The Arabidopsis Flavin-Dependent Monooxygenase FMO1 Is an Essential Component of Biologically Induced Systemic Acquired Resistance[1][OA]

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Upon localized attack by necrotizing pathogens, plants gradually develop increased resistance against subsequent infections at the whole-plant level, a phenomenon known as systemic acquired resistance (SAR). To identify genes involved in the establishment of SAR, we pursued a strategy that combined gene expression information from microarray data with pathological characterization of selected Arabidopsis (*Arabidopsis thaliana*) T-DNA insertion lines. A gene that is up-regulated in Arabidopsis leaves inoculated with avirulent or virulent strains of the bacterial pathogen *Pseudomonas syringae* pv *maculicola* (*Psm*) showed homology to flavin-dependent monooxygenases (FMO) and was designated as *FMO1*. An Arabidopsis knockout line of *FMO1* proved to be fully impaired in the establishment of SAR triggered by avirulent (*Psm avrRpm1*) or virulent (*Psm*) bacteria. Loss of SAR in the *fmo1* mutants was accompanied by the inability to initiate systemic accumulation of salicylic acid (SA) and systemic expression of diverse defense-related genes. In contrast, responses at the site of pathogen attack, including increases in the levels of the defense signals SA and jasmonic acid, camalexin accumulation, and expression of various defense genes, were induced in a similar manner in both *fmo1* mutant and wild-type plants. Consistently, the *fmo1* mutation did not significantly affect local disease resistance toward virulent or avirulent bacteria in naive plants. Induction of *FMO1* expression at the site of pathogen inoculation is independent of SA signaling, but attenuated in the Arabidopsis *eds1* and *pad4* defense mutants. Importantly, *FMO1* expression is also systemically induced upon localized *P. syringae* infection. This systemic up-regulation is missing in the SAR-defective SA pathway mutants *sid2* and *npr1*, as well as in the defense mutant *ndr1*, indicating a close correlation between systemic *FMO1* expression and SAR establishment. Our findings suggest that the presence of the FMO1 gene product in systemic tissue is critical for the development of SAR, possibly by synthesis of a metabolite required for the transduction or amplification of a signal during the early phases of SAR establishment in systemic leaves.

Plants generally possess multiple layers of defense to restrict the growth of potentially pathogenic microorganisms. Preformed mechanical or chemical barriers constitute an effective first line of defense against nonadapted or nonhost pathogens (Thordal-Christensen, 2003). Host pathogens that are able to overcome this first barrier provoke a whole set of inducible reactions. In specific or gene-for-gene resistance, plants rely on the presence of resistance gene products, which recognize matching avirulence factors from the pathogen to induce a multitude of protective responses (Dangl and Jones, 2001). Avirulent pathogens thus trigger rapid production of reactive oxygen species (ROS), accumulation of the defense signals salicylic acid (SA) and/or jasmonic acid (JA), increased expression of various defense-related genes, production of phytoalexins, and hypersensitive death of challenged cells (Kuć, 1995; Lamb and Dixon, 1997). Some of these responses also occur, albeit delayed, after infection with virulent pathogens, which manage to escape resistance protein recognition. Induced defenses thus limit the extent of pathogen spread not only in incompatible interactions to ensure specific resistance, but also in compatible interactions to centrally contribute to basal resistance (Parker et al., 1996).

Plant defense responses are initiated not only locally at the site of pathogen attack, but also in tissue distant from the site of infection (Cameron et al., 1994). These systemic resistance responses are generally subdivided into two broad categories, systemic acquired resistance (SAR) and induced systemic resistance. SAR develops in response to a pathogen that causes a necrotic lesion either as a consequence of a hypersensitive response (HR) or as a result of disease symptom development in the course of a compatible interaction (Hammerschmidt, 1999). Plants exhibiting SAR are generally resistant to a broad range of different pathogens. Establishment of SAR is dependent on the SA pathway and associated with both systemic increase of SA levels and systemic expression of pathogenesis-related (PR) genes (Ryals et al., 1996). By contrast, induced systemic resistance, a response to colonization of plant roots by certain rhizosphere bacteria, is dependent on JA and ethylene signaling (Pieterse et al., 2002).

The molecular mechanisms underlying SAR are under intensive study. The capability of plants to accumulate SA is known to be indispensable for SAR, as Arabidopsis (*Arabidopsis thaliana*) SA biosynthesis mutants

[1] This work was supported by the Deutsche Forschungsgemeinschaft (SFB 567).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.081257.

SA induction deficient 1 and 2 (*sid1* and *sid2*) and transgenic plants expressing the SA-degrading enzyme NahG are SAR defective (Gaffney et al., 1993; Nawrath and Métraux, 1999; Wildermuth et al., 2001; Nawrath et al., 2002). SA activates the SAR regulatory protein nonexpressor of PR genes (NPR1) through redox changes, which in turn drives systemic expression of antimicrobial PR proteins and facilitates their secretion by up-regulating protein secretory pathway genes (Mou et al., 2003; Wang et al., 2005). Several recent studies indicate that components of entirely distinct biochemical origin are necessary to realize SAR. Lipid metabolism turned out to play a central role in SAR signaling, as SAR is specifically compromised in Arabidopsis *defective in induced resistance 1* (*dir1*) and *suppressor of fatty acid desaturase deficiency 1* (*sfd1*), which bear mutations in a lipid transfer protein and a dihydroxyacetone phosphate reductase, respectively (Maldonado et al., 2002; Nandi et al., 2004). In addition, a peptide signal system mediated by the Asp protease constitutive disease resistance 1 (CDR1) appears to be essential for SAR long-distance signaling in Arabidopsis (Xia et al., 2004), and thiamine (vitamin B1) is capable of inducing SAR in a SA-dependent manner (Ahn et al., 2005). Moreover, ROS mediate a systemic signaling network that contributes to SAR (Alvarez et al., 1998). The complexity of systemic resistance regulation is further reflected by the fact that SAR establishment is subject to environmental and developmental plasticity. For instance, initiation of SAR has been demonstrated to occur in a light-dependent manner and the mechanisms of realizing SAR differ under variable light regimes (Zeier et al., 2004). Furthermore, leaf age influences the capability of initiating and executing SAR (Zeier, 2005).

Molecular events triggered by the primary infection process play a key role in SAR initiation. To identify uncharacterized genes involved in SAR establishment, we have selected Arabidopsis candidate genes up-regulated by SAR-inducing pathogens at the inoculation site, as indicated in microarray experiments publicly available from the Nottingham Arabidopsis Stock Centre (NASC) and from The Arabidopsis Information Resource (TAIR). T-DNA knockout lines corresponding to candidate genes were subsequently checked for an impaired SAR phenotype. This strategy revealed that the Arabidopsis *flavin-dependent monooxygenase 1* (*FMO1*) gene is essential for the establishment of SAR and systemic defense responses provoked both by an avirulent (*Psm avrRpm1*) and a virulent (*Psm*) strain of *Pseudomonas syringae*. By contrast, *FMO1* did not critically influence defense responses at the site of pathogen attack during these interactions.

