# A *TERMINAL FLOWER1*-Like Gene from Perennial Ryegrass Involved in Floral Transition and Axillary Meristem Identity[1]

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Control of flowering and the regulation of plant architecture have been thoroughly investigated in a number of well-studied dicot plants such as Arabidopsis, *Antirrhinum*, and tobacco. However, in many important monocot seed crops, molecular information on plant reproduction is still limited. To investigate the regulation of meristem identity and the control of floral transition in perennial ryegrass (*Lolium perenne*) we isolated a ryegrass *TERMINAL FLOWER1*-like gene, *LpTFL1*, and characterized it for its function in ryegrass flower development. Perennial ryegrass requires a cold treatment of at least 12 weeks to induce flowering. During this period a decrease in *LpTFL1* message was detected in the ryegrass apex. However, upon subsequent induction with elevated temperatures and long-day photoperiods, *LpTFL1* message levels increased and reached a maximum when the ryegrass apex has formed visible spikelets. Arabidopsis plants overexpressing *LpTFL1* were significantly delayed in flowering and exhibited dramatic changes in architecture such as extensive lateral branching, increased growth of all vegetative organs, and a highly increased trichome production. Furthermore, overexpression of *LpTFL1* was able to complement the phenotype of the severe *tfl1-14* mutant of Arabidopsis. Analysis of the *LpTFL1* promoter fused to the *UidA* gene in Arabidopsis revealed that the promoter is active in axillary meristems, but not the apical meristem. Therefore, we suggest that *LpTFL1* is a repressor of flowering and a controller of axillary meristem identity in ryegrass.

The life cycle of flowering plants in general can be divided in to three growth phases: vegetative, inflorescence, and floral (Poethig 1990). In the vegetative phase the shoot apical meristem (SAM) generates leaves that later will ensure the resources necessary to produce fertile offspring. Upon receiving the appropriate environmental and developmental signals the plant switches to floral, or reproductive, growth and the SAM enters the inflorescence phase ($I_1$) and gives rise to an inflorescence with flower primordia. During this phase the fate of the SAM and the secondary shoots that arise in the axils of the leaves is determined by a set of meristem identity genes, some of which prevent and some of which promote the development of floral meristems. Once established, the plant enters the late inflorescence phase ($I_2$) where the floral organs are produced. Two basic types of inflorescences have been identified in plants: determinate and indeterminate (Weberling, 1989). In determinate species the SAM eventually produces floral organs and the production of meristems is terminated with a flower. The SAM of indeterminate species is not converted to a floral identity and will therefore only produce floral meristems from its periphery, resulting in a continuous growth pattern. The regulation of meristem identity and plant architecture has been investigated in a number of dicotyledonous plants including Arabidopsis, *Antirrhinum*, tomato, and tobacco. However, in important seed crops such as wheat, barley, rice, forage grasses, and other monocotyledonous plants, information on how meristem determinacy is controlled is still limited. Therefore, we have undertaken a molecular investigation of the regulation of meristem identity and the control of floral transition in perennial ryegrass (*Lolium perenne*), a cool-season perennial forage grass native to Europe, temperate Asia, and North Africa.

In terms of plant development, the aerial parts of ryegrass are produced by the apex positioned on the base crown a few millimeters above the ground and surrounded by developing leaves (Fig. 1A). During vegetative growth the apical meristem generates lateral meristems initially recognized as semicircular ridges along the main axis. These become the leaf primordia. This morphological pattern does not change until the apex has been induced to flower by elevated temperatures and increasing day length. The minimal requirement for flower induction in perennial ryegrass is a vernalization period of 12 to 14 weeks below 5°C followed by secondary induction with long-day photoperiods (LD, 16 h of light, 8 h of darkness) and temperatures above 20°C. Upon transition to reproductive growth, the apical meristem and later also the lateral meristems start to expand and eventually turn into groups of inflorescences

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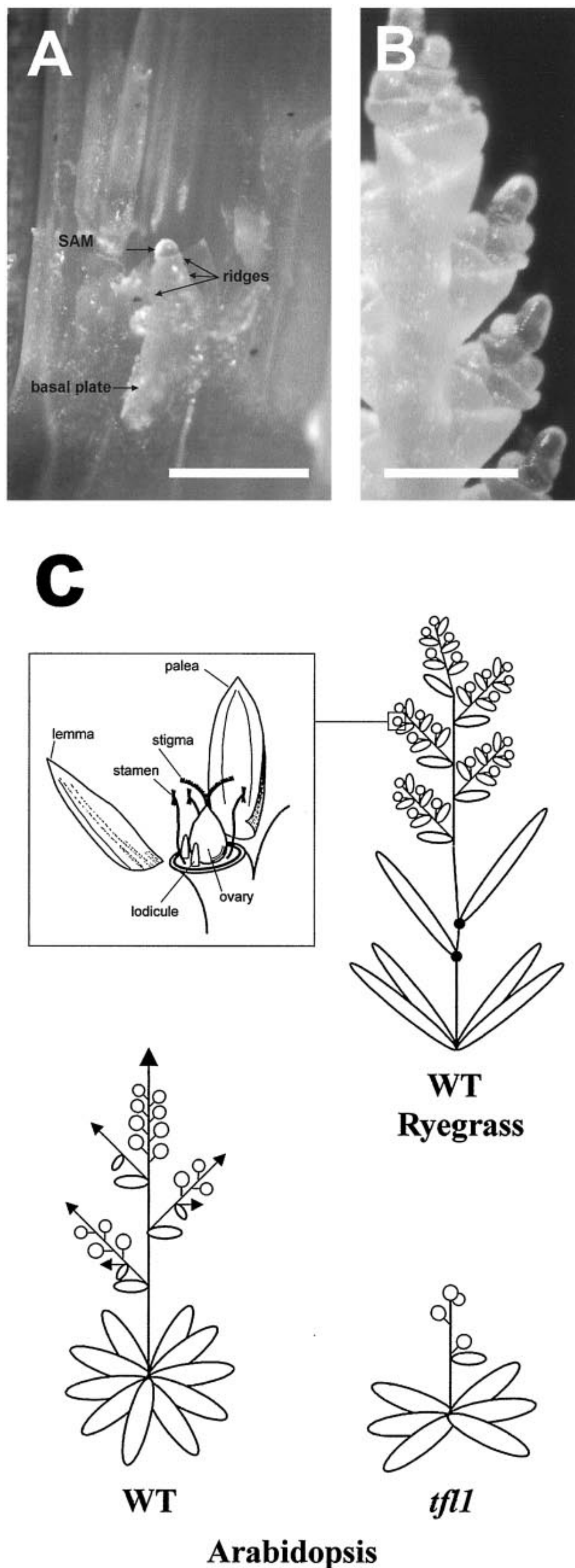
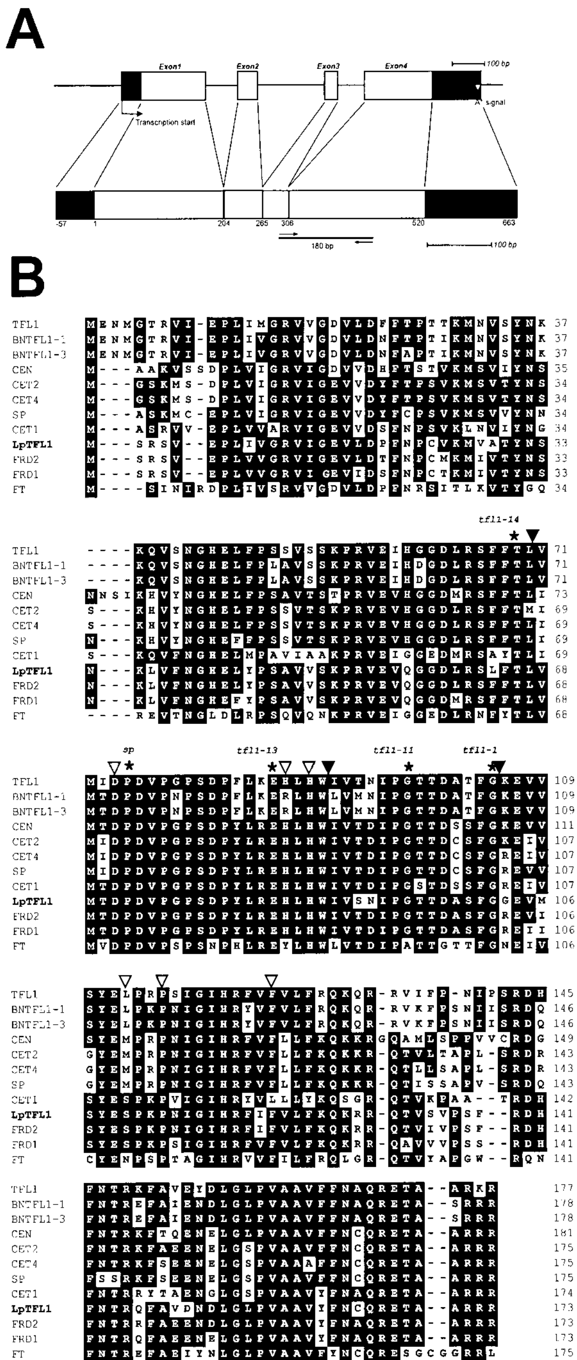



