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LEAFY expression and flower initiation in *Arabidopsis*

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SUMMARY

During the initial vegetative phase, the *Arabidopsis* shoot meristem produces leaves with associated lateral shoots at its flanks, while the later reproductive phase is characterized by the formation of flowers. The *LEAFY* gene is an important element of the transition from the vegetative to the reproductive phase, as *LEAFY* is both necessary and sufficient for the initiation of individual flowers. We have analyzed in detail the expression of *LEAFY* during the plant life cycle, and found that *LEAFY* is extensively expressed during the vegetative phase. In long days, *Arabidopsis* plants flower soon after germination, and this is paralleled by rapid upregulation of *LEAFY*. In short days, *Arabidopsis* plants flower several weeks later than in long days, but *LEAFY* expression increases gradually before flowering commences. Application of the plant hormone gibberellin, which hastens flowering in short days, enhances the gradual change in *LEAFY* expression observed in short days. Changes in *LEAFY* expression before the transition to flowering suggest that the time point of this transition is at least partly controlled by the levels of *LEAFY* activity that are prevalent at a given time of the life cycle. This assumption is borne out by the finding that increasing the copy number of endogenous *LEAFY* reduces the number of leaves produced before the first flower is formed. Thus, *LEAFY* combines properties of flowering-time and flower-meristem-identity genes, indicating that *LEAFY* is a direct link between the global process of floral induction and the regional events associated with the initiation of individual flowers.

Key words: *Arabidopsis*, flower development, floral induction, meristem identity, *LEAFY*

INTRODUCTION

Post-embryonic life of flowering plants is divided into two distinct phases: an initial vegetative phase, during which leaves with associated lateral shoots or paraclades are produced, and a subsequent reproductive phase, during which flowers are produced. The transition between the two phases is caused by a complex process termed floral induction, which is controlled by both endogenous and environmental signals. In *Arabidopsis*, a facultative long-day plant, flowering is promoted by long photoperiods, vernalization (transient exposure to cold), and higher growth temperatures (Napp-Zinn, 1985; Martínez-Zapater and Somerville, 1990; Koornneef et al., 1995).

Physiological approaches to study the molecular details of floral induction have recently been complemented by genetic studies, which have led to the identification of a large group of flowering-time mutations that either reduce or lengthen the time to flowering (Koornneef et al., 1991; Zagotta et al., 1992; Martínez-Zapater et al., 1994; Peeters and Koornneef, 1996). The genes inactivated in two late-flowering mutants, *luminidependens (ld)* and *constans (co)*, encode putative transcription factors (Lee et al., 1994; Putterill et al., 1995), each of which functions in a different floral induction pathway. Mutations in *LD* cause late flowering in both long and short days, and, consistent with this, the abundance of *LD* mRNA is not affected by photoperiod (Lee et al., 1994). In contrast, *co* mutants are only delayed in long days, and *CO* mRNA is more abundant in long than in short days (Putterill et al., 1995). A causal relation between *CO* RNA levels and flowering time has been confirmed by Simon and coworkers (1996), who showed that overexpression of *CO* causes photoperiod-independent early flowering.

Another group of genes involved in the floral transition are the flower-meristem-identity (or floral-initiation-process) genes. Among these, the *LEAFY (LFY)* gene stands out, because its expression precedes that of other meristem-identity genes with flower-specific expression, and because *lfy* loss-of-function mutations have the strongest effect on meristem identity (Mandel et al., 1992; Weigel et al., 1992; Jofuku et al., 1994; Ingram et al., 1995; Kempin et al., 1995). These observations suggest that *LFY* is responsible for the initial steps in flower initiation.

At the time when wild-type plants begin to produce flowers, *lfy* mutants continue to produce leaves and associated lateral shoots. In contrast to early-arising flowers, later-arising flowers have only a partial requirement for *LFY* activity due to the redundant activity of other genes such as *APETALA1 (AP1)*, and later flowers are replaced by structures that combine characteristics of both flowers and shoots. One indication of this mixed identity is that *lfy* mutant flowers are subtended by leaf-like organs, or bracts (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992). Conversely, while inacti-

vation of *LFY* causes the transformation of flowers into shoots or shoot-like structures, constitutive expression of *LFY* from the cauliflower-mosaic-virus 35S promoter causes a transformation of all lateral shoots into solitary flowers and a reduction in the number of leaves on the primary axis, particularly in short days (Weigel and Nilsson, 1995). In addition to its role in establishing flower-meristem identity, *LFY* functions also in maintaining meristem identity, and plants heterozygous for a *lfy* null mutation show a high rate of floral reversion when grown in short days (Okamuro et al., 1996).

The analysis of *35S::LFY* plants also revealed a second level of control of flower initiation, namely regulation of the competence of a meristem to respond to *LFY* activity, as all *35S::LFY* transformants make at least a few leaves before flowers without subtending leaves are produced (Nilsson and Weigel, unpublished results). That the *35S::LFY* phenotype is attenuated in short days indicates that acquisition of competence is at least partially dependent on environmental factors (Weigel and Nilsson, 1995).

To gain new insights into the molecular basis of flower initiation, we have studied in detail the activity of the promoter of the *LFY* gene, which has been previously reported to be expressed at high levels in flowers (Weigel et al., 1992). This study focuses on *LFY* expression during the entire life cycle of the plant, while the accompanying study by Hempel et al. (1997) describes in more detail the relationship between *LFY* expression and determination of floral fate during the transition to flowering. The two major conclusions from our studies are that the profiles of *LFY* expression during the *Arabidopsis* life cycle differ under different environmental conditions, and that the relative levels of wild-type *LFY* affect the timing of the transition to flowering.

MATERIALS AND METHODS

Construction of transgenes

All transgenes were constructed in the pCGN1547 transformation vector (McBride and Summerfelt, 1990). pDW130 contains a 5.3 kb genomic *LFY* fragment (nucleotides 464 to 5957; GenBank accession no. M91208; Weigel et al., 1992). pLS41 contains a 2.3 kb *Bam*HI promoter fragment fused at the downstream site (nucleotide 2755) to the *LFY* cDNA excised from pDW123, representing a longer splice form, but otherwise identical to pDW122 (Weigel et al., 1992). 3′ of the *LFY* cDNA is a 300 bp *Eco*ICR-*Eco*RI fragment from pBI221 (Clontech; Jefferson et al., 1987), encompassing the *nos* transcriptional terminator from *Agrobacterium*. pDW150 is derived from pDW137, a pCGN1547 derivative containing the *Hin*dIII-*Eco*RI fragment of pBI101.2, which encompasses the β-glucuronidase (GUS) coding region and the *nos* terminator (Clontech; Jefferson et al., 1987). The 2.3 kb *Bam*HI fragment spanning the *LFY* promoter was inserted in front of *GUS* in pDW137, such that the fusion gene uses the *LFY* initiation codon, which overlaps the downstream *Bam*HI site (ATGGATCC). The authentic *GUS* initiation codon is seven codons downstream from the *LFY* initiation codon.

