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Two genes encoding 1-aminocyclopropane-1-carboxylate synthase in zucchini (*Cucurbita pepo*) are clustered and similar but differentially regulated

(ethylene biosynthesis/gene structure/aminotransferases/auxin/wounding/cycloheximide)

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ABSTRACT **A 17-kilobase (kb) region of the zucchini (*Cucurbita pepo*) genome has been sequenced and contains two genes, *CP-ACC1A* and *CP-ACC1B*, encoding 1-aminocyclopropane-1-carboxylate synthase (ACC synthase; *S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14). The genes are transcribed convergently and are separated by a 5.7-kb intergenic region. Their coding regions are interrupted by four introns located in identical positions. While the DNA identity in their coding regions is 97%, their 5′ and 3′ flanking regions are highly divergent. Transcription of *CP-ACC1A* is rapidly induced by wounding in fruit and etiolated hypocotyls and by indoleacetic acid (IAA)/benzyladenine/LiCl only in fruit tissue. Conditions that induce *CP-ACC1B* expression have not been found. Protein synthesis inhibition derepresses the expression of *CP-ACC1A* and other unidentified ACC synthase genes, suggesting that they may be under negative control. The amino acid sequences deduced from the nucleotide sequences of the genes are 493 and 494 residues long with 95% identity. The most notable feature of the amino acid sequence is the presence of 11 of the 12 invariant amino acid residues involved in the binding of the substrate and pyridoxal-5′-phosphate in various aminotransferases. We conclude that ACC synthase is encoded by a multigene family of which certain members are differentially induced by auxin in a tissue-specific manner. Furthermore, ACC synthase, a pyridoxal-containing enzyme, may have an evolutionary relationship with the superfamily of aminotransferases.**

Ethylene is a plant hormone that influences many aspects of plant growth and development (1, 2). The enzyme whose activity limits ethylene production is 1-aminocyclopropane-1-carboxylate synthase (ACC synthase; *S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14), which catalyzes the formation of ACC, the immediate precursor of ethylene (2). The enzyme is induced by a diverse group of inducers, including ethylene and other plant hormones such as auxin, stress conditions, wounding, and various metals (1, 2). Studies with metabolic inhibitors have suggested that the enzyme induction is due to enhanced transcription (3–7). The question then arises how ACC synthase is regulated at the molecular level. For example, are there as many ACC synthase genes as inducers, or is there only one gene whose expression is somehow activated by all of the inducers? The ACC synthase genes should provide the opportunity to elucidate multiple signal transduction pathways. In addition, since ethylene evokes autocatalytic ethylene production (2), the genes can be used to help elucidate the mechanism of ethylene action.

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For these reasons, isolation of the ACC synthase genes is of paramount importance. As a first step towards this goal, we previously cloned a nearly full-length cDNA from *Cucurbita pepo* (8, 9). Here, we report the cloning and sequencing of a 17-kilobase (kb) chromosomal locus† harboring two homologous genes, *CP-ACC1A* and *CP-ACC1B*. We also report their expression characteristics after wounding and indoleacetic acid (IAA)/benzyladenine (BzlAde)/LiCl treatment in fruit and hypocotyl tissue. The effect of protein synthesis inhibition on gene induction has also been investigated to determine whether it constitutes a primary response to the inducers (10).

MATERIALS AND METHODS

Plant Material and Tissue Treatment. Five-day-old etiolated zucchini seedlings (*C. pepo* L. cv. Burpee hybrid) were grown in vermiculite at 25°C and 80% relative humidity. Eight-millimeter-long hypocotyl segments were incubated at 25°C in 1 mM citrate/1 mM Pipes/1 mM KCl/50 μg of chloramphenicol per ml, pH 6.0 (10) with or without IAA (0.5 mM) and BzlAde (0.1 mM). When 20 μM cycloheximide was used, the hypocotyls were abraded with Emery 305 powder. Intact fruits were purchased from a local supermarket, and 1-mm-thick slices were treated with or without 0.5 mM IAA/0.1 mM BzlAde/50 mM LiCl as described (8). Tissue treated with or without hormones has been termed "induced" or "uninduced," respectively. The term "wounding" is defined here as cutting intact etiolated seedlings to 8-mm-long segments or slicing intact fruit tissue to 1-mm-thick slices.

Construction and Screening of Genomic DNA Libraries. Genomic DNA was isolated from nuclei of etiolated hypocotyls by equilibrium sedimentation in CsCl gradients (11, 12). Fragments from partial *Sau*3A digests of this DNA were cloned by insertion into the *Bam*HI site of the EMBL3 λ-phage vector (13). The resulting libraries were screened by the method of Benton and Davis (14) with a ^{32}P-labeled pACC1 cDNA insert (8).

Other Methods. Total nucleic acids or poly(A)$^+$ RNA were isolated, electrophoretically separated, blotted, and hybridized as described (8). The transcription initiation site of the *CP-ACC1A* gene was defined by S1 nuclease analysis (15), primer extension with reverse transcriptase (16), and sequence determination of the primer extension product (17). DNA hybridization analysis was performed essentially as described by Davis *et al.* (12). The DNA sequence of both strands of the 17-kb region encompassing two ACC synthase genes and their flanking sequences was obtained by the

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid (carboxylate); IAA, indoleacetic acid; BzlAde, benzyladenine; AATase, aspartate aminotransferase.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M61195).

