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Divergence of *TERMINAL FLOWER1*-like genes in *Rosaceae*

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Abstract

Rosaceae is a large family, however, our understanding of its phylogeny is based largely on morphological observations. To understand the relationship between subfamilies *Rosoideae*, *Amygdaloideae*, *Maloideae* and *Spiraeoideae* at a molecular level, we isolated and compared the plant phosphatidyl ethanolamine-binding protein-like genes *TERMINAL FLOWER1* (*TFL1*)-like and *CENTRORADIALIS* (*CEN*)-like, which are involved in the control of shoot meristem identity and flowering time. A comparison of gene structures and phylogenetic tree analyses by the Neighbor-Joining method showed that each of the two *TFL1*-like (*MdTFL1-1* and *MdTFL1-2*) and *CEN*-like genes (*MdCENa* and *MdCENb*) in *Maloideae* were classified into two distinct clades. The *TFL1*-like and *CEN*-like genes of *Gillenia* in *Spiraeoideae* belonged to monophyletic *Maloideae* groups, suggesting that *Gillenia* and *Maloideae* have a common near ancestor. However, the *Gillenia TFL1*-like gene does not contain the insertion sequence of the third intron that is found in *MdTFL1-2*-like genes of the members of *Maloideae* such as apple, Korean whitebeam, quince, and Siberian mountain ash. Therefore, after the *Maloideae* ancestor genome became polyploid through hybridization between *Gillenia*-like species or genome doubling, an insertion sequence of the third intron of *MdTFL1-2*-like genes was generated.

Additional key words: *Amygdaloideae*, *CEN*, evolution, *Gillenia*, *Maloideae*, *Rosoideae*, *Spiraeoideae*, *TFL1*.

Introduction

The *Rosaceae* family includes several beneficial cultivars that are used for ornamentation and food. This family is divided into four subfamilies: *Rosoideae*, *Amygdaloideae* (*Prunoideae*), *Spiraeoideae* and *Maloideae* (*Pomoideae*) (Phipps *et al.* 1991). *Rosoideae* is composed of plants such as rose, strawberry, and raspberry that usually have a haploid chromosome number of x = 7. *Amygdaloideae* includes cherry, apricot, peach, plum, and almond having a haploid chromosome number of x = 8. *Spiraeoideae* includes genera such as Bowman's root (*Gillenia trifoliata*), Chinese sorbaria (*Sorbaria kirilowii*, x = 18), and Japanese spiraea (*Spiraea japonica*) and has a generic base haploid chromosome number of x = 9. *Maloideae* is composed of plants such as apple, pear, quince, cotoneaster, hawthorn, rowan, loquat, Korean whitebeam (*Aria alnifolia* [*Sorbus alnifolia*]), and Siberian mountain ash (*Sorbus sambucifolia*) and has a haploid chromosome number of mostly x = 17 (Goldblatt 1976, 1984). *Maloideae* diverged from the ancestral species with a polyploid genome. There are three hypotheses regarding the origin of the *Maloideae* polyploid-like genome. It was originally thought that the *Maloideae* genome (x = 17) was derived from an ancient allopolyploid genome resulting from the hybridization between *Amygdaloideae* (x = 8) and *Spiraeoideae* (x = 9) (Sax 1933, Challice and Kovanda 1981, Phipps *et al.* 1991). Another hypothesis suggests that the *Maloideae* genome resulted from the duplication of an ancient genome such as a *Spiraeoideae*-like species or was generated from hybridization between allogeneic *Spiraeoideae*-like species (Morgan *et al.* 1994, Evans and Campbell 2002, Potter *et al.* 2007). More recently, the apple genome project proposed that *Malus* originated through autopolyploidization (Velasco *et al.* 2010). In

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Abbreviations: *CEN* - *CENTRORADIALIS*; *FT* - *FLOWERING LOCUS T*; *MFT* - *MOTHER OF FT AND TFL1*; PEBP - phosphatidyl ethanolamine-binding protein; *TFL1* - *TERMINAL FLOWER1*; PCR - polymerase chain reaction.
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this way, the phylogenetic relationships among members of the *Rosaceae* family have become partially clear but are still poorly understood at the molecular level and are almost presented in traditional classifications based on morphological characteristics.