Plant material and growth conditions

The *lfy-12* and *lfy-26* mutant alleles have been described by Huala and Sussex (1992) and Lee et al., (1997); see also URL: http://www.salk.edu/LABS/pbio-w/leafyseq.html. Transgenic plants were generated with the vacuum-infiltration method (Bechtold et al., 1993). pDW130 was introduced into the Nossen ecotype; pLS41 into Nossen and Columbia ecotypes as indicated. pDW150 was introduced into the Columbia, Landsberg *erecta* and Nossen ecotypes, and lines used here were homozygous for the transgene.

Plants were grown at 23°C in long-day (16 hours light/8 hours dark) or short-day cycles (10 hours light/14 hours dark), under a mixture of Cool White and Gro-Lux/WS fluorescent lights (Osram, Sylvania). For GA treatments, plants were liberally sprayed twice weekly with a solution of 100 μM GA_3 (Sigma) in 0.02% Tween-20.

GUS staining and activity measurement

For histochemical analyses, dissected apices were incubated in 50 mM X-gluc staining solution (50 mM sodium phosphate buffer pH 7, 0.2% Triton X-100, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 20% methanol), first on ice under vacuum for 1-2 hours, then at 30°C for 16 hours. For whole-mount photographs, tissue was dehydrated in 70% ethanol and photographed under differential interference contrast optics on a Zeiss Axioskop microscope. For photographs of sections, tissue was dehydrated and embedded in Paraplast (Sherwood Medical). 12 μm thick sections were prepared on a Leica microtome, mounted, and, after removal of Paraplast with xylenes, photographed either under bright-field or dark-field illumination.

For quantitative measurements, we refined an assay using 4-methyl umbelliferyl glucuronide (MUG), which is converted by GUS enzyme into the fluorescent product 4-methyl umbelliferone (4-MU). Apices were incubated at 37°C for 16 hours in 1 mM MUG assay solution (50 mM sodium phosphate buffer pH 7, 10 mM EDTA, 0.1% SDS, 0.1% Triton X-100), in individual wells of a microtiter plate. After the reaction had been stopped by the addition of 0.3 M Na_2CO_3, fluorescence at 430 nm was measured on a luminescence spectrophotometer equipped with an ELISA plate reader (Perkin Elmer, model LS50B).

In situ hybridization

In situ hybridization using antisense RNA probes labeled with [^{35}S]-UTP as well as photography and image processing of hybridized sections were as described by Drews et al. (1991) and Lee et al. (1997). Template for the *LFY* probe was pDW124 (Weigel et al., 1992; URL: http://www.salk.edu/LABS/pbio-w/pDW124.html); for *GUS* it was pLS27, which contains the *GUS* coding region from pBI221 (Jefferson et al., 1987) subcloned into pBstKS+ (Stratagene).

Rescue experiments and genotyping

DW130 or LS41 transformants in Nossen ecotype were crossed to *lfy-26* in Landsberg *erecta*. To genotype F_2 plants, we developed a CAPS (cleaved amplified polymorphic sequence; Konieczny and Ausubel, 1993) marker that distinguishes between the Landsberg *erecta* and Nossen alleles of *LFY*. The two oligonucleotide primers used for polymerase chain reaction (PCR) were 5′-GCT GAT ATC GTT TAA CTA TCT TAA GAT ACA TGG-3′ and 5′-CGC TCA GTT GGT TGA CTC CGA CTC-3′. Since the upstream primer is located outside the region present in the transgenes, only endogenous *LFY* sequences are amplified. Amplification of Landsberg *erecta* DNA yields a 1.9 kb product that is digested by *Rsa*I into fragments of 970, 813 and 156 bp. The 970 bp fragment contains an additional *Rsa*I site in the Nossen ecotype, yielding two fragments of about 600 and 370 bp.

In the experiments with reduced copies of *LFY*, the *lfy-12* mutation was detected with a CAPS marker based on a *Mae*III site created by the *lfy-12* nucleotide change, which is the same as in *lfy-1* (Weigel et al., 1992; URL: http://www.salk.edu/LABS/pbio-w/caps.html).

RESULTS

LEAFY sequences required for in vivo function

lfy null mutations interfere with the formation of normal flowers. In positions where the first flowers are found on the inflorescence shoot of a wild-type plant, additional leaves and

lateral shoots are present in *lfy* mutants, while later-arising flowers are replaced by structures that have only partial shoot character. One indication of this mixed identity is that *lfy* mutant flowers are subtended by small leaves called bracts (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992).

To define the extent of genomic sequences required for in vivo function, we crossed four independent transgenic lines containing a 5.3 kb fragment of the *LFY* region (DW130) to plants carrying the strong *lfy-26* allele, and analyzed the phenotype of the F_2 progeny. In all cases, a single copy of the transgene was able to complement all defects of the *lfy* mutant, both in short and in long days. To further test whether regulatory sequences contained within the *LFY* promoter were sufficient for in vivo function, we constructed a mini-gene consisting of the *LFY* cDNA fused to 2.3 kb of *LFY* 5′ upstream sequences. This mini-gene (LS41) complemented the inflorescence and flower defects caused by the *lfy-26* mutation in two of three independent lines tested in both long and short days. One line showed no rescue in short days, but almost complete rescue in long days (Table 1). This result indicates that the 2.3 kb promoter can confer a pattern of *LFY* expression that is sufficient to support normal flower development, and that this promoter fragment is suitable for reporter gene studies.

