Regulation of *CLV3* Expression by Two Homeobox Genes in Arabidopsis[1]

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The ability of meristems to continuously produce new organs depends on the activity of their stem cell populations, which are located at the meristem tip. In Arabidopsis, the size of the stem cell domain is regulated by two antagonistic activities. The *WUS* (*WUSCHEL*) gene, encoding a homeodomain protein, promotes the formation and maintenance of stem cells. These stem cells express *CLV3* (*CLAVATA3*), and signaling of CLV3 through the CLV1/CLV2 receptor complex restricts *WUS* activity. Homeostasis of the stem cell population may be achieved through feedback regulation, whereby changes in stem cell number result in corresponding changes in *CLV3* expression levels, and adjustment of *WUS* expression via the *CLV* signal transduction pathway. We have analyzed whether expression of *CLV3* is controlled by the activity of *WUS* or another homeobox gene, *STM* (*SHOOT MERISTEMLESS*), which is required for stem cell maintenance. We found that expression of *CLV3* depends on *WUS* function only in the embryonic shoot meristem. At later developmental stages, *WUS* promotes the level of *CLV3* expression, together with *STM*. Within a meristem, competence to respond to *WUS* activity by expressing *CLV3* is restricted to the meristem apex.

The shoot apical meristem (SAM) of higher plants is formed during embryogenesis and gives rise to leaves and stem after germination (Steeves and Sussex, 1989). The side branches of angiosperms originate from axillary meristems that arise in the axils of leaves, whereas flowers are formed from secondary meristems that are initiated at the flanks of the SAM. The cells in these three types of meristems are arranged in three clonal layers (L1, L2, and L3). All organs and also floral meristems are produced at the flanks of the meristem dome in the peripheral zone. Loss of cells from the meristem during organ formation has to be compensated by divisions of cells in the central zone that act as pluripotent stem cells. When these stem cells divide, their daughter cells are displaced to the periphery, where they will be incorporated into organ primordia and eventually differentiate. Therefore, the ability of meristems to continuously produce new organs depends on the activity of their stem cell populations. Stem cells can be initiated repeatedly during plant development, whenever a new axillary or floral meristem is formed. The stem cells of floral meristems are not permanent, but lose their undifferentiated state when the inner set of floral organs is produced. Thus, stem cell identity may represent a flexible state that is subject to both positive and negative regulation.

A current model proposes that the size of the stem cell population in meristems is controlled by a negative feedback regulation between two pathways that promote or restrict stem cell number (Waites and Simon, 2000). The *WUS* (*WUSCHEL*) gene of Arabidopsis, encoding a nuclear-localized homeodomain protein, is expressed underneath the stem cell domain of shoot and floral meristems. In *wus* mutants, the cells in the central zone differentiate prematurely, indicating that *WUS* promotes the initiation and maintenance of stem cells at the apex (Laux et al., 1996; Mayer et al., 1998). However, *WUS* function is not required for meristem initiation per se because *wus* mutants still form axillary and floral meristems. The expression of *WUS* is controlled by the three *CLAVATA* genes (*CLV1*, *2*, and *3*) that act together in a signal transduction pathway and restrict stem cell fate (Brand et al., 2000; Schoof et al., 2000). The *CLV3* gene is expressed in the putative stem cells at the apex of shoot, floral, and axillary meristems (Fletcher et al., 1999). The CLV3 protein is proposed to be secreted from the stem cells and to act as a signaling molecule that binds to and thereby activates a heterodimeric receptor complex, consisting of the CLV1 and CLV2 proteins (Trotochaud et al., 2000). A consequence of this receptor activation is the restriction of *WUS* expression to a small domain in the deeper regions of the meristem (Brand et al., 2000). Increased expression of *CLV3* in transgenic plants causes a further repression of *WUS* expression, and a loss of stem cells (Brand et al., 2000). These results, together with studies on the formation of active CLV receptor complexes, indicate that the size of the stem cell population is controlled by the availability of *CLV3* to regulate *WUS* via the *CLV* pathway (Brand et al., 2000; Trotochaud et al., 2000).

The genes that control the expression levels and pattern of *CLV3* are not yet known, but several ob-

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servations suggest that one important activator of *CLV3* expression could be *WUS* itself. First, nondifferentiating cells that express *CLV3* accumulate in *clv1*, *clv2*, or *clv3* mutants due to unrestricted *WUS* expression (Fletcher et al., 1999; Brand et al., 2000). Second, transgenic plants that express *WUS* from the *CLV1* promoter in a larger domain within the meristem accumulate undifferentiated cells that express *CLV3*, resembling *clv* mutants (Schoof et al., 2000). Third, when *CLV3* expression in the meristem is under control of a heterologous promoter (the promoter of the *UFO* gene), the meristem fails to maintain stem cells and *CLV3* expression from its own promoter, indicating that *CLV3* expression and stem cell identity are subject to the same positive control mechanisms (Brand et al., 2000). Homeostasis of the stem cell population is then achieved through negative feedback regulation, whereby any increase or decrease in stem cell number results in a corresponding change in *CLV3* transcript levels, and an immediate adjustment of *WUS* expression via the *CLV* signal transduction pathway. A simple explanation for these observations is that *WUS* directly promotes both stem cell fate and *CLV3* expression. Alternatively, *WUS* could only be required to maintain a stem cell population, and *CLV3* expression in these stem cells is promoted by other factors.

A second candidate regulator of *CLV3* expression is the *STM* (*SHOOT MERISTEMLESS*) gene of Arabidopsis. *STM* encodes a homeodomain protein that is expressed throughout shoot and floral meristems, but is excluded from organ primordia (Long et al., 1996). Plants homozygous for loss-of-function mutations in the *STM* gene fail to initiate a recognizable SAM during embryogenesis (Barton and Poethig, 1993). In weak *stm* mutants, a shoot meristem can be formed that arrests prematurely, indicating that *STM* is also required at later stages for the maintenance of a self-renewing stem cell population in meristems (Endrizzi et al., 1996). Although both *STM* and *WUS* are independently activated in the embryonic shoot meristem, each appears to require the function of the other at the seedling stage (Mayer et al., 1998).

As a first step to investigate the regulation of *CLV3*, we have studied the dependence of *CLV3* expression on *STM* and *WUS* function because both genes are required for the formation and maintenance of stem cell populations in meristems.

