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Molecular Cloning and Characterization of A Novel 1-Aminocyclopropane-1-carboxylate Oxidase Gene Involved in Ripening of Banana Fruits

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One novel banana fruit ripening related 1-aminocyclopropane-1-carboxylate (ACC) oxidase gene quite different from ACC oxidase genes from other species was cloned. In contrast to other studies, the polypeptide encoded by this gene, named Mh-ACO1, lacks the putative leucine zipper motif which is conserved in all known ACC oxidases including the other previously reported banana ACC oxidase, Mh-ACO2. The *Mh-ACO1* locus consists of two nearly identical paralogous ACC oxidase genes arranged in opposite orientation and separated by a 3.1-kb intergenic region. The *Mh-ACO1* has only two introns, at positions identical to *Mh-ACO2*, which comprises a coding region interrupted by three introns. The predicted amino acid sequence of Mh-ACO1 shares less than 50% identity to those of ACC oxidase from other climacteric fruits, while that of Mh-ACO2 shows more than 65% homology. When expressed in *Saccharomyces cerevisiae Mh-ACO1*-encoded protein possessed the enzyme activity for ethylene conversion. The levels of mRNA corresponding to both *Mh-ACO1* and *Mh-ACO2* increased during fruit ripening and were induced by exogenous ethylene. We conclude that both *Mh-ACO1* and *Mh-ACO2* contribute to increased ethylene production in fruits and these two genes are differentially expressed in fruits and other organs in banana.

KEYWORDS: ACC oxidase; banana; leucine zipper; promoter

INTRODUCTION

The phytohormone ethylene regulates many stages of plant development such as seed germination, leaf abscission, fruit ripening, senescence of leaves and flowers, as well as responses to environmental stress caused by wounding, low temperature, drought, flooding, chemicals including herbicides, metals, ozone and SO_2, and pathogen attack (*1*−*3*). The pathway of ethylene synthesis is well established in higher plants (*4*). Ethylene is formed from methionine via *S*-adenosyl-L-methionine (AdoMet) and 1-aminocyclo- propane-1-carboxylic acid (ACC). ACC is synthesized from AdoMet by the action of ACC synthase, and the conversion of ACC to ethylene is carried out by ACC oxidase (ACO), also known as ethylene-forming enzyme (*1*). ACC oxidase cDNA was first isolated from tomato by Holdsworth et al. (*5*), designated as pTOM13. Introduction of the pTOM13 antisense cDNA into tomato successfully blocked the biosynthesis of ACC oxidase, causing inhibition of ethylene production (*6*). Heterologous expression of tomato ACC oxidase cDNAs either in *Saccharomyces cerevisiae* (*7*) or *Xenopus laevis* oocytes (*8*) conferred the conversion. Most of the recently published cDNAs or genes encoding ACC oxidase were isolated from climacteric fruits such as apple (*9*, *10*), avocado (*11*), banana (*12*−*14*), kiwifruit (*15*), melon (*16*), peach (*17*, *18*), tomato (*19*−*21*), or senescing flower petals such as carnation (*22*), orchid (*23*, *24*), and petunia (*25*, *26*). The similarity between any two amino acid sequences of known ACC oxidases is higher than 70%. All of them have 11 out of 12 conserved amino acids among members of the Fe(II) ascorbate superfamily of enzymes (*27*) including those three histidine residues proposed to be involved in the chelation of Fe(II) (*28*). A potential amphipathic α-helix to form the putative leucine zipper appears in the N-terminus of each ACC oxidase polypeptide (*29*).

On the basis of the ripening mechanisms, fruits can be divided into two groups: climacteric fruits which ripe with a peak in respiration and a concomitant burst of ethylene and nonclimacteric fruits with no dramatic change in respiration and low level of ethylene production (*30*). The quality and storage life of banana, a typical climacteric tropical fruit, are affected by ethylene, whether from exogenous or endogenous sources (*31*). There is commercial interest in regulating the ripening process so as to reduce the substantial losses caused by over-ripening of fruits. To achieve this, genetic manipulation of genes involved in ethylene biosynthesis proves to be the most promising. Hamilton et al. (*6*) demonstrated the inhibition of 97% ethylene production in tomato fruit by reducing ACC oxidase activity with antisense RNA. Less than 1% of ethylene production of control untransformed cantaloupe melon fruit could be detected in transgenic fruits expressing an antisense ACC oxidase gene,

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and the ripening process was blocked both on and off the vine. This phenotype could be reversed by exogenous ethylene treatment as in tomato (*32*). Antisense ACC oxidase gene was also used to inhibit yellowing of the head of broccoli associated with postharvest senescence. The transgenic broccoli showed 91% reduction in ethylene production 96 h after harvest in comparison to the nontransgenic control (*33*). The transgenic gene silencing approach expressing the sense ACC oxidase gene has also been proven to be effective in the reduction of enzyme activity in tomato fruits (*34*) and in tobacco flowers (*35*). Furthermore, the use of ripening mutants constructed by reverse genetic techniques using genes involved in ethylene biosynthesis has made it possible to better understand the ethylene-mediated fruit ripening. One of banana ACC oxidase genes, *Mh-ACO2* (GenBank accession number U80233), has been previously reported (*12*, *13*). Our data indicate that ACC oxidase is encoded by at least two divergent genes in banana. To understand the regulation of ethylene production involved in ripening of banana fruits, we isolated and characterized a novel fruit ripening-related ACC oxidase gene (*Mh-ACO1*) whose characteristics are quite different from ACC oxidase genes from other species. Interestingly, the ACC oxidase gene reported in this study, *Mh-ACO1*, encodes a polypeptide sequence without putative leucine zipper residues at the position analogous to the N-terminus of other ACC oxidase polypeptides. We further investigated the expression patterns of two ACC oxidase genes (*Mh-ACO1* and *Mh-ACO2*) during fruit ripening and in response to exogenous ethylene.

