

## Regulation of *APETALA3* floral homeotic gene expression by meristem identity genes

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### SUMMARY

The *Arabidopsis APETALA3* (*AP3*) floral homeotic gene is required for specifying petal and stamen identities, and is expressed in a spatially limited domain of cells in the floral meristem that will give rise to these organs. Here we show that the floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*API*) are required for the activation of *AP3*. The *LFY* transcription factor binds to a sequence, with dyad symmetry, that lies within a region of the *AP3* promoter required for early expression of *AP3*. Mutation of this region abolishes *LFY* binding in vitro and in yeast one hybrid assays, but has no obvious effect on *AP3* expression in planta. Experiments using a steroid-inducible form of *LFY* show that, in contrast to its direct

transcriptional activation of other floral homeotic genes, *LFY* acts in both a direct and an indirect manner to regulate *AP3* expression. This *LFY*-induced expression of *AP3* depends in part on the function of the *APETALA1* (*API*) floral homeotic gene, since mutations in *API* reduce *LFY*-dependent induction of *AP3* expression. *LFY* therefore appears to act through several pathways, one of which is dependent on *API* activity, to regulate *AP3* expression.

Key words: *APETALA3*, *LEAFY*, *APETALA1*, Floral homeotic gene, Meristem identity gene, Transcriptional regulation, *Arabidopsis thaliana*

### INTRODUCTION

*Arabidopsis* plants undergo a transition from vegetative growth, in which the shoot apical meristem produces leaves and axillary buds, to reproductive growth, when the meristem begins to form flowers. The meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*API*) are both necessary and sufficient for this transition to reproductive growth and the concomitant formation of flowers. Loss of *LFY* function leads to leaves and shoots in place of flowers, while constitutive expression of *LFY* results in precocious floral development (Weigel et al., 1992; Weigel and Nilsson, 1995). Similarly, constitutive *API* expression also results in premature flowering, and loss of *API* function results in a partial transformation of flowers to more inflorescence-like structures (Bowman et al., 1993; Irish and Sussex, 1990; Mandel and Yanofsky, 1995).

In turn, the development of floral structures depends on the action of three classes of floral homeotic genes, A, B and C. These ABC floral homeotic genes function in overlapping domains to specify different floral organ identities. *API*, in addition to its role as a meristem identity gene, has a second role as an A class gene and is required for the development of sepal and petal primordia (Bowman et al., 1993; Irish and Sussex, 1990; Mandel et al., 1992). The B class genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) specify petal and stamen identities (Bowman et al., 1989; Goto and Meyerowitz,

1994; Jack et al., 1992), while *AGAMOUS* (*AG*), the C class gene, is responsible for conferring stamen and carpel identities (Bowman et al., 1989; Yanofsky et al., 1990). To a large extent, the functions of these ABC genes in specifying different organ identities correspond to their domains of expression in the developing flower (Riechmann and Meyerowitz, 1997; Weigel and Meyerowitz, 1994).

*LFY* is required for the transcription of representatives of all three classes of ABC genes (Weigel and Meyerowitz, 1993). *LFY* encodes a nuclear-localized product that can bind to DNA and so could act directly to regulate transcription of the floral homeotic genes (Parcy et al., 1998). In fact, *LFY* protein has been demonstrated to bind to sequences in the *API* and *AG* enhancer regions that are required for normal levels of expression from these genes (Busch et al., 1999; Parcy et al., 1998). Ectopic expression of *LFY* is sufficient to induce the expression of *API* outside the flower (Parcy et al., 1998). Furthermore, the activation of early *API* expression by *LFY* is not dependent on protein synthesis, demonstrating that *LFY* is a direct transcriptional activator of *API* (Wagner et al., 1999). While ectopic expression of *LFY* is insufficient to ectopically activate *AG*, expression of a dominant, activated form of *LFY*, *LFY:VP16*, can induce *AG* expression in vegetative tissues (Parcy et al., 1998). These observations suggest that *AG* expression does not depend on *LFY* alone, but the requirement of other factors for *AG* activation can be bypassed by

*LFY:VP16* (Parcy et al., 1998). One such factor is the WUSCHEL (WUS) homeodomain protein, which cooperatively interacts with LFY to regulate *AG* expression (Lenhard et al., 2001; Lohmann et al., 2001).

The effects of *LFY* on *AP3* expression are more complex. *LFY* is required for *AP3* expression, since a loss-of-function *lfy-6* mutant shows a dramatic reduction in the levels and domain of *AP3* activation (Weigel and Meyerowitz, 1993). However, ectopic expression of either *LFY* or *LFY:VP16* does not significantly affect *AP3* expression (Parcy et al., 1998). These observations suggest that the regulation of *AP3* is considerably different from that of *AP1* or *AG*, and that LFY may not directly activate *AP3* expression. Alternatively, *LFY* activation of *AP3* may occur directly by binding to the *AP3* promoter, but other cofactors may be required for transcriptional activation to ensue.

*AP1* has also been implicated in the regulation of *AP3* gene expression. *AP1* encodes a MADS-domain containing protein that binds to sequences in the *AP3* promoter that are required for normal *AP3* expression (Hill et al., 1998; Tilly et al., 1998). While *AP3* expression is almost normal in *ap1* mutant flowers, plants containing both the strong *lfy-6* and *ap1-1* alleles show a complete abolition of *AP3* expression, reflecting the synergistic action of both *LFY* and *AP1* in activating *AP3* expression (Weigel and Meyerowitz, 1993). Furthermore, plants containing an activated form of *AP1*, *AP1:VP16*, display a partial transformation of medial first whorl organs into petals that is dependent on *AP3* function, supporting the idea that *AP1* positively regulates *AP3* (Ng and Yanofsky, 2001).