RESULTS

Construction of a *CLV3* Reporter Gene

To facilitate expression studies with *CLV3*, we constructed a *CLV3*::*GUS* transgene that expresses the *GUS* (*β-glucuronidase*) reporter gene under control of *CLV3* regulatory sequences. A transcriptional fusion of 1.5-kb genomic sequences immediately upstream of the translational start codon of *CLV3* to a *GUS* reporter gene, pBU16D7, was not sufficient to express the *GUS* reporter in the pattern typical for *CLV3*. However, the weak *clv3-3* allele carries a T-DNA insertion 175 bp downstream of the polyadenylation site, potentially disrupting or distancing an enhancer element (Fletcher et al., 1999). Therefore, we tested if additional sequences are required for *CLV3* expression by inserting the 1.2-kb DNA sequences 3′ to the *CLV3* translational stop codon downstream of the *GUS* gene in pBU16D7 to give pBU16 (*CLV3::GUS*). After plant transformation and selection of transgenic Arabidopsis, GUS activity in the plants was analyzed in whole-mount preparations and tissue sections. In wild-type Arabidopsis, *CLV3* RNA is detected from the heart stage of embryogenesis onwards in the presumptive SAM (Fletcher et al., 1999). During further development, *CLV3* remains expressed in the putative stem cells that are located in the central zone of the SAM, the axillary meristems, and in floral meristems. When *CLV3::GUS* transgenic plants were assayed for GUS activity, staining was first detectable at the heart stage of embryogenesis in the presumptive SAM (Fig. 1A). At later stages, a dark-blue GUS staining was visible in the central zone of the SAM, axillary, and floral meristems, coinciding with the *CLV3* RNA pattern (Fig. 1, B–F). Occasionally, a faint GUS staining was observed in cells immediately adjacent to the central zone (Fig. 1D). Because RNA in situ hybridizations confirmed that the pattern of *GUS* RNA in *CLV3::GUS* plants coincided with the *CLV3* RNA distribution (Fig. 1C), we assume that GUS staining in neighboring cells is due to the stability of the GUS protein in the descendants of *CLV3*-expressing cells. We concluded that the *CLV3*::*GUS* transgene can be used as a reliable reporter for *CLV3* expression in planta.

Requirement of *WUS* Function for *CLV3* Expression during Development

wus mutants fail to maintain a sufficient number of stem cells, resulting in a meristem arrest after formation of a few leaves (Laux et al., 1996). To test whether *WUS* activity is required not only for stem cell specification, but also for the early expression of *CLV3* during embryogenesis, we introduced the *CLV3::GUS* reporter into *wus-1* mutants. Plants homozygous for the *CLV3::GUS* transgene that carried the loss-of-function *wus-1* allele were obtained, and after self-fertilization, mature embryos were assayed for reporter gene activity. Of 259 embryos analyzed, 58 (22.4%, expected 25%) were identified as *wus-1* mutants by the lack of a SAM (Fig. 1, F and I). All *wus-1* embryos failed to express *CLV3::GUS*, indicating that *WUS* function is required for the early activation of *CLV3* expression in the embryo (Fig. 1G; Table I).

During further development, *wus*-1 mutant seedlings initiate leaves, axillary meristems, and inflores-

