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Isolation, Characterization, and Expression Analysis of the *MaMDH* Gene in Banana

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***Abstract*. A full-length cDNA isolated from banana (*Musa acuminata* L. *AAA group*) fruit was named *MaMDH*, containing an open reading frame encoding 332 amino acids that represents the gene for cytoplasmic malic dehydrogenase (MDH). Sequence analysis showed that *MaMDH* shares high similarity with *MDHs* from castor bean (XP_002533463), tobacco (CAC12826), peach (AAL11502), and chickpeas (CAC10208). Real-time quantitative polymerase chain reaction (PCR) analysis of *MaMDH* spatial expression showed that it was expressed in all organs examined: roots, rhizomes, leaves, flowers, and fruits. The expression was the highest in flowers followed by the fruits and roots, whereas the rhizomes and leaves displayed the lowest expression levels. Real-time quantitative PCR revealed that *MaMDH* exhibited differential expression patterns in post-harvest banana fruits correlating with ethylene biosynthesis. In naturally ripened banana fruits, *MaMDH* expression was in accordance with ethylene biosynthesis. In accordance, for banana fruits treated with the ethylene analog 1-methylcloprope ne (1-MCP), *MaMDH* expression levels were inhibited and remained constant. After treatment with ethylene, *MaMDH* expression in banana fruits significantly increased with ethylene biosynthesis and peaked 3 days after harvest, which was 11 days earlier than that in naturally ripened banana fruits. These results suggest that *MaMDH* expression is induced by ethylene to regulate post-harvest banana fruits ripening.**

Malic acid dehydrogenase (MDH) exists widely in living organisms and is a key enzyme in biological sugar metabolism that catalyzes the oxidation of malic acid to oxaloacetic acid in a reversible fashion. According to the coenzyme specificity, MDH can be divided into two subgroups that are dependent on either NAD (NAD-MDHs) or NADP (NADP-MDHs). MDH, an important metabolic enzyme in plants, participates in many physiological activities, including the TCA cycle, C4 circulation, respiration, fatty acid oxidation, nitrogen assimilation, amino acid synthesis, plant growth, pollen development, and improving plant tolerance to aluminum ability (Kochianl et al., 2004, Ma et al., 2001, Yao et al., 2011). As a result of its multifaceted activities, there are many MDH isozymes in plant cells. Depending on coenzyme specificity, cellular localization, and physiological function, these isozymes can be divided into five categories: chloroplast NAD-MDH, chloroplast NADP-MDH, mitochondria NAD-MDH (mMDH), microbody NAD-MDH (including glyoxysomal MDH and peroxisomal MDH), and cytoplasmic NAD-MDH (cyMDH) (Gietl, 1992; Ocheretina and Scheibe, 1997). At present there is a lot of research on cyMDH, but its function is not understood. In peaches, expression of cyMDH in ripe fruits was higher than that in fruits in the early developmental stages (Etienne et al., 2002). Likewise, the expression of the cyMDH in apples gradually increased during fruit development and maturation, resulting in enhanced cyMDH activity simultaneously (Yao et al., 2011b). Both malate and fructose content increased the effect of elevated *cyMDH* expression in apple calluses and tomatoes. In wheat, cyMDH mainly catalyzed synthesis of malic acid produced in response to stress conditions (Ding and Ma, 2004). Other studies reported that *cyMDH* played a role in enhancing plant resistance to aluminum, salt, and chilling injury as well as facilitating seed germination, cell growth, and pollen development (Wang et al., 2010; Yao et al., 2011a).

