In Planta Processing and Glycosylation of a Nematode CLAVATA3/ENDOSPERM SURROUNDING REGION-Like Effector and Its Interaction with a Host CLAVATA2-Like Receptor to Promote Parasitism

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Like other biotrophic plant pathogens, plant-parasitic nematodes secrete effector proteins into host cells to facilitate infection. Effector proteins that mimic plant CLAVATA3/ENDOSPERM SURROUNDING REGION-related (CLE) proteins have been identified in several cyst nematodes, including the potato cyst nematode (PCN); however, the mechanistic details of this cross-kingdom mimicry are poorly understood. Plant CLEs are posttranslationally modified and proteolytically processed to function as bioactive ligands critical to various aspects of plant development. Using ectopic expression coupled with nanoliquid chromatography-tandem mass spectrometry analysis, we show that the in planta mature form of proGrCLE1, a multidomain CLE effector secreted by PCN during infection, is a 12-amino acid arabinosylated glycopeptide (named GrCLE1-1Hyp4,7g) with striking structural similarity to mature plant CLE peptides. This glycopeptide is more resistant to hydrolytic degradation and binds with higher affinity to a CLAVATA2-like receptor (StCLV2) from potato (Solanum tuberosum) than its nonglycosylated forms. We further show that StCLV2 is highly up-regulated at nematode infection sites and that transgenic potatoes with reduced StCLV2 expression are less susceptible to PCN infection, indicating that interference of the CLV2-mediated signaling pathway confers nematode resistance in crop plants. These results strongly suggest that phytonematodes have evolved to utilize host cellular posttranslational modification and processing machinery for the activation of CLE effectors following secretion into plant cells and highlight the significance of arabinosylation in regulating nematode CLE effector activity. Our finding also provides evidence that multidomain CLEs are modified and processed similarly to single-domain CLEs, adding new insight into CLE maturation in plants.

Plants are vulnerable to attack by plant-parasitic nematodes. The cyst-forming endoparasitic nematodes (Globodera and Heterodera spp.) are among the most damaging plant pathogens, causing tremendous crop losses globally (Chitwood, 2003). Cyst nematodes have evolved an intimate parasitic relationship with their hosts by transforming normal root cells into a unique feeding structure called a syncytium that serves as the sole nutritive source required for subsequent growth and development (Hussey and Grundler, 1998; Davis et al., 2004). Cyst nematodes are soil-borne pathogens. Once infective juveniles hatch in the soil, they penetrate into the roots of host plants and select a single cell near the root vasculature to initiate a syncytium. The syncytium forms by the fusion of cells adjacent to the initial syncytial cell through extensive cell wall dissolution and develops into a large fused cell that is highly metabolically active and contains numerous enlarged nuclei and nucleoli (Endo, 1964). Like other plant pathogens, cyst nematodes use secrete effector proteins to facilitate plant parasitism. Effector proteins, originating from the nematode esophageal gland cells (two subventral and one dorsal) and secreted into root tissues through the nematode stylet (a mouth spear), represent important molecular signals that manipulate various host cellular processes to redifferentiate normal root cells into a syncytium (Davis et al., 2004; Mitchum et al., 2008, 2013).

Genes encoding effector proteins with sequence similarity to plant CLAVATA3/ENDOSPERM SURROUNDING REGION-related (CLE) proteins have recently been cloned from several cyst nematode species, including the potato.
cyst nematode (PCN [Globodera rostochiensis; Gr]; Wang et al., 2001, 2011; Gao et al., 2003; Lu et al., 2009), a regulated and devastating pest of potato (Solanum tuberosum [Sl]) and tomato (Solanum lycopersicum) crops. Plant CLE proteins, identified from diverse monocot and dicot species (Cock and McCormick, 2001; Oelkers et al., 2008), are a class of peptide hormones that regulate many aspects of plant growth and development (Yamada and Sawa, 2013). Plant CLE genes encode small proteins that contain an N-terminal signal peptide, an internal variable domain, and either a single or multiple conserved C-terminal CLE domain(s) (Cock and McCormick, 2001; Kinoshita et al., 2007; Oelkers et al., 2008). The Arabidopsis (Arabidopsis thaliana [At]) genome encodes at least 32 single-domain CLEs, of which CLAVATA3 (CLV3) is the best characterized member. CLV3 is found to interact with three major membrane-associated receptor complexes, CLV1, CLV2-CORYNE (CRN), and RECEPTOR LIKE PROTEIN KINASE2 (RPK2; Clark et al., 1993; Jeong et al., 1999; Müller et al., 2008; Kinoshita et al., 2010; Zhu et al., 2010), to control the fate of stem cells in the shoot apical meristem (Fletcher et al., 1999). Among the three CLV3 receptors, CLV1 and RPK2 are leucine-rich repeat (LRR) receptor-like kinases, whereas CLV2 is an LRR receptor-like protein that acts together with a membrane-associated protein kinase, CRN, to transmit the CLV3 signal. The 96-amino acid CLV3 precursor is proteolytically processed into a mature 13-amino acid arabinosylated glycopeptide derived from its CLE domain, in which one (at position 7) of the two Hyp residues (at positions 4 and 7) is further modified by the addition of three units of L-Ara (Ohyama et al., 2009). The mature CLV3 glycopeptide exhibits full biological activity and binds to the LRR domain of CLV1 more strongly than nonarabinosylated forms (Ohyama et al., 2009). Hyp arabinosylation, a posttranslational modification unique to plants, also has been observed in mature CLE2 and CLE9 peptides from Arabidopsis as well as in CLE-ROOT SIGNAL2, an Arabidopsis CLE2 ortholog that controls nodulation in Lotus japonicus (L; Ohyama et al., 2009; Shinohara et al., 2012; Okamoto et al., 2013), where the arabinosine chains are revealed to have important roles in biological activity, receptor binding, and peptide conformation (Shinohara and Matsubayashi, 2013). Many Arabidopsis CLE genes are expressed in roots (Sharma et al., 2003; Jun et al., 2010), and evidence is emerging that CLE-receptor signaling pathways regulate root meristem function (Stahl et al., 2009, 2013; Meng and Feldman, 2010).

