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Differential accumulation of transcripts for four tomato 1-aminocyclopropane-1-carboxylate synthase homologs under various conditions

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

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ABSTRACT **Degenerate oligonucleotide primers corresponding to conserved regions flanking the active-site domain of 1-aminocyclopropane-1-carboxylate (ACC) synthase (EC 4.4.1.14) were used for the polymerase chain reaction (PCR) to amplify DNA fragments from mRNA isolated from tomato fruit and tomato suspension cell culture. Antibodies raised against two conserved peptide sequences (TNPSNPLGTT and SLSKDLGLPGFRVG) were used to screen for positive colonies, after the PCR products were cloned into a Bluescript plasmid and expressed in *Escherichia coli*. Four distinct cDNA fragments encoding ACC synthase homologs were isolated. While pBTAS1 and pBTAS4 were obtained from fruit mRNA, cell culture mRNA yielded three sequences, pBTAS1, pBTAS2, and pBTAS3. Sequencing of these gene fragments revealed that pBTAS1 and pBTAS4 were identical to those full-length sequences previously reported by Van Der Straeten *et al.* [Van Der Straeten, D., Van Wiemeersch, L., Goodman, H. & Van Montague, M. (1990) *Proc. Natl. Acad. Sci. USA* 87, 4859–4863] and Olson *et al.* [Olson, D. C., White, J. A., Edelman, J., Harkin, R. N. & Kende, H. (1991) *Proc. Natl. Acad. Sci. USA* 88, 5340–5344] from tomato fruit, whereas pBTAS2 and pBTAS3 represent new sequences. Ribonuclease protection assays were used to examine the expression of these transcripts under three different conditions of enhanced ethylene production—namely, during fruit ripening, in response to mechanical wounding in fruit tissue, and auxin stimulation in vegetative tissue. Transcripts of pBTAS1 accumulated massively during ripening and wounding but only slightly in response to auxin treatment. Although pBTAS4 was associated with fruit ripening, it was unresponsive to auxin treatment in vegetative tissue. In contrast, the expression of pBTAS2 and pBTAS3 was greatly promoted in auxin-treated vegetative tissue but was absent from fruit tissue. While the expression of pBTAS2 was moderately dependent on wounding, pBTAS3 was unresponsive to wounding. These data support the view that ACC synthase is encoded by a multigene family and that the members are differentially expressed in response to developmental, environmental, and hormonal factors.**

1-Aminocyclopropane-1-carboxylate (ACC) synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase; EC 4.4.1.14), the key enzyme in ethylene biosynthesis, has been purified from several plant tissues (1–4). Recently, ACC synthase cDNA clones have been isolated and sequenced from wounded fruit tissues of tomato (5), winter squash (6), and zucchini (7) and from ripening apple (8) and tomato fruit (9).

Following the radiolabeling of the active site of tomato ACC synthase with *S*-adenosylmethionine (10) and purification of the protein by immunoaffinity chromatography (11, 12), we isolated and sequenced two highly conserved active-site tryptic peptides, which differed in only one amino acid residue (12). These results provided initial evidence for the existence of two isoforms of ACC synthase. The expression of two ACC synthase genes in wounded, ripe tomato pericarp was shown by the isolation of two different ACC synthase cDNA clones by Van Der Straeten *et al.* (5). One of these clones was full-length and the deduced active site sequence was identical to the sequence of our major active-site peptide (12). The other was a partial clone that did not cover the active-site region. Recently, Olson *et al.* (9) isolated a full-length clone that was identical to the partial clone previously isolated by Van Der Straeten *et al.* (5). These reports indicate that there are at least two ACC synthase genes expressed in tomato fruit.

Using appropriate primers, we have obtained four ACC synthase-related gene fragments by PCR amplification of cDNA derived from mRNA of tomato fruit and tomato suspension cell culture.‡ Employing the ribonuclease protection assay, we investigated the level of each transcript in response to ripening and wounding of tomato fruit and to auxin treatment of tomato seedlings.

