Identification of potential host plant mimics of CLAVATA3/ESR (CLE)-like peptides from the plant-parasitic nematode *Heterodera schachtii*

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SUMMARY

In this article, we present the cloning of two *CLAVATA3/ESR* (CLE)-like genes, *HsCLE1* and *HsCLE2*, from the beet cyst nematode *Heterodera schachtii*, a plant-parasitic cyst nematode with a relatively broad host range that includes the model plant *Arabidopsis*. CLEs are small secreted peptide ligands that play important roles in plant growth and development. By secreting peptide mimics of plant CLEs, the nematode can developmentally reprogramme root cells for the formation of unique feeding sites within host roots for its own benefit. Both *HsCLE1* and *HsCLE2* encode small secreted polypeptides with a conserved C-terminal CLE domain sharing highest similarity to *Arabidopsis* CLEs 1–7. Moreover, *HsCLE2* contains a 12-amino-acid CLE motif that is identical to AtCLE5 and AtCLE6. Like all other plant and nematode CLEs identified to date, *HsCLEs* caused *wuschel*-like phenotypes when overexpressed in *Arabidopsis*, and this activity was abolished when the proteins were expressed without the CLE motif. *HsCLEs* could also function *in planta* without a signal peptide, highlighting the unique, yet conserved function of nematode CLE variable domains in trafficking CLE peptides for secretion. In a direct comparison of *HsCLE2* overexpression phenotypes with those of AtCLE5 and AtCLE6, similar shoot and root phenotypes were observed. Exogenous application of 12-amino-acid synthetic peptides corresponding to the CLE motifs of *HsCLEs* and AtCLE5/6 suggests that the function of this class of CLEs may be subject to complex endogenous regulation. When seedlings were grown on high concentrations of peptide (10 μ M), root growth was suppressed; however, when seedlings were grown on low concentrations of peptide (0.1 μ M), root

growth was stimulated. Together, these findings indicate that AtCLEs1–7 may be the target peptides mimicked by *HsCLEs* to promote parasitism.

INTRODUCTION

Feeding cells, called syncytia, are a *de novo* cell type formed within host plant roots by obligate sedentary endoparasitic cyst nematodes (*Heterodera* and *Globodera* spp.) that serve as nutrient sinks to support nematode growth and development. These syncytial cells share developmental characteristics with a variety of different plant cell types, including meristematic cells, endosperm cells, transfer cells and developing xylem (Mitchum *et al.*, 2008). Secretory effector proteins originating in the oesophageal gland cells and delivered into root tissues via the nematode stylet are believed to provide the signals required for the induction and maintenance of the syncytium (Davis *et al.*, 2008). Cyst nematode-secreted CLAVATA3/ESR (CLE)-like effector proteins, shown to function as ligand mimics of plant CLE peptides (Lu *et al.*, 2009; Wang *et al.*, 2005, 2010), are strong candidates for a role in the developmental reprogramming of root cells for syncytium formation.

Plant CLEs have been shown to function as small secreted peptide ligands that bind to extracellular receptors and activate signalling cascades regulating aspects of plant growth and development, including shoot and floral meristem maintenance (Brand *et al.*, 2000; Clark *et al.*, 1995; Rojo *et al.*, 2002), root apical meristem maintenance (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2005; Hobe *et al.*, 2003) and vascular cell division (Etchells and Turner, 2010; Whitford *et al.*, 2008), in both monocotyledonous and dicotyledonous plants (Cock and

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McCormick, 2001; Oelkers *et al.*, 2008). Most CLEs promote the differentiation of stem cells; however, a subgroup of CLEs, including CLE41/44 and CLE42, display activity to inhibit tracheary element differentiation (Ito *et al.*, 2006). It has also been reported that CLE peptides in *Medicago truncatula* can locally and systemically control nodulation (Mortier *et al.*, 2010). However, the function of individual CLE proteins, including their recognition and processing, is for the most part unknown.

CLE-like proteins have been functionally characterized from the soybean cyst nematode (*Heterodera glycines*; HgCLEs) and potato cyst nematode (*Globodera rostochiensis*; GrCLEs), two of the most agronomically important cyst nematode species (Lu *et al.*, 2009; Wang *et al.*, 2005, 2010). However, neither of these cyst nematodes can parasitize model plant systems, such as Arabidopsis, Medicago or Lotus; thus, functional studies to decipher the role of nematode CLEs in syncytium formation are limited to the crop plants that they infect. Although the genome sequences for most crop species are available or in progress, many are highly complex, stable transformation is time-consuming and reverse genetic resources are limited. Therefore, the identification of CLE-like proteins from cyst nematodes that can parasitize model plant systems has the potential to accelerate our understanding of nematode CLE signalling.

