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Full Length Research Paper

Cloning and expression analysis of a partial *LEAFY* homologue from pineapple (*Ananas comosus* (L.) Merr.)

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***LEAFY*-like genes are crucial regulators of flowering in angiosperms. A partial homologue of *LEAFY*, designated as *AcLFY* (Genebank accession no HQ433335), was isolated from pineapple (*Ananas comosus* L. cv. Comte de Paris) by PCR. The conserved cDNA fragment of *AcLFY* is 256 bp in length and contained an open reading frame of 248 bp, which encodes 82 amino acids protein. The amino acid alignment showed that *AcLFY* had a high identity with the related fragment of *PhalLFY* in *Phalaenopsis* hybrid cultivar (91%), *LFY* in Oncidium (90%) and *OrcLFY* in *Orchis italica* (88%). RT-PCR analysis showed that the *AcLFY* could be expressed constitutionally. Moreover, it was expressed in the flesh of young fruit and bract at low levels, and was highly expressed in the fruit, stem, petal and sepal.**

Key words: Pineapple, cloning, expression, RT-PCR, *LEAFY*.

INTRODUCTION

Pineapple (*Ananas comosus* (L.) Merr.) was domesticated long before its first historic mention by Christophorus Columbus in 1493 (Morrison et al., 1973). It is a biennial tropical monocot herb of *Bromeliaceae* and is one of the commercially important tropical fruits constituting a major export item for some countries (Duval et al., 2001). It is the third most important fruit in the tropics and subtropics, after banana and citrus (Rohrbach et al., 2002), and fruits can be harvested in most months of the year. In 2009, pineapple world production reached 18.4 million tons and the production was 1.45 million tons in China (FAOSTAT, 2011).

Nutritionally, freshly harvested pineapple is an inexpensive source of vitamins A, B and C, calcium, phosphorus and iron. The fruit is used for canning and in the preparation of juice, jam, jelly and crystallized glaze fruit. The leaves, stem and fruits of the pineapple plant contain *bromelain* which is a mixture of industrially imp ortant proteolytic enzymes. That is the reason why several research groups are developing fundamental and applied studies to create new cultivars with better agronomic performance.

Environmental induction of inflorescence development that occurs prior to the scheduled forcing is a sporadic but widespread and important problem in pineapple-producing regions because the precocious flowering results in fruits that are either too small to be marketable or too few in number, which makes harvesting unprofitable (Bartholomew and Mal6zieux, 1994; Scott et al., 1993). Flower induction is a good way to make pineapple flower at the same time, however, some species are difficult to be used for force flowering.

Conventional breeding to develop improved pineapple varieties has proved to be difficult due to the high level of genomic heterozygosity and apparent genome instability (Kato et al., 2004). Moreover, it requires several generations of backcrossing which is too cumbersome and time consuming. Genetic engineering has a great potential to improve the horticultural characteristics of pineapple varieties by introducing very specific traits without altering other agronomic attributes. One of the

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most important traits of interest is the control of flowering. *LEAFY* (*LFY*) plays an important role in the reproductive transition and controls flower spatial patterning by inducing the expression of the ABC floral organ identity genes（Liu et al., 2009). The conserved helix-turn-helix structure of *LFY* presumes that this gene is a universal integrator of the external and internal signals that promote the induction of flowering and maintain the identity of floral meristems (Blázquez et al., 2000; Araki et al., 2001; Boss et al., 2004; Hamès et al., 2008). Expression of *Arabidopsis LFY* gene in *Citrus* induces flowering within the first year. This shortening of the juvenile period is stable in seedlings (Pena et al., 2001). Expression of loquat *LFY* gene (*ejLFY*) in *Arabidopsis* resulted in early-flowering too (Liu et al., 2008). So far, *LFY* family genes have been isolated in many plant species, including snapdragon, rice, wheat, pea and so on (Coen et al., 1990; Dornelas and Rodriguez, 2006; Hofer et al., 1997; Kelly et al., 1995; Kyozuka et al., 1998; Shitsukawa et al., 2006). The identified *LFY* family genes are highly conserved in both their structures and functions (Ma et al., 2005). In this study, a partial *LFY*-like gene *(AcLFY*) was cloned and its expression in different tissues was analyzed too.

MATERIALS AND METHODS

Plants of pineapple cultivar 'Comte de Paris' were planted in the greenhouse at natural temperature and light. When the young panicle of the plant was 4 to 5 cm in height, the apical meristems, flesh of young fruit, fruit stem, bract, leaf, petal and sepal were cut and frozen in liquid nitrogen immediately and stored at -80℃ until use.

