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The Stem Cell Population of *Arabidopsis* Shoot Meristems Is Maintained by a Regulatory Loop between the *CLAVATA* and *WUSCHEL* Genes

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Summary

The higher-plant shoot meristem is a dynamic structure whose maintenance depends on the coordination of two antagonistic processes, organ initiation and self-renewal of the stem cell population. In *Arabidopsis* shoot and floral meristems, the *WUSCHEL* (*WUS*) gene is required for stem cell identity, whereas the *CLAVATA1*, *2*, and *3* (*CLV*) genes promote organ initiation. Our analysis of the interactions between these key regulators indicates that (1) the *CLV* genes repress *WUS* at the transcript level and that (2) *WUS* expression is sufficient to induce meristem cell identity and the expression of the stem cell marker *CLV3*. Our data suggest that the shoot meristem has properties of a self-regulatory system in which *WUS*/*CLV* interactions establish a feedback loop between the stem cells and the underlying organizing center.

Introduction

The shoot meristem of higher plants is a dynamic stem cell system that repeatedly initiates organs during postembryonic life by integrating two fundamental functions. First, a small number of pluripotent stem cells that serve as the ultimate source of all shoot cells is maintained in the central zone (CZ) of slowly dividing cells. Their recent peripheral daughters themselves may act transiently as stem cells giving rise to a more restricted part of the shoot, until they become replaced through a division of a more apical stem cell (Stewart and Dermen, 1970). Second, the more rapidly dividing cells in the surrounding regions enter differentiation and initiate organs. At least in the small *Arabidopsis* shoot meristem, this occurs as soon as the cells have left the CZ, as indicated by the onset of marker gene expression for organ formation, e.g., *ZWILLE* (Lynn et al., 1999; Moussian et al., 1998) or *AINTEGUMENTA* (*ANT*) (Elliott et al., 1996).

The shoot meristem of angiosperms comprises three largely clonally independent cell layers, L1–L3. In *Arabidopsis*, the L1 and L2 cells predominantly divide anticlinally, giving rise to the epidermis and subepidermal tissue, respectively, whereas the L3 cells divide more randomly and form internal tissue. Clonal analysis has suggested that within each layer, all shoot cells are ultimately derived from 2–3 stem cells (e.g., Stewart and Dermen, 1970). The expression domain of the *CLV3* gene coincides with the presumed position of the apical stem cells and so far is the only molecular marker associated with stem cell identity (Fletcher et al., 1999).

How is the shoot meristem organization stably maintained while the resident cells constantly change? Cell fate in plants is dictated by a cell's position, indicating that cells respond to signals from their neighbors. This suggests that maintaining the shoot meristem organization requires regulation at two levels. First, distinct subpopulations must be delimited and second, different developmental programs must be specified within these subpopulations. Recently, several key genes involved in this regulation have been identified.

The homeobox gene *WUSCHEL* is required for specifying stem cell identity. Mutations in *WUS* result in the misspecification of stem cells and premature termination of shoot and floral meristems after a few organs have been formed (Laux et al., 1996). *WUS* expression is initiated at the 16-cell embryo stage, long before a shoot meristem is evident, and gradually becomes restricted to the center of the developing shoot meristem primordium by several asymmetric cell divisions (Mayer et al., 1998). In the active shoot meristem, *WUS* is expressed in a small cell group underneath the presumed position of the stem cells. The discrepancy between its expression domain and the cells where *WUS* function is required has led to the hypothesis that *WUS*-expressing cells act as an organizing center of the shoot meristem and signal to overlying neighbors to specify them as pluripotent stem cells (Mayer et al., 1998).

The *CLAVATA* (*CLV1, CLV2, CLV3*) genes promote the progression of meristem cells toward organ initiation. Mutations in any of these genes result in delayed organ initiation, leading to an accumulation of meristem cells and to a gradual increase in size of the shoot meristem dome (Clark et al., 1993, 1995; Kayes and Clark, 1998; Laufs et al., 1998b). Genetic and molecular analyses suggest that the three *CLV* genes encode components of a receptor kinase signaling pathway, in which CLV1 represents a putative receptor kinase (Clark et al., 1997), CLV2 a presumed accessory protein of the signaling complex (Jeong et al., 1999), and CLV3 a putative signaling peptide (Fletcher et al., 1999). The function of the *CLV* genes is antagonized by the *SHOOTMERISTEMLESS* (*STM*) gene (Clark et al., 1996), which is required to prevent the incorporation of central meristem cells into organ primordia (Endrizzi et al., 1996; Long et al., 1996).

Here we address the question of how the stem cell population is stably maintained in a changing cellular context. To this end, we have investigated the interactions between the *WUS* and the *CLV* genes at the genetic and molecular levels.

Results

Genetic Interactions between *CLV* and *WUS*

To study genetic interactions between *WUS* and the *CLV* genes, we analyzed double mutant combinations

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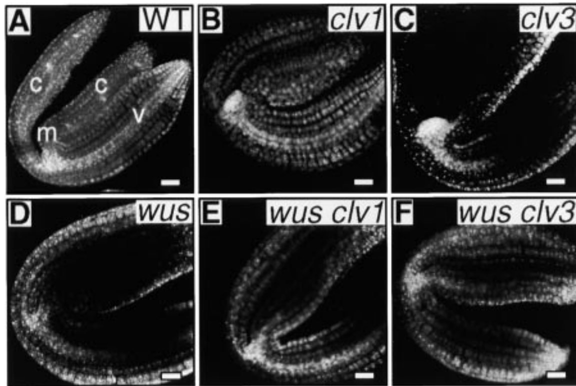

Figure 1. Shoot Meristem Phenotypes in Mature Embryos

(A) Wild type. The shoot meristem comprised of small cells gives a stronger signal, due to the density of brightly staining nuclei.

(B and C) The shoot meristem is enlarged in *clv1* and *clv3* embryos.

(D–F) No shoot meristem is recognized in *wus*, or *wus clv1* and *wus clv3* embryos.

Confocal laser-scanning microscopy images of embryos stained with the nuclear stain propidium iodide. c, cotyledon; m, shoot meristem; v, vasculature. Bars, 30 μm.

throughout development. We used the following single mutant lines: *wus-1* (Laux et al., 1996), *clv1-4* (Clark et al., 1993), *clv2-1* (Kayes and Clark, 1998), and *clv3-2* (Clark et al., 1995), all of which are likely to represent null alleles. We will focus on the analysis of *wus clv1* and *wus clv3* plants. *clv2* mutants display similar, but weaker defects when compared to *clv1* and *clv3* (Kayes and Clark, 1998), and *wus clv2* double mutants showed the same effects as *wus clv1* and *wus clv3* double mutants (data not shown).