We chose the reporter gene *uidA* encoding *E. coli* β-glucuronidase (GUS; Jefferson et al., 1987), because of the ease with which its expression is quantified, and generated 65 lines containing a *LFY::GUS* transgene (DW150) in the ecotypes Columbia, Landsberg *erecta* and Nossen. GUS expression was easily detectable by X-gluc staining in inflorescence apices of many lines, and several representative ones were chosen for further analysis after the transgene insertions had been made homozygous. We compared, in more detail, the pattern of *GUS* RNA expression to that of *LFY* RNA in inflorescence apices by in situ hybridization, and found *GUS* and *LFY* RNA expression to be similar (Fig. 1). Strong *GUS* expression is apparent in floral anlagen and in flowers of stages 1 and 2, in which *LFY* is thought to execute its role in promoting floral identity (Weigel et al., 1992). The pattern of *GUS* RNA from stage 3 on, however, deviates from that of endogenous *LFY*, with *GUS* RNA levels being strongest in prospective whorls two and three, whereas *LFY* RNA levels are highest in emerging sepal primordia. Although differences in RNA stability might contribute to the differences seen in RNA accumulation, it is likely that other elements in addition to the 2.3 kb promoter are needed to completely reproduce the *LFY* RNA pattern.

Activity of the *LEAFY* promoter during the vegetative phase

To determine the time course of *LFY* promoter activity under different photoperiods, we grew three independent Columbia and two Landsberg *erecta* lines homozygous for the *LFY::GUS* insertion in long and short days, and analyzed samples taken at different time points from germination until flower buds became visible to the naked eye. GUS activity was quantitatively measured in individual dissected apices, using the substrate 4-methyl umbelliferyl glucuronide (MUG), which is converted by GUS into the fluorescent product 4-MU. The procedure we used does not involve mechanical protein extraction, and GUS activity was determined on a per-apex basis. The quantitative data obtained with the MUG assays were complemented qualitatively by histochemical X-gluc analyses and by in situ hybridization to endogenous *LFY* RNA.

For all lines tested, the profiles of GUS activity were similar to the one shown in Fig. 2. In long days, *LFY::GUS* expression was sharply upregulated beginning after about 9 days, which correlates with the short time to flowering under this light regime (21 days to visible flower buds in Columbia). We expected a similar pattern in short days, that is, initial low activity, with a sharp increase at the time of the transition to flowering. The observed profile, however, differed strikingly from the expected one (Fig. 2). Starting from initially low levels, *LFY::GUS* expression increased gradually several weeks before flowers were initiated (56 days to visible flower buds).

Since plant hormones of the gibberellin (GA) class are known to accelerate flowering in short days (Langridge, 1957; Wilson et al., 1992), we also tested the effects of exogenously applied GA_3 on *LFY::GUS* expression. GUS activity in short-day grown plants that were continuously treated with GA_3 increased more rapidly than in control plants (Fig. 2). Since this was paralleled by a reduction in flowering time, from 56 to 43 days, the effects of GA on flowering time correlate with those on *LFY* promoter induction.

The quantitative measurements of whole apices did not indicate whether the observed changes in GUS activity were caused by a change in the number of *LFY::GUS* expressing cells, or an absolute change of *LFY::GUS* activity in a constant number of cells, or by a combination of both. To distinguish between these possibilities, and to determine the tissue in

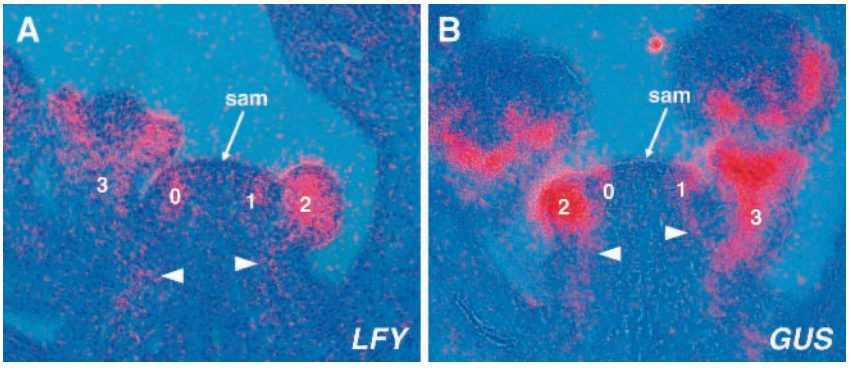

Fig. 1. Expression pattern of *LFY* and *GUS* RNA in inflorescences of *LFY::GUS* plants. RNA distribution in DW150-209 (Columbia) plants was localized by in situ hybridization to longitudinal sections of inflorescence apices. Silver grains have been colored red. Numbers indicate stages of flower development according to Smyth et al. (1990). (A) *LFY* RNA, which is absent from the shoot apical meristem (sam), is first detected in floral anlagen (0) and persists at high levels throughout floral primordia until stage 3, when expression abates in the center of the flower. Arrowheads indicate expression in what appear to be vascular traces in the stem. (B) *GUS* expression driven by the *LFY* promoter mimics that of the endogenous gene until stage 3, when RNA accumulates preferentially in presumptive whorls two and three. Both micrographs were taken at the same magnification.

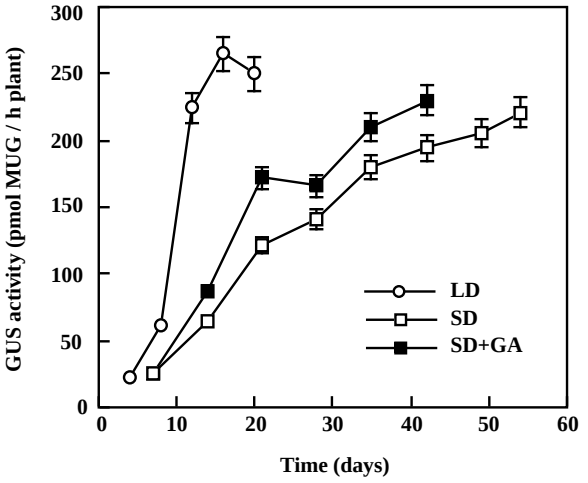

Fig. 2. Time-course of *LFY::GUS* activity under different growth conditions. Homozygous DW150-209 (Columbia) plants were grown in long days (LD), in short days (SD), or in short days and treated with GA_3 (SD+GA), until flower buds became visible to the naked eye. At each time point, GUS activity was measured in at least twelve individual apices, and the means ± s.e.m. (95% confidence interval) are given.

which the *LFY* promoter was active, we localized *LFY::GUS* expression by staining plants in the vegetative phase with the chromogenic substrate X-gluc (Fig. 3). GUS activity was already detectable in 4-day-old seedlings, both in short and long days. At this and later time points, GUS activity was present in the newly emerging leaf primordia, but absent from older leaves. In sections of 3-week-old plants, we detected weak GUS activity even in leaf anlagen (Fig. 3D).