In order to begin to dissect the molecular mechanisms by which these meristem identity genes function, we have analyzed the role of *LFY* and *AP1* in regulating *AP3* transcription. Previously, we have shown that the *AP3* promoter contains distinct *cis*-acting elements that are required for the different spatial and temporal aspects of *AP3* expression (Hill et al., 1998; Tilly et al., 1998). Here we show that LFY protein can bind to sequences within the *AP3* promoter that are required for early *AP3* expression. Furthermore, using an inducible form of LFY, we show that LFY acts both directly and indirectly to regulate *AP3* expression, and that the indirect pathway depends on the function of the *AP1* floral homeotic gene. Mutations of the LFY binding site in the *AP3* promoter fail to abrogate *AP3* expression in planta, suggesting that the indirect pathway may be sufficient to induce *AP3* expression in this context. Based on these observations, we propose a model for how these meristem identity genes act together to activate the expression of the *AP3* floral homeotic gene.

## MATERIALS AND METHODS

### Plant material, transformation and histochemical analysis

Plants were grown under 16 hours light/8 hours dark conditions. To generate *35S::LFY-GR*; *35S::UFO* seedlings, *35S::UFO/+* pollen was used to fertilize *35S::LFY-GR/35S::LFY-GR* plants and the resulting offspring used in bulk for experiments. Similarly, pollen from *35S::UFO/+* plants was used to fertilize *35S::LFY/35S::LFY* plants and pollen from *35S::UFO/+*; *ap1-1/ap1-1* plants was used to fertilize *35S::LFY*; *ap1-1/ap1-1*; the appropriate offspring were chosen by phenotype in the next generation. Pollen from *35S::LFY-GR* homozygous plants was used to fertilize *lfy-6/lfy-6* and *ap1-1/ap1-1* plants, the offspring allowed to self fertilize and the correct genotype

identified by PCR in the next generation. Transgenic lines were generated using the floral dip method (Clough and Bent, 1998) and selected on medium containing 30 mg/l kanamycin.  $\beta$ -glucuronidase (GUS) enzymatic activity was detected as previously described (Hill et al., 1998). At least three independent transgenic lines containing a single insert (as assessed by Southern analysis) were crossed into the various genetic backgrounds and were evaluated for their patterns of GUS expression.

### Electrophoretic mobility shift assays

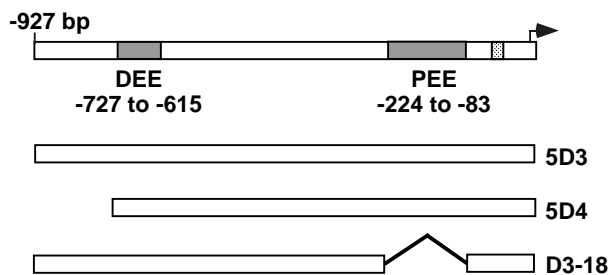
Proteins for EMSA were produced by *in vitro* transcription of the *LFY* cDNA which was translated *in vitro* using wheat germ extract (Promega, Madison, WI). Preparation of DNA probes, binding reactions, and gel conditions were as described previously (Hill et al., 1998). Fragments used for cold competitors in the binding reactions corresponded to the following sequences within the *AP3* promoter and were generated by polymerase chain reaction (PCR): competitor 1, -727 to -554; competitor 2, -705 to -587; competitor 3, -662 to -554; competitor 4 -727 to -626; competitor 5, -618 to -554; competitor 6, -727 to -678. Competitors were cleaned by ammonium acetate precipitation, quantitated, then added to the binding reaction in amounts of 10,000-fold molar excess over labeled probe. The following oligos were annealed with their complimentary oligos and cloned into the *EcoRV* site of the pBluescript SKII+ vector (Stratagene, La Jolla, CA): AP3I (site I), 5'-CTT AAA CCC TAG GGG TAA TA-3'; AP3Im, 5'-CTT AAA CCC TAT TATTAA TA-3'; AP3II (site II), 5'-TTC TAT TTT CCA AGG ATC TTT AGT TAA AGG C-3'; AP3IIm, 5'-TTC TAT TTT CCA ATT ATC TTT AGT TAA AGG C-3'; AP3I-II, 5'-CTT AAA CCC TAG GGG TAA TAT TCT ATT TTC CAA GGA TCT TTA GTT AAA GGC-3'; AP3Im-II, 5'-CTT AAA CCC TAT TAT TAA TAT TCT ATT TTC CAA GGA TCT TTA GTT AAA GGC-3'; AP3I-IIm, 5'-CTT AAA CCC TAG GGG TAA TAT TCT ATT TTC CAA TTA TCT TTA GTT AAA GGC-3'; AP3Im-IIm, 5'-CTT AAA CCC TAT TAT TAA TAT TCT ATT TTC CAA TTA TCT TTA GTT AAA GGC-3'. Mutated sequences are shown in bold. These fragments were released from the vector using *HindIII* and *EcoRI* to produce gel shift probes.

### Yeast one hybrid assays

An in frame GAL4AD:LFY fusion was created in the pGAD424 vector and transformed into yeast strain YM4271. The DEE promoter fragment was trimerized and cloned into the *EcoRI* site of the *lacZi* vector resulting in trimers fused in both the + and - orientations with respect to the *lacZ* reporter gene. The DEE-*lacZ* fusions were then integrated into the yeast strain YM4271 containing the GAL4AD:LFY construct. Yeast transformation protocols, yeast vectors and yeast strains were obtained from Clontech (Palo Alto, CA). To determine *lacZ* expression, yeast colonies were grown on selection medium for 2 days, and then 1 ml of the culture was resuspended in 2 ml of YPD and grown at 30°C. After 3-4 hours, the OD<sub>600</sub> of the culture was measured, and 1 ml of the culture was spun down and resuspended in 800  $\mu$ l of Z buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub>, 50 mM 2-mercaptoethanol, pH 7). One drop of 0.1% SDS and 2 drops of chloroform were added to each tube to lyse the cells. The suspension was then equilibrated at 30°C for 15 minutes. Subsequently, 160  $\mu$ l of 4 mg/ml ONPG (Sigma, St. Louis, MO) was added, and the reaction was allowed to take place for 2 hours. The reaction was stopped with 400  $\mu$ l of 1 M sodium carbonate, and the OD<sub>420</sub> and OD<sub>550</sub> of the suspension was noted. The units of  $\beta$ -gal activity were calculated using the following formula:  $U=1000 \times [(OD_{420}) - (1.75 \times OD_{550})]/[\text{time (minutes)} \times OD_{600}]$ . Replicate assays were conducted using five colonies of each construct tested per assay, and similar results were obtained in all assays.

