Phytol, a Constituent of Chlorophyll, Induces Root-Knot Nematode Resistance in *Arabidopsis* via the Ethylene Signaling Pathway

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Root-knot nematodes (RKNs; *Meloidogyne* spp.) parasitize the roots or stems of a wide range of plant species, resulting in severe damage to the parasitized plant. The phytohormone ethylene (ET) plays an important role in signal transduction pathways leading to resistance against RKNs. However, little is currently known about the induction mechanisms of ET-dependent RKN resistance. Inoculation of *Arabidopsis thaliana* roots with RKNs decreased chlorophyll contents in aerial parts of the plant. We observed accumulation of phytol, a constituent of chlorophyll and a precursor of tocopherols, in RKN-parasitized roots. Application of sclareol, a diterpene that has been shown to induce ET-dependent RKN resistance, to the roots of *Arabidopsis* plants increased phytol contents in roots accompanied by a decrease in chlorophyll in aerial parts. Exogenously applied phytol inhibited RKN penetration of roots without exhibiting nematicidal activity. This phytol-induced inhibition of RKN penetration was attenuated in the ET-insensitive *Arabidopsis* mutant *ein2-1*. Exogenously applied phytol enhanced the production of α -tocopherol and expression of *VTE5*, a gene involved in tocopherol production, in *Arabidopsis* roots. α -Tocopherol exerted induction of RKN resistance similar to that of phytol and showed increased accumulation in roots inoculated with RKNs. Furthermore, the *Arabidopsis vte5* mutant displayed no inhibition of RKN penetration in response to phytol. These results suggest that

exogenously applied phytol induces EIN2-dependent RKN resistance, possibly via tocopherol production.

Keywords: *Arabidopsis thaliana*, ethylene, hormones, induced resistance, nematode–plant interactions, plant resistance, root-knot nematode

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Plants cope with attacks from bacteria, fungi, viruses, nematodes, and herbivorous pests by producing low-molecular-mass compounds such as reactive oxygen species (ROS), secondary metabolites, and phytohormones that function as endogenous chemical signals to regulate defense responses (Bigeard et al. 2015; Yang et al. 2018). Identification of unknown defense-related chemical signals is important for elucidating the adaptation mechanisms of plants to harmful microorganisms.

Chloroplasts provide living plant cells with organic-molecule-based energy through photosynthesis. In addition to their role as an energy source, chloroplasts play an important role in plant defense. Biosynthesis of phytohormones such as jasmonic acid (JA) and salicylic acid (SA) and generation of ROS take place in chloroplasts (Fernandez and Burch-Smith 2019). Microbial attack causes reduction in chlorophyll content, changes in the enzymic activities of chloroplast-localized proteins, and alterations in the expression of genes encoding chloroplast proteins (Caplan et al. 2015; de Torres Zabala et al. 2015; Dong and Chen 2013; Ganusova et al. 2017; Seo et al. 2000). These morphological and physiological changes in chloroplasts have been implicated in the regulation of defense responses.

Chlorophyll degradation is a multistep process involving chemical and enzymic reactions. An early step in chlorophyll degradation is dissociation of the phytol tail and porphyrin ring of the chlorophyll molecule (Hörtensteiner and Kräutler 2011). Phytol-free porphyrin chlorophyllides exhibit toxicity against the larvae of *Spodoptera litura*, a herbivorous insect, suggesting that chlorophyllides play a role in plant defense (Hu et al. 2015). Phytol, an acyclic diterpene alcohol, is a precursor of tocopherols (vitamin E) (Dörmann 2007). Although phytol possesses antimicrobial and antitumor-sensing activities (Islam et al. 2015; Pejin et al. 2014, 2015), limited information is currently available on the physiological role of phytol in plant defense.

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Plant-parasitic nematodes parasitize the roots or stems of plants. Absorption of nutrients and moisture is inhibited in parasitized plants, resulting in damage such as wilting, stunting, yield loss, or plant death. Root-knot nematodes (RKNs; *Meloidogyne* spp.) parasitize more than 3,000 plant species and cause severe crop damage (Abad et al. 2003; Jones et al. 2013). Phytohormones, including ethylene (ET), JA, and strigolactone, regulate defense responses against RKNs (Bhattarai et al. 2008; Fudali et al. 2013; Fujimoto et al. 2011a,b; Xu et al. 2019). We previously reported that exogenous application of sclareol, a diterpene, induces RKN resistance in *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) through activation of ET biosynthetic and signaling pathways (Fujimoto et al. 2015). However, the induction mechanisms of ET-dependent RKN resistance currently remain unclear.

