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Full Length Research Paper

Cloning and expression analysis of a *LFY* homologous gene in Chinese jujube (*Ziziphus jujube* Mill.)

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LEAFY (LFY) homologous genes are necessary for the transition from vegetative to reproductive development in flowering plants. The full-length cDNA of a *LFY* homolog was successfully isolated from floral buds of Chinese Jujube (*Ziziphus jujube* Mill.) by degenerate reverse transcriptase-polymerase chain reaction (RT-PCR), rapid amplification of cDNA ends (RACE), and Blastn analysis. The *LFY* homologous gene was denominated as *ZjLFY* and deposited at the GenBank under accession JN165097. The full-length cDNA sequence of *ZjLFY* was 1517 bp in length and contained a completed open reading frame (ORF) of 1209 bp encoding 576 amino acids. Amino acid sequence comparison revealed that the ZjLFY had two variable regions and two conserved regions. At the amino acid level, the ZjLFY showed high similarity ranging from 57.5 to 82.9% with other *LFY* homologous members. Semi-quantitative RT-PCR and quantitative real-time PCR analysis revealed strong expression of *ZjLFY* in floral buds, but weak expression in fruit-bearing shoots, vegetative shoots, stems, mature leaves and axillary buds, suggesting that the *ZjLFY* played a role in both vegetative and reproductive development in Chinese jujube.

Key words: Chinese jujube, LFY, organ expression, semi-quantitative RT-PCR, quantitative real-time PCR.

INTRODUCTION

The transition from vegetative to flowering is one of the most important developmental switches in the life cycle of angiosperm that involves a series of endogenous and environmental signals induction. Recent researches on *Arabidopsis thaliana* showed that flowering is initiated by four genetic pathways including gibberellin, autonomous, vernalization and photoperiod pathways (Guo and Yang, 2008). A complicated network of flowering-time genes were found to tightly control the developmental processes leading to floral reproductive maturity. Three genes including CONSTANS (CO), FLOWERING LOCUS (FT) and GIGANTEA (GI) have been isolated, identified and considered to constitute a major genetic pathway in the photoperiodic regulation of flowering in *A. thaliana* (Putterill et al., 1995; Park et al., 1999; Samach et al., 2000; Blazquez et al., 2003; Huq et al., 2000). Of the three genes, CO and FT probably function as transcriptional activators, and directly or indirectly activate floral meristem identity genes (Simon et al., 1996).

As reported by Long and Barton (2000), floral meristem identity genes such as LEAFY (LFY) and APETALA1 (AP1) determine the fate of shoot meristems and originate the development of floral primordia instead of leaf primordia. *LFY* and *AP1* are responsible for development of flowers from floral primordia, of which *AP1* acts not only as a floral meristem identity gene, but also a floral organ

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Abbreviations: BLAST, Basic local alignment search tool; **NCBI,** National Center for Biotechnology Information; **ORF,** open reading frame; **RACE,** rapid amplified cDNA ends; **RT-PCR,** reverse transcriptase-PCR; **UTR,** untranslated region.

identity gene by specifying the development of sepals and petals (Huijser et al., 1992; Mandel et al., 1992). *LFY* has been demonstrated to play a crucial role in the transition from vegetative to reproductive development in *Arabidopsis* (Weigel et al., 1992; Weigel and Nilsson, 1995). Several LFY homologs have been isolated and characterized so far in some plant species including potato (*Solanum tuberosum*) (Guo and Yang, 2008), eucalyptus (*Eucalyptus grandis*) (Southerton et al., 1998; Dornelas et al., 2004), pine (Mellerowicz et al., 1998; Mouradov et al., 1998; Dornelas and Rodriguez, 2005a), *Populus trichocarpa* (Rottmann et al., 2000), kiwifruit (*Actinidia chinensis Planch)* (Walton et al., 2001), rubber tree (*Hevea brasiliensis*) (Dornelas and Rodriguez, 2005b), apple (*Malus x domestica*) (Wada et al., 2002), chestnut (*Castanea mollissima*) (Liu et al., 2011), and *Populus tomentosa* (An et al., 2011). The role of these LFY homologs in plant reproductive development was also demonstrated by mutations and genetic transformations. For instance, Loss-of-function mutations in *LFY* lead to plants in which vegetative shoots were initiated instead of floral organs (Weigel et al., 1992). Expression of *LFY* is first detectable in leaf primordia and reaches maximal level in young floral meristems (Blazquez et al., 1998). Constitutive expression of LFY gave rise to flowering of transgenic Arabidopsis plants *in vitro* in just 10 days (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Overexpression of *PtLFY* of *P. trichocarpa* in Arabidopsis accelerated flowering, but caused precocious flowering in a tiny proportion of transgenic lines in several poplar hybrids (Rottmann et al., 2000).

Chinese jujube (*Ziziphus jujube* Mill.), a member of the Rhamnaceae family, originated from China and has more than 4000-year history of cultivation. It is an economically important fruit tree species in China, well-known for its fruits with high dietary value and medical value. In contrast to other common fruit trees such as apple, pear, and citrus, the fruit-bearing shoots (‘Zaodiao’ in Chinese) of Chinese jujube tree show special properties of growth and development: they sprout from the mother fruit-bearing shoots, and fall off after fruits have ripened and leaves have fallen off during the growth season (Sun et al., 2009a). Meanwhile, floral buds geminate on the fruit-bearing shoots, complete differentiation and develop into mature fruits along with the fruit-bearing shoots. In particular, the tree has a short juvenile phase preceding the production of flowers and fruits in the reproductive phase. It can blossom and produce fruits in the same year the seedling is planted. Recently, a number of studies focusing on floral development have been carried out on Chinese jujube (Sun et al., 2009a, b). A homolog of SQUAMOSA/APETALA1, *ZjAP1*, was isolated from Chinese jujube by reverse transcriptase-polymerase chain reaction (RT-PCR) and rapid amplification of complementary DNA ends (RACE). In light of expression profile of *ZjAP1*, it was considered to play a role in both vegetative and reproductive development in Chinese jujube. However, these results are insufficient to fully explain the genetic mechanisms underlying the transition from vegetative to the reproductive phase in Chinese jujube.