### Plasmid constructions for plant transformation

The mutated promoter constructs were generated using PCR from



**Fig. 1.** Constructs used in this study. The *AP3* promoter, showing positions (in gray) of the distal early element (DEE) and proximal early element (PEE) which are required for early stage 3 to stage 5 expression of *AP3* (Hill et al., 1998). Constructs used in this study are shown. The stippled area represents the putative TATA element and the arrow indicates position of ATG.

plasmids p5D3, pD3-36 and pD3-18 (Hill et al., 1998) using the primers AP3IM-II, AP3I-IIM or AP3IM-IIM and vector primers. The resulting products were cloned into the TOPO TA vector (Invitrogen, Carlsbad, CA) and sequenced. In frame translational fusions at the ATG of the mutated *AP3* promoter constructs and the GUS reporter gene were created by cloning the various promoter constructs into the *Sall* and *Bam*HI sites of pBI101 (Clontech, Palo Alto, CA). Expression constructs were transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation.

#### Chemical treatments and real time RT-PCR conditions

Dexamethasone (DEX; Sigma, St. Louis, MO) was dissolved in ethanol and used at a final concentration of 1  $\mu$ M on seedlings. For inhibition of protein synthesis, 10  $\mu$ M cyclohexamide (CHX; Sigma, St. Louis, MO) was added simultaneously with the DEX treatment. For seedling treatments, wild-type or transgenic seedlings were grown for 5 days on growth medium then transferred to media containing either DEX or DEX/CHX for 16 hours. For inflorescence treatments, plants were grown on soil under long day conditions (16 hours light, 8 hours dark). The primary bolt was cut 1-2 days after the start of bolting and after 24-48 hours individual inflorescences were treated with 5  $\mu$ M DEX, 10  $\mu$ M CHX, or DEX/CHX as described previously (Wagner et al., 1999). RNA was extracted from seedlings and inflorescences using Trizol (GibcoBRL, Frederick, MD) according to the manufacturer's instructions. cDNA was synthesized using Superscript II RNase-Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real time PCR reactions were carried out using an ABI Prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA) in MicroAmp Optical 96-well reaction plates with optical covers, according to manufacturer's instructions. PCR reactions (final volume 50  $\mu$ l) contained TaqManMGB gene-specific probe and primers and the passive reference dye ROX, in order to normalize fluorescence across the plate. In all experiments, controls without template were used and at least two replicates using at least two independent RNA samples were used. *AP3*-specific TaqManMGB probe was conjugated to the fluorescent dye JOE and *API*-specific TaqManMGB probe was conjugated to the fluorescent dye FAM. Reaction conditions were: 50°C for 2 minutes, 94°C for 10 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 1 minute. Primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) to flank introns so genomic DNA contamination would not amplify. *AP3* primers: AP3F 5' CCACCAGAACCATCACCCTATT; AP3R 5' GTCAGAGGCAGAGGGTGCAT. *AP3* TaqManMGB probe: 5' CCCAACCATGGCCTT. *API* primers: APIF 5' TGAGCTGGAACCTAAGAGCTGAAGA; APIR 5' AACTGAGTCGTAATCTCCTCCATTG. *API* TaqManMGB Probe: 5' CCTCACTATGGACTACTAG. Relative quantification values and standard deviations

were calculated using the standard curve method according to the manufacturer's instructions (ABI Prism 7000 Sequence Detection System User Guide). Values were normalized to the mock treated sample and results analyzed with Microsoft Excel software.

## RESULTS

### LFY is required for expression from the *AP3* promoter

Previously we have identified discrete *cis*-acting elements in the *AP3* promoter which are required for different aspects of *AP3* expression (Hill et al., 1998). We defined two elements, the proximal early element (PEE) and distal early element (DEE), which are required for *AP3* expression during early stages of floral development, from stages 3 to 5 (Fig. 1). In addition, other promoter elements were identified that are required for petal or stamen-specific expression at later stages of development.

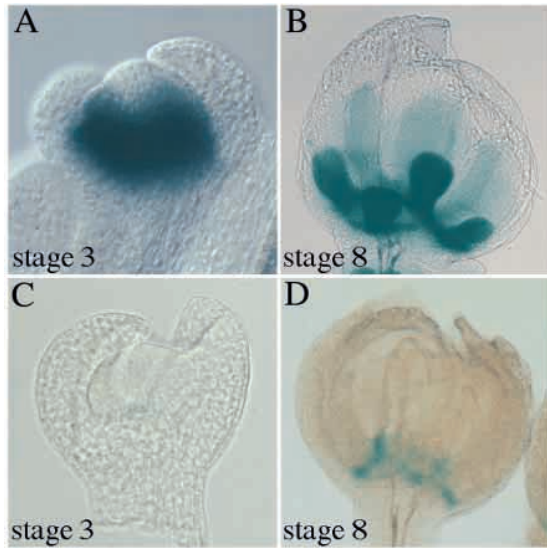
The 5D4 promoter fragment consists of 727 bp and contains all promoter elements required for *AP3* expression (Hill et al., 1998). We crossed a 5D4::*GUS* reporter gene construct into a *lfy-6* mutant background to test whether *LFY* acts via these *AP3* promoter sequences. The strong *lfy-6* mutation results in the transformation of flowers to a more inflorescence-like structure of spirally arranged organs that have leaf-like, sepaloid and/or carpeloid features (Weigel et al., 1992). Flowers homozygous for *lfy-6* lack stage 3-5 expression driven by the 5D4::*GUS* construct (Fig. 2A,C). This demonstrates that *LFY* is required for initial activation of *AP3* expression and this action is mediated by this 727 bp sequence within the *AP3* promoter. Disruption of 5D4::*GUS* expression is also seen at later stages in a *lfy-6* background, with GUS activity limited to just the base of the developing second and third whorl organs (Fig. 2B,D). This reduction in later *AP3* expression presumably reflects the lack of *AP3* autoregulation (Jack et al., 1992).

