A genetic framework for floral patterning

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The initial steps of flower development involve two classes of consecutively acting regulatory genes. Meristem-identity genes, which act early to control the initiation of flowers, are expressed throughout the incipient floral primordium. Homeotic genes, which act later to specify the identity of individual floral organs, are expressed in distinct domains within the flower. The link between the two classes of genes has remained unknown so far. Here we show that the meristem-identity gene *LEAFY* has a role in controlling homeotic genes that is separable from its role in specifying floral fate. On the basis of our observation that *LEAFY* activates different homeotic genes through distinct mechanisms, we propose a genetic framework for the control of floral patterning.

The development of multicellular organisms requires the repeated generation of complex tissue and organ patterns from fields of undifferentiated cells. A genetically tractable model for pattern formation in plants is the morphogenesis of individual flowers: a collection of undifferentiated cells termed the floral meristem produces four types of organ in a stereotypic fashion. A landmark in developmental genetics was the proposal of the ABC model, which describes how three classes of homeotic genes act in discrete domains to specify the identity of floral organ types[1,2] (Fig. 1a). Subsequent molecular analysis showed that region-specific activity of the ABC genes is largely regulated at the transcriptional level[3].

Little is known about how the initial pattern of ABC gene expression is generated. As region-specific expression of the *Arabidopsis thaliana* A-function gene *APETALA1* (*AP1*) is merely a consequence of repression by the C-function gene *AGAMOUS* (*AG*)[4], a minimal model for the generation of the ABC pattern needs to explain how *AP1* is initially activated throughout the flower, and how *AG*, as well as the B-function genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), are activated at a later time point in their specific domains.

Candidates for upstream regulators of flower-specific ABC genes are early-acting genes such as *LEAFY* (*LFY*). Unfortunately, it has been difficult to determine the directness of the interaction between *LFY* and ABC genes[5–7], because *LFY* affects the identity of the flower meristem itself, an event that precedes the activation of ABC genes. *lfy* null mutations cause a transformation of the first few flowers into leaves with associated shoots which do not express any of the ABC genes simply because these structures never acquired any floral identity[8–10]. Later-arising flowers are replaced by leaf-like bracts in *lfy* mutants, and abnormal flowers develop from the base of the bracts. In these flowers, *AP3* and *PI* expression is very much reduced; *AG* expression is delayed; and *AP1* expression is almost normal[7,11]. Again, it is difficult to decide whether these alterations are a consequence of missing *LFY* activity at the time that these genes are activated, or whether they result from an earlier defect in specification of the flower meristem. Moreover, expression of ABC genes in the abnormal flowers of *lfy* mutants indicates that *LFY* is not absolutely required for their activation and that ABC genes are redundantly regulated.

Here we show that the role of *LFY* in flower-meristem initiation can be separated from a role in the later activation of homeotic genes, and that different mechanisms are used in the activation of three representative members of each of the ABC gene classes. These observations allow us to propose a genetic framework for the patterning of flowers.

An activated form of LFY

In contrast to *LFY* RNA[9], LFY protein persists throughout the flower until floral stage 3, at which time the pattern of ABC genes develop (Fig. 1b). The sequence of LFY is not similar to that of other proteins with known biochemical function[9,12]. LFY localizes to the nucleus (Fig. 1b), binds DNA in a sequence-specific manner (Fig. 2a), and can, when fused to a heterologous activation domain, mediate transcriptional activation in yeast (Fig. 2b). Although these results indicate that LFY is a transcriptional regulator, *in vivo* targets of LFY have not been identified so far.

As at least some of its putative downstream genes are expressed in specific patterns within the flower, but LFY itself is not, LFY activity is likely to be regulated in some way. This regulation could occur at two different levels at least, affecting LFY's DNA-binding or transcriptional-activation potential. To determine whether any of the known ABC genes are likely targets of LFY, we generated a version of LFY whose transcriptional-activation potential should be constitutive. In this new allele, called *LFY:VP16*, a fusion of LFY to the strong activation domain from the viral transcription factor VP16 (refs 13, 14) is expressed under the control of normal *LFY* regulatory sequences[15] (Fig. 2c). The rationale for this experiment was as follows: if *LFY* acts only to specify flower meristem fate, *LFY:VP16* might affect the initial establishment of flower primordia, but not downstream events such as specification of floral organ identity. On the other hand, if *LFY* has a separate role in regulating

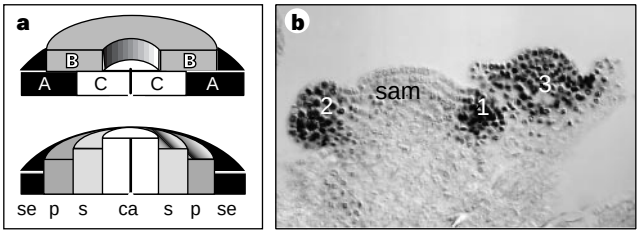

Figure 1 Activity domains of ABC floral homeotic genes and *LFY* expression. **a**, The ABC model[1]. Top, activity domains of ABC genes; bottom, readout of ABC gene activities in form of organ identity. se, sepal; p, petal; s, stamen; ca, carpel. **b**, Immunolocalization of LFY protein[26], which is found in nuclei and expressed fairly uniformly in flower primordia through stage 3 of development. Numbers indicate floral stages. sam, shoot apical meristem.

