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Isolation and characterization of the papaya MADS-box E-class genes, *CpMADS1* and *CpMADS3*, and a TM6 lineage gene *CpMADS2*

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ABSTRACT. Papaya (*Carica papaya* L.) plants are polygamous, with female, male, and hermaphroditic flowers. To understand the roles of MADS-box genes in flower development and sex determination, we cloned cDNAs of E-class genes *CpMADS1* and *CpMADS3* and a TM6 lineage of the B-class gene *CpMADS2* from young flower buds of papaya. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR analyses revealed that *CpMADS1* and *CpMADS3* were preferentially expressed in the carpel and also in petals and stamens. *CpMADS2* was expressed in both petals and stamens early during floral development. Comparison of 10 papaya genotypes of 5 different sex phenotypes - hermaphrodite, male, female, progeny-all-hermaphrodite, and progeny-all-male - by Southern blot analysis of genomic DNAs with probes of the 3 genes revealed similar restriction patterns and copy

number, suggesting a low relationship of the 3 *CpMADS* genes with sex expression of papaya plants at the genomic level.

Key words: *Carica papaya*; Floral sex expression; MADS-box genes

INTRODUCTION

Papaya (*Carica papaya* L.) is an economically important fruit. Papaya plants are polygamous, having female, male, and hermaphrodite reproductive organs (Figure 1). Storey (1953) proposed 3 alleles, *M*, M^h, and *m*, to determine plant sex types. The genotypes for female, hermaphrodite, and male plants are represented by *mm*, M^hm, and *Mm*, respectively. Papaya often exhibits male and imperfect hermaphrodite flowers because the floral organ development might be influenced by environmental and hormonal factors. Further, bisexual flowers could appear on male plants (Lange, 1961; Ghosh and Sen, 1975; Allan et al., 1987; Sharan et al., 1994). Despite various causes for the malformation of papaya fruits, hermaphrodite papayas are favored worldwide for economic production (Giacometti, 1987). However, bisexual flowers often possess unfavorable features, such as pistil degeneration and carpel-like anthers, which leads to fruit malformation, resulting in yield loss (Nakasone, 1986). A hermaphroditic papaya mutant (SR*) has been derived from the 'Sunrise' papaya cultivar. Genetic study revealed that SR* possesses the $M_{@}ml$ genotype, which suggests that it as a novel mutant. SR* is capable of producing all hermaphroditic papaya progenies (Chiu et al., 2003) and might provide an opportunity to improve all hermaphroditic papaya cultivars.

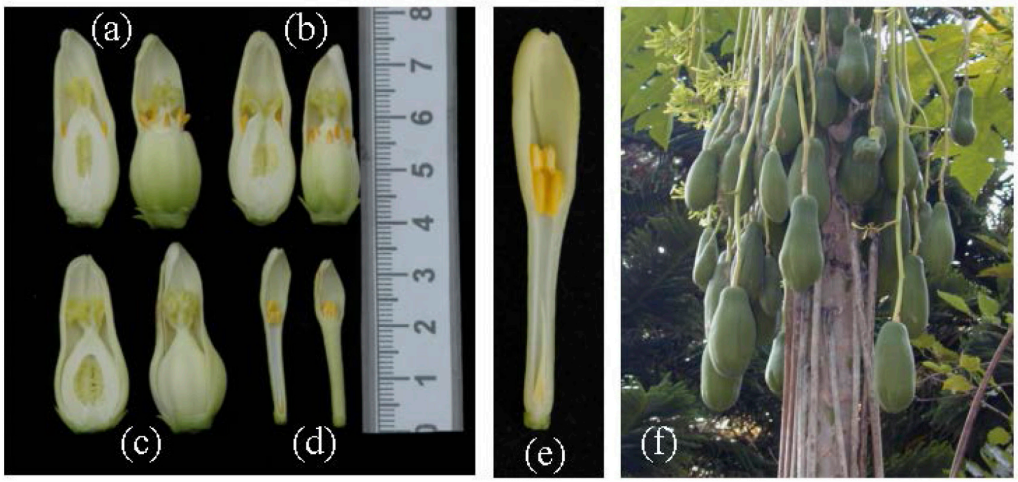

Figure 1. Morphology of flower buds from (**a**) all-hermaphroditic 'SR*-1-1-1-1', (**b**) hermaphroditic 'Sunrise', (**c**) female 'Sunrise', (**d**), (**e**) male 'Florida' papaya, and (**f**) the fruiting of male papaya.

Sex expression in papaya flowers is probably determined very early during floral differentiation (Arkle Jr. and Nakasone, 1984). Lange (1961) recognized that a hermaphrodite papaya might generate 15 floral phenotypes through pistil degeneration and the formation of

carpel-like anthers, with only one containing 5 carpels and 10 stamens producing a normal elongate hermaphrodite flower. Therefore, because of the complex sex phenotypes, papaya is a valuable source for studying genetic regulation of floral differentiation. Understanding the relationships between genes regulating floral organ differentiation and their upstream or downstream regulators is desirable (Ming et al., 2007).

