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Rapid paper

The *FLOWERING LOCUS T/TERMINAL FLOWER 1* Family in Lombardy Poplar

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Genes in the *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*) family have been shown to be important in the control of the switch between vegetative and reproductive growth in several plant species. We isolated nine members of the *FT/TFL1* family from Lombardy poplar (*Populus nigra* var. *italica* Koehne). Sequence analysis of the members of the *FT/TFL1* family revealed considerable homology within their coding regions both among family members and to the members of the same family in *Arabidopsis*, tomato and grapevine. Moreover, members of this family in all four species examined display a common exon–intron organization. Phylogenetic analysis revealed that the genes fall into four different clades: two into the *TFL1* clade; five into the *FT* clade; and one each into the *MOTHER OF FT AND TFL1* and *BROTHER OF FT AND TFL1* clades. One gene in the *TFL1* clade, *PnTFL1*, is expressed in vegetative meristems, and transgenic *Arabidopsis* that ectopically expressed *PnTFL1* had a late-flowering phenotype. The expression patterns of two genes in the *FT* clade, *PnFT1* and *PnFT2*, suggested a role for them in the promotion of flowering, and transgenic *Arabidopsis* that ectopically expressed either *PnFT1* or *PnFT2* had an early-flowering phenotype.

Keywords: Flowering control — *FLOWERING LOCUS T* (*FT*) — Poplar — *Populus nigra* — *TERMINAL FLOWER 1* (*TFL1*).

Abbreviations: *BFT*, *BROTHER OF FT AND TFL1*; EST, expressed sequence tag; *FT*, *FLOWERING LOCUS T*; LD, long day; *MFT*, *MOTHER OF FT AND TFL1*; N-J, Neighbor-Joining; ORF, open reading frame; PAR, photosynthetically active radiation; P35S, cauliflower mosaic virus 35S promoter; *PnFTL*, *Populus nigra FT/TFL1*-like; RACE-PCR, rapid amplification of cDNA ends-PCR; RT–PCR, reverse transcription–PCR; *TFL1*, *TERMINAL FLOWER 1*.

The nucleotide sequences reported in this paper have been submitted to the DDBJ database under accession numbers AB161110 (*PnFTL1* mRNA), AB183451 (*PnFTL1* genomic DNA), AB181183 (*PnTFL1* mRNA), AB369067 (*PnTFL1* genomic DNA), AB181185 (*PnFTL3* mRNA), AB181186 (*PnFTL3* genomic DNA), AB181241 (*PnFTL4* mRNA), AB369068 (*PnFTL4* genomic DNA), AB106111 (*PnFT1* mRNA), AB369069 (*PnFT1* genomic DNA), AB109804 (*PnFT2* mRNA), AB369070 (*PnFT2* genomic DNA), AB110612 (*PnFT3* mRNA), AB369071 (*PnFT3* genomic DNA), AB110612 (*PnFT4* mRNA), AB369073 (*PnFT4* genomic DNA) and AB369072 (*PnFTL9* genomic DNA).

Introduction

The features of the growth and development of woody plants differ from those of annual herbaceous plants, such as *Arabidopsis thaliana*. In particular, woody plants have a very long juvenile phase. In some species, the juvenile phase lasts as long as 40 years before flowering occurs for the first time (Hackett 1985, Greenwood 1987). This long juvenile phase has been an obstacle in the breeding of woody plants, including fruit trees. Some efforts at accelerating flowering by application of plant hormones have been made (Mullins et al. 1989, Meilan 1997), but the techniques are applicable to only limited species of woody plants and the mechanisms involved remain unknown. Therefore, it is important to characterize the molecular mechanisms of flowering in woody plants and to develop new breeding technologies for the acceleration of flowering by genetic engineering.

The genus *Populus* is an important resource, serving, in particular, as a source of raw materials for pulp, paper, board and lumber products (Mitchell et al. 1992). Due to this importance, an international team of researchers, funded by the US Department of Energy, determined the nucleotide sequence of the entire genome of black cottonwood (*P. trichocarpa*) one of the *Populus* species (Tuskan et al. 2006). The Lombardy poplar (*P. nigra* var. *italica* Koehne) is one of the most common species in the same genus and it has been planted worldwide. Furthermore, a system for transformation and a library of full-length enriched expressed sequence tags (ESTs) in this species have been established (Mohri et al. 1996, Nanjo et al. 2004, Nishiguchi et al. 2006). Therefore, the *Populus* species is

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generally chosen by researchers as the model genus of woody plants, and we selected the Lombardy poplar for the present study.

The genes that control flowering time in *Arabidopsis* have been isolated and characterized (for reviews, see Mouradov et al. 2002, Komeda 2004). In our efforts to understand the long juvenile phase of woody plants, we have focused on a family that includes the flowering-promoter gene, *FLOWERING LOCUS T* (*FT*) and the repressor gene *TERMINAL FLOWER1* (*TFL1*). In *Arabidopsis*, this family consists of six members: *FT*; *TFL1*; *MOTHER OF FT AND TFL1* (*MFT*); *ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOG* (*ATC*); *TWIN SISTER OF FT* (*TSF*); and *BROTHER OF FT AND TFL1* (*BFT*) (Kobayashi et al. 1999). The *FT* and *TFL1* genes have opposing effects on flowering time (Kardailsky et al. 1999, Kobayashi et al. 1999), and their effects have been related to the presence, in the gene products, of critical amino acid residues, namely Tyr85/Gln140 in FT and His88/Asp144 in TFL1 (Hanzawa et al. 2005, Ahn et al. 2006). Comparative and functional genomic research in several species of woody plants is starting to provide information on the conservation of flowering-regulatory pathways in both woody and herbaceous plants (Brunner and Nilsson 2004, Böhlenius et al. 2006, Hsu et al. 2006, Kotoda et al. 2006, Carmona et al. 2007). Böhlenius et al. (2006) and Hsu et al. (2006) each isolated an ortholog of *FT* from *Populus* species on the basis of information provided by the sequence of the entire genome of black cottonwood, and they showed that the orthologs function similarly in the induction of flowering. However, there are likely to be many more members of the *FT*/*TFL1* family in *Populus*, as well as in *Arabidopsis*. The isolation and functional analysis of such genes are now required for a full understanding of the molecular mechanisms of flowering in *Populus*.

We report here the cloning and identification of nine members of the *FT*/*TFL1* family from Lombardy poplar. We describe the genomic structure, phylogenetic relationships, patterns of expression of the *FT*/*TFL1* family, as well as the phenotypes of transgenic *Arabidopsis* that ectopically expressed individual genes in the *FT*/*TFL1* family of Lombardy poplar.

