Cloning and functional identification of the AcLFY gene in Allium cepa

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A B S T R A C T

Onion (Allium cepa L.) is one of the important vegetable crops in the world, usually with a two-year life cycle. The bulbs form in the first year after sowing, then bolting and flowering are induced by low temperature in the following year. Previous studies have shown that LEAFY gene is an inflorescence tissue specific gene, and that it is also the ultimate collection channel of all flowering pathway. In this study, using homologous gene cloning and reverse transcription-PCR (RT-PCR), we isolated an inflorescence meristem identity gene AcLFY cDNA, from onion. AcLFY contains a 1119 bp open reading frame, which encodes a putative protein of 372 amino acids, with ~70% homology to the daffodils LEAFY and >50% homology to LEAFY proteins from other higher plants. Fluorescence quantitative results showed that AcLFY gene has the highest expression level in inflorescence meristem during early bolting, and is still expressed in leaves after the formation of flower organs. Overexpression of AcLFY gene in Arabidopsis thaliana induced early bolting and flowering, whereas knockdown of the endogenous LEAFY gene by RNAi caused a significant delay in bolting. In addition, transgenic plants also exhibited significant morphological changes in rosette leaves, branches, and plant height.

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1. Introduction

Onion (Allium cepa L.) is one of the most important vegetables in the world, with a global annual production of more than 70 million tons. It is a typical biennial plant; usually in the first year the bulb swells into organ products under proper light cycle, and in the next year after the vernalization follows bolting and flowering to complete its life cycle. The bulb formation in onion determines its economic traits. Early bolting of onion will seriously affect the yield and quality. Therefore, studies on the mechanisms of bolting are of great significance to artificially manipulate the bolting and flowering. Studies with Arabidopsis and other flowering plants suggest that bolting and flowering in plants are induced by environmental factors such as light and temperature, and then through a series of signal transduction floral bud differentiation takes place under the synergistic effects of decision-making genes and many other genes, leading to the formation of flower organ [1]. Among many genes, the inflorescence meristem identity gene LEAFY is the key gene in flowering, which determines the direction of development of plants [2]. First found in Arabidopsis thaliana, LEAFY is a meristem specific gene that positively correlates with flower induction [3]. Through activating the expression of a downstream gene APELATA1 (AP1) to initiate the differentiation of flower [4,5], LEAFY forms a switch with AP1 to control the transition from vegetative growth to flowering period [4,6,7]. And after the formation of floral meristem, it continues to promote the expression of genes that determine flower organ traits, including AP3, PI, AG, SEP2 and SEP3 [8,9]. In regulation of other genes, LEAFY is also positively regulated by FLOWERING LOCUS (FT) and SUPPRESSOR OF OVEREXPRESS OF CO1 (SOC1) genes at the same time. Transgenic tobacco with the Arabidopsis LEAFY gene [10] shows an obvious phenomenon of early flowering, and in poplar [11] and other plants such as chrysanthemum, LEAFY gene also plays a role in regulating flowering time. Although onion is an important vegetable crop, the knowledge about its molecular biology has been advancing very slowly because of its huge genome and many repeat sequences, which is in contrast to the completion of genome sequencing of the majority of vegetable crops. In recent years, there have been a lot of studies on...
the regulation of onion flowering, but the structure and function of LEAFY gene remains undetermined.

Here we cloned a LEAFY homologous cDNA sequence, AcLFY (Genbank accession number: JX275962), from onion. Sequence analysis showed that AcLFY contains a 1119 bp open reading frame, encoding 372 amino acid residues. The putative amino acid sequence has typical structure characteristics of the LEAFY (FLO) family, with a proline rich region at the N-terminal, a leucine zipper structure, and a central acid region. Fluorescence quantitative analysis show that AcLFY gene has the highest expression in the inflorescence meristem of onion in early bolting, but only has trace expression in the flower organs. After the formation of the flower organs, expression of the gene can still be detected in the leaves. Transgenic experiments show that AcLFY gene can regulate the timing of plant bolting and flowering.

2. Materials and methods

2.1. Plant materials and growth conditions

The onion strain “1007” was used in this study. It was provided by Northeast Agricultural University research group of onion and garlic. The strain is a long day onion with yellow skin, superior traits, and high stability. The bulb of the strain was planted in the greenhouse NO.2 of horticultural experimental station of Northeast Agricultural University in November, 2012. Materials were taken during bolting and flowering in June, 2013. Early bolting is when the onion stems just spread out and the spathe begins to appear; bolting is when the stems are completely out while the spathe is not cracked and the flowers in the inflorescence are not open; flowering is the period when the spathe is cracking and small flowers appearing in the inflorescence.

