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A Molecular Link between Stem Cell Regulation and Floral Patterning in *Arabidopsis*

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Summary

The homeotic gene *AGAMOUS (AG)* has dual roles in specifying organ fate and limiting stem cell proliferation in *Arabidopsis* flowers. We show that the floral identity protein LEAFY (LFY), a transcription factor expressed throughout the flower, cooperates with the homeodomain protein WUSCHEL (WUS) to activate *AG* in the center of flowers. WUS was previously identified because of its role in maintaining stem cell populations in both shoot and floral meristems. The unsuspected additional role of WUS in regulating floral homeotic gene expression supports the hypothesis that floral patterning uses a general meristem patterning system that was present before flowers evolved. We also show that *AG* represses *WUS* at later stages of floral development, thus creating a negative feedback loop that is required for the determinate growth of floral meristems.

Introduction

Flowers contain four major types of organs, sepals, petals, stamens, and carpels, which are arranged in four concentric rings or whorls (Figure 1A). The combinatorial specification of floral organ identity by three classes of homeotic genes, termed A, B, and C, has been summarized in the ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991). Each class of homeotic genes is active in two adjacent whorls—class A in whorls one and two, class B in whorls two and three, and class C in whorls three and four.

In addition to specifying floral organ identity, several homeotic genes regulate other aspects of floral development. One well-studied example is the C function gene *AGAMOUS (AG)*, which not only specifies stamen and carpel identity, but also limits proliferation of floral stem cells (Bowman et al., 1989, 1991; Mizukami and Ma, 1995; Sieburth et al., 1995). Like shoots, flowers are derived from collections of undifferentiated cells called meristems. However, while a central pool of stem cells continuously replenishes the *Arabidopsis* shoot meristem, this pool is only transiently maintained in floral meristems, which therefore stop producing new organs after the carpels in whorl four have formed (Figure 1A). In *ag* mutants, organ formation does not terminate with the formation of fourth-whorl organs, but continues indeterminately. Thus, *ag* flowers have an indeterminate number of whorls containing only sepals and petals (Figure 1A).

Although *AG* has been cloned for over a decade (Yanofsky et al., 1990), there are still many gaps in our knowledge of *AG* regulation and function. Several factors that act together with *AG* in specifying organ identity and floral determinacy have been identified, as have several negative regulators of *AG* (for review, Irish, 1999; Ng and Yanofsky, 2001). However, it remains unknown how *AG*, whose RNA accumulates in whorls three and four (Drews et al., 1991), is activated specifically in the center of flowers, or which downstream targets mediate its role in organ identity and meristem determinacy. An important upstream regulator of *AG* is the DNA binding transcription factor LFY, which is expressed throughout the flower, and which directly activates both *AG* (Busch et al., 1999) and the A function gene *APETALA1 (AP1)* (Parcy et al., 1998; Wagner et al., 1999). Consistent with initial activation of *AP1* throughout the emerging flower, *AP1* activation does not appear to require flower-specific LFY coregulators, and high levels of LFY are sufficient to activate *AP1* in vegetative primordia (Parcy et al., 1998). In contrast, activation of *AG* by LFY requires at least one additional factor that appears to be present only in the center of floral meristems (Parcy et al., 1998; Busch et al., 1999).

We have identified the first direct region-specific regulator of *AG*, the homeodomain protein WUS, which was initially recognized because its loss of function causes meristem defects. The main shoot of *wus* mutants terminates after producing only a few leaves, although mutant plants can partially recover by producing adventitious shoots that repeat the pattern of the primary shoot (Laux et al., 1996). Occasionally, flowers are formed which do not produce the full complement of floral organs, but lack carpels and most stamens (Figure 1A). Consistent with a direct role of *WUS* in maintaining a central stem cell population in both shoot and floral meristems, *WUS* RNA is expressed in the center of both types of meristems (Mayer et al., 1998). The size of the *WUS*-dependent stem cell population is regulated through a negative feedback loop in which *WUS* induces expression of the *CLAVATA3 (CLV3)* gene, which in turn limits the *WUS* expression domain (Brand et al., 2000; Schoof et al., 2000).

Because *wus* mutant flowers do not show any homeotic organ transformations (Laux et al., 1996), a role of

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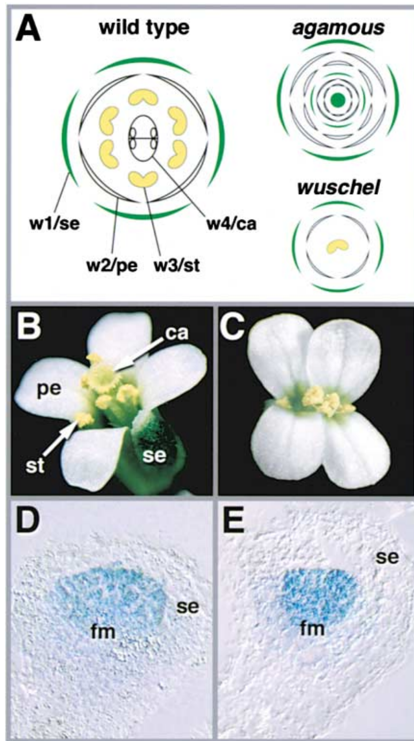

Figure 1. *WUS* Is Required for Activation of *AG* Enhancer Sequences

(A) Diagrams of wild-type and mutant flowers. Whorls (w1-4) and floral organ types, sepals (se), petals (pe), stamens (st), and carpels (ca), are indicated.

(B) Mature wild-type flower.

(C) Flower of a *UFO::CLV3* transgenic line lacking carpels but still producing several stamens, mimicking a weak *wus* mutant phenotype.

(D) Longitudinal section of a stage 5 wild-type flower, showing expression of KB31 *AG::GUS*.

(E) Reduced KB31 *AG::GUS* domain in a *UFO::CLV3* flower of similar stage as (D).

(fm) floral meristem. Floral stages are according to Smyth and colleagues (1990).

WUS in regulating homeotic gene expression has not been obvious. The previously unsuspected function of *WUS* in activating the floral homeotic gene *AG* now establishes a direct link between meristem function and floral patterning. We also show that, after *AG* expression is established in the flower, *AG* in turn represses *WUS*, thereby creating a negative feedback loop that regulates the balance between stem cell proliferation and differentiation in floral meristems.

