Original Research

Molecular Cloning, Expression Analysis and Subcellular Localization of LEAFY in *Juglans mandshurica* Maxim

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Abstract

   Juglans mandshurica is a precious timber and economic tree species in Northeast China. Because of the reproductive characteristics of this species, such as long childhood and dioecious, the flowering period of male and female flowers is different, which directly affects the sexual reproduction process such as flowering and pollination. At present, the molecular mechanism of dioecious flowering of *J. mandshurica* is not clear. In this study, the cDNA sequence of a crucial gene involved in plant flowering, LEAFY (*LFY*) was cloned from *J. mandshurica* by homologous cloning, and the expression in different organs at various developmental stages, and the subcellular localization of the gene were analyzed. *LFY* in *J. mandshurica* was found to possess high homology (over 79%), and a similar structure and function to LFY in other species, and was cloned and named *JmLFY* (GenBank: KX364241). The results of fluorescence quantitative real-time PCR analysis revealed that *JmLFY* was expressed in different organs, and that expression increased significantly before the early development stage of staminate inflorescence, the anther formation and pollen dispersal stage, pistillate inflorescence sprouting and flower development stage, and the pollination and late stage of zygotic embryo development. Expression of the green fluorescent protein fusion *JmLFY* gene in onion epidermal cells was localized to the nucleus and cytoplasm. Our results indicate that the *JmLFY* gene is the key transcription factor involved in flower formation and development in *J. mandshurica*. These findings provide information that may help to overcome the bottleneck caused by the long juvenile period and asynchronous female and male flowers of the species in the future.

Keywords: *Juglans mandshurica*, molecular cloning, expression analysis, *LEAFY*

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Introduction

Flowering symbolizes the transformation from vegetative to reproductive growth in plants, and is a complex physiological and biochemical process regulated by genetic and environmental factors, including hormone signaling, photoperiodic induction, and temperature changes [1-4]. Recently, considerable progress has been reported regarding the key genes involved in flower formation, and their correlation with flowering pathways [5-7]. The model plant *Arabidopsis thaliana* has been studied comprehensively, and used in research investigating the genetic mechanisms underlying plant flowering. *Arabdiopsis thaliana* flowering is regulated by a complex signaling network, which can be classified into four stages: (1) the plant responds to environmental and endogenous signals and initiates reproductive growth via vegetative growth, which is regulated by flowering time genes, such as CONSTANS (*CO*), FLOWERING LOCUS C (*FLC*), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (*SOC1*), and FLOWERING LOCUS T (*FT*) [8-13]; (2) floral meristem identity genes, such as LEAFY (*LFY*) and APETALA1 (*AP1*), are activated by signals from stage (1) [14-15]; (3) floral-determination genes, such as FRUITFULL (*FUL*) and APETALA3 (*AP3*) are activated by meristem identity genes [16-19]; and finally, (4) organ construction genes are activated, which arrange the relevant cells and tissues into four floral organs [20].

LFY is a crucial gene for plant flowering, and is involved in most of these four stages. *LFY* is expressed before other meristem identity genes [21-22]; it integrates flowering signals from the previous stage (such as long-day promotion and GA pathways, which differ from flowering time genes [23] and is a key activator of downstream *AP1* (functions as both a floral meristem identity gene and a floral determining gene) and the floral organ determining genes, subsequently inducing the expression of floral organ construction genes [24].

Real-time fluorescence quantitative PCR (qRT-PCR) has shown that *LFY* expression differs in a temporal and spatial manner, especially in different plants. FLORICAULA (*FLO*), a homolog of *LFY* in *Antirrhinum majus*, is expressed in a temporal manner in the floral meristem, sepal, petal, and carpel in sequence, but not in the stamen [25-27]. In *Populus tomentosa*, *PTLF* is expressed in developing inflorescence, and in the adjacent leaves and leaf buds [28]. BrpLFY from *Brassica rapa* subsp. *pekinensis* was detected in all tissues and all developmental stages from vegetative growth to completely differentiated flowers [29]. In *Carya cathayensis*, *CcLFY* was strongly expressed in flower buds, but was not detectable in the root and stem. Further detection of buds from short shoots indicated that *CcLFY* was expressed increasingly during the first 15 days and subsequently decreased [30].

LFY overexpression was shown to consume the shoot meristem during flower development, ultimately terminating shoot growth. In general, aspen species flower when they are at least 8-years-old; however, transgenic hybrid aspen plants were found to bloom at 7-months-old following transformation with the *Arabidopsis LFY* gene [31]. In *Fragaria x ananassa*, the asexual progeny appeared as an early flowering habit following overexpression of the *EjLFY*-1 genes [32]. The results of these studies suggest that the role of *LFY* in early flowering could be applied to breeding tree species with long juvenile periods.

The construction of prokaryotic expression vectors is a common method used to obtain protein products expressed by target genes in order to study their function. The functions of proteins are largely dependent on their cellular localization, and function in specific tissue parts in cells. Thus, determining the subcellular localization of proteins is a key step in the study of their function [33]. Green fluorescent protein (GFP) is commonly used to study the subcellular localization of proteins [33]. GFP fuses with the target protein and emits green fluorescence under blue light excitation, thus confirming the position of the protein based on the fluorescent signal [34].

Heterodichogamous characteristics are transitional during the evolution of plants from monoecism to dioecism, which appear protandry (the staminate inflorescence sprouts synchronously with leaf spreading, and the pistillate inflorescence sprouts after the pollen mature and disperse) or protogyny (the pistillate inflorescence sprouts synchronously with leaf spreading, and the staminate inflorescence develops slowly at first and then rapidly once the female flowers are pollinated) in a random manner in natural populations [35-38]. Heterodichogamous characteristics have attracted the interest of researchers because of their effects on sexual reproduction, including flowering and pollination [39].

