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RESEARCH ARTICLE

Molecular cloning and sequence analysis of an *LFY* homologous gene from *Juglans regia* L.

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Abstract **The existence of a long juvenile phase is found seriously to affect the early-stage economic benefits of later mature walnuts (*Juglans regia* L.). Studies on *LFY*, a key gene controlling floral transition and flower differentiation, may be helpful in solving the problem. This study reports the identification and characterization of a *JrLFY* gene from *Juglans regia* L., a homolog of *FLORICAULA/LFY*. The gene was isolated from flower buds of precocious walnut cultivar Zhonglin No. 5 by RT-PCR and RACE. The cDNA sequence of *JrLFY* (GenBank accession no. GU194836) was 1496 bp and contained an ORF of 1158 bp. Its corresponding genomic sequence (GenBank accession no. HQ019159) showed that the *JrLFY* contained three exons and two introns. The predicted amino acid sequence of the gene consisted of 385 amino acids and had a conserved region in the C-terminal when being aligned with sequences of other LFY homologs. Phylogenetic analysis showed that the LFY protein of walnut was close to those of hickory and chestnut. These studies will lay a foundation for understanding the mechanism of early fruiting and preparation for transfer of the *JrLFY* as a transgene to later mature walnuts.**

Keywords walnut, *JrLFY*, early mature, precocious, juvenility

Introduction

In perennial plants, the period between germination and flowering and fruit set can vary from one year to a few years or to several decades. The "Early Mature" (EM) walnut trees have the ability to flower within one year of germination, and their genotypes are characterized by successive flowering waves and large fruit sets that occur continuously in the following years (Breton et al., 2004). 'Qingxiang' cultivar, a later mature walnut, is popularized in China for its many excellent traits such as resistance to diseases, good flavor, suitable shell thickness and so on. However, the juvenility of Qingxiang is much longer than that of EM trees, which has impacted seriously on the early-stage economic benefits.

Plants show a wide variety of inflorescence morphologies, and the pattern of any particular inflorescence form is highly dependent on when and where primordial flowers arise in the shoot meristem (Benlloch et al., 2007; Prusinkiewicz et al., 2007). At the onset of flowering, the conversion of *Arabidopsis thaliana* meristem identity from secondary inflorescence to flower is largely dependent on endogenous and environmental cues, which eventually converge on the expression of the floral meristem identity gene *LEAFY* (*LFY*), encoding transcription factor (Mandel et al., 1992; Bäurle and Dean, 2006; Blázquez et al., 2006; Kobayashi and Weigel, 2007). *LFY*, a key gene controlling floral transition and flower differentiation, has been characterized in *Arabidopsis thaliana* and *Antirrhinum majus*, and many orthologous cDNAs cloned in tree species showed similar expression patterns (Tandre et al., 1995; Brunner et al., 2000; Sheppard et al., 2000; Rotem et al., 2007; Guo and Yang, 2008).

Constitutive expression of *LFY* is sufficient to convert branches into flowers, indicating that the gene is a critical factor for specifying floral meristem identity (Bowman et al., 1993; Mandel and Yanofsky, 1995). The LFY protein directly activates transcription of AP1 and its redundant homolog CAULIFLOWER (CAL) in the floral meristem (Parcy et al., 1998; Wagner et al., 1999; William et al., 2004). Over-expression of the *Arabidopsis LEAFY* advanced flowering time in *Populus tremula* × *P. tremuloides* (Weigel and Nilsson, 1995). In *Citrus*, the constitutive expression of

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Arabidopsis LEAFY allowed flowering and fruit set as early as one year among transgenic trees and their progenies (Peña et al., 2001).

The above results suggest that the attainment of high level of *LFY* expression is a key step for floral meristem specification. If the *JrLFY* of EM walnut was transferred into Qingxiang cultivar, it would be compensated for the shortcoming in early-stage economic benefits. As a first stage in this process, the flower buds from Zhonglin No. 5 cultivar were collected to clone the *JrLFY* from walnuts. To isolate the *JrLFY* cDNA sequence, we used both the RT-PCR and RACE techniques. The corresponding genomic sequence for *JrLFY* cDNA sequence can be obtained from direct PCR of genomic DNA. At the same time, the amino acid sequence of *JrLFY* can be predicted according to its ORF (open reading frame). In addition, the sequences cloned will be analyzed by sequence analysis software. These studies may help us know much about the early fruiting, *LFY* expression and potential for transgene in serotinous walnuts.

