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The 14–Amino Acid CLV3, CLE19, and CLE40 Peptides Trigger Consumption of the Root Meristem in *Arabidopsis* through a *CLAVATA2*-Dependent Pathway [W]

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***CLAVATA3* (*CLV3*), *CLV3/ESR19* (*CLE19*), and *CLE40* belong to a family of 26 genes in *Arabidopsis thaliana* that encode putative peptide ligands with unknown identity. It has been shown previously that ectopic expression of any of these three genes leads to a consumption of the root meristem. Here, we show that in vitro application of synthetic 14–amino acid peptides, CLV3p, CLE19p, and CLE40p, corresponding to the conserved CLE motif, mimics the overexpression phenotype. The same result was observed when CLE19 protein was applied externally. Interestingly, *clv2* failed to respond to the peptide treatment, suggesting that CLV2 is involved in the CLE peptide signaling. Crossing of the *CLE19* overexpression line with *clv* mutants confirms the involvement of CLV2. Analyses using tissue-specific marker lines revealed that the peptide treatments led to a premature differentiation of the ground tissue daughter cells and misspecification of cell identity in the pericycle and endodermis layers. We propose that these 14–amino acid peptides represent the major active domain of the corresponding CLE proteins, which interact with or saturate an unknown cell identity-maintaining CLV2 receptor complex in roots, leading to consumption of the root meristem.**

INTRODUCTION

In multicellular organisms, cell-to-cell communication is essential for coordinating growth and differentiation. In animals, peptides are known to be the major players in cell-to-cell communication (for review, see Alberts et al., 1994). This is in contrast with plants in which most intercellular communication is mediated by phytohormones, such as auxin, cytokinin, gibberellic acid, abscisic acid, ethylene, and brassinosteroids (Mandave, 1988; Kende and Zeevaart, 1997). However, in recent years, several putative peptide ligands have been identified in plants (for review, see Lindsey et al., 2002; Ryan et al., 2002) and have been shown to mediate signaling events during plant–pathogen interactions (Pearce et al., 1991), cell division (Matsubayashi and Sakagami, 1996), and anther–stigma interactions (Schopfer et al., 1999; Kim et al., 2003).

CLAVATA3 (CLV3) is a putative peptide ligand of *Arabidopsis thaliana* that interacts with a disulphide-linked CLV1/CLV2 receptor complex to restrict the stem cell population in the shoot apical meristem (SAM) in a non-cell-autonomous manner (Fletcher et al., 1999). CLV1 is a membrane-bound leucine-rich repeat receptor kinase, while CLV2 is a leucine-rich repeat receptor–like protein lacking a kinase domain (Clark et al., 1997; Jeong et al., 1999). The stem cells are marked by *CLV3* expression, while the SAM organizing center is marked by the expression of the *WUSCHEL* (*WUS*) stem cell-promoting transcription factor (Laux, 2003). A feedback regulation loop between CLV3 and WUS maintains the number of stem cells in the SAM (Brand et al., 2000; Schoof et al., 2000). As such, *clv1*, *clv2*, and *clv3* mutants have enlarged SAMs, while the *wus* mutant or *CLV3* overexpression terminates SAM development (Laux et al., 1996; Hobe et al., 2003). Although biochemical studies showed that CLV3 is required for the formation of a 450-kD functional CLV1/CLV2 receptor complex with several associated proteins (Trotochaud et al., 1999), the biochemical nature of the active ligand encoded by *CLV3* has not yet been elucidated.

CLV3 belongs to the *CLV3/ESR* (*CLE*) family of 26 genes in *Arabidopsis*, of which 25 are transcribed in one or more tissues (Cock and McCormick, 2001; Hobe et al., 2003; Sharma et al., 2003; Fiers et al., 2004). These genes encode small proteins that contain a putative secretion signal at their N termini and a conserved 14–amino acid motif (CLE motif) at or near their C termini (Cock and McCormick, 2001). In the *clv3-1* and *clv3-5* mutants, a single amino acid change (from G to A) in the CLE motif is enough to disrupt the function of CLV3 (Fletcher et al., 1999), indicating the importance of this motif. First identified in maize

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(*Zea mays*) as being expressed in endosperm regions surrounding the embryo, *ESR* genes encode extracellular proteins with unknown functions (Bonello et al., 2002). Such *CLE* genes have not only been found in many plant species, but also in parasitic nematodes (Wang et al., 2005).

One interesting feature of these CLE proteins is that they share very little sequence similarity outside the CLE motif (Cock and McCormick, 2001). Remarkably, *CLE40* from *Arabidopsis* and *Hg-SYV46* from nematode can fully complement the *clv3* mutant phenotype when expressed under the control of the *CLV3* and cauliflower mosaic virus *35S* (*35S*) promoter, respectively, illustrating the functional redundancy of these genes (Hobe et al., 2003; Wang et al., 2005). The redundancy could also be seen from the T-DNA insertional mutants of *CLE19* and *CLE40*, which gave no phenotype and a very subtle phenotype, respectively (Hobe et al., 2003; Fiers et al., 2004). By contrast, overexpression of several *CLE* genes, such as *CLV3*, *CLE19*, *CLE40*, or *Hg-SYV46*, under the control of the *35S* promoter induce striking developmental phenotypes in root and shoot development in *Arabidopsis* (Hobe et al., 2003; Fiers et al., 2004; Wang et al., 2005). Termination of root meristem development has been observed in transgenic plants overexpressing any of these four genes (Hobe et al., 2003; Fiers et al., 2004; Wang et al., 2005). These studies point to the existence of common and redundant signaling machinery in roots that responds to different *CLE* genes to trigger the differentiation of the root meristem.

Here, we report the development of a novel in vitro root assay to elucidate how *CLE* genes trigger the consumption of the root meristem. Application of chemically synthesized 14–amino acid peptides, CLV3p, CLE19p, and CLE40p (hereafter referred to as CLE peptides), corresponding to the conserved CLE motif of the related CLE proteins, led to consumption of the root meristem in a manner that closely resembles the overexpression phenotypes mentioned above. The same effect was achieved by application of heterologously produced full-length CLE19 protein. The *clv2* mutation abolished the sensitivity of roots to these peptides, suggesting that CLV2 is involved in the CLE signaling pathway. Using different marker lines, we demonstrate that the peptide treatments led to an inward shifting of cell identity in several cell layers and to a premature differentiation of the ground tissue daughter cells. We propose that the CLE peptides represent the major functional domain of the CLE proteins and that this domain interacts with CLV2 to trigger the termination of the root meristem.