Embryo Development

We analyzed the shoot meristem phenotypes of mature embryos using propidium iodide as a nuclear stain. Regions of small meristematic cells give areas of closely packed bright signals, whereas regions of large and vacuolated cells give dispersed signals. In wild type, the shoot meristem is recognized as a bulge of meristem cells between the cotyledons (Figure 1A). *clv1* and *clv3* embryos display a shoot meristem similar to wild type, which is however increased in size (Figures 1B and 1C). By contrast, *wus* embryos display a flat, weakly staining apex (Figure 1D). Previous histological studies have shown that *wus* apices do not have meristem cells at this stage (Laux et al., 1996). *wus clv1* and *wus clv3* embryo apices are indistinguishable from *wus* single mutants (Figures 1E and 1F).

Shoot Development

The wild-type shoot meristem gives rise to a rosette of leaves and subsequently forms an inflorescence of numerous floral meristems (Figure 2A). The dome-shaped *clv1* and *clv3* shoot meristems are enlarged relative to wild type, and inflorescence meristems initiate many flowers around their periphery (Figures 2B and 2C). By contrast, repeatedly initiated *wus* shoot meristems terminate prematurely in a flat apex (Figure 2D). Infrequently formed inflorescences give rise to no more than 3–4 flowers before termination (Figure 2D; Laux et al., 1996). *wus clv1* and *wus clv3* shoot meristems are indistinguishable from the *wus* single mutant in that they terminate prematurely in flat apices after 3–4 flowers have been initiated (Figures 2E and 2F). However, a minority of *wus clv1* and *wus clv3* inflorescences produced more than 10 flowers before termination (data not shown).

Flower Development

wus flowers lack most of the central organs, stamens, and carpels (Laux et al., 1996). To more precisely examine this phenotype, we compared a developmental series of *wus* floral meristems to wild type. The wild-type floral meristem arises from the inflorescence meristem as a bulge of cells (Figure 3A). At stage 3 of flower development (staging according to Bowman, 1994), it initiates a whorl of sepal primordia at its periphery (Figure 3B). After forming the second whorl of four petals and the third whorl of six stamens, the floral meristem terminates in a central gynoecium (Figure 3C and Table 1). Initially, *wus* floral meristems are indistinguishable from wild type (Figure 3D). However, when sepal primordia are formed, the *wus* floral apex assumes a flat structure (Figure 3E) in contrast to the dome-shaped wild-type meristem. After the sepals and petals have been

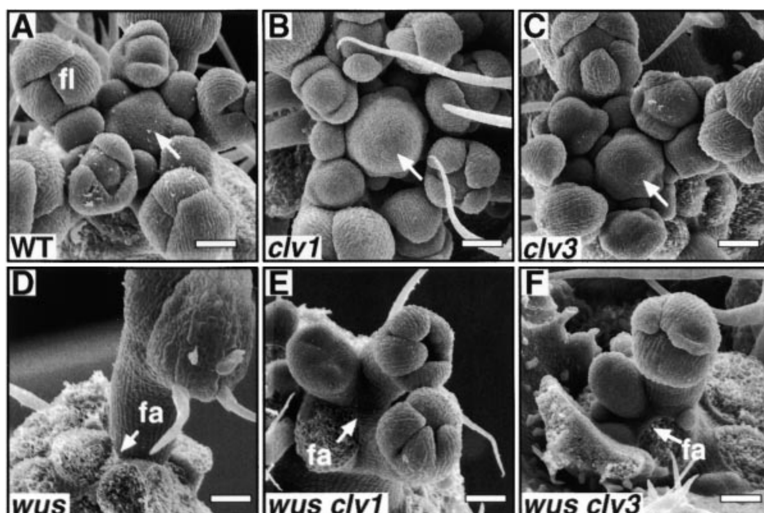

Figure 2. Inflorescence Phenotypes

(A) Wild type. The shoot meristem (arrow) is surrounded by floral meristems.

(B and C) The shoot meristem (arrow) is enlarged in *clv1* and *clv3* inflorescences.

(D–F) The shoot meristem terminates prematurely in a flat apex (arrows) after a few floral primordia are formed in *wus*, *wus clv1*, and *wus clv3* plants.

SEM images of inflorescences. fa, flat apex; fl, floral meristem. Bars, 1 mm.

