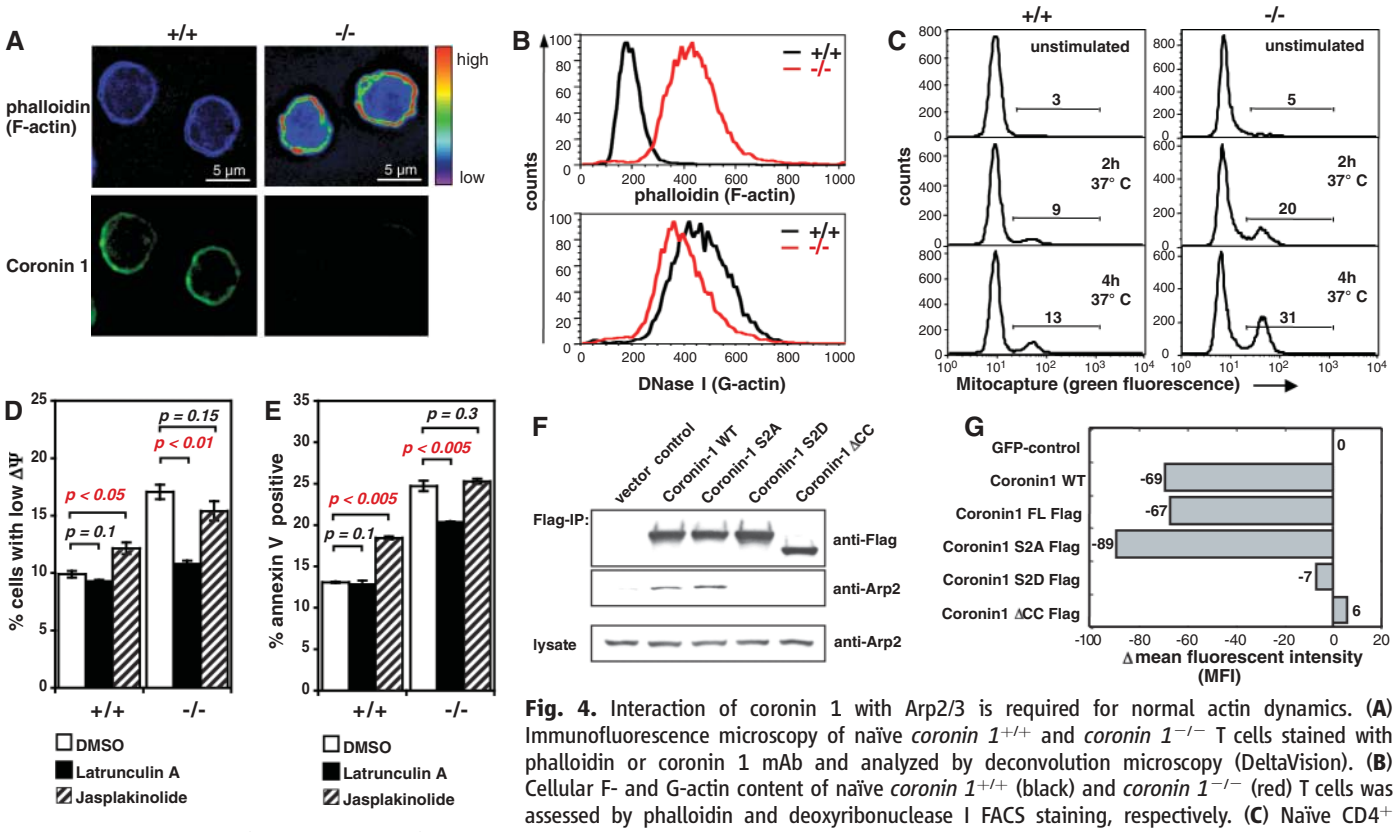

Fig. 4. Interaction of coronin 1 with Arp2/3 is required for normal actin dynamics. (**A**) Immunofluorescence microscopy of naïve *coronin* $1^{+/+}$ and *coronin* $1^{-/-}$ T cells stained with phalloidin or coronin 1 mAb and analyzed by deconvolution microscopy (DeltaVision). (**B**) Cellular F- and G-actin content of naïve *coronin* $1^{+/+}$ (black) and *coronin* $1^{-/-}$ (red) T cells was assessed by phalloidin and deoxyribonuclease I FACS staining, respectively. (**C**) Naïve CD4$^+$ DO11.10 TCR$^+$ *coronin* $1^{+/+}$ and *coronin* $1^{-/-}$ T cells were cultured at 37°C in normal medium for the indicated times, and changes in the MMP were assessed. (**D** and **E**) Naïve *coronin* $1^{+/+}$ and *coronin* $1^{-/-}$ T cells were preincubated on ice with 5 μg/ml latrunculin A, 1 μM jasplakinolide, or dimethyl sulfoxide (DMSO) carrier. Cells were cultured at 37°C for 2 hours, and loss of MMP (D) or annexin V$^+$ (E) was quantified by FACS. Error bars indicate SD from duplicate cultures. (**F**) Wild-type (WT) and mutant coronin 1 were expressed in A20 cells. Lysates were immunoprecipitated with Flag mAbs and immunoblotted with antibodies against Arp2 and Flag. (**G**) Basal F-actin of DO11.10 TCR$^+$ *coronin* $1^{-/-}$ cell lines, transfected with the indicated coronin 1 internal ribosomal entry site (IRES)–green fluorescent protein (GFP) expression constructs, was assessed by staining with phalloidin and quantified by FACS. Results represent the change in mean fluorescent intensity (ΔMFI) between GFP$^-$ and GFP$^+$ cells. Protein expression was assessed in fig. S7. The ΔMFI between *coronin* $1^{-/-}$ and *coronin* $1^{+/+}$ T cells was –122. Data are representative of two independent experiments.

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Dodeca-CLE Peptides as Suppressors of Plant Stem Cell Differentiation

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In plants and animals, small peptide ligands that signal in cell-cell communication have been suggested to be a crucial component of development. A bioassay of single-cell transdifferentation demonstrates that a dodecapeptide with two hydroxyproline residues is the functional product of genes from the *CLE* family, which includes *CLAVATA3* in *Arabidopsis*. The dodecapeptide suppresses xylem cell development at a concentration of 10^{-11} M and promotes cell division. An application, corresponding to all 26 *Arabidopsis* CLE protein family members, of synthetic dodecapeptides reveals two counteracting signaling pathways involved in stem cell fate.

