Plant CLE peptides from two distinct functional classes synergistically induce division of vascular cells

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The *Clavata3* (*CLV3*)/endosperm surrounding region (CLE) signaling peptides are encoded in large plant gene families. *CLV3* and the other A-type CLE peptides promote cell differentiation in root and shoot apical meristems, whereas the B-type peptides (CLE41–CLE44) do not. Instead, CLE41 inhibits the differentiation of *Zinnia elegans* tracheary elements. To test whether CLE genes might code for antagonistic or synergistic functions, peptides from both types were combined through overexpression within or application onto *Arabidopsis thaliana* seedlings. The CLE41 peptide (CLE41p) promoted proliferation of vascular cells, although delaying differentiation into phloem and xylem cell lineages. Application of CLE41p or overexpression of *CLE41* did not suppress the terminal differentiation of the root and shoot apices triggered by A-type CLE peptides. However, in combination, A-type peptides enhanced all of the phenotypes associated with *CLE41* gain-of-function, leading to massive proliferation of vascular cells. This proliferation relied on auxin signaling because it was enhanced by exogenous application of a synthetic auxin, decreased by an auxin polar transport inhibitor, and abolished by a mutation in the *Monopteros* auxin response factor. These findings highlight that vascular patterning is a process controlled in time and space by different CLE peptides in conjunction with hormonal signaling.

cambium | hypocotyl | RAM | SAM | TDIF

In higher plants, postembryonic organogenesis is mediated by meristems. These specialized structures provide a reservoir of undifferentiated stem cells as well as a limited population of proliferating cells, often referred to as transit-amplifying (TA) cells that are fated for differentiation (1). To date, molecular research has focused on the *Arabidopsis* primary meristems of root and shoot apices, but more recent studies have sought to dissect molecular programs underpinning the control of secondary meristems, including the vascular cambium (2, 3) that is a circumferential stem cell niche including the fusiform initials from which secondary xylem and phloem originate (2). During the course of differentiation, fusiform initial daughters take on a TA state to increase the population of xylem and phloem mother cells. Positioning and size of stem cell and TA cell populations, at least in root and shoot meristems, are controlled in a noncell-autonomous manner (1, 4, 5).

This noncell-autonomous control of stem cell size and positioning has best been described in *Arabidopsis* primary meristems where a stable pool of undifferentiated stem cells are maintained by a feedback loop mechanism involving the *Clavata* (*CLV*) signaling pathway and the *Wuschel* (*WUS*) homeodomain transcription factor (6, 7). Similar regulatory mechanisms may be at work within root meristems, where the transcription factor *WUS-related-homeobox 5* (*WOX5*) controls stem cell maintenance (8) and *CLV3* and related genes can influence root patterning (9–11), although to date the regulation of *WOX5* by a *CLV*-like pathway has not been demonstrated. Transcript profiling of the vascular cambium, either across the cambial zone of aspen (*Populus tremula*) (2, 12) or in secondary tissues of the *Arabidopsis* root-hypocotyl (3), showed that genes coding for several *CLV*-like leucine-rich repeat receptor-like kinases

(LRR-RLKs) and *CLV3*-related genes are differentially regulated across secondary meristem tissues, suggesting the involvement of a similar signaling system in vascular development as well.

Clavata3 (*CLV3*) represents the short-range mobile ligand of the *CLV1* receptor that together regulates differentiation (13) and belongs to a family of predicted plant proteins, named *CLV3*/endosperm surrounding region (CLE) (10, 14–16). All CLE proteins share common characteristics: They are small (<15 kDa), have a putative N-terminal secretion signal, and possess a conserved 14-aa CLE domain at or near their C terminus. Synthetic CLE domain-derived peptides can alter stem cell fate and cellular differentiation within primary shoot and root meristems (17). Furthermore, in situ mass spectrometry revealed that the bioactive domain derived from the *CLV3* preprotein is a modified dodecapeptide, overlapping with the CLE domain (18). Direct evidence for the involvement of CLE peptides in secondary meristems comes from the discovery of another dodecapeptide, corresponding to the CLE domain of CLE41, and identified in mesophyll cell cultures of *Zinnia elegans* as a tracheary element differentiation inhibitory factor (TDIF) (19).

