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Identification, characterization, and cloning of *Arabidopsis rhd1-4*: A UDP-glucose-4-epimerase mutant that exhibits hypersusceptibility to the sugar beet cyst nematode and altered root ethylene responses

by

Martin John Evers Wubben II

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

Program of Study Committee:
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2004

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TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION **1**

Introduction 1

Dissertation Organization 5

Literature Cited 6

CHAPTER 2. A Screen for *Arabidopsis thaliana* Mutants with Altered Susceptibility to *Heterodera schachtii* **10**

Acknowledgements 10

Abstract 10

Introduction 11

Materials and Methods 12

Results 13

Discussion 16

Literature Cited 19

Tables and Figures 23

CHAPTER 3. Susceptibility to the Sugar Beet Cyst Nematode is Modulated by Ethylene Signal Transduction in *Arabidopsis thaliana* **27**

Abstract 27

Introduction 28

Results 29

Discussion 33

Materials and Methods 36

Acknowledgements 39

Literature Cited 39

Tables and Figures 43

CHAPTER 4. *RHD1* encodes a UDP-glucose-4-epimerase that negatively regulates *Arabidopsis* susceptibility to the sugar beet cyst nematode **49**

Abstract 49

Introduction 49

Results 50

Discussion 51

Materials and Methods 54

Literature Cited 56

Figures 59

CHAPTER 5. Mutation of a UDP-glucose-4-epimerase alters nematode susceptibility and ethylene responses in *Arabidopsis* roots **62**

Abstract 62

Introduction 62

Results 65

Discussion 71

Experimental Procedures 76

Acknowledgements 80

Literature Cited 80

Tables and Figures 85

CHAPTER 6. GENERAL CONCLUSIONS **94**

General Conclusions 94

Literature Cited 99

ACKNOWLEDGEMENTS **101**

CHAPTER1. GENERAL INTRODUCTION

Plant-parasitic nematodes of the genus *Heterodera*, commonly known as cyst nematodes, are obligate biotrophs that parasitize host plant roots. Many economically important plant pathogens belong to this group of phytonematodes, such as the soybean cyst nematode (*H. glycines*), the sugar beet cyst nematode (*H. schachtii*), and the cereal cyst nematode (*H. avenae*). By far, the most damaging cyst nematode is *H. glycines* which accounted for ~50% of total soybean yield losses in the United States from 1996-98 (Wrather *et al.*, 2001). As recently as 2002, soybean yield losses due to *H. glycines* approached $780 million in the United States alone (Wrather *et al.*, 2003). *H. glycines* is the number one pathogen of soybean (Wrather *et al.*, 2003), therefore, there is considerable interest in understanding the mechanisms of cyst nematode parasitism at the molecular level in hope of devising novel transgenic-based strategies for cyst nematode resistance that can be used in conjunction with conventional control practices.

Cyst nematodes possess morphological and physiological characteristics that make them well suited for plant parasitism. First, the stylet, which is common to all plant parasitic nematodes, is a hollow mouth-spear that is able to penetrate plant cell walls. The stylet also functions as a hypodermic needle through which esophageal gland secretions are injected into plant cells (reviewed by Davis *et al.*, 2004). Second, cyst nematodes possess three large secretory glands, (2) subventral and (1) dorsal, that manufacture elicitors pertinent to plant parasitism which are delivered via the stylet (Davis *et al.*, 2004).

Cyst nematodes are endoparasites that become sedentary once feeding has been initiated inside the root by the infective second-stage juvenile (J2). The typical cyst nematode life-cycle begins when infective J2 nematodes hatch from eggs in the soil and infect host plant roots which the J2 locates by sensing specific host root exudates and secretions (Grundler *et al.*, 1991; Papademetriou and Bone, 1983). The J2 penetrates the root epidermis, usually in the zone of elongation or at sites of lateral root initiation, and migrates intracellularly toward the vascular tissues (Wyss and Zunke, 1986). Root epidermal penetration and intracellular migration are facilitated by mechanical force, i.e., perforation of the cell wall by the nematode's stylet (Wyss and Zunke, 1986), and by the activity of plant cell wall-degrading

enzymes that are secreted from the J2 subventral esophageal glands (Davis *et al.*, 2004). There are multiple lines of evidence that indicate that cyst nematodes secrete β-1,4-endoglucanases during root penetration and intracellular migration (Davis *et al.*, 2004; de Boer *et al.*, 1999; Goellner *et al.*, 2000; Smant *et al.*, 1998; Wang *et al.*, 1999). Sequences of gland-expressed genes with similarity to bacterial and fungal pectate lyases, another cell wall-degrading enzyme, have been identified in *Globodera rostochiensis* (potato cyst nematode) and *H. glycines* (de Boer *et al.*, 2002; Popeijus *et al.*, 2000; Gao *et al.*, 2003). Finally, there is recent evidence that cyst nematodes secrete plant expansin-like proteins which may act to make plant cell walls more susceptible to nematode-derived endoglucanases (Qin *et al.*, 2004).

The sedentary nature of cyst nematodes is dependent upon the formation of an elaborate feeding site, called a syncytium, whose function is to supply nutrients to the developing nematode until adulthood (Endo, 1986; reviewed by Williamson and Hussey, 1996). Syncytium formation is initiated when a J2 nematode selects an initial feeding cell, normally in proximity to xylem elements, by penetrating the cell wall with its stylet (Wyss and Zunke, 1986). At this point in syncytium formation, the J2 subventral esophageal glands decrease in activity while the dorsal esophageal gland enlarges and becomes considerably more active (Endo, 1986; Wyss, 1992). Secretions produced by the esophageal glands are released, via the stylet, within or around the initial feeding cell where they are believed to elicit the dramatic changes in host cell physiology and morphology that occur during syncytium formation (reviewed by Davis *et al.*, 2000, 2004).

Syncytia are characterized by widening of plasmodesmata leading to partial dissolution of the initial feeding cell wall. As a result, protoplast fusion occurs between the initial feeding cell and its neighbors. This process of partial cell wall dissolution continues in neighboring cells until hundreds of cells are incorporated into the expanding syncytium (Jones, 1981). Unlike root penetration and intracellular migration, partial cell wall dissolution in syncytia is not mediated by nematode-derived endoglucanases. Instead, plant endoglucanase genes are induced by the J2 to bring about the desired effect (Goellner *et al.*, 2001). Syncytia also exhibit increased metabolic activity in order to fulfill their function as a food source for the nematode. Increased metabolic activity in syncytia is characterized by

vacuolar disintegration, increased cytoplasmic density, and proliferation of ribosomes, mitochondria, and other organelles (Williamson and Hussey, 1996). Cyst nematodes also activate the cell cycle within syncytia, however, nuclear endoreduplication occurs without karyokinesis resulting in nuclear hypertrophy (Gheyson and Fenoll, 2002).

Once syncytium formation is initiated, J2 development proceeds through three molting events which give rise sequentially to the J3, J4, and adult stages (Wyss, 1992). Adult male cyst nematodes, which do not feed after the J3 stage, regain mobility and leave the root in search of adult females to fertilize. Upon fertilization, the female retains most eggs inside the uterus and subsequently dies, its body wall becoming a protective cyst for the eggs inside. However, some eggs are deposited outside the female in a gelatinous matrix where immediate hatching can occur and cause another round of infection.

Syncytium formation is a complex process that likely requires signal exchange between the nematode and the plant cell (reviewed by Williamson and Gleason, 2003). Evidence in support of this hypothesis has come from several sources. First, analyses of expressed sequence tags from a *H. glycines* esophageal gland-specific cDNA library revealed a relatively large number of genes coding for proteins with putative nuclear localization sequences in addition to the signal peptides that are required for secretion (Gao *et al.*, 2003). Upon further investigation, it appears that the products of some of these *H. glycines* secretory genes can be translocated into plant nuclei (Elling and Baum, unpublished results). Also, Tytgat *et al.* (2004) showed that the product of a dorsal gland-specific gene from *H. schachtii*, with similarity to ubiquitin extension proteins, is translocated into plant nuclei. . These observations indicate that a portion of nematode secretions, originating primarily from the dorsal esophageal gland (Gao *et al.*, 2003), are imported into plant nuclei where they may act to induce or repress the expression of host genes. In addition, a secretory gene from *H. glycines* has been identified that is similar to the CLAVATA3/ESR-related class of plant signal peptides which have been shown to promote cellular differentiation in plants (Davis *et al.*, 2004; Olsen and Skriver, 2003). Furthermore, a gene showing similarity to a bacterial chorismate mutase was isolated from *H. glycines* esophageal glands (Bekal *et al.*, 2003; Gao *et al.*, 2003). Chorismate mutase is a key enzyme in the shikimate pathway that is responsible for the synthesis of multiple plant compounds including aromatic amino acids, phytoalexins,

salicylic acid, and auxin (Strack, 1997). The chorismate mutase *Hg-cm-1* from *H. glycines* was shown to have chorismate mutase activity (Bekal *et al.*, 2003). *Hg-cm-1* was hypothesized to be a *H. glycines* virulence gene because different forms of *Hg-cm-1* present in different *H. glycines* inbred lines correlated with the ability of these inbred lines to infect resistant soybean plants (Bekal *et al.*, 2003). A chorismate mutase isolated from the root-knot nematode *Meloidogyne javanica* altered plant cell development when expressed heterologously, a phenotype that could be rescued by treatment with the auxin IAA (indole-3-acetic acid) (Doyle and Lambert, 2003; Lambert *et al.*, 1999), therefore, it is possible that chorismate mutase secreted by *H. glycines* may act to alter auxin levels in plant cells to promote feeding site formation. Additional evidence that cyst nematodes proactively modulate plant cellular hormone signaling comes from studies where cytokinin species were detected within exudates of *H. schachtii* J2 (de Meutter *et al.*, 2003). Finally, the discovery of cyst nematode secretory genes with similarity to plant genes involved in cell-cycle activation and protein degradation confirm the idea that syncytium formation is tightly regulated by the nematode, involving many aspects of nematode-induced modulations of plant cellular processes (Davis *et al.*, 2004).

Another line of evidence for host-nematode signal exchange are the numerous observations of altered host gene expression within cyst nematode-infected roots and/or syncytia (Alkharouf *et al.*, 2004; reviewed by Gheysen and Fenoll, 2002; Hermsmeier *et al.*, 1998, 2000; Mazarei *et al.*, 2002, 2003; Puthoff *et al.*, 2003; Vaghchhipawala *et al.*, 2001). These studies have identified genes in both soybean and *Arabidopsis thaliana* that show dramatic changes in their transcript levels following infection with *H. glycines* or *H. schachtii*, respectively. Other studies have also identified numerous genes that are differentially expressed within nematode feeding sites (reviewed by Gheyson and Fenoll, 2002).

While differential gene expression analyses of cyst nematode-infected host roots provide an idea of which host genes may be involved in syncytium formation, these analyses provide little information regarding the actual function of differentially expressed host genes in cyst nematode parasitism because only limited reverse genetic approaches have been completed. In contrast, a forward genetics experimental approach, which utilizes mutant

phenotypes to identify genes that are involved in a particular process, has the potential to identify specific host genes that are required for successful cyst nematode parasitism. The compatible interaction between *Arabidopsis thaliana* and the sugar beet cyst nematode, *Heterodera schachtii*, provides an excellent model system in which a forward genetics approach can be employed (Sijmons *et al.*, 1991). Mutagenized *A. thaliana* populations are available that can be screened for mutants that exhibit either hypersusceptibility or decreased susceptibility to *H. schachtii*. The characterization of such mutants, in addition to the identification of the underlying genetic defect, has the potential to single out individual genes and the signal transduction pathway(s) that are intimately involved in cyst nematode-plant interactions. The following chapters in this dissertation describe the identification, characterization, and cloning of *A. thaliana rhd1-4* that confers hypersusceptibility to *H. schachtii*. These chapters **i)** describe the first mutant identified based on altered cyst nematode susceptibility and **ii)** illustrate the potential and efficacy of a forward genetics experimental approach geared toward the identification of host genes required for cyst nematode parasitism.

Dissertation Organization

Following this introductory chapter (I), this dissertation is organized into four research chapters (II-V) and a final chapter of general conclusions (VI):

Chapter II discusses the identification of the *A. thaliana rhd1-4* mutant which shows hypersusceptibility to *H. schachtii*. Chapter II was published in the *Journal of Nematology* by Baum, T. J., Wubben II, M. J. E., Hardy, K., Su, H., and Rodermel, S. R. (2000; 32:166-173). M. Wubben was responsible for phenotypic characterizations of *rhd1-4*, including a portion of the preliminary mapping of *RHD1*, and co-authoring the manuscript. M. Wubben also supervised undergraduate hourly workers in the continuation of the mutant screen beyond that described in this chapter.

Chapter III discusses the detailed phenotypic characterization of the *rhd1-4* mutant and the role of ethylene signal transduction in mediating *A. thaliana* susceptibility to *H. schachtii*. Chapter III was published in *Molecular Plant-Microbe Interactions* by Wubben II, M. J. E., Su, H., Rodermel, S. R., and Baum, T. J. (2001; 14:1206-1212). M. Wubben was

responsible for the confirmation of preliminary experiments conducted by H. Su and performing all additional experimental work, statistical analyses of the ethylene mutant susceptibility data, and for authoring the manuscript.

Chapter IV discusses the effort to clone *RHD1* from *A. thaliana* using a map-based approach. M. Wubben was responsible for the fine mapping of *RHD1* and identification of *RHD1*. These data were not published because an independent cloning effort by another laboratory published the cloning of *RHD1* prior to submission of our manuscript (Seifert *et al.*, 2002).

Chapter V details the role of *RHD1* in *A. thaliana* root hormone signaling and attempts to explain the mechanism of *rhd1-4* hypersusceptibility to *H. schachtii*. Chapter V is published in *The Plant Journal* (2004) (Wubben II, M. J. E., Rodermel, S. R., and Baum, T. J.). M. Wubben was responsible for the design of all experiments and subsequent data collection. M. Wubben was also responsible for authoring the manuscript.

Chapter VI describes General Conclusions and ties together the four previous chapters and includes a discussion of future research directions.

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CHAPTER 2. A Screen for *Arabidopsis thaliana* Mutants with Altered Susceptibility to *Heterodera schachtii*

A paper published in the Journal of Nematology

T. J. Baum, M. J. E. Wubben II, K. A. Hardy, H. Su, and S. R. Rodermel

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ABSTRACT

Genetic approaches are a powerful means to elucidate plant-pathogen interactions. An *in vitro* screening protocol was developed to identify *Arabidopsis thaliana* mutants with altered susceptibility to *Heterodera schachtii*, the sugar beet cyst nematode. In an initial screen of approximately 5,200 ethyl methanesulfonate-generated mutant plants, two stable mutations were identified. Both mutant lines were backcrossed and were found to harbor single recessive mutant alleles. Mutant line 2-4-6 shows an approximately two-fold increase in sedentary and developing nematodes, while mutant line 10-5-2 exhibits a significant decrease in susceptibility that manifests itself only after nematodes become sedentary. Analyses of progeny from crosses of lines 2-4-6 and 10-5-2 indicated that the two mutations are not allelic. However, the mutant gene in line 2-4-6 was found to be allelic to the previously identified mutant *rhd1* and was termed *rhd1-4*. The mutant gene in line 10-5-2 was called *asc1* for altered susceptibility to cyst nematodes. Our results demonstrate the

feasibility of a nematological mutant screen and strengthen *A. thaliana* and *H. schachtii* as a model pathosystem.

INTRODUCTION

Cyst nematodes of the genus *Heterodera* are sedentary, obligate endoparasites of plants. These microscopic roundworms cause extensive losses in agricultural and horticultural crops worldwide (reviewed by Baldwin and Mundo-Ocampo, 1991). All *Heterodera* species share a common life cycle. Infective second-stage juveniles (J2) hatch from eggs and are attracted to the roots of host plants. After root penetration, J2 migrate intracellularly and then select initial feeding cells that they transform into nurse cell systems (Jones, 1981; Wyss, 1992; Wyss and Grundler, 1992). During this transformation process, the initial feeding cell fuses with neighboring cells by partial cell wall dissolution to form a syncytium. Characteristics of cells incorporated into the syncytium include increased size, enlarged nuclei, loss of the central vacuole, and cell wall ingrowths (reviewed by Jones, 1981; Williamson and Hussey, 1996). It is thought that syncytium formation is due to plant signal transduction mechanisms triggered by the direct or indirect perception of one or more cyst nematode esophageal gland secretions (reviewed by Williamson and Hussey, 1996).

Plant signal transduction events leading to cyst nematode establishment within host roots are not understood. Because a genetic approach is a powerful means to dissect signal transduction pathways, *Arabidopsis thaliana* has served as a model system to understand host-pathogen interactions in a number of pathosystems that did not involve nematodes (Delaney *et al.*, 1995; Glazebrook *et al.*, 1996, 1997; Parker *et al.*, 1996, 1997; Shah *et al.*, 1997; Yu *et al.*, 1998). One major advantage of *A. thaliana* is the relative ease of cloning genes known only by their mutant phenotypes (reviewed by Meyerowitz, 1994). *A. thaliana* became amenable to research involving plant-parasitic nematodes when monoxenic culture requirements for successful infection of *A. thaliana* roots by several plant-parasitic nematodes, including *H. schachtii* (the sugar beet cyst nematode), were established by Sijmons *et al.* (1991). These authors further recognized the potential of conducting screens for mutant plants with altered susceptibility to plant-parasitic nematodes, but such endeavors have met with only limited success (Niebel *et al.*, 1994; Sijmons *et al.*, 1994). In fact, to our

knowledge, no successful screen for *A. thaliana* mutants with altered susceptibility to *H. schachtii* has yet been reported.

Several problems aggravate the identification of mutants perturbed in cyst nematode-plant interactions. *H. schachtii* infects plant roots, which usually are not readily accessible for observation, i.e., roots need to be removed from the soil, frequently resulting in partial loss of the delicate *A. thaliana* root system. Assessment of nematode infection in the recovered *A. thaliana* roots is equally unreliable because parasitizing nematodes are easily dislodged and, therefore, lost for assessment. Furthermore, small changes in the root microenvironment or in inoculum delivery may profoundly affect infection numbers and rates. In a mutant screen, this variability will result either in overlooking interesting plants or in retaining too many false positives.

In this paper, we present an in vitro mutant screening procedure that overcomes these obstacles. The culture conditions are derived from the procedure reported by Sijmons *et al.* (1991). However, our method uses different culture vessels and growth conditions; we optimized these for high reproducibility. Furthermore, we established a large-scale surface disinfestation protocol for infectious nematodes and a new inoculation procedure capable of reproducible nematode delivery to each tested plant. Our method allows the successful screening for *A. thaliana* mutants with altered susceptibility to *H. schachtii.*

MATERIALS AND METHODS

The plant material in these experiments included wild-type Columbia *A. thaliana* (Col-0) and second generation progeny (M2) of ethyl methanesulfonate (EMS)-mutated Col-0 seeds (Lehle Seeds, Round Rock, TX). The batch of M2 seeds used in this study had a Mednik's P value for albino embryo mutations (Mednik, 1988) of 87/293 = 0.3, as determined by the supplier. The M2 seeds were in separate parental group bulks of 12,500 seeds each. Each parental group of M2 seeds was derived from approximately 1,577 M1 parents, i.e., approximately 8 M2 seeds had been harvested per M1 parent. The mutant screen presented in this paper used M2 seeds from three different parental groups in unequal proportions.

