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Nematode CLE signaling in Arabidopsis requires CLAVATA2 and CORYNE

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SUMMARY

Plant-parasitic cyst nematodes secrete CLAVATA3 (CLV3)/ESR (CLE)-like effector proteins. These proteins have been shown to act as ligand mimics of plant CLE peptides and are required for successful nematode infection; however, the receptors for nematode CLE-like peptides have not been identified. Here we demonstrate that CLV2 and CORYNE (CRN), members of the receptor kinase family, are required for nematode CLE signaling. Exogenous peptide assays and overexpression of nematode CLEs in Arabidopsis demonstrated that CLV2 and CRN are required for perception of nematode CLEs. In addition, promoter–reporter assays showed that both receptors are expressed in nematode-induced syncytia. Lastly, infection assays with receptor mutants revealed a decrease in both nematode infection and syncytium size. Taken together, our results indicate that perception of nematode CLEs by CLV2 and CRN is not only required for successful nematode infection but is also involved in the formation and/or maintenance of nematode-induced syncytia.

Keywords: CLAVATA2, CORYNE, nematode, CLE peptide, receptor, syncytium.

INTRODUCTION

Biotrophs are pathogens that establish intimate parasitic relationships with the host that they infect. Often these relationships involve some kind of modification or reprogramming of the host cell(s) to accommodate the pathogen's subsequent growth and development. Plant-parasitic nematodes are biotrophs that mainly attack the roots of plants and cause crop damage of over $100 billion annually (Sasser and Freckman, 1987). The most economically important plant-parasitic nematodes include the cyst-forming nematodes of *Heterodera* and *Globodera* spp. These sedentary endoparasitic nematodes form intimate parasitic relationships with their hosts by penetrating the root as motile juveniles and migrating intracellularly until they reach the root vasculature where they select a single cell to initiate a feeding site. The initial syncytial cell undergoes developmental changes to re-differentiate into a syncytium to support subsequent nematode growth and development in later sedentary stages (Davis *et al.*, 2004). The syncytium forms when neighboring cells fuse as a result of partial cell wall degradation (Endo, 1964), creating a permanent feeding cell that shares characteristics with plant cell types including meristematic cells, endosperm cells, transfer cells, and developing xylem (Mitchum *et al.*, 2008). Development and maintenance of the syncytium is dependent on the secretory effector proteins originating in the esophageal gland cells and delivered into the host root through the stylet of the plant-parasitic nematode (Davis *et al.*, 2008). Recently, the CLAVATA3 (CLV3)/ESR (CLE)-like effector proteins secreted by cyst nematodes have been shown to act as ligand mimics of plant CLE peptides, and are required for successful nematode infection (Wang *et al.*, 2005; Patel *et al.*, 2008; Lu *et al.*, 2009; Wang *et al.*, 2010a,b).

Plant CLEs are small peptide ligands involved in regulating a population of specialized cells, called stem cells, which allow post-embryonic organogenesis to occur (Simon and Stahl, 2006). These stem cell pools can be found in the shoot apical meristem (SAM), the root apical meristem (RAM), and the vascular cambium. Whether or not these stems cells remain in an undifferentiated state or differentiate into new plant tissues is tightly controlled by CLE signaling pathways. In Arabidopsis, the population of stem cells which resides in the organizing center (OC) of the SAM is maintained by the expression of the transcription factor WUSCHEL (WUS) (Laux *et al.*, 1996). Differentiation of those stems cells is promoted when the ligand–receptor pair of CLV3, a small extracellular peptide ligand in the CLE family (Fletcher *et al.*, 1999; Rojo *et al.*, 2002), binds to CLV1 (Ogawa *et al.*, 2008), a leucine-rich-repeat receptor-like kinase (LRR-RLK) and downregulates *WUS*. Previous models have suggested that CLV1 forms a receptor complex with the LRR-receptor-like protein (RLP) CLV2 (Clark *et al.*, 1993; Kayes and Clark, 1998; Jeong *et al.*, 1999; Trotochaud *et al.*, 1999). More recently, it has been suggested that CLV1 acts in parallel or together with the heterodimer receptor complex of CLV2 and CORYNE (CRN) (Miwa *et al.*, 2008; Muller *et al.*, 2008; Bleckmann *et al.*, 2010; Guo *et al.*, 2010; Zhu *et al.*, 2010). In comparison to the SAM, much less is known about the regulation of stem cells in the RAM. The quiescent center (QC) is the equivalent to the OC in the SAM. However, there are significant differences between the OC and the QC. In contrast to the OC, the cells surrounding the QC are maintained as stem cells. In addition, stem cells are differentiated in both proximal and distal directions. This indicates that there is a signaling ligand involved in cell–cell communication to maintain the cells surrounding the QC as stem cells, and a signal to promote differentiation (Sarkar *et al.*, 2007; Stahl *et al.*, 2009). Previous reports have identified that the WUS-related homeobox 5 (WOX5) transcription factor is expressed in the QC of the RAM and is required to maintain the distal stem cell pool (Sarkar *et al.*, 2007). Recently it has been shown that *CLE40*, the closest homolog to *CLV3*, is expressed in the columella cells and regulates expression of *WOX5* (Stahl *et al.*, 2009). The WOX5/CLE40 signaling pathway appears only to control the distal stem cell pool, indicating that other CLE signaling pathways may exist to control the proximal stem cell pool. Consistent with these observations, a number of Arabidopsis *CLE*s are expressed in roots (Sharma *et al.*, 2003), and when some of these *CLE*s are overexpressed they have been shown to cause premature termination of the primary root meristem (Fiers *et al.*, 2004; Strabala *et al.*, 2006; Meng *et al.*, 2010). In addition, the short root phenotype has been shown to be dependent on perception by CLV2 and CRN (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2005; Miwa *et al.*, 2008; Meng *et al.*, 2010). Taken together this indicates that a CLV-like and a CLE-controlled signaling pathway can act in the root.