Examination of X-gluc-stained apices showed also that the number of GUS-positive leaf primordia increased during the first 10 days under long-day conditions and the first 20 days under short day conditions (results not shown). It remained constant afterwards, suggesting that the overall increase in *LFY::GUS* expression after these time points primarily reflected increased activity of the *LFY* promoter, at least on a per-primordium basis.

To evaluate the validity of the *LFY::GUS* analyses, we monitored expression of endogenous *LFY* RNA by in situ hybridization. Wild-type Columbia plants were grown in short days, and harvested for in situ hybridization at 2, 4, 6 and 8 weeks after germination (Fig. 4). Detection of *LFY* RNA in apices of 2-week-old plants was close to the detection limit, but silver grain density was consistently higher over leaf primordia than over the shoot apical meristem (Fig. 4A). Expression of *LFY* RNA in emerging leaf primordia was readily apparent in 4- and 6-week-old plants, with higher levels in the older plants (Fig. 4B,C). While it is difficult to quantify expression levels by in situ hybridization, these qualitative data together with the quantitative MUG assays indicate that the relative levels of *LFY* RNA in emerging leaf primordia increased gradually during the first 6 weeks of vegetative growth under short-day conditions. Although we have not analyzed long-day-grown plants in detail, it has recently been reported that low levels of endogenous *LFY* RNA are found in 3-day-old long-day grown seedlings (Bradley et al., 1997).

We have detected GUS activity, but not *LFY* RNA, in leaf anlagen, and we detect GUS activity in more primordia than we detect *LFY* RNA. These differences likely reflect that detection of GUS activity is more sensitive than detection of *LFY* RNA by in situ hybridization, and that GUS protein is more stable than most RNAs. While higher sensitivity in the detection assay is not of concern, the higher stability of GUS protein means that the gradual increase in *LFY::GUS* activity should be viewed as a qualitative result.

Upregulation of *LEAFY* under inductive photoperiods

The more rapid increase of *LFY::GUS* expression in long days than in short days suggested that the *LFY* promoter is a target of photoperiodic regulation. Therefore, we measured GUS activity in *LFY::GUS* plants that were grown under short-day

Table 1. Complementation of a strong *lfy* mutation by a *LFY* minigene

		Organ number				
		Sepals	Petals	Stamens	Carpels	*n*
	Ler	4.0±0	4.02±0.03	5.9±0.1	2.0±0	60
	No-0	4.0±0	4.0±0	5.93±0.07	2.0±0	80
	lfy-26	8.8±0.5	0±0	0±0	5.1±0.4	80
LD	LS41-102	4.2±0.1	2.8±0.3	5.2±0.2	2.0±0	80
	LS41-207	4.03±0.03	4.00±0.04	5.93±0.05	2.01±0.03	160
	LS41-402	4.06±0.05	4.07±0.06	5.3±0.1	2.00±0.01	160
	Ler	4.03±0.06	4.01±0.03	5.7±0.1	2.0±0	70
	No-0	4.0±0	4.0±0	5.93±0.06	2.0±0	80
	*lfy-26**	–	–	–	–	–
SD	LS41-102*	–	–	–	–	–
	LS41-207	4.03±0.03	4.00±0.02	5.75±0.08	2.0±0	150
	LS41-402	5.7±0.1	4.5±0.2	5.3±0.2	2.0±0	110

Organ numbers in wild-type as well as *lfy-26* plants with and without the LS41 transgene (*LFY* promoter::*LFY* cDNA).Plants carrying the LS41 transgene in the Nossen (No-0) background were crossed to *lfy-26* plants in the Landsberg *erecta* (Ler) background. Transgenic F_2 plants were selected on kanamycin plates; homozygosity for the *lfy*-26 mutation was determined with a CAPS marker. On different plants, the organs in the first ten flowers were counted. Values are mean ± standard error (95% confidence interval).

n, total number of flowers counted; LD, long days; SD, short days.

*Organ number in these lines was not counted, because flower-like structures were not produced. Most lateral structures were leaf-bearing shoots, as is typical for *lfy* mutants grown in short days (Huala and Sussex, 1992).

Table 2. Time to flowering in relation to *LFY* copy number

Line genotype (*LFY* copies)		Leaf number					
		lfy-12			LS41-1203		
		*lfy/lfy** (0)	*LFY/lfy* (1)	*LFY/LFY* (2)	*LFY/LFY* (2)	+1 transgene (3)	+2 transgenes (4)
LD	RL	13.0±0.2†	13.2±0.2†	11.9±0.3	12.0±0.3	11.7±0.3	10.1±0.3†
	CL	12.5±0.4‡	4.3±0.2†	3.3±0.2	3.3±0.2	3.1±0.1	1.8±0.2‡
	TL	25.5±0.5‡	17.5±0.3‡	15.2±0.2	15.3±0.2	14.8±0.2†	11.9±0.2‡
SD	RL	34±1†	32±1	30±1	31±1	29±1	28.5±0.7
	CL	22±1‡	11.8±0.5†	9±1	9±1	7.7±0.5	6.5±0.5†
	TL	56±1‡	44±1‡	39±1	40±1	36±1‡	35±1‡

Flowering time is expressed as the number of leaves produced by the main shoot in segregating populations of a *lfy-12* (Columbia) line, and of a transgenic line (LS41-1203; Columbia) containing the *LFY* cDNA under the control of the *LFY* promoter.

Plants of the *lfy-12* segregating population ($n \geq 30$) were grown in long (LD) or short days (SD) as indicated. The genotype of individual plants was determined with a CAPS marker. Similarly, plants segregating for the *LFY* transgene ($n \geq 27$) were grown and genotyped by PCR for the presence of the transgene; plants homozygous for the transgene insertion were identified by progeny testing.

RL, rosette leaves; CL, cauline leaves (bracts); TL, total leaf number. Values are mean ± standard error (95% confidence interval).

*CL refers in this case to the number of leaves or bracts subtending both shoots and flower-like structures.

†$P<0.05$ compared to wild-type controls.

