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Cloning, genetic mapping, and expression analysis of an *Arabidopsis thaliana* gene that encodes 1-aminocyclopropane-1-carboxylate synthase

(development/ethylene/gene regulation/restriction fragment length polymorphism mapping/stress)

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ABSTRACT **A genomic clone of one member of the *Arabidopsis thaliana* (L.) Heynh. 1-aminocyclopropane-1-carboxylate (ACC) synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) gene family (*AT-ACC1*) was isolated and sequenced. A region of homology was found in the 5′-untranslated region with the promoter of a zucchini and a tomato ACC synthase gene. Comparison of its primary structure with other ACC synthases revealed conservation of seven peptide regions as well as similarity with 11 amino acids of the catalytic site of aminotransferases. Genomic DNA gel blotting suggested the existence of an ACC synthase multigene family in *Arabidopsis*, possibly with three other members, none of which is very closely related to *AT-ACC1*. The existence of at least one other gene was confirmed by the isolation of a cDNA (AT-ACC2) from a flower-specific cDNA library. The *AT-ACC1* gene was mapped on the *Arabidopsis* restriction fragment length polymorphism map and is located on the top of chromosome 1. This position does not correspond to any known mutation on the genetic map. Expression of the *AT-ACC1* gene was studied by reverse transcription-PCR on total RNA. Messenger accumulation was strong in young leaves and flowers. The gene was not induced by wounding of young leaves or in seedlings in the presence of auxin. Ethylene exposure of mature plants led to an induction of *AT-ACC1* gene expression. It is suggested that AT-ACC1 protein has a role in developmental control of ethylene synthesis.**

The gaseous plant hormone ethylene has a wide impact on plant growth and development (1). Its synthesis is induced during many stages of plant life including seed germination, leaf abscission, organ senescence, and fruit ripening. Its production also rises strongly upon exposure to various stresses. Finally, it is also induced by the plant hormone auxin. The direct precursor of ethylene in higher plants is the three-membered-ring amino acid 1-aminocyclopropane-1-carboxylic acid, the synthesis of which is catalyzed by 1-aminocyclopropane-1-carboxylate (ACC) synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) and which is the rate-limiting step in the pathway, dependent on pyridoxal 5′-phosphate as a cofactor (1).

The molecular aspects of ethylene biosynthesis and action have been recently reviewed (2). From a molecular point of view, it is intriguing how different conditions during development or exposure to external stress factors lead to induction of the same key regulatory enzyme, ACC synthase. In the past 2 years, ACC synthase genes have been cloned and sequenced from different species, including tomato (3–6), winter squash (7, 8), zucchini (9, 10), apple (11, 12), carnation (13), and mung bean (12, 14). In most of these species, the existence of ACC synthase multigene families has been demonstrated. The genes appear to be differentially regulated, although in some cases a certain coordination was observed. In the references mentioned above, the main focus has been on the expression after wounding or auxin exposure, or in ripening and senescence conditions. The importance of ethylene in the vegetative development from seedling to maturity has received little attention. Because *Arabidopsis thaliana* is a suitable object for developmental studies in plant biology, it was our goal to study the regulation of expression of ACC synthase genes in this species to improve our knowledge on the role of ethylene in plant development. In this paper, we present data on the isolation and the genetic and molecular characterization of one member of the *Arabidopsis* ACC synthase gene family.§

MATERIALS AND METHODS

Libraries, Gene Cloning, and Sequence Analysis. A cosmid library of *A. thaliana* (ecotype Columbia) was from N. Olszewski (University of Minnesota). Screening was done with the same oligonucleotides and under the same conditions as described (3). A flower-specific cDNA library of ecotype C24 was provided by M. H. Goldman and D. De Oliveira (Plant Genetic Systems, Ghent). The library was screened with a 2.2-kilobase (kb) *Bam*HI fragment of the *A. thaliana* ACC synthase gene (*AT-ACC1*), which covers the first part (140 amino acids) of the coding region and the two first introns.

DNA sequencing analysis was performed by using the Sanger method (15) on subclones in pUC18, and in the case of the *AT-ACC1* gene, internally positioned oligonucleotides were also used as primers.

