ARTICLE

Cloning and characterization of a *FLORICAULA/LEAFY* ortholog, *PFL*, in polygamous papaya

Qingyi YU[1, 2], Paul H. MOORE[3], Henrik H. ALBERT[3], Adrienne H.K. ROADER[4], Ray MING[1,*]

[1]*Hawaii Agriculture Research Center, Aiea, HI 96701, USA*
[2]*Department of Molecular Biosciences and Bioengineering, University of Hawaii, Honolulu, HI 96822, USA*
[3]*USDA-ARS, Pacific Basin Agricultural Research Center, Aiea, HI 96701, USA*
[4]*Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA 92093, USA*

ABSTRACT

The homologous genes *FLORICAULA* (*FLO*) in *Antirrhinum* and *LEAFY* (*LFY*) in Arabidopsis are known to regulate the initiation of flowering in these two distantly related plant species. These genes are necessary also for the expression of downstream genes that control floral organ identity. We used Arabidopsis *LFY* cDNA as a probe to clone and sequence a papaya ortholog of *LFY, PFL*. It encodes a protein that shares 61% identity with the Arabidopsis *LFY* gene and 71% identity with the *LFY* homologs of the two woody tree species: California sycamore (*Platanus racemosa*) and black cottonwood (*Populus trichocarpa*). Despite the high sequence similarity within two conserved regions, the N-terminal proline-rich motif in papaya *PFL* differs from other members in the family. This difference may not affect the gene function of papaya *PFL*, since an equally divergent but a functional *LFY* ortholog *NEEDLY* of *Pinus radiata* has been reported. Genomic and BAC Southern analyses indicated that there is only one copy of *PFL* in the papaya genome. *In situ* hybridization experiments demonstrated that *PFL* is expressed at a relatively low level in leaf primordia, but it is expressed at a high level in the floral meristem. Quantitative PCR analyses revealed that *PFL* was expressed in flower buds of all three sex types - male, female, and hermaphrodite with marginal difference between hermaphrodite and unisexual flowers. These data suggest that *PFL* may play a similar role as *LFY* in flower development and has limited effect on sex differentiation in papaya.

Keywords: divergence, floral meristem identity gene, flower development, gene expression, molecular phylogeny.

INTRODUCTION

Flower meristem identity genes control the developmental transition of plants from the vegetative to reproductive phase. Several flower meristem identity genes have been cloned from Arabidopsis; among them, *LEAFY* (*LFY*) is one of the earliest flower meristem identity expressed genes [1, 2]. *LFY* encodes a plant-specific transcription regulator [3, 4] and plays a major role in flower initiation [1, 2]. *LFY* transcripts are expressed at low levels during the vegetative phase, which are increased upon the transition from the vegetative to reproductive phase [5]. Mutations in *LFY* lead to the conversion of the flower meristems into inflorescence meristems and result in the replacement of flowers by lateral shoots [6-9]. Constitutive expression of *LFY* promotes reproductive transition and results in early flowering [10, 11]. In the complex flower development pathway, *LFY* is centrally located and acts as a link between the upstream flower timing genes and downstream floral homeotic genes [5]. *LFY* integrates environmental and endogenous signals to control flower timing [12]. It has also been shown that *LFY* activity is required for the activation of all the three classes of floral organ identity genes [4, 13-15].

Recent studies showed that the complex regulatory network of flower development is largely conserved across distantly related plants, especially among dicotyledonous plant species [16]. *LFY* homologs have been cloned from several species of gymnosperms and angiosperms, including both monocots and dicots [9, 17-24]. Their functions are highly conserved with slight variations among different species. Constitutive expression of *LFY* promoted flower

*Correspondence: Ray MING
Tel: +1-808-486-5374: Fax: +1-808-486-5020;
E-mail: rming@harc-hspa.com

initiation not only in Arabidopsis [11], but also in heterologous species, such as tobacco [25], aspen [11], rice [21] and citrus [26]. Ectopic expression of *LFY* homologous genes from other plant species in the Arabidopsis *lfy* mutant can partially or fully complement the *lfy* mutation [18, 27].

Papaya (*Carica papaya* L.) is a fruit crop grown widely in tropical and subtropical lowland regions. It is a dicotyledonous, diploid plant species and relatively close to the fully sequenced model plant Arabidopsis from the point of view of family level [28]. Papaya is one of the few plant species that produces three sex forms, male, female and hermaphrodite, and provides a unique opportunity to examine the applicability of the floral development pathway established in hermaphrodite to dioecious plants. Our previous study demonstrated that papaya has a primitive Y chromosome controlling sex determination [29]. Cloning and characterization of flower development genes from papaya will help us to understand the roles of floral meristem and organ identity genes in sex determination and differentiation in plants.

Here, we report the cloning and characterization of *LFY* ortholog in papaya, *PFL*. It shares a high similarity and identity with the *LFY* orthologs from other dicot species and its expression pattern is similar to that of other orthologous genes. Our results showed that *PFL* is a functional ortholog of the Arabidopsis *LFY* gene expressed in all three sex forms.

MATERIALS AND METHODS

Plant materials

Three different papaya cultivars, SunUp, Kapoho and AU9, were germinated and planted in Kunia station, Oahu. Young leaf tissues of SunUp, Kapoho and AU9 were collected for genomic DNA isolation. Hermaphrodite and female flower buds, young leaf tissues and root tissues were collected for total RNA isolation. Shoot apical meristem (SAM), floral meristem and leaf meristem tissues from SunUp and Kapoho were collected and fixed at different floral developmental stages for *in situ* hybridization.

cDNA library construction

Total RNA was isolated from hermaphrodite and female flower buds using the method described by Hall *et al* [30]. PolyA$^+$ RNA was isolated from the total RNA using PolyATtract mRNA Isolation System (Promega, WI, USA) according to manufacturer's instruction. About 5μg polyA$^+$ RNA was used to synthesize double stranded cDNA using the ZAP-cDNA Synthesis kit (Stratagene, CA, USA). Size-selected cDNA (100 ng) was ligated with 1μg *Eco*RI/*Xho*I digested Uni-ZAP XR lambda vector and packaged with GigapackII packaging extract (Stratagene, CA, USA). Libraries of 8×10^6 and 3.8×10^6 recombinants, respectively, were generated. A total of 18,432 (48×384) recombinant clones for each library were picked and archived in 384-well plates containing freezing medium. High-density (18,432 clones per 22.5 × 22.5 cm^2) and low-density (4,608 clones per 22.5×22.5 cm^2) membranes were made using a Genetix Q-BOT (Genetix, UK).

