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Full Length Research Paper

Expression analysis of banana *MaECHI1* during fruit ripening with different treatments

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The main function of endochitinase is believed to be pathogenesis related protein. However, more and more scientists reported the roles of endochitinase in plant growth and development. In order to investigate the role of endochitinase in postharvest banana fruit ripening, an endochitinase gene known as *MaECHI1* had been isolated from a suppression subtractive hybridization (SSH) complementary deoxyribonucleic acid (cDNA) library. *MaECHI1* was mainly expressed in banana fruit and flowers. Ethylene biosynthesis, gene expression and chitinase activities in different stages of postharvest banana fruit with or without ethylene and 1-methylcycle–propene (1-MCP) treatments were investigated. The results show that under ethylene treatment, banana ethylene production, gene expression, and chitinase activities increased markedly at the onset of banana ripening. Moreover, banana ethylene production and *MaECHI1* gene expression peaks appeared earlier with ethylene treatment than with other treatment. *MaECHI1* gene expression was markedly responsive to the fruit ripening process and to exogenous ethylene treatment.

Key words: Banana (*Musa acuminata* L.AAA), endochitinase gene expression, ethylene production fruit ripening.

INTRODUCTION

Chitinases (EC 3.2.1.14) are hydrolytic enzymes with three activity classes. One class is the β-(1, 4)-N-acetylglucosaminases, which cleave N-acetylglucosamine (NAG) oligomers and generate NAG monomers. A second class is the chitobiosidases, which cleave diacetylchito-biose units from the non-reducing end of the chitin chain and release disaccharides. The third class is the endochitinases, which cleave glycosidic linkages randomly at internal sites along the chitin chain and eventually provide a variety of low molecular mass NAG oligomers, like diacetylchitobioses and chitotrioses (Guthrie et al., 2005; Li and Greene, 2010). Although chitin does not exist in plant cell walls, chitinases are widely distributed in plants; this suggests that plants contain other uncharacterized targets (Kitajima et al., 2010; Neuhaus, 1999).

The main function of endochitinases is believed to be the hydrolysis of chitin, a structural polysaccharide, and to modify cell wall morphogenesis (Arakane and Muthukrishnan, 2010; Hossain et al., 2010; Sahai and Manocha, 1993). Some plant endochitinases are members of the pathogenesis-related (PR) proteins which are induced after systemic acquired resistance induction (biotic and abiotic) (Di Maro et al., 2010; Pulla et al., 2011; Ahmed et al., 2011; Tapia et al., 2011; Zhong et al., 2002). An increasing number of scientists have provided evidence for the roles of endochitinases in plant growth and development (Hermans et al., 2010). Most

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Abbreviations: 1-MCP, 1-Methylcyclopropene; **SSH,** suppression subtractive hybridization; **DAH,** days after harvest. **cDNA,** complementary deoxyribonucleic acid.

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plant endochitinases are inducible with wounding, cold, pathogen infections, or hormones, like ethylene, methyl jasmonate, and gibberellins (Zhong et al., 2002; Hedrick et al., 1998; Liu et al., 2010; López and Gómez-Gómez, 2009; Wu and Bradford, 2003; Yeh et al., 2000). This suggested that endochitinase may play different roles under different conditions.

Banana (*Musa acuminata* L.AAA) is a fruit crop of global economic importance. It is a primary source of nutrition for more than 4 billion people in the world. China produces approximately 10% of the world's yearly banana yield. Banana fruit is a typically climacteric fruit, characterized by a green-storage phase, followed by a burst in ethylene release. Consistent with the sharp peak in ethylene production, numerous physiological and biochemical changes occur in the banana fruit that result in ripening. Several genes have been isolated that were up-and down-regulated during this peak, including the chitinase gene. Medina-Suárez et al. (1997) discovered that a chitinase gene was down-regulated during banana fruit ripening. In the same year, Clendennen and May (1997) found that chitinase transcripts were abundant in ripening banana fruit. However, little is known about endochitinase activity in banana fruit ripening and ethylene production.

