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Interplay between MAMP-triggered and SA-mediated defense responses

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Summary

Plants respond to pathogen infection using an innate immune system with at least two distinct recognition mechanisms. One mechanism recognizes microbe-associated molecular patterns (MAMPs). The other is based on resistance (R) genes and specifically recognizes certain pathogen virulence factors, including those delivered through the type III secretion system (TTSS) of bacteria. Salicylic acid (SA)-mediated responses are an important part of the R gene-mediated defense. Substantial overlaps between MAMP-triggered and SA-mediated responses have been reported. However, interactions between MAMP-triggered and SA-mediated signaling mechanisms have not been well documented. Here we report intimate interactions between MAMP-triggered and SA-mediated signaling. We found that SA accumulated at a higher level 6 h after treatment with a MAMP, flg22 or inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) *hrcC* mutant, which is deficient in TTSS function. Disruptions of SA signaling components, such as *SID2* and *PAD4*, strongly affected MAMP-triggered responses monitored by expression profiling. We found two groups of genes that were induced by *Pst*DC3000 *hrcC* in an SA-dependent manner. One group was *SID2*-dependent at all time points, whereas the other was *SID2*-independent at early time points and *SID2*-dependent at later time points. Thus, the expression of the latter genes responds to MAMPs through both SA-independent and SA-dependent signaling mechanisms. Strong resistance to *Pst*DC3000 *hrcC* was dependent on SA signaling. These results indicate that the SA increase triggered by MAMPs is a major component of the MAMP-triggered signaling mechanism, explaining the overlapping spectra of MAMP-triggered and SA-mediated responses.

Keywords: MAMP, salicylic acid (SA), expression profiling, *SID2, PAD4, Arabidopsis thaliana*.

Introduction

Plants are subject to attack by a wide variety of microbial pathogens. In response, they express numerous defense mechanisms, many of which are induced by pathogen attack (Glazebrook, 2005). Perception of microbes by plants can be divided into three main phases, which appear to reflect steps of co-evolution in plant–pathogen interactions (Chisholm *et al.*, 2006; Nurnberger *et al.*, 2004). In the first phase, plants sense microbe-associated molecular patterns (MAMPs) that are characteristic molecular structures shared by large groups of microbes (Felix *et al.*, 1999; Kunze *et al.*, 2004). The perception of MAMPs leads to rapid activation of defense mechanisms, such as cell-wall reinforcement by callose deposition (Kim *et al.*, 2005), the production of reactive oxygen species (ROS; Nurnberger *et al.*, 2004), and the induction of numerous defense-related genes (Navarro *et al.*, 2004). In the second phase, successful pathogens acquire the ability to inhibit these MAMP-triggered basal defenses through the action of virulence factors (Hauck *et al.*, 2003; He *et al.*, 2006; Li *et al.*, 2005; de Torres *et al.*, 2006; Zhang *et al.*, 2007). In bacterial pathogens, these

virulence factors are known as type III effectors (TTEs) because they are transported into host cells through a type III secretion system (TTSS). In the third phase, plants acquire resistance (R) genes specialized to specifically detect these pathogen-derived virulence factors or their effects on host targets. R gene-mediated resistance is usually accompanied by rapid production of ROS, which is required for the hypersensitive response (HR), a type of programmed cell death thought to limit the access of the pathogen to water and nutrients. R gene-mediated resistance is also associated with activation of a salicylic acid (SA)-mediated signaling pathway that leads to the induction of many pathogenesis-related (PR) proteins, which are thought to contribute to resistance (Delaney *et al.*, 1994; Glazebrook, 2005).

The best-characterized MAMP among phytopathogens is flagellin, a structural component of the flagellum in Gram-negative bacteria (Felix *et al.*, 1999; Gomez-Gomez and Boller, 2002). In a series of seminal studies, a 22-amino-acid epitope of flagellin, flg22, was found to be recognized by the Arabidopsis FLS2 leucine-rich repeat (LRR) receptor kinase (Felix *et al.*, 1999; Gomez-Gomez and Boller, 2000; Gomez-Gomez *et al.*, 1999). Treatment of Arabidopsis leaves with flg22 activates multiple defense responses (Navarro *et al.*, 2004), including those mediated through mitogen-activated protein kinase (MAPK) cascades and WRKY transcription factors (Asai *et al.*, 2002), and inhibits the growth of subsequently inoculated *Pseudomonas syringae* (Zipfel *et al.*, 2004). Bacterial strains that have defects in the TTSS, such as an *hrcC* mutant (Yuan and He, 1996) of *P. syringae* pv. *tomato* DC3000 (*Pst*DC3000), induce responses in plants very similar to those triggered by MAMPs (Thilmony *et al.*, 2006; Truman *et al.*, 2006), because TTEs that could interfere with the MAMP-triggered response are not delivered into the plant cell.

SID2 encodes isochorismate synthase, and SA production is drastically reduced in *sid2* mutants, indicating that the vast majority of SA in Arabidopsis is produced from isochorismate, rather than through the phenylpropanoid pathway, in response to pathogen infection (Wildermuth *et al.*, 2001). *PAD4* and *EDS1* are required for activation of SA accumulation in response to some, but not all, SA-inducing stimuli (Falk *et al.*, 1999; Zhou *et al.*, 1998). *NPR1*, which acts downstream from SA, regulates defense responses, including the expression of several *WRKY* transcription factors through interaction with TGA transcription factors (Wang *et al.*, 2006).

Many genes are commonly induced or suppressed in plants after pathogen recognition via different mechanisms. In a model plant–pathogen system consisting of Arabidopsis and the bacterial pathogen *Pst*DC3000, gene expression changes induced by a virulent pathogen were qualitatively similar to those induced during an R gene-mediated resistance response (Tao *et al.*, 2003). Also, there is substantial overlap between gene expression changes induced by treatment with flg22 and those induced during an R gene-mediated defense response (Navarro *et al.*, 2004). Microarray data in public databases show that many genes are commonly induced or suppressed in plants treated with MAMPs or SA. For example, a flagellin-induced receptor kinase gene (*FRK1*) is induced after inoculation with *Pst*DC3000 *hrcC* (He *et al.*, 2006), and also after treatment with SA [benzothiadiazole (BTH)-treatment data; Integrated Microarray Database System (IMDS), http://ausubellab.mgh.harvard.edu/imds]. These results suggest that there is an interaction(s) between MAMP and SA signaling. However, detailed mechanisms underlying the interaction are unclear.

The goal of this study was to investigate interactions between MAMP-triggered and SA-mediated signaling mechanisms. We demonstrated that SA accumulates in a *SID2*-dependent manner after flg22 treatment. The elevated level of SA affects transcriptional regulation of SA-responsive genes. Analysis of responses using a custom DNA microarray (Sato *et al.*, 2007) revealed a group of genes that are induced by MAMPs in a *SID2*-dependent manner. Another group of MAMP-induced genes was *SID2* independent at early time points and *SID2* dependent at later time points. SA is important for MAMP-triggered resistance, as flg22 treatment failed to induce resistance against *Pst*DC3000 *hrcC* in *sid2* mutants. Thus, plants use multiple signaling mechanisms to achieve similar transcriptional responses elicited through different recognition mechanisms.