Results

WUS Is Required for *AG* Activation

To identify region-specific activators of *AG*, we examined candidate transcription factors expressed in the center of floral meristems. One candidate was the homeodomain protein WUS (Mayer et al., 1998). Strong *wus* mutants occasionally produce flowers, which contain a near normal number of sepals and petals, but lack all carpels and most stamens, the organs specified by *AG* (Laux et al., 1996). Because it was not known whether the loss of stamens and carpels was accompanied by a reduction in early *AG* expression, we wanted to determine the effect of *wus* mutations on *AG*. Unfortunately, most *wus-1* mutants never produced flowers under our growth conditions. Therefore, we used instead a transgenic line that expresses *CLV3*, a negative regulator of *WUS*, ectopically under the meristem-specific promoter of the *UFO* gene. As a result, *WUS* expression is reduced, but not abolished (Brand et al., 2000). As with *wus* mutants, flowers of this line lack carpels, but have more stamens than strong *wus* mutants (Figure 1C), indicating a weaker phenotype. To monitor *AG* activation, we crossed *UFO::CLV3* to an *AG::GUS* reporter line whose expression resembles that of endogenous *AG* (Busch et al., 1999). The *AG::GUS* domain was noticeably smaller in many *UFO::CLV3* flowers (Figure 1E), compatible with the notion that *WUS* is an activator of *AG*. That most flowers of strong *wus* mutants have at least one stamen (Laux et al., 1996) suggests that *WUS* is a partially redundant *AG* activator.

WUS Can Cause Ectopic Formation of Stamens and Carpels

As a more rigorous test for the ability of *WUS* to activate *AG*, we misexpressed *WUS* using *LFY* and *APETALA3 (AP3)* promoters. The *LFY* promoter is active throughout floral anlagen and young flowers up to stage 2 (Blázquez et al., 1997), at the end of which *AG* RNA expression is activated in the center of wild-type flowers (Drews et al., 1991). The *LFY* promoter is also weakly active in young leaf primordia. We chose the *LFY* promoter to reveal effects of *WUS* at early stages of flower development, and possibly in leaves. As a complement to *LFY::WUS*, we expressed *WUS* from the *AP3* promoter, which is active from stage 3 on in presumptive whorls two and three, and to some extent in whorl one, until late stages of floral development (Jack et al., 1994). We chose the *AP3* promoter to determine the effects of expressing *WUS* in a more restricted manner than from the *LFY* promoter.

Both *LFY::WUS* and *AP3::WUS* transgenes had dramatic effects on floral morphology (Figure 2). A common feature of all transgenic plants was an increase in floral organ number, and this was the main phenotype in weak lines of both *LFY::WUS* and *AP3::WUS*. Additional phenotypes, including homeotic organ transformations reminiscent of plants with ectopic *AG* expression (Drews et al., 1991; Mizukami and Ma, 1992; Parcy et al., 1998), were apparent in intermediate and strong lines. In wild-type, whorls two and three are occupied by petals and stamens, respectively (Figure 2A). In intermediate *AP3::WUS* lines, these whorls were occupied by supernumerary stamens and carpelloid stamens (Figure 2B). In strong *AP3::WUS* lines, organ number in whorls two and three was further increased, and all organs in these whorls consisted of carpelloid stamens (Figure 2C). First-whorl sepals and the carpels of the central gynoecium were largely normal. The whorl-specific effects suggested that the organ transformations were a direct consequence of *WUS* action, rather than an indirect consequence of an enlarged central zone of the floral meristem.

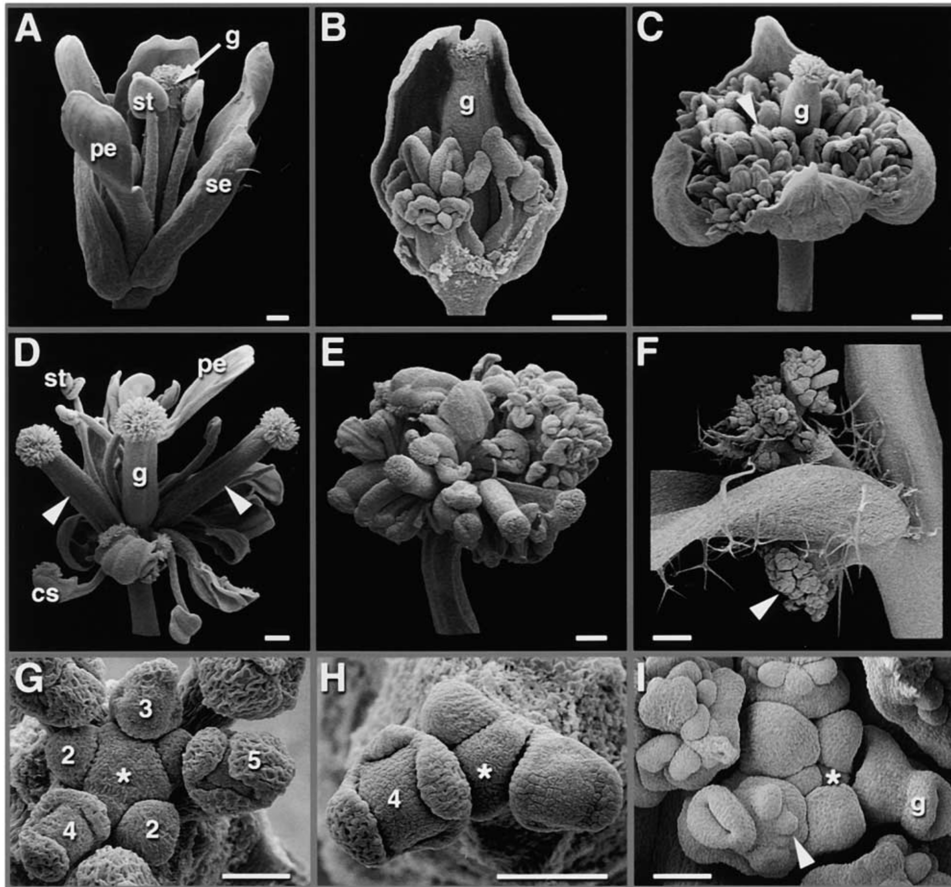

Figure 2. Phenotypes of Transgenic *AP3::WUS* and *LFY::WUS* Plants

(A) Scanning electron micrograph of mature wild-type flower, with sepals (se), petals (pe), stamens (st), and a central gynoecium (g) consisting of two congenitally fused carpels.

(B) Flower of an intermediate *AP3::WUS* line with two sepals removed. Petals are lost and supernumerary organs in the second and third whorl develop as stamens or carpelloid stamens.

(C) Flower of a strong *AP3::WUS* transgenic line. Inside of the largely normal first-whorl sepals, numerous carpelloid stamens develop (arrowhead indicates stigmatic papillae). The central gynoecium is also largely normal.

(D) Flower of an intermediate *LFY::WUS* line with a moderate increase in organ number. First-whorl sepals are missing and there are fewer petals. In addition to normal stamens, supernumerary carpelloid stamens (cs) develop. Extra gynoecia (arrowheads) surround the normal central gynoecium.

