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RESEARCH ARTICLE

Different Roles of Flowering-Time Genes in the Activation of Floral Initiation Genes in Arabidopsis

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We have analyzed double mutants that combine late-flowering mutations at four flowering-time loci (*FVE*, *FPA*, *FWA*, and *FT*) with mutations at the *LEAFY* (*LFY*), *APETALA1* (*AP1*), and *TERMINAL FLOWER1* (*TFL1*) loci involved in the floral initiation process (FLIP). Double mutants between *ft-1* or *fwa-1* and *lfy-6* completely lack flowerlike structures, indicating that both *FWA* and *FT* act redundantly with *LFY* to control *AP1*. Moreover, the phenotypes of *ft-1 ap1-1* and *fwa-1 ap1-1* double mutants are reminiscent of the phenotype of *ap1-1 cal-1* double mutants, suggesting that *FWA* and *FT* could also be involved in the control of other FLIP genes. Such extreme phenotypes were not observed in double mutants between *fve-2* or *fpa-1* and *lfy-6* or *ap1-1*. Each of these showed a phenotype similar to that of *ap1-1* or *lfy-6* mutants grown under noninductive photoperiods, suggesting a redundant interaction with FLIP genes. Finally, the phenotype of double mutants combining the late-flowering mutations with *tfl1-2* were also consistent with the different roles of flowering-time genes.

INTRODUCTION

Arabidopsis belongs to a group of species that grow as rosettes during their vegetative development. During this time, the shoot apical meristem proceeds through the reiterated production of primordia that will develop into vegetative organs (leaves with axillary meristems) (Haughn et al., 1995), which change in size and shape during the development of the rosette (Medford et al., 1992; Martínez-Zapater et al., 1995; Telfer et al., 1997). The genotype of the plant and the environmental conditions determine when the apical meristem changes its pattern of development and starts producing flowers (Martínez-Zapater and Somerville, 1990; Koornneef et al., 1991; Bagnall, 1993). In Arabidopsis, this transition, which we refer to as the floral transition, is followed by the elongation of the internodes or bolting, giving rise to the inflorescence shoot. Basal nodes of the inflorescences, which have already been initiated at the time of the floral transition, bear leaves with axillary shoot meristems, whereas nodes initiated after the floral transition lack leaves and axillary shoot meristems develop into flowers (Schultz and Haughn, 1991, 1993; Hempel and Feldman, 1994).

Arabidopsis is a quantitative long-day (LD) plant in which the time of floral transition is regulated by endogenous signals and environmental conditions, such as LD photoperiods and low temperatures (Napp-Zinn, 1985; Martínez-Zapater et al., 1994; Haughn et al., 1995). LD photoperiods accelerate flowering, although plants also flower under short days (SDs) after extended periods of vegetative development. For some genotypes, temporal exposure to low nonfreezing temperatures (vernalization) during germination or early vegetative development also accelerates floral transition (Napp-Zinn, 1987; Karlsson et al., 1993; Martínez-Zapater et al., 1994).

The isolation of late- and early-flowering mutants in Arabidopsis has led to the identification of genes that could be involved in the promotion or repression of the floral transition, respectively (Zagotta et al., 1992, 1996; Martínez-Zapater et al., 1994; Hicks et al., 1996). In addition, phenotypic characterization of some of the late-flowering mutants under

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different environmental conditions has revealed that they can be pooled into at least two phenotypic groups, depending on their response to enviromental cues (e.g., photoperiod and vernalization) (Martínez-Zapater et al., 1994). One group would include mutants such as *fca*, *fve*, *fpa*, and *fy*, which show a delayed-flowering phenotype under both LD and SD photoperiods but are strongly responsive to photoperiod and vernalization (Martínez-Zapater and Somerville, 1990; Koornneef et al., 1991). The other group would include mutants such as *constants* (*co*), *gigantea* (*gi*), *fwa*, and *ft*, which show lower responsiveness to those environmental conditions (Martínez-Zapater and Somerville, 1990; Koornneef et al., 1991). Among them, *co* and *gi* are completely insensitive to photoperiod and vernalization, whereas *ft* and *fwa* still show an important photoperiod response (Koornneef et al., 1991). Little molecular information is currently available about the nature of the late-flowering mutations because just a few of the corresponding genes have been cloned so far (Lee et al., 1994; Putterill et al., 1995; Macknight et al., 1997).

The floral transition at the shoot apical meristem results in the activation of the floral initiation process (FLIP) at the axillary shoot meristems (Schultz and Haughn, 1993). This program, which is also responsible for the inhibition of the development of leaf primordia, requires the activity of a set of genes that we refer to as the FLIP genes (Schultz and Haughn, 1993). Genetic and molecular analyses with Arabidopsis have identified two genes whose mutations severely impair the transition from a shoot meristem to a floral meristem–*LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Irish and Sussex, 1990; Schultz and Haughn, 1991; Huala and Sussex, 1992; Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Weigel and Meyerowitz, 1993). Moreover, overexpression experiments with transgenic plants have demonstrated that *LFY* and *AP1* are not only necessary but are sufficient to promote the initiation of flowers at the shoot meristems, with *AP1* acting downstream of *LFY*, at least for some flower meristem identity functions (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995).

Genetic analyses have also been used to identify another gene, *CAULIFLOWER* (*CAL*), whose function is the same as that of *AP1* and which encodes a highly homologous protein (Bowman et al., 1993; Kempin et al., 1995). Moreover, other genes such as *APETALA2* (*AP2*) (Kunst et al., 1989; Bowman et al., 1993; Jofuku et al., 1994; Kempin et al., 1995) and *UNUSUAL FLORAL ORGANS* (*UFO*) (Ingram et al., 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995; Lee et al., 1997) are also reportedly involved in the flower initiation program, as determined by their mutant phenotypes. Finally, mutations at the *TERMINAL FLOWER1* (*TFL1*) locus cause an early-flowering phenotype and the differentiation of the shoot apical meristems into terminal flowers (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997), suggesting that *TFL1* could be involved in delaying the commitment of Arabidopsis to flower and preventing the formation of a terminal flower at the shoot apical meristem.

The analysis of plants carrying mutations in several of these FLIP genes under different environmental conditions has led to a basic model for the establishment of FLIP (Weigel et al., 1992; Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993; Haughn et al., 1995). Although different versions of this model take into consideration the function of the flowering-time genes as controlling the activation of FLIP, the genetic interactions between FLIP and flowering-time genes have not been elucidated. Only recently, the controlled expression of *CO* in transgenic plants has shown that this gene is sufficient to activate *LFY* and *TFL* transcription but could require one additional pathway for the activation of *AP1* (Simon et al., 1996).

