

# Biochemical Diversity among the 1-Amino-cyclopropane-1-Carboxylate Synthase Isozymes Encoded by the *Arabidopsis* Gene Family\*

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1-Amino-cyclopropane-1-carboxylate synthase (ACS, EC 4.4.1.14) is the key enzyme in the ethylene biosynthetic pathway in plants. The completion of the *Arabidopsis* genome sequence revealed the presence of twelve putative ACS genes, *ACS1–12*, dispersed among five chromosomes. *ACS1–5* have been previously characterized. However, *ACS1* is enzymatically inactive whereas *ACS3* is a pseudogene. Complementation analysis with the *Escherichia coli* aminotransferase mutant DL39 shows that *ACS10* and *12* encode aminotransferases. The remaining eight genes are authentic ACS genes and together with *ACS1* constitute the *Arabidopsis* ACS gene family. All genes, except *ACS3*, are transcriptionally active and differentially expressed during *Arabidopsis* growth and development. IAA induces all ACS genes, except *ACS7* and *ACS9*; CHX enhances the expression of all functional ACS genes. The ACS genes were expressed in *E. coli*, purified to homogeneity by affinity chromatography, and biochemically characterized. The quality of the recombinant proteins was verified by N-terminal amino acid sequence and MALDI-TOF mass spectrometry. The analysis shows that all ACS isozymes function as dimers and have an optimum pH, ranging between 7.3 and 8.2. Their  $K_m$  values for AdoMet range from 8.3 to 45  $\mu\text{M}$ , whereas their  $k_{\text{cat}}$  values vary from 0.19 to 4.82  $\text{s}^{-1}$  per monomer. Their  $K_i$  values for AVG and sinefungin vary from 0.019 to 0.80  $\mu\text{M}$  and 0.15 to 12  $\mu\text{M}$ , respectively. The results indicate that the *Arabidopsis* ACS isozymes are biochemically distinct. It is proposed that biochemically diverse ACS isozymes function in unique cellular environments for the biosynthesis of  $\text{C}_2\text{H}_4$ , permitting the signaling molecule to exert its unique effects in a tissue- or cell-specific fashion.

The gas ethylene has been known since the beginning of the century to be used by plants as a signaling molecule for regulating a variety of developmental processes and stress responses (1). These include seed germination, leaf and flower senescence, fruit ripening, cell elongation, nodulation, wounding and pathogen responses. Ethylene production is induced by

a variety of external factors, including wounding, viral infection, elicitors, auxin treatment, and  $\text{Li}^+$  ions (2–7).

Ethylene is biosynthesized from methionine, which is converted to AdoMet<sup>1</sup> by the enzyme AdoMet synthetase. AdoMet is converted by the enzyme ACS to ACC, the precursor of ethylene (5, 7–9). ACC is oxidized to ethylene by ACO. ACS is a pyridoxal phosphate-containing enzyme (2, 5, 10) and its activity is regulated at the transcriptional (11–18) and post-transcriptional levels (19–21). The ethylene biosynthetic enzymes, AdoMet synthetase, ACS, and ACO, are encoded by multigene families in various plant species (5, 9, 16, 17, 22–25). The crystal structures of apple and tomato Le-ACS2 isozymes were recently elucidated (26, 27). The structures show that the enzyme is a homodimer, and its overall fold and active sites are similar to those of aminotransferases even though the two enzymes have completely different catalytic activities. The tertiary structures together with available biochemical data explain the catalytic roles of the conserved and non-conserved active site residues (28–31).

The sequencing of the *Arabidopsis* genome revealed that ACS genes are putatively encoded by twelve genes (32). The question immediately arises as to why there are so many ACS isozymes for synthesizing ethylene in *Arabidopsis*. It has been postulated that the presence of ACS isozymes may reflect tissue-specific expression that satisfies the biochemical environment of the cells or tissues in which each isozyme is expressed (15). For example, a group of cells or tissues with low concentration of the ACS substrate, AdoMet, would express a high affinity (low  $K_m$ ) ACS isozyme. Accordingly, the tissue-specific expression of distinct ACS isozymes, defined by their unique biochemical properties, would allow  $\text{C}_2\text{H}_4$  to be made in a tissue-specific manner to mediate various biological responses. Herein, we report the biochemical characterization of the *Arabidopsis* ACS gene family members as a first step toward understanding the role of multigene families in plants in general and of ACS in particular.

## EXPERIMENTAL PROCEDURES

### Materials

*pET22*, *pET28*, *pET32* vectors, and thrombin protease (restriction grade) were purchased from Novagen (Darmstadt, Germany). *pBAD-HisA* vector was from Invitrogen (Carlsbad, CA), and *Escherichia coli*

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<sup>1</sup> The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; ACS, 1-amino-cyclopropane-1-carboxylate synthase; ACC, 1-aminocyclopropane-1-carboxylic acid; ACO, ACC oxidase; AVG, aminoethoxyvinylglycine; CHX, cycloheximide; IAA, indoleacetic acid; PLP, pyridoxal-5'-phosphate; PMSF, phenylmethanesulfonyl fluoride; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; NDSB, non-detergent sulfobetaine; ORF, open reading frame; MES, 2-morpholinoethanesulfonic acid; NTA, nitrilotriacetic acid.

BL21-CodonPlus™(DE3)RIL strain was from Stratagene (La Jolla, CA). Superdex™-200 was from Amersham Biosciences. *pQE80* vector and Ni-NTA agarose were from Qiagen (Valencia, CA). Complete™-EDTA-free was purchased from Roche Applied Science (Indianapolis, IN). The AdoMet used in the enzyme assay (contains 59% of (S,S)-diastereomer form) was purchased from Roche Applied Science. Sinefungin and AVG were purchased from Sigma. All other chemicals used for biochemical analysis were of analytical grade. Oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA).

### Molecular Biology

Standard protocols were followed for DNA manipulations (33). Site-directed mutagenesis was carried out according to the QuikChange kit manual from Stratagene (34). DNA sequencing was used to confirm that no spurious mutations were introduced during mutagenesis.

### Expression of ACS

(a) *Plant Material*—*A. thaliana* ecotype Columbia seeds were surface sterilized for 8 min in 5% sodium hypochlorite, 0.15% Tween 20, excessively rinsed in distilled water, and plated on Petri dishes onto sterilized filter paper discs on top of 0.8% agar (Select Agar, Invitrogen) containing 0.5× Murashige-Skoog salts (Invitrogen), 0.5 mM MES, pH 5.7, 1% sucrose, 1× vitamin B5. The plates were incubated in the dark at 25 °C for 7 days after cold treatment at 4 °C for 2 days. Intact etiolated seedlings were removed and placed in Petri dishes containing 0.5× Murashige-Skoog salts solution buffered at pH 5.7 with 0.5 mM MES and supplemented with 20 μM IAA or 50 μM CHX. The seedlings were incubated for 2 h in the dark at room temperature with shaking (100 rev/min). Mock controls were incubated with an equal amount of solvent (ethanol) used to prepare the stock solution of the chemicals. Five g-fresh weight of seedlings were removed, briefly blotted dry, frozen in liquid nitrogen, and stored at -80 °C. Three-week-old light-grown plants were grown as described in the *Arabidopsis* Biological Resources Center (ABRC) manual. Roots, leaves, stems, flowers, and siliques were collected and quick-frozen in liquid nitrogen and stored at -80 °C.

*RT-PCR*—Total RNA was isolated from 7-day old etiolated seedlings, treated with or without 20 μM IAA or 50 μM CHX, and from various parts of light grown plants, using the RNeasy kit (Qiagen) or with the TRIzol reagent (Invitrogen). Genomic DNA was removed by treating 1–2 mg of total RNA with 10 units of RNase-free DNase I (Roche Applied Science) by incubating for 15 min at 37 °C in 10 μl of 1× DNase I buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>) containing 40 units of RNase inhibitor. 5 μg of DNase treated RNA primed with oligo (dT)<sub>24</sub> primer were used for first strand synthesis with SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

