Characterization of Peach TFL1 and Comparison with FT/TFL1 Gene Families of the Rosaceae

Yihua Chen and Peng Jiang
Horticulture Department, University of Georgia, Athens, GA 30602

Shivegowda Thammannagowda and Haiying Liang
Department of Genetics and Biochemistry, Clemson University, Clemson, SC 29634

H. Dayton Wilde1
Horticulture Department and Institute of Plant Breeding, Genetics and Genomics, University of Georgia, 1111 Plant Sciences, Athens, GA 30602

Additional index words. Prunus persica, gene expression, phylogenetics, ornamental, floral induction

Abstract. We investigated the FT/TFL1 family of peach (Prunus persica), a gene family that regulates floral induction in annual and perennial plants. The peach terminal flower 1 gene (PpTFL1) was expressed in a developmental and tissue-specific pattern that, overall, was similar to that of TFL1 orthologs in other woody Rosaceae species. Consistent with a role as a floral inhibitor, ectopic expression of PpTFL1 in Arabidopsis (Arabidopsis thaliana) delayed flowering and prolonged vegetative growth. Other members of the peach FT/TFL1 family were identified from the sequenced genome, including orthologs of flowering locus T, centroradialis, and mother of ft. Sequence analysis found that peach FT/TFL1 family members were more similar to orthologous genes across the Rosaceae than to each other. Together these results suggest that information on genes that regulate flowering in peach could be applied to other Rosaceae species, particularly ornamentals.

Received for publication 15 Aug. 2012. Accepted for publication 7 Oct. 2012.

We are grateful to Tetyana Zhebentyayeva for providing peach floral and vegetative buds.

1Corresponding author. E-mail: dwilde@uga.edu.

Genes that regulate the transition from vegetative to reproductive development are important to plant domestication and improvement. In crops such as tomato (Solanum lycopersicum) and soybean (Glycine max), cultivars have been developed with a determinate growth habit in which shoots are committed to flowering. Determinate growth in tomato and soybean is a recessive trait controlled by the loci self-pruning (sp) and determinate 1 (dt1), respectively (Woodworth, 1932; Yeager, 1927). Sp and dt1 have been identified as orthologs of the arabidopsis gene TFL1 (Pnueli et al., 1998; Tian et al., 2010). The protein encoded by TFL1 acts a repressor to maintain vegetative growth, and homozygous mutations of AtTFL1, GmTFL1, and SISP lead to reduced vegetative growth and early terminal differentiation. In an herbaceous perennial, woodland strawberry (Fragaria vesca), a mutation in FvTFL1 prevents long-day suppression of flowering, resulting in continuous blooms (Iwata et al., 2012; Koskela et al., 2012). Other genes that regulate the transition to flowering include flowering locus T (FT), which encodes a major component of florigen (Turck et al., 2008). FT and TFL1 have closely related sequences but opposing functions (Hanzawa et al., 2005).

In most woody plants, flowering is repressed during the juvenile phase and then regulated annually in the adult phase. TFL1 is integral to the control of both processes. The juvenile phase of apple (Malus ×domestica), pear (Pyrus communis), and hybrid poplar (Populus tremula ×alba) was shortened by years when the expression of TFL1 orthologs was suppressed (Frieman et al., 2011; Kotoda et al., 2006; Mohamed et al., 2010). The annual flowering habit of rose (Rosa sp.) has been altered by centuries of breeding for the recessive locus recurrent blooming (Iwata et al., 2012). Rb was recently identified as a TFL1 ortholog and rose cultivars with low RoTFL1 expression bloom repetitively instead of annually (Iwata et al., 2012; Wang et al., 2011b). Remontant rose cultivars also have a reduced juvenile phase, and apple and pear with suppressed TFL1 had repetitive blooms on new growth (Frieman et al., 2011; Iwata et al., 2012; Kotoda et al., 2006).

Genes such as TFL1 that regulate shoot meristem identity are targets for the improvement of woody ornamental plants by molecular breeding. Repetitive blooming is a valuable trait for woody ornamentals as shown by the commercial success of remontant azalea (e.g., Rhododendron sp. ‘Encore’), hydrangea (e.g., Hydrangea macrophylla ‘Endless Summer’), and rose (e.g., Rosa hybrida ‘Knockout’). Shoot meristem identity in woody plants is regulated by gene products interacting in response to environmental or endogenous cues (reviewed in Benloch et al., 2007; Wilkie et al., 2008). TFL1, for example, inhibits the transcription factor FD from turning on other floral regulatory genes (Hanano and Goto, 2011). In addition to genetic control, shoot phase change is further regulated through histone modification, DNA methylation, and microRNA production (Angel et al., 2011; Wang et al., 2011a; Yaish et al., 2011). As a result of their profound effect on the switch to determinate growth, TFL1 and FT are genes in this complex regulatory network that could give insight into the control of flowering patterns in woody ornamental plants.

