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Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits

(ethylene biosynthesis/*Lycopersicon esculentum* Mill./fruit ripening/wound stress)

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ABSTRACT **1-Aminocyclopropane-1-carboxylate synthase (ACC synthase; *S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) is the regulated enzyme in the biosynthetic pathway of the plant hormone ethylene. A full-length cDNA encoding this enzyme has been cloned from tomato fruits [Van Der Straeten, D., Van Wiemeersch, L., Goodman, H. M. & Van Montagu, M. *Proc. Natl. Acad. Sci. USA* (1990) 87, 4859–4863]. We report here the complete nucleotide and derived amino acid sequences of a cDNA encoding a second isoform of ACC synthase from tomato fruits. The cDNAs coding for both isoforms contain highly conserved regions that are surrounded by regions of low homology, especially at the 5′ and 3′ ends. Gene-specific probes were constructed to examine the expression of transcripts encoding the two ACC synthase isoforms under two conditions of enhanced ethylene formation—namely, during fruit ripening and in response to mechanical stress (wounding). The level of mRNA encoding both isoforms, ACC synthase 1 and 2, increased during ripening. In contrast, wounding caused an increase in only the level of mRNA coding for ACC synthase 1. Blot analysis of genomic DNA digested with restriction enzymes confirmed that ACC synthase 1 and 2 are encoded by different genes.**

Ethylene is involved in the regulation of many developmental and stress-induced processes in plants (1). These include fruit ripening, flower fading, promotion of growth in semiaquatic plants, and the synthesis of defense-related proteins in response to infection. Ethylene is synthesized from L-methionine via *S*-adenosyl-L-methionine and 1-aminocyclopropane-1-carboxylic acid (ACC; ref. 2). The rate-limiting enzyme of this pathway is, in most known instances, ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14; for a review see ref. 3). ACC synthase was first identified in homogenates of tomato pericarp tissue (4, 5). It was purified from such homogenates by chromatography and immunoadsorption and was shown to be a 50-kDa protein by SDS/PAGE (6, 7). A variety of chemical and environmental factors such as physical wounding, chilling, noxious chemicals, and the plant hormone indole-3-acetic acid stimulate ethylene formation by enhancing the activity of ACC synthase (3). One of the important problems regarding the regulation of ethylene biosynthesis concerns the question of whether diverse stimuli control the expression of one ACC synthase gene or whether there are several ACC synthase genes, each of which is regulated by a specific type of stimulus. A systematic analysis of enzyme preparations from wound-induced tomato pericarp tissue by SDS/PAGE and isoelectric focusing did not show size or charge variants of ACC synthase (8). However, sequencing of a tryptic fragment containing the active site of tomato ACC synthase provided evidence for the existence of two isoforms that differed, in this particular peptide, by one amino acid (9). The expression of two ACC synthase genes in wound-induced tomato pericarp tissue was also indicated by the isolation of two different cDNA clones encoding this enzyme (10). One of these cDNA clones contained the entire coding sequence of ACC synthase, whereas the other was a partial clone consisting of 420 base pairs (bp). In this paper, we report on the cloning of a cDNA encoding the entire amino acid sequence of the second ACC synthase isoform from tomato fruits and on the differential expression of the two ACC synthase genes in ripening and wound-induced tomato pericarp tissue.§

MATERIALS AND METHODS

Plant Material. Tomato plants (*Lycopersicon esculentum* Mill., cv. Duke) were grown in a greenhouse. For RNA determinations and enzyme assays, pericarp tissue was either frozen in liquid N_2 directly upon isolation and stored at −80°C until extraction or finely chopped with a razor blade (= wound induction) and incubated on moist filter paper in the dark at room temperature before freezing.

ACC Synthase Isolation and Enzyme Assays. Extraction, isolation, and assay of ACC synthase were according to Bleecker *et al.* (6). The enzyme was purified through the preparative ion-exchange chromatography step, followed by immunoaffinity purification.