To understand better the interaction between flowering-time and FLIP genes, we have constructed and analyzed double mutants that combine late-flowering mutations at four flowering-time loci (*FVE*, *FPA*, *FWA*, and *FT*), representing the two previously mentioned groups, with mutations at *LFY*, *AP1*, and *TFL1*. Double mutants between *ft-1* and *fwa-1* late-flowering mutations and *lfy-6* completely lack flowerlike structures, suggesting that both FT and FWA functions are redundant with LFY in the control of *AP1*. Late-flowering mutations at the *FVE* or *FPA* locus do not produce such an extreme phenotype when combined with *ap1-1* or *lfy-6* mutations, suggesting a more indirect effect on the FLIP genes. Taken together, these results suggest the existence of different and redundant ways of interaction between flowering-time and FLIP genes. The phenotypes of double mutants combining late-flowering mutations at the same four loci and *tfl1-2* mutations are also consistent with those results.

RESULTS

The Early-Flowering Phenotype of the *tfl1-2* Mutant Is Not Epistatic over the Late-Flowering Phenotype of *fve*, *fpa*, *ft*, or *fwa*

Genetic interactions between *TFL1* and some of the genes whose mutations produce a late-flowering phenotype were analyzed by studying the phenotypes of double mutants carrying late-flowering mutations at either one of the flowering-time loci, *FPA*, *FVE*, *FT*, and *FWA*, and at *TFL1*. When grown under LD conditions, Tfl1 plants flower slightly earlier than do wild-type plants and develop terminal flowers both in the apical inflorescence meristem and in the coflorescence meristems (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Schultz and Haughn, 1993), as shown in Figure 1A.

Flowering time, measured as the total number of leaves, was scored in the double mutants derived from crosses of the above-mentioned mutants. As shown in Table 1, under our experimental conditions, double mutants flowered with a total number of leaves that was close to the number of

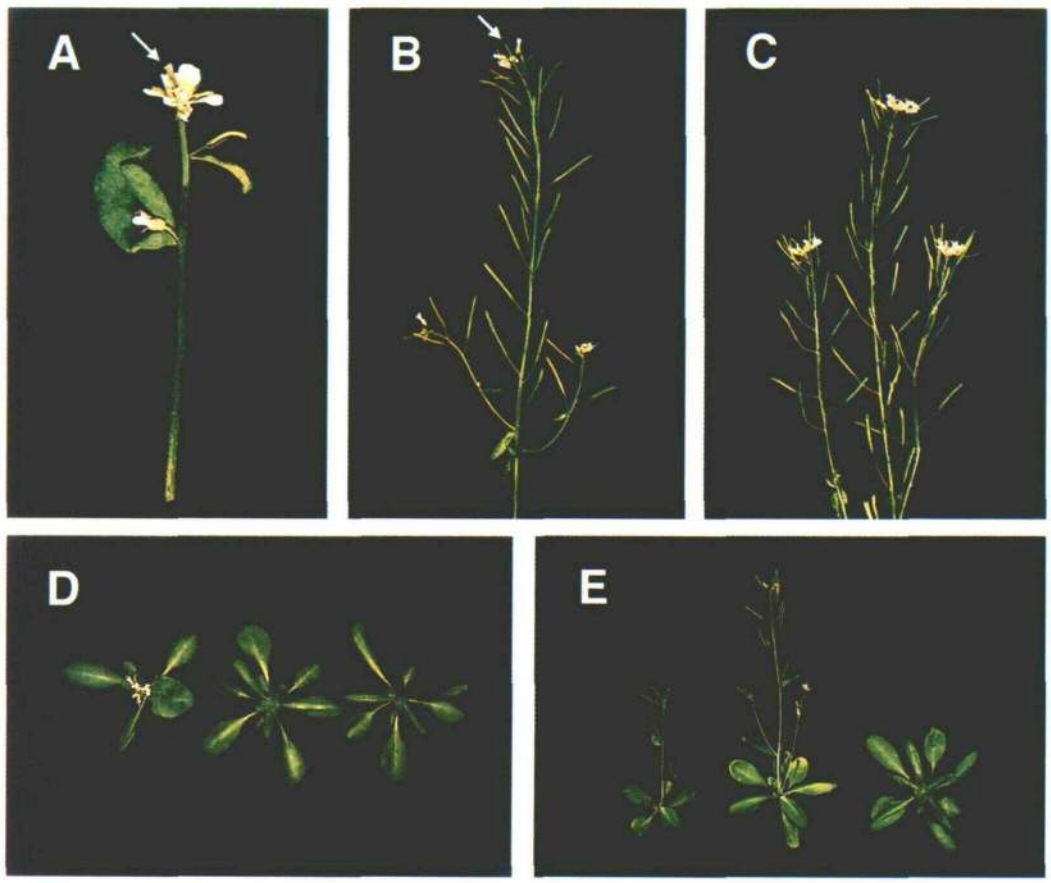

Figure 1. Flowering-Time Phenotypes and Inflorescence Structures of *tfl1-2* and *fve-2* and *fve-2 tfl1-2* Double Mutants.

(A) Inflorescence structure of a *tfl1-2* plant. The arrow indicates a silique developing from the terminal flower at the main inflorescence apex.
(B) Structure of the upper part of the *fve-2 tfl1-2* double mutant inflorescence. The position of a silique developing from the terminal flower is marked with an arrow.
(C) Upper part of the inflorescence of one *fve-2* plant.
(D) Rosettes of *tfl1-2* (left), *fve-2 tfl1-2* (center), and *fve-2* (right) plants grown for 3 weeks under LD conditions.
(E) Effect of 4 weeks of vernalization on the flowering time of *fve-2 tfl1-2* double mutants. The plants shown from left to right are nonvernalized *tfl1-2* and *fve-2 tfl1-2* vernalized for 4 weeks and nonvernalized *fve-2 tfl1-2*. Plants were grown for 4 weeks under LD conditions.

leaves of their late-flowering parents. The development of terminal flowers after 10 to 12 weeks of growth (Figure 1B) was the feature that allowed the distinction between the double mutants and their late-flowering parents (Figure 1C). However, in repeated experiments, we consistently found that double mutants *fve-2 tfl1-2* and *fpa-1 tfl1-2* bolted significantly later and flowered with a significantly higher number of leaves than did the single mutants *fve-2* or *fpa-1* in both LD (Table 1) and SD photoperiods (SD with a 10-hr photoperiod; data not shown). In contrast, double mutants *ft-1 tfl1-2* and *fwa-1 tfl1-2* showed an additive phenotype, bolting significantly earlier and flowering with a significantly lower number of leaves than did their corresponding late-flowering parents in both LD (Table 1) and SD photoperiods (data not shown). Thus, the phenotypes of the four double mutants are consistent with *TFL1* being involved in the same pathway as *FVE* and *FPA* and paralleling the functions of *FT* and *FWA*.

The late-flowering phenotype of *fve* and *fpa* mutants has been shown to be rescued by a vernalization treatment (Martínez-Zapater and Somerville, 1990; Koornneef et al., 1991). As expected from the vernalization response of the late-flowering parents *fve-2* and *fpa-1*, the results presented in Figures 1D to 1E and Table 2 show that the double mutants *fpa-1 tfl1-2* and *fve-2 tfl1-2* flowered earlier when they were vernalized at 4°C for 4 weeks and, like *tfl1-2* mutants, produced terminal flowers in place of lateral coflorescences.