Results

Cloning of genes in the FT/TFL1 family from Lombardy poplar

Since preliminary Southern blotting analysis revealed the presence of several members of the *FT*/*TFL1* family in Lombardy poplar, we performed degenerate reverse transcription–PCR (RT–PCR) with degenerate primers derived from the strongly conserved regions of plant members of this family (Fig. 1). We obtained partial cDNAs with seven unique sequences and designated them *PnFTL1* to *PnFTL7* (*P. nigra FT*/*TFL1*-like). The 5′ end and the 3′ end of open reading frames (ORFs) of each corresponding gene were predicted after rapid amplification of cDNA ends-PCR (RACE-PCR) and inverse PCR with the partial cDNA sequences and specific pairs of primers for each gene, as shown in Supplementary Table 1. We obtained nine cDNA clones and 17 genomic DNA clones, including full-length ORFs, from an individual female clone of Lombardy poplar (Fig. 1). Careful analysis of the sequences of these genes revealed that several of them were allelic variants and that the genes were derived from at least nine loci in the genome of Lombardy poplar. Comparison of the cDNA sequences with the genomic sequences showed that *PnFTL7* mRNA was generated from two loci. Therefore, for convenience, we designated the gene with the longer genomic sequence *PnFTL8*. Moreover, a novel gene, *PnFTL9*, which was amplified with the same set of primers as used to amplify *PnFTL5*/*PnFTL6*, was found among the genomic sequences. However, we failed to generate a cDNA clone of *PnFTL9*, even though we designed several specific sets of primers and extensively analyzed various organs from Lombardy poplar.

Identification of PnFTL genes

At both the nucleotide and the amino acid level, the *PnFTL* genes were strongly homologous to one another along their entire length (Fig. 1). The *PnFTL2* and *PnFTL3* genes were very similar to *TFL1*, while the *PnFTL5*, *PnFTL6*, *PnFTL7*, *PnFTL8* and *PnFTL9* genes were very similar to *FT* (Fig. 1 and Table 1).

To resolve the evolutionary relationships among members of the *FT*/*TFL1* family, we performed phylogenetic analysis of this family from Lombardy poplar, *Arabidopsis*, tomato (*Lycopersicon esculentum*), apple (*Malus* × *domestica*), citrus (*Citrus sinensis* and *C. unshiu*) and grapevine (*Vitis vinifera*). Neighbor-Joining (N-J) distance analysis, with the alignment of entire amino acid sequences, generated an unrooted tree with four major clades. *PnFTL2* and *PnFTL3* were in the *TFL1* clade; *PnFTL5*, *PnFTL6*, *PnFTL7*, *PnFTL8* and *PnFTL9* were in the *FT* clade; *PnFTL1* was in the *BFT* clade; and *PnFTL4* was in the *MFT* clade (Fig. 2).

Later, given the results of this phylogenetic analysis and subsequent analyses, we shall refer to several of the above-mentioned genes by their functional designations, as follows: the *PnFTL2*, *PnFTL5*, *PnFTL6*, *PnFTL7* and *PnFTL8* genes will be designated the *PnTFL1*, *PnFT1*, *PnFT2*, *PnFT3* and *PnFT4* genes, respectively (see, for example, Table 2).

The genomic clones of the *PnFTL* genes allowed identification of four exons and three introns (Fig. 3A). All genes showed evidence of conserved genomic organization and the exons were located in identical positions relative to the amino acid sequences of members of the