Wild-type Columbia Arabidopsis was used for genetic transformation. Arabidopsis plants (1 per pot) were grown in 60 × 80 mm pots containing a mixture of soil and vermiculite (1:1) at 22 °C under a 16 h/8 h light/darkness regime with 70% relative humidity.

2.2. RNA extract and synthesis of the first strand cDNA

Total RNA was extracted using Trizol reagent (Invitrogen), from inflorescence meristem and leaves during bolting, flower buds, flower stalks, receptacles and blades during early bolting, and flowers, flower stalks, receptacles and leaves during blooming, respectively. The first strand cDNA was synthesized using M-MLV Reverse Transcriptase (MBI).

2.3. Cloning of AcLFY gene

The middle fragment of AcLFY cDNA was amplified by PCR using two homologous primers LFYP-F and LFYP-R, which were designed using Primer Premier 5.0 based on the LEAFY conserved sequences. Subsequently, four primers GSP3-1, GSP3-2, GSP5-1, and GSP5-2 were designed to amplify the 5'- and 3'-end of AcLFY cDNA using 3'-Full RACE CoreSet Ver. 2.0 (TaKaRa) and 3'-Full RACE Kit (TaKaRa). The PCR products were cloned into pEASY- T3 vector, and positive colonies were identified by PCR and restriction enzyme digestion, and then subjected to sequencing. Full-length AcLFY cDNA was amplified with primers AcLFyORF1 and AcLFyORF2 using TaKaRa LA Taq DNA polymerase (Supplemental Table 1).

2.4. Analysis of AcLFY gene expression by qRT-PCR

A pair of specific primers AcLFY-F and AcLFY-R were used to amplify a 300-bp fragment of AcLFY. Both primers were designed based on a non-conservative region to avoid possible amplification of other genes of the LEAFY family. The relative expression levels of AcLFY were determined using the Bio-Rad IQ5 fluorescence quantitative PCR according to the SYBR® Premix Ex Taq™ II instructions. AcActin (GU570135) was used as an internal control [12].

2.5. Bioinformatics analysis of AcLFY

Homology comparison between AcLFY and its homologous genes was done with DNAMAN software. MEGA5.1 software was used to build phylogenetic tree of the homologous sequences. The analysis on the structure features and the physical and chemical properties of AcLFY was performed with SOPMA, TMHHM, SignalP 4.0 and NetPhos 2.0 programs on the CBS and NPS servers.

2.6. Construction of overexpression plasmid p3301-AcLFY and RNAi plasmid pTCK303-AcLFY

Using restriction endonucleases HindIII, BamHI, PstI, SacI, and EcoRI, the SSs promoter, AcLFY and T-nos fragments, were cloned into pCAMBIA3301 to construct the plant expression vector pCAM3301-AcLFY. For RNAi plasmid pTCK303-AcLFY, both the forward and reverse sequences of a 405-bp fragment of a conserved region in Arabidopsis LEAFY was amplified from the plasmid PT-AcLFY, respectively, and inserted into the carrier pTCK303. A diagram of the expression vectors is shown in Fig. S1.

2.7. Genetic transformation and identification of AcLFY

The plasmids were introduced into the root carcinoma agrobacterium EHA105 by freezing-thawing method. Inflorescence impregnation was used to transform A. thaliana. Transformed plants were harvested individually, and the seeds from transgenic plants overexpressing AcLFY were screened on MS medium supplemented with 5 mg/L of glufosinate (PPT). The seeds from transgenic plants of RNAi were screened on MS medium with supplemented 50 mg/L of hygromycin (Hyg B). After the screening, positive transgenic A. thaliana plants were identified by PCR. Three independent transgenic T2 lines of LFY-over-expression survived on MS medium with 5 mg/L of PPT, and three independent transgenic T2 lines of RNAi survived on MS medium with 50 mg/L of Hyg B. All these survived transgenic lines are homozygotes, and the seeds from T3 generation were used for the data collection.