Juglans mandshurica Maxim. is a precious timber and oil tree species with high economic [40-41], nutritional, medicinal [42-49], and collection value. *Juglans mandshurica* is mainly distributed in the Xiaoxing 'an Mountains, Wanda Mountains, Changbai Mountains, and the eastern mountainous areas of Liaoning, China [50-53], it has also been documented in Russia's Far East, in North Korea, and in Japan [54-56], and most of these resources are natural forests. Recently, the natural resources of the species have become endangered due to long-term over-logging and the imbalance of harvesting and breeding. As an endangered and key protected tree species in China [57], the yield and stock volume of *J. mandshurica* natural secondary forest are lower than those of coniferous timber forest, and the timber yield accounts for less than 1% of the total timber output in the forest region. However, the market price and demand for *J. mandshurica* timber and fruits has only increased over the years. Thus, a goal for forest management

and development of the species in northeast China is to utilize natural resources sufficiently and gain maximimum economic benefits.

As a monoecious species, heterodichogamous characteristics were observed when *J. mandshurica* germplasm resources were collected in northeast China. In *J. mandshurica*, the heterodichogamous characteristics and the imbalanced ratios of the pistillate inflorescence and staminate inflorescence (47.7:52.3% for protandry and 32.1:67.9% for protogyny, respectively) lead to asynchronous female and male flowers, which greatly affect the efficiency of pollination and fructification, and the subsequent fruit yield. In addition, the long juvenile period (usually 10-15 years from seed germination to flowering and fruiting) of *J. mandshurica* greatly restricts the development of plantation resources, and the fruit and wood dual-purpose forest industry.

To overcome the bottleneck of the long juvenile period and the asynchronous female and male flowers of *J. mandshurica*, and to elucidate the molecular mechanisms underlying flower formation, considering the important roles of *LFY* for plant flowering, we cloned the *LFY* gene in *J. mandshurica* by homologous cloning and analyzed the expression and subcellular localization of *LFY*. Our results provide a theoretical foundation and information for improving the fruit yield and quality of *J. mandshurica*, and for the development of genetic improvement strategies, which will have important theoretical and practical significance for production.

Materials and Methods

Plant Materials

Three healthy 30-year-old wild *J. mandshurica* were sampled from the Qingyuan region of Liaoning province (N41°47-2°28, E124°20-125°28), only three tree samples were selected as there were slight growth trait differences between sampled individuals under such tree age and the wild environmental conditions. Samples of leaf buds, leaves, female buds, male buds, pistils, and stamens were taken every 4-6 days from 2nd April to 21st May, 2017, representing the flower bud dormancy stage (April 2nd-26th), sprouting stage (30th April to 4th May), and development stage (8th-21st May). Fruit samples were collected on 4th June,

27th June, 27th July, and 27th August, during the globular, torpedo, cotyledon, and mature embryo stages of zygotic embryogenesis and development, respectively. The samples were wrapped with foil, frozen in liquid nitrogen, and stored in a refrigerator at –80ºC for RNA extraction, and subsequent gene cloning and expression.

Molecular Cloning of *JmLFY* Full-Length

Total RNA was extracted from leaves using the TIANGEN RNA kit, and cDNA was obtained using the TaKaRa Reverse Transcription System (RR047A) (TaKaRa, Dalian, China) [58].

Based on the mRNA of *Juglans regia LFY* protein, the complete coding sequence (CDS) of *J. regia LFY*, *C. cathayensis LFY*, and A. thaliana LFY in NCBI (http://www.ncbi. nlm.nih.gov/), multiple sequence alignments were applied in Clustal X2 to determine conserved sequences in the LFY gene. Primers designed to amplify LFY gene fragments and full-length LFY are listed in Table 1.

The reaction system used to amplify cDNA included cDNA 1 μL, LFY-F'/F (10 μmol⁻¹) 1 μL, LFY-R'/R (10 μmol⁻¹) 1 μL, LATaq Mix1 2.5 μL, and ddH₂O 9.5 μL. The reaction procedure was as follows: 94ºC for 5 min; 35 cycles of 94ºC for 40 s, 60.6ºC for 1 min, 72ºC for 2 min, and 72ºC for 5 min. The second round of PCR used the same primer, which produced a specific band. The final product was recovered using the MiniBEST Agarose Gel DNA Extraction Kit version 4.0(TaKaRa, Dalian, China), and the purified recovered product was ligated to the pGEM-T cloning vector with T4 DNA ligase using the pGEM-T cloning kit. The vector was then transferred into *Escherichia coli* DH5αcompetent cells. Then, IPTG 16 μL and X-gal 40 μL were added the competent cells, the mixture was transferred to solid LB medium containing Amp, and the plates were inverted and cultured at 37ºC for 12-14 h. Subsequently, five to eight single colonies were selected for PCR, and the positive clones were sequenced. The sequencing results were analyzed using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Clustal X2.0 was used for multiple sequence alignment. The complete sequence of the product was assembled into the full-length *JmLFY* cDNA sequence using DNAMAN software and then submitted to GenBank [58-60].

Table 1. Primers used for amplifying full-length and fragment cDNA of *LFY* gene in *Juglans mandshurica*.

