Mechanisms of Molecular Mimicry of Plant CLE Peptide Ligands by the Parasitic Nematode *Globodera rostochiensis*[1][C][W]

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Nematodes that parasitize plant roots cause huge economic losses and have few mechanisms for control. Many parasitic nematodes infect plants by reprogramming root development to drive the formation of feeding structures. How nematodes take control of plant development is largely unknown. Here, we identify two host factors involved in the function of a receptor ligand mimic, GrCLE1, secreted by the potato cyst nematode *Globodera rostochiensis.* GrCLE1 is correctly processed to an active form by host plant proteases. Processed GrCLE1 peptides bind directly to the plant CLE receptors CLV2, BAM1, and BAM2. Involvement of these receptors in the ligand-mimicking process is also supported by the fact that the ability of GrCLE1 peptides to alter plant root development in Arabidopsis (*Arabidopsis thaliana*) is dependent on these receptors. Critically, we also demonstrate that GrCLE1 maturation can be entirely carried out by plant factors and that the availability of CLE processing activity may be essential for successful ligand mimicry.

A number of genera and species of nematodes are serious plant pathogens causing enormous losses to agronomic crops worldwide (Sasser and Freckman, 1987). Several of the most damaging plant parasitic nematodes, including root-knot nematodes and cyst nematodes, infect plants by "hijacking" development of the root, leading the plant to form feeding sites for the nematode (Davis et al., 2008). Plant cyst nematodes modulate root developmental programs in hosts to form enlarged, multinucleate feeding cells called syncytia, which serve as the nutritive source (Davis et al., 2004). The formation of a syncytium is triggered by effector molecules secreted into plant tissues from the esophageal gland cells of cyst nematodes (Davis et al., 2004, 2008). As a result of large-scale screenings of the secretions synthesized by the esophageal gland cells, small secreted nematode proteins containing the long-thought plant-specific CLE domain suggest an important role of these proteins in the parasitic establishment of several cyst nematodes (Wang et al., 2001, 2005; Lu et al., 2009; Replogle et al., 2011).

CLE proteins are the best studied class of peptide ligands in plants. A large number of CLE proteins are broadly distributed across all land plant species, from the basal lycophyte *Selaginella* to both monocots and dicots (Cock and McCormick, 2001; DeYoung and Clark, 2001; Oelkers et al., 2008). The Arabidopsis (*Arabidopsis thaliana*) genome alone contains over 30 CLE-encoding genes. In addition, CLEs have been demonstrated to be expressed in diverse tissues (Sharma et al., 2003), where they presumably function in a large number of different pathways. CLE domain-containing proteins have been the subject of intense study and show extensive conservation of function in developmental patterning. CLE peptides act as ligands for a variety of receptor kinases that control stem cell specification, organogenesis, vascular differentiation, and nodulation (Hirakawa et al., 2008; Guo et al., 2010; Krusell et al., 2011). The founding CLE member, the Arabidopsis protein CLV3, activates the receptor kinases CLV1, CLV2, and BAM to control meristem development (Clark et al., 1995; Fletcher et al., 1999; Jeong et al., 1999). Significantly, CLE peptides can be added exogenously to plants to dramatically alter plant development, which makes these ligands potential tools for developmental manipulation (Fiers et al., 2005, 2006).

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Two lines of evidence suggest that the CLE proteins encoded by plant parasitic nematodes play a critical role in parasitism. First, RNA interference-driven inhibition of *hg-syv46*, a CLE protein-encoding gene of the soybean cyst nematode *Heterodera glycines*, reduced the ability of the nematodes to establish parasitic interactions, as indicated by the significantly reduced numbers of females present on the plants after inoculation (Bakhetia et al., 2007). Second, mutation of several CLE receptors in Arabidopsis affected both feeding structure formation and nematode infection by the beet cyst nematode *Heterodera schachtii* in Arabidopsis (Replogle et al., 2011). This second study also suggested that the nematode CLE proteins induce developmental reprogramming in roots via mimicking CLE peptide ligands in plants. In fact, several nematode CLE genes have been shown to be able to replace *CLV3* when expressed in Arabidopsis (Wang et al., 2005, 2011; Lu et al., 2009). However, direct evidence of the mechanism of nematode CLE function and interacting host factors is lacking.

CLE proteins are encoded as larger precursor proteins in which the CLE domain (12 or 13 residues) is found at the C terminus (Ito et al., 2006; Kondo et al., 2008; Ohyama et al., 2009). A variety of studies suggest that maturation of the CLE peptide is essential for function (Fiers et al., 2005, 2006; Ni and Clark, 2006). We have characterized a proteolytic processing activity, present in every plant species tested, that processes the N-terminal side of the CLE peptide at the identical position to that found in vivo (Ni and Clark, 2006; Ni et al., 2011). This processing activity is secreted from cells (Ni et al., 2011). CLV3 is among a minority of CLE proteins with a C-terminal extension beyond the CLE domain. In cauliflower (*Brassica oleracea*) extracts, we previously found evidence of progressive C-terminal trimming, suggestive of a carboxypeptidase (Ni et al., 2011). Interestingly, the overexpression phenotype of CLE19 in roots of Arabidopsis is suppressed by mutations in the SOL1 putative carboxypeptidase (Casamitjana-Martínez et al., 2003).