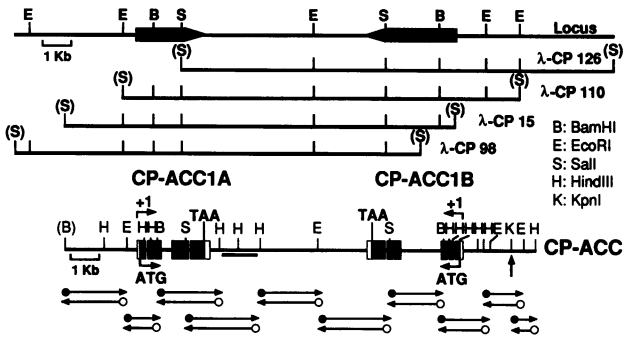

FIG. 1. Restriction maps, gene organization, and sequencing strategy. The four genomic segments, λ-CP 126, 110, 15, and 98, are aligned beneath a restriction map of the chromosomal DNA from which they derive. The sequences in the locus that hybridize to the pACC1 cDNA are shown by solid thick arrows. On the 17-kb locus, termed *CP-ACC*, reside two genes, *CP-ACC1A* and *CP-ACC1B*, respectively. The exons are solid blocks, and the 5′ and 3′ untranslated regions are open blocks. The lines connecting the five solid blocks represent the four introns. The small vertical arrow shows a nonunique *Kpn* I site (K) used for subcloning. The restriction sites shown in parentheses have been created during cloning. The solid bar near the 3′ end of *CP-ACC1A* indicates the region where a potential hairpin loop structure may be formed.

dideoxy chain-termination method (18) with Sequenase (United States Biochemical). Ten DNA fragments covering the entire 17-kb region (Fig. 1) were subcloned into phage M13mp18 and M13mp19 vectors (19). M13 universal primer and synthetic primers (20-mer) were used to sequence the single-stranded DNAs (ssDNAs).

Gene-Specific Probes. Probes were made to unique sequences in *CP-ACC1A* and *CP-ACC1B* genes. (*i*) CP-ACC1A-3′ is a 199-base pair (bp) *Ssp* I/*Eco*RI fragment from the 3′ end of the pACC1 cDNA (9). It hybridizes to 192 bp of the 3′ end of the *CP-ACC1A* gene [nucleotides 2275–2466 (shaded) in Fig. 2]. (*ii*) CP-ACC1B-3′ is a 344-bp *Eco*RI/*Bam*HI fragment that hybridizes to 340 bp of the 3′ end of the *CP-ACC1B* gene (nucleotides 10,693–11,032). The fragment was synthesized by the polymerase chain reaction, and its authenticity was verified by DNA sequence analysis. The *Eco*RI and *Bam*HI sites were artificially introduced to facilitate subcloning.

RESULTS AND DISCUSSION

Gene Structure and Organization. High-stringency hybridization of *C. pepo* genomic DNA with ^{32}P-labeled pACC1 cDNA was shown to detect a 14-kb *Bam*HI fragment and two smaller *Eco*RI fragments, suggesting the presence of one or two gene copies (8). To isolate these fragments, genomic libraries were constructed and screened with the pACC1 cDNA (8). Fig. 1 shows four overlapping genomic sequences isolated in this manner. They encompass ≈22 kb of chromosomal DNA and account for all of the hybridizing fragments. Restriction fragment mapping of the four genomic segments for sequences homologous to those in the cDNA probe demonstrated that two such sequences are confined to two *Eco*RI fragments of 6.9 and 6 kb. Fig. 1 shows that two genes, *CP-ACC1A* and *CP-ACC1B*, reside on the 17-kb locus, hereafter referred to as *CP-ACC*. The locus is present in a single copy in the haploid *Cucurbita* genome (data not shown). Both genes consist of five exons and four introns and are convergently transcribed (Fig. 1). The sequence of 16,646 nucleotides of the locus has been determined. Fig. 2 shows only 2760 nucleotides that contain *CP-ACC1A* and parts its 5′ and 3′ flanking regions. The nucleotide sequence of *CP-ACC1A* exons is identical to that of pACC1 cDNA (9) except for a single nucleotide change at position 1215 (Fig. 2), where cytidine is present instead of guanosine, encoding Arg-177 (instead of Gly-177). This difference probably reflects allelic variation or alteration during cloning.

Table 1 summarizes and compares the structural characteristics of *CP-ACC1A* and *CP-ACC1B*. Their exons are