Here, we demonstrate that phytol induces ET-dependent resistance to RKN in *Arabidopsis*, possibly through the production of α -tocopherol.

RESULTS

Identification of phytol as an RKN-induced metabolite in *Arabidopsis* roots.

Inoculation of roots of wild-type (Col-0) plants with RKNs decreased chlorophyll content in aerial parts of the same plant (Fig. 1). This result prompted us to test the hypothesis that chloroplast-related compounds accumulate in roots and induce the host defense against RKNs. We first examined metabolites in RKN-parasitized roots of wild-type *Arabidopsis* plants. RKN-inoculated roots were extracted with methanol, and the methanol extract was subjected to nontargeted gas chromatography–mass spectrometry (GC/MS) analysis (Supplementary Fig. S1A). We selected 23 chromatographic peaks with greater than twofold signal intensity over the control mock inoculation as candidates for RKN inoculation-induced metabolites. Metabolite identification was performed by searching mass spectral libraries and databases such as the National Institute of Standards and Technology Mass Spectral Library and MassBank. Although approximately half of the 23 peaks could not be assigned (Supplementary Table S1), we putatively identified peaks with

Fig. 1. Effects of root-knot nematode (RKN) inoculation on the accumulation of chlorophylls in *Arabidopsis* plants. Wild-type (Col-0) roots were inoculated with RKNs or water (Mock) as a control, and the amounts of chlorophylls *a* and *b* (Chl *a* and Chl *b*, respectively) in the leaves were assessed 7 days after inoculation. Values are means $\pm$ standard deviation ($n = 10$ biological replicates from two independent experiments). Asterisks denote significant differences from the mock inoculation (Tukey's honestly significant difference test, $P < 0.05$).

retention times of 13.24, 20.81, and 21.16 min as hexadecanoic acid, phytol (Supplementary Fig. S1B), and octadecanoic acid, respectively. These three compounds are biosynthesized or function in chloroplasts and plastids. Because limited information is currently available on the role of phytol in plant defense, we focused on this compound in subsequent experiments. Quantitative analysis of phytol confirmed that endogenous phytol in wild-type roots was increased at 7 days after inoculation with RKNs (Supplementary Fig. S1C).

Sclareol induces phytol accumulation in *Arabidopsis* roots.

Our previous study showed that penetration of RKNs into the roots of wild-type *Arabidopsis* plants was inhibited when roots were treated with sclareol (Fujimoto et al. 2015). However, application of sclareol to leaves did not inhibit the penetration of RKNs into roots of the same plant (data not shown). Application of 100 μ M sclareol, the minimal concentration required to inhibit RKN penetration (Fujimoto et al. 2015), to the roots of wild-type plants induced chlorosis-like symptoms on their leaves (Fig. 2A). This effect is consistent with previous findings that sclareol causes a stress response such as anthocyanin accumulation in plants (Van Den Brle et al. 2002). Consistent with the appearance of these symptoms, we observed a reduction in chlorophyll content in aerial parts of plants with sclareol-treated roots (Fig. 2B). Sclareol treatment resulted in an approximately 10-fold increase in phytol content in the roots (Fig. 2C).

Exogenous application of phytol inhibits the penetration of RKNs into *Arabidopsis* and tomato roots.

To examine whether phytol inhibits the penetration of RKNs into plant roots, we treated the roots of wild-type *Arabidopsis* plants with a solution containing various concentrations of phytol for 48 h. Treated roots were inoculated with infective

Fig. 2. Effects of sclareol on the accumulation of chlorophylls and phytol in *Arabidopsis* plants. **A**, Chlorosis-like symptoms on aerial parts of wild-type (Col-0) plants after 48 h of treatment with 100 μ M sclareol or 0.1% methanol (MeOH). Arrows indicate chlorosis-like symptoms. **B**, Amounts of chlorophylls *a* and *b* (Chl *a* and Chl *b*, respectively) in leaves after 48 h of treatment with 100 μ M sclareol. **C**, Amounts of phytol in roots after 48 h of treatment with 100 μ M sclareol. Values are means $\pm$ standard deviation of three (B) and six (C) biological replicates. Asterisks (*) denote significant differences from the methanol treatment ($P < 0.05$, Student's *t* test). The experiment was repeated two or more with similar results.