MATERIALS AND METHODS

Plant Materials. Banana (*Musa* spp., "Hsien Jin Chiao", AAA group) was used as the source of DNA and RNA. For RNA extraction, banana fruits at different degrees of ripeness were harvested according to the color changes taking place in the peel of the banana. The given index numbers referred to the color of peel were as it follows: 1, all green; 2, green with trace of yellow; 3, more green than yellow; 4, more yellow than green; 5, green tip; 6, all yellow; 7, yellow flecked with brown (*36*).

Extraction of Genomic DNA and Poly(A)$^+$ RNA from Banana. Banana genomic DNA was isolated according to Jofuku and Goldberg (*37*) followed by equilibrium sedimentation in CsCl gradients (*38*). After total RNA was extracted from the peel of mature green (stage 1) and ripening banana with increasing degree of maturation (from stages 2 to 7) by cetyltrimethylammonium bromide (*39*), poly(A)$^+$ RNA was separated by oligo-dT cellulose column chromatography as described (*40*).

Polymerase Chain Reaction (PCR). Gene fragments of ACC oxidase were generated by the PCR (*41*) using two degenerate oligonucleotides, EFE-1 (5′-ATCGAATTCAAC(T)TAC(T)CCA(CGT)-CC A(CGT)TGC(T)CC-3′) and EFE-2 (5′-CGAGGATCCA(G)AAA-(CGT)ACA(G)AAC(T) TTA(CGT)GGA(G)TA-3′), corresponding to peptides NYYPCP and YPKFVF for ACC oxidase conserved regions, respectively. The PCR was carried out by 1-min template denaturation at 94 °C, 2-min primer annealing at 50 °C, and 2-min primer extension at 72 °C for 35 cycles using 1 μg genomic DNA from banana leaves as the template. After digestion with *Eco*RI and *Bam*HI, the PCR products were subcloned into a pUC19 vector for sequence analysis by the dideoxy-chain-termination technique (*42*) using Sequenase Version 2.0 (United States Biochemical, Cleveland, Ohio).

Isolation of ACC Oxidase cDNAs and Genomic Clones. The construction of the cDNA library in λZapII (Stratagene, La Jolla, CA), the construction of banana genomic library, screening, hybridization, and wash condition followed the manufacturer's protocol (Stratagene). Genomic DNA of banana was partially digested by *Sau*3AI and separated by electrophoresis on a 0.7% (w/v) agarose gel. The gel segment corresponding to DNA fragments of 15 to 23 kb in sizes was cut out, eluted, and then inserted into *Bam*HI sites of the λEMBL3 replacement λ phage vector (Stratagene, La Jolla, CA) to create a banana genomic library. Approximately 1×10^6 nonamplified plaques of both libraries were screened with gene-specific probes, the PCR product described above for the cDNA library and the insert of pMAO1 (see below) for the genomic library. Probes were labeled with ^{32}P-dCTP by the random priming method (*43*), and purified by passage through a Sephadex G-50 column. Nucleotide and amino acid sequences were aligned using the AlignX program of Vector NTI Suite 6.0 software.

Southern and Northern Blot Analysis. Genomic DNA (10 μg) or λ phage DNA was digested with the appropriate restriction endonucleases and fractionated by electrophoresis on 0.7% (w/v) agarose gels. To identify all potential *Mh-ACO1*-related sequences, the insert of pMAO1 cDNA clone was used as a probe. To characterize the structure of Mh-ACO1 gene, special fragments of λBGE1 were used as probes. Ten μg poly(A)$^+$ RNA was denatured in 50% dimethyl sulfoxide, 10 mM Na_3PO_4 (pH 7.0), and 1 M deionized glyoxal (*44*) and separated on an 1% agarose gel. Nucleic acids were transferred onto a nylon filter (Hybond N, Amersham). Hybridization was performed overnight at 42 °C in 5× SSPE (0.18 M NaCl, 10 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.4 as 1×), 0.5% (w/v) SDS, 5× Denhardt's reagent (*45*), 250 μg/mL denatured fragmented salmon sperm DNA, and 50% formamide. The membranes were washed twice in 5× SSPE and 0.5% (w/v) SDS at room temperature for 10 min each, and in 2× SSPE, 0.5% (w/v) SDS at 65 °C for 15 min once. The hybridized membrane was exposed at −80 °C for 1 or more days (*46*). Amount of hybridization signals were counted by LabWorks Image Acquisition and Analysis Software (UVP, Inc., Upland, CA). Probes used for Northern blot analysis were pMAO1 (GenBank accession no. AF004839), pMAO2 (GenBank accession no. U80233), and pMACT1 (GenBank accession no. AF246288) cDNA inserts containing banana *Mh-ACO1*, *Mh-ACO2* (*12*, *13*), and actin genes, respectively. The expression level in each treatment for Northern blot analysis was normalized to the corresponding signal for actin gene.

Expression of Recombinant Protein in Yeast. The plasmid was constructed by ligating the 5.9-kb *Eco*RI−*Xho*I fragment of pYES2 (INVITROGEN, San Diego, CA) to the 1.2-kb *Eco*RI−*Xho*I fragment of pMAO1 cDNA. *Saccharomyces cerevisiae* INVSC2 strain was transformed with the plasmid using the lithium acetate method, with transformants being selected on NGcA plates containing 0.67% (w/v) bacto-yeast nitrogen base, 2% (w/v) glucose, 2% (w/v) bacto-agar, and 20 mg/L adenine hemisulfate. This gene was driven by *GAL1* promoter and induced by the addition of 5 mM galactose (*47*). Extraction of the total protein from yeast was according to Rose et al. (*47*) following 15 000 rpm centrifugation at 4 °C for 15 min. Both the extraction of DNA and RNA were as described by Rose et al. (*47*).

