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Full Length Research Paper

Three R2R3 MYB transcription factor genes from *Capsicum annuum* showing differential expression during fruit ripening

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Three R2R3-MYB genes, designated *CaMYB1*, *CaMYB2* and *CaMYB3*, were isolated from hot pepper (*Capsicum annuum*. L). *CaMYB1*, *CaMYB2* and *CaMYB3* encode polypetides consisting of 340, 262 and 345 amino acids respectively, containing R2R3 domain and the signature motif specific for the interaction between MYB and bHLH proteins in the R3 domain. Phylogenetic analysis based on the deduced amino acid sequences of these three R2R3 MYB transcription factor members revealed that CaMYB1 and CaMYB2 clustered together with the anthocyanin-related subgroup of R2R3 MYB proteins from other plants, while CaMYB3 did not. *CaMYBs* transcripts accumulation was detected in all stages of fruit development and in flower and leaves. Three *CaMYBs* transcription factors showed differential expression during fruit ripening. Anthocyanin biosynthetic gene expression patterns were quite different in young leaves, flower, and the four stages of fruit development. CaMYB1 and CaMYB2 may regulate anthocyanin biosynthesis in hot pepper.

Key words: Anthocyanin, *Capsicum annuum*, gene expression, R2R3 MYB transcription factor.

INTRODUCTION

The MYB transcription factors family is one of the most abundant classes of transcription factors in plants (Rosinski and Atchley, 1998). MYB transcription factors are classified into three sub-families; MYB1R, MYBR2R3 and MYB3R factors; depending on the number of imperfect repeats (R1, R2 and R3) in the DNA-binding domain, each consisting of about 50 amino acids and including a helix-turn-helix structure (Jin and Martin, 1999).

Among these MYB transcription factors, R2R3-MYBs constitute the largest transcription factors gene family in plants, with 126 R2R3 MYB genes identified in Arabidopsis (Stracke et al., 2001). The R2R3-MYB genes have been extensively studied and members of the MYB family have been found to be involved in diverse physiological and biochemical processes including regulation of meristem formation, floral and seed development (Penfield et al., 2001; Schmitz et al., 2002; Shin et al., 2002; Steiner-Lange et al., 2003), the control of cell morphogenesis (Lee and Schiefelbein, 1999, 2001; Higginson et al., 2003), the control of the cell cycle (Ito et al., 2001; Araki et al., 2004), and regulation of secondary metabolism (Borevitz et al., 2000; Jin et al., 2000; Nesi et al., 2001; Baudry et al., 2004).

Some were also involved in various defense and stress responses (Vailleau et al., 2002; Abe et al., 2003; Denekamp and Smeekens, 2003; Nagaoka and Takano, 2003) and in light and hormone signaling pathways

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Abbreviations: ANS, Anthocyanidin synthase; **bHLH,** basic-helix-loop-helix; **CHI,** chalcone isomerase; **CHS,** chalcone synthase; **DFR,** dihydroxavonol 4-reductase; **F3H,** flavanone 3-hydroxylase; **ORF,** open reading frame; **RACE,** rapid amplification of cDNA ends; **RT-PCR,** reverse transcriptase polymerase chain reaction; **UFGT,** UDP-glucose:flavonoid 3-O-glucosyltransferase; **UTR,** untranslated region.

(Gocal et al., 2001; Seo et al., 2003; Newman et al., 2004). Plant anthocyanin pigmentation in leaves, flowers, and fruit imparts violet to black color. Anthocyanins are the end product of the flavonoid biosynthetic pathway. Anthocyanin pigments have key roles in plants for their function in attraction of pollinators and seed dispersers. These compounds also function as ultraviolet protectants, antimicrobial agents, and feeding deterrents, in signaling between plants and microbes, and in male fertility of some species (Stommel et al., 2009).