second-stage juvenile RKNs and stained with acid fuchsin, a dye that stains nematodes, 7 days after inoculation. We estimated the degree to which phytol inhibits RKN penetration by counting the number of stained RKNs. Penetration of RKNs was inhibited by 10, 100, and 300 μM phytol to 26, 10, and 9%, respectively, of the control methanol (0 μM) treatment (Fig. 3A). Exogenously applied phytol was also effective for tomato (Fig. 3B); phytol at 1 μM reduced RKN penetration to approximately 50% that of the control. To establish whether the inhibition of RKN penetration by phytol could be attributed to its nematocidal activity, we tested the effects of phytol on the survival of RKNs. Second-stage juvenile RKNs were incubated in the presence of 100 μM phytol, and the number of surviving RKNs was counted at different time periods after the onset of incubation. Phytol had a negligible or no effect on the survival of RKNs over the time period tested (Fig. 3C). In subsequent experiments, phytol at a concentration of 300 μM was used.

Phytol-induced RKN resistance is dependent on EIN2.

The above results suggested that phytol induces defense responses in the host plant that inhibit RKN penetration. To examine which phytohormones are involved in phytol-induced RKN resistance, we assayed *Arabidopsis* mutants defective in the perception of specific phytohormones: SA-signaling mutant *NONEXPRESSOR OF PR GENES 1-1* (*npr1-1*), JA-signaling mutant *CORONATINE-INSENSITIVE1-1* (*coi1-1*), abscisic acid (ABA)-signaling mutant *ABA-INSENSITIVE1-1* (*abi1-1*), and ET-signaling mutant *ETHYLENE-INSENSITIVE2-1* (*ein2-1*). In *npr1-1* and *abi1-1*, similar to wild-type plants, the penetration of RKNs into roots was markedly inhibited by exogenously applied phytol (Fig. 4A). Phytol also induced inhibition of RKN penetration in *ein2-1* but to a lesser extent than that in the wild type. Mutant *coi1-1* also displayed marked inhibition

Fig. 3. Effects of phytol on the penetration of root-knot nematodes (RKNs) into *Arabidopsis* and tomato roots. Dose-dependent effects of phytol: **A**, *Arabidopsis* or **B**, tomato roots pretreated with the indicated concentrations of phytol 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 means $\pm$ standard deviation (SD) ($n = 10$ biological replicates from two independent experiments). Asterisks (*) denote significant differences from the 0 μM (0.1% methanol) control (Tukey's honestly significant difference test, $P < 0.05$). **C**, Nematicidal activity assay. Numbers of active second-stage juvenile RKNs after incubation in 100 μM phytol. Values are means $\pm$ SD of 10 biological replicates; n.s. indicates no significant difference from time 0 (Tukey's honestly significant difference test). The nematicidal activity assay was repeated twice with similar results.

of RKN penetration by phytol (Fig. 4A). However, this mutant exhibited decreased penetration of RKNs in the absence of phytol compared with wild-type plants (Fig. 4A, MeOH in *coi1-1* versus MeOH in WT), supporting a similar result observed in a previous study (Fujimoto et al. 2015) and suggesting that *coi1-1* has relatively low susceptibility to RKN penetration. Quantitative analysis indicated that exogenously applied phytol did not increase endogenous JA, SA, or ABA contents in roots (Supplementary Fig. S2). Therefore, we focused on elucidating the relationship between phytol and ET.

We used hydroponically grown whole plants to examine whether phytol enhances the production of ET, because of the difficulties associated with measuring ET without the influence of ET biosynthesis activated by wound stresses. Hydroponically grown wild-type *Arabidopsis* plants were sealed in a test tube, with the roots submerged in a solution containing phytol, and ET released from the whole plant was analyzed. Plants treated with phytol produced 2.8-fold more ET than those treated with methanol (Fig. 4B). Consistent with enhanced ET production, the accumulation of transcripts for the ET biosynthetic gene *1-aminocyclopropane-1-carboxylic acid synthase 2* (*AtACS2*) in roots was enhanced by phytol (Fig. 4C).

Phytol-induced RKN resistance may occur through tocopherol production.