RESULTS

FMO1 Is Expressed in Response to Virulent and Avirulent *P. syringae*

Gene expression profiling from two independent microarray datasets indicated that expression of the Arabidopsis *FMO1* gene (At1g19250) is increased 12 h post leaf infection of Arabidopsis ecotype Columbia (Col-0) with virulent *P. syringae* pv *tomato* DC3000 (*Pst*; Fig. 1, A and B). Inoculation with the isogenic avirulent *Pst avrRpm1* strain, which is recognized by Col-0

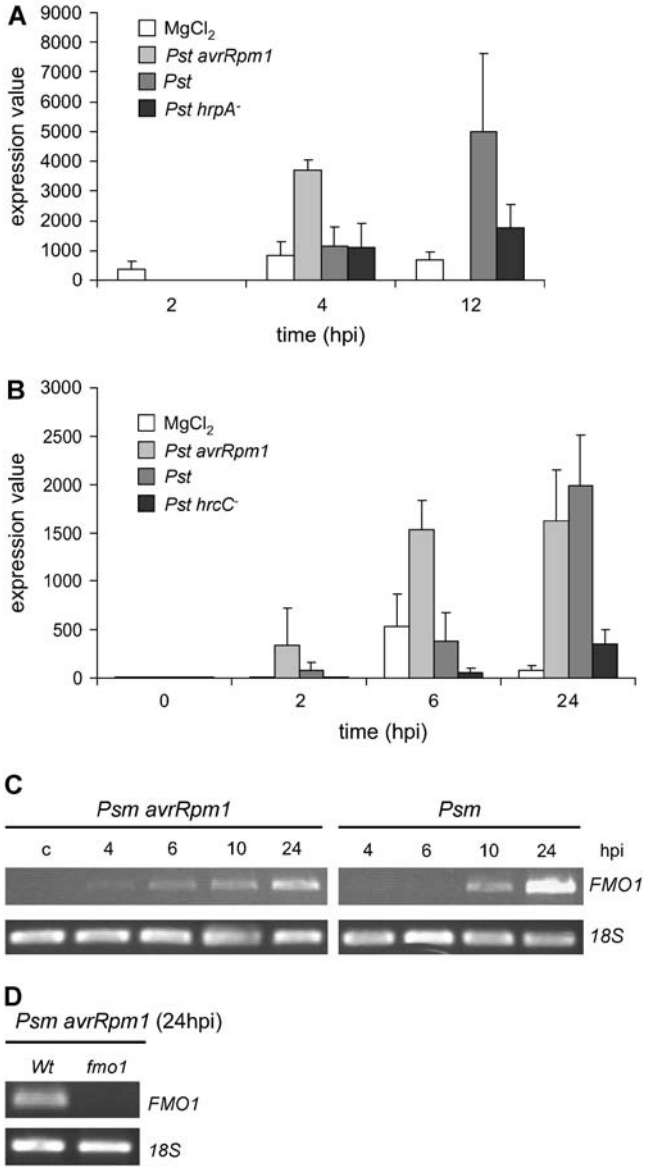

Figure 1. A and B, Expression levels of *FMO1* (At1g19250) in Arabidopsis leaves challenged with *Pst* according to microarray analyses. Means (±SD) of Affymetrix expression values originating from three independent replicates are given. The data were normalized according to the Affymetrix MAS 5.0 scaling protocol. A, Expression data from the NASC array *NASCARRAYS-59*: impact of type III effectors on plant defense responses. B, Expression data from TAIR (*TAIR-ME00331*: response to virulent, avirulent, type III secretion system-deficient and nonhost bacteria). C, RT-PCR analysis of *FMO1* expression triggered by *Psm* (virulent strain) and *Psm avrRpm1* (avirulent strain). Numbers indicate hpi. Control leaves (c) were infiltrated with 10 mM $MgCl_2$ for 24 h. 18S rRNA was amplified to standardize the transcript levels of each sample. D, Expression of *FMO1* in leaves of wild-type and *fmo1* mutant plants (T-DNA insertion line SALK_026163) 24 h after inoculation with *Psm avrRpm1*.

through the RPM1 resistance protein and consequently elicits an HR (Bisgrove et al., 1994), leads to earlier induction of *FMO1* expression, starting from about 4 h postinfection (hpi; Fig. 1, A and B). Upregulation of *FMO1* by *Pst* is largely dependent on a functional bacterial type III secretion system because the type III secretion-defective *Pst hrpA*$^-$ or *Pst hrcC*$^-$ strains only weakly induce its expression (Fig. 1, A and B). To experimentally verify the microarray data, we inoculated Col-0 with *Psm* ES4326, another virulent pathogen that induces similar defense responses as *Pst* (Dong et al., 1991). Reverse transcription (RT)-PCR analysis revealed that *FMO1* transcripts started to increase at about 6 hpi with avirulent *Psm avrRpm1* and at 10 hpi with virulent *Psm*, confirming that *FMO1* expression is triggered by host bacteria and that avirulent strains provoke an earlier transcription when compared with virulent strains (Fig. 1C).

Experiments investigating the kinetics of SAR establishment in the Arabidopsis-*Psm* interaction indicated that pathogen-treated primary leaves start to initiate SAR at least 1 d postinoculation. Moreover, the avirulent strain activated SAR faster than the virulent strain (data not shown). This tendency correlated with the expression pattern of *FMO1* in inoculated leaves (Fig. 1) and we thus postulated that FMO1 might play a role during SAR induction in primary leaves. A SALK insertion line (SALK_026163) harboring a T-DNA insertion in exon 4 of the *FMO1* gene in the Col-0 background was obtained from the NASC to examine whether FMO1 contributes to SAR establishment. In contrast to Col-0 plants, *fmo1* mutant plants failed to express *FMO1* after inoculation with *Psm* (Fig. 1D), demonstrating the knockout of *FMO1*.