Floral organ development of flowering plants is determined mainly by a group of MADS-box genes. An ABC model of flower development has been proposed from developmental and molecular studies of *Arabidopsis* and *Antirrhinum* (Coen and Meyerowitz, 1991). A-class MADS-box genes are expressed in sepals. A- and B-class genes interact to determine petal formation. B- and C-class genes control anther development, and C-class genes also determine carpel differentiation (Weigel and Meyerowitz, 1994). In addition to the ABC model of floral control, a D-class MADS-box genes was found to control ovule differentiation, as in *Petunia hybrida* (Angenent et al., 1995a,b; Colombo et al., 1995, 1997). Recent studies revealed that *SEPALLATA1/2/3* (*SEP1*/*2*/*3*) may interact with members of other MADS-box genes to regulate petal, stamen, and pistil development; therefore, these were grouped into E-class genes. The current concept of floral development has been refined as the ABCDE model (Pelaz et al., 2000; Theissen et al., 2000; Theissen, 2001; Honma and Goto, 2001).

The present study aimed to clone the MADS-box genes regulating floral organ development of papaya, especially the E-function genes. The expression pattern of the 2 E-class genes *CpMADS1* and *CpMADS3* was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR and compared with those of *CpMADS2*, a TM6 lineage gene. Papaya genotypes with different sex phenotypes were compared by genomic Southern hybridization to determine whether the MADS genes are related to sex types.

MATERIAL AND METHODS

Plant materials

Papaya plants grown in the field of the Pingtung Seed and Seedling Research Center of Taiwan Seed Improvement and Propagation Station were used. These included the hermaphroditic cultivars ‘Sunrise’, ‘Thailand’, and ‘Tainung No. 2’; the female plants of cultivars ‘Thailand’, ‘Tainung No. 2’, ‘Sunrise’, and ‘Florida’; the male plants of the cultivar ‘Florida’; the progeny-all-hermaphroditic ‘SR*-1-1-1-1’ (Chiu et al., 2003); and the progeny-all-male ‘mf-1-5-3-8’ (derived by cross-pollinating ‘SR*-1-1-1-1’ and capable of producing all male papaya progenies; Chiu CT, unpublished data). Samples of floral parts were used for total RNA isolation and samples of leaves were used for genomic DNA isolation. The floral sex phenotype of different cultivars is shown in Figure 1.

cDNA cloning and RT-PCR

Total RNA was extracted from floral parts and leaves of papaya. First-strand cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) and used as templates for PCR amplification of partial fragments of *CpMADS1*, *CpMADS2*, and *CpMADS3* with degenerate and oligo dT primers (Table 1). To obtain full-length cDNAs, 5'- and 3'-rapid amplification of cDNA ends (RACE) was performed using the SMART RACE

cDNA Amplification Kit (CLONTECH, Palo Alto, CA, USA) and gene-specific primers (Table 1). Gene expression levels were determined by extracting total RNAs (3 µg) from various papaya organs; these were reverse transcribed into first-strand cDNA and PCR-amplified using gene-specific primers (Table 1).

Table 1. Primer sets used for gene cloning, RT-PCR and real-time PCR.

	5' Primer	3' Primer
Gene cloning		
CpMADS1, *CpMADS3*	5'-CGAGAACWMGAYSAACCGGCAGG-3'	oligo dT
CpMADS2 cloning	5'-GGIMGIGGIAARATIGARATIAARMGIAT-3' (Kramer et al., 2004)	oligo dT
RACE gene-specific primer		
CpMADS1	5'-CTCGAGCTCCTTCGCTGGTTTGCTA-3'	5'-GCTCTGTGATGCTGAGGTGGCTCTC-3'
CpMADS2	5'-GTGGTGAAGGCGAAATGCTTGAAGG-3'	5'-TTGGAGGTCATACGCGAGCGAAAGT-3'
CpMADS3	5'-AGAGCTCCTTCAAGCTGCT TCTC-3'	5'-ACACAGAGCTGGTATCAGGAGGTAG-3'
Full-length cDNA		
CpMADS1	5'-GCGgaattcATGGGGAGAGGAAGAGTAGAGTT-3'	5'-GCGcccgggTCAAAGCATCCAGCCA-3'
CpMADS2	5'-GCGctcgagATGGGTCGTGGAAAGATTGAGAT-3'	5'-GCGggatccTCAAGCAAGACGAAGATCACTGC-3'
CpMADS3	5'-GCGctcgagATGGGGAGAGGAAGAGTGGAA-3'	5'-GCGggatccTCAAAGGAGCCATCCCTGG-3'
RT-PCR analysis		
CpMADS1	5'-GCGgaattcCCCTTTAAACACCAAAGAGCTG-3'	5'-GCGgagctcTTGGCCTTGACTTGTGGCTG-3'
CpMADS2	5'-GCGctcgagCCCCACCACCACGAC-3'	5'-GCGgagctcGTAATGTGGATCATTATGACATC-3'
CpMADS3	5'-GCGctcgagAGAACAAGATCAACAGACAAGTGA-3'	5'-GCGgagctcAGCTGCTTCTCAAGATTTTG-3'
Papain	5'-GGGCATTCTCAGCTGTTGTA-3'	5'-CGACAATAACGTTGCACTCC-3'
Real-time PCR analysis		
CpMADS1	5'-CGTATCTTATGGGCACCAACAC-3'	5'-GGTCTGAACCAACTGGGTTGTAC-3'
CpMADS2	5'-GAGATTAGGCGAAGAAATGGTGAA-3'	5'-TCGCGTATGACCTCCAAAGC-3'
CpMADS3	5'-CCACTTTGCACTTCACCCTTCT-3'	5'-GCCCCTCATCACTTCCAACA-3'
Papain	5'-CTACGGGTGCAATGGAGGTT-3'	5'-TTTCTCCCTTGAGCGACAATAAC-3'

Real-time PCR analysis

Four primer pairs (Table 1) were designed using the Integrated DNA Technologies network (http://www.idtdna.com/Scitools/applications/primerquest) to amplify the corresponding MADS-box genes. The cDNA templates for real-time PCR were the same as for RT-PCR. Reactions containing SYBR Green (Applied Biosystems) involved the use of the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). The PCR conditions were 2 min at 50°C, followed by 10 min at 95°C and 50 cycles of 95°C for 15 s and 60°C for 1 min. All PCR data were generated from at least 3 independent reactions for each biological replication. The *Papain* gene was used as a standard to control for sample variation in the total amount of RNA across different reactions.