Primers	Primer sequence (5'-3')	Usage
LFY-F'	ACCACCACCAATGCTCTTGAT	*Amplification for cDNA fragment*
LFY-R'	GMAGYTTKGTGGGWACATACCAA	*Amplification for cDNA fragment*
LFY-F	ATGGATCCCGACCCCTTTACTG	*Amplification for cDNA full-length*
LFY-R	GGTGATCACATGCCCCTCTAA	*Amplification for cDNA full-length*

Bioinformatics Analysis of *JmLFY*

The physicochemical parameters of the amino acid sequence encoded by *JmLFY* were analyzed using BioEdit and ProtParam ExPASy online software. BLAST (http://www.ncbi.nlm.nib.gov/blast) was used to compare homologous sequences, Clustal X2 was used to predict and analyze protein structure, and MAGE4.0 software was used to construct the neighbor joining (NJ) tree by calculating 1000 times repeatedly.

qRT-PCR Analysis

The expression of *JmLFY* in different organs was determined by real-time quantitative PCR (qRT-PCR). Total RNA representing different developmental stages was extracted from young leaves, mature leaves, female buds, male buds, pistils, stamens, and leaf buds by the cetrimonium bromide (CTAB) method. qRT-PCR primers were designed based in the sequence of *J. mandshurica JmLFY* using Primer Premier5.0 (Table 2). Seven common genes that have been used for other species or genera in Juglandaceae were screened for use as reference genes (Table 2). The stability of reference gene expression was analyzed in different *J. mandshurica* tissues using geNorm, NormFinder, and BestKeeper [60-61], and the relatively stable expression gene was used. Fluorescence signals were collected in triplicate for each sample. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method.

Expression Vector Construction and Prokaryotic Expression

A total of 100 μL bacterial solution of *JmLFY* cDNA stored at −80ºC was inoculated into 2 mL LB liquid medium. The culture was shaken at 37ºC and 200 rpm overnight (12 h), and plasmid was extracted using the Plasmid Small Extraction Kit (TIANGEN). Based on the obtained full-length cDNA sequence of the *JmLFY* gene, and the connection requirements of the PBM30 rapid cloning kit, primers were designed to construct the prokaryotic expression vector PBM30-*JmLFY*, as follows: *LFY*-F1,5′-CACCATGGATCCCGACCCCTTTAC-3′; *LFY*-R1: 5′-GAGGGGCATGTGATCACCC-3′. The plasmid template was amplified by PCR and the products were analyzed by 1% agarose gel electrophoresis. The target gel band was recovered, purified, and then connected to pBM30 using a pBM30 rapid cloning kit. The recombinant plasmid pBM30-*JmLFY* was transferred into *E. coli* DH5α competent cells, which were cultured and sequenced to confirm the presence of a successful vector construct. To verify whether the constructed recombinant vector expressed protein, plasmid DNA was extracted from the identified clones, and then transferred into BL21 (DE3) competent cells and cultured on LB solid medium containing kanamycin (50 μg.mL) overnight at 37ºC. Positive colonies were detected by PCR, and then induced by IPTG. The induced products were analyzed by SDS-PAGE polyacrylamide gel electrophoresis to confirm protein expression.

Subcellular Localization of *JmLFY* Gene

The PBM30-*JmLFY* positive clone vector was amplified using a high-fidelity thermostable DNA polymerase, and the product was separated and purified by agarose gel electrophoresis. The target fragment of *JmLFY* was fused with the pBM30-GFP fragment, which was cleaved twice by restriction endonuclease, using T4 DNA ligase (Takara). The recombinant pBM30-GFP-*JmLFY* plasmid DNA was purified using

Table 2. Primers used for real-time quantitation PCR of *JmLFY* gene.

Gene symbol	Gene description	Primer pair (5' to 3')
Actin1	*Caraya illinoensis* β-actin	F:ACCAACTGGGACGATATGGAGAAGA R:TACGACCAGAGGCATACAGGGACAA
Actin2	*Juglans regia* β-actin	F: GCTGAACGGGAAATTGTC R: AGAGATGGCTGGAAGAGG
Actin3	*Juglans regia* β-actin	F:GCCGAACGGGAAATTGTC R:AGAGATGGCTGGAAGAGG
18s1	*Juglans regia* 18S rRNA	F:GTAGTCATATGCTTGTCTC R:GTAGTCATATGCTTGTCTC
18s2	*Juglans regia.*'Xiangling' 18S rRNA	F:GGTCAATCTTCTCGTTCCCTT R:TCGCATTTCGCTACGTTCTT
HIS3	*Juglans sigillata,*Dode Histone H3	F:GATCGCACAGAGGTTGGTGTC R:CGTCCGTGAAATTGCACAAGA
EF1α	*Carya carthayensis* Elongation factor 1-α	F:AGGAGCTGGAGAAGGAGCCCA R:AACAGCAACGGTCTGGCGCA
qPCR-*LFY*	*Juglans mandshurica* Maxim *LFY* gene qPCR fragment	F:TCCGTTACAAWGAAAGGGTGCT R:CTTTGGTCGCCATAGCAGCAC

the DNA Maxiprep kit from TIANGEN. The skin of a fresh onion was disinfection, and the inner skin was removed using sterilized tweezers and spread on sterilized MS medium for 5-7 days' induction until the onion skin was slightly bulging. The onion epidermis was then infected by Agrobacterium containing pBM30-GFP-*JmLFY* and kanamycin, and then cultured at 28ºC until OD_{600} = 0.3. The cultures were then suspended in an equal volume buffer (10 mM MES, 800 μM acetosyringone, and 10 mM $MgCl_2$) for 6-12 h under a sterile dark state. The onion epidermis was then removed and placed on MS solid medium for 3-4 days, and visualized under a laser scanning confocal microscope (PERKINEIMER UITRAVIEW VOX).

Results

Cloning of *JmLFY* Gene

A specific band of approximately 1300 bp was obtained by PCR amplification using the full-length primer designed by Primer 5.0 and cDNA from young leaves of *J. mandshurica* as a template. The obtained target gene was then sequenced, and the results showed that the sequence was 1297 bp long, with a CDS of 1158 bp.