Results

MAMPs induce SA accumulation in a SID2*-dependent manner*

Many genes that show induced expression in response to MAMPs are also induced by SA, so we speculated that MAMPs might trigger SA accumulation. To test this idea, we monitored SA levels in wild-type Columbia (Col-0), *pad4-1* and *sid2-2* plants after treatment with MAMPs in the form of *Pst*DC3000 *hrcC* bacteria or flg22 peptide (Figure 1a). A suspension of *Pst*DC3000 *hrcC* bacteria in water, a solution of 10 μM flg22 peptide or water (mock) was infiltrated into leaves. In wild-type plants, SA levels in plants treated with *Pst*DC3000 *hrcC* or flg22 were significantly higher than in mock-treated plants at 6, 9 and 24 h post-infiltration (hpi; *q*-values < 0.05; Table S1). SA levels in treated plants ranged from two to 14 times the levels in mock-treated plants. SA levels in flg22-treated plants were significantly higher than in *Pst*DC3000 *hrcC*-treated plants (*q*-values < 0.01; Table S1). The highest SA levels were observed at 9 hpi for both *Pst*DC3000 *hrcC* and flg22. Accumulation of SA was almost completely abolished in *sid2-2* plants, and was impaired in *pad4-1* plants. This experiment shows that MAMPs induce

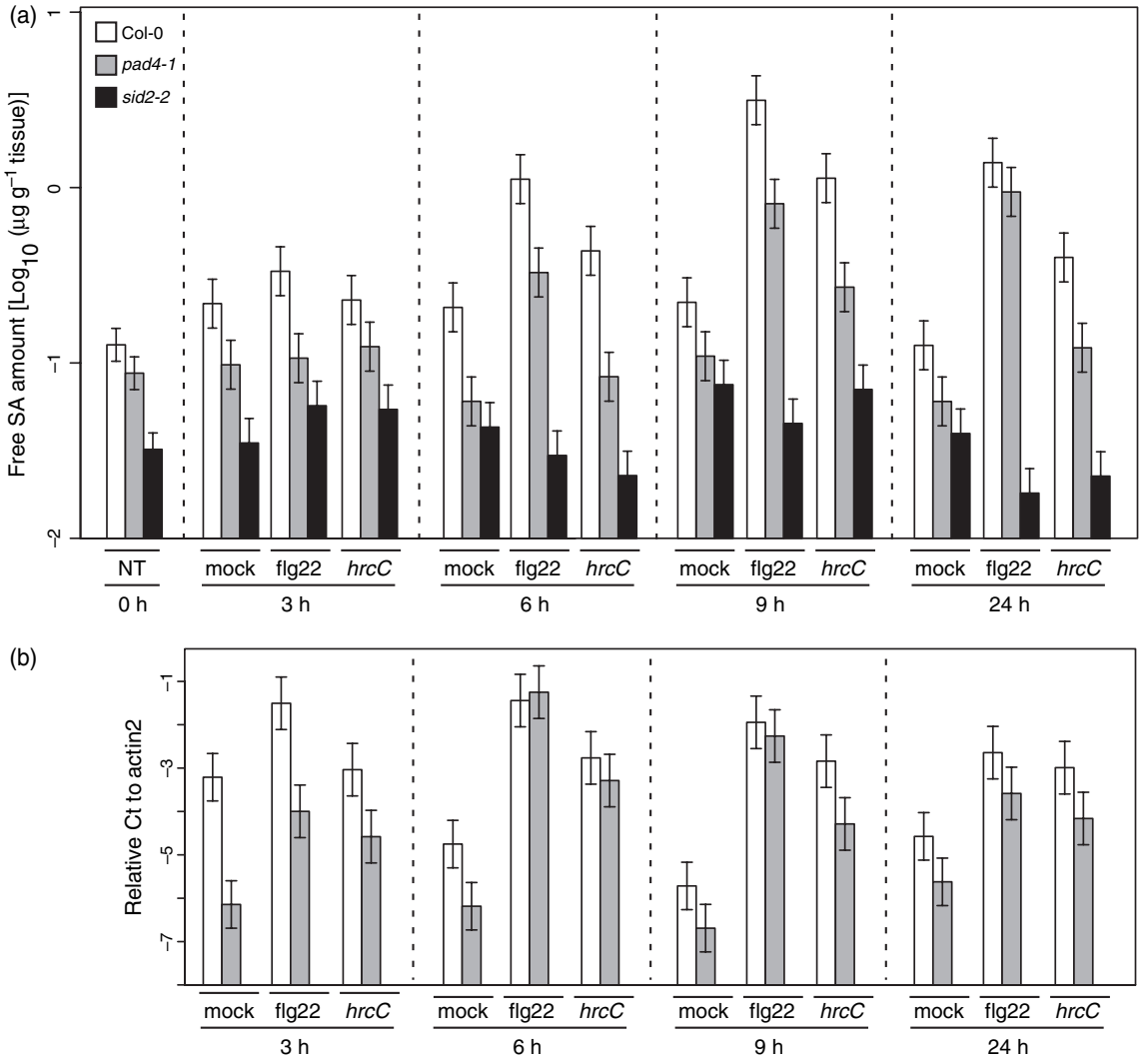


Figure 1. Salicylic acid (SA) accumulation and *SID2* expression induced by microbe-associated molecular patterns (MAMPs).
(a) SA accumulation. A *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) *hrcC* bacterial suspension (OD_{600} = 0.05; *hrcC*), water (mock) or 10 μM flg22 (flg22) was infiltrated into Col-0 (white bars), *pad4-1* (gray bars) or *sid2-2* (black bars) leaves. The free SA level in leaves was determined at the indicated time points. The SA level is shown on a $\log_{10}$ scale. Bars represent means and standard errors of two biological replicates calculated by ANOVA. For statistical analysis, see Table S1. (b) *SID2* mRNA levels. A *Pst*DC3000 *hrcC* bacterial suspension (OD_{600} = 0.05; *hrcC*), water (mock) or 10 μM flg22 (flg22) was infiltrated into Col-0 (white bars), *pad4-1* (gray bars) or *sid2-2* plants. *SID2* expression was analyzed by qRT-PCR. Bars represent means and standard errors of three replicates calculated by ANOVA. *SID2* mRNA was undetectable in *sid2-2* at all the time points. The vertical axis is the relative C_t, which is equivalent to the expression level on a $\log_2$ scale. For statistical analysis, see Table S5.

SA accumulation beginning between 3 and 6 hpi. MAMP-induced SA accumulation requires *SID2* and is partly dependent on *PAD4*.

The level of SID2 *mRNA is not always the limiting factor for flg22-induced SA accumulation*

We monitored *SID2* expression using qRT-PCR to test for a correlation between *SID2* mRNA and SA levels (Figure 1b). In Col-0, *SID2* expression was induced by treatment with either *Pst*DC3000 *hrcC* or flg22. *SID2* expression in *sid2-2* plants was undetectable, as expected because the *sid2-2* allele is a large deletion. The expression pattern of *SID2* was generally similar to the pattern of SA accumulation (Figure 1a,b). However, there is an important difference between them. Although *SID2* expression levels in *pad4-1* were comparable to wild type at 6 and 9 hpi with flg22, the SA levels in *pad4-1* were significantly lower than in wild type ($q < 0.001$; Table S1). This observation indicates that *SID2* induction triggered by flg22 is largely independent of *PAD4* by 6 hpi. It also suggests that something other than the *SID2* mRNA level is limiting for SA synthesis, and that *PAD4* affects this limiting factor.