Seeds were surface-sterilized with 2.6% sodium hypochlorite for five minutes, washed three times with sterile H_2O, then planted aseptically, one seed per well, into 12-well culture

plates (Falcon, Lincoln Park, NJ) containing 1 ml of sterile, modified Knop medium (Sijmons *et al.*, 1991) solidified with 0.8% Daishin agar (Brunschwig Chemie BV, Amsterdam, Netherlands). The plates were sealed with parafilm and placed in a 26 °C growth chamber with 12-hour light/12-hour dark days and approximately 150 µmol • m^{-2} • s^{-1} of light from cool white VHO fluorescent bulbs (Philips, Somerset, NJ). A field population of *H. schachtii*, designated TN101 (kindly provided by Dr. G. Tylka, Iowa State University, and originally obtained from Dr. T. Niblack, University of Missouri), was cultured in the greenhouse on cabbage or sugar beet plants. TN101 has been cultured for 10 years on cabbage and can be considered inbred by mass selection. *H. schachtii* eggs were isolated by breaking open females and cysts harvested from *H. schachtii*-infected roots. Infective J2 were hatched at 26 °C on modified Baermann pans in a 3.14 mM $ZnSO_4$ solution. The J2 were then surface-sterilized by a procedure that included washing three times in sterile H_2O, incubating for 12 minutes in an aqueous 0.01% $HgCl_2$ solution, and washing three more times in sterile H_2O. *A. thaliana* plants were inoculated individually with surface-sterilized J2 suspended in 50 µl sterile 1.5% low-melting point agarose (Gibco BRL, Grand Island, NY) maintained at 37 °C. The agarose-nematode suspension allowed even J2 distribution to each plant and facilitated J2 penetration into the solid growth medium. Immediately before inoculation, each well received 50 µl of an aqueous 20 mM penicillin-G solution (Fisher Scientific, Fair Lawn, NJ) to suppress occasional bacterial contamination. For the assessment of plant susceptibility to *H. schachtii*, 12-well plates were inverted and sedentary nematodes were studied using the dissecting microscope.

RESULTS

Mutant screen: Using the culture conditions established by Sijmons *et al.* (1991) as a starting point, we developed a screening protocol for the detection of mutants perturbed in nematode infection and development. Surface-sterilized *A. thaliana* seeds were grown, one seed per well, in 12-well culture plates containing 1 ml of a solid growth medium. The use of less medium led to wilting of plants over the growth period, while increasing the medium to 3 ml consistently reduced nematode infection success (data not shown). Two wild-type seeds and ten mutant seeds were arbitrarily positioned, one seed per well, in the 12-well plates.

Once the germinating seedlings were ten to 12 days old, each was inoculated with approximately 300 surface-sterilized *H. schachtii* second-stage juveniles (J2). Inoculated plants were maintained in controlled temperature and lighting regimes and were monitored for nematode infection.

For quantification of mutant susceptibility, we found that J2 that had infected wild-type *A. thaliana* roots were uniformly sedentary at 7 days after inoculation (dai) and could be identified at this time by their complete lack of movement and a slight body swelling (Fig. 1A). At 15 dai, the majority of sedentary nematodes had developed to the 4th juvenile stage (J4) on wild-type plants, and at this time it was easy to identify developing male versus female nematodes (Fig. 1B and 1C, respectively). We chose to assess the number of sedentary J2 at 7 dai and the number of females at 15 dai to quantify susceptibility of mutant plants. The number of developing males was not determined during the initial screen to minimize the time needed to assess individual plants. This decision was made because it was much easier to identify and count the relatively large and few J4 females than the smaller and more numerous males.

Using this protocol, we routinely obtained high numbers of sedentary J2 nematodes on wild-type plants at 7 dai (ranging from 30 to 40 per plant, depending on the experiment), which lead to the development of 4 to 8 females per plant at 15 dai. The remaining nematodes developed into males or failed to develop. This level of infection was stable within experiments and allowed the identification of plants with mutant phenotypes deviating from these normal parameters. Mutants whose numbers of infecting nematodes at either observation point were outside the range determined for wild-type plants were rescued by transplanting into soil to allow maximum seed set. Progeny of each transplanted mutant were retested following the same screening protocol as described above. Mutant lines that continued to exhibit altered susceptibility phenotypes in at least three generations were considered stable and retained for further genetic analyses.

In an initial mutant screen of approximately 5,200 *A. thaliana* M2 plants, we identified two mutant lines (designated lines 2-4-6 and 10-5-2) with altered susceptibility to *H. schachtii* whose phenotypes were stable in self-cross progenies. Backcrosses of these lines with wild-type Col-0 produced F1 plants with susceptibility phenotypes indistinguishable from the wild-

type (Table 1). F2 progenies derived from these crosses showed segregation ratios of the susceptibility phenotypes of 3 : 1 (wild-type : mutant) for both lines (Table 1). Therefore, lines 2-4-6 and 10-5-2 appear to harbor single recessive genes that are responsible for the observed altered susceptibility phenotypes. Furthermore, the F1 progeny from a cross of both mutant lines exhibited a susceptibility identical to wild-type plants (Table 1). This observation indicates that both mutant genes fall into different complementation groups, *i.e.*, they are not allelic.

Characterization of mutant line 2-4-6: This mutant is hypersusceptible to *H. schachtii* (Table 2, experiments #1 and 2). The hypersusceptibility is very apparent shortly after inoculation. At the first observation (7 dai), almost twice the wild-type numbers of J2 are sedentary on plants of this line. Plants of line 2-4-6 support increased numbers of sedentary nematodes throughout the nematode life cycle, with the result that there are significantly elevated numbers of females on roots of this line at 15 dai.

In addition to increased susceptibility, line 2-4-6 mutants show root-specific alterations in morphology. In particular, line 2-4-6 plant roots are shorter than normal and have more and longer root hairs and some of the root epidermal cells are deformed (Fig. 2). Because this mutant line has two distinct phenotypes, hypersusceptibility and altered root morphology, we assessed whether these phenotypes are due to the same mutation. In an F2 population derived from a backcross of line 2-4-6 to wild-type Col-0, both phenotypes cosegregated in 125 tested plants, confirming that a single genetic locus is responsible for the morphology and susceptibility phenotypes. Furthermore, we crossed mutant line 2-4-6 to the previously identified mutant *reb1-1* (Baskin *et al.* 1992), which shows similar root morphology changes. F1 progeny of 18 independent crosses all showed mutant root morphologies (data not shown). These findings establish allelism for the mutant gene of line 2-4-6 and *reb1*. Additionally, inoculation of *reb1-1* with *H. schachtii* revealed an approximately two-fold increased susceptibility of this mutant (Table 2, experiments #1 and 2). Moreover, *reb1-1* has recently been shown to be allelic to *rhd1* (G. Seifert, John Innes Centre, UK, pers. comm.), which also shows root morphology changes (Schiefelbein and Somerville 1990). We assessed the *H. schachtii* susceptibility phenotype of *rhd1* and found a significant increase in the number of parasitizing nematodes in this mutant as well (Table 2, experiment #3). The observation that

three independently isolated allelic mutants show similar morphology and susceptibility phenotypes confirms our cosegregation data (see above) that all phenotypes are caused by mutating a single gene. Following proper *Arabidopsis* nomenclature rules (Meinke and Koorneef 1997), we gave the mutant gene of line 2-4-6 the name *rhd1-4*, i. e., the fourth known mutant allele of the *RHD1* gene because a) the *rhd* name (Schiefelbein and Somerville 1990) predates the *reb* name (Baskin et al. 1992) and b) three alleles (*rhd1*, *reb1-1*, and *reb1-2*) had been reported at the time of submission of this manuscript.

Characterization of mutant line 10-5-2: Because mutant line 10-5-2 harbors a single recessive gene (see above) and because we were not aware of any prior mutants with a similar phenotype, we named the mutant gene of this line *asc1* for altered susceptibility to cyst nematodes. *asc1* does not show any obvious morphological alterations besides an increase in overall root size (data not shown). Shortly after inoculation, the numbers of sedentary J2 on *asc1* plants are not statistically different from those on wild-type plants (Table 2, experiment #4). However, at later assessments, it is apparent that the overall success of *H. schachtii* parasitism is reduced (Table 2, experiments #4 and 5). On average, less than 50% of the number of females observed on wild-type are found on *asc1* plants.

DISCUSSION

The model plant *A. thaliana* has been used extensively in studies of in planta nematode behavior (Wyss, 1992; Wyss and Grundler, 1992; Wyss et al., 1992), plant physiology during nematode parasitism (Böckenhoff and Grundler, 1994; Böckenhoff et al., 1996), ultrastructure of nematode-infected roots (Golinowski et al., 1996; Grundler et al., 1997, 1998), and nematode-elicited gene expression changes (Barthels et al., 1997; Favery et al., 1998; Goddijn et al., 1993; Hermsmeier et al., 2000; Møller et al., 1998; Puzio et al., 1998; Urwin et al., 1997 a, b). Despite these important advancements, there is only limited nematological research using *A. thaliana* for its main advantage, namely as a model system that is superbly suited to clone genes that are identified only by their mutant phenotype. Although the potential usefulness of *A. thaliana* for the identification of mutants impaired in their interactions with plant-parasitic nematodes (and the cloning of the mutated genes) was recognized early (Sijmons et al., 1991), no successful screens have been reported. Sijmons et

al. (1994) mentioned a screen for *A. thaliana* mutants with altered susceptibility to *H. schachtii*. However, the methodology of this screen was not disclosed, nor did it yield stable mutants. In a workshop, Niebel et al. (1994) described an *A. thaliana* mutant screen using the root-knot nematode *M. incognita*. The authors noted reproducibility problems of nematode infection levels in sand and suggested it was feasible to isolate mutants using an undisclosed in vitro screening procedure. Even though the preliminary identification of putative mutants was reported, no further data have been presented since.

Although the methodology reported in our paper may appear to differ only slightly from that reported by Sijmons et al. (1991), the adjustments we have made were crucial for reproducibility and success and allowed us to isolate what we believe to be the first *bona fide* *A. thaliana* mutants that were identified because of their altered susceptibility to *H. schachtii*. Our mutant screening protocol should be applicable to other nematode species with minor adjustments.

The two mutant lines we isolated exhibit different phenotypes. The hypersusceptibility of *rhd1-4* plants may indicate that the wild-type RHD1 gene product negatively influences *H. schachtii* infection success. Furthermore, the observation that *rhd1-4* plants are parasitized by an approximately two-fold number of J2 already at 7 dai suggests that RHD1 exerts its influence at a very early time point of the nematode-plant interaction.

Judging from the *asc1* phenotype, the wild-type ASC1 gene product may be involved in an important step of syncytium formation or function. This influence appears to be at a relatively late developmental time because normal numbers of nematodes are able to become sedentary on *asc1* plants but then fail to develop properly later in the process.

Naturally, many scenarios responsible for the observed phenotypes are possible. The observed mutations may influence plant susceptibility in ways that *per se* are independent from nematode parasitism. Such indirect effects could be, for example, an altered root system size, which would affect nematode parasitism for obvious reasons. Another example for an indirect effect is any condition influencing the general vigor of plants, which then could influence nematode development. On the other hand, a mutant phenotype may in fact be due to the impairment of a plant factor that is intrinsically involved in nematode parasitism. An example for such a direct effect is the mutation of signal transduction elements involved in

eliciting the formation or maintenance of the syncytium. Interestingly, *rhd1-4* roots are smaller than wild-type roots but are more susceptible, which would argue against root size as a strong determinant of susceptibility to the nematode. This mutant also shows more and longer root hairs than wild-type plants, which could indicate a so far unknown influence of root hairs on susceptibility to the nematode. The nature of a mutation's influence on nematodes can only be discerned after closer study and frequently only after the cloning of the mutated gene. Although mutants with a direct influence on nematode parasitism may appear to be more desirable, the observation that a plant line is altered in its susceptibility to *H. schachtii* will make this line's further characterization highly informative regardless of whether the effect on the nematode is of a direct or indirect nature.

Of the two mutants, *rhd1-4* is most amenable to map-based cloning and further analyses because this mutation can be scored by its obvious root phenotype (Fig. 2) without the need for assessment of susceptibility to *H. schachtii*. *asc1*, on the other hand, shows two obstacles for further analyses. First, *asc1* plants need to be inoculated with *H. schachtii* in order to score for mutant and wild-type phenotypes in mapping populations. Second, the variability in these inoculation assays will result in the scoring of a proportion of *asc1* as wild-type. Hence, mapping of the *asc1* mutant gene will be tedious. We are searching for more severe alleles, which could be more easily scored.

A concern when initiating this mutant screen was that plant genes involved in the compatible nematode-plant interaction might be essential and lethal when mutated. When considering the detrimental effect of nematode parasitism, a strong selective pressure against plant traits allowing the nematode to succeed should be expected. Therefore, the nematode most likely co-opted for its own use plant gene products with important functions. However, the isolation of the mutants presented in this paper documents that such plants can be viable. Identifying two independent mutants in this non-exhaustive screen of only 5,200 M2 plants predicts the presence of additional non-allelic mutations in our M2 mutant population. We are continuing our screen for additional mutants as well as for stronger alleles of *asc1*.

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TABLE 1. Genetic analyses of altered susceptibility mutant lines 2-4-6 and 10-5-2.

Cross	Generation	Number of tested plants	Number of progeny plants with mutant phenotype	Number of progeny plants with wild-type phenotype	P [a]
2-4-6 x Col-0	F_1	24	0	24	
	F_2	125	35	90	>0.3
10-5-2 x Col-0	F_1	12	0	12	
	F_2	112	23	89	>0.2
2-4-6 x 10-5-2	F_1	14	0	14	

[a] P values were obtained by χ^2 analyses comparing the observed F_2 segregation ratio with an expected 3 wild-type : 1 mutant phenotype. The non significant P values indicate that the observed segregation ratios are not significantly different from the expected 3 : 1 ratio.

TABLE 2. Susceptibility of *Arabidopsis thaliana* lines to *Heterodera schachtii*

	Line	# of plants tested	Sedentary J2	Females	Males
Experiment #1	wild-type	11	26.5	5.4	nd
	2-4-6	20	49.1**	11.8**	nd
	reb1-1	14	42.3**	10.2*	nd
Experiment #2	wild-type	28	nd	6.0	7.8
	2-4-6	16	nd	9.9**	9.3 ns
	reb1-1	17	nd	11.0**	9.6*
Experiment #3	wild-type	5	nd	6.8	15.4
	rhd1-1	22	nd	15.0**	21.6**
Experiment #4	wild-type	11	35.0	5.7	nd
	asc1	10	40.2 ns	1.6**	nd
Experiment #5	wild-type	28	nd	6.0	7.8
	asc1	16	nd	3.1**	4.5**

Data are average numbers per plant from five independent experiments. Second-stage juvenile (J2) counts were determined at 7 days after inoculation and female and male counts were determined at 15 days after inoculation. Mutant data were compared to wild-type data using a paired t-test. ns: non significant; *: $P \leq 0.05$; **: $P \leq 0.01$; nd: not determined.

Figure 1. Typical *Heterodera schachtii* development on roots of wild-type Col-0 *Arabidopsis thaliana* plants in the described in vitro culture system. Note that all life stages are visible outside the root. A) Sedentary second-stage juvenile (J2) 7 days after inoculation (dai). B) Male fourth-stage juvenile (J4) 15 dai. C) Female J4 15 dai.

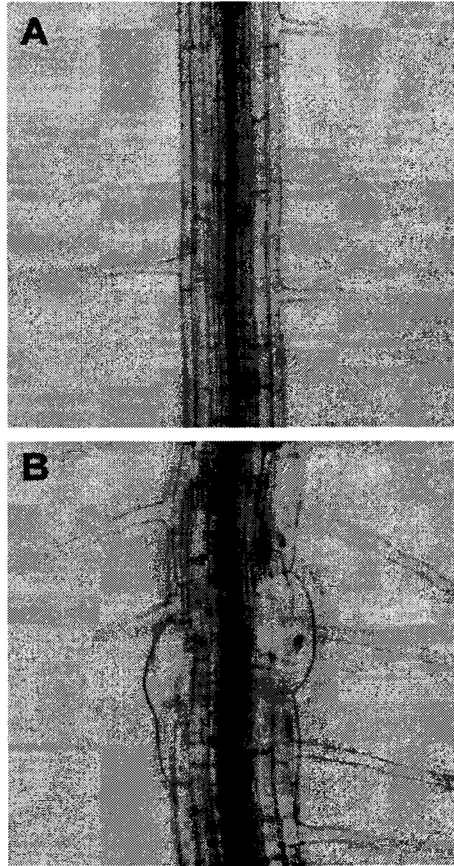

Figure 2. Root epidermal morphology of A) wild-type Col-0 compared to B) mutant line 2-4-6.

CHAPTER 3. Susceptibility to the Sugar Beet Cyst Nematode is Modulated by Ethylene Signal Transduction in *Arabidopsis thaliana*

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ABSTRACT

We previously 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 each exhibited, in addition to *H. schachtii* hypersusceptibility, decreased root length, increased root hair length and density, and deformation of the root epidermal cells when compared to wild-type Columbia (Col-0). Treatment of *rhd1-4* and Col-0 with the ethylene inhibitors 2-aminoethoxyvinylglycine (AVG) and silver nitrate ($AgNO_3$), as well as the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), suggested that the *rhd1-4* hypersusceptibility and root morphology phenotypes are due to an increased ethylene response. Assessment of known ethylene mutants further supported the finding that ethylene plays a role in mediating *A. thaliana* susceptibility to *H. schachtii*: mutants that overproduced ethylene (*eto1-1*, *eto2*, *eto3*) were hypersusceptible to *H. schachtii*, and mutants that were ethylene-insensitive (*etr1-1*, *ein2-1*, *ein3-1, eir1-1*, *axr2*) were less susceptible to *H. schachtii.* Because the ethylene mutants tested showed both 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 analysis of the *ttg* and *gl2* mutants. These mutants produce ectopic root hairs resulting in greatly increased root hair densities while maintaining normal ethylene signal transduction. The observed normal susceptibilities to *H. schachtii* of *ttg* and *gl2* indicated that increased root hair density per se does not cause hypersusceptibility. Furthermore, the results of nematode attraction assays suggested that the hypersusceptibility of *rhd1-4* and the ethylene-overproducing mutant *eto3* may be due to increased attraction of *H. schachtii* infective juveniles to root exudates of these

plants. Our findings suggest that a) *rhd1* is altered in its ethylene response and b) that ethylene signal transduction positively influences plant susceptibility to cyst nematodes.

INTRODUCTION

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* life cycle, an infective second-stage juvenile (J2) hatches from an egg in the soil and is attracted to the zone of elongation 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 this 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 (reviewed by Davis et al. 2000).

The successful completion of the *Heterodera* life cycle constitutes a compatible interaction. Exploration of a compatible cyst nematode-plant interaction using 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 plant genes necessary for successful *Heterodera* parasitism. We previously developed an in vitro mutant screening procedure and identified *Arabidopsis thaliana* mutants exhibiting altered susceptibility to the sugar beet cyst nematode, *H. 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), as well as to *reb1-1* and *reb1-2* (root epidermal bulger) (Baskin et al. 1992). As 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.

RESULTS

All *rhd1* alleles evaluated [i.e., *rhd1*, *reb1-1*, and *rhd1-4*; *reb1-2* was not assessed due to 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 two-fold increase in the number of J4 females able to develop when compared to wild-type 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; Fig. 1 A, B), and a portion of the *rhd1-4* root epidermal cells are deformed and exhibit a bulging phenotype (Fig. 1 C). 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 a root-specific effect of the *rhd1* mutation (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; Tanimoto et al. 1995; Pitts et al. 1998). 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.*

AVG: In planta ethylene production is inhibited by 2-aminoethoxyvinylglycine (AVG). AVG 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 between 0.05 and 2.5 μM. Root hair lengths and densities decreased in *rhd1-4* and Col-0 as AVG concentrations increased (data not shown). 1.0 μM AVG restored *rhd1-4* root hair length and density to approximately normal levels and completely suppressed the root epidermal cell deformation phenotype (Fig. 1 D). Furthermore, AVG-treated *rhd1-4* and Col-0 plants were less susceptible to *H. schachtii*, both genotypes supporting fewer sedentary J2 at 7 days after inoculation (dai) and fewer J4 females at 15 dai in comparison to 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 due an increased ethylene response.

