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Molecular cloning and expression analysis of four actin genes (*MiACT*) from mango

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Abstract

Actin is the most abundant protein in eukaryotic cells and is a key cytoskeletal component controlling cell morphology and motility. In this study, four *MiACT* genes were isolated from mango by homological cloning and designated as *MiACT1*, *MiACT4*, *MiACT7*, and *MiACT9*. Sequence alignments and phylogenetic analysis demonstrated that the four *MiACT* genes of mango were highly similar to each other at the nucleotide and amino acid levels. All of four MiACT proteins showed high similarity to the known actin proteins from other species. Reverse transcription polymerase chain reaction revealed that the four *MiACT* genes were constitutively and stably expressed in all organs tested. Application of plant growth regulators and four stress treatments had a remarkable effect on the expression of *MiACT4, MiACT7* and *MiACT9*, whereas expression of *MiACT1* was unresponsive. In contrast, the expression profiles of the four *MiACT* genes were not regulated by diurnal rhythms. Moreover, the expression of *MiACT1* was not affected by heavy metal treatments and the transcript level of *MiACT1* was rather stable in different days during the post-harvest period either under treatment or not. Our results suggest that the four actin genes play important roles throughout the entire life cycle of mango; the constitutively and stably expressed *MiACT1* is the best candidate as an internal standard for differential gene expression analysis in mango.

Additional key words: diurnal rhythm, heavy metals, *Mangifera indica*, NaCl, RT-PCR, temperature, waterlogging.

Introduction

Actins are ubiquitous and highly conserved proteins found in all eukaryotes as a principal and indispensable structural component of the cytoskeletons. As highly dynamic protein polymers, they are critical for numbers of processes such as maintaining cytoskeletal structure, cell motility, cell division, and intracellular movements which are of central importance to plant development (Pollard and Cooper 1986, Rubenstein 1990, Kabsch and Vandekerckhove 1992, McDowell *et al*. 1996a, Valentijn *et al*. 1999). Plants contain multiple actin isoforms encoded by gene families composed of several ancient and highly conserved gene classes (Baird and Meagher 1987, Meagher and McLean 1990). The actin gene families of petunia (Baird and Meagher 1987), soybean (Nagao *et al*. 1981), tobacco (Thangavelu *et al*. 1993), potato (Drouin and Dover 1990), rice (McElroy *et al*. 1990), *Arabidopsis* (Meagher and Williamson 1994), and *Gossypium hirsutum* (Li *et al*. 2005) contain dozens or even hundreds of distinct sequences.

In the model plant *Arabidopsis*, the *actin* gene family contains ten distinct members, eight are functional genes and two pseudogenes. Based on their phylogeny and expression patterns, those eight genes were grouped into two major classes, vegetative and reproductive. The reproductive *actins* were expressed at high levels in pollen, ovules, and developing embryos. The vegetative *actins* were strongly expressed in all plant organs including root, leaves, stems, sepals, and petals

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Abbreviations: ABA - abscisic acid; BA - benzyladenine; DAF - days after full bloom; f-flower - full flowering period; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; l-flower - late flowering period; PP - paclobutrazol; RT-PCR - reverse transcription polymerase chain reaction.
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(McDowell *et al.* 1996a, Kandasamy *et al*. 2002, McKinney *et al*. 2002).

Mango (*Mangifera indica* L) is one of the important tropical and subtropical crops belonging to *Anacardiaceae* family. We have been interested in the identification and functional analysis of differential gene expression in response to hormones and abiotic stresses and during fruit ripening as well as their regulation by hot water treatment. Many differentially expressed genes have been obtained in our previous study and gene expression pattern is a good topic for further research. So the requirements for proper internal reference gene for relative expression normalization have become much more stringent. Some *actin* genes are constitutive and stable expressed in all tissues and under different conditions. These are commonly used as endogenous internal controls for semi-quantitative RT-PCR. Here we report the first isolation and characterization of actin genes in mango and the best candidate for reference gene has been suggested.

