

ASC4, a Primary Indoleacetic Acid-responsive Gene Encoding 1-Aminocyclopropane-1-carboxylate Synthase in *Arabidopsis thaliana*

STRUCTURAL CHARACTERIZATION, EXPRESSION IN *ESCHERICHIA COLI*, AND EXPRESSION CHARACTERISTICS IN RESPONSE TO AUXIN*

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1-Aminocyclopropane-1-carboxylic acid (ACC) synthase is the key regulatory enzyme in the biosynthetic pathway of the plant hormone ethylene. The enzyme is encoded by a divergent multigene family in *Arabidopsis thaliana*, comprising at least five genes, ACS1–5 (Liang, X., Abel, S., Keller, J. A., Shen, N. F., and Theologis, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 11046–11050). In etiolated seedlings, ACS4 is specifically induced by indoleacetic acid (IAA). The response to IAA is rapid (within 25 min) and insensitive to protein synthesis inhibition, suggesting that the ACS4 gene expression is a primary response to IAA. The ACS4 mRNA accumulation displays a biphasic dose-response curve which is optimal at 10 μ M of IAA. However, IAA concentrations as low as 100 nM are sufficient to enhance the basal level of ACS4 mRNA. The expression of ACS4 is defective in the *Arabidopsis* auxin-resistant mutant lines *axr1–12*, *axr2–1*, and *aux1–7*. ACS4 mRNA levels are severely reduced in *axr1–12* and *axr2–1* but are only 1.5-fold lower in *aux1–7*. IAA inducibility is abolished in *axr2–1*.

The ACS4 gene was isolated and structurally characterized. The promoter contains four sequence motifs reminiscent of functionally defined auxin-responsive cis-elements in the early auxin-inducible genes *PS-IAA4/5* from pea and *GH3* from soybean. Conceptual translation of the coding region predicts a protein with a molecular mass of 53,795 Da and a theoretical isoelectric point of 8.2. The ACS4 polypeptide contains the 11 invariant amino acid residues conserved between aminotransferases and ACC synthases from various plant species. An ACS4 cDNA was generated by reverse transcriptase-polymerase chain reaction, and the authenticity was confirmed by expression of ACC synthase activity in *Escherichia coli*.

Ethylene, a major phytohormone, is one of the simplest organic molecules with biological activity and controls many as-

pects of plant growth and development (1, 2). The gas is endogenously produced during unique developmental stages such as growth, senescence, and abscission of leaves and flowers, development and ripening of fruits, and germination of seeds (2). Ethylene production is also induced by various stress conditions and chemical compounds, including wounding, temperature fluctuation, drought, anaerobiosis, viral infection, elicitor treatment, heavy metal exposure, or lithium ions (2). Ethylene serves as a signaling molecule to initiate and coordinate profound physiological changes and adaptations throughout the life cycle of a plant (1, 2).

Ethylene biosynthesis is stringently regulated during plant development (2, 3). The rate-limiting reaction catalyzed by the enzyme ACC¹ synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, EC 4.4.1.14) is responsible for the formation of the immediate ethylene precursor, ACC, from *S*-adenosylmethionine. The committed step is subject to control at the transcriptional and post-transcriptional level (2, 3). ACC synthase is a short-lived cytosolic enzyme (4) and is encoded by a highly divergent multigene family in a number of plant species including zucchini (5), tomato (6), mung bean (7), rice (8), and *Arabidopsis thaliana* (9, 10). Each member of the gene families is differentially expressed during plant development as well as in response to a distinct subset of environmental and chemical stimuli (5–10). For instance, the plant hormone auxin, typified by IAA, is a known inducer of ethylene production (2) and regulates specific members of each ACS multigene family in a tissue-specific manner (5, 8, 10–12).

We are interested in elucidating the multiple signal-transduction pathways leading to ACS gene activation by a diverse group of inducers, using biochemical, molecular, and reverse genetic approaches. Our particular goal is to understand how ACS genes are activated by auxin, by protein synthesis inhibition, and by lithium ions (6, 8, 10). Protein synthesis inhibitors such as CHX have been widely used as a tool to unmask regulatory mechanisms of early gene activation (13). Likewise, the lithium ion is known to interfere with phosphatidylinositol metabolism and signaling (14). On the other hand, auxin-inducible ACS genes provide a molecular probe to study mechanisms of auxin action and the intimate interrelationship of both plant hormones. As a first step toward this long term goal we have cloned ACS multigene families in tomato (6), rice (8), and *A. thaliana* (10). We are attempting to develop the molecular

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¹ The abbreviations used are: ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indole-3-acetic acid; ACS, ACC synthase; 2,4-D, 2,4-dichlorophenoxyacetic acid; PAA, phenylacetic acid; α -NAA, naphthalene-1-acetic acid; BA, benzyl adenine; CHX, cycloheximide; MES, 2-(*N*-morpholino)ethanesulfonic acid; nt, nucleotides; PCR, polymerase chain reaction; kb, kilobase(s).

genetic approaches in *Arabidopsis*, a model organism for a flowering plant (15). We have previously identified an auxin-regulated ACS gene in *A. thaliana*, ACS4 (10). Here, we report its structure and specific expression characteristics in response to IAA.