Homology of the cloned *LFY* gene sequence was compared with that of other species in the NCBI database using the BLAST tool. The results showed that *J. mandshurica LFY* shared 98 and 99% homology with *C. cathayensis* and *J. regia LFY*, respectively. Notably, *J. mandshurica LFY* shared more than 79% homology, even with some distantly related species, such as *Betula platyphylla*, *Betula luminifera*, *Castanea henryi*, and *Prunus mume*. This confirms that the flowering related *LFY* gene from *J. mandshurica* was successfully cloned, and was subsequently named *JmLFY* (GenBank: KX364241). The full-length cDNA sequence of the *JmLFY* gene obtained by sequence analysis using DNAMAN software carries a complete open reading frame (ORF), which can translate a peptide chain consisting of 385 amino acids. In order to categorize the *JmLFY* gene and its evolutionary relationship with other *LFY* genes, 18 registered amino acid sequences belonging to *LFY* genes were selected for cluster analysis.

Clustal X2 was used to compare the amino acid sequences of *JmLFY,* and MEGA4.0 was used to construct a phylogenetic tree of the 19 homologous amino acid sequences, including *JmLFY* (Fig. 1). Cluster analysis showed that *JmLFY* clustered together with *C. cathayensis CcLFY*, which then clustered with *J. regia* (*JrLFY*) as a branch. The branch was clustered to birch *B. platyphylla BpLFY* and *B. luminifera BlLFY*. It was also closely related to *Castanea henryi* (*ChLFY*), *Castanea mollissima* (*CmLFY*), *Fagus crenata* (*FcLFY*), and *Theobroma cacao* (*TcLFY*).
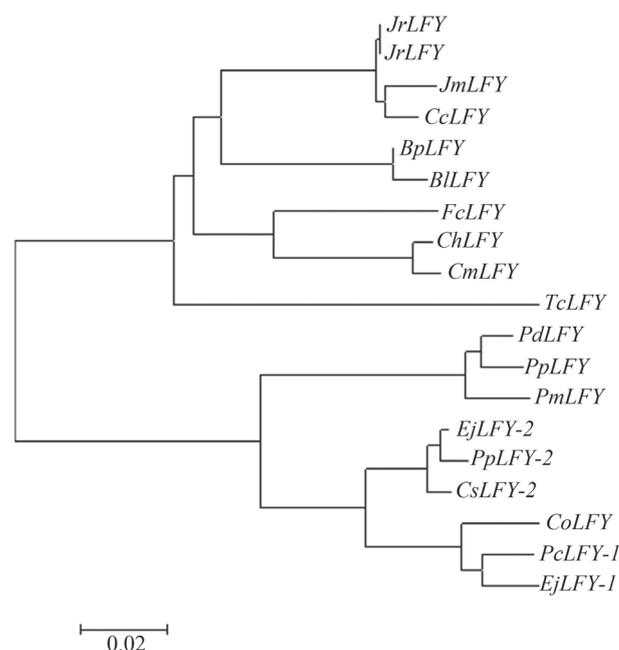

Fig. 1. Cluster analysis of the amino acids sequences encoded by *JmLFY* and other *LFY* homologous genes. Species resources of the LFY used are *Juglans regia* (Jrlfy, GU194836.4; Jrlfy JF520778.1); *Carya cathayensis* (CcLFY, DQ989225.1); *Betula platyphylla* (BpLFY, KP693587.1); *Betula luminifera*, (BlLFY, KP970616.1); *Fagus crenata* (FcLFY, AB674454.1); *Castanea henryi* (Chlfy, JQ825167.1); *Castanea mollissima* (Cmlfy, DQ270548.1); *Theobroma cacao* (TcLFY, XM_007038099.1); *Prunus dulcis* (PdLFY, AY947465.1); *Prunus persica* (PpLFY, EF175869.1); *Prunus mume* (PmLFY, KT803848.1); *Eriobotrya japonica* (EjLFY-2, AB162039.19); *Pyrus pyrifolia*, (PpLFY-2, AB162035.1); *Chaenomeles sinensis* (CsLFY, AB162038.1); *Cydonia oblonga* (CoLFY-1, AB162031.1); *Pyrus communis*, (PcLFY-1, AB162030.1); *Eriobotrya japonica* (EjLFY-1, AB162033.1).

Lower levels of homology were found when comparing *J. mandshurica* with Rosaceae species (nine LFYs). However, when the amino acid sequence encoded by the *LFY* gene was compared with that encoded by *JmLFY*, homology exceeded 79%, indicating that LFY is highly homologous among plant species.

The amino acid sequence encoded by *JmLFY* contained an N-terminal proline-enriched region, a central acid region, a leucine zipper structure, and conserved N- and C-terminal regions (Fig. 2). These results confirm that the full-length cDNA sequence of the *LFY* gene was successfully cloned, which is related to flowering in *J. mandshurica*.