Restriction Fragment Length Polymorphism (RFLP) Mapping. RFLP mapping analysis was done by the procedure detailed by Nam *et al.* (16) using the cosmid carrying the *AT-ACC1* gene as a probe. Segregation data were analyzed with the MAPMAKER computer program (17), and the maximum-likelihood recombination fractions for each pair of adjacent markers were transformed into centimorgan distances with the Kosambi function (18).

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AT-ACC1 and AT-ACC2, *Arabidopsis thaliana* ACC synthase cDNA 1 and 2, respectively; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcription PCR.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. Z12614 and Z12615).

Plant Material, Growth Conditions, and Stress Treatments. *A. thaliana* (ecotype C24) plants were grown at 22°C and 60% relative humidity under white-fluorescent light (photoperiod 16 hr light/8 hr dark, at a fluence rate of 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Young leaves from 3- to 5-week-old plants were wounded with a scalpel. Senescent leaves were from 8- to 12-week-old plants. Flower samples contained only flowers with white, nonsenescing petals. Mature-green siliques did not show any sign of chlorophyll loss. Mature-ripe siliques presented a yellow to light-brown coloration and had mature seeds.

Flowering *A. thaliana* plants (mature, 6- to 8-week-old) were either immediately frozen or, for ethylene inductions, placed in a sealed container and subjected to a continuous flow of ≈9 liters/hr of ethylene at 10 ppm for 2–12 hr.

Auxin treatments were done by soaking 7-day-old light-grown seedlings in 0.5 mM indoleacetic acid/50 mM sodium phosphate buffer, pH 7.0, for 4 hr.

RNA Isolation and Reverse Transcription-PCR (RT-PCR). Total RNA was isolated as reported (19).

RT-PCR was done as in Goblet *et al.* (20) with minor modifications. In summary, 10 μg of total RNA (accurately quantified) was mixed with the 3′ oligonucleotide in 50 μl of 1× buffer [67.2 mM Tris·HCl, pH 8.8/16.6 mM $(NH_4)_2SO_4$/2 mM $MgCl_2$/bovine serum albumin (nuclease free) at 1.68 mg/ml/0.72% 2-mercaptoethanol] and incubated at 85°C for 5 min, followed by 5 min at 45°C. The tubes were put on ice and 50 μl of a reaction mixture containing the 5′ oligonucleotide, 1× buffer, 2.5 units of *Taq* polymerase (Beckman), 16 units of avian myeloblastosis virus reverse transcriptase (Promega), 20 units of RNasin (Promega), and 0.2 mM of each dNTP were added. Reverse transcription was done by incubating the tubes at 40°C for 45 min immediately followed by the PCR reaction: 30–35 cycles of 1.5 min at 94°C, 1.5 min at 50°C, and 4.5 min at 72°C. Reactions were done in a PHC-2 thermocycler (Techne, Cambridge, U.K.).

The PCR products were analyzed by 0.8–1% agarose/TAE (40 mM Tris acetate/1 mM EDTA) gel electrophoresis and blotted on Hybond-N membranes. Three pairs of oligonucleotides were used as primers: *pair 1*: PCR14, 5′-TATAGTCTTTCTAAAGATATGGGACTT-3′ [base pairs (bp) 2953–2979] and PCR3, 5′-GTCGTCGGAAACTTAGTCGA-3′ (bp 3556–3537); *pair 2* (covering highly conserved regions, nonspecific): PCR20, 5′-CTCATTCCCTCCCCGTACTA-3′ (bp 2297–2316) and PCR19, 5′-CTCTAAAACCAGGAAGTCCC-3′ (bp 2992–2973); *pair 3* (specific for *AT-ACC1*): PCR22, 5′-TCGACTAAGTTTCCGACGAC-3′ (bp 3537–3556) and PCR24, 5′-GTCGAAATTGAATTATTCCA-3′ (bp 3758–3738). All data are the result of two independent experiments.

