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Termination of Stem Cell Maintenance in *Arabidopsis* Floral Meristems by Interactions between *WUSCHEL* and *AGAMOUS*

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Summary

Floral meristems and shoot apical meristems (SAMs) are homologous, self-maintaining stem cell systems. Unlike SAMs, floral meristems are determinate, and stem cell maintenance is abolished once all floral organs are initiated. To investigate the underlying regulatory mechanisms, we analyzed the interactions between *WUSCHEL* (*WUS*), which specifies stem cell identity, and *AGAMOUS* (*AG*), which is required for floral determinacy. Our results show that repression of *WUS* by *AG* is essential for terminating the floral meristem and that *WUS* can induce *AG* expression in developing flowers. Together, this suggests that floral determinacy depends on a negative autoregulatory mechanism involving *WUS* and *AG*, which terminates stem cell maintenance.

Introduction

Flowers develop from lateral meristems which are produced by the shoot apical meristem (SAM) (Steeves and Sussex, 1989). Floral meristems and SAMs are homologous stem cell systems, and their behavior is regulated by overlapping sets of genes, with many mutations affecting both in a similar manner. However, floral meristems and SAMs differ fundamentally regarding the temporal extent of their activity: while—at least in many species—SAMs are indeterminate, i.e., they can produce organs continuously, floral meristems are determinate and their activity is terminated when the full set of floral organs has been initiated. Therefore, floral meristems are faced with the problem of how to overcome the mechanisms that ensure stem cell maintenance at the proper developmental point.

In both *Arabidopsis* shoot and floral meristems, stem cells are specified by signals from an underlying cell group, the organizing center, that expresses the *WUSCHEL* (*WUS*) homeobox gene (Laux et al., 1996; Mayer et al., 1998). Loss-of-function mutations in *WUS* result in premature termination of both SAM and floral meristems after the formation of a few organs. Ectopic *WUS* expression, on the other hand, can abolish organ formation and instead induce stem cell identity, based on the expression of a stem cell marker, the *CLAVATA3* (*CLV3*) gene (Schoof et al., 2000). *CLV3* appears to act as a repressor of *WUS*, whose loss of function results in an enlarged *WUS* expression domain and an increase in stem cell number. These results suggest that the size of the stem cell population in the SAM and floral meristems is regulated by a negative feedback loop between the *WUS*-expressing cells of the organizing center and the *CLV3*-expressing stem cells (Brand et al., 2000; Schoof et al., 2000).

The differences between the SAM and floral meristems are determined by meristem identity genes, for example, *APETALA1* (*AP1*) or *LEAFY* (*LFY*) (Irish and Sussex, 1990; Schultz and Haughn, 1991; Weigel et al., 1992). The transcription factor *LFY* is both required and sufficient to specify lateral meristems as floral (Weigel and Nilsson, 1995). The determinate mode of growth of floral meristems is reflected in the pattern of *WUS* expression. During flower development, *WUS* is expressed from the initiation of a floral meristem onward, but is downregulated when carpel primordia form in the center of the meristem after stage 6 (Mayer et al., 1998; for stages of flower development, see Bowman, 1994). This suggests that the organizing center and the stem cells are maintained until a sufficient number of cells has been formed for complete floral organ development, after which *WUS* expression is terminated and the cells enter differentiation.

Termination of the floral meristem requires the *AGAMOUS* (*AG*) gene which codes for a MADS domain transcription factor (Bowman et al., 1989; Yanofsky et al., 1990). Mutations in *AG* cause the formation of indeterminate flowers in which the carpels are replaced by interior flowers. Gain-of-function studies analyzing the effects of constitutive *AG* expression with the cauliflower mosaic virus *35S* promotor have indicated that *AG* is sufficient to convert indeterminate into determinate meristems since in *35S::AG* plants, the inflorescence meristem terminated in a central flower (Mizukami and Ma, 1997). In addition to its role in meristem termination, *AG* is required to specify organ identity in whorls 3 and 4, stamens and carpels, respectively (Bowman et al., 1989).

In order to investigate the mechanisms of how stem cell maintenance is terminated in floral meristems, we have analyzed the interactions between *WUS* and *AG*, as these function as major regulators of the opposite modes of growth, i.e., indeterminate versus determinate.

Results

AG Is Required to Repress *WUS* at the End of Flower Development

Wild-type floral meristems are determinate and their activity ceases after the formation of four whorls of organs (Figure 1A). In addition to showing a homeotic transformation of stamens into petals, *ag* mutant flower meristems are indeterminate and produce interior flowers inside the third whorl (Figure 1B; Bowman et al., 1989). By contrast, *wus* mutant as well as *ag wus* double mutant flowers terminate prematurely with a central stamen

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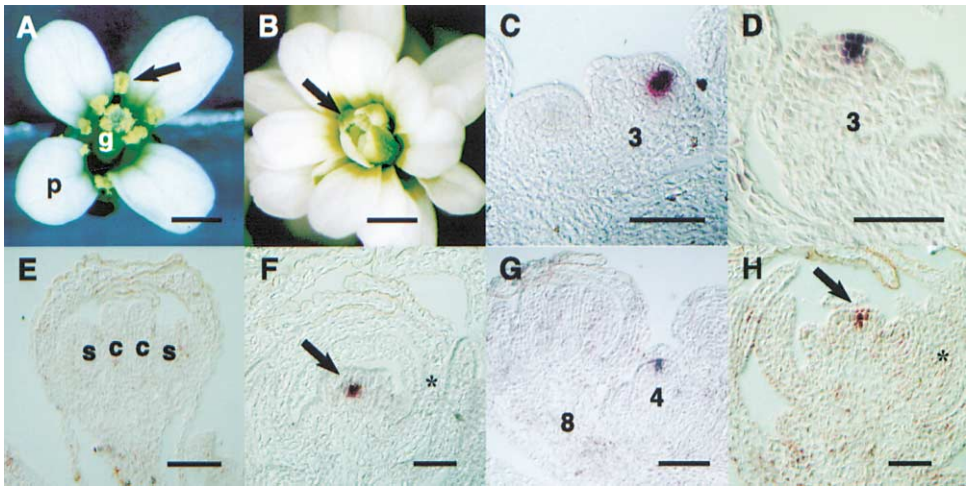

Figure 1. Expression of *WUS* and *CLV3* in Wild-Type and *ag* Mutant Flowers

(A and B) Micrographs of wild-type (A) and *ag* mutant (B) flowers. The wild-type flower (A) contains a central gynoecium (g) surrounded by six stamens of the third whorl (arrow). By contrast, *ag* mutants (B) form multiple interior flowers (arrow) inside the third whorl and show a homeotic transformation of stamens to petals. p: petal. Scale bars: 300 μm.