In this study, we isolated and characterized the *ZjLFY* gene by using a combination of reverse transcriptase-polymerase chain reaction (RT-PCR), rapid amplification of cDNA ends (RACE) and bioinformatic analysis from floral buds of Chinese jujube. In addition, we investigated spatio-temporal expression profile of the *ZjLFY* in the tree.

MATERIALS AND METHODS

Chinese Jujube cultivar, Jinsi No. 4, was used in this study. The trees were grown in Hengyang City, Hunan Province. For *LFY* gene isolation, floral buds were collected. For organ expression analysis, floral buds at different stages, fruit-bearing shoots, vegetative shoots, mature leaves, stems and axillary buds were used. Fruit-bearing shoots were collected before the appearance of basal floral buds. All samples were immediately frozen in liquid nitrogen and stored at −70°C.

E. coli strains BL21 and DH5α and plasmid pET-28a were maintained by our Laboratory. *Taq* DNA polymerase (TaKaRa), *Nde*I, *Eco*RI, T4 DNA ligase, protein marker, IPTG, EDTA, Tris-base, SDS, kanamycins and Coomassie Brilliant Blue R-250 were purchased from Ferm tech Bio Basic Inc., and TaKaRa.

Preparation of total RNA

Total RNA was isolated from the plant materials collected using Micro-to-Midi Total RNA Purification System (Invitrogen) according to the manufacturer’s instructions. The quality of the total RNA was determined by spectrophotometer analysis and agarose gel electrophoresis. Purity was assessed from the absorbency ratios A260/A230 and A260/A280, a measure of contamination by the polyphenols, carbohydrates and proteins, respectively.

Isolation of conserved region of *ZjLFY*

Degenerate RT-PCR was carried out to amplify partial *ZjLFY* fragment. First-strand cDNA was synthesized by using the First Strand cDNA Synthesis Kit (MBI). The RT was performed in a total volume of 20 µL containing 2 µL 10×RNA PCR Buffer, 4 µL $MgCl_2$ (25 mM), 2 µL dNTP Mixture (dATP, dCTP, dGTP and dTTP, each at 10 mM), 1 µL M-MuLV Reverse Transcriptase (5 U/µL), 0.5 µL RNase Inhibitor (40 U/µL), 1 µL Oligo$(dT)_{18}$ primer (2.5 µM), 2 µL RNA (around 1 µg), and 7.5 µL RNase-free water. The reaction was programmed in a thermal cycler (PE-9600, USA) and conducted under condition of 10 m at 30°C followed by 25 m at 50°C and 5 m at 95°C to stop the reaction. The second-step PCR amplification was performed using designed primers ZjLFYPF1 [5’-CAG(A/C)GGGAGCACCC(G/T)TTCATTGTGAC-3’, forward] / ZjLFYPR1[5’-GACGAAGCTT(C/G)GTGGG(A/T)ACATACCAAA(C/T)(A/G)GA-3’), reverse]. The primer pair were designed based on multiple amino acid sequence alignment of *LFY* homologs from other plants including *Carya cathayensis* (GenBank accession no. DQ989225), *C. mollissima* (GenBank accession no. DQ270548), *Cydonia oblonga* (GenBank accession no. AB162037) and *Vitis vinifera* (GenBank accession no. AF378126). The PCR cycling parameters were 38 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 5 min at 72°C.

Isolation of full-length cDNA of *ZjLFY*

To amplify the full-length cDNA of *ZjLFY* gene, RACE strategy was adopted according to the partial sequences acquired by RT-PCR. The 3' terminal of the gene were amplified with 3' RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Version E). Total RNA of Chinese jujube floral buds was reverse transcribed using SuperScript™ II Reverse Transcriptase with Adapter Primer provided in the system under condition of 10 min at 70°C, followed by 50 min at 42°C, 15 min at 70°C and 20 min at 37°C to stop the reaction. The second-step PCR amplification was conducted with primers ZjLFYGSP3F (5'-GCAGTGTCGTGATTTCTTGATTCAGGTC-3') and AUAP (5'-GGCCACGCGTCGACTAGTAC-3', provided in the 3' RACE System). The amplification program consisted of 35 cycles of 30 s at 94°C, 30 s at 62°C, 3 min at 72°C, and a final extension of 7 min at 72°C.

The 5' terminal of the gene were amplified with 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Version 2.0). First-strand cDNA was synthesized by RT using SuperScript™ II Reverse Transcriptase with primer ZjLFYGSP5R1 (5'-TAGAGAGACGAGGATGTG-3'). Then dc-tailed cDNA was amplified by using the Abridged Anchor Primer (AAP, 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3', provided in the System) and nested primer ZjLFYGSP5R2 (5'-CGCCCCAACATTCTCGCCTCTTTCCTTC-3'). Finally, the cDNA product was re-amplified using AUAP primer and nested primer ZjLFYGSP5R3 (5'-GCACATAGTGTCGCATTTTCGGCTTG-3'). The latter two PCR reactions were programmed as 3' RACE-PCR.

Cloning, sequencing and sequence analysis

RT-PCR and RACE-PCR products were estimated by 1.5% agarose gel. Subsequently, the target products were extracted and purified with Gel Extraction Kit (Ambiogen, China), TA-ligated into pMD18-T Vector (Takara, Japan) and transformed into *E. coli* strain DH5α. The positive clones were screened by the white/blue colony and identified by plasmid PCR with consensus primer pair M13PM4 (5'-GTTTTCCCAGTCACGAC-3') and M13PRV (5'-CAGGAAACAGCTATGAC-3'). Target clones were sequenced in both directions by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.