SAR Is Compromised in *fmo1* Mutants

To investigate the biological induction of SAR, leaves of a given plant were treated with *Psm avrRpm1* or *Psm* in a primary inoculation (designated as primary leaves) and 2 d later a secondary or challenge infection with virulent *Psm* was performed in rosette leaves located just above the primary leaves (systemic leaves). Bacterial growth was scored in systemic leaves 3 d later. After treatment of primary leaves with a control solution of $MgCl_2$, growth of *Psm* during the challenge infection was vigorous in both wild-type and *fmo1* mutant plants (Fig. 2A). When primary leaves of wild-type plants were preinoculated with *Psm avrRpm1* or *Psm*, we observed a significant reduction of bacterial growth in the subsequent challenge infection in systemic leaves, demonstrating the establishment of SAR in both cases. In marked contrast, SAR did not develop in *fmo1* mutant plants because growth of *Psm* in systemic leaves proved to be equally pronounced in plants pretreated in primary leaves with $MgCl_2$, *Psm avrRpm1*, or *Psm* (Fig. 2A).

Systemic accumulation of SA and enhanced expression of defense genes in systemic leaves are characteristic features of SAR (Ryals et al., 1996). When primary

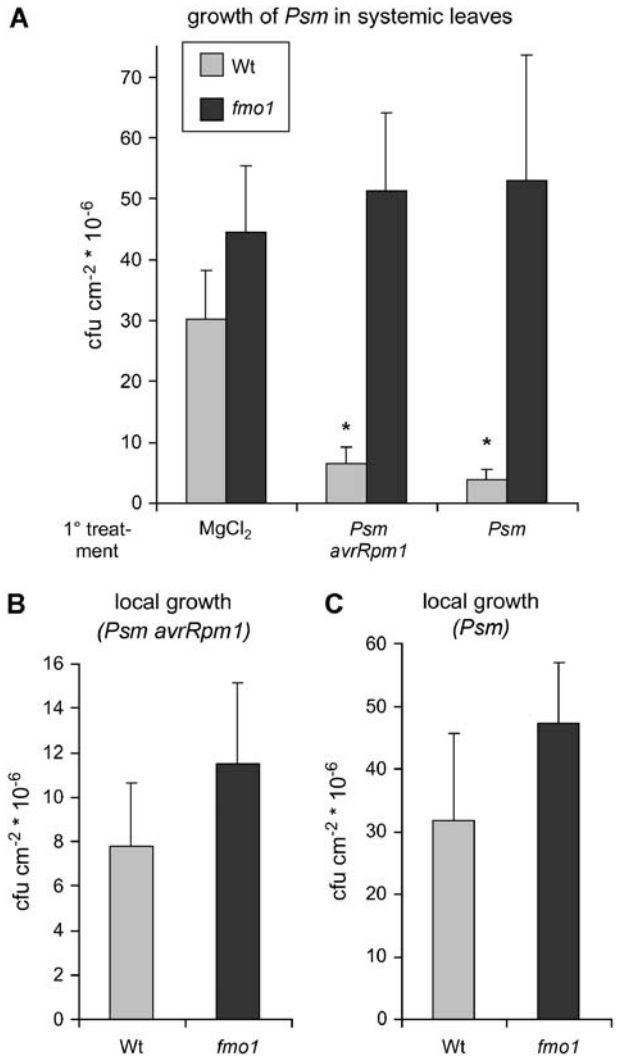

Figure 2. A, Bacterial growth quantification of *Psm* in systemic leaves to assess SAR in wild-type and *fmo1* mutant plants. Five-week-old Arabidopsis plants were pretreated with $MgCl_2$, *Psm avrRpm1*, or *Psm* (OD = 0.02 for each pathogen) in three primary leaves (1° treatment), and 2 d later, three systemic leaves located directly above the primary leaves were inoculated with *Psm* (OD = 0.002). Bacterial growth in systemic leaves was assessed 3 d (3 dpi) after infection of systemic leaves. Bars represent mean values (±SD) of colony-forming units per square centimeter from seven parallel samples each consisting of three leaf discs. Asterisks denote pathogen treatments with statistically significant differences to the respective $MgCl_2$ control ($P < 0.001$; Student's *t* test). Light bars, Wild-type plants; dark bars, *fmo1* plants. B and C, Quantification of bacterial growth to assess local resistance. B, Growth of *Psm avrRpm1* in leaves 3 d after inoculation with a bacterial suspension of OD = 0.005. C, Growth of *Psm* in leaves 3 d after inoculation (OD = 0.002). In both B and C, no statistical differences between the wild type and *fmo1* existed ($P > 0.05$; Student's *t* test). In addition, to ensure the uniformity of the experiments, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for comparable treatments in A, B, and C (data not shown).

leaves of wild-type plants were treated with *Psm avrRpm1* or *Psm*, systemic leaves exhibited about 5- and 7-fold higher levels of free SA, respectively, compared to naive plants that were pretreated with $MgCl_2$ solution only (Fig. 3A). Additionally, both avirulent

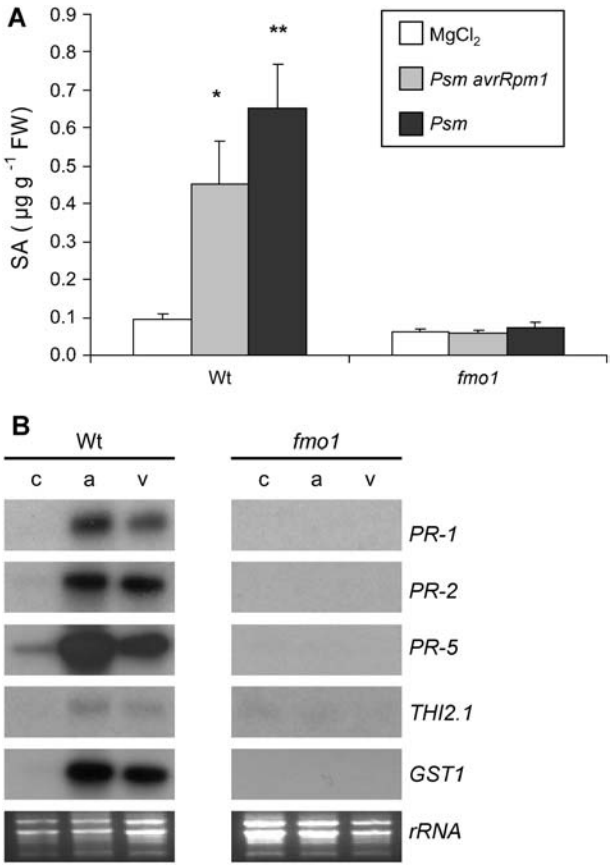

Figure 3. Systemic defense responses in wild-type and *fmo1* plants. Primary leaves of 5-week-old plants were treated as described in Figure 2A and untreated systemic leaves were harvested 2 d later for analysis. A, Systemic accumulation of SA. Bars represent mean values (±SD) of three independent samples. Each sample consisted of six leaves from two different plants. Asterisks denote pathogen treatments with statistically significant differences to the respective $MgCl_2$ control (*, $P < 0.02$; **, $P < 0.005$; Student's *t* test). White bars, $MgCl_2$ treatment; gray bars, *Psm avrRpm1* inoculation; black bars, *Psm* inoculation. B, Systemic expression of defense-related genes assessed by northern-blot analysis (c, $MgCl_2$ treatment; a, *Psm avrRpm1* inoculation; v, *Psm* inoculation).

