Ethylene Signaling Pathway Modulates Attractiveness of Host Roots to the Root-Knot Nematode *Meloidogyne hapla*

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Infective juveniles of the root-knot nematode *Meloidogyne hapla* are attracted to the zone of elongation of roots where they invade the host but little is known about what directs the nematode to this region of the root. We found that *Arabidopsis* roots exposed to an ethylene (ET)-synthesis inhibitor attracted significantly more nematodes than control roots and that ET-overproducing mutants were less attractive. *Arabidopsis* seedlings with ET-insensitive mutations were generally more attractive whereas mutations resulting in constitutive signaling were less attractive. Roots of the ET-insensitive tomato mutant *Never ripe* (*Nr*) were also more attractive, indicating that ET signaling also modulated attraction of root-knot nematodes to this host. ET-insensitive mutants have longer roots due to reduced basipetal auxin transport. However, assessments of *Arabidopsis* mutants that differ in various aspects of the ET response suggest that components of the ET-signaling pathway directly affecting root length are not responsible for modulating root attractiveness and that other components of downstream signaling result in changes in levels of attractants or repellents for *M. hapla*. These signals may aid in directing this pathogen to an appropriate host and invasion site for completing its life cycle.

Root-knot nematodes (RKN) (*Meloidogyne* spp.) are sedentary endoparasites that infect many plants and cause substantial crop losses worldwide. Infective, second-stage juveniles (J2) hatch from eggs deposited in the soil and must locate and invade host roots in order to complete their life cycle. They are preferentially attracted to the region of the root just above the root tip corresponding approximately to the elongation zone (Curtis 2007; Wyss et al. 1992). After host invasion, nematodes migrate between the root cells toward the vascular cylinder until they find host cells that, in response to signals from the nematode, differentiate into hypertrophied, multinucleated giant cells. These giant cells (three to six per nematode) serve as the only source of nutrients for the nematode and are a prerequisite for their further development and reproduction.

Because RKN are obligate parasites and feed only on cells of the root, the J2 must find an appropriate host before their

lipid reserves are depleted and they no longer have the energy to penetrate the roots. Little is known about what makes the roots attractive to the parasite (Curtis 2007; Perry 1997). However, RKN generally have a very broad host range spanning monocots and dicots, suggesting that signals commonly secreted from plant roots are responsible. Proposed attractants include volatile and water-soluble compounds that are secreted by roots or other organisms present in the rhizosphere (Curtis et al. 2009; Perry 2005). Volatile compounds are thought to play a role in long-distance attraction to the vicinity of the root, while water-soluble compounds may be more important for short-distance attraction to the zone of the root where invasion can take place (Perry 2005). Gradients of physical and chemical factors (e.g., temperature, redox potential, pH, and CO₂) present in the rhizosphere, but not specific to the roots of vascular plants, have been proposed to attract or repel nematodes (Diez and Dusenbery 1989; Robinson 2004; Robinson and Perry 2006). The existence of plant-specific allelochemicals that attract nematodes has been postulated; however, no compounds have been identified yet.

We have developed an attraction assay using a gel of Pluronic F-127 (PF-127) to investigate factors that modulate RKN attraction and behavior (Wang et al. 2009b). PF-127 is a copolymer with low toxicity that, as a 23% solution, is liquid at 15°C but forms a highly transparent gel at higher temperatures. Nematodes dispersed in the gel can perceive gradients and migrate in three dimensions as they do in the soil environment. The PF-127-based assay has been successfully used to investigate nematode response to both chemical gradients and plant roots (Reynolds et al. 2011; Wang 2009a and b, 2010). By establishing a pH gradient in PF-127, we demonstrated that J2 accumulate at pH 4.5 to 5.5, suggesting that a pH gradient might be one of the signals that guide the RKN to the elongation zone of the root (Wang et al. 2009a). However, it is likely that, in a soil environment, a pH gradient would be perceived over a very short distance at best and that additional factors are responsible for long-distance attraction. Moreover, these experiments indicated that the previously noted attraction of J2 to CO₂ might actually be due to acidification of the environment caused by soluble CO₂, rather than the CO₂ itself.

Phytohormones regulate many aspects of plant life, including developmental processes as well as plant response to biotic and abiotic stresses. Although many studies indicate that phytohormones are involved in nematode feeding-site formation (De Meutter et al. 2003; Goverse and Bird 2011; Goverse et al. 2000; Lohar et al. 2004; Wubben et al. 2001), their role in modulating root attractiveness has not received much attention thus far. In one study, root exudates from the *Arabidopsis* ethylene (ET)-overproducing mutant *eto3* were found to attract

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more J2 of the sugar beet cyst nematode *Heterodera schachtii* than exudates from the wild-type (WT) root (Wubben et al. 2001). However, cyst nematodes, in contrast to RKN, generally have a narrow host range and, thus, different factors might be responsible for their attraction to host roots. Here, we investigate the role of ET levels and signaling in attraction of the RKN *Meloidogyne hapla* to host roots. ET is a simple gaseous compound that regulates diverse plant processes, including seed germination, flowering and leaf senescence, and fruit ripening, as well as responsiveness to stress and pathogen attack (Abeles et al. 1992; Broekaert et al. 2006). Increased ET induces synthesis and accumulation of defense-associated proteins (Deikman 1997; Ecker and Davis 1987) and is essential for production of antimicrobial peptides and secondary metabolites, including phytoalexins (Nakazoto et al. 2000; Penninckx et al. 1998).