(E) Flower of a strong *LFY::WUS* line. There are many supernumerary organs, most of which are staminoid carpels or carpels.

(F) Ectopic inflorescence shoot or flower (arrowhead) induced close to the stem on the abaxial side of a cauline leaf in a strong *LFY::WUS* line. All organs are staminoid carpels, or carpels.

(G) Wild-type shoot apex with shoot apical meristem (asterisk) surrounded by young flowers. Asterisk indicates shoot apical meristem, and numbers indicate floral stages (Smyth et al., 1990).

(H) Shoot apex of strong *AP3::WUS* line. Early stages of flower development are normal.

(I) Shoot apex of strong *LFY::WUS* line. Floral meristems quickly grow larger than those of wild-type and obscure the shoot apical meristem. An aberrant central gynoecium (g) is the first primordium to arise, soon followed by other abnormal primordia (arrowhead) surrounding it.

Scale bars indicate 250 μm in (A)–(E) and 50 μm in (G)–(I).

Flowers of intermediate *LFY::WUS* lines had fewer or no sepals compared to wild-type, and fewer petals, which surrounded several whorls of stamens, staminoid carpels, and carpels. The latter often formed several normal gynoecia (Figure 2D). In strong *LFY::WUS* lines, floral development was further disrupted and new flowers consisting of stamens, staminoid carpels, and carpels formed within the primary flowers (Figure 2E). In the most extreme cases, the shoot meristem was also affected and became fasciated (not shown), possibly due to weak activity of the *LFY* promoter in floral anlagen, which had not yet separated from the main shoot meristem. Consistent with *LFY* promoter activity in young leaves, these plants also produced ectopic floral meristems on the abaxial side of cauline leaves (Figure 2F).

To determine the ontogeny of the *AP3::WUS* and *LFY::WUS* phenotypes, we examined developing flowers of intermediate and strong transgenic lines. Flowers of strong *AP3::WUS* lines were normal through stage 4 (Figures 2G and 2H). In contrast, *LFY::WUS* floral meristems grew much larger than those of wild-type did before the first organs were initiated. Furthermore, instead of first-whorl sepals being the first primordia to

form, the first structure to arise from these floral meristems was always a central gynoecium, which was surrounded by newly forming primordia and meristems (Figure 2I). This gradient of organ formation continued until later stages of development, and was also observed in strong *AP3::WUS* flowers (not shown). Thus, in more advanced flowers of strong *LFY::WUS* and *AP3::WUS* lines, the most mature organs were found in the center, while the periphery was occupied by less mature organs (Figure 2C).

WUS Acts through *AG* Regulatory Sequences Located in the Second Intron

Ectopic formation of stamens and carpels, which are the organs specified by *AG*, indicated aberrant *AG* activation in *AP3::WUS* and *LFY::WUS* flowers. To map *WUS*-responsive sequences, we examined the expression of several *AG::GUS* reporters in these transgenic lines. Sequences necessary and sufficient for normal *AG* expression in the center of flowers are located in a 3 kb HindIII restriction fragment that largely coincides with the second intron of *AG* (Sieburth and Meyerowitz, 1997; Busch et al., 1999; Deyholos and Sieburth, 2000). A reporter in which this fragment is placed upstream of a heterologous minimal promoter linked to the *GUS* coding sequence reproduces the endogenous *AG* expression pattern. This fragment, KB9, can be further divided into two nonoverlapping fragments, KB14 and KB31, which are both active in the center of young flowers (Busch et al., 1999; Deyholos and Sieburth, 2000). We found that expression of both KB9 and KB31 was affected in *AP3::WUS* and *LFY::WUS* (Figure 3). In *AP3::WUS* flowers, the onset of *AG::GUS* expression was not changed, but its levels were increased, especially outside the central gynoecium (Figures 3E and 3G). Strong *AG::GUS* expression continued until after stage 10, when *AG::GUS* expression subsides in wild-type. Persisting *AG::GUS* expression was most apparent in the zone of new organ initiation inside the first whorl (Figure 3H). These effects were dependent on transgene expressivity, and correlated with the severity of the mature phenotypes observed in different lines.

In addition to ectopic and increased *AG::GUS* expression, the *LFY::WUS* transgene caused precocious activation of both KB9 and KB31, even in weak lines (Figure 3L), consistent with the *LFY* promoter being active earlier than the *AP3* promoter. In strong *LFY::WUS* lines, the first primordia to separate from the shoot apical meristem already stained strongly for GUS (Figures 3I and 3K), while in a wild-type background, there were always several unstained floral primordia (Figures 3A and 3C). Importantly, ectopic activation was observed before ectopic meristems were well developed, indicating that ectopic activation of *AG::GUS* was not merely an indirect consequence of new floral meristems having formed. At later stages of development, *AG::GUS* continued to be expressed throughout the developing flowers, suggesting that we had created a positive feedback loop in which ectopic *WUS* caused the continuous formation of new floral meristems, thus maintaining *LFY* promoter activity. *AG::GUS* was also strongly and uniformly activated in the ectopic flowers that formed at the abaxial side of cauline leaves in extreme *LFY::WUS* lines (not shown).

In contrast to KB9 and KB31, there was no obvious effect of *AP3::WUS* on early KB14 *AG::GUS* activity (Figure 3F). KB14 was activated earlier in strong *LFY::WUS* lines, but the pattern of *AG::GUS* was different from wild-type, being confined to the periphery of floral meristems (Figure 3J). Although we had initially reported that KB14 and KB31 behave very similarly in young flowers (Busch et al., 1999), a more careful analysis by Deyholos and Sieburth (2000) showed that KB14 is preferentially active in stamens produced from the third whorl. In addition, the *lfy-12* mutation has more severe effects on KB14 than KB31 (Busch et al., 1999), possibly reflecting the fact that *lfy-12* mutants still make carpels, but no stamens (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992). Strong *LFY::WUS* lines produce mostly carpels and carpelloid organs, consistent with the divergent pattern of KB14 expression. In summary, these experiments indicated that *WUS* acts through KB31 sequences to activate *AG*, with KB14 sequences possibly affected more indirectly.

WUS Is a Direct Activator of *AG*

KB31 includes at least two sites to which LFY, the only known direct regulator of *AG*, binds in vitro. These binding sites are required for activity of the KB31 reporter in plants, as demonstrated with reporters containing mutant LFY binding sites (Busch et al., 1999). To study transcriptional activation by LFY, we have previously developed a heterologous transactivation assay using the yeast *Saccharomyces cerevisiae*. With this assay, we have shown that a fusion of LFY to the heterologous VP16 transcriptional activation domain is sufficient to activate a reporter linked to a LFY binding site from the promoter of the *AP1* gene (Parcy et al., 1998).