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tcatggtttgaactttgaaaagttgaatttttctacacgtttgattttcctggtaagaacttgatcttgttggatcttcctcactgcttataaattcactcaattctcttctttctttcctt   -1
ATCTTACAACCCAAAACCTCTCATTTTTAGGCACATCTCAACAACTTTCAAATGGGGTTTCATCAAATCGACGAAAGGAACCAAGCTCTTCTCTCGAAGATCGCCCTCGACGATGGCCAT   120
                                                    M  G  F  H  Q  I  D  E  R  N  Q  A  L  L  S  K  I  A  L  D  D  G  H    23
GGCGAGAACTCCCCGTATTTCGATGGGTGGAAAGCTTACGATAACGATCCGTTTCACCCTGAGAATAATCCTTTGGGTGTTATTCAAATGGGTTTAGCAGAAAATCAGgtttggtatatc
 G  E  N  S  P  Y  F  D  G  W  K  A  Y  D  N  D  P  F  H  P  E  N  N  P  L  G  V  I  Q  M  G  L  A  E  N  Q              59
gtgttttcgtgtttttcttatatgacttcacgtttgaaaatttcgctaactttgttttttttgtgaatttcgatagCTTTCCTTTGATATGATTGTTGACTGGATTAGAAAACACCCTGAA
                                                                                                  L  S  F  D  M  I  V  D  W  I  R  K  H  P  E    74
GCTTCGATTTGTACACCGGAAGGACTTGAGAGATTCAAAAGCATTGCCAACTTCCAAGATTACCACGGCTTACCAGAGTTTCGAAATgtacgagatatgatatactcttaactatatctg
 A  S  I  C  T  P  E  G  L  E  R  F  K  S  I  A  N  F  Q  D  Y  H  G  L  P  E  F  R  N                                  103
aactcaaaaggttaagttgatgggttatgataaaatttctttcttgtcagGCAATTGCAAATTTTATGGGGAAAGTAAGAGGTGGGAGGGTAAAATTCGACCCGAGTCGGATTGTGATGG   600
                                                    A  I  A  N  F  M  G  K  V  R  G  G  R  V  K  F  D  P  S  R  I  V  M    126
GTGGCGGTGCGACCGGAGCGAGCGAAACCGTCATCTTTTGTTTGGCGGATCCGGGGGATGCTTTTTTGGTTCCTTCTCCATATTATGCAGGgtgagttcttctttcatttccttttgttc
 G  G  G  A  T  G  A  S  E  T  V  I  F  C  L  A  D  P  G  D  A  F  L  V  P  S  P  Y  Y  A  G                              157
acttttctttaagtcaatattccttagtccaacctggaaagagaaagaagagagagaaagaaaccatttgacaaattaataactctacaaattcctctttgaaagtttgatgttttttttta
aggtcaaaacttcaaccattctcttgcaaagaaaaaaaaaagtcataattatatgaagaaaaaactaggccatccaagtcaacctttttaaatgctaataaagtcaatatgctttgtag
gtttaaaaaacaataaattgcttaatcatttcttaaattttaattaaaccctttgacttttatcattacccatttacataaattaacaatttattgctctttttgtagtaaaattaataa
aaaaaaagttaggtgtaaacgtacagtattatgttattgtaaaaatactgagaagtgttagtatgttgttttcagATTTGATCGAGACTTGAAATGGCGAACACGAGCACAAATAATTC   1200
                                                                                  F  D  R  D  L  K  W  R  T  R  A  Q  I  I    171
GGGTCCATTGCAACCGCTCGAATAACTTCCAAGTCACAAAGGCAGCCTTAGAAATAGCCTACAAAAAGGCTCAAGAGGCCAACATGAAAGTGAAGGGTGTTATAATCACCAATCCCTCAA
R  V  H  C  N  R  S  N  N  F  Q  V  T  K  A  A  L  E  I  A  Y  K  K  A  Q  E  A  N  M  K  V  K  G  V  I  I  T  N  P  S    211
ATCCCTTAGGCACAACGTACGACCGTGACACTCTTAAAACCCTCGTCACCTTTGTGAATCAACACGACATTCACTTAATATGCGATGAAATATACTCTGCCACTGTCTTCAAAGCCCCAA
N  P  L  G  T  T  Y  D  R  D  T  L  K  T  L  V  T  F  V  N  Q  H  D  I  H  L  I  C  D  E  I  Y  S  A  T  V  F  K  A  P    251
CCTTCACCAGCATCGCTGAGATTGTTGAACAAATGGAGCATTGCAAGAAGGAGCTCATCCATATTCTTTATAGCTTGTCCAAAGACATGGGCCTCCCTGGTTTTCGAGTTGGAATTATTT
T  F  T  S  I  A  E  I  V  E  Q  M  E  H  C  K  K  E  L  I  H  I  L  Y  S  L  S  K  D  M  G  L  P  G  F  R  V  G  I  I    291
ATTCTTACAACGATGTCGTCGTCCGCCGTGCTCGGCAGATGTCGAGCTTCGGCCTCGTCTCGTCCCAGACTCAACATTTGCTCGCCGCCATGCTTTCCGACGAGGACTTTGTCGACAAAT
Y  S  Y  N  D  V  V  V  R  R  A  R  Q  M  S  S  F  G  L  V  S  S  Q  T  Q  H  L  L  A  A  M  L  S  D  E  D  F  V  D  K    331
TTCTTGCCGAGAACTCGAAGCGTGTGGGCGAGAGGCATGCAAGgtttgttaaactacaccattattatttgtgggattgaaaagcattacaaaatgcaattaatttaagaatgtattaat   1800
F  L  A  E  N  S  K  R  V  G  E  R  H  A  R                                                                                346
caaattcagGTTCACAAAAGAATTGGATAAAATGGGGATCACTTGCTTGAACAGCAATGCTGGAGTTTTTGTGTGGATGGATCTACGGAGGCTATTAAAAGACCAAACCTTCAAAGCTGA
         F  T  K  E  L  D  K  M  G  I  T  C  L  N  S  N  A  G  V  F  V  W  M  D  L  R  R  L  L  K  D  Q  T  F  K  A  E    383
AATGGAGCTTTGGCGTGTGATTATCAATGAAGTCAAGCTCAATGTTTCTCCTGGCTCATCCTTTCATGTCACTGAGCCAGGTTGGTTTCGAGTTTGTTTCGCAAACATGGACGACAACAC
  M  E  L  W  R  V  I  I  N  E  V  K  L  N  V  S  P  G  S  S  F  H  V  T  E  P  G  W  F  R  V  C  F  A  N  M  D  D  N  T    423
CGTTGACGTTGCTCTCAATAGAATCCATAGCTTTGTCGAAAACATCGACAAGAAGGAAGACAATACCGTTGCAATGCCATCGAAAACGAGGCATCGAGATAATAAGTTACGATTGAGCTT
  V  D  V  A  L  N  R  I  H  S  F  V  E  N  I  D  K  K  E  D  N  T  V  A  M  P  S  K  T  R  H  R  D  N  K  L  R  L  S  F    463
CTCCTTCTCAGGGAGAAGATACGACGAGGGCAACGTTCTTAACTCACCGCACACGATGTCGCCTCACTCGCCGTTAGTAATAGCAAAAAATTAATTAAAAACATTTTTCAAAATATTCAT
  S  F  S  G  R  R  Y  D  E  G  N  V  L  T  N  S  P  H  T  M  S  P  H  S  P  L  V  I  A  K  N  *                          493
ACCATTCATATAGTTTTTTTTTTTTTTTTTTTTTTTTTTTGGGTCAATGTTGACTAAAGTTACCTATATTTTTTCCACAGTGGATATGATGTAAACTTCATATTTTTTTGGTTGCAATTGTAAAT   2400
GTAATGTATTTGGTTTTTGGCTTAGGGAACTCATACTTTATTTATAATCAATCAATGATTGTGATTTATGAattataattgtatatttttctttaaaagtatttattgcaaaatataaaaaag
tattatgaggaattgtaattgaatggaaaaggtatagagtcaaaggcataaaacatatattttatttttcttatggaagtttgttcatacttaaaatgtattatatttatggaaaactt
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FIG. 2. Complete DNA sequence of the *CP-ACC1A* gene including introns and parts of the flanking regions. The sequence of the mRNA transcribed by the gene is presented in capital letters; the remainder of the sequence is in small letters. The 11 boxed residues are the invariant amino acids conserved in various aminotransferases (20). The underlined dodecapeptide beginning at Ser-278 is part of the active site of ACC synthase (21). The underlined bases beginning at position +52 show the acc1 primer (30-mer) used for determining the start of transcription. The TATA box and three potential polyadenylylation signal sequences are boxed.