Southern analysis

Isolation of papaya genomic DNA from leaves was based on the procedure described by Tai and Tanksley [31] with minor modifications. Three restriction enzymes, *Hin*dIII, *Eco*RI and *Xba*I were used to digest papaya genomic DNA from the three cultivars, Kapoho, SunUp and AU9. Genomic DNA samples (10 μg) were incubated with 5 U of restriction enzyme/μg DNA in a total volume of 50 μl at 37°C for 4 h. Approximately 10 μg digested genomic DNA was electrophoresed through 0.8% agarose gel in 1×TBE buffer. After electrophoresis, the gel was blotted onto Hybond N+ membranes (Amersham, UK) using standard methods [32]. The probes were labeled by random primer labeling system (RediPrimer II, Amersham, UK). The procedure for hybridization and autoradiography were as described by Chittenden *et al* [33].

In situ hybridization

Apices, flower meristems, and leaf meristems of papaya were harvested at different stages and fixed in FAA fixation solution (3.7% formaldehyde, 5% acetic acid, 50% ethanol). Samples were then dehydrated in increasing concentrations of ethanol and embedded in paraffin (Paraplast Plus, VWR, PA, USA). Sections (8 μm thick) were cut and mounted onto ProbeOn Plus slides (Fisher Scientific, PA, USA). The amplified *PFL* cDNA fragment was cloned into TOPOII vector (Invitrogen, CA, USA). Antisense and sense RNA probes were generated with opposing SP6 and T7 promoters from the *PFL* cDNA subclone and labeled with digoxigenin (DIG) (Roche, Germany). The methods for tissue pretreatment and *in situ* hybridization were as described by Jackson [34].

RT-PCR analysis

Total RNA was treated with RNase-free DNase I (Promega, WI, USA) and quantified using a spectrophotometer (Shimadzu, Japan). About 5 μg of total RNA were reverse transcribed using RETROscript Kit (Ambion, TX, USA). PCRs were performed on the cDNA using the primer pair *PFL*-Sub10-3 (5'-CCA GAA CAT AGC CAA GGA GC) and *PFL* 2403 (5'-AAT GGC AAG ACG AGG ATG TG).

Real-Time quantitative RT-PCR assays

One primer pair (A1: 5'-ATA GTG CAG GCG CCA GTC AG; A2: 5'-GGC TCA AGC TGT TCA TCA TC) had a T_m of >55°C and was designed using Clone Manager 6 (Sci Ed Central) to produce a PCR product of 223 bp. About 2 μg of total RNAs were treated with RNase-free DNase I (Promega, WI, USA) and reverse transcribed using TaqMan RT kit (Applied Biosystems, CA, USA). cDNA products were diluted 5.5 fold for use in real-time RT-PCR amplification. Platinum Quantitative PCR SuperMix-UDG (Invitrogen, CA, USA) was used for real-time RT-PCR amplification. Reactions were run on a DNA Engine OPTICON 2 Real-Time PCR detection system (MJ research, MA, USA). Thermocycler conditions were 2 min at 50°C followed by 10 s at 95°C and 40 cycles of 15 s at 95°C, 20 s at 58°C, and 30 s at 72°C. For each sample, an RNA sample without reverse transcriptase was included to control for genomic DNA contamination. All presented PCR data were generated from a minimum of three independent reactions for each biological replication. *Actin* gene was used as a standard to control for sample variation in the total amount of RNA in different reactions.

Phylogenetic analysis

Multiple alignments of conceptual amino acid sequences were

generated by using Clustal W 1.81 (http://www.cmbi.kun.nl/bioinf/tools) with a gap open penalty of 10.00 and a gap extension penalty of 0.05. Phylogenetic analyses were conducted using the Neighbor-Joining (NJ) method as implemented by the PHYLIP program package. Pam matrix was used to generate distance. Bootstrap analysis (1,000 replicates) was performed to assess the support of individual branches.

RESULTS

Cloning of *PFL*

The Arabidopsis *LFY* cDNA was used as a probe to screen the papaya BAC library. Two positive BAC clones, 42B17 and 52E1, were identified and confirmed by southern hybridization. The sizes of these positive BAC clones were estimated as 60 kb and 175 kb, respectively. The smaller BAC clone, 42B17, was digested with *Hin*dIII and shotgun subcloned into *pPCR-Script* vector. The sub-clones were screened with the *LFY* probe and the positive sub-clone was sequenced from both ends. Direct sequencing of the BAC clone was carried out at the 3' end of the sub-clone where the third exon of *PFL* was interrupted by a *Hin*dIII site.

A full-length sequence of *PFL* was obtained through directly sequencing of BAC clone 42B17 via a primer walking approach. Three exons were predicted based on sequence homology and conserved splicing sites. The sizes of three exons were 429 bp, 315 bp and 357 bp, respectively.