MATERIALS AND METHODS

Isolation of mRNA. Ethylene production in tomato tissue (*Lycopersicon esculentum* Mill.) was measured according to the method of Su *et al.* (13) prior to RNA extraction. VFTN cherry tomato calyx suspension culture cells obtained from Betty Ishida (Agricultural Research Service, United States Department of Agriculture) were maintained in medium with Murashige and Skoog salts, 3% (wt/vol) sucrose, 0.1 μg of benzyladenine per liter, and 2.0 μg of 2,4-dichlorophenoxyacetic acid per liter, at pH 5.8. Fifty grams of fruit tissue or 5 grams of 5-day-old tomato suspension culture cells (subcultured every 2 weeks) was frozen in liquid N_2 and pulverized to a fine powder. The powdered tissue was homogenized in 100 ml of grinding buffer [4 M guanidinium chloride/8% (vol/vol) 2-mercaptoethanol/200 mM Tris·HCl, pH 7.5] with a Tissuemizer (Tekmar) for 2 min at top speed. The homogenate was centrifuged at 25,000 × *g* for 30 min, and the supernatant was extracted with an equal volume of phenol/chloroform, 1:1 (vol/vol). Total nucleic acids were precipitated from the aqueous supernatant overnight at −20°C with 0.3 M sodium acetate (pH 6.0) and 1 volume of 2-propanol. Poly(A)$^+$ RNA was then selected with poly(U)-Sepharose (Sigma) as described by Cashmore (14). Poly(A)$^+$ RNA was

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Abbreviation: ACC, 1-aminocyclopropane-1-carboxylate.

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M83318, M83320, M83322, and M83329).

similarly isolated from wounded, ripe fruit after the sliced tissue had been incubated at room temperature for 12 hr. Hypocotyls cut from 5-day-old seedlings were first incubated in 20 mM Mes buffer (pH 6.1) for 4 hr, during which time the wound-induced ethylene production subsided. After the addition of 0.5 mM indoleacetic acid, the tissues were further incubated with shaking for 5 hr. Two grams of hypocotyl segments was extracted by the method described above, and poly(A)$^+$ RNA was selected with the PolyATtract mRNA isolation kit from Promega.

Production of Antibodies Against Synthetic Peptides. For later convenience, a carboxyl-terminal cysteine was added to each of the two synthetic peptides representing a conserved region (TNPSNPLGTT) and the active-site region (SLSKDLGLPGFRVG) of ACC synthase. The synthetic peptides were conjugated to keyhole limpet hemocyanin (Calbiochem) with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (Pierce) as the coupling reagent (15). An estimated 2–4 mg of the synthetic peptide was conjugated to 5 mg of hemocyanin.

One milligram of each peptide conjugate in Freund's complete adjuvant was injected subcutaneously into 2.2-kg New Zealand White female rabbits. After two additional immunizations with 1 mg of each conjugate, the animals were sacrificed and the anti-peptide antibodies were purified with an immunoaffinity column in which the carboxyl-terminal cysteine of each peptide was immobilized to SulfoLink coupling gel from Pierce (16). Antibodies were eluted from the affinity gel with 100 mM triethylamine, pH 10.5/10% dioxane and were neutralized to pH 8.0 with 1 M Tris·HCl (pH 7.5). This purification step was found to be essential to reduce background and hence to improve sensitivity for screening *Escherichia coli* colonies.

