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# Identification of Natural Diterpenes that Inhibit Bacterial Wilt Disease in Tobacco, Tomato and Arabidopsis

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Regular Paper

**The soil-borne bacterial pathogen *Ralstonia solanacearum* invades a broad range of plants through their roots, resulting in wilting of the plant, but no effective protection against this disease has been developed. Two bacterial wilt disease-inhibiting compounds were biochemically isolated from tobacco and identified as sclareol and *cis*-abienol, labdane-type diterpenes. When exogenously applied to their roots, sclareol and *cis*-abienol inhibited wilt disease in tobacco, tomato and Arabidopsis plants without exhibiting any anti-bacterial activity. Microarray analysis identified many sclareol-responsive genes in Arabidopsis roots, including genes encoding or with a role in ATP-binding cassette (ABC) transporters, and biosynthesis and signaling of defense-related molecules and mitogen-activated protein kinase (MAPK) cascade components. Inhibition of wilt disease by sclareol was attenuated in Arabidopsis mutants defective in the ABC transporter AtPDR12, the MAPK MPK3, and ethylene and abscisic acid signaling pathways, and also in transgenic tobacco plants with reduced expression of *NtPDR1*, a tobacco homolog of *AtPDR12*. These results suggest that multiple host factors are involved in the inhibition of bacterial wilt disease by sclareol-related compounds.**

**Keywords:** ABC transporter • Diterpene • *Ralstonia solanacearum* • Wilt disease.

**Abbreviations:** ABC, ATP-binding cassette; BNH, benzothiodiazole; c.f.u., colony-forming units; DA, dehydroabietinal; d.p.i., days post-inoculation; ET, ethylene; HR, hypersensitive response; INA, 2,6-dichloroisonicotinic acid; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; MS medium, Murashige and Skoog medium; NMR, nuclear magnetic resonance; PDR, pleiotropic drug resistance; RT–PCR, reverse transcription–PCR; SA, salicylic acid; SAR, systemic acquired resistance; SPE, solid-phase extraction; TMV, *Tobacco mosaic virus*.

## Introduction

Plants defend themselves against infection from pathogens by producing a number of defense-related compounds such as phytohormones and antimicrobials. The phytohormones ethylene (ET) and jasmonic acid (JA) and the phenolic compound salicylic acid (SA) function as signaling molecules that induce expression of genes involved in disease resistance responses in plants (Dong 1998, Glazebrook 2005). Antimicrobials, including flavonoids, phenols and terpenes, are compounds that destroy or inhibit the growth of bacteria and fungi (Cowan 1999).

Bacterial wilt disease, one of the most devastating plant diseases, is caused by *Ralstonia solanacearum* and leads to substantial yield losses in several hundred important crops such as tomato, tobacco, potato, eggplant and banana (Hayward 1991). This soil-borne bacterium invades roots and multiplies in vascular systems, resulting in wilting of the host plant (Vasse et al. 1995). It is generally difficult to control this disease because of the genetic diversity of *R. solanacearum* and its ability to survive easily in adverse environments (Cook et al. 1989, Schell 2000).

Various techniques, such as soil amendments, biological control and cultural practices, have been developed to control

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bacterial wilt disease. Among these techniques, using the ability of plants to induce resistance to *R. solanacearum* is thought to be the most effective method for controlling the disease (Hayward 1991). For example, grafting commercial cultivars susceptible to bacterial wilt onto resistant rootstocks has been used for some crops such as tomato and eggplant; however, wilt often occurs in susceptible scions grafted onto resistant rootstocks (Grimault and Prior 1994). Another approach is control by non-antimicrobials that activate host defense to *R. solanacearum*. A well-known example of such chemical activators is probenazole, a thiazole compound, that induces resistance to fungal, bacterial and viral pathogens in rice and tobacco through activation of SA biosynthesis (Watanabe et al. 1977, Yoshioka et al. 2001, Nakashita et al. 2002). The synthetic SA analogs 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) induce resistance to a broad spectrum of pathogens in many plant species (Vernooij et al. 1995, Friedrich et al. 1996). Only a few examples have been reported for chemical activators of host defense to *R. solanacearum*. Exogenous applications of riboflavin (vitamin B2) or silicon inhibited bacterial wilt disease (Liu et al. 2010, Ghareeb et al. 2011), although the mechanism of bacterial wilt disease inhibition by these compounds is not fully understood.

Genetic and biochemical studies have identified host factors involved in defense responses of plants to *R. solanacearum*. Resistance to *R. solanacearum* in Arabidopsis (*Arabidopsis thaliana*) is conferred by the *RRS1-R* gene that encodes a nucleotide-binding site leucine-rich repeat-type protein (Deslandes et al. 2002). This *RRS1-R*-mediated resistance is partially dependent on SA. An ABA-related signaling pathway is also involved in defense responses of Arabidopsis to *R. solanacearum* (Hernández-Blanco et al. 2007). An ET-responsive transcription factor, mitogen-activated protein kinases (MAPKs) and a heat shock protein function as regulators of defense responses to *R. solanacearum* in tomato and *Nicotiana* plants (Zhang et al. 2004, Maimbo et al. 2007, Chen et al. 2009). These studies suggest that host defense to *R. solanacearum* is regulated through a complex mechanism involving multiple factors.

Diterpenes, a class of terpenes assembled from four isoprene units, are naturally occurring compounds. In higher plants, diterpenes have various functions as hormones, antioxidants, antimicrobials and defense-related molecules. Sclareol (**Fig. 1**), which was originally found in clary sage (*Salvia sclarea*), is a labdane-type of diterpene produced by a few plants including *Nicotiana* species. Sclareol exhibits antimicrobial activity against several plant pathogenic fungi and bacteria (Bailey et al. 1974, Bailey et al. 1975, Kennedy et al. 1992, Jackson and Danehower 1996). Exogenous application of sclareol also induces expression of defense-related genes, such as those encoding stress-responsive ATP-binding cassette (ABC) transporters, in *Nicotiana* plants and Arabidopsis (Jasiński et al. 2001, Campbell et al. 2003, Grec et al. 2003). Although these studies suggest that sclareol is involved in defense responses of plants to fungal and bacterial pathogens through inhibition of fungal and bacterial growth and, possibility, activation of host factors, there is no direct evidence demonstrating that activation of host factors by sclareol leads to disease resistance in plants.

Here, we report the isolation and identification of sclareol and its close analog *cis*-abienol as natural compounds that inhibit wilt disease caused by *R. solanacearum* in tobacco, tomato and Arabidopsis. We also provide evidence that an ABC transporter, ET, ABA and MAPK are required for the inhibition of bacterial wilt disease by sclareol.

## Results

### Isolation and identification of bacterial wilt disease-inhibiting compounds

Our aim was to explore natural compounds that inhibit wilt disease caused by *R. solanacearum*. The hypersensitive response (HR), a form of plant disease resistance response, is characterized by rapid and localized cell death at the site of pathogen invasion. HR is usually accompanied by production of defense-related compounds such as ET, JA, SA and antimicrobials (Dong 1998, Glazebrook 2005). The *R. solanacearum* strain 8266 is a strain incompatible with tobacco plants and its infiltration into tobacco leaves induces HR-like cell death, resulting in inhibition of bacterial growth (Takabatake et al. 2006). We assumed that tobacco leaves undergoing HR would produce defense-related compounds involved in HR-like cell death and/or resistance to *R. solanacearum*.