$AgNO_3$: *A. thaliana* ethylene perception is inhibited by silver ions (Ag^+), which inactivate at least one of the ethylene receptors (ETR1; Beyer 1976, 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 ranging from 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*, however, due to the strong effects of Ag^+ on general root morphology, 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). 1.0 μM ACC 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 due to 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 between 0.05 and 0.5 μM caused Col-0

seedlings to produce more and longer root hairs (Fig. 1 E, compare to *rhd1-4* treated with ACC in same figure), while simultaneously producing shorter root lengths (data not shown). Furthermore, 0.5 µM ACC caused an increase in *H. schachtii* J2 and J4 female numbers on Col-0 at 7 and 15 dai, 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 suffer 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 a) appears ethylene-independent and b) at most plays only 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, nevertheless 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 visually classified 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 *H. schachtii* susceptibility 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 supported more J4 females at 15 dai 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 *ctr1-1* (constitutive triple response) 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. Consequently, the *ctr1-1* 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 dai (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., with little or no effects on root hair development, were also assessed for their susceptibility to *H. schachtii*. However, the susceptibilities of these 'weak' mutants varied considerably between experiments such that they did not show a clear susceptility phenotype.

Throughout our experiments a strong positive correlation between root hair density/length and susceptibility to *H. schachtii* was apparent. However, it was not clear 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 due to 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* indicated 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: ethylene-mediated increases in root hair numbers and lengths were always 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 due to an increased attraction of infective juveniles to its roots, resulting in an increased number of sedentary J2 at 7 dai (Fig. 2). To test this hypothesis directly, we conducted J2 attraction assays using root exudates from *rhd1-4*, *eto3*, and Col-0 plants grown in vitro. 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 vs. a control lacking root exudates, *rhd1-4* vs. Col-0, and *eto3* vs. Col-0. Nematodes were placed in the center between cores, and attraction of J2 into the cores was assayed over time. Twelve replications of each comparison were performed. Results, shown in Table 3, indicate that *eto3* and *rhd1-4* root exudates attracted more nematodes than exudates from Col-0 roots, while root exudates 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 previously reported a fourth *rhd1* mutant allele termed *rhd1-4* (Baum et al. 2000). In this article we have shown 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 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). Modulation of *rhd1-4* and Col-0 ethylene production (via AVG and ACC) and ethylene perception (via $AgNO_3$) strongly indicated 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 indicated that the *rhd1-4* phenotypes are not due to 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 due to 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 due to mutation or ACC treatment results in hypersusceptibility. By the same token, ethylene-insensitivity due to mutation or by inhibition of ethylene production (via AVG) decreases susceptibility. However, we observed 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 due to ethylene-insensitivity or AVG-treatment, was 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, of the ACC- and AVG-treated seedlings, nor of the tested ethylene mutants. Rather, it appears that ethylene acts as a positive regulator of both 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 nematode attack elicits elevated levels of endogenous ethylene in plant roots (Glazer et al. 1983, 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* showed hypersusceptibility to *H. schachtii* and that these hypersusceptibilities were 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. While 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*, *ein3-1*) or through an applied chemical (AVG), inhibits plant susceptibility to a cyst nematode. While 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*, *rhd4*) indicated the RHD1 protein acts early in root hair formation (Schiefelbein and Somerville 1990). Our finding that *rhd1-4* is likely 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. 1995). While our data suggest that *RHD1* either directly or indirectly mediates the ethylene signal transduction pathway, how this 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 the deformation is ethylene-dependent. However, 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 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 have 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, for 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, Texas) and wild-type *A. thaliana* seeds, both of the Columbia (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, Netherlands) (Sijmons et al. 1991) in 9-cm-diameter petri dishes (Fisher Brand, Fisher Scientific, Pittsburgh, PA) or 12-well plates (Falcon Brand, Becton Dickinson, Lincoln Park, N. J.) that were sealed with parafilm (American National Can, Menasha, WI) and maintained at 26 °C on a 12 hour day/12 hour night cycle. A *H. schachtii* field population

designated TN101 (kindly provided by Dr. G. Tylka, Iowa State University, and originally obtained from Dr. T. Niblack, University of Missouri) was grown in greenhouse cultures on cabbage or sugar beet plants. This nematode culture had previously been propagated 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). 10- to 12-day-old *A. thaliana* plants were inoculated with approximately 300 surface-sterilized J2 suspended in sterile, 1.5 % low-melting point agarose (Gibco BRL, Grand Island, N. Y.) 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, therefore, these data were combined to increase sample size (i.e., mutant and Col-0 observations ranged from 11 to 21 except for *eto2* and *eto3* which had 4 and 6 observations, respectively, due to their absence in experiment 1). In experiment 3, mutant and Col-0 observations ranged from 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 25-28 total plants tested for each mutant. Significance was determined by paired t-test analysis ($P<0.05$). For the assessment of the ethylene mutants and *ttg* and *gl2*, tested plants were arbitrarily arranged along with wild-type controls in 12-well culture plates and assessed for sedentary J2 at 7 dai and J4 females at 15 dai using a dissecting microscope.

Root Measurements

Plants were allowed to germinate and grow for three days on horizontally placed petri dishes containing growth medium. All plates were then tilted at approximately 60° to promote unidirectional growth of all plant roots. All measurements were performed 10 days after germination. Root length (i.e., 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-long root segments that exhibited uniform root hair stands. Twenty-five such 1-mm-long segments were measured from 15 total plants for each *A. thaliana* line tested.

Ethylene inhibitors and precursor tests

AVG (Sigma, St. Louis, MO), $AgNO_3$ (Acros Organics, Fisher Scientific, Pittsburgh, PA) and ACC (Sigma, St. Louis, MO) stock solutions were made in distilled water and then filter-sterilized through 0.2-µm-pore filters (Nalge Nunc International, Rochester, N. Y.). 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 one day before planting. Plants were inoculated and assessed as described above. 10-12 plants were assessed for each *A. thaliana* line tested. Significant differences were determined by paired 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 µM, 10 µM, or 100 µM AVG. 4 tubes per treatment were prepared. Tubes were incubated at 26 °C for 3 hours after which an aliquot of the tube solution was spread on a microscope slide. Percent live J2 was determined by counting moving worms and dividing by the total number of worms present using a dissecting microscope. An identical count was conducted after a 26 hour incubation. There were no differences in percent live J2 between the AVG solutions and the water control at both observations. Significance was analyzed using a two sample t-test ($P<0.05$).

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 appropiate amounts of each compound into pre-cooled 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-diameter 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 in the text to 6-cm-diameter petri dishes (Fisher Brand; Fisher Scientific, Pittsburgh, PA) containing Knop medium. Transferred plugs were spaced 3.5 cm apart from edge to edge. 1.5 % low melting-point agarose was applied to seal the plug - growth medium boundaries and to displace trapped air. Approximately 50 surface-sterilized J2 in a drop of low melting point agarose were added to the center of the plates within equal distance to the two plugs. Nematodes attracted into either plug were counted after 20 hours in the dark at room temperature. Twelve replications were tested for each combination.

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Table 1. Root and root hair measurements of wild-type Col-0 and *rhd1-4*

Strain	Root length[1] (mm)	Root hair length[1] (mm)	Root hair density[1] (number/mm)
Col-0	53 ± 2	0.28 ± 0.02	21.2 ± 1.1
rhd1-4	31 ± 1	0.59 ± 0.03	27.9 ± 2.2

[1]Root length, root hair length, and root hair density of *rhd1-4* are significantly different from wild-type Col-0 as determined by paired t-test ($P<0.05$). The mean and standard error of the mean are presented.

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 after inoculation (dai).

Strains	Description	Root hair length	Root hair density	J4 Females at 15 dai: Experiments 1 and 2[a]	J4 Females at 15 dai: 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*	9.6 ± 1.0*
eto1-1 [1]	ethylene overproducer	increased	increased	13.6 ± 0.9*	7.1 ± 0.6
eto2 [2,3]	ethylene overproducer	increased	increased	11.3 ± 1.6*	7.2 ± 1.0
eto3 [2]	ethylene overproducer	increased	increased	12.7 ± 1.3*	7.5 ± 1.0
etr1-1 [4]	ethylene receptor	decreased	normal	3.0 ± 0.7*	2.8 ± 0.7*
ein2-1 [1]	ethylene insensitive	decreased	normal	1.4 ± 0.8*	1.4 ± 0.6*
ein3-1 [3,5]	ethylene insensitive	decreased	normal	3.9 ± 0.8*	ND
eir1-1 [5]	ethylene insensitive root	decreased	ND	3.5 ± 0.9*	1.5 ± 0.5*
axr2 [6]	auxin resistant	ND	decreased	4.2 ± 0.8*	3.0 ± 0.6*

[a] data from Experiments 1 and 2 were combined and then analyzed; [b] the 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. Means and standard errors of the means are shown as determined by least square means analysis. * significantly different relative to wild-type Columbia (Col-0) as determined by least significant difference analysis (two-tailed; $P<0.05$). Root hair length and root hair density of the mutants were compared to wild-type and determined to be decreased, normal, or increased. ND: not determined. References for individual mutants are [1]Guzman and Ecker 1990; [2]Kieber and Ecker 1993; [3]Kieber et al. 1993; [4]Bleeker et al. 1988; [5]Roman et al. 1995; [6]Wilson et al. 1990.

Table 3. The attraction of *Heterodera schachtii* second-stage juveniles (J2) to wild-type *Arabidopsis thaliana* ecotype Columbia-0 (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 between the means of attracted J2 for each pairwise combination as determined by one-tailed paired t-test ($P<0.05$).

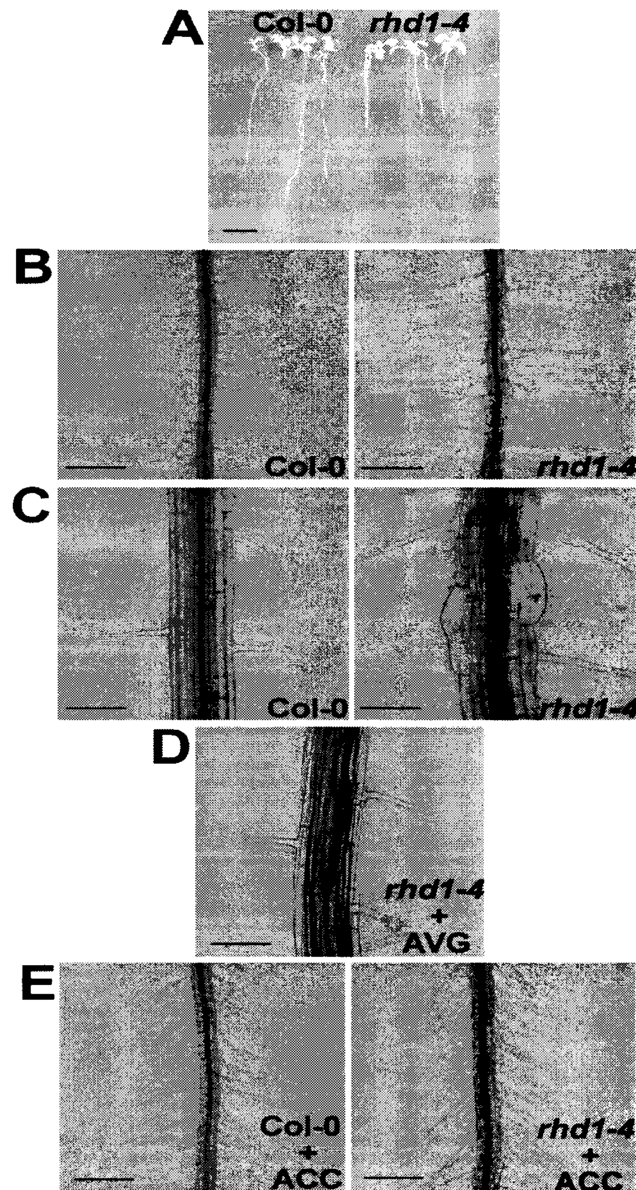

Figure 1. Comparison of wild-type Col-0 and *rhd1-4* with regard to growth habit and root hair and root epidermal cell morphology on normal medium and AVG- or ACC-supplemented medium. **A,** Macroscopic comparison of wild-type Col-0 and *rhd1-4*. Scale bar corresponds to 1000 μm. **B**, Comparison of wild-type Col-0 and *rhd1-4* root hair phenotypes. Scale bar corresponds to 400 μm. **C,** Comparison of root epidermal cell morphology of wild-type Col-0 and *rhd1-4*. Scale bar corresponds to 100 μm. **D**, Effect of 1.0 μM AVG on *rhd1-4* root epidermis and root hairs; compare to panel **C**. Scale bar corresponds to 100 μm. **E**, Effects of the ethylene precursor ACC on root hair development in wild-type Col-0 and *rhd1-4*; compare to panel **B**. Scale bars correspond to 400 μm.

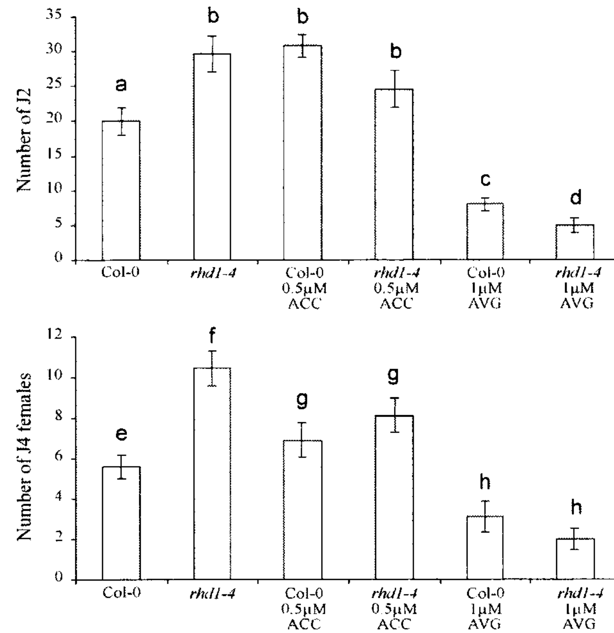

Figure 2. Effects of an ethylene inhibitor (AVG) and ethylene precursor (ACC) on nematode susceptibility in wild-type Col-0 (Col) and *rhd1-4*. Susceptibility was determined by counting sedentary second-stage juvenile nematodes (J2) at 7 days after inoculation (dai) and by counting female fourth-stage juvenile nematodes (J4) at 15 dai. Wild-type 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 t-test ($P<0.05$). Means sharing the same letter designation are not significantly different.

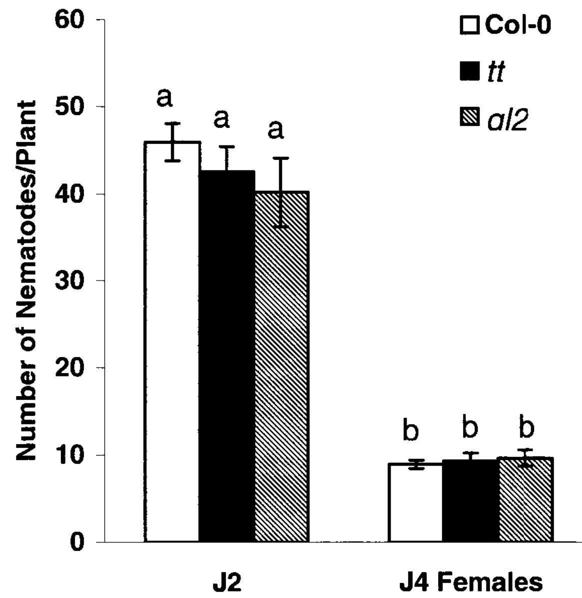

Figure 3. Susceptibility of the *ttg* and *gl2* epidermal cell fate mutants compared to wild-type Col-0. Sedentary second-stage juveniles (J2) and female fourth-stage juveniles (J4) were counted at 7 and 15 days after inoculation, respectively. Means and standard error of the means are shown. Significance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

CHAPTER 4. *RHD1* encodes a UDP-glucose-4-epimerase that negatively regulates *Arabidopsis* susceptibility to the sugar beet cyst nematode

ABSTRACT

In order to determine the underlying signal transduction events that mediate *rhd1-4* hypersusceptibility to *Heterodera schachtii* and its apparent increased ethylene response phenotype, an effort to clone *RHD1* was initiated. Using a map-based approach, *RHD1* was identified as gene At1g64440 at the bottom of chromosome 1. Single base-pair mutations were discovered in At1g64440 from all four *rhd1* alleles. RHD1 shares a high level of similarity with UGE1, a UDP-glucose-4-epimerase responsible for the conversion of UDP-glucose to UDP-galactose in the plant cytosol. Based on genomic location, *RHD1* corresponds to *UGE4*, one of five UDP-glucose epimerase isoforms present in *Arabidopsis thaliana*. UDP-galactose is utilized during the biosynthesis of arabinogalactan proteins (AGPs), xyloglucan, and rhamanogalaturan I, all of which have previously been shown to be absent or defective in *rhd1* roots. We hypothesize that defective AGP and/or cell wall architecture influences root ethylene signaling and host susceptibility to *H. schachtii*.

INTRODUCTION

The *Arabidopsis thaliana rhd1-4* mutant was identified as being hypersusceptible to the sugar beet cyst nematode, *Heterodera schachtii*, in a mutant screen geared toward identifying host genes that are required for successful cyst nematode parasitism (Baum *et al.*, 2000). Our initial characterization of *rhd1-4* suggested that increased ethylene production or sensitivity in the root mediated *rhd1-4* hypersusceptibility to *H. schachtii* in addition to multiple root-specific morphological phenotypes including decreased root length, increased root hair length, and root epidermal bulging (Baum *et al.*, 2000; Wubben *et al.*, 2001). In order to clarify the mechanism(s) that govern the *rhd1* mutant phenotypes, the molecular identity and predicted function of *RHD1* must be determined. Here, using a map-based approach, we present the identity of *RHD1* as gene At1g64440, which was shown to harbor

single base-pair mutations in all four *rhd1* alleles. RHD1 shares a high level of similarity to UDP-glucose-4-epimerases and may influence *A. thaliana* root ethylene responses via the synthesis of cell wall polymers and/or arabinogalactan proteins.

RESULTS

Cloning of *RHD1*

A *RHD1* map-based cloning project was initiated by crossing *rhd1-4* (ecotype Columbia-0 (Col-0)) to wild-type Landsberg erecta (Ler) and self-fertilizing the F_1 to generate a F_2 mapping population. The root epidermal bulging mutant phenotype was used to identify *rhd1-4* homozygotes within the F_2 population. Preliminary mapping localized *RHD1* to the bottom of chromosome 1, flanked by the simple sequence length polymorphism markers F19K23 and F5I14 (Baum *et al.*, 2000). F19K23 and F5I14 defined a locus of ~ 1.2 Mbp which was fully contained within a bacterial artificial chromosome (BAC) contig.

rhd1-4 homozygotes recombinant at either F19K23 or F5I14 were assessed with a battery of CAPS (cleaved amplified polymorphic sequence; Glazebrook *et al.*, 1998) markers that we designed within the F19K23 – F5I14 interval using available wild-type Col-0 genomic sequence data. Assessment of ~ 2200 *rhd1-4* homozygotes delimited *RHD1* to a 125 Kbp region bordered by the CAPS27 and CAPS31 markers (Figure 1A). This region was contained within two sequenced BACs, F15H21 and FIN19 (Figure 1A).

Twenty-three open reading frames (ORFs) were predicted to reside within the 125 Kbp region based on the BAC Genbank annotations and by comparison of the interval genomic BAC sequence to *A. thaliana* expressed sequence tag databases. Candidate ORFs were isolated from *rhd1-4* genomic DNA by polymerase chain reaction and sequenced. Each *rhd1-4* ORF sequence was then compared to its respective wild-type Col-0 sequence in search of mutations. Gene At1g64440 contained a single G/A_{1023} transition in *rhd1-4* (Figure 1B). None of the remaining ORFs from *rhd1-4* were polymorphic compared to wild-type Col-0.

Sequencing of At1g64440 from the remaining *rhd1* alleles revealed single basepair changes in each mutant line compared to wild-type Col-0: *rhd1-1* (G/A_{1260}), *rhd1-2* (C/T_{1833}), and *rhd1-3* (G/A_{436}) (Figure 1B). The identification of point mutations within the same gene in each *rhd1* allele allowed us to conclude that At1g64440 is *RHD1*. This identity for *RHD1*

was confirmed by an independent cloning effort by Seifert *et al.* (2002). Alignment of the *RHD1* coding sequence with the genomic DNA sequence revealed that *RHD1* is comprised of nine exons and eight introns (Figure 1B).