Protein Sequencing. Since the immunopurified protein was amino-terminally blocked, it was subjected to CNBr fragmentation (≈40 μg of protein in 0.2 ml of 0.1 M HCl containing 15 mg of CNBr at room temperature for 15 h). The resulting polypeptides were separated by a *N*-tris(hydroxymethyl)methylglycine SDS/PAGE system (11), blotted onto Immobilon membranes (Millipore), and stained with Coomassie blue. The individual polypeptide bands were excised and sequenced by using an Applied Biosystems model 475A gas-phase sequencer.

RNA Extraction and Poly(A)$^+$ RNA Isolation. RNA was extracted from tomato pericarp tissue according to Chirgwin *et al.* (12), with modifications. Frozen tomato tissue (30 g) was powdered in a coffee grinder chilled with dry ice. The powdered tissue was extracted in a buffer consisting of 5 M guanidinium isothiocyanate, 0.3 M Tris·HCl, 10 mM EDTA, and 8% 2-mercaptoethanol (pH 7.5), at a buffer-to-tissue ratio of 2:1 (13) by using a Brinkman Polytron homogenizer (three 30-sec bursts at the highest setting). The homogenate was centrifuged at 15,000 rpm in an SS34 rotor (Sorvall) for 20 min. The clarified supernatant was filtered through Miracloth

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylic acid.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M63490).

(Calbiochem), layered onto 5.7 M CsCl containing 0.1 M EDTA (pH 7.0) in three centrifuge tubes, and centrifuged in an SW27 rotor (Beckman) at 24,000 rpm and 20°C for 24 h. The RNA pellets were collected, dissolved in water, and precipitated with ethanol.

Since RNA preparations from tomato fruits often contain large amounts of polysaccharides, the extracted RNA was chromatographed on Sigmacell 50 (Sigma; ref. 14). Sigmacell (0.25 g) was first soaked in column buffer [0.5 M NaCl, 20 mM Tris·HCl, 1 mM EDTA, and 0.1% SDS (pH 7.5)] and then loaded into disposable columns. These were washed first with water, then with 0.1 M NaOH, and then again with water until the pH of the eluant fell below 7. After equilibration of the packed Sigmacell in column buffer, RNA dissolved in column buffer was passed through the column, followed by 5–10 ml of column buffer. The flow-through fraction was collected, heated at 65°C for 5 min, and then chilled on ice to 23°C. Poly(A)$^+$ RNA was isolated from the chromatographed RNA by two cycles of binding to and elution from oligo(dT)-cellulose as described by Aviv and Leder (15).

DNA Labeling. Plasmid DNA was digested with the appropriate endonucleases and separated by agarose gel electrophoresis. DNA bands containing the inserts Ben11, Ben17, or Ben18 (see Fig. 1) were collected by electrophoresis onto DE-81 paper (16). DNA was labeled with [α-^{32}P]dCTP by using the random primer method (17) as specified by the manufacturer of the DNA labeling kit (United States Biochemical). Radiolabeled DNA was separated from nucleotides by passage over Sephadex G-50.

RNA Blotting and Hybridization. RNA was subjected to electrophoresis through 1.2% agarose containing 2.2 M formaldehyde (18). The gels were stained with ethidium bromide (0.5 μg/ml) for 20 min and destained in distilled water at 23°C for 2–4 h. The RNA was transferred to Zeta-Probe membranes (Bio-Rad) in 10× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The membranes were heated at 60–80°C in a vacuum oven for 1 h. Prehybridization and hybridization to radiolabeled DNA were performed in 0.5 M sodium phosphate, 7% SDS (ultrapure; Boehringer Mannheim), and 1 mM EDTA (pH 7.2) at 65°C overnight and for 20–24 h, respectively (19). The membranes were washed twice with 5% SDS and twice with 1% SDS in 40 mM sodium phosphate (pH 7.2). All washes were for 30 min at 65°C. Membranes were air dried for 5 min, wrapped in a plastic film, and used to expose x-ray film (Kodak).

For quantitation of the relative levels of ACC synthase mRNAs in poly(A)$^+$ RNA preparations, RNA was denatured according to Sambrook *et al.* (16), diluted in 12× SSC containing 14.8% formaldehyde, and transferred to Zeta-Probe membranes by using a slot blot apparatus. The wells were washed with 1 ml each of 6× SSC and 7.4% formaldehyde. The membranes were rinsed in 2× SSC, air dried, and heated at 60–80°C in a vacuum oven for 1 h. Hybridizations to the probes Ben17 and Ben18 were performed as described above and were quantified with a Betascope radioactive blot analyzer (Betagen, Waltham, MA).