Organized tissue formation in multicellular organisms requires cell-cell communication. In animals, this process is mediated by many specific extracellular molecules, including peptides. Plant peptides that direct cell-cell communication have not previously been identified, rather a limited number of nonspecific mobile phytohormones such as auxins, cytokinins, and gibberellins direct plant tissue formation (*1*). The recent discovery of peptides in plants, as well as putative leucine-rich repeat (LRR) receptor kinases, suggests that the importance of peptide ligands in plant development may have been overlooked (*2*–*5*). One such signaling system involves both CLAVATA3 (CLV3), a putative peptide ligand, and CLAVATA1 (CLV1), a LRR receptor–like kinase, which determine cell fate in the shoot apical meristem (SAM) (*6*–*8*). LRR-containing receptors also function in cell-cell signaling in animals, although animal receptors do not contain a cytoplasmic kinase domain (*2*).

CLV3 belongs to a large gene family, *CLE* (for *CLV3/ESR*-related), that encodes small proteins with conserved carboxyl termini (*3*, *9*). Genetic and physiological analyses of transgenic plants overexpressing the *CLE* genes (*10*–*13*) and studies using synthetic peptides (*14*) have revealed the involvement of various CLE members in stem cell development; however, the un-

derlying mechanisms remain unknown, as no natural CLE peptide has been identified.

To identify signal molecules responsible for cell-cell interactions, we used a xylogenic culture system, in which isolated *Zinnia* (*Zinnia elegans* L.) mesophyll cells transdifferentiate into tracheary elements (the main conductive cells of the xylem), when cultured in medium containing both naphthaleneacetic acid as an auxin and benzyladenine as a cytokinin (*15*). Using this system, we previously identified xylogen, a unique arabinogalactan protein that mediates inductive cell-cell interaction in vascular development (*16*). During the isolation of xylogen, we noticed that tracheary element differentiation was inhibited by extracellular factor(s), which we isolated, characterized, and named tracheary element differentiation inhibitory factor (TDIF). TDIF was found in a 20% methanol fraction taken from the conditioned medium.

