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ORIGINAL ARTICLE

Functional characterization of three *TERMINAL FLOWER 1*-like genes from *Platanus acerifolia*

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Abstract

Key message ***TFL1*-like genes of the basal eudicot *Platanus acerifolia* have conserved roles in maintaining vegetative growth and inhibiting flowering, but may act through distinct regulatory mechanism.**

Three ***TERMINAL FLOWER 1*** (***TFL1***)-like genes were isolated and characterized from London plane tree (***Platanus acerifolia***). All genes have conserved genomic organization and characteristic of the phosphatidylethanolamine-binding protein (PEBP) family. Sequence alignment and phylogenetic analysis indicated that two genes belong to the ***TFL1*** clade, designated as ***PlacTFL1a*** and ***PlacTFL1b***, while another one was grouped in the ***BFT*** clade, named as ***PlacBFT***. qRT-PCR analysis showed that all three genes primarily expressed in vegetative phase, but the expression of ***PlacTFL1a*** was much higher and wider than that of ***PlacTFL1b***, with the latter only detected at relatively low expression levels in apical and lateral buds in April. ***PlacBFT*** was mainly expressed in young stems of adult trees followed by juvenile tissues. Ectopic expression of any ***TFL1***-like gene in ***Arabidopsis*** showed phenotypes of delayed or repressed flowering. Furthermore, overexpression of ***PlacTFL1a*** gene in petunia also resulted in extremely delayed flowering. In non-flowering ***35:PlacTFL1a*** transgenic petunia plants, the ***FT***-like gene (***PhFT***) gene was significantly upregulated and ***AP1*** homologues ***PFG***, ***FBP26*** and ***FBP29*** were significantly down-regulated in leaves. Yeast two-hybrid analysis indicated that only weak interactions were detected between PlacTFL1a and PlacFDL, and PlacTFL1a showed no interaction with PhFDL1/2. These results indicated that the ***TFL1***-like genes of ***Platanus*** have conserved roles in repressing flowering, but probably via a distinct regulatory mechanism.

Keywords London plane · Flowering regulation · PEBP · TFL1 · BFT

Introduction

London plane tree (*Platanus acerifolia* Willd.) is an interspecific hybrid of *P. orientalis* L. and *P. occidentalis* L., which are basal eudicot species in the family Platanaceae belonging to the order Proteales (Byng et al. 2016). It is widely planted and applied in the cities as an excellent landscape plant, praised as 'the king of street trees'. However, countless flowers and fruits with pollens and seed hairs, respectively, scatter everywhere in spring and early summer from the adult *Platanus* trees, which adversely impacts urban environment and human health and has become an increasingly serious problem in China that need to be resolved urgently (Liu et al. 2007; Lu et al. 2012). Breeding non-flowering or fruitless cultivars is desired for the species, so it is of great importance to investigate the molecular and genetic mechanisms involved in flowering regulation of *Platanus*.

It has been reported that genes of the phosphatidylethanolamine-binding protein (PEBP) family play crucial roles in controlling floral transition in plants (Wickland and Hanzawa 2015). In *Arabidopsis*, the PEBP (FT/TFL1) gene family includes six members: *FLOWERING LOCUS*

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T (*FT*), *TWIN SISTER OF FT* (*TSF*), *TERMINAL FLOWER 1* (*TFL1*), *BROTHER OF FT AND TFL1* (*BFT*), *ARABIDOPSIS CENTRORADIALIS HOMOLOGUE* (*ATC*), and *MOTHER OF FT AND TFL1* (*MFT*) (Kobayashi et al. 1999; Jin et al. 2020). *FT* and *TFL1* encode proteins that share highly conserved amino acid residues (~60% identity), but they have antagonistic functions in flowering regulation: *FT* is a florigen that induces flowering, while *TFL1* represses flowering (Hanzawa et al. 2005; Ahn et al. 2006; Corbesier et al. 2007). *TSF* is the closest *FT* homolog and participates in flowering induction (Yamaguchi et al. 2005; Lee et al. 2019). *BFT* and *ATC* are *TFL1*-like genes and function as repressors of flowering (Yoo et al. 2010; Huang et al. 2012). *MFT* is regarded as ancestral to *FT* and *TFL1* genes, as its orthologs exist in both basal land plants (like mosses and lycophytes) and seed plants (gymnosperms and angiosperms) (Hedman et al. 2009; Karlgren et al. 2011). In addition to promoting flowering, *MFT* also plays an important role in regulating seed germination (Yoo et al. 2004; Xi et al. 2010).

To date, *TFL1*-like genes have been identified and characterized in a wide variety of plant species including gymnosperms, monocots, and core eudicots (Wickland and Hanzawa 2015; Liu et al. 2016b). In *Arabidopsis*, *TFL1* is expressed in vegetative and inflorescence meristems to maintain their vegetative character and indeterminate state, and so to control flowering time and inflorescence architecture, respectively (Bradley et al. 1997). Loss of function of *TFL1* causes early flowering and determinate inflorescence by formation of a terminal flower, whereas its overexpression dramatically extends both the vegetative and reproductive phases (Ratcliffe et al. 1998). It was proposed that *TFL1* regulates inflorescence indeterminacy by repressing the flower meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*) in the center of the meristem, while *LFY* and *AP1* repress the transcription of *TFL1* in lateral floral primordia (Parcy et al. 2002). However, recent findings indicated that LFY is actually an activator of *TFL1* and only indirectly represses *TFL1* through *AP1* (Goslin et al. 2017; Serrano-Mislata et al. 2017). The function of flowering repression has been shown to be conserved for *TFL1*-like genes in lots of plant species (Wickland and Hanzawa 2015); many *TFL1* homologs also have conserved function in controlling inflorescence architecture, such as *CENTRORADIALIS* (*CEN*) in snapdragon (Bradley et al. 1996), *RCN1* and *RCN2* in rice (Nakagawa et al. 2002), *VvTFL1A* in grape (Fernandez et al. 2010), *GhSP* in cotton (McGarry et al. 2016; Si et al. 2018), and *TFL1*-like members in a number of legume crops (Foucher et al. 2003; Tian et al. 2010; Repinski et al. 2012; Liu et al. 2016a; Cheng et al. 2018). In addition, more diverse functions, including those involved in shoot branching and life history strategy, were characterized in *TFL1*-like genes (Perilleux et al. 2019). For instance, *SELF-PRUNIN* (*SP*), the *CEN* ortholog of tomato, regulates vegetative to reproductive switching of sympodial meristems (Pnueli et al. 1998); *StTFL1* is involved in tuberization regulation in potato (Guo et al. 2010); *AaTFL1* of *Arabis alpina* plays an important role in polycarpic development (Wang et al. 2011a, b); *HvCEN* promotes axillary bud initiation and tillering of barley (Bi et al. 2019a); *CsCEN* gene maintains the proliferative capacity of axillary meristems by antagonizing the thorn-specifying *THORN IDENTITY1* (*TI1*) gene in *Citrus* (Zhang et al. 2021); *TFL1* homolog *KSN* heterozygosity is associated with continuous flowering of *Rosa rugosa* Purple branch (Bai et al. 2021); and *DOTFL1* may controlled pseudobulb formation in the Orchidaceae family (Li and Zhang 2021). Although the PEBP family is extensively investigated, the functions of *TFL1*-like genes are yet to be explored in basal eudicot species, like London plane tree.

So far, there is very limited information available about the molecular mechanisms controlling flowering in basal eudicots and perennial woody species. In this study, we isolated and characterized three *TFL1*-like genes from London plane tree, aiming to improve our understanding of flowering regulation in *Platanus* and to provide support for its genetic improvement. The gene structures, phylogenetic relationship, spatial and temporal expression patterns, and protein interaction of the three genes were investigated, and their biological functions were further characterized by overexpressing them in *Arabidopsis* and petunia. The results provide valuable information for understanding the evolution of *TFL1*-like genes in basal eudicots and for creating non-flowering and fruitless varieties of *Platanus*.

Materials and methods

Plant materials and sample collection

Various samples were collected from two-year-old juvenile and/or over thirty-year-old adult London plane trees grown at the campus of Huazhong Agricultural University (Wuhan, China). Juvenile trees were sampled at June, including roots (JR), stems (JS), newly growing young leaves (JYL), fully expanded mature leaves (JML) and subpetiolar buds (JSB). As described in Fig. S1, the flower development of *Platanus* spans two growing seasons. During the first seasons, lateral buds are formed under the petiole base (namely subpetiolar buds) on developing shoots (April–May), followed by two developmental fates. Most subpetiolar buds of adult trees (frequently located at the middle and upper part of the shoots) differentiate inflorescence and secondary shoot meristems individually in the same bud, hereinafter referred to as mixed flower buds. While, some subpetiolar buds (frequently located at the bottom part of the shoots or lower shoots of the tree) can only differentiate shoot meristems without inflorescence meristems, hereinafter referred to as vegetative subpetiolar buds. To uncover the comprehensive gene expression patterns during the whole flower and fruit development process, samples from adult trees were collected along two consecutive growing seasons (from April to April of next year), including the stems (S), newly growing young leaves (YL), fully expanded mature leaves (ML), shoot apical buds (AB), lateral subpetiolar buds (SB), vegetative subpetiolar buds (VB), mixed flower buds (MB), vegetative tissues in mixed flower buds (MB-V), inflorescences in mixed flower buds (MB-F), male inflorescences (MF), female inflorescences (FF) and fruits (F) (The corresponding descriptions of the samples are also listed in Table S1). All samples were collected from three individual trees, respectively, and immediately frozen in liquid nitrogen and stored at – 80 ℃ until they were used for RNA extraction.