We describe the use of both synthetic CLE-derived peptides and *CLE* overexpression lines to classify *CLE* genes into 2 separate classes based on their ability to promote terminal differentiation of the primary shoot and root meristems in *Arabidopsis*. The synergistic interaction of these 2 classes of peptides to suppress differentiation and promote auxin-mediated cell proliferation within the secondary meristem (vascular cambium) demonstrates that specific *CLE* genes have dual functionality and cell-type-specific responsiveness.

Results

Root Growth Assays Define Two Functional Classes of CLE-Derived Peptides. To investigate the respective role of *CLE* genes in root development, *Arabidopsis* seedlings were grown on medium supplemented with one of 22 synthetic peptides encoded by the corresponding CLE domain. Measured root-growth rates defined two classes: 18 peptides arrested growth and 4 (CLE41p, CLE42p, CLE43p, and CLE44p) did not. They are designated A-type and B-type peptides, respectively [supporting information (SI) Fig. S1A and Table S1]. These quantitative results confirmed independent studies (11, 19, 20) with the caveat that the synthetic peptides CLE1 to CLE7 do not arrest root apical

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Fig. 2. Radial enlargement of the hypocotyl stele after CLE6p and CLE41p treatments. (A) Hypocotyl stele width of the wild type (Col-0) transferred 3 day and grown for 15 days in liquid medium supplemented with the indicated peptide(s) ($n = 15$). (B) Representative hypocotyls. Samples are aligned according to labels of abscissa in A. Arrows indicate the measured width. (Scale bar, 100 μm.) (C) Hypocotyl stele width of wild-type (Ler), *clv1-1*, and *clv2-1* seedlings transferred 3 day and grown for 10 days in liquid medium with or without combined CLE6p/CLE41p ($n = 10$). The asterisk indicates samples significantly different from control without peptide. (Student t test $P < 0.05$.)

CLE19p/CLE41p, CLE6p/CLE42p, CLV3p/CLE42p, and CLE19p/CLE42p (Fig. 1 C and D; data not shown). CLE peptides appeared to function locally, because transcriptional marker changes and vascular cell proliferation were restricted to the root (the sole organ in direct contact with the added peptides) in plantlets grown on solid medium only, but occurred in both root and shoot tissues in plantlets treated in liquid medium. The quantification of stele radial enlargement observed in wild-type hypocotyls grown in liquid medium with different combinations of CLE6p and CLE41p confirmed their synergistic activity on vascular development (Fig. 2 A and B).

Components acting downstream in the pathway(s) relaying the CLE6p/CLE41p signal are not known. However, we determined that *CLV1* and *CLV2*, coding for receptor-like proteins located in the plasma membrane and required for the perception of the CLV3 peptide in the SAM (27), are not necessary for CLE-induced hypocotyl enlargement, because *clv1-1* and *clv2-1* mutants had a response similar to that of the wild type after CLE6p/CLE41p binary treatment (Fig. 2C).

CLE Peptides Induce Vascular Proliferation and Delay Differentiation.

Because the increase in GUS activity induced by A/B peptide combinations in *CYCB1_{pro}:GUS* plantlets was homogeneous along the vasculature of the hypocotyl but not in the root (Fig. 1 B and C), we chose to use methodically the hypocotyl stele enlargement phenotype to examine the events leading to ectopic cell divisions.

Sections of *CYCB1_{pro}:GUS* hypocotyls grown without added peptides or with CLE6p alone were indistinguishable 7 or 10 days

Fig. 3. Cellular details of vascular tissues in hypocotyls treated with CLE peptides. Hypocotyls were sectioned directly above the collet and stained with ruthenium red. Seedlings were transferred 3 day and grown for 15 days in liquid medium supplemented with (A) no peptide, (B) CLE6p, (C) CLE6p and CLE41p, and (D) CLE41p ($n = 10$). (A, B, and D) Black arrows indicate phloem poles. Initial concentration of each added peptide was 10 μM. (Scale bars, 50 μm.)

after transfer (dat) in liquid medium (Fig. 1D and Fig. 3 A and B). Hypocotyls treated with CLE6p/CLE41p had unusually high GUS activity in most cells of the stele, except those at the phloem poles and in mature xylem (Fig. 1D). In these tissues, GUS staining coincided with a remarkably higher number of small cells and a disrupted arrangement of mature xylem bundles (Figs. 1D and 3C). CLE41p alone also caused the dispersion of mature xylem elements among the smaller and, presumably, actively dividing cells, but to a lesser extent than the CLE6p/CLE41p combination (Fig. 3D).