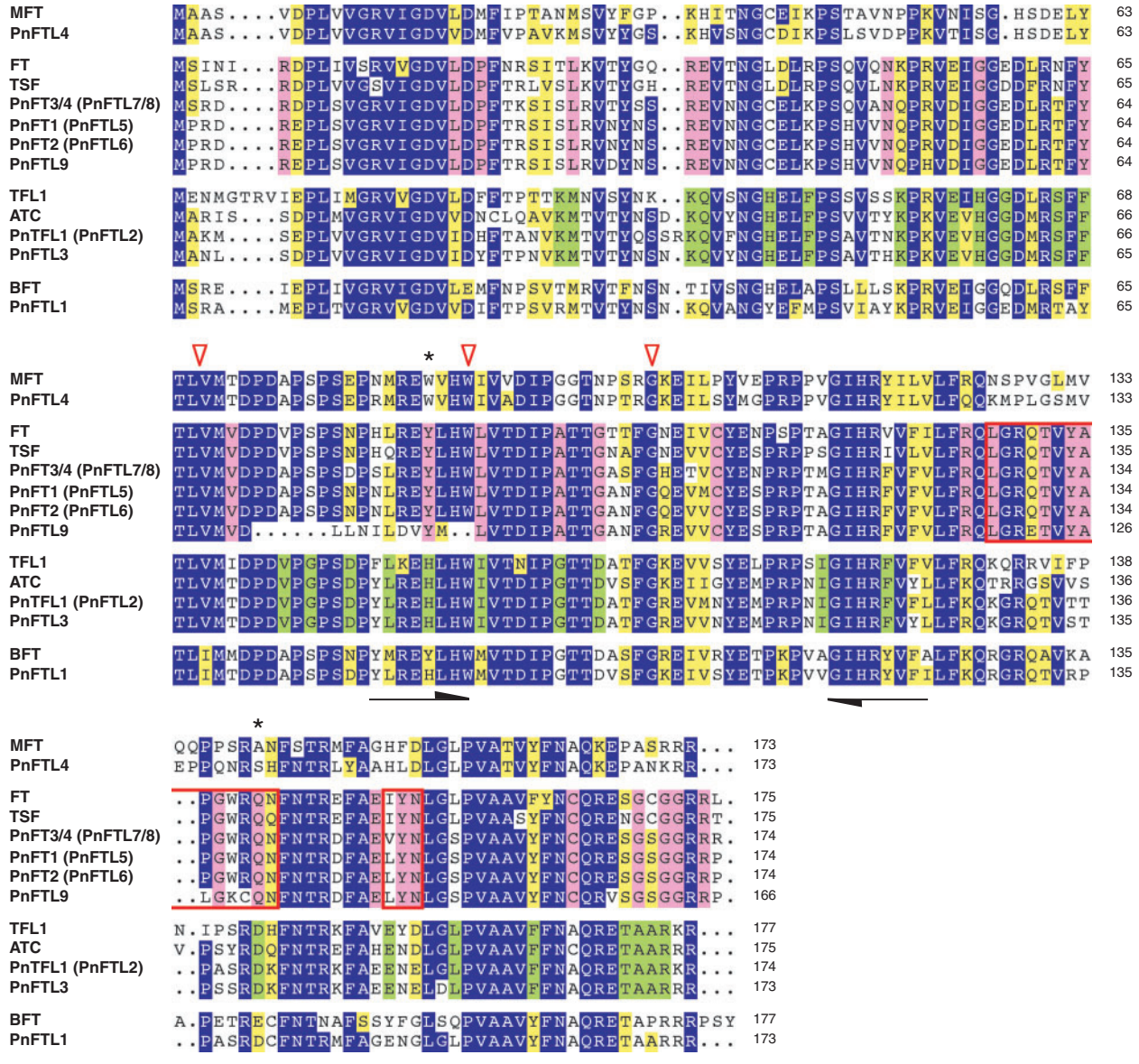

Fig. 1 Alignment of the deduced amino acid sequences of the products of the *FT*/*TFL1* family in Lombardy poplar and *Arabidopsis thaliana* [*FT*, AGI (Arabidopsis Genome Initiative; http://www.arabidopsis.org) code At1g65480; *TFL1*, AGI code At5g03840; *MFT*, AGI code At1g18100; *ATC*, AGI code At2g27550; *TSF*, AGI code At4g20370; and *BFT*, AGI code At5g62040]. All poplar sequences were derived from an individual female clone of Lombardy poplar (PnF-1). Amino acids in blue and in yellow are identical and similar, respectively, in at least 10 of the 14 members of the *FT*/*TFL1* family. Amino acids common to genes in the *FT* clade are shown in pink and those in the *TFL1* clade are shown in green. Dots indicate gaps introduced to maximize the extent of homology among sequences. The arabic numerals in the sequences represent the positions of amino acid residues from the site of initiation of translation. The multiple alignments were generated with ClustalW software. Half-arrows indicate the positions of degenerate primers for PCR that we used for the first cloning of the partial cDNAs. Arrowheads indicate the positions of introns. Asterisks indicate amino acids that are critical to the definition of proteins in the *FT* and *TFL1* clade. Two red boxes indicate the regions that are essential for FT-like activity in exon IV.

Arabidopsis FT/*TFL1* family (Figs. 1, 3A). In Lombardy poplar, exons I and IV varied in length from 195 to 204 bp and from 218 to 224 bp, respectively. In the case of exon II, that of *PnFTL9* was 38 bp long, whereas exon II of other genes was 62 bp long in every case. The length of exon III was 41 bp in all genes examined. However, the length of introns varied. As in *Arabidopsis*, the genes in the *TFL1* and *BFT* clades, namely *PnFTL1*, *PnTFL1* and *PnFTL3*, had short introns of 81–353 bp, while the genes in the *FT* and *MFT* clades, *PnFTL4*, *PnFT1*, *PnFT2*, *PnFT3*, *PnFT4* and *PnFTL9*, had long introns of 112–1,603 bp (Fig. 3A).

The genes in the *FT* clade of Lombardy poplar could be classified into two subgroups, with subgroup I consisting of *PnFT3* and *PnFT4*, and subgroup II consisting of *PnFT1*, *PnFT2* and *PnFTL9* (Fig. 3B). The sequences of introns were >85% homologous within each subgroup, but <50% homologous between subgroups. We also found that *PnFTL9* had a deletion of 393 bp in the region that corresponded to exon II and intron II of *PnFTL6* (Fig. 3B).

To compare the genes of Lombardy poplar and black cottonwood, we searched for *PnFTL* genes in the black cottonwood genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). We found homologs of each gene in the non-FT clade, namely the counterparts of *PnFTL1*, *PnTFL1*, *PnFTL3* and *PnFTL4* in black cottonwood (Table 2). In the case of genes in the *FT* clade, two genes in subgroup I were found in Lombardy poplar, whereas only one corresponding locus was found in black cottonwood (Table 2). In contrast, three genes in subgroup II were found in both species (Table 2).

We excluded *PnFTL9* from subsequent studies because the deletion and extensive substitutions in the regions essential for *FT*-like function suggested that the gene was not expressed and that its product would not be functional (Figs. 1, 3B).

Organ-specific patterns of expression of PnFTL genes

To explore the expression of *PnFTL* genes in Lombardy poplar, we performed an analysis by real-time RT–PCR using primers specific for each gene (Supplementary Table 2). In the *TFL1* clade, *PnTFL1* was expressed in hypocotyls, epicotyls and winter lateral buds of both juvenile and reproductive-phase plants (Fig. 4B), while *PnFTL3* was expressed mainly in the seeds (Fig. 4C). We also detected the expression of *PnFTL3* in winter lateral buds, but levels of expression were low and only one-thousandth of those of *PnTFL1* (Fig. 4C).

In the *FT* clade, we detected the expression of *PnFT1* in almost all organs, with particularly high levels of expression

Table 1 Identity and similarity of the deduced amino acid sequences encoded by *PnFTL* genes in comparison with those encoded by *FT* and *TFL1*

Gene	Identity/similarity (%)	
	to TFL1	to *FT*
PnFTL1	62.4/76.4	58.5/68.9
PnTFL1 (*PnFTL2*)	70.9/82.1	56.5/68.9
PnFTL3	71.3/83.7	56.8/67.6
PnFTL4	48.9/62.4	44.6/58.2
PnFT1 (*PnFTL5*)	55.9/66.5	76.6/85.1
PnFT2 (*PnFTL6*)	56.4/66.5	77.1/85.1
PnFT3 (*PnFTL7*)/ *PnFT4* (*PnFTL8*)	55.3/68.2	78.3/85.7
PnFTL9	48.6/59.2	65.7/74.3

Identity and similarity were calculated by ClustalW software (Thompson et al. 1994).

