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Biochemistry

The 1-aminocyclopropane-1-carboxylate synthase gene family of *Arabidopsis thaliana*

(ethylene biosynthesis/gene regulation/auxin/cycloheximide/gene evolution)

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ABSTRACT **Genomic sequences encoding five divergent 1-aminocyclopropane-1-carboxylic acid (ACC) synthase polypeptides (*ACC1, ACC2, ACC3, ACC4,* and *ACC5*) have been isolated from *Arabidopsis thaliana* by using heterologous cDNAs and PCR fragments amplified from genomic DNA with degenerate oligonucleotide primers. Each gene is located on a different chromosome in the *Arabidopsis* genome. The genes are differentially expressed during development and in response to environmental stimuli. Protein-synthesis inhibition derepresses the expression of all genes but most dramatically derepresses that of *ACC2*, suggesting that their expression may be under negative control. The sequence of *ACC2* was determined, and its transcription initiation site was defined. Authenticity of the polypeptide encoded by the gene was confirmed by expression experiments in *Escherichia coli*. The predicted size of the protein is 55,623 Da, and it contains the 11 invariant amino acid residues conserved between aminotransferases and ACC synthases from various plant species. Comparative analysis of structural and expression characteristics of ACC synthase genes from *Arabidopsis* and other plant species suggests that the sequence divergence of the ACC synthase genes and possibly the distinct regulatory networks governing the expression of ACC synthase subfamilies arose early in plant evolution and before the divergence of monocots and dicots.**

Ethylene is one of the simplest organic molecules with biological activity. It is involved in controlling many aspects of plant growth and development (1, 2). The rate-limiting step in the biosynthesis of ethylene is the conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene, catalyzed by the enzyme ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14). ACC synthase activity is highly regulated during plant development and is induced in response to a wide variety of environmental stimuli—including wounding, anaerobiosis, viral infection, and elicitor treatment (2–5). Ethylene itself, as well as other plant hormones, such as auxin, are also known to enhance ethylene production and ACC synthase activity (1, 2). Recent studies using cloned ACC synthase genes from several plants indicate that the induction of the enzyme activity is due to an increase in the accumulation of its mRNA (6–13). We are interested in elucidating the multiple signal-transduction pathways responsible for the enhanced ACC synthase gene expression by a diverse group of inducers and, in particular, by auxin and cycloheximide (CHX) (7) using biochemical and molecular genetic approaches (reverse genetics). We will attempt to develop the molecular genetic approaches in *Arabidopsis thaliana* (14, 15). As a first step toward this goal we report the isolation and expression characteristics of the multigene family encoding ACC synthase in *Arabidopsis*. Furthermore, the structure of the highly expressed gene *ACC2* has been determined.‡

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MATERIALS AND METHODS

Plant Material and Tissue Treatment. Five-day-old etiolated seedlings (*A. thaliana*, ecotype Columbia) were grown as described (16). The seedlings were incubated in liquid Murashige and Skoog media (GIBCO, with 1% sucrose and 0.5 mM *myo*-inositol) in the dark at 25°C in the absence or presence of 50 μM indoleacetic acid, 10 mM LiCl, or 50 μM CHX. For wound induction, 5-mm-long root and hypocotyl segments were incubated in liquid Murashige and Skoog medium at 22°C in the dark. Different organs from 25-day-old light-grown plants (16) were harvested and stored at −70°C.

