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Susceptibility to the Sugar Beet Cyst Nematode Is Modulated by Ethylene Signal Transduction in *Arabidopsis thaliana*

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Previously, we identified *Arabidopsis thaliana* mutant *rhd1-4* as hypersusceptible to the sugar beet cyst nematode *Heterodera schachtii*. We assessed *rhd1-4* as well as two other *rhd1* alleles and found that each exhibited, in addition to *H. schachtii* hypersusceptibility, decreased root length, increased root hair length and density, and deformation of the root epidermal cells compared with wild-type *A. thaliana* ecotype Columbia (Col-0). Treatment of *rhd1-4* and Col-0 with the ethylene inhibitors 2-aminoethoxyvinylglycine and silver nitrate and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid suggests that the *rhd1-4* hypersusceptibility and root morphology phenotypes are the result of an increased ethylene response. Assessment of known ethylene mutants further support the finding that ethylene plays a role in mediating *A. thaliana* susceptibility to *H. schachtii* because mutants that overproduce ethylene (*eto1-1*, *eto2*, and *eto3*) are hypersusceptible to *H. schachtii* and mutants that are ethylene-insensitive (*etr1-1*, *ein2-1*, *ein3-1*, *eir1-1*, and *axr2*) are less susceptible to *H. schachtii*. Because the ethylene mutants tested show altered susceptibility and altered root hair density and length, a discrimination between the effects of altered ethylene signal transduction and root hair density on susceptibility was accomplished by analyzing the *ttg* and *gl2* mutants, which produce ectopic root hairs that result in greatly increased root hair densities while maintaining normal ethylene signal transduction. The observed normal susceptibilities to *H. schachtii* of *ttg* and *gl2* indicate that increased root hair density, per se, does not cause hypersusceptibility. Furthermore, the results of nematode attraction assays suggest that the hypersusceptibility of *rhd1-4* and the ethylene-overproducing mutant *eto3* may be the result of increased attraction of *H. schachtii*-infective juveniles to root exudates of these plants. Our findings indicate that *rhd1* is altered in its ethylene response and that ethylene signal transduction positively influences plant susceptibility to cyst nematodes.

Cyst nematodes of the genus *Heterodera* are obligate, sedentary endoparasites of many important agricultural crops. These nematodes have developed highly evolved relationships with their respective hosts. In a typical *Heterodera* spp. life cycle, an infective second-stage juvenile (J2) hatches from an egg in the soil and is attracted to the elongation zone of the host root (Wyss and Zunke 1986; Wyss and Grundler 1992). Epidermal penetration and intracellular migration through the root cortex are facilitated by cell wall-degrading enzymes secreted by the nematode (De Boer et al. 1999; Smant et al. 1998; Wang et al. 1999). The J2 then selects an initial feeding cell in the vascular cylinder and pierces it with its stylet, a hollow, protrusible mouth spear (Wyss and Grundler 1992). The initial feeding cell undergoes significant physiological and morphological changes that culminate in the fusion of the initial feeding cell with neighboring cells by partial cell wall dissolution, forming an expanding syncytium (Jones 1981; Wyss 1992; Wyss and Grundler 1992). The syncytium provides the sedentary nematode with the nutrients needed to develop through the third-stage juvenile (J3) and fourth-stage juvenile (J4) into a reproductive male or female adult. Microscopic observations revealed that nematode secretions are injected through the stylet into or around the initial feeding cell where they may act as molecular signals triggering unknown signal transduction mechanisms that cause syncytium formation and maintenance (Davis et al. 2000).

The successful completion of the *Heterodera* life cycle constitutes a compatible interaction. Exploration of a compatible cyst nematode–plant interaction with mutant plants altered in their susceptibility to cyst nematodes bears promise to elucidate the molecular events of nematode–plant signal exchange as well as to identify the plant genes necessary for successful *Heterodera* parasitism. Previously, we developed an in vitro mutant screening procedure and identified *Arabidopsis thaliana* mutants exhibiting altered susceptibility to the sugar beet cyst nematode *Heterodera schachtii* (Baum et al. 2000). The hypersusceptible mutant line 2-4-6 was identified, and its mutant gene was determined to be allelic to *rhd1* (root hair defective) (Schiefelbein and Somerville 1990) and *reb1-1* and *reb1-2* (root epidermal bulger) (Baskin et al. 1992). Because it was the fourth-known *rhd1* allele, we named the mutant gene in line 2-4-6 *rhd1-4* (Baum et al. 2000). Here, we present an analysis of the physiological nature of the *rhd1-4* phenotypes, which led to the assessment of ethylene signal transduction as it pertains to cyst nematode parasitism.

