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ORIGINAL ARTICLE

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Isolation of *LEAFY* and *TERMINAL FLOWER 1* homologues from six fruit tree species in the subfamily Maloideae of the Rosaceae

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Abstract Flowering is an essential stage of fruit production. To understand the molecular mechanisms controlling flowering in maloid fruit tree species, we isolated and analyzed genes homologous to *Arabidopsis LEAFY* (*LFY*; flower meristem identity gene) and *TERMINAL FLOWER 1* (*TFL1*; inflorescence meristem identity gene) from six fruit tree species in the subfamily Maloideae of the Rosaceae; apple (*Malus* × *domestica*), Japanese pear (*Pyrus pyrifolia*), European pear (*Pyrus communis*), quince (*Cydonia oblonga*), Chinese quince (*Chaenomeles sinensis*), and loquat (*Eriobotrya japonica*). Two *LFY* homologues and two *TFL1* homologues were cloned from all six maloid species by rapid amplification of 3′ and 5′ cDNA ends, reverse transcription-PCR, and PCR with genomic DNA. Phylogenetic analysis by the neighbor-joining method showed that the two *LFY* homologues and two *TFL1* homologues were classified into two distinct clades. The presence of multiple copies of *LFY* and *TFL1* homologues is discussed with reference to the polyploid origin of the subfamily Maloideae.

Keywords Maloideae · *LEAFY* · *TERMINAL FLOWER 1*

The nucleotide sequence data reported here are available in the DDBJ/EMBL/GenBank database under the accession numbers AB162028 (*AFL1-fuji*), AB162029 (*PpLFY-1*), AB162030 (*PcLFY-1*), AB162031 (*CoLFY-1*), AB162032 (*CsLFY-1*), AB162033 (*EjLFY-1*), AB162034 (*AFL2-Fuji*), AB162035 (*PpLFY-2*), AB162036 (*PcLFY-2*), AB162037 (*CoLFY-2*), AB162038 (*CsLFY-2*), AB162039 (*EjLFY-2*), AB162040 (*MdTFL1-1*), AB162041 (*PpTFL1-1*), AB162042 (*PcTFL1-1*), AB162043 (*CoTFL1-1*), AB162044 (*CsTFL1-1*), AB162045 (*EjTFL1-1*), AB162046 (*MdTFL1-2*), AB162047 (*PpTFL1-2*), AB162048 (*PcTFL1-2*), AB162049 (*CoTFL1-2*), AB162050 (*CsTFL1-2*), AB162051 (*EjTFL1-2*).

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Introduction

Flowering is an essential stage for fruit production, and thus an understanding of the genetic mechanisms underlying the flowering event is important for efficient fruit production. During the last decade, molecular mechanisms of flowering have been studied extensively in herbaceous "model" plants such as *Arabidopsis* and snapdragon (*Antirrhinum majus*). Genetic models for flowering time, floral meristem identity, and flower differentiation have been proposed upon the identification of several transcriptional regulator genes (Araki 2001; Battey and Tooke 2002; Parcy et al. 1998; Ratcliffe et al. 1999; Liljegren et al. 1999). In contrast, studies on the molecular mechanisms of flowering in fruit trees have just begun. Several flowering-related genes have been cloned from grapevine (*Vitis vinifera*) (Carmona et al. 2002; Boss et al. 2001), kiwifruit (*Actinidia deliciosa*) (Walton et al. 2001), and apple (*Malus* × *domestica*) (Kotoda et al. 2000, 2002; Wada et al. 2002; Sung et al. 1999, 2000; Jeong et al. 1999). In this paper, we isolated genes homologous to *Arabidopsis LEAFY* (*LFY*) and *TERMINAL FLOWER 1* (*TFL1*) from six fruit tree species of the subfamily Maloideae of the Rosaceae.

LFY is a transcriptional regulation gene thought to play a primary role in determining flower meristem identity. *FLORICAULA* (*FLO*), a *LFY* homologue in snapdragon, has been shown to have almost the same role. Loss of function mutants of these genes result in the conversion of flowers into indeterminate secondary shoots (Coen et al. 1990; Weigel et al. 1992). Conversely, constitutive expression of *LFY* and its homologues has been shown to be sufficient to promote flower initiation and development in *Arabidopsis*, poplar (Weigel and Nilsson 1995), and citrus (Pena

et al. 2001). Furthermore, alternation of inflorescence architecture, from indeterminate to determinate, was observed in *Arabidopsis* transformed with a chimeric *LFY* gene expressed constitutively under the control of the cauliflower mosaic virus (CaMV) 35S promoter.

TFL1 encodes a protein that likely plays a role in signaling, perhaps as an inhibitor of mitogen-activated protein kinase pathways (Corbit et al. 2003), and is a putative regulator gene involved in the control of flowering time and floral architecture. The *Arabidopsis tfl1* mutant forms a terminal flower immediately after bolting and inflorescence architecture is altered from indeterminate to determinate. Furthermore, the mutants bolt and flower earlier than the wild type (Shannon et al. 1991; Bradley et al. 1997). *CENTRORADIALIS* (*CEN*), a *TFL1* homologue in snapdragon, has almost the same function as *Arabidopsis TFL1*, and the *cen* mutant shows altered inflorescence architecture (from indeterminate to determinate; Bradley and Meeks-Wagner 1996). A recent study in pea (*Pisum sativum*) revealed the presence of three homologues of *TFL1*. Interestingly, one of these is involved in inflorescence development and another in the transition from vegetative to reproductive phase (Foucher et al. 2003).

The Maloideae is one of the four subfamilies of the Rosaceae, which includes several important fruit tree species such as apple (*M.* × *domestica*), Japanese pear (*Pyrus pyrifolia*), European pear (*Pyrus communis*), quince (*Cydonia oblonga*), Chinese quince (*Chaenomeles sinensis*), and loquat (*Eriobotrya japonica*). Their floral morphological and phenological traits, such as inflorescence architecture and bloom season, vary. Pear forms a raceme inflorescence with eight to ten flowers. The pear inflorescence is considered to be indeterminate because side or lateral flowers bloom first. Apple also forms a raceme inflorescence with five flowers. In contrast to the pear inflorescence, that of apple is considered to be determinate because a terminal flower blooms first (Westwood 1978). Loquat forms a big inflorescence with 50–60 flowers, which is comprised of several panicles. In contrast to these species, quince and Chinese quince bear solitary flowers on shoot apexes without forming peduncles. The bloom season of maloid fruit tree species also varies, with apple, pear, and quince blooming in April and loquat from November to February in Japan.