The beet cyst nematode, *Heterodera schachtii*, is a close relative of the soybean cyst nematode, which infects the model plant Arabidopsis (Sijmons *et al.*, 1991). We have reported previously a CLE-like gene sequence isolated from *H. schachtii*, named *HsSYV46* (Patel *et al.*, 2008). mRNA *in situ* hybridization localized *HsSYV46* transcripts within the dorsal oesophageal gland cell of parasitic life stages of *H. schachtii*, similar to HgCLEs (Wang *et al.*, 2005) and GrCLEs (Lu *et al.*, 2009). Immunolocalization studies using a peptide antibody directed against HgCLE2 (formerly Hg4G12; Davis, 2009; Wang *et al.*, 2005) also cross-reacted with *HsSYV46*, and detected the protein in the dorsal gland cell and along the extension into the ampulla at the base of the nematode stylet (Patel *et al.*, 2008), indicating that the beet cyst nematode CLEs are probably secreted from the stylet. Transgenic Arabidopsis expressing dsRNA directed against *HsSYV46* was less susceptible to *H. schachtii*, indicating that nematode CLE peptides are required for the successful infection of host plant roots (Patel *et al.*,

2008). Nonetheless, no functional analyses have been performed on this CLE-like gene to date.

In this study, we report the identification of two new CLE-like genes from *H. schachtii* and conduct functional analyses of the encoded CLE peptides. Overexpression studies and peptide assays demonstrated that HsCLEs are biologically active plant CLE mimics in Arabidopsis, and identified AtCLEs1–7 as potential target peptides mimicked by HsCLEs to promote parasitism.

RESULTS

Identification of HsCLE1 and HsCLE2

Primers corresponding to the untranslated region (UTR) of the *H. glycines* CLE gene *HgCLE1* (Wang *et al.*, 2010) were designed and used to amplify CLE-like genes from cDNA generated from parasitic life stages of *H. schachtii* by polymerase chain reaction (PCR). A 0.5-kb amplified fragment was cloned into pCR4-TOPO vector and 48 clones were sequenced. Two different sequences were identified. The putative protein sequences shared more than 80% identity to HgCLEs, and were named HsCLE1 and HsCLE2. *HsCLE1* encoded a predicted protein of 139 amino acids containing a putative 24-amino-acid N-terminal signal peptide (SP) for secretion, determined using the SignalP program (Emanuelsson *et al.*, 2007). *HsCLE2* encoded a predicted protein of 138 amino acids containing a putative 21-amino-acid SP (Fig. 1a). The remainder of each protein consisted of a variable domain (VD) and a conserved 12-amino-acid C-terminal CLE motif (Fig. 1a).

Phylogenetic analysis of nematode and Arabidopsis CLEs

A phylogenetic analysis of the conserved 12-amino-acid CLE motif sequences of nematode CLEs and the 32-member Arabidopsis CLE family grouped the HsCLEs with Arabidopsis CLEs 1–7 (Fig. 1b). Based on the analysis by Oelkers *et al.* (2008), Arabidopsis CLEs 1–7 belong to group 2, one of the largest groups of plant CLEs. Our analysis also revealed that HsCLE2 shared an identical 12-amino-acid CLE motif with AtCLE5 and AtCLE6 (Fig. 1c), which indicated that HsCLE2 might have the same biological activity as these Arabidopsis CLEs. This was investigated further.

Fig. 1 Amino acid sequence analysis of HsCLEs. (a) Putative protein sequence alignment of HsCLE1 and HsCLE2 generated using the T-Coffee program (Notredame *et al.*, 2000). The sequences highlighted in grey correspond to the signal peptide sequences predicted by the SignalP program (Emanuelsson *et al.*, 2007). The 12-amino-acid CLAVATA3/ESR (CLE) motifs are indicated by a black line. (b) An unrooted neighbour-joining tree of nematode and Arabidopsis dodeca-CLE peptides generated using the PAUP program. Bootstrap values of 50% or higher are shown. The scale bar indicates the number of amino acid substitutions per site. At, *Arabidopsis thaliana*; Gr, *Globodera rostochiensis*; Hg, *Heterodera glycines*; Hs, *Heterodera schachtii*. (c) Amino acid sequence alignment of HsCLE2, AtCLE5 and AtCLE6 generated using the T-Coffee program. Sequences highlighted in grey correspond to signal peptide sequences. The 12-amino-acid CLE motifs are indicated by a black line.

a

Signal peptide

HsCLE1 MPNIFKILLIVLFAVISFSSSASTDGKKTANDGSGSNSSVGIGTKIKRIVTAGLLFTSLA 60
HsCLE2 MPNICKILLIVLLAVISFSLSDSTDGEKTANDGNGSNSSAGIGTKIKRIVTAGLLFTSLA 60
**** *****:***** * ****:*****.*****.*****.*****

HsCLE1 TGAQAIGRGNAQGVNAAGLVPSHVNTNRSMAPPPPPAQFEKGATRVEKMKAHLELAEKM 120
HsCLE2 AGGAEAIGRNNAQGGNAAGLVPSHVNTNRAMAPPPPPAQFEKGATRLEKMRASLLELSEKM 120
:***:***.*** *****:*****:*****:*****:***:*** **

HsCLE1 PPVNESKRLSPSGPDPQHH 139
HsCLE2 TD-KEPKRVSPGGPDPQHH 138
. :*.**:*.*.***:***

CLE motif

b **c**

Signal peptide

HsCLE2 MPN-ICKILLIVLLAVISFSLSDSTDGEKTANDGNGSNSSAGIGTKIKRIVTAGLLFTSL 59
AtCLE5 MATLILKQTLIILLIIFSLQTLSSQA-----RI-LRSYRAVSMGNM---- 40
AtCLE6 MANLILKQSLIILLIISTPILSSQA-----RI-LRTYRPTTMGDM---- 40
*.. * * **:* * : * . *