Southern hybridization of papaya genomic DNA

Genomic DNA from the 10 papaya genotypes was isolated from leaves by using the cetyltrimethylammonium bromide method (Doyle and Doyle, 1987). For Southern hybridization, 15 µg genomic DNA was digested with *Eco*RI (NEB, USA), size fractionated on 0.8% agarose gels, and transferred to Hybond N^+ membranes (Amersham International, Buckinghamshire, UK). Three DNA fragments specific for *CpMADS1*, *CpMADS2*, and *CpMADS3* were amplified from their respective cDNAs by adding α-^{32}P-dCTP to prepare radioactive probes. Southern hybridization was according to Sambrook and Russell (2001).

RESULTS

Identification and characterization of 3 MADS-box genes in papaya

To isolate MADS-box genes from papaya, we used a combination of RT-PCR and RACE. Sequence comparison led to the identification of several MADS-box genes. Three full-length cDNAs were cloned and named *CpMADS1* (EU659990), *CpMADS2* (EU659991), and *CpMADS3* (EU659992) (*Carica papaya* MADS). Each clone contained an open-reading frame with flanking 5'- and 3'-untranslated regions and a poly(A)$^+$ tail. The cDNAs for *CpMADS1*, *CpMADS2*, and *CpMADS3* were 735, 681, and 741 bp and encoded 245, 227, and 247 amino acid residues, respectively (Figure 2). The deduced amino acid sequences of *CpMADS1*, *CpMADS2*, and *CpMADS3* shared significant homology with other MADS-box proteins. *CpMADS1 s*howed 87% identity and 93% similarity to *SEPALLATA1* homolog (BAF95941, *Citrus unshiu*), *CpMADS2 s*howed 100% identity to *CpTM6-1* (ABQ51321, *Carica papaya*; Ackerman et al., 2008), and *CpMADS3 s*howed 81% identity and 89% similarity to apple MADS-box protein (CAA04325, *Malus* x *domestica*).

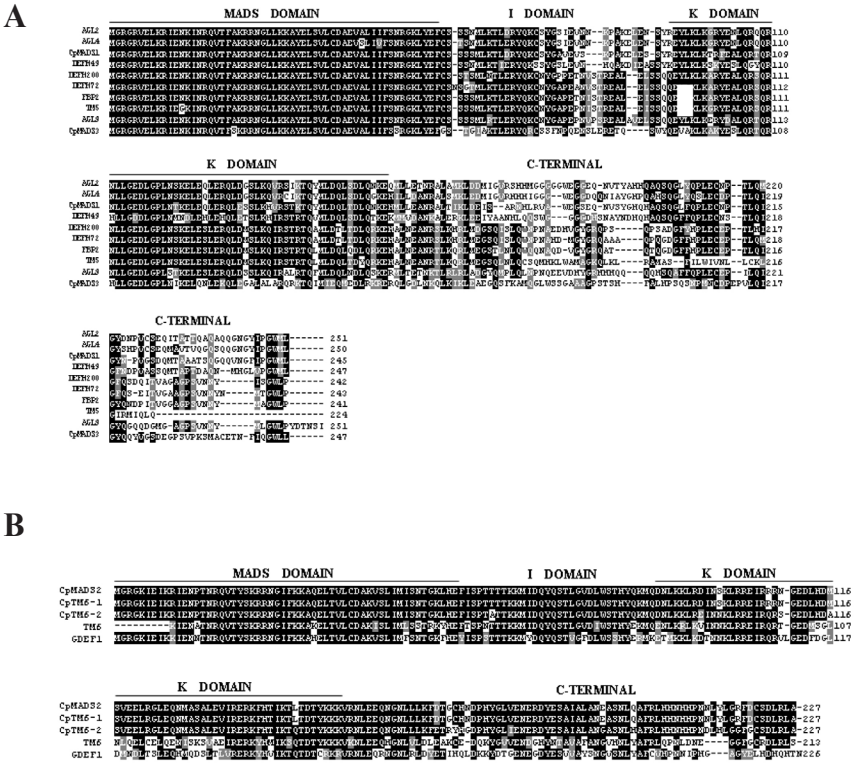

Figure 2. A. Alignment of amino acid sequence of *CpMADS1*, *CpMADS3* and related E-function MADS-box genes. *AGL2*, *AGL4* and *AGL9*, *Arabidopsis thaliana*; *CpMADS1* and *CpMADS3*, *Carica papaya*; DEFH49, DEFH200 and DEFH72, *Antirrhinum majus*; FBP2, *Petunia hybrida*; TM5, *Lycopersicon esculentum*. **B.** Alignment of amino acid sequence of *CpMADS2* and related TM6 lineage MADS-box genes. *CpMADS2*, *CpTM6-1* and *CpTM6-2*, *C. papaya*; *TM6*, *L. esculentum*; *GDEF1*, *Gerbera hybrida.*

The papaya MADS-box sequences were compared with those from Brassicaceae (*Arabidopsis*), Solanaceae (*Petunia* x *hybrida*), and others (Table 2) by using ClustalW multiple alignment and phylogenetic analysis (http://www.ebi.ac.uk/clustalw). *CpMADS1* and *CpMADS3* were assigned to E-class MADS-box genes and were closely related to *AGL2* (*SEP1*), *AGL4* (*SEP2*) of *Arabidopsis* or *FBP2* of petunia (Angenent et al., 1992). *CpMADS2* (Figure 3) belonged to B-class MADS-box genes and was identical to *CpTM6-1* (Ackerman, et al., 2008).