Cloning of the partial *AcLFY* cDNA

Total RNA was extracted from apical meristems using Column Plant RNAout 2.0 kit (Tiandz, Inc, China) following the manufacturer's instructions. First-strand cDNA was synthesized with M-MLV-reverse transcriptase from TAKARA according to the manufacturer's instructions.

To clone the conserved region of *AcLFY*, a pair of degenerate primers *LFY*-partial-F (5′CGGAYATIAAYAARCCIAARATGMGICAYTA3′) and *LFY*-partial-R (5′CGACGTGIC KIARIYKIGTIGGIACRTACCA3′) was designed according to the conserved regions of *LFY* homologues using the Primer Premier 5 software. The PCR amplification was carried out under the following condition: 1 cycle at 94℃ for 3 min; 32 cycles at 94℃ for 0.5 min, 52℃ for 1 min and 72℃ for 1 min and 1cycle at 72℃ for 10min. PCR products were isolated and cloned into pMD18-T vector (TAKARA, Japan) for sequence.

Sequence and phylogenetic analysis

Sequence chromatograms were examined and edited using Chromas version 2.23. The deduced *AcLFY* amino acid sequence was aligned with homologous sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov/ protein) using DNAMAN 6.0 program. Phylogenetic tree based on the amino acid sequences was constructed using DNAMAN 6.0. Gaps appearing in one sequence only were treated as nonconstant characters.

RT-PCR expression analysis

Total RNA was extracted from apical meristems, flesh of young fruit, fruit stem, bract, leaf, petal and sepal, and were reverse-transcribed. The resulting cDNAs from these tissues were then individually amplified using the primers LFYdl-F (5'-TGGTACCATGGACCCTGATGCACT TTC-3') and LFYdl-R (5'-GTGGATCCGAAACAAACTACCACAAACG-3'). The cycle condition was: 1 cycle at 94℃ for 3 min; 32 cycles at 94℃ for 0.5 min, 55℃ for 0.5 min and 72℃ for 1 min; a final extension at 72℃ for 10 min. *Ac18S rRNA* was used as the housekeeping gene. Ac18S rRNA-up was: ATGGTGGTGACGGGTGAC and *Ac18S rRNA*-dn was: AGACACTAAAGCGCCCG GTA. The PCR products were separated on a 1.0% agarose gel.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *AcLFY*

The conserved region of *LFY*-like gene was isolated from pineapple cDNA and designated as *AcLFY* (Genbank accession no. HQ433335). *AcLFY* is 256 bp in length and contained an open reading frame (ORF) of 248 bp, which encodes 82 amino acids protein. An alignment of the deduced amino acid sequence of *AcLFY (Ananas comosus), OrcLFY (Orchis italica,* BAC54955*), PhalLFY (Phalaenopsis* hybrid cultivar*,* AAY 40170*)* and *LFY* in Oncidium *(Oncidium hybrid cultivar,* ADL57240*)* was performed using the DNAMAN 6.0 program (Figure 1). The alignment showed that *AcLFY* had a high identity with the related fragment of *PhalLFY* (91%), *LFY* in Oncidium (90%) and *OrcLFY* (88%).

Phylogenetic analysis was conducted using the deduced pineapple *LFY* protein and the other amino acid sequences, and a rooted tree was generated by the maximum likelihood method with DNAMAN 6.0 program (Figure 2). The phylogenetic tree showed that *AcLFY* protein is more closely related to some monocot *LFY* proteins than to their dicot counterparts. *AcLFY* protein was clustered into the same subgroup 'Poales' with *ZlZFL1* in *Zea luxurians* (AAX35296), *SbFL* in *Sorghum bicolor* (AAX35325) and *RFL33* in *Oryza rufipogon* (AAY33631). The result suggests that *AcLFY* may potentially accelerate flowering.