3. Result

3.1. Bioinformatics analysis of AcLFY

Using a cDNA library synthesized from inflorescence meristem total RNA and a pair of degenerate primers, LFYP-F and LFYP-R, we first amplified a 324-bp fragment of AcLFY middle region by PCR. Based on the sequence of this fragment, we next designed primers for RACE (rapid amplification of cDNA ends) experiments. We obtained a 700-bp and a 600-bp fragments from 3’- and 5’-RACE, respectively. Full-length AcLFY cDNA was then amplified using two specific primers AcLFyORF1 and AcLFyORF2. The AcLFY cDNA, which contains an 1119-bp open reading frame, was registered in GenBank (access number: JX275962). NCBI database retrieval results show that AcLFY belongs to the FLO – LFY gene superfamily. AcLFY cDNA encodes a putative protein of 372 amino acids with an obvious proline-rich region at the N-terminal: 12 out of the total 22 proline residues are located within the first 33 amino acid residues. A leucine zipper structure with the “XEL…XTI…XEL…” motif is found between the 55th and the 86th residues, and an acidic region with continuously negatively charged glutamic acid and aspartic acid.
is found between the 68th and the 73rd resides. In addition, AcLFY protein also contains a lysine-rich region. The above structure features of AcLFY gene are characteristics of the FLO ~ LFY gene superfamily, suggesting that AcLFY is a typical inflorescence meristem gene of the FLO ~ LFY family.

Sequence analysis showed that AcLFY has 97% homology with shallot, 74.5% homology with Chinese narcissus, 70.8% homology with A. thaliana, mango, and walnut, suggesting that LEAFY genes are highly conserved between different plants. A common lysine-rich region was found in LEAFY proteins from monocotyledon plants but not in LEAFY proteins from most dicotyledonous plants (Fig. 1).

Using MEGA5.1 software, a bootstrap neighbor joining (NJ) tree was built between onion AcLFY amino acid sequences and 20 other LEAFY amino acid sequences from higher plants (Fig. 2). The results show that LEAFY amino acid sequences from onions and leeks have 97% homology. Besides that, AcLFY is also close to LEAFY proteins from Chinese narcissus and Tricyrtis hirta. These four plants are liliaceous plant, and are in parallel branches with other plants based on the homology of LEAFY proteins, suggesting that LEAFY is highly conserved between phylogenetically distant plants. The tree of system development also clearly shows that angiosperms and gymnosperms belong to two parallel branches in terms of LEAFY homology, suggesting that plants with LEAFY genes appeared early in evolution.

3.2. Analysis of spatial and temporal expression of AcLFY

The expression of AcLFY during reproductive growth was examined by real-time fluorescence quantitative PCR. Our results showed that the expression of AcLFY was highest in inflorescence meristem in early bolting, and trace AcLFY expression can be detected in receptacle, stalk and flower in bolting and flowering. It is worth mentioning that AcLFY was highly expressed in leaves throughout the reproductive growth period (Fig. 3).

3.3. Phenotypic analysis of AcLFY transgenic Arabidopsis

AcLFY transgenic Arabidopsis plants were significantly different from wild type (WT) plant in flowering time and morphology. The bolting of transgenic plants overexpressing AcLFY gene was 10 days earlier than that of the WT plants (Fig. 4B). Morphologically, overexpression of AcLFY gene reduced the number of rosette leaves by 10 (Fig. 4A, B), with no significant change in leaf area. The overexpression transgenic plant also showed a diminishing growth potential, slender stems, increased lateral branches capable of producing seeds with more pods (Fig. 4A), and were 2 cm taller than WT plants (Fig. 4B).

Compared with the WT, bolting of Arabidopsis plants with RNAi was postponed by about 50 days. The RNAi plants had 20 more leaves than the WT, with significant differences in leaf length, width and thickness (Fig. 4B). Leaf area in RNAi plants was 1.1 cm² larger than that in WT plants (Fig. 4B). After bolting, the performance of RNAi plants was more like vegetative growth. RNAi plants were 3 cm taller than the WT plants. Compared with the WT, RNAi plants had thicker scapes and collateral stems, less inflorescence with shorter and smaller pods. The pods of inflorescence in RNAi plants were easy to fall off during development, and most of the

![Fig. 1. Alignment of LEAFY amino acid sequences from different plants. AcLFY: Allium cepa, JX275962; AF LFY: Allium fistulosum, AGU12799.1; JLFY: Juglans regia, ADL61165.1; LEAFY: Arabidopsis thaliana, NP290993.1; MiLFY: Mangifera indica, ADX97193.1; NF LFY: Narcissus tazetta var. Chinensis, ADR78683.1; ThLFY: Tricyrtis hirta, BAN62509.1; VnLFY: Vitis vinifera, AAM40141.1.](image-url)
stage of leaf, plants had no leaf stalks after bolting (Fig. 4A). These observations show that plant RNAi still had many small leaves after bolting, whereas WT remaining pods were empty. Some RNAi plants even had no pods.