Isolation of *Platanus TFL1*-like genes

Modified CTAB method was used to extract the total RNA of London plane tissues according to the procedures described by Li et al. (2008). Two µg of total RNA was used for first-strand cDNA synthesis using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) following the protocol from the manufacturer and oligo (dT) primers. Degenerate primers PlacTFL1-dF and PlacTFL1-dR were used to amplify partial coding sequences of the *PlacTFL1a* and *PlacTFL1b* and designed according to nucleotide alignments of the *TFL1*-like genes from *Vitis vinifera*, *Populus nigra*, *Malus × domestica*, *Citrus sinensis*, and *Eriobotrya japonica*. The PCR was performed by denaturing cDNA at 95 ℃ for 3 min, followed by 35 cycles of 95 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. The partial gDNA sequences were also obtained using the same pair of primers. 5′ Tail-PCR and 3′ RACE were performed to amplify the 5′ and 3′ terminal regions of *PlacTFL1a*, respectively; 5′/3′ Tail-PCR was carried out to obtain 5′/3′ terminal sequences of *PlacTFL1b*. Primers for amplifying the partial sequence of *PlacBFT* gene were designed according to the transcriptome sequencing data of London plane (unpublished), and 3′ RACE was used to amplify its 3′ terminal region. The RACE conditions were 95 ℃ for 3 min, followed by 36 cycles of 95 ℃ for 30 s, 60 ℃ for 30 s, 72 ℃ for 1 min, and a final extension at 72 ℃ for 10 min. TAIL-PCR was performed using a modified method (Wang et al. 2011a, b). All primers were designed using Primer 5 software and are listed in Table S2. The full-length cDNA and gDNA sequences of the three genes were amplified and cloned into a pMD18-T vector (Takara), and 3–5 positive clones were randomly selected for sequencing.

Sequence alignment and phylogenetic analysis of *Platanus TFL1*-like genes

Amino acid sequences of representative FT/TFL1-like proteins from *P. acerifolia*, *Arabidopsis thaliana*, *Vitis vinifera* and *Nelumo nucifera* were aligned using Vector NTI version 11.5 (Invitrogen). Sequences of FT/TFL1 family genes for representative species (Table S3) deposited in the National Center for Biotechnology Information (NCBI) database were retrieved for the construction of a phylogenetic tree. The alignment of amino acid sequences was made with the default settings in MUSCLE implemented in MEGA version 6.0 (Kumar et al. 2004). Phylogenetic tree was constructed using MEGA v6.0 by the Neighbor-Joining (NJ) method with 1000 bootstrap replicates.

Expression analysis of *Platanus TFL1*-like genes

Semi-quantitative RT-PCR and quantitative real-time PCR (qRT-PCR) analyses were performed to detect the expression of the *Platanus TFL1*-like genes. Primers were designed within the non-conservative coding region and 3′ UTR (untranslated region) using Primer 5.0 software to amplify the products between 90 and 300 bp in size (Table S2). qRT-PCR was performed in a total volume of 10 µl containing 5 µl 2 × SYBR Green Master Mix, 0.2 µl of each forward and reverse primer (10 µmol/µl), 1 µl of the RT reaction mixture as template and water to a final volume. Reactions were carried out on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA). PCR efficiency for each primer pair was determined by a standard curve generated with serially diluted cDNA. The results from instrument onboard software Sequence Detector Version1.3.1 (PE Applied Biosystems) were further subjected to a custom-designed Microsoft Excel macro for analysis. Relative expression levels were calculated by Multiple Condition Solver REST-MCS v2 with *TPI* (*triose phosphate isomerase*) gene of *P. acerifolia* as a normalization (Lu et al. 2012). Calculations were based on three biological replicates and three technical replicates and data were shown as mean values ± SE (standard error).

Vector construction

To produce transgenic plants overexpressing the *Platanus TFL1*-like genes, we constructed *35S:PlacTFL1a*, *35S:PlacTFL1b* and *35S:PlacBFT* on the expression vector pCAMBIA2300. The full-length coding regions of *PlacTFL1a*/*b* or *PlacBFT* genes were subcloned by *Sal*I and *Kpn*I or *Sal*I and *BamH*I and ligation into pCAMBIA2300, which has cauliflower mosaic virus *35S* promoter (*CaMV35S*) and *NOS* terminator. The amplified sequences were confirmed by restriction digestions and

DNA sequencing. All generated constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 or AGL0 by the electroporation method.

Plant transformation and phenotype analysis

Genetic transformation of *35S:PlacTFL1a/b* and *35S:PlacBFT* genes into *A. thaliana* was performed using the floral dip method (Clough and Bent 1998). The seeds from infected plants were cultured on agar-solidified Murashige and Skoog (MS) medium with 50 μg ml^{-1} kanamycin and 50 μg ml^{-1} cefotaxime. Kanamycin-resistant seedlings were cultured in a growth incubator with a photoperiod of 16/8 h (light/dark) at 22 ± 1 ℃ Phenotypic alternations of transgenic lines including the numbers of rosette and cauline leaves, bolting time, and anthesis time were recorded in T_1 generation. For each gene constructions, homozygous transgenic lines defined by screening the kanamycin resistance of T_3 generation were selected for further study, except for the lines with severe phenotype that did not produce flowers and progeny. Sixteen plants for each homozygous line were used to record phenotypic changes.

Petunia hybrida 'W115' was transformed with *35S:PlacTFL1a* via the leaf disc method. First, 0.5% hypochlorite was used to sterilize the leaf explants for 20 min. Cut the leaves into approximately 0.5 × 0.5 cm squares and infected by *Agrobacterium tumefaciens* AGL0 containing *35S:PlacTFL1a* expression vector for 10–15 min. Co-cultivate the explants in MS plates (MS medium supplemented with 2.0 mg/ml 6-BAP, 0.1 mg/ml NAA, 1.0 mg/ml zeatin, and 1.0 mg/ml folic acid) without antibiotics for 2–3 days at 25 ℃ under dark condition, and then transfer them into selective medium plates containing 250 μg ml^{-1} carbenicillin and 250 μg ml^{-1} kanamycin. After the shoots appeared, excise and culture them on hormone-free MS medium supplemented with 1.0 mg/ml folic acid, 250 μg ml^{-1} carbenicillin and 50 μg ml^{-1} kanamycin until rooting. Transgenic plants were identified by PCR with a primer in the *35S* promoter (*35SF*) and a *PlacTFL1a*-specific primer (*PlacTFL1a-vR*) (Table S2).

Expression analysis of exogenous and endogenous genes in transgenic plants

To understand the functional conservation of *Platanus* TFL1-like proteins and whether key regulatory genes in *Arabidopsis* were affected by the transgene of *PlacTFL1*-like genes, we performed qRT-PCR experiment to investigate the expression levels of *AtAP1*, *AtFUL*, *AtLFY* and *AtSOC1*. Seedlings 21 d after sowing were sampled to isolate total RNA in wild type and transgenic *Arabidopsis*. Expression of petunia *FT*-like gene (*PhFT*; GenBank accession no. GU939627) and *AP1* homologues (*PFG*, *FBP26*, and *FBP29*) was investigated using qRT-PCR in apical buds and leaves of *35S:PlacTFL1a* transgenic petunia plants to understand the underlying mechanism of the repressed flowering phenotypes.

RNA extraction of wild-type and transgenic plants were conducted by the Trizol reagent (Takara, Japan) and reverse transcription were carried out using the method described above. *AtEF1α* and *PhEF1α* were used as the endogenous reference genes to normalize the data (Mallona et al. 2010). Primers are listed in Table S2.

Yeast two-hybrid assays

The full-length coding sequences of London plane *TFL1*-like and *FD*-like (*PlacFDL*, GenBank accession no. MH845055.1) genes, as well as petunia *FD*-like genes (*PhFDL1* and *PhFDL2*, Appendix S1), were cloned and introduced to the bait plasmid pGBKT7 and prey plasmid pGADT7, respectively. All constructions were confirmed by sequencing. Yeast cells were transformed using the Frozen-EZ Yeast Transformation II Kit (Zymo Research Corp, USA). All baits were tested for autoactivation capacity prior to the screening for potential protein–protein interactions, and none of them showed autoactivation. Co-transformed yeast cells were selected on SD plates lacking Leu and Trp. Interactions were determined by spotting assay on selective SD media lacking Leu, Trp, His and Ade, supplemented with X-α-Gal.

