Sclareol Induces Plant Resistance to Root-Knot Nematode Partially Through Ethylene-Dependent Enhancement of Lignin Accumulation

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The root-knot nematode (RKN) is one of the most devastating parasitic nematodes of plants. Although some secondary metabolites released by the host plant play roles as defense substances against parasitic nematodes, the mechanism underlying the induction of such defense responses is not fully understood. We found that sclareol, a natural diterpene known as an antimicrobial and defense-related molecule, inhibited RKN penetration of tomato and *Arabidopsis* roots. Sclareol induced genes related to ethylene (ET) biosynthesis and signaling and phenylpropanoid metabolism in *Arabidopsis* roots. In roots of *ein2-1*, an ET-insensitive mutant line, both sclareol-induced inhibition of RKN penetration and sclareol-induced enhancement of lignin accumulation were abolished. A mutant defective in lignin accumulation did not exhibit such inhibition. Sclareol also activated MPK3 and MPK6, *Arabidopsis* mitogen-activated protein kinases whose activation is required for triggering ET biosynthesis. Sclareol-induced inhibition of RKN penetration was exhibited by mutants of neither MPK3 nor MPK6. Treatment with a biosynthetic precursor of ET was insufficient compared with sclareol treatment to inhibit RKN penetration, suggesting the existence of an ET-independent signaling pathway leading to RKN resistance. These results suggested that sclareol induced resistance to RKN penetration partially through ET-dependent accumulation of lignin in roots.

Plants have evolved in complex environments in which they interact with many organisms. In order to survive in these environments, plants must overcome attack by harmful parasitic and phytophagous organisms such as bacteria, fungi, nematodes, and insects. Plants have developed defense systems with constitutive and inducible protective responses using different defense mechanisms against attack by specific types of organisms. The phytohormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) play important roles in all of these responses (Robert-Seilanian et al. 2011). These plant hormones regulate various plant signal pathways, and early transcriptional effects have been linked to hormone response (Kazan and Lyons 2014). One of the transcriptional reactions in defense response includes the accumulation and

release of metabolites, known as secondary metabolites. Secondary metabolites such as isoprenoids, phenylpropanoids, alkaloids, and fatty acids or polyketides play an important role in both constitutive and inducible direct defense of plants against their natural enemies (Cheng et al. 2007; Vogt 2010; Ziegler and Facchini 2008).

The isoprenoid pathway produces terpenes, which are composed of five carbon-isoprene units. These are classified as mono, sesqui-, di-, tri-, and higher-terpenes based on the number of isoprene units. Terpenes play roles in various physiological processes such as growth, development, responses to stresses, and disease resistance in plants (Cheng et al. 2007). Among the terpenes, the diterpene sclareol is of particular interest because of its range of physiological activities. Sclareol, which was originally isolated as a constituent of the essential oil in clary sage (*Salvia sclarea*), has been reported to be found in only a few plant species, including *Nicotiana* spp. (Caniard et al. 2012). Earlier studies showed that sclareol possessed direct antimicrobial activity against some types of plant-pathogenic bacteria and fungi (Bailey et al. 1975; Kennedy et al. 1992). Sclareol was demonstrated to be transported by NpABC1, a stress-responsive ATP-binding cassette (ABC) transporter, in *Nicotiana plumbaginifolia* (Jasiński et al. 2001). Treatment of *Nicotiana* spp. plants with sclareol induced the expression of various genes, such as those encoding proteins involved in metabolism, disease resistance, transport, and hormone biosynthesis and signaling (Campbell et al. 2003; Grec et al. 2003; Jasiński et al. 2001; Seo et al. 2012). Such induction of defense-related genes by exogenously applied sclareol was also observed in *Arabidopsis*, a plant species that does not produce sclareol (Campbell et al. 2003). This suggested that there are certain signaling pathways activated by sclareol that are common in plants. Furthermore, sclareol conferred resistance to the soilborne bacterial pathogen *Ralstonia solanacearum* in tobacco, tomato, and *Arabidopsis*; and transgenic tobacco with reduced expression of *NiPDR1*, a tobacco homolog of *NpABC1*, exhibited attenuated sclareol-induced resistance (Seo et al. 2012). These findings suggested that sclareol is involved in defense responses to biotic stresses in plants.

Plant-parasitic nematodes parasitize roots or stems of various plants, thereby inhibiting absorption of nutrients and moisture (Wyss et al. 1992). Root-knot nematodes (RKNs) (*Meloidogyne* spp.) deserve careful attention because they have a wide host range, and RKN parasitization causes severe crop damage. RKNs are sedentary endoparasitic nematodes. Second-stage juveniles (infective stage) penetrate root tips and migrate to the vascular cylinder, in which RKNs interact with the host plant. In susceptible host plants, RKNs induce a series of complicated changes in the plant cell architecture.

