

Maintenance of stem cell populations in plants

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Flowering plants have the unique ability to produce new organs continuously, for hundreds of years in some species, from stem cell populations maintained at their actively growing tips. The shoot tip is called the shoot apical meristem, and it acts as a self-renewing source of undifferentiated, pluripotent stem cells whose descendants become incorporated into organ and tissue primordia and acquire different fates. Stem cell maintenance is an active process, requiring constant communication between different regions of the shoot apical meristem to coordinate loss of stem cells from the meristem through differentiation with their replacement through cell division. Stem cell research in model plant systems is facilitated by the fact that mutants with altered meristem cell identity or accumulation are viable, allowing dissection of stem cell behavior by using genetic, molecular, and biochemical methods. Such studies have determined that in the model plant *Arabidopsis thaliana* stem cell maintenance information flows via a signal transduction pathway that is established during embryogenesis and maintained throughout the life cycle. Signaling through this pathway results in the generation of a spatial feedback loop, involving both positive and negative interactions, that maintains stem cell homeostasis. Stem cell activity during reproductive development is terminated by a temporal feedback loop involving both stem cell maintenance genes and a phase-specific flower patterning gene. Our current investigations provide additional insights into the molecular mechanisms that regulate stem cell activity in higher plants.

A major focus of present-day biological research is on the question of how stem cell fate is controlled during development. Plants make excellent model systems for studying this problem, because, unlike animals, plants maintain a reservoir of stem cells in their shoot and root apical meristems throughout their life spans. Thus plants provide an essentially endless supply of stem cells for study. Like animal stem cells, plant stem cells are self-renewing and have the potential to form many different organs and tissues. Stem cells in the shoot apical meristem (SAM) are the progenitors of all cells that make up stems, leaves, branches, and flowers. The root apical meristem is the source of all of the cells of the primary and lateral root system. Determining how stem cells are established and maintained in apical meristems is fundamental both for addressing the basic biological features of stem cell behavior and understanding many aspects of plant development. This article will discuss recent progress in our laboratory, and in numerous other laboratories, in unraveling the genetic regulatory mechanisms that maintain stem cell fate in the SAM of the model plant *Arabidopsis thaliana*.

Although mature plants have an enormous variety of forms, embryonic plant architecture is sufficiently limited that it is difficult to distinguish between embryos from different plant species based solely on morphological criteria. Likewise, a mature *Arabidopsis* embryo has a morphologically simple structure. It consists of an embryonic stem, two embryonic seed leaves called cotyledons, and a root apical meristem and a SAM at the basal and apical poles of the embryo, respectively. Because plants do not use cell migration or programmed cell death to

achieve morphogenesis, the patterning of the embryo occurs mainly through symmetric and asymmetric cell divisions in different places and planes and through cell expansion (1). Plants also do not set aside a dedicated germ line during embryogenesis; instead, germ cells form independently during the generation of each flower from the SAM.

The elaboration of plant architecture occurs mainly postembryonically. After germination, the *Arabidopsis* SAM begins to initiate vegetative organs, the leaves, that form a basal rosette. After several weeks of vegetative development the plant undergoes the transition to flowering, during which the stem elongates and begins to produce secondary branches and flowers. The primary SAM at this stage is called the inflorescence, or flower-bearing, meristem. Flowers are formed from floral meristems, which are derived from the flanks of the SAM. Floral meristems have the same general structure and organization as SAMs. Each floral meristem produces a flower consisting of four types of floral organs: sepals, petals, stamens (male reproductive organs), and carpels, the latter of which fuse to form the gynoecium (the fruit) that encloses the seeds of the next generation. One important difference between a SAM and a floral meristem is that a SAM grows indefinitely and produces an unspecified number of lateral organs, whereas a floral meristem produces a specified number of organs and terminates in the formation of the central carpels.

The *Arabidopsis* SAM is patterned gradually during embryogenesis (2), culminating in the formation of a highly organized structure with overlapping functional domains (Fig. 1). The self-renewing stem cell pool is confined to the most apical, central portion of the meristem (3). Infrequent cell divisions in this region, which is called the central zone, causes displacement of daughter cells outward into the peripheral region, where they begin to divide more frequently (4). These more rapidly dividing peripheral zone cells begin to undergo differentiation and become incorporated into organ primordia on the meristem flanks. Cells in the central zone also divide downward into the interior of the meristem, a region called the rib zone that contributes to the meristem pith.

The stem cell population consists of three clonally distinct cell layers (Fig. 1), as does the peripheral meristem region and each lateral organ primordium (3, 5). The epidermal layer, the L1, forms a single cell layer that remains distinct from the other layers because the cell divisions within it are always perpendicular to the plane of the meristem surface (anticlinal). The subepidermal L2 layer is also a single cell thick and divides exclusively in the anticlinal plane. The underlying L3 consists of

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Abbreviations: SAM, shoot apical meristem; LRR, leucine-rich repeat; LRR-RLK, LRR receptor-like kinase; KAPP, kinase-associated protein phosphatase; MAPK, mitogen-activated protein kinase; CLV, *Clavata*; WUS, *Wuschel*; AG, *Agamous*; LFY, *Leafy*; ULT, *Ultrapetala*.