A recent study indicated that CLE proteins secreted by nematodes are delivered to plant cells in the form of proproteins (Wang et al., 2010). If the nematode CLE precursors act as ligand mimics, a critical step would be the release of active CLE peptides through proteolytic maturation within plant tissues. Because the plant CLE processing activity is secreted (Ni et al., 2011), it is feasible for the apoplastic (Replogle et al., 2011) nematode CLE proteins to be processed by plant proteases. The GrCLE1 protein secreted by the potato cyst nematode (PCN) *Globodera rostochiensis* differs from most (but not all) plant CLE proteins in that it contains three tandem CLE domains near the C terminus (Lu et al., 2009). We have identified two host factors involved in GrCLE1 ligand mimicry: the plant CLE processing proteases that activate the nematode ligand mimic, and receptors to which the ligand mimic binds. These host factors make for novel and potentially effective targets to block the nematode infection process.

RESULTS

Nematode GrCLE1 Is Correctly Processed by a Protease from Cauliflower Extracts

We assessed the ability of plant CLE processing activity to recognize the three tandem CLE domains within the GRCLE1 precursor protein (Lu et al., 2009). Protein extracts isolated from cauliflower curds that consist of large quantities of meristem tissue have been used for efficient in vitro processing of the CLE protein CLV3 as well as other plant CLE precursor proteins (Ni and Clark, 2006; Ni et al., 2011). In vitro processing assays in which MBP-tagged GrCLE1 was incubated with cauliflower protein extracts revealed extensive C-terminal processing (Fig. 1A). If the incubation time was reduced, we could detect intermediate processed fragments (Supplemental Fig. S1), suggesting that the multiple CLE domains of GrCLE1 might be recognized separately. The processing of GrCLE1 was partially inhibited by excess CLV3 and by the protease inhibitor 4-(1-aminoethyl) benzenesufonyl fluoride hydrochloride (AEBSF) that blocks CLV3 processing (Ni et al., 2011; Fig. 1B). Moreover, CLV3 processing was partially inhibited by excess GrCLE1 (Fig. 1C), suggesting that the same enzyme carries out both CLV3 and GrCLE1 maturation.

To determine the processing sites on GrCLE1 by plant protease, a large-scale processing reaction for MBP-GrCLE1 with cauliflower extracts was performed. The sample was then subjected to mass spectrometry (MS) analysis to identify small peptides. Peptides with

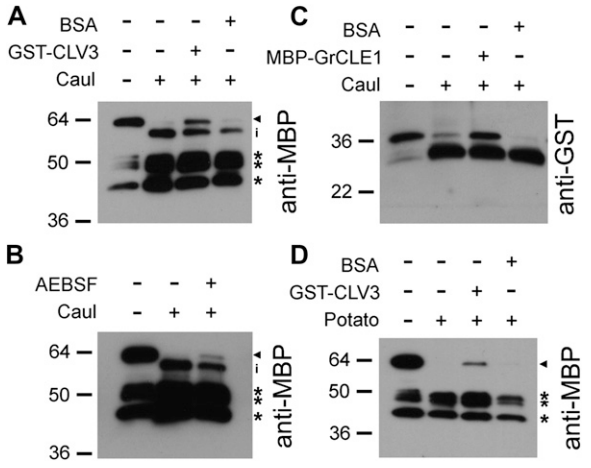

Figure 1. In vitro processing of GrCLE1 by plant protein extracts. A, Purified MBP-GrCLE1 incubated with cauliflower (Caul) extracts with or without excess GST-CLV3 or BSA. Full-length MBP-GrCLE1 protein is indicated with the arrowhead, and nonspecific proteins are indicated by asterisks. Note that processing in cauliflower extracts accumulates an intermediate protein (i). B, Purified MBP-GrCLE1 (arrowhead) incubated with cauliflower extracts with or without 10 mM AEBSF. C, GST-CLV3 incubated with cauliflower extracts with or without excess MBP-GrCLE1 or BSA. D, MBP-GrCLE1 incubated with potato (Michigan Purple) root extracts with or without excess GST-CLV3 or BSA. Note that no intermediate proteins accumulate in potato processing reactions.

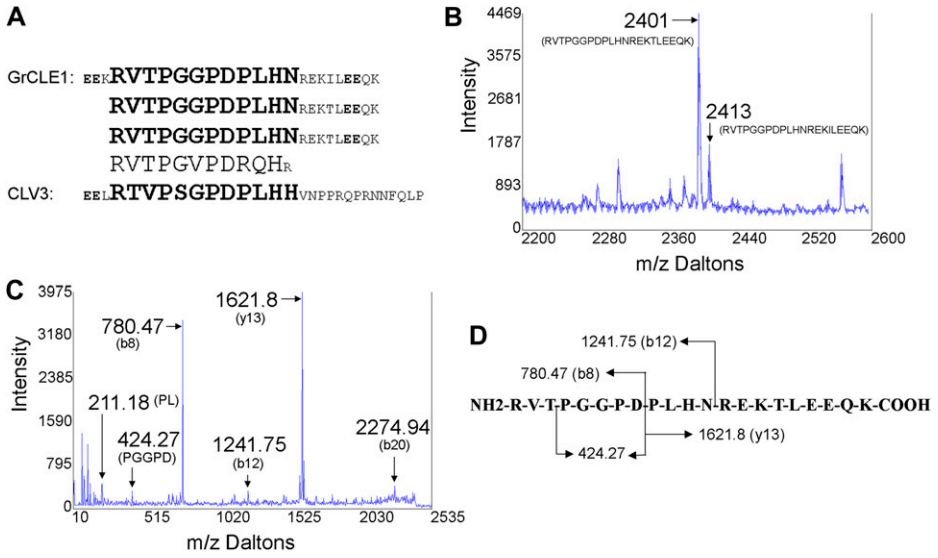

Figure 2. MS analysis of the processed products of GrCLE1 by cauliflower protein extracts. A, Alignment of amino acid sequences at the C termini of GrCLE1 and CLV3. CLE domains are highlighted. B, MS analysis of MBP-GrCLE1 after incubation with cauliflower extracts. The masses and predicted sequences of the major peptide fragments are indicated. The *y* axis is a measure of signal intensity. C, MS/MS analysis of the 2,401-D peptide identified in B. D, The 21-mer GrCLE1 CLE-containing peptide identified by MS/MS analysis of the in vitro processing reactions by cauliflower extracts. [See online article for color version of this figure.]