Materials and methods

Two cultivars of mango (*Mangifera indica* L.) were collected at Guangxi University, Nanning (longitude: 22° 13′ - 23° 32′; latitude: 107° 45′ - 108° 51′), Guangxi, China. One cultivar was cv. Chok Anand which can blossom several times a year. Mature leaves, young leaves, flowers (from both full and late flowering period), old stems, young stems, fruits (20, 40 and 60 DAF), and mature fruits were collected on 22 August 2010 and used for gene expression analysis in the organs. The other cultivar was Tainong No.1 which is a dominant mango cultivar in China. Leaves were collected every three hours for 24 h on 21 February 2011 to study the expression pattern of mango *MiACT* genes under day/night cycles.

Indole-3-acetic acid (IAA; 0.57 mM), abscisic acid (ABA; 0.38 mM), benzyladenine (BA; 0.44 mM), gibberellic acid (GA_3; 0.29 mM), or paclobutrazol (PP; 3.4 mM) were applied to the foliage of Tainong No.1 twice on 5 December and on 25 December 2010, whereas control plants were treated with distilled water. Leaves were sampled 3 d after the second treatment.

Abiotic stresses were applied to one-year-old plants of cv. Chok Anand grown in pots filled with soil under temperature of 25 °C, 16-h photoperiod, irradiance of 400 μmol m^{-2} s^{-1}, and relative humidity of 75 %. Waterlogging treatment was applied by submerging the roots of plants into water. Salt treatment was applied by watering plants with 300 mM NaCl. For application of cold and heat stresses, plants were transferred to growth chambers adjusted to either 4 or 40 °C. For heavy metal treatments, plants were watered with 10 mM $Pb(NO_3)_2$ or 10 mM $CoCl_2 . 6\,H_2O$, respectively. Samples were collected at 24, 48, and 72 h after the treatments.

Hot water treatment is widely used in many countries for insect and decay control (Anwar and Malik 2007). Ripe mango fruits of cv. Tainong No. 1 were harvested in July 2011. A part of the fruits were treated by hot water (50 °C) for 10 min, with no treatment fruits as control. All fruits were then packed into boxes and stored at 20 °C. Samples were collected at 1, 4, 7, 10, 13, and 15 d after the treatment. All samples were immediately dipped in liquid nitrogen and were stored at -80 °C until analysis.

The total RNA from mango was isolated according to the modified SDS method (Xiao *et al*. 2003) and was reverse transcribed by *M-MLV* (*Takara*, Dalian, China) with PCR primer (GGCCACGCGTCGACTAGTAC-$d(T)_{18}$) to synthesize the first strand cDNA. Two degenerate primers, ACTu1 (ATGGCCGAYRSYGA RGMTAT) and ACTu2 (ATGGCCGAYDSTGAGGA KATTCA), were combinded with 3′-RACE anchored primer (GGCCACGCGTCGACTAGTAC) to amplify the full-length *MiACT* cDNA. The PCR reaction was run in a *T-Professional* thermocycler (*Biometra*, Göttingen, Germany) using an initial denaturation step of 4 min at 95 °C, followed by 36 cycles of 40 s at 95 °C, 40 s at 57 °C and 2 min at 72 °C, and final extension at 72 °C for 10 min. The PCR products were cloned and sequenced. To detect the phylogenetic relationships, mango *actin* genes and their homologues from other plants were aligned using *ClustalX* (Thompson *et al.* 1997) with manual correction. Phylogenetic trees were constructed using *MEGA 4.0* software (Tamura *et al.* 2007).