Nucleic Acid Techniques. The *Arabidopsis* genomic library (ecotype Columbia) was from N. Crawford (University of California, San Diego). The DNA was cloned as partial *Sau*3A fragments into a *Bam*HI site of λ DASH (Stratagene). The library was screened under low-stringency conditions described by Rottmann *et al.* (13) with ^{32}P-labeled cDNA inserts of pACC1 [zucchini (6)], ptACC2 [tomato (13)], and *Arabidopsis* genomic PCR fragments (see below). The isolation, fractionation, and analysis of *Arabidopsis* genomic DNA and poly(A)$^+$ RNA, primer-extension with reverse transcriptase, and sequence determination of the primer-extended product were done essentially as described (6, 13, 17). The gene-specific probes used for RNA hybridization analysis shown in Fig. 2 were as follows: for *ACC1* and *ACC3*, the 4.5-kilobase (kb) *Sal* I–*Eco*RI fragment from λ clone AT-2 (Fig. 1 *II*, the genes show a high degree of homology in their coding regions, and thus their genomic fragments in λ clones AT-2 and λ AT-10 cross-hybridize); for *ACC2*, the 1.2-kb *Bam*HI fragment from λ clone AT-6; for *ACC4*, the 2.1-kb *Eco*RI fragment from λ clone AT-8, and for *ACC5*, the 1.7-kb *Eco*RI fragment from λ clone AT-15. Equal loading of mRNA was assessed by RNA hybridization by using a rice 17S ribosomal DNA probe (T. I. Zarembinski and A.T., unpublished work). The *ACC2* gene was sequenced by using the 4-kb *Eco*RI fragment from the genomic clone λ AT-6 (Fig. 1 *II*). Synthetic primers (20-mers) were used to sequence both strands of the double-stranded DNAs with [α-^{35}S]dATP (19) and Sequenase (United States Biochemical). Partial amino acid sequences deduced from PCR products corresponding to the N-terminal region of the ACC synthase polypeptide (amino acid residues 60–91, see Fig. 1*I*) were aligned to generate a phylogenetic tree by using parsimony after progressive alignment (PAPA) programs (20) from

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CHX, cycloheximide; nt, nucleotide(s).

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession numbers: M95594 (*ACC2*) and M95595 (pa*ACC2* cDNA).

R. F. Doolittle (University of California, San Diego). The aligned amino acid sequences were also used to generate a gene genealogy with a parsimony algorithm (PAUP) written for Macintosh computers (21).

PCR. The degenerate oligonucleotides TZ-1F (512-fold degeneracy) and TZ-1R (32-fold degeneracy) corresponding to the conserved amino acid sequences MGLAENQ and FQDYHG (see Fig. 1*I*) have been described (18). All reactions were done by using the *Taq* DNA polymerase from Perkin–Elmer/Cetus under conditions recommended by the supplier. Each reaction contained 10 ng of *Arabidopsis* genomic DNA or 1 ng of phage λ DNA and 200 ng of each primer and was done for 30 thermal cycles, each consisting of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C. The PCR fragments were purified twice by gel electrophoresis and adsorption to Schleicher & Schuell NA-45 paper, followed by PCR reamplification using the same set of primers to enrich the amplified DNA fragments for subsequent subcloning and labeling. The orientation of the genes was determined by PCR using plasmid (pUC18) subclones of the genomic sequences as a template and PCR amplimers containing one of the degenerate oligonucleotides (TZ-1F or TZ-2R) in combination with one of the M13 universal primers (forward or reverse). The paACC2 cDNA fragment was generated by PCR using a circular cDNA template synthesized with poly(A)$^+$ RNA from mature *Arabidopsis* plants, according to Huang *et al.* (22) and the following PCR amplimers (see Fig. 3 for location of amplimers): ACC2-5′, 5′-GTGCACTGCAGGCAAATGGTCTTCCGGG-3′ (90–107) and ACC2-3′, 5′-ACCGAATTCGTTATGCCTTAAGATTTTC-3′ (2281–2299). The cDNA was subcloned as a *Pst* I–*Eco*RI fragment into pUC18 and 19 vectors, and the two plasmids paACC2-8 (sense) and paACC2-9 (antisense) were used for expression experiment in *E. coli* as described (13). The sequence of one strand of the cDNA was also determined. An inverse PCR product was synthesized to determine the 3′ end of the *ACC2* gene with the same cDNA template and the following amplimers (see Fig. 3): ACC2R-5′, 5′-CTTCGACAAAACTGCACC-3′ (118–135) and ACC2R-3′, 5′-GATCGAGCTTTGGCCATAT-3′ (1943–1960). The product was subcloned into the *Eco*RV site of pIC20R and sequenced.