We used the yeast system to study the interaction of LFY and WUS with *AG* regulatory sequences. To confirm that LFY:VP16 could interact in yeast with *AG* sequences, as previously shown for *AP1* sequences, we placed a 287 bp fragment from KB31 upstream of a minimal promoter driving a *lacZ* reporter (FP50, Figure 4A). As with the reporter containing the *AP1* site (Parcy et al., 1998), we found that FP50 was activated in yeast by LFY:VP16, but not by unmodified LFY (Figures 4B and 4C). To test whether WUS can activate transcription from *AG* regulatory sequences in yeast, we expressed either WUS alone or in combination with LFY in yeast carrying FP50. Like LFY, WUS on its own was not sufficient to activate FP50. In contrast, coexpression of LFY and WUS resulted in robust reporter gene activity (Figure 4B).

To further delineate the sequences through which WUS acts, we tested other reporters including RH18, which contains a trimer of a 91 bp fragment that includes the two LFY binding sites (Figure 4A). LFY and WUS together strongly activated RH18 (Figure 4B). Inspection of the 91 bp fragment revealed two consensus binding sites for homeodomain proteins (Gehring et al., 1994) close to each LFY binding site. To test the importance of these putative WUS binding sites, we mutated them in the context of RH18. For mutations in LFY binding sites, there is a good correlation between effects on binding by LFY in vitro and activation by LFY:VP16 in yeast (Parcy et al., 1998; Busch et al., 1999; M.A.B.

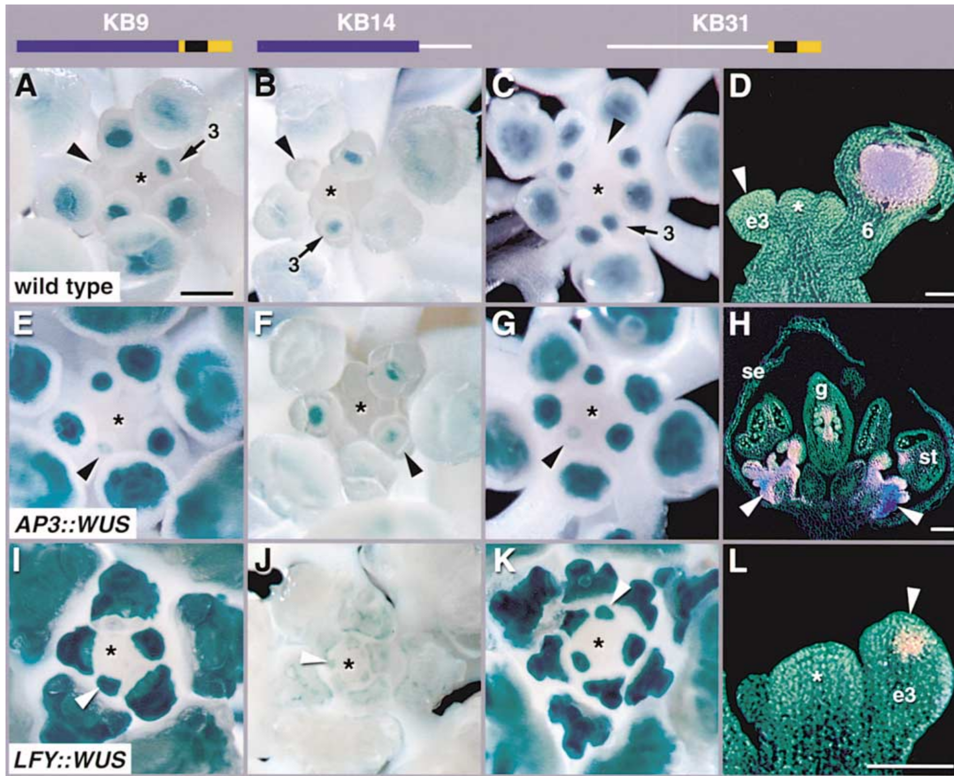

Figure 3. Activation of *AG::GUS* Reporters in *AP3::WUS* and *LFY::WUS* Plants

Diagrams of the *AG* enhancer sequences in the three reporters, KB9, KB14, and KB31, are shown on top. KB9 contains the entire regulatory region, while KB14 and KB31 contain complementary fragments of KB9 (Busch et al., 1999). The FP50 fragment used for yeast assays (Figure 4A) is indicated in black.

Asterisks indicate shoot apical meristems, numbers floral stages. Whole-mount preparations are shown except for (D), (H), and (L), which are longitudinal sections photographed under darkfield illumination. In darkfield, unstained tissue appears green, lightly stained tissue orange, and strongly stained tissue blue or purple.

(A–D) In wild-type plants, the first flower with obvious GUS activity is a mid-stage 3 flower, in which sepal primordia are clearly visible. An arrowhead in each panel indicates the next youngest floral primordium, lacking strong GUS staining.

(D) An early-stage 3 flower (e3), on which sepals are just starting to emerge, does not yet stain for GUS (arrowhead). Strong GUS expression is seen in a stage 6 flower. To maximize sensitivity of GUS detection, a reduced amount of ferro- and ferricyanide (2 mM) was used for this apex.

(E–G) Onset of *AG::GUS* expression in strong *AP3::WUS* lines is similar to that in wild-type; arrowheads indicate the first flowers with obvious GUS staining. KB9 and KB31 staining is stronger than in wild-type, especially at later stages of floral development.

(H) A glancing section through a stage 12 flower shows formation of new primordia, which have strong GUS activity (arrowheads), inside the first-whorl sepals.

(I–K) Onset of *AG::GUS* expression in strong *LFY::WUS* lines is earlier than in wild-type; the first floral primordia with obvious GUS staining are indicated by arrowheads. Note that KB14 staining is restricted to the periphery of floral primordia. For KB9 and KB31, staining is much stronger than in wild-type, and expanded throughout the flower.

(L) Even in a weak *LFY::WUS* line, which has very few floral defects, onset of *AG::GUS* expression is earlier than in wild-type. Compare the early-stage 3 flower (e3) to the one in (D).

Scale bar is 200 μm for all panels, except 50 μm for (D), (H), and (L).

and D.W., unpublished data). The JL51 reporter with mutations in the putative WUS binding sites was still activated by LFY:VP16 (Figure 4C), indicating that these mutations did not disrupt in vivo interaction of LFY with its binding sites, which are adjacent to the putative WUS binding sites. In contrast, the synergistic activation by WUS and unmodified LFY was abolished (Figure 4B). This observation indicates that the homeodomain consensus binding sites are required for interaction of WUS with the *AG::lacZ* reporter in vivo. Similarly, the LFY binding sites were required for synergistic activation of the *AG::lacZ* reporter by LFY and WUS, as shown by mutating them in the context of FP50 (Figure 4A). Mutating one of the two LFY sites (MX71 or MX72) had only modest effects, but mutating both sites (MX73) greatly reduced reporter activation by LFY plus WUS, as well as activation by LFY:VP16 (Figures 4B and 4C).