Selection and Stability Verification of Candidate Internal Reference Genes

The Ct value of each internal reference gene differed among samples (young leaves, mature leaves, female buds, male buds, pistils, stamens, and leaf buds) (Fig. 3). This wide range of CT values indicated that expression of internal reference genes is unstable, making them
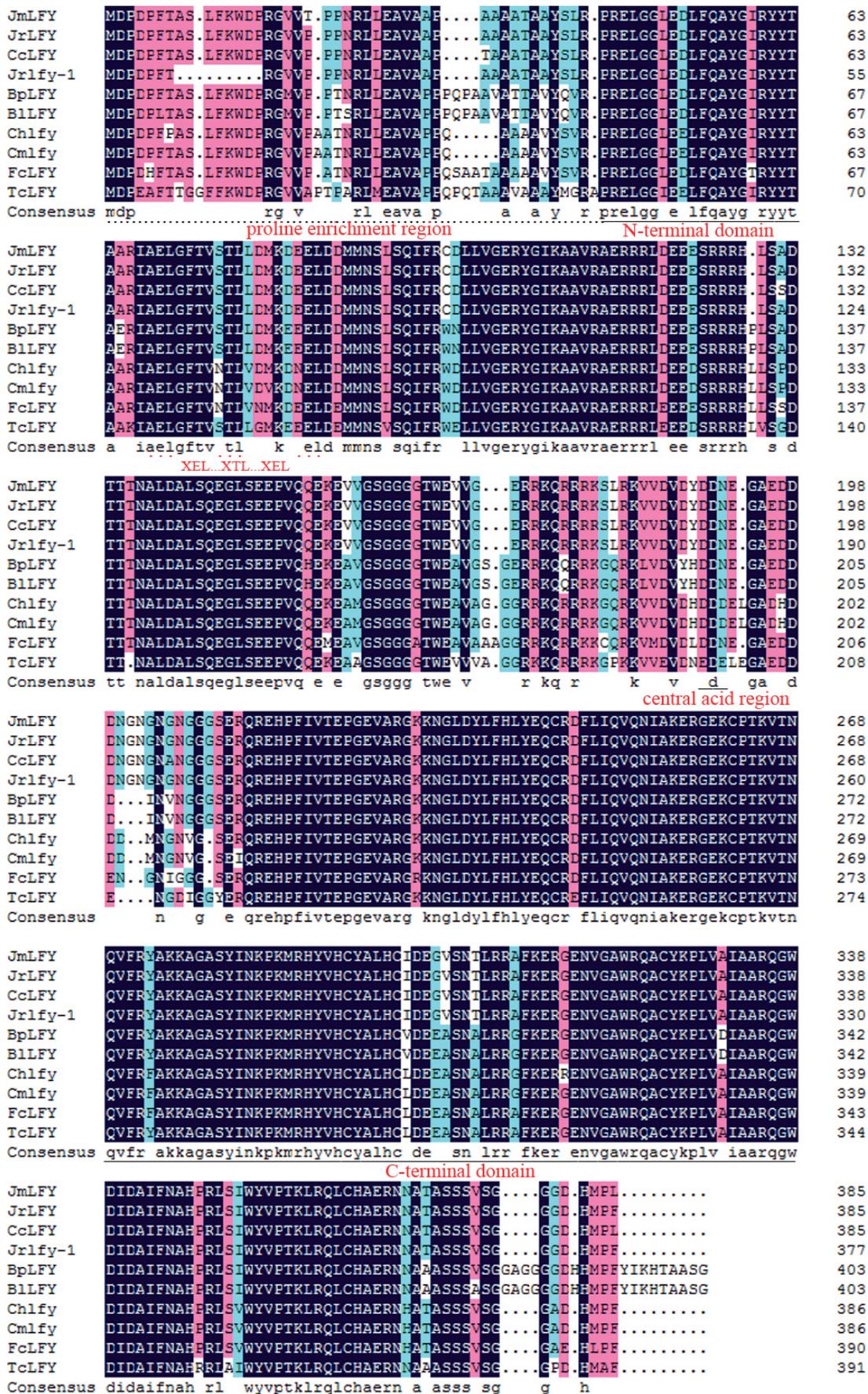
Fig. 2. Alignment of amino acids sequences encoded by *JmLFY* and other *LFY* homologous genes. The ellipsis meant the missing part of the sequences compared to others.
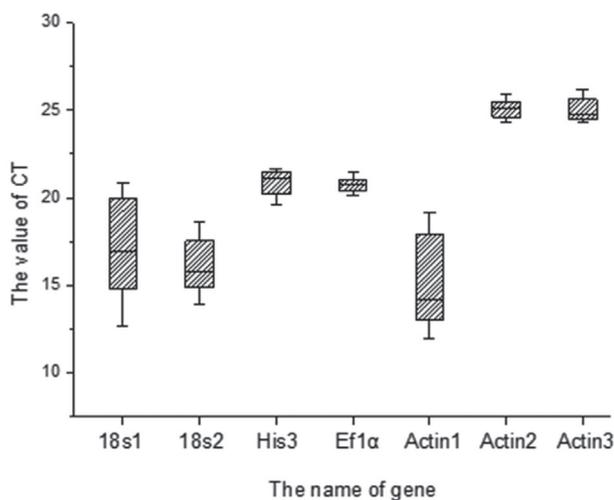
Fig. 3. Ct values of the candidate reference genes for RT-qPCR. A line across the box represented the median, and the "T" line under and below represented the maximum and minimum.

Fig. 4. Relative expression of JmLFY gene in male and female organs at different development stage.

Fig. 5. Relative expression of *JmLFY* gene in pistils experienced different development events on the same day (May 24th).

unsuitable for use as reference genes in this study. Thus, considering the Ct values and the results obtained with geNorm, NormFinder, and BestKeeper software (data not shown), EF1α was selected as a stably expressed internal reference gene for further study.

qRT-PCR Analysis

Expression of the *JmLFY* gene was relatively stable during the male flower bud dormant period (2nd-26th April), with relative expression levels less than 1.5 (Fig. 3). However, following the sprouting stage (30th April) of staminate inflorescences, expression of the *JmLFY* gene increased, peaking on May 4 and 17, which represented the later sprouting stage (or the pre-development stage) and the mid development stage, respectively, after which expression decreased sharply on May 21 (Fig. 3). These results indicated that the *JmLFY* gene plays an important role before the early development stage of staminate inflorescence and in the mid developmental stage of the flower (such as anther formation and pollen dispersal, based on phenological observations of flower organs for *J. mandshurica* in 2017). When the male flowers withered (May 21), *JmLFY* expression decreased sharply.