The availability of a collection of well-characterized mutants as well as extensive studies on these mutants have elucidated many aspects of the ET response pathway in *Arabidopsis* (Roman et al. 1995; Stepanova and Alonso 2009). In *Arabidopsis*, ET is perceived by a family of five transmembrane receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) that are localized in the membranes of the endoplasmic reticulum (Bleecker et al. 1988; Chang et al. 1993; Chen et al. 2002; Hua and Meyerowitz 1998; Hua et al. 1995; Sakai et al. 1998). These receptors act as negative regulators of ET response signaling that, in the absence of ET, activate another negative regulator, the Raf-like kinase CTR1 (Hua and Meyerowitz 1998; Kieber et al. 1993). Upon ET binding to these receptors, CTR1 is inactivated, leading to the derepression of EIN2, a key positive regulator of the signaling pathway (Guzman and Ecker 1990). The signal transduced by EIN2 activates EIN3 and EIN3-like transcription factors (Alonso et al. 2003; Chao et al. 1997). These transcription factors activate a transcriptional cascade network and, as a result, a multitude of target genes are activated or repressed. The diverse ET-mediated responses are modulated at multiple levels by internal and external signals, including interactions with other hormone-regulated signaling pathways as well as tissue-specific, developmental, and environmental factors. Mechanisms for fine-tuning the response include dynamic adjustments at transcriptional and posttranscriptional levels that are just beginning to be understood (Stepanova and Alonso 2009; Yoo et al. 2009).

In general, the identification of ET response mutants in *Arabidopsis* has been based on screening in seedlings for the “triple response,” which is characterized by inhibition of root and hypocotyl growth, radial thickening of hypocotyl, and curving of the apical hook (Guzman and Ecker 1990). Based on the triple-response assay, ET mutants can be divided into two broad classes (Ecker 1995; McGrath and Ecker 1998; Sakai et al. 1998; Solano et al. 1998). The first class comprises ET-insensitive mutants that are defective in their ability to perceive or respond to ET; these include gain-of-function (GOF) ET receptor mutants (e.g., ET-resistant [*etr1-1* and *etr1-3*] and ET-insensitive [*ein4-1*]) and loss-of-function (LOF) mutants in genes encoding positive regulators of the downstream signaling (e.g., *ein2*, *ein3-1*, *ein5-1*, *ein6*, and *ein7*). GOF ET receptor mutants are defective in binding ET and, thus, continue to inhibit the ET pathway even when ET is present (Bleecker et al. 1988; Guzman and Ecker 1990; Hall et al. 1999). Mutants in the second class display a constitutive triple-response phenotype due to either ET overproduction (ET-overproducing [*eto1*, *eto2*, and *eto3*]) or constitutive activation of the pathway, as in the case of the *constitutive triple response* mutant (*ctr1-1*).

Here, we investigate the role of ET signaling in the attractiveness of roots of *Arabidopsis* to J2 of the northern RKN *M.*

hapla in a PF-127-based assay. We selected these two organisms because both have been used as model organisms to study plant–nematode interactions. The genome of *M. hapla* has been sequenced and a genetic map has been constructed (Opperman et al. 2008). The available resources for *Arabidopsis* (*Arabidopsis* Genome Initiative 2000; Meyerowitz 2001) and the fact that many nematode species can complete their life cycle feeding on it (Sijmons et al. 1991) make this plant one of the most extensively used hosts to study aspects of nematode parasitism, including nematode behavior in vivo (Wyss and Grundler 1992), ultrastructure of nematode feeding sites (Golinowski et al. 1996; Grundler et al. 1997; Sobczak et al. 1998), alteration in cell cycle (De Almeida Engler et al. 1999, 2004; Niebel et al. 1996), involvement of plant hormones (Goverse et al. 2000), analysis of gene expression during nematode feeding-site formation (Barcala et al. 2010; Barthels et al. 1997; Favery et al. 1998; Goddijn et al. 1993; Hammes et al. 2005; Hermsmeier et al. 2000; Jammes et al. 2005; Urwin et al. 1997a and b), and determining functions of putative nematode effectors (Huang et al. 2006). In this study, we demonstrate that attractiveness of *Arabidopsis* roots to juveniles of *M. hapla* is affected by addition of an ET biosynthesis inhibitor; we assess the attractiveness of roots of *Arabidopsis* mutants altered in ET synthesis, perception, and signaling to explore what aspects of ET signaling may be involved. To investigate whether ET signaling modulates attractiveness of *M. hapla* to roots of other plant species, we examined the effect of the tomato ET receptor mutant *Never ripe* (*Nr*) on attractiveness to tomato roots.

RESULTS

Attraction of nematodes to *Arabidopsis* roots over time.

To establish the optimum time for assessing nematode attraction to roots in our PF-127 gel assay, we counted the number of nematodes touching root tips of seedlings of two ecotypes of *Arabidopsis*, Col-0 and Ws-2, at 2, 4, 6, 9, and 24 h after assay start. The number of J2 touching the root changed over time and similar changes were observed for both ecotypes (Fig. 1). The highest number of J2 was observed at 9 h. At 24 h after the initiation of the assay, the number of J2 around the root tip was dramatically lower, likely because J2 had invaded the host and were inside the root tissue, as we have previously noted for tomato (Wang 2009b). We chose the 9-h time point to assess attraction in the remaining experiments because, of the time points assessed, it gave the highest number of nematodes touching the roots for the WT ecotypes tested.

Effect of 2-aminoethoxyvinylglycine (AVG) on root attractiveness.

We next assessed the effect on nematode attraction to roots of supplementing the PF-127 gel with AVG, which reduces ET synthesis in the plant by inhibiting the enzyme ACC synthase (Yang and Hoffman 1984). *Arabidopsis* seedlings of two WT ecotypes (Col-0 and Ws-2) were pregrown without AVG, and AVG was added to the PF-127 solution to a final concentration of 1 μ M just before the assay was started. The presence of AVG in the gel significantly increased root attractiveness of both ecotypes (Fig. 2). Because ET levels can affect root growth, we measured root lengths of uninfected roots after 9 h of incubation in PF-127 without or with addition of 1 μ M AVG but did not find a significant difference between these treatments (Table 1).

