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A role for β-sitosterol to stigmasterol conversion in plant–pathogen interactions

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SUMMARY

Upon inoculation with pathogenic microbes, plants induce an array of metabolic changes that potentially contribute to induced resistance or even enhance susceptibility. When analysing leaf lipid composition during the *Arabidopsis thaliana–Pseudomonas syringae* interaction, we found that accumulation of the phytosterol stigmasterol is a significant plant metabolic process that occurs upon bacterial leaf infection. Stigmasterol is synthesized from β-sitosterol by the cytochrome P450 CYP710A1 via C22 desaturation. Arabidopsis *cyp710A1* mutant lines impaired in pathogen-inducible expression of the C22 desaturase and concomitant stigmasterol accumulation are more resistant to both avirulent and virulent *P. syringae* strains than wild-type plants, and exogenous application of stigmasterol attenuates this resistance phenotype. These data indicate that induced sterol desaturation in wild-type plants favours pathogen multiplication and plant susceptibility. Stigmasterol formation is triggered through perception of pathogen-associated molecular patterns such as flagellin and lipopolysaccharides, and through production of reactive oxygen species, but does not depend on the salicylic acid, jasmonic acid or ethylene defence pathways. Isolated microsomal and plasma membrane preparations exhibited a similar increase in the stigmasterol/β-sitosterol ratio as whole-leaf extracts after leaf inoculation with *P. syringae*, indicating that the stigmasterol produced is incorporated into plant membranes. The increased contents of stigmasterol in leaves after pathogen attack do not influence salicylic acid-mediated defence signalling but attenuate pathogen-induced expression of the defence regulator flavin-dependent monooxygenase 1. *P. syringae* thus promotes plant disease susceptibility through stimulation of sterol C22 desaturation in leaves, which increases the stigmasterol to β-sitosterol ratio in plant membranes.

Keywords: Arabidopsis, *Pseudomonas syringae*, stigmasterol, β-sitosterol, PAMPs, ROS.

INTRODUCTION

Several metabolic pathways are known to be activated in *Arabidopsis thaliana* leaves upon inoculation with the hemibiotrophic bacterial pathogen *Pseudomonas syringae*. Some metabolites accumulate, albeit with different temporal characteristics or to varying levels, in leaves that have been inoculated with non-adapted, virulent or avirulent bacterial strains, whereas others are only produced in a particular type of interaction (Mishina and Zeier, 2007a; Mishina *et al.*, 2008). Pathway activation may be triggered by the perception of pathogen-associated molecular patterns (PAMPs), the recognition of effector proteins secreted by the type III secretion system (TTSS), or a combination of both.

Biosynthesis of the phenolic defence hormone salicylic acid (SA), and its conversion to derivatives such as SA β-glucoside (SAG), SA glucose ester and methyl salicylate (MeSA), are major and general metabolic events in *P. syringae*-inoculated leaves (Dean and Delaney, 2008; Attaran *et al.*, 2009). By contrast, jasmonic acid (JA) and related oxylipins such as the JA biosynthetic precursor 12-oxo-phytodienoic acid and the non-enzymatically produced phytoprostanes are predominantly produced upon inoculation with avirulent, hypersensitive response (HR)-inducing strains, or with high inoculum densities of compatible strains (Grun *et al.*, 2007; Mishina and Zeier, 2007b). As such inoculation conditions result in strong tissue necrosis,

production of JA-related oxylipins appears to be associated with the disruption of leaf tissue (Mishina and Zeier, 2007b). Likewise, unsaturated C16 and C18 fatty acids such as linoleic and linolenic acid accumulate in *P. syringae*-treated Arabidopsis leaves (Yaeno *et al.*, 2004), with linolenic acid representing the initial precursor of JA biosynthesis. Moreover, virulent and avirulent but not non-host *P. syringae* strains invoke accumulation of the indole alkaloid camalexin, a tryptophan-derived phytoalexin of Arabidopsis that provides effective protection against necrotrophic but not (hemi)biotrophic pathogens (Thomma *et al.*, 1999; Zhou *et al.*, 1999). In addition to accumulation of phenolics (SA and its derivatives) and alkaloids (camalexin), the isoprenoid pathway, the third major metabolic pathway involved in plant secondary metabolite biosynthesis, is activated to produce the volatile terpenoids (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), β-ionone and α-farnesene (Attaran *et al.*, 2008). Finally, the levels of the phytohormone abscisic acid (ABA), which is involved in several plant responses to abiotic stress, increase in leaves infected with compatible *P. syringae* (de Torres Zabala *et al.*, 2009). Strikingly, among the various pathogen-induced metabolites mentioned above, the only compound unequivocally required for resistance against *P. syringae* infestation is SA (Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001). JA and ABA even favour pathogen susceptibility by interfering with SA biosynthesis and signalling (Kloek *et al.*, 2001; de Torres Zabala *et al.*, 2009).

The transcript levels of several hundred Arabidopsis genes are strongly increased in *P. syringae*-inoculated leaves, and many of these genes encode proteins with putative functions in primary and secondary metabolism (Zimmermann *et al.*, 2004). This suggests that several metabolic events induced in Arabidopsis leaves upon pathogen attack may await discovery. An important metabolic route for plant development and survival is the sterol biosynthetic pathway. Sterols are indispensable compounds in plants and other eukaryotes because they are structural constituents of membranes, in which they regulate fluidity and permeability (Schaller, 2003). Cell membranes of mammals and fungi are generally composed of one predominant sterol (cholesterol and ergosterol, respectively), but plants have a more complex sterol mixture (Hartmann, 1998). For instance, the sterol profile of plants of Arabidopsis accession C24 includes β-sitosterol as the major compound (64%), together with 24-methyl cholesterol (11%), stigmasterol (6%), isofucosterol (3%) and brassicasterol (2%), and several other minor sterols (Schaeffer *et al.*, 2001). It is not yet known whether a relationship exists between the sterol composition of plant membranes and plant defence or disease resistance.

We report here that C22 desaturation of the predominant Arabidopsis phytosterol β-sitosterol via the cytochrome P450 enzyme CYP710A1 and the concomitant accumulation of stigmasterol are significant metabolic processes in *P. syringae*-inoculated Arabidopsis leaves. Stigmasterol formation in leaves is triggered by perception of bacterial PAMPs and generation of reactive oxygen species (ROS), but is independent of the SA-, JA- or ethylene-associated signalling pathways. Through mutant analysis and exogenous sterol application, we show that an increased stigmasterol to β-sitosterol ratio in leaves attenuates specific plant defence responses, which results in enhanced susceptibility against *P. syringae*. Increased proportions of stigmasterol in microsomal membrane and plasma membrane isolates after bacterial attack suggest that pathogen-induced changes in the sterol composition of leaf membranes influence plant disease resistance and affect the outcome of particular plant–pathogen interactions.

RESULTS

β-sitosterol to stigmasterol conversion via CYP710A1 constitutes a significant metabolic process in *P. syringae*-inoculated Arabidopsis leaves

Publicly available microarray experiments indicate that the transcript levels of several hundred Arabidopsis genes are substantially increased in Arabidopsis leaves upon *P. syringae* inoculation, among them the cytochrome P450 gene *CYP710A1* (Zimmermann *et al.*, 2004; Figure S1a). The transcript level of *CYP710A1* in leaves is strongly increased after inoculation with both the avirulent HR-inducing strain *P. syringae* pv. *maculicola* ES4326 (*Psm*) *avrRpm1* and the virulent *Psm* strain (Figure 1a). CYP710A1 has been previously described as a sterol desaturase that introduces a double bond at the C22 position of the saturated sterol side chain of β-sitosterol to produce stigmasterol (Figure 1b,c) (Morikawa *et al.*, 2006; Arnqvist *et al.*, 2008). We thus determined the sterol composition of mock- and pathogen-treated Col-0 leaves by chloroform/methanol extraction and subsequent GC/MS analysis (Figure 1b,c). β-sitosterol is the predominant sterol in both control and *P. syringae*-treated leaves. However, although the levels of stigmasterol are low in control leaves (Figure 1b), we observed a significant peak of stigmasterol in extracts from *P. syringae*-treated leaves (Figure 1c), indicating induced production after bacterial treatment.

A detailed time-course analysis revealed that stigmasterol starts to accumulate in leaves between 10 and 24 h after inoculation (hpi) with both avirulent *Psm avrRpm1* and virulent *Psm* (Figure 2a). The leaf contents of stigmasterol increase to approximately 15 μg g^{-1} fresh weight (FW) until 48 hpi in both the incompatible and compatible interactions, and then remain essentially constant. In $MgCl_2$-infiltrated control leaves, the stigmasterol content does not change significantly during the course of analysis and remains low at 0.1–0.2 μg g^{-1} FW. Moreover, the leaf levels of β-sitosterol are constantly high at 150–200 μg g^{-1} FW, showing no

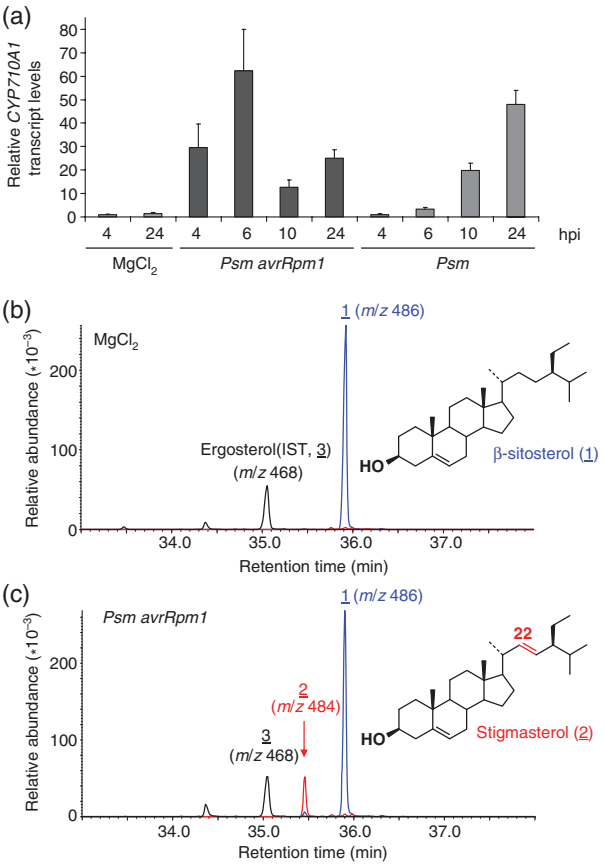

Figure 1. Transcript levels of *CYP710A1* and GC/MS-based determination of sterols in leaves of Arabidopsis ecotype Col-0 after bacterial inoculation.
(a) Quantitative real-time PCR determination of *CYP710A1* transcript levels in response to leaf inoculation with *Pseudomonas syringae* pv. *maculicola* (*Psm*; virulent strain) or *Psm avrRpm1* (avirulent strain) at indicated hours post-inoculation (hpi). Control leaves were infiltrated with 10 mM $MgCl_2$ and collected at 4 and 24 h after treatment. One sample consisted of two leaves. Transcript levels were normalized to those of the *UBQ10* reference gene (At4g05320), and expressed relative to the 4 h $MgCl_2$ sample. For each transcript value, three PCR replicates were performed. Values are means ± SD.
(b,c) GC/MS analyses of lipid extracts from Arabidopsis leaves. Ion chromatograms at *m/z* 486 (blue), *m/z* 484 (red) and m/z 468 (black) are shown. The retention times of β-sitosterol (1), stigmasterol (2) and ergosterol (3; used as internal standard) were 35.9, 35.5 and 35.1 min, respectively. (b) Extract from $MgCl_2$-treated control leaves (24 hpi) and chemical structure of β-sitosterol. (c) Extract from *Psm avrRpm1*-inoculated leaves (24 hpi) and chemical structure of stigmasterol.

