The signaling peptide-encoding genes CLE16, CLE17 and CLE27 are dispensable for Arabidopsis shoot apical meristem activity

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Abstract

The shoot apical meristem produces all of the leaves, stems and flowers of a flowering plant from a reservoir of stem cells at its growing tip. In Arabidopsis, the small polypeptide signaling molecule CLAVATA3 (CLV3), a member of the CLV3/EMBRYO SURROUNDING REGION-RELATED (CLE) gene family, is a key component of a negative feedback loop that maintains stem cell activity in shoot and floral meristems throughout development. Because in some plant species multiple CLE genes are involved in regulating shoot apical meristem activity, we tested the hypothesis that CLE genes other than CLV3 might function in stem cell homeostasis in Arabidopsis. We identified three Arabidopsis CLE genes expressed in the post-embryonic shoot apical meristem, generated loss-of-function alleles using genome editing, and analyzed the meristem phenotypes of the resulting mutant plants. We found that null mutations in CLE16, CLE17 or CLE27 affected neither vegetative nor reproductive shoot meristem activity under normal growth conditions, although CLE27 appears to slightly prolong vegetative growth. Our results indicate that the CLE16, CLE17 and CLE27 genes have largely redundant roles in the Arabidopsis shoot apical meristem and/or regulate meristem activity only under specific environmental conditions.

Introduction

Unlike animals, which develop their body plan predominantly during embryogenesis, the distinct architecture of plants is formed throughout the course of their lives. The growing tips of the plant, called the shoot and the root apical meristems, generate organs in a reiterative and continuous process. The shoot apical meristem (SAM) is organized during embryogenesis and produces all of the above ground elements of the plant [1]. Following the germination of the seed, the seedling SAM produces leaves from its flanks during the vegetative phase of development. In response to environmental and endogenous cues the SAM of the mature seedling undergoes the transition to flowering, the reproductive phase in which the shoot meristem is transformed into an inflorescence meristem (IFM) that produces axillary meristems followed...
by floral meristems that generate the flowers. Fertilization then enables the formation of seeds that transmit the genes to the next generation.

The organization of the SAM provides the capacity for plants to perform lifelong organogenesis. The SAM consists of a small reservoir of stem cells at the apex that is surrounded by a peripheral zone of cells that transition to more differentiated fates within discrete organ primordia. Beneath the stem cell reservoir resides a central domain called the organizing center (OC), which acts as a niche that maintains the fate of the overlying stem cell population. The activity of the OC sustains a relatively constant number of stem cells at the apex of the SAM despite the continuous differentiation of their descendants into organ and stem tissue on the flanks. The spatial and temporal control of gene activity and cellular function within these various domains relies on elaborate networks of phytohormones, transcription factors and intercellular signals to communicate information throughout the shoot apical meristem [2–4].

An intercellular signaling network known as the CLV-WUS pathway maintains stem cell homeostasis in Arabidopsis [5]. The CLV3 gene is expressed in the stem cells and encodes a small, secreted polypeptide signaling molecule [6] that moves through the apoplast into the cells of the underlying OC, where it is perceived by several receptor kinases complexes [7–11]. Signaling through the CLV pathway restricts the expression of the WUSCHEL (WUS) homeobox transcription factor gene to the cells of the OC [12]. WUS protein, in turn, moves through the plasmodesmata into the apical domain [13], where it sustains stem cell identity and directly induces CLV3 expression [14, 15]. WUS also integrates cytokinin signaling inputs in the interior of the SAM to stimulate cytokinin-mediated stem cell proliferation [16], while repressing the expression of genes that direct cell differentiation [17].