We assumed that the variation in inflorescence architecture in maloid fruit tree species could be at least partially regulated by *LFY* and *TFL1* homologues because *LFY* and *TFL1* homologues determine flower and inflorescence meristem identities and flowering time in many plant species as described above. In this paper, therefore, we have isolated and characterized *LFY* and *TFL1* homologues from six maloid fruit species as the

Table 1 Sequences of oligonucleotide primers. Degenerate primers designed from conserved regions of *LFY* and *TFL1* and their homologues were used to amplify partial sequence fragments of each homologue. For rapid amplification of cDNA ends (RACE), seven gene-specific primers were designed with the capability for nested PCR and used in combination with 3′- or 5′-GeneRacer adaptor-specific primers. Six gene-specific primers were designed from the 3′- or 5′- untranslated region of each homologue to isolate almost full length cDNA. For expression analysis by RT-PCR, gene-specific primers were designed and paired with the primers used in homologue isolation. Positions of amino acid sequences used to design the primers are indicated in Figs. 1 and 4

	Primer name	Sequence
PCR with genomic DNA		
LFY forward	LFY-F-1	CAGAGGGAGCATCCGTTTATCGTAAC
LFY reverse	LFY-R-1	GACGMAGCTTKGTKGGRACATACCA
TFL1 forward	TFL-F-1	AATGGCCATGAGCTCTTTCCTTC
TFL1 reverse	TFL-R-1	AACGYCTKCKRGCGGCRGTTTC
RACE		
Touch down PCR		
LFY 3′RACE forward	LFY-F-1	Same primer used in degenerate PCR
LFY 5′RACE reverse	LFY-R-2	ATGTCCCAGCCTTGGCCTGCTGCCTT
TFL1 3′RACE forward	TFL-F-2	TCCTGGCCCTAGTGATCCTTATC
TFL1 5′RACE reverse	TFL-R-2	AATGGATGGAGGAGTTCTGGGTACAGCTAC
Nested PCR		
LFY 3′RACE forward	LFY-F-2	GACAAACCAAGTGTTTAGGTATGC
LFY 5′RACE reverse	LFY-R-3	CTTGTTGATGTAGCTTGCCCCTGCCTT
TFL1 3′RACE forward	TFL-F-3	TGTGRCAGACATTCCAGG
TFL1 5′RACE reverse	TFL-R-3	GGCATCTGTGGTGCCTGGAATGTCTG
RT-PCR and PCR with genomic DNA		
LFY-1& LFY-2 forward	LFY-F-3	AYTGTGCTGTGYGGAGTTGTGGAAAATATG
LFY-1 reverse	LFY-R-4	ATTCAGTCTKCCCTAGCCTTAMTAGTACAY
LFY-2 reverse	LFY-R-5	GTAGATCATAACAGGATCCTAAAATATTG
TFL1-1 forward	TFL-F-4	GGARTGCTATTAGCTCCTCCTGAATTG
TFL1-1 reverse	TFL-R-2	Same primer used in touch down PCR for 5′RACE
TFL1-2 forward	TFL-F-5	GAAAAAGCAATATAAGAAGTACTACTCTCT
TFL1-2 reverse	TFL-R-4	TGAAAGTACGTAATAGTGGCCTAAT
Expression analysis by RT-PCR		
LFY-1and LFY-2 forward	LFY-F-4	GATCCAGGTCCAGAACATTGC
LFY-1 reverse	LFY-R-4	Same primer used in RT-PCR for gene isolation
LFY-2 reverse	LFY-R-5	Same primer used in RT-PCR for gene isolation
TFL1-1 forward	TFL-F-6	CCTCCTGAATTGACTTATCCATTAATCT
TFL1-1 reverse	TFL-R-2	Same primer used in touch down PCR for 5′RACE
TFL1-2 forward	TFL-F-5	Same primer used in RT-PCR for gene isolation
TFL1-2 reverse	TFL-R-4	Same primer used in RT-PCR for gene isolation
Actin forward	ACT-F-1	ATGGTGAGGATATTCAACCC
Actin reverse	ACT-R-1	CTTCCTGTGGACAATGGATGG