Table 2. Sequences used for phylogenetic analysis.

Classification	Taxa	Gene (Accession No.)	MADS-box class
Poaceae	*Zea mays*	ZAP1 (NP_001105333	A
		ZAG2 (CAA56504)	D
	Oryza sativa	OsMADS14 (P0C5B1)	A
		OsMADS4 (Q40703)	B (PI lineage)
		OsMADS16 (Q944S9)	B (Paleo Ap3 lineage)
		OsMADS13 (Q2QW53)	D
Liliaceae	*Lilium longiflorum*	LMADS1 (AAM27456)	B (Paleo Ap3 lineage)
Caryophyllaceae	*Silene latifolia*	SLM3 (CAA56657)	B (AP3 lineage)
Brassicaceae	*Arabidopsis thaliana*	AP1 (CAA78909)	A
		AGL8 (AAA97403)	A
		PI (P48007)	B (PI lineage)
		AP3 (AAD51903)	B (Ap3 lineage)
		AG (P17839)	C
		AGL1 (M5550)	C
		AGL11 (AAC49080)	D
		AGL9 (AAB67832)	E
		AGL2 (P29382)	E
		AGL4 (P29384)	E
	Brassica oleracea	BOI1AP3 (AAB08877)	B (Ap3 lineage)
Solanaceae	*Petunia x hybrida*	FBP3 (CAA50549)	B (PI lineage)
		PMADS2 (CAA49568)	B (PI lineage)
		FBP1 (Q03488)	B (PI lineage)
		PMADS 1 (Q07472)	B (Ap3 lineage)
		FBP6 (CAA48635)	C
		FBP11 (CAA57445)	D
		FBP7 (CAA57311)	D
		FBP2 (Q03489)	E
	Nicotiana tabacum	NAP1-1 (AAD01421)	A
		NAG1 (AAA17033)	C
	Lycopersicon esculentum	TM6 (DQ539419)	B (TM6 lineage)
		TAG1 (AAP35239)	C
		TM5 (Q42464)	E
Cucurbitaceae	*Cucumis sativus*	CUM1 (AAP35238)	C
		CUM10 (AAC08529)	D
Plantaginaceae	*Antirrhinum majus*	SQUA (CAA45228)	A
		DEFH49 (CAA64741)	E
		DEFH200 (CAA64743)	E
		DEFH72 (CAA64742)	E
Asteraceae	*Gerbera hybrida*	GGLO1 (AJ009726)	B (PI lineage)
		GLO (Q03378)	B (PI lineage)
		GDEF1 (AJ009724)	B (TM6 lineage)
		GDEF2 (AJ009725)	B (Ap3 lineage)
Fabaceae	*Medicago sativa*	NMH 7 (AAC15419)	B (Ap3 lineage)
Caricaceae	*Carica papaya*	CpPI (ABQ51323)	B (PI lineage)
		CpMADS2 (EU659991)	B (TM6 lineage)
		TM6-1 (ABQ51321)	B (TM6 lineage)
		TM6-2 (ABQ51322)	B (TM6 lineage)
		CpMADS1 (EU659990)	E
		CpMADS3 (EU659992)	E
Myrtaceae	*Eucalyptus globulus*	EAP1 (AAG24909)	A