CLV3 is a founding member of the CLV3/EMBRYO SURROUNDING REGION-RELATED (CLE) gene family, which is present throughout the plant lineage and in some plant parasitic nematodes [18, 19]. These genes encode polypeptides of less than 15 kDa in molecular mass that contain an amino-terminal signal peptide, a variable domain, and a conserved stretch of 14 amino acids near the carboxyl-terminus called the CLE domain that is processed to form the biologically active peptide [20–22]. Although the function of the vast majority of CLE genes is unknown, studies indicate that CLE peptides play key roles in stem cell homeostasis in Arabidopsis root and vascular meristems as well as in the SAM [23–25]. In some plant species, multiple CLE genes appear to be involved in the regulation of stem cell maintenance in shoot and floral meristems [26]. In rice, the CLV3-related FON2 and FOSI genes redundantly regulate stem cell activity within floral meristems [27], yet FON2 also affects inflorescence and axillary meristem maintenance [28] whereas FOSI and a third rice CLE gene, FCP1, are likely to be involved in vegetative SAM maintenance [27, 28]. In tomato, the SiCLV3 and SiCLE9 peptides both appear to affect vegetative meristem size [29], again illustrating potential redundancy within the CLE family. Thus, although they have not been identified in genetic screens, other members of CLE gene family may likewise function as additional signaling pathway components in the Arabidopsis SAM.

In this study, we identified three Arabidopsis CLE genes that are expressed within the vegetative and/or reproductive SAM. We generated loss-of-function mutations in each of the three genes and analyzed their meristem phenotypes throughout development. We determined that null mutations in the CLE16, CLE17 and CLE27 genes caused no measurable vegetative, inflorescence or floral meristem phenotypes under normal growth conditions, although CLE27 appears to slightly prolong the vegetative growth rate. Our data suggest that SAM-expressed CLE genes other than CLV3 act largely redundantly in the Arabidopsis meristem and/or function to regulate SAM activity only under certain environmental conditions.
Materials and methods

Plant materials and growth conditions

All Arabidopsis thaliana plants were in the Columbia-0 accession. The cle27-2 (SALK_077000) T-DNA insertion allele was generated by the SALK Institute [30] and was obtained from the Arabidopsis Biological Resource Center (ABRC), sequenced to confirm the location of the insertion site, and backcrossed three times to Col-0 prior to analysis. Plants were grown on soil (1:1:1 mixture of perlite:vermiculite:topsoil) under continuous light (120 μmol m\(^{-2}\) s\(^{-1}\)) at 21°C. Seeds were planted at a density of one seed per pot, except for the Col-0 and cle16 IFM histology experiment in which a density of two seeds per pot was used. Seeds were stratified at 4°C for 5 days before exposure to light. Seedlings were watered every day with a 1:1500 dilution of Miracle-Gro 20-20-20 fertilizer prior to flowering and once a week with fertilizer thereafter. Homozygous mutant plants were confirmed by PCR-based genotyping prior to analysis (primers listed in S1 Table).

Genome editing of CLE gene loci

CRISPR-Cas9 target gene sequences for CLE16 and CLE17 were identified using the CRISPR-P website [31]. The target sequences were amplified and cloned into the Gateway-compatible pSGR_pGEMT entry vector, which also harbored a Cas9 expression cassette. The pSGR_pGEMT constructs containing the Cas9 cassette as well as the CLE16 or CLE17 genomic target sequences were transferred into the pEarleyGate 301 binary vector using the LR enzyme mix (ThermoFisher Scientific), and sequenced. The recombinant pEarleyGate 301 constructs were then transferred into Agrobacterium tumefaciens GV3101 and transformed into wild-type Col-0 plants using the floral dip method [32]. The T1 seeds were sown and selected by spraying twice with 0.01% BASTA solution, 3–5 days apart. Resistant transformants were genotyped using the Cleaved Amplified Polymorphic Sequence (CAPS) method with gene-specific primers (primers listed in S1 Table). Heterozygous T1 mutant plants were self-fertilized and homozygous T2 individuals identified by genotyping, followed by sequencing to confirm the mutant allele.

Genotyping the CLE16 CRISPR alleles was performed by using forward and reverse primers (primers listed in S1 Table) in a Polymerase Chain Reaction (PCR) to amplify a 995 bp product. Digesting the PCR product with MspI yielded 779 bp and 216 bp bands from wild-type tissue, whereas the product from mutant tissue remained undigested. Genotyping the CLE17 CRISPR alleles was performed by using forward and reverse primers in a PCR reaction to amplify a 1016 bp product. Digesting the PCR product with BseI yielded 770 bp, 233 bp and 13 bp bands from wild-type tissue, whereas the product from mutant tissue remained undigested. Genotyping to confirm the absence of the Cas9 cassette from cle16 and cle17 mutant plants was performed using Cas9 forward and reverse primers (primers listed in S1 Table).