Preparation of Genomic DNA and DNA Blotting. Tomato leaves (10 g) were frozen, finely ground, and mixed with 75 ml of cold extraction buffer [0.01 M Tris·HCl, 1% triisopropylnaphthalene sulfonic acid, 6% *p*-aminosalicylic acid, 0.05 M NaCl, 0.02 M EDTA, and 6% butanol (pH 8.8)], followed by two phenol/chloroform extractions. After ethanol precipitation of the nucleic acids, the DNA was isolated by centrifugation in CsCl (8 g of CsCl per 7.5 ml of DNA solution) using a 50Ti rotor (Beckman) at 43,000 rpm and 4°C for 48 h. The DNA band was collected and dialyzed against 1× TE (0.01 M Tris·HCl/1 mM EDTA, pH 8) to remove the CsCl. DNA (5 μg) was digested to completion with *Eco*RI, *Bgl* II, or *Hin*dIII, and fractionated by electrophoresis on a 1% agarose gel. Transfer to nitrocellulose, hybridization, and washes were according to Sambrook *et al.* (16).

PCR. Sequences from genomic or cDNA were amplified by PCR using the appropriate oligonucleotide primers (2 μg of each), *Thermus aquaticus* (*Taq*) DNA polymerase, and a Perkin–Elmer/Cetus thermal cycler according to manufacturer's instructions. Twenty-five thermal cycles were performed, each consisting of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C.

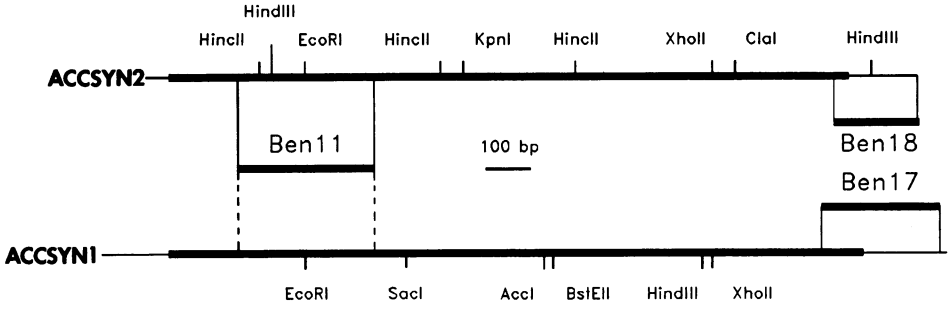
Cloning of cDNA and Sequencing. A λZAPII cDNA library (Stratagene) constructed by using poly(A)$^+$ RNA from ripe, unwounded tomato fruits (cv. UC82B) was screened for clones encoding ACC synthase. Plaque hybridization was carried out on nitrocellulose filters (Schleicher & Schuell) following the procedure in Ausubel *et al.* (20). Plaque purification and DNA isolation were performed according to Sambrook *et al.* (16). The cDNA inserts were subcloned as *Eco*RI fragments into pUC19. All sequencing followed the chain-termination method using supercoiled plasmid as template according to the Boehringer Mannheim sequencing handbook, except that the Klenow polymerization step was carried out at 50°C. DNA sequences were analyzed with the University of Wisconsin Genetics Computer Group computer programs (21). Protein sequence comparisons were performed with the MULTALIN program of Corpet (22).

RESULTS AND DISCUSSION

Sequencing of ACC Synthase. Sequencing of the polypeptides obtained by CNBr fragmentation yielded three amino acid sequences: GLA(K)NQL(R)L(T)LIED?IKRNP, AGGATGANETIIF?LADPGDAFLVPSPY, and EKTRGGRVRFDPERVV [from amino to carboxyl terminus; a letter in parentheses denotes a discrepancy compared to the derived amino acid sequence of Van Der Straeten *et al.* (10), and a question mark indicates that the respective amino acid could not be identified]. The amino acid sequence of tomato ACC synthase derived from the nucleotide sequence of cDNA clone pcVV4A of Van Der Straeten *et al.* (10) contains the above three sequences. This is conclusive evidence that the tomato ACC synthase identified by Van Der Straeten *et al.* (23) as a 45-kDa protein is identical to the ACC synthase originally purified and identified as a 50-kDa polypeptide by Bleecker *et al.* (6, 7).