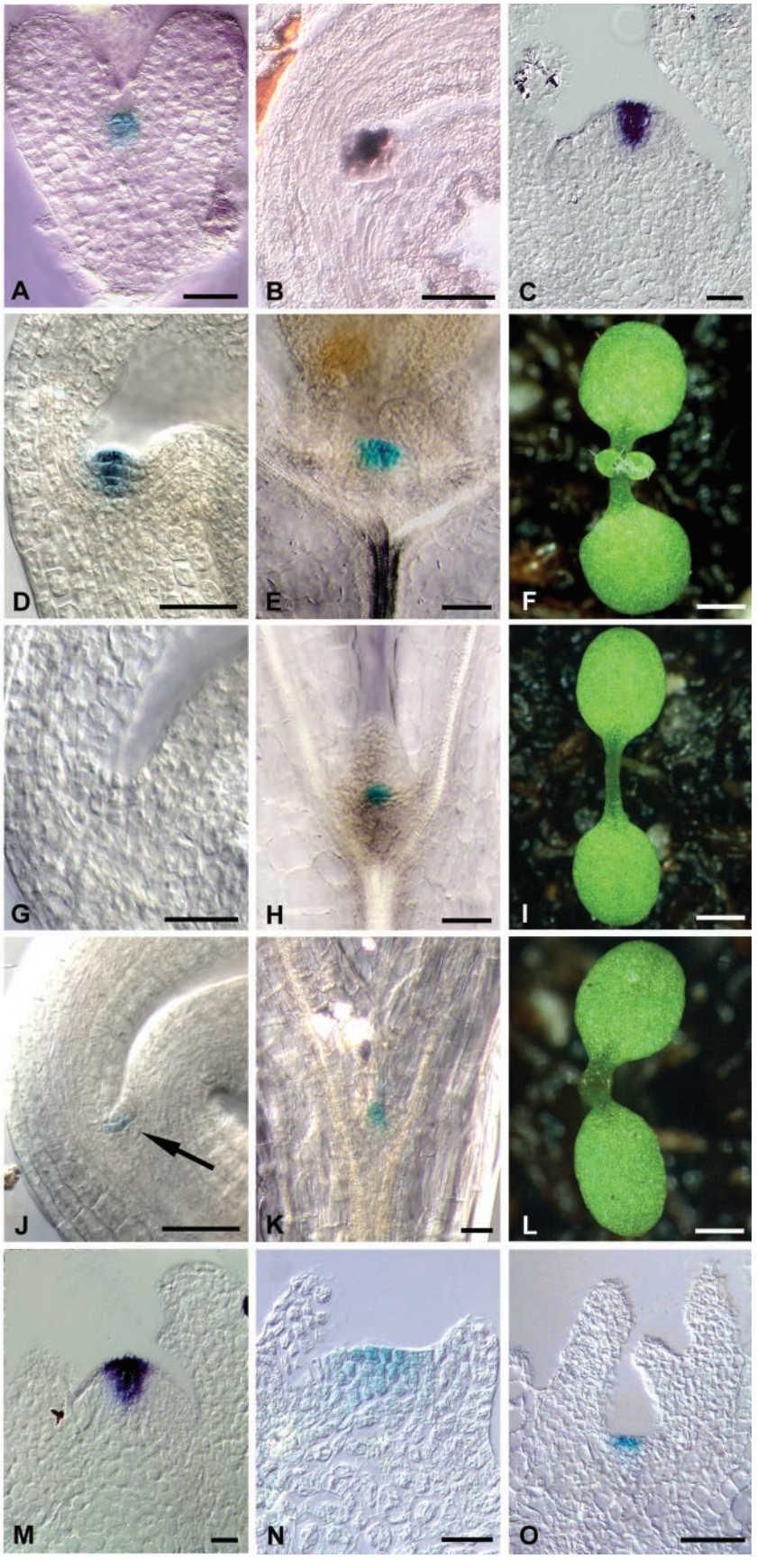

Figure 1. Dependence of *CLV3* expression on *WUS* and *STM*. A, GUS-stained heart stage *CLV3::GUS* embryo. B, *CLV3* expression in a bent cotyledon stage embryo, detected by in situ hybridization with a *CLV3* probe. C, *CLV3* expression in a *CLV3::GUS* inflorescence meristem, detected by in situ hybridization with a *GUS* probe. D, GUS-stained mature *CLV3::GUS* embryo; compare with B. E, GUS-stained wild-type seedling 10 d after germination (d.a.g.). F, Wild-type seedling 10 d.a.g. The first leaf pair is visible. G, GUS-stained mature *CLV3::GUS/wus-1* embryo. *CLV3* expression is not detectable. H, GUS-stained *CLV3::GUS/wus-1* seedling 10 d.a.g. I, *wus-1* seedling 10 d.a.g. J, GUS-stained mature *CLV3::GUS/stm-11* embryo showing *CLV3* expression (arrow). K, GUS-stained *CLV3::GUS/stm-11* seedling 10 d.a.g. showing *CLV3* expression. L, An *stm-11* seedling 10 d.a.g. has formed cotyledons, but no SAM is visible. M, Wild-type *CLV3::GUS* axillary meristem 21 d.a.g. *GUS* RNA is detected by in situ hybridization. N, GUS-stained *CLV3::GUS/wus-1* axillary meristem 21 d.a.g. O, GUS-stained *CLV3::GUS/stm-11* axillary meristem 21 d.a.g. Scale bars in F, I, and L = 1 mm; in all other figures, scale bars = 20 μm.

Table I. *CLV3::GUS expression in embryos and seedlings*

GUS staining of mature embryos or seedlings at 10 d.a.g. of the indicated genotypes, carrying the *CLV3::GUS* reporter. The no. of seedlings that showed a GUS signal (g.s.), percentage of the total (%), and the total of individuals analyzed (*n*) are tabulated. n.d., Not determined.

Genotype	Embryo			Seedling		
	n	g.s.	%	*n*	g.s.	%
wt	414	405	96	207	198	96
wus-1	58	0	0	106	83	78
stm-11	86	6	7	59	15	25
stm-5	–	n.d.	–	48	27	56

cences with a reduced number of floral organs (Laux et al., 1996). Therefore, we tested *wus-1* seedlings for expression of the *CLV3::GUS* marker, and found GUS-expressing cells at 9 to 10 d.a.g. (Fig. 1H; Table I). At this stage, a flattened SAM and two leaf primordia had been formed, and 83 of 106 *wus-1* seedlings expressed the *CLV3* reporter gene at low levels in a small group of cells in the center of the SAM. In later arising axillary meristems, strong GUS staining was observed in the central zone in a pattern comparable with wild-type meristems (Fig. 1, M and N). These results indicate a decreasing dependence of *CLV3* expression in *WUS* function during development: *WUS* is required to establish *CLV3* expression in the embryo, and for the up-regulation of *CLV3* in the SAM after germination. In axillary meristems, establishment of *CLV3* expression is *WUS* independent, and therefore may be controlled by other factors that could act redundantly with *WUS*.

WUS Promotes *CLV3* Expression at the Meristem Tip

To test whether *WUS* is sufficient to promote *CLV3* expression, we designed a transgene that allows the induction of *WUS* activity in the whole plant. A translational *WUS-GR* fusion was designed that is expressed under control of the *CaMV* (cauliflower mosaic virus) *35S* promoter in transgenic plants (*WUS-GR*). Nuclear entry of the fusion protein now depends on the addition of the synthetic glucocorticoid hormone dexamethasone (Dex; Lloyd et al., 1994). The *WUS-GR* transgene was transformed into Arabidopsis plants carrying the *CLV3::GUS* reporter gene. The expression of the *WUS-GR* transgene was confirmed by RNA in situ hybridization (data not shown). If the presence of the WUS protein in the nucleus is sufficient to activate *CLV3* expression, we would expect GUS activity throughout Dex-treated plants.

Untreated transgenic plants were phenotypically wild type and showed GUS-staining patterns typical for *CLV3*. However, seedlings that were treated with Dex during germination formed broad but flat shoot meristems that showed an intense GUS staining that was restricted to the apical cell layers of the SAM (Fig. 2A, compare with Fig. 1D).

When seedlings were sprayed with Dex 4 d.a.g., the cotyledons curled downwards and leaves that initiated during further development failed to expand (Fig. 2B). In addition to the staining in the shoot meristem, strong GUS staining was now found in axillary meristems that had formed on the adaxial side of leaves close to their base at 21 d.a.g. (Fig. 2. C–E). Compared with wild type (Fig. 1M), these axillary meristems did not form a typical meristematic dome, but appeared to be laterally expanded, thus resembling the SAM in Dex-treated *WUS-GR* plants. Activity of the *CLV3* reporter was highest in the meristematic cells of the apical cell layers, and decreased toward the deeper layers and the periphery, where initiation of new organ primordia was observed. Although leaf development was affected by the activation of the *WUS-GR* transgene, we never observed any GUS staining in leaf tissues. Thus, *WUS* expression is not sufficient to activate *CLV3* expression on its own, and additional localized factors may be required to cooperate with *WUS* in the activation of target genes.

Dependence of *CLV3* Expression on *STM*

To test whether *STM* function is required for expression of *CLV3*, we introduced the *CLV3::GUS* transgene into plants carrying the loss-of-function allele *stm-11* (Long et al., 1998). After self-fertilization, these plants segregated 25% *stm-11* mutant embryos, which were identified by the lack of a visible SAM. Plants homozygous for *CLV3::GUS* were identified in the next generation, and used for the expression studies. Although mature wild-type embryos showed the typical *CLV3::GUS* staining in the SAM, most *stm-11* mutant embryos did not express the *CLV3::GUS* reporter (Table I). However, in six of 86 *stm-11* mutant embryos analyzed (7% of the total), we observed weak GUS staining in two to four cells between the cotyledons where the SAM is formed in wild-type embryos, indicating that *STM* function is not absolutely required for *CLV3* expression in the embryo (Fig. 1J).