masses of 2,401 and 2,413 D were identified specifically in the processed sample (Fig. 2; Supplemental Fig. S2). The peptides match exactly the masses of peptides predicted from separate release of each of the three CLE domains. Two separate CLE-containing peptides, Arg-151 to Lys-171 and Arg-172 to Lys-192, are predicted to have a mass of 2,401 D, while one CLE-containing peptide, Arg-130 to Lys-150, is predicted to have a mass of 2,413 D (Fig. 2A). The predicted sizes of the CLE peptides also match well with the observation that the 2,401 peak signal intensity was approximately twice that of the 2,413 peak (Fig. 2B).

To definitely identify the 2,401-D peptide, the peak was subjected to further MS/MS analysis. Internal fragments revealed that this peak indeed corresponded to the Arg-151 to Lys-171 peptide (Fig. 2, C and D). These results indicated that each of the CLE domains of GrCLE1 was separately cleaved to generate three 21-mer CLE-containing peptides. This is similar to CLV3 in vitro processing, in which cleavage at the N-terminal end of the CLE peptide matches exactly that seen for mature CLE peptides in vivo, but the C-terminal trimming, likely carried out by a separate protease, is incomplete (Ni et al., 2011).

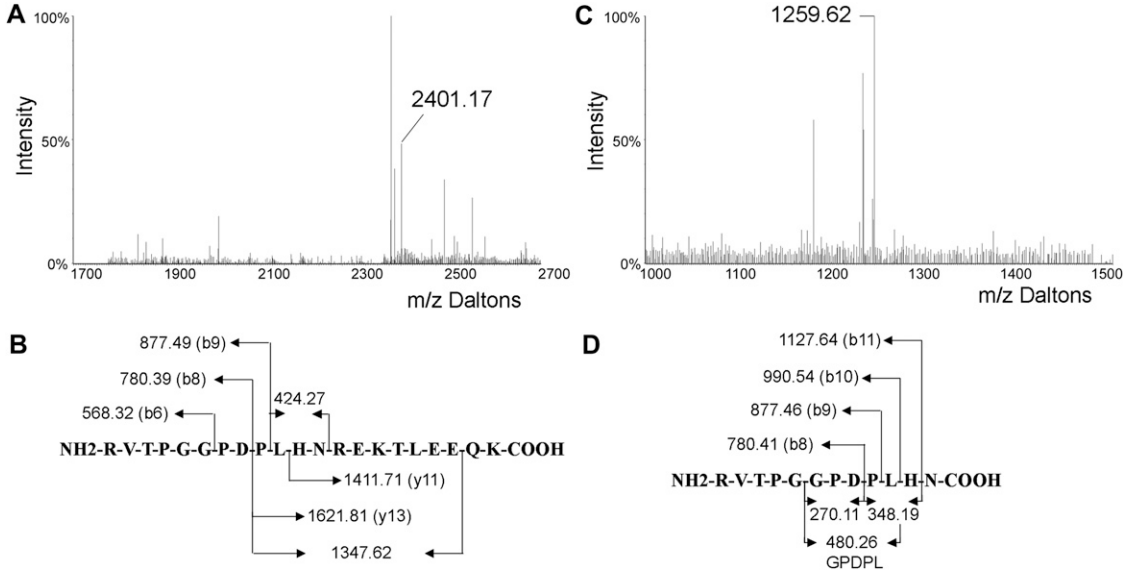

Figure 3. MS analysis of the processed products of GrCLE1 by protein extracts from potato roots. A, MS analysis showing the 2,401-D peak from MBP-GrCLE1 after incubation with potato root extracts. B, The 21-mer GrCLE1 CLE-containing peptide identified by MS/MS analysis of the 2,401-D peptide in A. C, MS analysis showing the 1,259-D peak. D, The 12-mer GrCLE1 mature peptide identified by MS/MS analysis of the 1,259-D peptide in C.

Potato Root Extracts Contain the GrCLE1 Processing Protease That Releases a 12-mer CLE Peptide

PCN is a root-infecting parasite. One critical question is whether the GrCLE1 precursor protein can be processed by proteases in roots. Here, we show that MBP-GrCLE1 was also efficiently processed by protein extracts from the roots of potato (*Solanum tuberosum*), a host for PCN (Davis et al., 2004). This processing activity was partially inhibited by excess CLV3 but not excess bovine serum albumin (BSA; Fig. 1D). To further test if the observed MBP-GrCLE1 proteolysis was the result of CLE processing or a nonspecific potato protease, we analyzed the proteolytic products by MS/MS. Here, we detected the 21-mer CLE-containing peptides that lacked C-terminal trimming as well as the fully processed 12-mer CLE peptide (RVTPGGPDPLHN) expected from each of the three GrCLE1 CLE domains (Fig. 3; Supplemental Fig. S3). Thus, potato root extracts contain correct CLE processing activities for both the N-terminal cleavage and the C-terminal trimming.

