Separate elements of the TERMINAL FLOWER 1 cis-regulatory region integrate pathways to control flowering time and shoot meristem identity

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ABSTRACT

TERMINAL FLOWER 1 (TFL1) is a key regulator of Arabidopsis plant architecture that responds to developmental and environmental signals to control flowering time and the fate of shoot meristems. TFL1 expression is dynamic, being found in all shoot meristems, but not in floral meristems, with the level and distribution changing throughout development. Using a variety of experimental approaches we have analysed the TFL1 promoter to elucidate its functional structure. TFL1 expression is based on distinct cis-regulatory regions, the most important being located 3' of the coding sequence. Our results indicate that TFL1 expression in the shoot apical versus lateral inflorescence meristems is controlled through distinct cis-regulatory elements, suggesting that different signals control expression in these meristem types. Moreover, we identified a cis-regulatory region necessary for TFL1 expression in the vegetative shoot and required for a wild-type flowering time, supporting that TFL1 expression in the vegetative meristem controls flowering time. Our study provides a model for the functional organisation of TFL1 cis-regulatory regions, contributing to our understanding of how developmental pathways are integrated at the genomic level of a key regulator to control plant architecture.

KEY WORDS: TERMINAL FLOWER 1 (TFL1), Plant architecture, Flowering time, Meristem identity, Inflorescence, Promoter, Arabidopsis thaliana

INTRODUCTION

Plant architecture reflects the activity of its meristems. Flowering species show great diversity in aerial architectures as a consequence of how meristem identity is controlled (Della Pina et al., 2014; Park et al., 2014). Upon seed germination, the shoot apical meristem (SAM) has a vegetative identity and generates leaves that often harbour meristems in their axils. Such axillary meristems may lie dormant, or acquire shoot or floral identity. Upon receiving appropriate signals, the main shoot converts to an inflorescence that generates floral meristems on its flanks, or itself converts to a floral meristem. Thus, the timing of events, and the integration of both developmental and environmental signals affecting meristem identity, give rise to the vast array of flowering forms seen in nature (Weberling, 1989). Many genes involved in these signalling pathways and meristem identity are known, but little is understood of how any key player integrates these signals at the genomic level.

TERMINAL FLOWER 1 (TFL1) is a key regulator of plant architecture in Arabidopsis thaliana, controlling flowering time and the fate of shoot meristems. tfl1 mutants flower early, after producing fewer leaves than wild type. Moreover, tfl1 shoot meristems convert to flowers (determinate growth), in contrast to the indeterminate growth of wild-type shoots (Fig. 1C,D) (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Schultz and Haughn, 1993). Thus, the inflorescence SAM forms a terminal flower after producing cauline leaves and a small number of flowers, and the meristems in the axils of the cauline leaves directly form solitary (axillary) flowers, rather that indeterminate cofilorescences, as in the wild type (Fig. 1C,D).

The pattern of TFL1 expression is complex and dynamic. Unlike general meristem genes, such as STM, WUS and members of the CLV family (Gailllochet et al., 2015), TFL1 is expressed in all shoot meristems but not in floral meristems. In the SAM, after germination, TFL1 is weakly expressed in the centre of the meristem during the vegetative phase and its expression is only strongly upregulated at the floral transition, as vegetative identity switches to inflorescence identity, still remaining restricted to the centre of the inflorescence meristem (Bradley et al., 1997). By contrast, in the shoot meristems in the axils of leaves, TFL1 expression appears strong from the start of emergence, becoming weaker and more restricted to their centre when the axillary shoots elongate (Ratcliffe et al., 1999; Conti and Bradley, 2007). Therefore, TFL1 expression discriminates between different developmental phases and different types of shoot meristems, which suggests that the TFL1 promoter responds to, and so integrates, different signals controlling developmental phase transitions and spatial meristem identity.

The expression pattern of TFL1 is thought to be pivotal for its function in the control of plant architecture. It has been suggested that TFL1 expression in the vegetative meristem is essential for its role in regulating the timing of the floral transition (Bradley et al., 1997), and the strong late flowering phenotype of plants with increased, ectopic TFL1 expression (via 35S::TFL1) agrees with this (Ratcliffe et al., 1998). Moreover, TFL1 is required in the inflorescence meristems to repress expression of the floral identity genes LFY and AP1 and, therefore, to maintain their indeterminate growth by preventing these meristems becoming floral meristems (Bowman et al., 1993; Bradley et al., 1997; Baumann et al., 2015).

The wild-type expression pattern of TFL1 is proposed to be essential for its function, but how TFL1 expression is regulated is