Results

Isolation and phylogenetic analysis of *Platanus TFL1*-like genes

Using 3' RACE combined with Tail-PCR methods, three *TFL1*-like genes were isolated from London plane tree. Sequence alignment and phylogenetic analysis indicated that two genes belong to the *TFL1* clade, designated as *PlacTFL1a* and *PlacTFL1b* (GenBank accession no. MG344736 and MG344737), while the other one belongs to the *BFT* clade, designated as *PlacBFT* (GenBank accession no. MG344738) (Figs. 1, 2). Comparison of their cDNA and genomic sequences revealed that *PlacTFL1a/b* and *PlacBFT* genes have identical gene structure (including four exons and three introns) and ORF (open reading frame) length (519 bp encoding 172 aa), and that the four exons of the three genes demonstrated consistent length of 198 bp, 62 bp, 41 bp and 218 bp, respectively (Fig. 1a). However, the length of introns in *PlacTFL1a*, *PlacTFL1b* and *PlacBFT* are different, and their gDNA are 1,632 bp, 1,355 bp, and 1,324 bp in length, respectively. *PlacTFL1a* and *PlacTFL1b* genes share high similarity, with the identity of 91.7% and 90.1%

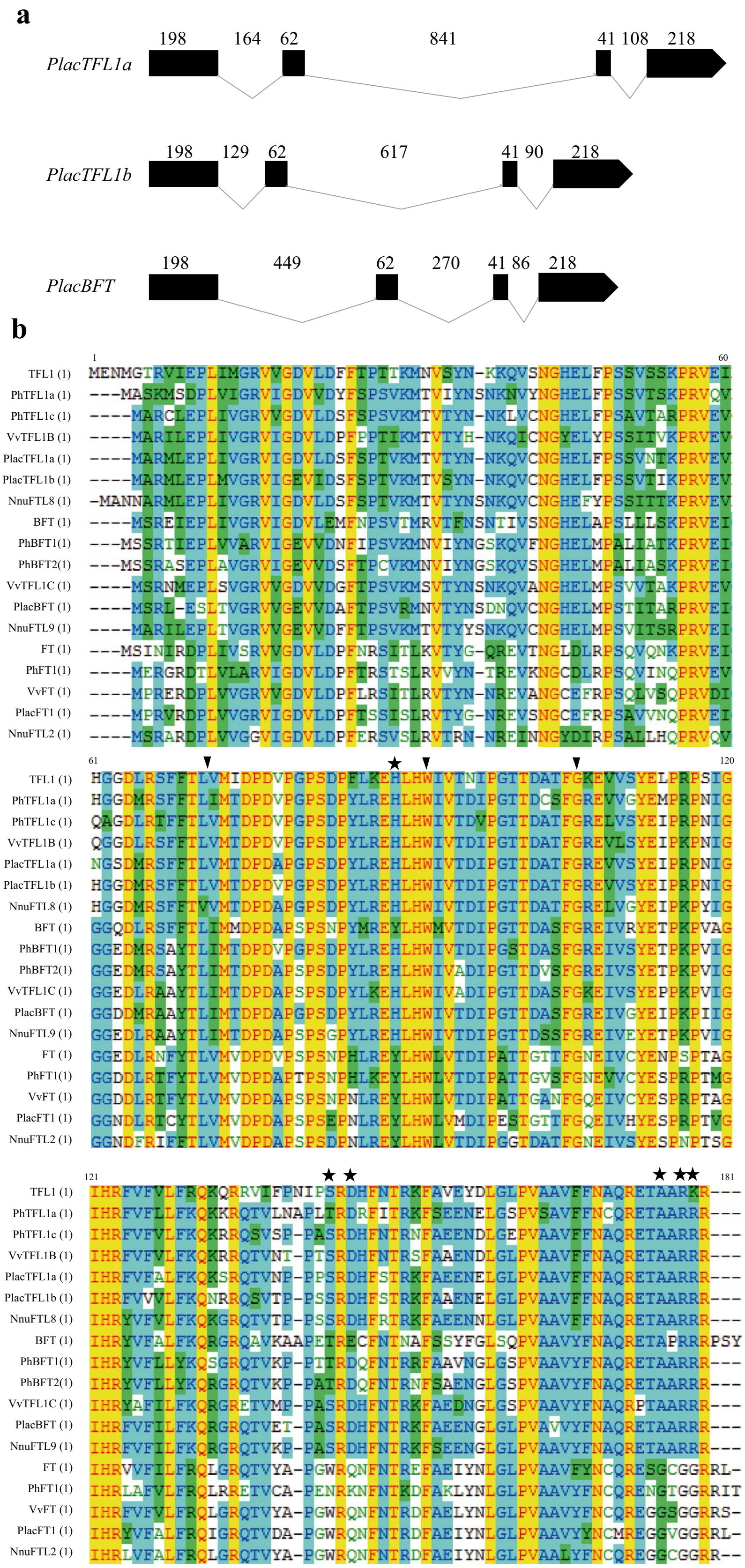


Fig. 1 Gene structure and sequence alignment of FT/TFL1 family genes. **a** Comparison of structure of the three *TFL1*-like gene sequences from London plane. Numbers indicate the base pairs in the exons (black boxes) and introns (thin lines). **b** Alignment of the deduced amino acid sequences of the products of FT/TFL1 family in *Platanus acerifolia*, *Arabidopsis thaliana*, *Petunia*, *Vitis vinifera* and *Nelumo nucifera*. Intron positions are indicated by black arrowheads. Asterisks indicate amino acids that are critical to the definition of proteins in the FT/TFL1 family

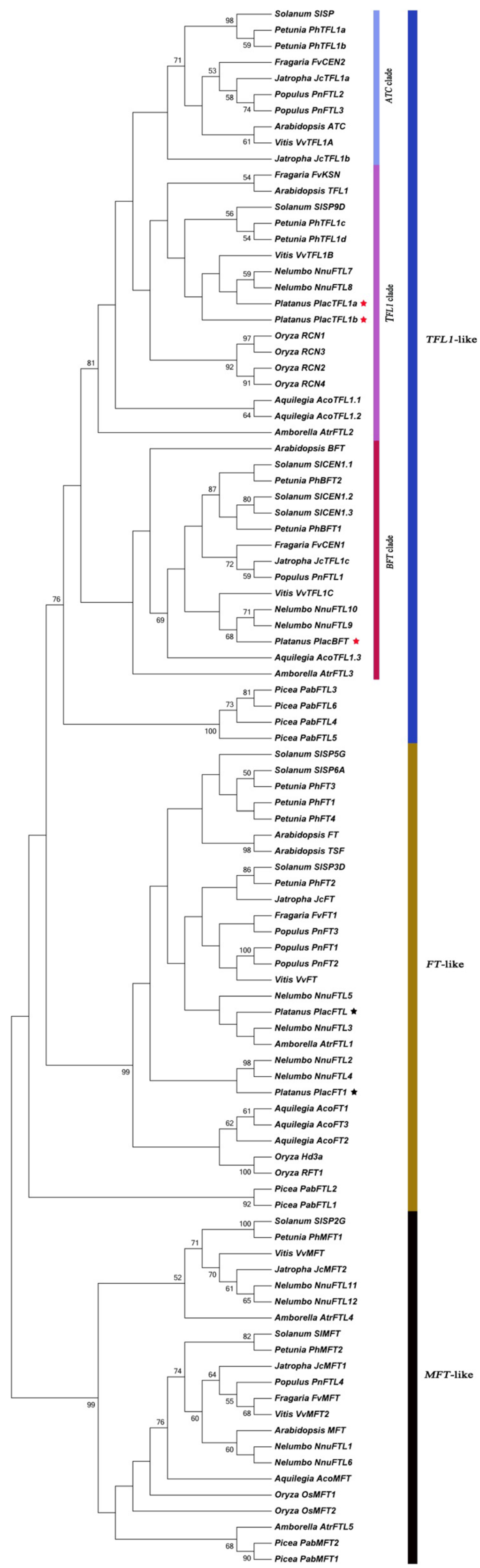


Fig. 2 Phylogenetic analysis of FT/TFL1 gene family. The tree was ▶ generated with MEGA v6.0 software, using the Neighbor-Joining (NJ) method and 1000 bootstrap replicates. Bootstrap values above 50% are indicated, and *Platanus FT*/*TFL1*-like genes are marked with stars

at nucleotide and amino acid levels, respectively, whereas *PlacBFT* was less closely related to *PlacTFL1a*/*b*, showing only 72.4% and 71.3% identity of nucleotide and 75.1% and 73.4% identity of amino acid with *PlacTFL1a* and *PlacTFL1b*, respectively (Fig. 1).

Multiple sequence alignment of the deduced amino acid sequences of the FT/TFL1-like proteins in *P. acerifolia* and several other species showed that PlacTFL1a/b and PlacBFT proteins had characteristic features of the TFL1-like proteins. The conserved key amino acid residues His88 and Asp144 of TFL1 (Ahn et al. 2006) were found at corresponding positions (His84 and Asp140) of PlacTFL1a/b and PlacBFT proteins, in addition, the amino acids Ser142, Ala173, Arg175, and Arg176 that were shown to be differently selected in TFL1-like proteins after the duplication resulting in FT and TFL1 clades (Wang et al. 2015) were also conserved in PlacTFL1a/b and PlacBFT (Fig. 1b). Phylogenetic analysis using the amino acid sequences of FT/TFL1 family proteins from London plane and other 12 representative species showed that the gene family consists of TFL1-like, FT-like, and MFT-like clades. TFL1-like clade is further divided into ATC, TFL1 and BFT subclades, and ATC subclade only contains genes from core eudicots (Fig. 2). PlacTFL1a/b and PlacBFT showed the closest relationship with their corresponding orthologs of *Nelumbo nucifera*, NnuFTL7/8 and NnuFTL9/10, respectively.

TFL1-like genes display distinct expression patterns in London plane

To get insight into the potential roles of the three *TFL1*-like genes in *Platanus*, we investigate their spatiotemporal expression patterns in juvenile (two-year-old) and adult (30-year-old) London plane trees. RT-PCR was performed to pretest the expression patterns of the three genes in various tissues and ontogenetic stages. The results showed that all three *Platanus TFL1*-like genes were primarily expressed in vegetative tissues, and no expression of them was detected in different development stages of inflorescences and fruits (Fig. S2).