Materials and methods

Experimental materials

The apical buds from Zhonglin No. 5 cultivar were cut and collected at the Agricultural University of Hebei, China, during the period of male flower differentiation. The cut apexes were frozen in liquid nitrogen immediately and stored at −70ºC until use.

Methods

Cloning of cDNA and genomic DNA of JrLFY

Total RNA was extracted from 0.1 g apical bud with the Total RNA isolation Reagent kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. First-strand cDNA was synthesized with M-MLV-Reverse Trancriptase from Promega following the manufacturer's instructions. To clone the conserved region of *JrLFY*, the PCR primers P1 (5′-GGTTGTCCGAAGAGCCG-3′) and P2 (5′-CAGCGTGA-CAAAGTTGACGAAGT-3′) were designed from the blasted conserved sequence based on *LFY* genes of other species. The PCR conditions were: first incubated at 94ºC for 3 min, followed by 30 cycles at 94ºC for 30 s, 55ºC for 30 s, and 72ºC, 2 min, with the final extension at 72ºC for 10 min. The PCR product was separated on 1% agarose gels and recovered by gel extraction, then cloned into pMD19-T vector (Takara Biotech, Dalian, China), and finally transformed into competent cells of *Escherichia coli* strain DH5α. White colonies were checked by PCR and the positive colonies were sequenced (Takara Biotech, Dalian, China).

To amplify the 3′ end of JrLFY cDNA, gene-specific primer P3 (5′-GCCTTTGGTCGCCATAGCAGCAC-3′) and P4 (5′-AGCAGCACGCCAAGGATGGGACA-3′) were designed based on the obtained homologous fragments. The first strand cDNA was synthesized according to manufacturer's instructions of the 3′-RACE system (Takara Biotech, Dalian, China). The first round PCR, using the 3′-RACE outer primer (5′-TACCGTCGTTCCACTAGT-GATTT-3′) and P3, was carried out according to the following conditions: initial denaturation at 94ºC for 3 min, followed by 30 cycles at 94ºC for 30 s, 60ºC for 30 s and 72ºC for 2 min, with the final extension at 72ºC for 10 min. An aliquot of 1 μL (1∶50 diluted) primary amplification product was used as a template for the 3′ end-nested amplification under the same PCR condition using inner primer (CGCGGATCCTCCAC-TAGTGATTTCACTATAGG) and P4. The PCR reaction yielded a product about 500 bp long. The PCR product was separated on 1% agarose gels and was recovered by gel extraction, then cloned into pMD19-T vector (Takara Biotech, Dalian, China), and finally transformed into competent cells of *E. coli* strain DH5α. White colonies were checked by PCR and the positive colonies were sequenced (Takara Biotech, Dalian, China).

To obtain 5′ end sequence of *JrLFY* cDNA, a primer P5 (5′-ATGGATCCCGACCCCTTTAC-3′) was designed according to the 5′ end of the sequence (Acc.no. DQ989225) from hickory (*Carya cathayensis*). Gene-specific primers P6 (5′-CACCGCCATTGCCATTAC-3′) and P7 (5′-TCCAC-CACTTTCCTCAGGC-3′) were designed based on the obtained homologous fragment. The primary PCR was carried out using P5 and P6 under the following PCR conditions: 94ºC for 5 min, followed by 30 cycles (94ºC for 40 s, 56ºC for 40 s and 72ºC for 1 min), with the final extension at 72ºC for 10 min. An aliquot of 1 μL (1∶50) diluted primary amplification product was used as a template for the 5′ end-nested amplification under the same PCR conditions using primers P5 and P7, and the PCR reaction yielded a product about 550 bp. The PCR product was separated on 1% agarose gels. The target DNA bands were recovered by gel extraction, then cloned into pMD19-T vector (Takara Biotech, Dalian, China), and finally transformed into competent cells of *E. coli* strain DH5α. White colonies were checked by PCR and the positive colonies were sequenced (Takara Biotech, Dalian, China).