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Fig. 1. 2.2 kb of the 5' region and 4.6 kb of the 3' region include all essential elements of the TFL1 promoter. (A) VISTA pairwise alignments of Brassicaceae TFL1 orthologues. The TFL1 promoters from Arabidopsis lyrata (Al), Capsella bursa-pastoris (Cb), Brassica rapa (Br) and Leavenworthia crassa (Lc) were aligned to the TFL1 promoter of Arabidopsis thaliana (At). Graphical output shows base pair identity (50-100% range) in a sliding window of 100 bp. Non-coding regions with >75% similarity are in red. TFL1 exons are in blue. Pink areas highlight conserved blocks. The scale indicates coordinates (in kb) in the A. thaliana TFL1 gene. At the top is shown the TFL1 genomic region in A. thaliana, including the TFL1 gene and the 5' and 3' intergenic regions, with conserved sequence blocks (A-G) in pink. (B) Constructs used in the experiments shown in this figure. (C) Diagrams of the plant architecture of wild-type (WT) and tfl1 mutant Arabidopsis plants. (D) Wild-type (Col) and tfl1-1 mutant plants grown under long-day conditions. Arrowheads mark terminal and axillary flowers in the tfl1-1 inflorescence. (E,F) Fully complemented tfl1-1 mutant plants after transformation with 2.2-T-4.6 or 2.2-cT-4.6 genomic constructs. (G,H) RNA in situ hybridisation for TFL1 or GUS in inflorescence apices of wild-type plants transformed with the 2.2-G-4.6 reporter construct. (I-L) GUS staining of 2.2-G-4.6 apices at different developmental stages: vegetative (I), floral transition (J), bolting (K) and 15 days after bolting (L). (M-P) Longitudinal sections of 2.2-G-4.6 apices at vegetative stage (M), floral transition (N), bolting (O) and 15 days after bolting (P). Arrowhead (O) marks GUS signal in axillary meristem. Scale bars: 100 µm.
poorly understood. Several regulators of its expression have been identified, most of them involved in the repression of TFL1 expression in flowers (Kaufmann et al., 2010; Moyroud et al., 2012; Winter et al., 2011; Liu et al., 2013; Pérez-Ruiz et al., 2015); nevertheless, little is known about the structure of the cis-regulatory regions that are targeted, apart from the observation that sequences in the 3′ intergenic region are important for TFL1 function (Ohshima et al., 1997; Kaufmann et al., 2010). Flowering time pathways are responsive to the environment and affect TFL1 expression, but again no model describes how they integrate pathways. For instance, TFL1 is controlled in the same way in all shoot meristems, through common elements in its promoter, suggesting a common mechanism and signalling pathway? Are the elements responsible for TFL1 expression in different shoot meristems located in different places in the promoter, which would suggest that different signals control these meristems? In addition, a functional map of the TFL1 promoter would facilitate identification of the transcription factors that regulate TFL1 expression, which should help in deciphering the genetic network that controls plant architecture.

Here, through a variety of experimental approaches, including phylogenetic shadowing, phenotyping of T-DNA insertion lines, complementation analysis and promoter::GUS fusions, we have carried out an intensive analysis of the TFL1 promoter. Our results reveal that TFL1 expression is based on distinct cis-regulatory regions that are required to direct expression to different meristems at the shoot apex to shape the architecture of the Arabidopsis plant.

RESULTS
The 3′ and 5′ intergenic regions of TFL1 contain all the elements required for correct expression

Although the cis-regulatory elements of plant gene promoters seem to be mostly frequently located in the 5′ intergenic region, in the case of TFL1, existing data indicated that some relevant cis-regulatory elements are located in the 3′ intergenic region (Ohshima et al., 1997; Kaufmann et al., 2010). As a first step to analyse the structure of the TFL1 promoter, we tested whether the 5′ and 3′ intergenic regions encompass all the regulatory elements required for correct expression.

In our study of the TFL1 promoter, we used three complementary approaches: phenotypic analysis of lines with T-DNA insertions, complementation of the tfl1 mutant with fragments from the TFL1 genomic region, and the expression analysis of reporter constructs with the GUS gene in wild-type plants. The design of genomic and reporter deletion constructs was based on the location of T-DNA insertions and of conserved regions (see below). TFL1 genomic constructs, for complementation, were named N1-T-N2 and GUS reporter constructs were named N1-G-N2, where N1 and N2 indicate the approximate length of the 5′ and 3′ flanking fragments, respectively (Fig. 1B, Fig. S1B, Fig. S2B).

Our first genomic construct, 2.2-T-4.6, contained the TFL1 gene sequence [coding sequence (CDS) plus introns] and its complete 3′ and 5′ flanking intergenic regions (2.2 kb and 4.6 kb, respectively) (Fig. 1B, Fig. S1B). The 2.2-T-4.6 construct was able to fully complement the phenotype of tfl1 mutant plants (Fig. 1E), as previously described (Sohn et al., 2007; Kaufmann et al., 2010). Substituting the TFL1 gene sequence by the TFL1 CDS in the construct 2.2-CT-4.6 (Fig. S1B) gave a similar degree of complementation (Fig. 1F).

When an equivalent reporter construct 2.2-G-4.6, in which the TFL1 gene sequence was replaced by the GUS gene (Fig. 1B, Fig. S2B), was transformed into wild-type plants it reproduced the TFL1 mRNA expression pattern previously reported (Bradley et al., 1997; Conti and Bradley, 2007). Consistent with this, in situ hybridisation on young inflorescence apices of these plants showed that the distribution of the mRNAs of the GUS gene and of the endogenous TFL1 gene were very similar, in the inflorescence meristem and the inflorescence stem vasculature (Fig. 1G,H). GUS staining assays in these plants showed that GUS signal was weak in the vegetative meristem (Fig. 1I,M) and was strongly upregulated in the inflorescence meristem with the floral transition (Fig. 1J,N).

After bolting, strong GUS signal was maintained in the inflorescence meristem, in young axillary shoot meristems and throughout the inflorescence stem (Fig. 1K,L,O,P).

Taken together, these results indicate that both the 5′ and 3′ intergenic regions contain the elements required for wild-type expression of TFL1, and suggest that intron sequences in the gene are not essential for TFL1 expression.

