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Research paper

Isolation and characterization of a *TERMINAL FLOWER 1* homolog from *Prunus serotina* Ehrh.

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Flowering control is one of the several strategies for gene containment of transgenic plants. *TERMINAL FLOWER 1* (*TFL1*) is known to be involved in the transcriptional repression of genes for inflorescence development. Two *TFL1* transcripts with different 3′ UTR were cloned from black cherry (*Prunus serotina* Ehrh.) using reverse transcription–polymerase chain reaction (RT–PCR) and rapid amplification of cDNA ends (RACE). Corresponding to the two *TFL1* transcripts, two *PsTFL1* gene sequences, 1248 bp and 1579 bp, were obtained and both contained the same 519 bp coding region which encoded a putative protein of 172 amino acid residues. The phylogenetic analysis of the amino acid sequences showed high identity of *PsTFL1* to *TFL1* orthologs of other *Prunus* species, including Yoshino cherry (*Prunus* × *yedoensis* Matsum.), peach (*Prunus persica* (L.) Batsch), apricot (*Prunus armeniaca* L.) and Japanese apricot (*Prunus mume* Sieb. et Zucc.). The real-time quantitative PCR detected a single copy of *PsTFL1* gene sequences in the black cherry genome with two alleles. The gene expression of *PsTFL1* was examined in several tissues including the stems, leaves, shoot tips, and vegetative and floral buds. The highest mRNA level was detected in shoot tips, and the lowest level in the leaves. Transgenic *Arabidopsis thaliana* (L.) Heynh. plants overexpressing *PsTFL1* showed significantly delayed flowering. These plants also showed largely increased vegetative growth, plant height, number of nodes, trichome density, and the conversion of flower to shoot was observed at each node and shoot apex.

Keywords: black cherry, ectopic expression, flowering, *Prunus serotina*, *PsTFL1*.

Introduction

Black cherry (*Prunus serotina* Ehrh.), also known as wild black cherry, rum cherry and mountain black cherry, is the only species in the genus *Prunus* that is of high commercial value for timber and sawlog production. The wood is valued for cabinets, furniture, veneer, architectural millwork and musical instruments (Marquis 1990). However, the damage caused by cambium-mining insect pests, such as the peach bark beetle (*Phloeotribus liminaris* Harris), triggers gummosis, a non-specific defensive response of black cherry to insect attack, pathogen infection or stress conditions. The deposition of resinous gum on the bark at the site of injury reduces the veneer quality of black cherry wood, and can reduce the value of an entire tree by as much as 90% (Barnd and Ginzel 2008). Genetic engineering to enhance host plant resistance offers an efficient approach to alleviate this problem compared with traditional breeding of trees, as it avoids the long juvenile period and enables transfer of traits of interest into selected genotypes (Peña and Séguin 2001). Gene containment must be achieved, however, since it is an environmental concern regarding planting of transgenic trees. To control flowering, a better understanding of the molecular mechanism of flowering in black cherry is necessary.

In plants, the transition from vegetative growth to reproductive growth is induced by various internal developmental signals and external environmental factors such as photoperiod, temperature and growing conditions (Boss et al. 2004).

Numerous genes involved in this transition have been cloned and characterized in *Arabidopsis thaliana* (L.) Heynh. Among these genes, *TERMINAL FLOWER1* (*TFL1*) and *FLOWERING LOCUS T* (*FT*) are two key regulators of flowering time and inflorescence development. These genes share a high similarity in amino acid sequence, and both belong to the family of phosphatidylethanolamine-binding proteins (PEBP) that are known to be involved in the signaling pathways regulating cell differentiation, but they have antagonistic functions. *TFL1* interacts with the bZIP transcription factor *FD* and represses the transcription of *FD*-dependent floral meristem identity genes such as *APETALA1* (*AP1*) and *AGAMOUS* (*AG*), while *FT* was involved in the transcriptional activation of those genes by interacting with *FD* (Banfield and Brady 2000, Abe et al. 2005, Hanano and Goto 2011). Therefore, *TFL1* suppresses the transition from juvenile to adult stage, whereas *FT* promotes flowering. Transcription factor *LEAFY* (*LFY*) acts in parallel with *FT* to activate downstream target genes such as *AG* and *AP1*, and *AP1* positively regulates *LFY* expression, but suppresses *TFL1* expression (Liljegren et al. 1999).

The molecular basis of flowering in trees has become an increasing focus in recent years because of the high economic value of tree species. *TFL1* homologous genes have been cloned and characterized from several tree species, including citrus (*Citrus sinensis* L. Osbeck) (Pillitteri et al. 2004), apple (*Malus* × *domestica* Borkh.) and Japanese pear (*Pyrus pyrifolia* (Burm. f.) Nakai) (Esumi et al. 2005), *Populus* (Igasaki et al. 2008, Mohamed et al. 2010) and Japanese apricot (*Prunus mume* Sieb. et Zucc.) (Esumi et al. 2010). The function of these genes in delaying flowering was also confirmed, which makes it a potential gene to control flowering by overexpressing it in the target species.

In the present study, *TFL1* homologous genes from black cherry were isolated and characterized. The function was examined by ectopic overexpression in *A. thaliana* under the control of the constitutive CaMV 35S promoter, and the transgenic plants showed significantly delayed flowering and abnormal inflorescence architecture.

Materials and methods

Plant materials

In vitro shoots of a mature elite genotype of black cherry (BC3) were maintained in culture as described by Liu and Pijut (2008). Plant tissues, including shoot tips, leaves and stems, were obtained from these in vitro shoot cultures for DNA and RNA extraction. Vegetative and floral buds were collected from the same clone at Martell Forest, Purdue University, West Lafayette, IN.

Wild-type *A. thaliana* (ecotype Columbia-0) seeds (kindly provided by Zhixiang Chen, Purdue University, West Lafayette, IN) and *tfl1-11* mutant (CS6235) seeds obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University (Columbus, OH) were stratified for 3 days at 4 °C to break seed dormancy, and then sowed in soil at 24 ± 2 °C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}). The plants were moved into a greenhouse with long-day (LD) conditions after four rosette leaves had developed.