Hot pepper (*Capsicum annuum* L.) fruits are usually used as vegetable foods and as spice. Hot pepper fruit color is important for culinary product quality. Anthocyanin content affects fruit color of pepper during its ripening stage (Stommel et al., 2009). Anthocyanin structural gene transcription requires the expression of at least one of each of three distinct transcription factor families: MYC, MYB, and WD40 (Griesbach, 2005; Lightbourn et al. 2007). To study the MYB transcription factors controlling anthocyanin accumulation in hot pepper, three R2R3-MYB homologs were cloned and characterized from hot pepper. The expression profiles of *CaMYBs* and anthocyanin biosynthetic genes in the hot pepper were also investigated. This study perhaps contributes towards an understanding of the relation of *CaMYBs* and anthocyanin biosynthesis in the hot pepper.

MATERIALS AND METHODS

Plant material

Hot pepper *(C. annuum* L) cv. Haijiao 4 (green-fruited) was planted at the Experimental Farm of the Chinese Academy of Tropical Agriculture (Hainan, China). Fruits of four development stages (stage 1, young fruit at 7 days post anthesis; stage 2, young fruit at 15 days post anthesis; stage 3, green-ripe fruit at 30 days post anthesis; stage 4, red-ripe fruit at 50 days post anthesis), or flower and young leaves of hot pepper were collected. Fruit flesh and other tissues were cut into small pieces, and immediately frozen in liquid nitrogen, and stored at -70℃ or were used immediately.

Isolation of RNA

Total RNA was extracted according to the Chang method (Chang et al., 1993). The quality and concentration of the extracted RNA was checked by agarose gel electrophoresis and measured by spectrophotometer (DU-70, Beckman, Fullerton, CA).

Internal conserved fragment cloning of *CaMYBs*

The degenerated primers, P1 (5'- CGGAATTCTTDATYTC RTTRTCNGT -3') and P2 (5'- CGGAATTCDSNAARAGYTGYCG -3'), were designed according to the conserved regions of R2R3 MYB transcription factor in the GenBank, where D is G,A or T, R is A or G, Y is C or T, S is G or C and N is A ,C,G or T. Total RNA (1 µg) was used in reverse transcription PCR (one-step RT-PCR kit, TAKARA, Dalian, China), under the following condition: 1 h reverse transcription at 50℃, 2 min denaturation at 94℃, followed by 32 cycles of amplification (94℃ for 30 s, 50℃ for 30 s, 72℃ for 30 s). The amplified product was purified (Tiangen, Beijing, China) and cloned into the pGEM-T easy vector (Promega, Madison, WI), followed by sequencing.

3'-RACE of *CaMYBs*

3'-ready cDNA was synthesized by reverse transcribing 1 µg of the total RNA with 3'-CDS primer A (provided in the kit). 3'-rapid amplification of cDNA ends (RACE) primer were designed (Table 1) and synthesized based on the cloned internal conserved fragment. 3'-RACE was carried out in a total volume of 25 µl containing 1 µl 3'-ready cDNA, and performed for 35 cycles of amplification (94℃ for 15 s, 68℃ for 30 s, and 72℃ for 1 min). The product was purified and cloned into pGEM-T easy vector (Promega), followed by sequencing.

5'-RACE of *CaMYBs*

An aliquot of 1 µg of the total RNA was reverse transcribed with 5'-CDS primer A and SMART II A oligonucleotide (provided in the kit) to obtain the 5'-ready cDNA, using the SMARTTM RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). 5'-RACE primer were designed (Table 1) and synthesized based on the cloned internal conserved fragment. Primary 5'-RACE PCR was performed with 5P1 and UPM primer (provided in the kit) in a total volume of 25 µl containing cDNA, and was denatured at 94℃ for 3 min, followed by 32 cycles of amplification (5 s denaturation at 94℃, 10 s annealing at 68℃, and 1 min extension at 72℃). The product of the primary PCR was diluted 50-fold, and was used as a template for nested PCR performed with primer 5P2 and NUP (provided in the kit). The product was purified and cloned into the pGEM-T easy vector (Promega), and sequenced.