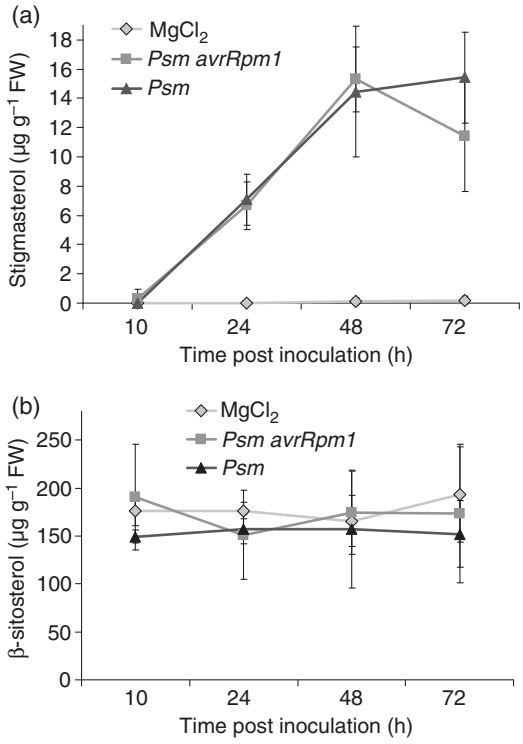

Figure 2. Time-course analyses of stigmasterol and β-sitosterol levels in Col-0 leaves after treatment with 10 mM $MgCl_2$, *Psm* or *Psm avrRpm1*.
(a) Leaf stigmasterol contents and (b) leaf β-sitosterol contents. Values are means ± SD (μg substance g^{-1} leaf fresh weight) from three samples, each consisting of six leaves.

significant alteration over time in $MgCl_2^-$, *Psm avrRpm1*- or *Psm*-treated leaves (Figure 2b). At 48 h after treatment, the molar ratio of stigmasterol to β-sitosterol is approximately 0.09 in *P. syringae*-inoculated leaves and 0.0007 in control leaves, reflecting a more than 100-fold pathogen-triggered increase in leaf stigmasterol content (Figure 3c).

According to the T-DNA Express Arabidopsis gene mapping tool (http://signal.salk.edu/cgi-bin/tdnaexpress), several Arabidopsis lines with putative T-DNA insertions in the *CYP710A1* gene exist. Using the PCR-based protocol described by Alonso *et al.* (2003), we identified two homozygous insertion lines from the SALK collection, *cyp710A1-1* (SALK_112491) and *cyp710A1-2* (SALK_014626), both with predicted T-DNA insertions in the promoter region of the *CYP710A1* gene. Neither insertion line shows any morphological or growth phenotype that would distinguish it from wild-type plants. However, compared to wild-type Col-0 plants, *cyp710A1-1* and *cyp710A1-2* are strongly impaired in the *P. syringae*-induced increase of *CYP710A1* transcript levels and accumulation of stigmasterol (Figure 3). This indicates that *P. syringae*-induced β-sitosterol to stigmasterol conversion results predominantly, if not exclusively, from pathogen-induced expression of *CYP710A1* and the concomitant increase in CYP710A1-mediated sterol C22 desaturase activity. A more diverse sterol analysis revealed that accumulation of stigmasterol is the most significant change in the overall sterol composition of Arabidopsis leaves upon pathogen inoculation, and that the only marked difference between wild-type and *cyp710A1* mutant plants is the strong attenuation of pathogen-triggered stigmasterol synthesis in *cyp710A1* (Table S1). Moreover, the pathogen-induced and CYP710A1-mediated C22 desaturation reaction is confined to the C24 ethylcholesterol β-sitosterol, because conversion of the structurally related 24-methylcholesterol (campesterol/24-*epi*-campesterol) to the corresponding

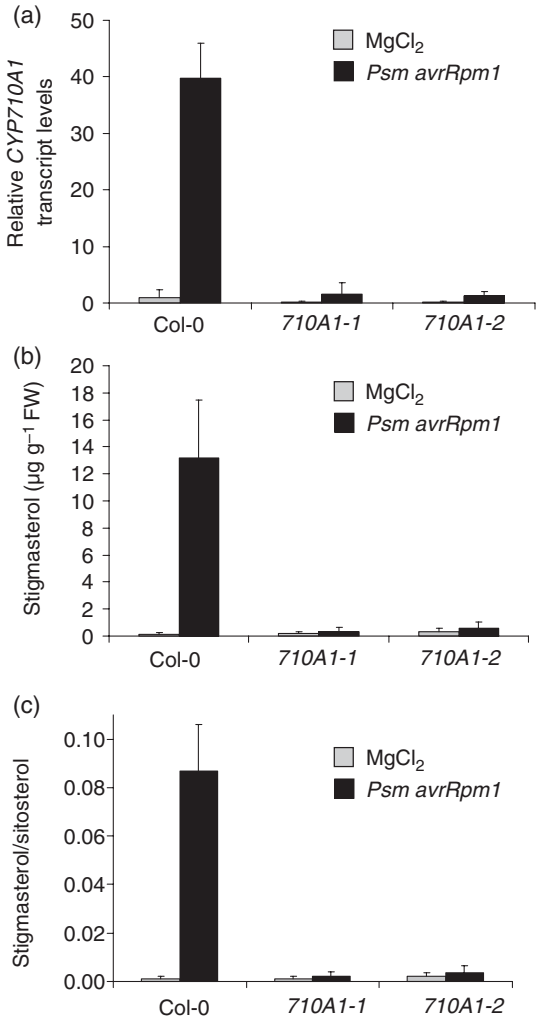

Figure 3. Transcript levels of the *CYP710A1* gene, stigmasterol content and stigmasterol/β-sitosterol ratios in leaves of Col-0, *cyp710A1-1* and *cyp710A1-2* plants at 48 h after treatment with $MgCl_2$ or *Psm avrRpm1*. Values are means ± SD of three leaf samples.
(a) Relative transcript levels of *CYP710A1*, as assessed by quantitative real-time PCR analysis. Transcript values were normalized to those of the *UBQ10* reference gene (At4g05320), and are expressed relative to the wild-type $MgCl_2$ sample. For each sample, three PCR replicates were performed and averaged.
(b) Leaf stigmasterol levels.
(c) Molar stigmasterol/β-sitosterol ratios.

24-methyl-Δ^{22}-cholesterol (crinosterol/brassicasterol) does not occur (Table S1).

Mutational defects in CYP710A1 lead to increased resistance towards *P. syringae*

To determine whether the considerable amount of stigmasterol that accumulates affects plant disease resistance, we determined the multiplication of various *P. syringae* strains in leaves of Col-0, *cyp710A1-*, and *cyp710A1-2* plants (Figure 4). Multiplication of the avirulent *Psm avrRpm1* strain was approximately five times lower in each of the *cyp710A1* mutant lines than in the Col-0 wild-type at 3 days post-inoculation (dpi) (Figure 4a). Similarly, the compatible *Psm* strain and another HR-inducing strain, *Psm avrRpt2*, showed significantly lower multiplication in the *cyp710A1* lines than in Col-0 (Figure 4b,c). By contrast, bacterial numbers of the non-adapted *Psg* strain were not statistically different in Col-0, *cyp710A1-1*, and *cyp710A1-2* leaves at 3 dpi (Figure 4d). Together, this suggests that the stigmasterol produced in response to *P. syringae* in wild-type plants favours bacterial multiplication of avirulent or virulent bacteria, and therefore enhances disease susceptibility towards adapted strains, whereas non-host resistance remains unaffected. We then tested whether exogenous application of stigmasterol at physiological concentrations would counteract the enhanced resistance phenotype observed in *cyp710A1* plants. We thus sprayed *Psm*- or *Psm avrRpm1*-inoculated leaves with a solution of 5 μM stigmasterol at 8 and 24 h after inoculation, and scored bacterial growth at 3 dpi. The enhancement of resistance observed in *cyp710A1* control plants was attenuated for both the incompatible and compatible interactions after stigmasterol application (Figure 5a,b). Moreover, exogenous stigmasterol application lowered the resistance of wild-type plants towards *Psm avrRpm1* (Figure 5a). By contrast, exogenous treatment of Col-0 leaves with β-sitosterol after pathogen inoculation had the opposite effect, and enhanced resistance to both *Psm avrRpm1* and *Psm* (Figure 5a,b). Thus, a higher ratio of stigmasterol to β-sitosterol in leaves appears to negatively affect resistance to virulent and avirulent *P. syringae* strains.

Upon local inoculation of *P. syringae*, systemic acquired resistance (SAR), a state of enhanced broad-spectrum disease resistance, develops throughout the whole plant (Cameron *et al.*, 1994; Mishina and Zeier, 2007b). To test whether sterol desaturation plays a role during the SAR process, we inoculated the lower (1°) leaves of Col-0 and *cyp710A1* mutant plants with the SAR-inducing *Psm* strain, and challenge-inoculated upper (2°) leaves 2 days later. After the 1° pathogen treatment, the *cyp710A1* lines were able to restrict bacterial growth during the 2° infection to the same extent as Col-0 plants, indicating that SAR is fully established in *cyp710A1* mutants and therefore does not depend on stigmasterol production (Figure 4e).