The expression pattern of *JmLFY* was similar during the developmental stage of the pistillate inflorescence. Expression was relatively stable during the dormant stage (2nd-26th April), and then increased markedly from the pistillate inflorescence sprouting stage to flower development (30th April to 13th May) (Fig. 3). Then, the expression of *JmLFY* in pistillate inflorescences decreased sharply (Fig. 3). These results suggest that *JmLFY* has an important role in pistillate inflorescence sprouting and flower development.

The expression of *JmLFY* in pistils on the same day (24th May) at different development stages was also analyzed. Higher expression was observed in
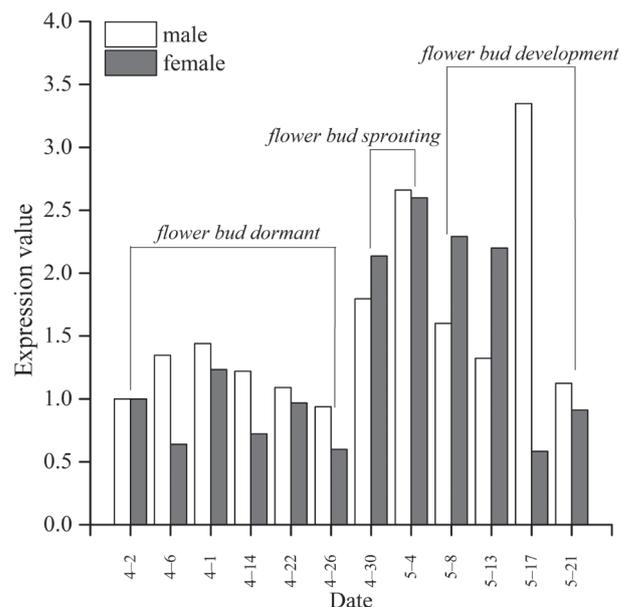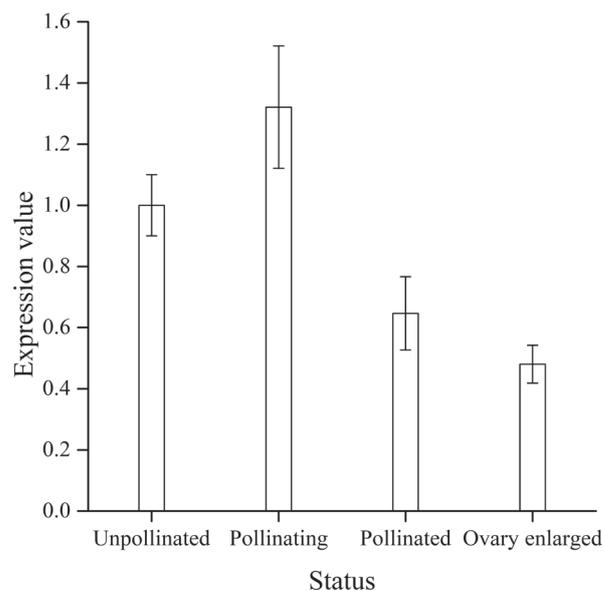
unpollinated pistils and pollinating pistils, while lower expression was observed in pollinated pistils and pistils with enlarged ovaries (Fig. 4). Analysis of *JmLFY* in zygotic embryos at different developmental stages revealed that expression was lower during the globular and torpedo embryo stages (4th-27th June) of zygotic embryogenesis compared with the cotyledon embryo stage (27th July and 27th August; Fig. 5). These results indicate that the *JmLFY* gene plays an important role in pollination and in the late stage of zygotic embryo development.
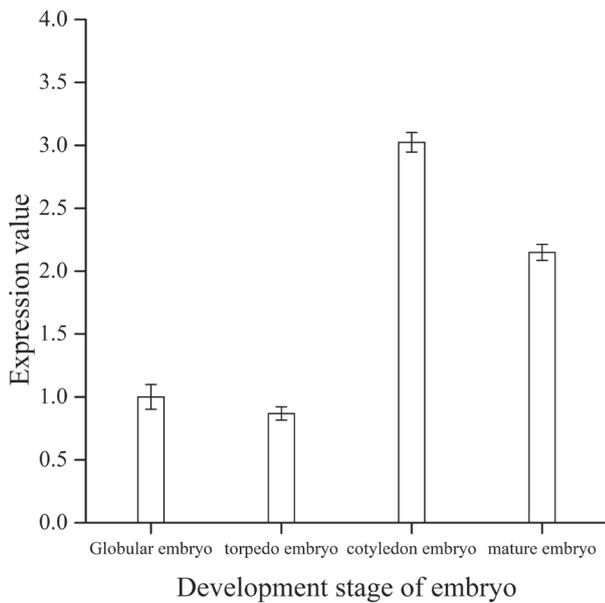
Fig. 6. Relative expression of *JmLFY* gene in zygotic embryos at different development stage.

Fig. 7. Relative expression of *JmLFY* gene in leave bud at different development stage.

The *JmLFY* gene was also expressed in leaf buds, and expression was stable from April 2-30 before increasing on May 4 and 8, which is consistent with the development of staminate and pistillate inflorescences (Fig. 6).

Prokaryotic Expression of *JmLFY* Gene

A prokaryotic expression vector was constructed using the pBM30 rapid cloning kit. Sequencing of positive colonies (1155 bp) confirmed that *JmLFY* was successfully included in the carrier pBM30. To verify protein expression driven by the recombinant vector, plasmid DNA was extracted from the identified clones. This DNA was then transferred to BL21 (DE3) competent cells and cultured on LB solid culture medium containing kanamycin (50 μg.mL^{-1}) overnight at 37°C. Positive colonies were detected by PCR, and then induced with IPTG. Following induction, products were detected by SDS-PAGE polyacrylamide gel electrophoresis. The results revealed protein bands of approximately 50 kDa in lanes containing bacterial liquid that was treated with IPTG, while no such band was found in the lane containing bacterial culture that was not treated with IPTG (Fig. 7). These results indicated that the pBM30-*JmLFY* recombinant vector could express protein successfully.