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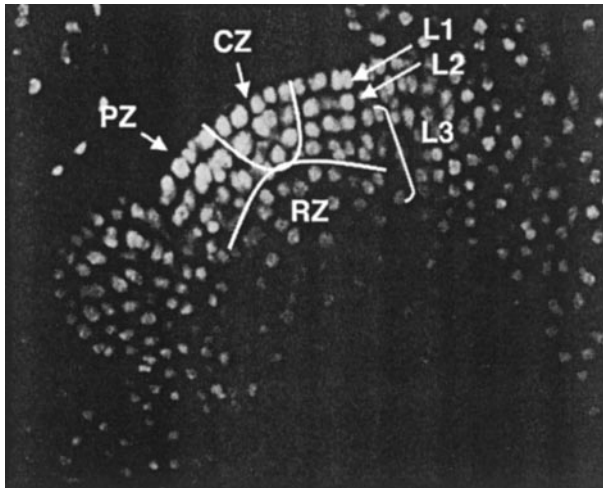


Fig. 1. Organization of the *Arabidopsis* SAM. Confocal laser scanning microscope optical section through a WT flowering SAM and developing floral meristem. The section shows the cell layers (L1, L2, and L3) and the histologically defined domains. The central zone (CZ) lies at the apex of the SAM and harbors the stem cell reservoir. The surrounding peripheral zone (PZ) consists of progenitor cells for lateral organs, and the underlying rib zone (RZ) consists of progenitor cells for the core of the stem. The SAM has been stained with propidium iodide to visualize the cell nuclei. [Modified with permission from ref. 33 (Copyright 2000, Elsevier).]

all of the cells in the interior of the SAM, which divide in all planes. Yet despite the fact that cell divisions in the SAM are highly regular, mosaic analyses have demonstrated that, beyond the general maintenance of the clonal layers, cell lineage patterns are not fixed (6–8). Instead, the cells in the SAM rely almost entirely on positional cues to determine their correct fates (9), and cell fate and cell proliferation information must be communicated both within and between the different meristem layers for proper development to occur. Thus, as in animals, the local environment of the plant meristem provides a niche for the maintenance of the stem cell population.

Mutations That Alter Stem Cell Fate

Our entrée into understanding the mechanism of plant stem cell maintenance has come from the analysis of *Arabidopsis* mutants that affect this process. Remarkably, loss-of-function *shootmeristemless* (*stm*) mutants, which lack a SAM and therefore contain no stem cells, survive embryogenesis and germinate normally (10), although they fail to produce any postembryonic lateral organs. Plants carrying loss-of-function mutations at the *WUSCHEL* (*WUS*) locus form an embryonic SAM, but it is not maintained after the production of a few lateral organs (11). These lateral organs often initiate ectopically from the center of the SAM, indicating that the stem cells at the shoot apex do not maintain their correct fate. New SAMs are established *de novo* at the base of the *wus* mutant cotyledons and leaves, but these meristems also terminate prematurely. This stop-and-start growth pattern, which culminates in a bushy or tousled-looking (*wuschel* in German) plant, continues through the floral phase. *wus* mutant inflorescence meristems produce a reduced number of flowers compared with WT plants, and most of the flowers terminate prematurely in a single stamen. Thus the *wus* mutant phenotypes define a gene whose WT function is required to promote shoot and floral meristem activity by maintaining the central stem cell reservoir.

WUS encodes a novel subtype of homeodomain protein that is nuclear localized and predicted to act as a transcription factor (12). *WUS* mRNA is first detected when embryos reach the 16-cell stage. At this time, *WUS* expression is confined to the four

inner cells of the apical region. As embryogenesis proceeds, asymmetric cell divisions lead to an asymmetric distribution of *WUS* transcripts and the gradual localization of the *WUS* expression domain to the internal layers of the developing SAM. *WUS* mRNA is confined to a small group of cells in the internal layers of shoot, and later, floral meristems, and is not detected in either the epidermal or subepidermal layers of these tissues. The *WUS* expression domain is maintained by the activity of the *FASCIATED1* (*FAS1*) and *FAS2* genes, which encode components of chromatin assembly factor-1 (13). *FAS1* and *FAS2* may therefore promote stable *WUS* gene transcription by facilitating the appropriate chromatin conformation.

Arabidopsis mutants with phenotypes that are opposite those of *wus* phenotypes have also been characterized. Plants carrying loss-of-function mutations at the *CLAVATA1* (*CLV1*), *CLV2*, or *CLV3* loci generate many excess SAM cells and form enlarged meristems that grow as a ring or a strap instead of as a point. From this phenotype we infer that the WT function of the three *CLV* genes is to restrict meristem cell accumulation. This requirement for *CLV* gene activity begins during embryogenesis, because the SAMs of mature *clv1* and *clv3* embryos are already significantly enlarged relative to the SAMs of WT embryos and contain more stem cells (14, 15). Excess stem cells continue to accumulate throughout the life of *clv* mutant plants, so that by the transition to flowering *clv* shoot meristems are greatly enlarged and produce many more floral meristems than WT plants.

The floral meristems produced by *clv* mutants are also enlarged compared with the WT and contain excess stem cells. The enlarged floral meristems lead to an increase in the number of organs in *clv* flowers. WT flowers are arranged into four concentric rings of organs, called whorls. In *Arabidopsis*, organ number in each whorl is nearly invariant: the vast majority of WT flowers consist of four sepals, four petals, five to six stamens, and two fused carpels (16). Flowers of *clv* null mutants contain many additional organs, including up to seven or eight carpels that fuse to form a club-shaped fruit. It is from this phenotype that the mutants are named “clavata,” from the Latin term “clavatus” meaning club-like. In addition to generating extra organs, the floral meristems of *clv1* and *clv3* null mutants often contain a mass of proliferating stem cells that erupt through the gynoecium. This phenotype is further evidence that the *CLV* loci are required to prevent unrestricted stem cell amplification in the center of the floral meristem.