Banana (*Musa acuminata* L. *AAA group* cv. Brazilian) is a typical climacteric fruit, which is largely cultivated in tropical and subtropical regions. The pattern of ethylene production during banana fruit ripening differs from other climacteric fruits with a sharp rise and fall in the rate of ethylene production occurring during the early climacteric rise in respiration (Liu et al., 1999). There are many physiological and biochemical changes in the post-harvest maturation process such as polyphenol and chlorophyll degradation, aromatic substance formation, and changes in carbohydrate levels. These changes all directly affect the quality, flavor, color, and post-harvest shelf life of banana fruits (Giovannoni, 2004). Therefore, research on the development of banana fruits and the regulation of post-harvest maturation is important for improving banana fruit quality and prolonging shelf life. At present, there is little research concerning basal metabolic enzymes in banana, and there are no reports on the *MDH* gene in banana. In this study, we cloned the cytoplasmic malic acid dehydrogenase gene *MaMDH* from banana fruits using cDNA probes with the quick end amplification method [rapid amplification of cDNA ends (RACE)] and analyzed the sequence using BLAST (<http://ncbi.nlm.nih.gov/blast>) and SOPMA (<http://npsa-pbil.ibcp.fr/cgi-bin/>). We also used fluorescent quantitative PCR to evaluate *MaMDH* expression in different organs of banana plants and post-harvest fruits exposed to different treatments. The result of this study provides a certain basis for further research on enzyme metabolism of banana fruits and contributes to understanding the molecular mechanism of banana and climacteric fruit during the maturation process.

Materials and Methods

Banana fruits and treatments. Banana (*M. acuminata* L. *AAA group*, cv. Brazilian) fruits were provided by the banana plantation of the Institute of Tropical Bioscience and Biotechnology (Chengmai, Hainan) and were harvested at the mature green stage (100 to 110 d after flower shooting). Banana fruit hands of similar developmental stages were selected and three fingers from the hands were divided into three groups for different treatments. For natural ripening, the group of banana fruits was kept at 25 °C and allowed

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to ripen naturally. For ethylene treatment, the group of banana fruits was treated with 100 μL·L^{-1} ethylene in an airtight container for 24 h at 25 °C to initiate ripening. For ethylene inhibition treatment, 1-MCP was used to interfere with ethylene responses; banana fruits were sealed in an airtight container and treated with 1 μL·L^{-1} 1-MCP for 24 h at 25 °C. The treated fruits were allowed to ripen at 25 °C and samples were then frozen in liquid N_2 and stored at –80 °C for subsequent extraction of total RNA at different ripening stages. The experiments were repeated at least three times.

Measurement of ethylene production. Ethylene evolution was measured by enclosing fruit samples in an airtight container for 2 h at 25 °C, withdrawing 1 mL of the headspace gas, and injecting the gas into a gas chromatograph (GC-2010; Shimadzu, Kyoto, Japan) fitted with a flame ionization detector and an activated alumina column. Ethylene production measurements were obtained as recommendations by the manufacturer. The experiments were repeated at least three times.

RNA extraction and cDNA synthesis. For the expression at the tissue-specific level, total RNA was separately extracted from the roots, rhizomes, leaves, flowers, and fruits of 8-month-old plants using the cetyltrimethylammonium bromide (CTAB) method (Asif et al., 2000).

For cloning of full-length cDNA, total RNA from banana fruit tissues (including peel and pulp) was isolated 2 d after harvest using the CTAB method. First-strand cDNA was synthesized using the SMART™ PCR cDNA Synthesis Kit and SMARTScribe reverse transcriptase (Clontech, Palo Alto, CA) according to the manufacturer's instructions.

Isolation of full-length MaMDH cDNA using RACE-PCR. Based on the entire 3′ cDNA of *MaMDH,* previously cloned by suppression subtractive hybridization (SSH) (Xu et al., 2007), RACE was used to obtain the 5′ end of the cDNA using double-stranded cDNA as a template. The 5′-RACE was performed using the BD SMARTer RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. The amplified products of the 5′ cDNA ends were inserted into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced by the Sangon Company (Shanghai, China) using an ABI 3770 DNA sequencer. According to the 5′- and 3′-cDNA regions, a pair of gene-specific primers was designed (5′-ATGG CAAAAGATCCGGTGC-3′ and 5′- CATG TATTCTTGCCTTGGGAC-3′) to obtain full-length cDNA. The sequence was analyzed by BLAST (<http://ncbi.nlm.nih.gov/blast>).

Quantitative real-time RT-PCR analysis of MaMDH expression. For real-time quantitative reverse transcription (RT)–PCR, total RNA was extracted from the roots, rhizomes, leaves, flowers, and fruit tissues (including peel and pulp) from naturally ripened banana fruits at 0, 2, 6, 10, 12, 14, and 16 d after harvest; from ethylene-treated fruits at 0, 1, 2, 3, 4, 5, and 6 d after harvest; and from 1-MCP-treated fruits at 0, 2, 6, 10, 12, 14, and 16 d after harvest. Poly(A)$^+$-mRNA (200 ng) was converted into cDNA using the SMART PCR cDNA Synthesis Kit (Clontech) in a final volume of 20 μL, which subsequently served as the template for real-time PCR.

