Expression analysis of the *CLE* signaling gene family in *Arabidopsis thaliana* and functional characterization of *CLE8* in seed development

by

Elisa Fiume

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Committee in charge:

Professor Jennifer C. Fletcher, Chair

Professor Robert L. Fischer

Professor Abby F. Dernburg

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ABSTRACT

Expression analysis of the *CLE* signaling gene family in *Arabidopsis thaliana* and functional

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In plants, as in all multicellular organisms, cell-to-cell communication is fundamental for coordinating growth and differentiation. Although non-peptide hormones have been long known to act as signaling molecules in plants, the sequencing of the *Arabidopsis* genome revealed the presence of many potential ligand-encoding genes and receptors suggesting that plants widely use them in different signaling pathways. In the past decade, signaling mediated by peptides in plants has become an emerging area of research and has been found to be utilized in a broad range of developmental processes. However, very few receptors and ligands have been functionally characterized. The *Arabidopsis CLAVATA3/EMBRYO SURROUNDING REGION* (*ESR*)-related (*CLE*) small polypeptide family is currently the best studied, nonetheless only few members have been assigned a function. To unveil possible developmental processes regulated by these putative signaling molecules, I undertook a systematic expression analysis of its members. These studies showed that all *Arabidopsis* tissues express one or more *CLE* gene, suggesting that CLE-mediated signaling regulates a wide range of developmental processes. Furthermore, no two *CLE* genes showed the same expression pattern, indicating that specificity of function is in part achieved at the regulatory level. On the other hand, many *CLE* genes have overlapping expression patterns, suggesting that there could be a a high degree of redundancy among the family members.

The putative functional redundancy of the *CLE* genes has been used to explain the lack of *cle* single mutant phenotypes, however, many other factors could also contribute. For example, *CLE* genes encode small proteins and generally lack introns making it difficult to obtain knock-out mutants using T-DNA insertional mutagenesis. Alternatively, the *CLE* genes might be regulating processes that when impaired, produce phenotypes that are overlooked in conventional screens because they are subtle or

conditional to environmental stimuli. In recent years, it has also been discovered that the *Arabidopsis* genome contains hundreds of essential genes, and because null mutations in these genes cause seed lethality, their functions are largely unknown. A number of signaling molecules has been implicated in regulating seed development, but no putative peptide ligand has been involved thus far.

In my research, I have isolated and studied a hypomorphic mutation in *CLE8*, the only member of the *CLE* family whose expression is limited to the young embryo and the endopserm. My phenotypic characterization of the mutant, together with molecular studies, have led me to discover that *CLE8* is necessary for proper seed development, indicating that most likely it is an essential gene. I demonstrate that *CLE8* is involved in patterning of the embryo basal domain, in elongation and patterning of suspensor cells, in endosperm maturation and thus ultimately regulates seed size. I found *CLE8* to be expressed in the embryo-proper and the endosperm and to have a non-cell-autonomous effect on suspensor cells. I showed that *CLE8* positively regulates the expression of the putative transcription factor *WOX8* both in the suspensor cells and in the endosperm, and that it likely evolved to specifically fulfill its role during seed development and diverged from other members of the family to solely interact with its receptor/s.

Furthermore, I have analyzed a partial loss-of-function allele of another essential gene: *EMB1611*. *EMB1611* encodes a large, novel protein with an N-terminal coiled-coil domain and two trans-membrane domains, but whose molecular function still remains elusive. From expression, phenotypic and molecular analysis I discovered that *EMB1611* functions to maintain cells in growing tissues in a proliferative or uncommitted state through maintaining the cellular organization of the shoot apical meristem and directly promoting stem cell fate. *EMB1611* null alleles are lethal at early stages of embryogenesis; thus, as for *CLE8*, the availability of a hypomorphic allele has been essential for studying the role of *EMB1611* in plant development.

CHAPTER 1

The CLE family of plant polypeptide signaling molecules

ABSTRACT

Polypeptide ligands have long been recognized as primary signaling molecules in diverse physiological processes in animal systems. Recent studies in plants have provided major breakthroughs with the discovery that small polypeptides are also involved in many plant biological processes, indicating that the use of polypeptides as signaling molecules in cell-to-cell communication is evolutionarily conserved. The CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION (ESR)-related (CLE) proteins are currently the best understood family of small polypeptides in plants. The recent isolation of MCLV3 from *Arabidopsis* and TDIF from a *Zinnia* cell culture system indicates that biologically active CLE polypeptides are produced by post-translational proteolysis and modification, similar to peptide hormone production in animals and yeast. Here, we review exciting discoveries involving the identification of the CLE proteins and their functions in various aspects of plant development, including restriction of stem cell accumulation by CLV3 and inhibition of xylem differentiation by TDIF.

INTRODUCTION

Cells in multicellular organisms must communicate with one another in order to regulate their growth and division and to coordinate their functions. Since the discovery of insulin in 1922 (Bliss, 1982) established the fact that polypeptides can be used as signaling molecules in a diverse range of physiological processes, extracellular peptide ligands have been found to be key mediators of cell-to-cell communication in animal systems (Brivanlou et al., 2003; van der Geer et al., 1994). For example, the transforming growth factor-beta (TGF-β) and epidermal growth factor (EGF) superfamilies consist of diverse groups of polypeptide growth factors that regulate cell duplication, migration, differentiation and survival (Feng et al., 2005; Harrisa et al., 2003). One feature of these polypeptide signals is that they are produced from larger pre-pro-proteins that undergo proteolytic cleavage of the signal peptide and the pro-domain before displaying biological activity (Todorovica et al., 2005). The active ligands are then perceived at the cell surface, most commonly by transmembrane receptor protein-tyrosine kinases (PTKs). Although each growth factor induces a distinct downstream signaling pathway, the common theme of receptor kinase signaling is that binding of the ligand to the receptor converts the latter into an active state, initiating a cascade of intracellular protein phosphorylation that transduces the signal inside the cell. The signal is propagated through intracellular mediators such as MAP kinases, tyrosine phosphatases and Ras proteins, ultimately targeting one or more nuclear transcription factors and leading to an output that alters the gene expression program of the cell (Brivanlou et al., 2002; Downward, 2001). In contrast to these well-understood animal polypeptide signaling systems, our historical understanding of intercellular signaling in plants has largely stemmed from the characterization of relatively simple lypophilic compounds called phytohormones, which include auxin and cytokinin (Gaspar et al., 2003). Plant hormones play a crucial role in controlling the way in which plants grow and develop and mediate their physiological responses to environmental stimuli. Thus, for many years, phytohormones have been considered to be the major plant signaling molecules. However, since 1991 (Pearce et al., 1991) when systemin was discovered as the first functional plant polypeptide, many secretory and non-secretory polypeptides in plants have been recognized as being involved in many biological processes. Systemin, an 18-amino-acid polypeptide derived from a larger precursor called prosystemin, was isolated by biochemical purification based on its proteinase inhibitor-inducing activity in tomato and plays a role in wounding responses (McGurl et al., 1992). A number of other families of plant polypeptides have subsequently been identified (Matsubayashi et al., 2006), including several with members that play roles in growth and development. Phytosulfokine (PSK) is a 5-amino- acid peptide with a sulfated tyrosine residue that regulates cellular dedifferentiation and redifferentiation in cooperation with auxin and cytokinin (Matsubayashi et al., 1996). POLARIS (PLS) appears to be required to maintain responsiveness to exogenous auxin and cytokinin during *Arabidopsis* root and vein development (Casson et al., 2002). INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) is involved in *Arabidopsis* floral abscission (Butenko et al., 2003), whereas the related ROTUNDIFOLIA4 (ROT4) and DEVIL1 (DVL1) polypeptides appear to control polar cell proliferation (Narita et al., 2004). Both IDA and ROT4/DVL1 are members of multi-gene families in *Arabidopsis* (Butenko et al., 2003; Narita et al., 2004).

The *CLE* family of plant-specific genes, named after its founding members *CLAVATA3* (*CLV3*) from *Arabidopsis* (Fletcher et al., 1999) and *EMBRYO SURROUNDING REGION* (*ESR*) from maize (Opshal-Ferstad et al., 1997), encode one of the largest families of plant polypeptides identified to date (Cock et al., 2001; Strabala et al., 2006). The *Arabidopsis* genome contains 32 *CLE* genes, and *CLE* family members are present in many other plant species including rice, alfalfa and tomato (Cock et al., 2001; Chu et al., 2006). The *CLV3* gene was isolated by genetic screening in *Arabidopsis* (Clark et al., 1995) and plays a key role in regulating plant growth and development (Fletcher et al., 1999; Clark et al., 1995). Although no functional information from mutant analysis is available for the vast majority of *CLE* genes, several CLE proteins act in different aspects of plant development. This review provides an overview of the CLE polypeptides, their activities and signal transduction systems, including potential receptors and downstream target genes.

Molecular characteristics of *CLE* gene family members

Members of the CLE family of signaling molecules share several common molecular characteristics (Cock et al., 2001) (Fig. 1). First, they encode small polypeptides of less than 15 kDa in molecular mass. Second, they contain a short stretch of hydrophobic amino acids at their amino termini that can target the polypeptide to the secretory pathway. Third, although at the DNA and protein level their sequences are largely unrelated, they share a conserved stretch of 14 amino acids, known as the CLE domain (Table 1), which is located close to or at the carboxyl terminus. Finally, despite their poor overall sequence conservation, all members of the family share certain structural parameters such as charge, hydrophilicity and length. The 32 *Arabidopsis CLE* genes are scattered across the genome, with loci on each of the five chromosomes. Their fairly large number is most likely the result of whole-genome duplications and reshufflings,which are known to have happened at least three times in *Arabidopsis* evolution (Bowers et al., 2003), as well as localized gene duplications followed by gene retention. The hypothesis that *CLE* gene number has risen at least in part thanks to localized gene duplication is supported by he presence of a tight cluster of highly similar *CLE* genes (*CLE4/5/6/7*) on chromosome 2 and by a looser clustering of *CLE* genes among repetitive sequences at the bottom of chromosome 1. In particular the presence of the *CLE4/5/6/7* cluster, which is a clear example of tandem repeats, is a sign of relatively recent gene duplication and suggests that these *CLE* genes may still be functionally redundant.

Phylogenetic analysis of the CLE proteins is challenging because of their low overall conservation and short length. As a result, trees are not very well supported, as illustrated by a phylogeny of the known plant CLE proteins in Figure 2. Nonetheless, some branches are supported by high bootstrap values and unveil putative couples of *Arabidopsis* paralogs, including CLE3 and CLE4, CLE5 and CLE6, CLE9 and CLE10, and CLE16 and CLE17. The phylogenetic analysis also suggests that CLE1 through CLE7 constitute a group of related proteins, as do CLE41, CLE42, CLE44 and CLE46.

CLE gene expression analysis provides valuable information for understanding the evolutionary forces shaping the family and the functions of the individual members. RT-PCR experiments performed with most of the *Arabidopsis CLE* genes (Sharma et al., 2003) reveal that many family members have a broad range of expression, whereas a few show more restricted distribution. All *Arabidopsis* tissues tested to date express multiple *CLE* genes and the overlap among different *CLE* gene expression patterns is conspicuous: most tissues analyzed express many members at the same time. Pollen is the tissue that expresses the lowest number of *CLE* genes, with only six represented. However, RT- PCR data only provide a rough picture of the expression domains. High-resolution spatial expression patterns of *CLE* mRNAs in tissues and group of cells obtained by RNA *in situ* hybridization and/or promoter-reporter fusions will give a much more precise idea of the extent of expression overlap and possible functional redundancy.

Taking into consideration the limits of RT-PCR data, it is nonetheless interesting to note that close relationships among *CLE* genes at the evolutionary level are not always coupled with shared expression patterns. For example, although *CLE3* and *CLE4* seem to be clear paralogs on the basis of phylogenetic analysis (Fig. 2), their expression patterns do not completely overlap, suggesting that sub-functionalization may have occurred mainly through cis-acting mutations in their regulatory sequences. Conversely, the putative paralogs *CLE5* and *CLE6* share the same expression patterns and are likely functionally redundant.

Biological functions of the *CLE* genes

CLV3

CLV3 plays a critical role in the maintenance of stem cell reservoirs in *Arabidopsis* shoot apical meristems (SAMs). Whereas most animal organs are formed during embryogenesis, higher plants develop post- embryonically from shoot and root apical meristems at their growing tips. These meristems contain niches that promote stem cell survival and the production of descendants destined for differentiation into lateral organs such as leaves and flowers. In order to maintain a functional SAM throughout development, coordination between the loss of stem cells via differentiation and their replacement through cell division must be precise (Clark, 2001). Insight into the regulation of normal SAM function has come from genetic studies in *Arabidopsis*. Loss-of-function mutations in *CLV3* cause excess stem cell accumulation in shoot apical and floral meristems (Clark et al., 1995). Since the SAM of a mature *clv3* embryo is already enlarged compared to that of a wild-type embryo, *CLV3* function is required from an early developmental stage. The SAM size increase becomes more dramatic with time and results in the formation of a strap-like, fasciated stem and the production of extra flowers and floral organs, indicating that *CLV3* acts to restrict stem cell proliferation throughout development (Clark et al., 1995).

The *CLV3* gene encodes a small 96-amino-acid polypeptide with a predicted 18-amino-acid secretion signal at the amino terminus (Fletcher et al., 1999). *CLV3* mRNA is expressed in the surface cell layers of the central zone (CZ) (Fletcher et al., 1999), which lie at the apex of the SAM and act as a reservoir of stem cells that replenish the peripheral zone (PZ) progenitor cells that populate lateral organ primordia. Genetic and immunological studies showed that CLV3 protein is transported through the secretory pathway and that secretion into the extracellular space is required for its normal function (Rojo et al., 2002). Recently, the endogenous mature CLV3 peptide (MCLV3) was identified by in situ matrix- assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses (Kondo et al., 2006). MCLV3 consists of a 12-amino-acid sequence (RTVPhSGPhDPLHH) generated from near the C- terminal end of CLV3, indicating that the biologically active CLV3 polypeptide is produced by post-translational proteolysis, as are peptide hormones in animals and yeast (Rehemtulla et al., 1992).

clv1 and *clv2* null mutants have similar but weaker phenotypes than *clv3* null mutants (Clark et al., 1993; Kayes et al., 1998), and epistasis analysis strongly indicates that the three *CLV* genes act in the same pathway to maintain SAM activity by restricting stem cell accumulation. *CLV1* encodes a receptor-like serine/threonine kinase that contains 21 leucine-rich repeats in the extracellular domain (Clark et al., 1997). *CLV2* encodes a receptor-like protein that is similar to CLV1 but lacks a kinase domain (Jeong et al., 1999), and data suggest that CLV2 forms a disulfide-linked heterodimer with CLV1 (Trotochaud et al., 1999). Although biochemical studies indicate that the CLV1, CLV2 and CLV3 proteins form an active receptor complex (Trotochaud et al., 1999), the model of direct ligand-receptor interaction remains to be confirmed. *CLV1* is expressed in a small group of cells just beneath the *CLV3* expression domain (Clark et al., 1997) and *CLV2* expression is also detected in shoots and flowers (Jeong et al., 1999), suggesting that CLV3 function might be mediated by activation of a CLV1/CLV2 receptor complex in neighboring cells. However, the demonstration that *clv1* null alleles confer weak phenotypes and strong *clv1* alleles have dominant negative activity suggests that other receptor-like kinases (RLKs) have overlapping functions with CLV1 to regulate shoot apical meristem function (Dievart et al., 2003). Several other intracellular pathway components have been identified that modulate CLV signaling (Fig. 3).

The type-2C kinase-associated protein phosphatase (KAPP) interacts directly with and dephosphorylates the CLV1 kinase domain, and acts as a negative regulator of CLV signal transduction (Stone et al., 1998; Williams et al., 1997). The protein phosphatase POLTERGEIST (POL) (Yu et al., 2003) was isolated from a genetic screen for modifiers of the *clv* meristem phenotype (Pogany et al., 1998). Mutations in either *POL* or a closely related gene *PLL1* provide partial, additive suppression of the *clv* stem cell accumulation defect (Yu et al., 2000). *POL/PLL1* over-expression inhibits differentiation and induces stem cell accumulation, especially in a *clv* background, suggesting that POL/PLL1 are intermediates downstream of the CLV1/CLV2 complex that indirectly promote *WUS* expression (Song et al., 2006). An additional component of the CLV complex is a Rho GTPase-related protein (Rop) that is related to the Ras GTPase superfamily in animals (Trotochaud et al., 1999). Ras GTPases are typically associated with cytosolic mitogen-activated protein kinase (MAPK) cascades (Downward et al., 2001), but whether such a kinase cascade lies downstream of the CLV1 receptor complex has not yet been determined.

The CLV pathway induces downstream signaling events that limit the size of the WUS expression domain. The *WUS* gene encodes a member of the WOX family of homeodomain transcription factors. *WUS* mRNA expression is confined to a group of cells in the deeper layers of the meristem, called the organizing center (OC), that specify the overlying cells as stem cells (Mayer et al., 1998). The *WUS* expression domain expands in clv shoot and floral meristems, indicating that *WUS* is negatively regulated by the CLV pathway (Fig. 3) (Brand et al., 2000). In fact, over-expression of *CLV3* results in complete repression of WUS expression throughout the meristem and conditions a *wus*-like phenotype of premature SAM termination. Using a *CLV3-GFP* fusion construct, Lenhard and Laux showed that CLV3 protein spreads laterally and downward from the stem cells to their neighbors. This non-cell- autonomous effect of CLV3 can be abolished by coexpression of *CLV1*, indicating that CLV1 sequesters the ligand and protects the *WUS*-expressing OC from the effects of CLV3 signaling (Lenhard et al., 2003). Conversely, *WUS* acts non-cell-autonomously to confer stem cell identity on the overlying cells and to promote *CLV3* expression by those cells (Schoof et al., 2000). Through this CLV-WUS negative feedback loop (Fig. 4), stem cell homeostasis is maintained in the shoot apical meristem.

Two live-imaging studies have revealed the dynamic activity of CLV3 in stem cell maintenance. One group used inducible *CLV3* over-expression to down-regu late *WUS* expression, which induced incorporation of the outermost cells of the CZ into organ primordia and resulted in a shift of the boundary between CZ and PZ identity (Muller et al., 2006). Conversely, when *WUS* expression was released from regulation by *CLV3* in *CLV3* interference (*CLV3i*) lines, expansion of the CZ due to the respecification of PZ cells to adopt a CZ fate was detected (Reddy et al., 2005). These results suggest that the balance between WUS and CLV3 is required both to regulate stem cell proliferation and to position the CZ/PZ boundary in the SAM. In sum, the CLV3 polypeptide is a key developmental mediator required to communicate cell fate information between the stem cells and their neighbors in the shoot apical meristem.

FON4/FON2

Recent studies of the rice *FON4/FON2* gene suggest that the genetic mechanism underlying the regulation of shoot apical meristem maintenance by the CLV3 signaling pathway is conserved in other plant species (Chu et al., 2006; Suzaki et al., 2006). The *fon4/fon2* mutation was isolated from rice based on its shoot apical meristem enlargement and increased floral organ number phenotypes. FON4/FON2 appears to be the structural and functional counterpart of CLV3 in rice. The two proteins have similar genomic structures and high sequence homology in the CLE domain, and *FON4/FON2* mRNA transcripts are detected in a small group of cells at the apex of shoot and floral meristems, similar to *CLV3*. In addition, *FON4/FON2* activity rescues the *clv3* mutant phenotype and over-expression of *FON4/FON2* in wild-type *Arabidopsis* plants conditions a *CLV3* over-expression phenotype (Table 1). Recently, the *FON1* gene encoding an LRR receptor-like kinase similar to CLV1 has been shown to function in rice floral meristem regulation (Suzaki et al., 2004).

Genetic interactions between *FON4/FON2* and *FON1* in rice as well as functional analysis of FON4/ FON2 activity in *fon1* plants clearly indicate that the rice FON4/FON2-FON1 pathway corresponds to the *Arabidopsis* CLV signaling system even though different effects of synthetic FON4p peptide and CLV3p peptide were observed in rice root apical meristems (Chu et al., 2006). The difference in defects caused by the *fon1* and *fon4/2* mutations suggests that FON1 is not the only receptor for FON4/FON2. OsLRK1, a close relative of FON1, may be a candidate for an additional receptor, because plants containing an antisense version of OsLRK1 display an increased floral organ number phenotype characteristic of *fon4/fon2* and *fon1* plants (Kim et al., 2000).

Because *Arabidopsis* and rice are distantly related plant species, the mechanism of shoot and floral meristem size regulation by the CLV signaling pathway may be functionally conserved in a wide range of flowering plants. Consistent with this notion, mutations in *thick tassel dwarf1* (*td1*) or *fasciated ear2* (*fea2*) cause enormous enlargement of the maize female reproductive (ear) meristem, and *TD1* and *FEA2* encode the putative maize orthologs of CLV1 and CLV2, respectively (Bommert et al., 2005; Taguchi-Shiobara et al., 2001). Yet although the maize mutants have highly enlarged reproductive shoot apical meristems, no reproductive SAM phenotype was observed in rice*fon1* mutants. Furthermore, the *td1* and *fea2* genes do not function in a single pathway (Bommert et al., 2005), unlike the *Arabidopsis CLV1* and *CLV2* genes. Thus despite its fundamental importance to plant development, the molecular mechanism of shoot apical meristem maintenance is not completely identical between species.

CLE40

CLE40 together with *CLV3* are the only *Arabidopsis CLE* family members for which loss-of-function phenotypes have been reported (Hobe et al., 2003). *CLV3* and *CLE40* differ from the other family members in having an intron that lies just after the signal sequence, and in both genes the intron and the two exons are comparable in size. Despite the common organization at the DNA level, the parallel between *CLV3* and *CLE40* ends when other molecular and functional features are taken into consideration. RT-PCR and RNA *in situ* hybridization experiments detected weak and ubiquitous expression of *CLE40* mRNA in all tissues (Sharma et al., 2003; Hobe et al., 2003). A loss-of-function *cle40-En* allele was isolated that carries a transposable element insertion immediately upstream of the CLE domain (Hobe et al., 2003). No above-ground phenotypes were observed in *cle40-En* plants, indicating that *CLE40* does not play a significant role in the CLV3-mediated meristem regulatory pathway. However, *cle40-En* roots exhibited a slightly waving pattern, grew shorter and were strongly slanted to the left, indicative of defective gravity perception and/or response. This observation not only implies that *CLE40* function is important for proper root growth, but also suggests that the CLE40 signaling pathway could integrate with the signaling pathways of the phytohormones auxin and ethylene which are known to play a role in the response of roots to gravity.

TDIF/CLE41/CLE42/CLE44

Whereas CLV3 and its orthologs act to restrict stem cell accumulation, several other CLE family members have been shown to inhibit cell differentiation (Ito et al., 2006). This function was first identified from *Zinnia elegans* mesophyll cell culture through the isolation of an extracellular factor that inhibited vascular tracheary element differentiation and promoted cell division.

The isolated small proteinaceous factor was a CLE- related dodecapeptide with two hydroxyproline (Hyp) residues –HEVHypSGHypNPISN– that was designated tracheary element differentiation inhibitory factor (TDIF). A full-length cDNA for *Zinnia* TDIF was isolated and found to encode a 132-amino- acid protein containing a 12-amino-acid sequence corresponding to TDIF at the carboxyl terminus. Thus TDIF, like CLV3, appears to be produced through post-translational processing and modification. The CLE domains of the *Arabidopsis* CLE41 and CLE44 proteins are identical to that of TDIF, and the CLE domain of CLE42 differs in only one amino acid residue. When the synthetic 12-amino-acid polypeptides corresponding to various CLE proteins were exogenously applied to *Zinnia* cell culture, only those dodecapeptides corresponding to the predicted sequences from CLE41/CLE44 and CLE42 exhibit strong TDIF activity (Table 1). Unexpectedly, application of mature CLV3 polypeptide to this cell culture system has the opposite effect, promoting the differentiation of the tracheary element (TE) cells (Ito et al., 2006). These observations reveal that two CLE pathways are active during *Zinnia* vascular development, one activated by TDIF that inhibits TE differentiation and one that can be activated by ectopic CLV3 that promotes TE differentiation.

Other CLE functions

The loss-of-function genetic approach is an extremely powerful tool in the identification of gene function. However, no functional information is available for the vast majority of the *CLE* genes because of their small size and the functional redundancy that can be anticipated based on the sequence similarity and overlapping expression domains of many *CLE* genes (Sharma et al., 2003). For example, no phenotypes were detected in *Arabidopsis* plants carrying a *CLE19* T-DNA null insertion allele (Fiers et al., 2004), suggesting the possibility of a functionally redundant partner or partners among closely related *CLE* family members. Although the fact that many *CLE* genes do not appear to be expressed in the shoot apical meristem (Sharma et al., 2003) suggests that they do not function in SAM maintenance, many CLE proteins can activate the CLV3 signaling pathway when ectopically expressed in the SAM (Strabala et al., 2006; Hobe et al., 2003; Ni et al., 2006). To date, ectopic expression of at least 12 *Arabidopsis CLE* genes produce phenotypes similar to those of *CLV3* over-expressing plants (Table 1), and a similar number completely or partially rescue the *clv3* phenotype. The ability of *CLE1* to rescue the *clv3* phenotype was shown to be largely dependent on CLV1, whereas that of CLE22 was CLV1 independent (Ni et al., 2006). This suggests that the CLE22 polypeptide is not recognized by the CLV1 receptor kinase. CLE4 and CLE40 also rescue the *clv3* phenotype in a putatively CLV1-independent fashion (Strabala et al., 2006; Hobe et al., 2003). However, these studies used strong *clv1* alleles that act in a dominant-negative manner, making the data difficult to interpret because the dominant-negative phenotype seems to mask the activity of functionally redundant receptors that also regulate shoot *Arabidopsis* stem cell accumulation (Dievart et al., 2003).

Novel pathways that can be activated by *CLE* gene activity have been revealed through gain-of-function analysis. For example, in addition to shoot apical meristem arrest, plants ectopically expressing *CLV3* also exhibited a root apical meristem termination phenotype (Table 1). Similar phenotypes were observed in extensive studies of *CLE19* and *CLE40* transgenic plants (Hobe et al., 2003; Fiers et al., 2004; Casamitjana-Martinez et al., 2003). Ectopic expression of *CLV3*, *CLE19* or *CLE40* under the control of the 35S or the RCH1 root-specific promoter in the root apical meristem caused progressive loss of root meristem cells and induction of cellular differentiation, as indicated by the formation of root hair cells closer than normal to the root tip. Application of chemically synthesized 14-amino-acid polypeptides, CLV3p, CLE19p, and CLE40p, corresponding to the conserved CLE motif, was sufficient to induce the root apical meristem consumption phenotype, whereas mutant *CLE* polypeptides engineered with single amino acid changes or deletions were not functional (Fiers et al., 2005). In recent bioassays using synthetic CLE dodecapeptides, all of the *CLE* genes except for *CLE1* through *CLE7*, *CLE41*, *CLE42* and *CLE44* showed strong inhibition of root meristem maintenance, suggesting that the CLE motif is the functional cue in this assay (Table 1) (Ito et al., 2006). These data indicate that a CLE signaling pathway may control cell fate in the root (Fig. 4). This is also supported by the identification of the *WUS-RELATED HOMEOBOX* (*WOX*) gene *WOX5*, which is required for stem cell maintenance in the root quiescent center (QC) (Sakar et al., 2007). Given that *WOX5* is a homolog of *WUS* and that the QC has an analogous role in the root to the OC in the shoot, it has been reasonably proposed that conserved CLE- mediated signaling pathways may control stem cell activity in both the SAM and the root apical meristem (RAM). Interestingly, the CLE-induced root meristem termination phenotype appears to be dependent on CLV2 function (Fiers et al., 2005), implying that CLV2 is involved in the perception of CLE polypeptides in roots. Since CLV2 has a relatively broad expression pattern, it will be interesting to determine if CLV2 is involved in a wide range of CLE-mediated signaling pathways.

Other *CLE* over-expression phenotypes have recently been characterized (Strabala et al., 2006). Plants that over-express *CLE19*, *CLE21*, or *CLE25* display miniature rosettes and inflorescences, often accompanied by developmental timing delays, apparent anthocyanin overproduction, and dwarfism. Plants over-expressing either *CLE42* or *CLE44* develop a shrub-like phenotype with small and rounded leaves and reduced apical dominance. Like the diverse *CLE* gene expression patterns, the variety of over-expression phenotypes indicate that CLE-activated signal transduction pathways are likely to operate in a number of *Arabidopsis* tissues. There seems to be a correlation between over-expression phenotype and CLE domain amino-acid sequence, because many *CLE* genes in the same clade showed similar over-expression phenotypes (Strabala et al., 2006).
However, because the CLE domain is highly conserved, it remains to be determined if the variable domain outside the CLE domain contributes to the specificity of any of the CLE proteins for receptor recognition and biological function.

Post-translational modifications and processing

A great deal of experimental evidence suggests that the CLE motif confers protein activity. The first clue toward this conclusion was furnished by the identification of two independent *CLV3* alleles, *clv3-1* and *clv3-5*. Both alleles carried a point mutation in the highly conserved glycine75 codon in the CLE domain (Fig. 1) that confers an intermediate *clv3* phenotype (Fletcher et al., 1999), revealing the importance of this domain for protein function. Moreover, application of synthetic 14-amino-acid polypeptides corresponding to the CLE motif of CLV3, CLE19 and CLE40 to

Arabidopsis roots mimics the over-expression phenotype of these *CLE* genes (Fiers et al., 2005). CLV3 deletion analysis showed that only the signal peptide and the CLE motif are necessary for function (Fiers et al., 2006). Furthermore, Ni and Clark (Ni et al., 2006) designed CLE domain swap experiments in order to assess whether CLE proteins that are not normally able to replace CLV3 function could acquire this ability when their CLE domains were replaced by that of CLV3. They also constructed a chimeric protein in which the CLV3 signal peptide and non- conserved sequences were replaced with sequences from the unrelated ERECTA receptor kinase, leaving only the C-terminal domain from CLV3. In all cases the chimeric proteins that contained only the CLE domain from CLV3 were able to complement the *clv3* phenotype (Ni et al., 2006). The outcome of these experiments strongly suggests that the CLE domain is the only functional region of the CLV3 protein.

Studies of two natural CLE peptides, TDIF in *Zinnia* and CLV3 in *Arabidopsis* (Kondo et al., 2006; Ito et al., 2006), clearly show that processing of full-length CLE protein into a 12-amino- acid polypeptide is crucial for function. This CLE dodecapeptide motif contains a number of conserved amino-acids. Two or three proline residues are always present and a glycine residue at position 6 is highly conserved, while a valine residue at position 3 and two asparagine residues at positions 8 and 12 are less conserved. The glycine, valine and asparagine residues as well as a proline residue at position 9 seem to be crucial for *Zinnia* TDIF activity (Ito et al., 2006). Although the isolated CLE peptides were hydroxylated at two proline residues, this post-translational modification seems not to be necessary for activity (Kondo et al., 2006; Ito et al., 2006). Although the mechanism through which the CLE proteins are processed has not yet been determined, a few hints have been gleaned from biochemical and genetic studies. First, a non-membrane-associated protease activity that can process both CLV3 and CLE1 has been detected in cauliflower extracts (Ni et al., 2006). Second, a suppressor of *CLE19* over-expression, *SOL1*, has been identified that encodes a putative Zn^{2+}-carboxypeptidase (Casamitjana-Martinez et al., 2003). Animal proteins homologous to SOL1 have been shown to cleave terminal R and K residues and play roles in prohormone and neuropeptide processing. These findings seem to suggest that some plant signaling molecules, like their animal analogs, are synthesized as inactive protein precursors from which the active molecules are liberated by proteolysis.

CLE genes in other organisms

CLE genes have been identified in the genomic and EST databases (Cock et al., 2001) of many different plant species, including *Brassica*, maize, tomato, soybean, *Medicago,* rice, cotton and wheat. There has not been an effort to identify all the *CLE* family members within plant species other than *Arabidopsis*, although recently 13 *CLE* family members were uncovered in the rice genome (Chu et al., 2006). Three *CLE* genes have been identified thus far in maize - *Esr1*, *Esr2* and *Esr3* - which are 80% to 90% homologous to one another at the nucleotide level (Bonello et al., 2002). They also share very similar expression patterns, being confined to the portion of the endosperm that surrounds the embryo between 4 and 28 days after pollination (Bonello et al., 2000). Although the roles of these proteins are not known, it is postulated that they are involved in signaling between the endosperm and embryo during early maize development. Six *CLE* genes have been recently identified in the lycophyte *Selaginella moellendorffii* and one in the moss *Physcomitrella patens* (Floyd et al., 2007), suggesting that the family originated soon after the evolution of land plants.

Although the *CLE* gene family is considered plant specific, a CLE-like protein called HG-SYV46 has been discovered in the soybean cyst nematode *Heterodera glycines* (Olsen et al., 2003; Wang et al., 2005). HG-SYV46 is believed to be an example of convergent evolution, and more specifically of adaptive molecular mimicry (Olsen et al., 2003). It is thought to be secreted by the parasitic nematode after its penetration in the soybean roots, in order to imitate the function of an endogenous CLE peptide and induce root cell proliferation and the consequent formation of syncytial feeding sites.

Perspectives and Frontiers

Many advances in the past few years have shed light on aspects of *CLE* gene structure, expression, activity and function. Even based on the limited functional data available, it seems clear that *CLE* family members play roles in a variety of activities during plant growth and development. Interestingly, some CLE proteins appear to be functionally equivalent and capable of activating the same pathways when ectopically expressed, suggesting that the specificity of *CLE* functions is mainly achieved by differential expression patterns (sub-functionalization at the level of regulatory sequences) rather than at the biochemical level (neo-functionalization). Given our knowledge of *CLE* gene expression patterns and the functional roles of a few of them, it seems reasonable to expect that a large number of different plant biological processes are under the control of this gene family.

The CLE family and other families of small putative signaling molecules are unique to plants, whereas, conversely, well-known animal signaling molecules such as Wnt, DPP, TGF-β and EGF have no plant homologs. Nonetheless, the general molecular mechanisms of signal transduction within the cell via intermediates such as receptor kinases, protein phosphatases and Ras superfamily members are shared between the two kingdoms. Thus, plants and animals have evolved similar strategies for transducing inter-cellular peptide signals, but the signaling molecules themselves appear to have been derived independently. The identification of downstream CLE signaling components remains a fundamental unresolved issue because very few players have been identified to date. At the moment, only CLV3 can be placed in a signaling pathway where a number of components are known. One major issue to be addressed is the identification of receptors. It is widely speculated that the RLK family represents the major receptor system for intercellular signaling in plants, due to the fact that it contains more than 400 members in *Arabidopsis* (Shiu et al., 2001) and largely overshadows the other plant receptor kinase family, the histidine kinases (Bleecker et al., 2000). On this premise, and on the basis of the CLV paradigm, RLKs are the primary candidate receptors for the CLE proteins. Some putative ligand-receptor pairs may be guessed at by comparing their expression patterns, because it is known that the CLV3 polypeptide moves only a few cell layers (Lenhard et al., 2003), so candidate ligands and receptors are expected to be expressed in close proximity to each other. However, it is quite possible that many CLE receptors will have much broader expression domains than their ligands, and could possibly interact with different CLE proteins in different tissues. Future studies will undoubtedly focus on the genetic and biochemical identification of receptor-ligand pairs through suppressor screens, pull-down assays, coimmunoprecipitation and bimolecular fluorescence complementation.

Many intercellular signaling components may be shared by multiple CLE pathways, although their downstream targets are likely to be unique. It is already known that CLV2 has a functional role in both the shoot and roots, and that the protein phosphatase KAPP interacts with both CLV1 and a related RLK called HAESA (Stone et al., 1994). Furthermore, the ubiquitous expression of *POL* (Yu et al., 2003) raises the possibility that this protein phosphatase may be recruited into different CLE signal pathways. Other plant LRR-RLKs use MAPK pathways for intercellular signal transduction, and this may be the case for the CLE-mediated signaling pathways as well. Another CLV3 pathway component, WUS, has homologs that could act as downstream targets of other CLE signaling pathways, and *WOX* genes have been shown to function in flower development (Matsumoto et al., 2001), cell division maintenance (Wu et al., 2005), root stem cell signaling (Sakar et al., 2007) and embryo development (Haecker et al., 2004). Determining the extent of functional overlap between the *CLE* gene family and the *WOX* gene family represents an important opportunity for the future.

Another unresolved fundamental issue concerning *CLE* gene function is the degree of redundancy among family members. Except for *CLV3* and *CLE40* (Fletcher et al., 1999; Hobe et al., 2003), the failure to observe a distinguishable phenotype in other *CLE* loss-of-function mutants (Fiers et al., 2004) has been interpreted as a symptom of full genetic redundancy. However, it must be noted that few *CLE* null mutants have been identified, probably due to their small gene size, and future mutant identification could revert this notion. Another possible explanation for the lack of loss-of-function phenotypes is the failure to detect them in a laboratory setting because they are conditional to environmental stimuli. Furthermore, it should be taken into consideration that mutant screens often focus on obvious strong phenotypes and do not always cover the whole plant life cycle. In cases where redundancy proves relevant to the study of *CLE* gene function, a number of strategies can be pursued, such as the generation of higher-order mutant combinations and the construction of artificial miRNA and RNAi vectors targeting multiple *CLE* genes at the same time.

