Phytosterols Play a Key Role in Plant Innate Immunity against Bacterial Pathogens by Regulating Nutrient Efflux into the Apoplast[1][C][W][OA]

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Bacterial pathogens colonize a host plant by growing between the cells by utilizing the nutrients present in apoplastic space. While successful pathogens manipulate the plant cell membrane to retrieve more nutrients from the cell, the counteracting plant defense mechanism against nonhost pathogens to restrict the nutrient efflux into the apoplast is not clear. To identify the genes involved in nonhost resistance against bacterial pathogens, we developed a virus-induced gene-silencing-based fast-forward genetics screen in *Nicotiana benthamiana*. Silencing of *N. benthamiana SQUALENE SYNTHASE*, a key gene in phytosterol biosynthesis, not only compromised nonhost resistance to few pathovars of *Pseudomonas syringae* and *Xanthomonas campestris*, but also enhanced the growth of the host pathogen *P. syringae* pv *tabaci* by increasing nutrient efflux into the apoplast. An Arabidopsis (*Arabidopsis thaliana*) sterol methyltransferase mutant (*sterol methyltransferase2*) involved in sterol biosynthesis also compromised plant innate immunity against bacterial pathogens. The Arabidopsis *cytochrome P450 CYP710A1*, which encodes C22-sterol desaturase that converts β-sitosterol to stigmasterol, was dramatically induced upon inoculation with nonhost pathogens. An Arabidopsis *Atcyp710A1* null mutant compromised both nonhost and basal resistance while overexpressors of *AtCYP710A1* enhanced resistance to host pathogens. Our data implicate the involvement of sterols in plant innate immunity against bacterial infections by regulating nutrient efflux into the apoplast.

A given plant species is normally susceptible to only a few pathogens although they are exposed to a vast number of potential pathogens in nature. Plants have evolved intricate defense mechanisms referred to as innate immunity to recognize and defend themselves against a wide array of pathogens (Hofius et al., 2007). Plant immunity involves pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI; Chisholm et al., 2006; Hofius et al., 2007). The resistance shown by all members of a plant species to the majority of potentially pathogenic microbes is known as nonhost resistance (Heath, 2000). Nonhost resistance is the most common and durable form of plant disease resistance in nature and is of great interest to agriculture (Ellis, 2006). Investigations of the molecular basis of nonhost resistance against bacterial pathogens are just emerging (Mysore and Ryu, 2004; Niks and Marcel, 2009).

Nonhost disease resistance involves two major components (Mysore and Ryu, 2004; Ellis, 2006). One is preformed defenses wherein the microbes are not able to overcome preexisting obstacles such as the cytoskeleton, antimicrobial compounds, and secondary metabolites. Another one is inducible defense responses that can be triggered by general elicitors from invading pathogens including PAMPs, such as lipopolysaccharides, flagellin and oligomers, or specific elicitors/effectors (Nürnberger and Lipka, 2005). ETI is also proposed to be a part of nonhost resistance (Schulze-Lefert and Panstruga, 2011). Many plant defense responses to pathogen infections are shared by nonhost and *R*-gene-mediated resistance (Heath, 2000; Mysore and Ryu, 2004; Freialdenhoven et al., 2005). For example, hypersensitive response (HR) is a typical resistance phenomenon that is associated with both *R*-gene and some nonhost resistance. Similar global gene expression profiles (Tao et al., 2003) and other induced responses (Zimmerli et al., 2004; Aghnoum and Niks, 2010; Zellerhoff et al., 2010; Hiruma et al., 2011) have been observed during nonhost resistance against fungal and bacterial pathogens.

Virus-induced gene silencing (VIGS) has been a popular approach to identify genes involved in disease resistance (Lu et al., 2003; Liu et al., 2004; del Pozo

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et al., 2004). One of the advantages of VIGS is that it allows the researcher to assess the function of genes where a null mutation might be lethal in sexually propagated plants or of genes that have functionally redundant family members (Senthil-Kumar et al., 2008; Senthil-Kumar and Mysore, 2011). In this study, we used VIGS-mediated fast-forward genetic screens (Lu et al., 2003; Anand et al., 2007) and identified several plant genes that play a role in nonhost disease resistance. We show squalene synthase (SQS), a key enzyme catalyzing the first enzymatic step in sterol biosynthesis, as a new player in nonhost disease resistance. Few studies have related the sterol metabolism to plant-microbe interaction (Devarenne et al., 1998, 2002; Griebel and Zeier, 2010). Previously, increased contents of stigmasterol in leaves through stimulation of sterol C22 desaturation after *Pseudumonas syringae* infection have been shown to attenuate pathogen-induced expression of the defense regulator flavin-dependent monooxygenase-1 (Griebel and Zeier, 2010). However, changes in membrane properties, due to alteration in sterols, leading to alteration in control of the efflux of cytoplasmic nutrients and solutes into apoplastic space where bacterial pathogens grow have not been explored (Sattelmacher, 2001; Bernsdorff and Winter, 2003; Hodzic et al., 2008; Melotto et al., 2008). Pathogens have mechanisms to derive nutrients from host plants, and it is speculated that there is a relationship between apoplastic nutrient levels and bacterial growth (Rico and Preston, 2008), but the mechanism is largely not studied. We systematically studied the relevance of membrane leakage and pathogen growth in two different plant species. Our results show that sterol content plays an important role in plant innate immunity against bacterial infections by regulating nutrient efflux into the apoplast.

RESULTS

Identification of Genes That Play a Role in Nonhost Resistance

A normalized *Nicotiana benthamiana* cDNA library (made from mixed-elicitor-treated leaf tissues; Anand et al., 2007) in tobacco rattle virus (*pTRV2*)-VIGS vector (Liu et al., 2002) was used in a forward-genetics screen to identify plant genes that play a role in nonhost resistance. Two leaves of each silenced plant were inoculated with a nonhost pathogen, *P. syringae* pv *tomato* T1 (*Pst* T1), expressing *GFPuv* (Wang et al., 2007) at a concentration of 1×10^5 colony forming units (cfu)/mL. Since the innate immunity limits the growth of nonhost pathogens, little or no green fluorescence emitted from *GFPuv*-expressing *Pst* T1 was observed in the wild-type and mock (TRV::00)-inoculated plant leaves under long-wavelength UV light (Fig. 1A). Of the 3,840 clones silenced, after three rounds of screening, nine of them showed varying levels of increased nonhost bacterial growth visualized by the intensity of the green fluorescence in the inoculated plant leaves (Fig. 1A).

To confirm whether the increase in green fluorescence correlated with the bacterial population, we conducted bacterial growth assays on *N. benthamiana* control (TRV::00) and gene-silenced plants that were vacuum infiltrated with *Pst* T1 (5×10^4 cfu/mL). As shown in Figure 1B, silenced plants accumulated 100- to 1,000-fold more bacteria by 7 d post inoculation (dpi) compared to the control. These results demonstrated the use of *GFPuv*-expressing bacteria in combination with VIGS-mediated forward genetics as a powerful approach to identify silenced plants that are compromised in nonhost disease resistance.

SQS Is Required for Plant Innate Immunity

The clone 6C8, that when silenced compromised nonhost resistance, was selected for further analyses. The insert in the 6C8 cDNA clone was sequenced, and this information was used to clone the full-length gene by RACE. The sequence was identical to a *N. benthamiana* gene (GenBank no. U46000) encoding SQS (NbSQS). Using real-time quantitative reverse transcription RT-PCR (qRT-PCR), we showed that *NbSQS* transcripts were significantly down-regulated in *N. benthamiana* plants silenced with the cDNA clone 6C8 (Supplemental Fig. S1A). The *SQS* gene is known to be involved in the biosynthesis of sterols in tobacco (*Nicotiana tabacum*; Devarenne et al., 2002). Consistent with its function, *NbSQS*-silenced plants showed a reduction in stigmasterol when compared to the control (TRV:00; Supplemental Fig. S1B).

We examined whether *NbSQS* is required for resistance against other nonhost pathogens and a host pathogen. Bacterial strains *Pst* T1, *P. syringae* pv *glycinea* (*Psg*), *Xanthomonas campestris* pv *vesicatoria* (*Xcv*), and *P. syringae* pv *tabaci* (*Pta*) expressing *GFPuv* were used to infiltrate *NbSQS*-silenced *N. benthamiana* and control plants using a needleless syringe. Strong green fluorescent spots produced by increased growth of *Pst* T1, *Psg*, and *Xcv* were observed in the *NbSQS*-silenced plant leaves, but not in the control plants, indicating that silencing of *NbSQS* compromised nonhost resistance to these bacteria (Fig. 1C). Silencing of this gene also resulted in the delayed occurrence of HR induced by nonhost pathogen *Pst* T1 (Supplemental Fig. S1C). Furthermore, the *NbSQS*-silenced leaf infiltrated with host pathogen *Pta* produced stronger green fluorescence than the control leaf (Fig. 1C). Estimation of bacterial number in planta by bacterial growth assay confirmed that there were more nonhost and host bacteria in the *NbSQS*-silenced plants than in the control plants (Fig. 1D). The silenced plants inoculated with *Psg* and *Xcv* also showed disease symptoms (Supplemental Fig. S1D). These results indicated that silencing of *NbSQS* not only compromised nonhost resistance, but also partially compromised basal resistance.