Stigmasterol formation is triggered by recognition of bacterial PAMPs

The conversion of β-sitosterol to stigmasterol in inoculated leaves is not only triggered by virulent and avirulent strains of *Psm* but also by the corresponding *Pst* strains (Figure 6a). Stigmasterol accumulation occurs only in pathogen-treated leaves and not in leaves distant from the initial inoculation site, suggesting that sterol C22 desaturation is a local not a systemic plant response (Figure 6a). Moreover, inoculation with the type III secretion-deficient *Pst hrpA*$^-$ strain or the

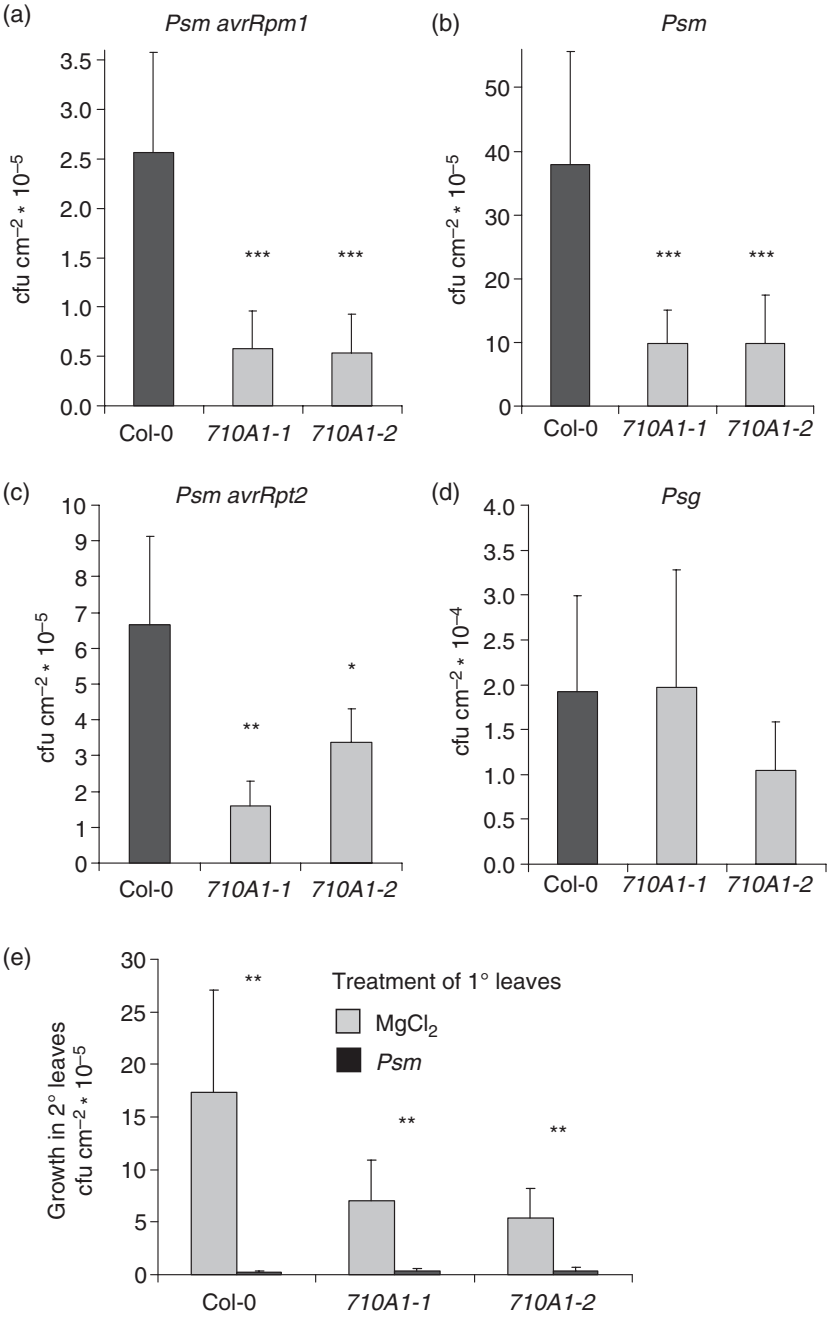

Figure 4. Resistance of Col-0, *cyp710A1-1* and *cyp710A1-2* plants to *P. syringae* leaf inoculation.

(a–d) Local resistance to avirulent, virulent and non-adapted *P. syringae*. Bacterial numbers for (a) *Psm avrRpm1* (applied in titres of OD 0.002), (b) *Psm* (OD 0.001), (c) *Psm avrRpt2* (OD 0.002) and *Psg* (OD 0.1) at 3 days after inoculation. Values are means ± SD [colony-forming units (cfu) per cm^2] from at least six parallel samples, each consisting of three leaf disks. Asterisks denote *cyp701A1* values that are statistically significantly different from the wild-type value (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's *t* test). To ensure uniformity of infiltrations, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for leaves of the different lines (data not shown).

(e) Systemic acquired resistance. Plants were pre-treated with either 10 mM $MgCl_2$ or *Psm* (OD 0.005) on three lower (1°) leaves. Two days later, three upper leaves (2°) were challenge-infected with *Psm* (OD 0.001). Bacterial growth in upper leaves was assessed 3 days after the 2° leaf inoculation. Asterisks indicate statistically significant differences of bacterial growth in 2° leaves between plants of a particular line pre-treated with *Psm* or $MgCl_2$ (**$P < 0.01$).

non-adapted *P. syringae* pv. *glycinea* (*Psg*) strain induced some local stigmasterol formation, which was statistically significant but lower than the accumulation in response to virulent and avirulent strains (Figure 6a).

The common responsiveness to various *P. syringae* pathovars and the TTSS-defective *Pst* strain indicate that β-sitosterol to stigmasterol conversion might initially be triggered by perception of conserved bacterial structures such as PAMPs rather than recognition of specific pathogen determinants such as TTSS effectors. We therefore investigated whether exogenous application of two well-characterized bacterial PAMPs, flagellin (the proteinaceous building unit of the bacterial flagellum) and lipopolysaccharide (LPS, a major component of the outer membrane of Gram-negative bacteria), promoted conversion of β-sitosterol to stigmasterol. Infiltration of 200 nM flg22, a peptide corresponding to the elicitor active epitope of flagellin (Gomez-Gomez *et al.*, 1999), into Col-0 leaves did result in considerable accumulation of stigmasterol at 48 h after treatment (Figure 6b). Significant increases in stigmasterol content also occurred when leaves were treated with gel-purified LPS preparations from *Pseudomonas*

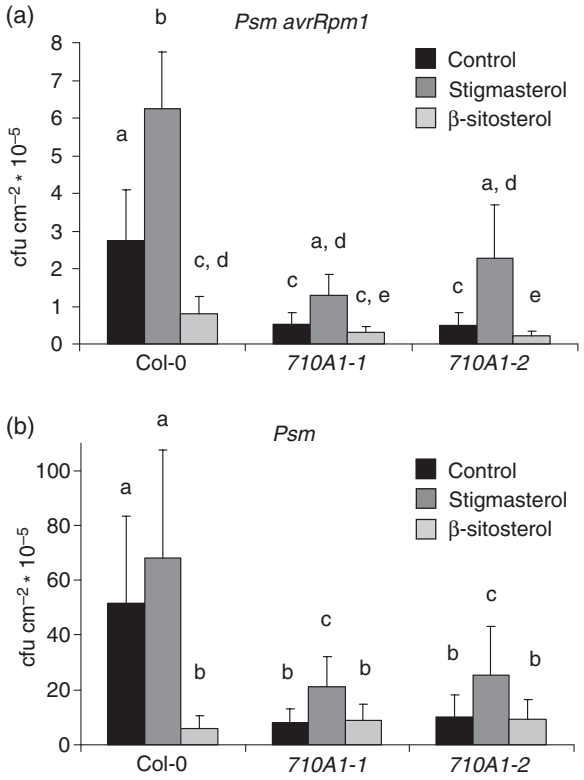

Figure 5. Influence of exogenous sterol application on Col-0, *cyp710A1-1* and *cyp710A1-2* resistance to *P. syringae*.
Inoculation experiments were performed as described in the legend to Figure 4. Inoculated leaves of plants were sprayed with 5 μM stigmasterol, 5 μM β-sitosterol, or a control solution (0.1% Tween/0.1% EtOH) at both 8 and 24 hpi, and bacterial growth was scored at 3 dpi. Values are means ± SD from at least eight parallel samples originating from one experiment. Values for bars not bearing the same letters are significantly different at $P < 0.05$ (Student's *t* test). Another biologically independent experiment yielded similar results.
(a) *Psm avrRpm1* (OD 0.002).
(b) *Psm* (OD 0.001).

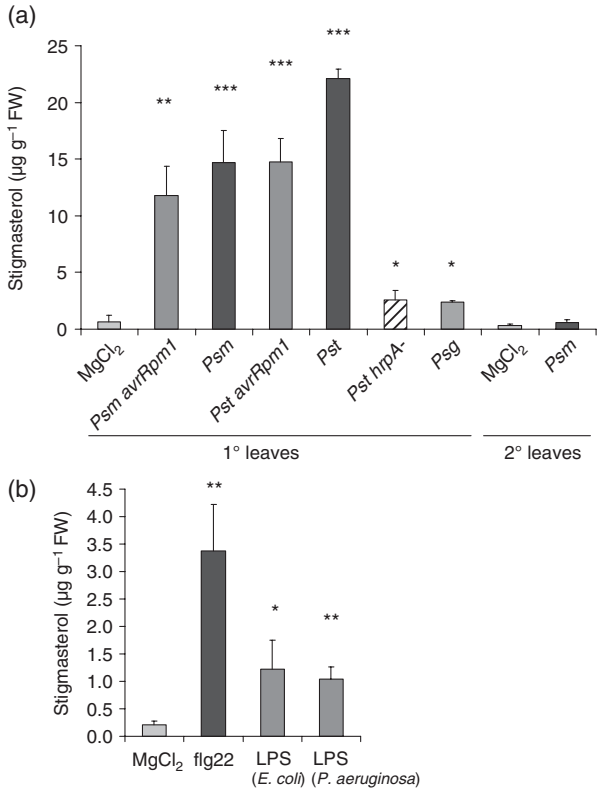

Figure 6. Leaf stigmasterol accumulation after inoculation with various *P. syringae* strains or bacterial PAMP treatments.
(a) Stigmasterol levels (means ± SD of three samples, in μg g^{-1} FW) in treated (1° leaves) or non-treated systemic leaves (2° leaves) of Col-0 plants at 48 h after 1° leaf infiltration. The 1° leaves were treated with 10 mM $MgCl_2$ or suspensions of *P. syringae* pv. *maculicola* ES4326 (*Psm*, *Psm avrRpm1*), *P. syringae* pv. *tomato* DC3000 (*Pst*, *Pst avrRpm1*), *Pst hrpA*⁻ (TTSS-defective) and *P. syringae* pv. *glycinea* race 4 (*Psg*; non-adapted).
(b) Stigmasterol levels (means ± SD of three samples) at 48 h after leaf treatment with 10 mM $MgCl_2$, 200 nM flg22 or 100 μg ml^{-1} lipopolysaccharide (LPS) purified from *Escherichia coli* or *Pseudomonas aeruginosa*.

aeruginosa or *Escherichia coli* at a concentration of 100 μg ml^{-1} (Figure 6b). Thus, single treatments of leaves with flagellin or LPS are sufficient to induce stigmasterol formation, suggesting that bacterial PAMP recognition initiates β-sitosterol C22 desaturation after *P. syringae* inoculation in Arabidopsis leaves.

Stigmasterol production is activated through increased ROS levels and occurs independently of SA, JA and ethylene signalling

To investigate whether induced stigmasterol biosynthesis is mediated by classical defence signalling pathways, we determined the leaf contents of stigmasterol in various well-characterized Arabidopsis defence mutants after *Psm avrRpm1* inoculation (Figure 7a). Wild-type-like stigmasterol production was observed in the SA pathway mutants *ics1* (*sid2*) and *npr1*, which are defective in SA biosynthesis and downstream signalling, respectively (Cao *et al.*, 1994; Nawrath and Métraux, 1999). Additionally, pathogen-induced stigmasterol levels in the leaves of the JA biosynthesis mutant *dde2* (Von Malek *et al.*, 2002), the JA signalling mutant *jar1* (Staswick *et al.*, 1992) and the ethylene-insensitive mutant *etr1* (Bleecker *et al.*, 1988) were similar to those of Col-0 plants. Likewise, the defence mutants *fmo1*, *ndr1* and *pad4*, which are compromised in SAR and certain local defence responses (Century *et al.*, 1995; Glazebrook *et al.*, 1997; Bartsch *et al.*, 2006; Mishina and Zeier, 2006), are still able to induce stigmasterol accumulation in a wild-type manner. Thus, stigmasterol production occurs independently of several well-described defence routes, including the SA-, JA- and ethylene-dependent pathways.