EXPERIMENTAL PROCEDURES

Arabidopsis Strains and Growth Conditions—The following *Arabidopsis* strains were used: wild type *A. thaliana* (L.) Heynh. ecotype Columbia, and auxin-resistant mutant lines *axr1-12* (16), *axr2-1* (17), and *aux1-7* (18). Seeds of the auxin-resistant mutant lines were kindly provided by Mark Estelle (Indiana University). To grow etiolated seedlings, seeds were surface sterilized for 8 min in 5% sodium hypochlorite (30% chlorox), 0.1% Triton X-100, excessively rinsed in distilled water, and plated in Petri dishes onto sterile filter paper discs on top of 0.7% agar (Bacto-agar, Difco) containing $0.5 \times$ Murashige-Skoog salts (Life Technologies, Inc.) at pH 5.6. After cold treatment at 4 °C for 3 days, the plates were incubated in the dark at 22 °C for 5–6 days.

Tissue Treatment—Intact etiolated seedlings (5–6 days old) were removed from the filter discs and placed in Petri dishes containing $0.5 \times$ Murashige-Skoog salt solution buffered at pH 5.6 with 0.5 mM MES and supplemented with the appropriate chemicals. The seedlings were incubated in the dark at room temperature with shaking (50–100 revolutions/min). Mock control incubations were supplemented with an equal amount of the solvent used to prepare the stock solution of the respective chemical. After the indicated time, aliquots (3–5 g fresh weight) of seedlings were removed, briefly blotted dry, frozen with liquid nitrogen, and stored at –80 °C. For the short time course experiment, intact etiolated seedlings (5–6 days old; 3–5 g fresh weight) were placed in 50-ml Falcon tubes. After the addition of 15 ml of $0.5 \times$ MS salts, 0.5 mM MES, pH 5.6, the tubes were moderately shaken by hand for the indicated period. The seedlings were immediately frozen with liquid N₂ after decanting the bathing solution and stored at –80 °C.

Plasmids—The following recombinant clones were used in this study: pAAA1 contains the 8.3-kb *EcoRI* fragment of λ AT-8 in pUC18. λ AT-8 is an *Arabidopsis* genomic clone containing the ACS4 gene (10). pAAA2 was derived from pAAA1 by deleting the 2.1-kb *SacI/EcoRI* fragment by *SacI* digestion and religation. pAAA3 contains the 2.1-kb *EcoRI* fragment of λ AT-8 in pUC18. pAAA4 was derived from pAAA3 by deleting the 1.1-kb *BamHI/EcoRI* fragment by *BamHI* digestion and religation. pAAA5 was constructed by PCR using sequencing primers A2A (5'-GAAGCCTACGAGCAAGCC-3') and T3D (5'-TTGTGCTGGGAGGAGAC-3') as amplimers and λ AT-8 phage DNA as the template. The 0.4-kb PCR product was subcloned into the *EcoRV* site of pIC20R. pAAA6 contains a PCR-generated, 1.4-kb ACS4 cDNA insert in the *BamHI* site of pUC19. Poly(A)⁺ RNA from IAA/CHX-treated etiolated *Arabidopsis* seedlings was reverse-transcribed (19). The single-stranded cDNAs were used as the template in a PCR (20) with amplimers CDA1, 5'-GGCCGGATCCAA ATG GTT CAA TTG TCA AGA AAA GC-3', and CDA2, 5'-GGCCGGATCCA CTA TCG TTC CTC AGC CTC ACG G-3' (*BamHI* recognition sites are underlined; start and stop codons are in bold-face type).

DNA Sequencing—Dideoxy sequencing of double-stranded DNA of pAAA plasmids was performed with universal and synthetic primers using [³⁵S]dATP (21) and the modified T7 DNA polymerase, Sequenase, according to the manufacturer's instructions (U.S. Biochemicals Corp., Cleveland, OH). DNA sequences were analyzed with the Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin).

Expression of ACS4 in *E. coli*—Plasmid pAAA6 was introduced into *Escherichia coli* DH5 α and M15[pREP4]. Expression conditions and measurements of ACC formation were as described previously (6).

Isolation of Nucleic Acids—Total nucleic acids were prepared from frozen *Arabidopsis* tissues. Typically, etiolated seedlings or other plant material (~3–5 g fresh weight in 50-ml Falcon tubes) were supplemented with 30 ml of extraction buffer (1 volume of 200 mM Tris-HCl, pH 7.5, 100 mM LiCl, 5 mM EDTA, 1% SDS, 1% β -mercaptoethanol, and 1 volume of phenol/chloroform/isoamyl alcohol, 25:24:1) and macerated with a polytron mixer (Brinkman) at the highest setting for 2 min. The homogenate was centrifuged, and the aqueous phase was reextracted with an equal volume of phenol/chloroform/isoamyl alcohol. After a second reextraction with chloroform, the aqueous phase was brought to 2 M LiCl and incubated overnight at 4 °C. Nucleic acids were recovered by centrifugation, dissolved in 0.5 ml of water, and reprecipitated with 2.5 volumes of ethanol after adjusting the salt concentration to 300 mM sodium acetate, pH 5.5. Poly(A)⁺ RNAs from larger batches of total nucleic acids were isolated by affinity chromatography using oligo(dT)

cellulose as described by Theologis *et al.* (22).