HsCLE2 AAGGAEAIGRNNAQGGNAAGLVPSHVNTNRAMAPPPPPAQFEKGATRLEKMRASLLELSEK 119
AtCLE5 -----DSQVL-----LHELGFDSLKFK----GHNER 62
AtCLE6 -----DSQVL-----LRELGIDLKFK----GQDER 62
*: * :.: . *.*: .*:

HsCLE2 MTDKEPKRVSPGGPDPQHH 138
AtCLE5 RFLVSSDRVSPGGPDPQHH 81
AtCLE6 RFLVDSERVSPGGPDPQHH 81
... *****

CLE motif

Fig. 2 Overexpression of *HsCLE1* and *HsCLE2* caused a range of *wuschel* (*wus*)-like phenotypes in *Arabidopsis*. (a, f) Representative of wild-type seedlings. (b–e, g–i) Representative *wus*-like phenotypes for *HsCLE1* and *HsCLE2* overexpression. (a) Wild-type seedling 3 weeks post-germination. (b) Three-week-old seedling exhibiting shoot apical meristem termination. (c, d) Ten-week-old seedling exhibiting inflorescence meristem termination. (e) A plant exhibiting floral meristem termination resulting in reduced silique production. The inset shows a close-up view of a silique from a wild-type plant (left) and *HsCLE* overexpression line (right). (f) Wild-type flower. (g–i) Flowers showing reduced floral organ numbers.

Construct	Phenotype of transgenic seedlings (T1)			Total no. of seedlings (%)
	Severe	Weak	No phenotype	
<i>HsCLE1</i>	47	11	98	156 (37)
<i>HsCLE1</i> ^{ASP}	56	29	71	156 (55)
<i>HsCLE1</i> ^{ΔCLE}	0	0	44	44 (0)
<i>HsCLE1</i> ^{ASPΔCLE}	0	0	52	52 (0)
<i>HsCLE2</i>	9	8	141	158 (11)
<i>HsCLE2</i> ^{ASP}	118	7	19	144 (87)
<i>HsCLE2</i> ^{ΔCLE}	0	0	52	52 (0)
<i>HsCLE2</i> ^{ASPΔCLE}	0	0	93	93 (0)

Table 1 Summary of *HsCLE1* and *HsCLE2* above-ground *wuschel* (*wus*)-like phenotypes in T1 transgenic seedlings.

HsCLEs function as ligand mimics of Arabidopsis CLEs

To study the function of *HsCLEs*, full-length *HsCLE1* and *HsCLE2* were cloned into the binary vector pMD1 under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and transformed into *Arabidopsis*. The phenotypes of the transgenic lines were characterized in the T1 generation. Transgenic plants overexpressing *HsCLE1* and *HsCLE2* showed similar above-ground

wuschel (*wus*)-like phenotypes, including premature termination of the shoot apical meristem (SAM) and reduced floral organ number compared with wild-type plants (Fig. 2, Table 1).

Previous studies have shown that a SP is required to target plant CLEs to the extracellular space to function (Meng *et al.*, 2010; Rojo *et al.*, 2002). In contrast, a SP is not required for *H. glycines* and *G. rostochiensis* CLE function *in planta* (Lu *et al.*, 2009; Wang *et al.*, 2010). To test whether a SP was required for

HsCLE function *in planta*, HsCLE1 and HsCLE2 without their respective SPs (*HsCLE^{ASP}*) were cloned into pMD1 and overexpressed in Arabidopsis. Similar to the other nematode CLE proteins, HsCLEs without a SP caused above-ground *wus*-like phenotypes when overexpressed in Arabidopsis. Interestingly, the percentage of plants exhibiting *wus*-like phenotypes was higher in plants overexpressing *HsCLE1^{ASP}* (55%) and *HsCLE2^{ASP}* (87%) relative to plants expressing *HsCLE1* (37%) and *HsCLE2* (11%) with a SP. In an earlier study, we demonstrated that the VD of HgCLEs can target CLE peptides to the extracellular space through an unknown pathway (Wang *et al.*, 2010). The fact that HsCLEs can function without a SP when overexpressed in Arabidopsis and the high level of sequence identity (86%) between the HgCLE and HsCLE VD sequences suggest that the trafficking function of nematode CLE VDs is conserved.

Previous studies have shown that the CLE motif is absolutely required for plant and nematode CLE function (Fiers *et al.*, 2006; Lu *et al.*, 2009; Meng *et al.*, 2010; Ni and Clark, 2006; Wang *et al.*, 2010). *HsCLE* genes lacking a CLE motif or both the CLE motif and SP were cloned into pMD1 and overexpressed in Arabidopsis. Deletion of the 12-amino-acid CLE motif abolished HsCLE activity *in planta* (Table 1). Taken together, these data indicate that both HsCLE1 and HsCLE2 are biologically active plant CLE mimics in Arabidopsis.