Isolation of TFL1 homologous genes from P. serotina

Total RNA was extracted from the leaves of in vitro shoot cultures using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Genomic DNA was removed by using the DNA-free DNase Treatment and Removal Kit (Life Technologies, Grand Island, NY, USA), and the first-strand cDNA was synthesized with AccuScript High-Fidelity cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA). Degenerate primers (Table S1 available as Supplementary Data at *Tree Physiology* Online) designed to amplify partial *TFL1* homologous sequence from Japanese apricot (Esumi et al. 2010) were used to perform nested polymerase chain reaction (PCR) to amplify partial *TFL1* cDNA sequence of black cherry using Taq DNA polymerase (5 PRIME). The PCR product was purified with QIAquick Gel Extraction Kit (Qiagen), and was inserted into a pGEM-T Easy vector (Promega, Fitchburg, WI, USA) for sequencing at the Purdue University Genomic Center (West Lafayette, IN, USA). Based on the partial cDNA sequence, both 5′ and 3′ inner and outer primers (Table S1 available as Supplementary Data at *Tree Physiology* Online) were designed to perform 5′ rapid amplification of cDNA ends (RACE), and 3′ RACE reactions (FirstChoice RLM-RACE; Life Technologies). The 5′ end and 3′ end cDNA sequences were assembled to obtain the full-length cDNA sequence by inserting into pGEM-T Easy vector for sequencing. Two different sequences of 3′ UTR were obtained by 3′ RACE. *PsTFL1-1* was designated as the one with longer 3′ UTR and *PsTFL1-2* as the shorter one (data not shown). The complete coding region of *PsTFL1* was then amplified using Vent DNA polymerase (New England Biolabs, Ipswich, MA, USA) and primers TFL1-5F and TFL1-5R (Table S1 available as Supplementary Data at *Tree Physiology* Online). The genomic sequences of *PsTFL1* were cloned from genomic DNA using the same primers. The gene structures were obtained by aligning the cDNA and genomic DNA sequences. The homologous genes from other plant species were searched by BLASTX based on the deduced amino acid sequence of *PsTFL1*, all the sequences were aligned by ClustalW (Larkin et al. 2007), and a phylogenetic tree was constructed using the p-distance parameter and neighbor-joining method in MEGA5 software (Tamura et al. 2011) based on the analysis of ClustalW.

Determination of gene copy number

Gene copy number of *PsTFL1* was determined by real-time quantitative PCR (qPCR), a method that was proven to be as

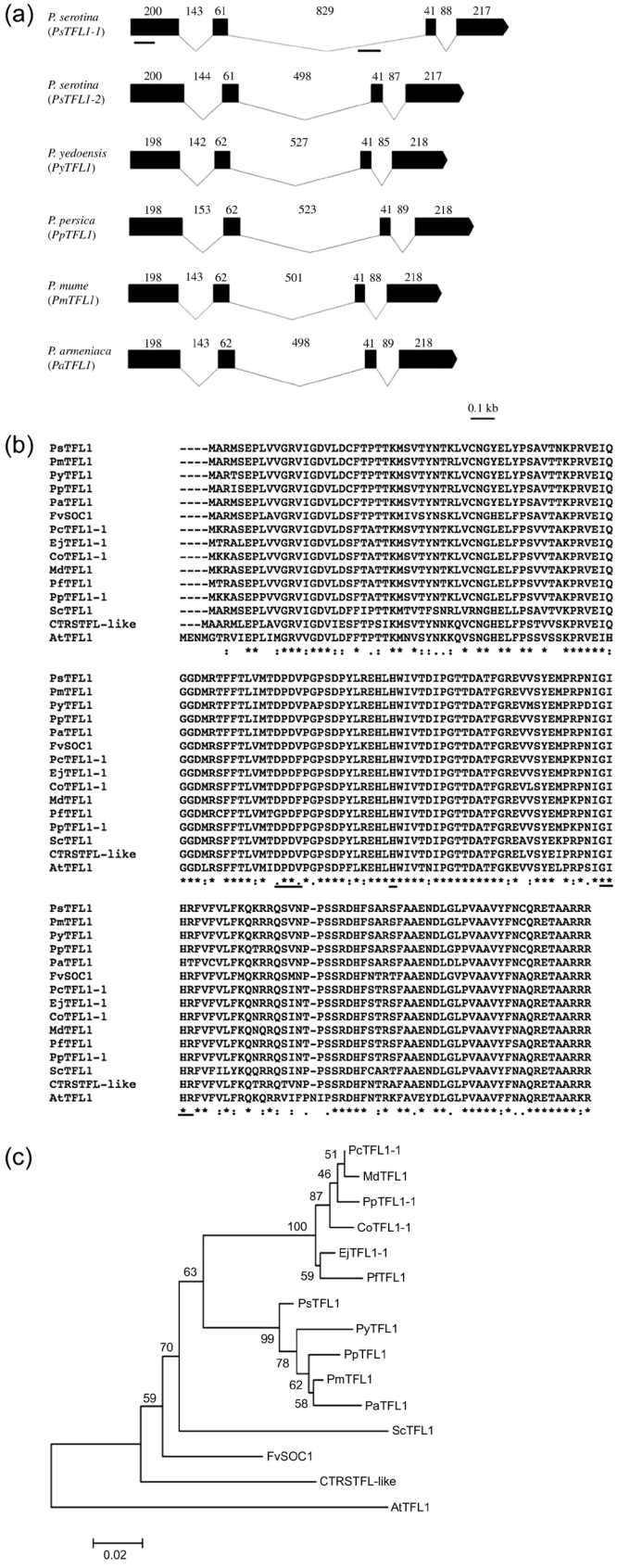

Figure 1. Gene structure and phylogenetic analysis of a *TFL1* homolog in black cherry. (a) Comparison of structure of two *PsTFL1* gene sequences and *TFL1* homologs from *Prunus* × *yedoensis* Matsum., *P. persica* (L.) Batsch, *P. mume* and *P. armeniaca* L. Numbers indicate the base pairs in the exons (black boxes) and introns (thin lines). Short lines indicate the two fragments amplified for gene copy number determination. (b) Alignment of the deduced amino acid sequences of *TFL1* homologs from *P. serotina* and other plant species including *P. mume* (*PmTFL1*, BAJ14521), *Prunus* × *yedoensis* (*PyTFL1*, AEO72023), *P. persica* (*PpTFL1*, ADL62867), *P. armeniaca* (*PaTFL1*, ADL62862), *Fragaria vesca* L. (*FvSOC1*, AEO72027), *Pyrus communis* L. (*PcTFL1-1*, BAD10963), *Eriobotrya japonica* (Thunb.) Lindl. (*EjTFL1-1*, BAD10966), *Cydonia oblonga* Mill. (*CoTFL1-1*, BAD10964), *Malus* × *domestica* (*MdTFL1*, BAD06418), *Pyracantha fortuneana* (Maxim.) Li (*PfTFL1*, AEO72026), *Pyrus pyrifolia* (Burm. f.) Nakai (*PpTFL1-1*, BAD10962), *Spiraea cantoniensis* Lour. (*ScTFL1*, AEO72025), *Citrus trifoliata* (L.) Raf. (*CTRSTFL1-like*, ABY91242) and *A. thaliana* (*AtTFL1*, AAB41624). Lines indicate important motifs and a key residue of *TFL1*. (c) A phylogenetic tree of *TFL1* homologs constructed by the neighbor-joining method based on the alignment results. Numbers at the nodes indicate bootstrap values calculated for 1000 replicates.