and virulent bacteria triggered systemic expression of the SA-inducible defense gene *PR-1* (Nawrath and Métraux, 1999), the jasmonate-dependent thionin gene *THI2.1* (Epple et al., 1995), and the SA- and JA-independent defense genes *PR-2* and *PR-5* in the wild type (Fig. 3B). Moreover, the *glutathione S-transferase* gene *GST1*, a reliable marker for ROS production during plant-pathogen interactions (Levine et al., 1994; Alvarez et al., 1998), was systemically up-regulated in wild-type plants inoculated with *Psm* or *Psm avrRpm1* (Fig. 3B). Unlike the wild type, *fmo1* mutant plants exhibited neither elevated systemic SA levels after a local infection with *Psm* or *Psm avrRpm1* nor increased systemic expression of any of the defense genes under examination (Fig. 3).

Local Resistance in *fmo1* Mutants Is Similar to Wild-Type Plants

To examine whether the loss of SAR in *fmo1* mutants was associated with compromised local disease resistance in the *P. syringae*-Arabidopsis interaction, we determined bacterial growth of *Psm avrRpm1* and *Psm* in naive plants. Bacterial multiplication of both avirulent and virulent isolates was similar in wild-type and *fmo1* mutants (Fig. 2, B and C). In some experiments, a slightly enhanced growth tendency of *fmo1* mutant plants could be observed for *Psm* or *Psm avrRpm1*, but this tendency was not statistically significant. These results indicate that specific or basal disease resistance in the examined interactions is not compromised in *fmo1*.

To further address this issue, we investigated typical defense responses that are induced by *Psm avrRpm1* in Col-0 wild-type plants at inoculation sites. SA and JA are well-characterized signaling molecules accumulating during incompatible interactions. Up-regulation of the SA biosynthesis gene *SID1* occurred in a similar manner in both wild-type and *fmo1* plants starting 4 hpi (Fig. 5). Accordingly, local SA accumulation in *fmo1* closely resembled SA elevation in wild-type plants at 10 hpi (Fig. 4A). Levels increased from about 0.15 $\mu g\ g^{-1}$ fresh weight in control leaves to about 1.5 $\mu g\ g^{-1}$ fresh weight in inoculated leaves. Likewise, *Psm avrRpm1* induced accumulation of JA to a comparable extent in wild-type and *fmo1* plants (Fig. 4B). Further downstream in these pathways, SA and JA trigger the expression of distinct sets of PR genes (Reymond and Farmer, 1998). Again, striking similarities were obvious in the expression patterns of the SA-inducible *PR-1* gene and the JA-inducible *PR-4* gene after *Psm avrRpm1* inoculation (Fig. 5). Moreover, *Psm avrRpm1*-induced transcription of the SA- and JA-independent defense genes *PR-2* and *PR-5* was detected in both wild type and *fmo1*, yet to a somewhat higher extent in the mutant. These data indicate that, at the site of pathogen inoculation, FMO1 is neither required for the execution of SA- and JA-dependent defense pathways nor for the accomplishment of pathways independent of these defense signals.

Increased production of secondary metabolites represents a further characteristic response to host pathogens. The indole derivative camalexin constitutes the major phytoalexin in Arabidopsis and accumulates in response to elicitor and pathogen treatment (Tsuji et al., 1992; Zhou et al., 1998). Camalexin was essentially absent in noninoculated leaves, but accumulated substantially in *Psm avrRpm1*-treated leaves already at 10 hpi (Fig. 4C). Again, no significant difference between wild type and *fmo1* existed. Moreover, expression of Phe ammonia lyase (PAL), the key enzyme of phenylpropanoid biosynthesis, is up-regulated upon infection with avirulent Pseudomonas (Zeier et al., 2004). *PAL1* transcripts were elevated at 4 to 6 hpi in both wild-type and *fmo1* leaves, indicating that the phenylpropanoid pathway is initiated independently from *FMO1*.

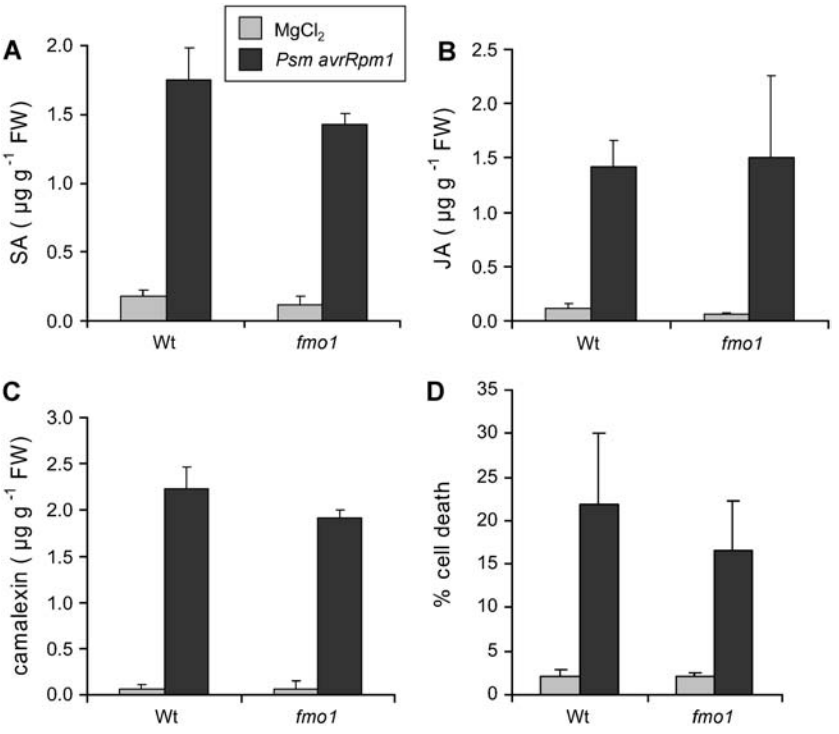

Figure 4. Local defense responses in wild-type and *fmo1* plants. A to C, Accumulation of signaling and antimicrobial compounds in leaves challenged with *Psm avrRpm1* (OD = 0.005). Control samples were treated with 10 mM $MgCl_2$. All samples were collected 10 h post treatment. A, SA levels. B, JA content. C, Accumulation of the phytoalexin camalexin. Mean values (±SD) of three independent samples are given. No statistical differences between equally treated wild-type and *fmo1* plants existed for each metabolite ($P > 0.05$; Student's *t* test). D, Quantification of microscopic HR lesions in leaves inoculated with *Psm avrRpm1* that were stained with trypan blue 24 hpi. Bars represent mean values (±SD) of dead cells in infiltrated areas from at least seven independent leaf samples. Light bars, Areas infiltrated with 10 mM $MgCl_2$; dark bars, *Psm avrRpm1*-infiltrated areas.