To reveal the expression pattern of the four *actin* genes, different samples were separately collected for isolating total RNA and 1 μg of the total RNA was utilized for the first strand cDNA synthesis. The RT-PCR primers were designed corresponding the sequences of *MiACT1*, *MiACT4*, *MiACT7*, and *MiACT9*, respectively. Gene specific primers were: MiACT1-F (GTTTCC CAGTATTGTGGGTAGG) and MiACT1-R (AGATCT TTTCCATATCATCCCAGTT) for *MiACT1*; MiACT4-F (CCCTTGACTATGAACAAGAGCTG) and MiACT4-R (GCGGTAATTTCCTTGCTCATCCTA) for *MiACT4*; MiACT7-F (CACTGAAGCACCTCTCAATCCTAA) and MiACT7-R (TCAGCAGTGGTAGTGAATGAG TAG) for *MiACT7*; and MiACT9-F (TGAGAAGAA CTATGAGCTGCCA) and MiACT9-R (CATGCTGCT TGGAGCAAGGGCT) for *MiACT9*. The expression pattern of AP2/ERF gene in response to different treatments was also investigated. The specific primers were AP2/ERF-F (GAGAAGGAAAAGCACTACAGA GGA) and AP2/ ERF-R (GGACCCTCTTCT GTCTCTTCGTTC). The RT-PCR were performed for

three replicates following the PCR program with denaturation step at 95 °C for 4 min followed by 32 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 6 min. RT-PCR products were resolved by 1.5 % (m/v) agarose gel electrophoresis. All the PCR products were then cloned and sequenced to confirm the transcripts of the four actin genes.

Results

Based on the RT-PCR strategy, four *actin* sequences were obtained and designated as *MiACT1* (*GenBank* accession No. JF737036), *MiACT4* (GenBank acc. No. JF737035), *MiACT7* (GenBank acc. No. HQ586000), and *MiACT9* (GenBank acc. No. HQ585999) from mango cv. Chok Anand. The whole *MiACT1* cDNA sequence was 1 455 bp in length, coding a 377 amino acid protein. The molecular mass and isoelectric point (pI) of the MiACT1 protein were 41.662 kDa and 5.16, respectively. The *MiACT4* cDNA sequence was 1 483 bp and encoded a 377 amino acid protein of 41.684 kDa with a pI of 5.16. The 1 362 bp *MiACT7* encoded a 377 amino acid protein