To test this assumption, strain 8266 was infiltrated into tobacco leaves and extracted with acetone followed by ethyl acetate (**Supplementary Fig. S1A**). Ethyl acetate-soluble basic, neutral and acidic fractions were tested for their activity to inhibit the development of disease caused by strain 8225, a compatible strain, using 3-week-old tobacco plants. Mature plants are generally used in bacterial wilt disease resistance assays; however, when a potential compound is applied to mature plants, a large volume of the sample is required. An assay using young plants was advantageous for reducing the sample volume and, hence, the amount of compound to be tested. Buffer (mock)-infiltrated leaves were used as a control. Each fraction was applied to roots of young tobacco plants, and the treated plants were inoculated with strain 8225. Inhibitory activity was found in the neutral fraction prepared from 8266-infiltrated leaves (**Supplementary Fig. S1B**). The neutral fraction from buffer-infiltrated leaves tended to exhibit slightly lower activity than 0.1% methanol used as a control; however, this difference was not significant. The acidic fraction from 8266-infiltrated leaves exhibited a slight, but not significant, inhibitory activity. The basic fraction from buffer- and 8266-infiltrated leaves tended to exhibit slightly enhanced activity for the development of disease. These results suggested that tobacco leaves produce compounds with the ability to inhibit bacterial wilt disease and this production is probably enhanced in response to infiltration with strain 8266.

To purify and isolate inhibitory activity, 8266-infiltrated leaves were extracted on a large scale. We focused on the

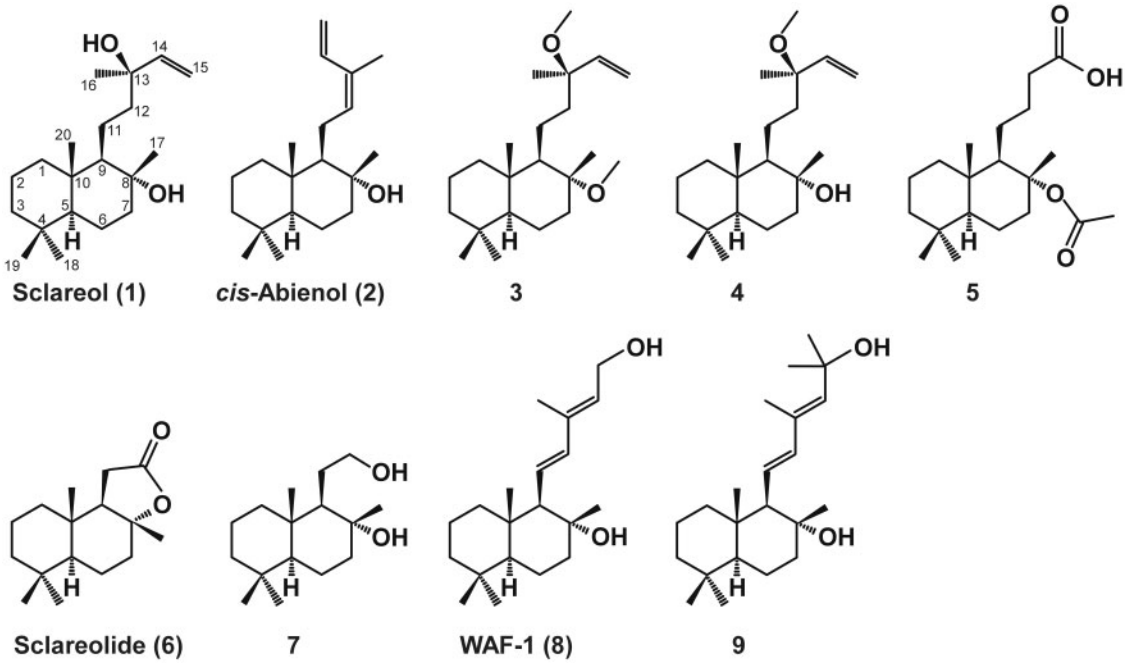


**Fig. 1** Chemical structures of sclareol and related compounds. Numbers shown on the sclareol molecule denote the International Union of Pure and Applied Chemistry numbering system for carbon atoms.

ethyl acetate-soluble neutral fraction for further purification. The neutral fraction after acetone extraction was fractionated by chromatography on a silica gel column followed by a $C_{18}$-based solid-phase extraction (SPE) cartridge column (**Supplementary Fig. S2A, B**). The active fractions obtained by SPE were finally fractionated by reversed-phase HPLC (**Supplementary Fig. S2C, D**). Activity was separated into two fractions: one fraction with a retention time of 25–30 min and another fraction with a retention time of 45–50 min (**Supplementary Fig. S3**). Peaks (A and B) corresponding to the activity in each fraction were collected. Resonances in $^{1}$H- and $^{13}$C-nuclear magnetic resonance (NMR) spectra for peak A and peak B were assigned to sclareol and *cis*-abienol, respectively (**Fig. 1**, **Supplementary Fig. S4**; see **Supplementary Text 1** for details of the spectral data). Sclareol and *cis*-abienol are labdane-type diterpenes that are produced in *Nicotiana* species. Previous studies have examined the antibacterial activity of sclareol and *cis*-abienol for *R. solanacearum*, although these compounds have been shown to exhibit little or no activity (Jackson and Danehower 1996). However, there are no detailed analyses on the effect of exogenously applied sclareol and *cis*-abienol on wilt disease caused by this bacterial pathogen.

## Exogenously applied sclareol and *cis*-abienol inhibit bacterial wilt disease in tobacco without exhibiting antibacterial activity

The effect of sclareol and *cis*-abienol on inhibition of bacterial wilt disease was examined using adult tobacco plants by the root-cutting inoculation method. Roots of 2.5-month-old plants were pre-treated with sclareol or with *cis*-abienol for 2 d, cut and inoculated with strain 8225 through the cut root. In plants pre-treated with methanol alone as a control, symptoms, such as yellowing or wilting, began to appear on leaves or stems 5–7 days post-inoculation (d.p.i.) and developed rapidly thereafter, and almost all inoculated plants wilted 14–16 d.p.i. (**Fig. 2A**, 0.1% methanol). In plants pre-treated with sclareol or with *cis*-abienol at 100 μM, disease symptoms appeared later than in control plants and developed gradually, and disease development ceased approximately 20 d.p.i. Growth of strain 8225 was inhibited in sclareol- and *cis*-abienol-pre-treated plants (**Fig. 2B**). The minimum concentration of sclareol and *cis*-abienol to exhibit inhibitory activity for wilt disease was 100 μM (**Fig. 2B**). When applied to leaves by spraying before root inoculation, neither sclareol nor *cis*-abienol showed inhibitory activity for wilt disease (data not shown). We confirmed that exogenous application of sclareol or *cis*-abienol at 100 μM to roots of adult (2.5-month-old) tobacco plants without inoculation did not cause necrosis or chemical injury, such as yellowing or browning of leaves, in the treated plant (data not shown). Sclareol and *cis*-abienol are known to inhibit plant growth (Cutler et al. 1977). Exogenous application of 100 μM sclareol to their roots for 2 d strongly inhibited root and shoot growth of young (3-week-old) tobacco plants, whereas application moderately inhibited root, but not shoot growth of adult (2.5-month-old) tobacco plants (data not shown).

