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Isolation and expression analysis of three different flowering genes (*TtLFY, TtAP1, and TtAP2*) from an unusual legume species, *Thermopsis turcica*

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Abstract: *LEAFY* (*LFY*), *APETALA1* (*AP1*), and *APETALA2* (*AP2*) genes encode three different transcription factors that control and regulate flower initiation and development in *Arabidopsis*. By using 3'- and 5'-RACE analysis, we isolated and sequentially characterized *TtLFY* (a LEAFY-like gene), *TtAP1* (a MADS-box–like gene), and *TtAP2* (an AP2/ERBF-like gene) in *Thermopsis turcica*, an unusual endemic legume species with three free carpellated flower structure. Semiquantitative RT-PCR analysis for 18 different vegetative and reproductive tissues of *T. turcica* indicated *TtLFY* transcripts mainly in the shoot tips and young floral buds and *TtAP1* transcripts in the sepals and petals; however, *TtAP2* transcripts were detected in all tissues. This is the first record for a *LFY*-like gene, *TtLFY*, expressed in the shoot tips of an underground plant section and for an *AP2*-like gene transcript found in all tissues, similar to a housekeeping gene.

Key words: *LEAFY*, *APETALA1*, *APETALA2*, *RACE*, legume sp.

1. Introduction

The switch from vegetative phase to flowering phase is one of the most important developmental transitions in the life cycle of angiosperms. The apical meristem produces leaves and lateral shoots during the vegetative phase, but it is converted to floral meristem due to environmental and internal clues such as temperature, light, plant growth regulators, and plant age (Jinghua et al., 2014). The transcription factors control transition from vegetative meristem to the floral meristem and induce floral meristem identity genes for initiation and development of floral organs. In *A. thaliana*, transcriptional factors *LEAFY* (*LFY*) (Weigel et al., 1992), *APETELA1* (*AP1*) (Mandel and Yanofsky, 1995), *APETAL2* (*AP2*) (Irish and Sussex, 1990; Jofuku et al., 1994), *AGAMOUS* (*AG*) (Yanofsky et al., 1990), and *SEPALLATA3* (*SEP3*) (Pelaz et al., 2001) are assigned as positive regulators of floral meristem initiation and development. Among these genes, *LFY* encodes a plant-specific transcription factor and plays a crucial role in the switch from vegetative to reproductive development. The mutated *LFY* results in secondary shoots in place of flowers, and overexpressed *LFY* causes early flowering or conversion of lateral shoots into flowers (Coen et al., 1990; Weigel et al., 1992). *LFY* is the master regulator of flowering establishment, as it is expressed throughout young floral meristems and activates various floral homeotic genes in combination with other regulators (Liu et al., 2009). *LFY* directly activates *AP1*, which has roles in the establishment of the floral meristem and determination of sepal and petal formation (Irish and Sussex, 1990; Bowman et al., 1993). *AP1* belongs to the A-class in the ABCDE model and is a MIKC-type MADS box transcription factor that regulates a diverse range of developmental producers in plants (Kaufmann et al., 2010; Chi et al., 2011). *LFY*, *AP1*, and *UNUSUAL FLORAL ORGANS* (*UFO*) have roles in the activation of a B-function gene, *APETALA3* (*AP3*), which together with *PISTILLATA* (*PI*), contributes to the determination of petals and stamens (Ng and Yanofsky, 2001). A *LFY* co-regulator, *SEP3*, plays an endogenous role in the transcriptional regulation of B-function (*AP3* and *PI*) and C-function (*AG*) genes under the regulation of three flowering-time genes, *SHORT VEGETATIVE PHASE* (*SVP*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), and *AGAMOUS-LIKE24* (*AGL24*) (Liu et al., 2009). In addition, *LFY* could also cooperate with a homeobox gene, *WUSCHEL* (*WUS*), to activate *AG*, which specifies the identity of stamens, carpels, and ovules and terminates floral meristems (Lohmann et al., 2001). *AP2* encodes a transcription factor containing two AP2 domains, and it has a role in the establishment of floral meristem, specification of floral organ identity, and regulation of floral gene expressions in plants (Irish and

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Sussex, 1990; Jofuku et al., 1994). *AP1*, *CAULIFLOWER* (*CAL*), and *FRUITFULL* (*FUL*) paralogs were the result of duplications in the AP1/FUL gene lineage in *Arabidopsis thaliana* (Pabón-Mora et al., 2013). *CAL* is mostly redundant with *AP1* and *FUL* plays unique roles in leaf and fruit development (Irish and Sussex, 1990; Bowman et al., 1993; Pabón-Mora et al., 2013).

Genetics and molecular mechanisms of plant flowering have been extensively studied in the model eudicots such as *Arabidopsis* and *Antirrhinum*, and the ABCDE model was proposed for floral organ identity specification (Theißen and Seadler, 2001). The pattern of floral initiation and development of floral organs are applicable to a wide range of eudicot plants but with variations in gene expression and/or function patterns (Ng and Yanofsky, 2001; Song et al., 2008). For instance, the floral organs are initiated in the order of sepal (whorl 1), petals (whorl 2), stamens (whorl 3), and carpels (whorl 4) in *Arabidopsis*. In the flowers of Papilionoideae, the largest of three subfamilies in the family Fabaceae, floral organs appeared in the order of sepal (whorl 1), carpel (whorl 3), and petals and stamens (whorl 2) (Tucker, 1989; Ferrándiz et al., 1999). Unlike the other eudicots, petals and stamens are initiated from the same concentric whorl in a papilionoid flower. Floral organ number (5 petals, 5 sepals, 10 stamens, and 1 carpel), arrangement, and initiation timing are generally common to the plants of Papilionoideae, which includes agriculturally important legume crops such as pea, bean, soybean, and clover (Tucker, 2003).

The genus *Thermopsis* R.Br., belonging to the subfamily Papilionoideae, is represented by the sole endemic *Thermopsis turcica* Kit Tan, Vural & Küçüködük in Turkey (Tan et al., 1983). The floral development of *T. turcica* is of particular interest because the number of free carpels produced by a flower is 3 or 4, which differs from the standard in papilionoid flowers. There are reports of different numbers of stamens or petals in some papilionoid species such as those belonging to the genus *Sophora* (Tucker, 2003; Song et al., 2008). The flowering mechanism of *T. turcica* is under investigation in order to understand the genetic and molecular mechanism of the flowering process and the unusual carpel numbers. In the present study, we aimed to isolate and functionally characterize *TtLFY*, *TtAP1*, and *TtAP2* from young floral buds of *T. turcica* by using reverse transcriptase polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The comparative analysis of *TtLFY*, *TtAP1*, and *TtAP2* with their homologs from other plant species was for structural characterizations. The expression analyses of *TtLFY*, *TtAP1*, and *TtAP2* were also studied in vegetative and reproductive tissues of *T. turcica* by real-time PCR to determine the spatial expressions of target genes.

2. Materials and methods

2.1. Plant material

The various vegetative and reproductive tissues of *T. turcica* were collected from a natural population near Lake Eber, Afyonkarahisar, Turkey, in May 2013 and 2014. The vegetative samples included young leaves (<0.5 cm^2), mature leaves (>1 cm^2), stem, and the tips of shoot apex developing on the rhizome stem under the soil. The reproductive samples included very young and semiopened floral buds and their floral organs (sepals, petals, stamens, and carpels), all the floral organs of pollinated flowers with their young seeds (<2 mm), and seed pods. The well-developed green seed pods (<1.5 cm in length) and their seeds (3–5 mm in diameter) were also sampled. All tissues were directly frozen in liquid nitrogen and stored at –86 °C until used.

2.2. The isolation of *TtLFY, TtAP1,* and *TtAP2*

Total RNA was extracted from vegetative and reproductive tissues using a modified isothiocyanate method (Strommer et al., 1993). DNase treatment of each total RNA sample was carried out by DNA-Free kit (Invitrogen) to remove any residual genomic DNA. The concentration of RNA was determined with Qubit RNA assay kit (Invitrogen, CA, USA) using the Qubit 2.0 fluorometer. RNA integrity was checked by visualization on a 2% (w/v) formaldehyde agarose gel. The total RNA isolated from young floral buds was used for the isolation of genes. The synthesis of first-strand cDNA was performed in a total volume of 20 µL containing 1 µg of total RNA, 20 pmol of oligo$(dT)_{18}$, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM each dNTP, 1 U RNase inhibitor, 200U MMLV Reverse Transcriptase (Clonetech), and DEPC-treated water. Because there was no previous molecular information on floral and housekeeping genes available for *T. turcica*, degenerate primers were used initially to isolate putative gene homologs of *LFY*, *AP1*, and *AP2*. The degenerate primers for *TtAP1* (dAP1F/dAP1R) and *TtbACTIN* (dbACTINF/dbACTINR) were as described by Song et al. (2008) and those of *TtAP2* (dAP2F/dAP2R) as described by Vahala et al. (2001) (Table). In case of *LFY* gene isolation, degenerate primers dLFYF and dLFYR were designed based on the amino acid and DNA sequences conserved among homologs from different legume species (Table). The annealing temperatures (A_t) used in PCR reactions were 72.0 °C for *TtLFY*, 60.4 °C for *TtAP1*, 61.2 °C for *TtAP2*, and 64.5 °C for Tt*b*ACTIN. Five microliters of 5-fold–diluted cDNA was used as template in PCR reactions with the following program: 2 min at 98 °C, 5 cycles (with 1 °C ramping time) of 10 s at 98 °C; 15 s at A_t – 5 °C and 30 s for 72 °C; 30 cycles of 10 s at 98 °C; 15 s at A_t °C and 30 s for 72 °C; and 5 min at 72 °C. The PCR products were checked on a 1.8% (w/v) agarose gel with ethidium bromide staining. The expected PCR products

Table. Degenerate and gene-specific primers used to amplify *TtLFY*, *TtAP1*, *TtAP2*, and Tt*bACTIN* fragments in routine PCR, RACE-PCR, and qPCR reactions.