The key regulator genes for flowering and morphology in plant species have diverged and evolved to uniquely adapt to different environmental conditions. Therefore, an analysis based on flowering or morphological genes will contribute to our understanding of plant evolution (De Bodt *et al.* 2003). For example, the second introns of *LEAFY*-like genes are often used as evolutionary markers in *Rosaceae* (Oh and Potter 2003, 2005, Esumi *et al*. 2005, Lo *et al.* 2007, 2009). The MADS-box gene *PISTILLATA* has also been used in *Amygdaloideae* phylogenetic relationship analysis because it has a low copy number in most plants (Lo *et al.* 2009). Plant phosphatidyl ethanolamine-binding protein (PEBP) encoding genes are involved in the development of reproductive tissues, control of shoot meristem identity, and flowering time. *Arabidopsis* has six PEBP-family members, which are classified into three subfamilies: *MOTHER OF FT AND TFL1* (*MFT*)-like, *FLOWERING LOCUS T* (*FT*)-like, and *TERMINAL FLOWER1* (*TFL1*)-like (Hedman *et al.* 2009). Analyses with plant *PEBP*-like genes have given considerably important insights into plant evolution. For example, the bryophytes and lycopods contain *MFT*-subfamily genes and not *FT*- or *TFL1*-subfamily genes. Therefore, *FT*- and *TFL1*-subfamily genes occurred during the evolution of seed plants (Hedman *et al.* 2009). In addition, a particular region within the C-terminal region of TFL1 (as a potential ligand-binding pocket, amino acid position, 131-145), evolves much more rapidly than that in the C-terminal region of FT (amino acid position, 128-141) (Ahn *et al.* 2006). Gentians, which are herbaceous perennial plants, bloom from summer to autumn but show naturally occurring genetic variation, such as a 1-month difference in flower initiation and a 2-month difference in blooming time. Recently, a gentian *TFL1*-like gene, *GtTFL1*, was isolated and characterized. Subsequently, a 320-bp insertion was found in the *GtTFL1* promoter region of early-flowering gentians. This insertion may affect *TFL1* expression levels and consequently alter flowering and blooming times (Imamura *et al.* 2011). The *Rosaceae* family has a worldwide distribution and a variety of flower initiation and blooming times. Therefore, PEBP-family members will be useful tools for understanding the flowering mechanism and evolution of the *Rosaceae* family.

In the apple (*Malus* × *domestica* Borkh.), several twin pairs of genes formed by polyploidy were identified; these include *LEAFY*-like genes, MADS-box genes, and plant *PEBP*-like genes (Yao *et al.* 1999, Wada *et al.* 2002, Esumi *et al.* 2005, Mimida *et al.* 2009). In particular, since loss-of-function of *TFL1*-like genes cause to the perpetual-like flowering in apple, rose and strawberry (Kotoda *et al.* 2006, Iwata *et al.* 2012), characterization of the *TFL1*-like gene is also particularly important for the horticultural *Rosaceae* species.

Apple *TFL1*-like, *MdTFL1-1* (*MdTFL1*) and *MdTFL1-2* (*MdTFL1a*) (acc. Nos. AB366639 and AB366640) genes regulate growth and flowering and are localized at the north end of the linkage groups (LG) 12 and LG 14, respectively (Mimida *et al.* 2009). Moreover, apple *FT*-like, *MdFT1* and *MdFT2* genes (AB161112 and AB458504), which possibly regulate floral initiation, are localized at the south end of LG 12 and LG 04, respectively (Kotoda *et al.* 2010). Interestingly, the whole of chromosome (Chr) 12, the north end of Chr 14, and south end of Chr 04 are derived from Chr 05 of the *Malus* ancestor (Velasco *et al.* 2010). Other *TFL1*-subfamily genes, *CENTRORADIALIS* (*CEN*)-like, *MdCENa* and *MdCENb* (AB366641 and AB366642), are involved in the development of proliferating tissues and are localized in the middle of LG 03 and LG 11, respectively (Mimida *et al.* 2009). It is likely that Chr 03 and Chr 11 are derived from an ancestor Chr 02 (Velasco *et al.* 2010). In this paper, we focused on the evolution of *Rosaceae TFL1*-subfamily genes and investigated the relationship among the members of *Rosaceae* species, particularly between *Maloideae* and *Spiraeoideae*.