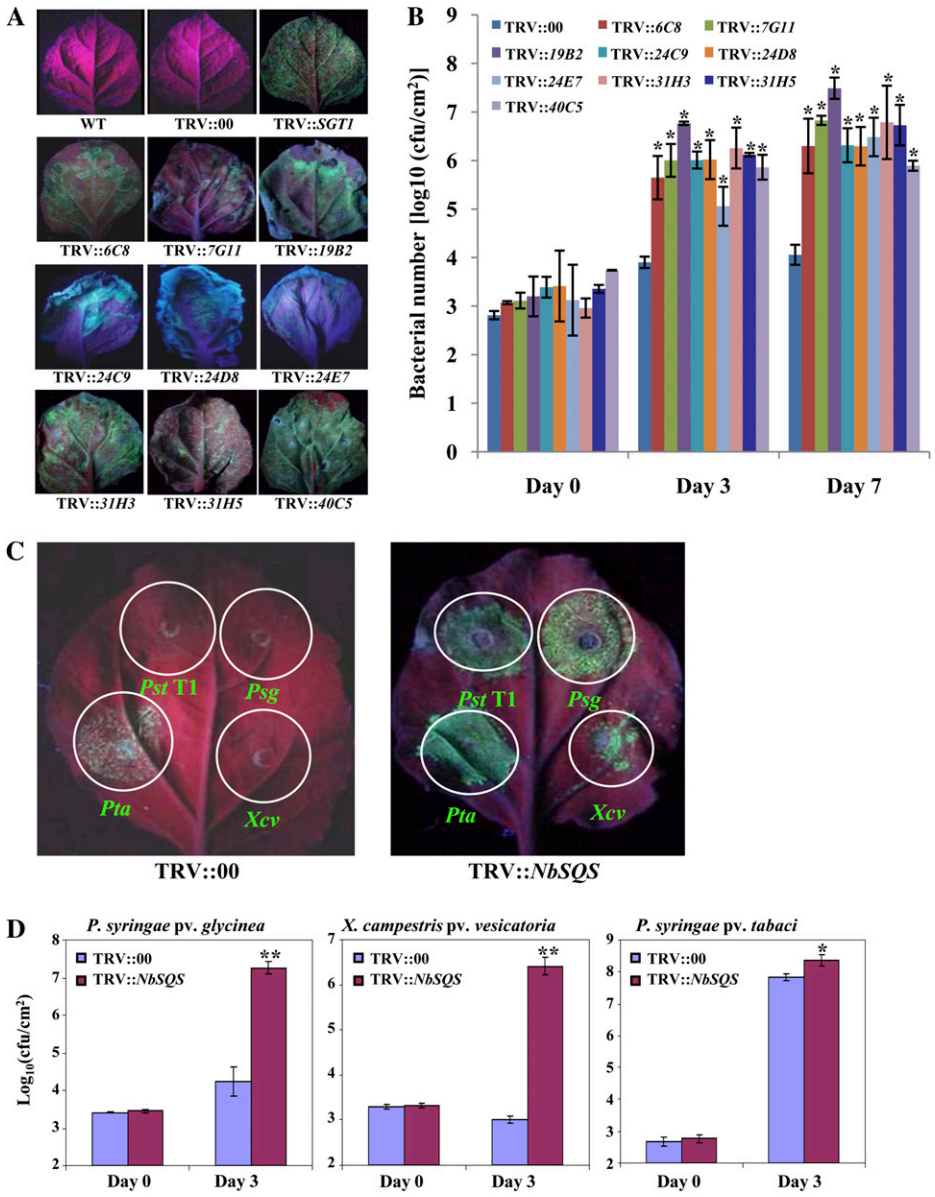

Figure 1. Identification of *NbSQS*-gene-silenced plants that compromise resistance to both host and nonhost pathogens. A, *N. benthamiana* plants (3 weeks old) were infiltrated with a mixture of *Agrobacterium tumefaciens* GV2260 carrying TRV1 and TRV2 containing *NbSGT1* (TRV::*NbSGT1*; positive control) or preselected TRV2 clones from the cDNA library or TRV2 empty vector (TRV::00; control). After 3 weeks, top leaves were syringe inoculated (in multiple spots) with a *GFPuv*-expressing nonhost pathogen, *Pst* T1, at a concentration of approximately 1×10^5 cfu/mL. Photographs were taken under long-wavelength UV light 4 dpi. B, Inoculation of *N. benthamiana* plants silenced by different cDNA clones was conducted as described above. Bacterial population was quantified by plating serial dilutions of leaf extracts. Asterisks indicate a significant difference from the control (Student's *t* test; $*P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. C, The *NbSQS*-silenced (TRV::*NbSQS*) and control (TRV::00) leaves were syringe inoculated with *GFPuv*-expressing nonhost pathogens *Pst* T1, *Psg*, and *Xcv*, and a host pathogen, *Pta*, at a concentration of 1×10^5 cfu/mL. Photographs were taken under long-wavelength UV light at 4 dpi. D, *NbSQS*-silenced and control plants were inoculated by vacuum infiltration with host and nonhost pathogens at a concentration of 1×10^5 cfu/mL. Bacterial population was quantified by plating the serial dilutions of leaf extracts at 0 and 3 dpi. Asterisks indicate a significant difference from the control (Student's *t* test; $*P < 0.05$ and $**P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. See also Supplemental Figures S1 and S2.

Silencing of *NbSQS* in *N. benthamiana* Results in Electrolyte Leakage and Nutrient Efflux

SQS is a key enzyme catalyzing the first enzymatic step in the biosynthesis of phytosterols, which are one of the major components of the cell membrane (Supplemental Fig. S2, A and B; Devarenne et al., 2002). Therefore, we tested through microscopy whether *NbSQS*-silenced plants have highly permeable membranes. Propidium iodide (PI), which binds to and stains cellular DNA, can only enter and stain cells with a permeable plasma membrane (Harrison and Vickers, 1990). In this study, PI was used to assess membrane permeability of control and *NbSQS*-silenced *N. benthamiana* cells. Leaf segments were stained with PI, and the entry of PI into plant cells was monitored under a confocal microscope. The *NbSQS*-silenced plants showed a significantly higher number of stained nuclei (red fluorescence) than the control plant cells (Fig. 2, A and B). Interestingly, the *NbSQS*-gene-silenced plants had larger cell size (Supplemental Fig. S2C). As a positive control, we showed that PI can enter and stain the cell nucleus much faster in heat-stressed tissues when compared to the control (Supplemental Fig. S2, D and E); this can be attributed to disruption of the plasma membrane due to heat stress (Wahid et al., 2007; Wang et al., 2011). In addition, we showed that the entry of FM1-43 dye was higher in the silenced cells (Supplemental Fig. S2F). These results indicate that the cell membrane of *NbSQS*-silenced plants is highly permeable.

We further examined whether silencing of *NbSQS* resulted in altered cell membrane permeability by measuring the electrolyte conductivity. *NbSQS*-silenced *N. benthamiana* leaves showed more than 50% ion leakage, while the control leaves had only approximately 20% ion leakage (Fig. 2C). This result indicates that silencing of *NbSQS* in *N. benthamiana* plants releases more ions from the cytoplasm into the apoplast. This is analogous to the situation wherein the host pathogen *Pta* manipulates the host cells to release more electrolytes in *N. benthamiana* and multiplies in planta, unlike nonhost pathogens (Fig. 2D). *Pst* T1 is known to induce HR cell death at early stages of infection and, therefore, slightly higher leakage can be observed in leaves inoculated with this nonhost pathogen (Fig. 2D).

To check whether the increased electrolyte leakage in *NbSQS*-silenced plants is associated with organic compounds, we performed metabolite profiling of apoplastic fluids from *NbSQS*-silenced and control plant leaves using gas chromatography-mass spectrometry (GC-MS; Broeckling et al., 2005). Interestingly, 420 $\pm$ 11 components were detected by GC-MS analyses in the apoplast from *NbSQS*-silenced *N. benthamiana*, while only 261 $\pm$ 5 were detected from the control plants. Among the commonly identified compounds, 35 compounds were at least 2-fold higher in the apoplast from *NbSQS*-silenced *N. benthamiana* than in the control plants (Supplemental Table S1). Out of these 35 compounds, 21 were sugars. In fact, we observed that *Pta* causes more efflux of nutrients into the apoplast of *N. benthamiana* when compared to nonhost pathogens that were similar to the mock treatment (Supplemental Table S2). These results indicate that, much like a virulent pathogen infection led to nutrient efflux, silencing of *NbSQS* in *N. benthamiana* leads to nutrient efflux from the plant cell into the apoplastic space that could serve as nutrients for multiplication of host and nonhost pathogens.