The expression level of *MaMDH* was determined by real-time RT-PCR analysis using Stratagene Mx3000P (Stratagene, CA) and SYBR Green as a fluorescent dye. The reaction mixture consisted of a 25-μL solution containing 12.5 μL 2 × SYBR Green PCR Master Mix, 0.5 μL ROX reference dye, and 100 ng reverse-transcribed RNA. The primer sets used and their optimal amounts were as follows: MaMDH5′: 5′-TGCTGGCG AATGGACAATC-3′ (10 pmol); MaMDH3′: 5′- GGCGGCAACAGAGTCAACC-3′ (10 pmol), actin5′: 5′-CGAGGCTCAATCAA AGA-3′ (10 pmol), actin3′: 5′-ACCAGCA AGGTCCAAAC-3′ (10 pmol). The thermal cycling conditions were 94 °C for 3 min followed by 40 cycles at 94 °C for 7 s, 55 °C for 15 s, and 72 °C for 20 s. Reactions were performed in triplicate and the data analyzed using MxPro™ QPCR software (Stratagene). Actin was used as a reference sample to which the *MaMDH* product amounts were compared. Differences in Ct values between the *MaMDH* and actin transcripts were expressed as fold changes relative to actin. The experiments were repeated at least three times.

Results

Cloning and sequence analysis of MaMDH from banana fruits. A cDNA fragment having high similarity to the *MDH* gene was previously obtained by SSH (Xu et al., 2007). RACE approaches were then used to isolate full-length *MDH* cDNA from banana fruits. Sequence analysis revealed that the cDNA was 999 bp in length, having an open reading frame encoding a single peptide of 332 amino acid residues with 48- and 250-bp 5′ and 3′ untramslated regions (UTRs), respectively. This gene was named *MaMDH.* The native molecular weight of the MaMDH protein was estimated to be 44.08 kDa with an isoelectric point of 6.26 (data not shown). Conserved domain analysis showed that MaMDH protein had 15 NAD-binding sites, eight malic acid-binding sites, and 16 dimer-binding sites. Meanwhile, MaMDH contained the catalytic group element "IWGNH" and NAD binding motif "TGAAGQI" (Fig. 1), which are both widely and highly conserved in cyMDHs from many species, suggesting that this gene encodes a cytoplasmic malate dehydrogenase.

The deduced amino acid sequence of MaMDH also had high sequence identity with MDHs from other plant species, including castor bean (92%), tobacco (92%), peach (92%), and chickpeas (92%) (Fig. 1). The 15 plant MDH amino acid sequences in the National Center for Biotechnology Information database were aligned by MEGA, which showed that, compared with other species, MaMDH is closer to cytoplasmic MDH. Once again, these suggested that the *MaMDH* gene encodes a cytoplasmic malate dehydrogenase (Fig. 2).

Hydropathy profile and secondary structure of MaMDH. Hydrophilicity analysis found that there was a strong hydrophobic domain at the N-terminal (Fig. 3A), which is another widespread feature of cyMDHs from other species. Protein secondary structure is a periodic conformation that arranges the polypeptide chain in a one-dimensional direction through the formation of hydrogen bonds, and its prediction and analysis aid the understanding of the protein's spatial structure. The MaMDH secondary structure was predicted from the amino acid sequence using Self Optimized Prediction Method from Alignment (SOPMA). The secondary structure of the putative MaMDH protein was composed of 45.2% α-helix, 5.7% β-turn, 17.2% extended strand, and 31.9% random coil. The C-terminal section of the protein had a greater amount of α-helix structure (Fig. 3B).

Temporal and spatial transcript accumulation of MaMDH. To clarify the patterns of *MaMDH* expression in different organs of the banana plant, real-time quantitative RT–PCR was carried out on cDNA from different organs. *MaMDH* was expressed at different levels in all organs examined: roots, rhizomes, leaves, flowers, and fruits with abundant *MaMDH* transcripts in flowers, lower expression levels in the roots, rhizomes, and fruits, and the lowest expression levels in leaves (Fig. 4).

