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# Genomic Organization of a Diverse ACC Synthase Gene Family in Banana and Expression Characteristics of the Gene Member Involved in Ripening of Banana Fruits†

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The banana is one of the typical climacteric fruits with great economic importance in agriculture. To understand the basic mechanism underlying banana ripening, gene clones for banana ACC synthase (EC 4.4.1.14), a key regulatory enzyme in the ethylene biosynthetic pathway, were characterized. Genomic clones were analyzed by restriction mapping, and the data in conjunction with sequence comparisons with the previously isolated PCR fragments indicated that at least nine ACC synthase genes (*MACS1*−*9*) exist in the banana genome. Southern blot analysis showed they are located in different regions of the banana genome. Three lambda genomic clones (GMACS-1, -9, and -12) were completely sequenced, and gene structures of *MACS1* (corresponding to alleles of GMACS-9 and -12) and *MACS2* (corresponding to GMACS-1) were elucidated. The coding regions of these three genes were all interrupted by three introns. The size and location of introns are similar to the ACC synthase genes from tomato and *Arabidopsis*. Northern analysis showed that only *MACS1* expressed during fruit ripening and was inducible by exogenous ethylene treatment, which indicates *MACS1* is a significant member of the ACC synthase gene family related to ripening in banana fruit. The transcription initiation site of GMACS-12 containing *MACS1* was defined. There is a TATTAAT sequence located at position −31 to −25 that qualifies as a TATA box. The delineation of transcription unit in *MACS1* will facilitate the promoter studies for this gene and allow its specific functions involved in fruit ripening to be determined.

**KEYWORDS: 1-Aminocyclopropane-1-carboxylate (ACC) synthase; ethylene; gene expression; multigene family**

## INTRODUCTION

Ethylene is one of the simplest organic molecules with biological activity. This hydrocarbon gas, known as the fruit-ripening hormone, profoundly influences the growth and development of higher plants. Ethylene production is induced during several developmental stages, including fruit ripening, seed germination, leaf and flower senescence, and abscission (*1*). It is also induced by external factors, including drought, wounding, chilling injury, elicitors, auxin treatment, and viral infection (*2*, *3*).

It has been recognized that ethylene plays a crucial role in the initiation and development of ripening in climacteric fruits (*4*), while the ACC synthase (EC 4.4.1.14) is the key regulatory enzyme in the biosynthetic pathway of ethylene (*5*). It is therefore important to understand how expression of the ACC synthase gene is regulated during fruit ripening. Cloning of its gene will open new horizons of investigation on the molecular mechanism of induction of the ACC synthase activity during fruit ripening. Furthermore, it is of great agronomical interest to be able to control fruit ripening and delay overripening for climacteric fruits.

In every plant species examined, ACC synthase is encoded by a multigene family in which each gene is differentially regulated by various environmental and developmental factors during the growth of a plant. For example, tomato has at least nine ACC synthase gene family members (*6*−*9*), which are differentially regulated by inducers such as auxin, fruit ripening, elicitors, and wounding. A multigene family for ACC synthase sequences has also been reported for zucchini (*10*), mungbean (*11*), rice (*12*), potato (*13*), carnation (*14*), tobacco (*15*, *16*), lettuce (*17*), and *Arabidopsis thaliana* (*18*).

From banana, a typical climacteric fruit which has great economic importance in agriculture (*19*, *20*), we have already isolated ACC synthase cDNA clones (accession numbers

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† Sequence data for the genomic clones have been deposited previously in the GenBank libraries under the following accession numbers: GMACS-1 (AF056162), GMACS-9 (AF056164), and GMACS-12 (AF056163). Accession numbers of the ACC synthase PCR fragments from banana genome described in this article are as follows: Facs52 (DQ343866), Facs44 (DQ343867), Facs2 (DQ343868), Facs3 (DQ343869), Facs4 (DQ343870), Facs8 (DQ343871), and Facs10 (DQ343872).

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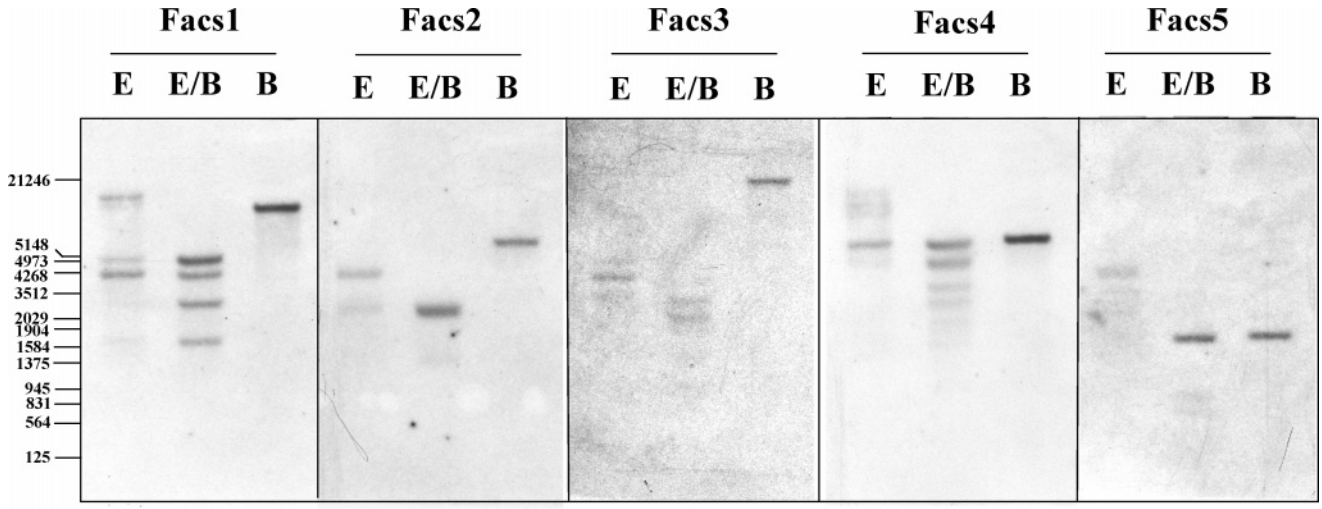



**Figure 1.** Southern analysis of banana genomic DNA. A 10 $\mu$g amount of DNA was digested with the indicated restriction endonucleases and probed with $^{32}$P-labeled Facs1, Facs2, Facs3, Facs4, and Facs5. E, *Eco*RI; B, *Bam*HI.

AF008941 and AF008942) corresponding to a group of genes (*MACS1*) which are expressed in ripening banana fruits. Thus far, many data show that ethylene, at least in plants, influences the expression of specific genes at the level of transcription (*21*−*24*). As a first step toward understanding the role of the ACC synthase multigene family in banana, we report in this paper the genomic organization and expression characteristics of the members of this family. Furthermore, we also present the complete nucleotide sequence and transcription start site of the *MACS1* gene, the location of possible *cis*-acting regulatory elements of its promoter, and a phylogenetic analysis of the ACC synthase genes from banana as well as from various other plants.