Cloning of the full-length cDNA of *CaMYBs*

After alignment and assembly of the sequences of the internal conserved sequence and the 3'-RACE and 5'-RACE products, the full-length cDNA sequence of the *CaMYBs* gene was deduced and subsequently obtained by PCR. The open reading frame (ORF) of *CaMYBs* was amplified by RT-PCR with the TAKARA RNA PCR kit. An aliquot of 1 µg of the total RNA was reverse transcribed according to the manufacturer's manual, and 2 µl of the cDNA was used in PCR in a total volume of 50 µl under the following condition: 94℃ for 2 min, followed by 32 cycles of amplification (94℃ for 15 s, 58℃ for 30 s, and 72℃ for 2 min). The PCR product was purified and cloned into the pGEM-T easy vector (Promega) followed by sequencing.

Multiple alignments and bioinformatic analyses

Comparative and bioinformatic analyses of *CaMYBs* were carried out online at http://www.ncbi.nlm.nih.gov. The nucleotide sequence, deduced amino acid sequence, and ORF encoded by *CaMYBs* were analysed, and sequence comparison was conducted through database searches using the BLAST program (http://www. ncbi.nlm.nih.gov). The phylogenetic analysis of CaMYBs and MYB transcription factors from other species was aligned with CLUSTAL W (1.82) using default parameters. Phylogenetic tree was constructed using MEGA version 2.1 from CLUSTAL W alignments (Kumar et al., 2001). The neighbor-joining method was used to construct the tree (Saitou and Nei, 1987).

Analysis of *CaMYBs* expression

RT-PCR for the analysis of *CaMYBs* expression was performed

Table 1. Primer sequences (nucleic sequences from 5' to 3').

3'RACE-PCR primer	Sequence
3CAMYB11	GTTGCAGGCTGAGGTGGATAAATTACT
3CAMYB12	GTGTTTTAGTGAACAGGAAGAAAGGAT
3CAMYB21	GCTGTAGATTGAGGTGGCTGAATTATC
3CAMYB22	GGCCGCATATAAAGAGAGGTGACTTTG
3CAMYB31	TCTTGCCGGTTACGATGGTGTAATCAG
3CAMYB32	TCTCCTCAAGTTGAACATCGGCCTCTC
5' RACE-PCR primers	
5CAMYB11	TCTTCACTTCATTATCAGTCCTGCCAG
5CAMYB12	ATTGGCTATTTGTGCCCATTTGTTTCC
5CAMYB21	CGAGTTCCAGTAGTTTTTCACATCGTT
5CAMYB22	GCAATCTCCCAGCAATAAGTGACCATC
5CAMYB31	CAGTGATTCTTTATTGCGTTATCGGTT
5CAMYB32	TCGCCCGTTAAGTAATCGGGCTATGGT
Semi-quantitative RT-PCR primers	
CaMYB11	GCAAGTCTTGCAGCATTGTCAC
CaMYB12	TTACATTCTCCGCGATTCGTCC
CaMYB21	CTTCTAGGCAACAGATGGTCAC
CaMYB22	CCCAGTACAAGTACTGCTCTTC
CaMYB31	CCGTCGATTGATATCTCATCTC
CaMYB32	ATTCCCATGCGATTAGCAGCCC
CaCHS1	CTTCGACCCTCAGTCAAACGAC
CaCHS2	GTGAGCGATCCAGAAGATAGAG
CaCHI1	AGAGTGCCGTTCCATTTCTTGC
CaCHI2	TCCAGCACCGCTTCTGACAGTT
CaF3H1	GCACACTGATCCTGGAACCATC
CaF3H2	TCTTCAGCTTGTATCTGCTGCT
CaDFR1	CTCTTGGCTTGTCATGAGACTC
CaDFR2	ACGCTCCAGCTGGTCTCATCAT
CaCNS1	GCCTTGTCTTCCAGTTTCATGC
CaCNS2	CAATCACTCTGTGCTCCACGCT
CaUFGT1	ACAAGGCAATGACACCCCTATT
CaUFGT2	TTGTGGCATGTCACTGATCCTT
ACT specific primers	
AF	CAGTGGTCGACAACTGGTAT
AR	TCCTCCAATCCAGACACTGT