Processed Nematode CLE Binds to Plant Receptors

Processed nematode CLE peptides need to be able to directly interact with plant receptor proteins to function to reprogram the root development of host plants by acting as ligand mimics. As the founding member of the CLE protein family, CLV3 has been well studied in signaling at the shoot meristem (Fletcher et al., 1999). CLV3 carries out this function through the ability of the mature CLV3 CLE domain to bind directly to the Leu-rich repeat receptor kinase CLV1 (Ogawa et al., 2008; Guo et al., 2010), the receptor-like protein CLV2, and the CLV1-related Leu-rich repeat receptor kinases BAM1 and BAM2 (Guo et al., 2010). Root expression of *CLV2*, *BAM1*, and *BAM2* (Jeong et al., 1999; DeYoung et al., 2006) makes these CLV3 receptors good candidates for nematode CLE binding. In a recent study, expression of *CLV2* and the CLV2-interacting membrane kinase gene *CRN* (Replogle et al., 2011) was detected in feeding sites induced by the CLE-expressing beet cyst nematode *H. schachtii* in Arabidopsis roots, and mutations on these receptors affected both feeding structure formation and nematode infection (Replogle et al., 2011). To test GrCLE1 peptide binding to these receptors, we compared the ability of cold GrCLE1 and CLV3 peptides to compete with ^{125}I-radiolabeled CLV3 CLE for binding to receptor proteins transiently expressed in tobacco (*Nicotiana benthamiana*)leaves, as described previously (Guo et al., 2010). For each receptor, the 12-mer GrCLE1 peptide (RVTPGGPDPLHN) showed specific binding indistinguishable from the CLV3 CLE peptide (Fig. 4). The longer 21-mer CLE-containing peptide (Arg-172 to Lys-192), which is a product of incomplete CLE maturation (Fig. 3, A and B), exhibited significantly reduced binding to all of the receptors (Fig. 4), suggesting that complete proteolytic maturation is critical for high-affinity receptor binding. The nonbinding, truncated, CLV3 CLE peptide CLV3S (Guo et al., 2010) acted as a negative control (Fig. 4).

The in Vivo Function of GrCLE1 Peptide Is Receptor Dependent

To test the function of the 12-mer and 21-mer GrCLE1 peptides in vivo, we assayed for the ability of the peptides to inhibit root growth in Arabidopsis in comparison with CLV3 CLE. Similar to previous reports (Lu et al., 2009), the GrCLE1 12-mer inhibited root growth effectively. At 10 nM concentration, the inhibition of GrCLE1 was even greater than CLV3 (Fig. 5A). The GrCLE1 21-mer, consistent with ligand-binding assays, exhibited measurable, but less effective, inhibition (Fig. 5A). The reduced binding and in vivo effectiveness of the GrCLE1 21-mer suggest that it is further processed in vivo.

To test if the ligand-receptor interactions observed in vitro correspond with the ability of the peptide to function in vivo, we tested GrCLE1 function in a

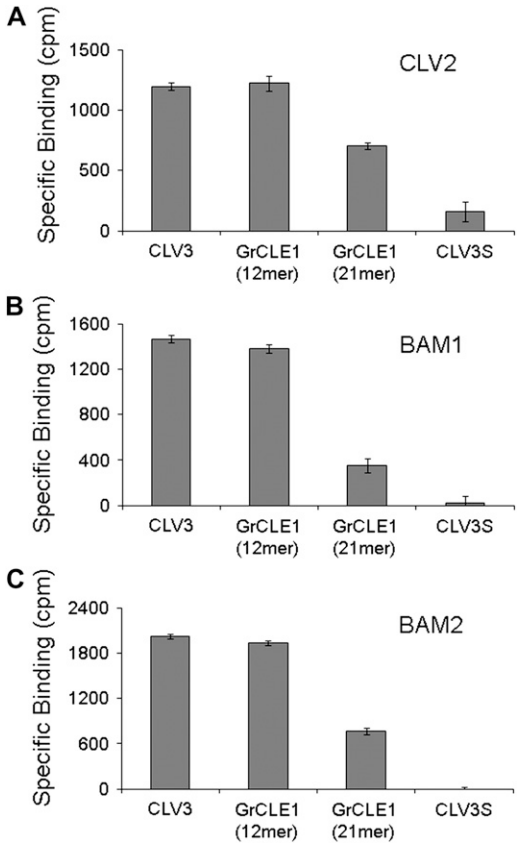

Figure 4. Receptor binding of the GrCLE1 peptide. Specific binding of the GrCLE1 12-mer and 21-mer peptides to Arabidopsis CLE receptor proteins transiently expressed in tobacco leaves. Shown are competitive replacements of ^{125}I-labeled CLV3 CLE peptide binding to detergent-washed membrane fractions for CLV2-MYC (A), BAM1-GFP (B), and BAM2-GFP (C). Data represent means ± SE over four replicates.