Table 1. Effect of Late-Flowering Mutations on the Total Number of Leaves and Shoot Morphology of *tfl1-2*

Genotype	Total Leaves[a]	Terminal Flower[b]
Landsberg *erecta*	8.6 ± 0.2	−
tfl1-2	6.7 ± 0.2[c]	+
fve-2	26.5 ± 0.7[c,d]	−
fve-2 tfl1-2	28.4 ± 0.6[c,d,e]	+
fpa-1	24.2 ± 0.5[c,d]	−
fpa-1 tfl1-2	26.8 ± 0.6[c,d,e]	+
ft-1	22.2 ± 0.5[c,d]	−
ft-1 tfl1-2	20.4 ± 0.6[c,d,e]	+
fwa-1	23.7 ± 0.3[c,d]	−
fwa-1 tfl1-2	22.3 ± 0.7[c,d,e]	+

[a] Rosette and inflorescence leaves. Values are expressed as mean ± SE.

[b] Presence (+) or absence (−) of a terminal flower at the inflorescence apex.

[c] Significantly different from Landsberg *erecta* at the 0.05 level.

[d] Significantly different from *tfl1-2* at the 0.05 level.

[e] Significantly different from the corresponding single late-flowering mutant at the 0.05 level.

In addition, the vernalized double mutants were significantly earlier than were the vernalized late-flowering parents, with the *tfl1-2* mutation having an additive effect on the floral promotion caused by the vernalization treatment. Vernalized double mutants still flowered with a significantly higher number of rosette leaves than did the vernalized Tfl1-2 or wild-type plants, indicating that the late-flowering phenotype was not completely rescued by the length of this vernalization treatment.

fve and *fpa* Mutations Have an Effect Similar to SDs on the Phenotype of *ap1* and *lfy* Mutants

Floral initiation requires the activity of a set of genes among which *LFY* and *AP1* seem to play a major role. In an attempt to understand the genetic interactions between flowering time and floral initiation, we studied the effect of combining strong alleles of either *LFY* (*lfy*-6) (Weigel et al., 1992) or *AP1* (*ap1-1*) (Bowman et al., 1993) with late mutations in the flowering-time loci *FVE*, *FPA*, *FT*, and *FWA*.

The combination of mutations *fve-2* or *fpa-1* with *ap1-1* gave rise to double mutant plants that were phenotypically very similar and flowered with a total number of leaves close to the number shown by the corresponding late-flowering parents (Table 3). Double mutants *fve-2 ap1-1* showed a significantly higher number of rosette leaves than did the *fve-2* parent, although this was not the case for the *fpa-1 ap1-1* double mutant. The most striking feature of both double mutants was their altered inflorescence structure, which was characterized by the presence of a much higher number of coflorescences not subtended by leaves, as shown in Figure 2D and Table 3. Thus, the production of floral meristems was delayed much more than in any of the mutant parents (Figures 2A to 2B). This phenotype is similar to that displayed by Ap1-1 plants when grown under SD conditions (Bowman et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993; Okamuro et al., 1997). However, when *fve-2 ap1-1* or *fpa-1 ap1-1* double mutants were grown under SD conditions, they showed an even more extreme phenotype than under LD conditions (data not shown), indicating that the effect of these late-flowering mutations and the effect of SD photoperiods are additive. As previously shown for the double mutants with *tfl1-2*, a vernalization treatment almost completely rescued the *ap1-1* mutant phenotype by correcting the effect of the late-flowering mutations (Table 3).

When the same two late-flowering mutations were combined with *lfy-6*, the resulting double mutants were again phenotypically very similar. Both double mutants bolted with the same number of rosette leaves as their corresponding late-flowering parent (Table 3). As described above for the double mutants with *ap1-1*, the number of coflorescences initiated before the formation of any flowerlike structure (which, in this case, was the typical *lfy-6* flowerlike structure; Figure 2C) in *fve-2 lfy-6* or *fpa-1 lfy-6* double mutants was much higher than in any of the mutant parents (Table 3 and Figure 2E). The phenotypes of these double mutants are also reminiscent of the phenotype displayed by *lfy* mutants when grown under SD conditions (Okamuro et al., 1993, 1996; Schultz and Haughn, 1993). In this case, the effect of the late-flowering mutations was also additive with respect to the effect of SD photoperiods, because double mutants have a much more extreme phenotype when grown under SD conditions (data not shown). Vernalization of the double mutants with *lfy-6* also corrected the effect of the late-flowering mutations, rendering plants with inflorescences that were phenotypically closer to the *lfy* inflorescences (Table 3).

Table 2. Total Number of Leaves in Vernalized *fve-2 tfl1-2* and *fpa-1 tfl1-2* Double Mutants and the Corresponding Single Mutants

Genotype	Total Leaves[a]
Landsberg *erecta*	6.6 ± 0.2
tfl1-2	6.4 ± 0.2
fve-2	13.1 ± 0.2[b,c]
fve-2 tfl1-2	9.0 ± 0.2[b,c,d]
fpa-1	11.5 ± 0.2[b,c]
fpa-1 tfl1-2	9.5 ± 0.3[b,c,d]

[a] Rosette and inflorescence leaves. Values are expressed as mean ± SE.

[b] Significantly different from Landsberg *erecta* at the 0.05 level.

[c] Significantly different from *tfl1-2* at the 0.05 level.

[d] Significantly different from the corresponding single late-flowering mutant at the 0.05 level.

Table 3. Effect of Late-Flowering Mutations *fve* and *fpa* and Vernalization on Leaf Number and Shoot Morphology of *ap1-1* and *lfy-6* Mutants

	Rosette Leaves[a]		Inflorescence Leaves[a]		Coflorescences[a,b]	
Genotype	NV[c]	V[d]	NV	V	NV	V
Landsberg *erecta*	7.3 ± 0.1	5.6 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	2.1 ± 0.2	2.0 ± 0.1
ap1-1	6.1 ± 0.1	6.3 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	4.3 ± 0.2	4.1 ± 0.2[e]
lfy-6	6.8 ± 0.2	6.8 ± 0.3	ND[f]	ND[f]	5.3 ± 0.2	4.8 ± 0.3[e]
fve-2	20.5 ± 0.8[e]	10.2 ± 0.2[e]	6.6 ± 0.3[e]	3.1 ± 0.3[e]	6.6 ± 0.3[e]	3.1 ± 0.3[e]
fve-2 ap1-1	24.1 ± 1.0[e,g,h]	8.3 ± 0.2[e,g,h]	6.4 ± 0.2[e,h]	2.4 ± 0.2	35.5 ± 1.7[e,g,h]	4.2 ± 0.2[e,g]
fve-2 lfy-6	19.3 ± 0.8[e,h]	10.0 ± 0.5[e,h]	ND[f]	ND[f]	31.5 ± 2.6	6.7 ± 0.4[e,g,h]
fpa-1	19.8 ± 0.5[e]	9.5 ± 0.1[e]	4.4 ± 0.2[e]	2.0 ± 0.2	4.4 ± 0.2[e]	2.0 ± 0.2
fpa-1 ap1-1	16.2 ± 0.4[e,g,h]	8.6 ± 0.2[e,g,h]	3.9 ± 0.3[e,f,h]	2.5 ± 0.2	32.6 ± 1.6[e,g,h]	4.4 ± 0.3[e,g]
fpa-1 lfy-6	18.9 ± 1.1[e,f,h]	9.4 ± 0.4[e,h]	ND[f]	ND[f]	30.1 ± 0.8[e,g,h]	5.8 ± 0.3[e,g,h]