**Figure 1.** Comparative morphology of perennial ryegrass and Arabidopsis. A, The ryegrass vegetative apex is very compact with the SAM and the semicircular ridges that later will give rise to leaves and tillers. It is positioned on the basal crown and surrounded by developing leaves. Bar = 1.0 mm. B, The ryegrass inflorescence consists of spikelets alternately attached to the main axis (rachis). Each spikelet consists of three to 10 flowers. Bar = 1.0 mm. C, Schematic diagrams of ryegrass and Arabidopsis. During vegetative growth the SAM of ryegrass and Arabidopsis produce very closely spaced leaves in a rosette. After the floral transition the SAM of both species elongate (bolt) and floral organs (circles) are produced along the main axis. In both plants secondary shoots arise from the axils of subtending leaves. In Arabidopsis wild type, flowers mature in an acropetal order and the SAM grows indefinitely (arrowheads), whereas in the *tfl1* mutant the SAM and the secondary shoots terminate in a flower. Like the *tfl1* mutant, the ryegrass SAM and secondary shoots also terminate in a flower. Maturation of flowers in the ryegrass inflorescence is basipetal, and all the secondary shoots formed below the apex also develop into arrays of flowers in a cymose pattern. The collar is a special meristematic region on the leaf blade in the junction between the leaf blade and the stem (black circles). An enlargement of a floret is shown (redrawn from K. Esau, Anatomy of Seed Plants, Ed 2. Wiley and Sons, New York, 1977). Each floret consists of four whorls of organs. The outermost whorl consists of the palea and the lemma surrounding the lodicules (whorl 2), the three stamens (whorl 3), and the ovary (whorl 4), which is interpreted as syncarpous, consisting of two or three carpels forming the ovary (C).

(spikelets), each containing three to 10 floral meristems. The spikelets are attached alternately and directly to the central axis (rachis; Fig. 1B). Each floret consists of four whorls of organs. The outermost whorl (1) consists of the palea and the lemma surrounding the lodicules (whorl 2), the three stamens (whorl 3), and the gynoecium (whorl 4), which is interpreted as syncarpous, consisting of two or three carpels forming the ovary (Fig. 1C). The latter arises at the apex as a single ring-like structure surrounding the emerging single ovule (Barnard, 1957).

The flowers of the ryegrass inflorescence are arranged in a cymose, always terminating apical growth with the production of a terminal flower. In this way ryegrass represent a determinate plant architecture also seen and described at the molecular level in dicot plants such as tobacco (Amaya et al., 1999) and tomato (Pnueli et al., 1998). In contrast, plants such as Arabidopsis and *Antirrhinum* have an indeterminate (racemose) inflorescence. The *TERMINAL FLOWER 1 (TFL1)* gene of Arabidopsis and its homolog *CENTRORADIALIS (CEN)* in *Antirrhinum* have been identified as a group of genes that specify an indeterminate identity of inflorescence meristems. Mutations in *TFL1/CEN* result in the conversion of the inflorescence into a terminal flower (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1996, 1997; Ohshima et al., 1997). In addition to its effect on meristem fate, *TFL1* also extends the vegetative phase of Arabidopsis (Shanon and Meeks-Wagner, 1991), but *CEN* does not seem to have a flowering time role in *Antirrhinum* (Bradley et al., 1996). CEN and TFL1 proteins have sequence similarity with mammalian phosphatidylethanolamine-binding proteins (PEBPs). These mammalian proteins were originally named for their ability to bind phospholipids in vitro (Grandy et al., 1990), but have recently been




demonstrated to be regulators of the central Raf1/MEK/ERK signaling pathway (Yeung et al., 1999). The *FLOWERING LOCUS T (FT)* gene also belongs to the family of plant PEBP genes, but has been shown to play an opposite role of *TFL1* in mediating flower inducing signals in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999).

Another group of genes specify a determinate floral meristem identity antagonistically to the *TFL1* gene. Well-characterized genes such as *LEAFY (LFY), APETALA1 (AP1),* and *CAULIFLOWER (CAL)* from Arabidopsis belong to this group (Irish and Sussex, 1990; Schultz and Haughn, 1991; Mandel et al., 1992; Weigel et al., 1992; 1993; Bowman et al., 1993; Gustafson-Brown et al., 1994). Mutations in *LEAFY* or *AP1* result in replacement of floral meristems by shoot meristems, and in accordance with their role, overexpression of *LFY* or *AP1* in Arabidopsis converts shoots into flowers (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Studies of expression patterns and combined analysis of mutants and overexpressing lines have clarified some of the interactions among meristem identity genes (Levy and Dean, 1998, Nilsson et al., 1998; Liljegren et al., 1999; Ratcliffe et al., 1999; Ferrándiz et al., 2000; Samach et al., 2000).

Antagonism between *TFL1* and *LFY, AP1* and *CAL1* is reflected in their complementary expression patterns and phenotypic effects. In wild-type Arabidopsis plants *TFL1* is expressed in the center of the subapical region, whereas *LFY, AP1*, and *CAL* expression are confined to the developing flowers (Mandel et al., 1992; Bradley et al., 1997; Ratcliffe et al., 1999). In the *tfl1* mutant the premature conversion of the apex into a floral meristem has been shown to be correlated with ectopic expression of *LFY* and *AP1/CAL* in the apex (Weigel et al., 1992; Bowman et al.,

**Figure 2.** Genomic organization of *LpTFL1* and similarity of the deduced protein with other plant PEBPs. A, The upper bar shows the genomic organization of the gene, including the untranslated (black boxes) and the translated (white boxes) regions. A 180-bp *TFL1*-like DNA fragment was isolated from ryegrass by RT-PCR. B, Comparison of the deduced protein sequence for the *LpTFL1* gene (accession no. AF316419) with those of *TFL1* (Bradley et al., 1997; Ohshima et al., 1997), *CEN* (Bradley et al., 1996), *SP* (Pnueli et al., 1998), *BNTFL1-1* and *BNTFL1-3* (Mimida et al., 1999), *CET1, CET2,* and *CET4* (Amaya et al., 1999), *FDR1* and *FDR2* (accession nos. AAD42896 and AAD42895, respectively), and *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999). CLUSTAL W program was used to make the alignment and the deduced distance tree. Identical residues are in black. Dashed lines indicate gaps introduced by the program to achieve maximum alignment. Identical intron positions among all species are marked with black arrowheads. White arrowheads indicate amino acids identified to be at the ligand-binding sites by crystallography (Banfield and Brady, 2000) and asterisks indicate amino acids in which point mutations were described for Arabidopsis (Bradley et al., 1997; Ohshima et al., 1997) and tomato (Pnueli et al., 1998). C, Distance tree of different plant PEBPs. The lengths of the horizontal lines are proportional to the similarity between the predicted protein sequences.