We noted that the Arabidopsis *cpr5* and *dnd1* mutants, which possess increased pathogen resistance because of constitutively activated defence responses (Bowling *et al.*, 1997; Yu *et al.*, 1998), show markedly increased leaf stigmasterol levels in the absence of a pathogen contact

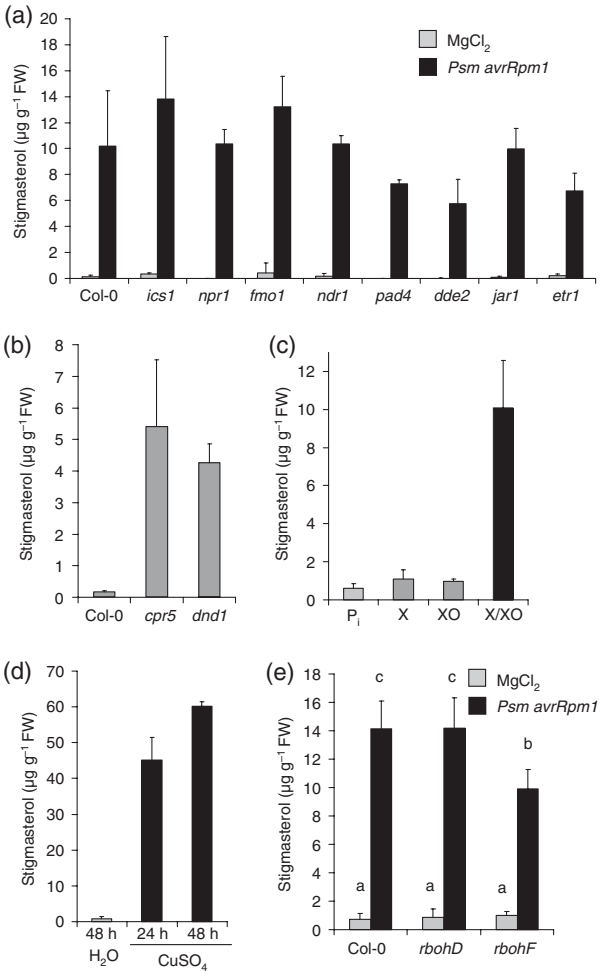

Figure 7. Induced stigmasterol synthesis in leaves of defence-related Arabidopsis mutants, and accumulation of stigmasterol in Col-0 leaves after treatment with ROS-generating substances (means ± SD of three samples, μg g^{-1} FW).

(a) Leaf stigmasterol levels of *Psm avrRpm1*- or mock-inoculated defence mutants and the Col-0 wild-type at 48 hpi.

(b) Constitutive levels of stigmasterol in leaves of untreated Col-0, *cpr5* and *dnd1* plants.

(c) Stigmasterol levels of Col-0 leaves treated with the superoxide-generating substrate/enzyme mix xanthine (X, 0.5 mM)/xanthine oxidase (XO, 0.5 U ml^{-1}), control buffer (20 mM sodium phosphate) or X or XO alone. Leaves were harvested from plants at 48 h after treatment.

(d) Stigmasterol levels of Col-0 leaves infiltrated with water or 10 mM $CuSO_4$. Leaves were harvested at the indicated times (h) after treatment.

(e) Leaf stigmasterol levels of *Psm avrRpm1*- or mock-inoculated *rboh* mutant and Col-0 wild-type plants at 48 hpi.

(Figure 7b). A common feature of *cpr5* and *dnd1* plants is constitutive enhancement of leaf ROS levels (Mateo *et al.*, 2006). We therefore tested whether exogenous ROS application alone is sufficient for the induction of stigmasterol biosynthesis. Infiltration of 0.5 mM xanthine and 0.5 U ml^{-1} xanthine oxidase, an enzyme/substrate mix that results in continuous production of O_2^- in the low micromolar range (Delledonne *et al.*, 1998), provoked accumulation of stigmasterol in Col-0 leaves at levels comparable to those found after *P. syringae* inoculation (Figure 7c). Moreover, infiltration of leaves with 10 mM copper sulfate, a treatment that leads to massive ROS production and oxidative stress (Drazkiewicz *et al.*, 2004), caused a vigorous increase in leaf stigmasterol content that exceeded the *P. syringae*-induced accumulation by a factor of three (Figure 7d). Thus, exogenous supply of ROS-generating compounds is sufficient for induction of stigmasterol biosynthesis in leaves.

A specific hallmark of the incompatible *P. syringae*–Arabidopsis interaction is the occurrence of an oxidative burst between 3 and 10 hpi, which is the consequence of resistance (R) protein-mediated recognition of a pathogen-derived avirulence factor (Lamb and Dixon, 1997; Zeier, 2005). The Arabidopsis respiratory burst oxidase homologue AtrbohD has been shown to be required for the *Pst avrRpm1*-triggered oxidative burst, with the related *AtrbohF* gene having a minor impact (Torres *et al.*, 2002). This was also the case for the *Psm avrRpm1*–Col-0 interaction, because *atrbohD* mutant plants were almost fully impaired in the early H_2O_2 burst at 4 hpi, whereas *atrbohF* plants showed only a slightly diminished H_2O_2 accumulation (Figure S2a). However, the leaf stigmasterol levels of both *atrbohD* and *atrbohF* plants are significantly increased after *Psm avrRpm1* inoculation, indicating that the early oxidative burst following avirulence protein recognition plays a minor role as a trigger for stigmasterol synthesis (Figure 7e).

Stigmasterol accumulation in *Botrytis cinerea*-infected leaves does not influence resistance against the fungal necrotroph

P. syringae is usually considered to be a biotrophic or hemibiotrophic phytopathogen (Glazebrook, 2005). To investigate whether β-sitosterol to stigmasterol conversion also plays a role in interactions of plants with pathogens that have a typically necrotrophic lifestyle, we performed leaf infection experiments with *Botrytis cinerea*, a fungus that causes soft rot disease on more than 200 plant species, including Arabidopsis. Inoculation of Arabidopsis leaves with *B. cinerea* spores resulted in early production of ROS, which is required for the pathogenicity of the fungal necrotroph (Govrin and Levine, 2000).

Spray inoculation of Col-0 leaves with *B. cinerea* strain B05.10 resulted in a marked increase in stigmasterol levels at 48 hpi, quantitatively similar to the increase detected after *P. syringae* infection. By contrast, stigmasterol accumulation was strongly attenuated in leaves of *B. cinerea*-infected *cyp710A1-1* plants (Figure 8a). To test whether the induced production of stigmasterol influenced the plants' susceptibility to *B. cinerea*, we droplet-inoculated the centre of Col-0 and *cyp710A1-1* leaves and followed the extent of tissue maceration, which was manifested as radial outgrowing lesions (Stefanato *et al.*, 2009). No apparent differences in the extent of disease lesions were obvious, however, and the

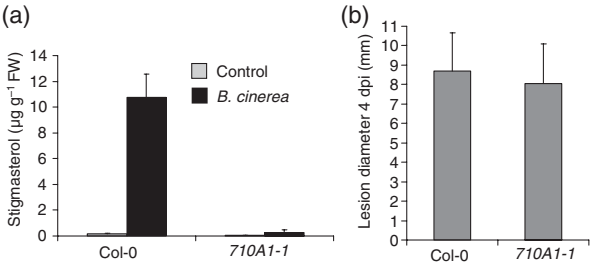

Figure 8. Stigmasterol accumulates upon *Botrytis cinerea* infection in Col-0 leaves without affecting resistance to the fungal necrotroph.
(a) Stigmasterol levels (means ± SD of three samples) in control leaves of Col-0 and *cyp710A1-1* plants and in leaves spray-inoculated with *B. cinerea* spores (48 h after treatment).
(b) Resistance of Col-0 and *cyp710A1-1* plants to *B. cinerea* droplet infection. The diameter of radially outgrowing lesions was determined at 4 dpi. Means ± SD of at least six leaf samples are given.

lesion diameters in Col-0 and *cyp710A1-1* leaves at 4 dpi showed no quantitative differences (Figure 8b). Thus, although *B. cinerea* infection induces sterol C22 desaturation in Arabidopsis leaves, the accumulating stigmasterol does not influence resistance against the necrotrophic fungus.

Stigmasterol production negatively affects expression of the positive defence regulator FMO1

To better understand the basis of the increased resistance of *cyp710A1* towards *P. syringae* infection, we examined the extent of inducible defence responses in Col-0 and *cyp710A1* plants. As the SA-associated defence pathway significantly contributes to restriction of *P. syringae* multiplication in leaves (Nawrath and Métraux, 1999), we first analysed SA accumulation and transcript levels of the SA-inducible *PR-1* gene. Compared to the wild-type, neither of the *cyp710A1* mutant lines showed constitutively increased levels of free SA, glycosidic SA or *PR-1* transcripts, excluding the possibility that the enhanced resistance phenotype of *cyp710A1* relies on constitutive activation of SA defence signalling (Figures 9a,b and 10a). Furthermore, synthesis of free and glycosidic SA in Col-0, *cyp710A1-1* and *cyp710A1-2* plants was induced upon *Psm avrRpm1* inoculation, and *PR-1* transcripts were increased to similar levels, suggesting that the SA pathway is not hyper-activated in *cyp710A1* mutants after pathogen contact (Figures 9a,b and 10a). The same is true for the oxidative burst, JA accumulation and the hypersensitive cell death response following *Psm avrRpm1* inoculation, because Col-0 and *cyp710A1* mutant plants induced these responses to similar extents (Figures 9c and 10c and S3a,b).

The flavin-dependent mono-oxygenase FMO1 positively regulates Arabidopsis pathogen resistance by an as yet unknown mechanism (Bartsch *et al.*, 2006; Mishina and Zeier, 2006). Expression of *FMO1* in pathogen-inoculated tissue occurs independently of SA signalling, and

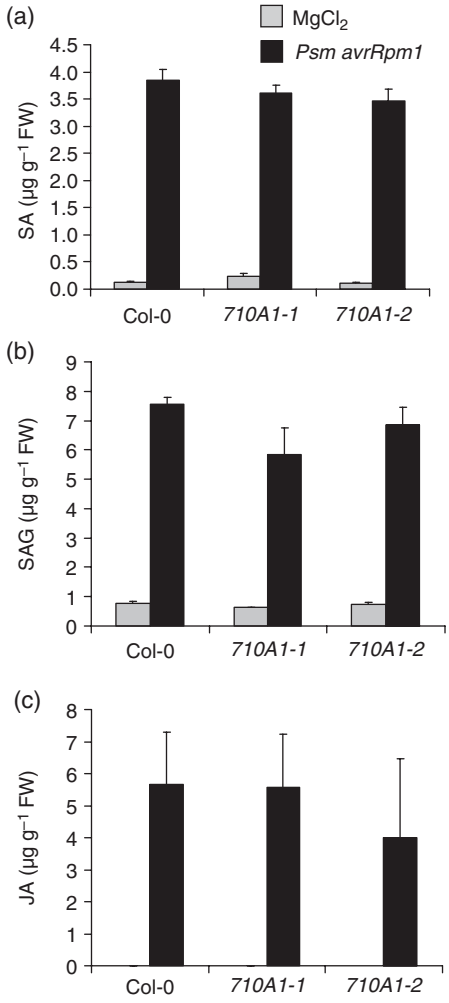

Figure 9. Defence responses in Col-0 and *cyp710A1* plants.
Accumulation of defence-related metabolites in leaves after *Psm avrRpm1* (OD 0.005) inoculation or $MgCl_2$ treatment. No statistically significant differences ($P < 0.05$; Student's *t* test) were detected between the values for pathogen-treated Col-0 and *cyp710A1-1* or *cyp710A1-2* mutants.
(a) Salicylic acid (SA) levels at 10 hpi (means ± SD of three samples).
(b) Levels of glucoside-bound SA (SAG) at 10 hpi (means ± SD of three samples).
(c) Jasmonic acid (JA) levels at 10 hpi (means ± SD of six samples).

over-expression of *FMO1* in Arabidopsis is sufficient to enhance plant resistance to *P. syringae* and *Hyaloperonospora arabidopsidis* (Bartsch *et al.*, 2006; Koch *et al.*, 2006). We found that the increase in *FMO1* transcript levels at 24 hpi was significantly higher in *Psm avrRpm1*-inoculated leaves of *cyp710A1* mutant plants than in Col-0 plants (Figure 10b), indicating that stigmasterol accumulation in Col-0 attenuates expression of this positive resistance regulator.