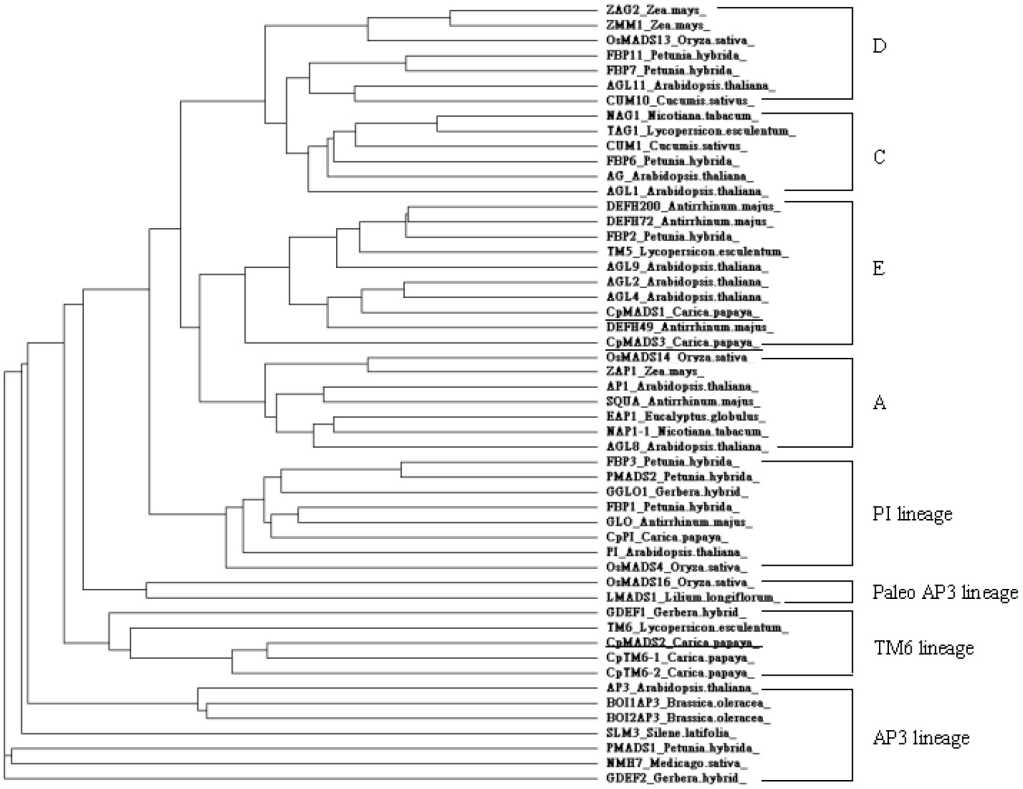

Figure 3. Phylogenetic analysis of representative plant A-, B-, C-, D-, and E-function MADS-box genes. On the basis of similarity of amino acid sequence, *CpMADS1* and *CpMADS3* were assigned to E-class MADS-box genes and were closely related to AGL2 and AGL4 of *Arabidopsis* or FBP2 of petunia. *CpMADS2* was assigned to B-class MADS-box genes and was the same as CpTM6-1. Names of *CpMADS1*, *CpMADS2* and *CpMADS3* are underlined. Names of the plant species for each MADS-box gene are after the protein names. The tree was generated by ClustalW multiple alignment program (http://www.ebi.ac.uk/clustalw/).

The *CpMADS1* and *CpMADS3* proteins contained sequences representing a complete MADS domain within the first 56 amino acids of their N terminus and the keratin-like coiled coil domain (K domain) within amino acids 91-157 (Figure 2A). The *CpMADS2* protein contained a sequence representing a complete MADS-box within the first 56 amino acid of its N terminus and the K domain within amino acids 89-153 (Figure 2B). All 3 genes belonged to type II MADS-box genes (MIKC-type genes), which contain a typical MADS-box domain, an intervening domain, a K domain, and a C terminal (Nam et al., 2004).

Southern analysis of *CpMADS1*, *CpMADS2*, and *CpMADS3*

Southern hybridization of *CpMADS1*, *CpMADS2*, and *CpMADS3* was performed on genomic DNA of the 10 varieties of papaya digested with *Eco*RI. Hybridization with the *CpMADS1*-specific probe detected 2 fragments of 1.3 and 5 kb. Because our *CpMADS2* sequence was identical to that of *CpTM6-1*, the Southern blot showed the same hybridization

pattern as was reported previously (Ackerman et al., 2008), revealing 3 bands at about 3.6, 2.3, and 1.2 kb (Figure 4). The genomic region of *CpMADS3* revealed a band only at about 6.6 kb. We found no genotypic or sex-phenotype difference by Southern hybridization with individual *CpMADS* probes.

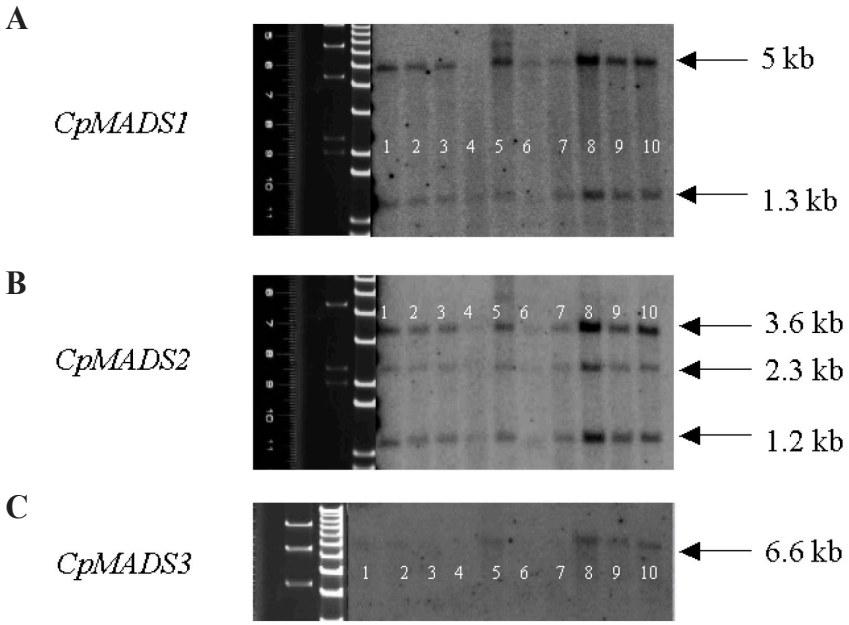

Figure 4. Southern blot analysis of 10 *Carica papaya* genomic DNA samples. *Lane 1* = hermaphroditic 'Thailand'; *lane 2* = female 'Thailand'; *lane 3* = female 'Tainung No. 2'; *lane 4* = hermaphroditic 'Tainung No. 2'; *lane 5* = female 'Sunrise'; *lane 6* = hermaphroditic 'Sunrise'; *lane 7* = progeny-all-hermaphroditic 'SR*-1-1-1-1'; *lane 8* = progeny-all-male 'mf-1-5-3-8'; *lane 9* = male 'Florida'; *lane 10* = female 'Florida'. Genomic DNA digested with *Eco*RI underwent Southern blot hybridization with radioactively labeled, full-length (**A**) *CpMADS1*, (**B**) *CpMADS2* and (**C**) *CpMADS3* DNA probe.

