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Redundant regulation of meristem identity and plant architecture by *FRUITFULL, APETALA1* and *CAULIFLOWER*

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SUMMARY

The transition from vegetative to reproductive phases during *Arabidopsis* development is the result of a complex interaction of environmental and endogenous factors. One of the key regulators of this transition is *LEAFY (LFY)*, whose threshold levels of activity are proposed to mediate the initiation of flowers. The closely related *APETALA1 (AP1)* and *CAULIFLOWER (CAL)* meristem identity genes are also important for flower initiation, in part because of their roles in upregulating *LFY* expression. We have found that mutations in the *FRUITFULL (FUL)* MADS-box gene, when combined with mutations in *AP1* and *CAL*, lead to a dramatic non-flowering phenotype in which plants continuously elaborate leafy shoots in place of flowers. We demonstrate that this phenotype is caused both by the lack of *LFY* upregulation and by the ectopic expression of the *TERMINAL FLOWER1 (TFL1)* gene. Our results suggest that the *FUL*, *AP1* and *CAL* genes act redundantly to control inflorescence architecture by affecting the domains of *LFY* and *TFL1* expression as well as the relative levels of their activities.

Key words: Inflorescence meristem, Flowering time, Developmental phases, Flower development, MADS-box, *LEAFY (LFY), TERMINAL FLOWER1 (TFL1), APETALA1 (AP1), CAULIFLOWER (CAL), FRUITFULL (FUL)*

INTRODUCTION

Postembryonic development in *Arabidopsis* proceeds through a series of phases, each characterized by the identity of the lateral primordia produced by the shoot apical meristem (SAM) (Poethig, 1990). During the vegetative phase, the SAM produces closely spaced leaf primordia, each subtending a secondary shoot meristem, to form a rosette. During the reproductive, or inflorescence (I) phase, the SAM produces determinate floral meristems on its flanks. The last few vegetative leaves produced are referred to as cauline leaves and become separated along the inflorescence stem by longer internode distances. Thus, the production of leaves can be considered to occur within two distinct subphases, V_1 (rosette) and V_2 (cauline).

Genes that promote flowering in *Arabidopsis* were identified as mutations that extend the duration of the V phase, increasing the number of leaves formed before the development of flowers, but generally not affecting the fate of the lateral primordia produced during the I phase (reviewed by Piñeiro and Coupland, 1998). Another group of genes, including *TERMINAL FLOWER1 (TFL1)*, act by delaying phase change and preventing the normally indeterminate SAM from becoming a flower (Alvarez et al., 1992; Shannon and Meeks-Wagner, 1991). In addition, several meristem-identity genes are responsible for conferring floral characteristics to the lateral primordia produced by the SAM during the I phase. Mutations in floral meristem identity genes cause primordia that would develop into flowers to instead develop shoot characteristics. The best characterized of these genes are *LEAFY (LFY)*, *APETALA1 (AP1)*, *APETALA2 (AP2)* and *CAULIFLOWER (CAL)* (for review, see Yanofsky, 1995). Only *lfy* and *ap1* mutants show dramatic flower-to-shoot phenotypes, especially in the most basal nodes. Furthermore, the nearly complete conversion of flowers into shoots observed in *lfy ap1* double mutants reveals that they act redundantly to specify meristem fate (Bowman et al., 1993; Huala and Sussex, 1992; Irish and Sussex, 1990; Schultz and Haughn, 1991; Shannon and Meeks-Wagner, 1993; Weigel et al., 1992). Together, the *LFY, AP1, CAL* and *AP2* genes appear to reinforce each other's activities leading to a sharp transition from vegetative to reproductive development.

The *FRUITFULL (FUL)* gene encodes a MADS-box protein that has previously been shown to be required for carpel and fruit development (Gu et al., 1998; Mandel and Yanofsky, 1995a). However, in addition to its expression domain during carpel and fruit development, the *FUL* gene is upregulated in the SAM at around the transition to flowering, suggesting that it may also play a role during this transition (Mandel and Yanofsky, 1995a; Hempel et al., 1997). *FUL* is closely related to the meristem identity genes *AP1* and *CAL*, suggesting the possibility of functionally redundant activities.

In this work we have undertaken a molecular genetic approach to uncover the possible roles of *FUL* in the transition to flowering as well as its interactions with different meristem identity genes. We have found that in addition to its role during carpel and fruit development, *FUL* acts as a flowering-time and meristem-identity gene. These studies provide new insights into the functional redundancy of MADS-box genes during the transition to flowering and on the upregulation of the *LFY* meristem identity gene.

MATERIALS AND METHODS

Plant material and growth conditions

The *ap1-1, ful-1, tfl1-2* and *lfy-26* alleles have been described previously (Bradley et al., 1997; Gu et al., 1998; Lee et al., 1997; Mandel et al., 1992). The *cal-5* allele was generated in a γ-irradiation mutagenesis experiment and contains a single base-pair deletion 33 bp downstream of the translation initiation codon that causes a frame shift and introduces a STOP codon 19 amino acids later (Savidge, 1996). 35S::LFY lines (DW151.2.5, in Landsberg *erecta* background; Weigel and Nilsson, 1995) and LFY::GUS (DW150.209, in Columbia; Blázquez et al., 1997) were kindly provided by Detlef Weigel. The 35S::AG line was obtained from Hong Ma (Mizukami and Ma, 1992). For all experiments, seeds were vernalized for 3-5 days at 4°C, then germinated and grown at 22-24°C under continuous light conditions.

Characterization of the molecular lesions in the *ful* alleles

For *ful-2, ful-4, ful-5* and *ful-6*, genomic DNAs were amplified by PCR with the primers OAM25 (5′-GGTCATTTCAGGGTTGT-CGGTT-3′) and OAM14 (5′-AATCATTACCAAGATATGAA-3′), which hybridize respectively 59 ncl upstream of the initiation codon and 202 ncl downstream of the STOP codon of the *FUL* gene. The amplification products of two independent reactions were sequenced and compared with the wild-type sequence for each allele. For *ful-5*, since the sequencing of the *FUL* genomic DNA only showed a silent change in the coding region, we analyzed the sequence of the transcribed RNA by performing a reverse transcription of the *ful-5* RNA using OAM14 as a primer, coupled with a PCR amplification using OAM25 and OAM14 as primers.

GUS activity measurements

For quantitative measurements of GUS activity in LFY::GUS *ful-2* plants, the assay described by Blázquez et al. (1997) was used.

In situ hybridizations

For in situ experiments at day 12 in *ap1 cal* and *ful ap1 cal* plants, genotyping for the presence of the *ful-1* allele was necessary since double and triple mutants were indistinguishable (see below).

Tissue was fixed for 2 hours at room temperature in FAE solution (ethanol:acetic acid:formaldehyde:water, 50:5:3.5:41.5, v/v/v/v), dehydrated, embedded and sectioned to 8 μm. After dewaxing in histoclear and rehydration, sections were treated for 20 minutes in 0.2 M HCl, neutralized for 10 minutes in 2× SSC and incubated for 30 minutes with 1 μg/ml Proteinase K at 37°C. Proteinase action was blocked with 5 minutes incubation in 2 mg/ml Gly and 10 minutes postfixation in 4% formaldehyde. Tissue sections were washed in PBS, dehydrated through an ethanol series and dried under vacuum before applying the hybridization solution (100 μg/ml tRNA; 6× SSC; 3% SDS; 50% formamide, containing approx. 100 ng/μl of antisense DIG-labeled RNA probe). Sections were hybridized overnight at 52°C, washed twice for 90 minutes in 2× SSC; 50% formamide at 52°C and the antibody incubation and color detection was performed according to the manufacturer instructions (Boehringer). The probes were synthesized as previously described using plasmids pDW122 (LFY; Weigel et al., 1992), pCIT565 (AG; Drews et al., 1991), pD793 (AP3, Jack et al., 1992) and pSL66 (TFL1; Liljegren et al., 1999).