Apoplastic Extracts from *NbSQS*-Silenced *N. benthamiana* Enhances Bacterial Multiplication in Vitro

To examine whether the nutrient-rich apoplastic fluid from the *NbSQS*-silenced *N. benthamiana* plants supports faster bacterial multiplication than the apoplastic fluids from control plants, we measured the multiplication rate of different bacterial strains including host, nonhost, and nonpathogenic bacteria using minimal growth medium (MGM) supplemented with 5% of apoplastic extracts from *NbSQS*-silenced and control plants. All tested bacteria, including nonpathogenic bacterium *Escherichia coli*, multiplied faster in MGM containing 5% apoplast from the *NbSQS*-silenced *N. benthamiana* plants than in MGM containing 5% apoplast from the control plants (Fig. 2E).

Silencing of *AtSQS* Compromises Plant Innate Immunity in Arabidopsis

Arabidopsis (*Arabidopsis thaliana*) contains two SQS-annotated genomic sequences: At4g34640 (*AtSQS1*) and At4g34650 (*AtSQS2*), which are located in tandem array in chromosome 4 and share 80.7% identity in amino acid sequences. The amino acid sequence of *NbSQS* shows 73.9% identity to *AtSQS1* and 72.8% to *AtSQS2*. Several potential Arabidopsis individual T-DNA knockout lines for *AtSQS1* and *AtSQS2* genes were obtained from the SALK mutant collection, but no homozygous mutants for either *AtSQS1* or *AtSQS2* knockouts could be isolated. Therefore, to examine the function of Arabidopsis *SQS* in disease resistance, we developed Arabidopsis *SQS* RNA interference (RNAi) lines. A recent study has demonstrated that Arabidopsis contains only a single gene (*AtSQS1*) encoding SQS since the expressed protein encoded by *AtSQS2* is unable to synthesize squalene from farnesyl diphosphate (Busquets et al., 2008). Therefore, the nucleotide sequence of *AtSQS1* was used to make a construct for development of *AtSQS1* RNAi lines. *AtSQS1* RNAi homozygous lines showed an elongated petiole compared to wild type (Supplemental Fig. S3A). Examination of the *AtSQS1* transcript in the RNAi lines by qRT-PCR indicated that four out of five transgenic lines tested showed a reduction in the expression of *AtSQS1*, but not *AtSQS2*, compared to the vector control (Supplemental Fig. S3B).

AtSQS1 RNAi lines were challenged with a nonhost pathogen, *Pta*, by leaf infiltration at a concentration of 1×10^6 cfu/mL. The disease symptoms were observed

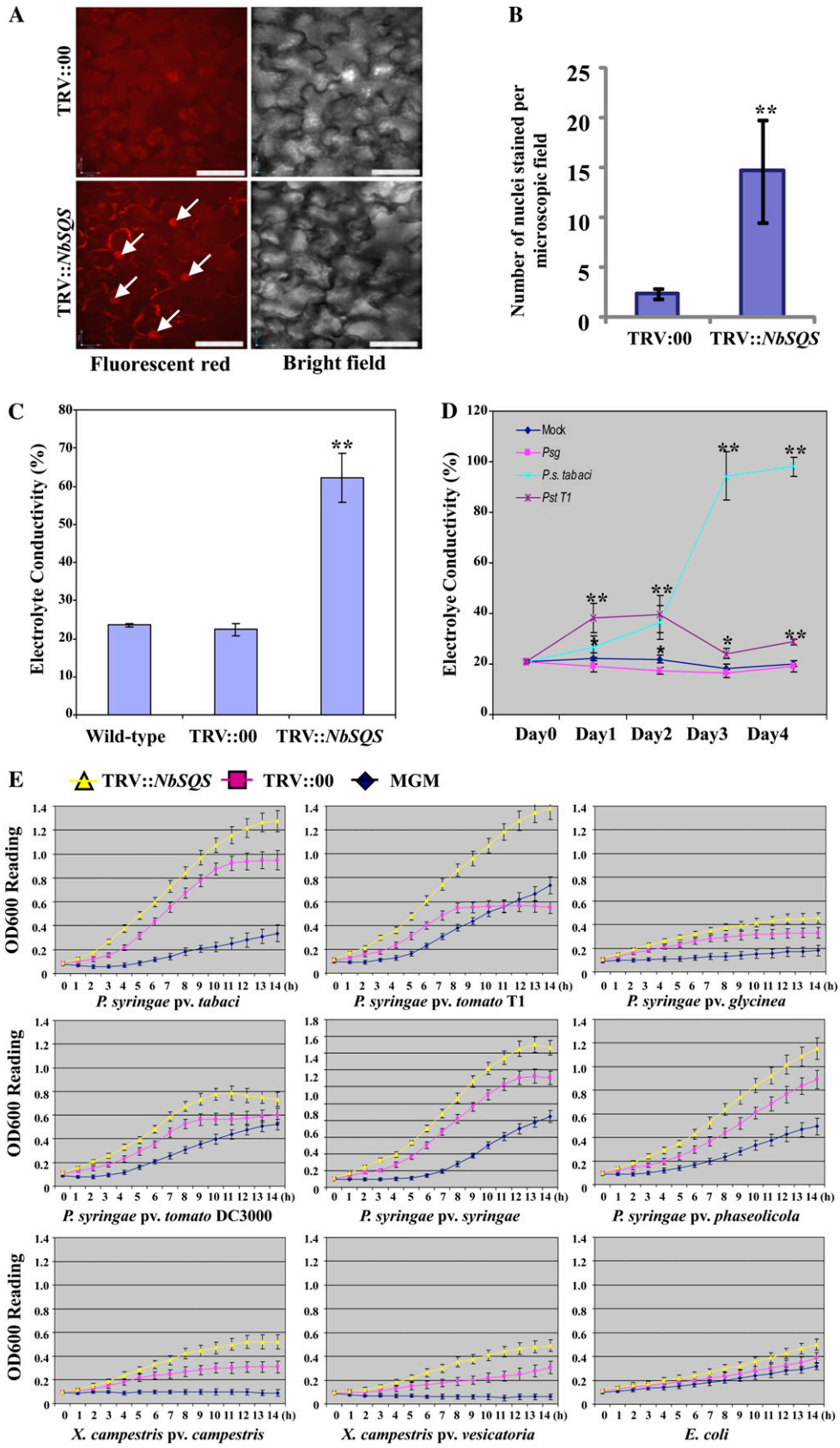

Figure 2. (*Legend appears on following page.*)

only in the transgenic *AtSQS1* RNAi lines of Arabidopsis that showed reduced transcription of *AtSQS1*, but not in the empty vector control line and an RNAi line (SSi1e) that did not reduce *AtSQS1* transcripts (Supplemental Fig. S3C). Correspondingly, bacterial growth assays indicated that the transgenic *AtSQS1* RNAi lines of Arabidopsis were susceptible to the nonhost pathogen *Pta* when compared to control plants (Supplemental Fig. S3D). These results demonstrated that the *AtSQS1* was required for plant nonhost resistance and is a functional analog of *NbSQS*.

Arabidopsis *sterol methyltransferase2* Compromises Plant Innate Immunity

Silencing of *AtSQS1* could affect multiple pathways involved in the biosynthesis of brassicasterol, brassinolide, and stigmasterol. Therefore, it is important to elucidate which phytosterol-specific pathway contributes to plant nonhost resistance. Sterol methyltransferase2 (SMT2), catalyzing the second methyl transfer reaction in the biosynthesis of phytosterols, serves as a branch point between in vivo synthesis of β-sitosterol/stigmasterol and brassinosteroids (Supplemental Fig. S2A). Thus, we tested the susceptibility of an Arabidopsis *smt2* mutant, previously identified as *cotyledon vascular pattern1* (*cvp1*; Carland et al., 2002), to nonhost pathogens. Upon challenging with nonhost pathogens *Pta* and *P. syringae* pv *syringae* (*Pss*), disease symptoms were developed on *AtSQS1* RNAi line and *smt2* mutant (*cvp1-4*) plant leaves, but not on the wild-type Columbia-0 (Col-0; Fig. 3A).

