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CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*

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SUMMARY

We have previously described the phenotype of *Arabidopsis thaliana* plants with mutations at the *CLAVATA1* (*CLV1*) locus (Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1993) *Development* 119, 397-418). Our investigations demonstrated that *clv1* plants develop enlarged vegetative and inflorescence apical meristems, and enlarged and indeterminate floral meristems. Here, we present an analysis of mutations at a separate locus, *CLAVATA3* (*CLV3*), that disrupt meristem development in a manner similar to *clv1* mutations. *clv3* plants develop enlarged apical meristems as early as the mature embryo stage. *clv3* floral meristems are also enlarged compared with wild type, and maintain a proliferating meristem throughout flower development. *clv3* root meristems are unaffected, indicating that *CLV3* is a specific regulator of shoot and floral meristem development. We demonstrate that the strong *clv3-2* mutant is largely epistatic to *clv1* mutants, and that the semi-dominance of *clv1* alleles is enhanced by double heterozygosity with *clv3* alleles, suggesting that these genes work in the same pathway to control meristem development. We propose that *CLV1* and *CLV3* are required to promote the differentiation of cells at the shoot and floral meristem.

Key words: organogenesis, pattern formation, cell division, cell differentiation, *Arabidopsis*, *CLAVATA3*, shoot meristem

INTRODUCTION

Meristems are central to higher plant development, as almost all post-embryonic organs, including roots, leaves, flowers and axillary meristems, are initiated by either shoot or root meristems. Two aspects of shoot meristem development are central to its function. First, the meristem must maintain a group of cells in the center that remain undifferentiated. The proliferation of these undifferentiated meristem cells is necessary to provide new cells for organ initiation. In *Arabidopsis*, for example, new organs are generated throughout the life span, requiring the plant to maintain a pool of undifferentiated cells from which to draw on for organ and new meristem initiation. Second, as the meristem apex moves away from these undifferentiated cells by continued cell division, the undifferentiated cells that are now on the flanks of the meristem must be allowed to enter a specific developmental pathway, such as leaf or flower development, leading to eventual differentiation. It is the balancing of these two features that allows for the continued growth of the meristem and its continuous initiation of new organs.

Proliferation of meristem cells balanced with their subsequent incorporation into organ primordia relates to the classical separation of the shoot meristem into a central zone (CZ) and surrounding peripheral zone (PZ; Brown et al., 1964; Vaughn, 1952; Vaughn and Jones, 1953). The CZ, at the very tip of the shoot meristem, would correspond to the region where cells are maintained in an undifferentiated state. In the PZ, daughter cells enter a specific developmental pathway and become incorporated into organ primordia. Molecular evidence corroborates this distinction. The floral meristem identity gene *LEAFY* is first expressed in subsets of cells in the PZ, presumably playing a role in directing those cells toward a floral fate (Weigel et al., 1992).

A number of mutants that disrupt meristem development have been described in *Arabidopsis* (Barton and Poethig, 1993; Clark et al., 1993; Komaki et al., 1988; Koornneef et al., 1983; Leyser and Furner, 1992; McKelvie, 1962; Medford et al., 1992). For this study, we focused on those genes that may play a specific role in the two processes outlined above, namely the maintenance of a pool of undifferentiated cells and the balance between the proliferation of these cells and their subsequent incorporation into organ primordia. The first *Arabidopsis* gene described in detail that may affect one of these two processes is *CLV1* (Clark et al., 1993; Leyser and Furner, 1992). In our previous report (Clark et al., 1993), we demonstrated that *clv1* mutations lead to an enlarged vegetative and inflorescence shoot meristem and an enlarged, indeterminate floral meristem. The fact that *clv1* mutants develop a much larger pool of undifferentiated meristem cells than is found in wild type suggests that *CLV1* might play a role in meristem development. A separate locus, *CLV2*, appears to exhibit similar mutant phenotypes based on brief descriptions (McKelvie, 1962; Koornneef et al., 1983). A gene required for the initiation of the embryonic meristem, *SHOOT MERISTEMLESS* (*STM*), has recently been described (Barton and Poethig, 1993). Plants

mutant for *stm* never develop a shoot meristem. The rest of the germinating seedling (root, hypocotyl, cotyledons) appears normal, but no new organs develop between the two cotyledons, because no meristem is present. While *STM* is required for the initiation of a meristem, it is unknown whether it is normally required for its continued maintenance. The homeobox genes *KNOTTED1* from maize (Sinha et al., 1993; Smith et al., 1992), and the similar *OSH1* from rice (Matsuoka et al., 1993) are both thought to be involved in shoot meristem development. Their role has been deduced from mRNA and protein expression patterns, and ectopic expression in other plant species. Expression of *KNOTTED1* coincides with regions of undifferentiated cells, largely in the shoot and floral meristems (Smith et al., 1992). In the shoot meristem, *KNOTTED1* is expressed in the center of the meristem, but not in leaf primordia. *KNOTTED1* is initially expressed throughout the floral meristem, but sequentially suppressed as organ primordia initiate. Ectopic expression of *KNOTTED1* causes a variety of defects including shortened, thickened leaves, additional cell divisions around the vasculature, shortened plants, and in the most extreme cases, formation of ectopic shoots from the leaves (Sinha et al., 1993). All of this suggests that *KNOTTED1* may play a role in maintaining cells in an undifferentiated state in maize. Overexpression of *OSH1* from rice causes many similar phenotypes in tobacco (Kanomurakami et al., 1993)

Here we present an analysis of mutations at the *CLAVATA3* (*CLV3*) locus in *Arabidopsis thaliana*. *clv3* mutant plants are similar in phenotype to *clv1* mutants (Clark et al., 1993). We provide evidence that *clv3* shoot apical meristems (SAMs) are enlarged compared to wild type in the mature embryo. *clv3* SAMs continue to enlarge throughout vegetative and inflorescence development, often becoming 1000-fold larger than wild type. *clv3-2* floral