RNA Hybridization Analysis—Northern analysis was essentially performed according to Ecker and Davis (23). Total nucleic acids were glyoxylated at 50 °C, electrophoresed on 1% agarose gels, and transferred to GeneScreen membrane (DuPont-NEN). After baking at 80 °C for 3 h, the membranes were prewashed in $0.1 \times$ SSPE, 0.1% SDS at 60 °C for 1 h. Prehybridization was performed in 50% formamide, $5 \times$ SSPE ($1 \times$ SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH 7.0, 1 mM EDTA), $5 \times$ BFP ($1 \times$ BFP is 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone ($M_r = 360,000$), 0.02% Ficoll ($M_r = 400,000$)), 1% SDS, and 100 μ g/ml denatured salmon sperm DNA at 42 °C for 4–6 h. The hybridization buffer contained in addition 5% dextran sulfate ($M_r = 500,000$) and the appropriate radioactively labeled probe. Probes were prepared from DNA restriction fragments by the random hexamer-primed synthesis method (24) to a specific activity of $\sim 1.0 \times 10^9$ counts/min/ μ g. Hybridizations were carried out with radiolabeled probes of 2×10^6 counts/min/ml hybridization solution at 42 °C for 16–20 h. The membranes were washed in 50% formamide, $5 \times$ SSPE, 0.1% SDS at 42 °C for 1 h followed by a final high stringency wash in $0.1 \times$ SSPE, 0.1% SDS at 60 °C for 1 h. The wet filters were exposed to Kodak XAR-5 film with a DuPont Cronex Lightning Plus intensifying screen at –80 °C. The autoradiograms were quantified using an LKB ultrascan laser densitometer (Bromma, Sweden). After exposure, the probe was removed by rinsing the filters in $0.1 \times$ SSPE, 0.1% SDS at 95–100 °C for 15–30 min.

Primer Extension Analysis—Primer extension was performed essentially according to the method of Boorstein and Craig (25). Approximately 5×10^4 counts/min of a 5'-end-labeled 29-mer synthetic oligonucleotide, DP1, complementary to nucleotides +146 to +174 of the ACS4 gene (Fig. 1B) was hybridized at 50 °C for 3 h to 15 μ g of poly(A)⁺ RNA from etiolated *Arabidopsis* seedlings treated with 20 μ M IAA and 50 μ M CHX for 2 h. The primer-RNA hybrids were incubated with 20 units of reverse transcriptase in 50 μ l of 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 100 μ M of each dNTP, 10 mM dithiothreitol, 60 μ g/ml actinomycin D for 1 h at 42 °C. The products were analyzed on a 6% denaturing polyacrylamide gel.

Other Molecular Techniques—Standard molecular techniques were performed according to Sambrook *et al.* (26).

RESULTS AND DISCUSSION

Isolation and Structural Characterization of the ACS4 Gene

Screening of a λ DASH *Arabidopsis* genomic library with the ptACS2 cDNA of tomato (6) as a probe under low stringency hybridization conditions resulted in the isolation of λ genomic clones for five ACC synthase genes ACS1–5 (10). The structure and expression characteristics of ACS2 have been reported (10). In this study, we present the structural characterization of the auxin-responsive gene ACS4 using the previously isolated λ AT-8 genomic clone (10). The 8.3-kb *EcoRI* fragment of the λ AT-8 clone (Fig. 1A) hybridizes to a specific PCR-generated sequence corresponding to the TZ region of ACC synthases (8, 10), indicating that this fragment contains the ACS4 gene (10). However, determination of the orientation of ACS4 by PCR in plasmid pAAA1 reveals that the 8.3-kb *EcoRI* fragment contains only part of the gene, coding for the N-terminal half of ACS4 (Fig. 1A). The 2.1-kb *EcoRI* fragment of λ AT-8 in pAAA3 codes for the C-terminal region and contains 3'-nontranslated sequences of ACS4 (Fig. 1A). To verify the immediate contiguity of both *EcoRI* fragments, an overlapping fragment was generated by PCR using λ AT-8 phage DNA as the template. The sequence of the subcloned fragment in pAAA5 is identical with flanking sequences of pAAA1 and pAAA3 inserts and excludes the possibility of an additional, closely positioned *EcoRI* site (Fig. 1A). The sequence of 3438 nt of the ACS4 genomic locus has been determined and is shown in Fig. 1B. The gene consists of four exons and three introns. The sequence also includes 1.3 kb of the 5'-flanking region and 0.5 kb of the 3'-nontranslated region. The predicted coding region of the ACS4 gene consists of 1,422 base pairs. The intron/exon junctions which are typical of donor and acceptor splice sites (27) have been established by reference to the sequence of ACS4