Because phytol is a precursor of tocopherols, we hypothesized that exogenously applied phytol may activate tocopherol

Fig. 4. Effects of phytol on root-knot nematode (RKN) penetration into roots of defense-related *Arabidopsis* mutants and the accumulation of ethylene. **A**, Roots of wild-type (WT) or mutant plants pretreated with phytol or 0.1% methanol (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 means $\pm$ standard deviation (SD) ($n = 10$ biological replicates from two independent experiments). Different letters indicate significant differences among treatments (Tukey's honestly significant difference test, $P < 0.05$). **B**, WT plants were incubated in a solution containing phytol in a sealed glass vial for 48 h, and ethylene was measured in a 1-ml sample withdrawn from the headspace. Values are means $\pm$ SD of four biological replicates. The ethylene measurement was performed twice with similar results. **C**, Quantitative reverse-transcription PCR analysis of *AtACS2* in WT roots after 48 h of treatment with phytol. Values are means $\pm$ SD of three biological replicates. The quantitative RT-PCR analysis was performed twice with similar results. Asterisks (*) denote significant differences from the control methanol treatment (Student's *t* test, $P < 0.05$).

biosynthesis. Phytol derived from chlorophyll degradation is phosphorylated to phytol phosphate, which, in turn, is phosphorylated to phytol diphosphate, a direct precursor of tocopherols. The first phosphorylation step is catalyzed by the phytol kinase VITAMIN E DEFICIENT5 (VTE5) (Valentin et al. 2006). To examine whether phytol activates tocopherol biosynthesis, we treated roots of wild-type *Arabidopsis* plants with phytol for 48 h and assessed the induction kinetics of the *AtVTE5* gene in roots. Phytol treatment enhanced the accumulation of *AtVTE5* transcripts in roots (Fig. 5A). Accumulation of α -tocopherol, the major form of vitamin E, was also increased in roots following exogenous application of phytol (Fig. 5B). These results prompted us to examine the effects of tocopherols on RKN resistance. Roots of wild-type plants were treated with α -tocopherol for 48 h and then inoculated with RKNs. Exogenously applied α -tocopherol inhibited the penetration of RKNs into the roots of *Arabidopsis* (Fig. 5C) and tomato (Fig. 5D) in a dose-dependent manner. A nematocidal activity assay showed that α -tocopherol had no effect on the survival of RKNs (Fig. 5E). We examined whether tocopherol accumulation was necessary for phytol-induced inhibition of RKN penetration using an *Arabidopsis* mutant for VTE5, *vte5* (Valentin et al. 2006). Phytol-induced inhibition of RKN penetration was completely abolished in roots of *vte5* (Fig. 5F).

Quantitative analysis indicated that endogenous α -tocopherol was increased in wild-type roots 7 days after inoculation with RKNs (Fig. 5G).

DISCUSSION

We identified accumulation of phytol in *Arabidopsis* roots in response to RKNs. Exogenously applied phytol inhibited the penetration of RKNs into roots without exhibiting any nematocidal activity. The phytol-induced inhibition of RKN penetration was attenuated in the ET signaling mutant *ein2-1*, while *vte5*, a tocopherol-deficient *Arabidopsis* mutant, exhibited no inhibition of RKN penetration in response to phytol. Accumulation of α -tocopherol was enhanced in response to not only exogenously applied phytol but also RKNs. These results suggest that exogenously applied phytol potentially activates EIN2-dependent resistance to RKN in *Arabidopsis* via tocopherol production.

Although the exact origin of phytol accumulation in RKN-parasitized or sclareol-treated roots remains unclear, it may result from degradation of chloroplasts or plastids in the roots or aerial parts of the same plant. In response to RKNs or exogenously applied sclareol, roots may produce a signal that is transmitted to the aerial parts to trigger chloroplast or plastid