Nematode CLE genes are expressed exclusively within the dorsal gland cell and encode secreted proteins with the characteristic CLE motif(s) at their C termini (Mitchum et al., 2008; Lu et al., 2009; Wang et al., 2011). Outside the conserved CLE motif, there is no sequence similarity between nematode and plant CLE proteins. The dramatic up-regulation of CLE genes in parasitic stages of the nematode life cycle (Wang et al., 2001, 2010b, 2011; Gao et al., 2003; Lu et al., 2009), along with the observation that transgenic plants expressing double-stranded RNA complementary to nematode CLE genes are less susceptible to nematode infection (Patel et al., 2008), have made it clear that CLE effectors play a critical role in nematode parasitism. Nematode-encoded CLE genes are the only CLE genes that have been identified outside the plant kingdom. Several lines of evidence suggest that nematode CLEs function as peptide mimics of endogenous plant CLEs. First, overexpression of nematode CLE genes in Arabidopsis generated phenotypes similar to those of plant CLE gene overexpression (Wang et al., 2005, 2011; Lu et al., 2009). Second, expression of nematode-encoded CLE genes in the shoot apical meristem of an Arabidopsis clv3-2 null mutant partially or completely rescued the mutant phenotypes (Lu et al., 2009; Wang et al., 2010b). Lastly, recent studies showed that Arabidopsis receptors, including CLV1, CLV2-CRN, and RPK2, are expressed in syncytia induced by the beet cyst nematode (BCN; Heterodera schachtii) and that receptor mutants fail to respond to BCN CLE peptides and show increased resistance to BCN infection (Replogle et al., 2011, 2013), further bolstering the notion of nematode-secreted CLE effectors as peptide mimics of endogenous plant CLEs and the importance of nematode CLE signaling in plant parasitism.

Plant CLE precursors undergo posttranslational modifications and proteolytic processing to become bioactive CLE peptides (Shinohara and Matsubayashi, 2010; Shinohara et al., 2012; Okamoto et al., 2013). To fulfill a role as peptide mimics of plant CLEs, nematode CLEs are presumably recognized by the existing host modification and processing machinery for maturation. However, until now, the bioactive form of nematode-secreted CLEs that acts in planta has not been described. In addition, cyst nematodes are specialist feeders. Many agriculturally important nematode species, such as PCN, the soybean cyst nematode (Heterodera glycines), and the cereal cyst nematode (Heterodera avenae), fail to infect Arabidopsis. The mechanism of perception of nematode-secreted CLEs in crop plants still awaits investigation. In this study, we explored the molecular basis of CLE mimicry in the PCN-potato pathosystem. Using ectopic expression coupled with nanoliquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) analysis, we determined that the in planta mature form of proGrCLE1, a representative and multidomain CLE effector secreted from PCN during infection (Lu et al., 2009), is a 12-amino acid arabinosylated glycopeptide (hereafter referred to as GrCLE1-1Hyp4,7g) similar in structure to bioactive plant CLE peptides. We further cloned a CLV2-like gene from potato (hereafter referred to as StCLV2). We found that the GrCLE1-1Hyp4,7g glycopeptide binds directly to the StCLV2 ectodomain with high affinity and that transgenic potato lines with reduced StCLV2 expression are less susceptible to PCN infection. Our data provide direct evidence that nematode-secreted CLE effectors can be recognized by existing host cellular machinery to become bioactive mimics of endogenous plant CLE signals and suggest that cyst nematodes tap into the conserved CLV2 signaling pathway to promote successful infection of crop plants.
RESULTS