(C–H) In situ hybridizations with *WUS* (C, E, and F) and *CLV3* (D, G, and H) antisense riboprobes.

(C) In stage 3 flowers (3), strong *WUS* expression is detected in a small cell group underneath the outermost two cell layers.

(D) *CLV3* is expressed in the presumed stem cells in the outer three cell layers of stage 3 flowers (3).

(E) No *WUS* expression is detected in a stage 10 flower, where carpels (c) occupy the center of the flower. s: stamen.

(F) A late stage *ag* mutant flower with several whorls of organs (asterisk) still shows strong *WUS* expression in the center of the floral meristem (arrow). As in wild-type floral meristems, expression is found underneath the outermost two cell layers.

(G) No *CLV3* expression is detected in the stage 8 flower (8), in contrast to the stage 4 flower (4).

(H) A late stage *ag* mutant flower with several whorls of organs (asterisk) shows continuing *CLV3* expression in the presumed stem cells in the outermost three cell layers of the floral meristem (arrow).

No signal was detected using either a *CLV3* or a *WUS* sense riboprobe (not shown). Scale bars in (C)–(H): 50 μm.

or petal, respectively (Laux et al., 1996). In view of the opposite single mutant phenotypes, the epistatic relation of *wus* over *ag* mutations with regard to meristem termination allows the hypothesis that *AG* functions as a negative regulator of *WUS*. To test this at the molecular level, we compared the expression of *WUS* in wild-type and in *ag* mutant flowers using RNA in situ hybridization (Figure 1).

During wild-type flower development, *WUS* is expressed in a small group of cells in the center of the floral meristem from stage 1 onward, i.e., as soon as the floral primordium arises on the flanks of the SAM (Mayer et al., 1998; stages according to Bowman, 1994). Although it is difficult to quantify expression levels by in situ hybridization, it consistently appeared that the *WUS* hybridization signal is strongest in stages 2 and 3 of flower development, that is when the floral meristem becomes separated from the SAM and forms the first whorl of organ primordia, respectively (Figure 1C). *WUS* expression is discontinued after stage 6 when carpel primordia are initiated and floral meristem activity ceases (Figure 1E; Mayer et al., 1998). In contrast to wild-type, *WUS* expression is not downregulated in *ag* mutant flowers, but *WUS* is continually expressed during the formation of many whorls of floral organs (Figure 1F).

Since *WUS* has been shown to be sufficient to induce expression of the stem cell marker *CLV3* in shoot apices (Schoof et al., 2000), its continued expression in *ag* mutant flowers suggests that these retain a population of undifferentiated stem cells. To test this, we investigated *CLV3* mRNA expression in wild-type and in *ag* mutant flowers. In wild-type flowers, *CLV3* mRNA was detected in the putative stem cells before carpel formation, but not thereafter (Figures 1D and 1G). By contrast, *ag* mutant floral meristems continued to express *CLV3* in late stages after the formation of several whorls of floral organs (Figure 1H).

Thus, *AG* is required to repress both *WUS* and *CLV3* at the transcript level when carpel primordia are initiated in the center of the floral meristem.

Prolonged *WUS* Expression Is Sufficient for Floral Meristem Indeterminacy

The epistasis of *wus* over *ag* mutations concerning floral meristem termination indicates that *WUS* is required for the indeterminate *ag* flower phenotype (Laux et al., 1996) and, consistent with this, *WUS* continues to be expressed in *ag* mutant flowers. Therefore, we asked whether the prolonged *WUS* expression could be responsible for the indeterminacy of *ag* mutant flowers and studied whether extending the period of *WUS* expression in a wild-type background is sufficient to produce indeterminate flowers. To do so, we expressed *WUS* under the control of the *AG cis*-regulatory region. *AG* starts to be expressed in the precursor cells of stamens and carpels in stage 3 flowers and continues to be expressed throughout developing stamens and carpels long after stage 6 (Drews et al., 1991; Sieburth and Meyerowitz, 1997), i.e., when endogenous *WUS* expression has subsided. In addition, since *AG* appears to repress *WUS*, placing *WUS* under the control of the promotor of its own repressor would activate expression of the

transgenic *WUS* copy as soon as the floral meristem represses the endogenous *WUS* gene.

For this and all subsequent misexpression experiments, we used the *pOpL* two component system, where expression of a synthetic transcriptional activator, *LhG4*, is driven by a promotor of interest (Moore et al., 1998). The gene of interest is expressed from a synthetic promotor, *pOp*, which can be activated by LhG4. None of the individual transgenic lines carrying either an activator or a target construct exhibited any phenotype, and the effects described were only observed after crossing activator and target lines. When appropriate, we will refer, for example, to plants of the genotype *AG::LhG4*; *pOp::WUS* as *AG::WUS* and to plants of the genotype *AG::LhG4*; *pOp::WUS-pOp::GUS*, which in addition carry a linked GUS reporter, as *AG::WUS*, *AG::GUS* for the sake of simplicity.

We expressed *WUS* and a linked *GUS* reporter gene under the control of the *AG cis*-regulatory region from the second intron, which confers the wild-type *AG* expression pattern (Busch et al., 1999; Deyholos and Sieburth, 2000). We will refer to these *cis*-regulatory sequences as the *AG* promotor for simplicity. To check the expression pattern of the transgenes, we stained the flowers for GUS activity. Initially weak GUS staining was observed specifically in the cells of the prospective third and fourth whorls from stage 4 (when sepals begin to overlie the floral meristem) onward (data not shown). In later stages, the signal became stronger and continued to be restricted to cells interior to the second whorl petal primordia (Figure 2B). Thus, with a slightly later onset of expression, the transgene mirrors the endogenous *AG* mRNA expression pattern.

AG::WUS expression results in a loss of floral meristem determinacy and a strong phenotype in whorls 3 and 4 (Figures 2A and 3A–3D): While *AG::WUS*-expressing flowers are indistinguishable from wild-type before stage 6 of flower development (data not shown), they begin to deviate from wild-type thereafter in that the center of the floral meristem is broadened and gives rise to carpel primordia with cells separating them (Figure 2F). By contrast, in wild-type flowers, the carpel primordia abut each other (Figure 2D). As flower development progresses, the gynoecium enlarges further, relative to wild-type, and shows ectopic meristematic tissue in the center (Figures 2E and 2G), eventually resulting in massively overproliferated gynoecial structures (Figure 2A). In addition, similar oversized gynoecia often develop from ectopic meristematic structures between the second and third whorl organs (Figures 2C and 3C). Stamen development is variably perturbed, ranging from a complete absence to the formation of more than six stamens. In many cases, third whorl stamens are partly fused to the central gynoecium (Figure 3D). As expected from the expression pattern of the transgene, sepals and petals are unaffected in *AG::WUS* plants (data not shown).