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ABC gene expression, then *LFY:VP16* might modify the expression of individual ABC genes and thus affect flower morphology. A caveat to this approach is that, in those cases in which binding of LFY to specific gene promoters is regulated, the VP16 fusion protein might not change expression of target genes.

LFY:VP16 plants resembled neither *lfy* mutants nor plants that express *LFY* ectopically[8,16]. The primary transformants, presumed to be hemizygous, fell into three phenotypic classes (Fig. 3). Plants with a weak phenotype had some flowers with staminoid petals in the second whorl. Plants with an intermediate phenotype had sepals that were carpelloid, petals that were converted into stamens and reduced in number, and a slight increase in carpel number in the fourth whorl. Finally, in plants with a strong phenotype, each flower was replaced by a carpelloid structure that lacked whorled phyllotaxy. The *LFY:VP16* phenotype was dependent on transgene copy number, such that an intermediate or strong phenotype was seen in homozygous progeny of weak or intermediate plants, respectively. Homozygous progeny of strong transformants could not be recovered because of sterility.

Several controls confirmed that the *LFY:VP16* phenotypes were related to endogenous *LFY* function. First, *LFY:VP16* interacted with wild-type *LFY* in a dosage-dependent manner, as the severity of the *LFY:VP16* phenotype increased when the copy number of endogenous *LFY* was reduced (by crossing *LFY:VP16* to plants with the *lfy*-12 null allele). This effect indicates that LFY:VP16 and wild-type LFY compete for the same targets. Furthermore, *LFY:VP16* rescued the flower initiation defects of homozygous *lfy-12* mutants. Finally, two mutant *LFY:VP16* versions (Fig. 2c), in which either the *LFY* coding sequence was truncated downstream to the VP16 domain (*mLFY:VP16*), or the normal VP16 domain was replaced by a mutant derivative shown to be inactive in other systems[17] (*LFY:mVP16*), did not cause any floral phenotype. The *LFY:mVP16* transgene complemented all floral defects of the *lfy-12* null allele, showing that insertion of a foreign protein domain did not inactivate the LFY protein.

Differential effects of *LFY:VP16* on ABC genes

As the homeotic transformations in *LFY:VP16* flowers indicated a change in ABC gene expression, we analysed RNA expression of representatives of each class of homeotic genes.

The A-function gene *AP1* is expressed uniformly in early wild-type flower buds, and later becomes restricted to the two outer whorls because of repression by *AG*[4,18]. In *LFY:VP16* flowers, the early pattern of *AP1* was unchanged, but the expression level was

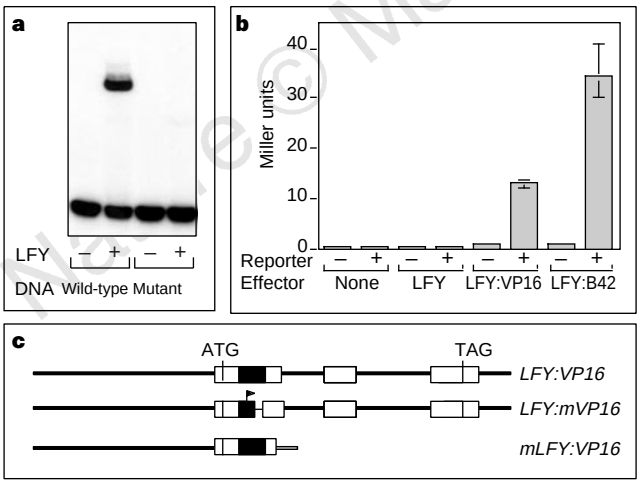

Figure 2 *In vitro* activity of LFY protein and LFY:VP16 constructs. **a**, Wild-type LFY binds *in vitro* to a double-stranded oligonucleotide (see Methods), as seen by a shift in electrophoretic mobility on a non-denaturing polyacrylamide gel. A two-base-pair change in the DNA sequence (mutant) abolishes DNA binding. The binding site was originally identified by immunoprecipitation of genomic DNA from the *AP1* locus. **b**, LFY activates gene expression in the yeast *Saccharomyces cerevisiae*. LFY was expressed together with a *lacZ* reporter, which was controlled by a yeast minimal promoter linked to a 194 bp DNA fragment spanning the LFY-binding site shown in **a**. The reporter is activated if LFY is fused to a strong transcriptional activational domain, such as the one from VP16 (ref. 13) or the synthetic domain B42 (ref. 44). Bars indicate the range of values from three replicate transformations. **c**, Diagram of *LFY:VP16* transgenes. Exons are indicated by rectangles. Sequences coding for the VP16 activation domain are shown in black. The flag in *LFY:mVP16* indicates a missense mutation in the mutant VP16 domain[17].