To identify conserved regions within the TFL1 promoter we compared the genomic sequences of TFL1 orthologues from a range of Brassicaceae species. Seven highly conserved sequence blocks (named A to G) were detected in the intergenic 5′ and 3′ regions of Arabidopsis thaliana, Arabidopsis lyrata, Capsella bursa-pastoris and Brassica rapa – species with a very similar inflorescence architecture (Fig.1A). The conserved sequence blocks, two in the 5′ region and five in the 3′ region, were named A (−1.1 to −0.9), B (−0.1 to ATG), C (stop to +0.5), D (+0.9 to +1.3), E (+1.5 to +2.0), F (+2.7 to +3.2) and G (+3.7 to +4.3) (Fig. 1A, Fig. S3). These conserved sequence blocks were used as a basis for the design of constructs for the promoter analysis described below. Note that coordinates indicate the distance from the start (−) or the end (+) of the TFL1 coding sequence.

Interestingly, the level of sequence conservation was reduced in the TFL1 orthologue of Leavenworthia crassa, a Brassicaceae species with a different plant architecture, known as rosette flowering, where plants lack an inflorescence stem and produce solitary flowers at the axil of rosette leaves (Liu et al., 2011). Noticeably, the conserved block F was missing from the L. crassa TFL1 orthologue (Fig. 1A, Fig. S3).

Both 5′ and 3′ promoter regions are necessary for the transcriptional regulation of TFL1

We asked whether both the 5′ and the 3′ intergenic regions are required for the transcriptional regulation of TFL1. The genomic construct 2.2-T-0.4, containing the complete 5′ but lacking the 5′ region, which was replaced by a minimal 35S promoter (Fig. 2A, Fig. S1B), failed to complement the tfl1 mutation (Fig. 2B) (Kaufmann et al., 2010). Accordingly, wild-type plants transformed with an equivalent reporter construct, 2.2-G-0.4 (Fig. S2B), mostly showed no GUS expression; the expression pattern was diverse among the plants showing some GUS staining assays in these plants showed that GUS signal was weak in the vegetative meristem (Fig. 2H-L),
resembling the expression in plants with the full-length reporter construct 2.2-G-4.6 (Fig. 1H-K).

These results show that both 5′ and 3′ intergenic regions are required for correct regulation of TFL1 expression. However, they also indicate that the 3′ region may be sufficient for correct spatial expression but that the 5′ region directs consistent and stable levels of expression.

The 5′ region of the promoter ensures high expression levels of TFL1

To understand the role of the 5′ region in the regulation of TFL1 and to delimit possible cis-regulatory regions, we tested complementation and reporter constructs with a series of deletions of the 5′ region and a set of T-DNA lines with insertions in this region (Fig. 3A, Fig. S1B, Fig. S2B, Fig. S4).

Most tfl1 plants (more than 70%) transformed with the genomic constructs 0.6-T-4.6 or 0.6-T-3.6, retaining only 0.6 kb from the 5′ region and therefore with conserved sequence block A deleted, but containing most of the 3′ region (Fig. 3A, Fig. S1B), showed full complementation (Fig. 3B). A very similar result was obtained with genomic construct 0.3-T-3.6, in which the 5′ region was deleted to −0.3 kb (Fig. S1A, Fig. 3A, C).

By contrast, only a small proportion of tfl1 plants (19%) transformed with the 0.2-T-3.6 construct, where the 5′ intergenic region was deleted to −0.2 kb (Fig. 3A, Fig. S1B), showed full complementation, and the most frequent architecture (37%) comprised plants that had determinate coflorescences that ended in terminal flowers, while the main inflorescence showed indeterminate growth (Fig. 3D). A construct in which the 5′ was further deleted to −0.16 kb, 0.16-T-3.6 (Fig. 3A, Fig. S1B), was unable to produce any fully complemented plants and the most frequent architecture (37%) comprised plants where the main inflorescence ended in a terminal flower after producing several determinate coflorescences (Fig. 3E). Finally, construct 0.1-T-3.6, in which the 5′ was deleted to −0.1 kb (Fig. 3A, Fig. S1B), was unable to rescue any of the tfl1 defects, and most plants showed a tfl1 phenotype, in which coflorescences were replaced by axillary flowers (Fig. 3F), as described for min-T-4.6 plants (Fig. 2G).

As a complementary approach to the genomic deletions, we studied the effect of T-DNA insertions in the 5′ and 3′ intergenic regions on the regulation of TFL1 expression. For the 5′ region, we analysed the phenotype of lines homozygous for T-DNA insertions at −0.3, −0.2 and −0.1 kb upstream of the ATG of the TFL1 gene (Fig. 3A, Fig. S1A). Unexpectedly, although tfl1 plants transformed with genomic constructs carrying 5′ deletions at −0.2, −0.16 and −0.1 kb showed a progressively simplified architecture, plants with T-DNA insertions at −0.3, −0.2 and −0.1 kb in the 5′ region showed a wild-type phenotype (Fig. S4).

Finally, wild-type plants transformed with a set of reporter constructs containing 5′ deletions, namely 0.6-G-4.6, 0.3-G-4.6, 0.2-G-4.6, 0.16-G-4.6, 0.1-G-4.6 and min-G-4.6 (Fig. S2B), showed a similar GUS pattern (Fig. 3G-K), strongly resembling that of the 2.2-G-4.6 reporter with the complete 3′ and 5′ regions (Fig. 1O). However, the intensity of the GUS signal gradually decreased in the deletion series (Fig. 3G-K). The decrease in GUS signal apparently started earlier in the lateral coflorescences before...
decreasing in the main inflorescence (Fig. 3L), correlating with the coflorescences forming terminal flowers before the main shoot. Together, these results indicate that the 0.3 kb 5′ region upstream of the TFL1 CDS contains cis-regulatory elements required for TFL1 to reach a suitable level of expression. This functionally defined region was named region I.

