**Separation of shoot and floral identity in Arabidopsis**

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**SUMMARY**

The overall morphology of an Arabidopsis plant depends on the behaviour of its meristems. Meristems derived from the shoot apex can develop into either shoots or flowers. The distinction between these alternative fates requires separation between the function of floral meristem identity genes and the function of an antagonistic group of genes, which includes TERMINAL FLOWER 1. We show that the activities of these genes are restricted to separate domains of the shoot apex by different mechanisms. Meristem identity genes, such as LEAFY, APETALA 1 and CAULIFLOWER, prevent TERMINAL FLOWER 1 transcription in floral meristems on the apex periphery. TERMINAL FLOWER 1, in turn, can inhibit the activity of meristem identity genes at the centre of the shoot apex in two ways; first by delaying their upregulation, and second, by preventing the meristem from responding to LEAFY or APETALA 1. We suggest that the wild-type pattern of TERMINAL FLOWER 1 and floral meristem identity gene expression depends on the relative timing of their upregulation.

Key words: Arabidopsis, Phase change, TERMINAL FLOWER 1 (TFL1), APETALA 1 (AP1), LEAFY (LFY), CAULIFLOWER (CAL), Shoot meristem, Floral meristem, Flower development

**INTRODUCTION**

The aerial parts of Arabidopsis are ultimately derived from the primary shoot apical meristem which is established during embryogenesis (Sussex, 1989; Evans and Barton, 1997). Over the plant life cycle, a series of growth phases reflect the activity of this meristem (Poethig, 1990; Schultz and Haughn, 1993; Ratcliffe et al., 1998). Following germination, the shoot apical meristem generates leaf primordia, which bear axillary shoot meristems. The duration of this vegetative phase (V) depends on environmental conditions and is controlled via an extensive network of flowering-time genes (Koornneef et al., 1991; Martinez-Zapater et al., 1994; Simon et al., 1996; Ruiz-Garcia et al., 1997; Nilsson et al., 1998). These genes regulate when the shoot switches to reproductive development and becomes an inflorescence (I). During a first-inflorescence phase (I₁), 2-3 cauline leaf primordia are produced, before a second-inflorescence phase (I₂) in which floral meristems are generated. During I₂, it is necessary for the shoot meristem to retain a distinct identity from the floral meristems it produces. A key question concerns how this separation is achieved (Shannon and Meeks-Wagner, 1993; Okamura et al., 1993).

The distinction between shoot and floral meristems is maintained by two complementary sets of genes. First, floral fate depends upon the action of meristem identity genes such as LEAFY (LFY), APETALA 1 (AP1), and CAULIFLOWER (CAL) (Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994; Kempin et al., 1995; Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995).

Secondly, a group of genes, including TERMINAL FLOWER 1 (TFL1), prevent the shoot from becoming a flower by retarding progression through all growth phases (Ratcliffe et al., 1998).

The opposing functions of TFL1 and floral meristem identity genes is reflected in their complementary expression patterns and phenotypic effects. In wild type, TFL1 and the floral meristem identity genes are expressed in separate domains (Fig. 1). Both types of genes are most strongly expressed during inflorescence development, with TFL1 in the centre of the apex and floral meristem identity genes on its periphery (Mandel et al., 1992; Weigel et al., 1992; Kempin et al., 1995; Bradley et al., 1997). A similar separation is also observed between low levels of TFL1 and LFY expression during vegetative growth (Bradley et al., 1997; Blazquez et al., 1997, 1998; Hempen et al., 1997).

If the activity of floral meristem identity genes is reduced, flowers develop with various shoot-like characteristics. (Irish and Sussex 1990; Schultz and Haughn 1991, 1993; Mandel et al., 1992; Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Shannon and Meeks-Wagner, 1993). In extreme cases, such as the lfy:ap1:cal triple mutant, inflorescence nodes comprise secondary shoots with subtending leaves, and flower-like structures are rarely made (Bowman et al., 1993). Compared to wild type, therefore, the lfy:ap1:cal shoot apex switches from the V to the I phase at about the normal time, but flowers of the I₂ phase are replaced by shoots. Conversely, when LFY or AP1 are constitutively expressed from a 35S CaMV promoter, all growth phases are shortened and shoot meristems are converted into flowers (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995).
The phenotypes produced by constitutive expression of LFY and AP1 are similar to the effects of a reduction in TFL1 activity. In tfl1 mutants, the V, I1 and I2 are dramatically shortened and the shoot enters a final floral (F) phase in which the apex itself becomes a floral meristem (Shannon and Meeks-Wagner 1991, 1993; Alvarez et al., 1992; Okamuro et al., 1993, Schultz and Haughn, 1991, 1993; Hicks et al., 1996; Bradley et al., 1997; Ohshima et al., 1997; Ratcliffe et al., 1998). Correlating with this, LFY and AP1 become prematurely and ectopically expressed in the tfl1 apex, indicating that TFL1 normally inhibits their activity in that region (Weigel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994; Bradley et al., 1997). By contrast, 35S TFL1 produces an extension in all growth phases, resulting in more highly branched plants which form flowers much later than wild type. Upregulation of LFY and AP1 is delayed in these plants, and an additional growth phase (I1*) occurs in which shoots lacking subtending leaves are formed (Ratcliffe et al., 1998).

To determine how TFL1 and floral meristem identity genes oppose each other, we have examined TFL1 expression in plants constitutively expressing LFY or AP1 and in mutants for lfy, ap1, or ap1cal. We show that LFY, AP1 and CAL can inhibit TFL1 at a transcriptional level. By contrast, inhibition of floral meristem identity gene expression by TFL1 occurs in two different ways. First, TFL1 retards upregulation of these genes by delaying phase progression at the shoot apex. Secondly, we demonstrate that TFL1 can prevent a response to LFY and AP1 even when they are expressed at high levels. We suggest that the final pattern of TFL1 and floral meristem identity gene expression in the wild-type shoot apex depends on the relative timing of their upregulation, and is maintained by distinctive mechanisms of mutual inhibition.