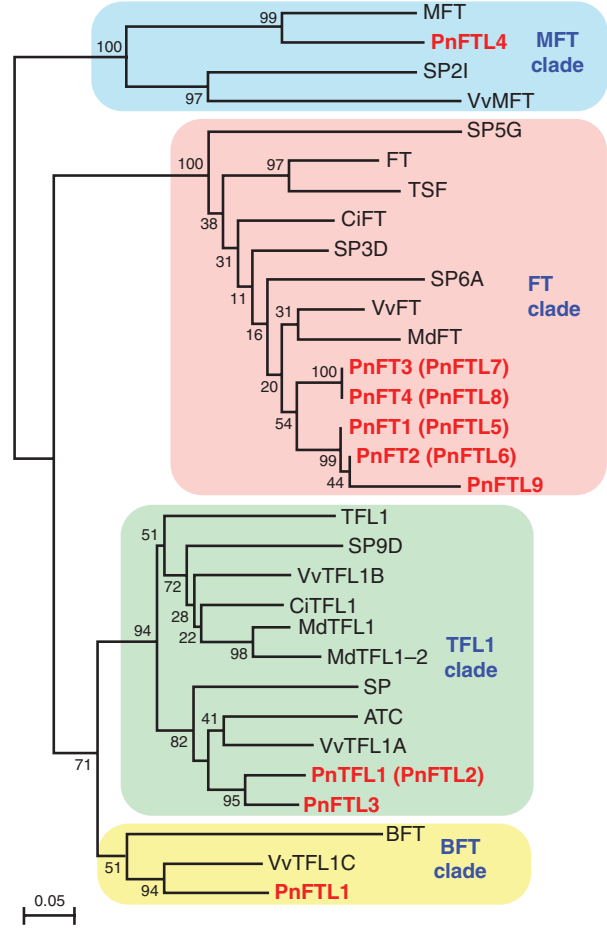

Fig. 2 Phylogenetic analysis of the members in the *FT*/*TFL1* family. The tree was constructed by the Neighbor-Joining (N-J) method for members of the *FT*/*TFL1* family in Lombardy poplar (*PnFTL1*, *PnTFL1*, *PnFTL3*, *PnFTL4*, *PnFT1*, *PnFT2*, *PnFT3*, *PnFT4* and *PnFTL9*), *A. thaliana* (*FT*, *TFL1*, *MFT*, *ATC*, *FT*, *TSF* and *BFT*), tomato (*SP*, accession No. U84140; *SP2I*, accession No. AY186734; *SP3D*, accession No. AY186735; *SP5G*, accession No. AY186736; *SP6A*, accession No. AY186737; and *SP9D*, accession No. AY186738), grapevine (*VvFT*, accession No. DQ871590; *VvTFL1A*, accession No. DQ871591; *VvTFL1B*, accession No. DQ871592; *VvTFL1C*, accession No. DQ871593; and *VvMFT*, accession No. DQ871594), apple (*MdTFL1*, accession No. AB162040; *MdTFL1-2*, accession No. AB162046; and *MdFT*, accession No. AB161112) and citrus (*CiFT*, accession No. AB027456; and *CiTFL1*, accession No. AY344244). The N-J unrooted dendrograms were generated from alignment of deduced amino acids with the ClustalW program, and the phylogenetic tree was displayed by the MEGA4 software package (Tamura et al. 2007). Bootstrap values for 100 resamplings are shown on each branch. The scale indicates the average number of substitutions per site. Genes in Lombardy poplar are in red.

in early-summer leaves and capsules of reproductive trees (Fig. 4E). Similarly, we detected strong expression of *PnFT2* in early-summer leaves and capsules of reproductive trees, while levels of expression in several vegetative organs, such as roots, stems and winter lateral (vegetative) buds, were very low compared with those of *PnFT1* (Fig. 4F). The pattern of expression of *PnFT3*/*4* was rather different. We detected strong expression of *PnFT3*/*4* in stems and winter lateral buds, whereas expression in early-summer leaves during the reproductive phase was barely detectable (Fig. 4G).

The *PnFTL1* gene, the ortholog of *BFT*, was expressed mainly in the organs of juvenile-phase trees (Fig. 4A), and *PnFTL4*, the ortholog of *MFT*, was expressed mainly in the seeds (Fig. 4D).

Table 2 Location of the counterparts of *PnFTL* genes in the genome of black cottonwood

Lombardy poplar gene	Location of counterpart in black cottonwood
PnFTL1	Chromosome XV
PnTFL1 (*PnFTL2*)	Scaffold_66[a]
PnFTL3	Chromosome IX
PnFTL4	Chromosome XV
PnFT1 (*PnFTL5*)	Chromosome X
PnFT2 (*PnFTL6*)	Scaffold_1409[a]
PnFTL9	Scaffold_1444[a]
PnFT3 (*PnFTL7*)	Chromosome VIII
PnFT4 (*PnFTL8*)	

[a]A scaffold is a portion of the genomic sequence that has been reconstructed from end-sequenced whole-genome shotgun clones; the actual location of each scaffold within the genome is unknown.

Seasonal changes in expression of PnFTL genes

To examine detailed patterns of expression of genes in the *FT* clade, namely *PnFT1*, *PnFT2* and *PnFT3*/*4*, we collected the leaves and lateral buds of branches from juvenile-phase trees and from healthy branches and probable floral regions of fruit-bearing branches of reproductive-phase trees. The result of monitoring the fate of lateral buds in probable floral region of fruit-bearing branches showed that approximately 86% of lateral buds in the region would differentiate into floral buds (data not shown). At the end of May, we detected the strong expression of *PnFT1* and of *PnFT2* in leaves of healthy branches and fruit-bearing branches from reproductive-phase trees; levels of expression were almost 1,000 times higher than those in branches of juvenile-phase trees. These high levels fell to basal levels by the middle of August (Fig. 5A, B). The levels