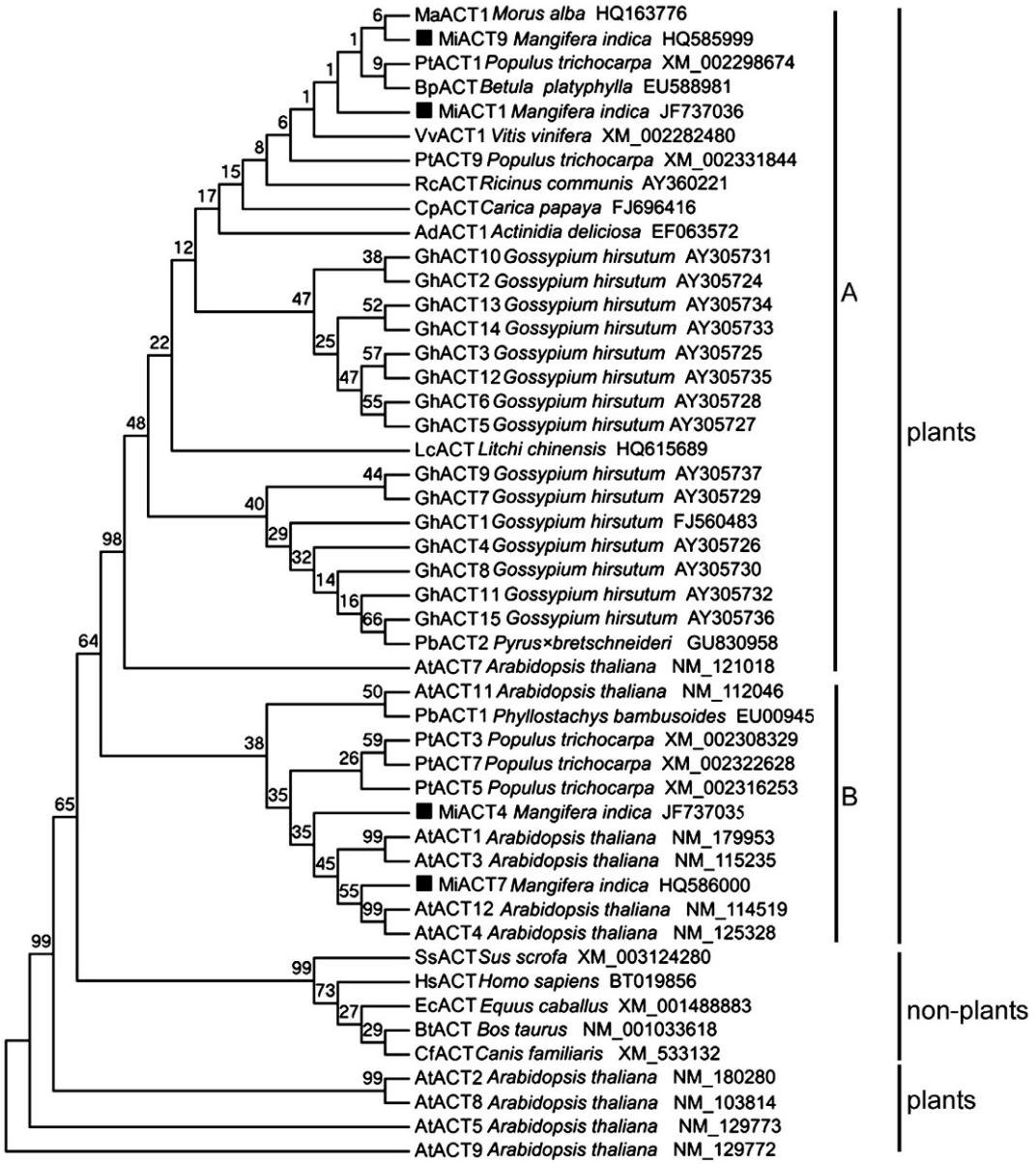

Fig. 1. Phylogenetic tree of actin proteins from various organisms. The numbers beside the branches represent bootstrap values based on 1 000 replications. MiACT were marked by *black box*.