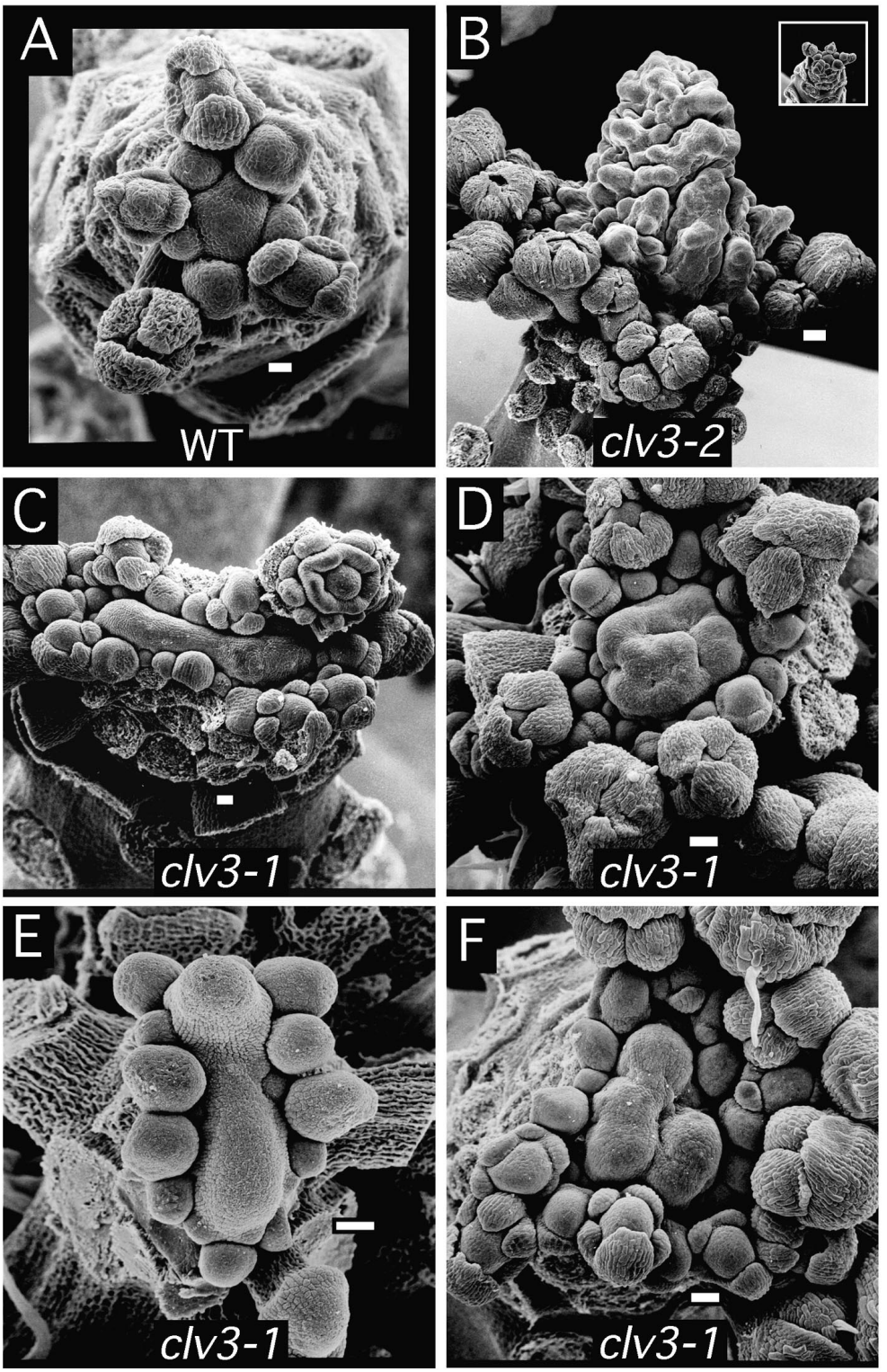


Fig. 1. Wild-type and *clv3* inflorescence apical meristems. Wild-type Landsberg (A), *clv3-2* (B) and *clv3-1* (C-F) inflorescences were prepared as described and observed under SEM (see Materials and methods). For each inflorescence, older flowers were removed to reveal the shoot apical meristem (SAM) and young flowers. SAMs of *clv3-2* (B) plants massively over-proliferate compared with wild-type (inset in B shows wild-type SAM at the same magnification). SAMs of *clv3-1* plants exhibit less dramatic line fasciation (C) or limited three-dimensional enlargement (D). (E,F) SAMs of *clv3-1* plants shortly after the transition to flowering (approx. 16 days after germination) revealing meristems that will likely fasciate (E) or enlarge in a three-dimensional fashion (F). Bars, 100 μm (B), and 10 μm for all other panels.

meristems are enlarged at the earliest stages of organ initiation and maintain an expanding pool of undifferentiated, proliferating meristematic cells throughout flower development. An examination of *clv3* roots reveal no differences from wild type, demonstrating that *CLV3* is a specific regulator of shoot and floral meristem development. We find genetic interactions between *clv1* and *clv3* mutants, suggesting that *CLV1* and *CLV3* function in the same pathway in their control of meristem development. Further evidence indicates that the CLV1 gene product may act as a multimer, and functions closely with CLV3. Finally, we present a model for the action of *CLV1* and *CLV3* in their regulation of meristem development.

MATERIALS AND METHODS

For all planting, seeds were sown on a 5-10 mm covering of Redi-earth peat-lite mix over a 1:1:1 mixture by volume of perlite:vermiculite:soil. Sown seeds were placed at 4°C for 6 days and then placed under lights. All plants were grown under 600 ft-candles of constant cool white fluorescent light at 23-25°C. Plants were fertilized approximately every 7 days. Seeds were sown at a maximum density of 1 seed per 4 cm^2.

Scanning electron microscopy (SEM) was performed as described by Bowman et al. (1989). Confocal laser scanning microscopy (CLSM) of flowers was performed as described previously by Clark et al. (1993). For microscopy of embryos, a modified procedure was developed (Running et al., 1995). Mature dry seeds were incubated overnight at 4°C in 15% ethanol. Seed coats were manually dissected from the embryo. Embryos were then stained, rinsed and cleared in an identical fashion to floral tissue. Note that no fixation was performed. For root cross-sections, roots were washed and embedded in agarose before hand sectioning and staining with fluorescent brightener 28 (Sigma).

SEM images on 35 mm negatives were digitized using a Nikon Coolscan. Digitized SEM images and converted CLSM images were combined in Adobe Photoshop where brightness and contrast were adjusted. Images were printed with a Kodak XLS8300 color printer.

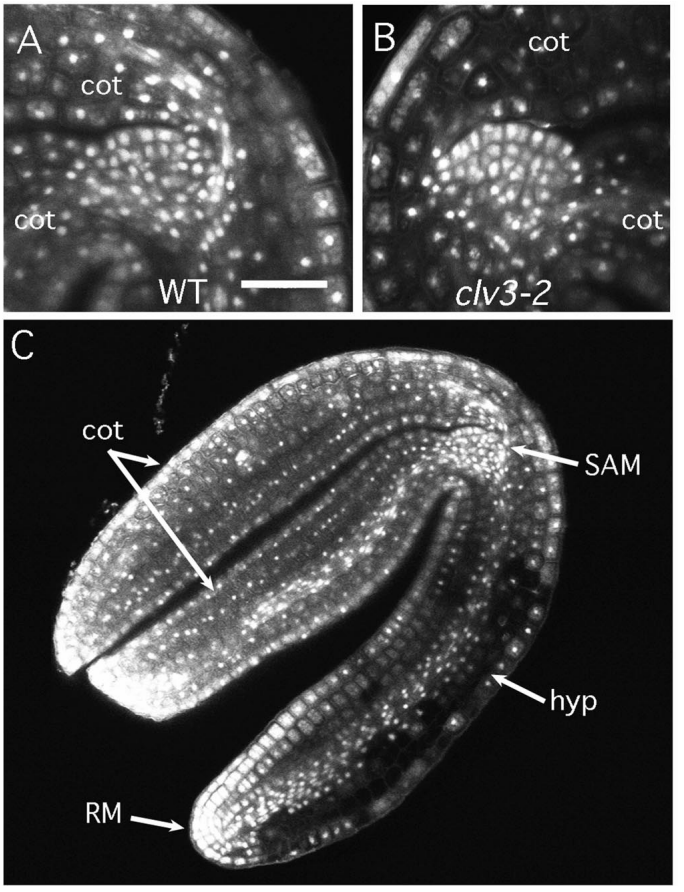
clv3-1 and *clv3-2* mutants acted as single, recessive mutations in all crosses, except those with *clv1*, where a semi-dominant phenotype was noted (see Results). *clv3-1* was mapped to chromosome 2. A three point cross of a *erecta clv3-1 hy1* triple mutant to wild-type Columbia revealed that *clv3-1* maps next to *erecta* and *hy1*, at a distance of 0.45±0.18 cM from *hy1*, and approximately 1 cM from *erecta*.

RESULTS

We have investigated two *clv3* alleles; both alleles were isolated by David Smyth. *clv3-1* was identified during an ethyl melthanesulfonate (EMS) mutagenesis of Landsberg *erecta* seeds. *clv3-2* was identified during a gamma-irradiation mutagenesis of Landsberg *erecta* seeds. Each allele was backcrossed three times to Landsberg *erecta* before analysis (see Materials and methods).

clv3 affects shoot apical meristem size throughout development

The inflorescence meristems of both *clv3-1* and *clv3-2* were enlarged compared to wild type (Fig. 1). Under optimal growing conditions (see Materials and Methods), the apical meristems of plants homozygous for *clv3-2* massively overproliferated and were clearly visible with the naked eye (Fig.