RESULTS AND DISCUSSION

Isolation of Genomic Sequences. We used two complimentary approaches for cloning *Arabidopsis* genomic sequences encoding ACC synthase. (*i*) Heterologous cDNAs from zucchini (6) and tomato (13) were used to screen an *Arabidopsis* genomic library at low stringency (13, 17). By screening 10^5 recombinant phages, 10 positive λ clones were isolated representing four groups of overlapping sequences. The partial restriction maps of four clones λ AT-2, λ AT-6, λ AT-10, and λ AT-8 representing the four groups are shown in Fig. 1*II*. (*ii*) Partially degenerate oligonucleotides were synthesized corresponding to amino acid sequences MGLAENQ and FQDYHG (Fig. 1*I*), which represent consensus sequences for discrete and highly conserved regions at the N terminus of various ACC synthases (13, 18). By using the degenerate primers and *Arabidopsis* genomic DNA, three distinct PCR fragments [P1, 320 base pairs (bp); P2, 250 bp; and P3, 150 bp] were obtained (Fig. 1 *IIIA*). The fragments were isolated, labeled, and used as probes for Southern analysis with genomic DNA. The results show that P1 and P3 fragments hybridize to single DNA fragments of *Arabidopsis* genome (Fig. 1, *III B* and *D*, respectively), whereas the P2 fragment hybridizes to three distinct DNA fragments (Fig. 1 *IIIC*). This result suggests that at least five genes in the *Arabidopsis* genome encode ACC synthase. Furthermore, Southern analysis with genomic DNA shows that the PCR fragments generated with degenerate oligonucleotides and

