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ORIGINAL ARTICLE

B-class MADS-box genes in trioecious papaya: two *paleoAP3* paralogs, *CpTM6-1* and *CpTM6-2*, and a *PI* ortholog *CpPI*

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Abstract In the ABC model of flower development, B function organ-identity genes act in the second and third whorls of the flower to control petal and stamen identity. The trioecious papaya has male, female, and hermaphrodite flowers and is an ideal system for testing the B-class gene expression patterns in trioecious plants. We cloned papaya B-class genes, *CpTM6-1, CpTM6-2*, and *CpPI*, using MADS box gene specific degenerate primers followed by cDNA library screening and sequencing of positive clones. While phylogenetic analyses show that *CpPI* is the ortholog of the *Arabidopsis* gene *PI*, the *CpTM6-1* and *CpTM6-2* loci are representatives of the paralogous *TM6* lineage that contain *paleoAP3* motifs unlike the *euAP3* gene observed in *Arabidopsis*. These two paralogs appeared to have originated from a tandem duplication occurred approximately 13.4 million year ago (mya) (bootstrap range 13.36 ± 2.42). In-situ hybridization and RT-PCR showed that the papaya B-class genes were highly expressed in young flowers across all floral organ primordia. As the flower organs developed, all three B-class genes were highly expressed in petals of all three-sex types and in stamens of hermaphrodite and male flowers. *CpTM6-1* expressed at low levels in sepals and carpels, whereas *CpTM6-2* expressed at a low level in sepals and at a high level in leaves. Our results showed that B-class gene homologs could function as predicted by the ABC model in trioecous flowers but differential expressions of *CpTM6-1*, and *CpTM6-2*, and *CpPI* suggested the diversification of their functions after the duplication events.

Keywords *Carica* · Molecular phylogeny · Nonparametric rate smoothing · *paleoAP3* motif · *Tomato MADS-box gene 6* (*TM6*)

Abbreviations

AP3	*APETALA3*
BAC	Bacterial artificial chromosome
DEF	*DEFICIENS*
DIG	Digoxigenin
GLO	*GLOBOSA*
ML	Maximum likelihood analyses
MP	Maximum parsimony
mya	Million year ago
NPRS	Nonparametric rate smoothing
PI	*PISTILLATA*
RACE	Rapid amplification of cDNA ends
TM6	Tomato MADS-box gene 6

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Introduction

Papaya (*Carica papaya* L.; Caricaceae) is a trioecious species that produces female, male, and hermaphrodite flowers. Although those three sex forms are genetically determined (Hofmeyr 1938), phenotypic expression of papaya sex is influenced by environmental factors including temperature, nutritional status, and moisture (Awada and Ikeda 1957; Awada 1958). Instability of papaya flower sex expression is common and sex reversal occurs in flowers of all three sex forms, but it is more pronounced in the hermaphrodite and male flowers. Incomplete sex reversal in the hermaphrodites results in a continuous graded series of flower types (Storey 1958). Thus, papaya provides a unique opportunity to study flower development of sex organs.

Most angiosperm flowers, including those of papaya, are made up of four types of organs that are arranged in concentric whorls (Coen and Meyerowitz 1991). Specific classes of genes working together are responsible for the development of these whorls (Coen and Meyerowitz 1991). According to the widely accepted ABC model of floral organ development, expression of the B-class genes, such as the *Arabidopsis PISTILLATA* (*PI*) and *APETALA3* (*AP3*) and the *Antirrhinum DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), is required for petal and stamen initiation and development (Sommer et al. 1990; Jack et al. 1992; Goto and Meyerowitz 1994). The *AP3* and *PI* proteins interact to form a heterodimer that stabilizes both B-class proteins (Samach et al. 1997; Thomas 2004). The B-class heterodimer is necessary, not only for petal and stamen development, but it may also play a role in establishing sex determination in both monoecious and dioecious flowers (Park et al. 2003).

The B-class genes *AP3* and *PI* are derived from a duplication of the ancestor of these genes approximately 260 million years ago (mya), shortly after the divergence of extant gymnosperms and angiosperms (Kim et al. 2004). A second duplication event occurred in the *AP3* lineage before the split of basal eudicots and core eudicots (Magallon et al. 1999; Irish 2003) and resulted in two paralogous lineages termed *euAP3* and *TM6*, the latter named after the first identified representative, TOMATO MADS-BOX GENE 6 (*TM6*; Pnueli et al. 1991; Kramer et al. 1998). The *PI* lineage does not have major duplications but several recent duplications were detected in genus or species level (Kim et al. 2004). The two *AP3* sublineages differ by their characteristic motifs in the C-terminal region caused by ancestral frameshift mutation (Kramer et al. 1998, 2006; Vandenbussche et al. 2003). The C-terminal motifs of the core eudicot *TM6* sublineage are similar to those of the *paleoAP3* lineage that are present in basal angiosperm, monocots, magnoliids, and basal eudicots, whereas the C-terminal motifs of *euAP3* sublineage are different from those of the *paleoAP3* lineage and are found exclusively in core eudicots (Kramer et al. 1998).

In petunia and tomato, the functions of *euAP3* and *TM6* have diversified by subfunctionalization—an evolutionary process partitioning the original gene function into two parts. Function of *euAP3* has been extensively studied in *Arabidopsis* and *Antirrhinum* and is critical in petal and stamen initiation and development (Bowman et al. 1989; Carpenter and Coen 1990; Sommer et al. 1990; Jack et al. 1994). Function of *TM6* has been recently studied in tomato and *Petunia* and its function as a B-class gene is mainly the determination of stamen identity (de Martino et al. 2006; Rijpkema et al. 2006). *TM6* is expressed in the third and fourth whorls like a C-class gene and is negatively regulated by the A-function gene *BLIND* in *Petunia* (Rijpkema et al. 2006). The duplication and divergence of the *AP3* lineage is believed to have contributed to the development of well-defined petals in the core eudicots (de Martino et al. 2006).

Papaya is an economically important fruit crop in tropical and subtropical regions. Papaya flower sex organ instability results in malformed, unmarketable fruit. It is important to elucidate the underlying mechanisms that contribute to this sexual instability and reversal. Cloning major genes controlling flower development, such as the B-class floral genes, would be the first step towards solving the problem of sexual instability in papaya flowers. Differences in expression patterns of the B-class floral genes that specify the male reproductive organs among the three different sex types in papaya will increase our understanding of sex differentiation in this specialty crop species.

Materials and methods

Plant materials

Gynodioecious papaya variety SunUp hermaphrodite and female plants and dioecious variety AU9 male and female plants were maintained at Hawaii Agriculture Research Center Kunia Station, Oahu. Genomic DNA and total RNA were isolated from each sex type of these two genotypes.

RNA extraction and cDNA library construction

RNA isolation and cDNA library construction were described previously (Yu et al. 2005).