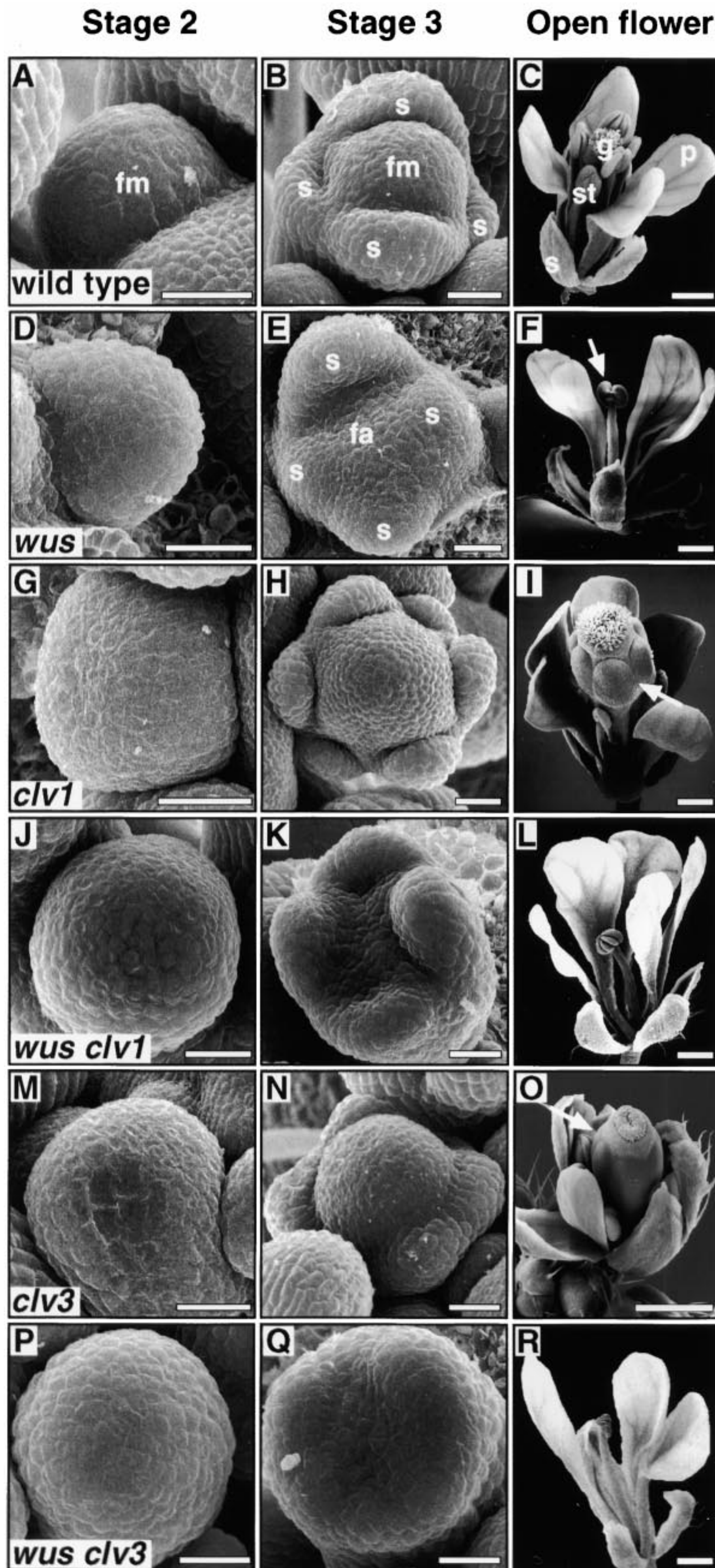

Figure 3. Flower Development

(A–C) Wild type. (A) Stage 2 flower: the floral meristem is a convex structure. (B) Stage 3 flower: the convex floral meristem is surrounded by four sepal primordia. (C) Open flower: sepals, petals, stamens, and the gynoecium are visible.

(D–F) *wus*. (D) Stage 2 flower: the floral meristem is a convex structure indistinguishable from wild type. (E) Stage 3 flower: sepal primordia are present, but the apex is flat, in contrast to wild type (B). (F) Open flower: sepals and petals are visible, but only one central stamen (arrow) and no female organs are present.

(G–I) *clv1*. (G and H) The floral meristem is convex throughout development, but increases in size relative to wild type. (I) Open flowers display supernumerary carpels (arrow).

(J–L) *wus clv1*. Flower development is indistinguishable from the *wus* single mutant.

(M–O) *clv3*. (M and N) The floral meristem is convex throughout development, but increases in size relative to wild type. (O) Open flowers display supernumerary carpels (arrow).

(P–R) *wus clv3*. Flower development is indistinguishable from the *wus* single mutant.

SEM images of developing flowers. fa, flat apex; fm, floral meristem; g, gynoecium; p, petal; s, sepal; st, stamen. Bars, 20 μm (A and B; D and E; G and H; J and K; M and N; P and Q) and 500 μm (C, F, I, L, O and R).

Table 1. Floral Organ Numbers in *wus*, *clv*, and *wus clv* Mutants

Genotype	n	Organ Numbers			
		Sepals	Petals	Stamens	Carpels
L*er*	10	4.0 ± 0.0	4.0 ± 0.0	6.0 ± 0.0	2.0 ± 0.0
wus-1	8	4.0 ± 0.0	4.0 ± 0.0	0.9 ± 0.4	0.0 ± 0.0
clv1-4	10	4.9 ± 0.7	4.6 ± 0.7	9.3 ± 1.0	4.5 ± 0.9
wus-1 clv1-4	29	4.2 ± 0.5	4.3 ± 0.8	1.2 ± 0.7	0.0 ± 0.0
clv2-1	14	4.1 ± 0.4	4.1 ± 0.3	6.5 ± 0.8	3.7 ± 0.8
wus-1 clv2-1	30	3.9 ± 0.8	3.7 ± 1.0	0.7 ± 0.6	0.0 ± 0.0
clv3-2	10	4.4 ± 0.7	4.5 ± 0.5	8.7 ± 0.8	5.4 ± 1.0
wus-1 clv3-2	18	4.1 ± 0.5	4.3 ± 0.8	0.8 ± 0.5	0.0 ± 0.0

The organ numbers and the standard deviations are given. n, number of flowers analyzed.

produced in the two outer whorls, the *wus* meristem terminates in a single central stamen without forming a female organ (Figure 3F and Table 1). Thus, *wus* floral meristem development deviates from wild type at about the time of sepal initiation, supporting the view that *WUS* is not required for the initiation of shoot and floral meristems, but for their maintenance during organ production (Laux et al., 1996; Mayer et al., 1998).

clv1 and *clv3* floral meristems resemble wild type in that they display a dome-shaped structure (Figures 3G and 3M), but they grow markedly larger (Figures 3H and 3N) and give rise to supernumerary organs, mainly stamens and carpels (Figures 3I and 3O and Table 1). Floral meristem development in *wus clv1* and *wus clv3* is indistinguishable from *wus* single mutants at all stages, terminating prematurely in a central stamen (Figures 3J–3L and 3P–3R). Although the organ numbers of double mutant flowers are more variable, on average they do not significantly deviate from the *wus* single mutant (Table 1).

In summary, *wus clv* shoot and floral meristems terminated prematurely, indistinguishable from *wus* single mutants. Given the opposite phenotypes of the single mutants, one possible interpretation is that the *CLV* genes act by negatively regulating *WUS*. However, the increased number of flowers formed in occasional *wus clv* double mutants, relative to *wus* single mutants, suggests that the *CLV* genes have additional functions independent of *WUS*.

WUS Is Ectopically Expressed in *clv* Mutants

To test whether the *CLV* genes negatively regulate *WUS* at the transcript level, we analyzed *WUS* expression in *clv* meristems by in situ hybridization.

Embryo Development

During wild-type development, *WUS* expression is initiated in 4 inner apical cells of the 16-cell embryo and by several asymmetric divisions becomes confined to the center of the developing shoot meristem (Mayer et al., 1998). At the heart stage of embryogenesis, there are two subepidermal cells that express *WUS*. These cells divide periclinally after which *WUS* expression is restricted to the basal daughters in the third cell layer (Figure 4A). We have never observed expression in both the apical and basal daughters (second and third cell layers; n = 47), indicating that asymmetric *WUS* expression is established during or rapidly after cell division. Eventually, mature wild-type embryos express *WUS* in a small cell group underneath the two outermost cell layers (Figure 4E). In contrast to wild-type heart and torpedo stage embryos, about one third of *clv1, 2,* and *3* embryos (9/25 for *clv1*, 5/20 for *clv2* and 9/22 for *clv3*) display *WUS* expression in both the second and the third cell layer (Figures 4B–4D). In addition, the *WUS* expression domain in *clv* heart stage embryos is often three cells wide (Figures 4B–4D), whereas in wild type it is always two cells wide (Figure 4A). Mature *clv* embryos express *WUS* underneath the two outermost cell layers similar to wild-type embryos, but *clv1* and *clv3* embryos display a broader expression domain (Figures 4F–4H). Negative control experiments using a sense *WUS* probe did not give any signal (not shown). Thus, *WUS* is expressed ectopically in *clv* embryos, although the misexpression in apical cell layers is only transient.