Finally, an as yet unexplored avenue of investigation is the extent to which the CLE polypeptide signaling pathways intersect and integrate with the phytohormone signaling pathways. Phytohormones such as auxin and cytokinin are known to act systemically, but also to produce a range of tissue- and stage-specific biological effects. The available evidence suggests that CLV3 and other CLE peptides may act over a much shorter range and could potentially refine the more global phytohormone signals into dynamic, coordinated decisions among neighboring groups of cells.

Fortunately, work on the *CLE* gene family is progressing rapidly, and further studies will undoubtedly yield new key insights into the mechanisms of cell-to- cell communication in plants.

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FIGURES

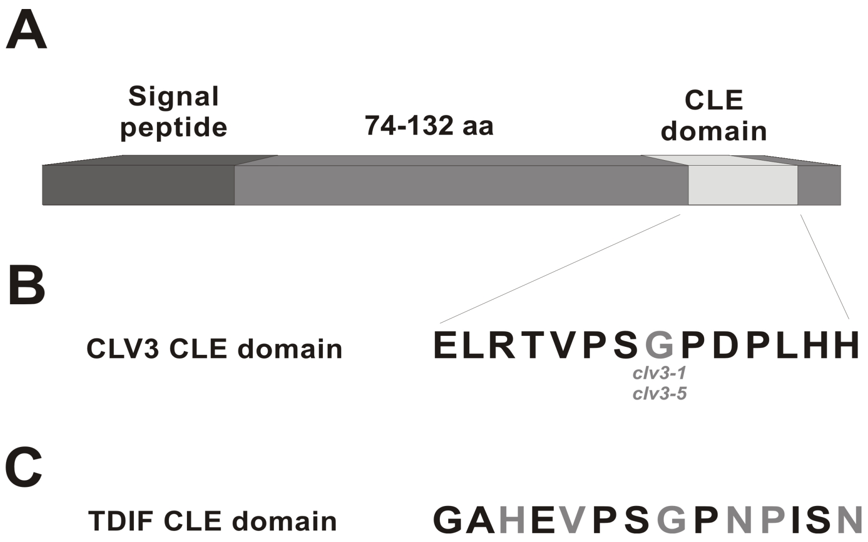

Figure 1. Molecular characteristics of the CLE proteins. (a) Schematic view of a generic CLE protein. (b) Amino acid sequence of the *Arabidopsis* CLV3 CLE domain. The natural product of the *CLV3* gene is a dodecapeptide corresponding to the central 12 amino acids of the CLE domain. Shown in light gray is the highly conserved glycine residue that is mutated in two clv3 alleles. (c) Amino acid sequence of the *Zinnia* TDIF CLE domain. The natural product of the *TDIF* gene is a dodecapeptide corresponding to the last 12 amino acids of the CLE domain. Shown in light gray are amino acids known to be crucial for TDIF peptide function.

Table 1. The CLE polypeptide family.

CLE	Sequence of dodecapeptide	AGI/ Accession No.	Size (aa)	Biological function			
				Loss-of-function	Gain-of-function		
					SAM	RAM	TE
CLV3	**RTVPSGPDPLHH**	At2g27250	96	shoot meristem	+	+	-
CLE1	**RLSPGGPDPRHH**	At1g73165	74	unknown	n.d.	-	-
CLE2	**RLSPGGPDPQHH**	At4g18510	75	unknown	+	-	-
CLE3	**RLSPGGPDPRHH**	At1g06225	83	unknown	+	-	-
CLE4	**RLSPGGPDPRHH**	At2g31081	80	unknown	+	-	-
CLE5	**RVSPGGPDPQHH**	At2g31083	81	unknown	+	-	-
CLE6	**RVSPGGPDPQHH**	At2g31085	81	unknown	+	-	-
CLE7	**RFSPGGPDPQHH**	At2g31082	86	unknown	+	-	-
CLE8	**RRVPTGPNPLHH**	At1g67775	86	unknown	n.d.	+	-
CLE9	**RLVPSGPNPLHN**	At1g26600	120	unknown	+	+	-
CLE10	**RLVPSGPNPLHN**	At1g69320	107	unknown	+	+	-
CLE11	**RVVPSGPNPLHH**	At1g49005	99	unknown	+	+	-
CLE12	**RRVPSGPNPLHH**	At1g68795	118	unknown	n.d.	+	-
CLE13	**RLVPSGPNPLHH**	At1g73965	107	unknown	+	+	-
CLE14	**RLVPKGPNPLHN**	At1g63245	80	unknown	n.d.	+	-
CLE16	**RLVHTGPNPLHN**	At2g01505	103	unknown	n.d.	+	-
CLE17	**RVVHTGPNPLHN**	At1g70895	99	unknown	n.d.	+	-
CLE18	**RQIPTGPDPLHN**	At1g66145	101	unknown	-	+	-
CLE19	**RVIPTGPNPLHN**	At3g24225	74	unknown	-	+	-
CLE20	**RKVKTGSNPLHN**	At1g05065	83	unknown	n.d.	+	-
CLE21	**RSIPTGPNPLHN**	At5g64800	106	unknown	-	+	-
CLE22	**RRVFTGPNPLHN**	At5g12235	103	unknown	n.d.	+	-
CLE25	**KVPNGPDPIHN**	At3g28455	81	unknown	-	+	-
CLE26	**RKVPRGPDPIHN**	At1g69970	118	unknown	-	+	-
CLE27	**RIVPSCPDPLHN**	At3g25905	91	unknown	n.d.	+	-
CLE40	**RQVPTGSDPLHH**	At5g12990	80	root	+	+	-
CLE41	**HEVPSGPNPISN**	At3g24770	99	unknown	n.d.	-	+
CLE42	**HGVPSGPNPISN**	At2g34925	88	unknown	-	-	+
CLE43	**RRIPSSPDRLHN**	At1g25425	96	unknown	n.d.	n.d.	n.d.
CLE44	**HEVPSGPNPISN**	At4g13195	112	unknown	-	-	+
CLE45	**RRVRRGSDPIHN**	At1g69588	124	unknown	n.d.	+	-
CLE46	**HKHPSGPNPTGN**	At5g59305	76	unknown	n.d.	-	-
OsFON4/2	**RSVPAGPDPMHH**	BAF41977	122	shoot meristem	+	-	n.d.
ZeTIDF	**HEVPSGPNPISN**	ABL67522	132	unknown	n.d.	n.d.	+

HgSYV46	RLSPSGPDPHHH	AF273728	139	unknown	+	+	n.d.

Table1. Alignment of the CLE dodecapeptides was performed using MUSCLE (Edgar, 2004). Each color represents a different amino acid residue. Gain-of- function phenotypes were observed in either *Arabidopsis* transgenic *CLE* over-expressing lines or plants treated with synthetic CLE peptides. SAM (shoot apical meristem) or RAM (root apical meristem) termination is denoted by +, whereas – indicates no meristem termination phenotype. The suppression of tracheary element (TE) differentiation is denoted by +, whereas – indicates a TE differentiation phenotype. n.d., not determined.

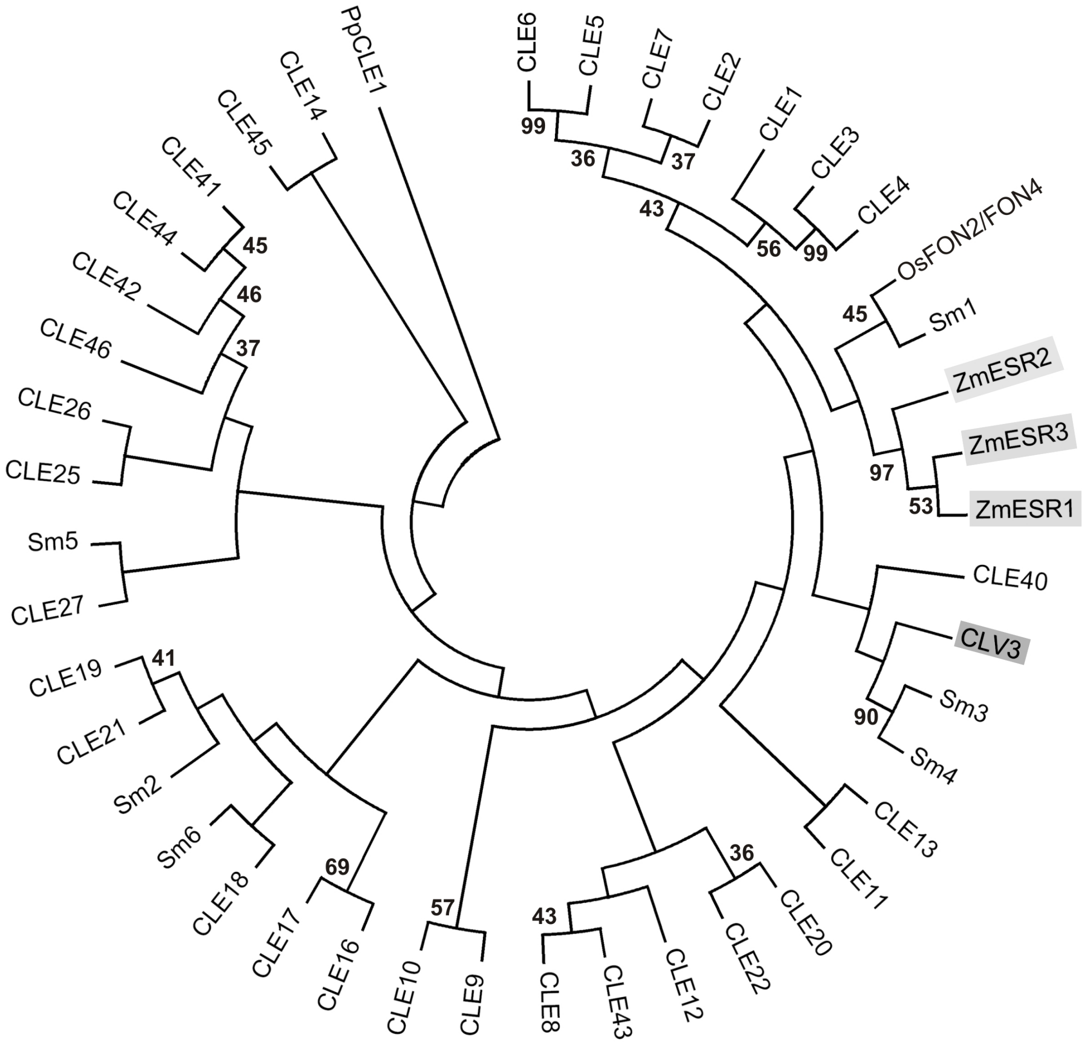

Figure 2. CLE protein phylogeny. The evolutionary history was inferred using the neighbor-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Multiple seuence alignment of CLE proteins was performed using MUSCLE (Edgar, 2004). Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) higher than 35 % are shown next to the branches. Phylogenetic analyses were conducted using MEGA4 software (Tamura et al., 2007).

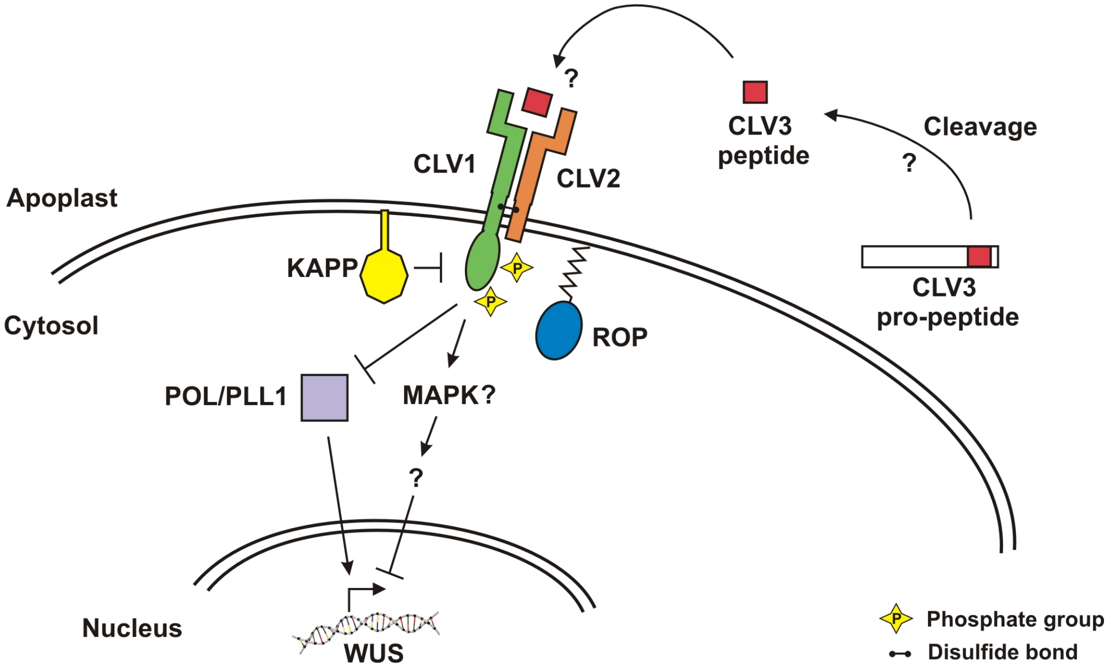

Figure 3. CLV3 signal transduction pathway. The CLV3 pro-peptide is secreted into the apoplast where it is likely processed to its mature form. CLV3 peptide acts as a ligand that is perceived by a CLV1 and CLV2 disulfide-linked heterodimeric receptor complex. In the presence of CLV3, the CLV1 kinase domain undergoes autophosphorylation and associates with the protein phosphatase KAPP, which can bind and dephosphorylate CLV1 and behaves as a negative regulator of the pathway. Another component of the active CLV complex is ROP, a Rho-like GTPase, the activation of which may initiate a MAPK signaling cascade to inhibit *WUS* at the transcriptional level. The largely redundant protein phosphatases POL and PLL1 act downstream in the pathway as putative positive regulators of *WUS* transcription that are attenuated by activity of the CLV complex.

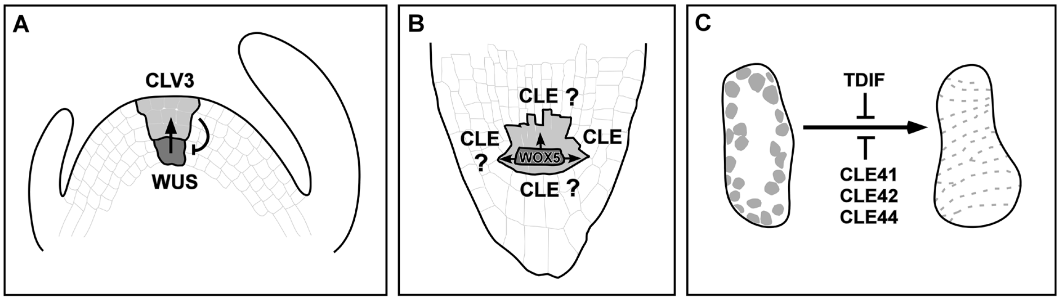

Figure 4. CLE functions in plants. (a) Schematic of the *Arabidopsis* shoot apical meristem. CLV3 mRNA is restricted to stem cells in the outer layers of the central zone, whereas WUS expression is detected in a few central cells called the OC (organizing center) in the deeper layer of the meristem. MCLV3 is released from the stem cells and moves inward to activate a CLV1/CLV2 receptor complex to limit WUS activity by restricting its expression domain. Non-autonomous WUS activity confers stem cell identity and positively regulates *CLV3* expression. This CLV-WUS negative feedback loop confers stem cell homeostasis on the shoot apical meristem. (b) Schematic of the *Arabidopsis* root apical meristem. *WOX5* expression is restricted to the QC cells and is required for maintenance of the surrounding root meristem cells. Expression of several *CLE* genes has been reported in the root, one or more of which may regulate the *WOX5* expression domain via a CLV3-like signal transduction pathway. (c) Schematic of trans-differentiation in a *Zinnia in vitro* model system. Mesophyll-cell-containing green chloroplasts (left) isolated from a *Zinnia* leaf trans-differentiates into a TE (tracheary element, right) in response to auxin and cytokinin in an *in vitro* cell culture system. *Zinnia* TDIF as well as the *Arabidopsis* CLE41, CLE42 and CLE44 dodecapeptides can specifically inhibit stage two of TE differentiation.

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CHAPTER 2

Comprehensive analysis of *CLE* polypeptide signaling gene expression and over-expression activity in *Arabidopsis*

ABSTRACT

Intercellular signaling is essential for the coordination of growth and development in higher plants. Although hundreds of putative receptors have been identified in *Arabidopsis thaliana*, only a few families of extracellular signaling molecules have been discovered and their biological roles are largely unknown. To expand our insight into the developmental processes potentially regulated by ligand-mediated signal transduction pathways, we undertook a systematic expression analysis of the members of the *Arabidopsis CLAVATA3/ESR-RELATED (CLE)* small signaling polypeptide family. Using reporter constructs we show that the *CLE* genes have distinct and specific patterns of promoter activity. We find that each *Arabidopsis* tissue expresses at least one *CLE* gene, indicating that CLE-mediated signaling pathways are likely to play roles in many biological processes during the plant life cycle. Some *CLE* genes that are closely related in sequence have dissimilar expression profiles, yet in many tissues multiple *CLE* genes have overlapping patterns of promoter-driven reporter activity. This observation, plus the general absence of detectable morphological phenotypes in *cle* null mutants, suggests a high degree of functional redundancy exists among *CLE* gene family members. Our work establishes a community resource of CLE-related biological materials and provides a platform for understanding and ultimately manipulating many different plant signaling systems.

INTRODUCTION

Plant growth and survival is critically dependent on the communication of information between cells. Intercellular signaling pathways convey cell fate information, regulate cell division and differentiation processes, propagate and amplify specific signaling states, and coordinate tissue responses and functions. The importance of cell-to-cell communication is underscored by the classification of 10% of the *Arabidopsis* proteome as playing roles in signal transduction (Initiative, 2000). Yet whereas more than 400 *Arabidopsis* genes encode receptor-like kinases (Shiu and Bleecker, 2001) that presumably function as transmembrane sensors for extracellular signals, only a few families of putative signaling molecules have been identified.

The *CLE* genes encode small polypeptides (<15 kD) that share several structural features. Each possesses either an amino terminal signal peptide or membrane anchor sequence, a large variable domain, and a highly conserved 14-amino-acid motif called the CLE domain near the carboxyl terminus (Cock and McCormick, 2001). Biochemical evidence indicates that the full-length CLAVATA3 (CLV3) polypeptide is proteolytically processed (Ni and Clark, 2006) to a mature active 12 or 13-amino-acid arabinosylated glycopeptide consisting of the CLE domain (Kondo et al., 2006; Ohyama et al., 2009). Synthetic peptides corresponding to the CLE motif of other CLE family members also show biological activity in various bioassays (Fiers et al., 2005; Ito et al., 2006), suggesting that such peptides are likely to be the functional *CLE* gene products.

The biological functions of only a few *CLE* genes are known. *CLV3* is a founding member of the family and plays a key role in the intercellular communication of stem cell fate during *Arabidopsis* development. *CLV3* is specifically expressed in the stem cell population of shoot and floral meristems (Fletcher et al., 1999). Secreted into the extracellular space (Rojo et al., 2002), the CLV3 polypeptide is perceived by CLAVATA1 (Clark et al., 1997; Ogawa et al., 2008) and other transmembrane receptors (Jeong et al., 1999; Muller et al., 2008) in the underlying cells. This signal transduction pathway is a core component of a negative feedback loop linking the stem cell reservoir and the underlying organizing center (Brand et al., 2000). Signaling through the CLV pathway restricts stem cell accumulation by limiting the expression domain of the *WOX* family transcription factor gene *WUSCHEL* (Laux et al., 1996), which promotes stem cell activity and *CLV3* expression (Schoof et al., 2000).

CLE gene activity also controls stem cell homeostasis in the root meristem. *CLE40* transcripts are present at low levels in all *Arabidopsis* tissues (Hobe et al., 2003), but in roots *CLE40* is specifically expressed in the stele and in differentiating columella cells (CCs) of the protective root cap (Stahl et al., 2009). CLE40 activity emanating from the CCs promotes distal root meristem differentiation by acting through the receptor kinase ARABIDOPSIS CRINKLY4 (ACR4) in a negative feedback loop that limits the expression domain of the *WUS*-related gene *WOX5* (Stahl et al., 2009). *WOX5* is present in the quiescent center (QC) and promotes columella stem cell fate in the distal domain of the root meristem. Thus CLE/WOX-mediated signaling modules regulate *Arabidopsis* stem cell fate in both the shoot and the root apical meristems.

Over-expression studies have uncovered other developmental processes that respond to CLE peptide activity. A number of *CLE* genes when mis-expressed in the root apical meristem gradually inhibit root meristem maintenance (Casamitjana-Martinez et al., 2003; Fiers et al., 2004; Fiers et al., 2005; Ito et al., 2006; Meng et al., 2010) via a signaling pathway that appears to be distinct from the CLE40 pathway. Plants over-expressing *CLE19*, *CLE21* or *CLE25* form miniature rosettes and inflorescences, and display anthocyanin overproduction and develop-mental delays (Strabala et al., 2006). Over-expression of *CLE42* or *CLE44* results in bushy, dwarfed plants with delayed development and reduced apical dominance, whereas *CLE18* or *CLE26* over-expression leads to enhanced root elongation (Strabala et al., 2006). Simultaneous over-expression of *CLE6* and *CLE41* produces stunted, bushy plants with increased hypocotyl vascular cell proliferation, suggesting that CLE peptides can function synergistically (Whitford et al., 2009). These phenotypes indicate roles for CLE family members in regulating many different aspects of development. Yet although many *Arabidopsis CLE* genes cause morpho-logical phenotypes when over-expressed, others still remain to be evaluated by this type of study.

Results from over-expression studies as well as *in vitro* bioassays revealed that multiple CLE peptides have the capacity to generate the same morphological phenotypes. More than a dozen CLE peptides can activate the CLV3 signaling pathway when ectopically expressed in the shoot meristem (Ni and Clark, 2006; Strabala et al., 2006; Jun et al., 2008; Meng et al., 2010). Application of 19 different synthetic CLE peptides in root growth assays can arrest root meristem growth (Ito et al., 2006; Strabala et al., 2006; Whitford et al., 2009), whereas the application of CLE41, CLE42, CLE43 or CLE44 peptides leads to suppressed xylem differentiation (Ito et al., 2006; Whitford et al., 2009). The CLE family has been divided into two functional classes on this basis, with A-type CLE peptides (CLV3, CLE1 through CLE27, and CLE40) being capable of inducing root and/or shoot meristematic cell differentiation whereas B-type CLE peptides (CLE41 through CLE44) are not (Whitford et al., 2009). These observations also raise the possibility that another key determinant of CLE peptide specificity, in addition to their primary amino acid sequence (Meng et al., 2010), may be their tissue distribution. Yet although RT-PCR studies show that *CLE* genes are transcribed in many different tissues (Sharma et al., 2003), their specific expression patterns remain to be characterized.

To obtain a more precise understanding of the extent of overlap between *CLE* genes and gauge their possible functional redundancy, we used reporter assays to obtain high resolution expression data for the entire *Arabidopsis* A-type *CLE* gene family. We observed highly specific *CLE* gene promoter activity patterns in roots, shoots, leaves, stems and flowers, with most tissues expressing multiple *CLE* promoters. We thus uncover a number of new biological processes that may be regulated by small peptide signal transduction pathways. In addition, we characterized the over-expression phenotypes of previously unstudied *CLE* genes, and identified hypomorphic or null insertion alleles for eight A-type *CLE* genes. Morphological examination of homozygous *cle* single mutant plants revealed no detectable growth or development phenotypes. Taken together, our expression and functional data indicate that *CLE* family members have diverse activities yet significant functional redundancy exists among them.

MATERIAL AND METHODS

Plant materials

Arabidopsis thaliana ecotype Columbia (Col) and Landsberg *erecta* (L*er*) plants were used in this study. Seeds were imbibed at 4°C for 3 days before sowing and were grown in a greenhouse under long days (16 h light and 8 h dark) with a day/night temperature cycle of 22°C/18°C. Seeds were surface sterilized for 10 min in 5% NaOCl and 0.1% Triton X-100, rinsed in distilled water, and plated on plates containing Murashige and Skoog medium with 0.8% type M agar (Life Technologies, Rockville, MD), 0.5 mM MES pH 5.7, 0.5% Sucrose, 13 B5 vitamins.

Construction of transgenic plants

To generate *CLE* promoter::GUS fusion constructs, the 5' upstream region (from 974 bp to 3398 bp) of each *CLE* gene was PCR-amplified from Col genomic DNA and cloned into a binary vector for transformation. For each construct, the binary vector in the *Agrobacterium tumefaciens* strain GV3101 or ASE was introduced into plants by the floral dip method (Clough and Bent, 1998). Primer sequences are listed in Table S3. Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Histochemical assays

GUS staining of transgenic plants was performed as described (Jefferson et al., 1987), with the modification that 0.5 mM potassium ferrocyanide and 2.5 mM potassium ferricyanide were used. Incubation times ranged from 2 to 16 hours following vacuum infiltration. Subsequent tissue embedding and sectioning were performed as described (Sieburth and Meyerowitz, 1997). For roots and some flower samples, whole-mount clearing was performed in Hoyer's medium (Liu and Meinke, 1998) and the samples were visualized using a Zeiss Axiophot microscope equipped with Nomarski optics. Whole seedlings, inflorescences and flower specimens were imaged using a Zeiss Stemi 2000-c and a Zeiss Stemi SV11 microscope. GFP fluorescence was visualized using a Leica DM LB fluorescence microscope or a Zeiss LSM 510 confocal microscope with 488 nm/530 nm excitation/emission light.

mRNA transcript analysis

cDNA was synthesized from 1~5 µg of total RNA using an oligo(dT_{18}) primer and SuperScript III reverse transcriptase (Invitrogen). For RT-PCR, one microliter of the first-strand cDNA reaction was used as a template. The annealing temperature for RT-PCR was 55-60^{o}C for all primer pairs and the number of PCR cycles was *EF1α*: 23 cycles; *TUB4*: 26 cycles; *CLE2*, *CLE16*, *CLE17*, *CLE27*: 30 cycles; *CLE1*, *CLE3*, *CLE4*, *CLE6*, *CLE7*, *CLE19*: 35 cycles; *CLE18:* 40 cycles. Primer sequences are listed in Table S3.

RESULTS

Analysis of A-type *CLE* promoter activity in vegetative tissues

Among the *Arabidopsis* A-type *CLE* genes, the expression patterns of *CLV3*, *CLE19* and *CLE40* have been already reported (Fletcher et al., 1999; Hobe et al., 2003; Fiers et al., 2004). To determine the expression patterns of the other A-type *CLE* genes, we generated *CLE* promoter:*GUS* or *GFP* fusion constructs using from 974 - 3398 base pairs of 5' genomic region upstream of each *CLE* coding sequence. At least 10 independent transgenic lines were analyzed for each gene promoter to monitor consistent reporter activity, with the exception of the *CLE11*, *CLE12* and *CLE13* promoters for which 4-8 independent transgenic lines were analyzed. Each *CLE* promoter drove GUS expression in one consistent pattern except for *CLE10.* We observed that all but one A-type *CLE* gene reporter is expressed in vegetative tissues (Table 1, Fig. 1-4).

Seedling expression patterns

We first analyzed *CLE* promoter activity in 11-day-old *Arabidopsis* seedlings (Fig. 1). From this analysis the *CLE* expression patterns could be divided into three groups: (1) those expressed in both shoot and root tissue (15 genes), (2) those expressed only in shoot tissue (2 genes), and (3) those expressed only in root tissue (5 genes) as listed in Table 1. *CLE8* was the only *CLE* gene for which expression was not detected at this stage of development.

Among the *CLE* genes with promoter activity in aerial tissues, *CLE12*, *CLE18*, *CLE22*, *CLE25* and *CLE26* all display GUS activity in the vascular tissues (Fig. 1A-E). Among these, *CLE12* staining is relatively weak in secondary and tertiary veins compared to primary veins, and is stronger in the root than in the shoot (Fig. 1A; Fig. 2C). *CLE18* and *CLE22* are expressed uniformly in vascular tissue throughout the rosette leaves, although *CLE18* promoter activity begins later during leaf growth than *CLE22* (Fig. 1B, C). The patterns of *CLE25* and *CLE26* promoter activity are complementary during leaf development. *CLE25* is strongly expressed in the vascular tissue of young leaf primordia, but is weaker in mature leaves (Fig. 1D; Fig. 2A). *CLE26* is initially detected in the leaf tip region where vein patterning initiates, and only later expands into the vasculature throughout the entire leaf (Fig. 1E; Fig. 2B).

The *CLE9*, *CLE16*, *CLE17* and *CLE27* promoters drive GUS activity in rosette leaf blade cells (Fig. 1F-I). *CLE9* is expressed specifically in the stomatal developmental lineage, including meristemoid cells, guard mother cells and young guard cells, throughout the aerial portions of the plant (Fig. 1F; Fig. 2I, J). *CLE16* promoter activity is detected throughout the blade pavement cells, whereas *CLE17* and *CLE27* promoter activity is broad in young leaves but becomes predominantly marginal as the leaves mature. *CLE16* and *CLE17* promoter activities are also detected in the trichomes (Fig. 1G, H; Fig. 2D, E), as is *CLE14* promoter activity (Fig. S1D). *CLE10* activity is restricted to the rosette leaf margins (Fig. 1J), particularly in the hydathode region (Fig. 2H). The *CLE5* and *CLE6* promoters also drive specific GUS staining in the hydathode region (Fig. 1K, L; Fig. 2F, G).

Other seedling tissues also express multiple *CLE* gene reporters. Hypocotyls display GUS activity driven by eight *CLE* promoters (Fig. 1A, C, E, G, H, I, M; Fig. 2L, M; Table 1). The shoot apex region exhibits GUS activity driven by the *CLE3-6*, *CLE10*, *CLE11*, *CLE16*, *CLE17*, *CLE21* and *CLE27* promoters (Fig. 1G-P). Among these, *CLE3* and *CLE11* show stipule-specific expression (Fig. 1N, O; Fig. 2K, L). *CLE4* is limited to the pith region (Fig. 1Q), whereas *CLE5*, *CLE6*, *CLE10*, and *CLE21* expression is restricted to the base of the rosette leaves and is excluded from the shoot apical meristem (Fig. 1J-M; Fig. 2M-P). *CLE16*, *CLE17* and *CLE27* are expressed in initiating leaf primordia (see below). GUS activity driven by the *CLE1*, *CLE2*, *CLE7* and *CLE13* promoters is specific to root tissue (Fig. 1Q-T).

Root expression patterns

The promoters of many *CLE* genes are active in the root system of 11-day-old seedlings. GUS activity from four *CLE* promoters is detected throughout the primary root cap (Fig. 3A-D). *CLE16* activity localizes to the root cap and the elongation zone but is absent from the meristematic division zone (Fig. 3E), whereas *CLE17* activity is absent from the root cap but localizes to the meristematic zone and the distal end of the elongation zone (Fig. 3F). *CLE22* activity is limited to a single file of newly differentiating vascular cells (Fig. 3G), whereas *CLE25* and *CLE26* activity is restricted to the vascular parenchyma (Fig. 3H, I). Vascular tissue in the elongation zone exhibits GUS activity from both the *CLE1* and *CLE18* promoters (Fig. 3A, D).

More mature root tissues express multiple *CLE* gene reporters in overlapping patterns. We examined two different areas of 11-day-old primary and lateral roots: a younger region with immature hair cells, as well as an older region with fully differentiated hair cells. We found that the stele in both areas of the root exhibit GUS activity driven by five different *CLE* promoters (Fig. 4A-H; Table 1). p*CLE20:CLE20-GFP* fusion protein activity is observed in the protoxylem and metaxylem (Fig. S1B), whereas *CLE25* and *CLE26* promoter activity is restricted to the metaxylem (Fig. S2A and data not shown). *CLE22* is strongly detected in the vascular parenchyma (Fig. S2B). Pericycle cells specifically display GUS activity from the *CLE4* and *CLE18* promoters (Fig. 4I-L; Fig. S2C), whereas both the pericycle and endodermis express *CLE7* (Fig. 4M, N; Fig. S2D). *CLE1* promoter activity is detected throughout the endodermis and the stele (Fig. 4O, P; Fig. S2E). In contrast, p*CLE14:GFP* activity is restricted to the root epidermis (Fig. S1F).

Several *CLE* promoters are active in a spatially and temporally restricted fashion in the primary root. In the less mature region of the root p*CLE16:GUS* and p*CLE17:GUS* activity are found exclusively in the epidermis (Fig. 4Q, S), whereas in the older region *CLE16* promoter activity localizes to the stele (Fig. 4R) and *CLE17* promoter activity expands throughout the root (Fig. 4T). *CLE3* promoter activity is patchy in the stele in the less mature root region (Fig. 4U), but becomes limited to the pericycle and endodermis in older tissue (Fig. 4V). Finally, *CLE5* promoter activity is not detected until the primary root is fully differentiated, when very weak activity is observed in the stele (Fig. 4W, X).

CLE promoter activity is highly dynamic during lateral root formation. *CLE27* promoter-driven GUS activity is detected in the cortex of the primary root at lateral root inception (Fig. S3A). As the lateral root cells begin to grow out, the *CLE27* reporter is expressed throughout the dome but most strongly in a ring around the base (Fig. S3B-C). As outgrowth continues, *CLE27* promoter activity is lost from the lateral root tip but remains detectable in the more basal region (Fig. S3D-E). In mature lateral roots, *CLE27* promoter activity is found at the base of the lateral root where it joins the primary root (Fig. S3F), as well as in the meristematic zone (Fig. S3G). *CLE2* and *CLE20* reporter expression is likewise detected at the base of initiating lateral roots (Fig. S3H, I; Fig. S1C, D), although at a slightly later stage than *CLE27*. As root outgrowth progresses, *CLE2* promoter activity becomes confined to the interior cells at the junction between the primary and lateral roots (Fig. S3J). Similarly *CLE11* promoter-driven GUS activity is observed in a small group of cells surrounding the initiating lateral roots (Fig. S3K) as well as at the root tip, and is sustained at the junction between the primary and lateral roots (Fig. S3L). *CLE5* and *CLE6* promoter activity is also seen specifically in the interior cells at the junction between the primary root and the mature lateral roots (Fig. S3M, N).

CLE promoter activity is also dynamic in the lateral root vasculature. *CLE22* is expressed earliest during lateral root formation (Fig. S3O-P), followed by *CLE25* (Fig. S3Q). p*CLE25:GUS* and p*CLE26:GUS* activity both localize to the vasculature of outgrowing lateral roots (Fig. S3Q-R), whereas *CLE4* (Fig. S3S) and *CLE12* (Fig. S3T) activity is restricted to the mature lateral root vasculature. *CLE7* promoter-driven activity is likewise limited to the mature lateral root vasculature, but unlike the others its expression is absent from the junction between the primary and lateral root (Fig. S3U). Thus the expression of a half dozen *CLE* reporters is induced at various stages during the development of the lateral root vasculature.

Lateral root tips initiate the expression of different *CLE* reporters at various stages during their formation. GUS activity driven by the promoters of both *CLE16* and *CLE17* is detected throughout the initiating lateral roots, before becoming confined to the tips and meristematic zones as the roots grow out (Fig. S4A-H). *CLE11* (Fig. S4I) and *CLE13* (Fig. S4K) activity initiates at the lateral root tip at a slightly later point during outgrowth, followed by *CLE18* promoter activity (Fig. S4M) and *CLE1* activity (Fig. S4O). The promoters of all six of these *CLE* genes are active in the root caps of mature lateral roots (Fig. S4D, H, J, L, N, P). Yet unlike the other five, which are localized in all root cap cells, *CLE1* promoter activity in lateral roots is restricted to the interior layers of the root cap. *CLE18* reporter signal is strong in the root cap and weaker in cells at the very distal end of the elongation zone. In addition, initiating vascular tissues display *CLE22* promoter activity (Fig. S4Q) and more mature vascular tissues display *CLE25* and *CLE26* promoter activity (Fig. S4R, S). Lateral roots therefore express different combinations of *CLE* gene reporters in overlapping patterns throughout their development.

Eight *CLE* genes are present on ATH1 microarray used to generate a spatiotemporal map of gene expression in the roots of 5-7 day old seedlings (Brady et al., 2007). Comparison of our results with this dataset using the eFP Browser (Cartwight et al., 2009) showed congruent profiles for *CLE3*, *CLE12* and *CLE17*. However, we did not detect activity of *CLE2* in the phloem, *CLE6* in phloem companion cells, *CLE21* in epidermal cells of the elongation region, or *CLE27* in primary root cortex and lateral root cap cells (Brady et al., 2007). Further, *CLE26* is reported in the metaphloem whereas we detect it in the metaxylem. These discrepancies likely reflect the increased sensitivity of transcriptional profiling compared to GUS reporter analysis, the use of insufficient *CLE* regulatory sequence for some reporter constructs, and/or the difference in developmental age between the samples evaluated.