Fig. 5. Importance of α -tocopherol in phytol-induced root-knot nematode (RKN) resistance. **A**, Expression of *AtVTE5* and **B**, concentration of α -tocopherol in wild-type (WT; Col-0) roots after 48 h of treatment with 300 μ M phytol or 0.1% methanol (MeOH). Values are means $\pm$ standard deviation (SD) of three biological replicates. **C**, *Arabidopsis* or **D**, tomato roots pretreated with the indicated concentrations of α -tocopherol for 48 h were inoculated with RKNs, and the number of RKNs that penetrated into the roots was counted 7 days after inoculation. **E**, Numbers of active second-stage juvenile RKNs after incubation in 100 μ M α -tocopherol. Values are means $\pm$ SD of 10 biological replicates; n.s. indicates no significant difference from time 0 (Tukey's honestly significant difference [HSD] test). The nematocidal activity assay was repeated twice with similar results. **F**, *Arabidopsis* WT (Nossen) or *vte5* roots pretreated with 300 μ M α -tocopherol for 48 h were inoculated with RKNs, and the number of RKNs that penetrated into the roots was counted 7 days after inoculation. **G**, Concentration of α -tocopherol in WT (Col-0) roots 7 days after inoculation with RKNs. For C, D, F, and G, values are means $\pm$ SD of 10 biological replicates from two independent experiments. Asterisks (*) denote significant differences from the methanol (MeOH or 0 μ M) treatment (A, B, and G, $P < 0.05$, Student's t test; C, D, and F, $P < 0.05$, Tukey's HSD test).

degradation. Another possibility is that phytol is biosynthesized de novo through the plastidic methylerythritol phosphate (MEP) pathway in roots in response to RKNs or exogenously applied sclareol. Phytol is a diterpene alcohol consisting of four isoprene units, and isoprenoids, including diterpenes, are biosynthesized via the MEP pathway in chloroplasts and plastids (Chen et al. 2011; Gutbrod et al. 2019). Because expression of *AtVTE5*, a gene involved in tocopherol production, was enhanced in phytol-treated roots, production of α -tocopherol from phytol in response to RKNs or exogenously applied sclareol may occur in roots.

Exogenously applied sclareol induces expression of genes involved in various physiological events in *Arabidopsis*, including defense responses, phytohormone responses, and secondary metabolism (Campbell et al. 2003; Seo et al. 2012). Only a few plant species such as tobacco (*Nicotiana tabacum*) and clary sage (*Salvia sclarea*) produce sclareol (Caniard et al. 2012), and it may act as an elicitor-like external stimulus for *Arabidopsis*, a plant that does not produce sclareol. Accumulation of phytol, a constituent of chlorophyll, was enhanced in response to RKNs or exogenously applied sclareol in *Arabidopsis*, suggesting a role for phytol as an endogenous defense-related signal. Further studies are warranted to investigate the role of endogenous phytol in RKN resistance and the effects of phytol on other plant-parasitic nematodes.

MATERIALS AND METHODS

Plant materials and growth conditions.

The *Arabidopsis thaliana* mutants *coil-1*, *npr1-1*, *abi1-1*, and *ein2-1* are in the Col-0 background and have been described previously (Fujimoto et al. 2015). Mutant *vte5* (resource number pst21655) is in the Nossen background and was obtained from the RIKEN BioResource Center.

All *Arabidopsis* plants were grown in either sand or a hydroponic system. In the sand system, 3-week-old seedlings were transferred individually to 220-ml plastic pots packed with autoclaved sand with an average particle size of 0.3 mm and grown at 22°C with 16 h of light. Liquid fertilizer (3,000-fold diluted Hyponex) was applied to the pots every 7 days. Ten-week-old plants were used for inoculation with RKNs or for chemical treatments.

In the hydroponic system, seed were sown on half-strength Murashige and Skoog medium supplemented with 0.8% (wt/vol) agar and grown at 22°C with 8 h of light. Two-week-old seedlings were transferred to holes (1 cm in diameter) in a polyethylene raft floating on 3,000-fold diluted Hyponex constantly aerated with an air pump. Seedlings had their roots submerged in the fertilizer and were grown at 22°C with 8 h of light. For chemical treatments, 8- to 10-week-old plants were used.

Tomato (*Solanum lycopersicum* ‘Momotaro’) was grown in the sand system as described above at 25 ± 2°C with 16 h of light. Tomato plants with two true leaves were used for inoculation with RKNs.

Inoculation and RKN penetration assay.