Phenotypic analysis

Whole seedlings, rosette leaves, inflorescences and flower specimens were imaged using Zeiss Stemi 2000-c and Zeiss Stemi SV11 microscopes, and images were acquired using a Canon D-40 digital camera. Inflorescence apices were prepared for scanning electron microscopy as described [33] and visualized on a Hitachi S4700 scanning electron microscope. Inflorescence apices were prepared for histology as described [34], stained for 25 seconds in a 0.1% Toluidine blue 0 dye solution (Sigma), de-stained through an ethanol series, and sectioned at 4 μm thickness. Sections were visualized using a Zeiss Axiosvert 200M microscope. Floral organ counting was performed as described [33].
Results

The starting point for our functional analysis was the identification of all CLE genes expressed in the SAM during vegetative or reproductive development. For the vegetative stage, we used promoter:GUS expression data gathered from the vegetative meristems of 10-day-old seedlings [35]. These data indicated that, in addition to CLV3, the promoters of both CLE16 and CLE17 drove expression in the vegetative meristem as well as in the adjacent organ primordia, although the pCLE16:GUS signal was much weaker than the pCLE17:GUS signal in the SAM itself [35]. For the reproductive stage, we mined published transcriptome data generated from laser micro-dissected IFMs [36] for CLE gene expression. The CLV3 gene was used as a positive control and appeared with an expression value of 31.27 RPKM (S1 Fig). In addition, the CLE17, CLE20, CLE27 and CLE42 genes were all detected as being expressed in the IFMs transcriptome dataset (S1 Fig). Among these, we omitted CLE20 from our analysis because our promoter:GUS data indicated that the promoter drove expression exclusively in the vasculature, including in the vascular strands directly beneath the SAM, but not within the SAM itself [35]. We also excluded CLE42 because a previous study reported that a loss-of-function cle42 T-DNA insertion allele displayed no shoot phenotype [37]. Consequently we focused on the functional analysis of the CLE16, CLE17 and CLE27 genes during Arabidopsis shoot development.

Generation of cle loss-of-function alleles

Two independent loss-of-function alleles of each of the three CLE genes were identified for functional characterization. Although CLE gene loci represent small targets for mutagenesis, a single allele of both CLE16 and CLE17 had already been reported (Table 1). The cle16-1 Ds transposon insertion in the CLE16 coding region acts as a transcriptional null allele; however, the genetic background is the Nossen accession [35]. The T-DNA insertion in the cle17-1 Col-0 allele is located in the 3’ untranslated region (UTR) downstream of the CLE17 coding region, and behaves as a hypomorphic, partial loss-of-function allele rather than a null allele [35].

Because CLE16 and CLE17 null alleles in the Col-0 accession were unavailable for comparative analysis, we generated new loss-of-function alleles of the two genes using CRISPR-Cas9 genome engineering (Table 1 and Fig 1). Transformation of wild-type Col-0 plants with an sgRNA targeted to the CLE16 coding sequence yielded multiple independent transformants. We detected 21 mutant individuals among the 24 T1 plants analyzed, a remarkable 87.5% mutation rate. Mutations in the T1 individuals were made homozygous in the T2 generation and confirmed by sequencing, and two were chosen for further study. One line contained an insertion of a “C” nucleotide after position +59 downstream of the translation start site (Fig 1A), and was designated cle16-2. A second line contained a deletion of a “G” nucleotide after

Table 1. Alleles of CLE genes expressed in the shoot apical meristem.