GrCLE1 Is Processed into a 12-Amino Acid Glycopeptide in Planta

Like plant CLEs, nematode-encoded CLEs contain a signal peptide for secretion, a variable domain (VD), and a single or multiple CLE domain(s) at their C termini (Mitchum et al., 2008; Lu et al., 2009; Wang et al., 2011). Previous studies have indicated that it is the proCLE protein (i.e. with the signal peptide being cleaved off prior to secretion through the nematode stylet; Mitchum et al., 2013), not a processed CLE peptide, that is delivered into the cytoplasm of syncytial cells during nematode infection. First, proHgCLE proteins were predominantly detected in parasitic life stages of soybean cyst nematode using an anti-HgCLE peptide antibody (Wang et al., 2010b). Second, all of the cyst nematode CLEs identified to date can mimic plant CLEs when overexpressed in plants as a proCLE protein (Lu et al., 2009; Wang et al., 2010b, 2011). In contrast, Arabidopsis plants expressing a 12-amino acid nematode-derived CLE peptide in the cytoplasm or apoplast showed no obvious phenotypic changes, further revealing a necessary role for the VD of nematode CLE proteins for function in planta (Wang et al., 2010b). Third, previous studies have provided convincing data to indicate that the VD of nematode CLEs has evolved a unique role in trafficking cytoplasmically delivered CLEs to the apoplast of plant cells posttranslationally through a yet to be discovered trafficking mechanism (Wang et al., 2010a, 2010b). Although accumulating evidence has indicated that nematode-secreted CLEs function as mimics of endogenous plant CLEs (Wang et al., 2005, 2010b, 2011; Mitchum et al., 2008; Lu et al., 2009; Wang et al., 2013), not a processed CLE peptide, that is delivered prior to secretion through the nematode stylet; Mitchum et al., 2008). Using strong cation-exchange chromatography followed by nanoLC-MS/MS analysis of extracted peptide fractions, we identified a triply charged ion with a mass-to-charge ratio of 563.3 that represents the 12-amino acid arabinosylated glycopeptide GrCLE1-1Hyp4,7g derived from the CLE domain of proGrCLE1. The GrCLE1-1Hyp4,7g glycopeptide contains two Hyp residues at positions 4 and 7, of which Hyp-7 is further modified by the addition of three units of Ara (Fig. 1B). Four additional 12-amino acid CLE-domain peptides (referred to as GrCLE1-1, GrCLE1-1Hyp7, GrCLE1-1Hyp4,7, and GrCLE1-1Hyp7g), present at low levels in the apoplastic extract (Supplemental Fig. S1A), also were identified. These four proGrCLE1-derived CLE peptides show varying degrees of modification: GrCLE1-1 contains no modification (Supplemental Fig. S1B); GrCLE1-1Hyp7 and GrCLE1-1Hyp4,7 contain either Hyp-7 or Hyp-4 and Hyp-7 (Supplemental Fig. S1B); and GrCLE1-1Hyp7g contains a Hyp-7 that is further modified with three units of Ara (Supplemental Fig. S1B). We suspect that these four CLE peptides represent products that did not complete the posttranslational modification process (Ohyama et al., 2008). No other endogenous plant CLE peptide or peptide related to the fourth atypical CLE motif of proGrCLE1 (Fig. 1A) was detected.

Overexpression and complementation studies in Arabidopsis have shown that PCN-secreted multidomain CLEs are functional in Arabidopsis (Lu et al., 2009), a nonhost of PCN. To test whether proGrCLE1 may undergo similar modification and processing in Arabidopsis, we analyzed apoplastic peptides accumulated in whole-plant submerged culture of Arabidopsis plants overexpressing $^{585}$GrCLE1. As in potato, GrCLE1-1Hyp4,7g was the predominant proGrCLE1-derived peptide detected in the culture medium (Supplemental Fig. S1C). No other endogenous plant CLE peptide was detected.

Isolation and Characterization of a CLV2-Like Gene from Potato

Several agriculturally significant cyst nematode species fail to infect Arabidopsis. Thus, investigating the mechanistic details of nematode CLE signaling, such as the perception of nematode CLE peptides in crop plant species, holds promise for more direct applications. In the Arabidopsis-BCN pathosystem, receptors including CLV1, CLV2-CRN, and RPK2 are indicated to be involved in perceiving BCN CLE peptides (Replogle et al., 2011, 2013), although the details of this CLE perception are unclear. A recent study showed that synthetic GrCLE1-1 peptide binds directly to the AtCLV2 receptor (Guo et al., 2011). Therefore, we hypothesized that orthologs of AtCLV2 in potato might be receptors for PCN CLE signals. Searching the potato genome (Xu et al., 2011) we identified a potato gene (named StCLV2) orthologous to AtCLV2 that encodes a 746-amino acid protein predicted to contain a signal peptide, a block of 20 LRRs interrupted by an 80-amino acid island region located between the 17th and 18th repeat units, a transmembrane region, and a 16-amino acid cytoplasmic tail (Fig. 2A). Phylogenetic analysis revealed that StCLV2 is closely related to AtCLV2 and its orthologs from three leguminous species (Krussel et al., 2011; Fig. 2B). To test the predicted membrane localization of StCLV2, we transiently expressed an StCLV2-GFP:HA (where HA stands for hemagglutinin) fusion protein in Nicotiana
leaves and found that it accumulated predominantly at the cell periphery and colocalized with the styryl dye FM4-64 plasma membrane counterstain (Fig. 2C). To determine whether StCLV2 is a functional receptor, we put StCLV2 under the control of the AtCLV2 native promoter and transformed the construct into an Arabidopsis clv2-1 mutant in the Landsberg erecta (Ler) genetic background (Kayes and Clark, 1998). One obvious phenotype of clv2-1 is increased carpel number. Wild-type Ler plants produce flowers with two carpels, whereas clv2-1 flowers generally contain four carpels (Kayes and Clark, 1998; Fig. 2D). We examined 61 independent T1 plants obtained from independent transformations for complementation of the clv2-1 mutant phenotype by analysis of carpel numbers and divided them into three groups based on the degree of restoration of the carpel number phenotype. Twenty-seven percent of T1 plants (group 1) could partially rescue the clv2-1 mutant, with a mean carpel number of 2.79 ± 0.18, close to that of wild-type Ler plants (Fig. 2D; Table I).