The 3′ region contains cis-regulatory elements for spatiotemporal expression of TFL1

To understand the role of the 3′ region in the regulation of TFL1 and to delimit possible cis-regulatory regions, we tested complementation and reporter constructs with a series of deletions and a set of T-DNA lines with insertions in this region (Fig. 4A, Fig. 5A, Fig. S1B, Fig. S2B).

As shown above, the genomic construct 0.6-T-3.6, in which the conserved region G was deleted (Fig. 4A, Fig. S1B), fully complemented the tfl1 mutant phenotype (Fig. 3B). Similarly, a genomic construct with a further deletion of the 3′ region, 0.6-T-3.3 (Fig. 4A, Fig. S1A), fully rescued the defects of tfl1 plants (Fig. 4B), although the percentage of fully complemented plants was markedly reduced (27%). Correlating with the deletion results, plants with a T-DNA insertion at +3.3 kb in the 3′ region (Fig. 4A, Fig. S1A) showed a wild-type phenotype (Fig. 4C). The expression patterns of equivalent reporter constructs 0.6-G-3.6 and 0.6-G-3.3 (Fig. S2) were very similar to that of the construct with the complete 5′ and 3′ intergenic regions, 2.2-G-4.6 (Fig. 4F-M, compare with Fig. 1M), although signal intensity was generally weaker in 0.6-G-3.3 plants, especially in the inflorescence SAM (Fig. 4L). These data indicate that the 3.6 kb fragment includes all of the 3′ regulatory elements required for TFL1 expression and suggest that the sequence between +3.3 and +3.6 kb (region V) contains elements required for an appropriate transcription level, mainly in the main inflorescence apex.

By contrast, genomic constructs 0.6-T-2.8 and 2.2-T-2.8, in which the 3′ region was further deleted to +2.8 kb thereby removing also conserved region F (Fig. 4A, Fig. S1B), were unable to complement the tfl1 mutant, and these plants showed determination of all inflorescence shoots (Fig. 4D, Fig. S4). Both the main inflorescence and the coflorescence apices produced cauline leaves with associated branches, like wild-type plants, but afterwards they only produced a few lateral flowers and then a terminal flower (Fig. 4D, Fig. S4D). In plants with equivalent reporter constructs 0.6-G-2.8 and 2.2-G-2.8 (Fig. S2B) the GUS signal was weak in the vegetative meristem, upregulated with the floral transition and...
strong in axillary meristems (Fig. 4N-P, Fig. S4E,F), as in the full-length reporter (Fig. 4F-M). However, for 0.6-G-2.8 and 2.2-G-2.8, expression was absent from the meristem of the main and axillary inflorescence (coflorescence) shoots after they started elongating, being observed only in the inflorescence stem (Fig. 4P,Q, Fig. S4G). These results indicate that the 3′ region between +2.8 and +3.3 kb (region IV) is essential to maintain TFL1 expression in the inflorescence meristem, so preventing the determination of inflorescence shoots. A reporter construct with a further deletion, 2.2-G-2.2 (Fig. 4A, Fig. S2B), had the same expression pattern as 2.2-G-2.8 (Fig. 4R-U), and plants with a T-DNA insertion at +2.2 kb (Fig. 4A, Fig. S1A) had essentially the same phenotype as plants complemented with 0.6-G-2.8 or 2.2-G-2.8 (Fig. 4D,E). This suggests that the region between +2.2 and +2.8 kb does not contain any significant regulatory elements.

Plants containing genomic construct 0.6-T-1.6 with a 3′ deletion that also removed conserved region E and plants with T-DNA insertions at +1.6 kb or +1.3 kb (Fig. 5A, Fig. S1) essentially showed the same phenotype. The main inflorescence was similar to that of plants with a T-DNA insertion at +2.2 kb, where a few flowers were made before a terminal flower. However, coflorescences were notably simplified (Fig. 5B-D, compare with Fig. 4D,E): they had only a few flowers, and eventually cauline leaves with solitary flowers, and a terminal flower (Fig. 5B-D). In plants with equivalent reporter constructs 0.6-G-1.6 and 2.2-G-1.3 (Fig. S2) GUS signal was observed in the vegetative meristem and was upregulated in the floral transition, but it was very weak or absent from axillary meristems (Fig. 5G-N). These results suggest that the region between +2.2 and +1.6 kb (region III) is required for strong expression in axillary meristems and, therefore, to form complex coflorescences. They also suggest that the region between +1.6 and +1.3 kb does not contain any significant regulatory elements.

Finally, plants with T-DNA insertions at +1.0 kb or +0.4 kb and plants containing genomic construct 0.6-T-0.4 with a deletion that removed conserved region D (Fig. 5A, Fig. S1) had a tfl1 mutant phenotype, with coflorescences converted into solitary axillary flowers (Fig. 5E,F). Most of the plants with equivalent reporter constructs 2.2-G-1.0 and 0.6-G-0.4 (Fig. S2B) did not show GUS
expression. In the few plants with expression, signal was absent from all shoot meristems, including vegetative meristems, and it was variable and ectopic in some lateral organs (Fig. 5O-V). These results suggest that the region between +1.3 and +1.0 kb (region II) is required for TFL1 expression in the vegetative meristem, for its upregulation with floral transition, and to repress its expression in lateral organs.