Shoot Meristems

In wild-type seedling and inflorescence shoot meristems, *WUS* is expressed in a small group of cells in the center of the meristem, underneath the three outermost cell layers (Figures 5A and 5E). By contrast, *clv* shoot meristems generally display *WUS* expression in the third and fourth cell layers of the shoot meristem, i.e., one cell layer up compared to wild type (Figures 5B, 5D, and 5F–5H). The only exception is *clv2* vegetative meristems where *WUS* is detected underneath the third cell layer (Figure 5C). In addition, the *WUS* expression domain is broader in all *clv* mutants than it is in wild type. In extreme cases of fasciation, i.e., when the *clv* meristems adopt an elongated rather than a circular shape, *WUS* expression extends laterally over more than 100 cells and is also present in the second cell layer (Figures 5I and 5J). In these cases epidermal cells are abnormal, being higher than wide (Figure 5J). The *WUS* expression domain in all cases exhibits a sharp boundary at the periphery and does not extend into the region where organ primordia are evident (Figure 5I). Negative control experiments using a sense *WUS* probe did not give any signal (not shown).

Although *WUS* expression was shifted one or two cell layers up in *clv* meristems, it still stretched over two cell layers only and was absent in deeper cells where it is expressed in wild type, giving a band of *WUS*-expressing cells. Histological sections show that *clv* inflorescence meristems are composed of a band of three to five layers of small, densely staining cells (Figure 5K) overlying large and highly vacuolated cells that appear to have undergone differentiation. Thus, all three *CLV*

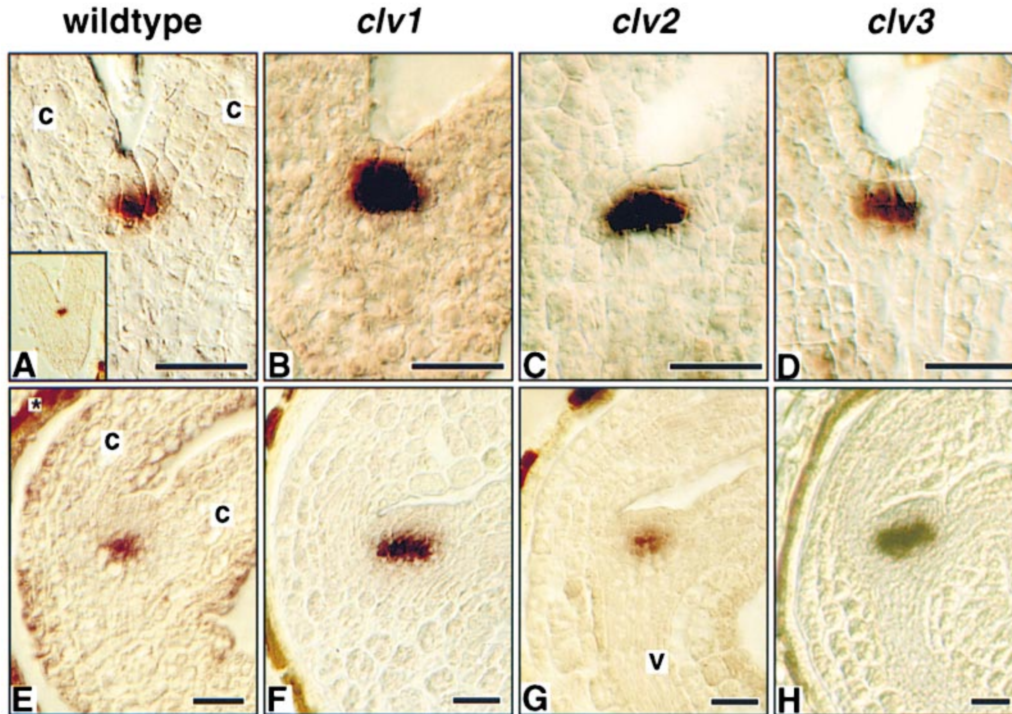

Figure 4. *WUS* mRNA Expression Patterns in Embryogenesis

(A–D) Heart stage embryos. (A) In wild type, *WUS* mRNA is detected in two cells of the third cell layer. Inset shows an overview. (B–D) *WUS* mRNA is detected in two to three cells of both the second and the third cell layers in *clv1, clv2*, and *clv3*.

(E–H) Mature embryos. *WUS* mRNA is detected in the third and fourth cell layers in wild type and *clv* mutants, but the expression domain is narrower in wild type (E) and *clv2* (G) than it is in *clv1* (F) and *clv3* (H).

WUS mRNA is detected by in situ hybridization to embryo sections as brown color.

c, cotyledon; v, vasculature. * brown color of seed coat cells independent of staining reaction. Bars, 20 μm.

genes are required to suppress *WUS* expression in apical cell layers and to restrict its lateral expansion.

Floral Meristems

In wild-type flower development, *WUS* is expressed from earliest stages on in a few cells in the floral meristem center, underneath the outermost two cell layers (Figure 5M). By contrast, in *clv1* and *clv3* floral meristems, *WUS* is expressed in the second and third layer, i.e., one cell layer up compared to wild type (Figures 5N and 5P) and in a broader domain. In *clv2* floral meristems (Figure 5O), however, no difference in the spatial expression pattern is detected compared to wild type. At the end of wild-type flower development, the floral meristem terminates in central carpel primordia and this step coincides with the termination of *WUS* expression in stage 6 flowers (Figure 5Q). By contrast, *clv1, 2*, and *3* floral meristems often do not terminate after carpel formation, but form proliferating tissue within the developing gynoecium. In these cases, *WUS* expression continues between the developing carpels (Figures 5R–5T). Negative control experiments using a sense *WUS* probe did not give any signal (not shown). In summary, the *CLV1* and *CLV3* genes are necessary to restrict *WUS* expression to inner cells in the center of the floral meristem in wild type, whereas *CLV2* is not detectably required for the correct spatial pattern. However, all three genes are required to switch off *WUS* expression at the end of wild-type flower development.