Nucleic Acid Hybridization Analysis. Nuclear DNA preparation, DNA gel blotting, probe preparation, and hybridizations were essentially as reported (3). For genomic DNA gel blots, a 2.2-kb *Bam*HI fragment of *AT-ACC1* (1–2279 bp) and a PCR-generated fragment covering 3537–3903 bp were used as probes. A 1.1-kb *Bam*HI fragment of the *AT-ACC1* gene (2279–3423 bp) that spanned the PCR-amplified sequences was used as a probe in the RT-PCR analysis when oligonucleotide pair 1 and pair 2 were used. Both hybridization and washes were under highly stringent conditions. An internal oligonucleotide was used for pair 3 (PCR23, 3614–3633 bp) and hybridized at 50°C.

RESULTS

Isolation and Characterization of *AT-ACC1* Genes. Screening of a genomic cosmid library of *Arabidopsis* with oligonucleotides derived from tomato ACC synthase peptide sequences (3) led to the isolation of the *AT-ACC1* gene and its identification by similarity to other ACC synthases. Fig. 1*A* presents the complete sequence of the *AT-ACC1* gene (5613 bp). The sequence covers 1432 bp upstream from the initiation codon and 1993 bp downstream from the stop codon. Putative CAAT and TATA boxes (21), as well as the potential polyadenylylation site (22), are indicated. The gene contains three introns; consensus dinucleotides are located at their boundaries (23). The position of the exon–intron junctions are identical to those in the tomato ACC synthase genes (5). When comparing the promoter region of *AT-ACC1* with the promoter of the *Cucurbita pepo*-ACC1A-encoding gene of zucchini (10), the highest similarity was found in the region from 525 to 730. Interestingly, this region is partially overlapping with the region of highest similarity with the *Lycopersicon esculentum LE-ACC2* promoter (ref. 5; 661–827 bp in *AT-ACC1*). A 13-bp-long sequence in *AT-ACC1* (903–915) was also found in the fourth exon of the zucchini gene and with 77% similarity, far upstream, in the promoter of the *LE-ACC2* gene. Comparison of the *AT-ACC1* promoter with ethylene-responsive promoters (for review, see ref. 2) revealed significant similarity (70–80%) in several short stretches of the 5′ region. The wound-inducible promoters, winla (24) and wunla (25), were 91% similar to *AT-ACC1* in the regions 918–929 bp and 1170–1180 bp, respectively. No significant similarities with auxin-responsive elements were found. It is worth noting that a *myc*-like binding site (26) resembling the G-box core is present at position 340, also found in several light-regulated promoters (27, 28), in the abscisic acid response element of the wheat *Em* gene (29), and in the *LE-ACC2* promoter (5). In addition, both the 5′-untranslated region and intron 2 of *AT-ACC1* contain long thymine (ref. 18; 36 nucleotides) and adenine stretches (15 nucleotides, close to the start codon).

The *AT-ACC1* gene encodes a polypeptide of 496 amino acids with a predicted molecular mass of 54.6 kDa and a pI of 7.3. The 12 amino acids of the active-site region are identical to those of tomato (TACC1 or pCVV4A; ref. 3), winter squash (7), and zucchini (9) ACC synthases. Moreover, in all ACC synthases so far cloned, 11 of the 12 amino acids involved in the binding of the substrate and the cofactor of aminotransferases are conserved. This result supports the hypothesis that ACC synthases and aminotransferases are evolutionarily related (5). Table 1 gives an overview of the amino acid and nucleotide sequence similarities of ACC synthases from various species of which full-length clones have been isolated.

A partial cDNA of another *Arabidopsis* ACC synthase gene was isolated from a flower-specific cDNA library using a 2.2-kb *Bam*HI fragment of *AT-ACC1* as a probe and was designated AT-ACC2. Its sequence is shown in Fig. 1*B*. The 159 nucleotides are 79% identical to the corresponding region of *AT-ACC1*. This level of sequence difference cannot be readily accounted for by the difference in ecotypes (Columbia vs. C24). Deduced amino acids in this region are 83% identical.