In order to investigate the role of endochitinase in postharvest banana fruit ripening, an endochitinase gene known as *MaECHI1* had been isolated from a suppression subtractive hybridization (SSH) complementary deoxyribonucleic acid (cDNA) library. This gene was abundant at the transcript level during the entire fruit ripening process (Xu et al., 2007). This led us to speculate that this gene played a role in banana fruit ripening. In the present study, we examined ethylene biosynthesis, differential gene expression patterns, and chitinase activities in banana fruit at different stages of postharvest, with or without treatments of ethylene and 1-methylcycle–propene (1-MCP). The results suggest that differential expression of banana *MaECHI1* may be involved in banana fruit ripening processes. Prior to this report, no published studies have addressed the expression of the *MaECHI1* during banana fruit ripening.

MATERIALS AND METHODS

Plant material and treatments

For tissue specific expression, banana seedlings were cultivated for 60 days and then harvested. The roots, stems, and leaves were separately frozen in liquid N_2 for ribonucleic acid (RNA) extraction. Mature green banana fruit (*Musa spp*. AAA group, cv. Brazilian) were harvested at 110 days after anthesis from a banana plantation (Lingao, Hainan province, China). Fruit clusters (banana hands) were separated into three groups; a control group was allowed to ripen naturally; the second and third groups were exposed for 12 h to 100 µl L^{-1} ethylene or 1 µl L^{-1} 1-MCP (Ethylblock, Rhom, and Haas, USA), respectively (Liu et al., 1999; Lohani et al., 2004). The three treatment groups were stored in separate chambers at 25°C; banana samples were obtained on a daily basis for enzyme assays and RNA extraction.

Gene isolation

MaECHI1 was isolated from a banana fruit cDNA library with a polymerase chain reaction (PCR) method (Xu et al., 2007) with the λTriplEx2 arm primers: Ptr5 CTCCGAGATCTGGACGAGC and Ptr3 TAATACGACTCACTCACTATAGGG.

Measurement of ethylene production

Ethylene production was measured by enclosing fruit samples in an airtight container for 2 h at 25°C, then withdrawing 1 ml of the headspace gas, and injecting the gas into a gas chromatograph (GC2014; Shimadzu, Tokyo, Japan). The area under the chromatograph peak indicated the amount of ethylene produced. Measurements were expressed as the ethylene produced per unit of sample per hour inside the container ($ng \cdot g^{-1} \cdot h^{-1}$).

Enzyme assay

The activity of endochitinase in a fruit sample was determined according to the method of Donzelli and Harman (2001). Briefly, fruit samples were combined with 0.1 mM (final concentration) of 4-methylumbelliferyl β-D-*N*,*N*' -diacetyl-chitobioside (Sigma) added as the substrate. The release of 4-methylumbelliferone after 30 min at 25°C was measured with a fluorescence (360-nm excitation, 460-nm emission) microtiter plate reader (Cytofluor II; PerSeptive Biosystems, Framingham, MA).

Semi quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from different banana tissues (root, stem, leaf, pulp, and peel) according to the method of Wan and Wilkins (1994). First strand cDNA synthesis (RT) was performed with Superscript^{TM}III reverse transcriptase (Invitrogen). This cDNA served as the template for PCR amplification. The PCR primers were designed to anneal to specific regions in the *MuACTIN* gene (GenBank accession: AF246288) and the endochitinase gene. The primer sequences were as follows: ACTINP1, 5′-GCCC TCCCTCATGCCATCCTTC-3′; ACTINP2, 5′-GAATACCAGCA-GCCTCCATGCC-3′; MaECHI1P1, 5′-GCTGTGCTG TAGCCA GTTCG -3′; MaECHI1P2, 5′-CACCAGGTCTGGGTTGTTGA -3′. 28 PCR cycles were performed in a programmed temperature control system (Biometra, Germany) under the following conditions: 30 s at 94°C, 40 s at 56°C, and 50 s at 72°C. Amplified products were analyzed by electrophoresis on a 1% (w/v) agarose gel.