Assay for Ethylene Emanation. Each 3 mL of host, untransformed and transformed yeast cells, were incubated with shaking at 30 °C until the A_{600} approximated 1.4. This culture was incubated with 30 mM sodium ascorbate, 0.1 mM $FeSO_4$, and 1 mM ACC for 1 h with shaking at 240 rpm (*48*). The ethylene produced was assayed using a SHIMADZU Gas Chromatograph GC 14A.

RESULTS

Characterization of Fruit-Ripening Related ACC Oxidase cDNAs from Banana. PCR was carried out to yield gene fragments of ACC oxidase using banana genomic DNA as the template and two degenerate oligonucleotides EFE-1 and EFE-2 as primers. After verification of its homology to known ACC oxidases, a 350-bp PCR fragment was used as a probe to screen banana cDNA library. Of the $\sim 10^6$ plaques screened, 464 positives were identified and 30 of them were isolated for further characterization. One of these cDNA clones, pMAO1, was selected for sequence determination. The complete nucleotide sequence has been deposited in the GenBank nucleotide sequence database under the accession number AF004839 and the open reading frame is designated as Mh-ACO1. The pMAO1 cDNA, 1190 bp long, encodes a polypeptide of 306 amino acids with a predicted molecular weight of 34 581 Da and a calculated isoelectric point of 5.26. Eleven of twelve amino acid residues

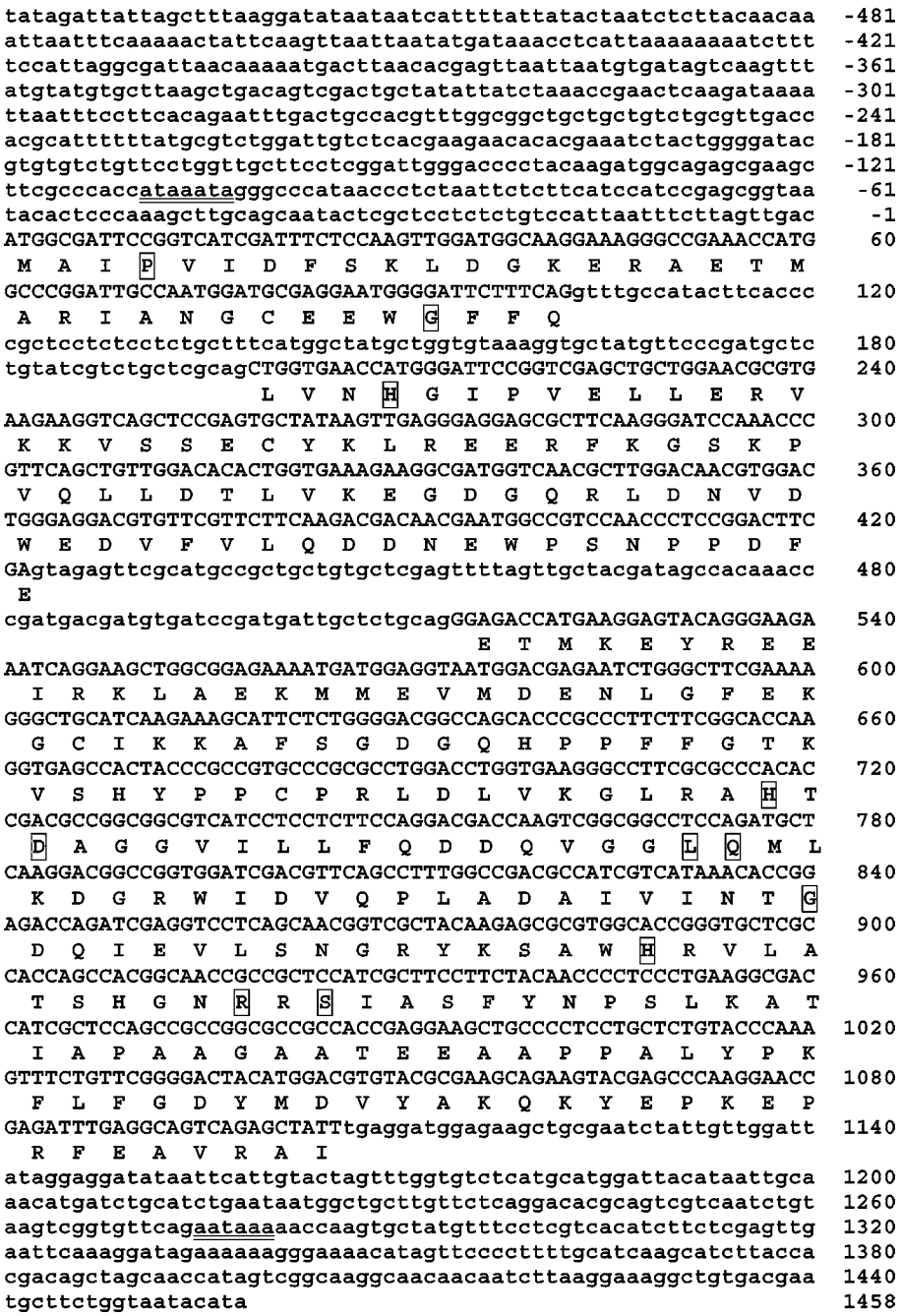

Figure 1. Complete nucleotide sequence of the *MAOG1A* gene, one of the two highly similar genes organized in the *Mh-ACO1* locus, including introns and the 5′-flanking region. The sequence from translation start site of the mRNA transcribed by the gene is in upper case, the remainder of the sequence is in lower case. Amino acids are indicated below their respective codons. The first nucleotide of the putative translational start codon is labeled as +1. The putative TATA box and polyadenylation signal are underlined. Amino acids conserved across all members of the Fe^{2+} and ascorbate requiring superfamily of enzymes are boxed.