The existence of several ACC synthase genes in *Arabidopsis* was confirmed by genomic DNA gel blots (Fig. 2). The *AT-ACC1* gene is a member of the ACC synthase gene family but without high similarity to any other member. Hybridization of the *AT-ACC1* 2.2-kb *Bam*HI fragment to both total and nuclear *Arabidopsis* DNA under high-stringency conditions revealed one *Bgl* II fragment at 2.7 kb and two *Eco*RI fragments of 5.0 and 6.5 kb, each with half the intensity of the *Bgl* II band (Fig. 2*A*). This is a single gene pattern corresponding to the restriction map of *AT-ACC1*. Confirmation was obtained by hybridizing *Arabidopsis* genomic DNA with the 1.1-kb *Bam*HI fragment (covering ≈75% of the coding region; data not shown), as well as with a 350-bp PCR-generated fragment, the sequence of which resides mostly in the 3′-untranslated region (Fig. 2*B*). In the latter cases a single band was observed at 6.5 kb in the *Eco*RI and at 12 kb in the *Xba* I lanes. However, when repeated under low-stringency conditions (53°C) with

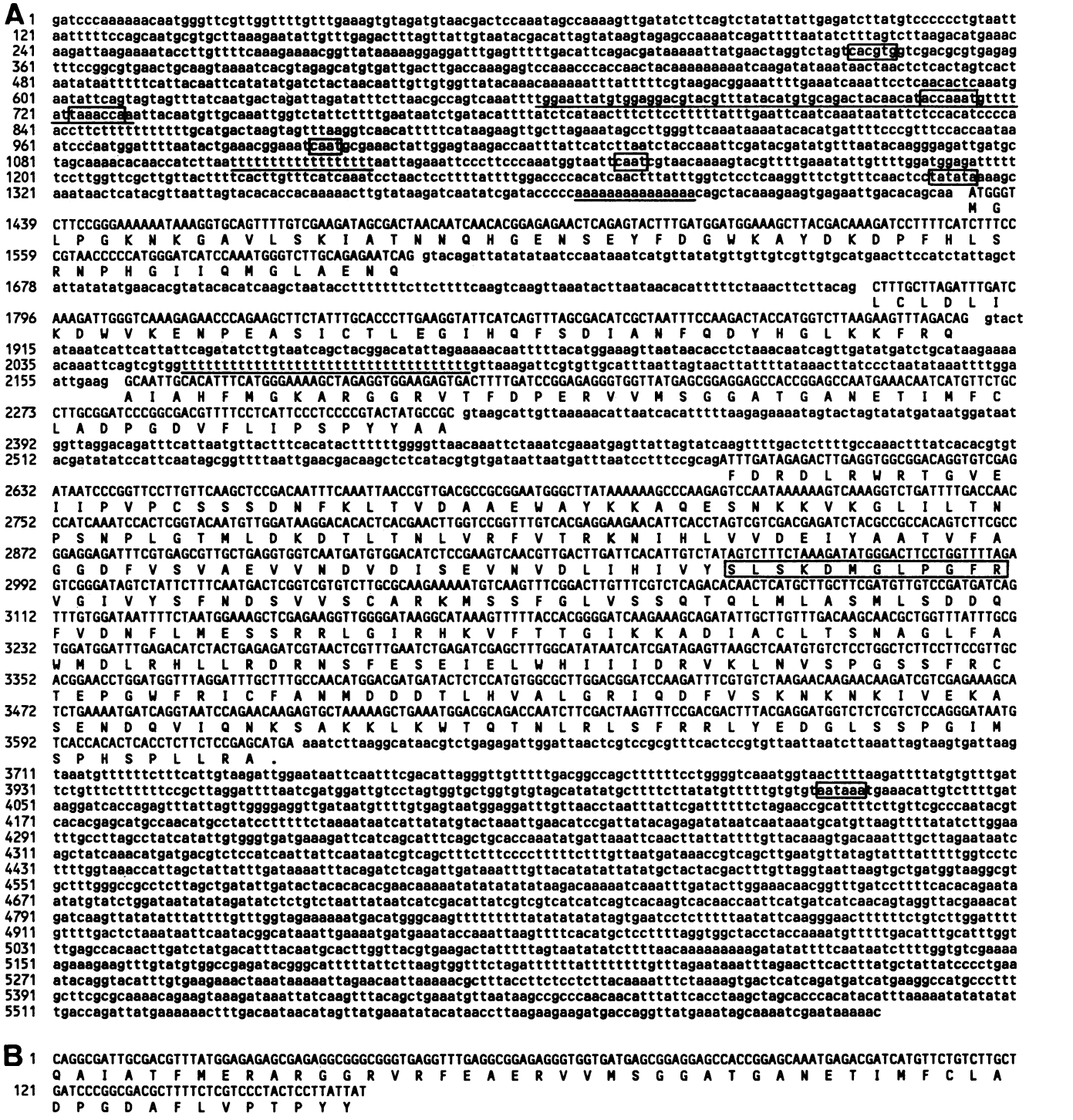

FIG. 1. Nucleotide and deduced amino acid sequences of the *A. thaliana* ACC synthase gene *AT-ACC1* and of the partial cDNA AT-ACC2. (*A*) DNA and amino acid sequences of *AT-ACC1*. Exons are in uppercase letters; noncoding regions are in lowercase letters. The G-box core element, the putative CAAT and TATA boxes, and polyadenylylation signals are boxed. The region of highest similarity with other ACC synthase promoters, containing a short inverted repeat, is underlined, as well as long thymine and adenine stretches. Deduced amino acids are in the one-letter code. The active-site region is boxed. (*B*) DNA and amino acid sequences of the AT-ACC2 cDNA.

the 2.2- and 1.1-kb *Bam*HI fragments covering the coding region as probes, several extra bands became apparent (data not shown), indicating the existence of related members (possibly three) of the ACC synthase gene family. In addition, it could be concluded that *AT-ACC1* is relatively divergent from ACC synthases in rice, tomato, and tobacco, because no cross-hybridization was visible under high-stringency conditions (Fig. 2*A*), whereas faint bands appeared under lower stringency (data not shown).