PCR was performed in the GeneAmp PCR System 9700 (Applied Biosystems). The total reaction volume was 25 μl containing 2.5 μl of 1st strand cDNA, 1 μl of primer solution (10 μM), 2.5 μl of 10× PCR buffer, 2 μl dNTP mix, and 0.2 μl ExTaq enzyme (Takara). The reaction mixtures were subjected to the following PCR conditions: 94 °C for 2 min, one cycle; 94 °C for 15 s, 62 °C for 15 s, and 72 °C for 3 min for 35 cycles; followed by one cycle of incubation at 72 °C for 3 min. The primers used are: *ACS1* F, 5'-TGTCTCAGGGTGCATGTGAGAATCAACTT-3' and R, 5'-AGCTCGAAGCAATGGTGAATGAGGAGACA-3'; *ACS2* F, 5'-GCGACTAACAAATCAACACGGAG-3' and R, 5'-ACATTATCCCTGGAGACGAGAGAC-3'; *ACS4* F, 5'-CCAAGTCTCTTCTGATTTCCCTT-3' and R, 5'-TAGTCGAAAACCCAGTTAGAGAC-3'; *ACS5* F, 5'-CCAGCTATGTTTCGATCTAATCGAGTTCATGGTTAAC-3' and R, 5'-TCCATGAAACCCGAAAACCCAGTTAGAGACTGTC-3'; *ACS6* F, 5'-TGACGGTACGGCGAGAATTCCTTATT-3' and R, 5'-CCTGAGGTTACTCTGCCAACACTTCTTCT-3'; *ACS7* F, 5'-AACAAACAACGTCGAGCTTCTCGAGT-3' and R, 5'-AGATCCCGGAGATATATTCAGGTTTCAGCT-3'; *ACS8* F, 5'-CGATCTCATTGAGTTCATGGCTGTGTAAGA-3' and R, 5'-ACGFTCCATCAACGAACTCTCAATCTA-3'; *ACS9* F, 5'-GGATGGGAAGAATACGAGAAGAACC-3' and R, 5'-ATCACTCTTCTACTATCTGTTGACTC-3'; *ACS10* F, 5'-AGGGTTATTGTTCCGTATACAAGTGTGGT-3' and R, 5'-TACGGAACCATCTGTTCCGATACAGTGA-3'; *ACS11* F, 5'-AACCTCGACTAACGAGACTCAATGTTCT-3' and R, 5'-ATGACACGATGAGCCTGGAGAGATGTTAA-3'; *ACS12* F, 5'-TTCGTCGGCTCTCTCATTCTTGTGTTCT-3' and R, 5'-ACCACACTCTCAGCACATGGTATTCTCA-3'.

For *ACT8*, the primers used were the same as described in Ref. 35.

### Construction of the ACS ORFs

Full-length open reading frame clones (ORFs) for all the putative ACS genes were constructed by: 1) PCR from preexisting full-length

cDNAs (*ACS1* U26543; *ACS2* M95595; *ACS4* U23481; *ACS5* L29261), using the following gene-specific primers: *ACS1*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGTCTCAGGGTGCATGTGAG-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTTAAGCTCGAAGCAGATGGTGA; *ACS2*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGGTCTTCCGGGAAAAAT-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTCATGTCTCGGAGAAGAGGTGA-3'); *ACS4*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGGTTCATGTCAAGAAA-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTATCGTTCCTCAGCCTCACG-3'); *ACS5*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGAAACAGCTTTCGACAAAA-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTCATCGTTCATCAGGTACAGC-3').

2) RT-PCR poly(A)<sup>+</sup> RNA from CHX-treated etiolated seedlings was purified by oligo(dT) cellulose (Qiagen) following the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of poly(A)<sup>+</sup> RNA with oligo dT<sub>18</sub> as a primer and 1000 units of SuperScript II RT enzyme (Invitrogen) as previously described (36). First-strand cDNA was re-suspended into 50 μl of dH<sub>2</sub>O after phenol/chloroform extraction followed by EtOH precipitation. Using gene-specific primers, 0.5 μl of 1st-strand cDNA and 3.75 units of Expand High Fidelity DNA polymerase (Roche Applied Science), cDNAs were amplified using the following conditions. After initial denaturation for 2 min at 94 °C, touch-down PCR (37) was performed, consisting of 12 cycles of 15 s denaturation at 94 °C, 30 s annealing at 63 °C with negative ramp 6 °C at 0.5 °C per cycles and 3 min at 68 °C, followed by 18 cycles of 15 s at 94 °C, 30 s at 57 °C and 3 min at 68 °C. A final elongation was undertaken at 68 °C for 7 min. Amplified cDNAs were purified with the Qiagen PCR purification kit.

The gene-specific primers used were: *ACS6*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGGTGGCTTTTGCAACAGAG-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTTAAGTCTGTGCA-CGGACTAG-3'); *ACS7*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGGTCTTCTCTAATGATG-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTCAAAACCTCTCTCGTGGTC-3'); *ACS8*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGGG-TCTTGTCAAAGAAA-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTATCGTTCCTCGGGTTCAGC-3'); *ACS9*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGAAACACTGTGAGAAA-A-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTCATCGTTCATCAGGTACAGC-3'); *ACS10*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGACCCGTACCGAACAAAC-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTCAATTTGAGATTACATGT-3'); *ACS11*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGTTGTCAAGCAAAGTTGTT-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTCAACGTTCTGATTCAAAAGT-3'); *ACS12*: F, (5'-GAATTCGGCCGTCGAAGGCCAGAAGGAGATATAACCATGAGGTTGATAGTACCTC-3') and R, (5'-AGTCGACGGCCCATGAGGCCCTCAAGATCTAAATGATTACAGC-3').

The ORFs were cloned into the *pUNI51* vector and sequenced. *pUNI51* was derived from *pUNI50* (38) by introducing a new polylinker containing two SfiI sites (A and B) that allow cloning of a full-length ORF unidirectionally by having the SfiI A and B sites at the 5'- and 3'-ends of the ORF, respectively. The sequences of all eleven putative ACS ORFs are error-free and can be transferred into any desirable expression vector by *in vitro* *crellox* recombination (38). Their sequences have been deposited in GenBank™. The clones are publicly available through the ABRC ([www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm](http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm)).

### Construction of *pBAD-Trx-His* Vector

All ACS isozymes were expressed in *E. coli* as N-terminal fusions with thioredoxin and His tags containing a thrombin cleavage site adjacent to the N terminus of ACS using the *pBAD* promoter (41). Fig. 1A shows the overall strategy for constructing the expression vector, *pBAD-Trx-His* for achieving this goal. The NdeI site present in front of the thioredoxin ORF was changed to the NcoI site by site-directed mutagenesis using the primer P1, 5'-CTTTAAGAAGGAGATATAC-CATGGGAAGCGATAAAAATTATTCACC-3' (underlined shows the NcoI restriction site) giving rise to *pET321*. The NdeI site present in front of the His tag was deleted and replaced with the MscI-BglII linker, 5'-CCATCATCATCATCACTCTCTGGA-3' (underlined shows the His<sub>6</sub> tag sequence) giving rise to *pET322*. A thrombin protease site and a NdeI site were introduced in front of the multicloning site (MCS) of *pET32* with a KpnI-BamHI linker, 5'-CGGTGGTGGCTCCGGTCTG-GTGCCACGGGT AGTCATATGGATATCG-3' (bold and underlined show thrombin and NdeI sites, respectively), replacing the KpnI/BamHI sites of *pET322*, giving rise to *pET323*.

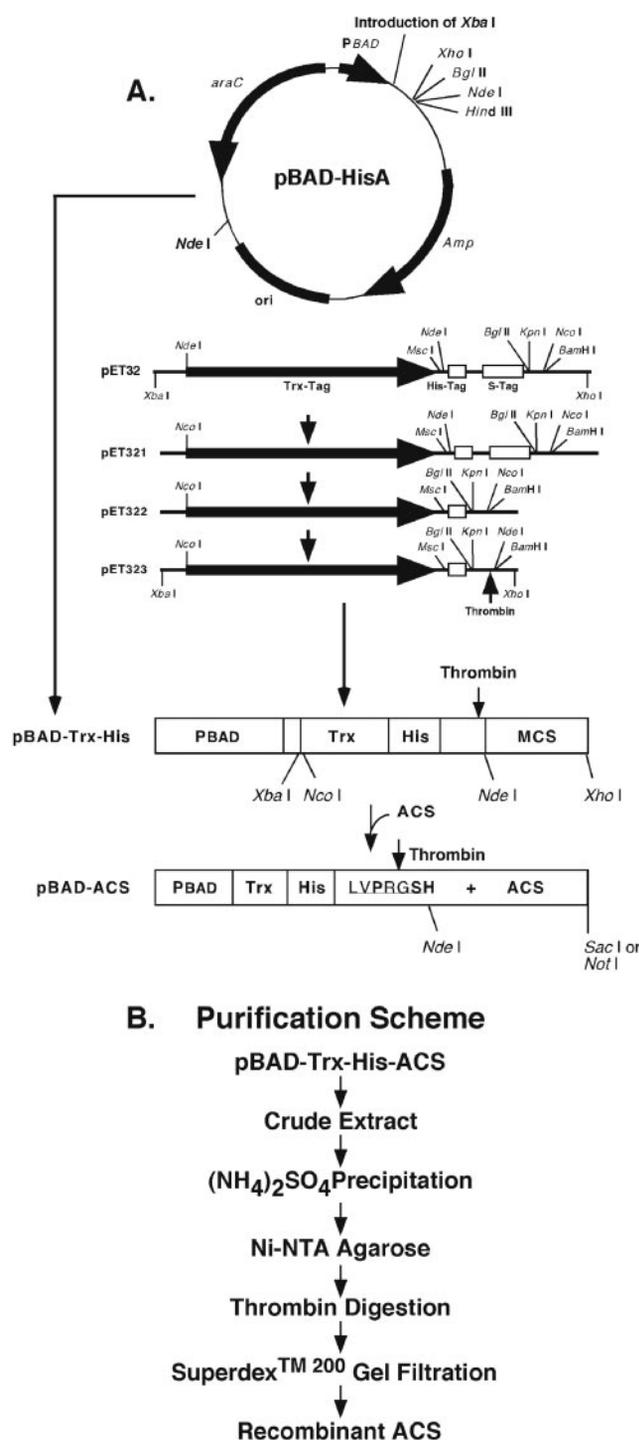


FIG. 1. Vector construction and purification scheme. *A*, construction strategy of the expression vectors *pBAD-Trx-His* and *pBAD-ACS* used for expression of ACS isozymes in *E. coli*. *B*, purification scheme of *E. coli* recombinant ACS proteins.