The full length of *JrLFY* cDNA was amplified with gene-specific primers P5 and P8 (5′-TTAGA AGGGCATGTGAT-CACC-3′), and the PCR conditions were: 94ºC for 4 min, followed by 33 cycles (94ºC for 40 s, 55ºC for 40 s and 72ºC for 1.5 min), with the final extension at 72ºC for 10 min. The PCR product was separated on 1% agarose gels, and target DNA bands were recovered by gel extraction, then cloned into pMD19-T vector (Takara Biotech, Dalian, China), and finally transformed into competent cells of *E. coli* strain DH5α. White colonies were checked by PCR and the positive colonies were sequenced (Takara Biotech, Dalian, China).

Meanwhile, *Juglans regia* genomic DNA was isolated by a modified cetyl-trimethyl-ammonium bromide-based method. The genomic DNA sequence of *JrLFY* was obtained from

direct PCR of genomic DNA using the three sets of primers used above (P5 and P7, P1 and P2, and P4 and P8). Genomic PCR was carried out under the following conditions: 94°C for 5 min, then incubated by a stepped program (94ºC, 40 s; 55ºC, 40 s; 72ºC, 1.5 min) for 35 cycles, and an extension at 72ºC for 10 min. All PCR products were separated on 1% agarose gels, and target DNA bands were recovered by gel extraction, then cloned into pMD19-T vector (Takara Biotech, Dalian, China), and finally transformed into competent cells of *E. coli* strain DH5α. White colonies were checked by PCR and the positive colonies were sequenced (Takara Biotech, Dalian, China).

Sequence analysis

Nucleotide and protein sequences of different LFY homologs were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and aligned with DNAMAN (Lynnon Biosoft, Vaudreuil, QC, Canada). Theoretical isoelectric point and mass values for the protein were also predicted and calculated using the DNAMAN program. For phylogenetic analysis, other 28 plant LFY protein sequences were retrieved from the GenBank database, and the proteins include *Arabidopsis lyrata* LFY (AlLFY, AAM27942), *A. thaliana* LFY (AtLFY, AAM27932), *Boechera stricta* LFY (BsLFY, AAW70551), *Antirrhinum majus* LFY (AmFLO, AAA62574), *Mangifera indica* LFY (MiLFY, ACN97632), *Prunus dulcis* LFY (PdLFY, AAY30859), *Pinus radiate* LFY (PrFLL, AAB51587), *Capsicum annuum* LFY (CaLFY, ABS18396), *Carya cathayensis* LFY (CcLFY, ABI58284), *Cedrela fissilis* LFY (CfLFY, AAT46474), *Clausena lansium* LFY (ClLFY, ABF61861), *Castanea mollissima* LFY (CmLFY, ABB83126), *Cydonia oblonga* LFY (CoLFY, BAD10958), *Citrus sinensis* LFY (CsLFY, AAR01229), *Dimocarpus longan* LFY (DlLFY, ABA39728), *Eriobotrya japonica* LFY (EjLFY, BAD10960), *Fortunella crassifolia* LFY (FcLFY, ABF61858), *Glycine max* LFY (GmLFY, ABE02270), *Hevea brasiliensis* LFY (HbLFY, AAT57872), *Idahoa scapigera* LFY (IsLFY, AAO73069), *Impatiens balsamina* LFY (IbLFY, CAI61979), *Nicotiana tabacum* FL2 (NtFL2, AAC48985), *Platanus racemosa* LFY (PrLFY, AAF77610), *Pyrus communis* LFY (PcLFY, BAD10957), *Silene coeli-rosa* LFY (ScLFY, CAC86163), *Solanum lycopersicum* LFY (SlLFY, AF197935), *S. tuberosum* LFY (StLFY, ABK56828), and *Vitis vinifera* LFY (VvLFY, AAN14527). A phylogenetic tree was constructed with Clustal W, and then the unrooted phylogenetic tree was generated.

Results

Cloning of cDNA and genomic DNA of *JrLFY*

The full length of the *JrLFY* cDNA was cloned from walnut (GenBank accession no. GU194836) and contained an ORF of 1158 bp coding a protein of 385 amino acids, corresponding to a 43.15-kDa polypeptide with an isoelectric point of 6.78. To obtain the complete genomic sequence and analyze the structure of the gene, three sets of primers were used for PCR amplification based on the walnut genomic DNA template. PCR products were sequenced and their sizes were about 1100 bp, 1800 bp and 300 bp in length, respectively, and then a *JrLFY* DNA of 2869 bp was obtained by assembling the above sequences and was deposited in GenBank (Acc. no. HQ019159). The BLAST of *JrLFY* ORF and its corresponding genomic sequence allowed the prediction of the gene structure (Fig. 1). There were three exons and two introns (first-exon 1–433, first-intron 434–978, second-exon 979–1340, second-intron 1341–2506 and third-exon 2507–2869). This exon–intron organization of *JrLFY* genomic sequence was similar to that of other plants, and the length of their exons did not remarkably vary; however, there were great differences in the length of the introns among them (Table 1).