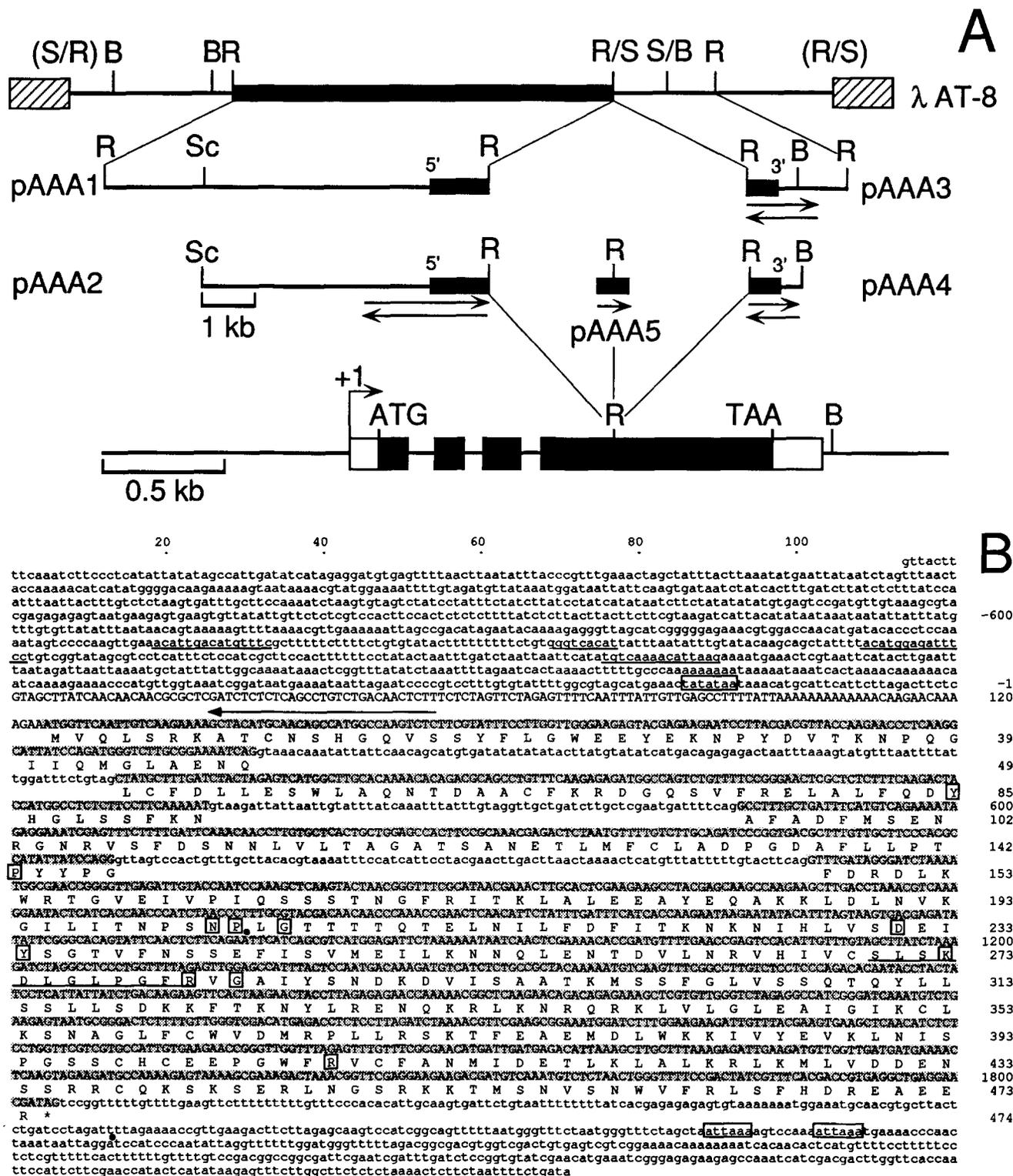


Fig. 1. Structure of the *ACS4* gene. A, gene organization of *ACS4* and structure of derived plasmid constructs. A partial restriction map of clone λ AT-8 and derived plasmids is shown (R, *EcoRI*; B, *BamHI*; S, *Sall*; Sc, *SacI*). Both strands of the indicated regions were sequenced by primer walking as described under "Experimental Procedures." The arrows indicate the extent of each sequence determination. The filled box in λ AT-8 is the fragment which hybridizes to the TZ-region of *ASC* genes (10). Filled boxes in the derived pAAA plasmids indicate the coding region of *ACS4*. The gene organization of *ACS4* is given below. Open blocks indicate the 5'- and 3'-untranslated regions. The exons are shown as filled blocks, and the connecting lines designate the introns. The +1 and arrow indicate the start and direction of transcription in *ACS4*. B, DNA sequence of the *ACS4* gene including introns and 5'- and 3'-flanking regions. The nucleotide at position +1 corresponds to the transcription initiation site (Fig. 2). The nucleotides upstream from the +1 position are negatively numbered. The sequence of the mRNA transcribed by the gene is shown in capital letters. The cDNA sequence is shaded; the remainder of the sequence is in small letters. The derived amino acid sequence is presented in the one-letter code below the DNA sequence and is numbered separately. The 11 boxed residues are the invariant amino acids conserved between *ACC* synthases and various aminotransferases (6). The underlined dodecapeptide beginning at Ser²⁷⁰ is part of the active center of *ACC* synthase (37). The arrow indicates primer DP1 used for determining the start of transcription (Fig. 2). The TATA box and putative polyadenylation signal sequences are boxed. Sequence motifs in the *ACS4* promoter reminiscent of functionally defined auxin-responsive elements are underlined. A dot indicates the *EcoRI* and *BamMI* site, respectively.