**MATERIALS AND METHODS**

**Plant growth conditions and microscopy**

Experiments were performed on Arabidopsis plants of the ecotype Columbia, except for the following: lfy-7 (Wassilewskija), cal1-lap1-1 (Wassilewskija; Landsberg erecta), and 35SLFY (Landsberg erecta). Seeds of mutant lines were ordered from the Arabidopsis Biological Resource Centre at Ohio State University. The 35SLFY and 35SAP1 lines were kindly supplied by Detlef Weigel and Martin Yanofsky. For all phenotypic analyses, seeds were imbibed and stratified for 5 days at 4°C in the dark, then germinated and grown on soil (1.5 parts John Innes No. 1, 1 part vermiculite, 1 part grit) in a glasshouse at 20°C under standard long day conditions (16 hours light/8 hours dark). During the winter, lighting in the glasshouse was supplemented and extended by high pressure sodium lights. Material for in situ hybridisation time courses was harvested from plants grown in a cabinet at 20°C under standard long day conditions. Light in the growth cabinet was supplied by banks of fluorescent tubes at an intensity between 90-120 μE/m²/second. Equivalent batches of plants from the growth cabinet and the glasshouse displayed no notable differences in growth.

The mean node number produced during different growth phases was determined for each batch of plants and a standard error was calculated with 95% confidence limits attached. Node numbers were scored for a minimum of 10 plants in each batch except in the cases of 35S TFL1;35SLFY and 35S TFL1;lfy-7 where only 4 and 2 plants were scored respectively (see below). Scanning electron microscopy was performed as detailed by Green and Linstead (1990).

**In situ hybridisation**

RNA in situ hybridisation was performed on tissue sections of 7 μm thickness as detailed by Ratcliffe et al. (1998) and Coen et al. (1990). RNA signal was detected as a dark blue/black colour on a light blue background when viewed under the light microscope. Double labelling was based on the method of Fobert et al. (1994). The two riboprobes were visualised sequentially; tissue sections were photographed to record localisation of the first probe, heat treated, and the location of the second probe visualised. Expression of the second gene could then be compared to that of the first.

**Construction of 35SAP1;35STFL1 plants**

Pollen from a hemizygous 35STFL1 plant of line J1.Atl1 was crossed to homozygous 35SAP1 plants of line 563.11.1A (Ratcliffe et al., 1998; Mandel and Yanofsky, 1995). Both parent lines contained a single copy of their respective transgenes in a Columbia background. Out of 23 plants examined in the F1 from this cross, 13 had a phenotype identical to that of the 35SAP1 parent line and 10 displayed a consistent novel phenotype. These 10 plants were confirmed to be doubly transgenic by RNA in situ hybridisation. Node numbers produced by these plants during each growth phase were recorded. Doubly transgenic plants in the F2 generation exhibited the same phenotypes as those observed during the F1.

**Construction of 35SLFY;35STFL1 plants**

Pollen from a hemizygous 35STFL1 plant from line J1.Atl1 was crossed to a homozygous 35SLFY plant of line 151.2.5 (Ratcliffe et al., 1998, Weigel and Nilsson, 1995). Twelve F1 plants were obtained, of which 8 exhibited the same phenotype as the 35SLFY parent. These individuals were confirmed as hemizygous for the 35SLFY transgene. Self-pollination of these plants yielded progeny that segregated 3:1 for kanamycin resistance and a 35SLFY versus wild-type phenotype. The 4 remaining F1 plants were shown to be doubly transgenic by in situ hybridisation, and all exhibited a consistent phenotype. Node numbers produced by these plants in each growth phase were recorded.

The 35SLFY transgene was in the Landsberg erecta background, whereas 35STFL1 was in a Columbia background. To verify that the 35STFL1 phenotype was not significantly affected in F1 hybrids derived from crosses between these different backgrounds, the 35STFL1 parent was also crossed to a wild-type segregant from the 35SLFY parent line. Plants containing the 35STFL1 transgene in the F1 from this cross exhibited the same overall phenotype as the parental line, but made slightly fewer rosette and cauline leaves (approximately 20 rosette and 12 cauline leaves in the parental line compared to 17 rosette and 11 cauline in the F1).

**Construction of the 35STFL1; lfy-7 plants**

Strong alleles for lfy prevent fertile flower formation, so a cross was made using a lfy-7 heterozygote as the parent. The lfy-7 allele was chosen since it has strong effects and contains a novel MsiI site, not found in the wild-type allele, which facilitates easy genotyping (Weigel et al., 1992). Primers, 5′-GTACGAGTAAATGTCA-TTGAC-3′ and 5′-TTCCGACCAGTCTTATGAATTG-3′ were designed flanking the MsiI site which generated a 482 bp fragment during PCR. If the lfy-7 allele was present, this fragment was cleaved into two smaller fragments on digestion with MsiI, whereas for wild type the fragment remained uncleaved.

Pollen from a lfy-7/+ plant was transferred to a hemizygous 35STFL1 plant of line J1.Atl1, and F1 seeds were germinated on kanamycin plates to eliminate those that lacked the transgene. After approximately 3 weeks, resistant F1 plants were transferred to soil and grown to maturity. The F1 plants were then genotyped to identify individuals carrying the lfy-7 allele and seed derived from self-pollination was collected. The inflorescences of these plants were more highly branched than siblings which lacked the lfy-7 allele. The F2 population was grown on soil and examined after 5 weeks. Of 108
plants, 70 had no visible bolt, whereas the remaining 38 had a well developed inflorescence. Amongst the 38 plants, 14 had a lfy phenotype. From the 70 plants that had not bolted after 5 weeks, 20 were grown to maturity, of which 2 developed a distinct phenotype from the remaining 18. These 2 plants were genotyped and shown to be homozygous for the lfy-7 allele.