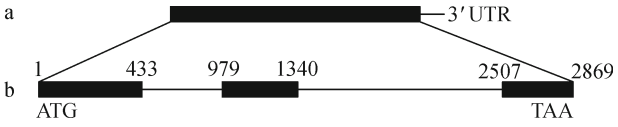

Figure 1 Schematic representation of *JrLFY* gene. a stands for schematic diagram of the JrLFY cDNA ORF (black box) with 3′UTR (thin lines); b stands for exon–intron organization of the *JrLFY* gene with location of exons (black boxes) and introns (thin lines). The number 1 indicates the location of the translational start codon as determined. Numbers delineate addresses in nucleic acid sequences.

Sequence analysis of the JrLFY protein

An alignment of the predicted amino acid sequence of *Juglans regia* JrLFY, *Carya cathayensis* CcLFY (GenBank accession no. ABI58284), *Antirrhinum majus* AMAFLO (GenBank accession no. AAA62574), *N. tabacum* NtFL2 (GenBank accession no. AAC48985) and *A. thaliana* AtLFY (GenBank accession no. NM_125579) was conducted using the DNAMAN program (Fig. 2), and the result showed that the deduced amino acid sequence of *JrLFY* had a higher identity with CcLFY (98.4%), AMAFLO (78.2%), NtFL2 (81.3%) and AtLFY (70.7%) at the overall amino acid level. A highly conserved region presented in the C-terminal half of the protein, hardly varied among the LFY homologs of these five different plant species. According to these results, we referred to the predicted protein as JrLFY.

To determine the phylogenetic relationship of JrLFY with LFYs from other species, the sequences of 28 LFY proteins were downloaded from GenBank and a phylogenetic tree was constructed. As shown in Fig. 3, a phylogenetic tree analysis for the LFY proteins was conducted by classifying them

Table 1 Organization of introns and exons in *LFY* genomic sequence from different plants

Organism	Accession number	Number of introns	Intron length (bp)	Number of exons	exon length (bp)	Number of amino acid residues
Juglans regia	HQ019159	2	545, 1166	3	433, 362, 363	385
Arabidopsis thaliana	AF466792	2	458, 907	3	457, 407, 411	424
Solanum tuberosum	EU371047	2	600, 572	3	477, 407, 378	418
Solanum lycopersicum	AF197936	2	309, 409	3	475, 389, 375	412
Capsicum annuum	EU000254	2	712, 2020	3	457, 413, 381	416
Chrysanthemum lavandulifolium	AY672542	2	583, 1083	3	463, 386, 390	412
Boechera stricta	AY734564	2	453, 1175	3	430, 404, 355	396
Orchis italica	AB088851	2	1070, 120	3	486, 444, 412	453
Vitis vinifera	AF378126	2	153, 663	3	451, 377, 381	402
Citrus sinensis	AY338976	2	408, 675	3	463, 353, 381	398
Idahoa scapigera	AY219229	2	556, 1070	3	445, 389, 333	389
Titanotrichum oldhamii	AY526319	2	421, 87	3	433, 377, 369	392
Serapias lingua	AB088466	2	100, 100	3	492, 438, 438	455
Cedrela fissilis	AY633621	2	176, 655	3	450, 378, 363	396
Arabidopsis lyrata	AF466802	3	441, 76, 876	4	457, 35, 390, 411	430
Solanum tuberosum	EF062357	2	600, 572	3	477, 407, 373	418

into four subgroups. JrLFY, CcLFY (hickory), CmLFY (chestnut), CoLFY (quince), EjLFY (loquat), and PcLFY (western pear) were clustered into the same subgroup, which showed that the JrLFY protein was closer to LFY proteins of hickory (Juglandales), chestnut (Fagales), loquat (Rosaceae), western pear (Rosaceae) and quince (Rosaceae). These results suggested that *JrLFY* was a *FLO/LFY* homolog gene.