RFLP Mapping of the *AT-ACC1* Gene. Using the restriction enzyme *Cla* I, one Columbia polymorphic band and one Landsberg erecta polymorphic band showed appropriate segregation and mapped to the same locus. This RFLP has been designated pvv4 and maps to the telomere proximal region at the top of chromosome 1 (data not shown) at 0.0 centimorgan on the revised RFLP map. It is ≈37.4 centimorgans above (telomere proximal) the RFLP 5972 in the published map of Nam *et al.* (16).

Table 1. Comparison of ACC synthases with AT-ACC1

Enzyme	Nucleotide identity, %	Amino acid identity, %	Ref.
TACC1 (tomato)	68	75	3
TACC2 (tomato)	66	71	4
CMW33 (winter squash)	67	71	7
CMA101 (winter squash)	58	64	8
CP-ACC1a (zucchini)	67	72	10
CAR-ACC3 (carnation)	65	73	13

Comparison is according to Myers and Miller (34) with PC/GENE version 6.01 (Intelligenetics).

Study of Expression of *AT-ACC1* as Determined by RT-PCR. Due to the lack of sensitivity of Northern (RNA) blotting in detecting very low-abundance mRNAs (as AT-ACC mRNAs), RT-PCR was used for analysis of mRNA levels (30, 31). To allow quantitative comparison within each experiment, the number of PCR cycles was kept low. In certain cases Northern blots were done with a probe predicted to produce constant signals under given induction conditions. Different sets of oligonucleotides derived from the coding sequences or the 3'-untranslated region of the *AT-ACC1* gene were synthesized to monitor levels of all ACC synthase mRNAs or AT-ACC1 mRNA alone. Fig. 3*A* presents a DNA gel blot of a RT-PCR reaction on different total RNA samples using primers PCR3 and PCR14, which most likely specifically amplify AT-ACC1 cDNA because primer PCR3 resides in the 3' end of the coding region where ACC synthases are known to be highly divergent. The signal was very high in young leaves and in flowers but barely visible in roots and absent in siliques and etiolated seedlings (Fig. 3*A*, lanes 1–7). The same pattern was found when the conserved oligonucleotides PCR19 and PCR20 were used (Fig. 3*B*, lanes 1–7).