Genetic analysis has revealed that the three *CLV* genes and *WUS* interact to control stem cell fate during development. *clv1* and *clv3* null mutants have nearly indistinguishable phenotypes, and *clv1 clv3* double mutants appear identical to either single mutant (15). Further, plants that are doubly heterozygous for *clv1* and *clv3* alleles display an enlarged meristem phenotype, indicating that each gene is sensitive to the dose of the other. *clv2* null mutants have slightly weaker shoot and floral meristem phenotypes than *clv1* and *clv3* null mutants, and *clv1* and *clv3* are epistatic to *clv2* with respect to those traits (17). However, *clv2* mutants, unlike *clv1* and *clv3* mutants, also display phenotypes in nonmeristematic tissues, indicating that *CLV2* functions more widely in development. Double mutants generated between *wus* null alleles and any *clv* allele have a *wus* mutant phenotype (11), revealing that *wus* is completely epistatic to *clv1*, *clv2*, and *clv3*. Thus all four genes act in the same genetic pathway to maintain the appropriate amount of stem cell accumulation in shoot and floral meristems.

A Short-Range Signaling Pathway Maintains Stem Cell Homeostasis

All three *CLV* genes have been cloned and found to encode components of a meristem signal transduction pathway. *CLV1* encodes a leucine-rich repeat (LRR) receptor-like kinase (LRR-

RLK) (18), one of 216 in the *Arabidopsis* genome (19). Members of this family contain multiple tandem extracellular LRRs, a transmembrane domain, and an intracellular serine/threonine kinase domain. The LRR-RLK family, which consists of 13 subclasses, is the largest class of receptor kinases in plants. The LRR-RLKs and other *Arabidopsis* RLKs form a monophyletic family that groups with *Drosophila* Pelle and other Pelle-like cytoplasmic kinases in phylogenetic analyses (19). Based on the fact that LRRs are considered to be protein-binding motifs, and that LRR-containing proteins in plants and animals are involved in protein-protein interactions and signal transduction (20), it has been proposed that the extracellular LRRs of the LRR-RLKs bind protein or peptide ligands. The functions of a half-dozen *Arabidopsis* LRR-RLKs have been defined to date. They play roles in various plant signaling pathways, including those regulating different aspects of development (21–23), hormone signaling (24), and disease resistance (25).

CLV2 and *CLV3* also encode members of large, plant-specific gene families. *CLV2* encodes a receptor-like protein with 21 extracellular LRRs, a transmembrane domain, and a short cytoplasmic tail (26). There are ≈ 30 *CLV2*-like genes in the *Arabidopsis* genome, but their functions are unknown. *CLV3* encodes a 96-aa predicted extracellular polypeptide (27) that is a founding member of the *CLV3*/ESR-related (*CLE*) protein family (28). The members of this family, which are found in many distantly related plant species, share common sequence elements including an amino-terminal signal peptide and a 14-aa region of homology at the carboxyl terminus. Two dozen *CLE* genes have been identified in *Arabidopsis* (28, 29). These genes are expressed in a wide range of tissues during development (29) and may function as ligands for RLKs in many plant signal transduction pathways.

The expression patterns of the *CLV3* and *CLV1* genes revealed important clues to their function. We found that, beginning during embryogenesis and continuing throughout the life of the plant, *CLV3* marks the stem cell population in the SAM (27). *CLV3* transcripts are detected predominantly in the superficial L1 and L2 cell layers at the apex of the meristem and in only a few underlying L3 cells. *CLV3* mRNA is not found in peripheral meristem cells, mature stem tissues, or lateral organs. *CLV3* is also expressed in the stem cell populations of all axillary shoot meristems and floral meristems. In floral meristems, *CLV3* is detected until the stem cell population is consumed in the formation of the carpel primordia (see below), when *CLV3* transcripts disappear from the flower. *CLV1* mRNA is also induced during embryogenesis, at the same time as *CLV3*, and is likewise restricted to shoot and floral meristem cells (18). However, *CLV1* transcripts are found predominantly in the L3 cells of the meristem, largely beneath the *CLV3* expression domain, and are not detected in the L1 epidermal layer. Thus *CLV1*-expressing cells and *CLV3*-expressing cells are neighbors, suggesting that the two meristem domains may communicate with one another through a *CLV3*/*CLV1* signal transduction pathway. *CLV2* is also expressed in shoots and flowers based on RNA gel blot analysis (26), but the precise *CLV2* expression domain in meristems has not been reported.

Important inroads have been made into determining the mechanism of *CLV* signal transduction. There is now solid biochemical evidence that *CLV1* and *CLV2* are bound together in a signaling complex (Fig. 2). The *CLV1* kinase domain is capable of both autophosphorylation and transphosphorylation on serine residues, indicating that *CLV1* is a bona fide receptor kinase (30, 31). *CLV1*, a predicted 105-kDa protein, is detected in size-fractionated meristem extracts in two different complexes: a 185-kDa complex and a 450-kDa complex (32). *CLV1* and *CLV3* are both components of the 450-kDa complex, whereas *CLV3* is not detected in the 185-kDa complex. The 450-kDa complex does not form in *clv3* mutants or *clv1* mutants

