

Genetic interactions of the *Arabidopsis* flowering time gene *FCA*, with genes regulating floral initiation

Tania Page^{1,†}, Richard Macknight^{1,‡}, Chang-Hsien Yang² and Caroline Dean^{1,*}

¹Department of Molecular Genetics, John Innes Centre, Colney, Norwich NR4 7UH, UK, and

²Graduate Institute of Agricultural Biotechnology, National Chung Hsing University, Taichung, Taiwan 40227, Republic of China

Summary

The genes controlling the timing of the transition from vegetative to reproductive growth are likely candidates for regulators of genes initiating floral development. We have investigated the interaction of one particular gene controlling flowering time, *FCA*, with the meristem identity-genes *TERMINAL FLOWER 1* (*TFL1*), *APETALA 1* (*AP1*) and *LEAFY* (*LFY*) and the floral repression gene *EMBRYONIC FLOWER 1* (*EMF1*). Double mutant combinations were generated and the phenotypes characterized. The influence of strong and intermediate *fca* mutant alleles on the phenotype conferred by a *35S-LFY* transgene was also analysed. The results support a model where *FCA* function promotes flowering in multiple pathways, one leading to activation of *LFY* and *AP1*, and another acting in parallel with *LFY* and *AP1*. Only the latter pathway is predicted to be non-functional in the intermediate *fca-4* allele. The results are also consistent with *AP1* and *TFL1* negatively regulating *FCA* function. Combination of Columbia *fca* and *emf1* mutant alleles confirmed that *FCA* is required for the early flowering of *emf1*. *EMF1* and *FCA* are therefore likely to operate in different floral pathways.

Introduction

The control of the transition from vegetative to reproductive development is being extensively studied in *Arabidopsis thaliana*. Genes involved in controlling the timing of the floral transition have been identified through mutagenesis and analysis of natural variation (Koornneef *et al.*, 1998b). Mutations that confer late flowering are considered to identify gene products whose wild-type function is to

promote flowering. The flowering time of some of the late flowering mutants, including *fca*, *fve*, *fy* and *ld* is accelerated in response to a long period of cold temperature (vernalization) but it is further delayed in restrictive short day photoperiods. These genes are considered to act in the autonomous floral promotion pathway (Koornneef *et al.*, 1991, 1998a; Martinez-Zapater and Somerville, 1990). The flowering time of other late flowering mutants, including *co*, *gi*, *fwa*, *fd*, *fe*, *ft* and *fha* is less responsive to both vernalization and photoperiod. These genes are considered to act in a genetic pathway controlling the long-day promotion of flowering (Koornneef *et al.*, 1991, 1998a; Martinez-Zapater and Somerville, 1990).

A likely function of the genes controlling the timing of the floral transition is the regulation of genes involved in formation of a floral meristem ('meristem-identity genes'). How this regulation might occur has been studied recently using different approaches. Analysis of double mutant combinations of the late flowering mutants *fve*, *fpa*, *fwa* and *ft* with the meristem-identity mutants *leafy* (*lfy*) and *apetala1* (*ap1*) mutations led to the division of *fwa* and *ft* into a different functional group with respect to other flowering time genes (Ruiz-Garcia *et al.*, 1997). *fwa lfy* and *ft lfy* double mutants did not produce any flower-like structures or *AP1* RNA. *FT* and *FWA* were therefore proposed to act redundantly with *LFY* to activate *AP1* and act as an intermediary step between some of the other floral promoters and floral meristem gene activation.

FWA and *FT* have also been found to control the competence to respond to *LFY* activity. Nilsson *et al.* (1998) analysed flowering time in *35S-LFY* transgene lines carrying different late flowering mutations and GUS activity from a *LFY-GUS* transgene in the same set of late flowering mutant backgrounds (Nilsson *et al.*, 1998). The data indicated that *CO*, *GI*, *FCA*, *FVE* and the genes involved in the gibberellin response pathway *GA1* and *GAI* all play a role in *LFY* activation. Most of the genes also seem to be required for the function of *LFY* in that the flowering time of the late flowering mutant *35S-LFY* line was intermediate between that of both parents. The situation was different, however, for *fwa*, *fe* and *ft*. The late flowering phenotype was epistatic to the early flowering conferred by the *35S-LFY* transgene showing that *FWA*, *FE* and *FT* function in parallel with or downstream of *LFY*.

Activation of meristem-identity genes has also been studied by Simon *et al.* (1996) who used a glucocorticoid-inducible system to activate *CO*. Induction of *CO* was sufficient to induce flowering in SD and to initiate transcription of *LFY* and *TFL1* as rapidly as when these genes are

Received 22 September 1998; revised 13 November 1998; accepted 20 November 1998.

*For correspondence (fax +44 1603 505725; e-mail caroline.dean@bbsrc.ac.uk).

†Present address: Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK.

‡Present address: Department of Biochemistry, University of Otago, Cumberland Street, Dunedin, New Zealand.

induced by LD photoperiods. *AP1* transcription, however, was induced more slowly by *CO* compared to LD.

EMBRYONIC FLOWER (EMF1) and *EMF2* genes also regulate meristem-identity gene activation and are considered to function as a central regulator repressing the transition to flowering (Chen *et al.*, 1997; Sung *et al.*, 1992). Strong *emf1* alleles bypass vegetative development and form carpelloid structures immediately after germination. *AP1* and *AGAMOUS* promoters are activated in germinating *emf* seedlings and constitutive expression of *LFY* in *emf1* mutants increased the severity of the *emf* phenotype. A reciprocal negative regulation between the *EMF* genes and the meristem-identity genes has therefore been suggested (Chen *et al.*, 1997). The role of *EMF* as a component of the central floral repression pathway is supported by the observation that *emf* mutations are epistatic to *gi* and *co* (Yang *et al.*, 1995).

In order to further dissect the relationship between meristem-identity and flowering time genes, we have analysed the interaction of *FCA* with genes involved in the formation of a floral meristem. Mutations in the *FCA* gene strongly delay the floral transition resulting in plants with much larger numbers of rosette leaves and cofilences. The increased vegetative phase is fully reversed when *fca* mutant plants are vernalized (Koornneef *et al.*, 1991). X-ray induced *fca* mutant sectors did not affect bolting or flowering, suggesting that *FCA* may act non-cell autonomously (Furner *et al.*, 1996). *FCA* encodes an RNA-binding protein with a WW protein interaction domain and the *FCA* transcript is alternatively spliced with only one form encoding the entire *FCA* protein (Macknight *et al.*, 1997). It appears, therefore, that *FCA* is a component of a post-transcriptional cascade regulating flowering time. A series of double mutants between *fca* and mutations affecting floral initiation have been made, using multiple combinations of different alleles wherever practicable. The phenotypes of the double mutants have been analysed with respect to flowering time and inflorescence phenotype.