conserved among all members of a superfamily requiring Fe(II) and ascorbate for activities (*27*) are found in Mh-ACO1 (**Figure 1**). Cysteine-165 and the latter two of three conserved histidine residues, H-177 and H-234, have been implicated in the ion binding (*28*, *29*). The amino acid identity and the degree of DNA homology in the coding regions between Mh-ACO1 and Mh-ACO2 are 47.3 and 59.0%, respectively. A phylogenetic analysis of amino acid sequences from various ACC oxidases of different climacteric fruits revealed that Mh-ACO1 from banana and CM-ACO2 from melon (GenBank accession number X95552) are clustered into another subgroup since their similarity is low (**Figure 2**). According to percentage indicated in **Table 1**, Mh-ACO1 had low sequence identity compared with other genes encoding ACC oxidase with 41.8−48.7% at amino acid level. The amino acid sequences of other ACC oxidases except Cm-ACO2 were more than 65% identical to each other (**Table 1**).

Identification of Mh-ACO1 as ACC Oxidase from Banana. To confirm that pMAO1 encodes a genuine ACC oxidase, the cDNA was cloned into expression vector pYES2 and transformed into yeast strain INVSC2. **Table 2** demonstrates the expression of ACC oxidase activity in the yeast cells containing Mh-ACO1 coding region after induction by galactose. The ability of ethylene conversion by clone pMAO1 is more than 66-fold greater than endogenous production of negative control pYES2. The absence of ascorbate or Fe^{2+} in the assay

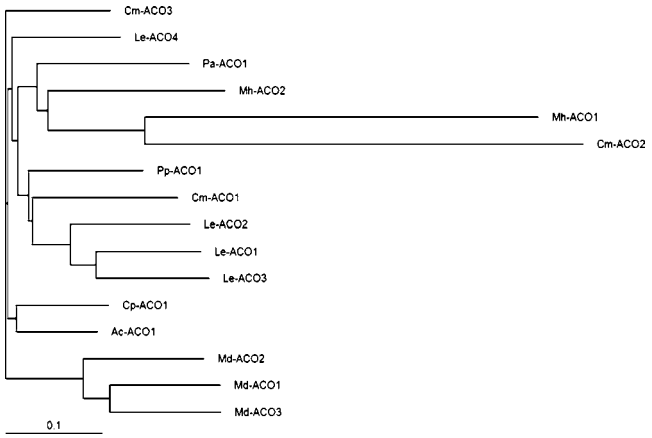

Figure 2. Amino acid sequence based dendrogram among ACC oxidase from banana (AF030411 for Mh-ACO1 and U86045 for Mh-ACO2), apple (X98627 for Md-ACO1, AF015787 for Md-ACO2, and AF030859 for Md-ACO3), avocado (M32692 for Pa-ACO1), kiwi (M97961 for Ac-ACO1), melon (X95551 for Cm-ACO1, X95552 for Cm-ACO2, and X95553 for Cm-ACO3), papaya (AF379855 for Cp-ACO1), peach (AF532976 for Pp-ACO1), and tomato (X58273 for Le-ACO1, Y00478 for Le-ACO2, Z54199 for Le-ACO3, and AB013101 for Le-ACO4). Amino acid sequences were aligned using the AlignX program of Vector NTI Suite 6.0 software. The scale below shows the percentage of difference.

mixture reduced the level of ethylene production by pMAO1 by approximately 54−62% (**Table 2**). This might result from a no limiting growth environment and adequate reserves in the yeast. These experiments demonstrate that the cDNA sequence of the clone pMAO1 encodes ACC oxidase.

Organization and Structure of *Mh-ACO1* Locus. The banana genomic library was screened with pMAO1 cDNA for homologous sequences by plaque hybridization. In 303 putative hybridized clones, 50 were chosen to be mapped by restriction endonucleases. One of the genomic clones, λBGE1, containing the complete Mh-ACO1 coding region, was selected for analysis of the whole sequence. Several hybridizing bands revealed in Southern blot analysis of banana genomic DNA (unpublished data), using entire cDNA clone of *Mh-ACO1* as a probe, indicated the presence of related members of an ACC oxidase gene family. Nucleotide sequence analysis of λBGE1 showed that two highly similar genes are organized in opposite directions, designated as *MAOG1A* (GenBank accession number AF030411) and *MAOG1B* (GenBank accession number AF030410), and separated by a 3.1-kb intergenic region (**Figure 1** and **3**). Both *MAOG1A* and *MAOG1B* genes possess three exons, interrupted by two introns. These two genes have functional splice sites (*50*) according to the intron−exon junction sequences (**Figure 1**). The only three mismatch nucleotide sequences and only one amino acid substitution (arginine to glutamine at position 96) between cDNA pMAO1 and *MAOG1A* were probably due to differences in triploid alleles of the same locus (*51*). Comparison between these two genes revealed nearly perfect sequence conservation, extending 274 bp upstream of the translation starting site. Indeed, a divergence was found at only one nucleotide, position 286 (A in *MAOG1A* and G in *MAOG1B*). Furthermore, nucleotide sequences of 3′-untranslated region 199 bp in length and 20 bp downstream the cleavage site for addition of poly(A) tail in these two genes were identical except one base deletion in *MAOG1A*. Both the purified phage DNA and genomic DNA of banana were digested with restriction enzymes and further characterized by Southern blot hybridization (**Figure 3A**) using part of intergenic region (indicated in **Figure 3B**) as probe. This analysis confirmed that