Gene	Primer	Sequence
TtLFY	dLFYF	TTCATYGTGACRGARCCTGGSGAA
	dLFYR	CTTGTAACAHGCTTGYCTCCAYGC
	$3LFYF1_{out}$	GGGAGGTTGCACGTGGCAAGAAGAA
	$5LFYR1_{out}$	AACATGCTTGTCTCCACGCTCCAAC
	$3LFYF2_{in}$	ACATCGCCAAAGACCGCGGTGAAAA
	$5LFYR2_{in}$	CCTCCTCGTCAAGACAGTGCAACGC
TtAP1	dAP1F	GGTAGRGTNCARYTGAAGMG
	dAP1R	GAGTCAGDTCVAGMTCRTTCC
	$3AP1F1_{out}$	TGCTCTGCGATGCTGAAGTCGCGTTG
	$5AP1R1_{out}$	GCAGCCTGCTGTGCTGCAACCTTCT
	$3AP1F2_{in}$	CCTATGCAGAGAGACAGATGGAGGCA
	$5AP1R2_{in}$	TCTGCTCCCATGTAATGCCTGTGGTT
TtAP2	dAP2F	CAGTAYMGMGGYGTYACNTT
	dAP2R	CRAAGTTGGTNACNGCNTCYT
	$3AP2F1_{out}$	GGGTGGATTTGACACAGCACATGCAGC
	$5AP2R1_{out}$	TGCTGCCTTGTCATAGGCCCTTGCT
	$3AP2F2_{in}$	TGCTCGTGCATATGATAGAGCGGCTA
	$5AP2R2_{in}$	TTGTCGGCGAAGTACGTGCACAAAT
Tt*bACTIN*	dbACTINF	TGAAGGAAAAACATGCSTAYAT
	dbACTINR	KGAACCACCACTCAAMACAATG
	bACTINF	AGCTCAGCTGTTGAGAAGAGC
	bACTINR	ACATCGCACTTCATGATCGAG

(330 bp for *TtLFY*; 667 bp for *TtAP1*; 447 bp for *TtAP2*; and 276 bp for *b-ACTIN*) were removed, purified from the agarose gel, ligated to the pJET1.2/blunt-end cloning vector (Thermo Fisher Scientific, USA), and transformed into *Escherichia coli* strain DH5□. Purified recombinant plasmids were sequenced by the dideoxy method using an ABI3730 automated sequencer (IONTEK, Turkey). At least three complete sequences were obtained with reverse and forward primers for each target gene. Both cDNA sequences and deduced amino acid sequences were BLAST searched against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

The partial cDNA sequences of *TtLFY*, *TtAP1*, and *TtAP2* were used to design two pairs (outer and inner) of gene-specific primers. The gene-specific primers were generated by using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table). Then 5' and 3' rapid amplification of cDNA ends (RACE) analyses were carried out in order to obtain the full length cDNA sequences of *TtLFY*, *TtAP1*, and *TtAP2*. Using SMARTer RACE cDNA amplification kit (Clontech, USA), according to the manufacturer's protocol, 5' and 3' RACE-ready cDNAs were amplified from total RNA extracts of young floral buds, and 5'- and 3'-RACE–ready cDNAs were used to amplify 5' and 3' RACE-PCR products, respectively. PCR reaction mixture (50 µL) included RACE-ready cDNA, a gene specific primer, universal primer A mix, dNTP, reaction buffer, and Advantage 2 Polymerase mix (Clontech, USA). The PCR cycles were 2 min at 94 °C, 5 cycles of 30 s at 94 °C, and 3 min at 72 °C; 5 cycles of 30 s at 94 °C, 30 s at 70 °C, and 3 min at 72 °C; and 20 cycles of 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C. PCR product (5 µL) was checked by 1.2% agarose gel electrophoresis, and the remaining PCR product was directly sequenced.

The cDNA sequences of *TtLFY*, *TtAP1*, and *TtAP2* were translated into amino acid sequences using a translation tool (http://www.fr33.net/translator.php). Physicochemical properties of target proteins were evaluated by the ProtParam tool (http://www.expasy.org/tools/protparam.html). Multiple sequence alignments were performed using Clustal Omega (Sievers et al., 2011) with a gap open penalty of 10.00 and a gap extension penalty of 0.05 (http://www.ebi.ac.uk/Tools/msa/clustalo/). The protein BLAST algorithm was used to search the NCBI GenBank databases (http://www.ncbi.nlm.nih.gov/) for homolog sequences, and amino acid sequences of selected species were downloaded for each target gene. For phylogenetic analysis, neighbor-joining trees with 10,000 bootstrap replicates were constructed with representative homologs for each of the target genes using MEGA6 program (Tamura et al., 2013).

2.3. Southern blot analysis

Southern blot analysis was performed as described by Sambrook et al. (1989). For this purpose, genomic DNA was extracted from young leaves of *T. turcica* by cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). Genomic DNA (30 µg) was digested with *Eco*RI, *Bam*HI, and *Hind*III restriction enzymes. DNA fragments were separated on a 1% (w/v) agarose gel, transferred to a positively charged nylon membrane, and hybridized with digoxigenin-labeled probes (Roche Diagnostics). RT-PCR products generated using gene-specific primers to *TtLFY*, *TtAP1*, and *TtAP2* were used for probe synthesis. The DNA probes were labeled with DIG using the DIG DNA labeling kit (Roche Diagnostics). After hybridization and washing, signals were detected using a CDP-Star kit (Roche Diagnostics) following the manufacturer's instructions.

2.4. Semiquantitative RT-PCR analyses of *TtLFY, TtAP1,* and *TtAP2*

The expression patterns of *TtLFY*, *TtAP1*, and *TtAP2* in 18 different vegetative and reproductive tissues were studied. The total RNA extraction, DNase treatment, RNA quantification, and RNA integrity for each RNA sample were as described above. The reverse primers (Table) were used for *TtLFY*, *TtAP1*, and *TtAP2* expressions. The size of PCR products was 150 bp for *TtLFY*, 122 bp for *TtAP1*, and 142 bp for *TtAP2*. bACTINF/bACTINR primer pair was designed to screen a 172 bp *TtbACTIN* fragment (GenBank accession number: KP781858). Total RNA (1 µg) was used in reverse transcription reactions using Maxima First Strand cDNA Synthesis kit (ThermoScientific) according to the manufacturer's instructions. The reaction mixture (25 µL) of real-time quantitative PCR consisted of 5 µL of cDNA, 1 µL of each forward/reverse gene specific primer, 5.5 µL of DEPC-treated and PCR-grade water, and 12.5 µL of Maxima SYBR Green qPCR Master Mix (ThermoScientific). Two biological replicates (with three technical replicates each) of each RNA source were used to screen the target and reference gene in each qPCR run. In addition, a nontemplate control (NTC) reaction (containing water instead of cDNA as template) with each primer pair was run in order to reveal the absence of primer dimers or contamination. The qPCR reaction conditions were 10 min at 98 °C and then 40 cycles of 10 s at 98 °C, 15 s at 60 °C, and 30 s at 72 °C. The melting-curve analysis was performed after every 40 cycles of amplification. Real-time PCR was performed using a BioRad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA), and the results were analyzed by CFX Manager software, version 1.0 (Bio-Rad Laboratories).

3. Results

After obtaining the partial sequences of the target genes using degenerate primers, gene-specific primers were prepared and used in 3'- and 5'-RACE analyses in order to obtain the full-length cDNA sequences of *TtLFY*, *TtAP1*, and *TtAP2*. The lengths of full cDNA sequences were 1518 bp for *TtLFY*, 1074 bp for *TtAP1*, and 2308 bp for *TtAP2*. These full-length nucleotide sequences were submitted to the NCBI GenBank database and given accession numbers KT163119 (*TtLFY*), KT163120 (*TtAP1*), and KT163121 (*TtAP2*).