To test whether an Arabidopsis *AtSQS1* RNAi line (SSi5e) and *smt2* mutant compromise basal resistance against virulent pathogens, 4-week-old seedlings were inoculated with *Pst* DC3000 and *P. syringae* pv *maculicola* (*Psm*). All plants were inoculated by dipping in 1×10^8 cfu/mL of bacteria. The SSi5e RNAi line and *smt2* mutant plants showed enhanced chlorotic and necrotic disease symptoms at 4 dpi, compared to wild-type Col-0 plants infected with the same pathogens (Fig. 3B). Bacterial growth assay confirmed that both *smt2* mutant and *AtSQS1* RNAi line supported higher levels of bacterial multiplication of *Pta* and *Pss* than the wild-type Col-0 plants in repeated experiments (Fig. 3C). Similarly, SSi5e and *smt2* mutant plants supported more *Pst* DC3000 and *Psm* bacterial multiplication than Col-0 plants (Fig. 3D). These results indicated that *smt2* plants were susceptible, to a similar degree as the *AtSQS1* RNAi line, to host and nonhost pathogens tested, suggesting that the SMT2-mediated phytosterol biosynthetic pathway plays a key role in plant innate immunity.

AtCYP710A1 Gene Positively Regulates Plant Innate Immunity

SQS catalyzes the first step in the biosynthesis of various phytosterols, while sterol methyltransferase is a key branching-point enzyme leading to biosynthesis of β-sitosterol and stigmasterol. Therefore, Arabidopsis *smt2* mutant and *AtSQS1* RNAi lines may contain altered levels of β-sitosterol and stigmasterol. Maintaining the correct balance of these phytosterols in plants may be related to plant defense because phytosterols can modulate permeability and fluidity of membrane (Schuler et al., 1991). To test this hypothesis, we first examined the content of β-sitosterol and stigmasterol in a *smt2* mutant and *AtSQS1* RNAi lines by GC-MS. As expected, β-sitosterol and stigmasterol in the *smt2* mutant and *AtSQS1* RNAi lines were reduced compared to the wild-type Arabidopsis (Fig. 4, A and B). To examine whether the levels of these sterols are affected when plants are infected with nonhost pathogens, we inoculated the plants by vacuum infiltration with a nonhost pathogen, *Pta*, at a concentration of 1×10^5 cfu/mL. The content of β-sitosterol was reduced in all tested lines, while stigmasterol was dramatically induced 12 h post inoculation (hpi; Fig. 4, A and B).

Figure 2. Silencing of *NbSQS* leads to leaky cell membrane and the apoplastic fluid of silenced plants supports more bacterial multiplication in vitro. A and B, Leaf tissues were collected from TRV::00 (control) and *NbSQS*-silenced plants, and incubated in a solution containing PI for 30 min. Sections were made and observed under a confocal microscope (Perkin Elmer ultra view ERS spinning disk confocal) for PI-stained fluorescent nuclei (left). Corresponding bright-field images were also taken at the same focal area (right). Scale bar in each image represent 50 micron. The number of stained nuclei were counted and represented in the graph. An asterisk indicates a significant difference from the control (Student's *t* test; $P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. C, Three weeks after silencing, two leaf discs (0.5-cm^2 each) of the *NbSQS*-silenced *N. benthamiana* plants were immersed into deionized water and electrolyte conductivity was measured. Electrolyte leakage was calculated as a percentage of the conductivity before autoclaving to the conductivity after autoclaving. Asterisks indicate a significant difference from the control (Student's *t* test; $P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. D, The wild-type *N. benthamiana* plant leaves were inoculated with *Pst* T1, *Psg*, and *Pta* at a concentration of approximately 5×10^4 cfu/mL. Electrolyte leakage was measured every 24 h after inoculation. Asterisks indicate a significant difference from the control (*$P < 0.05$ and **$P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. E, Apoplastic fluid was extracted from TRV::00 and TRV::*NbSQS*-silenced *N. benthamiana* leaves by the vacuum-centrifuge method. The filter-sterilized apoplastic fluid was added to MGM to make 5% apoplastic fluid. The bacterial population was quantified by measuring the optical density at 600 nm (OD_{600}) of bacterial cultures every hour. Diamond: MGM alone; square: MGM containing 5% apoplast extract from control plants; triangle: MGM containing 5% apoplast extract from *NbSQS*-silenced *N. benthamiana*. *x* axis represents time in hours. Error bars represent SDs of three technical replicates. Results were similar for two independent experiments. See also Supplemental Table S2.

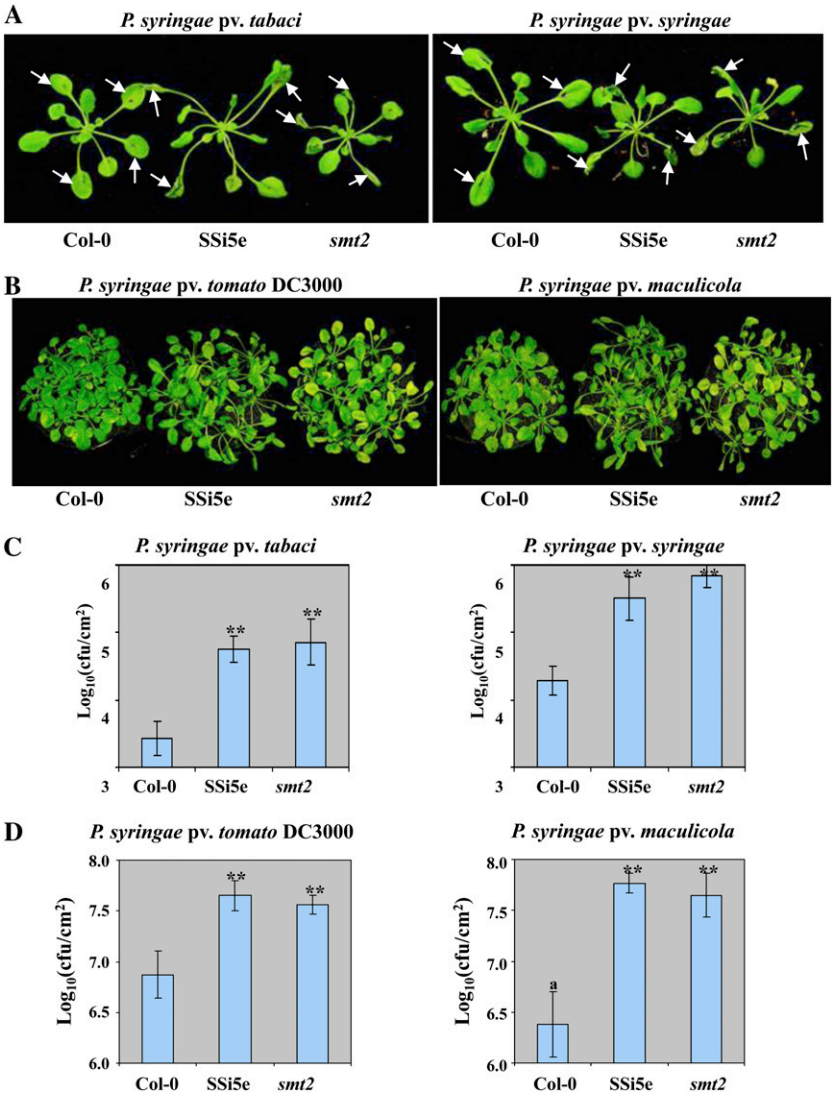

Figure 3. Silencing of the *AtSQS1* gene and *smt2* mutation in Arabidopsis leads to disease susceptibility. A and C, Four-week-old Col-0, *smt2* mutant, and *SQS1* RNAi (SSi5e) plant leaves were syringe infiltrated with nonhost pathogens *Pss* or *Pta* at a concentration of 1×10^7 cfu/mL or dip inoculated with host pathogens *Pst* DC3000 or *Psm* at a concentration of 1×10^8 cfu/mL. B and D, Four-week-old plant leaves were syringe inoculated with *Pta* or *Pss* at a concentration of 1×10^6 cfu/mL or dip inoculated with *Pst* DC3000 or *Psm* at a concentration of approximately 1×10^8 cfu/mL. Bacterial population was quantified by plating serial dilutions of plant leaf extracts at 3 dpi. Asterisks indicate a significant difference from the wild type (Student's *t* test; $P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. Photographs were taken at 4 dpi. See also Supplemental Figure S3.

Since Arabidopsis C22-sterol desaturase encoded by the gene *AtCYP710A1* (At2g34500) specifically converts β-sitosterol to stigmasterol (Morikawa et al., 2006), we examined the expression levels of *AtCYP710A1* in wild-type Col-0 by qRT-PCR in addition to the measurement of stigmasterol content at different time intervals after inoculation with different pathogens, including a nonhost pathogen, *Pta*, and a host pathogen, *Pst* DC3000. The transcript level of *AtCYP710A1* was dramatically increased 12 hpi with *Pta* and increased over 45-fold more than the control at 24 hpi (Fig. 4C). Correspondingly, the levels of stigmasterol were more than 12-fold higher at 24 hpi with *Pta* compared to the controls (Fig. 4D). An increase of *AtCYP710A1* expression and stigmasterol was also observed at 12 hpi with the host pathogen *Pst* DC3000 when compared to mock treatment (Fig. 4, C and D).