| CLE Gene | Mutant Allele | Type of Mutation | Source |
|----------|---------------|------------------|--------|
| CLE16    | cle16-1       | Ds transposon insertion at +37 bp | Jun et al 2010 |
| CLE16    | cle16-2       | Insertion of “C” nucleotide at +59 bp | This work |
| CLE16    | cle16-3       | Deletion of “G” nucleotide at +59 bp | This work |
| CLE17    | cle17-1       | T-DNA insertion at +412 bp | Jun et al 2010 |
| CLE17    | cle17-2       | Insertion of “A” nucleotide at +220 bp | This work |
| CLE17    | cle17-3       | Insertion of “T” nucleotide at +220 bp | This work |
| CLE27    | cle27-1       | T-DNA insertion at +149 bp | This work |
| CLE27    | cle27-cr1     | Insertion of “T” nucleotide at +173 bp | Yamaguchi et al 2017 |

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position +59 and was designated cle16-3. Each of these mutations generates a frame shift in the CLE16 coding sequence well upstream of the CLE domain, with the cle16-2 mutation also introducing several premature stop codons.

Transformation with an sgRNA targeted to the CLE17 coding sequence also yielded multiple independent transformants. We detected 5 mutant individuals among the 24 T1 plants analyzed, a 20.1% mutation rate. Mutations in the T1 individuals were made homozygous in the T2 generation and confirmed by sequencing, and two were chosen for further study. One line contained an insertion of an \"A\" nucleotide after position +220 downstream of the translation start site (Fig 1B), and was designated cle17-2. A second line contained an insertion of a \"T\" nucleotide after position +220, and was designated cle17-3. Each of these mutations generates a frame shift that introduces a premature stop codon in the CLE17 coding sequence upstream of the CLE domain. Due to the frame shift mutations none of these cle16 or cle17 alleles produces a functional CLE polypeptide, and thus they represent loss-of-function alleles.

In addition we identified a CLE27 T-DNA insertion allele in the Col-0 accession from the SALK collection [30], which to avoid confusion with the published CRISPR/Cas9 line described below we designate cle27-2. Sequencing indicated that cle27-2 carries a T-DNA insertion +149 base pairs (bp) downstream of the translation start site (Fig 1C), in the center of the CLE27 coding region (Table 1). The insertion site is located upstream of the CLE27 CLE domain, indicating that cle27-2 represents a loss-of-function allele. A second, independent CLE27 allele used was a CRISPR/Cas9-generated loss-of-function allele in the Col-0 accession designated cle27-cr1 [38]. This allele generates a frame shift that introduces a premature stop codon in the CLE27 coding sequence upstream of the CLE domain (Table 1), indicating that it is a null allele [38].

Analysis of SAM function during vegetative development

To determine whether the CLE16, CLE17 or CLE27 genes play a role in regulating shoot apical meristem activity during vegetative development, we analyzed the phenotypes of wild-type Col-0 as well as cle16-2, cle16-3, cle17-2, cle17-3, cle27-cr1 and cle27-2 seedlings from germination through the first four weeks of growth. We first measured the rate of rosette leaf initiation from the SAM beginning one day after germination (DAG), then again at 4 and 7 DAG, and weekly thereafter (Fig 2A). We found that all of the wild type and cle mutant seedlings had produced two rosette leaves between 4 and 7 DAG, and that by 14 DAG plants of all genotypes had produced an average of 5–6 rosette leaves (Fig 2A). The arrangement and morphology of the leaves was indistinguishable between the various genotypes at this stage of seedling development (Fig 2B–2H). The rate of leaf initiation from the SAM was not significantly different between Col and cle16, cle17 or cle27 seedlings during the first 28 days of vegetative growth (Fig 2A), after which time the plants began to undergo the transition to flowering.
Fig 2. Mutations in CLE16, CLE17 or CLE27 have no effect on the leaf initiation rate of the shoot apical meristem. (A) Leaf initiation rate in wild-type and cle mutant plants from 1 to 28 days after germination (DAG). Values shown are mean ± standard deviation (S.D.), n = 12 individuals per genotype. (B) Wild-type Col-0 rosette at 14 DAG. (C) cle16-2 rosette. (D) cle16-3 rosette. (E) cle17-2 rosette. (F) cle17-3 rosette. (G) cle27-cr1 rosette. (H) cle27-2 rosette. Scale bar, 1 cm.