‡$P<0.005$ compared to wild-type controls.

conditions for either 14 or 28 days, after which they were still in the vegetative phase, and then transferred to inductive long-day conditions. The inductive treatments had similar effects on 14- and 28-day-old plants, and upregulation of *LFY::GUS* was apparent after three inductive photoperiods (Fig. 5). A similar set of experiments was performed with 28-day-old wild-type Columbia plants grown in short days, and *LFY* expression was studied by in situ hybridization. Upregulation of *LFY* RNA upon transfer to long days occurred in the newly emerging primordia at the flanks of the shoot apex, as well as in already existing young leaf primordia (Fig. 6). We also studied the induction of *LFY::GUS* expression upon GA treatment of 21-day-old short-day grown plants. In this case, *LFY::GUS* expression after the beginning of the treatment increased faster than in untreated plants (not shown), but was not as dramatic as observed for plants transferred to long days.

Previous studies have shown that transient exposure to long days can stimulate flowering in plants grown in short days. The efficiency with which transient exposure to long days reduces flowering time depends on the age of the plants and on the number of long days before plants are returned to short days (Mozley and Thomas, 1995; Corbesier et al., 1996). To investigate whether transient inductive photoperiods affect the level of *LFY* expression only temporarily or, alternatively, promote an irreversible activation of *LFY*, we studied the effect of transient long-day treatments on *LFY::GUS* expression. *LFY::GUS* plants were grown in short days for 21 days and then exposed to 2, 4 or 6 consecutive long days before being returned to short-day conditions. In our conditions, 2 long days were ineffective in promoting flower initiation or increasing *LFY::GUS* expression. In contrast, both four and six inductive photoperiods led to a temporary increase in the rate of *LFY::GUS* expression for the duration of the treatment (Fig. 7). Both treatments reduced flowering time as measured by the number of leaves produced (37 and 35 leaves, respectively) when compared to control plants left in short days (39 leaves). Thus, there was a correlation between the level of *LFY* promoter expression and the lengths of the transient inductive treatments.

A more detailed study of the behavior of the *LFY* promoter upon transfer from non-inductive to inductive light conditions, and comparison of *LFY* upregulation with the time point of commitment to flowering, can be found in the accompanying manuscript (Hempel et al., 1997). The light conditions used here were different from those used by Hempel et al. (1997), who observed a more rapid increase in *LFY::GUS* activity upon induction by extended photoperiods than reported here. In addition, those authors observed a decrease in *LFY::GUS* activity in short-day grown plants during advanced stages of vegetative growth. Similarly, we have recently found that when plants are grown under light with an increased red-to-far-red ratio, total *LFY::GUS* activity increases only during the first 6 weeks of vegetative growth, with a substantial drop after this time period (Lee and Weigel, unpublished results).

Control of flowering time by the level of *LEAFY* expression

The gradual change in *LFY* expression during the vegetative phase suggested that the level of *LFY* expression might be an important determinant in flower initiation. This idea is partially supported by the precocious flowering of *35S::LFY* plants, which have also a reduced numbers of leaves, particularly under short days (Weigel and Nilsson, 1995). However, in the case of *35S::LFY* it is difficult to discern whether the effect on flowering time is a direct one or whether it is an indirect consequence of the formation of ectopic lateral flowers, which might in turn influence the initiation of leaves on the main shoot. To investigate whether in wild-type plants there is a critical level of *LFY* expression that turns a newly emerging primordium into a bract-less flower instead of a leaf with associated shoot, we attempted to change levels of endogenous *LFY* without changing the spatial expression pattern by varying the number of *LFY* wild-type copies from one to four. A standard indicator for flowering time is the number of leaves produced on the primary shoot before the first flower is initiated (Koornneef et al., 1991), and we counted leaf number in a segregating population of *lfy-12* mutants, which provided individuals with one or two copies of *LFY*, as well as in three transgenic lines segregating for a

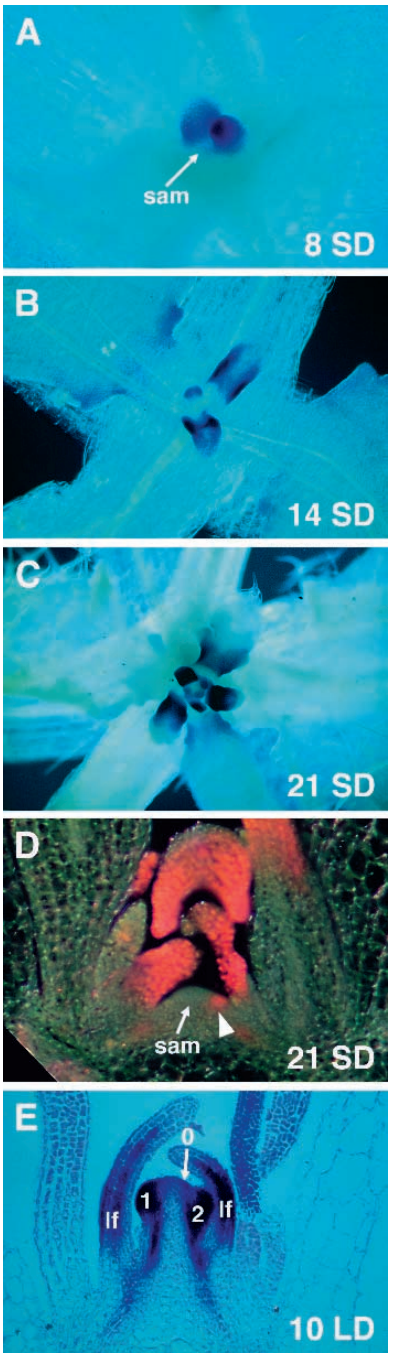

Fig. 3. Histochemical localization of GUS activity in *LFY::GUS* plants. Plants of the line DW150-209 (Columbia) (A-D) or DW150-307 (Landsberg *erecta*) (E) were grown for 8, 14 or 21 days under short-day, or for 10 days under long-day conditions, and stained with the substrate X-gluc. (A-C) Whole mount preparations, (D,E) longitudinal sections of shoot apices. (A-D) During vegetative growth, all newly formed leaves and leaf primordia surrounding the shoot apical meristem (sam) show GUS activity, indicated by blue staining, which appears orange under dark-field illumination (D). As the leaves grow, X-gluc staining first becomes confined to the basal margins of the leaves, and then disappears from older leaves. (D) Weak GUS activity is detected in a leaf anlage (arrowhead). (E) After the transition to flowering, strong *LFY::GUS* expression is observed in flower primordia and in previously formed leaves (lf). This section is lightly counterstained with eosin yellow. (A,D,E) are magnified two-fold relative to (B,C).