RESULTS

Expression of TFL1, LFY and AP1 in wild-type Arabidopsis

We compared the expression patterns of TFL1, LFY and AP1 at the primary shoot apex during its three different growth phases (Fig. 1). Corresponding to these growth phases, three different patterns of gene expression were discerned by RNA in situ hybridisation. During the vegetative phase, up to about 7 days after sowing, TFL1 was weakly expressed in the centre of the shoot meristem, and LFY was weakly expressed in young primordia at the periphery of the apex. No AP1 RNA was detected at this stage. At about 8-9 days, correlating with the I1 phase, TFL1 was upregulated in the centre of the shoot meristem. However, LFY expression remained weak and AP1 expression remained absent for about 2-3 days following TFL1 upregulation. Following the onset of the I2 phase at 10-12 days, high levels of LFY and AP1 expression were observed in groups of cells at the periphery, destined to form flowers, and TFL1 was still expressed at high levels in the centre of the apex. Expression of AP1 closely followed LFY upregulation, but whereas LFY was expressed throughout young floral meristems (Stage 1, Smyth et al., 1990), AP1 was first expressed only in an adaxial portion of these meristems at the junction with the shoot meristem. Slightly later, by stage 2-3, AP1 was present throughout floral meristems.

From the beginning of the I2 phase, the primary shoot was visibly elongated (bolting) when seen in longitudinal sections. At this point, secondary shoot meristems were seen in axils of the leaf primordia which had initiated during the V and I1 phases. These leaf primordia did not contain high levels of LFY or AP1, but strong TFL1 expression was observed in their young axillary shoot meristems. The axillary shoot meristems exhibited a similar expression pattern to the primary apex: high levels of TFL1 were established early on, and followed later by strong expression of LFY and AP1 in floral meristems at their periphery.

Over-expression of LFY inhibits TFL1

At all stages in the life cycle, TFL1 and LFY were expressed in separate domains of the shoot apex, with TFL1 in the central region and LFY in cells on the periphery. This raised the possibility that LFY might restrict TFL1 expression. We tested this by examining TFL1 expression in transgenic lines that constitutively express LFY from a CaMV 35S promoter (Weigel and Nilsson, 1995).

As described previously, 35SLFY plants produce flowers earlier than wild type (Weigel and Nilsson, 1995). The vegetative phase of the primary apex is 2-3 leaves shorter than in wild type, and many plants make a short bolt with an occasional cauline leaf, before terminating in a cluster of one or more flowers (Fig. 2). Other 35SLFY plants produce a terminal flower structure on a pedicel directly from the rosette.
Expression of TFL1 was not detected at any time, over a full range of developmental stages in the 3SLFY plants (Fig. 3B). More than 30 different 3SLFY plants were analysed, on alternate days between 2 and 16 days from sowing, and all lacked visible TFL1 expression. This result was verified in three separate in situ experiments. Wild-type segregants from the population afforded a positive control and exhibited normal TFL1 profiles (Fig. 3D). Thus, the lack of TFL1 RNA in the 3SLFY lines confirmed that LFY might restrict TFL1 expression.

**Constitutive TFL1 can overcome constitutive LFY activity**

The above results showed that constitutive LFY activity could inhibit TFL1 transcription. Nevertheless, it was unclear whether this revealed a functionally important aspect of LFY activity or whether the absence of TFL1 was an indirect consequence of all 3SLFY meristems rapidly assuming a floral meristem identity. To distinguish between these possibilities, we constructed plants that were doubly transgenic for a 3SLFY and a 3STFL1 transgene. In these plants, TFL1 would be constitutively expressed and uncoupled from transcriptional repression by LFY. If LFY activity was sufficient to confer floral meristem identity, then the double transgenics should be identical to the 3SLFY line. Alternatively, if the repression of TFL1 by LFY was also important, then the double transgenics should show an altered phenotype.

The 3STFL1 line was crossed to 3SLFY and the phenotype examined in the F1. The 3SLFY;3STFL1 double transgenics were clearly different to the 3SLFY parent line (Figs. 2, 3E). They produced approximately twice as many rosette leaves in the V phase as the 3SLFY parent. The V phase was also longer than for wild type but was shorter than in the 3STFL1 parent (Fig. 2). Following the V phase, the 3SLFY;3STFL1 apex did not directly terminate in a solitary flower, but entered an I1 phase during which about 5 cauline leaves with axillary shoots were initiated. This compared to about 0-2 cauline leaves in 3SLFY plants, 2-3 in wild type, and about 12 in 3STFL1. Next, 1-2 I1* nodes were made, before a large number of fertile flowers in the I2 phase. Unlike the 3SLFY parent, the 3SLFY;3STFL1 primary apex remained indeterminate. However, secondary apices occasionally terminated in a club-like fasciated cluster of fused carpels after production of 20-30 flowers (Fig. 3F). Additionally, secondary inflorescences sometimes twisted back on themselves to form a fasciated loop (not shown) before recovering and continuing indeterminate growth. Double transgenic plants in the F2 generation exhibited the same overall phenotype as the F1, but displayed a greater degree of variation, assumed to be due to relative copy number of the two transgenes. Nevertheless, all F2 double transgenics had an extensive primary inflorescence. In some cases, though, the I1 nodes consisted of a cauline leaf subtending a shoot which remained indeterminate. However, secondary apices occasionally terminated in a club-like fasciated cluster of fused carpels after production of 20-30 flowers (Fig. 3F). Additionally, secondary inflorescences sometimes twisted back on themselves to form a fasciated loop (not shown) before recovering and continuing indeterminate growth. Double transgenic plants in the F2 generation exhibited the same overall phenotype as the F1, but displayed a greater degree of variation, assumed to be due to relative copy number of the two transgenes. Nevertheless, all F2 double transgenics had an extensive primary inflorescence. In some cases, though, the I1 nodes consisted of a cauline leaf subtending a shoot which terminated in a flower-like structure. In other instances, the I1 nodes consisted of a cauline leaf and axillary shoot as seen in the F1.