D Genotype	SAM width (μm) Mean	Range	Mean L1 cells	n
WT	29 ± 3	21 - 35	7.0 ± 0.8	22
clv3-2	40 ± 6	33 - 61	9.8 ± 1.3	24

Fig. 2. Wild-type and *clv3-2* embryonic shoot apical meristems. Wild-type Landsberg and *clv3-2* seeds were imbibed overnight in 15% ethanol at 4 °C, and prepared for CLSM with a nuclear stain, propidium iodide (see Materials and methods). Optical sections of wild-type (A,C) and *clv3-2* (B) embryos reveal apical meristem cells that are small and brightly staining, and positioned between the base of the two cotyledons (cot). The shoot apical meristems (SAM) of *clv3-2* plants are consistently larger than those of wild type. Bar 25 μm (A and B are at same magnification). hyp, hypocotyl; RM, root meristem. (D) Wild-type and *clv3-2* embryonic SAM widths. Wild-type Landsberg and *clv3-2* seeds were prepared as described and analyzed by CLSM (see Materials and methods). Measurements of the width of the SAM were taken from optical sections through the center of the SAM of embryos in the orientation shown in C. The mean width of wild-type and *clv3-2* embryonic SAMs are presented, and the standard deviation for each mean is indicated. SAM cells were defined as small, brightly staining cells between the base of the cotyledons, and were easily distinguishable from surrounding cells. The number of cells in the L1 layer of the SAM in the central optical section was also determined. The number of seeds analyzed for each mean is indicated under *n*.

1B). When compared with *clv1-4*, which is the strongest reported allele of *clv1* (Clark et al., 1993), *clv3-2* was similar in the extent of meristem over-proliferation. *clv3-1* exhibited inflorescence meristem enlargement to a lesser degree (Fig. 1C-F). *clv3-1* plants displayed line fasciation (where the meristem is transformed from a point into a line), limited three-dimensional over-proliferation, or a combination of both. This indicates that *clv3-1* is stronger in phenotype than *clv1-1* (which only exhibits line fasciation; Clark et al., 1993), but weaker in phenotype than *clv3-2* or *clv1-4*.

We sought to determine the earliest developmental stage at which *clv3* affected shoot apical meristem size. Examinations of *clv3-1* and *clv3-2* plants prior to flowering revealed that *clv3* affects shoot apical meristem size throughout vegetative development (data not shown; see also Griffith, 1994). We then examined the shoot apical meristems of mature embryos by confocal scanning laser microscopy (see Materials and methods; Fig. 2C). As shown in Fig. 2, the shoot apical meristems (SAM) of the *clv3-2* embryos were larger than those of wild-type embryos. The average SAM size for *clv3-2* embryos is a third larger than that of wild type and has a correspondingly larger number of cells in the L1 layer as seen in central optical sections through the meristem (Fig. 2D). Thus, while the *clv3-2* SAM is 30% larger in a single dimension compared to wild type at germination, it continues to enlarge compared to wild type as development continues. As plant growth slows in conjunction with leaf senescence, our observations indicate that *clv3-2* SAM enlargement also slows or stops (data not shown).

clv3 floral meristems are larger throughout their development

We have examined the *clv3* floral meristem throughout development (Fig. 3). The first organ initiation events are at stage 3 (all stages according to Smyth et al., 1990), when sepals arise on the flanks of the floral meristem (Fig. 3A,B). At this stage, the average height (from the interior base of the medial sepals to the tip of the floral meristem) of *clv3-2* floral meristems (30 μm) is more than twice that of wild type (12 μm) (Table 1). Similarly to *clv1* stage 3 floral meristems (Clark et al., 1993), the *clv3-2* floral meristems, while being much taller, are only slightly wider than wild type on average. As the flowers continue to develop, *clv3-2* flowers initiate carpels as a ring of organs around a still-proliferating central dome (Fig. 3C-F). This dome will continue to develop, giving rise to additional organs, and to a large mass of meristem tissue (Fig. 4). The organs that are initiated on the flanks of this central dome later develop into carpels that are interior to the gynoecium formed by the whorl 4 carpels. The meristem region will often proliferate to such an extent that it will grow through the gynoecium (Fig. 4C,D). Also, in the most severely affected *clv3-2* flowers, there is often elongation of the floral stem between the attachment site of whorl 1, 2 and 3 organs (sepals, petal and stamens), and the base of the carpels (cf. Fig. 4D and E).

The larger *clv3* floral meristem develops into a flower with additional organs in each whorl (Fig. 5). *clv3-2* mutants are more affected than *clv3-1*. Stamen and carpel number are more dramatically increased than sepal and petal number in *clv3* flowers.