Ectopic Expression of a *WUS* Transgene Causes Meristem Overgrowth

To address the question of whether the altered *WUS* expression pattern in *clv* meristems causes their phenotypic defect, namely increased meristem size, we expressed a *WUS* transgene under the control of the *CLV1* promoter. We chose this promoter, because *CLV1* is normally expressed in those apical and lateral cells that express *WUS* ectopically in *clv* mutants (Clark et al., 1997). Using a two-component expression system (Moore et al., 1998), we established an activator line, expressing the synthetic transcription factor LhG4 under the control of a *CLV1* promoter fragment, and a target line, carrying the *WUS* coding region and *GUS* reporter gene each under the control of the pOp promoter, which is activated by LhG4 (see Experimental Procedures). Neither transgenic line showed any phenotype. The target line alone did not show activity of the reporter gene, indicating that in the absence of LhG4 the pOp promoter is not activated (not shown). After crossing activator and target lines, one quarter of the F1 progeny is heterozygous for both constructs and expresses *WUS* and *GUS* in the cells where the *CLV1* promoter is active, including the second and the third cell layer and cells at the periphery of the shoot meristem, as monitored by the expression of the *GUS* reporter gene (Figure 6E).

These transgenic seedlings displayed enlarged fasciated shoot meristems (Figure 6A) when compared to

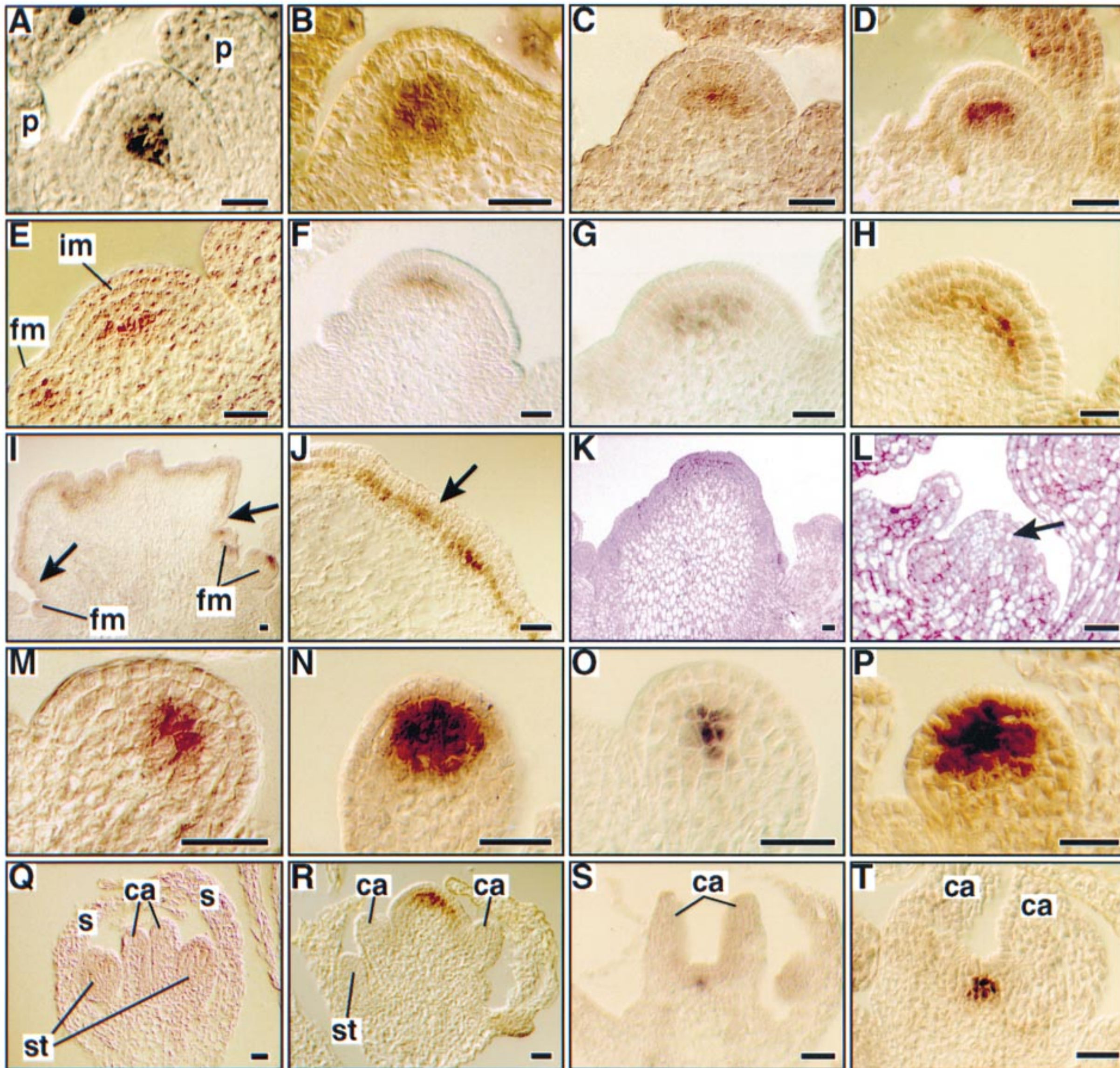

Figure 5. *WUS* mRNA Patterns in Shoot and Floral Meristems

(A–J and M–T) *WUS* mRNA is detected by in situ hybridization to tissue sections as brown color.

(A–D) Seedling shoot meristems. (A) In wild type, *WUS* mRNA is detected in a small cell group in the center of the shoot meristem underneath the three outermost cell layers. (B–D) *clv* mutants. *WUS* mRNA is detected one cell layer up compared to wild type in *clv1* (B) and *clv3* (D) meristems, but in *clv2* (C) the *WUS* expression is underneath the three outermost layers, similar to wild type. In all three *clv* mutants, the *WUS* domain is broader, corresponding to the enlarged meristem size.

(E–H) Inflorescence meristems. In wild type (E), *WUS* mRNA is detected in a small cell group in the center of the shoot meristem underneath the three outermost cell layers. In *clv1* (F), *clv2* (G), and *clv3* (H) mutants, *WUS* mRNA is detected in a domain that is one cell layer up and broader compared to wild type.

(I and J) Fasciated *clv1* shoot meristem. (I) Overview. *WUS* mRNA is detected across the elongated meristem underneath the epidermis, but is not detectable at the periphery above the emerging organ primordia (arrow). (J) Higher magnification. The epidermal cells are misshapen (arrow).

(K and L) Histological sections. (K) *clv1* meristems display about three layers of meristematic cells overlying enlarged, vacuolated cells. (L) The wild-type meristem contains five to six tiers of meristem cells in its center (arrow).

(M–P) Stage 2 floral meristems. In wild type (M), *WUS* mRNA is detected in a small cell group in the center of the floral meristem underneath the two outermost cell layers. In *clv1* (N) and *clv3* (P) meristems, *WUS* mRNA is detected in a domain that is one cell layer up and broader compared to wild type. In *clv2* (O) the *WUS* expression pattern is similar to wild type.