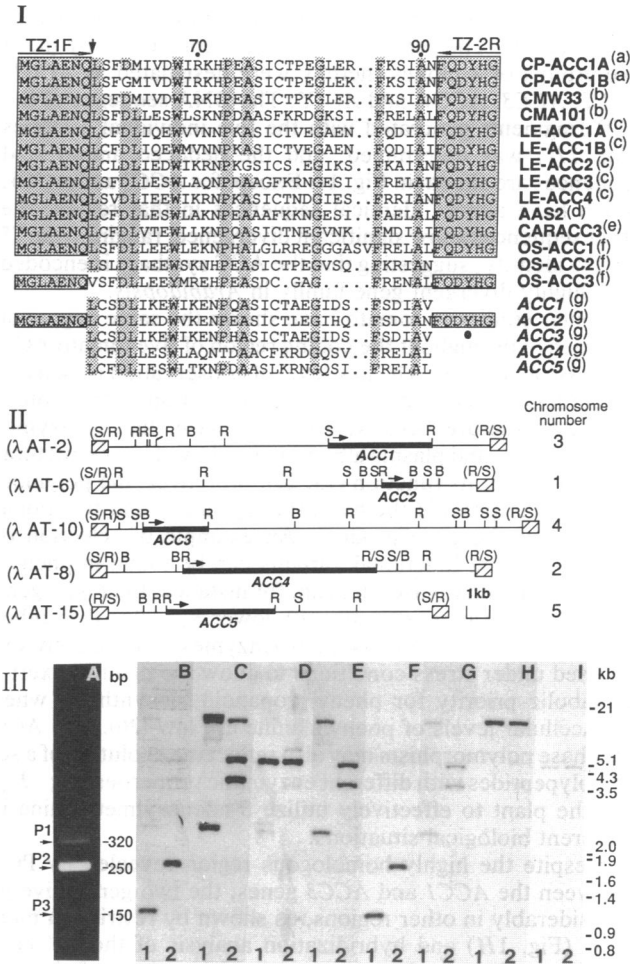

FIG. 1. Isolation of genomic sequences encoding ACC synthase in *Arabidopsis thaliana*. (*I*) Comparison of a highly conserved region (amino acid residues 53–97) of ACC synthase polypeptides among various plants. Right superscripts: a, zucchini (7), b, winter squash (10, 23); c, tomato (13); d, apple (11); e, carnation (24); f, rice (T. I. Zarembinski and A.T., unpublished work), and g, *Arabidopsis*. Completely conserved residues are shaded. Regions where degenerate primers have been designed (T. I. Zarembinski and A.T., unpublished work) are boxed. Vertical arrow indicates position of an intron; filled circle indicates one of the 11 invariant amino acids conserved between ACC synthase and aminotransferases (13). Numbering of residues is based on zucchini paACC1 polypeptide (7). The five *Arabidopsis* sequences were deduced from the DNA sequence of PCR products obtained with TZ-1F and TZ-2R primers and λ clones shown in *II*. (*II*) Restriction maps and chromosomal location of ACC synthase genes. Solid bars indicate fragments that contain the region corresponding to TZ-1F/TZ-2R PCR product. Hatched bars indicate vector. Direction of transcription is shown by arrow. Restriction sites are as follows: E, *Eco*RI; S, *Sal* I; and B, *Bam*HI. (*III*) Southern analysis. (*A*) PCR products generated with TZ-1F/TZ-2R primers and *Arabidopsis* genomic DNA. (*B–I*) Southern analysis of genomic DNA (4 μg per lane) digested with *Eco*RI/*Bam*HI (lanes 1) and *Eco*RI/*Sal* I (lanes 2). Probes are ^{32}P-labeled TZ-1F/TZ-2R PCR fragments generated either from genomic DNA: P1 (*B*), P2 (*C*), and P3 (*D*); or from individual λ clones: λ AT-2 (*E*), λ AT-6 (*F*), λ AT-10 (*G*), λ AT-8 (*H*), and λ AT-15 (*I*).

the individual isolated λ clones of Fig. 1*II* hybridize to the same genomic fragments as the P1 and P2 fragments. (Fig. 1, compare *III B–D* with *III E–H*). These results confirm that the four previously isolated genomic sequences correspond to PCR products present in the P1 (λ clone AT-6) and P2 (λ clone AT-8, λ AT-2, and λ AT-10) PCR fragments, respectively. Genomic sequences corresponding to the P3 genomic PCR product were isolated by screening a genomic library. A

partial restriction map of one of these clones is shown in Fig. 1*II* (λ AT-15). The genomic sequences represented by these five groups of λ clones thereby were designated as *ACC1*, *ACC2*, *ACC3*, *ACC4*, and *ACC5*. The DNA sequence of the PCR fragments generated with the five individual λ clones (Fig. 1*II*) was determined, and the deduced amino acid sequences are shown in Fig. 1*I*. Except for *ACC1* and *ACC3*, which have almost identical sequences in that region, the other sequences show significant divergence, ranging from 75 to 31% identity, suggesting that the ACC synthase is encoded by a highly divergent gene family in *Arabidopsis*.

Similar studies in tomato have shown the same degree of divergence throughout the coding region of five tomato ACC synthase genes (13). The low level of conservation is likely to reflect less selection pressure in the evolution of the protein primary structure because ACC synthase, like lysozyme, ribonuclease, and plasma albumin, may have fewer structural constraints for its function (13, 25). Moreover, the polymorphisms may provide the basis for regulatory and functional variants of the gene product. An example of selection of genes encoding functional variants can be found in phenylalanine ammonia-lyase. In bean, for instance, the *PAL3* gene that codes for an isoenzyme with lower K_m value than other phenylalanine ammonia-lyase isoenzymes is selectively expressed under stress conditions to allow the plant to exert a metabolic priority for phenylpropanoid biosynthesis when intracellular levels of phenylalanine are low (26). The ACC synthase polymorphism may also reflect the evolution of a set of polypeptides with different enzymatic properties (e.g., K_m) for the plant to effectively utilize *S*-adenosylmethionine in different biological situations.

Despite the highly homologous region revealed by PCR between the *ACC1* and *ACC3* genes, the two genes diverge considerably in other regions, as shown by restriction mapping (Fig. 1*II*) and hybridization analysis of their 3′ ends (data not shown). The chromosomal locations of the five genes were determined by restriction fragment length polymorphism analysis in Pablo Skolnik's laboratory (DuPont) and are shown in Fig. 1*II*. The data provide no evidence of clustering of the ACC synthase genes. This result contrasts markedly with the presence of a pair of closely linked and highly homologous genes in zucchini and tomato (7, 13). A possible explanation for this difference is that *Arabidopsis* may, by some unknown mechanism, constantly eliminate duplicated sequence repeats to maintain a compact genome.