## MATERIALS AND METHODS

**Plant Materials.** Aseptically maintained young plantlets of the banana cultivar "Hsian Jien Chiao" (*Musa* spp., AAA genome) were used to isolate genomic DNA. Prior to use, these plantlets were transferred to 15 cm plastic pots containing peat and vermiculite (1:1, v/v) medium. After reaching a height of 20 cm, the above-ground parts of plants were harvested, frozen with liquid nitrogen, and stored at −80 °C. A sample of banana fruit "Hsian Jien Chiao" was taken from farm-grown plants and classified into seven stages according to the surface coloration (*25*). Intact, mature green fruit was treated with 1000 $\mu$L/L $C_2H_4$ for 24 h and ripened at 25 °C and then frozen with liquid nitrogen for Northern analysis.

**Construction and Screening of Genomic DNA Library.** Genomic DNA was isolated from nuclei of young leaves and purified by equilibrium sedimentation in a CsCl gradient (*26*). A partial *Sau*3AI digest of the genomic DNA was size fractionated by electrophoresis on a 0.5% low-melting agarose gel. DNA fragments of 15−25 kb in size were cloned by insertion into the *Bam*HI site of the EMBL3 vectors (*27*) and packaged with Gigapack II gold (Stratagene). The primary library, $2.5 \times 10^6$ plaque-forming units/mL in size, transfected into *E. coli* XL1-Blue MRA(P2) was screened by the method of Benton and Davis (*28*) with a $^{32}$P-labeled pMACS-28 cDNA (accession number AF008942) insert. As the banana genome was estimated as $6.0 \times 10^8$ bp (*29*), $1.84 \times 10^5$ recombinant bacteriophages need to be generated and screened in order to achieve a 99% probability ($P = 0.99$) of having a given single-copy DNA sequence represented in a library of 15-kb fragments of banana genome based on the following calculation, where $f$ is the fractional proportion of the genome in a single recombinant (*30*).

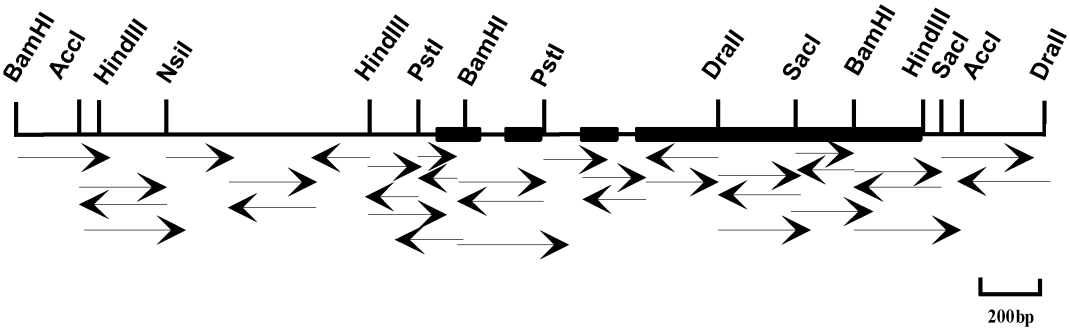
$$N = \frac{\ln(1-P)}{\ln(1-f)} = \frac{\ln(1-0.99)}{\ln(1-[1.5\times10^4/6\times10^8])} = 1.84\times10^5$$

Therefore, approximately $5.25 \times 10^5$ (equivalent to 3-fold theoretical number) nonamplified plaques of banana genomic library was screened with ACC synthase gene-specific probes to avoid sampling variation and preferential cloning of certain sequences. Hybridization was performed overnight at 42 °C in hybridization solution (5 × SSPE, 1 × Denhardt's reagent, 0.1% SDS, 100 $\mu$g/mL denatured fragmented salmon sperm DNA, 50% formamide) (*31*). Hybridization filters were washed twice at room temperature in 5 × SSPE, 0.1% SDS for 15 min each, then two times in 1 × SSPE, 0.5% SDS at 37 °C for 15 min each. Phage DNAs isolated from the positive clones were examined by polymerase chain reaction (PCR) with primer sets, ACS1 (5′-AACGAATTCATA(T/C)CAG(A)ATGGGT(A/C/G)T(C)T-3′) and ACS2 (5′-AGGGGATCCA(G)TGA(G)TAA(G)TCT(C)TGG(A)AA-3′), corresponding to highly conserved regions of ACC synthase amino acid sequences registered in the database from various plant sources. PCR was performed in a thermocycler using the conditions recommended by the manufacturer. The PCR parameters were 1 min template denaturation at 94 °C, 2 min primer annealing at 50 °C, and 2 min primer extension at 72 °C for 30 cycles. The DNA sequence was determined by the dideoxy-chain-termination technique (*32*). Sequence data were analyzed using the Vector NTI Suite 6.0 software.

**Determination of Transcription Initiation Site.** Primer extension analysis was performed according to Bond and Davidson (*33*). A 5′-end-labeled 30 mer synthetic oligonucleotide, acc1 (5′-TGATTTGGGT-GCTCCTCGCCGTAGATCCTC-3′) (0.2 pmol), complementary to nucleotides +77 to +106 of the GMACS-12 gene (**Figure 2**), was hybridized with 2 $\mu$g of poly(A)$^+$ RNA from mature green or ripening fruit at 55 °C for 6 h. The primer−RNA hybrids were incubated with 10 units of reverse transcriptase in 20 $\mu$L of a reaction mix (50 mM Tris-HCl (pH 8.5), 40 mM KCl, 8 mM $MgSO_4$, 0.4 mM dithiothreitol, 1 mM of each dNTP) at 42 °C for 1 h. For determination of the nucleotide sequence near the transcription initiation site, the sequencing reaction of the *Hin*dIII/*Bam*HI fragment of about 400 bp in size that contained about 200 bp of the promoter region of GMACS-12 was carried out with the same primer. The primer extension product was analyzed on a 6% (w/v) polyacrylamide sequencing gel. S1 nuclease analysis was performed as described by Buchman et al. (*34*). [5′-$^{32}$P]-acc1 primer was hybridized to the M13 mp19 ssDNA of the 5′ proximal *Hin*dIII/*Bam*HI fragment from GMACS-12 and extended with six units of Klenow fragment (*31*). The reaction was digested with *Mbo*I, which cleaves at position −120 (**Figure 2**). The 225 nt single-stranded probe was purified by denaturing polyacrylamide gel electrophoresis (*31*) and used for S1 digestions with 2 $\mu$g of poly(A)$^+$ RNA from mature green and ripening fruit. The protected fragments were separated on a 6% (w/v) polyacrylamide sequencing gel.