Attraction of nematodes to *Arabidopsis* ET mutants.

The finding that exposure of the roots to AVG increased their attractiveness to J2 suggested that ET or products of ET

signaling reduced their attractiveness. To investigate this further, we obtained *Arabidopsis* mutants affected in ET biosynthesis, perception, and downstream signaling. Activation of the ET response characteristically inhibits root elongation and induces ectopic root hair development (Dolan 1997; Kieber et al. 1993; Pitts et al. 1998; Tanimoto et al. 1995). To assess the impact of each mutation on root phenotype under our conditions, we compared root lengths of 8-day-old seedlings with the corresponding WT ecotypes (Table 1). As expected, ET-overproducing mutants *eto1*, *eto2*, and *eto3* and the constitutive triple-response mutant *ctr1-1* had significantly shorter roots than the corresponding WT plants. In contrast, most ET-insensitive mutants had significantly longer roots than the corresponding WT. The latter group consisted of all tested GOF mutants in ET receptors and LOF mutants in positive components of downstream signaling, except *ein5-1* and *ein7*. As expected, the two LOF ET receptor mutants containing T-DNA insertion *ers1-3* and *ers1(T-360)* did not differ in root length from the WT because this gene is functionally redundant with other receptors (Roman et al. 1995). However, the LOF mutant in *etr1-9* did show longer roots than the WT.

We compared the attraction of *M. hapla* J2 to roots of ET pathway mutants with their corresponding WT. The three ET-overproducing mutants (*eto1*, *eto2*, and *eto3*) and the constitutively signaling mutant *ctr1-1* attracted significantly fewer juveniles than the WT (Figs. 3A to C and 4). In contrast, GOF ET receptor mutants *etr1-3*, *ein4-1*, and *ers2-1* were significantly more attractive (Figs. 3A, D, and E and 5A and B). However, *etr1-1* and the LOF ET receptor mutants *ers1-3*, *ers1(T-360)* and *etr1-9* did not differ in attractiveness from the WT (Figs. 3J to L and 5C). Several ET-insensitive mutants that harbored LOF mutations in genes encoding positive regulators of ET response acting downstream from CTR1 in the signaling pathway were also assessed. Both mutant alleles in EIN2, *ein2*

(CS8844) of *ein2-1* (CS3071), attracted significantly more nematodes than the WT (Figs. 3A and F and 6), as did all other LOF mutants tested in genes encoding positive regulators of the pathway (*ein3-1*, *ein5-1*, and *ein7*) (Figs. 3A, G, and H and 6).

ET regulates root elongation through its effects on auxin biosynthesis and distribution (Negi et al. 2008; Růžicka et al. 2007). The ET insensitivity of mutant *eir1-1* is limited to its effect on root elongation (Roman et al. 1995), and molecular analysis revealed that this gene corresponds to PIN2, which encodes an auxin efflux carrier that is required for basipetal polar auxin transport in the root (Luschnig et al. 1998; Müller et al. 1998). Interestingly, the *eir1-1* mutant, even though it produces very long roots (Fig. 3I), did not have a significant effect on attractiveness of the root to *M. hapla* juveniles (Fig. 7). This result suggests that increased root length alone is not responsible for the higher attraction and that other components of the ET response pathway may be more important.

Extended attraction zone of ET-insensitive mutants.

In the course of the above experiments we noted that, for some ET receptor GOF mutants and ET-insensitive mutants, particularly *ein4-1* and *ers2-1*, the extent of the attractive zone of the root appeared to be increased. To assess this further, we counted separately the number of nematodes touching the root in the terminal 2 mm and in the region between 2 and 7 mm from the tip for *ein4-1*, *ein2-1*, and the WT (Table 2). In the WT, 79% of J2 touched the root within 2 mm of the root tip. In contrast, for *ein4-1* and *ein2-1* roots, the attraction zone of the root was extended and the two regions attracted similar numbers of nematodes (Table 2).

Nematode attraction

to the tomato ET-insensitive tomato mutant *Nr*.

To test whether ET signaling modulated attraction of *M. hapla* to other hosts, we examined the tomato mutant *Nr*,

Fig. 1. Attraction of juveniles of *Meloidogyne hapla* to *Arabidopsis* roots over time. Second-stage juveniles touching the terminal 7 mm of the root were counted at 2, 4, 6, 9, and 24 h after assay start in Pluronic F-127 gel for ecotypes Columbia (Col-0; white bar) and Wassilewskija (Ws-2; black bar). Each bar represents the mean, and error bars indicate standard error of the mean from one representative experiment ($n = 20$). This experiment was replicated, giving similar results.

Fig. 2. Effect of exposure of *Arabidopsis* roots to 2-aminoethoxyvinylglycine (AVG) on their attractiveness to nematodes. The number of nematode juveniles touching the terminal 7 mm of roots of *Arabidopsis* ecotypes Columbia (Col-0) and Wassilewskija (Ws-2) after 9 h of incubation in Pluronic F-127 gel containing second-stage juveniles without (white bar) or with (black bar) 1 μ M AVG was compared. Bars represent means and standard errors of the means from one experiment, $n = 20$. Bars with the same letter above are not significantly different ($P < 0.01$, Tukey's t test). The experiment was repeated with similar results.

which was first described to have a dominant mutation defective in fruit ripening even when exogenous ET was present (Lanahan et al. 1994). The ET-insensitive phenotype was later found to be due to a mutation in the ET receptor gene *LeETR3* (Wilkinson et al. 1995), which is normally expressed in shoots and roots of tomato seedlings (Lashbrook et al. 1998). After 9 h of incubation with nematodes, *Nr* roots attracted significantly more nematodes than those of the corresponding WT tomato (Fig. 8), supporting the importance of ET signaling in modulating attraction in tomato.