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Secondary metabolites in plants are also important for repelling and attracting RKNs. Although RKNs have an extensive host range, host plant species have significantly different levels of attractiveness for different RKN species (Wang et al. 2009). Although both the attractants and repellents for RKNs remain largely unknown, the basal levels or types of attractants and repellents differ according to the host species. Previous studies have suggested that JA and ET may participate in the attractiveness of plants to RKNs because the efficiency or degree of RKN penetration was shown to be changed in *Arabidopsis* or tomato mutants deficient in JA or ET signaling (Bhattarai et al. 2008; Fudali et al. 2013; Fujimoto et al. 2011a,b). JA and ET are involved in modulating herbivore-induced metabolite emissions (Schmelz et al. 2003). For example, the activation of ET biosynthesis led to the production of defense compounds such as antimicrobial substances (Pedras et al. 2000). Sclareol enhanced the expression of the *Arabidopsis* ABC transporter gene *AtPDR12*, the induction of which required activation of the ET signaling pathway (Campbell et al. 2003).

In the course of exploring the plant secondary metabolites involved in attracting or repelling RKNs, we found that sclareol inhibited its penetration. Here, we demonstrated that sclareol conferred resistance to RKN penetration by partially activating ET-dependent defense responses.

RESULTS

Exogenous application of sclareol inhibits penetration of RKNs into tomato roots.

To examine the effect of sclareol (Fig. 1) on the penetration of RKNs into plant roots, we used tomato, an extremely susceptible host plant for RKNs. The roots of 3-week-old tomato plants grown in sand in pots treated with different concentrations (50, 80, 100, or 200 μ M) of sclareol, incubated for 48 h, inoculated with RKNs, and stained with acid fuchsin, an agent that stains nematodes, 7 days after inoculation. The degree of inhibition of RKN penetration was estimated by counting the number of stained RKNs. The penetration of RKNs was inhibited by sclareol at 100 or 200 μ M to approximately 30% of the control methanol treatment (Fig. 2A). To determine the duration of treatment required to manifest the inhibitory effect of sclareol, tomato roots treated with 100 μ M sclareol were incubated for 24, 48, or 72 h and assayed for RKN resistance. Treatment for 24 h had no effect on inhibition of RKN penetration, whereas treatment for 48 h or longer had an inhibitory effect (Fig. 2B). Based on these results, we used sclareol at a concentration of 100 μ M and with a treatment period of 48 h for further analyses. To gain insight into its structure–activity relationship, we assayed sclareolide (Fig. 1), a naturally occurring sesquiterpene lactone with a chemical structure similar to sclareol. Sclareolide did not have any inhibitory activity for penetration of RKNs into tomato roots (Fig. 2C and D).

To determine whether inhibition of RKN penetration by sclareol was attributed to its nematicidal activity, the effect of sclareol on the survival of RKNs was tested. Sclareol had little or no effect on the survival of RKNs (Supplementary Table S1). Whether sclareol affected the infectivity of RKNs was also examined by inoculating a suspension of RKNs that had been previously incubated with sclareol, or methanol as a control, for 48 h on tomato roots, followed by counting the number of RKNs penetrated into the tomato roots (Supplementary Fig. S1A). No significant difference was observed in the number of RKNs between treatments (Supplementary Fig. S1B).

EIN2-dependent ET signaling is involved in inhibition of RKN penetration by sclareol.

The results from the RKN penetration assays using tomato (Fig. 2) suggested that inhibition of RKN penetration by sclareol

is due to defense responses induced or enhanced in the host plant by the chemical. As the first step to identify host factors involved in signaling of such defense responses, we analyzed the expression of known marker genes related to phytohormone signaling in *Arabidopsis*, a model plant for studying plant–nematode interactions, after treatment with sclareol. *Arabidopsis* plants were grown in two different growth conditions, and plants grown in a sand system were used for gene expression analysis (Fig. 3A). In addition, plants were grown in a hydroponic system, which provided intact roots for other biochemical and histochemical analyses. Roots of wild-type (WT) Columbia (Col-0) plants grown in the sand system were treated with sclareol, and the expression levels of marker genes in the treated roots were examined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR). Sclareol enhanced the accumulation of transcripts for the JA/ET signaling marker gene *PDF1.2*, whereas it had little or no effect on the accumulation of transcripts for *chiB*, another JA/ET signaling marker gene (Fig. 3B). None of the SA signaling marker genes (*BGL2* and *PR1*) or the JA signaling marker genes (*VSP2* and *LOX2*) responded to sclareol. Quantitative analysis of endogenous SA and JA in roots also showed that neither SA nor JA levels were changed by treatment with sclareol (Supplementary Fig. S2).

To further examine which phytohormone is involved, *Arabidopsis* mutants defective in the perception of specific phytohormones were assayed for resistance to RKN penetration. We first confirmed whether exogenously applied sclareol inhibits the penetration of RKNs into *Arabidopsis* roots using WT plants grown in the sand system. The number of RKNs penetrated into sclareol-treated roots was approximately half of that into methanol-treated roots (Fig. 4, WT). We next assayed using the SA signaling mutant *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1-1* (*npr1-1*), an abscisic acid (ABA) signaling mutant *ABA-INSENSITIVE1-1* (*abi1-1*), a JA signaling mutant *CORONATINE-INSENSITIVE1-1* (*coi1-1*), and an ET signaling mutant *ETHYLENE-INSENSITIVE2-1* (*ein2-1*). In *npr1-1* and *abi1-1*, sclareol reduced the number of RKNs that penetrated into the roots to the same degree as shown in WT plants (Fig. 4). *coi1-1* exhibited relatively low susceptibility to infection, as indicated by decreased penetration of RKNs in the absence of sclareol compared with WT plants, and there was no difference in the number of RKNs that penetrated into roots between treatments in this mutant. Inhibition of RKN penetration by sclareol was not observed in *ein2-1*. These results suggested that EIN2 is involved in sclareol-induced resistance to RKN penetration.