Table 1. Pairwise Comparison (%) of Deduced Amino Acid Sequences of ACC Oxidases from Banana (AF030411 for Mh-ACO1 and U86045 for Mh-ACO2), Apple (X98627 for Md-ACO1, AF015787 for Md-ACO2, and AF030859 for Md-ACO3), Avocado (M32692 for Pa-ACO1), Kiwi (M97961 for Ac-ACO1), Melon (X9551 for Cm-ACO1, C95552 for Cm-ACO2, and X95553 for Cm-ACO3), Papaya (AF379855 for Cp-ACO1), Peach (AF532976 for Pp-ACO1), and Tomato (X58273 for Le-ACO1, Y00478 for Le-ACO2, Z54199 for Le-ACO3, and AB013101 for Le-ACO4)

		banana	apple			avocado	kiwi	melon			papaya	peach	tomato		
species	gene name	Mh-ACO2	Md-ACO1	Md-ACO2	Md-ACO3	Pa-ACO1	Ac-ACO1	Cm-ACO1	Cm-ACO2	Cm-ACO3	Cp-ACO1	Pp-ACO1	Le-ACO1	Le-ACO2	Le-ACO3	Le-ACO4
banana	Mh-ACO1	47.3	48.7	47.3	48.1	46.6	46.6	45.0	41.8	46.3	47.8	46.4	46.7	46.9	45.8	46.9
	Mh-ACO2		68.0	66.6	68.0	71.6	76.3	68.8	43.4	70.2	72.5	70.9	70.8	69.4	68.8	69.8
apple	Mh-ACO1			87.9	98.7	68.5	77.7	73.9	43.4	77.8	79.2	74.3	77.1	76.6	75.2	75.6
	Mh-ACO2				87.6	65.8	75.2	69.4	42.5	73.1	75.5	70.7	73.4	72.0	73.0	74.1
	Mh-ACO3					68.8	77.1	73.9	43.0	78.4	79.2	74.3	77.8	76.9	75.5	75.9
avocado	Pa-ACO1						77.3	70.1	41.3	73.1	73.8	75.8	72.3	72.0	71.1	71.8
kiwi	Ac-ACO1							75.4	42.7	78.8	84.0	81.5	78.7	77.2	78.4	80.9
melon	Cm-ACO1								43.4	74.0	71.7	76.6	79.9	76.1	78.0	74.2
	Cm-ACO2									42.5	42.2	41.1	43.2	43.7	43.1	42.5
	Cm-ACO3										80.9	75.7	75.4	74.8	76.3	77.6
papaya	Cp-ACO1											79.6	80.3	77.8	79.0	77.8
peach	Pp-ACO1												82.1	79.7	79.7	78.8
tomato	Le-ACO1													89.6	93.4	76.9
	Le-ACO2														86.6	74.1
	Le-ACO3															76.9
	Le-ACO4															

Table 2. ACC Oxidase Activity Expressed and Compounds Influencing the Conversion of ACC to Ethylene in Yeast Transformed with Plasmid pMAO1 Containing ACC Oxidase *Mh-ACO1* cDNA

culture[a]	assay supplement: ascorbate	assay supplement: Fe^{2+}	ethylene production (nL 10^8 cells^{-1} h^{-1})	relative rate (% of pMAO1)
pMAO1	+	+	3.98 ± 0.13	100.0
pMAO1	−	+	2.47 ± 0.05	62.1
pMAO1	+	−	2.14 ± 0.11	53.8
pYES2	+	+	0.06 ± 0.01	1.5
INVSC2	+	+	0	0
−	+	+	0	0

[a] Yeast cells transformed with either pMAO1 or pYES2, and untransformed INVSC2 host cells were cultured in NGalA medium (*45*) until the OD_{600} of the suspension reached 0.89, 1.55, and 1.59, respectively. Each measurement represents the mean of 3 replicates.

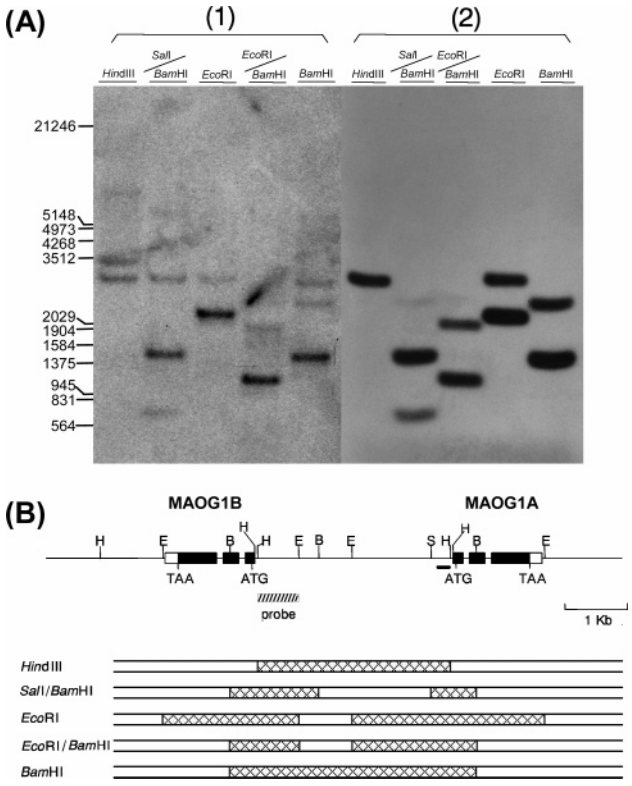

Figure 3. Southern blot analysis of banana genomic DNA and genomic clone λBGE1 DNA. (A) Each lane contains 10 μg of banana genomic DNA (1) or phage DNA (2) digested with the indicated restriction endonucleases and was hybridized with the probe. (B) Diagram of the restriction map and hybridization signals. The hatched box indicates the location of the probe, and hybridization signal is represented as reticular boxes. The line near the 5′-flanking region of *MAOG1A* represents the extent of identical nucleotide sequence with the probe.

the banana genome contained two closely linked and inverted copies of the ACC oxidase gene. Those faint bands appeared in Southern hybridization using banana genomic DNA as template could be cross reacted with 5′-flanking region of other member in the multiple gene family.