**Genomic Southern Blot Analysis.** Genomic DNA was extracted from banana seedlings as described by Dellaporta et al. (*26*) with modification. Ten micrograms of DNA was digested with the indicated

(A)




(B)

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ACTGCAGCAGCTGCTTCTCCTTCTTCTCTGCTCGCTTCAGCCTTTTCCGGTACGTACCTGAGATAACGGGTCACATGAGGATCTACGGCGAGGAGCACCCAAATCAGCAGATCCTCTCTC +120
                                                                M  R  I  Y  G  E  E  H  P  N  Q  Q  I  L  S
GGATCGCGACCAACGACGGCCATGGCGAGAACTCCTCCTACTTCGATGGGTGGAAGGCCTACGAGAAGGATCCTTTCCACCTCACCGACAACCCCACGGGGGTCATCCAAATGGGACTCG +240
R  I  A  T  N  D  G  H  G  E  N  S  S  Y  F  D  G  W  K  A  Y  E  K  D  P  F  H  L  T  D  N  P  T  G  V  I  Q  M  G  L
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A  E  N  Q
gCTTTCCCTCGACTTGATCCGAGACTGGATGAAGAAGAACCCACAGGCTTCGATCTGCACCGAAGAAGGGGTCTCAGAGTTCAAAGCAATTGCCAACTTTCAGGACTATCATGGCCTCCC +480
  L  S  L  D  L  I  R  D  W  M  K  K  N  P  Q  A  S  I  C  T  E  E  G  V  S  E  F  K  A  I  A  N  F  Q  D  Y  H  G  L  P
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  A  F  R  K
cgagccctgcctgctcaccctggataagggataagctaatgatggtgtgatttgctgacacgcgcagGCCATCGCCCAGTTCATGGAGAAGGTGAGAGGGGGACGAGCCAGATTTGACCC +720
                                                                      A  I  A  Q  F  M  E  K  V  R  G  G  R  A  R  F  D  P
AGACCGCATCGTGATGAGCGGTGGAGCCACCGGCGCTCAGGAAACCATCGCCTTTTGCCTGGCTGATCCTGGCGAGGCCTTCTTGATTCCAACGCCATATTATCCGGGgtaagtatttag +840
  D  R  I  V  M  S  G  G  A  T  G  A  Q  E  T  I  A  F  C  L  A  D  P  G  E  A  F  L  I  P  T  P  Y  Y  P  G
gtgtactaatctactgagttctttatccggcagaggatctaatggcatctgcatggtttccagATTCGATCGAGACTTCAGGTGGAGGACAGGAGTTCAGCTCCTCCCCATTCACTGCCA +960
                                                                        F  D  R  D  F  R  W  R  T  G  V  Q  L  L  P  I  H  C  H
CAGTTCCAACAAGTTCAAGATCACCCAAGCCGCACTGGAGACTGCTTACAGGAAGGCTCGAAACTCACACATTAGAGTCAAAGGAATACTGGTGACCAACCCATCGAACCCTCTGGGCAC +1080
  S  S  N  K  F  K  I  T  Q  A  A  L  E  T  A  Y  R  K  A  R  N  S  H  I  R  V  K  G  I  L  V  T  N  P  S  N  P  L  G  T
AACCATGGACAGAGAGACGCTGAGAACCCTAGTCAGCTTCGTCAACGAGAAAAGGATGCACTTGGTGTGCGACGAGATCTTCTCCGGAACCGTCTTCGACAAGCCGAGTTACGTGAGCGT +1200
  T  M  D  R  E  T  L  R  T  L  V  S  F  V  N  E  K  R  M  H  L  V  C  D  E  I  F  S  G  T  V  F  D  K  P  S  Y  V  S  V
CTCCGAGGTGATCGAAGACGATCCCTACTGCGACAGGGATCTGATTCACATCGCCTACAGCCTCTCCAAGGACCTGGGCGTCCCTGGCTTCCGCGTCGGCGTCATATACTCCTACAACGA +1320
  S  E  V  I  E  D  D  P  Y  C  D  R  D  L  I  H  I  A  Y  S  L  S  K  D  L  G  V  P  G  F  R  V  G  V  I  Y  S  Y  N  D
CGCCGTGGTCAGCTGCGCGAGGAAGATGTCGAGCTTTGGACTGGTCTCGTCGCAGACGCAGCACCTGCTCGCTTCCATGTTGGGAGACGAGGAGTTCACCACGAGTTTCTTAGCGACGAG +1440
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  L  S  L  P  R  R  R  F  E  D  P  T  I  M  T  P  H  L  M  S  P  H  S  P  L  V  Q  A  A  T  *
CAAAGAAGGTTAATTACCGTCTGATATGTTGCACATTTCTTTGTTCTTTGAATTATTTATTTATTTATTTTTTGGAAAAAATGGATTGAATGTTCCCACTAAGGTATATTAGATTGTTGT +2040
TCGGTCTCATTCATGTTATAGGAAACGAGGATAGAATTGCTTGCCTCTCTCTTTCTTTTATATATGGAAATATGTTGCAATTggcctaagcttatttgatgacattaatttcacaagaca +2160
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tcatgcttccgaggtgtcacctgaagattgaggttagaagggtttctcgacctgatccatccaacgctcaagatag                                              +2596
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**Figure 2.** (**A**) Partial restriction map and sequencing strategy for GMACS-12. The protein coding sequence is indicated by the solid bars; 5′ and 3′ flanking sequences are indicated by lines. The arrows indicate the extent of each sequence determination. (**B**) Complete DNA sequence of GMACS-12 and its flanking genomic region. The sequence of the mRNA transcribed by the gene is presented in capital letters. The remainder of the sequence is shown in small letters. The derived amino acid sequence is presented in the one-letter code below the DNA sequence. The transcription start site is boxed; CAAT box, TATA box, and three potential polyadenylation signal sequences are double underlined. The star identifies the stop codon. Several sequences found in the 5′ flanking region that may be involved in gene regulation are underlined.

restriction enzymes, separated by a 0.7% agarose gel electrophoresis, and then transferred onto a nylon filter (Hybond-N, Amersham). Blots were baked under vacuum for 2 h at 80 °C. DNA gel blots were probed with $^{32}$P-labeled PCR ACC synthase gene fragments (Facs) amplified with ACS1 and ACS2 as primers from banana genomic DNA: Facs1 (nucleotides 2100−2293 of AF056162), -2 (DQ343868), -3 (DQ343869),

**Table 1.** Genomic and cDNA Clones and Differential Expression of ACC Synthase Multigene Family in Banana

| gene class | genomic clones | PCR fragments | cDNA clones | response to fruit ripening | ethylene inducible |
|---|---|---|---|---|---|
| MACS1 | GMACS-3, -5, -9, -12, -14 | Facs5 | pMACS-25, 28 | + | + |
| MACS2 | GMACS-1, -10, -29, -31, -34, -36 | Facs1 | | − | − |
| MACS3 | GMACS-16, -27, -28, -52 | Facs52 | | − | − |
| MACS4 | GMACS-30 | Facs44 | | − | − |
| MACS5 | | Facs2 | | − | − |
| MACS6 | | Facs3 | | nd[a] | nd[a] |
| MACS7 | | Facs4 | | − | − |
| MACS8 | | Facs8 | | nd[a] | nd[a] |
| MACS9 | | Facs10 | | nd[a] | nd[a] |

[a] Not determined.

-4 (DQ343870), and -5 (nucleotides 1788−2005 of AF056163) separately. Hybridization was performed overnight at 42 °C in a solution that consisted of 5 × SSPE, 0.5% SDS, 5 × Denhardt's reagent, 250 μg/mL denatured fragmented salmon sperm DNA, and 50% formamide. The membrane was washed twice with 5 × SSPE and 0.5% SDS at room temperature for 10 min each and then washed once with 2 × SSPE and 0.5% SDS at 65 °C for 15 min. Autoradiography was performed at −80 °C using Kodak XAR-5 film and an intensifying screen. Exposure times for blots were on the order of one or more days. The PCR fragment was labeled to high specific activity with $^{32}$P-dCTP by random priming (*35*).