The BLAST algorithm was used to search the NCBI GenBank (http://www.ncbi.nlm.nih.gov/) databases for homologous sequences and identify the identity of target gene. Isoelectric point (Ip) and molecular weight (mW) of ZjLFY were predicted by using online ExPASy proteomics tools (http://expasy.org/tools/pi_tool.html). Hydrophobicity analysis was carried out by online ProScale tool (http://www.expasy.org/cgi-bin/protscale.pl). For phylogenetic analysis, a total of 20 completed amino acid sequences for LFY homologs including the newly cloned ZjLFY and the other 19 ones deposited in GenBank were aligned by Clustal X method. The 19 LFY genes include *A. thaliana LFY* (*AtLFY*, AF466792), *Capsicum annuum FLY* (*CaLFY*, EU000254), *C. cathayensis LFY* (*CcLFY*, DQ989225), *Cedrela fissilis LFY* (*CfLFY*, AY633621), *Chrysanthemum lavandulifolium LFY* (*ClLFY*, AY672542), *C. mollissima LFY* (*CmLFY*, DQ270548), *C. oblonga LFY-2* (*CoLFY-2*, AB162037), *Citrus sinensis LFY* (*CsLFY*, AY338976), *Eriobotrya japonica LFY-2* (*EjLFY-2*, AB162039), *Juglans regia LFY* (*JrLFY*, HQ019159), *Lycopersicon esculentum LFY* (*LeLFY*, AF197936), *M. domestica LFY2* (*MdLFY2*, DQ535886), *Orchis italica LFY* (*OiLFY*, AB088851), *Pyrus communis LFY-2* (*PcLFY-2*, AB162036), *Prunus persica LFY* (*PpLFY*, EF175869), *Salix discolor LFY* (*SdLFY*, AY230817), *Serapias lingua LFY* (*SlLFY*, AB088466), *S. tuberosum LFY* (*StLFY*, EU371047), and *V. vinifera LFY1* (*VvLFY1*, AF378126). Based on the multiple sequence alignment, an unrooted phylogenetic tree was generated.

Expression pattern of *ZjLFY* in different organs

The expression pattern of *ZjFLY* gene was studied by semi-quantitative RT-PCR and quantitative real-time PCR in different organs and in floral buds at different developmental stages, in which *Zjactin* gene was used as a control. The *Zjactin* fragment was amplified with ZjAT1PF (5′-GTGCTCGACTCTGGAGATGGTGTG-3′) and ZjAT1PR (5′-CGCTCATTACCGATTGTGATGACC-3′) that were designed based on *Z. jujube actin 1* cDNA sequences (GenBank accession No. EU251882), spanning the region of 308 bp. In semi-quantitative RT-PCR, the *ZjFLY* fragment was amplified by primer combination ZjLFYPF2 (5'-AGCACCCGTTCATTGTGACG-3') / ZjLFYPR2 (5'-CAGCCCCGGTAGAGACAGAG-3'), which spanned the region of 504 bp. The amplification program consisted of 40 cycles of 30 s at 94°C, 30 s at 60°C, 60 s at 72°C, and a final extension of 7 min at 72°C.

In quantitative real-time PCR, the *ZjFLY* fragment was amplified by primer combination ZjLFYPF3 (5'-CCTCTACGAGCAGTGTCGTG-3') / ZjLFYPR3 (5'-ATGTCCCAACCTTGTCTTGC-3'), which spanned the region of 303 bp. The PCR was performed in Mx3000P (Stratagene), and started with a denaturation stage at 95°C for 5 min, which was then followed by 40 cycles with each cycle composed of 95°C for 30 s, 58°C for 20 s, and 72°C for 20 s.

RESULTS

Cloning of full-length cDNA of *ZjLFY*

Following RT-PCR and Blastn analysis, a 445 bp-long nucleotide sequence corresponding to *LFY* gene was obtained (Figure 1a). Based on the nucleotide sequence of 445 bp, 3' terminal fragments of the *Z. jujube LFY* were successfully amplified by 3' RACE-PCR (Figure 1b). Furthermore, 5' terminal fragment of the *Z. jujube LFY* was produced by a nest PCR after 5' RACE-PCR (Figure 1c). Sequencing result showed that the 5' terminal fragment and 3' terminal fragment was 969 and 425 bp, respectively. The two nucleotide sequences shared the common region of 149 bp. The *ZjLFY* full-length cDNA was determined by overlapping the sequences of 5' and 3' ends accordingly and submitted to GenBank database (GenBank accession no. JN165097).

Sequence analysis of *ZjFLY*

The *ZjLFY* full-length cDNA was 1517 bp in length and contained a complete open reading frame (ORF) of 1209 bp, encoding 402 amino acids (Figure 2). It contained a 50 bp 5'-untranslated region (5' UTR). The putative initiation codon ATG was at positions 51-53. The termination codon TGA was present at positions 1257 to 1259, followed by 3' untranslated region (UTR), which contained the consensus polyadenylation signal AATAAA at positions 1284-1289. The tail-adding signal is likely to help mRNA get across nucleus, and plays an important role in steadying mRNA and improving efficiency of translation. The molecular