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RESULTS

All of the *rhd1* alleles evaluated, i.e., *rhd1*, *reb1-1*, and *rhd1-4* (*reb1-2* was not assessed as a result of its temperature-sensitive nature) (Baskin et al. 1992), are hypersusceptible to *H. schachtii* and are morphologically indistinguishable (Baum et al. 2000). This hypersusceptibility is manifested as an approximately twofold increase in the number of J4 females able to develop compared with wild-type *A. thaliana* cv. Columbia (Col-0) (Baum et al. 2000). The *rhd1-4* hypersusceptibility is especially interesting because the root of this mutant is significantly shorter than normal, resulting in less root tissue for the nematode to infect. In addition, *rhd1-4* develops more and longer root hairs relative to Col-0 (Table 1 and Fig. 1A and B), and a portion of the *rhd1-4* root epidermal cells are deformed and exhibit a bulging phenotype (Fig. 1C). All *rhd1-4* phenotypes can be attributed to a single recessive allele (Baum et al. 2000). We did not observe any alterations in the development or morphology of shoot tissues in light-grown or etiolated *rhd1-4* seedlings, suggesting that the *rhd1* mutation has a root-specific effect (data not shown).

Modulation of *rhd1-4* ethylene production and perception.

The shortened roots, increased root hair length, and increased root hair density phenotypes of *rhd1-4* were reminiscent of the phenotypes of wild-type *A. thaliana* seedlings grown in the presence of excess ethylene (Eliasson and Bollmark 1988; Pitts et al. 1998; Tanimoto et al. 1995). To evaluate the possible role of ethylene in mediating the *rhd1-4* phenotypes, we examined the effects of two ethylene inhibitors and an ethylene precursor on *rhd1-4* and Col-0 root morphology, root hair development, and susceptibility to *H. schachtii*.

2-Aminoethoxyvinylglycine (AVG).

In planta ethylene production is inhibited by AVG, which inhibits the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, slowing the conversion of S-adenosylmethionine to the ethylene precursor ACC (Yang and Hoffman 1984). AVG concentrations as low as 0.5 μM significantly inhibit root hair initiation in *A. thaliana* (Tanimoto et al. 1995). *rhd1-4* and Col-0 seedlings were germinated and grown on nutrient media containing AVG at concentrations of 0.05 to 2.5 μM. Root hair lengths and densities decreased in *rhd1-4* and Col-0 as AVG concentrations increased (data not shown). AVG (1.0 μM) restored *rhd1-4* root hair length and density to approximately normal levels and completely suppressed the root epidermal cell deformation phenotype (Fig. 1D). Furthermore, AVG-treated *rhd1-4* and Col-0 plants were less susceptible to *H. schachtii*, with both genotypes supporting fewer sedentary J2 at 7 days postinoculation (dpi) and fewer J4 females at 15 dpi compared with plants grown on medium lacking AVG (Fig. 2). The observation that AVG, an inhibitor of ethylene production, restored all *rhd1-4* phenotypes to normal suggests that the *rhd1-4* phenotypes are the result of an increased ethylene response.

Table 1. Root and root hair measurements of wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) and *rhd1-4*

Strains	Root length (mm)[a]	Root hair length (mm)[a]	Root hair density (no./mm)[a]
Col-0	53 ± 2	0.28 ± 0.02	21.2 ± 1.1
rhd1-4	31 ± 1	0.59 ± 0.03	27.9 ± 2.2

[a] Root length, root hair length, and root hair density of *rhd1-4* are significantly different from Col-0 as determined by paired Student's *t* test ($P < 0.05$). The mean and standard error of the mean are presented.

$AgNO_3$.

A. thaliana ethylene perception is inhibited by silver ions (Ag^+) that inactivate at least one of the ethylene receptors