Expression pattern of *CpMADS1*, *CpMADS2*, and *CpMADS3* in different tissues of papaya plants

The temporal and spatial expression patterns of *CpMADS1* and *CpMADS2* were determined by performing RT-PCR on total RNA extracted from young flower buds (1-20 mm), floral organs of mature flower buds, and vegetative tissues. The expression of all 3 MADS-box genes could be detected in 1-mm long flower buds and was retained at every developmental stage (Figure 5). In 35-mm long floral organs, *CpMADS1* and *CpMADS3* mRNA were expressed in petals, stamens, and carpel of hermaphroditic varieties (Figure 5a, c, e, g), petals and carpels of the female variety (Figure 5i, k), and petals and stamens of the male variety (Figure 5m, o). *CpMADS2* mRNA was expressed in petals and stamens of hermaphroditic and male flowers (Figure 5c, g, n) and petals of female flowers (Figure 5j). All 3 MADS-box genes were preferentially expressed in reproductive tissues. *CpMADS1*, *CpMADS2*, and *CpMADS3* were not expressed in roots and stems, except for a low level of expression of *CpMADS2* and *CpMADS3* in the leaves of some genotypes (Figure 5g, j, n).

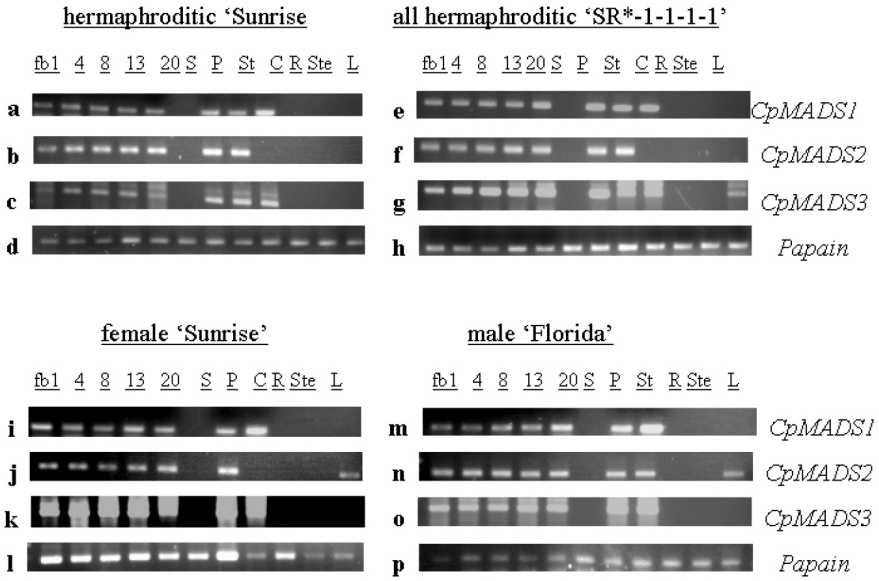

Figure 5. Detection of *CpMADS1*, *CpMADS2* and *CpMADS3* expression by RT-PCR. Flower buds (fb) at different developmental stages (1, 4, 8, 13, 20 mm), from flower organs, sepal (S), petal (P), stamens (St), and carpel (C) of 35-mm long floral buds, and from vegetative tissue root (R), stem (Ste) and leaf (L) of 4 papaya varieties: hermaphroditic 'Sunrise', all hermaphroditic 'SR*-1-1-1-1', female 'Sunrise', and male 'Florida' used as a template. *Papain* gene was used as positive control.

Quantitative real-time PCR was conducted to determine the relative expression level of *CpMADS1*, *CpMADS2*, and *CpMADS3* in hermaphroditic 'Sunrise' papaya flower buds and floral organs. The relative expression of all 3 genes was found during early flower bud development (1 mm; Figure 6). The transcript level decreased gradually with developmental stage until floral buds were 20 mm long, when the expression was increased. The relative mRNA level of *CpMADS1* and *CpMADS3* was higher in 20-mm floral buds than in 1-mm young buds. In floral parts, *CpMADS1* and *CpMADS3* were preferentially expressed in carpels, and then in petals and stamens (Figure 6A and C). *CpMADS2* was mainly expressed in stamens, then in petals, with a barely detectable level in carpels (Figure 6B).