[a] Values are expressed as mean ± SE.
[b] Lateral branches with indeterminate growth.
[c] NV, nonvernalized plants.
[d] V, vernalized plants. Vernalization was performed at 4°C for 4 weeks.
[e] Significantly different from Landsberg *erecta* at the 0.05 level.
[f] ND, not done. *lfy-6* and its double mutants produce an unlimited proliferation of inflorescence leaves that were not scored.
[g] Significantly different from the corresponding single late-flowering mutant at the 0.05 level.
[h] Significantly different from the corresponding *lfy-6* or *ap1-1* mutants at the 0.05 level.

Combinations of *ft* or *fwa* with *lfy* or *ap1* Produce New Inflorescence Phenotypes

Double mutants *fwa-1 ap1-1* and *ft-1 ap1-1* were phenotypically similar. The double mutants bolted at the same time as did the late-flowering parents (data not shown) and, as shown in Table 4, produced a similar number of rosette and inflorescence leaves. However, floral initiation at the lateral meristems of the inflorescence of those double mutants was dramatically delayed, giving rise to the altered inflorescence structures shown in Figures 3A to 3D. These structures are reminiscent of the inflorescences of *ap1-1 cal-1* double mutants grown under SD conditions (Bowman et al., 1993). As illustrated in Figure 3E, fertile Ap1-like flower structures were only observed on plants grown for >6 months once the apical meristem had produced >50 coflorescence-bearing nodes (Table 3). As a consequence of this strongly delayed floral initiation, the plants had a highly branched and bushy appearance (Figure 3E). Scanning electron microscopy of the apical inflorescence meristems of these double mutants (Figures 3F to 3I) confirmed that during the initial stages of the inflorescence development, the primary inflorescence meristem only produced additional inflorescence meristems giving rise to the small cauliflower structures observed (Figures 3C and 3H). Later in development, it was possible to detect the differentiation of floral meristems and flower organ primordia in their apex (Figure 3I). The inflorescence phenotype of double mutants between *ap1-1* and *ft-1* or *fwa-1* was therefore more extreme than that of the doubles with *fpa-1* or *fve-2*, which are described above.

The combination of either *ft-1* or *fwa-1* with *lfy-6* produced an even more extreme inflorescence phenotype than that of the double mutants with *ap1-1*. The data presented in Table 4 show that, as observed for the other double mutants described above, the number of rosette leaves and the bolting time (data not shown) of *ft-1 lfy-6* and *fwa-1 lfy-6* double mutants were similar to those of the late-flowering parents (Table 4). However, both double mutants showed a strongly altered inflorescence structure, which is illustrated in Figures 4A to 4D. These inflorescences never gave rise to the flowerlike structures produced by *lfy-6* mutant inflorescences (see Figure 2C) and showed a more dramatic phenotype than those described for the *lfy-6 ap1-1* double mutant (Weigel et al., 1992) and the *ap1-1 cal-1 lfy-6* triple mutant (Bowman et al., 1993). A similar phenotype has also been independently observed for the *fwa lfy-6* double mutants (A. Chaudhury, personal communication).

Scanning electron microscopy analyses showed that the apical meristems of these inflorescences and coflorescences continue producing caulinar leaves (Figures 4E to 4G) and leaf primordia with a spiral phyllotaxis (Figure 4H). The axillary meristems, which generally give rise to flowerlike structures in *lfy-6* mutants (Figure 4I), developed as lateral branches instead (Figure 4J). Consequently, these double mutants are highly branched (Figure 4D) and keep growing for months without producing any flowerlike structures. In some cases, the axillary meristems produced a filamentous structure with determinate growth (Figure 4K) or did not develop at all (Figure 4J). Only after 8 months of growth were we able to observe leaves with carpel-like features (stigmatic papillae, stylar tissue, or the development of ovules on their edges) at the apex of some lateral branches (Figures 4L

Figure 2. Inflorescence Structures of the *fve-2 ap1-1* and *fve-2 lfy-6* Double Mutants and Their Corresponding Parents.

Shown are the complete inflorescences of the plants. The inflorescences of the double mutants show a higher number of coflorescences than do any of the single mutants.

(A) *ap1-1.*
(B) *fve-2.*
(C) *lfy-6.*
(D) *fve-2 ap1-1.*
(E) *fve-2 lfy-6.*

to 4N). Such carpel-like structures were still organized in a spiral phyllotaxis and constituted the only evidence of floral organs observed in these plants. The virtual absence of floral structures in these plants suggests that the other genes responsible for floral initiation, mainly *AP1* and *CAL*, should be inactive in these double mutants or, in other words, that when *LFY* is absent, both *FT* and *FWA* are required for the function of FLIP genes such as *AP1* and *CAL*.

To test whether *ft-1* and *fwa-1* mutations had any effect on the level of *AP1* and *LFY* mRNAs in the inflorescence apex of single and double mutants, we performed an RNA gel blot hybridization. As shown in Figure 5 and consistent with the lack of flowerlike structures, the *AP1* mRNA could not be detected in the apices of double mutants *ft-1 lfy-6* or *fwa-1 lfy-6*. However, the sensitivity of the RNA gel blot hybridization experiments does not allow us to discount the possibility that *AP1* is expressed at very low levels or in a small number of cells. The *AP1* mRNA was present at wild-type levels in the single *lfy-6* and *ap1-1* mutants and in the double mutants *ft-1 ap1-1* and *fwa-1 ap1-1* (Figure 5). The *LFY* transcript, which was present at normal levels in the *lfy-6* mutant, as previously shown (Weigel et al., 1992), could still be detected in the *ft-1 lfy-6* or *fwa-1 lfy-6* double mutants (Figure 5), indicating that the *ft-1* and *fwa-1* mutations did not affect its expression level.