Results

Effect of fca mutations on the floral transition

Mutations in the *FCA* gene significantly delay the transition from vegetative to floral development. The six mutant alleles (*fca* 1–6) that have previously been described (all in the Landsberg *erecta* (*Ler*) background) show a range of flowering times (Koornneef *et al.*, 1991). Two of the *Ler fca* alleles, *fca-1* (one of the strongest alleles) and *fca-4* (an intermediate allele), were used in the double mutant analysis. In addition, *fca-9* (a Columbia (*Col*) allele) was used. The availability of mutant alleles in both ecotypes meant that double mutants could be made without mixing backgrounds, thus avoiding any complications of modifier

genes affecting the phenotype of the double mutant. In addition, observation of similar interactions between mutant alleles in different backgrounds strengthens the conclusions that can be made.

fca-1 and *fca-4* (the *Ler* mutant alleles) and the wild-type *Ler* parent were grown in a controlled environment cabinet (used to grow the majority of the double mutants) in which the plants were exposed to a long-day light regime of 10 h high intensity light, followed by a 6 h low intensity day extension. *fca-4* flowered with an average of 23 total (rosette + cauline) leaves compared to 29 for *fca-1* and 7 for *Ler* (Table 1). Both mutations significantly increased the number of cofilences (the lateral inflorescences arising from the main inflorescence, subtended by a cauline leaf) with *fca-1* showing a stronger effect than *fca-4*. The number of floral nodes was also significantly increased with both mutants producing approximately 50 as compared to 23 in wild-type *Ler* plants. *fca-9* flowered with approximately 37 rosette leaves and produced about eight cofilence nodes under greenhouse conditions (Table 1).

The strong *fca-1* allele carries a single nucleotide substitution (C to T) which introduces a stop codon within exon 13 (Macknight *et al.*, 1997) and production of a truncated *FCA* protein. The intermediate *fca-4* allele is the result of a large inversion that disrupts the *FCA* gene at the 3' end of exon 4 (C. Lister and C. Dean, unpublished results). The 3' fragment is expressed as a translational fusion in *fca-4* as determined by the presence of a polypeptide of 84 kDa (16 kDa larger than predicted to be encoded from the 3' *FCA* fragment) on Western blots probed with an antibody specific to the C-terminal half of the *FCA* protein (P Dijkwel and C. Dean, unpublished results). The resulting polypeptide, which is expressed at approximately the same level as wild-type *FCA* protein, would contain the second RNA-binding domain and the C-terminal region of the protein including the WW protein interaction domain (Macknight *et al.*, 1997). In comparison with a mutation in another strong allele (*fca-6*) that terminates the open reading frame very close to the break point in *fca-4*, we deduce that production of this partial protein accounts for the intermediate flowering time phenotype.

fca-9 (*Col*) flowers much later than *fca-1* (*Ler*) when grown side by side (C. Dean, unpublished results). The Columbia ecotype contains dominant alleles at *FLC* shown to enhance the late flowering phenotype of *fca* mutations (Sanda and Amasino, 1996) so it is difficult to directly compare the relative strengths of the *fca-1* and *fca-9* mutations. Our current analysis shows that *fca-9* produces a truncated protein shorter than that in *fca-1*, indicating that *fca-9* is a strong mutant allele (C. Lister and C. Dean, unpublished results).

Combination of fca and tfl1 mutations

Mutations in the *TFL1* gene result in conversion of the indeterminate apical meristem into a determinate floral

Table 1. Flowering time, leaf, coflorescence and floral node number of different Arabidopsis genotypes

	Flowering time	Rosette leaves	Cauline leaves	Coflorescence no. ^e	Floral nodes	No. of plants
<i>Ler</i>	22.9 ± 0.1	4.4 ± 0.3	2.4 ± 0.2	2.4 ± 0.2	22.9 ± 2.1	10
<i>fca-1</i>	47.5 ± 1.2	20.9 ± 0.8	7.8 ± 0.5	7.9 ± 0.5	51.9 ± 1.7	10
<i>fca-4</i>	42.9 ± 0.5	16.7 ± 0.5	6.2 ± 0.3	6.3 ± 0.3	48.5 ± 1.6	10
<i>tfl1-2</i>	21.1 ± 0.1	3.7 ± 0.2	2.1 ± 0.1	0.8 ± 0.4	5.0 ± 0.4	10
<i>fca-1 tfl1-2</i>	47.0 ± 0.9	24.0 ± 1.0 ^b	8.9 ± 0.5	9.3 ± 0.5	33.9 ± 0.8	10
<i>fca-4 tfl1-2</i>	43.4 ± 0.9	19.0 ± 1.1	5.6 ± 0.4	5.6 ± 0.4	30.6 ± 1.5	10
<i>lfy-5</i>	22.7 ± 0.2	5.5 ± 0.2 ^a	5.6 ± 0.3	6.0 ± 0.4	26.3 ± 3.1	10
<i>fca-1 lfy-5</i>	42.4 ± 0.8	22.9 ± 0.8 ^b	17.0 ± 0.8	20.8 ± 0.8	39.5 ± 1.4	10
<i>fca-4 lfy-5</i>	41.9 ± 1.3	18.0 ± 1.2	14.4 ± 1.2	21.2 ± 1.8	33.3 ± 1.8	20
<i>lfy-6</i>	38.0 ± 1.1	6.3 ± 0.4 ^a	7.8 ± 0.7	7.4 ± 0.6	13.0 ± 0.6	12
<i>fca-1 lfy-6</i>	59.1 ± 1.5	23.0 ± 0.1 ^b	42.5 ± 5.5	45.5 ± 4.5	28.5 ± 2.5	2
<i>fca-4 lfy-6</i>	58.8 ± 2.6	24.5 ± 3.3 ^c	42.9 ± 1.9	37.6 ± 1.9	21.6 ± 1.7	14
<i>ap1-1</i>	21.1 ± 0.1	3.2 ± 0.2 ^a	2.5 ± 0.2	4.4 ± 0.2	25.0 ± 2.1	10
<i>fca-1 ap1-1</i>	47.1 ± 2.1	22.9 ± 2.1	8.6 ± 0.8	18.1 ± 2.8	34.6 ± 3.5	9
<i>fca-4 ap1-1</i>	51.0 ± 0.5	27.4 ± 0.4 ^c	9.1 ± 0.6	22.4 ± 1.0	19.8 ± 1.9	20
<i>Col</i>	22.4 ± 2.1	11.2 ± 1.8	2.2 ± 0.4	2.2 ± 0.4	> 35	12
<i>fca-9</i>	58.5 ± 7.8	37.6 ± 5.3	8.4 ± 1.3	8.4 ± 1.3	> 35	20
<i>tfl1-1</i>	18.4 ± 1.2	5.3 ± 1.0 ^d	0.3 ± 0.4	0.3 ± 0.4	3.2 ± 0.9	15
<i>fca-9 tfl1-1</i>	54.8 ± 10.3	35.3 ± 6.9	5.4 ± 0.9	5.4 ± 0.9	18.6 ± 7.4	15
<i>lfy-2</i>	21.3 ± 1.9	6.8 ± 0.9		7.3 ± 1.1		15
<i>lfy-10</i>	23.3 ± 2.0	7.8 ± 1.1		7.3 ± 1.3		15
<i>fca-9 lfy-2</i>	56.7 ± 9.4	38.2 ± 7.1		40.9 ± 7.9		15
<i>fca-9 lfy-10</i>	62.2 ± 7.4	44.8 ± 6.4		40.5 ± 4.9		15
<i>ap1-3</i>	21.6 ± 1.6	7.7 ± 1.1	2.4 ± 0.5			15
<i>fca-9 ap1-3</i>	53.8 ± 7.9	33.1 ± 4.3	7.8 ± 1.4			15