T. turcica LFY gene (*TtLFY*) had 150 nt 5' UTR, 1170 nt coding sequence for 389 amino acids, 199 nt 3'UTR region with a polyadenylation signal (AATAAA), and a poly(A) tail. The predicted molecular weight and isoelectric point for the TtLFY protein were 44.53 kDa and 6.55, respectively. The deduced amino acid sequence of TtLFY had higher (78%–81%) sequence identity with LFY/FLO homologs of some legume species such as *Cicer arientinum*, *Pisum sativum*, *Acacia mangium*, *A. auriculiformis*, *Medicago truncatula*, *M. sativa*, *G. max*, *Lotus japonicus*, and *Phaseolus vulgaris* (Figure 1). The other selected nonlegume LFY/FLO sequences including *A. thaliana* LFY also had higher (65%–75%) sequence identities with TtLFY. Interestingly, the neighbor-joining (N-J) clustering analysis grouped TtLFY and *A. thaliana* LFY together, centered between legume and nonlegume FLO/LFY subgroups (Figure 1). The motifs are important indications for a transcription factor, and TtLFY had some conserved and variable motifs in its deduced structure. The alignment of TtLFY with its legume homologs indicated that it had two conserved regions at N-terminal (46–128 aa) and C-terminal (215–372 aa) and two variable regions at N-terminal (2–45 aa) and at mid-part (129–214 aa) (Figure 2). Seven of 45 (15.5%) amino acid residues in the first variable region were proline residues. The N-terminal conserved region had a putative short leucine zipper. Further structural analysis indicated that the second

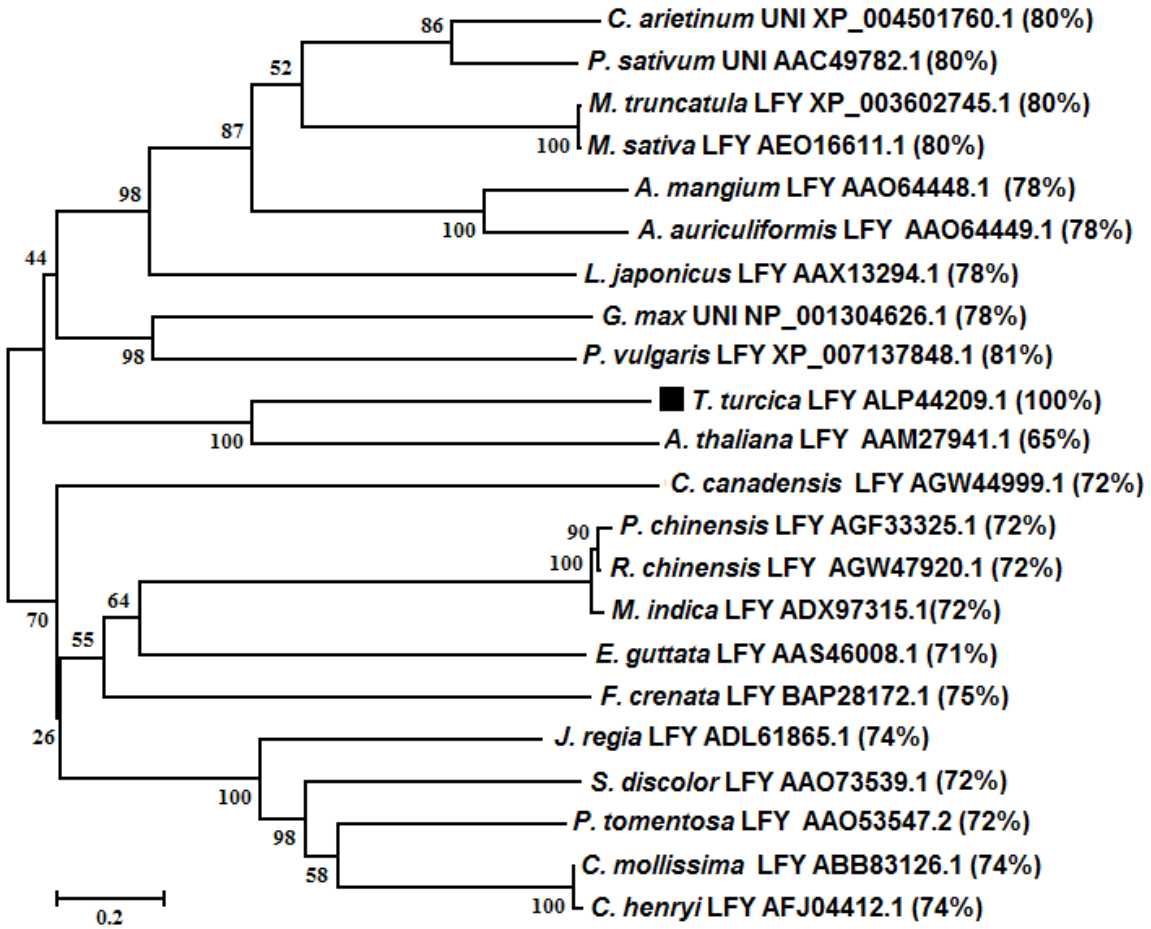

Figure 1. Neighbor-joining–based phylogenetic tree for *T. turcica* LFY and selected FLO/LFY protein sequences obtained from BLASTp analysis at NCBI. The percentage in parenthesis indicates sequence identity between TtLFY and its homologs. The percentages of replicate trees in the bootstrap test (10,000 replicates) are shown next to the branches (note: UNI indicates for UNIFOLIATA protein).

variable region had a central acidic domain consisting of Asp and Glu residues and a basic region consisting of Arg and Lys residues (Figure 2).

T. turcica AP1 (*TtAP1*) had a 57 nt 5'UTR and 714 bp protein coding sequence for 237 amino acids and 270 nt 3'UTR region with a polyadenylation signal and poly(A) tail. The predicted molecular weight and isoelectric point for the deduced TtAP1 protein were 27.65 kDa and 8.93, respectively. In the BLAST and N-J clustering analysis, in addition to legume AP1/FUL-like protein sequences, we selected AP1/FUL-like protein sequences of plant species (*Paeonia lactiflora*, *P. suffruticosa*, *Aquilegia coerulea*, *Nigella sativa*, *Jeffersonia diphylla*, and *Ranunculus bulbosus*) belonging to the families Paeoniaceae and Ranunculaceae, which are characterized by multi-free carpellated flowers (Figure 3). TtAP1 protein had higher sequence identity values (78%–88%) with selected AP1-like proteins of legume species and lower identity values (68%–63%) with AP1/FUL-like proteins of nonlegume species (Figure 3). The N-J clustering analysis clearly clustered the protein sequences based on AP1-like and FUL-like protein sequences. TtAP1 was grouped together with AP1-like proteins of legumes rather than AP1/FUL-like protein sequences of multi-free carpellated plant species (Figure 3). We aligned only TtAP1 and legume AP1 protein sequences in order to simplify the presentation of common motifs found in the deduced TtAP1 protein (Figure 4). TtAP1 protein was a typical MADS box transcription factor and had the MADS, I, K, and C domains. The amino acid compositions of these domains were highly similar. The last residues in C-terminal had EuAP1-like motif, a characteristic motif for AP1-like proteins (Figure 4).

T. turcica AP2 (*TtAP2*) had a 1638 bp coding sequence for 545 amino acids and a putative miR172 binding sequence. However, the polyadenylation signal (AATAAA) and poly(A) tail were not obtained in 3'UTR region of *TtAP2*. The molecular weight of deduced TtAP1

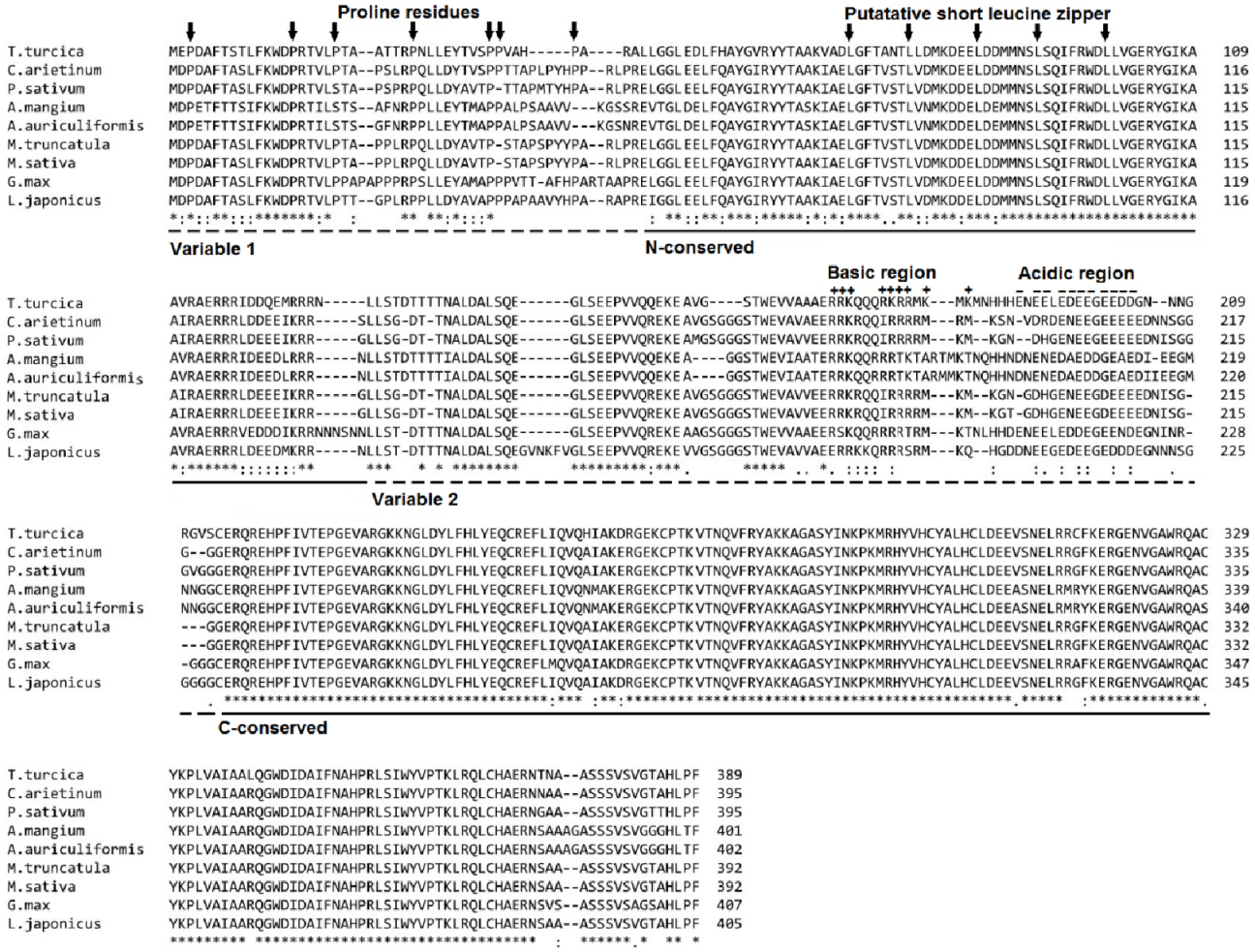

Figure 2. Amino acid sequence alignment for *T. turcica* LFY protein with selected legume LFY-like proteins (see Figure 1 for protein accession numbers). The dash and underline indicate two variable and two conserved regions, arrows indicate proline residues and leucine repeats, "+" and "-" are the signs of the basic and acidic regions, asterisks and dots indicate totally identical and similar amino acid residues, respectively. Dashes were used for introducing gaps to optimize alignment.

