African Journal of Biotechnology Vol. 10(20), pp. 3978-3985, 16 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2334
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Characterization of a chestnut *FLORICAULA/LEAFY* homologous gene

Tao Liu[1]*, Yun-qian Hu[2] and Xiao-xian Li[2]

[1]Faculty of Agronomy and Biotechnology, Yunnan Agricultural University, Kunming, Yunnan, 650201, China.
[2]Kunming Institute of Botany, the Chinese Academy of Sciences, Kunming, Yunnan, 650204, China.

Accepted 24 March, 2011

The *FLORICAULA/LEAFY* (*FLO/LFY*) homologues' genes are necessary for normal flower development and play a key role in diverse angiosperm species. In this paper, an orthologue of *FLORICAULA/LEAFY, CmLFY* (chestnut *FLO/LFY*), was isolated from the inflorescence of chestnut trees. Its expression was detected in various tissues. Furthermore, the flowering effectiveness of the gene was assessed with transgenic *Arabidopsis*. CmLFY protein showed a high degree of similarity to *PEAFLO* (78%), which is a homologue of *FLO/LFY* from pea. RT-PCR analysis showed that, *CmLFY* expressed at high levels in inflorescences, but not in young leaves, fruits or stems. The transgenic *Arabidopsis* with over-expressed *CmLFY* showed accelerated flowering, which supports that *CmLFY* encodes a functional orthologue of the *FLORICAULA/LEAFY* genes of angiosperms despite its sequence divergence. These results suggest that *CmLFY* is involved in inflorescence development in chestnut.

Key words: Chestnut, homologue, *FLORICAULA/LEAFY.*

INTRODUCTION

Two developmental programmes can be distinguished during the life cycle of flowering plants. During the initial vegetative phase, the apical meristem produces leaves and lateral shoot. After floral induction, the plant enters into a reproductive phase and the apical meristem changes its developmental pattern and initiates the production of flowers. In *Antirrhinum* and *Arabidopsis* this transition from vegetative to reproductive development pattern requires the establishment of floral meristem identity in the lateral meristems of the inflorescence, a process which has been demonstrated to be controlled by the homo- logous genes *FLORICAULA* (*FLO*) and *LEAFY* (*LFY*), respectively (Ma, 1998; Pidkowich et al., 1999). *LFY* is a transcriptional regulation gene thought to play a primary role in determining flower meristem identity. *FLORICAULA (FLO)*, a *LFY* homologue in snapdragon, has been shown to have almost the same role. Loss function mutants of these genes result in the conversion of flowers into indeterminate secondary shoots (Coen et al., 1990; Weigel et al., 1992). The over-expression of *LFY* under a constitutive promoter in *Arabidopsis* caused early flowering and converted all lateral shoots into solitary flowers. In heterologous plants such as aspen, *LFY* has been shown to have effects on the acceleration of flowering and induction of ectopic flowers (Weigel and Nilsson, 1995; Rottman et al., 2000), suggesting a conservation of *LFY* function across long phylogenetic distances within angiosperms. These reports strongly suggest that, the ability to control the expression of *LFY* or of its orthologs from other plants, could make it possible to artificially induce various plants to blossom and even skip or shorten the juvenile phase of woody plants.

Flowering is an essential stage for fruit production and thus, an understanding of the genetic mechanisms underlying the flowering event is important for efficient fruit production. During the last decade, molecular mechanisms of flowering have been studied extensively in herbaceous ‘‘model’’ plants such as *Arabidopsis* and *Antirrhinum*. In contrast, studies on the molecular mechanisms of flowering in fruit trees have just begun. Although, several flowering-related *FLO/LFY* orthologs have been cloned from kiwifruit (*Actinidia deliciosa*)

*Corresponding author. E-mail: beixue2007@gmail.com. Tel: +86-871 5227721.

Abbreviations: PCR, Polymerase chain reaction; **RT-PCR,** reverse transcriptase-PCR; **RACE,** rapid amplified cDNA ends.

(Walton et al., 2001), grapevine (*Vitis vinifera*) (Carmona et al., 2002), and apple (*Malus domestica*) (Kotoda et al., 2002), their specific role in the induction of the characteristic features of tree reproductive development is still being elucidated.

Trees are characterized by an extended adolescence. This is particularly important for commercial fruit/nut tree growers and breeders, because prolonged juvenile periods delay harvesting and the evaluation/breeding of new strains. Chestnut (*Castanea mollissima*) is one of the most commercially valuable fruit trees, with production once only annually. Chestnut has an extended juvenile-vegetative phase, during which vegetative growth is maintained. Attempts to exploit existing knowledge of the genetic control of flower development in *Arabidopsis* to engineer nut trees could have significant economic and scientific implications for the tree fruit industry. As yet, no studies have reported on the molecular mechanism underlying the development of chestnut flowers. Recently, MADS-box genes have been cloned and characterized in chestnut by us (Liu et al., 2006). Our results suggest that these chestnut MADS genes may be involved in floral organ and fruit development in chestnut. In this paper, we identified a chestnut *FLORICAULA/LEAFY* homologous gene involved in floral development. We described the isolation and characterization of *CmLFY* (DQ270548). The function of *CmLFY* was demonstrated by transgenic *Arabidopsis*, as expected for normal plants, transgenic plants showed earlier flowering phenotype, indicating that *CmLFY* may play a similar role of promoting flowering as the *FLORICAULA/LEAFY* genes in flower development.