Materials and methods

To analyze *TFL1*-like genes among *Rosaceae* species, we used several plants: *Rosoideae*: *Rosa rugosa* (local name Hamanashi), *Amygdaloideae*: *Prunus avium* (cv. Sato nishiki), *Spiraeoideae*: *Spiraea japonica*, *Gillenia trifoliata* and *Sorbaria kirilowii*, and *Maloideae*: *Aria alnifolia* and *Sorbus sambucifolia*. To assess the number of *MdTFL1*-like genes in *Rosaceae* species, we performed genomic Southern hybridization analysis with a full-length probe of *MdTFL1-1* (Fig. 1*A*). The restriction maps were matched for these *TFL1*-like sequences (Fig. 1), but some weak bands were detected in the samples of *S. japonica*, which did not contain restriction sites for *Eco*RI, *Xba*I, and *Hind*III in the *TFL1*-like gene. A similar result was observed for the samples of *S. kirilowii*. These weak bands may represent *CEN*-like genes, as the probe of the *Prunus mume TFL1*-like gene cross-hybridized weakly with the *CEN*-like sequence (Esumi *et al.* 2010). Therefore, it suggested that the *MdTFL1*-like gene is present as a single copy in *R. rugosa*, *P. avium*, *S. japonica*, *G. trifoliata*, and *S. kirilowii* and as two copies in *A. alnifolia* and *S. sambucifolia*.

In the course of characterizing the *TFL1*-like and *CEN*-like gene structures in *Rosaceae* species, polyme-

rase chain reaction (PCR) amplification was performed with DNA polymerase such as *KOD -plus* (*Toyobo*, Tokyo, Japan) or *Takara LA Taq* (*Takara*, Otsu, Japan) and the pair of primers: *TFL1*-exon1F (5′-GCCAAA CCTAGAGTTGAGATTCAAGGAGGG-3′) and *TFL1*-exon 1R (5′-CCCAGGTCATTTTCAGCGGCGAAGC-3′) or the primers: *TFL1*-degenerate-exon1F (5′-GTTGTT GGRAGAGTSATAGGAGATGTTCTTG-3′) and *TFL1*-degenerate-exon4R (5′-CTAGCGTCTTCTWGCWG CMBTTTCTC-3′) or *TFL1-2*-3′UTRR (5′-CATGAA AGTACGTAATAGTGGCCTAATGGCGAG-3′) for *TFL1*-like genes and a pair of primers: *CEN*-exon1F (5′-CCTTCCTCAGTAACCATCAAGCCTAAGGTTG AAG-3′) and *CEN*-exon4R (5′-GCAGGAGGGATC ACTGTCTGC CTG-3′) or *CEN*-degenerate-exon1F (5′-GTTGGGAGAGTKATTGGAGATGTTGTTGATT-3′) and *CEN*-degenerate-exon4R (5′-GCATTGAAG AADACAGCAGYCACHGGA-3′) for *CEN*-like genes. PCR was programmed for preheating at 94 °C for 3 min followed by 35 cycles of 96 °C for 10 s and 68 °C for 10 min. The amplified PCR products were cloned into the *pT7Blue T* vector (*Novagen*, Darmstadt, Germany) or *pBluescriptII SK*$^+$ (*Stratagene*, La Jolla, CA, USA), and these clones were sequenced.

Results and discussion

The used genes showed high identity (> 88 % in the coding regions) with *MdTFL1-1/MdTFL1-2* or *MdCENa*/*MdCENb* and were designated as *AaTFL1-1*/ *AaTFL1-2* (*A. alnifolia,* acc. Nos. AB636114/ AB636115), *GtTFL1* (*G. trifoliata*, AB636119), *PaTFL1* (*P. avium*, AB636121), *RrTFL1* (*R. rugosa*, AB636122), *SjTFL1* (*S. japonica*, AB636123), *SsTFL1-1*/*SsTFL1-2* (*S. sambucifolia*, AB636116/AB636117), *SkTFL1* (*S. kirilowii*, AB636125), *GtCEN* (AB636118), *PaCEN* (AB636120), and *SkCEN* (AB636124). We searched Rosaceae *TFL1*-like genes on the DNA database and found *CoTFL1-1*/*CoTFL1-2* (*Cydonia oblonga*, AB162043/AB162049), *CsTFL1-1*/*CsTFL1-2* (*Chaenomeles sinensis*, AB162044/AB162050), *EjTFL1-1*/ *EjTFL1-2* (*Eriobotrya japonica*, AB162045/AB162051), *PcTFL1-1*/*PcTFL1-2* (*Pyrus communis*, AB162042/ AB162048), *PmTFL1* (*Pyrus mume*, AB548832), and *PpTFL1-1*/*PpTFL1-2* (*Pyrus pyrifolia*, AB162041/ AB162047), *KSN* (*Rosa chinensis*, HQ174211), FM999796 (*Rosa luciae*) and *PmCEN* (*P. mume*, AB548820). Moreover, we also found PEBP-family members of strawberry (*Fragaria vesca*), peach (*Prunus persica*) and apple (*Malus* × *domestica* Borkh.) in the Genome Database for Rosaceae (GDR, http://www. rosaceae.org) (Figs. 1, 2). The plants and all analyzed *PEBP*-family genes are summarized in Table1.