Neither sclareol nor *cis*-abienol exhibited antibacterial activity (**Supplementary Fig. S5**). This is consistent with a previous finding showing that sclareol and *cis*-abienol had little or no

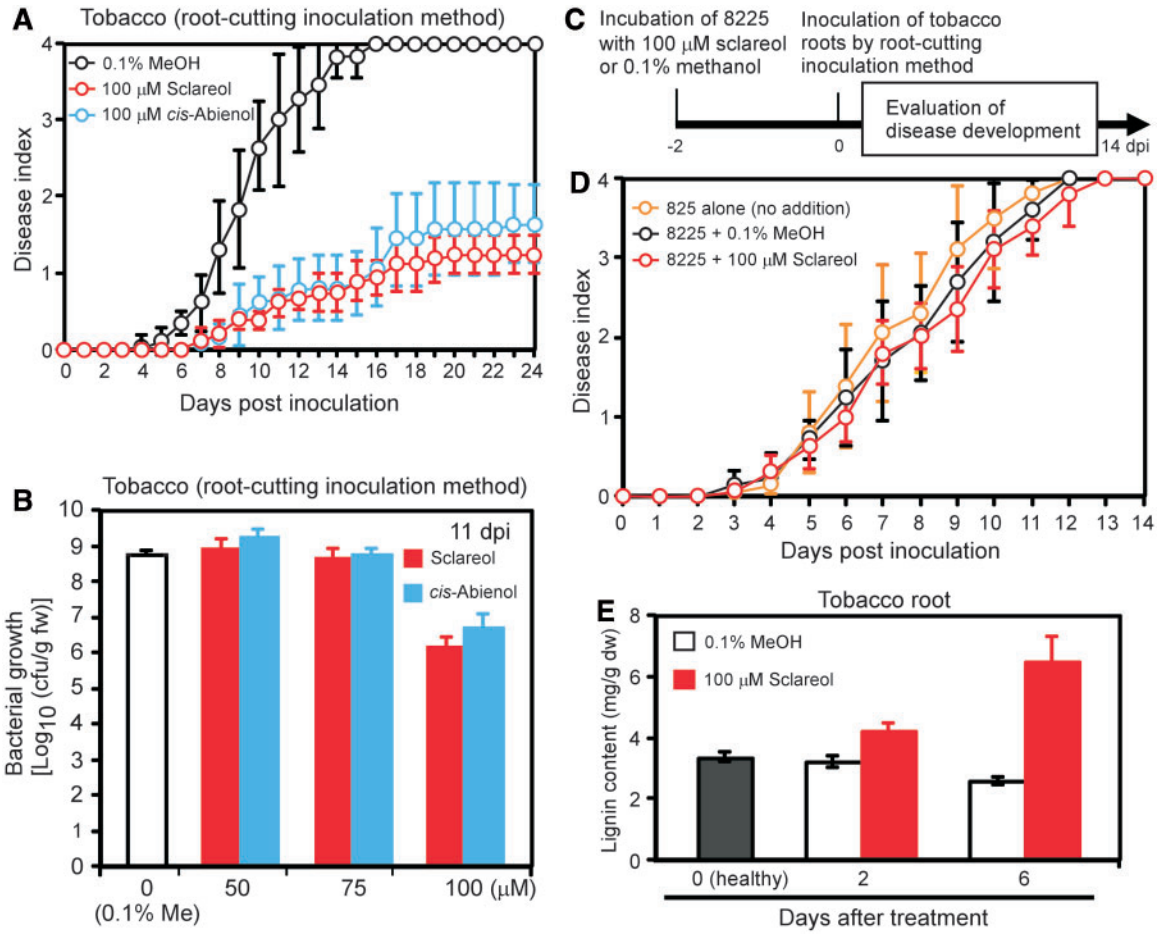


**Fig. 2** Effect of sclareol and *cis*-abienol on inhibition of wilt disease in adult tobacco. (A and B) Roots of adult tobacco plants were pre-treated with the indicated compounds for 2 d and inoculated with strain 8225. (A) Time course of disease symptom development. (B) Dose-dependent effect of sclareol or *cis*-abienol. (C) Timeline of the pathogenicity assay. (D) Effect of sclareol on the pathogenicity of strain 8225. Strain 8225, cultured in a medium that did not contain sclareol or methanol, was also used as a control. Data for A–D are the means ± SD from three independent assays with triplicate samples (each containing four plants). (E) Thioglycolic acid lignin concentrations in roots. Healthy roots (0) were also used. Data are the means ± SD from three independent measurements.

antibacterial activity for *R. solanacearum* (Jackson and Danehower 1996). The effect of these compounds on the pathogenicity of this pathogen was also tested. A bacterial suspension of strain 8225 was incubated with sclareol or with methanol and assayed by inoculating the bacterial suspension on tobacco roots (**Fig. 2C**). There was no major difference in the development of disease symptoms between treatments (**Fig. 2D**), suggesting that sclareol has little or no effect on the pathogenicity of strain 8225.

To study further the inhibitory effect of these diterpenes on wilt disease, we examined whether exogenously applied sclareol induces the accumulation of lignin, which plays an important role in defense against pathogen attack (Raes et al. 2003). Tobacco roots were treated with 100 μM sclareol, and endogenous contents of thioglycolic acid lignin were determined. Levels of thioglycolic acid lignin in sclareol-treated roots 2 and 6 d after treatment were higher by 1.3- and 2.5-fold, respectively, than those in methanol-treated roots (**Fig. 2E**).

## Exogenously applied sclareol and *cis*-abienol inhibit bacterial wilt disease in tomato plants

To examine whether sclareol and *cis*-abienol inhibit bacterial wilt disease in other plant species, tomato plants were tested. Roots of tomato plants were pre-treated with sclareol or *cis*-abienol for 2 d and assayed for 8107S, a strain with a high pathogenicity for tomato plants (Nakaho et al. 1996), using the root-cutting inoculation method. In plants pre-treated with methanol, symptoms began to appear 4 d.p.i. and developed rapidly thereafter, and almost all inoculated plants wilted 9–10 d.p.i. (**Fig. 3A**, 0.1% methanol). Pre-treatment with sclareol or with *cis*-abienol delayed the development of disease symptoms and reduced the incidence of disease symptoms (**Fig. 3A**). Growth of strain 8107S was also inhibited by pre-treatment with sclareol or *cis*-abienol at 100 μM (**Fig. 3B**). Another inoculation method was also used to test the inhibitory effect of sclareol on wilt disease. Tomato roots grown in

soil were treated by drenching the soil with a solution containing sclareol. Tomato roots were inoculated with strain 8107S by pouring the bacterial suspension into the drenched soil, and the development of disease symptoms was measured. Pre-treatment with 100 μM sclareol delayed the development of disease symptoms and reduced the incidence of disease symptoms, although the level of the reduction was lower than that observed using the root-cutting inoculation method (Fig. 3C). Pre-treatment with a 200 μM solution of sclareol had stronger inhibitory activity for wilt disease than a 100 μM solution.

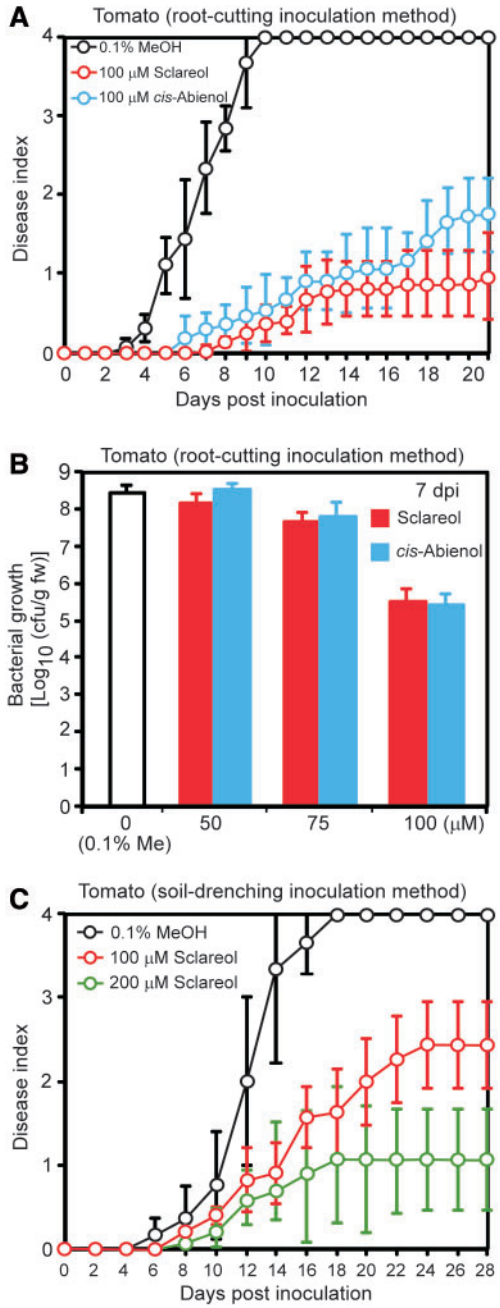


Fig. 3 Effect of sclareol and *cis*-abienol on inhibition of wilt disease in tomato. Tomato roots were pre-treated with the indicated compounds for 2 d and inoculated with strain 8107S. (A) Time course of disease symptom development. (B) Dose-dependent effect of sclareol or *cis*-abienol. (C) Effect of sclareol on the inhibition of wilt disease using the soil-drenching inoculation method. Data for A–C are the means ± SD from three independent assays with triplicate samples (each containing four plants).