1993; Gustafson-Brown et al., 1994; Bradley et al., 1997). In accordance with this data, delayed up-regulation of *LFY* and *AP1* is observed in 35S*TFL1* plants, resulting in an extended vegetative phase (Ratcliffe et al., 1998). There is increasing evidence to suggest that the ratio of *LFY/TFL1* activity in the SAM controls the developmental fate of the meristem (Ratcliffe et al., 1999; Ferrándiz et al., 2000). Thus, the lower the ratio, the longer the vegetative phase will continue and the longer the SAM retains an indeterminate phenotype producing shoot meristems in place of flowers.

To investigate the mechanism underlying flowering control and plant architecture in a widely distributed, agronomically important monocot crop plant we have isolated a homolog of the Arabidopsis TFL1 gene from perennial ryegrass, *LpTFL1*, and characterized it for its potential role in determining plant architecture and the vegetative-reproductive phase transition in grasses. Our results suggest that *LpTFL1* is a repressor of flowering in ryegrass with a unique expression pattern not reported before. Overexpression of *LpTFL1* in Arabidopsis results in a dramatic extension of the vegetative-inflorescence phase and a lateral branching that is consequently more extreme compared with overexpression of *TFL1* in Arabidopsis.

## RESULTS

### Isolation of a *TFL1*-Like Gene from Ryegrass

TFL1 was amplified from ryegrass inflorescence mRNA by reverse transcriptase- (RT) PCR using primers designed on the basis of an alignment of Arabidopsis TFL1, *Antirrhinum CEN*, tomato *SP*, and a related rice expressed sequence tag (EST; Fig. 2A). This fragment was used to screen a ryegrass flower cDNA library at moderate stringency for *TFL1*-like genes (*LpTFL1* genes). One full-length cDNA was identified. The coding region of this cDNA shows 87% and 85% DNA sequence identity to two rice genes, *FDR2* and *FDR1*, and 67% and 64% identity with *TFL1* and *CEN*, respectively. The region in the rice EST used to design the *LpTFL1*-specific primers is 86% identical to *LpTFL1*. On the protein level LpTFL1 shows 91% and 86% identity to the corresponding proteins, FDR2 and FDR1, respectively, and 71% and 68% identity to TFL1 and CEN (Fig. 2B). The *LpTFL1* cDNA-coding region shows 60% identity with the *FT* sequence, and the protein identity is 56%. Comparison of LpTFL1 sequence with other plant PEBP sequences found in the database revealed that LpTFL1 groups together with the two rice proteins and also CET1 from tobacco (Fig. 2C). Banfield and Brady (2000) have recently determined the three-dimensional structure of the *CEN* protein and identified the amino acids essential for a functional ligand-binding site. Other amino acids important for a functional protein have been identified by mutation (Bradley et al., 1997; Ohshima et al., 1997; Pnueli et al., 1998) (Fig. 2B). Of these 11 amino acids, LpTFL1 differs from CEN at only one position (110), having a Ser instead of a Met.

A DNA-blot analysis at moderate stringency using a full-length *LpTFL1* cDNA fragment as probe was performed to assess the number of *TFL1*-like genes in ryegrass. The results indicate that two *TFL1*-like genes are present in ryegrass (data not shown). To gain more information on the *LpTFL1* gene we screened a ryegrass genomic library with the full-length *LpTFL1* cDNA clone. Three independent genomic clones were retrieved and sequenced. All had an identical DNA sequence predicting the same open reading frames, which exactly matched the *LpTFL1* cDNA. The genomic organization of *LpTFL1* (Fig. 2A) is similar to that of the *TFL1* and *CEN* with three introns in the same positions (Fig. 2B), although the introns are of different sizes in ryegrass (100, 208, and 82 bp) compared with Arabidopsis (209, 205, and 86 bp). In the approximately 3.6-kb region upstream of the transcription start, no likely gene encoding open reading frames were found and therefore we assume this to be the *LpTFL1* promoter. The existence of regulatory elements further upstream of the approximately 3.6-kb fragment cannot be excluded; however, in Arabidopsis only a approximately 2.7-kb non-coding DNA fragment is found upstream the transcription start of *TFL1* .

### The *LpTFL1* Gene in Ryegrass Shows an Expression Pattern Different from *TFL1* in Arabidopsis

To determine the expression pattern of *LpTFL1* message in ryegrass we examined the mRNA levels in different tissues by a RNase protection assay. Ryegrass apices, however, are extremely small (<0.5 mm) until the secondary induction stage, where the inflorescence rapidly expands, so in addition we also performed RT-PCR on apex samples that were excised from plants at different stages during flowering induction. Each reaction was repeated three times independently.

*LpTFL1* message was detectable in most types of tissue, except for seeds (Fig. 3A). As early as seedling stage (1–2 visible leaves), *LpTFL1* message was detectable by the RNase protection assay. Expression of *LpTFL1* was also detected in leaves from secondary induced plants, and it reached approximately the same level as *LpTFL1* message in the mature flowers. Leaf expression of *TFL1* in Arabidopsis has not been reported (Bradley et al., 1997), however, detection of *CEN* message in *Antirrhinum* and *CET1* message in tobacco in other tissues than the meristematic regions have been reported by RT-PCR (Bradley et al., 1996; Amaya et al., 1999). After 6 weeks of vernalization less *LpTFL1* message was detected in the apex of vernalized plants compared with unvernalized plants (Fig. 3B). In contrast, we could detect a strong

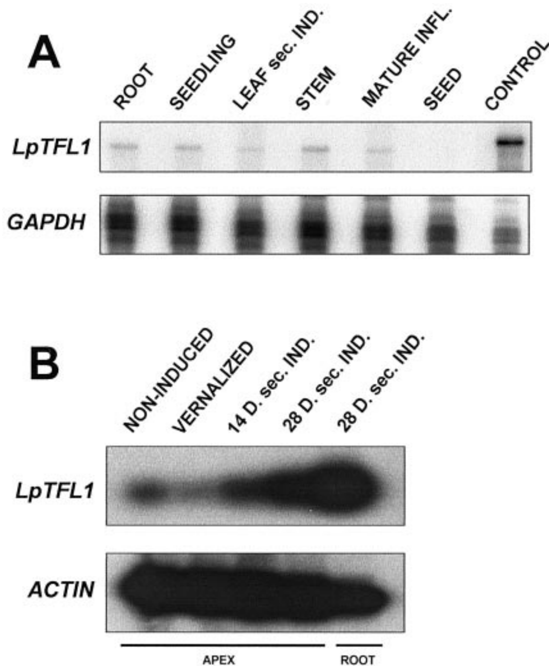



**Figure 3.** LpTFL1 mRNA levels in various tissues detected by ribonuclease protection assay (A) and RT-PCR (B). Fifteen micrograms of RNA was used from each kind of tissue that was hybridized with a 350-bp *LpTFL1* and a 180-bp *GAPDH* antisense riboprobe before RNase digestion. For a positive control, *LpTFL1* antisense probe was incubated with yeast RNA. B, Five micrograms of RNA from meristems and roots from different time-points during flowering induction was used for the RT-PCR.

up-regulation of *LpTFL1* message in the apex after the ryegrass plant had been transferred to the warmer conditions and LD photoperiods, which induce reproductive development. However, the level of *LpTFL1* message was lower in fully matured inflorescences (42 d sec. induction) compared with the stem and also the root (Fig. 3A). A high level of *LpTFL1* transcription was detected in roots from plants growing in LD for 4 weeks.