To better specify which cells proliferated when exposed to CLE peptides, we treated *APL_{pro}:GUS* and *ATHB8_{pro}:GUS* plants. These lines report the transcriptional activity of *Altered Phloem Development* (APL) marking protophloem, companion cells and metaphloem sieve elements (28), and *HOMEBOX GENE 8* marking procambium and protoxylem cells (29, 30; Y. Helariutta, personal communication), respectively. In *APL_{pro}:GUS* hypocotyls exposed to CLE41p, phloem strands lacked polar organization with respect to the stele compared to CLE6p or control samples. GUS activity was detected in hypocotyls treated with the combined CLE6p/CLE41p, but only in isolated irregular strands (Fig. 4A and Inset). On the contrary, GUS expression in *ATHB8_{pro}:GUS* hypocotyls significantly increased in the presence of CLE41p alone, and further increased and expanded to a larger domain in the presence of the combined CLE6p/CLE41p, compared with CLE6p or control samples (Fig. 4B).

To investigate on which pathways CLE41p (in combination with CLE6p) impinges to promote proliferation in the stele of the hypocotyl, we analyzed the occurrence of key transcriptional changes, relative to vascular development and cell division, after 3 h to 10 days of peptide exposure. Compared with untreated controls, the first changes in stele width were observed 2–4 days after the start of peptide treatment, regardless of the reporter lines (Fig. S4).

Fig. 4. Defects in vascular development after CLE peptide treatment. GUS expression in (A) *APL_{pro}::GUS* and (B) *ATHB8_{pro}::GUS* hypocotyls. Seedlings were transferred 3 day and grown for 15 days in liquid medium supplemented with the peptide(s) indicated, each at an initial concentration of 10 μM ($n = 10$). (Scale bars, 50 μm.) (Inset) Longitudinal section through a hypocotyl stele showing xylem (X) and phloem strands (PS). (Scale bar, 10 μm.)

The first transcriptional changes induced by CLE6p/CLE41p occurred much earlier (from 3 h onward) in the *ATHB8_{pro}::GUS* and *ET1335::GUS* lines marking the onset of procambium formation (31). CLE41p treatment alone induced transcriptional changes within 1 day in *ATHB8_{pro}::GUS* plants, whereas the *ATHB8* and *ET1335* markers were only visible at 4 and 7 days, respectively, in plants grown in the absence of added peptide (Fig. S4 A and B). A relative increase in cell proliferation reported by GUS activity within the stele of *CYCB1_{pro}::GUS* hypocotyls was detectable 7 days after CLE6p/CLE41p treatment, compared with 10 days in the untreated control (Fig. S4C). On the contrary, GUS activity reporting differentiation of phloem cells in *APL_{pro}::GUS* plants was delayed from days 7–10 by CLE6p/CLE41p (Fig. S4D).

In summary, CLE41p first increased *ATHB8* and *ET1335*, procambium and protoxylem cell-identity markers, and this induction was enhanced and advanced by the presence of CLE6p. The characterization of hypocotyls treated for ≥ 7 days suggested that CLE6p/CLE41p maintained an abnormally high number of cells in a proliferative mode (Fig. 3C and Fig. 4B), delaying downstream vascular differentiation. This observation is in agreement with the promotion of *Zinnia* mesophyll cell division and suppression of their transdifferentiation into tracheary elements by CLE41 (19).