### LFY protein binds to sequences in the *AP3* promoter

Since *LFY* has been shown to bind to *cis*-acting regulatory sequences in the *API* and *AG* floral homeotic genes (Busch et al., 1999; Parcy et al., 1998; Wagner et al., 1999), it seemed likely that *LFY* protein could also bind to *AP3* regulatory sequences. Using electrophoretic mobility shift assays (EMSA), we assayed the ability of *LFY* protein to bind to different sequences within the *AP3* promoter. *LFY* protein was found to bind to a promoter fragment that contains the distal early element (DEE) (Fig. 3A). *LFY* did not bind to any other *AP3* promoter fragments in EMSA, including the PEE that is also required for early *AP3* expression (Fig. 1; data not shown).

Competition assays demonstrated that *LFY* protein binding mapped to sequences contained within the DEE (Fig. 3A). Cold competitors corresponding to different regions of the -727 to -554 region of the *AP3* promoter were used to compete for *LFY* binding to the labeled -727 to -554 test fragment. The smallest region, as defined by various competitors, that is capable of competing for binding to *LFY* corresponded to the region from -705 to -626. Furthermore, the sequence from -727 to -678 did not interfere with *LFY* binding, delimiting the *LFY* binding site region to the 52 bp region from -678 to -626.

This 52 bp sequence could be divided into two regions,



**Fig. 2.** *LFY* is required for expression from the *AP3* promoter. GUS expression conferred by the *5D4::GUS* promoter construct. This construct drives expression in a wild-type background in the presumptive petal and stamen primordia at stage 3 (A) and expression is maintained in this domain in later stages of floral development; expression at stage 8 is shown (B). This pattern of expression is largely abolished in a *lfy-6* mutant background, with no detectable GUS expression at stage 3 (C). At stage 8, a limited patch of GUS expression is observed at the base of the developing second and third whorls (D).

termed site I (corresponding to basepairs  $-678$  to  $-658$ ) and site II ( $-657$  to  $-626$ ), each of which showed some sequence similarity to the *LFY* binding sequences found in the regulatory regions of the *API* and *AG* genes (Fig. 3B). These two sites each contain a palindromic sequence that overlaps the putative *LFY* binding sites. Mutated versions of site I and site II were generated that disrupted both the palindromic sequences and the presumptive *LFY* binding sites (Fig. 3B). We tested the ability of *LFY* to bind to these sites by assaying the wild-type and mutated versions of each site either individually or within the same 52 bp DNA fragment using several assay systems. First, we checked the ability of these sequences to bind to *LFY* in yeast one-hybrid assays (Fig. 3C). Trimerized versions of wild-type or mutated site I and/or site II sequences were fused to the *lacZ* coding sequence and introduced into the yeast genome; these strains were assayed for *lacZ* expression in the presence of a *LFY*-GAL4 activation domain (*LFY*-AD) fusion gene product. In addition, the wild-type and mutated oligonucleotide sequences were used in EMSAs to test their ability to bind to *LFY* (Fig. 3D). Both these assays gave similar results and indicated that site I is necessary and sufficient for *LFY* binding, while the site II sequence alone is not sufficient. However, *LFY* cannot bind when site II is mutated in the context of the entire fragment, indicating that intact site II is required for *LFY* binding in this context. This may reflect a requirement for a particular DNA conformation for *LFY* binding.

#### ***LFY* can act in both a direct and an indirect manner to activate *AP3* expression**

We assessed whether *LFY* acts directly or indirectly to activate

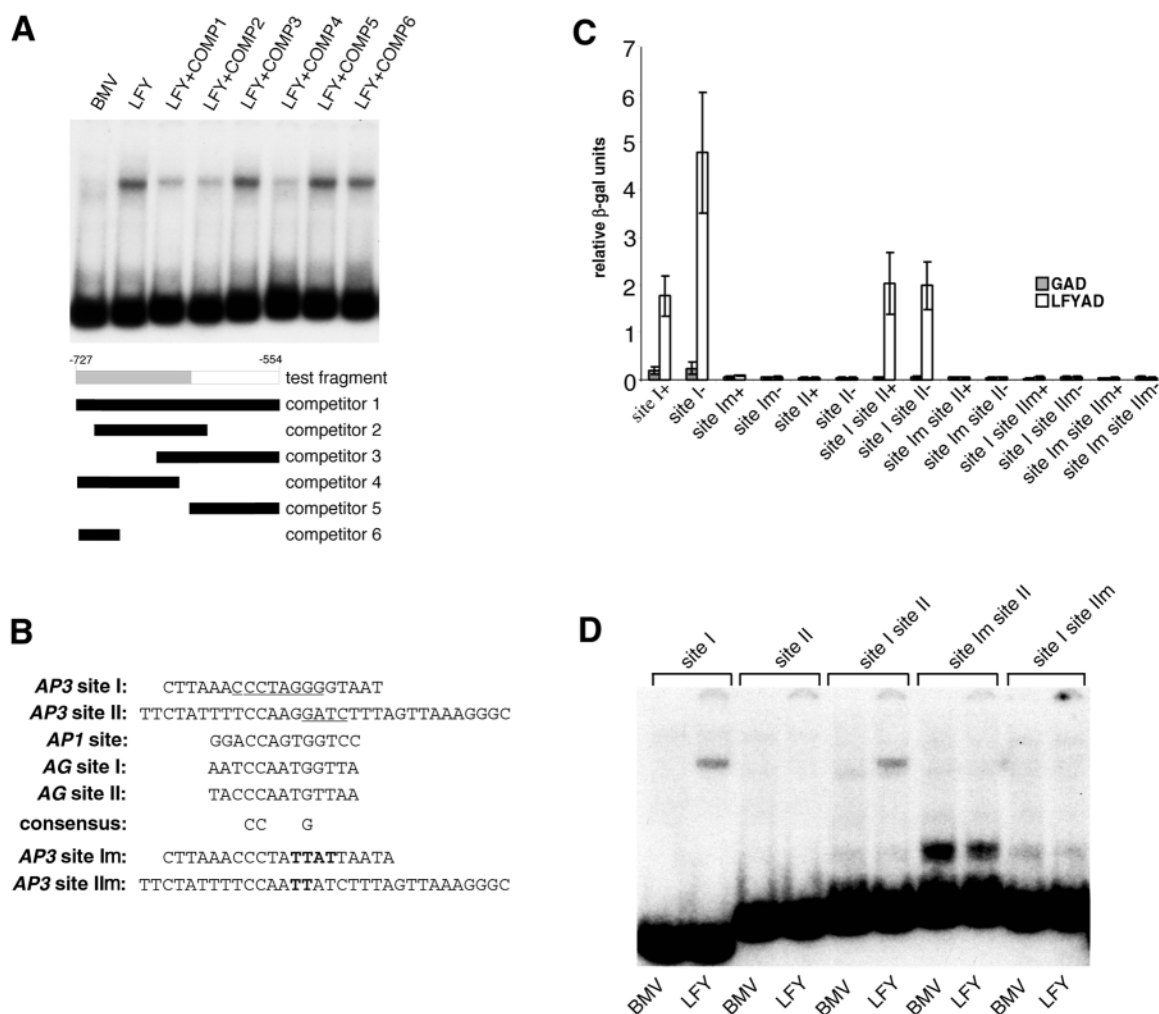
*AP3* transcription *in vivo* by utilizing a ubiquitously expressed inducible form of *LFY*, *35S::LFY-GR* (Wagner et al., 1999) to induce *AP3* expression in several contexts in the presence or absence of the protein synthesis inhibitor, cyclohexamide (CHX). The *LFY*-GR fusion protein is localized to the cytoplasm and thus is inactive, but can be induced to localize to the nucleus and function by treatment of plants with dexamethasone (DEX) (Wagner et al., 1999).