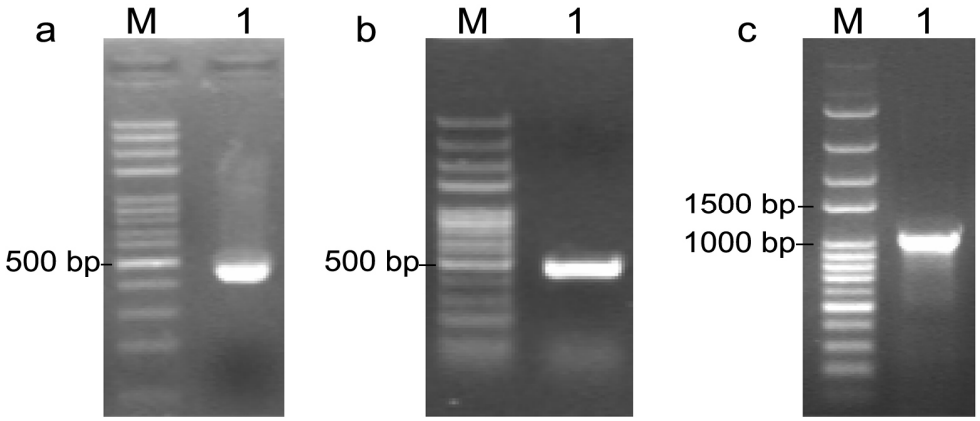

Figure 1. Cloning of *ZjLFY* cDNA. (a) Lane 1, Amplification of conserved region of *ZjLFY* by RT-PCR; (b) lane 1, amplification of 3' terminal of *ZjLFY* by 3' RACE; (c) lane 1, amplification of 5' terminal of *ZjLFY* by 5' RACE; M, DNA marker.

GAGCTAGCTGCAAATTAAGAAGTTGTAGACAGAAGGTTTTTGATCCATCG

ATGGATCCCGACCCCTTTACAGCGAGTTTGTTCAAGTGGGACCCACGCGGCGGCATGGTCGTACAGCCTAGCCGGTCGATCATGGAGCCACTG

M D P D P F T A S L F K W D P R G G M V V Q P S R S I M E P L

CAAGCAGCAACACAGCACACTGGTTCGTTGCCTGCGTCGGCGGCCACTGCATACTCAGTCAGGCAGTTGGGTGGTCTGGAAGAGCTGTTTCAG

Q A A T Q H T G S L P A S A A T A Y S V R Q L G G L E E L F Q

GCTTACGGTATCAGATACTACACGGCTGCGAAGATAGCTGAGCTTGGATTCACCGTGAGCACGCTGTTAGATATGAAAGACGACGAGCTCGAC

A Y G I R Y Y T A A K I A E L G F T V S T L L D M K D D E L D

GAGATGATGAACAGCCTCTCTCAGATATTTCGGTGGGAGCTGCTCGTCGGTGAACGATACGGTATTAAAGCTGCTGTTCGAGCTCACAGGAGA

E M M N S L S Q I F R W E L L V G E R Y G I K A A V R A H R R

CGCCTCGACGACGATGACTCCCGTCGGCGACACCTCATCGTCTCTGATACTCCCACCAACGCCCTCGATGCACTCTCACAAGAAGGACTGTCG

R L D D D D S R R R H L I V S D T P T N A L D A L S Q E G L S

GAGGAGCCGGTGCAAGAAGAGAAGGAGGTGGTGGGGAGTGTTGGCGGAGGGATGTGGGAGGTGGTGGCAGCAGGAGAAAGGAAGAAGAAGCAG

E E P V Q E E K E V V G S V G G G M W E V V A A G E R K K K Q

CGCCGAAGCTTGAAGGTTCACCGGAAAACGGTGGTGGAAAATCATGAAAATGATGCCGACGATGAGGACGACGAAGATGACGATAATGGAAAC

R R S L K V H R K T V V E N H E N D A D D E D D E D D D N G N

GGAAACGGACATGGTGGAATCAGTGAGAGACAAAGAGAGCATCCGTTCATCGTGACGGAGCCTGGGGAAGTGGCACGTGGCAAAAAGAACGGT

G N G H G G I S E R Q R E H P F I V T E P G E V A R G K K N G

CTGGATTATCTCTTCCACCTCTACGAGCAGTGTCGTGATTTCTTGATTCAGGTCCAGAATATTGCCAAGGAGCGAGGTGAAAAATGCCCCACT

L D Y L F H L Y E Q C R D F L I Q V Q N I A K E R G E K C P T

AAGGTTACAAACCAGGTGTTTAGATATGCAAAGAAAGCAGGAGCAAGCTATATAAACAAGCCGAAAATGCGACACTATGTGCACTGCTATGCG

K V T N Q V F R Y A K K A G A S Y I N K P K M R H Y V H C Y A

CTGCATTGCCTAGACGAGGAAGCATCGAATGCCCTCCGGCGAGCATTGAAGGAAAGAGGCGAGAATGTTGGGGCGTGGAGGCAGGCATGCTAC

L H C L D E E A S N A L R R A L K E R G E N V G A W R Q A C Y

AAGCCATTAGTGGCCATTGCAGCAAGACAAGGTTGGGACATTGATTCCATCTTCAATGCACATCCTCGTCTCTCTATTTGGTATGTTCCCACA

K P L V A I A A R Q G W D I D S I F N A H P R L S I W Y V P T

AAGCTTCGACAGCTTTGTCACGCCGAGCGCAACAATGCAACGCTTGCTGCTTCTAGCTCTGTCTCTACCGGGGCTGATCACTTGCCCTTTTGA

K L R Q L C H A E R N N A T L A A S S S V S T G A D H L P F *

AAAAGGAAAAGAGTTAGGATTGGT<u>AATAAA</u>TTTGTGTTGGAACTTGAGGAATTTAGGTCAGTGGGAATATGTGTTATTTTGTCCAATGTATTG

GACTTCGTTGATGCTTCTTGGATGGAAAATTAGCTTTTTGTGTTAGTTCACTATATGGAGTACAATGGGCTTGTGAAAATACTCTCGGCCTCA

AAAGCTGTACAACTATTAGTATTTTTGCGTTATTTGAAAGATATAGGCTTGTTTGAAAAAAAAAAAAAAAAA

Figure 2. Nucleotide and deduced amino sequences of *ZjLFY* cDNA. The asterisk indicates the termination codon. The consensus polyadenylation signal AATAAA is underlined. 5' UTR and 3' UTR are presented in italics.