Basal levels of *RHD1* transcript in various tissues were measured by quantitative real-time RT-PCR (Figure 2). *RHD1* transcript was detected in all tissues assayed, i.e., flowers, mature leaves, stems, and roots, with the highest level of *RHD1* expression in roots.

RHD1 sequence analysis

RHD1 encodes a predicted 348 amino acid protein that corresponds to the UDP-glucose-4-epimerase (UGE) isoform *UGE4* in *A. thaliana. UGE4* is one of five UGE isoforms that were predicted to reside in the *A. thaliana genome* (Reiter and Vanzin, 2001). UGEs catalyze the interconversion of UDP-glucose to UDP-galactose via an enzyme-bound UDP-4-keto-hexose intermediate (Maitra and Ankel, 1971). This enzyme represents the primary way that plants generate activated galactose, i.e., UDP-galactose, for use in subsequent biosynthetic pathways (Reiter and Vanzin, 2001).

RHD1 shares 61 – 77% identity at the amino acid level with the other four *A. thaliana* UGE isoforms including UGE1, which has been shown to possess UGE activity (Dörmann and Benning, 1996). As shown in Figure 3, alignment of RHD1 with UGE1 and UGEs from the bacterium *Moraxella catarrhalis*, the yeast *Saccharomyces cereviseae*, and *Homo sapiens* revealed a high level of conservation (~ 50% identity) across kingdoms. Predicted amino acid changes in the rhd1-1, rhd1-2, and rhd1-3 proteins occur at residues that are conserved between plants, bacteria, yeast and humans: rhd1-1 (Gly/Glu_{186}), rhd1-2 (Ala/Val_{250}) and rhd1-3 (Asp/Asn_{63}). *rhd1-4* contains a nonsense mutation (Trp/$STOP_{135}$) that would result in a severely truncated protein (Figure 3). PSORT (Nakai and Kanehiso, 1992) analysis predicted a cytosolic localization for RHD1, which is also where UDP-glucose and UDP-galactose substrates are presumed to accumulate (Bligny *et al.*, 1990; Dancer *et al.*, 1990).

DISCUSSION

Our initial characterization of *rhd1-4* suggested that ethylene-overproduction or increased ethylene sensitivity in the root mediated the observed *rhd1-4* mutant phenotypes

(Wubben *et al.*, 2001). In order to fully determine the cause of the *rhd1-4* phenotypes, the molecular identity of *RHD1* and its predicted protein sequence had to be determined, therefore, a map-based cloning protocol was employed to identify *RHD1*.

Sequencing of predicted open reading frames within a 125 Kbp interval at the bottom of chromosome 1 from *rhd1-4* yielded one gene (At1g64440) that showed a single base-pair change compared to the wild-type Col-0 sequence. This identity for *RHD1* was confirmed by observing single base-pair changes in At1g64440 in the *rhd1-1*, *rhd1-2*, and *rhd1-3* alleles. *RHD1* is predicted to encode a 348 amino acid protein that shows a high level of similarity to a family of five UDP-glucose-4-epimerases in *Arabidopsis thaliana*. In fact, *RHD1* corresponds to *UGE4*, which had been predicted to exist from previous *A. thaliana* genome sequence analyses (Reiter and Vanzin, 2001). An independent cloning effort and biochemical analysis of RHD1 by Seifert *et al.* (2002) substantiates our conclusions.

Predicted amino acid changes in the *rhd1* mutant protein sequences revealed that all mutations occur, with the exception of *rhd1-4*, at amino acids that are conserved between plants, bacteria, yeast, and humans. *rhd1-4* harbors a nonsense mutation that would result in a severely truncated protein which would lack ~ 47% of its C-terminus including large stretches of identity shared with other organisms. This suggests that *rhd1-4* is likely a null allele.

Our identification of *RHD1* as a UDP-glucose-4-epimerase (UGE) is in agreement with previous phenotypic characterizations of other *rhd1* alleles. UGE activity is required for the production of UDP-galactose, a primary component of arabinogalactan proteins (AGPs) (Reiter and Vanzin, 2001). AGPs are small proteoglycans that localize to the plasma membrane and cell wall (Knox *et al.*, 1991). AGPs have been implicated in regulating cell expansion via experiments utilizing $(\beta\text{-D-Glucose})_3$ Yariv reagent, a compound that inhibits AGP function by specifically binding to AGPs and causing them to aggregate within tissues (Willats and Knox, 1996). A link between AGPs and the *rhd1* root epidermal bulging phenotype was established when Ding and Zhu (1997) discovered that this *rhd1* phenotype could be phenocopied in wild-type plants by treating them with $(\beta\text{-D-Glucose})_3$ Yariv reagent. This observation suggested that the *rhd1* root epidermal bulging phenotype was due to aberrant AGP synthesis in the roots (Ding and Zhu, 1997). Additional analyses showed that *rhd1* roots contain substantially less AGPs than wild-type roots (Ding and Zhu, 1997). In

addition, immunohistochemical studies of *rhd1-2* showed that the presence of AGPs in root hair-forming cells was greatly reduced while non-hair forming cells showed normal AGP distributions (Andème-Onzighi *et al.*, 2002). Similarly, Seifert *et al.* (2002) showed that AGP abundance was greatly reduced in the *rhd1* root epidermis compared to wild-type.

Only the root hair-forming cells bulge in *rhd1* mutants (Andème-Onzighi *et al.*, 2002), which suggests that this phenotype is linked to root hair development, specifically, the process of root hair initiation. Investigations of root hair formation in maize showed that AGPs containing either glucoronic acid or $\beta(1 \rightarrow 6)$-galactan epitopes localized to the root hair surface and that this localization was especially strong during the initiation of the root hair bulge (Šamaj *et al.*, 1999). The authors postulated that AGPs might help to regulate the extent of bulge formation by rigidifying the outer cell wall of the root hair to combat increasing turgor pressure (Šamaj *et al.*, 1999).

It is unclear how a non-functional UDP-glucose-4-epimerase in *rhd1* would mediate hypersusceptibility to *H. schachtii* and alter root ethylene responses. AGPs have been implicated in cell-to-cell signaling and cell fate determination (Schultz *et al.*, 2002). Consequently, defective AGP biosynthesis in *rhd1* may result in alterations of these processes upon cyst nematode infection. It has been shown that pre-treatment of carrot AGPs with an endo-chitinase modifies their N-acetyl-glucoseamine components and enhances their ability to cause carrot cells in suspension culture to enter an embryonic state (van Hengel *et al.*, 2001). Chitinase gene expression has been detected in the stylet-associated esophageal gland cells in sedentary juvenile cyst nematodes that are beginning the process of syncytium formation (Gao *et al.*, 2002). Therefore, because syncytium formation requires such drastic changes in cell physiology and morphology, perhaps cyst nematode-derived chitinases modify host AGPs in a fashion similar to that described above to promote syncytium formation. Alternatively, AGPs whose synthesis is RHD1-dependent may directly inhibit cyst nematode pathogenesis. The expression of NaAGP4 in *Nicotiana alata* was found to be down-regulated upon fungal infection (Gilson *et al.*, 2001).

In addition to AGP biosynthesis, UDP-galactose is utilized during the synthesis of some cell wall polymers, including xyloglucan (XG) and rhamnogalacturonan I (RG I) (Moore *et al.*, 1991). It was shown that *rhd1* contains less cell wall-bound galactose in roots

than wild-type, specifically, galactose destined for incorporation into XG and RG I (Seifert *et al.*, 2002). Defects in cell wall architecture have been shown to alter ethylene production in *A. thaliana* tissues (Ellis *et al.*, 2002; Zhong *et al.*, 2002), therefore, it is possible that a similar event is occurring in *rhd1* roots due to altered D-galactose content in cell walls.

Our identification of RHD1 as an enzyme directly involved in AGP biosynthesis suggests that RHD1 may modulate root ethylene responses via AGPs or cell wall polymers, which are absent or defective in *rhd1*. Further characterization of *rhd1* mutants in light of RHD1 function will help clarify the relationship between *RHD1* and the ethylene signal transduction pathway.

MATERIALS AND METHODS

RHD1 cloning and sequence analysis

To clone *RHD1*, a F_2 mapping population was generated by crossing *rhd1-4* (Col-0) to wild-type Landsberg erecta (Ler) and self-fertilizing the F_1. F_2 seeds were surface-sterilized and sown in petri dishes. *rhd1-4* F_2 homozygotes were identified by the presence of root-hair cell bulging. Primary mapping was conducted using the F19K23 and F5I14 simple sequence length polymorphism (SSLP) markers. 5 mm^2 leaf samples were taken from one-week old *rhd1-4* F_2 homozygotes and placed in 0.6 ml thin-wall PCR tubes. The tissue was then treated with 40 µl 0.25 M NaOH (30 sec at 100°C), 40 µl 0.25 M HCl (30 sec at 100°C), 20 µl 0.5 M buffer (0.5 M Tris-HCl pH=8; 0.25% (v/v) Triton X-100) (2 min at 100°C). Tissue was used directly as template in PCR with SSLP marker primers. Genomic DNA was isolated from *rhd1-4* F_2 homozygotes recombinant at either SSLP marker for use in CAPS (cleaved amplified polymorphic sequence) marker analysis.

CAPS markers (Glazebrook *et al.*, 1998) were designed within the mapping interval defined by F19K23 and F5I14. 1.5-2.0 kb genomic sequences were amplified and digested with a battery of restriction endonucleases until a polymorphism was identified between wild-type Col-0 and wild-type Ler. CAPS marker primers and corresponding restriction endonucleases are as follows: m305 (forward-TGAAGCTAATATGCACAGGAG, reverse-TTCTCCAGACCACATGATTAT; Hae III), CAPS27 (forward-GGTTGGGATCTATACTAGACA, reverse-GGCTTAACACTTACCAAGACA; SspI);

CAPS26 (forward-CCCTTTGAGACCCATGAACTA, reverse-CTTGGGCTGTCGGTGCTTA; Hae III), CAPS31 (forward-GCCCAGACGAAGACCAGAAA, reverse-CCCTCTCCTTCTTACACAGT; NdeI), CAPS16 (forward-, reverse-; Hinf I). No recombinants were identified at CAPS26, indicating the position of *RHD1* within a 125 kb region bordered by CAPS27 and CAPS31.

Putative open reading frames (ORFs) within the 125 kb region were isolated from *rhd1-4* by PCR. Primers were designed at least 200 bp upstream and downstream of the 5' and 3' ends of the ORFs, respectively. PCR products were visualized on 0.8% agarose gels by electrophoresis to verify successful amplification. PCR products were purified using the QIAquick PCR purification kit (Qiagen; Valencia, CA) and submitted for sequencing at the Iowa State University DNA Sequencing and Synthesis Facility. *rhd1-4* ORF sequences were compared to the corresponding wild-type Col-0 ORF sequences using Vector NTI software. Only At1g64440 showed a single base-pair difference between *rhd1-4* and wild-type. At1g64440 was isolated from *rhd1-1*, *rhd1-2*, and *rhd1-3* plants as described above and sequenced.

Multiple sequence alignment of UDP-glucose-4-epimerase proteins was accomplished using CLUSTAL W (Thompson *et al.*, 1994). Ascension numbers for the proteins analyzed are as follows: RHD1/UGE4 (AAG51709), UGE1 (Q42605), *Moraxella catarrhalis* (AAF91338), *Saccharomyces cerevisiae* (GAL 10; NP_009575), and *Homo sapiens* (NP_000394).

Quantitative real-time RT-PCR

Total RNA was isolated from 20-50 mg of frozen ground tissue using the Gentra Total RNA Isolation Kit (Gentra Systems; Minneapolis, MN). DNase treatment of 1-1.5 μg total RNA was accomplished using the DNA-Free Kit (Ambion, Inc.; Austin, TX). DNase-treated total RNA was then used for first-strand cDNA synthesis using Superscript II Reverse Transcriptase (Invitrogen; Carlsbad, CA) following manufacturer's instructions. To use as template for qRT-PCR, cDNAs were diluted to volumes equivalent to 10 ng total RNA/μl with nuclease-free water. RHD1 and 18S coding sequence-specific primers were designed as described in Puthoff *et al.* (2003). Primer sequences are as follows. RHD1: forward (5'-

AAAGCTGAACGCGAACTAAACTGGAA-3'), reverse (5'-CCGTAACCGAAAGGATTGTTGCTTGC-3'); 18S: forward (5'-GACAGACTGAGAGCTCTTTCTTGA-3'), reverse (5'-ACGTAGCTAGTTAGCAGGCTGAG-3'). qRT-PCR reaction conditions and cycling program were as described in Puthoff *et al.* (2003). A standard curve (four-fold dilution series; five total dilutions) was made using the RNA sample having the highest level of RHD1 or 18S expression. Cycle threshold and relative mRNA starting quantities for each unknown sample were calculated from this standard curve using iCycler software (Bio-Rad; Hercules, CA). RHD1 mRNA starting quantity values were normalized using 18S mRNA starting quantity values.

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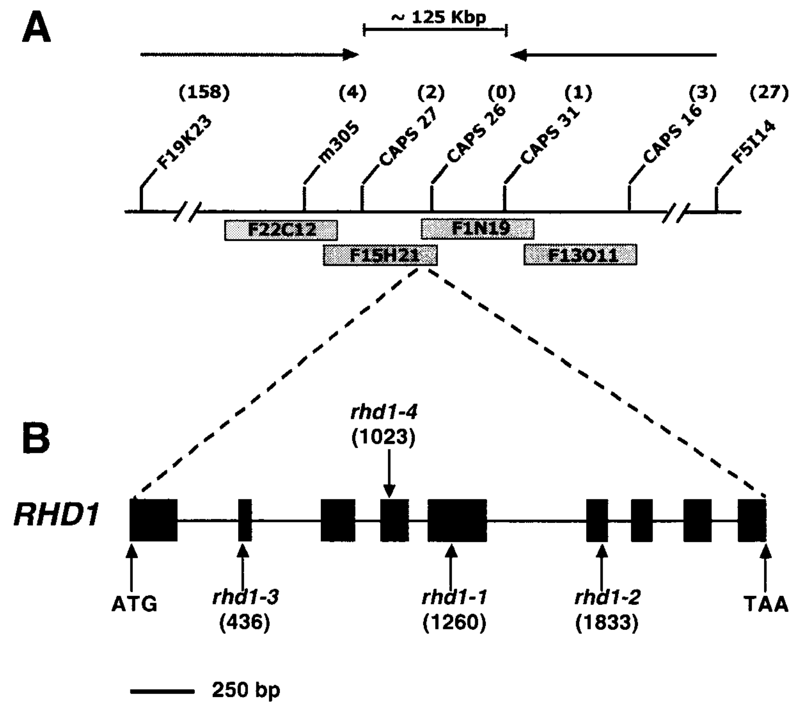