WUS and LFY Bind DNA Independently

That both the LFY and the homeodomain binding sites were required for transcriptional activation in yeast suggested that LFY and WUS bind DNA independently. To test this directly, we prepared extracts from yeast strains expressing either LFY or WUS or both proteins, and used these in electrophoretic mobility shift assays (EMSAs). The probe used included one of the previously

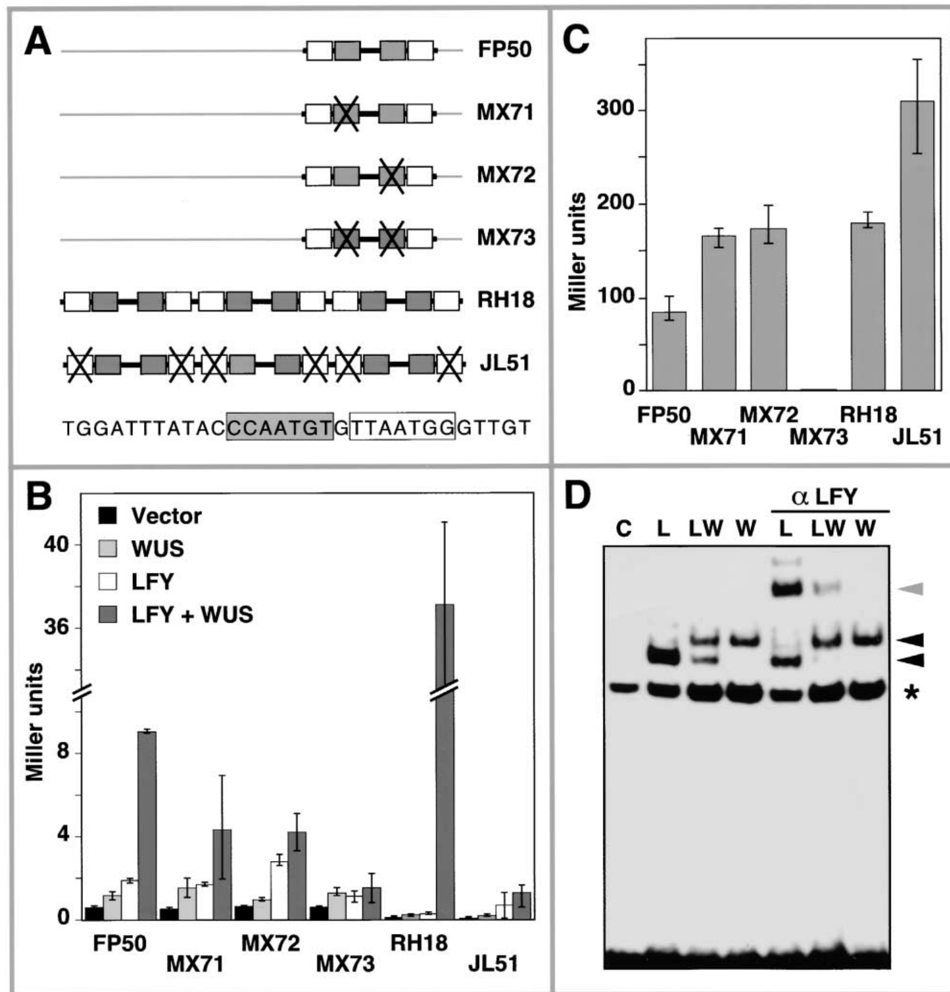

Figure 4. Interaction of LFY and WUS with *AG* Regulatory Sequences in Yeast and In Vitro

(A) Diagram of yeast reporter constructs and sequence of probe used in (D). Gray boxes indicate LFY binding sites, white boxes WUS binding sites, and crosses mutated binding sites. LFY binding sites were changed from CCAATG(G/T) to AAAATG(G/T), WUS binding sites from TTAAT(G/C)(G/C) to TTCCT(G/C)(G/C). The position of the FP50 fragment is indicated as a black box in Figure 3.

(B) β-galactosidase activity of yeast transformed with different combinations of reporter and effector vectors. Average is from three independent transformants, bars indicate range.

(C) Reporter activity in yeast expressing LFY:VP16.

(D) EMSA with extract from yeast containing empty expression vector (C), or expressing LFY (L), or WUS (W), or both (LW). Asterisk indicates complex with an endogenous yeast protein seen also in the control. Black arrowheads indicate specific protein-DNA complexes observed with extracts containing LFY and WUS. EMSAs that include anti-LFY antiserum (α-LFY) are shown in the three right-hand lanes. LFY-containing protein-DNA complexes are supershifted (gray arrowhead).

identified LFY binding sites in the *AG* enhancer (*AG* II; Busch et al., 1999) and the adjacent putative WUS binding site (Figure 4A). We found that WUS, like LFY, could bind on its own to this probe (Figure 4D). This result was confirmed using WUS and LFY produced by coupled in vitro transcription/translation (not shown).

When we used WUS and LFY together in the EMSA, the result was additive. We detected two DNA-protein complexes, which appeared to be identical to the individual LFY and WUS complexes (Figure 4D). This result was obtained both with extract from yeast expressing LFY and WUS simultaneously and with mixed extracts from strains expressing each protein individually. That only one of the two complexes seen in the reaction with LFY and WUS contained LFY protein was confirmed with supershifts using anti-LFY antibodies (Figure 4D). The absence of a new abundant complex containing both LFY and WUS suggested that the two proteins do not bind DNA cooperatively. To further investigate this, we performed coimmunoprecipitation experiments in the presence and absence of the DNA fragment used for the EMSAs. In neither case did we observe a strong interaction between the two proteins (not shown). It is possible that the proximity of the individual LFY and WUS binding sites is fortuitous, rather than reflecting a need for the two proteins to interact directly. It is known that synergistic transcriptional activation can not only be achieved through cooperative DNA binding, but also through independent contacts of two proteins with the basic transcription machinery (Carey et al., 1990; Lin et al., 1990; Oliviero and Struhl, 1991; Sauer et al., 1995).

