Scientia Horticulturae 124 (2010) 482–489

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Characterization and expression analysis of *PhalLFY*, a homologue in *Phalaenopsis* of *FLORICAULA*/*LEAFY* genes

Jian-Xia Zhang [a,b,1], Kun-Lin Wu [a,1], Song-Jun Zeng [a], Jun Duan [a,*], Li-Ning Tian [c]

[a] Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Leyiju, Guangzhou 510560, China
[b] Graduate School of the Chinese Academy of Sciences, Beijing 100039, China
[c] Southern Crop Protection and Food Research Centre Agriculture and Agri-Food Canada, London, Ontario N5V 4T3, Canada

ARTICLE INFO

Article history:
Received 25 November 2009
Received in revised form 4 February 2010
Accepted 4 February 2010

Keywords:
PhalLFY
Cloning
Flower development
Flowering
Expression
Phalaenopsis hybrida

ABSTRACT

FLORICAULA/*LEAFY* (*FLO*/*LFY*) homologues are floral meristem identifying genes and play a key role in flower development among diverse species. *Phalaenopsis* is one of the world's most popular flowers. To understand the molecular mechanism of the reproduction process, *PhalLFY*, a *FLO*/*LFY* homologue gene was cloned from *Phalaenopsis hybrida* (cv.wedding promenade) and its expression pattern was analysed during vegetative as well as reproductive development. The full cDNA of *PhalLFY* was 1655 bp in length with an open reading frame (ORF) of 1422 bp encoding 424 amino acids. The analysis based on amino acid sequence alignment showed that *PhalLFY* shared a high degree of similarity to *LFY*-like (68%) from *Anacamptis*, *LFY*-like (61%) from *Hyacinthus*, *RFL* (60%) from *Oryza sativa* and *LFY* (54%) from *Arabidopsis*. A transient expression assay using PhalLFY/GFP fusion protein showed that *PhalLFY* was nucleus-localized protein. Semi-quantitative RT-PCR analysis and *in situ* hybridization results showed that *PhalLFY* was expressed both in the vegetative organs as well as the reproductive organs. Its transcripts accumulated at higher levels in the stem during the transition from vegetative to reproductive growth, especially in the inflorescence meristems, floral meristems, floral primordia and all floral organs during the floral developmental stage of *P. hybrida*. Moreover, *PhalLFY* transcript was lower in the young phase than in the mature phase. These results suggest that *PhalLFY* is a *FLO*/*LFY* homologue probably involved in the control of *Phalaenopsis* reproductive development.

1. Introduction

Transition from vegetative phase to reproductive phase is the first step in plant flower development. Vegetative meristem first switches to inflorescence meristem which then converts into floral meristem. Studies on *Arabidopsis* have indicated that the floral meristem is initiated by a set of floral meristem identity genes that include *LEAFY*(*LFY*), *APETALA1*(*AP1*), *CAULIFLOWER*(*CAL*), *APETALA2*(*AP2*), and *UNUSUAL FLORAL ORGANS*(*UFO*) (Mandel et al., 1992; Jofuku et al., 1994; Kempin et al., 1995; Yanofsky, 1995). Among them, *LFY* plays a central role on flower meristem identity (Schultz and Haughn, 1991; Weigel et al., 1992; Pidkowich et al., 1999). At the same time, *LFY* also plays a role in regulating the inflorescence shape and controlling the flowering time (Coen et al., 1990; Nilsson et al., 1998).

LFY homologues have been cloned from a number of species. All of these homologues exist as single or low copy number in genomes and contain two introns in conserved positions (Ma et al., 2005). The protein sequences are most highly conserved in the C-terminal region, whereas the N-terminus is more variable (Shu et al., 2000; Wada et al., 2002). Most of the *FLO*/*LFY* homologues share proline-rich region, while it is absent in the homologues of eucalyptus and *Malus domestica* (Mouradov et al., 1998; Southerton et al., 1998; Dornelas et al., 2004).