Functional similarity between HsCLE2 and Arabidopsis AtCLE5 and AtCLE6

The finding that HsCLE2 shared an identical 12-amino-acid CLE motif with AtCLE5 and AtCLE6 led us to test the functional similarity between these plant and nematode CLEs. Each gene, with and without a SP, was cloned into pMD1 and overexpressed in Arabidopsis. The phenotypes of transgenic seedlings were characterized in the T1 generation. Consistent with previous reports, the overexpression of full-length AtCLE5 and AtCLE6 in Arabidopsis caused the premature termination of shoot and floral meristems (Meng *et al.*, 2010; Strabala *et al.*, 2006; Table 2). In this study, we observed above-ground *wus*-like phenotypes in 78% (AtCLE5) and 84% (AtCLE6) of transgenic plants (Table 2), similar to the 87% of plants expressing *HsCLE2* without SP (Table 1). However, unlike the HsCLEs, deletion of the AtCLE5 and AtCLE6 SPs abolished their activity, highlighting the functional differences between the plant and nematode CLE VDs.

It has been reported previously that the overexpression of different plant and nematode CLEs can have different effects on plant root growth (Lu *et al.*, 2009; Meng *et al.*, 2010; Strabala *et al.*, 2006; Wang *et al.*, 2010). In this study, we examined the root growth of transgenic plants overexpressing *HsCLE1* and *HsCLE2* without their SPs, alongside plants overexpressing full-length AtCLE5 and AtCLE6. Transgenic T2 seeds derived from T1 plants representing multiple, independent events and displaying severe above-ground *wus*-like phenotypes were plated onto vertical plates to observe root growth. Plants were genotyped for the presence of the transgene and root growth was measured at 10 days after sowing. The average root length of nontransgenic seedlings was compared with the average root length of transgenic seedlings and analysed by Student's unpaired *t*-test in Excel. Two of the five AtCLE5 overexpression lines and one of the five AtCLE6 overexpression lines exhibited a short root phenotype. Although root growth suppression was not severe, the slight reduction in root length was correlated with the presence of the transgene and statistically significantly different ($P \leq 0.05$) from wild-type plants (Table 3). Similarly, three of the five *HsCLE1^{ASP}* and *HsCLE2^{ASP}* overexpression lines displayed short roots comparable with AtCLE5 and AtCLE6 (Table 3).

Concentration-dependent effects of exogenous synthetic HsCLE motif peptides on root growth

To examine the effects of CLE motif peptides on Arabidopsis root growth, seeds were germinated on vertical plates on medium containing individual synthetic 12-amino-acid peptides at concentrations of 0.1, 1 and 10 μ M. Root length was measured at 9 days post-germination. The 12-amino-acid peptides corresponding to the CLE motifs of AtCLE19 (Ito *et al.*, 2006) and HgCLE1/2 (Wang *et al.*, 2010) were included for comparison. Consistent with previous reports (Fiers *et al.*, 2005; Ito *et al.*, 2006), the AtCLE19 peptide caused severe suppression of root growth, resulting in a short-root phenotype. The AtCLE19 peptide was highly effective at 0.1 μ M and root growth was suppressed further with increasing concentrations of peptide in the medium (Fig. 3a). Exogenous treatment of Arabidopsis roots with the HgCLE peptide from *H. glycines* (a close relative of *H. schachtii* that does not parasitize Arabidopsis) also resulted in a short-root phenotype that increased in severity with increasing peptide concentration (Fig. 3a). Interestingly, the HsCLE1 and HsCLE2/

Table 2 Summary of AtCLE5 and AtCLE6 above-ground *wuschel* (*wus*)-like phenotypes in T1 transgenic seedlings.

Construct	Phenotype of transgenic seedlings (T1)			Total no. of seedlings (%)
	Severe	Weak	No phenotype	
<i>AtCLE5</i>	21	8	8	37 (78)
<i>AtCLE5^{ASP}</i>	0	0	82	82 (0)
<i>AtCLE6</i>	55	66	23	144 (84)
<i>AtCLE6^{ASP}</i>	0	0	97	97 (0)

Table 3 Summary of *HsCLE* and *AtCLE* root phenotypes in T2 transgenic lines.