We also observed a dosage-dependent induction of stigmasterol that correlates with the amount of pathogen inoculums. We inoculated the wild-type Arabidopsis Col-0 with a nonhost pathogen, *Pta*, at different concentrations. As shown in Supplemental Figure S4, at 12 hpi the transcripts of *AtCYP710A1* gene were increased 115- or 15-fold when inoculated at 1×10^6 cfu/mL or 1×10^5 cfu/mL, respectively. Similarly, at 24 hpi the *AtCYP710A1* gene was induced 272-fold or 45-fold at 1×10^6 cfu/mL or 1×10^5 cfu/mL, respectively.

AtCYP710A1 Gene Is Induced during Both PTI and ETI Responses

We examined whether *AtCYP710A1* could be induced upon inoculation with other host and nonhost pathogens. Four-week-old Arabidopsis (Col-0) seedlings were individually inoculated with different pathovars of *P. syringae* and nonpathogenic *Pseudomonas fluorescens* at a concentration of 1×10^6 cfu/mL by leaf infiltration. qRT-PCR analysis indicated that *AtCYP710A1* was increased by 24 hpi with most bacteria

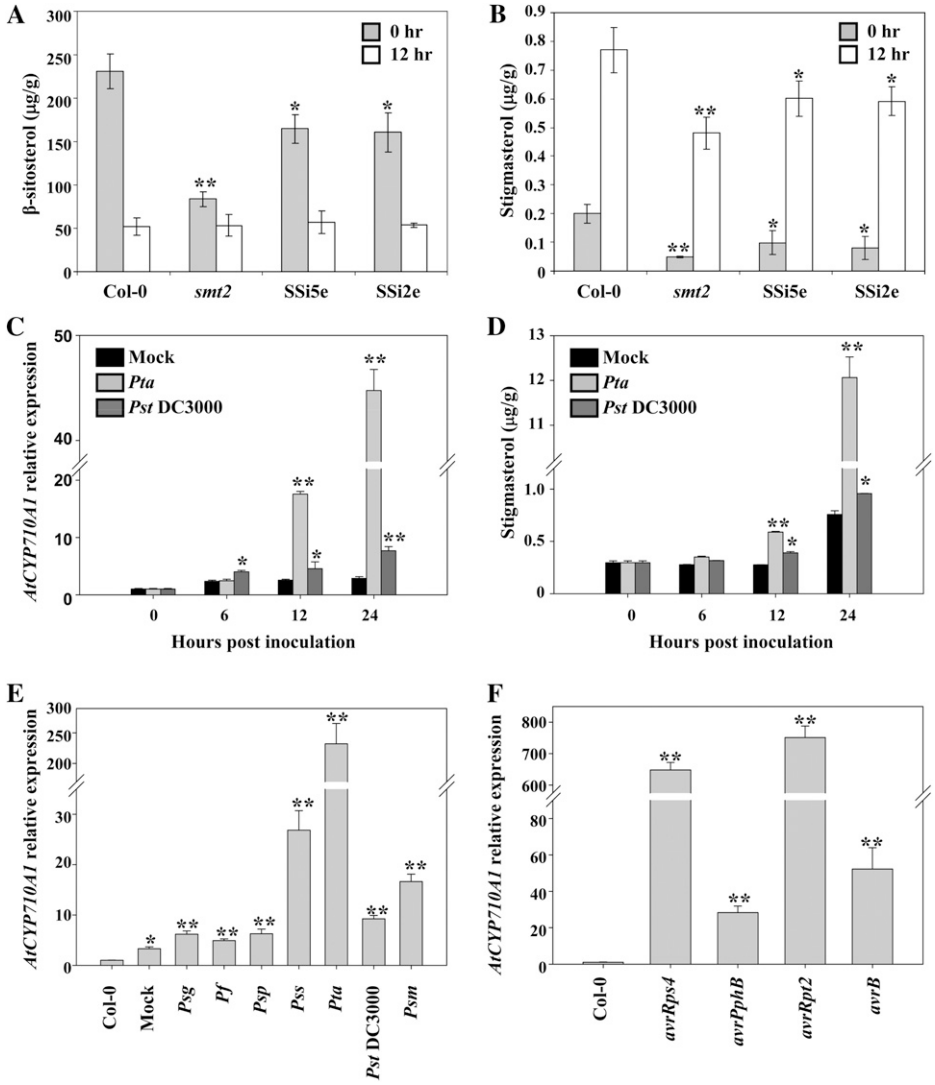

Figure 4. Differential expression of the *AtCYP710A1* gene and accumulation of stigmasterol due to plant defense. A and B, Four-week-old Arabidopsis wild-type (Col-0), *smt2* mutant, and *SQS1* RNAi (SSi5e and SSi2e) plants were vacuum infiltrated with a nonhost pathogen, *Pta*, at a concentration of 1×10^5 cfu/mL. Sterols were extracted from the leaves of inoculated plants and quantified at 12 hpi by GC-MS. C, Col-0 plants were vacuum infiltrated with 10 mM $MgCl_2$ (mock), *Pta*, and *Pst* DC3000 at a concentration of 1×10^5 cfu/mL. Transcript levels of *AtCYP710A1* were measured by qRT-PCR and the expression levels relative to 0 h (1.0) are shown. D, Stigmasterol level in the plant leaves was determined as described in B. E, Col-0 leaves were syringe infiltrated with various bacterial pathogens at a concentration of 1×10^6 cfu/mL. The relative expression of *AtCYP710A1* to uninoculated Col-0 (1.0) was determined at 24 hpi by qRT-PCR. Col-0: Uninoculated; mock: 10 mM $MgCl_2$; Pf: *P. fluorescens*; Psp: *P. syringae* pv *phaseolicola*. F, Col-0 plants were syringe inoculated with *Pst* DC3000 containing different avirulence genes (*avrRps4*, *avrPphB*, *avrRpt2*, and *avrB*) at a concentration of 1×10^5 cfu/mL. The relative expression of *AtCYP710A1* to uninoculated Col-0 (1.0) was determined. Asterisks indicate a significant difference from the wild type (Student's *t* test; $*P < 0.05$ and $**P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. See also Supplemental Figures S4 and S5.

tested, compared to the mock control (Fig. 4E). These data demonstrated that *AtCYP710A1* is commonly induced during both basal and nonhost defense responses with variation in the degree of induction.

Since stigmasterol is a component of cell membranes, we speculated that the *AtCYP710A1* gene might also play a role in ETI. *Pst* DC3000 expressing *AvrB*, *AvrPphB*, *AvrRps4*, or *AvrRpt2* was used to inoculate the wild-type Arabidopsis Col-0 (has the corresponding *R* genes) individually by leaf infiltration at a concentration of 1×10^5 cfu/mL. *AvrRps4* and *AvrRpt2* expressing *Pst* DC3000 induced *AtCYP710A1* by approximately 600-fold, while *AvrB* and *AvrPphB* expressing *Pst* DC3000 induced *AtCYP710A1* by approximately 30-fold at 24 hpi when compared to mock inoculation (Fig. 4F). These data suggested that *AtCYP710A1* is induced during ETI.