robust as Southern blot analysis in the determination of gene copy number (McAndrew et al. 1997, De Preter et al. 2002). Mandelonitrile lyase isoform 2 (*MDL2*) was selected as a single-copy endogenous reference gene confirmed by Hu and Poulton (1999) using Southern blot analysis. Primers—TFL1-6F and TFL1-6R; TFL1-8F and TFL1-8R (Table S1 available as Supplementary Data at *Tree Physiology* Online)—were designed to amplify a common fragment shared by both of the sequences and a fragment contained only by *PsTFL1-1* in the second intron, respectively. All the amplicon lengths were 100–120 bp. Genomic DNA from the leaves of black cherry was extracted using the DNeasy Plant Mini Kit (Qiagen). The qPCR was performed with the Stratagene Gene MX 3000 PM. Each reaction contained the following reagents in 20 µl: 2 µl DNA (around 100 ng), 1 µM of each primer, 10 µl Brilliant SYBR Green QPCR Master Mix (Agilent Technologies), and sterile water was added to the final volume. The cycling conditions consisted of DNA polymerase activation at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and followed by a melting curve analysis from 55 to 95 °C. Each sample was replicated three times and the relative standard curve of the serial dilution of genomic DNA for both the target and reference gene was constructed, and gene copy number was analyzed following the calculation reported by Weng et al. (2004).

Transformation of A. thaliana with 35S::PsTFL1

The cDNA sequence of *PsTFL1* was inserted into the kanamycin-selectable binary vector pBI121 where the GUS gene was replaced under the control of the CaMV 35S promoter. The construct was then transformed into *Agrobacterium tumefaciens* strain EHA105 by heat-shock (Hofgen and Willmitzer 1988) and then introduced into the wild-type *A. thaliana* ecotype Columbia-0 and the *tfl1-11* mutant using the floral dip method (Clough and Bent 1998). The surface-disinfested progeny seeds were germinated on agar plates containing half-strength Murashige and Skoog medium (1962) containing 50 mg l^{-1} kanamycin as the selection agent. The plates were

incubated at 25 °C under a 16-h light photoperiod (80 μmol m^{-2} s^{-1}). The plants were screened for 2–3 weeks until six leaves developed, and then the plants were transplanted into soil and placed in the greenhouse under long day (LD) conditions. Wild-type and mutant plants were germinated in soil at the same time as the transgenic plants were germinated, and were grown under the same conditions. The number of days from sowing to the formation of 1-cm-long inflorescence and the number of rosette and cauline leaves at that stage were recorded. Statistical analyses were performed using Student's *t*-test ($P < 0.05$). The integration of *PsTFL1* in the transgenic plants was confirmed by PCR analysis.

Expression analysis of PsTFL1 in black cherry and transgenic Arabidopsis

The relative expression of total *PsTFL1* in the stems, leaves, shoot tips and vegetative and floral buds of black cherry and in *35S::PsTFL1 Arabidopsis* plants were investigated by RT-qPCR. Primers, TFL1-6F and TFL1-6R (Table S1 available as Supplementary Data at *Tree Physiology* Online), were designed to specifically amplify a 113-bp fragment shared by both *PsTFL1-1* and *PsTFL1-2*. RNA extraction and reverse transcription were conducted as described previously. Each reaction contained the following reagents in 20 μl: 5 μl cDNA, 1 μM of each primer, and 10 μl Brilliant SYBR Green QPCR Master Mix (Agilent Technologies), and sterile water was added to the final volume. The cycling conditions consisted of DNA polymerase activation at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 58 °C for 30 s and 72 °C for 1 min, and followed by a melting curve analysis from 55 to 95 °C performed with the Stratagene Gene MX 3000 PM. *Actin* in black cherry and *Arabidopsis* was used as a reference gene to normalize the data, and each sample was replicated three times. The relative expression levels were calculated using the delta-delta Ct method with efficiency correction (Pfaffl 2001). The *PsTFL1* mRNA levels in each transgenic *Arabidopsis* individual and their flowering-related traits including days to flowering and the number of rosette and cauline leaves were plotted as *x*–*y* pairs to detect any correlation between them.

Expression analysis of AtFT, AtTFL1 and PsTFL1 in transgenic Arabidopsis

Thirty transgenic *Arabidopsis* plants were grouped into three classes (Class I, II or III) according to their inflorescence and floral architecture. The average of the relative expression levels of *AtFT, AtTFL1* and *PsTFL1* genes in the leaf tissue of the transgenic *Arabidopsis* Class I to III was quantified and compared by reverse transcription (RT)–qPCR. Leaves were collected from the 30 T_1 plants when 1-cm inflorescences had developed. An equal amount of cDNA from individual plants of the same class was combined. Primers—AtFT-F and AtFT-R; AtTFL1-F and AtTFL1-R; TFL1-9F and TFL1-6R (Table S1 available as Supplementary Data at *Tree Physiology* Online)—were designed to specifically amplify a 100–120-bp-long fragment of each gene, and TFL1-9F was designed to specifically amplify *PsTFL1* without cross-amplifying *AtTFL1*. *Arabidopsis Actin2* was used as the reference gene. The reaction conditions were the same as previously described.

Results

Isolation of TFL1 homologous genes from P. serotina

Only one 5′ end cDNA sequence was obtained by 5′ RACE, whereas two 3′ end cDNA sequences with identical coding region, but different 3′ UTR length, were found. Therefore, it was assumed that there were two cDNA sequences of *PsTFL1* which shared identical coding regions, but different UTR. Two different genomic sequences of *PsTFL1* were obtained, 1579-bp long (*PsTFL1-1*) and 1248 bp (*PsTFL1-2*), respectively. Sequencing analysis indicated that *PsTFL1-1* had the longer 3′ UTR (data not shown). The alignment of cDNA and genomic sequences showed that both sequences consisted of four exons and three introns, and shared identical exon regions (Figure 1a). Therefore, both of them contained the same 519-bp coding region which encoded a putative protein of 172 amino acid residues. The deduced protein sequence showed 74% identity with the *Arabidopsis TFL1*. It also contained D-P-D-x-P (70–74), a His residue at position 86, and G-x-H-R motifs (115–118) that were found to be highly conserved throughout the family of PEBP, and were believed to contribute to the conformation of the ligand binding site of CENTRORADIALIS (CEN), another *TFL1* homolog (Banfield and Brady 2000) (Figure 1b). The phylogenetic analysis also revealed high identity of *PsTFL1* amino acid sequence to *TFL1* orthologs of other *Prunus* species, including Yoshino cherry (*Prunus* × *yedoensis*), peach (*Prunus persica*), apricot (*Prunus armeniaca*) and Japanese apricot (*Prunus mume*) which formed a sub-clade (Figure 1c).

