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 trachyphyllelement. 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 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.

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 Arabidopsis and related genes can influence 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. SL4 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 proliferation.

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. SL4 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 proliferation.

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meristem (RAM) growth at 1 μM (19, 20), but do at 10 μM (compare Fig. S1 A and B).

To confirm genetically the results obtained with exogenous application of synthetic CLE peptides, we characterized available transgenic plants that overexpress representative A- and B-type genes, CLE6OE and CLE41OE, respectively, under the control of the CaMV 35S promoter. In brief, CLE6OE T1 plants exhibited a short-root phenotype and microscopic analysis revealed that the activity of the RAM ceased gradually over time, similarly to its arrest observed after A-type peptide treatment. The shoot apical meristem (SAM) of CLE6OE seedlings also ceased activity gradually. In contrast, CLE41OE T1 plants had normal roots, but grew as compact dwarf plants and produced numerous small leaves, although the size and structure of their primary SAM seemed unaffected. Our results agree with separate studies of closely related CLE genes ([10, 21–23]; for additional details, see SI Text).

**Structure of Primary Meristems Treated with CLE Peptides.** To better understand how the CLE peptides triggered root-growth arrest, representatives of either classes were applied to Arabidopsis transcriptional marker lines reporting mitotic divisions [CYCB1.1pro:GUS; (24)] and auxin response [DR5pro:GUS; (25)]. Compared with control plants (without added peptide), the primary root basal meristem of CLE41p-treated seedlings had identical structure and GUS pattern in both lines (Fig. S2 A, B, G, H, L, and M). In contrast, CYCB1.1pro:GUS seedlings treated with CLE6p, CLV3p, or CLE19p (all A-type peptides) had very low GUS levels in the shorter meristem, indicating that the gradual cessation of root growth correlated with the inhibition of cell division within the RAM (Fig. S2 C–E). CLE6p-treated roots also had a disorganized cellular structure with unusually large cells and differentiated vascular strands close to the meristem center (SI Text; Fig. S2N), in agreement with the previously observed terminal differentiation of the RAM induced by CLV3p, CLE19p, and CLE40p (11). None of the tested CLE-peptide treatments altered the position of the GUS maximum in DR5pro:GUS root tips, although GUS activity was lowered after A-type peptide treatments, suggesting that RAM arrest may be associated with decreased auxin response (Fig. S2 I–K).

To determine whether the initial distinction between A- and B-type CLE peptide activity is also reflected in other organs, we studied how CLE peptides affected the SAM. SAM size or structure of plants grown in liquid culture and treated with or without CLE41p did not differ significantly, but all seedlings treated with CLE6p had a reduced SAM size, resulting in a smaller and flatter region separating emerging leaf primordia (Fig. S3).

To summarize, the RAM and SAM of plants treated with the A-type CLE6p were consumed, whereas those treated with the B-type peptide CLE41p were indistinguishable from the wild type.

**A-Type and B-Type CLE Peptides Act Synergistically on Vasculature Development.** Phenotypes resulting from single CLE gene overexpression, heterologous complementation of clv3 mutations by other CLE genes, or single CLE peptide treatment suggested that subfamily members have redundant functions (Fig. S1) (25, 26). In addition, we postulated that CLE peptides might have antagonistic or synergistic modes of action. To test this hypothesis, we assayed root growth after binary treatment with A/B CLE peptides. CYCB1.1pro:GUS seedlings transferred to solid media containing both CLE6p (at a constant concentration of 10 μM) and CLE41p (at concentrations ranging from 1–100 μM) had the same root-growth arrest as seedlings treated with CLE6p alone (Fig. L4). In the presence of CLE6p, whether or not combined with CLE41p, GUS activity gradually decreased in the primary root meristem, until it disappeared in arrested plants (Fig. S2 C and F). Therefore, CLE41p did not influence CLE6p-induced RAM arrest. In contrast, the binary peptide treatment resulted in a striking CYCB1.1pro:GUS activity within the stele of the mature portion of the primary root: GUS level increased with CLE41p concentration and was the highest close to the base of extended lateral roots or the cotlet (Fig. LB). Furthermore, cell proliferation induced by the CLE6p/CLE41p combination resulted in radial enlargement of the mature portion of the primary root (Fig. 1 B and D).