At the seedling stage, *stm-11* mutants form partially fused cotyledons (Fig. 1L) and, even in the absence of an embryonic SAM, malformed leaves can

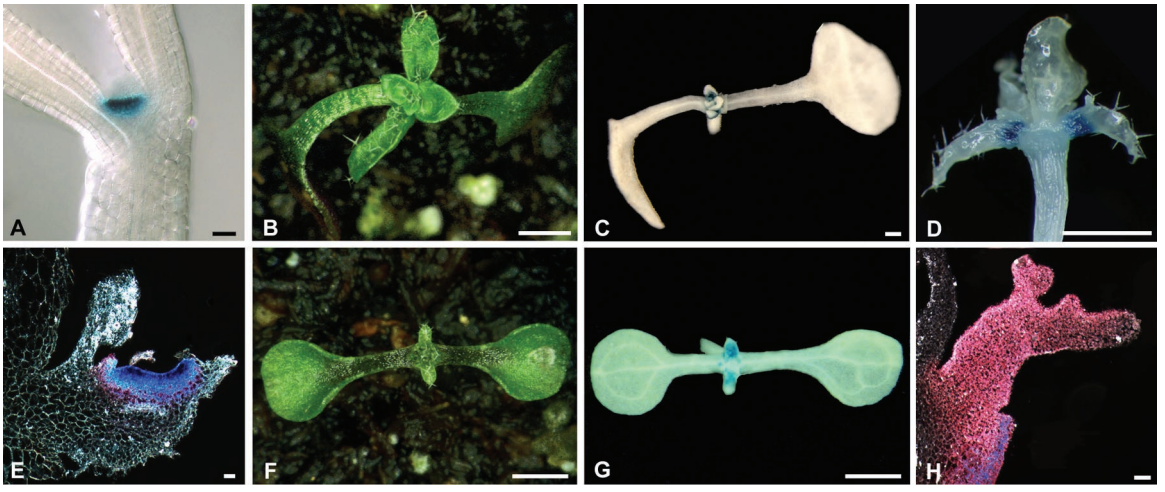

Figure 2. Induction of *CLV3* expression by *WUS*. A, GUS-stained *CLV3::GUS/WUS-GR* seedling 2 d.a.g., induced with Dex during germination. B, *CLV3::GUS/WUS-GR* seedling 24 d.a.g., induced with Dex at 4 d.a.g. C, GUS-stained seedling, as in B. D, GUS-stained *CLV3::GUS/WUS-GR* seedling 28 d.a.g., induced with Dex at 4 d.a.g. The cotyledons have been removed and GUS staining is visible in the leaf axils. E, Longitudinal section through a GUS-stained leaf, as in D; strong GUS expression is found in the apical cell layers of the laterally expanded axillary meristem. F, *CLV3::GUS/WUS-GR/STM-GR* seedling 24 d.a.g., induced with Dex 4 d.a.g. (compare with B). G, GUS-stained seedling, as in F; GUS expression is found in leaves. H, Section through a GUS-stained primary leaf of G, viewed with dark-field illumination. GUS signal (red) is found in all leaf cells. Scale bars in A, E, and H = 20 μm; in B through D, F, and G, scale bars = 1 mm.

be initiated at the cotyledon bases (Long and Barton, 1998; data not shown). *stm-11* mutants were then tested for activity of *CLV3::GUS* 10 d.a.g., and GUS-positive cells were detected in the basal region of the cotyledons in 15 of 59 mutant seedlings (25% of the total; Table I; Fig. 1K). *CLV3::GUS* expression was also found in the axillary meristems of *stm-11* mutants 21 d.a.g. (Fig. 1O).

To exclude effects due to the genetic background of *stm-11*, we also analyzed *CLV3* expression in another strong *stm* mutant, *stm-5* (Endrizzi et al., 1996). Under our growth conditions, *stm-5* mutants are phenotypically weaker than *stm-11*. In plants homozygous for the *stm-5* allele, GUS expression was observed in 27 of 48 seedlings (56% of the total) at 10 d.a.g. (Table I).

Taken together, our observations indicate that *STM* is not generally required for *CLV3* expression in embryos and young seedlings. However, *CLV3* expression levels are reduced in *stm* mutants, suggesting a role for *STM* in the up-regulation of *CLV3* expression.

Ectopic *STM* Expression Is Not Sufficient to Activate *CLV3* in Non-Meristematic Tissues

To further analyze the role of *STM* in the regulation of *CLV3*, we constructed a transgene that allows ectopic expression of *STM*. Previous studies have shown that transgenic plants expressing *STM* under control of the constitutive *CaMV35S* promoter are severely stunted with a highly disorganized shoot meristem, and arrest development at an early seedling stage (Williams, 1998). To control ectopic *STM* activity during development, we fused the *STM* coding region with the hormone-binding domain of the rat glucocorticoid receptor. This *STM-GR* fusion protein is constitutively expressed from the *CaMV35S* promoter throughout the plant. The *STM-GR* transgene was introduced into Arabidopsis plants carrying the *CLV3::GUS* reporter. If *STM* can act on its own to promote *CLV3* expression, we expected to observe blue GUS staining in all tissues after *STM* activation by Dex addition.

The expression of the *STM-GR* transgene was confirmed by reverse transcriptase (RT)-PCR and RNA in situ hybridization (data not shown). Without Dex application, transgenic lines were indistinguishable from the wild type and expressed the *CLV3* reporter gene in a normal pattern. Plants that were treated with Dex during germination carried more rounded cotyledons and small, lobed leaves with ectopic stipules along the leaf margin and fully differentiated trichomes (Fig. 3, A and B). On the adaxial (upper) leaf surface, ectopic meristems originated that again initiated new leaves, resulting in the formation of a dense array of small leaves and meristems. In some plants, ectopic meristems were also found on the surface of the cotyledons. Strong expression of the *CLV3* reporter gene was detected in the SAM and ectopic meristems, in a pattern comparable with *CLV3* expression in wild-type shoot meristems (Fig. 3, C and D). Occasionally, weak GUS staining was observed in the vasculature of the cotyledons and in stipules. Sensitive fluorometric GUS assays of leaf samples revealed an increase in GUS activity above background levels not before 5 d after Dex treatment (not shown). One explanation for this temporal delay