**Northern Blot Analysis.** Total RNA isolation was carried out by the method of Taylor and Powell (*36*). After glyoxylation in the solution (50% DMSO, 10 mM $Na_3PO_4$ buffer pH 7.0, 1 M deionized glyoxal, 25 μg of RNA), total RNAs were separated on a 1% agarose gel electrophoresis and transferred onto nylon membrane (Hybond-N, Amersham). The membrane was hybridized and washed as described in Southern blot analysis.

## RESULTS

**Isolation of the Banana ACC Synthase Genes.** Screening a lambda-EMBL3 banana genomic library with the pMACS-28 cDNA as a probe under high stringency hybridization conditions led to the isolation of 29 lambda genomic clones. According to the restriction mapping for 29 genomic clones, 16 representative clones containing complete gene body and promoter sequences were chosen for further characterization. The data showed they can be grouped into four classes of genes: *MACS1* with five overlapping genomic sequences (GMACS-3, -5, -9, -12, and -14), *MACS2* with six overlapping genomic sequences (GMACS-1, -10, -29, -31, -34, and -36), *MACS3* with four overlapping genomic sequences (GMACS-16, -27, -28, and -52), and *MACS4* with one genomic sequence (GMACS-30). The 16 clones were partially sequenced, and the data showed that genes from the same class were slightly different from each other, which may be due to the triploidy nature of the banana genome.

To determine whether these four classes of genes encode ACC synthase, phage DNAs isolated from the clones were examined by PCR with primer sets ACS1 and ACS2. The PCR fragments were sequenced, and their sequences were compared with the previously isolated and database-registered PCR fragments (Facs) from banana genomic DNA: Facs1 (nucleotides 2100−2293 of AF056162), -2 (DQ343868), -3 (DQ343869), -4 (DQ343870), -5 (nucleotides 1788−2005 of AF056163), -8 (DQ343871), -10 (DQ343872), and -44 (DQ343867). The data show that *MACS1* corresponds to Facs5, *MACS2* to Facs1, and *MACS4* to Facs44 (**Table 1**). However, *MACS3* is newly discovered, and its PCR fragment was named Facs52 (DQ343866). Moreover, *MACS1* also corresponds to the previously isolated cDNA sequences pMACS-25 (AF008941) and pMACS-28 (AF008942).

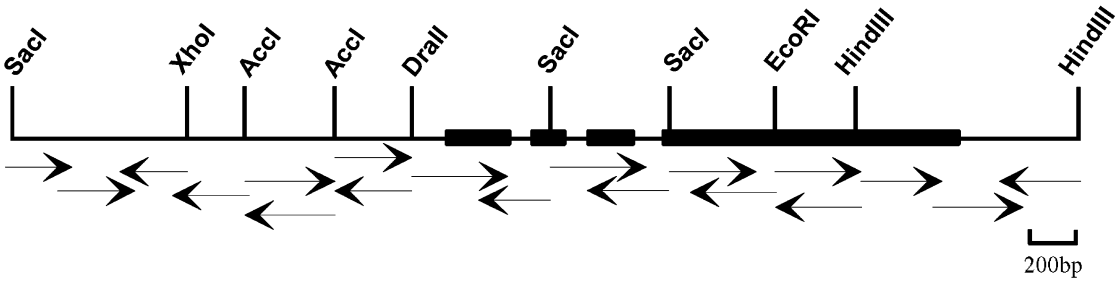
**Genomic Organization of the ACC Synthase Genes.** To verify the organization of ACC synthase genes in the banana genome, Southern analysis was performed at high stringency with Facs1, -2, -3, -4, and -5 (**Figure 1**). The data indicated that they are located in different regions of the banana genome. The blots probed with Facs2, -3, and -5 all yield a single band on each lane, which indicates that these three genes exist as single-copy genes in the banana genome. However, several bands were observed in *Eco*RI and *Eco*RI/*Bam*HI digests on the blots probed with Facs1 and Facs4, which indicates that an additional two or more genes are homologous to Facs1 and Facs4 exist in the banana genome.

**Gene Structure and Organization of *MACS1* and *MACS2*.** Three lambda genomic clones, GMACS-1 (AF056162), GMACS-9 (AF056164), and GMACS-12 (AF056163), were completely sequenced. The sequences of GMACS-9 and GMACS-12 are highly conserved because they belong to the same gene class (*MACS1*). The coding sequences of GMACS-9 and GMACS-12 are 100% identical to pMACS-25 and pMACS-28, respectively. The sequence of GMACS-12 spanning 1548 bp upstream from the initiation codon and 702 bp downstream from the stop codon is presented in **Figure 2**. GMACS-9 contains 1897 bp upstream from the initiation codon and 726 bp downstream from the stop codon. Comparison of the 5′ sequences (−1 to −1400 bp) of these two genes revealed significant similarity (93%). The sequence of GMACS-1 includes 1958 bp of 5′ and 841 bp of 3′-flanking regions (**Figure 3**), and the identity to GMACS-12 is 53.2% at the protein level and 56.2% in the nucleic acid of the transcription region. The promoter of GMACS-1 does not show high similarity to GMACS-9 or GMACS-12 in general, but it does show significant similarity (90−100%) to both in several short sequences (6−20 bp) of the 5′ region. Besides, these three genes all contain four exons and three introns, which are all less than 300 bp long. The splice junction sequences in these three ACC synthase genes show a high degree of conservation.

**Determination of Transcription Initiation Site.** We have determined the transcription initiation site of GMACS-12 containing *MACS1* both by S1 nuclease analysis and primer extension using reverse transcriptase. **Figure 4** shows that the probe for S1 nuclease analysis protects RNA isolated from ripe fruit but not from green fruit. Primer extension analysis gave similar results. Although primer extension yielded three bands, the strongest one corresponds to the result of S1 nuclease analysis, indicating that the initiation site of GMACS-12 is the base adenine at 104 bp upstream the translation start codon ATG. There is a TATTAAT sequence starting at position −31 to −25 (**Figure 2**) that qualifies as a TATA box, and a potential CAAT box was present at position −128 to −125. The 3′ end of the gene was determined by comparing its nucleotide sequence with the nearly full-length cDNA, pMACS-28. Moreover, comparison of the 5′-untranslated region (+1 to +74) between GMACS-9 and GMACS-12 revealed that GMACS-9 has three bases (CTT) more than GMACS-12 (**Figure 4**). These data explain why primer extension yielded another two bands. The upper band may result from the expression of GMACS-9, which would indicate that the initiation site of GMACS-9 is also adenine.