Because auxin and cytokinin are differently involved in the program of tracheary element transdifferentiation (*15*, *17*), we prepared the 20% methanol fraction from the conditioned medium of cultures containing different combinations of naphthaleneacetic acid and benzyladenine (Fig. 1A). We bioassayed TDIF activity in the 20% methanol fraction by adding it at the start of culture to the tracheary element differentiation–induced culture containing both naphthaleneacetic acid and benzyladenine (D culture). The 20% methanol fraction prepared from the culture containing only naphthaleneacetic acid (CN culture) exhibited the highest activity (Fig. 1, A and B). The amount of inhibitory activity in the D culture was much less than that in CN culture. When the TDIF fraction from the conditioned medium of CN culture was added at the start of culture, tracheary element differentiation was almost completely inhibited (Fig. 1C), whereas cell division was promoted (Fig. 1D). These opposing biological activities suggest that TDIF specifically suppresses the tracheary element differentiation pathway, but does not affect general physiological activity.

To address which precise processes were suppressed during tracheary element differentiation, we added the TDIF fraction to D culture at different times. The process of tracheary element differentiation in culture is divided into three stages; stage one (0 to 24 hours) in which mesophyll cells dedifferentiate, stage two (24 to 48 hours) in which dedifferentiated cells differentiate into procambium cells as stem cells, and stage three (48 to 96 hours) in which stem cells differentiate into tracheary elements (*15*). The addition of TDIF completely inhibited tracheary element differentiation until 36 hours after culture initiation (fig. S1A). RNA gel-blot analyses with stage-specific marker genes (*18*) suggested that TDIF preferentially suppressed the entry into stage 3; the transition of procambial cells to tracheary elements (Fig. 1E).

To characterize the chemical properties of TDIF, we treated the biologically active fraction with pronase E, trypsin, proteinase K, and denatured proteases (fig. S1B). Proteinase K did not affect TDIF activity; pronase E, but not its denatured form, eliminated most activity; and trypsin slightly reduced activity. These results indicate that TDIF is a proteinaceous factor. TDIF was hypothesized to be a small peptide because it passed through a 5-kD cutoff membrane (Biomax-5, Millipore) during ultrafiltration experiments (fig. S1C) and was not inactivated when exposed to 100°C for 10 min (fig. S1D).

We fractionated TDIF by high-performance liquid chromatography in association with a bioassay (Fig. 2, A to C) and finally isolated a peptide that appeared as a single peak (Fig. 2C). The combination of tandem mass spectrometry analysis and amino acid sequencing revealed that TDIF is a dodecapeptide with two hydroxyproline residues (Hyp), HEVHypSGHypNPISN (fig. S2) (*19*, *20*). Synthetic TDIF at 30 pM, corresponding to the concentration in the CN medium, inhibited tracheary element differentiation by 50% (Fig. 3A). The full-length cDNA corresponding to TDIF was isolated from *Zinnia* cells cultured in CN medium (Fig. 2D). The cDNA potentially encodes a

