Identification of a Genomic Clone to ACC Oxidase from Papaya (*Carica papaya* L.) and Expression Studies[†]

RODOLFO LÓPEZ-GÓMEZ,*,‡ FRANCISCO MORALES-DOMÍNGUEZ,§
OMAR MENDOZA ALCÁZAR,‡ AND MIGUEL ANGEL GÓMEZ-LIM#

Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo,
Edificio B3 Ciudad Universitaria, Morelia, Mich. CP 58060, Mexico; Departamento de Química,
Centro Básico, Universidad Autónoma de Aguascalientes, Avenida Universidad 940,
C.P. 20 100, Aguascalientes, Ags. Mexico; and CINVESTAV−Irapuato,
Km 9.6 carretera Irapuato-León, Apartado Postal 629, Irapuato GTO, Mexico

In this paper are presented structural analysis and expression studies of one genomic clone encoding a 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase) from papaya. Using RT-PCR amplification of ACC oxidase cDNAs from ripe papaya, a product of 800 bp was obtained, which after sequence analysis was found to code for a protein highly homologous to ACC oxidase proteins. This PCR product was used as a probe for screening a genomic library, and two different groups of clones were obtained as indicated by restriction mapping. One clone (CPACCO-1) was selected for further study and fully sequenced. Comparison of this sequence with the PCR product and other cloned ACC oxidase genes revealed that CPACCO-1 encoded the transcript in four exons interrupted by three introns. Southern blot analysis showed one or two major bands hybridized to the PCR probe, suggesting that the ACC oxidase gene is present in one or two copies in the papaya genome. By northern blot analysis it was found that the ACC oxidase transcripts appear in the pulp earlier than in the peel, suggesting a developmental regulation. A wounding experiment revealed the highest expression of this gene by 2 h. Transcriptional regulation by ethylene could be due to the presence of a putative GCC box in the promoter region.

KEYWORDS: Climacteric; ethylene; fruit ripening; papaya; ACC oxidase

INTRODUCTION

Ethylene is synthesized in higher plants from methionine via *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylic acid (ACC). The two key enzymes of the pathway are ACC synthase and ACC oxidase; their roles during fruit ripening have been the focus of research of many groups over several years. In climacteric fruits, such as papaya, the rise in ethylene production parallels the respiration rate and reaches a maximum at the same time as the respiratory climacteric (*1*).

Studies on ethylene production in papaya fruit have focused on measurements of ACC oxidase activity. The highest level of activity was found in the exocarp of 75% ripe fruit (*2*), whereas in mature green fruit, the highest levels of ACC oxidase activity were determined in the placental and dorsal bundle (*3*). The level of ACC, the substrate for ACC oxidase, is initially low in the mesocarp, increasing 3-fold when the peak of ethylene synthesis occurs; this result suggests that at the peak of ethylene synthesis, ACC oxidase activity may be limiting (*4*).

Papaya is a major economic crop in many tropical countries, with a world production of 5,591,692 Mt in 2002 (*5*). The fruit is a valuable export commodity that generates hard currency earnings in many developing countries. Papaya shipments arriving at terminal markets have a range of disorders associated with over-ripeness, mechanical injury, and parasitic diseases (*6*). Papaya fruit are not harvested until the skin color shows some yellowing (*7*); however, by this time the fruit is actively producing ethylene, which shortens the shelf life (*8*).

Storage studies on papaya harvested at different harvest maturities have identified softening as a key quality factor to be controlled (*9*). In this respect, enzymes involved in ethylene synthesis represent key targets for research. The papaya fruit is very susceptible to over-ripening caused by ethylene, and all of the strategies in use today to extend the shelf life of papaya are based on the control of ethylene action and production. The availability of fruits with low endogenous ethylene production might substantially reduce the losses caused by this gas. To aid in our understanding of the control of ethylene production in papaya, we report here the isolation of a genomic sequence for papaya ACC oxidase and its expression during normal ripening and wounding. This is the first reported cloning of a papaya ACC oxidase gene.

* Author to whom correspondence should be addressed [telephone/fax (443) 326-57-88; e-mail lgomez@zeus.umich.mx].
† The nucleotide sequence reported in this paper has been deposited in GenBank under accession no. AF379855
‡ Universidad Michoacana de San Nicolás de Hidalgo.
§ Universidad Autónoma de Aguascalientes.
# CINVESTAV-Irapuato.

Genomic Clone to ACC Oxidase from Papaya

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MATERIALS AND METHODS

Plant Material. Papaya fruits were obtained from a local market. Ethylene production of individual fruits was monitored by GC, and fruits were selected at different ripening stages according to the production of the gas.

PCR and Sequence Analysis. Total RNA was extracted from both peel and pulp at each ripening stage according to the method described by López-Gómez and Gómez-Lim (*10*). Complementary DNA was synthesized from pulp using reverse transcriptase (AMV, Roche Molecular Biochemicals). Sequences for papaya ACC oxidase were amplified from cDNA by Polymerase Chain Reaction (PCR), using degenerate primers for ACC oxidase 5′ GC(A/T/G/C) TG(C/T) GA-(A/G) AA(T/C) TGG GG(G/A/C/T) TT 3′ forward and 5′ AA(A/G) TT(C/T) CA(G/A) GC(A/C/G/T) AA(A/G) GA(A/G) 3′ reverse (*11*). A PCR product of appropriate length, 816 bp, was cloned into the PCRII vector (InvitroGen, Carlsbad, CA), and it was sequenced using the Sequenase enzyme according to the manufacturer's instructions (United States Biochemical), with deoxyadenosine 5′-[α-(^{35}S)thio]triphosphate (Amersham). The entire cloned PCR product was sequenced on both strands.