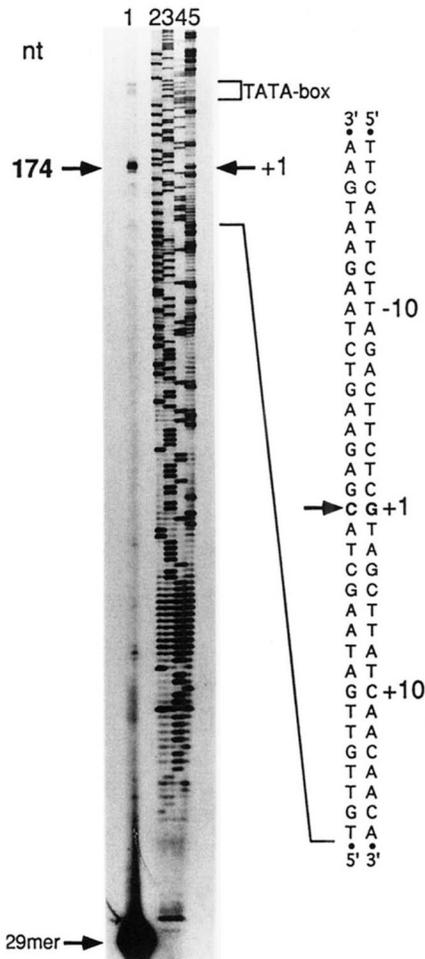


FIG. 2. Determination of the transcription initiation site of *ACS4*. ³²P-labeled primer DP1 (Fig. 1B) was hybridized with 15 μg of poly(A)⁺ RNA from etiolated *Arabidopsis* seedlings treated with 20 μM IAA and 50 μM CHX for 2 h, and extended with reverse transcriptase (lane 1). Lanes 2–5 are reactions G, A, T, and C, respectively. The arrow and bold residue represent the major start site of transcription.

cDNA (see below). The number and size of exons and the location of introns are similar to other ACS genes isolated from zucchini, tomato, rice, and *Arabidopsis* (5, 6, 8, 10). However, the zucchini twin genes *CP-ACS1A/1B* have five exons (5), and *LE-ACS4* from tomato and *VR-ACS4* and *VR-ACS5* from mung bean contain only three exons (6, 7).

Determination of the Transcription Initiation Site

The start site of transcription was determined by primer extension analysis using reverse transcriptase and the primer DP1 that is complementary to the 5'-end of the *ACS4* mRNA (Fig. 2). One major primer extension product of 174 nucleotides was obtained with poly(A)⁺ RNA from auxin-treated etiolated *Arabidopsis* seedlings. These data define the size of the 5'-nontranslated region of *ACS4* mRNA to be 124 nucleotides long (Fig. 1B, Fig. 2). The sequence at position -35 to -29, TATATAA, qualifies as a TATA box (27), and a CAAT sequence (27) is present further upstream at position -122 to -119 (Fig. 1B). The *ACS4* promoter contains four sequence motifs reminiscent of functionally defined *cis*-elements of early genes regulated by auxin in a primary fashion, *PS-IAA4/5* from pea (28, 29) and *GH3* from soybean (30) (Fig. 1B). A comparison in Fig. 3A shows that the sequence motif at position -411 to -404 of *ACS4* resembles the auxin-responsive element (*AuxRE*) of the auxin-responsive domain A in *PS-IAA4/5* from pea (28, 29). An

(-414)	<u>GTGGGTCACATTATTTAA</u>	(-397)	<i>ACS4</i>	A
.....	<u>GTGGGTCACATTATTTAA</u>	<i>rol b/c</i>	
(-190)	<u>ATATGTCCCATTCTTGTC</u>	(-173)	<i>PS-IAA4/5</i>	
(-256)	<u>ACGTGTCACAAACCCATT</u>	(-239)	<i>GmAux28</i>	
(-221)	<u>TGCTGTCCCACGTTTCCA</u>	(-204)	<i>OS-ACS1</i>	
(-373)	<u>TACA-TGG--AGATTTCC</u>	(-358)	<i>ACS4</i>	B
(-463)	<u>AACATTGA-CATGTTTCC</u>	(-447)	<i>ACS4</i>	
(-280)	<u>CACA-TGCTCATGTTTCC</u>	(-264)	<i>PS-IAA4/5</i>	
(-276)	<u>CACA-TGG-CATGTTTCC</u>	(-261)	<i>PS-IAA6</i>	
(-269)	<u>CACA-TGG-CATGTTTCT</u>	(-254)	<i>GmAux22</i>	
(-285)	<u>TGTCAAaacATTAAG</u>	(-271)	<i>ACS4</i>	C
(-176)	<u>TGTCTCctCAATAAG</u>	(-162)	<i>GH3</i>	
(-130)	<u>TGTCTC---AATAAG</u>	(-119)	<i>GH3</i>	
(-176)	<u>TGTCACcccTATAAG</u>	(-162)	<i>PS-IAA4/5</i>	

FIG. 3. Putative auxin-responsive elements in the promoter of *ACS4*. Sequence motifs of the *ACS4* promoter (underlined in Fig. 1B) are compared with functionally defined auxin-responsive *cis*-elements in *PS-IAA4/5* from pea (28, 29) and *GH3* from soybean (30). In addition, similar motifs of selected, early auxin-responsive genes are compared. Nucleotides conserved between *ACS4* sequence motifs and functionally characterized as well as putative auxin-responsive elements are shaded. A, comparison with the *AuxRD* A of *PS-IAA4/5* (28, 29). B, comparison with the *AuxRD* B of *PS-IAA4/5* (28, 29). C, comparison with the conserved motif in two functionally characterized auxin-responsive elements in *GH3* (30). The source of the sequences is the alignment by Oeller *et al.* (31) and, for *GH3*, the report by Li *et al.* (30).