RESULTS

In Vitro Application of CLE Peptides Causes a Short Root Phenotype

It has been reported previously that overexpression of *CLV3*, *CLE19*, *CLE40*, or *Hg-SYV46* in *Arabidopsis* under the control of *35S* promoter resulted in termination of root meristem development (Hobe et al., 2003; Fiers et al., 2004; Wang et al., 2005). The same phenotype was observed when *CLE19* was expressed under the control of the root meristem–specific promoter *RCH1* (Casamitjana-Martinez et al., 2003). The amino acid sequences of CLV3, CLE19, and CLE40 were compared to identify common motifs. The only conserved sequence among these proteins resides in the CLE motif (Figure 1A). We therefore examined whether a chemically synthesized 14–amino acid peptide of CLE19p (Figure 1B), corresponding to the conserved CLE motif of CLE19, is also able to trigger the consumption of the root meristem. As a control, we used a 16–amino acid peptide corresponding to the C terminus of AGAMOUS that has no similarity with the CLE peptides (AGp, Figure 1B). *Arabidopsis* seeds were germinated on vertical plates with media containing different concentrations of the individual peptides. The length of the primary root was measured after 7 d (Figure 2A). Although no clear effect on root growth was observed when CLE19p was applied at the 0.1 to 1 μM concentration range, a dramatic inhibitive effect was observed at 10 μM or higher (Figure 2A). Further increase of the CLE19p concentration from 10 to 100 μM gave only a slight decrease in root length (Figure 2A). AGp had no significant effect on root length. As such, we used a concentration of 10 μM for the subsequent analyses. These results gave us the first indication that the conserved motif of CLE19 is sufficient to mimic the short root phenotype generated by overexpression of the *CLE19* gene.

Furthermore, CLV3p and CLE40p (Figure 1B), corresponding to the CLE motifs of CLV3 and CLE40, respectively, were tested for their effect on root growth. Unlike CLV3, CLE40 is expressed in roots (Hobe et al., 2003). Several peptides, including a mutant CLV3 peptide (CLV3m, with a G-to-A conversion as in *clv3-1* and *clv3-5* mutants), two truncated peptides (CLV3t and CLE19t, with five amino acids removed the C termini of CLV3p and CLE19p, respectively), and a shuffled CLV3 peptide (CLV3s, the same amino acid composition as CLV3p but with a shuffled sequence), were chemically synthesized as controls (Figure 1B). The peptides were added individually to medium at a concentration of 10 μM. Seedlings treated with CLV3p and CLE40p, similar to those treated with CLE19p, showed a clear reduction in root length, as compared with the controls with either no peptide or treated with CLV3m, CLV3s, CLV3t, or CLE19t, which did not have a significant effect on root length (Figure 2B). Statistical analysis showed no significant differences among treatments with CLV3p, CLE19p, and CLE40p.

To investigate if all peptides derived from the CLE motif of the CLE proteins are able to produce the short root phenotype, we synthesized a 14–amino acid peptide (CLE5p) of CLE5. The CLE motif of CLE5 differs from that of CLV3, CLE19, and CLE40 (Figure 1B). CLE5 is expressed in roots (Sharma et al., 2003). CLE5p shares between 43 and 50% identity with the CLE peptides. Treatment with CLE5p did not cause any reduction in root length (Figure 2B), indicating that the sequence of the peptide is critical for the function of the CLE peptides in triggering the consumption of root meristem, and not all peptides with a CLE motif from one of the 26 *CLE* genes results in a similar response.

To examine if CLV3p application in solid media could complement the *clv3* phenotype, we measured the size of the *clv3* SAMs using Nomarski optics after 4, 8, and 14 d of treatment. We failed to detect any changes, suggesting that either CLV3p cannot be transported from the root to the SAM, or the peptide is not able to function in the SAM.

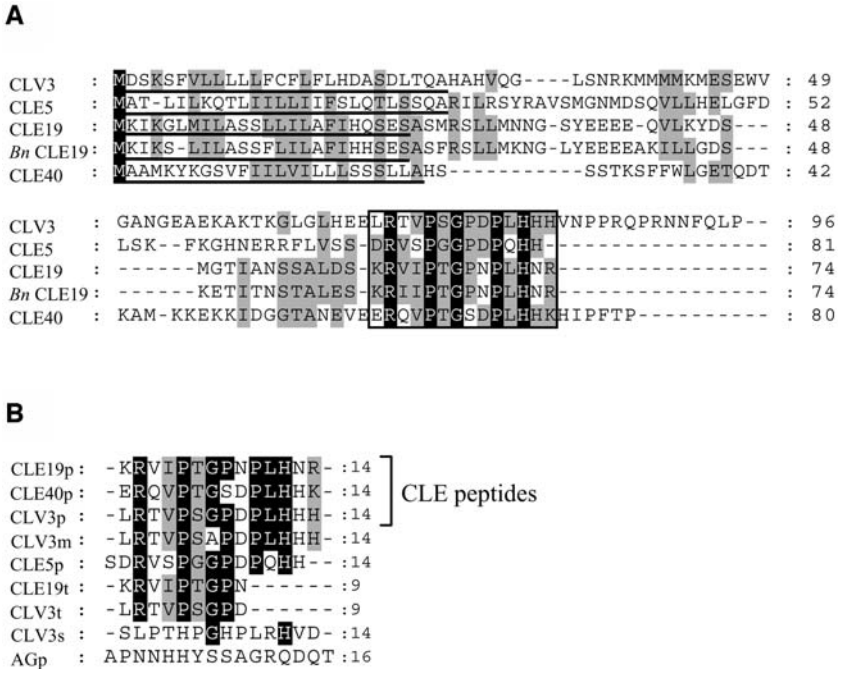

Figure 1. The CLE Motif and the Peptides Used in the Root Assays.

(A) Alignment of CLV3, CLE5, CLE19, *Bn* CLE19, and CLE40. The signal sequences are underlined, and the CLE motif is framed.
(B) Chemically synthesized peptides used in the root assays.
Identical amino acids in five or more proteins/peptides are shaded in black, and amino acids that are similar or identical in at least three proteins are highlighted in gray.

The CLE Peptides Trigger the Consumption of the Root Meristem in a Manner Similar to *CLE19* Overexpression

We next investigated if the short root phenotype generated after the CLE peptide treatment resembles the phenotype observed after overexpression of *CLE19* (Fiers et al., 2004). Using Nomarski optics, we observed that roots treated for 14 d with CLV3s, CLV3m, and CLE5p were morphologically indistinguishable from roots grown on plates without peptide (Figures 3A to 3D). These roots have a cell division zone that consists of a population of cytoplasm-dense cells, followed by gradually enlarged elongated cells. By contrast, roots treated with the active CLE peptides were much thinner, with a significantly decreased number of meristematic cells (Figures 3E to 3G). These roots seem to have an equal number of cell layers along the radial axis, as in the wild type, except for a region above the quiescent center (QC) where the formation of ground tissue appeared to be delayed. The thinner root phenotype seems to be caused by reduction in cell expansion. In CLE peptide-treated roots, only a few cytoplasm-dense cells could be recognized, which were immediately followed by elongated and highly vacuolated cells above this region. This consumption of the root meristem closely resembles the phenotype observed in roots of *CLE19* overexpression lines (Casamitjana-Martinez et al., 2003; Fiers et al., 2004).