^aSignificantly different from Landsberg *erecta* parent (all significance tests done at 0.05 level). ^bSignificantly different from *fca-1*. ^cSignificantly different from *fca-4*. ^dSignificantly different from Columbia parent. Wild-type and mutant alleles in Landsberg *erecta* were grown in controlled environment rooms whereas wild-type and mutant alleles in Columbia were grown under greenhouse conditions. ^eLateral inflorescences arising from the main inflorescence, not including secondary inflorescences subtended by rosette leaves.

meristem (Shannon and Meeks-Wagner, 1991). They also cause an acceleration in flowering time, the extent differing in different alleles (Alvarez *et al.*, 1992). In order to analyse the interaction of *FCA* and *TFL1*, the double mutants *fca-1 tfl1-2* (a strong *Ler* allele), *fca-4 tfl1-2* and *fca-9 tfl1-1* (a strong *Col* allele) were generated.

The flowering time, leaf number, coflorescence and floral node number of the single and double mutant combinations are shown in Table 1. *tfl1-2* flowered with an average of 5.8 leaves, approximately one leaf less than the *Ler* parent. However, the flowering time of the double mutants, *fca-1 tfl1-2* (Figure 1b) and *fca-4 tfl1-2* was later than the respective *fca* parents. Coflorescence and cauline leaf number on the main inflorescence of the double mutants was similar to the *fca* parent. The number of floral nodes produced was less than the *fca* parent but > sixfold greater than in the *tfl1-2* mutant. Production of the terminal flower was suppressed in the double mutants but it did occur eventually in all cases. As in the single mutant, the terminal flower produced in the double mutant was abnormal, generally lacking sepals and petals, having six stamens and a deformed gynoecium (Figure 1f). A greater number of secondary inflorescences (arising from axillary meristems

and subtended by rosette leaves) were observed in *fca-1 tfl1-2* as compared to *fca-1* (Figure 1b). *tfl1-1* flowered with approximately eight total leaves less than the *Col* parent, agreeing with the previous findings that this allele results in a greater acceleration of flowering time than *tfl1-2* (Alvarez *et al.*, 1992). The number of leaves produced in *fca-9 tfl1-1* and *fca-9* were not significantly different. Formation of the terminal flower was significantly delayed and the coflorescence number in the double mutant was 18-fold greater than in *tfl1-1*. Where analysed, short day photoperiod effects on the *tfl1-2* phenotype were additive with those of *fca* mutations (data not shown).

All the *fca* alleles therefore showed a similar interaction with the different *tfl1* alleles. The late flowering phenotype was epistatic to the early flowering conferred by *tfl1* alleles and the formation of the terminal flower was significantly delayed.

Combination of *fca* and *lfy* mutations

The role of *FCA* in floral initiation as promoted by *LFY* was examined. *LFY* acts in combination with *AP1* and other genes to promote floral meristem identity. In addition, *LFY*