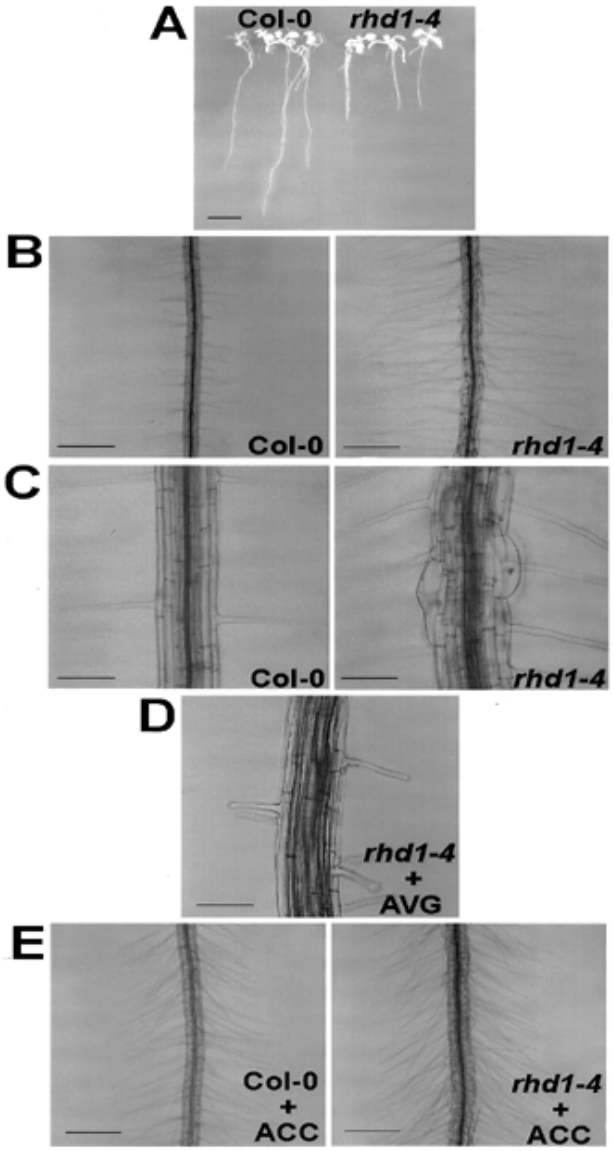

Fig. 1. Comparison of root length, root hair development, and root epidermal cell morphology of wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) and *A. thaliana* mutant *rhd1-4* plants grown on normal medium and 2-aminoethoxyvinylglycine (AVG)- or 1-aminocyclo-propane-1-carboxylic acid (ACC)-supplemented medium. **A,** Macroscopic comparison of Col-0 and *rhd1-4*. Scale bar = 1,000 μm. **B,** Comparison of Col-0 and *rhd1-4* root hair phenotypes. Scale bar = 400 μm. **C,** Comparison of root epidermal cell morphology of Col-0 and *rhd1-4*. Scale bar = 100 μm. **D,** Effect of 1.0 μM AVG on *rhd1-4* root epidermis and root hairs (compare to **C**). Scale bar = 100 μm. **E,** Effects of the ethylene precursor ACC on root hair development in Col-0 and *rhd1-4* (compare to **B**). Scale bars = 400 μm.

(ETR1) (Beyer 1976; Beyer 1979; Rodriguez et al. 1999). *A. thaliana* root hair initiation is inhibited by 1.0 μM Ag^+ (Tanimoto et al. 1995). We found that Ag^+ concentrations of 1.0 to 10.0 μM progressively reduced root hair lengths and root hair densities of *rhd1-4* and Col-0 seedlings growing in nutrient medium (data not shown). These observations are consistent with the idea that *rhd1-4* exhibits an increased ethylene response. In contrast to the AVG data, Ag^+ did not suppress the root epidermal cell deformation phenotype in *rhd1-4*. Attempts were made to determine the susceptibility of Ag^+-treated plants to *H. schachtii*. As a result of the strong effects of Ag^+ on general root morphology, however, a meaningful susceptibility analysis could not be conducted.

ACC.

Increasing the availability of the ethylene precursor ACC elevates endogenous ethylene concentrations in plants (Yang and Hoffman 1984). ACC (1.0 μM) promotes root hair development in *A. thaliana* (Tanimoto et al. 1995), and increased ethylene concentrations inhibit root elongation (Eliasson and Bollmark 1988). If the *rhd1-4* phenotypes are the result of an increased ethylene response, as suggested by the AVG and Ag^+ data, then the *rhd1-4* phenotypes should be phenocopied in Col-0 plants supplemented with ACC. In support of this notion, we observed that ACC concentrations of 0.05 to 0.5 μM caused Col-0 seedlings to produce more and longer root hairs (Fig. 1E; compare to untreated *rhd1-4* in Fig. 1B) while simultaneously producing shorter root lengths (data not shown).

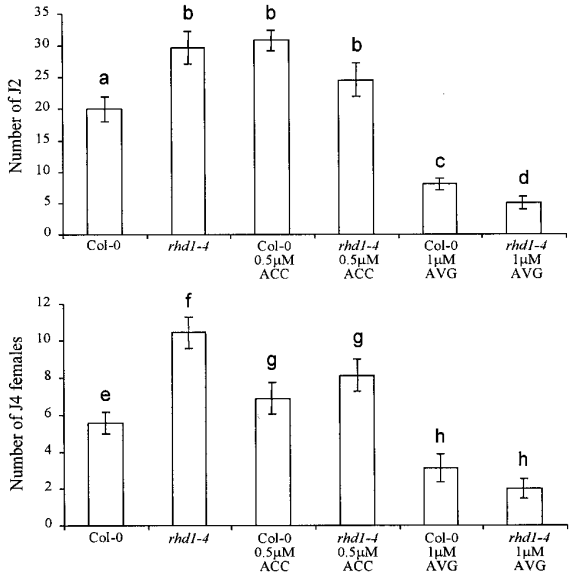

Fig. 2. Effects of an ethylene inhibitor 2-aminoethoxyvinylglycine (AVG) and ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) on nematode susceptibility in wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) and *A. thaliana* mutant *rhd1-4*. Susceptibility was determined by counting sedentary second-stage juvenile nematodes (J2) at 7 days postinoculation (dpi) and counting female fourth-stage juvenile nematodes (J4) at 15 dpi. Col-0 and *rhd1-4* control plants received appropriate amounts of sterile distilled water instead of AVG or ACC solution. Means and standard error of the means are shown. Significance was determined by paired Student's *t* test ($P < 0.05$). Means sharing the same letter designation are not significantly different.