using total RNA from hot pepper tissues, and amplified with *CaMYBs* specific primers (Table 1). Specific primers were designed from the low homology regions of *CaMYBs* coding sequences and the 3'- untranslated region (UTR). The *ACT* gene was used as an internal control parallel in the reactions, amplified with *ACT* specific primers AF (5'-CAGTGGTCGACAACTGGTAT-3') and AR (5'-ATC CTCCAATCCAGACACTGT-3'). PCR reaction was carried out in 22 cycles of programmed temperature control for 30 s at 95°C, 30 s at 50°C and 1 min at 72°C with a 5 min preheat at 95°C and a 10 min final extension at 72°C. The PCR products were analysed by agarose gel electrophoresis with ethidium bromide staining.

Determination of anthocyanin content

Anthocyanin content was quantified by the pH differential spectrophotometry method (Niu et al., 2010). Three measurements for each biological replicate sample were performed.

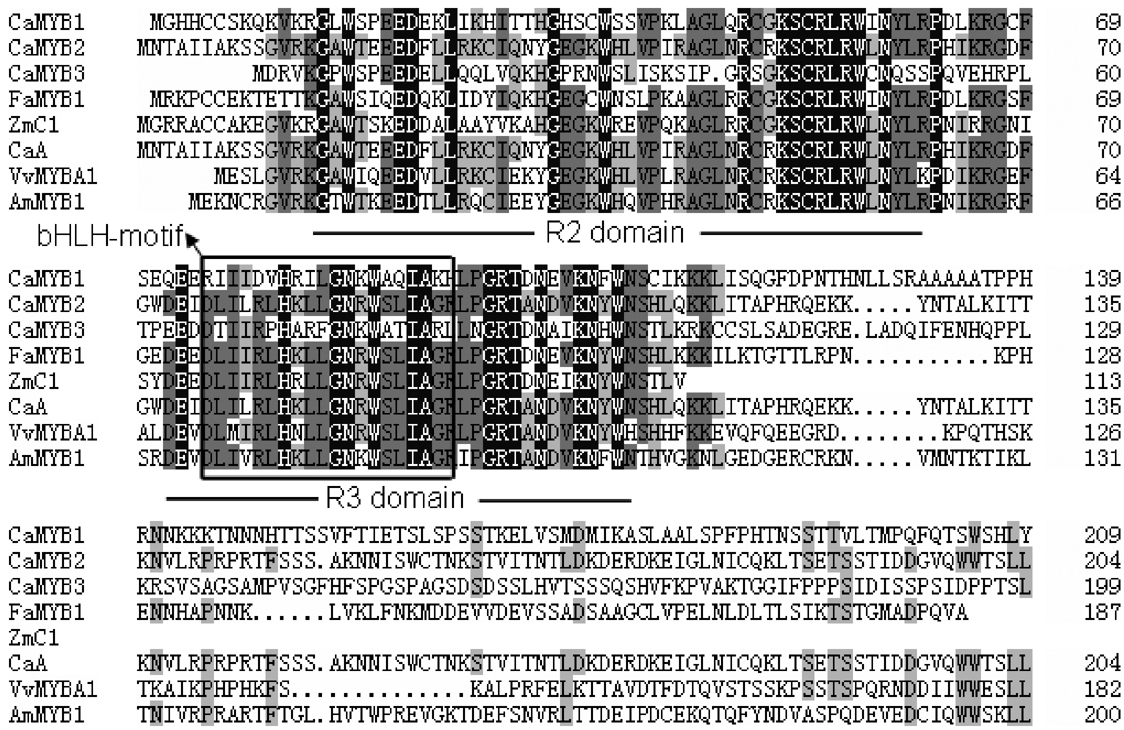

Figure 1. The deduced amino acid sequences of CaMYBs are compared with anthocyanin-related MYB proteins of other species. Amino acid residues that are identical in all seven sequences are shaded darkly, while well-conserved residues are shaded in gray. The R2R3- binding domain is underlined. The box indicates specific residues that form the motif implicated in bHLH co-factor interaction. The accession numbers of these proteins, or translated products, in the GenBank database are as follows: AmMYB1 (*Antirrhinum majus*, ABB83826), VvMYBA1 (*V. vinifera*, AB242302), CaA (*Capsicum annuum*, AJ608992), ZmC1 (*Zea mays*, AAK81903) and FaMYB1 (*Fragaria ananassa*, AAK84064).

Analysis of anthocyanin biosynthetic genes expression