### *LpTFL1* Delays or Prevents Flowering in Arabidopsis

*LpTFL1*, one of the two *TFL1*-like genes in ryegrass, has a function similar to the Arabidopsis TFL1. We used the maize ubiquitin promoter (Christensen and Quail, 1996) to drive overexpression of the *LpTFL1*-coding region in Arabidopsis. Following transformation with UBI::*LpTFL1*, 33 BASTA-resistant Arabidopsis plants were obtained. All the transformants showed remarkable vegetative characteristics and were much delayed in flowering compared with the wild type (Figs. 4 and 5). Whereas wild-type plants bolted 10 d after they were moved from SD (short day) to LD photoperiod, even the earliest flowering UBI::*LpTFL1* plants required another month in LD before they bolted. After 3 months, more than one-half of the plants had not produced a single flower (Fig. 4C). Overexpression of *LpTFL1* affected the vegetative and the early inflorescence stage of Arabidopsis, as observed by the increased number of nodes produced before and after bolting. During the vegetative phase, wild-type Arabidopsis plants produced 16 ± 1.9 rosette leaves, whereas the UBI::*LpTFL1* plants grown under the same conditions produced 33.9 ± 8.9 rosette leaves (not shown). After the plants had bolted, the UBI::*LpTFL1* plants produced 26 ± 14.3 cauline leaves on the main stem before flower-

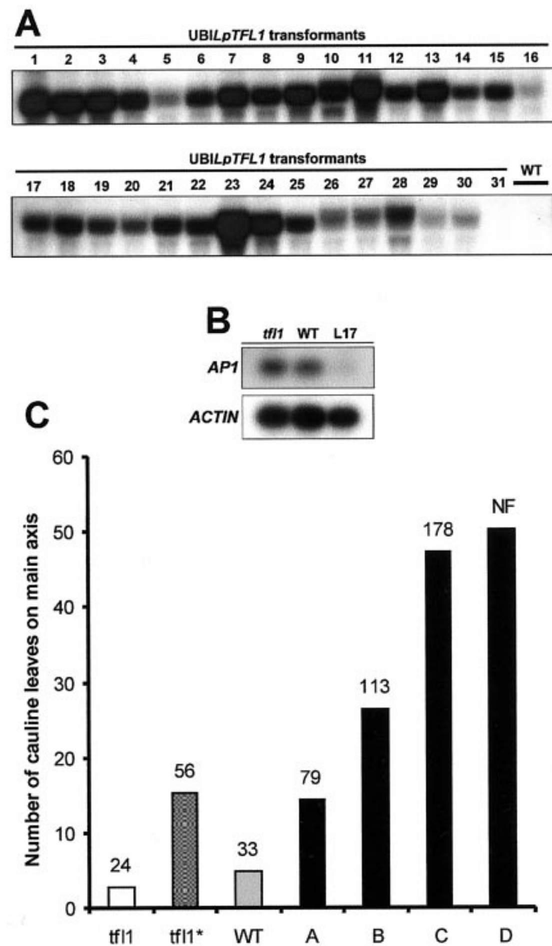



**Figure 4.** UBI-*LpTFL1* dramatically alters the duration of the vegetative phase of Arabidopsis. A, RNA gel-blot analysis of primary transformants (lines 1–30) and wild-type plants (WT). Fifteen micrograms of RNA from rosette leaves was blotted and probed with a *LpTFL1* cDNA probe. Transgenic lines 5, 16, 29, and 30 have single-copy insertions as detected by DNA-blot analysis (not shown). Lines 2, 7, 9, 11, and 13 were non-flowering. B, Expression of *AP1* and *ACTIN* in a *tfl1-14* mutant line, wild type, and in a UBI::*LpTFL1* plant (line 17) as detected by RT-PCR on 5 $\mu$g of RNA from each plant. C, Number of cauline leaves produced on the main stem in *tfl1* mutant, the complemented mutant (*tfl1**), wild type (WT), and UBI-*LpTFL1* primary transformants (groups A–D). Each bar represents the mean value of the plants within the specific group. Numbers above the bar indicates the total number of days from germination till the onset of the first flower. The plants were grouped according to the time to flowering: (A ≥ 75 d; B ≥ 100 d; C ≥ 150 d; D non-flowering [NF]) The number of plants in each group is *tfl1*, 6; *tfl1**, 6; WT, 6; A, 6; B, 13; C, 5; and D, 5.

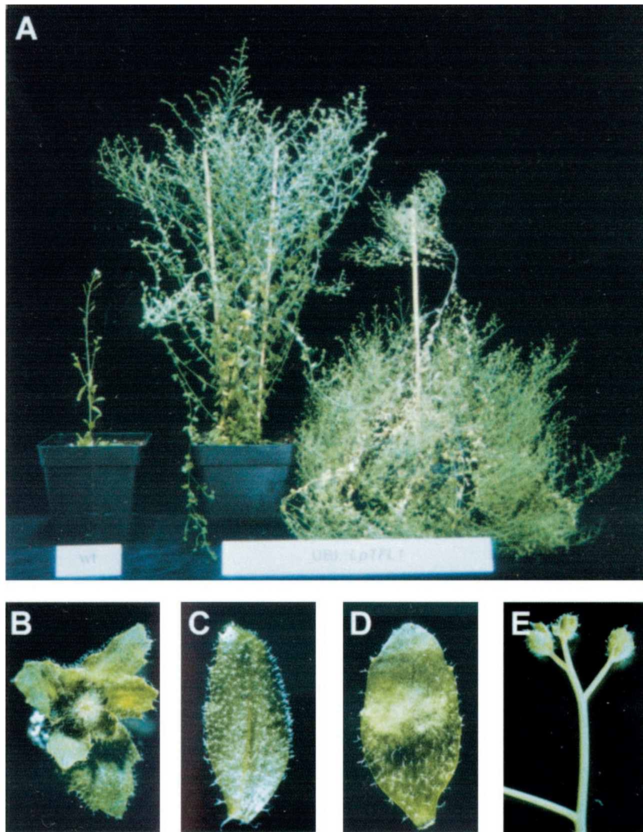



**Figure 5.** The effect of UBI-*LpTFL1* on the morphology of Arabidopsis. A, The UBI::*LpTFL1* Arabidopsis primary transformants, line 1 and 2 (right-hand side), showing extensive vegetative growth and up to fourth-order branching 4 months after germination compared with a 1-month-old flowering wild-type plant (left side). Line 2 (middle) was non-flowering after 7 months of growth. B, The SAM of most UBI::*LpTFL1* Arabidopsis lines is compact, filled with leaf primordia, and covered with trichomes. C and D, Trichome distribution on the adaxial surface of the uppermost cauline leaves on the main stem of UBI::*LpTFL1* (C) compared with wild-type cauline leaves at same age (D). E, In the UBI::*LpTFL1* plants leafy shoots filled with trichomes are produced in place of normal flowers on the upper coflorescences.