For double labeling experiments, a DIG-labeled *TFL1* probe and a fluorescein-labeled *LFY* probe were both added to the hybridization solution. Washes, DIG-antibody incubation and color detection with NBT-BCIP as substrates were performed as described above to reveal *TFL1* expression as a blue precipitate. Slides were treated in 2× SSC for 2 hours at 65°C to inactivate the alkaline phosphatase coupled to the DIG-antibody and then incubated with fluorescein-antibody. Color detection was performed according to the manufacturer instructions (Boehringer) using as a substrate Fast Red tablets to reveal *LFY* expression as a red signal.

Scanning electron microscopy (SEM)

Inflorescences were collected, fixed and observed as previously described (Gu et al., 1998).

Generation and identification of multiple mutants

In all combinations the *ful-1* allele was used, except for the generation of LFY::GUS *ful-2* lines. *ful-1* carries a Ds:GUS element that allows the identification of the mutant allele by assaying GUS activity in cauline leaves. *ful-1* was crossed as female to homozygous mutants *ap1-1*, *ap1-1 cal-5* and *tfl1-2* and double/triple mutants were identified in F_2 populations as new/additive phenotypes segregating in a 1:16 ratio (1:64 for *ful ap1 cal*).

Since *LFY* and *FUL* are closely linked (approximately 1 cM), *ful-1* pollen was crossed onto *lfy-26* homozygous plants and GUS detection was performed on F_2 plants with *lfy* phenotype until one positive was found, and pollinated with *ful-1* pollen. In the F_1 from this cross, plants with *ful* phenotype were selected as *ful-1 lfy-26/+* and selfed, and F_2 *lfy* plants were assumed to be homozygous for both mutations.

lfy-26 ap1-1 cal-5 ful-1 were generated by crossing *ap1-1 cal-5* onto *ful-1 lfy-26*. Plants with *ap1 cal* phenotypes in the F_2 generation were tested for GUS activity, and the positives were selfed and assumed to have an *ap1 cal (ful lfy)/++* genotype. The F_3 populations resulting from these selfed individuals segregated plants with *ap1 cal* and *ap1 lfy*-like phenotypes in a 3:1 ratio, and the latter ones were assumed to be the quadruple mutant combination, since given the close linkage between *LFY* and *FUL*, 99% of the F_3 *lfy* plants would be *ful* homozygous.

For the generation of *tfl1-2 ap1-1 cal-5 ful-1* quadruple mutants, *tfl1-2 ful-1* plants were used as females for *ap1-1 cal-5* pollen. *ful* phenotypes were selected and selfed in the F_1 generation and in the F_2, since no new phenotypes were observed, the presence of the *cal-5* allele was analyzed in plants with *ap1 ful tfl* phenotype as described below.

For 35S::LFY *ap1-1 cal-5 ful-1*, 35S::LFY plants were used as pollen donors to fertilize *ap1-1 cal-5 ful-1/+* emasculated flowers. 35S::LFY phenotypes with GUS activity were selected and selfed in the F_1 generation, and in the F_2, plants with *ful* fruit phenotypes were genotyped for the presence of 35S::LFY, *ap1-1* and *cal-5* and further reconfirmed by the segregation of the selfed F_3 progenies. For 35S::AG *ap1-1 cal-5 ful-1*, the same strategy was adopted, except that in the F_2 population, a new phenotype was identified as the quadruple combination.

Genotyping

To genotype plants for the presence of the *ap1-1* allele, we used dCAPS markers (Neff et al., 1998). The *ap1-1* mutation introduces a change from G to A at the acceptor site before exon 4 (Mandel et al., 1992). For genotyping, genomic DNA was amplified by PCR using the primers 5′-GCAAGTCTTCCCCAAGATAAGGC-3′ and 5′-GACAGCTTATTGCACCTGAG-3′. The restriction enzyme *Stu*I cleaves only wild-type DNA yielding a 290 and a 22 bp fragment.

For *cal-5* genotyping, a *Fok*I RFLP was used. Genomic DNA was amplified by PCR with primers 5′-ATGGGAAGGGG-TAGGGTTG-

3′ and 5′-ATTCAGAG-GAGTACTCGAAC-3′. Digestion with *Fok*I generates two fragments of 42 and 140 bp in *cal-5* DNA, while wild-type DNA is not digested.

ful-1 plants were genotyped by PCR on genomic DNA with the primer AGL8PG: 5′-TGTATTCACGTCACATACCG-3′, located in the promoter region of the *FUL* gene, and primers AGL8MG: 5′-CTCATGAGC-TTTCTTGAGC-3′ and GUS3: 5′-CTTGTAACG-CGCTTTCCC-3′, located respectively in the coding regions of the FUL gene and the GUS gene of the Ds-element inserted in the *ful-1* allele. *ful-1* homozygotes were identified by the detection of an amplification product with primers AGL8PG/GUS3, and no product with the pair AGL8PG/AGL8MG.

The presence of the 35S:LFY transgene was assessed by PCR on genomic DNA with the primers 5′-ACCCAAGCTTCT-TCGCAAGACCCTTCCTCT-3′, located in the 35S promoter region, and 5′-AACTAGAAACGCAAGTCG-3′, located in the *LFY* coding region, which only amplified the transgene and no endogenous sequences.

RESULTS

The *fruitfull* phenotypes

The *ful-1* mutation affects several aspects of plant development. The most dramatic effect is observed within the carpel where, from stage 12 (Smyth et al., 1990), the valve cells fail to elongate and differentiate, and the replum cells grow with an altered morphology. As a result, silique development is severely affected, leading to short fruits with small crowded seeds (Fig. 1A,J,N; Gu et al., 1998). In addition to the fruit phenotype, *ful-1* cauline leaves are also affected in shape, cell organization and vascular differentiation (Gu et al., 1998).

We analyzed five new *ful* alleles (see Materials and Methods) and examined their phenotypes. *ful-4*, *ful-5* and *ful-6* were isolated after EMS mutagenesis in the Landsberg *erecta* (L*er*) background. The strong *ful-5* allele, which has a phenotype very similar to *ful-1*, has a single base pair change from C to T in position 156 of the first exon. This mutation results in the generation of an alternate donor splicing site at position 154 that causes a 31 base deletion in the messenger RNA, a frame shift and a truncated protein. *ful-4* and *ful-6* contain single base pair mutations from G to A in the 5th and 6th exon-intron boundaries respectively. *ful-4* has an intermediate phenotype, with a similar arrested development of the valves, but a less pronounced defect in replum morphology (Fig. 1D,L,P). *ful-6* is a weak allele, which causes the valve cells differentiate and expand to almost the same extent as the wild type, and the replum cells undergo mild enlargement, allowing the formation of a functional dehiscence zone in the valve-replum boundaries (Fig. 1E,M,Q).