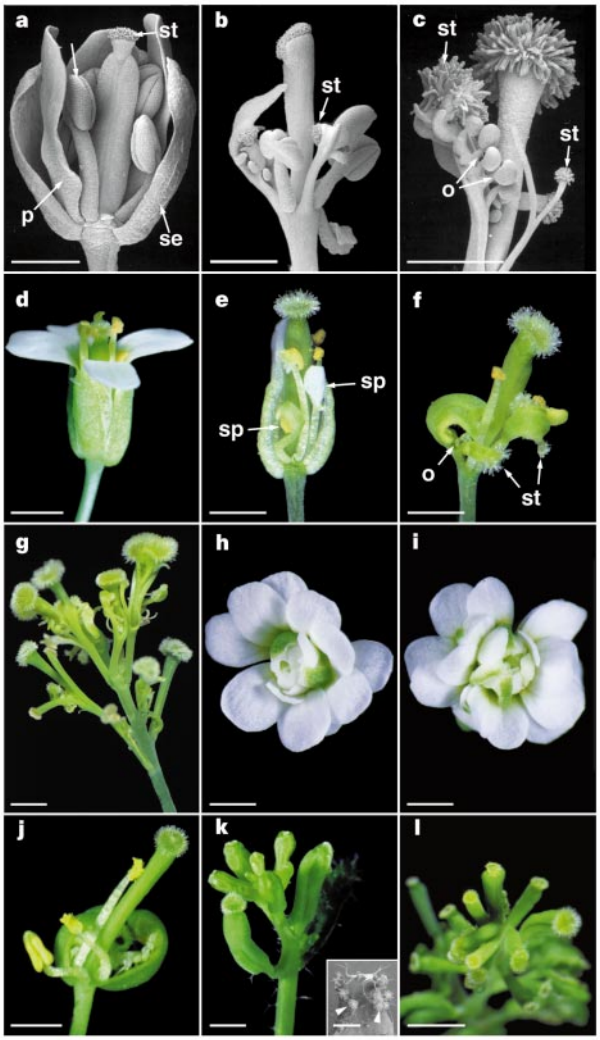

Figure 3 Phenotypes of wild-type, mutant and transgenic plants. **a**, Scanning electron micrograph (SEM) of a wild-type flower, with one sepal, petal and stamen removed. **b**, SEM of a flower with an intermediate *LFY:VP16* phenotype. Note the carpellody of first-whorl organ. **c**, SEM of a flower with a strong *LFY:VP16* phenotype. Most organs are tipped with stigmatic tissue. Ovules are visible on one organ. **d**, Light micrograph of a wild-type flower. **e**, Flower with a weak *LFY:VP16* phenotype, with one sepal removed. Second-whorl petals show partial conversion into stamens. **f**, Flower with an intermediate *LFY:VP16* phenotype. Some of the first-whorl sepals are carpelloid, indicated by ovules and stigmatic tissue. **g**, Inflorescence with a strong *LFY:VP16* phenotype. **h**, *ag-1* flower. **i**, *LFY:VP16 ag-1* flower. **j**, *ap2-2* flower. **k**, *LFY:VP16 ap2-2* inflorescence. The inset shows a cauline leaf with stigmatic tissue on its margins (arrow heads). **l**, *35S::LFY ap2-2* inflorescence. se, sepal; p, petal; s, stamen; st, stigmatic tissue; sp, staminoid petal; o, ovule. Scale bars, 500 μm in **a–c** and inset in **k**, and 1 mm in **d–l**.

greatly increased (Fig. 4a, e).

The C-function gene *AG* begins to be expressed in the centre of wild-type flowers during stage 3 (ref. 19). Both the spatial and the temporal patterns of *AG* RNA were changed markedly in *LFY:VP16* flowers. *AG* expression began earlier and was detected throughout the flower (Fig. 4b, f). Comparison of *AP1* and *AG* expression patterns indicates that *LFY:VP16* may be able to override repression of *AP1* by *AG*[4], but probing of adjacent sections for *AP1* and *AG* expression will be needed to confirm this. In addition to changes in the pattern of expression of *AG*, the levels of *AG* expression were increased in *LFY:VP16* plants.

As the conversion of sepals into carpels and of petals into stamens in *LFY:VP16* plants was similar to the phenotype of plants that express *AG* constitutively[20], we crossed plants with the strong *ag-1* allele to *LFY-VP16* plants. Sepal and petal identity were restored in *LFY:VP16 ag-1* plants, showing that ectopic *AG* expression was the main cause of the *LFY:VP16* phenotype (Fig. 3h, i).

The B-function gene *AP3* is first expressed in wild-type flowers during stage 3, and its expression is largely restricted to the presumptive second and third whorls[21]. *LFY:VP16* had only minor effects on *AP3* expression. *AP3* expression was normal in the lateral regions of flowers, but reduced in medial regions, correlating with missing second- and third-whorl organs in these positions (Fig. 4c, g). *AP3* expression in first-whorl organs of advanced *LFY:VP16* flowers was also more extensive than in wild-type flowers (results not shown).