Lignin is involved in EIN2-mediated RKN resistance induced by sclareol.

To identify the factors that function downstream of EIN2 in sclareol-induced RKN resistance, we used microarray data on sclareol-responsive *Arabidopsis* root genes (Seo et al. 2012). Our previous microarray analysis identified many genes with a

Fig. 1. Chemical structures of sclareol and sclareolide.

role in phytohormone responses, defense responses, and metabolism. Among them, we examined the possibility of genes for lignin biosynthetic enzymes and ET-responsive factors (ERFs) as such downstream factors, because lignin plays a role in defenses in roots and because ERFs function in ET signaling. If these genes function downstream of EIN2, it is expected that their expression would not be induced by sclareol in the *ein2-1* mutant. Roots of *ein2-1* mutant plants grown in the sand system were treated with sclareol or methanol, and the induction kinetics were assessed for three lignin biosynthetic genes—phenylalanine ammonia-lyase (*PAL1*), trans-cinnamate 4-monooxygenase (*C4H*), and cinnamoyl-CoA reductase (*CCR2*)—and an ERF gene (*ERF2*). There was no difference in the degree of accumulation of transcripts for *PAL1*, *C4H*, and *CCR2* between treatments in *ein2-1*, whereas accumulation of transcripts for these three genes was enhanced by sclareol in WT plants (Fig. 5, *ein2-1* and WT). To confirm whether the behavior of *PAL1*, *C4H*, and *CCR2* observed in *ein2-1* was specific to this mutant, their induction kinetics in other phytohormone-related mutants were also examined. In *npr1-1*, *abil-1*, and *coi1-1*, as in WT plants, the *PAL1*, *C4H*, and *CCR2* genes responded substantially to sclareol. Accumulation of transcripts for *ERF2* was enhanced by sclareol in the WT but not in *ein2-1* (data not shown). We also checked the expression of *AtPDR12*, a gene that was previously identified as the most highly induced gene by microarray analysis (Seo et al. 2012), in *ein2-1* after treatment with sclareol. The degree of *AtPDR12* transcript accumulation was almost equal between treatments in *ein2-1*. This was consistent with a previous report showing that EIN2 regulates the expression of *AtPDR12* (Campbell et al. 2003).

The result shown in Figure 5 suggested the possible involvement of lignin in EIN2-mediated RKN resistance induced by sclareol. To further test the possibility, we first examined whether sclareol induced accumulation of lignin. Roots of WT

plants grown in the sand system were treated with sclareol, and the endogenous content of thioglycolic acid lignin in the treated roots was determined. Sclareol increased thioglycolic acid lignin levels by approximately threefold compared with the control methanol treatment (Fig. 6A, WT). Such an increase was also detected in roots of the *npr1-1*, *abil-1*, and *coi1-1* mutants. In contrast, roots of *ein2-1* exhibited no increase in thioglycolic acid lignin in response to sclareol. To analyze the localization of lignin in roots, roots of hydroponically grown plants were treated with sclareol and stained with phloroglucinol, an agent that stains lignin. In sclareol-treated roots of WT plants, lignin staining was detected in the root hair zone in the root tip (Fig. 6B, sclareol in WT). Such staining was undetectable in the corresponding zone in *ein2-1* (Fig. 6B, sclareol in *ein2-1*). We also examined the effect of 1-aminocyclopropane-1-carboxylic acid (ACC), the biosynthetic precursor of ET, on accumulation of lignin. Exogenously applied ACC induced the accumulation of lignin in roots of WT but not *ein2-1*, although the degree of induction was lower than that by sclareol (Fig. 6B, ACC in WT and *ein2-1*).

Finally, we assessed whether lignin accumulation was necessary for sclareol-induced inhibition of RKN penetration using an *Arabidopsis* mutant for *CCR2*, *ccr2* (Nossen background). Quantitative analysis of lignin confirmed that roots of *ccr2* failed to accumulate thioglycolic acid lignin in response to sclareol, whereas roots of the WT with the same genetic background did (Fig. 6C). Roots of *ccr2* exhibited, in response to sclareol, no inhibition of RKN penetration (Fig. 6D) and no enhancement of *PAL1*, *C4H*, and *CCR2* transcript accumulation (Supplementary Fig. S3).

Activation of ET biosynthesis is also important for sclareol-induced RKN resistance.