Quantitative real-time RT-PCR

For real-time quantitative RT-PCR, each total RNA was extracted separately from naturally ripened banana fruit and 1-MCP-treated banana fruit at 0, 4, 8, 12, 18, 20 and 25 days after harvest (DAH); from ethylene-treated fruit at 0, 1, 2, 3, 4, 5 and 6 DAH. Each 200 ng Poly(A)$^+$ messenger ribonucleic acid (mRNA) 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 *MaECHI1* was determined by real-time RT-PCR analysis using Stratagene Mx3000P (Stratagene, CA, USA) and SYBR Green as a fluorescent

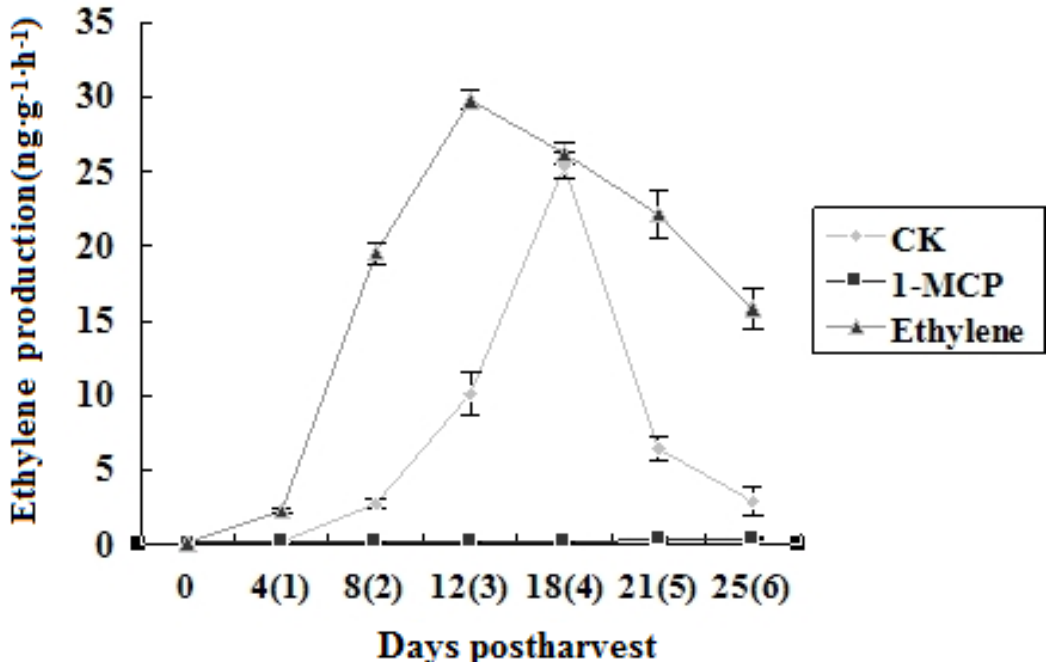

Figure 1. Changes in ethylene production in naturally ripened bananas, ethylene-treated bananas and 1-methylcycle–propene (1-MCP) treated bananas. Ethylene production was measured by gas chromatography. Vertical bars indicate the standard error (SE) of three replicates. When absent, the SE bars fall within the dimensions of the symbol. The X-axis represents the days postharvest, and numbers in parentheses represent the days after harvest with ethylene treatment.

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: MaECHI1P1, 5′-GCTGTGCTGT AGCCAGTTCG-3′(10 pmol);MaECHI1P2,5′-CACC-AGGTCTGGGTTGTTGA-3′(10 pmol), ACTINP1,5’-GCCCTC-CCTCATGCCATCCTTC-3’,ACTINP2,5’-GAATACCAGCAGCCTCC-ATGCC-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 standard and control sample to which the *MaECHI1* product amounts were compared. Differences in Ct values between the *MaECHI1* and actin transcripts were expressed as fold-changes relative to actin.

RESULTS

Gene isolation

To investigate differentially expressed banana genes in the very early stages after harvest, a full-length chitinase gene of 1051 bp was isolated from a suppression subtractive hybridization (SSH) cDNA library (Xu et al., 2007). The result of an NCBI BLASTx search (http://www.ncbi.nlm.nih.gov/) indicated that this gene was the same as an endochitinase from banana (GenBank accession: AF416677) called *MaECHI1*.