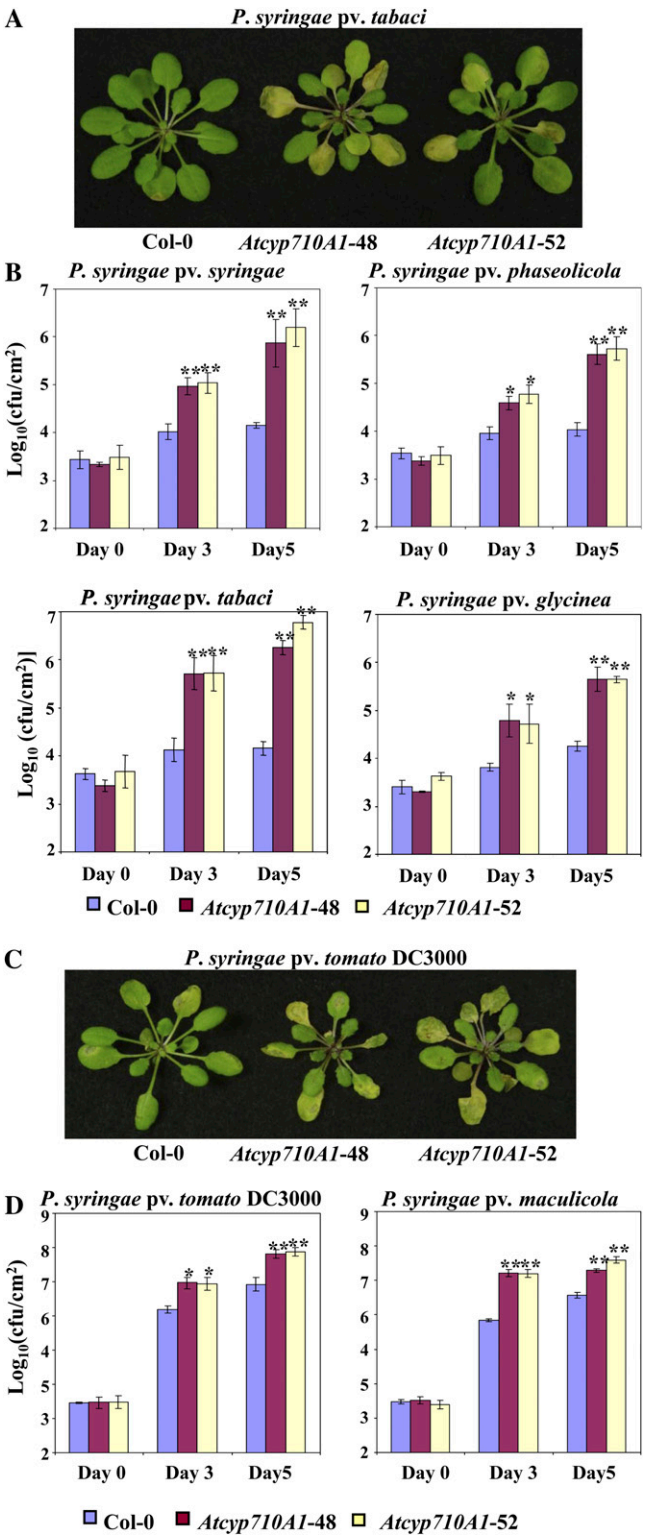

Figure 5. Arabidopsis *Atcyp710A1* mutant compromises disease resistance. A and C, Arabidopsis wild type (Col-0) and *Atcyp710A1* mutants (48 and 52) were syringe inoculated with a nonhost pathogen, *Pta*, or a host pathogen, *Pst* DC3000, at a concentration of 1×10^6 cfu/mL. Photographs were taken at 3 dpi. B and D, Plant leaves were syringe infiltrated with various host and nonhost pathogens at a concentration of approximately 1×10^6 cfu/mL. Bacterial population was quantified at 0, 3, and 5 dpi. Asterisks indicate a significant difference from the wild type (Student's *t* test; $*P < 0.05$ and $**P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. See also Supplemental Figures S5 to S7.

To check the induction of *AtCYP710A1* during PTI, we examined the expression of *AtCYP710A1* in Arabidopsis leaves inoculated with various elicitors in the publicly available gene expression database, GENEVESTIGATOR (www.genevestigator.com). Induction of *AtCYP710A1* was seen with PAMP elicitor FLG22 (Supplemental Fig. S5A). Taken together, our data and already-published results demonstrated that *AtCYP710A1* is induced during both PTI and ETI.

Arabidopsis *Atcyp710A1* Mutant Compromises Plant Innate Immunity

To further investigate the function of the *AtCYP710A1* gene in plant innate immunity, Arabidopsis *Atcyp710A1* T-DNA insertion mutants were obtained from SALK (SALK_112491 and SALK_014626) and the European Arabidopsis Stock Center (GK-325E09). In both the SALK lines, the T-DNA insertion was in 5′ untranslated region and did not have a complete loss of transcripts and therefore was not characterized further. In the GK-325E09 mutant, the T-DNA was inserted in the coding region (i.e. 98 nucleotides after the start codon) of *AtCYP710A1* gene and was a null mutant (Supplemental Fig. S5, B and C). Two homozygous plants (*Atcyp710A1*-48 and *Atcyp710A1*-52) were selected for characterization. The mutant plants had no obvious developmental abnormalities (Supplemental Fig. S5D). *Atcyp710A1* mutant plants along with wild-type Col-0 were inoculated (by leaf infiltration using needleless syringe) with nonhost pathogens *Pta*, *Pss*, *Psp*, and *Psg*, as well as host pathogens *Psm* and *Pst* DC3000. The results showed that all tested nonhost pathogens multiplied more, and some even produced disease symptoms in the *Atcyp710A1* mutant plant leaves compared to Col-0 at 3 and 5 dpi (Fig. 5, A and B). The virulent pathogens *Psm* and *Pst* DC3000 also grew up to 15-fold more in the *Atcyp710A1* mutant and produced enhanced disease symptoms compared to Col-0 plants (Fig. 5, C and D; Supplemental Fig. S5E). Silencing of the *AtCYP710A1* gene by VIGS in wild-type *N. benthamiana* and tomato (*Solanum lycopersicum*) plants also leads to susceptibility upon inoculation with nonhost pathogens *Pst* T1 and *Pta*, respectively (Supplemental Fig. S6, A and B).

Apoplastic Extracts from Arabidopsis *Atcyp710A1* Mutant Plants Support Higher Bacterial Multiplication

To investigate whether the Arabidopsis *Atcyp710A1* mutant also has increased nutrient efflux into the apoplast, similar to *N. benthamiana SQS*-silenced plants, both virulent and avirulent pathogens were

grown in vitro in MGM containing 5% apoplastic fluid extracted from wild-type (Col-0) and mutant (*Atcyp710A1*-48 and *Atcyp710A1*-52) leaves. The results indicated that the apoplastic fluid from the mutant leaves supported more multiplication of virulent and avirulent pathogens compared to apoplastic fluid from Col-0 leaves (Supplemental Fig. S7A). Consistent with this data, higher membrane permeability, as reflected by higher entry of PI, was also seen in *Atcyp710A1* mutant cells (Supplemental Fig. S7, B–D). These results suggest that the *AtCYP710A1* gene plays a crucial role in controlling nutrient efflux into the apoplast by maintaining membrane integrity.

Overexpression of the *AtCYP710A1* Gene Confers Disease Resistance in Arabidopsis

Since the expression of the *AtCYP710A1* gene increased during both compatible and incompatible interaction with pathogens, we reasoned that *AtCYP710A1* is a plant defense gene and its overexpression might confer disease resistance to host pathogens. High levels of *AtCYP710A1* gene transcripts were confirmed by qRT-PCR in Arabidopsis *AtCYP710A1* gene overexpression lines (Fig. 6A; Morikawa et al., 2006; Arnqvist et al., 2008). We challenged these plants through dip inoculation of host pathogen *Psm* (1×10^8 cfu/mL). As speculated, disease symptoms and bacterial multiplication were suppressed in *AtCYP710A1* gene overexpression lines when compared to wild-type Col-0 (Fig. 6, B and C). *AtCYP710A1* gene overexpression plants also showed a mild decrease in host-pathogen-induced ion leakage at 12 hpi (Supplemental Fig. S8). These results further suggested that the *AtCYP710A1* gene positively mediates plant innate immunity.

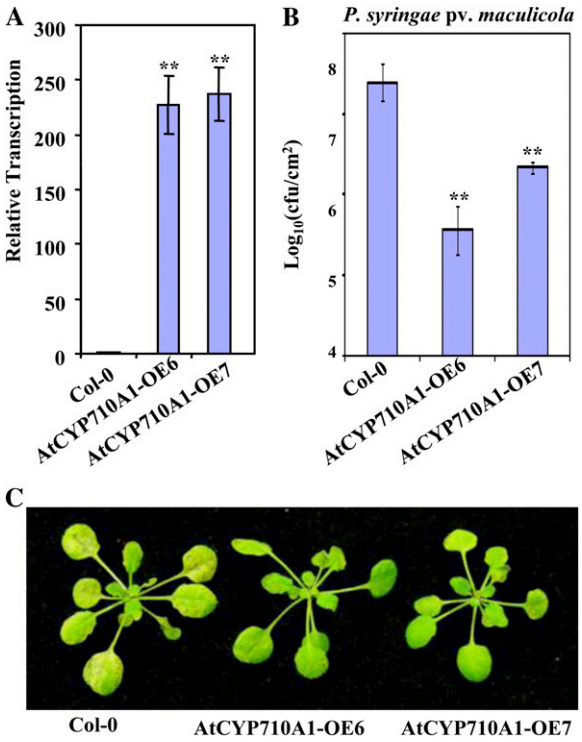

Figure 6. Overexpression of the *AtCYP710A1* gene in Arabidopsis confers disease resistance. A, Transcripts of *AtCYP710A1* in two independent *AtCYP710A1* overexpressor lines (AtCYP710A1OE-6 and AtCYP710A1OE-7) were determined by qRT-PCR. Expression of *AtCYP710A1* in the overexpressors relative to expression in wild type (Col-0; 1.0) is shown. Values for individual plants from each line are shown here. B, Arabidopsis Col-0 and *AtCYP710A1* gene overexpressors were inoculated with a host pathogen, *Psm*, by spraying at a concentration of 1×10^8 cfu/mL. Bacterial population in plants shown in B was quantified at 3 dpi. C, Photographs of disease symptoms were taken at 3 dpi. Asterisks indicate a significant difference from the wild type (Student's *t* test; $*P < 0.05$ and $**P < 0.01$). Data were pooled from two independent experiments representing four biological replicates. See also Supplemental Figure S8.