Construct	T2 line	Root length (mean $\pm$ SE)	<i>P</i> value
Wild-type		63.83 $\pm$ 1.15 (<i>n</i> = 44)	
<i>HsCLE1^{ASP}</i>	T2#1-6	62.17 $\pm$ 1.97 (<i>n</i> = 9)	0.54
	T2#2-3	33.35 $\pm$ 5.21 (<i>n</i> = 11)	≤ 0.05
	T2#4-6	65.74 $\pm$ 1.68 (<i>n</i> = 11)	0.42
	T2#7-6	54.64 $\pm$ 2.32 (<i>n</i> = 10)	≤ 0.05
	T2#9-2	33.87 $\pm$ 4.31 (<i>n</i> = 9)	≤ 0.05
<i>HsCLE2^{ASP}</i>	T2#2-4	66.94 $\pm$ 1.41 (<i>n</i> = 11)	0.2
	T2#4-2	51.08 $\pm$ 4.59 (<i>n</i> = 8)	≤ 0.05
	T2#6-1	52.26 $\pm$ 1.36 (<i>n</i> = 8)	≤ 0.05
	T2#7-6	53.53 $\pm$ 1.48 (<i>n</i> = 8)	≤ 0.05
	T2#8-5	58.39 $\pm$ 1.65 (<i>n</i> = 8)	0.06
<i>AtCLE5</i>	T2#1-3	61.01 $\pm$ 1.25 (<i>n</i> = 5)	0.42
	T2#4-3	61.78 $\pm$ 2.14 (<i>n</i> = 8)	0.48
	T2#5-1	40.9 $\pm$ 3.33 (<i>n</i> = 11)	≤ 0.05
	T2#6-4	54.67 $\pm$ 0.74 (<i>n</i> = 9)	≤ 0.05
	T2#6-5	58.35 $\pm$ 2.54 (<i>n</i> = 7)	0.08
<i>AtCLE6</i>	T2#1-3	66.32 $\pm$ 1.18 (<i>n</i> = 9)	0.35
	T2#3-3	61.25 $\pm$ 3.43 (<i>n</i> = 6)	0.45
	T2#4-3	66.12 $\pm$ 2.94 (<i>n</i> = 9)	0.46
	T2#5-8	57.72 $\pm$ 1.17 (<i>n</i> = 9)	≤ 0.05
	T2#6-3	60.36 $\pm$ 2.97 (<i>n</i> = 9)	0.23

AtCLE5/AtCLE6 CLE peptides caused different effects on Arabidopsis root growth, depending on the peptide concentration in the medium. At low concentration (0.1 μ M), the *HsCLE1* and *HsCLE2/AtCLE5/AtCLE6* synthetic CLE motif peptides stimulated Arabidopsis root growth. When the peptide concentration was increased to 1 μ M, the *HsCLE1* peptide had no effect on root growth, but the *HsCLE2/AtCLE5/AtCLE6* peptide caused a slight suppression of root growth. When peptide concentrations were increased to 10 μ M, all peptides caused severe root growth phenotypes (Fig. 3a and Fig. S1). On microscopic examination, the short roots were morphologically thinner than controls, with a significantly decreased number of meristematic cells (Fig. 3b,d). In contrast, roots exposed to 0.1 μ M *HsCLE1* and *HsCLE2/AtCLE5/AtCLE6* peptides were morphologically indistinguishable from controls (Fig. 3b,c).

DISCUSSION

CLE-like genes that have been identified and characterized from phytopathogenic cyst nematodes (Gao *et al.*, 2003; Lu *et al.*, 2009; Patel *et al.*, 2008; Wang *et al.*, 2001, 2005, 2010) encode secreted polypeptides that are delivered into host plant cells (Wang *et al.*, 2010). These polypeptides are processed to produce CLE peptide mimics possibly involved in the activation and/or redirection of plant CLE signalling pathways to initiate and maintain syncytia in host roots (Mitchum *et al.*, 2008). Our current understanding of the function of plant CLEs in growth and development has been primarily investigated using the model plant Arabidopsis (Fiers *et al.*, 2007). Unlike many crop plant species,

all CLE family members have been identified in Arabidopsis, several have been characterized in detail and the corresponding receptors for some of the plant CLE peptide ligands have been identified and studied (Brand *et al.*, 2000; Clark *et al.*, 1995; Etchells and Turner, 2010; Fiers *et al.*, 2005; Hobe *et al.*, 2003; Muller *et al.*, 2008; Rojo *et al.*, 2002; Strabala *et al.*, 2006; Whitford *et al.*, 2008). Thus, Arabidopsis provides a genetically tractable model system for the study of nematode CLE signalling. Overexpression of nematode CLE genes in Arabidopsis provided the first evidence that nematode CLEs could function as ligand mimics of plant CLEs (Wang *et al.*, 2005). More recently, the use of Arabidopsis has been exploited for structure–function studies of nematode CLE proteins, and has revealed a potential role for ligand mimicry in determining the host range of cyst nematodes (Wang *et al.*, 2010). However, these studies are limited in scope because the nematode CLEs were isolated from the soybean cyst nematode (*H. glycines*) and potato cyst nematode (*G. rostochiensis*), two plant-parasitic nematodes that do not parasitize Arabidopsis. The fact that these CLE peptides would never be present in Arabidopsis root cells makes it impossible to determine the exact function of the nematode CLE peptides in syncytium formation using a nonhost plant. Although hairy roots of host plants, such as soybean and potato, have been used for nematode CLE studies (Lu *et al.*, 2009; Wang *et al.*, 2010), the production of stable, single-insertion transformants for detailed, reproducible characterization is impossible. Moreover, controlled *in vitro* hairy root assays lack the above-ground part of the plant, which might affect signal transduction.