Cloning of cDNA Encoding ACC Synthase 2. Initially, we used mixed oligonucleotide primers corresponding to two of our known peptide sequences for the amplification of a nucleotide sequence from tomato genomic DNA by PCR. The amino acid sequence derived from the DNA sequence of the PCR product showed several discrepancies compared to the amino acid sequence obtained by direct sequencing of the protein. Upon publication of the nucleotide and derived amino acid sequences of ACC synthase by Van Der Straeten *et al.* (10), it became evident that our PCR product was contained in their second, partial cDNA clone designated pcVV4B. In continuing this work, we aimed at cloning the full-length cDNA that encodes the second isoform of ACC synthase, henceforth called ACC synthase 2, and at investigating the expression of the genes for ACC synthase 1 [corresponding to pcVV4A of Van Der Straeten *et al.* (10)] and ACC synthase 2.

First, we amplified by PCR a 288-bp fragment from a cDNA library prepared from poly(A)$^+$ RNA of ripe, unwounded tomato fruits (Stratagene) by using two oligonucleotide primers, 5′-TAAAAAAGCATCACCAGGATC-3′ and 5′-G(CTGCAG)GTCTCGCG[A]AAAATCAGCTT-3′ (Fig. 1; the nucleotides in parentheses enclose an added *Pst* I site; the [A] differs from the G specified for the same position in ref. 10 because our protein sequence data indicated a lysine instead of a glutamic acid residue). The 288-bp PCR product

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                                                  M  D  L  E  T  S  E  I  S  N  Y  K  S  S  V  V  L
100  TCTAAGTTGGCTAGTAACGAACAACATGGTGAAAACTCACCATATTTTGATGGGTGGAAAGCATACGATAACGATCCTTTCCACTTGGTGAATAATTTG  198
     S  K  L  A  S  N  E  Q  H  G  E  N  S  P  Y  F  D  G  W  K  A  Y  D  N  D  P  F  H  L  V  N  N  L
199  AATGGGGTTATTCAGATGGGTCTCGCGGAAAATCAGCTTTCAGTTGACTTGATTGAAGAATGGATTAAGAGAAATCCAAAAGCTTCCATTTGTACAAAT  297
     N  G  V  I  Q  M  G  L  A  E  N  Q  L  S  V  D  L  I  E  E  W  I  K  R  N  P  K  A  S  I  C  T  N
298  GATGGAATTGAATCTTTCAGGAGAATTGCCAACTTTCAAGATTATCATGGATTGCCTGAATTCACAAATGCGATTGCAAAATTTATGGAGAAAACAAGA  396
     D  G  I  E  S  F  R  R  I  A  N  F  Q  D  Y  H  G  L  P  E  F  T  N  A  I  A  K  F  M  E  K  T  R
397  GGTGGTAAGGTTAAGTTTGATGCTAAACGTGTAGTAATGGCTGGTGGAGCTACTGGAGCTAATGAGACTCTCATACTTTGTTTGGCTGATCCTGGTGAT  495
     G  G  K  V  K  F  D  A  K  R  V  V  M  A  G  G  A  T  G  A  N  E  T  L  I  L  C  L  A  D  P  G  D
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     A  F  L  V  P  T  P  Y  Y  P  G  F  N  R  D  L  R  W  R  S  G  V  Q  L  L  P  I  S  C  K  S  C  N
595  AATTTCAAAATTACAATAGAAGCTATCGAAGAGGCCTATGAAAAAGGTCAACAAGCAAATGTCAAAATCAAAGGCTTGATTTTGACCAACCCTTGTAAT  693
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     S  T  Q  T  Q  H  L  L  A  F  M  L  S  D  D  E  F  V  E  E  F  L  I  E  S  A  K  R  L  R  E  R  Y
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1486 TGTCTCCTAATTCTCCAATTCCTCGCTGTAGTGCGAGCAAACAAAATTATTGTTGTACAATCAAATAAAGTTATAAAGCTTGTAAATAAACTAGATTC  1584
1585 GCCCAGGCTCGTGCTAAAGCACGGGCCCAACATAGTTAATTTTAGTGTCCTAAAAAAAAAAAAAAAA  1652
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FIG. 1. (*Upper*) Comparison of the partial restriction maps of cDNAs encoding ACC synthase 2 (ACCSYN2) and ACC synthase 1 (ACCSYN1) (10). Thick lines on the restriction maps represent coding sequences, and thin lines represent noncoding sequences. The positions of the hybridization probes used in this work (Ben11, Ben17, and Ben18) are indicated between the two restriction maps. Ben11 is homologous to the cDNA encoding ACC synthase 2 and is 84% similar to the cDNA encoding ACC synthase 1. (*Lower*) Composite nucleotide and deduced amino acid sequence of ACC synthase 2. The positions of the oligonucleotides used as PCR primers to amplify the Ben11 probe are underlined, as are the two polyadenylylation signals.