Nematode interaction triggers ET response.

Invasion of viruses, bacteria, and fungi on plant tissues generally enhances ET biosynthesis (De Laat and van Loon 1983; Huang et al. 2005), as does invasion of tomato roots by the RKN *M. javanica* (Glazer et al. 1983). To test whether ET is induced at early stages of the interaction, we used an *Arabidopsis* line transformed with a construct in which the reporter gene β -glucuronidase (*GUS*) is under control of a synthetic EIN3-responsive promoter (Stepanova et al. 2007). Reporter line plants were incubated in PF-127 gel containing J2 or without added J2 for 12 h and then subjected to histochemical staining. Plants incubated in the absence of nematodes showed no visible staining of root tips or root hair zone (Fig. 9A and B). However, in plants that were incubated with J2 for 12 h, *GUS* activity staining was observed mainly in the epidermis (trichoblasts and atrichoblasts) located in the root hair zone

(Fig. 9C and D). We did not see any J2 inside the roots at the 12-h time point, indicating that little, if any, successful invasion had yet occurred.

DISCUSSION

ET modulates attractiveness through the ET-signaling pathway.

The increased attractiveness of the roots in the presence of the ET biosynthesis inhibitor AVG and the reduced attraction to ET-overproducing mutants (*eto1*, *eto2*, and *eto3*) indicated that ET itself or ET signaling modulated attractiveness of the seedling roots to *M. hapla*. The observation that roots of lines with the constitutive ET-response mutant *ctr1-1* attracted fewer nematodes indicated that an active ET response pathway rather than levels of hormone itself was responsible for the lower attractiveness of the root. In addition, the level of root attractiveness was higher in all but one of the ET-insensitive receptor mutants tested (Table 1). Roots of seedlings with GOF mutants in ET receptors *etr1-3*, *ein4-1*, and *ers2-1* were significantly more attractive to nematodes than those of the WT. LOF mutations in ET receptors *ETR1* (*etr1-9*) and *ERS1* (*ers1-3* and *ers1(T-360)*) failed to show a significant difference in attraction, presumably because they are functionally redundant with other receptors (Roman et al. 1995). In addition, roots of *Arabidopsis* LOF mutants in positive components of the ET-signaling pathway (e.g., EIN2, EIN3, EIN5, EIN6, and EIN7) also

Table 1. Root length and attractiveness of *Arabidopsis* lines used in the study

Genotype	ABRC number ^a	WT ^b	Phenotype or mutation	Root length (cm) ^c	Att. ^d	References
Col-0	...	–	WT, untreated	1.33 ± 0.07	–	–
Col-0	...	–	WT, treated with 1 μ M AVG	1.32 ± 0.06	↑	–
Col-0	...	–	WT	1.54 ± 0.04	–	–
<i>eto1-1</i>	CS3072	Col-0	CTR due to ET overproduction	0.50 ± 0.03*	↓	Guzman and Ecker 1990; Wang et al. 2002
<i>eto2</i>	CS8059	Col-0	CTR due to ET overproduction	0.70 ± 0.05*	↓	Kieber et al. 1993
<i>eto3</i>	CS8060	Col-0	CTR due to ET overproduction	0.30 ± 0.01*	↓	Kieber et al. 1993
<i>ctr1-1</i>	CS8057	Col-0	CTR/LOF mutation in negative regulator	0.40 ± 0.04*	↓	Kieber et al. 1993
<i>etr1-1</i>	CS237	Col-0	EIN/GOF receptor mutant	3.10 ± 0.11*	ns	Bleecker et al. 1988
<i>etr1-3</i>	CS3070	Col-0	EIN/GOF ET receptor mutant	2.72 ± 0.07*	↑	Guzman and Ecker 1990
<i>ein4-1</i>	CS8053	Col-0	EIN/GOF ET receptor mutant	2.19 ± 0.08*	↑	Roman et al. 1995
<i>ein2</i>	CS8844	Col-0	EIN/LOF mutation in positive regulator	3.18 ± 0.13*	↑	Guzman and Ecker 1990
<i>ein2-1</i>	CS3071	Col-0	EIN/LOF mutation in positive regulator	3.10 ± 0.10*	↑	Guzman and Ecker 1990
<i>ein3-1</i>	CS8052	Col-0	EIN/LOF mutation in transcription factor	2.32 ± 0.08*	↑	Chao et al. 1997
<i>ein5-1</i>	CS8054	Col-0	EIN/LOF mutation in regulator of Tf	1.88 ± 0.10	↑	Roman et al. 1995; Olmedo et al. 2006
<i>ein7</i>	CS8056	Col-0	EIN/LOF mutation in regulator of Tf	1.62 ± 0.05	↑	Roman et al. 1995
<i>eir1-1 (pin2)</i>	CS8058	Col-0	EIN and agravitropic roots due to mutation in auxin transporter PIN2	2.03 ± 0.10*	ns	Roman et al. 1995
Ws-2	...	–	Wild-type	1.45 ± 0.04	–	–
<i>etr1-9</i>	CS6374	Ws-2	LOF mutation in ET receptor	2.09 ± 0.26*	ns	Chang et al. 1993; Qu et al. 2007
<i>ers1-3</i>	CS6373	Ws-2	LOF mutation in ET receptor	1.32 ± 0.25	ns	Qu et al. 2007
<i>ers1 (T-360)</i>	CS3978	Ws-2	LOF mutation in ET receptor	1.37 ± 0.03	ns	...
No-0	...	–	WT	1.24 ± 0.06	–	–
<i>ers2-1</i>	CS8854	No-0	EIN/GOF ET receptor mutant	2.22 ± 0.18*	↑	Hua et al. 1995
<i>Ler</i>	...	...	WT	1.17 ± 0.05	–	–
<i>ein6</i>	CS8055	Ler	EIN due to LOF mutation	1.80 ± 0.03*	↑	Roman et al. 1995

^a *Arabidopsis* Biological Resource Center (ABRC) stock number.