**Expression Characteristics.** To understand how the members of this gene family are involved in fruit ripening, Northern analysis of six genes (*MACS1*, *-2*, *-3*, *-4*, *-5*, and *-7*) was performed with RNA isolated from banana peel tissue at

**(A)**




**(B)**

```
gagctcatcgagggtttgaagaagctttacaacagagaaggaaaatggcagagtagccgaacattacccccctgagaaatcttcggaagcaaagaggaagagcgagaagggacagagtcac 120
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ggttaagcaggtctcttgtccaacatcaggggcccagtagattttgtgtcggcaggtgtatctaaattgcttcatctgaactaaatccagcaccattctatctgtctgtaaaaacaatcc 1800
gtgattgtgttacttcgaggattctataaatgcctactcctccaccttccaagcatcaccaaaatctctcttttctctctctctctctctctctctctctctctctctcgttcttttgt 1920
acatacagcgtctctcaatctcttctcagcctaccagaATGAATCAGATGCTGCTCTCCAGAAAAGCTGCATGCAACATCCATGGGCAGGACTCCTCCTATTTCCTGGGGTGGCAGGAGT 2040
                                        M  N  Q  M  L  L  S  R  K  A  A  C  N  I  H  G  Q  D  S  S  Y  F  L  G  W  Q  E
ACGAGAAGAACCCCTATGATCCAATCACTAACCCGACAGGGATCATTCAGATGGGTCTCGCAGAGAACCAGgttggttctctcactcagacaagttctcctcactcaagtcacttgtgac 2160
Y  E  K  N  P  Y  D  P  I  T  N  P  T  G  I  I  Q  M  G  L  A  E  N  Q
agtaactaatattaaggcctctcgttcatgcttgcagCTCTGCTTTGATCTCATCGAATCGTGGCTTGAGAACCATCCCGACCCAGCTGCATTCAAGAAAGATGGAGCACTACTATTCCG 2280
                                        L  C  F  D  L  I  E  S  W  L  E  N  H  P  D  P  A  A  F  K  K  D  G  A  L  L  F  R
GGAGCTCGCTTTGTTCCAGGACTACCATGGCTTGCCAGCCTTCAAGCGTgtaagtccataaacttaaactaatttaccggggatatatcagaaggatcacatccattctcgcatatatca 2400
  E  L  A  L  F  Q  D  Y  H  G  L  P  A  F  K  R
acagaatctgctgggttactaattgcagtccgcaatgcatacatctgcagGCATTGGCTAAATACATGGGAGAAGTAAGAGGAAACAAAGTAGCTTTCGATCCCAACAGGCTCGTCCTCA 2520
                                                    A  L  A  K  Y  M  G  E  V  R  G  N  K  V  A  F  D  P  N  R  L  V  L
CGGCTGGTGCTACTTCTGCCAATGAGACTCTCATGTTTTGTCTTGCCGAACCTGGAGAAGCATTCCTTCTGCCTACTCCATACTACCCAGGgtagtacacatccacaacagagccttgtc 2640
T  A  G  A  T  S  A  N  E  T  L  M  F  C  L  A  E  P  G  E  A  F  L  L  P  T  P  Y  Y  P  G
gaccaattactctacccatgcacgcgatcggttccatgtacttgaattgccatggtgacacactcacctttcccatgtgtgcctgacagATTCGACAGAGACCGCAAATGGCGAACCGGA 2760
                                                                                                F  D  R  D  R  K  W  R  T  G
GCGGAGATCGTTCCCATCCATTGTTCGAGCTCTAACGGCTTCAGGATCACCAAACCGGCCCTGGAAGCTGCATACCAAGATGCGCAGAAGCGTAGCCTCAGAGTGAAAGGTGTGCTGGTG 2880
 A  E  I  V  P  I  H  C  S  S  S  N  G  F  R  I  T  K  P  A  L  E  A  A  Y  Q  D  A  Q  K  R  S  L  R  V  K  G  V  L  V
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 T  N  P  S  N  P  L  G  T  T  L  T  R  H  E  L  D  I  L  V  D  F  V  V  S  K  D  I  H  L  I  S  D  E  I  Y  S  G  T  N
TTCGACTGGCCGGGGTTCATCAGCATTGCAGAGGCCACAAAGGACAGGAACAACGTCTCCCATCGGATTCACATCGTGTGCAGCCTCTCTAAAGATCTCGGCCTCCCAGGTTTTCGTGTC 3120
 F  D  W  P  G  F  I  S  I  A  E  A  T  K  D  R  N  N  V  S  H  R  I  H  I  V  C  S  L  S  K  D  L  G  L  P  G  F  R  V
GGTGCAATCTATTCGGAGAATGAAGCAGTCGTGTCTGCTGCTACTAAGATGTCAAGCTTTGGGATGGTCTCTTCTCAAACCCAGTACCTCCTCGCGGCGTTGTTATCCGACAAAGAATTC 3240
 G  A  I  Y  S  E  N  E  A  V  V  S  A  A  T  K  M  S  S  F  G  M  V  S  S  Q  T  Q  Y  L  L  A  A  L  L  S  D  K  E  F
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 T  D  K  Y  L  L  E  N  Q  K  R  L  K  E  R  H  D  M  L  V  E  G  L  R  R  I  G  I  G  C  L  K  G  S  A  A  L  F  C  W
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 V  D  M  R  H  L  L  K  S  N  T  F  K  G  E  M  E  L  W  K  K  I  V  Y  Q  V  G  L  N  I  S  P  G  S  S  C  H  C  D  E
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 P  G  W  F  R  V  C  F  A  N  M  S  E  D  T  L  T  L  A  M  Q  R  L  K  S  F  V  D  S  G  D  C  G  S  N  H  D  S  G  H
CAGCGCCCCAGAAAGCCATTCTTGACCAAGTGGGTCCTCCGCTTGTCGTCCACCGATCGCAAGTCCGAGCGATGAtaacaccggattgctgcagcgaatgcacgatcgatcattctttac 3720
 Q  R  P  R  K  P  F  L  T  K  W  V  L  R  L  S  S  T  D  R  K  S  E  R  *
atttttacgccaaacagcatgtttctttttctcctcgtgtctttttgtcttggatcacgcgatctgtctggtttttgagtgcccaaggcttcatgatcgagagagattgttcacttcctc 3840
attccatgaagttattcgatttctcagtaacagtatgtacgataacttttgttcttgtttcttctaagaaagcattcagtatggaacatgacactgttcttgttgctaccatagataaata 3960
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aacggcggccgaacgaggcaaggacggatttttgagatgaggtgcattctgttctcatctagcttggaacgagaacatgatcaagacatcatgctgatctctcgcagtgtttacttctac 4440
ccggagatggagatggacactcggtcactatcgtcgacatctgctgtctcggttgtcgacgtcacgtcaaaagctt                                             4516