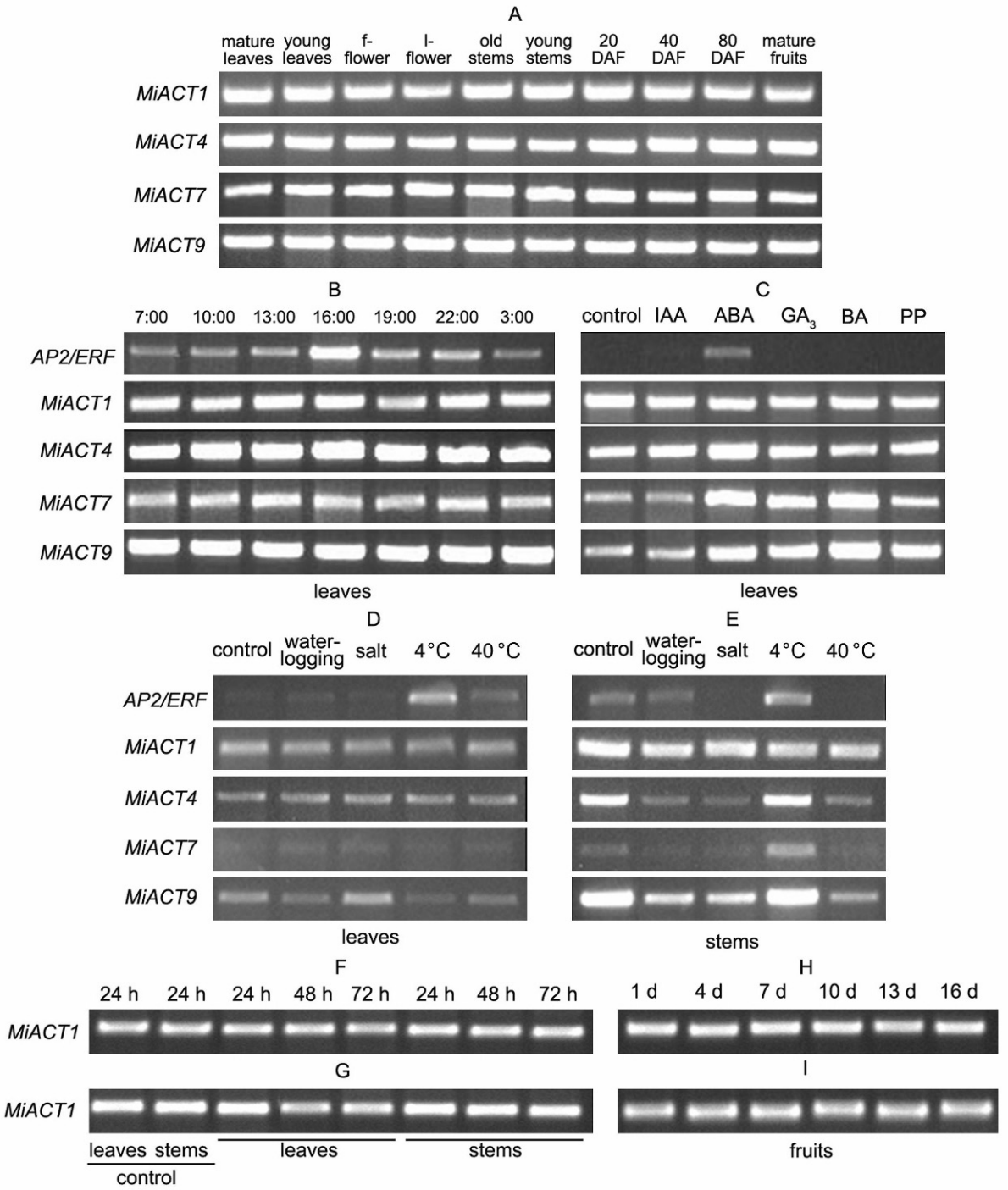

Fig. 2. Expression analyses of the four *MiACT* genes in mango. Expression patterns of *MiACT* genes in different organs under normal condition (*A*); in leaves over 24 h (*B*); in response to phytohormones and paclobutrazol (*C*); in response to four stresses in leaves (*D*) and in stems (*E*); in response to $Pb(NO_3)_2$ in leaves and stems (*F*); in response to $CoCl_2$ in leaves and stems (*G*); in fruits after harvest under normal conditions (*H*); and in fruits after harvest under hot water treatment (*I*). Expression analysis of *AP2/ERF* in response to different treatments are shown in *B*, *C*, *D*, and *E*.

of 41 656 kDa with a pI of 5.16. Finally, the *MiACT9* cDNA was 1 579 bp in length coding a 377 amino acid protein of 41 656 kDa with a pI of 5.16. The four actin gene sequences were 81 to 93 % identical at the nucleotide level and 98 to 99 % identical at the amino acid level.

BLAST analysis revealed that the nucleotide sequences of four *MiACT* genes were highly similar to other actin sequences present in the *GenBank* database. To investigate the evolutionary relationships between

actins, a phylogenetic tree was constructed using neighbor-joining (NJ) algorithm of *MEGA 4* version. In the phylogenetic tree, most protein sequences were clustered into three major clades except two pseudogenes *AtACT5* and *AtACT9* which formed a single group separated from other organisms. Plant and non-plant actin encoding proteins formed two distinct clades in the tree; most plants grouped into a big cluster and were further subdivided into two classes, A and B. The MiACT1 and MiACT9 proteins were closely related to MaACT1 from *Morus alba*, PtACT1 from *Populus trichocarpa,* BpACT from *Betula platyphylla,* and VvACT1 from *Vitis vinifera* which all grouped together in class A. The proteins MiACT4 and MiACT7 were more closely related to AtACT1, AtACT3, AtACT4, and AtACT12 from *Arabidopsis thaliana* and grouped into class B (Fig. 1). The bootstraps in the tree were mostly lower than 50 % based on the amino acid sequences which was also found in previous reports (Deng *et al*. 2010, Peng *et al*. 2010).