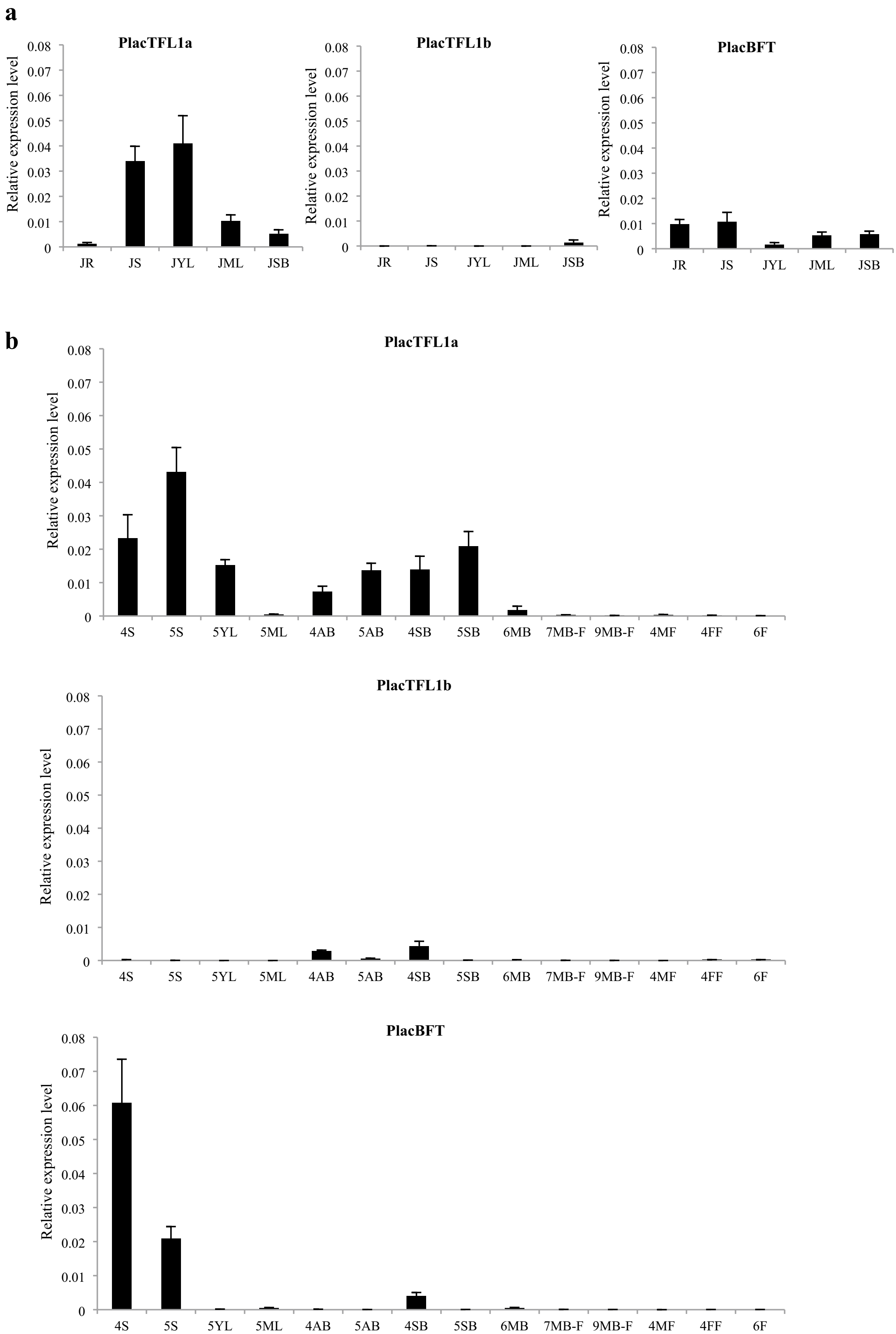
The expression levels were further detected in vegetative tissues and several development stages of inflorescences and fruits from juvenile and/or adult trees by qRT-PCR, which showed that *PlacTFL1a* was expressed in all sampled tissues of juvenile plants, with high expression in young leaves and stems, low expression in mature leaves and SBs, and weak expression in roots (Fig. 3a). In adult trees, *PlacTFL1a* was expressed mainly in stems, young leaves, shoot apical buds, VBs, SBs before inflorescence initiation (April and May), and vegetative tissues in MBs, but was rarely detected in mature leaves, inflorescences, and fruits (Fig. 3b). The expression levels of *PlacTFL1a* in stems, shoot apical buds, and SBs increased at May compared to April, but its expression in SBs suddenly dropped at June when the inflorescences began to differentiate. In the VBs that did not form inflorescences, *PlacTFL1a* maintains high expression levels at July, and declines during later months, till very weak or no expression being detected during dormant period (Dec to Jan); after the dormancy release, expression of PlacTFL1a resumed gradually again at Feb to March (Fig. 4). In the vegetative tissues of MBs, *PlacTFL1a* displayed the same expression pattern as in the VBs (Fig. 4). Compared to *PlacTFL1a*, *PlacTFL1b* showed much lower levels as well as narrower regions of expression. For instance, it was only weakly expressed in SBs and not detected in roots, stems, and young or mature leaves of juvenile trees (Fig. 3a); in adult trees, *PlacTFL1b* was mainly expressed in apical and subpetiolar buds at April (Fig. 3b), with less transcripts in VBs and vegetative tissues of MBs during the growing period (Fig. 4). *PlacBFT* was also expressed in all detected tissues of juvenile plants, but predominantly in stems and roots followed by SBs and mature leaves (Fig. 3a). In adult trees, *PlacBFT* was predominantly expressed in stems (April and May), and lower expression was detected in SBs at April and VBs at March, with almost no expression in other tissues (Figs. 3b; 4).

Ectopic expression of *Platanus TFL1*-like genes in *Arabidopsis* inhibits flowering

To figure out the potential functions of *Platanus TFL1*-like genes in flowering regulation, *CaMV35S* was used to ectopically express *PlacTFL1a/b* and *PlacBFT* genes in *Arabidopsis*. Thirty-eight, fifty-one and thirty-three independent transgenic lines were achieved for *35S:PlacTFL1a*, *35S:PlacTFL1b* and *35S:PlacBFT*, respectively. Overexpression of the three *Platanus TFL1*-like genes in *Arabidopsis* resulted in similar phenotypic alterations in delaying or repressing flowering (Fig. 5a–d, k; Table S4). Among the transgenic lines, five from *35S:PlacTFL1a*, eight from *35S:PlacTFL1b*, and three from *35S:PlacBFT* displayed the strongest phenotypes, which produced an average of 50–54 rosette leaves before bolting under long day conditions (16/8 h, day/night), with the maximum number of rosette leaves up to 75 (Fig. 5e). After bolting, the inflorescence meristems of these plants maintained the status to form secondary and tertiary inflorescences reiteratively rather than to produce flowers until death (Fig. 5f), so we cannot obtain seeds and progenies from these strong phenotypic lines. For the transgenic lines with moderate or weak phenotypes, homozygous plants were chosen for further observation. We found most transgenic lines with moderate or weak phenotypes enhanced late flowering in their homozygous generation. For instance, homozygous lines #20 and #24 of *35S:PlacTFL1a* and #19 of *35S:PlacTFL1b* bolted with twenty to forty rosette leaves, but they always maintained at the inflorescence-producing status without flowers (Fig. 5g, k; Table S4). Transgenic lines with relatively weak phenotypes ultimately converted to flowering when inflorescences developed to a certain degree (Fig. 5h). In addition, overexpression of the three *PlacTFL1*-like genes in *Arabidopsis* also showed higher plant stature, larger leaves, thicker inflorescence stems, and more cauline leaves compared with the wild-type plants (Fig. 5d–f, i, k; Table S4). qRT-PCR analysis of transgenes in wild-type *Arabidopsis* (Col-0) and transgenic lines indicated that the phenotypic variations of the three *PlacTFL1*-like transgenic plants are related in a certain extent to the expression levels of the transgenes, namely higher expression levels tend to result in more severe phenotypic changes such as later bolting and flowering time or more rosette leaves (Fig. 5j, k; Table S4). Expression analysis of the flowering time related genes in *35S:PlacTFL1*-like transgenic plants showed that *AP1*, *FUL*, *LFY* and *SOC1* were intensively down-regulated in 21-day-old seedlings of *35S:PlacTFL1a* line 20 and *35S:PlacTFL1b* line 19, especially *AP1* and *FUL* genes showed the strongest downregulating (Fig. 6). It is worth noting that only *AP1* and *FUL* genes were significantly declined in *35S:PlacBFT* line 26 (Fig. 6).