It is likely that the continuing proliferation of cells in the center of *AG::WUS* flowers is the result of prolonged temporal expression of *WUS* in whorl 4 rather than a consequence of the ectopic expression in whorl 3 since in an additional experiment, ectopic expression of *WUS* in whorls 2 and 3 did not affect floral meristem determinacy (see below).

The effects of prolonged *WUS* expression in flowers could not be mimicked when *SHOOTMERISTEMLESS* (*STM*), which is required to maintain undifferentiated meristem cells (Barton and Poethig, 1993; Endrizzi et al., 1996), was expressed under control of the *AG* promotor (data not shown), suggesting that the observed effects are specific to *WUS*.

These results suggest that repression of *WUS* is a critical and specific regulatory switch to terminate floral meristems.

AG Can Counteract *WUS* at Different Levels

Our observation that *WUS* expression from a heterologous promotor can abolish the *AG*-dependent termination of floral meristems suggests that *AG* mainly represses *WUS* at the level of transcription. However, in contrast to the SAM where *WUS* expression in organ primordia prevents differentiation (Schoof et al., 2000), stamen and carpel structures still differentiate in *AG::WUS* flowers, even though *WUS* is expressed in the respective organ primordia. Since differentiation of stamens and carpels is regulated by *AG* in wild-type, this suggested that *AG* could counteract *WUS* function not only at the level of *WUS* transcription, but also when *WUS* is expressed from a heterologous promotor.

Thus, we tested whether the formation of stamens and carpels in *AG::WUS* plants was due to *AG* activity. We did so by decreasing the dose of *AG* and analyzed flowers of 40 *AG::WUS*-expressing plants which were heterozygous *ag-1/AG* as determined by PCR (data not shown). In wild-type, *ag-1* is a strong recessive loss-of-function allele of *AG* (Bowman et al., 1989). In all cases, the flowers developed a proliferating mass of cells with a meristematic appearance interior to the petals, and virtually no differentiation occurred (Figures 3C–3F).

Thus, even if *WUS* is expressed from a heterologous promotor, *AG* appears to be able to counteract its effects and allow differentiation. Possible mechanisms could include a posttranscriptional influence of *AG* on *WUS* activity or opposite regulation of common downstream processes.

Ectopic *WUS* Expression Induces *AG*-Dependent Organ Transformations

The continued GUS staining throughout the excess proliferating cells of old *AG::WUS*, *AG::GUS* flowers suggested that the *AG* promotor is still active there at a time when it has long been switched off in wild-type flowers, except for few specialized cell types (Figure 2C; Bowman et al., 1991). This suggests that *WUS* is able to maintain expression of the *AG* promotor.

To study whether *WUS* is sufficient to induce ectopic *AG* expression, we expressed *WUS* in whorls 2 and 3 of developing flowers from the *APETALA3* (*AP3*) promotor (Jack et al., 1992). If *WUS* is sufficient to induce *AG* expression in whorl 2, this would be predicted to repress the organ identity genes *AP1* and *APETALA2* (*AP2*) (Mizukami and Ma, 1992; Gustafson-Brown et al., 1994) and perturb petal identity, similar to the effects of *AP3::AG* expression (Jack et al., 1997).

In fact, *AP3::WUS* transgenic plants produce flowers with transformed second whorl organs. Second whorl primordia begin to deviate from wild-type development after

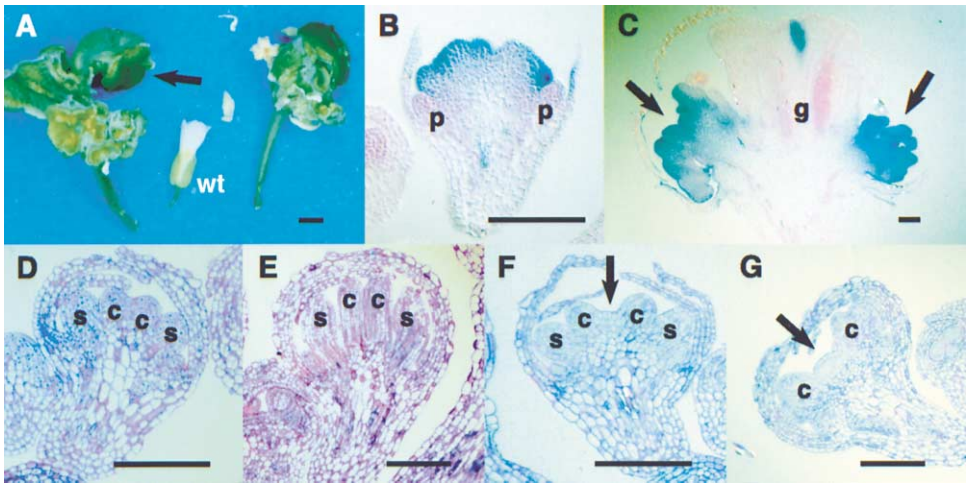

Figure 2. Effect of *AG::WUS* Expression

(A) Micrograph of a wild-type (wt) and two *AG::WUS*, *AG::GUS*-expressing flowers. Note the massive overproliferation of gynoecial structures in the latter (arrow). Scale bar: 1 mm.

(B) Longitudinal section through a GUS-stained stage 7 *AG::WUS*, *AG::GUS* transgenic flower in the plane of petal primordia. Staining is restricted to cells interior to the petal primordia (p) of the second whorl.

(C) Longitudinal section through a GUS-stained mature *AG::WUS*, *AG::GUS* transgenic flower. Staining is observed in the ectopic proliferating gynoecia (arrows) and in a small region at the tip of the central gynoecium (g). Weak blue staining in sepals adjacent to strongly stained interior tissues in (B) and (C) most likely results from diffusion of the reaction intermediate of GUS staining. Red color in (B) and (C) is a result of EosinY staining during the embedding procedure.

(D and E) Histological sections through stage 6 (D) and stage 9 (E) wild-type flowers, stained with toluidine-blue. The entire center of the floral meristem is consumed in the formation of the carpel primordia.