Tissue-Specific Expression and Induction of Gene Expression by Fruit Ripening and Ethylene. To clarify the roles of two banana ACC oxidase genes, *Mh-ACO1* and *Mh-ACO2*, expressed in fruit ripening, mRNA was extracted from both peel and pulp of banana for Northern blot analysis. No *Mh-ACO1* mRNA was detectable in both the peel and pulp of mature green banana at ripening stage 1 (**parts A and B of Figure 4**).

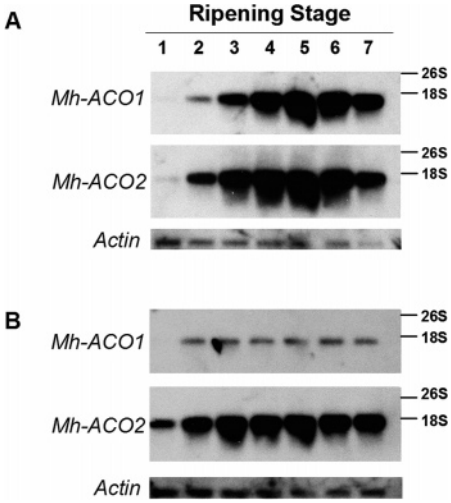

Figure 4. Gene expression of banana ACC oxidase genes *Mh-ACO1* and *Mh-ACO2* during various stages of Hsien Jin Chiao fruit ripening. Poly(A)$^+$ RNA was isolated from pericarp (A) and pulp (B) tissues of mature green (stage 1) and ripening banana fruits with increasing degree of maturation (from stages 2 to 7). Each lane contained 10 μg of poly(A)$^+$ RNA and was separated by electrophoresis on a 1% agarose gel and blotted onto Hybond N membrane. The filter was hybridized sequentially with the indicated ^{32}P-labeled probes. The exposure times of *Mh-ACO1* and *Mh-ACO2* were 24 and 6 h, respectively. Hybridization of the filter with actin cDNA in each lane was shown for internal control.

Accumulation of *Mh-ACO1* and *Mh-ACO2* mRNA increased gradually during ripening after stage 2 and was most abundant at stage 6 in peel (**Figure 4A**). Gene expression of *Mh-ACO1* was greatly induced at early stage of fruit ripening in pulp and showed maximal expression at stage 6. The abundance of *Mh-ACO2* mRNA transcripts in pulp had increased dramatically by stage 6 (**Figure 4B**). By consideration of the different exposure time of Northern analysis, the expression level of *Mh-ACO2* was higher than that of *Mh-ACO1* no matter in peel or pulp (**Figure 4**). Stamen tissue contained the greatest amount of transcripts homologous to *Mh-ACO1*, followed by an easily detectable signal for ripened fruit, bract, and petal, whereas ripened fruit accumulated largest amount of *Mh-ACO2* transcripts relative to other organs (**Figure 5A**). Gene expression of *Mh-ACO1* and *Mh-ACO2* were both induced by 10 μL/L and saturated by 100 μL/L of exogenous application of ethylene (**Figure 5B**).

DISCUSSION

The amino acid sequence of putative banana ACC oxidase Mh-ACO1 only shares less than 50% homology with ACC oxidases from other climacteric fruits (**Table 1**) and is more divergent from others as shown in the phylogenetic dendrogram (**Figure 2**). According to the alignment among known ACC oxidases, 11 out of 12 amino acid residues conserved among all members of a superfamily requiring Fe(II) and ascorbate for activities (*27*) can be found in the sequence of Mh-ACO1. Comparison between primary structure of banana ACC oxidase Mh-ACO1 and Mh-ACO2 (*13*) indicates that Mh-ACO2, like most of other plant ACC oxidases, has all 12 of these conserved amino acids (**Figure 6**). The second conserved amino acid, alanine-27, is replaced by glycine in Mh-ACO1 and indicates that this alanine is not required for ACC oxidase activity (**Table 2**). The lower than expected conservation in CM-ACO2, less than 44% identity to other ACC oxidases, was explained by the fact that CM-ACO2 may be subject to fewer structural

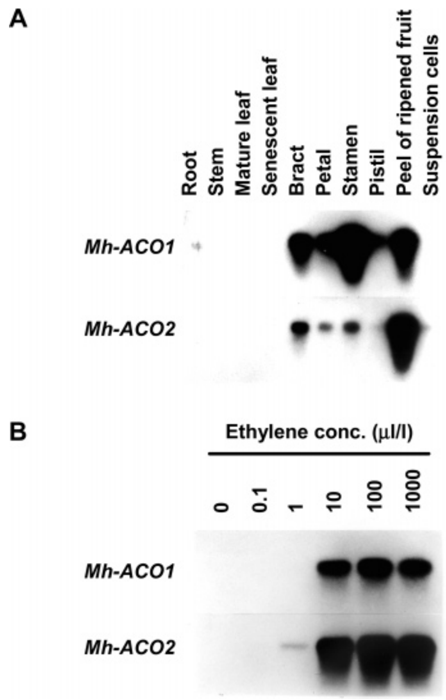

Figure 5. Spatial expression and the effect of exogenous application of different ethylene dose on gene expression of *Mh-ACO1* and *Mh-ACO2*. mRNA was extracted from different organs of banana (A) and pericarp of mature green banana fruits treated with indicated concentration of ethylene for 3 days (B). Each lane contained 10 μg of poly(A)$^+$ RNA and was separated by electrophoresis on a 1% agarose gel and blotted onto Hybond N membrane. The filter was hybridized sequentially with ^{32}P-labeled pMAO1 and pMAO2 cDNA.