Table 4. Effect of Late-Flowering Mutations *ft* and *fwa* on Leaf Number and Shoot Morphology of *ap1-1* and *lfy-6* Mutants

Genotype	Rosette Leaves[a]	Inflorescence Leaves[a]	Coflorescences[a,b]
Landsberg *erecta*	6.8 ± 0.2	2.0 ± 0.1	2.0 ± 0.1
ap1-1	5.9 ± 0.1[c]	2.0 ± 0.0	4.1 ± 0.2[c]
lfy-6	6.9 ± 0.2	ND[d]	6.1 ± 0.2[c]
ft-1	16.6 ± 0.3[c]	5.6 ± 0.3[c]	5.6 ± 0.3[c]
ft-1 ap1-1	14.8 ± 0.5[c,e,f]	5.7 ± 0.2[c,f]	>50
ft-1 lfy-6	17.0 ± 1.5[c,f]	ND[d]	>50
fwa-1	16.2 ± 0.3[c]	7.5 ± 0.2[c]	7.5 ± 0.2[c]
fwa-1 ap1-1	17.5 ± 0.5[c,e,f]	6.0 ± 1.0	>50
fwa-1 lfy-6	21.5 ± 0.6[c,e,f]	ND[d]	>50

[a] Values are expressed as mean ± SE.
[b] Lateral branches with indeterminate growth.
[c] Significantly different from Landsberg *erecta* at the 0.05 level.
[d] ND, not done. *lfy-6* and its double mutants produce an unlimited proliferation of inflorescence leaves that were not scored.
[e] Significantly different from the corresponding single late-flowering mutant at the 0.05 level.
[f] Significantly different from the corresponding *lfy-6* or *ap1-1* mutants at the 0.05 level.

DISCUSSION

Previous genetic models illustrating the control of flowering time in Arabidopsis (Martínez-Zapater et al., 1994; Haughn et al., 1995) have been based on the strong early-flowering phenotype of *embryonic flowering* (*emf*) mutants (Sung et al., 1992) to predict a negative control of the floral transition. Over this negative control, several genes, identified by late-flowering mutations, would have a promotive effect on floral transition (Haughn et al., 1995). Given that at least two of the genes, *AP1* and *LFY*, have been shown to be both necessary and sufficient switches for the initiation of flowers (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995), we decided to test the hypothesis that genes required for the acceleration of the floral transition would interact with FLIP genes through a common mechanism (Madueño et al., 1996).

The phenotypic analyses of double mutants carrying late-flowering and either *lfy* or *ap1* strong mutations indicate that late-flowering mutations with different phenotypes differentially affect the phenotypes of Lfy or Ap1 plants and suggest the existence of at least two different types of interaction among the genes analyzed, as represented in Figure 6. On the one hand, both *FT* and *FWA* seem to function redundantly with *LFY* in controlling other FLIP genes, such as *AP1*. On the other, *FVE* and *FPA* would be involved in *LFY* and *AP1* activation, although their effect on *AP1* could be mediated either through LFY or FT and FWA functions. Based on the SD phenotypes of double mutants with either *fve-2* or *fpa-1* mutations, we suggest that the function of these genes, which are part of the constitutive pathway, would be redundant to the function of other genes such as *CO* that are responsible for the photoperiodic induction (Figure 6).

Double mutants combining late-flowering mutations at the *FT* or *FWA* locus with a strong mutation at the *LFY* locus showed a virtual lack of flowerlike structures, indicating that in the absence of *LFY*, both *FWA* and *FT* are required for *AP1* function, the other major gene responsible for FLIP. In addition, the fact that the inflorescence phenotype observed in *ft-1 lfy-6* and *fwa-1 lfy-6* is even stronger than in the *lfy-6 ap1-1* double mutants (Weigel et al., 1992) and in the *ap1-1 cal-1 lfy-6* triple mutant (Bowman et al., 1993) suggest that *FWA* and *FT* could also be required for the function of other genes that have been implicated in FLIP, such as *CAL*, *AP2*, or *UFO* (Schultz and Haughn, 1991; Lee et al., 1997). Mutations *ft-1* and *fwa-1* do not seem to alter the levels of the *LFY* message in the apex of *ft-1 lfy-6* or *fwa-1 lfy-6* double mutants, indicating that other gene functions distinct from FT and FWA are required for the regulation of *LFY* expression, as illustrated in Figure 6.

If *FT* and *FWA* were also involved in the activation of other FLIP genes, double mutants *ft-1 ap1-1* and *fwa-1 ap1-1* should show a more extreme phenotype than the simple addition of late-flowering and Ap1 inflorescence and flower phenotypes. In fact, these two double mutants combine a late-flowering phenotype with the production of cauliflower-like structures, resulting from the reiterative production of inflorescence meristems, that are reminiscent of the phenotype of *ap1 cal1-1* double mutants grown under SD conditions (Bowman et al., 1993). Despite this strong inflorescence