Table 1. Structural characteristics of the ACC synthase genes

Region	Length, bp		Identity,* %
	CP-ACC1A	*CP-ACC1B*	
5′ flanking	2500	2500	51
5′ untranslated	51	64	94.1
Exon 1	177	177	93.8 (94.9)
Intron 1	87	87	92
Exon 2	132	132	92.4 (90.9)
Intron 2	83	88	83.1
Exon 3	161	161	93.8 (94.9)
Intron 3	465	1258	81.3
Exon 4	567	567	97.9 (97.9)
Intron 4	86	86	100
Exon 5	445	448	98.4 (97.3)
3′ untranslated	214	214	69.1
3′ flanking	1900	1900	50

*The number in parentheses is the protein sequence identity.

highly homologous (92–98%) and generate amino acid sequences that are 95% similar. Their coding regions are interrupted at identical positions by four highly conserved introns of comparable length, except for intron 3 whose length differs greatly (Table 1). The sequences at the intron/exon boundaries are typical of donor and acceptor splice sites (22). The conservation of nucleotide sequence and intron number and position implies that the paired gene organization arose via duplication and subsequent gene inversion of a primordial gene. The inverted orientation of closely spaced homologous genes may place several useful constraints on DNA evolution by preventing gene loss by unequal crossing-over (23). Furthermore, the virtual sequence identity between the coding regions of the two genes suggests that the gene pair has evolved in a concerted fashion by recurrent gene conversion (24).

We have determined the transcription initiation site of *CP-ACC1A* by S1 nuclease protection (Fig. 3*A*), primer extension (Fig. 3*C*), and sequence determination of the primer-extended product (Fig. 3*D*) using poly(A)$^+$ RNA from wounded (control) and induced fruit tissue. *CP-ACC1B* is not transcribed under these conditions (see Fig. 4). The 5′ untranslated region of the mRNA defined by the data in Fig. 3 is present in the full-length cDNA pACC2 (unpublished sequence) except for the first nucleotide. There is a TATAAAT sequence starting at position −26 (Fig. 2) that qualifies as a TATA box (22). The 3′ end of the gene was determined by comparing its nucleotide sequence with that of two nearly full-length cDNAs, pACC1 and pACC2. pACC2 has two additional nucleotides at the end of its 3′ untranslated region compared with pACC1, followed by a poly(A) tail 83 residues long. A potential polyadenylylation signal sequence AATGAA (25) is present 17 nucleotides upstream from the polyadenylylation site (Fig. 2). Thus, the mRNA transcribed by the *CP-ACC1A* gene is 1743 nucleotides long, close to the reported size of ACC synthase mRNA of 1900 nucleotides (8). The structure of *CP-ACC1B* shown in Fig. 1 is tentative. It awaits the isolation of full-length mRNA transcribed by the gene. We have not yet found developmental stages or inducers that activate it. However, its DNA sequence does not reveal any characteristics of a pseudogene (23). The 5′ and 3′ flanking regions of the genes are quite divergent (Table 1), which raises the possibility of differential regulation (see Fig. 4). Thirteen arrays of direct repeats ranging in size from 8 to 30 bp are present in the 5′ upstream region of *CP-ACC1B* and may be possible control elements. The most striking feature of the intergenic region is the presence of a long (≈500 bp), imperfect, inverted repeat that could form a hairpin structure (see Fig. 1). A prominent feature of the *CP-ACC* locus is the presence of long oligo(dT) tracts (26).