Thus an activated version of *LFY*, *LFY:VP16*, has no pronounced effect on one ABC gene, *AP3*, but causes increased activation of another ABC gene, *AP1*, and both increased and ectopic activation of a third ABC gene, *AG*. If *LFY* controlled homeotic gene expression only through its role in specifying flower meristem fate, we would expect that changing the transcriptional-activation potential of LFY might affect flower initiation but not the later activation of ABC genes. As an alteration in the activation potential of LFY affects ABC gene expression, we propose that *LFY* has a role in activating ABC genes that is separable from its role in specifying flower meristem identity. Furthermore, the differential effects of *LFY:VP16* on different ABC genes indicate that *LFY* may interact with these genes through different mechanisms.

Flower-independent activation of *AP1*

LFY is expressed in both leaf and flower primordia, at lower levels in the former[15]. As ABC genes are not normally expressed in leaves, we tested whether this was due to insufficient levels of *LFY*, or to the absence of other, flower-specific factors, whose activity could possibly be mimicked by adding the VP16 domain to LFY. We introduced a transgene that causes *LFY* overexpression in vegetative tissues (*35S::LFY*; ref. 16) into an *AP1::GUS* reporter line, and found that the *AP1* promoter is induced in young *35S::LFY* seedlings before there is any sign of ectopic flower formation (Fig. 5a–c). We verified that endogenous *AP1* RNA was induced in young *35S::LFY* seedlings by reverse transcription followed by polymerase chain reaction (RT–PCR) (Fig. 5c). Thus the role of *LFY* in inducing *AP1* can be separated from its role in the specification of flower meristems.

Morphological studies have shown that, after floral induction in wild-type *Arabidopsis*, primordia that would otherwise have become leaves develop into flowers instead[22]. Interestingly, *AP1* promoter activity in *35S::LFY* seedlings was mainly confined to young leaf primordia (Fig. 5c), indicating that *LFY* may interact with other differentially active factors to induce *AP1* expression preferentially in primordia that have the potential to adopt a floral fate.

Flower-independent activation of *AG*

In contrast to *AP1*, *AG* is not induced in vegetative tissue of *35S::LFY* plants, as shown by *in situ* hybridization (results not shown) and with an *AG::GUS* reporter line (Fig. 5e), indicating that *AG* activation does not depend on LFY levels only. However, the ability of *LFY:VP16* to activate *AG* throughout the flower meristem indicated that the VP16 domain may allow LFY to function largely independently of other factors that regulate *AG*. If this were correct, *LFY:VP16* should be able to induce *AG* expression in vegetative tissue. Like endogenous *LFY*, *LFY:VP16* should be expressed, albeit at low levels, in leaf primordia, especially in those giving rise to cauline leaves, which are initiated just before the first flower is formed[15]. Consistent with a weak effect of *LFY:VP16* on *AG* in cauline leaves, we occasionally observed stigmatic tissue, normally found only at the tip of carpels, along the margin of cauline leaves (results not shown). The appearance of stigmatic tissue in leaves was much more pronounced when *LFY:VP16* was introduced into *ap2* mutants (Fig. 3k). *AP2* is functionally a flower-specific repressor of *AG*, although *AP2* is expressed throughout the plant[23]. The enhancement of the *LFY:VP16* leaf phenotype in *ap2* mutants indicates that low levels of *LFY:VP16* present in leaves do not normally overcome repression of *AG* by *AP2*.

To test more directly whether *LFY:VP16* can induce *AG* in any vegetative tissue, we tried to generate plants in which *LFY:VP16* was