As a result of these two alleles a striking elongation of the style was observed in genetic backgrounds different from L*er*. *ful-2* is an EMS mutagenized line in the Columbia (Col)

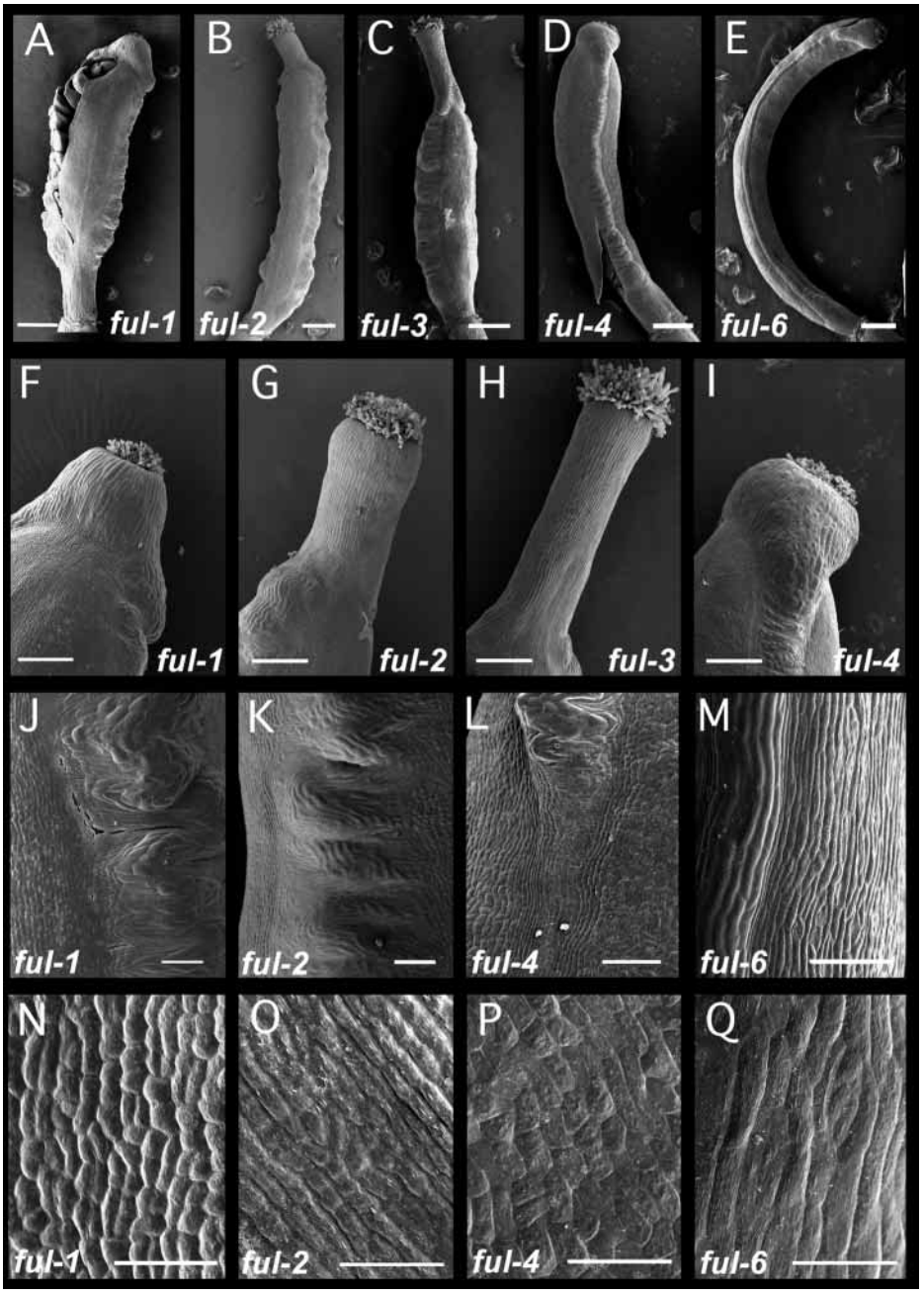

Fig. 1. Scanning electron micrographs of *fruitfull* mutants showing fruits 10 days after pollination (late stage 17; according to Smyth et al., 1990). (A-E) *ful* allelic series. (F-I) Style phenotypes from four different *ful* alleles. (F and I) *ful-1* and *ful-4* respectively, both in the Landsberg *erecta* background. (G) A *ful-2* style, in Columbia background and (H) a *ful-3* style, in Nossen background. Close-up of (J-M) the valve-replum boundary and (N-Q) the valve epidermal cells in four different *ful* mutants as indicated. Scale bars, 500 μm (A-E), 200 μm (F-I), 100 μm (J-M) and 50 μm (N-Q).

Table 1. Time to flowering under continuous light conditions

Genotype	No. rosette leaves	No. cauline leaves	Total leaves
Landsberg *erecta*	7.9±0.6	2.9±0.4	10.8±0.6
*ful-1**	9.7±0.4	4.2±0.3	13.9±0.4
Columbia	11.3±1.0	3.3±0.3	14.3±1.1
ful-2	12.9±1.1	5.0±0.5	17.9±1.2
*lfy-26**	8.7±0.7	7.1±1.1	15.8±1.2
*ful-1 lfy-26**	9.9±0.5	10.2±1.1‡	20.3±1.1
*LFY/lfy-26**	9.0±0.5	3.9±0.3	12.9±0.5
*ful-1 LFY/lfy-26**	9.5±0.6	5.9±0.5‡	15.4±0.5

Flowering time is expressed as the number of leaves produced by the main shoot.

LFY/lfy-26 and *ful-1 LFY/lfy-26* plants were identified by genotyping of the progenies of segregating populations with a CAPS marker (see Materials and Methods).

Values are mean ± standard error. $n \geqslant 15$ in all cases.

*All in Landsberg *erecta* background.

‡Refers to the number of leaves subtending shoots.

background, and contains a nonsense mutation in exon 3 (W91 to STOP). *ful-3* is a Nossen line with a Spm transposon element inserted into the last intron. The *ful-2* and *ful-3* styles of late stage 17 fruits are 2-3 times longer than the Col wild type (Fig. 1F-H). *ful-2* siliques have a less severe phenotype in the valves, whose cells elongate to a small extent allowing a modest expansion of the fruit, although, as in *ful-1*, no differentiation of stomata is observed. *ful-3* is a strong allele producing a similar phenotype to *ful-1* in valves and replum. Even though a gradation in the silique phenotypes was observed in the allelic series, the cauline leaves in all mutants were affected similarly, suggesting that the *FUL* function in leaf morphology requires its complete activity (results not shown).

Mutations in *FRUITFULL* affect flowering time

Since *FUL* is strongly upregulated in the shoot apical meristem shortly after the transition to flowering, and in response to photoinductive conditions, a possible role for *FUL* in promoting the initiation of flowers has been proposed (Hempel et al., 1997; Mandel and Yanofsky, 1995a). To investigate this possibility, we quantified flowering time in *ful* mutants grown under continuous light. *ful* plants showed a small, significant delay in the time to flower when measured both in number of leaves (Table 1) and days to bolt (results not shown). The increase in leaf number was observed for both rosette and cauline leaves, and no significant effect of the *erecta* mutation was observed, since both *ful-1* (L*er*) and *ful-2* (Col) mutants flowered later than the corresponding wild-type ecotypes. These results suggest that *FUL* acts to promote the flowering pathway, although, given the slight effect in flowering time, it may be involved in a highly redundant network of signaling.

FUL and *LFY* act in parallel pathways

One candidate for a gene that interacts with *FUL* in this flower-promoting pathway is *LFY*, as *LFY* levels have been shown to be important for the transition to flowering (Blázquez et al., 1997). *lfy* mutants have an extended V_2 phase and are defective in floral meristem identity specification (Huala and Sussex, 1992; Schultz and Haughn, 1991; Weigel et al., 1992). In response to photoinductive treatments, *LFY* and *FUL* are upregulated at the shoot apex in an overlapping pattern (Blázquez et al., 1997; Hempel et al., 1997).