was cloned into pUC19 to yield pBen11 (Fig. 1) and was sequenced. The nucleotide sequence of the 288-bp insert (Ben11) is contained in pcVV4B (10) and encodes, therefore, ACC synthase 2. Ben11 was used to probe at moderately high stringency (0.5× SSC, 65°C) 250,000 plaques of the same tomato cDNA library for full-length clones encoding ACC synthase 2. Five strongly hybridizing plaques were identified of which three were selected for further analysis based on the size of their inserts.

Clone 1 was sequenced in its entirety on both strands. It consisted of 1523 bp (nucleotides 97–1619; Fig. 1) but fell short of the initiator ATG and the 3′ poly(A) tail. Clone 2 was composed of 1594 bp (nucleotides 59–1652; Fig. 1); it contained the poly(A) tail but not the initiator ATG. Clone 3 was composed of 1576 bp (nucleotides 1–1576; Fig. 1) and contained the entire coding region of the gene as well as 48 bp of the 5′ untranslated region and 97 bp of the 3′ untranslated region. However, it fell short of the 3′ poly(A) tail. To assure that all three clones represented the same transcript, five regions of clone 2 (total of 930 bases) and four regions of clone 3 (total of 929 bases), all overlapping with clone 1, were also sequenced. The overlapping sequences of clones 1, 2, and 3 were identical except for three base discrepancies, which probably resulted from cloning artifacts. The partial ACC synthase clone pcVV4B of Van Der Straeten *et al.* (10) is homologous to the corresponding region of clones 1, 2, and 3 with the exception of five consistent base changes. These discrepancies may be due to differences between the tomato cultivars used.

ACC Synthase 2 Nucleotide Sequence. The complete nucleotide sequence of the cDNA encoding ACC synthase 2 and its derived amino acid sequence are shown in Fig. 1. The full-length composite sequence including the 5′ and 3′ untranslated regions is 1652 bp long. The overall DNA sequence similarity of the coding regions for ACC synthase 1 (clone pcVV4A; ref. 10) and ACC synthase 2 (Fig. 1) is 75%. Both cDNAs contain highly conserved regions that are surrounded by regions of lower homology, especially at the 5′ and 3′ ends. Of the restriction sites shown in Fig. 1, only the *Eco*RI and the *Xho* II sites are shared between the two clones.

Comparison of ACC Synthase 2 to Other ACC Synthase Proteins. The derived molecular masses of ACC synthase 1 and 2 are 54.7 and 53.5 kDa, respectively, corresponding to 485 and 476 amino acid residues (Fig. 1 and ref. 10). SDS/PAGE analysis of ACC synthase from tomato pericarp tissue yielded a molecular mass of 45–50 kDa (6–8, 23, 24). From the discrepancy between the derived amino acid sequence of ACC synthase 1 and the molecular mass of the extracted enzyme (10) as well as from a direct comparison of the *in vitro* translation product and the *in vivo* labeled enzyme (24), it has been concluded that nascent ACC synthase in tomato pericarp tissue is processed to a lower molecular mass enzyme. This processing most likely involves proteolytic cleavage at the carboxyl end of the protein and is probably not related to targeting or activation of the enzyme (10, 25). It appears to be analogous to proteolytic processing of ACC synthase in winter squash and zucchini (25, 26). Since a thorough search for size and charge variants of ACC synthase in tomato pericarp homogenates by SDS/PAGE and isoelectric focusing did not result in the identification of isoforms of the enzyme, it appears that ACC synthase 1 and 2 are not distinguishable by these methods (8). At optimal alignment, the homology between the derived amino acid sequences of ACC synthase 1 (10) and 2 is 68%.