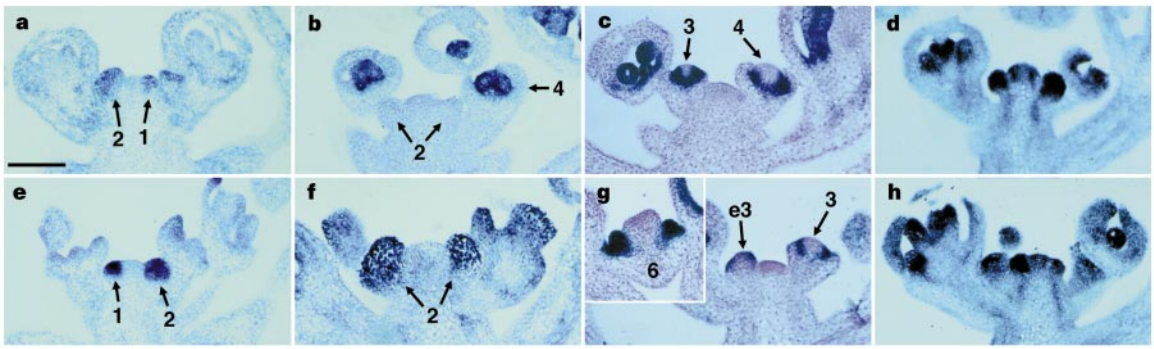

Figure 4 Expression of *AP1*, *AG*, *AP3* and *LFY* in wild-type (**a–d**) and *LFY:VP16* transgenic (**e–h**) plants. Longitudinal sections of flowering apices that were hybridized *in situ* are shown. *LFY:VP16* sections were from plants with intermediate (**e**, **g**, **h**) or strong (**f**) phenotypes. **a**, **e**, *AP1* expression during stages 1 and 2 is increased in *LFY:VP16* plants. **b**, **f**, *AG* RNA is first expressed in the centre of wild-type flowers during stage 3. In *LFY:VP16* plants, *AG* expression is detected during stages 1 and 2 and extends into the subapical region of the shoot meristem, reminiscent of the *LFY* expression pattern (**d**, **h**). *AG* and *AP1* RNAs (**e**) seem to overlap; however, the sections probed for *AP1* and *AG* were from *LFY:VP16* lines of different phenotypic strengths. **c**, **g**, *AP3* expression, which in wild-type plants begins during early stage 3 (e3), is restricted to whorls 2 and 3 and the base of whorl 1. In *LFY:VP16* flowers, *AP3* expression is unchanged in the lateral part of the meristem (inset) and slightly reduced in the medial part (main panel). **d**, **h**, *LFY* expression is similar in wild-type and *LFY:VP16* inflorescences. Scale bar, 100 μm.

constitutively expressed (*35S::LFY:VP16*). We never recovered adult transformants, so we vacuum-infiltrated[24] an *Agrobacterium* strain containing the *35S::LFY:VP16* vector directly into homozygous *AG::GUS* reporter plants. About 30,000 seeds were collected, germinated and stained for GUS activity. Although no GUS activity was detected in 20,000 control *AG::GUS* seedlings, about 30 seedlings derived from vacuum infiltration with *35S::LFY:VP16* were growth-arrested and showed GUS activity in all organs (Fig. 5f). This frequency, 0.1%, concurs with our average transformation efficiencies of between 0.05% and 2%. We conclude that, once strongly expressed, LFY:VP16 can induce *AG* independently of flower initiation.

The observation that the VP16 activation domain enables LFY to induce *AG* ectopically, both in vegetative tissues and in flowers, does not indicate whether LFY is normally an activator or a repressor of *AG*. It has been suggested that *LFY* may activate *AG* during early flower development but contributes subsequently to repression of *AG* in the outer whorls[6,7,12]. We therefore re-examined *35S::LFY* plants, in which *LFY* levels are increased throughout the flower[16]. Although most *35S::LFY* flowers were phenotypically wild type, the most apical ones produced in short days had carpelloid organs in the first whorl and lacked some petals, indicating increased *AG* expression. This was even more obvious in plants resulting from the cross of *35S::LFY* to *ap2-2* and *ap2-1* mutants, in which a synergistic effect was seen (Fig. 3l; results not shown). *35S::LFY ap2-2* flowers resembled those of *LFY:VP16 ap2-2* plants (Fig. 3k, l). The fact that increased carpellody is caused by *LFY* overexpression in *ap2* mutants is complementary to the observation that eliminating *LFY* function in *lfy ap2* double mutants reduces carpellody as compared with *ap2* single mutants[9,10]. It therefore seems that the primary role of *LFY* is activation of *AG*.

Why does LFY, which is present throughout the flower, normally induce *AG* only in the centre of the flower? In the prevailing view of *AG* regulation, meristem-identity genes such as *LFY* activate *AG* throughout the flower, but *AG* is specifically repressed in the outer whorls by A-function genes such as *AP2* (refs 1, 7). An alternative, formally equivalent model of *AG* regulation holds that A-function genes are general repressors of *AG*, and that this repression is selectively overcome in the centre of the flower during stage 3. We propose that a combination of the two models is most likely to reflect the *in vivo* situation. For example, if *AG* were uniformly activated throughout the flower, and if absence of *AG* RNA from outer whorls was caused by repression by *AP2*, then *AG* should be uniformly expressed in *ap2* mutant flowers. However, *AG* expression is weaker in the first whorl of *ap2* mutants than in interior whorls[19]. Furthermore, although phenotypic analysis indicates that *AP2* functions throughout the flower in *ag* mutants, *AP2* cannot repress *AG* RNA expression in the centre of these flowers[1,4]. These results indicate that *AG* may be induced to a greater extent in the centre of the flower than in the periphery. We propose that this differential effect involves the interaction of *LFY* with a co-activator that aids LFY in overcoming *AP2*-mediated repression of *AG* in the centre of the flower. The VP16 activation domain may render LFY independent of other regulators of *AG*, thus allowing LFY to induce *AG* throughout the flower even in the presence of repressors such as *AP2*.