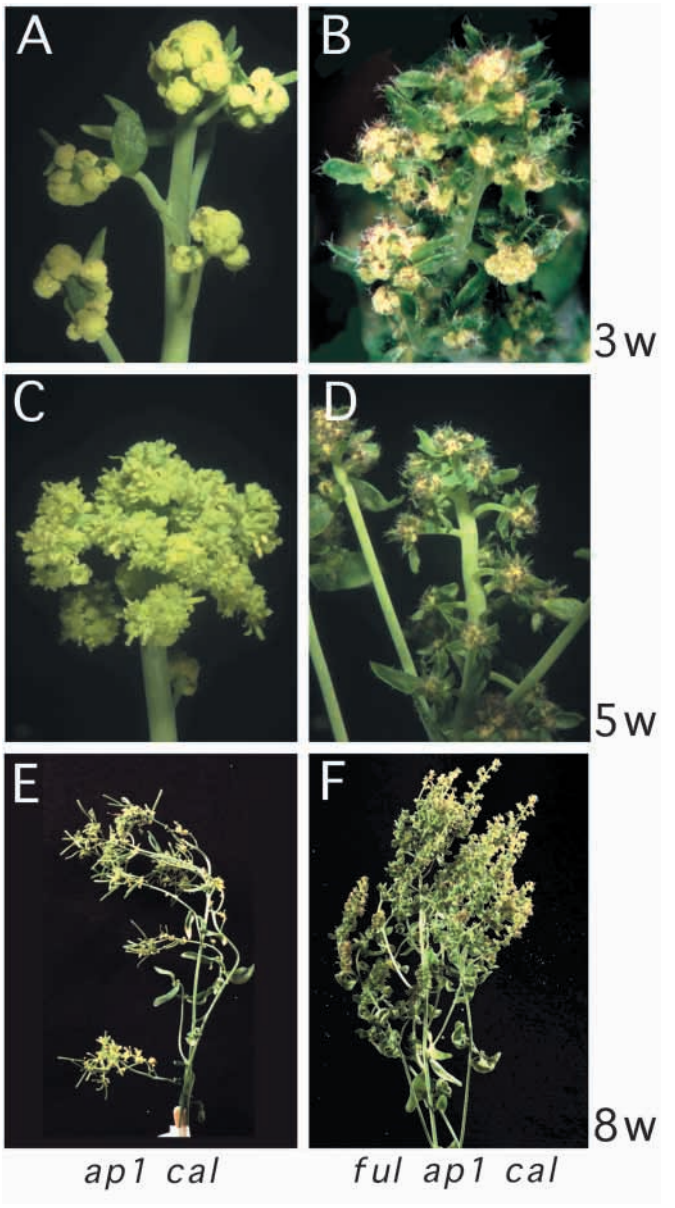

Fig. 2. Mutant phenotypes of *ful ap1 cal* and *ap1 cal* plants. (A-B) Apical inflorescences of 3-week-old *ap1 cal* (A) and *ful ap1 cal* (B) plants. (C-D) Apical inflorescences of 5-week-old *ap1 cal* (C) and *ful ap1 cal* (D) plants. Mature floral organs are visible in *ap1 cal*, but absent in *ful ap1 cal* inflorescences. (E) An *ap1 cal* plant, 8 weeks after germination. Mature siliques have already developed. (F) A *ful ap1 cal* plant, 8 weeks after germination. The inflorescence structures have branched out producing numerous leafy shoots, but no flower structures are evident.

To investigate whether the late flowering phenotype in *ful* mutants might be caused by a delay in *LFY* upregulation or a reduction in its levels, we introduced a LFY::GUS transgene into *ful-2* plants (see Methods). We did not observe any effect of the *ful* mutation on the upregulation of *LFY* (not shown), suggesting that either *LFY* is not directly regulated by *FUL*, or that other genes can substitute for *FUL* to upregulate *LFY*. In addition, we generated *ful-1 lfy-26* double mutants, which

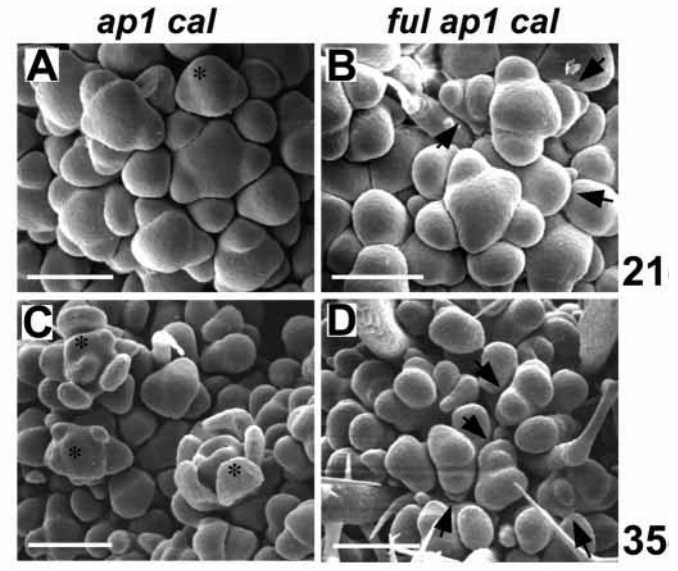

Fig. 3. Scanning electron micrographs of *ap1 cal* (A,C) and *ful ap1 cal* (B,D) apical meristems. In 21-day-old *ap1 cal* inflorescences (A), floral meristems (*) up to stage 3 of development (according to Smyth et al., 1990) are visible. *ful ap1 cal* apices of the same age (B) initiate a higher number of cauline leaves with axillary meristems (arrows) and no floral meristems are visible. 35-day-old *ap1 cal* inflorescences (C) are composed of floral meristems at several developmental stages (*), some with characteristics of inflorescence meristems, such a spiral phyllotaxy. 35-day-old *ful ap1 cal* apices (D) show a proliferation of mainly leaf primordia with axillary meristems (arrows), with no recognizable phyllotactic arrangement. Scale bars, 100 μm.

flowered later than the corresponding *lfy-26* and *ful-1* single mutants (Table 1). Moreover, a similar quantitative effect was observed in *ful-1* plants heterozygous for *lfy-26*, which flowered significantly later than either *ful-1* mutants or *LFY*/*lfy* plants, with a more dramatic increase in the duration of the V_2 phase as scored by the number of cauline leaves produced (Table1), suggesting that *LFY* and *FUL* act in parallel pathways or at least influence the same process independently. *ful-1 lfy-26* plants also showed an additive phenotype in the floral identity defects, with the same kind of flower-to-shoot transformations as in *lfy-26*, and the *ful-1* phenotype in the carpel-like organs that eventually formed (not shown).

Flowering is eliminated in *ful ap1 cal* triple mutants

The *AP1* gene is required for both flower meristem and flower organ identity. (Irish and Sussex, 1990; Bowman et al., 1993).