Yip *et al.* (9) identified the active site of ACC synthase from apple and tomato fruits by covalent labeling with *S*-adenosyl-L-methionine and pyridoxal phosphate. Both substrate and cofactor bind to a lysine residue contained within the tryptic dodecapeptide SLSKD(M/L)GLPGFR. Yip *et al.* (9) found two active-site dodecapeptides in tryptic digests of tomato ACC synthase. These had the same amino acid sequence except for the residue at position 6, which was either me-

thionine or leucine. They suggested that wound-induced pericarp tissue from ripening tomatoes contains two similar forms of ACC synthase, one of which is induced during ripening (the isoform with the leucine-containing active-site peptide) and one by wounding (the isoform with the methionine-containing active-site peptide). These two ACC synthase isoforms are immunologically related since they were both isolated by immunoadsorption using the monoclonal antibodies of Bleecker *et al.* (6, 7). ACC synthase 1 corresponds to the methionine-containing form of the enzyme (SLSKDM). However, ACC synthase 2 does not appear to correspond to the leucine-containing isoform identified by Yip *et al.* (9), even though methionine is replaced by leucine in the active-site domain (SLSKDL). First, the dodecapeptide of Yip *et al.* (9) contains leucine in position 8 while ACC synthase 2 contains phenylalanine in the corresponding position (Fig. 1). Second, the amino acid preceding the SLSK sequence is serine (SSLSK) in ACC synthase 2 and tyrosine in ACC synthase 1 (YSLSK). Trypsin would not cleave between two serine residues but it would do so between tyrosine and serine. Therefore, tryptic digestion could yield the active-site dodecapeptide isolated by Yip *et al.* (9) from ACC synthase 1 but not from ACC synthase 2. Thus, it has to be assumed that ripening tomato fruits contain at least three isoforms of ACC synthase.

Differential Regulation of ACC Synthase 1 and 2. Gene-specific probes were prepared to compare the expression of the two ACC synthases in tomato pericarp tissue. Since the 3′ ends of the two cDNAs are highly divergent, we amplified by PCR a region specific to each clone. We synthesized one oligonucleotide, 5′-G(GAATTC)ATGTATGATGAAAGTGTT-3′ (the nucleotides in parentheses indicate an added *Eco*RI site), which was used to prime both probes at the 5′ ends. It corresponds to bases 1546–1563 of ACC synthase 1 and to bases 1452–1469 of ACC synthase 2. Even though this oligonucleotide contained four mismatches to the respective ACC synthase 2 nucleotide sequence, it still served as primer under the conditions used. With the above oligonucleotide as one primer, 5′-GCATTCATTAATTAGCTAAGAC-3′ (corresponding to bases 1807–1786 of ACC synthase 1) as the primer at the 3′ end, and tomato genomic DNA as template, we obtained a 267-bp fragment, which was cloned into pUC19 to give pBen17 (Fig. 1); its insert (Ben17) was used as a gene-specific probe for ACC synthase 1. The probe for ACC synthase 2 was amplified by using the common primer (see above), 5′-TCTAGA$(dT)_{20}$-3′ as the second primer, and the cloned 3′ end of clone 2 as template. The resulting 209-bp fragment was cloned into pUC19 to yield pBen18 (Fig. 1); its insert (Ben18) served as a gene-specific probe for ACC synthase 2. The identities of both probes were confirmed by nucleotide sequencing.