MATERIALS AND METHODS

Plant materials

The chestnut and *Arabidopsis* ecotype Columbia (Col) were used in this study. Chestnut examples were collected from Kunming, Yunnan, People's Republic of China and were frozen in liquid nitrogen immediately and stored at -80°C.

RNA extraction and cDNA cloning

Total RNA was extracted from the inflorescences using a modified CTAB method (Zeng and Yang, 2002). The cDNA was prepared using Smart cDNA library construction kit (BD Biosciences Clontech, American). Full-length *CmLFY* cDNA was obtained by the 5′ and 3′ rapid amplified cDNA ends (RACE) method (Chenchik et al., 1996). The first amplified chestnut cDNA contained about 440 bp between primers CmS and CmA, designed from *Arabidopsis thaliana LFY* (Weigel et al., 1992) and *Antirrhinum majus FLO* (Coen et al., 1990) cDNA sequences. The 3′ RACE was carried out between cassette CDSIII/3' primer and CmS2 primer. The DNA fragment amplified was cloned into $_{P}$MD18-T vector (Takara, Japan). The 5′ RACE was carried out between cassette CDSIII/5' primer and CmA2 primer. Full-length cDNAs were amplified with the sense primer LFY-ORF5 and antisense primer CDSIII/3' primer. These clones were sequenced completely by the dideoxy method using ABI3730 automated sequencer (ABI Company, USA).

Phylogenetic comparisons

Protein sequences of different *LFY* homologs were retrieved from GenBank and aligned with CLUSTAL. Phylogenetic relationships among these genes were inferred by NJ analysis (MEGA Ver.3.1).

Plant transformation

The full length *CmLFY* (including start codon ATG) was amplified between LFY-ORF5 and LFY-ORF3 (Figure 1). Then, the amplified cDNA was blunted and KpnI and SalI linkers were ligated both ends, cloned into a *pCambia2301-101* binary vector in a sense-oriented manner under the CaMV 35S promoter. An *Agrobacterium tumifaciens* GV3101 strain (Van-Larebeke et al., 1974) was used for the transformation of the *A. thaliana* Columbia ecotype by the floral-dip method (Clough and Bent, 1998). For the selection of transformed plants, resultant seeds were planted on a 1/2MS culture medium containing kanamycin (50 μg mL^{-1}) as selective antibiotics and then, transferred to 22°C under LD conditions (16 h light, 8 h dark). Transformants were identified as kanamycin-resistant when seedlings in the medium produced green leaves and well-established roots. Resistant transformants were transplanted to moistened potting soil composed of vermiculite and perlite (1:1 (v/v^{-1})) after two to four adult leaves had developed and their flower phenotypes were observed under long-day conditions.

RNA analysis of transgenic plants by RT-PCR

Detection of *CmLFY* transcripts was performed by RT-PCR. RNA was isolated from whole plants of *Arabidopsis* by a method using CTAB described previously described. *CmLFY* specific transcripts were identified using 1 μg of total RNA as a template and the following primers: a sense primer LFY-ORF5 and an antisense primer LFY-ORF3 for *CmLFY* (Table 1), giving rise to about 1300 bp long PCR product. PCR reactions were run for 35 cycles at 58°C for *CmLFY*. The PCR products were run on 1.5% (w/v^{-1}) agarose gels stained with ethidium bromide.

RESULTS

Cloning and sequence analyses of CmLFY

A comparison of *Arabidopsis LFY* (Weigel et al., 1992) and *Antirrhinum FLO* (Coen et al., 1990) showed the presence of several conserved regions. Two of these regions, CmS and CmA, were used to design degenerate oligo- nucleotide primers for RT-PCR. Using these primers with cDNA prepared from an inflorescence of chestnut, we obtained a PCR amplified fragment containing both primers and other conserved sequences. This fragment was named *CmLFY (*chestnut *FLORICAULA/LEAFY* homologue). To obtain a full-length *CmLFY* cDNA, several primers for 5'/3' RACE were designed. Primer sites and the nucleotide sequences of primers used for cloning were shown in Figure 1 and Table 1, respectively.

The coding region of CmLFY is 1,161 bp, encoding 386 amino acids. The deduced amino acid sequence of CmLFY cDNA has 78% homology with PEAFLO, 79% with PtFL and 59.9% with PlaraLFY. NLF, PRFLL, PEAFLO,

Table 1. Primer sequences used in PCR cloning of *CmLFY*.