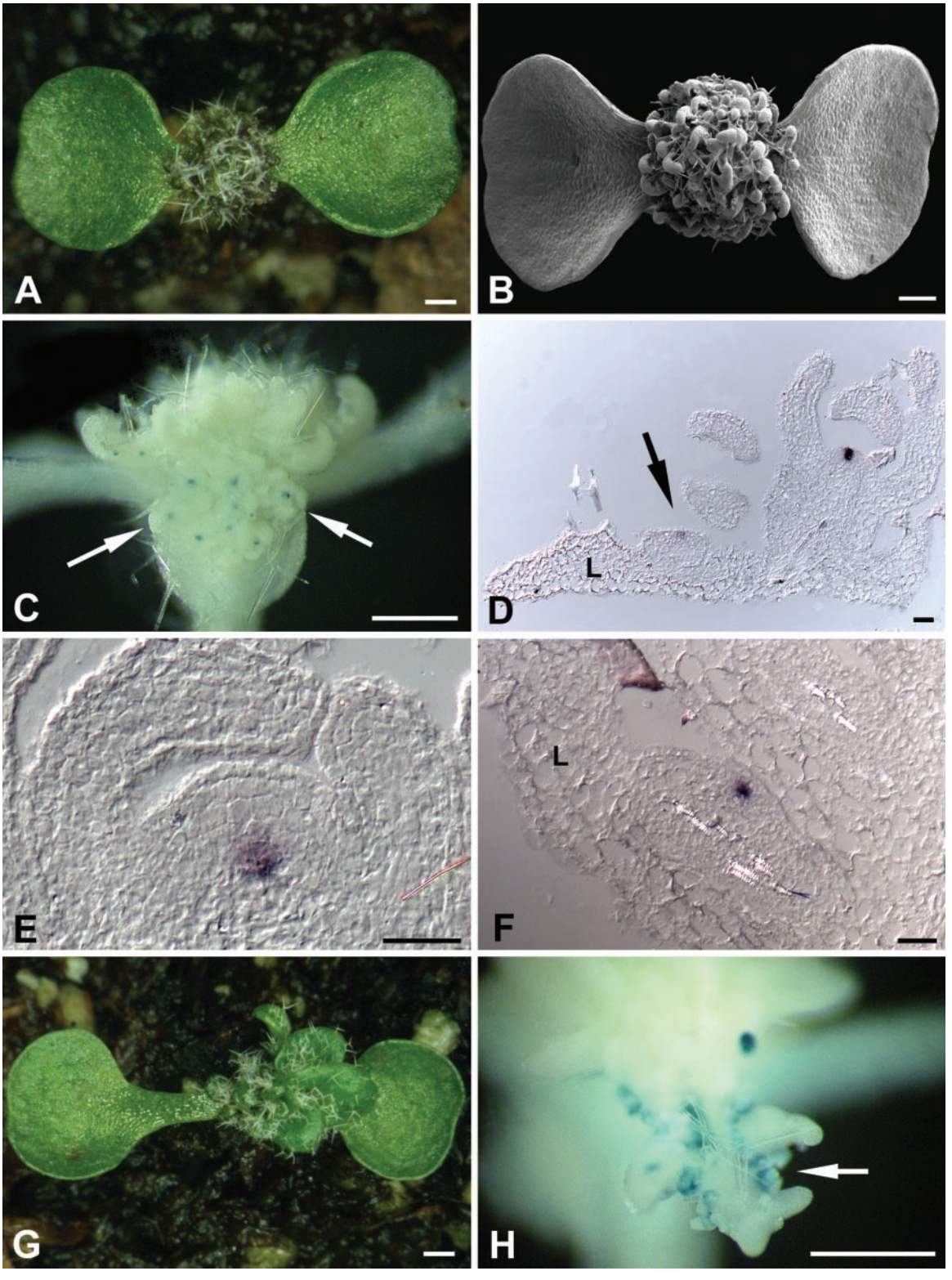

Figure 3. Induction of *CLV3* expression by *STM*. A, *STM-GR* seedling 24 d.a.g., induced with Dex at 4 d.a.g.; a dense array of leaves with new meristems is formed. B, Scanning electron micrograph of a seedling, as in A. C, GUS-stained *CLV3::GUS/STM-GR* seedling, as in A; ectopic meristems are initiated on the adaxial leaf surface, GUS signal is found in the center of these meristems (arrow). D, Section through a leaf of a *STM-GR* seedling, as in A. Expression of *CLV3* is detected by RNA in situ hybridization in the central zone of ectopic meristems (arrow). E, Section through the SAM of a *STM-GR* seedling, as in A. *WUS* expression, detected by RNA in situ hybridization, is found in the inner regions of the meristem,

(*Legend continues on facing page.*)

in *CLV3* activation is that *STM* does not act as a direct activator of *CLV3*. Instead, *STM* promotes the formation of new meristems, where *CLV3* expression may be activated by other meristem-specific genes, e.g. *WUS*. We analyzed the expression pattern of *WUS* and *CLV3* in *STM-GR* plants by RNA in situ hybridization. *WUS* was expressed in a small group of cells underneath the *CLV3* expression domain, comparable with the expression pattern in wild-type meristems (Fig. 3, D–F). To test if *WUS* function is required for *CLV3* expression, we introduced the *STM-GR* transgene into *wus* loss-of-function mutants. After crossing the *STM-GR* transgene into *wus-1* mutants and self-fertilization, homozygous *wus-1* seedlings were identified in the next generation and sprayed with Dex to activate *STM*. The leaves that were initiated during the first 3 weeks after Dex application were lobed and carried ectopic stipules, resembling leaves of *STM-GR* plants (Fig. 3, G and H). However, ectopic meristems were only occasionally formed, indicating that *WUS* function supports their induction by *STM*. *CLV3::GUS* expression was observed occasionally in ectopic stipules, but not in other non-meristematic tissues of these plants (Fig. 3H).

Co-Expression of *WUS* and *STM* Induces *CLV3* Expression in Leaves

Because ectopic expression of either *WUS* or *STM* was not sufficient to activate *CLV3* expression in non-meristematic regions, we then analyzed the consequence of misexpressing both genes together. After crossing of *STM-GR* transgenic plants to *WUS-GR* plants, the resulting F_1 plants were allowed to self-fertilize, and seedlings of the F_2 generation were induced with Dex 4 d.a.g. Within 2 d after Dex application, seedlings carrying only the *STM-GR* transgene can be distinguished from *WUS-GR* seedlings by their cotyledon shape. We identified *STM-GR/WUS-GR* seedlings as a new phenotypic class, characterized by upward curling of the cotyledons (Fig. 2F, compare with Figs. 2B and 3A). During the next 20 d after Dex treatment, small leaves were initiated that failed to expand. In contrast to *STM-GR* or *WUS-GR* plants, expression of the *CLV3* reporter gene was detected throughout the leaves of the *STM-GR/WUS-GR* plants, indicating that both *STM* and *WUS* function are required and sufficient to activate *CLV3* expression in non-meristematic cells (Fig. 2, G and H).