## Correlation between the chemical structures of sclareol-related compounds and their inhibitory activities for bacterial wilt disease

To determine the chemical moieties of sclareol-related compounds responsible for inhibitory activity against bacterial wilt disease, the structure–activity relationship of sclareol derivatives was studied. We used tomato as a test plant in the disease resistance assay because the efficiency of tomato infection by strain 8107S was higher than tobacco infection by strain 8225 under our experimental conditions. Because *cis*-abienol is oxidatively decomposed (Hieda et al. 1982), sclareol was used as the starting material for chemically synthesizing derivatives. Sclareol contains two hydroxyl (OH) groups at carbon (C) positions 8 and 13 (see Fig. 1). We focused on the role of these OH groups in our structure–activity assays and synthesized derivatives with modifications at C8, C13 or both (Fig. 1, compounds 3–5). Compound 4 exhibited a high level of inhibitory activity toward the growth of strain 8107S in tomato, comparable with that of sclareol and *cis*-abienol (Fig. 4). No such activity was observed for compound 3 or compound 5.

Other compounds structurally related to sclareol were also examined in the same assay. Sclareolide (Fig. 1), a sesquiterpene lactone naturally occurring in tobacco, and its derivative (Fig. 1, compound 7) did not have any inhibitory activity (Fig. 4). We previously isolated WAF-1, a labdane-type diterpene, as an

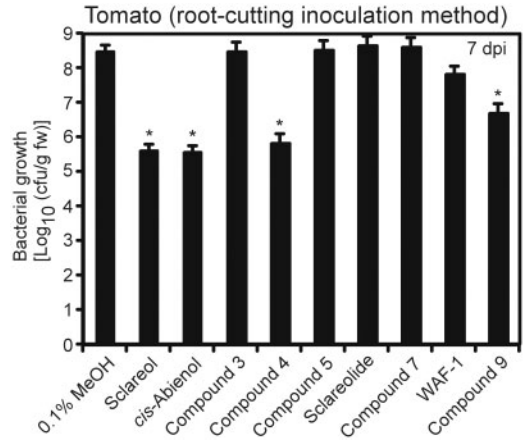


Fig. 4 Structure–activity correlation analysis of sclareol and its related compounds. Roots of tomato plants were pre-treated with the indicated compounds at 100 μM for 2 d and inoculated with strain 8107S. Bacterial growth was measured at 7 d.p.i. As a control, 0.1% methanol was used (Control). Data are the means ± SD from three independent assays with triplicate samples (each containing four plants). Asterisks indicate significant differences from the control ($P < 0.05$).

activator of stress-responsive tobacco MAPKs (Fig. 1; Seo et al. 2003). WAF-1 exhibited only a slight, but not significant, inhibitory activity (Fig. 4). Because WAF-1 easily undergoes oxidative degradation in air, resulting in a loss of its biological activity (Seo et al. 2003), an air-stable derivative was synthesized by introducing two methyl groups at C15 (Fig. 1, compound 9), the position where initial oxidation occurs. Compound 9 exhibited lower inhibitory activity than sclareol.

## Microarray analysis of sclareol-responsive Arabidopsis genes

To study the mechanism of bacterial wilt disease inhibition by sclareol-related compounds, the model plant *A. thaliana* was used. Although Arabidopsis does not produce sclareol and *cis*-abienol, it responds to exogenously applied sclareol (Campbell et al. 2003), suggesting the existence of a mechanism for responding to sclareol, and is a model system for studying bacterial wilt disease (Deslandes et al. 1998). We mainly used sclareol as the test compound for the study because *cis*-abienol undergoes oxidative decomposition as described above. We first examined whether sclareol inhibits wilt disease caused by *R. solanacearum* in Arabidopsis. Roots of Arabidopsis Columbia (Col-0) plants were pre-treated with sclareol and assayed for infection by strain RS1000 using the root-cutting inoculation method. In plants pre-treated with methanol, disease symptoms, such as yellowing or wilting of leaves, began to appear 7–9 d.p.i. and continued developing until almost all inoculated plants wilted 21–24 d.p.i. (Fig. 5A, 0.1% methanol). Pre-treatment with 100 μM sclareol delayed the development of disease symptoms and inhibited growth of the strain (Figs. 5A, B), suggesting that sclareol has an inhibitory effect on bacterial wilt disease for Arabidopsis, similar to tobacco and tomato.

To identify host factors involved in inhibiting bacterial wilt disease by sclareol, we performed a microarray analysis of sclareol-responsive Arabidopsis genes using an Agilent Arabidopsis 4 Oligo Microarray. Because sclareol showed inhibitory activity only when applied to roots, roots were used as the organ for identifying potential candidates for host factors. Arabidopsis roots were treated with 100 μM sclareol or with 0.1% methanol as a control for 2 h. This time point was designated to identify early responsive genes. We selected up-regulated genes with a *P*-value $<0.01$ and a fold change $>2$ over the methanol treatment and identified 659 genes (Supplementary Table S1). Approximately 30% of identified genes were those with an unknown function, and the others included genes with roles in metabolism, signaling, transcription and replication, stress response, disease resistance, transport, and hormone biosynthesis and signaling (Fig. 6). One of the most highly induced genes was the gene encoding AtPDR12. In *Nicotiana plumbaginifolia*, NpPDR1 functions as a transporter for sclareol (Jasiński et al. 2001), and AtPDR12 has the closest sequence homology to NpPDR1 and is transcriptionally induced when Arabidopsis plants are floated on a solution containing sclareol (Campbell et al. 2003). Genes

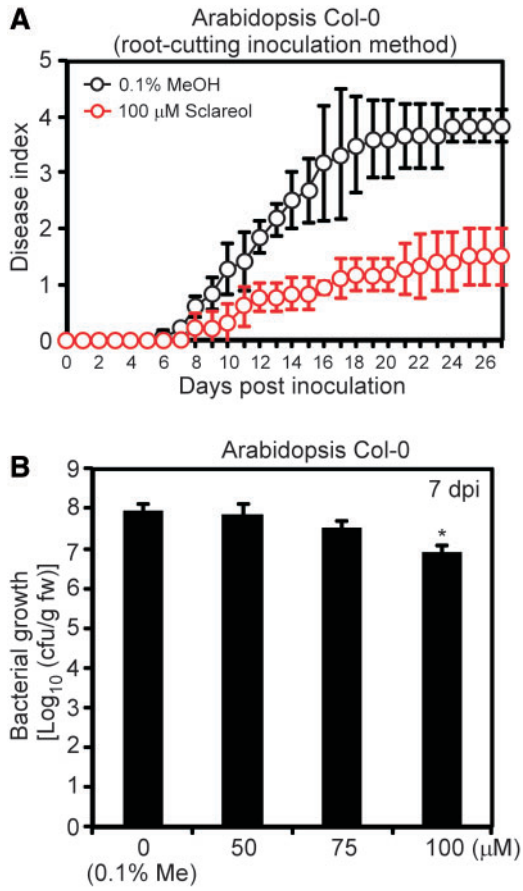


**Fig. 5** Effect of sclareol on the inhibition of wilt disease in Arabidopsis. Roots of Arabidopsis Col-0 plants were pre-treated with the indicated compounds for 2 d and inoculated with strain RS1000. (A) Time course of disease symptom development. (B) Dose-dependent effect of sclareol. Data for A and B are the means ± SD from three independent assays with triplicate samples (each containing 4–5 plants). Asterisks indicate significant differences from the control ($P < 0.05$).