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**Figure 3.** (**A**) Partial restriction map and sequencing strategy for GMACS-1. The protein coding sequence is indicated by the solid bars; 5′ and 3′ flanking sequences are indicated by lines. The arrows indicate the extent of each sequence determination. (**B**) Complete DNA sequence of GMACS-1 and its flanking genomic region. Coding regions are in uppercase letters; noncoding regions are in lowercase letters. The derived amino acid sequence is presented in the one-letter code below the DNA sequence. The putative CAAT and TATA boxes and potential polyadenylation signal sequences are underlined. The star identifies the stop codon.

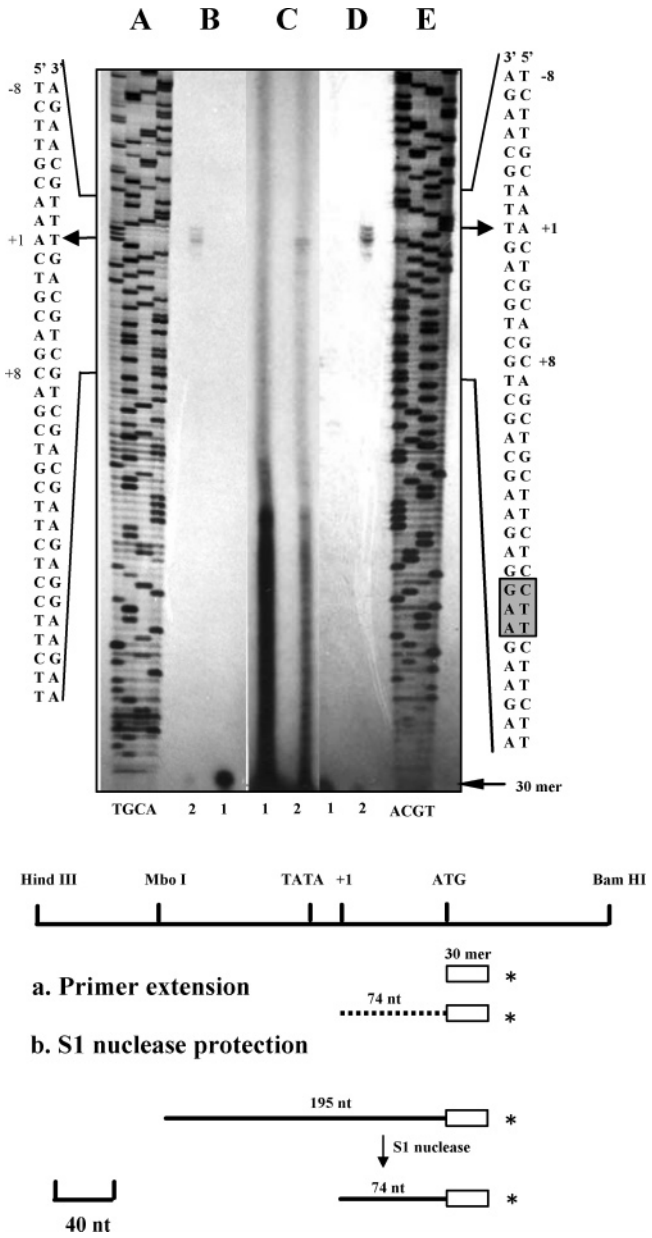


**Figure 4.** Determination of the transcription initiation site of GMACS-12. (A and E) Dideoxy sequencing reactions using acc1 as primer with a *Hin*dIII/*Bam*HI fragment from GMACS-12 and GMACS-9, respectively. (B and D) Primer extension analysis. A 5′-end-labeled 30-mer synthetic oligonucleotide, acc1, complementary to nucleotides +77 to +106, was hybridized with 2 $\mu$g of mRNA from mature green fruit (lane 1) and ripening fruit (lane 2). (C) S1 nuclease protection. A 5′-labeled 195 nt single-stranded probe was used for S1 digestion with 2 $\mu$g of mRNA from mature green fruit (lane 1) and ripening fruit (lane 2). Extra three bases (CTT) present in GMACS-9 compared with the corresponding region in GMACS-12 are boxed and shaded.

different stages of fruit ripening or from fruit postexposure for 24 h with various amounts of ethylene. The results are summarized in **Table 1**. Only *MACS1* gene expressed during fruit ripening and was inducible by exogenous ethylene treatment. The levels of *MACS1* mRNA accumulation during banana fruit ripening are shown in **Figure 5A**. The amount of mRNA was undetectable in mature green fruit; however, it increased dramatically at ripening stage 2 and reached a peak at ripening stage 5. A dose-dependent increase of *MACS1* gene expression was observed in mature green fruit after treatment with various amounts of ethylene for 24 h (**Figure 5B**). The inducible mRNA

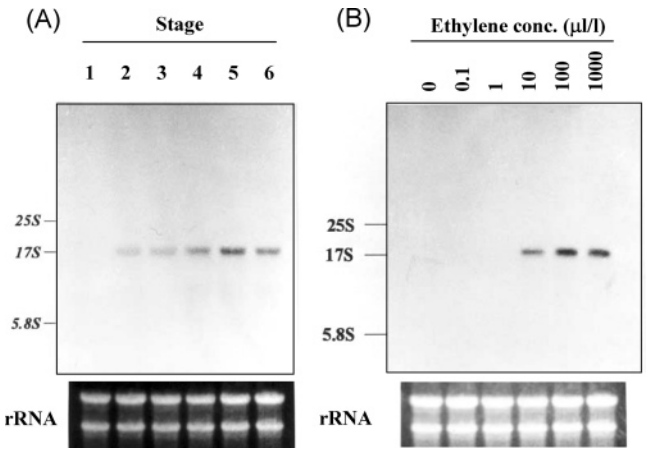


**Figure 5.** Expression of *MACS1* gene during various stages of fruit ripening (**A**) and in response to exogenous ethylene (**B**). Each lane was loaded with 25 $\mu$g of total RNA isolated from peel tissue of banana fruit in different development stages (from stage 1 to stage 6) and from the peel tissue of mature green fruit after treatment with indicated concentrations of ethylene for 24 h. RNAs were electrophoretically separated on an agarose gel and blotted onto Hybond-N membrane. The level of rRNA was used as a reference to ensure equal loading of total RNA. The filter was hybridized with a $^{32}$P-labeled gene-specific probe Facs5.

was detected with as little as 10 $\mu$L/L of $C_2H_4$ and saturated at 100 $\mu$L/L. The other five genes (*MACS2*, *-3*, *-4*, *-5*, and *-7*) were not detected by Northern analysis, suggesting that either these genes were not expressed or their expressions were at an extremely low level in banana fruit.

## DISCUSSION

Recent studies revealed that ACC synthase gene expression is induced by a diverse group of inducers, including ethylene (*6*, *7*, *21*, *37*, *38*), auxin (*11*, *39*−*41*), wounding (*10*, *42*, *43*), and flooding (*9*, *44*). From these data, it can be argued that there are many ACC synthase genes to reflect the diversity of inducers. The existence of a multigene family in banana was confirmed by cloning nine classes of ACC synthase genes (**Table 1**). Southern analysis (**Figure 1**) indicated that they are located in different regions of the banana genome and exist as single-copy or low-copy genes in the banana genome. Northern analysis revealed that only the *MACS1* gene expresses during fruit ripening and was inducible by exogenous ethylene treatment, which indicates that *MACS1* is a significant member of the ACC synthase gene family related to ripening in banana fruit. This result was also found by Liu et al. (*38*). The other five genes (*MACS2*, *-3*, *-4*, *-5*, and *-7*) were not detected by Northern analysis. They can possibly express in other developmental stages of banana or be regulated by other inducers such as auxin or stress conditions. Their function remains to be proved.