DISCUSSION

Unlike *R*-gene-mediated resistance, which is specific for a particular bacterial pathovar, nonhost resistance is governed by a broad defense mechanism that can defend against a wide range of bacterial pathovars (Heath, 2000; Mysore and Ryu, 2004; Ellis, 2006). Nonhost resistance involves many aspects of plant defense mechanisms such as production of antimicrobial compounds and HR (Nürnberger and Lipka, 2005). In addition, many of the subsequent plant defenses that determine the success or failure of pathogenic bacteria take place in the apoplast, the final battlefield of plant-bacterial interactions (Hoefle and Hückelhoven, 2008). The leaf apoplast constantly exchanges components with the cytosol and contains minimal nutrients, mostly transferred from the cell due to membrane permeability (Sattelmacher, 2001). Biotrophic and hemibiotrophic bacterial pathogens colonize a host by growing in the apoplast and absorbing nutrients from the cytoplasm (Melotto et al., 2008). A recent report indicated that to get nutrients from cells, pathogen virulence factors directly bind promoters of sugar transporters like SWEET11 and SWEET14 in Arabidopsis, and efflux sugars from the cytosol (Chen et al., 2010). Therefore, restricting the availability of nutrients to pathogens can be a potential plant defense mechanism; such a strategy can be exploited for broad spectrum disease resistance.

We developed a VIGS-based novel approach to identify plant genes that play a role in nonhost disease resistance. Based on the multiplication of bacteria directly placed in the apoplast, we identified the role of SQS in limiting the multiplication of nonhost bacteria. Our results showed that *SQS*-silenced *N. benthamiana* plants leaked more nutrients into the apoplast, thus supporting increased bacterial multiplication. We demonstrated that other sterol biosynthetic pathway genes like *AtSMT2* and *AtCYP710A1* are also involved in plant defense by regulating nutrient efflux into the apoplast. Arabidopsis mutant lines of these genes were compromised in nonhost and basal disease resistance.

After a pathogen manages to overcome preformed constitutive and inducible barriers, it is subjected to recognition at the plasma membrane (Alfano and Collmer, 1996; Hoefle and Hückelhoven, 2008). At this point, the PAMP can be recognized and plants induce PTI (Hofius et al., 2007). A recent study (Griebel and Zeier, 2010) showed that *AtCYP710A1* gene-mediated biosynthesis of stigmasterol is induced upon inoculation with a virulent pathogen, *Psm*, avirulent pathogen *Psm-avrRpm1*, nonhost pathogen, *Psg*, and PAMPs (FLG22). Similarly, data from the publicly available gene expression database GENEVESTIGATOR showed induction of this gene upon FLG22 treatment. Therefore, plants have a strategy to manipulate sterol synthesis to prevent nutrient efflux into the apoplast. β-Sitosterol and stigmasterol are the predominant sterols in plant membranes (Hodzic et al., 2008). Since plant sterols are structurally related to cholesterol and control membrane mechanical properties (Hodzic et al., 2008), changes in sterol content and composition can affect membrane permeability, hydrocarbon chain order, condensation efficacy, and elastic behavior (Bernsdorff and Winter, 2003).

The fact that both *SQS*-gene-silenced *N. benthamiana* and the Arabidopsis *Atcyp710A1* mutant increased nutrient efflux into the apoplast indicates the relevance of sterols to prevent nutrient efflux into the apoplast. Our results also suggest that plant cells can induce defense mechanisms to deprive potential pathogens of the nutrients available in the apoplast. The *SQS*-gene-silenced plants were susceptible to a wide range of pathogens and consistently showed more nutrients in the apoplast compared to wild-type plants. This is mainly due to excessive nutrient efflux into the apoplast. Although specific transporters are known to be involved in efflux of nutrients into the apoplast upon pathogen infection (Chen et al., 2010), our data suggest that membrane permeability also plays an important role in nutrient efflux into the apoplast. To defend against pathogens effectively, we speculate that plants have to reduce excess nutrient efflux immediately after pathogen infection and well before the start of disease.

Stigmasterol levels in Arabidopsis transgenic lines with *AtCYP710A1* gene overexpression were increased up to 32-fold (Morikawa et al., 2006). We believe that Arabidopsis *AtCYP710A1* gene-overexpressing plants have a less-permeable membrane and hence resisted pathogen growth while the *Atcyp710A1* mutant compromised both host and nonhost resistance. Recent studies also showed the relevance of stigmasterol in plant-pathogen interaction (Griebel and Zeier, 2010; Wang et al., 2012). Consistent with our results, resistance of 3-hydroxy-3-methylglutaryl-CoA synthase overexpressed Arabidopsis plants to *Botrytis cinerea* was shown to be due to up-regulation of *AtCYP710A1* gene expression and stigmasterol content (Wang et al., 2012). However, contrary to our results, study by Griebel and Zeier (2010) indicated less host bacterial growth in different Arabidopsis *Atcyp710A1* mutants, Salk_112491, and Salk_014626. In the Salk_112491 line, T-DNA is inserted nearly 780-bp upstream of the start codon of the *AtCYP710A1* gene. The Salk_014626 line has two T-DNA insertions where one of the T-DNA is inserted in the potential promoter region (480-bp upstream of the start codon) of the *AtCYP710A1* gene and another T-DNA is inserted in the exon of a gene that encodes isopentenyl diphosphate isomerase 2. The *Atcyp710A1* mutant used in this study has T-DNA insertion in the exon and is a null mutant. At this point, we are unable to explain the exact reason for the discrepancy in our results. It is possible that the reduced *AtCYP710A1* expression in the SALK lines

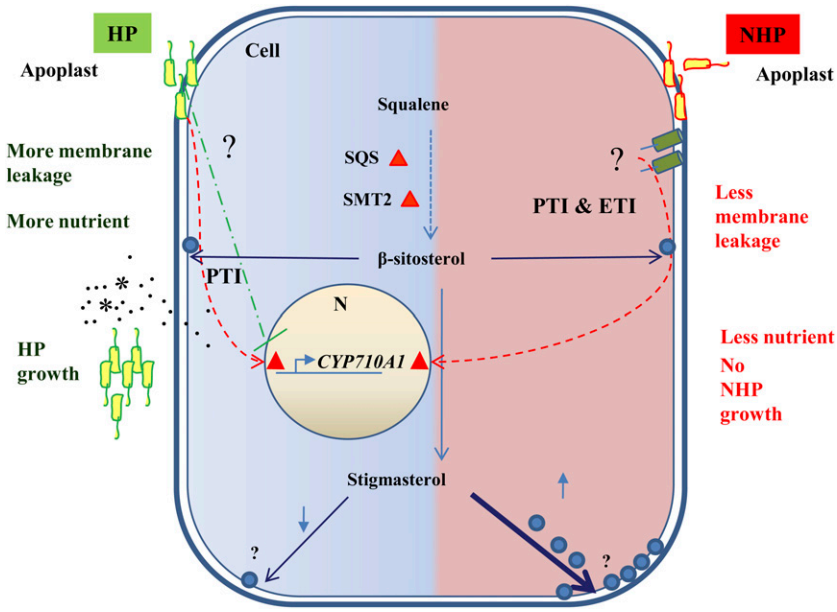

Figure 7. Sterols play a role in nonhost resistance and basal resistance by regulating nutrient efflux into the apoplast. Successful plant pathogenic bacteria multiply in the apoplast by drawing nutrients from cytoplasm. During attack by nonhost bacteria, the plant cell initiates signaling events leading to enhanced expression of genes involved in sterol biosynthesis. An unknown mechanism makes the cell reduce nutrient efflux, thereby depriving nutrient availability in the apoplast, thus arresting nonhost pathogen growth. When pathogenic bacteria attack their host plants, bacterial PAMPs trigger a general plant defense response, leading to induction of stigmasterol. However, unknown virulence factors of host pathogenic bacteria might suppress the production of stigmasterol by inhibiting the expression of the *AtCYP710A1* gene, thus allowing nutrient efflux from cytoplasm to the apoplastic space. HP, Host pathogen; NHP, nonhost pathogen. [See online article for color version of this figure.]

may have other unknown pleiotropic effects on the plant, contrary to the null mutant.