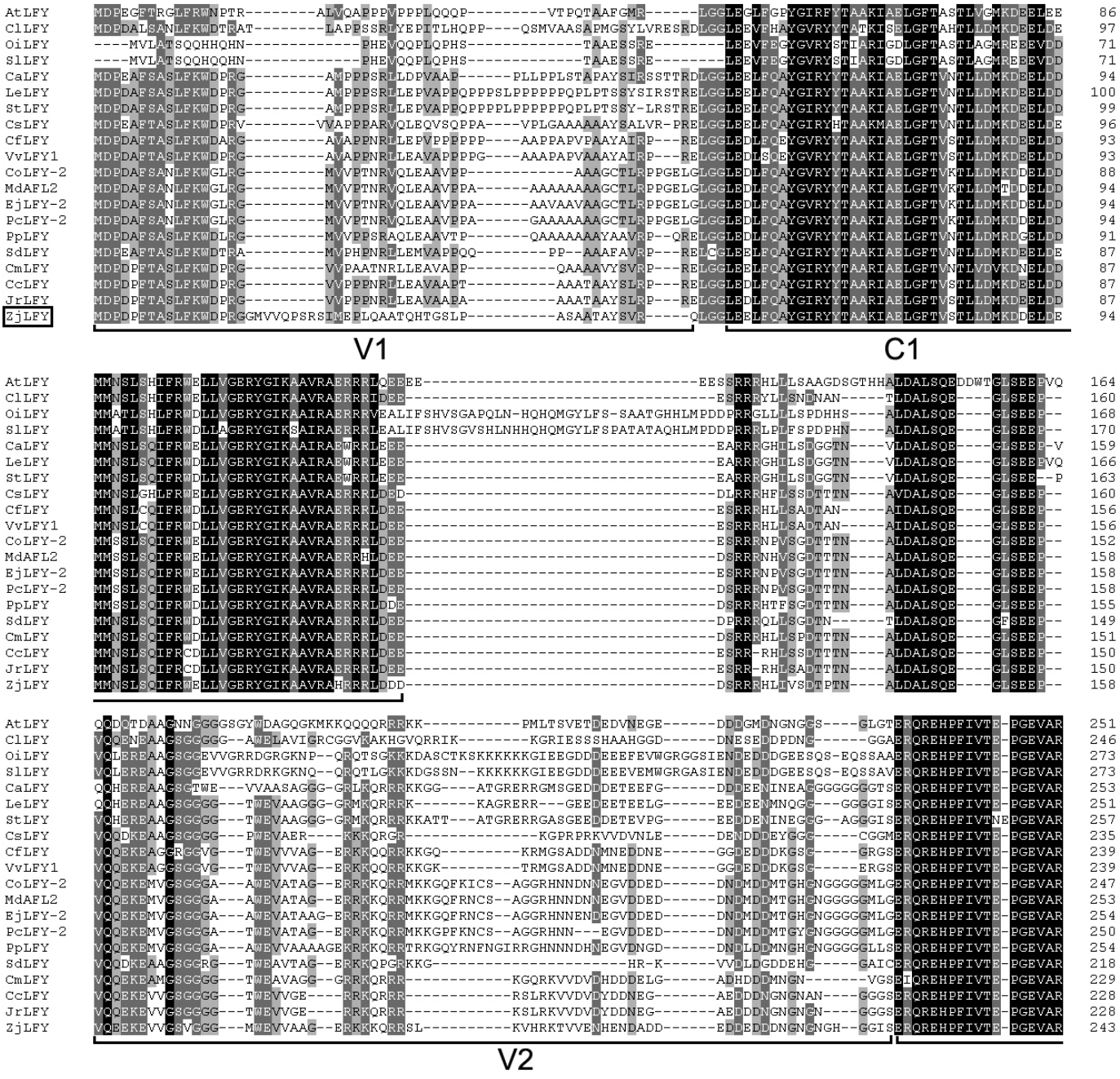

Figure 3. Amino acid sequence alignment of LFY homologs from different plant species. Two variable regions (V1 and V2) and two conserved regions (C1 and C2) are underlined. The ZjLFY is boxed.

weight and isoelectric point of *ZjLFY* were predicted to be 45.22 kDa and 6.29, respectively. Analyzed by ProScale, most of the amino acid sequences of *ZjLFY* were hydrophilic regions. In addition, a total of 20 amino acid sequences for LFY homologs were aligned by Clustal X method (Figure 3). The ZjLFY had two variable regions (V1 and V2) and two conserved regions (C1 and C2) as presented in other LFY homologs.

Compared with StLFY and LoLFY, the ZjLFY lacked a proline-rich region in the variable region (roughly the first 40 amino acids). Similarity analysis indicated that the ZjLFY exhibited a higher similarity with JrLFY (82.9%), CcLFY (82.3%), CmLFY (80.3%) and SdLFY (80.2%), and a lower with OiLFY (57.5%) and SlLFY (58.2%) at the overall amino acid level. Based on the multiple amino acid sequence alignment, an unrooted phylogenetic tree was generated (Figure 4). The phylogenetic tree analysis for the 20 LFY homologs classified them into four subfamilies.