The oxidative burst at the site of pathogen ingress and the subsequent hypersensitive cell death response represent hallmarks of incompatible plant-pathogen interactions (Lamb and Dixon, 1997). During the oxidative burst, ROS are produced that contribute to triggering the HR in infected cells and driving expression of protective genes in neighboring tissue (Levine et al., 1994). The expression of *GST1* is triggered by ROS produced during the oxidative burst (Alvarez et al., 1998; Zeier et al., 2004). Enhanced *GST1* expression was observed from 4 to 10 h after *Psm avrRpm1* inoculation in wild-type leaves and a similar pattern was evident in *fmo1* mutant leaves. *FMO1* has recently been described as a marker gene for cell death pathways in plants (Olszak et al., 2006). To investigate whether FMO1 contributes to hypersensitive cell death lesion formation upon infection with *Psm avrRpm1*, we performed trypan blue-staining experiments with inoculated leaves (Zeier et al., 2004). At 24 hpi, wild-type plants exhibited a considerable amount of stained cells inside the pathogen-treated leaf area (Fig. 4D) and similar staining patterns were observed in inoculated *fmo1* mutant leaves. Thus, FMO1 does not play a critical role in the regulation of the oxidative burst or the hypersensitive cell death response at the site of pathogen attack.

Local and Systemic Expression of *FMO1* in Arabidopsis Defense Mutants

The SA-signaling pathway is essential for the full establishment of local and systemic disease resistance in the Arabidopsis-*P. syringae* interaction (Nawrath and Métraux, 1999). To examine whether expression of *FMO1* is dependent on SA signaling and SA-related defense pathways, we checked the pathogen-induced up-regulation of *FMO1* in different Arabidopsis defense mutants (Fig. 6). *Psm avrRpm1* or *Psm* induced *FMO1* expression to a similar extent in the SA biosynthesis mutant *sid2*, in the SA-insensitive mutant *npr1*, and in wild-type plants at the site of inoculation (Fig. 6A), demonstrating that local *FMO1* expression

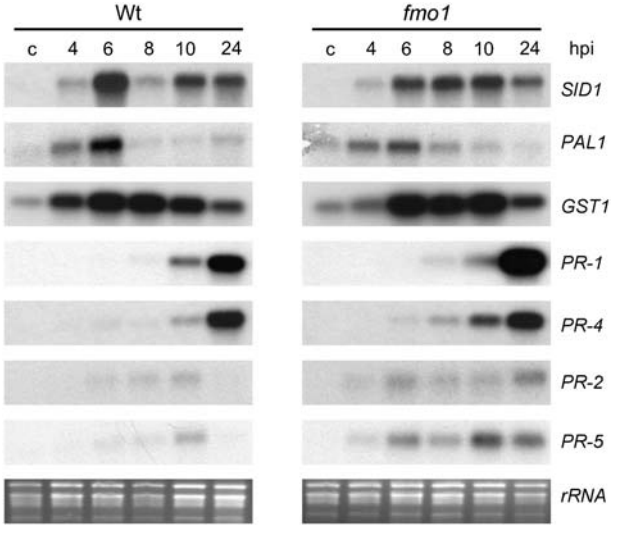

Figure 5. Local defense responses in wild-type and *fmo1* plants. Expression of defense-related genes in leaves challenged with *Psm avrRpm1* (OD = 0.005), as assessed by northern-blot analysis. Numbers indicate hpi. Control leaves (c) were treated with 10 mM $MgCl_2$ (4 h).

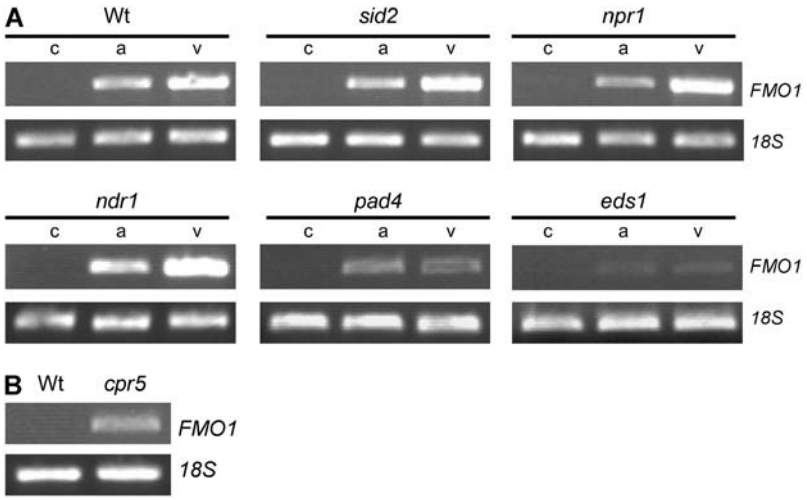

Figure 6. A, Expression of *FMO1* at the site of pathogen inoculation in wild-type plants and Arabidopsis defense mutants (24 hpi) as assessed by RT-PCR analysis (c, $MgCl_2$ treatment; a, *Psm avrRpm1* inoculation; v, *Psm* inoculation; OD = 0.005 for each pathogen). B, Levels of *FMO1* transcripts in untreated leaves of wild-type and *cpr5* mutant plants.

does not require SA signaling. Additionally, *FMO1* was strongly expressed in the *no disease resistance 1* (*ndr1*) defense mutant (Century et al., 1995) upon *P. syringae* infection. In the defense-signaling mutants *phytoalexin-deficient 4* (*pad4*; Zhou et al., 1998) and *enhanced disease susceptibility 1* (*eds1*; Parker et al., 1996), pathogen-induced FMO1 expression was clearly attenuated or entirely suppressed, respectively. Moreover, the *constitutive expression of PR 5* (*cpr5*) mutant that constitutively exhibits resistance in both an *npr1*-dependent and -independent manner (Bowling et al., 1997) exhibited marked constitutive *FMO1* expression (Fig. 6B).