protein was 60.74 kDa, and its isoelectric point was 6.67. In comparison to the deduced TtLFY and TtAP1 proteins, TtAP2 had lower (80%–59%) sequence identity with its AP2 homologs of legume and nonlegume species. There was no species-specific clustering for the full-protein length of AP2 homologs. Therefore, the highly conserved portions of selected AP2 proteins were used in phylogenetic analysis, because the N- and C-terminals of AP2 protein family have very low sequence identity (Figures 5 and 6). In total, 168 amino acid residues found in NLS AP2-R1 and AP2-R2 domains (Figure 6) were used for the construction of phylogenetic relationships among the selected AP2 proteins. N-J clustering analysis clearly grouped AP2 proteins in a species-specific manner (Figure 5); TtAP2 was grouped with AP2-like proteins of legume species (*C. arietinum*, *G. max*, *G. soja*, *M. truncatula*, *P. sativum*, *P. vulgaris*, *Vigna angularis*, and *V. radiata*) rather than AP2-like protein sequences of multi-free carpellated plant species *P. lactiflora* and *P. suffruticosa*. The alignment of TtAP2 with its legume and nonlegume homologs indicated that the numbers of amino acids found in AP2 proteins were quite different, and TtAP2 had a longer amino acid sequence than its homologs. In the deduced TtAP2 structure, motif 1 (MWDLND), motif 2 (VTRQFFP), and motif 3 (LDLSDL); nuclear localization sequence (motif KKSR); and two repeats (R1 and R2) of AP2 motifs (also known as YRG/RAYD motifs) with their linker were well conserved; however, the other parts of the deduced TtAP2 protein, especially C-terminal, were poorly conserved (Figure 6).

The copy numbers of *TtLFY*, *TtAP1*, and *TtAP2* in *T. turcica* were determined by genomic Southern blot

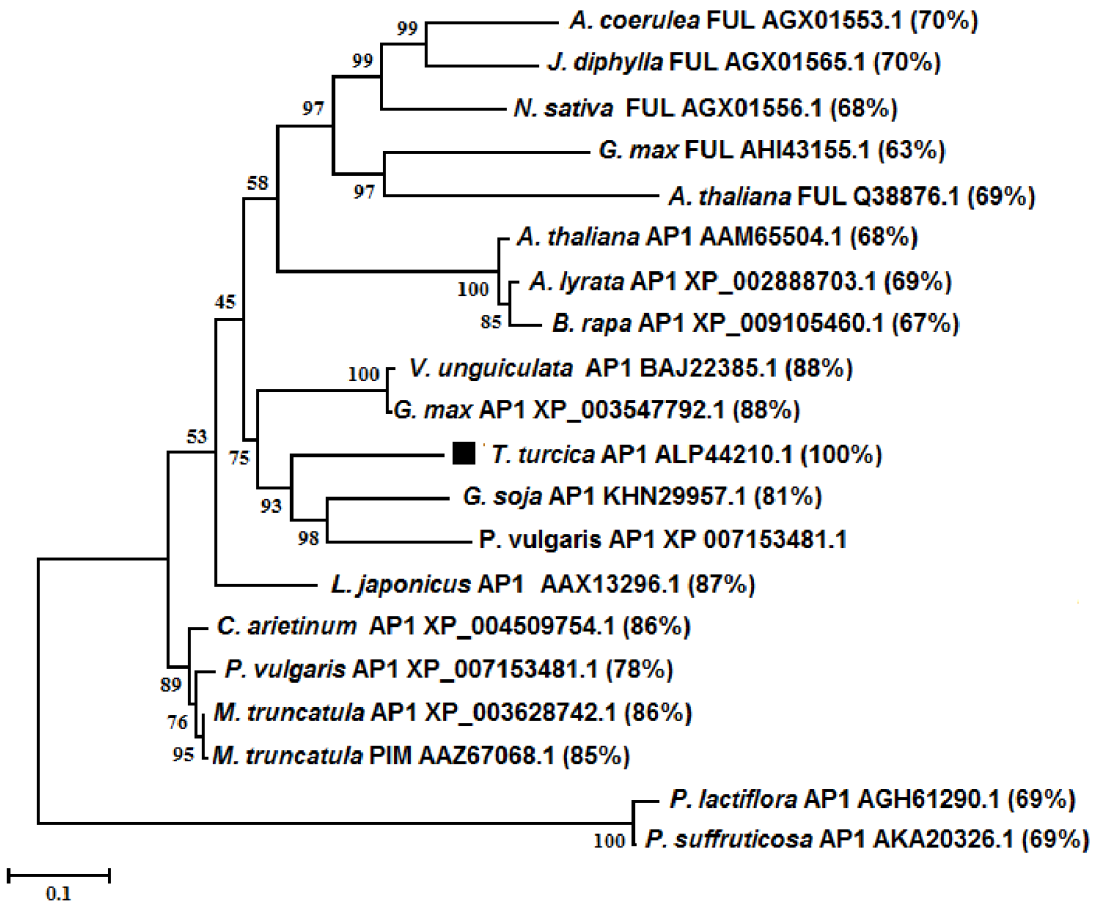

Figure 3. Neighbor-joining–based phylogenetic tree for *T. turcica* AP1 and selected AP1/FUL-like protein sequences obtained in BLASTp analysis at NCBI. The percentage in parenthesis indicates the ratio for sequence identity between TtAP1 and its homologs. The percentages of replicate trees in the bootstrap test (10,000 replicates) are shown next to the branches.

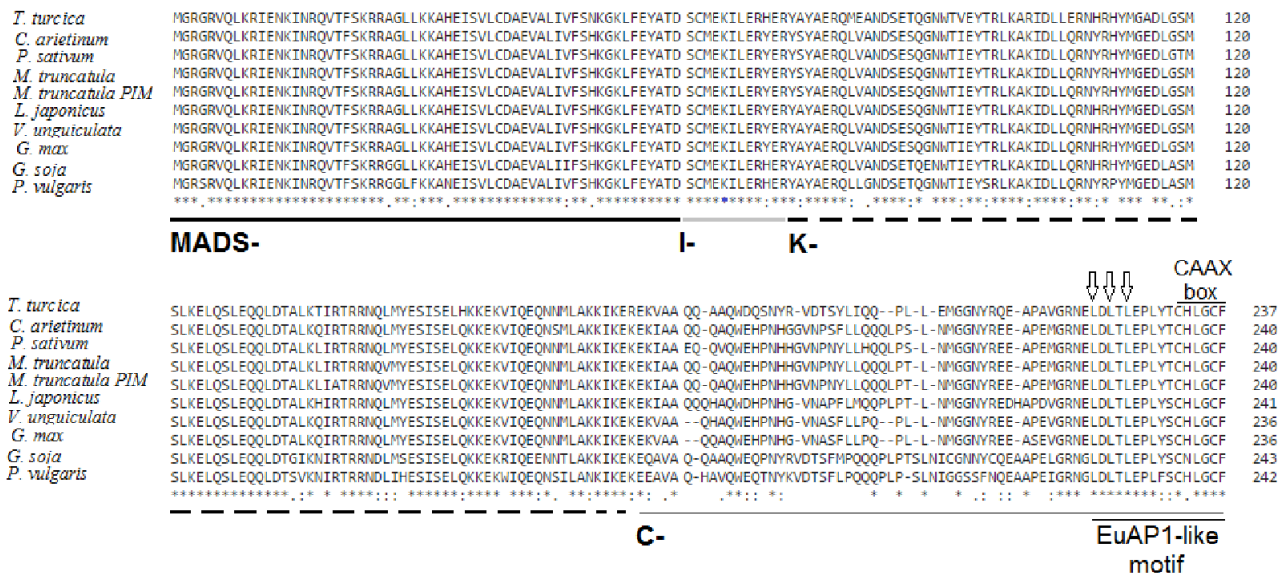

Figure 4. Amino acid sequence alignment for *T. turcica* AP1 with selected legume AP1 -like proteins (see Figure 3 for protein accession numbers). MADS-, I-, K-, and C-domains were underlined. The asterisks and dots indicate totally identical and similar amino acid residues, respectively. Dashes were used for introducing gaps to optimize alignment.