RNA was isolated from tomato pericarp tissue at four stages of ripening: mature green, breaker (showing the first signs of coloration), pink, and light red. RNA was also isolated from pericarp tissue at these same ripening stages after 4 h of wounding. Poly(A)$^+$ RNA prepared from each of the eight RNA samples was analyzed by Northern blot hybridization using the gene-specific probes Ben17 and Ben18 to detect transcripts encoding ACC synthase 1 and 2, respectively (Fig. 2). In intact pericarp tissue, the level of mRNA encoding both isoforms of ACC synthase was very low at the mature-green stage. It increased with the onset of ripening at the breaker stage, was highest in pink fruits, and was lower again at the light-red stage (Fig. 2). In several experiments, hybridization of Ben17 and Ben18 to mRNA from fully ripened tomatoes was very low, indicating that the level of ACC synthase mRNA was much reduced in ripe fruits (results not shown). Mechanical wounding greatly increased the mRNA level for ACC synthase 1, as evidenced by the strong hybridization signal obtained with Ben17 (Fig. 2 *Top*). The effect of wounding on the expression of mRNA corresponding to Ben17 increased with ripening from the

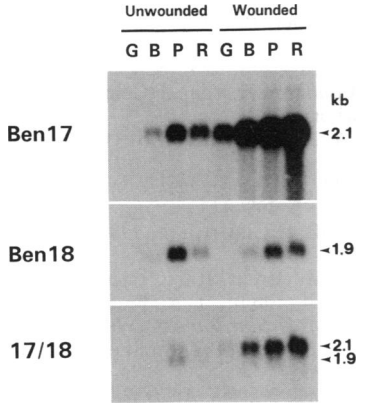

FIG. 2. Differential expression of the genes encoding ACC synthase 1 and 2. Poly(A)$^+$ RNA isolated from pericarp tissue of mature-green (G), breaker (B), pink (P), and light-red (R) tomato fruits at time 0 (unwounded) or 4 h after wounding was separated by electrophoresis on agarose gels (2 μg per lane) and blotted onto Zeta-Probe membranes. The filters were probed with ^{32}P-labeled Ben17, the gene-specific probe for ACC synthase 1 (*Top*); with ^{32}P-labeled Ben18, the gene-specific probe for ACC synthase 2 (*Middle*); or with a mixed probe consisting of ^{32}P-labeled Ben17 and Ben18 (*Bottom*). Autoradiographs were exposed with an intensifying screen for 3 days (*Top* and *Middle*) or 5 days (*Bottom*). kb, kilobase(s).

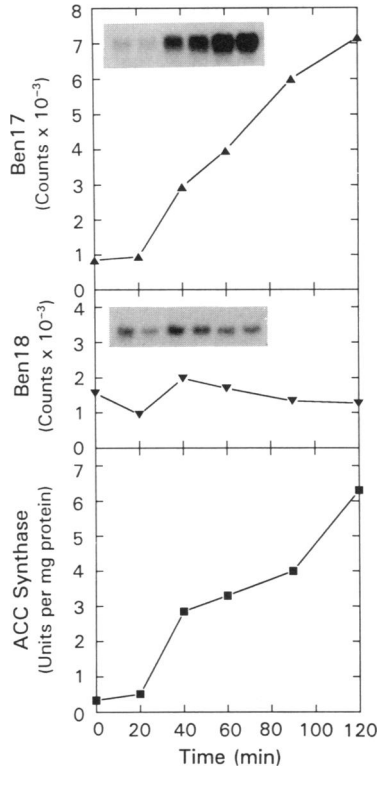

FIG. 3. Time course of appearance of mRNA encoding ACC synthase 1 and 2 and of ACC synthase activity after wound induction. Total RNA was isolated from pericarp tissue of pink tomato fruits at the indicated times, separated by electrophoresis on agarose gels (10 μg of RNA per lane), blotted onto Zeta-Probe, and probed with ^{32}P-labeled Ben17 (*Top*) or Ben18 (*Middle*). The autoradiograms were exposed with intensifying screens for 5 days, and the radioactivity associated with each band was quantified by using a Betascope radioactive blot analyzer. (*Insets*) Corresponding Northern blots. (*Bottom*) ACC synthase activity (1 unit = 1 nmol of ACC per h at 30°C) was assayed in the same tissue samples.