ing, in contrast to the wild type, which produced only 4.8 $\pm$ 0.4 cauline leaves (Fig. 4C). Thus, in terms of time and the number of nodes produced before flowering, the majority of the UBI::*LpTFL1* plants appeared to be arrested in the early inflorescence phase. Similar observations were made in Arabidopsis plants in which overexpression of *TFL1* was driven by the 35S cauliflower mosaic virus (CaMV) promoter (Ratcliffe et al., 1998). However, the 35S::*TFL1* plants produced only two-thirds of the number of rosette leaves and one-half of the number of cauline leaves compared with the UBI::*LpTFL1* plants, when grown under continuous light (Ratcliffe et al., 1998). Five UBI::*LpTFL1* plants (lines 2, 7, 9, 11, and 13) remained in the early inflorescence stage throughout their life cycle and failed to produce flowers before they senescenced and died (after 7 months). Non-flowering individuals has also been observed in 35S::*TFL1* Arabidopsis; however, this was only when these plants were grown under SD condition (Ratcliffe et al., 1998) and not under LD conditions, as reported here.

In addition to the main SAM, Arabidopsis plants transformed with UBI::*LpTFL1* also exhibit abnormal axillary meristem development. The development of coflorescences with developing flowers in the axils of the cauline leaves normally observed in wild-type Arabidopsis was rarely seen in the UBI::*LpTFL1* plants. However, in the place of floral organ formation, a "leafy" branch was produced, resulting in a highly branched, bushy, and dramatic phenotype (Fig. 5A). Third-order branching was a common trait among the UBI::*LpTFL1* plants, and fourth-order branching was observed in a single plant (Fig. 5A, right-hand plant). A reiterative series of leaves was continuously produced from the SAM of the UBI::*LpTFL1* plants, most of them with a high density of trichomes (Fig. 5B). The trichome distribution on the surface of the cauline leaves was in general much more dense than in the wild type (Fig. 5, C and D). Increased trichome production in relation to *TFL1* overexpression in Arabidopsis has not previously been reported, although the disappearance of adaxial trichomes is a marker for loss of juvenility (Chien and Sussex, 1996; Telfer et al., 1997). Compared with the wild type, most of the UBI::*LpTFL1* plants produced remarkably more and longer internodes on the main stem, and also on the coflorescences. In contrast to the wild-type plants, the uppermost coflorescences without the subtending cauline leaf of the UBI::*LpTFL1* plants did not consist of normal solitary flowers, but instead a leaf-like shoot (Fig. 5E).

Based on the time to flowering, the transformants could be grouped into four classes (A–D) displaying a phenotype from late flowering (Fig. 4C, group A) to never flowering (Fig. 4C, group D). RNA gel-blot analysis revealed that most of the UBI::*LpTFL1* plants showed strong expression of *LpTFL1* (Fig. 4A, lines 1–31). Overall, the severity of the UBI::*LpTFL1* plant phenotypes was positively correlated with the level of *LpTFL1* expression in the corresponding plants, although the two lines (1 and 23) with the highest *LpTFL1* expression level did flower after 174 and 182 d, respectively. The expression level of *LpTFL1*, in turn, was positively correlated with the total number of gene copies inserted in the genome, as determined by DNA gel-blot analysis (data not shown). In plants with a single-copy insertion (Fig. 4A, lines 5, 16, and 29–30), the *LpTFL1* RNA levels were reduced compared with other lines and consequently the phenotype was less severe, but the time to flowering was still significantly longer than in the wild type (Fig. 4C, group A). Three BASTA-resistant plants in which *LpTFL1* expression was not detected by gel-blot analysis looked similar to wild-type plants with respect to their morphology, but flowered 10 d later than the wild type (not shown).

Detection of the floral meristem identity genes *AP1* was performed by RT-PCR on mRNA isolated from Arabidopsis wild type, the *tfl1-14* mutant, and the hemizygote second generation UBI::*LpTFL1* line 17. The plants were harvested when the mutant flowered and the first floral organs were visible in the wild type. *AP1* transcript was detectable in the *tfl1-14* mutant and the wild type, but was not detectable in the UB:*LpTFL1* line 17 (Fig. 4C), suggesting that *LpTFL1*, like *TFL1*, is capable of suppressing or delaying the transcription of *AP1* in Arabidopsis.

### *LpTFL1* Overexpression in a *tfl1-14* Mutant Background

To further address the functional similarity between LpTFL1 and Arabidopsis TFL1 we asked if LpTFL1 is able to complement the Arabidopsis tfl1-14 strong mutant allele. In this mutant a C to T mutation leads to a Thr → iso-Leu substitution at position 69 (Fig. 2B). The *tfl1-14* mutant has a short vegetative phase and exhibits reduced plant height with few nodes, increased number of inflorescence arising from the rosette axillary meristems, and a determinate growth pattern (Bradley et al., 1997; Ohshima et al., 1997). The construct used for transformation of the Arabidopsis wild type was also used for transformation of the *tfl1-14* mutant. More than 100 independent UBI::*LpTFL1-tfl1-14* primary transformants were obtained from each mutant line after selection for the binary plasmid. All the plants displayed a variety of phenotypes from wild type to the same extended vegetative phenotype seen in the UBI::*LpTFL1* wild-type background. On average (taken only from the first six plants flowering) the UBI::*LpTFL1-tfl1-14* plants produced 15.2 ± 3.5 cauline leaves on the main stem and flowered 33 d later than the *tfl1-14* mutant and 23 d later than the wild type (Fig. 4C). All the UBI::*LpTFL1-tfl1-14* plants grew indefinitely and the production of terminal flowers and rosette inflorescence, which is always seen in the *tfl1-14* mutants, was never observed in the transformants. Thus, the *LpTFL1* rescued the Arabidopsis tfl1-14 mutant in terms of morphology, and the extended vegetative appearance we observe is presumably due to the force of the relatively strong maize ubiquitin promoter.

### *LpTFL1* Is a Potential Regulator of Axillary Meristem Development

We examined the properties of the approximately 3.6-kb putative *LpTFL1* promoter by fusing it to the *Uid*A gene and transforming it into Arabidopsis. More than 100 BASTA-resistant lines were obtained. Ten seedlings of primary transformants with six to seven rosette leaves were tested for GUS expression. Three out of the 10 transformants showed β-glucuronidase (GUS) expression, and in all three plants the expression was confined to a very narrow area in the axillary meristems of the rosette leaves (Fig. 6A). Another test for GUS activity was performed after the plants had bolted and had produced four to five cauline leaves with flowers in the axils. Fifteen primary transformants were tested and GUS activity was detected in three of these plants. At this stage GUS activity was still detected in the axillary meristems of the rosette leaves, but it was also detected in some of the axillary meristems of the cauline leaves, although weaker (Fig. 6B). In this area, however, GUS activity remained even after the formation of a new flower (Fig. 6B). No GUS expression was detected in the apical meristem or in Arabidopsis leaves, although *LpTFL1* is expressed in ryegrass leaves. Analysis of GUS-positive $T_2$ lines showed similar results (not shown). Furthermore, it showed that GUS expression was restricted to the axillary meristems of rosette leaves and the three to five most basal cauline leaves, which were initiated during the vegetative phase. Thus, we find that the activity of the approximately 3.6-kb *LpTFL1* promoter used in this experiment is regulated differently from the expression of *LpTFL1* in ryegrass, and that the GUS expression pattern is similar to *TFL1* expression only in the secondary shoot meristems formed during the vegetative phase.