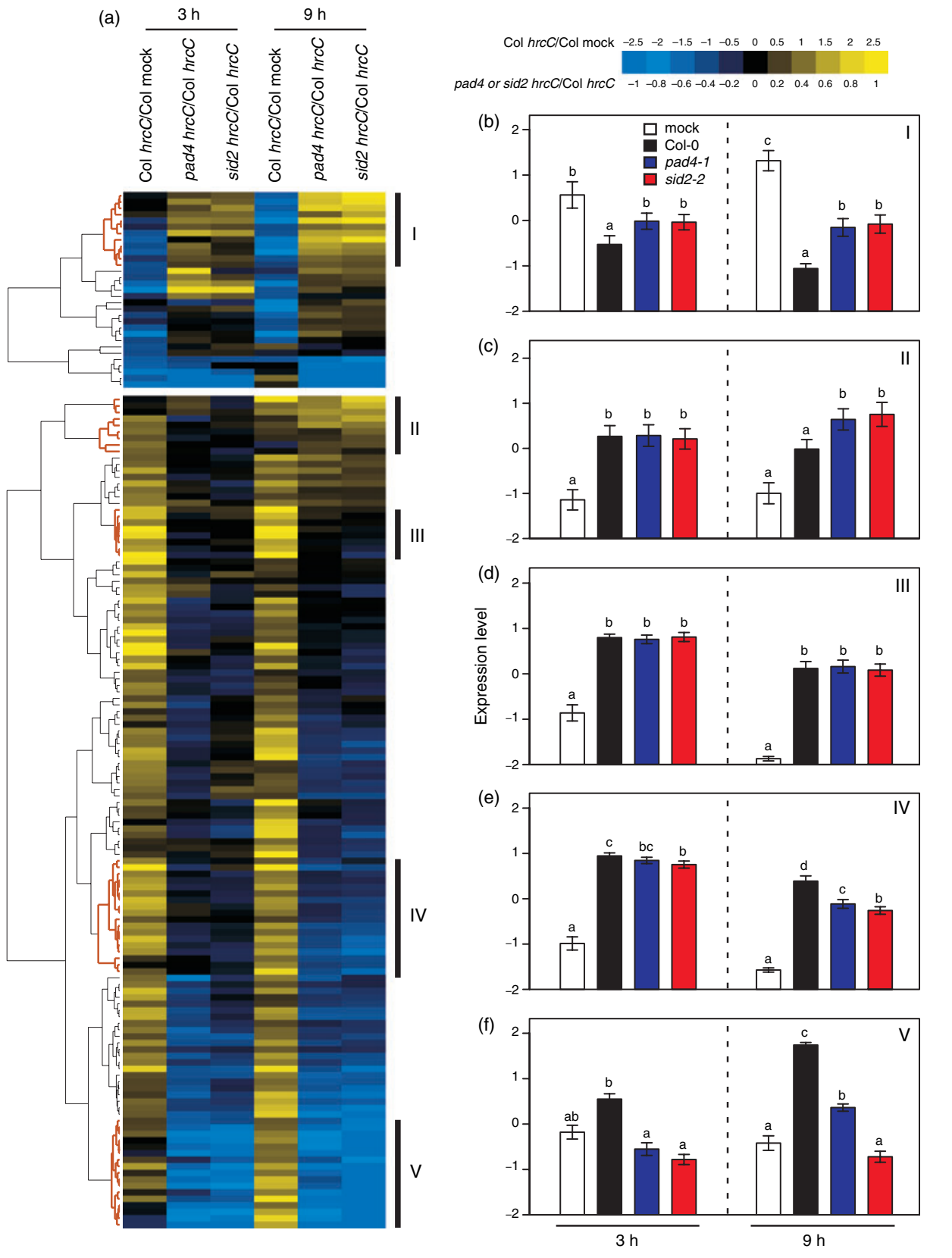
Identification of clusters of genes that share characteristic regulatory patterns

We recently developed a custom DNA microarray that was designed for study of Arabidopsis responses to pathogen infection. Expression levels measured using this microarray were shown to be accurate and reproducible (Sato *et al.*, 2007). We used the microarray to investigate how MAMP-triggered and SA-mediated signaling interact. We first conducted a preliminary microarray experiment, in which leaves of wild-type or *sid2-2* plants were infiltrated with *Pst*DC3000 *hrcC* (*hrcC*), and wild-type plants were also infiltrated with water (mock), in order to determine the most informative time points for further DNA microarray experiments. Samples were collected after 3, 6, 9 and 24 h and profiled using the microarray (Figure S1). Two time points (3 and 9 h) were chosen based on the following criteria: (i) the effect of *sid2-2* should be small at one time point and large at the other; (ii) expression patterns should be different in *hrcC* samples, but similar in water-treated samples.

Wild-type, *pad4-1* and *sid2-2* plants were infiltrated with *Pst*DC3000 *hrcC*, and samples were collected after 3 or 9 h. Water-infiltrated samples were collected from wild-type plants. An expression profile was obtained for each sample using the microarray. The entire experiment was repeated three times independently. Data were processed as described in Experimental procedures. Among the 571 genes monitored by the microarray, we found 272 genes that were differentially expressed (q-values < 0.05; Table S2) at 3 or 9 h in at least one of the comparisons between Col-0 *hrcC* and Col-0 mock, between *pad4-1 hrcC* and Col-0 *hrcC* or between *sid2-2 hrcC* and Col-0 *hrcC*. Of these 272 genes, genes that were induced (128 genes) or suppressed (31 genes) by more than twofold in Col-0, in response to DC3000 *hrcC*, were selected for further analysis. For these 159 genes, the $\log_2$ ratios of Col-0 *hrcC* to Col-0 mock (Col *hrcC*/Col mock), *pad4-1 hrcC* to Col-0 *hrcC* (*pad4 hrcC*/Col *hrcC*) and *sid2-2 hrcC* to Col-0 *hrcC* (*sid2 hrcC*/Col *hrcC*) at 3 and 9 h were subjected to complete-linkage agglomerative hierarchical clustering (Eisen *et al.*, 1998). The $\log_2$ ratios of Col *hrcC*/Col mock were weighted by a factor of 0.25 to reduce their effects on the clustering pattern. Clusters of interest are labeled as clusters I–V in Figure 2a. We defined these clusters in a restrictive manner to illustrate distinct patterns of gene expression. Many genes not assigned to a particular cluster have intermediate expression patterns. For example, many inducible genes located between clusters III and V in the heatmap show intermediate levels of dependence on *PAD4* and *SID2*. We did not assign these genes to one of the flanking clusters.