Ethylene biosynthesis during postharvest banana fruit ripening

Changes in ethylene production were examined during postharvest ripening of the banana. In naturally ripened fruit, ethylene production began to increase at eight DAH, reached a peak of 25.4 $ng \cdot g^{-1} \cdot h^{-1}$ at 18 DAH, and then decreased rapidly. The application of exogenous ethylene strongly promoted banana ethylene biosynthesis. In ethylene-treated bananas, ethylene biosynthesis began at one to two DAH, or six days earlier than the controls, and rapidly reached a peak of 29.7 $ng \cdot g^{-1} \cdot h^{-1}$ at three DAH. In contrast to exogenous ethylene treatment, a very low level of ethylene production was detected during the whole stage in 1-MCP- treated fruits (Figure 1).

MaECHI1 expression and enzyme activity stimulated by exogenous ethylene

The transcript levels of *MaECHI1* were examined in several tissues from banana plants (Figure 2). Based on the RT-PCR results, the transcript was undetectable in roots and stems; however, it was expressed at low levels in leaves, at elevated levels in flowers, and at the highest levels in the fruit. The expression level of *MaECHI1* in the fruit was further investigated. Under natural conditions,

Figure 2. Differential expression of the endochitinase gene (*MaECHI1*) in different banana tissues. PCR products are shown from amplifications of reverse transcribed cDNA samples isolated from root, stem, leaf, flower and fruit. The lower line shows the PCR amplification of the actin gene in each sample (positive control).

the level of gene expression in fruit gradually increased and peaked at 18 DAH and reached to 22.88 (Figure 3a), which was consistent with the process of ethylene biosynthesis. The enzymatic activity of *MaECHI1* showed a pattern similar to that found in the transcripts; that is, the enzymatic activity peaked at 20 DAH in naturally ripened banana fruit, which was two days later than the transcripts (Figure 4).

When the banana fruit was treated with ethylene, *MaECHI1* transcription rapidly increased and reached a maximum at four DAH; then, transcription quickly declined. The expression peak appeared 14 days earlier than that of naturally ripened fruit. The maximum gene expression level was nearly 300–folds higher than that of naturally ripened fruits (Figure 3b). Similarly, chitinase activity changed markedly during the ripening process. The endochitinase activity in fruit increased from 10.8 $U \cdot g^{-1} \cdot h^{-1}$ at 0 DAH to 161.9 $U \cdot g^{-1} \cdot h^{-1}$ at five DAH. The peak appeared 15 days earlier than that of naturally ripened fruits (Figure 4).

MaECHI1 expression and enzyme activity repressed by 1-MCP

The effect of 1-MCP, an inhibitor of ethylene biosynthesis, on both gene expression and endochitinase activity was investigated. From 0 to 25 DAH, the level of gene expression in the fruits remained consistently lower than gene expression after the other treatments (Figure 3c). Furthermore, the endochitinase activity in the fruit also remained constant during the period of 0 to 25 DAH (Figure 4).

DISCUSSION

In plants, endochitinase was first found to function in disease resistance (Sela-Buurlage et al., 1993). More recently, increasing evidence has shown that endochitinases play important roles in plant growth and development, including germination (Cordero et al., 1994; Wu et al., 1994), seedling growth (Hossain et al., 2010; Zhong et al., 2002; Hermans et al., 2010), flowering (Neale et al., 1990; Takakura et al., 2000), reproduction (Leung, 1992), and somatic embryogenesis (Dong and Dunstan, 1997; Kragh et al., 1996). To the best of our knowledge, only one report systematically showed that an abundant class III chitinase homolog behaved as a fruit-specific vegetative storage protein and served as a source of amino acids for the synthesis of ripening-associated proteins (Peumans et al., 2002).

The present study was the first to show that the endochitinase gene played a role in postharvest banana fruit ripening. It was found that *MaECHI1* was expressed primarily in banana fruit, which suggested that this gene played an important role in banana fruit ripening. This result was consistent with the reported results by Xu et al. (2007), who analyzed gene expression with the micro-array technique. The appearance of a peak in ethylene release is a primary characteristic of postharvest banana fruit ripening. This peak was previously shown to be markedly stimulated by exogenous ethylene (Liu et al., 1999, 2009).