As a final test for the independent action of the two

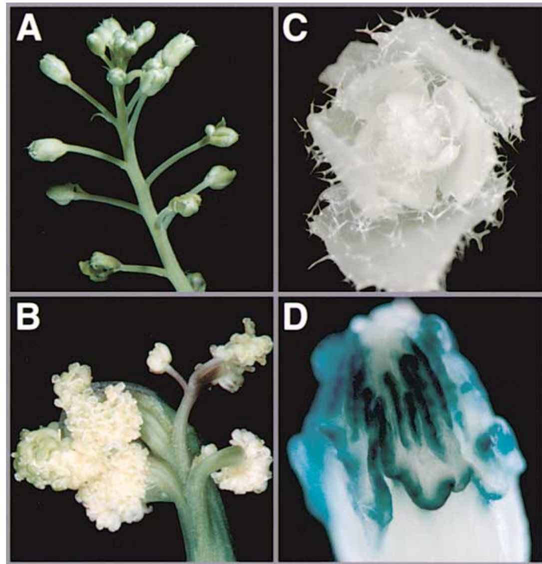

Figure 5. *LFY*-Independent Effects in *LFY::WUS* Plants

(A) *lfy-12* inflorescence with flowers containing leaves, sepals, carpels, and intermediate organs.

(B) Inflorescence of a *lfy-12* mutant carrying an intermediate *LFY::WUS* transgene, with dramatic proliferation of floral organs, all of which are carpelloid. The stem is fasciated, as are older flowers.

(C) KB31 *AG::GUS* staining is absent in this young *lfy-12* inflorescence.

(D) KB31 *AG::GUS* reporter is strongly activated in the meristems arising on the flanks of the grossly overproliferating shoot apical meristem of a young *lfy-12 LFY::WUS* inflorescence.

proteins, we overexpressed WUS in *lfy-12* null mutants. Whereas *lfy-12* flowers contain leaves, sepals, carpels, and intermediate organs (Huala and Sussex, 1992), *LFY::WUS lfy-12* flowers comprised only carpelloid organs (Figures 5A and 5B). That the *LFY::WUS* construct was effective in modifying the *lfy-12* floral phenotype suggested that overexpression of WUS could reduce the requirement for LFY in *AG* activation. Indeed, the KB31 reporter was only weakly active in a nontransgenic *lfy-12* background (Figure 5C), but strongly activated in *LFY::WUS lfy-12* (Figure 5D). That overexpression of WUS alone is sufficient to activate *AG* in plants but not in yeast likely reflects both the action of additional regulators that are only present in flowers and the fact that the enhancer tested in yeast is smaller than the one tested in plants. Furthermore, that WUS overexpression can reduce the requirement for LFY in *AG* activation indicates partially redundant action of these two transcription factors. Partially redundant action of WUS and LFY can also be deduced from the observation that *wus lfy* double mutants appear to have a more than additive phenotype (Laux et al., 1996).

WUS Binding Sites Are Required for *AG* Activation in Plants

Having shown that WUS can interact with the *AG* enhancer in vitro and in yeast, we went on to test the importance of the WUS binding sites in plants. We mutated the two WUS binding sites in the KB31 *AG::GUS* reporter, and scored the level of GUS activity in young flowers as described previously (Busch et al., 1999). Of 48 transgenic lines carrying the mutated reporter, JL49, none had strong GUS activity in flowers, only 1 (2%) had intermediate activity, 2 (4%) had weak activity, and the vast majority, 45 (94%), had no GUS activity. This contrasts with our previous results for 35 lines of the parental KB31 reporter, of which 10 (29%) had strong GUS activity, 6 (17%) had intermediate, 12 (37%) had weak, and only 7 (20%) had no GUS activity (Busch et al., 1999) (Figure 6). Thus, the WUS binding sites identified in vitro are important for activity of the *AG* enhancer in vivo.

AG Is a Negative Regulator of *WUS*

Having identified WUS as an activator of *AG*, we were intrigued by the fact that the floral meristem continues to proliferate in *ag* mutants, which is a phenotype opposite to that of *wus* mutants, in which the floral meristem terminates prematurely (Figure 1A) (Bowman et al., 1989, 1991; Laux et al., 1996). This phenotype is dependent on *WUS* since a *wus* mutation is epistatic to *ag* with respect to floral meristem proliferation (Laux et al., 1996). Furthermore, mild overexpression of *WUS* in the center of flowers causes partial indeterminacy of floral meristems similar to that seen in plants with partially compromised *AG* function (Mizukami and Ma, 1995; Sieburth et al., 1995; Schoof et al., 2000). To investigate possible feedback regulation of *WUS* by *AG*, we examined *WUS* expression in *ag* mutants. In wild-type, *WUS* is expressed initially in floral meristems in a pattern similar to that of shoot meristems, but it is not maintained past stage 6, when the floral meristem is consumed in the formation of the central gynoecium (Figure 7A) (Mayer et al., 1998; Schoof et al., 2000). In contrast, we found that *WUS* persisted in the indeterminate meristem of *ag* flowers, which continue to produce new organs in an indeterminate fashion (Figure 7B).

Apart from *AG*, *WUS* is also negatively regulated by the *CLV* pathway (Brand et al., 2000; Schoof et al., 2000). However, the *AG* and *CLV* pathways are at least partially independent since the effects of *ag* and *clv1* mutations on floral meristem determinacy are additive (Clark et al., 1993). Consistent with this observation, *WUS* expression was increased more strongly in *ag-1 clv1-4* double mutants compared to *ag-1* single mutants (Figure 7C).

Discussion

At least two ABC homeotic genes, *AP1* and *AG*, are directly activated by the LFY transcription factor, which specifies floral identity (Parcy et al., 1998; Busch et al., 1999; Wagner et al., 1999). Based on their different expression patterns, we have proposed that the response of different target genes to LFY is modified by region-specific factors (Parcy et al., 1998). Here, we have investigated how the homeodomain protein WUS, which was initially identified because it regulates stem cell proliferation in both shoot and floral meristems (Mayer et al., 1998), contributes to activation of the LFY target gene *AG*.