Fifty-two percent of T1 plants (group 2) showed a lesser degree of restoration, with a mean carpel number of 3.37 ± 0.21 (Table I). The degree of restoration of the clv2-1 mutant was found to correlate with the level of StCLV2 expression in flowers from AtCLV2P:StCLV2/clv2-1 transgenic plants (Supplemental Fig. S2A). Plants that exhibited almost no restoration of the carpel number phenotype (group 3) consistently had the lowest level of StCLV2 expression (Supplemental Fig. S2A). We further examined StCLV2 expression in potato. Similar to AtCLV2, StCLV2 was found to be expressed in multiple tissues, including root tissue (Supplemental Fig. S2B). Collectively, these data reveal that StCLV2 is a functional CLV2-like receptor.

GrCLE1-1Hyp4,7g Is the Bioactive Form of proGrCLE1 That Functions in Planta

We were able to purify a small quantity of the GrCLE1-1Hyp4,7g glycopeptide (Supplemental Fig. S3) from whole-plant submerged culture of Arabidopsis
plants overexpressing ΔSP GrCLE1 and used this for functional characterization. We first tested the activity of GrCLE1-1Hyp4,7g on potato root growth. Exogenous application of synthetic nonmodified GrCLE1-1 peptide at a 10 μM concentration to potato roots has been shown to suppress root growth (Lu et al., 2009). Interestingly, the GrCLE1-1Hyp4,7g glycopeptide was found to be more effective in suppressing root growth than GrCLE1-1 and GrCLE1-1Hyp4,7 at a 10 nM concentration (Fig. 3A), revealing that Hyp arabinosylation is critical for nematode CLE peptides to have high biological activity. In contrast, the hydroxylated GrCLE1-1Hyp4,7 peptide was indistinguishable from GrCLE1-1 in root growth suppression (Supplemental Fig. S4), in agreement with a previous finding that Pro hydroxylation per se has no effect on CLE peptide activity (Kondo et al., 2006). It is generally recognized that glycosylation can protect glycopeptides from proteolytic attack (Seitz, 2000), but this has not been tested for arabinosylated CLE glycopeptides. The enzymes responsible for processing CLE precursors are largely unknown, but members of the subtilase group of Ser proteases have been implicated (Ni et al., 2011). We treated both purified GrCLE1-1Hyp4,7g and synthetic GrCLE1-1Hyp4,7 peptides with either

<table>
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<tr>
<th>Genotype</th>
<th>Mean Carpel No. ± SE</th>
<th>No. of Siliques Examined</th>
<th>P Compared with cvl2-1</th>
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<tr>
<td>Ler</td>
<td>2.00 ± 0.000</td>
<td>100</td>
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<tr>
<td>cvl2-1</td>
<td>3.86 ± 0.049</td>
<td>100</td>
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<tr>
<td>AtCLV2P:StCLV2/cvl2-1 (group 1)</td>
<td>2.79 ± 0.18</td>
<td>800</td>
<td>2.01E-12</td>
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<tr>
<td>AtCLV2P:StCLV2/cvl2-1 (group 2)</td>
<td>3.37 ± 0.21</td>
<td>1,600</td>
<td>1.55E-09</td>
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<tr>
<td>AtCLV2P:StCLV2/cvl2-1 (group 3)</td>
<td>3.80 ± 0.087</td>
<td>650</td>
<td>0.086</td>
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Refer to Table 1 for the mean carpel number per silique from three groups of AtCLV2P:StCLV2/cvl2-1 transgenic plants compared with those from Ler and cvl2-1 plants. Fifty siliques per plant for each group (n = 2 for Ler, n = 2 for cvl2-1, n = 16 for group 1 transgenic plants, n = 32 for group 2 transgenic plants, and n = 13 for group 3 transgenic plants) were counted for mean carpel numbers. The mean carpel numbers from group 1 and 2 transgenic plants were significantly different from that of cvl2-1 as determined by Student's t test (P < 0.001).
subtilisin A (Ser endoproteinase) or a protein extract from potato roots as a proxy for hydrolytic conditions experienced in planta. We found that the arabinosylated GrCLE1-1Hyp4,7g glycopeptide is more resistant to hydrolytic degradation than GrCLE1-1Hyp4,7 with both treatments (Fig. 3B; Table II). Our results reveal that glycosylation indeed helps protect CLE peptides from pro tease degradation; this protection is likely critical for nematode CLE peptides to remain active in the plant cell apoplast, which contains many proteases (van der Hoorn, 2008).