involved in the biosynthesis or signaling of defense-related signaling molecules were identified; these genes included those for SA biosynthesis (e.g. *EDS5* and *PAD4*), JA biosynthesis (e.g. *OPR1* and *AOC3*) and signaling (e.g. *JAS1/JAZ10* and *AtMYC2*), ET biosynthesis (*ACS6*) and signaling (e.g. *ERF2*) and ABA signaling (*AtMYC2*). Genes encoding MAPK cascade components, such as *MAPKKK17*, *MAPKKK18*, *MPK3* and *MPK17*, were also found.

## Inhibition of bacterial wilt disease by sclareol is attenuated in some defense-related Arabidopsis mutants

We further studied the roles of AtPDR12, defense-related signal molecules and MAPKs in the inhibition of bacterial wilt disease by sclareol using mutants defective in their function, biosynthesis or perception. Compared with wild-type (Col-0) plants, inhibition of bacterial growth by pre-treatment with sclareol was attenuated in the AtPDR12 mutant *atpdr12-2*, the ET signaling mutant *ethylene-insensitive2-1* (*ein2-1*), the ABA

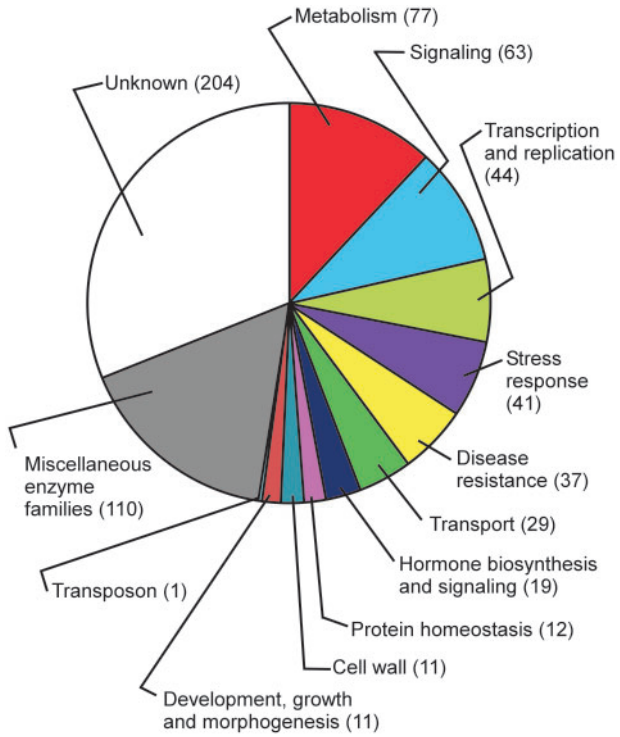


**Fig. 6** Categorization of up-regulated genes in Arabidopsis roots treated with sclareol. The number of genes in each class is shown in parentheses.

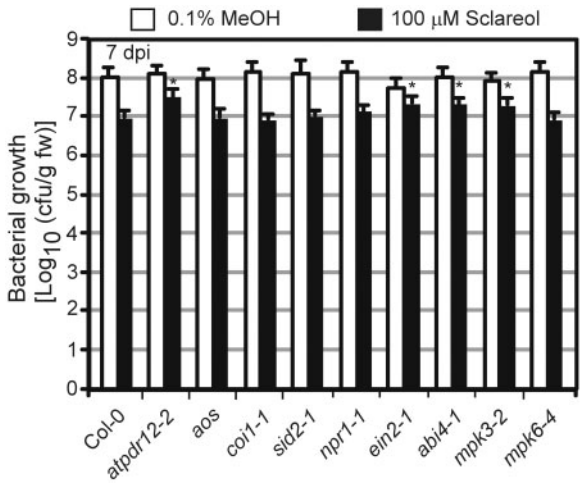


**Fig. 7** Effect of sclareol on the inhibition of wilt disease in defense-related Arabidopsis mutants. Roots of Arabidopsis Col-0 and mutant plants were pre-treated with the indicated compounds for 2 d and inoculated with strain RS1000. Bacterial growth was measured at 7 d.p.i. Data are the means ± SD from three independent assays with triplicate samples (each containing 4–5 plants). Asterisks indicate significant differences from the sclareol treatment in Col-0 plants ($P < 0.05$).

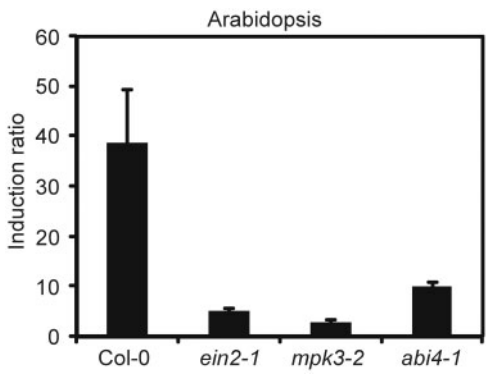


**Fig. 8** Analysis of *AtPDR12* induction by sclareol in defense-related Arabidopsis mutants. Quantitative RT–PCR analysis of *AtPDR12* transcript accumulation in the roots of Arabidopsis wild-type (Col-0) and mutant plants treated with 100 μM sclareol or with 0.1% methanol for 2 h. The induction ratio was calculated as the ratio of *AtPDR12* transcript levels in sclareol-treated roots relative to methanol-treated roots. Data are the means ± SD from three independent measurements.

signaling mutant *ABA-insensitive4-1* (*abi4-1*) and the MPK3 mutant *mpk3-2* (**Fig. 7**). *ein2-1* plants exhibited a slightly decreased susceptibility to the strain in the absence of sclareol. No such attenuation was observed in JA biosynthesis [*allene oxide synthase* (*aos*)] and signaling [*coronatin-insensitive1-1* (*coi1-1*)] mutants, SA biosynthesis [*SA induction-deficient2-1* (*sid2-1*)] and signaling [*nonexpressor of PR genes1-1* (*npr1-1*)] mutants or *mpk6-4*, another MAPK mutant.

The results shown in **Fig. 7** suggested that activation of AtPDR12 by sclareol is important for the inhibition of bacterial wilt disease in Arabidopsis. To assess whether activation of AtPDR12 by sclareol is mediated by EIN2, MPK3 or ABI4, the induced expression of *AtPDR12* by sclareol was examined in the roots of *ein2-1*, *mpk3-2* and *abi4-1* mutants. Expression of *AtPDR12* was not induced in *ein2-1*, *mpk3-2* and *abi4-1* mutant plants to the same high level as shown in wild-type plants (**Fig. 8**).