CLE-like genes from nematodes have been reported in the soybean cyst nematode (SCN; *Heterodera glycines*) (Wang *et al.*, 2005, 2010a), the beet cyst nematode (BCN; *Heterodera schachtii*) (Patel *et al.*, 2008; Wang *et al.*, 2010b), and the potato cyst nematode (PCN; *Globodera rostochiensis*) (Lu *et al.*, 2009). Beet cyst nematode CLEs have been detected in the dorsal gland ampulla, indicating they are probably secreted from the stylet into host cells (Patel *et al.*, 2008). More recently, SCN CLEs have been shown to be secreted directly to the syncytial cytoplasm where the variable domain is thought to redirect the nematode CLE peptides to the apoplast (Wang *et al.*, 2010a). These findings suggest that when delivered to the apoplast, nematode CLEs would be available to interact with extracellular host receptors to function as ligand mimics of plant CLE signaling pathways. Overexpression studies have shown that nematode CLEs can trigger plant CLE signaling pathways (Wang *et al.*, 2005; Lu *et al.*, 2009; Wang *et al.*, 2010a,b), but the identity of the receptors and downstream signaling pathways that are activated to initiate developmental cascades required for the re-differentiation of root cells to form syncytia are currently unknown.

In this paper we describe the use of synthetic CLE peptides, nematode *CLE* overexpression lines, promoter–reporter lines, and nematode infection assays of receptor mutants to investigate a role for CLV2 and CRN in nematode CLE signaling. Our results indicate that the CLV2/CRN signaling pathway is required for successful nematode infection and syncytium development.

RESULTS

CLV2 and CRN are required for nematode CLE perception

We have previously shown that exogenously applied 12-amino-acid (aa) peptides corresponding to the CLE motifs of the SCN (HgCLEs) and the BCN (HsCLEs) CLEs can function as plant CLE peptide mimics causing termination of the primary root meristem in a concentration-dependent manner (Wang *et al.*, 2010b). In fact, HsCLE2 was found to share an identical 12-aa CLE motif with Arabidopsis CLEs 5 and 6 (Wang *et al.*, 2010b). In Arabidopsis, it has been shown that the short root phenotype caused by overexpression or exogenous application of some plant CLE peptides is dependent on CLV2 signaling (Fiers *et al.*, 2005; Miwa *et al.*, 2008; Muller, 2008; Meng *et al.*, 2010). More recent evidence indicates that CLV2 forms a complex with CRN and can transmit the signal from CLV3 binding in a CLV1-independent manner (Miwa *et al.*, 2008; Muller *et al.*, 2008; Bleckmann *et al.*, 2010; Zhu *et al.*, 2010). To determine whether or not CLV2 and CRN might play a role in perception of cyst nematode CLEs we screened the Arabidopsis *clv2-1* null mutant and the *crn-1* amorphic allele for resistance to the HgCLE, HsCLE1, and HsCLE2

12-aa peptides. Seeds were grown on vertical plates in the absence of exogenous peptide or in the presence of 1 μM HgCLE or 10 μM of the HsCLEs and roots were measured 9 days after germination. Wild-type seedlings [Landsberg *erecta* (L*er*)] had statistically shorter roots when grown on plates with any of the CLE peptides in comparison with the no-peptide control (Figure 1a). In contrast, root growth in *clv2-1* and *crn-1* was relatively unimpaired in the presence of the different CLE peptides (Figure 1a). The same observation was made with *sol2-1*, another mutant allele of *CRN* (Miwa *et al.*, 2008) (Figure S1 in Supporting Information). Previous reports have indicated that the short root phenotype can be attributed to a decrease in the number of meristematic cells in the RAM (Fiers *et al.*, 2005). Using Nomarski optics, we confirmed that *clv2-1* and *crn-1* were insensitive to peptide application, resulting in root meristems that were indistinguishable from the no-peptide control (Figure 1b–d).

Nematode CLEs function *in planta* through a CLV2- and CRN-dependent pathway

Overexpression of *HgCLE2*, *HsCLE1*, and *HsCLE2* in wild-type Arabidopsis has been shown to cause a *wus*-like phenotype similar to other plant CLEs (Strabala *et al.*, 2006; Meng *et al.*, 2010; Wang *et al.*, 2005, 2010a,b). If CLV2 and/or CRN are involved in perception of nematode CLEs then we would expect the phenotypes to be diminished or abolished when overexpressed in *clv2-1* and/or *crn-1*. Each of the nematode *CLE* genes were cloned into an overexpression vector and transformed into the mutant backgrounds. Transgenic seedlings in the T_1 generation were screened and characterized in soil. In contrast to the overexpression