Analysis of A-type *CLE* promoter activity in reproductive tissues

With a few exceptions, such as those for *CLE2* and *CLE8*, most A-type *CLE* gene reporters are expressed during reproductive growth (Table 2, Fig. 5-7). As in seedlings, each reproductive tissue expresses more than one *CLE* gene reporter. Conversely, most *CLE* gene reporters are expressed in more than one reproductive tissue, yet their individual expression patterns are highly specific and restricted.

Inflorescence expression patterns

The promoters of many *CLE* genes are active in inflorescence tissues. GUS activity from the *CLE16* promoter is found specifically in the epidermis (Fig. 5A). *CLE12, CLE13, CLE20, CLE22, CLE25* and *CLE26* reporters are expressed in the stem vasculature (Fig. 5B-F and data not shown), in each case more strongly in the primary inflorescence branching points than elsewhere in the stem. Activity from 7 *CLE* promoters is largely restricted to the primary branching points of the stem (Fig. 5G-M; Table 2), although *CLE17* promoter activity is also present in the stem trichomes. Consistent with its expression in vegetative tissues, signal from the *CLE9* reporter is detected in the stomata of stem epidermal cells (Fig. 5N). Pedicels display promoter-driven GUS activity from *CLE16* in the epidermis (Fig. 5P), from *CLE22* and *CLE26* in the vasculature (Fig. 5Q, R), and from *CLE27* throughout (Fig. 5S).

Eight *CLE* promoters are active in the cauline leaves. *CLE12* and *CLE22* promoter activity is strong in the proximal vasculature and at the very tip of the cauline leaf (Fig. 5B, D). The *CLE26* promoter drives patchy vascular expression in the blade (Fig. 5F). *CLE18*-driven expression is detected in the vasculature and at the leaf margins (Fig. 5O), whereas *CLE10* and *CLE17* promoter-driven GUS activity is limited to the leaf margins (Fig. 5J, K). *CLE9* expression is only detected in stomatal cells (Fig. 5N), and *CLE3* expression is restricted to stipules (data not shown). Promoter activity from all eight genes is also detected in rosette leaves.

Flower expression patterns

Floral tissues display highly complex *CLE* promoter activity patterns (Fig. 6). Sepals and petals express nine different *CLE* gene reporters. *CLE9* promoter activity is detected in the sepal stomatal cells, consistent with what is observed in vegetative tissues (Fig. 2J). The *CLE14* reporter is expressed in sepal trichomes (Fig. S1G). *CLE16* promoter activity is detected in sepal and petal vasculature throughout flower development, although stronger at the distal ends in young flowers, (Fig. 6A) and becomes confined to these regions in fully mature flowers (Fig. 6H). *CLE18* and *CLE26* reporters are expressed in sepal veins, more strongly in their distal portion (Fig. 6B, C). *CLE22* promoter activity is observed uniformly in the vasculature of both sepals and petals, although it becomes more pronounced in the sepal veins in fully developed flowers (Fig. 6D). *CLE17* promoter activity is detected along the margins of sepals and petals in young flower buds (Fig. 6E). *CLE5* and *CLE6* reporters are expressed at the base of each flower organ type, above the abscission zone (Fig. 6F, G).

The male reproductive structures express a variety of *CLE* gene reporters (Fig. 6).

Each stamen-expressed reporter is present either in the anther or in the filament but not in both. Five *CLE* reporters are expressed in the filament (Table 2). *CLE16* and *CLE18* are uniformly detected throughout the entire filament (Fig. 6H, I). In contrast, *CLE6* promoter activity is detected very weakly in the distal region of the filament (Fig. 6G), whereas *CLE4* and *CLE26* promoter activity is limited to the distal tip of the filament, where it connects to the anther (Fig. 6J, K). *CLE4* is the only one of these that is expressed exclusively in the filament and not in other floral tissues.

Anthers express six different *CLE* gene reporters (Table 2). *CLE1* promoter activity is found in both pollen grains and tapetum cells of anthers from their emergence in stage 6 floral buds until maturation (Fig. 6L). The *CLE25* reporter is expressed throughout the anthers in young developing flowers (Fig. 6M). In mature flowers *CLE25* expression become more restricted, and overlaps with that of *CLE7* in the central region of the anther sacs, along the boundaries with the connective tissue (Fig. 6N). *CLE11*, *CLE12* and *CLE13* promoter activity is evident solely in pollen grains. The *CLE12* reporter is expressed throughout all stages of pollen development (Fig. 6O). However, *CLE11* and *CLE13* reporters show a dynamic and complementary expression pattern: the *CLE11* reporter is expressed only in fully mature pollen grains (Fig. 6P, Q), whereas the *CLE13* reporter is expressed only in young developing anthers (Fig. 6R, S).

The female reproductive structure exhibits *CLE* promoter activity in a variety of complex patterns (Fig. 7). The *CLE10* reporter is the most broadly expressed, its activity being detected in the stigma, style, transmitting tract, septum, and ovules (Fig. 7A). The style also displays promoter activity from *CLE1, CLE5,* and *CLE11*. Among these, *CLE5* and *CLE1* reporters are expressed in the transmitting tract of the style (Fig. 7B, C), whereas *CLE11* reporter expression is observed in a central region that appears to correspond to the transmitting tract or vascular fans (Fig. 7D). Uniquely, *CLE17* promoter activity is detected in a ring at the margin between the stigmatic tissue and the style, in the transmitting tract, and in the septum (Fig 7E). *CLE21* promoter activity is observed in the valve margins (Fig. 7F), whereas *CLE16* promoter activity occurs in the valves (Fig. 7G). The vasculature of the gynoecium exhibits *CLE22*, *CLE25* and *CLE26* promoter activity (Fig. 7H, I, J). *CLE25* reporter expression is additionally detected in the septum, funiculi and at the proximal end of the ovules (Fig. 7K), whereas the *CLE27* reporter is expressed throughout the funiculi and ovules of fully mature flowers (Fig. 7L). Finally, the abscission zone exhibits promoter activity from *CLE10* (Fig. 5J), *CLE12* (Fig. 6O), *CLE13* (Fig. 6R, S), *CLE16* (Fig. 6H), *CLE21* (Fig. 7G), *CLE22* (Fig. 7H), *CLE26* (Fig. 7J), and *CLE27* (Fig. 7M). These promoter activity patterns are consistent throughout gynoecium and silique development, with two exceptions. First, *CLE17* reporter expression shifts from the stigma/style region in carpels to the valve margins in siliques (Fig. 7N). Second, *CLE4* reporter expression becomes detectable specifically in the silique receptacle (Fig. 7O). In sum, our observations indicate that different *CLE* promoters drive GUS activity in complex, overlapping spatial and temporal patterns, particularly in the reproductive tissues.

Detailed analysis of *CLE* promoter activity in shoot apices

Promoter activity from ten different *CLE* genes is detected around the shoot apex region of seedlings (Fig. 1, 2). We examined their expression patterns in greater detail by sectioning 10-day-old GUS-stained seedlings. We found that *CLE21* promoter activity is restricted to the stipules (Fig. S5A), whereas *CLE10* promoter activity is detected in stipules and in a proximal, adaxial domain of leaf primordia (Fig. S5B). *CLE4* and *CLE26* promoter activity is located in the pith and the ground cells of the hypocotyl, respectively (Fig. S5C, D).

In contrast, the *CLE16*, *CLE17,* and *CLE27* reporter constructs show consistent activity in or adjacent to the shoot apical meristem (SAM) in multiple independent transgenic lines. Compared to untransformed wild-type plants (Fig. $8A_1$) and p*CLV3:GUS* plants (Fig. $8B_1$), *CLE16* promoter activity is detected throughout initiating leaf primordia on the shoot apical meristem flanks (Fig. $8C_1$). As the leaf primordia develop, *CLE16* GUS activity is stronger in the proximal than the distal region, and in the L1 cells (Fig. $8C_1$; Fig. S6A, B). A similar pattern is observed in p*CLE17*:*GUS* leaf primordia (Fig. $8D_1$). However, *CLE17* promoter activity is also detected in the SAM (Fig. $8D_1$). Transgenic plants with weak *CLE17* promoter activity show GUS staining in the outer layers of the CZ and the PZ (Fig. S6C). Transgenic plants with strong *CLE17* promoter activity exhibit GUS staining throughout the SAM and initiating leaf primordia, most strongly in the PZ and the outer cell layers (Fig. S6D). *CLE27* promoter activity is exclusively detected in the epidermal layer of developing leaf primordia and young rosette leaves, and in the pith region beneath the rib meristem (Fig. $8E_1$; Fig. S6E, F). However, *CLE27* reporter expression is excluded from the SAM and the initiating leaf primordia on the meristem flanks. These results are consistent with *Arabidopsis* SAM transcription profiling data showing that *CLE27* mRNA is absent from the SAM, but that *CLE17* is expressed at low levels in the CZ and OC and at moderate levels in the PZ (Yadav et al., 2009). *CLE16* is not represented in the profiling dataset.

Because *CLE17* reporter expression in the SAM overlapped with the *CLV3* expression domain, we tested whether *CLE17* could activate the *CLV* signaling pathway by analyzing the phenotypes of p*35S:CLE17* transgenic plants. Ectopic expression of *CLV3* causes SAM arrest early during vegetative development (Fig. $8B_2$, B_3) as well as premature floral meristem termination (Brand et al., 2000). In contrast, ectopic expression of *CLE17* does not confer a SAM termination phenotype (Fig. $8D_2$, D_3), nor is floral organ formation affected. These data indicate that CLE17 cannot activate the CLV3 signaling pathway in either shoot or floral meristems. Instead, the rosette leaves of *CLE17* over-expressing plants show a delayed growth rate, and smaller and epinastic morphology compared to wild-type rosette leaves (Fig. $8D_2$, D_3). Developmental timing is also delayed and apical dominance appears to be reduced. In addition, *CLE17* over-expression (Fig. 8H) causes root apical meristem termination (Fig. $8D_4$, D_5), as has been observed in plants that have been treated with exogenous CLE peptides or over-express other *CLE* genes, such as *CLV3* (Fig. $8B_4$, B_5). Transgenic plants over-expressing either *CLE16* or *CLE27* (Fig. 8G, I) display phenotypes that closely resemble those of p*35S:CLE17* plants (Fig. $8C_{2-5}$, E_{2-5}).

Alignment of the CLE16, CLE17 and CLE27 peptides with that of CLV3 reveals altered amino acids at key residues in the CLE domain. Compared to CLV3, CLE16 and CLE17 both contain a histidine instead of a proline at position 4, and CLE27 contains a cysteine instead of the highly conserved glycine at position 6 (Fig. 8F). These observations suggest that the failure of these three proteins to activate the CLV3 signaling pathway when over-expressed in the SAM may be due to differences in the composition of their CLE motifs.

CLE over-expression phenotypes in shoots and roots

Despite extensive over-expression studies that uncovered other developmental processes responding to CLE peptide activity, the over-expression phenotypes a few *CLE* genes remain undetermined. To fill in this gap, we generated transgenic plants expressing the coding region of *CLE8*, *CLE12* or *CLE22* under the control of the CaMV 35S promoter, and scored them for shoot and root meristem arrest phenotypes (Table S1). We found that p*35S:CLE8* plants showed neither shoot nor root meristem defects (Fig. S7B), whereas both p*35S:CLE12* and p*35S:CLE22* plants displayed SAM termination (Fig. S7C, D). Reduced root growth and RAM arrest was also observed in *CLE12* and *CLE22* over-expressing plants (Fig. S7E, F). Our data are consistent with previous work except in the case of *CLE8*, which has been reported to trigger RAM consumption when over-expressed (Ito et al., 2006). Combined with earlier studies, our results indicate that 16 of 26 A-type *CLE* genes, including *CLV3*, can induce SAM termination when over-expressed, and that 18 (or 19) A-type *CLE* genes can induce RAM termination.

Mutational analysis of A-type *CLE* loci

To begin to determine the functions of additional A-type *CLE* genes, we obtained *cle* T-DNA insertion alleles from the publically available collections. We identified one allele each with an insertion within the *CLE1, CLE10, CLE16* and *CLE18* coding regions, as well as one allele each with an insertion in the *CLE3, CLE7, CLE13* and *CLE19* 5’ UTR, and one allele with an insertion in the *CLE17* 3’ UTR (Fig. 9). We performed RT-PCR to examine *CLE* mRNA transcript levels in plants homozygous for each of these *cle* alleles. No transcripts were detected in *cle1-1, cle7-1, cle16-1,* and *cle18-1* plants (Fig. 9A-D), indicating that they represent null alleles. Reduced transcript levels were detected in *cle3-1, cle10-1, cle13-1, cle17-1* and *cle19-1* plants (Fig. 9E-I), showing that these are hypomorphic alleles. For *cle3-1*, we observed a minor but reproducible decrease in *CLE3* mRNA levels (Fig. 9E). In addition, we identified T-DNA insertion alleles within 700 base pairs upstream or downstream of the *CLE2*, *CLE4*, *CLE6*, *CLE9*, *CLE17* and *CLE21* coding regions, but RT-PCR experiments detected no significant reduction in transcript levels (Table S2). Detailed inspection of seedling, inflorescence, flower and root development revealed no detectable morphological defects in plants homozygous for any of these *cle* alleles, suggesting that substantial functional redundancy occurs among A-type *CLE* family members.

DISCUSSION

Dozens of potential intercellular signaling molecules as well as hundreds of putative receptors have been cataloged in the *Arabidopsis* genome, yet relatively little is known about their individual expression patterns or functions. Several members of the A-type CLE family of small secreted polypeptides act to maintain cell fate in shoot and root apical meristems; however, the dearth of mutations in the other small *CLE* coding sequences has limited our insight into their biological activities. In addition, only a single study of A-type *CLE* mRNA transcription profiles exists (Sharma et al., 2003), and few *CLE* genes are represented on microarrays, another source of detailed expression data. To address these deficiencies we performed a comprehensive characterization of A-type *CLE* promoter expression during *Arabidopsis* vegetative and reproductive development, and identified null or hypomorphic alleles of seven *CLE* genes.

One important finding from our work is that most *Arabidopsis* tissues express one or more *CLE* gene reporters (Fig. 10). This includes highly specialized cell types such as stomata, trichomes and stipules. Primary roots display *CLE* promoter-driven expression in the root cap, the root apical meristem, and in each radial cell layer (Fig. 3, 4), and lateral root formation is associated with the dynamic activity of multiple *CLE* promoters (Fig. S3). The vasculature is characterized by the expression of 14 *CLE* reporters, half of which are specific to either the root or the shoot vasculature. Multiple *CLE* gene reporters also are expressed in different tissues of the inflorescence stem (Fig. 5), as well as in each floral organ. In particular, the reproductive organs express a variety of *CLE* gene reporters in specific spatial and temporal patterns (Fig. 6, 7). Seven different *CLE* reporters are expressed at the base of the flower, where the cognate genes may be involved in the signaling process(es) that controls floral organ abscission. These observations indicate that, beyond their functions in shoot and root apical meristems, CLE-mediated signal transduction pathways are likely to play roles in a wide variety of different biological processes.

Another intriguing finding is that the A-type *CLE* gene promoters drive highly distinct and specific patterns of expression. For example, the promoters of *CLE3, CLE5, CLE16* and *CLE17* are active in a spatially and temporally restricted fashion in the primary root (Fig. 4), whereas the *CLE1*, *CLE5*, *CLE11*, *CLE16*, *CLE17* and *CLE21* promoters are active in unique sub-domains of the developing gynoecium (Fig. 7). No two *CLE* promoters drive expression in identical patterns throughout the plant, and indeed we observe that even *CLE* genes with very similar sequences have divergent reporter expression patterns. This is exemplified by CLE3 and CLE4, which have an identical CLE motif and pair together in the published phylogenies (Ito et al., 2006; Strabala et al., 2006; Jun et al., 2008; Mitchum et al., 2008). We detect *CLE3* promoter activity in leaf stipules (Fig. 2K) and the pericycle and endodermis of mature roots (Fig. 4U, V), whereas *CLE4* promoter activity coincides with *CLE3* in the root pericycle (Fig. 4I, J) but is also found in the hypocotyl pith (Fig. S5C), inflorescence branch points (Fig. 5G), stamen filaments (Fig. 6J) and receptacle (Fig. 7O). Thus while these two genes may function interchangeably in the pericycle, they would appear to have unique activities in the other tissues. The importance of the location of gene expression in conferring functional specificity has also been shown with closely related members of the MADS domain transcription factor family (Pinyopich et al., 2003).

Although many CLE proteins act interchangeably when ectopically expressed in shoots or roots, indicating that tissue distribution important for their functional specificity, the location of gene expression is not the only determinant of CLE function. Studies have shown that the CLE motif itself determines much of the functional specificity of the proteins in different plant tissues (Fiers et al., 2005; Fiers et al., 2006; Ito et al., 2006; Kondo et al., 2006; Ni and Clark, 2006; Meng et al., 2010). We found that the *CLE17* promoter is active in the SAM in a domain that overlaps with *CLV3*, yet its over-expression fails to induce a *CLV3* over-expression SAM termination phenotype (Fig. 8). The CLE peptide of the SAM-expressed *CLE17* gene differs from that of CLV3 at several residues, including the C-terminal residue (^{12}His) that plays an essential role in CLV3 peptide function and binding to the CLV1 receptor kinase (Kondo et al., 2008). These data provide an additional piece of evidence that the CLE motif plays a critical role in determining CLE activity and receptor binding specificity *in planta*. Other factors contributing to CLE signaling specificity are likely to include the tissue distribution of their cognate receptors as well as of the enzymes that process the CLE proteins to the active arabinosylated peptides (Ohyama et al., 2009).

To date, assessing the biological roles of small signaling peptide gene families has proven to be a significant challenge, primarily due to a lack of hypomorphic or null alleles. These are not available for most CLE family members because the small size of the genes reduces the target size for T-DNA insertion, and because the mature molecule consists of only a short stretch of amino acids. We have identified null mutations in the *CLE1*, *CLE7*, *CLE16* and *CLE18* genes, but in each case the homozygous plants lack detectable morphological phenotypes, indicating that their function may be conditioned by environmental factors and/or masked by redundancy with other CLE peptides. In the future, alternative strategies will be required to specifically target *CLE* genes for down-regulation. Possibilities include artificial microRNAs (Schwab et al., 2006) and increased efficiency homologous recombination (Osakabe et al., 2010; Zhang et al., 2010).

Although each *CLE* promoter is active in a unique spatial and temporal pattern during *Arabidopsis* development, we observe that multiple *CLE* promoters are active in overlapping patterns within a given tissue. These overlapping *CLE* genes are most likely to have redundant functions and thus our complete gene family promoter expression analysis serves as a guide to identify potential redundant *CLE* peptides within specific tissues. However, it should be noted that overlapping expression patterns do not necessarily guarantee redundant activities and that *CLE* genes expressed in the same cell types could potentially send opposite signals, as has been observed for members of the EPF family of small peptide ligands during stomatal development (Abrash and Bergmann, 2010).

Finally, our elucidation of *CLE* promoter activity throughout *Arabidopsis* development provides a resource for predicting candidate receptors based on their overlapping or neighboring expression patterns. For instance, the *PXY* gene encodes an LRR receptor kinase that belongs to the same clade of LRR-RLK subclass XI proteins as CLV1 (Hirakawa et al., 2008). *PXY* is expressed in dividing vascular procambium cells

(Fisher and Turner, 2007) and interacts with the B-type CLE peptide CLE41 (Etchells and Turner, 2010). We find that 14 different *CLE* promoters drive GUS activity in the vasculature (Table 1). Thus the products of one or more of these *CLE* genes could act as ligands for PXY, and/or for VASCULAR HIGHWAY1 (VH1), another LRR-RLK expressed in procambial cells throughout the plant (Clay and Nelson, 2002). An LRR-RLK encoded by the *EMS1/EXS* gene is expressed in the sporogenous and parietal cells of the developing anther, where it controls microsporocyte differentiation and tapetal identity (Canales et al., 2002; Zhao et al., 2002). The *CLE1, CLE7, CLE12, CLE13* and *CLE25* promoters are all active during early anther formation, making these genes candidates to encode EMS1/EXS ligands. *EMS1/EXS* is also expressed in developing leaf primordia, inflorescence meristems and young flower buds (Canales et al., 2002). Yet among the *CLE* promoters active in young anthers, only those of *CLE12, CLE13* and *CLE25* are also active in leaf primordia and none is active in or adjacent to the inflorescence meristem. Thus broadly expressed receptors such as EMS1/EXS may potentially bind different CLE ligands deriving from different cell types. In conclusion, our systematic analysis of the *CLE* gene family illustrates the complex expression dynamics of these signaling molecules throughout the *Arabidopsis* life cycle and provides a foundation for identifying and characterizing many ligand-receptor mediated signaling pathways during plant development.

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FIGURES

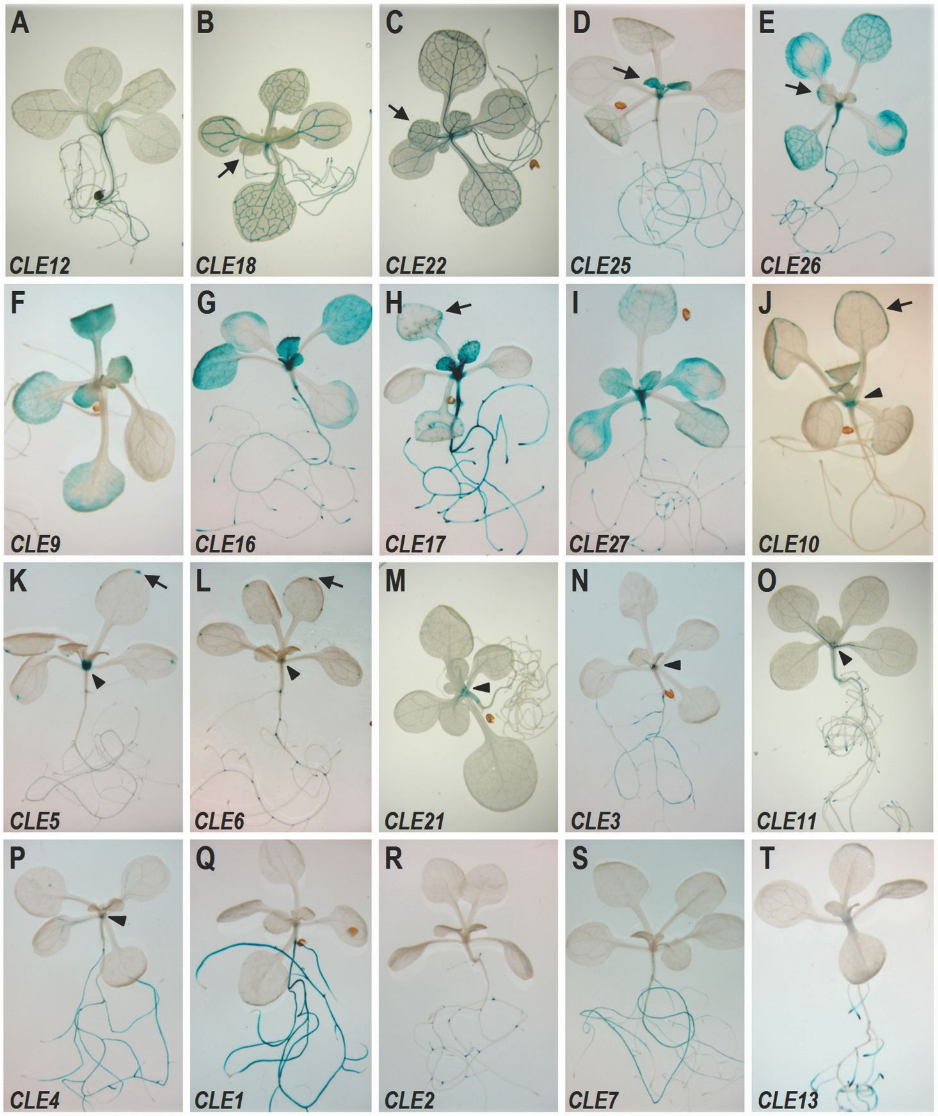


Figure 1. *CLE* promoter activity in 11-day-old-seedlings. (A) *CLE12*. (B) *CLE18*. (C) *CLE22*. (D) *CLE25*. (E) *CLE26*. (F) *CLE9*. (G) *CLE16*. (H) *CLE17*. (I) *CLE27*. (J) *CLE10*. (K) *CLE5*. (L) *CLE6*. (M) *CLE21*. (N) *CLE3*. (O) *CLE11*. (P) *CLE4*. (Q) *CLE1*. (R) *CLE2*. (S) *CLE7*. (T) *CLE13*. Arrows indicate GUS activity in the vasculature of young leaves in (A-D), leaf margins in (H, J) and hydathodes in (K, L). Arrowheads in (J-P) indicate GUS activity in the shoot apex.

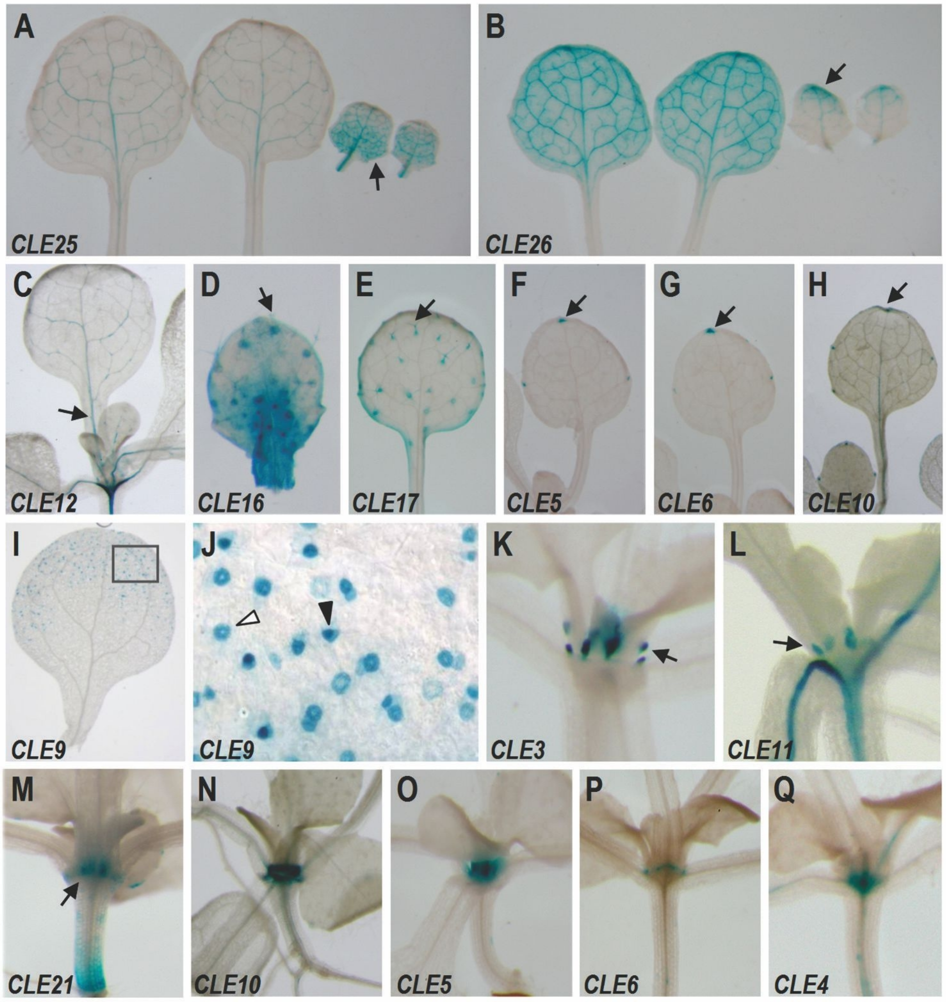


Figure 2. *CLE* promoter activity in 11-day-old-seedlings. (A) *CLE25*, (B) *CLE26* and (C) *CLE12* in leaf vascular tissue. (D) *CLE16* in trichomes and leaf blade. (E) *CLE17* in trichomes and leaf margin. (F) *CLE5*, (G) *CLE6*, and (H) *CLE10* in hydathodes. (I) *CLE9* in stomata. (J) Magnified view of the region boxed in (I). (K) *CLE3*, (L) *CLE11* and (M) *CLE21* in stipules. (N) *CLE10*, (O) *CLE5* and (P) *CLE6* in the leaf base. (Q) *CLE4* in the pith. In (A, B), first to fourth rosette leaves are arranged in order from the left. Arrows indicate GUS activity in the vasculature of the basal and apical regions of young leaves in (A) and (B), respectively, and in the midvein in (C), trichomes in (D, E), hydathodes in (F-H) and stipules in (K-M). Black and white arrowheads in (J) indicate a meristemoid cell and a young guard cell, respectively.

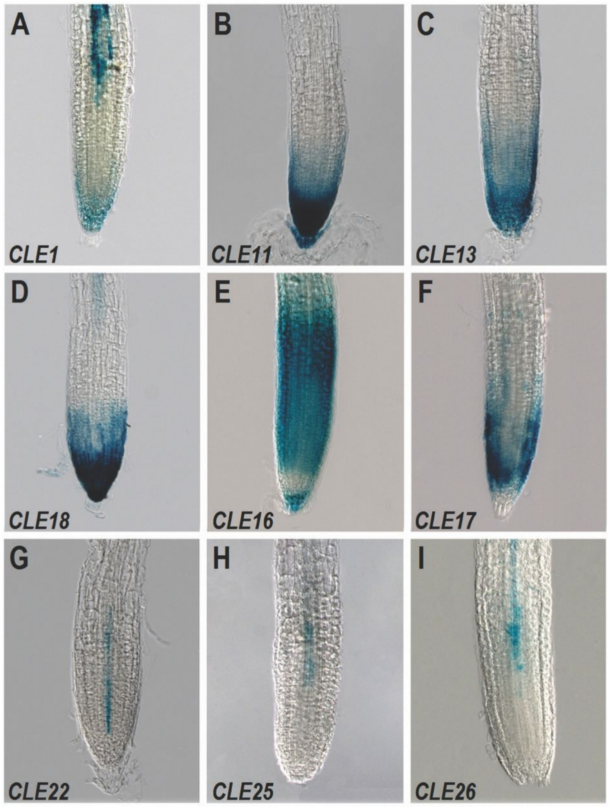


Figure 3. *CLE* promoter activity in the primary root tips of 11-day-old-seedlings. (A) *CLE1* in the root cap and vascular parenchyma. (B) *CLE11*, (C) *CLE13*, and (D) *CLE18* in the root cap and apical meristem. (E) *CLE16* in the root cap and throughout the elongation zone. (F) *CLE17* in the root apical meristem. (G) *CLE22* in the newly differentiating vascular tissue. (H) *CLE25* and (I) *CLE26* in the vascular parenchyma.

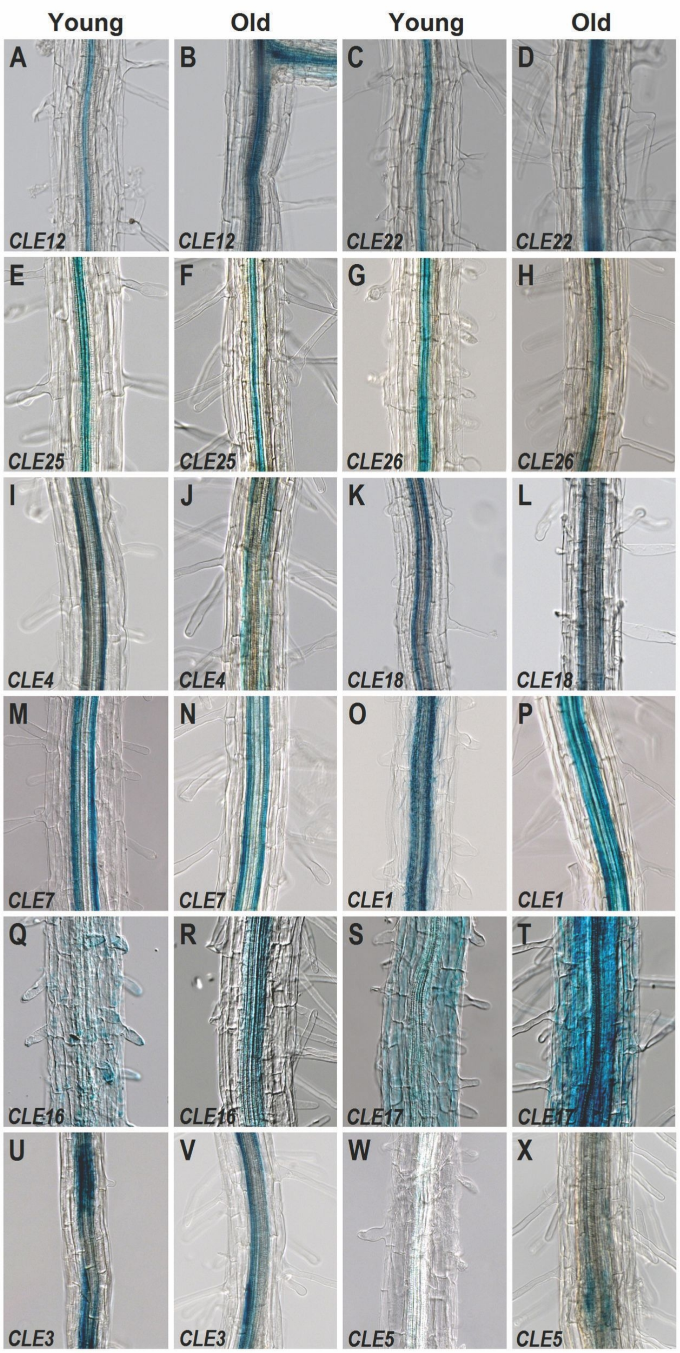


Figure 4. *CLE* promoter activity in primary root cell files.

(A, B) *CLE12*, (C, D) *CLE22*, (E, F) *CLE25* and (G, H) *CLE26* in the stele. (I, J) *CLE4* and (K, L) *CLE18* in the pericycle. (M, N) *CLE7* and (O) *CLE1* in the pericycle and endodermis. (P) *CLE1* in the stele and endodermis. (Q) *CLE16* in the epidermis. (R) *CLE16* in the stele. (S) *CLE17* in the epidermis. (T) *CLE17* throughout the root. (U, V) *CLE3* and (W, X) *CLE5* in patches around the vascular bundle. Images in (A, C, E, G, I, K, M, O, Q, S, U, W) were taken from regions of 11-day-old primary or lateral roots where the root hair cells are not fully mature. Images in (B, D, F, H, J, L, N, P, R, T, V, X) were taken from the primary root maturation zone with fully differentiated root hair cells.

Table 1. Summary of p*CLE*:*GUS* activity during vegetative development.

CLE Gene	Shoot					Root				
	Shoot apex[a]	Hypo-cotyl	Vasculature	Leaf blade	Other[b]	Tip[c]	Vasculature	Ground tissues	Epidermis	Other[d]
CLE1						+	+	+		
CLE2										+
CLE3					+		+	+		
CLE4					+		+			
CLE5					+		+			+
CLE6					+					+
CLE7							+	+		
CLE8										
CLE9					+					
CLE10					+	+/-[e]				
CLE11		+			+	+				+
CLE12		+	+				+			
CLE13						+				
CLE14									+	+
CLE16	+	+		+	+	+	+		+	+
CLE17	+	+		+	+	+	+	+	+	+
CLE18			+			+	+			
CLE20							+			+
CLE21		+			+					
CLE22		+	+			+	+			
CLE25			+			+	+			
CLE26		+	+			+	+			
CLE27	+	+		+		+				+

[a] Shoot apex includes shoot apical meristem and rosette leaf primordia
[b] Other includes pith, stipules, stomata, hydathodes, leaf margins, trichomes and the leaf base
[c] Root tip includes root cap, root apical meristem and cell division zone
[d] Other includes root hair cells and lateral root branch points
[e] Eight of 15 p*CLE10*:*GUS* lines showed root tip expression

Table 2. Summary of p*CLE*:*GUS* activity during reproductive development.