We investigated TFL1 in peach, which has potential as a genetic model for woody ornamental plants for several reasons: 1) it has a small, diploid genome (230 Mb) that has been sequenced; 2) complete, self-fertile flowers are produced after a relatively short juvenile stage of two to three years; and 3) several floral regulatory genes have been characterized, including peach orthologs of agamous, apetalal, leafy, plena, seedstick, sepallata, and short vegetative phase (An and Li, 2008; Arús et al., 2012; Martin et al., 2006; Tadiello et al.,
2009; Tani et al., 2009; Xu et al., 2008; Yamane et al., 2011; Zhang et al., 2008). The genomic sequence of *PpTFL1* of peach has been identified (Li et al., 2010) and the allelic diversity of *PpTFL1* was found to be low among cultivars bred for the eastern United States (Chen and Wilde, 2011).

We examined *PpTFL1* function by characterizing its native expression in peach and the effect of its ectopic expression in arabidopsis. We identified *PpFT* and related gene family members and compared the *FT/TFL1* family of peach with that of other species of Rosaceae. These studies allowed us to examine the reliability of applying genetic information from peach to other woody rosaceous plants.

**Materials and Methods**

**Plant materials and nucleic acid preparation.** Leaves, floral organs, and fruit were obtained from peach trees (*Redhaven*) grown at the University of Georgia Horticulture Farm (Watkinsville, GA). Shoot tip samples were collected in May and August and dormant buds (floral and vegetative) were collected in November and March. Seedling material was obtained from two-month-old plants maintained in a growth room (22°C, 16 h light/8 h dark). Total RNA was isolated from peach and arabidopsis tissues using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD). After treatment with RNase-free DNase, RNA was quantified with a spectrophotometer (NanoDrop 8000; Thermo Scientific, Wilmington, DE) and I μg RNA was used in each reverse transcription reaction. cDNA synthesis was performed with M-MLV reverse transcriptase and oligo(dT)15 primers as described by Chen et al. (2003). The cDNA from peach and arabidopsis was diluted 1:25 and 1:100, respectively, for polymerase chain reaction (PCR) analysis. Plant genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen).

**Reverse transcription–polymerase chain reaction primers and reaction conditions.** The *PpTFL1* genomic sequence (GenBank accession GU591895) was used to design PCR primers (Fig. 1). The primers TE1F (5'–CACCCTCTCTCCATTTCTGTCC-3') and TE4R (5'–GAGGAGCTTTGGGTTTGAGTAG-3') were designed from 5' and 3' UTR sequences, respectively, for gene-specific amplification of the complete *PpTFL1* coding sequence. Amplification of exons 3 and 4 was carried out with TE3RTF (5'–GAGCAGATATTCGGGCCACACAG-3') and TE4RTR (5'–ACGGCCAGCGACAGAGGACC-3') in peach and with TE3RTF and TE4R in transgenic arabidopsis. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a reference gene (Tong et al., 2009) and was amplified by the primers GAPDH2 (5'–TGAGGGCAAGCTGAAAGGTATCTT-3') and GAPDH2 (5'–TCAAGTCAACCAACAGGGTACTGT-3'). Primers were synthesized by MWG Operon (Huntsville, AL).

Reverse transcription–PCR was conducted with 0.2 μM of each primer, 30 ng cDNA, 1X PCR buffer, 2.5 mm MgCl2, 0.2 mm dNTPs, and 1 U Taq polymerase. For the amplification of *PpTFL1* from genomic DNA, reactions were denatured at 94°C for 5 min followed by 45 cycles of 94°C for 20 s, 65°C for 25 s, 72°C for 3 min, and a final extension at 72°C for 5 min. Similar parameters were used for analysis of cDNA, except for the extension time (30 s) and annealing temperatures, which were 66°C for *PpTFL1* and 60°C for GAPDH. The number of PCR cycles for cDNA template was 38 and 35, respectively, for *PpTFL1* and GAPDH in peach and 32 cycles for *PpTFL1* in arabidopsis.

**Transformation of arabidopsis with *PpTFL1*.** The arabis- dopsis *actin2* promoter and terminator sequences (An et al., 1996) of vector pACT2 were subcloned into the binary plasmid pCAMBIA1300. The full-length *PpTFL1* gene was amplified from peach genomic DNA by PCR and *NcoI* and *ApaI* restriction sites were added to the 5' and 3' ends, respectively. After confirmation by sequencing, the gene was ligated into cleaved *NcoI* and *ApaI* restriction sites located between the *AtACT2* regulatory sequences (Fig. 1). The binary plasmid was introduced by heat shock into Agrobacterium tumefaciens EHA105 that was then used to transform arabidopsis (Columbia) by the floral dip method (Clough and Bent, 1998). The resulting seeds were surface-sterilized and selected on agar plates containing half-strength Murashige and Skoog medium and 50 mg·L⁻¹ hygromycin. The plates were kept at 4°C for 3 d and then transferred to a 22°C growth room with 16 h light provided by fluorescent lights (160 μmol·m⁻²·s⁻¹). Transgenic seedlings and nontransgenic controls were transplanted into soil and allowed to flower in the growth room. The number of rosette leaves produced and the days until flower primordia first became visible were recorded.