A well-described response of plant cells upon perception of bacterial elicitors (e.g. flagellin or elongation factor Tu) or fungal elicitors (e.g. ergosterol or chitin) is extracellular

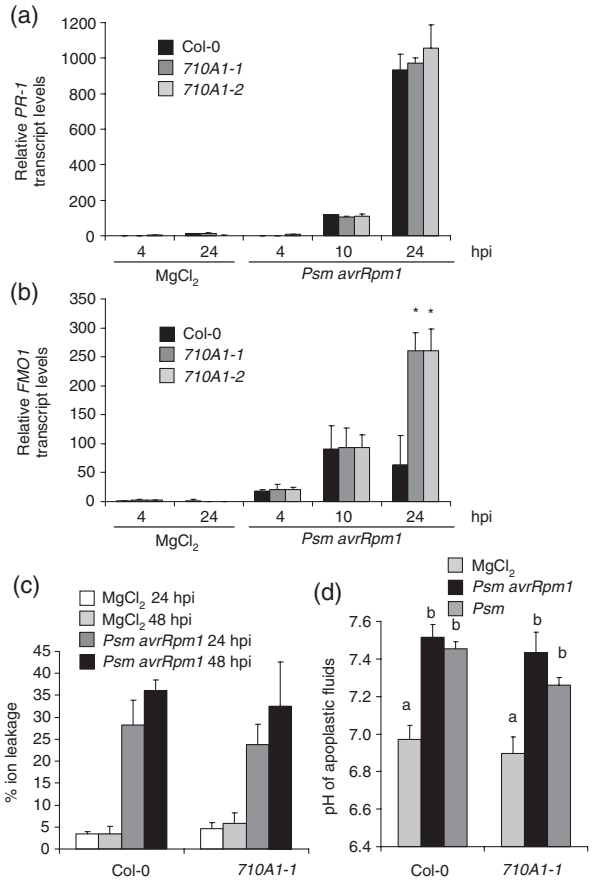

Figure 10. Defence responses in Col-0 and *cyp710A1* plants.
Transcript levels of defence-related genes, ion leakage from leaves, and alkalinization of apoplastic fluids after *P. syringae* inoculation.
(a,b) Relative transcript levels of defence-related genes, as assessed by quantitative real-time PCR analyses (see Figure 1a for details). (a) Transcript levels of the SA-inducible gene *PR-1*.
(b) Transcript levels of *FMO1*.
(c) Ion leakage from leaves at the indicated times after $MgCl_2$ or *Psm avrRpm1* treatment to assess the hypersensitive cell death response. The measured values are expressed as a percentage of the values obtained after leaf boiling (100% values). Values are means ± SD of four samples.
(d) pH values of apoplastic washing fluids from leaves at 2 days after treatment with 10 mM $MgCl_2$, *Psm* or *Psm avrRpm1*. Values are means ± SD of three samples. Different letters indicate statistically significant differences ($P < 0.05$).

alkalinization, which has been measured in the growth medium of suspension-cultured cells or directly in the leaf apoplast (Granado *et al.*, 1995; Felix *et al.*, 1999; Kunze *et al.*, 2004; Felle *et al.*, 2005). The fungal sterol ergosterol triggers extracellular alkalinization in tomato cells at picomolar concentrations. The side-chain double bond at C22 is a structural element common to ergosterol and stigmasterol. In contrast to other plant or animal sterols, stigmasterol also elicits medium alkalinization in tomato suspension cells, although the required concentrations are in the low micromolar range and thus much higher than for fungal ergosterol (Granado *et al.*, 1995). This prompted us to test whether plant-derived stigmasterol synthesized after pathogen attack acts directly as an elicitor and contributes to a medium alkalinization response in *P. syringae*-inoculated Arabidopsis leaves. We therefore determined the pH values of apoplastic washing fluids obtained from leaves after mock, *Psm avrRpm1* or *Psm* treatment. Inoculations with both *Psm avrRpm1* and *Psm* triggered a marked pH increase of 0.4–0.5 units in apoplastic fluids from both Col-0 and *cyp710A1-1* leaves, with no statistically significant difference between Col-0 and *cyp710A1-1* (Figure 10d). This suggests that *P. syringae* inoculation evokes apoplastic alkalinization of Arabidopsis leaves independently of stigmasterol accumulation, and that stigmasterol is not a direct elicitor of the alkalinization response.

Pathogen-induced stigmasterol is integrated into plant membranes

As sterols are characteristic constituents of biological membranes, we investigated whether the pathogen-induced accumulation of stigmasterol in leaves manifests itself in a higher content of the C22 unsaturated sterol in isolated plant membrane fractions after inoculation. We therefore isolated microsomal membrane fractions by leaf homogenization and ultracentrifugation, and subsequently enriched these fractions for plasma membranes (PMs) by two-phase partitioning (Laloi *et al.*, 2007). Microsomal and PM isolates were then subjected to chloroform/methanol extraction in order to determine their sterol composition by GC/MS. Similar to the situation in whole-leaf extracts (Figure 3c), the stigmasterol/β-sitosterol ratio strongly increased in both the microsomal and PM fractions of Col-0 leaves upon *Psm avrRpm1* inoculation from approximately 0.002 to 0.06, whereas the ratios in microsomal or PM isolates from *cyp710A1-1* leaves remained at basal values (Figure 11a,b). This indicates that the stigmasterol synthesized after pathogen contact in wild-type leaves is predominantly incorporated into plant membranes.

DISCUSSION

We describe here the conversion of β-sitosterol to stigmasterol, a plant metabolic process that is triggered after

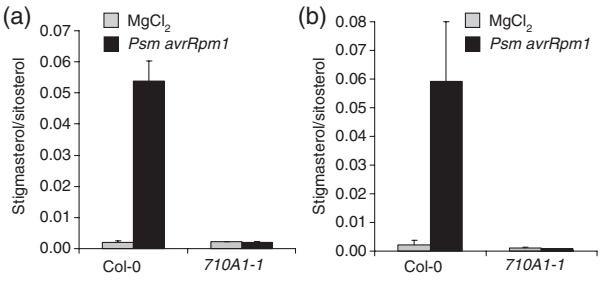

Figure 11. Stigmasterol/β-sitosterol ratios in microsomal membrane pellets (a) and plasma membrane fractions (b) isolated from Col-0 and *cyp710A1-1* leaves 48 h after mock or *Psm avrRpm1* treatment. Values are means ± SD from three samples.

bacterial and fungal pathogen infection. During the interaction of Arabidopsis plants with virulent and avirulent *P. syringae* strains, stigmasterol is produced in leaves between 10 and 48 h after inoculation and reaches levels of approximately 15 μg g^{-1} FW (Figure 2a). For comparison, increases in total levels of the defence hormone salicylic acid (i.e. the sum of free SA, glycoside-bound SA and MeSA) in *P. syringae*-inoculated Col-0 leaves amounted to 10–15 μg g^{-1} FW at 48 hpi under our routine experimental conditions (Mishina *et al.*, 2008; Attaran *et al.*, 2009). Thus, the *P. syringae*-induced accumulation of leaf stigmasterol is quantitatively similar to the increase in total SA, illustrating that, on a quantitative basis, the C22 desaturation of β-sitosterol constitutes a significant metabolic process in *P. syringae*-challenged Arabidopsis leaves. Stigmasterol production is not only evoked upon leaf inoculation with the hemibiotrophic bacterium *P. syringae*, but also after infection with the necrotrophic fungus *B. cinerea* (Figure 7a). As increased transcript levels of *CYP710A1* are also found in Arabidopsis leaves infected with the biotrophic fungus *Golovinomyces cichoracearum* or the hemibiotrophic oomycete *Phytophtora infestans* (Zimmermann *et al.*, 2004; Fabro *et al.*, 2008), it is likely that stigmasterol is produced in a range of mechanistically different plant–pathogen interactions.

In recombinant protein assays and *in planta* over-expression studies, the CYP710A family members CYP710A1, CYP710A2 and CYP710A4 are able to catalyse the C22 desaturation of β-sitosterol to stigmasterol (Morikawa *et al.*, 2006; Arnqvist *et al.*, 2008). Our *cyp710A1-1* and *cyp710A1-2* mutant analyses show that CYP710A1 is predominantly, if not exclusively, responsible for the pathogen-induced synthesis of stigmasterol in Arabidopsis leaves (Figures 3, 7a and 10). This may be explained by the fact that *CYP710A1* transcript levels are strongly increased upon pathogen inoculation (Figure 1a and S1a), whereas *CYP710A2* and *CYP710A4* are not pathogen-responsive and are expressed in leaves at comparatively low basal levels (Figure S1b,c). Moreover, our analysis of the overall sterol composition of wild-type and *cyp710A1* plants suggests that CYP710A1 specifically catalyses the conversion of β-sitosterol to stigmasterol *in planta*, but not the C22 desaturation of other sterols such as 24-methylcholesterols (Table S1). This is consistent with previous *in vitro* activity studies indicating that CYP710A1 has strict substrate specificity and can distinguish between distinct 24-alkyl groups (Morikawa *et al.*, 2006). Further, the wild-type-like growth phenotype of the *cyp710A1* plants suggests that brassinosteroid contents are not affected by CYP710A1, which acts at the end of the general sterol biosynthetic pathway in a branch that does not affect brassinolide biosynthesis. CYP710A-mediated C22 desaturation of β-sitosterol to stigmasterol is evolutionary conserved among land plants, as several functional isoforms of higher plants and the moss *Physcomitrella patens* have been characterized (Morikawa *et al.*, 2009). Interestingly, whereas stigmasterol is a minor sterol in the leaves of unstressed higher plants, it is the major sterol in *P. patens*, suggesting taxonomical differences in the stigmasterol/β-sitosterol ratio that may have functional implications (Morikawa *et al.*, 2009). However, the biological relevance of β-sitosterol C22 desaturation in higher and lower plants has not been clear until now.