To predict *TFL1*-like gene structures of *Rosaceae*, proper splicing of exon-intron junctions was confirmed by comparison with *MdTFL1-1* and *MdTFL1-2* or *MdCENa* and *MdCENb*. The *TFL1*-like gene structures of *Rosaceae* were conserved, and they consisted of possibly four exons interspersed with three introns, but the *F. vesca TFL1*-like gene (acc. No. in GDR: mrna30188, here, referred to as *FvTFL1*) may consist of five exons interspersed with four introns (Fig. 1*B*). The lengths of the second and third introns differed considerably between *Maloideae* and other *Rosaceae* (Fig. 1*B*). The third intron of the *MdTFL1-2* gene contains an insertion sequence of about 900 bp (Mimida *et al.* 2009). Similarly, other *Maloideae MdTFL1-2*-like genes such as *AaTFL1-2*, *CsTFL1-2*, and *SsTFL1-2* had an insertion sequence in the third intron but the *TFL1*-like genes in *Amygdaloideae*, *Rosoideae,* and *Spiraeoideae* did not (Fig. 1*B*). The third intron of *MdTFL1-2* contains a similar sequence (approximately 150 bp in length) indicated by -IS1′ and IS1″, which is also found in its promoter region (IS1) (Fig. 1*B*, Mimida *et al*. 2009). As a result of the database search, the IS1-like sequences, which may be small intronic non-coding RNAs (ncRNAs) or transposable elements, are observed to be expressed in various tissues and are present widely in the genome of apples but not in that of *F. vesca*, *P. persica*, or in expressed sequence tags (ESTs) of *Rosa* species such as *R. chinensis*, *R. wichurana*, or *Rosa* hybrid cultivar (data not shown). Therefore, IS1-like sequences may be specifically present in *Maloideae*. The insertion sequences of the third introns of the *Maloideae MdTFL1-2*-like genes are likely to have occurred after *Maloideae* genome duplication (polyploidization) and their divergence from the common ancestor of *Gillenia* and *Maloideae.* On the other hand, the structures of *CEN*-like genes are relatively conserved among *Rosaceae* species, but not in *F. vesca* (Fig. 1*B*). Comparing *Rosaceae TFL1*-subgroup genes, length variation within the second and third introns of *Maloideae MdTFL1-1*-like and *MdTFL1-2*-like genes could be observed.

We performed alignment and phylogenetic tree analyses using the *PEBP*-like genes of *Rosaceae* and *Arabidopsis*. The nucleotide sequences of *Rosaceae PEBP*-like genes could be divided into the following five distinct clades in our phylogenetic tree: *MFT*-clade, *TFL1*-clade, *CEN*-clade, *BFT*-clade and *FT*-clade (Fig. 2*A*). Interestingly, *F. vesca* has two additional *FT*-like genes, mrna28920 and mrna04645, which are different from a gene cluster of mrna21482 (*F. vesca*), ppa012320m (*P. persica*), and *MdFT1* and *MdFT2* (*M.* × *domestica*) (Fig 2*A*). Tree topology with two *FT*-like gene clusters suggests that *M.* × *domestica* and *P. persica* may have lost cluster genes of mrna28920 and mrna04645 after the divergence with *F. vesca.* In Arabidopsis, a facultativelong-day plant, Ft protein is produced in leaves in response to photoperiod and