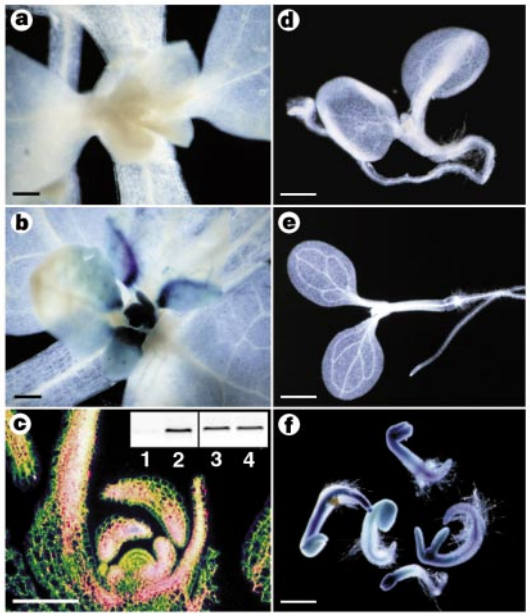

Figure 5 *AP1::GUS* and *AG::GUS* expression outside flowers. **a–c**, AP1::GUS activity in 11-day-old plants. **a**, Wild-type plants have no detectable AP1::GUS activity. **b**, *35S::LFY* plants express AP1::GUS throughout young leaf primordia and at the base of older leaves. **c**, Longitudinal section through the shoot apex of a *35S::LFY* plant. GUS staining, which appears pink under dark field microscopy, is present at high levels in leaves. It is either absent from the shoot apical meristem, or present at much lower levels. (Meristematic cells in the centre are not vacuolated and appear yellow even in control plants.) The inset shows detection of RNA by RT–PCR in young seedlings (six days after germination, grown in short days). Lanes 1, 2, *AP1* in wild-type and *35S::LFY* plants, respectively; lanes 3, 4, control amplification of *eIF4A* in wild-type and *35S::LFY* plants, respectively. **d–f**, AG::GUS activity is absent from 5-day-old wild-type (**d**) and *35S::LFY* (**e**) plants, but is detected in all organs of *35S::LFY:VP16* seedlings, which are growth-arrested (**f**). Scale bars, 200 μm in **a**, **b**, 100 μm in **c**, and 500 μm in **d–f**.

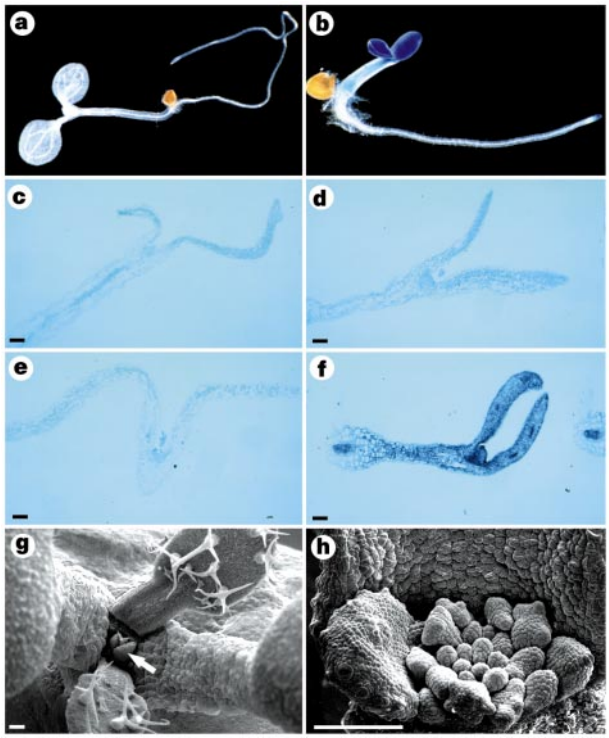

Figure 6 *LFY* and *UFO* together induce *AP3* expression in seedlings. **a**, **b**, *AP3::GUS* is expressed in *35S::LFY 35S::UFO* seedlings (**b**), but not in wild-type seedlings (**a**). **c–f**, *In situ* hybridization does not detect *AP3* RNA in 5-day-old seedlings with no transgene (**c**), only *35S::LFY* (**d**), or only *35S::UFO* (**e**). In contrast, *AP3* RNA is detected in *35S::LFY 35S::UFO* seedlings (**f**). **g**, SEM of a shoot apex of a 5-day-old non-transgenic seedling. Leaf primordia cover the shoot meristem (arrow). **h**, SEM of a shoot apex of a *35S::LFY 35S::UFO* seedling. The shoot meristem has been consumed by primordia whose epidermal cells are reminiscent of petals or stamens. Scale bars, 1 mm in **a**, **b**, and 100 μm in **c–h**.