Notably, whereas plants with T-DNA insertions at either +1.6 or +1.3 kb flowered at the same time as the wild type, the flowering time of plants with T-DNA insertions at either +1.0 or +0.4 kb was early, as in the tfl1 mutant (Fig. 5W). This indicates that regulatory elements contained in region II (+1.0/+1.3) are required to control flowering time. As deletion of conserved sequence block D (region II) already resulted in no complementation, and plants with insertions at +1.0 kb or +0.4 kb had a tfl1 mutant phenotype, deletion analysis of the 3′ region was not pursued further from +0.4 kb.

### Regulatory regions sufficient for TFL1 expression

The data presented above define discrete cis-regulatory regions 5′ and 3′ of the TFL1 gene (Figs 6 and 7, regions I-V), overlapping
with conserved sequence blocks B, D, E and F (Fig. 6A), that are necessary to control its expression. To assess whether these regions are also sufficient to direct correct TFL1 expression, we tested the performance of a genomic and a reporter construct that combined these regions (Fig. 6B).

The genomic construct 0.6-T-regs contains the TFL1 gene, a 3′ fragment −0.6 kb/ATG, which includes regulatory region I and overlaps conserved sequence block B, regulatory regions II, III and IV, and a 0.5 kb 3′ fragment downstream the TFL1 stop codon, which overlaps with conserved block C (Fig. 6A,B). Out of nine tfl1 plants transformed with 0.6-T-reg, two showed full complementation (Fig. 6C). This proportion of fully complemented tfl1 plants was similar to that obtained when using 0.6-T-3.3 (27%), with the same regulatory regions as 0.6-T-reg but in the context of the full promoter. Also, the expression of the equivalent reporter construct 0.6-G-regs (Fig. 6A,B) was the same as that of 0.6-G-3.3, these being the shortest reporter constructs that gave a wild-type expression pattern (Fig. 6D-F, compare with Fig. 4L,M). These results indicate that a combination of the 5′ and 3′ regulatory regions defined by our analysis plus 0.5 kb downstream of the TFL1 CDS is sufficient to direct correct TFL1 expression.

**DISCUSSION**

**Spatial and functional structure of the TFL1 promoter**

Our results define five major functional cis-regulatory regions required for proper expression of the Arabidopsis TFL1 gene, four of which substantially overlap with blocks of high sequence conservation in TFL1 orthologues from other Brassicacea species (Fig. 7).

The 5′ proximal region mainly contains elements controlling expression level, with region I (−0.3/ATG) absolutely required for TFL1 expression (Figs 3 and 7). In addition, some non-essential expression level elements seem to be located at the 3′, in region V (+3.3/+3.6). Interestingly, in contrast to most T-DNA insertions in the 3′ region, neither T-DNA insertions at the 5′ proximal region nor at +3.3 in the 3′ region cause a tfl1-like phenotype. This seems to fit with the classical definition of transcriptional enhancers, which are considered to be active independently of the distance to their target genes (Shlyueva et al., 2014).

The 3′ intergenic region contains regulatory elements for spatiotemporal expression of TFL1. Thus, region IV (+2.8/+3.3) is required for maintenance of TFL1 expression, although not for its initial expression, in the inflorescence meristems. Its absence causes the main inflorescence and coflorescences to terminate prematurely but remain complex, producing secondary and tertiary branches (Figs 4 and 7). Region III (+1.6/+2.2) is required for strong initial expression in lateral axillary meristems and, consistent with this, plants with deletions to (or insertion at) +1.6 only form very simplified coflorescences (Figs 5 and 7). Finally, region II (+1.0/+1.3) contains elements required for expression in the vegetative meristem and for its upregulation in the inflorescence meristem, as well as for repression in leaves and flowers (Figs 2, 5 and 7).

Previous studies have shown that LFY and different MADS-box transcription factors directly bind to the cis-regulatory regions identified in this work (Fig. S5). This allows us to speculate as to how the different TFL1 expression phases could be established. MADS-box factors SOC1, AGL24, SVP and SEP4, which bind through interaction with AP1 to sequences that are present in cis-regulatory regions II and III, have been shown to contribute to TFL1 repression in flowers (Kaufmann et al., 2010; Liu et al., 2013). This would explain why ectopic TFL1 expression in flowers is observed when these regions are deleted. XAANTAL2 (XAL2, or AGL14), a

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**Fig. 6. Conserved cis-regulatory regions are sufficient to drive correct TFL1 expression.** (A) The TFL1 genomic region. Dotted lines indicate the fragments in constructs employed in the experiments in this figure. Conserved sequence blocks (A-G) are in pink. The functional cis-regulatory regions identified in this work (I-V) are marked with blue rectangles. Coordinates indicate the distance in kb from the start (−) or end (+) of the TFL1 coding sequence. (B) The constructs used in the experiments in this figure. (C) Main inflorescence shoot of a tfl1-1 mutant complemented by transformation with the 0.6-T-regs genomic construct. (D-F) GUS signal in inflorescence shoot apices of wild-type plants transformed with the 0.6-G-regs reporter construct. Whole-mount of a main inflorescence bolting apex (D), and longitudinal sections through the apices of a main inflorescence at bolting (E) or a coflorescence (F). Scale bars: 100 µm.
MADS-box protein with sequence similarity to SOC1, has been proposed to act as an activator of TFL1 expression (Pérez-Ruiz et al., 2015). XAL2 has also been shown to bind to TFL1 genomic regions (Pérez-Ruiz et al., 2015), to sequences that locate to cis-regulatory regions I, II and III, which are required for TFL1 expression level (I) and the upregulation of TFL1 expression in the SAM (II) and lateral inflorescence meristems (III), therefore supporting the proposed role of XAL2 as a TFL1 activator. Finally, only LFY has thus far been shown to bind to sequences that locate to cis-regulatory region IV, which is required for maintenance of TFL1 expression in inflorescence meristems. Although LFY is considered to repress TFL1 expression in flowers (Ratcliffe et al., 1999), this does not preclude a role as a positive regulator of TFL1 expression in the inflorescence meristems. In fact, although LFY expression is mainly detected in flowers, LFY protein has been shown to move and act non-autonomously, and immunolocalisation results might indicate the presence of LFY protein at low levels in the SAM (Sessions et al., 2000); moreover, although the dual role of LFY as an activator and a repressor of TFL1 expression in different domains might appear contradictory, such context-dependent activity of transcription factors is in fact very common (for example, see Kaufmann et al., 2010). In addition, it is very likely that other, as yet unidentified factors also play crucial roles in the establishment of the TFL1 dynamic expression pattern.