The expression patterns of the genes in each cluster are summarized in Figures 2b-2f. To create these summaries, the expression values ($\log_2$) of each gene across samples were transformed to adjust the mean to zero and the standard deviation to one. Then, the means and standard errors of the transformed expression values of all the genes in each cluster were calculated for each sample type. The resulting graphs show the mean expression patterns for the sets of genes in each cluster. The expression patterns of individual genes within each cluster may deviate somewhat from the mean expression pattern of the cluster. Significant differences were determined using a paired two-tailed Student's t-test. The resulting q-values are shown in Table S4. All of the effects described below were significant at q-values < 0.01. Genes in cluster I (12 genes) were repressed in response to *PstDC3000 hrcC* in a manner that was dependent on both *PAD4* and *SID2* (Figure 2b). This effect was particularly evident at 9 hpi. Clusters II–V contained genes that were induced in response to *PstDC3000 hrcC*. Expression levels of genes in cluster II (nine genes) were significantly higher in *pad4-1* and *sid2-2* compared with Col-0 at 9 hpi (Figure 2c), indicating that SA signaling suppresses expression of these genes. The induced expression of the genes in cluster III (eight genes) was not significantly affected by either *pad4-1* or *sid2-2* (Figure 2d), indicating that the genes in this cluster are regulated by a MAMP-triggered signaling mechanism that is independent of SA. The genes in cluster IV (18 genes) were induced by *PstDC3000 hrcC* at 3 and 9 hpi (Figure 2e). At 3 hpi expression was modestly reduced in *sid2-2* plants, but not significantly affected in *pad4-1* plants. At 9 hpi, expression levels were reduced in both *sid2-2* and *pad4-1* plants. The genes in cluster V (17 genes) were induced by *PstDC3000 hrcC* at 9 hpi, and expression was strongly dependent on *SID2* and *PAD4* at both time points (Figure 2f). The expression patterns of clusters I, IV and V indicate that MAMP-triggered SA accumulation is required for many MAMP-triggered responses. A requirement for SA in the expression of cluster V genes was observed at 3 hpi, even though no increase in SA was observed at 3 hpi with flg22 or *Pst*DC3000 *hrcC* (Figures 2f and 1a). However, *PAD4*- and *SID2*-dependent gene induction at 3 hpi could be explained by the lower

Figure 2. Expression patterns identified by agglomerative hierarchical clustering analysis.
(a) Hierarchical clustering. The $\log_2$ ratios of each indicated sample comparison were used for the analysis. Clustering was performed with CLUSTER (Eisen *et al.*, 1998) using uncentered Pearson correlation and complete linkage clustering, and was visualized with TREEVIEW (Eisen *et al.*, 1998). Blue indicates negative values, yellow indicates positive values and black indicates zero.
Expression values ($\log_2$) of genes: (b) cluster I, 12 genes; (c) cluster II, nine genes; (d) cluster III, eight genes; (e) cluster IV, 18 genes; (f) cluster V, 17 genes in each cluster were Z-transformed, and means and standard errors of each sample were calculated. According to two-tailed Student's t-tests with Benjamini and Hochberg false discovery rate (BH-FDR) multiple testing corrections (q-values < 0.01), expression values at each time point differ significantly if they are marked with different letters, such as a and b. A list of the genes in each cluster is presented in Table S3. Results of the statistical analysis are presented in Table S4.



basal SA levels in *pad4-1* and *sid2-2* plants (Figure 1a). For the genes in clusters I and IV, the effect of *sid2-2* is larger at 9 hpi than at 3 hpi (Figure 2b,e). This is likely to reflect SA accumulation after 3 hpi (Figure 1a).

Detailed expression patterns of particular genes

We performed more detailed expression analysis for particular genes using qRT-PCR. Leaves of 4-week-old Col-0, *pad4-1* or *sid2-2* plants were inoculated with *Pst* DC3000 *hrcC*, 10 μM flg22 or water (mock), and were harvested at 3, 6, 9 or 24 hpi. The experiments were conducted three times and the data were processed as described in Experimental procedures. A table showing *q*-values is provided in Table S5. These experiments were completely independent of the microarray experiments.

Photosystem I (PSI) type-II chlorophyll *a*/*b*-binding protein (At1g19150, cluster I) was suppressed by *hrcC* and flg22 treatments (Figure 3a). Its suppression was *SID2* dependent at 24 hpi with both flg22 and *hrcC* (*q*-values < 0.001; Table S5), and was *PAD4* dependent at 9 and 24 hpi with *hrcC* (*q*-values < 0.05; Table S5). *PDF1.2a* (At5g44420, cluster II) is known to be jasmonic acid (JA) and ethylene (ET) regulated (Thomma *et al.*, 1998). *PDF1.2a* expression was induced by *hrcC* at 6 and 9 hpi, but not by flg22. Although the expression level induced by *hrcC* returned to the basal level in Col-0 and *pad4-1* at 24 hpi, it remained higher in *sid2-2* compared with Col-0 (*q*-value < 0.05; Figure 3b). This result suggests that a factor(s) of *hrcC* other than flagellin contributes to the induction of *PDF1.2a*, and that SA signaling suppresses gene expression. The factor is likely to be coronatine as it is known that *Pst*DC3000 *hrcC* overproduces coronatine (Penaloza-Vazquez *et al.*, 2000), which mimics JA-Ile (Thines *et al.*, 2007), and SA and JA signaling negatively regulate each other (Glazebrook *et al.*, 2003; Kunkel and Brooks, 2002). Interestingly, the expression of *PDF1.2a* was significantly higher in *sid2-2* treated with flg22 at 24 hpi compared with Col-0 (*q*-value < 0.05; Figure 3b), suggesting that flg22 induces JA and/or ethylene signaling, and that the induction is normally suppressed in Col-0 by SA signaling. A putative *chitinase* (At2g43620, cluster III) was prominently induced by treatment with *hrcC* or flg22, and this was unaffected by *pad4-1* and *sid2-2* mutations at all time points (Figure 3c). This result suggests SA-independent MAMP-triggered defense signaling. *PENETRATION 1* (*PEN1*; At3g11820, cluster IV) expression was strongly induced after *hrcC* and flg22 treatment, and was neither *PAD4*- nor *SID2*-dependent at 3 or 6 hpi. The *hrcC* induction was *SID2* dependent at 9 (*q*-value < 0.05; Table S5) and 24 hpi (*q*-value < 0.001; Table S5), and was *pad4* dependent at 24 hpi (*q*-value < 0.05; Table S5; Figure 3d). This suggests that the SA increase triggered by MAMPs contributes to maintenance of *PEN1* expression. The difference between *sid2-2* and Col-0 was not significant after flg22 treatment (Figure 3d). *PR-1* (At2g14610, cluster V) was almost completely *SID2*- and partly *PAD4*-dependent in response to flg22 and *hrcC* infiltration at all time points (Figure 3e), and the expression pattern was highly correlated with the pattern of SA accumulation (Figure 1a). This strong dependence of *PR-1* expression on SA is consistent with previous observations (Delaney *et al.*, 1994).