AuxRD A element is present in the promoters of a number of early auxin-responsive genes, including *PS-IAA4/5*-related genes from various plant species, *SAUR* genes from soybean, *OS-ACS1* from rice, T-DNA gene 5 and *rol b/c* from *Agrobacterium* (31). Noteworthy, nucleotides flanking the *AuxRD* A-like sequence in *ACS4* and *rol b/c* are remarkably conserved (Fig. 3A). Two sequence motifs of *ACS4*, at position -462 to -448 and at position -372 to -359, display a high degree of identity with the *AuxRD* B element of the auxin-responsive domain in *PS-IAA4/5* and related genes (28, 29, 31) (Fig. 3B). The *AuxRD* B motif functions as an auxin-specific enhancer element in *PS-IAA4/5* (28, 29). In addition, a third sequence element at position -285 to -271 of *ACS4* is strikingly similar to a motif conserved in two independently acting auxin-responsive elements of the soybean *GH3* promoter (30). The presence of putative auxin-responsive elements in *ACS4* with similarities to functionally defined *cis*-elements in other early auxin-regulated genes suggests, at least in part, utilization of analogous *trans*-acting factors for signaling auxin-mediated *ACS4* gene activation.

Isolation of *ACS4* cDNAs

An *ACS4* cDNA was obtained by reverse transcription-coupled PCR amplification using primers corresponding to deduced nontranslated genomic sequences and poly(A)⁺ RNA from auxin-treated etiolated *Arabidopsis* seedlings as the template. The sequence of the PCR-generated *ACS4* cDNA in pAAA6 comprises 1,422 base pairs and is identical with the deduced coding region of the genomic *ACS4* clone. To determine the 3'-end of the *ACS4* gene, the insert of pAAA6 was used as a probe to screen two *Arabidopsis* cDNA libraries (32, 33). After screening of approximately 800,000 plaques, two hybridizing *ACS4* cDNA clones were purified. However, both cDNAs do not contain poly(A) tails. A second approach using inverse PCR to clone flanking *ACS4* cDNA sequences repeatedly failed for unknown reasons. Nonetheless, we note two potential polyadenylation signals, ATTTAA, approximately 210 base pairs downstream of the translational stop codon. The *ACS4* mRNA is predicted to be about 1,800 nt long (5'-untranslated region, 124 nt; coding region, 1,422 nt; 3'-untranslated

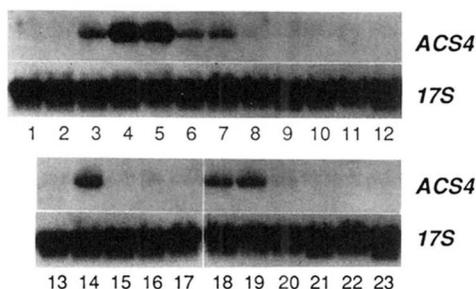


FIG. 4. **Specificity of ACS4 mRNA expression.** Total RNA (25 μ g) from 6-day-old etiolated seedlings treated for 1 h with various chemicals and conditions, if not otherwise indicated, were hybridized with the 32 P-labeled DNA insert of pAAA6. The lanes are: 1, untreated; 2 and 13, control-treated; 3 and 14, 20 μ M IAA; 4, 50 μ M CHX; 5, 20 μ M IAA + 50 μ M CHX after 30 min of pretreatment with 50 μ M CHX only; 6, 20 μ M 2,4-D; 7, 20 μ M α -NAA; 8, 20 μ M PAA; 9, 20 μ M L-tryptophane; 10, wounding; 11, 0.5 M sorbitol; 12, heat treatment at 42 $^{\circ}$ C for 15 min followed by 45 min recovery at room temperature; 15, 20 μ M abscisic acid; 16, 20 μ M gibberellic acid; 17, 20 μ M BA; 18, 20 μ M IAA + 20 μ M BA; 19, 20 μ M IAA + 20 μ M BA + 50 mM LiCl; 20, 50 mM LiCl; 21, 10 parts/million ethylene; 22, N₂; 23, air control.

region, \sim 250 nt), close to the size of 1,850 nt detected by RNA hybridization analysis (data not shown).

Properties of ACS4 and Expression in *E. coli*

Conceptual translation of the coding region of ACS4 mRNA yields a polypeptide of 474 amino acid residues (53,795 Da, pI 8.2), close to the size of other ACC synthase isoenzymes from various plant species (34). The ACS4 protein also contains all the conserved regions found in other ACS isoenzymes as well as the 11 invariant amino acids conserved between ACC synthases and aminotransferases (Fig. 1B). The dodecapeptide sequence characteristic of the pyridoxal phosphate binding site is also present (Fig. 1B). The ACS4 amino acid identity to other ACS isoenzymes varies from 49 to 73% (35). ACS4 is most similar in primary sequence to *Arabidopsis* ACS5, winter squash CM-ACS2, apple MS-ACS1, and to tomato LE-ACS3 (35). Together with the rice OS-ACS1 (8), these ACS isoenzymes comprise a major lineage in the ACC synthase phylogenetic tree (35). Interestingly, most of the genes in this lineage are auxin regulated in vegetative tissues indicating a striking correlation between their phylogenetic relationship and their pattern of expression (8, 35).

Authenticity of the polypeptide encoded by ACS4 mRNA was confirmed by expression experiments in *E. coli*. Plasmid pAAA6 contains in pUC19 a PCR-generated cDNA corresponding to the coding region of ACS4 mRNA, fused to the N terminus of the β -galactosidase gene in sense orientation. Transformants bearing the pAAA6 plasmid produce 2.8 nmol of ACC/10⁸ cells after 4 h of incubation in the presence of 1 mM isopropyl- β -D-thiogalactoside. *E. coli* cells containing only pUC19 do not accumulate detectable ACC.