constraints than most other ACC oxidases (*18*). Most of the ACC oxidase genes possess three introns located at the relatively identical positions except that Mh-ACO1 (GenBank accession no. AF030411), CM-ACO2 (GenBank accession no. X95552), and CM-ACO3 (GenBank accession no. X95553) are interrupted by two introns only (**Figure 7**). As members of same subgroup in dendrogram, *Mh-ACO1* and *CM-ACO2* seem to be derived from *Mh-ACO2* and *CM-ACO1*, respectively, and each of them has lost the third intron whereas *CM-ACO3* has lost the second one (*18*). Although with similar gene structure, amino acid sequences of polypeptide deduced from Mh-ACO1 and CM-ACO2 only share the lowest identity, namely, 41.8% (**Table 1**). On the other hand, the region between phenylalanine-108 and glutamic acid-132 of Mh-ACO1 represents a potential amphipathic α-helix, as does the region between leucine-105 and lysine-144 of Mh-ACO2, but Mh-ACO1 lacks the putative leucine zipper which is conserved in all known ACC oxidases including Mh-ACO2 (**Figure 6**). The putative leucine zipper with dimerization potential might be involved in binding of ACC oxidase to membrane (*29*). Moreover, the organization of ACC oxidase *Mh-ACO1* genes and overall homology of 99.8% between MAOG1A and MAOG1B support the possibility that this gene cluster arose by a duplication event similar to ACC oxidase genes from petunia (*26*).

ACC oxidase, which exhibits a requirement for Fe(II) and ascorbate, catalyzes ACC in the presence of O_2 into ethylene, CO_2, HCN, dehydroascorbate, and two molecules of water. Furthermore, the activity of ACC oxidase is activated by CO_2 (*52*). Previously, ACC oxidase cDNAs from tomato (*6*) and apple (*48*) have conferred ethylene-forming ability in transformed yeast cells with enzymatic activity having similar characteristics to those observed in in vivo (*1*) and in vitro (*9*, *53*) plant systems. Moreover, ACC oxidase cDNA from tomato (pHTOM5) was identified by functional expression in oocytes of *Xenopus laevis* (*26*). Here, we demonstrate that Mh-ACO1 was also successfully synthesized in eukaryotic yeast expression system with the enzyme activity of conversion from ACC to ethylene (**Table 2**). The enzyme activity was lower than apple ACC oxidase expressed in yeast probably because the ability of transformed yeast carrying ACC oxidase cDNA to convert

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                1                                               50
Mh-ACO1     (1) M-AIPVIDFSKLDGKERAETMARIANGCEEWGFFQLVNHGIPVELLERVK
Mh-ACO2     (1) MDSFPVIDMEKLLGRERGAAMEILRDACEKWGFFEILNHGISHDLMDEVE
                    ●                     ●   ●       ●

                51                                             100
Mh-ACO1    (50) KVSSECYKLREERFKGSKPVQLLDTLVKEGDGQRLDNVDWEDVFVLQDDN
Mh-ACO2    (51) KVNKEQYNKCREQKFNEFANKALENADSEIDHLDWESTFFLRHLPVSNIS

                101                                            150
Mh-ACO1   (100) EWPSNPPDFEETMKEYREEIRKLAEKMMEVMDENLGFEKGCIKKAFSGDG
Mh-ACO2   (101) EIPDLDDQYRKAMKEFAAAIEKLAERLLDLLGENLELEKGYLKKAFS-NG
                               *      *    *        *

                151                                            200
Mh-ACO1   (150) QHPPFFGTKVSHYPPCPRLDLVKGLRAHTDAGGVILLFQDDQVGGLQMLK
Mh-ACO2   (150) SKGPTFGTKVSSYPPCPRPDLVKGLRAHTDAGGIILLFQDDQVSGLQFLK
                                           ● ●               ●●

                201                                            250
Mh-ACO1   (200) DGRWIDVQPLADAIVINTGDQIEVLSNGRYKSAWHRVLATSHGNRRSIAS
Mh-ACO2   (200) DGEWLDVPPMRHAIVVNLGDQLEVITNGKYKSVVHRVVAQTDGNRMSIAS
                                  ●               ●         ● ●

                251                                            300
Mh-ACO1   (250) FYNPSLKATIAPAAGAATEEAAPP-ALYPKFLFGDYMDVYAKQKYEPKEP
Mh-ACO2   (250) FYNPGSDAVIFPAPALVEKEAEEKKEVYPRFVFEDYMKLYVGHKFQAKEP