(Q–T) Stage 6 flowers. (Q) In wild type, *WUS* expression in the floral meristem is discontinued after the initiation of carpels. In all *clv* mutants (R–T), *WUS* mRNA is detected within the developing gynoecium.

ca, carpel; fm, floral meristem; im, inflorescence meristem; p, leaf primordium; s, sepal; st, stamen. Bars, 20 μm.

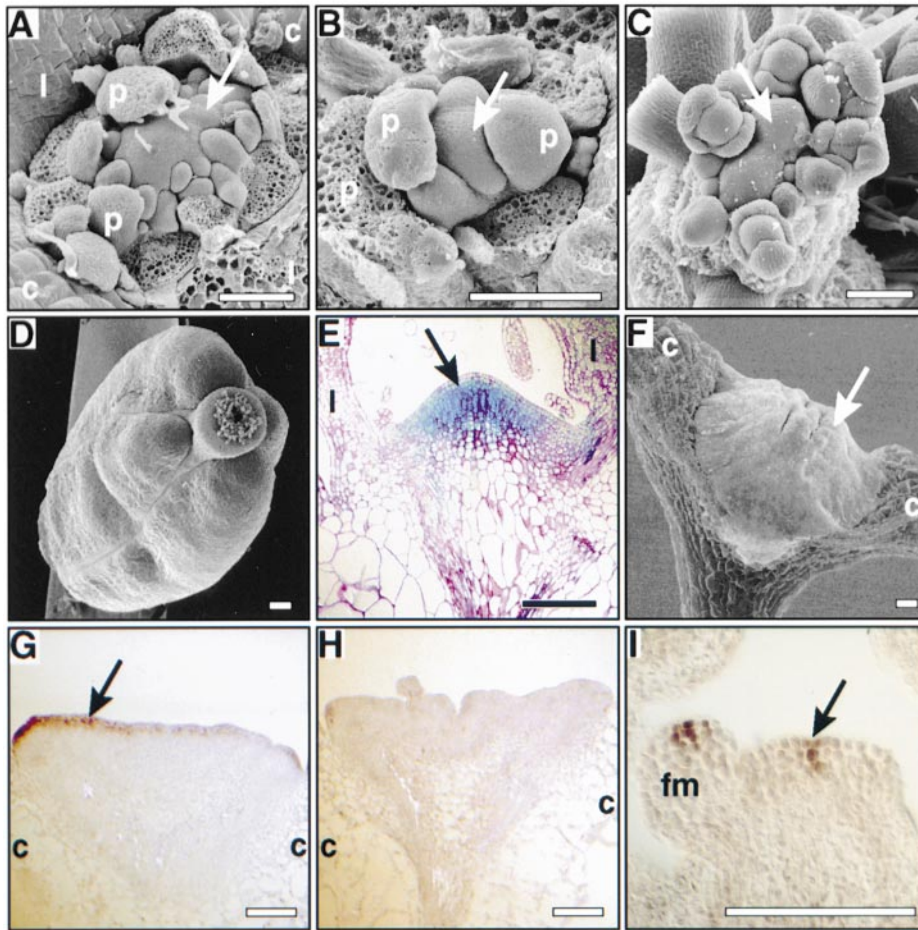

Figure 6. Phenotypes of *CLV1::WUS* and *ANT::WUS* Plants

(A) SEM image of a 2-week-old *CLV1::LhG4 (pOp::WUS, pOp::GUS)* plant, showing an enlarged shoot meristem (arrow).

(B) SEM image of a sibling plant of the one shown in (A), not expressing the transgenic copy of *WUS*. The shoot meristem (arrow) is indistinguishable from wild type (not shown).

(C) SEM image of a fasciated *clv3* shoot meristem.

(D) SEM image of a silique of a *CLV1::LhG4 (pOp::WUS pOp::GUS)* plant with four carpels.

(E) Expression of the *GUS* reporter gene in a histological section of a 2-week-old *CLV1::LhG4 (pOp::WUS pOp::GUS)* plant. The meristem dome (arrow) is enlarged and fasciated. GUS activity is shown as blue color in a region comprising most of the shoot meristem dome, with weak to no staining in the epidermis.

(F) SEM image of a two-week-old *ANT::LhG4 (pOp::WUS pOp::GUS)* plant. A mass of meristem cells (arrow) but no leaves are formed.

(G–I) in situ hybridization to tissue sections of apices of two-week-old *ANT::LhG4 (pOp::WUS pOp::GUS)* plants or wild-type plants. Signal is detected as brown color. (G) *CLV3* mRNA is detected in the three outermost cell layers (arrow) throughout the massively enlarged shoot apex. (H) Negative control experiment. No signal is obtained with a *CLV3* sense probe. (I) In wild-type shoot and floral meristems, *CLV3* mRNA is detected in the presumptive stem cell region, the three outermost cell layers of the meristem center (arrow).

c, cotyledonary petiole; fm, floral meristem; l, leaf; p, leaf primordium. Bars, 100 μm.

wild type (Figure 6B), but were otherwise normal. This phenotype is very similar to *clv* shoot meristems (Figure 6C). Histological sections of the shoot meristem showed several layers of small densely staining cells overlying a corpus of large vacuolated cells (not shown). In strongly fasciated meristems epidermal cells were misshapen, being higher than wide (not shown). In cases where inflorescences were formed, the flowers displayed supernumerary organs, most notably carpels (Figure 6D). On average, gynoecia of transgenic plants possessed 3.6 carpels (n = 25) compared to 2.0 in wild type (n = 30) and 4.5 in *clv1* mutants (see Table 1). All plants with this phenotype showed the predicted GUS staining pattern (Figure 6E), whereas their siblings that were phenotypically wild type, did not display GUS staining (not shown). The same phenotypes were obtained in a second independent experiment using a construct in which the *CLV1* promoter and the *WUS* coding region were fused directly (see Experimental Procedures; data not shown). Transgenic plants expressing a mutant version of the *WUS* gene in which the coding region was disrupted by stop codons in all reading frames were phenotypically wild type, indicating that the observed phenotypes were caused by WUS protein (see Experimental Procedures; data not shown).

In summary, expression of the *WUS* gene in an enlarged domain is sufficient to cause a phenotype very similar to *clv* mutants. This, together with our genetic results, suggests that the *clv* meristem phenotype is caused by the deregulation of *WUS* expression and thus

that in wild type the *CLV* genes regulate meristem size by restricting *WUS* expression.