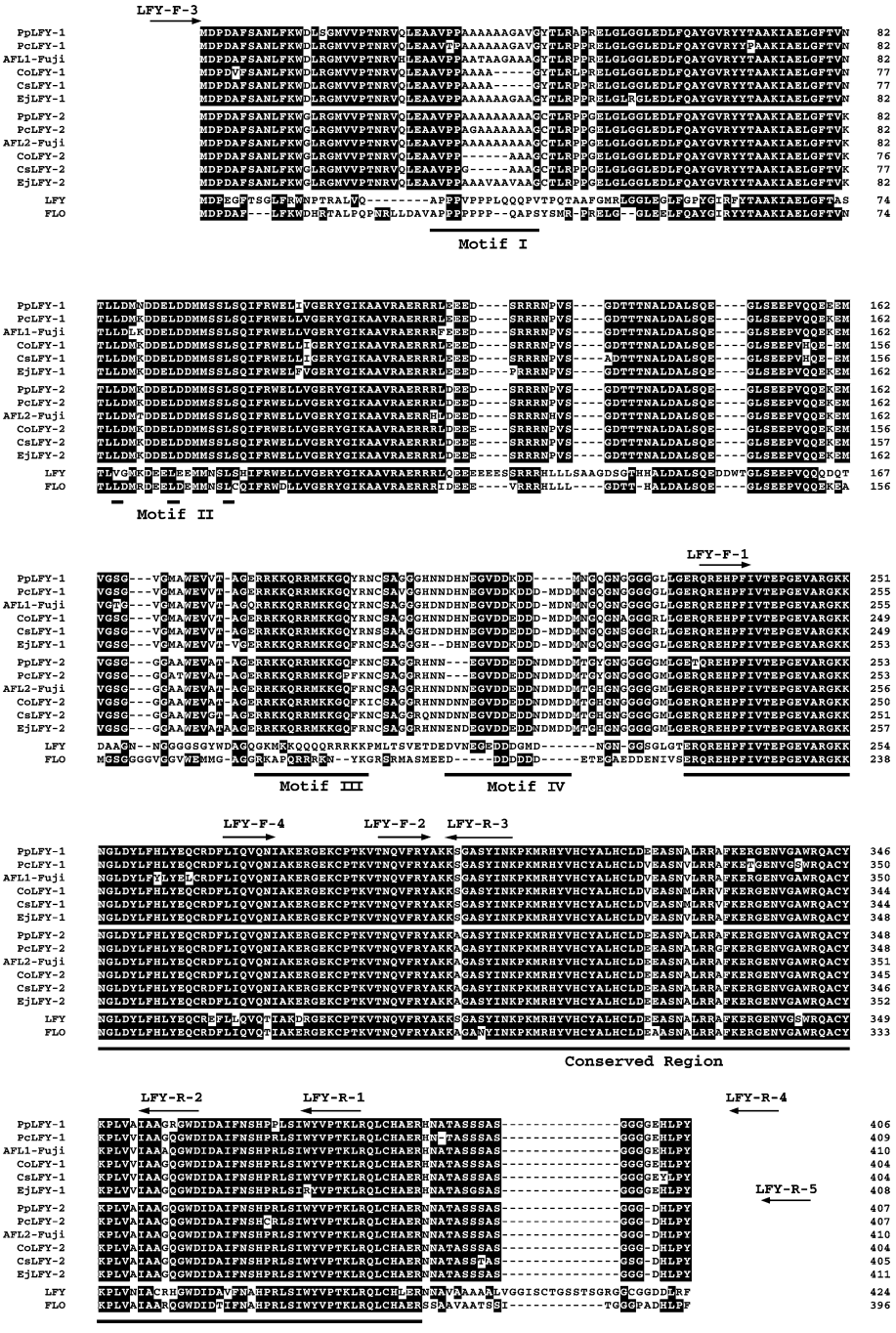

Fig. 1 Amino acid sequence alignment of maloid *LFY* homologues, *Arabidopsis LFY*, and snapdragon *FLO*. Plant species from which sequence data are derived are denoted by initials of their scientific name followed by the name of the group of *LFY* homologues, e.g., *PpLFY-1* means *LFY-1* of *Pyrus pyrifolia*. *AFL1-Fuji* and *AFL2-Fuji* are *LFY* homologues of *Malus* × *domestica* cv. Fuji (see text for details). *Lines below alignment* Highly conserved region and four motifs (see details in text), *arrows above alignment* positions of amino acid sequences used to design primers. Residues conserved in more than 11 sequences are *shaded*

first step towards our ultimate goal of understanding the molecular mechanism controlling flowering and inflorescence development in maloid fruit species.

Materials and methods

Plant materials

Apple (*M.* × *domestica*) cv. Fuji, Japanese pear (*P. pyrifolia*) cv. Housui, European pear (*P. communis*) cv. Bartlett, quince (*C. oblonga*) cv. Smyruna, Chinese quince (*C. sinensis*) cv. unknown, and loquat (*E. japonica*) cv. unknown were used. All plant materials were collected from adult trees grown in the field. Buds before floral differentiation in June, floral buds in September and December, mature leaves, hypanthiums with calyx lobes and ovaries, styles with stigmas, stamens, petals, and peduncles were collected for RNA isolation. Young leaves for DNA isolation were collected in spring. They were stored at −80°C until use.

RNA and DNA isolation

Total RNA was isolated from 0.3–0.5 g plant material by the modified cetyltrimethylammmonium bromide (CTAB) method, as described by Kotoda et al. (2000). Single strand cDNA was synthesized from 1 μg total RNA with oligo-dT adaptor primer by reverse transcriptase ReverTra Ace (Toyobo, Japan) after DNase I treatment (Takara Bio, Japan). Genomic DNA was isolated from 2 g leaves by the CTAB method (Doyle and Doyle 1987) and purified by PEG precipitation (Rowland and Nguyen 1993).

Cloning of homologues by PCR

We used a combination of polymerase chain reaction (PCR) of genomic DNA, reverse transcription (RT)-PCR, and rapid amplification of cDNA ends (RACE) techniques to isolate *LFY* and *TFL1* homologues (Table 1). First, partial sequences of *LFY* and *TFL1* homologues were isolated by RT-PCR with degenerate primers LFY-F-1/LFY-R-1 and TFL-F-1/TFL-R-1, which were designed from conserved regions of *LFY* and *TFL1*, respectively. PCR was performed with TaKaRa Ex *Taq* DNA polymerase (Takara Bio) and PCR products were cloned in pGEM-T easy vector (Promega, Madison, Wis.). DNA sequences of the clones were determined with BigDye (Applied Biosystems, Foster City, Calif.) or Dynamic (Amersham Biosciences, Piscataway, N.J.) terminator cycle sequencing using ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Several *LFY* and *TFL1* homologue gene-specific primers for further experiments were designed based on the partial DNA sequences (Table 1).

3′- and 5′-RACE to obtain full length clones were performed using cDNA of floral buds with a GeneRacer kit (Invitrogen, Carlsbad, Calif.). Touchdown PCR was performed with five cycles of 94°C for 30 s, 72°C for 60 s, followed by five cycles of 94°C for 30 s, 70°C for 60 s and finally 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s using gene-specific primers (LFY-F-1, LFY-R-2, TFL-F-2, and TFL-R-2) paired with GeneRacer primers. Nested PCR was performed with 30 cycles of 94°C for 30 s, 56°C for 30 s, 70°C for 60 s using gene-specific primers (LFY-F-2, LFY-R-3, TFL-F-3, and TFL-R-3) paired with GeneRacer nested primers.