The numbers of meristematic cells in the primary root of wild-type and CLE peptide-treated roots were quantified by counting the number of nonelongated cytoplasm-dense cells along the cortex layer, starting from the QC after 14 d of treatment. In wild-type seedlings, the number of meristematic cells was on average 81, while only one to four such cells could be found from seedlings germinated in the presence of a CLE peptide ($n = 10$; Figures 3E to 3H).

Previous reports showed that ectopic expression of *CLV3*, *CLE19*, or *CLE40* caused consumption of the root meristem without immediately disturbing auxin distribution in the columella initial cells or QC function (Casamitjana-Martinez et al., 2003; Hobe et al., 2003; Fiers et al., 2004). To determine if this was also the case after the treatments with CLE peptides, seedlings of *DR5*:*β-glucuronidase* (*GUS*) (a reporter line showing auxin distribution; Sabatini et al., 1999) and *QC25* (QC-specific marker; Casamitjana-Martinez et al., 2003) marker lines were examined. Although a clear reduction in root length and a decreased number of meristematic cells were observed, no visible difference was observed in the *DR5*:*GUS* and *QC25* (see Supplemental Figure 1 online) expression patterns. This observation revealed that neither the peptide treatment nor the overexpression acts primarily on the QC.

CLV2 Is Involved in Perception of the CLE Peptides in Roots

To identify components of the signal transduction pathway involved in perception of the CLE peptide signal in roots, we determined whether the CLV signaling pathway is involved in the short root phenotype by treating *clv1*, *clv2*, and *clv3* mutants

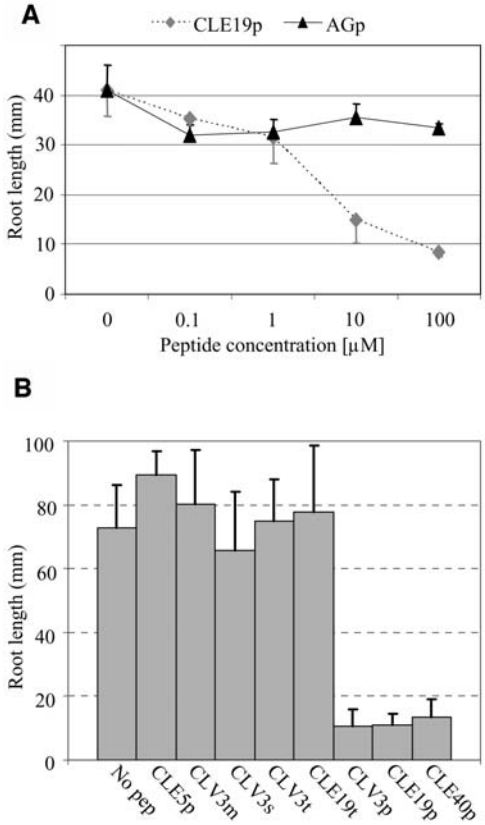

Figure 2. Treatment with CLE Peptides Gave a Short Root Phenotype in *Arabidopsis*.

(A) Effect of different concentrations of CLE19p and AGp (control) on wild-type (*Col-0*) root development. The lengths of the main roots were measured after 7 d of growth on peptide-containing media ($n = 8$ for each treatment).

(B) Effects of different peptides on the root lengths of *Arabidopsis* (L*er*). The roots were measured after 14 d of growth on media with 10 μM various peptides. Note that CLE5p, CLV3m, CLV3t, and CLE19t did not inhibit root growth ($n = 10$ for each treatment).

Data and error bars represent mean ± SD.

with the CLE peptides. Seedlings of the wild type (Landsberg *erecta* [L*er*]), *clv1-1*, and *clv3-2* grown on plates with any of the CLE peptides showed a significantly shorter root length and decreased number of meristematic cells (Figures 4A and 4B). For example, the number of meristematic cells in *clv1-1* roots grown on peptide-free media was 47 ± 12 mm, which was reduced to 2 ± 2 mm after treatment with CLE19p (Figure 4B).

clv2-1, however, showed significantly less sensitivity to the CLE peptides (Figures 4A and 4B). In the absence of peptide application, we did not detect any defects in the root of the *clv2*, using both Nomarski optics and confocal microscopy (see Supplemental Figure 2 online). The primary roots of *clv2* mutants treated with CLE peptides also maintained a normal meristem morphology (Figure 4E), in contrast with fully differentiated root meristems of the wild type, *clv1-1*, and *clv3-2* after the peptide treatments (Figures 4C, 4D, and 4F). Seedlings of *clv2-1* treated with CLV3p, CLE19p, or CLE40p had 51 to 83 meristematic cells, as compared with two to four such cells for *clv1-1* seedlings and one to three for *clv3-2* (Figure 4B).

Heterologously Produced CLE19 Protein Also Triggers a Consumption of the Root Meristem

To examine if the full-length CLE protein functions like the CLE peptides, a construct was made in which the sequence encoding the *Brassica napus* (*Bn*) CLE19 protein without the putative secretion signal peptide was fused to the C terminus of glutathione *S*-transferase (GST) and expressed in *Escherichia coli*. After cleaving with thrombin and purification (Figure 5A), the *Bn* CLE19 protein was evaluated for its effect on *Arabidopsis* roots. Application of the *Bn* CLE19 protein at a concentration of 10 μM strongly inhibited root growth of *Arabidopsis* seedlings, as compared with the control GST protein, which did not have any detectable effect (Figure 5B). After 10 d of treatment, the average root length in the GST-treated seedlings was ~34 mm, while for the *Bn* CLE19-treated seedlings it was ~7 mm (Figure 5B). Nomarski-based observations showed that *Bn* CLE19 protein-treated roots exhibited a similar phenotype as the CLE peptide-treated or *CLE19* overexpression roots. No additional phenotypes were observed as compared with the treatment with CLE19p, demonstrating a functional similarity between the CLE peptides and the full-length protein.

Genetic Analysis Confirms That CLV2 Is Involved in Perceiving the CLE19 Signal in Roots

After establishing the role of CLV2 in perception of CLE peptides in roots, we studied if CLV2 is also needed for the perception of the ectopically expressed *CLE19* in transgenic *Arabidopsis*. We crossed a P_{35S}:Bn *CLE19* overexpression line (as described in Fiers et al., 2004) with L*er*, *clv1-1*, *clv2-1*, and *clv3-2*. In the F1 generation, we identified seedlings with short roots and transferred them to the greenhouse to obtain F2 seeds. Plants with long roots were selected from each family of F2 seedlings. We reasoned that plants with long roots should not carry the P_{35S}:Bn *CLE19* transgene, unless the line carries a mutation that can suppress the function of the transgene. As shown in Table 1, all plants from the F2 populations of P_{35S}:Bn *CLE19* × L*er*, P_{35S}:Bn *CLE19* × *clv1-1*, and P_{35S}:Bn *CLE19* × *clv3-2* with a long-root phenotype did not carry the transgene. Plants with both long roots and the transgene were only identified in the F2 population of P_{35S}:Bn *CLE19* × *clv2-1* (Table 1). All these 11 plants also displayed the *clv2* mutant phenotype in siliques. A similar result was obtained with the P_{35S}:At *CLE19* transgenic line (data not shown), demonstrating that the *clv2* mutation also suppressed the short-root phenotype produced by the At *CLE19* transgene. Consistent with this conclusion, none of the short-root plants obtained from the F2 population of P_{35S}:Bn *CLE19* × *clv2-1* were homozygous for the *clv2* mutation (data not shown), further confirming that the homozygous *clv2* mutation was needed to suppress the short-root phenotype induced by *Bn CLE19* overexpression.