Detection of PsTFL1 gene copy number

When using the primers targeting the common fragment of two *PsTFL1* sequences, qPCR analysis revealed a single copy of *PsTFL1* in the genome of black cherry compared with the single-copy gene *mdl2* (Table 1). However, when using the primers targeting *PsTFL1-1*, only half copy was detected which further indicated that PsTFL1 had a single copy in the black cherry genome, and *PsTFL1-1* and *PsTFL1-2* are two allelic gene sequences with identical exon regions. The relative standard curves of the serial dilution of genomic DNA for both target and reference genes are shown in Figure 2a–c.

Expression analysis of PsTFL1 in black cherry tissues

The relative expression level of total *PsTFL1* was examined in the stems, leaves, shoot tips, and vegetative and floral buds of black cherry using RT–qPCR. The mRNA level of *PsTFL1* in shoot tips was the highest which was 2.12-fold higher than that

Table 1. The slopes and intercepts, C_T values, and the estimated copy number of the reference gene, *MDL2*, and the target genes, *PsTFL1-common* and *PsTFL1-1*.

	Slope (*S*)	Intercept (*I*)	C_T	4′ (X_0/R_0)	Estimated no. copies
MDL2	–3.332	29.689	18.66 ± 0.20		4
PsTFL1-common	–3.052	27.932	17.78 ± 0.06	4.152 ± 0.20	4
PsTFL1-1	–2.892	28.299	19.48 ± 0.12	2.20 ± 0.23	2

Values are means ± SD. Each qPCR reaction was replicated three times.

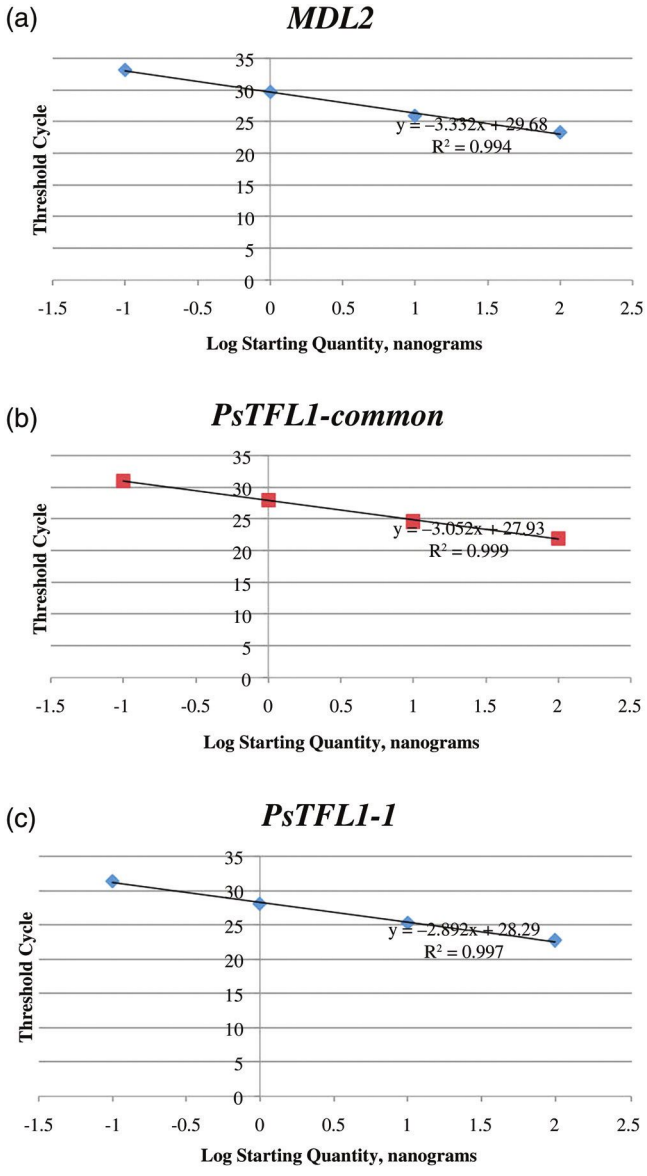

Figure 2. The qPCR relative standard curve of the reference gene, *MDL2* (a), and the target genes, *PsTFL1-common* (b) and *PsTFL1-1* (c), obtained by plotting the threshold cycle (C_T) value versus the log of each initial concentration of genomic DNA.

in the stems. Its level in vegetative buds was the second highest and was higher than that in floral buds. The lowest expression level was in the leaves which was only 33% of that in the stems (Figure 3). The protein level of *TFL1* in shoot tips is critical to the maintenance of juvenility and suppression of inflorescence development, which explained its high expression level in this tissue.

Ectopic expression of PsTFL1 in A. thaliana

The binary vector pBI121 containing *35S::PsTFL1* was constructed for the transformation of the wild-type *Arabidopsis* ecotype Col-0 (Figure 4a), and a total of 30 independent T_1 transgenic plants were obtained. To verify the integration of *PsTFL1* in the genome of these plants, PCR analysis was conducted to amplify a 519-bp fragment of *PsTFL1* cDNA from the genomic DNA of each individual (Figure 4b). Furthermore, the relative expression level of *PsTFL1* in the leaves was also investigated by RT–qPCR (Figure 4c). The results revealed that there was no correlation between the mRNA level of *PsTFL1* and flowering time, the mRNA level of *PsTFL1* and the number of rosette leaves, and the mRNA level of *PsTFL1* and the number of cauline leaves (Figure 5). For example, the individual #30 had the most severe delay in flowering. However, its level of *PsTFL1* was relatively low compared with the rest of the transgenic individuals. This was consistent with what Pillitteri et al. (2004) found in transgenic *Arabidopsis* overexpressing *CsTFL1*, a *TFL1* homolog from citrus (*C. sinensis*). In their study, no strict correlation between high *CsTFL1* mRNA levels and severity of delay in flowering was observed.