Specificity of ACS4 mRNA Accumulation

We have previously shown that ACS4 is differentially expressed in mature *Arabidopsis* plants. ACS4 mRNA is detectable by RNA blot analysis in roots, leaves, and flowers only (10). Interestingly, ACS4 transcript accumulation is inducible by auxin. In this study, we have analyzed the auxin response of ACS4 in detail.

Response to IAA and CHX—The effect of IAA and of the protein synthesis inhibitor CHX was examined (Fig. 4). Treatment of 5-day-old intact etiolated *Arabidopsis* seedlings with 20 μ M IAA for 1 h increases the steady-state ACS4 mRNA level about 10-fold, relative to untreated or mock-treated seedlings (compare lane 3 with lanes 1 or 2). The response to auxin is

highly potentiated (about 50-fold) by 50 μ M CHX (compare lane 3 with lane 5). Application of 50 μ M CHX alone has the same effect (compare lane 3 with lane 4). At this concentration, CHX effectively prevents protein biosynthesis in *Arabidopsis* seedlings (data not shown). These results qualify ACS4 as a primary response gene which activation is independent of *de novo* protein synthesis. Induction of gene expression by protein synthesis inhibition can be explained by transcriptional activation via depletion of a short-lived repressor polypeptide and/or by mRNA stabilization (13, 36). Both effects have been described for CHX-mediated PS-IAA4/5 gene activation.²

Effect of IAA Analogs—The effect of various IAA analogs on steady-state levels of ACS4 mRNA is shown for 20 μ M 2,4-D (lane 6), 20 μ M α -NAA (lane 7), 20 μ M PAA (lane 8), and 20 μ M L-tryptophane (lane 9). As compared with the response to 20 μ M IAA (compare lane 1 or lane 2 with lane 3; or lane 13 with lane 14), the synthetic auxin 2,4-D and the natural auxin α -NAA are similar effective to induce accumulation of ACS4 transcripts. The weak natural auxin PAA is less effective, and the structural analog tryptophane has no effect.

Effect of Other Plant Hormones—Other plant hormones such as 20 μ M abscisic acid (lane 15), 20 μ M gibberellic acid (lane 16), 20 μ M BA (lane 17), or 10 parts/million ethylene (lane 21) do not increase ACS4 mRNAs above control levels (as compared with mock treatment in lane 13, or with an air control in lane 23 for the ethylene treatment). Furthermore, a combination of 20 μ M IAA with 20 μ M BA neither augments nor attenuates the response to auxin (compare lane 13 with lane 18).

Effect of Various Stress Conditions—The effect of several stress conditions on ACS4 gene expression was studied. Wounding (lane 10), osmotic shock (lane 11), heat shock (lane 12), and anaerobiosis (lane 23) fail to induce ACS4 transcript accumulation. LiCl has no effect, neither when applied alone (lane 20) nor in combination with 20 μ M IAA and 20 μ M BA (lane 19). Taken together, these results are very similar to the expression profile of early auxin-inducible IAA genes of *Arabidopsis*³ and demonstrate specificity of ACS4 gene expression for auxin and protein synthesis inhibitors such as CHX. A similar expression characteristics has been described for CM-ACS2 from winter squash (12), OS-ACS1 from rice (8), and for auxin-inducible LE-ACS genes from tomato.⁴

Kinetics of ACS4 mRNA Accumulation

We monitored ACS4 mRNA accumulation in intact etiolated seedlings for up to 8 h after addition of 20 μ M IAA (Fig. 5). The ACS4 gene responds rapidly to exogenous auxin by increasing transcript levels within the first 30 min of treatment (Fig. 5A). ACS4 mRNA accumulate steadily and reach a 20-fold higher steady-state level after 8 h of exposure to IAA (Fig. 5A). A short time course experiment reveals first detectable increases in ACS4 mRNA after 25 min of auxin treatment (Fig. 4B). A comparable induction kinetics in response to IAA has been reported for CM-ACS2 which transcripts start to accumulate 20 min after auxin addition (12) and for the auxin-inducible LE-ACS genes in tomato.⁴

Dose Response to IAA

Next, we studied the effect of different IAA concentrations on the expression of ACS4. A dose-response curve ranging from 1 \times 10⁻⁸ to 5 \times 10⁻⁴ M of IAA was obtained after an auxin exposure of intact seedlings for 2 h (Fig. 6). The ACS4 dose-response curve shows a characteristic bimodal shape. Optimal ACS4 transcript accumulation occurs at 1 \times 10⁻⁵ M of IAA.

² T. Koshiba and A. Theologis, unpublished data.

³ Abel, S., Nguyen, M., and Theologis, A. (1995) *J. Mol. Biol.*, in press.

⁴ K. Kawakita and A. Theologis, unpublished data.

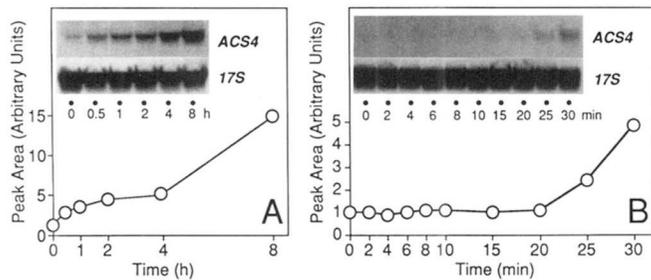


FIG. 5. Kinetics of *ACS4* mRNA accumulation in response to IAA. Intact etiolated *Arabidopsis* seedlings (5 day old) were incubated in the presence of 20 μ M IAA. After the indicated periods, total RNA was isolated and separated in a 1% agarose gel (25 μ g for each time point), transferred to a nylon membrane, and hybridized with the 32 P-labeled DNA insert of pAAA6 or with a 32 P-labeled 17 S rDNA insert. The results are shown graphically relative to the mRNA level of the zero time control (arbitrary value of "1"). The original autoradiogram is also shown.