Figure 1. Positional cloning of *RHD1*. **(A)** Schematic representation of the bottom of chromosome 1 and corresponding *RHD1* locus. Arrows at top of figure indicate direction of chromosome walk from the F19K23 and F5I14 simple sequence length polymorphism markers. Approximate cleaved amplified polymorphic sequence (CAPS) marker positions are shown relative to chromosome 1 and the underlying bacterial artificial chromosome contig. Numbers of recombinant *rhd1-4* F2 homozygotes identified at each marker are designated above marker labels. **(B)** Schematic representation of the *RHD1* gene showing positions and sizes of exons (black boxes) and introns (intervening black lines). Start (ATG) and stop (TAA) codon positions are indicated by arrows. Positions of *rhd1* allele nucleotide mutations are indicated by arrows. Scale bar corresponds to 250 basepairs.

```
RHD1              (1) ----------------------MVGNILVTGGAGYIGSHTVLQLLLGGYNTVVIDNLDNS
UGE1              (1) ------------------MGSSVEQNILVTGGAGFIGTHTVVQLLKDGFKVSIIDNFDNS
M. catarrhalis    (1) MTNTGTTDMKNIDTSPSNSEHYLPKKILVTGGAGYIGSHTLIELIQAGFVPVVYDNLSNS
S. cerevisiae     (1) -------------MTAQLQSESTSKIVLVTGGAGYIGSHTVVELIENGYDCVVADNLSNS
H. sapiens        (1) ----------------------MAEKVLVTGGAGYIGSHTVLELLEAGYLPVVIDNFHNA

                                                                   *
RHD1             (39) S------LVSIQRVKDLAG-DHGQNLTVHQVDLRDKPALEKVFSETKFDAVMHFAGLKAV
UGE1             (43) V------IEAVDRVRELVGPDLSKKLDFNLGDLRNKGDIEKLFSKQRFDAVIHFAGLKAV
M. catarrhalis   (61) S------FVAVQRVEQIVG----KHIEFIQGDVLDKTHLDAVFKAHQFFAVVHFAGLKAV
S. cerevisiae    (48) T------YDSVARLEVLTK----HHIPFYEVDLCDRKGLEKVFKEYKIDSVIHFAGLKAV
H. sapiens       (39) FRGGGSLPESLRRVQELTG----RSVEFEEMDILDQGALQRLFKKYSFMAVIHFAGLKAV

                                                                 *
RHD1             (92) GESVAKPLLYYNNNLIATITLLEVMAAHGCKKLVFSSSATVYGWPKEVPCTEESPL----
UGE1             (97) GESVEKGRRYFDNNLVGTINLYETMAKYNCKMMVFSSSATVYGQPEKIPCMEDFEL----
M. catarrhalis  (111) GESTKNPLKYYQNNVVGTLSLLELMAKYGVKNCIFSSSATVYGSSNRLPITEDMPR----
S. cerevisiae    (98) GESTQIPLRYYHNNILGTVVLLELMQQYNVSKFVFSSSATVYGDATRFPNMIPIPEECPL
H. sapiens       (95) GESVQKPLDYYRVNLTGTIQLLEIMKAHGVKNLVFSSSATVYGNPQYLPLDEAHPTG---

                                                            *
RHD1            (148) SGMSPYGRTKLFIEDICRDVQRGDP-EWRIIMLRYFNPVGAHPSGRIGEDPCGTPNNLMP
UGE1            (153) KAMNPYGRTKLFLEEIARDIQKAEP-EWRIILLRYFNPVGAHESGSIGEDPKGIPNNLMP
M. catarrhalis  (167) FCTSPYGQSKLMVEHILEDLVNADD-TWNVVCLRYFNPIGAHESGRIGEDPTDIPNNLMP
S. cerevisiae   (158) GPTNPYGHTKYAIENILNDLYNSDKKSWKFAILRYFNPIGAHPSGLIGEDPLGIPNNLLP
H. sapiens      (152) GCTNPYGKSKFFIEEMIRDLCQADK-TWNVVLLRYFNPTGAHASGCIGEDPQGIPNNLMP

                                                                  *
RHD1            (207) YVQQVVVGRLPNLKIYGTDYTTKDGTGVRDYIHVVDLADGHICALQKLD-DT--EIGCEV
UGE1            (212) YIQQVAVGRLPELNVYGHDYPTEDGSAVRDYIHVMDLADGHIAALRKLFADP--KIGCTA
M. catarrhalis  (226) YISQVAVGNLKQLSVFGN?YETPDGTGV???IHVVDLAKGHVAALHYLVGQSV-GIGFCP
S. cerevisiae   (218) YMAQVAVGRREKLYIFGDDYDSRDGTPIRDYIHVVDLAKGHIAALQYLEAYNENEGLCRE
H. sapiens      (211) YVSQVAIGRREALNVFGNDYDTEDGTGVRDYIHVVDLAKGHIAALRKLKEQ----CGCRI

RHD1            (264) YNLGTGKGTTVLEMVDAFEKASGMKIPLVKVGRRPGDAETVYASTEKAERELNWKANFGI
UGE1            (270) YNLGTGQGTSVLEMVAAFEKASGKKIPIKLCPRRSGDATAVYASTEKAEKELGWKAKYGV
M. catarrhalis  (285) INLGTGQGTSVLQLIKAFESNTGQSVPYVITSRRTGDIAAYYASADKAKAILNWTAELDI
S. cerevisiae   (278) WNLGSGKGSTVFEVYHAFCKASGIDLPYKVTGRRAGDVLNLTAKPDRAKRELKWQTELQV
H. sapiens      (267) YNLGTGTGYSVLQMVQAMEKASGKKIPYKVVARREGDVAACYANPSLAQEELGWTAALGL

RHD1            (324) EEMCRDQWNWASNNPFGYGSSPNST-----------------------------------
UGE1            (330) DEMCRDQWKWAFNNPWGY--Q-NKL-----------------------------------
M. catarrhalis  (345) ERMCVDTWRWQSNNPKGYLS----------------------------------------
S. cerevisiae   (338) EDSCKDLWKWTTENPFGYQLRGVEARFSAEDMRYDARFVTIGAGTRFQATFANLGASIVD
H. sapiens      (327) DRMCEDLWRWQKQNPSGFGTQA--------------------------------------
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Figure 2. Alignment of the predicted RHD1 protein sequence with UDP-glucose-4-epimerase (UGE) proteins from diverse organisms. Shown are the *A. thaliana* RHD1/UGE4 (AAG51709) and UGE1 (Q42605) protein sequences aligned with UGEs from *Moraxella catarrhalis* (AAF91338), *Saccharomyces cerevisiae* (GAL 10; NP_009575), and *Homo sapiens* (NP_000394). Areas of identity are highlighted in black while blocks of similarity are gray. * denotes positions of amino acid changes resulting from the rhd1 allele mutations. Multiple protein sequence alignment was accomplished using CLUSTAL W (Thompson et al., 1994).

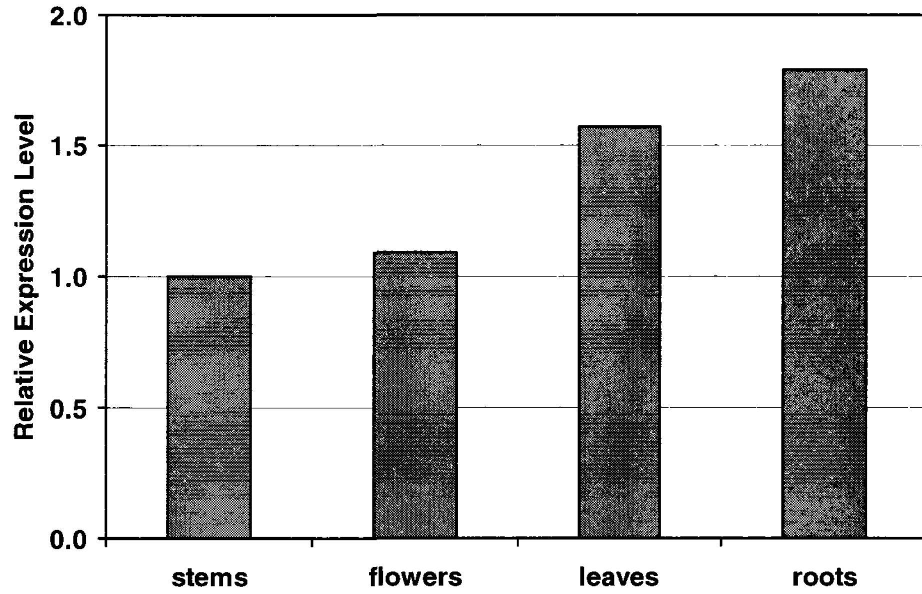

Figure 3. Relative expression of *RHD1* in wild-type Col-0 tissues as determined by quantitative real-time RT-PCR. Presented are the *RHD1* mean mRNA starting quantities in flowers, mature leaves, and roots relative to that of stems which is set equal to 1.0 for ease of presentation.

CHAPTER 5. Mutation of a UDP-glucose-4-epimerase alters nematode susceptibility and ethylene responses in *Arabidopsis* roots

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ABSTRACT

In *Arabidopsis*, mutation of RHD1, a UDP-glucose-4-epimerase, causes root-specific phenotypes, including hypersusceptibility to the cyst nematode *Heterodera schachtii*, increased root hair elongation, decreased root length, and root epidermal bulging. Previous experiments suggested that increased ethylene sensitivity or production mediated the *rhd1-4* phenotypes. In the present study, double mutant analyses revealed that only *rhd1-4* hypersusceptibility to *H. schachtii* and increased root hair elongation were dependent upon the ethylene signaling genes *EIN2* and *EIN3* but not upon ethylene signaling mediated by the auxin efflux carrier *EIR1*. In contrast, the *rhd1-4* short root and root epidermal bulging phenotypes did not require *EIN2*, *EIN3*, or *EIR1*. A time-course analysis of *RHD1* transcript levels in wild-type plants treated with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid showed a root-specific down-regulation of *RHD1* expression by ethylene. This observation was corroborated by our finding of increased *RHD1* transcript levels in roots of the ethylene-insensitive mutants *etr1* and *ein2*. In addition to ethylene, auxin is known to strongly influence *H. schachtii* susceptibility and root hair elongation. Therefore, we investigated the sensitivity of *rhd1-4* roots to indole-3-acetic acid (IAA). Equivalent IAA concentrations caused a greater reduction in *rhd1-4* root elongation compared to wild-type roots. Finally, *H. schachtii* parasitism was found to strongly down-regulate *RHD1* expression in the root three days after inoculation. We conclude that *RHD1* is a likely target of root-specific negative regulation by ethylene and that loss of RHD1 function results in a heightened sensitivity of root tissues to both ethylene and auxin.

INTRODUCTION

Cyst nematodes are obligate, sedentary, endoparasites that infect the roots of their hosts and cause significant yield reductions in agricultural crops. A unique characteristic of cyst nematode parasitism is the formation of a specialized feeding site, called a syncytium, within the host root (Endo, 1986). Syncytium formation is a complex process that likely involves the modulation of host signal transduction pathways by the nematode. In fact, numerous studies have demonstrated that dramatic changes in host gene expression occur within nematode-infected tissues or within nematode-induced feeding sites (reviewed by Gheysen and Fenoll, 2002; Hussey *et al.*, 2002; Puthoff *et al.*, 2003; Williamson and Gleason, 2003). Some of the effectors of nematode-induced changes in host cell physiology are believed to be esophageal gland secretions delivered, via the nematode's stylet, into or in direct proximity to the initial feeding cell (Davis *et al.*, 2000, 2004).

While many host signaling pathways are presumably involved in nematode-plant interactions, it has become increasingly clear that successful cyst nematode parasitism hinges on those pathways governing host responses to the phytohormones ethylene and auxin. In *Arabidopsis thaliana*, which is infected by the sugar beet cyst nematode *Heterodera schachtii*, the auxin-inducible *AtPGM* promoter was specifically up-regulated within cyst nematode-induced feeding sites (Mazarei *et al.*, 2003). In addition, experiments involving the *A. thaliana* auxin transport genes *AUX1* and *PIN2* (also called *EIR1*) suggest a local increase in auxin concentration occurs during feeding site induction and maintenance (Goverse *et al.*, 2000; Mazarei *et al.*, 2003).

Mutant and hormone inhibitor analyses have been invaluable in elucidating the pertinent ethylene and auxin signaling components required for cyst nematode parasitism. The *A. thaliana* ethylene-insensitive mutants *etr1*, *ein2*, and *ein3* were substantially less susceptible to *H. schachtii* than wild-type plants (Wubben *et al.*, 2001). Chemical inhibition of ethylene synthesis in wild-type *A. thaliana* plants by AVG (1-aminoethoxyvinylglycine) caused decreased susceptibility to *H. schachtii* (Wubben *et al.*, 2001). Auxin-insensitivity, as manifested in the *A. thaliana* mutants *axr2* and *eir1*, also results in decreased cyst nematode susceptibility (Goverse *et al.*, 2000; Wubben *et al.*, 2001). In contrast, *A. thaliana* mutants that overproduce ethylene (*eto*) were consistently more susceptible to *H. schachtii* (Goverse *et al.*, 2000; Wubben *et al.*, 2001). Syncytia formed within *eto* mutant roots were more

extensive than syncytia within wild-type roots, and larger female nematodes developed on *eto* roots compared to wild-type roots (Goverse *et al.*, 2000). Finally, treatment of wild-type *A. thaliana* plants with the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) caused increased *H. schachtii* susceptibility compared to untreated plants (Wubben *et al.*, 2001).

We previously identified an allele of the *A. thaliana* mutant *rhd1*, i.e., *rhd1-4*, during a screen for mutants that exhibit altered susceptibility to *H. schachtii* (Baum *et al.*, 2000). *rhd1* mutant alleles exhibit hypersusceptibility to *H. schachtii* in addition to increased root hair elongation and decreased root length (Wubben *et al.*, 2001), as well as root epidermal bulging (Baskin *et al.*, 1992; Schiefelbein and Somerville, 1990). The *rhd1* mutant phenotypes, with the exception of root epidermal bulging, were consistent with the effects of either increased ethylene production, sensitivity, or signaling on root development and *H. schachtii* susceptibility in *A. thaliana* (Eliasson and Bollmark, 1988; Pitts *et al.*, 1998; Tanimoto *et al.*, 1995; Wubben *et al.*, 2001). *rhd1-4* seedlings treated with the ethylene synthesis inhibitor AVG exhibited wild-type levels of *H. schachtii* susceptibility, root hair elongation, and root epidermal morphology (Wubben *et al.*, 2001). In contrast, the ethylene precursor ACC caused the *rhd1-4* increased root hair length, decreased root growth, and *H. schachtii* hypersusceptibility phenotypes to appear in wild-type plants. These data suggested that a root-specific alteration in ethylene production or sensitivity mediated the *rhd1-4* phenotypes (Wubben *et al.*, 2001). Although root hair development and *H. schachtii* susceptibility exhibited a tight positive correlation, analyses of epidermal cell fate mutants, which show ectopic root hair development in the absence of altered ethylene signaling, confirmed that root hair development by itself does not influence *H. schachtii* susceptibility (Wubben *et al.*, 2001).

RHD1 encodes a member of the UDP-glucose-4-epimerase (UGE) gene family in *A. thaliana* (Seifert *et al.*, 2002). UGEs are responsible for UDP-galactose synthesis from UDP-glucose in plant cells. Consequently, treatment of *rhd1* mutants with exogenous galactose resulted in the suppression of the root epidermal bulging phenotype (Seifert *et al.*, 2002). It has recently been shown that exogenous ethylene can also suppress this phenotype in *rhd1* mutants (Seifert *et al.*, 2004). In contrast to our findings, analyses of *rhd1* mutants, including

the *rhd1-4* allele, conducted by other laboratories did not reveal a discernable increased root hair length phenotype compared to wild-type (Baskin *et al.*, 1992; Schiefelbein and Somerville, 1990; Seifert *et al.*, 2004). This lack of consensus is puzzling because increased root hair elongation is consistently observed in our experiments with *rhd1-4* and other *rhd1* alleles.

In the present study, we addressed these discrepancies in the reported root hair length phenotype of *rhd1* by investigating *rhd1* root hair elongation in response to different plant nutrient media. In addition, we further explored the relationship between *RHD1* and ethylene by implementing double mutant, gene expression, and hormone sensitivity analyses. Our results indicate that *RHD1* is a target of negative regulation by ethylene and that loss of *RHD1* function causes an increase in root sensitivity to ethylene and auxin. This increase in root sensitivity to ethylene likely mediates the observed increased root hair length and *H. schachtii* hypersusceptibility phenotypes exhibited by *rhd1* mutants which are manifested to a greater extant on specific plant nutrient media.

RESULTS

Effect of MS and Knops nutrient media on *rhd1-4* and wild-type root hair elongation

Knops nutrient medium provides the most consistent and reliable results with respect to *H. schachtii* susceptibility analyses of wild-type and mutant *A. thaliana* genotypes when compared to a wide variety of media, including Murashige-Skoog (MS; Murashige and Skoog, 1962) medium (Baum *et al.*, 2000; Sijmons *et al.*, 1991). *rhd1-4* was identified as being hypersusceptible to *H. schachtii*, therefore, additional experiments involving *rhd1-4* were conducted on Knops medium. This circumstance resulted in the discovery of an *rhd1-4* increased root hair length phenotype (Wubben *et al.*, 2001). However, this phenotype has not been corroborated by other laboratories which predominantly utilized MS nutrient medium for their analyses of *rhd1* mutants (Schiefelbein and Somerville, 1990; Seifert *et al.*, 2004). In order to reconcile these observations, we investigated the effect of MS medium on *rhd1-4* and wild-type Columbia-0 root hair elongation in comparison to Knops medium.

MS and Knops media share many nutritional components, however, the majority of nutrients are more concentrated in MS medium. A comparison of anion and cation

concentrations between MS and Knops media is presented in **Table 1**. *rhd1-4* and wild-type seedlings grown on Knops medium developed elongated root hairs with *rhd1-4* showing an increased root hair length phenotype compared to wild-type, as previously described (**Figure 1**) (Wubben *et al.*, 2001). Compared to Knops medium, MS medium exerted an inhibitory effect on root hair elongation in both *rhd1-4* and wild-type. However, elongated root hairs were occasionally observed on *rhd1-4* roots grown on MS medium, while this was not the case for wild-type roots (data not shown). MS medium also increased the severity of the *rhd1-4* root epidermal bulging phenotype compared to Knops medium. We conclude that while Knops medium appears to promote root hair elongation, this effect is not restricted to *rhd1-4* because wild-type seedlings also exhibit a dramatic increase in root hair lengths when grown on Knops medium compared to MS. However, *rhd1-4* appears to be more sensitive than wild-type to the stimulatory effect of Knops medium on root hair elongation.

Genetic interactions between *RHD1* and ethylene signal transduction genes

Inhibition of ethylene synthesis, via AVG, suppressed the *rhd1-4* increased root hair length, root epidermal bulging, and *H. schachtii* hypersusceptibility phenotypes, suggesting that these phenotypes were mediated by either increased ethylene production or sensitivity (Wubben *et al.*, 2001). Unfortunately, the biological effect of AVG-treatment is not limited to ethylene synthesis inhibition (Abel, 1985). In order to confidently determine i) the relevance of ethylene signal transduction in general and ii) the specific ethylene signaling genes, if any, involved in mediating the *rhd1-4* mutant phenotypes, we constructed double mutants between *rhd1-4* and known ethylene signal transduction mutants. For these experiments, *rhd1-4* was used because the *rhd1-4* allele contains a nonsense mutation which would result in a severely truncated protein and, therefore, *rhd1-4* can be considered a null allele (Seifert *et al.*, 2002).