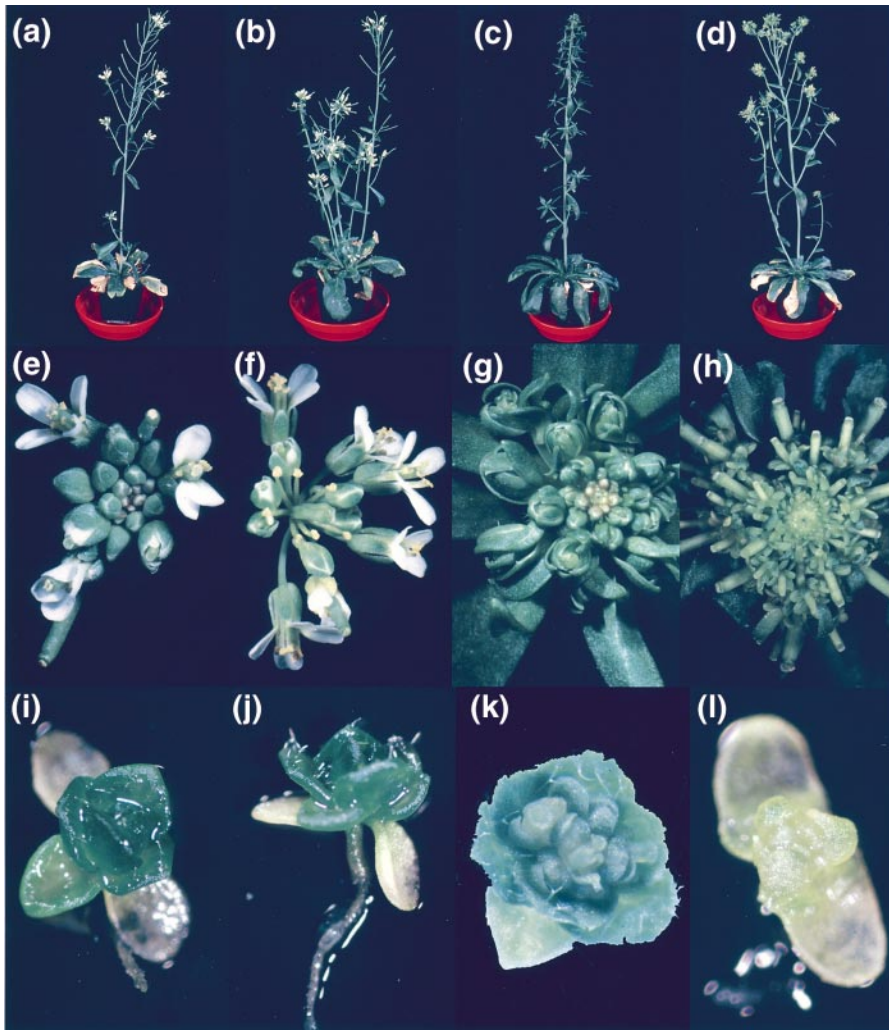


Figure 1. Phenotypes of double mutant combinations.

(a) Wild-type *Ler*; (b) *fca-1 tfl1-2* in LD; (c) *fca-1 lfy-5* in LD; (d) *fca-1 ap1-1* in LD; (e) apex of wild-type *Ler* plant in LD; (f) apex of *fca-1 tfl1-2* in LD; (g) apex of *fca-1 lfy-6* in LD; (h) apex of *fca-1 ap1-1* in LD; (i) *fca-9 emf1-1* 25 d after germination; (j) *fca-9 emf1-1*, showing trichomes on the adaxial (upper) surface of the sessile leaves; (k) *fca-9 emf1-1* 40 d after germination; and (l) *fca-9 emf1-2* 25 d after germination.

functions in the formation of normal flowers as *lfy* mutants form flower-like structures that lack petals and stamens (Huala and Sussex, 1992; Weigel *et al.*, 1992). *LFY* is considered to act as a link between the timing of the floral transition and the initiation of individual flowers (Blazquez *et al.*, 1997). Double mutant combinations of *fca-1 lfy-5* (a weak allele), *fca-1 lfy-6* (a strong allele), *fca-4 lfy-5* and *fca-4 lfy-6* were generated and analysed in the LD controlled growth cabinet. *fca-9* in combination with *lfy-2* and *lfy-10* (both weak alleles) were analysed under greenhouse conditions (Table 1). *lfy* mutations increased rosette leaf number at flowering in the single and in all the double mutant combinations. The combination of *lfy-6* with *fca-4* resulted in a strong delay in flowering with *lfy-6 fca-4* plants producing nine leaves more than the *fca-4* parent. In many *fca* and *lfy* combinations there was a large (fivefold) increase in the number of cymosecences formed

(Figure 1c). Thus, production of nodes resulting in the typical *lfy* flower-like structure was significantly delayed in *fca* mutants. Grown side by side in the LD cabinet, the phenotype of an *fca-1 lfy-5* double mutant was indistinguishable from that of a *co2 lfy-5* double mutant (Simon and Coupland, 1996). One feature of the *fca-1 lfy-6* double mutant was the lack of secondary inflorescences produced compared to *fca-1* (Figure 1c). Where analysed, the effects of short day photoperiods were additive to the effects of *fca* mutations (data not shown).

The individual flowers of the *lfy fca* double mutants closely resembled the flowers of the respective *lfy* parent (Figure 1g) (Weigel *et al.*, 1992). The flowers of the *lfy 5 fca* double mutants were fertile, unlike flowers of the strong allele *lfy-6*, showing that any enhancement of the *lfy5* floral phenotype by *fca* mutations was only moderate. Grown under greenhouse conditions, *fca-9 lfy-2* and *fca-9 lfy-10*

flowers appeared to show a slightly more extreme *lfy* phenotype than their respective *lfy* parent. Petals and stamens were absent, carpels were unfused and the plants were sterile.

In summary, *fca* alleles enhanced various aspects of the *lfy* phenotype, most notably a large increase in the number of shoot-like structures on the inflorescence. As this enhancement is seen with null *lfy* mutations, the effects of the *fca* mutations cannot be due to reduction of residual *LFY* activity. Combination of *fca* and *lfy* alleles caused the plants to flower even later, with the greatest delay caused by *lfy-6* in an *fca-4* background.

Combination of *fca* and *ap1* mutations

In order to analyse the interaction of *FCA* and *AP1*, different combinations of *fca* mutations with *ap1* alleles (Bowman *et al.*, 1993; Mandel *et al.*, 1992) were generated. The strong *ap1-1* allele was combined with *fca-1* and *fca-4*. The relatively weak allele, *ap1-3*, was combined with *fca-9*. In the LD cabinet conditions, the *ap1-1* mutant flowered earlier and with fewer rosette leaves than the *Ler* parent (in agreement with (Schultz and Haughn, 1993)). The *fca-1 ap1-1* double mutant plants flowered at a similar time to the *fca-1* parent, with a similar number of rosette leaves (Figure 1d), but the *fca-4 ap1-1* plants flowered considerably later than *fca-4* (27.4 versus 16.7 leaves). Under greenhouse conditions *ap1-3* flowered significantly earlier than the *Col* parent but the *fca-9 ap1-3* double mutant flowered with approximately the same number of leaves as *fca-9*.

The number of cauline leaves and floral nodes were unchanged in *ap1-1* in the LD conditions relative to *Ler*, but the number of coflorescences increased almost two-fold. The coflorescence number was approximately two- and 3.5-fold greater in the double mutants *fca-1 ap1-1*, *fca-4 ap1-1* compared to the respective *fca-1*, *fca-4* parents. The number of floral nodes was greatly reduced in *fca-4 ap1-1*.

The phenotypic abnormalities of the individual flowers were similar in the single and double mutants. The characteristic determinate branched flowers previously described for *ap1-1* mutations (Bowman *et al.*, 1993) were present and these formed a dense mass of floral-like structures at the inflorescence apex in the late flowering backgrounds (Figure 1h). The fertility of the *fca-9 ap1-3* double mutant was slightly reduced under greenhouse conditions compared to its parents.