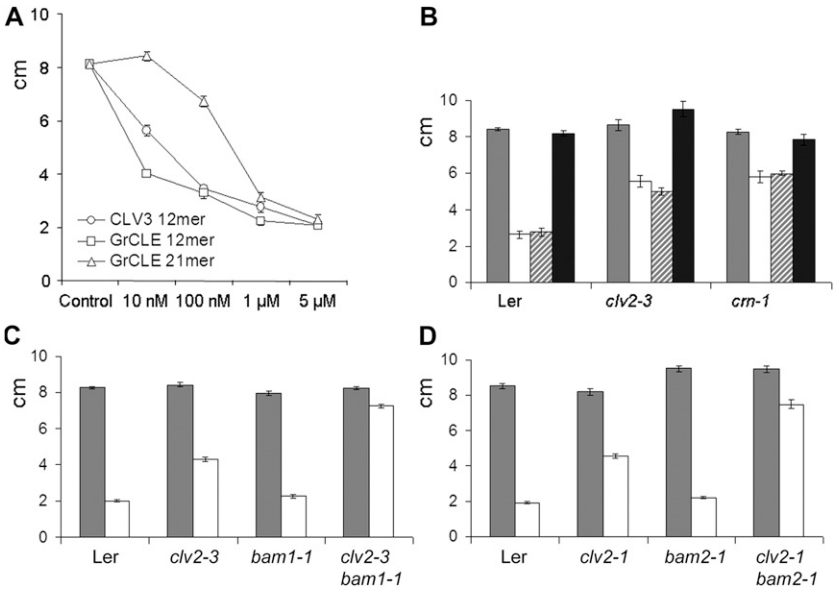

Figure 5. Root inhibition effects of the GrCLE1 peptide. A, Root length of Arabidopsis ecotype Landsberg *erecta* 10 d after growing on vertical plates containing GrCLE1 12-mer and 21-mer and CLV3 CLE peptides at various concentrations. Data represent means ± SE (n = 10–12). B, Root length of 10-d-old Arabidopsis Landsberg *erecta*, *clv2-3*, and *crn-1* plants on control plates (gray bars) or plates containing 1 μM CLV3 (white bars), GrCLE1 12-mer (hatched bars), or CLV3S (black bars) peptides. Data represent means ± SE (n = 5–27). C and D, Root length of Landsberg *erecta*, Arabidopsis mutants *clv2*, *bam1*, and *bam2*, and the *bam clv2* double mutant after growing for 10 d on vertical plates with (white bars) or without (gray bars) 1 μM GrCLE1 12-mer peptide. Data represent means ± SE (n = 11–40). Note that BAM1 and BAM2 are redundant and each single mutant is phenotypically wild type.

variety of Arabidopsis mutant backgrounds. *CLV2*, *CRN*, *BAM1*, and *BAM2* are all expressed in the root; indeed, CLV2 has been reported necessary for the impact of CLV3 and other CLE peptides on the root (Fiers et al., 2005; Miwa et al., 2008; Meng et al., 2010). At 1 μM concentration, *clv2* and *crn* mutants were partially resistant to both the GrCLE1 and CLV3 12-mer peptides to nearly identical extents (Fig. 5B). While *bam1* and *bam2* single mutants are not resistant to peptide treatment, *clv2* resistance to the GrCLE1 peptide was enhanced by the *bam1* or *bam2* mutation (Fig. 5, C and D), indicating that both of these factors

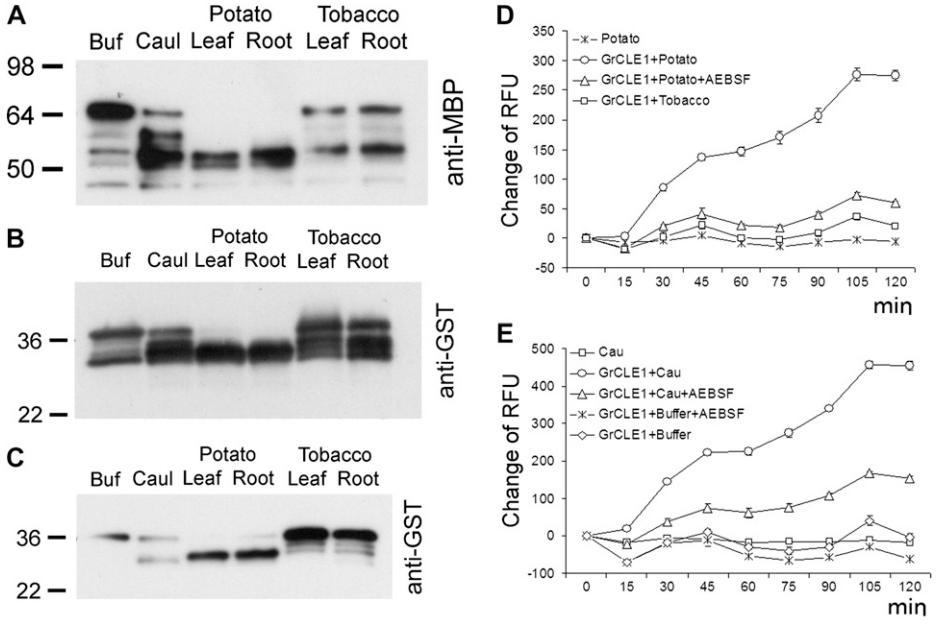

Figure 6. In vitro proteolytic processing of GrCLE1 and other CLE precursors by protein extracts from potato and tobacco plants. A, Purified MBP-GrCLE1 incubated with cauliflower (Caul) extracts or leaf and root extracts of potato and tobacco. B, Purified GST-CLV3 incubated with cauliflower extracts or leaf and root extracts of potato and tobacco. C, GST-ERCLV3 incubated with different protein extracts or leaf and root extracts of potato and tobacco. D, Synthetic Dabcyl/EDANS-tagged GrCLE1 substrate peptide or DMSO was incubated with root protein extracts from the host species potato or the nonhost species tobacco at room temperature with or without the presence of the protease inhibitor AEBSF. E, Synthetic Dabcyl/EDANS-tagged GrCLE1 substrate peptide or DMSO was incubated with cauliflower protein extracts or buffer at room temperature with or without the presence of the protease inhibitor AEBSF. The fluorescence intensity at 493 nm was measured every 15 min for 2 h. Values shown are means ± SE (n = 4) of change in fluorescence intensity over time. RFU, Relative fluorescence units.