## Inhibition of bacterial wilt disease by sclareol is attenuated in *NtPDR1*-silenced tobacco

Analyses using microarray and Arabidopsis mutants identified several candidates for host factors involved in the inhibition of bacterial wilt disease by sclareol. To examine whether these candidates function similarly in the inhibition of bacterial wilt disease by sclareol in other plant species, we focused on NtPDR1, a tobacco homolog of AtPDR12 (Sasabe et al. 2002), and studied its role using *NtPDR1*-silenced tobacco plants. An expression analysis confirmed that exogenous application of sclareol to tobacco roots enhanced accumulation of *NtPDR1* transcripts (**Supplementary Fig. S6**). To silence *NtPDR1*, an inverted repeat (IR) construct that included a portion of the coding and 5′-untranslated region from *NtPDR1* cDNA was introduced into tobacco plants using *Agrobacterium tumefaciens* (**Supplementary Fig. S7**). Three transgenic lines (lines NtPDR1IR-16, NtPDR1IR-17 and NtPDR1IR-20) having lower levels of *NtPDR1* transcript accumulation (**Fig. 9A**) were

assayed for wilt disease. The degree of bacterial growth inhibition by pre-treatment with sclareol in all *NtPDR1*-silenced lines was lower than in the control line carrying the vector alone (**Fig. 9B**).

## Discussion

Sclareol and *cis*-abienol have antimicrobial activity, and exogenously applied sclareol induces expression of defense-related genes (Bailey et al. 1974, Bailey et al. 1975, Kennedy et al. 1992, Campbell et al. 2003). However, evidence linking these compounds directly to disease resistance against pathogens was insufficient. In the current study, bacterial wilt disease-inhibiting compounds were biochemically isolated from tobacco and were identified as sclareol and *cis*-abienol. Pre-treatment with sclareol or *cis*-abienol resulted in a delay in the development of disease symptoms caused by *R. solanacearum* and a reduction in bacterial growth in tobacco, tomato and Arabidopsis (**Figs. 2**, **3**, **5**). These observations suggested that sclareol and *cis*-abienol function in delaying or reducing development of wilt disease through inhibition of bacterial growth. Because neither sclareol nor *cis*-abienol exhibited any antimicrobial activity for *R. solanacearum* nor altered its pathogenicity, it is possible that inhibition of bacterial growth is due to host defense-like responses elicited or enhanced in the plant after treatment with these compounds. One such response is probably the accumulation of lignin. Microarray analysis of sclareol-responsive genes identified lignin biosynthetic genes such as those encoding cinnamoyl-CoA reductases (**Supplementary Table S1**; Raes et al. 2003), suggesting that the induction of lignin accumulation is regulated at the transcriptional level.

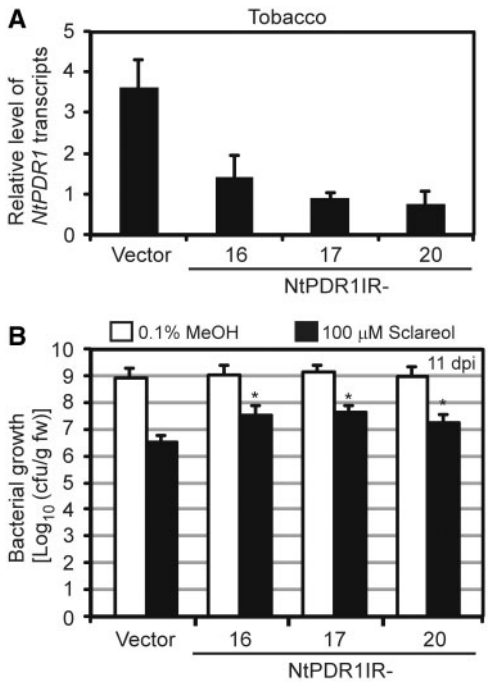


**Fig. 9** Attenuation in inhibition of wilt disease by sclareol in *NtPDR1*-silenced tobacco plants. *NtPDR1*-silenced and vector control lines were used for each analysis. (A) Quantitative RT–PCR analysis of *NtPDR1* in each line. Data are the means ± SD from three independent measurements. (B) Roots of adult tobacco plants were pre-treated with the indicated compounds for 2 d and inoculated with strain 8225. Bacterial growth was measured at 11 d.p.i. Data are the means ± SD from three independent assays with triplicate samples (each containing four plants). Asterisks indicate significant differences from the sclareol treatment in the vector control line ($P < 0.05$).

Genetic analysis indicates that AtPDR12 and NtPDR1 contribute to inhibition of wilt disease by sclareol. However, because this inhibition was not completely abolished in the Arabidopsis *atpdr12-2* mutant and *NtPDR1*-silenced tobacco plants, the contribution of AtPDR12 and NtPDR1 may be minor. The plant ABC transporter superfamily is a large protein group consisting of many subfamilies (Rea 2007). The sclareol transporter NpPDR1 of *N. plumbaginifolia* belongs to the pleiotropic drug resistance (PDR) subfamily (Jasiński et al. 2001). Although AtPDR12, NtPDR1 and NpPDR1 are members of the same subfamily, a recent study has shown that AtPDR12 functions in transporting ABA rather than sclareol in Arabidopsis (Kang et al. 2010). It is possible that sclareol enhances transport of ABA through activation of AtPDR12/NtPDR1, resulting in activation of a pathway that leads to defense. In this model, activation of AtPDR12/NtPDR1 by sclareol would be mediated partially through EIN2-dependent ET signaling, ABI4-dependent ABA signaling and MPK3 signaling pathways. This hypothesis is partially supported by a previous report showing that fungal infection-induced expression of *AtPDR12* requires the ET signaling pathway (Campbell et al. 2003). A positive role for ABA and MAPKs in resistance to *R. solanacearum* has also been reported (Hernández-Blanco et al. 2007, Chen et al. 2009). EIN2 is also involved in susceptibility of the host plant to *R. solanacearum* (Hirsch et al. 2002). EIN2 may have different roles in sclareol-induced host responses and infection-induced host responses. Possibly, *cis*-abienol has the same mode of action as sclareol.

We have previously shown that exogenous application of WAF-1 to tobacco leaves results in activation of both WIPK and SIPK, tobacco orthologs of MPK3 and MPK6, respectively, and enhancement of local resistance to *Tobacco mosaic virus* (TMV; Seo et al. 2003). However, a recent study has shown that WIPK and SIPK function to regulate local resistance to TMV negatively (Kobayashi et al. 2010). This finding suggests that enhancement of TMV resistance by WAF-1 occurs independently of MAPK activation by WAF-1. Inhibition of bacterial wilt disease by sclareol requires MPK3. It is possible that the inhibitory mechanism for bacterial wilt disease is different from the induction mechanism of TMV resistance by WAF-1.

Despite sclareol's ability to induce transcription of genes with roles in SA and JA biosynthesis and signaling pathways, mutants defective in these pathways exhibited no attenuation in inhibition of wilt disease by sclareol, suggesting that this

inhibition is independent of SA and JA biosynthesis and signaling pathways. The simplest explanation for these observations is that the induction of gene expression by sclareol is due to a secondary or side effect of the compound. We cannot exclude the possibility that the up-regulated genes identified by microarray analysis include genes indirectly induced by such an effect of sclareol. In our microarray analysis, we selected genes up-regulated 2 h after treatment with sclareol to identify genes involved in the early stage of sclareol signaling. Further expression analysis revealed that sclareol-induced expression of *AtPDR12* also occurs at 2 d, a time point of challenge inoculation with *R. solanacearum*, after treatment with sclareol (data not shown). However, such induction at 2 d did not occur for *MPK3*, another up-regulated gene (data not shown). Although not all genes identified by our microarray analysis are expressed 2 d after treatment with sclareol, our results suggest that the microarray analysis at the time point (2 h) was effective in screening genes involved in inhibition of wilt disease by sclareol. Further studies to investigate the function of the candidate genes identified in the microarray analysis are required.