The involvement of ET biosynthesis in sclareol-induced RKN resistance was also investigated. We first examined

Fig. 2. Effect of sclareol and sclareolide on the penetration of root-knot nematodes (RKNs) into tomato roots. **A** and **C**, Dose-dependent effect of sclareol and sclareolide, respectively. Tomato roots pretreated with the indicated concentrations of sclareol for 48 h were inoculated with RKNs, and the number of RKNs that penetrated into the roots was counted 7 days after inoculation. **B** and **D**, Tomato roots pretreated with 100 μM sclareol or sclareolide for 24, 48, or 72 h were inoculated with RKNs, and the number of RKNs that penetrated into the roots was counted 7 days after inoculation. **A** to **D**, Methanol (MeOH) (0.1%) was used as a control. Values are the mean ± standard deviation from two independent experiments, each performed in five replicates. Asterisks denote statistically significant differences compared with the 0.1% MeOH control (Tukey's honestly significant difference test; $P < 0.05$).

whether sclareol activates ET biosynthesis by measuring endogenous amounts of ET in WT *Arabidopsis* plants after treatment with sclareol. Because of the difficulty in measuring ET from roots without applying any wound stresses that allow its biosynthesis to activate, hydroponically grown WT plants were sealed in a test tube, with the roots submerged in a solution containing sclareol, and ET released from the whole plant was analyzed. Plants treated with sclareol produced 3.5-fold more ET than plants treated with methanol (Fig. 7A). Sclareolide caused only a slight increase in ET emission (Supplementary Fig. S4). Consistent with enhanced ET biosynthesis, accumulation of transcripts for the ACC synthase (ACS) genes *ACS2* and *ACS6* in WT roots was enhanced by sclareol (Fig. 7B). These results suggested that sclareol activates biosynthesis of ET.

Stress-induced activation of ET biosynthesis requires activation of mitogen-activated protein kinases (MAPKs) in plants. In *Arabidopsis*, external stimuli such as wounding cause a rapid activation of two MAPKs, MPK3 and MPK6, which results in triggering biosynthesis of ET through phosphorylation of stress-responsive ACS such as *ACS2* and *ACS6* by the activated MPK3 and MPK6 (Li et al. 2012). We examined whether MPK3 and MPK6 are involved in sclareol-induced RKN resistance. MAPK activity can be detected by measuring the phosphotransferase activity for the target substrate in an immune complex between the MAPK and a MAPK-specific

antibody. To detect the activities of MPK3 and MPK6, we generated anti-MPK3 and anti-MPK6 antibodies against antigen peptides corresponding to the C-terminal amino acid regions of each MAPK (see Materials and Methods for details). The specificity of these antibodies was assessed by determining the enzymatic activities of MPK3 and MPK6 in *mpk3-2* and *mpk6-4* (*Arabidopsis* mutants for MPK3 and MPK6, respectively) after wounding. Total extracts from

Fig. 4. Effect of sclareol on penetration of root-knot nematodes (RKNs) into roots of defense-related *Arabidopsis* mutants. Roots of wild-type (WT) (Col-0) or mutant plants pretreated with 100 μ M sclareol for 48 h were inoculated with RKNs, and the number of RKNs that penetrated into the roots was counted 7 days after inoculation. Values are the mean $\pm$ standard deviation from two independent experiments, each performed in five replicates. Asterisks denote statistically significant differences compared with the 0.1% methanol (MeOH) control of the WT (Tukey's honestly significant difference test; $P < 0.05$).

Fig. 3. Analysis of expression of defense-related genes in *Arabidopsis* roots after treatment with sclareol. **A**, Plant growth systems used. **B**, Real-time reverse-transcriptase polymerase chain reaction analysis of salicylic acid (SA)- (*BGL2* and *PR1*), jasmonic acid (JA)- (*VSP* and *LOX2*), and JA/ethylene (ET)-responsive (*PDF1.2* and *ChiB*) genes in wild-type (Col-0) roots 48 h after treatment with 100 μ M sclareol or 0.1% methanol (MeOH). Values are the mean $\pm$ standard deviation from three independent measurements. Asterisks denote statistically significant differences compared with the 0.1% MeOH control (Student *t* test; $P < 0.05$).

wounded leaves of *mpk3-2* or *mpk6-4* mutant plants grown in the hydroponic system were immunoprecipitated with anti-MPK3 or anti-MPK6 antibodies, and kinase activities in the immune complexes using myelin basic protein (MBP) as an artificial substrate were determined. Little wounding-induced activity of MPK3 was detected in *mpk3-2*, whereas little wounding-induced activity of MPK6 was detected in *mpk6-4* (Supplementary Fig. S5). Immunoblotting analysis indicated

Fig. 5. Gene expression analysis of roots of defense-related *Arabidopsis* mutants after treatment with sclareol. Real-time reverse-transcriptase polymerase chain reaction analysis of defense-related genes in roots of wild-type (WT) (Col-0) and mutants 48 h after treatment with 100 μ M sclareol or 0.1% methanol (MeOH). Values are the mean $\pm$ standard deviation from three independent measurements. Asterisks denote statistically significant differences compared with the MeOH control (Student *t* test; $P < 0.05$). Each value represents the relative degree of expression.

that a protein that reacted with the anti-MPK3 antibody was undetectable in *mpk3-2*, whereas a protein that reacted with the anti-MPK6 antibody was undetectable in *mpk6-4*. Thus, the anti-MPK3 and anti-MPK6 antibodies we generated can be used to specifically detect the activities of MPK3 and MPK6, respectively.