**Identification and sequence comparison of FT/TFL1 family members.** Gene sequences of *PpFT* family members in Rosaceae species were obtained from the GenBank database and by screening the draft genomes (v1.0) of peach, apple, and strawberry (*F. vesca*) (Jung et al., 2008) with orthologous arabidopsis and *Vitis vinifera* sequences. Multiple sequence alignment was carried out with the predicted protein sequence from peach *PpTFL1* family members and TFL1 of *Prunus mume* (BAJ14521), *M. domestica* (BAD06418), *P. communis* (BAD10963), *F. vesca* (AEP23097), and *Rosa chinensis* (ADO64261). ClustalW (Thompson et al., 1994) was used for pairwise alignment with the Gonnet protein weight matrix, a gap penalty of 10, and gap extension of 0.1. The multiple sequence alignment output file obtained in FASTA format was subjected to sequence identity and similarities comparison using the software developed at University of Alberta, Canada (Stothard, 2004).

Phylogenetic relationships between FT/TFL1 family members of Rosaceae species were established by maximum likelihood analysis of mRNA sequences using MEGA4 software (Tamura et al., 2007). A total of 1000 bootstraps were performed and bootstrap support values over 50% are indicated in the results. Predicted mRNA sequences were taken, without adjustment, from the peach genome v1.0, the apple genome v1.0, the strawberry (*F. vesca*) genome v1.0 (build 8), and the

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**Fig. 1.** Polymerase chain reaction (PCR) primers and gene construct for *PpTFL1* analysis. PCR primer locations are shown above exons (E) on the *PpTFL1* gene diagram. For transformation, the genomic sequence was subcloned between arabidopsis *actin2* promoter and terminator sequences inserted into the T-DNA of pCAMBIA1300. Gray boxes show untranslated regions of exons; RB and LB = right and left T-DNA borders; hygR = hygromycin-resistance marker CaMV35SP:HPT.
floral induction, differentiation, or mature floral bud stages expression in the peach shoot apex was not detected during 
PpTFL1 the flowers open, usually in late March to mid-April.

The final phases of floral development take just before exposure to cool temperatures, bud dormancy is broken in the developing floral buds enter dormancy by late summer. After observed in flower petals (Mimida et al., 2009).

The expression of PpTFL1 in peach tissues was examined by reverse transcription–PCR (Fig. 2). PpTFL1 transcripts were detected in seedling roots and leaves and young leaves of adult trees, but were not detected in peach embryos, mature leaves, flower buds, and flower petals (Fig. 2A). Detection of a spliced product with primers flanking an intron (Fig. 1) confirmed that PCR products were derived from mRNA, not genomic DNA.

Like PpTFL1, the genes PmTFL1 of japanese apricot (P. mume) and MdTFL1-1 of apple are expressed in seedling tissues and young leaves of adult plants (Esumi et al., 2010; Mimida et al., 2009). As a result of its polyploid origin, apple has a second gene, MdTFL1-2, which is expressed in seedling roots and stems but not leaves of seedlings or adult plants. In contrast to PpTFL1 and MdTFL1-2, expression of MdTFL1-1 was observed in flower petals (Mimida et al., 2009).

In peach, flowering is induced starting in late May and the developing floral buds enter dormancy by late summer. After exposure to cool temperatures, bud dormancy is broken in the spring. The final phases of floral development take just before the flowers open, usually in late March to mid-April. PpTFL1 expression in the peach shoot apex was not detected during floral induction, differentiation, or mature floral bud stages (Fig. 2B). PpTFL1, however, was expressed during vegetative bud development.

Similar to peach, TFL1 expression was absent or low during floral differentiation in buds of apple (Hattasch et al., 2008; Mimida et al., 2009, 2011), quince [C. oblonga (Esumi et al., 2008)], and rose [Rosa wichurana (Iwata et al., 2012)]. However, in pear (P. pyrifolia, P. communis), one of the two TFL1 genes was expressed during floral differentiation (Esumi et al., 2005, 2008). Like in peach, expression of TFL1 was observed in vegetative buds of japanese apricot (Esumi et al., 2010) and apple (Mimida et al., 2009).