## DISCUSSION

Perennial ryegrass is a forage grass with a high agronomic value, since it is a low-cost crop, it is perennial, and it is widely used for feeding cattle. One of the major goals in crop improvement is the control of reproductive growth and flower development. Molecular information on these events is very limited in this species. We have isolated a *TFL1*-like gene from perennial ryegrass, which is likely to be a repressor of flowering and involved in control of axillary meristem identity.

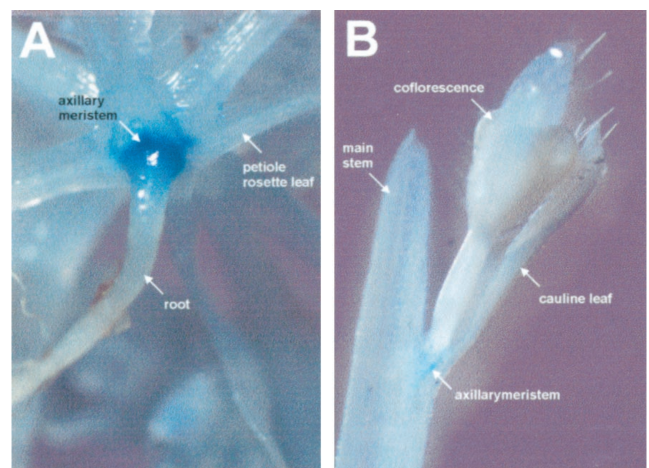



**Figure 6.** LpTFL1 promoter activity in Arabidopsis revealed by GUS expression. A, Thirteen-day-old seedling; B, lower stem section bearing a coflorescence subtended by a cauline leaf 7 d after bolting,

### *Lolium* LpTFL1 Is a New Member of the Plant PEBP Family

The ryegrass *TFL1*-like gene, *LpTFL1*, encodes a protein with high homology to a group of plant proteins that share structural similarities to mammalian PEBPs. Based on these similarities the plant PEBPs are predicted to play a role in the regulation of signaling cascades as has been shown for the mammalian PEBPs (Young et al., 1999; Banfield and Brady, 2000). The two proteins most similar to LpTFL1 are the rice FDR2 and FDR1 with 91% and 86% identity, respectively. In a multiple comparison including TFL1-like proteins from different species, as well as FT from Arabidopsis, LpTFL1 is grouped together with the two rice proteins and a tobacco CEN-like protein, CET1. No data on *FDR2/FDR1* expression patterns and functions in rice has been reported, and for *CET1*, expression has been reported to be detectable in vegetative and inflorescence shoots, but only by RT-PCR (Amaya et al., 1999). Compared with the Arabidopsis PEBP sequences, LpTFL1 shows 71% identity to TFL1 and 56% identity to FT. FT, which is 56% identical to the TFL1 protein, also belongs to the family of plant PEBPs, however, in contrast to TFL1, FT has been shown to mediate flowering-inducing signals in Arabidopsis (Kardailsky et al., 1999; Kobayashi et al., 1999). In this process FT acts in parallel with and under the influence of the *CONSTANS (CO)* gene, which is a mediator of the LD-induction pathway (Samach et al., 2000). LpTFL1 shows 50% identity to a partial *FT*-like region on a rice clone (nbxb0035E07r), but although the DNA-blot analysis indicates the existence of another *LpTFL1*-like gene, no ryegrass *FT*-like cDNA with a higher homology to this partial rice *FT*-like sequence has yet been identified. Overexpression of *LpTFL1* in Arabidopsis results in significantly delayed flowering in combination with a dramatic large and bushy phenotype, suggesting that LpTFL1 is more TFL1-like than FT-like.

In spite of the high degree of homology between the plant PEBPs, constitutive expression of these proteins in different plants leads to different phenotypes. The dramatic impact of *LpTFL1* overexpression on floral transition and plant architecture in Arabidopsis is more extreme than that previously reported by overexpressing *TFL1* in Arabidopsis (Ratcliffe et al., 1998). One possible explanation for the more severe phenotype observed in our study may be that the activity of the maize ubiquitin promoter is stronger than the 35S CaMV promoter in Arabidopsis. If this is the case, the monocot ubiquitin promoter shows a remarkably strong activity not previously reported in a dicot plant. Alternatively, our observation may be due to differences in the protein sequence and conformation of LpTFL1 compared with TFL1. Overexpression of *CEN* in tobacco has also been reported to significantly delay the floral transition, as well as to change the plant architecture (Amaya et al., 1999). In contrast, there was no effect of overexpressing *TFL1* in tobacco (Amaya et al., 1999). These results, together with our results, indicate that differences in the protein sequences among the plant PEBPs are likely to account for some of the differences observed in the overexpressing plants.

Eleven amino acid residues in the plant PEBP sequences have so far been identified as essential for a functional protein (Fig. 2B) by crystallography (Banfield and Brady, 2000) or by mutations (Bradley et al., 1997; Ohshima et al., 1997; Pnueli et al., 1998). At these residues, LpTFL1 differs from the consensus at one position (110), which is also the position with the highest degree of amino acid variation between species. It is interesting that the variation in amino acid residues at position 110 exactly matches the grouping of plant PEBP by the clustalW alignment (except for FT). One group comprising TFL1, BNTFL1–1, and BNTFL1–3 has a Leu at this position, and another group comprising CEN, CET2, CET4, and SP has a Met, and a third group, which includes LpTFL1, FDR2, FDR1, and CET1, has a Ser at this position. The immediate assumption that the amino acid differences at this position can be linked to the variance in phenotype severeness of plants overexpressing different *TFL1*-like genes would suggest that overexpression of BNTFL1–1/BNTFL1–3, like TFL1, also has no effect in species like tobacco, and that overexpression of CET1, FDR2/FDR1, like LpTFL1, might have a significant effect on plant architecture and flowering time in species like Arabidopsis. Future results on overexpression of *TFL1*-like genes in different species would contribute to clarify the correlation between protein sequence and the effect on morphology. In any case, our results show that the effects of different PEBPs cannot solely be explained by genetical diversity, since ryegrass is more distantly related to any of the dicot species, and yet LpTFL1 has a strong and unequivocal effect on the Arabidopsis.

### Control of Floral Transition

The dramatic phenotype of Arabidopsis plants overexpressing *LpTFL1* suggests that in ryegrass, *LpTFL1* may play a role in controlling meristem identity and in the transition from vegetative to reproductive growth. In ryegrass, *LpTFL1* message is detected at all stages from germination to maturity. It is found at the apex, in the inflorescence, and also in leaves, stems, roots, and mature flowers. However, expression of *LpTFL1* in the ryegrass apex is not constitutive. Levels of *LpTFL1* message changed during flower induction with a reduction after 6 weeks of vernalization, followed by a strong up-regulation during secondary induction until the structures of the spikelets were visible. Data on cold-induced down-regulation of *TFL1*-like genes has not been reported, presumably because vernalization is nor-

mally not required for flowering induction in many of the tested species. Analysis of the expression pattern of *FLOWERING LOCUS C (FLC)* in Arabidopsis, a gene encoding a MADS-box repressor of flowering that is down-regulated by vernalization (Sheldon et al., 1999), suggests that the physiological role of vernalization is to alleviate a block in flowering. Since *LpTFL1* may function as a repressor of flowering in perennial ryegrass, a species that requires at least 12 weeks of cold treatment to flower, down-regulation of *LpTFL1* during vernalization may be necessary for ryegrass to proceed to the reproductive phase. It is intriguing that the late-flowering phenotype of 35S::*TFL1* Arabidopsis can be suppressed by a vernalization treatment, suggesting that *TFL1* may repress the up-regulation of floral meristem identity genes by increasing FLC activity or expression (Ratcliffe et al., 1998; Simpson et al., 1999). In unvernalized perennial ryegrass an analogous system may be operating in which the level of a FLC-like protein might be high due in part to induction by *LpTFL1*. Down-regulation of *LpTFL1* upon vernalization, and perhaps an FLC-like protein as well, would then be necessary for the meristem to be competent to flower. This would represent an important additional role for *LpTFL1* in flowering and finer dissection of *LpTFL1* message level during vernalization will be required to substantiate this role.