Flower-independent activation of *AP3*

Activation of the A-function gene *AP1* or of the C-function gene *AG* can be separated from flower initiation by overexpression of normal LFY or of an activated form of LFY. However, in neither of these situations did we observe a marked change in activation of the B-function gene *AP3*, indicating that regulation of *AP3* by *LFY* relies on a mechanism different from the ones used for *AP1* and *AG*. A partially redundant co-regulator that might act together with *LFY* in activating *AP3* is *UNUSUAL FLORAL ORGANS (UFO)*. When expressed constitutively, *UFO* causes ectopic activation of *AP3* within flowers[25]. All *UFO* loss- and gain-of-function phenotypes are masked in a *lfy* null background[25–27], indicating that *UFO* activity requires functional LFY protein. Furthermore, *LFY* and *UFO* expression domains overlap at the time of *AP3* induction, indicating that LFY and UFO protein may act together, with UFO providing much of the positional information for *AP3* activation. The molecular function of UFO and of its snapdragon orthologue FIMBRIATA is unknown, but the presence of a functional F-box indicates that they may be involved in targeting other proteins for ubiquitin-mediated degradation[28–30]. Thus, although it is unlikely that UFO itself is a transcriptional regulator, like some other F-box proteins UFO might regulate the activity of transcription factors[31,32].

To determine whether LFY and UFO together can induce *AP3* independently of flower initiation, we generated doubly transgenic seedlings that constitutively expressed both *LFY* and *UFO*. *35S::LFY 35S::UFO* seedlings were growth-arrested and did not form any mature leaves. The combination of *35S::LFY* and *35S::UFO* transgenes was sufficient to induce *AP3* in vegetative tissues of seedlings (Fig. 6). Thus activation of *AP3*, like that of *AP1* and *AG*, can be separated from flower initiation.

A genetic framework for floral patterning

We have shown, for representatives of each of the three ABC classes of floral homeotic genes, that their activation by *LFY* can be uncoupled from the initiation of flowers, and we conclude that *LFY* not only confers flower-meristem identity at an early stage of flower development, but also has a different role in the later activation of ABC genes. Because of the early role of *LFY* in flower development, and because of redundancy of *LFY* with other genes, we used untraditional approaches, such as using transgenic plants that express an activated version of LFY, to dissect the different roles of LFY. This strategy, applied previously to transcription factors from *Caenorhabditis elegans*[33] and *Drosophila melanogaster*[34], has proven very informative, and could be used in the study of many plant transcription factors. However, our gain-of-function studies were guided by previous loss-of-function analyses, and loss-of-function data together with careful controls are essential to validate results generated with the kind of approaches used here. Interestingly, the VP16 fusion did not equally affect all known functions of *LFY*, including flower initiation or activation of B-function genes. This could be because the binding of LFY to target sites in certain promoters is regulated, or because a minimal concentration of LFY is required for cooperative DNA binding to selected sites, in which case adding the VP16 domain would have no effect below a threshold of LFY.

Our results indicate that different mechanisms may regulate each of the three ABC genes that we have identified as *LFY* targets (Fig. 7). The A-function gene *AP1* is initially expressed uniformly throughout floral primordia, where it is activated shortly after *LFY* activation[9,18]. As *LFY* can induce *AP1* very early during the formation of any primordium that could potentially become a flower, no region- or flower-specific co-regulator is needed for *AP1*. In contrast to *AP1*, both *AG* and *AP3* are activated in region-specific patterns within flowers[19,21]. Activation of *AP3* seems to rely mainly on the combination of LFY, which is uniformly expressed in flower meristems, and other factors such as UFO, which is expressed in a region-specific pattern in both shoot and flower meristems. In this case, *LFY* provides flower-meristem specificity and *UFO* provides region specificity. A similar situation could apply to *AG*, that is, LFY may act in combination with another factor that is expressed in the centre of both shoot and flower meristems. Note that shoot meristems resemble stage 2 flower meristems, both morphologically and at the level of gene expression. In addition to *UFO*, several other genes are expressed in similar patterns in shoot and stage 2 flower meristems, although mutations in these genes—in contrast to mutations in *UFO*—disrupt development of both shoots and flowers[35–37].

A scenario that emerges from these observations is that the patterning of ABC gene expression is achieved by co-opting a meristem patterning system that evolved before flowers appeared. To this underlying pattern, which is common to shoot and flower meristems, are added a few flower-specific factors that are not region-specific themselves, such as LFY, and the combination of these two classes of factors provides region specificity within flowers. This model may explain why the search for specific regulators of ABC gene expression has met with limited success. If most factors controlling region-specific expression of ABC genes also affect shoot development, it might be difficult to uncover them in conventional genetic screens. Other unresolved problems include the molecular mechanisms by which LFY controls the expression of target genes; the mechanistic basis of redundancy of *LFY* and *UFO* with other meristem-identity and patterning genes; and the nature of the interaction of *LFY* with other important floral regulators, such as *AP2*, *LEUNIG* or *CURLY LEAF*[23,38,39]. □

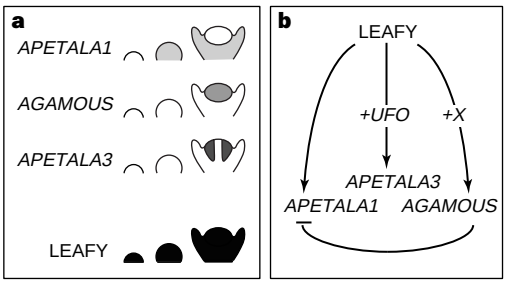

Figure 7 Model for ABC gene activation by LFY. **a**, Comparison of the expression profiles of LFY protein and ABC gene RNAs. Left, stage 0 flower; middle, stage 1 and 2 flower; right, stage 3 flower. **b**, Effect of LFY on different targets. The VP16 activation domain makes LFY independent of factor 'X'.