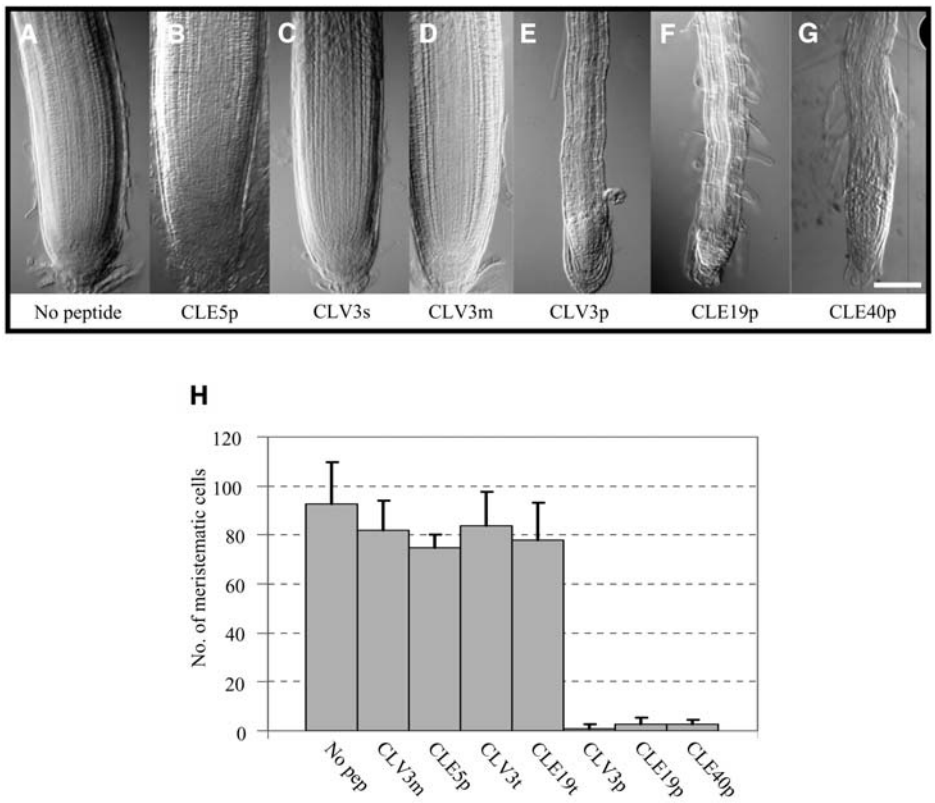

Figure 3. Effect of CLE Peptides on Root Meristems.

(A) to **(G)** The morphology of the primary roots of *Arabidopsis* (L*er*) 14 d after treatments with different peptides. Note the consumption of the root meristem (**[E]** to **[G]**) after treatments with CLE peptides. The bar in **(G)** = 50 μm for **(A)** to **(G)**.

(H) Number of meristematic cells in roots of L*er* seedlings after 14 d of treatment with different peptides. Data and error bars represent mean ± SD ($n = 10$).

Treatment with CLE Peptides Leads to a Misspecification of Cell Identities

To study the effect of CLE peptides on root development, three tissue-specific marker lines were selected, treated, and inspected by confocal microscopy. These lines were (1) *J0571*, which shows green fluorescent protein (*GFP*) expression in cortex and endodermis layers, including the ground tissue initials and their daughter cells, and occasionally in the QC; (2) P_{SCR}:*GFP*, which reflects the expression of the *SCARECROW* (*SCR*) transcription factor (Wysocka-Diller et al., 2000) and shows *GFP* expression in the endodermis layer, QC, and ground tissue initials; and (3) promoter cortex2-driven nuclear-localized Histone 2B:yellow fluorescent protein (P_{CO2}:*YFP-H2B*), which shows *YFP* expression in cortex cells, but not in their initials, nor in the QC (Heidstra et al., 2004). These reporter lines were grown on media containing either CLE peptides or controls (CLV3s, CLV3m, or without peptide).

In all these lines, *GFP* expression was localized in the expected cell layer in the absence of peptides (Figures 6A, 6E, and 6I) or with control peptides (Figures 6B, 6F, and 6H). In all samples examined, a maximum of one ground tissue daughter cell was observed above the single ground tissue initial cell (Figure 6A, labeled with an arrowhead). In a number of samples, neither visible ground tissue daughter cell nor ground tissue initial cell could be detected (Figure 6I). When seedlings of *J0571* were treated with CLE peptides for 4 d, a single-file, with up to four ground tissue daughter cells, was observed (Figures 6C and 6D, labeled with arrowheads). More than 50% of the plants treated with CLE peptides exhibited this phenotype. This is an early defect, since at this stage the reduced root growth was not yet evident. After an 8-d treatment with CLE peptides, these single-file ground tissue daughter cells were enlarged and elongated, but the number of cells in the file did not increase further (see Supplemental Figure 3 online), suggesting that the division of these cells was impaired.

What is the identity of this single file of cells located at the position of the ground tissue daughter cell? The *GFP* expression in the *J0571* marker line (Figures 6C and 6D, indicated by arrowheads) suggested that they still maintained their ground tissue identity, despite the fact that the division pattern was altered. Similarly, when P_{SCR}:*GFP* seedlings were treated with CLE peptides, *GFP* expression was observed in this single file, suggesting that it still maintains an endodermis and ground tissue identity (inset in Figure 6G, marked by arrowheads). Since the *SCR* promoter is not active in the cortex layer (Figure 6E), we could exclude the possibility that these cells obtained a full