					Stamens		Gynoecium				
CLE gene	Stem/ pedicels	Branching points	Cauline leaves	Sepals/ petals	Anthers	Filament	Stigma	Style	Ovary	Ovules	Flower base[a]
CLE1					+			+			
CLE2											
CLE3			+								
CLE4		+				+					+[c]
CLE5		+						+			+
CLE6		+				+					+
CLE7					+						
CLE8											
CLE9	+		+	+[d]							
CLE10		+	+				+	+	+	+	
CLE11					+			+			
CLE12	+[e]		+		+						+
CLE13	+[e]				+						+
CLE14				+[d]							
CLE16	+			+[e]		+			+		+
CLE17	+	+	+	+			+	+	+[c]		
CLE18			+	+[d,e]		+					
CLE20	+[e]										
CLE21		+							+		+
CLE22	+[e]		+	+[e]					+[e]		+
CLE25	+[e]				+				+[e]	+	
CLE26	+[e]		+	+[d,e]		+			+[e]		+
CLE27		+								+	+

[a] Includes receptacle and abscission zone

[b] Includes valves, replum and septum

[c] Siliques only

[d] Sepals only

[e] Vasculature

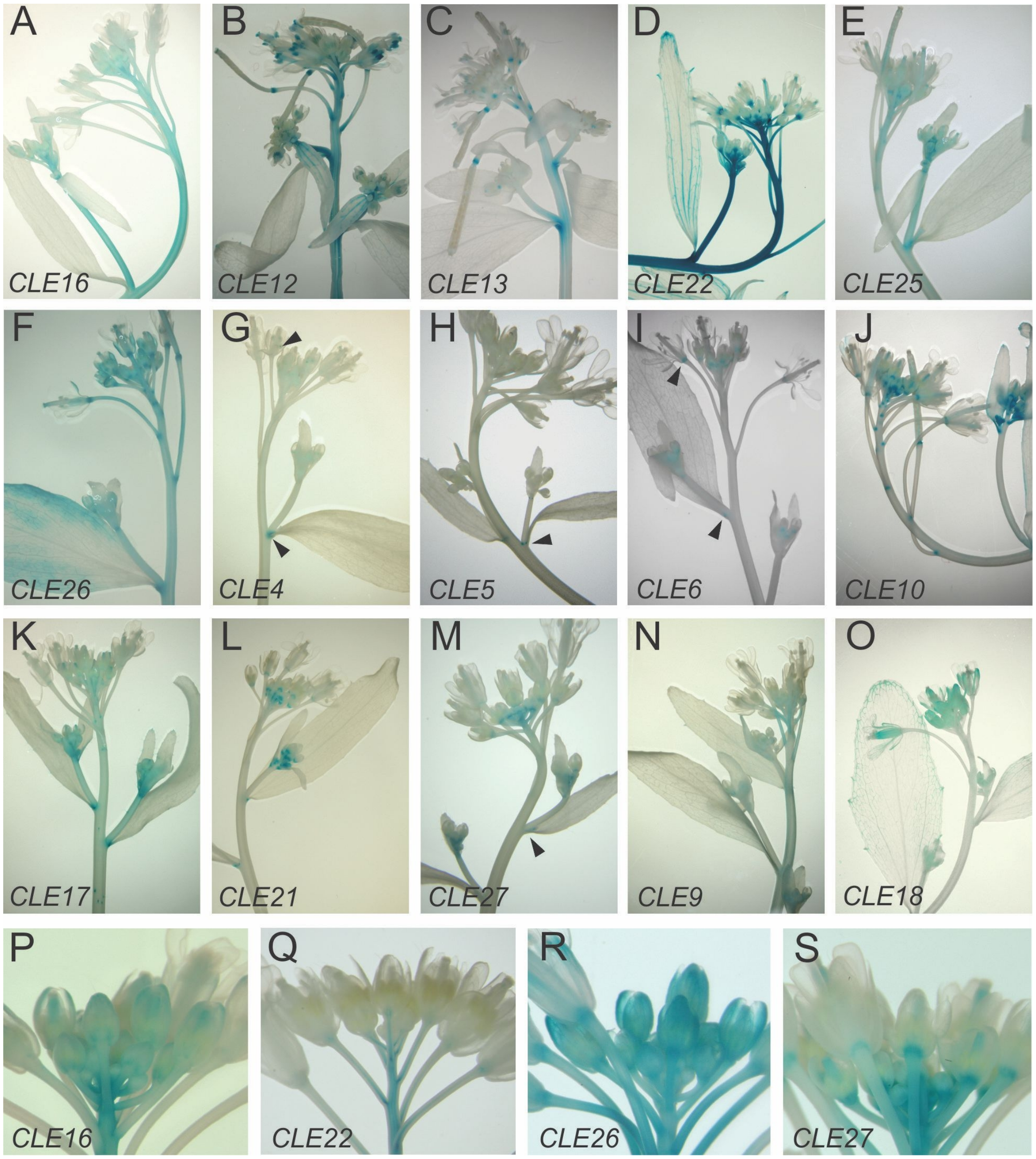


Figure 5. *CLE* promoter activity in inflorescences and cauline leaves.
(A) *CLE16* in the epidermis. (B) *CLE12*, (C) *CLE13*, (D) *CLE22*, (E) *CLE25* and (F) *CLE26* in the vasculature. The *CLE22* stem is over-stained to visualize GUS activity in the cauline leaf. (G) *CLE4*, (H) *CLE5*, (I) *CLE6*, (J) *CLE10*, (K) *CLE17*, (L) *CLE21* and (M) *CLE27* in the inflorescence branching points. (N) *CLE9* in the stomata. (O) *CLE18* in the cauline leaf vasculature and marginal cells. (P) *CLE16* in the epidermis. (Q) *CLE22* and (R) *CLE26* in the vasculature. (S) *CLE27* throughout the pedicels.

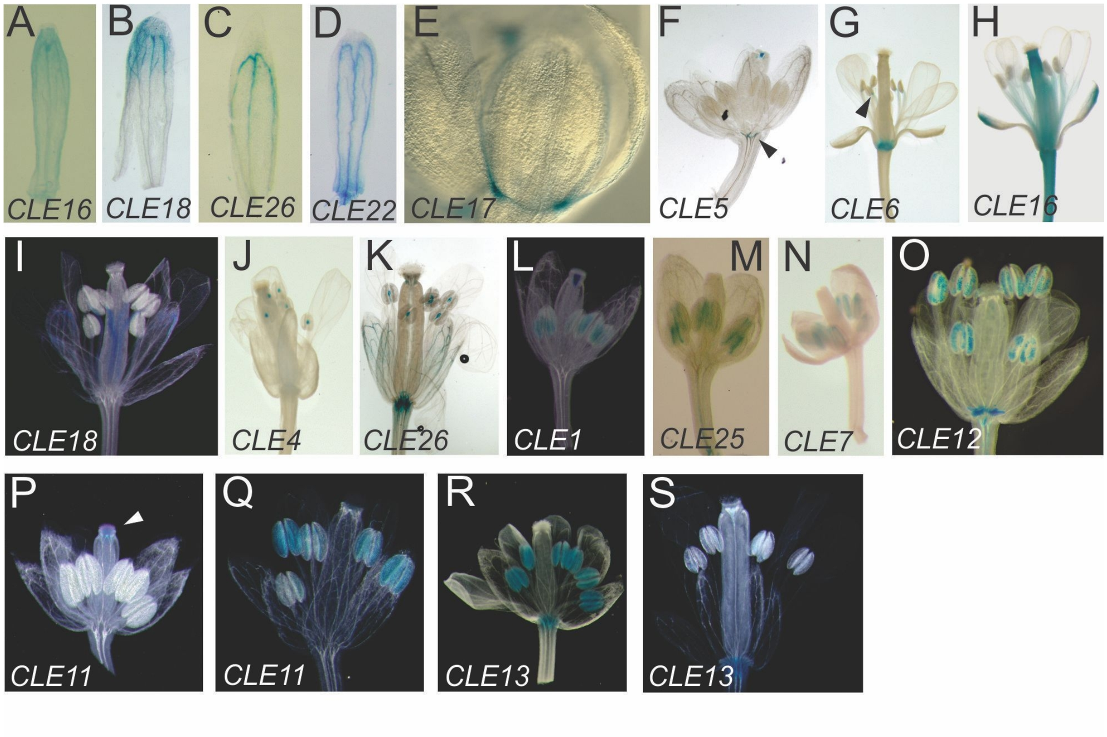


Figure 6. *CLE* promoter activity in flowers.
(A) *CLE16*, (B) *CLE18*, (C) *CLE26* and (D) *CLE22* in sepal vasculature. (E) *CLE17* in sepal and petal margins. (F) *CLE5* and (G) *CLE6* at the base of the flower. (H) *CLE16*, (I) *CLE18*, (J) *CLE4*, and (K) *CLE26* in the stamen filaments. (L) *CLE1*, (M) *CLE25* and (N) *CLE7* in the anthers. (O) *CLE12*, (P, Q) *CLE11* and (R, S) *CLE13* in the pollen grains.

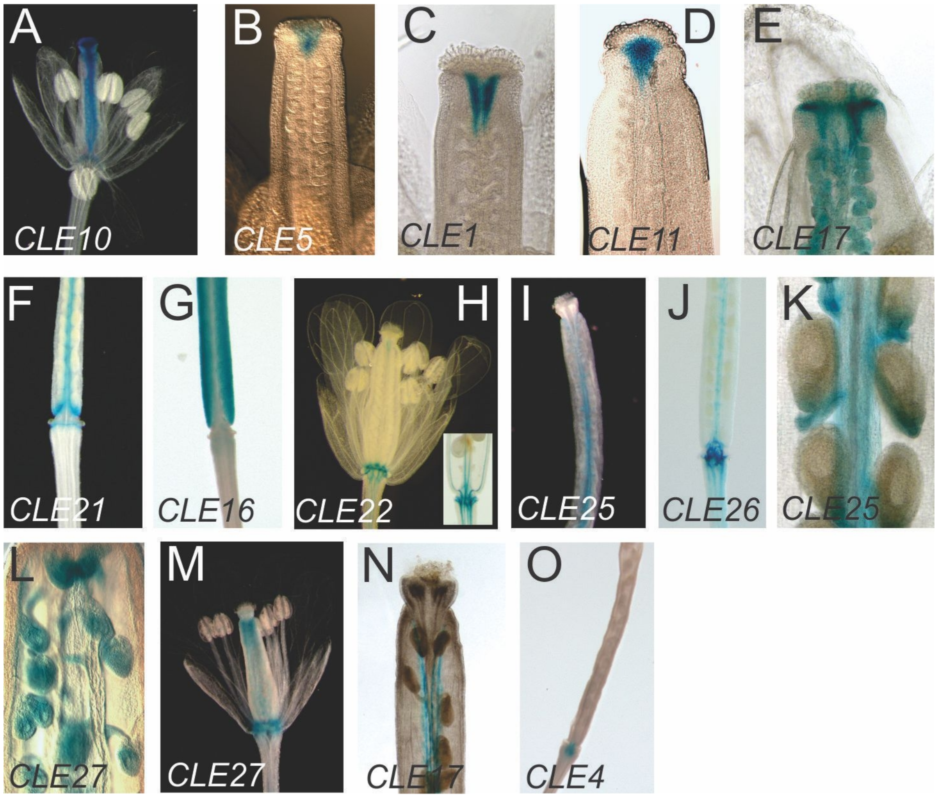


Figure 7**.** *CLE* promoter activity in the gynoecium.
(A) *CLE10* in the stigma, style and central tissues. (B) *CLE5*, (C) *CLE1* and (D) *CLE11* in the style. (E) *CLE17* at the stigma/style boundary. (F) *CLE21* in the valve margins. (G) *CLE16* in the valves. (H) *CLE22*, (I) *CLE25* and (J) *CLE26* in the vasculature. Inset in (H) shows *CLE22* in the basal vasculature and abscission zone. (K) *CLE25* in the funiculi and ovules. (L, M) *CLE27* in the ovules and abscission zone, respectively. (N) *CLE17* in the silique valve margins. (O) *CLE4* in the silique receptacle.

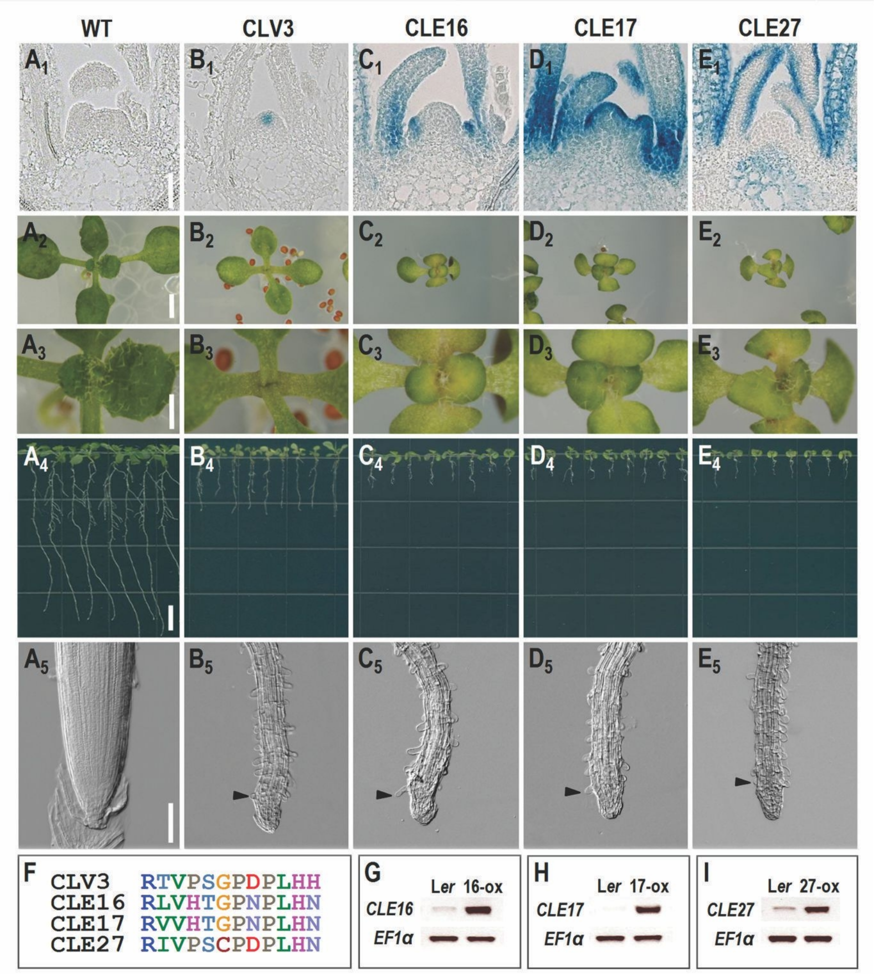


Figure 8. *CLE16*, *CLE17* and *CLE27* over-expression phenotypes. (A_1-E_1) Longitudinal section of 10-day-old wild-type, p*CLV3*:*GUS*, p*CLE16*:*GUS*, p*CLE17*:*GUS* and p*CLE27*:*GUS* plant shoot apices, respectively. (A_2-A_5) 13-day-old L*er* seedlings. (B_2-B_5) 13-day-old p*35S*:*CLV3* seedlings. (C_2-C_5) 13-day-old p*35S*:*CLE16* seedlings. (D_2-D_5) 13-day-old p*35S*:*CLE17* seedlings. (E_2-E_5) 13-day-old p*35S*:*CLE27* seedlings. (F) Comparison of CLE peptide sequences. Alignment was performed using MUSCLE (Edgar, 2004). Each color represents a different amino acid residue. (G-I) RT-PCR analysis of *CLE* expression in transgenic plants. *EF1a* is used as a control. (A_3-E_3) are the magnified views of shoot apex in (A_2-E_2). Nomarski images of the roots in (A_5-E_5) show root hair differentiation (arrowheads) close to the root tip. Bars: A_1-E_1 and A_5-E_5 100 µm; A_2-E_2 2.5 mm; A_3-E_3 1 mm; A_4-E_4 5 mm.

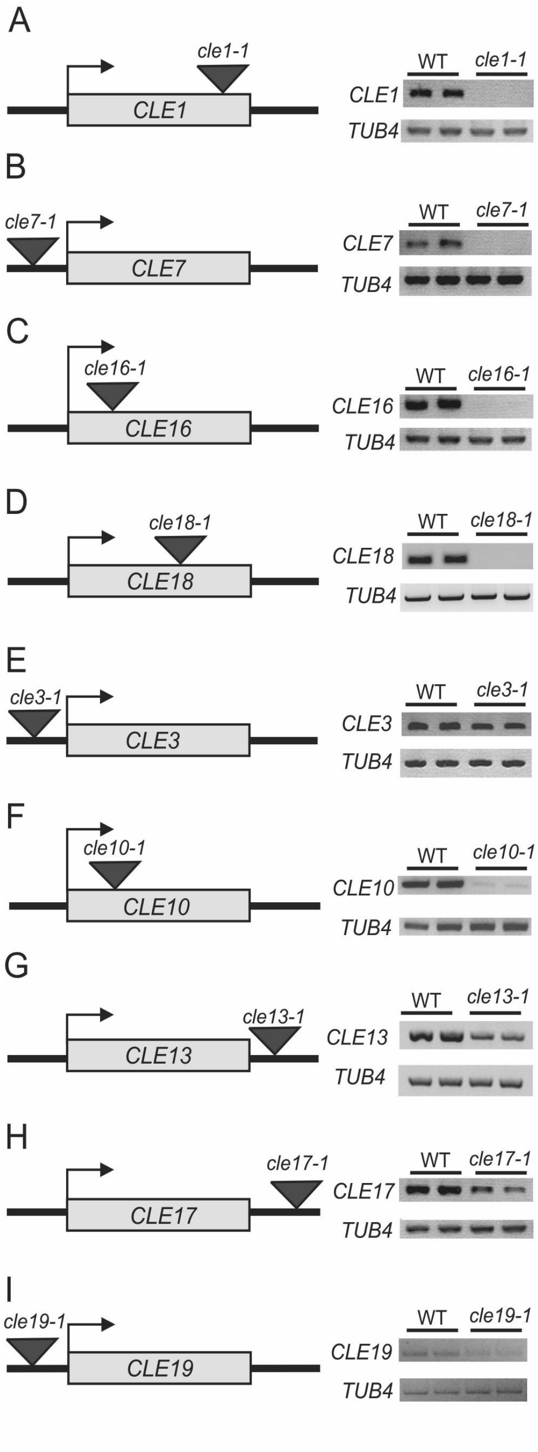


Figure 9. Characterization of *CLE* insertion alleles. Location of the insertion allele relative to each *CLE* coding region (grey box), and *CLE* mRNA transcript levels in two individual wild-type (WT) and homozygous *cle* mutant plants. *TUBULIN4 (TUB4)* is used as a control.

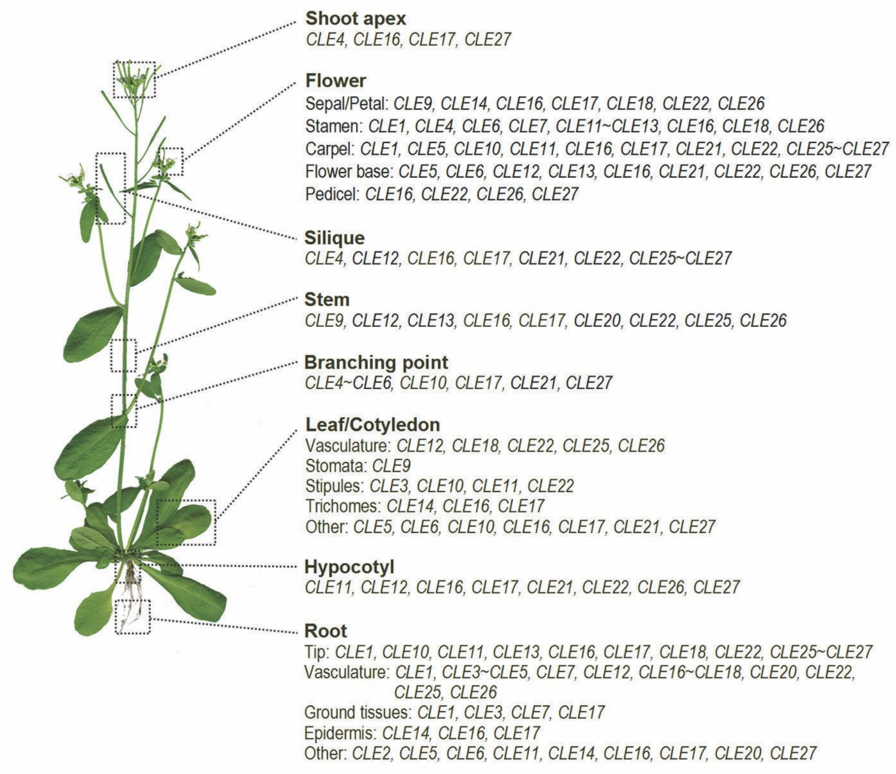
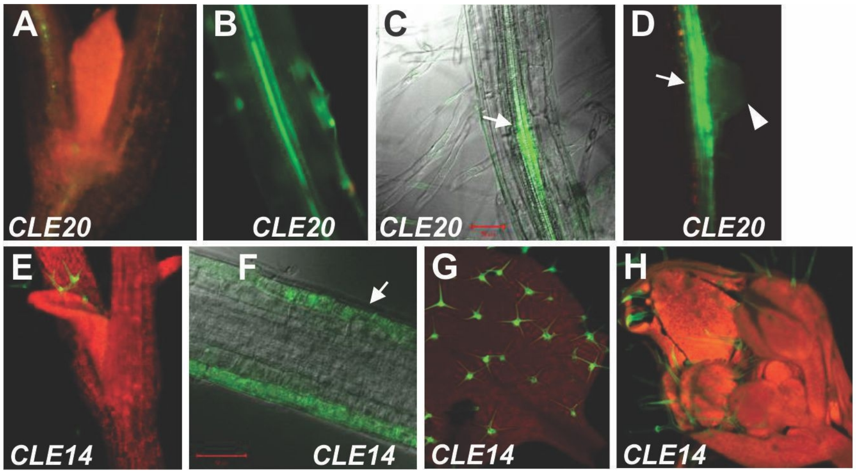
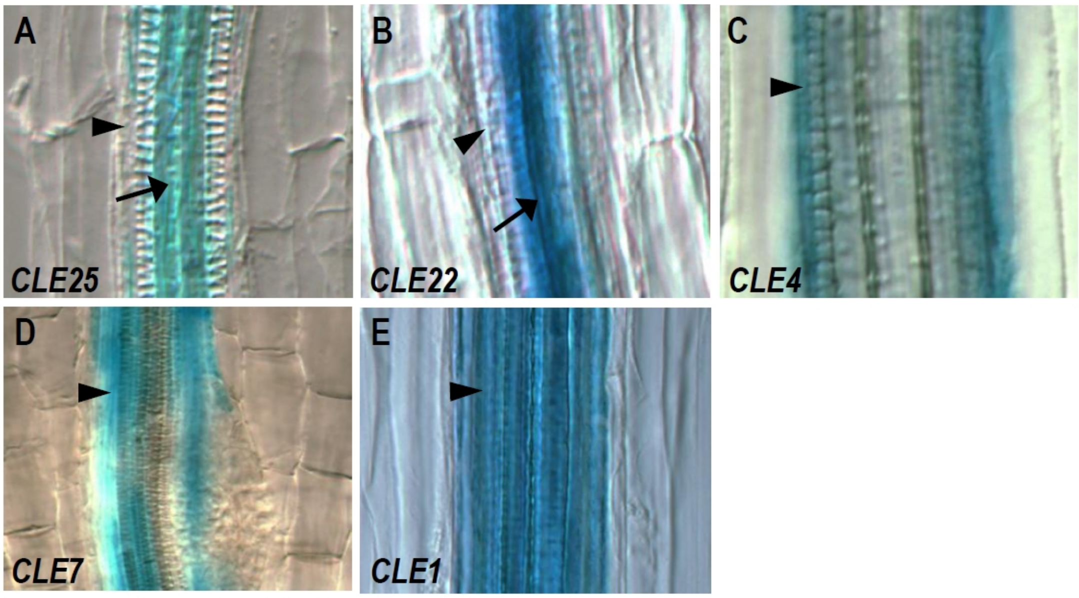


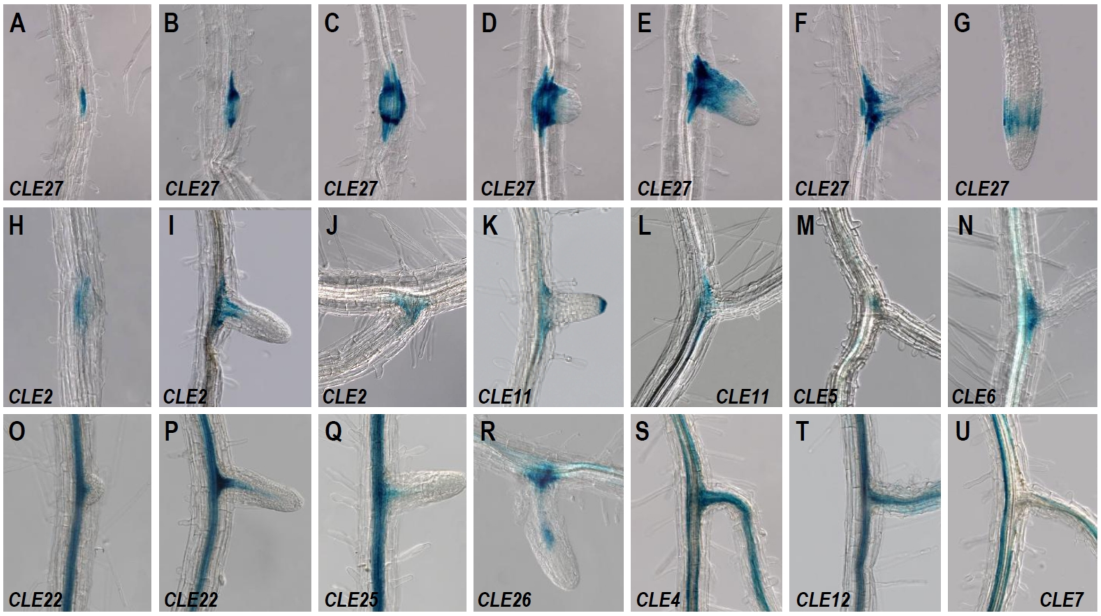
Figure 10. Summary of A-type *CLE* promoter activity in *Arabidopsis*. Shown is a list of the *CLE* genes expressed in the various tissues of a mature *Arabidopsis* plant.



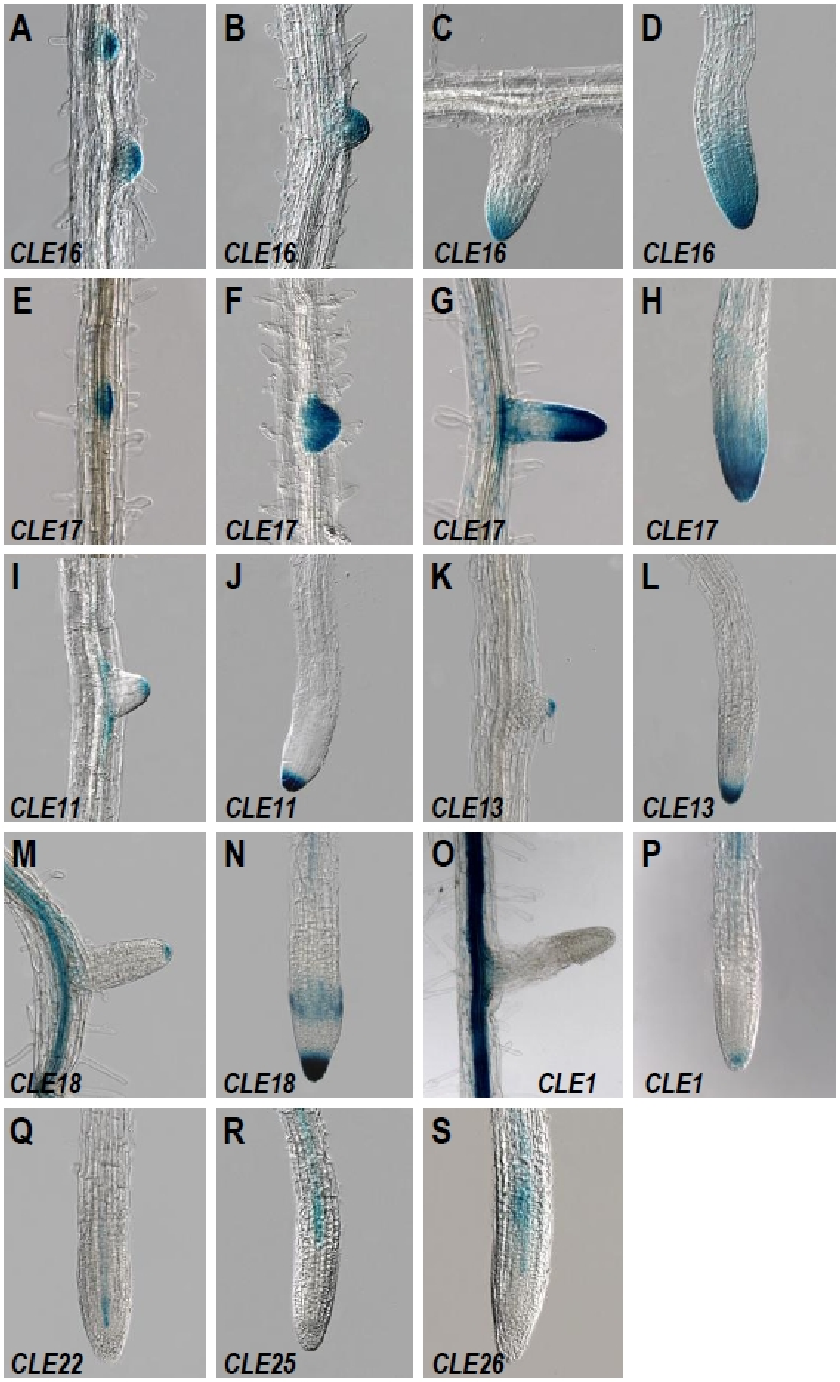
Supplemental Figure 1. CLE14 and CLE20 promoter activity. (A) CLE20 is not expressed in the shoot apex or leaves of a 10-day-old seedling. (B) CLE20 in the root vascular protoxylem and metaxylem. (C, D) CLE20 in lateral root primordia. (E) CLE14 in the trichomes of a 10-day-old seedling. (F) CLE14 in root epidermal cells. (G) CLE14 in the trichomes of a rosette leaf. (H) CLE14 in the sepal trichomes on the inflorescence apex. Arrows in (C, D) and arrowhead in (D) indicate lateral root initiation point and lateral root tip, respectively. Arrow in (F) indicates the root epidermal layer.



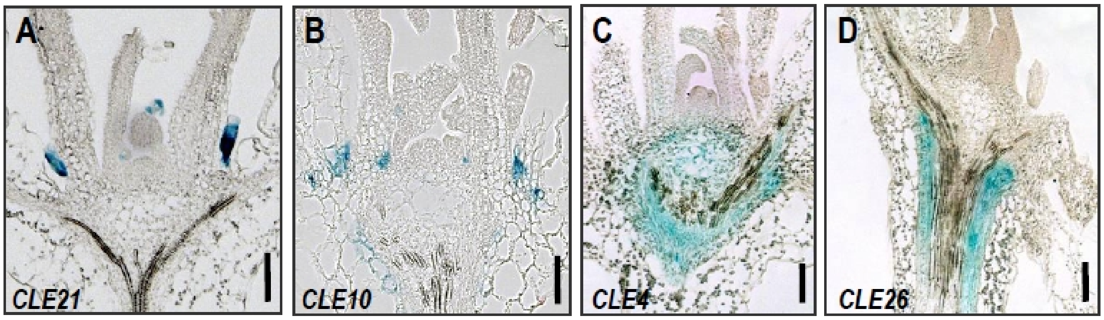
Supplemental Figure 2. Examples of *CLE* promoter activity in primary root vasculature. (A) *CLE25* in the metaxylem cells. (B) *CLE22* in the vascular parenchyma. (C) *CLE4* in the pericycle. (D) *CLE7* in the pericycle and endodermis. (E) *CLE1* in the stele and endodermis. Arrows in (A, B) indicate the phloem. Arrowheads indicate protoxylem vessels.



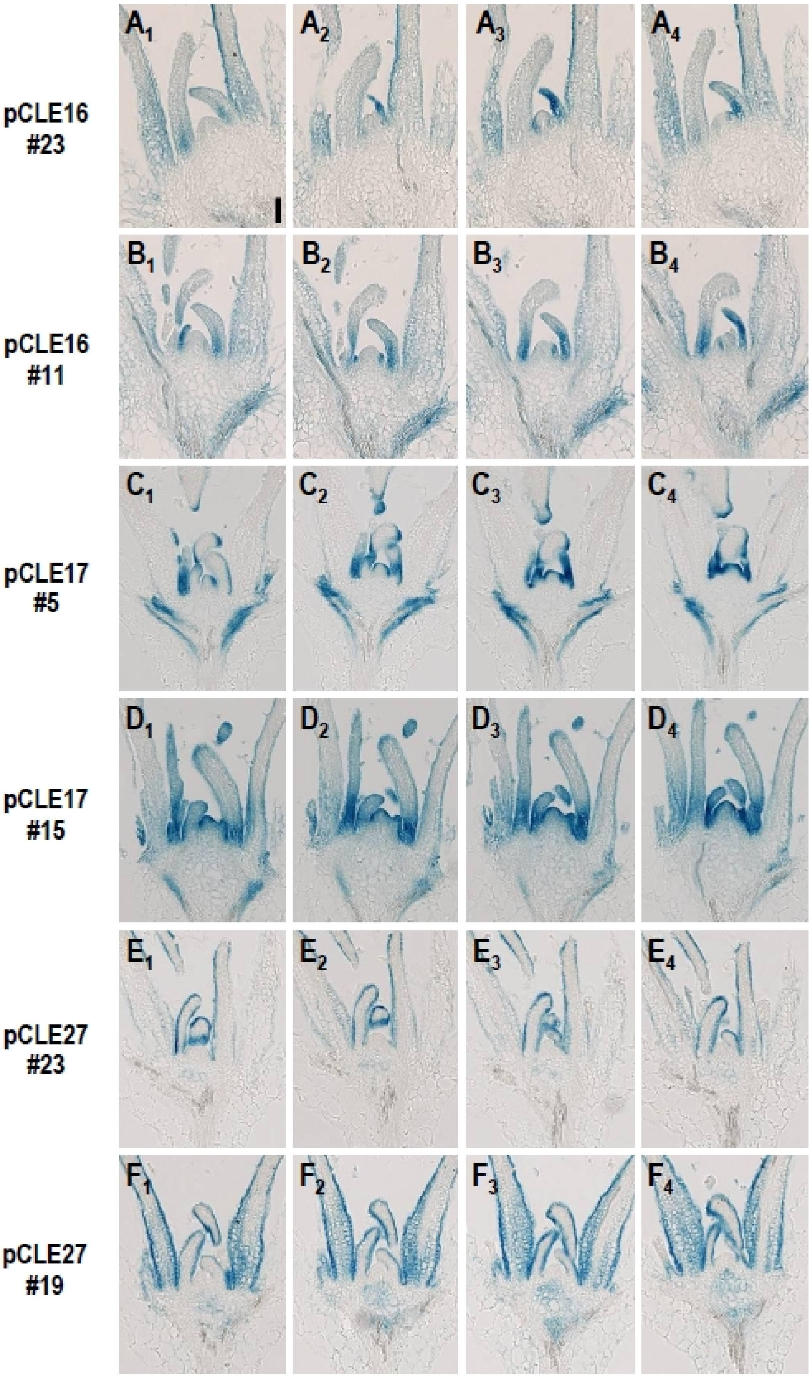
Supplemental Figure 3. *CLE* promoter activity during lateral root development. (A-C) *CLE27* in an initiating lateral root. (D-E) *CLE27* in an elongating lateral root. (F-G) *CLE27* in a mature lateral root. (H-I) *CLE2* in an initiating lateral root. (J) *CLE2* at the junction between the primary and lateral root. (K) *CLE11* in an elongating lateral root. (L) *CLE11*, (M) *CLE5* and (N) *CLE6* at the junction between the primary and lateral root. (O, P) *CLE22*, (Q) *CLE25* and (R) *CLE26* in initiating or elongating lateral roots. (S) *CLE4*, (T) *CLE12* and (U) *CLE7* in mature lateral roots.



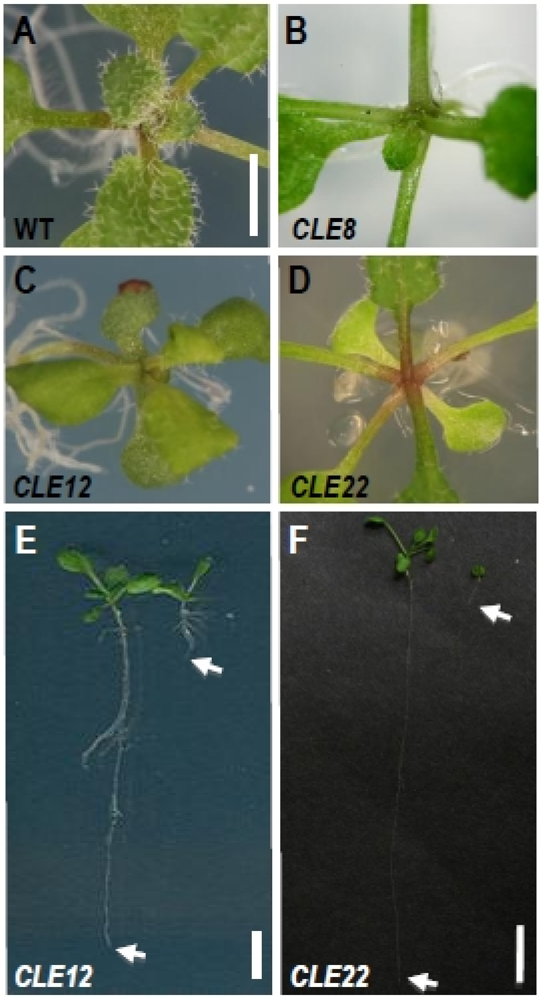
Supplemental Figure 4. *CLE* promoter activity in lateral root tips of 11-day-old seedlings. (A-D) *CLE16* and (E-H) *CLE17* throughout the root cap and apical meristem. (I, J) *CLE11*, (K, L) *CLE13*, (M, N) *CLE18* and (O, P) *CLE1* in the root cap. (Q) *CLE22* in newly differentiating vascular tissue. (R) *CLE25* and (S) *CLE26* in the vascular parenchyma.