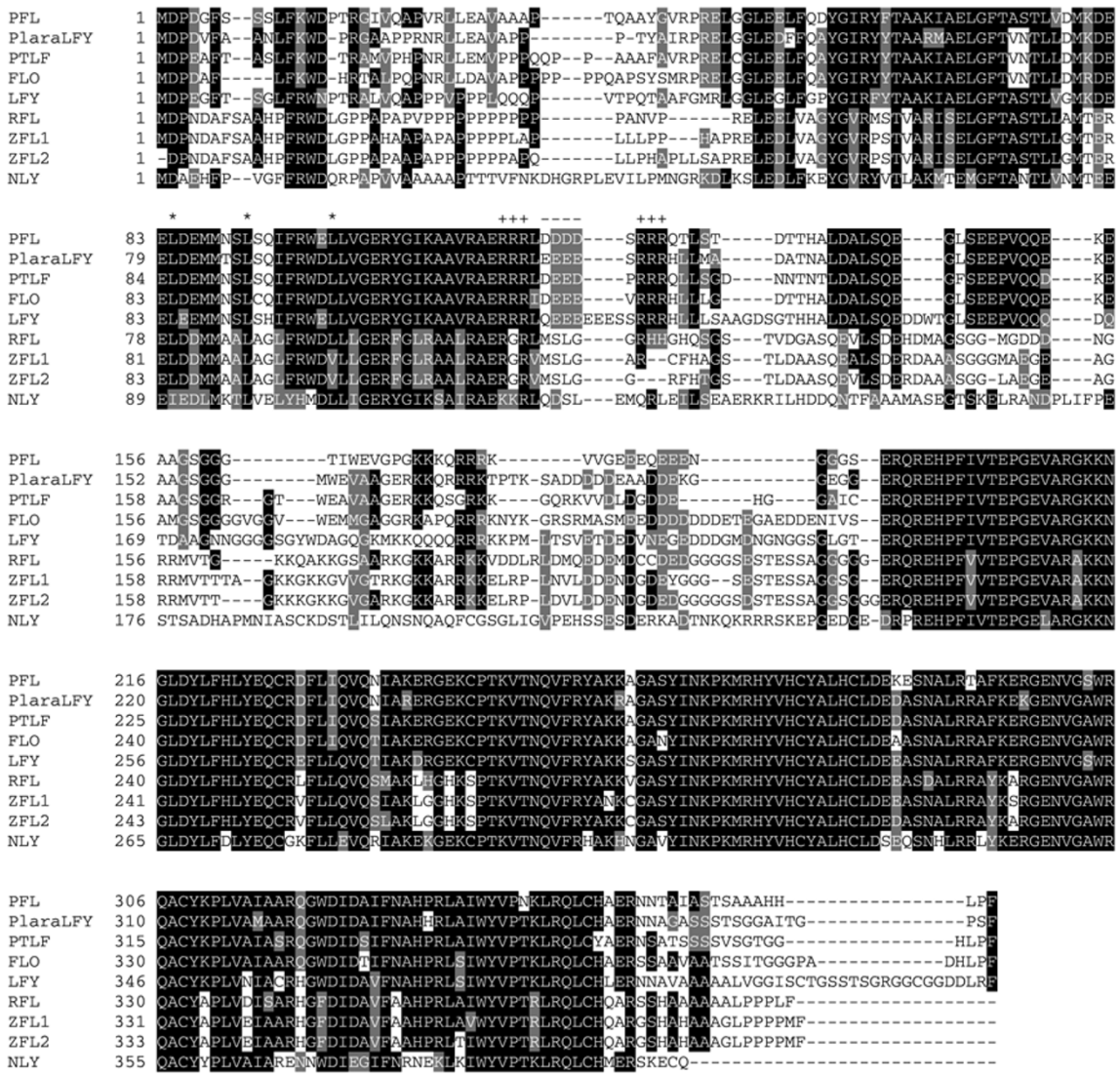

Fig. 1 Sequence comparison of *FLO/LFY*-like proteins. The leucine repeats are marked with "*". The basic- and acidic-regions are labeled as "+" and "-", respectively.

Tab. 1 Comparison of amino acid sequences between papaya and other *FLO/LFY* homologs

FLO/LFY homologs	Percentage of Identity	Percentage of Similarity
Arabidopsis thaliana (LFY)	61%	66%
Snapdragon (FLO)	67%	70%
California Sycamore (*PlaraLFY*)	71%	76%
Black Cottonwood (*PTLF*)	71%	75%
Rice (*RFL*)	48%	57%
Maize (*ZFL1*)	49%	57%
Maize (*ZFL2*)	49%	57%
Pine (*NLY*)	44%	55%

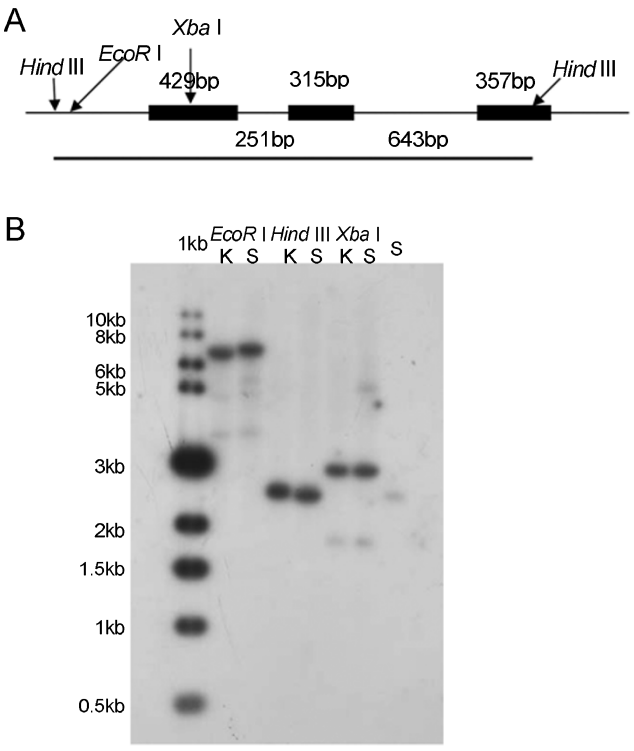

Fig. 2 (A) Restriction map of genomic clone of *PFL* of selected sites. The probe used in the southern hybridization was indicated in the line underneath. **(B)** Southern hybridization result of Kapoho (K) and SunUp (S) papaya genomic DNA digested with three different enzyme, *Eco*RI, *Hin*dIII, and *Xba*I, and labeled with *PFL* probe. S: size control. The same fragment with the probe was used as the size control.