Ectopic expression of PpTFL1 in Arabidopsis. To examine the function of PpTFL1, arabidopsis was transformed with the gene construct AtACT2: PpTFL1. Twenty-nine T1 transgenic plants were produced, which varied in flowering time, and four independent transformants with strong phenotypes were characterized further (Fig. 3). The gene construct contained the genomic sequence of PpTFL1, which had to be spliced after transcription in Arabidopsis. Reverse transcription–PCR analysis with primers from exons 3 and 4 (Fig. 1) found that the correctly spliced product was present in transgenic plants TC1, TC2, TC7, and TC29 (Fig. 3A). Proper splicing of the entire PpTFL1 transcript to the predicted 574 bp size was observed in TC1 (Fig. 3B) and the other three transgenic lines (not shown).

Constitutive expression of PpTFL1 in Arabidopsis prolonged vegetative growth compared with nontransgenic plants, resulting in an increased number of basal rosette leaves. Figure 3D–F shows a range of transgenic phenotypes caused by PpTFL1 expression. Figure 3F shows the most severe phenotype, in which 29 rosette leaves were produced before flowering was initiated. The average for this line (TC1) was 21 leaves compared with

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Table 1. Phenotype of T2 transgenic arabidopsis plants with PpTFL1 gene.

| Plant lines | Time to flowering [mean ± se (d)] | Rosette leaves [mean ± se (no.)] | No. |
|-------------|-----------------------------------|-----------------------------------|-----|
| WT (Col)*   | 35.4 ± 1.0                        | 13.2 ± 0.6                        | 9   |
| ACT2-PpTFL1 |                                   |                                   |     |
| TC1         | 39.6 ± 2.2                        | 21.0 ± 2.2                        | 10  |
| TC4         | 39.3 ± 1.6                        | 17.8 ± 1.0                        | 8   |
| TC7         | 40.6 ± 2.7                        | 17.8 ± 1.1                        | 8   |
| TC29        | 39.8 ± 2.4                        | 17.8 ± 1.1                        | 8   |

*Wild-type control (WT) was the arabidopsis Columbia (Col) ecotype. Transgenic lines TC1, TC4, TC7, and TC29 contained the terminal flower 1 gene of peach driven by the arabidopsis actin 2 promoter.

required for comparative genomics (Shulavev et al., 2008). To examine the reliability of a single genetic model for a conserved function like flowering, peach FT/TFL1 family members can be compared with orthologs in Rosaceae. There is a high degree of sequence conservation between peach FT/TFL1 orthologs and those of other Rosaceae species. The phenotype of transgenic arabidopsis with PpTFL1 was similar to that with PmTFL1, MdTFL1-1, or MdTFL1-2 (Esumi et al., 2010; Mimida et al., 2009). The pattern of expression of TFL1 in peach was closely matched in Japanese apricot, but comparison with orthologs in apple and pear was complicated by the expanded genomes of species in the Maloideae subfamily.

Apple and pear have two TFL1 paralogs that exhibited tissue-specific (MdFTL1-2) or developmental (PpFTL1-1) expression patterns that differed from PpTFL1 and the other paralog. This may be a result of subfunctionalization of the TFL1 paralogs, like in the case of the two apple FT paralogs (Kotoda et al., 2010). The prediction of candidate gene identity and function from peach data may be complicated by the divergence of paralogs. On the other hand, early flowering in pear (P. communis) transformed with citrus FT [Citrus unshiu (Matsuda et al., 2009)] and in plum (Prunus domestica) transformed with poplar FTI [Populus trichocarpa (Srinivasan et al., 2012)] demonstrates functional conservation between divergent FT genes. Recent studies of TFL1 in F. vesca (Iwata et al., 2012; Koskela et al., 2012) illustrate the potential of this sequenced, herbaceous perennial to contribute to an understanding of the flowering process. For the study of floral genes in woody ornamentals, an advantage of peach is the simplicity of its small, diploid genome in which the floral genes examined to date have been found to be single-copy (An and Li, 2008; Martin et al., 2006; Tadie et al., 2009; Tani et al., 2009; Xu et al., 2008; Zhang et al., 2008).

An obstacle to the use of peach as a genetic model is the lack of an efficient transformation system for conducting functional genomics. There are alternative approaches being developed for the analysis of genes in peach. Virus-induced gene silencing (VIGS) is being investigated in peach and apricot using apple latent spherical virus (ALSV) vectors (Kawai et al., 2012). An ALSV vector containing an MdTFL1-1 fragment was able to induce early flowering in apple through VIGS (Sasaki et al., 2008).
Viruses have also been used to deliver gene-specific nucleases for targeted mutation (Marton et al., 2010). TILLING is another functional genomics strategy (Wang et al., 2012) that can be applied to peach, and an EMS-mutagenized peach TILLING population is being established (V. Decroocq, personal communication). These techniques could allow the genetic control of floral induction to be examined in peach for application to other woody ornamentals.

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