Many of the genes that are induced by Pst*DC3000* hrcC *in a* SID2*-dependent manner are BTH inducible*

To determine whether SA is sufficient for induced expression of the genes induced by *hrcC* in a *SID2*-dependent manner, we investigated expression changes induced by treatment with BTH, an analog of SA. Expression data for BTH-treated plants was obtained from a public database of Affymetrix ATH1 array data (IMDS, http://ausubellab.mgh.harvard.edu/imds), and was analyzed to determine the genes that were induced by BTH 8 h after treatment (> twofold changes and *q*-values < 0.05). Among 93 genes that were identified as *Pst*DC3000 *hrcC* inducible at 9 h in the mini-array experiment (> twofold changes and *q*-values < 0.05): 27 genes, including *SID2* (At1g74710), *PR-1* (At2g14610) and *PEN1* (At3g11820), were *SID2* dependent and BTH inducible; eight genes were *SID2* dependent and not BTH inducible; 20 genes were not *SID2* dependent but were BTH inducible; 38 genes were neither *SID2* dependent nor BTH inducible (Table S6). These results indicate a significant correlation between genes that are *Pst*DC3000 *hrcC* inducible in a *SID2*-dependent manner and BTH-inducible genes ($P = 9.4 \times 10^{-5}$ by Fisher's exact test). These results strengthen the conclusion that SA accumulation triggered by MAMPs causes induction of a subset of MAMP-inducible genes.

SA-mediated signaling is necessary for flg22-triggered defense

To test for a role of SA signaling components in MAMP-triggered defense, we performed a bacterial growth assay. To induce MAMP-triggered defense, 5-week-old plants were infiltrated with 1 μM flg22. Control plants were treated with water. After 1 day, leaves were infiltrated with a suspension of *Pst*DC3000 *hrcC* bacteria ($OD_{600} = 0.001$). The bacterial titer was measured at 0, 2 and 4 days after inoculation (dpi). The experiment was conducted twice and analyzed as described in Experimental procedures. Comparison of bacterial growth between mock pre-treated and flg22 pre-treated wild-type plants at 2 and 4 dpi confirmed that the pre-treatment with flg22 confers resistance to the *hrcC* strain (Figure 4a and *q*-values < 1×10^{-8}; Table S7). Bacterial titers in mock pre-treated *pad4-1* and *sid2-2* plants were different from mock pre-treated Col-0 at 4 dpi, illustrating the enhanced susceptibility phenotypes of these mutants

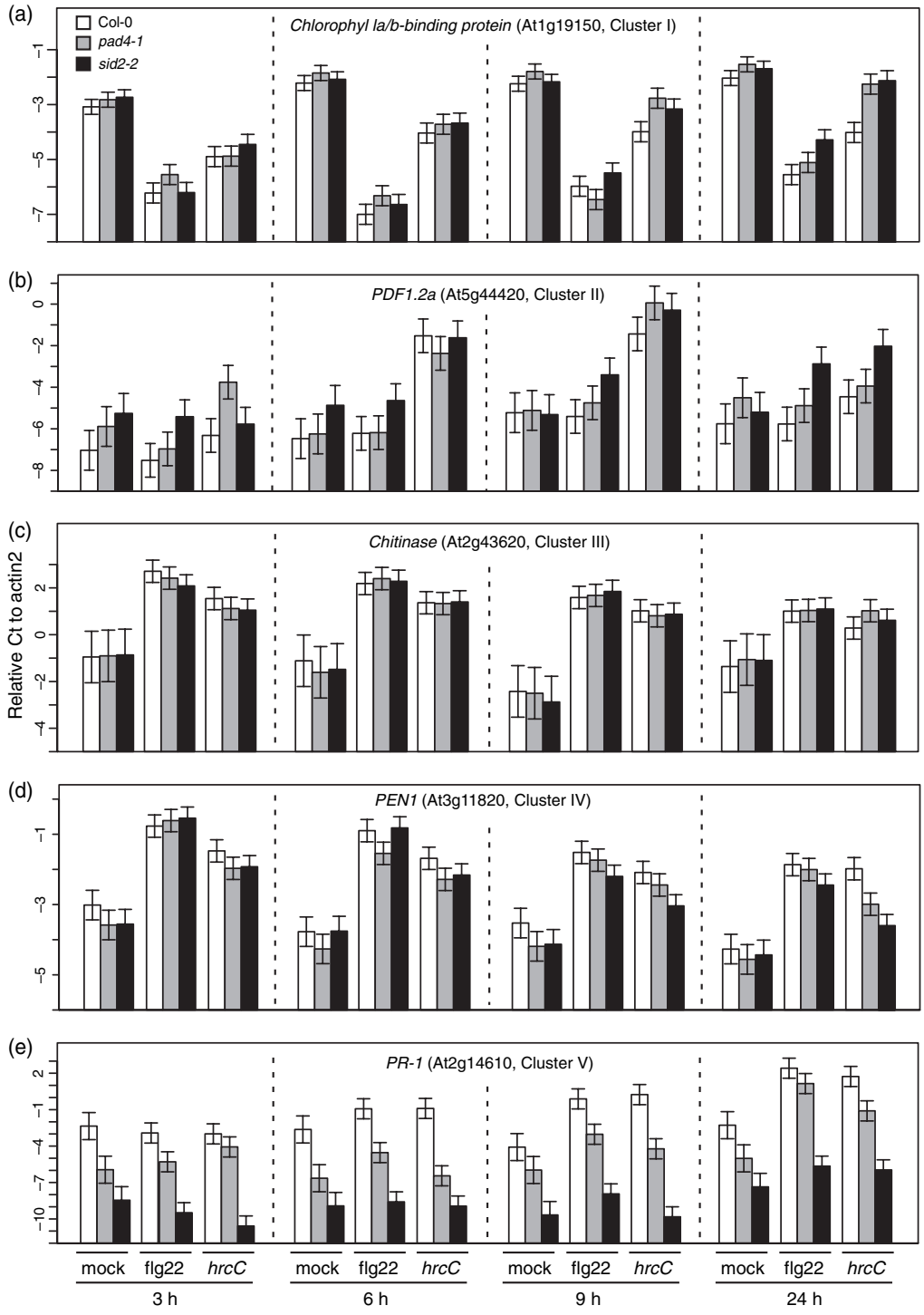


Figure 3. Expression patterns of five genes of interest.
A *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) *hrcC* bacterial suspension (OD_{600} = 0.05; *hrcC*), water (mock) or 10 μM flg22 (flg22) was infiltrated into Col-0 (white bars), *pad4-1* (gray bars) or *sid2-2* (black bars) leaves. Expression levels were measured by qRT-PCR.
(a) *PSI type II Chlorophyll a/b-binding protein* (At1g19150).
(b) *PDF1.2a* (At5g44420).
(c) *Chitinase* (At2g43620).
(d) *PEN1* (At3g11820).
(e) *PR-1* (At2g14610).
Bars represent means and standard errors of three replicates calculated by ANOVA. The vertical axis is the relative C_t (threshold cycle), which is equivalent to the expression level on a $\log_2$ scale. For statistical analysis, see Table S5.