(F and G) Histological sections through stage 6 (F) and stage 9 (G) *AG::WUS*-expressing flowers. The carpel primordia are separated by several cells (F, arrow) which continue to proliferate and push the carpel primordia apart (G, arrow).

c: carpel, s: stamen. Scale bars in (B)–(G): 100 μm.

stage 9 (Figures 4A–4E): they form additional structures which mostly differentiate into anthers, i.e., the pollen-bearing parts of stamens, and sometimes also show carpeloid characteristics, such as stigmatic tissue (data not shown), both suggestive of ectopic *AG* function in whorl 2. In some cases, second whorl primordia give rise to a normal-looking gynoecium and surrounding stamens, suggesting that they have been completely transformed into floral meristems (Figure 4F). In addition, third whorl stamens were sometimes also affected, with organs arising from the abaxial side of the filament (data not shown). Staining for activity of the linked GUS reporter confirmed expression of the *AP3::WUS*, *AP3::GUS* transgenes in whorls 2 and 3 from stage 5 onward (Figure 4I). As expected from this expression pattern, sepal and carpel development is indistinguishable from wild-type (data not shown).

To confirm that the transformation of second whorl organs of *AP3::WUS* flowers is caused by ectopic *AG* expression, we analyzed the effect of *AP3::WUS* expression in *ag-1* homozygous mutant plants, whose genotype was determined by PCR for the *ag-1* allele (data not shown). These plants exhibited a novel floral phenotype: cells in the second and third whorls did not differentiate into staminoid or carpeloid organs as in *AP3::WUS* flowers in a wild-type background, but instead behaved as floral meristems (Figures 4G and 4H). These initiated a whorl of sepals and further interior floral meristems, which eventually led to a complex structure of meristems within meristems.

Thus, ectopic expression of *WUS* causes a transformation of second whorl organs suggestive of ectopic *AG* function.

WUS Can Induce *AG* Expression

To directly show that ectopically expressed *WUS* activates the *AG* promotor, we expressed *AP3::WUS* in plants which carried an *AG::GUS* reporter gene (*pAG-I::GUS*) and stained for GUS activity (Sieburth and Meyerowitz, 1997). In wild-type flowers, the *pAG-I::GUS* reporter is only active in whorls 3 and 4 (Figures 5C and 5D). By contrast, in developing *AP3::WUS*; *pAG-I::GUS* flowers, we observed ectopic GUS staining in whorl two from approximately stage 6 onward (Figure 5A), that is shortly after expression of the *AP3::WUS* transgene could be detected and well before any morphological changes occurred (see above). Reporter gene expression was maintained throughout development of the transformed second whorl organs, and the differentiated ectopic anthers still showed strong GUS staining (Figure 5B). In order to compare the expression level of the *AP3::WUS* transgene to that of the endogenous *WUS* gene, we performed RNA in situ hybridization using a *WUS* antisense probe. The expression level from the *AP3::WUS* transgene was similar to that of the endogenous *WUS* gene in young flower meristems (Figure 5E).

The early onset of ectopic *pAG-I::GUS* expression shortly after the *AP3::WUS* transgene is activated and before any morphological changes are observed in second whorl organs indicates that the ectopic activation of the *AG* promotor is not simply a consequence of the establishment of new floral meristems.

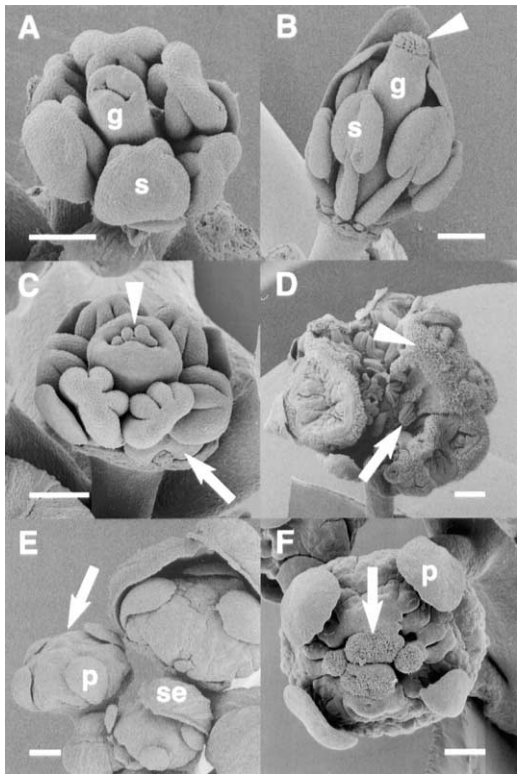

Figure 3. The Phenotype of *AG::WUS*-Expressing Flowers Is Sensitive to *AG* Function

(A–F) Scanning electron micrographs. Sepals and petals were partly removed to reveal interior structures.

(A and B) Stage 9 (A) and stage 12 (B) wild-type flowers. Note the presence of stigmatic tissue on top of the gynoecium (arrowhead in [B]). g: gynoecium, s: stamen.

(C) Stage 9 *AG::WUS*-expressing flower. Excess tissue is present inside the gynoecium (arrowhead). Additional outgrowths develop between second whorl petals and third whorl stamens (arrow).

(D) Mature *AG::WUS*-expressing flower. Differentiation of stigmatic tissue (arrowhead) and stamens (arrow) is apparent, which were often fused to the enlarged gynoecium.

(E) *AG::WUS*; *ag-1/AG* flowers of approximately stages 9 to 12. No organ differentiation is seen inside the second whorl petals (arrow). p: petal, se: sepal.

(F) Old *AG::WUS*; *ag-1/AG* flower. Only small patches of stigmatic tissue (arrow) can be seen on top of the callus-like mass. p: petal. Scale bars: 100 μm.

To confirm this, we analyzed the expression of the stem cell marker *CLV3* in developing *AP3::WUS* flowers by RNA in situ hybridization. In a total of 43 sections in which a neighboring inflorescence meristem or young floral meristem exhibited clear endogenous *CLV3* expression, we never detected any hybridization signal in whorls 2 or 3 of *AP3::WUS* flowers (Figure 5F). This implies that activation of *CLV3* expression by *WUS* can only occur in competent cells, as has also been observed in leaves (M.L. and T.L., unpublished data). In a control experiment, we readily detected *WUS* mRNA produced from the transgene in these cells (Figure 5E), indicating that they are accessible to ribonucleotide probes.

In summary, we conclude that normal levels of *WUS* can induce expression from the *AG* promotor, independently of the establishment of ectopic floral meristems.

Endogenous *WUS* Does Not Activate *AG* Expression in *lfy* Mutant Floral Meristems

AG-dependent termination of stem cell maintenance is restricted to determinate floral meristems, while the SAM does not express *AG* and can thus be active indeterminately (Drews et al., 1991). However, if *WUS* is sufficient to induce and maintain *AG* expression in developing flowers, why does it not do so in the SAM? One possible explanation is that induction of *AG* expression by *WUS* requires additional factors which are only provided in floral meristems, but are absent from the SAM.