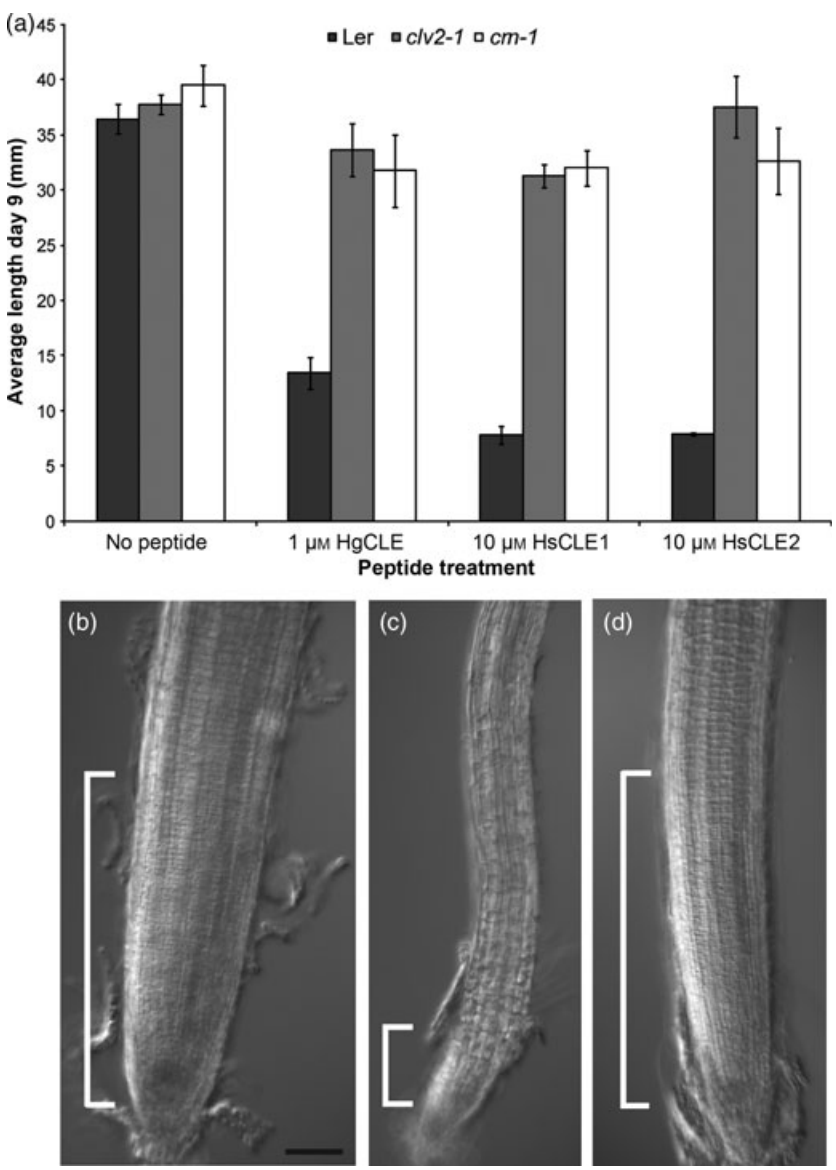

Figure 1. Effect of cyst nematode CLAVATA3 (CLV3)/ESR (CLE) peptides on receptor mutants.
(a) Average root length of wild-type (L*er*), *clv2-1*, and *crn-1* seedlings grown for 9 days on media with or without the synthetic nematode dodecapeptide CLE motif. Data represent the mean ± SE, $n = 10$. Asterisks indicate statistically significant differences in root length of peptide-treated mutants compared with the respective peptide-treated L*er* root by Student's *t*-test ($P < 0.05$). Data are representative of three independent experiments.
(b–d) Representative roots tips of seedlings grown on media with or without synthetic CLE peptides for 10 days and visualized with differential interference microscopy: (b) no peptide; (c) sensitive to peptide, and (d) resistant to peptide. Brackets indicate the region of the root apical meristem containing dividing cells. Scale bar, 50 μm.

Table 1 Summary of nematode *CLE* overexpression phenotypes in *clv2-1* and *crn-1*

		T_1 shoot phenotypes			
Background	Construct	*wus*-like (%)	WT (%)	T_1 lines (no.)	Total T_1 (no.)
WT	*HgCLE2*[a]	85	15	9	80
	HsCLE1[b]	37	63	9	156
	HsCLE2[b]	11	89	9	158
clv2-1	*HgCLE2*	0	100	12	96
	HsCLE1	0	100	9	67
	HsCLE2	0	100	9	28
crn-1	*HgCLE2*	0	100	7	85
	HsCLE1	0	100	7	41
	HsCLE2	0	100	7	37

[a]Wang *et al.*, 2010a.
[b]Wang *et al.*, 2010b.
WT, wild type.

phenotypes seen in wild-type Arabidopsis, where a high percentage of *wus*-like phenotypes were observed when the peptides were correctly targeted to the apoplast (Wang *et al.*, 2010a,b), no *wus*-like phenotypes were observed when *HgCLE2*, *HsCLE1*, and *HsCLE2* were overexpressed in *clv2-1* or *crn-1* (Table 1). These results demonstrate that mutations in *CRN* and *CLV2* abolish nematode *CLE* overexpression phenotypes.

Spatial and temporal relationship between *CLV2, CRN,* and nematode feeding sites

Cyst nematodes enter the root near the zone of elongation, migrate through root cortical cells using their stylet to puncture through cell walls, and begin feeding from a single cell near the vascular cylinder. CLE peptides, originating from the highly active dorsal esophageal gland cell, are delivered through the feeding stylet to the cytoplasm of the host root cell (Wang *et al.*, 2010a). In order for CLV2 and CRN to be able to perceive the nematode CLEs as ligand mimics they must be expressed in the correct spatial and temporal context.

Using a *CRN:GUS* transgene in Arabidopsis, *CRN* expression was previously shown to be expressed throughout the root including the vasculature where the nematode initiates feeding (Figure 2a–c; Muller *et al.*, 2008). To confirm whether *CRN* is expressed in nematode feeding sites, transgenic Arabidopsis seedlings expressing *CRN:GUS* were infected with BCN and monitored during nematode development. Expression of GUS was detected in feeding sites as soon as early second-stage juveniles (J2) began to feed. (Figure 2d). The expression of GUS reached its peak once nematodes reached late J2 parasitic stages, but remained detectable in the feeding sites of third-stage juvenile (J3) parasitic nematodes (Figure 2e–g). By the time the nematodes reached the fourth juvenile life stage (J4), GUS expression was either weak or absent in feeding sites (Figure 2h).