When mature plants were exposed to a continuous flow of ethylene at 10 ppm an early induction could be seen (2 hr, Fig. 3*A*, lane 9) but the induction almost returned to basal level after 8 hr (lane 11). Remarkably, control plants did not show any signal (Fig. 3*A*, lane 8). The same pattern was observed when oligonucleotide pair 3 was used (data not shown). When the nonspecific set of oligonucleotides was used, a biphasic induction occurred; a first peak occurred at 2 hr, and a second, strong induction occurred at 12 hr. Exposure of

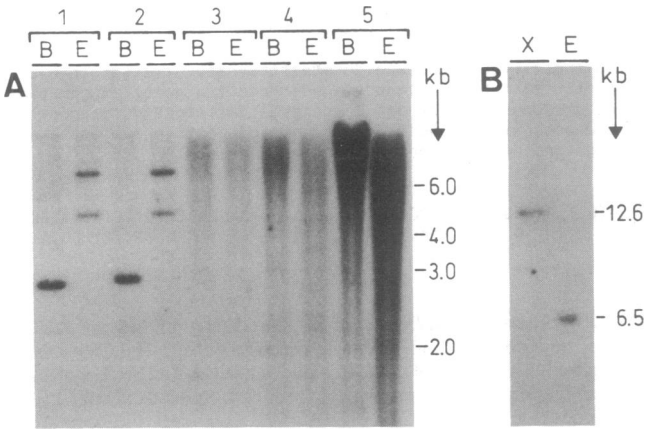

FIG. 2. Genomic DNA gel blot analyses of *A. thaliana*, *Oryza sativa* (cv. Taipei), *Lycopersicon esculentum* (cv. Orlando), and *Nicotiana tabacum*. (*A*) Lanes: 1, *A. thaliana* (ecotype C24) (1 μg of total DNA); 2, *A. thaliana* (1 μg of nuclear DNA); 3, rice (3 μg of nuclear DNA); 4, tomato (5 μg of total DNA); 5, tobacco (20 μg of nuclear DNA). B, *Bgl* II; E, *Eco*RI. The filter was hybridized with a 2.2-kb *Bam*HI fragment of the *AT-ACC1* gene. (*B*) *A. thaliana* (ecotype Landsberg erecta) *Xba* I (X) and *Eco*RI (E) digests. The filter was hybridized at 60°C with a PCR fragment, most of which covered the 3'-untranslated region (3540–3905 bp).

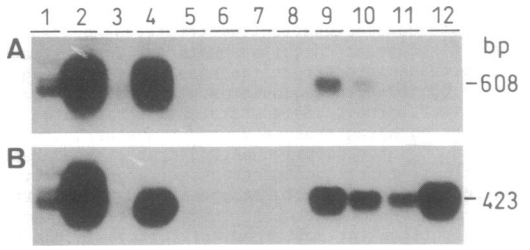

FIG. 3. RT-PCR analysis of expression of the *AT-ACC1* gene on total RNA from different organs of *Arabidopsis* and from mature plants after ethylene exposure. (*A*) Primers specific for *AT-ACC1* (PCR3 and PCR14). Lanes: 1, 500-bp marker; 2, young leaves; 3, roots; 4, flowers; 5, mature-green siliques; 6, ripe siliques; 7, etiolated seedlings; 8, mature plants; 9, as for 8, but after 2-hr ethylene exposure; 10, 4 hr; 11, 8 hr; 12, 12-hr ethylene exposure. (*B*) Nonspecific primers (PCR19 and PCR20). Lanes are numbered as for *A*.

7-day-old light-grown seedlings to indoleacetic acid did not lead to any significant induction of the *AT-ACC1* gene (data not shown).