In this study, we found that, in naturally ripened fruit, *MaECHI1* expression gradually increased, consistent with the timing of the ripening process. This suggested that this gene was up-regulated during ripening, which correlated with previous work performed with this gene expression library (Xu et al., 2007). Under ethylene treatment, the gene expression peak sharply appeared and was 14 days earlier than it appeared in naturally ripened fruit. Moreover, the chitinase activity peak in the fruit appeared 15 days earlier than that peaks appeared in naturally ripened fruit. Under 1-MCP treatment, both gene expression and chitinase activity were greatly suppressed, and no significant peak appeared from 0 to 25 DAH.

These results demonstrate that the expression of this endochitinase gene was markedly regulated by exogenous ethylene and 1-MCP. Thus, these exogenous regulators could accelerate or suppress chitinase activity. Ethylene response element binding factor (ERF) proteins are plant-specific transcription factors that have been shown to play important roles in plant responses to various hormones or environmental cues. Onate-Sanchez and Singh reported that, in most cases, the induction of ERF gene expression preceded the mRNA accumulation of a chitinase gene (Onate-Sanchez and Singh, 2002).

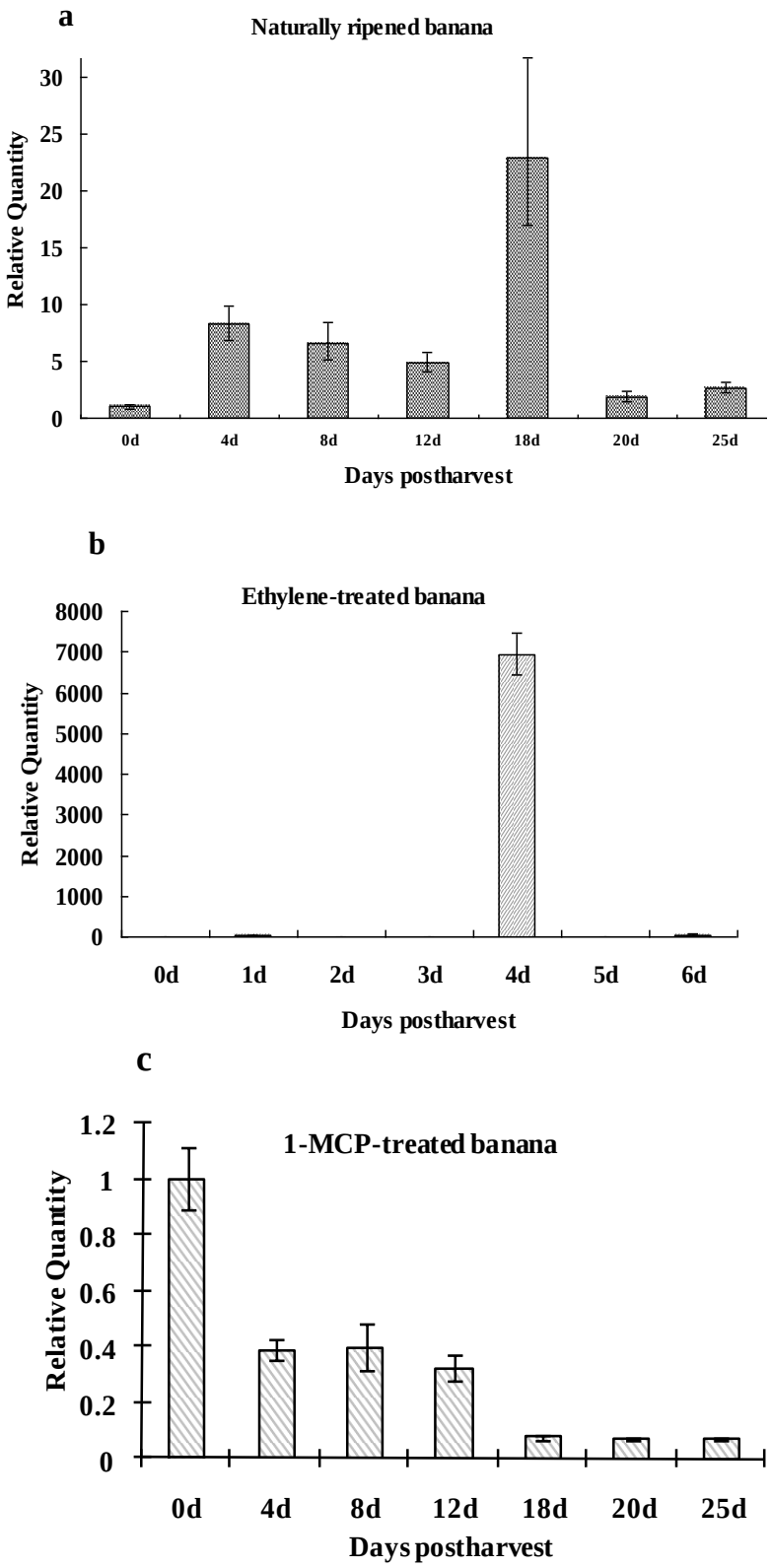

Figure 3. Expression of an endochitinase gene (*MaECHI1*) in the banana fruit. a) Naturally ripened bananas. b) Ethylene-treated bananas. c) 1-MCP- treated bananas. Vertical bars indicate the standard error (SE) of three replicates. When absent, the SE bars fall within the dimensions of the symbol. The X-axis represents the days postharvest. Y axis represents relative expression of *MaECHI1* with respect to actin.