Two primers were designed at the beginning of the first predicted exon and the 3'-end of the third predicted exon.

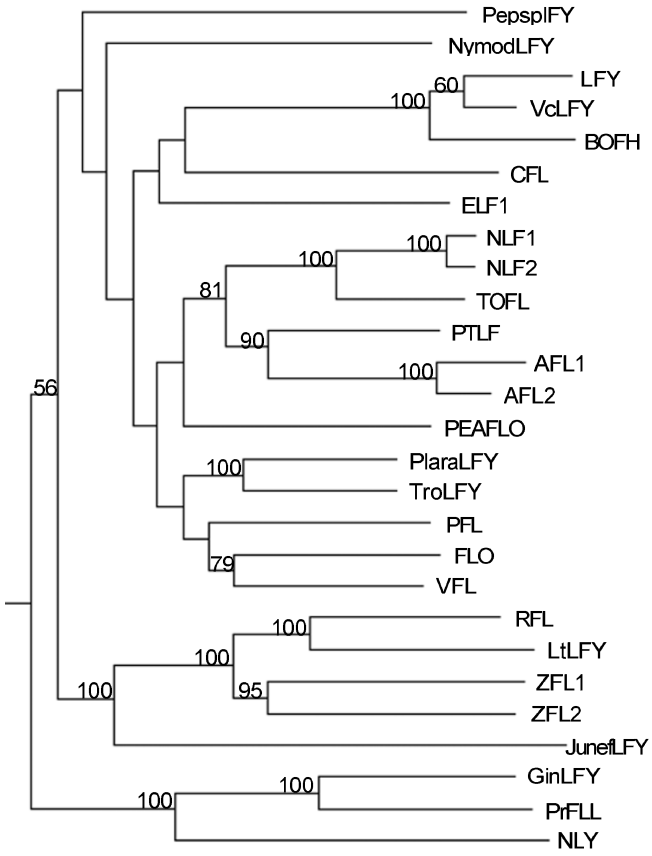

Fig. 3 Phylogenic relationship among *FLO*/*LFY*-like proteins. Bootstrap analysis (1,000 replicates) was performed to assess the support of each branch. Bootstrap values (percentages) are shown on branches when over 50. Branch lengths are proportional to the number of amino acid substitutions.

The cDNA fragment of *PFL* was obtained by amplification of the cDNA from papaya hermaphrodite flower buds using these two primers. This cDNA fragment was cloned into pCRII-TOPO vector (Invitrogen, CA, USA) and sequenced. The sequence result matched exactly the predicted open reading frames. Comparison between *PFL* cDNA and genomic sequence confirmed that *PFL* has three exons and two introns and that the splicing sites are highly conserved among *PFL*, *FLO*, and *LFY*.

PFL cDNA was about 1.1 kb and predicted to encode a protein of 367 amino acids. The deduced amino acid sequence of *PFL* was aligned with other *FLO/LFY*-like proteins (Fig. 1). This alignment revealed two highly conserved regions and two short variable regions. A proline-rich region near the amino terminus and an acidic central region were found in the variable regions of most of angiosperm *FLO/LFY*-like proteins. The proline-rich region was not found in the papaya *PFL*. However, a highly acidic region was observed in *PFL* between glutamate 182 and

Tab. 2 List of *FLO/LFY* homologs cloned in different plant species

Ortholog	Gene Bank Accession No.	Family	Genus	Species	Chr. No.	Ploidy	Genome Size (Mbp)	Note
PFL	DQ054794	Caricaceae	Carica	papaya	18	2	372	Eudicot
LFY	M91208	Cruciferae	Arabidopsis	thaliana	10	2	172	Eudicot
FLO	M55525	Scrophulariaceae	Antirrhinum	majus	16	2	1568	Eudicot
PlaraLFY	AF106842	Platanaceae	Platanus	racemosa				Eudicot
PTLF	U93196	Salicaceae	Populus	balsamifera	38	2		Eudicot
NFL1	U16172	Solanaceae	Nicotiana	tabacum	48	4	5733	Eudicot
NFL2	U16174	Solanaceae	Nicotiana	tabacum	48	4	5733	Eudicot
BOFH	Z18362	Cruciferae	Brassica	oleracea			760	Eudicot
PEAFLO	AF010190	Leguminosae	Pisum	sativum	14	2	4337	Eudicot
VFL	AF450278	Vitaceae	Vitis	vinifera	38	2	417	Eudicot
TroLFY	AF230078	Trochodendraceae	Trochodendron	aralioides				Eudicot
TOFL	AF197934	Solanaceae	Lycopersicon	esculentum	24	2	1005	Eudicot
ELF1	AF034806	Myrtaceae	Eucalyptus	globulus		2	564	Eudicot
AFL1	AB056158	Rosaceae	Malus	Malus x domestica	14	2	2205	Eudicot
AFL2	AB056159	Rosaceae	Malus	Malus x domestica	14	2	2205	Eudicot
CFL	AF059320	Cucurbitaceae	Cucumis	sativus	14	2	882	Eudicot
VcLFY	AF184589	Cruciferae	Jonopsidium	acaule				Eudicot
NymodLFY	AF105110	Nymphaeaceae	Nymphaea	odorata				Basal Angiosperm
PepspLFY	AF106843	Piperaceae	Peperomia	HBG70537	22	2	613	Basal Angiosperm
RFL	AB005620	Gramineae	Oryza	sativa	24	2	490	Monocot
ZFL1	AY179882	Poaceae	Zea	mays	20	2	2671	Monocot
ZFL2	AY179881	Poaceae	Zea	mays	20	2	2671	Monocot
LtLFY	AF321273	Gramineae	Lolium	temulentum	14	2	3063	Monocot
JunefLFY	AF160481	Juncaceae	Juncus	effusus	46	2	294	Monocot
PrFLL	U92008	Pinaceae	Pinus	radiata	24	2	21560	Gymnosperm
NLY	U76757	Pinaceae	Pinus	radiata	24	2	21560	Gymnosperm
GinLFY	AF108228	Ginkgoaceae	Ginkgo	biloba	24	2	9751	Gymnosperm

glutamate 188 where it was preceded by a short basic region (between lysine 171 and lysine 178), which is similar to other *FLO/LFY*-like proteins. *PFL* also shares with other dicot *FLO/LFY*-like proteins a region containing leucines repeated every 7 or 8 amino acids (positions leucine 69 to leucine 99) and a region with alternating basic-acidic-basic stretches (positions arginine 115 to arginine 126).