^b Wild type (WT).

^c AVG = 2-aminoethoxyvinylglycine, CTR = constitutive triple response, ET = ethylene, LOF = loss of function, and GOF = gain of function.

^d The mean of 10 seedlings and standard error of the mean. Rows between horizontal lines were considered together for statistical comparisons of root length. Values marked with asterisks (*) are significantly different from the corresponding WT line as determined by Tukey's honestly significant difference test for mutants in Col-0 and Ws-2 background or Student's *t* test for mutants *ers2-1* and *ein6*; *P* < 0.05. Col-0 = Columbia, Ler = Landsberg erecta, No-0 = Nossen, and Ws-2 = Wassilewskija.

^e Attractiveness compared with WT: ↑, more attractive; ↓, less attractive; ns, no significant difference.

attracted significantly more nematodes than the WT. Together, these results support the observation that downstream components of ET signaling rather than ET levels are responsible for the differences in attraction of the root to *M. hapla*.

The only exception was that roots of the GOF receptor mutant *etr1-1* did not show increased attractiveness. Interestingly, another mutant allele, *etr1-3*, in the same gene did show increased attractiveness. The *etr1-1* mutation results in Cys to Tyr substitution in the N-terminal domain of the receptor and causes inability to bind the Cu(I) cofactor required for ET binding (Bleecker et al. 1988). In the *etr1-3* mutant, a point mutation results in impaired but not completely eliminated ET binding; these mutants exhibit partial ET insensitivity (Guzman and Ecker 1990; Hall et al. 1999). Although we cannot explain

why mutants with these alleles differ in their effects on attraction, the assay results for the remaining mutants and the increased attractiveness of tomato roots with the GOF ET receptor mutant *Nr* (LeETR3) support the importance of ET perception and signaling in modulating root attractiveness in *Arabidopsis* and other host plants.

Dissecting the ET-signaling pathway.

Recent studies have shown that the downstream components of the ET-signaling responses are part of a complex and highly regulated response network involving cross talk with additional inputs, including hormone-signaling pathways (Stepanova and Alonso 2009; Yoo et al. 2009; Zhao and Guo 2011). ET, together with jasmonic and salicylic acid, is induced upon

Fig. 3. Response of juveniles of *Meloidogyne hapla* to ethylene (ET) mutants of *Arabidopsis*. Representative images showing the root tips of *Arabidopsis* wild-type ecotypes and ET mutants after 9 h of incubation of each seedling in 1 ml of Pluronic F-127 gel containing 200 juveniles of *M. hapla*. A, Columbia (Col-0); B, *etol1*; C, *eto3*; D, *etr1-3*; E, *ein4-1*; F, *ein2*; G, *ein3-1*; H, *ein5-1*; I, *eir1-1*; J, Wassilewskija (Ws-2); K, *etr1(T-360)*; L, *ers1-3*. Black bar in each panel is 500 μ m.

pathogen attack (Broekaert et al. 2006). The crosstalk between ET and auxin has been noticed for decades. Depending on the developmental process, these two hormones can act antagonistically—for example, during abscission of fruit and flowers (Brown 1997)—or synergistically—for example, during regulation of root growth (Pickett et al. 1990; Rahman et al. 2001; Růžicka et al. 2007; Stepanova et al. 2007) and root hair formation (Pitts et al. 1998). Two aspects of ET root responses that could affect root attractiveness are i) changes in the root morphology or architecture and ii) changes in the composition of root exudates mediated by some of the multitude of transcriptional changes that occur in response to ET. Although, in general, mutations with increased root length show increased attraction to nematodes (Table 1), there are exceptions, and these exceptions provide clues regarding which aspects of the ET-signaling pathway modulate root attractiveness.

Active ET signaling causes reduced root length and ectopic root hair formation and promotes radial expansion (Abeles et al. 1992; Dolan 1997; Smalle and Van Der Straeten 1997). Inhibiting root growth results in decreased length of the zone of elongation and, possibly, reduction of the area from which attractants are emitted. Earlier work indicated that attractiveness to nematodes was proportional to the rate of root growth (Lavalley and Rohde 1962; Weiser 1955) and, when the root growth was inhibited or slowed, attraction was also reduced (Lownsbery and Viglierchio 1961). In support of this explanation, mutants *eto1*, *eto2*, *eto3*, and *ctr1-1* had significantly shorter roots than the WT and they attracted fewer nematodes. In addition, many of the ET-insensitive mutants that attracted more nematodes have significantly longer roots than the corresponding WT (Table 1). However, there are exceptions that argue against this simple model. In experiments with AVG,

this inhibitor was added to the gel just prior to assay start and did not affect root length significantly during the 9-h incubation period. However, more nematodes were observed around the roots with AVG than in the control. In addition, for the *eir1-1/pin2* mutant, which is mutated in a gene encoding a basipetal polar auxin transporter (Luschnig et al. 1998; Müller et al. 1998) and is characterized by roots significantly longer than the WT, attraction of nematodes was not increased.