Polymerase Chain Reaction. PCR primers containing restriction-site sequences are listed in Table 1. DNA fragments were synthesized by PCR by the method of Wang *et al.* (17), with modifications. Briefly, cDNA was synthesized in a 10-μl reaction mixture containing 2 μg of poly(A)$^+$ RNA, 1× PCR buffer (20 mM Tris·HCl, pH 8.3/50 mM KCl/2.5 mM $MgCl_2$ with bovine serum albumin at 10 μg/ml), 1 mM dithiothreitol, 0.5 mM dNTPs, 15 units of RNasin (United States Biochemical), 0.3 μg of $(dT)_{15}$ (United States Biochemical), and 150 units of avian myeloblastosis virus reverse transcriptase (BRL). The mixture was incubated at 37°C for 2 hr, heated to 95°C for 10 min, and then quickly chilled on ice. PCR was performed in 50 μl of 1× PCR buffer with 30 μM dNTPs, 10 μM primers, and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin–Elmer/Cetus). The amplification was carried out for 30–35 cycles with denaturation at 94°C for 1 min, primer annealing at 42°C for 2 min, and extension at 72°C for 2 min. Reactions that lacked reverse transcriptase were performed as negative controls and did not yield any PCR products.

Cloning and Screening the PCR Products. Amplified PCR products were extracted twice with phenol/chloroform, 1:1, and precipitated with ethanol. PCR products were then digested with *Bam*HI and *Hin*dIII (BRL) and ligated into the corresponding sites in pBluescript II SK(+) (Stratagene). Primer 1 (Table 1) was designed so that PCR products could be inserted in frame with the β-galactosidase gene of the plasmid. *E. coli* XL-1 Blue was transformed with the ligation mixture, plated onto selection plates, and incubated overnight at 37°C. Recombinant white colonies were spotted onto duplicate nitrocellulose filters and were further incubated for 12 hr. Colonies on one filter were treated with isopropyl β-D-thiogalactopyranoside for 5 hr and lysed for 30 min with 1% SDS in a chloroform-saturated chamber. Filters were washed and subsequently blocked with 3% non-fat dry milk in Tris-buffered saline (25 mM Tris·HCl, pH 7.5/154 mM NaCl). Filters were incubated for 2 hr with a mixture of affinity-purified antibodies diluted 1000-fold in Tris-buffered saline containing 3% bovine serum albumin. After three washes with 0.1% Tween-20 in Tris-buffered saline and incubation with goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) for 50 min, positive colonies were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride color reagents (Bio-Rad). Plasmids were isolated from immunopositive colonies and digested with *Bam*HI and *Hin*dIII, and the insert size was determined by electrophoretic separation in a horizontal 1.2% agarose gel.

Sequencing of PCR Products. Double-stranded plasmid DNA isolated from immunopositive colonies was alkali-denatured and sequenced with the Sequenase 2.0 kit using either the T3 or T7 sequencing primers according to the manufacturer's instructions (United States Biochemical).

Ribonuclease Protection Assay. The relative levels of ACC synthase mRNAs in poly(A)$^+$ RNA preparations were examined using the ribonuclease protection assay kit from Ambion (Austin, TX) according to the manufacturer's instructions. The general principles of this assay have been described (18). [α-^{32}P]UTP-labeled antisense RNA probes were transcribed from *Bam*HI-digested plasmid DNA (1 μg) and were subsequently purified by 8 M urea/5% polyacrylamide gel electrophoresis. The probe was eluted from the gel and 1.2 × 10^5 cpm was then hybridized with 0.5–2 μg of poly(A)$^+$ RNA. The hybridization products were treated with a mixture of ribonucleases A and T1 and were then analyzed by polyacrylamide gel electrophoresis and autoradiography.

Table 1. Amino acid sequences of two conserved peptides of ACC synthase and their corresponding nucleotide sequences used as PCR primers

Peptide sequence	PCR primer No.	PCR primer Sequence
SNPLGTT	1	5′-CT(GGATCC)G-TCAAAYCCNYTRGGCACNAC-3′
MSSFGLVS	2	5′-CTC(AAGCTT)-ACNARNCCRAARCTYGACAT-3′

Oligonucleotide primers were constructed based on the peptide sequences. Nine bases were added to the 5′ end of each primer that include the restriction sites shown in parentheses. Primer 1 is a sense primer with a *Bam*HI site, and primer 2 is an antisense primer with a *Hin*dIII site.