ein2-1 mutant seedlings are completely insensitive to ethylene (Guzman and Ecker, 1990; Roman *et al.*, 1995) and exhibit root phenotypes contrary to those present in *rhd1-4*, including decreased *H. schachtii* susceptibility and decreased root hair elongation (Alonso *et al.*, 1999; Pitts *et al.*, 1998; Wubben *et al.*, 2001). EIN2 is an integral membrane protein whose hydrophobic portion shares a modest level of similarity with the Nramp family of

divalent cation transport proteins, however, a comparable function for EIN2 in *Arabidopsis* has not been demonstrated (Alonso *et al.*, 1999; reviewed by Hirayama and Alonso, 2000). Genetic epistasis experiments have shown that *EIN2* acts downstream of the *CTR1* Raf-like kinase and upstream of the nuclear-localized transcriptional activator *EIN3* in the ethylene signaling pathway (Roman *et al.*, 1995; Chao *et al.*, 1997; reviewed in Guo and Ecker, 2004).

EIN3 belongs to a family of transcriptional activators, the other members of which have been termed *EIN3*-like (*EIL*) (Chao *et al.*, 1997; Solano *et al.*, 1998). *ein3* mutants exhibit ethylene-insensitivity (Guzman and Ecker, 1990), however, loss of EIN3 function does not completely block ethylene signaling because *ein3* plants retain a low level of ethylene responsiveness (Roman *et al.*, 1995). This residual ethylene responsiveness in *ein3* is due to the presence of various EIL proteins which may serve redundant functions for some aspects of ethylene signaling (Chao *et al.*, 1997; Solano *et al.*, 1998). Despite this fact, *ein3-1* seedlings exhibit decreased *H. schachtii* susceptibility and decreased root hair elongation compared to wild-type (Pitts *et al.*, 1998; Wubben *et al.*, 2001).

The *eir1-1* mutant exhibits root-specific ethylene-insensitivity (Roman *et al.*, 1995). This mutant is less susceptible to *H. schachtii* and exhibits decreased root hair elongation and increased root length compared to wild-type (Luschnig *et al.*, 1998; Wubben *et al.*, 2001). Molecular analyses revealed EIR1 likely functions as an auxin efflux carrier required for basipetal polar auxin transport in the root (Luschnig *et al.*, 1998; Müller *et al.*, 1998). Therefore, *eir1* root ethylene-insensitivity is likely due to low levels of intracellular auxin in root cells because of defective polar auxin transport (Luschnig *et al.*, 1998; Müller *et al.*, 1998; Rahman *et al.*, 2001). Double mutant plants homozygous for *rhd1-4* and *ein2-1* (*rhd1-4 ein2-1*), *rhd1-4* and *ein3-1* (*rhd1-4 ein3-1*), and *rhd1-4* and *eir1-1* (*rhd1-4 eir1-1*) were constructed and assessed as follows:

<u>*H. schachtii* susceptibility</u>: In these experiments, *rhd1-4* was hypersusceptible to *H. schachtii* while *ein2-1*, *ein3-1*, and *eir1-1* were less susceptible than wild-type, confirming our initial analyses of these mutants (Figure 2a, b, and c, respectively) (Baum *et al.*, 2000; Wubben *et al.*, 2001). *rhd1-4 ein2-1* plants were consistently less susceptible to *H. schachtii*, i.e., they showed *ein2-1* susceptibility (Figure 2a). The susceptibility of *rhd1-4 ein3-1* to *H. schachtii* was intermediate between the respective single mutants (Figure 2b). In contrast,

rhd1-4 eir1-1 plants were comparable to *rhd1-4* in their susceptibility to *H. schachtii* (Figure 2c). These observations indicate that *rhd1-4* hypersusceptibility to *H. schachtii* is dependent upon ethylene signaling mediated by *EIN2*. The observation that *ein3-1* was not able to completely suppress the effects of the *rhd1-4* mutation on *H. schachtii* susceptibility in the *rhd1-4 ein3-1* double mutant likely reflects the inability of the *ein3-1* mutation to completely abolish ethylene signal transduction. Ethylene signaling mediated by polar auxin transport as determined by *EIR1* appears to be dispensable for *rhd1-4* hypersusceptibility to *H. schachtii.*

Root hair elongation and root epidermal bulging: The single mutants *ein2-1*, *ein3-1*, and *eir1-1* developed shorter root hairs than wild-type while *rhd1-4* exhibited increased root hair length (Figure 3). Root hair elongation in *rhd1-4 ein2-1* seedlings was severely reduced, resembling the *ein2-1* mutant, while *rhd1-4 eir1-1* seedlings produced root hairs similar in length to those observed on *rhd1-4* roots. Although root hair elongation was dramatically increased in *rhd1-4 ein3-1* seedlings compared to *ein3-1*, this increase was not equal to *rhd1-4* alone. These data indicate that increased root hair elongation in *rhd1-4* requires *EIN2* but not *EIR1*. Again, the intermediate phenotype exhibited by *rhd1-4 ein3-1* is most likely due to the incomplete ethylene-insensitivity of *ein3-1*.

The *rhd1-4* root epidermal bulging phenotype was present in all three double mutants tested (*rhd1-4 ein2-1, rhd1-4 ein3-1,* and *rhd1-4 eir1-1*) (Figure 3). This result indicates that root epidermal bulging in *rhd1-4* does not require ethylene signal transduction as defined by *EIN2*, *EIN3*, or *EIR1*.

Root elongation: As in previous experiments (Wubben *et al.*, 2001), *rhd1-4* showed decreased root elongation compared to wild-type Columbia (Col-0) (Figure 4). All three double mutants tested here (*rhd1-4 ein2-1, rhd1-4 ein3-1,* and *rhd1-4 eir1-1*) showed the *rhd1-4* short root phenotype (Figure 4a, b, and c, respectively). These results indicate that the *rhd1-4* short root phenotype is not mediated by ethylene.

Ethylene negatively regulates *RHD1* expression in roots

Our finding that ethylene is required for the *rhd1-4 H. schachtii* hypersusceptibility and increased root hair length phenotypes prompted us to explore whether ethylene itself regulates *RHD1* expression. Two-week-old wild-type Col-0 seedlings were treated with the

ethylene precursor ACC or a control solution. Shoot and root tissues were harvested from these seedlings at 24 and 48 hours after treatment. Total RNA from these tissues was converted to single-strand cDNA, which functioned as template for quantitative real-time polymerase chain reaction (qRT-PCR) using *RHD1*-specific gene primers.

Wild-type roots showed significantly reduced *RHD1* transcript levels at both 24 and 48 hours after ACC-treatment compared to control roots (Figure 5). In contrast, ACC-treatment exerted little influence on shoot *RHD1* transcript levels at either time-point (Figure 6), indicating the repressive effect of ACC on *RHD1* expression is restricted to the root.

Because ACC treatment resulted in decreased *RHD1* transcript abundance in roots, we investigated steady-state levels of *RHD1* transcript in shoots and roots of ethylene-insensitive mutants compared to wild-type. Shoot and root tissues of two-week-old wild-type Col-0, *etr1*, *ein2*, and *ein3* plants were collected and analyzed as described above. This experiment revealed significantly higher *RHD1* transcript levels in roots of *etr1* and *ein2*, but not *ein3*, compared to wild-type roots (Figure 6). Interestingly, the effects of the *etr1* and *ein2* mutations on *RHD1* expression were restricted to the root because no significant alteration in *RHD1* transcript levels was detected in shoot tissues of these mutants (Figure 6). As stated previously, *ein3* mutants retain a low level of ethylene responsiveness (Roman *et al.*, 1995), which may explain the lack of change in *RHD1* mRNA abundance in *ein3* roots.

rhd1-4 **roots are more sensitive to exogenous IAA**

The complete *EIN2*-dependency and partial *EIN3*-dependency of the *rhd1-4* increased root hair length and *H. schachtii* hypersusceptibility phenotypes, in conjunction with our finding that *RHD1* expression is negatively regulated by ethylene, strongly suggest that *RHD1* is a target of that branch of the ethylene signaling pathway which governs root hair elongation and *H. schachtii* susceptibility. Given the function of RHD1 as a UDP-glucose epimerase (Seifert *et al.*, 2002), increased root sensitivity to ethylene, as opposed to ethylene-overproduction, is the most likely result of a loss of RHD1 function.

In addition to ethylene, auxin has been shown to positively regulate root hair elongation and *H. schachtii* susceptibility in *Arabidopsis* (Goverse *et al.*, 2000; Masucci and Schiefelbein, 1996; Pitts *et al.*, 1998; Wubben *et al.*, 2001). One way in which interactions

between ethylene and auxin signal transduction have been demonstrated is by the identification of auxin-resistant mutants which also show reduced sensitivity to ethylene (reveiwed by Stepanova and Ecker, 2000). For these reasons, loss of RHD1 function may affect root sensitivity to auxin in addition to ethylene.

Exogenous application of the auxin IAA (indole-3-acetic acid) inhibits root elongation in *A. thaliana* (King *et al.*, 1995; Luschnig *et al.*, 1998). Consequently, altered auxin sensitivity in *rhd1-4* can be detected by comparing proportional mutant root elongation, in response to a range of IAA concentrations, to proportional wild-type root elongation. Results of this experiment are shown in **Figure 7**. Treatment of wild-type and *rhd1-4* seedlings with 0.3 nM IAA did not cause a significant reduction in root elongation for either genotype. However, higher IAA concentrations revealed dramatic differences in root IAA sensitivity between wild-type and *rhd1-4*. Treatment of wild-type seedlings with 0.6 nM IAA caused a slight stimulation in root growth, an effect of low IAA concentrations that has been reported (Mulkey *et al.*, 1982). *rhd1-4* plants, however, showed a significant reduction in root elongation at this IAA concentration. *rhd1-4* seedlings continued to exhibit increased root growth inhibition compared to wild-type over the course of continually higher concentrations of IAA, ranging from 1.3 to 10 nM. These data show that *rhd1-4* is more sensitive than wild-type to the inhibitory effect of IAA on root elongation.

Effect of cyst nematode infection on *RHD1* expression

Loss of *RHD1* function results in *H. schachtii* hypersusceptibility, therefore, we may expect that *RHD1* mRNA would be a target for down-regulation by the nematode during the infection process. To address this issue, we isolated total RNA from roots and shoots of wild-type seedlings three days after mock-inoculation or inoculation with *H. schachtii*. *RHD1* transcript levels were determined by qRT-PCR as described. As hypothesized, *RHD1* expression was strongly down-regulated in *H. schachtii*-infected roots when compared to the uninfected control, however, no change in *RHD1* transcript levels was detected in shoots from infected plants (**Figure 8**). This result is consistent with the observation that loss of *RHD1* function, as in *rhd1-4*, causes *H. schachtii* hypersusceptibility. It is also interesting that *H.*

schachtii-induced down-regulation of *RHD1* expression is root-specific as is the case for the down-regulation of *RHD1* by ethylene.

DISCUSSION

The hormone ethylene regulates a multitude of developmental and stress response signaling events in plants. *A. thaliana* mutants that exhibit aberrant ethylene production, perception, or downstream signal transduction have been indispensable towards unraveling the complex network that comprises the ethylene signal transduction pathway (reviewed by Guo and Ecker, 2004). Here, we present evidence based on double mutant, gene expression, and hormone sensitivity analyses which indicates that *RHD1* is a target of root-specific negative regulation by ethylene and that loss of RHD1 function causes increased root sensitivity to ethylene and auxin. Furthermore, we resolve apparent discrepancies regarding the *rhd1* root hair elongation phenotype that have been reported.

rhd1-4 was identified from a screen for mutants that exhibit altered susceptibility to the sugar beet cyst nematode, *Heterodera schachtii*. For the purpose of this screen, Knops nutrient medium was used to maximize *H. schachtii* infection (Baum *et al.*, 2000; Sijmons *et al.*, 1991). These experiments also revealed that *rhd1* mutants developed longer root hairs than wild-type, a phenotype that has not been corroborated by other laboratories (Baskin *et al.*, 1992; Schiefelbein and Somerville, 1990; Seifert *et al.*, 2002, 2004). We were initially puzzled by these contradictory observations until we noticed that studies in other laboratories used different nutrient media, predominantly MS nutrient medium (Murashige and Skoog, 1962), for *rhd1* characterization. In light of this information, we investigated whether the *rhd1* increased root hair length phenotype was conditional upon the nutrient medium used.

We found that *rhd1-4* and wild-type Col-0 root hair elongation was severely reduced when MS medium was used compared to Knops medium. In fact, this effect of MS medium was so dramatic that *rhd1-4* exhibited no discernable change in root hair length compared to wild-type. Knops medium prompted root hair elongation in both *rhd1-4* and wild-type relative to MS, with *rhd1-4* showing an increased root hair length phenotype compared to wild-type, as was previously described (Wubben *et al.*, 2001). In addition, root epidermal bulging was more severe in *rhd1-4* plants grown on MS relative to Knops media. The

positive effect of Knops medium on root hair elongation is non-specific because both wild-type and *rhd1-4* were affected. One possible explanation for this phenomenon involves the effect of iron nutrition on root hair development.

In *Arabidopsis*, iron-deficiency induces root hair initiation and elongation in an ethylene-dependent manner (Moog *et al.*, 1995; Schmidt and Schikora, 2001). While both MS and Knops media are iron-supplemented, MS medium utilizes Fe^{2+}, a form of iron readily imported by plant roots. In contrast, Knops medium contains Fe^{3+} which needs to be reduced to Fe^{2+} by the plant before uptake can occur (reviewed by Curie and Briat, 2003). While Knops medium is not iron-deficient as defined by the presence or absence of iron, it may be considered as such in comparison to MS medium which contains a more bioavailable form of iron at a higher concentration. It is tempting to speculate that the ethylene-dependent induction of root hair elongation by iron-deficiency may intimately involve EIN2. The similarity of the N-terminal hydrophobic domain of EIN2 with the Nramp family of metal ion transporters led to the hypothesis that a divalent metal cation may act to regulate EIN2 and ethylene signaling (Alonso *et al.*, 1999). However, attempts to demonstrate either metal ion binding or transport for EIN2 have been unsuccessful (reviewed by Hirayama and Alonso, 2000).

Previous experiments using the ethylene synthesis inhibitor AVG suggested that *rhd1-4* hypersusceptibility to *H. schachtii*, increased root hair length, and root epidermal bulging phenotypes were ethylene-dependent (Wubben *et al.*, 2001). To verify the ethylene-dependence of these phenotypes, we introduced the *rhd1-4* allele into different ethylene-insensitive genotypes. *EIN2* is genetically positioned between the ethylene receptor *ETR1* and the transcriptional activator *EIN3* (Chao *et al.*, 1997; Roman *et al.*, 1995; reviewed in Guo and Ecker). EIN2 plays a critical role in ethylene signal transduction because loss of EIN2 function causes complete ethylene-insensitivity (Guzman and Ecker, 1990). *ein2-1* mutant plants exhibit phenotypes in direct opposition to *rhd1-4*, namely, decreased susceptibility to *H. schachtii* and dramatically shortened root hairs compared to wild-type (Rahman *et al.*, 2002; Wubben *et al.*, 2001). Analyses of *rhd1-4 ein2-1* double mutants revealed that *ein2-1* is epistatic to *rhd1-4* with regard to *H. schachtii* susceptibility and root hair length. These results indicate that *rhd1-4* hypersusceptibility to *H. schachtii* and

increased root hair elongation requires *EIN2*-mediated ethylene signal transduction, supporting our hypothesis that these *rhd1-4* phenotypes are mediated by increased ethylene sensitivity. The *rhd1-4* root epidermal bulging phenotype, which could be suppressed by AVG treatment (Wubben *et al.*, 2001), was retained in *rhd1-4 ein2-1*, indicating this phenotype is actually ethylene-independent. Ethylene-independence was also observed for the *rhd1-4* short root phenotype.