Primer	Oligonucleotide
Primers for an internal fragment	
CmS sense primer	5'- GGGAGCACCCGTTCATTGTGACTG -3'
CmA antisense primer	5'-GA/CAGCTTG/TGTG/TGGGACATACCAGAC-3'
Cassette primers	
CDSIII/5' primer	5'-AAGCAGTGGTATCAACGCAGAGT-3'
CDSIII/3' primer	5'-ATTCTAGAGGCCGAGGCGGCCGACATG-3'
3' RACE primer	
CmS2 primer	5'-CGGCCTTGATTACCTCTTCCATCTC -3'
5' RACE primer	
CmA2 primer	5'- CACCGCGCTCCTTGGCAATGTTCTGT -3'
CmLFY specific primers	
LFY-ORF5	5'-GGTACCAAGCTAGCTTCATTGATG -3'
LFY-ORF3	5'-CGGTCGACTAGAAATGCAAATTTTTCTC-3'

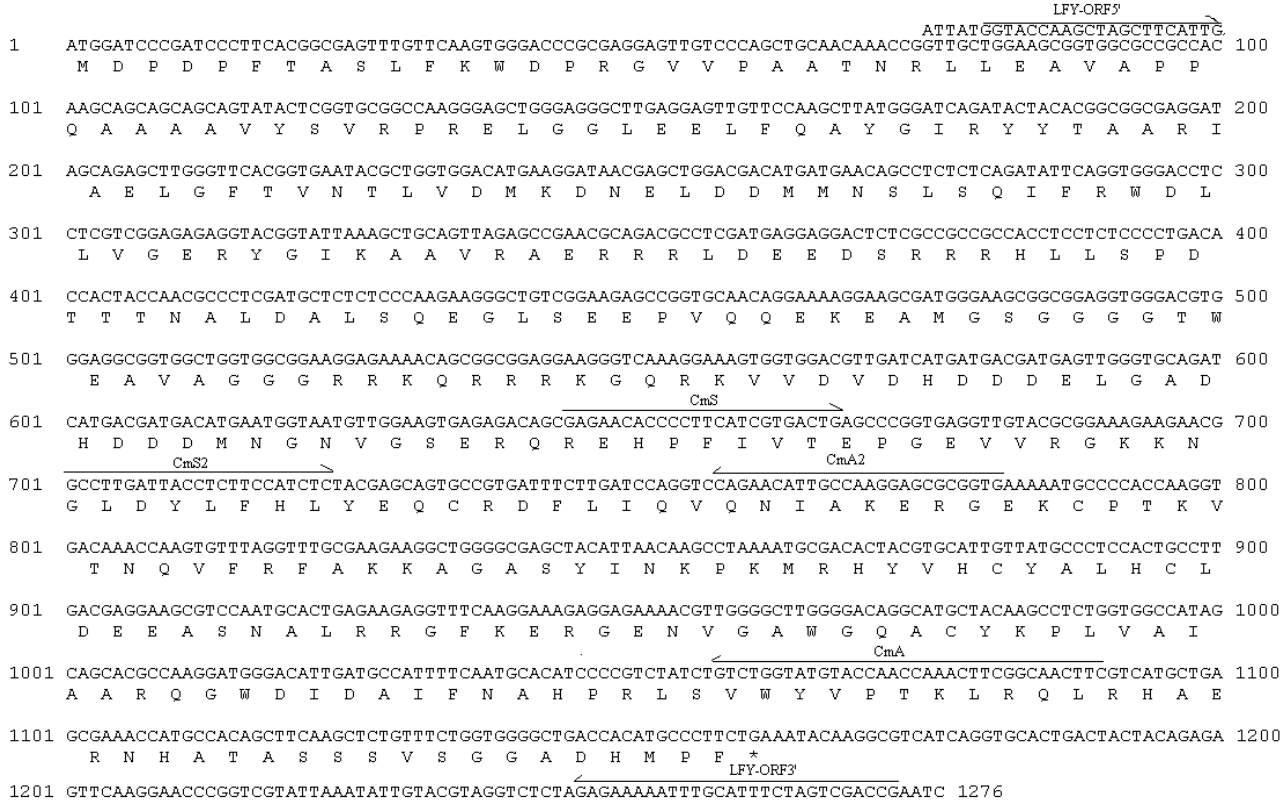
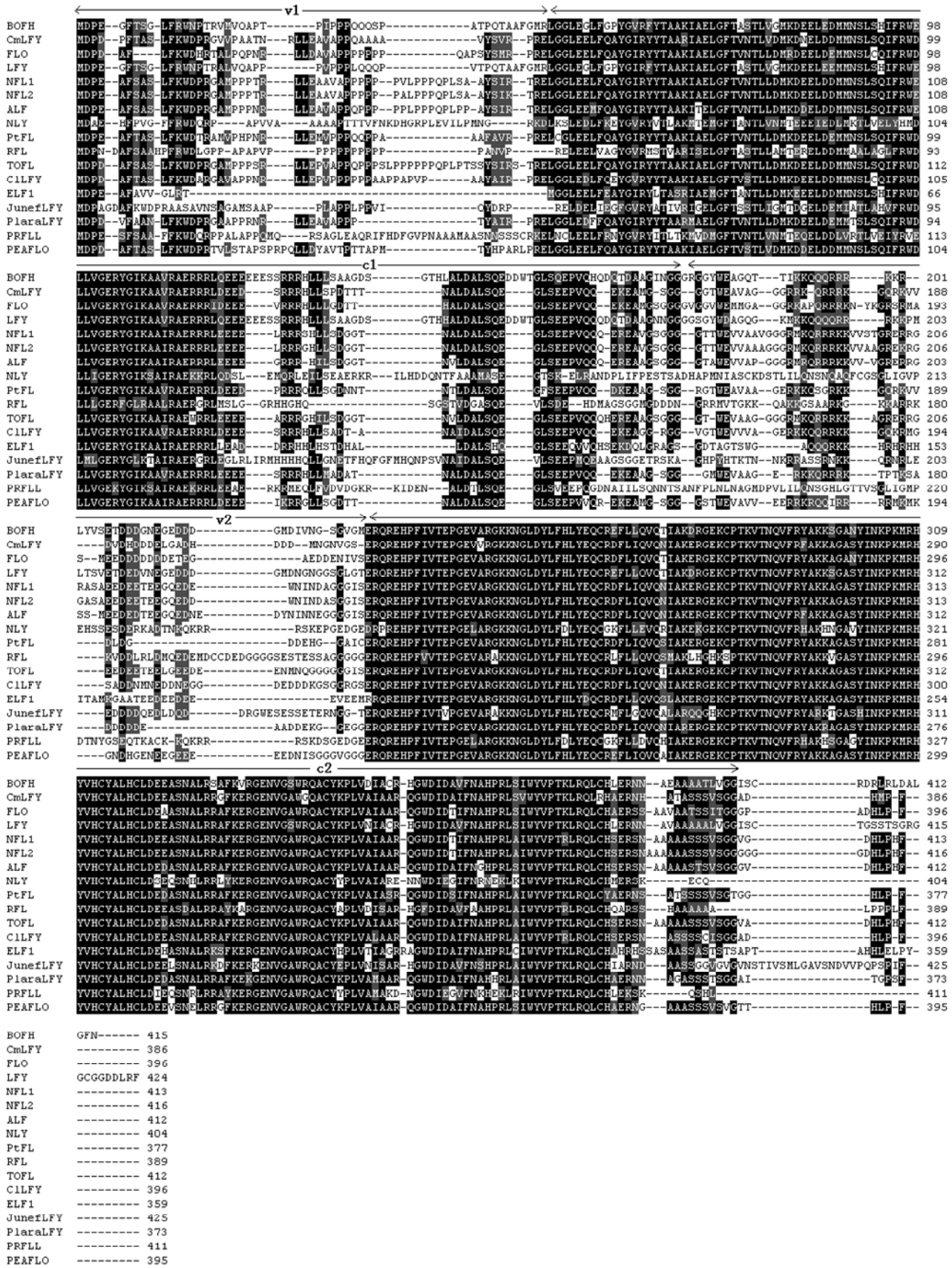
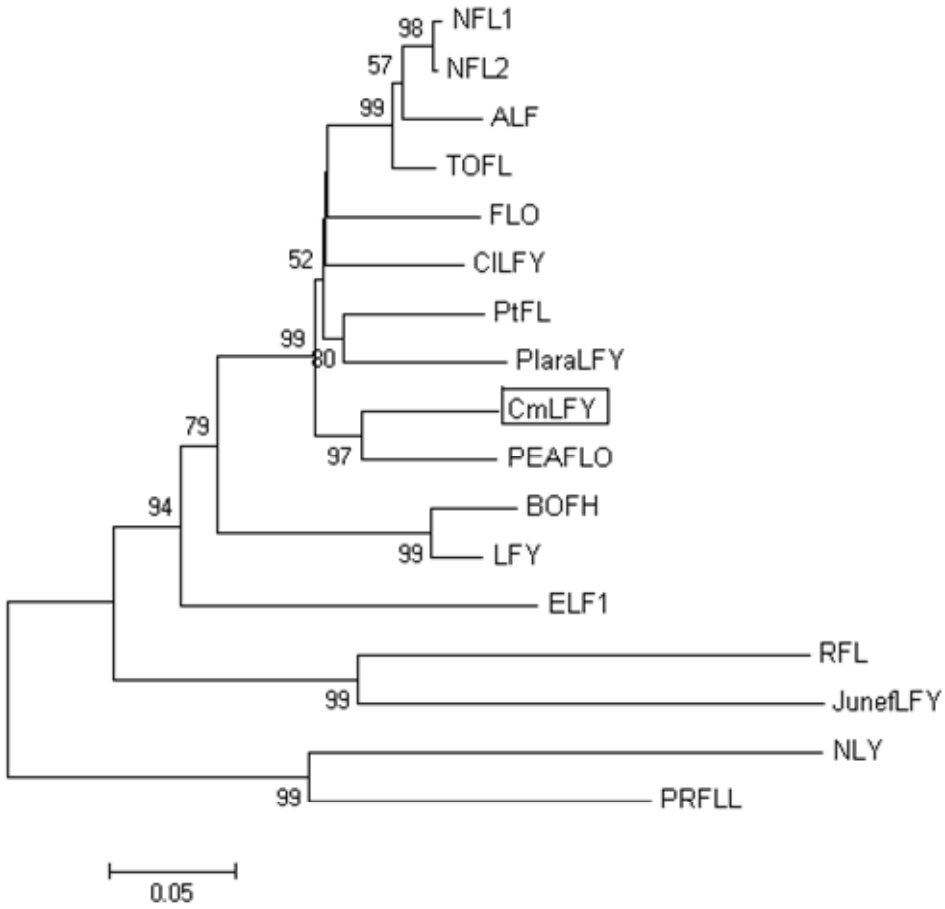