The comparison of *PFL* proteins to other *FLO/LFY*-like proteins showed that *PFL* protein is more closely related to the other dicot *FLO/LFY* proteins than to their monocot counterparts or to gymnosperm *FLO/LFY*-like proteins. *PFL* protein shares 66% and 70% similarity with *LFY* and its snapdragon counterpart *FLO*. It shares a higher similarity with *LFY* homolgous proteins of two woody tree species, California sycamore (*Platanus racemosa*) and black cottonwood (*Populus trichocarpa*) at 75% and 76%, respectively (Tab. 1).

Southern hybridization analysis of papaya genomic DNA was performed using the subcloned *PFL* genomic fragment as a probe to determine whether *PFL* is a single copy gene in papaya. Only one hybridizing band was seen with *Hin*d III digestion (Fig. 2). One strong band with some weak bands were seen with *Eco*RI digestion and *Xba*I digestion. Since the sequencing result showed there are *Eco*RI and *Xba*I restriction sites within the *PFL* gene and only one cDNA fragment was amplified from the mRNA of papaya hermaphrodite flower buds, it is likely that only one copy of *PFL* gene exists in the papaya genome. This same probe was also used to label the papaya BAC library (data not shown). Nine positive BAC clones were obtained. All nine positive BAC clones showed the same pattern after they were digested with *Hin*dIII and *Xho*I

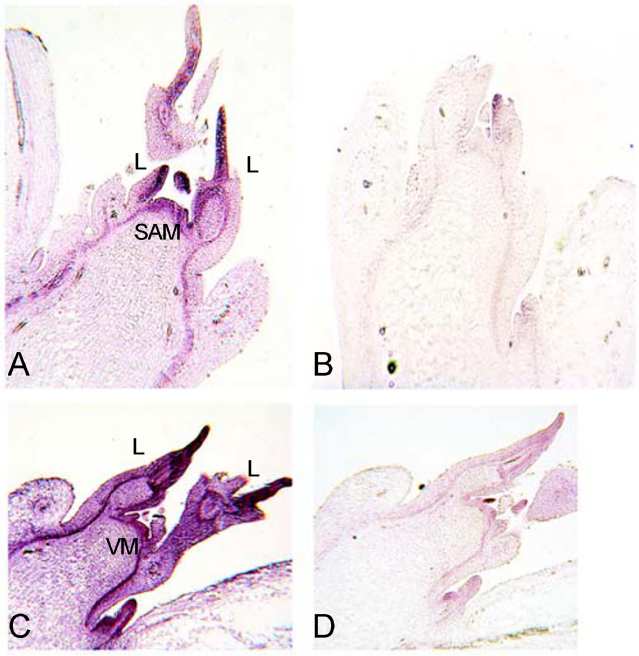

Fig. 4 *In situ* hybridization of *PFL* transcripts in vegetative tissues of papaya. **(A)** Longitudinal section of SAM and leaves hybridized by the antisense *PFL* RNA probe. Expression was detected in the SAM and in the adaxial face and margins of leaves (L). **(B)** A consecutive section of a was hybridized by the sense *PFL* RNA probe as control. **(C)** Longitudinal section of vegetative meristem hybridized by the antisense *PFL* RNA probe. Expression was observed in vegetative meristem (VM) and leaf primordia. **(D)** A consecutive section of c was hybridized by the sense *PFL* RNA probe as control.

and labeled with the *PFL* probe. This result reinforced our conclusion that only one copy of *PFL* exists in the papaya genome.

A phylogenetic tree was constructed using the predicted protein sequences to determine the evolutionary relationships between *PFL* and other *FLO/LFY*-like proteins (Tab. 2, Fig. 3). The phylogenetic tree shows three *FLO/LFY*-like proteins from two different gymnosperm species, *NLY, PrFLL* and *GinLFY*, clustered together and quite separate from the proteins of the angiosperms. Five *FLO/LFY*-like proteins from monocot species, *RFL*, *LtLFY*, *ZFL1, ZFL2* and *JunefLF* cluster in a group separate from the dicot species. *LFY*, *VcLFY* and *BOFH* from three different species within Cruciferae formed a distinct cluster. Similar to this, *NFL1*, *NFL2* and *TOFL* from two different species within the Solanaceae formed another sub-cluster. The general phylogenetic relationships found in this tree are consistent with the currently accepted species phylogenies. The *PFL* protein is closely related to other *FLO/LFY*-like proteins in dicots, but it is very different from its monocot or gymnosperm counterparts.

Expression of *PFL* during papaya flower development

The flowering times of different papaya genotypes are strictly related to the number of nodes produced before flowering [35]. Three papaya genotypes were chosen to test whether the flowering time of papaya is related to the expression of *PFL*. Saipan Red, an early-flowering genotype, flowers at 17-20 nodes. Kapoho, a late-flowering genotype, flowers at 45-47 nodes. SunUp is intermediate and flowers at 32-34 nodes. The SAM tissues from these three genotypes were collected at 5 node intervals beginning at the 5-node stage. These tissues were used to determine the stages at which the *PFL* would be turned on using *in situ* hybridization. The *in situ* hybridization revealed that *PFL* mRNA was already detected in the SAM of young seedlings of all three genotypes at the 5-node stage (Fig. 4).