Strain MAFF number 108258 of RKN (*Meloidogyne incognita*) used in the present study has been described previously (Fujimoto et al. 2011a,b). Maintenance and preparation of the strain and inoculation were performed in accordance with the procedure described by Fujimoto et al. (2011a). Briefly, RKNs were collected 7 to 10 days after hatching, and their infective second-stage juveniles were used for inoculation. A suspension solution containing 500 RKNs (for *Arabidopsis*) or 200 RKNs (for tomato) per plant was injected into the sand surrounding the roots of each plant grown in a pot. RKN-

inoculated plants were grown under the conditions described above. Seven days after inoculation, plant roots were washed with water to remove sand and stained with acid fuchsin, as described previously (Fujimoto et al. 2011a). Total numbers of stained RKNs were counted using a light microscope. The combination of 500 RKNs (for *Arabidopsis*) or 200 RKNs (for tomato) and one inoculated plant was regarded as one biological replicate. The RKN penetration assays were independently performed twice with five biological replicates.

Chemical treatments.

Phytol (Sigma), α -tocopherol (Tokyo Chemical Industry Co.), or sclareol (Sigma) dissolved in methanol was stored at -20°C as a stock solution (100 mM) and diluted in water to various concentrations immediately before use. Methanol concentrations did not exceed 0.1% in any experiment.

Roots of plants grown in the sand system were treated by immersing the pot itself into a solution containing the indicated concentrations of each individual chemical in a tray. At 48 h after immersion, treated plants were transferred to a new tray containing water and used for inoculation with RKNs.

Hydroponically grown plants were removed from the fertilizer, transferred to a Petri dish containing water with their roots submerged in the water, and preincubated for 48 h prior to chemical treatment to diminish the influence of any wound stresses associated with removal. After discarding the water with a pipette, plant roots were treated by gently adding a solution containing the indicated concentrations of each individual chemical to the dish until the roots were completely submerged in the solution. Plants were incubated for 48 h and then used for phytohormone measurements, gene expression analysis, or measurements of chlorophyll concentration in sclareol-treated plants.

Nematicidal activity assay.

Suspension solution (1 ml) containing 100 RKNs was added to an equal volume (1 ml) of solution containing 200 μ M phytol or 0.2% (vol/vol) methanol as a control in one well of a 24-well polystyrene plate and incubated in the dark at 25°C. The numbers of motile and nonmotile RKNs were counted at 1, 2, 3, 6, 12, 24, and 48 h after the onset of incubation. During these trials, nonmotile RKNs at the second juvenile stage were prodded with NaOH at 1 mol/liter to check for a response, as described by Chen and Dickson (2000). One well containing 100 RKNs was regarded as one biological replicate, and 10 replicates were used for each chemical treatment (1,000 RKNs).

Nontarget GC/MS analysis.

Roots of six *Arabidopsis* Col-0 plants grown in sand in pots were inoculated with RKNs (500 RKNs per plant) or water alone as a control (mock). Roots were harvested 7 days after inoculation and combined as one sample. Each sample was homogenized in three volumes of cold methanol using a mortar and pestle and placed on ice for 30 min. The extract solution was centrifuged at 10,000 × g at 4°C for 10 min, and the supernatant was transferred to a test tube and dried under a stream of nitrogen gas. The remaining residue was dissolved in ethyl acetate and subjected to GC-MS analysis. Analyses were performed on an Agilent 7890A GC coupled with an Agilent mass selective detector (MSD) 5975C. Separation was performed on a capillary column (HP-1MS, 30-m length, 0.25-mm inner diameter, 0.25- μ m thickness; Agilent) with He as the carrier gas at a flow rate of 1 ml/min. Other GC conditions were as follows: column oven temperature was held at 50°C for 1 min, increased to 300°C at 12°C/min, and held for 1 min; injection port temperature was 280°C; transfer line temperature was

280°C; injection mode was splitless; ionization mode was electron impact (EI; 70 eV); and scan range was m/z 50 to 500. Peaks with a fold change >2.0 over the control mock inoculation were selected and analyzed by matching their mass spectra with those of reference compounds in a chemical library (National Institute of Standards and Technology, United States) or mass spectral databases (MassBank and the MassBank of North America). The nontarget GC-MS analysis was repeated twice.

Measurements of phytol, α -tocopherol, and phytohormones.