The DNA sequence of the ACC oxidase cDNA clone thus identified, cCPACO-1, was further characterized using the Sequence Analysis software package of the Genetics Computer Group (University of Wisconsin).

Library Construction and Screening. A papaya genomic library was constructed with DNA isolated from green leaves in the Lambda EMBL3 vector (Stratagene, Palo Alto, CA) using the procedures of Ausubel (*12*) and the Stratagene protocol. Approximately 2×10^5 primary plaques from the genomic library were plated, blotted, and hybridized with the ^{32}P-labeled insert from the ACC oxidase cDNA clone (cCPACCO-1). Three successive hybridizations identified several positive clones, from which DNA was isolated and analyzed by restriction mapping. The genomic clone, CPACCO-1, corresponding to the cDNA clone, was subcloned into pBluescript (Stratagene), and the coding and flanking regions were sequenced and analyzed as before.

RNA Isolation and RNA blots. Total RNA was extracted from papaya fruit tissue according to the method described by López-Gómez and Gómez-Lim (*10*). Samples of total RNA (10 μg) were subjected to electrophoresis in a formaldehyde-containing gel, followed by transfer to a Hybond-N membrane (Amersham) and hybridization to the ^{32}P-labeled cCPACCO-1 insert (*13*). The insert was labeled to a specific activity of 10^8-10^9 cpm/μg by random priming (Gibco BRL). Blots were washed at 65 °C two times (15 min) at high stringency (1 mM Na$_2$EDTA, 40 mM NaHPO$_4$, 1% SDS) and subsequently autoradiographed at -70 °C using Kodak XAR-5 film.

For the wounding experiment disks of ~1 cm in diameter were cut from papaya leaves, soaked in 0.4 M mannitol, and incubated for 15 min in 3 mL of 100 mM ACC in 0.4 M mannitol. After the incubation, the ACC solution was removed and the tissue was incubated for an additional 0, 6, or 24 h at 30 °C. At the end of each interval, ethylene production was monitored. The tissue was subsequently frozen and RNA extracted as described before.

DNA Hybridization. Papaya genomic DNA was isolated from young leaves as described by Dellaporta et al. (*14*). Ten micrograms of DNA was digested with the appropriate restriction enzymes and electrophoresed in a 0.8% agarose gel. The gel was blotted to a Hybond-N membrane (Amersham) and hybridized to the labeled insert of cCPACCO-1 at 65 °C using the phosphate buffer method (*12*). Blots were washed at 65 °C two times (15 min) at high stringency (1 mM Na$_2$EDTA, 40 mM NaHPO$_4$, 1% SDS). The blots were subsequently autoradiographed at -70 °C using Kodak XAR-5 film using intensifying screens.

RESULTS

Ethylene Production in Papaya. Ethylene production in whole fruit is negligible in preclimacteric papaya but increases until reaching a value of ~7.5 μL kg $^{-1}$ h^{-1}, and then it declines as ripening progresses (data not shown). On the other hand, ethylene production by wounded leaves is clearly detectable by
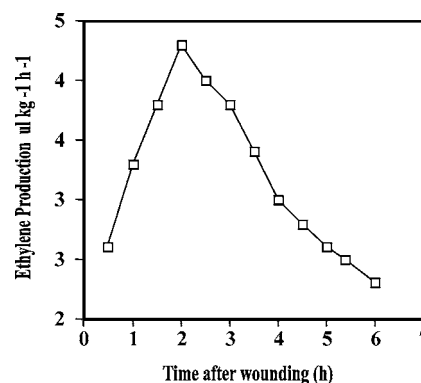

Figure 1. Pattern of ethylene production by papaya leaf disks. Disks of ~1 cm in diameter were cut from young papaya leaves, weighed, and placed in a vacuum flask. The disks were infiltrated for 2 min under vacuum with a solution containing 10 mM amino oxyacetic acid (AOA) and 10 mM ACC in 100 mM sodium phosphate, pH 5. The disks were then transferred to sealed tubes for 15 min, and a 1 mL sample was removed from the headspace and analyzed by GC. The tubes were flushed with air, and after 1 h, the tubes were sealed for 15 min and a sample was removed as before. This procedure was repeated every 30 min.

30 min, reaching a value of ~48 μL kg $^{-1}$ h^{-1} by 2 h and then declining to basal levels (**Figure 1**).

Cloning of Papaya ACC Oxidase and Organization in the Genome. PCR amplification of fruit pulp cDNA, with degenerate primers corresponding to conserved regions of other ACC oxidase genes, yielded a product of appropriate length. Sequencing of the 816 bp product revealed (cCPACCO-1) high identity to the ACC oxidase genes (data not shown).