In Fig. 4, the effect of wounding on *Arabidopsis* ACC synthase mRNA levels is shown. Fig. 4*A* presents a DNA gel blot of samples treated with the oligonucleotide pair 3, which specifically amplifies AT-ACC1 mRNA. The signals obtained between 30-min and 8-hr wounding did not vary significantly. However, a clear induction was seen when the nonspecific set of oligonucleotides was used (pair 2); a peak occurred 4 hr after wounding (Fig. 4*B*).

DISCUSSION

We report the cloning of a gene encoding a member of the *A. thaliana* ACC synthase gene family (*AT-ACC1*) and one partial cDNA corresponding to another ACC synthase (AT-ACC2). The existence of a multigene family in *Arabidopsis* was confirmed by genomic DNA gel blots that also indicate that the *AT-ACC1* gene is distantly related to other family members. An analysis of the promoter sequence revealed similarities with several other promoters. Most noteworthy was the homology with the promoter of the tomato ACC synthase *LE-ACC2* gene (5) and the zucchini *CP-ACC1a* gene (10), in the region 661–730 bp. In addition, a G-box core element was found far upstream (340–345 bp). In both cases, their possible involvement in gene regulation remains to be proved. Comparison of the predicted amino acid sequence with other ACC synthase proteins revealed the same common features as presented earlier (11), and similarities ranged between 71 and 75% (Table 1).

The major biological question with respect to ethylene biosynthesis concerns the molecular mechanism of induction

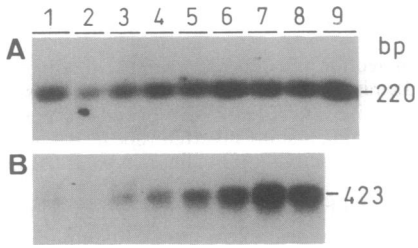

FIG. 4. RT-PCR analysis of expression of the *AT-ACC1* gene done on total RNA from wounded, young *Arabidopsis* leaves. (*A*) Primers specific for *AT-ACC1* (PCR22 and PCR24). Lanes: 1, Young leaves; 2, after 30-min wounding; 3, 60 min; 4, 90 min; 5, 2 hr; 6, 3 hr; 7, 4 hr; 8, 8 hr; 9, control on 5 ng of cosmid DNA, carrying *AT-ACC1*. (*B*) Nonspecific primers (PCR19 and PCR20). Lanes are numbered as for *A*.

of ACC synthase genes, either in a coordinated or in a differential fashion. From physiological data (1), it can be argued that at least three classes of ACC synthases could exist—one ripening and senescence-related, one auxin-induced, and one stress-induced. However, a recent report on ACC synthase genes in tomato has indicated that the individual genes are not assigned to only one function (6). The data presented here support the existence of a complex regulatory pattern of the ACC synthase gene family in *Arabidopsis*. The *AT-ACC1* gene was expressed prominently in young leaves and flowers but was not expressed in leaves of mature plants nor in mature green or ripe siliques (Fig. 3). The *AT-ACC1* gene was also shut down in rosette leaves with the onset of the reproductive phase (data not shown). Interestingly, AT-ACC1 mRNA levels seemed to be influenced by ethylene itself. Upon ethylene exposure of mature plants, the AT-ACC1 mRNA accumulated after 2 hr, whereas the ACC synthase gene family showed a biphasic activation with peaks after 2- and 12-hr treatment (Fig. 3). Although the *AT-ACC1* gene appeared to be switched off in senescent leaves (data not shown), whether it has any role in the onset of senescence or in the early senescence phase remains to be determined. In addition, *AT-ACC1* was shown not to be wound-inducible, at least not in young tissue. This contrasted with the pattern of accumulation observed for the ACC synthase mRNAs in general, where a peak was detected after 4 hr (Fig. 4).

Physiological data have documented the role of ethylene in inhibition of cell elongation and subsequent determination of cell size and shape in etiolated seedlings (32, 33). Nevertheless, our knowledge on the role of ethylene in development from the young seedling to a mature plant remains extremely poor. A detailed analysis of the molecular phenomena governing the tissue and cell-specific expression of *AT-ACC1* and other members of the ACC synthase gene family in *Arabidopsis* will provide clues about the role of ethylene in controlling plant development.

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