Since two kinds each of *LFY* and *TFL1* homologues were isolated from Japanese pear, quince, and loquat by RACE, two different gene-specific primer sets that could be used to amplify almost full length *LFY* and *TFL1* homologues were designed from conserved regions present at the 5′- and 3′- untranslational regions of the cDNAs (Table 1). RT-PCR was performed with 35 cycles of 94°C for 30 s, 30 s of designated annealing temperatures for the genes to be amplified, 72°C for 60 s. The annealing temperatures used were 54°C for *LFY-1*, 55°C for *LFY-2*, 55°C for *TFL1-1*, and 50°C for *TFL1-2*. *TFL1-2* homologues of apple, quince, and Chinese

Table 2 Identity matrix of deduced amino acid sequences of *LFY* homologues. Plant species for which sequence data are used are denoted by the initials of their scientific name followed by the name of the group of *LFY* homologues, e.g., *PpLFY-1* means *LFY-1* of *Pyrus pyrifolia*. *AFL1-Fuji* and *AFL2-Fuji* are *LFY* homologues of *Malus* × *domestica* cv. Fuji (see text for details). *LFY* and *FLO* mean *LEAFY* (M91208) of *Arabidopsis thaliana* an *FLORICAULA* (M55525) of *Antirrhinum majus*. Amino acid identity scores (%) were calculated by the CLUSTAL X program (Thompson et al. 1997)

	PpLFY-1	PcLFY-1	AFL1-Fuji	CoLFY-1	CsLFY-1	EjLFY-1	PpLFY-2	PcLFY-2	AFL2-Fuji	CoLFY-2	CsLFY-2	EjLFY-2	LFY
PpLFY-1													
PcLFY-1	94												
AFL1-Fuji	94	93											
CoLFY-1	95	94	94										
CsLFY-1	94	94	94	98									
EjLFY-1	95	94	95	95	94								
PpLFY-2	90	89	89	90	89	90							
PcLFY-2	89	88	88	89	89	89	98						
AFL2-Fuji	90	88	89	90	89	90	98	97					
CoLFY-2	90	89	89	90	90	90	97	96	97				
CsLFY-2	90	89	89	90	89	89	96	95	96	98			
EjLFY-2	89	88	88	89	88	89	97	96	97	97	96		
LFY	63	61	62	62	62	62	62	62	62	63	64	62	
FLO	68	68	68	68	68	68	69	69	69	70	70	70	65

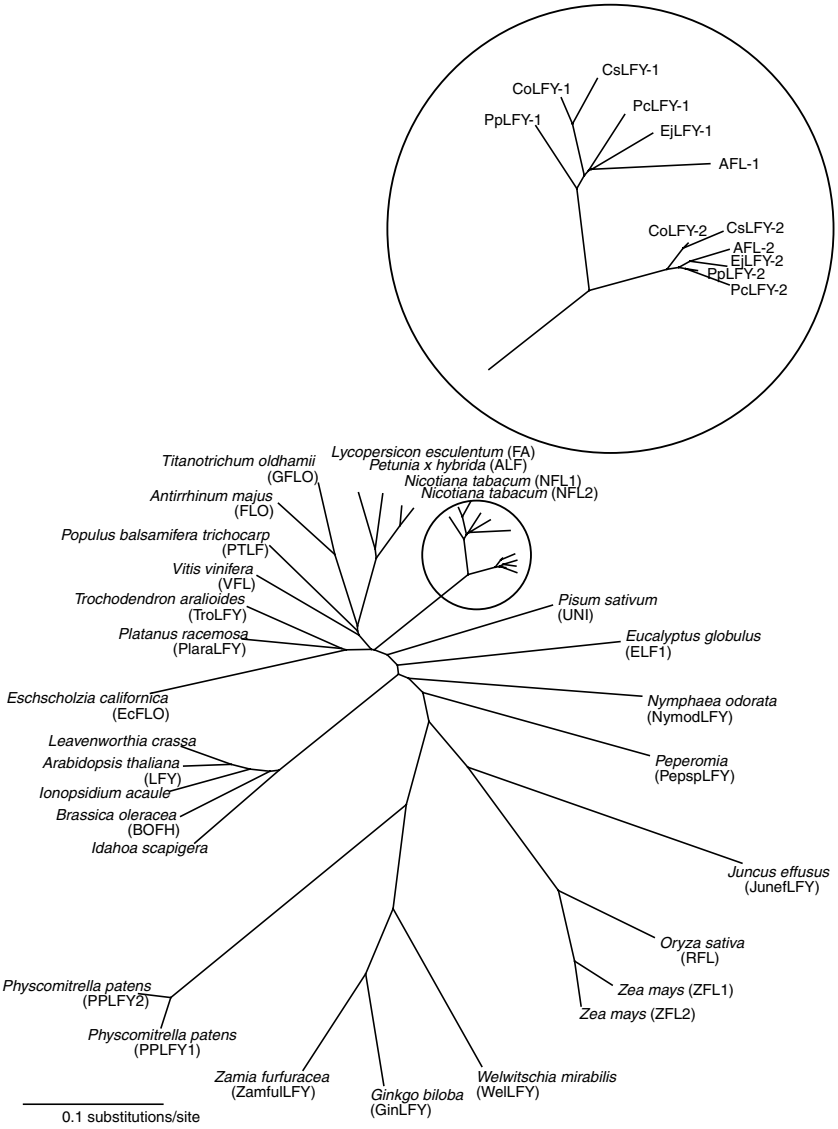

Fig. 2 Phylogenetic tree of *LFY* homologues constructed by the neighbor-joining method (Saitou and Nei 1987). Maloid *LFY* homologues are all located in the portion of the tree indicated by the *circle*, an enlarged version of which is shown above the tree. Abbreviations for maloid *LFY* homologues as in Fig. 1. Abbreviations and accession numbers of other *LFY* homologues in the tree: *Antirrhinum majus* (*FLORICAULA*, M55525), *Arabidopsis thaliana* (*LEAFY*, M91208), *Brassica oleracea* (*BOFH*, Z18362), *Escholzia californica* (*EcFLO*, AY188789), *Eucalyptus globules* (*ELF1*, AF034806), *Ginkgo biloba* (*GinLFY*, AF108228), *Ionopsidium acaule* (AY219226), *Idahoa scapigera* (AY219228), *Juncus effuses* (*JunefLFY*, AF160481), *Leavenworthia crassa* (AY219227), *Lycopersicon esculentum* (*FALSIFLORA*, AF197934), *Nicotiana tabacum* (*NFL1* and *NFL2*, U15798 and U15799), *Nymphaea odorata* (*NymodLFY*, AF105110), *Oryza sativa* (*RFL*, AB005620), *Peperomia* (*PepspLFY*, AF106843), *Petunia* × *hybrida* (*ALF*, AF030171), *Physcomitrella patens* (*PPLFY1* and *PPLFY2*, AB052251 and AB052252), *Pisum sativum* (*UNIFLOLIATA*, AF03516), *Platanus racemosa* (*PlaraLFY*, AF106842), *Populus balsamifera* (*PTLF*, U93196), *Titanotrichum oldhamii* (*GFLO*, AY526319), *Trochodendron aralioides* (*TroLFY*, AF230078), *Vitis vinifera* (*VFL*, AF450278), *Welwitschia mirabilis* (*WelLFY*, AF109130), *Zamia furfuracea* (*ZamfulLFY*, AF105107), *Zea mays* (*ZFL1* and *ZFL2*, AY179882 and AY179881)

quince were cloned by PCR with the genomic DNA using the same primers with 35 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 120 s.