RT-PCR for the analysis of *CaMYBs* expression was performed using total RNA from hot pepper tissues, and amplified with anthocyanin biosynthetic genes specific primers (Table 1). The *ACT* gene was used as an internal control parallel in the reactions, amplified with *ACT* specific primers AF(5'-CAGTGGTCGA CAACTGGTAT-3') and AR (5'-ATCCTCCAATCCAGACACTGT-3'). PCR reaction was carried out in 25 cycles of programmed temperature control for 30 s at 95°C, 30 s at 50°C and 1 min at 72°C with a 5 min preheat at 95°C and a 10 min final extension at 72°C. The PCR products were analysed by agarose gel electrophoresis with ethidium bromide staining.

RESULTS

Cloning and characterization of *CaMYBs*

About 180-bp PCR products were amplified with degenerated primers P1 and P2, and then PCR product was purified and cloned into the pGEM-T easy vector followed by sequencing. Based on sequences from 20 individual recombinant plasmids, three candidate fragments were obtained, which showed similarity with other plant R2R3 MYB transcription factor genes as revealed by a BlastX search. After sequence extension by 3'-RACE and 5'-RACE, sequences of 1093, 1086 and 1045 bp in length were obtained for *CaMYB1*, *CaMYB2* and *CaMYB3*, respectively.

CaMYB1, *CaMYB2* and *CaMYB3* encode polypeptides consisting of 340, 262 and 345 amino acids respectively; containing R2R3 domain and the signature motif specific for the interaction between MYB and bHLH proteins in the R3 domain (Figure 1). Blast analysis revealed that deduced protein sequences are already deposited in database at NCBI (*CaMYB1 -AAQ05796*.1; *CaMYB2 -CAE75745*.1; *CaMYB3- ABN11121*.1). Phylogenetic analysis based on the deduced amino acid sequences of these three R2R3 *MYB* transcription factor members revealed that CaMYB1 and CaMYB2 clustered together with the anthocyanin-related subgroup of R2R3 MYB proteins from other plants, while *CaMYB3* did not (Figure 2). *CaMYB1* and *CaMYB2* maybe regulate anthocyanin biosynthesis in hot pepper.

Expression of *CaMYBs* transcription factors during fruit ripening

In order to study the *CaMYBs* transcription pattern, total RNA was isolated from young leaves, flower, and the four