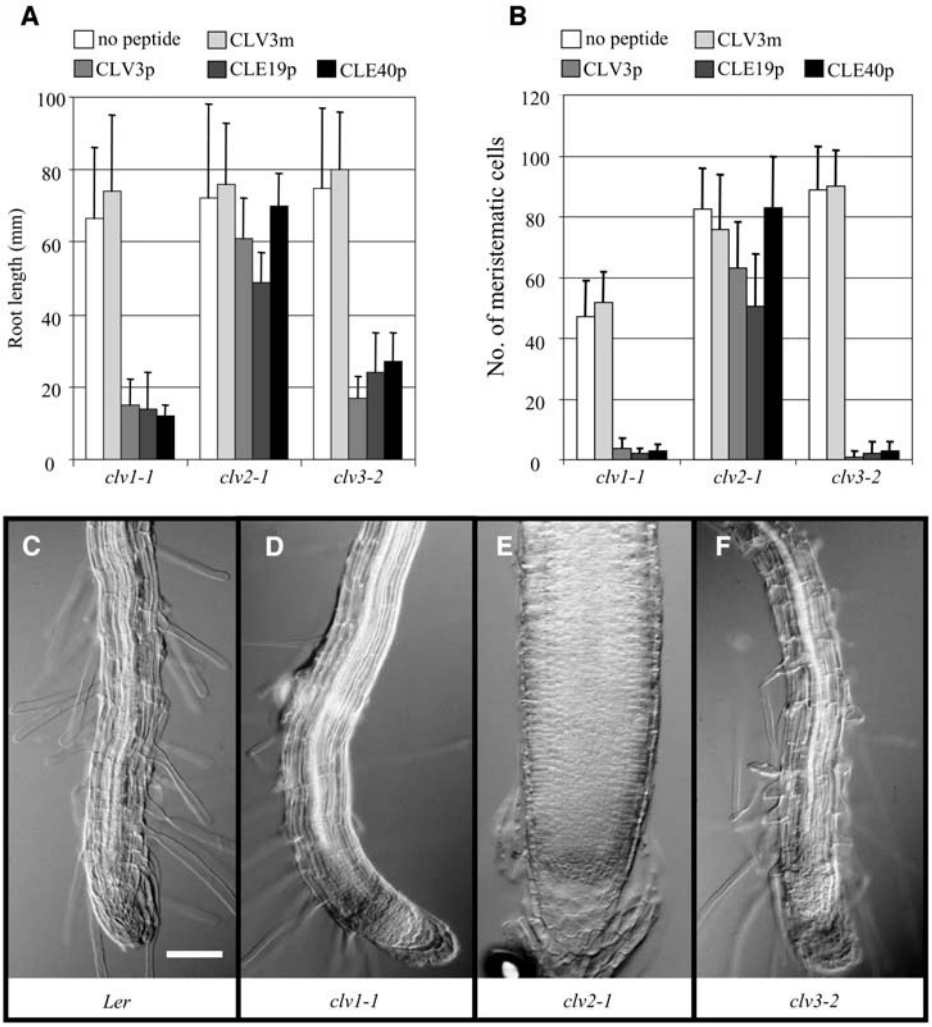

Figure 4. Effect of Peptides on the Root Growth in Different Mutants.

All roots were measured after 2 weeks growing on media containing different peptides.

(A) The length of the primary roots of *clv1-1*, *clv2-1*, and *clv3-2* grown on media with different peptides. Note that the CLE peptides did not give a significant inhibition in *clv2-1* ($n = 10$).

(B) Number of meristematic cells in roots of *clv1-1*, *clv2-1*, and *clv3-2* grown on media with different peptides ($n = 10$). Data and error bars in **(A)** and **(B)** represent mean ± SD.

(C) to **(F)** The morphology of roots of L*er* and *clv* mutants treated with CLE19p, showing that *clv2-1* is not sensitive to the peptide. Bar in **(C)** = 50 μm for **(C)** to **(F)**.

cortex identity. To further clarify the identity of these cells, the cortex-specific marker line P_{CO2}:*YFP-H2B* (Figure 6I) was examined upon peptide treatment. Interestingly, the single file of cells showed *YFP* expression (Figures 6J to 6M, indicated by arrowheads). We also noticed that the *YFP* expression was excluded from the ground tissue initial cells (Figures 6J and 6M, marked by arrows). As such, we concluded that the ground tissue daughter cells had obtained a cortex/endodermis double identity before the asymmetrical periclinal division occurs. This phenotype partially resembles the phenotype of the *scr* mutant in which P_{CO2}:*YFP-H2B* and *SCR* are expressed in the single-layered ground tissue (Heidstra et al., 2004).

Examination of the cell layers across the root meristem showed that, beside the changes in the ground tissue, all other cell layers were morphologically recognizable. However, in both the *J0571* and P_{SCR}:*GFP* marker lines treated with CLE peptides for 4 d, we observed the expression of *GFP* in the pericycle layer (Figures 6C, 6D, 6G, and 6H, indicated by asterisks). This ectopic *GFP* expression was observed in >70% of roots treated with CLE19p and ~40% treated with CLV3p and CLE40p. The pericycle initial cells (located next to QC) and the adjacent few cells in the same layer often showed strong GFP signal, while GFP levels decreased in the above cells (Figures 6C, 6D, 6G, and 6H). In a few cases, noncontinuous *GFP* expression was

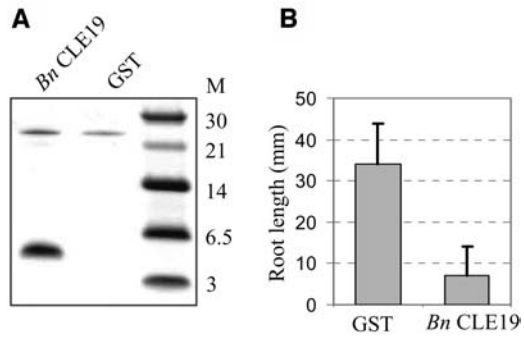

Figure 5. Heterologous Production of CLE19 Protein and Its Effect on Root Development.

(A) SDS-PAGE gel of thrombin-cleaved GST-Bn CLE19 and GST protein produced in *E. coli* with a mass of ~5 and 24 kD, respectively. The molecular mass (in kD) is shown at the right.
(B) Effect of purified the *Bn* CLE19 or GST proteins on root development. Wild-type (*Col-0*) seedlings were grown for 10 d on plates containing 10 μM *Bn* CLE19 or GST. Data and error bars represent mean ± SD ($n = 8$).

observed in this layer (Figure 6C). The number of pericycle cells with *GFP* expression at one focal plane ranged from 1 to 50. These cells were often distributed asymmetrically along the two sides of the root (Figures 6C, 6D, 6G, and 6H). Interestingly, in 8-d-old seedlings treated with CLE peptides, *GFP* expression in the pericycle cells was no longer detectable (see Supplemental Figure 3 online).

Similarly, upon the treatment with CLE peptides, we observed the expression of the cortex marker in the endodermis layer (Figures 6J to 6L, indicated by asterisks). Approximately 70% of roots treated with the CLE19p showed *YFP* expression in the endodermal layer, while the frequency of *YFP* expression was ~40% for CLV3p- and CLE40p-treated roots.