The impetus for this study was to isolate and characterize CLE genes from *H. schachtii*, the beet cyst nematode, a parasite on Arabidopsis, to facilitate studies directed at the understanding of the function of nematode CLEs in syncytium formation. We identified two CLE-like genes, *HsCLE1* and *HsCLE2*, and, using overexpression and peptide assays, demonstrated that they are biologically active plant CLE mimics in Arabidopsis. Overexpression of *HsCLEs* resulted in a range of *wus*-like phenotypes similar to those observed in plant CLE overexpression studies (Strabala *et al.*, 2006). Moreover, *HsCLE* activity was abolished in the absence of the conserved CLE motif. In addition, our study highlights the functional diversification of plant and nematode CLE VDs. Unlike plant CLEs, *HsCLEs* could function *in planta* without a SP, providing further evidence that the VD of nematode CLE proteins can target cytoplasmically delivered CLEs to the apoplast in order to function as ligand mimics (Wang *et al.*, 2010). The high percentage of sequence similarity between the VD sequences of *HsCLEs* and *HgCLEs* suggests that secreted *HsCLE* and *HgCLE* polypeptides might traffic to the extracellular space through the same pathway in plant cells.

Remarkably, we found that *HsCLE2* shared an identical 12-amino-acid C-terminal CLE motif with Arabidopsis *AtCLE5* and *AtCLE6*, providing further support for the idea that nema-

Fig. 3 Effect of CLAVATA3/ESR (CLE) peptides on Arabidopsis root growth. (a) Average root length of Arabidopsis seedlings grown on medium containing no peptide, or 0.1, 1 or 10 μ M synthetic dodecapeptide corresponding to the indicated CLE motif. Data represent the mean $\pm$ SE ($n \geq 16$ except for 10 μ M HsCLE1 which had only $n = 7$). Broken line indicates the average growth of roots after 9 days in the absence of peptide. Asterisks indicate statistically significant differences compared with no peptide treatment at a probability level of $P \leq 0.05$. Peptide assays were conducted three independent times with similar results. (b–d) Representative root tips of Arabidopsis seedlings grown on medium with or without synthetic CLE peptides for 14 days and visualized with differential interference microscopy. Scale bar represents 50 μ m. (b) No peptide. (c) Stimulated root growth for 0.1 μ M HsCLE1 and HsCLE2/AtCLE5/AtCLE6. (d) Terminated root growth for 0.1–10 μ M AtCLE19p12, 1–10 μ M HgCLEp12, 10 μ M HsCLE1p12 and 10 μ M HsCLE2p12.

tode CLE genes have co-evolved to mimic host plant CLEs, such that the distinct host preferences of cyst nematodes may be a product of ligand mimicry (Mitchum *et al.*, 2008; Wang *et al.*, 2010). Although cell type-specific expression patterns for *AtCLE5* and *AtCLE6* have not yet been reported, both genes have been shown to be expressed in roots (Sharma *et al.*, 2003). In a comparative analysis, overexpression of *HsCLE2*, *AtCLE5* and *AtCLE6* resulted in very similar phenotypes in both shoots and roots. In addition, overexpression of *HsCLE1*, which has a CLE domain that is also closely related to AtCLEs 1–7, resulted in similar phenotypes to *AtCLE5* and *AtCLE6*. The functional similarity between HsCLEs and AtCLEs 1–7 suggests that these may

be the target peptides mimicked by HsCLEs to promote parasitism. Phenotypic characterization of *AtCLE5* and *AtCLE6* knockout lines has not yet been reported, possibly because functional redundancy among CLE family members may complicate this type of analysis. Transgenic plants expressing a hairpin RNA of *CLE6* did not show any phenotypes (Whitford *et al.*, 2008). If phenotypic abnormalities are reported in knockout lines, functional complementation of *atcle5* and *atcle6* mutant lines will provide additional information concerning ligand mimicry of HsCLEs.

Synthetic peptide assays have been used widely to study the effects of CLE peptides on plant root growth. Fiers *et al.* (2005)

reported that the 14-amino-acid CLE domain peptide of AtCLE5 had no effect on Arabidopsis root growth at 10 μ M. Their result was further confirmed by Ito *et al.* (2006) and Kinoshita *et al.* (2007) using a 12-amino-acid AtCLE5/6 CLE motif peptide at 1 μ M. Recently, Whitford *et al.* (2008) reported that the CLE domain peptide of AtCLE6 suppressed root growth at 10 μ M. Here, we tested the activity of different 12-amino-acid peptides at various concentrations. At 10 μ M, the HsCLE2/AtCLE5/AtCLE6 peptide strongly suppressed root growth, consistent with the results of Whitford *et al.* (2008). At 1 μ M, this peptide caused a slightly shorter root phenotype that was statistically significantly different from untreated roots, which differs from earlier reports (Ito *et al.*, 2006; Kinoshita *et al.*, 2007). Slight differences in peptide concentrations in these studies could account for the differences observed at 1 μ M. Interestingly, however, at 0.1 μ M, the HsCLE2/AtCLE5/AtCLE6 peptide caused a stimulation of root growth. The effects of the 12-amino-acid HsCLE1 CLE motif peptide were similar to those observed with the HsCLE2/AtCLE5/AtCLE6 peptide. In contrast, synthetic peptides corresponding to the CLE motifs of AtCLE19 and HgCLE1/2 (CLE peptides of *H. glycines*) caused a short-root phenotype that increased in severity with increasing peptide concentration. These results indicate that Arabidopsis responds to this group of peptides in a dosage-dependent manner and the endogenous regulation of CLE peptide levels may be one mechanism used by the plant to modulate signal transduction. In addition, the peptide assays suggest that HsCLEs and HgCLEs may be signalling differently in Arabidopsis.