Because *AP3* is not normally expressed in seedlings, we took advantage of the fact that constitutive expression of *LFY* in conjunction with *UFO* results in ectopic *AP3* transcription in seedlings (Parcy et al., 1998). Inducing *LFY* expression at the seedling stage provides an ideal way to directly assess the effects of *LFY* expression on *AP3* transcriptional activation. *35S::LFY-GR; 35S::UFO* seedlings were grown without DEX for 5 days, then treated with 1  $\mu$ M DEX. Upon DEX treatment, these seedlings arrest their normal development, but recover if DEX is removed. The *35S::LFY-GR; 35S::UFO* seedlings were treated with DEX alone, or concomitantly with 10  $\mu$ M CHX at day 5, and seedling tissue was harvested after 16 hours of treatment and examined for levels of *AP3* expression. The relative levels of *AP3* expression with the various treatments were assessed using a quantitative real time reverse transcription PCR (RT-PCR) approach. The treatment of *35S::LFY-GR; 35S::UFO* seedlings with DEX posttranslationally activated *LFY* and resulted in induction of *AP3* expression (Fig. 4A). CHX treatment somewhat reduced the levels of *AP3* expression in these DEX-induced seedlings, but these levels are significantly above that of seedlings treated with CHX alone (Fig. 4A). These observations indicate that part of the *LFY*-dependent activation of *AP3* expression in the seedling requires protein synthesis, and implies that the activation of *AP3* by *LFY* in this context is indirect. In addition, the fact that we could observe significant levels of *AP3* expression in these DEX/CHX-treated seedlings indicates that *LFY* can also function in a direct manner to activate *AP3* expression.

We also examined whether *API* expression can be directly induced in the presence of DEX and CHX in *35S::LFY-GR; 35S::UFO* seedlings. *API* is directly activated in response to *LFY* in floral tissue (Wagner et al., 1999). In contrast to what we observed for *AP3*, the levels of DEX-inducible *API* expression in the presence of CHX was equivalent to that of treating with DEX alone (Fig. 4B). This implies that all *API* expression in these seedlings is dependent on direct activation by the *LFY* protein.

Since *LFY* directly activates *API* expression in seedlings, we examined whether *LFY*-dependent expression of *AP3* required *API* activity. *35S::LFY; 35S::UFO; ap1-1* seedlings were examined for the presence of *AP3* transcripts using quantitative real time RT-PCR. An examination of the relative levels of *AP3* ectopic activation in a *35S::LFY; 35S::UFO; ap1-1* background, as compared to a *35S::LFY; 35S::UFO* background indicates that overall levels of *AP3* expression are significantly reduced when *API* function is absent (Fig. 4C). However, some *AP3* expression is still detectable in the *35S::LFY; 35S::UFO; ap1-1* seedlings, supporting the idea that *LFY* can act independently of *API*, and presumably directly, to activate *AP3* transcription.

We also examined *AP3* expression induced by *35S::LFY-GR* in floral tissue. DEX application to young *35S::LFY-GR* flower