Penetration of CLE19p into Roots

To determine whether the CLE peptides enter the root or function on the root surface, a labeled CLE19p (R-CLE19p) was synthesized with a lissamine rhodamine fluorophore coupled to the N-terminal Lys. Wild-type (*Columbia-0* [*Col-0*]) seedlings were incubated with 10 μM R-CLE19p, free lissamine rhodamine, or propidium iodide (PI) and analyzed by confocal microscopy. PI is a vital dye that is widely used for staining of the cell wall. It can only enter the cells if the membrane integrity is disrupted (Robinson et al., 2002). R-CLE19p was able to enter the root. R-CLE19p fluorescence was observed in all cell layers of the roots within 1 min after peptide application. The fluorescence was located predominantly in the intercellular spaces (Figure 7A). After 4 min the signal was more intense, but still only located in the intercellular spaces (Figure 7D). Interestingly, the free lissamine rhodamine entered the roots with a lower efficiency, as indicated by the weak fluorescence (Figures 7B and 7E). PI entered the roots with the highest efficiency (Figures 7C and 7F), taking only 1 min to reach the saturation level (Figures 7C and 7D).

DISCUSSION

Previous studies suggested that CLV3 acts as a secreted protein that is required for the formation of the active CLV1/CLV2 signaling complex (Fletcher et al., 1999; Trotochaud et al., 1999; Rojo et al., 2002). However, the precise molecular identity of the functional CLV3 protein is still unknown. Several attempts have been made to identify the mature CLV3 protein using antibodies (Rojo et al., 2002), but so far only a CLV3-T7 fusion protein could be detected (Rojo et al., 2002). Recently, we and other labs have demonstrated that ectopic expression of *CLV3*, *CLE19*, *CLE40*, or *HgSYV46* leads to a consumption of the root meristem (Casamitjana-Martinez et al., 2003; Hobe et al., 2003; Fiers et al., 2004; Wang et al., 2005). Sequence alignment showed that the similarity between the putative proteins encoded by these genes was restricted to the CLE motif. Both *CLV3* and *CLE19* appeared to be not expressed in root meristems (Fletcher et al., 1999; Fiers et al., 2004), suggesting that the phenotype of ectopic expression represents a gain-of-function phenotype. We developed an in vitro root assay in *Arabidopsis* to examine how these proteins might generate the same phenotype in roots. The in vitro assay showed that three chemically synthesized 14–amino acid CLE peptides, corresponding to the conserved CLE motif of CLV3, CLE19, and CLE40, were able to mimic the overexpression phenotype when applied in vitro.

Three CLE peptides share only 50% (between CLV19p and CLE3p or CLE40p) to 64% (between CLV3p and CLE40p) sequence identity. The similar phenotype observed suggests that a common signaling pathway is involved and that the receptor(s) is able to recognize multiple ligands with certain

Table 1. Analysis of the F2 Plants with Long Roots from Crosses between P_{35S}:Bn *CLE19* and L*er* or Different *clv* Mutants (*clv1*, *clv2*, and *clv3*)

Cross Combinations	F2 Plants with Long Roots Examined	Plants with Multiple Carpels[a]		Plants with Wild-Type Carpels	
		Without P_{35S}:Bn *CLE19*	With P_{35S}:Bn *CLE19*	Without P_{35S}:Bn *CLE19*	With P_{35S}:Bn *CLE19*
P_{35S}:Bn *CLE19* × L*er*	12	0	0	12	0
P_{35S}:Bn *CLE19* × *clv1-1*	12	2	0	10	0
P_{35S}:Bn *CLE19* × *clv2-1*[b]	60	5	11	33	0
P_{35S}:Bn *CLE19* × *clv3-2*	13	3	0	10	0

[a] Multiple carpels in the pistil is the typical phenotype for all *clv* mutants.
[b] Two independent experiments were performed, and the data were combined in sum.

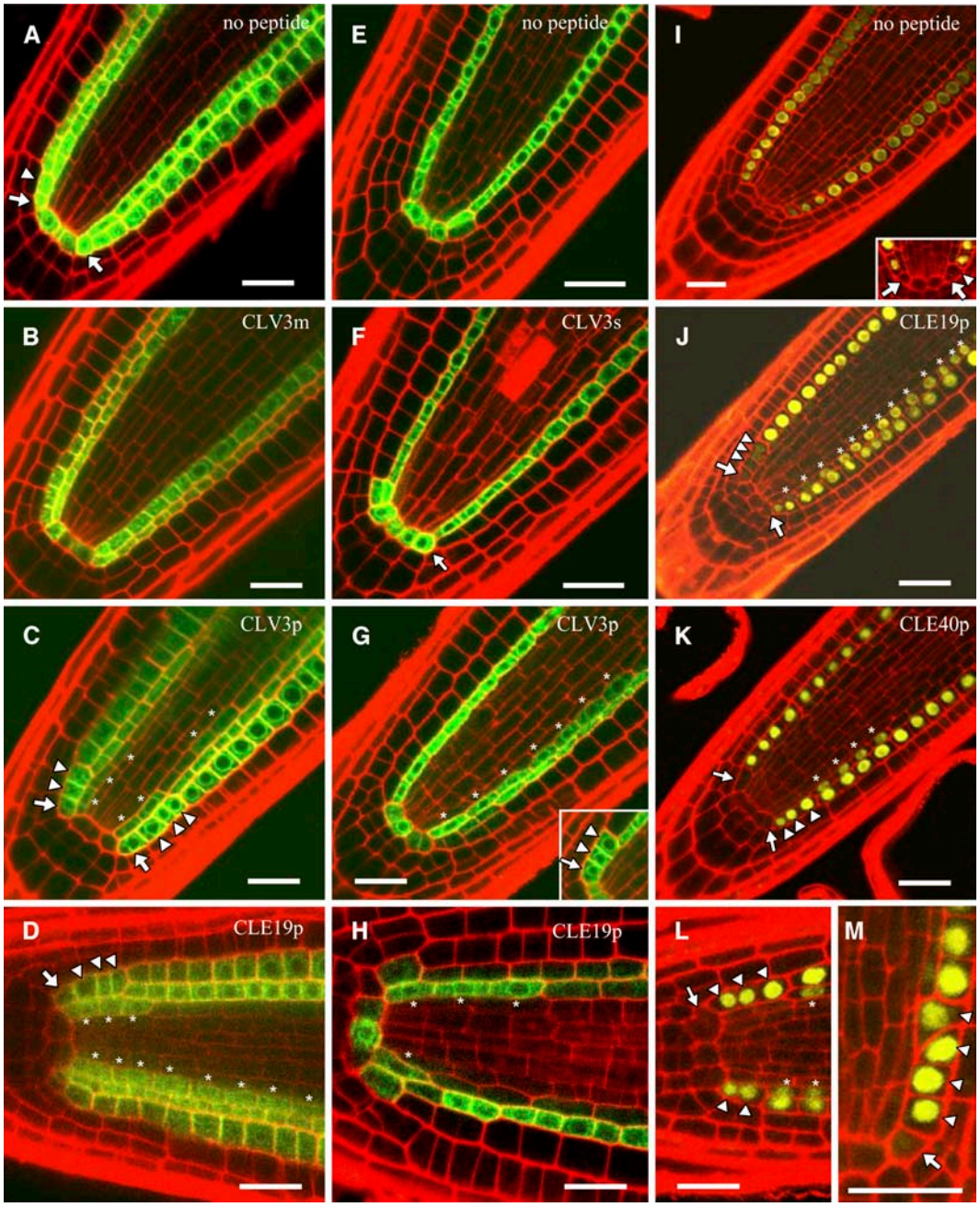

Figure 6. Effects of CLE Peptides on the Cell Identity of Roots.