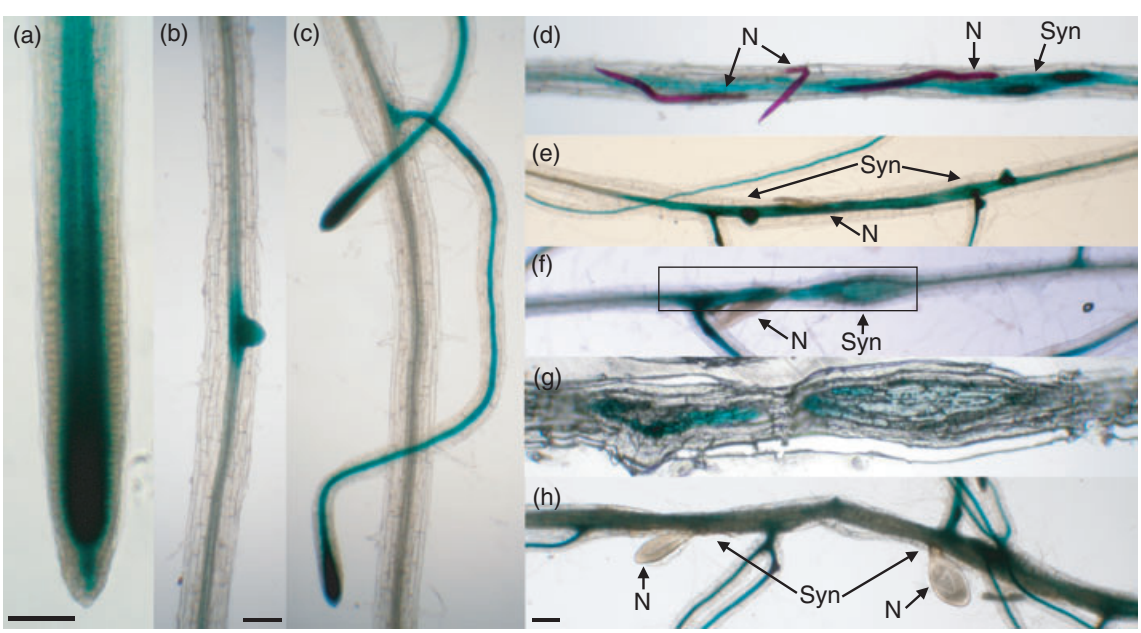

Figure 2. *CRN:GUS* expression during nematode infection.
(a–c) The GUS expression in uninfected Arabidopsis root tips (a), middle section of the root showing GUS staining in the vascular tissue and a lateral root primordium (b), and GUS staining in the junction of a lateral root in the upper section of the root towards the hypocotyl (c).
(d–h) *CRN:GUS* expression in response to *Heterodera schachtii*; early parasitic second-stage juvenile (J2), 2 days post-inoculation (dpi) (d), late parasitic J2, 4 dpi (e), parasitic third-stage juvenile (J3), 6 dpi (f), longitudinal section through the syncytium (g) corresponding to the region of the root highlighted by the black box in (f), parasitic fourth-stage juvenile (J4), 10 dpi (h).
Abbreviations: N, nematode; Syn, syncytium. Scale bar, 50 μm.

Similar to *CRN*, *CLV2* is expressed in many different vegetative tissues (Jeong *et al.*, 1999). However, little is known about the expression pattern of *CLV2* in roots. For an ongoing study to visualize *CLV2* expression in roots, mCherry was fused to the C-terminus of the Arabidopsis *Histone 2B* (*H2B*) gene and placed under the transcriptional control of the *CLV2* promoter (AB and RS, unpublished data). The H2B protein has been shown to be a valid marker for chromatin organization in plant nuclei and has been used to describe development of the syncytial endosperm in Arabidopsis (Boisnard-Lorig *et al.*, 2001). In uninfected roots, *CLV2*:*H2B-mCherry* fluorescence was detected throughout the root vasculature with the strongest expression detected in lateral root primordia and the zone of elongation extending down to the root apical meristem (Figure S2). Expression of *CLV2* under the control of the endogenous promoter, using 1252 bp of the *CLV2* 5′ region was sufficient to rescue the *clv2-1* mutant in all isolated lines (n = 20). We utilized the Arabidopsis *CLV2*:*H2B-mCherry* transgenic line to evaluate *CLV2* expression during nematode infection. Upon nematode infection, increased expression of *CLV2*:*H2B-mCherry* fluorescence was detected in the nuclei of syncytia fed upon by parasitic J2s (Figure 3a,b). At the J3 life stage, *CLV2*:*H2B-mCherry* continued to be specifically expressed within