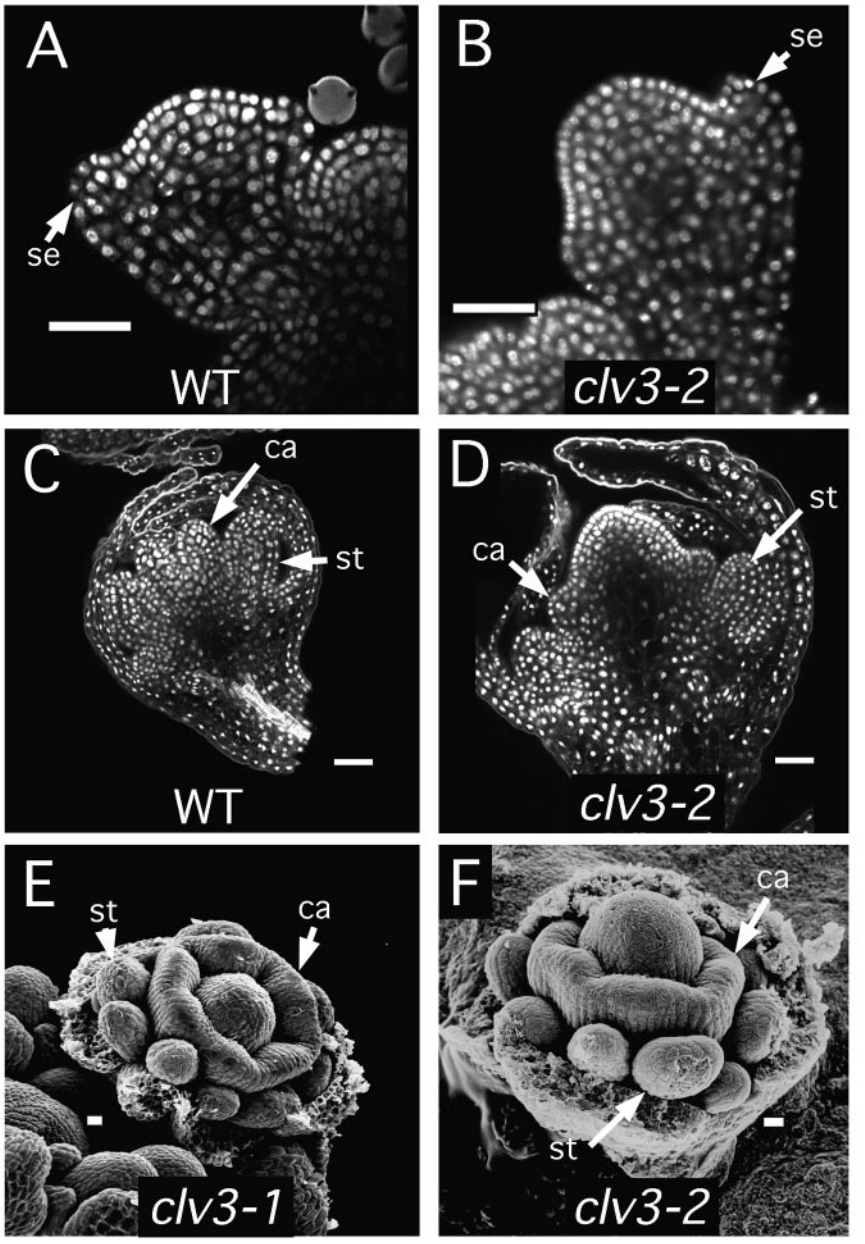


Fig. 3. Early development of wild-type, *clv3-1* and *clv3-2* flowers. Wild-type Landsberg (A,C) and *clv3-2* (B,D) flowers were prepared for CLSM as described (see Materials and methods). (A,B) Optical sections were taken through stage 3 flowers. Note the taller floral meristem in the *clv3-2* floral primordium (B). (C,D) Optical sections were taken through stage 6 flowers. Note that the carpels in the *clv3-2* flower (D) formed on the flanks of a central dome not present in wild type (C). *clv3-1* (E) and *clv3-2* (F) flowers were prepared as described and observed under SEM. Note the central dome growing out of the ring of carpels primordia (ca) is larger in *clv3-2* (F). se, sepal primordia; st, stamen primordia. Bars, 25 μm (A-D) and 10 μm (E,F).

clv3 roots are unaffected

Because *CLV3* plays a role in controlling meristem cell proliferation in both shoot and floral meristems, we sought to determine whether *CLV3* plays a general role in cell division control, or a more specific role in shoot and floral meristem development. We therefore examined a separate region of active cell division, the root meristem. Root meristems are postulated to have a fundamentally different structure than shoot or floral meristems, with specific sets of initials proposed to give rise to individual cell types (Dolan et al., 1993). In addition, the root meristem does not initiate lateral organs in the same fashion as the shoot or floral meristem. Thus, we hypothesized that if *CLV3* plays a specific role in shoot- and floral-type meristem development then the *clv3* roots would be unaffected. If, however, *CLV3* plays a general role in cell division control, then the *clv3* root meristem should be disrupted.

We examined the root tip and root shaft of 21-day old wild-type and *clv3-2* plants under CLSM (Fig. 6A-D). We also analyzed cross-sections from 8-day old wild-type and *clv3-2* plants (Fig. 6E,F). We observed no difference in the organization of initials and the root tip, nor any changes in the number of cell layers at the root shaft (Fig. 6). Layers corresponding to the epidermis, cortex, endodermis and pericycle in the exterior, and 5-6 layers of procambial cells in the interior were observed in both wild-type and *clv3-2* roots (Fig. 6). While

Table 1. Floral meristem sizes at stage 3

Genotype	Width (μm)	Height (μm)	*n*
wild-type	49±6	12±3	23
clv3-2	60±6	31±7	18

Stage 3 wild-type and *clv3-2* flowers were analyzed by CLSM and 6 μm paraffin sections. All measurements were based on central floral meristem sections through the medial sepals. Width measurements were taken between the base of the medial sepal primordia. Height measurements were taken from the inner base of the medial sepal primordia to the top of the flower. The mean width and height of wild-type and *clv3-2* flowers are presented, and the standard deviation for each mean is indicated. The number of flowers analyzed for each mean is indicated under *n*.

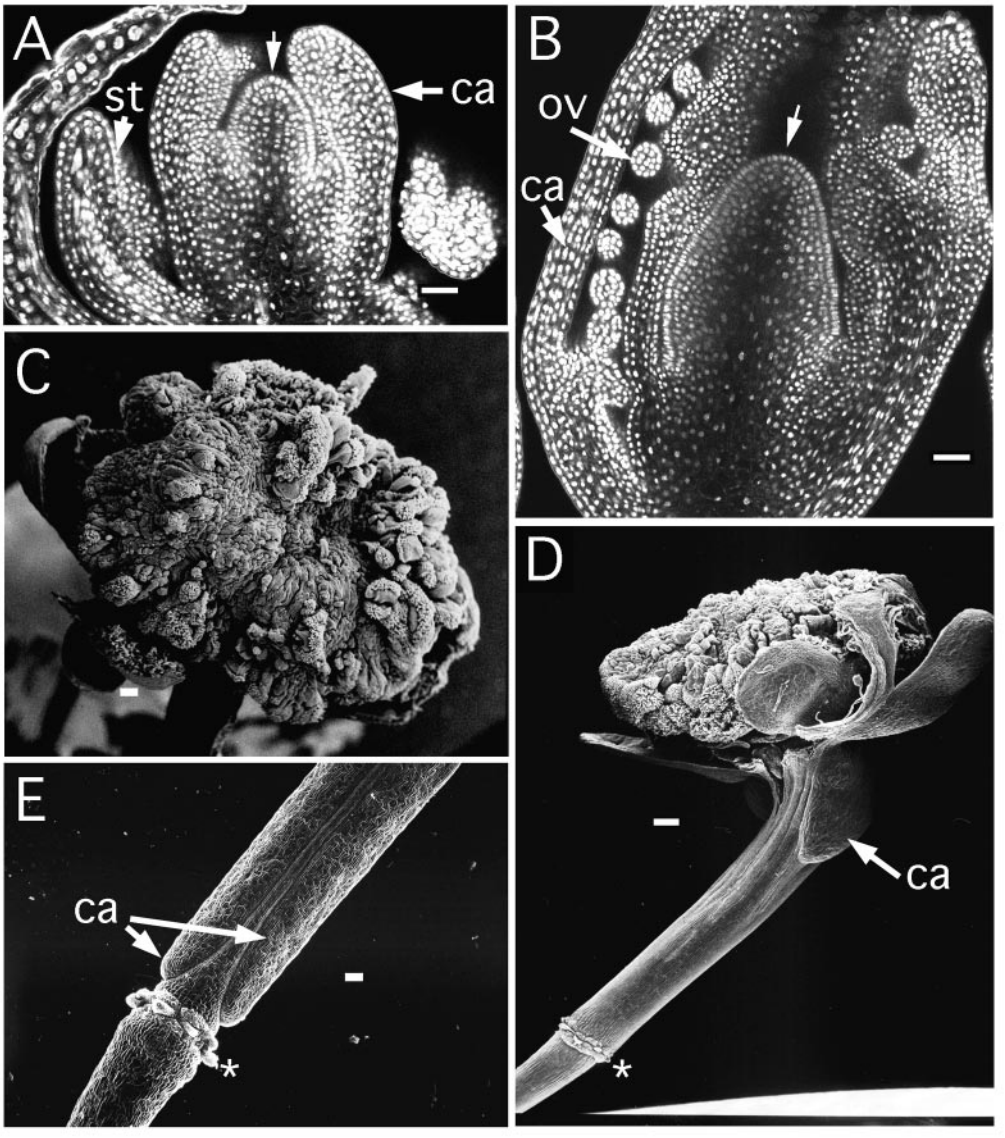


Fig. 4. Late development of *clv3-2* flowers. *clv3-2* flowers (A,B) were prepared for CLSM as described. *clv3-2* (C,D) and wild-type Landsberg (E) siliques were prepared for SEM as described. Optical sections of flowers of stages 9 through 12 reveal that the central dome in *clv3-2* flowers (arrow) continues to proliferate and continues to generate additional organs on its flank (A,B). In *clv3-2* siliques this meristem dome can grow through the carpel walls, breaking the gynoecium open (C, top view; D, side view). This meristem region is composed of undifferentiated cells and patches of stigmatic tissue. In some *clv3-2* flowers, there is elongation between the base of the carpels and the site of attachment of whorl 1, 2 and 3 organs compared with wild type. ca, carpel; st, stamen; ov, ovule; *, site of attachment of whorl 1, 2 and 3 organs. Bar, 50 μm (A,B); 100 μm (C,E) and 200 μm (D).