A likely candidate for such an additional factor is *LFY*, which is both required and sufficient for floral meristem identity and can bind regulatory elements in the *AG* second intron (Weigel et al., 1992; Weigel and Nilsson, 1995; Busch et al., 1999). In late arising *lfy* mutant floral meristems, which develop into more flower-like structures, the onset of *AG* mRNA expression is delayed and initially restricted to a smaller domain than in wild-type (Weigel and Meyerowitz, 1993; Busch et al., 1999).

We asked whether *WUS* required *LFY* to act on the *cis*-regulatory elements of the *AG* second intron, as it does in the wild-type (Figure 2C), and introduced the *AG::WUS*, *AG::GUS* constructs into a *lfy* mutant background. If the endogenous *WUS*, which is expressed normally in *lfy* mutant flowers (Figure 6A), could activate the *AG* promotor, even if only weakly, this would be predicted to result in a self-amplification of the signal and produce strong GUS staining and ectopic cell proliferation, similar to what was observed with the same constructs in wild-type. However, if no activation is observed, this would indicate that endogenous *WUS* cannot activate the *AG* promotor in the absence of *LFY*.

We crossed *AG::LhG4/−; lfy-6/+* and *pOp::WUS-pOp::GUS/−; lfy-6/+* plants. Since both transgenes were unlinked to the *LFY* locus (data not shown), we expected 56% (9/16) wild-type plants, 19% (3/16) unaltered *AG::WUS* phenotypes, 19% (3/16) *lfy* mutant phenotypes, and 6% (1/16) plants homozygous mutant for *lfy* and carrying both transgenes. The results of the same cross in a *LFY* wild-type background indicated that *AG::WUS*, *AG::GUS* expression was initiated in all flowers of plants carrying the constructs and confirmed that the *AG* promotor used is responsive to *WUS* activity (Figure 2C; data not shown).

In a total of 490 F1 plants, we observed 56.7% wild-type plants and 15.9% *AG::WUS*, *AG::GUS* phenotypes that were indistinguishable from *AG::WUS*, *AG::GUS* flowers in a wild-type background. 25.3% of the plants showed the unaltered *lfy-6* mutant phenotype, and no GUS staining was detected in any of their flowers (61 plants tested). Only a few plants (ten plants; 2% of the total) displayed a *lfy-6* phenotype and additional alterations in isolated flowers (one to six per plant) which exclusively arose late in development and were only partly transformed into shoots (Figures 6B–6G). In these, the central gynoecium was enlarged and contained fields of small meristematic cells which showed strong GUS staining (6 out of 6 inflorescences analyzed), indicating that the *AG::WUS*, *AG::GUS* transgenes were expressed and produced a similar phenotype as in a wild-type background. Even in these ten plants, early arising flowers were unaffected by the transgenes and did not show GUS staining (Figure 6B).

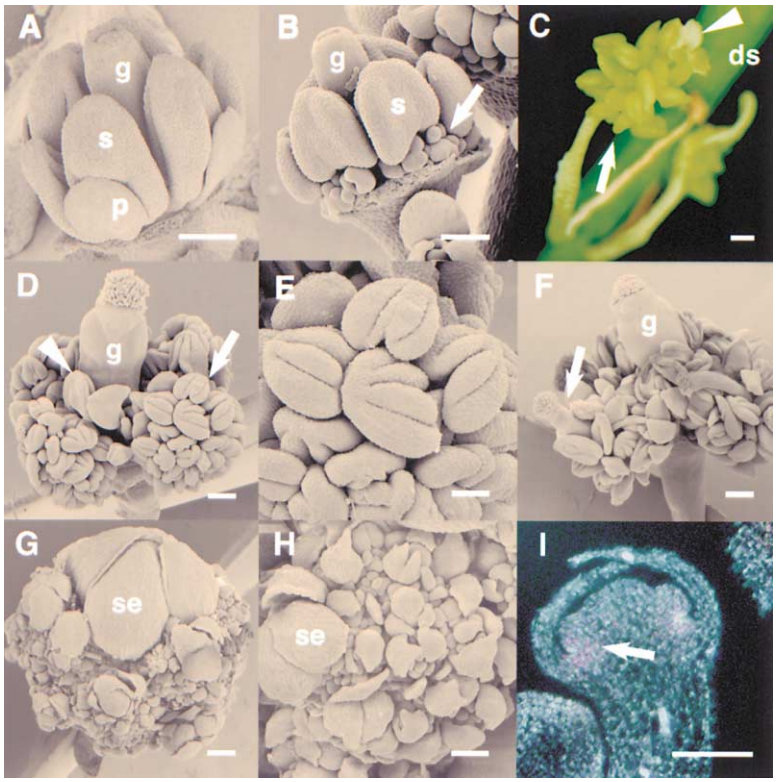

Figure 4. Phenotype of *AP3::WUS*-Expressing Flowers

(A, B, and D–H) Scanning electron micrographs. Outer sepals have been removed to reveal interior structures.

(A) Stage 10 wild-type flower. g: gynoecium, s: stamen, p: petal.

(B) Stage 10 *AP3::WUS*-expressing flower. In place of the petals, additional undifferentiated structures are visible (arrow). g: gynoecium, s: stamen.

(C) Second whorl organ of an old *AP3::WUS*-expressing flower. Several anthers have formed on the abaxial side of the organ (arrow), which still shows white petaloid tissue at the tip (arrowhead). ds: developing silique (in background).

(D) Old *AP3::WUS*-expressing flower. Numerous anthers have developed from the second whorl primordia (arrow). Third whorl stamens appear largely unaltered (arrowhead). g: central gynoecium of the original flower.

(E) Higher magnification view of the ectopic anthers indicated by the arrow in (D).

(F) Old *AP3::WUS*-expressing flower. The second whorl primordium has given rise to an additional gynoecium (arrow) surrounded by supernumerary stamens. g: central gynoecium of the original flower.

(G) Old *AP3::WUS*; *ag-1/ag-1* flower. No differentiation of carpels or stamens from second whorl primordia has occurred. Rather, numerous floral meristems with whorls of sepals have been formed. se: sepal.

(H) Higher magnification view of the excess meristems surrounded by whorls of sepals that develop in the second and third whorls of *AP3::WUS*; *ag-1/ag-1* flowers. se: sepal.

(I) Section of a GUS-stained stage 5 *AP3::WUS*, *AP3::GUS* flower viewed under dark-field illumination. GUS staining, seen in red, is visible in the second and third whorl organ primordia (arrow).

Scale bars in (A)–(D), (F), and (G): 100 μm. Scale bars in (E), (H), and (I): 50 μm.