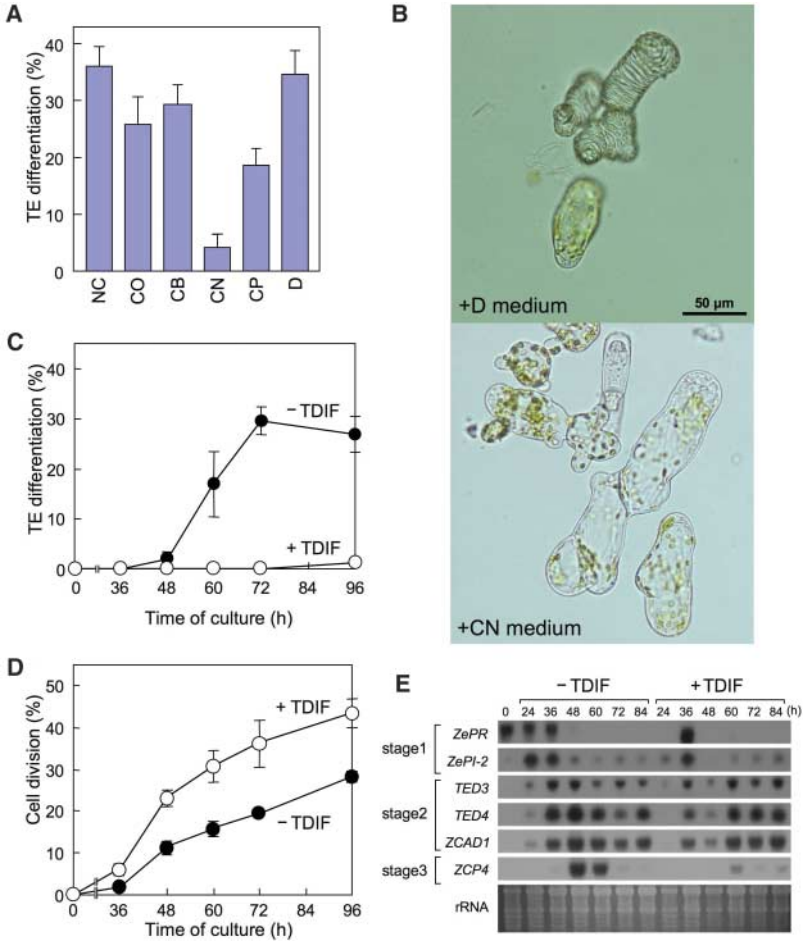

Fig. 1. TDIF activity in cultured medium. (**A**) TDIF activity varies among different cultures (CO, hormone free; CB, only benzyladenine (BA); CN, only naphthaleneacetic acid (NAA); CP, low BA and NAA; D, both BA and NAA). *Zinnia* mesophyll cells were cultured in CO, CB, CN, CP, and D media for 72 hours. A 20% methanol fraction of each medium was added to D culture at the start of culture, at a final concentration equivalent to threefold the original concentration. NC, no addition. (**B**) Tracheary element differentiation is inhibited by the 20% fraction of CN culture medium, but not D culture medium. (**C**) TDIF inhibits tracheary element differentiation. (**D**) TDIF increases cell division over time. (**E**) TDIF suppresses mRNA accumulation of a stage three–specific gene, but not those specific to stages one or two. The progress of tracheary element differentiation was monitored by the expression of stage-specific marker genes, *ZePR* (*Zinnia elegans* pathogenesis-related gene, AB091075), *ZePI-2* (gene for *Zinnia elegans* protease inhibitor-2, AB091074), *TED3* (tracheary element differentiation–related gene 3, D30801), *TED4* (tracheary element differentiation–related gene 4, D30802), *ZCAD1* (*Zinnia* cinnamyl alcohol dehydrogenase gene, D86590), and *ZCP4* (*Zinnia* cysteine protease gene 4; AB091070), as described (*18*).