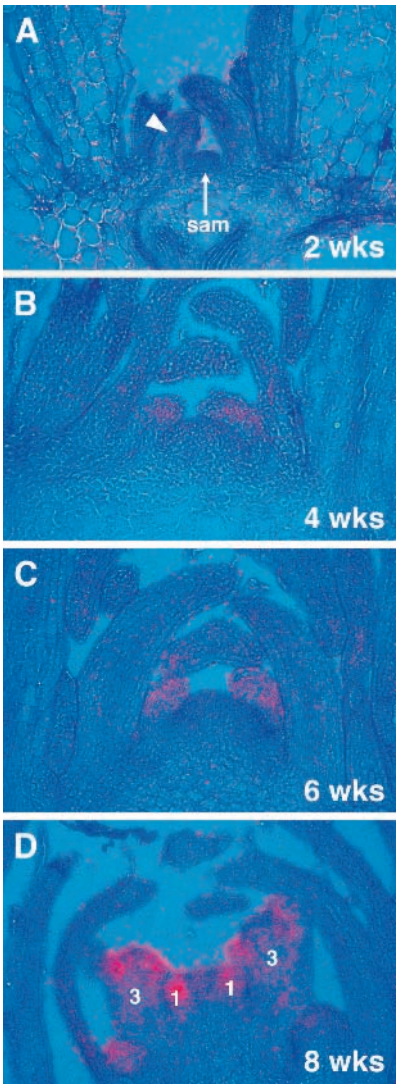

Fig. 4. *LFY* RNA expression during the vegetative phase. Columbia wild-type plants were grown in short days for 2, 4, 6 or 8 weeks. (A) *LFY* expression in leaf primordia flanking the shoot apical meristem (sam) is barely detectable after 2 weeks. Compare density of silver grains over leaf primordia (arrowhead) to that over shoot apical meristem. (B) *LFY* expression is readily apparent in leaf primordia flanking the shoot apical meristem after 4 weeks. (C) *LFY* expression has increased further after 6 weeks, at which time there is still no indication of flower formation. (D) After 8 weeks, high levels of *LFY* RNA are detected in the few flower primordia that have just formed. Numbers refer to floral stages. All panels are at the same magnification.

LFY transgene that contains the *LFY* cDNA under the control of the *LFY* promoter, which provided individuals with two, three or four copies of *LFY*. Homozygous *lfy* mutants (zero copies) were also included in the experiment, although in this case normal flowers were never initiated and leaf counts are not immediately comparable.

In both long and short days, we observed statistically significant differences between wild-type plants and plants with reduced or increased copy number of *LFY* (Table 2). Plants with one copy of *LFY* flowered later than wild-type plants, while

plants with one or two extra copies flowered earlier. Varying the copy number of *LFY* affected primarily the number of cauline leaves (bracts), although the variation in rosette-leaf number was also significant. The presence of two extra copies of the *LFY* cDNA driven by the *LFY* promoter caused precocious flower initiation in all three transgenic lines tested, but with varying efficiency, most likely due to position effects of the integration sites.

Our results with *lfy-12* plants are at variance with those of Schultz and Haughn (1991), who reported for *lfy-1*, which carries the same mutation as *lfy-12* and was induced in the same ecotype, that there is a small, statistically insignificant reduction in rosette leaf number. However, Mizukami and Ma (1997) recently reported an increase in rosette leaf number in plants homozygous for the weak *lfy-5* allele.

DISCUSSION

The switch from the formation of leaves with associated lateral shoots to the formation of flowers is but one example of the many changes that occur during the *Arabidopsis* life cycle, and which are collectively known as phase change (Poethig, 1990). Several of these changes, which include altered patterns of trichome distribution on leaves, an increase in the rate of cell division in the apex, the release of lateral shoots, and internode elongation on the main stem, are closely associated with the transition between the vegetative and reproductive phases of growth (Napp-Zinn, 1985; Hempel and Feldman, 1994; Chien and Sussex, 1996; Telfer et al., 1997). These processes, along with flowering, are normally synchronized and are triggered by the same environmental signals, but can be uncoupled under specific circumstances (Chien and Sussex, 1996; Telfer et al., 1997). To understand the control of phase change, we have to learn how the genes that execute the different processes are regulated. Here, we have analyzed how the expression level of a gene involved in flower initiation, *LFY*, is controlled by environmental and endogenous factors, and how levels of *LFY* activity in turn determine floral fate.

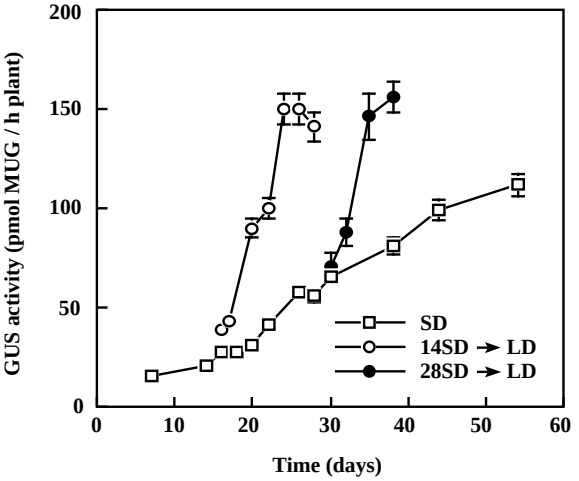

Fig. 5. Effect of inductive photoperiods on *LFY::GUS* activity. DW150-104 (Columbia) plants were grown for 14 or 28 days under short-day conditions and then transferred to long days (LD), or left in short days (SD) as control, until flower buds were visible to the naked eye. At each time point, GUS activity was measured in at least twelve individual apices, and the means ± s.e.m. (95% confidence interval) are given.