All of the 30 T_1 plants showed significantly delayed flowering and increased the number of rosette and cauline leaves under LD conditions compared with the wild-type plants (Table 2). The extension of the vegetative stage led to an increased production of rosette leaves, so that the number of rosette leaves was positively correlated with the delay of flowering. The much denser trichome distribution on the surface of the cauline leaves and abnormal inflorescence architecture of the transgenic plants were also observed (Figure 6). The transgenic plants produced an increase in the number of nodes and plant height, and the flowers of many plants were converted into shoot-like structures (Figure 6). Based on the flowering time and inflorescence morphology, T_1 plants were classified into three groups (Table 3). Class I plants had slightly delayed flowering (10.13-day delay) and produced normal flowers. However, almost no axillary inflorescences were developed, and the number of nodes and cauline leaves were much more than those of the wild type (Figure 7). Class II plants produced

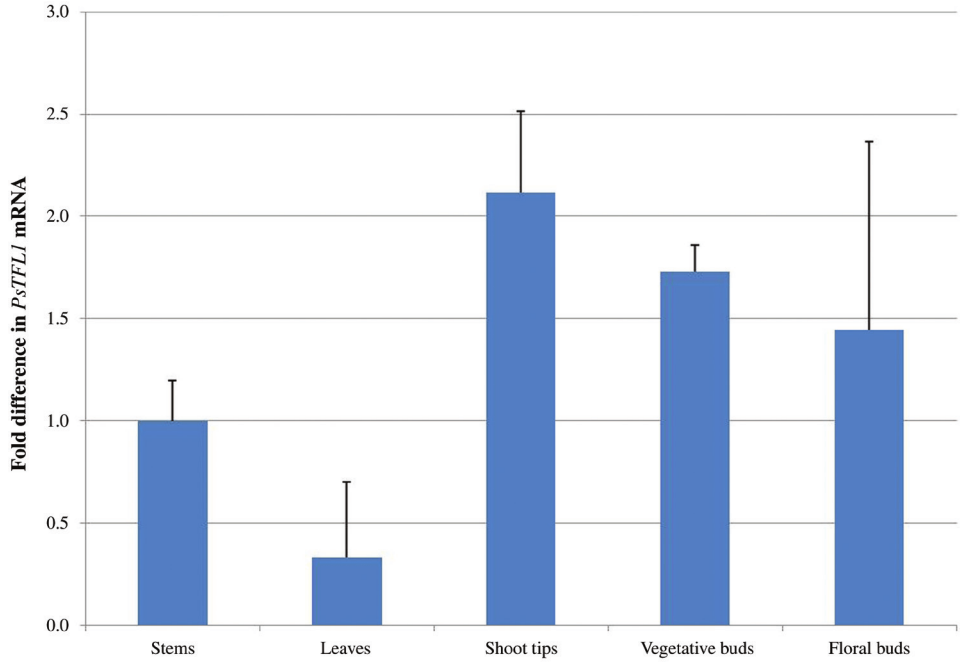

Figure 3. Expression of *PsTFL1* in various tissues of black cherry by qPCR analysis. *β-actin* in black cherry was used as a reference gene. Three replicates were assayed for each sample and error bars represent the standard deviation.

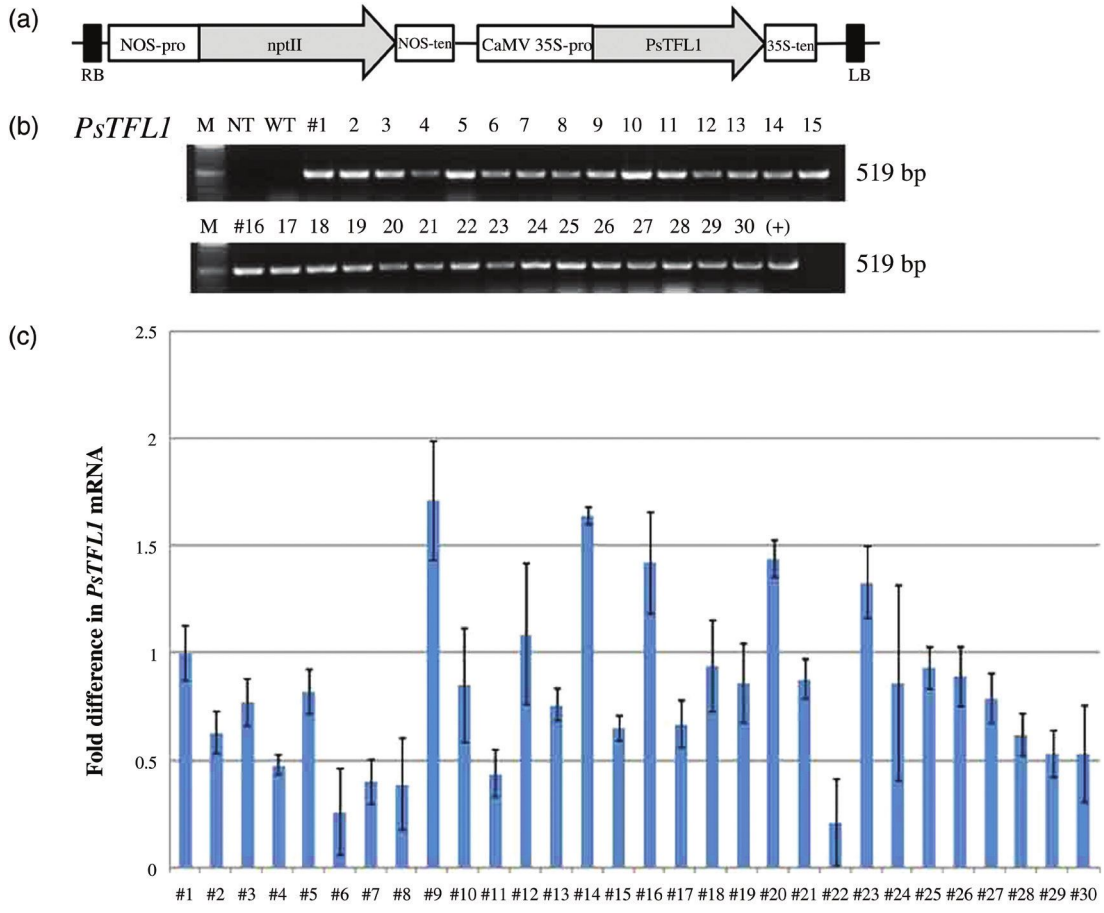

Figure 4. Analysis of transgenic *Arabidopsis* plants overexpressing *PsTFL1*. (a) Diagram of the construct pBI121 containing *35S::PsTFL1*. (b) Polymerase chain reaction analysis of the integration of *PsTFL1* in the genome of 30 transgenic plants. (c) The expression levels of *PsTFL1* in each individual plant by qPCR. *AtActin2* was the reference gene.