Amplifying target genes using degenerate primers

Previously reported MADS-box gene specific degenerate primers were used to amplify papaya B-class genes

(Kramer et al. 1998; Kim et al. 2004). The cDNA was synthesized from male papaya flower total RNA at various stages of development. This cDNA was used as a PCR template for amplifying the papaya *AP3* and *PI* homologs. The amplified cDNA fragments were excised from an agarose gel and cloned into the TOPO vector (TOPO TA cloning kit; Invitrogen).

Southern hybridization of target genes to papaya genomic DNA

Genomic DNA of AU9 male, SunUp female, and SunUp hermaphrodite was digested with *Eco*RI, *Hin*dIII, and *Xba*I and transferred to nylon membranes. Southern hybridization was performed using three different washing stringencies of 55, 60, and 65°C to identify possible additional copies of B-class genes in papaya. Positive BAC clones of *CpTM6-1* and *CpTM6-2* were digested by *Hin*dIII and hybridized with *CpTM6-2*. We used cDNA probes by RT-PCR with the primers *CpTM6-1*F (5′-GGGTCGTGGA AAGATTGAGA-3′)/*CpTM6-1*R (5′-TTTTCCGATAGT AATCGCAGTT-3′); and *CpTM6-2*F (5′-CCTACTGCCA CGACGAAGAA-3′)/*CpTM6-2*R (5′-GGTGGTGGTTAT GGTGAAGA-3′).

Phylogenetic analysis

To verify the subfamily identities of newly identified genes from C. papaya and to address their orthology to previously reported genes, we analyzed the newly identified papaya genes together with previously reported angiosperm AP3 (31 sequences) and PI (20 sequences) genes. Amino acid sequences of these genes were aligned using CLUSTAL X (v 1.83; Thompson et al. 1997) initially with the default options and then adjusted manually. To produce a consistent cDNA alignment, the program AA2DNA was used to generate a cDNA alignment on the basis of protein alignment (http://www.bio.psu.edu/People/Faculty/Nei/Lab/software.htm). Maximum likelihood analyses (ML; Felsenstein 1981) were performed for AP3 and PI matrices separately with PHYML (Guindon and Gascuel 2003). On the basis of Model Test v3.06 (Posada and Crandall 1998), we selected the GTR + I + Γ model of molecular evolution. Support values for nodes on the ML tree were estimated with 100 bootstrap replicates (Felsenstein 1981). The maximum parsimony (MP) analyses were also performed for the DNA data set using PAUP* 4.0b10 (Swofford 2001). The search strategy involved 100 random addition replicates with TBR branch swapping, saving all optimal trees. To assess support for each node, bootstrap analysis (Felsenstein 1981) was performed using 100 replicate heuristic searches each with 100 random taxon addition sequences and TBR branch swapping, saving all optimal trees. For both ML and MP analyses, sequences of Amborella, which is a sister to all other angiosperms (Qiu et al. 1999; Soltis et al. 1999; Zanis et al. 2002), are used as outgroup.

Estimation of divergence time

Because a duplication and subsequent diversification at the base of core-eudicots in the *AP3* lineage generated two sublineages (*euAP3* and *TM6*), we used reduced data set for the estimation of divergence time of two papaya *AP3* paralogs containing only *TM6* sequences of core-eudicots (seven sequences), a basal eudicot sequence (Ranunculales), and four outgroup sequences, *Persea* (Laurales), *Magnolia* (Magnoliales), *Asarum* (Piperales), and monocot. We calculated ML branch length and optimized these using PAUP* 4.0b10 (Swofford 2001) onto an organismal phylogenetic tree based on recent multiple gene studies (Qiu et al. 1999; Soltis et al. 1999; Zanis et al. 2002). Trees with branch length were transformed into ultrametric trees using nonparametric rate smoothing (NPRS; Sanderson 1997) as implemented in TREEEDIT (ver. 1.0 alpha 10 by A. Rambaut and M. Charleston at University of Oxford). The characteristic pollen of the eudicots, combined with their extensive fossil record, places the origin of the eudicots at 125 mya (Hughes 1994), one of the firmest dates in the paleobotanical record. This minimum age for eudicots was used to calibrate the tree. To compute error estimates for the ages, we reapplied the NPRS procedure to 100 bootstrapped matrices obtained by resampling the data irrespective of codon position using SEQBOOT in PHYLIP package (ver. 3.5C; http://evolution.genetics.washington.edu/phylip.html).

RT-PCR Analysis

Total RNA was isolated from roots and leaves, as well as from petals, stamens, and carpels of immature and mature flowers from male, female, and hermaphrodite plants. Standard hot phenol extraction methods were used for RNA isolation (Sambrook et al. 1989). The RT-PCR analysis was performed using the TaqMan kit according to the manufacturer's instructions (Applied Biosystems). The following gene-specific primer pairs were used to distinguish the expression patterns of the two papaya *AP3* orthologs and one papaya *PI* ortholog: *CpTM6-1*F (5′-GGGTCGTGGAAAGATTGAGA-3′)/*CpTM6-1*R (5′-TTTTCCGATAGTAATCGCAGTT-3′), *CpTM6-2*F (5′-CCTACTGCCACGACGAAGAA-3′)/*CpTM6-2*R (5′-GGTGGTGGTTATGGTGAAGA-3′), and *CpPI*-F (5′-GTTCTGGCAAGATGCATGAG-3′)/*CpPI*-R (5′-TCGCGATCTCCTGTTGT-3′).

In-situ hybridization

Papaya flowers buds of 1–3 mm in length were fixed and sectioned using standard techniques. To detect the localization of mRNAs of papaya *AP3* and *PI* orthologs, in-situ hybridization was performed using digoxigenin (DIG)-labeled RNA probes according to the manufacturer's instructions (Boehringer Mannheim). Because of the high sequence similarity between *CpTM6-1* and *CpTM6-2*, we used single probe to detect signals from both paralogs. We used a DNA probe by PCR with the primers designed in C-terminal region of *CpTM6-1* [CpTM6-1F (5′-GCAA GCTCCATGAGTTCATC-3′) and CpTM6-1R (5′-GAAG GCAACGAGAGTTC-3′)], which is the most variable region comparing to other *AP3* orthologs.