The *pBAD-HisA* vector was modified as follows: the *NdeI* site present downstream of the origin of replication was deleted by site-directed mutagenesis using the primer P2, 5'-CGGTATTTTCACACCGCATATC-GTGCACCTCTCAGTACAATC-3' (bold shows mutated base). The other *NdeI* site present in the MCS of *pBAD-HisA* was deleted by *BglII*/*HindIII* digestion and filling in with T4 DNA polymerase. An *XbaI* site was introduced between the *pBAD* promoter and the ribosome-binding site (*rbs*) by site-directed mutagenesis using the primer P3, 5'-CATACCCGTTTTTTGGTCTAGAAGGAGGAATTAACCATG-3' (underlined shows the *XbaI* site). Finally, the *XbaI*/*XhoI* fragment of *pET323* containing the *rbs*, *Trx* tag, *His* tag, *thrombin* site, and MCS was subcloned into the *XbaI*/*XhoI* sites of the modified *pBAD-HisA* giving rise to the *pBAD-Trx-His* vector used in this study.

*Expression in E. coli*—The ACS ORFs were subcloned into *pBAD-Trx-His* and *pET22* vectors as *NdeI*/*SacI* fragments (ACS2, -4, -5, -6, -8, -9, and -12) or as *NdeI*/*NotI* fragments (ACS7, -10, and -11) (42) giving rise to the *pBAD-ACS2-12* and *pET22-ACS2-12* plasmids, respectively. The *pBAD-Trx-His* expression system produces His-tagged proteins that can be purified by affinity chromatography. The *pET22* expression system (42) produces native proteins that can be purified by conventional purification procedures (non-affinity chromatography). We decided to use the *pBAD* expression system because it gave the same levels of enzyme activities as the *pET22* system (see Table I) while allowing protein purification by affinity chromatography. Attempts to purify the ACS isozymes using the IMPACT I (Intein mediated purification with an affinity chitin-binding tag; New England Biolabs) expression system were unsuccessful.

*E. coli* transformants harboring the expression vectors *pET22-ACS2-12* and *pBAD-ACS2-12* were cultured in LB and RM media (1× M9 salts-2% Casamino acids-1 mM MgCl<sub>2</sub>) plus 0.2% glucose, respectively, with 150 μg/ml ampicillin at 37 °C in a buffered flask with constant shaking at 300 rpm, until the cell cultures reached an OD<sub>600</sub> of 0.8. Isopropyl-1-thio-β-D-galactopyranoside (*pET* plasmid) or L-(+)-arabinose (*pBAD* plasmid) was added to each culture to a final concentration of 1 mM or 0.2% (w/v), respectively, and the cell cultures were then allowed to grow for an additional 4 h at 30 °C. Cells were pelleted by centrifugation at 3,000 × *g* for 15 min, washed once in half of the original volume with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, and stored at -70 °C. For large scale protein preparation, a single colony containing *pBAD-ACS* plasmid was first inoculated into 100 ml of RM plus glucose medium, incubated until OD<sub>600</sub> reached 1.0, then transferred into 3 to 6 liters of the same medium containing 150 μg/ml ampicillin, and induced under the same conditions. Cells were harvested by centrifugation at 1,500 × *g* for 20 min at 4 °C.

#### Assay for ACS Activity

ACS activity was assayed in a reaction mixture (total volume, 0.4 ml) containing 100 mM HEPES buffer, pH 8.0–10 μM PLP-120 μM AdoMet and an appropriate enzyme fraction. The mixture was incubated at 30 °C for 20 min, and the reaction was terminated with 50 μl of 10 mM HgCl<sub>2</sub>. The amount of ACC formed was determined according to (43). One unit of enzyme activity converts 1 μmol of AdoMet to ACC per hour at 30 °C.

#### Protein Purification

The overall protein purification scheme is shown in Fig. 1*B*. Frozen cell pellets were resuspended in 1:20 of the original volume in extraction buffer containing 50 mM potassium phosphate, pH 8.0, 10 μM PLP, 0.1 M NaCl, 1 mM PMSF, 2 mM EDTA, 2 mM NDSB201-100 μl/ml lysozyme, and proteinase inhibitor Complete™ (1 tablet per 50 ml of buffer). The cells were lysed by sonication. An aliquot of the cell extract was analyzed on SDS-PAGE. After centrifugation at 20,000 × *g* for 30 min, the supernatant was used as crude ACS solution. Twenty percent of the streptomycin sulfate solution was added to the crude ACS solution to the final concentration of 1.5% to precipitate genome DNA. The precipitate was removed by centrifugation. The supernatant was precipitated with ammonium sulfate (100% saturation), and the resulting precipitate was collected by centrifugation in a small volume of 50 mM potassium phosphate buffer, pH 8.0, 10 μM PLP, 1 mM PMSF, 0.5 M NaCl, 2 mM NDSB201-Complete™ and desalted on a Sephadex-25 column (2.5 × 30 cm) equilibrated with the same buffer. Imidazole solution, 1 M, pH 8.0, was added to the fraction with ACS activity, to a final concentration of 10 mM, and applied to Ni-NTA agarose column (1 × 3 cm) equilibrated with the same buffer. Weakly bound proteins were washed from the resin with 50 mM potassium phosphate buffer, pH 8.0, 10 μM PLP, 1 mM PMSF, 0.5 M NaCl, 20 mM imidazole, 2 mM NDSB201-Complete™. The His<sub>6</sub>-tagged protein was eluted with 50 mM potassium phosphate buffer, pH 8.0, 10 μM PLP, 1 mM PMSF, 0.5 M NaCl, 250 mM imidazole, 2 mM NDSB201-Complete™. Eluted fractions with ACS activity were buffer-exchanged on a Sephadex-25 m in 20 mM Tris-HCl buffer, pH 8.0, 10 μM PLP, 150 mM NaCl, 2 mM NDSB201-2.5 mM CaCl<sub>2</sub>. Subsequently, thrombin protease (1 unit/mg ACS) was added to the thioredoxin-His<sub>6</sub>-tag-ACS solution and digested at 20 °C for 16 h. The digest was precipitated with ammonium sulfate and the resulting precipitate was dissolved in a small volume of 50 mM potassium phosphate buffer, pH 8.0, containing 10 μM PLP, 0.1 mM PMSF, 0.3 M NaCl, 2 mM NDSB201, and fractionated on a Superdex™-200 column (1 × 30 cm) equilibrated with the same buffer at a flow rate of 0.4 ml/min using an Amersham Biosciences FPLC system.

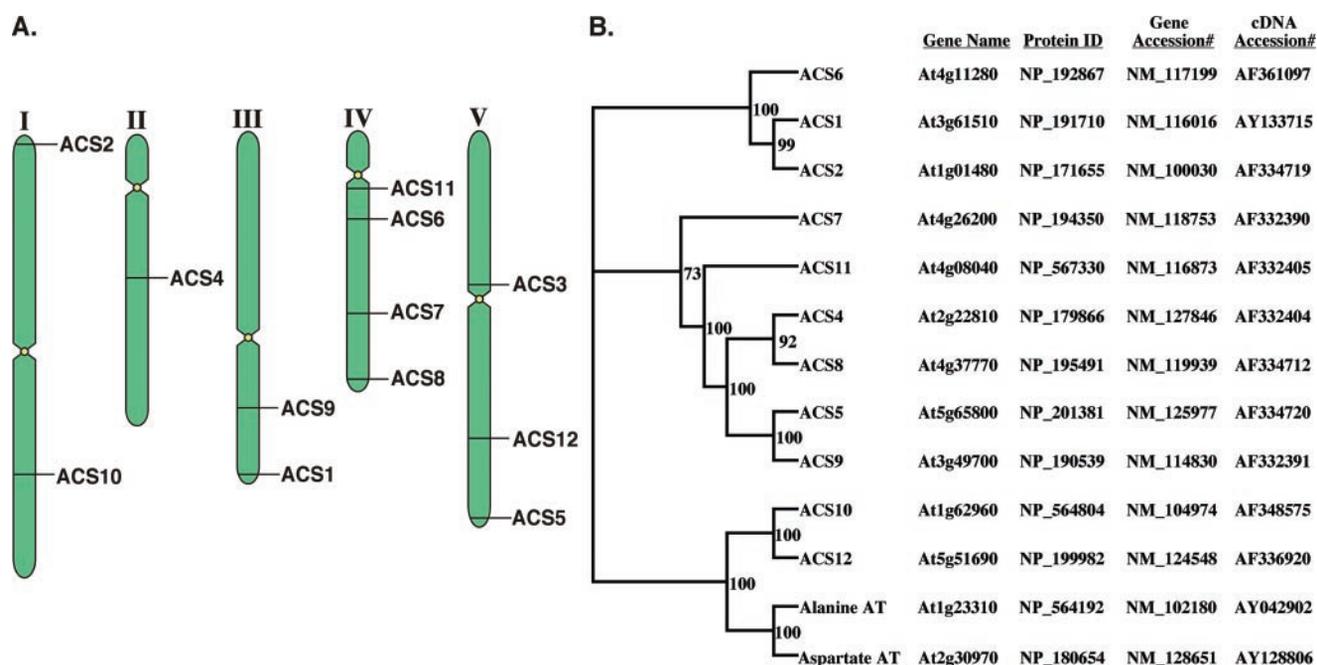


FIG. 2. Chromosomal location and phylogenetic analysis of ACS genes. A, the location of 12 putative ACS genes on the five (I–V) *Arabidopsis* chromosomes is shown according to the V4.0 version of the *Arabidopsis* genome annotation submitted to GenBank™. B, an unrooted dendrogram was generated using ClustalW (39). Treeview was used to generate the graphical output (40). The numbers at the branching points indicate the percentage of times that each branch topology was found during bootstrap analysis ( $n = 1000$ ). The gene names, accession numbers, protein i.d., and the accession numbers of the full-length ORFs of each gene are also shown.