DISCUSSION

The complete sequencing of the *Arabidopsis* genome has revealed more than 100 different MADS-box genes in this species (West et al., 1998). With the progress in understanding of the control of MADS-box genes in organ differentiation of flowers in *Arabidopsis*, studies have been conducted to elucidate the relationship between the MADS-box genes and sex determination in economic crops with gender specificity, such as monoecious cucumber (Kater et al., 2001) and corn (Heuer et al., 2001; Schreiber et al., 2004), dioecious asparagus (Park et al., 2003), and trioecious papaya (Ackerman et al., 2008). Trioecious papaya is an economically important sub-tropical fruit crop, with its regular hermaphroditic flowers and production yield is emphasized in hermaphrodite papaya cultivation (Arkle Jr. and Nakasone,

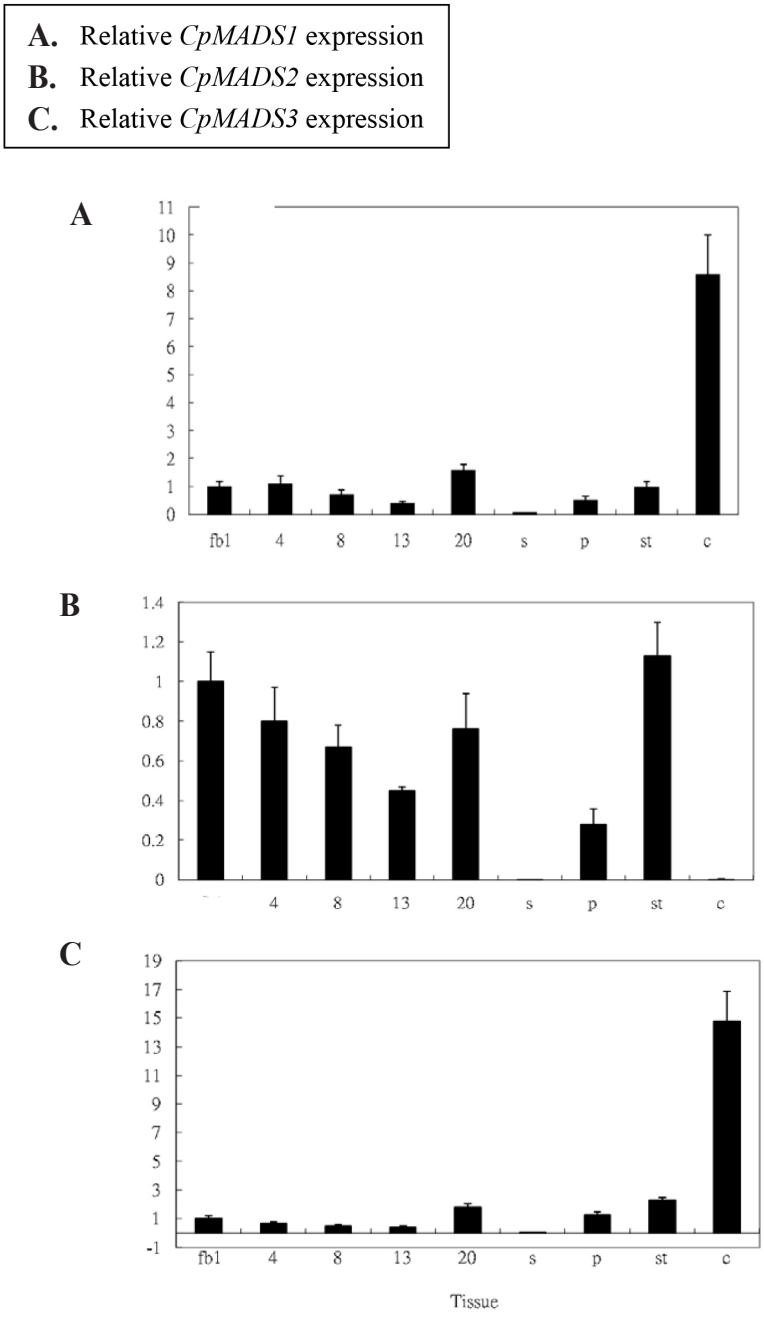

Figure 6. A. *CpMADS1*, **B.** *CpMADS2* and **C.** *CpMADS3* expression analysis in different developmental stages of flower buds (fb 1, 4, 8, 13, 20 mm), and flower organs, sepal (s), petal (p), stamens (st), and carpel (c) of 35-mm long floral buds of hermaphroditic 'Sunrise' papaya by quantitative real-time PCR. Error bars represent standard deviations calculated from 3 replications.

1984). Recently, 3 B-class MADS-box genes of papaya, *CpTM6-1*, *CpTM6-2*, and *CpPI*, were isolated and shown to be involved in the differentiation and development of petals and stamens (Ackerman et al., 2008). No other MADS-box genes of papaya have been reported. In the present study, we cloned 2 E-class MADS-box genes, *CpMADS1* and *CpMADS3*, and 1 B-class gene *CpMADS2*. Multiple alignment and phylogenetic analysis of the papaya MADS-box genes suggest a broad conservation of floral homeotic gene functions between papaya and other species (Figures 2 and 3).

The relatively high transcript level of both *CpMADS1* and *CpMADS3* genes in the carpel suggests their potential role in carpel development (Figures 5 and 6). In addition, *CpMADS1* and *CpMADS3* were specifically expressed in the inner 3 whorls of the papaya flower, similar to *SEP3* (Mandel and Yanofsky, 1998) and *SEP4* (Ditta et al., 2004) of *Arabidopsis* and their orthologs: *FBP2* in petunia (Angenent et al., 1992), *TaSEP-3* and *TaSEP-4* in wheat (Paolacci et al., 2007), and *TM5* in tomato (Pnueli et al., 1994). These genes have been thought to be involved in the regulation of organ differentiation of petals, stamens, and carpels by interacting with A- and/or B- and/or C-class MADS-box genes (Pelaz et al., 2000; Honma and Goto, 2001). The *SEP*-class genes have been reported to play a role in ovule and fruit development (Busi et al., 2003; Favaro et al., 2003). We found that *CpMADS2* was expressed mainly in petals and stamens (Figures 5 and 6), as was *CpTM6-1* (Ackerman et al., 2008). Real-time PCR analysis indicated a considerably higher *CpMADS2* transcript level in stamens than in petals (Figure 6), which suggests the involvement of the TM6 lineage gene in stamen development.