Double mutant plants homozygous for *rhd1-4* and *ein3-1* showed *H. schachtii* susceptibility and root hair elongation phenotypes intermediate between the respective single mutants, suggesting a partial EIN3-dependence of these phenotypes in *rhd1-4*. Loss of EIN3 function does not completely abolish ethylene signaling (Roman *et al.*, 1995), presumably because *EIN3* belongs to the *EIL* (EIN3-like) family of transcription factors (Chao *et al.*, 1997; Solano *et al.*, 1998). Over-expression of *EIL1* or *EIL2* can rescue *ein3* ethylene-insensitivity. In addition, over-expression of either *EIN3* or *EIL1* in a wild-type background results in a constitutive ethylene signaling phenotype, similar to that of the *ctr1* mutant (Chao *et al.*, 1997). Finally, *eil1* mutants show an ethylene-insensitive phenotype roughly equal to that of *ein3*, i.e., less severe than *ein2-1*, however, *ein3 eil1* double mutants are completely insensitive to ethylene and are able to fully suppress the *ctr1* constitutive ethylene response phenotype (Alonso *et al.*, 2003). Therefore, it is feasible that root hair elongation and *H. schachtii* susceptibility in *A. thaliana* are similarly regulated in a cooperative fashion by EIN3 and other EIL proteins. Therefore, complete suppression of the increased root hair length and *H. schachtii* hypersusceptibility phenotypes in *rhd1-4* may require loss-of-function mutations in not only *EIN3*, but also in *EIL1* and possibly other *EIL* genes. Although *rhd1-4* is most likely a null allele (Seifert *et al.*, 2002), the interaction between RHD1 and EIN3 may be further clarified by analyzing double mutants made between *ein3-1* and other *rhd1* alleles. The *rhd1-4* root epidermal cell bulging and decreased root elongation phenotypes were unaffected by the *ein3-1* mutation, supporting our conclusion that these phenotypes are ethylene-independent.

eir1 mutant roots are moderately insensitive to exogenous ethylene (Luschnig *et al.*, 1998; Roman *et al.*, 1995). *EIR1* encodes a protein that is similar to bacterial membrane transporters and is believed to mediate auxin efflux in the root. Therefore, *eir1* ethylene-

insensitivity is likely due to low levels of intracellular auxin in root cells because of defective polar auxin transport (Luschnig *et al.*, 1998; Müller *et al.*, 1998; Rahman *et al.*, 2001). *eir1-1* plants display decreased susceptibility to *H. schachtii* and greatly shortened root hairs compared to wild-type (Wubben *et al.*, 2001). We found that *rhd1-4 eir1-1* double mutant plants retained all *rhd1-4* associated phenotypes. These results indicate the *rhd1-4* phenotypes do not require *EIR1*. This result was surprising because auxin has been shown to play a pivotal role in both root hair elongation (Masucci and Schiefelbein, 1996; Pitts *et al.*, 1998) and *H. schachtii* susceptibility (Goverse *et al.*, 2000; Wubben *et al.*, 2001). These results support our hypothesis that ethylene is the primary mediator of *rhd1-4* hypersusceptibility to *H. schachtii* and increased root hair elongation. However, introduction of *rhd1* into additional auxin-resistant genetic backgrounds would need to be performed to verify the possible role of auxin in regulating the *rhd1* mutant phenotypes.

Experiments using qRT-PCR revealed that ethylene exerts a negative effect on *RHD1* expression, specifically in the root. We observed that ethylene treatment, in the form of ACC, caused an approximate 2.5-fold reduction in *RHD1* mRNA expression in wild-type roots. To the contrary, *RHD1* transcript levels were increased in roots of the ethylene-insensitive mutants *etr1* and *ein2*. The root-specificity of these ethylene effects on *RHD1* expression correlates with the root-specific nature of the *rhd1* mutant phenotypes. These expression data also parallel the fact that ACC-treatment of wild-type plants causes both *H. schachtii* hypersusceptibility and increased root hair elongation, i.e., *rhd1-4* phenotypes, and that the *etr1* and *ein2* mutants are less susceptible to *H. schachtii* and exhibit shorter root hairs than wild-type (Pitts *et al.*, 1998; Tanimoto *et al.*, 1995; Wubben *et al.*, 2001). Finally, these expression data support the idea that *RHD1* is a target of root-specific negative regulation by ethylene and that loss of RHD1 function would result in an increased sensitivity of the root to ethylene.

The observation that the effects of *rhd1-4* on root hair elongation and *H. schachtii* susceptibility could be completely suppressed by *ein2-1* and partially so by *ein3-1* strongly suggest a causal role for ethylene in mediating the *rhd1-4* increased root hair length and *H. schachtii* hypersusceptibility phenotypes. However, root hair elongation and *H. schachtii* susceptibility are modulated by auxin as well as ethylene. Examination of *rhd1-4* root

sensitivity to exogenous indole-3-acetic acid (IAA) revealed that *rhd1-4* seedlings are more sensitive to the inhibiting effects of IAA on root elongation compared to wild-type. Therefore, it appears that loss of RHD1 function causes an increased sensitivity to both ethylene and auxin. The fact that the auxin efflux carrier mutant *eir1-1* was not able to suppress the *rhd1-4* increased root hair length and *H. schachtii* hypersusceptibility phenotypes suggests that auxin does not play a direct role in mediating these phenotypes in *rhd1-4* plants, even though *rhd1-4* shows an altered sensitivity to auxin.

Another link between *RHD1* and ethylene has recently been reported by Seifert *et al.* (2004). These authors determined that ethylene, applied exogenously or via introduction of the *ctr1* mutation, could suppress the root epidermal bulging phenotype exhibited by *rhd1* mutants. The authors postulated that the malformed cell walls which develop in *rhd1* roots may initiate an ethylene-mediated stress response whose function is to suppress the effects of the *rhd1* mutation on root epidermal bulging (Seifert *et al.*, 2004). There is additional evidence showing a relationship between cell wall composition and ethylene signal transduction. Mutation of *cev1*, which corresponds to the cellulose synthase CeSa3, causes constitutive ethylene production and decreased root cellulose content (Ellis *et al.*, 2002). In addition, it was shown that mutation of an A. thaliana chitinase-like gene induced ethylene overproduction and altered cell wall composition (Zhong *et al.*, 2002). These observations parallel our hypothesis that *rhd1-4* seedlings, which develop malformed root cell walls, show a heightened root-specific sensitivity to ethylene.

We hypothesize the *rhd1-4 H. schachtii* hypersusceptibility and increased root hair length phenotypes we observe are directly caused by increased ethylene sensitivity in *rhd1-4* roots due to aberrations in cell wall structure and nucleotide sugar flux. Furthermore, we hypothesize the morphological effects of this increased root ethylene sensitivity, i.e., increased root hair length, are more readily observed on Knops rather than MS media. If i) Knops medium promotes ethylene-mediated root hair elongation and ii) ethylene suppresses *rhd1* root epidermal bulging, then it is not surprising that we observe root epidermal bulging to be less severe in *rhd1-4* plants grown on Knops medium compared to MS. Increased ethylene sensitivity in *rhd1-4* roots renders the mutant more sensitive to this characteristic of Knops medium than wild-type, resulting in increased root hair elongation and *H. schachtii*

hypersusceptibility. The fact that we found these two phenotypes to be ethylene-dependent agrees with this assertion. The ethylene-independence of the *rhd1-4* root epidermal cell bulging and shortened root phenotypes indicates that these phenotypes are the direct result of a loss of *RHD1* function.

We also determined that *H. schachtii* infection causes a root-specific down-regulation in *RHD1* transcript levels three days after infection. ACC-treatment also causes a root-specific down-regulation of *RHD1* expression in wild-type, therefore, it is possible that *H. schachtii*-mediated down-regulation of *RHD1* expression occurs via an ethylene signal elicited in the host root by the nematode during infection. In support of this idea, the induction of ethylene-regulated genes has been observed at this time-point during *H. schachtii* infection. The plant defensin *PDF1.2* is induced by ethylene (Penninckx *et al.*, 1996) and shows a dramatic up-regulation in *H. schachtii*-infected roots (Wubben *et al.* unpublished data).

Our results presented here further highlight the relationship between *RHD1* and ethylene signal transduction. We have provided evidence which points to a role for *RHD1* as a target of ethylene signaling with respect to the processes of root hair elongation and modulation of *H. schachtii* susceptibility. Furthermore, it appears that the nutrient medium optimized for cyst nematode infection highlights the altered sensitivity of *rhd1-4* to ethylene which would not be apparent if alternative plant growth media were used.

EXPERIMENTAL PROCEDURES

Plant Material and Nematode Inoculations

All *Arabidopsis thaliana* plants (Columbia-0 ecotype) were grown from surface-sterilized seeds planted on Knops nutrient medium (1.0% sucrose; pH 6.4) solidified with 0.8% Daishan agar (Brunschwig Chemie; Amsterdam, Netherlands) (Baum *et al.*, 2000; Sijmons *et al.* 1991) in 9-cm-diameter petri dishes, 10-cm-square petri dishes (Fisher Brand; Fisher Scientific, Pittsburgh, PA), or 12-well plates (Falcon Brand; Becton Dickinson, Lincoln Park, N. J.) at 25°C under 16 hours light / 8 hours darkness unless noted otherwise. For experiments requiring MS media (Murashige and Skoog, 1962), MS Salt Mixture (Gibco; Grand Island, NY) was adjusted to pH 5.7 (1.0% sucrose; 0.8% granulated agar (Fisher; Fair

Lawn, NJ)). Nematode inoculations were performed as previously described (Wubben *et al.*, 2001). Least square means and corresponding standard errors were calculated to account for unequal replications within each inoculation experiment. *ein2-1*, *ein3*-1, and *eir1-1* mutant lines were obtained from the Arabidopsis Biological Resource Center at The Ohio State University.

Double mutants

rhd1-4 ein2-1 and rhd1-4 eir1-1: *rhd1-4* was used as a female in crosses to *ein2-1* and *eir1-1*. F_1 seeds were germinated on Knops medium to observe F_1 root morphology. Presentation of wild-type root morphology in the F_1 indicated a successful cross since the respective genes belong to different complementation groups. F_1 seedlings were transplanted to soil and allowed to self-fertilize. The ethylene-insensitive phenotypes of *ein2-1* and *eir1-1* were used to identify *RHD1_ / ein2-1 ein2-1* and *RHD1_ / eir1-1 eir1-1* F_2 classes by germinating ~ 20 F_2 seeds from each cross on Knops medium containing 0.5 µM 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma, St. Louis, MO). Seedlings insensitive to ethylene were transplanted to soil and allowed to self-fertilize. F_3 progeny were collected and planted on Knops medium in petri plates. Plants showing root hair cell bulging were observed in segregating F_3 classes from both crosses. Such plants were transplanted to soil and maintained as double mutants.

rhd1-4 ein3-1: *rhd1-4* was used as a female in crosses to *ein3-1*. F_1 hybrid plants were identified as described above and self-fertilized. Plants homozygous for *rhd1-4* and *ein3-1* were identified from a segregating F_2 population by using allele-specific CAPS (Cleaved Amplified Poloymorphic Sequence; Konieczny and Ausubel, 1993) markers that could differentiate between the *RHD1* and *rhd1-4* alleles and the *EIN3* and *ein3-1* alleles. Genomic DNA was isolated from ~ 20 individuals and used as template for CAPS marker PCR (polymerase chain reaction) amplification and restriction analyses. RHD1**:** forward (5'-TCCAGAGATGTGATTTAGTGCTC-3'), reverse (5'-TAGCCTCCCAACAACGACTT-3'), product size = 646 bp, PCR product is polymorphic for the MscI restriction enzyme which does not digest the *rhd1-4* allele product but does digest the *RHD1* allele product. EIN3**:** forward (5'-AGGCAGTCTCAAGAGCAAGC-3'), reverse (5'-

CATTCATCAGAAGCGAGCAA-3'), product size = 727 bp, PCR product is polymorphic for the BstEII restriction enzyme which does not digest the *EIN3* allele product but does digest the *ein3-1* allele product. Annealing temperatures for *RHD1* and *EIN3* CAPS marker primer pairs are 55 °C. F_2 individuals showing homozygous *rhd1-4* and *ein3-1* restriction patterns were allowed to self-fertilize to generate F_3 seed which was used for subsequent experiments.

Root length measurements

Seeds of wild-type Col-0, *rhd1-4*, *ein2-1*, *eir1-1*, *ein3-1*, *rhd1-4 ein2-1*, *rhd1-4 ein3-1*, and *rhd1-4 eir1-1* were surface-sterilized and grown on square petri plates containing Knops medium. One seed of each genotype was planted per plate, the position of which was randomly assigned. Plates were tilted near vertical and root lengths (i.e., distance from the crown to the tip of the main root) were determined 10 days after planting using a reticle placed in the eyepiece of a dissecting light microscope. 12-15 seedlings of each genotype were assessed in each experiment.

IAA sensitivity analysis

Square petri plates containing IAA-supplemented Knops medium were prepared as follows. Knops medium was prepared as described above and divided into 200 ml aliquots. After autoclaving, 100μl of an appropriate IAA stock solution (suspended in DMSO) was added to each aliquot to achieve the desired final concentration of IAA within the media. Control medium received 100 μl of DMSO. Seeds of wild-type Columbia-0 and *rhd1-4* were surface sterilized and planted on two plates for each IAA treatment, seven seeds of each per plate, randomly arranged within the plate. Plates were incubated in the dark, due to the photosensitive nature of IAA, at 25°C at a near vertical orientation. Root lengths were measured seven days after planting using a reticle placed in the eyepiece of a dissecting microscope. 10-14 seedling roots were measured for each treatment in three separate experiments.

RHD1 Quantitative real time RT-PCR (qRT-PCR)

Total RNA was isolated from 20-50 mg of frozen ground tissue using the Gentra Total RNA Isolation Kit (Gentra Systems; Minneapolis, MN). DNase treatment of 1-1.5 μg total RNA was accomplished using the DNA-Free Kit (Ambion, Inc.; Austin, TX). DNase-treated total RNA was then used for first-strand cDNA synthesis using Superscript II Reverse Transcriptase (Invitrogen; Carlsbad, CA) following manufacturer's instructions. To use as template for qRT-PCR, cDNAs were diluted to volumes equivalent to 10 ng total RNA/μl with nuclease-free water. RHD1 and 18S coding sequence-specific primers were designed as described in Puthoff *et al.* (2003). Primer sequences are as follows. RHD1: forward (5'-AAAGCTGAACGCGAACTAAACTGGAA-3'), reverse (5'-CCGTAACCGAAAGGATTGTTGCTTGC-3'); 18S: forward (5'-GACAGACTGAGAGCTCTTTCTTGA-3'), reverse (5'-ACGTAGCTAGTTAGCAGGCTGAG-3'). qRT-PCR reaction conditions and cycling program were as described in Puthoff *et al.* (2003). A standard curve (four-fold dilution series; five total dilutions) was made using the RNA sample having the highest level of RHD1 or 18S expression. Cycle threshold and relative mRNA starting quantities for each unknown sample were calculated from this standard curve using iCycler software (Bio-Rad; Hercules, CA). RHD1 mRNA starting quantity values were normalized using 18S mRNA starting quantity values.

ACC treatment time-course analysis

Wild-type Columbia seeds were surface-sterilized and planted on petri plates containing Knops nutrient medium at 15-20 seeds/plate (24 plates total). Two-weeks after planting, 12 plates were chosen at random to be treated with ACC (Sigma; St. Louis, MO) while the remaining 12 plates were treated with a control solution. For ACC treatment, 3 ml of a 250 μM ACC solution (suspended in water) was applied to the surface of the plate. Plates were then sealed with Parafilm and put back into the original growth chamber. For each treatment, i.e., 24 and 48 hours post ACC or control treatment, three plates were chosen

at random from which shoot and root tissues were collected and pooled together. Tissues were flash frozen in liquid nitrogen and stored at -80°C until further use.

Upon request , all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes.

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Table 1 Ion concentrations within 1X MS and Knops media

		MS (μM)	Knops (μM)	MS/Knops
Anions	BO_3^{3-}	100	9	11.1
	Cl^-	6,000	23.55	255
	I^-	5	0	n/a
	MoO_4^{2-}	1	0.06	16.7
	NO_3^-	39,800	2,540	15.7
	PO_4^{2-}	1,250	1,500	0.83
	SO_4^{2-}	1,748	200	8.74
Cations	Ca^{2+}	2,800	1,270	2.2