Despite conversion to stigmasterol, the levels of the CYP710A1 substrate β-sitosterol do not decrease but essentially remain constant after pathogen inoculation (Figure 2b). It is thus likely that the proportion of β-sitosterol desaturated after pathogen contact is synthesized *de novo* through the sterol biosynthetic pathway. The first pathway-specific reaction of the sterol branch of the isoprenoid pathway in all eukaryotes is catalysed by squalene synthase, which converts two molecules of farnesyl diphosphate into the linear C30 terpenoid squalene. This is followed by epoxidation of squalene to 2,3-oxidosqualene through squalene epoxidase (Benveniste, 2004). Until recently, the next step was believed to differ in photosynthetic and non-photosynthetic eukaryotes. Whereas 2,3-oxidosqualene is known to be converted to lanosterol in fungi and animals, it had been assumed to be specifically cyclized into cycloartenol in plants (Schaller, 2004). However, Ohyama *et al.* (2009) recently identified lanosterol synthase 1 (LAS1) in Arabidopsis, and described a dual biosynthetic pathway in higher plants leading to phytosterols, in which the major part of the metabolic flux occurs via cycloartenol, and a minor part takes place via LAS1 and lanosterol. Several enzymatic steps involving methyl transferases, reductases, isomerases, demethylases and desaturases are required to convert cycloartenol or lanosterol to β-sitosterol (Schaller, 2004). Publicly available microarray data indicate that, other than *CYP710A1*, the transcripts of only a few Arabidopsis genes supposedly involved in phytosterol biosynthesis (listed in Benveniste, 2004) are increased in leaves upon *P. syringae* inoculation: the transcript levels of the two squalene epoxidase isoforms *SQE5* (At5g24150) and *SQE6* (At5g24160), as well as *LAS1* (At3g45130) are moderately increased at 24 h post-infection with avirulent or virulent *Pst* strains (Figure S4). It is thus possible that pathogen-induced *de novo* synthesis of β-sitosterol takes place via enhanced expression of squalene epoxidase and lanosterol synthase genes, and thus proceeds via the recently discovered plant lanosterol pathway.

Our data reveal that induced stigmasterol formation is independent of the SA and JA/ET defence pathways (Figure 7a). Moreover, exogenous application of two typical bacterial PAMPs, flagellin and LPS, is a sufficient trigger for stigmasterol production (Figure 6b). Stigmasterol also accumulates when Arabidopsis leaves are exogenously supplied with ROS-generating substances (Figure 7c,d). During the incompatible Arabidopsis–*Psm avrRpm1* interaction, ROS

are massively produced around 4 hpi, and this early oxidative burst is triggered via recognition of the bacterial effector AvrRpm1 by the plant resistance protein Rpm1 (Figure S2a) (Bisgrove *et al.*, 1994). By contrast, the early oxidative burst is absent in the compatible Arabidopsis–*Psm* interaction (Figure S 2b). Our finding that stigmasterol is produced with similar kinetics in response to the compatible and incompatible *Psm* and *Psm avrRpm1* strains between 10 and 48 hpi (Figure 2a) argues against a marked function for the early oxidative burst as a trigger of stigmasterol biosynthesis. This is corroborated by the fact that *atrbohD* mutants, in which the early *Psm avrRpm1*-induced H_2O_2 accumulation is strongly attenuated (Figure S2a), exhibit wild-type-like stigmasterol production after avirulent *P. syringae* inoculation. Both avirulent and virulent *P. syringae* possess bacterial PAMPs, and PAMP perception is known to initiate the endogenous generation of ROS (Felix *et al.*, 1999; Meyer *et al.*, 2001). Adapted *P. syringae* bacteria strongly multiply in the leaf apoplast between 10 and 48 hpi (Katagiri *et al.*, 2002; Mishina and Zeier, 2007b), and the concomitant exposure to bacterial PAMPs may give rise to a continuous production of ROS at later infection times. Consistent with this assumption, non-enzymatically produced oxylipins, whose formation mirrors increases in ROS, are formed in later stages of both the incompatible and compatible Arabidopsis–*P. syringae* interactions (Grun *et al.*, 2007). We thus propose that persistent, PAMP-induced ROS formation at later stages (between 10 and 48 hpi) after *P. syringae* inoculation is a likely stimulus for β-sitosterol C22 desaturation. A similar ROS trigger probably also occurs in *B. cinerea*-infected leaves, as the necrotrophic fungus evokes ROS formation during infection (Govrin and Levine, 2000).

We have shown that the induced accumulation of leaf stigmasterol and an overall increase in the ratio of stigmasterol to β-sitosterol in leaves favour apoplastic *P. syringae* multiplication and thus lead to enhanced disease susceptibility (Figures 4a–c and 5). The increased levels of stigmasterol do not influence the SA and JA defence pathways (Figure 9), and stigmasterol does not act as a direct elicitor of the extracellular alkalinization response evoked by *P. syringae* (Figure 10d). However, the pathogen-induced increase in stigmasterol in leaves weakens expression of the flavin-dependent monooxygenase FMO1 that acts as a positive regulator of Arabidopsis disease resistance (Figure 10) (Bartsch *et al.*, 2006; Koch *et al.*, 2006; Mishina and Zeier, 2006; Schlaich, 2007), and this may be sufficient for resistance attenuation. Thus, the stigmasterol produced may interfere with specific plant defence signalling pathways and thus increase susceptibility. Alternatively, stigmasterol may directly benefit the growth or virulence of plant pathogens. For instance, oomycetes of the genus *Phytophthora* lack sterol biosynthetic pathways and acquire sterols exogenously from their environment or host to support growth and sexual reproduction. For *Phytophtora sojae*, stigmasterol application has been shown to enhance hyphal growth in culture and attenuate the transcript levels of elicitins that putatively act as avirulence signals (Yousef *et al.*, 2009). Whether a similar impact of specific plant sterols exists for phytopathogenic bacteria such as *P. syringae* is not known.

Arabidopsis plants develop SAR in upper leaves approximately 2 days after inoculation of lower leaves with avirulent or virulent *P. syringae* (Cameron *et al.*, 1994; Mishina and Zeier, 2007b; Mishina *et al.*, 2008). The putative lipid transfer protein DIR1 is an essential SAR component that is necessary for the generation or translocation of a mobile long-distance signal (Maldonado *et al.*, 2002). DIR1 has been suggested to act as a chaperone for a potential lipid signal (Grant and Lamb, 2006). Sterols may be good candidates for transport by lipid transfer proteins because they can bind to elicitins, small cysteine-rich fungal proteins with structural similarity to plant lipid transfer proteins (Mikes *et al.*, 1998). In this context, it is interesting to note that stigmasterol formation and SAR establishment are induced by the same molecular determinants: PAMPs and ROS (Figures 6b and 7b,c) (Alvarez *et al.*, 1998; Mishina and Zeier, 2007b). Thus, we initially considered stigmasterol, which accumulates at the site of pathogen infection, as a good candidate for a mobile SAR signal. However, the SAR-positive phenotype of *cyp710A1* mutant plants clearly argues against a role for stigmasterol during SAR. Moreover, our finding that stigmasterol levels are not increased in systemic non-infected leaves confirms that the sterol is only involved in the modulation of local resistance, but not in SAR (Figure 6a).

Remarkably, the enhanced stigmasterol/β-sitosterol ratio in leaves was also found in microsomal membrane isolates and the plasma membrane fraction thereof (Figure 11). This suggests that the stigmasterol produced after pathogen inoculation is integrated into plant membranes, and thus alters the membrane sterol composition. As a varying ratio of individual sterols can alter the physical characteristics of lipid bilayers (Hartmann, 1998), we propose that the negative effect of induced stigmasterol production on resistance relies on an influence on the biophysical properties of plant membranes rather than on a direct signalling effect of free sterol molecules. Our findings that stigmasterol accumulation modulates resistance to *P. syringae* but not to *B. cinerea* might be related to the different lifestyles of the two pathogens, which are hemibiotrophic and necrotrophic, respectively.

How might an increased stigmasterol/β-sitosterol ratio in the membrane affect plant disease resistance? Compared to β-sitosterol, stigmasterol has an additional double bond in the sterol side chain. This renders the alkyl chain less flexible, and therefore influences the solubility and packing properties, and the ability to be accommodated in lipid bilayers. Experimental evidence exists that incorporation of stigmasterol in di- and monosaturated bilayers has a

reduced ordering effect on the bilayer hydrocarbon chains compared with integration of β-sitosterol (Hodzic *et al.*, 2008). Likewise, stigmasterol reduces the permeability of soybean phosphatidylcholine bilayers much less efficiently than β-sitosterol does (Schuler *et al.*, 1991). Distinct sterol molecules may also differently affect the activities of membrane-situated enzymes (Hartmann, 1998). For instance, stigmasterol was found to stimulate the activity of maize root H^+-ATPase, whereas its C22-saturated counterpart β-sitosterol did not (Grandmougin-Ferjani *et al.*, 1997). Several reports have proposed the existence of sterol- and sphingolipid-enriched membrane microdomains in plants that can be isolated as detergent-resistant membranes (Mongrand *et al.*, 2004; Bhat and Panstruga, 2005; Borner *et al.*, 2005; Laloi *et al.*, 2007). These so-called lipid rafts may play a role as platforms for the recruitment of molecular components involved in plant defence signalling (Bhat *et al.*, 2005). A prerequisite for the existence of membrane rafts is the ability of sterols to induce liquid-ordered membrane phases (Zappel and Panstruga, 2008). Whether individual phytosterols have different microdomain-inducing abilities has not yet been established. However, it is conceivable that a pathogen-induced change in the stigmasterol/β-sitosterol ratio can influence the number and/or physicochemical properties of ordered membrane (micro) domains and thereby modulate plant defence signalling.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana L. Heynh. plants were grown in a controlled environmental chamber (J-66LQ4, Percival, http://www.percival-scientific.com) with a 9 h day/15 h night cycle (daytime photon flux density 70 μmol m^{-2} sec^{-1}) and relative humidity of 70% (Griebel and Zeier, 2008). Growth temperatures during the day and night period were 21 and 18°C, respectively. Experiments were performed with 6-week-old, naïve and unstressed plants exhibiting a uniform appearance.

The *cyp710A1-1* and *cyp710A1-2* mutants corresponding to the SALK T-DNA insertion lines SALK_112491 and SALK_014626, respectively, both have a Col-0 background. To identify homozygous T-DNA insertion lines by PCR, the method described by Alonso *et al.* (2003) was applied, using the following gene specific primers: *CYP710A1-1* forward, 5′-CAAATTGCAATGGTATACCGG-3′; *CYP710 A1-1* reverse, 5′-TTGTGTTTTATACGACATTACTCGTG-3′; *CYP7 10A1-2* forward, 5′-AGAGAGTTGCTCGACGAGAAG-3′; *CYP710A1-2* reverse, 5′-GCGCTATACGC-AACTTGAAAC-3′. The T-DNA-specific primers were as proposed by Alonso *et al.* (2003).

Further mutants are described in Mishina and Zeier (2007b), except for *etr1* (Bleecker *et al.*, 1988), *cpr5* (Bowling *et al.*, 1997), *dnd1* (Yu *et al.*, 1998), *rbohD* and *rbohF* (Torres *et al.*, 2002). Wild-type plants in the Col-0 background were used as control plants.