Partial sequences of 16 genomic clones revealed a small difference among genes of the same class. We propose that this is due to allelic difference in the banana genome. Moreover, there is significant divergence (50−80%) between different classes of genes in banana. Similar studies in tomato (*6*, *7*) and *Arabidopsis thaliana* (*18*, *45*) have shown the same degree of divergence throughout the coding region of different ACC synthase genes. A dendrogram of nine banana ACC synthase gene fragments (Facs1, -2, -3, -4, -5, -8, -10, -44, and -52 corresponding to gene family *MACS2*, *-5*, *-6*, *-7*, *-1*, *-8*, *-9*, *-4*, and *-3*, respectively) isolated in this study, two banana ACC synthase genes (*MaACS5*, accession number AJ223186, and *Ma-*

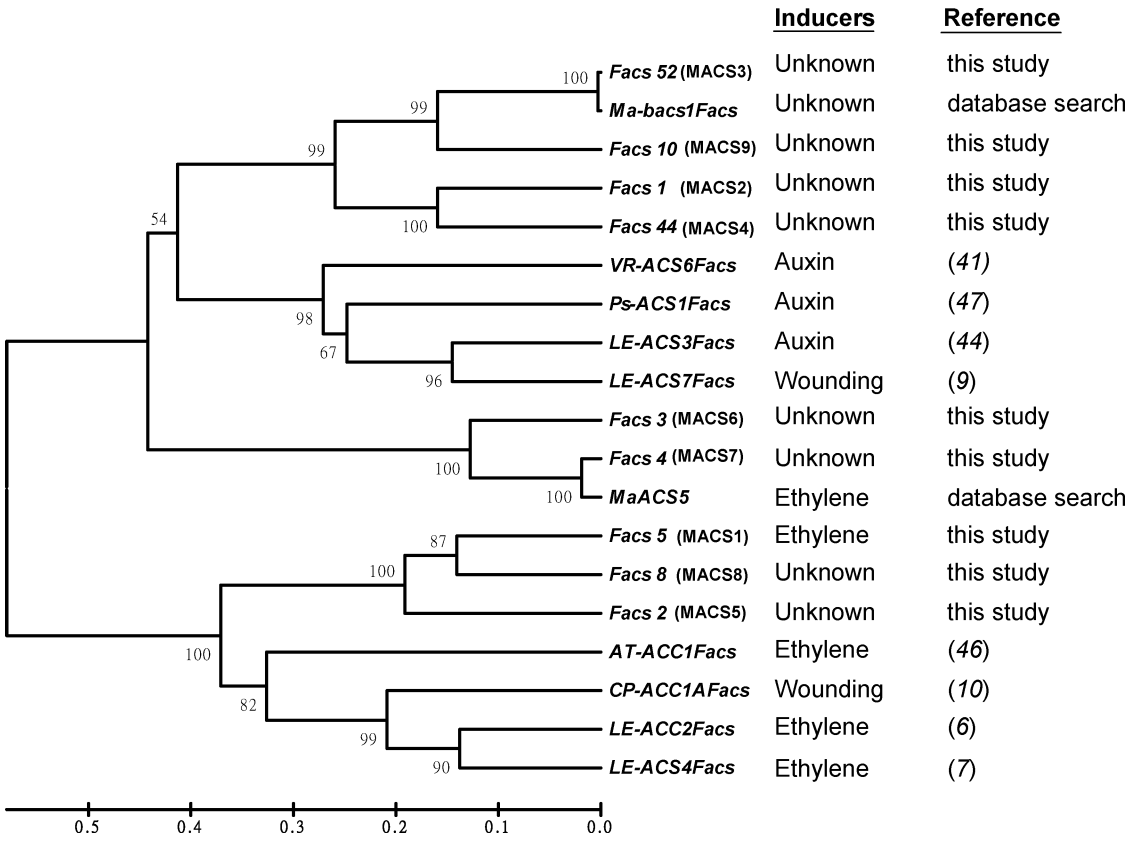


**Figure 6.** Nucleotide sequence-based dendrogram among nine banana ACC synthase gene fragments amplified by PCR using ACS1 and ACS2 as primers in this study (Facs1, -2, -3, -4, -5, -8, -10, -44, and -52), two banana ACC synthase genes (*MaACS5*, accession number AJ223186, and *Ma-bacs1*, accession number X96946) from database search, as well as eight ACC synthase genes with the same fragment from other species including zucchini *CP-ACC1A* (*10*), *Arabidopsis thaliana AT-ACC1* (*46*), tomato *LE-ACC2* (*6*), tomato *LE-ACS3* (*44*), tomato *LE-ACS4* (*7*), tomato *LE-ACS7* (*9*), pea *Ps-ACS1* (*47*), and mungbean *VR-ACS6* (*41*). Nucleotide sequences were aligned using MEGA package and the UPGMA program. Scale bar and phylogenic distances were calculated according to the number of differences. Numbers next to the node refer to the bootstrap values expressed as a percentage after 500 replicates. Only values >50% are presented.

*bacs1*, accession number X96946) from database search, as well as eight ACC synthase genes with the same fragment from other species including zucchini, tomato, *Arabidopsis*, pea, and mungbean was obtained using the MEGA package and the UPGMA program (http://www.megasoftware.net) and is shown in **Figure 6**. Phylogenetic analysis reveals that the genes fall into two branches. One major lineage contains *MACS1*, *-5*, and *-8* isolated from banana in this study as well as *Arabidopsis AT-ACC1* (*46*), zucchini *CP-ACC1A* (*10*), tomato *LE-ACC2* (*6*), and *LE-ACS4* (*7*). Most of the genes in this sublineage are ethylene regulated, which indicates a striking correlation between their phylogenetic relationship and their pattern of expression. This result was also found by Lincoln et al. (*7*). The other major lineage contains three sublineages. The first one contains *MACS2*, *-3*, *-4*, and *-9* isolated from banana in this study and another banana ACC synthase *Ma-bacs1* registered in the database, and among these *MACS3* shows high similarity (98%) to *Ma-bacs1*. The second one contains tomato *LE-ACS3* (*44*) and *LE-ACS7* (*9*), pea *Ps-ACS1* (*47*), and mungbean *VR-ACS6* (*41*); three of them are auxin regulated. The third one contains two banana ACC synthase genes (*MACS6* and *-7*) isolated in this study and one (*MaACS5*) from database search. Interestingly, *MACS7* also shows high similarity (96.4%) to *MaACS5*.

The structure of three ACC synthase genes of banana (GMACS-1, GMACS-9, and GMACS-12) contains four exons and three introns. The intron/exon junctions of GMACS-9 and GMACS-12, which are typical of donor and acceptor splice sites, have been established by reference to pMACS-25 and pMACS-28, respectively, and the intron/exon junctions of GMACS-1 have been established by reference to other ACC synthase genes. ACC synthase genes can be divided into four classes based on the number and position of introns (*6*). They have either two, three, or four introns (**Figure 7**), but the position of each intron is exactly conserved among ACC synthase genes. Thus far, only zucchini ACC synthase (*10*) has four introns, and most ACC synthase genes including banana *MACS1* and *MACS2*, tomato *LE-ACC2* (*6*), *LE-ACS3* (*44*), and *Arabidopsis AT-ACC1* (*46*) contain three introns. Some genes contain two introns but at different sites. For example, *LE-ACS 4* (*7*) and *LE-ACS7* (*9*) have intron II and intron III at corresponding positions, but *Ps-ACS1* (*47*) and *VR-ACS6* (*41*) have intron I and intron III.