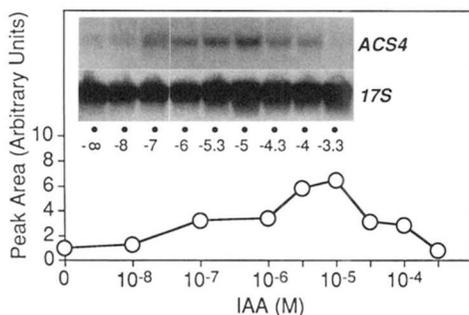


FIG. 6. Dose-response of *ACS4* to IAA. Intact etiolated *Arabidopsis* seedlings (5 day old) were treated with various concentrations of IAA for 2 h. Total nucleic acids were isolated and separated in a 1% agarose gel (25 μ g for each IAA concentration), transferred to a nylon membrane, and hybridized with the 32 P-labeled DNA insert of pAAA6 or with a 32 P-labeled 17 S rDNA insert. The results are shown graphically relative to the mRNA level of the no auxin control (arbitrary value of "1"). The original autoradiogram is also shown.

Higher IAA concentrations are suboptimal, and IAA at 5×10^{-4} M completely inhibits *ACS4* mRNA accumulation. However, IAA concentrations as low as 100 nM are sufficient to elevate basal *ACS4* mRNA levels. A similar biphasic dose-response curve has been described for a number of other primary auxin-responsive genes, such as *IAA* genes in *Arabidopsis* seedlings,³ *PS-IAA4/5* in pea protoplasts (28), *GH1* in soybean seedlings (38), or the *SAUR* promoter-GUS transgene in tobacco (39). Interestingly, the bimodal response of auxin-induced gene expression is paralleled by biphasic characteristics of other auxin-elicited processes such as stem elongation, stomatal opening, or potassium influx into stomatal guard cells (40).

Expression of *ACS4* in Auxin-resistant Mutants

Expression of *ACS4* was studied in the *Arabidopsis* auxin-resistant mutant lines *axr1-12* (16), *axr2-1* (17), and *aux1-7* (18). These mutants have a pleiotropic though auxin-related phenotype and are cross-resistant to several other plant hormones, including ethylene (41). Intact etiolated wild type and mutant seedlings were treated in the presence or absence of 20 μ M IAA for 2 h (Fig. 7). Relative to the expression in mock-treated and auxin-treated wild type tissue, steady-state *ACS4* mRNA levels are severely reduced (greater than 10-fold) in respectively treated *axr1-12* and *axr2-1* seedlings. In contrast to *axr1-12* seedlings, auxin inducibility of *ACS4* mRNA accumulation in *axr2-1* plants is abolished. Unlike in the *axr1-12* and *axr2-1* mutant lines, *ACS4* gene expression is only mod-

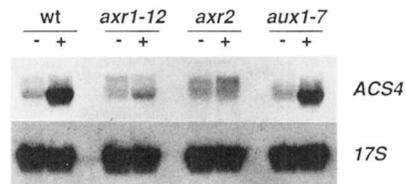


FIG. 7. Expression of *ACS4* mRNA in etiolated auxin-resistant seedlings. Intact etiolated *Arabidopsis* seedlings (5 day old), wild type or the mutant lines *axr1-12*, *axr2-1*, or *aux1-7*, were incubated in the presence (+) or absence (-) of 20 μ M IAA for 2 h. Total RNA was isolated, separated (25 μ g) in a 1% agarose gel, transferred to a nylon membrane, and hybridized with the 32 P-labeled DNA insert of pAAA6 or with a 32 P-labeled 17 S rDNA insert.

estly affected in *aux1-7* seedlings (Fig. 7). *ACS4* transcript levels are 1.5-fold reduced, however, auxin inducibility is retained. A similar defective expression in these three mutant lines has been described for members of the IAA multigene family³ and for the *SAUR-AC1* gene (42, 43). Inhibition of three classes of genes with different auxin-responsive elements supports the previous notion that the mutations act early in an auxin response pathway and probably affect general components in hormone signaling (41). In view of the specificity of *ACS4* induction for auxin, defective expression in auxin-resistant mutant plants suggests a role of *ACS4* in auxin action and demonstrates the interrelationship of both plant hormones. This interpretation is corroborated by the observation that expression of auxin-regulated *LE-ACS* genes is defective in the *diageotropica* mutant of tomato.⁴ The *diageotropica* mutant expresses only low levels of an auxin-binding protein which presumably is an auxin receptor (44).

CONCLUSIONS

We have shown that *ACS4* of *A. thaliana* is an early auxin-responsive gene which is activated by the hormone in a primary fashion. The expression characteristics of *ACS4* are intriguingly similar with the hormonal response of early *IAA* genes in *Arabidopsis*.³ Consistently with this observation is the presence of four putative auxin-responsive *cis*-elements in the *ACS4* promoter that are similar to functionally characterized *cis*-elements of early auxin-inducible genes (28–31). The specificity of induction for auxin treatment and the defective expression of *ACS4* in auxin-resistant *Arabidopsis* mutants qualify *ACS4* as a probe to study molecular aspects of the intimate interrelationship of auxin and ethylene action.

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