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results indicate that neither CLE16, CLE17 nor CLE27 individually functions in regulating SAM activity during the early stages of Arabidopsis vegetative development.

Next we quantified the rosette diameter of wild-type and cle mutant plants at the floral transition, when the mature seedlings ceased producing vegetative organs. Compared with wild-type Col-0 plants (Fig 3A and 3B), the diameter of cle17 rosettes (Fig 3A, 3E and 3F) and cle27 rosettes (Fig 3A, 3G and 3H) was unaltered. The diameter of cle16-3 rosettes was slightly but significantly larger than those of Col-0 and cle16-2 rosettes (Fig 3A, 3C and 3D); however, that

Fig 3. Mutations in CLE16, CLE17 or CLE27 have no effect on the rosette diameter of mature seedlings. (A) Rosette diameter of wild-type and cle mutant plants at the floral transition. Values shown are mean ± standard deviation (S.D.). Asterisks indicate a significant difference from wild-type at p <0.001 (two-tailed Student’s t test). n = 14–20 individuals per genotype. (B) Wild-type Col-0 rosette at the floral transition. (C) cle16-2 rosette. (D) cle16-3 rosette. (E) cle17-2 rosette. (F) cle17-3 rosette. (G) cle27-cr1 rosette. (H) cle27-2 rosette. Scale bar, 1 cm.

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of cle16-2 plants was indistinguishable from the wild type (Fig 3A and 3C). Because only one of the two cle16 null alleles has this effect we conclude that neither CLE16, CLE17 nor CLE27 is likely to play an independent role in rosette growth.

A major developmental event that alters the activity of the shoot apical meristem is the floral transition. This is when the SAM integrates endogenous signals as well as environmental signals from the leaves into broad transcriptional alterations that change the identity of the meristem from vegetative to reproductive. The reproductive, or inflorescence meristem, then initiates a number of axillary meristems followed by floral meristem primordia from its flanks. To determine whether the SAM-expressed CLE genes played any role in the transition of the meristem from vegetative to reproductive activity, we measured the number of days to bolting, total leaf number and axillary meristem number in wild-type and cle mutant plants.

We observed no difference in either mean days to bolting or total leaf number in cle17 plants compared to wild-type Col-0 plants (Fig 4A and 4B), indicating that CLE17 activity does not affect the floral transition. We detected a small decrease in the number of days to bolting in plants homozygous for either cle27 allele. Both cle27-cr1 and cle27-2 plants flowered an average of one day earlier than wild-type when grown under constant light conditions: 32.63±1.63 days for Col-0 compared to 31.22±2.29 for cle27-cr-1 and 31.39±1.79 days for cle27-2 plants (Fig 4A). However, the total number of leaves at flowering was unchanged in cle27-cr1 and cle27-2 plants (Fig 4B), suggesting that CLE27 may slightly prolong the plant growth rate over time rather than specifically affecting the floral transition [39]. Conversely, cle16-2 and cle16-3 plants both generated one to two more leaves than wild-type plants prior to flowering, and the cle16-2 allele also slightly delayed the time to bolting (Fig 4A and 4B). However, an independent experiment performed using identical growth conditions showed no significant difference between the two cle16 alleles and wild-type Col-0 with respect to either days to bolting or total leaf number (S2 Fig). Thus these data indicate that CLE16, CLE17 and CLE27 have no significant effect on the transition to flowering under constant light conditions.

During the transition to flowering the Arabidopsis primary SAM produces a small number of axillary meristems from the axils of the cauline leaves. Under our growth conditions wild-type Col-0 plants generated an average of 5.2±0.66 axillary meristems per SAM (Fig 4C). Neither cle16 nor cle27 plants displayed altered axillary meristem number (Fig 4C). A very slight increase in axillary meristem number, to an average of 5.7±0.73, was detected in cle17-2 plants (Fig 4C). However, because such an increase was not observed in cle17-3 plants we conclude that CLE17 also has no significant effect on axillary meristem formation. These results indicate that neither CLE16, CLE17 nor CLE27 contributes to regulating the process of axillary meristem formation by the shoot apical meristem.