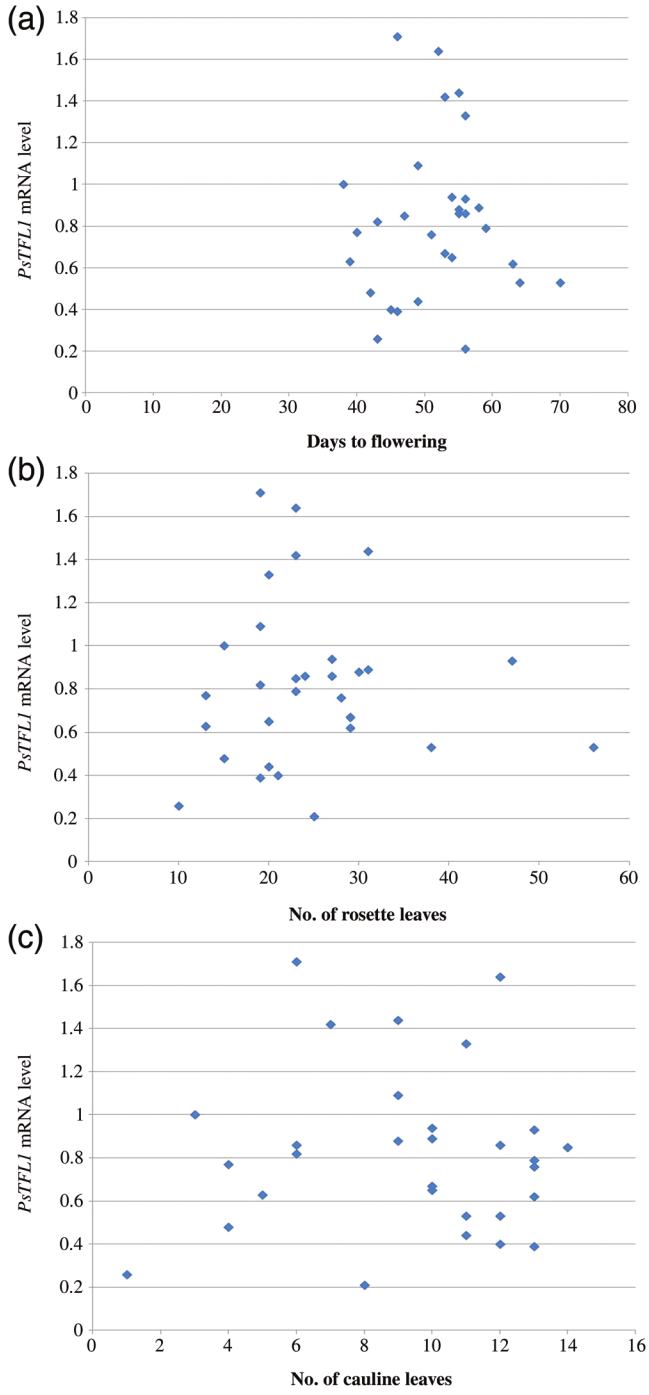

Figure 5. Regression analysis between the mRNA level of *PsTFL1* and flowering time (a), the mRNA level of *PsTFL1* and the number of rosette leaves (b), the mRNA level of *PsTFL1* and the number of cauline leaves (c) of 30 transgenic *35S::PsTFL1 Arabidopsis* lines.

1-cm inflorescence 18.43 days later than the wild type. The characteristic of single stem and increased node number was also observed in Class II plants, but the flowers formed shoot-like structures in which no petal, stamen or carpel could be found, and leaves were formed in the place of petals. All Class II plants failed to form siliques. Class III plants showed the most significant delay in flowering (25.95-day delay) and the morphological change in the inflorescence was also the most dramatic in terms of flower-to-shoot conversions (Figure 7). The single inflorescence stems were wider and the sizes of the cauline leaves were larger compared with Class I, II and the wild type. In addition, larger inflorescence meristems were produced with the sepals and petals replaced by leaves, and the stamens and carpels were missing. None of the Class III plants produced seeds. The phenotype of Class II plants seemed to be the intermediate of Class I and Class III plants. To further confirm the function of *PsTFL1* in regulating flowering time, complementation analysis was conducted in a *TFL1* mutant, *tfl1-11*. The *tfl1-11* mutant plants show early bolting with fewer rosette leaves and determinate inflorescences (Bradley et al. 1997). Nine independent *35S::PsTFL1/tfl1-11* transgenic *Arabidopsis* plants were obtained and all of them displayed a significant delay in flowering with increased number of rosette leaves compared with the *tfl1-11* plants under LD conditions (Table 2, Figure 6). On an average, these transgenic plants produced 1-cm inflorescence at 38 days with 18.2 rosette leaves, whereas the *tfl1-11* mutants flowered at 24.8 days with 8.5 rosette leaves. The results indicated that *PsTFL1* was able to complement the phenotype of *Arabidopsis tfl1-11* mutant and had a similar function as that of *Arabidopsis TFL1*.

Expression patterns of FT and TFL1 in transgenic Arabidopsis Class I, II and III plants

Since the flowering time is not simply determined by the mRNA level of *TFL1* as the results showed, the average mRNA levels of *FT* in the three classes of transgenic *Arabidopsis* plants were investigated, because *FT* is known to play a role antagonistic to *TFL1* in inflorescence development. The *FT* mRNA was the highest in Class I and the lowest in Class III, which correlated well with the delay in flowering, even though there were only slight differences among them (Figure 8a). The relative expression levels of both *PsTFL1* and endogenous *AtTFL1* were quantified as well. Surprisingly, the mRNA level of *PsTFL1* was the lowest in Class III plants that had the most significant delay in flowering (Figure 8b). But, the expression level of endogenous *AtTFL1* was the highest in Class III (Figure 8c). The results indicated that the overexpression of *PsTFL1* in *Arabidopsis* plants caused changes in the complex network of flowering through the interactions between *TFL1* and the floral meristem identity genes, so that *FT* levels were influenced and the phenotype of delay in flowering was the consequence of all signals coordinated.

Discussion

Gene structure and sequence similarity of PsTFL1 with its orthologs

Two *PsTFL1* sequences were cloned from the black cherry genome, which was consistent with the finding of two *PsTFL1*

Table 2. Flowering characteristics of transgenic *Arabidopsis* overexpressing *PsTFL1*.