Other exceptions to the correlation of root length and attraction provide further clues. Seedlings with mutations *ein5-1* or *ein7* attracted more nematodes but did not show significantly longer roots in our study (Table 1) though, in another study, increased root length was noted (Roman et al. 1995). These mutations both lie within gene *XRN4*, which encodes an exoribonuclease and is thought to function by indirectly modulating levels of the transcription factor EIN3 (Olmedo et al. 2006; Potuschak et al. 2006). Interestingly, only a few differences in transcript accumulation have been noted for *ein5* mutants compared with the WT (Souret et al. 2004), and the level of ET-induced genes is not reduced as strongly in *ein5* mutants as in *ein2* (Olmedo et al. 2006). This suggests that only a subset of the EIN3-regulated genes may be affected in *ein5* mutants, and that characterization of such genes may direct us to signals that are important for nematode attraction. ET biosynthesis leads to activation of defense compounds, including antimicrobial secondary metabolites such as phytoalexins (Pedras et al. 2000) and pathogenesis-related proteins. Analysis of additional *Arabidopsis* mutants may be useful to further narrow down candidate attractants or repellents. In addition, although *M. hapla* and other RKN have extensive host ranges, host species differ significantly in their level of attractiveness (Bird 1959; Wang 2009b), suggesting that the basal levels or types of attractants and repellents differ.

Fig. 4. Attraction of juveniles of *Meloidogyne hapla* to *Arabidopsis* ethylene (ET)-overproducing mutants and *ctr1-1* mutant. The number of second-stage juveniles touching the root within the terminal 7 mm was counted at 9 h after starting the assay in two experiments. **A**, In the first experiment, ET-overproducing mutants were compared with the wild-type (WT) ecotype Columbia (Col-0) and **B**, in the second experiment, *ctr1-1* and Col-0 were compared. Both experiments were repeated with similar results. Bars represent means and standard error of the means from one representative experiment, $n \geq 14$. Asterisks above the bars indicate significant differences ($P < 0.01$) between mutants and corresponding WT based on **A**, Tukey's honestly significant difference or **B**, Student's *t* test.

Does the ET pathway modulate attractant or repellent levels?

The existence of both plant-specific nematode attractants and repellents has been postulated but the chemical nature of such compounds is largely unknown (Curtis et al. 2009; Weiser 1955). Root diffusates originating from different root zones may attract nematodes, repel them, or result in quiescence (Diez and Dusenbery 1989; Zhao et al. 2000). The net attraction or repulsion may depend on the qualitative and quantitative composition of root secretions. The differences in attraction that we see could indicate that active ET signaling is repressing levels of one or more attractants or increasing levels of one or more repellents. The increase in the span of the root that is highly attractive in *ein4-1* and *ein2* mutants compared with the WT could be due to the higher level of an attractant in a more extended region or to a lower level of repellent. It is likely that gradients formed by mixtures of root exudates from different zones of the root direct the nematode to the elongation zone.

Herbivore attack elicits major changes in the volatile compounds that are released by plants (Dicke and Baldwin 2010), and ET together with jasmonic acid signaling is involved in modulating herbivore-induced volatile emissions (Schmelz et al. 2003). Roots of citrus damaged by weevil larvae and, in some cases, undamaged roots have been shown to be attractive to both entomopathogenic nematodes and the plant-parasitic nematode *Tylenchulus semipenetrans* (Ali et al. 2011). However, thus far, the effect of subterranean volatiles on RKN has not been explored.

Cyst nematodes versus RKN.

Our results on the effect of *Arabidopsis* mutants in ET response on root attractiveness to RKN are opposite from results obtained with the sugar beet cyst nematode *H. schachtii*

(Wubben et al. 2001). In that study, root exudates from the ET-overproducing mutant *eto3* were found to be more attractive to cyst nematodes than exudates from the WT. In addition, ET-overproducing mutants were hypersusceptible whereas a num-

ber of EIN mutants (*etr1-1*, *ein2-1*, *ein3-1*, and *ein1-1*) were less susceptible to this species of nematode than the WT. These disparities could be simply due to differences in experimental design; we tested living roots, not “medium plugs”

Fig. 5. Attraction of juveniles of *Meloidogyne hapla* to *Arabidopsis* ethylene receptor mutants. The number of second-stage juveniles touching the root within the terminal 7 mm was counted at 9 h after starting the assay. Mutants were assessed together with the corresponding wild-type (WT) ecotype: **A**, Columbia (Col-0) in the case of *etr1-1*, *etr1-3*, and *ein4-1*; **B**, Nossen (No-0) in the case of *ers2-1*; or **C**, Wassilewskija (Ws-2) in the case of *etr1-9*, *ers1-3*, and *ers1(T-360)*. Bars represent means and standard error of the means from one representative experiment, $n \geq 12$. Asterisks above the bars indicate significant differences between mutants and corresponding WT based on **A** and **C**, Tukey's honestly significant difference test ($P < 0.01$) or **B**, Student's *t* test ($P < 0.01$). Experiments were repeated with similar results.