RT-PCR analysis of *AcLFY* in pineapple tissues

Organ-specific expression patterns of *AcLFY* in pineapple were investigated by RT-PCR. Total RNA was extracted from the apical meristems, flesh of young fruit, fruit stem, bract, leaf, petal and sepal, and was reverse-transcribed. The resulting cDNAs were used as templates for analysis. The results showed that *AcLFY* was expressed in the flesh of the young fruit and bract at low levels, in fruit stem, petal and sepal at high levels, and in the apical

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Pineapple HQ433335          MNKPKMRHYVHCYALHCLDEEASNALRRAFKERGENVGA  39
Orchis BAC54955          KKVGASYINKPKMRHYVHCYALHVLDEDASNSLRRVFKERGENVGA 366
Phalaenopsis AAY40170    K.VGASYINKPKMRHYVHCYALHVLDEEASNALRRAFKERGENVGA 354
Oncidium ADL57240        K.VGASYINKPKMRHYVHCYALHVLDEEASNALRRAFKERGENVGA 298

Pineapple HQ433335       WRQACYNPLVAISARHGWDIDAVFNAHPRLSIWYVPTNLRHVE     82
Orchis BAC54955          WRLACYKPLVAISASHSFDIDAVFNAHPRLSIWYVPTKLRQLCHLA 412
Phalaenopsis AAY40170    WRLACYKPLVAISAAHSFDIDAVFNAHPRLSIWYVPTKLRQLCHLA 400
Oncidium ADL57240        WRLACYKPLVGISAAHAFDIDVVFNAHPRLSIWYVPTKLRQLCHLA 344
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Figure 1. Alignment of the deduced *AcLFY* (Genebank accession no. HQ433335) protein sequence from *Ananas comosus* with other angiosperm *LFY* -like proteins. Identical amino acid residues in this alignment are shaded in black, 75% or more similar amino acid residues are shaded in red and black dots denote gaps. The alignment was performed with the DNAMAN 6.0 program.