An interesting conclusion of our study is that most of the important cis-regulatory elements of the TFL1 promoter are located in the 3′ intergenic region of the gene, coinciding with blocks of sequence conserved among TFL1 orthologues. Although examples exist of essential cis-regulatory elements located at the 3′ end of plant genes, they are relatively scarce (Larkin et al., 1993; Brand et al., 2002; Konishi and Yanagisawa, 2011; Raatz et al., 2011). As discussed by Raatz et al. (2011), this could reflect the fact that not enough attention has been paid to possible 3′ cis-regulatory elements in studies on plant promoters. Other studies (Castaings et al., 2014), however, indicate that sequence conservation in the 3′ end of orthologous plant genes is not so common, which might suggest that relevant cis-regulatory elements are not frequently located in their 3′ intergenic regions.

**The TFL1 promoter has a functional modular structure**

Our results indicate that distinct cis-regulatory regions of the TFL1 promoter are required for expression in different spatiotemporal domains at the Arabidopsis shoot apex. A similar promoter structure, with distinct cis-regulatory regions controlling

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**Fig. 7. Regulatory structure of the TFL1 promoter.** The TFL1 genomic region (top). Phylogenetically conserved sequence blocks are in pink. Blue rectangles (I-V) mark the cis-regulatory regions identified in this work, with coordinates indicating the distance in kb from the start (−) or end (+) of the TFL1 coding sequence. The defects in the TFL1 expression pattern caused by the deletion of each cis-regulatory region are indicated beneath. The diagrams in the first three rows illustrate the GUS expression pattern (blue) in shoot apices during development (from top to bottom per column: vegetative, floral transition and inflorescence bolting), observed in wild-type plants transformed with reporter constructs with deletions of the cis-regulatory regions (indicated with a dotted line). The bottom row illustrates the phenotypes observed for Arabidopsis tfl1-1 mutant plants transformed with genomic constructs with deletions of the corresponding cis-regulatory regions. The green dashed arrows and lines indicate indeterminate or determinate branches not represented in detail.
expression in different domains, has also been observed for other developmental regulators, such as the Arabidopsis CRABS CLAW (CRC) or SPATULA (SPT) genes, which, like TFL1, show expression in different domains that changes during development (Lee et al., 2005; Groszmann et al., 2010). This suggests that this type of promoter structure might help achieve a dynamic expression pattern. For the CRC promoter the identified cis-regulatory regions act, to some extent, as independent regulatory modules (Yuh et al., 1998, Lee et al., 2005). Elucidating whether this is also the case for the cis-regulatory regions in the TFL1 promoter requires further analysis.

A functional modular structure to the promoters of developmental regulators might facilitate gene evolution to generate a diversity of plant forms. Thus, different combinations of cis-regulatory modules in different species could lead to different expression patterns of TFL1-like genes, as has been predicted to contribute to different inflorescence architectures (Prusinkiewicz et al., 2007). For instance, it is interesting that in the Arabidopsis relative L. crassa, in which changes in TFL1 have been proposed to contribute to its distinct inflorescence architecture (Liu et al., 2011), the conserved block F (region IV) is absent from the 3’ of its TFL1 gene (Fig. 1A).

Different promoter regions control TFL1 expression in different shoot meristems

Our results suggest that regulation of TFL1 in the SAM and lateral shoot meristems is controlled through the activity of different cis-regulatory regions in the TFL1 promoter. On the one hand, TFL1 expression in the main inflorescence specially requires region IV. In plants with genomic deletions or insertions that remove region IV, the main inflorescences only produce a few flowers and end in a terminal flower, as in tfl1 mutants. This agrees with the absence of expression of equivalent TFL1::GUS deletions in the inflorescence meristem in elongating inflorescences. By contrast, lateral inflorescences of these plants (without region IV) are much more complex than in tfl1 mutants (where they are replaced by solitary flowers), and expression of TFL1::GUS with deletions of region IV is strong in emerging lateral shoot meristems (Figs 4 and 7). On the other hand, TFL1 in lateral inflorescences seems to particularly require region III. Deletion of region III causes strong simplification of coflorescences, without further affecting the main inflorescence, and this correlates with a substantial decrease in the expression of TFL1::GUS with deletions of region III in lateral shoot meristems (Figs 5 and 7).