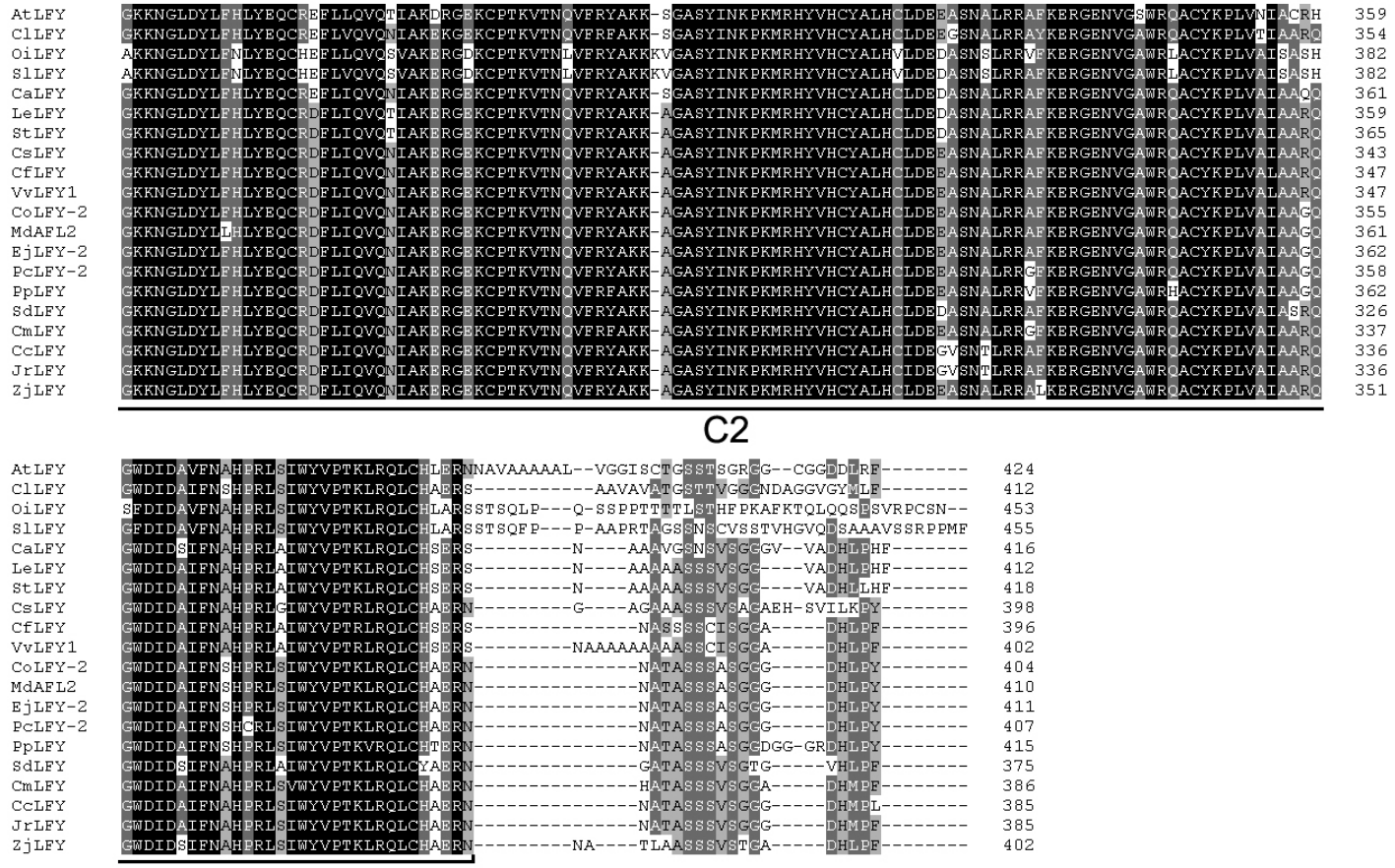

Figure 3. Continue

Accordingly, ZjLFY, CoLFY-2, EjLFY-2, MdAFL2, PcLFY-2 and PpLFY clustered into the same subgroup. This dendrogram revealed that the ZjLFY was closer to LFY of woody species such as *C. oblonga*, *E. japonica*, *M. domestica*, *P. communis*, and *P. persica*.

Spatio-temporal expression of *ZjLFY* gene

To clarify the spatio-temporal expression pattern of *ZjLFY* in Chinese jujube tree, semi-quantitative RT-PCR and quantitative real-time PCR were carried out with cDNA from different organs. As shown by semi-quantitative RT-PCR, the *ZjLFY* gene was expressed at all stages of floral bud development, showing high expression level (Figure 5a1). However, the expression level decreased with floral buds approaching to maturity. A slight transcript level of *ZjLFY* was also detected in different vegetative organs including fruit-bearing shoots, vegetative shoots, stems, mature leaves, and axillary buds (Figure 5b1), whereas *ZjLFY* mRNA accumulation was higher in fruit-bearing shoots than in the other four organs. The quantitative real-time PCR produced coincident results for *ZjLFY* expression with the semi-quantitative RT-PCR (Figures 5a2 and b2). During developmental stages of floral buds, for instance, the relative expression amount of *ZjLFY* reached the highest level on April 10, and was three times what it was on April 24 (Figures 5a2 and b2). The profile of *ZjLFY* expressed suggested that expression levels of *ZjLFY* fluctuated in different organs with higher expression level in reproductive organs than in vegetative organs.