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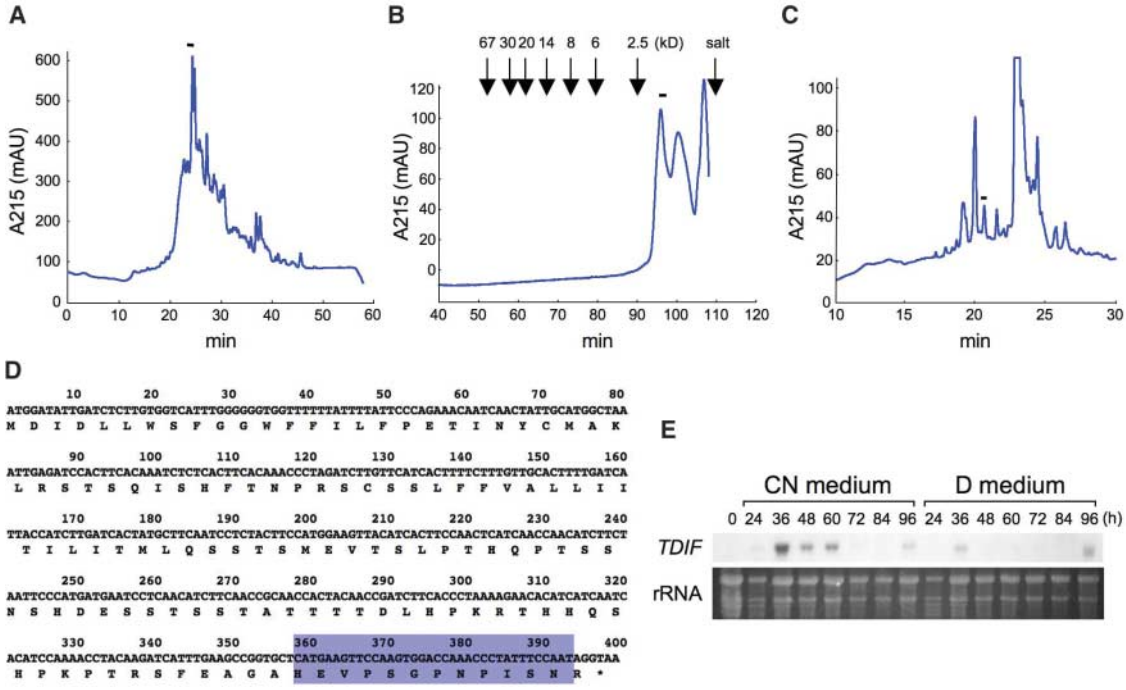

Fig. 2. Identification of TDIF. (**A**) Poly(2-sulfoethyl aspartamide) ion-exchange column chromatography. The horizontal bar indicates an active fraction. (**B**) Gel-filtration column chromatography profile using tandem Super SW 3000 columns. (**C**) An inertsil ODS3 reversed-phase column chromatography profile. (**D**) The deduced amino acid sequence of a TDIF gene from *Zinnia elegans*. (**E**) Time course of TDIF mRNA accumulation in cells cultured in CN and D medium.