Genotype	Individual plant	No. of rosette leaves	No. of cauline leaves	Days to flowering
Wild type (Col-0)	30	11.63 ± 0.45	2.93 ± 0.28	32.87 ± 0.37
Tfl1-11	10	8.50 ± 0.54	1.90 ± 0.28	24.80 ± 0.49
35S::PsTFL1/tfl1-11	9	18.22 ± 0.83	5.67 ± 0.33	38.00 ± 0.67
35S::PsTFL1	#1–30	24.57 ± 1.77	9.23 ± 0.64	51.57 ± 1.40
	#1	15	3	38
	#2	13	5	39
	#3	13	4	40
	#4	15	4	42
	#5	19	6	43
	#6	10	1	43
	#7	21	12	45
	#8	19	13	46
	#9	19	6	46
	#10	23	14	47
	#11	20	11	49
	#12	19	9	49
	#13	28	13	51
	#14	23	12	52
	#15	29	10	53
	#16	23	7	53
	#17	20	10	54
	#18	27	10	54
	#19	24	6	55
	#20	31	9	55
	#21	30	9	55
	#22	25	8	56
	#23	20	11	56
	#24	27	12	56
	#25	47	13	56
	#26	31	10	58
	#27	23	13	59
	#28	29	13	63
	#29	38	12	64
	#30	56	11	70

Values are mean ± SE for wild-type, mutant, and transgenic *A. thaliana*.

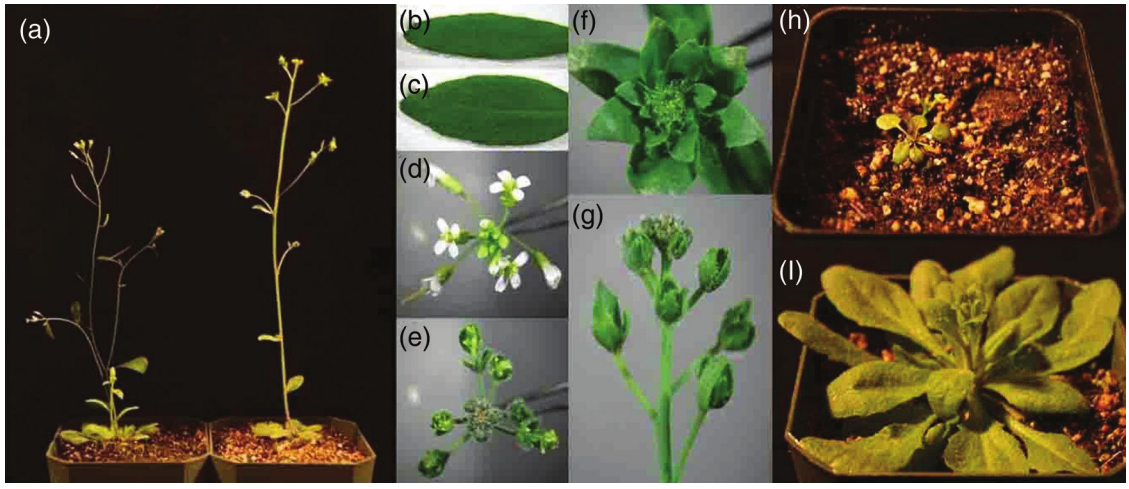

Figure 6. Phenotypes of *Arabidopsis* Col-0 wild-type (WT), the *tfl1-11* mutant and transgenic plants overexpressing *PsTFL1*. (a) Six-week-old WT (left) and *35S::PsTFL1* (right; #1). (b and c) Trichome distribution on the adaxial surface of cauline leaves of WT (b) and *35S::PsTFL1* (c). (d and e) Inflorescence architecture of WT (d) and *35S::PsTFL1* (e). (f) Floral architecture of *35S::PsTFL1* Class III plants. (g) Flower-to-shoot conversion observed at each node of Class II and Class III plants. (h) A 23-day-old *tfl1-11* mutant plant with 1-cm-long inflorescence. (i) A 37-day-old *35S::PsTFL1/tfl1-11* plant showed significantly late flowering.

Table 3. Flowering characteristics of transgenic *Arabidopsis* Class I, II, and III.

Genotype	Days to flowering	No. of leaves	No. of plants
Wild type	32.87 ± 0.37	14.57 ± 0.56	30
35S::PsTFL1 Class I	43.00 ± 1.25	22.00 ± 2.54	9
35S::PsTFL1 Class II	51.30 ± 1.13	34.10 ± 1.39	10
35S::PsTFL1 Class III	58.82 ± 1.48	43.18 ± 3.41	11

Values are mean ± SE.

Figure 7. Phenotypes of *Arabidopsis* Col-0 wild-type (WT) and transgenic plants Class I, II and III (from left to right).

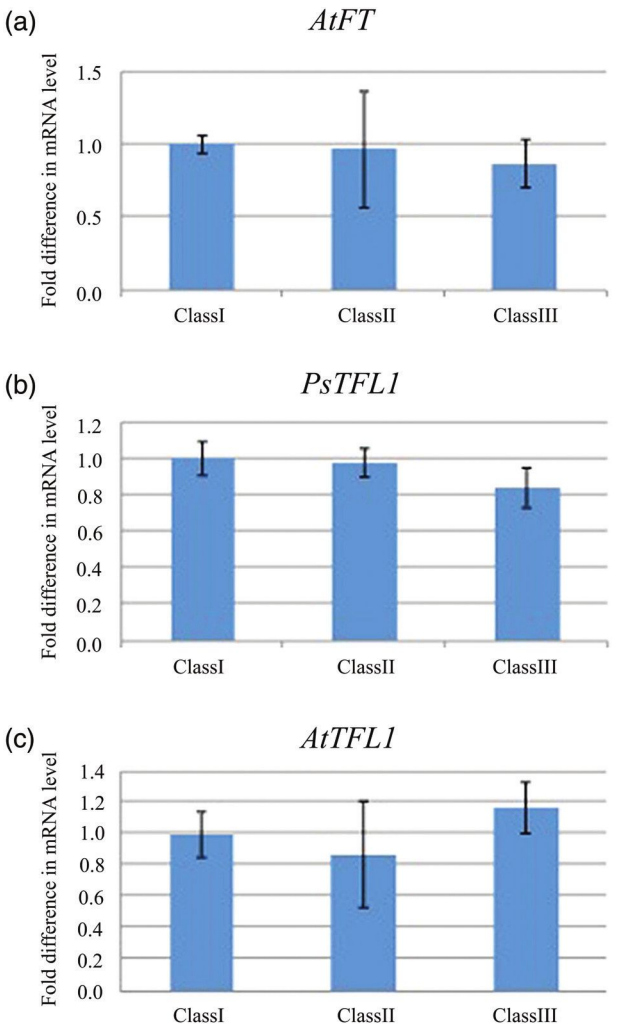

Figure 8. Relative expression levels of *AtFT* (a), *PsTFL1* (b) and *AtTFL1* (c) in transgenic *Arabidopsis* Class I, II and III plants. Expression levels were normalized to *AtActin2* in all cases. Three replicates were assayed for each sample and error bars represent the standard deviation.

cDNA sequences with slight differences in the 3′ UTR. The alignment of the genomic and cDNA sequences showed that both genomic sequences consisted of four exons and three introns. This gene structure was shared by all the *TFL1* homologs cloned from other plant species to date. In addition to the highly conserved motifs that are important to the function of *TFL1*, *PsTFL1* also contained His84. His84 was reported to be an important residue that surrounds the potential binding pocket of the protein and the function of *TFL1* could be switched to that of *FT* when this residue was changed to the corresponding amino acid of *FT* in *Arabidopsis* (Hanzawa et al. 2005). Phylogenetic analysis showed high similarity of *PsTFL1* to the homologs in *Arabidopsis* and other plant species. The qPCR analysis detected the presence of a single copy of *TFL1* in the black cherry genome as in other *Prunus* species, such as *P. avium*, *P. armeniaca*, *P. mume* and *P. persica*. In our study, the two alleles of *PsTFL1* were detected with identical coding sequence and the genomic sequences shared 93% similarity.