A comparison of observed and expected frequencies reveals that the proportion of *AG::WUS*, *AG::GUS* phenotypes is reduced at the expense of *lfy* mutant phenotypes. Since one quarter of the homozygous *lfy* mutants should carry both transgenes, this finding, together with the absence of GUS staining in all of the unaltered *lfy* mutant inflorescences and in most flowers of the ten plants with isolated affected flowers, suggests that in *lfy* mutant floral meristems, the transgenes are generally not expressed. This indicates that endogenous *WUS* cannot efficiently activate the *AG::WUS*, *AG::GUS* transgenes in the absence of *LFY* function.

In the ten plants where sporadic activation of the transgenes was found, it was restricted to isolated floral meristems formed late in the plant's life, suggesting that a stochastic process was involved in this activation. In these cases, *AG::WUS* expression produced similar effects to those seen in wild-type, suggesting that *WUS* was able to maintain expression of the transgenes.

Discussion

Floral meristems are considered to be modified SAMs, and both share a number of regulatory genes and mechanisms (Steeves and Sussex, 1989). One important common feature is the existence of an apical population of undifferentiated stem cells with the ability to self-maintain. Despite this similarity, the temporal extent of meristem activity differs markedly: The SAM in *Arabidopsis* is active indeterminately and produces an in principle unlimited number of lateral organs, indicating that stem cell maintenance is continually active. In contrast, floral meristems only form a fixed complement of organs after which their activity ceases, and to this end, stem cell maintenance has to be switched off. This raises the questions of how stem cell maintenance is terminated and how the time point of this is regulated.

We have analyzed the interactions between two major regulators of the indeterminate and the determinate modes of growth, *WUS* and *AG*, respectively. We present evidence that *WUS* is able to induce *AG*, which in turn represses *WUS*, and thus terminates the floral meristem. This occurs only in floral meristems due to a requirement for *LFY* activity, allowing the SAM to be active indeterminately.

WUS as an Activator of *AG* Expression

Our results indicate that ectopic *WUS* activity can induce *AG* expression. This occurs independently of the induction of ectopic stem cell identity and at levels of *WUS* expression which are normally found in developing floral meristems.

These observations raise the question of whether endogenous *WUS* plays a role in activating *AG* expression

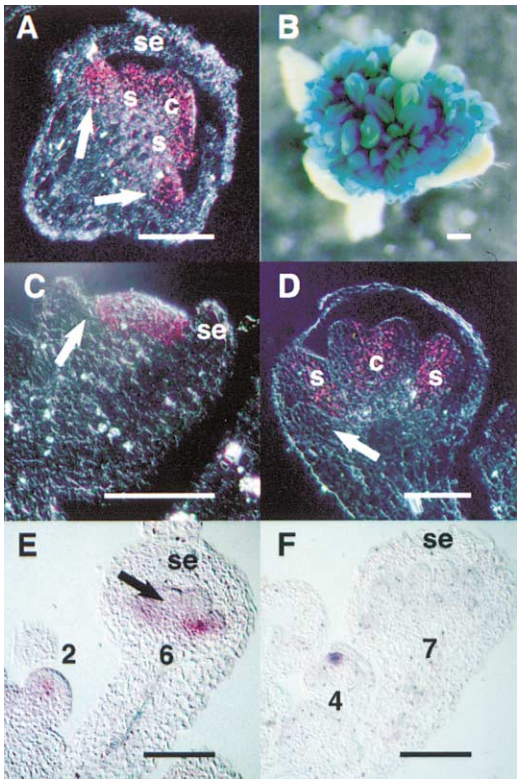

Figure 5. *WUS* Can Induce Ectopic *AG* Expression

(A) Oblique section of a GUS-stained stage 6 *AP3::WUS*; *pAG-I::GUS* flower viewed under dark-field illumination. GUS staining, seen in red, is visible in the primordia of the second, third, and fourth whorls. Arrows mark ectopic staining in second whorl primordia. c: carpel, s: stamen, se: sepal.

(B) Old *AP3::WUS*; *pAG-I::GUS* flower stained for GUS activity. Strong staining is detected in the ectopic anthers, which have developed from the second whorl organ primordia.

(C) Section of a GUS-stained stage 3 *pAG-I::GUS* flower viewed under dark-field illumination. GUS staining, seen in red, is restricted to the precursor cells of the third and fourth whorl, but is excluded from the cells that will give rise to the petals (arrow). se: sepal primordium.

(D) Section of a GUS-stained stage 7 *pAG-I::GUS* flower viewed under dark-field illumination. GUS staining, seen in red, is detected in the stamen (s) and carpel (c) primordia, but is absent from petal primordia (arrow).

(E) *WUS* expression in *AP3::WUS* flowers as detected by in situ hybridization. The expression level from the transgene in the second and third whorl organ primordia (arrow) of the stage 6 flower (6) is similar to that of endogenous *WUS* in the center of the stage 2 floral meristem (2). This was confirmed by comparing the signal strength in many young floral meristems and older *AP3::WUS* flowers, similar to the one shown here, hybridized on the same slides. se: sepal.

(F) *CLV3* expression in *AP3::WUS* flowers as detected by in situ hybridization. No staining is detected in the second and third whorl organ primordia of the stage 7 flower (7), yet a strong signal is seen in the presumed stem cells of the adjacent stage 4 flower (4). se: sepal.

Scale bars in (A), (C), and (D): 50 μm. Scale bars in (B), (E), and (F): 100 μm.

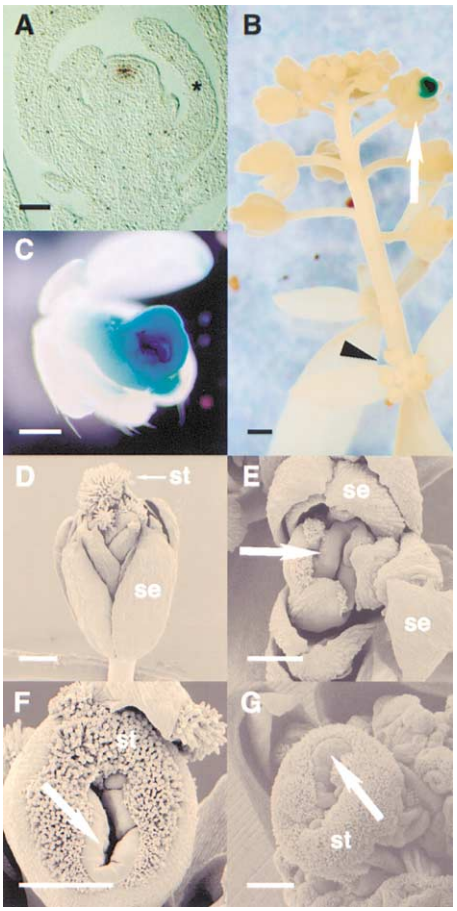

Figure 6. Induction of *AG* Expression by *WUS* Requires *LFY*

(A) *WUS* expression in a transformed *lfy-6* mutant flower. Expression is detected in the meristem center underneath the outermost cell layers. The meristem has given rise to numerous leaf-like lateral organs (asterisk).