Measurement of ethylene evolution during post-harvest banana fruit ripening. Changes in the rate of ethylene production were examined during post-harvest banana fruit ripening. In naturally ripened banana fruits (Fig. 5A), ethylene production began to increase 8 d after harvest, peaked sharply at 14 d, and then rapidly decreased. For ethylene-treated banana fruits (Fig. 5B), endogenous ethylene production started 1 d after harvest and reached a maximum 3 d after harvest, which is 7 and 11 d, respectively, before that of naturally ripened banana fruits. In addition, the maximal level of ethylene production was higher for ethylene-treated banana fruits compared with naturally ripened banana fruits. However, on treatment with the ethylene inhibitor 1-MCP (Fig. 5C), ethylene production started 14 d after harvest and but its level remained very low.

Differential expression of MaMDH during banana fruit ripening. Changes in *MaMDH* expression during post-harvest ripening were examined using real-time RT-PCR. Changes in *MaMDH* expression in naturally ripened fruits differed from ethylene-treated fruits and occurred at different stages of ripening (naturally ripened: 14 d, ethylene-treated: 3 d) (Fig. 6A). There were marked changes in MaMDH expression levels in ethylene-treated banana fruits during post-harvest ripening with MaMDH expression increasing sharply during the period 0 to 3 d after harvest (Fig. 6B). For 1-MCP-treated fruits, *MaMDH* expression was essentially constant 0 to 14 d after harvest and peaked on Day 16 (Fig. 6C).

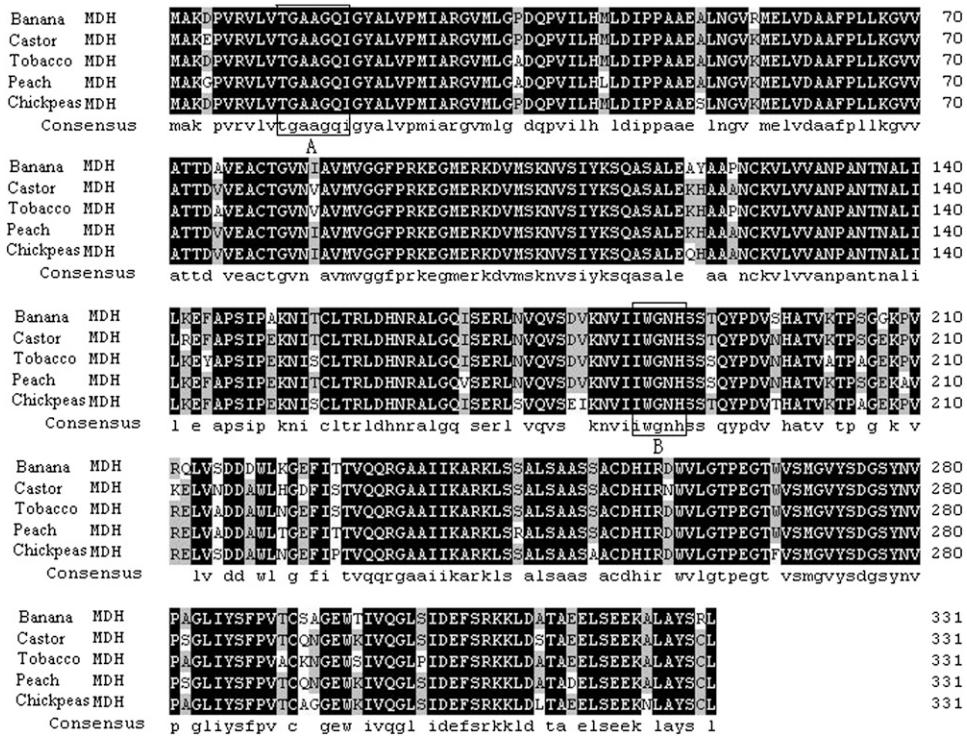

Fig. 1. Alignment of predicted amino acid sequences of MDH genes from different plants. (**A**) Domain: NAD-combined primitives; (**B**) domain: catalytic primitives. MDH = malic dehydrogenase.