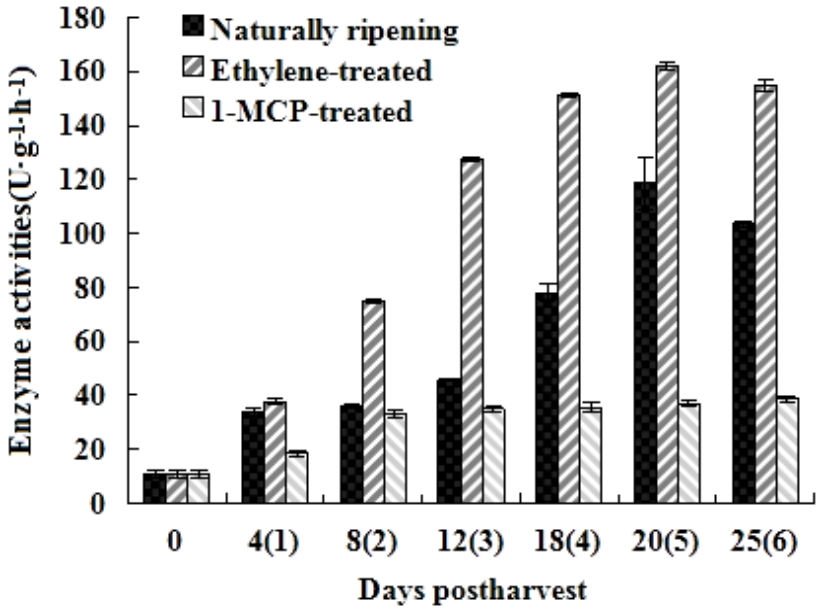

Figure 4. Postharvest changes of endochitinase activities in the banana fruit with different treatments. *Vertical bars* indicate the standard error (SE) of three replicates. When absent, the SE bars fall within the dimensions of the symbol. The X-axis represents the days postharvest, and numbers in parentheses represent the days after harvest with ethylene treatment.

Recently, another report showed that another endochitinase (*CHIB*) gene in Arabidopsis was closely related to a different ethylene receptor (ERS1). Over expressing ERS1 receptor protein in etr2 ein4 ers2 mutants substantially elevated CHIB expression (Liu et al., 2010). Therefore, we speculate that the endochitinase gene described here might also be a potential downstream target for ethylene response factors, based on our observations that banana fruit ripening was accelerated or delayed by exogenous ethylene and 1-MCP (Liu et al., 1999; Lohani et al., 2004; Gupta et al., 2006; Jiang et al., 2001; Jiang et al., 2004).

Conclusion

MaECHI1 gene expression and activity increased markedly at the onset of ripening in postharvest banana fruit. Ripening could be markedly accelerated or suppressed by exogenous ethylene or 1-MCP, respectively, and the expression levels of *MaECHI1* coincided with the timing of banana fruit ripening. Further studies will mainly focus on revealing the detailed biological mechanisms of *MaECHI1* involvement of banana fruit postharvest ripening.

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