Overexpression of *PlacTFL1a* in petunia represses flowering and promotes branching

Due to the similar and strong phenotypes in *Arabidopsis* overexpressing *PlacTFL1a/b* and *PlacBFT*, we further investigated the function of *PlacTFL1a* by ectopic expression in petunia. After verified by PCR amplification of the transgene, twenty-five independent T_0 transgenic plants were obtained, but only three of them (#3, #6, and #23) showed evident expression via RT-PCR detection (Fig. 7a). Phenotypic observation and statistics analysis indicated that #6 and #23 lines showed obviously late flowering with more leaves and branches (Fig. 7b, c). In particular, the transgenic individual #6 maintained vegetative growth for more than one year and did not flower after several generations of cutting propagation, whereas wild-type W115 plants usually blossom within two months after planted (Fig. 7c). Finally, no seeds and sexual progenies can be obtained from #6 that was so excluded from the statistical analysis (Fig. 7b). Transgenic line #23 showed moderate phenotype of delayed flowering. It is worth mentioning that the main shoots of #23 usually return to vegetative growth after forming the first flower (Fig. 7c, d). RT-PCR analysis revealed that plants


a
PlacTFL1a
Relative expression level
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
JR
JS
JYL
JML
JSB
PlacTFL1b
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PlacTFL1b
PlacBFT

◂**Fig. 3** Expression profiling of *TFL1*-like genes in London plane. **a** Relative expression of *TFL1*-like genes in juvenile plants. **b** Relative expression of *TFL1*-like genes in adult trees. *JR* roots of juvenile, *JS* stems of juvenile, *JYL* young leaves of juvenile, *JML* mature leaves of juvenile, *JSB* subpetiolar buds of juvenile, *S* stems, *YL* young leaves, *ML* mature leaves, *AB* shoot apical buds, *SB* subpetiolar buds, *MB* mixed flower buds, *MB-F* inflorescences in mixed flower buds, *MF* male inflorescences, *MF-P* fleshy peduncles of male inflorescences, *FF* female inflorescences. The numbers indicate the sampling month of the tissues. The level of expression was normalized to London plane *TPI* gene. Error bars represent SE for three replicates

showing severe phenotype had higher expressed levels of the transgene compared with the plants exhibiting moderate or weak phenotype (Fig. 7), indicating that the transgene is responsible for the phenotypic alterations.

To study the underlying mechanism of repressed flowering in *PlacTFL1a*-overexpressing plants, expression of petunia *FT*-like gene (*PhFT*) and *AP1* homologues (*PFG*, *FBP26*, and *FBP29* genes) were investigated in apical buds and leaves of the transgenic line #6 and wild-type 'W115'. The results indicated that *PhFT* gene was up-regulated in apical buds of *35S:PlacTFL1a* transgenic plant, whereas *PFG*, *FBP26* and *FBP29* genes were down-regulated, especially *FBP26* was significantly repressed in both apical buds and leaves (Fig. 8).

Interactions between London plane TFL1-like and FD-like proteins

A yeast two-hybrid analysis was performed to evaluate whether the TFL1-like proteins of *Platanus* can interact like the most situation in other species with its FD-like proteins. The coding sequences of *PlacTFL1a*/*b*, *PlacBFT*, *PlacFDL* and *PhFDL1*/*2* genes were cloned into the pGADT7 (prey) and pGBKT7 (bait) domains, respectively, and used for interactional analysis. The results indicated that only PlacTFL1a had a weak interaction with PlacFDL in *P. acerifolia* (Fig. 9). Based on the ability of overexpressing *PlacTFL1a* to repress flowering in petunia, the interactions between *P. acerifolia* TFL1-like proteins and the FD-like proteins of petunia were also investigated. As a result, no interaction was detected between PlacTFL1a and petunia FD-like proteins (Fig. 9). However, the interaction of PlacBFT and PhFDL1 was detected in one direction (Fig. 9).

Discussion

Phylogenetic evolution of the *FT/TFL1*-like genes in basal eudicots

Previous phylogenetic analyses indicated that the PEBP gene family undergone two ancient duplications that generated three clades: *MFT*-like, *FT*-like and *TFL1*-like (Karlgren et al. 2011; Liu et al. 2016b). The first duplication giving rise to the *MFT*-like and *FT/TFL1*-like clades was suggested to take place in the common ancestor of seed plants or even earlier; the second duplication happened before the divergence of seed plants, resulting in the *FT*-like and *TFL1*-like clades (Liu et al. 2016b). After the separation of gymnosperms and angiosperms, further duplications occurred in each group. In angiosperms, an early whole-genome duplication (WGD) event has produced duplicate genes in each clade, and so most angiosperms contain approximately a half-dozen PEBP genes (Table S3). According to the evolutionary history of FT/TFL1 gene family, basal eudicots like *P. acerifolia* should possess *MFT*-like, *FT*-like and *TFL1*-like homologues. Previously, our research group have identified two *Platanus FT*-like genes *PaFT* and *PaFTL* that exhibited the function of promoting flowering in transgenic *Arabidopsis* or tobacco plants (Zhang et al. 2011; Cai et al. 2019). And we isolated here another three genes of the family, which belong to *TFL1*-like clade (Figs. 1, 2).

In accordance with previous studies, our phylogenetic tree divided the PEBP proteins from 13 species—into MFT-like, FT-like, and TFL1-like groups. MFT-like clade was further separated into two subgroups, and two or more genes are present in most species except *Aquilegia coerulea*, *Arabidopsis*, *Fragaria vesca* and *Populus nigra* that may have lost one copy (Fig. 2). In *Platanus*, two *MFT*-like genes were speculated, which was supported by the transcriptome data wherein two distinct *MFT*-like transcripts were found (data not shown). TFL1-like clade was also grouped into two classes, BFT-like and TFL1-like, both of which consist of genes from all included basal angiosperm, basal eudicots, and core eudicots (Fig. 2), indicating that the duplication generating these two lineages should occur in the common ancestor of angiosperms. The members from rice are present only in TFL1-like other than BFT-like lineage, suggesting that monocots may have lost the *BFT*-like genes. Furthermore, the phylogenetic tree shows that the genes from core eudicots in the *TFL1*-like lineage form two subgroups, *ATC* -like and *TFL1*-like (Fig. 2), indicating that *TFL1*-like clade experienced another duplication during the evolution of core eudicots. Whereas, some species like *Populus* and *Jatropha* have lost the *TFL1*-like genes.

In basal eudicots, *Nelumbo nucifera* and *Platanus* belong to the same order, Proteales (Byng et al. 2016). In contrast to 7 PEBP members in *Platanus* (two *MFT*-like, three *TFL1*-like, and two *FT*-like), *N. nucifera* contains more *FT/TFL1* genes, including four *MFT*-like, four *FT*-like and four *TFL1*-like genes (Fig. 2), which should be resulted from a recent whole-genome duplication in *N. nucifera* (Ming et al. 2013; Wang et al. 2013). Another species of the basal eudicot, *A. coerulea*, contains one *MFT*-like, three *TFL1*-like, and three *FT*-like genes, indicating it has lost one *MFT*-like member

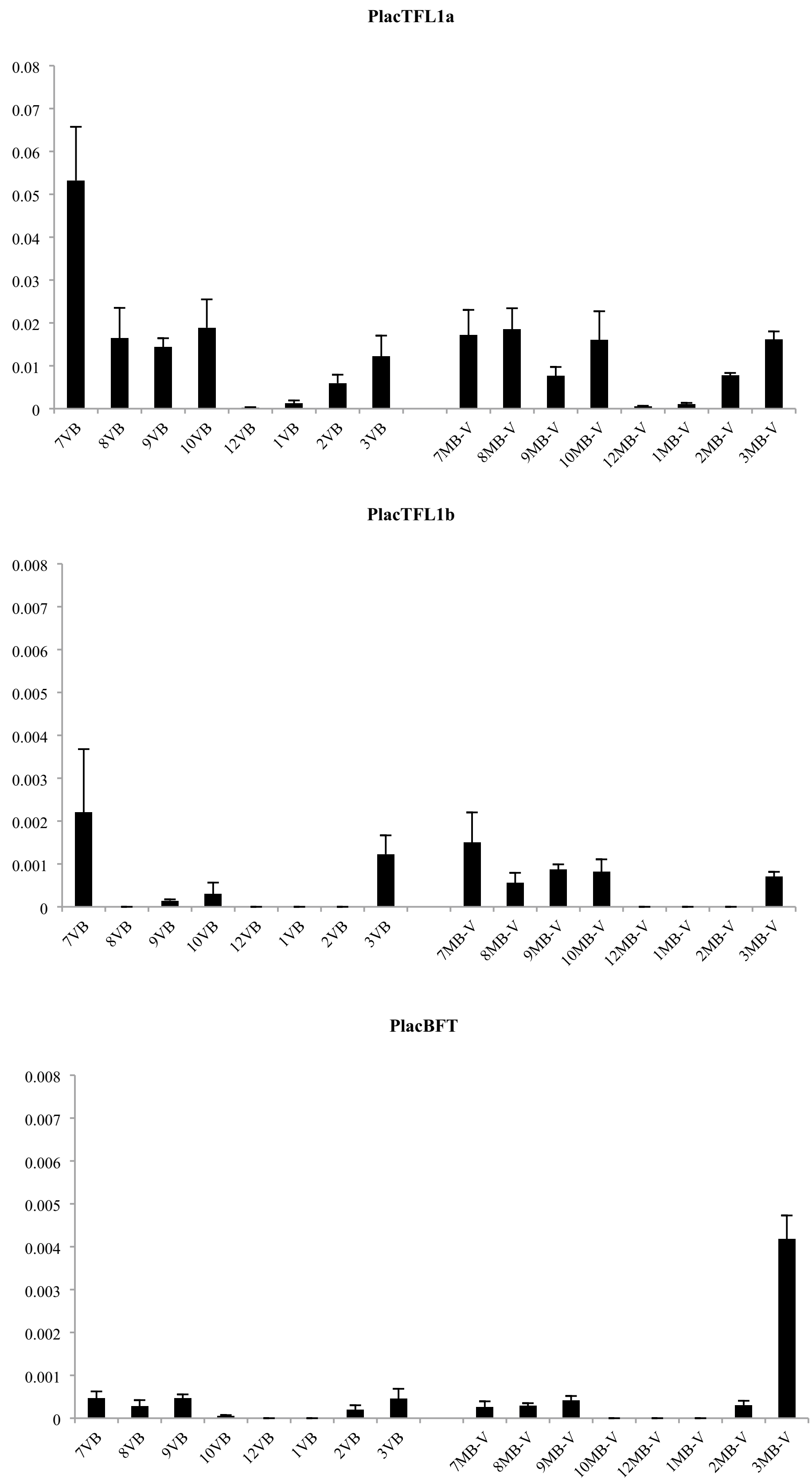

Fig. 4 Expression of *PlacTFL1a/b* and *PlacBFT* genes in different stages of VB and MB-V. VB, vegetative sub-petiolar buds; MB-V, vegetative tissues in mixed flower buds. The numbers indicate the sampling month of the tissues. The level of expression was normalized to London plane *TPI* gene. Error bars represent SE for three replicates