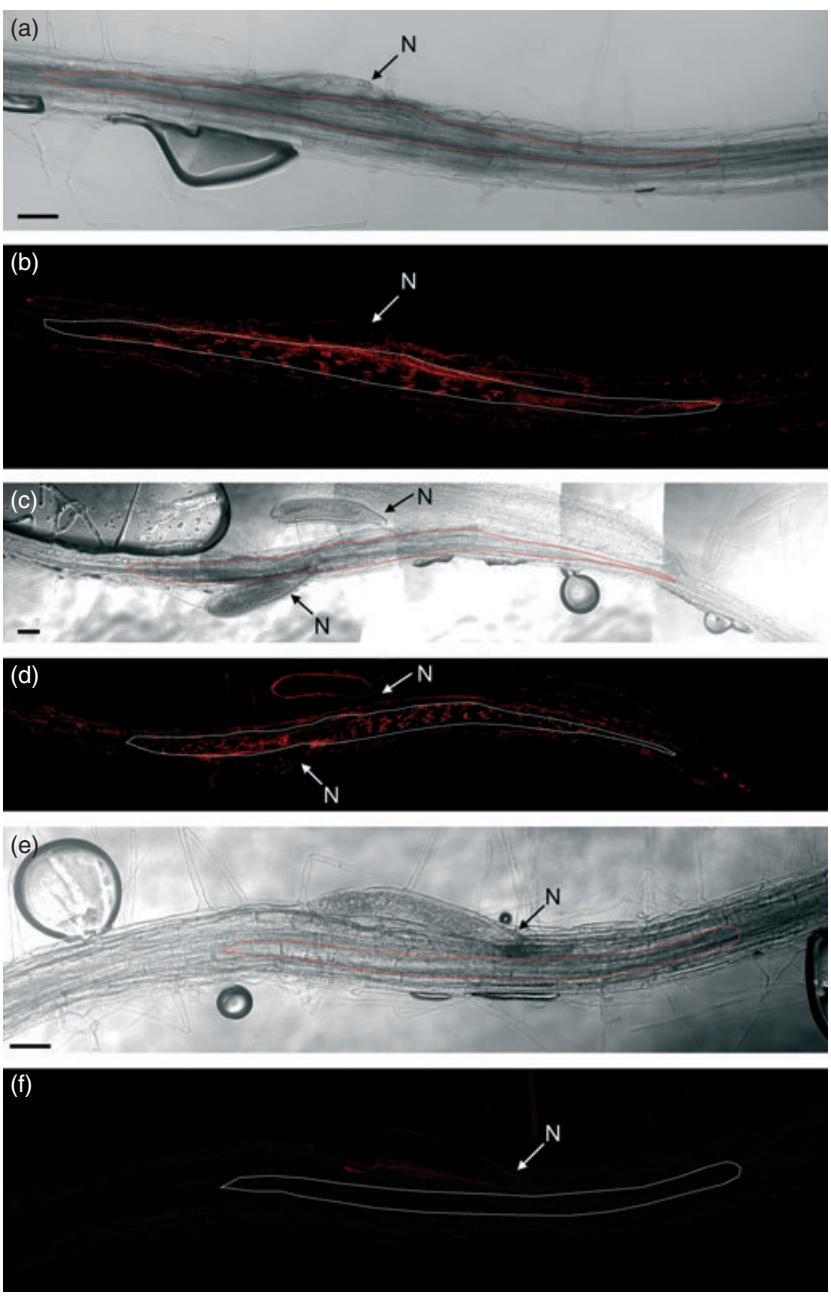

Figure 3. Confocal and differential interference contrast (DIC) images of *CLV2:H2B-mCherry* expression during nematode infection.
(a) Parasitic second-stage juvenile (J2) parasitic with DIC, 4 days post-inoculation (dpi), *CLV2:H2B-mCherry* transgenic line.
(b) Parasitic J2 with mCherry fluorescence, 4 dpi, *CLV2:H2B-mCherry* transgenic line.
(c) Parasitic third-stage juvenile (J3) with DIC, 6 dpi, *CLV2:H2B-mCherry* transgenic line.
(d) Parasitic J3 with mCherry fluorescence, 6 dpi, *CLV2:H2B-mCherry* transgenic line.
(e) Parasitic J2 with DIC, 4 dpi, wild-type plants.
(f) Parasitic J2 with mCherry fluorescence, 4 dpi, wild-type plants.
Abbreviations: N, nematode. Outline indicates the area of the syncytium. Scale bars, 50 μm.

feeding sites (Figure 3c,d). No autofluorescence was detected in the nuclei of syncytia fed upon by parasitic J2s in wild-type plants (Figure 3e,f).

Mutant alleles of *CLV2* and *CRN* cause a reduction in nematode infection and defects in syncytial size

By using an RNA interference (RNAi) approach targeting nematode *CLE* genes, previous reports have shown that nematode CLE peptides are important for successful infection of host plant roots (Bakhetia *et al.*, 2007; Patel *et al.*, 2008). To determine if perception of nematode CLEs by CLV2 or the CLV2/CRN complex is required, root infection assays with nematodes were performed on the *clv2-1* and *crn-1* single mutants and the *crn-1 clv2-1* double mutant. According to Muller *et al.* (2008), *crn-1 clv2-1* is morphologically indistinguishable from either of the single mutants, indicating that they act in the same pathway. The mutant alleles and the wild-type L*er* were randomized in 12-well plates and grown on modified Knop's medium. Two weeks after germination, seedlings were inoculated with infective J2s. J4 females were counted at 14 days post-inoculation (dpi) and adult females were counted at 30 dpi. Both the single and double mutants showed a statistically significant reduction in nematode infection with the exception of *crn-1* at 14 dpi (Figure 4a). At 30 dpi nematode infection was reduced by approximately 25% in all receptor mutants tested. A similar reduction in nematode infection across all mutant lines supports the hypothesis that CLV2 and CRN are acting in the same signaling pathway. Using *sol2-1*, we observed a 40% reduction in nematode infection