Stele radial enlargement induced by binary peptide treatment was not restricted to the root. Indeed, ectopic cell proliferation was also observed in the vascular cells of shoot organs (cotyledons, leaves, and hypocotyls) when CYCB1.1pro:GUS seedlings were grown in liquid culture supplemented with all binary A/B combinations tested: CLE6p/CLE41p, CLV3p/CLE41p,
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;1pro::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;1pro::GUS hypocotyls grown without added peptides or with CLE6p alone were indistinguishable 7 or 10 days 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 APLpro::GUS and ATHB8pro::GUS plants. These lines report the transcriptional activity of Altered Phloem Development (APL) marking protophloem, companion cells and metaphloem sieve elements (28), and HOLOBOX GENE 8 marking procambium and protoxylem cells (29, 30; Y. Helariutta, personal communication), respectively. In APLpro::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. 4 A and Inset). On the contrary, GUS expression in ATHB8pro::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).
The first transcriptional changes induced by CLE6p/CLE41p occurred much earlier (from 3 h onward) in the ATHB8pro:GUS and ET1335:GUS lines marking the onset of procambium formation (31). CLE41p treatment alone induced transcriptional changes within 1 day in ATHB8pro: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 stelar of CYCB1pro: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 APLpro: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 into a proliferative mode (Fig. 3C and 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 CLE6OE, CLE41OE, and wild-type lines. When compared with wild-type plants, hemizygous CLE41OE and CLE6OE 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 CLE6OE and CLE41OE transgenes displayed a strongly stunted and bushy phenotype (Fig. S5A). This dramatic synergistic effect extended to vascular development: When compared to the wild-type, CLE6OE or CLE41OE plants, hypocotyl sections from CLE6OE;CLE41OE 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 CLE6OE;CLE41OE 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 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: DR5pro:GUS, IAA2pro:GUS (33), PIN1pro:GUS (34), and PIN3pro:GUS (35). In the absence of peptide, GUS staining was detected only in the stele of PIN3pro:GUS hypocotyls at 7 and 10 days after treatment. Upon CLE6p/CLE41p treatment, GUS staining was observed earlier (day 4) in PIN3pro:GUS hypocotyls, and was detectable at day 7 in the other three 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 ATTH8 expression suggesting that the dividing cells have procambium or protaxylem 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 ATTH8 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 disorganized 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 mesistematic 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 leptomere 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 μmol·m−2·s−1). All synthetic peptides (>70% purity; Pepscan Systems) were dissolved in sterile sodium phosphate buffer (50 mM, pH 6).
Histochemical and Histochemical 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 μM 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 μm were cut with a rotation microtome 2040 (Leica Microsystems), dried on Vectabond-coated object glass slides (Vector Laboratories). GUS-stained samples were treated 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 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 × objective, and imaged with an Axiocam camera and Axiosvision 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 scanner 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/TDF is secreted from the phloem and suppresses the differentiation of vascular stem cells into xylem cells through the LRR-RLK PXVYDTR.

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Results and Discussion

**CLE6 Overexpression Phenotypes.** Twenty of 27 CLE6OE T1 seedlings exhibited a short-root phenotype. Microscopic examination of these plants revealed that RAM activity ceased gradually over time, similarly to the RAM arrest observed upon A-type peptide treatment. Root hairs of short arrested roots were abnormally close to the root tip. CLE6OE roots stopped growing later (~16 dag) than wild-type roots treated with A-type peptide (10 μM; ~3.5 days after transfer on peptide medium). Similar results were reported in separate studies of transgenic plants overexpressing A-type CLV3 (3), CLE19 (4), and CLE40 (5, 6). Our observations confirmed that CLE6 indeed alters root meristem homeostasis.

Further analysis of CLE6OE seedlings showed that the SAM also ceased activity gradually, so that only a few leaves developed from the primary apex. However, SAM arrest released primary and secondary axillary bud development (Fig. S1B). Meristem arrest most probably resulted from a gradual reduction in SAM size (Fig. S7E), as was also observed upon exogenous A-type peptide treatment (Fig. S5C). CLE6OE T1 lines had various levels of meristematic activity inhibition and altered phyllotaxis with reduced rosette area when compared with wild-type (Fig. S8). Additional mutant phenotypes included thick and deformed leaves appearing before meristem consumption (observed in all 27 T1 transformants) (Fig. S7B); enlarged cells in the leaf vasculature (Fig. S7K); fasciated inflorescences carrying aerial rosettes on axillary stems (15:27) (Fig. S9B); occasional ectopic leaf organogenesis from the petiole (2:27) (Fig. S9C); cup-shaped terminal leaf (4:27) (Fig. S9D); and inflorescence meristems prematurely terminated, resulting in small filamentous structures harboring trichomes (7:27) (Fig. S9E). These developmental abnormalities were similar to those observed for other A-type CLE overexpression lines (3–7).