To study the structural features controlling expression of CPACCO-1 during papaya fruit ripening, a papaya genomic library was prepared to allow isolation of the corresponding genomic clone. Three successive hybridizations with the cCPACO1 insert yielded three positive plaques; restriction mapping revealed they were different. One of them, clone CPACCO-1, was subcloned in pBluescript and sequenced. Analysis of the primary structure of the pCPACCO-1 indicated that the clone of 4911 bp contained the whole coding region of papaya ACC oxidase for 318 amino acid residues, interrupted by three introns at positions 1435−1562, 1791−1941, and 2275−2708 (**Figure 2**).

When comparing the amino acid sequence of the coding region of CPACCO-1 with those of other ACC oxidases reported previously, we found homology values of 79.87% with the melon and pyrus fruits ACC oxidases and 66.98% with the papaya gene isolated by Lin et al. (*28*) (**Figure 3**).

Southern blot analysis of genomic DNA was performed to determine the number of ACC oxidase genes in the papaya genome. Papaya genomic DNA digested with *Eco*RI, *Bam*HI, and *Hin*dIII was hybridized to the 816 bp PCR fragment. The probe hybridized to a band of ~4 kb in the *Eco*RI and *Hin*d III digestions and to two fragments of about 3.6 and 2.5 kb in the *Bam*HI/*Hin*dIII double digestion (**Figure 4**). These results suggest that ACC oxidase may be encoded by more than one gene and possibly two in the papaya genome.

Flanking Sequences of Papaya ACC Oxidase. The ATG at position 1330 is likely to be the translation initiation codon because the nucleotide sequence flanking that conforms to the consensus for translation start sites in higher plants (*15*); furthermore, the high homology of CPACCO-1 to other identified ACC oxidase clones starts at the stated methionine. There is a putative TATA box found at position 1104, but we do not