Results

Cloning B-class MADS-box genes in papaya

Arabidopsis *AP3* and *PI* cDNA clones were first used to screen two papaya flower cDNA libraries constructed from female and hermaphrodite flower buds, respectively (Yu et al. 2005). Sequencing of selected positive cDNA clones proved they were false positive. Then degenerate primers designed from conserved sequences of *AP3* and *PI* orthologs provided by Elena Kramer were used to clone B-class genes in papaya (Kramer et al. 1998 and personal communication). Because expression of B-class genes was expected primarily in the second and third flower whorls, we postulated that they might be expressed to the greatest extent in male flowers that lack a fourth whorl. In addition, since the papaya male inflorescence is more extensive and easier to sample than that of hermaphrodites, we isolated total RNA from differently sized male papaya flowers for isolating the B-class genes using rapid amplification of cDNA ends (RACE). The amplified fragments were transferred to a nylon membrane for Southern hybridization using the *Arabidopsis AP3* and *PI* genes as probes. Portions of the cDNA with homology to the *AP3* and *PI* genes were cloned into the PCR II-TOPO vector (Invitrogen) and used to screen the papaya bacterial artificial chromosome (BAC) and cDNA libraries. Screening and sequencing of positive cDNA clones resulted in two paralogs of *AP3*, named *CpTM6-1* and *CpTM6-2* (see detailed phylogenetic analysis below), and one ortholog of *PI*, named *CpPI* (GenBank Accession Nos. *CpTM6-1*: EF562498; *CpTM6-2*: EF562499; *CpPI*: EF562500). Full genomic sequences were obtained from positive BAC clones by primer walking.

Southern hybridization of *CpTM6-1* and *CpTM6-2* was performed on papaya genomic DNA digested with three restriction enzymes using three different washing stringencies. The *CpTM6-2* positive BAC clones digested by *Hin*dIII were hybridized with *CpTM6-2* cDNA probe. The different washing stringencies produced the same result. Based on their *Eco*RI restriction map, *CpTM6-1* should produce three bands at 3,952, 2,630, and 1,346 bp; while *CpTM6-2* should produce one band at 3,841 bp. Indeed, *CpTM6-1* detected the three predicted bands (Fig. 1a), while *CpTM6-2* revealed a strong band at the predicted size of 3,841 bp, likely also the 3,952 bp band of *CpTM6-1* due to high degree of sequence homology. *CpTM6-2* also detected the two smaller *CpTM6-1* fragment but the signals were rather weak. For *Hin*d III digestion, *CpTM6-1* should produce two bands at 2,070 and 2,087 bp; while *CpTM6-2* should produce one band at 5,606 bp. Since 2,070 and 2,087 bp are very close to each other, they were showed with one robust banding combining both 2,070 and 2,087 bp fragments. *CpTM6-1* hybridized strongly to the lower band and weakly to the upper *CpTM6-1* (Fig. 1a), and the opposite was true for the probe *CpTM6-2* (Fig. 1b). The banding pattern and signal intensity of the image hybridized but combining these two probes further confirmed the prediction (Fig. 1c). There is no *Xba*I restriction site within both *CpTM6-1* and *CpTM6-2* genes. We could not predict the sizes of fragments since we don't have enough extended sequences beyond these two genes. When *Hin*dIII digested *CpTM6-1* and *CpTM6-2* positive BAC clones were hybridized with *CpTM6-2* cDNA probe, seven of the ten BACs contained both *CpTM6-1* and *CpTM6-2* fragments, and the remaining three BACs contained only *CpTM6-1* fragment (Fig. 1d).

Phylogentic analyses of *AP3*- and *PI*-like genes in papaya

Blast searches in GenBank identified these genes as putative members of the *AP3* and *PI* subfamilies of MADS-box genes, respectively. An amino acid sequence alignment of the two papayas *AP3* paralogs, together with representatives of previously reported *AP3*-like genes, showed that these two *AP3* paralogs have a *paleoAP3* motif instead of an *euAP3* motif at the C-terminal end (Kramer et al. 1998; Fig. 2). Phylogenetic analyses clearly showed that these two genes are grouped together with previously reported *TM6*-like genes including *TM6* (*Lycopersicon*), *PTD* (*Populus*), and *Gu.ti.AP3s* (*Gunnera*) rather than *euAP3* genes. These genes form a clade (*TM6* lineage) with 87 and 77% of bootstrap supports in ML and MP analyses, respectively (Table 1; Fig. 3). The other genes in the eudicots clade of our phylogenetic tree (Fig. 3) form the strongly supported clade (*euAP3* lineage). Clearly, these two papaya *AP3* lineage genes are *TM6* orthologs and are thus named *CpTM6-1* and *CpTM6-2*. Out of the 684 bp of the *CpTM6-1* cDNA sequence, 61 bp are different from those of *CpTM6-2* that

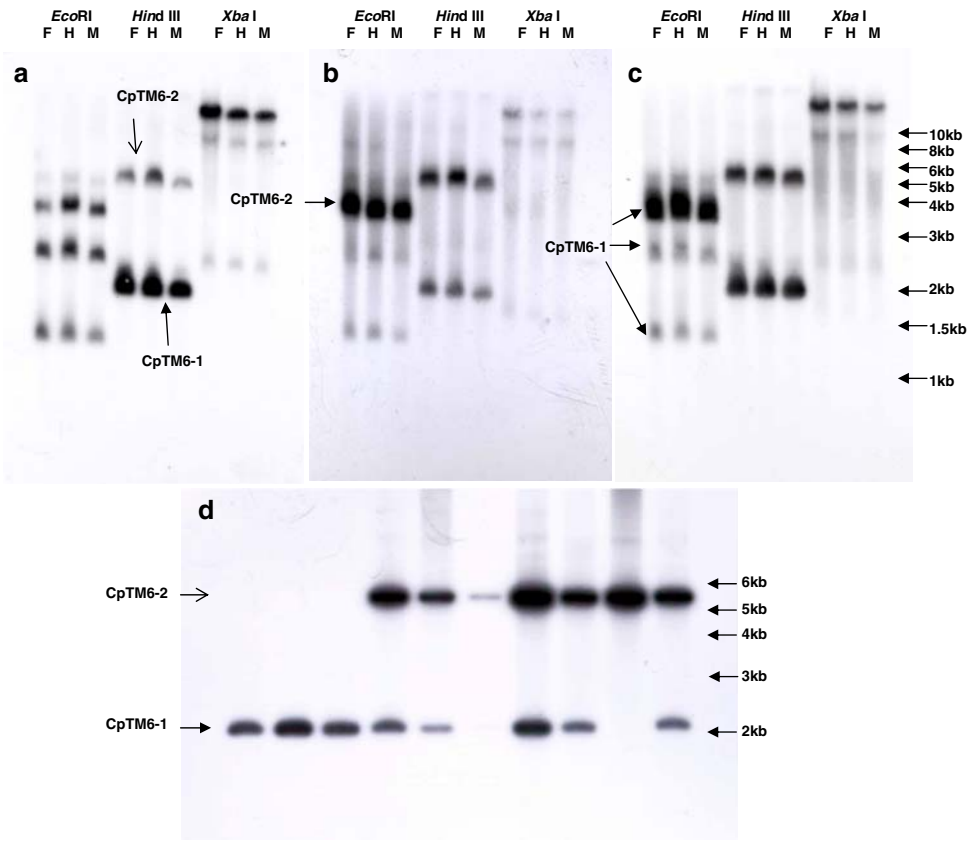

Fig. 1 Southern hybridization of *CpTM6-1* and *CpTM6-2* to the genomic DNA and positive BAC clones. The genotypes are *F* SunUp female, *H* SunUp hermaphrodite, and *M* AU9 male. The Southern hybridization was performed using three different washing stringencies (55, 60, and 65°C). The results of the three washing stringencies were the same and only the images with washes at 55°C were shown. **a–c** Papaya genomic DNA of three sex types was digested using three restriction enzymes and hybridized with *CpTM6-1* cDNA probe (**a**), *CpTM6-2* cDNA probe (**b**), and both *CpTM6-1* and *CpTM6-2* cDNA probes (**c**). **d** Ten *CpTM6-2* positive BAC clones were digested with *Hind*III and hybridized with *CpTM6-2* cDNA probe. The membrane was washed at 65°C

translates to a change of 18 amino acids. These two paralogs share 91% sequence identity.