Both E-class genes, *CpMADS1* and *CpMADS3*, were actively expressed in young flower buds at the 1-mm stage (Figure 4A). The B-class *CpMADS2* gene was also detectable at the same stage (Figures 5, 6) as were *CpTM6-2* and *CpPI* MADS-box genes (Ackerman et al., 2008). In young flowers longer than 1 mm, the 4 whirls were considered fully differentiated (Arkle Jr. and Nakasone, 1984). Therefore, we could detect the expression of both B- and E-class MADS-box genes in such buds. The determination of their exact temporal expression, especially the E-class MADS-box genes, requires flower buds less than 1 mm long for *in situ* hybridization, such as that reported by the dynamic expression analysis of floral genes in snapdragon (Zachgo et al., 2000). Southern blot analysis of genomic DNA from the 10 papaya genotypes of 5 different sex phenotypes with probes for *CpMADS1*, *CpMADS2*, and *CpMADS3* revealed similar restriction patterns and copy number, regardless of genotype and sex type (Figure 4). Thus, the 3 *CpMADS* genes might not directly participate in the determination of plant sex of hermaphrodite, male, female, progeny-all-hermaphrodite, and progeny-all-male papaya cultivars. Previous study on *CpTM6-1*, *CpTM6-2*, and *CpPI* MADS-box genes also revealed the same Southern blot pattern in normal hermaphrodite, male, and female papaya cultivars (Ackerman et al., 2008). Other mechanisms such as sex-chromosome or sex-determination genes might be directly involved in determining papaya plant sex. The relationship between MADS-box genes and sex-chromosome or sex-determination genes remains to be investigated. In the dioecious plant *Silene latifolia*, a complete MADS-box gene, *SlAP3Y*, was found to be duplicated onto the Y chromosome (Matsunaga et al., 2003). In the past 7 decades, the hypotheses about sex determination in papaya have been extended from a single gene with 3 alleles to an X and Y sex chromosome system with 2 slightly different Y chromosomes (Storey, 1953; Ming et al., 2007). Yu et al. (2007) used chromosome fluorescence *in situ* hybridization to decipher the Y chromosome of papaya and a male-specific

region located near the centromere of the Y chromosome and concluded that papaya plant sex determination was controlled by the XY chromosome system. The authors designated the sex chromosomes of female, hermaphrodite, and male plants as XX, XY^h, and XY, respectively (Ming et al., 2007). Furthermore, Sondur et al. (1996) proposed a model to explain the regulation of flower organ development inspired by the ABC model, whereby factors that promote the stamen but suppress the carpel induce the stamen and reduce carpel size, and a null allele determining the gender of papaya were designated *SEX1-M*, *SEX1-H*, and *sex1-f*, respectively. Ming et al. (2007) suggested the existence of 2 genes involved in sex determination in papaya: a carpel suppressor in male flowers and a stamen suppressor in female flowers. The former gene was proposed to be a downstream regulator that aborts the carpels at a later developmental stage because the aborted gynoecium remains attached in the male flower (Figure 1d, e). Occasionally, carpel development was reversed in some flowers of a male papaya plant, and the plant subsequently self-pollinated to set fruits, especially in the terminal or axillary portions of an inflorescence (Figure 1f). This male-to-female conversion in papaya suggests the possibility of an upstream regulator of MADS-box genes. Ming et al. (2007) and Ackerman et al. (2008) suggested that the female sex determination gene is probably an upstream regulator of the B-class genes *AP3* and *PI* because of no evidence of stamens in female flowers (Figure 1c).

In our study, we also compared the expression patterns of all 3 genes, *CpMADS1*, *CpMADS2*, and *CpMADS3*, by real-time PCR in 10 papaya genotypes (data not shown). In the developing floral buds from 1 to 20 mm, the expression of all 3 genes in all cultivars decreased gradually and then increased before the maturation of the buds. In the floral organs of the mature buds, the B-class genes were expressed in the second and third whorls and mainly in stamens, and in general, the 2 E-class genes were expressed in the inner 3 whorls and mainly in the carpel. The result also indicated that the expression of *CpMADS2* might peak before the bud reached 1 mm, with a similar level of expression between the 1-mm bud and stamens in mature buds (Figure 6). The expression of B-class genes is required for petal and stamen initiation and development (Ming et al., 2007). At the 1-mm bud stage, the initiation of the floral bud is complete, and the 4 whirls of the papaya flower bud have just differentiated (Arkle Jr. and Nakasone, 1984), which might explain the decreased expression of *CpMADS2* in developmental buds at an early stage. On the other hand, the reversed expression at a later development stage might be due to a rapid increase of organ tissue. Further, we found that the transcript level in the 20-mm bud, petals, and stamens did not exceed that of 1-mm buds, which indicated that the B-class genes should be expressed before the initiation of floral organ development and show the highest expression before the completion of differentiation. Therefore, we indirectly provide molecular data to support the hypothesis raised from the morphological characters of female flowers that the female sex determination gene may have to abort stamens before the initiation of stamen primordia (Ming et al., 2007).

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