In summary, all *fca* alleles enhanced the *ap1* inflorescence phenotype but did not significantly affect floral development in the *ap1* flowers. As *ap1-1* is a strong allele this suggests that *FCA* functions to promote the formation of flowers in pathways that act redundantly with *AP1*. The early flowering phenotype of *ap1-1* mutations indicates

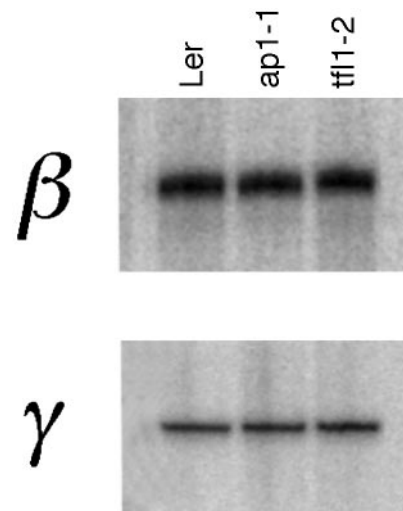


Figure 2. RNase protection analysis of RNA isolated from *Ler*, *ap1-1* and *tf11-2* seedlings grown in LD conditions.

The probes used to assay *FCA* transcript β and γ are described in Macknight *et al.* (1997).

that *AP1* functions as a repressor of the floral transition and the epistasis of the late flowering phenotype of *fca-1* over the early flowering of *ap1-1* suggests that it acts via inhibiting *FCA* function. In contrast to the early flowering conferred by *ap1-1* in an *Ler* background, *ap1-1* produced a significant delay in flowering time in an *fca-4* background.

Analysis of *FCA* RNA abundance and splicing in *tf11-2* and *ap1-1* mutants

One interpretation of the epistasis with respect to flowering time of *fca* mutations in *fca tf11* and *fca ap1* combinations is that *TFL1* and *AP1* negatively regulate *FCA* function. This could be through modulation of *FCA* expression or through a downstream antagonistic action. The *FCA* gene is alternatively spliced and four different *FCA* transcripts have been characterized (Macknight *et al.*, 1997). Transgenic plants carrying fusions where the *FCA* gene is driven by the strong, constitutive 35S cauliflower mosaic virus promoter accumulate a short *FCA* transcript, termed transcript β , which does not encode a full-length functional *FCA* protein (Macknight *et al.*, 1997). It is possible, therefore, that the splicing of the *FCA* gene could be regulated to limit *FCA* protein levels. We investigated whether the levels of *FCA* or the splicing of the *FCA* transcript were altered in *tf11-2* or *ap1-1* mutants. RNase protection experiments were used to assay the two major *FCA* transcripts, transcript β which constitutes approximately 55% of the *FCA* message in wild-type plants and transcript γ , which constitutes approximately 35% of the transcripts and is the transcript that would encode the full-length *FCA* protein (Macknight *et al.*, 1997). The level and ratio of the *FCA* transcripts was found to be the same in RNA from *ap1-1*, *tf11-2* and *Ler* seedlings (Figure 2). Thus, the repressive effects of *AP1*

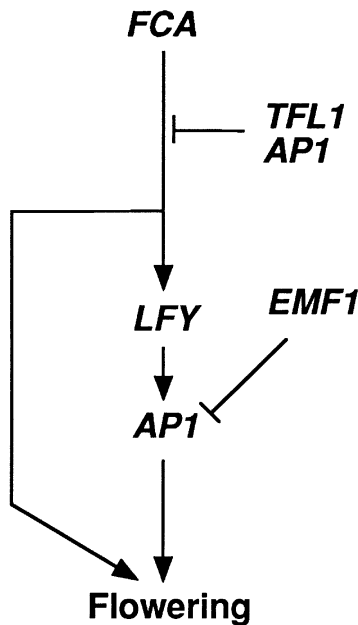


Figure 3. Model showing possible interactions of *FCA* with meristem-identity genes.

EMF1 is shown negatively regulating *AP1* based on the results of Chen *et al.* (1997). Later functions of *TFL1* are not included. This is based on the hypothesis that *fca-4* produces a product that is active in only a subset of pathways in which wild-type *FCA* functions. The model needs to be tested by introduction of transgenes expressing truncated *FCA* proteins into different genetic backgrounds.

and *TFL1* on *FCA* function that influence the timing of the floral transition do not appear to act through changes in the gross levels or splicing of the *FCA* message.

Combination of *fca-9* with *emf1* alleles

Mutations in the *EMF1* gene result in flowering in the absence of an obvious vegetative phase (Sung *et al.*, 1992). Plants carrying the weak allele *emf1-1* produce just a small inflorescence with cauline leaves and one or two flowers with incomplete floral organ development. Plants carrying *emf1-2* show a much stronger phenotype and produce only carpelloid structures, capped with stigmatic papillae. To assess the interaction of *EMF1* and *FCA*, *fca-9 emf1-1* and *fca-9 emf1-2* double mutants were generated (only the Col *fca* allele was used to avoid mixing genetic backgrounds which was a complication in the previous analysis (Haug and Yang, 1998). *fca-9 emf1-1* double mutants displayed the *emf1* phenotype during early development, for example, small shoot size, sessile cotyledons and leaves, short hypocotyl and oval-shaped cotyledons. However, the majority of the *fca-9 emf1-1* double mutants did not flower and produced significantly more sessile leaves (> 10) than the *emf1-1* single mutant (Figure 1i–k) before senescing. Among those double mutants which did flower, the flowering time was delayed, the number of sessile leaves was higher (approximately 10), and several flower-

Table 2. Effect of a 35S *LFY* transgene on flowering time of *Ler*, *fca-1* and *fca-4*

Genotype	Total leaf number at flowering	
	non-transgenic control	+ 35S-LFY
<i>Ler</i>	11.6 ± 0.2	7.2 ± 0.1
<i>fca-1</i>	39.8 ± 0.8	25.3 ± 0.7
<i>fca-4</i>	24.6 ± 0.4	23.1 ± 0.2

Values are means ± standard errors from populations of between 15 and 40 plants. Plants were grown in similar but not identical controlled environment conditions to those shown in Table 1, thus the relative flowering times are the same but the absolute values are different.

like structures with multiple pistils were produced (Figure 1k). Thus, the early appearance of flowers in *emf1-1* is dependent on *FCA* function. The sessile leaves carried trichomes on both surfaces, although the density was much higher on the adaxial (upper) surface (Figure 1j). The production of leaves with trichomes on both surfaces is a characteristic of leaves produced in the adult phase of vegetative development (Telfer *et al.*, 1997).

emf1-2 fca-9 double mutants showed the early *emf1-2* phenotype with small, sessile cotyledons, although later in development the presence of the *fca-9* allele caused a significant reduction in the extent of the carpelloid structures produced (Figure 1l). Thus, loss of *FCA* function delayed the formation of reproductive structures in both *emf1* alleles.