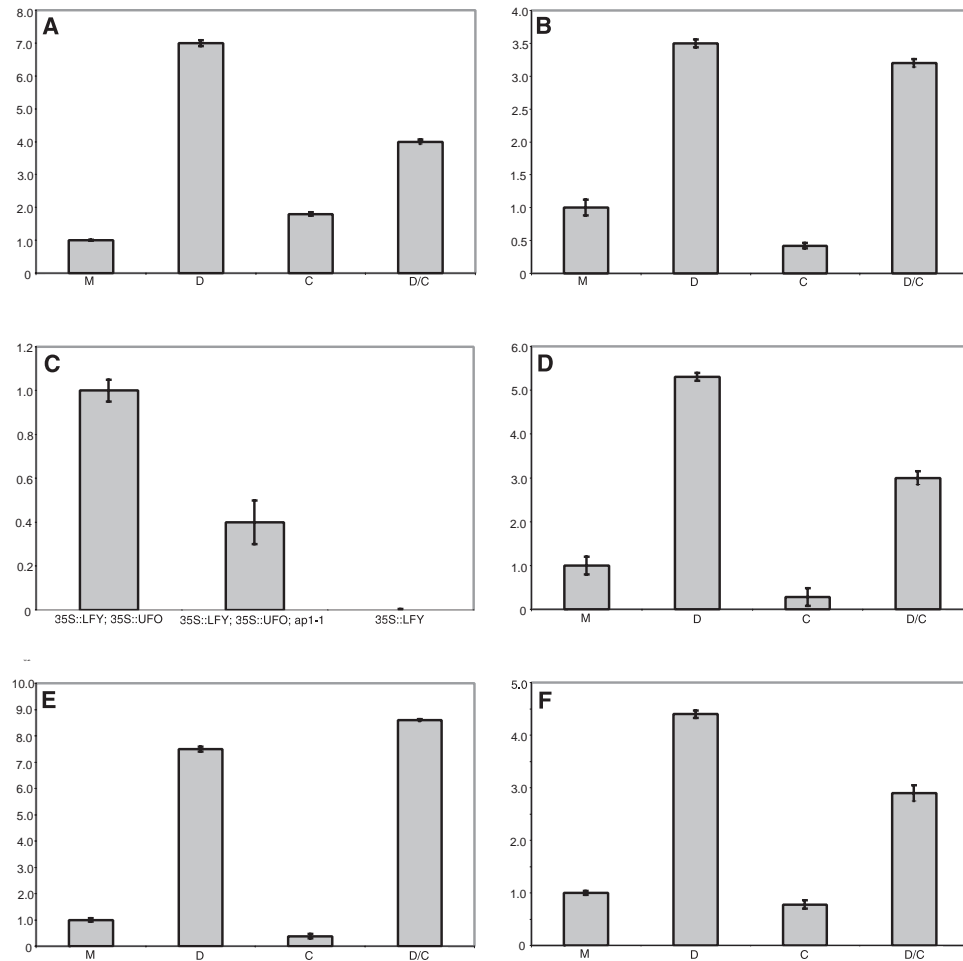


**Fig. 3.** LFY binds to sequences within the distal early element of the *AP3* promoter. (A) Electrophoretic mobility shift assay demonstrates that in vitro translated LFY gene product binds to a labeled sequence corresponding to the *AP3* promoter region from  $-727$  to  $-554$  (test fragment) which contains the DEE (shown in gray). The ability of various cold competitors to block LFY binding is shown. Competitor 1 contains sequences from  $-727$  to  $-554$  and corresponds to the test fragment; competitor 2,  $-705$  to  $-587$ ; competitor 3,  $-662$  to  $-554$ ; competitor 4  $-727$  to  $-626$ ; competitor 5,  $-618$  to  $-554$ ; and competitor 6,  $-727$  to  $-678$ . The brome mosaic virus (BMV) in vitro translation reaction was used as a non-specific control. (B) The region to which LFY binds was further subdivided into site I ( $-678$  to  $-659$ ) and site II ( $-658$  to  $-627$ ). Palindromic sequences within each of these sites are underlined; these sites are aligned with LFY binding sites defined in the *AP1* and *AG* floral homeotic gene regulatory regions (Busch et al., 1999; Parcy et al., 1998). Sequences mutated in *AP3* site I and *AP3* site II are shown in bold. (C) Yeast one-hybrid assays demonstrate that LFY binds to *AP3* site I. Trimerized versions of *AP3* site I, site II or the 52 bp fragment containing both sites (site I site II) were fused in a normal (+) or inverted (–) orientation to the *lacZ* reporter gene and introduced into yeast. Similar constructs were generated containing the mutated site Im or site Ilm versions described in B. The ability of the GAL4 activation domain alone (GAD) or the GAL4 activation domain fused to LFY (LFYAD) to activate *lacZ* expression was assayed in five replicates. Standard errors for each construct are shown. (D) Electrophoretic mobility shift assays demonstrate that LFY binds to site I. The ability of in vitro translated LFY protein to bind to labeled sequences corresponding to site I, site II, or the mutated versions was assessed. BMV, non-specific control.

buds did not induce *AP3* expression above endogenous levels (data not shown). In order to eliminate *AP3* expression dependent on endogenous LFY-dependent pathways, we examined the induction of *AP3* expression in a *35S::LFY-GR*; *lfy-6/lfy-6* background. In these flower buds, DEX application induced *AP3* expression, and simultaneous DEX/CHX application somewhat reduced *AP3* expression, but did not abrogate this expression completely (Fig. 4D). This is similar to what we observed in *35S::LFY-GR*, *35S::UFO* seedlings (Fig. 4A) and implies that LFY can act in both a direct and an indirect manner to activate *AP3* expression in flowers. As a

control, we also examined the levels of AP1 expression induced in *35S::LFY-GR*; *lfy-6/lfy-6* flowers (Fig. 4E). Similar to what has been observed previously, LFY appears to act directly to activate AP1 transcription in these tissues (Wagner et al., 1999).

To determine whether LFY-dependent induction of *AP3* depended on AP1 activity in flowers, we examined the levels of *AP3* expression in *35S::LFY-GR*; *ap1-1/ap1-1* flower buds. DEX treatment of *35S::LFY-GR*; *ap1-1/ap1-1* plants (Fig. 4F) resulted in levels of *AP3* expression that were higher than the level produced in the presence of DEX and CHX, indicating