Analysis of SAM function during reproductive development

Next we determined whether the CLE16, CLE17 or CLE27 genes functioned in regulating shoot apical meristem activity during reproductive development by comparing inflorescence and floral meristem activity between wild-type Col-0 and cle16, cle17, and cle27 plants. It is known that clv3 plants form enlarged inflorescence meristems that produce many more flowers than wild-type plants in a random rather than a spiral phyllotaxy [40]. Using scanning electron microscopy, we examined the tips of wild-type and cle mutant inflorescence meristems harvested when the length of the stem reached 1 cm. The morphology of the cle16, cle17 and cle27 IFMs was indistinguishable from that of wild-type Col-0 inflorescences, as was the rate of floral meristem initiation (Fig 5). The phyllotaxy, or arrangement, of floral meristem formation from the IFM flanks was also unaffected, with successive floral primordia initiating in a spiral pattern in both wild-type and cle mutant plants (Fig 5).
Fig 4. Mutations in CLE16, CLE17 or CLE27 have no significant effect on the floral transition. (A) Days to bolting of wild-type and cle mutant plants. (B) Total leaf number of wild-type and cle mutant plants at the transition to CLE genes and shoot meristem development.
We analyzed wild-type and cle inflorescence meristem morphology and size in greater detail by histological sectioning. The wild-type Col-0 inflorescence meristem is dome-shaped and is composed of three cell layers (Fig 6A and 6D). The cells in the outermost two cell layers, L1 and L2, divide in a strictly anticlinal orientation and form the epidermal and sub-epidermal layers [1], respectively. The underlying L3 cells divide in all orientations and provide the girth of the IFM. The morphology of cle16, cle17 and cle27 IFMs was indistinguishable from that of wild-type IFMs, and the layering of the meristem was intact in all genotypes (Fig 6B, 6C and 6E–6H).

clv3 mutant inflorescence meristems contain many more cells and are both wider and taller than wild-type IFMs [12], so the diameter and height of Col-0, cle16, cle17 and cle27 IFMs was measured. Two experiments were performed, one comparing cle16 homozygous IFMs to Col-0 and the other comparing cle17 and cle27 IFMs to Col-0. Although the mean Col-0 IFM size differed between the two experiments due to slightly different cultivation conditions (see Methods), the mean size of the cle16, cle17 and cle27 IFMs was not significantly different from that of the corresponding Col-0 IFMs (Fig 6I and 6J). These observations show that, unlike CLV3, CLE16, CLE17 and CLE27 individually have no effect on inflorescence meristem activity under normal growth conditions.

Finally, we quantified the number of floral organs in wild-type and cle mutant flowers as a readout for potential alterations in floral meristem size. Compared to wild-type flowers, which consist of four sepals in the first whorl, four petals in the second whorl, 5–6 stamens in the third whorl and two carpels in the fourth whorl (Fig 7A and 7B), clv3 flowers produce supernumerary organs in all four whorls, particularly the inner two, and can generate additional organs within the carpel whorl [6, 40]. The extra floral organs are a product of enlarged floral meristems and the extra whorls of organs result from reduced floral meristem determinacy [40]. In contrast to clv3 mutants, the mean number of sepals, petals and stamens produced by plants carrying null mutations in CLE16, CLE17 or CLE27 was indistinguishable from the wild type (Fig 7A and 7C–7H). Carpel number in all genotypes was invariant at two. These data indicate that individually CLE16, CLE17 and CLE27 are dispensable for regulating floral meristem activity.
The aim of our study was to identify additional members of the Arabidopsis CLV3-related CLE gene family that are expressed in above-ground meristems and to determine whether they played a role in regulating shoot apical meristem activity. We identified CLE16 and CLE17 as being expressed in both vegetative and inflorescence meristems, and CLE27 as being expressed in inflorescence meristems and vegetative leaf primordia. Our functional analysis indicates that loss-of-function mutations in CLE16, CLE17 or CLE27 have no significant effect on vegetative leaf initiation rate or rosette diameter, on inflorescence meristem morphology, phyllotaxy or size, or on floral organ number. CLE27 does, however, seem to play a small role in prolonging the vegetative growth rate, as cle27 plants flower on average one day earlier than wild-type plants (Fig 4). Thus unlike CLV3, the CLE16, CLE17 and CLE27 genes are largely dispensable for shoot apical meristem maintenance on their own. Consistent with this result,
Fig 7. Mutations in CLE16, CLE17 or CLE27 do not affect floral organ number. (A) Floral organ number in wild-type and cle mutant plants. Values shown are mean ± standard error (S.E.), n = 80 flowers per genotype. (B) Col-0 buds and open flowers. (C) cle16-2 buds and open flowers. (D) cle16-3 buds and open flowers. (E) cle17-2 buds and open flowers. (F) cle17-3 buds and open flowers. (G) cle27-cr1 buds and open flowers. (H) cle27-2 buds and open flowers. Scale bar, 0.5 cm.