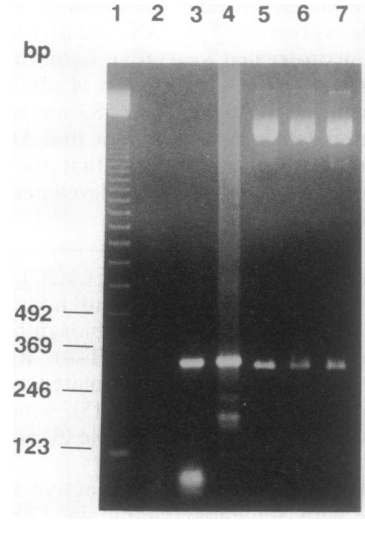

FIG. 1. Agarose gel electrophoretic analysis of PCR products amplified from tomato poly(A)$^+$ RNA. Lane 1, molecular size markers (bp, base pairs); lane 2, mature green fruit; lane 3, ripe fruit; lane 4, cell culture; lane 5, insert released from pBTAS1; lane 6, insert released from pBTAS2; lane 7, insert released from pBTAS3.

RESULTS AND DISCUSSION

Polyclonal antibodies were individually raised against two synthetic peptides representing a conserved region (TNPSNPLGTTC) and the active-site region (SLSKDLGLPGFRVGC) of ACC synthase. The cross-reactivity of these antibodies with apple ACC synthase was examined. Neither antiserum was capable of precipitating ACC synthase activity from a crude enzyme preparation. However, when a crude enzyme preparation was mixed with a sample of ^{3}H-labeled ACC synthase (12) and subjected to SDS/polyacrylamide gel electrophoresis and Western blot analysis, these antibodies specifically recognized the 48-kDa ^{3}H-labeled ACC synthase (data not shown). Similar results were obtained when these antibodies were tested against tomato fruit ACC synthase. These data suggested that the antibodies recognized denatured but not native enzyme.

ACC synthase gene fragments encompassing the active-site domain were amplified by PCR using degenerate oligonucleotides as primers (Table 1) and the cDNAs synthesized from poly(A)$^{+}$ RNA isolated from green tomato fruit, ripe tomato fruit, and tomato cell culture as templates (Fig. 1). Three putative ACC synthase gene sequences were obtained, as judged by their deduced amino acid sequence homology with known ACC synthase sequences (5–9).

One major product was obtained from a poly(A)$^{+}$ RNA preparation isolated from ripe fruit; no similar-sized PCR product was observed with green tomato fruit poly(A)$^{+}$ RNA. Because ACC synthase activity can be detected only in ripening but not green tomato fruit (13, 19), this result is consistent with the view that ACC synthase activity is regulated at the transcriptional level (5, 6, 8, 13, 20). The PCR product was cloned into a plasmid vector so that it could be expressed in *E. coli* and the recombinant colonies obtained were screened with the anti-peptide antibodies. Of 10 immunopositive clones isolated from ripe tomato fruit PCR products, 9 were found to be of the pBTAS1 sequence, while the 10th differed from pBTAS1 by only three bases. This sequence was not listed in Fig. 2, because these changes could have resulted from errors in the amplification process. Additionally, PCR was performed with a ripe tomato cDNA library as template and yielded 5 immunopositive clones, all identical to pBTAS1.

PCR products derived from tomato cell culture mRNA yielded 18 immunopositive clones, among them 12 were

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A. cDNA sequences

pBTAS1    TTTGGACAAAGACACACTGAAAAGTGTCTTGAGTTTCACCAACCAACACA     50
pBTAS2    ACTAAACAGAAACGAGCTTGAACTTCTTCTTACATTCGTCGACGAAAAAG
pBTAS3    AATGTCACGAAACGAACTAAACATTCTAAACACATTTGCCATGACCAAAA
pBTAS4                                    AAAATCTCCACCTTCACTAACG

pBTAS1    ACATCCACCTTGTTTGTGACGAAATCTACGCAGCCACTGTCTTTGACACG    100
pBTAS2    GCATTCACCTCATCAGTGACGAGATCTATTCAGGGACTGTTTTTAACTCA
pBTAS3    ATATTCATATAGTTAGCGACGAAATATACGCTGGTACGTATTCTGATTCG
pBTAS4    AACATAATATCCATCTTGTTTGCGACGAAATATATGCTGCTACCGTATTC