**CLE41 Overexpression Phenotypes.** CLE41OE T1 transformants exhibited phenotypes markedly distinct from CLE6OE plants. CLE41OE plants sustained normal root growth (n = 18), but developed as compact dwarf plants producing numerous small leaves. The severity of this mutant phenotype was highly variable, although displayed by all overexpressors (18:18; Fig. S7C). Furthermore, T1 CLE41OE plants did not exhibit any SAM arrest and microscopic examination showed that their SAM size and structure were indistinguishable from those of wild-type controls (Fig. S7F). These developmental abnormalities were similar to those observed for other B-type CLE overexpression lines (6).

Vascular enlargement was observed in sectioned hypocotyls and leaf veins of CLE41OE plants (Fig. S7 I and L), which is reminiscent of the phenotype induced by binary treatments with synthetic A- and B-type peptides (Fig. 3 C and D). Secondary growth was highly disorganized within the hypocotyls of the strongest CLE41OE mutants. In such plants, secondary xylem was dispersed throughout the stele whereas it is normally located toward the center of the vascular cylinder (compare RoLDpre;GFP control and CLE6OE; Fig. S7 G–I). These secondary xylem islands were surrounded by numerous cytoplasm-dense cells, possibly originating from the procambium and responsible for the stele radial enlargement.

**CLE6 and CLE41 Loss-of-function Phenotypes.** The A-type CLE6 ORF was cloned into the hairpin RNA expression vector pHELLSGATE 12 (8). No plant with detectable mutant phenotypes was identified among 15 independent CLE6KD T1 transformants. Seeds carrying a transgene designed for the expression of a hairpin RNA silencing the B-type CLE41 gene was obtained from the AGRIKOLA consortium [www.agrikola.org; (9)]. Again, no plant showed detectable mutant phenotypes among 12 independent CLE41KD transformants either in the T1 or in the subsequent T2 generations (M. Weingartner; personal communication).

Few loss-of-function phenotypes have been described to date for the CLE gene family (cle40-En and cle3-2) (5, 10). Considering that this family consists of 31 members (27 A-type and 4 B-type), it is likely that no mutant phenotype was observed in CLE6KD or CLE41KD transgenic lines because of high functional redundancy shared by multiple members.

**Reduced Fertility and Phenotype Stability of Gain-of-Function cle Mutants.** As observed in studies of other A-type CLE genes, CLE6OE T1 transgenic plants had reduced fertility because they produced flowers containing pin-shaped pistils with few or no anthers (16:27) (3–5). CLE6OE flowers had larger outer whorls, whereas CLE41OE flowers were structurally normal, but reduced in size (Fig. S9A). All CLEOE lines displayed delayed flowering. Under our experimental conditions, CLE6OE, CLE41OE, and control T1 transformants reached growth stage 5.10 (first flower buds visible on the emerging inflorescence) (11) at 55 ± 10.6 dag (n = 27), 49.9 ± 7.2 dag (n = 18), and 30.0 ± 2.9 dag (n = 20), respectively. Note that 5 (of 18) T1 CLE41OE plants examined never set any viable seed but lived for over 4 months.

The reduced fertility and seed viability caused by CLE6 or CLE41 overexpression explains that only transgenic plants with milder phenotypes could be propagated by self-pollination and why descendants often had weaker phenotypes compared with their parents (three generations examined). Consequently, most phenotypes presented here were analyzed on T1 transformants and crosses were obtained with plants exhibiting mild phenotypes.