These data indicated that constitutive TFL1 activity could prevent LFY from conferring a floral identity on young meristems, even though LFY was expressed in comparable quantities to that in the 3SLFY parent (Fig. 3A,G,H). As the
plant aged, however, TFL1 became less able to prevent a response to LFY and normal flowers formed. This might account for the 35SLFY:35STFL1 plants having a less severe phenotype than the 35STFL1 parent, and for the occasional termination of secondary shoots in carpels.

**LFY is involved in inhibition of TFL1 in floral meristems**

The phenotype of the 35SLFY;35STFL1 plants demonstrated that TFL1 activity could interfere with the response of meristems to LFY. During the I2 phase of wild type, therefore, it is important that TFL1 expression is prevented in cells at the flanks of the apex which express LFY. The absence of TFL1 RNA from the 35SLFY plants suggested that LFY itself might have such an inhibitory function. To assess this possibility, we analysed TFL1 expression in a strong lfy mutant. In a wild-type inflorescence, LFY is expressed in very young floral meristems (from stages 0-1). If LFY acted to inhibit TFL1, we predicted that ectopic TFL1 expression might be observed in equivalent groups of cells at the periphery of the lfy mutant apex.

A prominent feature of strong lfy mutants is an expanded I2 phase in which an increased number of cauline leaves subtending secondary shoots are produced. Eventually, infertile carpelloid flower-like structures form rather than shoots, but normal fertile flowers never develop (Schultz and Haughn, 1991, 1993; Weigel et al., 1992; Blazquez et al., 1997). Since lfy homozygotes do not set seed, expression studies were performed on a segregating population of plants derived from a self-fertilised lfy-7 heterozygote.

At early time points, lfy mutants were morphologically indistinguishable from wild type and TFL1 was upregulated in a normal pattern in all plants examined. Later, at 14-16 days, longitudinal sections revealed that the primary apices were bolting (Fig. 4A-F). At this time, when wild-type segregants were initiating floral meristems of the I2 phase, lfy mutants could be recognised through their altered inflorescence morphology and increased numbers of cauline leaf primordia (Fig. 4E,F). TFL1 RNA was present in the primary shoot apex of the lfy mutant, in the same position as in the wild-type apex (Fig. 4A,C). Nevertheless, despite the absence of functional LFY, TFL1 was not ectopically expressed in the youngest groups of cells on the periphery of the apex which were equivalent to stage 0-1 wild-type floral meristems (arrows, Fig. 4A,C). A double-labelling experiment using a TFL1 probe followed by AP1, verified that these individuals were lfy mutants; AP1 RNA was either absent or very much reduced (arrow, Fig. 4B). By contrast, double-labelling on plants with a wild-type morphology from the same time points, showed strong AP1 expression in young floral meristems (arrow, Fig. 4D). Although TFL1 was not expressed in the youngest nodes on the periphery of the lfy apex, it was expressed in the axillary meristems of older nodes by the time the cauline leaf

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**Fig. 3.** Effects of constitutive LFY activity in a wild-type versus a 35STFL1 background. (A-D) TFL1 and LFY expression in 35SLFY plants (A,B) compared to wild-type segregants (C, D). Longitudinal sections taken for RNA in situ hybridisation are shown at a representative time point of 6 days. (A) LFY expression in 35SLFY. (B) Section from same 35SLFY apex shown in A probed with TFL1. (C) weak LFY expression in leaf primordia of wild-type control. (D) TFL1 expressed in apex of wild-type control. (E) 35STFL1, 35SLFY and 35SLFY:35STFL1 plants at approximately 40 days after sowing. Individuals shown were taken from an F2 population. Note that the 35SLFY plant is senescing and siliques are amongst the rosette leaves, whereas the 35SLFY:35STFL1 plant is still growing and producing shoots (arrow) rather than flowers in the axils of cauline leaves. (F) Carpelloid structure which occasionally terminated secondary shoots of 35SLFY:35STFL1 plants. (G,H) Longitudinal sections from a 35SLFY:35STFL1 shoot probed with TFL1 or LFY. Similar levels of expression are observed from both transgenes, although expression from the 35S promoter is not completely homogeneous. Note that crosses were also made to produce 35STFL1:tfl1 plants (data not shown); these displayed no obvious phenotypic differences to the 35STFL1 plants. It is likely, therefore, that the endogenous TFL1 gene made only a minimal contribution to the 35SLFY:35STFL1 phenotype compared to the effect of the 35STFL1 transgene. Scale bars, approximately 100 μm.
The primordium and axillary shoot meristem were distinct (Fig. 4E,F). In wild type, only 2 to 3 such I₁ nodes were made before single flowers, but in the lfy mutant, more than 10 of these nodes were made before carpelloid flowers. Therefore, by this criterion, TFL1 was ectopically expressed in lfy mutants, being present in axillary shoot meristems at the positions where floral meristems developed without TFL1 expression in wild type.

At later time points, from about 20 days, the lfy inflorescence apex produced nodes which formed carpelloid flowers. Expression of TFL1 was not observed in the meristems that formed these structures, but AP1 expression was now detected (Fig. 4G,H), confirming the delay in AP1 upregulation in lfy mutants (Mandel and Yanofsky, 1995). A reduction in TFL1 expression was noted in the inflorescence meristem at these later time points (Fig. 4G). Wild-type segregants also often showed reduced levels of TFL1 in the main apex, but this was unusually less marked than in the lfy mutants (Fig. 4I,J). This suggested that TFL1 levels might decline more rapidly in the lfy mutant, correlating with the eventual formation of carpelloid terminal structures in the inflorescence (Weigel et al., 1992). Secondary shoots harvested from mature lfy inflorescences at 30-40 days from sowing did not show marked TFL1 expression in their apices, although expression was still detected in tertiary shoot meristems (Fig. 4K). By contrast, AP1 RNA was expressed throughout the tip of such secondary shoots, in accordance with them terminating in carpelloid flowers (Fig. 4L).