pBTAS1    CCTCAATTCGTCAGTATAGCTGAAATCCTCGATGAACAGGAAATGACTTA    150
pBTAS2    CCAGGCTTCGTTAGTGTCATGGAAGTTCTAATTGAAAAGAACTACATGAA
pBTAS3    CCTAAATTCGTAAGTATAATCGACGCATTAATCGATAGAAAACTCGAAAA
pBTAS4    AATCCTCCAAAATTCGTTAGCATCGCTGAAATTATCAACGAAGATAATTG

pBTAS1    CTGCAACAAAGATTTAGTTCACATCGTCTACAGTCTTTCAAAAGACATGG    200
pBTAS2    AACCCGAGTTTGGGAACGAGTTCACATTGTTTACAGTCTCTCAAAAGATC
pBTAS3    ATCAAAAATGTGGAACCAAGTTCACATTGTATCGAGTCTATCGAAGGATT
pBTAS4    TATCAATAAAGATTTAGTACACATTGTGTCTAGTCTTTCCAAGGACTTAG

pBTAS1    GGTTACCAGGATTTAGAGTCGGAATCATATATTCTTTTAACGACGATGTC    250
pBTAS2    TCGGTCTCCCCGGTTTTCGCATTGGTGCAATCTATTCCAACGATGAGATG
pBTAS3    TAGGCCTTCCAGGTTTCAGAGTTGGCATGATTTATTCGAACAACGAAACT
pBTAS4    GTTTTCCAGGATTTCGAGTGGGAATTGTGTACTCGTTCAACGATGATGTT

pBTAS1    GTTAATTGTGCTAGAAAA                                    268
pBTAS2    GTTGTCTCCGCGGCCACAAAA
pBTAS3    CTTATACATGCCCAAACAAAG
pBTAS4    GTTAACTGTGCTAGAAAA

B.  Aligned amino acid sequences

                ▼                              ▼ ▼
pBTAS1    snplgttLDKDTLKSVLSFTNQHNIHLVCDEIYAATVFDTPQFVSIAEILDE   52
pBTAS2    snplgttLNRNELELLLTFVDEKGIHLISDEIYSGTVFNSPGFVSVMEVLIE
pBTAS3    snplgttMSRNELNILNTFAMTKNIHIVSDEIYAGTYSDSPKFVSIIDALID
pBTAS4                    KISTFTNEHNIHLVCDEIYAATVFNPPKFVSIAEIINE
                           . .*.  ..**...****..*  ..* ***. . . .

                                  ▼       ▼
pBTAS1    QEMTYC-NKDLVHIVYSLSKDMGLPGFRVGIIYSFNDDVVNCARKmssfglv  103
pBTAS2    KNYMKTRVWERVHIVYSLSKDLGLPGFRIGAIYSNDEMVVSAATKmssfglv
pBTAS3    RKLEKSKMWNQVHIVSSLSKDLGLPGFRVGMIYSNNETLIHAQTKmssfglv
pBTAS4    DN---CINKDLVHIVSSLSKDLGFPGFRVGIVYSFNDDVVNCARKmssfglv
                   . **** *****.*.****.* .** .. ..  . *
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FIG. 2. Nucleotide sequences of four DNA fragments representing tomato ACC synthase homologs and their deduced amino acid sequences. pBTAS1, pBTAS2, and pBTAS3 are PCR products derived from mRNA isolated from tomato fruit or tomato cell suspension culture by using the mixed oligonucleotide primers 1 and 2 (see Table 1). pBTAS4 is the PCR product derived from ripe tomato fruit mRNA by using a gene-specific primer derived from the ACC synthase 2 sequence of Olson *et al.* (9) and primer 2. While the DNA sequences derived from the degenerate primers are not included, their deduced amino acid sequences are shown in lowercase letters. Stars and periods indicate identical or similar amino acids, respectively. Filled triangles indicate invariant amino acid residues that correspond to those involved in the binding of pyridoxal phosphate coenzyme in aminotransferases (21). The conserved active-site regions that encompass the active-site Lys-71 are underlined. Gaps (–) are introduced to optimize the alignment, which was produced by the multiple sequence alignment program (IntelliGenetics) using a gap penalty of 5 and an open and unit gap cost of 10.