#### Protein Determination and N-terminal Sequence Analysis

Protein concentration was determined with the bicinchoninic acid method (44). N-terminal sequence analysis was done at the Stanford Protein and Nucleic Acid Facility.

#### Molecular Size Determination

SDS-PAGE was carried out with 10% acrylamide gel according to (45) using marker proteins:  $\beta$ -galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa). Superdex™-200 (1 × 30 cm) column was calibrated with following marker proteins (Sigma): apoferritin (443 kDa),  $\alpha$ -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (29 kDa). MALDI-TOF mass analysis was carried out on a Bruker Reflecton II MALDI-TOF mass spectrometer. Samples were prepared by mixing one part of protein sample with nine parts of matrix (saturated sinapinic acid or cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid, 30% acetonitrile), and 1  $\mu$ l of this solution was applied onto the sample probe. External calibration was carried out using two ion peaks from bovine serum albumin:  $[M + 2H]^{2+}$ , 33,216;  $[M + H]^+$ , 66,432.

#### Determination of Kinetic Parameters for ACS

ACC synthase activity was measured at different AdoMet concentrations ranging from 3 to 200  $\mu$ M, in a reaction mixture containing 100 mM buffer-10  $\mu$ M PLP-1 mM dithiothreitol. The buffers used were: pH 5.0–5.5, sodium acetate buffer; pH 6–7, MES buffer; pH 7–8.5, HEPES buffer; pH 8.5–9.0, EPPS buffer; pH 10–11, glycine-NaOH buffer. The amount of protein used for each reaction was ACS2, 77 ng; ACS4, 80 ng; ACS5, 21 ng; ACS6, 9.8 ng; ACS7, 210 ng; ACS8, 10 ng; ACS9, 9.8 ng; and ACS11, 87 ng, respectively.  $K_m$  and  $K_i$  measurements were carried out at the optimum pH of each isoenzyme. The  $K_m$  value for AdoMet was calculated from Lineweaver-Burk plots. Each ACS was incubated with various concentrations of AVG (46, 47) or sinefungin (48), and the rate of ACC formation was determined. The  $K_i$  values were determined using Dixon plots.

#### Complementation Experiment

The ACS ORFs were subcloned downstream of the T5 promoter in the His tag-containing *pQE80* vector. ACS1,2,4,5,6,8,9,12 were subcloned as NdeIblunt/SacI fragments into BamHIblunt/SacI-digested vectors. ACS10 was subcloned as a NdeIblunt/NotIblunt fragment into BamHIblunt/SalIblunt-digested *pQE80*. ACS11 was subcloned as

NdeIblunt/SspIblunt into BamHIblunt/SacIblunt *pQE80* vector. Each expressed ACS protein contains a His tag at its N terminus. The AATase cDNA, *pAAT37* (49) from the alfalfa nodule was used as a positive control. The *E. coli* aspartate aminotransferase auxotroph DL39 is devoid of aminotransferase activity for tyrosine, phenylalanine, and aspartate (50, 51). *E. coli* DL39 was transformed with the *pQE-ACS* plasmids and the transformants were plated on M9 medium plus various amino acids and incubated at 30 °C for 2 days, and photographed. The transformants were also grown in liquid M9 medium in the presence of various amino acids at 30 °C for 24 h, and the absorbance at 600 nm was determined.

## RESULTS

**The Arabidopsis ACS Gene Family**—The *Arabidopsis* genome contains twelve annotated ACS genes (32), scattered among the five chromosomes (Fig. 2A). Five of them have been previously cloned and structurally characterized (52). ACS1 is enzymatically inactive (52), ACS3 is a pseudogene (52) and ACS2, 4, and 5 encode enzymatically active ACS (22, 52). The remaining ACS genes are newly discovered, except for ACS6, which had been previously cloned as a cDNA (53). Phylogenetic analysis reveals that the genes fall into three branches (Fig. 2B). ACS10 and 12 are phylogenetically related to alanine and aspartate ATases, respectively (Fig. 2B).

The polypeptides of the ACS gene family are quite similar in size, ranging between ~50.9 (ACS7) to ~61.2 kDa (ACS10) (Table I). ACS3 is not included in the analysis because it is a truncated polypeptide (52). The molecular sizes of the ACS family are similar to those reported from other plant species (8, 15). Alignment of the eleven putative ACS polypeptides is shown in Fig. 3. All ACS isozymes contain the seven conserved boxes (boxes 1–7) found in ACS from other plant species (56). In addition, the eleven conserved residues between ACS and ATases are also present in the *Arabidopsis* ACS gene family members, except for the tyrosine (Y) residue in box 2, which is part of the PLP-binding site (14, 15, 57, 58), and the glycine (G) residue in box 3, which has been replaced by serine (S) and phenylalanine (F), respectively, in ACS10 and ACS12 (Fig. 3, compare residues with open circles). The conserved glutamate (E) residue in box 1, which is responsible for substrate speci-

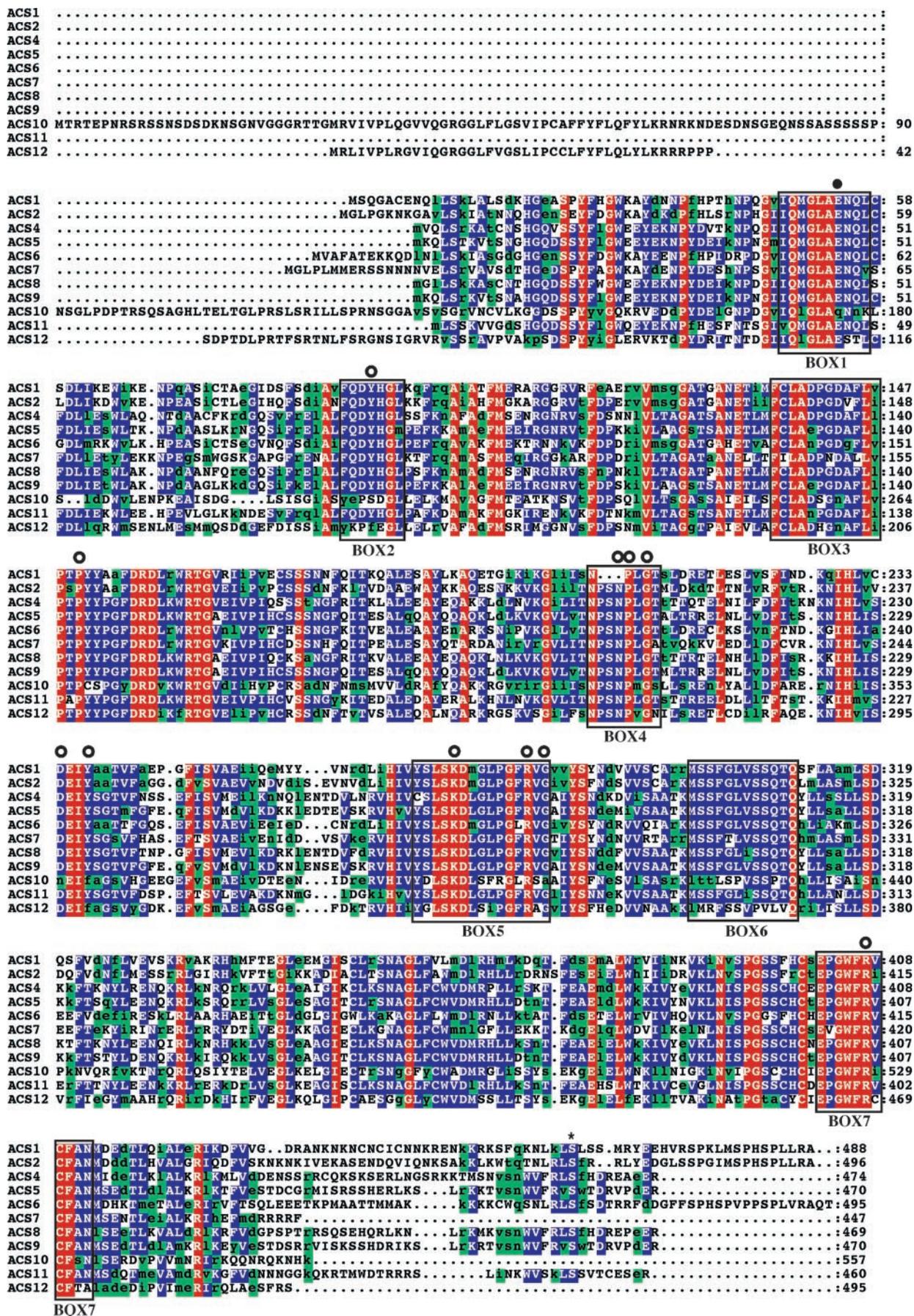


FIG. 3. Amino acid sequence alignment of 11 putative ACS polypeptides. ACS3 is not included because it is a truncated polypeptide (Ref. 52, Gene number At5g28360; annotation v4.0). The open circles indicate the 11 invariant amino acids conserved among ACS isozymes and various amino transferases (15). The conserved glutamate residue (E) marked with a filled circle is involved in substrate specificity (54). The serine (S) residue marked with an asterisk is a putative phosphorylation site (55). The seven conserved domains of the ACS isozymes are marked as boxes 1–7.