Fig. 6. Attraction of juveniles of *Meloidogyne hapla* to *Arabidopsis* ethylene-insensitive mutants downstream of CTR1. Second-stage juveniles touching the root within the terminal 7 mm were counted 9 h after starting the assay. Attractiveness of the roots was compared with the corresponding wild-type (WT): **A**, Columbia (Col-0) in the case of *ein2*, *ein2-1*, *ein3-1*, *ein5-1*, and *ein7*; or **B**, Landsberg erecta (Ler) in the case of *ein6*. Bars represent means and standard error of the means from one representative experiment, $n \geq 14$. Asterisks above the bars indicate significant differences between mutants and corresponding WT based on **A**, Tukey's honestly significant difference test ($P < 0.01$) or **B**, Student's *t* test ($P < 0.01$). Experiments were repeated with similar results.

containing root exudates. Alternatively, despite the fact that both cyst nematodes and RKN are sedentary endoparasites, these two nematode groups belong to evolutionarily diverged taxa and differ in many aspects of parasitism (Holterman et al. 2009; Williamson and Gleason 2003). Thus, root attractants or repellents might differ for these two groups of parasites. Although the *eir-1/pin2* mutant did not differ from the WT in attraction to *M. hapla* in our studies, it did have reduced susceptibility to cyst nematodes (Goverse et al. 2000). Although, we cannot assume that susceptibility and attraction are equivalent, this may mean that basipetal auxin transport (and increased auxin) has a role in susceptibility to cyst nematodes but not attraction for RKN, suggesting that different parts of the ET response pathway are involved in the two responses.

Importance of attractants and repellents in susceptibility.

In order to complete its life cycle, the sedentary parasite needs to establish a feeding site in a host from which it can derive sufficient nutrients to produce progeny, a process requiring a month or longer. Low levels of ET signaling may indicate a host that is healthy, growing well, not senescent, and unstressed. The tests with the EBS:GUS reporter line indicate that roots incubated with RKN J2 show an ET response by 12 h after assay start. At this time point, we did not observe any J2

inside the roots, and staining indicated that activation of the ET response occurred predominantly in epidermal cells of the maturation zone of the root. Many studies have shown that tissue wounding rapidly induces a transient increase of ET levels and that mechanical stimulation of plants by touching, rubbing, brushing, or even wind also can induce ET biosynthesis (Hyodo 1991). Early steps of nematode invasion are characterized by mechanical probing and wounding of epidermal cells (Wyss and Grundler 1992). Such interactions may induce an ET response. This ET signaling may indicate to approaching J2 that the host is already being attacked by nematodes or other pathogens and, thus, is not a suitable host, possibly reducing overinfection.

The simplicity and reproducibility of PF-127 gel tests together with availability of *Arabidopsis* mutants opens a new approach to discovering regulatory mechanisms underlying root attractiveness and for identification of the compounds involved. It would be interesting to check whether other hormones associated with defense response (e.g., salicylic and jasmonic acids) are involved in regulation of root attraction by using mutant studies. ET or jasmonic acid treatment of rice plants has been recently reported to induce defense genes and result in reduced

Fig. 7. Attraction of juveniles of *Meloidogyne hapla* to *Arabidopsis eir1-1* mutant. Second-stage juveniles touching the root within the terminal 7 mm from the root tip were counted 9 h after starting the assay. Experiment was repeated with similar results. Bars represent means and standard error of the means from one representative experiment, $n > 12$. Similar letters represent data sets not significantly different according to Student's *t* test ($P < 0.01$).

Fig. 8. Comparison of attractiveness of *Meloidogyne hapla* juveniles to *Never ripe* (*Nr*) and wild-type (WT) tomato roots. The number of nematodes touching the roots of *Nr* tomato and WT 'Rutgers' was counted for each seedling after 9 h of incubation in Pluronic F-127. Bars and error bars represent means and standard error of the means, respectively ($n = 9$). Different letters above the bars indicate that the means are significantly different based on Student's *t* test ($P < 0.01$).

Table 2. Comparison of the attraction of nematodes to root region between 0 and 2 mm from tip with that between 2 and 7 mm from the tip

Line	Number of J2 touching root ^a			J2 touching root (%) ^b	
	0 to 7 mm	0 to 2 mm	2 to 7 mm	0 to 2 mm	2 to 7 mm
Col-0	7.1 ± 2.3	5.6 ± 1.5	1.5 ± 1.3	79	21
<i>ein4-1</i>	15.5 ± 4	9 ± 4.1	6.5 ± 3.2	58	42
<i>ein2-1</i>	19.6 ± 6.4	8.5 ± 3.1	11.1 ± 4.8	43	57

^a Mean number of second-stage juvenile (J2) nematodes touching each region of the root and standard errors from one representative experiment ($n > 12$). Experiment was repeated with similar results.

^b Percentage of total J2 touching the indicated region compared with the entire zone 0 to 7-mm region.

infection of *M. graminicola* (Nahar et al. 2011). Although active defense may be detrimental to establishing a successful feeding site or directly damaging to the nematode, at least part of the lower infection rate may be due to reduced attractiveness of roots of the treated plants. Identification of the responsible plant products may lead to new strategies for control of RKN that do not compromise plant growth or development.

MATERIALS AND METHODS

Nematode culture.

M. hapla VW9 was propagated in the greenhouse on tomato (*Solanum lycopersicum*) 'VFNT' using conditions described by Liu and Williamson (2006). The eggs were collected by root bleaching, concentrated by 36% (wt/vol) sucrose flotation, and surface sterilized with bleach (Branch et al. 2004; Wang et al. 2009b). Eggs were hatched in sterile water, and J2 were collected every other day and used immediately for assays.

Plant material and growing condition.