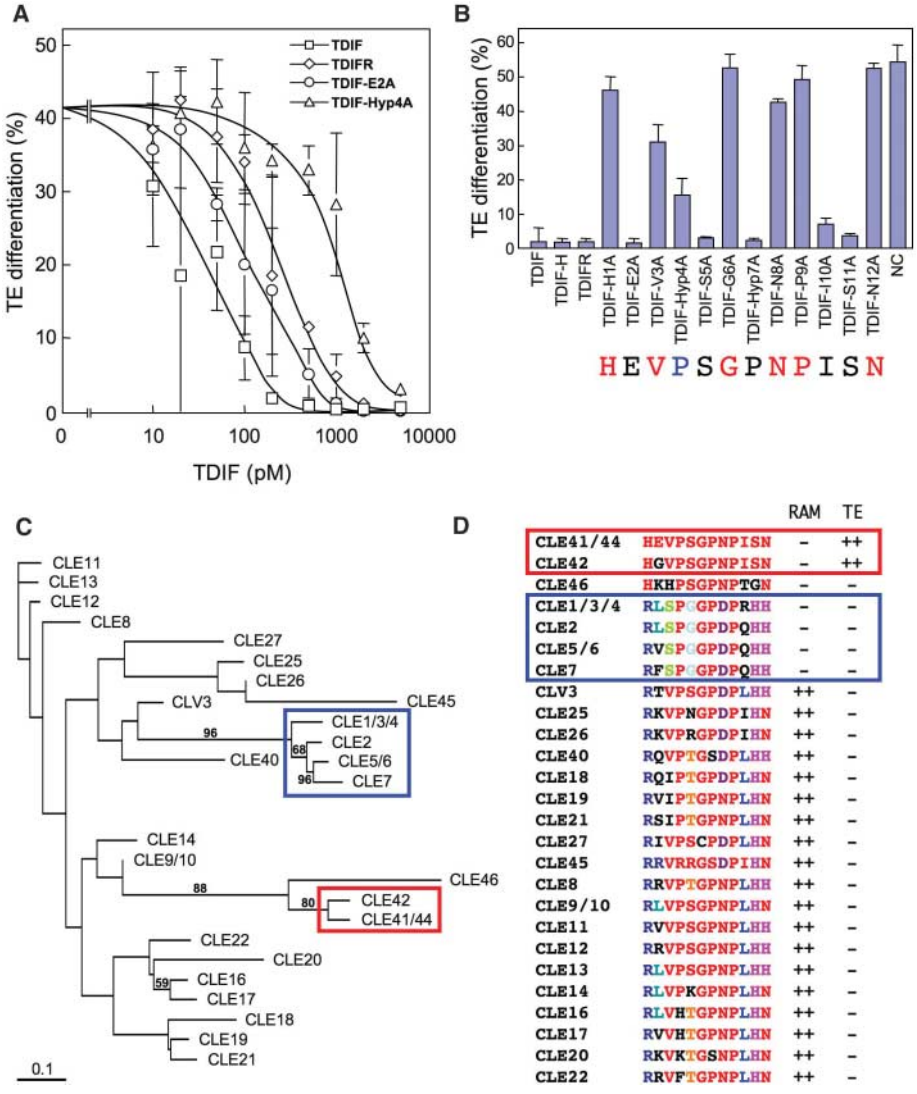

Fig. 3. Functional analysis of dodeca-CLE peptides. (**A**) Kinetics of the inhibition of tracheary element differentiation by TDIF and TDIF derivatives. (**B**) The inhibition of tracheary element differentiation by TDIF derivatives at 8 nM. Each amino acid residue of TDIF was replaced by alanine (TDIF-H1A to TDIF-N12A). TDIF-R had an additional arginine added at the C terminus. In TDIF-H, the two Hyp residues were replaced by proline. NC, no addition. (**C**) An unrooted neighbor-joining tree of *Arabidopsis* dodeca-CLE peptides (table S1). Bootstrap values of 50% and above from the neighbor-joining method, with Kimura's correction (*25*), are shown. The scale bar indicates the number of amino acid substitutions per site. (**D**) Amino acid composition and function of the 26 synthetic dodeca-CLE peptides with two hydroxyproline residues. The suppression of tracheary element differentiation (TE) and root apical meristem (RAM) formation (*14*) by the dodeca-CLE peptides was examined.

protein of 132 amino acids, but only 12 from H^{120} to N^{131} match the TDIF sequence. This result indicates that TDIF is produced through the removal of residues M^1 to A^{119} at the N terminus and R^{132} at the C terminus. The level of transcripts for the TDIF precursor was high between 36 and 60 hours in CN-cultured cells and present at lower levels in D-cultured cells after 36 and 96 hours (Fig. 2E). This result is consistent with the appearance of the TDIF activity in CN culture (fig. S1E) and the weak TDIF activity in D culture (Fig. 1A).

Through homology searches, the TDIF sequence was found to be the same as the *Arabidopsis* C-terminal 12 amino acids CLE41 and CLE44, and highly homologous to CLE42 and CLE46 (fig. S3). Although many studies have suggested that the *CLE* gene family produces functional small peptides (*3*, *9*–*14*), there has been no direct evidence of CLE peptides in situ. The identification of TDIF is direct evidence that a 12–amino acid peptide functions as an extracellular signaling molecule in plants.

To deduce the chemical basis of CLE function, we made TDIF derivatives and compared their relative activities (Fig. 3, A and B). Hydroxylation of the two proline residues was not necessary for TDIF activity; however, addition of an arginine at the C terminus, which is the precursor sequence, reduced activity by 1/7th compared with the wild-type peptide. Alanine scanning experiments indicated that replacement of the amino acids at positions 2, 5, 7, 10, and 11 within the peptide did not affect TDIF activity (Fig. 3B). In contrast, substitution of alanine for H^1, V^3, G^6, N^8, P^9, and N^{12} all caused a severe loss of TDIF activity. A substitution of the 4th amino acid reduced activity by 1/30th (Fig. 3A).