TABLE I  
Activity of the *Arabidopsis* ACS isozymes in *E. coli*

Gene	Amino acid no.	Predicted mol. mass	Isoelectric pt.	<i>pET22</i>	<i>pBAD</i>
		<i>Da</i>		<i>nmol/100 μlh at 30 °C</i>	
ACS1	488	55,002.36	7.16	0	0
ACS2	496	55,794.03	7.26	4.7	6.6
ACS4	474	54,075.91	8.17	17	28
ACS5	470	53,593.45	7.49	89	227
ACS6	495	55,808.79	6.23	204	270
ACS7	447	50,946.11	5.92	7	124
ACS8	469	53,651.29	7.82	202	165
ACS9	470	53,450.24	6.79	133	114
ACS10	557	61,184.32	7.67	0	0
ACS11	460	52,079.41	6.35	63	28
ACS12	495	55,213.12	7.04	0	0

TABLE II  
Percent amino acid and nucleotide sequence identity of the *Arabidopsis* ACS gene family members

The numbers in parentheses show the nucleotide sequence identity.

	ACS1	ACS2	ACS4	ACS5	ACS6	ACS7	ACS8	ACS9	ACS10	ACS11	ACS12
ACS1		65 (67)	50 (53)	51 (54)	60 (61)	54 (58)	53 (55)	49 (55)	34 (46)	49 (55)	34 (45)
ACS2			49 (55)	50 (56)	60 (54)	52 (58)	51 (55)	52 (58)	34 (43)	50 (55)	34 (45)
ACS4				74 (72)	49 (53)	56 (58)	79 (79)	74 (72)	36 (50)	64 (62)	40 (47)
ACS5					51 (55)	55 (57)	77 (73)	91 (84)	37 (49)	65 (62)	39 (47)
ACS6						55 (59)	52 (55)	51 (57)	32 (34)	53 (58)	33 (32)
ACS7							57 (60)	56 (59)	37 (39)	56 (57)	41 (38)
ACS8								77 (73)	38 (49)	67 (64)	40 (47)
ACS9									38 (47)	65 (63)	41 (47)
ACS10										35 (48)	51 (57)
ACS11											39 (49)
ACS12											

ficity (54), is present in all the members of the ACS gene family, except ACS10 where it has been replaced by glutamine (Q) (Fig. 3). This indicates that this E residue is not the sole determinant for ACS substrate specificity because ACS12 has the E47 residue but is lacking ACS activity. Furthermore, the serine (S) residue in the hypervariable C terminus, which is a phosphorylation site (55), is present in all ACS isozymes except ACS7, ACS10, and ACS12, because all three have a truncated C terminus compared with the other ACS isozymes (Fig. 3). ACS10 and 12 have longer N termini than all the other ACS isozymes (Fig. 3). The amino acid and nucleotide sequence identity among the various members of the ACS gene family varies from 32 to 91% and 34 to 84%, respectively, indicating that the ACS gene family is quite divergent (Table II).

The authenticity of the various ACS isozymes was verified by expression experiments in *E. coli*. Full-length ORFs were subcloned into *pET22* and *pBAD-Trx-His* vectors, and ACS activity was determined as described under "Experimental Procedures." Table I shows that all ACS isozymes are enzymatically active, except for ACS1, ACS10, and ACS12. The inactivity of ACS1 is due to the absence of the tripeptide PSN in the conserved box 4 (Fig. 3 and Ref. 52). The inactivity of ACS10 and ACS12 was quite puzzling because both proteins have most of the hallmarks of the authentic ACS isozymes. We entertained two possibilities: (a) ACS10 and 12 are pseudogenes like ACS1 or (b) they encode another enzymatic activity, preferably aminotransferases, a close relative to ACS (58). The first possibility was tested by constructing hybrid or modified ACS10 and 12 proteins to determine whether they become enzymatically active. We tested whether the presence of their N-terminal extension or the absence of their C-terminal peptides are responsible for their inactivity. We constructed a N-terminal-truncated ACS12 (N-ACS12; Arg<sup>66</sup>-Ser<sup>495</sup>, 430 amino acid residues) and N-ACS12 plus the C-terminal peptide of ACS8 (C8; Thr<sup>434</sup>-Arg<sup>469</sup>), N-ACS12+C8 (466 amino acid residues). Both hybrid proteins (N-ACS12 and N-ACS12+C8/ACS8) were expressed in *E. coli*, and their ACS activity was nil. We also

constructed and tested mutants of ACS12, N-ACS12, and N-ACS12+C8 by altering their F92Y and box 2 sequence from YKPFEGGL to YQDYHGL (underlined amino acids show the amino acids changed by site-directed mutagenesis to the same residues as in other ACS isozymes). The activity of all six mutants was nil. A clue to the possible function of ACS10 and 12 was provided by the phylogenetic tree (Fig. 3). Their phylogenetic resemblance with the alanine and aspartate ATases raised the prospect they may encode ATases (Fig. 1B). This possibility was tested by complementation experiments of the *E. coli* ATase mutant DL39 (50, 51). The results of Fig. 4 show that ACS10 and ACS12 are ATases with broad specificity for aspartate and aromatic amino acids such as tyrosine and phenylalanine. Their activity for branched chain amino acids is nil. All functional ACS isozymes do not have ATase activity (Fig. 4). The data of Fig. 4 have also been confirmed by growth of each transformant in liquid media supplemented with various amino acids (data not shown). Accordingly, the *Arabidopsis* ACS gene family consists of eight functional (ACS2, 4–9, and 11) and one non-functional (ACS1) members.

**Expression of the ACS Genes**—Expression studies using RT-PCR show that IAA induces six ACS genes in 7-day-old etiolated seedlings (compare lanes 1 and 2 with lane 3 in Fig. 5A). It has been previously reported that ACS5 is not auxin inducible (22). This discrepancy is attributed to the longer period incubation of the tissue with auxin reported in (22). The possibility exists, however, that the induction of ACS5 by auxin is transient and detectable only by short auxin treatment. CHX treatment of etiolated seedlings strongly enhances mRNA accumulation of all ACS genes (compare lanes 1 and 2 with lane 4 in Fig. 5A). The expression of ACS1 is nil in the presence of auxin or CHX (compare lanes 1 and 2 with lanes 3 or 4 in Fig. 5A). RT-PCR analysis of mRNAs from various parts of light grown seedlings reveals differential expression among the various ACS genes during plant growth and development (Fig. 5). Specifically, all ACS genes except ACS1 and ACS4 are expressed in the roots (lane 1, Fig. 5B). ACS1 and ACS5 are

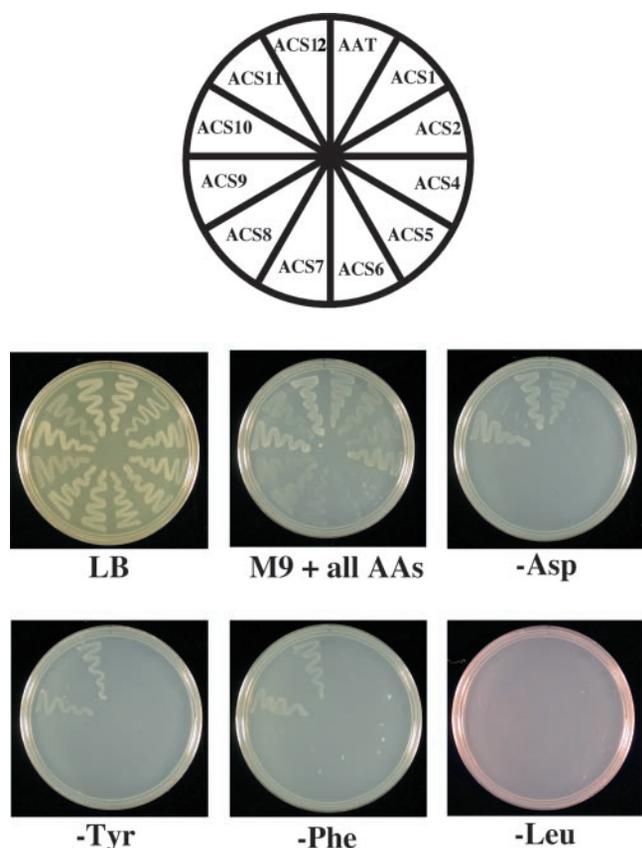


FIG. 4. Complementation of the *E. coli* aminotransferase mutant DL39 with various ACS and AspAT cDNAs. *pQE-ACS* and *pQE-AspAT* plasmids were used to transform *E. coli* DL39 as described under "Experimental Procedures." The streaked sections correspond to the cDNAs shown in the top diagram. The plates were grown in the various media shown for 2 days at 30 °C and then photographed.

expressed in leaves, ACS6 in flowers, and ACS1, -2, -5, -8, -9, and -11 in siliques (Fig. 5B).