The 12-amino acid GrCLE1-1 peptide was previously demonstrated to bind directly to AtCLV2 (Guo et al., 2011). To test whether the potato receptor StCLV2 interacts specifically with proGrCLE1-derived CLE peptides, we conducted a competitive ligand-binding assay by incubating $^{125}$I-Tyr-GrCLE1-1 and competitor peptide with membrane fractions derived from N. benthamiana leaves expressing StCLV2-GFP:HA (Guo et al., 2011). No-peptide treatment and an unrelated 12-amino acid GrCEP12 peptide (Chronis et al., 2013) were included as negative controls. All of the proGrCLE1-derived CLE peptides showed specific binding to StCLV2, with GrCLE1-1Hyp4,7g glycopeptide exhibiting the highest binding affinity (Fig. 3C). The hydroxylated GrCLE1-1Hyp4,7 peptide displayed a slightly stronger binding affinity to StCLV2 than the nonmodified GrCLE1-1 peptide (Fig. 3C), revealing that Pro hydroxylation also exerts a small degree of effect on receptor-binding affinity. The modified Tyr-GrCLE1-1 peptide showed binding affinity similar to GrCLE1-1 (Fig. 3C). Taken together, our analyses indicate that the GrCLE1-1Hyp4,7g glycopeptide is the bioactive form of proGrCLE1 that interacts with StCLV2 in planta.

**Nematode CLE Signaling through StCLV2 Is Required for Plant Parasitism**

As shown above, proGrCLE1-derived CLE peptides bind directly to StCLV2 in vitro. For StCLV2 to perceive PCN-secreted CLE peptides, we reasoned that it should be expressed in the appropriate root tissue during nematode infection. To test this, we generated transgenic potato plants expressing an StCLV2P:GUS transgene and found that StCLV2 is preferentially expressed in the root apical meristem (RAM) and the base of emerged lateral roots in uninfected roots (Supplemental Fig. S5A). We further monitored StCLV2 expression during the course of PCN infection. Strong GUS expression was detected in feeding sites as soon as the second-stage juveniles began to feed, and the expression level remained high in feeding sites associated with parasitic nematodes at later developmental stages (Fig. 4). The induced expression of StCLV2 in nematode feeding sites demonstrates that StCLV2 is available to interact with PCN-secreted CLE peptides during nematode infection. To further evaluate the role of StCLV2 in nematode parasitism, we prepared an artificial microRNA construct specifically targeting StCLV2 and transformed it into potato. Screening of over 10 independent transgenic lines identified two (lines 27 and 29) where StCLV2 expression was reduced in roots by more than 70% compared with wild-type potato (Fig. 5A). Both roots and aboveground parts of the two StCLV2 knockdown lines were examined, and no obvious phenotypic difference was observed in comparison with the control plants. We then tested nematode infection on these two lines along with wild-type plants and a transgenic line (line 6) that exhibited no
suppression of StCLV2 (Fig. 5A). At 36 d postinoculation, nematode females were counted. Our results showed that the two StCLV2 knockdown lines are more resistant to nematode infection, because the number of nematode females recovered from these two lines was approximately 47% to 55% less than those from the control lines (Fig. 5B). The decreased susceptibility to PCN implies that StCLV2 is involved in nematode parasitism.

Exogenous application of synthetic GrCLE1-1 peptide to potato roots has been shown to result in a phenotype of root meristem consumption (Lu et al., 2009). To further establish that StCLV2 is involved in perceiving PCN-secreted CLE signals in planta, we evaluated the effect of the GrCLE1-1 peptide on roots of StCLV2 knockdown lines. In contrast to the control lines, which showed a dramatically reduced size of the RAM after treatment with the synthetic GrCLE1-1 peptide (at a concentration of 10 μM), RAMs of the treated knockdown lines were only slightly smaller than those of untreated lines (Fig. 5C; Supplemental Fig. S5B). The small degree of RAM reduction of the knockdown lines might result from residual StCLV2 activity or, alternatively, suggests the involvement of additional receptors in perceiving the GrCLE1-1 peptide signal. The latter is consistent with recent findings in the Arabidopsis-BCN pathosystem (Replogle et al., 2011, 2013). In summary, our data reveal that StCLV2 is directly involved in perceiving PCN-secreted CLE signals and that nematode CLE signaling through StCLV2 facilitates PCN infection of potato.

DISCUSSION

Plant-parasitic nematodes have evolved unique effectors that mimic plant peptide hormones. The underlying mechanisms of this cross-kingdom mimicry, however, have remained largely unknown. In this study, ectopic expression was used to mimic the in planta secretion of CLE effectors during nematode infection, which enabled the identification of the in planta mature form of a PCN-secreted multidomain CLE effector. We determined that the mature PCN CLE peptide is a 12-amino acid arabinosylated glycopeptide (GrCLE1-1Hyp4,7g; Fig. 1B) that shares striking structural similarity with mature plant CLE peptides and demonstrated that this glycopeptide binds with high affinity to a CLV2-like receptor (StCLV2) from potato (Fig. 3C), a host of PCN.