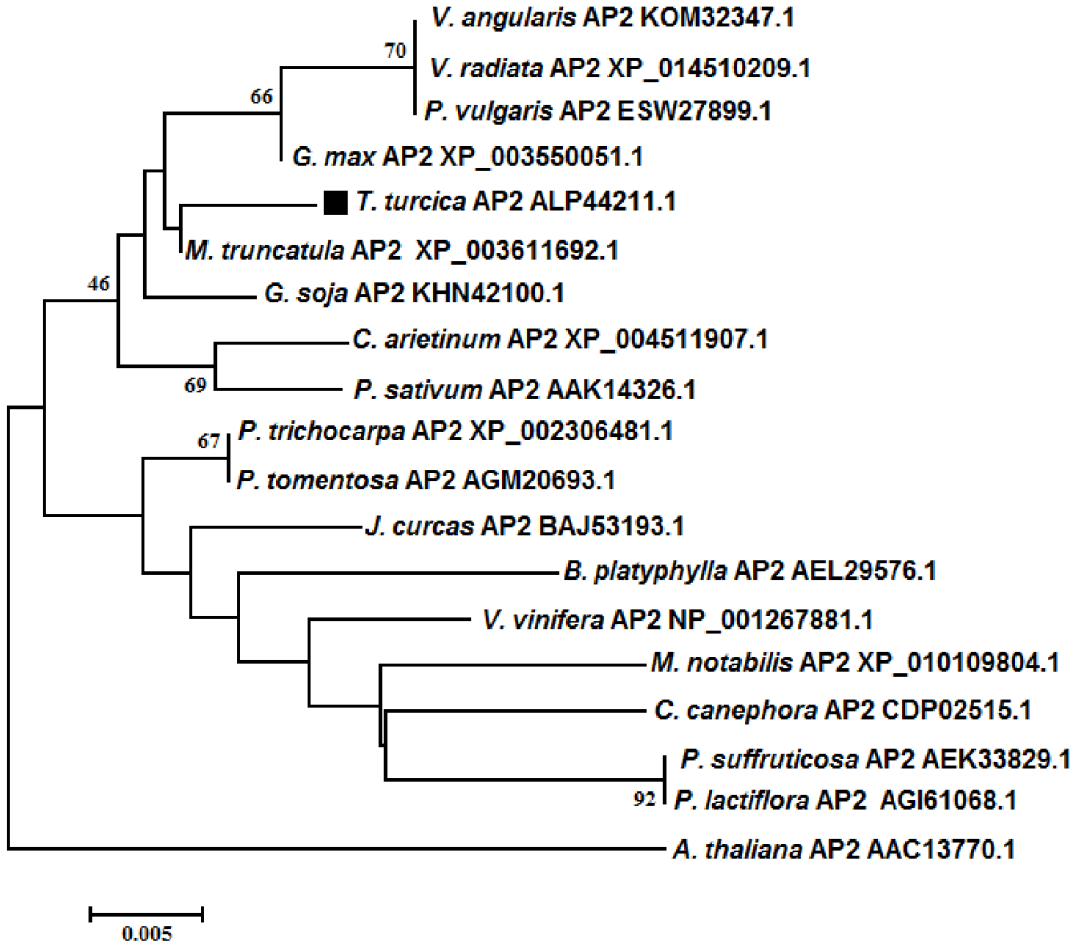

Figure 5. Neighbor-joining–based phylogenetic tree for *T. turcica* AP2 and selected AP2 family protein sequences obtained in BLASTp analysis at NCBI. The highly conserved 168 amino acid sequence of AP2 proteins (NLS-AP2-Linker-AP2 domains; see Figure 6) were used in phylogenetic analysis, because N- and C-terminals of AP2 family have very low sequence identity among the plant species. The sequence identities of the compared protein portion were higher than 95%. Bootstrap values higher than 40% (10,000 replicates) were shown next to the branches.

analysis (Figure 7) using *T. turcica* DNA digested with three restriction enzymes, *Eco*RI, *Bam*HI, and *Hind*III. The use of gene-specific probes produced only a single band for each gene under low stringency conditions. This result suggests that each of the *TtLFY*, *TtAP1*, and *TtAP2* genes occurs as a single copy in the genome of *T. turcica*.

We carried out the relative expression analysis in different vegetative and reproductive tissues of *T. turcica* by using real-time PCR in order to functionally characterize *TtLFY*, *TtAP1*, and *TtAP2* genes. Figure 8 shows expression analysis of three different genes in 18 different vegetative and reproductive tissues of *T. turcica*. The results indicated that *TtLFY* transcripts were obviously abundant in the shoot apex and in the floral bud. The level of *TtLFY* transcripts was low in the sepal and carpel tissues of semi-opened flowers and in the leaf tissues (both young and mature). On the other hand, *TtLFY* transcripts were almost absent in all remaining samples including stem tissue, petal, and stamen tissues of semi-opened flowers; all floral tissues of pollinated flowers; and all seeds and seedpods of young and mature fruits. *TtAP1* transcripts were mainly abundant in the sepals, petals, and floral buds. The expression analysis also indicated that a low level of *TtAP1* transcripts was detected in the young leaf tissue, shoot apex, and carpel tissues. Its expression was very low or absent in the stamen tissues and in the seeds and seed pods. Unlike the *TtLFY* and *TtAP1*, *TtAP2* expression was obviously detectable in all examined tissues. The highest *TtAP2* transcript level was determined in sepals of semiopened flowers. *TtAP2* transcripts were also higher in some vegetative tissues such as mature seedpods, mature leaf tissue, and shoot apex.

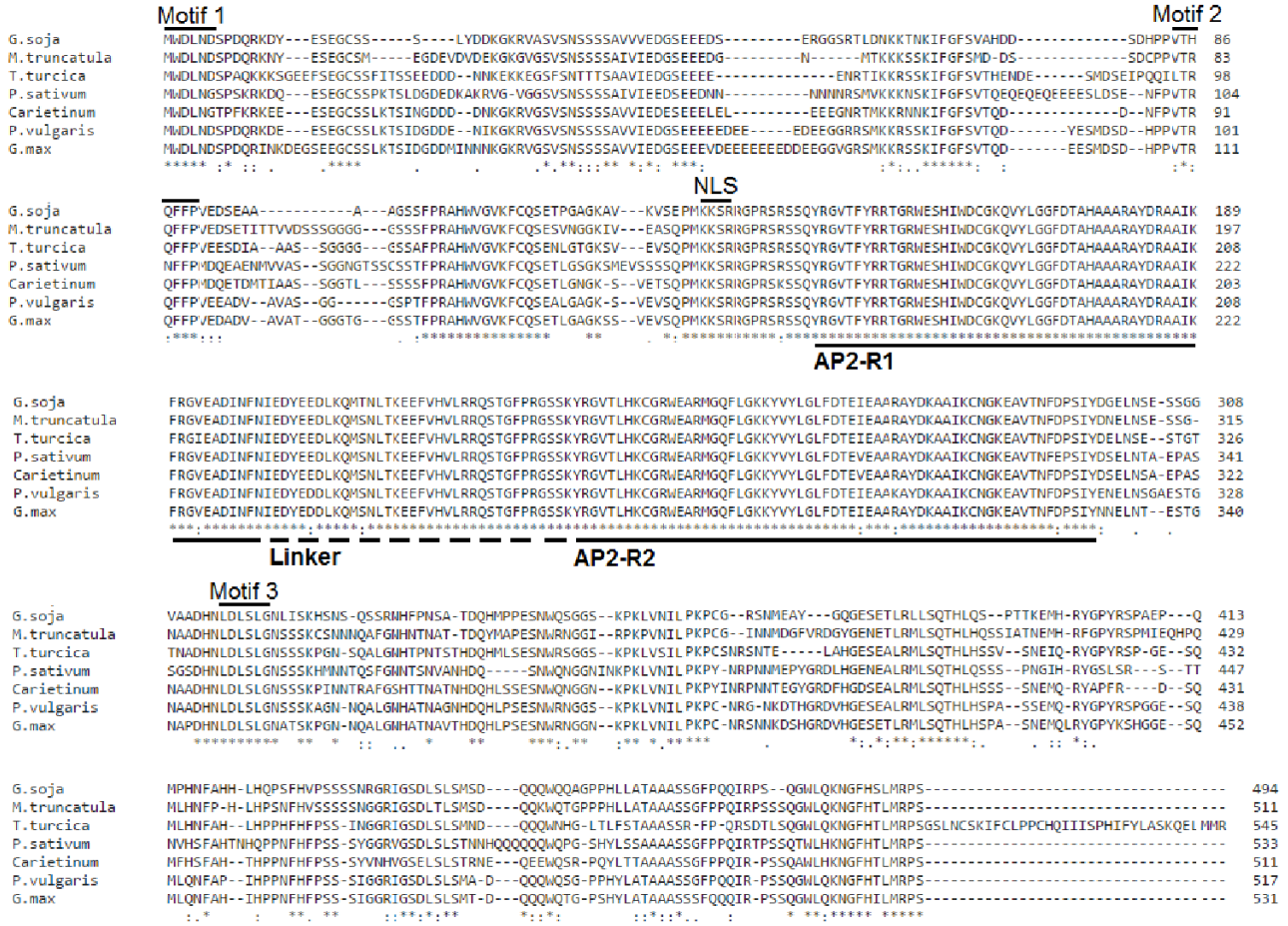

Figure 6. Amino acid sequence alignment for *T. turcica* AP2 with legume AP2-like proteins (see Figure 5 for protein accession numbers). The motifs were underlined (see text for information). The straight lines indicate repeats (R1 and R2) of AP2 domain. The linker between AP2-R1 and AP2-R2 were dash-lined. Dashes were used for introducing gaps to optimize the alignment. The asterisks and dots indicate totally identical and similar amino acid residues, respectively.