identical to pBTAS1, 3 to sequence pBTAS2, and the remaining 3 to sequence pBTAS3. The diversity of ACC synthase gene fragments amplified from tomato cell culture mRNA is conceivable because cultured cells are actively dividing and are subjected to various stimuli that are known to influence ACC synthesis, such as incubation period, level of auxin in the medium, and oxygen deficiency.

Recently Olson *et al.* (9) isolated from a ripe tomato cDNA library a full-length ACC synthase clone (ACC synthase 2) that covers the partial sequence previously reported by Van Der Streaten *et al.* (5). This gene transcript accumulates during fruit ripening but not in response to wounding. This clone encodes the active-site sequence SLSKDLGFPGFR, which differs from that encoded by pBTAS1, pBTAS2, or pBTAS3. In comparing the sequences of our primers 1 and 2 (see Table 1) with the equivalent regions for the ACC synthase 2 gene reported by Olson *et al.* (9), there is agreement with respect to primer 2, but there are four mismatched bases in primer 1 (as indicated by underlines, TGTAATC CATTAGGTACCAT). This would explain why we were unable to obtain this particular sequence by using primers 1 and 2 in our PCR amplification procedure. Employing a gene specific primer [ATGGT(GGATCCG)CAAAATCTC-CACCTTCACTAAC] containing a *Bam*HI restriction site (shown in parentheses) for subsequent cloning and primer 2, we amplified one fragment, pBTAS4, from ripe tomato fruit mRNA (Fig. 2). The DNA sequence of pBTAS4 is identical to the sequence reported by Olson *et al.* (9), except for three mismatched bases, only one of which results in a change in amino acid sequence, at position 26, from a serine in their sequence to a proline in our sequence.

ACC synthase is known to be induced by a variety of developmental as well as environmental factors such as ripening, the plant hormone auxin, and mechanical wounding (22). We used the ribonuclease protection assay to study the expression of each ACC synthase transcript in unwounded and wounded ripe tomato fruit and in tomato hypocotyls after auxin treatment (Figs. 3 and 4). The amount of poly(A)$^+$ RNA used in Fig. 3 was 4 times that in Fig. 4. pBTAS1, the major ACC synthase transcript in ripe tomato fruit, was regulated by ripening and wounding, as shown by previous investigators (5, 9). Because the probe was purified before use, we assume that the smearing of the signal on the autoradiograms was largely due to degradation of the probe. pBTAS1 was a minor transcript in cell culture (Fig. 3) and was slightly induced by auxin in hypocotyls (Fig. 4). Because a low level of pBTAS1 was also present in green tomato fruit and control seedlings (Figs. 3 and 4), it might be responsible for the basal, "system 1" ethylene production (23) in green tomato fruit as well as in vegetative tissue. pBTAS2, the major transcript in cell culture, was absent from ripening fruit, but its level moderately increased in mechanically wounded fruit tissue (Fig. 3). In tomato seedlings, the ethylene production rate was less than 0.5 nl·g^{-1}·hr^{-1} in the control tissue but increased to 30 nl·g^{-1}·hr^{-1} in the auxin-treated tissue. This auxin-dependent ethylene production was accompanied by a substantial accumulation of pBTAS2 and pBTAS3 transcripts (Fig. 4). Although the levels of pBTAS1, pBTAS2, and pBTAS3 transcripts increased in auxin-treated hypocotyls, the induction was most conspicuous with pBTAS3, which was a minor transcript in cell culture and did not