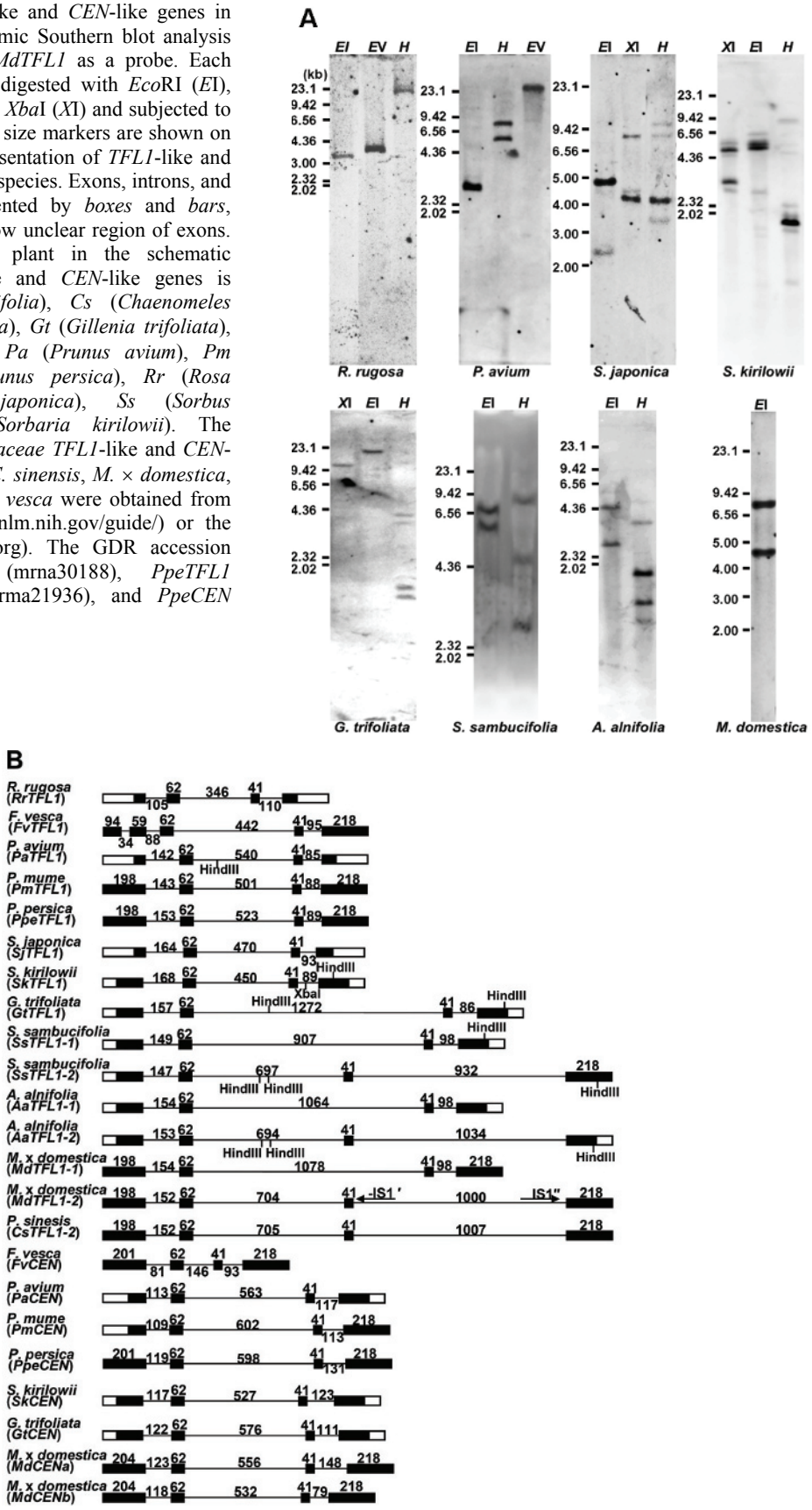

Fig. 1. Structure of *TFL1*-like and *CEN*-like genes in *Rosaceae* species. *A* - Genomic Southern blot analysis with full-length cDNA of *MdTFL1* as a probe. Each genomic DNA sample was digested with *Eco*RI (*E*I), *Eco*RV (*E*V), *Hind*III (*H*), or *Xba*I (*X*I) and subjected to hybridization. The molecular size markers are shown on the left. *B* - Schematic representation of *TFL1*-like and *CEN*-like genes in *Rosaceae* species. Exons, introns, and interval regions are represented by *boxes* and *bars*, respectively. Open boxes show unclear region of exons. The abbreviation of each plant in the schematic representation of *TFL1*-like and *CEN*-like genes is shown by *Aa* (*Aria alnifolia*), *Cs* (*Chaenomeles sinensis*), *Fv* (*Fragaria vesca*), *Gt* (*Gillenia trifoliata*), *Md* (*Malus* × *domestica*), *Pa* (*Prunus avium*), *Pm* (*Prunus mume*), *Ppe* (*Prunus persica*), *Rr* (*Rosa rugosa*), *Sj* (*Spiraea japonica*), *Ss* (*Sorbus sambucifolia*), and *Sk (Sorbaria kirilowii*). The nucleotide sequences of *Rosaceae TFL1*-like and *CEN*-like genes such as those in *C. sinensis*, *M.* × *domestica*, *P. mume*, *P. persica*, and *F. vesca* were obtained from the *NCBI* (http://www.ncbi.nlm.nih.gov/guide/) or the *GDR* (http://www.rosaceae.org). The GDR accession numbers are *FvTFL1* (mrna30188), *PpeTFL1* (ppa012369m), *FvCEN* (mrma21936), and *PpeCEN* (ppa012343m).