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none of the three genes activates the CLV3 signaling pathway when over-expressed in the SAM [35].

Comparison of the three CLE peptides with CLV3 reveals differences at key residues. The CLE16 and CLE17 peptides are identical except at position 2 [41], and both differ from the CLV3 peptide at several residues, including the C-terminal histidine residue that has an essential role in CLV3 peptide function and binding to the receptor kinase CLV1 [42]. The CLE27 peptide is also divergent, differing from CLV3 at the 2\textsuperscript{nd} and 12\textsuperscript{th} positions and also containing a cysteine residue in place of the highly conserved glycine residue at position 6 that when mutated in CLV3 causes a moderate stem cell accumulation phenotype [6]. These observations suggest that the CLE16, CLE17 and CLE27 peptides are not perceived by the receptor kinase complexes that interact with CLV3, and instead may have functions within the SAM that are not related to maintaining stem cell homeostasis via the CLV-WUS pathway.

There may be several reasons why, unlike CLV3, the CLE16, CLE17 and CLE27 genes individually have no discernable developmental phenotypes under standard growth conditions. One possibility is that the three CLE genes have redundant functions in Arabidopsis meristems. The CLE gene family consists of 32 members in Arabidopsis [18, 43], only a few of which exhibit single mutant phenotypes [24, 35, 37, 44]. In addition, most Arabidopsis tissues express multiple CLE genes in overlapping patterns [35], and many CLE peptides act interchangeably when ectopically expressed in roots or shoots [20, 43, 45, 46]. These observations suggest that CLE gene functional redundancy may be widespread. Consistent with this notion, we have observed no meristem-related phenotypes among cle16 cle17, cle16 cle27, or cle17 cle27 double mutant plants. Therefore generating even higher order mutant combinations among SAM-expressed CLE genes may be required to uncover meristem-related phenotypes. An important corollary to our study is that targeting the orthologous CLE16, CLE17 or CLE27 genes one by one in agricultural plant species is unlikely to be sufficient to enhance yield, but that targeting the genes in combination may prove a more effective strategy for crop improvement to benefit agricultural productivity.

A second, and non-exclusive, explanation for the absence of phenotypes is that the three CLE genes regulate SAM activity only under specific environmental conditions. To date only a handful of studies describing the effect of different environmental states on Arabidopsis CLE gene activity have been published. In roots, induction of CLE14 expression under phosphorus limiting conditions causes terminal differentiation of the root apical meristem [47], while a CLE-CLV1 signaling module is proposed to prevent the expansion of the lateral root system in nitrogen-poor environments [48]. Above ground, CLE45 has been shown to play a role in prolonging pollen tube growth only at high temperatures [49]. With the recent availability of null alleles for all Arabidopsis CLE genes generated using genome-editing technology [38], we are rapidly developing the tools needed to determine the significance of these small but important signaling molecules for Arabidopsis biology.

Supporting information

S1 Fig. Mean CLE gene expression levels in inflorescence meristems (IM).
(TIF)

S2 Fig. Mutations in CLE16 have no significant effect on the floral transition.
(TIF)

S1 Table. Primer sequences used in the study.
(DOCX)
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