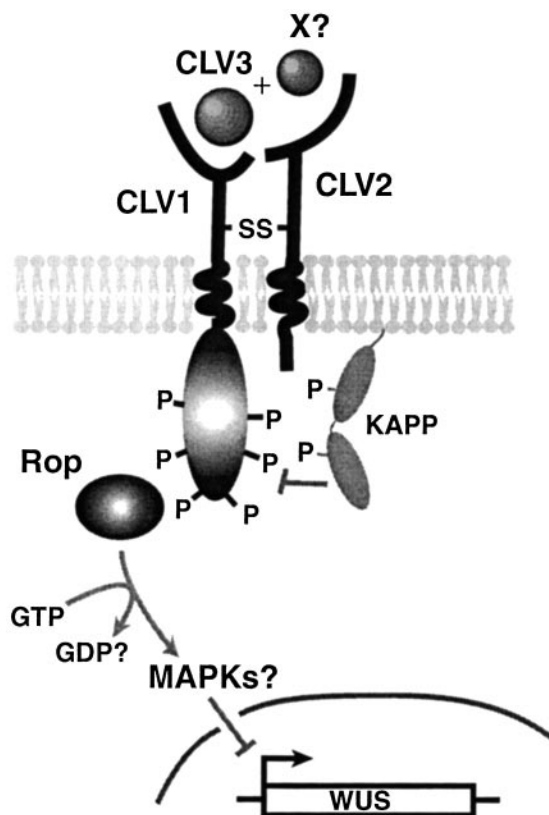


Fig. 2. Schematic of the *CLV* signaling complex. The *CLV1* LRR-RLK forms a heteromeric complex with the *CLV2* receptor-like protein at the plasma membrane of interior SAM cells. Binding of the *CLV3* polypeptide, possibly in association with another protein (X), is proposed to stimulate assembly of an active signaling complex that also contains a phosphatase (KAPP) and a Rho-like GTPase (Rop). The signal is relayed from the cytosol to the nucleus, potentially via a MAPK cascade, to limit *WUS* expression. P, phosphorylation site; SS, disulfide bond. [Reprinted with permission from ref. 68 (Copyright 2002, Annual Reviews, www.annualreviews.org).]

that lack an active kinase domain. The 450-kDa fraction is therefore likely to represent the active *CLV* receptor signaling complex, whereas the 185-kDa complex is inactive. Although specific antibodies for *CLV2* have not yet been reported, its presence in the 450-kDa complex is inferred from the observation that this complex does not form in *clv2* mutants although high levels of *CLV1* and *CLV3* transcripts are present (26). Mutations in *CLV2* also reduce the accumulation of the 185-kDa complex, suggesting that *CLV2* may also be a subunit of the inactive complex that forms a disulfide-linked heterodimer with *CLV1*.

The active *CLV* signaling complex contains several additional cytosolic proteins. One is a type 2C kinase-associated protein phosphatase (KAPP), which interacts with several different LRR-RLKs (25, 34, 35). *KAPP* is expressed in *Arabidopsis* meristems in a broad region that encompasses the *CLV1* expression domain (30), and *KAPP* protein binds to and dephosphorylates the phosphorylated form of the *CLV1* kinase domain (30, 31). *KAPP* binds to the *CLV1* kinase domain through its forkhead-associated (FHA) domain, and site-directed mutagenesis of conserved FHA residues abolishes the ability of *KAPP* to interact with RLKs (36). *KAPP* overexpression phenotypes mimic those of weak loss-of-function *clv* alleles (30), whereas reduction in *KAPP* transcription levels can suppress the *clv1* mutant phenotype (31). These data are consistent with *KAPP* acting as a negative regulator of *CLV* signaling by dephospho-

rylating CLV1. AtSERK1, a plasma membrane-localized LRR-RLK, was recently shown to become sequestered in intracellular vesicles when transiently coexpressed with KAPP (35). KAPP dephosphorylation of threonine residues in the AtSERK1 kinase domain A loop was found to play an active role in the receptor internalization. These results suggest that KAPP is an integral part of an endocytosis mechanism that internalizes AtSERK1, and possibly other LRR-RLKs such as CLV1, during receptor-mediated signaling.

An additional cytoplasmic component of the active CLV complex, identified through coimmunoprecipitation experiments (32), is a member of the plant-specific Rop subfamily of Rho GTPases (37). Members of the Rho GTPase family, which includes CDC42, Rac, and Rho, are key cytosolic components of cell surface-receptor signaling cascades that regulate cytoskeletal organization, polarized cell growth, and many other cellular processes in animals and fungi (38–40). *Arabidopsis* contains at least 10 Rop GTPases (41, 42), some of which have defined roles in mediating tip growth in pollen tubes and root hairs (37, 43) and others of which act in various developmental processes (44). It is not yet clear which Rop protein(s) participates in CLV signaling, because the antibody used in the biochemical experiments cross-reacts with multiple Rop isoforms (32).

How is the signal relayed downstream of the CLV complex? Although the exact mechanism is currently unknown, a model has been proposed based on precedents in other systems (32). Rho/Rac GTPases are members of the Ras superfamily of cytoplasmic signal transduction molecules, many of which relay signals through a mitogen-activated protein kinase (MAPK) pathway. The *Arabidopsis* genome contains multiple genes encoding MAPK, MAPKK, and MAPKKK components of this pathway (45). A complete MAPK cascade has recently been reported (46) to function downstream of the *Arabidopsis* flagellin receptor FLS2, which encodes an LRR-RLK (25), in innate immune responses. Based on this paradigm, once the CLV1 kinase domain becomes activated via phosphorylation, it might associate with and activate the Rop GTPase, which in turn could activate a MAPK cascade that ultimately leads to changes in target gene expression. Functionally redundant MAPK signaling proteins were discovered at several steps in the FLS2 pathway, and several of the components of the FLS2 cascade may also function in other pathways (46). If this is a common theme for other LRR-RLK signaling pathways, it could account for the lack of MAPK cascade mutants identified to date in the CLV signaling pathway.