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   1 gagtatttaattaaaaatgccgtatgagaaggaattagaggtgtacgtatatgtgcatatatatatatatatac
  75 acatattatatgtaagtaaatgcagtggaagaattagagagagagacattatattaaatgaattaagcagctaaa
 152 agccacᴄcaat｜ggtgttgttttgtcaactttctcaacgatgaatatttatagaattaaagagagattgaagattc
 226 agggagtcacctcaattgtgggtccactttacacaatatatacatacgtgtgtatatatatatatatatatata
 300 tatgtatacacacatacattaccccctatctcaagttcaaattatatctttaattttactatttaaaattttttt
 374 aaattaataaaaatgaagatttttgagtattttttatacaaatatattttttaaagttgaatttaaactaaatta
 448 aattaattttttaatttataattaaaaaaattaataaatataaaccaaatatttatatatatatatatgcatt
 522 aattcagaatattatgagttaaatagaatattagtcgtagaaaaatgaatgtttggaggagagataagactgaa
 596 gaaaagaaaaaaagggaaaaagtaaagggggcacagatcacaagcagatgacgatatggtaacaggcaggacaa
 670 aagaggtaggggtccgacgggtgtgatgtacatacatgctccacgattcactgcttttttcttttttacaaactt
 744 atatttctcttattatatatatatatatatgtatgtacacacacacatatgtatatgtatatgtatatgt
 818 atgtgtacatgttcttgccttctgtgagtgttggcagtggcagaaagaaagtctttagtcaaagtttaccacac
 892 ｜caat｜tgtatgcactggtttgatagtcatgaggcaaaaagacttgtattttgtatgaaatgtttggaataattttt
 966 gtgttatactaattaaaatcataatataatataatatagatgatatgtatacaataaagagtaggaggctattg
1040 ggggaattccattattacccctttgaagctctttcattttactaaattgacattcaaattttggc｜tata｜aattc
1113 caagttcccttcatcaccattacacaaacaccaccacaggacagcaacactgttttctaactcccccagaaaa
1186 agggaaagacaagagcagtgtcaacc｜caat｜agt｜taaagagc｜aaaccttctccttcttctttgtttgattgaa
1259 aaaaagagaaaagcaaaaaacagggagagatttagcagacaagaaagaaagaaagaaagaaagaaagtttta
1330 ATGGAGAACTTCCCAGTTATTGATTTGTCAAAGCTCAATGGAGAAGAGAGAGCATTAACCATGGAG
          M   E   N   F   P   V   I   D   L   S   K   L   N   G   E   E   R   A   L   T   M   E
1396 TTGATCCATGATGCCTGTGAAAACTGGGGCTTCTTTGAGgtatttctaaataaaccatctataacttaca
          L   I   H   D   A   C   E   N   W   G   F   F   E
1466 tttttacacacacacacacacacgctcatgtatatgtatagttatgtatatgtgagtagcagctgaaa
1536 ctgaatgtttgattttggtttttgaaagTTGGTGAACCATGGGATCTCTCATGACCTGATGGACACTGTGG
                                       L   V   N   H   G   I   S   H   D   L   M   D   T   V   E
1606 AGAGGCTGACAAAGGAGCATTACATGAAGTGTATGGAGCAGAGATTCAAAGAAATGGTGGAAAGTAATGG
          R   L   T   K   E   H   Y   M   K   C   M   E   Q   R   F   K   E   M   V   E   S   N   G
1676 TCTTGAGGCTGTTCAGTCTGAAATCAATGATATGGATTGGGAAAGTACCTTCTTCTTGCGCCATCTTCCA
          L   E   A   V   Q   S   E   I   N   D   M   D   W   E   S   T   F   F   L   R   H   L   P
1746 GCTTCAAACATGCATGAAATTCCTGATCTTGAAGATGACTACAGGttccccttctctcccaagtccatat
          A   S   N   M   H   E   I   P   D   L   E   D   D   Y   R
1816 tttttttaatctgattgatgaaaatatcgaaaacccagaagaaaaaaatgaaattagagaaatgggttct
1886 gttgtttttcaagtttctgatttgggcttttctgggttgattgtgatgaaaacaggAAGGCAATGAAGGA
                                                                      K   A   M   K   E
1956 GTTTGCAGTGGGGCTGCAGAAACTTGCAGAGCAAATGTTAGACTTGTTGTGTGAGAATCTTGGGTTAGAG
          F   A   V   G   L   Q   K   L   A   E   Q   M   L   D   L   L   C   E   N   L   G   L   E
2026 AAAGGGTATTTGAAGAAAGTATTTTATGGGTCAAAGGGTCCTAATTTTGGGACAAAGGTTAGCAACTATC
          K   G   Y   L   K   K   V   F   Y   G   S   K   G   P   N   F   G   T   K   V   S   N   Y   P
2096 CTCCATGTCCTAAACCAGATCTTATCAAGGGACTCAGAGCCCACACAGATGCAGGTGGCATCATCTTGTT
          P   C   P   K   P   D   L   I   K   G   L   R   A   H   T   D   A   G   G   I   I   L   L
2166 GTTCCAAGATGACAAGGTCAGTGGCCTCCAGCTCCTCAAGGATGACCAGTGGGTTGATGTCCCTCCCATG
          F   Q   D   D   K   V   S   G   L   Q   L   L   K   D   D   Q   W   V   D   V   P   P   M
2236 AAACATTCCATTGTCATCAACCTTGGTGATCAACTTGAGgtatacatttttttaattataaagattatta
          K   H   S   I   V   I   N   L   G   D   Q   L   E
2306 tatttgtggtgtatattatatatatcacccacataagcaaattcttaacttgggtttaattatattatat
2376 tggtaattaacttcttaacaatattattcctgtaatgatgtgatgctatttgtattattatacatgtcac
2446 ttgctcttagatttactgtaaaccccgtaaagctgcttttctatattaaaggggtccattttcctttttg
2516 tttaagcaacttaaggaatatattgagattttctatgcaataatttctgggttcctttctggttcagcat
2586 tgtccccttctgtactataaaagagaatccaaataggaactaattttttttttatttgaatttgataaa
2656 attttaaatcactaacattcttattattattatttttttggttgtttatatagGTGATTACTAACGGTAA
                                                                  V   I   T   N   G   K
2726 ATACAAGAGTGTAATGCACAGAGTTATAGCACAGACAGATGGGAACAGAATGTCACTAGCCTCATTCTAC
          Y   K   S   V   M   H   R   V   I   A   Q   T   D   G   N   R   M   S   L   A   S   F   Y
2796 AATCCTGGAGATGATGCTGTGATCTACCCAGCACCATCTCTGGTAGAGAAAGAAGCAGAGAAGAATCAGA
          N   P   G   D   D   A   V   I   Y   P   A   P   S   L   V   E   K   E   A   E   K   N   Q   I
2866 TTTACCCAAAATTTGTGTTTGATGATTACATGAAACTTTATGTTGGGTTGAAATTTCAGGCTAAGGAGCC
          Y   P   K   F   V   F   D   D   Y   M   K   L   Y   V   G   L   K   F   Q   A   K   E   P
2936 AAGATTTGAAGCCATGAAAGCCATGGAGTCTACTGTAACTCCTGGTGCCATTGCAACTGTTTGAaagaaa
          R   F   E   A   M   K   A   M   E   S   T   V   T   P   G   A   I   A   T   V   *
3006 gaagaaaaaaccccttcttagaaactcaaaaaaaggaaagctgggtttttactcttttatgtgttgggtgtg
3076 tttgtgttggtgaaaattaatactacaaaaatgatgtggctttttttttcttactgttcttttacctttttag
3146 tgtcaaggaattatgccctaatcatggtgtttggaagttgattggcttagcaaggaacttactagcttaa
3216 ｜aataa｜ccctatgggtttctgttgtgctttctgtgtgtaatgtattctgtctagtggcctttggtgggttt
3286 ctgttgtataaagtaataattttaagattctaattatgctttttcttttaagtatgatttgttttatcatg
3356 tctttccaacttaatttaatgtttaattgattcatattaattattactatacgtataaaatatcgataaa
3426 aaataaaatattttttaaaatacaaatgagtcgatgaactttataaatataattgatttttatcatatttat
3496 aaagtttgtcaatttatttatattttttaaaatacttaaaagttaattgattattattattttttgaaata
3566 aaaaaaaatttaagtcaattcttttaacccgttatgattatagagtgttctttttcatctaagtacgtcat
3636 aattaattggaatcaacaaagaccaacttggtcacgacaactctaccaacacattgaactaagttcttaa
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3706  ttttgtcaaatcaaatatataaaccaacgattaccaacctataatacactcacagagtaataagttctat
3776  attttcaataatataaaaaatttaacaactaaactgtcaagtcgaaccaaccactgtcaacatataatac
3846  attcacaaaataataagttcgatactctcaataaaatttaactttcaaacatatgaaccaacttataata
3916  actactgtcaacctaaaatacactaagctcttaagtttgttaaatcaaacatacgaatcaactactgtta
3986  acatataacacactcacaaattaacaagttctgtattctcagtaaaattcaaactcgtaattctcaaata
4056  taatataaaaaatttaacatctaaaactatcacgttgatatgctaaaagtgtttcggagcattttgctaa
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4196  agtatggaaaaatagccttctacttttttgttgagaaggaaatgttcttgatgcttcatcttcttccttt
4266  tcttatttctttttttaattttttctacacaaattctttttatcctcatctgcaaatttgttggcctcatc
4336  tcatcaaaccagtttcagaaagtggaatactgattgaatccacttcaacaccaccaagcaaatcttgtgg
4406  tgacatcaaatttctacatatatttatttgattttttcaaagtgaaaatctatatatattatatggtttag
4476  ataaatatatatacattaatcataactacaatcatctaatgagaacccatgagctgtaccaataaaaaaa
4546  gaagatgaaaataaatactgatagctcttattcacatccacatgcacatacaaaatgtgacttatacgtc
4616  tgcatagagaatgtggtagtcaattttttttccccccttaaagaagttcatattcgggggcaaacattactt
4686  gtacagtgtatgtatgtatatgaatggatatgtaaaagcactgaggcaagtaccaatagttagatgagaa
4756  taagggccaccagcatctcagaaacaaaatagacaagagcaagtacgaagtgtagctttcaatatagacc
4826  accagagtaagtgattcaagagaacaatatgatctctttcagataacgatggcctgtttggaatgagaga
4896  aaagaaaggaaaagga
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Figure 2. Complete nucleotide and deduced amino acid sequence of the genomic clone of CPACO1. The coding sequence is capitalized, whereas the introns and 5′ and 3′ untranslated regions are indicated in lower case. The three introns are between the position 1435–1562, 1791–1941, and 2275–2708 nucleotides. The derived amino acid sequence is presented below the DNA sequence. The asterisk indicates the stop codon. The CAAT boxes, the putative GCC box, and the pseudo TATA box are indicated in boxes. The possible polyadenylation site is in a box, too. Partial cDNA sequence obtained by RT-PCR is underlined.