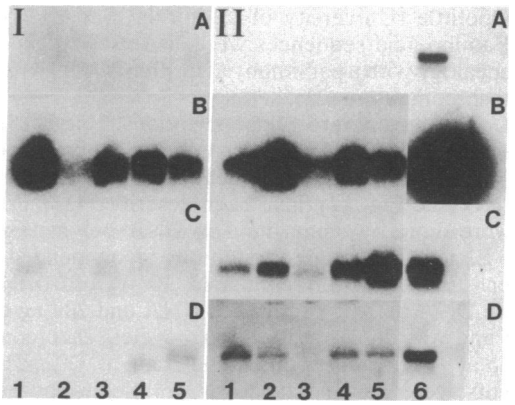

FIG. 2. Expression characteristics of the ACC synthase genes. (*I*). RNA hybridization analysis of mRNA isolated from various organs of light-grown plants (25-day-old). Lanes: 1, roots; 2, stems; 3, leaves; 4, flowers; and 5, siliques. (*II*) RNA hybridization analysis of mRNA isolated from 5-day-old etiolated seedlings treated with various inducers. Lanes: 1, control; 2, wounding; 3, anaerobiosis; 4, LiCl; 5, indoleacetic acid; 6, CHX. Duration of treatment was 8 hr. RNA filters in *I* and *II* were probed with ^{32}P-labeled gene-specific probes as follows: (*A*) *ACC1/3*, (*B*) *ACC2*, (*C*) *ACC4*, and (*D*) *ACC5*. Each lane in *I* and *II* contains 3 μg of poly(A)$^+$ RNA.

Differential Expression. Levels of the various *A. thaliana* ACC synthase mRNAs in different organs of light-grown plants are shown in Fig. 2*I A–D*. The ACC2 mRNA is expressed at high levels in roots, at moderate levels in leaves and flowers, at low levels in siliques, and is almost undetectable in stems (Fig. 2*IB*, lanes 1–5). On the other hand, the expression of the ACC1/ACC3, ACC4, and ACC5 mRNAs is much lower than that of ACC2 mRNA in all these organs (Fig. 2*I*, compare *A*, *C*, and *D* with *B*). However, differential patterns of expression are seen upon longer exposure. For