In situ hybridization was also carried out to study *PFL*'s spatial expression pattern (Fig. 5). *PFL* mRNA was detected at a very high level in flower primordia and at a relatively lower level in leaf primordial. Expression of *PFL* was observed in the adaxial face and margins of leaf and

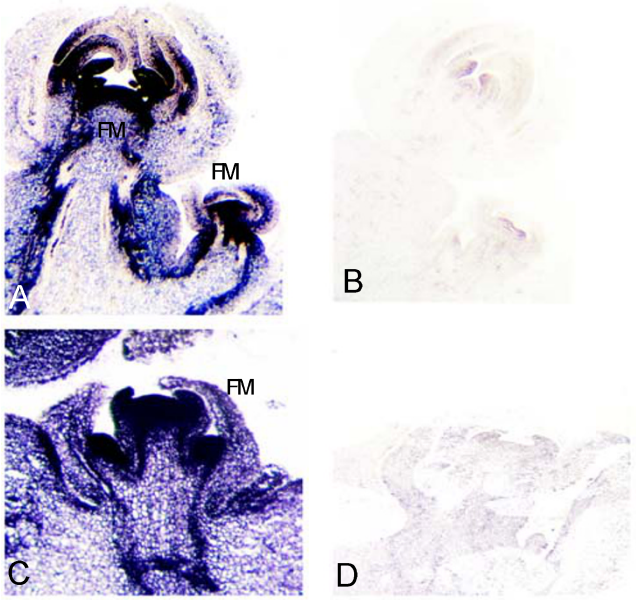

Fig. 5 *In situ* hybridization of *PFL* transcripts in floral tissue of papaya. **(A)** Longitudinal section of a floral branch meristem (FM) hybridized with the antisense *PFL* RNA probe. Hybridization signals were observed in floral meristems (FM) at different developmental stages. **(B)** A consecutive section of **(A)** was hybridized with the sense *PFL* RNA probe as control. **(C)** Longitudinal section of inflorescence hybridized with the antisense *PFL* RNA probe. Expression is detected in all floral organ primordia. **(D)** A consecutive section of c was hybridized with the sense *PFL* RNA probe as control.

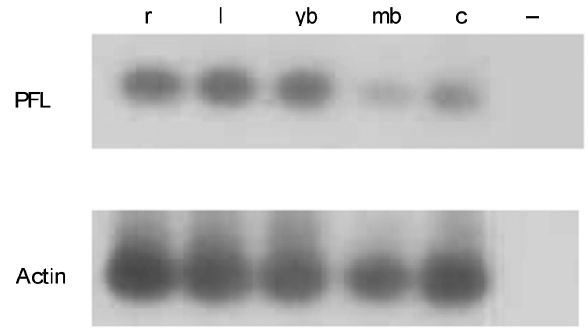

Fig. 6 RT-PCR result. *Actin* gene was used as positive control. R: root; l: leaf; yb: young flower buds; mb: mature flower buds; c: carpel; -: negative control.

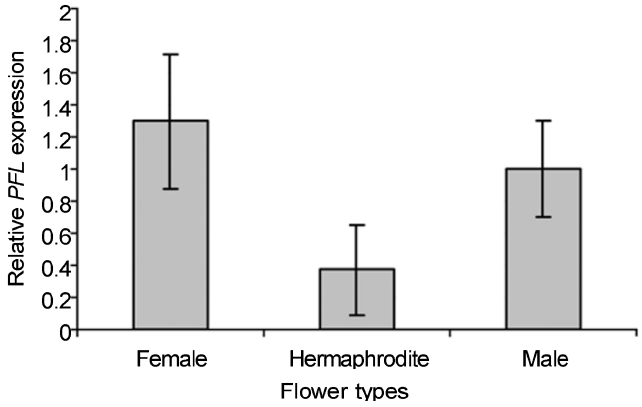

Fig. 7 *PFL* expression analysis in papaya different sex-type flowers by quantitative Real-Time PCR. Error bars represent standard deviations calculated from three replications.

axillary buds. *PFL* was also expressed in developing floral organs but at lower levels than in the flower primordia. At later stages of flower development, *PFL* expression was restricted to the two inner whorls of the flower.

RT-PCR results showed that *PFL* is expressed not only in reproductive stage, but also in vegetative stage (Fig. 6). *PFL*'s expression was found in root and leaf tissue as well as flower tissue. Quantitative Real-time PCR was conducted to test *PFL*'s expression levels in papaya flowers of three different sex types. The expression levels of PFL were the same between male and female flowers, but Marginal difference was detected between hermaphrodite and unisexual flowers ($P< 0.05$) with slightly elevated expression level in unisexual flowers (Fig. 7).

DISCUSSION

Papaya is a perennial semi-woody tropical fruit tree that flowers throughout the year once flowering is initiated. The pattern of flowering in papaya is different from seasonal flowering annual and perennial plant species. *PFL* expression was found in roots, SAM, leaf primordia, leaves and axillary buds throughout the vegetative phase. This is consistent with documented observations that all known *FLO/LFY* orthologs, except *FLO* from snapdragon, are expressed in both vegetative and floral meristems. However, during the reproductive phase development, *PFL* expression in the flower primordia is at a significantly elevated level, suggesting that *PFL* has a role in initiating flowers and regulating floral organ identity genes similar to other *LFY*/*FLO* homologues. This also suggests that there might be regulatory genes interacting with *PFL* to elevate *PFL* expression to a critical level in order to initiate flowering.

Papaya flowers are formed in the axils of leaves. After the completion of transition from vegetative phase to reproductive phase, its SAM continuously initiates new leaves and new flowers. In contrast, the SAM of Arabidopsis and *Antirrhinum* only produce flowers and bracts after the transition. It is not clear whether *PFL* functions in maintenance of an indeterminate phase so that the SAM can continuously produce leaf and floral primordia as proposed for the *LFY* orthlogs in pea, grapevine and tomato [19, 22, 36]. Such a role would be in line with the result that *PFL* expressed in both vegetative and reproductive phases and the observation that a vegetative phase coexists with a reproductive phase after flower initiation.