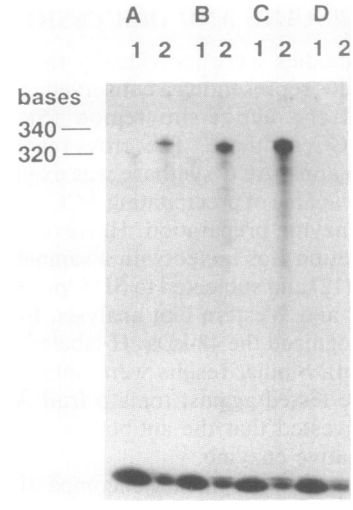

FIG. 4. Expression of four members of the ACC synthase gene family in auxin-treated tomato seedling hypocotyls as assessed by the ribonuclease protection assay (see Fig. 3). Probes were pBTAS1 (*A*), pBTAS2 (*B*), pBTAS3 (*C*), and pBTAS4 (*D*). Lanes 1, 0.5 μg of poly(A)$^+$ RNA from untreated hypocotyls; lanes 2, 0.5 μg of poly(A)$^+$ RNA from hypocotyls treated with 0.5 mM indoleacetic acid.

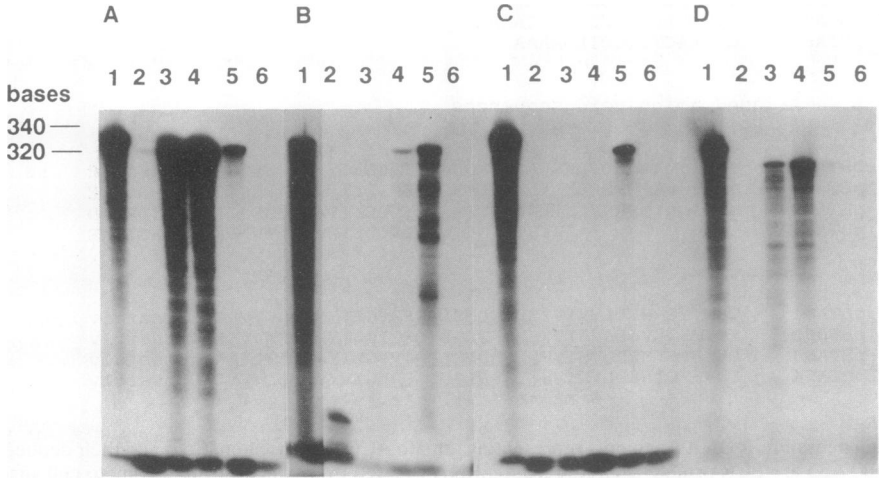

FIG. 3. Expression of four genes of the ACC synthase family in tomato fruit and tomato cell culture as assessed by the ribonuclease protection assay. [α-^{32}P]UTP-labeled antisense RNA probes were transcribed from *Bam*HI-linearized plasmid DNA (1 μg) of each clone with T7 RNA polymerase. The purified probes (1.2 × 10^5 cpm) were hybridized with 2 μg of poly(A)$^+$ RNA from various sources and digested with a mixture of ribonucleases A and T1. Probes were pBTAS1 (*A*), pBTAS2 (*B*), pBTAS3 (*C*), and pBTAS4 (*D*). Lanes 1, undigested probe without poly(A)$^+$ RNA; lanes 2, poly(A)$^+$ RNA from green tomato fruit; lanes 3, poly(A)$^+$ RNA from ripe tomato; lanes 4, poly(A)$^+$ RNA from wounded ripe tomato; lanes 5, poly(A)$^+$ RNA from 5-day-old suspension cell culture; lanes 6, digested probe without poly(A)$^+$ RNA.

appear in fruit tissue at either ripening stage (Fig. 4). In agreement with the observation of Olson *et al.* (9), our ribonuclease protection assays (Figs. 3 and 4) indicate that the level of pBTAS4 transcript increased moderately in ripe fruit as well as in wounded ripe fruit. Although we did not examine wounded tissue of other ripening stages, Olson *et al.* (9) concluded that the level of this transcript did not increase in unripe fruit tissue following wounding. The pBTAS4 transcript was not detected in green fruit, in cell culture, or in seedling hypocotyls before or after auxin treatment.