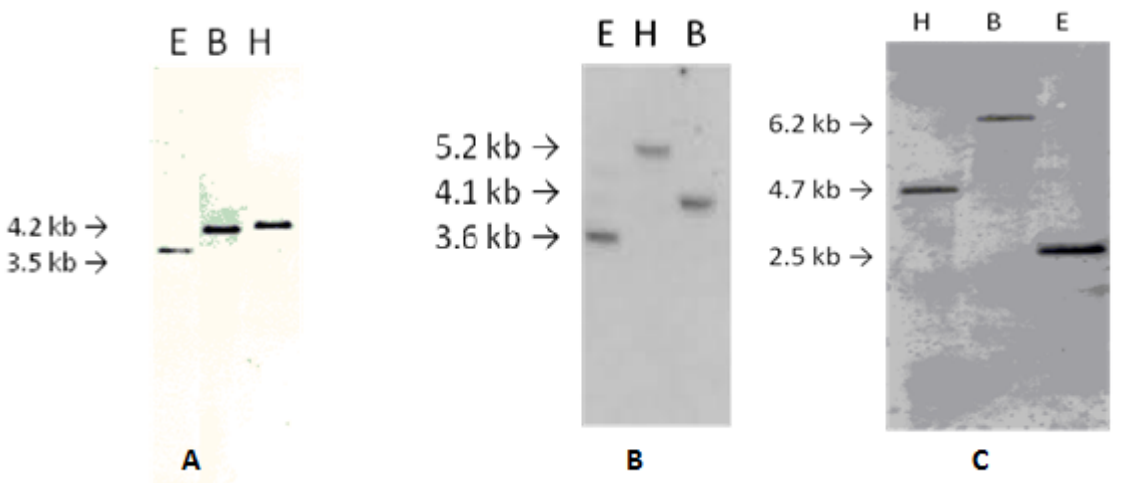

Figure 7. Genomic Southern blot analysis of the *TtLFY* (A), *TtAP1* (B), and *TtAP2* (C) genes. Genomic DNA was isolated from the leaves of *T. turcica*. DNA samples (30 µg) were digested with each restriction enzyme and separated on a 1% agarose gel. The gel was blotted onto a positively charged nylon membrane. The blots were probed with DIG-labeled fragments from the cDNAs of each of the genes. The positions of DNA standards are indicated on the left. E = *Eco*RI, B = *Bam*HI, H = *Hind*III.

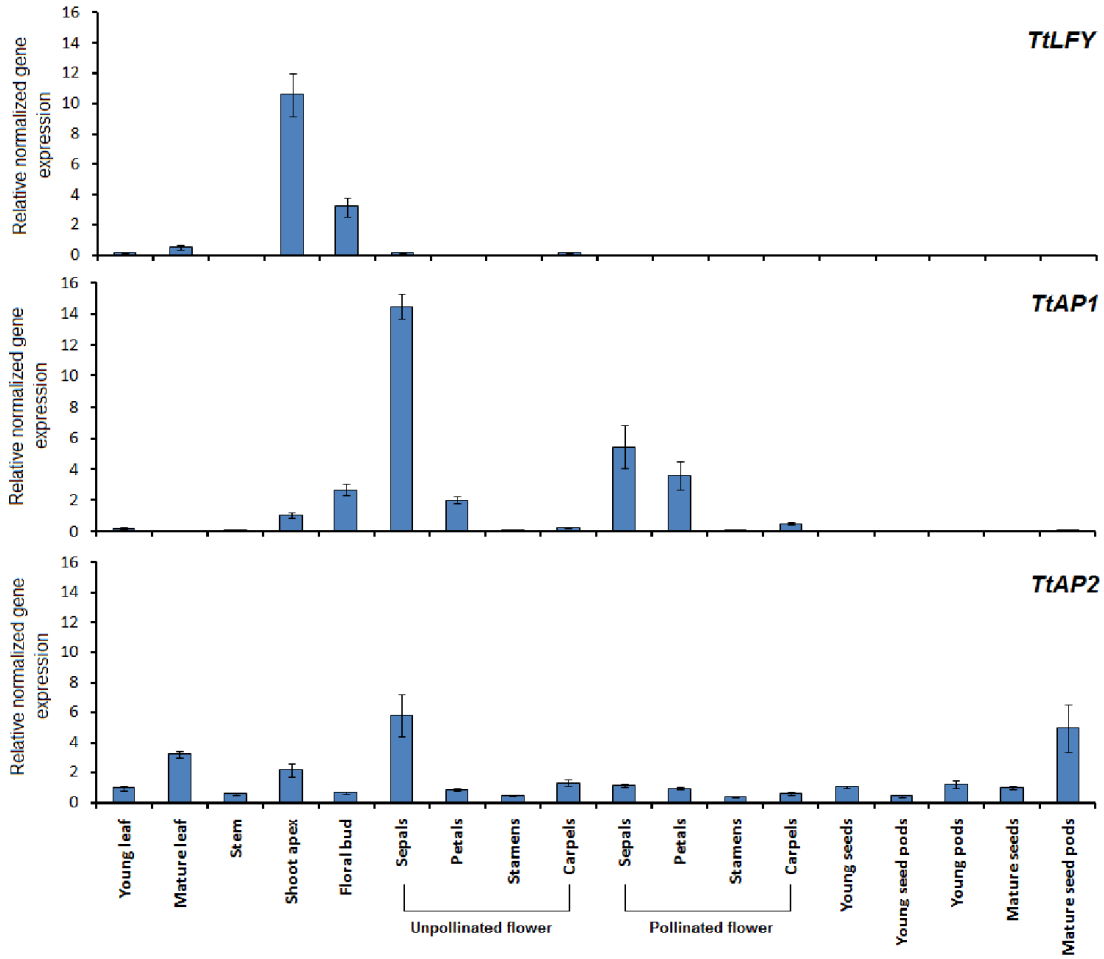

Figure 8. Expression of *TtLFY*, *TtAP1*, and *TtAP2* in different vegetative and reproductive tissues of *T. turcica.*

4. Discussion

Molecular mechanisms of flowering and fruit setting have been extensively studied in herbaceous annual plants such as *Arabidopsis* and *Antirrhinum* and in some woody plants. However, very little is known about the underlying molecular mechanisms of flowering, floral organ development, and fruit setting in herbaceous perennials. The endemic legume *T. turcica* is an herbaceous perennial plant with a blooming season from May to July. Its flowers are large, golden yellow, and zygomorphic and have 5 sepals, 5 petals, 10 free stamens, and three free carpels (Tan et al., 1983). The setting of three fruit or pods from a single flower is an important exception for Fabaceae plants (Tan et al., 1983). The *T. turcica* with its unusual morphological characteristic could be an important genetic resource. The structural and functional characteristics of some important homeotic genes such as *LFY*, *AP2*, and MADS box genes responsible for flower setting in *T. turcica* were investigated to identify the molecular mechanism controlling this unusual morphological feature.

The sequence and structural analysis of the isolated gene and its deduced protein clearly indicated that the *TtLFY* gene is a *FLO/LFY* gene homolog found in *T. turcica*. The predicted amino acid sequence of TtLFY had higher similarities with other LFY/FLO homologs (Meng et al., 2007; Zhang et al., 2010; Wang et al., 2012). The TtLFY protein has two conserved and two variable regions similar to other LFY/FLO proteins. Two conserved regions at N- and C-terminals were important for FLO/LFY function (Zhang et al., 2010). It was suggested that the proline-rich region in the first variable region may be important for transcriptional activation (Coen et al., 1990); however, the deduced TtLFY protein lacks the proline-rich region, which was also absent in some LFY/FLO proteins such as *Pinus radiata* and *Phalaenopsis hybrida* LFY proteins (Zhang et al., 2010). The proline-rich region of LFY/FLO proteins may have been subjected to evolutionary changes as it is located within the variable region. TtLFY has a putative short leucine zipper in the

first conserved region and acidic and basic regions in the second variable region. These regions are characteristics of some transcriptional activators and important for the function of FLO/LFY-like proteins (Coen et al., 1990; Weigel et al., 1992). The structural model for C-terminal conserved region based on SWISS-MODEL Workspace (figure not shown) indicated that the TtLFY protein has a seven alpha-helix fold similar to *A. thaliana* and *Carya cathayensis* LFY proteins (Hamès et al., 2008; Wang et al., 2012). The conserved region at the C-terminal has a cooperative binding mechanism to DNA and plays a role in the induction of downstream floral meristem genes in flowering, such as *AP1* (Hamès et al., 2008).