To examine whether exogenously applied sclareol activates MPK3 and MPK6 in roots, we used plants grown in the hydroponic system because pulling the root out of the pot gives the plant a wound that causes rapid activation of MAPKs (Rodriguez et al. 2010). Roots of hydroponically grown WT plants were treated with sclareol and subjected to immunoprecipitation with anti-MPK3 or anti-MPK6 antibodies followed by the immune complex MBP kinase assay. The MBP kinase activities of MPK3 and MPK6 began to increase 5 min after treatment, reached a peak at 30 min, and thereafter returned to the basal level (Fig. 7C). Methanol treatment did not increase the kinase activities of these two MAPKs. The protein levels of MPK3 and MPK6 were almost equal over the time course, suggesting that rapid activation of MPK3 and MPK6 by sclareol occurs at the posttranslational level.

Neither *mpk3-2* nor *mpk6-4* exhibited inhibition of RKN penetration in response to sclareol (Fig. 7D). We also examined whether ACC possesses the same inhibitory effect on RKN penetration as sclareol. When roots of WT *Arabidopsis* grown in the sand system were treated with 50 μ M ACC and inoculated with RKNs, the number of penetrating RKNs was decreased compared with methanol-treated roots, although the inhibitory effect was smaller than sclareol treatment (Fig. 7D, sclareol versus ACC in WT). None of genes *ein2-1*, *mpk3-2*, or *mpk6-4* exhibited inhibition of RKN penetration in response to ACC.

DISCUSSION

Exogenously applied sclareol did not show any nematocidal activity for RKNs or alter their infectivity, suggesting that inhibition of RKN penetration by sclareol was due to host defense responses elicited or enhanced in the plant after treatment with this chemical. Several lines of evidence demonstrate that a signal transduction pathway leading to such host responses is mediated by ET and its downstream factor, EIN2. First, *ein2-1* exhibited a complete absence of inhibition of RKN penetration by sclareol (Fig. 4). Second, sclareol enhanced both the production of endogenous ET and the expression of ET-responsive or biosynthetic genes (Fig. 7A and B). Third, sclareol activated MPK3 and MPK6 (Fig. 7C), MAPKs whose activation is required for triggering ET biosynthesis. Fourth, neither *mpk3* nor *mpk6* exhibited inhibition of RKN penetration in response to sclareol (Fig. 7D). These results suggested that, in roots of *Arabidopsis* and, possibly, tomato, exogenously applied sclareol activates MPK3 and MPK6 which, in turn, triggers biosynthesis of ET.

Invasion of pathogens, such as viruses, bacteria, and fungi, enhances ET biosynthesis in plants (Wang et al. 2002). Plant-parasitic nematodes also elicited elevated levels of endogenous ET in plant roots (Glazer et al. 1983, 1985; Volkmar 1991). Fudali and associates (2013) reported that, compared with WT *Arabidopsis*, ET-overproducing mutants (*eto1*, *eto2*, and *eto3*) were less attractive to RKNs (*Meloidogyne hapla*), whereas an ET-insensitive mutant (*ein2-1*) was more attractive to the same nematode. In plant-RKN interactions, ET may play a positive role in resistance to RKNs. However, the ET biosynthesis and signaling pathways seemed not to be the sole pathway that led to inhibition of RKN penetration, because the inhibitory effect of exogenously applied ACC, a direct biosynthetic precursor of ET, on RKN penetration was lower than that of sclareol

(Fig. 7D). It is likely that activation of ET biosynthesis and signaling pathways is necessary but not sufficient to inhibit of RKN penetration. Sclareol does not necessarily activate the whole ET signaling pathway, because *chiB*, an ET-responsive gene, did not respond to sclareol (Fig. 3B). It remains unclear whether the ET signaling pathway that is not activated by sclareol is involved in resistance to RKN, and further studies to resolve this issue are required.

SA is unlikely to be involved in sclareol-induced resistance because *npr1-1*, an SA-signaling mutant, exhibited enhanced sclareol-induced inhibition of RKN penetration and because treatment with sclareol did not alter endogenous SA content or expression of SA-responsive genes. Such inhibition was also abolished in the *coi1-1* mutant, a JA-signaling mutant. However, this abolition might be due to the phenotypic profile of reduced susceptibility of this mutant to RKN penetration. It has been shown that RKNs need an intact JA pathway when they parasitize a host plant (Bhattarai et al. 2008; Fujimoto et al. 2011b). The results that sclareol did not alter endogenous JA content or expression of JA-responsive genes suggested that JA is not involved in sclareol-induced resistance.

There are two possibilities to explain a reduction in RKN penetration in roots treated with sclareol. One possibility is that lignin induced by sclareol is partially involved in inhibiting penetration of RKNs into roots. Lignin is synthesized via the phenylpropanoid pathway and its synthesis has been shown to be regulated at the transcriptional level (Raes et al. 2003). Sclareol enhanced both expression of the phenylpropanoid pathway genes *PAL1*, *C4H*, and *CCR2* and accumulation of lignin. In general, RKNs penetrate into meristematic and elongation zones in the root tip (Wyss et al. 1992). We could not detect lignin staining at such sites of RKN penetration; lignin staining was detected mainly in the root hair zone behind the elongation zone (Fig. 6B). It is possible that accumulation of lignin occurred in the meristematic and elongation zones in response to sclareol but the accumulation failed to be detected histochemically due to a relatively low detection limit of the staining method we used. The histochemical localization of lignin did not correlate with the site of RKN penetration in our experimental conditions. Nevertheless, our results from the RKN penetration assay using *ccr2*, a mutant defective in lignin accumulation, indicated the importance of lignin in sclareol-induced RKN