Expression Characteristics. Wounding intact fruit causes the accumulation of ACC synthase mRNA, and this is greatly enhanced by IAA/BzlAde/LiCl (ref. 8; and Fig. 4*a* lanes 4–6 in *A*). Using gene-specific probes, we determined that the induced mRNA is the transcriptional product of *CP-ACC1A; CP-ACC1B* appears not to be expressed (Fig. 4*a*, compare lanes 5 and 6 in *B* with those in *C*). Wounding etiolated hypocotyls also induces ACC synthase mRNA (Fig. 4*a*, compare lanes 1 and 2 in *A*). This wound-induced mRNA is also the transcriptional product of *CP-ACC1A* (Fig. 4*a*, compare lanes 2 and 3 in *B* with those in *C*). However, while treatment of hypocotyls with IAA/BzlAde enhances ethylene evolution 30-fold (data not shown), the ACC synthase mRNA level is not enhanced under these conditions (Fig. 4*a*, compare lanes 2 and 3 in *A*). These data suggest that IAA/BzlAde may induce another nonhomologous gene(s) in hypocotyl tissue. This proposition is supported by the following experimental evidence: (*i*) low-stringency hybridization of genomic DNA with pACC1 cDNA detects numerous DNA fragments (data not shown), indicating the presence of a multigene family in

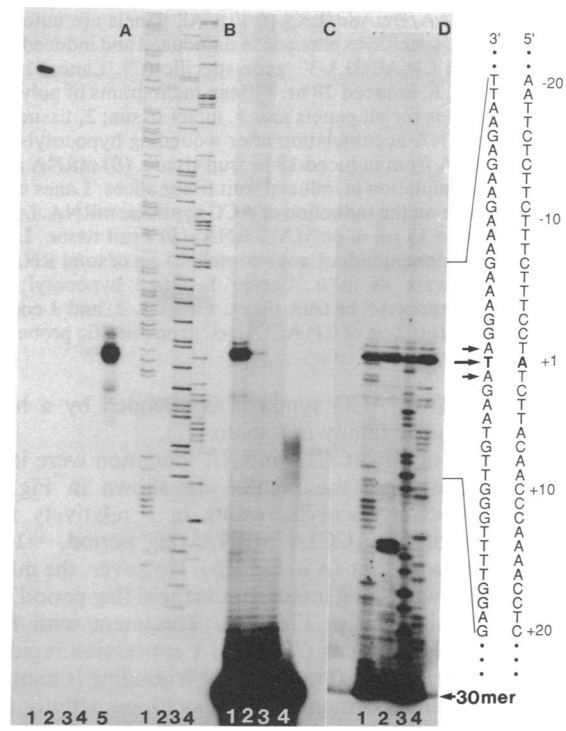

FIG. 3. Determination of the transcription initiation site of *CP-ACC1A*. (*A*) S1 nuclease protection. A 5′-end-labeled 30-mer synthetic oligonucleotide, acc1, complementary to nucleotides +52 to +81 (Fig. 2) was hybridized to M13mp18 single-stranded DNA of the 5′ proximal 1.1-kb *Eco*RI/*Bam*HI fragment from *CP-ACC1A* (Fig. 1) and extended with the Klenow fragment. The reaction was digested with *Bst*NI, which cleaves at position −70 (Fig. 2). The 151-nt single-stranded probe was recovered and used for S1 digestions with 2 μg of poly(A)$^+$ RNA from intact fruit (lane 3), uninduced-18 hr (lane 4), and induced-18 hr tissue (lane 5). Lanes 1 and 2 contain the undigested and S1-digested probe alone, respectively. (*B*) Dideoxy sequencing reactions with the 1.1-kb *Eco*RI/*Bam*HI fragment (see *A*) after priming with the acc1 primer. Lanes 1–4: reactions A, C, G, and T, respectively. (*C*) Primer extension analysis. 5′-end ^{32}P-labeled acc1 primer was hybridized with 2 μg of mRNA from induced-18 hr (lane 1), uninduced-18 hr fruit tissue (lane 2), and intact fruit (lane 3), and was extended with reverse transcriptase (16). Lane 4 is the control without RNA. (*D*) Nucleotide sequencing of the 5′ end of mRNA. 5′ end ^{32}P-labeled acc1 primer was hybridized with 30 μg of poly(A)$^+$ RNA from induced-18 hr fruit tissue and extended with reverse transcriptase in the presence of dNTP/ddNTPs (17). The lanes 1–4 are as in *B*. The bold residue represents the major start of transcription.