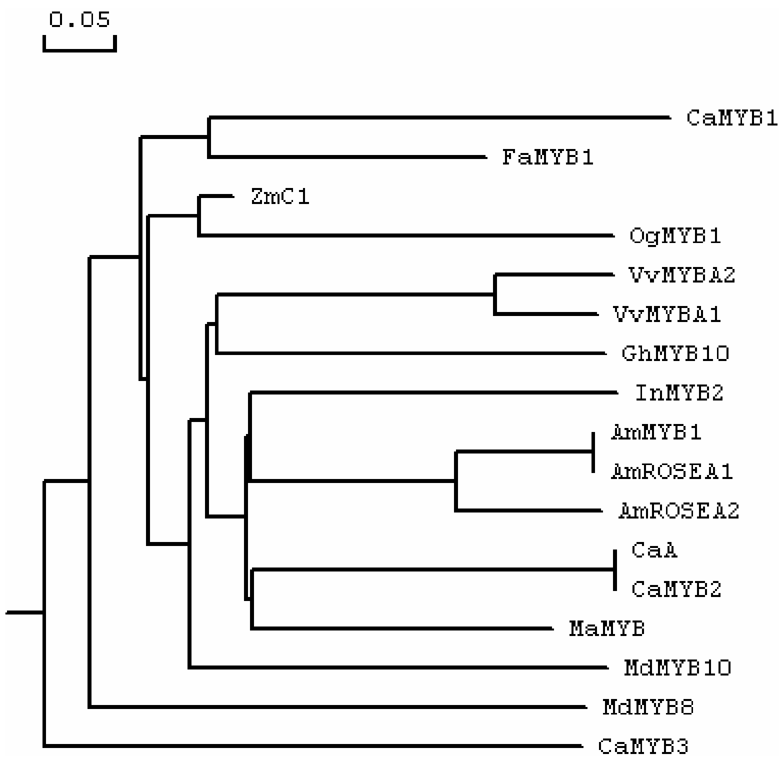

Figure 2. Phylogenetic analysis of CaMYBs and other species by MEGA version 2.1 from CLUSTAL W alignments. The neighbor-joining method was used to construct the tree. The anthocyanin-related MYB proteins of other species used in the evolutionary analysis are retrieved from Genbank including InMYB2 (*Ipomoea nil*, BAE94709), MaMYB (*Mimulus aurantiacus* ACA04006), OgMYB1 (*Oncidium* Gower Ramsey, ABS58501), GhMYB10 (*Gerbera* hybrid, CAD87010), AmROSEA1 (*Antirrhinum majus*,ABB83826), AmROSEA2 (*Antirrhinum majus*, ABB83827), MdMYB8 (*Malus domestica* ,ABB84756.1), MdMYB10 (*Malus domestica*, ABB84753.1), VvMYBA1 (*V. vinifera* ,AB242302), VvMYBA2 (*V. vinifera* ,AB097924), ZmC1 (*Zea mays* , AAK81903), FaMYB1 (*Fragaria ananassa*, AAK84064) and CaA (*Capsicum annuum*, AJ608992).

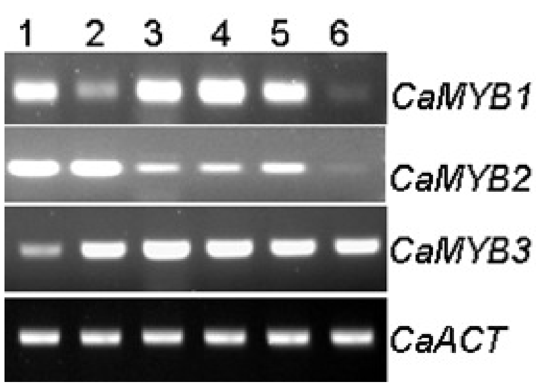

Figure 3. Expression of *CaMYBs* transcription factors in young leaves (1), flower (2), and the four stages of fruit development (3 to 6).

stages of fruit development, and subjected to semi-quantitative RT-PCR analysis. *CaMYBs* transcripts accumulation was detected in all stages of fruit development and in flower and leaves. Three *CaMYBs* transcription factors showed differential expression duringfruit ripening (Figure 3). The highest expression level of *CaMYB1* during fruit development was detected in DAF30 fruit, the lowest expression level of *CaMYB1* was detected in ripen fruit. A high level of expression of *CaMYB1* was also detected in leaf tissue. The profile of *CaMYB2* was consistent of *CaMYB2* during fruit development, but the expression level of *CaMYB1* was higher than those of *CaMYB1* during fruit development. A high level of expression of *CaMYB2* was detected in flower. The expression level of *CaMYB3* decreased during fruit development.