Different signalling appears to control TFL1 in the SAM and lateral meristems, suggesting two non-exclusive models. In one model, TFL1 expression in lateral shoot meristems requires activation by different effectors than in the main shoot. Alternatively, TFL1 expression might be regulated by the same effectors in SAM and lateral meristems; for instance, if they are at a higher level in the lateral meristems than in the SAM. In this second model, activation in only region III would be sufficient for expression in lateral meristems, whereas expression in the SAM would require activation in both region III and region IV. That main and lateral inflorescences are differently regulated is supported by the specific expression of genes such as BRANCHED1 in shoot auxiliary meristems but not in the SAM (Aguilar-Martinez et al., 2007).

A 3’ cis-regulatory region is required for control of flowering time by TFL1

A remarkable finding of our study is that the +1/+1.3 region of the TFL1 promoter (region II) is strictly required for a wild-type flowering time. The +1/+1.3 region is required for TFL1 expression in the vegetative SAM and its upregulation at the floral transition. One hypothesis proposes that TFL1 expression in the vegetative meristem controls (represses) flowering. This was based on the observation that the main difference between Arabidopsis TFL1 and the Antirrhinum majus homologue CENTRORADIALIS (CEN) gene is that whereas TFL1, which controls flowering time and determination of the inflorescence, is expressed both in the vegetative and the inflorescence SAM, CEN, which only controls determination, is expressed only in the inflorescence SAM (Bradley et al., 1993). Our result links TFL1 expression to the vegetative meristem and floral transition and provides support for this hypothesis.

TFL1 function in pea (Pisum sativum) is split between two TFL1 homologues: PtTFL1a/LATE FLOWERING (LF), which controls flowering but not determination, and PtTFL1a/DETERMINE (DET), which controls determination of the inflorescence but not flowering (Foucher et al., 2003). Interestingly, DET, like CEN, is expressed in the inflorescence but not in the vegetative apex, while LF is expressed in the inflorescence and in the vegetative apices (Foucher et al., 2003; Berbel et al., 2012). Although knowledge of the LF expression pattern is scarce (only from RT-PCR data), the data also suggest that regulation of flowering by TFL1-like genes is related to their expression in the vegetative apex. It would be interesting to elucidate whether the subfunctionalisation of the pea TFL1 genes derives from divergence in their expression patterns.

TFL1-like genes have been studied in many plants with different inflorescence architectures and, frequently, as in the examples described above, their expression patterns seem to correlate well with their function in shaping plant architecture (Benlachou et al., 2007; Megarry and Ayre, 2012; Lifschitz et al., 2014; Wickland and Hanzawa, 2015). It will be important to understand the extent to which divergence in the expression patterns of TFL1-like genes has contributed to different plant and inflorescence architectures and how the cis-regulatory regions of these genes have evolved to generate these distinct expression patterns.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana plants of Columbia (Col) ecotype were used unless otherwise stated. The tfl1-1 (Col) and tfl1-2 (Landsberg erecta, Ler) mutants have been described previously (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992).

The T-DNA insertion lines characterised in this study were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (Alonso et al., 2003) and are described in Table S1. The position of the T-DNA insertions in the TFL1 genomic region was confirmed by PCR. Primers used for genotyping are listed in Table S2.

Plants were grown under controlled temperature (21-23°C) and long-day photoperiods (16 h light/8 h dark), as previously described (Fernández-Nohales et al., 2014). Plants used for flowering-time measurements were grown in the same conditions but under short-day photoperiods (8 h light/16 h dark).

Plasmid construction

Genomic and GUS reporter constructs in this study derive from a TFL1 clone isolated from an Arabidopsis Ler genomic library (Whitlam et al., 1993). The genomic clone included the TFL1 gene plus 5.5 kb of the 5’ and 8.7 kb of the 3’ flanking sequences. This 15.3 kb fragment was subcloned as a NotI fragment into the pBluescript II SK+ vector (Agilent), giving rise to plasmid pBSKTF1.

For genomic constructs, different fragments from the TFL1 genomic region in pBSKTF1 were subcloned into the binary vector pBIN19 (Bevan, 1984). These fragments were obtained by restriction digestion at sites in the TFL1 genomic region, or by PCR, or by a combination of both methods.
For reporter constructs, the β-glucuronidase gene (GUS) from plasmid pB101 (Jefferson et al., 1987) was subcloned into the pGEM-T Easy vector (Promega) as a HindIII/EcoRI fragment, giving rise to plasmid pG101. Different fragments of the 5’ and 3’ TFL1 flanking regions were obtained from pBSKTF1, as described above, and subcloned into pG101 upstream or downstream of the GUS gene. The promoter::GUS fusions generated were subcloned into the binary vector pBIN19.

The minimal 46 bp 35S promoter from cauliflower mosaic virus (Benfey and Chua, 1990) was obtained by PCR using plasmid pDW294 (Busch et al., 1999) as a template. PCR reactions were carried out with high-fidelity PfuTurbo DNA polymerase (Stratagene) using the primers listed in Table S2.

Plant transformation and analysis of transgenic lines
Constructs based on the pBIN19 plasmid were introduced into Agrobacterium tumefaciens strain C58. Arabidopsis thaliana plants were transformed by vacuum infiltration as described (Bechtold et al., 1993).