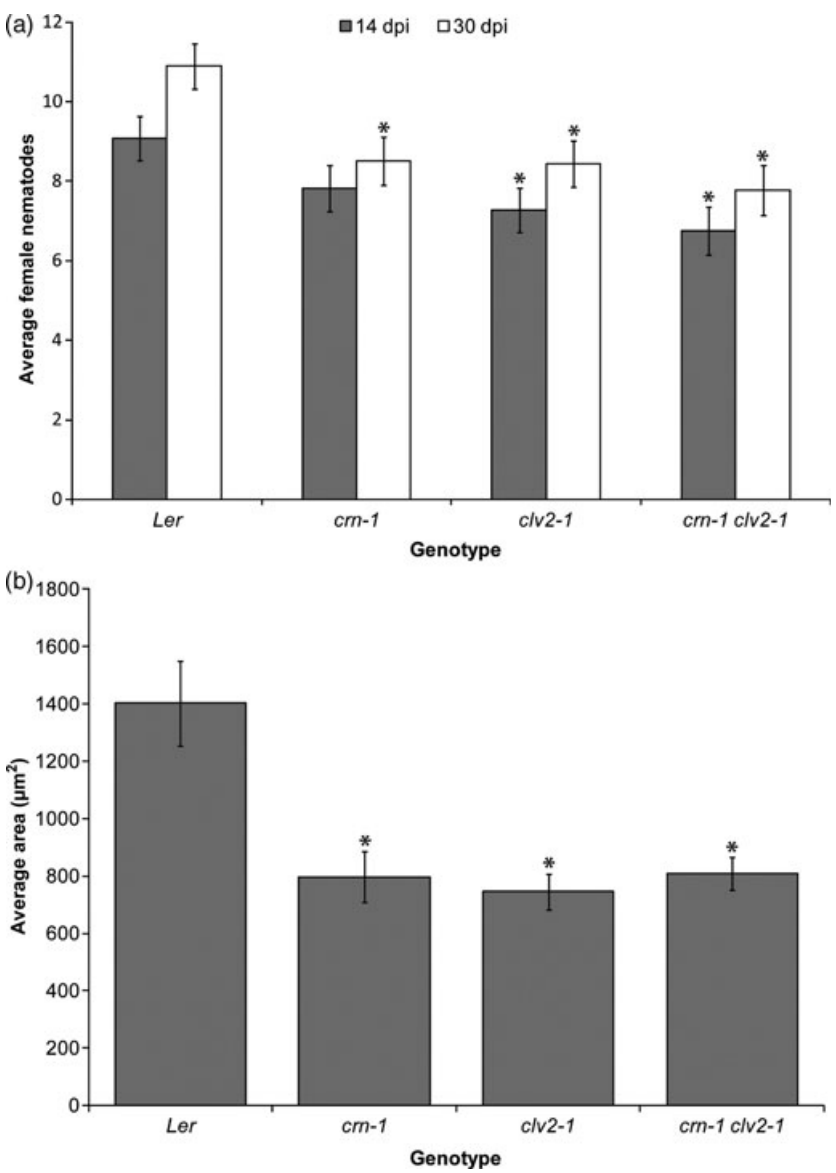

Figure 4. Effect of *clv2-1* and *crn-1* mutant alleles on *Heterodera schachtii* infection.
(a) Fourth-stage juvenile (J4) females were counted at 14 days post-inoculation (dpi) and adult females were counted at 30 dpi. Data represent mean ± SE, *n* = 35 for L*er*, 32 for *crn-1*, 34 for *clv2-1*, and 29 for *crn-1 clv2-1*. Data are representative of three independent experiments.
(b) Seedlings were grown on vertical square plates for 10 days and inoculated with 10 second-stage juveniles (J2s) per root. At 14 dpi, syncytia that fed only one nematode and appeared translucent were microscopically examined and their area was determined. Data represent mean ± SE, *n* = 11 for L*er* and *crn-1*, 14 for *clv2-1*, and 12 for *crn-1 clv2-1*.
Asterisks indicate statistically significant differences compared with L*er* by Student's *t*-test ($P < 0.05$).

(Figure S3a). Since the establishment of a feeding site is required for nematode development and reproduction, the above observations motivated us to determine if there were any defects in syncytial size between the receptor mutants and the wild type. The mutant alleles and the wild-type L*er* were grown on vertical square plates and inoculated with infective J2s. At 14 dpi, syncytia that were transparent and fed upon by only one nematode were measured. The average area of wild-type L*er* syncytia was 1402 ± 147 μm^2 (Figure 4b). In contrast, a statistically significant reduction of 40% in syncytium size was observed in the receptor mutants. The average area of *crn-1*, *clv2-1*, and *crn-1 clv2-1* was 797 ± 89, 745 ± 61, and 808 ± 57 μm^2, respectively (Figure 4b). The same reduction in syncytium size was seen in the *sol2-1* mutant allele (Figure S3b).

DISCUSSION

Nematode *CLE* genes have been found to be upregulated in the dorsal esophageal gland cell at the onset of parasitism and remain on through the adult female life stage. *CLE* genes are turned off in adult males that are no longer feeding (Wang *et al.*, 2005; Patel *et al.*, 2008; Lu *et al.*, 2009; Wang *et al.*, 2010a). In SCN and BCN, immunolocalization studies have localized nematode CLEs along the dorsal gland extension and in the ampulla at the base of the nematode stylet, indicating they are secreted into host plant roots via the stylet (Wang *et al.*, 2005; Patel *et al.*, 2008; Wang *et al.*, 2010a). Consistent with these results an immunofluorescence study found that SCN CLEs are secreted directly into the root cytoplasm of the host plant (Wang *et al.*, 2010a). The variable domain of SCN CLEs is then able to redirect the proteins into the apoplast where they can act as plant CLE ligand mimics by interacting with extracellular membrane-bound plant CLE receptors. However, thus far, host plant receptors that perceive nematode CLE signals have not been identified.

Many studies have used synthetic CLE peptides to help determine the roles that plant CLE peptides play in plant growth and development. Previous studies have shown that nematode CLE peptides cause root growth phenotypes similar to other plant CLEs (Lu *et al.*, 2009; Wang *et al.*, 2010a,b). Other studies have also shown that these peptide screens can identify receptors that may be involved in certain CLE signaling pathways by utilizing receptor mutants (Fiers *et al.*, 2005; Stahl *et al.*, 2009; Meng *et al.*, 2010). To identify potential nematode CLE receptors we tested mutants of plant CLE receptors implicated in CLE signaling in the RAM. In the root, exogenous peptide assays and overexpression studies have shown that CLV2 is required for proper proximal meristem function (Stahl *et al.*, 2009; Meng *et al.*, 2010). In addition, a new member of the receptor kinase family, CRN, forms a heterodimer with CLV2 and is required for proper localization of the CLV2/CRN complex to the plasma membrane (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010). In Arabidopsis, *CRN* has been found to be widely expressed in both shoot and root tissues, suggesting dual roles in shoot and root development (Muller *et al.*, 2008). *CLV2* has been found to be expressed in shoot tissues (Jeong *et al.*, 1999), but less is known about its expression in the root. In this work we screened a null mutant allele of *CLV2* and an amorphic mutant allele of *CRN* for resistance to the nematode CLE peptides. Both *clv2-1* and *crn-1* were resistant to HgCLE, HsCLE1, and HsCLE2 peptides (Figures 1 and S1). Similar to synthetic peptide assays, overexpression of *HgCLE*, *HsCLE1*, and *HsCLE2* in the *clv2-1* and *crn-1* mutant backgrounds abolished the *wus*-like phenotypes seen when the nematode CLEs are overexpressed in wild-type backgrounds (Wang *et al.*, 2005, 2010a,b). Taken together, the peptide assays and overexpression data indicate that CLV2 and CRN are required for perception of nematode CLE.

In order to serve as a receptor complex for nematode CLE peptides, *CLV2* and *CRN* would most likely need to be expressed in feeding cell initials as well as the developing feeding sites. Using promoter–reporter lines we confirmed that both *CLV2* and *CRN* were expressed in nematode-induced syncytia (Figures 2 and 3), consistent with a role in nematode CLE perception. The recent detection of *CLV2* and *CRN* expression from microaspirated syncytial contents at 5 dpi by microarray analysis (Szakasits *et al.*, 2009) supports this finding. It is also possible that nematode CLE receptors are expressed in the cells adjacent to the expanding syncytium. As the nematode CLEs are redirected to the host root apoplast, extracellular receptors of the adjacent cells that are primed for incorporation could trigger plant CLE signaling pathways needed to fully form the syncytium. In the future it will be interesting to more precisely localize the CLV2 and CRN proteins within syncytia using immunofluorescence techniques. This will aid in determining whether or not these nematode CLE receptors are localized within the cell wall openings that occur during syncytium formation or if they are localized on the outer plasma membrane of the syncytium and/or adjacent cells.