WUS Can Activate *CLV3* within the Same Cells

In the wild type, a small group of cells underlying the stem cell domain expresses both *WUS* and *STM*. However, *CLV3* RNA is not found in these cells, but in a separate domain at the tip of the meristem. The spatial separation of the *WUS* expression domain from the stem cell domain expressing *CLV3* suggests that either the *WUS* protein itself, or a *WUS*-dependent signal, is transmitted to the cells at the apex of the meristem (Mayer et al., 1998). Is this non-cell autonomy of *WUS* required for *CLV3* activation?

We tried to circumvent a non-cell-autonomous function of *WUS* by expressing *WUS* directly in the stem cell domain under control of the *CLV3* promoter. We replaced the *GUS* reporter gene in pBU16 with a *WUS* cDNA, and the resulting transgene *CLV3*::*WUS* was introduced into wild-type Arabidopsis. Four weeks after germination, transgenic plants had formed an apparently normal hypocotyl and cotyledons. Whereas non-transgenic control plants had already initiated six visible leaves, the *CLV3*::*WUS* transgenic plants produced only large domes of cells between the cotyledons (Fig. 4A). These apices resembled enlarged meristems that lacked organ primordia. The outer cell layers consisted of small, meristematic cells that covered an inner mass of large and vacuolated cells. Interestingly, we never observed any vascular tissue in these aberrant meristems. RNA in situ hybridization revealed that *CLV3* was now expressed not only in a few cells at the meristem apex, but at very high levels throughout the outer three cell layers, thus covering the apical dome (Fig. 4, C and E). *WUS* RNA was found only in cells that expressed *CLV3* (Fig. 4, B and D), but not in its normal expression domain underneath the stem cells. This could be explained if *WUS* activity in the stem cell domain can directly induce *CLV3* expression, resulting in a strong activation of the *CLV* signal transduction pathway, and repression of *WUS* transcription from its own promoter. Undifferentiated cells were still confined to the three outer meristem layers of the *CLV3*::*WUS* transgenic plants, indicating that *WUS* can promote stem cell fate in the same cells where it is expressed. Importantly, *WUS* fails to induce *CLV3* expression in deeper regions of the meristem. We conclude that non-cell autonomy of *WUS* is not a prerequisite for *CLV3* activation in the meristem.

Figure 3. (*Legend continued from facing page.*)
similar to wild type. F, Section through an axillary meristem of a *STM-GR* seedling, as in A. The domain of *WUS* expression resembles the expression in the SAM. E and F, No ectopic expression of *WUS* is observed. G, *CLV3::GUS/STM-GR/wus-1* seedling 24 d.a.g.; Dex induction commenced 7 d.a.g. when the seedlings could be phenotypically identified as wus mutants. H, GUS-stained seedling as in G; GUS signal was detected in ectopic stipules that occasionally formed on the leaf margins (arrow). Note the absence of ectopic meristems. L, Leaf. Scale bars in A through C, G, and H = 500 μm; in D through F, scale bars = 20 μm.