Supplemental Figure 5. *CLE* promoter activity in the shoot apex region. Longitudinal sections of 10-day-old (A) p*CLE21*:*GUS*, (B) *pCLE10:GUS*, (C) p*CLE4:GUS* and (D) p*CLE26:GUS* seedlings. Bars: 100 µm.



Supplemental Figure 6. *CLE16*, *CLE17* and *CLE27* promoter activity in the shoot apex. (A, B) Serial sections of 10-day-old p*CLE16*:*GUS* seedlings. (C, D) Serial sections of 10-day-old p*CLE17*:*GUS* seedlings. (E, F) Serial sections of 10-day-old p*CLE27*:*GUS* seedlings. The numbers at the left column indicate independently transformed lines. Bar: 100 µm.



Supplemental Figure 7. *CLE8*, *CLE12* and *CLE22* over-expression phenotypes.
(A) A 13-day-old wild-type seedling. (B) A 13-day-old *p35S*:*CLE8* seedling. (C) A 13-day-old *p35S*:*CLE12* seedling. (D) A 13-day-old *p35S*:*CLE22* seedling. (E) Comparison of root development between a 13-day-old wild-type (left) and a *p35S*:*CLE12* (right) seedling. (F) Comparison of root development between a 13-day-old wild-type (left) and a *p35S*:*CLE22* (right) seedling. Arrows indicate the locations of the root tips. Bars: A-D 2.5 mm; E-F 5 mm.

Supplemental Table 1. *CLE* over-expression meristem phenotypes.

CLE Gene	Dodecapeptide Sequence	AGI/ Accession No.	Size (aa)	p*35S*:*CLE* Phenotype	
				SAM	RAM
CLE8	**RRVPTGPNPLHH**	At1g67775	86	-	-
CLE12	**RRVPSGPNPLHH**	At1g68795	118	+	+
CLE16	**RLVHTGPNPLHN**	At2g01505	103	-	+
CLE17	**RVVHTGPNPLHN**	At1g70895	99	-	+
CLE22	**RRVFTGPNPLHN**	At5g12235	103	+	+
CLE27	**RIVPSCPDPLHN**	At3g25905	91	-	+

Each color in the CLE dodecapeptide sequence represents a different amino acid residue. SAM (shoot apical meristem) or RAM (root apical meristem) termination is denoted by a plus sign (+), whereas a minus sign (–) indicates no meristem termination phenotype.

Supplemental Table 2. *CLE* insertion alleles.

CLE Gene	Allele Name	Insertion Site Relative to ATG	Insertion Line ID Number	Affect on Transcript Levels
CLE1 (At1g73165)	*cle1-1*	+210 bp (CDS)	RATM12-0322-1_H	Absent
CLE2 (At4g18510)	*cle2-1*	+360 bp	SALK_109358	None
	cle2-2	+256 bp (3'UTR)	GK_471C05	None
CLE3 (At1g06225)	*cle3-1*	-36 bp (5' UTR)	RATM15-0143-1_G	Slightly reduced
CLE4 (At2g31081)	*cle4-1*	+505 bp	SALK_048896	None
CLE6 (At2g31085)	*cle6-1*	-289 bp	GK_521E09	Slightly increased
CLE7 (At2g31082)	*cle7-1*	-56 bp (5' UTR)	SAIL 108_G05	Absent
CLE9 (At1g26600)	*cle9-1*	-62 bp (5' UTR)	WisDsLox297300_04B	Slightly increased
CLE10 (At1g69320)	*cle10-1*	+72 bp (CDS)	GARLIC_1147_A10	Nearly absent
CLE13 (At1g73965)	*cle13-1*	+405 bp (3' UTR)	GK_549B08	Reduced
CLE16 (At2g01505)	*cle16-1*	+37 bp (CDS)	RATM11-1864-1_G	Absent
CLE17 (At1g70895)	*cle17-1*	+412 bp (3'UTR)	SALK_094989	Reduced
	cle17-2	-689 bp	SALK_103714	Slightly increased
CLE18 (At1g66145)	*cle18-1*	+188 bp (CDS)	GK_479A03	Absent
CLE19 (At3g24225)	*cle19-1*	-49 bp (5' UTR)	GK_232C03	Reduced
CLE21 (At5g64800)	*cle21-1*	-248 bp	SALK_088408	None
	cle21-2	-108 bp	GK_203E06	None

The ATG site is denoted as +1 bp. bp, base pairs; CDS, coding sequence; UTR, untranslated region.

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CHAPTER 3

Functional characterization of *CLE8* in seed development

INTRODUCTION

Among the different groups belonging to the plant kingdom, the Spermatophyta (seed plants) is exceedingly the most diverse and successful one. The seed evolved to be a complex structure whose development requires a stringently regulated growth of different tissues. The coordination of tissue-specific developmental programs is believed to be achieved through signal transduction pathways that integrate information originating from different tissues.

In Angiosperms, seeds develop from the ovules, structures that consist of the nucellus (megasporangium) enclosed by two layers of maternal diploid tissue referred to as the seed envelope or integument(s) (Linkies A et al., 2010). Inside the nucellus, a megaspore develops into a female gametophyte, the megagametophyte. In most Angiosperms, including the model plant *Arabidopsis thaliana*, the mature megagametophyte, the embryo sac, is seven-celled and eight-nucleate, referred to as the Polygonium-type (Floyd et al., 2000). The embryo sac is a polar structure that consists of two cells that are competent for fertilization (the haploid egg cell and the homodiploid central cell) and haploid sterile cells (synergids and antipodals) whose number varies depending on the species. Angiosperms are characterized by the process of double fertilization: the megagametophyte receives a pollen tube that delivers two sperm cells. One sperm cell fertilizes the egg cell giving rise to the zygote that develops into an embryo, whereas the second sperm cell targets the central cell polar nuclei leading to the formation of the endosperm. The products of the double fertilization process are not genetically equivalent: in the case of diploid plants (such as *Arabidopsis*) the embryo, which makes up the next generation, is diploid whereas the endosperm is triploid (Dumas et al., 2008). Both the newly-formed zygote and endosperm are enclosed by the maternally-derived integument layers. The concerted growth and development of the two products of fertilization and the surrounding maternal tissues is a hallmark of Angiosperm development and is referred to as seed development (Chaudhury et al., 2001).

Embryo development

Upon fertilization, the zygote elongates and undergoes an asymmetric cell division that gives rise to a large basal cell and a small apical cell. This cell division establishes the plant primary apical-basal axis. The apical cell and its daughter cells undergo isotropic proliferation giving rise to an 8-celled spherical embryo (octant stage). The basal cell and its daughters elongate and divide only transversely to produce a file of cells, the suspensor. Only the upper cell of the suspensor (the hypophysis) is eventually included in the embryo and contributes to the formation of the primary root meristem, whereas the rest of the cells form the extra-embryonic suspensor (Jenik et al., 2007). It is still not clear how early apical-basal polarity is established in developing embryos. Recent findings suggest that both cell-autonomous and position-dependent processes may be essential for the establishment of the basal cell lineage and suspensor development (Haecker et al., 2004; Bayer et al., 2009). In *Arabidopsis*, the *WUSCHEL-RELATED HOMEOBOX* (*WOX*) transcription factor genes are dynamically expressed during early embryogenesis and seem to confer specific transcriptional programs upon the cell expressing them. For example, *WOX2* and *WOX8* are expressed in the zygote, and, after its division, *WOX2* is expressed in the apical cell and *WOX8* in the basal cell; *WOX9* does not appear to be expressed in the zygote but is expressed in the basal cell and possibly also in the apical cell. These expression patterns reveal a level of transcriptional control that clearly parallels the anatomical patterns of cell division (Haecker et al., 2004; Wu et al., 2007; Breuninger et al., 2008). The plant radial axis is established at the transition between the octant and the dermatogen stage, when the 8 cells divide tangentially giving rise to an outer cell layer called the protoderm which eventually will differentiate into the epidermal cell layer. After few cell divisions, when the embryo contains around 100 cells, its overall shape is still round (globular stage). At late globular stage, embryonic leaves (cotyledons) start to grow and protrude out at two opposite positions in the apical region (heart stage embryo). At this time, both a root and a shoot apical meristem are specified at the opposite ends of the vasculature primordia (provasculature). During the subsequent phases, the cotyledons and the hypocotyl elongate (torpedo stage and bent cotyledon stage), the radial pattern is further elaborated and ultimately the embryo undergoes developmental arrest (mature stage) and becomes dormant (Capron et al., 2009; De Smet et al., 2010).

Endosperm and integuments development

The fertilized central cell starts its first mitotic division before the zygote and undergoes approximately 4 rounds of division before the first zygotic division occurs (Boisnard-Lorig, 2001). The endosperm nuclei initially divide without cell wall formation, giving rise to a coenocyte. This is often referred to as the syncytial endosperm phase even though the multinucleate cytoplasm is not derived from cell fusion. The first three rounds of nuclear division are highly synchronized and give rise to eight endosperm nuclei that are distributed in the periphery of the embryo sac, surrounding a large central vacuole. As the ovule grows to accommodate the developing embryo and endosperm it bends giving rise to a horseshoe shape, with the micropylar and the chalazal zones becoming adjacent (Brown et al., 1999). The following nuclei divisions are not synchronized and this allows the definition of three mitotic domains in the developing endosperm: the micropylar endosperm where nuclei still divide synchronously, the peripheral endosperm (in the central chamber) and the chalazal endosperm where divisions stop earlier than in the other domains. At the globular embryo stage the syncytial cytoplasm of the micropylar endosperm surrounds the developing embryo and is often referred to as embryo-surrounding-region (ESR), whereas the peripheral syncytium is a thin layer with evenly spaced nuclei (Olsen, 2004). When the embryo reaches the heart stage the endosperm cellularization process starts in the micropylar region and proceeds rapidly in a wave towards the peripheral endosperm and then the chalazal endosperm (Sorensen et al., 2002). This results in a completely cellularized endosperm, except for a small portion of the chalazal endosperm that is depleted gradually as the embryo grows so that in mature seeds the embryo fills the embryo-sac and only a single endosperm layer (the aleurone layer) persists (Brown et al., 1999).

Tissue-tissue cross-talk during seed development

It is widely accepted that there is a strong sporophytic effect influencing seed growth and size (Roach et al., 1987, Galloway et al., 2009) and that seed development is profoundly influenced by the balance of paternal and maternal genomes (Pennington et al., 2008; Scott et al., 1998). The maternal influence is exerted via the endosperm both through the cytoplasm of the central cell as well as through specific activation of maternal alleles and silencing of paternal alleles resulting in maternal genome imprinting (Berger et al., 2006). The triploid endosperm has a natural imbalance of two maternal genomes and a paternal one. If this balance is perturbed by performing interploidy crosses, the developing seeds either abort prematurely or show some developing defects (Scott et al., 1998, Pennington et al., 2008). In *Arabidopsis*, crosses between diploid and tetraploid parents give rise to progeny with either maternal genome excess (embryo 2m:1p, endosperm 3m: 1p) or paternal genome excess (embryo 1m:2p, endosperm 2m:2p). Seeds with parent genome imbalance show complementary phenotypes. Seeds with maternal genome excess show inhibition of endosperm development due to premature cellularization, which causes a delay and/or arrest of embryo development. Seeds with paternal genome excess show endosperm over-proliferation due to a delay in cellularization and an acceleration of mitosis, and ultimately bigger embryos (Scott et al., 1998). The fact that endosperm repartitions nutrients from the mother to the developing embryo has led to the hypothesis that these effects are an example of parental conflict (Pennington et al., 2008). Although the molecular mechanism driving this phenomenon has not yet been characterized, it unveils the tight interconnection and communication happening among the tissues that compose the seed and puts the endosperm in the spot as a key integrator of seed growth and development (Costa et al., 2004, Berger et al., 2006).

Because the proper development of a seed requires the coordinated growth of so many different tissues, the existence of physical connections among the tissues and of signal transduction pathways that would make intercellular communication possible has always been speculated. Cytoplasmic connections between cells of the female gametophyte and also between the female gametophyte and the sporophytic nucellar cells have been observed at various stages of ovule development (Stadler et al., 2005). However, it is generally believed that in *Arabidopsis,* after cellularization of the female gametophyte and degeneration of the micropylar nucellus, there are no plasmodesmata connections between cells at the micropylar pole of the gametophyte and integuments or between the central cell and the chalazal nucellus. Before or during fertilization it is generally accepted that the female gametophyte becomes symplastically isolated from the maternal sporophyte, but plasmodesmata are still observed between most cells of the gametophyte (Stadler et al., 2005). During endosperm development in *Arabidopsis,* the plasma-membrane of the un-cellularized endopserm is juxtaposed with the cell wall of the developing embryo. Upon fertilization and the onset of embryo and endosperm development, the embryo seals off the endosperm depositing a layer of new cell wall material on its surface. This is followed by the deposition of a lipidic cuticle at around the globular stage of embryo development, so that endosperm and embryo represent independent symplastic fields from very early in seed development even if the timing has not been precisely documented (Ingram, 2010). Given the lipid nature of the cuticle around the embryo proper it is believed it is a barrier to the movement of apoplastic solutes, proteins, peptides and amino acids, which could still be available to the embryo proper through the suspensor. The suspensor, like the embryo-proper, is thought to be symplastically isolated from the endosperm and maternal sporophytic tissue. Remarkably, unlike the embryo-proper, the suspensor lacks a cuticle deposition, which makes it a likely candidate to integrate signals emanating from endosperm, embryo-proper and maternal tissues and ultimately to orchestrate embryo-proper development by acting as a conduit for nutrients and growth regulators from the maternal tissues (Kawashima et al., 2010).

Further evidence for the importance of cell–cell signaling events in basal cell specification and suspensor differentiation has been obtained from studies with the Arabidopsis *YODA* (*YDA*) and *SHORT SUSPENSOR* (*SSP*) genes. These genes encode a mitogen-activated protein kinase kinase (MPKK) (Lukowitz et al., 2004) and an interleukin-1 receptor-associated kinase/Pelle-like kinase (Bayer et al., 2009), respectively. Mutant *yda* and *ssp* zygotes do not elongate and produce a small basal cell following zygotic division that does not develop into a suspensor. These observations indicate there is an early signal, whose origin is still unknown, responsible for the specification of suspensor cell fate.

Genetic and molecular studies in recent years have started to shed some light on the signaling mechanisms that regulate seed growth and development. The *Arabidopsis HAIKU2* (*IKU2*) encodes a leucine-rich repeat (LRR) kinase that is expressed in the endosperm of developing seeds (Luo et al., 2005). *IKU* mutants display precocious endosperm cellularization, and a decrease in endosperm size that ultimately results in a smaller embryo and a decrease in seed size. Mutations in *iku* genes do not cause maternal sporophytic effects; however, the reduction in seed size causes a precocious arrest in cell elongation in the integuments (Garcia et al., 2003). On the other hand, mutations in the *WRKY* transcription factor gene *TRANSPARENT TESTA GLABRA2* (*TTG2*) show reduced cell elongation in the integuments and, as a consequence, restrict endosperm and seed growth (Garcia et al., 2005). Interestingly, mutations that cause an increase in the number of integument cells also display an increase in endosperm proliferation (Canales et al., 2002). These findings demonstrate that there is a two-way communication between the endosperm and the maternal tissues that is important to coordinate seed development and that this communication happens, at least in part, through classic signal transduction pathways.

Mutations in the *iku* genes are not informative about possible signaling between the embryo and the endosperm because their reduced embryo growth is likely to be a direct outcome of reduced endosperm proliferation and consequent decrease in the amount of nutrients delivered to the embryo. It is not yet known what molecular mechanisms adjust the growth of the embryo to the space available, but the *iku* phenotype indicates that some cross-talk happens between the embryo and the endosperm.

A first clue about communication from the embryo to the endosperm has been provided in *Arabidopsis* through studying the mutations in *CDC2A*, a homolog of the cyclin dependent kinase CDKA;1 (also known as Cdc2A). *cdc2a* null mutants fail to progress through the second mitotic division during male gametophyte development, so that the resulting pollen carries a vegetative cell and only one (instead of two) sperm cell that specifically targets the female egg cell and not the central cell (Nowack et al., 2006). Surprisingly, upon fertilization of the egg cell the central cell also undergoes a few rounds of free-nuclear divisions, starting endosperm development, but eventually the seeds abort due to paternal effect (Nowack et al., 2006). These observations suggested that a signal from the newly formed zygote initiates proliferation of the central cell and commences endosperm development. Very recently, the *cdc2a* phenotype has been analyzed in more depth using a new *in vivo* imaging protocol for fertilization as well as markers of karyogamy and paternal expression. This study revealed that signaling events associated with sperm cell fusion with the female gametophyte, rather than signaling arising from the zygote, are involved in triggering mitotic divisions of the central cell (Aw et al., 2010).

Based on the observation that in *Arabidopsis* the fertilized central cell undergoes approximately 4 rounds of mitotic division before the first zygotic division (Boisnard-Lorig, 2001), it is believed that early endosperm development is independent of embryo development. The isolation of mutants that show proliferation of the unfertilized central cell confirms this view. All the mutants that fall in this group are loss-of-function alleles of members of the chromatin remodeling Polycomb group (PcG) (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999; Kohler et al., 2003). In wild-type plants, these proteins are involved in the arrest of the female gametophyte development upon fertilization (Berger et al., 2006), suggesting a dependence of embryo development upon endosperm proliferation. However, the characterization of mutants impaired in central cell fertilization (Ungru et al., 2008; Ngo et al., 2007) and other evidence collected over the years (Weijers et al., 2003; Vernon et al., 1994) revealed that early embryo development and patterning is at least independent of endosperm proliferation. Based on the data collected to date, it can be inferred that even though very early events in both embryo and endosperm seems to be largely independent, a coordination in development of these two organisms is necessary to produce viable seeds.

The CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-related polypeptide family and their potential role in signaling during seed development

The ESR was first defined in studies carried out in maize (Opsahl-Ferstad et al., 1997). This group of endosperm cells differs from other endosperm cells in terms of cytological characteristics (van Lammeren, 1986) and gene expression profile (Opsahl-Ferstad et al., 1997 and Bonello et al., 2000) in most studied species including *Arabidopsis*. Among the genes specifically expressed in the ESR are genes involved in sucrose transport and metabolism (Bate et al., 2004), which gives a hint that this region is fundamental in transferring nutrients to the embryo, probably through the suspensor.

Even more interestingly, three maize putative signaling molecules belonging to the CLAVATA3/ESR-related (CLE) family are expressed (Opsahl-Ferstad et al., 1997) and secreted (Bonello et al., 2002) by cells of the ESR right at the interface with the developing embryo, suggesting they might play a role in signaling between these two tissues. The CLE family of plant-specific genes was named after its founding members CLAVATA3 (CLV3) from *Arabidopsis* and the ESRs from maize. With 32 members in *Arabidopsis*, it is the largest family of plant polypeptides identified so far. The *CLV3* gene was isolated by genetic screening and plays a key role in the maintenace of the stem cell reservoir in shoot apical and floral meristems (Fletcher et al., 1999). Although no functional information from mutant analysis is available for the vast majority of *CLE* genes, several CLE proteins have been implicated in regulating different aspects of plant development (Jun et al., 2008; Wang et al., 2010). Ironically, despite these genes has been named based on their expression pattern in maize, no *Arabidopsis CLE* gene has been reported to be expressed in the endosperm. In this study I have identified *CLE8* as an *Arabidopsis CLE* gene specifically expressed in the endosperm and in young developing embryos, and demonstrated that it plays a role in basal embryo patterning and endosperm differentiation.

MATERIAL AND METHODS

Plant material

All *Arabidopsis thaliana* genotypes were in the Columbia (Col) ecotype background and were grown on either Murashige and Skoog medium (with or without proper selective chemicals) or soil (1:1:1 mixture of perlite:vermiculite:topsoil) under continuous light (120 $\mu mol\cdot m^{-2}\cdot s^{-1}$) at 22°C. Seeds were stratified at 4°C for 4 days before exposing them to light. Before in-vitro culture, seeds were surfaced sterilized for 40 min with chlorine gas.

Plant transformation

Stable transformation: *Agrobacterium tumefaciens* strain GV3101 was used to transform *Arabidopsis* plants by the floral dip method (Clough et al., 1998).

Transient transformation: onion epidermis cells were transformed by particle bombardment using a Biolistic PDS-1000/He unit (BioRad, Richmond, CA), as previously described (Sanford et al., 1993).

DNA extraction

Preparation of Arabidopsis genomic DNA for PCR analysis was conducted as previously described (Edwards et al., 1991).

Preparation of Arabidopsis genomic DNA for cloning was performed using the Dneasy plant kit (Qiagen, USA).

RNA extraction

Total RNA isolated by using the RNeasy plant kit (Qiagen) was treated with RNase-free DNase (Roche) for 30 min at 37°C and then purified with phenol/chloroform.

RT-PCR

The first-strand cDNA synthesis was performed on 1 µg of total RNA by using Superscript III RNase H^- reverse transcriptase (GIBCO/BRL) and an oligo dT primer (18mer), according to the manufacturer's instructions. The annealing temperature was 58-60°C for all primer pairs. *CLE8* coding sequence was amplified using the following primers:

CLE8_ATG_fw 5'-ATGAAAGTGTTGAAGAGAGATTC-3'
CLE8_STOP_rv 5'-TCAGTGATGTAATGGATTAGGAC-3'

The *ACTIN2* coding sequence was amplified using the following primers:

ACT2_fw 5'-GTTAGCAACTGGGATGATATGG-3'
ACT2_rv 5'-CAGCACCAATCGTGATGACTTGCCC-3'

qPCR

Quantification of *CLE8* and *WOX8* cDNAs by real-time PCR was performed using the SYBR Green PCR master mix (Applied Biosystems) and a MyIQ Thermal Cycler (BioRad). The primers used were the following:

qTub4Fw 5'-GGTCAATACGTCCGGGATTC-3'
qTub4Rv 5'-TCTGACCGAACGGACCAGAT-3'
qCLE8Fw 5'-TCCTTTTCTGGTTGGAGTCG-3'
qCLE8Rv 5'-GAAGATGAGGTTTCGCCTTG-3'
qWOX8Fw 5'-CCGGCTTCAAGAATATGGTC-3'
qWOX8Rv 5'-GGGCTTTTGTGATGAACACG-3'

The specificity of the amplification was determined by performing a dissociation curve analysis. Three technical replicates were done for each sample. Relative quantification (RQ) values were calculated using the $2^{-\Delta Ct}$ method (Livak et al., 2001). The Ct was calculated using the *TUB4* gene as the endogenous control. Three biological replicas were run for each sample.

Cloning

PCR amplified products were cloned into pENTR/D-TOPO (Invitrogen) according to the manufacturer manual and then moved into the appropriate destination vector from the pMDC series (Curtis et al., 2003) or pBGWFS7 (Karimi et al., 2002) through an LR reaction (Invitrogen) according to the manufacturer manual. *CLE8* promoter sequence was PCR amplified using the primers listed below and cloned into the binary vector pBGWFS7 for expression analysis.

CLE8proTopoFW 5'-CACCAAGAAGCATAAACTACAAGAAAGG-3'
CLE8proRV 5'-TTTCTCTCC$(T)_{12}$ACTTTTAGTTTCTCATGTACTTCTTTTTAGC-3'

CLE8 coding sequence was PCR amplified using the primers listed below and cloned into the binary vector pMDC32 for over-expression analysis and pMDC83 for protein sub-cellular localization analysis.

CLE8 ATG TopoFW 5'-CACCATGAAAGTGTTGAAGAGAGATTC-3'
CLE8_STOP_rv 5'-TCAGTGATGTAATGGATTAGGAC-3'
CLE8_noSTOP_rv 5'-GTTCCTAGCATAGTTTAGACTTC-3'

CLE8 genomic sequence containing the promoter and the coding region was PCR amplified using the primers CLE8proTopoFW and CLE8_noSTOP_rv (see above) and cloned into the binary vector pMDC107 for *cle8-1* complementation analysis.

CLE8 transcript was PCR amplified using the primers listed below and cloned into the pGEM-T Easy vector (Promega, USA) for *in vitro* transcription.

CLE8 insitu FW 5'-GGAGAGAAAATGAAAGTGTTGAAGA-3'
CLE8 insitu RV 5'-TGATCTAGAACAATGGTTGATGTTT-3'

RNA *in-situ* hybridization

RNA *in situ* hybridization analyses were conducted according to Carles et al (Carles et al., 2010) using *CLE8* full length coding sequence as probe.

GUS histochemical assays

Whole mount: β-glucoronidase activity has been tested as described (Jefferson, 1989) with the modification that 1mM potassium ferrocyanide and 1mM potassium ferricyanide were used. Incubation times ranged from 8 to 12 hours following vacuum infiltration. Subsequent tissue embedding and sectioning were performed as described (Sieburth et al., 1997).

On slide: developing seeds were isolated and placed directly in 100ul of staining solution on a microscope slide. A cover slip was put on the sample and some pressure applied in order to squeeze embryos out of the seeds. The slides were incubated at 37 °C in a tight container to prevent drying.

Microscopy

Nomarski microscopy analysis was performed using a chloral hydrate solution (containing glycerol, chloral hydrate, and water in a ratio of 1:8:3) on a Zeiss Imager A1 equipped with DIC optics. For fluorescent analysis, the prepared ovules were placed in 10% glycerin and analyzed using the Zeiss filters 46HE for YFP and 38HE for GFP.

GFP visualization in onion epidermal peels was performed 24-36 hours after bombardment using a Zeiss Axiophot microscope.

RESULTS

CLE8 expression analysis

As I undertook a systematic expression analysis of members of the *Arabidopsis* CLE gene family (Jun et al., 2010), I became interested in discovering possible maize *ESR* orthologs. A preliminary expression analysis of *Arabidopsis CLE* genes was already available (Sharma et al., 2003) and suggested *CLE8* as a strong candidate.

I performed an initial RT-PCR analysis of *CLE8* expression using RNA extracted from different tissues throughout *Arabidopsis* development. I detected *CLE8* transcripts uniquely in siliques samples (Fig.1A). To understand if *CLE8* expression would be modulated or steady during seed development, I analyzed *CLE8* expression at different stages of silique formation. RT-PCRs were run on RNA extracted from siliques containing seeds in four different developmental stages: zygote to early globular, early globular to triangular, heart to torpedo and torpedo to mature. *CLE8* is expressed throughout seed development with a peak of expression in siliques containing seeds with embryos at the globular stage (Fig.1B). RNA in situ hybridization experiments were conducted on silique tissue using the entire *CLE8* coding sequence as a *CLE8* probe. Expression was detected in embryos from the zygote to the globular stage (Fig.1C-E), whereas no expression was detected in more developed embryos (Fig.1F). A sense probe used as a negative control did not show any embryo staining (Fig.1G).

An evaluation of *CLE8* endosperm expression could not be performed through this technique because endosperm, together with the seed coats, tend to brown during the experimental procedure (Fig.1G). To overcome this limitation I cloned the *CLE8* putative promoter region upstream of the *uid-A* reporter gene and stably transformed the construct into *Arabidopsis* plants. The promoter region was chosen to include the entire genomic region upstream of the *CLE8* start codon, up to the STOP codon of the next upstream gene (2959 bp). β-glucoronidase (GUS) activity was detected in the endosperm throughout seed development (Fig.1H, J and K) and in embryos (Fig.1H and I) younger than the heart stage (Fig.1O). Suspensor cells seemed to lack GUS staining (Fig.1J). In young seeds the ESR completely surrounds the suspensor cells making it challenging to determine if the GUS staining comes from the endosperm only or from both endosperm and suspensor cells. To overcome this experimental limitation, I performed GUS assays on slides (see methods) on isolated embryos. In these conditions the GUS staining solution can permeate only one cell layer, so it is enough to assess possible suspensor cell expression. Suspensor cells did not exhibit any GUS reporter expression (Fig.1M and N), nor was staining visible in ovules prior to fertilization (Fig.1O). The promoter sequence used in these experiments was also used to drive a CLE8-GFP fusion construct that complemented the *cle8-1* phenotype (see below), indicating that GUS expression is indicative of the native *CLE8* expression pattern.

This extended expression analysis strongly suggests that *CLE8* might play a role in embryo and endosperm development, making it the first identified *Arabidopsis* ortholog of the maize *ESR* genes.

CLE8 sub-cellular localization

CLE8 protein, like other CLE family members, is predicted to be secreted to the apoplast by protein subcellular localization prediction softwares such as SignalP (http://www.cbs.dtu.dk/services/SignalP/). CLE8 contains a hydrophobic stretch at the 5` portion of the protein that is likely to act as a signal peptide, and the rest of the protein is predicted soluble and lacks any known retention signals (Fig.2A). To confirm this hypothesis I created a CLE8 translational fusion to the green fluorescent protein (GFP) and transiently transformed epidermal onion cells. As a control I transformed onion cells with GFP alone that localizes in the cytoplasm and the nucleus (Fig.2B and C). When GFP was fused to CLE8 the fluorescence was detected in the apoplast of transformed cells, including between cells that were not transformed (Fig.2D-G), indicating that CLE8 enters the secretory pathway and it is secreted to the extracellular space.

Phenotypic analysis of *cle8-1* plants

To obtain a mutant impaired in *CLE8* gene function, a TILLING for EMS-induced mutations was performed. An allele was isolated and named *cle8-1*. It exhibits a C to T nucleotide substitution at position 200 in *CLE8* coding region that results in a non-conservative amino-acid substitution of the second proline residue of the CLE domain (amino acid position +67 in the protein) with a leucine residue (Fig.3A). This proline is believed to be important for CLE peptide function because it has been shown to be subject to post-translational modifications (Kondo et al., 2006; Ohyama et al., 2009) and furthermore it is conserved in all *Arabidopsis* A-type *CLE* genes except for *CLE40* (Jun et al., 2008). This mutation is adjacent to another important amino-acid in the CLE domain, a glycine that is highly conserved (*CLE27* and *43* are the only exceptions in *Arabidopsis*). Plants carrying *CLV3* alleles mutated in this glycine were shown to exhibit a loss-of-function phenotype of moderate intensity (Fletcher et al., 1999). These previously published results indicated that *cle8-1* could be a potentially informative loss-of-function allele. To confirm this hypothesis I conducted an in-depth phenotypic analysis of *cle8-1* homozygous plants.

A careful morphological analysis of *cle8-1* homozygous plants throughout vegetative and reproductive growth did not reveal any aberrant phenotype. Homozygous plants were also analyzed for any deviation from normal timing of major developmental processes such as growth rate and flowering time and no defect was found. Mature but not yet drying siliques of wild-type Col and homozygous *cle8-1* plants were analyzed for the presence of aberrant seeds. Almost all wild-type siliques carried a full set of normally developing seeds (Fig.3B), whereas plants homozygotes for the *cle8-1* mutation showed siliques carrying defective seeds (Fig.3C). The range of macroscopic defects I observed in *cle8-1* seeds varied from wrinkled seeds drying prematurely, to seeds aborted at early stages of development (Fig.3C). Some seeds were yellowish (Fig.3C) and did not contain any embryos (data not shown), whereas others were abnormally sized (Fig.3C) and contained under-developed embryos.

To better quantify the *cle8-1* seed defect, I analyzed the morphology of dried mature seeds. Wild-type plants produced only a small percentage (0.38%, n=670) of macroscopically defective seeds (wrinkled seeds and seeds obviously smaller than average). In contrast, 3.5% (n= 517) of *cle8-1* seeds were wrinkled or obviously smaller than normal (table1). The difference between the two seed sets is statistically significant ($p<0.05$). A statistically significant difference between wild-type and *cle8-1* seeds was also evident in a germination test. Only 2% of wild-type seeds (n=150) did not germinate after 7 days of light exposure, whereas more than 14% (n= 177) of *cle8-1* seeds exhibited germination defects (table2). Most *cle8-1* non-germinating seeds did not show any sign of germination at all, and a few showed only root emergence, a defect that was never observed in wild-type seeds. A precise analysis of the defective seeds revealed that *cle8-1* seeds were significantly smaller than wild-type seeds ($p<0.05$). Mean wild-type seed length is 0.44±0.032mm and width is 0.28±0.018mm (n=100), whereas *cle8-1* seed length is 0.41±0.04mm and width is 0.26±0.02mm (n=100) (Fig.4).

To further understand the nature of the *cle8-1* defects, I isolated seeds containing developing embryos zygote stage to early globular stage and analyzed their morphological phenotype with Normaski microscopy. In this developmental window, wild-type plants carried normal looking seeds with both embryo and endosperm following the usual pattern of growth (Fig.5A-D, I). A relatively low frequency of defective seeds was observed in wild-type background (1.21%, n= 577), and the majority of those defective seeds arrested at early stages of development. In contrast, around 15% (n=572) of *cle8-1* seeds displayed strong deviations from wild-type seed development (Fig.5E-F, J-K, table3). The developmental defects observed in *cle8-1* seeds were divided into three categories depending on the tissue affected: embryo-proper, suspensor and endosperm defects (table4). Most aberrant individuals showed defects in the suspensor (41%). Wild-type embryos in the developmental window analyzed, are subtended by suspensors made of a single file of elongated cells (Fig.5A-D), whereas in *cle8-1* seeds I observed suspensor cells failing to elongate (Fig.5E and F) or top suspensor cell acquiring the wrong planes of cell division giving rise to embryos whose boundary between the embryo proper and the suspensor is unclear (Fig.5G and H). Suspensor length was measured at the dermatogen stage and found to be significantly reduced ($p<0.05$) in *cle8-1* compared to wild-type plants (Fig.6). More than 34% of *cle8-1* defective seeds showed embryo defects (table4). All wild-type embryos progressed through early development following the normal pattern of cell divisions, and their boundary with the suspensor was always recognizable (Fig.5A-D). In contrast, *cle8-1* embryo defects ranged from embryos that prematurely ceased developing (Fig.5E) to embryos undergoing uncontrolled cell division in their basal portion (Fig.5G). Among *cle8-1* defective seeds, more than 24% showed some defects in the developing endosperm (table4). In wild type seeds, at the globular embryo stage, the syncytial cytoplasm of the ESR surrounds the developing embryo and the multinucleate peripheral endosperm syncytium is a thin peripheral layer with evenly spaced nuclei (Fig.5I). In corresponding *cle8-1* seeds, I observed endosperm tissue that either lacked this organization and showed uneven distribution of the free nuclei (Fig.5K), or seeds that altogether lacked the peripheral endosperm nuclei and showed only a few nuclei at the micropilar and chalazal pole of the embryo-sac (Fig.5J).

This phenotypic analysis of *cle8-1* plants allowed me to conclude that *CLE8* plays a fundamental role in *Arabidopsis* seed development. *CLE8* regulates suspensor cell elongation and patterning, preventing the cells from acquiring the wrong plane of division, and is required to regulate proper cell proliferation in the basal domain of the embryo proper. In the endosperm, *CLE8* promotes proliferation and the organized distribution of the free endosperm nuclei.

cle8-1 complementation test

To confirm that the *cle8-1* phenotype is caused by the point mutation in *CLE8* and not by another unidentified mutation linked to the *CLE8* locus, I transformed homozygous mutant plants with a binary vector carrying the *CLE8* coding region under the control of the *CLE8* promoter sequence (*pCLE8::CLE8-GFP*). Seeds of transgenic plants were analyzed and the number of aberrant seeds was found to be significantly ($p<0.05$) lower than in untransformed *cle8-1* plants and not significantly ($p<0.05$) different from wild-type plants (table 5). Because *CLE8* genomic region was able to rescue the *cle8-1* phenotype I conclude that the aberrant phenotypes observed are solely due to the mutation in the *CLE8* coding region.

Genetic characterization of the *cle8-1* allele

Flowers from wild-type and *cle8-1* plants were hand-crossed to perform both self and reciprocal crosses between the two genotypes. Four days after pollination, I isolated seeds developing from successful crosses, cleared them in Hoyer`s solution and visualized their phenotypes (table6). As previously observed, seeds from self pollinated *cle8-1* flowers showed a statistically significant ($p<0.05$) higher level of aberrant phenotypes (12.8%, n=219) than seeds from wild-type flowers (0.46%, n=226). Heterozygous seeds from both reciprocal crosses showed a lower percentage of aberrant seeds (3.66% n=191, and 5% n=179) than *cle8-1* homozygotes, but still significantly higher percentage than wild-type. The two heterozygous seed populations are not statistically different ($p<0.05$), and thus *cle8-1* does not show a maternal sporophytic effect. These results, together with the mutant complementation analysis, revealed that *cle8-1* is a recessive allele, characterized by incomplete penetrance.