The ribonuclease protection data indicate that pBTAS1 is the major transcript in ripe as well as wounded ripe fruit (Fig. 3). Previously, we isolated and sequenced two tryptic active-site peptides of ACC synthase from wounded ripe tomato fruit (12). The sequence of the major tryptic active-site peptide (SLSKDMGLPGFR) differed from the minor peptide only in the replacement of the methionine at position 6 by leucine. The deduced active-site amino acid sequence of pBTAS1 is identical to that of the major tryptic active-site peptide. Although pBTAS2 and pBTAS3 contain DNA sequences encoding the minor tryptic active-site peptide SLSKDLGLPGFR, further examination suggests that this minor peptide is probably derived from pBTAS2, but not from pBTAS3. The amino acid residue preceding the active-site peptide is tyrosine in pBTAS2, but serine in pBTAS3 (Fig. 2*B*). Commercial trypsin is often contaminated with chymotrypsin, which cleaves next to aromatic residues such as tyrosine but not serine. Thus, the polypeptide encoded by pBTAS2, but not by pBTAS3, would yield the active-site dodecapeptide we previously isolated from wounded ripe tomato fruit (21). This proposal is also consistent with the observation that pBTAS2, but not pBTAS3, was expressed in wounded ripe tomato fruit (Fig. 3 *B* and *C*).

Comparison of the available amino acid sequences of ACC synthase (Fig. 2*B* and refs. 5–9) reveals that there are regions of sequence that are strikingly conserved. For example, between amino acid residues 15 and 103 (Fig. 2*B*), a sequence identity of 41% and a similarity of 29% are found. Most notable is the active-site region from Ser-68 to Arg-79 (amino acid residues are numbered based on pBTAS1 as shown in Fig. 2*B*) encompassing the active-site Lys-71, which forms a Schiff base with pyridoxal 5′-phosphate (12). In addition, there are a number of invariant amino acid residues or sequences, including Leu-13, Ile-25, Asp-30, Glu-31, Ile-32, Tyr-33, Thr-36, Pro-41, Phe-43, Ser-45, Tyr-84, and Ser-85. By comparing the deduced amino acid sequences of 16 pyridoxal phosphate-dependent aspartate, tyrosine, and histidinol-phosphate aminotransferases, Mehta *et al.* (21) have identified six invariant amino acid residues that are involved in the binding of pyridoxal 5′-phosphate. It is interesting that five of those six invariant amino acid residues are present in all four of our ACC synthase gene fragments, as indicated in Fig. 2*B*. The other invariant residue is tyrosine, which is located at position 70 of aspartate aminotransferases (21). Although this residue is located outside our fragments, it can be found in all known full-length ACC synthase clones (5–9, 24). Another notable feature of these ACC synthase homologs is the sequence diversity observed within a 25-amino acid segment immediately preceding the active-site Lys-71 (Fig. 2*B* and ref. 8). This region should be useful for the construction of gene-specific probes for future mapping experiments. Additionally, such probes could be useful for determining the sizes of the transcripts corresponding to pBTAS2 and pBTAS3, which was not provided by the present ribonuclease protection assays.

Although the PCR primers we employed in this study do not amplify all ACC synthase-related genes, this technique allowed the isolation of ACC synthase gene fragments from auxin-treated mungbean hypocotyls (25) and from ripening peach and avocado fruit (J.-G. Dong and S.F.Y., unpublished work).

In summary, by employing the PCR technique we obtained four different ACC synthase gene fragments, which were used to examine the expression of different ACC synthase transcripts under three different conditions of enhanced ethylene production. Our data as well as those of others (5, 9) indicate that tomato ACC synthase is encoded by a multigene family and that the expression of each gene is differentially activated by different developmental, environmental, and hormonal factors.

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