Subcellular Localization of *JmLFY* Gene

To determine the subcellular localization of the *JmLFY* protein, the *JmLFY* gene was transiently expressed in onion epidermal cells and imaged under a laser confocal microscope by constructing a fusion vector comprising *JmLFY* and enhanced green

fluorescent protein pBM30-GFP. Detection of the pBM30-GFP-LFY fusion vector using a dual-turntable laser confocal real-time imaging analyzer, revealed that the *JmLFY* protein was expressed in the nucleus and cytoplasm (Fig. 8 and Fig. 9).
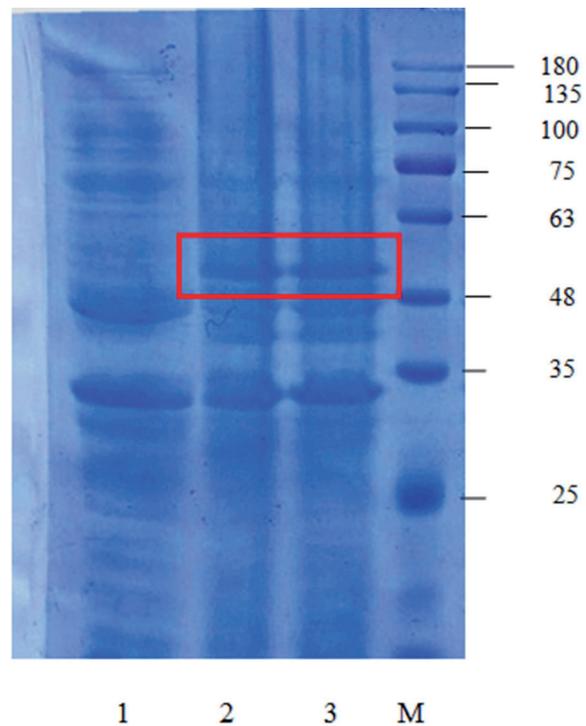
Fig. 8. SDS polyacrylamide gel electrophoresis. Lane 1, treated without IPTG; lane 2-3, treated with IPTG.
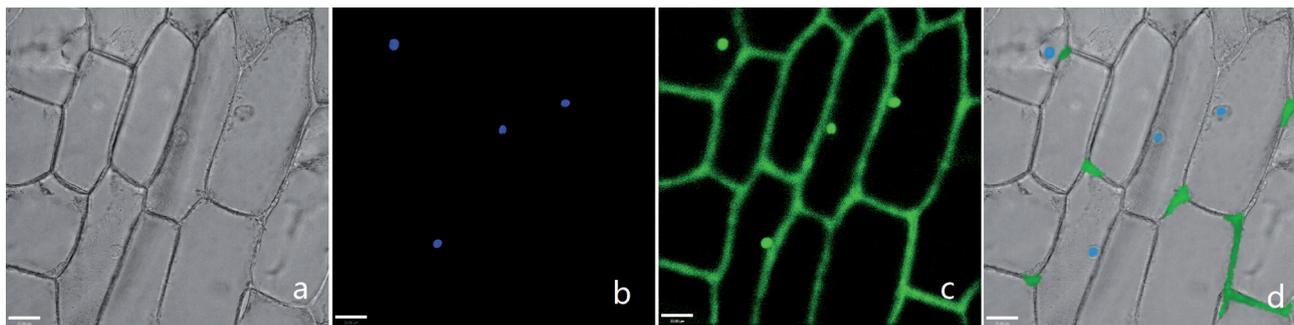
Fig. 9. Subcellular Localization of the pBM30-GFP-JmLFY. a) bright field; b) nucleus stained by DAPI (excitation at 405 nm); c) subcellular localization represents fluorescence signal (excitation at 488nm); d merged image of panels.

Discussion

Dioecy is a transition type in the evolution process of plants from monoecy to dioecy. In the evolution process of flowering plants from monoecy to dioecy, the reproductive system evolves through the special alienation of male and female flowers to form two mating type groups, namely, female-premorphic and male-premorphic [62-66]; Because the hermaphroditism of plants will directly affect the sexual reproduction process such as flowering and pollination, the reproductive characteristics of plants have aroused widespread interest of researchers at home and abroad [67–69]. It is very important to study the spatiotemporal expression of male and female flowers in higher plants at the molecular level to reveal the regulation mechanism of sex expression in higher plants. At present, research in this field mainly focuses on the cloning and expression analysis of flower development related genes. Through deep sequencing [68-70]. In conclusion, the expression pattern of flower development-related genes may play an important role in the regulation of sex differentiation. In all flowering plants, LFY gene is an important gene in the regulation of flower organs and one of the earliest identified flowering genes. It is a highly conserved plant-specific transcription factor [58]. In this study, BLAST sequence alignment showed that LFY homologous genes cloned from *Juglans mandshurica* showed high similarity to LFY gene sequences of plants such as Upland cotton.The amino acid sequence encoded by the flower-related jMLFY gene of *Juglans mandshurica* was compared with that of other species. LFY gene also had proline rich region at the N-terminal, central acid region, leucine zipper structure, and conserved regions at the N-terminal and C-terminal. These amino acid structures are consistent with those of LFY gene studied by previous researchers [60], which are sufficient to prove that we have successfully cloned the full-length cDNA sequence of LFY gene related to flowering of *Juglans mandshurica*. These results not only indicate the high homology of these LFY/ Flo gene families, but also suggest that these proteins may have similar functions. Both the PMLFY1 gene transducing plum to Arabidopsis thaliana [52] and the ACLFY gene transplanting onion to Arabidopsis thaliana can promote the early flowering of *Arabidopsis thaliana*, which proves that the over expression of LFY gene can promote the flowering of plants, showing the significant role of LFY gene in promoting the flowering of plants. It is also more direct proof that the flower-related LFY gene can promote the early flowering of plants. According to the high homology of LFY/ Flo family genes, it could be predicted that the same result could be obtained if JMLFY was transferred into *Arabidopsis thaliana*. It can be used as an experimental direction to verify the function of JMLFY gene.