Growth and inoculation of *Pseudomonas syringae*

The *P. syringae* strains used in this study, the bacterial culture and the inoculation experiments have been described in detail previously (Mishina and Zeier, 2007b). For determination of defence responses, bacterial suspensions of OD 0.005 (determination of transcript and metabolite levels), OD 0.002 (*Psm avrRpm1* growth assays) or OD 0.001 (*Psm* growth assays) were infiltrated into three full-grown leaves per plant. Bacterial growth was assessed 3 days after infiltration (Mishina and Zeier, 2007b).

Exogenous sterol application

A 5 mM stigmasterol (S2424; Sigma-Aldrich, http://www.sigma-aldrich.com) or β-sitosterol (S1270; Sigma-Aldrich) solution in EtOH was prepared and diluted in 0.1% Tween to a concentration of 5 μM. The final sterol solution was sprayed onto the surface of leaves at 8 and 24 h after *P. syringae* inoculation. As a control treatment, a solution of 0.1% Tween/0.1% EtOH in water was applied.

Growth and inoculation of *Botrytis cinerea*

The *Botrytis cinerea* strain B05.10 was cultured on potato dextrose agar as described previously (Stefanato *et al.*, 2009).

flg22 and LPS treatments

The flg22 peptide and purified LPS from *E. coli* and *P. aeruginosa* were diluted in 10 mM $MgCl_2$ to final concentrations of 200 nM (flg22) and 100 μg ml^{-1} (LPS), and infiltrated into leaves. The flg22 peptide, representing the elicitor active domain of bacterial flagellin (Felix *et al.*, 1999), was synthesized by Mimotopes (http://www.mimotopes.com). Chromatographically purified LPS preparations were acquired from Sigma-Aldrich (L3024 and L8643). Control infiltrations were performed with 10 mM $MgCl_2$.

Copper sulfate and xanthine/xanthine oxidase treatments

Copper sulfate (Merck, http://www.merck-chemicals.com) was infiltrated into the sample leaves at a concentration of 10 mM to induce high oxidative stress in the leaves. Control treatments were performed with deionized H_2O. To investigate the influence of ROS, the O_2^--producing combination of xanthine and xanthine oxidase was applied at a concentration of 0.5 mM xanthine and 0.5 units ml^{-1} xanthine oxidase in 20 mM sodium phosphate buffer (pH 6.5). Xanthine oxidase from buttermilk and xanthine were obtained from Sigma-Aldrich. Control treatments were performed with sodium phosphate buffer, as well as with xanthine or xanthine oxidase individually.

Determination of phytosterol content

For the determination of phytosterols, plant lipids were extracted from 150 mg FW of frozen leaf samples. Leaf tissue was homogenized with methanol:chloroform (2:1 v/v), an internal standard (10 μg ergosterol) was added, and lipids were extracted for 0.5 h at 70°C. After addition of 500 μl of H_2O, the mixture was thoroughly shaken and centrifuged for 1 min at 12 000 ***g*** for phase separation. The lower organic phase was removed, and the phase separation step was repeated. The collected organic phases were combined, dried over Na_2SO_4, and the volume was reduced in a stream of nitrogen. Samples were subject to a vapour-phase extraction procedure as described previously (Mishina and Zeier, 2006). Samples were eluted from the vapour-phase extraction column using 1 ml CH_2Cl_2, the volume was reduced to 80 μl, and sterols were converted to trimethylsilyl derivatives by adding of 10 μl of pyridine and 10 μl of BSTFA (*N,N-bis*-trimethylsilyltrifluoroacetamide). A 3 μl aliquot was subjected to GC/MS analysis (Mishina and Zeier, 2006). For quantification of sterols, peaks emanating from selected ion chromatograms [*m/z* 486 for β-sitosterol, *m/z* 484 for stigmasterol, *m/z* 380 for 24-methyl-Δ^{22}-cholesterol (brassicasterol), *m/z* 382 for 24-methyl-cholesterol (campesterol), *m/z* 329 for cholesterol, *m/z* 386 for (iso) fucosterol, *m/z* 393 for cycloartenol, *m/z* 468 for ergosterol] were integrated, and the corresponding peak areas were expressed

relative to the peak area of the internal standard ergosterol. Experimentally determined correction factors for each sterol/standard combination were taken into account.

Analysis of gene transcript levels

For analysis of gene transcript levels, RNA samples and the corresponding cDNA were prepared from frozen leaves as described by Mishina and Zeier (2006). Expression of *CYP710A1* (At2g34500), *PR-1* (At2g14610) and *FMO1* (At1g19250) was investigated using quantitative real-time PCR as described by Attaran *et al.* (2009). Transcript levels were analysed using the following gene-specific primers: 5′-AAGAAGCTCTTCGGTGACCA-3′ (*710A1* forward), 5′-GCTGGA GGG-CAGAGTAAGTG-3′ (*710A1* reverse), 5′-GTGCTCTTGTTCTTC CCTCG-3′ (*PR-1* forward), 5′-GCCTGGTTGTGAACCCTTAG-3′ (*PR-1* reverse), 5′-TCTTCTGCGTGCCGTAGTTTC-3′ (*FMO1* forward), 5′-CGCCATTTGACAAGAAGCATAG-3′ (*FMO1* reverse). The *UBQ10* gene (At4g05320), which is non-responsive to *P. syringae* inoculation, was used as the reference gene (Czechowski *et al.*, 2005).

Determination of defence metabolites

Determination of free SA, glycosidic SA and jasmonic acid levels in leaves was performed using vapour-phase extraction and subsequent GC/MS analysis as described by Mishina and Zeier (2006).

Assessment of the HR by ion leakage

Infected and control leaves were cut from plants and washed with deionized water. Individual leaves were put in small glass vessels filled with deionized water and slightly shaken for 2 h. The conductivity of the water solution was measured using a B173 conductivity meter (Horiba Instruments Ltd, http://www.horiba.com). Afterwards, samples were boiled for 0.5 h, and the conductivity after total leaf collapse was measured. The percentage ratio between the first measurement and the second measurement was used to assess the pathogen-induced level of membrane damage compared to the level of total damage after boiling.

Collection and pH determination of apoplastic washing fluids

Treated leaves were cut from plants 48 h after infection, and washed several times with deionized water. After covering them with fresh water, leaves were subjected to vacuum infiltration (vacuum pump MZ 2C, Vaccubrand, http://www.vacuubrand.com) for 20 min. Water on the leaf surfaces was removed with paper tissue, and apoplastic washing fluids were collected by centrifugation of the leaves at 500 ***g*** for 5 min. For each sample, 60 leaves from 20 different plants were used, and their washing fluids were combined. pH values were determined using an inoLab pH meter (pH Level 1, http://www.wtw.com).

Isolation of membranes

Microsomal membrane and plasma membrane isolates were obtained as described previously (Laloi *et al.*, 2007) with some modifications. Leaf material was homogenized in the presence of a buffer containing 330 mM sucrose, 1 M Tris/HCl, 0.5 M EDTA, 1 mM DTT and complete protease inhibitor cocktail tablets (Roche, http://www.roche.com). The homogenate was filtrated and centrifuged at 15 000 ***g***. The supernatant was again centrifuged at 100 000 ***g*** for 1 h yielding microsomal pellets that were either resuspended in 10 mM KH_2PO_4 (pH 8.2) for phytosterol determination or in a buffer containing 10% sucrose, 6 mM KCl, 5 mM K_2HPO_4 (pH 7.8) and protease inhibitor tablets for plasma membrane isolation. Plasma membranes were isolated by two-phase partitioning of the microsomal pellet samples between 6.4% PEG (Applichem, http://www.applichem.com) and 6.4% dextran T500 (Sigma-Aldrich) in 3 mM KCl, 10% sucrose and 50 mM K_2HPO_4 (pH 7.8). Centrifugation at 1500 ***g*** for 5 min resulted in phase partitioning, with an upper PEG-enriched phase containing the plasma membranes. For purification, phase-partitioning was repeated for both phases. The PEG-enriched phase was centrifuged for 1 h at 100 000 ***g***. The resulting plasma membrane pellets were resuspended in a buffer containing 1 mM DTT and 50 mM Tris/HCl (pH 7.4) and used for phytosterol determination.

Quantification of microscopic HR lesions and assessment of H_2O_2 production

The extent of microscopic HR lesion formation and H_2O_2 production were assessed using trypan blue and diaminobenzidine (DAB) staining procedures, respectively, as described by Zeier *et al.* (2004).

Reproducibility of experiments and statistical analyses

The data shown in the figures generally resulted from a single biological experiment. Unless otherwise stated, the results were similar in three biologically independent experiments. Statistical analyses were performed using Student's *t* test.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Transcript levels of *CYP710A* isogenes in Arabidopsis leaves upon challenge with various strains of *Pseudomonas syringae*.

Figure S2. Diaminobenzidine (DAB) staining to assess the oxidative burst in Arabidopsis leaves upon *Psm* and *Psm avrRpm1*) inoculation.

Figure S3. Assessment of the oxidative burst and microscopic HR lesions in Col-0 and *cyp710A1-1* leaves after *Psm avrRpm1* inoculation.

Figure S4. Sterol biosynthesis pathway genes with increased transcript levels in Arabidopsis leaves upon challenge with various strains of *Pseudomonas syringae* pv. *tomato*.

Table S1. Sterol composition of $MgCl_2$- or *Psm avrRpm1*-infiltrated leaves from Col-0, *cyp710A1-1* and *cyp710A1-2* plants.

REFERENCES

Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.

Alvarez, M.E., Pennell, R.I., Meijer, P.-J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell*, **92**, 773–784.

Arnqvist, L., Persson, M., Jonsson, L., Dutta, P.C. and Sitbon, F. (2008) Overexpression of *CYP710A1* and *CYP710A4* in transgenic Arabidopsis plants increases the level of stigmasterol at the expense of sitosterol. *Planta*, **227**, 309–317.

Attaran, E., Rostás, M. and Zeier, J. (2008) *Pseudomonas syringae* elicits emission of the terpenoid (E,E)–4,8,12-trimethyl-1,3,7,11–tridecatetraene in Arabidopsis leaves via jasmonate signaling and expression of the terpene synthase TPS4. *Mol. Plant Microbe Interact.* **21**, 1482–1497.

Attaran, E., Zeier, T.E., Griebel, T. and Zeier, J. (2009) Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in Arabidopsis. *Plant Cell*, **21**, 954–971.

Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J.L., Bautor, J. and Parker, J.E. (2006) Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monoxygenase FMO1 and the nudix hydrolase NUDT7. *Plant Cell*, **18**, 1038–1051.

Benveniste, P. (2004) Biosynthesis and accumulation of sterols. *Annu. Rev. Plant Biol.* **55**, 429–457.

Bhat, R.A. and Panstruga, R. (2005) Lipid rafts in plants. *Planta*, **223**, 5–19.

Bhat, R.A., Miklis, M., Schmelzer, E., Schulze-Lefert, P. and Panstruga, R. (2005) Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. *Proc. Natl Acad. Sci. USA*, **102**, 3135–3140.

Bisgrove, S.R., Simonich, M.T., Smith, N.M., Sattler, A. and Innes, R.W. (1994) A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. *Plant Cell*, **6**, 927–933.

Bleecker, A.B., Estelle, M.A., Somerville, C. and Kende, H. (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science*, **241**, 1086–1089.