Fig. 6. Analysis of lignin accumulation and root-knot nematode (RKN) penetration in *Arabidopsis* roots treated with sclareol. **A**, Thioglycolic acid lignin concentrations in roots of wild-type (WT) (Col-0) and defense-related mutants 3 days after treatment with 100 μ M sclareol or 0.1% methanol (MeOH). **B**, Histochemical localization of lignin in the root tips of WT (Col-0) and an *ein2-1* mutant 3 days after treatment with 100 μ M sclareol or 50 μ M 1-aminocyclopropane-1-carboxylic acid (ACC). Dashed lines indicate where lignin was detected. Bars indicate 100 μ m. **C**, Thioglycolic acid lignin concentrations in roots of WT (Nossen) and *ccr2* mutant 3 days after 100 μ M sclareol. **D**, Roots of WT (Nossen) or *ccr2* mutant plants pretreated with 100 μ M sclareol for 48 h were inoculated with RKNs, and the number of RKNs that penetrated into the roots was counted 7 days after inoculation. Values are the mean $\pm$ standard deviation (SD) from two independent experiments, each performed in five replicates. MeOH (0.1%) was used as a control. A and C, Values are the mean $\pm$ SD from five replicates. A, C, and D, Asterisks denote statistically significant differences compared with the 0.1% MeOH control of the WT (Tukey's honestly significant difference test; $P < 0.05$).

resistance. Lignin produced in response to sclareol may function as a physical barrier for the invasion of RKNs. Because sclareol-induced enhancement of lignin accumulation was abolished in *ein2-1*, it is likely that lignification induced by sclareol occurs downstream of EIN2.

Another possibility is a partial involvement of metabolites induced by sclareol. Sclareol enhanced the expression of many genes, including *PAL1*, related to biosynthesis of secondary metabolites such as flavonoids, alkaloids, and phenolics (Fig. 5) (Campbell et al. 2003; Grec et al. 2003; Jasiński et al. 2001; Seo et al. 2012). Flavonoids are known to act as repellents for nematodes

and inhibit their motility and hatching (Wuyts et al. 2006). It is possible that roots produce metabolites in response to sclareol, and some of them function as inhibitors for RKN penetration.

The phenomena observed in the present study were similar to those in a previous study with the combination of *Arabidopsis* and *R. solanacearum* (Seo et al. 2012); exogenously applied sclareol conferred resistance to *R. solanacearum* without exhibiting any antibacterial activity, and the resistance required EIN2 and MPK3. These findings suggested that defense mechanisms induced by sclareol are extremely common in many plant species. However, there are few plant species that

Fig. 7. Analysis of importance of ethylene (ET) biosynthesis in sclareol-induced root-knot nematode (RKN) resistance. **A**, Concentrations of ET emitted from wild-type (WT) (Col-0) plants 48 h after treatment with 100 μ M sclareol. **B**, Real-time reverse-transcriptase polymerase chain reaction analysis of ET biosynthetic genes in WT (Col-0) roots 48 h after treatment with 100 μ M sclareol or 0.1% methanol (MeOH). **A** and **B**, Values are the mean $\pm$ standard deviation (SD) from three independent measurements. **C**, Analysis for immune complex myelin basic protein (MBP) kinase activity of MPK3 and MPK6 and immunoblotting analysis of their protein levels using anti-MPK3 (α -MPK3) or anti-MPK6 (α -MPK6) antibodies. Arrows indicate phosphorylated myelin basic protein (P.MBP). Ponceau S staining is shown for equal loading of samples. M indicates a molecular marker. **D**, Roots of WT (Col-0) or mutant plants pretreated with 100 μ M sclareol, 50 μ M ACC, or 0.1% MeOH for 48 h were inoculated with RKNs, and the number of RKNs that penetrated into the roots was counted 7 days after inoculation. Values are the mean $\pm$ SD from two independent experiments, each performed in five replicates. **A**, **B**, and **D**, Asterisks denote statistically significant differences compared with the 0.1% MeOH control of WT (Student's *t* test for **A** and **B**, $P < 0.05$; Tukey's honestly significant difference test for **D**, $P < 0.05$).

produce sclareol, and both tomato and *Arabidopsis*, the plant species used in this study, do not produce sclareol. Therefore, sclareol is not an endogenous signaling substance that mediates defense responses in tomato and *Arabidopsis* but an elicitor-like substance that activates such responses. Further studies, including the identification of metabolites induced by sclareol, will help us to understand the mechanisms of plant defense against nematodes and pathogens.

MATERIALS AND METHODS

Plant material and growth conditions.