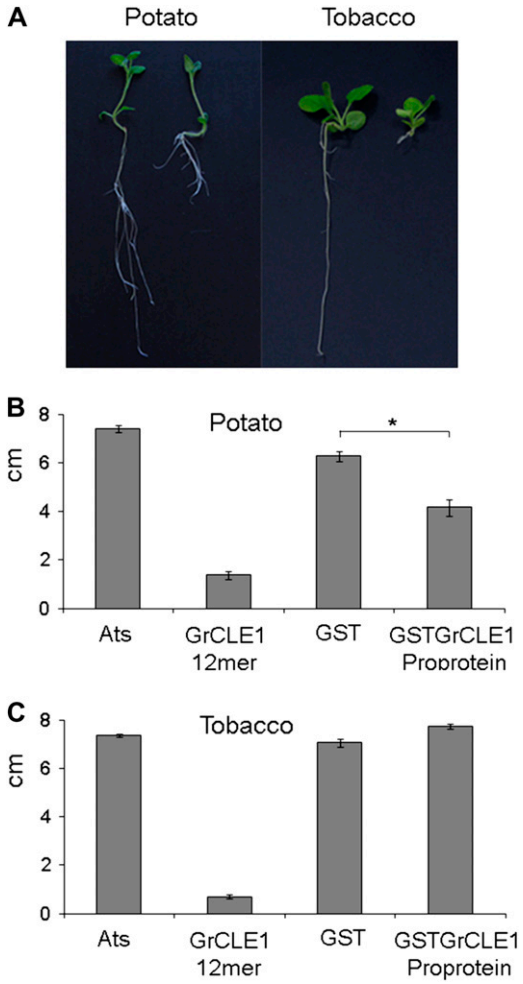

Figure 7. Effects of GrCLE1 precursor protein on root development of potato and tobacco plants. A, Potato (Atlantic × MSR041-3) and tobacco grown on vertical plates with (right) or without (left) 1 μM GrCLE1 12-mer peptide. B, Root length of potato plants after growing for 7 d on control plates (Arabidopsis media + Suc [Ats]) or plates containing 1 μM GrCLE1 12-mer peptide, purified GST protein, or purified GST-GrCLE1 protein. Data represent means ± SE (n = 10–11). * P = 0.0001 by two-tailed Student's t test. C, Root length of tobacco plants after growing for 7 d on control plates or plates containing 1 μM GrCLE1 12-mer peptide, purified GST protein, or purified GST-GrCLE1 protein. Data represent means ± SE (n = 10–12). [See online article for color version of this figure.]

contribute to the root responses to CLE peptides. These results are fully consistent with the recent studies suggesting that CLV2 and CRN form a CLE-binding complex distinct from that of BAM (Guo et al., 2010). This result implicates the cognate orthologs of *CLV2*, *CRN*, *BAM1*, and *BAM2* in host species as potential mediators of the nematode ligand mimicry.

In Planta Processing Is Sufficient for in Vivo Function of GrCLE1 Proprotein

When assaying CLE processing activity, we observed great variability in the extent of CLE processing activity present in various tissue types from different plant species (Supplemental Fig. S4). Mature, differentiated tissues from tobacco, for example, showed little or no processing activity on CLE proproteins including GrCLE1, while tissues of similar developmental stages from potato were able to process the CLE proteins as efficiently as cauliflower meristematic tissues (Fig. 6, A–C). CLE proteins characterized to date act in young, developing tissues, not mature differentiated regions (Jun et al., 2008; Mitchum et al., 2008; Meng et al., 2010); thus, many mature tissues may not require abundant CLE processing activity. The difference of CLE processing activity between tobacco and potato was further confirmed through a fluorescence resonance energy transfer-based protease assay using a Dabcyl/EDANS-tagged peptide consisting of the GrCLE1 motif with seven upstream and two downstream residues (K[Dabcyl]-TLEEQKRVTPGGPDPLHNRE-EDANS). CLV3 CLE processing only requires four residues upstream of the CLE motif (Ni et al., 2011), so this peptide should be recognized by the CLE processing protease. When this fluorogenic substrate was incubated with protein extracts from potato roots and cauliflower curds, AEBSF-sensitive peptide processing was detected (Fig. 6, D and E). By contrast, when the same amount of protein extracts from roots of tobacco was used, no processing was detected (Fig. 6D).