Figure 3. Alignment of predicted CPACO 1 protein with ACC oxidase (ACCO) from other plants; all similar amino acids are boxed. The protein sequences shown in this diagram are listed in the GenBank database under the following accsession numbers: ACCO papaya (CPACCO-1) (AF379855), apple (Q00985), banana (CAA11200), muskmelon (P54847), peach (S41880), and papaya (L76283).

believe that this is a bona fide TATA box. The entire 5′ flanking sequence was examined for elements thought to regulate expression of other ethylene-regulated genes, such as chitinase (*16*), E4 (*17*), Eth1 (*18*), and ACO1 (*19*). There was little similarity to the 5′ flanking sequences of the different genes analyzed; however, we found a putative GCC box (TAAA-GAGCC) at position 1219. This element has been shown to be an ethylene responsive motif that is both necessary and sufficient for the regulation of transcription by ethylene (*20*, *21*). We also found three CCAAT boxes at positions 157, 891, and 1210. These boxes are commonly found in the 5′ noncoding region of many eucaryotic genes (*22*). Furthermore, we also detected the sequence TATTTAAT at position 4, which belongs to the cis regions that have been previously identified as controlling the spatial or developmental specificity of the expression of some genes (*23*).

ACC Oxidase Expression during Papaya Fruit Ripening and Wounding. To determine the pattern of ACC oxidase expression during papaya fruit ripening, gel blot analysis of total RNA isolated from papaya peel and pulp from naturally ripened fruit at different ripening stages was performed. **Figure 5** shows the RNA blot probed with the cCPACCO-1 fragment. In preclimacteric fruit, the ACC oxidase message is detectable at very low levels in the peel is highly abundant in the pulp. The message becomes quite noticeable in the peel of climacteric fruit, which also contains high levels of ACC oxidase mRNA in the pulp. In postclimacteric fruit the message is detectable both in the peel and in the pulp, but the levels are clearly lower than in climacteric fruit.