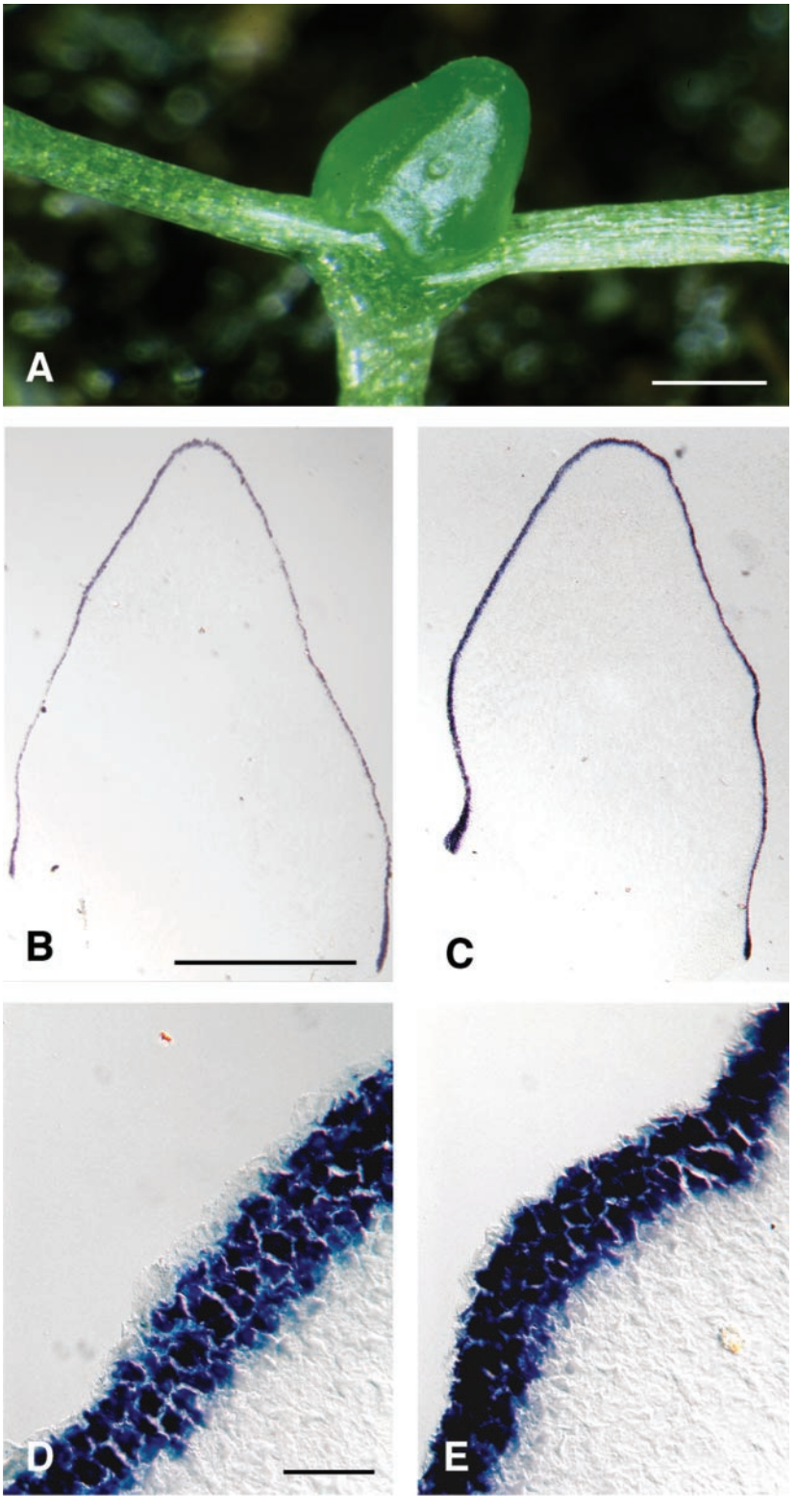

Figure 4. Cell-autonomous induction of *CLV3* by *WUS* in the stem cell domain. A, *CLV3*::*WUS* seedling 28 d.a.g.; a large apical dome is formed between the cotyledons, but organs are missing. B, In situ detection of *WUS* RNA in sections through the apical dome of a *CLV3*::*WUS* seedling. *WUS* RNA is found only in three apical cell layers, but not in the deeper regions of the meristem. D, High magnification view of the outer cell layers in B. C, As in B, but detection of *CLV3* RNA. *CLV3* and *WUS* are expressed in the same cells. E, High magnification of C. Scale bars in A and B = 500 μm; scale bars in D = 10 μm.

DISCUSSION

Pluripotent stem cells serve as a source for new cells to compensate for cell loss from the meristem during organ formation. Homeostasis of the shoot meristem requires a continuous adjustment of stem cell number, which can be achieved through the antagonistic activities of *WUS* and the *CLV* signal transduction pathway. A current model proposes that *WUS* induces both stem cell fate and *CLV3* expression; the size of the stem cell population is then

controlled by a simple feedback system, where *CLV3* activates a signal transduction pathway that results in a down-regulation of *WUS* expression, corresponding to *CLV3* levels (Brand et al., 2000; Schoof et al., 2000).

Our analysis of *CLV3* expression in wild-type and *wus* mutant embryos showed that *WUS* function is required for activation of *CLV3* expression during embryogenesis. This could suggest that *WUS* is involved in the transcriptional control of *CLV3*. However, the lack of *CLV3* expression could also be an indirect consequence of *wus* mutant embryos failing to initiate the cells that normally express *CLV3*. Activity of *WUS* is not confined to the embryonic SAM because inflorescence and floral meristems of *wus* mutants do not maintain permanent stem cells and abort organ formation prematurely (Laux et al., 1996). Importantly, the ability of *wus* mutants to initiate meristems at all reflects the activity of additional regulators that promote meristem function. We found that *CLV3* is expressed in the abnormal shoot meristems of *wus* mutant seedlings and also in later arising axillary meristems in a pattern comparable with wild type, indicating that *WUS* is required for *CLV3* expression only during embryogenesis. During seedling development, expression of *CLV3* in the SAM and axillary meristems is promoted by additional factors that act partially redundantly with *WUS* to control stem cell fate.

Misexpression of *WUS* in the whole plant did not result in a widespread up-regulation of *CLV3* expression. Instead, increased *CLV3* expression was observed only in the SAM and axillary meristems, and within these meristems, high levels of *CLV3* expression were found in the apical cell layers. This observation indicates that only cells at the tip of established meristems are competent to respond to *WUS* expression or to a *WUS*-derived signal (an alternative, but less likely explanation would be that *CLV3* expression is actively repressed in all other cells). Consistent with a requirement for additional activators, Lenhard et al. (2001) reported that misexpression of *WUS* in whorls 2 and 3 of flowers, under control of the *AP3* promoter, was insufficient to activate *CLV3* expression in developing organ primordia.

In wild type, the *STM* gene is expressed in the entire meristem, but down-regulated at sites of organ formation (Long et al., 1998). To test the role of *STM* in the regulation of *CLV3* expression, we first analyzed *CLV3* expression in *stm* mutant embryos and seedlings. Although *stm* mutants are not able to form a functional SAM, we detected low levels of *CLV3* expression in embryos, seedlings, and axillary meristems. In all cases, the patterns of *CLV3* expression resembled those found in wild-type plants. At least part of this residual *CLV3* expression could be due to *WUS* activity because *WUS* is initially expressed at a position corresponding to the shoot meristem in *stm* mutant embryos (Mayer et al., 1998). However, no *WUS* RNA was detected in the apices of *stm* seedlings (Mayer et al., 1998), suggesting that neither *STM* nor *WUS* are required to define the expression domain of *CLV3*.

Ectopic expression of *STM* resulted in the formation of lobed leaves and new, functional meristems on the leaf surface that expressed *CLV3* in a wild-type pattern. In addition, *CLV3* was occasionally expressed in the vasculature of some leaves and in ectopic stipules. The gain-of-function phenotype of an *STM*-related homeobox gene, *KNAT1*, shows a close association between ectopic meristems and leaf veins (Chuck et al., 1996). Thus, expression of *CLV3* in vasculature tissue of *STM-GR* plants may reflect an increased capacity of vein tissue for meristem formation. Stipules originate during leaf formation in pairs at the leaf base, and several mutant backgrounds or transgenes that cause misexpression of *knox* genes in leaves result in the co-induction of ectopic stipules along the leaf margin and ectopic meristems, which has led to an interpretation of stipules as an indicator for meristem-like activity (Ori et al., 2000). However, expression of the *CLV3*::*GUS* reporter in *STM-GR* plants was not observed throughout leaves or hypocotyls, indicating that *STM* expression is not generally sufficient to activate *CLV3* expression in non-meristematic tissues. Within ectopic meristems, we found both *WUS* and *CLV3* expression in a wild-type pattern, suggesting that *STM* promotes *CLV3* expression only indirectly by establishing a meristem-specific program of gene expression. We were not yet able to resolve whether the expression of *CLV3* in ectopic meristems of *STM-GR* plants depends on *WUS* because the number of ectopic meristems that initiated in a *wus* mutant background was insufficient for a detailed analysis.