Anthocyanin content and expression of biosynthetic genes during fruit ripening

Anthocyanin content varied among the four stages of fruit

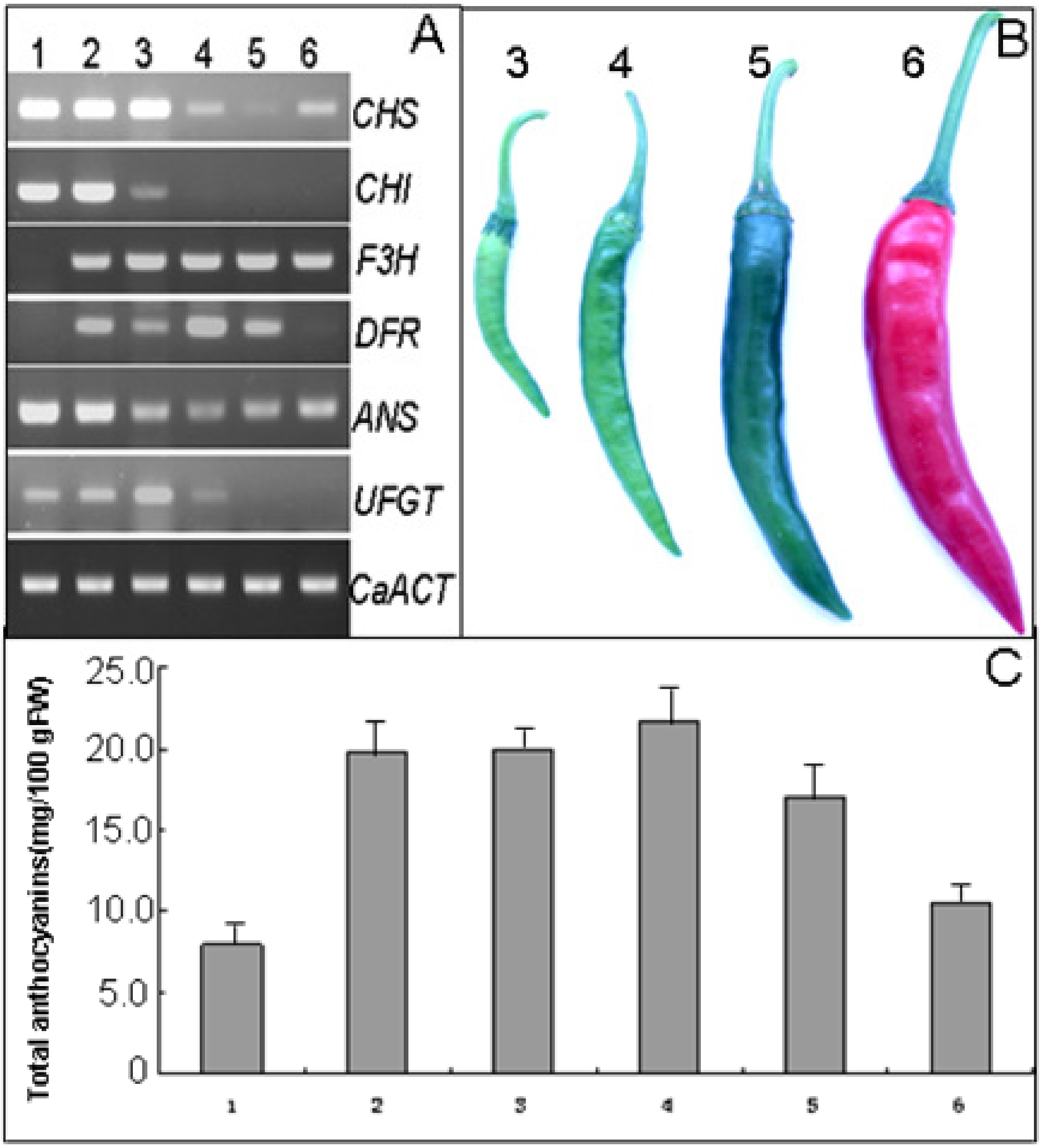

Figure 4. RT-PCR analysis of anthocyanin biosynthetic genes in the fruit. Actin was used as an internal control. Total RNA was extracted from young leaves, flower, and the four stages of fruit development. A) Six target genes were investigated including CHS (FJ705842), CHI (FJ705843), F3H (FJ705844), DFR (FJ705846), ANS (FJ705847) and UFGT(FJ705848). B) The four stages of fruit development. C) Anthocyanin content in young leaves (1), flower (2), and the four stages of fruit development (3 to 6). Error bars represent ± SE (n = 3).