The phenotype of *LFY*/*FLO* mutants in dicotyledonous plants share certain similarity. For example, *alf* mutants of petunia led to aberrant leaves and flowers (Souer et al., 1998), tomato *fa* mutations altered the development of the inflorescence, resulting in the replacement of flowers by secondary shoots and a late-flowering phenotype (Molinero-Rosales et al., 1999), and *zfl1* and *zfl2* from maize brought a disruption of floral organ identity and defects in inflorescence architecture and in the vegetative to reproductive phase transition (Bomblies et al., 2003). These results indicate that *LFY*/*FLO* homologues play a universal role in the specification of flower organs and the vegetative to reproductive phase transition. However, the phenotype of *LFY*/*FLO* homologues mutations is different in some species. For example, mutation in the *Arabidopsis LFY* gene resulted in a partial conversion of flower into inflorescence (Weigel et al., 1992), whereas *flo* mutant in the *Antirrhinum* showed a complete conversion of flowers into inflorescence shoots (Coen et al., 1990). The inactivation of *PEAFLO*

* Corresponding author. Tel.: +86 20 37252978; fax: +86 20 37252978.
E-mail address: duanj@scib.ac.cn (J. Duan).
[1] These authors made equal contribution to the paper.

0304-4238/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.scienta.2010.02.004

in the pea mutant (uni) not only caused a floral phenotype, but also changed the morphology of pea leaves (Hofer et al., 1997). Likewise, overexpression of *LFY* could promote flower initiation and development from shoot apical and axillary meristems and cause early flowering (Blazquez et al., 1997; Wada et al., 2002; Kim et al., 2008). Moreover, overexpression of *LFY* in citrus produced fertile flowers and fruits in the first year and notably shortened their juvenile phase (Pena et al., 2001).

Phalaenopsis belongs to the orchid family which is one of the most diverse plant families. *Phalaenopsis* is well known for ornate flower shapes and in the global orchid trade dominance. In China, *Phalaenopsis* is commonly purchased in the New Year. It must have flowers to be marketable for the holiday. Despite the production and value as well as its specific marketed dates, little is known about the molecular mechanisms of floral development of Phalaenopsis. Based on the previous studies in other plants, *LFY* may be an important gene involving in flower development in *Phalaenopsis*. To elucidate the structure and function of *LFY* gene in *Phalaenopsis* and to understand the molecular mechanism of genes regulating the *Phalaenopsis* floral phase transition, we have isolated a putative *FLO*/*LFY*-like homologue gene in *Phalaenopsis*. We analyzed gene structure and expression with respect to plant floral development. The study will contribute to revealing of the mechanisms of flowering of this ornamental plant.

2. Materials and methods

2.1. Plant materials

In this study, we used *P. hybrida* (cv.wedding promenade). The plants were grown in the greenhouse with standard conditions. Samples of leaves, roots, stems, pedicels, inflorescence, buds and each floral organ (inner and outer sepals, petals, lips, polliniums, floral shoot and columns) were collected and dissected. The samples were frozen in liquid nitrogen immediately after collection and stored at −80 °C.

2.2. cDNA cloning and sequencing of LFY/FLO homologue from Phalaenopsis

Total RNA was isolated from inflorescence tissues using TRIzol® reagent (Invitrogen) and RT-PCR was performed using the degenerate forward primer 5′ CGGAYATIAAYAARCCIAARATGMGICAYTA 3′ and reverse primer 5′ CGACGTGICKIARIYKIGTIGGIACRTACCA 3′ (Frohlich and Meyerowitz, 1997). The amplified cDNA fragment was independently cloned at least twice into pGM-T Easy vector (Promega) and sequenced. After sequencing, the sequence was submitted to GenBank to identify similar genes. To obtain the full-length cDNA sequence of *PhalLFY*, a set of specific primers were designed for the 5′ and 3′ RACEs (Rapid Amplification of cDNA Ends) by using the SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). These primers were: PF1: 5′ TACAAGCCCCTCGTCGCCATCT 3′; PF2: 5′ GACATCGACGCCGTCTTCA 3′; PR1: 5′ GGCAGAGCTGACG-GAGTTTGGTG 3′; PR2: 5′ GGACGTAGTGTCGCATCTTAGGCTTGTT 3′. The amplified sequences were cloned and sequenced. The full length cDNA containing ORF for *PhalLFY* was obtained by PCR with forward primer (5′ AATGGATCCATGGACCCAAGCGACGCCTTC 3′) and reverse primer (5′ CATGGATCCAAGCATGGAAGGAGGGATTCC 3′). The primers contained the generated BamHI recognition site (GGATCC) to facilitate the cloning of the cDNAs.