Expression of *LEAFY* and phase change in *Arabidopsis*

Two models have been used to describe the transition to flowering in *Arabidopsis*. The traditional view has been that the apical meristem dictates the fate of lateral meristems, and that in order for the shoot meristem to produce flowers, it has to switch from a vegetative to an inflorescence phase (Sussex, 1989). The other model postulates that phase change affects

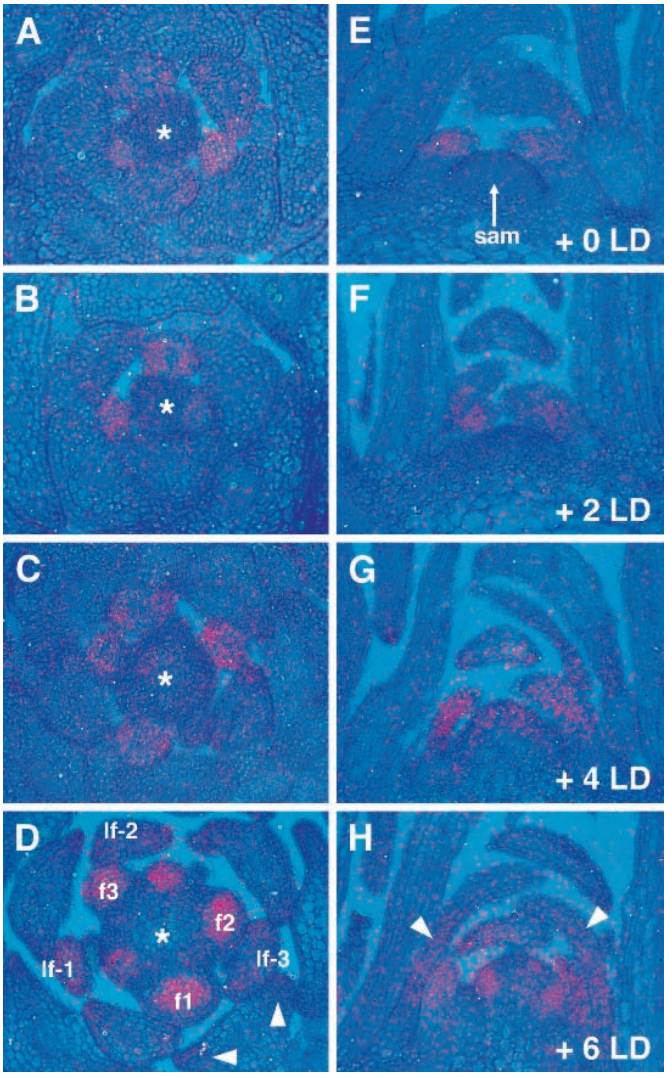

Fig. 6. Effect of inductive photoperiods on *LFY* RNA accumulation. Columbia plants were grown in short days for 4 weeks, and then transferred to long days. Samples were taken before the shift and at 2, 4 or 6 days after transfer. (A-D) Cross, and (E-H) longitudinal sections of shoot apices. (A,E) *LFY* RNA is present in young leaf primordia surrounding the shoot apical meristem (sam; asterisk) before the transfer. (B,C,F,G) An increase in *LFY* RNA accumulation is first apparent 4 days after transfer. (D,H) Six days after transfer, flower primordia with high levels of *LFY* RNA are apparent. (D) The first flower primordium that has formed (f1) has higher *LFY* RNA level than the last leaf primordium formed (lf-1). Note also that more leaf primordia (in this apex, at least seven) express *LFY* RNA than at the beginning of the long-day induction. *LFY* RNA is generally restricted to the leaf margins, which is particularly obvious in older leaves (arrowheads). All panels are at the same magnification, which is the same as in Fig. 4.

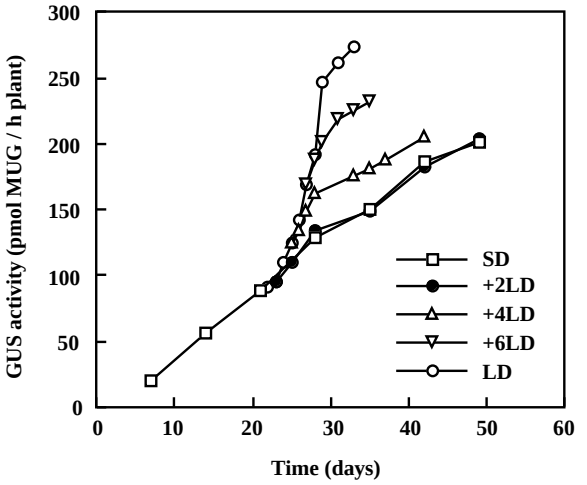

Fig. 7. Effect of transient long-day exposure on *LFY::GUS* expression. DW150-209 (Columbia) plants were grown in short days for 3 weeks. Plants were transferred for 2, 4 or 6 days to long-day conditions (+2LD, +4LD, +6LD), and then moved back to short days. Control plants were either kept in short days (SD) or not returned to short days after transfer to long days (LD) until flowering occurred. At indicated time points, GUS activity was measured in at least twelve individual apices. The values are the mean ± s.e.m. (95% confidence interval).

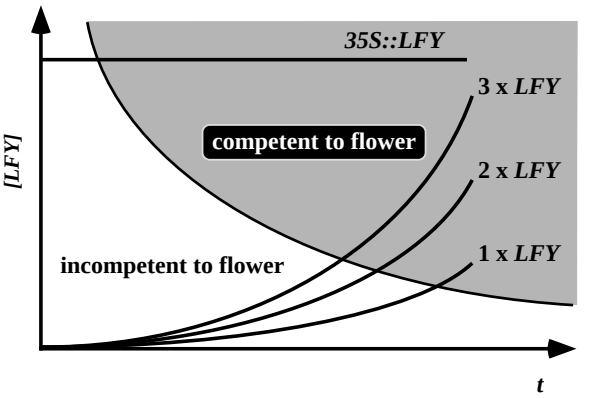

Fig. 8. A generic model for the determination of flower initiation in *Arabidopsis*. The threshold of *LFY* expression required for flower initiation changes with the age of the plant. The particular level of *LFY* expression required at different time points in the life cycle defines a competence gradient, which is regulated by environmental and genetic factors. Young wild-type plants are essentially incompetent to initiate flowers. In this model, the effects of increasing or decreasing *LFY* copy number, or of constitutive *LFY* expression, which are very much dependent on the shape of the competence gradient, here arbitrarily depicted as hyperbolic.

lateral primordia directly, causing primordia that would otherwise develop into leaves with associated shoots (paraclades) to adopt a floral fate, with the shoot meristem proper being of little, if any, importance in this transition (Hempel and Feldman, 1994, 1995). Morphological changes in the central, undifferentiated zone of the shoot apical meristem, as well as the rapid induction of genes expressed in the center of the shoot apical meristem support the first model (Miksche and Brown, 1965; Mandel and Yanofsky, 1995; Menzel et al., 1996). Our observation that *LFY* expression in lateral primordia is continuous from the vegetative to reproductive phase and merely changes in intensity appears to fit well with the second model, although it does not exclude the former. This observation is also compatible with the previous suggestion that the activity of upstream-acting genes that control phase switching would increase gradually during the *Arabidopsis* life cycle (Schultz and Haughn, 1993; Yang et al., 1995). However, the connection between changes in activity of these upstream factors and *LFY* expression might be complex, as indicated by the observation that the initial increase of *LFY::GUS* activity during the vegetative phase is not maintained when plants are grown under light with a high red-to-far-red ratio (Hempel et al., 1997; Lee and Weigel, unpublished observations).