Discussion

Long juvenility has impacted seriously on the early-stage economic benefits of serotinous walnuts like the Qingxiang cultivar. The EM walnut flowering within one year of germination was useful to study the genetic cues controlling flowering and sexual maturity in woody perennials (Breton et al., 2004). Overexpression of the *Arabidopsis LEAFY* gene advanced flowering time in *Populus tremula* × *P. tremuloides* (Weigel and Nilsson, 1995). Therefore, it seems possible that the problem of late flowering could be overcome if the *JrLFY* of EM walnut was transferred into Qingxiang cultivar. Expression of *LFY* is weak in leaf primordia during the vegetative phase but is strongly activated in floral meristems at the onset of flowering (Hempel et al., 1997). Therefore, we collected the flower buds from Zhonglin No. 5 cultivar to clone the *JrLFY* in this article.

In this study, we isolated the full-length cDNA and DNA of *JrLFY* from Zhonglin No. 5 cultivar. The BLAST of *JrLFY* ORF and its corresponding genomic sequence allowed the prediction of the gene structure (three exons and two introns). This exon–intron organization of *JrLFY* genomic sequence was similar to gene organization of *LFY* gene of other plants including *Populus tomentosaLFY* (An et al., 2010), *Capsicum annuum LFY* (Kim et al., 2008), *S. tuberosum LFY* (Guo and Yang, 2008), *B. stricta LFY* (Baum et al., 2005), *Serapias lingua LFY* (Montieri et al., 2004) and so on. Sequence analysis of the JrLFY protein indicated that it contained a highly conserved C-terminal region which has also been described in other plants (Weigel et al., 1992; Carmona et al., 2002). The differences in the conserved region at the protein level have originated from various origins, evolutionary histories and different selection and breeding levels of different species. According to the J. Hutchinson System (an evolutionary theory), Juglandales, Fagales and Rosaceae located in the same branch. Among the three orders, the relationship between Juglandales and Fagales is closer and both are more advanced than *Rosaceae*. Phylogenetic analysis showed that the JrLFY protein was closer to LFY proteins of hickory (Juglandales), chestnut (Fagales), loquat (Rosaceae), western pear (Rosaceae) and quince (Rosaceae). Walnut and hickory were clustered first, then with chestnut, and together with the loquat, western pear and quince at last. This is consistent with the J. Hutchinson System.

In conclusion, this study was undertaken to analyze the *JrLFY* by cloning the cDNA and its genomic DNA and determining the phylogenetic relationship. Using the information obtained from these studies, we will enrich the theory of early fruiting and provide theoretical guidance for understanding the gene expression of *JrLFY* in walnut and potential effects of a transgene *JrLFY* in serotinous walnuts.