To further analyze the function of FMO1 during SAR, we tested whether *FMO1* is systemically expressed upon local *Psm avrRpm1* inoculation (Fig. 7). The pathogen-induced SAR response was associated with an up-regulation of *FMO1* in systemic leaves of wild-type plants (Fig. 7, A and B). In SA-signaling mutants *sid2* and *npr1*, however, SAR was fully compromised and *FMO1* failed to be expressed systemically. The *ndr1* mutant constitutes a further SAR-deficient mutant, and systemic expression of *FMO1* was not enhanced upon *Psm avrRpm1* infection. The *eds1* mutation, by contrast, did not abolish *Psm avrRpm1*-triggered SAR, and systemic *FMO1* up-regulation still took place in *eds1*, albeit to a lesser extent than in the wild type. Moreover, the camalexin-deficient mutant *pad3* exhibited a wild-type-like SAR response and showed a systemic *FMO1* expression pattern similar to the wild type. Thus, establishment of SAR closely correlated with systemic elevation of *FMO1* transcript levels in the lines under investigation and, in contrast to the expression characteristics at the site of pathogen attack (Fig. 6A), systemic *FMO1* expression was dependent on an intact SA-signaling pathway.

DISCUSSION

SAR can be activated in many plant species by necrotizing pathogens and, once established, it confers long-lasting resistance toward a broad spectrum of different pathogens (Durrant and Dong, 2004). SAR turns out to be under complex molecular regulation because several components of entirely distinct biochemical origin are necessary for its induction in Arabidopsis (Nawrath and Métraux, 1999; Maldonado et al., 2002; Nandi et al., 2004; Xia et al., 2004; Wang et al., 2005). We show here that FMO1, whose expression is induced by virulent and avirulent strains of *P. syringae* both at the site of pathogen ingress and in systemic tissue, constitutes a further component essential for the successful activation of SAR in Arabidopsis because *fmo1* knockout mutants proved to be totally compromised in the activation of systemic defense responses and the establishment of SAR (Figs. 2A and 3).

Currently, a central role for FMO1 in plant disease resistance is emerging. In fact, the *FMO1* gene has recently been recognized by distinct approaches to be involved in plant defense (Bartsch et al., 2006; Koch et al., 2006; Olszak et al., 2006). *FMO1* was demonstrated to be up-regulated in Arabidopsis *acd11*, a mutant exhibiting constitutively activated SA-, PAD4-, and EDS1-dependent defenses and spontaneous HR lesions (Brodersen et al., 2002; Olszak et al., 2006). In addition, it was shown that *FMO1* expression is enhanced in the runaway cell death *lesion-simulating disease 1* (*lsd1*) mutant (Dietrich et al., 1997), but not in the constitutive defense-signaling mutants *ctr1*, *cev1*, *mpk4*, and *cpr6* that do not develop spontaneous cell death (Olszak et al., 2006). Thus, *FMO1* was suggested as a marker gene for certain forms of defense and cell death. In a screen for genes whose expression depends on EDS1 and PAD4, Bartsch et al. (2006) showed the requirement of functional FMO1 in the execution of basal resistance against a virulent isolate of the oomycete pathogen *Hyaloperonospora parasitica* and of specific resistance against *H. parasitaca* isolate Noco2 or *P. syringae* carrying the *avrRps4* avirulence gene. Moreover, an activation-tagging approach identified an Arabidopsis line constitutively overexpressing *FMO1*,

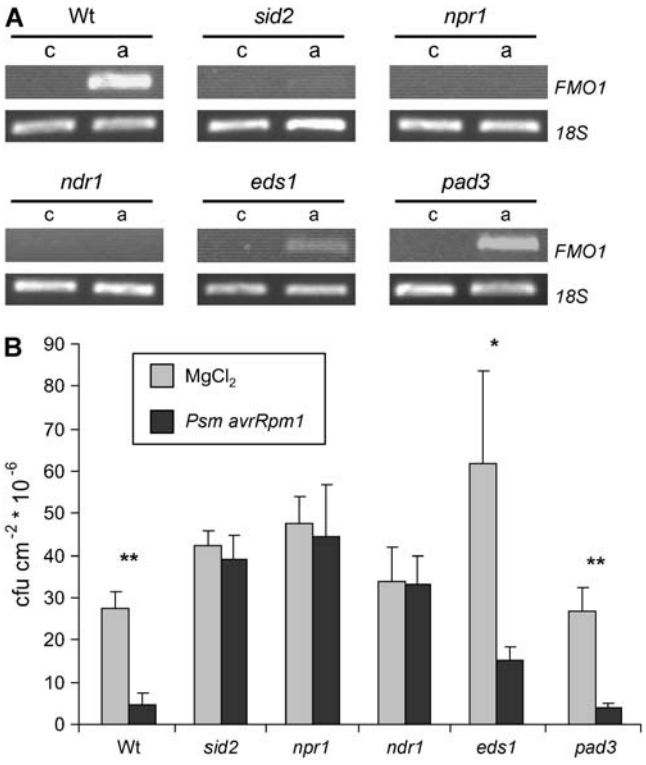

Figure 7. Correlation of systemic *FMO1* expression and SAR establishment. A, Systemic expression of *FMO1* in wild-type plants and Arabidopsis defense mutants in response to *Psm avrRpm1* as assessed by RT-PCR analysis (c, $MgCl_2$ treatment; a, *Psm avrRpm1* inoculation; OD = 0.02). For further details, see legend to Fig. 3. B, Growth quantification of *Psm* in systemic leaves (3 dpi) to assess SAR induced by *Psm avrRpm1* in wild-type and Arabidopsis defense mutants. For further details, see legend to Fig. 2A. Asterisks denote lines with statistically significant differences between plants pretreated with $MgCl_2$ and *Psm avrRpm1* (*, $P < 0.01$; **, $P < 0.001$; Student's *t* test).

which is characterized by enhanced disease resistance against *P. syringae* (Koch et al., 2006).

Basal resistance is triggered by a multitude of relatively unspecific pathogen-associated molecular patterns to limit the growth of virulent pathogens to a certain extent (Nürnberger and Lipka, 2005). By contrast, resistance gene-mediated resistance is based on specific recognition events and two subclasses of resistance proteins with distinct signaling requirements are generally distinguished, depending on the presence of either an N-terminal coiled-coil domain (CC-NB-LRR) or a domain with similarity to the Drosophila Toll and mammalian interleukin-1 receptors (TIR-NB-LRR; Aarts et al., 1998). We have demonstrated that, at the site of pathogen inoculation, basal resistance against virulent *Psm* and specific resistance against *Psm avrRpm1* are not compromised in *fmo1* mutants (Fig. 2, B and C). Moreover, various characteristic defense responses locally triggered by *Psm avrRpm1*, including oxidative burst, accumulation of SA, JA, and camalexin, and expression of defense genes as well as the hypersensitive cell death response, are not significantly altered in *fmo1* (Figs. 4 and 5). In line with these findings, Bartsch et al. (2006) showed that resistance to *Pst avrRpm1* is not affected in *fmo1* mutants, using the same T-DNA insertion line (SALK_026163). Because the AvrRpm1 avirulence protein is recognized by RPM1, a CC-NB-LRR-type resistance protein, we conclude that basal resistance to *P. syringae* and specific resistance mediated by CC-NB-LRR receptors are largely FMO1 independent. By contrast, resistance to *Pst avrRps4* has been reported to be attenuated in *fmo1* and therefore FMO1 is required for specific resistance against *P. syringae* mediated by TIR-NB-LRR resistance proteins (Bartsch et al., 2006). The contribution of FMO1 to basal resistance against *H. parasitica*, specific resistance against *Pst avrRps4*, and SAR triggered by *Psm* reveals that overlapping molecular principles exist in distinct kinds of resistance within different pathosystems.