Previous reports have demonstrated that SCN and BCN CLEs are important for nematode parasitism by showing a reduction in nematode infection after knocking down *CLE* expression in the worm using RNAi approaches (Bakhetia *et al.*, 2007; Patel *et al.*, 2008). To directly test for a role of CLV2/CRN in nematode CLE perception we performed infection assays on the receptor mutants. We showed that a reduction in nematode infection occurs on the receptor mutants (Figures 4a and S3). Concurrently, we also saw a reduction in syncytium size in the receptor mutants (Figures 4b and S3). The fact that we saw a similar reduction in both nematode infection and syncytium size in both the single and double mutants is consistent with genetic and biochemical data that CLV2 and CRN are acting in the same pathway (Muller *et al.*, 2008; Bleckmann *et al.*, 2010; Zhu

et al., 2010). These data indicate that not only is nematode CLE perception by CLV2 and CRN important for successful nematode infection, but demonstrates that CLE signaling also plays a role in feeding cell formation.

The involvement of CRN in nematode CLE signaling also opens up the interesting possibility that nematode CLE signaling may be directly or indirectly suppressing host plant defense responses. In root tips of *sol2-1*, another mutant allele of *CRN*, plant disease resistance-related and stress responsive genes were upregulated (Miwa *et al.*, 2008). Therefore, when nematode CLEs are secreted they could activate the CLV2/CRN signaling pathway leading to a suppression of plant disease resistance-related and plant stress responsive genes. One might speculate that the main target for nematode CLEs is a signaling pathway which allows developmental programming of root cells for syncytium formation to occur and that suppression of plant defense responses is just an added benefit to the nematode. Alternatively, the nematode may require suppression of plant defense responses through plant CLE signaling in order for the syncytium to form properly. Further studies will need to be performed to investigate this possibility.

Several possibilities exist for why we only see a partial reduction in nematode numbers and syncytium size in the *clv2-1* and *crn-1* mutant backgrounds. First, besides CLEs, nematodes secrete many different effectors that probably play an important role in feeding cell formation (Wang *et al.*, 2001; Gao *et al.*, 2003). For example, when BCN *CLE*s were targeted with RNAi a similar partial reduction in nematode infection was observed (Patel *et al.*, 2008), either as a consequence of limited reductions in transcript levels or an indication that the other effectors still active in the nematode allow infection to proceed. A second possibility for the partial reduction in the receptor mutants is that there could be multiple nematode CLE receptors. The nematode *CLE*s reported so far belong to gene families (Lu *et al.*, 2009; Wang *et al.*, 2010a,b). In addition, PCN CLEs have multiple CLE motifs that may be simultaneously processed to release different CLE peptides (Lu *et al.*, 2009). This leaves the possibility that nematode CLE peptides may activate multiple plant CLE signaling pathways concurrently to function in an antagonistic or synergistic fashion as reported for plant CLEs (Whitford *et al.*, 2008). The current plant CLV3 signaling pathway in the shoot indicates that there are parallel signaling pathways. Genetic evidence indicates that CLV1 acts in a separate pathway from the CLV2/CRN pathway (Muller *et al.*, 2008). In support of the genetic data, recent reports using luciferase complementation assays and FRET analysis have shown that CLV1 forms a homodimer and that CLV2 and CRN form a heterodimer without CLV3 stimulation (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010). There is also evidence for CLV1 interacting with the CLV2/CRN complex, leading to the possibility that different signaling pathways could be activated depending on which receptor in the complex interacts with the CLE ligand (Bleckmann *et al.*, 2010; Guo *et al.*, 2010; Meng and Feldman, 2010; Zhu *et al.*, 2010). Recently, various plant CLE peptides were used as cold competitors for radiolabeled CLV3 CLE binding to CLV1, CLV2, and the CLV1-related BARELY ANY MERISTEM (BAM) 1 and BAM2 (Guo *et al.*, 2010). Arabidopsis CLE5, which has an identical CLE motif to the recently identified nematode CLE, HsCLE2 (Wang *et al.*, 2010b), was included in this study. CLE5 provided either full or partial competition to CLV3 binding for all four receptors (Guo *et al.*, 2010), providing direct evidence that the nematode CLE and CLV2 can form a receptor–ligand complex. However, the binding was not specific to CLV2. Thus it is possible that in the *crn-1 clv2-1* double mutants, nematodes are still able to signal through other receptors in the roots.

Additional candidate receptors could include CLV1 and/or BAM1 and BAM2. Unlike *CLV2*, which has a broad expression pattern in plants, *CLV1* expression is thought to be restricted to the center of the SAM and its function is thought to be confined to stem cell specification in the shoot (Clark *et al.*, 1997; Fletcher *et al.*, 1999). Therefore, in order to utilize CLV1 as a receptor, nematodes would have to activate *CLV1* expression in the roots. Recently, BAM1 and BAM2 have been shown to act redundantly in the SAM and are widely expressed throughout the plant, including in root tissues (DeYoung *et al.*, 2006; DeYoung and Clark, 2008). We have found that *bam1* is also resistant to exogenous application of synthetic nematode CLE peptides (AR, S. Chen, XW and MGM, unpublished data). Furthermore, there are over 200 LRR-RLKs in Arabidopsis and only a few receptor–CLE ligand pairs have been characterized (Shiu and Bleecker, 2001). Thus, further studies using reporter fusions and a combination of mutants will need to be performed to investigate the possible involvement of other host plant receptors in nematode CLE signaling.

To date, the exact function of nematode CLE proteins in syncytium formation is unresolved. However, this paper has shown that nematode CLE signaling through the CLV2/CRN receptor complex is important for proper syncytium formation and ultimately successful nematode infection. These findings open the door for identifying the downstream signaling components regulated by CLV2/CRN to uncover the role that nematode CLE signaling plays in syncytium formation.