**Cellular Analysis of the CLE6 and CLE41 Overexpression Leaf Phenotypes.** To understand the CLE6 and CLE41 overexpression leaf phenotypes, we examined ploidy and microscopic cellular details of fully extended 5th leaves harvested on representative T1 plants of the same age (25 dag) (Fig. S10 A–F). Notably, CLE6OE and CLE41OE leaves contained cells with an overall higher and lower DNA content (Fig. S10 G–I) (12), and their mesophyll palisade cells were larger and smaller, respectively (Fig. S10 J–L), than those of controls. Final cell size and ploidy suggest that CLE6 promotes cell differentiation in the leaf lamina, whereas CLE41 has an opposite action. These trends are reminiscent of those observed in apical or cambial meristems, respectively. The leaf lamina cellular phenotypes might result from the local action of the encoded peptide, although we cannot exclude that they are
indirect consequences of altered vascular development in the case of CLE41.

Next, we tested genetically whether an A-type CLE gene enhanced phenotypes that were induced by a B-type CLE gene. For this purpose, we characterized F1 plants resulting from independent crosses between homozygous T3 parents carrying either a CLE6OE or CLE41OE single locus transgene. CLE6OE plants were the pollen donors because they formed nonfunctional pin-shaped pistils. The CLE41OE parental plants were not descendants of the strongest gain-of-function T1 mutants because such plants were partly infertile (see above). The T3 parents were also outcrossed to wild type to account for a possible dosage effect of the respective transgenes, and the resulting hemizygous F1 plants were included in the study. Real time quantitative RT-PCR showed that the levels of CLE6 or CLE41 transcripts were significantly higher in the parents than in the wild type, and confirmed all CLE6OE, CLE41OE, and wild-type crosses (data not shown).

Analyses of hemizygous CLE6OE and CLE41OE F1 plants confirmed data obtained with the corresponding T1 plants regarding rosette development (Fig. S11 A–C), fifth fully extended leaf size (Fig. S11 E–G), ploidy (Fig. S11 I–K), palisade cell size (Fig. S11 M–O), and vascular cell size and number (Fig. S11 Q–T). Furthermore, in all aspects, the combined overexpression of the two genes in CLE6OE/CLE41OE F1 plants resulted in the dramatic enhancement of the CLE41OE leaf phenotypes (Fig. S11 D, H, L, P, and T).

To conclude, our characterization of the CLE gene overexpression leaf and vascular phenotypes and the study of the plant responses to exogenously applied synthetic CLE peptides, all indicate that A-type and B-type CLE gene products act synergistically to enhance B-type CLE gain-of-function phenotypes.

Materials and Methods

CLE6 and CLE41 Transgenic Lines. Full-length CLE6 and CLE41 ORFs were PCR-amplified from genomic DNA with modified gene-specific oligonucleotides and cloned with Gateway technology into pDONR221 (Invitrogen) (2). The resulting ORF entry clones were sequence validated.

CLE6 and CLE41 ORFs were cloned downstream of the CaMV 35S promoter into pK7WG2 (13). The CLE6 hairpin RNA expression vector (CaMV 35S promoter) was generated by cloning the corresponding ORF into pHELLSGATE12 (8). Resulting binary vectors were transferred to Agrobacterium tumefaciens strain GV3101 for transformation into wild-type Arabidopsis thaliana (L.) Heyn. (Col-0) plants via flower dip (14). All transgenic plants were selected for their resistance to kanamycin.