In the *Arabidopsis* genome, more than 100 MADS box genes were reported, and they were categorized in nine subgroups based on their sequence and functional similarities (Theißen et al., 2000). *Arabidopsis AP1* and *Antirrhinum SQUAMOSA* (*SQUA*) belong to SQUA clade (also called AP1/AGL9 clade) of MADS box genes (Theißen et al., 2000; Tsaftaris et al., 2004). The *AP1*/SQUA is a floral meristem identity gene and has a key regulatory role in the establishment of floral meristem along with *LFY*/*FLO*. *AP1*/*SQUA* and *AP2*, as A-class gene of the ABCDE model, is also a floral organ meristem identity gene and has a role in the establishment and development of sepals and petals. Analysis of PEAM4, the pea AP1 functional homolog, demonstrated that the dual function of AP1/SQUA in both *Arabidopsis* and *Antirrhinum* has been extended to the species leading to diverse floral morphogenesis (Berbel et al., 2001). The partial or full cDNA sequences of *AP1*/*SQUA* gene homologs have been isolated and characterized from various plant species such as *A. thaliana* (Bowman et al., 1993), *A. majus* (Carpenter and Coen, 1990), *G. max* (Chi et al., 2011), *C. sativus* (Tsaftaris et al., 2004), *C. persicum* (Tanaka et al., 2011), *S. tetraptera* (Song et al., 2008), *C. sinesensis* (Pllitteri et al., 2004), *Nymphaea* spp. (Luo et al., 2011), *M. truncatula* (Benlloch et al., 2006), *P. lactiflora* (Ge et al., 2014), and *P. sativum* (Berbel et al., 2001). The deduced TtAP1 protein has MADS-, I-, K-, and C-domains and shows high sequence identity with AP1-like proteins, especially those of legumes. The C-domain of the TtAP1 protein has a clear EuAP1-like motif consisting of RRNaLAT/NLa and farnesylation signal CAAX. The C-domain is the least conserved domain among the MADS-box proteins (Litt and Irish, 2003) and contains an EuAP1-like motif which is well characterized for the AP1-like proteins (Chi et al., 2011) such as AP1, FUL, and CAL in *Arabidopsis* and SQUA in *Antirrhinum* (Berbel et al., 2001; Tsaftaris et al., 2004). The farnesylation motif CAAX was also reported for AP1-like proteins isolated from other legumes such as *L. japonicus*, *P. sativum*, and *M. truncatula* (Berbel et al., 2001; Benlloch et al., 2006), but not for AP1-like GmAP1 protein isolated from *G. max* (Chi et al., 2011). The results indicated that the posttranslational regulation of deduced TtAP1 protein could be essential for AP1 function in *T. turcica*.

The expression of *AP2* transcripts in various floral organs of *Arabidopsis* is under miR172 regulation as a translational repressor (Tsaftaris et al., 2012). A 21-nucleotide miR172 (UAGCAUCAUCAAGAUUUUUAU) has the capacity to bind to their possible target site on the *AP2* transcripts (Aukerman and Sakai, 2003). For accurate target recognition, there must be 100% complementation to seed region base pairs 2–7 on the 5' end of miR172 (Tsaftaris et al., 2012). The *TtAP2* transcript has a possible binding site at a position between 1442 and 1463 nt, near the 3' end of the open reading frame for miR172, where there is a 100% match to 2–16 nucleotides. Bioinformatic analyses indicated that *TtAP2* encodes a putative AP2-related protein that belongs to the large AP2/EREBP family. The deduced TtAP2 sequence contains two well-conserved repeats of AP2 domains and the linker region between them as other AP2-like proteins (Jofuku et al., 1994). The nuclear localization signal motif (KKSR), motif 1 (MLDLN), motif 2 (VTRQFFP), and motif 3 (LDLSLG) were the other conserved regions in TtAP2 protein, similar to *Arabidopsis* AP2 (Jofuku et al., 1994) and *C. sativus* AP2 (Tsaftaris et al., 2012). The functions of motif 1, motif 2, and motif 3 are not known, but they may be important to protein structure or the activation of transcription (Vahala et al., 2001).

The sequencing of cloned PCR products from degenerate primers could not detect any paralogous sequences for *TtLFY*, *TtAP1*, or *TtAP2*. In addition, the Southern blot analyses indicated that *TtLFY*, *TtAP1*, and *TtAP2* exist as single-copy genes in the genome of *T. turcica*. Therefore, these three flowering genes of *T. turcica* occur as a single copy, similar to most angiosperms (Coen et al., 1990; Weigel et al., 1992; Song et al., 2008), which suggests that these genes were in the form of the ancient genome and not duplicated as in some gymnosperms and angiosperms.

In order to obtain the transcript level of cloned and isolated *TtLFY*, *TtAP1*, and *TtAP2* genes in different vegetative and reproductive tissues of *T. turcica*, relative expression analysis was performed using semiquantitative RT-PCR. The *TtLFY* transcripts were found largely in the shoot apex, moderately in the floral buds, and few in the leaves. The *TtLFY* transcripts were almost undetectable in the stem, developed floral organs, and fruit tissues. Transcripts of *LFY*-like genes were determined abundantly at the beginning of floral development and slightly in vegetative tissues such as shoot apices and leaves of angiosperms (Kotoda et al., 2000; Wang et al.,

2012; Jinghua et al., 2014). In addition to control of the transition from vegetative phase to reproductive phase, *LFY* homologs have functions in regular leaf development in pea and tomato plants (Jinghua et al., 2014). That the highest transcript level of *TtLFY* is in the underground shoot apex of *T. turcica* could indicate that *TtLFY* has a strong function in the development of vegetative meristem. Strong *LFY* expression in the vegetative meristem was also reported for *LFY* homologs of *P. persia* and *Phalaenopsis* (Zhang et al., 2010). Although some reports indicated that the transcripts of *LFY* homolog genes were also detectable in floral organs in some plants such as soybeans (Meng et al., 2007) and *Phalaenopsis* (Zhang et al., 2010), *TtLFY* transcripts were absent in the sepals, petals, stamens, carpels, seeds, and seed pods of *T. turcica*. An expression pattern similar to *TtLFY* was reported for *C. mollissima LFY* (Liu et al., 2011). These results showed that *TtLFY* is functioning in meristematic floral and vegetative tissues as well as in the leaves of *T. turcica*, but not in developed floral organs.

Many studies reported that the presence of *AP1* transcripts is specific to reproductive tissues rather than vegetative tissues (Berbel et al., 2001; Pillitteri et al., 2004). In this study, in agreement with the literature, *TtAP1* transcripts were mostly detected in the sepals and petals of semiopened and fertilized flowers and in young floral buds. The highest level of *TtAP1* transcripts was found in the sepals of semiopened flowers, and its level declined over time. The petal tissues of *T. turcica* also had higher amounts of *TtAP1* transcripts, which proves A-function of *AP1*, which is responsible for sepal and petal induction and development, as described for *A. thaliana* (Bowman et al., 1993) and *P. lactiflora* (Ge et al., 2014). There are some reports regarding the higher expression of AP1 homologs in stamen and carpel tissues (Pillitteri et al., 2004); however, *TtAP1* expression was very low in the carpels and not detectable in the stamens. We detected a smaller amount of *TtAP1* transcripts in some vegetative tissues such as shoot apex and young leaves. A similar result was also reported for *C. sativus* (Tsaftaris et al., 2004). There were no *TtAP1* transcripts in the seeds and seed coats of *T. turcica*, as reported for *S. tetraptera* (Song et al., 2008), suggesting that *TtAP1* has no function in fruit development.

In the present study, the transcripts of *TtLFY* and *TtAP1* were specific to few tissues of *T. turcica*, as described above. On the other hand, *TtAP2* transcripts were clearly detected in each vegetative and reproductive tissue of *T. turcica* under investigation (Figure 7). Similar findings were reported for *AP2* transcripts in reproductive and vegetative tissues of *Arabidopsis, Hordeum* sp., *Triticum* sp., *Picea abies*, and *P. lactiflora* including floral buds; floral organs; and leaf, stem, and root tissues (Jofuku et al., 1994; Vahala et al., 2001; Gil-Humanes et al., 2009; Ge et al., 2014). The detection of *AP2* transcripts in a different plant tissue or in a different developmental stage does not always mean *AP2* functioning. *AP2* expression is under posttranslational control of a microRNA, miR172 (Aukerman and Sakai, 2003), and *TtAP2* has a putative miR172 binding site in 3'-end of its mRNA. However, the transcript amount of *TtAP2* was higher in the sepals and mature seed pods of *T. turcica*. *AP2* is well known for initiation and development of sepals (Jofuku et al., 1994). Ripoll et al. (2011) found that *AP2* acts to prevent replum overgrowth by negatively regulating BP and RPL, two genes that normally act to promote replum formation. *TtAP2* could act in the development of sepal tissue and in the control of fruit size in *T. turcica*, but it needs miR172 confirmation.

The plant species of the genus *Thermopsis* are mainly spread throughout the mountainous and humid regions of Central Asia and North America (Wojciechowski, 2003), but the genus is represented solely by the endemic *T. turcica* in Anatolia with its unusual multiple free carpellated flowers (Tan et al., 1983). This morphologic feature offers an alternative strategy for increasing the crop production in agriculturally important legumes. However, the production of multiple free carpels or fruits from a single flower is not common among the plant species. There is no available information explaining or covering the molecular mechanisms underlying this unusual morphological phenotype in the literature. Indeed, a multiple (four or more) free carpellated flower plan is a remarkable feature of some plant species belonging to the families Crassulaceae, Paeoniaceae, and Ranunculaceae (Davis et al., 1965). However, their multiple free carpels were not of great interest as these plant families are not agriculturally important. On the other hand, the nucleotide and deduced protein sequences of floral genes were not available for Crassulaceae. There are few studies describing the isolation and characterization of flower genes including LFY, AP1/FUL, and AP2 genes in Paeoniaceae and Ranunculaceae (Ballerini and Kramer, 2011; Pabón-Mora et al., 2013; Ge et al., 2014). The N-J clustering analyses (Figures 1, 3, and 5) indicated that deduced *T. turcica* LFY, AP1, and AP2 proteins were more similar to protein homologs of legumes than plant species with multiple free carpellated flowers. Isolation and characterization of more flowering genes for Crassulaceae, Paeoniaceae, and Ranunculaceae might help illuminate the molecular mechanism of multiple free carpellated flower architecture in plants. The structural and functional analysis of *TtLFY*, *TtAP1*, and *TtAP2* genes have provided important molecular data for the flowering, floral organ development, and fruit setting in *T. turcica*; however, the available data were insufficient to describe the molecular mechanisms regulating and controlling

multiple carpellated flower formation. Nevertheless, the research on *T. turcica* BCDE class MADS box genes and/or the genes related to floral organ numbers is still in progress.