Divergence between *CpTM6-1* and *CpTM6-2* is caused by amino acid substitution. There are no insertions or deletions between these two copies within the 684 bp coding sequence. The C-terminal domain of *CpTM6-2* is the same as the *paleoAP3* motif of tomato, *Petunia*, and poplar (Fig. 2), but the C-terminal domain of *CpTM6-1* has one amino acid substitution. There is a 6 amino acid difference in the K domain of *CpTM6-1* and *CpTM6-2*, whereas the remaining 11 amino acids differences are found in the non-conserved regions. The conserved MADS domain of *CpTM6-1* and *CpTM6-2* remained exactly the same.

As previously reported, the amino acid sequences of the papaya *PI*-homolog (*CpPI*) clearly have the *PI* motif at the end of the C-terminal region (Kramer et al. 1998; Fig. 4). In the phylogenetic tree *CpPI* is placed in the eudicots clade with 91% of supporting values in the MP analysis and 100% supporting values in ML analysis (Fig. 5). In the eudicots clade, *CpPI* is grouped together with *GGLO1* (*Gerbera*) and *Ri.sa.PI* (*Ribes*) in the MP analysis (not shown) and is sister to a clade of *Cucumis*, *Ribes*, *Gerbera*, *Petunia*, *Nicotiana*, *Antirrhinum*, and *Syringa* in the ML analysis (Fig. 5), but the supporting values of these relationships in both analyses were less than 50%.

Estimation of duplication time of two papaya *AP3*-like genes

Using our *AP3* data set and NPRS (Sanderson 1997), we estimated that the duplication that produced *CpTM6-1* and *CpTM6-2* occurred approximately 13.4 mya (bootstrap range: 13.36 ± 2.42; Fig. 6). The estimated divergence time between papaya *TM6* genes and *Populus TM6* ortholog *PTD* is about 58.5 my (bootstrap range: 58.49 ± 5.90; Fig. 6).

Gene expression analysis of *CpTM6-1*, *CpTM6-2, and CpPI*

The sequences of *CpTM6-1* and *CpTM6-2* are highly similar and a probe designed from *CpTM6-1* was used for in-situ hybridization for detection of expression of these two paralogs (*CpTM6*) in early flower development. *CpTM6* mRNA was detected in floral organ primordia of papaya flowers of all three-sex types that were less than 0.5 mm in length. Expression of *CpTM6* at this stage was detected throughout the entire undifferentiated sex organ primordia as well as in the petal primordia, but not in the surrounding sepal or vegetative bract primordia (Fig. 7a–c). At the 1 mm stage of papaya female flowers, the sepals, petals,

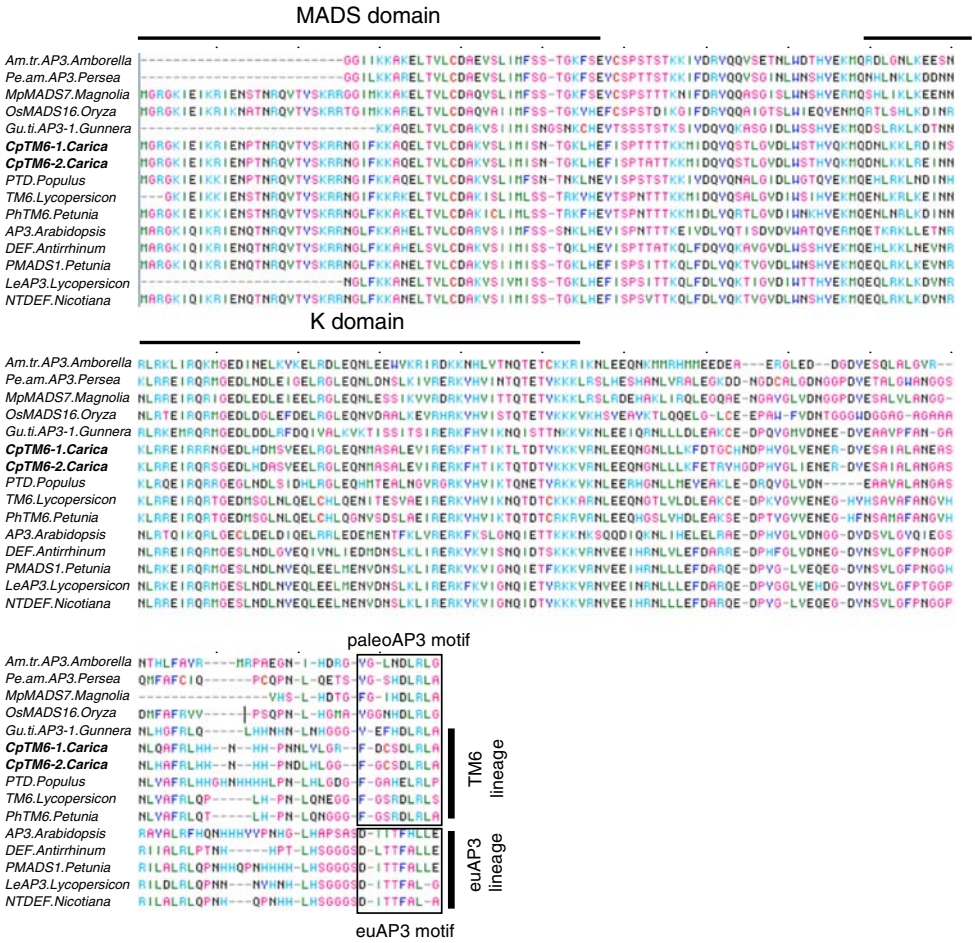

Fig. 2 A protein alignment of two papaya *AP3* ortholog sequences and representatives of other angiosperm *AP3* genes. Two papaya *AP3* genes have *paleoAP3* motif

and carpels were differentiated. Expression of *CpTM6* was detected in petals and carpels of female flowers but not in sepals (Fig. 7d). Similar expression patterns with reduced levels of expression were observed at 3 mm stage of female flowers (Fig. 7e). In male flowers and hermaphrodite flowers, at 3.0 mm stage, *CpTM6* mRNA was detected at high levels in the stamen and anther tissues, low levels in the petals and carpels, and not detectable in sepals (Fig. 7f). In-situ hybridization of PI revealed intense signals in sepal and undifferentiated floral primordia in early stage of male flowers (Fig. 7g) and robust signals in sepals and stamens in 2 mm male and hermaphrodite flowers (Fig. 7h, i).