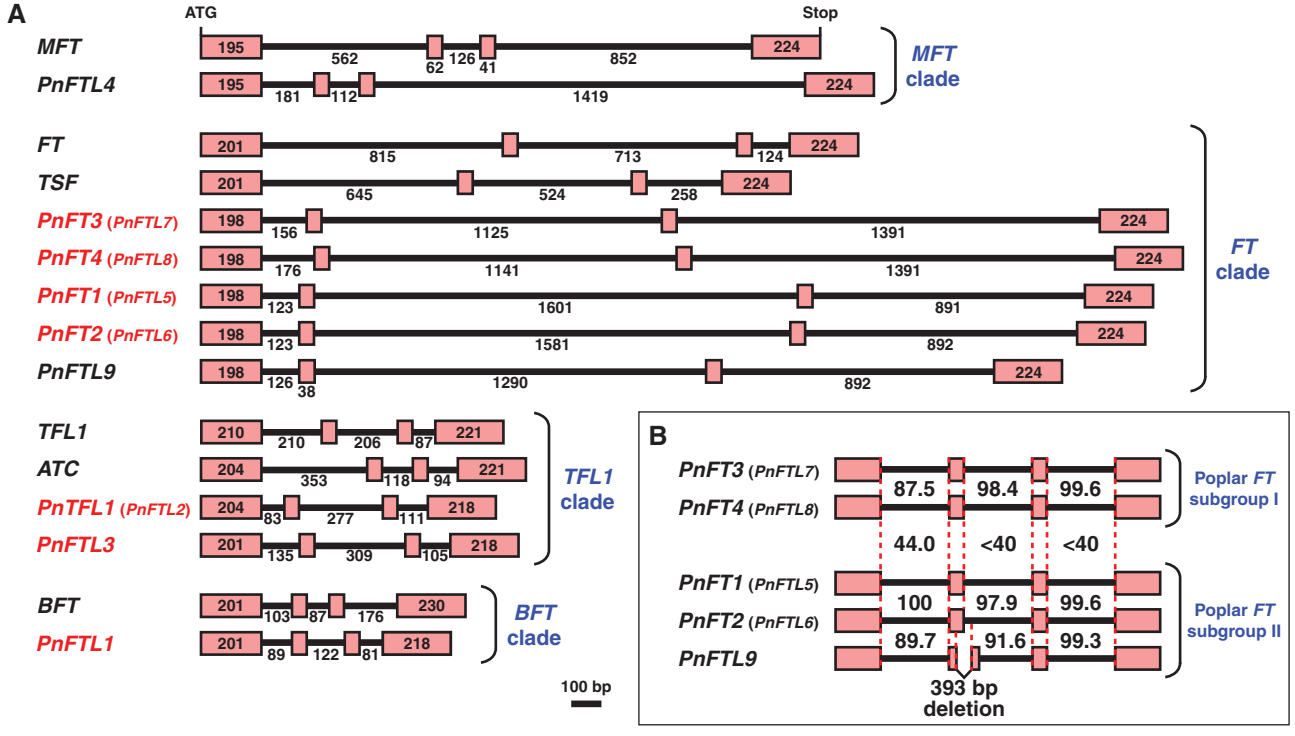

Fig. 3 (A) Genomic organization of members of the *FT*/*TFL1* family in Lombardy poplar and *Arabidopsis*. Boxes represent exons and lines represent introns. Numbers indicate the lengths of exons and introns (bp). (B) Comparison of the introns in genes in the *FT* calde. The similarities between introns (as percentages) are shown.

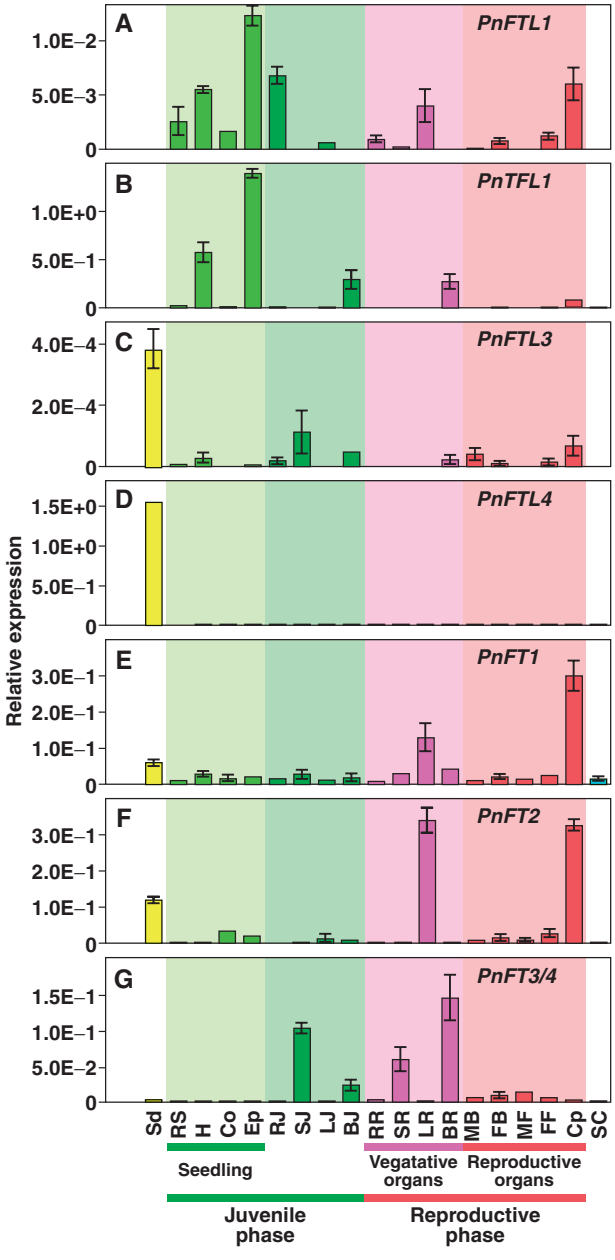

Fig. 4 Expression of genes in the *FT*/*TFL1* family in various organs and cells of Lombardy poplar. An aliquot of first-strand cDNA mixture, corresponding to 50 ng of total RNA, was amplified by PCR with gene-specific primers, as listed in Supplementary Table S1. Levels of detected amplicons were normalized by reference to amplified products that corresponded to the gene for polyubiquitin in Lombardy poplar (*PnUB1*; accession No. AB182939). Values are means ± SE from the results of 3–6 replicates. Sd, seeds; RS, H, Co and Ep, roots, hypocotyls, cotyledons and epicotyls of 2-week-old seedlings; RJ, SJ, LJ and BJ, roots, stems, early-summer leaves and winter lateral buds of juvenile-phase plants; RR, SR, LR, BR, MB, FB, MF, FF and Cp, roots, stems, early-summer leaves, winter lateral buds, male flower buds, female flower buds, male flowers, female flowers and capsules of reproductive phase plants; and SC, cells of Lombardy poplar in suspension culture.