Arabidopsis CLE genes, *AtCLE5* and *AtCLE6*, have been studied previously *in planta* by different groups (Meng *et al.*, 2010; Strabala *et al.*, 2006; Whitford *et al.*, 2008). In all studies, it was reported that overexpression of *AtCLE5* and *AtCLE6* caused above-ground *wus*-like phenotypes, including premature termination of shoot and floral meristems; however, inconsistent results were reported for the effects on root growth. Strabala *et al.* (2006) reported that the overexpression of *AtCLE5* and *AtCLE6* stimulated the growth of Arabidopsis roots, resulting in longer roots compared with the control, but this phenotype was not quantified. In addition, T1 generation seedlings were used to characterize root growth. In our experience, transgenic T1 seedlings selected by growth on antibiotics always exhibit variable root growth, and it is difficult to accurately measure root length after seedlings are transferred to vertical plates. More recently, Meng *et al.* (2010) reported that *AtCLE6* had no effect on root growth in overexpression studies. Of the 37 seedlings measured, only 21 exhibited above-ground *wus* phenotypes, indicating that the peptide levels varied among seedlings. This variation may have compromised the detection of a phenotype. Here, we first scored the above-ground phenotypes of our T1 transformants, and advanced those exhibiting a severe *wus*-like phenotype to the T2 generation for the investigation of root growth. Two of the five

independent *AtCLE5* lines and one of the five independent *AtCLE6* overexpression lines exhibited a statistically significant short-root phenotype compared with wild-type plants. These data, combined with the peptide assays, suggest that plants may employ an endogenous mechanism to regulate the developmental responses to these CLE peptides in a dosage-dependent manner.

The identification and characterization of CLE-like genes from the beet cyst nematode, a *bona fide* parasite of Arabidopsis, enables the use of the *H. schachtii*–Arabidopsis model pathosystem to accelerate studies to elucidate the role of nematode CLE peptides in syncytia formation. In addition, knockout lines of putative receptor genes are available in Arabidopsis. Peptide screening, complementation and overexpression studies, as well as infection assays of available mutants, will aid in the identification of the target endogenous CLEs being mimicked and the potential receptors involved in nematode CLE peptide signalling.

EXPERIMENTAL PROCEDURES

Nematode and plant material

The beet cyst nematode (*H. schachtii*) was propagated on glasshouse-grown sugar beet (*Beta vulgaris* cv. Monohi). Parasitic life stages were extracted from infected roots as described by Goellner *et al.* (2000) and frozen at -80°C . The *A. thaliana* Columbia (Col-0) ecotype was used for all overexpression experiments in this study.

RNA isolation and gene cloning

Nematode and plant RNA isolation and first-strand cDNA synthesis were conducted according to Wang *et al.* (2010). Primers corresponding to the UTR sequences of the predicted *HgCLE1* (Wang *et al.*, 2010) cDNA sequence were designed as follows to clone CLE-like genes from *H. schachtii*: SYV46F (5'-GATCCGA AAAAATGCCAAAC-3') and SYV46R1 (5'-TCCTCCGTTAGATCC ATCCA-3'). PCR products were cloned into the pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and multiple clones were sequenced.

Construct generation

The following primers were used: HsCLEAXhoF (5'-AAACTCG AGATGCCAAACATTTCAAATCCT-3') and 03G07CKpn (5'-AAAGGTACCTTAATGATGACGTGGGTCGG-3') to clone *HsCLE1*; HsCLEAsXhoF (5'-AAACTCGAGATGGATGGCAAAAACTGCTA ATG-3') and 03G07CKpn to clone *HsCLE1^{ΔSP}*; HsCLEAXhoF and HsCLEAcR (5'-TTATTCATTGACCGGCGGCATT-3') to clone *HsCLE1^{ΔCLE}*; HsCLEAsXhoF and HsCLEAcR to clone *HsCLE1^{ΔSPΔCLE}*; HsCLEBXhoF (5'-AAACTCGAGATGCCAAACATATGCAAAATCC-3') and 03E03CKpn (5'-AAAGGTACCCTAATGATGTTGTGGGTC GG-3') to clone *HsCLE2*; HsCLEBsXhoF (5'-AAACTCGAGATG

GATTCCACTGATGGCGAAAAA-3') and 03E03CKpn to clone *HsCLE2^{ASP}*; *HsCLEBxhoF* and *HsCLEBcR* (5'-TTACTCTTTGTCCGTCATTTCTC-3') to clone *HsCLE2^{ΔCLE}*; *HsCLEBsXhoF* and *HsCLEBcR* to clone *HsCLE2^{ASPΔCLE}*.