Some of the Arabidopsis plants overexpressing *LpTFL1* never flowered before senescence. Similar vegetative non-flowering Arabidopsis plants were obtained by combining mutations in *AP1, CAULIFLOWER (CAL)*, and *FRUITFULL (FUL)*, all three MADS-box genes (Ferrándiz et al., 2000). The vegetative growth of the triple mutant was correlated with a low *LFY:TFL1* transcript ratio and with *TFL1* becoming ectopically expressed in the laterally arising meristems in an overlapping pattern with *LFY* expression. The SAM of the *ap1, cal, ful* triple mutant is arrested in the vegetative→$I_1$ phase, producing only cauline leaves with axillary meristems that in turn repeat this pattern forming "leafy" cauliflower along the main inflorescence (Ferrándiz et al., 2000). Similar cauliflower-like structures were not observed in our UBI::*LpTFL1* plants because the repeated formation of meristems was slower. However, an additional morphological characteristic of the UBI::*LpTFL1* plants was the high density of trichomes that covered the leaves and the SAM (Fig. 5). In Arabidopsis, disappearance of the trichomes from the adaxial surface of cauline leaves has been shown to be tightly linked to floral induction and in support for this observation, it was shown that *tfl1* leads to accelerated loss of adaxial trichomes in Arabidopsis (Telfer et al., 1997). In agreement with this observation we find that the expression of a *TFL1*-like gene in Arabidopsis prevents the loss of adaxial trichomes. By this criterion the UBI::*LpTFL1* plants were less competent to flower compared with the triple mutant. However, the fact that most of the UBI::*LpTFL1* plants do flower and produce normal flowers after an extended vegetative phase suggests that a delayed, but otherwise normal expression of *LFY*, *AP1, CAL*, and the floral organ identity genes has occurred. Therefore, the level of LpTFL1 activity can be decreased over time or additional factors override LpTFL1 function and ensure the proper transcription of meristem and organ identity genes. One possible factor is FT, which is able to up-regulate floral meristem identity gene like AP1 and *LFY* (Kobayashi et al., 1999; Samach et al., 2000).

**A Potential Molecular Mechanism for Determinate Plant Architecture in Perennial Ryegrass**

Perennial ryegrass and Arabidopsis represent two different forms of plant architecture: determinate and indeterminate, respectively. A molecular basis for indeterminate growth has been proposed for Arabidopsis (Bradley et al., 1996, 1997; Ratcliffe et al., 1998, 1999; Liljegren et al., 1999) in which indeterminate plant architecture is correlated with expression of *TFL1* in the center of the SAM. In this central region as well as in the uppermost layers of the SAM, TFL1 activity is capable of excluding the expression of *AP1* and *LFY*, and therefore the formation of a terminal flower. Several parallels can be drawn between the Arabidopsis data and our results. Like *TFL1* in Arabidopsis, *LpTFL1* message is present in the ryegrass apex at the vegetative stage. Furthermore, a significant up-regulation of *LpTFL1* occurs in the apex upon LD induction, which is similar to the up-regulation of *TFL1* observed in Arabidopsis when the plants enter the $I_1$ phase (Ratcliffe et al., 1999). Like *TFL1*, overexpression of *LpTFL1* results in a repression of *AP1* transcription (Fig. 4C). Localization of *LpTFL1* expression in the ryegrass apex awaits characterization, but our initial data based on the analysis of the *LpTFL1* promoter driving GUS expression in Arabidopsis showed strong activity in the axillary meristems of the rosette leaves during the vegetative phase (Fig. 6A). At later stages, when plants had bolted, GUS activity was still detectable in the axillary meristems of the rosette leaves, as well as in the axillary meristems of those cauline leaves that were initiated during the vegetative phase (Fig. 6B). Although weaker, the GUS activity remained in this position after the shoots developed. No visible GUS activity was detected in the SAM where *TFL1* is normally expressed. The axillary expression of *LpTFL1*::GUS is reminiscent of *CET2/CET4* expression in tobacco, a species with determinate plant architecture, in which *CET2/CET4* is restricted to the axillary meristems and excluded from the SAM (Amaya et al., 1999). The restricted expression pattern of *CET2/CET4* was found to be correlated with expression of the tobacco *LFY* homolog *NFL* in the center of the inflorescence apical meristem (Amaya et al., 1999). A similar *LFY*-like mediated exclusion of *LpTFL1* from the center of

the ryegrass SAM still needs to be confirmed. Our observed *LpTFL1*::GUS expression suggests that some regulatory elements are missing in the approximately 3.6-kb *LpTFL1* promoter fragment or that the elements in the monocot promoter is not fully recognized in Arabidopsis. Ohshima et al. (1997) found that insertion of a T-DNA 458 bp downstream of the putative *TFL1* polyadenylation signal leads to the production of an Arabidopsis tfl1 mutant phenotype, suggesting the existence of a regulatory element 3′ of the *TFL1* gene. In an alternate manner, the lack of GUS expression in the SAM may be due to a different set of transcription regulatory factors associated with the determinate architecture of ryegrass. In summary, ryegrass architecture may be the result of lack of *LpTFL1* expression in the SAM resulting in determinacy combined with *LpTFL1* expression in the meristematic ridges of the ryegrass apex (the axillary meristems) resulting in repression of floral identity genes and specification of an indeterminate shoot meristem identity allowing the formation of tillers.

Analysis of transgenic ryegrass overexpressing *LpTFL1* is in progress, and based on the data presented here we can speculate the following scenario for *LpTFL*-mediated control of floral transition and plant architecture: Shortly after germination, *LpTFL1* expression is established in the meristematic ridges of the apex to maintain the production of vegetative organs such as leaves and tillers. During vegetative growth, a high level of *LpTFL1* and other flowering repressors, perhaps similar to *FLC*, is maintained to avoid precocious flowering before the winter season. During the winter vernalization period, levels of *LpTFL1* and other flowering repressors decreases, allowing the plants to become competent for flowering. As the temperature increases and the photoperiods lengthen in spring, *LpTFL1* expression is up-regulated in the apex to promote lateral branching of the main axis. In this way, a maximum number of spikelets are produced. Expression of *LpTFL1* subsequently becomes progressively more restricted to vegetative tissues such as stem and root, and the ryegrass plant finishes its life cycle by the production of the last uppermost seed in the top spike.

Dissection of the molecular mechanisms underlying the floral transition and flower formation in important monocot crop plants like perennial ryegrass is in its infancy. By analyzing one of the main components in this multifactorial process, a *TFL1*-like gene in perennial ryegrass, we have just begun to address the question of how the green common ryegrass growing on millions of acres is transformed into flowering plants each year following a winter season. Further studies will perhaps confirm and certainly modify the scenario for *LpTFL1* function that we propose here.