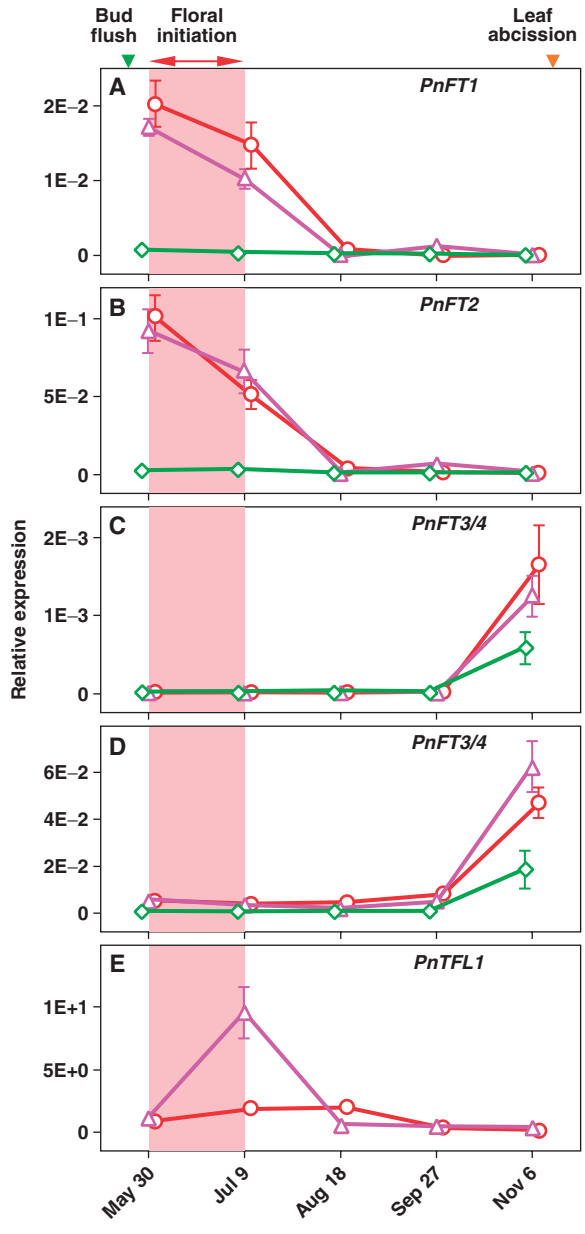

Fig. 5 Seasonal variations in the expression of genes in the *FT*/*TFL1* family of Lombardy poplar. (A–E) The expression of *PnFT1* (A), *PnFT2* (B) and *PnFT3/4* (C) in the leaves and the expression of *PnFT3/4* (D) and *PnTFL1* (E) in the lateral buds of Lombardy poplar trees at the juvenile phase (5 years old; diamonds) and on healthy branches (triangles) and fruit-bearing branches (circles) of trees in the reproductive phase (>30 years old). Shaded areas indicate the probable period for initiation of flowers, and arrowheads indicate the periods of bud flush and leaf abscission. Five mature leaves and three sets of lateral buds (approximately 200 mg each) were collected and pooled at 2 p.m. on the date indicated. An aliquot of first-strand cDNA mixture, corresponding to 0.5 µg of total RNA, was amplified by PCR with gene-specific primers. Levels of detected amplicons were normalized by reference to amplified products that corresponded to *PnUB1*. Values are means ± SE of results from five replicates. Each symbol without a bar indicates that the SE fell within the symbol.

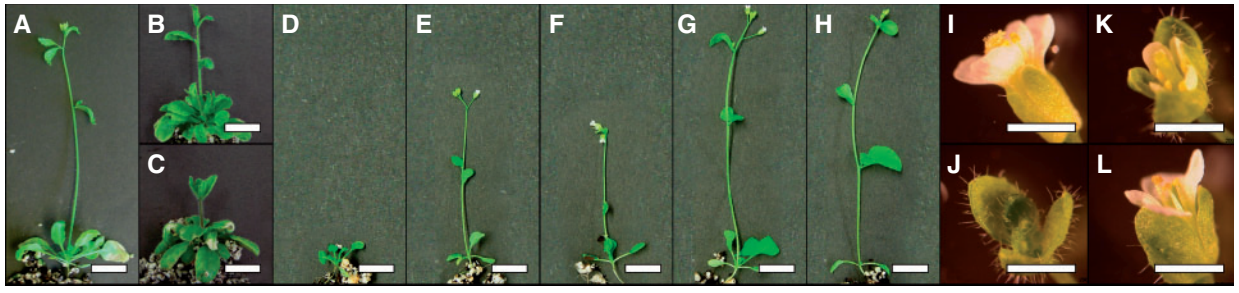

Fig. 6 Phenotypes of transgenic *Arabidopsis* ectopically expressed genes in the *FT*/*TFL1* family from Lombardy poplar under long-day conditions. (A–H) A 21-day-old wild-type plant (A; control), 35-day-old transgenic plants that harbored P35S::*PnTFL1* (B) and P35S::*PnFTL3* (C), a 14-day-old wild-type plant (D; control) and 14-day-old transgenic plants that harbored P35S::*FT* (E), P35S::*PnFT1* (F), P35S::*PnFT2* (G) and P35S:: *PnFT3/4* (H). (I–L) Flowers of wild-type (I) and transgenic P35S::*PnTFL1* plants (J–L). Bars in (A)–(H) represent 1 cm and those in (I)–(L) represent 1 mm.

Table 3 Comparison of flowering time and number of rosette leaves in transgenic *Arabidopsis* plants that ectopically expressed individual *PnFTL* genes

Construct	Days to flowering	No. of rosette leaves
WS (control)	22.7 ± 0.5^{b}	6.7 ± 0.5^{b}
P35S::*FT*	14.5 ± 0.5^{a}	3.5 ± 0.4^{a}
P35S::*PnFT1* (*PnFTL5*)	12.0 ± 0.4^{a}	2.1 ± 0.1^{a}
P35S::*PnFT2* (*PnFTL6*)	11.7 ± 0.5^{a}	2.4 ± 0.2^{a}
P35S::*PnFT3/4* (*PnFTL7/8*)	13.0 ± 0.5^{a}	2.9 ± 0.2^{a}
P35S::*TFL1*	31.7 ± 1.9^{c}	8.5 ± 0.6^{c}
P35S::*PnTFL1* (*PnFTL2*)	40.3 ± 5.7^{c}	10.6 ± 0.4^{c}
P35S::*PnFTL3*	36.8 ± 4.4^{c}	10.0 ± 0.6^{c}
P35S::*PnFTL1*	20.3 ± 0.4^{b}	6.4 ± 0.1^{b}
P35S::*PnFTL4*	22.8 ± 0.4^{b}	6.8 ± 0.4^{b}

Plants were grown under LD. Values are means ± SE of the results from 12 independent lines of transgenic plants. Scheffé's multiple comparison test was used to compare the effects of treatments. Superscripts letters indicate a statistically significant difference from results with other letters in the same column ($P < 0.05$).

in leaves of branches at the juvenile phase did not change significantly during this period (Fig. 5A, B). However, the levels of expression of *PnFT1* and of *PnFT2* were quite low in the lateral buds of all branches, and we did not observe any seasonal changes (data not shown).

The patterns of expression of *PnFT3/4* were different from those of *PnFT1* and *PnFT2* (Fig. 5C). Levels of expression of *PnFT3/4* had risen 20- to 100-fold in the leaves of each branch examined by the beginning of November, when senescence was induced in leaves of Lombardy poplar (Fig. 5C). Similarly, in the lateral buds of each branch, levels of expression of *PnFT3/4* had risen almost 10-fold by the beginning of November (Fig. 5D).