EXPERIMENTAL PROCEDURES

Peptide assays

Arabidopsis seeds were sterilized using the chlorine gas method (Wang *et al.*, 2010b). Sterilized seeds were germinated on vertical plates in a growth chamber at 22°C under long-day conditions (16 h light/8 h dark) containing synthetic peptides (Sigma-Genosys, http://www.sigmaaldrich.com) as previously described (Wang *et al.*, 2010b). The *clv2-1* mutant in the L*er* background (Koornneef

et al., 1983) was obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). The *crn-1* mutant in the L*er* background (Muller, 2008) and the *sol2-1* mutant in the Utr background (Miwa *et al.*, 2008) have been described previously. The HgCLEp, HsCLE1p, and HsCLE2p peptides used in this study were as described (Wang *et al.*, 2010b). Two days after germination, root length was marked each day for 9 days. Plates were scanned using an Epson Perfection V200 PHOTO scanner (http://www.epson.com/) and total root length was determined using Scion Image. Primary root tips of Arabidopsis were mounted on glass slides and visualized with an Olympus Vanox AHBT3 microscope (http://www.olympus.com/) equipped with Nomarski optics.

Overexpression in mutant backgrounds

The *CLE* gene sequences from the SCN (*HgCLE2*^{4SP}) and the BCN (*HsCLE1* and *HsCLE2*) used to generate the overexpression constructs were previously described (Wang *et al.*, 2010a,b). Constructs were transformed into the mutant backgrounds using the Arabidopsis floral dip method (Clough and Bent, 1998). Seeds from primary Arabidopsis transformants (T_1) were selected on 0.5 × MS medium [MS basal nutrients salts (Caisson Laboratories, http://www.caissonlabs.com/), 2% sucrose, 0.8% Type A agar (Sigma, http://www.sigmaaldrich.com/), pH 5.7] containing 50 μg ml^{-1} timentin (GlaxoSmithKline, http://www.gsk.com/) to control *Agrobacterium* contamination and 50 μg ml^{-1} kanamycin and grown under the same conditions as above. Seedlings resistant to kanamycin were transplanted to soil 7 days after germination. Two weeks after transplanting to soil the shoot phenotypes were observed.

PROMOTER-REPORTER LINES

CRN:GUS has been previously described and characterized (Muller *et al.*, 2008). To generate *CLV2:H2B-mCherry*, vector pMDC99 (Curtis and Grossniklaus, 2003) was modified by introducing the CDS of chimeric construct mCherry-H2B at the 3′ site of the gateway cassette using the unique *Pac*I restriction site to give pAB149. To analyze the expression of *CLV2* 1252 bp of the 5′ region and 9 bp of the CDS were amplified using the primers AB_CLV2_Pro_F (5′ CACCAGACACAAAGCCCTTTCCATTGTC 3′) and AB_CLV2_Pro_R (5′ CTTTATCATAGCTCAGAGGA 3′) to give a CACC-TOPO-containing amplicon, which was cloned into pENTR/D-TOPO® (Invitrogen, http://www.invitrogen.com/). This entry clone was used in a LR reaction with pAB149 to give pAB183 (*CLV2:H2B-mCherry*).

Nematode infection of promoter–reporter lines

The BCN *H. schachtii* was propagated on greenhouse-grown sugar beets (*Beta vulgaris* cv Monohi). The eggs of the BCN were isolated and hatched as previously described (Mitchum *et al.*, 2004). After 2 days, second-stage juveniles (J2) were collected and surfaced-sterilized according to Wang *et al.* (2007), except that 0.004% mercuric chloride, 0.004% sodium azide, and 0.002% Triton X-100 were used. Sterilized seeds were grown on modified Knop's medium with Daishin agar (Brunschwig Chemie, http://www.brunschwig-ch.com/) (Sijmons *et al.*, 1991). Ten days after germination, seedlings were inoculated with 20 sterilized J2 per root.

Histochemical β-glucuronidase (GUS) assays

At the indicated timepoints, freshly excised *CRN:GUS* tissues were infiltrated with GUS substrate buffer [0.5 mM 5-bromo-4chloro-3-indolyl glucuronide, 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), pH 7.0, 50 mM NaCl, 0.06% Triton X-100, 3 mM potassium ferricyanide) and incubated overnight at 37°C (Jefferson *et al.*, 1987). Stained roots were placed in glass Petri dishes and visualized with a Nikon Eclipse TS100 inverted microscope (http://www.nikon.com/).

Confocal microscopy

CLV2:H2B-mCherry seed was sterilized, grown, and inoculated with nematodes as described above. At the indicated timepoints, infected roots were mounted on glass slides and visualized with a 510 META confocal scanning microscope (Carl Zeiss, http://www.zeiss.com/) excited at 543 nm.

Infection assay with receptor mutants

Sterilized receptor mutants were plated in 12-well Falcon tissue culture plates (BD Biosciences, http://www.bdbiosciences.com/) containing modified Knop's medium with 0.8% Daishin agar in a randomized block design. Plants were grown at 24°C with a 12-hour photoperiod. Fourteen days after germination, seedlings were inoculated with 200 surface-sterilized BCN J2. The J4 females were counted at 14 dpi and adult females were counted at 30 dpi. The average values were calculated and significant differences were determined by using Student's *t*-test ($P < 0.05$). To measure syncytium size, receptor mutants were germinated on modified Knop's medium in vertical square plates and inoculated at 10 days after germination with 10 surface-sterilized BCN J2. At 14 dpi, syncytia that were transparent and fed upon by only one nematode were visualized with a Nikon Eclipse TS100 inverted microscope and photographed using a Nikon COOLPIX 5000 digital camera. The syncytia were outlined using the Adobe Photoshop CS5 magnetic lasso tool and the area of the longitudinal section was calculated by the software. This is similar to the approach others have recently taken to measure the area of syncytia (Siddique *et al.*, 2009; Hofmann *et al.*, 2010). Significant differences were determined by using Student's *t*-test ($P < 0.05$).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Response of wild-type (Utr) and *sol2-1* seedlings to the synthetic 12-aa nematode CLE peptide.

Figure S2. Confocal and differential interference contrast (DIC) images of *CLV2:H2B-mCherry* expression in uninfected roots.

Figure S3. Effect of *sol2-1* mutant allele on *Heterodera schachtii* infection.

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