DISCUSSION

Flowering is an essential stage for fruit setting, and thus the genetic mechanisms of the flowering event are therefore important for efficient fruit production. To date, molecular mechanisms of flowering have been studied extensively in herbaceous plants especially in model plants such as *Arabidopsis* and *Antirrhinum*, with much progress being made. However, few studies on the molecular mechanisms of flowering in woody fruit trees were conducted, partially owing to their specific characteristics being different from herbaceous plants, that is, long juvenile phases, bud dormancy, and seasonally alternating vegetative and reproductive development. *FLY* homologous genes were considered to play a key role in the transition from vegetative to reproductive phase and

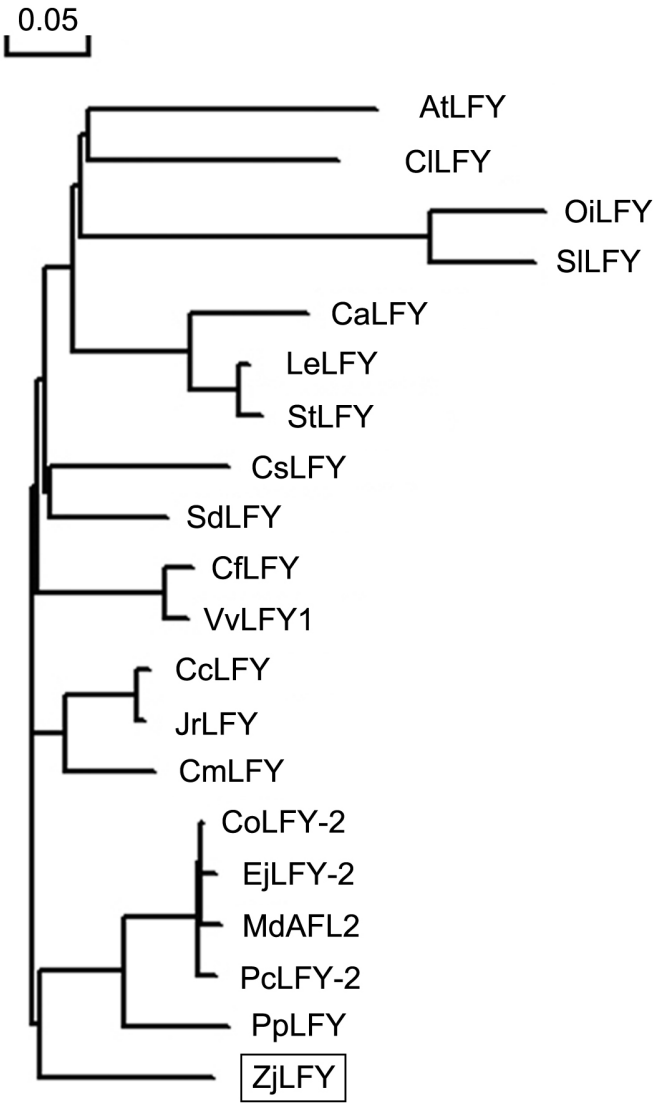

Figure 4. Phylogenetic analysis of LFY homologs from different plant species. The tree was displayed as a phylogram in which branch lengths are proportional to distance.

elucidation of flowering mechanisms should be attributed to functions of the *LFY* gene. Therefore, several LFY genes have been cloned from fruit trees including kiwifruit (Walton et al., 2001), grapevine (Carmona et al., 2002), apple (Kotoda et al., 2002), and chestnut (Liu et al., 2011). However, their specific role in the induction of the characteristic features of tree reproductive development is still unknown.

As one member of woody fruit trees, Chinese jujube is favored by consumers and markets due to the appearance, quality, flavor and storability of the fruit. Since the Chinese jujube has shorter juvenile phases than most other fruit trees, it has the potential to be the 'model' woody plant for studying flowering mechanisms. Moreover, an understanding of the flowering mechanisms of Chinese jujube could be useful for breeding new varieties with early flowering and fruiting in other long-juvenile fruit trees. In this study, the *ZjLFY* was successfully isolated and characterized from Chinese jujube. As with other LFY homologs, the ZjLFY had two large conserved regions (C1 and C2) and two shorter variable regions (V1 and V2). These domains are typical markers for transcriptional activators and may be functionally important for LFY proteins (Weigel et al., 1992; Coen et al., 1990). As observed in CmLFY, no proline-rich region (roughly the first 40 amino acids) was present in the ZjLFY. However, as reported by Guo and Yang (2008), StLFY, SlLFY and AtLFY had a proline-rich domain of approximately 40 amino acids in the V1 region. Determination of the complete coding sequence of ZjLFY allowed us to conduct a phylogenetic analysis for LFY homologs.

The phylogenetic tree further confirmed that the ZjLFY belonged to a LFY member although its function in Chinese jujube tree has not elucidated. The ZjLFY shared a higher similarity with LFY homologs from *C. oblonga*, *E. japonica*, *M. domestica*, *P. communis* and *P. persica*, which was also reflected on the phylogenetic tree. In addition, LFY homologs from herbaceous plants and those from woody plants formed two independent groups on the tree. Several studies on phylogenetic evolution of the LFY have been performed (Mellerowicz et al., 1998; Mouradov et al., 1998; Dornelas et al., 2004; Dornelas and Rodriguez, 2005a; Guo and Yang, 2008; An et al., 2011; Liu et al., 2011), but no similar result was reported. The next work for us is to search the direct proof that the LFY evolution is related to type of their species. The expression characters of a gene are usually closely related to its role in the tree. According to our results, the *ZjLFY* can be expressed in both vegetative and reproductive organs. Semi-quantitative RT-PCR analysis showed that the *ZjLFY* was strongly expressed in floral buds especially at the early stage of floral formation, but weak expression was also detected in vegetative organs. Similarly, in eucalyptus and grape the expression of the *LFY* homologs was also observed in vegetative organs (Southerton et al., 1998; Dornelas et al., 2004). However, the expression pattern of *ZjLFY* is different from *CfLFY* and *CmLFY*. The expression of *CfLFY* was restricted to reproductive tissues, and *CfLFY* transcripts accumulated in the floral buds and floral organs. The *CmLFY* was only expressed in inflorescences but not in vegetative organs such as stems and tender leaves.