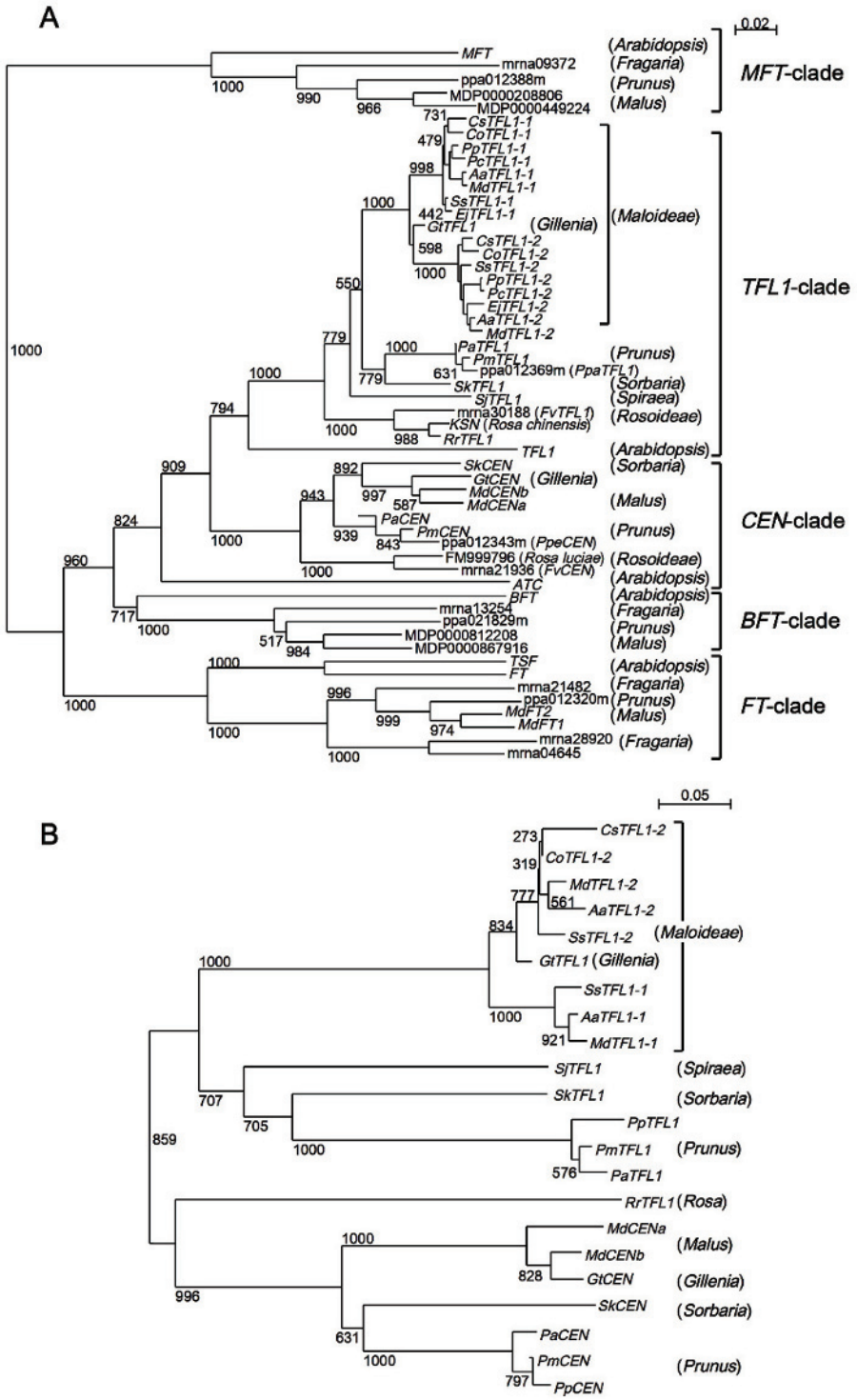

Fig. 2. The phylogenetic analysis of *PEBP*-like nucleotide sequences in *Rosaceae* species. *A* - Phylogenetic relationship tree of *Rosaceae PEBP*-like genes. The abbreviation is shown by *Co* (*Cydonia oblonga*), *Ej* (*Eriobotrya japonica*), *Pc* (*Pyrus communis*), and *Pp* (*Pyrus pyrifolia*). *PEBP*-family members of *F. vesca* (mrna), *P. persica* (ppa), and *M.* × *domestica* (MDP) which were obtained from the *GDR*, are represented by their accession number. The *TFL1*-like gene sequences of *C. oblonga*, *C. sinensis*, *E. japonica*, *M.* × *domestica*, *P. communis*, *P. mume*, *P. pyrifolia*, *Rosa chinensis* and *Rosa luciae* were obtained from *NCBI*. *B* - Phylogenetic relationship tree of first intron regions in *Rosaceae TFL1*-like genes. Phylogenetic relationship tree constructed using the *Clustal X* multiple sequence alignment program *v. 1.83* (Jeanmougin *et al.* 1998) and the *Njplot* program (Perrière and Gouy 1996). The branches with bootstrap values are shown on the nodes. Numbers indicate bootstrap values. The units for the *scale bars* indicate branch lengths (0.02 or 0.05 substitutions/site).

Table 1. The *Rosaceae* family is divided into four subfamilies: *Rosoideae, Amygdaloideae* (*Prunoideae*), *Spiraeoideae* and *Maloideae* (*Pomoideae*) which include many important species with different haploid chromosome number (x). This table summarizes all analyzed *PEBP*-family genes with relevant accession numbers.

Rosaceae	Species	x	Genes for PEBP family
Rosoideae	rose (*Rosa chinensis*)	7	*KSN* (HQ174211
	Japanese rose (*Rosa rugosa*)	7	*RrTFL1* (AB636122)
	memorial rose (*Rosa luciae*)	7	(FM999796)