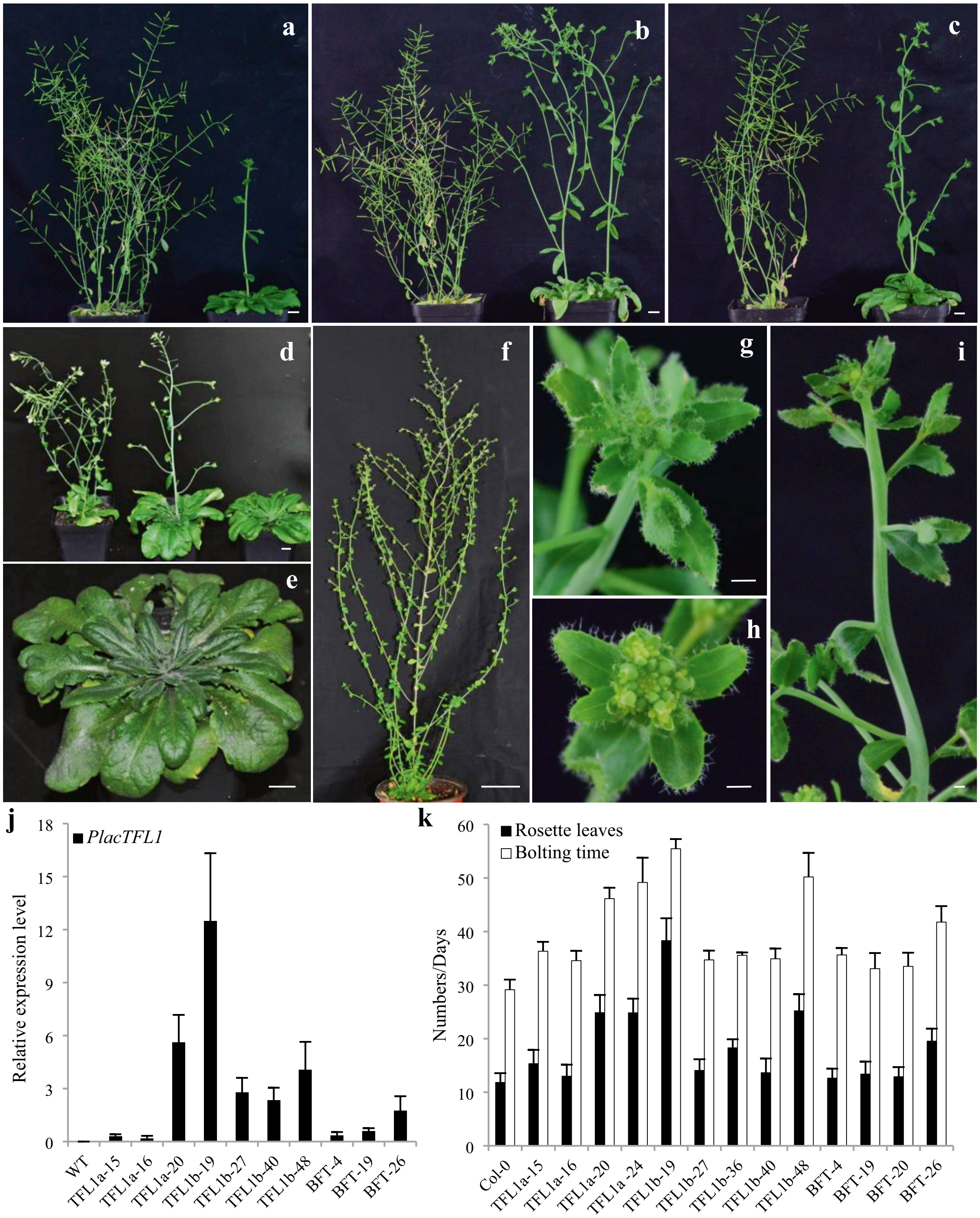

Fig. 5 Phenotype analysis of transgenic *Arabidopsis* plants overexpressing *PlacTFL1a/b* or *PlacBFT* genes. **a–c** Wild-type (left) and *35S:PlacTFL1a* transgenic line #10, *35S:PlacTFL1b* transgenic line #48, *35S:PlacBFT* transgenic line #26 (right), respectively. **d** Wild-type (left) and *35S:PlacTFL1a* transgenic plants with moderate (line #15, middle) and strong (line #10, right) phenotype. **e** Rosette leaves of *35S:PlacTFL1a* transgenic line #10. **f** Strong phenotypic lines of *35S:PlacTFL1a* (line #10). **g**, **h** The top of inflorescences in *35S:PlacTFL1a* transgenic plants with strong (**g**, line #20) and moderate (**h**, line #15) phenotype. **i** Inflorescence stem of *35S:PlacTFL1a* transgenic line #20. **j** qRT-PCR analysis of transgenes in Col-0 and transgenic *Arabidopsis* lines overexpressing *PlacTFL1a/b* and *PlacBFT* genes. **k** Numbers of rosette leaves before bolting and days of bolting time in Col-0 and transgenic lines. Line #10 displayed the strong phenotypes, which we cannot obtain seeds and progenies from it that was so excluded from the statistical analysis. Bars: 10 mm (**a–f**), 1 mm (**g–i**)

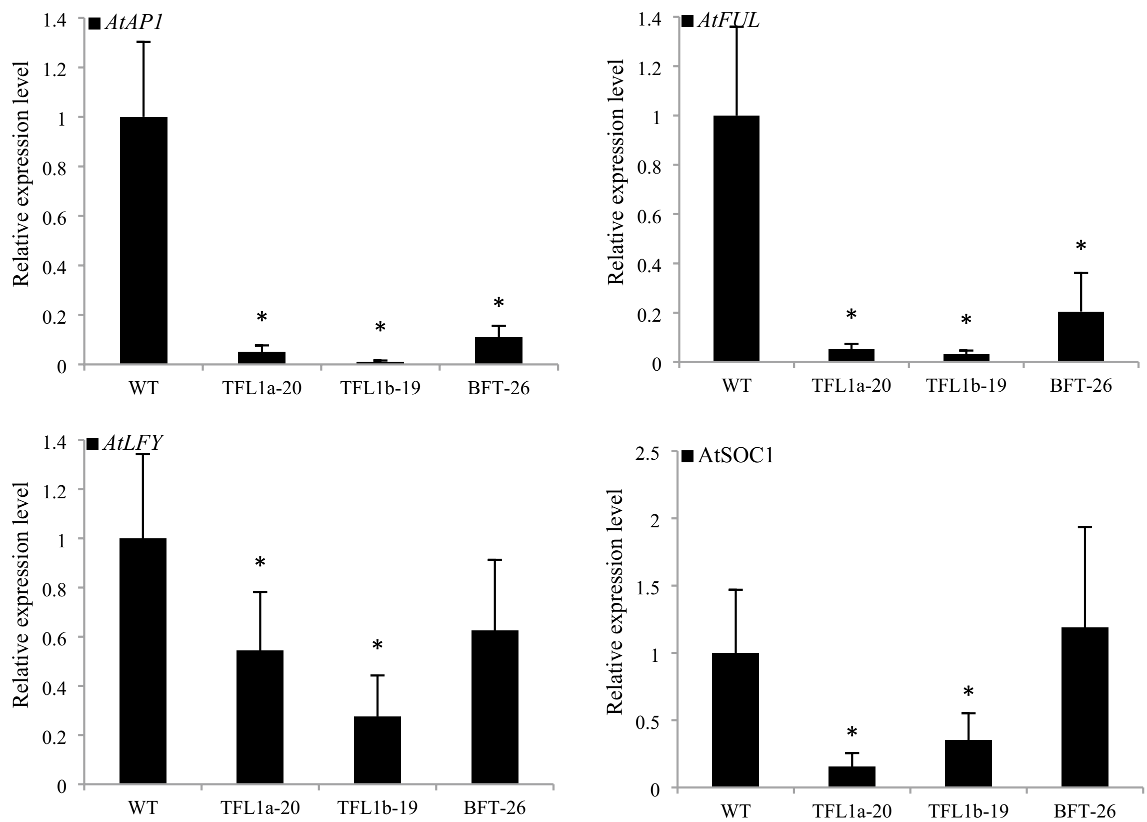

Fig. 6 qRT-PCR analysis of endogenous flowering-related genes in 21-day-old seedlings of *Arabidopsis* wild-type and *35S:PlacTFL1*-like transgenic lines. The numbers after the genes indicate transgenic lines of the three *35S:PlacTFL1*-like genes, respectively. Data represent the mean ± SE from three biological replicates, and *AtEF1α* was used as internal control. WT, wild-type seedlings. The asterisks indicate significant differences compared with the WT plants ($P < 0.05$)

and experienced further duplication of the *TFL1*-like and *FT*-like genes.