**Fig. 4.** Activation of *AP3* expression by *LFY* in vivo. (A) Results from real time RT-PCR amplification of RNA isolated from *35S::LFY-GR; 35S::UFO* seedlings mock treated with 0.1% ethanol and 0.015% Silwet (M), or treated with dexamethasone (D), cycloheximide (C), or dexamethasone and cycloheximide together (D/C). Amplifications were carried out with primers and probe corresponding to *AP3*, and normalized to the mock-treated control. Standard deviations are indicated. (B) Results from real time RT-PCR amplification of *35S::LFY-GR; 35S::UFO* seedling RNA using primers and probe corresponding to *API*; treatments and labels as in A. (C) Results from real time RT-PCR amplification of seedling RNA from the indicated genotypes, using primers and probe corresponding to *AP3*. Standard deviations are indicated. (D) Results from real time RT-PCR amplification of *35S::LFY-GR; lfy-6/lfy-6* young floral tissue RNA using primers and probe corresponding to *AP3*; treatments and labels as in part (A). (E) Results from real time RT-PCR amplification of *35S::LFY-GR; lfy-6/lfy-6* young floral tissue RNA using primers corresponding to *API*; treatments and labels as in A. (F) Results from RT-PCR amplification of *35S::LFY-GR; ap1-1/ap1-1* young floral tissue RNA using primers and probe corresponding to *AP3*; treatments and labels as in A.

that *LFY* can act via an *API*-independent indirect pathway to activate *AP3* expression in the flower.

#### Intact *LFY* binding sites at the DEE are not required for *AP3* expression in planta

Because our real time RT-PCR results indicated that *LFY* acts in both a direct and an indirect manner to regulate *AP3* transcription, we chose to test the significance of the in vitro defined *LFY* binding sites in planta. We generated transgenic plants in which site I and/or site II sequences were mutated in the context of several diagnostic *AP3* promoter constructs (Fig. 1). The same site I and/or site II mutations used in the EMSA

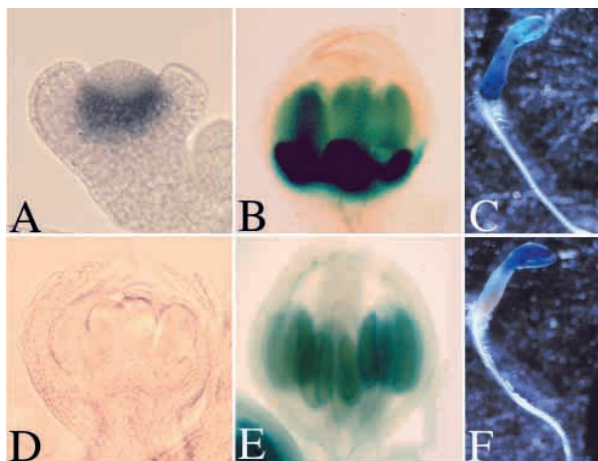
and yeast-one hybrid assays were introduced into the 5D3 and the D3-18 (lacking the PEE but containing the DEE) *AP3* promoter constructs (Fig. 1) and fused to the *GUS* reporter gene. These mutated reporter gene constructs were stably transformed into *Arabidopsis* plants, and at least three independent single insertion lines for each construct were analyzed for reporter gene expression in flowers. We also examined the expression of these reporter constructs in *35S::LFY; 35S::UFO* seedlings. Mutation of sites I and II individually, or of both sites simultaneously, has no obvious effect on reporter gene expression in either the wild-type or *35S::LFY; 35S::UFO* backgrounds (Fig. 5 and data not shown). This is quite surprising in the case of the site I and site II mutated D3-18 construct, since this construct is mutated for the *LFY* binding site in DEE as well as being deleted for the PEE, the other promoter region required for stage 3-5 *AP3* expression (Hill et al., 1998). Based on these observations, these results suggest that *LFY*-dependent activation of *AP3* does not require that the identified *LFY* binding site be intact.

## DISCUSSION

### *LFY* binds to DEE sequences in the *AP3* promoter

*LFY* is required for the onset of normal *AP3* expression, since mutations in *lfy* result in significantly reduced levels of *AP3* transcripts during early

stages of floral organogenesis (Weigel and Meyerowitz, 1993). Using both in vitro electrophoretic mobility shift assays and in vivo yeast one hybrid assays, we have demonstrated that *LFY* protein binds to specific regulatory sequences in the *AP3* promoter. This binding site lies within the DEE, which is required for stage 3 to 5 expression from the *AP3* promoter (Hill et al., 1998). This binding is sequence specific, since mutation of this region in the *AP3* promoter abolishes binding in vitro and in yeast one hybrid assays. Comparisons of the *LFY* binding sites in the *AG* and *API* regulatory regions defines a consensus that is only loosely matched by the binding site we have defined in the *AP3* promoter (Fig. 3B). The



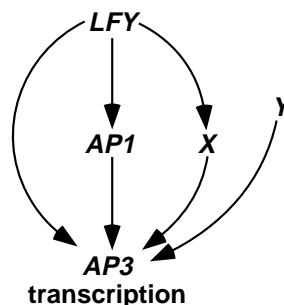
**Fig. 5.** Mutation of the LFY binding site in the *AP3* promoter does not disrupt expression in planta. The *5D3::GUS* construct containing site Im and site IIIm mutations (*5D3-ImIIIm::GUS*) confers GUS expression in the presumptive petal and stamen primordia in a wild-type background both at stage 3 (A) and at stage 8 (B), similar to that seen for the unmutated version of *5D3::GUS* (Hill et al., 1998). *5D3-ImIIIm::GUS* also confers GUS activity in *35S::LFY*, *35S::UFO* seedlings (C). Expression of the *D3-18* construct containing site Im and site IIIm mutations (*D3-18-ImIIIm*) in a wild-type background at stage 6 (D) and stage 10 (E). This pattern of GUS expression recapitulates that conferred by the unmutated *D3-18::GUS* construct (Hill et al., 1998). Furthermore, *D3-18-ImIIIm::GUS* is expressed in seedlings containing the *35S::LFY*, *35S::UFO* transgenes (F).

binding of LFY to regulatory sequences is therefore presumably quite sensitive to sequence context, similar to the context dependence of many other transcription factors. LFY does not appear to bind to any other sequences within the *AP3* promoter in vitro (data not shown).

### A model for regulation of *AP3*

Based on our results, we propose a model for LFY and *API* activation of *AP3* transcription (Fig. 6). We suggest that there are at least four separate pathways that regulate the onset of *AP3* expression in the flower.