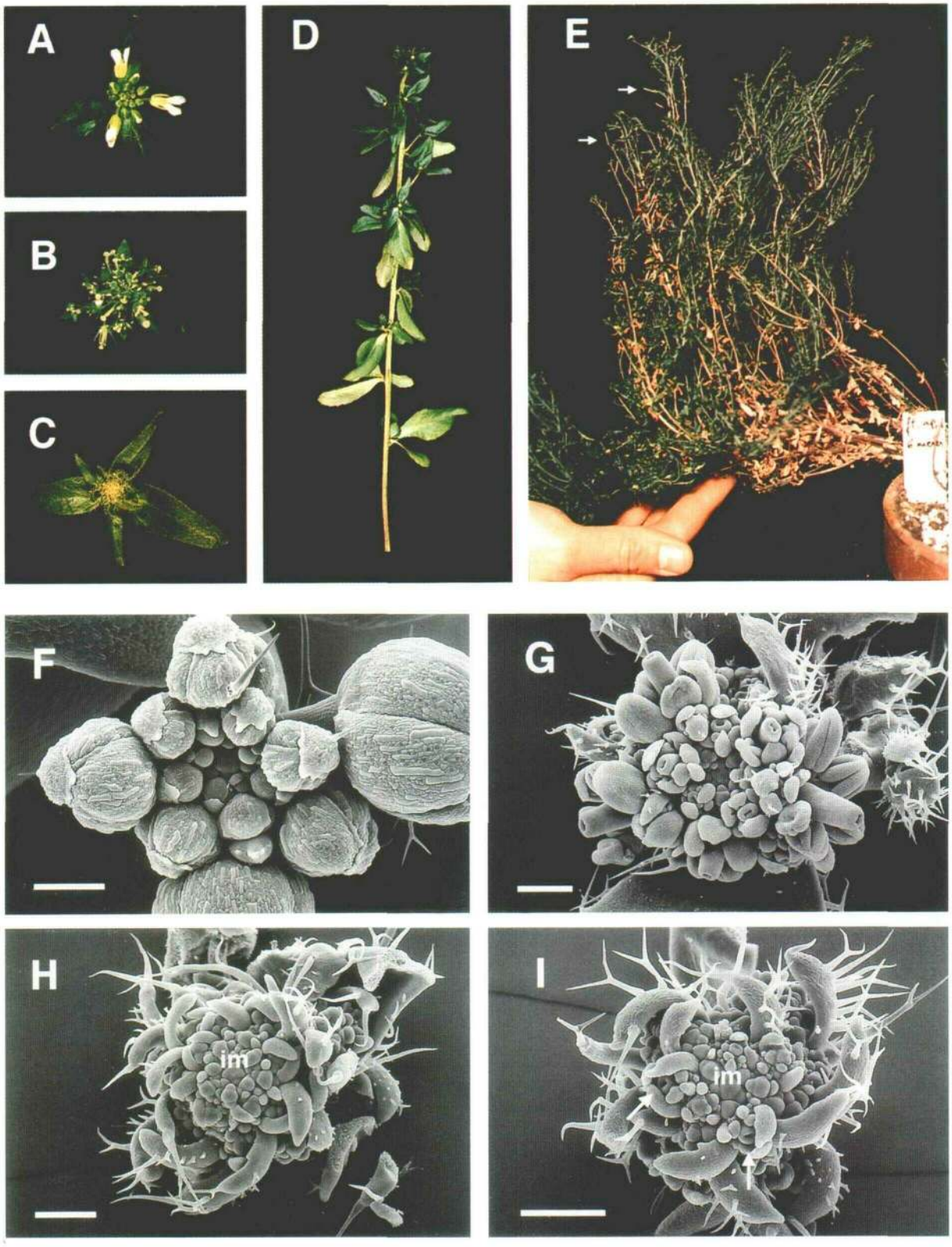

Figure 3. Structure of the Inflorescences and the Inflorescence Apices of *ft-1* and *ap1-1* and *ft-1 ap1-1* Double Mutants.

phenotype, *ft-1 ap1-1* and *fwa-1 ap-1* double mutants do not produce a higher number of inflorescence leaves than do their late-flowering parents and are still able to produce some fertile Ap1-like flowers. These phenotypes indicate that in these double mutants, *LFY* still functions in the inhibition of leaf primordia initiation and that similar to what has been described for *ap1 cal* double mutants, it finally overcomes the negative effect of *fwa* or *ft* mutations on *CAL* and other FLIP genes, promoting the initiation of flower development. RNA gel blot hybridization analyses allowed the detection of both the *LFY* and *AP1* transcripts in apices of mature plants of these double mutants, as has been observed in apices of adult *ap1 cal* plants (Bowman et al., 1993). The fact that *ft* and *fwa* single late-flowering mutants produce regular flowers indicates that a wild-type LFY function is sufficient to achieve *AP1* and other FLIP gene activity, although with some delay represented by the increased production of coflorescences in the *ft* and *fwa* mutants (Figure 3).

Mutations at the *FVE* and *FPA* loci seem to have a similar effect on the phenotype of Ap1 and Lfy plants. These plants had a much higher number of coflorescences than did any of the single mutants. Despite this strong inflorescence phenotype, double mutant inflorescences produced the same flowerlike structures observed either in the *lfy-6* or in the *ap1-1* single mutant parents, suggesting that the *fve* and *fpa* mutations delay the appearance of both the LFY and AP1 functions in the lateral meristems. Interestingly, in double mutants *fve-2 ap1-1* and *fpa-1 ap1-1*, the increase in the number of coflorescences was not accompanied by an increase in the number of caulinar leaves, suggesting that enough LFY activity is already present to inhibit leaf primordia initiation but not to determine floral meristem identity. This hypothesis is consistent with the phenotypes observed for plants heterozygous for *lfy-6* under SD (Okamuro et al., 1993, 1996) and with the phenotype shown by plants homozygous for weak *lfy* alleles (Weigel et al., 1992).

The inflorescence phenotypes observed in double mutant combinations with either *fve* or *fpa* late-flowering mutations are reminiscent of those caused by SD photoperiods on single *lfy-6* or *ap1-1* plants (Bowman et al., 1993; Okamuro et al., 1993; Schultz and Haughn, 1993; Shannon and Meeks-Wagner, 1993). However, experiments conducted under SD conditions indicate that the effects of late-flowering mutations (*fve* and *fpa*) and SD conditions on the Lfy or Ap1 phenotypes are additive (data not shown). Because the effect of SDs can be due mainly to the lack of function of the LD pathway, which is composed of *CO* and other genes such as *GI* (Coupland, 1995), we believe that both the constitutive and the LD pathways would act additively in their interactions with *LFY* and *AP1*, as shown in Figure 6. This additive interaction of both pathways is consistent with the fact that *fve-2 ap1-1* and *fve-2 lfy-6*, and *fpa-1 ap1-1* and *fpa-1 lfy-6*, are phenotypically similar to the reported double mutants *co-2 ap1-1* and *co-2 lfy-5* (Putterill et al., 1995).

Given the fact that no *AP1* function was observed in double mutants *ft-1 lfy-6* and *fwa-1 lfy-6*, any effect of the constitutive or LD pathway on *AP1* should take place indirectly either through *LFY* or *FT* and *FWA* (Figure 6). This conclusion is in agreement with recent published results on the effect of *CO* on *LFY* and *AP1* expression, indicating that *CO* function leads to rapid induction of *LFY* and a more delayed induction of *AP1* and hinting at the existence of other genes required for *AP1* expression (Simon et al., 1996). This additional pathway required for *AP1* activation could be constituted by genes such as *FT* and *FWA*. Thus, although *CO* was originally placed in the same phenotypic group as *FWA* and *FT*, based on its SD phenotype, its interaction with FLIP genes could be more similar to that of the *FVE* and *FPA* interaction demonstrated in this work. Therefore, it is reasonable to believe that *CO* and other loci required under LD conditions could act through both *LFY* and the *FT* and *FWA* genes. This would provide the plant with a redundant

Figure 3. (continued).

All plants were grown under LD photoperiods.

(A) Close-up of the apex of the primary inflorescence of *ft-1*.

(B) Close-up of the apex of the primary inflorescence of *ap1-1*.

(C) Close-up of the apex of the primary inflorescence of *ft-1 ap1-1*.

(D) Inflorescence structure of an *ft-1 ap1-1* mutant after 2 months of growth.

(E) Inflorescence structure of an *ft-1 ap1-1* mutant after 6 months of growth. The arrows indicate the presence of fertile siliques.

(F) Inflorescence apex of an *ft-1* plant grown for 2 months.

(G) Inflorescence apex of an *ap1-1* plant after 1 month of growth.

(H) Inflorescence apex of an *ft-1 ap1-1* plant after 2 months of growth. The inflorescence meristem (im) is continuously producing lateral inflorescence meristems.

(I) Inflorescence apex of an *ft-1 ap1-1* plant after 6 months of growth. Some of the flowers developing from secondary inflorescence meristems are marked by arrows. im, inflorescence meristem.

Bars in **(F)** to **(I)** = 250 μm.