Comparison of the 5′ flanking sequence of GMACS-12 with those of other ethylene-regulated genes such as *E4*, *E8* (*48*), *HRGP* (*49*), *LE-ACC2* (*6*), and *MAO2* (*50*) shows that several sequences of 6−12 bp are identical in the GMACS-12 promoter (**Table 2**). Moreover, the sequence of the promoter was compared with a database of known *cis*-acting regulatory elements at the PlantCARE (*51*) (http://sphinx.rug.ac.be:8080/PlantCARE/). While some similarity with many elements was found in the search, exact matches were found for six particular elements. A G-box core (GCCACGTTGCA) appearing at position −1532 to −1522 (**Figure 2**) is identical with the core consensus element from sbp-CAM1 involved in light responsiveness (*52*). A second element (ATTTCAAA) located at position −1117 to −1110 is also found in the promoter region

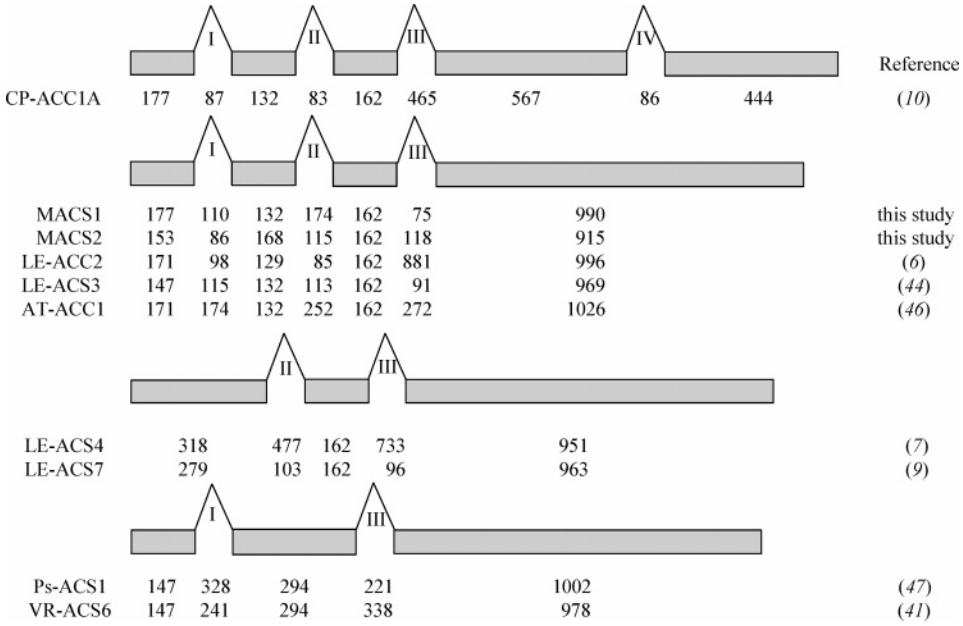


**Figure 7.** Schematic diagrams of different ACC synthase genes from banana (*MACS1* and *MACS2*, this study), tomato (*LE-ACC2*, *LE-ACS3*, *LE-ACS4*, and *LE-ACS7*), *Arabidopsis thaliana* (*AT-ACC1*), zucchini (*CP-ACC1A*), pea (*Ps-ACS1*), and mungbean (*VR-ACS6*). Protein-coding exons are indicated by boxes, and introns are indicated by bent lines. Numbers below boxes or lines represent the length of base pairs.

**Table 2.** Common Sequences Present in *MACS1*-Containing GMACS-12 and Other Ethylene-Related Gene Promoters

| ethylene-related gene | sequences | position of GMACS-12 | ref |
|---|---|---|---|
| LE-ACC2 | ATATTA | –1448 to –1443 | *6* |
| | AAAGTA | –1236 to –1231 | |
| | ACAAACAA | –1203 to –1196 | |
| | AAGGCAA | –988 to –3982 | |
| | TCCGATC | –868 to –8628 | |
| | AACTTGA | –444 to –438 | |
| | TTTTCC | –93 to –88 | |
| MAO2 | AACCCA | –1455 to –1450 | *50* |
| | AATATT | –1447 to –1442 | |
| | CACCAA | –1192 to –1187 | |
| | AGATCATGC | –1104 to –1096 | |
| | GTTTCT | –1088 to –1083 | |
| | GATACG | –966 to –961 | |
| | ACATGA | –776 to –771 | |
| | AAAGGTG | –639 to –633 | |
| | AAACTTTT | –604 to –597 | |
| | TGCTTC | –568 to –563 | |
| | AACACCTCA | –516 to –508 | |
| | TACAAT | –297 to –262 | |
| | CGAGTCGAAAG | –250 to –240 | |
| | ATCGCCAAT | –153 to –145 | |
| | GGAATCGACTC | –17 to –7 | |
| E4 | TTTCTAT | –962 to –956 | *48* |
| | ATAAAGAATATA | –924 to –913 | |
| E8 | CCGACAAAG | –1241 to –1233 | *48* |
| HRGP | TATTTGTCGT | –188 to –179 | *49* |

of carnation *GST1*, which has been identified as an ethylene-responsive element (*53*). The sequence motif CGTCA which has been shown to be involved in methyl jasmonate-responsive expression for lipoxygenase 1 gene in barley grain (*54*) is found three times in the promoter of GMACS12 at positions −768 to −764, −219 to −215, and −181 to −177. The sequence motif AACCTAA which was shown to be a MYB binding site involved in light responsiveness (*55*) is found at position −593 to −587 in the promoter of GMACS12. At position −550 to −542 the sequence CTACGTGAT is identical with the core consensus element from cor15a gene of *Arabidopsis thaliana* involved in abscisic acid responsiveness (*56*). Furthermore, the sequence motif TTGACC which has been identified as a fungal elicitor-responsive element (*57*) is found twice in the promoter of GMACS12, namely, at positions −527 to −522 and −70 to −65. Besides, a GCC box homologous motif GCGGCC is found at position −209 to −204. The ethylene-responsive factors bind the GCC box (GCCGCC) element that is present in the promoters of many PR genes (*58*, *59*). In all of these cases, their possible involvement in gene regulation and signal transduction remains to be proved.

Large losses of fruits are incurred due to ethylene's effects on plant senescence before they can reach the consumer. Therefore, the ability to manipulate the time of fruit ripening would reduce spoilage during shipping and storage and hence extend the shelf life of fruits (*2*, *3*, *60*). In this report we have characterized a diverse ACC synthase gene family in banana and identified an ACC synthase gene activated during fruit ripening and in ethylene-treated, mature green fruit. Several strategies of genetic engineering could be employed to reach the aim above, such as RNA interference of ACC synthase gene caused by antisense, sense, or hairpin construct (*61*). Furthermore, those constructs could be driven by fruit-specific and ethylene-inducible ACC synthase gene promoter to retard ripening of banana fruits and extend their storage life.

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