RT-PCR analyses were performed on mRNA samples from flowers, leaves, and roots validate the in situ observations with *CpTM6-1, CpTM6-2*, and *CpPI*. None of the three genes was expressed in roots; *CpTM6-2* was the only gene expressed in leaves; and all three genes expressed in flowers (Fig. 8). Flower organs were dissected from immature flowers of male, female, and hermaphrodite papaya plants. In the dissected flower organs, *CpPI* was expressed in petals and stamens of hermaphrodite flowers, in petals of female flowers, and petals and stamens of male flowers. *CpTM6-1* transcripts were abundant in petals and stamens of hermaphrodite flowers, petals of female flower, and petals and stamens of male flowers. *CpTM6-1* expression was detectable in sepal and carpels of hermaphrodite flowers and carpels of female flowers. *CpTM6-2* transcripts were also abundant in petals and stamens of hermaphrodite flowers, petals of female flower, and petals and stamens of male flowers. *CpTM6-2* expression was detectable in sepal of hermaphrodite and male flowers but not in carpels of hermaphrodite or female flowers. Another notable difference of expression pattern among B-class genes is that *CpTM6-2* is highly expressed in leaves while *CpTM6-1* and *CpPI* are not.

Discussion

Southern hybridization of *CpTM6-1* and *CpTM6-2* to papaya genomic DNA did not detect additional genes of the *AP3* lineage. We searched our papaya flower EST database consisting of 8,571 unique genes derived from five papaya flower cDNA libraries (unpublished data), and only found ESTs matching *CpTM6-1*, *CpTM6-2*, and *CpPI* and didn't find any additional B class genes. Moreover, the degenerate

Table 1 List of Sequences used in the phylogenetic analyses. GenBank accession number of each gene is indicated in the parenthesis after the gene names

Classification	Taxa	PI-homologs	AP3-homologs	Reference
Amborellaceae	*Amborella trichopoda*	Am.tr.PI (AY337760)		Kim et al. (2004)
			Am.tr.AP3-1 (AY337743)	Kim et al. (2004)
Nymphaeaceae	*Nuphar variegatum*	Nu.va.PI (AY337737)		Kim et al. (2004)
			Nu.va.AP3-1 (AY337745)	Kim et al. (2004)
Lauraceae	*Persea americana*		Pe.am.AP3 (AY337748)	Kim et al. (2004)
Magnoliaceae	*Liriodendron tulipifera*	LtPI (AF052864)		Kramer et al. (1998)
	Magnolia kobus		MpMADS7 (AB050649)	Unpublished
Aristolochiaceae	*Asarum europaeum*		AeAP3-1 (AF230697)	Kramer and Irish (2000)
Monocots				
Alismataceae	*Sagittaria montevidensis*	SmPI (AF230712)		Kramer and Irish (2000)
Orchidaceae	*Orchis italica*	OrcPI (AB094985)		Unpublished
Poaceae	*Oryza sativa*	OSMADS2 (L37526)		Chung et al. (1995)
			OSMADS16 (AF077760)	Moon et al. (1999)
	Zea mays	ZMM16 (AJ292959)		Münster et al. (2001)
Eudicots				
Ranunculaceae	*Ranunclus bulbosus*		RbAP3-1 (AF052876)	Kramer et al. (1998)
Gunneraceae	*Gunnera tinctoria*		Gu.ti.AP3-1 (AY337753)	Kim et al. (2004)
			Gu.ti.AP3-2 (AY337754)	Kim et al. (2004)
			Gu.ti.AP3-3 (AY337755)	Kim et al. (2004)
Caryophyllaceae	*Silene latifolia*	SLM2 (X80489)		Hardenack et al. (1994)
			SLM3 (X80490)	Hardenack et al. (1994)
Polygonaceae	*Rumex acetosa*		RAD1 (X89113)	Ainsworth et al. (1995)
Myrtaceae	*Eucalyptus grandis*	EGM2 (AF029976)		Southerton et al. (1998)
Saxifragaceae	*Ribes sanguineum*	Ri.sa.PI (AY337742)		Kim et al. (2004)
			Ri.sa.AP3-1 (AY337758)	Kim et al. (2004)
Cucurbitaceae	*Cucumis sativus*	CUM26 (AF043255)		Unpublished
Juglandaceae	*Juglans regia*	JrAP3 (AJ313089)		Unpublished
Salicaceae	*Populus trichocarpa*	PTD (AF057708)		Sheppard et al. (2000)
Rosaceae	Malus domestica	MdPI (AJ291490)		Yao et al. (2001)
	Rosa rugosa	MASAKO BP (AB038462)		Kitahara et al. (2001)
Brassicaceae	*Arabidopsis thaliana*	PI (D30807)		Goto and Meyerowitz (1994)
			AP3 (AF115814)	Purugganan and Suddith (1999)
	Brassica oleracea		BobAP3 (U67456)	Carr and Irish (1997)
			Boi1AP3 (U67453)	Carr and Irish (1997)
Caricaceae	*Carica papaya*	**CpPI (EF562500)**		This study.
			CpTM6-1 (EF562498)	This study.
			CpTM6-2 (EF562499)	This study.
Oleaceae	*Syringa vulgaris*	SvPI (AF052861)		Kramer et al. (1998)
			SvAP3 (AF052869)	Kramer et al. (1998)
Scrophylariaceae	*Antirrhinum majus*	GLO (X68831)		Tröbner et al. (1992)
			DEF (X52023)	Sommer et al. (1990)
Solanaceae	*Hydrangea macrophylla*		HmAP3 (AF230702)	Kramer and Irish (2000)
			HmTM6 (AF230703)	Kramer and Irish (2000)
	Lycopersicon esculentum		LeAP3 (AF052868)	Kramer et al. (1998)
			TM6 (X60759)	Pnueli et al. (1991)
	Nicotiana tabacum	NTGLO (X67959)		Hansen et al. (1993)
			NTDEF (X96428)	Davies et al. (1996)

Table 1 continued

Classification	Taxa	PI-homologs	AP3-homologs	Reference
	Petunia hybrida	FBP1 (M91190)		Angenent et al. (1992)
		PMADS2 (X69947)		Kush et al. (1993)
			PMADS1 (X69946)	Kush et al. (1993)
			PhTM6 (AF230704)	Kramer and Irish (2000)
	Solanum tuberosum		STDEF (X67511)	Garcia-Maroto et al. (1993)
Apiaceae	*Daucus carota*		DcMADS3 (AJ271149)	Unpublished
Asteraceae	*Gerbera hybrida*	GGLO1 (AJ009726)		Yu et al. (1999)
			GDEF1 (AJ009724)	Yu et al. (1999)
	Hieracium piloselloides		HPDEF1 (AF180364)	Guerin et al. (2000)