To determine if GrCLE1 maturation could occur independent of all other nematode factors, we tested the ability of the GrCLE1 precursor protein to alter root development. We exploited the observed differences in in vitro CLE processing activity from potato and tobacco to simultaneously ask whether this processing activity could facilitate GrCLE1 activation in vivo. Both tobacco and potato roots respond similarly to Arabidopsis when treated with the mature GrCLE1 12-mer (Fig. 7A). By contrast, only the potato roots,

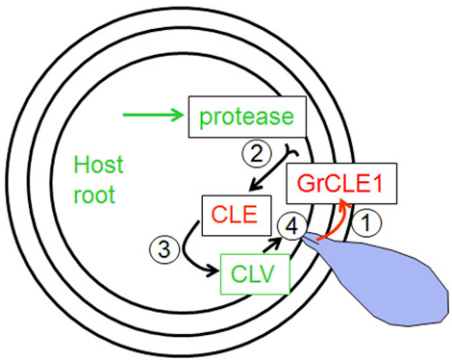

Figure 8. A model for the role of host factors in nematode parasitism. Step 1. The nematode secretes GrCLE1 precursor protein into the host root tissue. Step 2. Host CLE processing activities provide proteolytic maturation of GrCLE1 to release the mature CLE peptide. Step 3. Nematode-derived mature CLE peptides bind to host receptors. Step 4. Host receptor signaling contributes to the formation of feeding structures and nematode parasitism. [See online article for color version of this figure.]

with their high levels of CLE processing activity, responded to GrCLE1 proprotein treatment (Fig. 7, B and C). Thus, GrCLE1 proprotein was converted to an active signaling molecule exclusively by plant factors, and this conversion was correlated with the availability of CLE processing activity.

DISCUSSION

We find evidence that nematode CLE-containing proteins are correctly processed by host plant enzymes to maturation and that the processed nematode CLE peptides can bind directly to plant receptor proteins (Fig. 8). Alteration of root development in Arabidopsis by the mature GrCLE1 is dependent on the same receptors to which it can bind in vitro. Signaling in vivo may occur in host plants through orthologs of the Arabidopsis *CLV2/CRN/BAM1/BAM2* receptors. Importantly, GrCLE1 maturation can be carried out entirely by plant factors. A critical plant factor in this regard appears to be the availability of CLE processing activity.

It has long been suggested that nematode CLE proteins act as ligand mimics in plants (Olsen and Skriver, 2003; Mitchum et al., 2008), and genetic evidence indicated that several nematode CLEs can replace the function of CLV3, both in transgenic plants overexpressing nematode CLEs (Wang et al., 2005, 2010; Lu et al., 2009) and in root inhibition assays using synthetic CLE peptides (Lu et al., 2009; Replogle et al., 2011). It has also been reported recently that CLV2 is required for the function of nematode CLEs in vivo (Replogle et al., 2011). This study provides biochemical evidence demonstrating the mechanistic interactions between GrCLE1 and plant components. Our results suggested that at least two plant factors are involved in the molecular mimicry of nematode CLEs: the protease that processes the precursor CLE protein, and the receptors that bind and relay the signal from the CLE ligand mimics. Because nematode-encoded CLE proteins are important for parasitism (Bakhetia et al., 2007) and their maturation depends on host plant proteases, the host plant factors are potential targets to limit nematode infection. Specifically, if reducing the availability of CLE processing activity prevents efficient nematode CLE maturation, nematode infection may be compromised.

BAM1 and BAM2 are close homologs of CLV1 and have been characterized as CLV3 receptors that act together with CLV1 and in parallel with CLV2-CRN in regulating stem cell homeostasis at the SAM (Guo et al., 2010). To our knowledge, the function of BAM receptors in roots as factors for nematode molecular mimicry has not been previously described. We showed that both BAM1 and BAM2 can bind the 12-mer GrCLE1 peptides and that both are required for nematode-derived CLE to alter Arabidopsis root development (Fig. 5, C and D). CLV2 similarly binds to the mature nematode CLE and is required for its action in Arabidopsis. Thus, BAM1, BAM2, and CLV2 may normally act to control root growth. Because the phenotypes of *clv2* and *bam* are additive for resistance to CLE inhibition of root growth, they may function at the RM similarly as they do at the SAM: BAM and CLV2/CRN act as independent receptor complexes in perceiving and relaying the same CLE signals (Guo et al., 2010). In fact, overexpression of *BAM2* in *clv2* mutants restored sensitivity to CLE peptides (Supplemental Fig. S5).

MATERIALS AND METHODS

Plant Growth

Arabidopsis (*Arabidopsis thaliana* Landsberg *erecta*), potato (*Solanum tuberosum*) varieties Missaukee, Atlantic, Michigan Purple, and Snowden, tobacco (*Nicotiana benthamiana*), soybean (*Glycine max* Skylla), eggplant (*Solanum melongena* Black Beauty), and tomato (*Solanum lycopersicum* Big Boy) plants were grown at 22°C under constant light. Potato seeds and tubers were kindly provided by Dr. David Douches (Michigan State University). Plants in pots were grown on a soil mixture of Metro-Mix 360 (Sun Gro), vermiculite, and perlite (2:1:1) supplemented with Scott's Osmocote 14-14-14 fertilizer. Plants in petri dishes were grown on half-strength Murashige and Skoog salts (Sigma) with 0.8% (w/v) phytoagar. Seeds were kept for 4 d at 4°C after imbibition. Sprouted potato seed tubers were planted directly in pots.

Expression Constructs

To express MBP- and glutathione *S*-transferase (GST)-fused mature GrCLE1 protein in *Escherichia coli*, primers 5′-CGAATTCACAAATGAAAAGGATGA-3′ and 5′-CGTCGACTCAGCGATGTTGT-3′ were used to amplify the GrCLE1 coding region without the signal peptide. The PCR products were cloned into pMAL-C2 (New England Biolabs) and pGEX-5X-1 (GE Healthcare) with the engineered restriction sites *Eco*RI and *Sal*I. GST-tagged mature CLV3 and GST-tagged ERCLV3 for expression in *E. coli* (Ni et al., 2011) and CLV2-MYC, BAM1-GFP, and BAM2-GFP for transient expression in tobacco leaves (Guo et al., 2010) were previously described.