The application of ACC also caused *rhd1-4* to produce more root hairs (Fig. 1E) and further decreased the *rhd1-4* root length (data not shown). Additionally, 0.5 μM ACC caused an increase in *H. schachtii* J2 and J4 female numbers on Col-0 at 7 and 15 dpi, respectively (Fig. 2). The susceptibility of ACC-treated *rhd1-4* plants was not increased, possibly indicating a saturation of the ethylene effect on nematode susceptibility. The observed decrease in root length, increase in root hair length and density, and hypersusceptibility in ACC-treated Col-0 plants further support the notion that an increased ethylene response is responsible for the *rhd1-4* phenotypes, with the exception that ACC-treated *rhd1-4* plants did not show an increase in susceptibility.

The root epidermal cell morphology of *rhd1-4* and Col-0 seedlings was not affected by the ACC concentrations used in this study, which indicates that the root epidermal cell deformation phenotype appears ethylene independent and, at most, only plays a minor role in mediating *rhd1-4* hypersusceptibility to *H. schachtii* because ACC-treated Col-0 plants, which lack the root epidermal cell deformation phenotype, are hypersusceptible. More importantly, observing decreased susceptibility to *H. schachtii* of AVG-treated Col-0 plants and increased susceptibility of ACC-treated Col-0 plants in these studies strongly implicates ethylene in modulating plant susceptibility to this nematode.

Susceptibility of *A. thaliana* ethylene and epidermal cell fate mutants.

The influence of ethylene on plant susceptibility to *H. schachtii* was further investigated by examining the susceptibilities of *A. thaliana* mutants that either overproduce ethylene or are ethylene insensitive (Table 2). The ethylene-mediated changes in root hair density and length in these mutants were classified visually as increased, decreased, or normal, relative to Col-0 (Table 2). The root hair phenotypes of most of these mutants have been evaluated previously and are in agreement with our observations (Masucci and Schiefelbein 1996; Pitts et al. 1998; Schneider et al. 1997).

A positive correlation between root hair development and susceptibility to *H. schachtii* was apparent upon assessment of the ethylene mutants (Table 2). The ethylene-overproducing mutants *eto1-1*, *eto2*, and *eto3*, each having more and longer root hairs, consistently support more J4 females at 15 dpi, relative to Col-0. This observation is even more remarkable considering that *eto* mutants produce root systems that are approximately one-third to one-half the size of Col-0 plants (data not shown). In addition, attempts were made to determine the susceptibility of the constitutive triple response (*ctr1-1*) mutant (Guzman and Ecker 1990). *ctr1-1* shows an increased ethylene response that is more pronounced than the ethylene response of the *eto* mutants, resulting in the development of a very small root system. This root system was too small to support nematode development, although large numbers of J2 were attracted to it (data not shown). Conversely, the ethylene-insensitive mutants *etr1-1*, *ein2-1*, *ein3-1*, *eir1-1*, and *axr2*, which have decreased root hair lengths and/or densities, consistently hosted fewer J4 females at 15 dpi (Table 2). *ein2*, which exhibits the highest level of ethylene insensitivity, always showed the greatest reduction in J4 female numbers. Weaker ethylene-insensitive mutants (*ein5*, *ein6*, and *ein7*), i.e., those with little or no effects on root hair development,

also were assessed for their susceptibility to *H. schachtii*. The susceptibilities of these "weak" mutants, however, varied considerably among experiments such that they did not show a clear susceptibility phenotype.

Throughout our experiments, a strong positive correlation between root hair density and/or length and susceptibility to *H. schachtii* was apparent. It was not clear, however, whether root hair alterations and susceptibility changes were independent pleiotropic effects of the *rhd1-4* mutation or whether susceptibility to the nematode was, in fact, conditioned by increased root hair density and length. To determine whether increased root hair density alone would elevate plant susceptibility, we assessed *A. thaliana* mutants altered in root epidermal cell fate. In the *ttg* and *gl2* mutants, virtually all root epidermal cells are fated to become hair cells, which greatly increases root hair density in the absence of altered ethylene production or signal transduction. This ectopic root hair formation is hypothesized to be the result of a lack of negative regulation of hair cell formation during epidermal cell fate determination (Masucci and Schiefelbein 1996). The observed wild-type level susceptibilities of *ttg* and *gl2* indicate that root hairs do not directly influence plant susceptibility to *H. schachtii* (Fig. 3). Therefore, the altered susceptibilities observed for *rhd1-4* and the ethylene mutants are root hair independent but appear highly dependent on ethylene signal-transduction because ethylene-mediated increases in root hair numbers and lengths always were associated with increased susceptibilities to *H. schachtii*. Similarly, the opposite relationship held true.