The L-Ara chain in mature plant CLE peptides is critical for receptor binding and full biological activity (Ohyama et al., 2009; Okamoto et al., 2013). Our finding that the arabinosylated GrCLE1-1Hyp4,7g glycopeptide had higher receptor-binding affinity and was more effective in root growth suppression than the corresponding nonarabinosylated forms (Fig. 3A and C) not only demonstrates the potency of this in planta processed GrCLE1-1Hyp4,7g glycopeptide as a ligand mimic of endogenous plant CLE signals but substantiates the physiological importance of Hyp arabinosylation in CLE peptide signaling. A recent study using chemically synthesized CLV3 glycopeptide revealed that the Ara chain is important for the biological activity of the peptide and also contributes to the conformation of the peptide backbone (Shinohara and Matsubayashi, 2013). Notably, we found that the GrCLE1-1Hyp4,7g glycopeptide was more resistant to hydrolytic degradation than the GrCLE1-1Hyp4,7 peptide with only Pro hydroxylation (Fig. 3B; Table II). This finding suggests a new role of arabinosylation in stabilizing CLE peptides. This stability gained through glycosylation may help ensure
an efficient activity of nematode-derived CLE peptides when they are exposed to numerous proteases present in the plant cell apoplast. To date, the mature structure of bioactive CLE peptides has only been determined for a limited number of plant CLEs (Ito et al., 2006; Ohyama et al., 2009; Shinohara et al., 2012; Okamoto et al., 2013), all of which contain a single CLE motif. The proGrCLE1 effector secreted by PCN is unique in that it contains tandem CLE motifs (Fig. 1A). Multidomain CLEs also have been identified in several plant species, including two crop species, rice (Oryza sativa) and wheat (Triticum aestivum; Kinoshita et al., 2007; Oelkers et al., 2008); however, it remains to be determined whether these multidomain plant CLEs can be processed to become functional CLE peptides. Our results reveal that a multidomain CLE is modified and processed similar to single-domain CLEs, indicating that plants use conserved mechanisms to generate bioactive CLE peptides from both single-domain and multidomain CLE precursors. Thus, it is reasonable to assume that bioactive CLE peptides are generated from multidomain plant CLEs, although the significance of the evolution of multidomain CLEs awaits to be uncovered.

Except for the CLE motif domain, there is no sequence similarity between plant and nematode CLE proteins. Thus, the detection of the GrCLE1-1Hyp4,7g glycopeptide in the plant culture medium indicates that cyst nematodes have evolved to coopt existing posttranslational trafficking, modification, and processing pathways of host cells to generate functional CLE effectors. Post-translational modification (PTM) was recently recognized to play crucial roles in the regulation of effector activity, localization, and target binding (van der Heijden and Finlay, 2012), emerging as an exciting area in the field of effector research. Lipidation is a common PTM occurring in a number of type III effectors of bacterial pathogens. For example, upon translocation into the plant cell, the AvrPphB effector from the phytopathogen Pseudomonas syringae undergoes a self-cleavage process to expose its myristoylation and palmitoylation motif, which subsequently results in lipidation and proper localization to the plasma membrane (Dowen et al., 2009). The regulation of effector activity by host-mediated phosphorylation is also observed for bacterial effectors (van der Heijden and Finlay, 2012). The type III effector AvrPto of P. syringae was phosphorylated when expressed in plant leaves, and the phosphorylated residue was found to promote the AvrPto-mediated virulence and avirulence of P. syringae in tomato leaves (Anderson et al., 2006). Ubiquitination and cleavage by host protease caspase3 are two additional PTMs that are known to regulate effectors of the Salmonella spp. pathogen during infection (van der Heijden and Finlay, 2012). In this study, we discovered a new example of host-mediated PTM of pathogen effectors: the Hyp arabinosylation found in the GrCLE1-1Hyp4,7g glycopeptide derived from the proGrCLE1 effector. Considering the fact that all nematode CLEs characterized to date are functional in planta, host-mediated Hyp arabinosylation is likely to be a common PTM of mature nematode CLE peptides for their activation following delivery into host cells to function as ligand mimics of endogenous plant CLE signals. This finding further highlights the significance of host-mediated PTM in effector regulation.

Figure 5. StCLV2 is involved in nematode CLE signaling to promote parasitism. A, Transgenic potato lines with reduced StCLV2 expression. An artificial microRNA construct targeting StCLV2 was generated and transformed into potato. Quantitative RT-PCR was used to evaluate StCLV2 expression in the resulting transgenic lines, and two (lines 27 and 29) were found to have dramatically reduced expression of StCLV2 compared with the wild-type (WT) plant and transgenic line 6. Data represents means ± s.e of three biological replicates, normalized to the potato POLYUBIQUITIN-LIKE (UBI) gene (XM_006360024) and relative to expression in the wild-type plant (**P < 0.001, Student’s t test). B, StCLV2 knockdown lines show decreased susceptibility to nematode infection. Wild-type and transgenic lines (10 vegetatively propagated plantlets for each line) were inoculated with nematode juveniles, and nematode females were counted 5 weeks postinoculation. Values are means ± s.e of three independent experiments (***P < 0.001, Student’s t test). C, Roots of StCLV2 knockdown lines become less responsive to the GrCLE1-1 peptide. Potato plantlets (four for each line) were grown for 15 d on medium with or without the GrCLE1-1 peptide (10 μM). The length of the RAM of individual roots (five roots for each plantlet) was measured microscopically. Data represent means ± s.e (n = 20 for each line). Means with different letters are significantly different (P < 0.001, Student’s t test).
Moreover, the detection of the GrCLE1-1Hyp4,7g glycopeptide in the plant culture medium supports the unique role of the VD in trafficking nematode-secreted CLE effectors from the cytoplasm to the apoplast of host cells (Wang et al., 2010a, 2010b). How the VD of nematode CLE effectors is recognized by the host cellular machinery that allows for Hyp arabinosylation followed by proteolytic processing is an area of great interest for future study.