Our finding that FMO1 represents a critical component of SAR in Arabidopsis is further underlined by recent work demonstrating that constitutive overexpression of *FMO1* leads to enhanced disease resistance toward *P. syringae* (Koch et al., 2006). FMO1 might function in the induction of SAR in inoculated tissue, in the propagation of a mobile signal to distant tissue, in the perception of this long-distance signal in systemic tissue, or in the potentiation of defense responses in systemic tissue. Induced expression of *FMO1* in inoculated leaves is attenuated in *eds1* and *pad4* mutants, confirming that FMO1 contributes to the EDS1/PAD4 pathway in local defense signaling (Bartsch et al., 2006). In contrast, FMO1 expression is not affected in *sid2*, *npr1*, and *ndr1* mutants, demonstrating that local *FMO1* induction is independent of the SA-signaling pathway and NDR1-mediated signaling (Fig. 6). However, in contrast to the wild type, these three mutants fail to express *FMO1* systemically (Fig. 7A), and this is associated with a loss of SAR (Fig. 7B). In the *eds1* mutant, pathogen-induced *FMO1* expression is abolished at the site of inoculation, yet still observable in systemic tissue, and a significant SAR response is established in *eds1*. Thus, the failure to systemically rather than locally up-regulate *FMO1* transcription correlates with the development of SAR in all investigated lines. Additionally, *fmo1* mutants, despite exhibiting unaltered local defenses, are totally compromised in any of the examined systemic responses (Fig. 3). These include systemic SA accumulation, systemic expression of SA-dependent and -independent PR genes, as well as up-regulation of *GST1*, a reliable marker for ROS generation (Levine et al., 1994). Moreover, *FMO*1 transcripts are up-regulated in the absence of a pathogen in defense of the mutant *cpr5*, which exhibits constitutive disease resistance (Fig. 6B; Bowling et al., 1997). Taking these findings together, we propose a model in which the presence of FMO1 in systemic tissue is critical for the realization of SAR. A metabolite generated by FMO1 might be necessary during the early phase of SAR establishment in systemic leaves, presumably for the transduction or amplification of a long-distance signal originating from primary leaves.

Feedback loops, including SA and ROS, exist to amplify plant defense responses (Shirasu et al., 1997) and oxidative microbursts in systemic tissue have been shown to mediate a reiterative signal network during SAR (Alvarez et al., 1998). Moreover, superoxide has been demonstrated to induce *FMO1* expression (Olszak et al., 2006). We thus propose that FMO1 contributes to a signal amplification loop involving ROS, SA, NPR1, and NDR1 that is required to potentiate SAR responses in systemic tissue.

Although SA represents a central and necessary signaling component for the establishment of SAR, there are controversial data as to whether it functions as a mobile signal that moves from infected leaves to systemic tissue. $^{18}O_2$ feeding experiments in tobacco mosaic virus-infected tobacco (*Nicotiana tabacum*) demonstrate that about 60% to 70% of the SA detected in systemic leaves originates from inoculated tissue, with the remainder resulting from de novo synthesis (Shulaev et al., 1995). Similarly, ^{14}C-labeling experiments in cucumber (*Cucumis sativus*) plants inoculated with tobacco necrosis virus showed that SA accumulation in systemic leaves results both from transport and from de novo synthesis (Mölders et al., 1996). Although SA transport from inoculated to systemic tissue is feasible in these species, SA does not necessarily represent the SAR long-distance signal. In cucumber, removal of pathogen-treated leaves led to systemic resistance induction before a rise in SA levels was detectable in petiole exudates of inoculated leaves (Rasmussen et al., 1991). Moreover, grafting experiments using transgenic tobacco expressing the salicylate hydroxylase NahG indicate that SA is not the long-distance signal during SAR, but it is required for signal transduction in systemic tissue (Vernooij et al., 1994). Considering Arabidopsis, Kiefer and Slusarenko (2003), by applying ^{14}C-SA to rosette leaves, have demonstrated that exogenous SA is able to move from source to sink tissue. On the other hand, we have shown here that Pseudomonas-infected *fmo1* mutant plants locally accumulate wild-type levels of SA, whereas no SA accumulation occurs systemically (Figs. 3A and 4A). A similar trend is observed in the SAR-defective mutants *ndr1* and *npr1* (Fig. 7B; T.E. Mishina and J. Zeier, unpublished data). This indicates that systemic accumulation of SA that is normally observed during biologically induced SAR in Arabidopsis is not due to transport of SA produced at the site of infection, but is largely caused by de novo synthesis in systemic tissue in which the above proposed feedback loop, including FMO1 and SA, might operate.

Mammalian FMO either contribute to oxidative xenobiotic metabolism or catalyze the oxygenation of endogenous metabolites, i.e. biogenic amines (Krueger and Williams, 2005). Besides *FMO1*, the only plant FMO genes characterized so far represent Arabidopsis *YUCCA* and its petunia (*Petunia hybrida*) ortholog *FLOOZY*, which are involved in auxin biosynthesis (Zhao et al., 2001; Tobena-Santamaria et al., 2002). YUCCA has been demonstrated to catalyze the hydroxylation of the amino group in tryptamine. A challenging future task represents the identification of the putative metabolite generated by FMO1 and the clarification of its role in disease resistance.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) L. Heynh. plants were grown in a mixture of soil (Fruhstorfer Pflanzenerde), vermiculite, and sand (9:1:1) in a controlled environment chamber (J-66LQ4; Percival) with a 9 h day (photon flux density 70 μmol m^{-2} s^{-1})/15 h night cycle and 70% relative humidity. Growth temperatures were set to 22°C during the day and 18°C during the night.