The three genes reported in this manuscript are in bold font

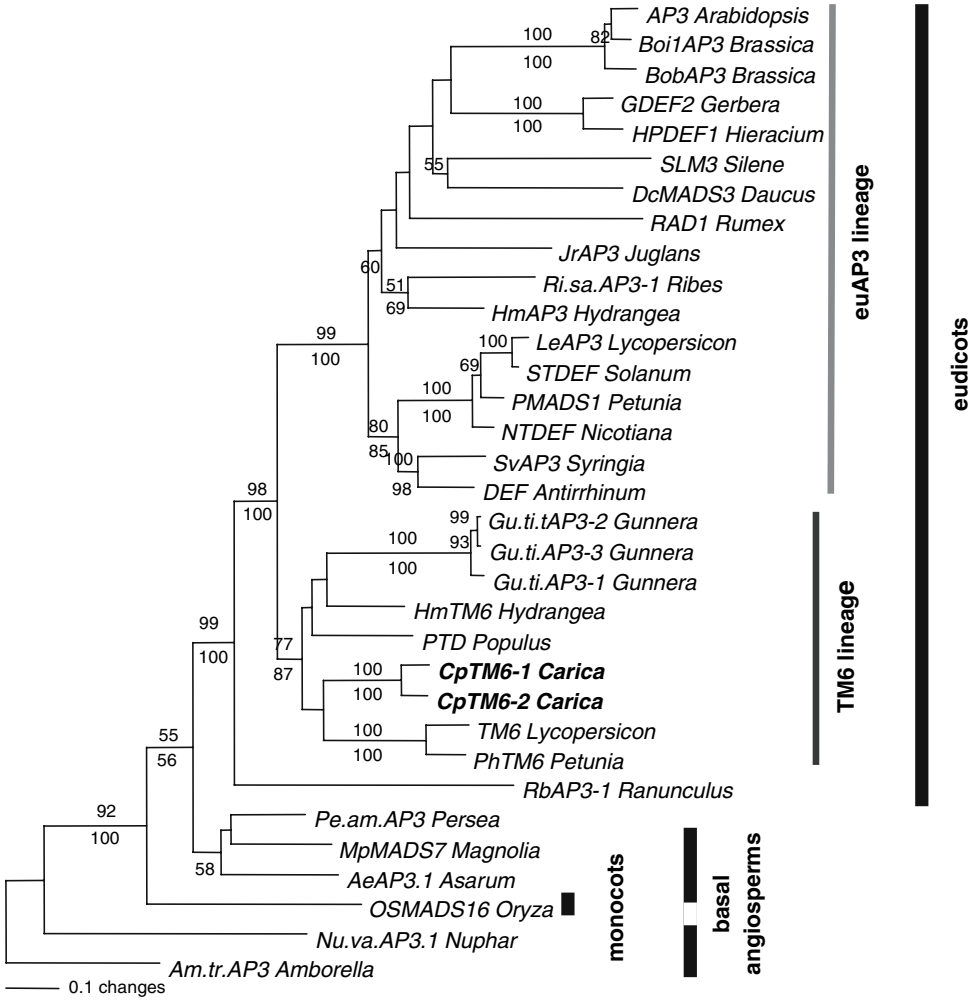

Fig. 3 Maximum likelihood tree of 33 representative *AP3* genes. Numbers above/below the nodes indicate bootstrap values of parsimony and maximum likelihood analyses. Only values over 50% are indicated. Two papaya *AP3* genes were clustered in the *TM6* lineage

primer of *euAP3* (provided by Elena Kramer) was used to amplify cDNA of papaya male and hermaphrodite flowers. The PCR products were cloned and sequenced and didn't yield additional genes of the *AP3* lineage. All these indicated that *euAP3* ortholog might not exist in papaya. The identical restriction patterns among three sex types excluded the possibility that these genes are on the male specific region of the Y chromosome in papaya (Liu et al. 2004). Since *Arabidopsis* has an *euAP3* but no *TM6* ortholog, the loss of *euAP3* in papaya and *TM6* in *Arabidopsis* likely occurred after the divergence of *Arabidopsis* and papaya from a common ancestor about 72 mya (Wikström et al. 2001).

The detection of both *CpTM6-1* and *CpTM6-2* from seven of the ten *CpTM6-2* positive BACs suggested that they located within a BAC. The origin of these two paralogs appeared to be from a tandem duplication occurred

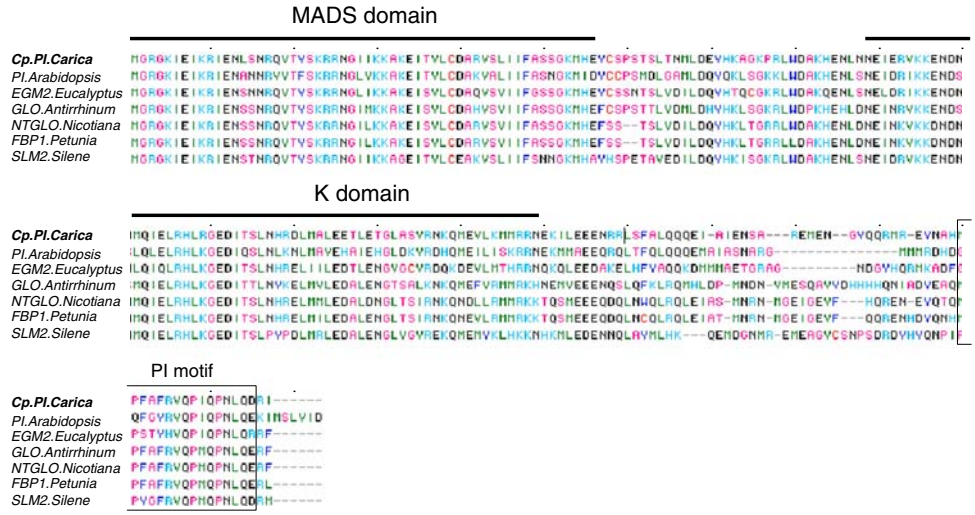

Fig. 4 A protein alignment of papaya *PI* sequence and representatives of other angiosperm *PI* sequences