The synergistic action of A- and B-type peptides was confirmed genetically in F1 plants resulting from crosses between *CLE6^{OE}*, *CLE41^{OE}*, and wild-type lines. When compared with wild-type plants, hemizygous *CLE41^{OE}* and *CLE6^{OE}* plants had a retarded rosette growth and a typical A-type CLE gain-of-function SAM arrest phenotype, respectively. The F1 plants carrying both the *CLE6^{OE}* and *CLE41^{OE}* transgenes displayed a strongly stunted and bushy phenotype (Fig. S5A). This dramatic synergistic effect extended to vascular development: When compared to the wild-type, *CLE6^{OE}/-*, or *CLE41^{OE}/-* plants, hypocotyl sections from *CLE6^{OE}/-;CLE41^{OE}/-* F1 plants had a mass of cytoplasm-dense small cells and a disrupted arrangement of secondary xylem with dispersed clusters of mature xylem vessels, as also observed in vascular bundles of hypocotyls treated with CLE6p/CLE41p (Fig. S5B).

However, the SAM arrest coupled with the activation of axillary meristems observed in *CLE6^{OE}/-;CLE41^{OE}/-* plants suggested that CLE6-induced SAM arrest was not suppressed by CLE41 overexpression. This result is in agreement with the inability of synthetic CLE41p to suppress CLE6p-induced SAM or RAM arrest, and confirms that CLE6 and CLE41 are not

Fig. 5. Interaction between CLE peptide and hormone signaling. (A) Hypocotyl stele width of whole or decapitated wild-type seedlings transferred at 3 and 5 day, respectively, and for 10 days into liquid medium supplemented with the indicated peptide(s) ($n = 15$). (B) Treatments combining CLE peptides, auxin, and auxin transport inhibitor. Ten seedlings were transferred 3 day and grown for 10 days either with NAA (1 mM) or NPA (1 μM), in combination with no peptide, CLE6p, CLE41p, or CLE6p/CLE41p. Initial concentration of each added peptide was 10 μM in both experiments. The asterisk marks samples significantly different from mock-treated controls. (Student t test $P < 0.05$.)

antagonistic with regard to apical meristem function (for additional details, see SI Text).

Auxin Mediates A/B CLE-Induced Proliferation. Polar auxin transport is a key process in vascular development (32). Therefore, we investigated the potential interplay between auxin and CLE actions. A shoot-derived signal was found to be required for CLE-induced cell proliferation, because stele enlargement was not enhanced in wild-type seedlings decapitated 5 days after germination (dag) and treated with CLE6p/CLE41p for 10 days (Fig. 5A). We compared peptide-treated seedlings grown in liquid culture supplemented with either the synthetic auxin 1-naphthaleneacetic acid (NAA, 1 μM) or the polar auxin transport inhibitor 1-naphthylphthalamic acid (NPA, 1 μM). CLE6p/CLE41p-driven enlargement was markedly enhanced by NAA, but reduced by NPA (Fig. 5B), consistent with the hypothesis that auxin is involved in CLE-induced vascular proliferation.

Four additional reporter lines were tested to study auxin-related transcriptional changes during CLE-induced radial enlargement of the hypocotyl stele: *DR5_{pro}::GUS*, *IAA2_{pro}::GUS* (33), *PIN1_{pro}::GUS* (34), and *PIN3_{pro}::GUS* (35). In the absence of peptide, GUS staining was detected only in the stele of *PIN3_{pro}::GUS* hypocotyls at 7 and 10 days after treatment. Upon CLE6p/CLE41p treatment, GUS staining was observed earlier (day 4) in *PIN3_{pro}::GUS* hypocotyls, and was detectable at day 7 in the other 3 lines. The transcriptional activation of the *DR5*, *IAA2*, *PIN1*, and *PIN3* genes in the enlarged steles (Fig. S4 E–H)

hinted at an auxin response concomitant with increased cell proliferation.

Auxin stimuli are relayed transcriptionally by auxin response factors (ARFs). In particular, *MONOPTEROS(MP)/ARF5* mediates auxin responses involved in vascular development (36). Therefore, we tested its involvement in CLE6p/CLE41p-induced cell proliferation. In wild-type leaves, numerous small cells parallel to mature xylem appeared after 10 days of binary peptide treatment, but under the same conditions these cells are absent in leaves homozygous for *mpG92*, a weak *monopteros* mutant allele, even though such leaves form rudimentary vascular-cell files (Fig. S6).

To conclude, we propose that our observations can be explained by the following sequence of events: A and B-type peptides act synergistically to prime specific vascular cell types for division; auxin level or auxin sensitivity increase in these cells, resulting in proliferation and delaying vascular differentiation into phloem and xylem. This model is in agreement with the observation that, in trees, auxin concentration peaks within the actively dividing zone of the cambial meristem and that the radial width of the auxin concentration gradient correlates with cambial growth rate (37). Further information, including Figs. S7–S11, is available in the *SI Text*.