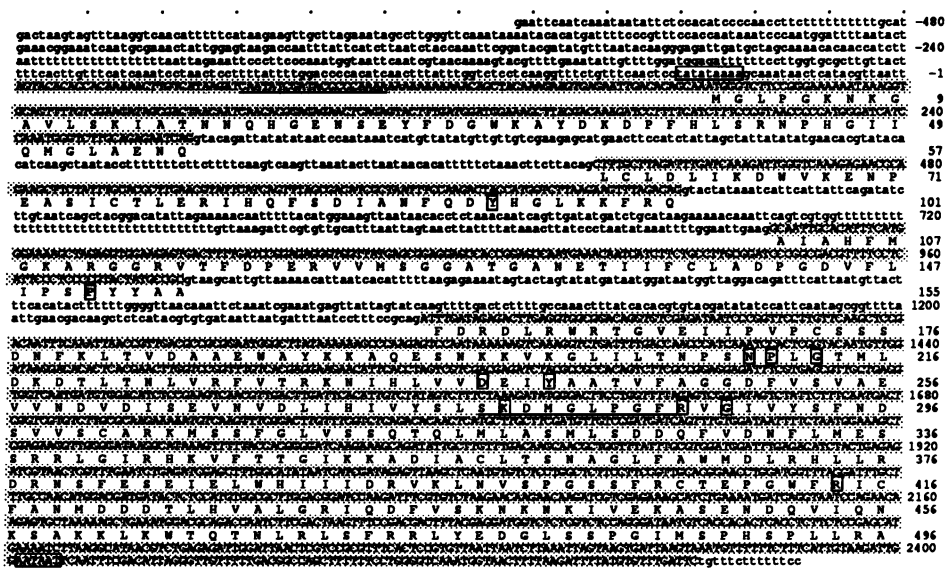

FIG. 3. Complete DNA sequence of *ACC2* including introns and parts of flanking regions. The sequence corresponding to mRNA is shaded and in uppercase letters. The dodecapeptide-containing part of the ACC synthase active site (32) is underlined. The 11 boxed amino acids are the invariant residues conserved between ACC synthase and various aminotransferases (7). Sequence corresponding to atacc2 primer (20-mer) used for primer extension and RNA sequencing (beginning at +31) is underlined. TATA box and a potential polyadenylylation signal sequence are boxed. The nucleotide sequence of the cDNA differs at 1-nt position from the genomic sequence: the gene has a cytosine at position +926, whereas the cDNA has a guanine at that position, encoding *Met*-136 (instead of *Ile*-136). Because the cDNA was obtained by PCR, this difference probably reflects an error during PCR amplification.

example, the ACC1/ACC3 mRNA(s) is expressed only in siliques (Fig. 2 *IA*); the ACC4 mRNA is expressed only in roots, leaves, and flowers (Fig. 2 *IC*); and the ACC5 mRNA is expressed only in flowers and siliques (Fig. 2 *ID*). We also determined the expression of *A. thaliana* ACC synthase-encoding genes in response to various known inducers of ACC synthase gene expression, such as wounding, N_2, Li^+, indoleacetic acid, and CHX in etiolated seedlings (7, 13); the results are shown in Fig. 2*II A–D*. Wounding greatly induces the ACC2 mRNA and moderately affects the levels of ACC4 mRNA (Fig. 2*II*, compare lanes 1 and 2 in *B* and *C*). However, ACC5 mRNA decreases upon wounding (Fig. 2 *IID*). Anaerobiosis suppresses the expression of all ACC synthase genes in etiolated seedlings (Fig. 2*II B*, *C*, and *D*, lane 3). Treatment with 10 mM LiCl induces only the ACC2 and ACC4 mRNAs (Fig. 2*II B* and *C*, compare lanes 1 and 4) and does not significantly affect expression of the other genes (Fig. 2*II A* and *D*). The plant hormone auxin induces only the ACC4 mRNA (Fig. 2 *IIC*, compare lanes 1 and 5). The ACC1/ACC3 mRNA(s) is undetectable in etiolated *Arabidopsis* seedlings and is not induced by any of these inducers. However, CHX, a protein-synthesis inhibitor, derepresses *ACC1/ACC3* gene expression as well as the rest of the ACC synthase genes (Fig. 2*II A–D*, compare lanes 1 and 6).

Expression of *ACC2* differs greatly from that of other ACC synthase genes studied so far (6–13) in that ACC2 mRNA accumulates at considerable levels in vegetative tissues, such as roots and leaves (Fig. 2*I*). Particularly interesting is the marked accumulation of ACC2 mRNA in roots. The result is reminiscent of some observations relating ethylene to root geotropic growth (27). Inhibition of protein synthesis causes a striking accumulation of ACC2 transcript (Fig. 2*II*), an effect also seen for various hormonally regulated mammalian genes (28) as well as some auxin-regulated genes in pea and soybean (29, 30). One proposed mechanism is that the gene is under the control of a short-lived repressor protein that is decreased in amount or function by CHX addition (29, 31). Protein-synthesis inhibition may also prevent mRNA degradation by preventing synthesis of a labile nuclease. Genetic evidence for the repressor model controlling ethylene biosynthesis is the ethylene-overproducing mutant *eto-1* in *A. thaliana* (16). The *ACC2* gene should provide a unique system for molecular genetic analysis of the mechanism underlying gene activation by protein-synthesis inhibitors.