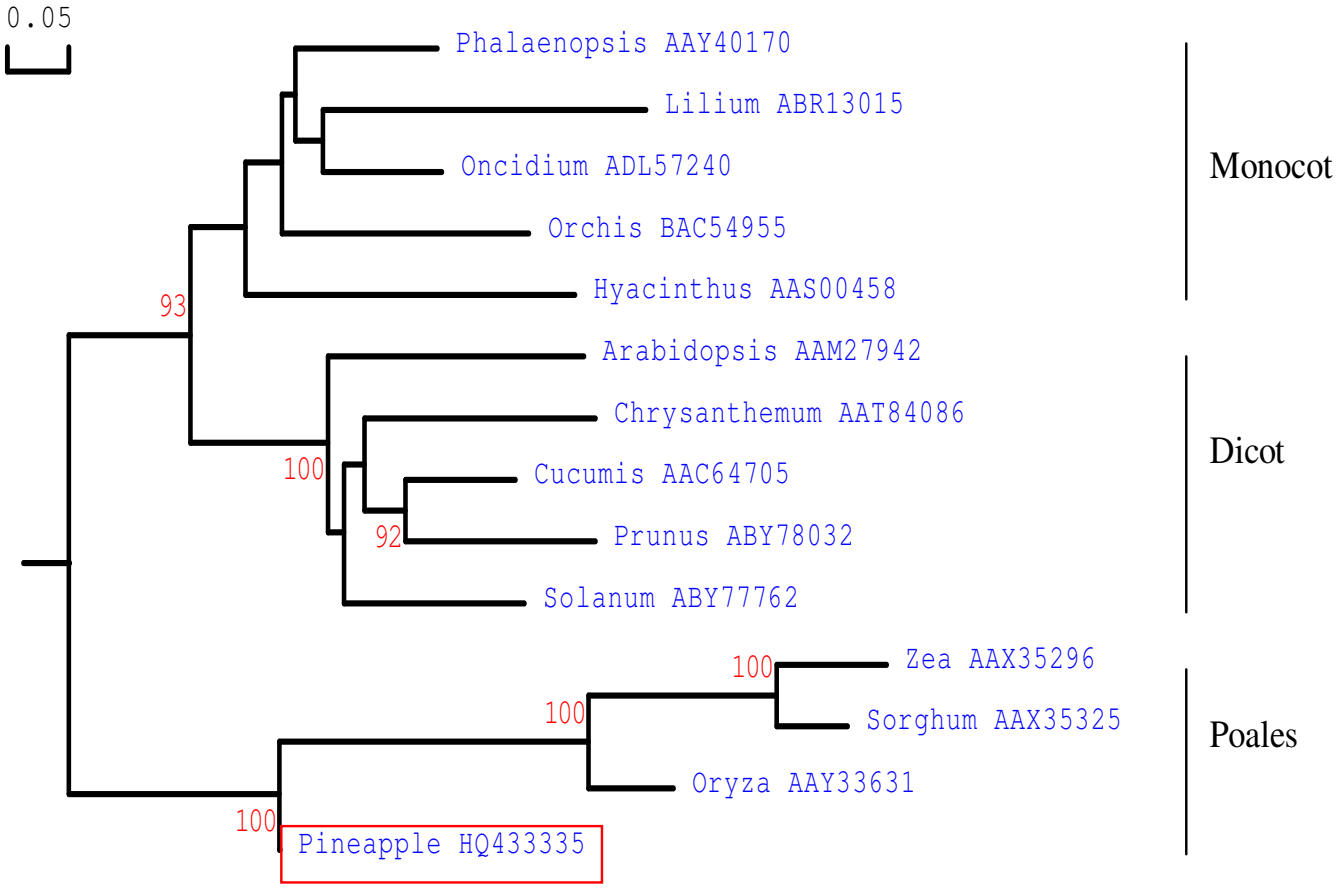

Figure 2. Phylogenetic analysis of the *LFY* homologous genes from different plant species. Protein sequences of the entire coding region were obtained from the NCBI database: AAX35296 (*Zea luxurians*), AAX35325 (*Sorghum bicolor*), AAY33631 (*Oryza rufipogon*), AAY40170 (*Phalaenopsis* hybrid cultivar), ABR13015 (*Lilium longiflorum*), ADL57240 (*Oncidium hybrid cultivar*), BAC54955 (*Orchis italica*), AAS00458 (*Hyacinthus orientalis*), AAM27942 (*Arabidopsis lyrata*), AAT84086 (*Chrysanthemum lavandulifolium*), AAC64705 (*Cucumis sativus*), ABY78032 (*Prunus persica*) and ABY77762 (*Solanum tuberosum*). The numbers next to the nodes give bootstrap values from 1000 replicates and the branch lengths are proportional to the distance.

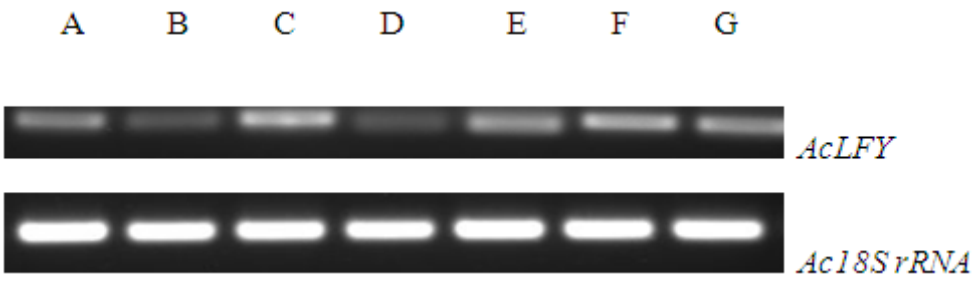

Figure 3. RT-PCR analysis of *AcLFY* expression pattern in different tissues. RNA transcripts from different tissues. A, apical meristems; B, flesh of young fruit; C, fruit stem; D, bract; E, leaf; F, petal; G, sepal. *Ac18S rRNA* transcripts were used as the control signals.

meristems and leaf at moderate levels (Figure 3). So, it was inferred that *AcLFY* gene could be expressed constitutionally.

The expression patterns of *LFY* homologues are diverse in different plants. In *Arabidopsis* plants, *LFY* expression is observed in lateral meristem initials through the whole vegetative phase and the inflorescence meristem (Weigel et al., 1992). *LiLFY1* in *Lilium longiflorum Thunb* is expressed in young flower buds and shoot apical meristem but not in the roots, shoots, mature leaves and mature floral organs (Wang et al., 2008). *Ginlfy* gene in *Ginkgo biloba* was expressed in different organs such as the root, stem, leaf, young fruit and in the male flower bud and female flower bud in different stages. However, *GinNdly* in *Ginkgo biloba* was only expressed in the leaf, male flower bud and female flower bud in different stages (Guo et al., 2005). Analysis of *StLFY* expression at the mRNA level by RT-PCR showed that it was slightly expressed in the apical buds, floral buds and initial stolons (Guo et al., 2008). *EcFLO* mRNA was expressed during the vegetative phase of the shoot apical meristem and in developing dissected leaves (Andrea et al., 2003). The expression difference of *LFY* homologues is probably because of the change of the promoter sequence during evolution.

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