Divergent expression and function of the *TFL1*-like genes in *P. acerifolia*

In general, gene expression pattern has significant relationship with their functions. The expression patterns of *TFL1*-like genes show obviously changes among different members and/or plant species, and their functions also exhibit significant divergence and diversity (Wickland and Hanzawa 2015). For instance, *Arabidopsis TFL1* transcripts are present in vegetative and inflorescence meristems to repress flowering and maintain inflorescence indeterminacy (Bradley et al. 1997; Serrano-Mislata et al. 2016), its paralog *BFT* is expressed in the shoot apical meristem, young leaf, and axillary inflorescence meristem (Yoo et al. 2010), whereas another paralog *ATC* was only detected in the hypocotyl of young plants, and not in the inflorescence meristem (Mimida et al. 2001). Apple *TFL1*-like genes *MdTFL1* and *MdTFL1a* are expressed in the vegetative tissues in both the adult and juvenile phases; *MdCENa* (*ATC* ortholog) is mainly expressed in fruit receptacles, cultured tissues, and roots, while *MdCENb* is silenced in most organs (Mimida et al. 2009). The three *TFL1*-like genes in *Jatropha curcas* also show distinct expression patterns: *JcTFL1a* and *JcTFL1c* are mainly expressed in the roots of juvenile plants, whereas *JcTFL1b* transcripts are abundantly accumulated in the fruits and stems (Li et al. 2015, 2017).

Like most *TFL1*-like genes in other species, the three *TFL1*-like genes of London plane are preferentially expressed in vegetative tissues, but they have distinct spatiotemporal expression patterns. *PlacTFL1a* was widely expressed in vegetative organs of both juvenile and adult plants, including stems, leaves, apical buds, VBs, and the vegetative tissues of MBs (Figs. 3, 4). The expression of *PlacTFL1a* in SBs increased gradually prior to the inflorescence initiation (from April to May), but dramatically decreased during the inflorescence differentiation period (June), suggesting that *PlacTFL1a* play a crucial role in maintaining the vegetative growth and repressing the reproductive development of London plane, which is further supported by the highest expression level of *PlacTFL1a* in the VBs at July when the inflorescences are developing

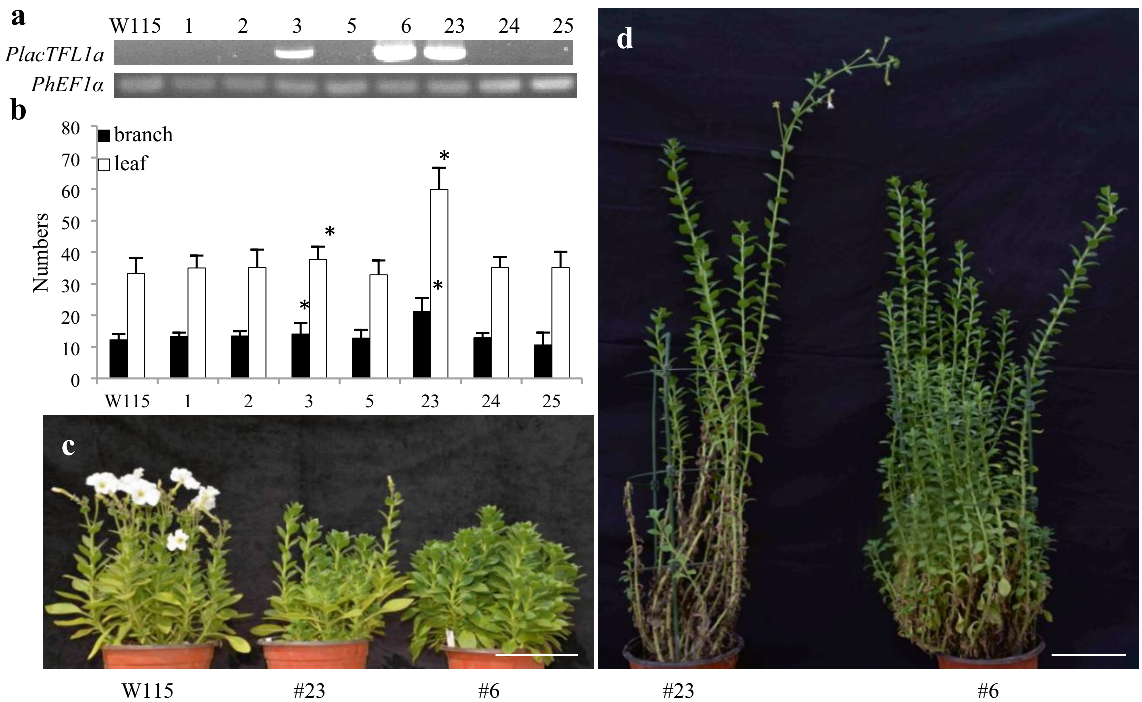

Fig. 7 Phenotype analysis of transgenic petunia plants ectopically expressing *PlacTFL1a* gene. **a** RT-PCR analysis of transgenes in wild-type petunia (W115) and transgenic lines. **b** Numbers of branches and leaves on the principal shoot before flowering in W115 and T_1 transgenic lines. **c** W115 (left) and *35S:PlacTFL1a* transgenic lines #23 (middle) and #6 (right) after cultivated for two months. **d** T_0 *35S:PlacTFL1a* transgenic lines #23 (left) and #6 (right) after transplanted for seven months. *Significantly difference compared to Col-0 ($P<0.05$). Bars: 10 cm

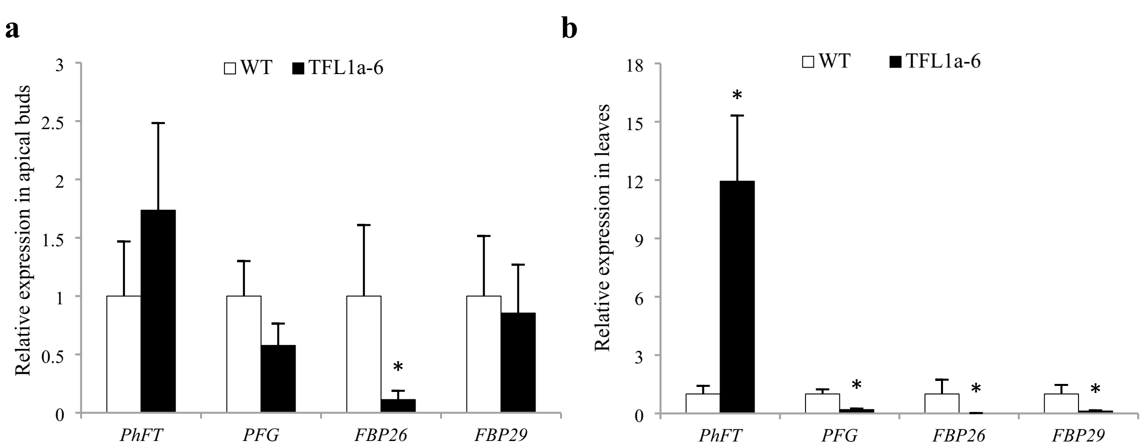

Fig. 8 qRT-PCR analysis of endogenous flowering-related genes in *35S:PlacTFL1a* transgenic line (#6). **a**, **b** The expression levels of *PhFT*, *PFG*, *FBP26*, and *FBP29* in apical buds (**a**) and leaves (**b**) of W115 (WT) and *35S:PlacTFL1a* transgenic line. *PhEF1α* was used as internal control; the asterisks indicate significant differences compared with the WT plants ($P<0.05$)

(Fig. 4). Based on this hypothesis, we speculate that higher expression level of *PlacTFL1a* should be present in the VBs at June, but at that moment the subpetiolar buds maintaining vegetative status could not be distinguished visibly from those undergoing flower bud differentiation, and so not detected. It is interesting that the expression levels of *PlacTFL1b* is significantly lower than *PlacTFL1a* and only weak expression is detected in a few tissues (Figs. 3, 4), although they are very closely related in terms of the coding sequences, with the identity of 91.7% at nucleotide level. In general, functional evolution of genes depends on two aspects: the change of gene coding sequences and alteration