DNA sequence and phylogenetic analysis

cDNA and genomic DNA sequences were aligned using GENETYX (GENETYX, Tokyo, Japan) with manual adjustment. Putative introns in the genomic DNA sequences were determined and excluded from the analyses. Amino acid sequences were aligned and identity scores were calculated using a CLUSTAL X program (Thompson et al. 1997). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei 1987).

Expression analysis

Transcription of *LFY* and *TFL1* homologues of Japanese pear, European pear, apple, and quince were

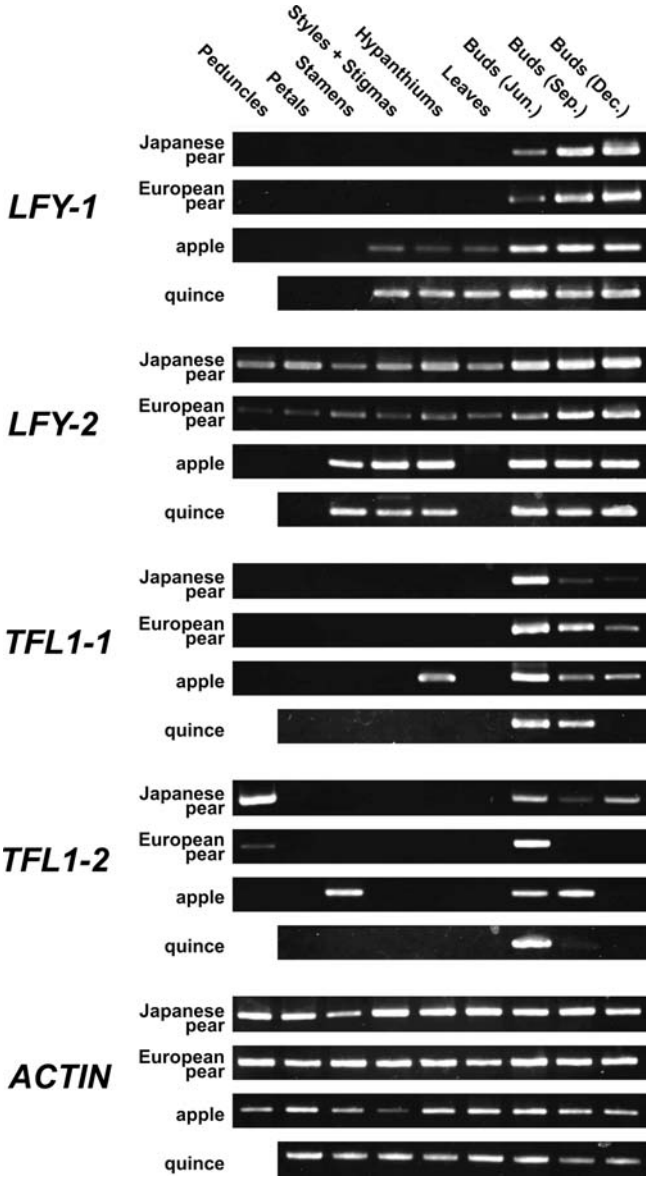

Fig. 3 Transcription of *LFY* and *TFL1* homologues in various organs of Japanese pear, European pear, apple, and quince. RT-PCR amplification of *LFY-1* homologues (LFY-F-4 and LFY-R-4), *LFY-2* homologues (LFY-F-4 and LFY-R-5), *TFL1-1* homologues (TFL-F-6 and TFL-R-2), *TFL1-2* (TFL-F-5 and TFL-R-4), and an actin gene (ACT-F-1 and ACT-R-1) was performed. Total RNA was isolated from peduncles, petals, stamen (anthers and filaments), styles/stigmas, hypanthiums with ovaries and calyx lobes, mature leaves, buds in June (before floral differentiation), floral buds in September, and floral buds in December. In quince, there is no peduncle because a solitary flower attaches directly to the end of the short shoot

determined by RT-PCR. Single strand cDNAs synthesized as described above were used as template. PCR was performed using gene specific primers with an amount of cDNA equivalent to the amount synthesized from 25 ng total RNA (Table 1) and a program consisting of 40 cycles of 94°C for 30 s, 30 s of the appropriate annealing temperatures for the genes to be amplified, 72°C for 45 s. The annealing temperatures used were 54°C for *LFY-1*, 55°C for *LFY-2*, 55°C for *TFL1-1*, and 50°C for *TFL1-2*. As a reference, actin gene-specific primers, ACT-F-1 and ACT-R-1, were used with a PCR program consisting of 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 45 s. The PCR products were electrophoresed on a 1% (w/v) agarose gel, stained with ethidium bromide, and detected using a UV illuminator. The experiment was repeated six times to confirm reproducibility.

Results and discussion

LFY homologues in six maloid fruit tree species

Two different types of cDNAs for *LFY* homologues were isolated from each maloid species used in this study; *PpLFY-1* and *PpLFY-2* for Japanese pear, *PcLFY-1* and *PcLFY-2* for European pear, *CoLFY-1* and *CoLFY-2* for quince, *CsLFY-1* and *CsLFY-2* for Chinese quince, and *EjLFY-1* and *EjLFY-2* for loquat. Since the two apple *LFY* homologues isolated in this study showed more than 99.5% amino acid sequence identity to those already reported (Wada et al. 2002) and seemed to be their alleles, we used the nomenclature of Wada et al. (2002) with the name of the cultivar used, i.e., *AFL1-Fuji* and *AFL2-Fuji*.