We do not believe that *LFY* has a positive role in leaf development, based on the absence of leaf defects in *lfy* mutants. However, *LFY* suppresses the outgrowth of leaves during the reproductive phase, since all lateral structures on the inflorescence shoot of a strong *lfy* mutant are subtended by small leaves called bracts (Schultz and Haughn, 1991; Weigel et al., 1992). Therefore, we propose that *LFY* is expressed in emerging leaf/paraclade primordia because this is the place where floral induction is effective, as these primordia have the potential to adopt the alternative floral fate, once *LFY* activity reaches a critical level.

LEAFY as a link between flowering time and floral identity

One of the questions that still remains to be solved concerns the molecular mechanism by which *LFY* exerts its role and initiates flower formation at the shoot apex. Previous work has demonstrated that flower initiation is promoted by a combination of *LFY* expression and competence to respond to *LFY* activity (Weigel and Nilsson, 1995). We have extended this observation and shown that the time to flowering is critically affected by levels of *LFY* expression in its normal pattern, as determined with plants that carry one, two, three or four copies of wild-type *LFY*. Since altering copy number of wild-type *LFY* changes flowering time, *LFY* can be said to have characteristics of both flowering-time and flower-meristem-identity genes, which is the behavior expected for a gene that integrates the signals from the environment and then promotes a switch in meristem identity. A related finding is that *FLO*, the *LFY* ortholog in *Antirrhinum*, affects development of both individual flowers and of full inflorescence traits (Bradley et al., 1996b).

Our observations also provide additional support for the idea that *LFY* is a rather direct target of floral-induction signals, because upregulation of *LFY* in long days is not restricted to future flower primordia, but includes previously initiated leaf primordia (Fig. 6H). Apparently, there is no need for precise spatial regulation of *LFY*, because more advanced leaf primordia are insensitive to *LFY* activity. Furthermore, the different responses of the *LFY* promoter to long and short days suggest that the pathways defined by these two conditions are separable at the *LFY* promoter. Simon et al. (1996) have recently obtained at least circumstantial evidence that the signal transduction path from one specific element in the long-day pathway, the transcription factor encoded by the flowering-time gene *CO*, to the *LFY* promoter might indeed be very short. They showed that, irrespective of day length, induction of *CO*

activity in transgenic plants triggers both immediate flowering and upregulation of *LFY*, while upregulation of other meristem-identity genes lagged, when compared to induction by long days.

Control of *LEAFY* expression in different species

Expression of *LFY* and its orthologs has now been studied in detail in wild-type plants of three species, *Nicotiana* (Kelly et al., 1995), *Antirrhinum* (Bradley et al., 1996b), and *Arabidopsis* (this work), and all three species have been found to behave differently.

In *Nicotiana*, the apparent *LFY* ortholog *NFL* is expressed constitutively, with expression in the vegetative phase being confined to defined regions of the apical meristem including emerging leaf primordia (Kelly et al., 1995). Although a *NFL* mutant is not available, it is likely that *NFL/LFY* plays a role in flower initiation, as overexpression of the *Arabidopsis LFY* gene in *Nicotiana* induces early flowering (Weigel and Nilsson, 1995). It thus appears that the main check point controlling the transition to flowering in *Nicotiana* is the competence to respond to *LFY* activity. One interpretation for the phenotype of *35S::LFY Nicotiana* plants is that high levels of *LFY* activity can compensate for low competence at a young age.

In *Antirrhinum*, expression of the *LFY* ortholog *FLO*, whose loss-of-function phenotype is similar to that of *LFY* in *Arabidopsis* (Coen et al., 1990), is tightly correlated with floral induction, as measured by very sensitive polymerase-chain-reaction assays (Bradley et al., 1996b). *FLO* is not detected at all during the early vegetative phase under either inductive or non-inductive photoperiods, and its expression after floral induction is confined to newly emerging floral primordia. However, in older plants, weak *FLO* expression is occasionally detected at a low level in vegetative tissue (Bradley et al., 1996b). Since the effects of *FLO* overexpression in *Antirrhinum* are unknown, we cannot evaluate the importance of competence to respond to *FLO* in this species, but it is not unlikely that competence plays only a minor role in *Antirrhinum*.

Arabidopsis, then, appears to be a case that lies in between the two more extreme cases represented by *Nicotiana* and *Antirrhinum*. While *LFY* is expressed at low levels in leaf primordia, its RNA expression is regulated in response to environmental cues and plant age. This is paralleled by the finding that increasing *LFY* expression levels by increasing the copy number of wild-type *LFY* accelerates flowering. These observations confirm that transcriptional regulation of *LFY* is an important determinant of flowering time. However, the phenotype of plants that express *LFY* constitutively is still affected by environmental conditions, demonstrating that competence to respond to *LFY* activity represents a second level for the control of flowering time (Weigel and Nilsson, 1995; Fig. 8).

It has been proposed that the indeterminate architecture of the *Arabidopsis* inflorescence is the result of the interplay between genes that confer floral identity, including *LFY*, and genes that promote shoot identity, including *TERMINAL FLOWER 1* (*TFL1*), such that *TFL1* represses *LFY* expression in shoots and *LFY* represses *TFL1* expression in flowers (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Weigel et al., 1992; Bradley et al., 1997). Similarly to *LFY*, *TFL1* is expressed not only in the reproductive phase, but also during the vegetative phase, and *tfl1* mutants flower early (Shannon and Meeks-Wagner, 1991; Bradley et al., 1997). These observations are in agreement with a role of *TFL1* in the regulation of *LFY* expression during the vegetative phase. In *Antirrhinum*, on the other hand, expression of the *LFY* ortholog *FLO* and of the *TFL1* ortholog *CENTRORADIALIS (CEN)* is largely restricted to the reproductive phase (Coen et al., 1990; Bradley et al., 1996a). It will be interesting to determine what constitutes the evolutionarily derived condition – vegetative and reproductive, or exclusively reproductive expression of *LFY/FLO* and *TFL1/CEN*.

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