**Expression and Purification of the ACS Isozymes**—A number of *E. coli* expression systems were tested prior to deciding on the use of the *pBAD-Trx-His* expression vector (see "Experimental Procedures"). The expressed proteins are N-terminal fusions of ACS with thioredoxin and His<sub>6</sub>. The 16-kDa thioredoxin tag enhances the solubility of the fusion protein and allows easy identification of its removal after thrombin digestion by SDS-PAGE (size reduction of the digested fusion polypeptide). Fig. 6A shows a Coomassie-stained gel of total protein extracts from *E. coli* strains expressing various ACS proteins. All ACS isozymes are efficiently expressed and their molecular sizes range from 60–65 kDa indicating that they are indeed fusions of thioredoxin and His<sub>6</sub>. (Fig. 6A; compare lane V *E. coli* extract to those expressing the various ACS isozymes). The expression level of each fusion protein is approximately the same (Fig. 6A; compare the intensity of the bands marked with a solid circle). The majority of the fusion proteins were localized in the insoluble fraction of the total *E. coli* extracts. Among the various ACS isozymes, some are more soluble than others. The order of decreased solubility is: ACS7 > 6 > 2 > 4 > 8 > 9 > 5 > 11. The various ACS isozymes were purified from the soluble fraction by Ni-affinity chromatography. Subsequently, the Trx-His-ACS were digested with thrombin protease and purified by filtration chromatography on Superdex<sup>TM</sup>-200. Fig. 7 shows the elution profiles of the various ACS isozymes. ACS2, -6, -7, and -9 are eluted as a single peak whereas ACS4, -8, -5, and -11 have a broad elution profile (two or more active peaks). We collected the last active peak with a retention time of 33–35

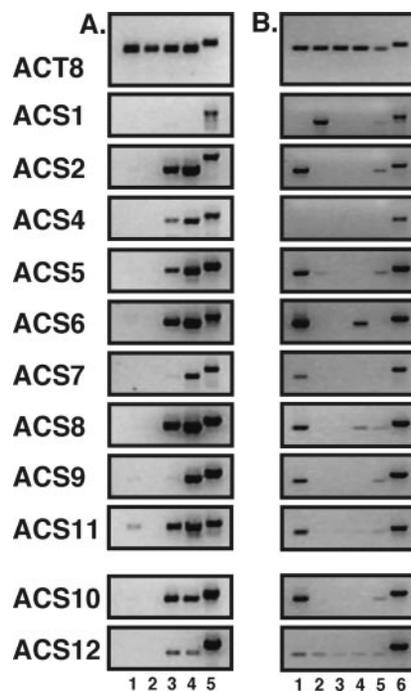


FIG. 5. Expression characteristics of the ACS genes in 7-day-old etiolated seedlings and mature light grown plants. A, etiolated seedlings were treated with or without IAA or CHX and the expression of each gene was accessed by RT-PCR as described under "Experimental Procedures." The lanes are: 1, control-EtOH; 2, control+EtOH; 3, IAA 20  $\mu$ M; 4, CHX 50  $\mu$ M; 5, genomic DNA (control). Duration of treatment, 2 h. B, light grown. The lanes are: 1, roots; 2, leaves; 3, stems; 4, flowers; 5, siliques; 6, genomic DNA. *ACT8* was used as a nondifferential-expressed gene (35).

min for further analysis. ACS activity eluted at this retention time corresponds to a molecular size of ~100 kDa (dimer) as determined using known sized proteins. Fig. 6B shows that the purified ACS isozymes consist of a single band on a SDS-PAGE and stained by Coomassie Blue. The yield of the various ACS isozymes varies from 0.03 to 17 mg per 3 liters of *E. coli* culture (Table IV). Table III summarizes the results of the ACS isozyme purification. The purification scheme developed allows purification of the ACS isozymes to homogeneity with 10–600-fold purification depending on the ACS isozyme. Thrombin digestion yields a polypeptide with three additional amino acids, GSH, at the N terminus.

**Molecular Mass and Subunit Structure**—The molecular size of the eight functional ACS isozymes, estimated by SDS-PAGE, ranges from 45 to 50 kDa, whereas their sizes determined by gel filtration on a Superdex<sup>TM</sup>-200 column ranges from 93 to 105 kDa (Table IV), indicating these functional ACS proteins are dimers. Since the size of the purified proteins determined by SDS-PAGE is smaller than the predicted size (see Table IV), the prospect is raised that the purified ACS isozymes have undergone partial proteolysis during purification. This may be due to nonspecific proteolytic degradation by thrombin or during purification. The authenticity of the N termini was determined by sequencing the N terminus of the purified polypeptides. The results presented in Table IV indicate that all ACS isozymes have the correct and expected N-terminal sequence. The possibility of a proteolytic degradation at the C termini was determined by mass spectrometry using a MALDI-TOF mass spectrometer as described under "Experimental Procedures." The molecular size of each ACS determined by this method is shown in Table IV. The data show that the size of ACS4 and ACS7 is the same as that predicted. However, the size of ACS2, -5, -6, -8, -9, and -11 was smaller by 2–5 kDa than the predicted size for each protein, suggesting a cleavage at the

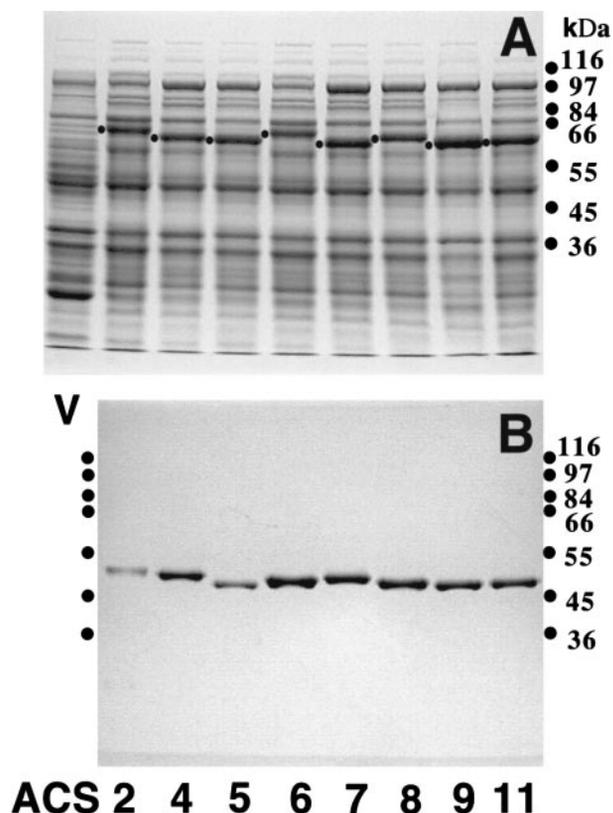


FIG. 6. Expression of ACS isozymes in *E. coli*. SDS-PAGE of total protein extracts from *E. coli* strains expressing various ACS isoenzymes (A) and purified ACS isozymes (B). Approximately equal amounts of protein were loaded on each lane (10  $\mu$ g), separated on a 10% polyacrylamide gel (45), and stained with Coomassie Blue. The lane marked V represents protein from *E. coli* with vector alone. The black dots in A mark each recombinant ACS expressed as a fusion polypeptide. The reference proteins are shown on the right:  $\beta$ -galactosidase, 116 kDa; phosphorylase b, 97 kDa; fructose-6-phosphate kinase, 84 kDa; bovine serum albumin, 66 kDa; glutamate dehydrogenase, 55 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa.

C terminus. Fig. 8 shows the putative cleavage sites at the C termini that occurred during purification. For example, the experimentally determined size of 51,695 Da for ACS2 corresponds to an ACS2 polypeptide that arises from GSH-(Met<sup>1</sup>-Lys<sup>460</sup>). The cleavage of the Lys<sup>460</sup>-Lys<sup>461</sup> bond in the intact ACS2 protein results in a 51,642 Da protein very similar in size to the experimentally determined value of 51,695 Da. Using similar calculations, we determined that purified ACS5, -6, -8, -9, and -11 arise from the cleavages of Lys<sup>450</sup>-Lys<sup>451</sup>, Glu<sup>441</sup>-Glu<sup>442</sup>, Arg<sup>436</sup>-Ser<sup>437</sup>, Arg<sup>444</sup>-Ile<sup>445</sup>, and Arg<sup>439</sup>-Arg<sup>440</sup> bonds, respectively (Fig. 8). These results suggest that the purified ACS isozymes, except ACS4 and -7, are cleaved at the C-terminal region of the molecule during purification despite the presence of the protease inhibitors, Complete<sup>TM</sup> and PMSF, during the purification procedure.