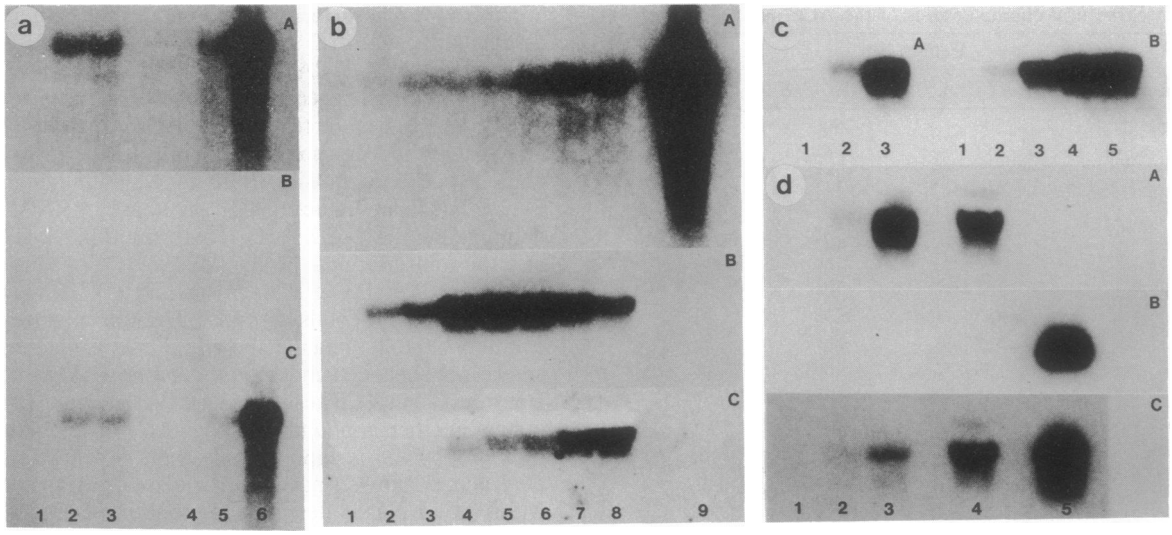

FIG. 4. Expression of ACC synthase genes in etiolated hypocotyls and fruit tissue after wounding and treatment with IAA + BzlAde (hypocotyl) or IAA/BzlAde/LiCl (fruit). All panels are autoradiograms of RNA filters hybridized with ^{32}P-labeled pACC1 or gene-specific probes. (*a*) Gene-specific expression in uninduced and induced hypocotyl and fruit tissue slices. Probes: pACC1 cDNA (*A*); CP-ACC1B-3′, gene-specific (*B*); and CP-ACC1A-3′, gene-specific (*C*). Lanes: 1, intact hypocotyl; 2, uninduced-6 hr; 3, induced-6 hr; 4, intact fruit tissue; 5, uninduced-18 hr; 6, induced-18 hr. Fifteen micrograms of poly(A)$^+$ RNA are in lanes 1, 2, and 3, and 2 μg are in lanes 4, 5, and 6. (*b*) Induction kinetics. The lanes for all panels are: 1, intact tissue; 2, tissue treated 0 hr; 3, 1 hr; 4, 2 hr; 5, 3 hr; 6, 4 hr; 7, 5 hr; 8, 6 hr (0 hr = 10 min from cutting). (*A*) mRNA accumulation after wounding hypocotyls. Lanes contain 20 μg of poly(A)$^+$ RNA except for lane 9, which contains 1 μg of poly(A)$^+$ RNA from induced-18 hr fruit tissue. (*B*) mRNA accumulation after wounding intact fruit. Lanes contain 20 μg of poly(A)$^+$ RNA. (*C*) mRNA accumulation in induced fruit tissue slices. Lanes contain 25 μg of total RNA. The probe for all panels was pACC1 cDNA. (*c*) Effect of cycloheximide on the induction of ACC synthase mRNA. (*A*) Hypocotyl tissue. Lanes: 1, intact tissue; 2, uninduced-5 hr; 3, cycloheximide-5 hr. Lanes contain 15 μg of poly(A)$^+$ RNA. (*B*) Fruit tissue. Lanes: 1, intact tissue; 2, uninduced-5 hr; 3, induced-5 hr; 4, cycloheximide-5 hr; 5, induced + cycloheximide. Lanes contain 25 μg of total RNA. Probe: pACC1 cDNA. (*d*) Gene-specific expression in cycloheximide-induced tissue. Probes were as in *a*. Lanes: 1, intact hypocotyl; 2, uninduced-5 hr hypocotyl; 3, cycloheximide-induced-5 hr hypocotyl; 4, cycloheximide-induced-5 hr fruit slices. Lanes 1, 2, and 3 contain 15 μg of poly(A)$^+$ RNA per each and lane 4 contains 25 μg of total RNA. Lane 5 in *B* contains 2 ng of CP-ACC1B-3′ gene-specific probe and in *C* contains 1 μg of induced fruit poly(A)$^+$ RNA for positive controls; there is no lane 5 in *A*.

Cucurbita; and (*ii*) ACC synthase is encoded by a highly divergent multigene family in tomato (27).

The kinetics of CP-ACC1A mRNA induction were investigated in detail, and the results are shown in Fig. 4*b*. Wounding intact hypocotyls results in a relatively rapid accumulation of CP-ACC1A mRNA (lag period, ≈1 hr), which is linear with time (*A* in Fig. 4*b*). However, the mRNA induction after wounding intact fruit is rapid (lag period, ≈10 min) and transient (*B* in Fig. 4*b*). Treatment with IAA/BzlAde/LiCl also induces *CP-ACC1A* expression rapidly in fruit (lag period, ≈1 hr; *C* in Fig. 4*b*). Wounding is known to result in rapid enhancement of ACC synthase activity (3, 5, 6). Our data clearly show that this is due to the enhancement of ACC synthase mRNA (*B* in Fig. 4*b*). The rapidity of mRNA accumulation in fruit tissue is the fastest reported for any wound-induced mRNA (28–31) and is comparable to that reported for the auxin-induced gene expression (10). We attribute the mRNA accumulation to transcriptional activation (32); however, a posttranscriptional event may also be involved. There is a considerable history of the influence of ethylene and wounding on metabolic processes in plants that indicates that these distinct signals activate similar stress responses (2, 33). Foremost amongst identified genes activated by ethylene and/or by wounding are those encoding defense-response proteins (28–32). The possibility exists that the wound-induced ethylene production is the trigger for the wound-induced gene activation. This can be tested by inhibiting wound-induced ACC synthase gene expression by antisense RNA (34) or by gene transplacement (35).