Thus, at lower nodes of the lfy inflorescence, TFL1 was ectopically expressed by the time shoot meristems were distinct from their subtending cauline leaf primordia. However, at the upper nodes of the lfy inflorescence, TFL1 was not found in the meristems that expressed AP1 and formed carpelloid flowers. This indicated that AP1 could also be involved in the inhibition of TFL1 expression in floral meristems.

**Inhibition of TFL1 in young floral meristems requires AP1/CAL**

To check whether AP1 was required to inhibit TFL1 expression, we analysed a strong ap1 mutant. In ap1 mutants, the vegetative phase is slightly reduced, the I₁ phase is of
similar length to wild type, and during the I2, flowers develop with shoot-like characteristics (Irish and Sussex, 1990; Bowman et al., 1993; Schultz and Haughn, 1993). At about 14 days after sowing, LFY was strongly expressed in meristems at the periphery of the I2 shoot apex, as has been previously described (Weigel et al., 1992). However, TFL1 was not ectopically expressed in these regions, even though AP1 activity was compromised (Fig. 5A-C). Furthermore, TFL1 expression was not detected in shoots developing within flowers of the ap1 mutant (Fig. 5B). Strong TFL1 expression was observed only in wild-type positions: in the centre of the primary apex and in secondary shoot meristems developing in the axils of leaf primordia formed during the V and I1 phases.

These observations show that AP1 activity is not needed to prevent TFL1 expression in floral meristems. However, the AP1 gene is known to show functional redundancy with a highly homologous gene, CAL (Bowman, 1992; Bowman et al., 1993; Kempin et al., 1995). This raised the possibility that CAL could also contribute to the inhibition of TFL1. We therefore investigated TFL1 expression in cal;ap1 double mutants.

In ap1;cal plants the V and I1 phases are similar to wild type, but the meristems initiated during the I2 phase do not behave as determinate floral meristems; instead they remain indeterminate and initiate further meristems. Depending upon the environmental conditions, the whole apex rapidly proliferates and assumes the appearance of a cauliflower. Eventually, however, flowers are produced and viable seed is set (Bowman, 1992; Bowman et al., 1993).

At day 8, no difference was detected in the early expression of TFL1 and LFY between cal;ap1 mutants and wild type. By about 12 days, cal;ap1 mutant apices had entered the I2 phase and sections showed that the primary shoot was elongating. As with the ap1 mutant, high levels of LFY expression were detected throughout meristems produced by the apex during the I2 (Fig. 5F), confirming the observations of Bowman et al. (1993). However, the pattern of TFL1 expression was markedly different from that in the ap1 mutant: TFL1 was ectopically expressed in young meristems that were equivalent to floral meristems of about stage 1 (Fig. 5E). At later time points, cal;ap1 apices were so extensively proliferated that it was very difficult to distinguish the primary shoot meristem. At these stages, TFL1 expression was visible as a pattern of dots, at the centres of the supernumerary meristems (Fig. 5G,I). Scrutiny of serial sections (not shown) cut through entire cal;ap1 apices revealed that TFL1 and LFY were both present at high levels in all these meristems (individual sections contained TFL1 RNA in only a limited number of meristems because the expression domain of TFL1 usually only spans 2-3 sections). The results showed that the action of AP1/CAL prevents TFL1 expression in meristems generated during the I2 phase.

**Fig. 5.** Expression patterns of TFL1 and LFY in ap1 and cal;ap1 mutants. (A-D) Expression in the ap1-7 mutant. (A) Expression of TFL1 in the ap1 mutant apex at 14 days (early I2). Expression is present in the primary apex and axillary shoot meristems (ax) but is absent from the youngest nodes equivalent to young stage 1-2 floral meristems. (B) TFL1 expression in ap1 apex at 16 days. Note that TFL1 is not expressed in the shoot-like flowers (arrow) produced by ap1. (C,D) Double labelling to compare TFL1 and LFY expression. Apex shown at 20 days but similar patterns were observed from start of I2. (C) TFL1 expression. (D) Section from C after double labelling with LFY. Note strong expression of LFY in youngest nodes equivalent to stage 1-2 floral meristems. (E-J) Expression in the cal;ap1 mutant. Expression of TFL1 (E) and LFY (F) in young inflorescence at 13 days. Note that TFL1 and LFY are now expressed in young meristems (1-2; equivalent to stage 1-2 floral meristems). (G,H) Expression of TFL1 and LFY at 16 days. (I,J) Expression of TFL1 and LFY at 21 days. Both genes are expressed as a pattern of dots through the cauliflower-like proliferation. Scale bars, approximately 100 μm in A-H and approximately 500 μm in I,J.
**TFL1 is transiently upregulated in the tfl1 mutant**

We have shown that floral meristem identity genes prevent expression of TFL1 in meristematic cells produced on the periphery of the shoot apex during I2. In a complementary manner, TFL1 acts within the shoot meristem to retard transition through growth phases and hence delays the expression of floral meristem identity genes on the periphery of the shoot apex (Ratcliffe et al., 1998). Whether a meristem develops as a flower or a shoot could therefore depend on whether TFL1 or floral meristem identity gene expression is established first. In the tfl1 mutant, LFY and AP1 are expressed throughout the main apex, which eventually develops into a flower (Mandel et al., 1992; Weigel et al., 1992; Bradley et al., 1997). To investigate how the timing of TFL1 compared to LFY and AP1 expression, we performed in situ hybridisations on the tfl1 mutant.