The ground tissue initial cells are marked by arrows, and the ground tissue daughter cells, including cells at this position, are labeled with arrowheads. Abnormal expression of *GFP* or *YFP* in other cell layers is marked by asterisks.

(A) to **(D)** Confocal analysis after 4-d treatment of the roots of *J0571*. Note that treatments with CLV3p **(C)** or CLE19p **(D)** lead to a delayed separation of cortex and endodermis and expression of *GFP* in pericycle cells. No peptide **(A)**, 10 μM CLVm **(B)**, CLV3p **(C)**, and CLE19p **(D)**.

(E) to **(H)** Confocal analyses of P_{SCR}:*GFP* seedlings after 4-d treatments. No peptide **(E)**, 10 μM CLV3s **(F)**, 10 μM CLV3p **(G)**, and 10 μM CLE19p **(H)**. The inset in **(G)** shows the delayed separation of the cortex and endodermis cells (with *GFP* expression).

(I) to **(M)** P_{CO2}:*YFP-H2B* marker line treated with different peptides. Note the *YFP* expression in the ground tissue daughter cells and the pericycle cells, but not in ground tissue initial cells.

(I) No peptide, 4 d after germination. The inset shows the absence of *YFP* expression in the QC, ground tissue initial, and ground daughter cells.

(J) 10 μM CLE19p, 4 d after treatment.

(K) 10 μM CLE40p, 5 d after treatment.

(L) and **(M)** 10 μM CLE19p, 5 d after treatment.

Bars = 30 μm.

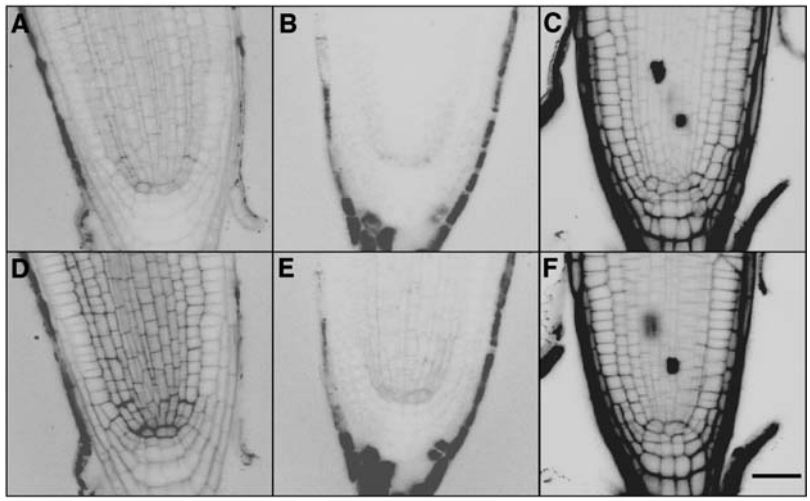

Figure 7. Penetration of Fluorescence-Labeled CLE19p in Roots.

The roots of *Arabidopsis* (*Col-0*) were incubated for 1 min (**[A]** to **[C]**) or 4 min (**[D]** to **[F]**) with 10 μM R-CLE19p (**[A]** and **[D]**), 10 μM lissamine rhodamine (**[B]** and **[E]**), or 10 μM PI (**[C]** and **[F]**).
Bar in **(F)** = 30 μm for **(A)** to **(F)**.

sequence variations. It is possible that in the endogenous situation the specificity of *CLV3*, *CLE19*, and *CLE40* is defined by the regulation of expression. The control peptides, including CLV3s, CLV3m, CLV3t, and CLE19t, did not affect root development. CLE5p, a peptide corresponding to a distant member of the *CLE* genes expressed in roots, was also unable to trigger the consumption of the root meristem. These observations suggest that the CLE peptides act in a sequence-specific manner.

Experiments with a labeled peptide showed that this peptide is able to efficiently enter the intercellular spaces of the root. The fluorescence signal was located predominately in the cell wall, suggesting that active transport is not likely to be involved. Although the labeled peptide penetrated into roots faster than the free fluorescence dye, PI, which cannot enter the intact plasma membrane (Robinson et al., 2002), enters into the roots even faster. The differences in penetration efficiency can be explained either by the differences in hydrophobicity or molecular masses.

Using a QC marker and the *DR5*:*GUS* line, we observed that application of the CLE peptides resulted in a consumption of the root meristem without directly interfering with the QC function or auxin distribution in roots. This conclusion is further supported by the observation that the ground tissue initial cells located next to the QC and tightly regulated by the QC (van den Berg et al., 1995, 1997) were not affected. These data are in agreement with previous observations that overexpression of *CLV3*, *CLE19*, or *CLE40* in *Arabidopsis* causes a termination of root meristems without acting primarily on the QC (Casamitjana-Martinez et al., 2003; Hobe et al., 2003; Fiers et al., 2004).

To determine if the sequence before the CLE motif could influence the activity, *Bn* CLE19 protein (with its N-terminal secretion signal removed) was produced in *E. coli* and tested in the root assay. The full-length CLE19 protein appeared to be functional in *Arabidopsis* in a similar fashion as CLE19p, suggesting that the extra sequence before the CLE motif does not affect its function. This is consistent with what is known for other peptide ligands in plants, such as phytosulphokine and flagellin (Felix et al., 1999; Yang et al., 1999). Although the pre-proteins for phytosulphokine in rice (*Oryza sativa*), *Arabidopsis*, and asparagus (*Asparagus officinalis*) are different in sequence, the functional peptides are believed to be the same (Yang et al., 1999). Since no endogenous CLE ligand has been identified yet, we cannot exclude the possibility that the endogenous peptides are longer or shorter than the CLE peptides tested.

For the identification of signaling components involved in perception of these CLE peptides, we examined whether components of the *Arabidopsis* CLV signaling pathways, specifically CLV1, CLV2, and CLV3, are involved in CLE peptide signaling. Interestingly, the *clv2* mutant was insensitive to CLE peptide treatment, suggesting that CLV2 is functionally involved in perception of the CLE peptides in roots. Genetic analysis demonstrated that CLV2 is also involved in the perception of the P_{35S}:Bn *CLE19* transgene, suggesting that the same machinery is involved in the perception of the transgenic *Bn CLE19* and CLE peptides. Among these *CLV* genes, *CLV2* is the only one expressed in *Arabidopsis* roots (Birnbaum et al., 2003). It seems that CLV2 not only participates in the CLV1/CLV2 receptor complex to transmit the CLV3 signal in the SAM (Jeong et al., 1999), but also functions in an unidentified CLV1-like receptor kinase complex in roots to perceive the CLE peptides. The fact that *clv2* does not show any root phenotype suggests that either CLV2 is redundant in roots while the redundant protein(s) cannot sense the CLE peptides, or CLV2 is not functional in roots and the CLE peptides act ectopically to generate a gain-of-function phenotype.