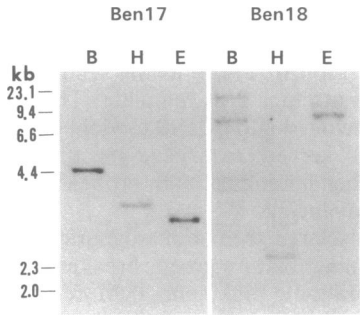

FIG. 4. Blot analysis of tomato genomic DNA. Each lane contained 5 μg of DNA digested with *Bgl* II (B), *Hind*II (H), or *Eco*RI (E). The filters were probed with ^{32}P-labeled Ben17 or Ben18. Exposure was for 84 h with an intensifying screen.

mature-green to the light-red stage. This trend is consistent with the level of wound-induced ethylene formation, which also increases with ripening (27). In contrast, wounding had little, if any, effect on the level of mRNA encoding ACC synthase 2 (Fig. 2 *Middle*). Molecular size determinations indicated that the mRNAs hybridizing to Ben17 and Ben18 differed in length. To verify this, we used Ben17 and Ben18 together as a mixed hybridization probe of the blot shown in Fig. 2. Two bands of hybridization were evident (Fig. 2 *Bottom*), indicating that Ben17 and Ben18 indeed recognized ACC synthase mRNAs of different lengths. We estimate that the sizes of the mRNAs corresponding to Ben17 and Ben18 are 2.1 and 1.9 kb, respectively. Using slot-blot analysis and taking into account the specific radioactivities of the Ben17 and Ben18 cDNAs, we calculated that the relative ratio of the mRNAs encoding ACC synthase 1 and 2 was between 1.5:1 and 3:1 in unwounded breaker and pink tomato pericarp tissue, respectively.

The level of mRNA encoding ACC synthase 1 (Ben17) increased in pink tomato pericarp tissue within 40 min of wounding (Fig. 3 *Top*). A similar increase in the activity of ACC synthase was found in the same tissue (Fig. 3 *Bottom*). The level of mRNA encoding ACC synthase 2 (Ben18) did not increase as a result of wounding (Fig. 3 *Middle*), even when the incubation period was extended to 8 h (results not shown).

DNA Blot Analysis. Southern blot analysis was performed on tomato genomic DNA digested with *Bgl* II, *Hin*dIII, or *Eco*RI. The filters were probed with either Ben17 or Ben18 and were washed at moderately high stringency (0.5× SSC, 65°C). Ben17 hybridized to a 4.2-kb band in the *Bgl* II digest, a 3.2-kb band in the *Hin*dIII digest, and a 3-kb band in the *Eco*RI digest (Fig. 4). Van Der Straeten *et al.* (10) using pcVV4A as a probe found bands of similar size in *Eco*RI and *Bgl* II digests. Ben18 hybridized to bands of 9.3 and ≈23 kb in the *Bgl* II digest, 2.4 kb in the *Hin*dIII digest, and 9.4 kb in the *Eco*RI digest and also weakly to several other bands in each of these three digests. These same hybridization patterns have been observed in three separate experiments and may reflect limited homology of Ben18 to other genomic DNA sequences. Using gene-specific probes, we could, thus, demonstrate that the genes encoding ACC synthase 1 and 2 have different restriction patterns. This result does not agree with the conclusion of Van Der Straeten *et al.* (10), who proposed on the basis of hybridization with pcVV4A that the two genes for ACC synthase have the same *Eco*RI and *Bgl* II restriction patterns. The pcVV4A probe, which encodes ACC synthase 1, may not have detected DNA sequences encoding ACC synthase 2 if used at too high stringency.

In summary, cDNA clones encoding ACC synthase have now been obtained for tomato (10), winter squash (25), zucchini (26), and apple (28). In this report, we describe the full-length nucleotide sequence of a cDNA encoding a second isoform of ACC synthase from tomato fruits and its derived amino acid sequence. Using gene-specific probes, we have demonstrated the differential regulation of two genes encoding an enzyme of ethylene biosynthesis. The mRNA levels for both isoforms, ACC synthase 1 and 2, increase during ripening, whereas mechanical stress (wounding) enhances the expression of the ACC synthase 1 transcript only.

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