The expression pattern of *MiACT* in different organs of cv. Chok Anand under normal conditions was examined by RT-PCR. The results showed that the four *MiACT* genes are ubiquitously expressed in all organs tested (Fig. 2*A*).

In a previous study of actin gene in *Ulva pertusa* (*ACT*) was highly expressed during cell growth and development in the light period and repressed under darkness. The results suggested that the accumulation of transcripts was positively regulated by photoperiod (Kakinuma *et al*. 2004). Whether the diurnal rhythm also affects the expression pattern of the mango *MiACT* was addressed by expression analysis of leaves harvested at different times of day. Leaves from cv. Tainong No.1 were collected every three hours for 24 h on 21 February 2011. We found the four *MiACT* genes widely expressed in leaves at different time points. However, *MiACT4* expression was much higher than other three *MiACT* genes and *MiACT7* expression was the lowest. There were no significant differences in expression between any time points for any of the four *MiACT* genes (Fig. 2*B*).

To determine whether these four ubiquitously expressed *MiACT* genes are suitable as internal reference genes, semi-quantitative RT-PCR was used to identify the expression pattern of the four *MiACT* genes in Tainong No.1 leaves after several different treatments. After the application of ABA, GA_3, BA, and pacloputrazol, the four *MiACT* genes were extensively expressed in leaves. The expression of *MiACT1* was little affected by these treatments. In contrast, the transcription of *MiACT4* was up-regulated by ABA treatment. Both *MiACT7* and *MiACT9* genes were similarly up-regulated by ABA, GA, and 6-BA treatments, whereas greatly elevated expression of *MiACT9* was detected after pacloputrazol treatment. Thus, three of the four genes were responsive to hormonal and growth factor regulation (Fig. 2*C*).

The expression patterns of the four *MiACT* genes in Chock Anand leaves and stems in response to various stresses were also obtained in the present study. In leaves, the expression patterns of *MiACT1*, *MiACT4*, and *MiACT7* were not affected by stress treatments. The transcription of *MiACT9* was up-regulated by NaCl, and *MiACT7* had the lowest transcription level than any other *MiACT* gene (Fig. 2*D*). In stems, the *MiACT4, MiACT7* and *MiACT9* showed similar expression patterns and were down-regulated by waterlogging, NaCl, and high temperature. However, *MiACT1* expression in stem was little affected by these stresses (Fig. 2*E*). The *MiACT1* expression was not induced by $Pb(NO_3)_2$ and $CoCl_2 \cdot 6\ H_2O$ in both leaves and stems (Fig. 2*F*,*G*). The basic expression level of *MiACT1* was relatively high in Tainong No.1 fruits under normal condition. However, after hot water treatment the expression of *MiACT1* was slightly inhibited. The transcript level of *MiACT1* was rather stable in different days during the post-harvest period egardles of hot water treatment or not (Fig. 2*H*,*I*).

The expression patterns of *AP2/ERF* gene in response to different treatments were studied in mango. *AP2/ERF* gene expressed higher at 16:00 than any other time points in leaves of cv. Tainong No.1 (Fig. 2*B*). The expression level of *AP2/ERF* was up-regulated by ABA in leaves of cv. Tainong No.1 (Fig. 2*C*) and by low temperature in stems and leaves of cv. Chok Anand (Fig. 2*D*,*E*), and down-regulated by NaCl and high temperature in stems (Fig. 2*E*).

Discussion

In this work, we first reported the cloning the actin genes from mango. Four novel actin genes were obtained by using RT-PCR and RACE method. The four *MiACT* genes displayed high sequence similarity within their coding regions but highly divergent sequences in 3′-untranslated regions (data not shown). *BLAST* analysis revealed that the four *MiACT* genes were highly similar to other actin sequences present in the GenBank database.