(B and C) GUS-stained inflorescence (B) and flower (C) of an *AG::WUS*, *AG::GUS*; *lfy-6*/*lfy-6* plant. Note the isolated GUS-positive flower (arrow in [B]) and the absence of GUS staining in the early arising, fully transformed lateral meristems (arrowhead in [B]).

(D–G) Scanning electron micrographs.

(D) Nontransgenic *lfy-6* mutant flower. se: sepal, st: stigmatic tissue.

(E) *AG::WUS*, *AG::GUS*; *lfy-6*/*lfy-6* flower. Note the enlarged gynoecium in the center (arrow). se: sepal.

(F) Higher magnification view of a phenotypically altered *AG::WUS*, *AG::GUS*; *lfy-6*/*lfy-6* flower to show the fields of small cells inside the enlarged gynoecium (arrow). st: stigmatic tissue.

(G) Late stage *AG::WUS*, *AG::GUS* flower in a wild-type background, showing fields of small, dense cells inside a gynoecial structure (arrow). st: stigmatic tissue.

Scale bar in (A): 50 μm. Scale bar in (B): 1 mm. Scale bars in (C)–(G): 100 μm.

to terminate the floral meristem. Since *WUS* appears not to be required for *AG* expression in developing stamens and carpels (Laux et al., 1996; M.L. and T.L., unpublished), this function would be specific for the center of the floral meristem. Unfortunately, it is not possible to use simple *wus* loss-of-function analyses to answer this question because *wus* mutant floral meristems are unable to maintain stem cells and terminate prematurely, irrespective of *AG* activity (Laux et al., 1996).

However, some observations are consistent with an important role for *WUS* in activating *AG* expression in the floral meristem center. *LFY*, the only known direct activator of *AG* expression (Busch et al., 1999), is not sufficient to induce *AG*, but appears to require additional factors; *LFY* is expressed throughout the floral meristem

and well before the onset of localized *AG* expression in the center of stage 3 flowers (Drews et al., 1991; Weigel et al., 1992), and constitutive *LFY* expression did not result in ectopic *AG* expression (Parcy et al., 1998). Furthermore, termination of the floral meristem center seems to require higher levels of *AG* activity than differentiation of stamens and carpels because plants with weak loss of *AG* function formed flowers with stamens and carpels, but which had lost determinacy (Mizukami and Ma, 1995; Sieburth et al., 1995). This suggests that additional activators may be required in the center of the floral meristem to achieve high levels of *AG* expression, especially because the mRNA expression level of *LFY* declines there after late stage 3 (Weigel et al., 1992). *WUS* is a good candidate for such an additional factor because it is expressed both in the right place, the floral meristem center, and at the right time: its expression level peaks just before the onset of *AG* expression and continues up to stage 6, albeit at slowly decreasing levels (Mayer et al., 1998).

Activation of *AG* Expression by *WUS* Is Restricted to Floral Meristems

If *WUS* activates *AG* expression in determinate floral meristems, what prevents it from doing so in the SAM and thus inappropriately terminating its activity? Our results indicate that in the absence of *LFY* function, endogenous *WUS* cannot initiate expression of the *AG* promotor. This interpretation is supported by the indeterminate development of early arising *lfy* mutant flowers, which suggests that in the absence of *LFY*, *AG* is not sufficiently activated by *WUS* or any other gene to terminate the meristem (Schultz and Haughn, 1991; Weigel et al., 1992). Since *LFY* is only expressed in floral meristems, but not in the SAM (Weigel et al., 1992), this will prevent *WUS* from activating its repressor *AG* there (see below), allowing indeterminate growth of the SAM.

What does the requirement for *LFY* mean mechanistically? One possibility is that *WUS* can only maintain *AG* expression after *AG* has been activated by LFY. However, this seems unlikely because *WUS* can ectopically induce the *AG* promotor in second whorl cells where no detectable previous activation of *AG* expression by LFY has taken place (Drews et al., 1991; see Figure 4I). It also seems unlikely that *WUS* activates *LFY* expression, which would then indirectly induce *AG* expression, because *LFY* is expressed normally in *wus* mutants and no ectopic *LFY* expression could be detected in plants with ectopic *WUS* activity (R. Gross-Hardt, M.L., and T.L., unpublished data). Thus, it appears that *WUS* requires additional factors to activate *AG* expression. This could either be *LFY* itself or yet others that are only provided once floral meristem identity has been specified by *LFY*.

The requirement for *LFY* does not seem to be absolute since *WUS* was apparently able to maintain *AG* expression in a few *lfy* flowers arising late in plant development. This finding is in line with previous observations that such *lfy* flowers are only weakly transformed into inflorescence meristems, which had been taken to suggest that other factors can substitute for *LFY* function late in a plant's life. Genetic analyses indicated that one of these could be *AP1* (Weigel et al., 1992; Weigel and Meyerowitz, 1993).

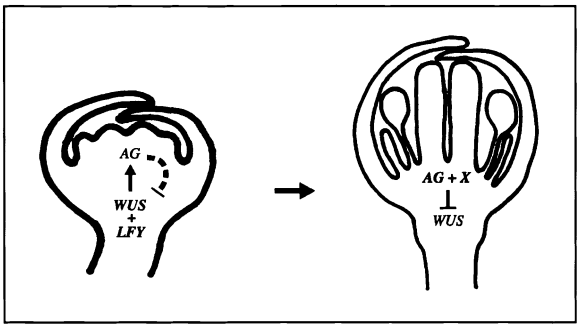

Figure 7. A Model for Termination of Stem Cell Maintenance in Floral Meristems

In early stages of flower development, *WUS* in combination with *LFY* induces *AG* expression in the center of the floral meristem (left). After stage 6, *AG* together with other factors (X) represses *WUS* and thus terminates stem cell maintenance and allows gynoecium differentiation (right).

Mechanism and Temporal Control of Floral Meristem Termination

Genetic analysis has demonstrated that *AG* is required for termination of floral meristem activity. As our results indicate, the critical step for this is that *AG* represses *WUS*: *WUS* expression is not downregulated in *ag* mutant flowers, its function is required for their indeterminate growth, and prolonged *WUS* expression in wild-type flowers is sufficient to render them indeterminate (this study and Laux et al., 1996). Although some level of posttranscriptional regulation may also play a role, downregulation of *WUS* seems to occur mainly at the level of transcription because *AG* was no longer able to terminate the floral meristem when *WUS* was expressed from a heterologous promotor.