We were interested in determining whether the gene that we had identified was fruit-specific. To that end we decided to use a 478 bp fragment from the 3′ untranslated regions as a gene-
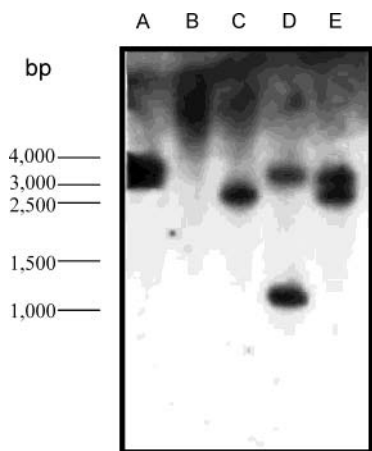
Figure 4. Genomic Southern blot analysis of papaya ACC oxidase. High molecular weight DNA was purified from papaya leaves, digested with *Eco*R1 (**A**), *Bam*HI (**B**), *Hin*dIII (**C**), *Eco*RI–*Bam*HI (**D**), or *Eco*RI–*Hin*dIII (**E**), fractionated on a 0.7% agarose gel, and blotted to a Hybond-N membrane (Amersham). The membrane was hybridized to the labeled cCPACO1 fragment at 65 °C for 16 h. Blots were washed at 65 °C at high stringency (1 mM Na$_2$EDTA, 40 mM NaHPO$_4$ pH 7.2, SDS 1%) two times for 15 min each and subsequently autoradiographed at −70 °C. The numbers on the left indicate molecular weight markers.
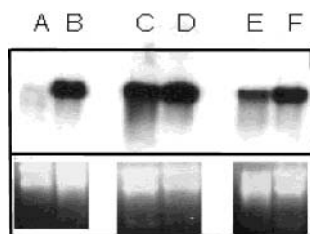

Figure 5. RNA blot showing the level of the CPACO1 transcript at different ripening stages: (**A**) RNA from peel at preclimacteric stage; (**B**) RNA from pulp at preclimacteric stage green fruit; (**C**) RNA from peel at climacteric stage; (**D**) RNA from pulp at climacteric stage; (**E**) RNA from peel at postclimacteric stage; (**F**) RNA from pulp at postclimacteric stage; (top) hybridized filter; (bottom) agarose gel stained with ethidium bromide. Total RNA was isolated from papaya pulp and peel at the ripening stages indicated and separated by formaldehyde agarose gel electrophoresis (10 μg), followed by transfer to a Hybond-N membrane (Amersham) and hybridization to ^{32}P-labeled cCPACO1 as described in **Figure 3**.

specific probe. Although most members of the ACC oxidase gene family are highly homologous throughout the protein coding regions, they show a degree of sequence divergence within the 3′ untranslated region. This approach has been successfully used before to study differential expression within a gene family (*24*). Total RNA was extracted from papaya root, stem, leaf, peel from preclimacteric fruit, pulp from preclimacteric fruit, peel from climacteric fruit, and pulp from climacteric fruit, and a gel blot analysis was performed using the 478 bp fragment.

As **Figure 6** illustrates, there is a strong signal in mRNA from climacteric fruit (pulp and peel) and there is hardly any in RNA from other tissues.

To study further the expression of ACC oxidase, we analyzed the levels of the ACC oxidase message in wounded tissue. Papaya leaves were sectioned with a scalpel and incubated for different periods of time; total RNA was then extracted, blotted, and probed with cCPACCO-1. As shown in **Figure 7**, a strong signal was detectable at 2 h after wounding, and at 6 h this signal was reduced. A fair message was detectable at 0 h.
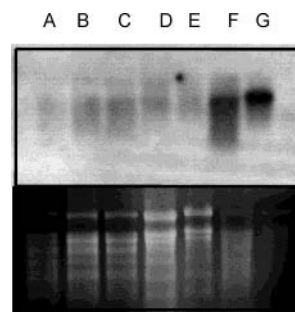

Figure 6. RNA blot showing the level of the CPACO1 transcript in different papaya organs: (**A**) RNA from root, (**B**) stem, (**C**) young leaf, (**D**) old leaf, (**E**) flower, (**F**) peel at postclimacteric stage, and (**G**) pulp at climacteric stage; (top) hybridized filter; (bottom) agarose gel stained with ethidium bromide. Total RNA was isolated from the organs indicated and separated by formaldehyde agarose gel electrophoresis (10 μg), followed by transfer to a Hybond-N membrane (Amersham) and hybridization to a ^{32}P-labeled 478 bp fragment from the 3′ untranslated regions contained in a *Hin*dIII fragment, as described in **Figure 3**.
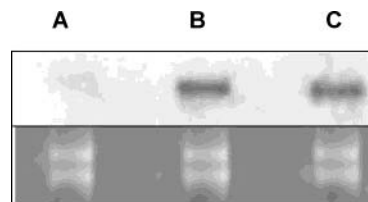

Figure 7. RNA blot showing the level of the CPACO1 transcript after mechanical wounding: (**A**) 0 h; (**B**) 2 h; (**C**) 4 h; (top) hybridized filter; (bottom) agarose gel stained with ethidium bromide. Papaya leaves were sectioned with a scalpel and incubated for different periods of time. Subsequently, total RNA was extracted, blotted, and probed with cCPACO1, as described in **Figure 5**.

DISCUSSION

In this paper we report the cloning, expression, and structural characterization of a gene encoding ACC oxidase in papaya. The expression of this gene was correlated with ethylene production in fruit and wounded tissue. During papaya fruit ripening there is an increase in ethylene production, and we have shown that this increase is associated with increased ACC oxidase activity and transcript level. We could see a dramatic increase in the messenger signal during the highest ethylene production. An examination of the ACC oxidase message level in the peel and pulp revealed that the message is detectable in the pulp at all times, although with different intensities. The message is detectable in the peel at very low levels in preclimacteric fruit and at very high levels in climacteric and postclimacteric fruit. These results suggest that in papaya fruit ripening, the ACC oxidase message is developmentally regulated and that ripening proceeds from inside out, as in bananas (*11*). These results are in agreement with the carotenoid development (*1*) and tissue softening (*25, 26*) during papaya fruit ripening.