AtCLE5 and *AtCLE6* were cloned directly from Arabidopsis cDNA. The following primers were used: *AtCLE5EcoRF* (5'-AAAGAATTCATGGCGACTTTGATCCTCAAG-3') and *AtCLE5HindR* (5'-TTTAAGCTTTCAATGGTGTGTGGATCGG-3') to clone *AtCLE5*; *AtCLE5EΔSpEcoRF* (5'-AAAGAATTCATGCGAATCCTCCGTTTCATATCG-3') and *AtCLE5HindR* to clone *AtCLE5^{ASP}*; *AtCLE6EcoRF* (5'-AAAGAATTCATGGCGAATTTGATCCTTAAGC-3') and *AtCLE6HindR* (5'-TTTAAGCTTTCAATGGTGTGTGGATCAGG-3') to clone *AtCLE6*; *AtCLE6EΔSpEcoRF* (5'-AAAGAATTCATGCGAATCCTCGTACATATCG-3') and *AtCLE6HindR* to clone *AtCLE6^{ASP}*.

To generate CaMV 35S overexpression constructs, blunt end PCR products were amplified with *Pfu* turbo (Stratagene, La Jolla, CA, USA) and cloned into the *SmaI* digestion site of the pMD1 vector (Tai *et al.*, 1999), a derivative of pBI121 (CLONTECH, Mountain View, CA, USA). All constructs were confirmed by sequencing and transformed into *Agrobacterium tumefaciens* GV3101 prior to transformation of Arabidopsis by the floral dip method (Clough and Bent, 1998).

Plant growth

Arabidopsis seeds were sterilized with chlorine gas in a desiccator in open 1.5-mL microcentrifuge tubes for 6 h by mixing 200 mL of household bleach with 3 mL of 12.1 M hydrochloric acid in a beaker and placing it in the desiccator with the seeds. Seeds were aerated in a laminar flow hood for 30 min following gas treatment and then cold stratified at 4 °C for 2–3 days. Arabidopsis was grown in MetroMix 200 soil mixture (Sungro Horticulture Bellevue, WA, USA) in a growth chamber at 22 °C under long-day conditions (16 h light/8 h darkness). The floral dip method was used for Arabidopsis transformation.

For the selection of primary Arabidopsis transformants, sterilized seeds were plated onto 0.5 × Murashige and Skoog (MS) medium [MS basal nutrient salts (Caisson Laboratories, North Logan, UT, USA), 2% sucrose, 0.8% Type A agar (Sigma, St. Louis, MO, USA), pH 5.7] supplemented with 50 µg/mL timentin (Glaxo-SmithKline, Research Triangle Park, NC, USA) to control for *Agrobacterium* contamination, plus 50 µg/mL kanamycin, and placed in a growth chamber at 22 °C under long-day conditions. After 7 days of growth, the transgenic seedlings were transplanted to MetroMix 200 soil mixture and grown under the same conditions.

Phenotypic analysis

Phenotypes of transgenic plants were monitored beginning at 2 weeks after transplantation to soil. Seedlings showing SAM termination were characterized as severe *wus* phenotypes. Seedlings with normal SAM development, but exhibiting defects in floral

meristem development (no carpels and decreased stamen number) at later stages, were characterized as weak *wus* phenotypes. Photographs of the plants were taken with a Nikon (Melville, NY, USA) Coolpix 5000 digital camera. For root phenotype analysis, T2 seeds were germinated on square plates containing 0.5 × MS medium supplemented with 2% sucrose, and grown vertically. The growth of the primary roots was marked for 10 days and measured with a Scion Image Alpha 4.0.3.2 program (Scion, Frederick, MD, USA). Standard error calculations and Student's *t*-test were performed in Excel.

Peptide assay

Synthetic peptides (Sigma-Genosys, The Woodlands, TX, USA) with a purity of >70% were dissolved in 1 M filter-sterilized sodium phosphate buffer, pH 6.0. The following peptides were designed: *AtCLE19p*, RVIPTGNPLHN; *HgCLEp*, RLSPSGDPHHH; *HsCLE1p*, RLSPSGDPHHH; *AtCLE5/6/HsCLE2p*, RVSPGGPD-PQHH. Peptides were added to 0.5 × MS medium with 2% sucrose to achieve concentrations of 0.1, 1 and 10 µM. Arabidopsis root length was measured from the base of the hypocotyl to the tip of the primary root by marking the root length each day for 9 days. Root length was quantified using Scion Image. Primary root tips of Arabidopsis were analysed by mounting on a slide and viewing with an Olympus Vanox (Hirschfeld Instruments, St. Louis, MO, USA) microscope equipped with Nomarski optics. Peptide assays were conducted three independent times.

Phylogenetic analysis

Sequence alignment and the unrooted consensus tree with bootstrap values were generated using the PAUP program.

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ACCESSION NUMBERS

The accession numbers for the sequences used in this study are as follows: *HsCLE1* mRNA (HM588679); *HsCLE2* mRNA (HM588680); *AtCLE5* mRNA (NM_179828) *At2g31083*; *AtCLE6* mRNA (NM_128664) *At2g31085*; *AtCLE19* mRNA (NM_148747) *At3g24225*; *HgCLE1* mRNA (AF273728) protein (ACT32609); *HgCLE2* mRNA (AF473827) protein (ACT32610).

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SUPPORTING INFORMATION

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

Fig. S1 Effect of CLAVATA3/ESR (CLE) peptides on Arabidopsis root growth. Arabidopsis seedlings grown for 9 days on medium containing no peptide (a), 0.1, 1 or 10 μ M HsCLE1 peptide (b–d), or 0.1, 1 or 10 μ M HsCLE2 peptide (e–f).

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