Flowering affects the reproductive success of plants and is an important part of the life cycle of plants. The transformation of plants from vegetative development stage to reproductive development stage is regulated by a variety of complex molecular networks, in which many genes are involved, such as LFY, CO, FT, AP1, TFL1, etc. These flowering-related genes regulate and restrict each other [53]. Among these regulatory genes, LFY gene is a key gene related to flower formation. Studies have shown that many different plants contain LFY gene related to flower formation, and LFY gene is expressed to varying degrees in roots, stems, leaves and other organs except in flower organs, and with the change of time. The expression level of LFY gene in different organs also varies with it, showing a certain period of time [49]. Therefore, the study of the expression characteristics of LFY gene is helpful to find the expression rules of LFY gene, and further prove the relationship between LFY gene and flower formation, which plays an important role in the study of the function of LFY gene. In this study, we selected female flower buds, male flower buds, pistils, stamens, leaf buds and fruits of different developmental stages of *Juglans mandshurica* for real-time fluorescence quantitative expression analysis of JMLFY gene. The results showed that JMLFY gene was expressed in different organs and tissues of *Juglans mandshurica*, and there were differences in the expression levels of the same organ at different stages. The expression level of JMLFY gene in floral related organs was significantly higher than that in non-floral organs. These results indicate that JMLFY gene is indeed

a key gene related to flower formation, and its regulatory mechanism in flower formation pathway and its role in non-flower organs deserve further study. Therefore, the prokaryotic expression vector of JMLFY gene in *Juglans mandshurica* was constructed, and the protein products were obtained. The protein structure of the expressed protein products was analyzed, which laid a foundation for exploring the function and regulation of JMLFY related to flowering at the protein level. Protein function is closely related to its localization in cells. Proteins can only play their functions when they mature and must be allocated to specific parts of the cell tissue. The subcellular localization information of proteins reflects their real biological functions. Subcellular localization is a key step in the study of protein function, and green fluorescent protein gene (GFP) is currently the most widely used [38]. In this study, in order to further determine the subcellular localization of JMLFY protein, JMLFY was fused with the enhanced green fluorescent protein of PBM30-GFP to construct the fusion expression vector PBM30-GFP-LFY, which was introduced into onion epidermal cells by gene gun. It was found that the fusion expression vector of JMLFY-GFP could see green fluorescence in the nucleus, which further proved that the protein had the general characteristics of transcription factor. This result is consistent with the reported nuclear localization of LFY gene in upland cotton, alfalfa and carrot [32]. This suggests that the homologous LFY gene has similar functions and functions in regulating flower initiation

LFY is crucial for plant flowering, and its expression differs between organs, such as the root, stem, leaf, and flower, and between developmental stages. In the present study, the *JmLFY* gene was cloned successfully and its expression was analyzed in different organs and at different developmental stages. The homology with other species was more than 80%. Additionally, the structure of the amino acid sequence was similar to that reported in other species, implying that the function of *JmLFY* is similar to that of *LFY* in other species. The high homology of *LFY/FLO* family genes suggests that the same results will be obtained for *JmLFY*, which may be used to verify the function of *JmLFY* in future studies. Wang et al. (2014) studied the expression of *CcLFY* in different organs and in different developmental stages of *C. cathayensis* female flower buds [37]. The results revealed that *CcLFY* was highly expressed in both immature leaves and flower buds, and the morphological expression was asynchronous to gene expression. Similar results were found in our study; we showed that *JmLFY* is expressed in different organs and that the expression level differs in the same organ at different development stages. *JmLFY* expression increased before the early development stage of the staminate inflorescence, anther formation and pollen dispersal stage, pistillate inflorescence sprouting and flower development stage, and the pollination and late stage of zygotic embryo development.

Conclusions

In this study, we successfully cloned the cDNA sequence of the *LFY* gene in *J. mandshurica*. The CDS sequence was 1158 bp long and encoded 385 amino acids. Homology analysis of the nucleotide and amino acid sequences encoded by *JmLFY* revealed high homology between *JmLFY* and *LFY* genes from other species, particularly in related species. This confirmed that *LFY* was indeed obtained in our study. The gene was named *JmLFY* and the full-length CDS was submitted to the NCBI database (GenBank: KX364241). qRT-PCR analyses revealed that the *JmLFY* gene is expressed constitutively in male and female organs of *J. mandshurica*. The expression of *JmLFY* increased significantly during flower bud sprouting, flower induction, early fruit formation, and fruit maturation. The relative expression of *JmLFY* also increased in leaf buds during the flower bud sprouting stage. A vector (pBM30-*JmLFY*) was successfully constructed for the prokaryotic expression of *JmLFY*, which was transformed into the BL21 (DE3) competent state and induced by IPTG. The product was detected by SDS-PAGE polyacrylamide gel electrophoresis, and the results revealed a protein with the expected size of ~50 kDa, confirming that the pBM30-*JmLFY* vector could express protein. Subcellular localization of JmLFY protein revealed that it is located in both the nucleus and cytoplasm, which differs from previous reports. Our results demonstrate that *JmLFY* was cloned successfully. In addition, we verified the high level of structural homology, and similarity of function and expression of *JmLFY* with *LFY* genes in other species.

Acknowledgments

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