Seed of tomato (*Solanum lycopersicum* 'Momotaro'), which carries the *Mi*-gene (Milligan et al. 1998; Nombela et al. 2003), was purchased from a seed company (Takii & Co. Ltd., Kyoto, Japan). Seedlings (7 to 10 days old) were transferred to autoclaved sand with an average particle size of 0.3 mm in diameter, packed in 220-ml plastic pots, and grown at 23 to 27°C with 16 h of light. A commercial liquid fertilizer (Hyponex) diluted 3,000-fold with water was applied to the pots every 3 days. Unless otherwise stated, tomato plants with two true leaves that had fully expanded were used.

Arabidopsis thaliana with Columbia (Col-0) and Nossen backgrounds was used. All the mutants except for *ccr2* were in the Col-0 background. *coil-1*, *npr1-1*, *abil-1*, and *ein2-1* have been described previously (Abe et al. 2008; Leon-Reyes et al. 2009; Uno et al. 2000), as have *mpk3-2* and *mpk6-4* (Seo et al. 2012). *ccr2* (resource number pst20225), a transposon-tagged mutant line of *CCR2* gene in a Nossen background, was provided by the RIKEN BioResource Center.

Because *coil-1* is male sterile (Xie et al. 1998), an F₂ population segregating for this mutation was used to select homozygous *coil-1* plants. The *coil-1* mutant has a single base substitution at nucleotide position 1,663, resulting in conversion to a nucleotide sequence of GAATGCN, which is recognized by the restriction enzyme *BsmI* (Xie et al. 1998). For selection, genomic DNA purified from a single rosette leaf of each plant was amplified using DNA polymerase (ExTaq, Takara Bio, Shiga, Japan) and the set of primer 5'-CTATCTGAGACAAGGCGGCTTAACCGAC-3' and 5'-CAATTTTGT AACCGCTGCAGCGATTGCTCG-3'. After digesting with *BsmI*, the reaction mixture was analyzed by agarose gel electrophoresis. The plants in which digested bands were observed were regarded as homozygous *coil-1* plants.

All *Arabidopsis* plants were grown in either the sand system or the hydroponic system. For the sand system, 3-week-old seedlings were transferred individually to autoclaved sand with an average particle size of 0.3 mm in diameter, packed in 220-ml plastic pots, and grown at 22°C with 16 h of light. Hyponex, diluted 3,000-fold, was applied to the pots every 5 to 7 days. Ten-week-old plants were used for each analysis.

For the hydroponic system, seed were sown on half-strength Murashige and Skoog medium (Wako Pure Chemical, Osaka, Japan) supplemented with 0.7% agar and grown at 20 to 22°C with 8 h of light. Seedlings (10 to 14 days old) were grown on a polyethylene raft through holes (1 cm in diameter) floating on 3,000-fold diluted Hyponex that was constantly aerated with an air pump, with their roots submerged in the fertilizer. For each analysis, 8- to 10-week-old plants were used.

Preparation and maintenance of nematodes.

A strain (collection number 108258) of RKNs (*M. incognita*), which possesses the RKN resistance gene *Mi*, was provided from the MAFF gene bank of the National Institute of Agrobiological Sciences of Japan and has been used in previous studies (Fujimoto et al. 2011a,b). Maintenance and preparation of the strain were in accordance with the procedure described by Fujimoto and associates (2011a). RKNs collected 7 to 10 days after hatching were used for each analysis.

Chemical treatments.

Stock solutions (100 mM) of sclareol (Sigma) and sclareolide (Sigma) in methanol and a stock solution (100 mM) of ACC (Sigma) in water were diluted in water to various concentrations. Methanol concentrations did not exceed 0.1% in all the experiments.

Roots of plants grown in the sand system were treated by soaking the pot itself in a solution containing various concentrations of each chemical in a tray, with their roots submerged in the solution. After soaking for 24, 48, or 72 h, the treated plants were transferred to a new tray containing water and used for RKN penetration assays; or the roots were pulled out of the pots, washed with water, and used for real-time RT-PCR analysis or quantification of thioglycolic acid lignin.

For treatment of the roots of hydroponically grown plants, plants were pulled out of the fertilizer, transferred to a petri dish containing water with their roots submerged in the water, and preincubated for 48 h to diminish the influence of any wound stresses that could be caused by removal. After discarding water using a pipette, the plant roots were treated by gently adding a solution containing various concentrations of each chemical to the petri dish until the roots were submerged in the solution, incubated for adequate time periods, and used for phytohormone measurements, immune complex kinase assays, or histochemical analysis of lignin.

RKN penetration assay.

In total, 200 and 500 second-stage juvenile RKNs were used for tomato and *Arabidopsis*, respectively. Inoculation of roots with RKNs was performed in accordance with the procedure described by Fujimoto and associates (2011a). Briefly, roots of plants were inoculated with RKNs by injecting a suspension solution containing RKNs into the sand surrounding the roots

Table 1. List of primers and the corresponding clones used for real-time reverse-transcriptase polymerase chain reaction analysis