The tfl1-I allele contains a point mutation which disrupts TFL1 function but which might not prevent transcription (Shannon and Meeks-Wagner, 1991; Bradley et al., 1997). TFL1 expression was not detected in tfl1-I mutant apices during the vegetative phase of development. However, by day 6, TFL1 was strongly upregulated in the tfl1-I shoot apex, about 2 days earlier than in wild type (Fig. 6A). By day 8-9, TFL1 was no longer visible, and LFY was seen throughout the main apex, correlating with its floral fate (data not shown; Bradley et al., 1997). High levels of LFY expression were not observed before upregulation of TFL1.

The transient early expression of TFL1 in the tfl1 mutant can be explained in the following way. The reduction in TFL1 activity instigates a premature exit from vegetative growth, therefore the TFL1 gene responds by being upregulated earlier than in wild type. However, in the absence of TFL1 activity, floral meristem identity genes also become rapidly upregulated in the shoot apex and inhibit TFL1 transcription. Therefore, if TFL1 activity was not established in a shoot apex before floral meristem identity genes are upregulated, those genes could inhibit its expression.

**TFL1 is transiently upregulated in 35SAP1 plants**

As described previously (Mandel and Yanofsky, 1995), plants with constitutive AP1 expression resemble tfl1 mutants: they develop very rapidly under long day conditions, usually only making 3–4 rosette leaves, and then a single cauline leaf before terminating in a cluster of 3–4 flowers (Figs 2, 6F). If the phenotype exhibited by 35SAP1 plants was due to inhibition of TFL1 transcription, we anticipated that 35SAP1 plants would lack high levels of TFL1 RNA.

TFL1 RNA was not detected in very young 35SAP1 seedlings at 2–4 days from sowing (not shown). By day 6, however, TFL1 was strongly expressed in the 35SAP1 shoot apex, as seen in the tfl1 mutant (Fig. 6B). This strong expression persisted for a narrow time interval and had disappeared from the main apex by days 8–10 (Fig. 6C). At this time, longitudinal sections clearly showed that the 35SAP1 plants were bolting and that LFY was expressed in the main shoot apex in accordance with its floral fate (Fig. 6D). Strong TFL1 expression was seen in secondary meristems in axils of leaf primordia at days 8–10 (Fig. 6C). However, by day 12, TFL1 expression was not discerned anywhere in the plant (not shown).

The window of high TFL1 levels in the 35SAP1 plants implied that AP1 alone was unable to prevent upregulation of TFL1 in a shoot meristem at early stages of development. It was likely therefore, that the shortened growth phases observed in the 35SAP1 plants were not entirely due to repression of TFL1 transcription and were at least partly caused by the effects of ectopic AP1 on other factors which determine flowering time or interfere with TFL1 action.

**Constitutive TFL1 can overcome constitutive AP1 activity**

To determine the extent to which the 35SAP1 phenotype was due to repression of TFL1, we constructed plants containing...
both 35STFL1 and 35SAP1. If the factors responsible for the accelerated flowering of 35SAP1 acted purely by interfering with the action of TFL1 (i.e. events downstream of TFL1 transcription), we anticipated that the double transgenics would show the same phenotype as the 35SAP1 line.

The phenotype of the 35SAP1;35STFL1 double transgenic was markedly different from that of the 35SAP1 parent line in terms of vegetative phase duration and inflorescence morphology (Figs 2, 6E). First, the V phase of 35SAP1;35STFL1 plants was 2-3 times longer than in the 35SAP1 parent line, being about the same duration as in wild-type controls. Next, the 35SAP1;35STFL1 apex underwent an I1 phase, producing several cauli leaves which subtended meristems that developed as shoots rather than flowers. This I1 phase was much longer than in the 35SAP1 parent and was 0.75 times the length of the 35STFL1 I1 phase (Fig. 2). Subsequently, the 35SAP1;35STFL1 apex entered a brief I1* phase (where 1-2 flower-like shoots lacking cauli leaves were initiated) prior to an I2 in which a large number of flowers were made. All shoots on the 35SAP1;35STFL1 plants remained indeterminate.

The 35STFL1 transgene offset the early flowering of 35SAP1 such that bolting in the 35SAP1;35STFL1 plants occurred at the same time as in wild type (Fig. 2). Therefore, the factors causing early flowering of 35SAP1 plants could not all act downstream of TFL1. However, the observation that the 35SAP1;35STFL1 phenotype was less severe than that of the 35STFL1 parent confirmed that 35SAP1 did influence other components in addition to TFL1 transcription. This was also consistent with the observation that the 35SAP1 line had an even shorter vegetative phase than the tfl1 mutant (Fig. 2). As with 35SLFY;35STFL1, the absence of terminal flowers in the 35SAP1;35STFL1 plants showed that once a shoot meristem had switched to reproductive growth, AP1 expression was not sufficient to confer floral identity if that group of cells also expressed TFL1.

**Constitutive TFL1 prevents AP1 activation in the lfy mutant**

In a wild-type plant, upregulation of LFY is closely followed by AP1 expression on the periphery of the shoot apex. In the lfy mutant, however, there is a delay between initiation of inflorescence development and the onset of AP1 expression. This shows that LFY has a role in AP1 activation but that AP1 can eventually be upregulated even if LFY is absent (Weigel et al., 1992; Mandel et al., 1992; Gustafson-Brown et al., 1994; Simon et al., 1996). In 35STFL1 plants there is a delay in the onset of both LFY and AP1 expression (Ratcliffe et al., 1998). To determine whether the delay in AP1 expression was solely a consequence of the delay in LFY expression we introduced the 35STFL1 transgene into a strong lfy mutant background. If the effects on AP1 were mediated via LFY alone, it was anticipated that 35STFL1; lfy plants would eventually form carpeloid flowers that expressed AP1, at a similar position to the flowers in the 35STFL1 parent. Plants containing the 35STFL1 transgene in a lfy-7 mutant background had a vegetative phase of approximately the same length as that in 35STFL1 (Fig. 2). However, 35STFL1; lfy plants never formed the infertile carpeloid flowers seen on the lfy mutant. Instead, the 35STFL1; lfy apex continued indefinitely to produce I1 nodes comprising a cauli leaf subtending a shoot (Fig. 7A,B). More than 110 I1 nodes were produced by the primary apex of 35STFL1; lfy plants and they survived for about 5 months, senescing 1-2 months later than sibling 35STFL1 plants which had LFY activity (Fig. 2). To check whether the secondary shoots produced by the 35STFL1; lfy plants at an equivalent position to flowers on the 35STFL1...
prevented through the action of floral meristem identity genes are active. On the periphery of the shoot by delaying the onset of growth phases that floral meristem identity genes are active. On the periphery of the shoot apex (Fig. 8). The repression of these structures is prevented through the action of AP1/CAL and LFY.