Root Inhibition Assays

Arabidopsis seeds were directly sown and grown vertically on Ats plates (Estelle and Somerville, 1987) with or without peptides. Root length was measured 10 d after germination. For tobacco and potato root assays, seeds for tobacco or for potato hybrid Atlantic × MSR041-3 were germinated on half-strength Murashige and Skoog plates. Seedlings with similar size were transferred to the above-mentioned Ats plates. Root length was measured after 7 d on vertical plates.

Proteolytic Processing of CLE Domain Proteins

Cauliflower (*Brassica oleracea*) meristematic tissues were collected, and protein extracts were prepared as described (Ni and Clark, 2006). Using the same extraction buffer (50 mM HEPES, pH 7.4, 10 mM EDTA, and 2% plant-specific protease inhibitor cocktail [Sigma]), protein extracts were prepared from roots or leaves of various plant species. Tissues were harvested from 3-week-old plants and were ground in cold extraction buffer with a pestle and mortar. The homogenates were filtered through one layer of Miracloth (Calbiochem) and centrifuged at 3,300*g* for 10 min at 4°C to remove tissue debris. Protein extracts were further centrifuged at 100,000*g* for 1 h at 4°C, and the resulting supernatants were used for proteolytic processing of CLE domain proteins. Protein concentrations were determined using BSA standards.

GST- or MBP-tagged CLE domain proteins were expressed in *E. coli* (BL21) and purified using Glutathione-Sepharose 4B (GE Healthcare) or Amylose (New England Biolabs) resins according to each manufacturer's instructions. Purified recombinant proteins were used in proteolytic processing reactions as described (Ni and Clark, 2006). Approximately 0.5 μg of CLE domain proteins was incubated with 10 μg of the indicated plant protein extracts or mock

solution (elution buffers for individual tagged proteins) for 2 h at room temperature with rotation. For processing competition assays, 25-fold excessive competitor substrate protein or BSA was added in the reactions. Protease inhibitor AEBSF (Sigma) was used at a concentration of 10 mM as described (Ni et al., 2011).

Protein gel-blot analysis was performed using chicken anti-GST antibodies (from Ken Cadigan) and horseradish peroxidase-conjugated rabbit anti-chicken antibodies (Promega) for GST-fused proteins and anti-MBP monoclonal antibodies (New England Biolabs) and goat anti-mouse antibodies (Bio-Rad) for MBP-fused proteins.

MS and MS/MS analyses were performed as described (Ni et al., 2011). Completed proteolytic processing reactions were subjected to 14,000*g* centrifugation through Microcon YM-10 centrifugal filters (Millipore) for 30 min at 4°C. Flow-through fractions were collected and sent to the Michigan Proteome Consortium (University of Michigan) for intact MS analysis. Peaks of interest were further characterized by MS/MS analysis.

Fluorescence Resonance Energy Transfer Assay of Proteolytic Processing

The fluorogenic substrate K(Dabcyl)-TLEEQKRVTPGGPDPLHNRE-EDANS was synthesized by LifeTein with a purity of greater than 90%. A stock solution of 1 mM was made in dimethyl sulfoxide (DMSO), and the final concentration of 50 μM was used in all proteolytic processing assays. A total of 100 μg of plant protein extract from cauliflower, potato (Atlantic), or tobacco roots was used in a 100-μL processing reaction with or without 10 mM AEBSF. Protein extracts plus DMSO and fluorogenic substrate plus protein extraction buffer were used as controls. The reactions were set up on 96-well opaque Costar plates, and fluorescence was measured immediately. The measurements were taken every 15 min after shaking for 5 s by a Spectramax M2 plate reader (Molecular Devices) set to 335-nm excitation and 493-nm emission.

Radioiodination of CLV3 and Binding Assays

Radioiodination of the CLV3 peptide, expression of receptor proteins, and ligand binding assays were performed as described (Guo et al., 2010). Receptor proteins were transiently expressed in tobacco leaves, and proteins were extracted 2 d after infiltration. Totals of 200 μg of BAM1-GFP, 200 μg of BAM2-GFP, and 500 μg of CLV2-MYC were used in binding assays. Twenty micromolar cold CLV3, GrCLE1 12-mer, GrCLE1 21-mer, or CLV3S peptide was added to the reactions for competition with [^{125}I]CLV3. Radioactivity associated with the protein pellets after washing was determined with an automatic γ-counter (Micromedic 4/600 Plus; ICN Biomedicals). Specific binding was calculated by subtracting from total binding the background binding when 20 μM cold peptide was present.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Time course of in vitro processing of GrCLE1.

Supplemental Figure S2. Controls in MS analysis of MBP-GrCLE1 after incubation with cauliflower protein extracts.

Supplemental Figure S3. MS/MS data and controls in MS analysis of MBP-GrCLE1 after incubation with potato root protein extracts.

Supplemental Figure S4. In vitro proteolytic processing of GrCLE1 by protein extracts from hosts and non-hosts of *Globodera rostochiensis.*

Supplemental Figure S5. Ectopic BAM2 can replace CLV2 function in vivo in CLE peptide root inhibition assays.

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