Figure 1. Nucleotide and deduced amino sequences (single-letter code) of *CmLFY* cDNA. The asterisk (*) indicates a stop codon. Arrows above the sequences indicate the primers used for RACE-PCR.

ELF1 and CmLFY lacked a proline-rich region in the variable region (roughly the first 40 amino acids). To more closely determine the evolutionary relationship between FLO/LFY-like proteins, a phylogenetic tree was con- structed (Figure 2b). This tree showed that, the topology of these genes seems to be concordant with the topology of the species phylogeny and suggests that *CmLFY* is an angiosperm ortholog of *FLO*/*LFY*-like genes.


v1
c1
v2
c2
BOFH
CmLFY
FLO
LFY
NFL1
NFL2
ALF
NLY
PtFL
RFL
TOFL
C1LFY
ELF1
JunefLFY
PlaraLFY
PRFLL
PEAFLO

a



b

Figure 2. (a) Sequence comparison of FLO/LFY-like proteins (accession numbers in parentheses): NLY and PRFLL from *P. radiata* (U76757 and U92008, respectively); CmLFY from chestnut (DQ270548); BOFH from *B. oleracea* (Z18362); FLO from *A. majus* (M55525); LFY from *A. thaliana* (M91208); NFL1 and NFL2 from *N. tabacum* (U16172 and U16174, respectively); ALF from *P. hvbrida* (AF030171); PtFL from *P. balsamifera* (U93196); RFL from *O. sativa* (AB005620); TOFL from *L.esculentum* (AF197934); PEAFLO from *P. sativum* (AF010190); ClLFY from *C. glaziovii* (AY633622); ELF1 from *Eucalyptus globules* (AF034806); JunefLFY from *J. effusus* (AF160481) and PlaraLFY from *P. hispanica* (AF106842). Black boxes indicate identical amino acids, shaded boxes indicate amino acids with similar properties, and dots indicate gaps introduced to optimize alignment. c1 and c2, conserved regions; v1 and v2, variable regions; (b) Protein sequence comparisons of CmLFY to other LFY/FLO homologs. The deduced amino acid sequence of CmLFY was compared with some published LFY homologs. Box characters represent chestnut protein.

Expression patterns of CmLFY

CmLFY mRNA expression was analyzed in various tissues by RT-PCR. Total RNA was isolated and PCR primers specific to *CmLFY* were used to detect the expression patterns in several tissues. The result showed that *CmLFY* mRNA was expressed in inflorescence tissues, but not in young leaves, fruits and stems (Figure 3). The RT-PCR of *CmLFY* -specific primers resulted in amplification of a single band. Increasing the PCR cycle did not change these expression patterns. The amplified DNA fragments were the expected length.

Ectopic expression in *Arabidopsis*

To further verify the function of *CmLFY*, we constructed a binary vector *pCambia2301-101-CmLFY* containing full-length *CmLFY* coding area inserted in the sense-oriented direction under the control of the Cauliflower 35S promoter. The *pCambia2301-101-CmLFY* was introduced into wild- type *Arabidopsis* plants by *Agrobacterium*-mediated transformation. Fourteen independent transgenic plants that survived on kanamycin were identified (Table 2). Five (lines one-five) of them flowered earlier than the wild-type plants by five-seven

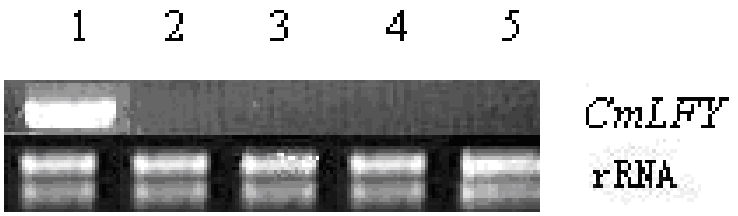


Figure 3. Expression pattern of *CmLFY* gene revealed by RT-PCR analysis. Lane 1: inflorescences; Lane 2: stem; Lane 3: tender leaves with no flowers; Lane 4: tender leaves with flowers; Lane 5: fruit. rRNA is used as RNA standard. Amplification of *CmLFY* was performed for 30 cycles.