development. As shown in Figure 4a, the content was low in ripe fruit and high in DAF30 fruit. Anthocyanin biosynthetic gene expression patterns were quite different in young leaves, flower, and the four stages of fruit development (Figure 4b).

DISCUSSION

R2R3 *MYB* genes have been cloned and characterized from horticulture crops, such as grape (Kobayashi et al., 2002), apple (Ban et al., 2007), pear (Feng et al., 2010), Chinese bayberry (Niu et al., 2010), mangosteen (Palapol et al., 2009), strawberry (Aharoni et al., 2001), and a few other species. The first characterized plant R2R3-MYB was *C1* from maize, which regulates genes encoding enzymes of the anthocyanin biosynthetic pathway (Cone et al., 1993). Similar to *C1*, some MYB transcription factors have been proved to regulate anthocyanin biosynthesis (Ban et al., 2007; Feng et al., 2010; Niu et al., 2010; Palapol et al., 2009; Allan et al., 2008; Espley et al., 2007; Takos et al., 2006).

In this study, we reported on the cloning and characterization of three genes encoding R2R3 *MYB* protein. The deduced amino acid sequence of CaMYBs showed extensive similarity to their counterparts in other species, containing R2R3 domain and the signature motif specific for the interaction between MYB and bHLH proteins in the R3 domain. Phylogenetic analysis based on the deduced amino acid sequences of these three R2R3 MYB transcription factor members revealed that *CaMYB1* and *CaMYB2* clustered together with the anthocyanin-related subgroup of R2R3 MYB proteins from other plants, while *CaMYB3* did not. In addition, the deduced amino acid of *CaMYB2* shared 96% identity with the CaA, controlling anthocyanin accumulation encode a MYB transcription factor in pepper (purple-fruited) (Borovsky et al., 2004). Based on sequence similarity, we hypothesize that CaMYB1 and CaMYB2, maybe regulate anthocyanin biosynthesis in hot pepper (green-fruited).

Anthocyanin content varied among the four stages of fruit development in hot pepper *(C. annuum* L) cv. Haijiao 4 (green-fruited), the content was low in ripe fruit and high in DAF30 fruit. Only after about 10 days post anthesis does anthocyanin become visible, peaking 20 days post-anthesis in pepper 5226 (purple-fruited). At ripening, anthocyanin disappears and the fruit turns orange due to the accumulation of carotenoid pigments (Borovsky et al., 2004). Plant anthocyanin biosynthetic genes have been identified or cloned in many plant species (Grotewold, 2006) with CHS, CHI, F3H, F3'H, DFR, ANS and UFGT, participating in the biosynthesis of anthocyanin. The transcription of genes in the anthocyanin biosynthesis pathway in hot pepper was also investigated by RT-PCR. In hot pepper, *CaDFR* is expressed in a correlative way with *CaMYB1* and *CaMYB2* expression, suggesting *CaMYB1* and *CaMYB2* may activate the expression of *CaDFR*.

In this study, the transcription of *CaMYB1* and anthocyanin biosynthetic genes declined during late stages of fruit development, in agreement with the lower efficiency of anthocyanin synthesis. The lower efficiency of anthocyanin synthesis and the degradation of anthocyanin may explain the fast decline in anthocyanin content. In summary, we have cloned three genes encoding R2R3 MYB transcription factors from hot pepper. Based on sequence similarity and expression profile, we hypothesize that *CaMYB1* and *CaMYB2* may regulate anthocyanin biosynthesis in hot pepper.

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