The in situ CLV3 peptide is 12 amino acids long and contains two hydroxyproline residues (*21*). Tracheary element differentiation and root growth were investigated with bioassays of 26 synthetic peptides of 12 amino acids, including the two hydroxyprolines, corresponding to the predicted products of all of the 31 *Arabidopsis CLE* genes (Fig. 3, C and D, and table S1). Dodecapeptides from CLE42 and CLE41/CLE44 have strong TDIF activity (Fig. 3D), but did not inhibit root growth. On the other hand, the dodecapeptides of all other CLE proteins lacked TDIF activity. Therefore, CLE42 and CLE41/CLE44 may play a specific role in xylem differentiation.

Like CLE42 and CLE41/CLE44, five peptides (CLE46, CLE1/CLE3/CLE4, CLE2, CLE5/CLE6, and CLE7) did not suppress root growth, whereas the others did so strongly (Fig. 3D). In accordance with overexpression of the *CLV3* (*8*) and various *CLE* genes except the *CLE1* to *CLE7* genes (*10*–*14*), our results demonstrate that most dodeca-CLE peptides suppress root growth. Nevertheless, the clade composed of CLE42 and CLE41/CLE44 displayed distinctive functions; the clade composed of CLE1/CLE3/CLE4, CLE2, CLE5/CLE6, and CLE7 did not exhibit any detectable activity in our bioassay, although the CLE1 to CLE7 peptides have been reported to function in the shoot apical meristem (*12*, *13*).

Alanine scanning mutagenesis, along with comparison of the amino acid sequence among CLE peptides, has identified the amino acids necessary for both general and specific activity of CLE peptides. G^6 and P^9, which are absolutely conserved in almost all *Arabidopsis* CLE peptides, and the slightly less conserved residues, V^3, N^8, and N^{12}, were essential for TDIF activity. Therefore, these amino acids may be required for the general activity of CLE peptides. H^1 may confer distinct biological activity as it is unique among CLE peptides with TDIF activity. Our data, along with in situ identification of the CLV3 peptide (*21*), indicate that the active form of CLE proteins is a dodecapeptide containing two hydroxyprolines.

Previous analysis indicates that the major function of CLV3, which is a putative ligand to the CLV1 or CLV1-CLV2 receptor complex, is the suppression of stem cell proliferation in the meristem (*2*, *22*, *23*). Excess CLV3 seems to promote differentiation from stem cells (*8*, *14*). In contrast, TDIF suppresses the differentiation of xylem cells from stem cell–like procambial cells and promotes cell division. Indeed, a functional CLV3 peptide promotes xylem cell differentiation in *Zinnia* cell culture (fig. S4). Therefore, there are two counteracting pathways in CLE signaling, one that promotes and one that inhibits stem cell differentiation in vascular development. It will be interesting if two similar counteracting pathways function in meristems.

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19. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
20. Hydroxylation of proline is a frequent modification of secretory proteins and peptides in plants. We have reported the motifs necessary for proline hydroxylation and hydroxyproline glycosylation in plant secretory proteins (*24*). The hydroxylation, but not glycosylation, of the two prolines of TDIF conforms well to this rule. Although hydroxylation of prolines is not necessary for activity of TDIF, the hydroxylation may render the peptide more hydrophilic, facilitating its movement and its resistance against proteolysis in apoplastic space.
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Supporting Online Material

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A Plant Peptide Encoded by *CLV3* Identified by in Situ MALDI-TOF MS Analysis

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The *Arabidopsis CLAVATA3* (*CLV3*) gene encodes a stem cell–specific protein presumed to be a precursor of a secreted peptide hormone. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) applied to in situ *Arabidopsis* tissues determined the structure of a modified 12–amino acid peptide (MCLV3), which was derived from a conserved motif in the CLV3 sequence. Synthetic MCLV3 induced shoot and root meristem consumption as cells differentiated into other organs, displaying the typical phenotype of transgenic plants overexpressing *CLV3*. These results suggest that the functional peptide of CLV3 is MCLV3.

Several lines of evidence indicate that peptides are important for plant growth and development. Three plant peptide hormones, systemins, phytosulfokine, and SCR/SP11, have been biochemically identified. These bioactive peptides are originated and posttrans-