Attraction assays.

Casual observations suggested that infective *H. schachtii* juveniles found the roots of *rhd1-4*, *ctr1-1*, and *eto* mutant plants as well as ACC-treated Col-0 plants in a shorter period of time than those of Col-0 plants grown on normal medium. These findings suggest that the hypersusceptibility of *rhd1-4* may be the result of an increased attraction of infective juveniles to its roots, resulting in an increased number of sedentary J2 at 7 dpi (Fig. 2). To directly test this hypothesis, we used root exudates from *rhd1-4*, *eto3*, and Col-0 plants grown in vitro to conduct J2 attraction assays. Growth medium cores from the immediate vicinity of the plant roots were transferred to new medium plates and placed in pairwise combinations of two cores per plate. The following combinations of root exudates were tested: Col-0 versus a control lacking root exudates, *rhd1-4* versus Col-0, and *eto3* versus Col-0. Nematodes were placed in the center between cores, and the attraction of J2 into the cores was assayed over time. Twelve replications of each comparison were performed. Results (Table 3) indicate that *eto3* and *rhd1-4* root exudates attracted more nematodes than exudates from Col-0 roots, whereas root exudates

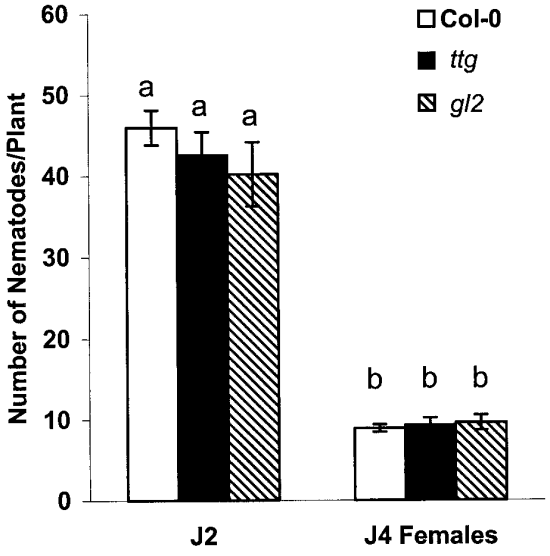

Fig. 3. Susceptibility of the *ttg* and *gl2* epidermal cell fate mutants compared with wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0). Sedentary second-stage juveniles (J2) and female fourth-stage juveniles (J4) were counted at 7 and 15 days postinoculation, respectively. Means and standard error of the means are shown. Significance was determined by paired Student's *t* test ($P < 0.05$). Means sharing the same letter designation are not significantly different.

Table 2. Susceptibility of *Arabidopsis thaliana* ethylene mutants to *Heterodera schachtii* as determined by the total number of fourth-stage juvenile (J4) females at 15 days postinoculation (dpi)

Strains	Description	Root hair length	Root hair density	J4 females at 15 dpi[a]		Reference
				Experiments 1 and 2	Experiment 3	
Col-0	Wild-type strain			6.4 ± 0.7	6.0 ± 0.7[b]	
rhd1-4	Root hair defective	Increased	Increased	9.3 ± 0.7[c]	9.6 ± 1.0[c]	
eto1-1	Ethylene overproducer	Increased	Increased	13.6 ± 0.9[c]	7.1 ± 0.6	Guzman and Ecker 1990
eto2	Ethylene overproducer	Increased	Increased	11.3 ± 1.6[c]	7.2 ± 1.0	Kieber and Ecker 1993; Kieber et al. 1993
eto3	Ethylene overproducer	Increased	Increased	12.7 ± 1.3[c]	7.5 ± 1.0	Kieber and Ecker 1993
etr1-1	Ethylene receptor	Decreased	Normal	3.0 ± 0.7[c]	2.8 ± 0.7[c]	Bleecker et al. 1988
ein2-1	Ethylene insensitive	Decreased	Normal	1.4 ± 0.8[c]	1.4 ± 0.6[c]	Guzman and Ecker 1990
ein3-1	Ethylene insensitive	Decreased	Normal	3.9 ± 0.8[c]	Not determined	Kieber et al. 1993; Roman et al. 1995
eir1-1	Ethylene insensitive root	Decreased	Not determined	3.5 ± 0.9[c]	1.5 ± 0.5[c]	Roman et al. 1995
axr2	Auxin resistant	Not determined	Decreased	4.2 ± 0.8[c]	3.0 ± 0.6[c]	Wilson et al. 1990

[a] Data from experiments 1 and 2 were combined and analyzed. Means and standard errors of the means are shown as determined by least square means analysis.