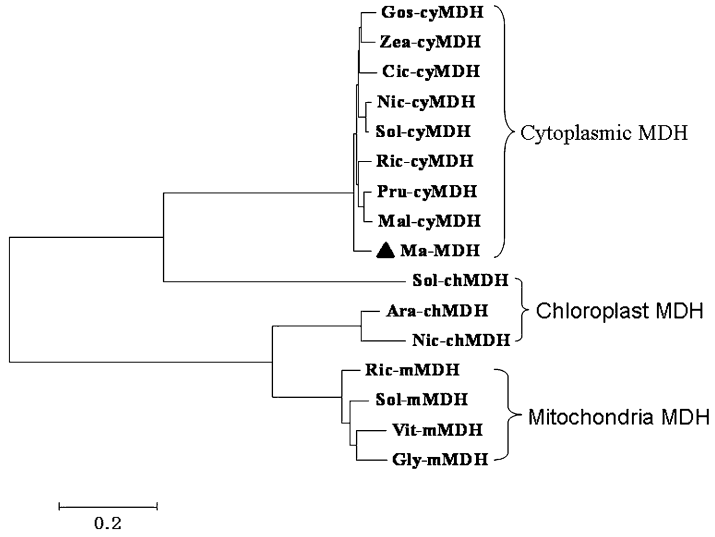

Fig. 2. Phylogenetic tree analysis of the deduced amino acid sequences of malic dehydrogenase (MDH) in different species. The tree was constructed using the MEGA method. cyMDH = cytosolic NAD-dependent MDH; mMDH = mitochondrial NAD-dependent MDH; chMDH = chloroplast NADP-dependent MDH; Pru-cyMDH (AAL11502); Mal-cyMDH (ABB36659); Ric-cyMDH (XP-002533463); Nic-cyMDH (CAC12826); Gos-cyMDH (ACJ11738); Zea-cyMDH (NP-001105603); Cic-cyMDH (CAC10208); Sol-cyMDH (ABC01890); Ara-chMDH (CAA74320); Nic-chMDH (CAB45387); Sol-chMDH (NP001234009); Gly-mMDH (AAD56659); Ric- mMDH (XP-002524262); Vit-mMDH (AF195869); Sol-mMDH (AAU29198). MaMDH (banana).

Discussion

We cloned the malic acid dehydrogenase gene from the cDNA library of banana fruits and found that *MaMDH* had a NAD-binding site, malic acid-binding site, and dimer-binding site that are consistent with other members of the cyMDH family. In addition, MaMDH carried the catalytic sequence "IWGNH" and NAD combined site "TGAAGQI," which are two basic elements that are highly conserved in the cyMDHs of many species. These features together indicate that MaMDH may be a member of the cytoplasmic-type malic acid. Homology analysis found that *MaMDH* encoded a protein that showed high amino acid sequence identity and sequence conservation with several cyMDHs from plants. In terms of cellular localization, MDHs from the same organelles have higher sequence similarity. According to the endosymbiotic theory for the origin of organelles, different MDH family members may have arisen as a result of bacterial invasion of eukaryotic cells (Gray et al., 2001; McAlister-Henn, 1988). However, the relationship between different kinds of MDH is complex, and understanding their evolutionary origins will require future research.

A strong hydrophobic domain 26 amino acids downstream from the first five N-terminal amino acids of MaMDH was found in all cyMDHs from plants, animals, and bacteria. Computer analysis indicated that this domain was unlikely to exist as a presequence or signal peptide. Furthermore, in alfalfa, the N-terminal amino acid sequence of cyMDH protein matched exactly that predicted from the cDNA sequence (Susan et al., 1998). This excludes the possibility of proteolytic of cyMDH peptides. Whether this implies that cyMDHs interact with some membrane system is an interesting hypothesis warranting further investigation.

The secondary structure analysis by molecular modeling MaMDH was mainly constituted with α-helix and random coil, which were interlaced with β-turn and extended strand. The structures of bend, turn, and random coil are essential for the MDH reaction, because both the catalytic site and NAD-binding domain are located in these structural domains.