Co-expression of *STM-GR* and *WUS-GR* resulted in GUS staining throughout leaf tissue, implying that activity of both homeobox genes is necessary and sufficient to activate *CLV3*. The *STM* gene product itself, or target genes that are controlled by *STM*, therefore may be required to cooperate with *WUS* to promote *CLV3* expression. This would explain why expression of *WUS* in the *AP3* domain was not sufficient to control *CLV3* expression in floral organs, where *STM* transcripts are down-regulated (Lenhard et al. , 2001). Notably, the levels of *CLV3* expression in the leaves of *STM-GR/WUS-GR* plants were lower than in wild-type meristems. Given that co-expression of *STM* and *WUS* suffices to activate basic levels of *CLV3* expression, we may speculate on what accounts for the separation of the *CLV3* expression domain (at the tip of the meristem) from the *WUS* domain (in deeper cell layers) in meristems. One possible explanation is that *WUS* acts only non-cell-autonomously in meristems, and causes the production or activation of a signal that is perceived by competent cells at the meristem apex. When we ex-

pressed *WUS* under control of the *CLV3* promoter, thus obliterating any requirement for a signaling process, the number of non-differentiating cells increased dramatically compared with wild type. This can be explained if *WUS* can promote stem cell fate and activate *CLV3* expression cell autonomously at the meristem apex. As a consequence, *WUS* expression levels controlled by the *CLV3* promoter increase further, thus creating a positive feedback loop of gene expression. *WUS* expression from its own promoter in deeper regions of the meristem is then expected to be switched off due to increased *CLV3* dependent signaling via the *CLV* pathway (Brand et al., 2000). If *WUS* can act cell autonomously and no signaling process is required, the lack of *CLV3* expression within the *WUS* expression domain could be explained with a requirement for localized cofactors to cooperate with *WUS*, allowing for *CLV3* expression only at the apex. Further support for this view comes from the observation that constitutive expression of *WUS* throughout the plant activates high levels of *CLV3* expression only in the apical cell layers of meristems. However, we found that *STM*, together with *WUS*, can activate *CLV3* expression at low levels in leaves. This could indicate that the regulation of *CLV3* expression also involves negative control mechanisms that are acting only in meristems.

In conclusion, we have shown here that only early *CLV3* expression in the embryonic SAM depends on *WUS* function. During later stages of development, neither *WUS* nor *STM* are required to establish the pattern of *CLV3* expression in the central zone of the SAM and axillary meristems. The role of *WUS* is now confined to increase the levels of *CLV3* expression, together with *STM*. Within a meristem, only cells at the meristem apex can express *CLV3*, indicating that localized (and as yet unidentified) factors or a specific cellular competence are required to respond to *WUS* activity.

MATERIALS AND METHODS

Plant Material and Genetics

The Arabidopsis Columbia ecotype was used for *Agrobacterium tumefaciens*-mediated vacuum transformation (Clough and Bent, 1998). Transgenes were introduced into mutant backgrounds by crosses or by direct transformation. The *stm-5*, *stm-11*, and *wus*-1 mutants used in this study were described previously (Endrizzi et al., 1996; Laux et al., 1996; Long et al., 1998).

GUS Staining

We used the GUS staining protocol of Sieburth and Meyerowitz (1997) with minor modifications. Plants were incubated in a staining solution [50 mM $NaPO_4$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 10 mM X-Gluc] at 37°C for at least 3 h. The tissue was then fixed, cleared, and embedded in paraffin or visualized as whole mount. Sections (7 μM) were cut from the embedded tissue.

Molecular Methods

In situ hybridizations were performed as described previously (Brand et al., 2000). To construct the *CLV3::GUS* marker gene, we isolated the *CLV3* regulatory sequences by PCR amplification from vector gE5.5*CLV3* (Brand et al., 2000) carrying a genomic DNA fragment comprising the *CLV3* gene. The 1.5-kb DNA promoter fragment 5′ to the transcriptional start of the *CLV3* gene was amplified using the 3′primer 551 up-(5′CCCCCTGCAGAGAG-AAAGTGA-CTGAGTGAG3′), introducing a *Pst*I site, and the 5′primer forward-(GTAAAAGGACGGCCAG) that binds to vector sequences. The enhancer fragment 1.2 kb downstream of the transcriptional stop was amplified with the 5′primer 553dow-(5′AAAAGCGGCCGCCCTAATCTCTTGTTGCTTTAA3′), introducing a *Not*I site, and the 3′primer 554dow-(5′CCCCGAGCTCTATGTGTGTTTTTTCTAAACAA3′), which introduces a *Sac*I site. Both fragments were cloned into the pGreen vector (Hellens et al., 2000) using the *Eco*RI/*Pst*I and *Not*I/*Sac*I sites to give pBU14. The *uidA* (*GUS*) coding sequence was cloned between these two fragments into the *Sma*I site of pBU14 to give pBU16 (*CLV3::GUS*). Transgenic plants are selected by Basta resistance.

To obtain a *CaMV35S*::*STM-GR* transgene, we amplified a *STM* cDNA by RT-PCR using the following primers: *STM*5′-(5′GGGGTCTAGAGATGGAGAGTGGTTCCAA-CAGCA3′), which introduces an *Xba*I site 5′ to the translation start codon; and *STM*3′-(5′GGGGGGATCCG-CAAGCATGGTGGAGGAGATGTGA3′), which replaces the stop codon with a *Bam*HI site. The correct PCR products were cloned into the *Xba*I and *Bam*HI sites of the pBIΔGR vector (Lloyd et al., 1994), in frame with the hormone-binding domain of the glucocorticoid receptor to give pRS4 (*STM-GR*). Transgenic plants were selected for kanamycin resistance.

The *CaMV35S*::*WUS-GR* transgene was constructed by PCR amplification of a *WUS* cDNA using the primers *WUS*5′-(5′TAGAGGATCCTATGGAGCCGCCACAGCATCAG3′) and *WUS*3′-(5′TTCAGGATCCTCGTTCAGACGTAGCT-CAAGAG3′). The *WUS*5′primer introduces a *Bam*HI site 5′to the translation start codon. The *WUS*3′ primer replaces the stop codon with a *Bam*HI site. PCR products were cloned into the *Bam*HI site of the pBIΔGR vector to give pMG10 (*WUS-GR*). Transgenic plants were selected for kanamycin resistance. Dex inductions were carried out by spraying plants with a solution containing 1 μM Dex (Sigma D8893).

To express the *WUS* coding region under control of *CLV3* regulatory sequences, we isolated a *WUS* cDNA by RT-PCR using the primers *WUS*Bam-(5′CCCAGGATCCAACACA-CATG-GAGCCGCCA3′) and *WUS*Spe-(5′AAAGACTAGT-GGCGTAAGAGCTAGTTCAG3′), which introduce *Bam*HI and *Spe*I sites, respectively, and inserted it into the *Bam*HI and *Spe*I sites of pBU14 to give pMG7 (*CLV3::WUS*). Transgenic plants were selected for Basta resistance.

All plasmid constructs were verified by restriction digests and DNA sequencing to rule out any amplification

errors. A minimum of 50 transgenic plants was obtained for each construct.

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