## MATERIALS AND METHODS

### Plant Growth Conditions

Ryegrass (*Lolium perenne*) plants (clone F6, DLF-TRIFOLIUM) were grown in soil in a greenhouse with daylight at 21 and 18°C, day and night temperature, respectively. For the primary induction (vernalization) plants were grown in a growth chamber at or below 5°C for at least 12 weeks. During vernalization, the light period was decreased to 8 h per day. Following vernalization, plants were grown under 16 h of light at 22°C and 18°C, day and night temperature, respectively, for secondary induction. For RNA analysis plants were harvested before vernalization, after 6 weeks of vernalization, and after 14 and 28 d of secondary induction and meristems were excised. Samples from other tissues like leaves, stems, seeds, and roots were also harvested for expression analysis.

Arabidopsis seeds were stratified for 2 to 3 d at 4°C and then grown in soil in growth chambers at 22°C and 18°C , day and night temperature, respectively. During the first 2 weeks plants were grown at SD conditions (8 h of light per day) and then moved to LD conditions (16 h of light per day). In the Arabidopsis time-course experiment rosette leaves were counted when plants started to bolt and the number of leaf nodes were counted from the most basal cauline leaf to the uppermost leaf proximal to the inflorescence. The number of days from germination to the production of the first flower-like structure was also scored.

### Screening of cDNA and Genomic Library

To isolate *TFL1*-like genes from ryegrass, a set of primers partially conserved between *TFL1* of Arabidopsis, *CEN*of *Antirrhinum*, and a rice EST (RICR2918A; accession no. 428842) were designed. Primer RY2 N (5′-GGTTATGACA-GACCCAGATGTG-3′) was used in combination with primer RY4V (5′-CGAACCTGTGGATACCAATG-3′) to amplify a 180-bp fragment by RT-PCR. Preparation of RNA for the RT-PCR used the FastRNA, GREEN Kit RNA isolation system (Bio101, Carlsbad, CA). The 180-bp fragment corresponding to a putative ryegrass *TFL1*-like fragment was used to screen a cDNA library (Stratagene, La Jolla, CA) made of ryegrass inflorescences for full-length *TFL1*-like cDNAs. Approximately 800,000 recombinants were screened at moderate stringency of 60°C , with washes at 60°C in 2× SSC (0.3 M NaCl and 0.1 M sodium citrate, pH 7.4) and 0.1% (w/v) SDS. Three positive clones were isolated, and plasmids were isolated from single plaques by in vivo excision. All cDNA clones were sequenced and contained identical sequences with similarity to *TFL1* and *CEN* and were named ryegrass *TFL1*-like, *LpTFL1* (GenBank accession no. AF316419).

A λEMBL3 SP6/T7 genomic library (CLONTECH, Palo Alto, CA) made from a partial *Sau3A* digest of ryegrass DNA was screened for *TFL1*-like genes. Approximately 1,000,000 recombinants were screened at moderate stringency (as described above) with the full-length *LpTFL1* cDNA clone. Nine positive clones were isolated and digestion of the λ DNA clones with *Bam*HI, *Sal*I, *Xba*I, and *Sac*I

revealed three unique clones. These clones were partially sequenced and all three had identical sequence from 4.0 kb upstream and 2.0 kb downstream of the *LpTFL1* sequence. The sequence of the exons of the genomic clones, as well as the 5′ and 3′ untranslated region were identical to *LpTFL1*. DNA sequencing was performed using the ABI Prism system (Perkin-Elmer, Foster City, CA), and sequence analysis and alignments were produced using Gene Codes Sequencer software, version 4.02.

## RNA/DNA Analysis

For detection of *LpTFL1* RNA level in different organs of ryegrass, 15 $\mu$g of RNA was hybridized to a 350-bp riboprobe corresponding to the 5′ end of *LpTFL1* antisense. The riboprobe was synthesized by in vitro transcription from a T7 priming site fused to the 350-bp *LpTFL1* fragment by a T7 RNA polymerase in the presence of [$\alpha$-$^{32}$P]CTP (800 Ci/mMol). Hybridization, RNase digestion, and precipitation was performed as described in the system kit (RPA III, Ambion, Austin, TX) End products were electrophoresed on a 6% (w/v) polyacrylamide gel. The level of transcript was determined by autoradiography. A riboprobe corresponding to 180 bp of a ryegrass *GAPDH* antisense fragment was used as control.

Changes in *LpTFL1* RNA levels in meristems during the flowering induction were detected by RT-PCR. Poly-(A)$^+$ mRNA was isolated from 5 $\mu$g of total RNA and all mRNA was used in the reverse transcription. Two internal primers, INS5 (5′-CACATTGGTTATGACGGACC-3′) and INS3 (CTCCCCCCCAAATGAAGC-3′), were used in the subsequent PCR reaction to amplify a 200-bp *LpTFL1* fragment from the first strand cDNA templates. PCR products were electrophoresed, blotted, and hybridized to a *LpTFL1*-specific probe using standard blotting techniques. For a positive control, primers for ryegrass *ACTIN*: AC5 (5′-GAGAAGATGACCCARATC-3′) and AC3 (5′-CACTTCATGATGGAGTTGT-3′) were used. The PCR reaction was repeated three times. Detection of *LpTFL1* RNA levels in transformed Arabidopsis was performed by standard RNA gel-blot analysis. Detection of *AP1* RNA levels in the 4-week-old *tfl1-14* mutant, wild type, and the UBI::*LpTFL1* line 17 was performed by RT-PCR using two 3′-end specific *AP1* primers AP05 (5′-CCCCCTCTGCCACCG-3′) and AP03 (5′-AGGTTGCAGTTGTAAACGGG-3′).

## Construction of UBI::*LpTFL1* and the Transformation of Arabidopsis Wild Type and *tfl1* Mutants

The coding region of *LpTFL1* cDNA was amplified using primers B0 (5′-GGATCCCCATGTCTAGGTCTGTGGAG-3′) and B550 (5′-GGGATCCCACAACTGGGATAGCCA-3′) and recombinant *pfu* polymerase. The fragment was blunt ligated into vector pAHC27 (Christensen and Quail, 1996) containing the maize *Ubiquitin* promoter, an exon:intron region, and the NOS terminator. The entire cassette (UBI::EXintron::*LpTFL1*::NOS) was excised from the plasmid by digestion with *Hin*dII and *Eco*RI and was ligated into the *Eco*RI-*Hin*dII site of the binary vector pCAMBIA3300 (Jefferson, Australia), which confers BASTA resistance, to give pCAMLPTFL1. Arabidopsis plants (Columbia and *tfl1-14* mutants) were transformed with *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) harboring the pCAMLPTFL1 (for *LpTFL1* overexpression) using the floral dip method described by Clough and Bent (1998).

## Construction of LpTFL1::*GUS* Arabidopsis Plants

A 3.6-kb *Sac*I-*RcaI* DNA fragment upstream the start codon of *LpTFL1* was ligated into the *Sac*I-*Nco*I site of the binary vector pCAMBIA3301 (Jefferson), replacing the CaMV 35S promoter in front of the GUS first intron to give pCAMLpTFL1GUS. This vector also confers resistance to BASTA. Arabidopsis plants (Columbia) were transformed with pCAMLPTFL1GUS and with the original pCAMBIA3301 using the method described above. The colorimetric 5-bromo-4-chloro-3-indolyl-$\beta$-glucuronic acid assay was used to determine the localization of GUS expression in Arabidopsis tissue (Jefferson, 1987). Leaves, stem, inflorescence, and axillary meristems were vacuum infiltrated and samples were incubated in the solution overnight at 37°C in the dark. Tissues were bleached in 96% (w/v) ethanol and GUS expression was recorded with a stereo microscope (Nikon, Tokyo).

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