Recapitulation of the *cle8-1* phenotype by *CLE8 amiRNA* transgenic plants

To test the hypothesis that *cle8-1* is a hypomorphic allele, I attempted to obtain *CLE8* loss-of-function plants by expressing an artificial microRNA (amiRNA) specifically targeting *CLE8* (Ossowski et al., 2008). The *CLE8 amiRNA* was designed using the publicly available on-line tool WMD3 (http://wmd3.weigelworld.org) and cloned under the control of a double CaMV35S promoter in a binary vector. Wild-type *Arabidopsis* plants were transformed and more than 40 transgenic lines analyzed by RT-PCR, but none completely lacked *CLE8* transcription (Fig.7A). Nontheless, transgenic plants with reduced *CLE8* transcript levels produced aberrant seeds that phenocopied *cle8-1*. They had an unclear suspensor-embryo proper boundary (Fig.7C), endosperm defects (Fig.7D), short suspensors (Fig.7E) and basal embryo patterning defects (Fig.7E). The frequency of aberrant seeds in independent *CLE8 amiRNA* transgenic lines was not statistically different ($p<0.05$) from that observed in *cle8-1* plants, but was significantly higher ($p<0.05$) than that observed in wild-type background (table7). The analysis of *CLE8 amiRNA* transgenic plants is in accordance with *cle8-1* being a hypomorphic allele.

Expression of marker lines in *cle8-1* background

To characterize the development of *cle8-1* seeds at the molecular level, I compared the expression of cell fate markers in the mutant and wild-type backgrounds. *WOX8gΔ:NLS-venusYFP*$_3$ was chosen as a reporter gene for the basal lineage. In wild-type embryos this marker gene is expressed in all suspensor cells and at later stages of embryo development it is expressed in all suspensor cells and in the hypophysis (Fig.8A and B) as previously reported (Breuninger et al., 2008). I observed in *cle8-1* seeds that the *WOX8gΔ:NLS-venusYFP*$_3$ marker gene was only expressed in the basal cells of suspensor (Fig.8C) or its expression was strongly reduced in the upper cells of the suspensor (Fig.8D). Because of the incomplete penetrance of the allele, some *cle8-1* seeds showed the same marker expression pattern as wild-type seeds. Interestingly, in wild-type seeds the *WOX8gΔ:NLS-venusYFP*$_3$ marker was expressed in the endosperm cells of the ESR (Fig.8E-G). *cle8-1* seeds either lacked *WOX8* endosperm expression or it was restricted to few cells of the micropilar endosperm (Fig.8H-J). This analysis suggests that *CLE8* functions to establish proper gene expression programs in both the embryo basal lineage and the ESR.

To analyze early stages of endosperm development, a *MEA::GUS* reporter was chosen as a marker. In wild-type plants the *MEA::GUS* marker is expressed in the female gametophyte prior to fertilization (Fig.9A), and upon fertilization it is expressed throughout the endosperm (Fig.9C) until two days after fertilization when it becomes confined to the posterior pole (Luo et al., 2000). *MEA::GUS* marker expression in *cle8-1* unfertilized ovules was comparable to what I observed in wild-type ovules (Fig.9B). However, upon fertilization *cle8-1* seeds displayed weak *MEA::GUS* expression throughout the endosperm at a higher levels in the posterior pole, reminiscent of endosperm at later stages of development (Fig.9D). *MEA::GUS* expression analysis revealed that *cle8-1* endosperm undergoes an accelerated acquisition of characteristics of more mature endosperm, thus indicating *CLE8* acts in the endosperm to prevent premature differentiation.

CLE8 overexpression analysis

To determine if over-expressing *CLE8* would affect development, I cloned its coding region downstream of a double CaMV35S promoter and transformed this construct into wild-type *Arabidopsis* plants. Independent transgenic lines showing the highest amount of *CLE8* transcript up-regulation (Fig.10E) were analyzed. Surprisingly, no pleiotropic phenotypes were observed in *35S::CLE8* plants as are observed in plants over-expressing other *CLE* family members (Strabala et al., 2006; Strabala, 2008). However, the *35S::CLE8* seeds were significantly bigger ($p<0.05$) than those of wild-type plants grown in the same conditions (Fig.10A-D). To analyze *CLE8* over-expressing plants at the molecular level, I performed quantitative RT-PCRs on independent lines. *CLE8* over-expressing plants showed a statistically significant ($p<0.05$) up-regulation of *WOX8* transcription (Fig.10F), whereas they did not show any change in the expression levels of *WOX9*, the closest homologue of *WOX8* (data not shown). Furthermore, *WOX8* up-regulation in *35S::CLE8* plants is opposite of what was observed for the *WOX8gΔ:NLS-venusYFP*$_3$ marker in *cle8-1* seeds. Based on these data, I conclude that *CLE8* acts to specifically and positively regulate the transcription factor gene *WOX8*.

DISCUSSION

Although the CLE proteins constitute the largest known family of plant-specific signaling peptides, only a few members have been assigned a clear function in *Arabidopsis*. *CLV3* is necessary to maintain the stem cell reservoirs in apical meristems (Fletcher et al., 1999), *CLE40* function is required for organized cell divisions in the root meristem (Hobe et al., 2003), whereas CLE41 promotes the proliferation of procambial/cambial stem cells (Hirakawa et al., 2010). In this work I show that *CLE8* plays an important role during seed growth regulating different processes such as the patterning of the suspensor and the basal domain of the embryo, as well as endosperm differentiation.

CLE8 is secreted by the embryo-proper and/or the endosperm

CLE8 transcripts accumulate in the embryo-proper from the zygote to the triangular stage, as well as in the endosperm throughout seed development. Remarkably, both *cle8-1* hypomorphic seeds and *CLE8 amiRNA* transgenic seeds with reduced *CLE8* expression show defects in suspensor cell elongation and patterning, indicating a non-cell autonomous role for *CLE8* in this tissue. CLE8::GFP fusion is indeed targeted to the apoplast of transiently transformed onion cells, confirming that CLE8 can enter the secretory pathway and reach the extracellular space *in planta*, where it can activate receptors on the surface of nearby cells. Consistent with these findings, I clearly observed down-regulation of the *WOX8* marker gene for the embryo basal lineage in the upper cells of *cle8-1* suspensors, where *CLE8* is not expressed. Thus, most likely CLE8 peptide is secreted by the endosperm and/or young embryos and travels in the extracellular space to bind its ligand(s) on the surface of suspensor cells. These findings are in accordance with genetic studies that show how CLV3 protein acts as an intercellular signal that is able to move few cell layers in the shoot apical meristem and negatively regulates *WUS* transcription in neighboring cells (Lenhard et al., 2003). Further experiments will be necessary to elucidate if both the endosperm and the embryo-proper are a source of CLE8 signal targeting the suspensor cells, and if CLE8 signal emanating from the endosperm also targets adjacent maternal tissues influencing their proliferation and differentiation.

CLE8 regulates cell elongation and proliferation in the basal embryo and suspensor

The majority of *cle8-1* aberrant seeds show defects only in suspensor cell elongation and patterning, and basal embryo proliferation, even though *CLE8* transcripts accumulate throughout the embryo-proper until the heart stage. This indicates that high levels of CLE8 signal are only necessary to trigger proper development in the basal embryo, suggesting that other *CLE* genes might act redundantly with *CLE8* in the apical embryo and/or completely eliminating *CLE8* function might be required to see an effect on the apical embryo.

CLE8 function in endosperm proliferation and differentiation

Almost 25% of *cle8-1* aberrant seeds show defects in endosperm development. In most cases the endosperm starts its development but fails to persist beyond the embryo globular stage. In a few cases the distribution of the free endosperm nuclei in the embryo-sac lacks organization and the nuclei are bigger than normal. Most likely, these seeds will eventually abort or give rise to non-viable seeds. The analysis of the early endosperm-specific marker *MEA::GUS* in *cle8-1* seeds confirms this hypothesis because they displayed GUS staining in very young seeds in a fashion reminiscent of what is observed in wild-type seeds at later stages (Luo et al., 2000), suggesting *cle8-1* endosperm undergoes a faster rate of maturation. Consistent with the *MEA::GUS* marker analysis, *35S::CLE8* transgenic plants produce bigger seeds, probably due to a delay in endosperm maturation that leads to endosperm over-proliferation. Endosperm size is known to be the main determinant of embryo size, integument elongation and ultimately seed size (Garcia et al., 2003; Garcia et al., 2005). This observations suggest *CLE8* functions to promote endosperm proliferation and organization.

CLE8 downstream target

It is already known that CLE/WOX-mediated signaling modules regulate *Arabidopsis* stem cell fate in both the shoot (CLV3/WUS), the root (CLE40/WOX5) and the vasculature (CLE41/WOX4) meristems (Schoof et al., 2000; Stahl et al., 2009; Hirakawa et al., 2010), suggesting that similar regulatory mechanisms are generally responsible for stem-cell maintenance. *CLV3* and *CLE40* are known to induce transcriptional repression of *WUS* and *WOX5*, respectively. In this study I discovered that a CLE/WOX-mediated signaling module is involved in embryo and endosperm development indicating that the same module has been repeatedly recruited during evolution to regulate a variety of developmental processes. But, unlike the previously known cases, *CLE8* positively regulates *WOX8* as inferred from the reduced expression of the *WOX8gΔ:NLS-venusYFP*$_3$ marker in *cle8-1* seeds and up-regulation of *WOX8* in *35S::CLE8* transgenic plants. Notably, even though independent transgenic plants can over-express *CLE8* up to three hundred times the basal levels, *WOX8* up-regulation is only two to three times higher than normal levels in these plants. This may be indicative of saturation of the signaling pathway linking CLE8 to WOX8, perhaps at the receptor/s and/or effector/s level.

WOX8 and *WOX9* have been implicated in the patterning and proliferation of the basal part of the embryo. Embryos impaired in both *WOX8* and *WOX9* function undergo irregular cell division or form enlarged, misshapen cells in the basal lineage. Early *wox8wox9* embryos are also constituted by highly vacuolized cells that develop into finger-like structures that eventually arrest their development (Breuninger et al., 2008; Wu et al., 2007). All these phenotypes are reminiscent of *cle8-1* aberrant embryos and suspensor cells. It is important to underline that down-regulation of the *WOX8gΔ:NLS-venusYFP*$_3$ marker in *cle8-1* seeds was observed also in morphologically wild-type looking suspensors, indicating that it is not merely a secondary effect of a morphological defect, but rather an informative molecular characteristic of *cle8-1* seeds.

In my analysis I found the *WOX8gΔ:NLS-venusYFP*$_3$ marker strongly expressed in the ESR in all wild-type seed stages analyzed (zygote to torpedo). Even though early studies on *WOX8* showed it to be expressed in the endosperm by RNA in situ hybridization (Haecker et al., 2004), no mention of a possible role of this gene in endosperm development has been made in subsequent studies. Because *WOX* gene expression and function studies suggest they are generally involved in the promotion of cell division and/or prevention of cell differentiation (van der Graaff et al., 2009), it is reasonable to speculate that *WOX8* might play a role in promoting ESR proliferation. In this scenario, *cle8-1* seeds, which lack normal *WOX8* levels of expression, might exhibit a slower rate of endopserm proliferation which ultimately leads to the observed overall decrease in seed size. Conversely, plants over-expressing *CLE8* and, as a consequence, also *WOX8*, would show an over-proliferation of endosperm cells contributing to the observed increase in embryo and seed size. Alternatively, down-regulation of *WOX8* could interfere with the differentiation of the ESR, affecting the availability of nutrients for the embryo that consequently would grow at a slower rate and give rise to smaller seeds.

Implications for the identification of CLE8 receptor/s and other downstream effectors

Many studies have shown that over-expression of almost every *CLE* gene results in termination of the shoot and root meristems or other pleiotropic phenotypes (Strabala et al., 2006, Ni et al., 2006, Jun et al., 2010) implying that many different CLE peptides can activate non-cognate receptors and that specificity of function is in part achieved by sub-functionalization at the level of regulatory sequences. Interestingly, *CLE8* behaves differently than other *CLE* genes when over-expressed and does not show any aberrant phenotypes in tissues other than seeds, where it is normally expressed. Furthermore, *CLE8* is the only member of the family whose expression is restricted to seed development (Sharma et al., 2003; Jun et al., 2010). Thus, it could be speculated that *CLE8* evolved to specifically fulfill its role in seed development and diverged from other members of the family to solely interact with its receptor/s. This piece of information will be very useful in trying to identify CLE8 cognate receptor/s as I expect them to be specifically and uniquely expressed during seed development.

There are more than 610 receptor-like kinases (RLKs) in *Arabidopsis* (Shiu et al., 2001) but only a handful of them have been functionally characterized. Among those, a few have been implicated in embryo patterning and seed development. RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2) are redundantly required for central domain radial pattern formation and basal pole differentiation during the globular stages of embryogenesis. *RPK1* is expressed in the central domain and basal domain of the embryo proper, as well as the suspensor at the dermatogen stage, whereas *TOAD2* is expressed in the central and basal domains of late globular stage embryos (Nodine et al., 2007). However, *RPK1* is also expressed in vegetative tissues and implicated in abscisic acid signaling (Osakabe et al., 2005) and *toad2*/*rpk1* loss-of-function phenotypes are not similar to *cle8-1* defects, suggesting that these two RLKs are not likely to be CLE8 receptors. For the same reasons the SERK family of RLKs can be excluded as potential receptors (Li, 2010). Instead, the IKU2 RLK could be a potential receptor for CLE8 because it is solely expressed in the endosperm, and *iku2* mutants show small seed phenotypes associated with reduced growth and early cellularization of the endosperm (Luo et al., 2005). Interestingly, the MAPKK kinase YODA (YDA) and the interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinase SHORT SUSPENSOR (SSP) have been found to act in the same pathway and to be implicated in zygote elongation and basal cell linage patterning (Lukowitz et al., 2004; Bayer et al., 2009). The loss-of-function *yda* and *ssp* embryo patterning phenotypes (Lukowitz et al., 2004) are reminiscent of *cle8-1* defects, suggesting these kinases could be downstream effectors of a signal transduction pathway where CLE8 acts as a ligand.

Conclusions and perspectives

The lack of a *CLE8* loss-of-function allele, the gene dosage effect of *cle8-1* allele and the lack of amiRNA plants displaying dramatic *CLE8* down-regulation, are all consistent with the idea that *CLE8* function is essential in *Arabidopsis*. Most likely, CLE8 peptide levels are finely tuned to achieve a coordinated growth of different seed tissues. Future experiments will be aimed at dissecting *CLE8* function in endosperm and embryo development in order to understand signaling routes in developing seeds. Is *CLE8* endosperm expression alone able to rescue both the endosperm and basal embryo defects in *cle8-1*? Or is *CLE8* embryo expression fundamental to rescue the basal embryo defects? Answering these questions will contribute to unveil possible directionality of signaling among different seed tissues.

My *CLE8* over-expression results, together with the analysis of the *WOX8gΔ:NLS-venusYFP*$_3$ marker in the *cle8-1* background, support the idea that *CLE8* acts upstream of *WOX8* to induce its expression. This implies that during evolution, a CLE/WOX signaling module has also been recruited as a key mechanism to regulate seed development. It would very interesting to investigate if, as in apical meristems, the signaling module consists of a feedback regulation in which *WOX8* regulates *CLE8* expression as well as *CLE8* inducing *WOX8* expression.

Last but not least, I would explore the hypothesis that IKU2 acts in the CLE8 pathway through genetics and eventually check their possible physical interaction through biochemistry studies.

I believe that identifying the CLE8 receptor/s and, as a consequence, knowing its/their tissue localization, will be another key step in understanding how different seed tissues can communicate and coordinate their growth.

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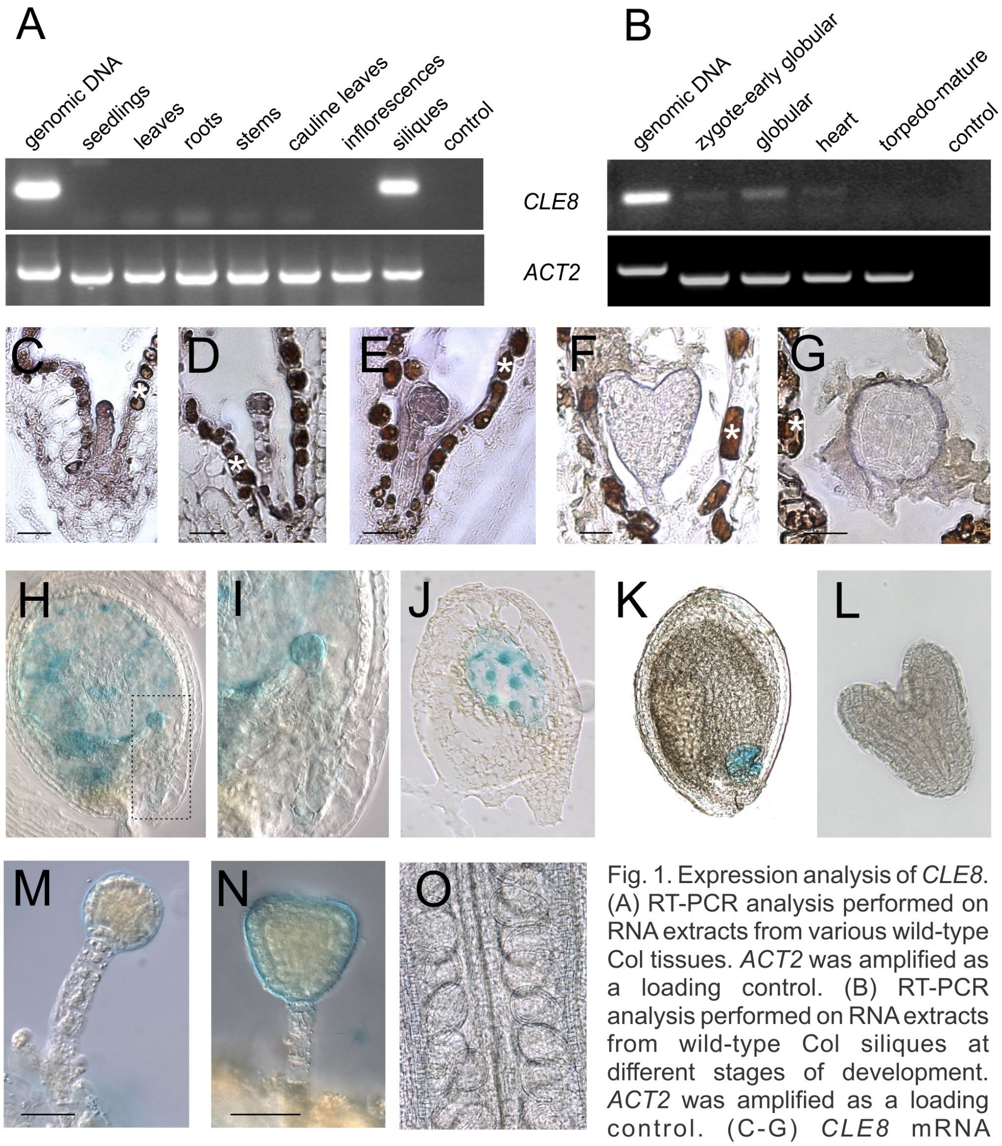

Fig. 1. Expression analysis of *CLE8*. (A) RT-PCR analysis performed on RNA extracts from various wild-type Col tissues. *ACT2* was amplified as a loading control. (B) RT-PCR analysis performed on RNA extracts from wild-type Col siliques at different stages of development. *ACT2* was amplified as a loading control. (C-G) *CLE8* mRNA expression pattern in wild-type developing seeds. *CLE8* mRNA is localized throughout the embryo-proper from one cell stage (C) to the globular stage (D and E). No signal was detected from the heart stage on (F). (G) Control hybridization with a *CLE8* sense probe. White stars indicate background browning of the seed coat. (H-O) *CLE8promoter-GUS* activity in developing seeds and unfertilized ovules. GUS staining is detected in the endosperm throughout seed development (H, J and K), in young embryos (I, M and N) until the heart stage (L). No signal has been detected in unfertilized ovules (O). Scale bars: 20µm.

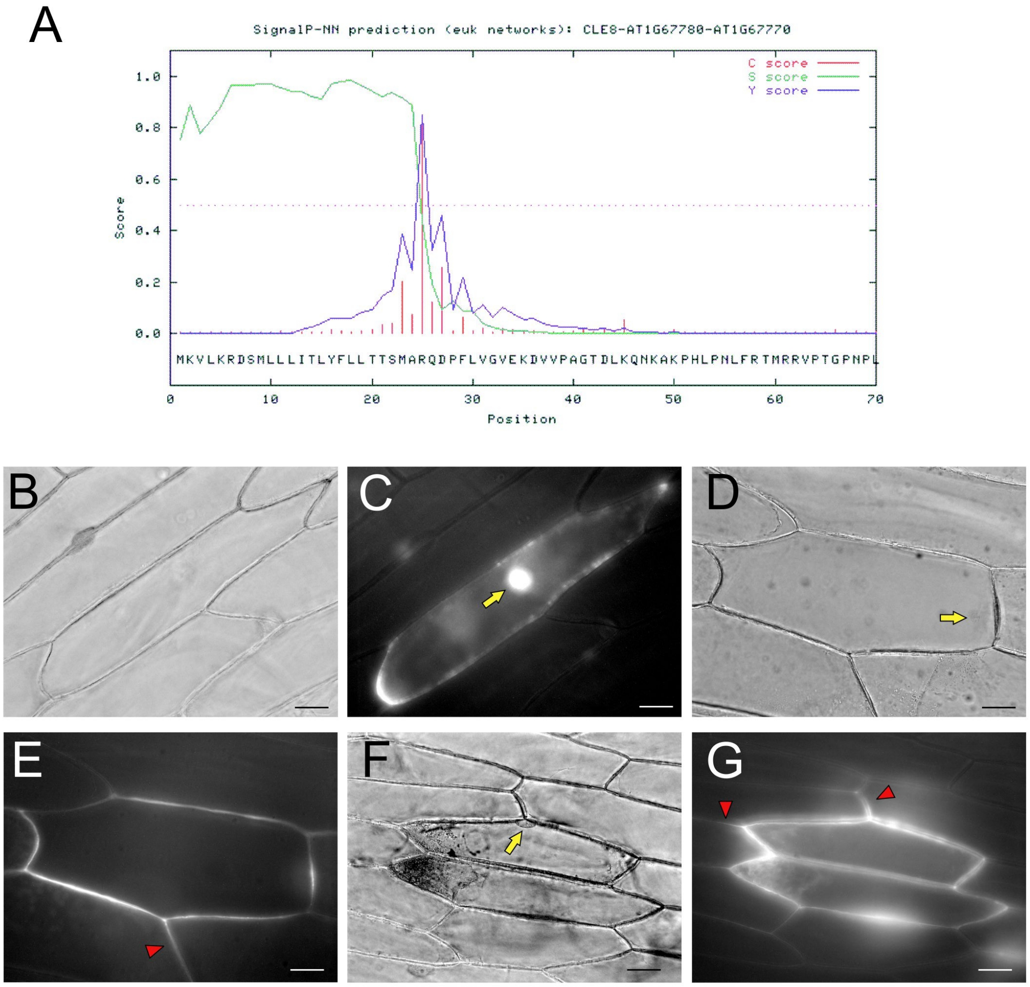

Fig. 2. CLE8 subcellular localization. (A) graphical output of CLE8 amino-acid sequence analysis performed with SignalP. (B-C) bright field (B) and dark field (C) exposure of an onion epidermal cell transiently expressing GFP. (D-G) bright field (D and F) and dark field (E and G) exposure of onion epidermal cells transiently expressing CLE8-GFP fusion. Yellow arrows indicate nuclei, red arrowheads indicate GFP signal spreading out of transformed cells via apoplast.Scale bars: 40μm.

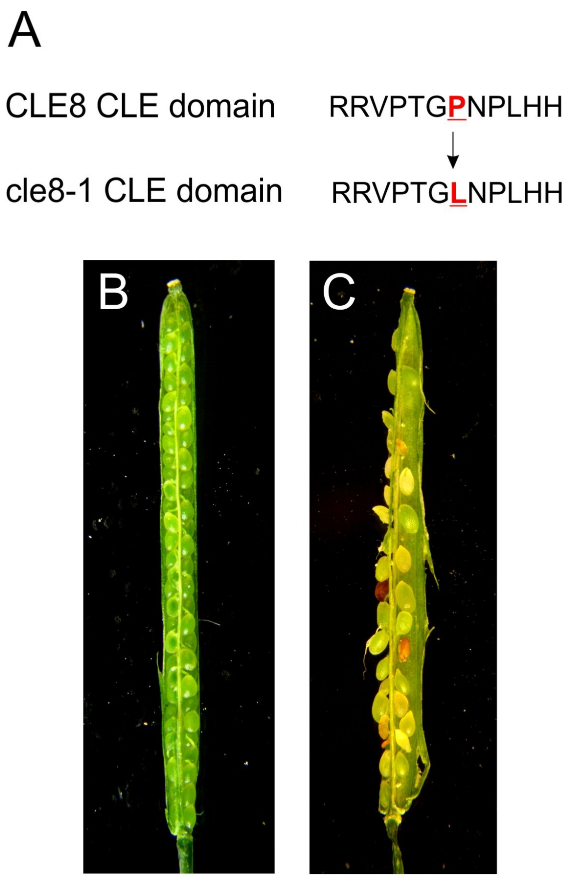

Fig. 3. *cle8-1* mutation. (A) CLE8 CLE domain amino-acid sequence in wild-type (top) and mutant (bottom) CLE8. (B) A wild-type open silique containing mature seeds. (C) A *cle8-1* open silique showing aberrant seeds.

Table 1. Seed Morphology

Seed Genotype	n	% of Defective Seeds
Col	517	0.38
cle8-1	670	3.46

n= number of seeds analyzed. Difference between values is statistically significant ($p<0.05$). Observations made on mature dry seeds.

Table 2. Seed Germination

Seed Genotype	n	% of Non-germinating Seeds
Col	150	2.00
cle8-1	177	14.10

n= number of seedlings analyzed. Difference between values is statistically significant ($p<0.05$). Observations made 7 days after germination.

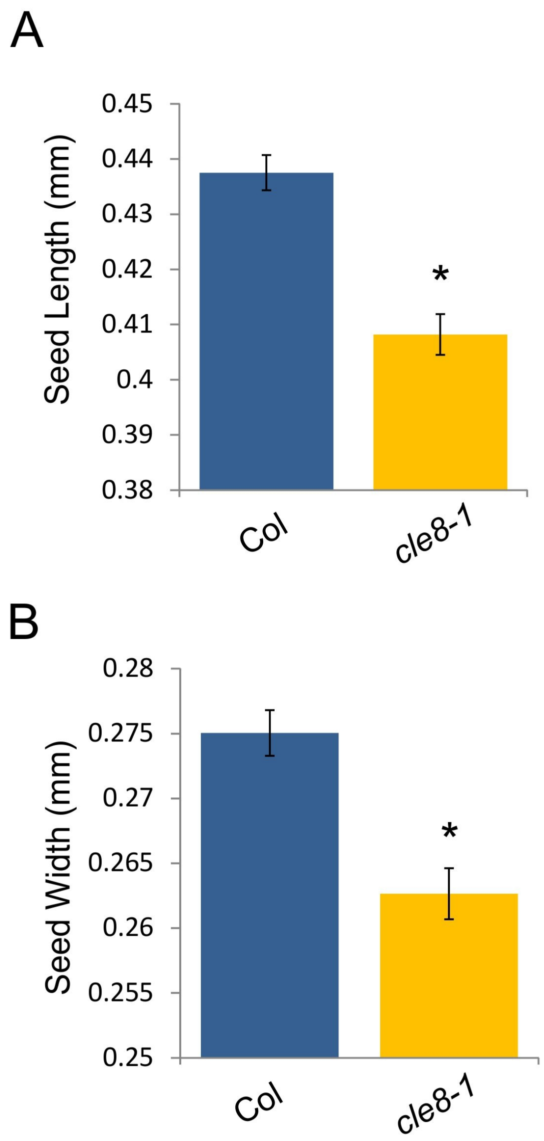

Fig. 4. *cle8-1* seed morphology. (A) Length of wild-type (blue bar, n=100) and *cle8-1* (yellow bar, n=100) seeds. (B) Width of wild-type (blue bar, n=100) and *cle8-1* (yellow bar, n=100) seeds. The standard error is indicated. *statistically significant (p<0.05).

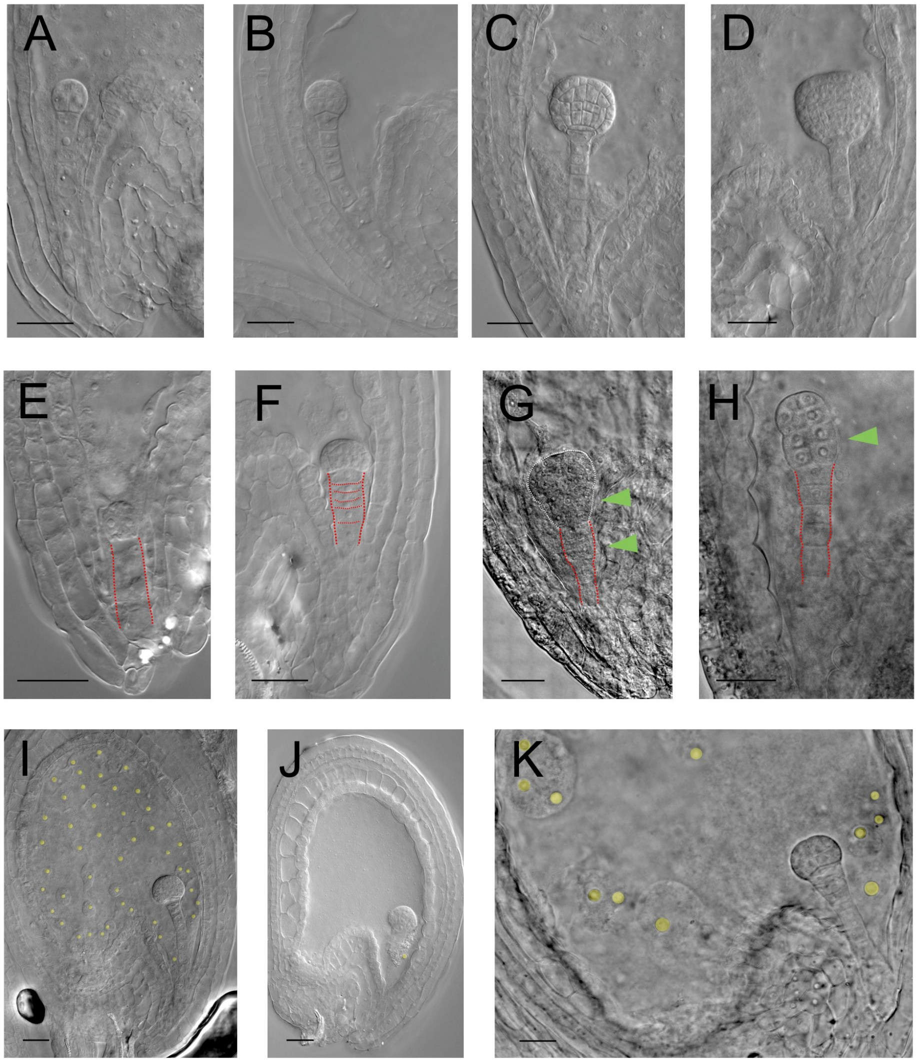

Fig. 5. *cle8-1* embryo and endosperm defects. (A-D) wild-type embryos from 2-cell stage to triangular stage. (E-H) *cle8-1* defective embryos. Red dotted lines indicate suspensors. White dotted lines outline embryo-proper shape. Green arrowheads indicate aberrant planes of cell division. (I) wild-type seed showing a regular distribution of the free endosperm nuclei. (J-K) *cle8-1* aberrant seeds almost completely lacking endosperm (J) and showing uneven distribution of the free endosperm nuclei (K). Free endosperm nuclei are highlighted in yellow. Scale bars: 20μm.

Table 3. *cle8-1* Phenotype in Developing Seeds

Genotype of Parent Plant	n	% of Defective Seeds
Col	577	1.21
cle8-1	572	15.38

n= number of seeds analyzed. Difference between values is statistically significant ($p>0.05$). Developmental window: early globular to triangular.

Table 4. *cle8-1* Phenotypic Distribution

Defective Tissue	Frequency
embryo proper	34.66%
suspensor	41.00%
endosperm	24.43%

Table 5. *cle8-1* Complementation Experiment

Genotype of Parent Plant	n	% of Defective Seeds
Col	145	2.80
cle8-1	118	14.40
*CLE8*p::*CLE8* line 8*	185	$4.30^{\dagger}$
*CLE8*p::*CLE8* line 13*	187	$6.40^{\dagger}$

*in *cle8-1* background. †Difference is statistically significant ($p<0.05$) when compared to *cle8-1* value and not when compared to wild-type value. n= numbers of seeds analyzed.

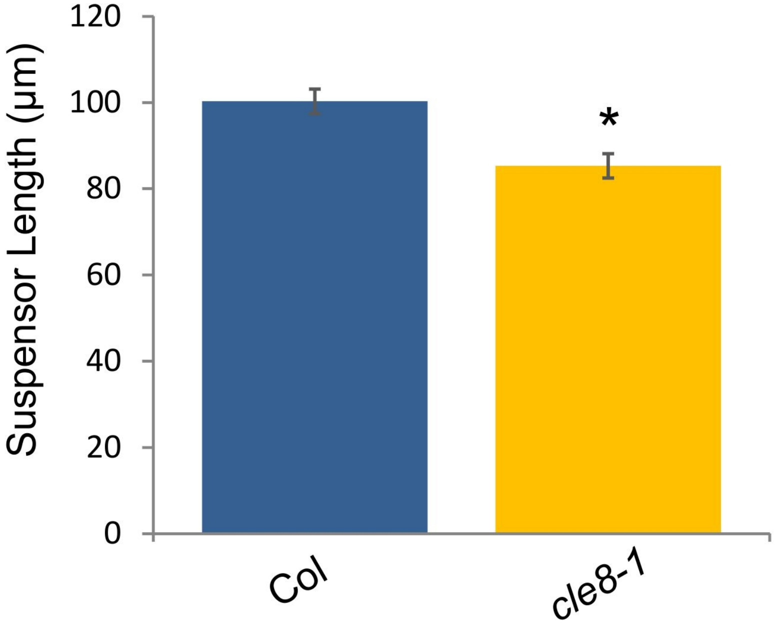

Fig. 6. *cle8-1* suspensor length. Length of wild-type (blue bar) and *cle8-1* (yellow bar) suspensors at the dermatogen stage of embryo development (n>25). The standard error is indicated. *statistically significant ($p<0.05$).

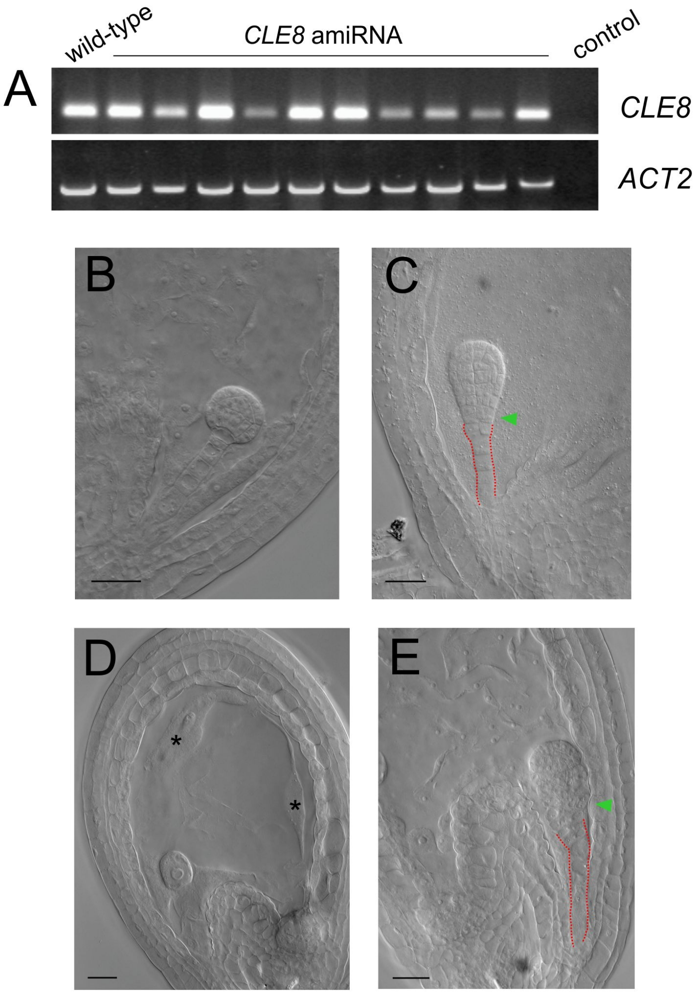

Fig. 7. *CLE8* amiRNA. (A) RT-PCR analysis performed on RNA extracts from siliques of wild-type and various independent transgenic *CLE8* amiRNA lines. *ACT2* was amplified as a loading control. (B) wild-type seed. (C-E) transgenic seeds showing aberrant development. Red dotted lines indicate suspensors. Green arrowheads indicate aberrant planes of cell division. Black stars indicate abnormal endosperm. Scale bars: 20μm.