We also analyzed expression of *PnTFL1* in the lateral buds of each branch. The level of expression of *PnTFL1* had risen 10-fold in the lateral buds of healthy branches examined by the beginning of July, and fell to basal levels by the middle of August (Fig. 5E). A similar pattern was observed in lateral buds of branches from juvenile-phase trees (data not shown). On the other hand, in the lateral buds from fruit-bearing branches, the level of expression of *PnTFL1* had risen approximately 2-fold by the beginning of July (Fig. 5E).

Phenotypes of transgenic Arabidopsis plants that expressed ectopic PnFTL genes

To determine the functions of *PnFTL* genes, we introduced the ORF of each gene under the control of the cauliflower mosaic virus 35S promoter (P35S) into *Arabidopsis*. Then, we selected 12 independent homozygous lines, in the third generation for each construct, for phenotypic analyses (i.e. $n = 12$ in all cases below).

Transgenic *Arabidopsis* that ectopically expressed the P35S::*PnTFL1* and P35S::*PnFTL3* constructs (*TFL1* clade), required 40.3 ± 5.7 and 36.8 ± 4.4 d, respectively, for flowering, whereas wild-type plants flowered within 22.7 ± 0.5 d under long-day (LD; 16 h light/8 h dark) conditions (Fig. 6A–C and Table 3). In addition, in the case of the first generation of P35S::*PnTFL1* and P35S::*PnFTL3* plants, two lines in each case did not flower during >5 months of growth and failed to produce any seeds (data not shown). Each gene affected the vegetative and the early-inflorescence stage of *Arabidopsis*, as revealed by the increased numbers of nodes produced before and after bolting. During the juvenile phase, wild-type *Arabidopsis* plants produced 6.7 ± 0.5 rosette leaves under LD conditions, whereas the P35S::*PnTFL1* and P35S::*PnFTL3* plants produced 10.6 ± 0.4 and 10.0 ± 0.6 rosette leaves, respectively (Table 3). The numbers of cauline leaves on the main stems were also higher in both types of transgenic plant than in wild-type plants after the plants had bolted (data not shown). In addition, we frequently observed abnormal flowers with leaf-like sepals (Fig. 6J–L) and

occasionally inflorescences that were developing on flowers themselves.

In contrast, transgenic *Arabidopsis* that expressed P35S::*PnFT1*, P35S::*PnFT2* and P35S::*PnFT3*/*4* constructs (*FT* clade) had a dramatic early-flowering phenotype (Fig. 6F–H and Table 3). Flowering of P35S::*PnFT1*, P35S::*PnFT2* and P35S::*PnFT3*/*4* plants occurred after 12.0 ± 0.4, 11.7 ± 0.5 and 13.0 ± 0.5 d, respectively, and each plant had 1.9 ± 0.1, 2.2 ± 0.2 and 2.6 ± 0.2 rosette leaves, respectively (Table 3).

PnFTL1 and *PnFTL4* did not affect the flowering, flower morphologies and inflorescences of transgenic *Arabidopsis* under LD conditions. Flowering of P35S::*PnFTL1* and P35S::*PnFTL4* plants occurred after 20.3 ± 0.4 and 22.8 ± 0.4 d, respectively, and each plant had 6.4 ± 0.1 and 6.8 ± 0.4 rosette leaves, respectively (Table 3).

Discussion

Collectively, we have identified nine members of the *PnFTL* gene family, as revealed by comparisons of sequences and genomic organization (Table 1 and Fig. 3). We thought that we had isolated and identified all members of the *FT*/*TFL1* gene family in Lombardy poplar for the following reasons. Enough numbers of genes in the *FT*/*TFL1* family were isolated with the comparison of the numbers of those in *Arabidopsis*, tomato, rice (*Oryza sativa*) and grapevine (Kobayashi et al. 1999, Carmel-Goren et al. 2003, Chardon and Damerval 2005, Carmona et al. 2007), and with the result of the comparison with the genome of black cottonwood (Table 2). Our phylogenetic analysis showed that the nine genes fall into four clades: two into the *TFL1* clade; five into the *FT* clade; and one each into the *MFT* and *BFT* clades (Fig. 2). Our results provide the first comprehensive description of the *FT*/*TFL1* family in *Populus* species, and to our knowledge this is the first report to demonstrate the function of genes in the *TFL1* clade in poplar.

The expression of *PnTFL1* indicated that this gene is probably associated with meristematic regions, such as lateral buds (Fig. 4B), and the levels of expression of this gene increased remarkably in vegetative buds during the probable period for initiation of flowers (Fig. 5E). The pattern of expression of *PnFTL3* resembled that of *PnTFL1*, although the patterns did not overlap completely (Fig. 4B, C). Both genes, however, prevented determination of the floral meristem in transgenic *Arabidopsis* (Fig. 6B, C and Table 3). Moreover, we observed abnormal flowers with leaf-like sepals on both P35S::*PnTFL1* and P35S::*PnFTL3* transgenic *Arabidopsis* plants (Fig. 6J–L). Similar observations have been reported in *Arabidopsis* plants that ectopically expressed genes in the *TFL1* clade, namely *TFL1* of *Arabidopsis* (Ratcliffe et al. 1998), *LpTFL1* of ryegrass (Jensen et al. 2001) and *VvTFL1A* of grapevine (Carmona et al. 2007). Thus, our results suggest that these genes might play a role in the maintenance of meristematic functions during vegetative development in Lombardy poplar, as well as in other plants. Moreover, the *TFL1*-like genes in other woody plants, citrus and apple appear to be involved in the regulation of the juvenile phase (Pillitteri et al. 2004, Kotoda et al. 2006). We plan to examine whether suppression of the expression of *PnTFL1* might reduce the length of the juvenile phase in Lombardy poplar. Further analyses of these genes, using transgenic poplar, might provide information about their involvement in the transition from the juvenile to the reproductive phase.