Phylogenetic analysis revealed that the four MiACT proteins formed two separate groups with MiACT1 and MiACT9 in one and MiACT4 and MiACT7 in another. MiACT1 and MiACT9 were grouped into class A along with many previously described plant actins. Fifteen distinct members of the actin gene family in cotton (*Gossypium hirsutum*) were also classified into class A and all these *GhACT* genes except *GhACT6* were expressed in vegetative and reproductive organs including leaf, stem, cotyledon, root, anther, fiber, and petal, and the transcript levels of these *GhACT* genes were different in all tested tissues (Li *et al*. 2005). The transcriptions of *MiACT1* and *MiACT9* were constitutively and stably expressed in both vegetative and

reproductive organs. *MiACT4* and *MiACT7* are more closely related to *AtACT1*, *AtACT3*, *AtACT4*, and *AtACT12* from *Arabidopsis thaliana* and grouped into class B. In *Arabidopsis,* the eight highly expressed actins are divided into two ancient classes, vegetative (*AtACT2*, *AtACT7*, and *AtACT8*) and reproductive (*AtACT1*, *AtACT3*, *AtACT4*, *AtACT11*, and *AtACT12*), that are thought to have diverged from a common ancestral actin sequence more than 500 million years ago (McDowell *et al*. 1996b, McKinney and Meagher 1998). The *AtACT1*, *AtACT3*, *AtACT4*, and *AtACT12* genes were strongly expressed in mature pollen but at very low levels in the other major organs (An *et al.* 1996a, Huang *et al*. 1996), whereas *AtACT2*, *AtACT7,* and *AtACT8* were coordinately and strongly expressed in vegetative organs (An *et al*. 1996b, McDowell *et al*. 1996a). The transcripts of *MiACT4* and *MiACT7* were ubiquitously expressed in vegetative and reproductive organs, including leaves, flowers, stems, and fruits. However, *MiACT4* and *MiACT7* did not group with *AtACT2*, *AtACT7*, or *AtACT8* in the phylogenetic tree. A similar discrepancy between expression pattern and the phylogenetic analysis was also reported in the previous studies (Peng *et al*. 2010).

Quantitative PCR is much more accurate to investigate gene expression than semi-quantitative RT-PCR. The quantitative PCR require the special pair of primers but we could not obtain these primers when we analyze the high sequence similarity genes. Therefore, the semi-quantitative RT-PCR may be the best choice to analyze these genes. For RT-PCR to measure accurately relative expression among organs across time points or in response to different treatments, specific PCR conditions and an appropriate internal control must be determined (Dean *et al*. 2002). In the present study, semi-quantitative RT-PCR was performed to examine the expression patterns of the four *MiACT* genes in different organs of two mango cultivars in response to plant hormones, paclobutrazol, and various stresses. We also studied the PCR conditions for each of four *MiACT* genes to avoid saturated amplification of the genes and 32 cycles is the best choice for the RT-PCR analyses of four *MiACT* genes (date not shown). The obtained results indicated that the expression profiles of all the four *MiACT* genes were constitutive and stably expressed in all tested tissues under normal condition and not regulated by diurnal rhythms. *MiACT7* and *MiACT9* were significantly induced by exogenous plant hormones and by paclobutrazol whereas *MiACT4* was up-regulated only by ABA. These results imply that *MiACT7*, *MiACT9*, and *MiACT4* might be involved in hormone signalling pathways. The four *MiACT* genes except *MiACT1* showed similar expression patterns and were regulated by stress treatments.

The *AP2/ERF* protein family contains transcription factors that play a crucial role in plant growth and development and in response to biotic and abiotic stressors (Licausi *et al*. 2010). Indeed, expression was induced by dehydration, NaCl, and ABA treatments (Yang *et al*. 2009, Zhu *et al*. 2010). In our experiments, the expression level of each of four *MiACT* genes was used as a control for the expression of *AP2/ERF*. We found that circadian rhythm, ABA, NaCl and temperature regulate the expression level of *AP2/ERF* gene in mango.

The *MiACT1* gene was ubiquitously and stably expressed in different tissues, not regulated by diurnal rhythms, little affected by plant hormones, paclobutrazol, stress treatments, and in different days during the post-harvest period either under hot water treatment or under normal condition. These results suggested that *MiACT1* is the best candidate as a reliable reference gene for gene expression studies in mango.

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