We showed that the StCLV2 receptor is important for nematode CLE signaling and ultimately for successful PCN infection of potato. Notably, nematodes often produce/secrete CLE effectors with different CLE motifs (Lu et al., 2009; Wang et al., 2011; Alenda et al., 2013). Based on studies of the Arabidopsis-BCN pathosystem (Replogle et al., 2011, 2013) as well as a recent finding that the nonmodified GrCLE1-1 peptide could bind physically to BARELY ANY MERISTEM1 (BAM1) and BAM2 in Arabidopsis (Guo et al., 2011), it is likely that PCN and other cyst nematodes may utilize their secreted CLE effectors to activate multiple CLE signaling pathways through different host receptors to promote successful infection. Thus, disruption of nematode CLE perception or the associated CLE signaling pathways may provide a powerful means to generate improved nematode resistance in crop plants.

**MATERIALS AND METHODS**

### Nematode Culture and Inoculation

Maintenance of the PCN (Glomus ristochiiensis) culture and nematode inoculation on cultivated potato (Solanum tuberosum) plantlets was conducted as described previously (Lu et al., 2008; Chronis et al., 2013).

### DNA Constructs

The 35S:StCLV2 construct (previously named 35S:GrCLE1ASp) was described by Lu et al. (2009). The StCLV2 gene was identified in the annotated potato genome sequence PGSC0003DM400027288 and amplified from complementary DNA derived from potato roots using primers StCLV2_ATGF and StCLV2_TGAR (Supplemental Table S1), then cloned into the pGEMT-Easy vector (Promega), resulting in pGEMT-StCLV2. For the subcellular localization study, StCLV2 was amplified from pGEMT-StCLV2 using primers StCLV2_XhATGF and StCLV2_BamHI_nrTGAR and then cloned into the pBIN61-GFP:HA binary vector (Sacco et al., 2009) at the XhAt and BamHI sites to generate the StCLV2-GFP:HA fusion construct. For the chv1-1 complementation study, the 1,252-bp promoter sequence of aCLV2 (Replogle et al., 2011) was amplified from genomic DNA of Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 with primers aACL2_PstI_1252F and aACL2_SalI_1252R and cloned into the binary vector pCAMBIA1300 (Cambia) at the PstI and SalI sites to generate pACL2P1252. The pACL2 coding sequence was then cloned into pACL2P1252 at the SalI and BamHI sites to make pACL2P1252/StCLV2. To generate the pACL2P1252/GUS construct, a 1,874-bp sequence upstream of the StCLV2 was amplified from genomic DNA of potato ‘Désirée’ using primers StCLV2_SalI_1246F and StCLV2_BamHI_R and cloned into the binary vector pBI101-2 (Jefferson et al., 1987) at the SalI and BamHI sites. To make the artificial microRNA construct, a 21-mer sequence (5'-TTTACTGTATTTGTCGCC-3') targeting StACL2 (nucleotide positions 1,234-1,253), designed according to the Web artificial microRNA designer interface WMD3 (wmd3.weigelworld.org), was engineered into the miR319a precursor by replacing the endogenous miR319a sequence in pRS300 (Ossowski et al., 2006) using overlap PCR (Schwab et al., 2006) with the primers listed in Supplemental Table S1. The new amiR319a precursor was then cloned into the modified binary vector pMD1 (its 35S promoter was replaced by the superpromoter; Lee et al., 2007) at the XhAt and SacI sites to make the pSMD1-StACL2ami artificial microRNA construct.

### Plant Materials and Transformations

Plant materials used include Arabidopsis ecotypes Columbia-0 and Ler, the chv1-1 mutant (Kayes and Clark, 1998), potato ‘Désirée’ and Nicotiana benthamiana. Transgenic Arabidopsis plants overexpressing 35S:StCLV2 and transgenic chv1-1 plants expressing AtCLV2P/StCLV2 were generated via the floral dip method (Clough and Bent, 1998). Agrobacterium rhizogenes 15834 was transformed with 35S:StCLV2 and used to generate potato hairy roots (Wang et al., 2007; Lu et al., 2009). Calli were induced by culturing transgenic hairy roots at 22°C in the dark on callus induction medium containing 1X Murashige and Skoog salts (Sigma-Aldrich), 26.7 µM G4, 4 µM nicotinic acid, 2.4 µM pyridoxine HCl, 1.2 µM thiamine HCl, 0.5 µM folic acid, 0.2 µM t-biotin, 0.01% (w/v) myoinositol (Acros), 3% (w/v) Suc, 0.002% (w/v) 2,4-dichlorophenoxyacetic acid, 50 µg L⁻¹ lanamycin, 238 µg L⁻¹ lincomycin (Gibco/SmithKline), and 1% (w/v) agar. Transgenic potato lines expressing StCLV2GFP or pSMD1-StCLV2ami were generated as described (Chronis et al., 2013).

### Discovery of in Planta Processed proGrCLE1 Peptide

Submerged culture of transgenic potato calli or transgenic Arabidopsis plants overexpressing 35S:StCLV2 was used for the discovery of proGrCLE1-derived peptides in plants. Experimental details on the detection, purification, and quantitation of the GrCLE1-1Hyp4,7g arabinosylated glycopeptide are given in Supplemental Materials and Methods S1.

### Subcellular Localization

Agrobacterium tumefaciens C58C1 transformed with StCLV2-GFP:HA was infiltrated into N. benthamiana leaves (Chronis et al., 2013). Two days after infiltration, leaves were collected and stained in FM4-64 solution (Invitrogen). The leaf sections were then visualized with an SPS Leica confocal microscope.