Activation of *AG*

Four lines of evidence—the effects of manipulating *WUS* in plants, yeast transactivation assays, in vitro DNA binding studies, and analysis of WUS binding sites in plants—show that WUS is a direct activator of *AG*. Our

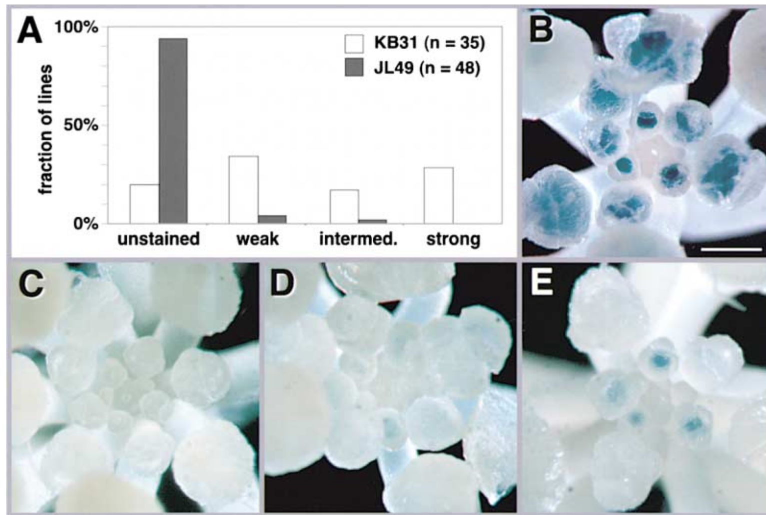

Figure 6. WUS Binding Sites Are Required for *AG* Enhancer Activity in Plants

(A) Distribution of GUS activity levels in lines carrying KB31 (wild-type WUS sites) or JL49 (mutant WUS sites). KB31 data are from Busch and colleagues (1999).
(B) KB31 apex with strong GUS activity.
(C–E) JL49 apices with no (C), weak (D), or intermediate GUS activity (E).
Scale bar in (B) is 200 μm for all panels.

observations indicate that region-specific activation of *AG* in the center of wild-type flowers relies on the integration of a flower-specific activity provided by floral identity proteins such as LFY with region-specific activities such as the one provided by the stem cell regulator WUS. That WUS is a potent activator of *AG* was demonstrated most prominently by the ability of WUS overexpression to reduce the requirement for LFY in *AG* activation. However, the role of WUS in activating *AG* is likely to be a partially redundant one because even strong *wus* mutants produce often at least one stamen, an organ type requiring *AG* expression. Notably, *WUS* is also partially redundant in the maintenance of shoot and floral meristems, as deduced from the observation that strong *wus* mutants can form shoots and that the number of missing floral organs in *wus* mutants is variable (Laux et al., 1996).

Interestingly, the effect of mutating the two WUS binding sites in the *AG* enhancer (Figure 6) was more dramatic than the effect of the *UFO::CLV3* transgene on *AG* enhancer activity (Figure 1E). Although this could simply be due to residual WUS activity in *UFO::CLV3* plants, the surprisingly strong effect may indicate that the WUS binding sites are not only used by WUS, but also by other activators of *AG*. Apart from the identity of additional positive regulators, an unresolved question is how activators such as LFY and WUS interact with the many other factors that have overlapping and partially redundant roles in *AG* repression (Jofuku et al., 1994; Goodrich et al., 1997; Byzova et al., 1999; Conner and Liu, 2000).

Evolution of Floral Pattern

The flower is an evolutionary novelty that characterizes the most successful group of vascular plants, the angiosperms. The origin of floral organs was contemplated more than 200 years ago by Goethe (1790), who proposed that floral organs are modified vegetative leaves. This hypothesis received important experimental support in the 1990s from the analysis of floral homeotic mutants (Bowman et al., 1991; Coen and Meyerowitz, 1991), and more recently from the dramatic finding that a small number of MADS domain transcription factors is sufficient to convert vegetative leaves into floral organs (Honma and Goto, 2001; Pelaz et al., 2001). The phylogenetic analysis of MADS box genes, which include most floral homeotic genes, has indicated that radiation and divergence of this gene family preceded floral evolution. The patterned expression of several MADS box genes is a largely conserved feature of flowers, and some of these patterns are already seen in the simpler reproductive structures of gymnosperms (for review, Irish, 1999; Theissen et al., 2000; Ng and Yanofsky, 2001).

In contrast to floral organ identity functions, the evolutionary origin of the prepattern that is interpreted by MADS box genes has been less clear. We have previously proposed that the pattern of ABC gene expression is achieved through co-option of a more general patterning system that was present in shoots before flowers evolved (Lee et al., 1997; Parcy et al., 1998). This hypothesis, that floral organ patterning is derived from shoot meristem patterning, extends Goethe's (1790) assertion of floral organs being modified leaves. In support of our hypothesis, we have now shown that WUS, a bona fide meristem-patterning factor, directly controls expression of the floral homeotic gene *AG*. Whether co-option of meristem-patterning factors is a general principle in activation of homeotic genes requires, however, further study.

In flowers, WUS was apparently co-opted as a region-specific transcription factor, and combined with a factor providing floral specificity, LFY, to produce a flower- and region-specific pattern of *AG* expression. A conceptually related mechanism for the generation of evolutionary novelty has been proposed for butterflies, where a system for proximal-distal patterning of appendages such as legs has been co-opted for the elaboration of wing eyespots (Carroll et al., 1994; Keys et al., 1999).

Regulation of Floral Stem Cells

Vegetatively growing shoots are distinguished from flowers both by their growth habit and the types of organs they produce. Shoots produce an indeterminate number of leaves, whereas flowers produce a determi-

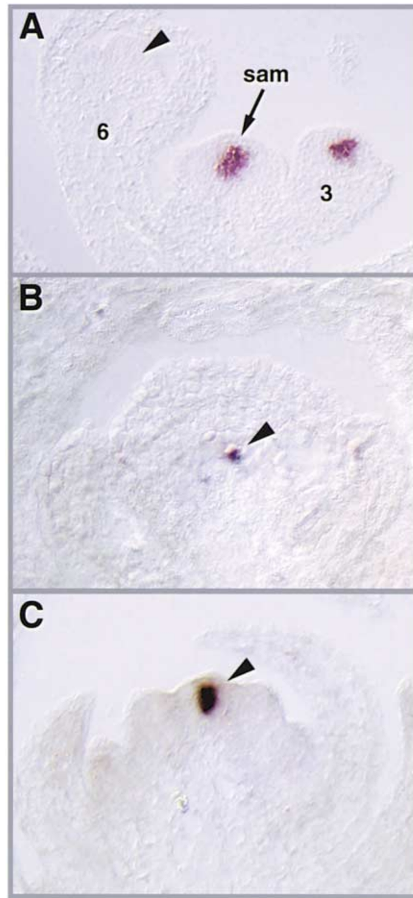

Figure 7. *AG* represses *WUS* Expression

(A) Expression of *WUS* RNA, as detected by in situ hybridization, in the shoot apical meristem (sam) of a wild-type inflorescence, and in a stage 3 flower. By floral stage 6, when the gynoecium has begun to form, *WUS* RNA is no longer detected (arrowhead).

(B) Close-up of the floral meristem in an *ag-1* mutant flower that had already formed at least five whorls of organs, including the two organs directly overlaying the floral meristem. This flower is roughly equivalent to a stage 10 wild-type flower. *WUS* RNA expression (arrowhead) persists in a small domain in the indeterminate floral meristem.