For the rapid mRNA induction to qualify as a primary response, it has to be insensitive to protein synthesis inhibition (10). *A* in Fig. 4*c* shows that cycloheximide not only does not inhibit the wound-induced mRNA accumulation in hypocotyl tissue but also actually superinduces it (Fig. 4*c*, compare lanes 2 and 3 in *A*). Similarly, cycloheximide alone induces ACC synthase mRNA in fruit tissue (Fig. 4*c*, compare lanes 2 and 4 in *B*) and does not prevent the IAA/BzlAde/LiCl-mediated mRNA induction (Fig. 4*c*, compare lanes 3 and 5 in *B*). Using gene specific probes, we were able to demonstrate that the cycloheximide-induced mRNA is the transcriptional product of the *CP-ACC1A* gene in fruit tissue (Fig. 4*d*, compare lane 4 in *B* and *C*). However, in hypocotyl tissue *CP-ACC1A* is a minor contributor of cycloheximide-induced mRNA (Fig. 4*d*, compare lanes 3 and 4 in *A* with those in *C*). These results suggest that cycloheximide induces other unidentified ACC synthase genes in hypocotyl tissue.

Protein synthesis inhibition has been shown to enhance the level of various hormonally regulated mammalian genes (36) as well as some auxin-regulated genes in pea and soybean (10, 37). One possibility is that *CP-ACC1A* may be under the control of a short-lived repressor protein. Addition of cycloheximide could result in a decrease in the level of the repressor protein (10); or, it may alter its function (38). Genetic evidence for the repressor model is the ethylene-

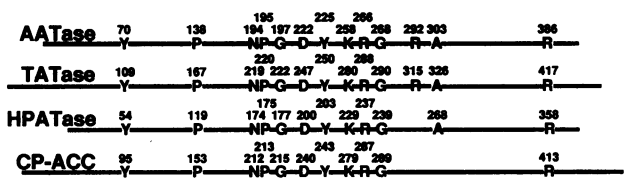

FIG. 5. Amino acid residues conserved among aminotransferases and ACC synthase. The AATase, tyrosine aminotransferase (TATase), and histidinol phosphate (HPATase) residues are numbered according to cytosolic pig, rat, and *E. coli* enzymes, respectively (20). The ACC synthase residues are those encoded by the *CP-ACC1A* gene (Fig. 2).

overproducing recessive mutant *etol-1* in *Arabidopsis thaliana* (39). Protein synthesis inhibition may also prevent mRNA degradation by preventing the synthesis of a labile nuclease. Thus, cycloheximide may have a dual effect—activation of transcription and mRNA stabilization.

ACC Synthase Coding Sequences. The amino acid sequences of CP-ACC1A and CP-ACC1B polypeptides, deduced from the DNA sequence of the genes, are comprised of 493 (M_r 55,878; pI 7.07) and 494 (M_r 55,922; pI 7.68) amino acids, respectively, and show 95% identity. Their identity to a *Cucurbita maxima* enzyme (ref. 40; 493 amino acids, M_r 55,895; pI 6.81) is 95%; their identity to a tomato enzyme (ref. 41; 485 amino acids, M_r 54,694; pI 7.71) is 67%. The evolution of the ACC synthase polypeptide is discussed elsewhere (27). The size of the predicted polypeptide agrees with that estimated by *in vivo* labeling and immunoprecipitation studies (9). The *C. pepo* enzyme exists *in vivo* as a homodimer of ≈111,600 Da (9).

ACC synthase is a pyridoxal phosphate-requiring enzyme, and most such enzymes have a lysine residue in their active site (42). There are three invariant lysine residues among 10 ACC synthase genes so far sequenced from various plants (27). It has been shown (21) that *S*-adenosylmethionine can inactivate ACC synthase by covalently binding to one of these lysine residues. The amino acid sequence of a dodecapeptide at the site of attachment was found to be Ser-Leu-Ser-Lys-Asp-Leu-Gly-Leu-Pro-Gly-Phe-Arg (21). This sequence is present in CP-ACC1A (Fig. 2) and CP-ACC1B (data not shown) polypeptides and contains Lys-279, one of the three invariant lysine residues. Interestingly, the pyridoxal phosphate-binding site of several aminotransferases contains some of the residues surrounding Lys-279 (Fig. 5). In particular, the pig aspartate aminotransferase (AATase) is about 45 kDa and binds pyridoxal phosphate at Lys-258, which is roughly at the same region of the polypeptide as in ACC synthase (Fig. 5). The most homologous sequence containing Lys-258 of ACC synthase is found in the mammalian mitochondrial AATases (Ser-Tyr-Ala-Lys-Asn-Met-Gly-Leu-Tyr-Gly-Glu-Arg-Val-Gly, ref. 20; identical residues are underlined, see Fig. 2).

Among various aminotransferases, such as AATase, tyrosine aminotransferase, and histidinol-phosphate aminotransferase, only 12 amino acid residues are completely conserved (20). Remarkably, all but 1 of these amino acids are found in the identical places in all known ACC synthases (Fig. 5). From x-ray crystallographic studies of AATase, Lys-258 and the neighboring Arg-266 and Gly-268 (Lys-279, Arg-287, and Gly-289 in ACC synthase, Fig. 5) are known to be involved in binding of pyridoxal phosphate (20). It is also possible that Tyr-95, Asn-212, Asp-240, and Tyr-243 of CP-ACC1A may correspond to Tyr-70, Asn-194, Asp-222, and Tyr-225 of AATase (Fig. 5). Arg-413 of ACC synthase, which is in the middle of the largest block of completely conserved amino acids (27), may function as Arg-386 of AATase in binding the carboxyl group of the substrate. The three remaining residues, Pro-153, Pro-213, and Gly-215, would therefore correspond to Pro-138, Pro-195, and Gly-197 of AATase to maintain the proper three-dimensional structure of the enzyme (20). It should be pointed out that if AATase is used as a model for the structure of ACC synthase, it is predicted that the synthase must dimerize to function because the active site is shared between two subunits. This prediction is supported by experimental evidence that the *C. pepo* enzyme is a homodimer (9). However, there is no recognizable homology between aminotransferases and ACC synthase other than the residues discussed. Consequently, analysis of the three-dimensional structure of ACC synthase will be necessary to substantiate an evolutionary relationship between these enzymes.