Borner, G.H., Sherrier, D.J., Weimar, T., Michaelson, L.V., Hawkins, N.D., Macaskill, A., Napier, J.A., Beale, M.H., Lilley, K.S. and Dupree, P. (2005) Analysis of detergent-resistant membranes in Arabidopsis. Evidence for plasma membrane lipid rafts. *Plant Physiol.* **137**, 104–116.

Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F. and Dong, X. (1997) The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell*, **9**, 1573–1584.

Cameron, R.K., Dixon, R.A. and Lamb, C.J. (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. *Plant J.* **5**, 715–725.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X.N. (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell*, **6**, 1583–1592.

Century, K.S., Holub, E.B. and Staskawicz, B.J. (1995) NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc. Natl Acad. Sci. USA*, **92**, 6597–6601.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R. (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiol.* **139**, 5–17.

Dean, J.V. and Delaney, S.P. (2008) Metabolism of salicylic acid in wild-type, *ugt74f1* and *ugt74f2* glucosyltransferase mutants of *Arabidopsis thaliana*. *Physiol. Plant*, **132**, 417–425.

Delledonne, M., Xia, Y., Dixon, R.A. and Lamb, C. (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature*, **394**, 585–588.

Drazkiewicz, M., Skorzynska-Polit, E. and Krupa, Z. (2004) Copper-induced oxidative stress and antioxidant defence in *Arabidopsis thaliana*. *Biometals*, **17**, 379–387.

Fabro, G., Di Rienzo, J.A., Voigt, C.A., Savchenko, T., Dehesh, K., Somerville, S. and Alvarez, M.E. (2008) Genome-wide expression profiling Arabidopsis at the stage of *Golovinomyces cichoracearum* haustorium formation. *Plant Physiol.* **146**, 1421–1439.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**, 265–276.

Felle, H.H., Herrmann, A., Hückelhoven, R. and Kogel, K.H. (2005) Root-to-shoot signalling: apoplastic alkalinization, a general stress response and defence factor in barley (*Hordeum vulgare*). *Protoplasma*, **227**, 17–24.

Glazebrook, J. (2005) Contrasting mechanisms of defence against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmidt, R. and Ausubel, F.M. (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics*, **146**, 381–392.

Gomez-Gomez, L., Felix, G. and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* **18**, 277–284.

Govrin, E.M. and Levine, A. (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* **10**, 751–757.

Granado, J., Felix, G. and Boller, T. (1995) Perception of fungal sterols in plants (subnanomolar concentrations of ergosterol elicit extracellular alkalinization in tomato cells). *Plant Physiol.* **107**, 485–490.

Grandmougin-Ferjani, A., Schuler-Muller, I. and Hartmann, M.A. (1997) Sterol modulation of the plasma membrane H^+-ATPase activity from corn roots reconstituted into soybean lipids. *Plant Physiol.* **113**, 163–174.

Grant, M. and Lamb, C. (2006) Systemic immunity. *Curr. Opin. Plant Biol.* **9**, 414–420.

Griebel, T. and Zeier, J. (2008) Light regulation and daytime dependency of inducible plant defenses in Arabidopsis: phytochrome signaling controls systemic acquired resistance rather than local defense. *Plant Physiol.* **147**, 790–801.

Grun, C., Berger, S., Matthes, D. and Mueller, M.J. (2007) Early accumulation of non-enzymatically synthesized oxylipins in *Arabidopsis thaliana* after infection with *Pseudomonas syringae*. *Funct. Plant Biol.* **34**, 1–7.

Hartmann, M.-A. (1998) Plant sterols and the membrane environment. *Trends Plant Sci.* **3**, 170–175.

Hodzic, A., Rappolt, M., Amenitsch, H., Laggner, P. and Pabst, G. (2008) Differential modulation of membrane structure and fluctuations by plant sterols and cholesterol. *Biophys. J.* **94**, 3935–3944.

Katagiri, F., Thilmony, R. and He, S.Y. (2002) The *Arabidopsis thaliana-Pseudomonas syringae* interaction. In *The Arabidopsis Book* (Somerville, C.R. and Meyerowitz, E.M., eds). Rockville, MD: American Society of Plant Biologists, doi 10.1199-tab.0039.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F. and Kunkel, B.N. (2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana coronatine-insensitive* (*coi1*) mutation occurs through two distinct mechanisms. *Plant J.* **26**, 509–522.

Koch, M., Vorwerk, S., Masur, C., Sharifi-Sirchi, G., Olivieri, N. and Schlaich, N.L. (2006) A role for a flavin-containing mono-oxygenase in resistance against microbial pathogens in Arabidopsis. *Plant J.* **47**, 629–639.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. and Felix, G. (2004) The N-terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell*, **16**, 3496–3507.

Laloi, M., Perret, A.M., Chatre, L. *et al.* (2007) Insights into the role of specific lipids in the formation and delivery of lipid microdomains to the plasma membrane of plant cells. *Plant Physiol.* **143**, 461–472.

Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 251–275.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C. and Cameron, R.C. (2002) A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature*, **419**, 399–403.

Mateo, A., Funck, D., Mühlenbock, P., Kular, B., Mullineaux, P.M. and Karpinski, S. (2006) Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. *J. Exp. Bot.* **57**, 1795–1807.

Meyer, A., Puhler, A. and Niehaus, K. (2001) The lipopolysaccharides of the phytopathogen *Xanthomonas campestris* pv. *campestris* induce an oxidative burst reaction in cell cultures of *Nicotiana tabacum*. *Planta*, **213**, 214–222.

Mikes, V., Milat, M.L., Ponchet, M., Panabières, F., Ricci, P. and Blein, J.P. (1998) Elicitins, proteinaceous elicitors of plant defense, are a new class of sterol carrier proteins. *Biochem. Biophys. Res. Commun.* **245**, 133–139.

Mishina, T.E. and Zeier, J. (2006) The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiol.* **141**, 1666–1675.

Mishina, T.E. and Zeier, J. (2007a) Interactions of Arabidopsis with non-adapted *Pseudomonas syringae* strains: possible determinants of bacterial non-host resistance. *Physiol. Plant*, **131**, 448–461.

Mishina, T.E. and Zeier, J. (2007b) Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. *Plant J.* **50**, 500–513.

Mishina, T.E., Griebel, T., Geuecke, M., Attaran, E. and Zeier, J. (2008) New insights into the molecular events underlying systemic acquired

resistance. In *Biology of Plant-Microbe Interactions Volume 6* (Lorito, M., Woo, S.L. and Scala, F., eds). St Paul, MN: International Society for Molecular Plant–Microbe Interactions, paper 81.

Mongrand, S., Morel, J., Laroche, J., Claverol, S., Carde, J.P., Hartmann, M.A., Bonneu, M., Simon-Plas, F., Lessire, R. and Bessoule, J.J. (2004) Lipid rafts in higher plant cells: purification and characterization of Triton X-100-insoluble microdomains from tobacco plasma membrane. *J. Biol. Chem.* **279**, 36277–36286.

Morikawa, T., Mizutani, M., Aoki, N. *et al.* (2006) Cytochrome P450 *CYP710A* encodes the sterol C-22 desaturase in Arabidopsis and tomato. *Plant Cell*, **18**, 1008–1022.

Morikawa, T., Saga, H., Hashizume, H. and Ohta, D. (2009) *CYP710A* genes encoding sterol C22-desaturase in *Physcomitrella patens* as molecular evidence for the evolutionary conservation of a sterol biosynthetic pathway in plants. *Planta*, **229**, 1311–1322.

\Nawrath, C. and Métraux, J.P. (1999) Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell*, **11**, 1393–1404.

Ohyama, K., Suzuki, M., Kikuchi, J., Saito, K. and Muranaka, T. (2009) Dual biosynthetic pathways to phytosterol via cycloartenol and lanosterol in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **106**, 725–730.

Schaeffer, A., Bronner, R., Benveniste, P. and Schaller, H. (2001) The ratio of campesterol to sitosterol that modulates growth in Arabidopsis is controlled by STEROL METHYLTRANSFERASE 2;1. *Plant J.* **25**, 605–615.

Schaller, H. (2003) The role of sterols in plant growth and development. *Prog. Lipid Res.* **42**, 163–175.

Schaller, H. (2004) New aspects of sterol biosynthesis in growth and development of higher plants. *Plant Physiol. Biochem.* **42**, 465–476.

Schlaich, N.L. (2007) Flavin-containing monooxygenases in plants: looking beyond detox. *Trends Plant Sci.* **12**, 412–418.

Schuler, I., Milon, A., Nakatani, Y., Ourisson, G., Albrecht, A.M., Benveniste, P. and Hartman, M.A. (1991) Differential effects of plant sterols on water permeability and on acyl chain ordering of soybean phosphatidylcholine bilayers. *Proc. Natl Acad. Sci. USA*, **88**, 6926–6930.

Staswick, P.E., Su, W. and Howell, S.H. (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proc. Natl Acad. Sci. USA*, **89**, 6837–6840.

Stefanato, F.L., Abou-Mansour, E., Buchala, A., Kretschmer, M., Mosbach, A., Hahn, M., Bochet, C.G., Métraux, J.P. and Schoonbeek, H.-J. (2009) The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*. *Plant J.* **58**, 499–510.

Thomma, B.P., Nelissen, I., Eggermont, K. and Broekaert, W.F. (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant J.* **19**, 163–171.

de Torres Zabala, M., Bennett, M.H., Truman, W.H. and Grant, M.R. (2009) Antagonism between salicylic and abscisic acid reflects early host–pathogen conflict and moulds plant defence responses. *Plant J.* **59**, 375–386.

Torres, M.A., Dangl, J.L. and Jones, J.D. (2002) Arabidopsis *gp91phox* homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proc. Natl Acad. Sci. USA*, **99**, 517–522.

Von Malek, B., van der Graaff, E., Schneitz, K. and Keller, B. (2002) The Arabidopsis male-sterile mutant *dde2-2* is defective in the *ALLENE OXIDE SYNTHASE* gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. *Planta*, **216**, 187–192.

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*, **414**, 562–565.

Yaeno, T., Matsuda, O. and Iba, K. (2004) Role of chloroplast trienoic fatty acids in plant disease defense responses. *Plant J.* **40**, 931–941.

Yousef, L.F., Yousef, A.F., Mymryk, J.S., Dick, W.A. and Dick, R.P. (2009) Stigmasterol and cholesterol regulate the expression of elecitin genes in *Phytophtora sojae*. *J. Chem. Ecol.* **35**, 824–832.

Yu, I.-c., Parker, J. and Bent, A.F. (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. *Proc. Natl Acad. Sci. USA*, **95**, 7819–7824.

Zappel, N.F. and Panstruga, R. (2008) Heterogeneity and lateral compartmentalization of plant plasma membranes. *Curr. Opin. Plant Biol.* **11**, 632–640.

Zeier, J. (2005) Age-dependent variations of local and systemic defense responses in Arabidopsis leaves towards an avirulent strain of *Pseudomonas syringae*. *Physiol. Mol. Plant Pathol.* **66**, 30–39.

Zeier, J., Pink, B., Mueller, M.J. and Berger, S. (2004) Light conditions influence specific defence responses in incompatible plant-pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. *Planta*, **219**, 673–683.

Zhou, N., Tootle, T.L. and Glazebrook, J. (1999) Arabidopsis *PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *Plant Cell*, **11**, 2419–2428.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiol.* **136**, 2621–2632.