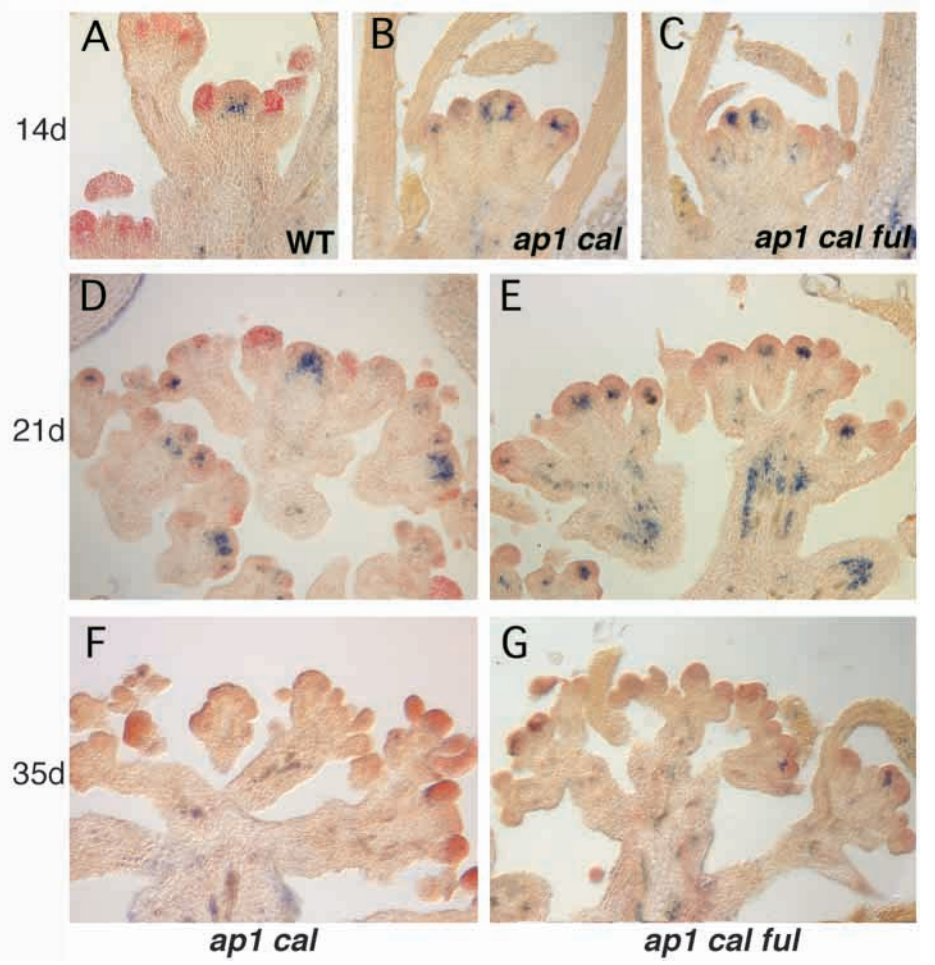

Fig. 4. Expression of *TFL1* and *LFY* in wild-type, *ap1 cal* and *ful ap1 cal* plants. Sections of wild-type apices at day 14 (A), *ap1 cal* plants at days 14 (B), 21 (D) and 35 (F) and *ful ap1 cal* plants at days 14 (C), 21 (E) and 35 (G) probed simultaneously with *LFY* (red label) and *TFL1* (blue label) antisense RNA. In day-14 wild-type plants (A) *LFY* is strongly expressed in flower meristems arising laterally from the inflorescence apex. *TFL1* is expressed below the inflorescence meristem in a region not overlapping with *LFY*. In day-14 *ap1 cal* plants (B), *LFY* is weakly expressed and *TFL1* is ectopically detected below the lateral meristems initiated. *LFY* and *TFL1* domains of expression overlap in some regions, as described by Ratcliffe et al. (1999). In day-14 *ful ap1 cal* meristems (C), *LFY* and *TFL1* expression is similar to *ap1 cal* at the same time point. In day-21 *ap1 cal* inflorescences (D,E), *LFY* is detected at high levels in some meristems, presumably those already committed to a floral fate. *TFL1* is not found in highly *LFY*-expressing meristems, and is strongly detected in those putatively behaving as inflorescence meristems. Some regions still show the early pattern of *LFY* and *TFL1* coexpression. Day-21 *ful ap1 cal* plants still show very low levels of *LFY* expression, and *TFL1* can be detected below most of the meristems initiated, in a similar pattern to earlier time points. At 35 days, *ap1 cal* inflorescences show high *LFY* expression in floral meristems and *TFL* is barely detectable. In 35-day-old *ful ap1 cal* plants (G), *LFY* can be detected at similarly low levels, whereas *TFL1* expression is reduced and restricted to a few meristems.

Mutations in the closely related and partially redundant *CAL* gene enhance the *ap1* mutant phenotype, such that *ap1 cal* double mutants proliferate inflorescence meristems in positions that would normally be occupied by flowers, resulting in a 'cauliflower' appearance. Eventually, however, flower meristems are specified and *ap1*-like flowers are produced (Bowman et al., 1993; Kempin et al., 1995; Fig. 2A,C,E). The *AP1* gene also plays a role in negatively regulating *FUL* in emerging flower primordia, as *FUL* is ectopically expressed in *ap1* and *ap1 cal* mutant flower meristems (Mandel and Yanofsky, 1995a; M. A. Mandel, C. F. and M. F. Y, unpublished observations).

The close sequence similarity of *FUL*, *AP1* and *CAL* (Mandel and Yanofsky, 1995a), together with the fact that *FUL* is expressed throughout the proliferating meristems of *ap1 cal* double mutants, suggested the possibility that the ability of *ap1 cal* mutants to eventually form flowers could be the result of ectopic *FUL* activity. In order to test this hypothesis, we introduced the *ful-1* mutation into *ap1-1 cal-5* mutant plants. We found that mutations in *ful* dramatically enhanced the *ap1 cal* double mutant phenotype such that the triple mutant plants proliferated leafy shoots and failed to flower. After bolting, the *ful ap1 cal* shoot apical meristem gave rise to cauline leaves with associated axillary meristems which repeated this pattern to form leafy shoots with small axillary 'cauliflowers' along the main inflorescence, which as a consequence, branched out extensively to adopt a bushy appearance (Fig. 2B,D,F).

A closer inspection by SEM of *ap1 cal* and *ful ap1 cal* inflorescences revealed differences in the behavior of the proliferating meristems. Early in their development, *ap1 cal* double mutants displayed the conversion of flowers into inflorescence meristems. In 21-day-old plants, fifth order inflorescence meristems arranged in a spiral phyllotaxy were apparent and some of the older meristems had visibly acquired a flower identity (Fig. 3A). After 35 days of growth, flowers with developing stamens and carpels were visible in the lower branches, and in the more apical positions new flowers were differentiating (Figs 2C, 3C). After 2 months of growth, the meristems had stopped proliferating and fully developed siliques were observed (Fig. 2E).

By contrast, the *ful ap1 cal* meristems at 21 days typically produced cauline leaf primordia with associated axillary meristems as well as two or three additional meristems not subtended by leaves, yielding a phyllotaxy intermediate between spiral and cruciform (Fig. 3B). At these and all later time points, no floral structures were evident, and the proliferating meristems gave rise to new meristems and cauline leaves at a lower rate, losing all phyllotactic arrangement (Fig. 3D). The *ful ap1 cal* plants kept under our standard growth room conditions failed to produce any kind of flower structures even six months after germination, and remained in this vegetative state until they died. Interestingly, we did observe on one occasion, when accidental overheating of the growth room occurred, that some of these plants formed a few floral organs after several months of vegetative development. These results suggest that there may be a threshold required to induce flowering, and that although the triple mutant is normally just below this threshold, conditions such as heat stress can allow the requirement for *FUL/AP1/CAL* to be overcome.

In contrast to the enhancement of the *ap1 cal* double mutant phenotype, we did not observe enhancement of the *ap1* mutant phenotype when *ful-1 ap1-1* double mutants were generated. The phenotype of the double mutants was found to be strictly additive for both flowering time and flower and fruit development (results not shown). Likewise, *ful-1 cal-5* double mutants were indistinguishable from the *ful-1* single mutants, consistent with previous observations showing that mutations in *CAL* do not confer a mutant phenotype in the presence of a functional copy of *AP1*. These results indicated that both *AP1* and *CAL* are able to compensate for the loss of *FUL* function in specifying floral meristem identity.

Overexpression of *LFY* rescues the non-flowering phenotype of *ful ap1 cal* plants

The non-flowering phenotype of *ful ap1 cal* mutants is likely the result of reduced activity of the *LFY* meristem identity gene product, and there are two distinct explanations for how *FUL/AP1/CAL* can contribute to LFY activity. One scenario is that *LFY* RNA expression in the *ful ap1 cal* triple mutant may not reach the threshold required for flower specification. An alternative scenario is that the activity of the LFY protein may require one of the AP1, CAL or FUL functions, for example as a cofactor, even though *LFY* RNA levels in the triple mutant may be sufficient to promote flowering.

To distinguish between these possibilities, we compared the expression of *LFY* in *ap1 cal* double mutants to that in *ful ap1 cal* triple mutants grown under continuous light. In both *ap1 cal* and *ful ap1 cal* plants 14 days after sowing (d14), *LFY* RNA levels were much lower than in the corresponding wild-type controls (Fig. 4A-C). In d21 *ap1 cal* inflorescences, floral meristems and flowers up to stage 5 of development could be easily distinguished, whereas in *ful ap1 cal* mutants, no floral characteristics appeared (Fig. 3A,B). *LFY* expression was detected at high levels in all floral meristems of *ap1 cal* plants. In contrast, *LFY* RNA levels were significantly reduced in *ful ap1 cal* triple mutants, although the accumulation of *LFY* RNA was still readily detected (Figs 4E, 6A,B). These results suggests that *AP1*, *CAL* and *FUL* play a redundant role in boosting *LFY* RNA levels but that other factors are capable of inducing *LFY* expression initially.