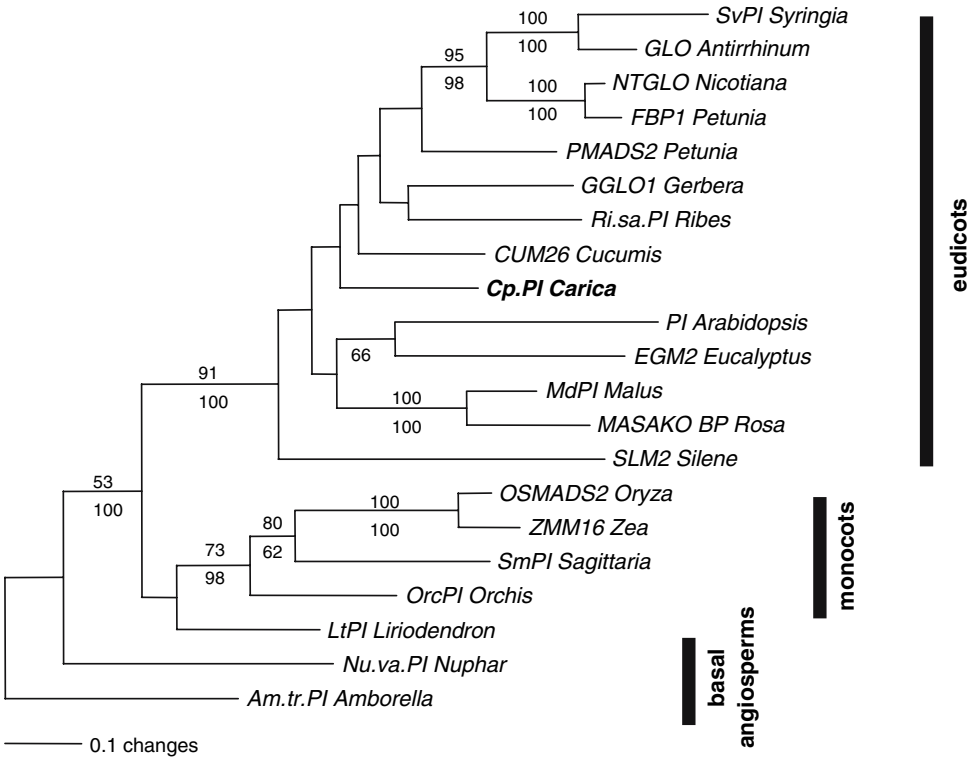

Fig. 5 Maximum likelihood tree of 20 representative *PI* genes. Numbers above/below the nodes indicate bootstrap values of parsimony and maximum likelihood analyses. Only values over 50% are indicated

about 13.4 mya as estimated. The three BACs containing only the *CpTM6-1* likely resulted from digestion at a *Hin*dIII site between these two paralogs

The expression patterns of *CpTM6-1* and *CpTM6-2* validated the potential of *TM6* as a fully functional B-class gene as had been suggested by ectopic overexpression of *TM6* in *tap3* background in tomato and in *def* background in *Petunia* (de Martino et al. 2006; Rijpkema et al. 2006). In species having both *euAP3* and *TM6* paralogs, their functions appeared to have diverged and are partially redundant; the function of *TM6* as a B-class gene is primarily in the third whorl, whereas *euAp3* functions in both second and third whorls. In tomato, RNAi-induced loss of *TM6* function caused a homeotic conversion of stamens to carpel-like organs (de Martino et al. 2006). However, these TM6i lines showed little effect on petal development other than a reduced overall size (de Martino et al. 2006), possibly caused by reduced cell proliferation (Sheppard et al. 2000). In *Petunia*, *TM6* also functions as a B-class gene in determination of stamen identity, but *TM6* is regulated as a C-class gene and is expressed in third and fourth whorls (Rijpkema et al. 2006). In papaya, both *CpTM6-1* and *CpTM6-2* are expressed in petals and stamens and detectable low-level expression in sepals. The strong expression in petals contracts with what has been observed for *TM6* orthologs in tomato and *Petunia* (de Martino et al. 2006;

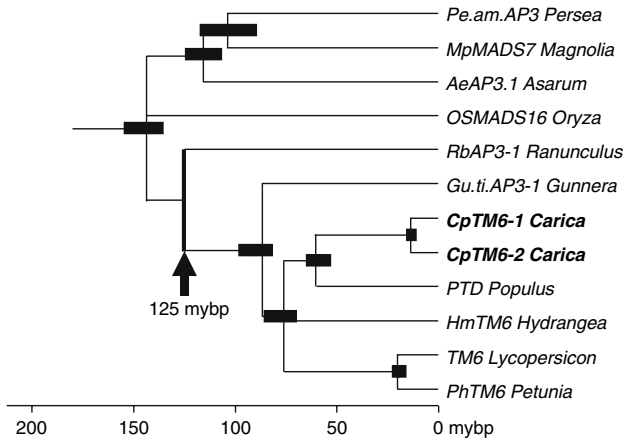

Fig. 6 *AP3*-like gene chronogram calibrated with the origin of eudicots at 125 mya (*arrow*). *Boxes* indicate standard deviation range of bootstrap analyses

Rijpkema et al. 2006). However, this expression pattern is similar to what has been detected for the two *TM6* orthologs in apple (Kitahara et al. 2004).

The differences are the robust expression of *CpTM6-2* in leaves and the detectable low level expression of *CpTM6-1* in carpels (Fig. 7). The C-terminal domain of *CpTM6-2* is exactly the same as that of the tomato and *Petunia TM6* genes. However, *CpTM6-2* did not express in the fourth whorl of papaya as was shown in *Petunia* and tomato. This fact indicates that the subfunctionalization of B-class gene paralogs varies among species, particularly in those species without a paralog of the *euAP3* sublineage such as papaya.

Lamb and Irish (2003) reported that the *euAP3* and *paleoAP3* C-terminal motifs are critical for the functional specificity of the *AP3* protein and that each of these motifs was sufficient to confer distinct *AP3* functions. De Martino et al. (2006) and Rijpkema et al. (2006) provide additional evidence that the *AP3* orthologs with only *paleoAP3* motifs are functionally divergent from the orthologs containing only the *euAP3* motifs, or both *euAP3* and *paleoAP3*. This intriguing research area, stemming from the study by Kramer et al. (1998), warrants more detailed expression studies on *CpTM6-1* and *CpTM6-2* to determine how their functions evolved without an *euAP3* paralog by comparing them with other orthologs having *paleoAP3* motifs. For example, the highest signals for *PhTM6* transcripts (a *Petunia* ortholog with a *paleoAP3* motif) were detected in early stage (5 mm) carpels and stamens, whereas expression levels observed in petals and sepals were much lower. In very late flower development stages (40–50 mm), *PhTM6* expression levels remained high in the fourth whorl while declining in the third whorl stamens (Vandenbussche et al. 2004).