Our results suggest that inhibition of bacterial wilt disease by sclareol occurs independently of SA and JA biosynthetic and signaling pathways, but is partially dependent on ET and ABA signaling pathways. Sclareol and *cis*-abienol seem to have a mode of action different from that of the chemicals known to be capable of activating host resistance to pathogens such as probenazol, INA and BTH, which function in SA biosynthetic or signaling pathways, and β-aminobutyric acid, which functions in different signal transduction pathways depending on the type of pathogen (Ton and Mauchi-Mani 2004, Eschen-Lippold et al. 2010). Recently, dehydroabietinal (DA), an abietane-type diterpene, was demonstrated to function as an activator of systemic acquired resistance (SAR) in plants (Chaturvedi et al. 2012). Activation of SAR induced by DA requires SA accumulation and signaling, suggesting that DA has a different mode of action from that of sclareol and *cis*-abienol, labdane-type diterpenes.

To study the structure–activity relationship of sclareol-related compounds, we mainly focused on their OH groups. Compound 3 and compound 5, which lack the OH group at C8, did not possess inhibitory activity for bacterial wilt disease, whereas compound 4, which contains an OH group at the same carbon position, exhibited activity comparable with that of sclareol and *cis*-abienol. However, it has been reported that 2-hydroxy manool, which lacks the OH group at C8, has inhibitory activity for *R. solanacearum* (Jackson and Danehower 1996). Although the exact role of the OH group at C8 in promoting the biological activities of compounds remains unknown, our results suggest that this moiety plays a role in the activity of sclareol-related compounds. Among the derivatives with modifications of the side chain at C9, compound 7 and WAF-1 exhibited little or no activity and compound 9 had lower activity than sclareol. Thus, modifications of the side chain at C9 altered the level of activity, suggesting that both the length of the side chain at C9 and the kind or number of functional group(s) bound to the chain are also important for the biological activities of sclareol-related compounds. These findings will be useful in the synthesis of more potent inhibitors.

Based on the hypothesis that tobacco plants undergoing HR would produce compounds conferring resistance to *R. solanacearum*, we evaluated compounds from 8266-infiltrated leaves on which HR-like cell death responses had occurred, and identified sclareol and *cis*-abienol as two active compounds. We expected that sclareol and *cis*-abienol would function as endogenous signals in HR-like cell death induced by infection with strain 8226. Sclareol and *cis*-abienol are produced in trichome glands on the leaf surface (Guo et al. 1994, Guo and Wagner 1995), but little is known about their exact localization in other cellular compartments. We therefore measured the endogenous content of sclareol in extracts from leaves or roots after infection. Since exact quantification of *cis*-abienol failed due to its degradation during the extraction process, only sclareol was quantified. Infection with strain 8266 resulted in a 2.5-fold increase in the sclareol content over that with mock inoculation. However, healthy tobacco leaves contained 594 nmol $g^{-1}$ FW of sclareol, a concentration much higher than the minimum concentration (100 μM) that inhibited bacterial wilt disease (**Supplementary Fig. S7A**). Actually, tobacco plants produce high concentrations of sclareol and *cis*-abienol on the leaf surface (Bailey et al. 1974). Interestingly, despite containing such a large amount of sclareol and *cis*-abienol, the ethyl acetate-soluble neutral fraction from mock-inoculated leaves exhibited only slight inhibitory activity for wilt disease. One possible explanation is the existence of an unknown substance capable of reducing the biological activity of sclareol and *cis*-abienol in the fraction. The concentration (approximately 10 nmol $g^{-1}$ FW) of sclareol in roots after infection was only one-tenth the minimum effective concentration (100 μM) of sclareol when applied to roots (**Supplementary Fig. S7B**). Although we were unable to obtain direct evidence for the role of sclareol and *cis*-abienol as endogenous signals in defense responses during HR-like cell death, the possibility of such defense signals cannot be excluded. Analysis of transgenic plants deficient in these diterpenes will solve this issue. Nevertheless, our results clearly indicate that exogenously applied sclareol and *cis*-abienol have the ability to inhibit bacterial wilt disease. The identification of sclareol and *cis*-abienol as bacterial wilt disease-inhibiting compounds using the assay system with young tobacco plants demonstrates the effectiveness of this system for screening such compounds. It will be interesting to explore further bacterial wilt disease-inhibiting compounds from other resources.

## Materials and Methods

### Plant materials, plant growth conditions and bacterial strains

Tobacco (*Nicotiana tabacum* cv Samsun NN) and tomato (*Solanum lycopersicum* cv Ponderosa) plants were used in

this study. All *A. thaliana* wild-type and mutant plants used are in the Columbia background. *atpdr12-2* (SALK_005635; Alonso et al. 2003), *aos* (SALK_017756) and *abi4-1* (seed stock number CS8104) were obtained from the Arabidopsis Biological Resource Center (ABRC). *sid2-1*, *npr1-1*, *coi1-1* and *ein2-1* have been described elsewhere (Abe et al. 2008, Leon-Reyes et al. 2009). *mpk3-2* (GABI_697F07) and *mpk6-4* (Takahashi et al. 2007) were a gift from K. Ichimura (Kagawa University), F. Takahashi, K. Shirasu and K. Shinozaki (RIKEN, Japan).

For the culture of young tobacco plants, 2-week-old seedlings grown on half-strength Murashige and Skoog (MS) medium (Wako Pure Chemical) supplemented with 0.7% agar were transferred to plastic vats containing a section of polyethylene fabric with a mesh size of 3 mm, soaked with a commercial liquid fertilizer (Hyponex; Hyponex Japan), diluted to 1 : 2,000 and grown with aeration for another 7–10 d under a cycle of 16 h of light and 8 h of darkness at 80–100 μmol photons $m^{-2}$ $s^{-1}$ at 25°C.

For the culture of adult tobacco plants, 7- to 10-day-old seedlings were transferred to peat moss pellets (Jiffy-7C, Jiffy Products of America Inc.) and grown for 2 months under the same conditions used for young tobacco plants.

For the culture of tomato plants, 7- to 10-day-old seedlings were transferred to Jiffy-7C or to pots (12 cm in diameter, 13 cm in height) filled with a commercial soil (Super Mix A; Sakata Seed Co.) and grown for 40 d under the conditions used for young tobacco plants.

Arabidopsis plants were grown as described previously (Deslandes et al. 1998). Briefly, 7-day-old seedlings grown in MS medium were transferred to Jiffy-7C and grown under a cycle of 10 h of light and 14 h of darkness at 100–120 μmol photons $m^{-2}$ $s^{-1}$ at 20°C for 4 weeks.

The *R. solanacearum* strains 8225 and 8266 have been described elsewhere (Takabatake et al. 2006). Strain 8107S has also been described elsewhere (Nakaho et al. 2004). Strain RS1000 was obtained from the MAFF gene bank of the National Institute of Agrobiological Sciences of Japan. All strains were cultured in PSA (0.5% Bacto peptone, 2% sucrose and 0.05% L-glutamic acid) or CPG (1% Bacto peptone, 0.1% yeast extract, 0.5% glucose and 0.1% casamino acids) medium at 28°C.

## Purification, isolation and identification of bacterial wilt disease-inhibiting compounds

Ten kilograms (fresh weight) of tobacco leaves were infiltrated with a bacterial suspension [$10^8$ colony-forming units (c.f.u.) $ml^{-1}$] of strain 8266 and incubated at 25°C for 48 h. The inoculated leaves on which HR-like lesions formed were homogenized in 4 vols. of cold 80% (v/v) acetone and extracted overnight at 4°C. Ethyl acetate-soluble acidic, neutral and basic fractions from the acetone extract were prepared according to the procedure described previously (Seo et al. 2003). The ethyl acetate-soluble neutral fraction was separated on a column (3 cm × 50 cm) of silica gel (Wakogel C-200; Wako Pure Chemical) eluted with increasing concentrations of ethyl acetate in hexane starting with 10% (v/v) ethyl acetate, and finally with 100% methanol. Fractions eluted with 10–20% ethyl acetate in hexane were combined, evaporated and separated on a reversed-phase cartridge column (Sep-Pak Vac $C_{18}$; Waters) eluted with increasing concentrations of methanol in water starting with 10% (v/v) methanol. Fractions eluted with 80–100% methanol were combined and finally separated on a reversed-phase HPLC column (CAPCELL PAK $C_{18}$, 5 μm particle size, 10 mm × 15 cm; Shiseido Co.). Elution was performed with a linear gradient from 25 to 100% acetonitrile in water for 50 min, then 100% acetonitrile for 5 min at a flow rate of 6 ml $min^{-1}$, with monitoring at 200 and 238 nm. Peaks with retention times of 28.4 (peak A) and 49.2 min (peak B) were collected and individually subjected to $^1$H-NMR and $^{13}$C-NMR analysis. $^1$H-NMR and $^{13}$C-NMR spectral data are described in **Supplementary Text 1**.