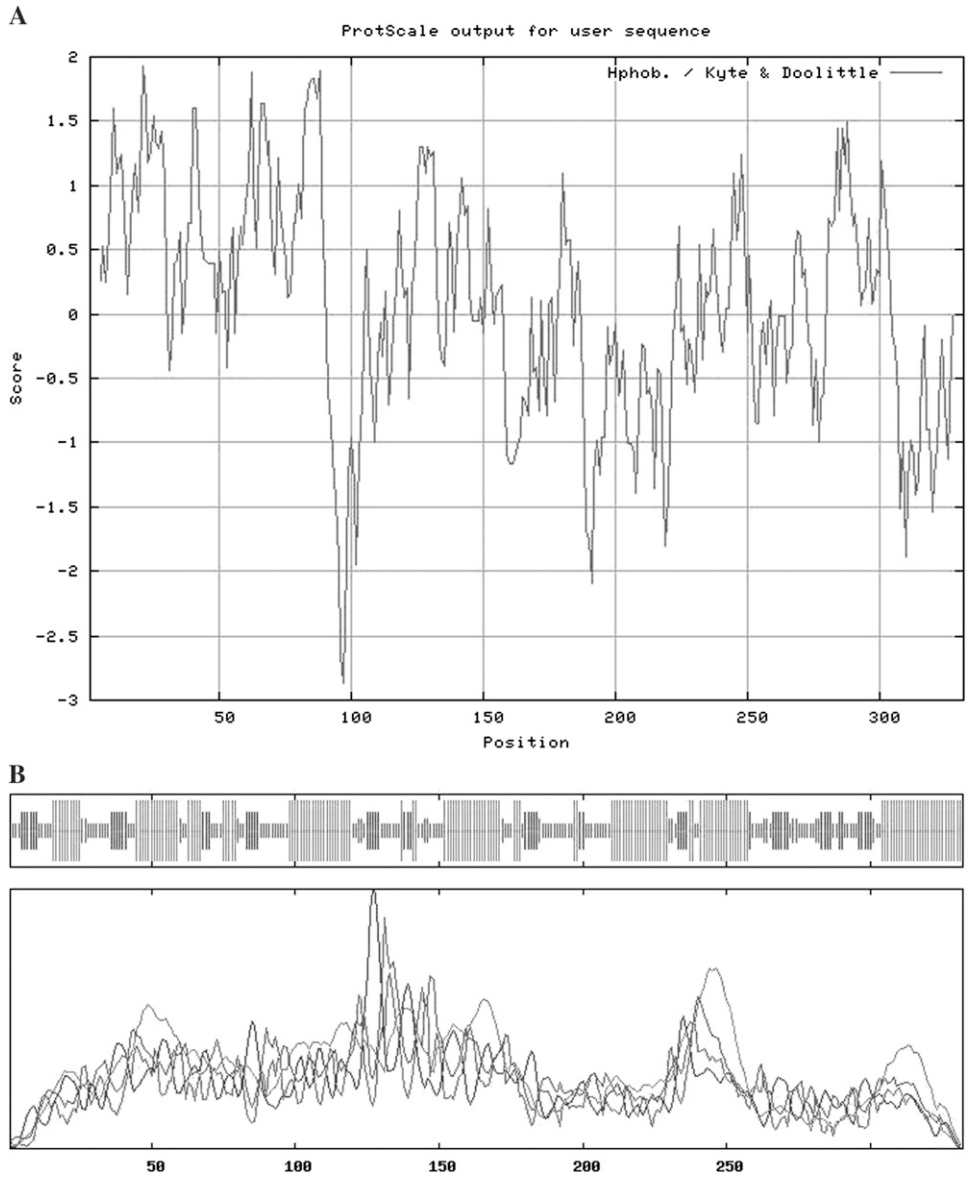

Fig. 3. (**A**) Hydropathy profile of MaMDH. Hydropathy was analyzed using ProtScale. Hydrophobic domains are those that are above the zero line, whereas the hydrophilic domains are those that are below the zero line. (**B**) MaMDH secondary structure prediction. The α-helix was represented in blue. The β-turn was represented in green. The extended strand was represented in red. The random coil was represented in orange.