The reduced levels of *LFY* RNA accumulation in the triple mutant provide molecular evidence that suggests that the failure to upregulate *LFY* may be the cause of the non-flowering phenotype. To demonstrate that this is indeed the case, we introduced a constitutively expressed *LFY* transgene (35S::LFY) into *ful ap1 cal* triple mutants. In contrast to the triple mutant, 35S::LFY *ful ap1 cal* plants were able to produce many *ful ap1*-like flowers, indicating that high levels of *LFY* expression could overcome the lack of *AP1/CAL/FUL* functions (Fig. 5A). Taken together, these data demonstrate that the inability of *ful ap1 cal* plants to flower is due to a reduction of *LFY* expression.

It has been previously described that *ap1* mutations can largely suppress the early flowering phenotype conferred by the 35S::LFY transgene (Mandel and Yanofsky, 1995b; Weigel and Nilsson, 1995). The phenotypes of the 35S::LFY, 35S::LFY *ap1*, 35S::LFY *ap1 cal* and 35S::LFY *ful ap1 cal* showed a gradation in flowering time (Table 2) and number of flowers (not shown) produced by the shoot apical meristem before terminating. These results suggest that the early flowering and shoot-to-flower conversion caused by the

Table 2. Effect of the *ap1/cal/ful* mutations on the 35S::LFY phenotype

Genotype	No. rossette leaves	No. cauline leaves	Total leaves
Landsberg *erecta*	8.1±0.5	2.3±0.6	10.4±0.6
35S::LFY	6.1±0.5	–	6.1±0.5
35S::LFY *ap1*	6.8±0.5	2.3±0.5	9.1±0.5
35S::LFY *ap1 cal*	8.9±0.6	4.2±0.5	13.1±0.6
35S::LFY *ful ap1 cal*	9.3±0.6	10.3±1.1	19.3±1.1

The number of leaves produced by the main shoot is used as a measure of flowering time.
Values are mean ± standard error. $n \geqslant 15$ in all cases.
All in Landsberg *erecta* background.

35S::LFY transgene is mostly due to the subsequent activities of FUL/AP1/CAL.

Because low levels of *LFY* RNA accumulate in *ful ap1 cal* triple mutants, it was unclear if *LFY* was still active in the triple mutant. We therefore introduced the strong *lfy-26* allele into the triple mutant and found the quadruple mutants to be very similar to *lfy ap1* doubles or *lfy ap1 cal* triples (Bowman et al., 1993). The plants showed enhanced vegetative characteristics, developing secondary shoots in place of flowers, subtended by the typical *ful-1* cauline leaves (Gu et al., 1998). The proliferation of 'leafy cauliflowers' that occurs in *ful ap1 cal* triple mutant does not occur in *lfy ful ap1 cal* quadruple mutants. Instead, the quadruple mutant develops vegetative shoots, indicating that some *LFY* activity is needed to cause a reiterative pattern of meristem proliferation. In addition, the similar phenotypes of *lfy ap1* and *lfy ful ap1 cal* indicate that in an *ap1 lfy* background, *FUL* and *CAL* are not able to specify floral meristem identity.

Mutations in *TFL1* suppress the nonflowering phenotype of *ful ap1 cal* plants

A number of studies have demonstrated an antagonistic interaction between *TFL1* and the floral meristem identity genes (Mandel and Yanofsky, 1995b; Savidge, 1996; Weigel and Nilsson, 1995; Weigel et al., 1992; Lijegren et al., 1999; Ratcliffe et al., 1999). To further explore these interactions, we compared the expression patterns of *TFL* and *LFY* in the meristems of *ap1 cal* mutants to that in the *ful ap1 cal* triple mutants. *LFY* is strongly expressed in wild-type flower meristems 14 days after sowing (d14), but is only expressed at low levels in equivalently staged *ap1 cal* double mutants. One week later (d21), when flower meristems become distinct in *ap1 cal* mutants, *LFY* RNA is detected at higher levels and its expression increases at later time points when flowers are already apparent (d35) (Figs 4B,D,F, 6A). In contrast, *LFY* is only detected at low levels in *ful ap1 cal* mutants, even after two months of growth (Figs 4C,E,G, 6B).

TFL1 is normally expressed in the center of the inflorescence apex and is not detected in the lateral meristems committed to a floral fate (Fig. 4A). *TFL1* expression was found at comparable levels and pattern in *ap1 cal* and *ful ap1 cal* plants at d14, below the shoot apex and, ectopically, in the laterally arising meristems (Fig. 4B,C). Similar domains of *TFL1* expression were found in *ap1 cal* and *ful ap1 cal* meristems at d21; in both cases, *TFL1* was expressed more strongly in basal positions and at lower levels in the more apical meristems At d35, *TFL1* was almost undetectable in *ap1 cal* inflorescences, where *LFY* expression had reached its highest level, whereas some small domains of *TFL* expression and lower levels of *LFY* could still be seen in *ful ap1 cal* (Fig. 4F,G). Thus, the mutually exclusive patterns of *LFY* and *TFL1* expression became overlapping both in *ap1 cal* and *ful ap1 cal* meristems, even though the ratio of *LFY* to *TFL1* RNA expression was clearly higher in *ap1 cal* than in *ful ap1 cal* meristems from d21 (Fig. 4D,E).

Since *TFL1* RNA accumulates in the *ful ap1 cal* triple mutant, we examined the activity of *TFL1* in this context by introducing the *tfl1* mutation into the triple mutant. *ful ap1 cal tfl1* plants were only distinguishable from *ap1 tfl1* plants by the *ful* fruit phenotype, indicating that the lack of *TFL1* completely abolished the proliferation of meristems observed in *ful ap1 cal* plants (Fig. 5B). We also observed that, as in wild type, in the *ful ap1 cal* background, the *tfl1* mutation had a semidominant phenotype. *ful ap1 cal TFL1/tfl1* plants were initially indistinguishable from the *ful ap1 cal* triples, but, after 2 months of growth, they all were able to form some flowers and set seeds. The quantitative effect of *TFL1* on the *ful ap1 cal* phenotype might reflect a threshold ratio of *LFY:TFL1* activities that has to be reached to induce flowering.

ful ap1 cal meristems are not competent to respond to *AG* floral inductive activity

We have shown that *LFY* RNA levels are significantly reduced in *ful ap1 cal* triple mutants, and previous studies have demonstrated that *LFY* plays a key role in the upregulation of the *AP3* and *AG* organ identity genes. We therefore compared the expression of *AP3* and *AG* in the *ful ap1 cal* triple mutant to that in the *ap1 cal* double mutant to determine if the expression of these organ identity genes requires *AP1/CAL/FUL*. Whereas the accumulation of *AP3* and *AG* RNAs was readily detected in 21-day-old *ap1 cal* meristems, these RNAs were not detected in *ful ap1 cal* plants of the same age (Fig. 6C-F). Even after more than 2 months of growth (not shown), no expression of *AP3* and *AG* could be detected in the triple mutant. These results demonstrate that *FUL, AP1* and *CAL* have overlapping functions in the upregulation of *AP3* and *AG*, most likely mediated at least in part through the upregulation of *LFY*.