Wounding papaya leaves also induced the expression of ACC oxidase message, which was more abundant by 2 and 6 h after wounding. This period corresponds to the time of highest wound ethylene production in leaves (*27*).

From the experiment using the 3′ untranslated region, we found faint signals in all of the tissues probed. We do not believe this to be bona fide hybridization, and therefore it is likely that our gene is expressed only in the fruit in the presence of ethylene and during development.

The positions and numbers of introns in papaya ACC oxidase gene are identical to those in carnation, tomato, and banana genes previously characterized.

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We found a putative TATA box at position 1104, but we do not believe that this is a bona fide TATA box. It is too far from the translation initiation codon; normally, the TATA box is located <40 bp from that codon (*28*). Furthermore, it does not conform to the consensus TATA box region (*15*). We believe that CPACCO-1 does not contain a TATA box. There are 130 identified genes lacking a TATA box (*28*). Therefore, the fact that our gene does not present one should not be surprising. The CAAT box found, on the other hand, does conform to the consensus region in terms of homology and distance to the translation initiation codon. The putative GCC box found in the 5′ flanking region of this gene is intriguing because it suggests that another point of regulation is at the transcriptional level by ethylene. This sequence has 8 of the 11 bases reported for the classical GCC box (TAAGAGCCGCC) (*20*, *21*).

From sequence analysis we found that the identified gene contains a complete open reading frame of a 318 amino acid polypeptide. This sequence has an identity of 78% with the previous papaya ACC oxidase cDNA reported by Lin et al. (*29*) and presents all 12 residues conserved among all of the ferrous ion and ascorbate requiring superfamily of enzymes (*30*). These two genes isolated from papaya fruit suggest the participation of at least two ACC oxidase genes during papaya fruit ripening. This has been suggested indirectly by the biphasic kinetic behavior of the EFE enzyme inactivation in papaya exocarp during papaya heat treatment (*31*).

That southern blot analysis revealed at least two bands suggests the existence of a small multigene family with at least two ACC oxidase genes in papaya, as in mung bean (*32*), tomato (*33*), and *Petunia hybrida* (*30*).

Because the two ACC oxidase papaya genes isolated to date come from the fruit, these genes are probably involved in the papaya fruit ripening process, as in tomato (*24*).

Considering that papaya fruit suffers postharvest losses of up to 75% (*34*), the modification of papaya by genetic engineering may have a dramatic impact on the commercialization of the fruit. Experiments are in progress to produce papaya fruit with delayed ripening.

LITERATURE CITED

(1) Paull, R. E.; Chen, N. J. Postharvest variation in cell wall degrading enzymes of papaya (*Carica papaya*) during ripening. *Plant Physiol.* **1983**, *72*, 382−385.

(2) Chan, H. T.; Couey, H M.; Sakay, W. S. Distribution of the ethylene forming enzyme in ripening *Carica papaya. Proceedings of the 7th World Congress of Food Science and Technology*, Singapore, Sept 29, 1987; International Union of Food Science and Technology: Ontario, 1990.

(3) Paull, R. E. Pineapple and papaya. In *Bichemistry of Fruit Ripening*; Seymour, G., Taylor, J., Tucker, G., Eds.; Chapman and Hall: London, U.K., 1993; pp 291−323.

(4) Paull, R. E.; Chen, N. J. Heatshock response in field-grown ripening papaya fruit. *J. Am. Soc. Hortic. Sci.* **1990**, *115*, 623−631.

(5) FAO. 2003; http://apps.fao.org/cgi-bin/nph-db.pl?subset=agriculture.

(6) Capellini, R. A.; Ceponis, M. J.; Lighter, G. W. Disorders in apricot and papaya shipment to New York market 1972−1985. *Plant Dis.* **1988**, *72*, 366−368.

(7) Akamine, E. K.; Goo, T. Relationship between surface color development and total soluble solids in papaya. *HortScience* **1971**, *6*, 567−568.

(8) Akamine, E. K.; Goo, T. *Simulated Hawaii Papaya Surface Shipping Test*; Departmental Paper 45; Hawaii Agricultural Experiment Station, University of Hawaii: 1977; p 9.

(9) Paul, R. E. Pineapple and papaya. In *Biochemistry of Fruit Ripening*; Seymour, G., Taylor, J., Tucker, G., Eds.; Chapman and Hall: London, U.K., 1993; pp 291−323.

(10) López-Gómez, R.; Gómez-Lim, M. A. A method for extracting intact RNA from fruits rich in polysaccharides using ripe mango mesocarp. *HortScience* **1992**, *27* (5), 440−442.

(11) López-Gómez, R.; Campbell, A.; Dong, J. G.; Yang, S. F.; Gómez-Lim, M. A. Ethylene biosythesis in banana fruit: Isolation of a genomic clone to ACC oxidase and expression studies. *Plant Sci.* **1997**, *123*, 123−131.