All fractions obtained were evaporated and dissolved in methanol, and aliquots of the solution were diluted with water to adequate concentrations and used in the assay for testing bacterial wilt disease-inhibiting compounds.

## Inoculation, evaluation of disease development and bacterial growth assay

For the assay testing bacterial wilt disease-inhibiting compounds, roots of 4–5 young tobacco plants were completely immersed in a solution containing adequate concentrations of fractions in a Petri dish (9 cm in diameter) and incubated at 25°C for 2 d. A solution containing 0.1% methanol was used for the control. After briefly washing with tap water, roots were trimmed 1–2 cm from the tips and immediately immersed for 10 min in a bacterial suspension ($10^8$ c.f.u. $ml^{-1}$) of strain 8225. After brief washing with tap water to remove excess bacterial suspension, plants were transferred to plastic vats containing a commercial liquid fertilizer (Hyponex) diluted to 1 : 2,000, grown with aeration for 10 d under a cycle of 16 h of light and 8 h of darkness at 80–100 μmol photons $m^{-2}$ $s^{-1}$ at 30°C and evaluated for disease development.

For root-cutting inoculation, roots of adult tobacco, tomato and Arabidopsis seedlings grown in Jiffy-7C pellets were completely immersed in a solution containing adequate concentrations of chemicals and incubated at 25°C for 2 d. A solution containing 0.1% methanol was used for the control. After briefly washing with tap water, roots extending from the pellets were trimmed 3–6 cm from the tips, and then the pellets were completely immersed in a bacterial suspension ($10^8$ c.f.u. $ml^{-1}$) for 30 min. After briefly washing with tap water to remove excess bacterial suspension, plants were incubated at 30°C for tobacco and tomato or 26°C for Arabidopsis and subjected to evaluation of disease development or measurement of bacterial growth.

For soil-drenching inoculation, adequate concentrations of sclareol were applied to roots of tomato grown in soil in a pot by slowly pouring the chemical solution (300 ml 1,000 $cm^{-3}$ soil) over the soil surface and incubated at 25°C. A newly prepared chemical solution was applied to the roots as described

above 1 d after treatment and incubated for a further 1 d. Inoculation was conducted by pouring $5 \times 10^7$ c.f.u. $ml^{-1}$ of a bacterial suspension (300 ml 1,000 $cm^{-3}$ soil) over the soil surface. Inoculated plants were incubated at 30°C and subjected to evaluation of disease development.

Development of disease symptoms was evaluated using a disease index ranging from 0 to 4: 0, no wilted leaves; 1, up to 25% wilted; 2, up to 50% wilted; 3, up to 75% wilted; and 4, entirely wilted.

For the bacterial growth assay, sections (1–2 cm long) of the middle stem of inoculated tobacco and tomato plants or all rosette leaves of inoculated Arabidopsis plants were collected at selected time points after inoculation using the root-cutting inoculation method. Collected samples were extracted with sterilized distilled water and diluted to adequate concentrations. Aliquots of the diluted solution were plated onto CPG agar medium containing tetrazolium chloride and incubated at 28°C for 2 d, and colonies with pink centers were counted.

For quantitative analysis of sclareol in roots shown in **Supplementary Figure S8B**, roots were inoculated by immersion in a bacterial suspension ($10^7$ c.f.u. $ml^{-1}$) of strain 8226 and incubated without cutting the roots to prevent sclareol leakage.

## Pathogenicity assay

For the pathogenicity assay, strain 8225 was cultured with 100 μM sclareol or with 0.1% methanol in CPG medium at 28°C for 2 d and inoculated on tobacco roots using the root-cutting inoculation method. Inoculated plants were subjected to evaluation of disease development.

## Preparation and synthesis of compounds

Of the compounds used in our study (see **Fig. 1**), sclareol and sclareolide are commercially available. For the experiments shown in **Figs. 2–9**, sclareol and sclareolide were purchased from Sigma-Aldrich. *cis*-Abienol freshly purified from tobacco leaves according to a simple purification procedure (Hieda et al. 1982) was used for each analysis. WAF-1 was synthesized as described previously (Seo et al. 2003). Experimental details for the synthesis of compounds 3, 4, 5, 7 and 9 are described in **Supplementary Text 1**.

## Chemical treatments

All compounds were dissolved in methanol and diluted to appropriate concentrations with water. Methanol concentrations did not exceed 0.1% in all experiments. Roots of adult tobacco, tomato and Arabidopsis plants grown in Jiffy pellets were immersed in a solution containing appropriate concentrations of compounds and incubated for 2 d for the disease resistance assay or for 2 or 6 h for gene expression analyses. For the controls, a solution containing 0.1% methanol was used.

## Lignin measurement

Adult tobacco roots treated with 100 μM sclareol or with 0.1% methanol for 2 d were harvested (referred to as 2 d in **Fig. 2G**) or transferred to water, incubated for a further 4 d and harvested (referred to as 6 d in **Fig. 2G**). Harvested samples were divided into sections (0.5–1 cm long) and the amount of thioglycolic acid lignin was measured as described previously (Suzuki et al. 2009).

## Microarray analysis

For microarray analysis, roots of Arabidopsis Col-0 plants were treated with 100 μM sclareol or 0.1% methanol and incubated for 2 h. Root samples from each treatment were harvested in triplicate. Total RNA was extracted using TRIzol reagent (Invitrogen) followed by RNA purification columns (RNeasy, Qiagen) and labeled with cyanine dye 3 (Cy3) using Quick Amp Labeling (Agilent) for a one-color experiment. Individually labeled cRNAs were subjected to gene expression analysis using microarrays. Further experimental details are described in **Supplementary Text 1**. Microarray data were deposited in the public Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) and have been assigned the accession number GSE31230.

## RNA extraction and quantitative real-time RT–PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Quantitative real-time reverse transcription–PCR (RT–CR) analysis was performed in a two-step reaction using a SYBR Green kit (Bio-Rad) and gene-specific primers (**Supplementary Text 1**). The levels of expression of *AtPDR12* and *NtPDR1* were normalized to the expression levels of *β-actin* and *Ntactin*, respectively.

## Vector construction and tobacco transformation

A 500 bp region of *NtPDR1* cDNA (corresponding to positions 151–650) was amplified by PCR, and the resulting products were inserted into the *Bam*HI and *Kpn*I sites of a modified binary vector, pBE2113 (Ohtsubo et al. 1999), separated by a 989 bp region of the *β-glucuronidase* (*GUS*) gene (corresponding to positions 821–1,809), to obtain the NtPDR1IR construct.

The NtPDR1IR construct was used to transform tobacco by the leaf disc co-cultivation method using *A. tumefaciens* LBA4404. Kanamycin-resistant transformants were selected for reduced *NtPDR1* expression by quantitative real-time RT–PCR analysis. Three independent lines with the greatest reduction in *NtPDR1* expression were isolated, self-pollinated and used for further analysis.

## Supplementary data

**Supplementary data** are available at PCP online.

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