Gg + G:S_{gs} + G:R_{gr} + \varepsilon_{gsr},$$

where y , μ , v , R , γ , G , S , and ε represent expression value, a constant, a constant, replicate, residual from the first model, gene, sample, and residual from the second model, respectively. R , γ and ε were random effects and the rest were fixed effects.

Case B: for data generated in multiple experimental sets, each having three biological replicates:

$$\log_2(y_{gsrb}) = \mu + B_b + B:R_{br} + \gamma_{gsrb}$$

And then, for each g th gene, the next model was fit separately:

$$\gamma_{gsr} = Gg + G:S_{gs} + G:(B + B:R)_{grb} + \varepsilon_{gsr},$$

where y , μ , Gg , B , R , γ , G , S , and ε represent expression value, a constant, a constant, experiment, replicate, residual from the first model, gene, sample, and residual from the second model, respectively. B , R , γ and ε were random effects and the rest were fixed effects. R was nested in B .

Case C: for comparisons with two fixed factors:

$$\log_2(y_{gyir}) = \mu + R_r + \gamma_{gyir}$$

And then, for each g th gene, the next model was fit separately:

$$\gamma_{gyir} = Gg + G:Y_{gy} + G:I_{gyi} + G:R_{gr} + \varepsilon_{gyir},$$

where y , μ , Gg , R , γ , G , Y , I , and ε represent expression value, a constant, replicate, residual from the first model, gene, condition, inoculum, and residual from the second model, respectively. R , γ and ε were random effects and the rest were fixed effects.

All correlation values are uncentered Pearson correlations.

Clustering.

Figure 4 was created by clustering of the $\log_2$ fold-change values of mutants versus Col-0. The set of 205 miniarray genes significantly affected by ES4326 infection of wild-type plants ($q < 0.05$) and significantly different in at least one mutation in the study set relative to Col-0 ($q < 0.05$) were subjected to complete linkage hierarchical clustering of only the mutants, using Cluster (Eisen et al. 1998). This provided the order and

dendrogram for the mutants. Then, the set of 205 genes was separated into the set of genes induced by ES4326 infection of Col-0, and the set repressed. Each set of genes was subjected to complete linkage hierarchical clustering of only the genes. This provided the order and dendrograms for the genes. The $\log_2$ fold-change values for Col-0 versus mock were scaled by a factor of 0.4 and added as the last column of the figure. Note that these values played no part in the clustering.

Microarray data accession numbers.

The original (.CEL) data files for the 18 ATH1 hybridizations from the ES4326 timecourse experiment are available from NASC Arrays as NASC-414. The files for the 21 ATH1 hybridizations from the wild-type and mutants comparison are available as NASC-454 (accession number pending). The miniarray files are available from Gene Expression Omnibus as GSE11009.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- Nottingham Arabidopsis Stock Centre (NASC) arrays:
affymetrix.arabidopsis.info
 National Center for Biotechnology Information Gene Expression
 Omnibus: www.ncbi.nlm.nih.gov/geo