(C) Floral meristem in an *ag-1 clv1-4* double mutant flower of similar stage as the one shown in (B). The *WUS* domain is larger than in the *ag-1* single mutant.

nate number of floral organs. At the molecular level, these differences are apparent in the expression patterns of the stem cell regulator *WUS* and of floral homeotic genes including *AG*. Expression of *WUS*, which promotes stem cell proliferation, persists in shoot meristems, but is only transiently maintained in flowers (Mayer et al., 1998). Conversely, floral homeotic genes are not expressed in shoots, but they are activated in young flowers, and their expression persists until late stages of floral organ development (for review, Weigel and Meyerowitz, 1994).

A key factor in distinguishing flowers from shoots is the floral identity protein LFY, which can convert shoot meristems into floral meristems (Weigel and Nilsson, 1995). One of the genes acting downstream of LFY is *AG*, which is activated in the center of flowers through the combined action of LFY and the meristem regulator WUS. Importantly, not only does WUS have dual roles in floral development—transient promotion of stem cell proliferation and activation of a floral homeotic gene—, but so does *AG*, which specifies floral organ identity as well as determinate growth of the floral meristem. The latter function is achieved through a feedback loop in which *AG*, once established, represses the *AG* activator *WUS* and thereby prevents further maintenance of a floral stem cell population. Importantly, it has been shown that *AG* activity is required in the cells that express *WUS* to determine floral meristem determinacy (Sieburth et al., 1998), indicating that the *AG/WUS* interaction is relatively direct.

The negative feedback between *WUS* and *AG* is reminiscent of the one in which *WUS* induces expression of *CLV3*, which in turn represses *WUS* expression through a signal transduction cascade that involves binding of the secreted CLV3 ligand to the CLV1/CLV2 receptor complex (Brand et al., 2000; Schoof et al., 2000; Trotochaud et al., 2000). An important difference between the *WUS/AG* and the *WUS/CLV* regulatory loops is that the former takes place in the same cells, with activation and repression temporally separated, while the latter takes place between adjacent cells, with activation and repression occurring simultaneously.

Experimental Procedures

Plants

lfy-12, *ag-1*, *clv1-4*, *UFO::CLV3*, and *AG::GUS* have been described (Bowman et al., 1989; Huala and Sussex, 1992; Clark et al., 1993; Busch et al., 1999; Brand et al., 2000). Plants were grown in long days (16 hr light).

Plant Vectors and Transformation

WUS sequences were amplified from first-strand cDNA of Columbia wild-type with *Pfu* Turbo polymerase (Stratagene) and primers JL0004 (TGA TCT TAT TTA CCG TTA ACT TTG TGA) and JL0005 (CGA AAG AGA GAG AGA GAG GAA AGA). The product was cloned and sequenced (pJL4). An *AP3::WUS::ocs3′* cassette with a 1.3 kb fragment of the *AP3* promoter (Jack et al., 1994) was created in pART7 derivative pBJ36 (Gleave, 1992) (pJL2). The cassette was shuttled into pART27 derivative pMLBART (Gleave, 1992) (pJL1). For *LFY::WUS*, we used the 2.2 kb promoter (Blázquez et al., 1997) with the initiation codon changed to TTG. The *AP3* promoter in pJL2 was replaced with the *LFY* promoter (pJL7), and the resulting *LFY::WUS::ocs3′* cassette shuttled into pMLBART (pJL8).

For pJL49, the WUS binding sites, TAAT(G/C)(G/C), were mutated to TCCT(G/C)(G/C) in the context of pKB22, which contains a 750 bp BamHI/HindIII *AG* fragment in pBstKS+ (Busch et al., 1999). After sequencing, the insert was shuttled into pDW294 (Busch et al., 1999).

pJL1 and pJL8 were transformed (Bechtold and Pelletier, 1998) into kanamycin-resistant *AG::GUS* lines (Busch et al., 1999). Transformants were selected using Finale® (AgrEvo). More than 100 independent transgenic lines were generated for each construct. Plants transformed with pJL49 were selected on kanamycin medium.

Yeast Vectors and Assays

For expression constructs, p423 and p424 vectors with *GAL1* promoters were used (Mumberg et al., 1994). pFP13 (LFY) and pFP14 (LFY:VP16) have been described (Parcy et al., 1998). The WUS coding sequence was cloned into p423 (pJL36). *lacZ* reporters were in pKF1, a derivative of pLG718 (Guarente and Mason, 1983) with a SmaI cloning site. For pFP50, a 287 bp fragment of the *AG* enhancer was amplified with FP1038 (GGT CTG AAC ATG TCT AGG GTT TC) and FP1039 (TAA TAT GTC ATT GTA ATA CG). For pMX71, pMX72, and pMX73, the same primers were used to amplify the equivalent

fragments from plasmids with mutant LFY binding sites (Busch et al., 1999). For pRH18, a 91 bp fragment was amplified using RH1001 (TCA CTC GAG TTT AAA TTT AAT CCA ATG) and RH1002 (TCG TCG ACA ACA ACC CAT TAA CAC ATT G), and trimers isolated after ligation in the presence of XhoI and SalI. The same strategy was used for pJL51, with mutagenic primers JL0041 (TCA CTC GAG TTT AAA TTT CCT CCA ATG) and JL0042 (TCG TCG ACA ACA ACC CAG GAA CAC ATT G).

For each experiment, effectors and reporters were transformed (Gietz et al., 1995) simultaneously into *S. cerevisiae* strain EGY48 (Golemis et al., 1996). β-galactosidase measurements were as described (Golemis et al., 1996).

Histology

Scanning electron microscopy, GUS staining, and in situ hybridization were as described (Bowman et al., 1991; Blázquez et al., 1997; Fletcher et al., 1999). To increase the specificity of GUS staining, 10 mM ferro- and ferricyanide were used (Sessions et al., 1999), except for the KB31 apex shown in Figure 3D.

Electrophoretic Mobility Shift Assays

LFY and WUS were expressed in *S. cerevisiae* EGY48 containing plasmids pFP13 and pJL36, respectively. 1:1000 dilutions of saturated cultures were grown in medium containing 2% galactose for 15 hr, and protein extracted by standard methods. 31mers were end-labeled with [γ-^{32}P] ATP before annealing and purification over a polyacrylamide gel. The binding reaction, with 100 fmol of target DNA, was incubated for 20 min on ice in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.25 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 20 mM $MgCl_2$, and 12.5 ng μl^{-1} double stranded fish sperm DNA (Roche Molecular Biochemicals). For supershifts, 1 μl of a 1:10 dilution of anti-LFY antiserum (Parcy et al., 1998) was added to the reaction after 10 min.

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