	Co^{2+}	0.192	0.024	8
	Cu^{2+}	0.094	0.06	1.6
	Fe^{2+}	100	0	n/a
	Fe^{3+}	0	20	n/a
	K^+	20,055	2,518.1	8
	Mg^{2+}	1,500	200	7.5
	Mn^{2+}	100	1.5	66.7
	Na^+	222	22.06	10.1
	NH_4^+	21,000	0	n/a
	Zn^{2+}	48	0.141	340.4

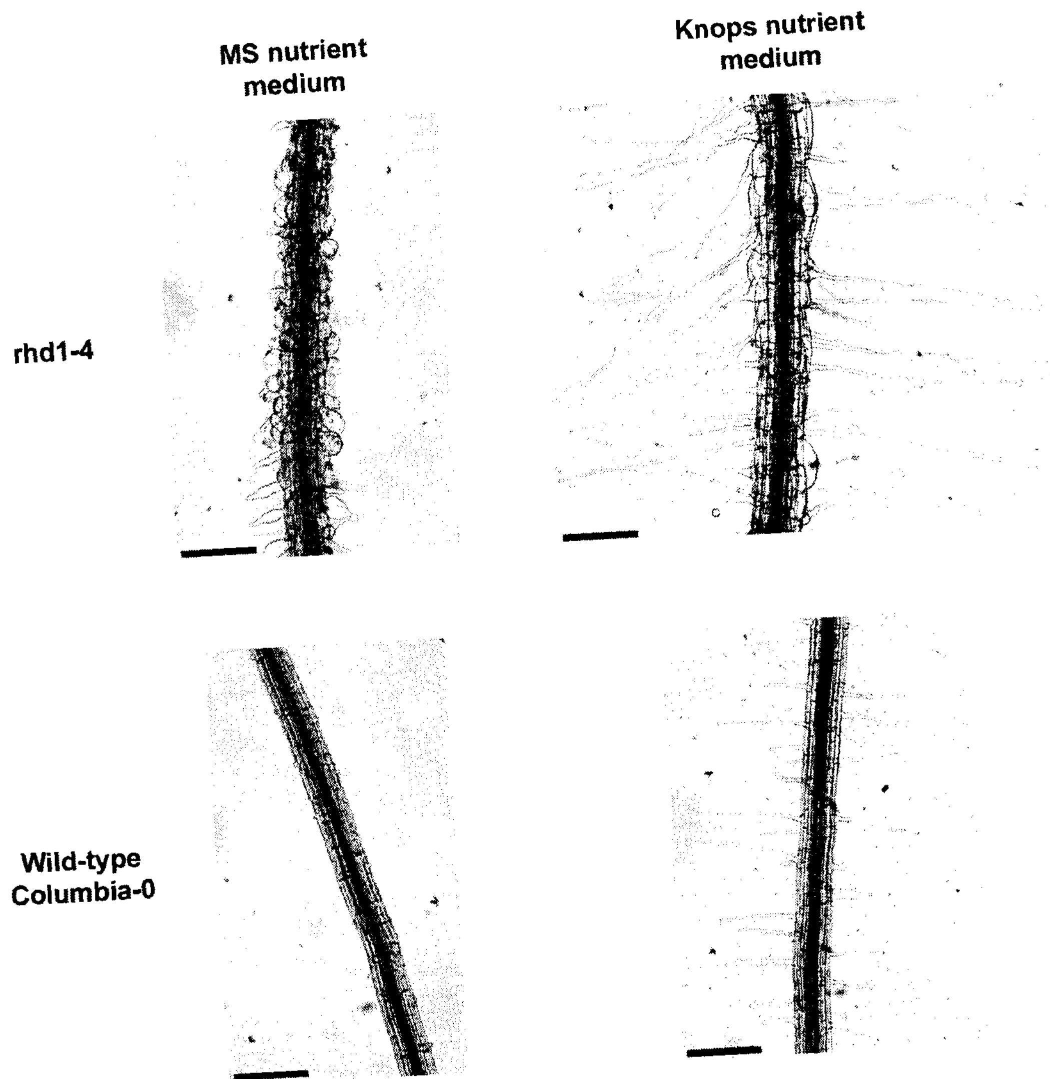

Figure 1. Root hair elongation of 8-day-old *rhd1-4* and wild-type Columbia-0 seedlings grown on Murashige and Skoog (MS) versus Knops nutrient media. Scale bars correspond to 100 μm.

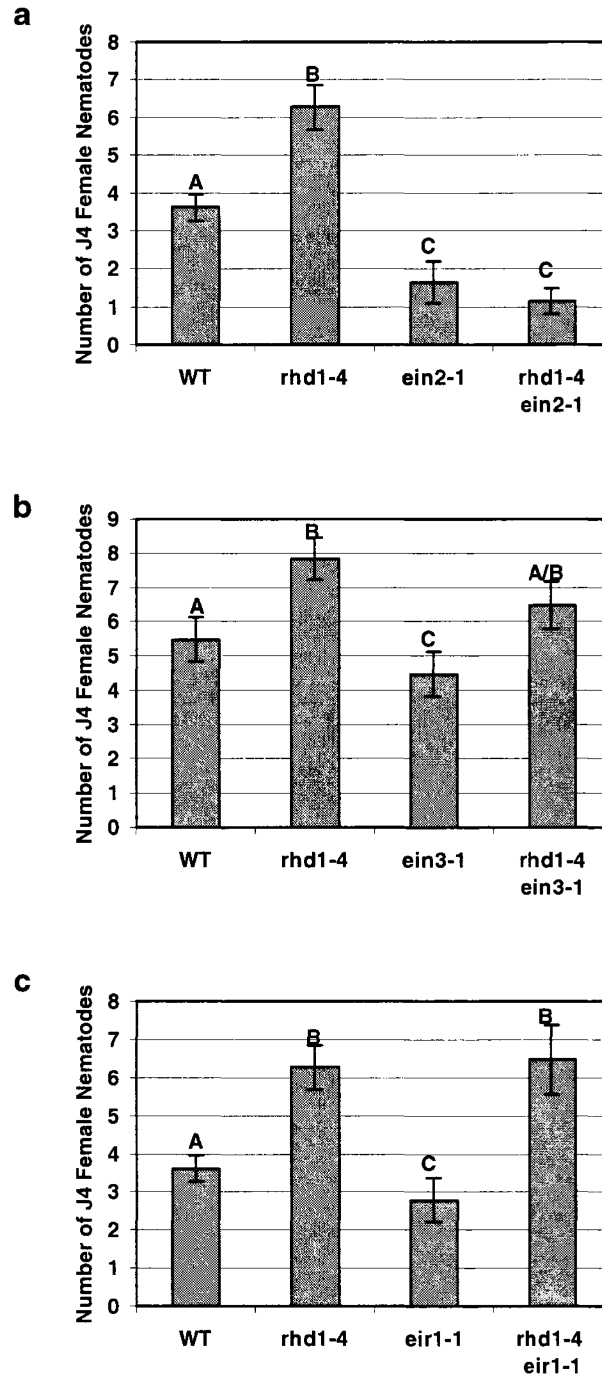

Figure 2. *Heterodera schachtii* susceptibility of wild-type Col-0 (WT) and *rhd1-4* compared to (a) *ein2-1* and *rhd1-4 ein2-1*, (b) *ein3-1* and *rhd1-4 ein3-1*, and (c) *eir1-1* and *rhd1-4 eir1-1*. Fourth-stage juvenile (J4) female nematodes were counted 14 days after inoculation. Data are presented as the least squared mean ± standard error. Means sharing the same letter designation are not significantly different as determined by unadjusted paired t-test ($P<0.05$).

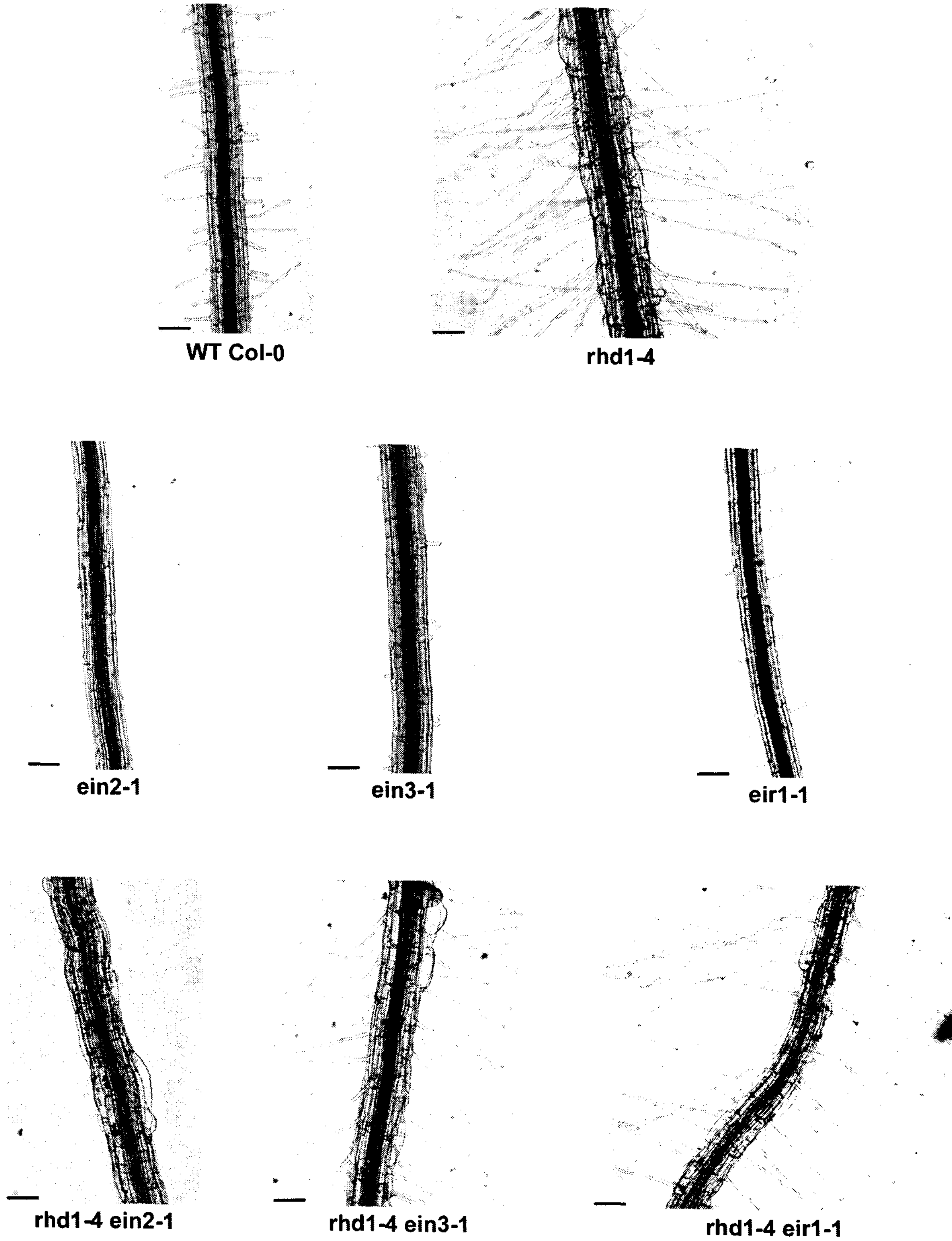

Figure 3. Assessment of wild-type Columbia-0 (WT Col-0), single mutant, and double mutant root hair elongation and root epidermal bulging. Scale bars correspond to 100 μm.

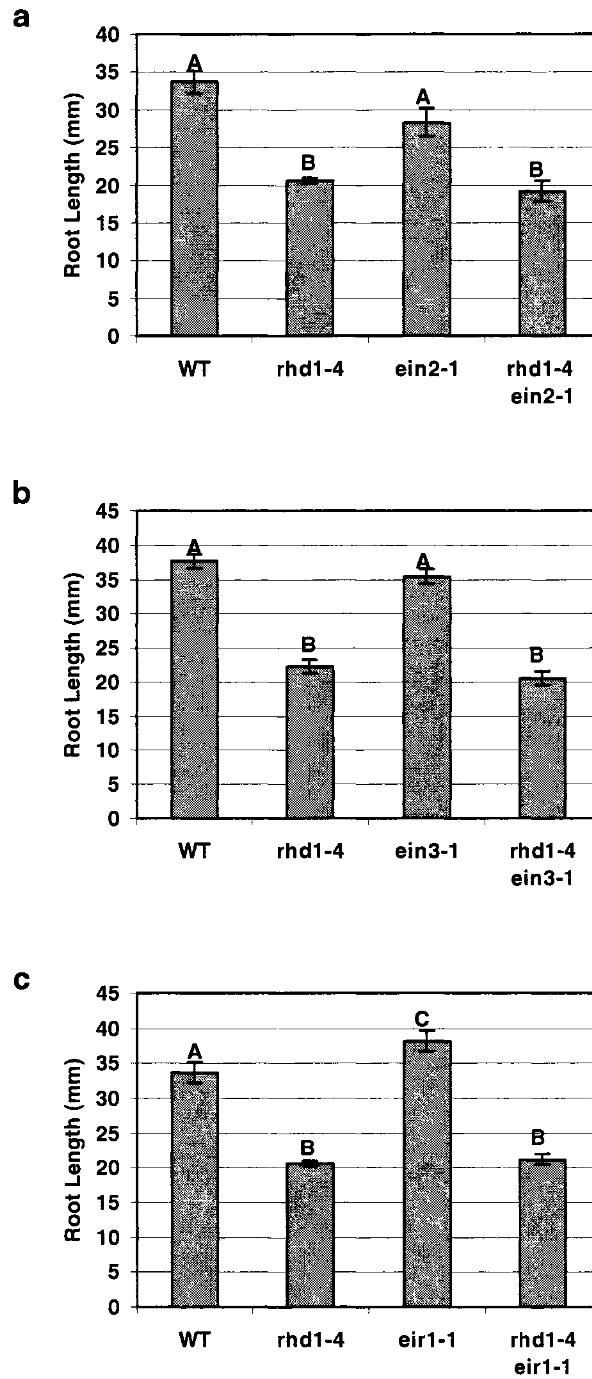

Figure 4. Root length assessment of wild-type Columbia-0 (WT) and *rhd1-4* compared to (a) *ein2-1* and *rhd1-4 ein2-1*, (b) *ein3-1* and *rhd1-4 ein3-1*, and (c) *eir1-1* and *rhd1-4 eir1-1*. Data are presented as the mean ± standard error. Means sharing the same letter designation are not significantly different as determined by paired t-test ($P<0.05$).

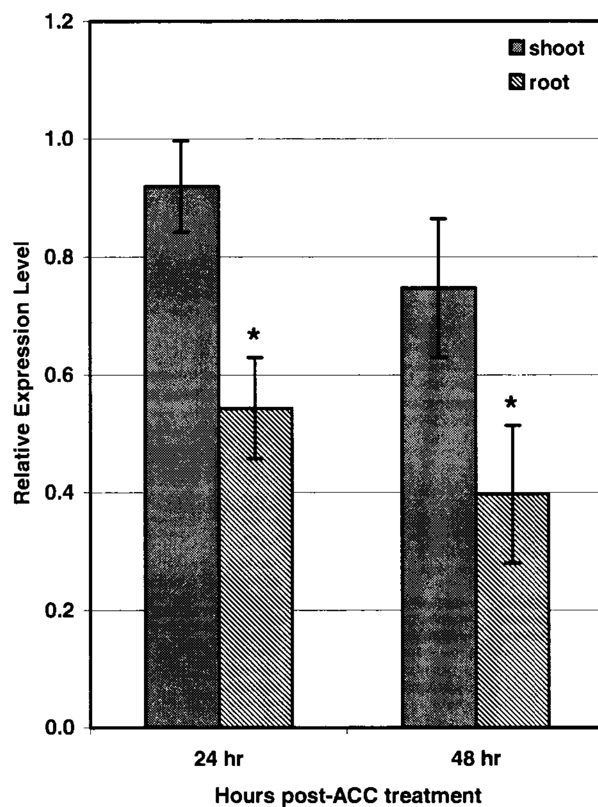

Figure 5. Measurement of *RHD1* transcript levels by quantitative real-time PCR in wild-type Columbia-0 shoot and root tissues in response to ACC treatment. ACC-treated and control tissues were collected at 24 and 48 hr post-ACC treatment. Presented are mean *RHD1* transcript levels ± standard error, from three independent biological replications, in ACC-treated tissues relative to *RHD1* transcript levels in corresponding control tissues (set to 1.0). Means significantly different from 1.0 are denoted by (*) as determined by paired t-test ($P<0.05$).

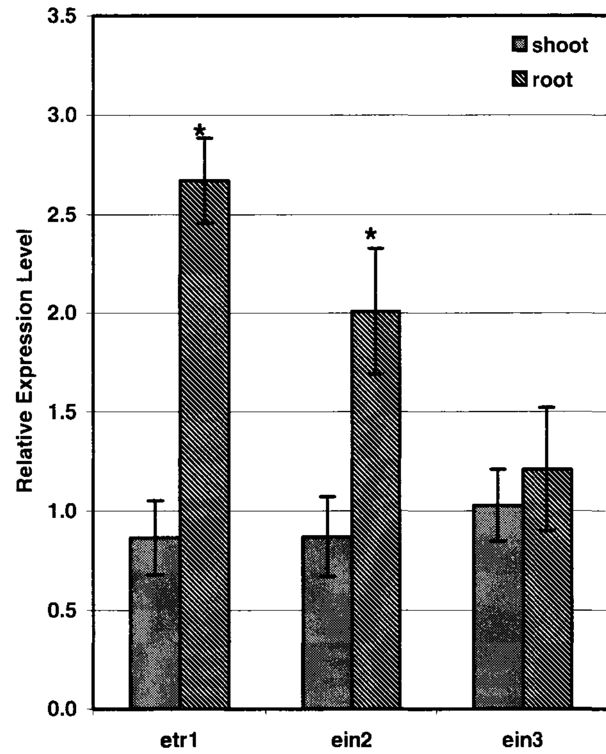

Figure 6. *RHD1* expression as determined by quantitative real-time PCR in shoot and root tissues of *etr1*, *ein2*, and *ein3* relative to wild-type Columbia-0 (set to 1.0). Tissue was collected from two-week-old seedlings. Presented is mean RHD1 transcript levels ± standard error from four independent biological replications. Means significantly different from 1.0 are denoted by (*) as determined by paired t-test ($P<0.05$).

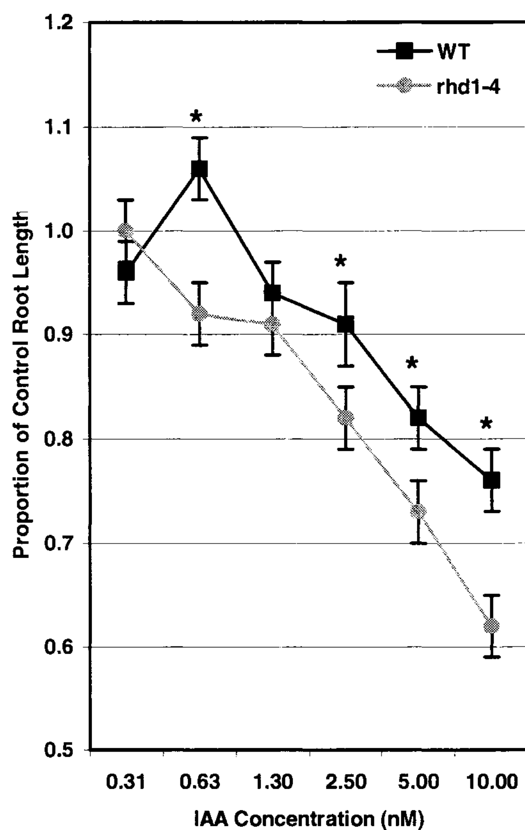

Figure 7. Sensitivity of wild-type Columbia-0 (WT) and *rhd1-4* seedlings to exogenous indole-3-acetic acid (IAA). Seedling root lengths were measured seven days after planting on control and IAA-supplemented Knops media. Presented is the least squared mean (lsmean) ± standard error proportional decrease in root growth in response to a range of IAA concentrations. *rhd1-4* lsmeans significantly different from the corresponding WT lsmeans, as determined by unadjusted paired t-test ($P<0.05$), are denoted by (*).

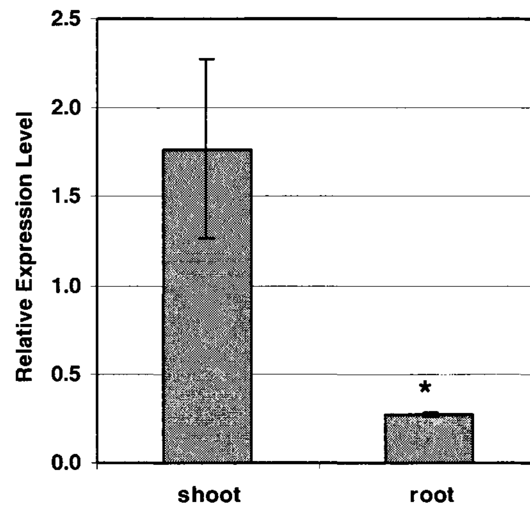

Figure 8. Assessment of *RHD1* expression in shoot and root tissues of wild-type Columbia-0 (WT) plants infected with *Heterodera schachtii*, the sugar beet cyst nematode, quantitative real-time PCR. 12-day-old WT plants were inoculated with *H. schachtii* infective second-stage juveniles or mock-inoculated. The mean ± standard error *RHD1* mRNA starting quantity in infected WT tissues, from three independent biological replications, is shown relative to the mock-inoculated control (set to 1.0). Means significantly different from 1.0, as determined by paired t-test ($P<0.05$), are denoted by (*).

CHAPTER 6. GENERAL CONCLUSIONS

The *Arabidopsis thaliana – Heterodera schachtii* compatible interaction is an ideal model system for the identification of determinants of host susceptibility to cyst nematodes (Sijmons *et al.*, 1991). We hypothesize that the characterization of *A. thaliana* mutants that show altered susceptibility to *H. schachtii* and the identification of the causal mutant gene will aid in unraveling the complex signal transduction events that occur between host and cyst nematode during parasitism. In Chapters II-V of this dissertation, I described the isolation (Baum *et al.*, 2000) and characterization (Wubben *et al.*, 2001, 2004) of *rhd1-4*, an *A. thaliana* mutant whose hypersusceptibility to *H. schachtii* is likely mediated by root-specific increased ethylene sensitivity. I also described the cloning of *RHD1* and its predicted function as a UDP-glucose-4-epimerase.

The role of ethylene in plant-cyst nematode interactions

Our initial phenotypic characterization of *rhd1-4* revealed that in addition to *H. schachtii* hypersusceptibility, *rhd1-4* exhibited increased root hair elongation, decreased root growth, and bulging of the root epidermal cells (Baum *et al.*, 2000; Wubben *et al.*, 2001). Increased root hair elongation and decreased root growth are typical responses of wild-type *A. thaliana* plants to ethylene treatment (Eliasson and Bollmark 1988; Tanimoto *et al.* 1995; Pitts *et al.* 1998), therefore, we set out to determine whether the *rhd1-4* phenotypes were due to an alteration in ethylene signal transduction. Chemical inhibition of ethylene synthesis in *rhd1-4* (Wubben *et al.*, 2001) or blocking ethylene signaling via introduction of the genetic null *ein2-1* into *rhd1-4* (Wubben *et al.*, 2004) showed that *rhd1-4* hypersusceptibility to *H. schachtii* and increased root hair length phenotypes were ethylene-dependent and indicated these phenotypes were mediated by increased ethylene sensitivity in *rhd1-4* roots. We also observed that pre-treatment of wild-type plants with an ethylene precursor resulted in increased *H. schachtii* susceptibility (Wubben *et al.*, 2001).

A central role for ethylene in promoting *A. thaliana* suceptibility to *H. schachtii* was confirmed by our analyses of mutants perturbed in ethylene production, perception, or down-stream signal transduction. In general, mutants that over-produced ethylene in their tissues

(*eto1*, *eto2*, *eto3*) were hypersusceptible to *H. schachtii*, while mutants unable to either perceive ethylene (*etr1*) or transduce an ethylene signal (*ein2*, *ein3*, *eir1*) showed dramatically decreased susceptibility to *H. schachtii* compared to wild-type (Wubben *et al.*, 2001).

The phytohormone ethylene regulates many aspects of plant growth and development (reviewed by Bleecker and Kende, 2000). Therefore, it may not be surprising that host susceptibility to cyst nematodes is strongly influenced by the basal level of ethylene signaling in the host, i.e., ethylene-overproduction versus ethylene insensitivity. However, the identities of ethylene-regulated host genes that promote cyst nematode parasitism are largely unknown. One possible role for ethylene during syncytium formation is the promotion of partial cell wall dissolution between the initial syncytial cell and its neighbors. Plant β-1,4-endoglucanase genes are induced by the sedentary second-stage juvenile (J2) at an early timepoint during syncytium formation where they are believed to degrade host cell walls and promote cellular fusion (Goellner *et al.*, 2000). Many of these host endoglucanases are regulated by ethylene, therefore, ethylene may be required for proper syncytium development. This idea is supported by Goverse *et al.* (2000) who observed that syncytia induced by *H. schachtii* within roots of ethylene-overproducing mutants were more extensive than syncytia induced in wild-type roots.

Ethylene is an important regulatory hormone with a broad range of function in plants and, therefore, any modulation of ethylene signaling by the cyst nematode has the potential to elicit numerous host responses. In other words, ethylene signaling may be one of several 'master switches' that the nematode manipulates in order to bring about desirable host responses during syncytium formation. This type of strategy for altering host gene expression would present several advantages to the nematode. For example, the number of nematode-derived elicitors required for syncytium formation would likely be less if ethylene itself, as opposed to specific ethylene-regulated targets, were modulated by the nematode. Such a strategy would decrease the chance that a host species would mutate into a nonhost species because the core ethylene signaling pathway is relatively well conserved across plant genera.

A surprising observation that arose during our double mutant analysis of *rhd1-4* was the inability of *eir1-1* to suppress *rhd1-4* hypersusceptibility to *H. schachtii* (Wubben *et al.*, 2004). *eir1-1* plants show decreased susceptibility to *H. schachtii* (Goverse *et al.*, 2000;

Wubben *et al.*, 2001, 2004) and are ethylene-insensitive only in the root (Roman *et al.*, 1995). *eir1-1* ethylene-insensitivity, however, is believed to be due to low levels of intracellular auxin because *EIR1* encodes an auxin efflux carrier that is required for polar auxin transport (Luschnig *et al.*, 1998; Müller *et al.*, 1998). There is mounting evidence that syncytium formation requires a local increase in intracellular auxin levels (Goverse *et al.*, 2000; Karczmarek *et al.*, 2004; Mazarei *et al.*, 2003). How, then, does *rhd1-4* retain the ability to promote *H. schachtii* hypersusceptibility in the presence of *eir1-1*? One possibility is that the increased ethylene sensitivity conferred by *rhd1-4* negates any requirement of the syncytium for auxin. On the other hand, because *rhd1-4* root epidermal cells exhibit altered cell wall architecture (Seifert *et al.*, 2002, 2004) and polar auxin transport occurs via the cortical and epidermal cell layers (Müller *et al.*, 1998), there may be alternative methods of auxin mobilization in *rhd1-4* roots. Introducing specific ethylene-responsive and auxin-responsive promoter :: reporter gene constructs into *rhd1-4 eir1-1* will help clarify the mechanism of *rhd1-4* epistasis to *eir1-1* and, possibly, the sequential order of action of auxin and ethylene during syncytium formation.

The *ein2-1* allele, which confers total ethylene-insensitivity, completely suppressed *rhd1-4* hypersusceptibility to *H. schachtii* (Wubben *et al.*, 2004). In contrast, the *ein3-1* allele only partially suppressed this phenotype in *rhd1-4 ein3-1* plants (Wubben *et al.*, 2004). *EIN3* encodes a nuclear-localized transcriptional activator that belongs to a family of proteins of which the other members are termed EIN3-like (EIL) (Chao *et al.*, 1997; Solano *et al.*, 1998). The *ein3-1* and *eil1* mutants retain a low level of ethylene responsiveness, but when combined into an *ein3 eil1* double mutant, complete ethylene-insensitivity is achieved (Chao *et al.*, 1997). Therefore, *H. schachtii* susceptibility analyses of *eil1* and *ein3 eil1* should be conducted so that the relative importance of these ethylene signaling components to *H. schachtii* parasitism is thoroughly measured. An *rhd1-4 ein3 eil1* triple mutant should also be constructed and analyzed to see if *rhd1-4* hypersusceptibility to *H. schachtii* is completely suppressed. The above described experiments would help to identify specific subsets of the ethylene signal transduction pathway that are required for syncytium formation by *H. schachtii*.

We determined that *RHD1* is a target of negative regulation by ethylene specifically in root tissues. This conclusion is based on our observations that treating wild-type plants with an ethylene precursor caused a root-specific decrease in *RHD1* transcript levels and that *RHD1* expression was elevated in roots of the ethylene-insensitive *etr1* and *ein2* mutants (Wubben *et al.*, 2004). It is interesting that ethylene influences *RHD1* expression only in roots despite the fact that *RHD1* transcript is also detected in shoot tissues. Together, these data suggest that mechanisms of gene regulation by ethylene differ between the shoot and root. However, it should also be remembered that, despite being systemically expressed in the plant, loss of *RHD1* function only results in root-associated phenotypes. Therefore, the negative regulation of *RHD1* by ethylene is likely tied directly to specific functions that *RHD1* serves in root tissues. Otherwise, we would expect to see a systemic down-regulation in *RHD1* expression in plant tissues upon ethylene treatment. The root specific nature of the *rhd1* mutant phenotypes and ethylene-mediated negative regulation of *RHD1* expression supports our conclusion that loss of RHD1 function alters root ethylene responses.

In addition to ethylene, *H. schachtii* infection causes a decrease in *RHD1* transcript levels three days after inoculation. It is possible that *H. schachtii*-induced down-regulation of *RHD1* expression is mediated by ethylene because induction of ethylene-responsive genes has been observed in cyst nematode-infected *Arabidopsis* roots (Puthoff *et al.*, 2003; Wubben and Baum, unpublished results). Therefore, it is tempting to speculate that *H. schachtii* infection elicits an *Arabidopsis* ethylene response in the root that mediates a down-regulation in RHD1 expression that, in turn, promotes plant susceptibility to the nematode

Possible mechanisms of increased ethylene sensitivity in *rhd1-4* roots

As described in Chapter IV, *RHD1* was identified as a UDP-glucose-4-epimerase, an enzyme that catalyzes the conversion of UDP-glucose to UDP-galactose. It is not intuitively obvious how the mutation of *RHD1* would result in a root-specific increase in ethylene sensitivity. However, there are several possible scenarios that would explain this *rhd1* phenotype given the function of *RHD1*.

One possibility is that *rhd1*-mediated defects in cell wall architecture result in an ethylene-mediated stress response. The cell wall, once thought of as a rigid and stagnant

structure, has since been recast as a dynamic part of the plant cell with roles in various aspects of signal transduction (reviewed by Kohorn, 2000). Other studies have revealed that defects in cell wall structure can result in ethylene over-production (Ellis and Turner, 2001; Zhong *et al.*, 2002). Therefore, it may be possible that aberrant cell wall structures present in the cortical and epidermal cell layers of *rhd1* mutant roots elicit a similar type of ethylene stress response. This idea is supported by the observation that root epidermal bulging is more severe in *rhd1-4 ein2-1* plants than in *rhd1-4* alone, implying that ethylene signaling, which requires *EIN2*, may act to suppress this phenotype in *rhd1-4* roots (Seifert *et al.*, 2004; Wubben *et al.*, 2004).

Another possible explanation for the effect of *rhd1* on ethylene sensitivity is that of defective arabinogalactan protein (AGP) synthesis in *rhd1* roots. AGPs are implicated in cell-to-cell signaling and cell differentiation (reviewed by Nothnagel, 1997), processes that likely require hormone signal transduction. Unfortunately, there is limited knowledge regarding the precise functions of specific AGPs, let alone information about signaling events within plant cells that are mediated by AGPs. *Arabidopsis* is predicted to contain 15 AGP-encoding genes (Schultz *et al.*, 2000). Due to concerted efforts by the *Arabidopsis* community, T-DNA knock-out lines of almost all *A. thaliana* genes will soon be available. Characterization of AGP loss-of-function mutants may provide a link between *rhd1*, AGPs, and ethylene signal transduction.

In conclusion, the results of our extensive characterization of the *rhd1* mutant highlight the important role that ethylene signal transduction plays in mediating host susceptibility to cyst nematodes. It is most probable that increased ethylene sensitivity in the *rhd1* root, propagated by aberrant cell wall architecture, mediates *rhd1* hypersusceptibility to the sugar beet cyst nematode, *Heterodera schachtii*. However, it is rarely the case that a hormone acts independently from other hormones toward eliciting a specific response. For example, ethylene and auxin are known to interact, both synergistically and antagonistically, during many plant development processes. Therefore, futher studies into the role of ethylene in governing host susceptibility to cyst nematodes need to incorporate possible interactions between ethylene and other hormones.

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