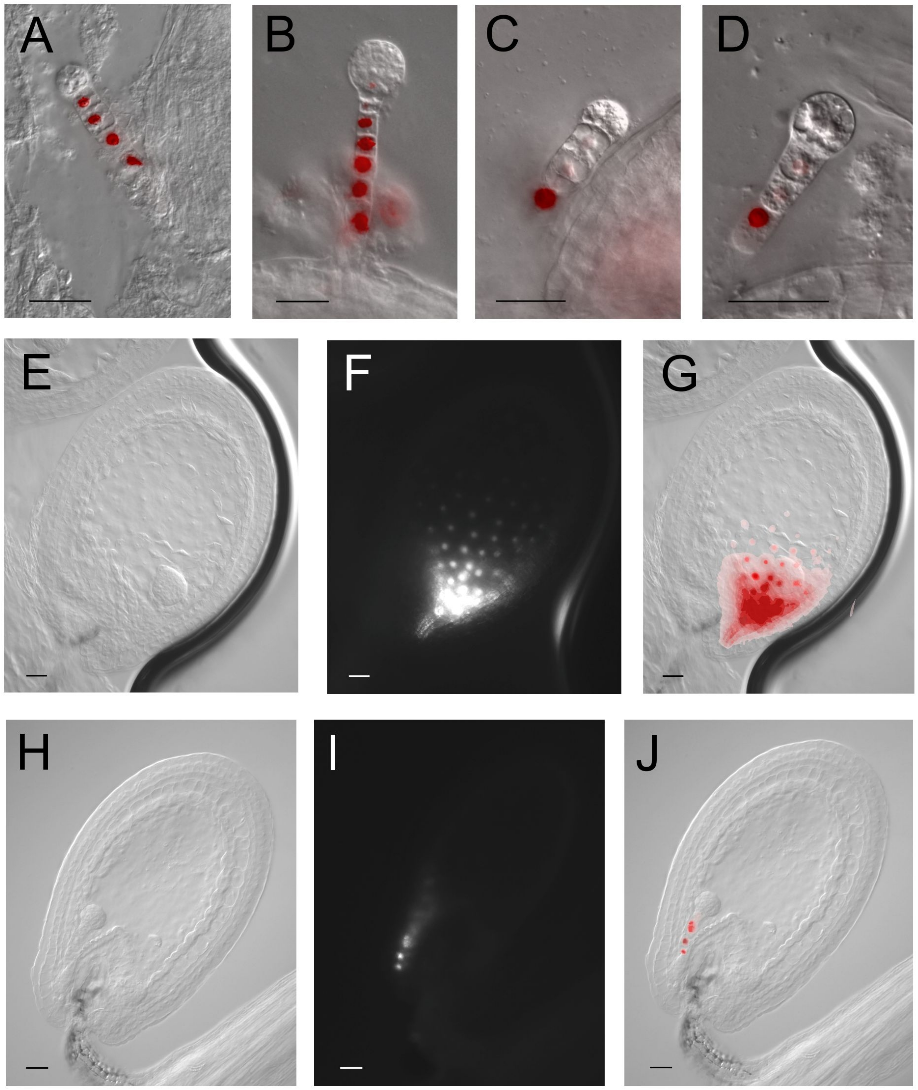

Fig. 8. *WOX8* marker expression in *cle8-1*. (A-D) Merged pictures of Nomarski images and fluorescent marker expression. In wild-type seeds *WOX8gΔ:NLS-venusYFP*$_3$ is expressed in all suspensor cells (A and B), whereas in *cle8-1* seeds the marker is only expressed in the basal cells of the suspensor (C-D). (E-J) Nomarski, florescent marker expression and merged images showing *WOX8* expression in the ESR. Wild-type seeds showing strong *WOX8gΔ:NLS-venusYFP*$_3$ expression in the ESR (E-G) and *cle8-1* seeds showing weak marker expression in the ESR (H-J). Scale bars: 20µm.

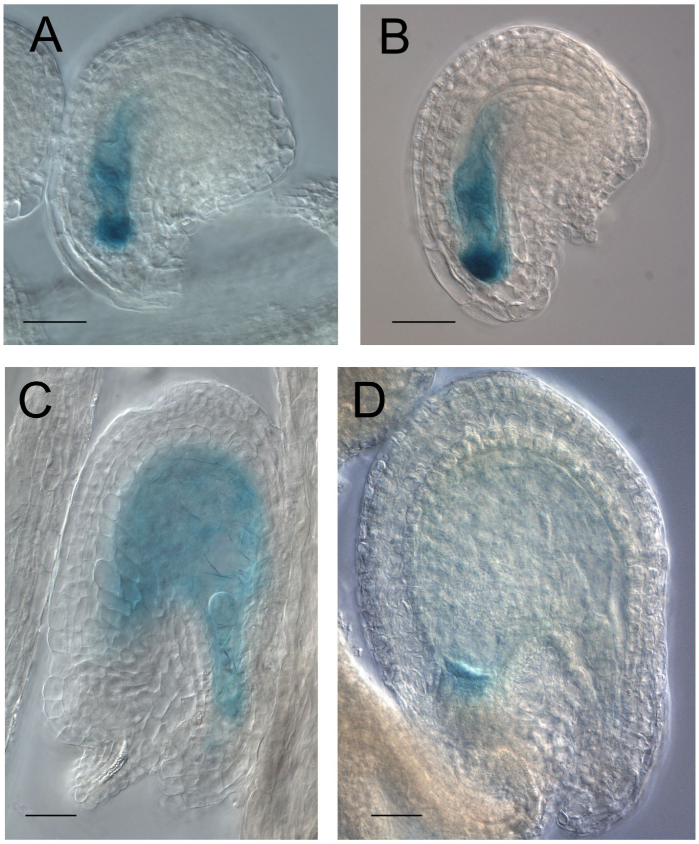

Fig. 9. *MEA::GUS* marker expression in *cle8-1*. (A and B) *MEA::GUS* expression in unfertilized wild-type (A) and *cle8-1* (B) ovules. (C and D) *MEA* marker expression throughout the endosperm in a wild-type seed (C) and *MEA* marker expression limited to the chalazal endosperm in a *cle8-1* seed (D). Scale bars: 20µm.

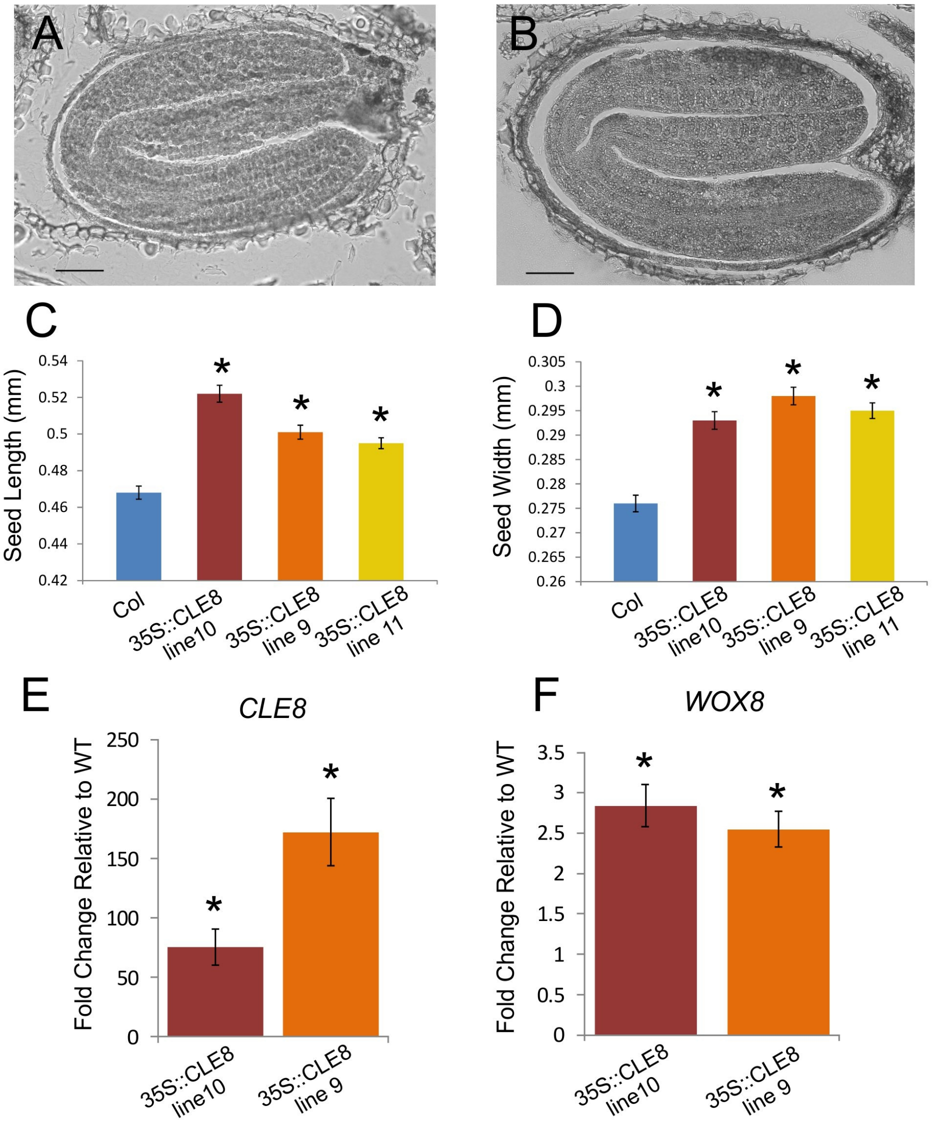

Fig. 10. Phenotypic and molecular characterization of *CLE8* over-expressing plants. (A) longitudinal section of a wild-type seed. (B) longitudinal section of a *35S::CLE8* transgenic seed. Scale bars: 50µm. (C) Length of wild-type (blue bar, n=100) and *35S::CLE8* (red, orange and yellow bars, n=100) seeds. (D) Width of wild-type (blue bar, n=100) and and *35S::CLE8* (red, orange and yellow bars, n=100) seeds. (E and F) *CLE8* (E) and *WOX8* (F) mRNA transcription levels in two independent *35S::CLE8* lines determined by real time qRT-PCR. The error bars indicate the standard error. *Statistically significant ($p<0.05$).

CHAPTER 4

The essential gene *EMB1611* maintains shoot apical meristem function during *Arabidopsis* development

ABSTRACT

The *Arabidopsis thaliana* genome contains hundreds of genes essential for seed development. Because null mutations in these genes cause embryo lethality, their specific molecular and developmental functions are largely unknown. Here we identify a role for *EMB1611/MEE22*, an essential gene in *Arabidopsis*, in shoot apical meristem maintenance. *EMB1611* encodes a large, novel protein with amino-terminal coiled-coil regions and two putative transmembrane domains. We show that the partial loss-of-function *emb1611-2* mutation causes a range of pleiotropic developmental phenotypes, most dramatically a progressive loss of shoot apical meristem function that causes premature meristem termination. *emb1611-2* plants display disorganization of the shoot meristem cell layers early in development, and an associated stem cell fate change to an organogenic identity. Genetic and molecular analysis indicates that *EMB1611* is required for maintenance of the *CLV-WUS* stem cell regulatory pathway in the shoot meristem, but also has *WUS*-independent activity. In addition, *emb1611-2* plants have reduced shoot and root growth and their rosette leaves form trichomes with extra branches, a defect we associate with an increase in endoreduplication. Our data indicate that EMB1611 functions to maintain cells, particularly those in the shoot meristem, roots and developing rosette leaves, in a proliferative or uncommitted state.

INTRODUCTION

All organisms contain a core suite of genes that are required for viability. Many essential genes have been identified in *Arabidopsis thaliana*, mainly by mutational analysis of embryo-defective (*emb*) mutants (McElver et al., 2001). At least 250 *EMB* genes are required for normal *Arabidopsis* embryo development (Tzafrir et al., 2004). These genes are thought to encode proteins for basic cellular functions, and mutants with disrupted embryogenesis have been shown to display defects in cell division or differentiation, metabolism, growth, or patterning [e.g. (Patton et al., 1998; Sparkles et al., 2003; Steinborn et al., 2002)]. However, beyond having embryo lethal phenotypes, it is not known how the vast majority of essential *Arabidopsis* genes specifically affect plant development nor is their mechanism of activity understood.

In flowering plants, essential genes may be necessary for post-embryonic growth and development in addition to their critical roles during embryogenesis. In fact, a key feature of plant embryogenesis is the establishment of root and shoot apical meristems that provide the cells for post-embryonic development. The root apical meristem (RAM) is the source of all cells for the root system. The shoot apical meristem (SAM) produces the above-ground leaves, stems and flowers while maintaining a reservoir of pluripotent stem cells for future organ formation. The *Arabidopsis* SAM is established gradually during embryogenesis through activation of the *SHOOT MERISTEMLESS (STM)* and *CLAVATA-WUSCHEL (CLV-WUS)* pathways. The homeobox transcription factor gene *STM* is required to establish and maintain the meristem cell population, acting to prevent the expression of lateral organ identity genes in the SAM (Byrne et al., 2000). The distantly related homeobox gene *WUS* is expressed in the meristem interior (Mayer et al., 1998) and confers stem cell identity on the overlying cells (Schoof et al., 2000). The stem-cell-promoting activity of *WUS* is antagonized by the secreted polypeptide CLV3 (Fletcher et al., 1999; Rojo et al., 2002), which is produced by the stem cells and activates a signal transduction pathway in the underlying cells that limits the size of the *WUS*-expressing cell population (Brand et al., 2000). The CLV-WUS pathway also maintains the stem cell reservoir in floral meristems until the specification of the carpels terminates stem cell activity (Lenhard et al., 2001; Lohmann et al., 2001).

We have analyzed a partial loss-of-function mutant that illuminates the activity of an essential *Arabidopsis* gene, *EMB1611*, which encodes a large, novel protein. *EMB1611* transcripts are widely detected during development and null alleles are lethal at early stages of embryogenesis. We demonstrate that the hypomorphic *emb1611-2* mutation causes a dramatic shoot termination defect that is associated with loss of stem cell fate and is partially *WUS*-dependent. *emb1611-2* plants have pleiotropic developmental phenotypes and show an increase in DNA ploidy levels in leaf cells, indicating that *EMB1611* is necessary to limit endoreduplication. We propose that *EMB1611* functions to maintain cells in growing tissues in a proliferative or uncommitted state.

MATERIAL AND METHODS

Mapping and cloning

An *Arabidopsis thaliana* F2 mapping population from a cross between *emb1611-2* in Columbia and the Landsberg *erecta* ecotype was used to determine the chromosomal position of *EMB1611* relative to available CAPS markers (Konieczny and Ausubel, 1993). Additional CAPS markers were created using the TIGR Landsberg *erecta* random sequence database (www.tigr.org) and a list of single nucleotide polymorphisms (SNP) between Col and L*er* (Cereon Genomics, www.cereon.com). Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Perkin Elmer, www.perkinelmer.com) according to the manufacturer’s instructions. Sequence analysis was performed using VectorNTI Suite software (Invitrogen, www.invitrogen.com).

RT-PCR

RNA was extracted from various tissues using the RNeasy Plant Mini Kit (Qiagen, www.qiagen.com), and 2 μg of RNA was used with reverse transcriptase (Invitrogen) in an RT reaction to create cDNA. 1/40th of the RT product was used in a

PCR reaction for *EMB1611* and *EF1α*, using 35 and 22 cycles of amplification, respectively. The primers used for amplification of *EMB1611* were EMBRTFSplit (5'-ccttttggcccaggaaag-3') and EMBRTR (5'-ttgatcagttagagcgattt-3'). The EMBRTFSplit primer was designed to cross an intron so that cDNA but not genomic DNA would be amplified.

EMB1611 cDNA sequencing

Six sets of primers were used to produce six overlapping PCR fragments from cDNA created by reverse transcriptase. The six PCR fragments were sequenced and aligned to create the full sequence of the *EMB1611* cDNA. Primer sequences are available upon request.

35S:: *EMB1611* construction

The *35S::EMB1611* construct was created by cloning *EMB1611* coding sequence from two PCR fragments. The 5' and 3' halves of the *EMB1611* cDNA were amplified from a cDNA library and cloned adjacent to one another into the pCR-BLUNT vector (Invitrogen). This full-length cDNA clone was sequenced and cut with ClaI and BamHI, then ligated into the pCD223 binary vector (Carles et al., 2005). *Arabidopsis* transformation was performed using the floral dip method (Clough and Bent, 1998).

Genotyping emb1*611-2* and *emb1611-3* alleles

Sequences were PCR amplified using primers close to the deletion site, which gave a 456 bp band for Col and a 418 bp for the *emb1611-2* mutation. The *emb1611-3* allele was PCR amplified using the pROK2 T-DNA vector right border primer and the genomic primer cEMB4F (5'-cctgataggcaaggtgg-3'). The wild-type *EMB1611* allele was PCR amplified using the cEMB4F and the cEMB4R (5'-agttatgcagcaacaagga-3') primers to cross the T-DNA insertion site.

Plant growth conditions

Plants were grown in 24-hour constant light at 200 µmol m^{-2} s^{-1} at 21°C. Plants were grown on MS plates for 14-17 days and transplanted to soil. The *wus-1* mutation was identified by PCR genotyping (Mayer et al., 1998). Because *EMB1611* and *WUS* are linked, a DNA marker (T4C15-86.3kb) closely linked to the *emb1611-2* mutation was used to genotype *emb1611-2* plants in the cross with *wus-1*.

Tissue sectioning and GUS staining

Tissue was fixed in 3.7% formaldehyde: 50% ethanol: 5% acetic acid for 1-2 hours, followed by dehydration and embedding in Paraplast X-tra embedding medium (Fisher Scientific, www.fishersci.com). Samples were sectioned at 8 µm thickness and placed onto slides, then stained for 1-2 minutes in 0.1% toluidine-blue stain (Sigma-Aldrich, www.sigmaaldrich.com) containing 0.1% sodium borate. The slides were dehydrated and mounted, viewed under a compound microscope and photographed with a digital camera. GUS staining, tissue embedding and sectioning were performed as described previously (Carles et al., 2005).

RNA *in situ* hybridization

Tissue fixation and hybridization were performed as described in the Nonradioactive In Situ Hybridization Application Manual (Roche, www.roche.com). The *WUS* antisense probe was generated as described previously (Mayer et al., 1998) using a digoxigenin-labeling mix (Roche). The *EMB1611* probe was generated by cloning the first 1001 bp of the coding sequence into pGEMTeasy (Promega,www.promega.com).

Scanning electron microscopy (SEM)

Scanning electron microscopy was performed as described previously (Bowman et al., 1989) using a Hitachi 4700 scanning electron microscope.

Root growth measurements

Roots were grown vertically and photographs taken starting on day 6 post-germination and ending on day 14. The images were measured using NIH Image software. Lines were drawn along the roots and then the length of each line converted to cm. Lateral roots were counted as the number of visible lateral roots in the images taken.

Flow cytometry

Rosette leaf nuclei were extracted as described (Galbraith et al., 1983). Samples were filtered through a 20 µm nylon mesh and centrifuged for 5 min at 1000Xg. The pellet was resuspended in 500 µl of 50 µg/ml propidium iodide (PI) (Sigma-Aldrich) and incubated for 5 min on ice (Johnston et al., 1999). The nuclei were re-pelleted and resuspended in 300 µl PI. 1/10 dilutions were made with the 50 µg/ml PI stain and analyzed by flow cytometry. Approximately 10,000 events were counted and the proportion of nuclei of each size determined as a percentage of all nuclei counted. The ploidy of each peak was determined by comparison with the 2C peak of a tomato leaf sample.

RESULTS

Characterization of the Essential Gene *EMB1611*

At2g34780 is an essential gene in *Arabidopsis* that consists of 8 exons and 7 introns (Figure 1A, B). A mutation in this gene was identified previously in a screen for embryo-lethal mutants (McElver et al., 2001) and named *emb1611*. This *emb1611-1* allele is a T-DNA insertion into the fifth intron (Figure 1B) that causes lethality at the preglobular stage of embryo development (www.seedgenes.org). Another mutation in *At2g34780* was detected in a large-scale screen for defects in female gametophyte development (Pagnussat et al., 2005) and called *mee22*. The *mee22* allele is a Ds insertion into an unknown location in the last exon that reportedly causes early embryo lethality and arrested endosperm development (Pagnussat et al., 2005).

The *At2g34780* locus encodes a novel 1297 amino acid protein. Structural analysis programs (Lupas et al., 1991) predict the presence of amino-terminal coiled-coil domains (Figure 1C) and, within this region, an InterPro prefoldin domain similar to those present in myosin heavy chain protein (Rayment et al., 1993) and the molecular chaperone prefoldin (Siegert et al., 2000). In addition, transmembrane topology prediction programs (Hofmann and Stoffel, 1993; Rost *et al.*, 1996; Tusnady and Simon, 1998) indicate the presence of two putative transmembrane (TM) domains near the carboxyl terminus (Figure 1C). Database searches revealed homologs of the predicted *At2g34780* protein in *Oryza sativa* and *Medicago truncatula*. The predicted protein from Oryza locus *Os01g0768700* consists of 374 amino acids and is 26% identical and 43% similar to the carboxyl terminus of the *At2g34780* predicted protein. However, this annotated Oryza product lacks a start codon and is likely to be an incomplete sequence. The predicted protein from Medicago (ABE90049) contains 1284 amino acids and is 28% identical and 46% similar to the full-length *At2g34780* predicted protein.

emb1611-2 is a recessive mutation causing pleiotropic phenotypes (see below) that was discovered in a line (SAIL 1147_A10) of T-DNA-mutagenized plants of the Columbia (Col) ecotype. Although the line contained at least three T-DNA insertions, none was linked to the mutant phenotypes. Consequently, we used positional cloning (Konieczny and Ausubel, 1993) to map the *emb1611-2* mutation to the bottom half of chromosome 2 between the Cleaved Amplified Polymorphic Sequence (CAPS) markers COP1 and M429 (Figure 1A). Additional markers were used to fine map the locus between CAPS markers on BACs T29F13 and F19I3. Sequencing of candidate genes in the region revealed a 38-base-pair deletion in *At2g34780* that was linked to the *emb1611-2* phenotypes. This deletion causes a frameshift near the carboxyl terminus that adds twenty novel amino acids and then introduces a stop codon that truncates the protein, deleting the last 137 amino acids (Figure 1C).

We isolated an additional *EMB1611* allele*, emb1611-3,* that confirmed that the gene is essential for viability. The *emb1611-3* allele is a T-DNA insertion (Alonso et al., 2003) at the end of exon four, which adds 23 novel amino acids before introducing a premature stop codon (Figure 1B, C). In the selfed progeny of a heterozygous *emb1611-3/+* plant, homozygous *emb1611-3* plants were never recovered. The genotype of the progeny was a very strong fit to a 2:1 ratio of heterozygous plants (30/44) to homozygous wild-type plants (14/44). Thus, *emb1611-3* embryos either fail to form or abort early enough to abort seed production.

We used complementation to confirm the identity of *At2g34780* as *EMB1611*. Because of the large size of the genomic region we over-expressed the *At2g34780* coding sequence under the control of the Cauliflower Mosaic Virus 35S promoter, which restored both the *emb1611-2* phenotype (Figure 3B, C) and the *emb1611-3* phenotype to wild type. Thus, from the mapping data and rescue of the mutant phenotypes, we conclude that *At2g34780* corresponds to the *EMB1611* gene. Introduction of 35S*::EMB1611* into wild-type Col plants did not cause a detectable phenotype, although *EMB1611* steady-state transcript levels were increased to 3-4 times the normal amount. Therefore, *Arabidopsis* plants are not sensitive to this amount of elevated *EMB1611* expression.

EMB1611 mRNA is Broadly Expressed

EMB1611 has a broad yet specific expression pattern in *Arabidopsis* tissues. The gene is transcribed at low but equivalent levels in all tissues based on RT-PCR (Figure 2A). During early embryogenesis *EMB1611* transcripts are found in the endosperm and throughout early globular stage embryos (Figure 2B), but become more concentrated in the inner tissues of late globular, heart and torpedo stage embryos (Figure 2C-E). By the bending cotyledon stage, *EMB1611* transcripts are uniformly distributed throughout the embryo (Figure 2F). After germination we observe *EMB1611* mRNA expression throughout the shoot apical meristem and young leaf primordia of developing seedlings (Figure 2H). Following the transition to flowering, we continue to observe specific *EMB1611* mRNA expression throughout the inflorescence meristems and in developing floral meristems (Figure 2J). As flower formation progresses *EMB1611* transcripts become cleared from the outer layers of maturing sepals and petals but remain detectable throughout the developing stamens and carpels (Figure 2K). *EMB1611* is also expressed in roots (Figure 2A), but we did not explore its precise root expression pattern because the affect of *EMB1611* on shoot development was our primary focus. Thus, *EMB1611* is specifically expressed in embryos, and above-ground in shoot and floral meristems as well as developing organ primordia.

emb1611-2 Mutation Reveals a Role for *EMB1611* in Stem Cell Maintenance

The *emb1611-2* mutation caused the premature termination of the reproductive shoot apical meristem (SAM) in a multiple floral branch point, similar to the botanical structure known as an “umbel” (Figure 3B). This structure consisted of two to four flowers produced at the same terminal node, with the concomitant loss of the inflorescence meristem. Compared to wild-type inflorescence meristems (Figure 3A) that produced more than 50 flowers in total, *emb1611-2* inflorescence meristems generated an average of 6.35 +/- 0.41 flowers (range is 3 to 12), including the flowers at the terminal point. Analysis of the terminal structures of *emb1611-2* plants revealed that the terminus was a branched structure consisting of multiple pedicels, with no terminal flower but instead a flat apex at the shoot tip (Figure 3D, E). No SAM structures were detected at the apex of these terminated shoots and no dome of meristematic cells or floral buds was observed (Figure 3E), suggesting that the SAM is completely consumed in the production of the flowers at the terminal point.

Analysis of mature Col and *emb1611-2* embryos using confocal laser scanning microscopy revealed no detectable differences in their embryonic SAMs. We next analyzed the SAM during seedling growth to determine if the *emb1611-2* mutation affects vegetative meristem size (Figure 4A-F). The mean SAM width of 7 day old *emb1611-2* seedlings (41.74 +/- 1.75 μm, n=11) was slightly, but significantly, smaller than that of wild-type seedlings (47.09 +/- 1.35 μm, n=12; $p<0.05$). The mean SAM height of 7 days old *emb1611-2* seedlings (7.15 +/- 0.33 μm, n=11) was also smaller than that of wild type seedlings (10.33 +/- 0.75 μm, n=12; $p<0.05$). At 17 days after germination, *emb1611-2* SAMs began to be displaced from their apical position (Figure 4E), and we could observe reduction in the size of the primary inflorescence meristem to that of a floral meristem

(Figure 4F), consistent with the final meristem termination in an “umbel” of flowers.

Sections revealed disorganization of the cells within *emb1611-2* vegetative meristems. Wild-type shoot apical meristems are organized into three clonally distinct cell layers (Figure 4A). These layers consist of two single-cell-thick tunica layers (L1, L2), in which the cells maintain their clonal lineages by dividing in the anticlinal plane, and an underlying corpus (L3) in which the cells divide in any plane. The individual cells, particularly in the L1 and L2, are regular in size and shape. In contrast to the distinct layering and regular size of wild type SAM cells, the clonal layering of *emb1611-2* vegetative meristems appeared disrupted, with no clear delineation between the L2 and L3 cells, and the cells themselves displayed irregular sizes and shapes (Figure 4B, C). These phenotypes were observed as early as 7 days after germination. Thus, the cells of *emb1611-2* shoot apical meristems become disorganized and misshapen early during vegetative development.

The functional zones of the SAM were also disrupted in *emb1611-2* plants. We utilized a *pSTM::uidA* reporter line (McConnell and Barton, 1998) as a marker to visualize the specific activity of the GUS reporter in the peripheral zone of the meristem (Carles et al., 2005), and analyzed its expression in wild type and *emb1611-2* seedlings. The pattern of *pSTM::uidA* activity in the periphery of the meristem was largely unaltered in 7-day-old *emb1611-2* seedlings (Figure 4H) compared to wild-type seedlings (Figure 4G); however, a few *emb1611-2* individuals showed light GUS staining in the center of the SAM (Figure 4I). Some 13-day-old *emb1611-2* seedlings showed a normal pattern of *pSTM::uidA* activity while others displayed *pSTM::uidA* activity throughout the SAM. In 17-day-old *emb1611-2* plants, *pSTM::uidA* activity was invariably detected throughout the meristem (Figure 4J-L). Serial sections confirmed that the GUS signal was uniformly distributed throughout the SAM, showing that all of the cells had peripheral zone identity. These results suggest that as the *emb1611-2* SAM ages, the central stem cell domain assumes a peripheral zone fate.

Because *pSTM::uidA* activity gradually spreads throughout the SAMs of *emb1611-2* plants and they undergo premature termination, we hypothesized that the *CLV-WUS* stem cell feedback loop was not sustained in *emb1611-2* SAMs. We tested this hypothesis by using *in situ* hybridization to examine the expression of *WUS*, which is transcribed in the central, interior region of the SAM (Figure 4M, P) and confers stem cell identity on the overlying cells (Schoof et al., 2000). Among 7-day-old *emb1611-2* seedlings, 50% had a normal *WUS* expression domain (Figure 4N) whereas the other 50% displayed no *WUS* expression in the SAM (Figure 4O). Among 14-day-old *emb1611-2* seedlings, 50% showed *WUS* expression in a reduced domain and at lower levels than in wild-type seedlings (Figure 4Q), whereas the other 50% displayed no *WUS* expression in the SAM (Figure 4R). Thus, the failure of *emb1611-2* plants to maintain their inflorescence meristems correlates strongly with reduction or absence of the *WUS*-expressing cell population that promotes the fate of the overlying cells as stem cells.

We note that *emb1611-2* inflorescence meristems but not floral meristems undergo premature termination. One simple explanation is that *emb1611-2* maintains both types of meristems, however only the inflorescence meristems manifest a premature termination phenotype because the floral meristems differentiate into carpels before the *emb1611-2* mutation can trigger a complete cell fate switch. If so, prolonging the period in which *emb1611-2* floral meristems are indeterminate would be expected to ultimately cause termination in an abnormal branch point. We tested this hypothesis by crossing *emb1611-2* to *ag-3* plants, which contain a null mutation in the floral homeotic gene *AGAMOUS (AG)*. The *ag-3* mutation transforms the normally determinate floral meristems into indeterminate structures that produce reiterations of sepals and petals in place of stamens and carpels (Bowman et al., 1991). We found that *emb1611-2 ag-3* flowers consisted of reiterating whorls of sepals and petals and were indistinguishable from *ag-3* flowers (Figure S1). We therefore conclude that the *EMB1611-1* mutation likely affects inflorescence but not floral meristems because of intrinsic differences in their cellular structures or molecular compositions.

Maintenance of the *CLV-WUS* Pathway Depends on *EMB1611*

The *CLV* signal transduction pathway is a key element of a negative feedback loop that regulates stem cell accumulation in the shoot apical meristem. *clv1-4* mutants have enlarged shoot and floral meristems (Figure 5A) due to excessive stem cell accumulation (Clark et al., 1993). *clv1-4* is a dominant negative allele of *CLV1* that exhibits the most severe *clv1* phenotypes (Dievart et al., 2003). *clv3-2* mutants have virtually the same phenotype as *clv1-4* mutants (Clark et al., 1995), although *clv3-2* is a recessive, null allele of *CLV3* (Fletcher et al., 1999). Because *EMB1611* is required to maintain shoot apical meristem stem cell fate and high levels of *CLV3* and *WUS* transcription, we hypothesized that a reduction in *EMB1611* function might affect the function of the *CLV-WUS* regulatory pathway.

We tested this hypothesis by creating *emb1611-2 clv1-4* and *emb1611-2 clv3-2* double mutant plants. Remarkably, *emb1611-2 clv1-4* plants terminated in an *emb1611-2* (Figure 5B) manner, with several floral pedicels emerging from a terminal point (Figure 5C). The timing of termination in the *emb1611-2 clv1-4* double mutants was similar to *emb1611-2* single mutants, indicating that the *clv1-4* mutation is unable to rescue the *emb1611-2* SAM termination phenotype. In contrast, the double mutant flowers resembled *clv1-4* flowers, containing supernumerary organs in all whorls (Figure 5C-E). This result is consistent with *emb1611-2* having little or no effect on floral development. *emb1611-2 clv3-2* plants also failed to show rescue of the *emb1611-2* shoot termination phenotype and produced an "umbel" flowering structure. The double mutant flowers displayed the *clv3-2* phenotype of supernumerary organs in each whorl. Thus, wild-type *EMB1611* activity is required in order for excess stem cells to accumulate in *clv1-4* and *clv3-2* inflorescence, but not floral, meristems.

The major target of negative regulation by the *CLV* pathway is *WUS* (Brand et al., 2000; Schoof et al., 2000). *wus-1* is a strong allele that causes failure to maintain the SAM stem cell reservoir and production of a terminal organ(s) at the shoot apex through consumption of the remaining meristematic cells (Laux et al., 1996). Ectopic SAMs are continuously initiated but not maintained by *wus-1* plants, and their inflorescence meristems terminate abnormally in a small number of defective flowers (Figure 4F). Because both *wus-1* and *emb1611-2* plants (Figure 5G) terminate inflorescence meristem activity prematurely, we generated *emb1611-2 wus-1* plants to determine if the two genes act in the same genetic pathway.

The phenotype of *emb1611-2 wus-1* double mutant plants was more severe than that of either single mutant alone. *emb1611-2 wus-1* plants (Figure 5H) were smaller than wild-type and smaller than either *wus-1* or *emb1611-2* plants. Under our growth conditions 50% (n=45) of *wus-1* plants produced inflorescence structures and reproductive organs (Figure 5F), whereas the remainder arrested during vegetative development. In contrast, 100% (n=26) of *emb1611-2 wus-1* plants arrested during vegetative development and never produced inflorescences or flowers (Figure 5H). This result indicates that *EMB1611* is required for the partial shoot rescue observed in *wus-1* plants. Yet, because both single mutants had the capability to produce an inflorescence meristem at least half of the time, the double mutant phenotype appears to be synergistic in nature. Thus *EMB1611* and *WUS* have partially overlapping functions in maintaining shoot apical meristem activity.

Activity of the *TERMINAL FLOWER1 (TFL1)* CETS family gene (Pnueli et al., 2001) prevents the inflorescence meristem from acquiring floral meristem identity (Liljegren et al., 1999). *tfl1-1* is a strong allele (Ohshima et al., 1997) that causes early flowering and SAM termination though the production of a flower structure directly from the inflorescence apex (Alvarez et al., 1992; Shannon and Meeks-Wagner, 1991). Because both *tfl1-1* (Figure 5I) and *emb1611-2* plants undergo premature inflorescence meristem termination, we determined whether the two genes act in the same genetic pathway. *emb1611-2 tfl1-1* plants appeared indistinguishable from *emb1611-2* plants except for the primary inflorescence meristem, which produced a terminal flower at the apex like *tfl1-1* plants (Figure 5J). Although the primary SAM in the *emb1611-2 tfl1-1* mutant resembles that of a *tfl1-1* mutant, it likely does not represent a case of genetic epistasis but rather an issue of developmental timing. Because *tfl1-1* plants are committed to making a terminal flower by eight days of growth (Shannon and Meeks-Wagner, 1991), SAM termination occurs earlier in *tfl1-1* than in *emb1611-2* plants. Additionally, we observed that *tfl1-1* axillary meristems produced more flowers prior to terminating than did the primary meristem, and in *emb1611-2 tfl1-1* plants the “umbel” phenotype was observed in some of these axillary meristems. Therefore, it is most likely that *tfl1-1* appears phenotypically epistatic to *emb1611-2* in the primary SAM because the *tfl1-1* mutation converts the inflorescence to a floral meristem before the *emb1611-2* mutation can induce complete termination.

emb1611-2 Plants Display Pleiotropic Phenotypes

emb1611-2 plants displayed abnormal vegetative growth. To characterize this phenotype we followed the development of 100 Col and 100 *emb1611-2* plants from germination through a time course of landmark events: radicle emergence, cotyledon emergence, cotyledon expansion, emergence of the first two true leaves, emergence of leaves three through eight, and bolting time (Figure S2). We found that the timing of these events became progressively delayed in *emb1611-2* plants. *emb1611-2* seeds were delayed by 24 hours compared to wild-type seeds in radicle emergence, cotyledon expansion and first leaf emergence. The time at which 50% of *emb1611-2* plants reached a certain developmental landmark lagged one, two, and then three days behind wild-type plants, and the range in timing of event occurrence was far larger for *emb1611-2* than for wild-type plants.