Table 2. Comparison of flowering time, number of rosette leaves in T_1 transgenic and wild-type *Arabidopsis* (Col) plants in LD conditions.

Transgenic line	Days to flowering[a]	Rosette leaves at time of flowering[b]	Note
1	18	4	Early flowering
2	16	3	Early flowering
3	18	4	Early flowering
4	20	5	Early flowering
5	20	4	Early flowering
6	24	7	
7	24	8	
8	25	9	
9	22	8	
10	24	8	
11	25	8	
12	26	9	
13	26	10	
14	25	8	
wt[c]	25.5[d]	9.5[d]	(n=10)[e]
v-wt[f]	26[d]	9.5[d]	(n=8)[e]

[a] Days to flowering is defined as the time when flower primordial were first visible to the naked eye;[b] rosette leaves were counted on the day that flower primordial were first visible;[c] w wild-type *Arabidopsis* Columbia plants;[d] Mean number;[e] Number of plants;
[f] vector-transformed plants.

days and produced only four to five rosette leaves when they flowered (Figure 4 and Table 2). Most of the T1 transgenic plants could self-pollinate and grow siliques normally and the resultant seeds also had the ability to germinate except for the lines 1, which is so small that it was difficult to obtain its seeds. The early flowering were inherited in the next generation and co- segregated with the kanamycin-resistant genes. The expression of the *CmLFY* mRNA in T1 plants was confirmed by RT-PCR, all putative transformed lines showed the expected products of *CmLFY*. No amplifi- cation was observed for the cDNA prepared from non-transformed *Arabidopsis* plants and vector-trans- formed *Arabidopsis* plants. The transgenic plants are stably transformed and the siblings which segregate without the transgene had no amplification of *CmLFY* and flowered at the same time as wild-type plants.

DISCUSSION

Despite the importance of understanding the regulation of the flowering process in woody perennials for the management and improvement of woody species, very little is known about the underlying molecular mechanisms. Regulation of flowering in woody perennials shows remarkable differences in contrast to herbaceous species, that is, long juvenile phases, bud dormancy and the alternating vegetative and reproductive development according to the season.

To investigate the molecular mechanism in chestnut flowering, we cloned CmLFY, a putative homologue of Arabidopsis LFY. A comparison (Figure 2a) of amino acid sequences with *Arabidopsis* LFY (Weigel et al., 1992), *Antirrhinum* FLO (Coen et al., 1990) and other FLO/LFY homologs (*Brassica oleracea*. BOFH.

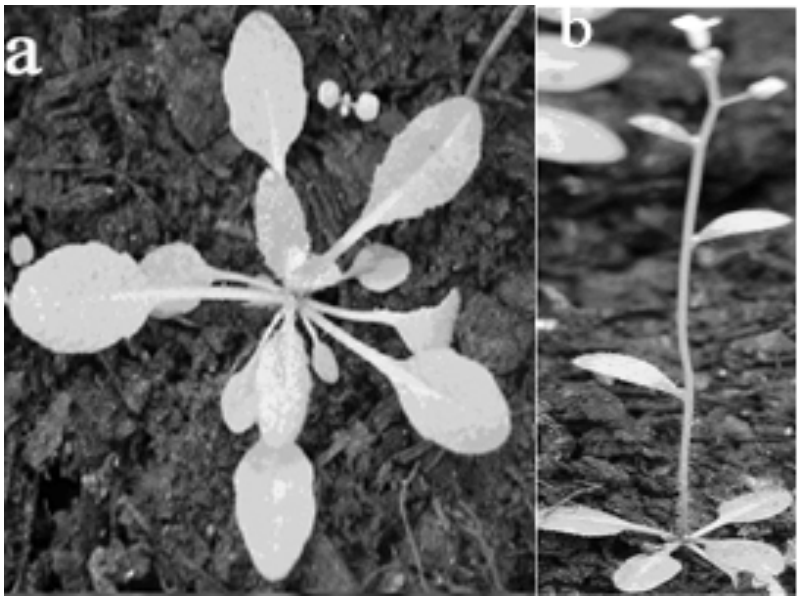


Figure 4. Transgenic and wild-type Arabidopsis plants grown under LD photoperiods for 20 days. Arabidopsis plants were grown in one-half strength of medium for 9 days and transferred to potting soil. (a) wild-type Arabidopsis Columbia. (b) Transgenic Arabidopsis carrying a 35S::*CmLFY* gene.