Deduced amino acid sequence alignment of *LFY* homologues revealed several species-specific insertions/deletions (indels) (Fig. 1). At around position 35, there are species-specific differences in the number of Ala residues. The number of Ala residues in *CoLFY-1*, *CoLFY-2*, *CsLFY-1*, and *CsLFY-2* was less than in the others. Deletion of a few residues specific to *CoLFY-1*, *CoLFY-2*, *EjLFY-1*, *PpLFY-1*, *PpLFY-2*, and *PcLFY-2* was observed in the central region. At position 180, a single residue insertion was observed in *EjLFY-2*.

LFY and its homologues in many plant species contain several characteristic motifs such as a single highly conserved region and four characteristic motifs as described below (Weigel et al. 1992; Frohlich and Meyerowitz 1997). The highly conserved region present in the C-terminal half of the protein shows very low variation among *LFY* homologues of different plant species. This conserved region is also found in maloid *LFY* homologues cloned in this study (Fig. 1, conserved region). The first characteristic motif found in *LFY* homologues of many plant species is the Pro-rich region located at the N-terminal end and known to be well conserved in *LFY* homologues of angiosperm species, while it is not well conserved in those of gymnosperm (see alignment data of Carmona et al. 2002; Frohlich and Meyerowitz 1997). In maloid *LFY* homologues, however, this region contains only one or two Pro residues followed by a continuous stretch of Ala residues (Fig. 1, motif I). The second characteristic motif is a short putative Leu zipper structure with regular Leu residues. This motif appeared to be completely conserved in the maloid species tested (Fig. 1, motif II). The third characteristic motif is a basic

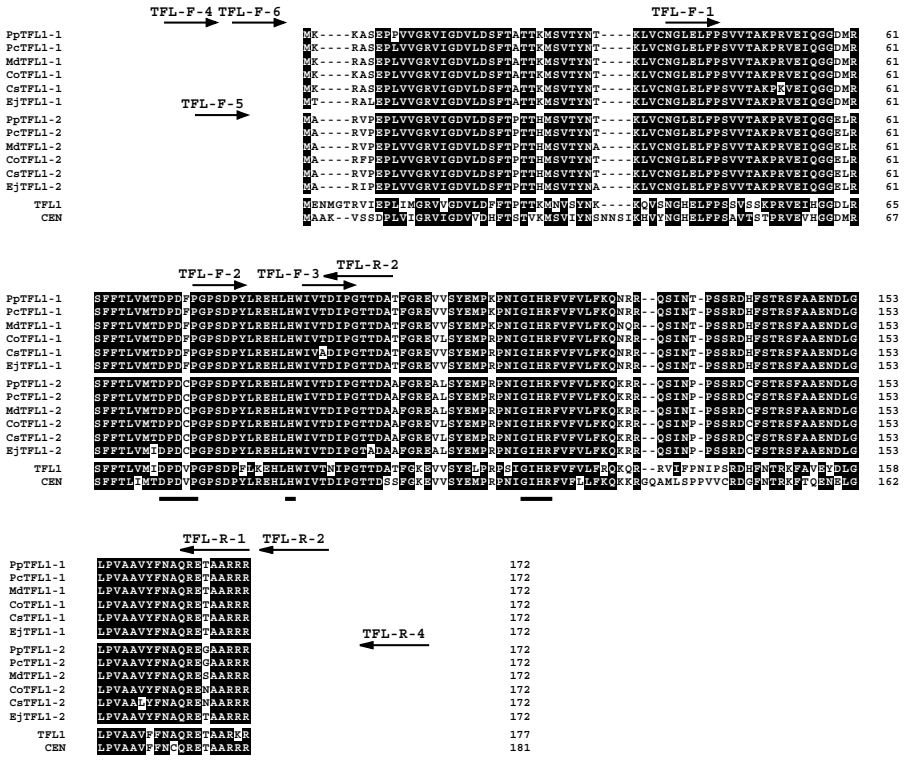

Fig. 4 Amino acid sequence alignment of maloid *TFL1* homologues, *Arabidopsis TFL1*, and snapdragon *CEN*. Plant species from which sequence data are derived are denoted by the initials of their scientific names followed by the name of the group of *TFL1* homologues, e.g., *PpTFL1-1* means *TFL1-1* of *Pyrus pyrifolia*. *Lines below alignment* Amino acid residues important for ligand binding: D-P-D-X-P motif (70–74), His residue at position 86, and G-X-H-R motif (116–119) (Banfield and Brady 2000); *arrows above alignment* positions of amino acid sequences used to design primers. Residues conserved in more than 11 sequences are *shaded*

region consisting mainly of Arg and Lys residues, and the fourth motif is an acidic region consisting mainly of Asp and Glu residues. These two motifs are positioned centrally within the protein and are known to be conserved in *LFY* homologues of angiosperms (Frohlich and Meyerowitz 1997). They were also found in the maloid *LFY* homologues and consisted of nine basic residues and about ten acidic residues, respectively (Fig. 1, motifs III and IV).

A phylogenetic tree based on deduced amino acid sequences of maloid *LFY* homologues revealed two distinct clades: groups *LFY-1* and *LFY-2*. About 95 and 97% amino acid sequence identity was observed among homologues in groups *LFY-1* and *LFY-2*, respectively, while there was about 90% identity between homologues in different groups (Table 2, Fig. 2). These different groups were also discriminated by specific differences in amino acid residues in the sequence, e.g., Asp (*LFY-1*) and Glu (*LFY-2*) at position 14, Tyr and Cys at position 44, Arg and Glu at position 50, and Asn and Lys at position 82, etc. (Fig. 1).

Since *LFY* homologues have generally been identified as single copy genes in diploid plant species such as *Arabidopsis* (Weigel et al. 1992), snapdragon (Coen et al. 1990), tomato (Morinero-Rosales et al. 1999), rice (*Oryza sativa*) (Kyozuka et al. 1998), and grapevine (Carmona et al. 2002), the presence of two different *LFY* homologues in maloid plants may reflect the polyploid origin of Maloideae. The origin of Maloieae has been discussed extensively (Sax 1933; Challice 1974, 1981; Phipps et al. 1991). Evidence from isozyme analyses of apples supports an allopolyploid origin (Chevreau et al. 1985; Weeden and Lamb 1987). Recently, phylogenetic analysis of Maloideae species based on DNA sequences for the granule-bound starch synthase gene apparently demonstrated their polyploid origin (Evans and Campbell 2002). As in Maloideae, multiple copies of *LFY* homologues were found in tobacco (*Nicotiana tabacum*) (Kelly et al. 1995) and maize (*Zea mays*) (Bomblies et al. 2003), both of which are tetraploids.