These results raise the question of whether the inability to produce floral structures in *ful ap1 cal* mutants is due simply to the reduced *LFY* expression, or is due in part to the loss of organ-identity gene activation. Related to this is the observation that *AG* promotes floral identity even in the absence of *LFY* and *AP1* functions and is necessary to maintain floral identity under non-inductive conditions (Mizukami and Ma, 1997). To test whether the lack of the *AG* floral promoting activity was the direct cause of the non-flowering phenotype, we introduced a 35S::AG transgene in *ful ap1 cal* plants. Constitutive expression of *AG* did not induce the *ful ap1 cal* plants to flower, indicating that the loss of flowering in the triple mutant is not simply due to a failure to upregulate *AG* (Fig. 5C,D) and that *AG* requires at least one of the *FUL/AP1/CAL* activities to promote a floral fate.

DISCUSSION

FUL controls several aspects of plant development

We have shown that *FRUITFULL* is involved in several distinct processes during *Arabidopsis* development, as was suggested

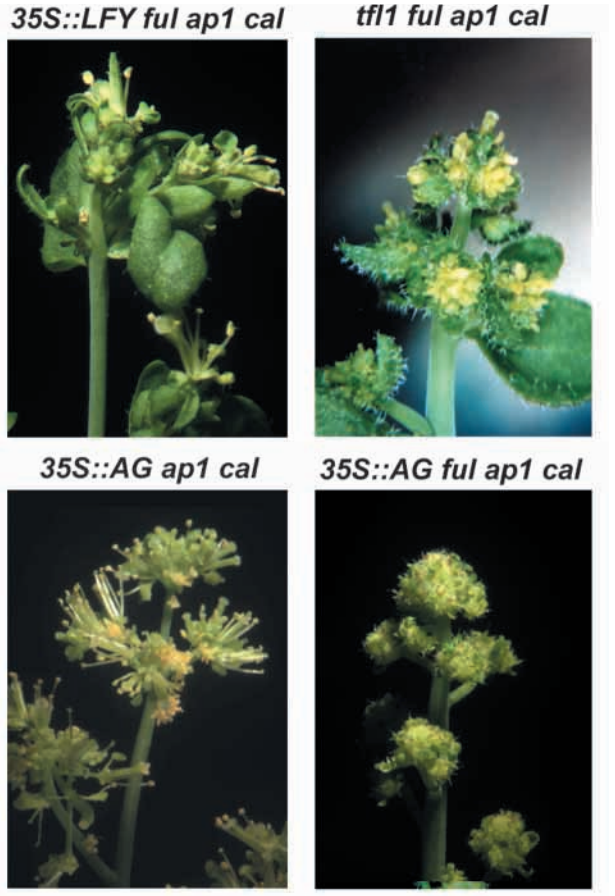

Fig. 5. Phenotype of the *35S::LFY ful ap1 cal, tfl1 ful ap1 cal, 35S::AG ap1 cal* and *35S::AG ful ap1 cal* plants. All plants were grown for 4 weeks. (A) *35S::LFY ful ap1 cal* inflorescences produced flowers composed of bract-like sepals, stamens and *ful*-like carpels, subtended by typical *ful* cauline leaves. (B) The quadruple *tfl1 ful ap1 cal* mutants produced flowers similarly to *tfl1 ap1* plants. The *35S::AG* transgene accelerates flowering in *ap1 cal* inflorescences (C), which form *ap1*-like flowers without producing 'cauliflower' structures first (as described in Mizukami and Ma, 1997). *35S::AG ful ap1 cal* plants (D) did not flower even after several months of growth. The inflorescences resemble those of *ful ap1 cal* triple mutants, but the leafy organs are reduced in size and curled upwards, a typical effect of the ectopic *AG* activity.

by its complex pattern of expression (Mandel and Yanofsky, 1995a). *FUL* has an early function in controlling flowering time, meristem identity and cauline leaf morphology, and has a later role in carpel and fruit development that affects valve, replum and style morphology. These phenotypic effects correlate well with the biphasic pattern of *FUL* expression (Gu et al., 1998; Mandel and Yanofsky, 1995a) and reveal a certain degree of non-autonomy of the *FUL* function since the mutants have a clear phenotype in the replum region where no *FUL* RNA is detected. Moreover, the study of an allelic series shows that these different roles are separable. All of the mutant alleles have a similar effect on flowering time (C. F. and M. Y., unpublished observations) and cauline leaf morphology. However, the weak alleles display a much less severe phenotype in valve and replum morphology as compared to strong alleles.

AP1/CAL/FUL act redundantly to upregulate *LFY* in floral meristems

It has been proposed that a threshold level of *LFY* expression is required for the vegetative-to-floral transition (Blázquez et al., 1997). Our results indicate that this threshold level is not reached in *ful ap1 cal* plants, leading to a dramatic non-flowering phenotype. The initial activation of *LFY* does not depend on *FUL*/*AP1*/*CAL*, but these activities are necessary for its subsequent upregulation, since *LFY* expression in *ful ap1 cal* meristems never exceeds its initially low levels. Moreover, constitutive expression of *LFY* restored flowering in the *ful ap1 cal* plants, reinforcing the idea of *LFY* levels as the switch to reproductive development. Our results agree with previous studies that identified *AP1* and *CAL* as direct or indirect activators of *LFY*. These studies showed that *LFY* is ectopically expressed in the converted flower meristems of 35S::AP1 plants (Liljegren et al., 1999) and that the initial expression of *LFY* is significantly reduced in *ap1 cal* mutants (Bowman et al., 1993).

It is interesting to note that although constitutive expression of *LFY* suppressed the non-flowering phenotype of *ful ap1 cal* triple mutants, many of the phenotypes caused by the ectopic *LFY* expression were suppressed in the triple mutant background. It has been previously reported that the flower-to-shoot conversion in 35S::LFY plants is largely suppressed by mutations in *AP1*, although the plants are still early flowering (Weigel and Nilsson, 1995). We found that the flowering time in 35S::LFY *ap1 cal* plants is further increased and that 35S::LFY *ful ap1 cal* plants flower significantly later than wild-type plants (Table 2). This suggests that the threshold of *LFY* required to induce flowering is higher in the *ful ap1 cal* background, or alternatively, that another factor may be required to accumulate when *AP1/CAL/FUL* are not present, to act with *LFY* in promoting flowering.

Similar phenotypes to those found in *ful ap1 cal* plants have been reported for several mutant combinations. For example, the *ft ap1* and *fwa ap1* double mutant inflorescences resemble the *ful ap1 cal* 'leafy cauliflowers', although they are able to flower after several months of growth (Ruiz-García et al., 1997). Similarly, *ld ap1 cal* triple mutants form proliferating leafy shoots at the apex and are unable to flower (Aukerman et al., 1999). The *ap1 cal* double mutants also show an enhancement of vegetative characteristics when grown in short days or at 16°C (Bowman et al., 1993). *LFY* fails to be upregulated both in *ld ap1 cal* and *ap1 cal* grown in non-inductive conditions (Aukerman et al., 1999; Bowman et al., 1993). The similarities among these mutant phenotypes and the *ful ap1 cal* inflorescences may reflect a possible role of the *FT, FWA* and *LD* genes in the competence of the meristems to respond to reduced levels of *LFY*, and/or indicate their possible function as *LFY* activators. It will be interesting to test whether *FUL* fails to be upregulated at low temperatures or in the *ft, fwa* or *ld* backgrounds, thereby preventing *LFY* activation.

AP1/CAL/FUL may control the transition between developmental phases by modulating the ratio of LFY/TFL activities

It has been suggested that the overlapping expression domains of *LFY* and *TFL1* in emerging lateral primordia cause the meristem proliferation of *ap1 cal* inflorescences (Ratcliffe et al., 1999). Support for this idea comes from genetic studies,

which show that this proliferation does not occur when *lfy* or *tfl1* mutations are introduced into the *ap1 cal* double mutant. Thus, the role for *CAL* in an *ap1* mutant background is to activate *LFY* and repress *TFL1* expression in lateral meristems, thus preventing their overlapping activities (Bowman et al., 1993; Ratcliffe et al., 1999).