What is the biological basis for signaling through the CLV complex in meristematic cells? Genetic and molecular evidence has led to the conclusion that the CLV signaling pathway is part of a negative feedback loop that controls stem cell fate in the *Arabidopsis* shoot apex. Our first insight came from an experiment designed to determine what happens to *CLV3* expression in the absence of the CLV1 receptor kinase. Because *clv1* mutant phenotypes are very similar to *clv3* mutant phenotypes, we tested the possibility that the *clv1* phenotypes might be caused by a reduction in either the domain or level of *CLV3* expression. In contrast to our prediction, we observed that *CLV3* was expressed at very high levels in a greatly enlarged domain in *clv1* SAMs (27). This result indicates that *CLV3* expression is not reduced in *clv1* mutant plants, but instead that *CLV1* normally acts to restrict the number of *CLV3*-expressing cells in WT meristems. Similarly, the *CLV1* expression domain is enlarged in *clv3* mutant plants. In fact, in *clv1*, *clv2*, or *clv3* mutants the expression domains of *CLV1* and *CLV3* enlarge coordinately (17, 27). From these data we inferred the presence of two opposing stem cell regulatory pathways. The expansion of the *CLV* expression domains would be normally controlled by a positive, stem cell-promoting pathway, which in turn would be negatively regulated by the stem cell-restricting *CLV* pathway. The stem

cell-promoting pathway would then also promote the expression of the *CLV* genes by causing the enlargement of the cell populations that express them.

We were able to separate these two pathways genetically by expressing *CLV3* constitutively in *Arabidopsis* plants under the control of the cauliflower mosaic virus 35S promoter. Transgenic 35S::*CLV3* plants expressing high levels of *CLV3* mRNA germinated normally, but stopped initiating organs after producing a few leaves (47). These results indicate that stem cells are not correctly maintained when *CLV3* is constitutively expressed at high levels. They further demonstrate that *CLV3* is sufficient to restrict stem cell accumulation throughout the SAM. Flower meristems could be produced by lines expressing lower levels of constitutive *CLV3*. Such meristems failed to initiate the inner organ types, again demonstrating an inability to maintain stem cells. The level of *CLV3* protein therefore appears to be the critical factor that determines the amount of stem cells that are produced in both shoot and floral meristems. In addition, when the 35S::*CLV3* transgene was introduced into the *clv1* or *clv2* mutant background, the resulting plants constitutively expressed *CLV3* at high levels but exhibited the typical *clv* mutant phenotype. Thus *CLV3* signaling requires functional *CLV1* and *CLV2* proteins, and the terminal meristem phenotypes observed in the transgenic 35S::*CLV3* plants result from enhanced *CLV3* signaling through the *CLV1* receptor complex.

WUS is a key target of regulation by the CLV signal transduction pathway, as determined by examining its expression pattern in *clv3* mutant and *CLV3* overexpressing plants (47). In WT plants, *WUS* mRNA is restricted to the internal layers of shoot and floral meristems, beneath the *CLV3* domain, and is not detected in either the epidermal or subepidermal layers. Transgenic *CLV3* overexpression lines phenocopy the *wus* mutant phenotype, suggesting that *WUS* activity is down-regulated in response to signaling through the CLV complex. Consistent with this hypothesis, the *WUS* expression domain expands laterally and into the subepidermal layer of *clv3* mutant meristems (47). Conversely, *WUS* mRNA is not detected in the arrested meristems of 35S::*CLV3* plants, indicating that one outcome of enhanced CLV signaling is a severe reduction in the level of *WUS* transcription. *WUS* therefore appears to respond directly, but negatively, to the level of CLV3 signaling: a high level of *CLV3* reduces the size of the *WUS* expression domain, whereas a low level allows it to expand.

The positive, stem cell-promoting pathway depends on activity of the *WUS* gene. Driving *WUS* expression ectopically in all initiating organ primordia on the flanks of the SAM results in seedlings that form normal cotyledons and an apex that consists entirely of undifferentiated meristematic cells (48). *CLV3* mRNA, which in WT plants is found only at the very apex of the meristem, can be detected on the periphery of this mound of cells. This result indicates that *WUS* misexpression is sufficient to induce *CLV3* transcription and stem cell fate. Based on these data, *WUS* is proposed to enable the cells that express it to act as an “organizing center” that confers stem cell fate on the overlying cells of the meristem (12). *WUS* is therefore a key component of the stem cell-promoting pathway, which functions to control the stem cell-restricting CLV pathway by regulating *CLV3* transcription. The regulation of *CLV3* by *WUS* is probably indirect, because the *WUS* expression domain does not overlap with the *CLV3* domain and there is no evidence that *WUS* protein moves out of the cells in which it is made.