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1. Abeles, F. B. (1973) *Ethylene in Plant Biology* (Academic, Orlando, FL).
2. Yang, S. F. & Hoffman, N. E. (1984) *Annu. Rev. Plant Physiol.* **35**, 155–189.
3. Boller, T. & Kende, H. (1980) *Nature (London)* **286**, 259–260.
4. Yu, Y. B., Adams, D. O. & Yang, S. F. (1979) *Plant Physiol.* **63**, 589–590.
5. Yu, Y. B. & Yang, S. F. (1980) *Plant Physiol.* **66**, 281–285.
6. Konze, J. R. & Kwiatkowski, G. M. K. (1981) *Planta* **151**, 327–330.
7. Hyodo, H., Tanaka, K. & Watanabe, K. (1983) *Plant Cell Physiol.* **24**, 963–969.
8. Sato, T. & Theologis, A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6621–6625.
9. Sato, T., Oeller, P. W. & Theologis, A. (1991) *J. Biol. Chem.* **226**, 3752–3759.
10. Theologis, A., Huynh, T. V. & Davis, R. W. (1985) *J. Mol. Biol.* **183**, 53–68.
11. Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1**, 19–21.
12. Davis, R. W., Botstein, D. & Roth, J. R. (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
13. Frischauf, A. M., Lehrach, H., Poustka, A. & Murray, N. (1983) *J. Mol. Biol.* **170**, 827–842.
14. Benton, W. D. & Davis, R. W. (1977) *Science* **196**, 180–182.
15. Buchman, A., Fromm, M. & Berg, P. (1983) *Mol. Cell. Biol.* **1**, 1900–1914.
16. Bond, B. J. & Davidson, N. (1986) *Mol. Cell. Biol.* **6**, 2080–2088.
17. MacDonald, R. J., Swift, G. H., Quinto, C., Swain, W., Pictet, R. L., Nicovits, W. & Rutter, W. J. (1982) *Biochemistry* **21**, 1453–1463.
18. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
19. Messing, J. (1983) *Methods Enzymol.* **101**, 20–78.
20. Mehta, P. K., Hale, T. I. & Christen, P. (1989) *Eur. J. Biochem.* **186**, 249–253.
21. Yip, W. K., Dong, J. G., Kenny, J. W., Thompson, G. A. & Yang, S. F. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7930–7934.
22. Breathnach, R. & Chambon, P. (1981) *Annu. Rev. Biochem.* **50**, 349–383.
23. Snyder, M., Hunkapiller, M., Yuen, D., Silvert, D., Fristrom, J. & Davidson, N. (1982) *Cell* **29**, 1027–1040.
24. Hicket, D. A., Cuif, L. B., Abukashawa, S., Payant, V. & Benkel, B. F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1611–1615.
25. Nevins, J. R. (1983) *Annu. Rev. Biochem.* **52**, 441–465.
26. Struhl, K. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8419–8423.
27. Rottmann, H. R., Peter, F. G., Oeller, W. P., Keller, A. J., Shen, F. N., Nagy, P. B., Taylor, P. L., Campbell, A. D. & Theologis, A. (1991) *J. Mol. Biol.*, in press.
28. Bell, J. N., Ryder, T. B., Wingate, V. P. M., Bailey, J. A. & Lamb, C. J. (1986) *Mol. Cell. Biol.* **6**, 1615–1623.
29. Ecker, J. R. & Davis, R. W. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5202–5206.
30. Dyer, W. E., Henstrand, J. M., Handa, A. K. & Herrmann, K. M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7370–7373.
31. Graham, J., Hall, G., Pearce, G. & Ryan, C. A. (1986) *Planta* **169**, 399–405.
32. Lawton, M. A. & Lamb, C. J. (1987) *Mol. Cell. Biol.* **7**, 335–341.
33. Theologis, A. & Laties, G. G. (1982) *Plant Physiol.* **69**, 1031–1035.
34. Ecker, J. R. & Davis, R. W. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5372–5376.
35. Scherer, S. & Davis, R. W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4951–4955.
36. Atwater, J. A., Wisdom, R. & Verma, I. M. (1990) *Annu. Rev. Genet.* **24**, 519–541.
37. Franco, A. R., Gee, M. A. & Guilfoyle, T. J. (1990) *J. Biol. Chem.* **265**, 15845–15849.
38. Sen, R. & Baltimore, D. (1986) *Cell* **47**, 921–928.
39. Guzman, P. & Ecker, J. R. (1990) *Plant Cell* **2**, 513–523.
40. Nakajima, N., Mori, H., Yamazaki, K. & Imaseki, H. (1990) *Plant Cell Physiol.* **31**, 1021–1029.
41. Van der Straeten, D., Van Wiemeersch, L., Goodman, H. M. & Van Montagu, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4859–4863.
42. Hayashi, H., Wada, H., Yoshimura, T., Esaki, N. & Soda, K. (1990) *Annu. Rev. Biochem.* **59**, 87–110.