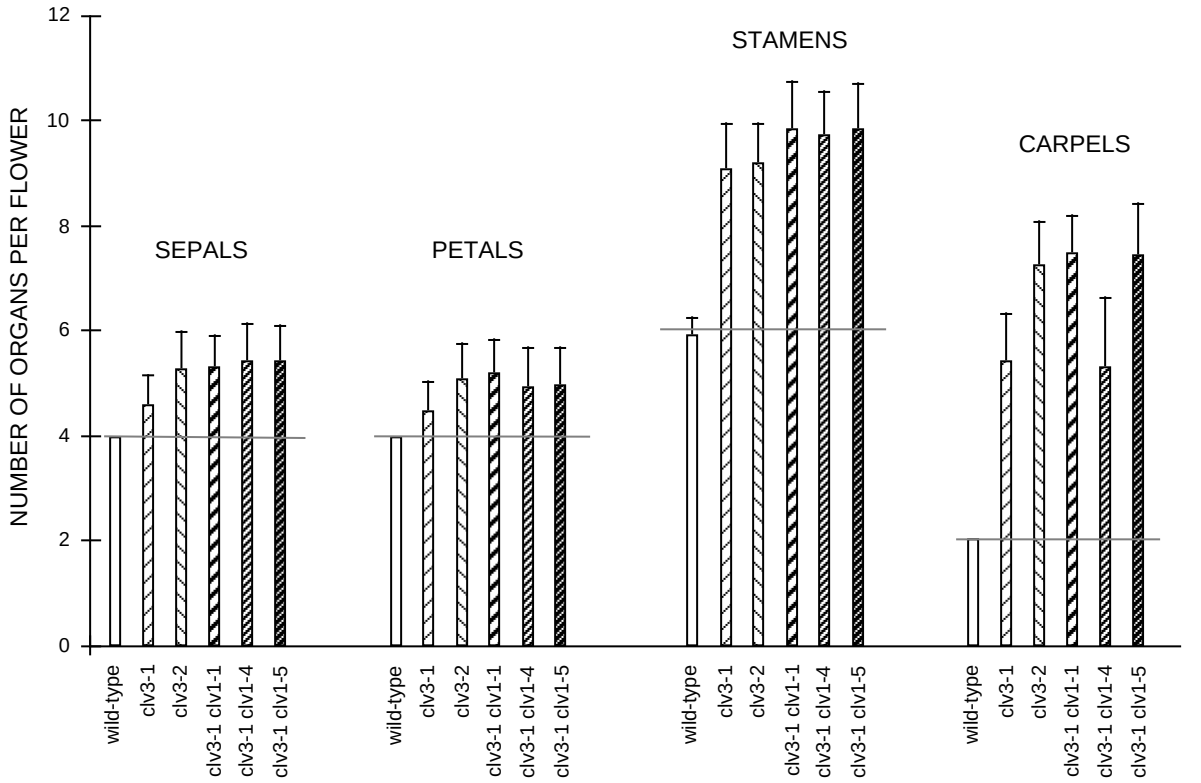


Fig. 5. The number of organs in wild-type, *clv3* and *clv1 clv3* double mutant flowers. Bars represent the mean number of indicated organs for flowers of wild-type Landsberg, *clv3-1*, *clv3-2*, *clv3-1 clv1-1*, *clv3-1 clv1-4*, and *clv3-1 clv1-5* plants. At least 100 flowers were counted for each mean calculated. Only the first ten flowers of any given plant were analyzed. Standard deviations are indicated by vertical black lines. The dotted horizontal lines represent the normal wild-type organ number. Note that the upper limit of the mean plus standard deviation for the number of stamens for wild-type flowers exceeds six due to flowers with 4 and 5 stamens, but no wild-type flowers were observed with more than six stamens. Whorl 2 and 3 organs that developed as filamentous organs (see text) were included in the counts of petals and stamens, respectively.

subtle differences may have gone unnoticed, the dramatic changes seen in the structure of *clv3-2* shoot and floral meristems are not seen in the root.

clv1 and *clv3* mutants display genetic interactions

Because *clv1* and *clv3* mutants both result in similar phenotypes, we sought to test whether they demonstrate any genetic interactions. We first analyzed *clv1 clv3* double mutant plants. We hypothesized that if *CLV1* and *CLV3* work in the same pathway to control meristem development, then *clv1 clv3* double mutant plants should have a phenotype similar to a strong *clv1* or *clv3* single mutant. If, however, *CLV1* and *CLV3* work in different pathways to control meristem development, then a *clv1 clv3* double mutant might display novel or exaggerated phenotypes. To confirm the successful construction of *clv1 clv3* double mutant plants, each potential double mutant was backcrossed to both *clv1* and *clv3*.

All *clv1 clv3* double mutant plants, including those constructed with weaker alleles, were similar in phenotype to *clv1-4* and *clv3-2* single mutants plants (Figs 5 and 7, Table 2). Only the *clv1-1 clv3-1* double mutant displayed a distinctive phenotype. *clv1-1* single mutants develop approximately 50% more leaves than wild-type plants over the same period of time (7.9±1.2 rosette leaves for wild type versus 12.6±1.7 for *clv1-1*); this increase in leaf number was enhanced in the *clv1-1 clv3-1* double mutant.

Table 2. *clv1 clv3* double mutant phenotypes

	***CLV1* GENOTYPE**			
***CLV3* GENOTYPE**	CLV1	clv1-5	clv1-1	clv1-4
CLV3	Wild Type	Intmed	Intmed	Strong
clv3-1	Intmed	Strong	Strong	Strong
clv3-2	Strong	ND	Strong	Strong

The phenotypes of *clv1* single mutants, *clv3* single mutants and *clv1 clv3* double mutants (all homozygous) are indicated. Phenotype classification is based on the extent of shoot meristem proliferation, the number of floral organs, and the extent of floral indeterminacy. ND, not determined; Intmed, intermediate phenotype.

A second method for investigating *CLV1* and *CLV3* interactions was to analyze *clv1*/+ *clv3*/+ double heterozygotes.

Because *clv1* is semi-dominant, we hypothesized that if *CLV1* and *CLV3* function together, then the semi-dominance of *clv1* might be enhanced by the addition of a heterozygous *clv3* allele. Various *clv1* and *clv3* alleles were crossed to wild-type Landsberg and to each other. The resulting F_1s were planted, and the number of organs on 100 flowers of each genetic combination were counted. Table 3A presents the number of flowers for each genetic combination with gynoecia composed of two carpels, three carpels or four carpels. Sepal and petal number were not affected in *clv*/+ plants, and stamen number was only slightly affected (data not shown). For *clv1*/+ plants, those with the *clv1-1* and *clv1-5* alleles exhibited the strongest dominance, despite the fact that these alleles are not the strongest alleles when homozygous, suggesting some type of dominant interference. *clv3-1* was recessive, and the semi-dominance of *clv3-2* was barely detectable. For plants doubly heterozygous, the effect of the added *clv3* allele was compared to plants heterozygous for *clv1* only (Table 3B). χ^2 tests indicate that *clv3-1* and *clv3-2* both significantly enhanced the semi-dominance of *clv1*/+ plants.

Unusual organs

clv1 and *clv3* mutant plants can develop unusual organs (data not shown). Occasionally filamentous organs replace stamens or, rarely, petals. This filament substitution occurs most commonly in the adaxial positions, less commonly in the abaxial positions, and rarely in lateral positions. The inflorescence apical meristems of strong *clv* mutants (*clv1-4*, *clv3-2* and *clv1 clv3* double mutants) occasionally develop reduced flowers and filamentous organs. The reduced flowers have 2-4 sepals and few or no internal organs. The filamentous organs are similar to those seen in *clv1-4 lfy* mutants (Clark et al., 1993).