Overexpression of CLE6 and/or CLE41 in transgenic and crossed plants was confirmed by real-time quantitative PCR (oligonucleotide sequences available on request) following normalization to the stable CDKA1;1 and eEF-1A transcript levels and scaling with the lowest respective expression level.
Fig. S1. Differential effect of CLE peptides on Arabidopsis root growth. (A) Seedlings were transferred at 3 dag to media each complemented with the indicated CLE synthetic peptide (10 \mu M) (see Table S1 for amino acid sequences). (B) Same as (A), but with a peptide concentration of 1 \mu M. Primary root length (cm) was measured every day for 6 days (n = 10 for each peptide treatment). By definition, peptides in A-type arrested root growth at 10 \mu M, but roots exposed to B-type peptides exhibited the same growth rate as controls without added peptide or with a randomized peptide (rCLE19p).
Fig. S2. Effects of CLE peptides on RAM. Seedlings germinated for 3 days on agar medium without peptide were transferred to peptide-supplemented media. (A–F) Expression of CYCB1,1pro:GUS and (G–K) DR5pro:GUS in primary root tips after 7 days of growth on MS media without peptide (A and G), or with CLE41p (B and H), CLE6p (C and I), CLV3p (D and J), CLE19p (E and K), and CLE6p and CLE41p combined (10 μM of each peptide) (F). (L–N) Confocal microscopic images of plasma membranes (FM4–64 dye) of wild-type primary root tips grown for 7 days without peptide or with CLE41p or CLE6p. Arrow indicates boundary of differentiated vascular tissue that is abnormally close to the root tip. Initial concentration of each added peptide was 10 μM. [Scale bar, 60 μm (A–K) and 40 μm (L–N).]
Fig. S3. SAMs after CLE peptide treatment. Arabidopsis seedlings germinated on solid medium were transferred 3 dag into liquid culture supplemented with or without synthetic CLE peptides for 14 days. Representative longitudinal sections through SAM ($n = 5$) grown with (A) no peptide added, (B) CLE41p, (C) CLE6p, or (D) both CLE6p and CLE41p. Initial concentration of each added peptide was 10 $\mu$M. (Scale bar, 50 $\mu$m.)
Fig. S4. Transcriptional changes induced over time by CLE6p and CLE41p. Hypocotyl stele width (Left) and representative whole-mount hypocotyl images (Right) showing GUS activity in the marker lines: (A) ATHB8pro:GUS, (B) ET1335:GUS, (C) CYCB1,1pro:GUS, (D) APLpro:GUS, (E) DRSpro:GUS, (F) IAA2pro:GUS, (G) PIN1pro:GUS, and (H) PIN3pro:GUS. For each treatment, 10 seedlings were germinated for 3 days on solid media (0 h) and grown in peptide-supplemented liquid media (CLE41p or CLE6p-CLE41p at 10 μM each) for 3 h, 6 h, 1 day, 2 days, 4 days, 7 days, and 10 days. Initial concentration of each added peptide was 10 μM. (Scale bar, 100 μm.) Red frames indicate transcriptional marker lines with GUS activity in the hypocotyl stele at the corresponding time point(s). For each individual marker line, GUS staining incubation times were optimized to highlight differences between the peptide treatments and their respective controls without peptides. The asterisk marks samples significantly different from controls without peptide (np). (Student t test P < 0.05.)
Fig. S5. Combined CLE genes overexpression phenotypes. (A) Representative F1 plants resulting from crosses between wild-type, CLE6OE, and CLE41OE plants, compared with the wild type (Col-0) (Pot radius, 8 cm). (B) Toluidine blue-stained transverse sections of hypocotyls (35 dag) showing xylem (X), phloem strands (PS), and the vascular cambium (VC). Samples are aligned according to labels in top panels. (Scale bars, 50 μm.)
Fig. 56. Requirement of the MONOPTEROS auxin response factor for CLE-induced radial vascular enlargement. (A) DIC images of vascular strands containing 1 or 2 differentiated xylem vessels in wild-type (Ler) and monopteros (mpG92) mutant leaves on binary CLE6p-CLE41p treatment compared to controls without peptide. Arrows indicate cells adjacent to the mature xylem, consisting of large cells (LC) in wild-type untreated leaves, but in numerous small cells (NSC) in treated ones. In comparison, no cell proliferation was observed in treated mpG92 leaves. (Scale bar, 50 μm.) (B) Representative whole-mount leaf vascular images (Left) showing increased GUS activity for the ATHB8pro::GUS marker lines on CLE6p-CLE41p treatment compared to controls without peptide. Ruthenium red counterstained transverse sections through leaf blade vascular bundles of GUS-stained ATHB8pro::GUS (Center) and CYCB1,1pro::GUS (Right) marker lines showing xylem (X), phloem (P), and procambial (PC) vascular strands. Comparative analysis of ATHB8pro::GUS and CYCB1,1pro::GUS leaf blades, with or without CLE6p/CLE41p treatment, showed that the elongated NSC cells directly adjacent to mature xylem vessels correspond to procambial cells. (Scale bar = 10 μm.) All seedlings were germinated on solid medium and transferred 3 dag into liquid culture supplemented with or without CLE6p and CLE41p for 10 days. Initial concentration of each added peptide was 10 μM.