The effect of *fca* mutations on the acceleration of flowering conferred by 35S-LEAFY

The putative roles of *FCA* downstream of *TFL1* and *AP1*, and the requirement for *FCA* for early flowering of *emf1* mutants, prompted us to look at the effect of *fca* mutations on the acceleration of flowering conferred by a 35S-*LFY* transgene. Ectopic expression of *LFY* results in an acceleration of the transition to flowering and all lateral shoots being converted into flowers (Weigel and Nilsson, 1995). A transgenic line (in *Ler* and therefore avoiding any complications due to segregation of modifiers) carrying the 35S-*LFY* transgene was crossed to *fca-1* and *fca-4* and the leaf number at flowering scored in plants doubly homozygous for the mutation and the transgene (Table 2). The leaf number of *fca-1 35S-LFY* was intermediate with that of both parents (agreeing with data from Nilsson *et al.*, 1998), whereas leaf number of *fca-4 35S-LFY* was not significantly different to *fca-4*. The *fca-1 35S-LFY* and *fca-4 35S-LFY* lines showed the conversion of axillary nodes into flowers, the shortening of the inflorescence, the terminal flower and the twisting and curling of the leaves typical of the parent 35S-*LFY* line. However, even in the

presence of the *35S-LFY* transgene, *fca* mutations delay the floral transition at the apex and the two *fca* alleles show different interactions.

Discussion

The interaction of *FCA* with *TFL1*, *LFY*, *AP1* and *EMF1* has been analysed with respect to effects on inflorescence and flower formation and changes in flowering time. With respect to inflorescence formation, *fca* mutations showed non-additive interactions with *tfl1*, *lfy* and *ap1* mutations. Formation of a terminal flower in the *fca tfl1* combinations was significantly delayed, demonstrating that *FCA* function is required for the early transition of the apex into a determined floral meristem. *fca* mutations also enhanced the number of coflorescence nodes in *lfy* and *ap1* backgrounds. This enhancement occurs in a background where *LFY* or *AP1* activity is absent, suggesting that *FCA* is required for the activation or function of the genes that act redundantly with *LFY* or *AP1* to affect the initiation of flowers. This interaction is very similar to that described for *FVE* and *FPA* (Ruiz-Garcia *et al.*, 1997). There was very little effect of *fca* mutations on flower structure.

Analysis of the flowering time of the different double mutant combinations yielded informative interactions. In *Ler*, *AP1* appears to repress the floral transition as *ap1-1* mutant plants (both in *Ler* and *Col*) flowered earlier and with fewer leaves than wild-type, in agreement with Schultz and Haughn (1993). In contrast, *ap1-1 fca-1* combinations flowered as late as *fca-1*. Why *ap1-1* mutants flower early is unclear as *AP1* overexpression resulting from a *35S-AP1* transgene results in early flowering (Mandel and Yanofsky, 1995) and expression of *AP1* appears to be an indicator of floral determination (Hempel *et al.*, 1997). It is possible that *AP1* is expressed at very low levels early in development and it is this activity that acts to repress flowering. Whatever the mechanism, the early flowering phenotype of *ap1-1* requires *FCA* which suggests that *AP1* may repress the floral transition through a repressive effect on *FCA* function.

The late flowering phenotype of *fca* was also found to be epistatic to the early flowering of *tfl1* alleles. As with *AP1*, this would indicate that *TFL1* acts in the same genetic pathway as *FCA* and represses *FCA* function. Loss of *TFL1* activity would result in earlier flowering due to decreased repression of *FCA* function. A similar interaction has been found between *tfl1-2* and mutant alleles of *FVE* and *FPA* (Ruiz-Garcia *et al.*, 1997). In contrast, the double mutant phenotype of *tfl1* and mutant alleles of *FT*, *FWA* and *SIN1* is considered to be additive (Ray *et al.*, 1996; Ruiz-Garcia *et al.*, 1997), the double mutant phenotype flowering slightly but significantly earlier than the late flowering parent. This then places *FT*, *FWA* and *SIN1* in a different floral promotion pathway to *FCA*, *FVE* and *FPA*. These results are consistent with previous divisions of *FCA*, *FVE*

and *FPA* as part of the autonomous floral promotion pathway, and *FT* and *FWA* as part of the long day promotion pathway (Koornneef *et al.*, 1998a). How *TFL1* functions in the autonomous promotion pathway remains to be established. The lack of detectable change in *FCA* transcript level and splicing suggests that the repression on *FCA* function is not through a direct effect on *FCA* transcription or splicing. *FCA* RNA has been detected throughout the plant and at different developmental stages (Macknight *et al.*, 1997). *TFL1* expression was detected in subapical meristematic cells and throughout the inflorescence stem (Bradley *et al.*, 1997). The expression patterns could indicate that *TFL1* function acts to antagonize the promotive effects of *FCA* in meristematic cells.

The early flowering of *emf1* mutants was also affected by the lack of *FCA* function. However, in contrast to the epistasis observed with *ap1* and *tfl1*, *emf1-1 fca-9* double mutants showed a phenotype that could be interpreted as additive to that of both parents. The early *emf1* characteristics were present but many more leaf-like structures were produced before floral-like structures appeared in a proportion of the double mutants. This phenotype was more extreme than that found in *emf1-1 fca-1* double mutants (Haug and Yang, 1998) where 5–6 sessile leaves were produced. The attenuated phenotype of *emf1 fca-1* as compared to *emf1 fca-9* is probably the result of the combination of modifiers inherited from the *Ler* and *Col* parents used in the cross. However, both cases show that *FCA* function is required for the early transition to reproductive development in *emf1* mutants, suggesting that *FCA* operates in an independent pathway to *EMF1*. *emf1-1 gi-3* and *emf1-1 co* double mutants showed the *emf1-1* phenotype (Haug and Yang, 1998; Yang *et al.*, 1995). In contrast, the combination of *fwa* and *fld-2* with *emf1* alleles produced plants with more sessile leaves than *fca emf 1* double mutants (Chou and Yang, 1998; Haug and Yang, 1998). *EMF1* would appear, therefore, not to act in a central repression pathway to flowering but acts as a repressor within the long-day promotion pathway, with *GI* and *CO* functioning as upstream repressors of *EMF1*. The similar phenotype of *emf1* in long and short day photoperiods would support placing *EMF1* in the *CO*, *GI* pathway (Bai and Sung, 1995).