(Fig. 7C), had any floral characteristics, we examined such secondary shoots by SEM (Fig. 7D,E). Secondary shoots, harvested (after 100 days) from the 35STFLI; Ify primary apex at about node 90, showed no floral characteristics and were reiterating the growth pattern of the primary apex by initiating leaf primordia at their periphery.

It was shown previously that 35STFLI plants express high levels of LFY and AP1 by the time they develop normal flowers (Ratcliffe et al., 1998). To examine if carpelloid flowers failed to form in 35STFLI; Ify because TFL1 prevented the expression of AP1, we examined secondary shoots by in situ hybridisation at the same position as the SEM analysis. These secondary shoots displayed constitutive TFL1 expression and contained high levels of LFY RNA (comparable to levels observed in the Ify-7 inflorescence; not shown) but AP1 was not expressed (Fig. 7F-H). By contrast, AP1 was expressed in sections taken from a comparable position on a mature Ify-7 mutant inflorescence (Fig. 4L). This result implied that in the absence of LFY function, constitutive TFL1 could inhibit the expression of AP1.

The lack of AP1 expression in 35STFLI; Ify plants reveals that TFL1 can delay AP1 activation by pathways other than through LFY (Fig. 8). Such pathways could involve activation of AP1 by flowering-time genes such as FT and FWA (Ruiz-Garcia et al., 1997; Nilsson et al., 1998). When mutations in these genes are combined with Ify mutations, very similar characteristics are generated to those of the 35STFLI; Ify-7 plants (Ruiz-Garcia et al., 1997). The repression of AP1 activation by TFL1 might therefore involve inhibition of FT and FWA.

**DISCUSSION**

We have shown that the activities of TFL1 and floral meristem identity genes are maintained in separate regions of the shoot apex by distinct mechanisms of mutual inhibition. Expression of TFL1 is prevented at the periphery of the apex through the action of LFY or AP1 and CAL. Conversely, TFL1 acts to inhibit activity of floral meristem identity genes at the centre of the shoot apex (Fig. 8).

**Restriction of TFL1 activity**

Inhibition of TFL1 occurs at the level of the RNA transcript; either transcription of the TFL1 gene or the accumulation of its message is prevented. This conclusion is supported by complementary results: TFL1 expression becomes ectopic in mutants for LFY or AP1/CAL, and is inhibited when these floral meristem identity genes are constitutively expressed.

In Ify mutants, TFL1 is ectopically expressed at lower nodes of the inflorescence, which form secondary shoots rather than flowers. When these nodes are initiated, instead of forming a floral meristem, cells at the periphery of the apex become partitioned into a leaf primordium and axillary meristem (Schultz and Haughn, 1991; Weigel et al., 1992; Coen and Carpenter, 1993). TFL1 is not expressed at the earliest stages of these nodes, but is observed in the axillary meristems by the time they are distinct from their subtending leaf primordia. This delay could be rationalised if some transcription factor, required for TFL1 expression, is active only in meristems and not leaf primordia. At upper nodes of the Ify inflorescence, carpelloid flowers form instead of shoots, but TFL1 expression is not observed in the meristems from which such floral structures develop. By these later nodes, however, AP1 and CAL have been activated, suggesting that they can prevent TFL1 expression even in the absence of LFY activity.

Ectopic TFL1 expression is observed in the calap1 inflorescence, showing that AP1 and CAL are indeed needed to inhibit TFL1. In the calap1 mutant, cells on the periphery of the inflorescence become meristems and do not form subtending leaf primordia. TFL1 is established at very early stages in the development of these meristems. Because LFY is upregulated as normal, these structures might be considered equivalent to the floral meristems which are initiated in the wild-type I2 phase. Therefore, the co-expression of LFY and TFL1 demonstrates that LFY alone is not sufficient to prevent TFL1 transcription in young meristems. At later stages, though, flowers are formed by the calap1 inflorescence, showing that TFL1 activity is eventually inhibited.

TFL1 transcription is not detected in 35SLFY and is only briefly observed in 35SAP1 plants, strengthening our conclusion that TFL1 is inhibited by floral meristem identity genes (Fig. 8). The lack of TFL1 expression during the vegetative phase of 35SLFY and 35SAP1 suggests that high levels of floral meristem identity genes can prevent low levels of TFL1 transcription. Alternatively, vegetative TFL1 transcription might be present in 35SAP1 and 35SLFY but below the threshold of detection. The period of strong TFL1 expression in 35SAP1 plants, corresponding to the I1 phase, differentiates them from 35SLFY plants in which no TFL1 expression is detected. If AP1 and LFY can both repress TFL1 transcription, why is TFL1 upregulation observed in 35SAP1 but not 35SLFY plants? One possible explanation is that other factors are required with AP1 to act as co-repressors of TFL1 transcription. Such factors could be absent during the I1 phase, and only appear during later phases. Constitutive LFY activity, on the other hand, might directly induce such factors and possibly AP1 and CAL as well, allowing inhibition of TFL1 transcription at all developmental stages. Supporting this possibility, it has recently been demonstrated that LFY can transcriptionally activate AP1 (Parcy et al., 1998).