In apple, kiwifruit and grape, the expression levels of their LFY homologs increase in the proliferating inflorescence meristems generating inflorescence branches with the highest levels detected in young floral meristems (Walton et al., 2001; Carmona et al., 2002). Therefore, the highest levels of *ZjLFY* expression correspond to the emerging floral buds, supporting a role for the *ZjLFY* gene product in this process. Furthermore, the expression of *ZjLFY* declines as organs expand, as described for Arabidopsis, pea, and Eucalyptus (Weigel et al., 1992; Hofer et al., 1997; Southerton et al., 1998). *ZjLFY* is also expressed in fruit-bearing shoots despite the low level of

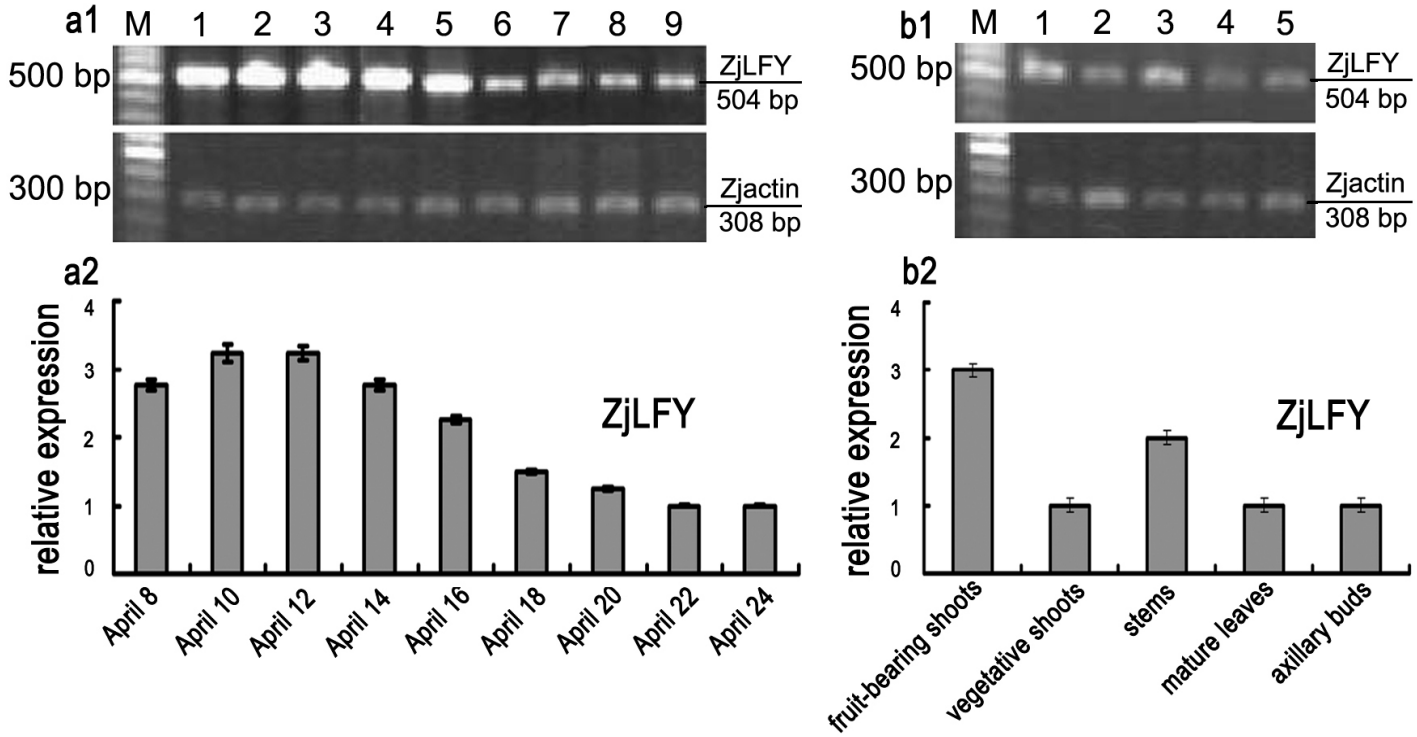

Figure 5. Semi-quantitative RT-PCR and quantitative real-time PCR analysis of *ZjFLY* gene in floral buds (a) and in different organs (b). M, DNA marker; a1, lanes 1 to 9 correspond to floral buds sampled on April 8, April 10, April 12, April 14, April 16, April 18, April 20, April 22, and April 24; b1, lanes 1 to 5 correspond to different organs and sampling dates (in parentheses): fruit-bearing shoots, April 5; vegetative shoots, May 5; stems, May 5; mature leaves, August 2; axillary buds, May 5.

expression, implying that it is also functionally important during the development of fruit-bearing shoots. In summary, expression of *ZjLFY* in vegetative organs and developing floral buds suggests that *ZjLFY* plays an important role during Chinese jujube reproductive and vegetative development.

Conclusion

The breeding of most fruit trees often requires more than 20 years, including periods of cross pollination, seedling selection and regional trials, to produce varieties that meet the demands of consumers. One of the limited factors is the long juvenile phase of at least several years. It had been found that transgenic approaches of *LFY* introduction could reduce the juvenile phase of *Populus* and the resulting transgenic poplar flowered in five months after regeneration (Weigel and Nilsson, 1995). The Chinese jujube tree has a relatively short juvenile phase, and can flower and set fruits in the first year. As a gene closely related to floral development, the *ZjLFY* homolog could be one of the tools available for studying the specific mechanisms of early flowering. In addition, the *ZjLFY* gene could be introduced into other fruit trees with long juvenile phase for early flowering and fruiting by transgenic approach.

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