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

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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 *a*ltered *s*usceptibility to *c*yst nematodes. Our results demonstrate the feasibility of a nematological mutant screen and strengthen *A. thaliana* and *H. schachtii* as a model pathosystem.

Key words: asc, altered susceptibility, *Arabidopsis thaliana,* EMS, *Heterodera schachtii,* in vitro, mutant, *reb1, rhd1,* signal transduction, syncytium.

Cyst nematodes of the genus *Heterodera* are sedentary, obligate endoparasites of plants. These microscopic roundworms cause extensive losses in agricultural and horticultural crops worldwide (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 (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 (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 (Meyerowitz, 1994). *Arabidopsis 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

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with altered susceptibility to plant-parasitic nematodes, but such endeavors have met with only limited success (Niebel et al., 1994; Sijmons et al., 1994). To our knowledge, no successful screen for *A. thaliana* mutants with altered susceptibility to *H. schachtii* has yet been reported.

Several problems are impediments to the identification of mutants perturbed in cyst nematode-plant interactions. *Heterodera 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). Quantifying 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 5 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 plants were sealed with parafilm and placed in a 26 °C growth chamber with 12-hour-light/12-hour-dark days and approximately 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of light from cool white VHO fluorescent bulbs (Philips, Somerset, NJ). A field population of *H. schachtii,* designated TN101 (kindly provided by G. Tylka, Iowa Sate University, and originally obtained from 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. *Heterodera 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. *Arabidopsis 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 aque-

ous 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 10 mutant seeds were arbitrarily positioned, one seed per wall, in the 12-well plates. Once the germinating seedlings were 10 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 quantifying 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 fourth juvenile stage (J4) on wild-type plants, and at this time it was easy to identify developing male vs. 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.

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 led 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 were retained for further genetic analyses.

In an initial mutant screen of approximately 5,200 *A. thaliana* M2 plants, we iden-

Fig. 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.

tified 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, 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

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 × Col-0	F_1	24	0	24	
	F_2	125	35	90	> 0.3
10-5-2 × Col-0	F_1	12	0	12	
	F_2	112	23	89	> 0.2
2-4-6 × 10-5-2	F_1	14	0	14	

[a] P values 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	Number 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.

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 (i) the *rhd* name (Schiefelbein and Somerville, 1990) predates the *reb* name (Baskin et al., 1992) and (ii) 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 *a*ltered *s*usceptibility to *c*yst 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.

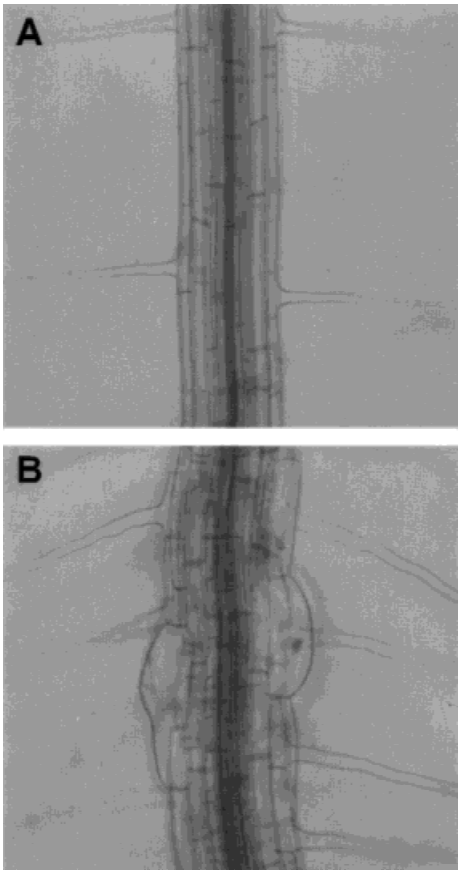

FIG. 2. Root epidermal morphology of A) wild-type Col-0 compared to B) mutant line 2-4-6.

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., 1997a,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 bonafide *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 twofold number of J2 already at 7 dai suggests that RHD1 exerts its influence at a very early time point of the nematode-plant interaction. In contrast to RHD1, 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 are independent from nematode parasitism. Such indirect effects could be, for example, an altered root system size, which would affect nematode parasitism by increasing or decreasing host targets. Another example of 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 longer root hairs than wild-type plants, which might suggest an unknown influence of root hairs on susceptibility to the nematode. How a specific host mutation may influence nematode parasitism can be addressed by cloning the mutated gene. Whether or not the mutations described here have a direct effect on nematode parasitism, the observation that the plant lines described here are altered in their susceptibility to *H. schachtii* will make further characterization highly informative.

Of the 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*. Alternatively, *asc1* 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, any variability in 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 that could be more easily scored.

A concern when initiating our 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 alternative alleles of *asc1* with more stable and scorable phenotypes.

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