There appeared to be differences in transcriptional pattern between the two *LFY* homologues; however, both homologues were transcribed extensively in buds where floral differentiation takes place (Fig. 3). Although *LFY-1* homologues in pear, *PpLFY-1* and *PcLFY-1*, were transcribed only in the buds, those of apple and quince, *AFL1-Fuji* and *CoLFY-1*, were transcribed not only in the buds but also in the leaves, hypanthiums, and styles/stigmas. *LFY-2* homologues in pears, *PpLFY-2* and *PcLFY-2*, were transcribed in all tissues examined, while *AFL2-Fuji* and *CoLFY-2* were transcribed only in buds, hypanthiums, styles/stigmas, and stamens. Although the expression pattern of *AFL2-Fuji* in this study was the same as that previously reported for *AFL2*, a putative allele of *AFL2-Fuji* (Wada et al. 2002), some differences between the expression patterns of *AFL1-Fuji* and *AFL1*, a previously reported putative allele of *AFL1-Fuji* (Wada et al. 2002), were found. In contrast to the results of Wada et al (2002), slight transcription of *AFL1-Fuji* was found in leaves, hypanthiums, and styles/stigmas in our experiment. Differences in PCR sensitivities could have led to the discrepancy between our results and the previous report (Wada et al. 2002). The dif-

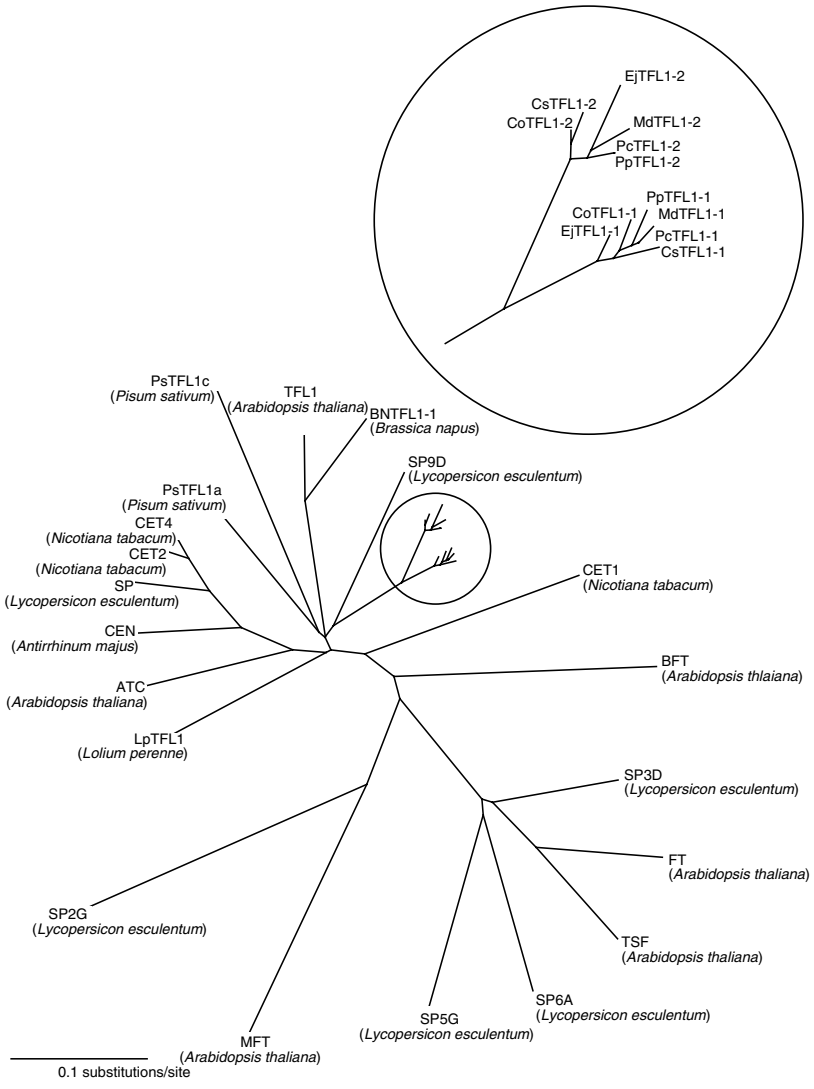

Fig. 5 Phylogenetic tree of *TFL1* homologues constructed by the neighbor-joining method (Saitou and Nei 1987). Maloid *TFL1* homologues are all located in the portion of the tree indicated by the *circle*, an enlarged version of which is shown above the tree. Abbreviations for maloid *TFL1* homologues as in Fig. 4. Abbreviations and accession numbers for other *TFL1* homologues included in the tree: *Antirrhinum majus* (*CEN*, S81193), *Arabidopsis thaliana* (*TFL1*, NM_120465; *ATC*, NM_128315; *FT*, NM_105222; *TSF*, NM_118156; *MFT*, NM_101672; *BFT*, NM_125597), *Brassica napus* (*BNTFL1-1*, AB017525), *Nicotiana tabacum* (*CET1*, AF145259; *CET2*, AF145260; *CET4*, AF145261), *Lolium perenne* (*LpTFL1*, AF316419), *Pisum sativum* (*PsTFL1a*, AY340579; *PsTFL1c*, AY343326), *Lycopersicon esculentum* (*SP*, U84140; *SP2G*, AY186734; *SP5G*, AY186736; *SP6A*, AY186737; *SP3D*, AY186735; *SP9D*, AY186738)

ferent transcriptional patterns observed between the two *LFY* homologues, as well as differences between species, may be due to differences in regulatory sequences. Nonetheless, regardless of the types and species, the two *LFY* homologues were expressed in buds where flower primordia are formed, suggesting that both homologues could play an important role in floral bud formation in Maloideae.