2.3. Phylogenetic analysis

Phylogenetic comparisons of amino acid sequences of different *LFY*/*FLO* homologues were obtained from GenBank (http://www.ncbi.nlm.nih.gov/) and aligned with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). Phylogenetic trees based on the complete sequences were generated using MEGA4 and constructed by the neighbor joining (NJ) method. Bootstrap values were derived from 1000 replicate runs.

2.4. Semi-quantitative RT-PCR

Total RNA was extracted from leaves, roots, stems, pedicels, buds and floral organs using TRIzol® from Invitrogen following manufacturer's protocol. Each RNA sample was treated with RNase-Free DNase (Promega) following manufacturer's protocol in an effort to remove any residual genomic DNA (gDNA). DNase treated RNA was subjected to reverse transcriptase reactions using oligo-dT primer and PrimeScript™ Reverse Transcriptase (Takara) according to manufacturer's protocol.The gene-specific primers used in RT-PCR were 5′ ATCCCCGCCTCTCAATCT 3′and 5′ TCTCATCCGCACCAGTTC 3′. As a control, the cDNA sequence of the actin gene was amplified by using the two primers 5′ TGGAAACTGCCAAGACG 3′ and 5′ GCAGCGAAGATTCAAAA 3′. The following thermocycling conditions were employed: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 51 °C for 45 s, and 72 °C for 1 min; final extension at 72 °C for 10 min. The amplified products were separated on a 1.5% agarose gel, visualised and photographed.

2.5. Construction of 35S:GFP::PhalLFY fusion proteins

The PCR product of the full-length cDNA for *PhalLFY* was first cloned in pGM T-vector and then digested with BamHI. It was further cloned in the pUC/GFP to produce a C-terminal GFP tagged fusion protein. The pUC/GFP vector was made by ligating the 35S promoter from pBIm and GFP from pBI101-GFP into pUC18 in frame. After sequence confirmation by PCR, these constructed vectors were bombarded into onion epidermal cells. Then the epidermal cells were observed under the Axioplan2 fluorescence microscopy with 488 nm wavelength.

2.6. RNA in situ hybridization

Inflorescences, floral buds, floral organs (sepals, petals, lips, columns, pollinarium and floral shoots), roots, leaves, pedicels and ovaries were collected from *Phalaenopsis* and fixed in buffer containing 4% paraformaldehyde, 0.25% glutaraldehyde, and 50 mM sodium phosphate (pH 7.2) at 4 °C for 24 h. They were then dehydrated by ethanol dilution series, after which the ethanol was replaced by 100% butanol. The samples were subsequently embedded in paraffin (Sigma). Paraffin-embedded tissues were cut into 8-μm thick sections using a rotary microtome and were placed on glass slides coated with polylysine hydrobromide.

Digoxigenin (DIG)-labeled antisense was transcribed from Sph I-digested plasmid DNA in which the 1000 bp fragment of *PhalLFY* was cloned in pGM-T using Sp6 RNA polymerase, and the sense RNA probes as controls were transcribed from Spe I-digested plasmid DNA using T7 RNA polymerase. The tissue sectioning was done as described by Zhang et al. (2005).

3. Results

3.1. Cloning and sequence analysis of PhalLFY

The full cDNA was 1655 bp in length with an ORF of 1422 bp encoding 474 amino acids. The analysis using ClustalW2 based amino acid sequence alignment (Fig. 1) showed *PhalLFY* shared a high identity with *LFY* (68% and 67%) from *Anacamptis* and *Orchis*, respectively. It shared identity with *LFY*-like (61%) from *Hyacinthus*, *RFL* (60%) from *Oryza*, *LFY1* (59%) from *Lilium*, *FLO* (59%) from *Antirrhinum* and *LFY* (54%) from *Arabidopsis*. The amino acid