Roots collected from five plants inoculated with RKNs or treated with a chemical were combined as one sample and used to measure phytol, α -tocopherol, JA, SA, and ABA. One sample was regarded as one biological replicate. The quantification of phytol and α -tocopherol was performed using an Agilent GC-MS system (7890A GC coupled with MSD 5975C) according to procedures described previously (Ischebeck et al. 2006; Zhang et al. 2016). Quantification of JA, SA, and ABA was performed using the Agilent GC-MS system as described previously (Seo et al. 2016). For the quantification of phytol, α -tocopherol, JA, SA, and ABA, the extract from each sample was dissolved in ethyl acetate or hexane and divided into two or more aliquots (technical replicates) before being subjected to GC-MS. Quantification was performed with one technical replicate for one biological replicate. The remaining aliquots were subjected to the GC-MS system to determine experimental variability on different days. We confirmed that there was no statistically significant difference in quantitative values between the technical replicates from the same sample. We used 3 to 10 biological replicates for each treatment.

For measurement of ET, five 8-week-old plants grown in the hydroponic system were placed in a solution containing 300 μ M phytol or 0.1% (vol/vol) methanol, with their roots submerged in the solution, in a sealed 40-ml glass vial and incubated at 25°C with 16 h of light. After 48 h of incubation, a 1-ml sample was withdrawn from the headspace using a gas-tight syringe and injected into a GC equipped with a flame ionization detector (Shimadzu Co.), as described previously (Seo et al. 2016). Five plants were regarded as one biological replicate. The ET measurement was repeated twice.

Chlorophyll measurement.

Leaves harvested from five plants inoculated with RKNs or treated with a chemical were combined as one biological replicate and used to extract chlorophyll. Each sample was extracted with 80% (vol/vol) acetone. After centrifuging at $10,000 \times g$ for 5 min, the absorbance of the supernatant was measured at 646 and 663 nm using a spectrophotometer. Chlorophyll *a* and *b* contents were calculated according to the formula described by Porra et al. (1989). The acetone extract containing chlorophylls from each sample was divided into two or more aliquots (technical replicates) before being subjected to chlorophyll measurement. Quantification of chlorophylls was performed with one technical replicate for one biological replicate. The remaining aliquots were subjected to chlorophyll measurement to determine experimental variability on different days. We confirmed that there was no statistically significant difference in quantitative values between the technical replicates from the same sample. We performed two independent experiments with five biological replicates each for the inoculation with RKNs. For the chemical treatment, three biological replicates were used and the experiment was repeated twice.

Quantitative reverse-transcription PCR analysis.

Roots collected from five plants inoculated with RKNs or treated with a chemical were combined as one biological

replicate and used to extract total RNA. Quantitative reverse-transcription PCR (RT-PCR) analysis using total RNA was performed in accordance with the procedure described by Fujimoto et al. (2011a) using the following primer sets: forward 5'-GGATGGTTTAGGATTGCTTTG-3' and reverse 5'-GCAC TCTTGTTCTGGATTACCTG-3' for *AtACS2* (At1g01480), forward 5'-TGCTTTTGTTCGTTAGTGA-3' and reverse 5'-GGTGAACGACCTTAATCTTCAT-3' for *AtVTE5* (At5g04490), and forward 5'-ACTGTGCCTATCTACGAGGGTTTC-3' and reverse 5'-CCCCTTCTGCTGTTGTGGT-3' for *AtActin8* (At1g49240). Expression levels of *AtActin8* were used to normalize those of the target genes. The cDNAs prepared from each sample were divided into three or more aliquots (technical replicates) before subjecting them to quantitative RT-PCR analysis. Quantitative RT-PCR analysis was performed with one technical replicate for one biological replicate. The remaining aliquots were subjected to quantitative RT-PCR to determine experimental variability different days. We confirmed that there was no statistically significant difference in quantitative values between the technical replicates from the same sample. The quantitative RT-PCR analysis was repeated two or more times.

Statistical analyses.

All data were subjected to statistical analyses to estimate the influence of differences between treatments (Sokal and Rohlf 1995). The Student's *t* test was used to compare the significant differences between the means of two treatments. Two-way analysis of variance followed by Tukey's test was also used to evaluate statistical significance. The mean of the dependent variable (i.e., number of RKNs in the root) was logarithmically transformed to obtain normality and homoscedasticity. These analyses were performed using JMP software (version 9.0.2; SAS Institute Inc., Cary, NC, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

RIKEN BioResource Center:

<https://www.brc.riken.jp/lab/epd/Eng/catalog/transp.shtml>

MAFF number 108258: http://www.gene.affrc.go.jp/about_en.php

MassBank: <http://www.massbank.jp/>

MassBank of North America: <https://mona.fiehnlab.ucdavis.edu/>

LITERATURE CITED

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