Three cell layer identity markers were examined to further study the effects of the peptide treatment. First, we observed that the treatments led to the accumulation of multiple cells at the

position of the ground tissue daughter cell. This is striking since in the wild-type situation an asymmetrical division always occurs immediately after the ground tissue daughter cell is produced, to form an outer cortex cell and an inner endodermis cell (van den Berg et al., 1995, 1997). This tightly regulated cell division pattern is controlled by SCR in a cell autonomous manner, and the function of SHORT ROOT (SHR) is required for the activation of SCR, which in turn induces the rotation of the division plane (Helariutta et al., 2000; Heidstra et al., 2004).

Detailed analyses showed that two ground tissue markers (*J0571* and P_{SCR}:*GFP*) were expressed in the row of ground tissue daughter cells, suggesting that these cells still maintain their ground tissue identity. In the absence of CLE peptide application, *YFP* expression in the cortex-specific marker line (P_{CO2}:*YFP-H2B*) was excluded from the ground tissue initials and the ground tissue daughter cells (see inset in Figure 6I; Heidstra et al., 2004), However, upon treatment with CLE peptides, *YFP* expression was observed in the single file of cells located at the position of the ground tissue daughter cell (Figures 6J to 6M), indicating that these cells obtained a cortex identity prior to the asymmetrical division. Since *SCR* was expressed in these cells, we conclude that they have obtained a cortex/endodermis double identity. This single cell layer of ground tissue with a double identity is similar to that in *scr* but different from the *shr* mutant, in which the single layer has only cortex identity (Di Laurenzio et al., 1996; Helariutta et al., 2000). Interestingly, both *scr* and *shr* mutants have a short root phenotype in which the root meristem gradually differentiates after germination, suggesting that interrupted cell layer formation could have a direct effect on the maintenance of meristematic activity in roots. Whether CLE peptides interfere with SCR signaling components remains to be studied. Additionally, it is also not known if the CLE peptides first inhibit the division of the ground tissue daughter cells, which then leads to the differentiation of these cells, or vice versa.

Another striking effect observed after treatment with CLE peptides is the confusion in cell layer identity. The ground tissue genes (P_{SCR}:*GFP* and J0571) were transiently activated in the pericycle layer, and a cortex gene was active in the endodermal layer. The common feature is that the inner cell layers take the identity of the outer cell layer. Such a confusion or misspecification of layer identity could be a consequence of the failure of cells to sense the presence of neighboring cells. It is well known that positional signals are used by plant cells to define their identity (van den Berg et al., 1995, 1997). As such, we believe that these CLE peptides and the CLV3, CLE19, and CLE40 transgenes act in a dominant-negative fashion to interact with or saturate an unknown cell identity–maintaining CLV2 receptor complex. This in turn blocks intercellular communication among different cells and cell layers in the root, which leads to consumption of the root meristem.

METHODS

Plant Growth Conditions and Plant Strains

The marker lines *J0571* (made by Jim Haseloff) and P_{SCR}:*GFP* (Wysocka-Diller et al., 2000) and the mutants of *clv1-1*, *clv2-1*, and *clv3-2* were provided by the Nottingham Arabidopsis Stock Centre. P_{CO2}:*YFP-H2B*, *DR5*:*GUS*, and *QC25* were used as previously described (Casamitjana-Martinez et al., 2003; Heidstra et al., 2004).

Root Assay

Seeds were gas-sterilized in a desiccator for 1 h with 100 mL of bleach (4% NaClO) mixed with 3 mL of HCl in a beaker. For peptide treatments, the sterilized seeds were plated at a distance of 0.5 cm on media containing different concentrations of peptides, half-strength Murashige and Skooge microelements and macroelements (Duchefa), 1% (w/v) sucrose, and 0.5 g/L MES, pH 5.8, with 1.5% (w/v) agar. Peptides and proteins were added to the sterilized media before the medium was solidified. Plates were first incubated at 4°C in the dark for 2 d and then transferred to a room with a temperature of 23°C, 16 h light per day, and cultured nearly vertically. The root length was measured from the base of the hypocotyl to the tip of the primary root. GUS analysis was as described by Fiers et al. (2004), and GFP analyses were performed after 4, 6, and 8 d of growth. Peptides were ordered from Mimopopes with a purity of >70% and dissolved in a filter-sterilized sodium phosphate buffer (50 mM, pH 6).

To examine the penetration of the peptide into roots, CLE19p with a lissamine rhodamine fluorophore coupled to the N-terminal Lys (R-CLE19p) was ordered (Mimopopes). Four-day-old seedlings of *Arabidopsis thaliana* (*Col-0*) were incubated in either 10 μM R-CLE19p, 10 μM lissamine rhodamine, or 10 μg/mL PI by incubating the roots for different time periods, after which they were examined using confocal microscopy.

Microscopy

Roots and dissected SAMs were cleared following the protocol of Sabatini et al. (1999) and analyzed using a Nikon microscope equipped with Nomarski optics. For confocal microscopy, roots were counterstained with 10 μg/mL PI (Sigma-Aldrich) and analyzed with a Leica SP2 inverted confocal microscope following the protocol of Heidstra et al. (2004).

Protein Purification

Two primers were designed (5′-TATGGATCCGCTTCATTTCGGAGTTTG-3′ and 5′-ATACTCGAGTTACCTGTTGTGAAGTGGA-3′) to amplify *Bn CLE19* (without the signal sequence) from the cDNA. The PCR fragment was digested with *Bam*HI and *Hin*dIII (Invitrogen) and cloned into the *pGEX4T-2* vector, and the fusion protein was purified as described by the manufacturer (Amersham Biosciences). The *Bn* CLE19 and the GST control were tested using SDS-PAGE and quantified with Coomassie Brilliant Blue plus protein assay reagent (Pierce).

Genetic Analysis

The F1 of the crosses between a P_{35S}:Bn *CLE19* plant and individual *clv* mutants (*clv1-1*, *clv2-1*, and *clv3-2*) or the wild type (L*er*) were examined using the root assay mentioned above for a normal or short root phenotype. F2 seeds were harvested from plants with short roots and assayed again for individuals with a long or short root phenotype. These two groups were transplanted separately to soil and checked for the presence of the transgene with a PCR for the *NPTII* gene (5′-TGGGCACAACAGACAATCGGCTGC-3′ and 5′-TGCGAATCGGGAGCGGCGATACCG-3′). Homozygous *clv* mutants were identified from each group by their carpel phenotype.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers AT2G27250 (*CLV3*), AT2G31082

(*CLE5*), At3g24225 (*CLE19*), AF343656 (*Bn CLE19*), and AT5G12990 (*CLE40*).

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