TFL1 homologues in six maloid fruit tree species

As with *LFY*, two different types of cDNA for *TFL1* homologues were isolated from each maloid species used in this study, namely *PpTFL1-1* and *PpTFL1-2* for Japanese pear, *PcTFL1-1* and *PcTFL1-2* for European pear, *MdTFL1-1* and *MdTFL1-2* for apple, *CoTFL1-1* and *CoTFL1-2* for quince, *CsTFL1-1* and *CsTFL1-2* for Chinese quince, and *EjTFL1-1* and *EjTFL1-2* for loquat. All homologues consisted of 172 deduced amino acid residues and no species-specific indels were found (Fig. 4). However, a total of 25 substituted residues in the sequence alignment of maloid homologues were found. Among them, 17 substitutions (positions 2, 3, 4, 5, 8, 33, 52, 69, 90, 96, 104, 105, 111, 128, 134, 159, 167) were species-specific, while 8 were specific to the type of *TFL1* homologue (positions 23, 26, 59, 60, 73, 99, 127, 140), *TFL1-1* or *TFL1-2*.

The maloid *TFL1* homologues were classified into two distinct clades in a phylogenetic tree based on the deduced amino acid sequences. The *TFL1* homologues belonging to the two different clades, *TFL1-1* and *TFL1-2*, shared around 90% identity, while homologues belonging to the same clade had identities of 98% (*TFL1-1*) and 97% (*TFL1-2*) (Fig. 5, Table 3). As with multiple copies of *LFY* homologues, the presence of two distinct *TFL1* homologues in the maloid species tested in this study could be attributed to the polyploid origin of Maloideae.

TFL1-like proteins are similar to mammalian phosphatidylethanolamine binding proteins (PEBPs), also known as Raf-1 kinase inhibitor protein (RKIP). A

Table 3 Identity matrix of deduced amino acid sequence of *TFL1* homologues. Plant species for which sequence data are used are denoted by the initials of their scientific name followed by the name of the group of *TFL1* homologues, e.g., *PpTFL1-1* means *TFL1-1* of *Pyrus pyrifolia*. *TFL1* and *CEN* mean *TERMINAL FLOWER 1* (U77674) of *Arabidopsis thaliana* and *CENTRORADIALIS* (S81193) of *Antirrhinum majus*. Amino acid identity scores (%) were calculated by the CLUSTAL X program (Thompson et al. 1997)

	PpTFL1-1	PcTFL1-1	MdTFL1-1	CoTFL1-1	CsTFL1-1	EjTFL1-1	PpTFL1-2	PcTFL1-2	MdTFL1-2	CoTFL1-2	CsTFL1-2	EjTFL1-2	TFL1
PpTFL1-1													
PcTFL1-1	98												
MdTFL1-1	98	99											
CoTFL1-1	98	98	97										
CsTFL1-1	97	98	97	97									
EjTFL1-1	97	98	97	97	97								
PpTFL1-2	89	90	90	91	90	91							
PcTFL1-2	89	90	90	91	90	91	100						
MdTFL1-2	88	90	89	90	89	90	98	98					
CoTFL1-2	90	91	90	91	90	91	98	98	97				
CsTFL1-2	89	90	90	91	90	91	98	98	97	98			
EjTFL1-2	88	89	88	90	88	90	97	97	97	96	95		
TFL1	73	74	75	73	73	75	73	73	73	73	73	73	
CEN	73	74	74	74	73	73	71	71	69	72	71	70	69

crystal structure of *CEN*, a *TFL1* homologue in snapdragon, revealed several important motifs that could be involved in interaction with kinase by ligand binding (Banfield and Brady 2000). *SP*, a *TFL1* homologue in tomato (*Lycopersicon esculentum*), has also been shown to encode a modular protein with the potential to interact with a variety of signaling pathways (Pnueli et al. 2001). Since the maloid *TFL1* sequences contain key amino acid residues that are conserved in *TFL1* homologues of other genera, *TFL1* homologues of maloid species may function in the same way as *TFL1* and *CEN*.

The function of *TFL1* in *Arabidopsis* involves determination of inflorescence meristem identity as well as floral transition (Bradley et al. 1997). *CEN* is also involved in inflorescence development in snapdragon (Bradley et al. 1996). *SP* in tomato, however, is expressed in vegetative apical meristem and controls regulation of the vegetative-reproductive switch (Pnueli et al. 1998). Furthermore, in pea, *PsTFL1a* and *PsTFL1c* were identified as *TFL1* homologues and have distinct functions. The former plays a role in inflorescence development and the latter is involved in flower initiation (Foucher et al. 2003). Although, as with *PsTFL1a* and *PsTFL1c*, the two different types of maloid *TFL1* homologues could have distinct functions, we assume that the two maloid types can be attributed to the polyploid nature of maloid species, and that they have the same function. This assumption could be supported by the fact that maloid *TFL1-1* and *TFL1-2* groups are placed very close in the phylogenetic tree as compared with *PsTFL1a* and *PsTFL1c*.

Maloid *TFL1* homologues were transcribed mainly in buds (Fig. 3). In all species examined, both *TFL1* homologues, *TFL1-1* and *TFL1-2*, were expressed at high levels in buds before floral differentiation, and their expression seemed to decrease after floral differentiation. These observations could support the idea that *TFL1* homologues play an important role in floral differentiation. Species-specific patterns of organ-specific expression were also observed, e.g. the presence of *MdTFL1-1* transcripts in hypanthiums of apple, *MdTFL1-2* transcripts in stamens of apple, and those of *PpTFL1-2* and *PcTFL1-2* in peduncles of pears. It is unclear whether *TFL1* homologues are involved in other pathways in floral organs. Further analysis will be needed to reveal the functions of *TFL1* homologues in tissues other than buds.

In conclusion, in this study we have cloned two clones each for *LFY* and *TFL1* homologues from six maloid fruit tree species with different flower architecture and flowering habit. There appeared to be several indels and substitutions of amino acid residues in maloid *LFY* homologues. As compared to *LFY* homologues, maloid *TFL1* homologues appeared to be highly conserved, although they contain several substituted amino acid residues. The difference in maloid *LFY* homologues may reflect functional differences, which could result in the differences in flowering habit and inflorescence archi-

tectures observed among maloid species. These differences will be analyzed in future studies once transformation systems have been established for all maloid species tested in this study. In the short term, it may be useful to see if any difference can be observed when the homologous genes are expressed heterologously in model plant species such as *Arabidopsis*, as has been done with *AFL1* and *AFL2* (Wada et al. 2002).

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