DISCUSSION

We have investigated the effects of mutations at the *CLV3* locus on *Arabidopsis thaliana* development. The effects of *clv3* appear to be limited to shoot and floral meristem development, with root meristem development unaffected. *clv1 clv3* double mutant plants are similar in phenotype to *clv1* and *clv3* strong allele single mutant plants. In addition, the semi-dominance of *clv1* alleles is enhanced by heterozygosity at the *clv3* locus.

CLV3 is a specific regulator of shoot and floral meristem development

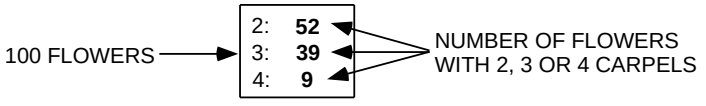
We observed that the *clv3-2* shoot apical meristem (SAM) is larger than wild type in the mature embryo and continues to enlarge compared to wild type such that the *clv3-2* inflorescence SAM can become 1000-fold larger (in volume) than a similar stage wild-type inflorescence SAM. The direct result of a *clv3* mutation is to increase the number of undifferentiated cells at the shoot meristem. While wild-type *Arabidopsis thaliana* has one of the smallest shoot apical meristems known among the flowering plants, the shoot apical meristems of *clv3-2* mutants are larger than almost all known shoot meristems among flowering plants (Gifford and Corson, 1971). This implies that the role of wild-type *CLV3* is to regulate the pool of meristematic cells in the wild-type shoot meristem.

clv3 floral meristems (FMs) are also enlarged compared to wild type. The *clv3* FM not only undergoes additional cell proliferation in early stages compared to wild type, but also maintains a continually enlarging meristem. The wild-type FM, however, fails to maintain a pool of meristematic cells, as the SAM does, and as a result is a determinate structure. In this sense, the *clv3* FM is fundamentally different from the wild-type FM: it maintains a pool of meristematic cells and is indeterminate. These observations suggest that one role of *CLV3* is in restriction and eventual elimination of meristematic cellular proliferation in the wild-type floral meristem.

Table 3. Phenotypes of *clv1/clv3* double heterozygotes

A

	+ / +	clv1-1 / +	clv1-4 / +	clv1-5 / +	clv1-6 / +	clv1-7 / +
+ / +	2: **99** 3: **1** 4: **-**	2: **74** 3: **20** 4: **6**	2: **95** 3: **5** 4: **-**	2: **77** 3: **20** 4: **3**	2: **99** 3: **1** 4: **-**	2: **100** 3: **-** 4: **-**
clv3-1 / +	2: **99** 3: **1** 4: **-**	2: **45** 3: **38** 4: **17**	2: **76** 3: **21** 4: **3**	2: **75** 3: **20** 4: **5**	2: **89** 3: **8** 4: **3**	2: **99** 3: **1** 4: **-**
clv3-2 / +	2: **94** 3: **5** 4: **1**	2: **27** 3: **35** 4: **38**	2: **70** 3: **29** 4: **1**	2: **52** 3: **39** 4: **9**	2: **100** 3: **-** 4: **-**	2: **96** 3: **4** 4: **-**



B

	2 carpels	3 carpels	4 carpels
clv1/+ CLV3	**445**	**46**	**9**
clv1/+ clv3-1/+	**384**	**88**	**28**
clv1/+ clv3-2/+	**345**	**107**	**48**

CLV3 vs. clv3-1/+ χ^2 $P < 0.001$

CLV3 vs. clv3-2/+ χ^2 $P < 0.001$

Plants homozygous for various *clv1* alleles were crossed to wild-type or *clv3* homozygous plants. In addition, *clv3* plants were crossed to wild type. (A) For each cross, F_1 seeds were planted and 100 flowers of the resulting plants were counted. Each box represents the number of those 100 flowers with gynoecia composed of two carpels (2), three carpels (3) or four carpels (4). (B) The counts in A are combined for all *clv1* alleles in a *CLV3*, *clv3-1*/+ and *clv3-2*/+ backgrounds to determine the effect of *clv3* heterozygousity in *clv1*/+ plants. χ^2 tests (DF = 2) were then performed to determine if the differences between *clv3-1*/+ and *CLV3*, and the differences between *clv3-2*/+ and *CLV3* were significant. *P* values for both comparisons were <0.001, and Fisher's Exact Test (2 tail) *P* values were 8.27E-07 (for *clv3-1*/+) and 3.27E-15 (for *clv3-2*/+).