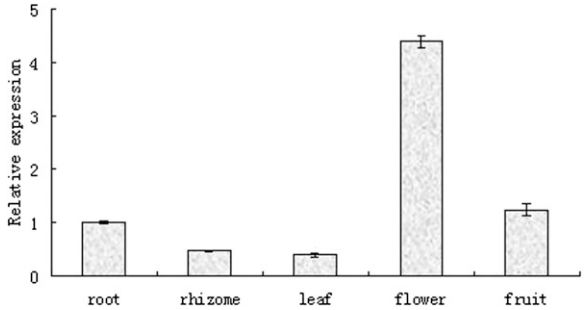

Fig. 4. Expression of *MaMDH* in different organs by real-time reverse transcription–polymerase chain reaction. Samples were obtained from the roots, rhizomes, leaves, flowers, and fruits of 8-month-old plants. The means were generated from three independent measurements and the vertical bars indicate the SES. When absent, the SE bars fall within the dimensions of the symbol.

In most climacteric fruits, ethylene evolution begins to increase at the onset of the climacteric period and continues to increase until the final decrease as the fully ripe stage is reached. As a typical climacteric fruit, banana fruits exhibit different ethylene biosynthesis patterns with a sharp rise and fall in ethylene production during the early climacteric rise in cell respiration (Liu et al., 1999). In this study there was no detectable ethylene production in naturally ripened banana fruits during the pre-climacteric period (Fig. 5A). A sharp peak in ethylene production at the onset of the climacteric period has been recognized as a characteristic ripening feature of banana fruits (Inaba et al., 2007). In ethylene-treated banana fruits, ethylene biosynthesis was activated immediately after ethylene treatment (Fig. 5B). 1-MCP inhibits ethylene perception (Sisler and Serek, 1997) and also influences ethylene biosynthesis by feedback inhibition of acc synthase gene (ACS) and acc oxidase gene (ACO) enzyme expression (Blankenship and Dole, 2003). Compared with ethylene-treated banana fruits, ethylene biosynthesis was almost completely inhibited in 1-MCP-treated banana fruits.

At present, there are many studies concerning MDH (Berkemeyer et al., 1998; Cvetić

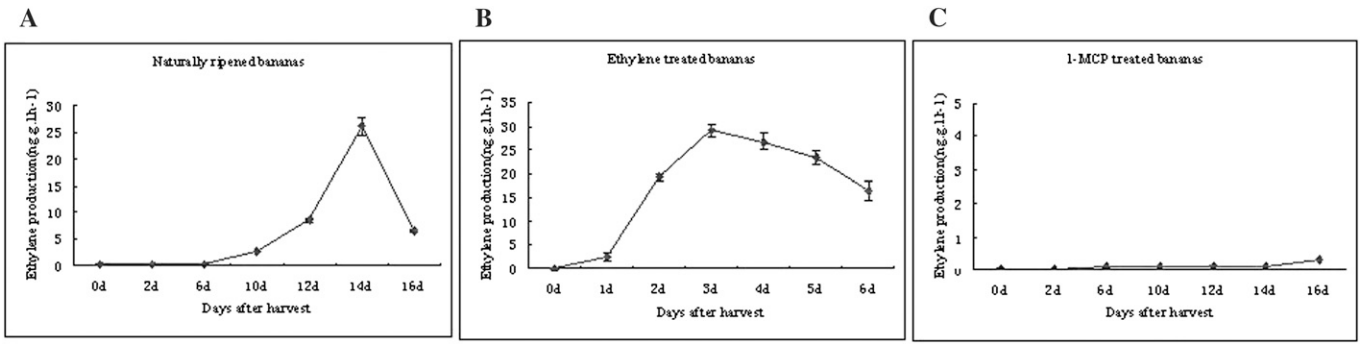

Fig. 5. Changes in ethylene production in naturally ripened (**A**), ethylene-treated (**B**), and 1-MCP-treated (**C**) bananas. Vertical bars indicate the SE of three replicates. When absent, the SE bars fall within the dimensions of the symbol. 1-MCP = 1-methylcyclopropene.