For complementation analyses, tfl1-1 mutant plants were transformed and T1 plants selected on the basis of kanamycin resistance and transferred to soil. At least seven independent T1 lines were analysed for each construct. tfl1-1 and Col wild-type plants transformed with an empty pBIN19 vector and with the 2.2-G-6.4 construct, respectively, were used as controls in the complementation analysis. Quantitative data of the range of phenotypes obtained for the different genomic constructs are presented in Table S3. For analyses of the expression pattern of GUS reporter constructs, Col wild-type plants were transformed. A minimum of 15 kanamycin-resistant T1 lines were selected per construct and transferred to soil. GUS activity was first analysed in T1 plants, in whole-mounts of inflorescences at bolting or 15 days after bolting (mature inflorescences). Quantitative data of the range of expression patterns observed for the different GUS reporter constructs in T1 plants are presented in Table S4. The data in Fig. 3L correspond to an independent experiment in which at least 12 T1 plants for each reporter construct were selected and analysed simultaneously. Bolting apices were stained for GUS (at high stringency, see below) and reporter activity in the shoot apical dome of the main inflorescence and the most apical (older) cofloraeces was determined.

Representative lines for each GUS reporter construct were identified and subsequently tested in the next generation for single-locus insertion of the transgene, based on a 3:1 segregation on selective medium. In this way, three to four independent T2 lines were selected for each construct and GUS expression pattern in the main shoot was analysed in microscopy sections at different developmental stages: vegetative apices (after 6–8 days under long-day conditions), floral transition (12-day plants; this day was determined as described by Bradley et al., 1997), bolting apices (after 20–25 days) and mature inflorescence apices (35–40 days). Expression patterns observed for the different representative lines for each construct were consistently similar.

GUS assays
Samples were stained for GUS as described (Benlloch et al., 2011) with minor modifications. Two staining buffers with different concentrations of ferrocyanide and ferricyanide were employed: 2 mM ferro-ferricyanide (medium stringency) for samples with weak GUS activity, and 10 mM ferro-ferricyanide (high stringency) for samples with strong GUS activity (Sessions et al., 1999).

Images of GUS-stained whole-mount apices in 70% (v/v) ethanol were taken with a Nikon SMZ800 stereomicroscope. Samples were then stained in Eosin and embedded in paraffin as described (Benlloch et al., 2011). Sections (12 µm) were obtained with a Leica RM-2025 microtome and photographed with a Nikon Eclipse 600 light microscope.

RNA in situ hybridisation
Plasmids pJAM2045 (for TFL1) and SS018 (for GUS) were linearised and antisense probes were transcribed in vitro using the DIG RNA Labelling Kit and T7 RNA polymerase (Roche). The RNA probes were then subjected to mild alkali hydrolysis as described (Coen et al., 1990). RNA in situ hybridisations were performed on 8 µm sections of inflorescence apices as described (Ratcliffe et al., 1999) and imaged using a Nikon Eclipse 600 microscope.

Determination of TFL1 transcription initiation site
The transcription start site of TFL1 was determined by three independent rapid amplification of cDNA ends (RACE) experiments as being 45 bp upstream of the start codon. For this purpose, mRNA was extracted from bolting inflorescences with the QuickPrep Micro mRNA Purification Kit (Amersham) and 5’-RACE was carried out using the GeneRacer Kit (Invitrogen) according to the manufacturer’s instructions and the TFL1-specific primers ASMO70 and ASMO71 (Table S2).

Sequence analysis of the TFL1 promoter
For phylogenetic shadowing of TFL1 cis-regulatory sequences (Boffelli et al., 2003; Hong et al., 2003), genomic regions of TFL1 orthologues from different species were isolated and sequenced. CsTFL1 was isolated from a Capsella bursa-pastoris BAC library obtained from Dr R. Schmidt (Leibniz Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany) using the TFL1 cDNA as probe. BrTFL1 from Brassica rapa was identified in the BAC KBH96810 using GBrowse [at TAIR (www.arabidopsis.org)]. The sequence of the genomic fragment containing LtTFL1 from Leavenworthia crassa (GenBank accession GU136396) was provided by Dr D. Baum (University of Wisconsin-Madison, USA).

The alignment of the non-coding sequences from the different TFL1 orthologues and the identification of the conserved regions were carried out using mVISTA (http://genome.lbl.gov/vista/index.shtml) and CHAOS-DIALIGN (http://dialign.tibs.de/chaos-dialign-submission) (Brudno et al., 2003, 2004; Frazer et al., 2004). Because the results of both algorithms were essentially identical, only mVISTA results are shown in this work. A shuffle-LAGAN alignment with a window of 100 bp and a minimum sequence identity of 75% were used for the mVISTA analysis.

For identification of putative MADS domain (CARG boxes) and LFY transcription factor binding sites, we scanned the conserved regions of the Arabidopsis TFL1 genomic region in the JASPAR database (Sandelin et al., 2004) using its specific section for plants. For CARG boxes, we selected all the available JASPAR matrix models classified as ‘MADS-box factors’ using default parameters and we finally selected those with a score higher than 10 and a relative score higher than 0.8 that were conserved in at least two species. For LFY binding sites, we selected the JASPAR matrix model for LFY using default parameters but with a relative score of 0.75, and we finally selected those with a score higher than 7.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
F.M., D.B. and A.S.-M. designed the work; A.S.-M., M.J.D., P.F.-N., F.M. and Y.H. performed the experiments described (Ratcliffe et al., 1999) and imaged using a Nikon Eclipse 600 microscope.

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Data availability

Sequences of the genes used in this study can be found in the NCBI GenBank database under the following accession numbers: Arabidopsis thaliana Ler TFL1 (KX139000), Capsella bursa-pastoris TFL1 (KX139002) and Brassica rapa TFL1 (KX139001).

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.135269.supplemental

References