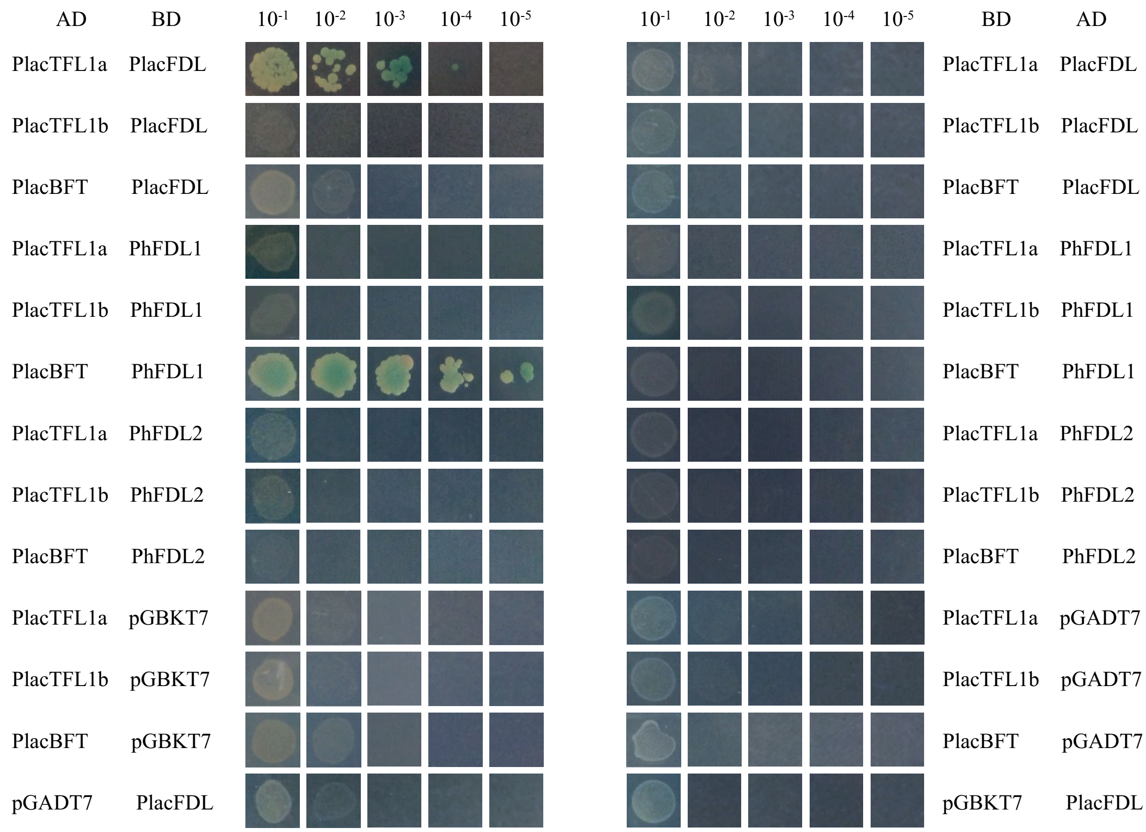

Fig. 9 Interactions between *Platanus* TFL1-like proteins and the FD-like proteins. Tenfold serial dilutions from 10^{-1} to 10^{-5} of each culture were spotted on the selected SD-Leu/-Trp/-His/-Ade plates with X-α-gals

of gene expression patterns. Overexpression of *PlacTFL1a* and *PlacTFL1b* in *Arabidopsis* resulted in comparable phenotypic changes (Fig. 5), indicating that *PlacTFL1b* has retained its function in point of protein sequence but may has lost most functions in London plane due to expression degeneration after duplication, similar to above-mentioned apple *MdCENb* (Mimida et al. 2009). Expression pattern of *PlacBFT* is also significantly different from that of *PlacTFL1a*, with predominant expression in stems and roots, and weak in growing SBs (Fig. 3), suggesting *PlacBFT* may have undergone subfunctionalization during evolution.

Unlike some *TFL1*-like genes that are strongly expressed in developing inflorescences, such as *Arabidopsis TFL1* (Bradley et al. 1997), *Antirrhinum CEN* (Bradley et al. 1996), and *HvTFL1s* in rubber tree (Bi and Tahir 2019), no *TFL1*-like genes of London plane were expressed evidently in inflorescences with various developmental stages (Fig. 3; Fig. S2). Given that the expression of *TFL1*-like genes in the inflorescences is related to their functions in the control of inflorescence architecture (Bradley et al. 1996, 1997; Nakagawa et al. 2002; Fernandez et al. 2010; Perilleux et al. 2019), we speculate that the *TFL1*-like genes of London plane may not involve in inflorescence development. To verify their functions, the three *TFL1*-like genes of *Platanus* were further investigated by transgenic studies in *Arabidopsis* and petunia. Overexpression of each gene delayed or repressed flowering, increased the number of leaves and nodes in transgenic plants compared to their wild-type counterparts, as reported in other species that constitutively express *TFL1*-like genes, confirming their highly functional conversation in flowering regulation among different plant species.

Potential mechanism of *PlacTFL1a* function

In model plants, *TFL1* functions via directly repressing flowering-related genes, such as *AP1*, *FUL*, and *LFY* (Bradley et al. 1997; Ratcliffe et al. 1999; Hanano and Goto 2011). In contrast to *PlacTFL1a*, the expression of London plane *AP1* homologs (*FUL*-like genes, *PlacFLs*) increased in SBs at the stage of inflorescence initiation (June) and maintained their expression level during the inflorescence developing process (Zhang et al. 2019), suggesting that *PlacTFL1a* inhibits reproductive development and flowering probably through repressing the expression of *FUL*-like genes in *Platanus*, which is consistent with the results reported in pear in which the expression of *TFL1*-like genes (*PpTFL1-1a* and *PpTFL1-2a*) rapidly decrease in reproductive meristems followed by upregulation of *PpAP1* and *PpFUL* genes (Bai et al. 2017). Furthermore, significant downregulation of *PFG*, *FBP26*, and *FBP29* genes were detected in *35:PlacTFL1a* transgenic petunia plants that displayed the phenotype of severely repressed flowering (Fig. 8). It has been reported previously that knockdown of *PFG* and *FBP26* genes (two *FUL* orthologs) represses the transition from vegetative to reproductive development in petunia, resulting in a phenotype exactly similar to the *35:PlacTFL1a* transgenic plant *#23* in our study (Immink et al. 1999), which indicates that *35:PlacTFL1a* represses flowering at least partially through regulating the expression of *AP1/FUL*-like genes in petunia. All these results support a probably conserved regulatory mechanism between *TFL1* and *AP1/FUL*-like genes in flowering regulation. However, our transgenic individual *#6* showed drastically non-flowering phenotype even after several generations of propagation by cutting lasting for approximate two years, which is much more late flowering than the *PFG* and *FBP26* down-regulated plants, even than a quadruple mutant of all the petunia *AP1/FUL*-like genes, *pfg fbp26 fbp29 euap1* (Morel et al. 2019), suggesting that *35:PlacTFL1a* must have regulated other flowering-related genes besides the *AP1/FUL*-like genes. Indeed, we found a *FT*-like gene (*PhFT*) was significantly up-regulated in the leaves of *35S:PlacTFL1a* transgenic line #6 (Fig. 8). However, *FT* and its orthologs in most plant species were proved to function as florigens that promote flowering (Wickland and Hanzawa 2015). Interestingly, our recent study indicated that *PhFT* (corresponding to *PhFT1* therein) might function as a repressor of flowering in petunia, because its overexpression in *Arabidopsis* resulted in significantly late flowering (Wu et al. 2019). The functions of repressing flowering have also been reported for several *FT*-like genes in other species, such as *BvFT1* in sugar beet (Pin et al. 2010), *HaFT1* in sunflower (Blackman et al. 2010), *NtFT1/2/3* in tobacco (Harig et al. 2012), and *SlSP5G(2/3)* in tomato (Cao et al. 2015). In summary, *PlacTFL1a* represses flowering in petunia might through activating the flowering repressor *PhFT1* and inhibiting the flowering promotors *AP1/FUL*-like genes, and some other unknown regulators if any.

It is well known that *TFL1* repress flowering probably via interacting with FD to compete with FT (Hanano and Goto 2011; Ho and Weigel 2014; Zhu et al. 2020). Besides *Arabidopsis*, TFL1-like proteins interacting with FD homologues have been identified in *Rosa chinensis*, kiwifruit and so on (Varkonyi-Gasic et al. 2013; Randoux et al. 2014; Kaneko-Suzuki et al. 2018). The results of yeast two-hybrid analysis demonstrated that only PlacTFL1a has weak interaction with PlacFDL, while both PlacTFL1b and PlacBFT have no interaction with PlacFDL (Fig. 9). Even so, ectopic expression of both *PlacTFL1b* and *PlacBFT* in *Arabidopsis* still can delay flowering. Two hypotheses could be used to explain this result: one possibility is that PlacTFL1b and PlacBFT are able to interact with other FD-like members in London plane, as well as *Arabidopsis* FD protein; alternatively, interaction between *Platanus* TFL1-like protein and FD-like protein is not necessary for its function in repressing flowering. The latter assumption is supported by the fact that overexpression of *PlacTFL1a* in petunia results in repressed flowering, but no interaction between PlacTFL1a as well as PlacTFL1b and petunia FD-like proteins (PhFDL1 and PhFDL2) was detected (Fig. 9), while PlacBFT does interact with one of the petunia FD-like protein PhFDL1. A recent genome-wide ChIP-seq analysis demonstrated that TFL1 may interact with other DNA-binding proteins, besides FD, to regulate the expression of downstream genes (Goretti and Silvestre 2020). In summary, our results confirmed the function of *Platanus TFL1*-like genes in repressing flowering, but probably via a distinct regulatory mechanism.

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Author contributions LGF and BMZ designed the experiments. ZSS, ZQ, YXY, LYJ and SMM performed the experiments. ZSS, WJQ, JJ and NCR analyzed the data. ZSS and LGF wrote and revised the manuscript. All authors participated in the research and approved the final manuscript.

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Availability of data and materials All data used in this research are included in this published article and its supplementary information files.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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