Thus in WT *Arabidopsis* plants, stem cell maintenance is mediated by short-range signaling events. The CLV3 signal is likely to be released from the stem cells at the apex of the meristem and bind a CLV1/CLV2 receptor complex in underlying cells. Signaling through the CLV pathway limits *WUS* activity by restricting its expression to a narrow domain of cells in the deeper layers of the meristem. Constitutive signaling through *CLV3* enhances this negative pathway, causing down-

regulation of *WUS* and complete loss of stem cells. Disruption of the negative pathway in *clv* mutants causes the *WUS* expression domain to expand laterally and upward into the subepidermal cell layer. Activity of the positive pathway, mediated by *WUS*, promotes expression of *CLV3* and maintenance of the stem cell domain. This mutual regulation, involving both positive and negative interactions, provides a feedback system required to maintain an appropriately sized stem cell reservoir throughout *Arabidopsis* development.

This negative feedback loop elegantly compensates for the departure of cells from the meristem during organ formation. As stem cell daughters enter the transition zone and become incorporated into organ primordia, the decrease in stem cell number leads to a reduction in the level of the *CLV3* signal. The drop in negative signaling through the *CLV* pathway causes the expansion of the *WUS*-expressing cell population. This, in turn, triggers the amplification of the stem cell population via the stem cell-promoting pathway, until the level of *CLV3* produced by the stem cells rises sufficiently to restrict further expansion of the *WUS* domain and equilibrium is once again attained.

Signaling Via *CLV3*

One major question to be addressed is how the *CLV1/CLV2* receptor complex is activated by *CLV3*. Based on its sequence, *CLV3* is predicted to be exported through the secretory pathway to the extracellular space by the default pathway for soluble plant proteins. *CLV3* has been shown to act in a cell nonautonomous fashion (27), but the expression data do not exclude the possibility that activation of the *CLV* pathway occurs intracellularly within those few cells in the interior of the SAM that express both *CLV3* and the receptor complex. In collaboration with Enrique Rojo, Valentina Kovaleva, and Natasha Raikhel at the University of California, Riverside, we devised several assays to determine whether the *CLV3* protein is secreted and whether secretion is required for its function *in vivo* (49).

To test the prediction that the *CLV3* protein is secreted, we used two translational fusions of *CLV3* to the amino terminus of β -glucuronidase, one full length and the other lacking the putative signal peptide, in transient subcellular localization assays. The fusion protein lacking the signal peptide was detected in the cytoplasm, whereas the full-length *CLV3*-GUS fusion protein was detected only in the extracellular space. Thus the *CLV3* protein is exported through the secretory pathway, and the presence of its signal peptide is required for its extracellular localization. Additional transient assays, in which full-length *CLV3*-GUS was cobombarded together with a nuclear-localized protein as a marker for transformed cells, show that *CLV3* is capable of moving beyond the cells in which it is made (V.K.S. and J.C.F., unpublished data). We confirmed that these results were relevant *in vivo* by overexpressing full-length and truncated *CLV3*-GFP fusion proteins in *clv3* mutant plants. We found that plants transformed with the full-length 35S::*CLV3*-GFP fusion protein rescued the *clv3* mutant phenotype and showed GFP fluorescence only in the extracellular space. Plants transformed with the truncated version of *CLV3* fused to GFP did not rescue the *clv3* phenotype and showed GFP fluorescence only in the cytoplasm. Based on these data, we conclude that *CLV3* is a secreted protein both *in vitro* and *in planta*.

Next, we asked whether *CLV3* secretion is essential for its activity in shoot and floral meristems. We tested whether preventing the *CLV3* protein from reaching the cell surface would block its function in restricting stem cell accumulation (Fig. 3). Overexpression constructs were generated in which *CLV3* was tagged with a vacuolar sorting signal to direct the protein to the vacuole rather than the extracellular space (Vac constructs). We also generated several overexpression constructs in which *CLV3* was tagged with a mutated vacuolar

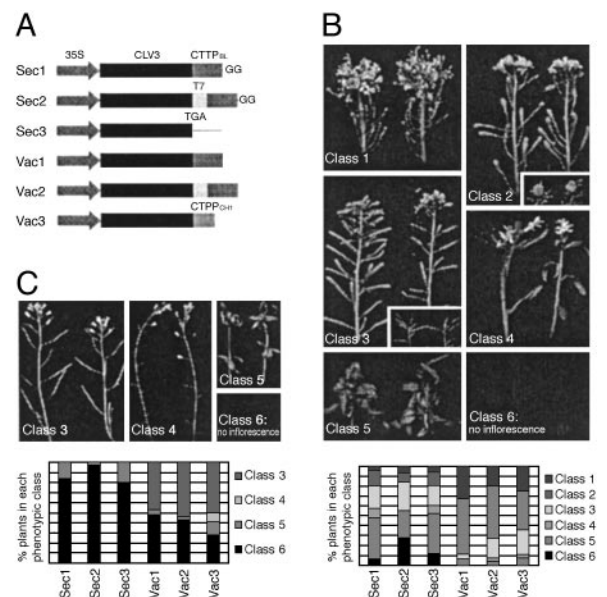


Fig. 3. Targeting to the vacuole blocks the activity of *CLV3*. (A) Scheme of the fusion constructs. The three Vac constructs contain fusions of *CLV3* to the C-terminal vacuolar sorting signal from barley lectin (ctVSSBL) or tobacco chitinase A (ctVSSCH). The ctVSSBL with two additional Gly residues (GG), which no longer functions as a vacuolar-sorting signal, was attached to the carboxyl terminus of *CLV3* (Sec1) or *CLV3*-T7 (Sec2). Sec3 contains the full-length *CLV3* protein with no additional tag. (B) *clv3-2* plants transformed with the fusion constructs. Primary transformants were grouped into six phenotypic classes according to the severity of the meristem phenotype. Class 1 plants showed no transgene activity and resembled untransformed *clv3-2* plants. Class 6 plants showed the strongest transgene activity and exhibited a gain-of-function phenotype in which the SAM of each plant terminated prematurely before flowering. Plants in the other classes fell between these two extremes. The graph at the bottom shows for each construct the percentage of transformed plants recovered in each phenotypic class. (C) WT plants transformed with the fusion constructs. Primary transformants were grouped into classes 3, 4, 5, and 6. The graph at the bottom shows for each construct the percentage of transformed plants recovered in each phenotypic class. [Reprinted with permission from ref. 49 (Copyright 2002, American Society of Plant Biologists).]