Anthony et al., 1993; *Nicotiana tabacum*. NFL. Kelly et al., 1995; *Oraza sativa*. RFL. Kyozuka et al., 1998; *Pinus radiata*. NLY. Mouradov et al., 1998 ; *Petunia hybrida*. ALF. Souer et al., 1998; *Eucalyptus globulus*, ELF1, Southerton et al., 1998 ; *Lycopersicon esculentum*. TOFL. Molinero-Rosales et al., 1999; *Juncus effusus*. JunefLFY. Frohlich and Parker., 2000; *Platanus hispanica*. PlaraLFY. Frohlich and Parker., 2000; *Populus balsamifera*. PTFL. Rottmann et al., 2000; *Cedrela glaziovii*. ClLFY. Marcelo and Adriana, 2006) showed the presence of conserved regions including two large conserved regions (c1 and c2) and two shorter regions of lower similarity (variable regions v1 and v2). These domains are typical markers for transcriptional acti- vators and may be functionally important for FLO/LFY-like proteins (Weigel et al., 1992; Coen et al., 1990). The phylogenetic tree showed that the topology of these genes seems to be concordant with the topology of the species phylogeny and suggests that *CmLFY* is an angiosperm ortholog of *FLO*/*LFY*-like genes (Figure 2b).

The expression of *CmLFY* was only found in inflorescences, but not in tissues such as fruits, stems and tender leaves (Figure 3). It supports that the *CmLFY* is a functional homologous gene of chestnut. In the species such as apple, kiwifruit and grape, the expression levels of their FLO/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 high levels of *CmLFY* expression in inflorescence suggests that *CmLFY* plays a role during chestnut reproductive development as it has been suggested for most FLO/LFY-like genes studied.

To further demonstrate the function of *CmLFY*, we have produced transgenic *Arabidopsis* plants expressing *CmLFY.* They flowered earlier and had a shorter inflorescence and reduced number of rosette leaves compared with the controls (Figure 3). Based on the result that several transgenic *Arabidopsis* with 35S::*CmLFY* flowered earlier than the controls, the expression of *CmLFY* should have some relationship with the early flowering phenotypes, although, the mechanism of flower-bud formation in chestnut might be different from that in *Arabidopsis*. Of course, to be more certain that the *CmLFY* gene functions similarly to *LFY*, *LFY* mutant-rescue experiments will be required. The *CmLFY* gene was not expressed strongly in transformants showing early flowering relative to those that were wild type in appearance, which suggests that the severity of phenotype in transformants does not depend solely on the level of gene expression in this case, although the exact cause is still unknown. Some reports are available that describe transgenic *Arabidopsis* plants expressing *LFY* homologs from other plant species. For example, *Arabidopsis* transgenic lines expressing *NEEDLY* from Radiata pine and *ELF1* from Eucalyptus showed early flowering (Mouradov et al., 1998; Southerton et al., 1998). Until now, however, our ability to manipulate fruit/nut tree strains through genetics has been limited by the extended maturation period of these plants. Both taking long time to flowering and the fact that, no characterized flowering mutants have been described in this genus make genetic studies in chestnut more difficult. Nevertheless, advances in the establishment of transformation protocols for

chestnut may allow us to use reverse genetic approaches and to define more clearly the role played by *CmLFY* in the reproductive development.

The breeding of fruit trees of chestnut 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 limit factors of chestnut breeding is the long juvenile phase of at last several years. It had been found that transgenic approaches of LFY introducing could reduce the juvenile phase of *Populus* and the transgenic poplar flowered in 5 months after regeneration (Weigel and Nilsson, 1995). Therefore, probably we can anticipate that these techniques are likely applicable to fruit trees of chestnut in future years and believed that, the transgenic approach would be a useful breeding strategy for reducing the time required for generation among woody plants. However, the efficiency of producing early flowering transgenic lines with *Arabidopsis LFY* gene seems to be low in woody plants in some case (Pena et al., 2001). This suggests that regulatory genes such as *LFY* do not always function beyond species as well as expected. Since environmental and genetic factors controlling flower development in chestnut have not been made clear so far, the *CmLFY* gene could at least, be one of the tools available for studying the mechanism of flower development in tree fruits such as the chestnut.

ACKNOWLEDGMENTS

This research was financially supported by the Faculty of Agronomy and Biotechnology (SJCX09012 to T Liu) and the National Natural Science Foundation of China (NSFC, 30800062 to XX Li).

REFERENCES

Anthony RG, James PE, Jordan BR (1993). Cloning and sequence analysis of a *FLO/LFY* homologue isolated from cauliflower (*Brassica oleracea L. var. botrytis*). Plant Mol. Biol. 22: 1163-1166.