(12) Ausubel, F. M.; Brent, R.; Kingston, R. E.; More, D. D.; Seidman, J. G.; Smith, J. A.; Sthul, K. *Current Protocols in Molecular Biology*; Greene Publishing Associates and Wiley: New York, 1991; pp 5.3.2−5.3.8.

(13) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: a Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; pp 7.43−74.5.

(14) Dellaporta, S. L.; Wood, J.; Hicks, J. B. A plant DNA minipreparations: Version II. *Plant Mol. Biol.* **1983**, *1*, 19−21.

(15) Joshi, C. P. An inspection of the domain between putative TATA box and translation start site in 79 plant genes. *Nucleic Acids Res.* **1987**, *15*, 6643−6653.

(16) Broglie, K. E.; Biddle, P.; Cressman, R.; Broglie, R. Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tabacco. *Plant Cell* **1989**, *1*, 599−607.

(17) Montgomery, J.; Goldman, S.; Deikman, J.; Margosian, L.; Fischer, R. L. Identification of an ethylene-responsive region in the promoter of a fruit ripening gene. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5939−5943.

(18) Holdsworth, M. J.; Schuch, W.; Grierson, D. Nucleotide sequence of an ethylene-related gene from tomato. *Nucleic Acids Res.* **1987**, *15*, 10600.

(19) Tang, X.; Gomes, A. M. T. R.; Bhatia, A.; Woodson, W. R. Pistil specific and ethylene-regulated expression of 1-aminocyclopropane-1-carboxylate oxidase genes in petunia flowers. *Plant Cell* **1994**, *6*, 1227−1239.

(20) Ohme-Takagi, M.; Shinshi, H. Ethylene-inducible DNA binding proteins that interact with ethylene-responsive element. *Plant Cell* **1995**, *7*, 173−182.

(21) Shinshi, H.; Usami, S.; Ohme-Takagi, M. Identification of an ethylene-responsive region in the promoter of a tabacco class 1 chitinase gene. *Plant Mol. Biol.* **1995**, *27*, 923−932.

(22) Hanley, B. A.; Schuler, M. A. Plant intron sequence: evidence for distinct groups of introns. *Nucleic Acids Res.* **1988**, *16*, 7159−7175.

(23) Forde, B.; Freeman, J.; Oliver, J. E.; Pineda, M. Nuclear factor interacts with conserved A/T-rich elements upstream of a nodule-enhanced glutamine synthetase gene fom French bean. *Plant Cell* **1990**, *2*, 925−939.

(24) Barry, C. S.; Blume, B.; Bouzayen, M.; Cooper, W.; Hamilton, A. J.; Grierson, D. Differential expression of the 1-aminocyclopropane-1-carboxylate oxidase gene family of tomato. *Plant J.* **1996**, *9* (4), 525−535.

(25) Chan, H. T., Jr.; Tam, S. Y. T.; Seo, S. T. Papaya polygalacturonase and its role in thermally injured ripening fruit. *J. Food Sci.* **1981**, *46*, 190−193.

(26) Lazan, H.; Ali, Z. M.; Liang, K. S.; Yee, K. L. Polygalacturonase activity and variation in ripening of papaya fruit with tissue depth and heat treatment. *Physiol. Planta.* **1989**, *77*, 93−98.

(27) Yang, S. F.; Pratt, H. K. The physiology of ethylene in wounded plant tissues. In *The Biochemistry of Wounded Plant Tissues*; Khal, G., Ed.; de Gruyter: New York, 1978; pp 595−621.

(28) Shahmuradov, I. A.; Gammerman, A. J.; Hancock, J. M.; Bramley, P. M.; Solovyev, V. V. PlantProm: a database of plant promoter sequences *Nucleic Acids Res.* **2003**, *31*, 114−117.

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(29) Lin, C. T.; Lin, M. T.; Shaw, J. F. Cloning and characterization of a cDNA for 1-aminocyclopropane-1-carboxylate oxidase from papaya fruit. *J. Agric. Food Chem*. **1997**, *45*, 526−530.

(30) Tang, X.; Wang, H.; Brandt, A. S.; Woodson, W. R. Organization and structure of the 1-aminocyclopropane-1-carboxilate oxidase gene family from *petunia hybrida*. *Plant Mol. Biol*. **1993**, *23*, 1151−1164.

(31) Chan, H. T., Jr. Ripeness and tissue depth effects on heat inactivation of papaya ethylene-forming enzyme. *J. Food Sci*. **1991**, *59* (4), 996−998.

(32) Kim, W. T.; Yang, S. F. Structure and expression of cDNAs encoding 1-aminocyclopropane-carboxylate oxidase homologs isolated from excised mung bean hypocotyls. *Planta* **1994**, *194*, 223−229.

(33) Bouzayen, M.; Cooper, W.; Barry, C.; Zegzouti, H.; Hamilton, A. J.; Grierson, D. EFE multigene family in tomato plants: Expression and characterization. In *Cellular and Molecular Aspects of the Plant Hormone Ethylene*; Peach, J. C., Latche, A., Balague, C., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1993; pp 76−81.

(34) Paull, R. E.; Nishijima, W.; Reyes, M.; Cavaletto, C. Postharvest handling and losses during marketing of papaya (*Carica papaya* L.). *Postharvest Biol. Technol*. **1997**, *11*, 165−179.