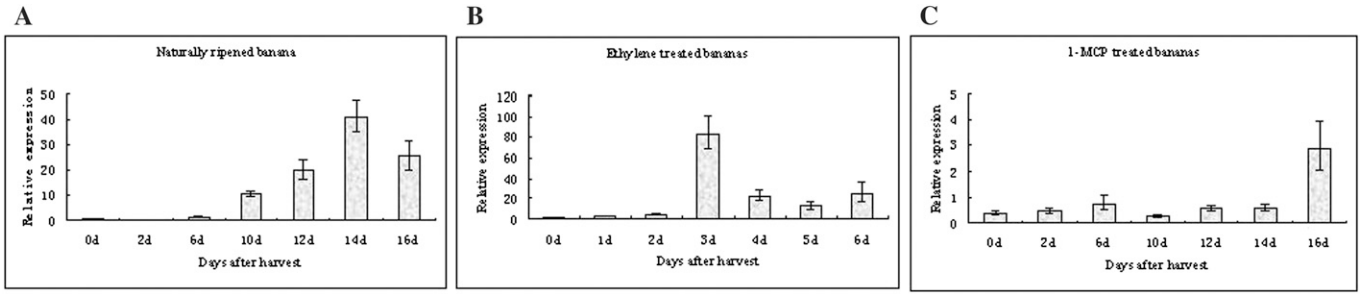

Fig. 6. Relative *MaMDH* expression as determined by real-time reverse transcription–polymerase chain reaction in naturally ripened (**A**), ethylene-treated (**B**), and 1-MCP-treated (**C**) bananas. The means were generated from three independent measurements and the vertical bars indicate the SEs. When absent, the SE bars fall within the dimensions of the symbol. 1-MCP = 1-methylcyclopropene.

et al., 2008; Hebbelmann et al., 2012), but its function is not understood. One of the available studies about the various MDH types showed that *cyMDH* expression in apples was higher in leaves, stems, and fruits accompanied by low expression in roots (Yao et al., 2011b). In this study, we followed *MaMDH* expression in banana tissues using fluorescent quantitative PCR and found that the gene was expressed in roots, rhizomes, leaves, flowers, and fruits with the highest expression levels in the flowers followed by expression in roots and fruits and relatively low expression in rhizomes and leaves. Our results thus have similarities to that of Yao et al. (2011b), but some differences were also observed. Like apples, cytoplasmic malic acid dehydrogenase gene was expressed in all organs, but *MaMDH* expression in the roots was considerably high; only flower- and fruit-specific expression preceded it. This difference may be a species characteristic in banana, but may also be the result of different mechanisms found in climacteric fruit.

To date, there are more studies on the decomposition and anabolic metabolism regulated by MDH, but the role of MDH in plant development and fruit ripening has not been fully clarified yet and our knowledge is still limited concerning cytoplasmic malate dehydrogenase gene expression during climacteric fruit ripening. We studied *MaMDH* gene expression in naturally ripened banana fruits, 1-MCP-treated banana fruits, and ethylene-treated banana fruits (Fig. 6). In naturally ripened banana fruits, *MaMDH* expression increased after harvest reaching a maximum on the same day as ethylene production peaked; in ethylene-treated banana fruits, *MaMDH* expression rose sharply along with ethylene biosynthesis; in 1-MCP-treated banana fruits, *MaMDH* expression was almost completely suppressed. This result suggests that *MaMDH* expression may be developmentally regulated by ethylene biosynthesis during post-harvest banana fruit ripening. The relative maximal expression of *MaMDH* during post-harvest ripening in ethylene-treated, naturally ripened, and 1-MCP-treated banana fruits was 83.1, 41.0 and 2.9, respectively. Exogenous ethylene treatment caused four-fold or greater and 28.6-fold or greater increases in maximum *MaMDH* expression compared with naturally ripened and 1-MCP-treated banana fruits, respectively (Fig. 6). These results strongly suggest that exogenous ethylene stimulated maximal *MaMDH* expression. However, although endogenous ethylene biosynthesis is induced and peaked at Day 3, which is in line with *MaMDH* expression, the expression of *MaMDH* in ethylene-treated banana fruits changed sharply relative to the naturally ripened banana fruits. According to previous study (Liu et al., 1999), the change in ethylene production by the naturally ripened and ethylene-treated fruits was different and may be the result of different mechanisms involved in regulating ethylene biosynthesis. *MaMDH* expression is likely induced by ethylene and plays an important role in the fruit ripening process, but additional data are needed to support this conclusion.

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