Methods

Plasmid constructs. Plant expression: *LFY:VP16* was generated by inserting a segment encoding the VP16 activation domain (amino acids 413–490), amplified by PCR from plasmid pRG50 (ref. 14), into a *LFY* genomic fragment (nucleotides 465–2,844, 2,845–5,937 of GenBank accession number M91208 (ref. 9). The *LFY:VP16* chimaera was cloned into the transformation vector pCGN1547 (ref. 40), producing pDW245. *LFY:mVP16* (pFP21) was generated in the same way, using the sequence encoding a mutant, truncated VP16 activation domain (amino acids 413–456), originating from plasmid pMSVP16 ΔC119/FP442 (ref. 17). To generate *mLFY:VP16* (pFP17), we used PCR to replace the region 3′ to *VP16* sequences in pDW245 by a stop codon followed by the transcriptional terminator of the nopaline synthase gene. For *35S::LFY:VP16*, a chimaera containing *LFY* complementary DNA and the wild-type *VP16* activation domain was generated in pBluescriptKS$^+$ (pFP10). The *LFY:VP16* cDNA was then inserted into the 35S vector pCGN18 (ref. 41), to yield pFP15.

Bacterial expression: The *LFY* open-reading frame, encoding a variant with a deletion of unconserved amino acids 391–421, was cloned into the *Escherichia coli* expression vector pET28a$^+$ (Novagen), to yield pMX013. The deletion has minimal effects on LFY activity, as assayed by constitutive expression in plants (pIL12), but allows LFY expression in bacteria.

Yeast expression: We cloned a 194-base-pair (bp) *Bgl*II/*Sal*I fragment from the *AP1* promoter into pLG178 (ref. 42), to generate the reporter pFP30; *LFY* and *LFY:VP16* cDNAs were subcloned into p424 (ref. 43), to generate the *LFY* and *LFY:VP16* effector constructs (pFP13 and pFP14, respectively). We cloned a *LFY* cDNA into pJG4-5 (ref. 44), to generate the LFY:B42 fusion (pDW188).

***In vitro* studies.** LFY protein with amino- and carboxy-terminal hexahistidine tags was expressed in *E. coli* BL21-DE3 cells. Insoluble protein was isolated as inclusion bodies, denatured, affinity-purified over a Ni-NTA (Qiagen) column, and renatured by stepwise dialysis against decreasing concentrations of urea. Oligonucleotides were labelled with T4-polynucleotidekinase and [γ-^{32}P]ATP. The binding reaction, with 25 fmol of target DNA, was done for 10 min on ice in 20 mM Tris pH 7.5, 150 mM NaCl, 0.25 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 20 mM $MgCl_2$, 0.02% NP-40 and 12.5 ng μl^{-1} poly(dI·dC). The wild-type sequence for the electrophoretic mobility shift assay was 5′-TTG GGG AAG GA*C* *C*AG TGG TCC GTA CAA TGT-3′, the italicized bases were changed to AA in the mutant. Yeast transformations and β-galactosidase activity measurements were done as described[44,45].

Plant material. The following transgenic lines were used: DW151.2.5L and DW151.2.5C (*35S::LFY*) (refs 16, 46); DW229.5.3 (*35S::UFO*) (ref. 25); AM154.5c (*AP1::GUS*) (ref. 47); 1008.5 (*AP3::GUS*) (ref. 48); and pAG-I::GUS (*AG::GUS*) (ref. 49). GUS assays[15] were done with material grown in short days.

Expression analyses. We generated antisense RNA probes for *in situ* hybridization from plasmids pDW119 (*LFY*) (ref. 9), pCIT565 (*AG*) (ref. 19), pD793 (*AP3*) (ref. 21), and pAM128 (*AP1*) (ref. 18). Hybridization and signal detection were as described[50] (see also http://www.wisc.edu/genetics/CATG/barton/protocols.html), except that RNase treatment was omitted and counter-staining of some sections was done with basic fuchsin. For RT–PCR, RNA was extracted from 6-day-old seedlings grown in short days using the RNeasy Plant Mini Kit (Qiagen). RT–PCR was carried out with the Titan RT–PCR kit (Boehringer). An *AP1* cDNA fragment was detected using oligonucleotides 5′-GCA CAT CCG CAC TAG AAA AAA CCA AC-3′ and 5′-CTT CTT GAT ACA GAC CAC CCA TGT-3′. As a control, a fragment from the gene encoding eIF4A was amplified using 5′-TTC TCA AAC CAT AAG CAT AAA TAC CC-3′ and 5′-AAA CTC AAG GAA GTA CTT GAG GGA CAA-3′. Aliquots were analysed after 25 cycles.

Scanning electron microscopy. Tissue was prepared as described[1], and viewed in a Cambridge S360 microscope at an accelerating voltage of 10 kV.

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