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

Heiko Schoof,² Michael Lenhard,² Achim Haecker, Klaus F. X. Mayer,³ Gerd Jürgens, and Thomas Laux*

Introduction

The shoot meristem of higher plants 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.

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 post-embryonic 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; Mousian 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 antically, 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 sub-populations 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
Cell
636

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 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.
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).
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 expression 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 3H) 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 3I - 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 fascination, 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

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Sepals</th>
<th>Petals</th>
<th>Stamens</th>
<th>Carpels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ler</td>
<td>10</td>
<td>4.0 ± 0.0</td>
<td>4.0 ± 0.0</td>
<td>6.0 ± 0.0</td>
<td>2.0 ± 0.0</td>
</tr>
<tr>
<td>wus-1</td>
<td>8</td>
<td>4.0 ± 0.0</td>
<td>4.0 ± 0.0</td>
<td>0.9 ± 0.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>clv1-4</td>
<td>10</td>
<td>4.9 ± 0.7</td>
<td>4.6 ± 0.7</td>
<td>9.3 ± 1.0</td>
<td>4.3 ± 0.9</td>
</tr>
<tr>
<td>wus-1 clv1-4</td>
<td>29</td>
<td>4.2 ± 0.5</td>
<td>4.3 ± 0.8</td>
<td>1.2 ± 0.7</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>clv2-1</td>
<td>14</td>
<td>4.1 ± 0.4</td>
<td>4.1 ± 0.3</td>
<td>6.5 ± 0.8</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>wus-1 clv2-1</td>
<td>30</td>
<td>3.9 ± 0.8</td>
<td>3.7 ± 1.0</td>
<td>0.7 ± 0.6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>clv3-2</td>
<td>10</td>
<td>4.4 ± 0.7</td>
<td>4.5 ± 0.5</td>
<td>8.7 ± 0.8</td>
<td>5.4 ± 1.0</td>
</tr>
<tr>
<td>wus-1 clv3-2</td>
<td>18</td>
<td>4.1 ± 0.5</td>
<td>4.3 ± 0.8</td>
<td>0.8 ± 0.5</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

The organ numbers and the standard deviations are given. n, number of flowers analyzed.
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 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. * brown color of seed coat cells independent of staining reaction. Bars, 20 μm.
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.

c, 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).

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 and older ones are displaced to the periphery (Figure 7).

**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.

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 conceivable 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 non-functional 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., 1998). 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 Ler 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 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 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 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 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 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 embryos (Endrizzi et al., 1996) and in situ hybridization experiments (Mayer et al., 1998) were done as previously described.
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 Ler 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 by a novel

All antigens were identified by a protocol kindly communicated by D. Weigel. Plant material was prefixed at by a novel

Plant Cell 8 (1997). Development

GUS Staining
Staining for GUS activity was performed according to a protocol kindly communicated by D. Weigel. Plant material was prefixed at by a novel

Plant Cell 8 (1997). Development

GUS Staining
Staining for GUS activity was performed according to a protocol kindly communicated by D. Weigel. Plant material was prefixed at by a novel

Plant Cell 8 (1997). Development

GUS Staining
Staining for GUS activity was performed according to a protocol kindly communicated by D. Weigel. Plant material was prefixed at by a novel