Our data imply that a number of distinct regulatory networks govern the expression of different ACC synthase genes under various biological situations. This fact is reminiscent of the situation for several other plant gene families (18, 37–40). Thus, the differential pattern of accumulation of ACC synthase mRNAs probably reflects the ability of the ethylene biosynthetic pathway to respond to a diverse array of developmental and environmental signals associated with the production of ethylene, which influences many aspects of plant growth, development, disease resistance, and senescence. However, the current data likely do not reveal the full complexity of regulation because ACC synthase genes may differ further with respect to the developmental timing of expression in different organs, the kinetics of induction by specific environmental stimuli, and the tissue- and cell-type-specific pattern of expression.

Structural Characterization of *ACC2*. Among the five ACC synthase genes, *ACC2* is the most highly expressed during normal development, and its expression is greatly potentiated by CHX (Fig. 2*IIB*). Because we are interested in developing molecular genetic approaches for elucidating the mechanism of CHX inducibility, *ACC2* was chosen for further structural characterization. The sequence of 3040 nucleotides (nt) of the gene has been determined (Fig. 3); the sequence includes parts of the 5′ and 3′ flanking regions. The coding region consists of 1491 bp that are interrupted by three introns. The intron/exon

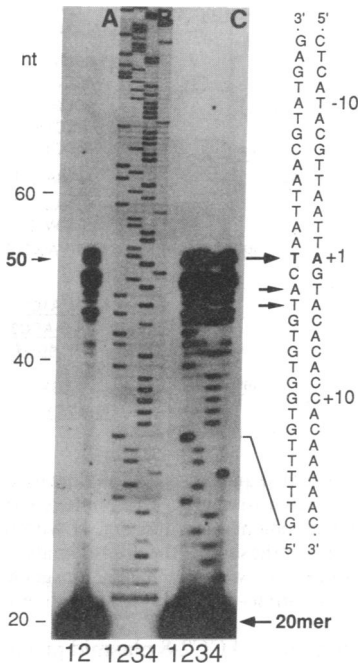

FIG. 4. Determination of transcription initiation site of *ACC2*. (*A*) Primer-extension analysis. 5′-^{32}P-acc2 primer (Fig. 3) was hybridized with 5 μg of poly(A)$^+$ RNA from etiolated seedlings induced with CHX for 8 hr (lane 2) and was extended with reverse transcriptase. Lane 1 is the control without mRNA. (*B*) Dideoxynucleotide chain-termination sequencing reactions with 4 kb of the *Eco*RI fragment (Fig. 1*II*) after priming with same primer. Lanes: 1–4, reactions G, A, T, and C, respectively. (*C*) Nucleotide sequencing of 5′ end of mRNA. Primer-extension reaction (see *A*) was done with 20 μg of poly(A)$^+$ RNA from the same induced tissue and extended with reverse transcriptase and dNTP/ddNTPs (7). Lanes 1–4 are as in *B*. The boldface residue represents the first major start of transcription.

junctions, which are typical of donor and acceptor splice sites, have been established by reference to paACC2 cDNA sequences (see below) (33). Number and size of exons and location of introns are similar to other ACC synthase genes isolated from tomato (13) and zucchini (7), except that zucchini ACC synthase has an additional intron within the last exon (7). We have determined the transcription initiation site of *ACC2* by primer extension and by sequencing the primer-extended product (Fig. 4 *A* and *C*). The mRNA sequence revealed by RNA sequencing is identical to the genomic sequence found in the 5′ untranslated region of *ACC2* (Fig. 4 *B* and *C*), indicating that no additional intervening sequence appears in that region. There is a sequence beginning at position −31 (Fig. 3) that qualifies as a TATA box (33). Sequence of the 3′ end of the gene was established by comparing genomic sequence to a cDNA fragment generated by inverse PCR (see *Materials and Methods*). A potential polyadenylylation signal (AATAAT) is found 91 nt upstream from the polyadenylylation site (Fig. 3). Thus, the mRNA transcribed by *ACC2* is predicted to be 1796 nt long (5′, 94 nt; coding region, 1491 nt; 3′, 211 nt) without poly(A) tail, close to the 1900-nt size detected by RNA-hybridization analysis. The polypeptide encoded by the ACC2 mRNA contains 496 amino acids (55,623 Da, pI 7.28), close to the size of other ACC synthase isoenzymes from various plant species (7–10, 13). The ACC2 amino acid identity to other ACC synthase isoenzymes varies from 55 to 70%.