Because LFY and AP1/CAL are thought to behave as transcription factors they could potentially restrict TFL1 expression by binding to its promoter (Weigel et al., 1992;
Mandel et al., 1992; Kempin et al., 1995; Parcy et al., 1998). Alternatively, the inhibition of TFL1 might be less direct and mediated via other factors which are transcriptionally regulated by the LFY and AP1/CAL proteins. Future characterisation of the TFL1 promoter should distinguish between these possibilities and reveal whether or not it contains binding sites for LFY, AP1 or CAL.

**Restriction of floral meristem identity gene activity**

TFL1 can inhibit the floral meristem identity genes at two levels. First, TFL1 can retard the upregulation of these genes. Previous studies on 35S TFL1 and tfl1 plants suggest that TFL1 acts in the shoot meristem to retard phase changes over the entire life cycle (Schultz and Haughn, 1993; Bowman et al., 1993; Ratcliffe et al., 1998). As such, it delays the onset of phases where LFY and AP1 are expressed on the periphery of the apex, and ultimately, their expression in the shoot meristem itself. Thus, when high levels of TFL1 are present, as in 35S TFL1 plants, LFY and AP1 are upregulated much later than in wild-type. Conversely, when TFL1 function is reduced, as in the tfl1 mutant, LFY and AP1 are upregulated prematurely and ectopically in the shoot apex.

Secondly, we demonstrate here that TFL1 can prevent a response to floral meristem identity genes. This is revealed by the phenotype of plants constitutively expressing both TFL1 and floral meristem identity genes. In these plants, TFL1 and LFY or AP1 are free from normal transcriptional regulation. Immediately following the vegetative phase, the main apex and axillary meristems do not develop into flowers, as occurs in the 35S LFY and 35S AP1 parent lines. Instead the primary and axillary meristems develop as shoots; TFL1 therefore prevents a response to LFY and AP1 even though they are present at high levels. Although TFL1 and floral meristem identity genes are not normally co-expressed, this interference by TFL1 might be significant in dampening any mis-expression of LFY/AP1 in the apex, ensuring that these genes remain functional in distinct apical and peripheral domains.

Normal flowers are produced at later inflorescence nodes of plants constitutively expressing both TFL1 and floral meristem identity genes. By this time, therefore, the ability of TFL1 to prevent a response has diminished. Furthermore, flowers are formed earlier than in the 35S TFL1 parent line, indicating that constitutive expression of LFY or AP1 does, to some extent, attenuate the extension of growth phases caused by TFL1. This attenuation could be due to the effects of constitutive LFY and AP1 on other components which influence growth phase duration independently or downstream of TFL1.

Taken together, the above results show that TFL1 can prevent a meristem from assuming floral identity both by retarding the upregulation of LFY and AP1/CAL and by preventing a response to these genes. It is unlikely, however, that TFL1 behaves as a direct repressor of transcription. More probably, TFL1 associates with membrane protein complexes, since it has similarity to animal proteins with such properties (Bradley et al., 1996, 1997; Oshshima et al., 1997; Pnueli et al., 1998). In this way, the TFL1 protein could act non-autonomously as part of a system transmitting signals from the centre to the periphery of the apex. Such a mechanism could indirectly influence whether floral meristem identity genes are upregulated or functional in peripheral cells. This might be clarified by studying the cellular localisation of the TFL1 protein, and analysis of its binding affinity for putative target molecules.

**Regulation of meristem fate**

The separation of flower and shoot meristem identities requires the floral meristem identity genes and TFL1 to be expressed in distinct domains (Shannon and Meeks-Wagner, 1993; Okamura et al., 1993). Mutual inhibition between these genes raises the question of how their expression domains are established. One possibility is that the final pattern of expression observed at the shoot apex reflects the relative timing of TFL1 and floral meristem identity gene upregulation: whichever gene activity is established first in a region would prevent upregulation of the other (Fig. 8).

It appears that TFL1 is upregulated in the centre of the wild-type shoot apex during the I1 phase before LFY and AP1/CAL are strongly expressed on the periphery. By the I2 phase, when AP1 and LFY are upregulated, the prior establishment of TFL1 could prevent their expression within the shoot meristem. Therefore, LFY and AP1/CAL upregulation would be restricted to cells on the periphery of the shoot apex which had not established TFL1 activity. Under the influence of LFY, these peripheral cells would form floral meristems rather than the leaf primordia of earlier phases. Activity of CAL/AP1 along with LFY would then prevent TFL1 transcription in these floral meristems, allowing them to develop into flowers. In addition to the primary apex, meristems formed in the axils of leaves before the I2 phase, would also establish TFL1 expression prior to LFY and AP1/CAL upregulation, accounting for their development as secondary shoots.

The relative timing of TFL1 and floral meristem identity gene expression could also account for the transient high levels of TFL1 transcription observed in the tfl1 mutant. In the mutant, the V phase is shortened and TFL1 is upregulated earlier than in a wild-type apex. Therefore, in addition to maintaining the duration of V, TFL1 is itself upregulated in response to the switch to I1. However, once TFL1 has been upregulated, the mutant TFL1 protein cannot then prevent expression of LFY and AP1 within the shoot meristem. The activity of these genes therefore switches TFL1 off. A period of strong TFL1 expression is observed in the mutant apex because there is a time-lag before LFY and CAL/AP1 become upregulated and inhibit TFL1 transcription. It is possible that similar mechanisms of mutual inhibition between TFL1 homologues and floral meristem identity genes ensure separation of shoot and flower identities in other species. Thus, the relative timing of upregulation between these genes could account for some of the variation in inflorescence architecture observed amongst different angiosperm plants (Coen and Nugent, 1994).

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