	strawberry (*Fragaria vesca*)	7	*FvTFL1* (mrna30188), *FvCEN* (mrma21936), mrna13254, mrna09372, mrna04645, mrna21482, mrna28920
	raspberry (*Rubus* sp.)	7	
Amygdaloideae (*Prunoideae*)	cherry (*Prunus avium*)	8	*PaTFL1* (AB636121), *PaCEN* (AB636120)
	apricot (*Prunus armeniaca*)	8	
	Japanese apricot (*Prunus mume*)	8	*PmTFL1* (AB548832), *PmCEN* (AB548820)
	peach (*Prunus persica*)	8	*PpeTFL1* (ppa012369m), *PpeCEN* (ppa012343m), ppa012369m, ppa012388m, ppa012320m, ppa021829m, ppa012343m
	plum (*Prunus* sp.)	8	
	almond (*Prunus dulcis*)	8	
Spiraeoideae	Bowman's root (*Gillenia trifoliata*)	9	*GtTFL1* (AB636119), *GtCEN* (AB636118)
	Japanese spiraea (*Spiraea japonica*)	9	*SjTFL1* (AB636123)
	Chinese sorbaria (*Sorbaria kirilowii*)	18	*SkTFL1* (AB636125), *SkCEN* (AB636124)
Maloideae (*Pomoideae*)	apple (*Malus* × *domestica*)	17	*MdTFL1-1* (AB366639), *MdTFL1-2* (AB366640) *MdCENa* (AB366641), *MdCENb* (AB366642), *MdFT1* (AB161112), *MdFT2* (AB458504), MDP0000449224, MDP0000867916, MDP0000208806, MDP0000812208
	European pear (*Pyrus communis*)	17	*PcTFL1-1* (AB162042), *PcTFL1-2* (AB162048)
	Japanese pear (*Pyrus pyrifolia*)	17	*PpTFL1-1* (AB162041), *PpTFL1-2* (AB162047)
	Chinese Quince (*Chaenomeles sinensis*)	17	*CsTFL1-1* (AB162044), *CsTFL1-2* (AB162050)
	quince (*Cydonia oblonga*)	17	*CoTFL1-1* (AB162043), *CoTFL1-2* (AB162049)
	cotoneaster (*Cotoneaster* sp.)	17	
	hawthorn (*Crataegus* sp.)	17	
	rowan (*Sorbus* sp.)	17	
	loquat (*Eriobotrya japonica*)	17	*EjTFL1-1* (AB162045), *EjTFL1-2* (AB162051)
	Korean whitebeam (*Aria alnifolia*)	17	*AaTFL1-1* (AB636114), *AaTFL1-2* (AB636115)
	Siberian mountain ash (*Sorbus sambucifolia*)	17	*SsTFL1-1* (AB636116), *SsTFL1-2* (AB636117)