Authenticity of the polypeptide encoded by ACC2 mRNA was confirmed by expression experiments in *E. coli*. A full-length cDNA, paACC2, corresponding to ACC2 mRNA was synthesized by PCR and fused to the N terminus of the β-galactosidase gene in pUC18 in sense (paACC2-8) and in pUC19 in antisense (paACC2-9) orientation. *E. coli* trans-

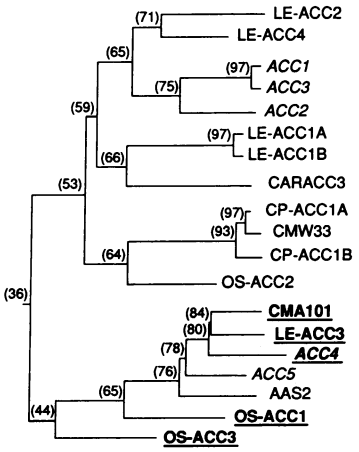

FIG. 5. Amino acid sequence-based phylogeny of ACC synthase family (distances drawn to scale). The phylogenetic tree was constructed from the alignment shown in Fig. 1*I* with the PAPA method (20). Numbers in parentheses are the approximate percent identities of sequences between the two sublineages. The genes underlined and in boldface type are auxin-inducible in vegetative tissues.

formants bearing the paACC2-8 plasmid produce 0.6 nmol of ACC per 10^8 cells after 3 hr of incubation in the presence of 1 mM isopropyl B-D-thiogalactoside. *E. coli* containing only pUC18 or paACC2-9 do not accumulate ACC.

Evolution of ACC Synthase. Phylogenetic analysis of amino acid sequences deduced from the PCR products using PAPA methods (20) show that some ACC synthase genes are more similar to genes of other species than to other ACC genes in the same species (Fig. 5). A dendrogram of identical topology was also obtained by using the PAUP program (21). One major lineage contains *ACC4* and *ACC5* along with five other genes from winter squash (23), tomato (13), rice (T. I. Zarembinski and A.T., unpublished work), and apple (11). Similar analysis using the complete coding sequence of some ACC synthase genes (refs. 7, 10, 13, 23, 24; T. I. Zarembinski and A.T., unpublished work) reveals a nearly identical gene genealogy (data not shown). From the results of phylogenetic analysis, we propose that the polymorphisms of ACC synthase arose before the time of divergence of monocotyledonous and dicotyledonous plants and is probably present in all higher plants. An early origin of gene family in plant evolution has also been found in phytochrome, the most ancient regulatory photoreceptor family in plants (34, 35). The ACC-to-ethylene pathway has been detected in cycads and in certain ferns (ref. 36; A. D. Campbell, X.L., and A.T., unpublished work). Thus, the invention of ACC synthase could have occurred >350 million years ago, much earlier than the estimated 125–150 million years ago that monocots and dicots diverged. Moreover, the current data indicate a striking correlation for some ACC synthase genes between their phylogenetic relationship and their expression pattern. For example, the genes shown to be induced by auxin in vegetative tissues, including *ACC4* from *Arabidopsis* (this paper), *CMA101* from winter squash (23), *OS-ACC1* and *OS-ACC3* from rice (T. I. Zarembinski and A.T., unpublished work), and *LE-ACC3* from tomato (J. E. Lincoln and A.T., unpublished work) all fall into one major lineage (Fig. 5). This result is likely to reflect that the generation of separate regulatory networks controlling the expression of ACC synthase subfamilies was also an early evolutionary event preceding the divergence of monocots and dicots. Because the emerging genealogy and regulatory networks revealed by the current data represent a minimum of complexity, a more complex hierarchy of correlations between a phylogenetic sublineage and a particular pattern of expression may be seen as more ACC synthase genes from plants of diverse groups are isolated and their structural and expression characteristics are elucidated.

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