Discussion

Misexpression of A-type CLE genes or in vitro treatment with A-type synthetic CLE peptides (CLV3p, CLE6p, CLE19p, and CLE40p) stimulate terminal differentiation of RAM and SAM, whereas B-type genes and peptides (CLE41p) suppress differentiation into tracheary elements. Although a simple interpretation would be that the 2 peptide classes encode opposite functions, their interaction seems more complex because we demonstrated that combined A/B-type CLE peptides are not directly antagonistic, but instead act synergistically in a cell-specific context. All our phenotypic observations converge to show that A-type peptides potentiate the action of B-type peptides.

Most prominently, combined A/B CLE treatments resulted in the ectopic division of vascular cells observed in root, leaf, and hypocotyl bundles. Massive proliferation was marked by *ATHB8* expression suggesting that the dividing cells have procambium or protoxylem characteristics. Cells belonging to the phloem lineage, marked by *APL* expression, were still present, but their relative contribution to stele radial enlargement was minor, considering that the increase in *ATHB8* promoter activity was not paralleled by that of *APL*. Combined A/B CLE exposure or overexpression perturbed the balance between proliferation and differentiation, but also the orientation of the cell division plane, resulting in disjointed phloem strands and disorganized vascular patterning. This may be explained by the anisotropic distribution of CLE peptides that act directionally, from secretion to perception sites, in normal tissues.

To explain the role of CLE peptides in vascular development, we propose that signaling mechanisms at play in cambial meristems, across different zones and cell layers, might be similar to those controlling stem cell homeostasis in SAM and RAM, where TA cells derive from the rarely dividing pluripotent stem cells and increase the population of actively dividing meristematic cells. TA cells have a limited proliferative capacity and, unlike stem cells, are restricted in their differentiation potential (5). Considering that the primary effect of CLE41 is to suppress phloem and xylem differentiation although maintaining vascular cells in a proliferative mode, we propose that CLE41, and possibly other B-type peptides, are key signals determining the TA cell fate in the cambial meristem.

That vascular development might involve complex CLE signaling is supported by the identification of CLE (including CLE6 and CLE41) and LRR-RLK genes differentially transcribed

across vascular cell types at distinct stages of differentiation (2, 3). The vascular proliferation induced by A/B CLE may be mediated by separate signaling pathways, possibly controlled by different receptors. However, we showed that neither CLV1 nor CLV2 is required for this vascular proliferation response (Fig. 2D), even though *clv1* and *clv2* mutants suppress CLV3 gain-of-function (6). A mutation in the *CORYNE* gene coding for a membrane-associate kinase also suppresses CLV3 overexpression phenotypes. Genetic evidence indicates that *CORYNE* cooperates with CLV2 to transmit the CLV3 signal independently from CLV1, and is therefore unlikely to relay the A/B CLE response (38).

Other LRR-RLK candidates are to be considered as potential receptors on the basis of sequence similarity, expression domain, and associated vasculature phenotypes. The somatic embryogenesis receptor kinase 1 (SERK1) increases the competence of cultured cells for somatic embryogenesis (39). Interestingly, *SERK1* expression is detected in procambium cells—in the vasculature of root, hypocotyl, and inflorescence stem—and correlates with the onset of ectopic cell divisions occurring in vascular bundles on prolonged exposure to the synthetic auxin 2,4-dichloro-phenoxyacetic acid, prompting the hypothesis that *SERK1* is a marker of TA cells (40). Barely any meristem 1 (*BAM1*) and *BAM3* code for the LRR-RLKs most closely related to CLV1, and are expressed in a wide range of tissues, including cambium. *Bam* loss-of-function reduces the stem cell niche, contrary to the phenotype of *clv* mutants, which is compatible with the hypothesis that BAMs may relay the A/B CLE peptide signal, but *bam* loss-of-function does not lead to root phenotypes, possibly because of redundancy with other RLK homologs (3, 41). Mutations in the *PXY* gene, encoding another membrane-bound RLK, result in disorganized vasculature with interspersed phloem and xylem strands, procambium cells persisting and dividing throughout vascular development, and a reduction in metaxylem cell number per vascular bundle. Such phenotypes are reminiscent of the phenotypes reported here, but are caused by *pxy* loss-of-function and thus unlikely to result from activation of PXY signaling (42). Alternatively, in specific instances, CLE ligands might target receptors via antagonistic interactions competing with or replacing the inductive signal. Testing such a hypothesis will require challenging biochemical and genetic studies considering the complexity of both the LRR-RLK and CLE families.