[b] Mutant strains assessed in experiment 3 were divided into three separate inoculation events to increase the number of observations for each mutant, therefore, each inoculation event had its own wild-type mean. The presented wild-type mean is from one such inoculation event.

[c] Significantly different from wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) as determined by least significant difference analysis (two tailed; $P < 0.05$).

from Col-0 were more attractive than the control. These findings suggest that an increased attraction of J2 to the roots of *rhd1-4* and *eto3* may contribute to their hypersusceptibility.

DISCUSSION

We reported previously on a fourth *rhd1* mutant allele, termed *rhd1-4* (Baum et al. 2000). In this article, we show that, in addition to hypersusceptibility to *H. schachtii*, plants with the *rhd1-4* allele exhibit shorter roots, increased root hair density and length, and a deformation of the root epidermal cells. In addition, the absence of any alteration in the development and morphology of *rhd1-4* shoot tissues suggests that the effects of the *rhd1* mutation are root specific.

Shorter roots and increased root hair density and length are hallmarks of plant development mediated by elevated ethylene concentrations (Cao et al. 1999; Eliasson and Bollmark 1988; Tanimoto et al. 1995). The modulation of *rhd1-4* and Col-0 ethylene production (via AVG and ACC) and ethylene perception (via $AgNO_3$) strongly indicate an increased ethylene response as a cause for the *rhd1-4* phenotypes. The fact that AVG, an inhibitor of ethylene production, could rescue all *rhd1-4* phenotypes indicates that the *rhd1-4* phenotypes are not the result of constitutive activation of the ethylene signal transduction pathway because phenotypes of constitutive ethylene response mutants such as *ctr1* are not affected by the application of inhibitors of ethylene production like AVG (Guzman and Ecker 1990).

The inoculation of known ethylene mutants with altered root hair phenotypes resulting from ethylene overproduction or ethylene insensitivity also revealed a positive correlation between ethylene effects and susceptibility to the nematode (Table 2). These results are in agreement with the susceptibilities recorded for ACC- and AVG-treated Col-0 and *rhd1-4* plants. In general, the overproduction of ethylene resulting from mutation or ACC treatment results in hypersusceptibility. By the same token, ethylene insensitivity resulting from mutation or from inhibition of ethylene production (via AVG) decreases susceptibility. We observed, however, that altered root hair development, i.e., increased root hair density and length upon ethylene overproduction or ACC treatment and decreased root hair density and length resulting from ethylene insensitivity or AVG treatment, also correlated with susceptibility changes. Interestingly, assessment of the *ttg* and *gl2* mutants, which have large numbers of ectopic root hairs but are not perturbed in either ethylene production or signal transduction, revealed that increased root hair density alone does not result in hypersusceptibility to *H. schachtii*. Therefore, we conclude that ethylene-mediated root hair development, per se, is not the cause of the altered susceptibilities of the *rhd1* alleles, the ACC- and AVG-treated seedlings, nor the tested ethylene mutants. Rather, it appears that ethylene acts as a positive regulator of root hair development and susceptibility to *H. schachtii* in *A. thaliana*.

We determined that the hypersusceptibilities of *rhd1-4* and of the ethylene-overproducing mutant *eto3* could be attributed, at least in part, to an increased attraction of infective nematode juveniles to the roots of these plants. The observation that *H. schachtii* attraction is influenced by the ethylene signal transduction pathway could indicate that, through coevolution with host plants, the nematode has developed a response behavior to a (by)product of an important and ubiquitous plant signal transduction pathway. Furthermore, when considering that a nematode attack elicits elevated levels of endogenous ethylene in plant roots (Glazer et al. 1983; Glazer et al. 1985; Volkmar 1991), one could envision a feedback loop leading to increased attractiveness and, therefore, increased susceptibility of host roots under attack. Responsiveness to a (by)product of the ethylene signal transduction pathway would thereby confer an evolutionary advantage to the nematode.

Our finding that ethylene plays a role in the reproductive success of cyst nematodes agrees with recent observations that the phytohormone auxin is required for the formation of syncytia (Goverse et al. 2000). In addition to demonstrating the importance of auxin in syncytium development, Goverse et al. (2000) observed that *eto1*, *eto2*, and *eto3* show hypersusceptibility to *H. schachtii* and that these hypersusceptibilities are indicative of their relative levels of ethylene overproduction, e.g., *eto3* is the strongest *eto* mutation and was observed to host the greatest number of female nematodes. Although our data do not reflect such a relationship among the *eto* mutants, our data do clearly show a hypersusceptibility of the *eto* mutants to *H. schachtii*. Research by Goverse et al. (2000) has shown that a lack of auxin-inducible ethylene production in the form of the *axr2* mutant hinders successful cyst nematode parasitism. Additionally, we have shown here that inhibited ethylene signal transduction, either through genetic lesions (*etr1-1*, *ein2-1*, and *ein3-1*) or through an applied chemical (AVG), inhibits plant susceptibility to a cyst nematode. Whereas Goverse et al. (2000) showed changes in the morphology of syncytia in ethylene mutants, our results add increased root attraction to the growing list of ethylene effects on *A. thaliana* susceptibility to *H. schachtii*.