One pathway appears to be the direct activation of *AP3* transcription by LFY. Two lines of evidence support the existence of this pathway. First, we can demonstrate, using *35S::LFY-GR* to induce *AP3* expression in the presence of cycloheximide, that LFY protein can function in the absence of protein synthesis to activate *AP3* transcription. Second, the DNA binding studies indicate that LFY can bind directly to sequences in the *AP3* promoter that are required for early expression. However, mutation of the defined LFY binding site in planta does not abrogate *AP3* expression (Fig. 5), which would appear to contradict the idea that LFY acts directly by binding to *AP3* promoter sequences. We can suggest two possibilities to reconcile these observations. First, we may not have identified the relevant binding site, although we have not been able to detect LFY binding to any other sequences in the *AP3* promoter using EMSA (data not shown). Alternatively, LFY may act to directly activate *AP3* transcription through the binding site we have defined, but this pathway may be redundant with the indirect LFY-dependent pathways leading to *AP3* activation. Therefore, loss of LFY binding to the *AP3*



**Fig. 6.** Model for activation of *AP3* expression. Initiation of *AP3* expression in the flower depends on multiple regulatory cascades. LFY appears to act through at least three pathways; one pathway is direct, one pathway requires the function of *API*; another indirect pathway depends on an as yet unidentified factor (X). In addition, a LFY-independent mechanism requiring unknown factor(s) (Y) functions in certain tissues to promote *AP3* transcription.

promoter may not be sufficient to disrupt LFY-dependent activation of *AP3* in planta.

The second LFY-dependent pathway we have defined is indirect and depends on the activity of *API* to activate *AP3* transcription. Loss of *API* function significantly reduces the level of *AP3* transcription that can be induced by LFY action (Fig. 4C). *API* has been previously shown to be a positive regulator of *AP3* (Hill et al., 1998; Krizek and Meyerowitz, 1996; Ng and Yanofsky, 2001; Weigel and Meyerowitz, 1993). Since LFY directly activates *API* (this work) (Wagner et al., 1999), and *API* has been shown to bind to sequences within the PEE (Hill et al., 1998), this short regulatory cascade may activate transcription through the PEE sequences. LFY direct activation of *API* expression appears to be limited to early stages of floral development, when the pattern of *API* expression is largely coincident with that of LFY in the floral meristem (Parcy et al., 1998; Wagner et al., 1999). At later stages, spatially restricted *API* expression depends on other factors, which may include *AG* (Gustafson-Brown et al., 1994; Parcy et al., 1998; Wagner et al., 1999).

The fact that mutation of *API* does not completely abolish LFY-dependent expression of *AP3* suggests that LFY also regulates *AP3* via a third pathway that is independent of *API* (Fig. 4F). This appears to be an indirect pathway of activation via an unknown factor 'X' (Fig. 6). This indirect pathway potentially could depend on the products of the *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*) genes, which are both paralogs of *API* and appear to have overlapping functions (Ferrandiz et al., 2000); and so may act in a partially redundant fashion to weakly activate *AP3* in the absence of *API*.

A number of other identified genes are also candidates for being involved in this third, LFY-dependent indirect pathway of *AP3* activation. One such gene is *UFO*, which has been shown to encode a region-specific factor that is required in conjunction with LFY to activate *AP3* (Lee et al., 1997). *UFO* does not appear to have DNA binding activity, and so presumably does not act as part of the transcriptional machinery (data not shown). It is more likely that *UFO* acts as part of an SCF (SKP1-Cullin-F-box) complex and targets specific proteins for ubiquitin-dependent degradation, since *UFO* encodes an F-box containing protein that has been shown to interact with *SKP1*-like gene products (Bai et al., 1996; Ingram et al., 1995; Samach et al., 1999). This postulated role of *UFO* has led to a model whereby *UFO* acts to promote the degradation of a putative negative regulator of *AP3* (Samach et al., 1999). One possibility to explain the role of *UFO* in the

indirect pathway regulating *AP3* expression would be that *LFY* activates the transcription of the putative negative regulator while *UFO* acts to target it for degradation.

At least one other candidate gene has been identified which may act in this *LFY*-dependent indirect pathway activating *AP3* transcription. A myb-domain containing DNA binding protein has recently been identified that binds to *AP3* promoter sequences and appears to act as a positive regulator of *AP3* transcription in vivo (C. Juarez, E. Chae, Q. K.-G. T. and V. F. I., unpublished data).

Finally, a fourth pathway that is independent of *LFY* can be defined, which requires an as yet unidentified factor or factors ('Y', Fig. 6). Low levels of *AP3* expression are detectable at the base of the second and third whorls in *lfy-6* mutant plants, indicating that not all *AP3* expression is dependent on *LFY* function (Fig. 2). This pathway also appears to be independent of *UFO* as well as *ASK1*, a putative subunit of a *UFO*-containing SCF ubiquitin ligase complex, since mutations in either *UFO* or *ASK1* still result in *AP3* expression at the base of the second and third whorls (Levin and Meyerowitz, 1995; Zhao et al., 2001).

### Floral homeotic gene regulation by *LFY*

*LFY* has now been implicated in activating the transcription of representatives of all three A, B, and C classes of floral homeotic genes (Busch et al., 1999; Wagner et al., 1999; Weigel and Meyerowitz, 1993) (this work). Despite this global control of floral homeotic gene expression by *LFY*, each of these organ identity genes is expressed in a different spatially limited domain, implying that *LFY* acts in conjunction with other factors to delimit ABC gene activation. Our results suggest that *LFY* acts to regulate expression of *AP3* in a manner distinct from that of *API* or *AG*. The multiple *AP3* regulatory pathways we have defined could act as a failsafe mechanism to ensure appropriate expression of *AP3* and may reflect a requirement for the strict temporal control of expression of this floral homeotic gene. In light of these results, it seems likely that there are multiple complex regulatory interactions that serve to reinforce the precise spatial and temporal control of floral homeotic gene expression, which in turn is critical for normal floral patterning.

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