**Enzymatic Properties of ACS Isozymes**—Table IV shows the enzymatic properties of the Arabidopsis ACS isozymes. The pH optima vary from 7.3 to 8.2. These values are smaller than those previously reported for various ACS isozymes purified from *E. coli* or plant tissues (compare pH values in Table IV with Table V). The  $K_m$  values range from 8.3 to 45  $\mu$ M whereas the  $V_{max}$  ranges from 13 to 324  $\mu$ mol of ACC/mg of protein/h at 30  $^{\circ}$ C. The inhibitor constants for 2 known inhibitors, AVG and sinefungin, vary from 0.019–0.80  $\mu$ M and 0.15–12  $\mu$ M, respectively. AVG is a more effective inhibitor of ACS activity than sinefungin.

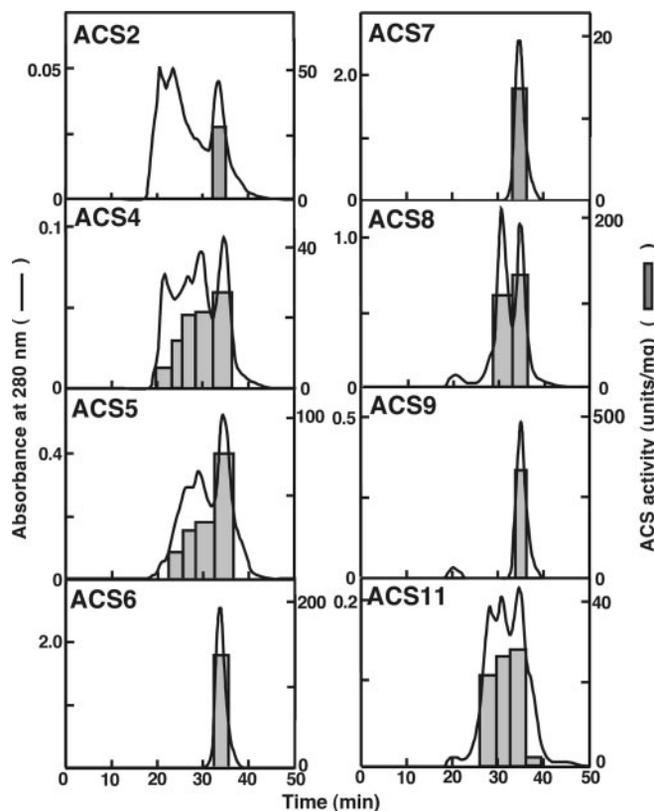


FIG. 7. Elution profiles of ACS isozymes during purification on Superdex-200 column chromatography. Fractions containing ACS were digested with thrombin protease (see “Experimental Procedures”), applied on a Superdex-200 column (1  $\times$  30 cm) equilibrated with 50 mM potassium phosphate buffer, pH 8.0, containing 10  $\mu$ M PLP, 0.1 mM PMSF, 0.3 M NaCl, and 2 mM NDSB201. The elution was carried with the same buffer at a flow rate of 0.4 ml/min in an Amersham Biosciences FPLC system at 4  $^{\circ}$ C. For all panels: curves denote absorbance at 280 nm; histograms, ACS activity.

## DISCUSSION

A hallmark discovery of the *Arabidopsis* genome sequencing project was the finding that many gene products encode isoforms of the same polypeptide (32). The biological significance of multigene families in general and of the ACS gene family in particular is unknown. While the various ACS isozymes catalyze the same biochemical reaction, it is not known whether their biological function(s) are distinct or overlapping. Genetic evidence (19) and evolutionary considerations (71, 72) support the view that each member of the ACS gene family may have a distinct biological function. It has been postulated that tissue specific expression of a particular ACS isozyme satisfies the biochemical environment of the cells and tissues in which each isozyme is expressed (15). Accordingly, the distinct biological function of each isozyme is defined by its biochemical properties. Such a concept enhances the physiological fine-tuning of the cell and demands that the enzymatic properties of each isozyme be distinct (73).

The availability of the *Arabidopsis* genome sequence provided the opportunity to experimentally test this proposition with a complete ACS family. The *Arabidopsis* ACS family consists of nine isozymes. Eight of them are enzymatically active and one, ACS1, is enzymatically inactive (52). ACS1 is missing the highly conserved tripeptide Thr-Asn-Pro (TNP) between Ile<sup>204</sup> and Ser<sup>205</sup>. Introduction of TNP into ACS1 restores activity (52). ACS1 is enzymatically active, however, as a heterodimer with some members of the ACS gene family.<sup>2</sup> The

<sup>2</sup> A. Tsuchisaka and A. Theologis, unpublished data.

TABLE III  
Purification of ACS isozymes from *E. coli*

Step	Protein	Activity <sup>a</sup>	Specific activity	Yield	Purification
	mg	units	units/mg	%	fold
ACS2					
Ammonium sulfate	1608.00	84.00	0.052	100.00	1.0
Ni-NTA agarose	1.50	4.00	2.700	4.80	51.9
Superdex 200	0.03	0.91	30.300	1.10	582.0
ACS4					
Ammonium sulfate	1470.00	150.00	0.100	100.00	1.0
Ni-NTA agarose	2.40	8.20	3.400	5.50	34.0
Superdex 200	0.04	1.12	28.000	0.75	280.0
ACS5					
Ammonium sulfate	1380.00	330.00	0.240	100.00	1.0
Ni-NTA agarose	5.20	135.00	25.900	40.90	108.0
Superdex 200	0.29	22.50	77.600	6.80	323.0
ACS6					
Ammonium sulfate	1354.00	1000.00	0.730	100.00	1.0
Ni-NTA agarose	7.00	340.00	48.600	34.00	66.6
Superdex 200	2.90	262.00	90.300	26.20	123.7
ACS7					
Ammonium sulfate	1250.00	2040.00	1.630	100.00	1.0
Ni-NTA agarose	22.50	292.00	12.900	14.30	7.9
Superdex 200	16.70	225.00	13.500	11.00	8.3
ACS8					
Ammonium sulfate	1530.00	780.00	0.510	100.00	1.0
Ni-NTA agarose	9.50	528.00	55.600	67.70	109.0
Superdex 200	0.80	107.00	133.000	13.60	261.0
ACS9					
Ammonium sulfate	956.00	765.00	0.800	100.00	1.0
Ni-NTA agarose	3.10	175.00	56.400	22.90	70.0
Superdex 200	0.20	64.90	324.00	8.50	405.0
ACS11					
Ammonium sulfate	1068.00	192.00	0.180	100.00	1.0
Ni-NTA agarose	1.90	38.40	20.200	20.00	112.0
Superdex 200	0.07	1.76	25.200	0.92	140.0

<sup>a</sup> Unit = 1  $\mu$ mol of ACC/h at 37 °C.

TABLE IV  
Enzymatic properties of the *Arabidopsis* ACS isozymes

Properties are based on the concentration of (S,S)- Ado Met.  $K_m$ ,  $V_{max}$ ,  $k_{cat}$ , and  $K_i$  values were measured at the optima pH of each ACS.

Gene	Molecular mass				pI	Yield	Optimal pH	$K_m$	$V_{max}$	$k_{cat}$	$K_i$ ( $\mu$ M)		N-terminal sequence
	Predicted	Mass spec.	SDS-PAGE	Gel filtration							AVG	Sinefungin	
	Da	Da	kDa	kDa							$\mu$ M	$\mu$ M	
ACS1	55,002.36				7.16								
ACS2	55,794.03	51,695	50.0	105	7.26	0.03	8.2	45	32.00	0.50	0.800	12.0	GSHMGLPGKKNKA-
ACS4	54,075.91	54,533	48.5	97	8.17	0.03	7.8	15	31.20	0.47	0.042	0.52	GSHMVQLSRKATX-
ACS5	53,593.45	51,192	46.0	95	7.49	0.29	7.8	37	81.70	1.22	0.063	0.65	GSHMKQLSTKVTS-
ACS6	55,808.79	49,697	47.0	105	6.23	3.50	7.3	23	120.60	1.87	0.370	1.80	GSHMVAFATEKKQ-
ACS7	50,946.11	51,938	48.5	95	5.29	16.7	8.0	8.3	13.50	0.19	0.027	0.35	GSHMGLPLMMERS-
ACS8	53,651.29	49,460	48.0	97	7.82	3.00	8.2	15	143.00	2.13	0.019	0.15	GSHMGLLSKKAS-
ACS9	53,450.24	50,203	46.5	93	6.79	0.20	8.0	40	324.50	4.82	0.068	0.55	GSHMKQLSRKVTS-
ACS10	61,184.32				7.67								
ACS11	52,079.41	49,677	46.5	98	6.35	0.07	8.0	25	25.20	0.37	0.045	1.50	GSHMLSSKVVGDS
ACS12	55,213.12				7.04								

genome also contains three annotated genes, ACS3, ACS10, and ACS12, with great resemblance to the various members of the ACS gene family. ACS3 is a pseudogene representing a truncated version of ACS1 (52). ACS3 is located on the upper arm of chromosome 5 (Fig. 1A). The original mapping of ACS3 on chromosome 4 was incorrect (52). ACS10 and ACS12 encode aminotransferases (Table I).