sorting signal that forces the protein into the secretory pathway (Sec constructs). When these constructs were introduced into *clv3* null mutant or WT plants, several phenotypic classes were distinguished. Most of the Sec-containing plants showed complete rescue of the *clv3* phenotype or had a gain-of-function phenotype, whereas the majority of Vac-containing plants had little or no rescue of the phenotype. A few Vac-containing plants showed a gain-of-function phenotype, which could be attributed to saturation of the vacuolar sorting pathway by high levels of *CLV3*.

To localize the *CLV3* protein in the different transgenic lines, we performed immunogold labeling with an antibody-directed, T7-tagged *CLV3* protein. *CLV3* protein was detected in the cell wall of Sec-containing plants, but not in Vac-containing plants that failed to rescue the *clv3* phenotype. *CLV3* was also detected in the cell wall of Vac-containing plants that displayed a gain-of-function phenotype. This observation confirmed that the vacuolar sorting pathway became saturated in these lines, and thus the excess *CLV3* protein was secreted and able to interact with the *CLV1* complex. Therefore we conclude from this work that *CLV3* functions as a secreted protein that activates the *CLV* stem cell signaling pathway in the extracellular space.

A Temporal Feedback Loop Terminates Stem Cell Activity in Flowers

Flowers are produced from floral meristems, which are derived from the SAM and likewise harbor a stem cell reservoir that

provides cells for the generation of the floral organs. Each floral meristem forms as a primordium on the flank of the SAM. As it grows the floral meristem initiates three concentric rings of organs (sepals, petals, and stamens) and then the stem cells are consumed in the formation of the carpels that comprise the central gynoecium. Thus unlike SAMs, in which the stem cell population is maintained indefinitely to provide progeny cells for continuous organogenesis, floral meristems ultimately terminate stem cell production to permit the female reproductive organs to differentiate in the center of the flower.

Floral meristem activity is regulated by several overlapping sets of regulatory factors. The CLV signaling pathway is active at the floral meristem apex until carpel initiation, when *CLV1*, *CLV3*, and *WUS* all are down-regulated to undetectable levels. Floral meristem identity is conferred by transcription factors such as LEAFY (*LFY*) and APETALA1 (*AP1*) (50, 51), which can convert shoot meristems into floral meristems (52, 53). The floral meristem identity genes induce the transcription of flower-specific homeotic genes in overlapping spatial domains of the floral meristem (54, 55). Activity of the floral homeotic gene products in four different combinations then specifies the identity of the sepals, petals, stamens, and carpels (56).

The floral homeotic gene *AGAMOUS* (*AG*) encodes a MADS-domain transcription factor (57) that plays key roles in both floral organ identity specification and stem cell termination. *ag* mutations cause the transformation of stamens into petals and replacement of the central carpels with an entirely new flower (58). The resulting *ag* flowers consist entirely of sepals and petals and resemble shoots because they retain a stem cell population at their apex (59) and continue to produce organs indefinitely. *AG* overexpression has the opposite effect: instead of maintaining indeterminate growth, the SAMs of 35S::*AG* plants terminate in a solitary flower (60). Thus *AG* is required to terminate stem cell activity in the center of the developing flower and is sufficient to convert a shoot meristem into a floral meristem.

ag mutants and *wus* mutants have opposite flower phenotypes, and genetic experiments demonstrate that these two genes play antagonistic roles in regulating floral meristem activity. *WUS* expression persists in *ag* mutant flowers even after the formation of many organs (59, 61). *AG* is therefore a negative regulator of *WUS* transcription and, consequently, of stem cell maintenance. Stem cell termination is restored in *ag wus* double mutant flowers, which resemble *wus* single mutant flowers, indicating that the capability of *ag* flowers to continuously produce new organs depends on the ectopic activity of *WUS*. Thus prolonged *WUS* expression is sufficient to permit indefinite floral meristem activity, and a key role of *AG* is to down-regulate *WUS* and terminate stem cell maintenance in the center of the developing flower.