Given that both A-type and B-type CLE peptides affect cellular differentiation in a noncell-autonomous and dose-dependent manner, and that positional signals relayed by receptor-like kinases are primarily determined by their expression domains, we propose that ligand gradients and receptor specificity across the cambial meristem determine the rate at which vascular-fated cells are released from a TA cell state on their path to terminal differentiation. Our findings suggest that certain cells undergoing division in the developing vasculature might be located in domains where the A- and B-type peptide signals overlap. They highlight that vascular patterning is a process that is controlled in time and space by hormonal signaling and can be perturbed by diverse CLE peptides that impinge on these hormonal signaling pathways.

Materials and Methods

Peptide Assays. Sterilized seeds were germinated after stratification in vertical plates on media containing 0.5 × Murashige and Skoog microelements and macroelements (Duchefa Biochemie B.V.), 1% (wt/vol) sucrose, pH 5.8, with 1.5% (wt/vol) agarose. Seedlings were transferred at 3 dag to the same medium, either solid or liquid, but supplemented with peptide(s). For solid medium assays, root length was measured each day. For liquid medium assays, seedlings (10 in 5 mL per tube) were incubated in 50-mL Falcon tubes on an orbital shaker. All seedlings were grown at 22 °C under continuous light (100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). All synthetic peptides (>70% purity; Pepscan Systems) were dissolved in sterile sodium phosphate buffer (50 mM, pH 6).

Histochemical and Histological Analyses. GUS staining was assayed as described (43). Whole-mount microscopic samples were cleared and mounted in 90% lactic acid (Acros Organics) (44) and analyzed by DIC microscopy (Leica DM LB; Leica Microsystems). For fluorescence microscopy, whole seedlings stained with 3.5 μ M FM 4–64 (Invitrogen) were illuminated at 543 nm and imaged at 600 nm. All fluorescence images were collected on a confocal microscope 100M with software package LSM 510 version 3.2 (Zeiss).

For leaf, root, and hypocotyl anatomical sections, untreated and GUS-stained samples were fixed overnight at 4 °C with 1% glutaraldehyde and 4% paraformaldehyde in 50 mM phosphate buffer, pH 7. Samples were ethanol-dehydrated and embedded in Technovit 7100 resin (Heraeus) according to the manufacturer's protocol. Thin sections of 5 μ m were cut with a rotation microtome 2040 (Leica Microsystems), dried on Vectabond-coated object glass slides (Vector Laboratories). GUS-stained samples were cell wall counterstained with 0.05% wt/vol ruthenium red for 8 min (Sigma-Aldrich), whereas untreated samples were stained with 0.05% wt/vol toluidine blue O (Sigma-Aldrich), in 0.1 M phosphate buffer, pH 7.0 for 20 s; all samples were rinsed in tap water for 30 s to 1 min. After drying, the sections were mounted in DePex medium (VWR) and covered with cover slips. Thin sections on slides were analyzed with a light microscope (Leica DM LB) equipped with a 40 $\times$ objective, and imaged with an AxioCam camera and Axiovision software version 3.1

(Zeiss). Transverse hypocotyl sections were taken immediately above the collet.

Image Analysis. Plants were photographed with a CAMEDIA C-3040 zoom digital camera (Olympus) and plates were scanned with a Hp scanjet 5500c (Hewlett-Packard). Photographs and scans were measured with ImageJ (<http://rsb.info.nih.gov/ij/>).

Note Added in Proof. While this manuscript was under review, Hirakawa *et al.* (45) reported that CLE41/TDIF is secreted from the phloem and suppresses the differentiation of vascular stem cells into xylem cells through the LRR-RLK PXY/TDR.

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