We have found that the expression domains of *LFY* and *TFL1* overlap in the *ful ap1 cal* triple mutants, as was observed for *ap1 cal* meristems (Ratcliffe et al., 1999; this work). Accordingly, the *ful ap1 cal* plants formed meristems in a reiterate pattern, and this proliferation was not observed when *LFY* or *TFL* were also mutated. In the *ap1 cal* background, where *FUL* is expressed at the apex and ectopically in all lateral meristems, *FUL* was required for the increase of *LFY* expression, whereas it seemed to have little effect on *TFL1* regulation. Thus, in the *ful ap1 cal* inflorescences, the ratio of *LFY:TFL1* expression was always lower than in *ap1 cal* double mutants.

Our data suggest that the levels of *LFY* as well as the relative levels of *LFY* and *TFL1* control plant architecture and meristem behavior. When *ap1 cal* SAMs enter the inflorescence developmental phase, they give rise laterally to new meristems that also behave as inflorescence apical meristems. In *ful ap1 cal* plants, the SAMs seem to proliferate as V_2 meristems, producing cauline leaves with axillary meristems that in turn repeat this pattern forming the 'leafy cauliflowers' (Figs 2, 3). Thus, lower *LFY* to *TFL1* relative levels, together with their overlapping expression, would result in the reiteration of a more vegetative phase in *ful ap1 cal* plants. In contrast, in *ap1 cal* double mutants, a slightly higher *LFY* to *TFL1* ratio, together with higher *LFY* levels, would allow the transition to the inflorescence phase, that in turn would cause the reiterative behavior of the meristems. The subsequent upregulation of *LFY* in *ap1 cal*, mediated by *FUL*, would raise the *LFY:TFL1* ratio to the required levels for floral specification. The late formation of floral structures in *ful ap1 cal* inflorescences in a *TFL1* heterozygous background seems to support this hypothesis, since in this situation, a reduced *TFL1* activity could eventually lead to the transition to floral commitment.

As noted above, the proliferating *ful ap1 cal* meristems appear to be arrested in the V_2 phase, in contrast to the reproductive character of the *ap1 cal* cauliflowers. Besides the morphological evidence, additional data support this conclusion. For example, constitutive *AG* expression failed to promote floral identity in the *ful ap1 cal* meristems whereas it was able to promote flowering in *ap1 cal* double mutants (Mizukami and Ma, 1997). The lack of floral specification in 35S::AG *ful ap1 cal* plants suggests that *AG* may act through *AP1/CAL/FUL* to promote floral fate, or, alternatively, that the *ful ap1 cal* background prevents the meristems from becoming competent to respond to *AG*, perhaps by keeping them in a vegetative state.

FUL has a floral promoting activity independent of *LFY*

We have demonstrated that *FUL* plays a redundant role with *AP1* and *CAL* in *LFY* upregulation, thus promoting floral meristem specification. Our data also suggest that *FUL* is involved in phase transition during development in a pathway that is independent of *LFY*. This is clearly illustrated by the observation that the delay in flowering caused by mutations in *LFY* is further enhanced in *ful lfy* double mutants (Table 1). In addition, small but significant increases in V_1 and V_2 phases are found in *ful* single mutants, even though levels of *LFY* expression are not noticeably affected. Furthermore, flowering time is dramatically reduced in plants that constitutively

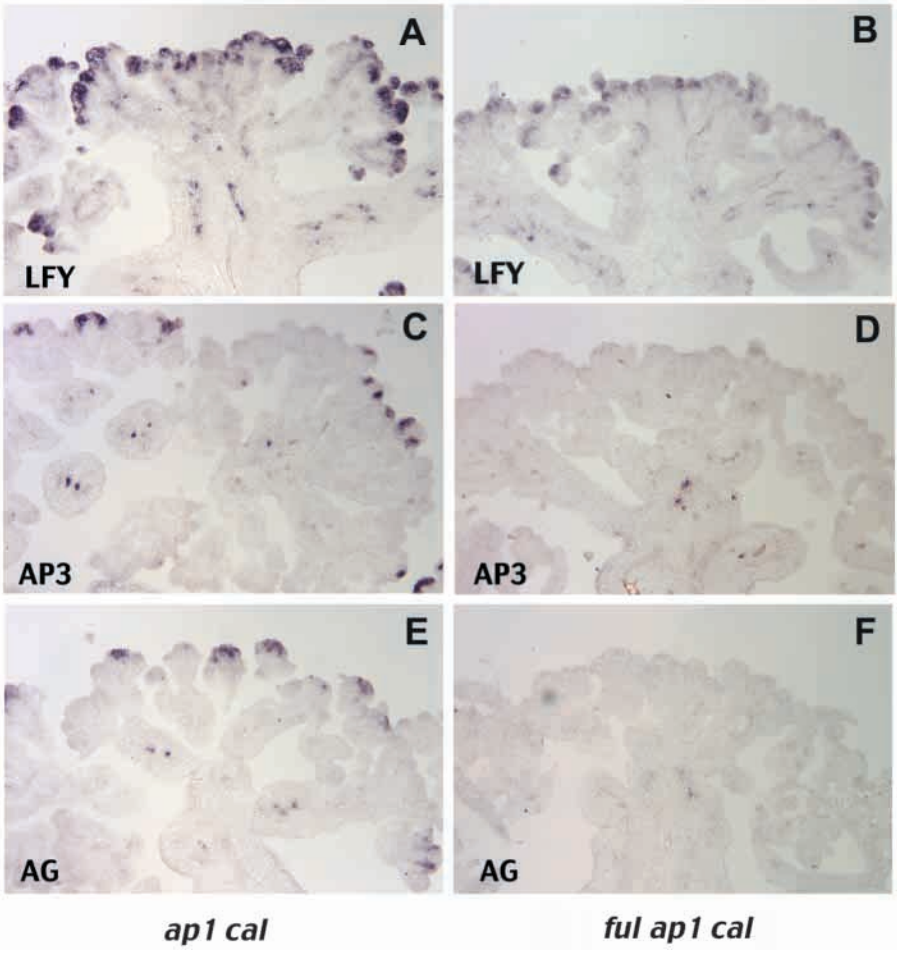

Fig. 6. Expression of *LFY, AP3* and *AG* in *ap1 cal* and *ful ap1 cal* plants. Sections of 21-day-old *ap1 cal* (A,C,E) and *ful ap1 cal* (B,D,F) plants probed with *LFY* (A,B), *AP3* (C,D) and *AG* (E,F) antisense RNA are shown. Sections in A,C,E and B,D,F are from single inflorescences. *LFY* is strongly expressed in the presumed floral meristems of *ap1 cal* inflorescences. In *ful ap1 cal* meristems, *LFY* is detected at much reduced levels. (C,G) Some meristems in *ap1 cal* inflorescences show *AP3* (C) and *AG* (E) patterns of expression similar to those found in early stages (4-5) of wild-type development. (D,F) *AP3* (D) and *AG* (F) are not detected in *ful ap1 cal* meristems.

express *FUL* under the control of the 35S promoter, and this floral-promoting activity is independent of *LFY*, since is not affected in the *lfy* mutant background (C.F. and M.F.Y., unpublished results). It is interesting to note that the *LFY*-independent role of *FUL* in promoting the phase transition requires *AP1*, since *FUL* is unable to promote flowering in a *lfy ap1* background. However the small effect on flowering time caused by *ful* mutations, and the rapid and strong *FUL* upregulation in the SAM after the induction of the reproductive phase, suggest that its flower promoting activity might be largely obscured by other highly redundant activities. Good candidates for genes that act redundantly with *FUL* in the SAM are the *AGL20* and *AGL24* MADS-box genes that share a similar pattern of upregulation (S. Gold and M. Yanofsky; C. Gustafson-Brown, M. Yanofsky and W. Crosby; unpublished results). Regardless of whether additional MADS-box genes or as yet unidentified genes are involved, it is clear that *FUL* acts in a highly redundant pathway to control the transition to flowering.

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