Hermaphrodite and female flowers of papaya are determinate while male flowers are partially determinate. Flowering in papaya has no effect on leaf morphology, although a novel function of *FLO/LFY* orthologs in regulating leaf morphogenesis has been reported previously [19, 22]. However, initiation of male flowers results in production of long and branched peduncle with many clusters of male flowers. This unique trait in male trees is more likely associated with the sex determination gene and less likely affected by *PFL* since there is no difference in expression levels between male and female flowers.

With male, female, and hermaphrodite co-existing in the same species, papaya is an ideal system to examine the roles of floral meristem and organ identity genes in sex determination and differentiation and to test the applicability of ABCE model in dioecious species. Our preliminary analyses resulted in marginal difference between hermaphrodite and unisexual flowers. This difference might reflect the role of *PFL* in regulation of unisexual organ development, or simply due to the difference of developmental stages of the same sized hermaphrodite and unisexual flowers.

Homologous genes in species controlling the basic path-

ways have evolved with variation in their amino acid sequences and modified functions of their original roles. For flower meristem identity, the early events of flower development appear to be conserved mechanisms that are retained in a wide variety of plant species. Sequence analysis showed *PFL* was more closely related to with *LFY* homolgous proteins of two woody tree species, California sycamore (*Platanus racemosa*) and black cottonwood (*Populus trichocarpa*). Phylogenetic analyses of this target gene also revealed that *PFL* was not as closely related to *LFY* as suggested by previous systematic study on angiosperm families [28]. This is likely due to different rate of divergence among genes under different selection pressures in the genome due to their roles in various developmental pathways. Comparison between *PFL* and other *FLO/LFY* homologs showed that the gene structure and splicing sites are highly conserved among the angiosperms. Moreover, *PFL* shares two highly conserved regions with other *FLO/LFY* homologs, indicating that these two-conserved regions might relate to the basic functions of *FLO/LFY*-like proteins. *LEAFY* protein regulates the expression of downstream floral homeotic genes by binding the sequences in their enhancers [4, 13, 37, 38]. The two-conserved regions are involved in DNA binding [39] and the divergent functions between *FLO/LFY* homologs correspond to substitutions in these two-conserved regions [39].

Proline-rich motifs and acidic-rich motifs are typical for transcriptional activators and may be important for the function of *FLO/LFY*-like proteins. A proline-rich region near the amino terminus and an acidic central region are found in most angiosperm *FLO/LFY*-like proteins. Since both of these features are also found in certain types of transcription factors, these two domains are thought to be important for the function of *FLO/LFY*-like proteins. *PFL* lacks the proline-rich region and has a short acidic region at corresponding position. Since these two domains are located at variable regions, they might not be functionally significant. Similarly, the *FLO/LFY*-like proteins from pine, apple and *Eucalyptus* also lack the proline-rich region [18, 40, 41]. Ectopic expression of these *FLO/LFY* orthologs in *Arabidopsis* causes the premature conversion of shoots into flowers, as does *35S:LFY* [18, 40, 41]. These regions may be subject to evolutionary changes so that different species formed their own genetic regulation system to adapt to the specific environment.

Our results showed that *PFL* was expressed in flower buds of all three sex types, likely due to its role as a positive activator of *AP3* and *AG* [3, 4]. The marginal difference of *PFL* expression between hermaphrodite and unisexual flowers revealed by quantitative PCR was not sufficient to suggest a role of *PFL* as a regulatory element for sex differentiation in papaya. Further functional analyses of *PFL* and its interaction with other regulatory genes would clarify its role in flower development and sex differentiation in papaya.

ACKNOWLEDGEMENTS

We thank Marc CREAPAU, Peizhu GUAN, Denise STEIGER and Jim CARR for technical assistance, Elliot MEYEROWITZ for providing the Arabidopsis *LFY* cDNA clone, and Mingli WANG for review the manuscript. This work was supported by a USDA-ARS Cooperative Agreement (CA 58-3020-8-134) with the Hawaii Agriculture Research Center.

Received, Jun 5, 2005
Revised, July 27, 2005
Accepted, Aug 3, 2005

REFERENCES

1 Liljegren SJ, Gustafson-Brown C, Pinyopich A, Ditta GS, Yanofsky MF. Interactions among *APETALA1*, *LEAFY*, and *TERMINAL FLOWER1* specify meristem fate. Plant Cell 1999; **11:** 1007-18.
2 Wagner D, Sablowski RWM, Meyerowitz EM. Transcriptional activation of *APETALA1* by *LEAFY*. Science 1999; **285:**582-4.
3 Weigel D, Meyerowitz EM. Activation of floral homeotic genes in *Arabidopsis*. Science 1993; **261:**1723-26.
4 Parcy F, Nilsson O, Busch MA, Lee I, Weigel D. A genetic framework for floral patterning. Nature 1998; **395:**561-6.
5 Blázquez MA, Soowal L, Lee I, Weigel D. *LEAFY* expression and flower initiation in *Arabidopsis*. Development 1997; **124:** 3835-44.
6 Haughn GW, Somerville CR. Genetic control of morphogenesis in *Arabidopsis*. Dev Genet 1988; **9:**73-89.
7 Meyerowitz EM, Bowman JL, Brockman LL, *et al*. A genetic and molecular model for flower development in *Arabidopsis thaliana*. Dev Suppl 1991; **1:**157-67.
8 Schultz EA, Haughn GW. *LEAFY*, a homeotic gene that regulates inflorescence development in *Arabidopsis*. Plant Cell 1991; **3:** 771-81.
9 Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM. *LEAFY* controls floral meristem identity in *Arabidopsis*. Cell 1992; **69:**843-59.
10 Mandel MA, Yanofsky MF. A gene triggering flower formation in *Arabidopsis*. Nature 1995; **377:**522-4.
11 Weigel D, Nilsson O. A development switch sufficient for flower initiation in diverse plants. Nature 1995; **377:**495-500.
12 Blázquez MA, Weigel D. Integration of floral inductive signals in *Arabidopsis*. Nature 2000; **404:**889-92.
13 Busch MA, Bomblies K, Weigel D. Activation of a floral homeotic gene in *Arabidopsis*. Science 1999; **285:**585-7.
14 Blázquez MA. Flower development pathways. J Cell Sci 2000; **113:**3547-48.
15 Ng M, Yanofsky MF. Three ways to learn the ABCs. Curr Opin Plant Biol 2000; **3:**47-52.
16 Ma H, de Pamphilis C. The ABCs of floral evolution. Cell 2000; **101:**5-8.