Sequence definition	AGI number	Forward primer (5'–3')	Reverse primer (5'–3')
<i>BGL2</i> ; β-1,3-glucanase 2	At3g57260	GCCGACAAGTGGGTTC AAGA	AACCCCCCACTGAGGGTT
<i>PR1</i> ; pathogenesis-related genes 1	At2g14610	GTTGCAGCCTATGCTCGGAG	CCGCTACCCAGGCTAAGTT
<i>VSP2</i> ; vegetative storage protein 2	At5g24770	GTTAGGGACCGGAGCATCAA	AACGGTCACTGAGTATGATGGGT
<i>LOX2</i> ; lipoxygenase 2	At3g45140	TTGCTCGCCAGACACTTGC	GGGATCACCATAAACGGCC
<i>PDF1.2</i> ; plant defensin 1.2	At5g44420	CCATCATCACCTTATCTTCGC	TGTCCCACTTGGCTTCTCG
<i>chiB</i> ; basic chitinase B	At3g12500	ACGGAAGAGGACCAATGCAA	GTTGGCAACAAGGTCAAGGT
<i>AtPDR12</i> ; ABC transporter G family member 40	At1g15520	ATGCTTTCGCTCAGGTACTTCA	CTTCACCGCCGTCCACTC
<i>PAL1</i> ; phenylalanine ammonia-lyase 1	At2g37040	TTCCTCTCTCTACATCGCC	ACACCATTGACGCCATTCC
<i>C4H</i> ; trans-cinnamate 4-monooxygenase	At2g30490	TCCAGCAGAAAGCAAAATCCTT	GCACATACCTGAAGTCATTACC
<i>CCR2</i> ; cinnamoyl-CoA reductase	AT1G80820	GACGATCCCGTAAGTAGACAATT	ACAATGGCTTGAGTGTACACG
<i>CBP20</i> ; cap-binding protein 20	At5g44200	CCTTGTGGCTTTTGTTCGTC	ACACGAATAGGCCGGTCATC

of each plant. After 7 days of inoculation, the roots of plants were washed free of all sand and stained with acid fuchsin, as described previously (McBeth et al. 1941). The total numbers of stained RKNs were counted using a light microscope.

Nematicidal activity assay and infectivity assay.

For the nematicidal activity assay, approximately 100 RKNs in 1 ml of nematode suspension were added to 1 ml of 200 μ M sclareol solution or control solution (0.2% methanol) in 24-well tissue culture plates and incubated in the dark at 25°C. At 1, 3, 6, 12, 24, and 48 h after the onset of incubation, the numbers of motile and nonmotile RKNs were counted. During these trials, nonmotile RKNs at the second stage were prodded with NaOH at 1 mol/liter to check for a response, as described by Chen and Dickson (2000).

For infectivity assays, approximately 200 RKNs were soaked with a solution containing 100 μ M sclareol or 0.1% methanol in a petri dish. Two days after soaking, RKNs were used for RKN penetration assays using tomato plants.

Phytohormone measurement.

For the measurement of ET released from *Arabidopsis* plants, five 10-week-old plants (Col-0) grown in the hydroponic system were placed in a solution containing 100 μ M sclareol or 0.1% methanol, with the roots submerged in the solution in a sealed 40-ml glass vial, and incubated in a chamber maintained at 25°C with 16 h of light. After 48 h, a sample was withdrawn from the headspace and analyzed for ET, as described previously (Seo et al. 2007).

Extraction and quantification of SA and JA was performed in accordance with the procedure described by Seo and associates (2007).

RNA extraction and real-time RT-PCR.

Total RNA (3 μ g) extracted from roots using TRIzol reagent (Invitrogen) was reverse transcribed into cDNA using random oligo-hexamers and Superscript III reverse transcription in accordance with the manufacturer's instructions (Invitrogen). Real-time RT-PCR was carried out using SYBR Green PCR Master Mix (Applied Biosystems) using the first-strand cDNA as a template on a sequence detector system (ABI Prism 7900HT; Applied Biosystems). Expression of *CBP20* was used for normalization as a standard control gene of *Arabidopsis*. Nucleotide sequences of the gene-specific primers are described in Table 1. Each treatment had three replicates (five plants per sample).

Protein extraction, immune complex kinase assay, and immunoblotting analysis.

Roots were harvested at the indicated time intervals, immediately frozen in liquid N₂, and stored at -80°C until use. Protein extraction was performed in accordance with the procedure described by Seo and associates (2007). Anti-MPK3 and anti-MPK6 antibodies were generated from rabbits immunized with synthetic peptides corresponding to amino acid residues 359 to 370 (QEAIALNPTYG) for MPK3 and 384 to 395 (REALAFNPEYQQ) for MPK6, and purified using each antigen. The affinity-purified antibodies were used for each analysis. Immune complex kinase assays using 50 μ g of total protein and immunoblotting analysis using 25 μ g of total protein were performed as described previously (Seo et al. 2007).

Lignin analysis.

Harvested roots were used to either quantify thioglycolic acid lignin (Suzuki et al. 2009) or histochemically detect lignin with phloroglucinol (Caño-Delgado et al. 2000). Each treatment had three replicates (five plants per sample).

Statistical analyses.

The number of RKNs detected in roots in each experiment was adapted to two-way analysis of variance followed by Tukey's test, and Student's *t* test was used to estimate the influence of such differences (Sokal and Rohlf 1995). The mean of the dependent variables (i.e., number of RKNs in the root or egg masses on the root) was logarithmically transformed to obtain normality and homoscedasticity. These analyses were performed using JMP software (version 10.0.2; SAS Institute Inc., Cary, NC, U.S.A.).

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