In some of the interactions, *fca-1* and *fca-9* showed different effects to *fca-4*. *fca-4 lfy-6* and *fca-4 ap1-1* flowered significantly later than *fca-4*, whereas *fca-1 lfy-6*, *fca-1 ap1-1* and *fca-9 lfy-2*, *fca-9 ap1-3* flowered at about the same time as *fca-1* and *fca-9*, respectively. *LFY* and *AP1* function must therefore still be acting to promote flowering in *fca-4*. A *35S-LFY* transgene caused accelerated flowering in *fca-1* but not in *fca-4* such that *35S-LFY fca-4* flowered at the same time as *35S-LFY fca-1*. The molecular basis of the intermediate phenotype conferred by *fca-4* is unknown. The *fca-4* allele does not produce one of the *FCA* transcript

forms, δ or the protein isoform derived from it. In addition, a truncated FCA polypeptide is made consisting of a translational fusion to an unknown protein of the second of the two RNA-binding domains, the glutamine-rich C terminus and the WW protein interaction domain (C. Lister, P. Dijkwel and C. Dean, unpublished results). This chimaeric protein does not appear to create a gain of function or dominant negative allele as *fca-4* is recessive. The *fca-4* allele may produce an FCA protein which functions to activate the same targets as wild-type but with reduced activity. If these targets were *LFY* and *AP1*, this would lead to partial activation of *LFY* and *AP1* explaining the lateness in *fca-4 lfy* or *ap1* combinations. However, this would not account for the late flowering conferred by *fca-4* being epistatic to the early flowering from the *35S-LFY* transgene. If *FCA* acted downstream of *LFY* and *AP1*, the late flowering of the strong *fca-1* allele would be epistatic to the early flowering from the *35S-LFY* transgene rather than additive. A third possibility is that *FCA* acts in a parallel pathway to *LFY* and *AP1*. However, it would then be hard to explain the different interactions of strong *lfy* and *ap1* mutations with the *fca-1* and *fca-4* alleles.

A model that can account for all the results on interactions affecting flowering time and inflorescence formation is one in which *FCA* functions in a parallel pathway to *LFY* and *AP1* as well as in the activation of *LFY* and *AP1* (Figure 3). Strong *fca* alleles, for example *fca-1* and *fca-9*, would represent loss of both pathways, whereas the partial activity in *fca-4* would represent loss of the parallel pathway only. Activation of *LFY* and *AP1* would still occur in *fca-4*, which would explain why *lfy* and *ap1* mutations result in later flowering in an *fca-4* background. The similar flowering times of *35S-LFY fca-4* and *35S LFY fca-1* genotypes would indicate that the loss of the parallel pathway could not be compensated for by overexpression of *LFY*. This model is heavily dependent on the interpretation of the results from the rather unusual allele, *fca-4*, and therefore needs to be tested and elaborated by introduction of transgenes expressing truncated FCA proteins into different genetic backgrounds. It does have the attraction that it may provide an explanation for the presence of the multiple transcripts and FCA protein isoforms in wild-type plants. Further analysis is required to determine if the multiple FCA isoforms produced in wild-type plants are functionally important.

Experimental procedures

Plant lines

Landsberg *erecta*, *fca-1*, *fca-4* (*Ler* alleles) were obtained from Prof. M. Koornneef. *tfl1-2*, *ap1-1* (*Ler* alleles) were from the Nottingham stock centre. *lfy-5* and *lfy-6* (*Ler* alleles) and the *35S-LFY* transgenic line DW151.2.5 (in *Ler*) were obtained from Dr D.

Weigel. *tfl1-1*, *lfy-2*, *lfy-10*, *ap1-3* (*Col* alleles) were from the Ohio stock centre. *fca-9*, an *fca* allele in the Columbia background, was isolated by C.-H.Y. whilst in Dr R. Sung's laboratory (University of California, Berkeley, USA). Double mutant lines were isolated from *F₂* populations generated by cross-pollinating parental lines homozygous for individual mutations. In the case of the *emf1* and *lfy-6* mutants, heterozygous lines were used as mutant parents. Double mutants were easily identified segregating in the expected 1/16th ratio in the *F₂* progeny. A line showing a typical double mutant phenotype (as compared to the other double mutants segregating in the *F₂*) was selfed and *F₃* seed collected. Flowering time, leaf number, coflorescence and floral node number were scored on *F₃* individuals grown alongside relevant controls. For *fca lfy-6* and *fca emf1*, where the double mutant plant was sterile, *F₂* individuals homozygous for *fca* and heterozygous for either *lfy-6* or *emf1* were selfed and the phenotype was scored on the 1/4 of the progeny that were homozygous for both mutations. The plants were regularly moved around in the growth room to minimize effects of microenvironment in the chamber.

Growth conditions

Plants were grown in soil (mixture of Levingtons M3 compost with grit) either in a greenhouse (for *fca-9* analyses) or a Sanyo Gallenkamp controlled environment room under short day or extended short day conditions (for the *fca-1*, *fca-4* analyses). Light conditions for the short-day room were 10 h illumination by 400 W Wotan metal halide power star lamps, PAR 113.7 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ and an R/FR ratio of 2.41. Light for extended short-days was as for short-days (10 h) followed by 8 h illumination with tungsten halide lamps only, PAR 14.27 $\mu\text{mol m}^{-2} \text{sec}^{-1}$, and an R/FR ratio of 0.66. Plants were transferred to partitioned trays at about the four-leaf stage.

RNAse protection assays

RNA isolation and RNAse protection assays for *FCA* transcripts β and γ were performed as described in Macknight *et al.* (1997).