The biochemical characterization of the ACS isozymes requires an expression system that provides high levels of active enzymes. We carried out a number of preliminary experiments for determining the best *E. coli* expression system to be used. We expressed the ACS8 protein using the *pET28* (His<sub>6</sub>-tagged ACS8) and *pET32* (Trx-His<sub>6</sub>-tagged ACS8) expression vectors, both containing the *T7* promoter (42). The enzyme activities of the crude extracts were 56 and 40 nmol/100  $\mu$ l/h at 30 °C for *pET28-ACS8* and *pET32-ACS8*, respectively. These activities were one-fourth to one-fifth of those obtained with the *pET22*

(native ACS8) and the *pBAD* (Trx-His<sub>6</sub>-tagged ACS8) expression vectors containing the *T7* and *pBAD* promoter, respectively (Table I). Accordingly, we used the *pBAD* instead of the *T7* promoter containing expression vector because it allows protein purification by affinity chromatography. The enzyme activities recovered with the eight ACS isozymes varied greatly ranging from 0.75% (ACS4) to 26.2% (ACS6) (Table III). This was due to the solubility differences among the various isozymes and not due to differential stability of the isozymes. The yield of ACS6, the most soluble member, was 35 times higher than that of ACS4 (Table III). This was quite evident after digestion of Trx-His<sub>6</sub>-tagged ACS4, -5, and -11 with thrombin protease, which led to a large amount of insoluble proteins. In addition gel filtration of the digested ACS4, -5, and -11 gave many peaks with enzyme activity on a Superdex<sup>TM</sup>-200 column (Fig. 7). Thrombin digestion resulted in the precise removal of the N-terminal polypeptide (thioredoxin) used for

FIG. 8. Determination of the cleavage sites in the purified ACS polypeptides by MALDI-TOF mass spectrometry. The open circle indicates the conserved arginine (R) residue in all ACS isozymes. The asterisk marks the putative phosphorylation site. Several gaps have been introduced for optimum alignment. Vertical arrows indicate the cleavage site during purification, and the numbers above the arrows indicate the molecular mass of each ACS determined by mass spectrometry. The amino acid residue at the cleavage site is numbered and marked with a solid circle.

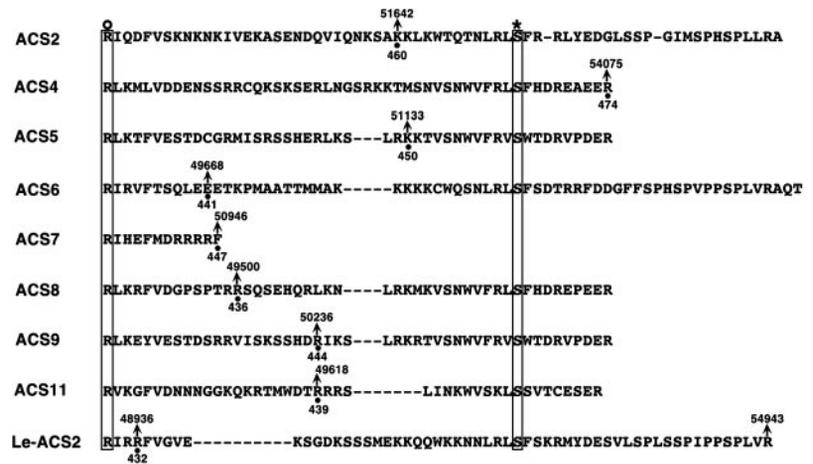


TABLE V  
Enzymatic properties of ACSes from various plant species: a comparison

Protein source	Optimum pH	$K_m$	$k_{cat}$	$K_i$ ( $\mu M$ )		Ref.
				AVG	Sinefungin	
Recombinant		$\mu M$	$s^{-1}$			
Apple/pSKACS1	8.3	12	9			28
Tomato/Le-ACS2		22	1.47			59
Zucchini/pACC1	9.5	17				60
Tissue <sup>a</sup>						
Mung bean/hypocotyl	8.5	60				61
Tomato/pericarp		20				62
Tomato/fruit	9.0	32.3		1.0		63
Tomato/fruit				2.0		64
Tomato/pericarp		17		10.0		65
Tomato/fruit	8.5	13				66
Tomato/red fruit		27			2.5	67
Tomato/green fruit				1.0	1.0	48
Tomato/red fruit				1.7	25.0	48
Tomato/pericarp		13		0.2		46
Tomato/pericarp	8.5	20				47
Winter squash/hypocotyl	9.0–8.5	13		0.12		68
Winter squash/mesecarp	9.5	12.1		2.50		69
Winter squash/mesocarp		13.3		2.10		70

<sup>a</sup> All ACSes are crude enzyme preparations.

affinity purification, as determined by amino acid sequence of the purified enzymes (Table IV). In addition mass spectrometry was used to determine whether the purified proteins were intact. All purified ACS isozymes were truncated at the C terminus except for ACS4 and -7 (Fig. 8). The putative proteolytic cleavage sites were in the hypervariable C-terminal region defined after the conserved arginine (R) residue among all ACS isozymes in box 7 (Fig. 3). The C termini contain many amino acids with positive (Arg and Lys) and negative (Asp and Glu) charges. These amino acids are potential targets of endogenous trypsin- and acid protease-like proteases despite the presence of protease inhibitors, Complete<sup>TM</sup> and PMSF, during the purification procedure. Also the possibility exists that the excess amount of thrombin protease used resulted in secondary cleavage of arginine residues at the C terminus. ACS4 and ACS7 were purified as intact polypeptides because ACS4 does not have proteolytic susceptible sites like the remaining ACS isozymes and ACS7 is missing the C-terminal hypervariable region (Fig. 3).

The variable region of the C terminus is the domain responsible for phosphorylation (55) and post-transcription regulation of ACS. The conserved serine (S) residue at the C terminus (Fig. 3) is responsible for phosphorylation of ACS by a Nt-CDPK2-like kinase.<sup>3</sup> More importantly, phosphorylation of

the serine residue does affect enzyme activity (55). This suggests that the enzyme properties determined in this study with C-terminal-truncated proteins may represent the values of the intact proteins. Phosphorylation of the C terminus regulates the protein stability of the enzyme mediated by the ETO1 gene product. Phosphorylated ACS is more stable than non-phosphorylated ACS because it prevents protein-protein interaction between ACS and ETO1 (21). Furthermore, mutations in the C terminus such as *eto2* and *eto3* enhance the stability of ACS5 and ACS9, respectively, by preventing the interaction of ETO1 with the ACS protein. It remains to be determined whether ACS7 is also post-transcriptionally regulated because the regulatory C-domain responsible for protein stability is missing.

The enzymatic properties of the ACS isozymes support Rottman's proposition regarding the multiplicity of ACS isozymes (15). They are distinct when compared within the ACS gene family and within those from other plant species (Tables IV and V). The ACS isozymes are not only biochemically divergent but also their genes have a divergent pattern of expression and response to the plant hormone auxin (Fig. 5). Promoter GUS fusions with the members of the ACS gene family reveal a spectacular and highly divergent pattern of expression throughout the life cycle of *Arabidopsis*.<sup>2</sup> Yeast microarray data show that expression divergence is the major reason for maintaining duplicated genes in a genome. More importantly, a large number of duplicated genes have diverged quickly in

<sup>3</sup> H. Mori, M. Iwata, T. Fukaya, and M. Tatsuki, unpublished data.

expression, and the vast majority of gene pairs eventually become divergent in expression (74). Such biochemical and expression specialization provides a unique biological function. For example, the tomato plant has at least ten ACS genes,<sup>4</sup> two of which, *Le-ACS2* and *Le-ACS4*, are expressed in the tomato fruit and are responsible for fruit ripening (75). The remainder are unable to provide ACC in the ripening fruit because they are expressed elsewhere, restricted by their tissue specific expression. Determination of the AdoMet concentration in single cells or group of cells using laser capture microdissection (76) coupled with nanocapillary electrophoresis (77, 78) will provide additional experimental support to elucidate these relationships. Nearly 25 years ago Harris (79) put forward a similar concept regarding multilocus enzymes in man: "Although in general the different isozymes which make up these various multilocus sets are very similar to one another in their catalytic functions, differences in their kinetics, their inhibition characteristics and other properties such as stability have been noted in quite a number of cases. It is difficult, in viewing the striking tissue differentiations that occur, not to conclude that the detailed enzymic properties of the different isozymes of the set have been tailored in the course of evolution so they are appropriate for the specific metabolic roles they subserve in the particular intracellular environment of the tissues in which they are found. However, in most cases the exact nature of such presumed functional differences has not been clearly defined and this is a major task for the future."

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<sup>4</sup> A. Theologis, unpublished data.