17 Coen ES, Romero JM, Doyle S, *et al*. *floricaula*: A homeotic gene required for flower development in *Antirrhinum majus*. Cell 1990; **63:**1311-22
18 Mouradov A, Glassick T, Hamdorf B, *et al*. *NEEDLY*, a *Pinus radiata* ortholog of *FLORICAULA/LEAFY* genes, expressed in both reproductive and vegetative meristems. PNAS 1998; **95:** 6537-42.
19 Molinero-Rosales N, Jamilena M, Zurita S, *et al*. The tomato orthologue of *FLORICAULA* and *LEAFY*, controls flowering time and floral meristem identity. Plant J 1999; **20:**685-93.
20 Kelly A, Bonnlander MB, Meeks-Wagner DR. *NFL*, the tobacco homolog of *Floricaula* and *Leafy*, is transcriptionally expressed in both vegetative and floral meristems. Plant Cell 1995; **7:**225-34.
21 Kyozuka J, Konishi S, Nemoto K, Izawa T, Shimamoto K. Down-regulation of *RFL*, the *FLO/LFY* homolog of rice, accompanied with panicel branch initiation. PNAS 1998; **95:**1979-82
22 Hofer J., Turner L., Hellens R, *et al*. *UNIFOLIATA* regulates leaf and flower morphogenesis in pea. Curr Biol 1997; **7:**581-7.
23 Gocal GFW, King RW, Blundell CA, *et al*. Evolution of floral meristem identity genes. Analysis of *Lolium temulentum* genes related to *APETALA1* and *LEAFY* of *Arabidopsis*. Plant Physiol 2001; **125:**1788-801.
24 Bomblies K, Wang R -L, Ambrose BA, *et al*. Duplicate *FLORICAULA/LEAFY* homologs zfl1 and zfl2 control inflorescence architecture and flower patterning in maize. Development 2003; **130:**2385-95.
25 Ahearn KP, Johnson HA, Weigel D, Wagner D Ry. *NFL*1, a *Nicotiana tabacum LEAFY*-Like gene, controls meristem initiation and floral structure. Plant Cell Physiol 2001; **42:**1130-9
26 Peña L, Martin-Trillo M, Juarez J, *et al*. Constitutive expression of *Arabidopsis LEAFY* or *APETALA*1 genes in citrus reduces their generation time. Nat Biotechnol, 2001; **19:**263-7.
27 Chujo A, Zhang Z, Kishino H, Shimamoto K, Kyozuka J. Partial conservation of *LFY* function between rice and *Arabidopsis*. Plant Cell Physiol 2003; **44:**1311-9.
28 Bremer K, Chase MW, Stevens PF. An ordinal classification for the families of flowering plants. Ann Mo Bot Gard 1998; **85:** 531-53.
29 Liu Z, Moore PH, Ma H, *et al*. A primitive Y chromosome in papaya marks incipient sex chromosome evolution. Nature 2004; **427:**348-52.
30 Hall TC, Buchbinder BU, Pyne JW, Sun SM, Bliss FA. Messenger RNA for G1 protein of French bean seeds: cell-free translation and product characterization. Proc Natl Acad Sci U S A 1978; **75:**3196-200.
31 Tai TH, Tanksley, SD. A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. Plant Mol Biol Rep 1990; **8:**297-303.
32 Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989.
33 Chittenden LM, Schertz KF, Lin YR, Wing RA, Paterson AH. A detailed RFLP map of *Sorghum bicolor x S. propinquum*, suitable for high-density mapping, suggests ancestral duplication of *Sorghum* chromosomes or chromosomal segments. Theor Appl Genet 1994; **87:**925-33.
34 Jackson D. *In-situ* hybridization in plants. In: Bowles, DJ, Gurr SJ and McPherson, M. Eds. Molecular plant pathology: A practical approach. Oxford University Press: London 1991:163-74.
35 Nakasone HY, Storey WB. Studies on the inheritance of fruiting height of *Carica papaya* L. Proc Amer Soc Hort Sci 1955; **66:** 168-82.
36 Carmona MJ, Cubas P, MartÍnez-Zapater JM. *VFL*, the grapevine *FLORICAULA/LEAFY* ortholog, is expressed in meristematic regions independently of their fate. Plant Physiol 2002; **130:** 68-77.
37 Lamb RS, Hill TA, Tan QKG, Irish VF. Regulation of *APETALA3* floral homeotic gene expression by meristem identity genes. Development 2002; **129:**2079-86.
38 William DA, Su Y, Smith MR, *et al*. Genomic identification of direct target genes of *LEAFY*. Proc Natl Acad Sci U S A 2004; **101:**1775-80.
39 Maizel A, Busch MA, Tanahashi T, *et al*. The floral regulator *LEAFY* evolves by substitutions in the DNA binding domain. Science 2005; **308:**260-3.
40 Southerton SG, Strauss SH, Olive MR, *et al*. *Eucalyptus* has a functional equivalent of Arabidopsis floral meristem identity gene *LEAFY*. Plant Mol Biol 1998; **37:**897-910.
41 Wada M, Cao Q-F, Kotoda N, Soejima J-I, Masuda T. Apple has two orthologues of *FLORICAULA/LEAFY* involved in flowering. Plant Mol Biol 2002; **49:**567-77.