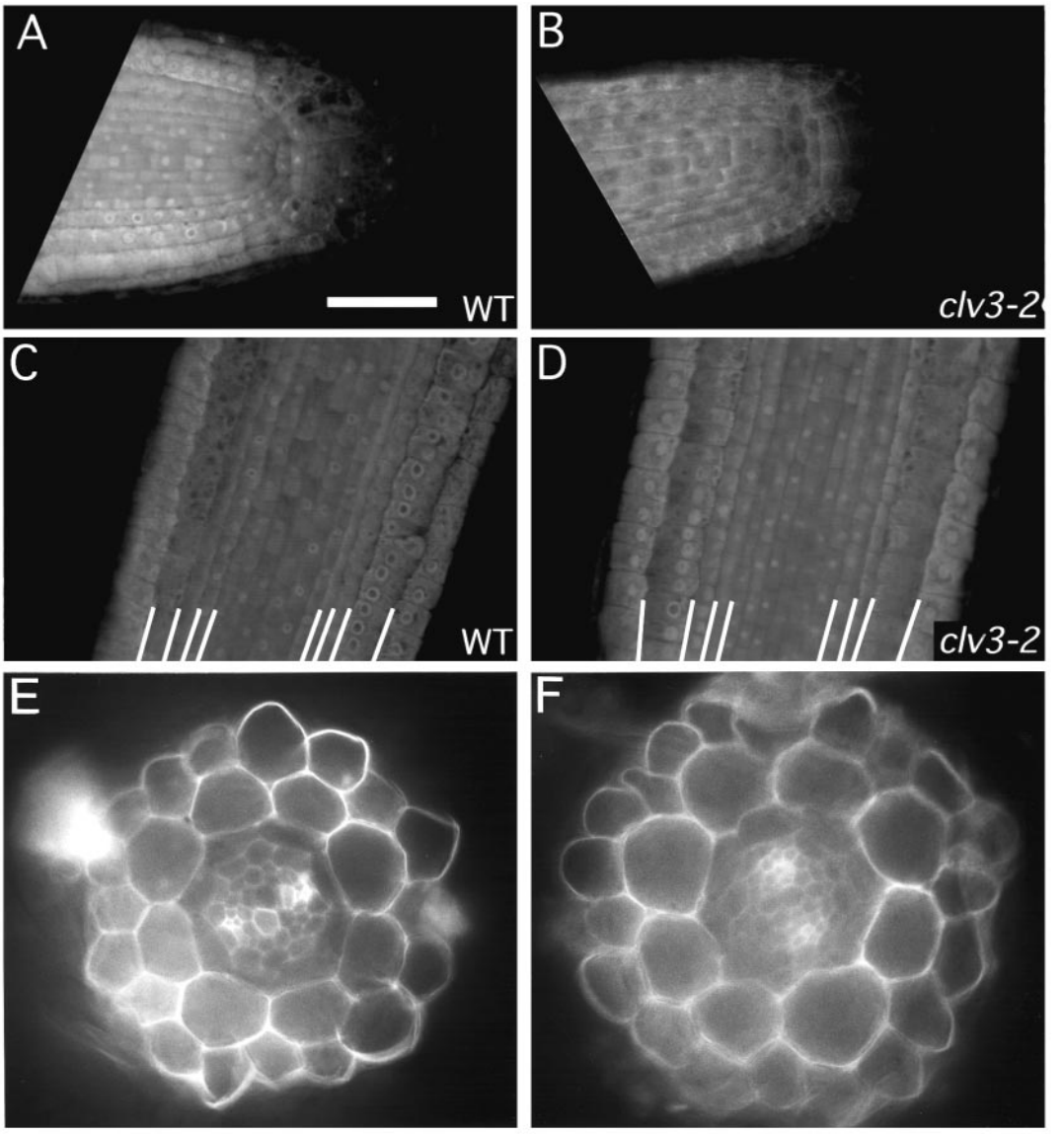


Fig. 6. Analysis of *clv3* roots. Roots from 21-day old wild-type (A,C) and *clv3-2* (B,D) roots were prepared for CLSM. Optical sections through root tips (A,B) and root shafts (C,D) are shown. (E,F) Hand cut sections of 8-day old wild-type and *clv3-2* roots are also shown (see Materials and methods). No differences were observed between the *clv3-2* root tips (C) and those of wild type (A). The *clv3-2* root shafts (D,F) had identical cell layers and cell numbers as the wild-type root shafts (C,E). Because root shaft images were taken at a variable distance from the tip, variations in root shaft thickness were observed. Bar, 25 μm (A-D).

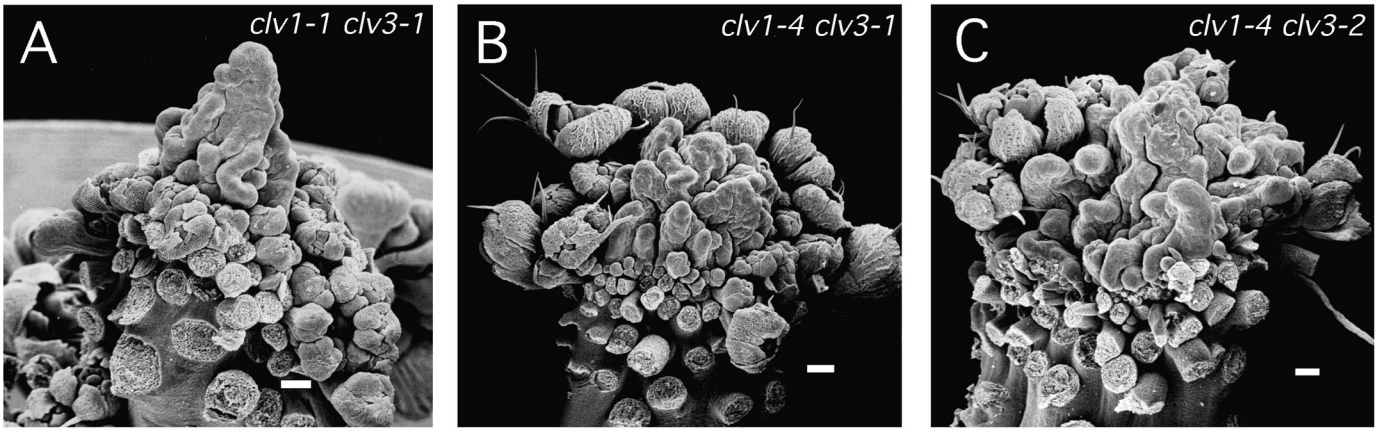


Fig. 7. *clv1 clv3* double mutant plants. Scanning electron micrographs of shoot apical meristems of *clv3-1 clv1-1*, *clv3-1 clv1-4* and *clv3-2 clv1-4* plants. The shoot meristem is the large mass of cells in the center of each inflorescence. Note that all double mutants have massive over-proliferation of the shoot meristem similar to *clv3-2* (Fig. 1B) and *clv1-4* (Clark et al., 1993) single mutants. Bar, 100 μm.

Allele	clv1-1	clv1-4	clv1-5	clv1-6	clv1-7	clv3-1	clv3-2
Homozyg. Phenotype	Intmed	Strong	Intmed	Weak	Weak	Intmed	Strong
Heterozyg. Phenotype	Yes	Slight	Yes	No	No	No	Slight

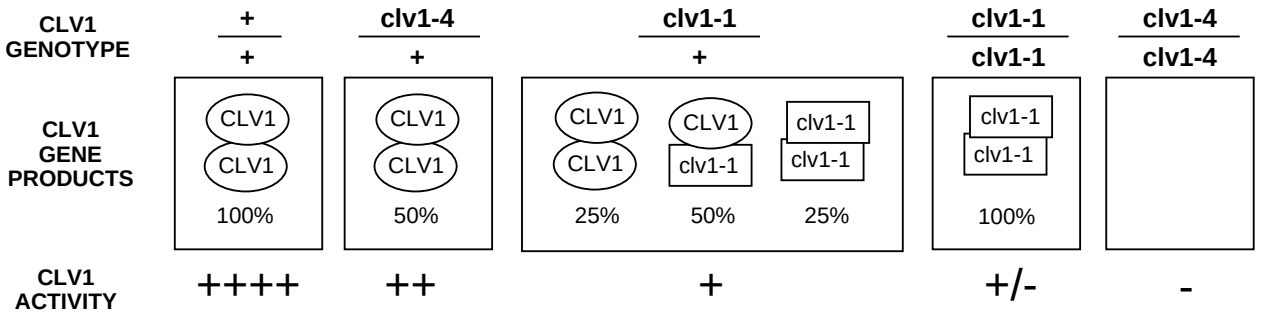


Fig. 8. Model for *clv1* semi-dominance. The table indicates the severity of homozygous and heterozygous mutant phenotypes for various *clv1* and *clv3* alleles. Below is a model to explain the observation that the weak and intermediate *clv1* alleles *clv1-5* and *clv1-1* display the strongest semi-dominance. Shown below each genotype are the complement of CLV1 multimers and the resulting level of activity. This assumes that CLV1 acts as a dimer, that the clv1-1 gene product is able to bind to and interfere with CLV1 subunits, and that *clv1-4* is either a null allele or encodes a protein with no activity and incapable of binding to wild-type subunits. Intmed, intermediate phenotype.

One question we wished to address was whether *CLV3* plays a specific role in shoot and floral meristem development, or whether it is a more general regulator of cell division patterns. In an attempt to distinguish between these two possibilities, we examined another region of active cell division: the root meristem (RM). We hypothesized that if *CLV3* is generally involved in cell division control, then the *clv3* roots would be severely affected, because the root appears to rely on coordinated cell division patterns in the different sets of initials (Dolan et al., 1993). If, however, *CLV3* is a specific regulator of shoot and floral meristem development, then we would predict that the *clv3* root would be unaffected. In examination of *clv3-2* and wild-type roots, we found no difference in the *clv3* roots, supporting the notion that *CLV3* is a specific regulator of shoot and floral meristem development.

Another gene hypothesized to play a regulatory role in meristem development, *KNOTTED1* from maize, is expressed in both the shoot and floral meristem, but not in the root meristem (Smith et al., 1992). In addition, *stm* mutants of *Arabidopsis* fail to form a shoot meristem, but the root meristem develops normally (Barton and Poethig, 1993). This raises the possibility that the root meristem development is controlled by a different set of genes, and is regulated independently of the shoot meristem.

CLV1 and *CLV3* closely interact to affect the same processes

clv1 clv3 double mutants are identical in phenotype to strong *clv1* or *clv3* single mutants. The simplest interpretation is that *CLV1* and *CLV3* work in the same pathway: a strong homozygous *clv1* mutation eliminates the functioning of that pathway so that a further mutation at *clv3* has no effect, and vice versa.