                301             319
Mh-ACO1   (299) RFEAVRAI-----------
Mh-ACO2   (300) RFEAMKAMEAVATHPIATS
```

Figure 6. Optimal alignment of ACC oxidases Mh-ACO1 and Mh-ACO2 from banana. Identical amino acids are shown in black boxes. Amino acids conserved across all members of the Fe^{2+} and ascorbate requiring superfamily of enzymes are marked with black dots. The amino acid residues marked with star form a potential leucine zipper. Gaps have been introduced to maximize the alignment.

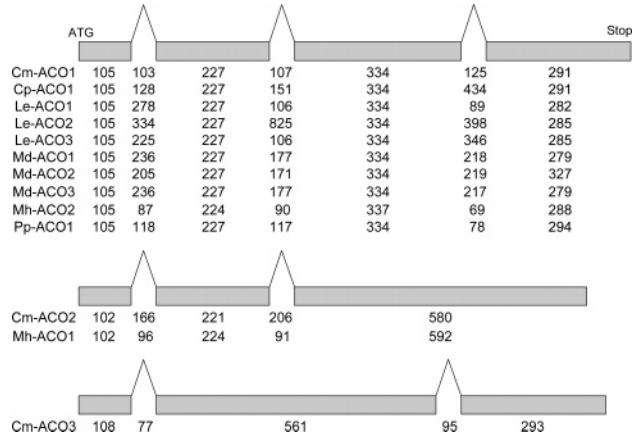

Figure 7. Schematic diagrams of different ACC oxidase genes from banana (AF030411 for Mh-ACO1 and U86045 for Mh-ACO2), apple (X98627 for Md-ACO1, AF015787 for Md-ACO2, and AF030859 for Md-ACO3), avocado (M32692 for Pa-ACO1), kiwi (M97961 for Ac-ACO1), melon (X95551 for Cm-ACO1, X95552 for Cm-ACO2, and X95553 for Cm-ACO3), papaya (AF379855 for Cp-ACO1), peach (AF532976 for Pp-ACO1), and tomato (X58273 for Le-ACO1, Y00478 for Le-ACO2, Z54199 for Le-ACO3, and AB013101 for Le-ACO4). Protein-coding exons are indicated by boxes, and introns are indicated by bended lines. Numbers below boxes or lines represent the length of base pairs.

ACC to ethylene depends on the growth phase of the culture in galactose-containing medium (*48*). More conceivable, different ACC oxidase isoforms may exist in climacteric fruits such as apple and banana, and each isoform may represent characteristic enzyme kinetics (*54*). Hence, the ripening process of climacteric fruits may be regulated by variations in enzyme activity and the ability to convert the ACC to ethylene.

Ethylene production of climacteric fruits has been proposed to be regulated by two systems. System I is responsible for producing basal ethylene levels with a low production rate in the preclimacteric phase to provide sufficient quantities of ethylene for induction of ripening prior to the rise of respiration, and system II operates an autocatalytic biosynthesis and rapid increase of ethylene production rate during fruit ripening (*55*, *56*). Color changes, softening, and conversion of starch to sugar are also associated with the ripening process (*57*). As a typical climacteric fruit (*58*), banana is supposed to have several different ACC oxidase genes participating in systems I and II. According to the results of Northern analysis, *Mh-ACO2* expressed earlier than *Mh-ACO1* at rather low levels in stage 1 of the preclimacteric phase, but it seems to be sufficient for onset of fruit ripening. Low levels of ACC existed prior to preclimacteric phase (*59*) might act as the substrate for Mh-ACO2 to produce small quantities of ethylene in system I. Therefore, system I ethylene production in ripening banana fruit might be contributed by Mh-ACO2 and ripening proceed from inside out as predicted previously (*14*, *60*). After accumulating enough ethylene catalyzed by Mh-ACO2, ethylene probably diffused from pulp into peel (*61*, *62*) and resulted in a dramatic change on the gene expression of both *Mh-ACO1* and *Mh-ACO2*. The activity of ACC oxidase in peel did arise after that took place in the pulp (*59*). Large amount of *Mh-ACO2* transcripts were induced and accumulated in both pulp and peel, and its expression level in peel was higher than in pulp. Free ACC in pulp increased at the same time as production of ethylene raised (*59*) and was coincident with the abrupt great increase of ACC synthase transcripts (*63*). Expression of *Mh-ACO1*, mainly restricted to the tissue associated with floral organs and ripening fruits in banana, was spatially and developmentally regulated (**Figures 4** and **5**). All three ACC oxidase genes of tomato were also spatially regulated throughout flower development such as *Le-ACO1* (GenBank accession number X58273), which was predominantly expressed in the petals, stigma, and style, whereas *Le-ACO3* transcripts accumulated in all of the floral organs examined apart from the sepals (*64*). In the climacteric and postclimacteric phase, accumulation of *Mh-ACO1* transcripts elevated throughout the final stage. Expression pattern of *LE-ACO1*, similar to that of *Mh-ACO2*, was expressed in preclimacteric phase and increased the higher levels upon commencement of ripening (*21*). Low concentration of exogenous ethylene as 1 μL/L was sufficient to induce gene expression of *Mh-ACO2* but not *Mh-ACO1*. The threshold concentration of exogenous ethylene on induction effect of gene expression of both *Mh-ACO1* and *Mh-ACO2* was between 1 μL/L and 10 μL/L (**Figure 5**), and that was probably the reason the abundance of *MA-ACO1* mRNA remained at a high level until the full-ripened stage after treatment of 100 μL/L ethylene (*63*).

Except for a combination of gene transcript levels for both *Mh-ACO1* and *Mh-ACO2* that are involved in banana fruit ripening, it is clear that the modulation of enzyme activity for both Mh-ACO1 and Mh-ACO2 are also involved in this ripening process. The enzyme kinetics of Mh-ACO1 and Mh-ACO2 awaits further study to clarify the relationship between these two isoforms during banana fruit ripening. Nevertheless, the data presented in this study have given the structure, the function and the organization of a novel member of ACC oxidase gene and shown that the *Mh-ACO1* is one of the pivotal contributors of this multigene family that regulate banana fruit ripening in a rather sophisticated temporal and spatial expression manner.

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