Carmona MJ, Cubas P, Martinez-Zapater JM (2002). *VFL*, the grapevine *FLORICAULA/LEAFY* ortholog, is expressed in meristematic regions independently of their fate. Plant Physiol. 130: 68-77.

Chenchik A, Diachenko L, Moqadam F, Tarabykin V, Lukyanov S, Siebert PD (1996). Full-length cDNA cloning and determination of 5′ and 3′ ends by amplification of adaptor-ligated cDNA. Biotechniques, 21: 526-534.

Clough SJ, Bent AF (1998). Floral dip: a simplified method for *agrobacterium*-mediated transformation of *arabidopsis thaliana*. Plant J. 16: 735-743.

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

Frohlich MW, Parker DS (2000). The mostly male theory of flower evolutionary origins: from genes to fossils. Syst. Bot. 25: 155-171.

Kelly AJ, Bonnlander MB, Meeks-Wagner DR (1995). *NFL*, the tobacco homolog of *FLORICAULA* and *LEAFY*, is transcriptionally expressed in both vegetative and floral meristems. Plant Cell. 7: 225-234.

Kotoda N, Wada M, Kusaba S, Kano-Murakami Y, Masuda T, Soejima J (2002). Overexpression of *MdMADS5*, an *APETALA1*-like gene of apple, causes early flowering in transgenic *arabidopsis*. Plant Sci. 162: 679-687.

Kyozuka J, Konishi S, Nemoto K, Izawa T, Shimamoto K (1998). Down-regulation of *RFL*, the *FLO/LFY* homolog of rice, accompanied with panicle branch initiation. Proc. Natl. Acad. Sci USA, 95: 1979-1982.

Liu T, Li XX, Xu PZ, Zhou ZK, Hu YQ (2006). Cloning and expression of a MADS-box Protein Gene (*CmMADS3*) from *Castanea mollissima.* Acta Bot. Yunnan, 28: 295-299.

Ma H (1998). To be, or not to be, a flower-control of floral meristem identity. Tig. 14: 26-32.

Marcelo CD, Adriana PMR (2006). The tropical cedar tree (*Cedrela fissilis Vell. Meliaceae*) homolog of the arabidopsis *LEAFY* gene is expressed in reproductive tissues and can complement arabidopsis leafy mutants. Planta. 223: 306-314.

Molinero-Rosales N, Jamilena M, Zurita S, Gomez P, Capel J, Lozano R (1999). *FALSIFLORA*, the tomato orthologue of *FLORICAULA* and *LEAFY*, controls flowering time and floral meristem identity. Plant J. 20: 685-693.

Mouradov A, Glassick T, Hamdorf B, Murphy L, Fowler B, Marla S, Teasdale RD (1998). *NEEDLY*, a *pinus radiata* ortholog of *FLORICAULA/LEAFY* genes, expressed in both reproductive and vegetative meristems. Proc Natl Acad. Sci. USA, 95: 6537-6542.

Pena L, Martin-Trillo M, Juarez J, Pina JA, Navarro L, Martinea-Zapater JM (2001). Constitutive expression of arabidopsis *LEAFY* or *APETALA1* genes in citrus reduces their generation time. Nat Biotechnol. 19: 263-267.

Pidkowich MS, Klenz JE, Haughn GW (1999). The making of a flower: control of floral meristem identity in arabidopsis. Plant Sci. 4: 64-70.

Rottmann WH, Meilan R, Sheppard LA, Brunner AM, Skinner JS, Ma C, Cheng S, Jouanin L, Pilate G, Strauss SH (2000). Diverse effects of overexpression of *LEAFY* and *PTFL*, a poplar (*Populus*) homolog of *LEAFY/FLORICAULA*, in transgenic poplar and *arabidopsis*. Plant J. 22: 235-245.

Souer E, Krol A, Kloos D, Spelt C, Bliek M, Mol J, Koes R (1998). Genetic control of branching pattern and floral identity during *petunia* inflorescence development. Development, 125: 733-742.

Southerton SG, Strauss SH, Olive MR, Harcourt RL, Decroocq V, Zhu X, Llewellyn DJ, Peacock WJ, Denhis ES (1998). *Eucalyptus* has a functional equivalent of the *arabidopsis* floral meristem identity gene *LEAFY*. Plant Mol Biol. 37: 897-910.

Van-Larebeke N, Engler G, Holsters M, Elsacker S, Zaenen L, Schilperoort RA, Schell J (1974). Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature, 252: 169-170.

Walton EF, Podivinsky E, Wu RM (2001). Bimodal pattern of floral gene expression over the two seasons that kiwifruit flowers develop. Plant Physiol. 111: 396-404.

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

Weigel D, Nilsson O (1995). A developmental switch sufficient for flower initiation in diverse plants. Nature, 37: 495-500.

Zeng Y, Yang T (2002). RNA isolation from highly viscous samples rich in polyphenols and polysaccharides. Plant Mol. Biol. Rep. 20: 417a-417e.