Arabidopsis ecotype Col-0 was used as the wild type in all experiments. The *fmo1* line represents the Salk T-DNA insertion line SALK_026163 in the Col-0 background. Homozygous insertion mutants were identified by PCR, using a gene-specific and a T-DNA-specific primer (Alonso et al., 2003), and used for experiments. Further, the following Arabidopsis defense mutants were used in this study: *sid2-1* (Nawrath and Métraux, 1999), *npr1-2* (NASC ID no., N3801), *ndr1* (Century et al., 1995), *pad3-1* (Glazebrook and Ausubel, 1994), *pad4-1* (Glazebrook et al., 1997), *eds1-2* (Aarts et al., 1998), and *cpr5-2* (Bowling et al., 1997).

Growth of Plant Pathogens and Infection

Pseudomonas syringae pv *maculicola* ES4326 lacking (*Psm*) or carrying (*Psm avrRpm1*) the *avrRpm1* avirulence gene were grown at 28°C in King's B medium containing the appropriate antibiotics (Zeier et al., 2004). Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to a final optical density (OD) concentration of 0.02, 0.005, or 0.002. The bacterial suspensions were infiltrated from the abaxial side into a sample leaf using a 1-mL syringe without a needle. Control inoculations were performed with 10 mM $MgCl_2$. Bacterial growth was assessed by homogenizing discs originating from infiltrated areas of three different leaves in 1 mL 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28°C for 2 d.

All pathogen experiments depicted in the figures were repeated at least twice with similar results.

Characterization of Systemic Resistance Responses

Plants were first infiltrated into three lower leaves with a suspension of *Psm* or *Psm avrRpm1* (OD = 0.02), or with 10 mM $MgCl_2$ as a control. Two days after the primary inoculation, nontreated upper leaves were harvested for SA determination and gene expression analysis or plants were inoculated on three upper leaves with virulent *Psm* (OD = 0.002). Growth of *Psm* in upper leaves was scored 3 d later.

Quantification of Microscopic HR Lesions

The extent of microscopic HR lesion formation was assessed by trypan blue staining, light microscopy, and quantification of stained cells as described by Zeier et al. (2004).

Northern-Blot Analysis

Total RNA was isolated from frozen leaves using peqGOLD RNAPure reagent (peqLab) following the manufacturer's instructions. For each sample, two leaves from different plants of the same treatment were used. One microgram of total RNA was loaded on formaldehyde-agarose gels, separated by electrophoresis, and blotted on nylon membranes (Hybond-N; Amersham). RNA-blot hybridization was performed with specific ^{32}P-labeled DNA probes generated by PCR using appropriate oligonucleotide primers. The probes represented the following Arabidopsis genes: *SID1* (Arabidopsis annotation At4g39030), *PAL1* (At2g37040), *GST1* (At1g02930), *PR-1* (At2g14610), *PR-2* (At3g57260), *PR-4* (At3g04720), *PR-5* (At1g75040), and *THI2.1* (At1g72260).

RT-PCR Analysis

One microgram of extracted RNA was treated with DNase I (Fermentas) for 30 min at 37°C to remove genomic DNA, the DNase inactivated by incubation at 70°C for 5 min in the presence of 2.5 mM EDTA, and cDNA synthesized in a final reaction volume of 20 μL at 42°C for 1 h using random primer mix, ribonuclease inhibitor (RNaseOUT; Invitrogen), and reverse transcriptase (SuperScript II; Invitrogen). After another enzyme inactivation step for 15 min at 70°C, the cDNA mixture was diluted in water (1:10) and 3 to 10 μL of the final dilution used in a 30-μL RT-PCR reaction (3 μL for 18S rRNA, 10 μL for *FMO1*). The following primers were used for the amplification of cDNA derived from 18S rRNA and *FMO1* mRNA, respectively: 5′-AAACGGCTACCACATCCAAG-3′ (18S-forward), 5′-ACCCATCCCAAGGTTCAACT-3′ (18S-reverse), 5′-CTTCTACTCTCCTCAGTGGCAAA-3′ (*FMO1*-forward), and 5′-CTAATGTCGT-CCCATCTTCAAAC-3′ (*FMO1*-reverse). The PCR reaction was performed as follows: 95°C for 10 min, 25 (18S) or 30 (*FMO1*) cycles of 92°C for 60 s, 60°C for 90 s, 72°C for 90 s, and a final extension step at 72°C for 5 min. Ten microliters of each PCR reaction were visualized by agarose gel electrophoresis with ethidium bromide staining.

Gas Chromatographic Determination of SA, JA, and Camalexin

The determination of SA, JA, and camalexin levels in leaves was performed by a modified vapor-phase extraction method (Schmelz et al., 2004). Briefly, 150 mg of frozen leaf tissue were homogenized with 600 μL of extraction buffer (water:1-propanole:HCl = 1:2:0.005). After addition of internal standards (D_4-SA, dihydrojasmonic acid, and indolepropionic acid; 100 ng each) and 1 mL of methylene chloride, the mixture was shaken thoroughly and centrifuged at 14,000 rpm for phase separation. The lower, organic phase was then removed, dried over Na_2SO_4, and treated with 2 μL of 2 M trimethylsilyldiazomethane in hexane (Sigma-Aldrich) for 5 min at room temperature to convert carboxylic acids into their corresponding methyl esters. After stopping the methylation reaction with 2 M acetic acid in hexane, the sample was subjected to a vapor-phase extraction procedure using a volatile collector trap packed with Super-Q absorbent (VCT-1/4X3-SPQ; Analytical Research Systems). The final evaporation temperature was set to 200°C, and samples were eluted from the collector trap with 1 mL methylene chloride. Finally, the sample volume was reduced to 50 μL in a stream of nitrogen, and the sample was subjected to gas chromatography-mass spectrometry analysis. The sample mixture (2 μL) was separated on a gas chromatograph (GC 6890 N; Agilent Technologies) equipped with a fused silica capillary column (DB-1; Fisons), and combined with a 5975 mass spectrometric detector (Agilent Technologies). For quantitative determination of metabolites, peaks originating from selected ion chromatograms were integrated. The area of a substance peak was related to the peak area of the corresponding internal standard (SA/D_4-SA; JA/dihydrojasmonic acid, camalexin/indolepropionic acid). Experimentally determined correction factors for each substance/standard pair were considered.

ACKNOWLEDGMENTS

We thank Volker Lipka and Juriaan Ton for the donation of Arabidopsis mutant seeds, and Michael Rostás, Markus Riederer, and Volker Lipka for proofreading the manuscript. We are grateful to M. de Torres Zabala, M. Grant, B. Kemmerling, and T. Nürnberger for publicly sharing their microarray data.

Received April 2, 2006; revised June 1, 2006; accepted June 1, 2006; published June 15, 2006.

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