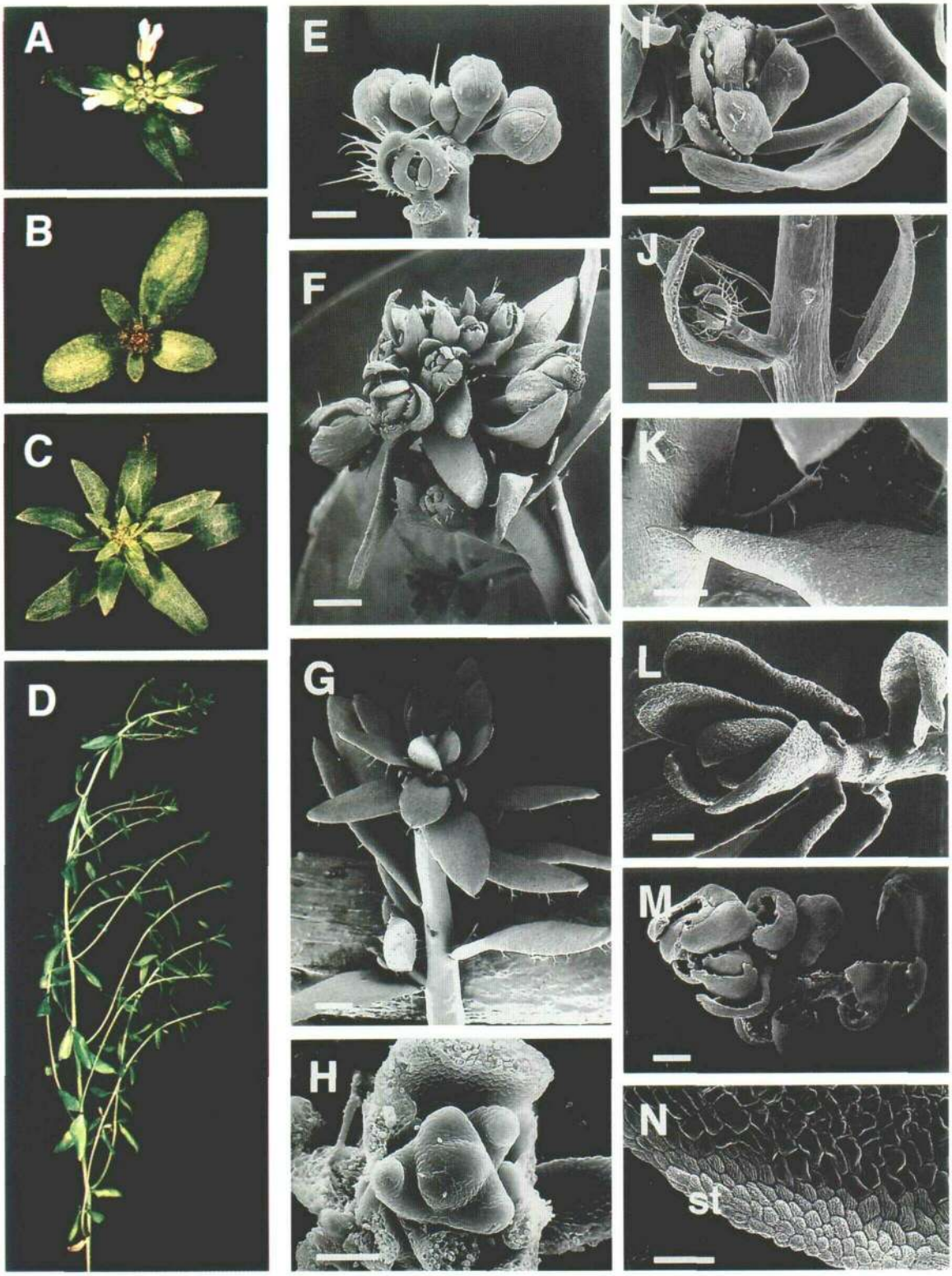

Figure 4. Structure of the Inflorescences and the Inflorescence Apices of *ft-1* and *lfy-6* and *ft-1 lfy-6* Double Mutants.

response mechanism that, acting through different genes, would warrant flowering under floral inductive conditions. Additionally, *LFY* on one side and *FT* and *FWA* on the other side would be responsive to both constitutive (*FVE* and *FPA*) and photoperiod-dependent (*CO*) floral inductive pathways.

The phenotypes of double mutants bearing a late-flowering mutation and a strong mutant allele at the *TFL1* locus (*tfl1-2*) are consistent with the existence of two different groups of late-flowering mutations. The epistatic late-flowering phenotype of double mutants *fve-2 tfl1-2* and *fpa-1 tfl1-2* would suggest that, acting in the same pathway as *FVE* and *FPA*, *TFL* would negatively regulate them. Whether this negative effect of *TFL1* is exerted by repressing their expression or by preventing their function in those cells in which *TFL1* is expressed (Bradley et al., 1997) cannot be elucidated from our results. The participation of *FVE*, *FPA*, and *TFL1* in the same developmental pathway is also consistent with two additional observations: (1) late-flowering mutants *fpa* and *fve* and early-flowering *tfl1* mutants are all photoperiod responsive (Koornneef et al., 1991; Schultz and Haughn, 1993); and (2) the vernalization treatment, which represents an alternative pathway for FPA and FVE functions (Martínez-Zapater and Somerville, 1990; Koornneef et al., 1991), also acts as an alternative pathway for TFL1 function, as deduced from the additivity observed between the effect of vernalization and *tfl1-2* mutations in double mutants *fve-2 tfl1-2* and *fpa-1 tfl1-2* (Table 2). The increased late-flowering phenotype of these double mutants could be explained by the existence of negative regulation of *FVE* and *FPA* genes on *TFL1* or redundant TFL functions.

In conclusion, the results of the double mutant analysis are consistent with the existence of constitutive and LD-dependent pathways that would promote FLIP. Both pathways could perform their function by controlling *LFY* and other genes, such as *FWA* and *FT*, that have also been identified by late-flowering mutations. *FWA* and *FT* would act redundantly with *LFY* in controlling downstream genes, such as *AP1* and other FLIP genes. Although our work offers new views on the role of some flowering-time genes and their interactions with FLIP genes, many questions have yet to be answered to have a complete understanding of the regulation of floral transition. Of special interest are those questions relating to the points of interaction of different environmental stimuli inducing flowering, such as vernalization and photoperiod, and to the molecular nature of those interactions.

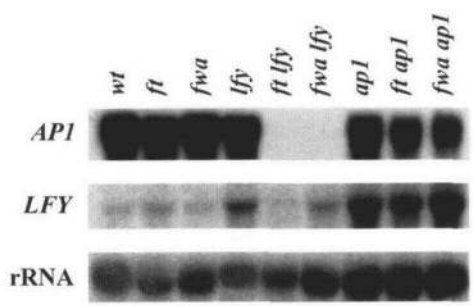

Figure 5. Expression of *AP1* and *LFY* mRNAs in the Inflorescence Apex of Double Mutants Bearing *fwa* or *ft* Mutations.

Each lane contains 40 μg of total RNA from the inflorescence apices of the different double mutants and their corresponding parental lines. All plants were grown under LD conditions. Total RNA was prepared as described in Methods. As a loading control, the membrane was hybridized with an rRNA probe, as described in Methods. *wt*, wild type.

Figure 4. (continued).