WUS Is Sufficient to Induce *CLV3* Expression

Next we asked whether *WUS* expression is sufficient to induce stem cell identity. For this purpose we expressed *WUS* under the control of the *ANT* promoter that confers expression in organ primordia and developing organs (Brian Kwan and David Smyth, personal communication; Elliott et al., 1996), using again the two-component system. After activating the *WUS* transgene, leaf formation was essentially abolished in *ANT::LhG4, pOp::WUS* transgenic seedlings and a large bulge of cells similar to meristem cells was formed (Figure 6F). Transgenic plants expressing the mutant version of the *WUS* gene (see above) were phenotypically wild type, indicating that the observed phenotypes are caused by WUS protein (not shown). To determine whether stem cell identity was induced, we performed in situ hybridizations with the *CLV3* gene as a molecular marker (Fletcher et al., 1999). *ANT::LhG4, pOp::WUS* plants specifically express *CLV3* in the three outermost layers throughout the enlarged apex (Figure 6G), indicating that *WUS* is sufficient to induce *CLV3* expression at the correct position and by this criterion to induce stem cell identity.

Discussion

The higher-plant shoot meristem is a dynamic stem cell system that maintains its size while continually initiating organ primordia at the periphery. To determine mechanisms underlying the regulation of shoot meristem homeostasis, we have analyzed the interactions between antagonistic gene activities, the *WUS* gene and the group of *CLV* genes. The *WUS* gene is required to maintain stem cell identity, whereas the *CLV* genes encode components of a signaling pathway that limits the size of the shoot meristem. Our results show that *WUS* is expressed in an enlarged domain in *clv* mutants, indicating that the *CLV* genes act as negative regulators of *WUS* at the transcript level. Our analyses of double mutant combinations and transgenic plants indicate that ectopic *WUS* expression is necessary and sufficient for overgrowth of the shoot meristem in *clv* mutants. We also show that *WUS* expression is sufficient to induce shoot meristem cell identity and correct expression of the stem cell marker *CLV3* in cells destined to form organ primordia. These findings suggest a model in which the size of the stem cell population is maintained in the active shoot meristem by a regulatory feedback loop between the stem cells and the organizing center mediated by *WUS* and *CLV* activities (Figure 7).

A Model for the Self-Regulatory Properties of the Shoot Meristem

The shoot meristem can be divided into four functionally distinct cell groups: (1) stem cells and (2) their immediate daughter cells in the apical region of the central zone, (3) a subjacent organizing center, and (4) founder cells for organ initiation in surrounding regions. This partitioning is maintained by signaling between shoot meristem regions while the resident cell population is continually turned over as new cells are born in the central zone

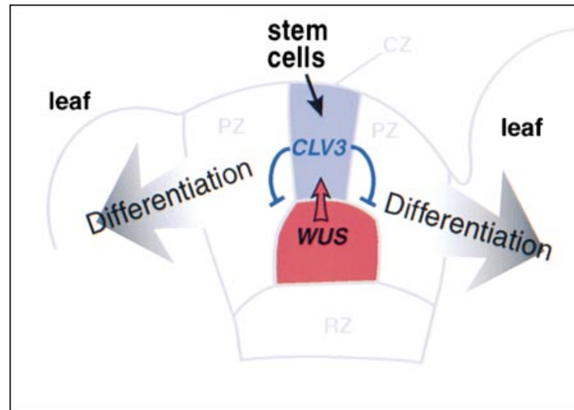

Figure 7. Model for Shoot Meristem Maintenance

Stem cell identity is specified by signaling (red arrow) from a subjacent organizing center (red) requiring *WUS* activity. The stem cells (blue) restrict the range of *WUS* expression via CLV3 signaling (dark blue). Cells that have passed the boundary defined by *CLV* function establish organ founder cell populations. CZ, central zone; PZ, peripheral zone; RZ, rib zone.

and older ones are displaced to the periphery (Figure 7). A signal emanating from the *WUS*-expressing organizing center maintains stem cell identity of the overlying cells. The stem cells in turn signal back to the organizing center mediated by *CLV3*, which encodes a putative ligand for the CLV1 receptor kinase. *CLV1* is expressed not only in the *WUS* domain, but also in apically and laterally adjacent cells (Clark et al., 1997). The CLV3/CLV1 interaction activates a signaling pathway resulting in repression of the *WUS* gene in these adjacent cells and thus restricts the size of the organizing center.

It is conceiveable that the stem cells also provide an activating signal to maintain the *WUS* expression domain at a defined distance from the apex although the overlying L3 cells divide. It should be noted that if the activating and repressing signals emanate from the stem cells, they are effective in different cells: the activity of the short range inhibitor CLV3 is limited to the second and third cell layers, whereas the hypothesized activating signal reaches the subjacent cells. A candidate for a positive regulator of *WUS* expression is the *STM* gene: *STM* has been suggested to antagonize *CLV* function, based on genetic evidence (Clark et al., 1996), and *STM* is necessary to maintain *WUS* expression (Mayer et al., 1998). However, since *STM* is expressed throughout the meristem and not specifically in the stem cell region, it may be a more general regulator of meristem cell development.

Our model accounts for the gradual enlargement of the shoot meristem when either CLV3 or CLV1 are nonfunctional or *WUS* is expressed under the control of the *CLV1* promoter. In these cases, the *WUS* transcription domain would expand apically and laterally, resulting in an enlarged population of stem cells that would produce more daughter cells than are consumed as founder cells for organ initiation. As the enlarged stem cell population would no longer repress *WUS* in a *CLV*-dependent manner, the hypothesized activating signal would promote expansion of the *WUS* expression domain. As development continues, this positive feedback loop between

stem cells and organizing center would amplify an initially small surplus of stem cells and their daughters, eventually resulting in a vastly oversized shoot meristem, consistent with the increasingly more severe phenotype in developing *clv* plants.

Organs are still initiated at the periphery of *clv* shoot meristems, suggesting that the transition of cells from the meristem center toward the status of organ primordia cells requires two separable steps (Lenhard and Laux, 1999). In the first step, stem cell daughters exit stem cell identity by leaving the range of *WUS* activity, the boundaries of which are defined by *CLV*-mediated restriction of *WUS* expression. However, as indicated by the *wus* mutant phenotype, the absence of *WUS* function per se is not sufficient for organ initiation (Laux et al., 1996). In a second step, cells become recruited into organ primordia at the periphery of the shoot meristem, and this step may be regulated, e.g., by the *MGOUN* genes (Laufs et al., 1998a). Ectopic expression of *WUS* under the control of the *ANT* promoter converts organ primordia into shoot meristem cells, suggesting that *WUS* has to be switched off for primordia initiation to occur.