Analysis of *clv1* and *clv3* semi-dominance provides evidence that these genes function together in this pathway. *clv1-1*, *clv1-4* and *clv1-5* alleles have a detectable semi-dominant phenotype (Table 3). Curiously, the *clv1* allele that has the strongest phenotype when homozygous (*clv1-4*) has a weaker semi-dominant phenotype than two alleles (*clv1-1* and *clv1-5*) that are weaker in phenotype when homozygous (Fig. 8). This suggests that the *CLV1* gene product may act as a

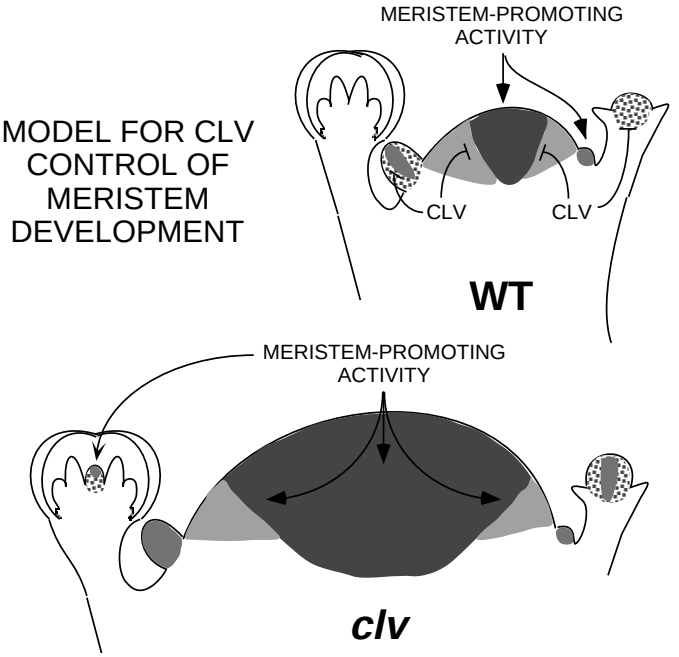


Fig. 9. Model for *CLV* action. The model postulates that there is a meristem-promoting activity (MPA) in shoot meristems and young flowers that maintains cells in a proliferative, undifferentiated state. We propose that *CLV* acts to restrict this MPA to the center of the shoot meristem, so that cells on the flanks of the meristem can enter a specific developmental pathway. Similarly, in the young floral meristem there is a meristem-promoting activity (not necessarily the same MPA as in the shoot) that provides for floral meristem proliferation. This floral MPA is first restricted, then eliminated by the action of the *CLV* loci (see also text). Higher levels of MPA are indicated by darker shading and lower levels by lighter shading.

multimer and that the weaker alleles, when heterozygous, exhibit dominant interference. A possible scenario for this is shown in Figure 8. Here the *CLV1* protein has been hypothesized to act as a dimer. Underneath each genotype is shown the amount of *CLV1* dimer that would be formed, and the resulting level of activity. This level of activity mirrors the phenotype seen for each genotype.

Further analysis of *clv1*/*clv3* double heterozygotes revealed that *clv3* heterozygosity increased the dominant phenotype of the *clv1* heterozygotes. Thus, the level of activity of *CLV1* is very sensitive to the level of activity of *CLV3*. This suggests that these genes function closely together, perhaps by forming a heteromultimer or acting in adjacent steps in a linear pathway.

A more complicated interpretation is that *CLV1* and *CLV3* function in parallel pathways that converge on the same point. This scenario, however, would require that each of the two pathways in question have no function in the absence of the other (i.e., *CLV1* has no function in a *clv3* mutant). In addition, each pathway could not have independent branch points, because *clv1* and *clv3* mutants have no independent phenotypes. Finally, *CLV1* and *CLV3* would have to function near enough to the endpoints to provide the dosage sensitivity seen in the *clv1 clv3* double heterozygous plants. In either case, *CLV1* and *CLV3* exert their influence on the same process in meristem development.

Model for *CLV* action

We have developed a preliminary model for *CLV* (*CLV1* and *CLV3*) action, based on the observation that *CLV* acts to regulate the amount of undifferentiated cells in both the shoot and floral meristem. As shown in Fig. 9, we postulate that there is a meristem-promoting activity (MPA) in shoot meristems and young flowers that maintains cells in a proliferative, undifferentiated state. We propose that *CLV* acts to restrict this MPA to the center of the shoot meristem, so that cells on the flanks of the meristem can enter a specific developmental pathway. It is in this flank region, for example, that *LEAFY* is first expressed, sending cells down a floral developmental pathway (Weigel et al., 1992). Similarly, in the young floral meristem there is a meristem-promoting activity (not necessarily the same MPA as in the shoot) that provides for floral meristem proliferation. This floral MPA is first restricted, then eliminated, by the action of the *CLV* loci. In the *clv* shoot meristem, high levels of MPA extend to the flanks of the shoot meristem, preventing these cells from entering a specific developmental pathway. Thus, the cells on the flanks of the shoot meristem become part of the meristem, enlarging it. This is reiterated, leading to the 1000-fold larger *clv* shoot meristem. In the young *clv* floral meristem (stages 1-3), failure to properly restrict the MPA leads to a larger floral meristem because of additional meristem activity. As the *clv* flowers develop, the MPA is not eliminated from the floral meristem, so a pool of undifferentiated cells continues to proliferate throughout flower development.

Indirect evidence that supports the model comes from examination of cells in the center of the *clv* flower versus cells in the center of the wild-type flower. The model would predict that *clv* flowers are not simply larger than wild type, but that the MPA present in the center of the *clv* flower would make these cells qualitatively different. A test of this difference might be to examine *AGAMOUS* expression. *AGAMOUS* is a floral organ identity gene responsible for the correct development of the stamens and carpels (Bowman et al., 1989; Yanofsky et al., 1990). *AGAMOUS* is expressed throughout the center of the flower beginning at stage 3, and continues to be expressed in stamen and carpel primordia during their initiation and differentiation (Drews et al., 1991). The model would predict that in the center of *clv* flowers high MPA would retain the cells in an undifferentiated state, and thus prevent the expression of a specific developmental regulator. Indeed, previous experiments have indicated that *AGAMOUS* is not expressed in the center of *clv1* flowers, and is not expressed in the center of the meristem dome as late as stage 12 (Clark et al., 1993). *AGAMOUS* expression is similarly altered in *clv3* flowers (S. E. C. and E. M. M., unpublished data). Thus cells in the center of *clv* flowers are qualitatively different from those in the center of a wild-type flower.

A second possibility, which is not mutually exclusive with the one outlined above, is that *CLV* acts as a molecular 'switch' in cells of the shoot meristem. CLV might be present in all cells of the shoot meristem, and be responsible for detecting that the shoot apex has grown further away and/or that adjacent basal cells have begun to differentiate. This signal, perhaps transmitted via cell-to-cell communication, would allow the cells to enter a specific developmental pathway. Thus CLV would act to promote the switch of cells from an undifferentiated toward a differentiated state in both the shoot and floral meristem.

Finally, *CLV1* and *CLV3* could also act in the center of the shoot, instead of at the flanks as proposed in the first model. *CLV1* and *CLV3* might act to restrict the rate of division of cells in the center of the shoot meristem, so that in *clv* mutants, these cells would divide faster, providing for a larger pool of undifferentiated cells. However, it is difficult to explain the floral phenotype with this model. If the larger *clv* floral meristem is simply the result of additional cell division, then why would cells in the center not express *AGAMOUS*? and why would they fail to differentiate?

At this stage it is difficult to distinguish between these models. Identification of the *CLV1* and *CLV3* genes and examination of their expression patterns may shed light on this question.

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