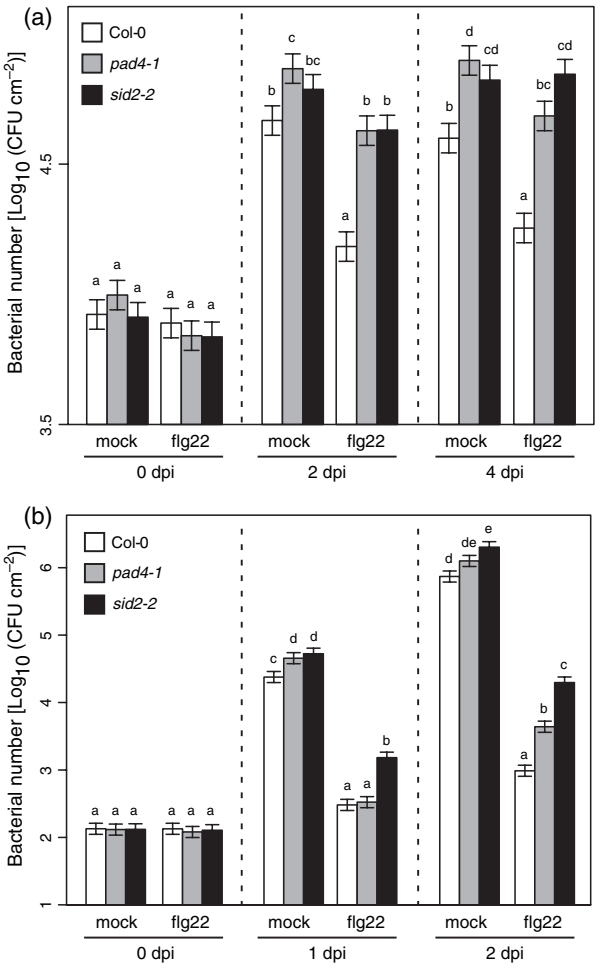


Figure 4. Salicylic acid (SA) signaling is required for flg22-triggered defense against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) *hrcC*.
A *Pst*DC3000 *hrcC* bacterial suspension of OD_{600} = 0.001 (a) and OD_{600} = 0.0001 (b) was infiltrated into Col-0, *pad4-1* or *sid2-2* plants 1 day after treatment with water (mock) or 1 μM flg22 (flg22). Bars represent means and the standard errors of two replicates determined by ANOVA. Within data for each time (dpi), significant differences are indicated by different letters, such as a and b (*q*-values < 0.005). For statistical analysis, see Table S7.

(Figure 4a and *q*-values < 0.0005; Table S7). Importantly, the bacterial titers in flg22 pre-treated *pad4-1* and *sid2-2* plants were clearly higher than flg22 pre-treated Col-0 at 2 and 4 dpi (Figure 4a and *q*-values < 1×10^{-12}; Table S7). This difference in bacterial growth is larger than the difference between mock pre-treated mutant and wild-type plants. In *sid2-2* plants, there was no significant difference between mock and flg22 pre-treated plants at 4 dpi, indicating that the effect of flg22 pre-treatment on resistance to the *hrcC* strain is almost entirely dependent on *SID2*. Thus, SA-mediated signaling is required for flg22-triggered resistance against *Pst*DC3000 *hrcC*.

We also performed a bacterial growth assay using *Pst*DC3000. One day after flg22 treatment, leaves were infiltrated with a suspension of *Pst*DC3000 bacteria (OD_{600} = 0.0001). The bacterial titer was measured at 0, 1 and 2 dpi. The experiment was conducted twice and analyzed as described in Experimental procedures. Comparison of bacterial growth between mock pre-treated and flg22 pre-treated wild-type plants at 1 and 2 dpi confirmed the previous observation (Zipfel *et al.*, 2004) that pre-treatment with flg22 confers resistance to *Pst*DC3000 (Figure 4b and *q*-values < 10^{-15}; Table S7). Bacterial titers in mock pre-treated *sid2-2* plants were slightly higher than mock pre-treated Col-0 at 1 and 2 dpi, illustrating the enhanced susceptibility phenotype of this mutant (Figure 4 and *q*-values < 0.00005; Table S7). Importantly, the bacterial titers in flg22 pre-treated *sid2-2* plants were significantly higher than flg22 pre-treated Col-0 at 1 and 2 dpi (Figure 4b and *q*-values < 10^{-12}; Table S7). However, in *sid2-2* plants there was still a large difference between mock and flg22 pre-treated plants at 1 and 2 dpi, indicating that the effects of flg22 pre-treatment on resistance to *Pst*DC3000 are only partially dependent on SA signaling.

Discussion

The perception of MAMPs by plants leads to the activation of many defense responses (Kim *et al.*, 2005; Navarro *et al.*, 2004; Nurnberger *et al.*, 2004). Recently, Mishina and Zeier (2007) showed that MAMP treatments, including flg22, caused local and systemic SA accumulation 2 days after treatment. However, their focus was on the activation of systemic acquired resistance, rather than on the interplay between MAMP-triggered and SA-mediated effects on gene expression changes and pathogen growth. We demonstrated that MAMPs induced SA accumulation within 6 h in a *SID2*-dependent manner. We performed expression profiling of Arabidopsis wild-type and SA-related mutants, and discovered that some MAMP-induced genes are SA independent, whereas other genes become SA dependent at later time points. These two mechanisms can explain why MAMP-triggered responses and SA-mediated responses are qualitatively highly overlapping. Using a bacterial growth assay, we showed that SA signaling is required for MAMP-triggered resistance to *Pst*DC3000 *hrcC* and contributes to MAMP-triggered resistance to *Pst*DC3000.

Zipfel *et al.* (2004)reported that resistance to *Pst*DC3000 triggered by flg22 pre-treatment did not require SA signaling, based on their observation that the *pad4-1* mutation and the *NahG* transgene did not affect resistance. We observed a small but significant effect of *pad4-1* (Figure 4b). We speculate that the reason we detected the *pad4-1* effect is because of a difference in statistical power, as we employed an experimental design that allows us to fit a linear model to the data (Experimental procedures). We also observed a clear effect of *sid2-2* on resistance, which was larger than the *pad4-1* effect (Figure 4b). It is not surprising that *sid2-2* and

the *NahG* transgene had different effects on the resistance phenotype. The effect of *NahG* on expression profiles after challenge with *P. syringae* pv. *maculicola* ES4326 is very similar to that of *pad4-1*, but very different from those of *sid2-2*, *eds5-1* and *eds5-3* (Glazebrook *et al.*, 2003). NahG also affects non-host resistance to *P. syringae* pv. *phaseolicola* NPS3121, whereas *sid2-2* does not (Van Wees and Glazebrook, 2003). As *SID2* has been well established as an essential biosynthetic gene for pathogen-induced SA synthesis (Wildermuth *et al.*, 2001) and the overall effects of *NahG* are not clear, we conclude that resistance to *Pst*DC3000 triggered by flg22 pre-treatment is partially, but significantly, dependent on SA signaling.

It is intriguing that the resistance to *Pst*DC3000 *hrcC* triggered by flg22 pre-treatment was almost entirely dependent on SA signaling, whereas the resistance to *Pst*DC3000 was comprised of SA-dependent and larger SA-independent components (Figure 4). The main difference between *Pst*DC3000 and *Pst*DC3000 *hrcC* is that *Pst*DC3000 can deliver TTEs into plant cells, but *Pst*DC3000 *hrcC* cannot. This should explain the large difference in the capacity of these strains to grow in untreated plants. The fact that *Pst*DC3000 *hrcC* can only grow poorly in untreated plants suggests that there is another layer of defense beyond the SA-dependent mechanism that can be observed with *Pst*DC3000 *hrcC* in flg22 pre-treated plants. It is conceivable that this putative second layer of defense can be efficiently negated by TTEs delivered by *Pst*DC3000 in untreated plants, but can be effective against *Pst*DC3000 when triggered in advance by flg22 pre-treatment. If this is the case, the observations in Figure 4 can be explained by assuming that SA signaling is mostly unnecessary for the second layer of defense.