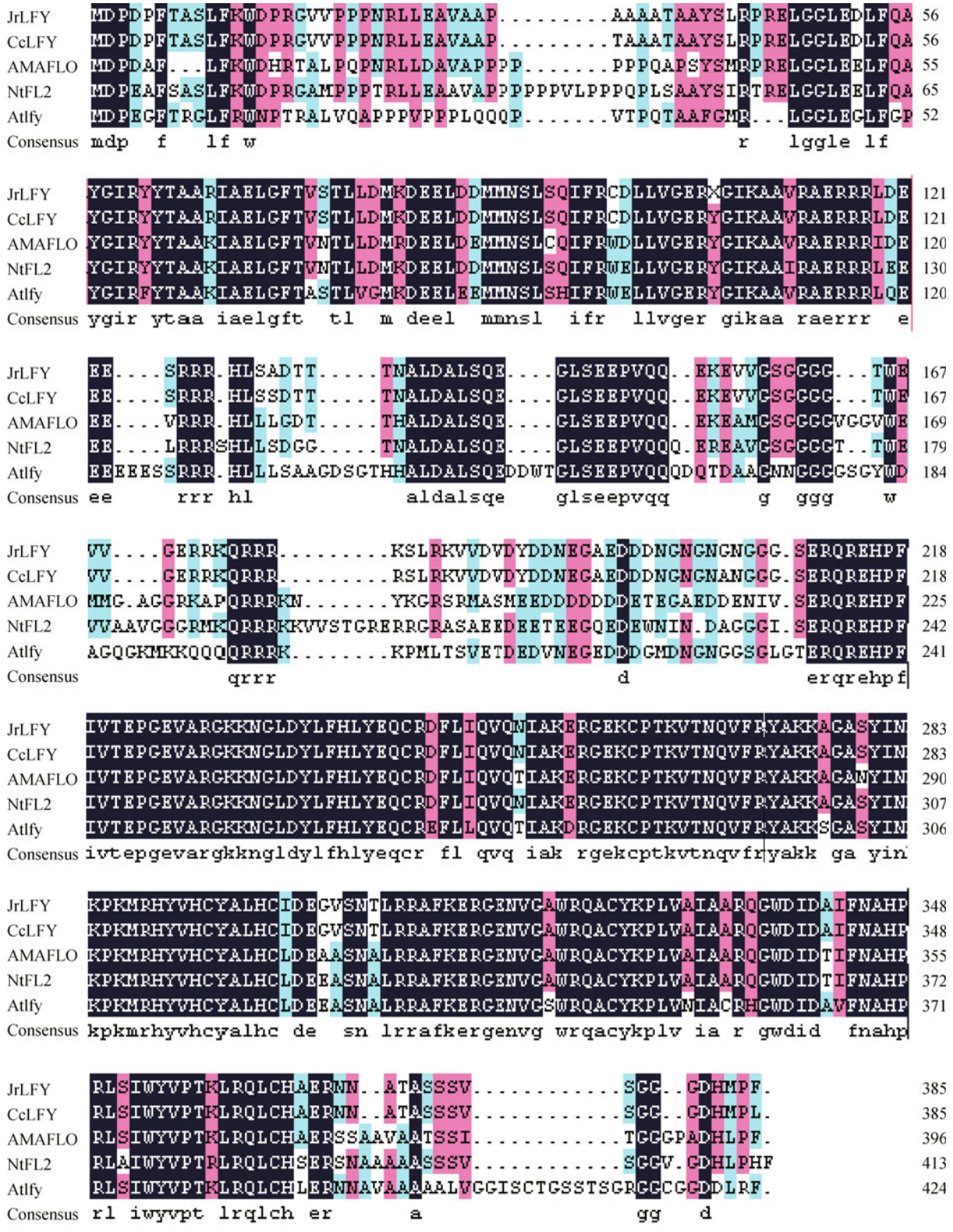

Figure 2 Alignment of amino acid sequence of *Juglans regia* LFY (JrLFY, ACZ48701) with those of *C. cathayensis* LFY (CcLFY, ABI58284), *Antirrhinum majus* LFY (AMAFLO, AAA62574), *Nicotiana tabacum* FL2 (NtFL2, Q40505) and *Arabidopsis thaliana* LFY (AtLFY, NM_125579). Identical amino acid residues in this alignment are shaded in black, and similar amino acid residues are shaded in red (higher similarity) and blue (lower similarity). The alignment was performed with the DNAMAN program.

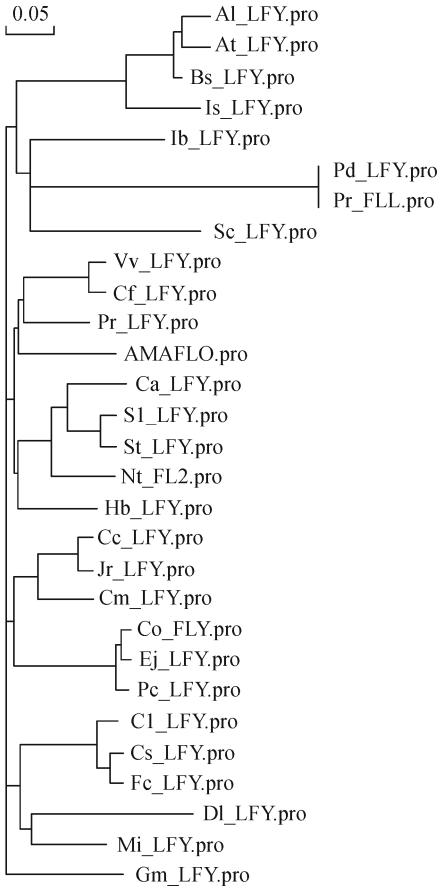

Figure 3 Phylogenetic analysis of the 29 LFY proteins. Individual data are derived from GenBank except for *JrLFY* provided by this research. The tree was displayed as a phylogram in which branch lengths are proportional to distance.

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