Fig. S7. CLE6 and CLE41 overexpression phenotypes. (A–C) From left to right, representative RolD<sub>pro</sub>:GFP (control, 35 dag), CLE6<sup>OE</sup> (40 dag), and CLE41<sup>OE</sup> (90 dag) T1 transformants. Samples are in the same order for all image rows. Pot diameter, 8 cm. (D–F) Toluidine blue-stained longitudinal sections of SAM (25 dag). Arrows indicate boundary between leaf primordia and SAM. (G–I) Hypocotyl transverse sections (25 dag). (J–L) Large cells and numerous small cells (NSC; outlined except in Insets) adjacent to paired differentiated xylem vessels within the vascular strands (VS). In all cases, the image represents a portion of a fully expanded 5th true leaf (25 dag). [Scale bars, 30 μm (D–F); 100 μm (G–I); and 50 μm (J–L).]
Fig. S8. Variability observed in T1 transgenic plants overexpressing CLE genes. Phenotypes of 26 dag CLE6OE and CLE41OE plants (Left and Right, respectively) compared with wild-type plants (Center). (Pot radius, 5.5 cm.)
Fig. S9. Particular CLE6 and CLE41 overexpression phenotypes. (A) Flowers of RolD<sub>pro</sub>:GFP, CLE6<sup>OE</sup>, and CLE41<sup>OE</sup> T1 transformants (left to right). CLE6<sup>OE</sup> T1 transformants showing (B) aerial rosette, (C) ectopic leaf meristem, (D) terminal cup-shaped leaf-like structure, and (E) trichome-bearing filament marking terminal differentiation of the inflorescence meristem.
Fig. S10. Single CLE gene overexpression leaf phenotypes. Representative soil-grown T1 transformant plants (25 dag). (A) Rol\textsubscript{pro}:GFP (control). (B) CLE6\textsuperscript{OE}. (C) CLE41\textsuperscript{OE} (Pot radius, 5.5 cm). Samples are in the same order for all rows. (D–F) Fifth leaf dissected from the plants shown above. (G–L) All data for the corresponding 5th leaf. (G–I) DNA content measured by flow cytometry ($n = 5$). (J–L) Palisade cells. (Scale bar, 25 $\mu$m.)
Fig. S11. Leaf phenotypes resulting from combined CLE gene overexpression. Representative soil-grown wild-type and F1 plants (25 dag). (A) Wild-type (Col-0). (B) CLE6<sup>OE</sup>/-. (C) CLE41<sup>OE</sup>/-. (D) CLE41<sup>OE</sup>/-; CLE6<sup>OE</sup>/-. (Pot radius, 5.5 cm.) Samples are in the same order for all rows. (E–H) Fifth leaf dissected from the plants shown above. (Scale bar, 5 mm.) (I–T) All data for the corresponding 5th leaf. (I–L) DNA content measured by flow cytometry (n = 5). (M–P) Palisade cells. (Scale bar, 25 μm.) (Q–T) Large cells and numerous small cells (NSC; outlined, except in insets) adjacent to paired differentiated xylem vessels within the vascular strand (VS). (Scale bar, 50 μm.) In all cases, a portion of a fully expanded 5th true leaf (25 dag) is presented; only leaves of CLE41<sup>OE</sup>/-; CLE6<sup>OE</sup>/- show NSC.
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*In this study, the synthetic CLE peptides assayed are delimited by the first basic (Arg, His, or Lys) residue flanking either side of the conserved CLE domain in a given predicted CLE preprotein, assuming that such residues mark potential proteolytic cleavage sites.

†In a similar *in vitro* root growth assay, Ito et al. (2) and Kinoshita et al. (3) determined that CLE1–7p did not induce RAM arrest at 1 μM. Our repetition of the same assay confirms the earlier report. However, CLE1–CLE7p do induce RAM arrest at 10 μM (Fig. S2A and B). Because peptide bioactivity depends on peptide stability and dosage (4), and given our positive meristem arrest phenotype, it is reasonable to assume that the results previously reported for CLE1–CLE7 are false negatives. Additionally, CLE5p—annotated as CLE7p in TAIR8 and in the table—has been demonstrated to reduce SAM size (4), providing independent evidence that CLE5p can be classified as an A-type peptide.

‡All CLE peptides in this study were defined by AGI accession and associated gene name as per TAIR8. Note that the CLE5 and CLE7 peptide coding sequences have been inverted in previous studies (1, 2, 4).