A model for the involvement of SA signaling in MAMP-triggered responses is presented in Figure 5. Induction of the cluster III genes was not affected by the *sid2-2* or *pad4-1* mutation (Figures 2d and 3c), so they are placed under the SA-independent signaling pathway. As induction of the cluster-V genes was totally dependent on *SID2* at all times (Figures 2f and 3e), they are placed under the SA-dependent signaling pathway. Induction of the cluster-IV genes, including *PEN1*, was mostly independent of *SID2* at early time points, but was clearly dependent on *SID2* at later time points when SA was present at a high level (Figures 1a, 2e and 3d). Repression of the cluster I genes was weakly dependent on *SID2* at early time points and more dependent on *SID2* at later time points, so the cluster I genes appear to have a similar regulatory pattern to the cluster IV genes. This pattern of gene expression regulation, which is SA-independent early and SA-dependent late, suggests that the perception of MAMPs by plants leads to the induction of early responsive genes through an SA-independent signaling mechanism and initiates SA accumulation. When SA has accumulated to a sufficiently high level, expression of some of the early responsive genes is kept high by an SA-dependent signaling mechanism. This suggests that plants exploit SA-mediated signaling to maintain MAMP-triggered defense expression.

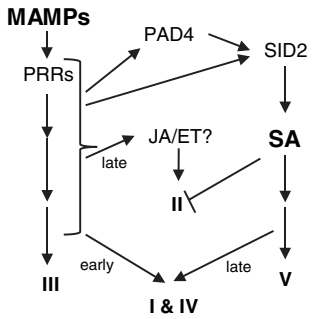


Figure 5. A proposed model for the contribution of salicylic acid (SA) in microbe-associated molecular pattern (MAMP)-triggered defense.
MAMPs are recognized by pattern recognition receptors (PRRs), which induce the expression of the genes in cluster III and the early expression of the genes in cluster IV, and reduce expression of the genes in cluster I, mainly in an SA-independent manner. Recognition also induces SA accumulation via *SID2*. SA accumulation affects expression of the genes in cluster V and late expression of the genes in clusters I and IV. *PAD4* affects *SID2*-dependent SA accumulation, although *PAD4* is not necessary for induction of *SID2* mRNA. MAMPs also induce JA/ET-regulated genes in cluster II that are normally suppressed by SA signaling.

MAMP-triggered induction of the cluster II genes, including a common JA/ET-marker gene *PDF1.2a*, in *sid2-2* plants (Figure 3b), suggests that JA and/or ET signaling mechanisms are also part of the MAMP-triggered defense response (Figure 5). However, both JA- and ET-deficient mutants exhibit intact flg22-induced resistance (Zipfel *et al.*, 2004). JA and ET signaling may not be important for resistance against *Pst*DC3000. Alternatively, they may play redundant roles. Combining mutations for multiple known signaling mechanisms could reveal such redundant regulatory mechanisms.

As *sid2-2* totally abolished MAMP-triggered SA accumulation (Figure 1a), *SID2* is absolutely required for MAMP-triggered SA accumulation. On the other hand, *pad4-1* only partially blocked SA accumulation (Figure 1a). Therefore, we speculate that there are two regulatory pathways from MAMP signaling to *SID2*, one via *PAD4* and one bypassing *PAD4*. However, there might be differences in how these pathways regulate *SID2*-dependent SA accumulation. Induction of *SID2* mRNA was largely independent of *PAD4* (Figure 1b), so the pathway bypassing *PAD4* appears to play a major role in the induction of *SID2* mRNA. It seems likely that the pathway via *PAD4* affects SA levels by regulation of something other than the *SID2* mRNA level (e.g. substrate concentration or modification of SID2). We cannot test this possibility because we do not presently have a way to block the *PAD4*-bypassing pathway.

The fact that MAMP-triggered signaling autonomously turns on SA-mediated signaling raises questions about the evolution of defense-signaling mechanisms. The SA-mediated signaling mechanism may have evolved as a part of MAMP-triggered defense. R gene-mediated defense may have evolved subsequently, resulting in efficient activation of pre-existing defense-signaling mechanisms, including SA-mediated signaling. Although it is conceivable for pathogen virulence factors to target particular plant defense effectors, based on present knowledge, targeting of plant defense regulatory mechanisms seems to be the rule. This is probably because the overall plant defense against a particular pathogen is typically composed of multiple, quantitative defense effectors: nullifying just a single plant defense effector may not contribute much to the virulence of the pathogen. It is more effective if a pathogen virulence factor targets a plant defense regulatory mechanism that controls multiple plant defense effectors, so that multiple defense effectors get negated at once. A drawback of this scenario for the pathogen is that it leaves the plant defense effectors potentially effective against the pathogen, if they are activated. Evolution of a better regulatory mechanism would restore plant resistance without acquisition of new defense effectors. Thus, although exceptions exist, a major part of plant defense evolution from the MAMP-triggered defense to the R gene-mediated defense might be evolution of regulatory mechanisms, whereas the sets of defense effectors used in both defenses might have remained relatively similar.

Experimental procedures

Plant growth, bacterial inoculation and flg22 treatment

Wild-type (WT) Col-0, *pad4-1* (At3g52430; Zhou *et al.*, 1998; Jirage *et al.*, 1999) and *sid2-2* (At1g74710; Wildermuth *et al.*, 2001) Arabidopsis plants were grown on soil in a growth chamber at 22°C with a 12-h photoperiod and 75% relative humidity for 4–5 weeks before bacterial inoculation. *Pst*DC3000 and *Pst*DC3000 *hrcC* were grown overnight at room temperature (~25°C) in King's B medium with 25 $\mu g\ \mu l^{-1}$ of rifampicin. Bacteria were harvested by centrifugation, washed and diluted to the desired density with water. Arabidopsis leaves were infiltrated using a needleless syringe (Katagiri *et al.*, 2002). Flg22 peptide was purchased from EZBiolab Inc. (http://www.ezbiolab.com). Either 1 or 10 μM flg22 was infiltrated in the same way.

Custom microarray analysis

Four-week-old Arabidopsis leaves were infiltrated with *Pst*DC3000 *hrcC* (OD_{600} = 0.05; 5×10^7 CFU ml^{-1}) or water. Samples were collected at 3 and 9 hpi, frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted and profiled using the custom DNA microarray described by Sato *et al.* (2007). Three independent experiments (replicates) were performed. All the microarray data obtained in this study were submitted to Gene Expression Omnibus. All the microarray data were normalized together by SBQ (Sato *et al.*, 2007). Results were analyzed using a two-staged mixed ANOVA model (Appendix S1). The obtained estimated means and standard errors were used in a two-tailed Student's *t*-test, and the *q*-values were calculated using the Benjamini and Hochberg false discovery rate (BH-FDR; Benjamini and Hochberg, 1995) from the *P*-values obtained in the *t*-test. Genes with expression values below that of a negative control probe (spiking control 5; Sato *et al.*, 2007) in each sample were ignored, because this indicates that the expression value measurement is at the limit of sensitivity. The remaining genes were subjected to further analysis (361 genes). Table S2 shows the entire data set, including expression values, *P*-values and *q*-values.