The *emb1611-2* mutation caused significant growth arrest or reduction during seedling development. Only 2% of wild-type but 11% of *emb1611-2* plants died during the vegetative stage, representing a significantly higher rate of death among *emb1611-2* than Col seedlings ($chi^2=41.33$, $p<0.001$). Another 12 *emb1611-2* plants failed to produce an inflorescence meristem, and thus 23% (23/100) of all *emb1611-2* plants failed to reach the reproductive phase. The number of rosette leaves at the time of flowering was smaller in *emb1611-2* (9.31 +/- 1.05 S.D.; range 8 to 13) than in wild-type plants (17.68 +/- 2.17; range 14 to 21). *emb1611-2* plants also had smaller rosette diameters (13.5 +/- 3.29 mm) than wild-type plants (32.27 +/- 3.67 mm) when measured at 24 days after germination (Figure 6A, B), and the overall size of mature *emb1611-2* plants (Figure 6D) was much smaller than that of Col plants (Figure 6C). Yet despite their delayed and reduced growth, *emb1611-2* plants flowered earlier than wild-type plants. *emb1611-2* plants that did not terminate in the rosette stage bolted an average of one week earlier than wild-type plants, with 50% of *emb1611-2* plants bolting by 24 days and 50% of the Col plants bolting by 31 days (Figure S2).

Flower pedicel length was also affected by the *emb1611-2* mutation. Wild-type *Arabidopsis* pedicels were fairly uniform in length and formed a bell-shaped curve around the mean (Figure 6F). In contrast, *emb1611-2* pedicel length exhibited a large degree of variation and did not cluster around a mean (Figure 6G, H). Mean pedicel length was 0.55 +/- 0.09 cm for wild-type and 1.04 cm +/-0.41 for *emb1611-2* plants, and wild-type pedicels ranged in length from 0.35 to 0.75 cm whereas *emb1611-2* pedicels ranged in length from 0.56 to 2.80 cm (Figure 6H). The *emb1611-2* pedicel cells themselves were not enlarged, suggesting that the partitioning of excess cells into the pedicel primordia may underlie the increase in *emb1611-2* pedicel length. In contrast, *emb1611-2* flowers did not differ greatly from wild-type flowers (Figure 6I), with only occasional irregularities observed (Figure 6J). In contrast to *emb1611-2* inflorescence meristems, we did not detect premature termination of *emb1611-2* floral meristems. *EMB1611* activity thus appears to be largely dispensable for flower development.

The root system of *emb1611-2* plants was dramatically reduced compared to wild-type plants. Full-grown *emb1611-2* plants had shorter roots and an overall less-branched root system. To analyze root growth during development, we grew Col and *emb1611-2* seedlings vertically on plates and measured root length at 6, 8, 10, 12 and 14 days post-germination (Figure 7A). We found that both wild type and *emb1611-2* roots grew at a linear rate, but the ~0.25 cm/day growth rate of *emb1611-2* roots was significantly less than the ~0.78 cm/day growth rate of wild-type roots (Figure 7A, B). Yet unlike the shoot apical meristems, *emb1611-2* root apical meristems did not undergo termination. The roots of 9-day-old *emb1611-2* plants had a normal morphology and their organization and layering was indistinguishable from that of wild-type roots (Figure 7D, E). The cells of *emb1611-2* and wild-type roots were similar in size, ruling out defects in root patterning or cell expansion as primary causes of the short root phenotype. Together, these results suggest that *emb1611-2* roots are shorter than wild-type roots due to a reduced cell division rate.

Wild-type Col plants have an extensive system of lateral roots when mature, whereas the number of branches produced by *emb1611-2* roots was greatly reduced (Figure 7C). Lateral roots in *emb1611-2* plants emerged normally from the primary root. However, *emb1611-2* plants formed an average of 0.15 lateral roots/cm in 10-day-old *emb1611-2* plants versus 1.01 lateral roots/cm in Col plants. These data indicate that *emb1611-2* plants produce far fewer lateral roots than normal, and that their reduced number is not merely a consequence of the roots being shorter.

EMB1611 is Required to Limit Endoreduplication

emb1611-2 leaves were smaller and narrower than wild-type leaves, and had considerably shorter petioles and blades (Figure 8A, B). We compared wild type and *emb1611-2* leaf surfaces using SEM and found that the cells on both the abaxial and adaxial surfaces of *emb1611-2* leaves appeared similar to wild-type cells, with no significant difference in shape or size. However, *emb1611-2* leaves displayed trichome defects. Trichomes are single-celled hairs on the *Arabidopsis* epidermis that grow outward and develop a branched morphology. Wild-type trichomes contain either three or four branches (Figure 8C), with a mean of 3.15 +/- 0.36 branches per trichome (n=74). *emb1611-2* trichomes formed at least three and as many as six branches (Figure 8D, E), with a mean of 3.72 +/- 0.64 branches per trichome (n=229), a statistically significant increase (t-stat= 9.61; $p<0.01$).

Trichomes undergo multiple rounds of endoreduplication, DNA replication without cell division. Because hyper-branched trichomes have been associated with excessive amounts of DNA in trichome cells (Hulskamp et al., 1994), we tested the hypothesis that *emb1611-2* leaves have excessive DNA content. DAPI-stained *emb1611-2* trichome nuclei (Figure 8G) appeared slightly brighter than wild-type nuclei (Figure 8F), suggesting a higher chromosomal content. Wild-type leaf pavement cells have ploidy levels from 2C to 16C (Melaragno et al., 1993). Analysis of whole leaf nuclear DNA extracts using flow cytometry revealed a reduction in the number of low (2C and 4C) DNA content nuclei and a relative increase in the number of higher (8C and 16C) DNA content nuclei in *emb1611-2* compared to wild-type leaves (Figure 8H). This result shows that normal *EMB1611* activity is required to control the extent of endoreduplication in wild-type leaf cells.

DISCUSSION

The *EMB1611/MEE22* gene is required for early embryogenesis in *Arabidopsis*. Here we report the characterization of a partial loss-of-function allele, *emb1611-2*, that sheds light on the role of this essential gene throughout *Arabidopsis* development. Unlike the embryo lethal *emb1611-1* and *emb1611-3* alleles, which are caused by T-DNA insertions into exons midway through the gene, the *emb1611-2* allele introduces a small deletion near the carboxyl-terminus that leaves the coiled-coil domains and one putative transmembrane domain intact. This truncated EMB1611-2 protein appears to retain sufficient function to allow the mutant plants to progress through embryogenesis, but not to support proper post-embryonic development. The death of greater numbers of *emb1611-2* plants as vegetative growth progresses suggests that the lack of full EMB1611 function becomes increasingly difficult for the plants to compensate for over time.

We isolated the *emb1611-2* allele in a screen to identify novel regulators of shoot apical meristem-derived growth, and evidence from morphological, molecular and genetic analyses indicates that *EMB1611* has an important function in sustaining shoot apical meristem activity during post-embryonic development. *emb1611-2* plants undergo premature SAM termination, and *emb1611-2* vegetative and inflorescence meristems are smaller than wild-type meristems. Progressive disorganization of the clonal cell layers is observed in *emb1611-2* SAMs, and the individual cells become misshapen and enlarged. These results indicate that *EMB1611* is required to maintain the cellular organization of the SAM. The *emb1611-2* allele indicates that the initial organization of the SAM occurs normally in the presence of reduced *EMB1611* activity, but that such reduced activity is not sufficient for SAM maintenance over time. Contrastingly, *EMB1611* activity appears to be dispensable for the maintenance of floral meristems, even when they are rendered indeterminate.

Several complementary lines of evidence demonstrate that *EMB1611* plays a key role in maintaining SAM cell fate. First, analysis of *pSTM::uidA* reporter gene expression reveals that the stem cells in the center of *emb1611-2* SAMs gradually assume the fate of peripheral zone cells. Second, neither *WUS* nor *CLV3* expression is sustained in the interior of *emb1611-2* SAMs. Third, mutations in CLV signal transduction components do not rescue the *emb1611-2* SAM phenotype, indicating that *EMB1611* is required for amplification of the stem cell pool in *clv1* and *clv3* SAMs. These data are consistent with *EMB1611* maintaining the identity of the stem cells upon which the CLV-WUS feedback loop acts, and/or promoting their proliferative capacity. In the absence of *EMB1611*, the *WUS*-dependent stem-cell-promoting signal gradually diminishes as the plants age, causing premature meristem termination as the stem cell population differentiates to produce a cluster of floral meristems across the shoot apex. However, unlike *emb1611-2* and *wus-1* plants, *emb1611-2 wus-1* plants never form inflorescence meristems. Thus *EMB1611* also acts partially independently of *WUS* to sustain SAM activity. The involvement of *EMB1611* in promoting SAM function is likely an indirect consequence of its requirement to maintain the cellular organization of the SAM, rather than a direct regulatory effect.

The trichome phenotype found in *emb1611-2* rosette leaves revealed a defect at the cellular level. Wild-type *Arabidopsis* trichomes are single-celled hairs that consist of three (occasionally four) branches, whereas most *emb1611-2* trichomes form more than three branches. During their development trichomes undergo several rounds of endoreduplication, and the number of trichome branches depends on the ploidy level of the cell (Hulskamp, 2004). Tetraploid *Arabidopsis* plants, in which the DNA content of each cell is doubled, produce hyper-branched trichomes (Hulskamp et al., 1994). Mutants such as *triptychon, kaktus* and *rastafari* form hyper-branched trichomes that have a higher DNA content than wild-type trichomes (Perazza et al., 1999). Conversely, *glabrous3* trichomes have fewer branches and contain less DNA than wild type (Hulskamp et al., 1994). We observed a higher proportion of 8C and 16C cells in *emb1611-2* than in wild-type leaves, showing that a significant proportion of *emb1611-2* leaf cells went through at least one additional round of endoreduplication after mitotic arrest. This may account for the extra branch formation in *emb1611-2* trichomes.

The endoreduplication defect observed in *emb1611-2* rosette leaves provides insight into a potential molecular mechanism for EMB1611 function during development. Endoreduplication is a unique form of cell cycling in which the chromosomal DNA of the cell is replicated without intervening mitoses. This process, a common mechanism for increasing cell size in plants, is under precise genetic control and is tightly linked with cell fate specification (Sugimoto-Shirasu and Roberts, 2003). The extra endocycles in *emb1611-2* leaf cells suggest two possible mechanisms for EMB1611 activity. One is that *emb1611-2* cells fail to terminate the endocycle at the correct ploidy level, in which case EMB1611 may be part of a mechanism that limits the extent of endoreduplication. The other is that *emb1611-2* cells prematurely switch from the mitotic cycle to the endocycle, in which case EMB1611 might be required to delay the onset of endoreduplication. With respect to the latter, an attractive explanation for the progressive disorganization, cell fate alteration, and early termination of *emb1611-2* SAMs is that the stem cells prematurely exit the active proliferation cycle, either entering or triggering pathways leading to differentiation. A similar link between cell cycle control and shoot meristem integrity has been made by Andersen et al. (Anderson *et al.*, 2008), who showed that disruption of the cyclin-dependent kinase genes *CDKB2;1* and *CDKB2;2* affected SAM organization and led to premature meristem termination. Like *emb1611-2* plants, plants with reduced *CDKB2* expression had significantly higher nuclear DNA content; thus, our data and theirs together illustrate the importance of coordinating meristem organization and strict cell cycle regulation to maintain proper SAM activity during development.

Endoreduplication often occurs during the differentiation of other specialized cell types, including endosperm (Boisnard-Loriga et al., 2001), and thus the arrested endosperm phenotypes of *mee22* plants may also be linked to defects in endoreduplication and/or cell cycle progression. The *mee22 Ds* insertion site is reportedly in the last exon of *EMB1611* and confers partial gametophytic lethality (Pagnussat et al., 2005). In contrast, the *emb1611-1* and *emb1611-3* alleles contain T-DNA insertions in the fifth intron and fourth exon, respectively, and behave genetically as simple embryo-lethal alleles with no gametophytic effects. One possible explanation for this discrepancy is that the *mee22* gene product causes neomorphic activity in the gametophytes. Alternatively, the *mee22* segregation ratio (Pagnussat et al., 2005) is consistent with *mee22* conferring partially-penetrant, dominant lethality. Further investigation of the nature of the *mee22* allele should provide insight into the reason for the phenotypic disparity between *mee22* and the *emb1611-1* and *emb1611-3* alleles.

The *EMB1611* gene is essential for *Arabidopsis* embryo development, and potentially for endosperm development as well. The embryo-lethal phenotypes caused by the severe *emb1611-1* and *emb1611-3* alleles and the pleiotrophic developmental phenotypes of *emb1611-2* plants are consistent with a key requirement for EMB1611 in a general cellular process, and our results suggest a role for this gene in promoting cell division and cell proliferation, likely by modulating endoreduplication. Although the precise mechanism remains to be defined, analysis of this hypomorphic mutant provides insights into the control of shoot apical meristem organization and maintenance, and reveals the activity of an essential *Arabidopsis* gene.

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FIGURES

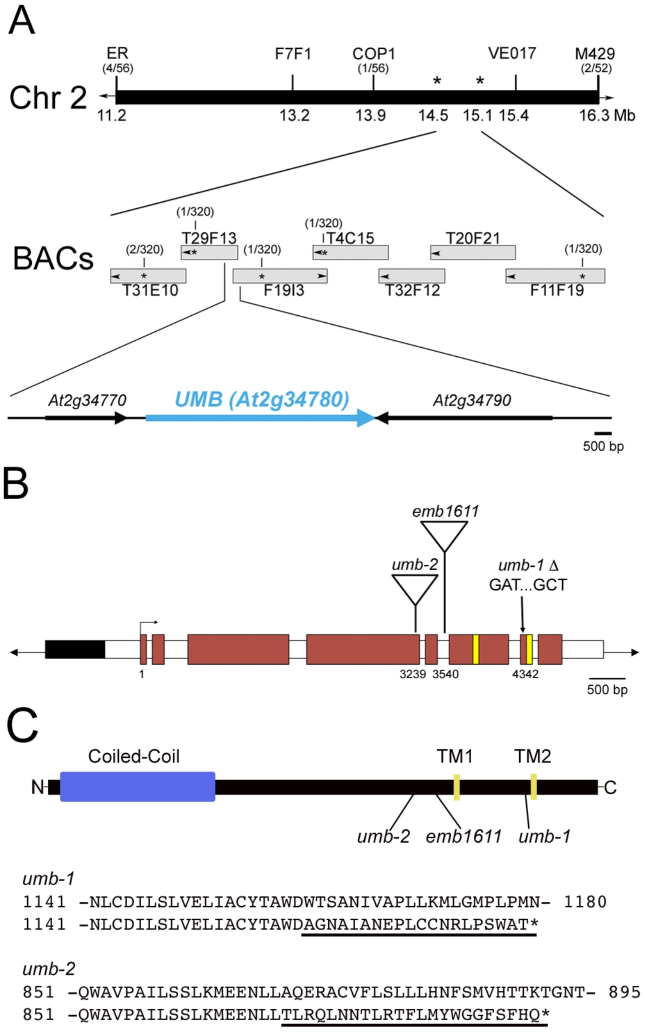

Figure 1. Identification of *At2g34780* as *EMB16*.

(A) Mapping and cloning of the *EMB1611* gene. The relative positions of cleaved amplified polymorphic sequence (CAPS) markers on chromosome 2 are shown on the top line. Asterisks indicate the positions of the CAPS markers used for fine mapping. The number of recombinant chromosomes is denoted in parenthesis. Arrowheads indicate the directions of the bacterial artificial chromosomes (BACs), and arrows indicate annotated transcription units. (B) The *EMB1611* gene structure is represented by red boxes for exons, and by white boxes for untranslated regions (UTRs) and introns. Yellow boxes denote the locations of the nucleotides encoding the two putative transmembrane (TM) domains. The black box indicates the last exon of the nearest upstream gene. The positions of the *emb1611-2* deletion mutation, and the *emb1611- 3* (SALK_33170) and *emb1611-1* (SAIL_30945) insertion mutations, are shown. (C) Schematic diagram of the

EMB1611 structural domains and locations of the mutations. The amino acid alterations caused by the *emb1611-2* and *emb1611-3* mutations are underlined. The numbers represent the amino acid positions relative to the translational start site, and the asterisks indicate stop codons.

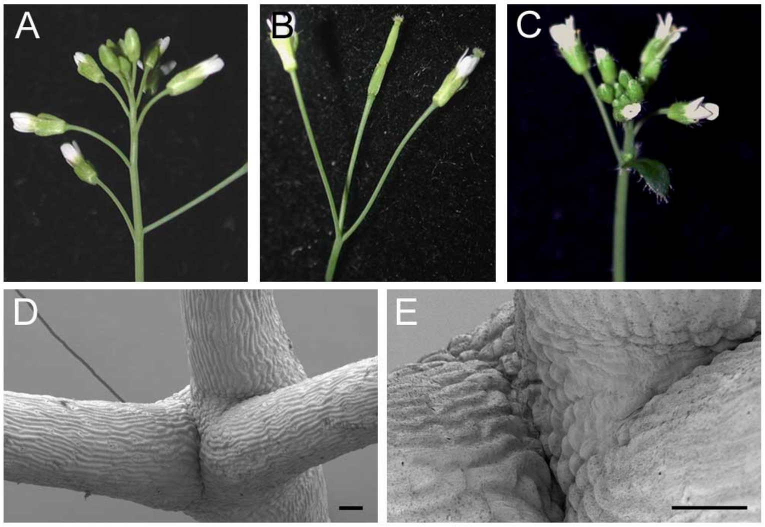

Figure 2. Meristem termination phenotypes of *emb1611-2* plants.

(A) Wild-type Columbia (Col) inflorescence meristem. (B) *emb1611-2* inflorescence meristem. (C) Rescue of *emb1611-2* inflorescence termination phenotypes by a *35S::EMB1611* construct. (D) Micrograph of an *emb1611-2* apex. (E) Higher magnification of the apex in (D). Scale bars: 50 µm.

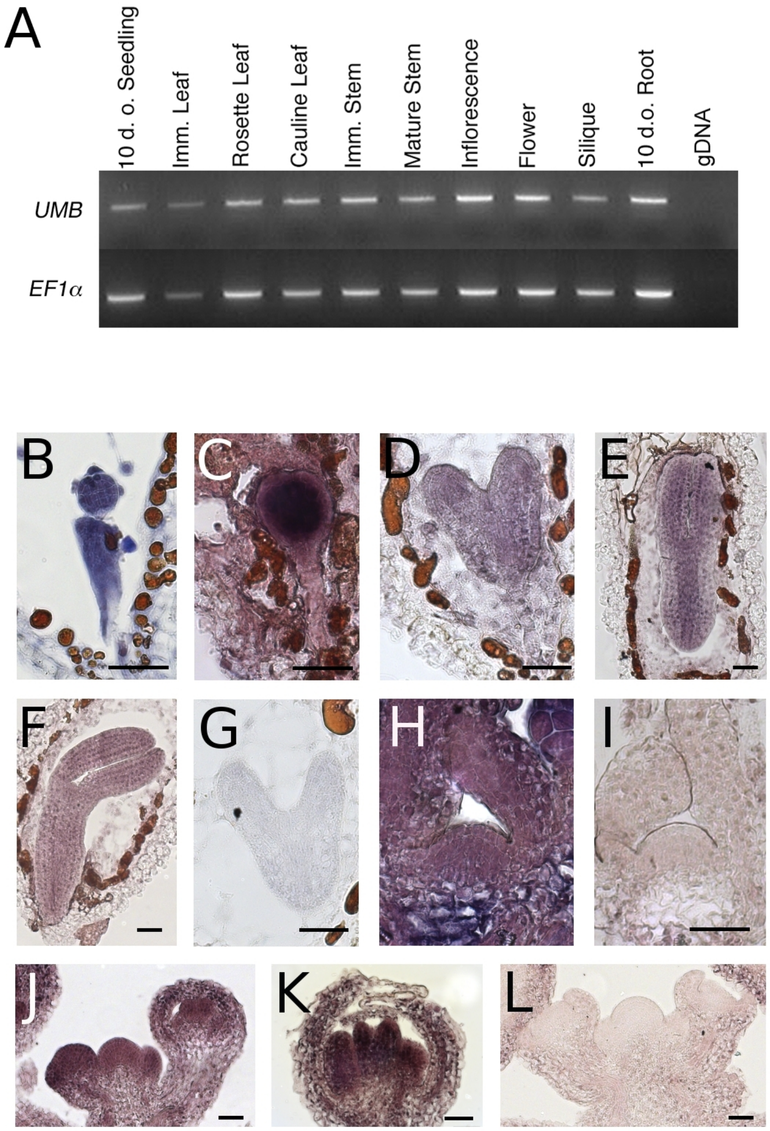

Figure 3. Expression analysis of *EMB1611*.

(A) *EMB1611* expression in various *Arabidopsis* tissues during development. *EF1α* is shown as a loading control. (b–l) *EMB* mRNA expression patterns in wild-type Columbia (Col) embryos, seedlings and inflorescences. B) Early globular stage. (C) Globular stage. (D) Heart stage. (E) Torpedo stage. (F) Bending cotyledon stage. (G) *EMB* sense probe. (H) Vegetative SAM and developing leaf primordia of 7-day-old seedlings. (I) *EMB* sense probe. (J) Inflorescence meristem and young floral meristems. (K) Stage-9 flower. (L) *EMB* sense probe. Scale bars: 25 µm.

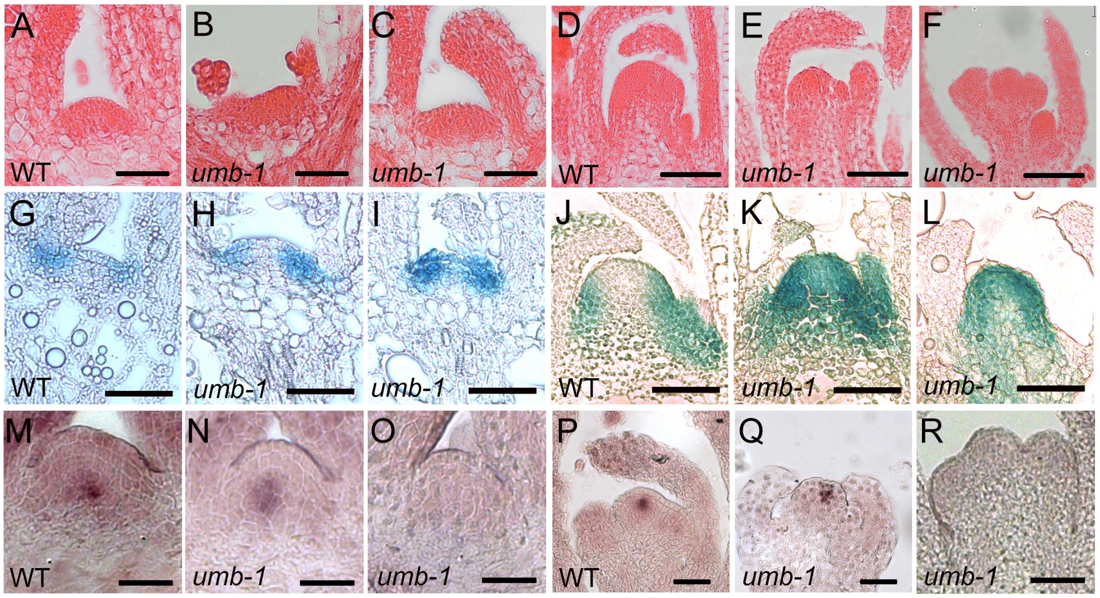

Figure 4. Meristem phenotypes during *emb1611-2* development.

(A–C) Sections of 7-day-old Columbia (Col) (A) and *emb1611-2* (B, C) vegetative meristems. (D–F) Sections of 17-day-old Col (D) and *emb1611-2* (E, F) meristems. (G–I) Sections of 7-day-old meristems from Col (G) and *emb1611-2* (H, I) plants containing a pSTM::GUS marker for the peripheral zone. (J–L) Sections of 17-day-old meristems from Col (J) and *emb1611-2* (K, L) plants containing a pSTM::GUS marker. (M–O) In situ expression analysis of *WUS* in 7-day-old Col meristems (M), and in 7-day-old *emb1611-2* meristems in which *WUS* expression is present (N) or absent (O).

(P–R) *WUS* expression in 14-day-old Col meristems (P), and in 14-day-old *emb1611-2* meristems in which *WUS* expression is present (Q) or absent (R). Scale bars: 25 μm (A–C, M–R); 50 μm (D–L).

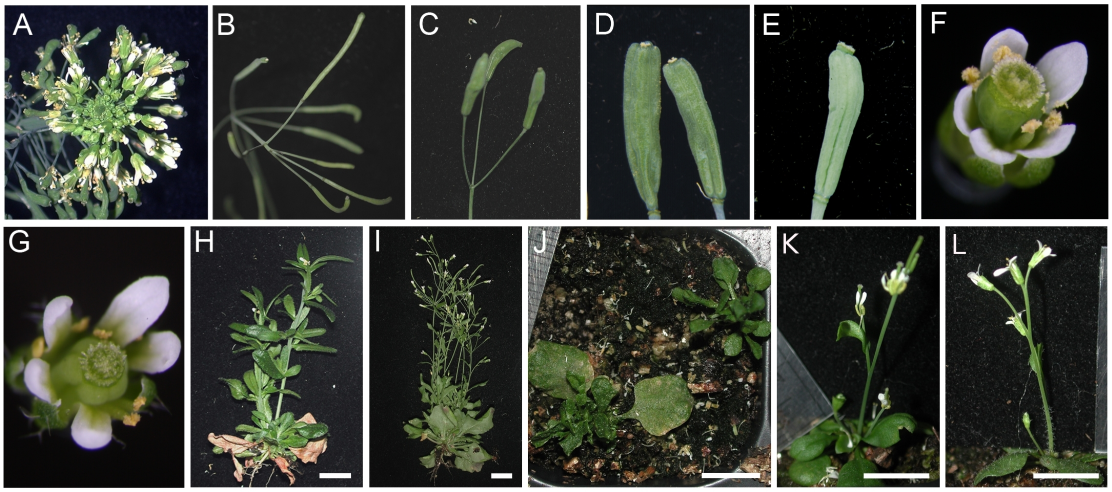

Figure 5. Double mutants between *emb1611-2* and alleles of meristem genes.
(A) *clv1-4* enlarged inflorescence. (B) *emb1611-2* terminated inflorescence. (C) *emb1611-2 clv1-4* terminated inflorescence. (D) *clv1-4* flower. (E) *emb1611-2 clv1-4* flower. (F) Mature *wus-1* plant. (G) Mature *emb1611-2* plant. (H) Mature *emb1611-2 wus-1* plants. (I) *tfl-1* plant. (J) *emb1611-2 tfl-1*plant. Scale bars: 1 cm.

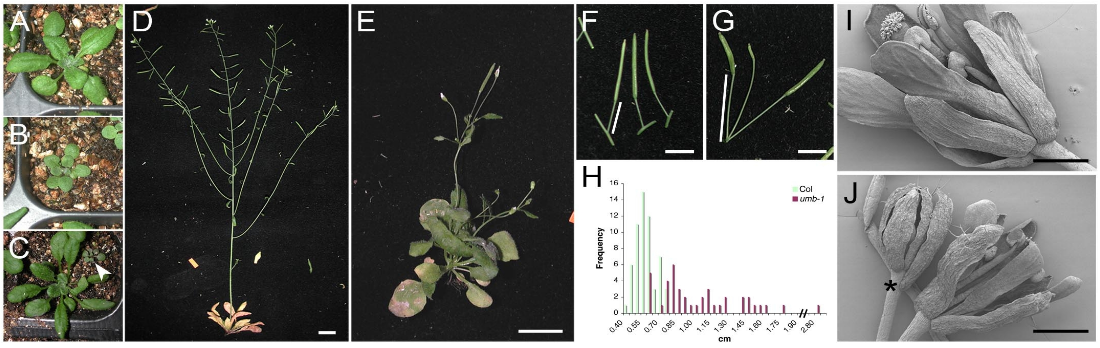

Figure 6. Morphological phenotypes of *emb1611-2* plants.

(A) Columbia (Col) rosette at 20 days old. (B) *emb1611-2* rosette at 20 days old. (C) *emb1611-2*/emb1611-3 rosette at 20 days old (arrowhead). (D) Mature Col plant. (E) Mature *emb1611-2* plant. (F) Pedicels (white bar) from a Col plant. (G) Elongated pedicels (white bar) from an *emb1611-2* plant. (H) Distribution of pedicel length in Col (green, n = 60) and *emb1611-2* plants (magenta, n = 40). The frequency of pedicels of the indicated length is shown. (I) Micrograph of a Col flower. (J) Micrograph of an *emb1611-2* flower subtended by a sepal structure (asterisk). Scale bars: 1 cm (D, E); 5 mm (F, G); 50 μm (I, J).

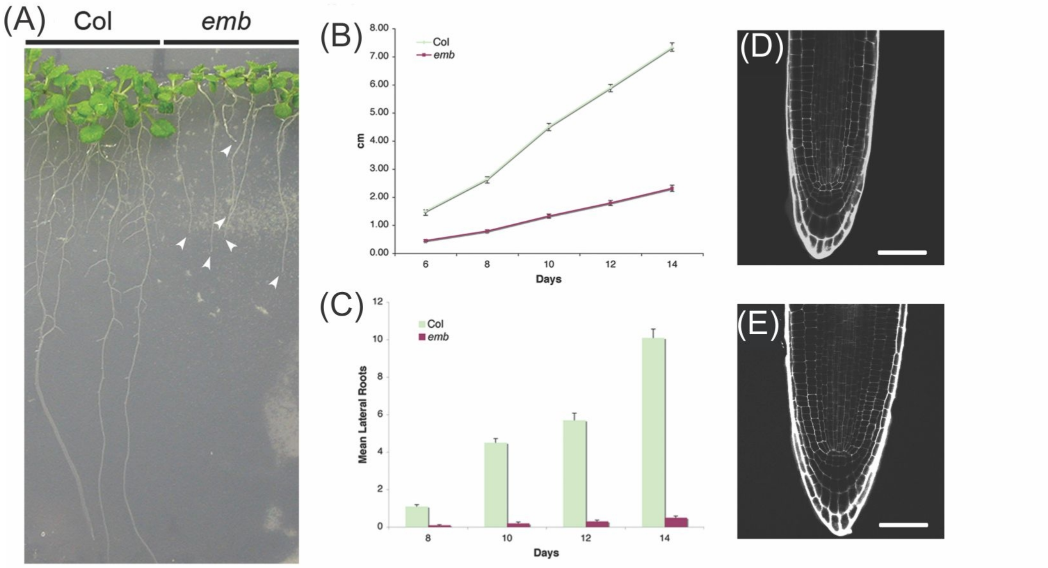

Figure 7. Root phenotypes of *emb1611-2* plants.

(A) Root growth of Columbia (Col) and *emb1611-2* seedlings. Arrowheads denote the *emb1611-2* root tips. (B) Root length in Col (green) and *emb1611-2* (magenta) seedlings (n = 20). Bars represent standard errors. (C) Lateral root formation in developing Col (green) and *emb1611-2* (magenta) seedlings (n = 20). Bars represent standard errors. (D) Confocal laser scanning micrograph of a Col root tip. (E) Confocal laser scanning micrograph of an *emb1611-2* root tip. Scale bars: 50 μm.

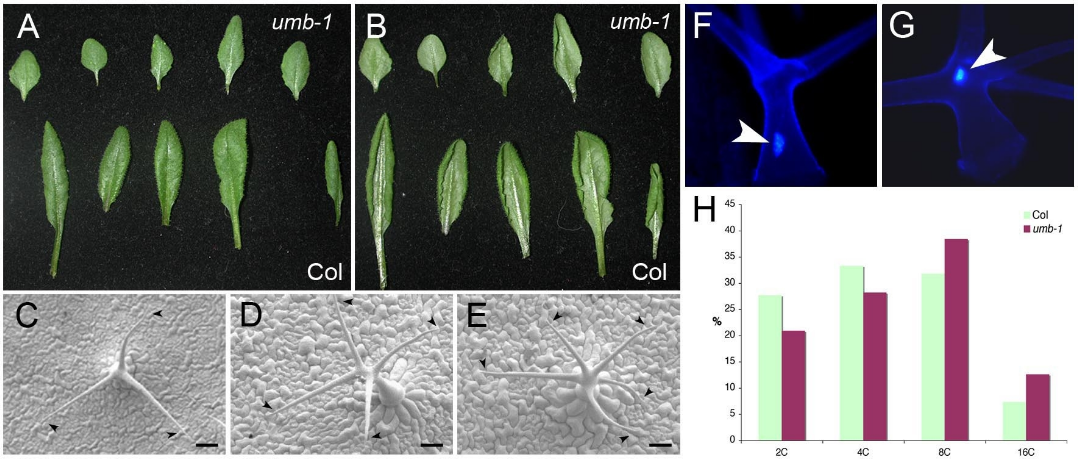

Figure 8. Leaf phenotypes and mechanism of *EMB1611* activity.

(A) Adaxial surfaces of *emb1611-2* and Columbia (Col) rosette leaves. (B) Abaxial surfaces of *emb1611-2* and Col rosette leaves. (C) Scanning electron micrograph of a Col trichome with three points (arrowheads). (D) *emb1611-2* trichome with four points. (E) *emb1611-2* trichome with five points. (F) Col trichome with 4',6-diamidino-2-phenylindole (DAPI)-stained nucleus (arrowhead). (G) *emb1611-2* trichome with nucleus that stains more brightly with DAPI than the wild type. (H) DNA ploidy distribution in Col (green) and *emb1611-2* (magenta) mature leaves. Scale bars: 50 µm.

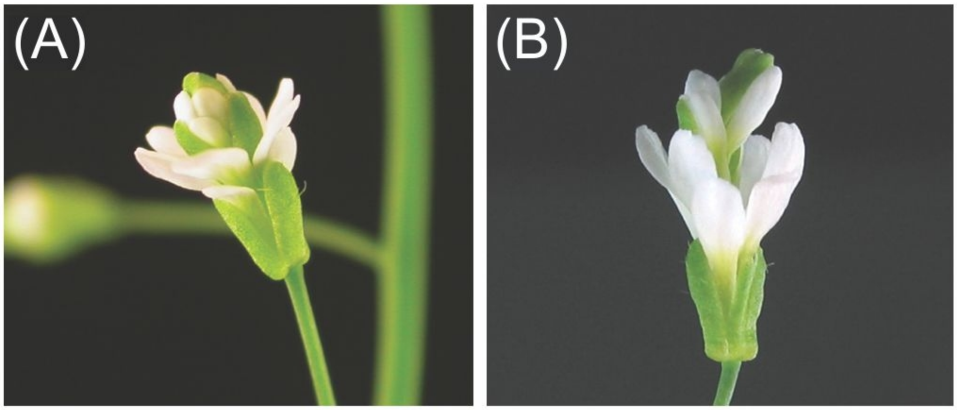

Figure S1. Double mutants between *emb1611-2* and *ag-3*.(a) Indeterminate *ag-3* Col flower consists of reiterating whorls of sepals and petals, with slight internode elongation between them.(b) Indeterminate *emb1611-2 ag-3* Col flower that likewise consists of reiterating whorls of sepals and petals.

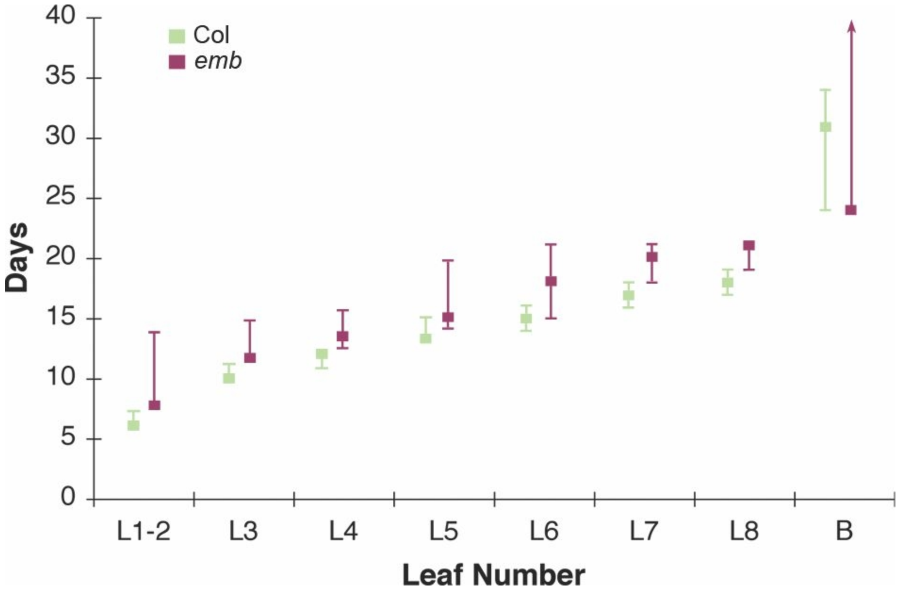

Figure S2**.** Growth rate of Col and *emb1611-2* seedlings. Histogram shows the number of days (boxes) at which 50% of the Col (green) and *emb1611-2* (magenta) plants scored had formed the specified number of leaves (*n* = 100). The bars represent the range at which all the plants scored reached the developmental event. B, bolting.

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