Acknowledgements

This work was funded through the BBSRC CSG grant to the JIC, BBSRC Plant Molecular Biology II programme and EC Bridge grant (BIOT CT 90-0207). C.-H.Y. was supported by a grant from the National Science Council, Taiwan, Republic of China, grant number NSC 85-2321-B-005-076. The authors thank Dr Detlef Weigel for transgenic lines and extremely useful discussion. They also thank Drs Jose Martinez-Zapater, George Coupland and Gordon Simpson for comments on the manuscript.

References

- Alvarez, J., Guli, C.L., Yu, X.-H. and Smyth, D.R. (1992) *terminal flower*: a gene affecting inflorescence development in *Arabidopsis thaliana*. *Plant J.* **2**, 103–116.
- Bai, S. and Sung, Z.R. (1995) The role of *EMF1* in regulating the vegetative and reproductive transition in *Arabidopsis thaliana* (Brassicaceae). *Am. J. Bot.* **82**, 1095–1103.
- Blazquez, M.A., Soowal, L.N., Lee, I. and Weigel, D. (1997) *LEAFY* expression and flower initiation in *Arabidopsis*. *Development*, **124**, 3835–3844.

- Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M. and Smyth, D.R.** (1993) Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. *Development*, **119**, 721–743.
- Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R. and Coen, E.** (1997) Inflorescence commitment and architecture in *Arabidopsis*. *Science*, **275**, 80–83.
- Chen, L., Cheng, J.-C., Castle, L. and Sung, Z.R.** (1997) *EMF* genes regulate *Arabidopsis* inflorescence development. *Plant Cell*, **9**, 2011–2024.
- Chou, M.-L. and Yang, C.-H.** (1998) *FLD* interacts with genes that affect different developmental phase transitions to regulate *Arabidopsis* shoot development. *Plant J.* **15**, 231–242.
- Furner, I.J., Ainscough, J.F.-X., Pumfrey, J.A. and Petty, L.M.** (1996) Clonal analysis of the late flowering *fca* mutant of *Arabidopsis thaliana*: cell fate and cell autonomy. *Development*, **122**, 1041–1050.
- Haug, M.-D. and Yang, C.-H.** (1998) *EMF* genes interact with late-flowering genes to regulate *Arabidopsis* shoot development. *Plant Cell Physiol.* **39**, 382–393.
- Hempel, F.D., Weigel, D., Mandel, M.A., Ditta, G., Zambryski, P., Feldman, L. and Yanofsky, M.** (1997) Floral determination and expression of floral regulatory genes in *Arabidopsis*. *Development*, **124**, 3845–3853.
- Huala, E. and Sussex, I.M.** (1992) *LEAFY* interacts with floral homeotic genes to regulate *Arabidopsis* floral development. *Plant Cell*, **4**, 901–913.
- Koornneef, M., Alonso-Blanco, C., Blankestijn-de Vries, H., Hanhart, C.J. and Peeters, A.J.M.** (1998a) Genetic interactions among late flowering mutants of *Arabidopsis*. *Genetics*, **148**, 885–892.
- Koornneef, M., Alonso-Blanco, C., Peeters, A.J.M. and Soppe, W.** (1998b) Genetic control of flowering time in *Arabidopsis*. *Annu. Rev. Pl. Phys. Pl. Mol. Biol.* **49**, 345–370.
- Koornneef, M., Hanhart, C.J. and Van der Veen, J.H.** (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **299**, 57–66.
- Macknight, R., Bancroft, I., Page, T. et al.** (1997) *FCA*, a gene controlling flowering time in *Arabidopsis*, encodes a protein containing RNA-binding domains. *Cell*, **89**, 737–745.
- Mandel, M., Gustafson-Brown, C., Savidge, B. and Yanofsky, M.F.** (1992) Molecular characterization of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature*, **360**, 273–277.
- Mandel, M.A. and Yanofsky, M.F.** (1995) A gene triggering flower formation in *Arabidopsis*. *Nature*, **377**, 522–524.
- Martinez-Zapater, J.M. and Somerville, C.R.** (1990) Effects of light quality and vernalization on late-flowering mutants of *Arabidopsis thaliana*. *Plant Physiol.* **92**, 770–776.
- Nilsson, O., Lee, I., Blazquez, M.A. and Weigel, D.** (1998) Flowering-time genes modulate the response to *LEAFY* activity. *Genetics*, **150**, 403–410.
- Ray, A., Lang, J.D., Golden, T. and Ray, S.** (1996) *Short integument (SIN1)*, a gene required for ovule development in *Arabidopsis*, also controls flowering time. *Development*, **122**, 2631–2638.
- Ruiz-Garcia, L., Madueno, F., Wilkinson, M., Haughn, G., Salinas, J. and Martinez-Zapater, J.M.** (1997) Different roles of flowering time genes in the activation of floral initiation genes. *Plant Cell*, **9**, 1921–1934.
- Sanda, S.L. and Amasino, R.M.** (1996) Interaction of *FLC* and late-flowering mutations in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **251**, 69–74.
- Schultz, E.A. and Haughn, G.W.** (1993) Genetic analysis of the floral initiation process (FLIP) in *Arabidopsis*. *Development*, **119**, 745–765.
- Shannon, S. and Meeks-Wagner, D.R.** (1991) A mutation in the *Arabidopsis TFL1* gene affects inflorescence meristem development. *Plant Cell*, **3**, 877–892.
- Simon, R. and Coupland, G.** (1996) *Arabidopsis* genes that regulate flowering time in response to daylength. *Seminars Cell Dev. Biol.* **7**, 419–425.
- Simon, R., Igeno, M.I. and Coupland, G.** (1996) Activation of floral meristem identity genes in *Arabidopsis*. *Nature*, **282**, 59–62.
- Sung, Z.R., Belachew, A.T., Shunong, B. and Bertrand-García, R.** (1992) *EMF*, an *Arabidopsis* gene required for vegetative shoot development. *Science*, **258**, 1645–1647.
- Telfer, A., Bollman, K.M. and Poethig, S.** (1997) Phase change and regulation of trichome distribution in *Arabidopsis thaliana*. *Development*, **124**, 645–654.
- Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F. and Meyerowitz, E.M.** (1992) *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell*, **69**, 843–859.
- Weigel, D. and Nilsson, O.** (1995) A developmental switch sufficient for flower initiation in diverse plants. *Nature*, **377**, 495–500.
- Yang, C.-H., Chen, L.-J. and Sung, Z.R.** (1995) Genetic regulation of shoot development in *Arabidopsis*: role of the *EMF* genes. *Dev. Biol.* **169**, 421–435.