Affymetrix ATH1 microaray data analysis

Salicylic acid (SA)-responsive genes were identified using the BTH time course data from the IMDS (http://ausubellab.mgh.harvard.edu/imds). The CEL files were pre-processed using RMAEXPRESS with quantile normalization (Irizarry *et al.*, 2003). The data from the BTH treatment (8 h) and untreated control (0 h) were analyzed using Significance Analysis of Microarrays (Tusher *et al.*, 2001; two class-paired). The genes that showed expression levels significantly higher or lower at 8 h than those at 0 h (fold changes > 2, *q*-values < 0.05) were identified.

Quantitative RT-PCR analysis

*Pst*DC3000 *hrcC* bacterial suspension (OD_{600} = 0.05; 5×10^7 CFU ml^{-1}; *hrcC*), water (mock) or 10 μM flg22 (flg22) were infiltrated into 4-week-old Col-0, *pad4-1* or *sid2-2* plants. Four leaves from two plants per sample were collected 3, 6, 9 and 24 hpi, frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from leaves using TRIzol reagent (Invitrogen, http://www.invitrogen.com). Real-time PCR analysis was carried out using an ABI7500 Real Time PCR system (Applied Biosystems, http://www.appliedbiosystems.com) and the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen). Total (60 ng) RNA was used for each reaction. The thermal cycling program was 50°C for 10 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. *Actin2* (At3g18780) was used as the internal reference. The primer sequences are shown in Table S8. Three independent experiments (biological replicates) were performed. Data was analyzed using the ANOVA models (Appendix S1). The relative C_t (threshold cycle) values were compared for each gene at each time point using two-tailed Student's *t*-tests and BH-FDR. Table S5 shows all the data with relative C_t, *P*-values and *q*-values.

Isolation and quantification of endogenous SA

A *Pst*DC3000 *hrcC* bacterial suspension (OD_{600} = 0.05; 5×10^7 CFU ml^{-1}; *hrcC*), water (mock) or 10 μM flg22 (flg22) were infiltrated into 4-week-old Col-0, *pad4-1* or *sid2-2* leaves. Twelve leaves from three plants per sample were collected 0 (untreated), 3, 6, 9 and 24 hpi, frozen in liquid nitrogen and stored at −80°C.

Salicylic acid was extracted and analyzed by GC-MS with a modification of the methods described by Dewdney *et al.* (2000) and Ribnicky *et al.* (1998). Tissues (200 mg) were ground in liquid nitrogen to a fine powder using a pestle and mortar and extracted with 1 ml of 90% methanol. 2-Hydroxybenzoic-3,4,5,6-d4 acid (SA-d4; CDN Isotopes Inc., http://www.cdnisotopes.com) was dissolved in methanol and then diluted with water to the

final concentration of 3% methanol, and 2 μg of it was added to each sample as an internal standard. Samples were vortexed vigorously, incubated at 4°C for 1 h and then centrifuged at 10 000 *g* at room temperature (RT) for 5 min. The supernatant was transferred to a new tube, and the pellet was re-extracted with 1 ml of 100% methanol. The supernatant was transferred to a new tube, and the pellet was re-extracted with 0.5 ml of 100% methanol. Half of the combined supernatant was vacuum-dried in a centrifugal concentrator. The dried samples were resuspended in 500 μl of 5% trichloroacetic acid, vortexed vigorously and centrifuged at 10 000 *g* at RT for 5 min. The supernatant was extracted three times with 700, 500 and 400 μl of a 100:99:1 (v/v) mixture of ethyl acetate:cyclopentane:isopropanol. The organic phases were combined and vacuum-dried in a centrifugal concentrator. The dried samples were resuspended in 1 ml of 1% acetic acid and vortexed. The samples were then applied to a conditioned (rinsed with 2 ml each of methanol, water and 0.5% acetic acid) 1-ml C18 SPE column (Fisher Scientific, http://www.fisher.co.uk), rinsed with 2 ml of water, and eluted with 1 ml of acetonitrile. The samples were vacuum dried, resuspended in 100 μl of methanol, vortexed vigorously and centrifuged at 13 000 *g* at RT for 5 min. The supernatant was transferred into a glass vial, methylated with ethereal diazomethane, as described by Cohen (1984), and brought to near-dryness using a stream of nitrogen gas. The samples were resuspended in 50 μl of ethyl acetate and analyzed by GC-MS (HP5890/HP5970; Agilent, http://www. agilent.com) as described by Ribnicky *et al.* (1998). The monitored ions for SA (native, enriched and internal standard) were (*m*/*z*) 120, 124, 152 and 156. Full scans of each compound were also performed to provide precise chemical identity.

The quantity of SA was calculated based on the ratio of the major fragment ion (*m*/*z* 120) of the methyl ester of native SA and the corresponding fragment ion (*m*/*z* 124) of the ^{2}H-labeled internal standard in a method similar to that described by Cohen *et al.* (1986) for indole-3-acetic acid (IAA).

The experiment was repeated twice and the data from 0 to 3–24 h were analyzed separately using an ANOVA model (Appendix S1). The $\log_{10}$-transformed SA values, as this transformation made the data variance homogenous, were compared at each time point using two-tailed Student's *t*-tests and BH-FDR. Table S1 shows all the data with SA quantities, *P*-values and *q*-values.

Bacterial growth assay

*Pst*DC3000 (OD_{600} = 0.0001, 1×10^5 CFU ml^{-1}) and *Pst*DC3000 *hrcC* bacterial suspensions (OD_{600} = 0.001, 1×10^6 CFU ml^{-1}) were infiltrated into 5-week-old plants 1 day after treatment with water or 1 μM flg22. Each sample consisted of two leaf discs (total surface 0.57 cm^2) taken from a single leaf. Leaf discs were pulverized in 400 μl of 5 mM $MgSO_4$ and dilution series were made. Of each dilution, 10 μl was streaked on King's B plates containing 25 μg ml^{-1} of rifampicin. The leaf bacterial titer was measured at 0, 1 and 2 dpi for *Pst*DC3000 or at 0, 2 and 4 dpi for *hrcC*. From this data, the $\log_{10}$-transformed colony-forming units (CFU) per cm^2 leaf surface area was calculated. Two independent experiments (biological replicates) were performed. Each genotype:treatment:time:replicate interaction level contained 16 measurements. Results were analyzed using an ANOVA model (Appendix S1). The $\log_{10}$-transformed bacterial titer values were compared at each time point using two-tailed Student's *t*-tests and BH-FDR. Table S7 shows all the data with CFU-values, *P*-values and *q*-values.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Pairwise comparisons of preliminary microarray data.
Table S1. Statistical analysis for Figure 1a.
Table S2. Complete microarray data.
Table S3. Genes represented in clusters.
Table S4. Statistical analysis for Figure 2b–f.
Table S5. Statistical analysis for Figures 1b and 3.
Table S6. Comparison between *hrcC* and benzothiadiazole (BTH) inducible genes.
Table S7. Statistical analysis for Figure 4.
Table S8. Primers used for qRT-PCR.
Appendix S1. Linear models used in the study.

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