Arabidopsis WT and mutant seed (Table 1) were obtained from the *Arabidopsis* Biological Resource Center. The EBS:GUS line was generated by A. N. Stepanova in J. Ecker's lab (Stepanova et al. 2007) and was a generous gift from J. Alonso (Department of Genetics, North Carolina State University, Raleigh, U.S.A.). Seed were surface sterilized and suspended in 0.1% agarose (Sigma-Aldrich, St Louis) as described by Wang and associates (2009b). The seed were sown on petri plates with modified Murashige and Skoog medium (0.5× Murashige and Skoog basal medium with Gamborg's vitamins) (Sigma-Aldrich), 0.5% sucrose, and 1.2% agar (Fisher Scientific, Fair Lawn, NJ, U.S.A.) covered with circles of number 1 Whatman filter paper (Florham Park, NJ, U.S.A.) to prevent root growth into the agar medium. Plates with seed were incubated for 2 days at 4°C and then moved to a culture room with a photoperiod of 16 h of light and 8 h of darkness at 24°C. Petri plates were kept at an angle of 60° to promote unidirectional root growth. After 8 days of growth, *Arabidopsis* plants were used for the root-length measurements or the attraction assays. Tomato seed of *Nr* and 'Rutgers' were a generous gift from R. M. Bostock (Department of Plant Pathology, University of California, Davis, U.S.A.). Seed of tomato (*S. lycopersicum*) were surface sterilized in 70% (vol/vol) ethanol for 3 min followed by treatment with bleach and water (2:1) for 10 min and rinsed in water three times for 5 min each time. Seed were germinated on 1.2% (wt/vol) water agar (Fisher Scientific, Fair Lawn, NJ, U.S.A.) in the dark at 24°C. After 4 days, seedlings were used for attraction assays.

Root-length measurements.

Root length was measured for 10 plants representing each *Arabidopsis* line or treatment. Measurements were taken after 8 days of growth at 24°C and a photoperiod of 16 h of light and 8 h of darkness. Tukey's honestly significant difference (HSD) or Student's *t* test was performed to compare each mutant with the corresponding WT or treated with untreated seedlings using JMP 8.0 software (SAS Institute Inc., Cary, NC, U.S.A.). Differences with $P < 0.01$ were considered significant.

Attraction assays.

Time-course experiments were carried out in 23% (wt/vol) PF-127 (Sigma-Aldrich) prepared according to Wang and associates (2009b) using 12-well tissue culture plates (Corning Inc., Corning, NY, U.S.A.). In all, 1 ml of 23% PF-127 containing 200 J2 was distributed into each well at 15°C; then, one 8-day-old *Arabidopsis* plant was added to each well. The plates were transferred to room temperature to allow the gel to

solidify. The roots were observed at indicated times, and the number of J2 touching the terminal 7 mm of the root was counted. Each experiment was repeated. In the first experiment, 30 plants were used and, in the second experiment, 20 replicates were used.

For tests with ET inhibitor AVG (Sigma-Aldrich), stock solutions of 10 mM AVG were prepared in water. AVG was added to the 23% PF-127 solution for 1 μM final concentration just before starting the assay; then, nematode juveniles (200 juveniles per 1 ml) and 8-day-old *Arabidopsis* seedlings were added. The number of nematodes touching the terminal 7 mm of the root was counted 9 h after initiating the assay. Data were submitted to Tukey's HSD test to assess significant differences ($P < 0.01$).

Assays with *Arabidopsis* mutants, were carried out in 12-well tissue culture plates using 23% (wt/vol) PF-127 as described above for the time-course experiment. After 9 h, nematodes touching the terminal 7 mm of the root were counted. For each WT and mutant, between 12 and 20 plants were used, and each experiment was repeated at least once. One-way analysis of variance (ANOVA) was performed using JMP software (SAS Institute Inc.). Results were considered to be significant or not significant based on Student's *t* test or Tukey's

Fig. 9. Expression of the reporter gene β -glucuronidase (*GUS*) under control of a synthetic EIN3-responsive promoter in *Arabidopsis* roots in Pluronic F-127 (PF-127) gel in the absence and the presence of *Meloidogyne hapla* juveniles. Roots are shown after 12 h of incubation of each seedling in **A** and **B**, PF-127 gel only or **C** and **D**, PF-127 gel containing juveniles of *M. hapla*. **A** and **C**, Root region encompassing root tip, elongation zone, and the beginning of the root-hair zone. **B** and **D**, Section of the root representing the root-hair zone. Arrows indicate *GUS* activity, visible as blue staining in epidermis. Images are representative from three independent experiments where at least 12 seedlings were examined for each treatment. Bars: 200 μm (**A** and **C**) and 100 μm (**B** and **D**).

HSD ($P < 0.01$). Pictures were made using a Nikon SMZ-U dissecting microscope, digital camera (Micropublisher 3.3 RTV; QImaging, Burnaby, BC, Canada), and SimplePCI high performance imaging system (Compix Inc., Sewickley, PA, U.S.A.).

Tomato attraction assays were performed in six-well tissue culture plates. In all, 3 ml of 23% PF-127 containing approximately 1,000 J2 and one tomato seedling was added to each well. In each experiment, nine seedlings of mutant and WT were used. The nematodes touching the root tip were counted after 9 h of incubation. The results were submitted to ANOVA (Student's t test, $P < 0.01$) using JMP software.

GUS staining.

The plants of EBS:GUS lines were treated and grown as described above for ET mutants and, after 8 days of growth, were used for the assay. Each seedling was incubated in 1 ml of 23% PF-127 containing 200 J2 or in 23% PF-127 alone in wells of a 12-well tissue culture plate at 24°C. After 12 h of incubation, plates were placed at 4°C for 3 to 5 min to allow Pluronic gel to "melt" and plants were collected into the GUS reaction buffer (2 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, and 0.2% [vol/vol] Triton X-100 in 50 mM sodium phosphate, pH 7.0) and incubated for 12 h at 37°C. The results of histochemical analysis (GUS staining) were analyzed on a Leica DM5000 B microscope equipped with a digital camera (Leica DFC310 FC) and driven by analysis software (Leica Application Suite version 3.6.0; Leica Microsystems, Heerbrugg, Switzerland). At least 12 transgenic plants were analyzed for each treatment and representative plant images were presented.

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