The amino acid alignments between *CpPI* and *PI* revealed a relatively high identity (60%). We can therefore

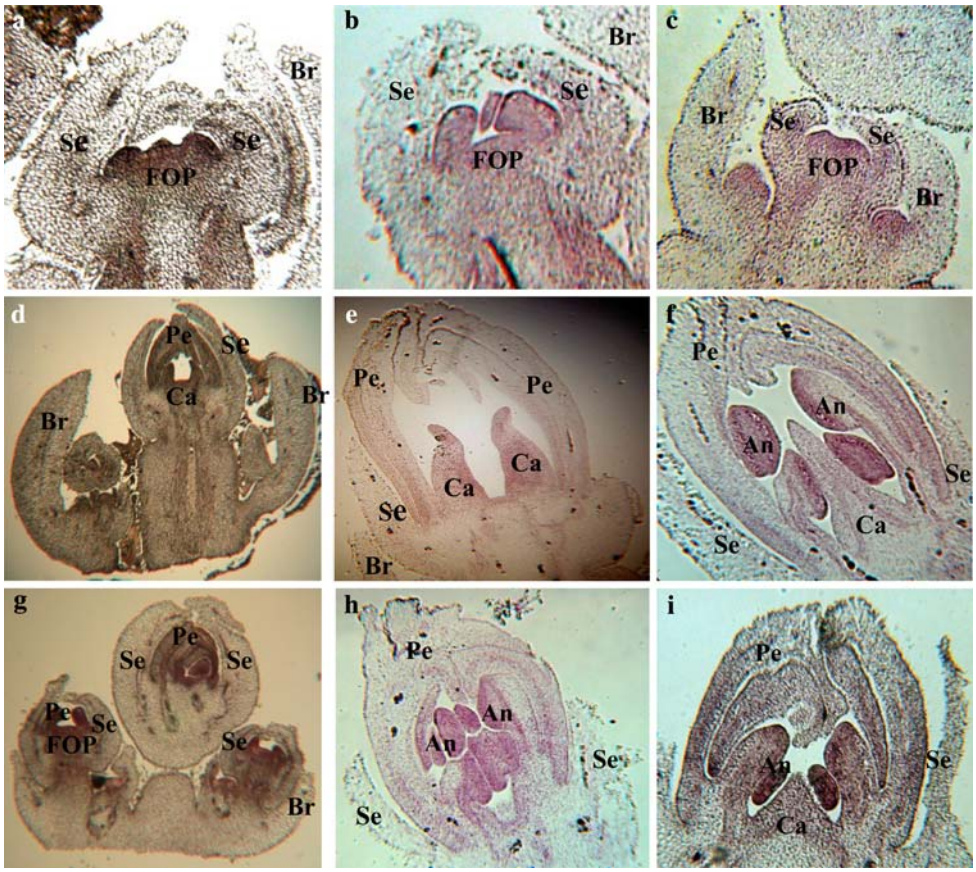

Fig. 7 In-situ hybridization of *CpTM6-1* and *PI* transcripts in floral tissues of SunUp papaya. All figures are longitudinal sections of floral organ primordia (*FOP*) in papaya flowers representing all three sex types. **a–c** FOP consists of the petal primordia (outer bulges in the FOP) and the sexual organ primordia (inner bulge of the FOP). *CpTM6-1* expression in <1 mm hermaphrodite flower buds (**a**), in <1 mm female buds (**b**), in <1 mm male flower buds (**c**), in a 1 mm female flower bud (**d**), in a 3 mm female bud (**e**), in a 3 mm hermaphrodite flower bud (**f**). *PI* expression in <1 mm male flower buds (**g**), in a 2 mm male flower bud (**h**) and in a 2 mm hermaphrodite flower bud (**i**). *Br* bracts, *Se* sepals, *Pe* petals, *Ca* carpels, *An* roots, *AC* aborted carpels. The images were taken with ×40 magnification

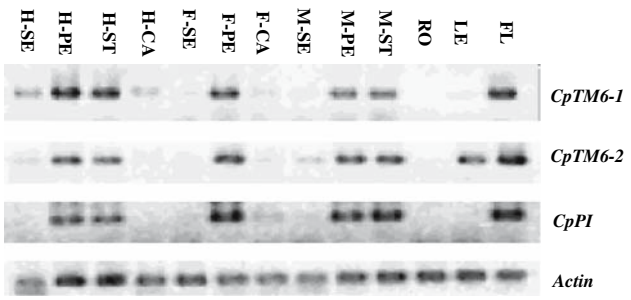

Fig. 8 RT-PCR test of the expression patterns of *CpTM6-1*, *CpTM6-2*, and *CpPI*. *H* hermaphrodite flowers, *F* female flowers, *M* male flowers, *SE* sepals, *PE* petals, *ST* stamens, *CA* carpels, *RO* roots, *LE* leaf, *FL* flowers. Papaya *actin* gene was used as positive control

assume that these two orthologs have not diverged as much as the corresponding *AP3* homologs of these two species, i.e., papaya and *Arabidopsis*. Not only do *CpPI* and *PI* share higher overall identity, and nearly 100% identity in the MADS-box region, they are also seen to be more closely related to each other in overall phylogeny (Fig. 5).

The general expression patterns of *CpTM6-1*, *CpTM6-2*, and *CpPI* were similar to the B-class genes in other eudicots, but differ somewhat from the expression patterns of their *Arabidopsis* homologs (Jack et al. 1992; Goto and Meyerowitz 1994). Our in-situ data shows that *CpTM6* shares expression patterns similar to those of the *Antirrhinum DEF* ortholog (Sommer et al. 1990; Schwarz-Sommer et al. 1992). Strong expression of *CpTM6* was seen in floral organ primordia during the early flower development. Vandenbussche et al. (2004) detected low levels of *PhDEF* expression in the first and fourth whorls of *Petunia* using RT-PCR. Similar expression patterns have been observed in papaya *CpTM6*, tobacco *NTDEF* (Davies et al. 1996), and tomato *TM6* genes (Pnueli et al. 1994).

We tested the expression of *CpTM6* in all three-sex types of papaya and confirmed no differences among male, female, and hermaphrodite flowers at early developmental stage before the differentiation of floral organ primordia. The expression of B- and C-class genes is expected in early developmental stage of male flowers, because males are formed by carpel abortion at a relatively late stage with visible remnants of aborted carpels and occasionally sex reversal with fully functional carpels. Abortion of stamens in papaya female flowers occurs very early leaving no trace of the stamens. The expression of *CpTM6* in 0.5 mm flowers of all three-sex types raises the possibility that the abortion of stamens might not occur from inception as it appears based on female flower morphology. The suppression of B class gene expression that was observed in 1–2 mm female flowers indicated that the sex determination genes have been turned on by this developmental stage. Whether the B class genes are the direct targets of the sex determination genes remains to be tested. Analyses of B class genes in dioecious species sorrel, white campion, asparagus, and *Thalictrum dioicum* revealed no common role for homologs of B class genes in sex determination (Hardenack et al. 1994; Ainsworth et al. 1995; Park et al. 2003; Di Stilio et al. 2005). Our findings in papaya are consistent with this observation.

The estimated divergence time of 58.5 my between papaya and black cottonwood (*Populus trichocarpa*) *TM6* orthologs is about half the divergence time of 109 my between papaya and *Populus* (Wikström et al. 2001). This discrepancy might be explained by variation of molecular evolution among individual genes and/or the reduced rate of sequence evolution in black cottonwood because it is a long-lived and vegetatively propagated species with the potential to contribute gametes to multiple generations (Tuskan et al. 2006).

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