Although each of the genes in the *FT* clade had a similar *FT*-like activity in transgenic *Arabidopsis* (Fig. 6F–H and Table 3), the patterns of expression differed between subgroup I (*PnFT3* and *PnFT4*) and subgroup II (*PnFT1* and *PnFT2*). The genes in subgroup II were expressed mainly in the early-summer leaves of Lombardy poplar during the reproductive phase and they were not expressed during the juvenile phase, although the patterns of expression of *PnFT1* and *PnFT2* did not overlap completely (Fig. 5A, B). These observations resemble those reported for one of the counterparts of these genes in eastern cottonwood (*P. deltoides*) by Hsu et al. (2006) that an increase in the expression of this gene might be important for transition from the juvenile to the reproductive developmental stage in poplar. Thus, in poplar, as in *Arabidopsis* and rice, the FT protein produced in the leaves might move to the meristem and promote flowering (Corbesier et al. 2007, Tamaki et al. 2007). However, we failed to find any differences in terms of the patterns and levels of expression of these genes between healthy branches, which seldom produce flowers, and fruit-bearing branches (Fig. 5A, B). It seems likely, therefore, that the state of branches (vegetative or reproductive) might be controlled by other factors in addition to the patterns and levels of the expression of *FT* and related genes, and we had thought that the patterns and levels of expression of *PnTFL1* indicated that it is one of the candidates for the factors of floral initiation. In contrast, the expression of *PnFT3*/*4*, which was classified into another subgroup, increased in senescent leaves of both reproductive and juvenile-phase plants late in the year (Fig. 5C). These results support the suggestion by Böhlenius et al. (2006) that an increase in the expression of the counterpart of *PnFT3*/*4* in European aspen (*P. tremula*) might be important for short-day-induced cessation of growth. However, this latter gene is also expressed in early-summer leaves, an observation that suggests that floral initiation might also be controlled by this gene in European aspen (Böhlenius et al. 2006). Although the European aspen is very different in this respect from Lombardy poplar, differences among species or sections within the genus *Populus* would not be unexpected.

Böhlenius et al. (2006) and Hsu et al. (2006) demonstrated that the acceleration of flowering is simply dependent on increasing levels of endogenous functional FT protein by the analyses of the transgenic *Populus* that overexpressed an *FT* gene in each subgroup.

Neither the *PnFTL1* nor the *PnFTL4* gene influenced flowering time in transgenic *Arabidopsis* that expressed each respective gene ectopically (Table 3). However, since both genes exhibited restricted patterns of expression and included sequences conserved in each clade that had been isolated from several other species, we can assume that these genes are functional. Further analysis is now necessary to validate this assumption.

In Lombardy poplar and black cottonwood, we found five and four genes in the FT clade (Table 2). In other species, the number of genes in the *FT* clade was 1–3; namely, in *Arabidopsis*, tomato, rice and grapevine, there were two, three, two and one genes, respectively (Kobayashi et al. 1999, Carmel-Goren et al. 2003, Chardon and Damerval 2005, Carmona et al. 2007). This considerable redundancy of genes in the *FT* clade suggests that these genes might play important roles in the life cycle in the genus *Populus*.

In summary, we isolated nine members of the *FT*/*TFL1* family from Lombardy poplar and identified the functions of the some of their products. Our goal is to clarify not only mechanisms of flowering but also phenomena that control flowering in woody plants in order to exploit them for breeding purposes.

Materials and Methods

Plant materials

Lombardy poplar trees that had been planted on the grounds of Hokkaido University (Sapporo, Japan) were used as source material. A clonal strain of Lombardy poplar, propagated by cutting, was grown in a phytotron at 25°C and at 70% relative humidity under metal halide lamps [500 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR); 16 h photoperiod].

Arabidopsis thaliana seeds were stratified for 3–4 d at 4°C and then grown on a solid medium that contained Murashige and Skoog's basal salts (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968) and 1% sucrose, supplemented with 0.4% gellan gum (MSB5 medium), in a growth chamber at 22°C under cool white fluorescent light (50 μmol m^{-2} s^{-1} of PAR, 16 h photoperiod). A 7–10 d after sowing, seedlings were transferred to soil and grown under the same conditions.

Isolation of total RNA

Total RNA was extracted from various organs and tissues of Lombardy poplar with hexadecyltrimethylammonium bromide (Shinohara and Murakami 1996), and purified with a Wizard® SV Total RNA Isolation kit (Promega, Madison, WI, USA). First-strand cDNA was synthesized from 5 μg of total RNA by reverse transcriptase with an oligo$(dT)_{20}$ primer according to the instructions supplied with ReverTra Ace® (Toyobo, Tokyo, Japan).

Isolation and sequencing of FT/TFL1 genes from Lombardy poplar

We performed PCR using the sets of primers shown in Supplementary Table S1. Partial *PnFTL* genes were amplified with degenerate primers whose design was based on conserved regions of plant *FT*/*TFL1* genes. The ends of the ORFs of genes were determined by 3′ and 5′ RACE-PCR (Frohman et al. 1988) or inverse PCR (Ochman et al. 1988). All products of PCR were subcloned and sequenced (ABI Prism 3100 and 3100-Avant DNA sequencer; Applied Biosystems, Foster City, CA, USA). The sequences of full-length ORFs and genomic sequences were determined by sequencing of subcloned products of PCR that had been amplified with first-strand cDNA or genomic DNA derived from an individual female clone as template. To exclude the possibility of recombination or mutation by PCR, we performed triplicate experiments and sequenced >100 clones per gene.

Sequence alignment and construction of a phylogenetic tree

Sequences were aligned by the N-J method with ClustalW software (Saitou and Nei 1987, Thompson et al. 1994). An N-J tree was produced from the results of 1,000 bootstrap replicates using ClustalW, and the phylogenetic tree was displayed with the MEGA4 software package (Tamura et al. 2007).

Analysis of gene expression

We performed real-time PCR for detection of the expression of genes in the *FT*/*TFL1* family in Lombardy poplar using the LightCycler™ system (Roche, Penzberg, Germany) with a 'LightCycler™ FastStart DNA Master SYBR Green I' kit (Roche). The specific transcript of each Lombardy poplar *FT*/*TFL1* family member was identified with gene-specific primers, as shown in Supplementary Table S1, and primers specific for the Lombardy poplar gene for polyubiquitin (*PnUB1*) were used for the control. Reactions were allowed to proceed for 45 cycles, as follows: 10 s at 95°C and 16 s at 66°C, with each set of *PnFTL* gene-specific primers (Supplementary Table S2). Values were normalized on the basis of the amount of *PnUB1* cDNA.

Transformation of Arabidopsis

Agrobacterium tumefaciens GV3101 (pMP90), harboring a binary vector, was used to transform *Arabidopsis* plants by the floral dip method (Clough and Bent 1998). All binary vectors used in this study were constructed by introducing *FT*/*TFL1* cDNA into the hygromycin-selectable binary vector pSMAH621 (Igasaki et al. 2000). For selection of transformed plants, sterilized seeds were plated on MSB5 medium supplemented with 20 mg l^{-1} hygromycin (Wako Pure Chemical Industries, Osaka, Japan) and allowed to germinate in the growth chamber. Hygromycin-resistant transformants were transplanted to soil on the tenth day after sowing. The expression of introduced genes in selected transformants was confirmed by RT–PCR. The day of sowing was taken as day 0. We analyzed the morphology of plants in the third generation.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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