Growth Control of the Floral Meristem

The floral meristem is subject to the same *CLV*/*WUS*-mediated growth control as the shoot meristem. In contrast to the shoot meristem, however, the floral meristem is determinate, giving rise to a specific set of floral organs. The floral homeotic gene *AGAMOUS* (*AG*), which determines organ identity in whorls 3 (stamens) and 4 (carpels), and the *CLV* genes have been implicated in terminating growth of the floral meristem (Yanofsky et al., 1990; Clark et al., 1993, 1995). Both *wus* and *wus clv* flowers lack carpels and terminate in a centrally positioned stamen, whereas *clv* flowers produce proliferating cells, some of which express *WUS*, within the gynoecium. This prolonged expression of *WUS* could simply be a consequence of the surplus of *WUS*-expressing cells in *clv* floral meristems, which are not consumed during carpel formation. However, the prolonged expression of *WUS* in *clv2* floral meristems, where no previously expanded expression domain is detectable, argues against this interpretation. Therefore, an alternative explanation is that the *CLV* genes are also required to terminate *WUS* expression at the end of flower development, maybe in concert with other genes such as *AG*. The differences in *WUS* expression and in the severity of phenotypes in *clv1,3* versus *clv2* meristems suggest that in *clv2* mutants CLV1/CLV3 signaling is still partially functional and able to repress *WUS* in outer cell layers.

Initiation of a Self-Regulatory Shoot Meristem

The shoot meristem is initiated very early in embryogenesis as indicated by the onset of *WUS* expression in four inner cells of the 16-cell embryo (Mayer et al., 1998). By the heart stage, *WUS* expression has been narrowed down to the prospective organizing center of the shoot meristem. Our results show that during this process, the *CLV* genes are required to rapidly downregulate *WUS* in apical daughter cells after cell division: in *clv* mutants this downregulation takes longer, resulting in about a third of the embryos that at a given time point still express *WUS* in apical daughter cells. Nevertheless, albeit delayed, this downregulation eventually takes place in *clv* mutants, indicating that, even if the correct *WUS* expression pattern is not established in time, the daughter cells can still assess their position and respond properly. At later stages, *clv* embryos display an enlarged *WUS* expression domain that correlates with the enlarged shoot meristem in *clv* embryos. Thus, the regulatory feedback loop between *WUS* and *CLV* activities appears to be functional in advanced-stage embryos.

Biological Significance of the *CLV*/*WUS* Interaction

The size regulation of the shoot meristem critically depends on a balance between the production and consumption of cells, i.e., stem cell proliferation and organ initiation. The feedback loop proposed here would have the properties of a self-regulatory system: if the *WUS*-dependent signal is too weak, fewer stem cells are specified, resulting in too weak a *CLV3*-dependent signal for *WUS* repression, and the consequently enlarging *WUS* expression domain would specify more stem cells. Conversely, if the *WUS*-dependent signal is too strong, more stem cells are specified, resulting in too strong a *CLV3*-dependent signal for *WUS* repression, and the reduced *WUS* expression domain would specify fewer stem cells. Thus, the size of the stem cell population in the active shoot meristem could be continually checked by the *CLV/WUS* interaction.

Experimental Procedures

Plant growth, genetic experiments, histological sections, and scanning electron microscopy analyses were done as previously described (Laux et al., 1996). All mutations have been induced in the L*er* ecotype, which we used as wild-type reference. Confocal analysis of embryos (Endrizzi et al., 1996) and in situ hybridization experiments (Mayer et al., 1998) were done as previously described. To generate the *CLV3* antisense and sense riboprobes, the *CLV3* coding region was amplified from reverse-transcribed mRNA of inflorescence meristems using the primers CLV3XHO5 (5′-CTCTCGAGCAGTCACTTTCTCTC-3′) and CLV3BAM3 (5′-ACAAGGGATCCGGTCAAGGG-3′), digested with BamHI and XhoI and inserted into pBluescript II KS(−). The antisense probe was transcribed using T7-RNA polymerase and the sense probe was transcribed using T3-RNA polymerase as described (Mayer et al., 1998).

CLV1::WUS and *ANT::WUS* Expression Constructs

To generate the *CLV1::LhG4* construct, the coding region for LhG4 was excised from pBINPLUS:LhG4 (a gift from Ian Moore) and inserted into the unique BamHI-site of pKR126 (a gift from Robert Williams and Elliot Meyerowitz). pKR126 contains 5642 bp of *CLV1* 5′ promoter region and 729 bp of *CLV1* 3′ genomic region, with the *CLV1* ORF replaced by a BamHI site. The resulting *CLV1::LhG4* fragment was inserted into pBarA, a derivative of pGPTV-BAR (Becker et al., 1992) to yield plasmid MT134. For the *ANT::LhG4* construct, a genomic fragment (a gift from David Smyth) encompassing 6.5 kb of upstream sequence from the *ANT* locus and including the first 29 bp of the *ANT* coding sequence was fused in frame to the *LhG4* coding sequence. The resulting *ANT::LhG4* fragment was inserted into pBarA to give plasmid MT76.

For the *Op::WUS-Op::NLSGUS* tandem reporter, the full-length *WUS* cDNA was inserted into pUBOP (a gift from Ian Moore). The resulting *Op::WUS* fragment was excised and inserted into pBarA to yield MT69. Similarly, the coding region for NLSGUS (van der Krol and Chua, 1991) was excised from pVIP35 (a gift from Alexander van der Krol), and ligated into pUBOP. The resulting *Op::NLSGUS*

fragment was then cloned into MT69 to give MT72. Details of the cloning are available upon request.

To generate the *CLV1::WUS* expression construct, the *WUS* coding sequence was inserted as a HpaI fragment into the blunt-ended BamHI site of plasmid pKR126. For the negative control constructs, an analogous fusion was created, using a modified version of the *WUS* cDNA, which contains several stop codons in all three reading frames immediately following the *WUS* start codon.

Plant Transformation

All plasmids were introduced into *Agrobacterium* strain GV3101(pMP90) (Koncz and Schell, 1986) by electroporation and transformed into L*er* wild-type plants by the floral dip method (Clough and Bent, 1998).

GUS Staining

Staining for GUS activity was performed according to a protocol kindly communicated by D. Weigel. Plant material was prefixed at room temperature in 90% acetone for 20 min, rinsed in staining buffer without X-Gluc and infiltrated with staining solution (50 mM $NaPO_4$, pH 7.2; 2 mM potassium-ferrocyanide; 2 mM potassium-ferricyanide; 0.2% Triton X-100; 2 mM X-Gluc) under vacuum on ice for 15 min and incubated at 37°C for 3 hr. After dehydration in an ethanol series up to 60% EtOH, tissue was postfixed in FAA (50% EtOH, 5% formaldehyde, 10% acetic acid) for 30 min at room temperature, dehydrated completely, and embedded in LR-White (London Resin Company) according to the manufacturer's instructions.

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