rhd1-1 was found in a screen designed to identify *A. thaliana* mutants altered in root hair development. Epistasis analysis between *rhd1-1* and other root hair defective mutants (*rhd2*, *rhd3*, and *rhd4*) indicated that the RHD1 protein acts early in root hair formation (Schiefelbein and Somerville 1990). Our finding that *rhd1-4* likely is altered in its ethylene response supports the notion that *RHD1* is a regulator of root hair development because ethylene has been shown to promote root hair development in *A. thaliana* (Tanimoto et al.

Table 3. The attraction of *Heterodera schachtii* second-stage juveniles (J2) to wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0), *rhd1-4*, and *eto3* root exudates at 20 h after J2 placement, equidistant to root exudates tested in pairwise combinations

	Col-0 versus Control		***rhd1-4* versus Col-0**		***eto3* versus Col-0**	
Total[a]	25	14	49	21	112	51
Mean[b]	2.1 ± 0.3[c]	1.2 ± 0.3	4.1 ± 0.8[c]	1.8 ± 0.5	9.3 ± 2.4[c]	4.3 ± 1.3

[a] Total J2 attracted over 12 replications. Data are from one representative experiment.
[b] Mean number of J2 attracted per replication ± the standard error.
[c] Denotes significant difference ($P < 0.05$) between the means of attracted J2 for each pairwise comparison as determined by one-tailed paired Student's *t* test.

1995). Whereas our data suggest that *RHD1* directly or indirectly mediates the ethylene signal transduction pathway, how this process occurs is unknown. Epistasis analyses between *rhd1-4* and known hormone signal transduction mutants are currently underway in order to elucidate the position of *RHD1* relative to genes known to reside in the ethylene signal transduction pathway.

The nature of the *rhd1-4* root epidermal cell deformation phenotype is not resolved at this time. The observation that AVG suppresses this phenotype in *rhd1-4* suggests that the deformation is ethylene dependent. The observations that $AgNO_3$ is not able to suppress the epidermal cell deformation in *rhd1-4* and that ACC is not able to induce the epidermal cell deformation phenotype in wild-type plants, however, contradict the AVG experiments and suggest that the epidermal cell deformation phenotype is ethylene independent. One possibility is that AVG also affects a pathway that leads to epidermal cell expansion. This conclusion is supported by the observation that none of the ethylene mutants had an epidermal cell deformation phenotype that we could observe. Also, because ACC-treated Col-0 and ethylene-overproducing mutants are hypersusceptible in the absence of epidermal cell deformation, we conclude that the epidermal cell deformation is without influence on susceptibility.

In the studies described here, we present an example of a plant signal transduction pathway that is directly involved in mediating susceptibility to the cyst nematode *H. schachtii*. We also presented a strong case that RHD1 is involved in ethylene signaling. Further characterization of this association is not only of academic interest but also of practical relevance because understanding this compatible interaction may open avenues to generate plants that are less susceptible to cyst nematodes.

MATERIALS AND METHODS

Plant and nematode materials and inoculations.

Second-generation progeny (M2) of ethyl methanesulfonate (EMS)-mutated (Lehle Seeds, Round Rock, TX, U.SA.) and wild-type *A. thaliana* seeds, both Col-0 ecotype, were used in the mutant screen that identified *rhd1-4*. The screen itself is presented elsewhere (Baum et al. 2000). All *A. thaliana* plants were grown from surface-sterilized seeds on modified Knop medium solidified with 0.8% Daishin agar (Brunschwig Chemie, Amsterdam, The Netherlands) (Sijmons et al. 1991) in 9-cm petri dishes (Fisher Scientific, Pittsburgh, PA, U.S.A.) or Falcon 12-well plates (Becton Dickinson, Lincoln Park, NJ, U.S.A.), which were sealed with Parafilm (American National Can, Menasha, WI, U.S.A.) and maintained at 26°C on a cycle of 12 h day–12 h night. A *H. schachtii* field population, designated TN101 (provided by G. Tylka, Iowa State University, and obtained originally from T. Niblack, University of Missouri), was grown in greenhouse cultures on cabbage or sugar beet plants. This nematode culture had been propagated previously for 10 years on cabbage and can be considered inbred by mass selection. Eggs were isolated, and J2 were hatched and surface sterilized, as described in Baum et al. (2000). Ten- to twelve-day-old *A. thaliana* plants were inoculated with approximately 300 surface-sterilized J2 that were suspended in sterile, 1.5% low-melting-point agarose (GIBCO-BRL, Grand Island, NY, U.S.A.) at 37°C. *A. thaliana* ethylene and root hair mutants were obtained from the Arabidopsis Biological Resource Center at The Ohio State University.