Functions of PsTFL1

TFL1 and *FT* are homologous genes that belong to the PEBP family and are conserved in monocots and dicots (Hanzawa et al. 2005). *TFL1* was first cloned by T-DNA tagging of an *Arabidopsis tfl1* mutant (Ohshima et al. 1997). In monocots, Nakagawa et al. (2002) analyzed the functions of rice *TFL1* homologs, *RCN1* and *RCN2*, by overexpression in both *Arabidopsis* and rice, and observed a delay of flowering and a highly branching phenotype. A *TFL1* homolog from perennial ryegrass (*Lolium perenne* L.), *LpTFL1*, was isolated and characterized by Jensen et al. (2001). It was able to cause a significant delay of flowering in *Arabidopsis* plants overexpressing it, and also complemented the phenotype of *tfl1-14* mutants. Similar results were reported in several fruit tree species as well, including citrus (*C. sinensis*) (Pillitteri et al. 2004), apple (*Malus* × *domestica*) (Kotoda and Wada 2005) and Japanese apricot (*P. mume*) (Esumi et al. 2010). In addition to the delay of flowering time, all of the previous studies found dramatic changes in plant architecture associated with mutations in *TFL1*.

In our study, *Arabidopsis* plants overexpressing *PsTFL1* also showed significantly delayed flowering in both wild-type and *tfl1-11* plants. Twenty-one T_1 plants in wild-type background even exhibited severely altered inflorescence and floral architecture that resulted in sterility. Similar results were observed in *Arabidopsis* overexpressing *FT/TFL1*-like genes from *Picea engelmannii* Parry ex Engelm. × *Picea glauca* (Moench) Voss and *Picea sitchensis* (Bong.) (Klintenäs et al. 2012). Therefore, *TFL1* was involved not only in the regulation of flowering time but also in the development of inflorescence meristems. The high expression levels of *PsTFL1* in shoot tips and vegetative buds of black cherry also supported its function in the maintenance of the vegetative stage in those tissues. However, the mRNA levels of *PsTFL1* in individual transgenic *Arabidopsis* plants were not positively correlated with flowering time. Mohamed et al. (2010) observed a positive correlation between expression level of *PopCEN1*, a *CEN-TFL1* homolog in *Populus*, and delayed spring bud flush in *35S::PopCEN1* transgenic poplar. In our study, some of the transgenic *Arabidopsis* plants had an extraordinarily high level of *PsTFL1*, but had only a slight delay in flowering, which raised a question of whether the entire *PsTFL1* mRNA was translated to protein. Therefore, an examination of *PsTFL1* protein level in those individuals is necessary to correlate *PsTFL1* with flowering time.

FT is known as one of the key transcriptional regulators on which several floral pathways converge to promote floral transition. FT is mainly expressed in the phloem tissue of leaves, and its function specifically requires *FD* activity (Abe et al. 2005). *FD* and *FT* interact in the nucleus of the shoot apex to upregulate the expression of *AP1,* which is also a target of *LFY* on *AP1* (Liljegren et al. 1999). Class III transgenic *Arabidopsis* plants had the most flowering time delay. The level of *FT* in their leaves was also lower than the plants in Class I and II. This finding agrees well with the function of *FT* in promoting flowering, and was consistent with the observation that *FT* mRNA accumulation was correlated with early flowering (Kobayashi et al. 1999). It was also concluded that *FT* activity might be more important than *TFL1* in the timing of flowering. Since *TFL1* and *FT* have opposite functions in regulating flowering time, *TFL1* expression was expected to be the highest in Class III and the lowest in Class I plants. Surprisingly, *PsTFL1* mRNA levels in three classes of plants were exactly opposite to what was expected. Interestingly, the expression of endogenous *AtTFL1* was the highest in Class III plants. As the constitutive expression of *PsTFL1* was free from transcriptional regulation, differences in the expression level of *PsTFL1* may be a result of the difference in transgene copy number or integration region (Pröls and Meyer 1992, Hobbs et al. 1993). Conti and Bradley (2007) reported that *TFL1* protein had a broader distribution than the mRNA in the meristems and moves in both inflorescence and vegetative meristems. Therefore, the pattern of *PsTFL1* protein in the shoot apex might be different from the pattern of *PsTFL1* mRNA in the leaves, which requires further investigation. Therefore, it is not the absolute level of *TFL1* mRNA, but its distribution and interaction with floral meristem identity genes that caused a delay in flowering.

In the *35S::PsTFL1 Arabidopsis* plants, the time to flower was closely associated with inflorescence and floral architecture. With few exceptions, Class I plants flowered first, followed by Class II plants and eventually Class III plants. Only Class I plants were able to develop normal floral organs and set seeds, whereas Class II and III plants showed severe, abnormal phenotypes in which flower-to-shoot conversion at every node was observed. During plant growth and development, various signaling networks coordinate and determine meristem identity and, consequently, architectural form (Conti and Bradley 2007). *TFL1* is obviously one of the key signals involved in this process, and hence its expression also strongly affects the morphology of the whole plant. The phenotype of flower-to-shoot conversion could be explained by the functions of *TFL1* in the repression of floral genes and in the maintenance of vegetative growth. Therefore, it could be concluded that the two *PsTFL1* sequences were orthologs of *Arabidopsis TFL1*.

Conclusions

In summary, a *TFL1* homolog was isolated from black cherry and its function in delaying flowering and maintaining normal inflorescence architecture was characterized in *Arabidopsis*. Further work is needed to elucidate more detailed mechanisms that regulate the phase transition and flowering in hardwood tree species. Transgenic black cherry shoots containing *35S::PsTFL1* were obtained and the expression levels of *PsTFL1* in those transgenic shoots are being evaluated. The *PsTFL1*-overexpressing shoots will then be rooted, plants acclimatized, and further investigated for floral-related phenotypic traits.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared. Mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty of the

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