Does AG repress *WUS* by binding directly to its promotor? This seems unlikely because wild-type *AG* function in the *WUS*-expressing central cells was not sufficient for meristem termination when the cell layer above, i.e., the second cell layer of the meristem, was mutant for *ag* (Sieburth et al., 1998). This observation suggests that either a non-cell-autonomous step may be required for *AG* to repress *WUS* or that additional target genes may have to be repressed by *AG* in the second cell layer to terminate the floral meristem.

Is *AG* alone sufficient to repress *WUS*? Overexpression analysis of *AG* suggests that this is not the case (Mizukami and Ma, 1997): *35S::AG* plants show a transformation of the inflorescence meristem into a determinate flower, but no direct termination of the SAM similar to that which results from ectopic expression of another repressor of *WUS*, *CLV3* (Brand et al., 2000; M.L. and T.L., unpublished data). Thus, it appears that *AG* requires additional factors to repress *WUS*. Candidates are the genes of the *CLV* pathway, whose loss of function also compromises floral determinacy and leads to prolonged *WUS* expression in flowers (Schoof et al., 2000).

The time of floral meristem termination must be precisely controlled to ensure complete flower development. This could be executed at the level of *WUS* regulation: premature repression of *WUS* causes precocious termination of meristem activity before carpels are formed (M.L. and T.L., unpublished data), whereas pro-

longed expression results in prolonged meristem activity and disturbs gynoecium differentiation. In line with this argument, the onset of *AG* expression in stage 3 correlates with the beginning decrease in *WUS* expression levels. However, *WUS* is not immediately repressed, but rather the expression of *WUS* and *AG* overlaps until stage 6, i.e., for more than a day (Bowman, 1994). Thus, the time of floral meristem termination may depend not only on when *AG* expression starts, but also on when it attains a sufficient level to repress *WUS*.

A Model for an Autoregulatory Mechanism in Floral Meristem Termination

Our data suggest that stem cell maintenance in floral meristems is terminated by a temporal autoregulatory mechanism involving *WUS* and *AG* (Figure 7): in young flower primordia, *WUS* specifies the most apical cells as stem cells—as it does in the SAM—and thus allows the formation of the full complement of organs. In addition, *WUS* contributes to the expression of *AG* in the center of the floral meristem. In combination with other factors, *AG* in turn then represses *WUS*. This eventually results in termination of stem cell maintenance. Since the activation of *AG* expression by *WUS* requires *LFY*, this negative autoregulation is restricted to determinate floral meristems.

Conceptually, this model for the regulation of floral meristem determinacy bears resemblance to the negative feedback loop between *WUS* and *CLV3* which regulates size homeostasis of the stem cell population in shoot and floral meristems (Schoof et al., 2000). Thus, it is conceivable that similar autoregulatory mechanisms govern spatial and temporal aspects of stem cell regulation.

Experimental Procedures

Mutant Lines and Growth Conditions

The wild-type reference used in all experiments was the Landsberg *erecta* (L*er*) ecotype. The *ag-1* mutant has been described previously (Bowman et al., 1989), as well as the *lfy-6* mutant (Weigel et al., 1992). Plant growth conditions were as described (Laux et al., 1996).

Histology, Scanning Electron Microscopy (SEM), and GUS Staining

Preparation of histological sections from LR-White embedded material and SEM were done as described (Laux et al., 1996; Schoof et al., 2000). GUS staining was performed as described (Schoof et al., 2000), and FAA-fixed and dehydrated material was embedded in Paraplast Plus (Oxford Labware, St. Louis, MO) and sectioned using a rotary microtome. Seven micrometer sections were placed on SuperFrost Plus slides (Menzel Gläser, Braunschweig, Germany), dewaxed by immersion in Histoclear, and mounted with Eukitt (Plano, Wetzlar, Germany).

Images were acquired using a Zeiss Axiophot microscope (Zeiss, Jena, Germany) and a Nikon Coolpix 990 digital camera (Nikon, Düsseldorf, Germany). Contrast and color balance were adjusted using Adobe Photoshop 4.0 (Adobe, Mountain View, CA).

PCR-Based Genotyping

Plants were genotyped for the *ag-1* allele using primers AG1FOR (5′-GGA CAA TTC TAA CAC CGG ATC-3′) and AG1BACK (5′-CTA TCG TCT CAC CCA TCA AAA GC-3′) at an annealing temperature of 55°C. Digestion of the PCR products with HindIII produces two bands of 297 bp and 20 bp from the product of the *ag-1* allele, while the product from the wild-type allele of 317 bp is not digested.

Construction of Transgenes and Plant Transformation

Generation of the *pOp::WUS* (MT69) and *pOp::WUS-pOp::GUS* (MT72) constructs and transgenic lines was described before (Schoof et al., 2000). We found that fortuitously both *pOp::WUS-pOp::GUS* constructs used in this study were closely linked to the *AG* locus (data not shown), precluding an analysis of *AG::WUS* expression in an *ag-1* homozygous mutant background.

For the *AG::LhG4* construct, the *AG* second intron was isolated from pMD992 (provided by L. Sieburth; Deyholos and Sieburth, 2000) by partial digestion with HindIII and NcoI, blunted by treatment with S1-nuclease, and subcloned into pBin-LhG4 (provided by I. Moore), which had been digested with BamHI and SalI and blunt-ended with T4-DNA-polymerase. The resulting *AG::LhG4* fragment was excised from pBin:*AG::LhG4* by digesting with AscI and PacI, blunt-ended with T4-DNA-polymerase, and subcloned into pBarA, a derivative of pGPTV-BAR (Becker et al., 1992), treated with HindIII and T4-DNA-polymerase, to yield plasmid MT84.

MT84 was introduced into *Agrobacterium* strain GV3101 (pMP90) (Koncz and Schell, 1986) by electroporation. *Arabidopsis* wild-type plants were transformed by floral dip (Clough and Bent, 1998).

We initially tested several combinations of independent *AG::LhG4* and *pOp::WUS-pOp::GUS* transgenic lines, which all produced qualitatively the same phenotype. All subsequent analyses were performed using a combination of lines giving a strong phenotype.

AP3::LhG4 transgenic plants were kindly provided by Y. Eshed and J.L. Bowman, and the *pAG-I::GUS* line was obtained from L. Sieburth (Sieburth and Meyerowitz, 1997).

In Situ Hybridization

In situ hybridization for *WUS* was performed as described in Mayer et al. (1998). The *CLV3* probe has been described in Schoof et al. (2000).

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