**Discussion**

In this study, we isolated a LEAFY homologous gene AcLFY from onion, and examined the expression and function of this gene in onion bolting and flowering. Previous studies have shown that LEAFY genes have distinct expression patterns in different plants. For example, in A. thaliana LEAFY gene is expressed throughout the whole cycle of floral meristem to activate specific genes in different parts of the flower [13,14]. It is still expressed at high level in the vegetative organs after flower development [4]. In rice, LEAFY gene is highly expressed in young spike, but not in mature leaves [15]. In garlic, galFY is strongly expressed in differentiating flower primordia, but is down-regulated when most flowers are formed [16]. In orchid, the PhapLFY of LEAFY homologous genes have different expression patterns from LEAFY genes in above plants: it is abnormally expressed in the flower organs, but is highly expressed in leaves in the vegetative growth stage. However, when the leaves grow old, its expression is greatly reduced, suggesting that its function is associated with leaf development process [17]. The orthologous LEAFY genes have been found to be expressed in leaf primordium in a variety of perennial woody plants, including two kinds of eucalyptus [18], the giant eucalyptus [18], poplar [19], and kiwi fruit [20]. The expression of LEAFY genes in leaves is strongly expressed in differentiating flower primordium, but is down-regulated when most flowers are formed [16]. In orchid, the PhapLFY of LEAFY homologous genes have different expression patterns from LEAFY genes in above plants: it is abnormally expressed in the flower organs, but is highly expressed in leaves in the vegetative growth stage. However, when the leaves grow old, its expression is greatly reduced, suggesting that its function is associated with leaf development process [17]. The orthologous LEAFY genes have been found to be expressed in leaf primordium in a variety of perennial woody plants, including two kinds of eucalyptus [18], the giant eucalyptus [18], poplar [19], and kiwi fruit [20]. The expression of LEAFY genes in leaves is strongly expressed in differentiating flower primordium, but is down-regulated when most flowers are formed [16]. In orchid, the PhapLFY of LEAFY homologous genes have different expression patterns from LEAFY genes in above plants: it is abnormally expressed in the flower organs, but is highly expressed in leaves in the vegetative growth stage. However, when the leaves grow old, its expression is greatly reduced, suggesting that its function is associated with leaf development process [17]. The orthologous LEAFY genes have been found to be expressed in leaf primordium in a variety of perennial woody plants, including two kinds of eucalyptus [18], the giant eucalyptus [18], poplar [19], and kiwi fruit [20]. The expression of LEAFY genes in leaves is strongly expressed in differentiating flower primordium, but is down-regulated when most flowers are formed [16]. In orchid, the PhapLFY of LEAFY homologous genes have different expression patterns from LEAFY genes in above plants: it is abnormally expressed in the flower organs, but is highly expressed in leaves in the vegetative growth stage. However, when the leaves grow old, its expression is greatly reduced, suggesting that its function is associated with leaf development process [17].

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other flowering related genes to inhibit the growth and development of meristem cells and the formation of floral meristem. Overexpression of AcLFY gene in A. thaliana caused early bolting and flowering. In addition, many reports have proposed that LEAFY and its homologous genes are not only related to bolting and flowering, but are also multi-functional and associated with various forms of morphogenesis [32,14]. In our study, AcLFY not only greatly influenced the bolting and flowering in transgenic Arabidopsis, but also affected the growth performance of the transgenic plants. Knockdown of LEAFY in A. thaliana using RNAi led to no or very late bolting, and even if bolting does occur, morphological performance of inflorescence was similar to that of the vegetation, indicative of a tendency of reversion from reproductive growth to vegetative growth. These observations suggest that AcLFY may be necessary for the normal development of the leaves in onion.

Gene knockout is the most direct and compelling approach to study gene function. In this study, we cloned AcLFY gene from onion, and investigated its function in transgenic A. thaliana. We found that AcLFY overexpression in Arabidopsis induced early bolting and flowering, and caused a series of changes in

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**Fig. 4.** Morphological changes in transgenic Arabidopsis plants with overexpression of AcLFY or RNAi. A: a) Rosette leaves of wild type (left), over-expression (middle), and RNAi (right) Arabidopsis during vegetative growth period; b) Wild type, over-expression, RNAi Arabidopsis a week after bolting; c) Leaves from wild type (left), over-expression (middle), and RNAi (right) Arabidopsis; d) Pod status of wild type (left), over-expression (middle), and RNAi (right) Arabidopsis. B: Statistical analysis of leaf number, leaf area, bolting time and height in transgenic Arabidopsis plants.
morphology. Ideally, we should knock out this gene from onion genome to better understand its function. However, it is extremely challenging to establish genetic transformation system in onion. We were unable to obtain transgenic onion plants with multiple tries. Besides LEAFY gene, flowering regulation in plants involves many environmental and intrinsic factors. Our future research will focus on the interaction between LEAFY and FT, CO, and SOC1, and the regulation of bolting and flowering in onion.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.04.022.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.04.022.

References