All plants were grown under LD photoperiods.
(A) Close-up of the apex of the primary inflorescence of *ft-1*.
(B) Close-up of the apex of the primary inflorescence of *lfy-6*.
(C) Close-up of the apex of the primary inflorescence of *ft-1 lfy-6*.
(D) Complete inflorescence structure of an *ft-1 lfy-6* plant after 2 months of growth.
(E) Scanning electron microscopy of the primary inflorescence apex of an *ft-1* plant. Bar = 250 μm.
(F) Primary inflorescence apex of a *lfy-6* plant. Bar = 500 μm.
(G) Primary inflorescence apex of an *ft-1 lfy-6* plant. Bar = 500 μm.
(H) Upper view of the primary inflorescence meristem of an *ft-1 lfy-6* plant grown for 6 months. Developed leaf primordia have been removed to show the apical meristem. Note the spiral-patterned appearance of the primordia and the typical shape of the leaf primordia. Bar = 50 μm.
(I) Typical flowerlike structure developing from an axilary meristem of a *lfy-6* plant. Bar = 500 μm.
(J) Structures produced in axillary positions of the inflorescence shoot of an *ft-1 lfy-6* plant. At left, a caulinar leaf subtends a lateral branch constituted by leaves. At right, no structure has developed in the axil of the leaf. Bar = 500 μm.
(K) Filamentous structure of determinate growth sometimes appearing in the axil of caulinar leaves of *ft-1 lfy-6* plants. Bar = 250 μm.
(L) Structures appearing in the apices of lateral branches of *ft-1 lfy-6* plants after 8 months of growth. Note the development of carpelloid features. Bar = 250 μm.
(M) Structures from the same apices as shown in **(L)** with more pronounced carpelloid features. These structures show the presence of stigmatic and placental tissue. Bar = 500 μm.
(N) Epidermal cells of a structure similar to the one shown in **(L)** exhibiting a sector of stylar tissue (st). Bar = 50 μm.

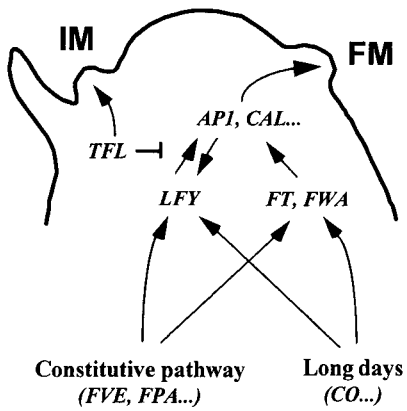

Figure 6. Schematic Representation of the Hypothetical Genetic Interactions Taking Place in the Apex among the FLIP and the Flowering-Time Genes Analyzed, as Deduced from the Phenotype of Double Mutants.

FM, floral meristem; IM, inflorescence meristem.

These and other double mutant analyses, in combination with the growing availability of molecular tools, will help us to understand how floral transition is regulated.

METHODS

Plant Material

Plants (*Arabidopsis thaliana*) used in this work belong to the ecotype Landsberg *erecta.* The origin and properties of the mutant lines used as parental plants are summarized in Table 5. Plants were grown at 18°C under long-day (LD) conditions (16 hr of light) illuminated by cool-white fluorescent lamps (140 μE m^{-2} sec^{-1}) in a mixture of sand–vermiculite–sphagnum (1:1:1 [v/v]) and irrigated with mineral nutrient solution (Haughn and Somerville, 1986) at weekly intervals. Phenotypic analyses were performed with plants grown under a 16-hr photoperiod (LD conditions). For vernalization treatments, seeds were sown in pots and exposed to 4°C in the dark for 4 weeks before being transferred to the growth chambers (Martínez-Zapater et al., 1995).

Construction of Double Mutants

Double mutant lines were isolated from F_2 populations generated by cross-pollinating parental lines homozygous for individual mutations. In the case of the *lfy-6* mutation, which causes complete sterility, a heterozygous line was used as the mutant parent. Double mutants were identified by their specific phenotypes segregating at the expected ratio. The double mutant nature of the plants was confirmed by analyzing their progeny for a true breeding phenotype and the progeny of backcrosses to the parental lines.

Phenotypic Analyses

Leaf number was counted for at least 10 plants of each genotype under each growing condition. Rosette leaves were scored by periodically monitoring the plants, marking the leaves initiated from the main rosette shoot with waterproof ink, and counting. All of the leaves present on the main shoot after internode elongation were considered as inflorescence leaves. Lateral inflorescence branches showing indeterminate growth, either with or without subtending leaves, were considered as coflorescences. Bolting time was measured as the number of days from sowing time until the main shoot had elongated up to 1 cm.

For morphological characterization, coflorescences were taken from plants of each genotype at various positions within the inflorescence shoot. This characterization was performed with at least 10 plants of each genotype. Scanning electron microscopy of the inflorescence shoot apex and the apices of coflorescences was performed as described by Schultz and Haughn (1991).

Table 5. Origin and Properties of the Mutant Lines Used

Locus	Mutant Phenotype	Allele Used	Reference
AP1	Partial transformations of flowers into inflorescences	1	Bowman et al. (1993)
LFY	Partial transformations of flowers into inflorescences and inflorescence leaves proliferate	6	Weigel et al. (1992)
TFL1	Determinate inflorescence with terminal flowers and early flowering	2	Alvarez et al. (1992)
FVE	Late flowering under LD and SD conditions, reversible by vernalization	2	Koornneef et al. (1991)
FPA	Late flowering under LD and SD conditions, reversible by vernalization	1	Koornneef et al. (1991)
FT	Late flowering under LD conditions, but not reversible by vernalization	1	Koornneef et al. (1991)
FWA	Late flowering under LD conditions, but not reversible by vernalization	1	Koornneef et al. (1991)

RNA Extraction and Gel Blot Hybridization

Both the inflorescence shoot apex and the apices of secondary shoots, which developed after excision of the main shoot apex, were used as plant material for the extraction of total RNA. In general, only flower buds developing after the last coflorescence leaves were considered within these apices. Mutants homozygous for *lfy-6* (*lfy-6*, *ft-1 lfy-6*, and *fwa-1 lfy-6*) constantly developed additional leaf primordia. For this reason, we collected apices from plants that could represent a similar developmental stage as the apices of *ap1-1* double mutants. Apices were collected shortly after bolting when inflorescences were <1 cm in length, except in the case of double mutants with *ft-1* and *fwa-1*, for which adult plants (grown for 2 months) were used as the source of the apices. RNA extractions were performed as previously described (Jarillo et al., 1993).

Total RNA (40 μg) was electrophoresed in formaldehyde–agarose gels, transferred to Hybond N^+ membranes (Amersham), and hybridized with ^{32}P-labeled probes under standard conditions (Sambrook et al., 1989). The probes used were the SacI-HincII restriction fragment of the 3′ end of the *AP1* cDNA (to avoid cross-hybridization to other MADs transcripts; Mandel et al., 1992) and the KpnI-BamHI restriction fragment from pDW122 for the *LFY* gene (Weigel et al., 1992). As a probe for rRNA, we used a 0.3-kb EcoRI fragment corresponding to the 18S rDNA of cauliflower (J.M. Franco-Zorrilla, unpublished results).

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