### Peptide Bioassays

Peptides with a purity of greater than 80% were synthesized (Sigma-Aldrich) and dissolved in filter-sterilized 50 mM sodium phosphate buffer. Tested peptides included the chemically synthesized peptides of GrCLE1-1 and GrCLE1-1Hyp4,7 and the purified GrCLE1-1Hyp4,7g glycopeptide. True potato seeds were surface sterilized and plated on eight-well square plates (Sigma-Aldrich) containing medium with each peptide at a 10 nM concentration (Lu et al., 2009).

### Enzymatic Hydrolysis Assay

Potato roots (approximately 5 g) of 14-d-old seedlings were collected and ground in liquid nitrogen, then in 10 mL of extraction buffer (50 mM HEPES [pH 7.4] and 10 mM EDTA). The homogenate was centrifuged at 16,000g for 10 min at 4°C, and the recovered supernatant was further centrifuged at 100,000g for 1 h at 4°C followed by lyophilization. The dried protein extract was resuspended in 0.5 mL of extraction buffer and used for the enzymatic digestion assay. The purified GrCLE1-1Hyp4,7g glycopeptide and synthetic GrCLE1-1Hyp4,7 peptide each was treated with subtilisin A (Sigma-Aldrich; 0.0125 μg of subtilisin A for 100 fmol of peptide) in 0.1 M NH₄HCO₃, at room temperature for 0, 10, 30, and 50 min or with potato root extract (40 μL of potato root extract for 1 pmol of peptide) at room temperature for 0 and 2 h. Each sample was boiled for 5 min immediately after treatment. Subtilisin-treated samples were analyzed directly. Potato root extract-treated samples were further purified by centrifugation at 16,000g for 10 min and then by ultrafiltration through a 3-kD cutoff ultrafilter (Millipore). Samples were desalted and reconstituted in 0.5% (v/v) formic acid containing 3 fmol μL⁻¹ GrCLE1-1Hyp4 peptide for nanoliquid chromatography-multiple reaction monitoring (MRM).
quantitative analysis. The synthetic GrCLE1-1Hyp4,7 peptide was used for optimizing MRM parameters and creating a standard curve. The triply charged parent ions to both b6 and y4 product ions for each peptide were used to generate transition ion pairs in MRM analysis.

Binding Assay

*Arabidopsis plants overexpressing 35S: AtCLV2P:StCLV2/ clv2-1 plants was extracted and used to assess StCLV2 expression by quantitative real-time PCR (RT-PCR). mRNAs from roots, leaves, and stems of cultured potato plants expressing SACLVP2GFPHA were collected, infiltrated with GUS substrate buffer (100 μM Tris, pH 7.5, 50 mM NaCl, 1 μM 5-bromo-4-chloro-3-indolyl-glucuronic acid, 0.06% [v/v] Triton X-100, and 1 mM potassium ferricyanide [pH 7]), and incubated for 12 to 14 h at 37°C (Jefferson et al., 1987). To further stain nema-
todes, GUS-stained roots were incubated in 1% (v/v) hypochlorite for 7 min, rinsed in water for 20 min, and then boiled in acid fuchsin solution (138 μM acid fuchsin and 0.5% [v/v] acetic acid) for 1 min. Stained roots were kept in the solution for 30 min and then rinsed in 100% ethanol. Stained roots were mounted on glass slides and visualized with a Nikon Eclipse TS100 inverted microscope.

Reverse Transcription-PCR and Quantitative Reverse Transcription-PCR

mRNAs from flowers of wild-type Ler, clv2-1 mutant, and transgenic A. thaliana CS8C1 transformed with StCLV2-GFP:HA were used for determining SACLVP2 expression by reverse transcription (RT)-PCR. The Arabidopsis Glyceraldehyde-3-phosphate dehydrogenase A ( AtGPDH ) gene ( At5g06420 ) was used as an endogenous reference. mRNAs from roots, leaves, and stems of cultured potato plants expressing pSMID1-StCLV2ami as well as wild-type 'Désirée' plantlets were used and extracted used to quantify SACLVP2 expression by reverse transcription-PCR as described previously ( Lu et al., 2008 ). The potato UBI gene ( XM_006360024 ) was used as an endogenous reference. All primers used are listed in Supplemental Table S1.

Phylogenetic Analysis

Phylogenetic analysis of A. thaliana and its orthologs, and candidate CLE receptors, was conducted as described previously ( Lu et al., 2008 ). Full-length protein sequences were used for the analysis.

Supplemental Data

The following supplemental materials are available.

Host-Mediated Activation of Nematode CLE Effectors

Supplemental Figure S1. Identification of additional proGrCLE1-derived CLE peptides in plants.

Supplemental Figure S2. Characterization of SCLV2.

Supplemental Figure S3. Nanoliquid chromatography UV chromatograms showing the purity of the isolated GrCLE1-1Hyp4,7 glycopeptide.

Supplemental Figure S4. Effects of different concentrations of synthetic nonglycosylated proGrCLE1-derived CLE peptides on potato root growth.

Supplemental Figure S5. Characterization of SCLV2 using transgenic potato lines.

Supplemental Table S1. List of primers used in this study.

Supplemental Materials and Methods S1. Discovery of in planta processed proGrCLE1 peptide.

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