Our results showed that the *AtCYP710A1* gene also plays a role during *R*-gene-mediated plant disease resistance. The Arabidopsis Col-0 ecotype encodes many resistance proteins that can recognize their cognate avirulence effectors, such as AvrB, AvrPphB, AvrRps4, and AvrRPt2, leading to HR. The *AtCYP710A1* gene was slightly induced upon infection with the host pathogen *Pst* DC3000, and the induction was up to 600-fold more when the *Pst* DC3000 expressed one of the corresponding avirulence genes, suggesting that *AtCYP710A1* is also induced during ETI.

Based on our data showing the up-regulation of sterol biosynthetic genes during both PTI and ETI, we propose a model shown in Figure 7 for the role of sterols (especially stigmasterol) in plant defense. During an incompatible interaction (nonhost or *R*-gene-mediated resistance), either PTI or ETI, or both together, induces sterol pathway genes like *AtCYP710A1* and prevents nutrient efflux to the apoplast. During a compatible interaction, stigmasterol biosynthesis is initially induced by PTI, but suppressed by pathogens. We speculate that the virulence proteins (effectors) injected by the host pathogens into the plant cell will interfere with the induction of stigmasterol biosynthesis to combat plant defense mechanisms and favor nutrient efflux into the apoplast. A host pathogen is also known to manipulate alkalinization of the apoplast to favor nutrient efflux (Atkinson and Baker, 1987).

In conclusion, our results show that plants alter sterol biosynthesis upon infection by a potential bacterial pathogen through PTI and/or ETI. This restricts nutrient transfer from cytosol to the apoplast. Overexpression of the *AtCYP710A1* gene improved plant immunity against pathogens. Our results show promise for a novel strategy to engineer plants for broad spectrum disease resistance by manipulating sterol levels. The exact role of stigmasterol or β-sitosterol in membrane permeability and the relevance of sterols as signaling molecules warrant further investigation.

MATERIALS AND METHODS

Plant Materials, Bacterial Pathogens, and Growth Conditions

Nicotiana benthamiana seeds were germinated as previously described (Anand et al., 2007). Arabidopsis (*Arabidopsis thaliana*) *Atsqs1* T-DNA mutants (Salk_034266, 087515, 034431, and 077057), *Atsqs2* T-DNA mutants (Salk_018690 and 018698), and *Atcyp710A1* mutants (SALK_112491 and SALK_014626) were obtained from the Arabidopsis Biological Resource Center. *Atcyp710A1* (locus: At2G34500) T-DNA mutant (GK-325E09; Nottingham Arabidopsis Stock Centre ID N376345-62) was obtained from Nottingham Arabidopsis Stock Centre. Arabidopsis *AtCYP710A1* overexpression lines (Morikawa et al., 2006) were provided by Dr. Daisaku Ohta, Osaka Prefecture University, Osaka, Japan. *Atsmt2* mutant (Carland et al., 2002; *cvp1-3*) was obtained from Dr. Timothy Nelson, Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT. Arabidopsis seeds were cold treated for 3 to 4 d at 4°C and germinated in Professional Blend soil in a growth chamber at 20°C to 22°C under short-day conditions (8-h light). *Agrobacterium tumefaciens* GV2260 was grown in Luria-Bertani medium at 28°C. *Pseudomonas syringae* strains were grown in King's B medium at 28°C. Bacterial growth was monitored as described (Wang et al., 2007).

VIGS in *N. benthamiana*

For screening cDNA library, VIGS was carried out as described earlier (Mysore and Ryu, 2004; Ryu et al., 2004; Anand et al., 2007) with slight modifications. See Supplemental Materials and Methods S1 for more details.

Plant Inoculation, Disease Assay, and Quantification of Bacteria

Bacterial cells of *P. syringae* strains were collected by centrifugation of overnight culture at 5,000 rpm for 10 min, washed twice, and resuspended to the desired concentration in 10 mM $MgCl_2$ with 0.01% (v/v) Silwet L-77 (Osi Specialties). The bacterial suspension was used to inoculate fully expanded leaves of gene-silenced *N. benthamiana* plants 2 weeks after TRV infection or 4-week-old Arabidopsis plants by dipping or leaf infiltration using a needleless syringe or vacuum, and incubated in growth chambers at 20°C to 22°C. Two leaf discs (0.5-cm^2 each) of each plant were homogenized and serial diluted in 10 mM $MgCl_2$ and plated on King's B agar medium for quantification of in planta bacterial growth. Bacterial population was calculated as cfu per square centimeter of leaf area. Experiments were performed with at least four replicates.

Extraction of Apoplastic Fluid and Metabolite Profiling

N. benthamiana leaves were submerged in a beaker containing 1,000 mL milliQ water and a vacuum was applied for 1 min to remove the air pockets in the apoplast. Vacuum-infiltrated leaves were then blotted dry, cut into two pieces along the midrib to remove major leaf veins, carefully rolled, and placed into a 15-mL tube with a hole punched at the bottom leading to a 1.5-mL collection tube. This setup was then inserted into a 50-mL conical tube (Corning) for spinning. Apoplast fluid was extracted by centrifugation at 500*g* for 10 min at 4°C. The fraction collected in the 1.5-mL tube was transferred to a fresh tube and centrifuged for 10 min at 3,000*g* at 4°C. The supernatant was then filter sterilized (to remove bacterial contamination) and was either used for further analyses immediately or stored at −80°C. Apoplastic fluid from Arabidopsis was extracted similarly except centrifugation was at 2,000*g* for 30 min at 4°C. Metabolite profiling of apoplastic fluid was performed as previously described (Broeckling et al., 2005) with minor modifications. In brief, 300 μL of apoplastic fluid was transferred to a 4-mL glass vial and lyophilized in a freeze drier (VirTis) overnight. HPLC grade water (1.5 mL) containing 25 μg/mL ribitol (internal standard) and 1.5 mL of chloroform was added, vortexed, and incubated for 45 min at 50°C. The samples were then centrifuged and the polar phase was transferred (1 mL) to an autosampler vial and dried by vacuum centrifugation at room temperature. The dried extracts were methoximated in 50 μL of pyridine with 15 mg/mL of methoxyamine hydrochloride, sonicated, and incubated at 50°C until the residue was resuspended. The samples were then derivatized with 50 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane for 1 h at 50°C. One microliter of the solution was analyzed by GC-MS.

Phytosterol Extraction and Analysis

Phytosterols were extracted by chloroform/methanol as previously described (Morikawa et al., 2006) with minor modifications. Leaf tissues were frozen in liquid nitrogen and lyophilized in a freeze drier (VirTis) for 3 d. The homogenized dry tissue (20 mg) was used to extract the total sterols in 5-mL chloroform/methanol (1:2, v/v) with gentle shaking at room temperature as described in detail in Supplemental Materials and Methods S1.

Statistical Analysis

Student's *t* test was conducted for statistical analysis of the data presented in the figures. Error bars indicate the SE values of replicate means. Asterisks indicate a significant difference from the wild type (*$P < 0.05$ and **$P < 0.01$).

Supplemental Data

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

Supplemental Figure S1. Endogenous *NbSQS* gene transcript levels, sterol levels, occurrence of HR, and disease symptoms in *NbSQS*-gene-silenced plants.

Supplemental Figure S2. Simplified biosynthetic pathway for plant sterols and structure of β-sitosterol and stigmasterol.

Supplemental Figure S3. Silencing of the *AtSQS1* gene and *smt2* mutation in Arabidopsis leads to disease susceptibility.

Supplemental Figure S4. Dosage-dependent expression of the *AtCYP710A1* gene upon nonhost pathogen inoculation.

Supplemental Figure S5. *AtCYP710A1* gene expression upon elicitor treatment and details of Arabidopsis *Atcyp710A1* mutants.

Supplemental Figure S6. Silencing of the *AtCYP710A1* gene by VIGS supports more multiplication of nonhost bacteria in *N. benthamiana* and tomato.

Supplemental Figure S7. Analysis of pathogen growth in apoplastic fluid and membrane permeability characteristics.

Supplemental Figure S8. Membrane leakage in *AtCYP710A1* overexpression plants upon host pathogen infection.

Supplemental Table S1. Increased compounds in apoplast extracted from the *SQS*-silenced *N. benthamiana* compared to the control.

Supplemental Table S2. Metabolite profiling of apoplast.

Supplemental Materials and Methods S1. Additional details for identification and characterization of *SQS*, *SMT2*, and *CYP710A1* genes in *N. benthamiana* and Arabidopsis.

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