The expression of *AG* is regulated by the floral meristem identity pathway and also the stem cell signaling pathway (Fig. 4). *AG* is initially transcribed in the center of developing floral meristems in the cells that will ultimately become specified as stamens and carpels, and *AG* expression in these organs persists until the late stages of flower development (57). *AG* is directly activated by *LFY* (55), which binds to regulatory elements in the second intron of the gene. However, *LFY* protein is distributed throughout the floral meristem (62), and thus does not confer region-specific *AG* induction. This function is provided by *WUS*, which is expressed in the center of floral meristems in a subset of cells that eventually express *AG*. Although *wus* mutant flowers do not display homeotic organ transformations they lack all carpels and most stamens, the organ types that are specified by *AG*. In addition, misexpression of *WUS* in flowers causes the formation of ectopic stamens and carpels as a consequence of ectopic *AG* activation. Recent reports have shown that *WUS* is also a direct activator of *AG*, acting through a homeodomain

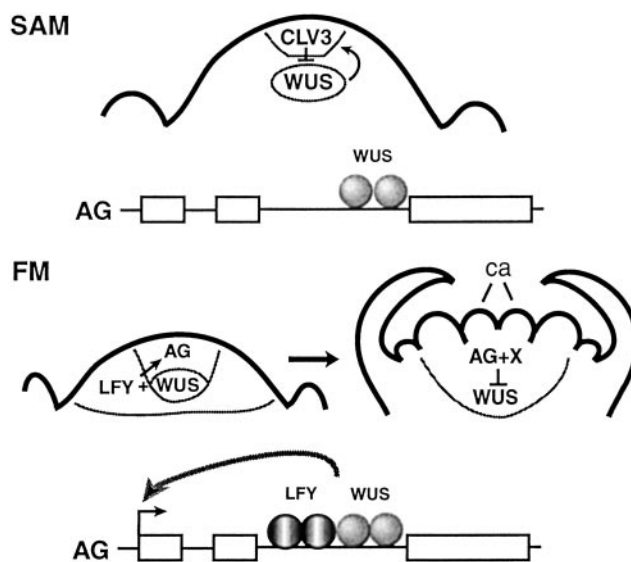


Fig. 4. Temporal feedback loop regulating stem cell termination in determinate floral meristems. (Upper) Schematic of an indeterminate SAM, showing the interaction between *CLV3* and *WUS* in their respective domains (gray circles). In the SAM, *LFY* is absent and *AG* expression is not induced. (Lower) Schematic of a determinate floral meristem over time. *LFY* is present throughout the young floral meristem. Both *LFY* and *WUS* bind to enhancer sequences and cooperate to induce *AG* transcription in the center of the developing flower. At the time of carpel (ca) initiation, *AG* and an additional factor (X) repress *WUS* expression to terminate stem cell activity. [Reprinted with permission from ref 68 (Copyright 2002, Annual Reviews, www.annualreviews.org).]

protein consensus binding site in the second intron (59, 61). These sites are adjacent to the *LFY* binding sites, although the two proteins bind DNA independently (61). Thus *LFY* provides the flower specificity and *WUS* provides the regional specificity for *AG* induction in the central region of floral meristems.

A third gene that appears to be involved in *AG* activation in *Arabidopsis* floral meristems is *ULTRAPETALA* (*ULT*). We have shown that mutations in *ULT* cause shoot and floral meristem enlargement, leading to the production of extra flowers from the SAMs and extra floral organs from the floral meristems (63). *ult* mutant floral meristems also produce some additional organs in the center of the flower before terminating, reminiscent of *ag* mutants. *AG* activation is delayed in the very center of *ult* floral meristems (63), whereas overexpression of *ULT* leads to premature and ectopic *AG* induction (C.C. and J.C.F., unpublished data). Thus *ULT*, which encodes a member of a plant-specific family of novel proteins (C.C. and J.C.F., unpublished data), is required under normal circumstances for the correct timing of *AG* induction in floral meristems.

Thus the termination of stem cell activity in floral meristems is mediated by a temporal negative feedback loop requiring both flower patterning and stem cell maintenance genes. As in SAMs, *WUS* promotes maintenance of the stem cell domain in floral meristems to permit the generation of the full complement of floral organs. In addition, *WUS* and the flower-specific factor *LFY* bind independently to *AG* regulatory sequences and cooperate to direct *AG* transcription in the center of developing floral meristems, with *ULT* providing temporal specificity. Once activated, *AG* feeds back to repress *WUS*, thus terminating stem cell activity and allowing the differentiation of the central cells into carpel primordia. Unlike the other target of *WUS* induction, *CLV3*, *AG* does not require the constant presence of *WUS* to maintain its expression and consequently *AG* transcription persists even after *WUS* has been switched off.

Conclusions and Final Thoughts

Long-term maintenance of a stem cell population is critical for the particular developmental habit of plants, that is, continuous organ formation to achieve maximal growth under constantly changing environmental conditions. The past 10 years have produced a quantum leap in our understanding of plant stem cell activity from a descriptive to a mechanistic level. We and others have demonstrated that stem cell fate in shoot and floral meristems is controlled by a signal transduction pathway consisting of the CLV receptor complex and the WUS transcription factor. We have shown that this pathway consists of both plant-specific proteins and those with similarity to animal proteins, and that activation of the pathway occurs extracellularly. We have also determined that the CLV/WUS pathway functions as a homeostatic feedback loop that elegantly corrects for fluctuations in stem cell number that occur as a consequence of organogenesis. Finally, studies in several labs are providing insights into how a temporal negative feedback loop terminates stem cell activity to permit the

correct patterning of flowers during the reproductive phase of plant development.

Many of the principles of stem cell regulation elucidated in *Arabidopsis* are also likely to apply to other plants, even those distantly related to the rose family of which *Arabidopsis* is a member. Mutants with *clv*-like fasciated meristem phenotypes have been identified in a number of plant species (64–66), and the cloning of some of the affected genes reveals that the CLV signaling pathway may be conserved among higher plants (65, 67). The challenges for the future will be to characterize all of the components of the CLV signaling pathway, including the targets of WUS transcriptional regulation, to obtain a comprehensive understanding of how the output of this pathway controls stem cell fate and to integrate stem cell signaling into an overall picture of meristem activity.

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