Assessment of the ethylene mutants was performed over three experiments. The experimental error of experiments 1 and 2 was determined by *F* statistic to not differ significantly. These data were, therefore, combined to increase sample size (i.e., mutant and Col-0 observations were 11 to 21, except for *eto2* and *eto3*, which had four and six observations, respectively, as a result of their absence in experiment 1). In experiment 3, mutant and Col-0 observations were 16 to 28 plants per line. The means and standard error of the means were determined by least square means analysis to account for unequal observation numbers between the mutants and wild-type Col-0. Significance was determined by least significant difference analysis ($P < 0.05$). One inoculation experiment for the *ttg* and *gl2* mutants was performed, comprising 25 to 28 total plants tested for each mutant. Significance was determined by paired Student's *t* test analysis ($P < 0.05$). To assess the ethylene mutants and *ttg* and *gl2*, tested plants were arranged arbitrarily with wild-type controls in 12-well culture plates and assessed for sedentary J2 at 7 dpi and J4 females at 15 dpi with a dissecting microscope.

Root measurements.

Plants were allowed to germinate and grow for 3 days on horizontally placed petri dishes containing growth medium. All plates were then tilted to approximately 60° to promote unidirectional growth of all plant roots. All measurements were performed 10 days after germination. Root length (distance from the crown to the tip of the main root) and root hair length were measured with a reticle installed in an eyepiece of a dissecting microscope. Fifteen plants for each tested *A. thaliana* line were measured for root length. Root hairs were chosen arbitrarily from regions of roots that had fully formed root hairs. Twenty root hairs were measured from each of seven plants per *A. thaliana* line tested. Root-hair densities were determined by counting the root hairs in 1-mm root segments that exhibited uniform root hair stands. Twenty-five of the 1-mm segments were measured from 15 total plants for each *A. thaliana* line tested.

Ethylene inhibitors and precursor tests.

AVG (Sigma, St. Louis, MO, U.S.A.), $AgNO_3$ (Fisher Scientific), and ACC (Sigma) stock solutions were made in distilled water, then filter sterilized through 0.2-µm pore filters (Nalge Nunc International, Rochester, NY, U.S.A.). For the inoculation experiments, 50 µl of appropriately diluted solutions were added to the surface of individual wells of 12-well plates (1 ml of Knop medium per well) and allowed to absorb for 1 day before planting. Plants were inoculated and assessed as described above. Ten to twelve plants were assessed for each *A. thaliana* line tested. Significant differences were determined by paired Student's *t* test ($P < 0.05$). The effect of AVG on J2 viability was determined by incubating freshly hatched J2 in sterile culture tubes with water containing either 0, 10, or 100 µM AVG. Four tubes per treatment were prepared. Tubes were incubated at 26°C for 3 h, after which an aliquot of the tube solution was spread on a microscope slide. A dissecting microscope was used to determine the percent of live J2 by counting moving worms and dividing by the total number of worms present. An identical count was conducted after a 26-h incubation. There were no differences in the per-

cent of live J2 between the AVG solutions and the water control at both observations. Significance was analyzed with a two-sample Student's *t* test ($P < 0.05$).

The evaluation of wild-type and *rhd1-4* root morphology resulting from growth on AVG-, $AgNO_3$-, or ACC-supplemented medium was accomplished by first incorporating appropriate amounts of each compound into precooled Knop medium to obtain the desired final concentration. Petri plates were then poured, and seeds of each *A. thaliana* line were planted as described above. Root and root hair lengths and densities were determined as described above.

Attraction assays.

Seeds of wild-type Col-0, *rhd1-4*, and *eto1-1* were planted and allowed to grow for 10 days in 9-cm petri dishes, as described above. After this growth period, plants were carefully removed. A cork borer was used to remove approximately 7-mm-diameter growth medium plugs from areas that had been immediately adjacent to roots and, therefore, contained root exudates. Medium plugs were transferred in pairwise combinations, as described above, to 6-cm petri dishes (Fisher Scientific) containing Knop medium. Transferred plugs were spaced 3.5 cm apart from edge to edge, and 1.5% low-melting-point agarose was applied to seal the plug-growth-medium boundaries and displace trapped air. Approximately 50 surface-sterilized J2s in a drop of low-melting-point agarose were added to the center of the plates within equal distance to the two plugs. Nematodes that were attracted into either plug were counted after 20 h in the dark at room temperature. Twelve replications were tested for each combination.

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