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References

Aukerman MJ, Sakai H (2003). Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-like target genes. The Plant Cell Online 15: 2730-2741.

Ballerini ES, Kramer EM (2011). Environmental and molecular analysis of the floral transition in the lower eudicot *Aquilegia formosa*. EvoDevo 2: 1-20.

Benlloch R, d'Erfurth I, Ferrandiz C, Cosson V, Beltrán JP, Cañas LA, Ratet P (2006). Isolation of *mtpim* proves *Tnt1* a useful reverse genetics tool in *Medicago truncatula* and uncovers new aspects of *AP1*-like functions in legumes. Plant Physiol 142: 972-983.

Berbel A, Navarro C, Ferrándiz C, Cañas LA, Madueño F, Beltrán JP (2001). Analysis of *PEAM4*, the pea *AP1* functional homologue, supports a model for *AP1*-like genes controlling both floral meristem and floral organ identity in different plant species. Plant J 25: 441-451.

Bowman JL, Alvarez J, Weigel D, Meyerowitz EM, Smyth DR (1993). Control of flower development in *Arabidopsis thaliana* by *APETALA 1* and interacting genes. Development 119: 721-743.

Carpenter R, Coen ES (1990). Floral homeotic mutations produced by transposon-mutagenesis in *Antirrhinum majus*. Gene Dev 4: 1483-1493.

Chi Y, Huang F, Liu H, Yang S, Yu D (2011). An *APETALA1*-like gene of soybean regulates flowering time and specifies floral organs. J Plant Physiol 168: 2251-2259.

Coen ES, Romero J, Doyle S, Elliott R, Murphy G, Carpenter R (1990). *floricaula*: a homeotic gene required for flower development in *Antirrhinum majus*. Cell 63: 1311-1322.

Davis PH (1965). Flora of Turkey and the East Aegean Islands (Vol. 1). Edinburgh, UK: Edinburgh University Press, pp. 95-206.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Ferrándiz C, Navarro C, Gomez MD, Canas LA, Beltran JP (1999). Flower development in *Pisum sativum*: from the war of the whorls to the battle of the common primordia. Dev Genet 25: 280-290.

Ge J, Zhao D, Han C, Wang J, Hao Z, Tao J (2014). Cloning and expression of floral organ development-related genes in herbaceous peony (*Paeonia lactiflora* Pall.). Mol Biol Rep 41: 6493-6503.

Gil-Humanes J, Pistón F, Martín A, Barro F (2009). Comparative genomic analysis and expression of the *APETALA2*-like genes from barley, wheat, and barley-wheat amphiploids. BMC Plant Biol 9: 66.

Hamès C, Ptchelkine D, Grimm C, Thevenon E, Moyroud E, Gérard F, Martiel JL, Benlloch R, Parcy F, Müller CW (2008). Structural basis for LEAFY floral switch function and similarity with helix turn-helix proteins. EMBO J 27: 2628-2637.

Irish VF, Sussex IM (1990). Function of the *apetala-1* gene during *Arabidopsis* floral development. Plant Cell 2: 741-753.

Jinghua D, Fangdong L, Hongyan D, Lin Z, Deyi Y, Yanzhi F, Lvyi M (2014). Cloning and expression analysis of a *LFY* homologous gene in Chinese jujube (*Ziziphus jujube* Mill.). African J Biotech 11: 581-589.

Jofuku KD, Den Boer BG, Van Montagu M, Okamuro JK (1994). Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. Plant Cell Online 6: 1211-1225.

Kaufmann K, Wellmer F, Muiño JM, Ferrier T, Wuest SE, Kumar V, Riechmann JL (2010). Orchestration of floral initiation by *APETALA1*. Science 328: 85-89.

Kotoda N, Wada M, Komori S, Kidou SI, Abe K, Masuda T, Soejima J (2000). Expression pattern of homologues of floral meristem identity genes *LFY* and *AP1* during flower development in apple. J Amer Soc Hort Sci 125: 398-403.

Litt A, Irish VF (2003). Duplication and diversification in the *APETALA1/FRUITFULL* floral homeotic gene lineage: implications for the evolution of floral development. Genetics 165: 821-833.

Liu C, Xi W, Shen L, Tan C, Yu H (2009). Regulation of floral patterning by flowering time genes. Develop Cell 16: 711-722.

Liu T, Hu YG, Li XX (2011). Characterization of a chestnut *FLORICAULA/LEAFY* homologous gene. Afr J Biotechnol 10: 3978-3985.

Lohmann JU, Hong RL, Hobe M, Busch MA, Parcy F, Simon R, Weigel D (2001). A molecular link between stem cell regulation and floral patterning in *Arabidopsis*. Cell 105: 793-803.

Luo H, Chen S, Jiang J, Chen Y, Chen F, Teng N, Huang C (2011). The expression of floral organ identity genes in contrasting water lily cultivars. Plant Cell Rep 30: 1909-1918.

Mandel MA, Yanofsky MF (1995). A gene triggering flower formation in *Arabidopsis*. Nature 377: 522-524.

Meng Q, Zhang C, Huang F, Gai J, Yu D (2007). Molecular cloning and characterization of a *LEAFY*-like gene highly expressed in developing soybean seeds. Seed Sci Res 17: 297-302.

Ng M, Yanofsky MF (2001). Activation of the *Arabidopsis* B class homeotic genes by *APETALA1*. The Plant Cell Online 13: 739-753.

Pabón-Mora N, Hidalgo O, Gleissberg S, Litt A (2013). Assessing duplication and loss of *APETALA1/FRUITFULL* homologs in Ranunculales. Front Plant Sci 4: 358.

Pelaz S, Gustafson-Brown C, Kohalmi SE, Crosby WL, Yanofsky MF (2001). *APETALA1* and *SEPALLATA3* interact to promote flower development. Plant J 26: 385-394.

Pillitteri LJ, Lovatt CJ, Walling LL (2004). Isolation and characterization of a *TERMINAL FLOWER* homolog and its correlation with juvenility in citrus. Plant Physiol 135: 1540-1551.

Ripoll JJ, Roeder AH, Ditta GS, Yanofsky MF (2011). A novel role for the floral homeotic gene *APETALA2* during *Arabidopsis* fruit development. Develop 138: 5167-5176.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning, Vol. 1, No. 7.58. New York, NY, USA: Cold Spring Harbor Laboratory Press.

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Higgins DG (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol Systems Biol 7: 539.

Song J, Clemens J, Jameson PE (2008). Quantitative expression analysis of the ABC genes in *Sophora tetraptera*, a woody legume with an unusual sequence of floral organ development. J Exp Bot 59: 247-259.

Strommer J, Gregerson R, Vayda M, Glick BR, Thompson JE (1993). Isolation and characterization of plant mRNA. In: Glick BR, Thompson JE, editors. Methods in Plant Molecular Biology and Biotechnology. Boca Raton, FL, USA: CRC, pp. 49-65.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725-2729.

Tan K, Vural M, Kucukoduk M (1983). An unusual new *Thermopsis* from Turkey. Notes Roy Bot Gard Edinburgh 40: 515-518.

Tanaka Y, Yamamura T, Terakawa T (2011). Identification and expression analysis of the *Cyclamen persicum* MADS-box gene family. Plant Biotechnol 28: 167-172.

Theißen G, Becker A, Di Rosa A, Kanno A, Kim JT, Münster T, Winter KU, Saedler H (2000). A short history of MADS-box genes in plants. Plant Mol Biol 42: 115-149.

Theißen G, Saedler H (2001). Plant biology: floral quartets. Nature 409: 469-471.

Tsaftaris AS, Pasentsis K, Iliopoulos I, Polidoros AN (2004). Isolation of three homologous *AP1*-like MADS-box genes in crocus (*Crocus sativus* L.) and characterization of their expression. Plant Sci 166: 1235-1243.

Tsaftaris AS, Pasentsis K, Madesis P, Argiriou A (2012). Sequence characterization and expression analysis of three *APETALA2*-like genes from saffron crocus. Plant Mol Biol Rep 30: 443-452.

Tucker SC (1989). Overlapping organ initiation and common primordia in flowers of *Pisum sativum* (Leguminosae: Papilionoideae). Amer J Bot 714-729.

Tucker SC (2003). Floral development in legumes. Plant Physiol 131: 911-926.

Vahala T, Oxelman B, von Arnold S (2001). Two *APETALA2*-like genes of *Picea abies* are differentially expressed during development. J Exp Bot 52: 1111-1115.

Wang ZJ, Huang JQ, Huang YJ, Chen FF, Zheng BS (2012). Cloning and characterization of a homologue of the *FLORICAULA/LEAFY* gene in hickory (*Carya cathayensis* Sarg). Plant Mol Biol Rep 30: 794-805.

Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM (1992). *LEAFY* controls floral meristem identity in *Arabidopsis*. Cell 69: 843-859.

Wojciechowski MF (2003). Reconstructing the phylogeny of legumes (Leguminosae): an early 21st century perspective In: Klitgaard BB, Bruneau A, editors. Advances in Legume Systematics, Part 10, Higher Level Systematics. Kew, UK: Royal Botanic Gardens, pp. 5-35.

Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM (1990). The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors. Nature 346: 35-39.

Zhang JX, Wu KL, Zeng SJ, Duan J, Tian LN (2010). Characterization and expression analysis of *PhalLFY*, a homologue in *Phalaenopsis* of *FLORICAULA/LEAFY* genes. Sci Hort 124: 482-489.