then is translocated to the shoot apex (Corbesier *et al.* 2007, Jaeger and Wigge 2007). In contrast, in apple, an autonomous flowering plant (Wilkie *et al.* 2008), since *MdFT1* and *MdFT2* are expressed in shoot apex during the flower initiation, their proteins may not be a transmissible floral inducer across the tissues or layers (Mimida *et al.* 2011). Therefore, the photoperiodic flower induction process in apple is different from that in *Arabidopsis*. On the other hand, there are a variety of commercial strawberry cultivars (*Fragaria* × *ananassa*) classified as long-day, short-day, and day-neutral types in response to photoperiod for flower induction (Durner and Poling 1988), suggesting the photoperiodic pathway of strawberry is active to induce flower initiation. To provide an evolutionarily comparative view of the photoperiod flowering pathway in the *Rosaceae* family, the strawberry *FT*-like genes will be necessary to further investigation.

We also performed alignment and phylogenetic tree analyses using the relatively conserved first introns of our isolated genes and other *TFL1*-like genes in the *Rosaceae* family, but we did not use those of *F. vesca* genes because the structures of these genes are different from those of other *Rosaceae TFL1*-like genes (Fig. 1*B*). Overall, these phylogenetic trees were similar (Fig. 2). In the *Maloideae* subfamily, *A. alnifolia* is commonly classified into the *Sorbus* group. Our result showed that *AaTFL1-1* and *AaTFL1-2* were almost identical to

MdTFL1-1 and *MdTFL1-2* (99.5 and 99.1 % nucleotide identity in coding regions, respectively), more so than *SsTFL1-1* and *SsTFL1-2* (94.0 and 98.9 %) and other orthologs in the encoding regions and the first introns (Fig. 2). Moreover, in comparison with other regions, the third intron nucleotide sequence of *AaTFL1-2* showed 92.9 % identity to that of *MdTFL1-2* and 89.7 % to that of *SsTFL1-2*. However, the relationships among *Maloideae* genes differ for each of the analyzed target genes (Esumi *et al.* 2005). Further, it will be necessary to study the relationships among the *Sorbus* genera. In *Spiraeoideae*, *GtTFL1* was more similar to *MdTFL1-1* and *MdTFL1-2* (95.8 and 96.6 % identity in coding region, respectively) than to *SjTFL1* (87.3 ~ 88.4 %) and *SkTFL1* (90.2 %) and constituted one sub-clade with two *TFL1*-like genes of *Maloideae* (Fig. 2). A similar result was observed for *CEN*-like genes, *GtCEN* is closer to *MdCENa* and *MdCENb* (94.1 and 95.1 % identity in coding region, respectively) than to *SkCEN* (89.7 %) (Fig. 2). As a result of the possible fusion of two chromosomes into one in the *Maloideae* ancestor, the number of most *Maloideae* chromosomes is x = 17 (Velasco *et al.* 2010). The number of *S. kirilowii* chromosomes is x = 18, which is likely to be the same number as in the *Maloideae* ancestor. However, our results suggest that both of *TFL1*-like and *CEN*-like genes of *Maloideae* were more similar to those of *G. trifoliata* than to those of *S. kirilowii* (Fig. 2). Consequently, *S. kirilowii* does not share a common direct ancestor with *Maloideae.*

In this study, we cloned some *TFL1*-like genes from *Rosaceae* species. We investigated *Spiraeoideae TFL1*-like genes that show a high degree of variation. This suggested that *Maloideae* diverged from a common ancestor with *Spiraeoideae Gillenia*. This result supports the hypothesis of Evans and Campbell (2002) and Velasco *et al.* (2010) that *Maloideae* originated from ancestors of *Gillenia*. However, the third intron of *Maloideae TFL1-2* contains an insertion sequence that is not found in that of *Gillenia TFL1*. In future, by investigating the insertion sequences of the third introns of *Spiraeoideae TFL1*-like genes, we may be able to establish when the divergence of the *Maloideae* and *Spiraeoideae* subfamily occurred and how the *Maloideae* genome generated and developed.

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