

Molecular Characterization of a Heteromeric ATP-Citrate Lyase That Generates Cytosolic Acetyl-Coenzyme A in Arabidopsis^{1[w]}

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Acetyl-coenzyme A (CoA) is used in the cytosol of plant cells for the synthesis of a diverse set of phytochemicals including waxes, isoprenoids, stilbenes, and flavonoids. The source of cytosolic acetyl-CoA is unclear. We identified two Arabidopsis cDNAs that encode proteins similar to the amino and carboxy portions of human ATP-citrate lyase (ACL). Coexpression of these cDNAs in yeast (*Saccharomyces cerevisiae*) confers ACL activity, indicating that both the Arabidopsis genes are required for ACL activity. Arabidopsis ACL is a heteromeric enzyme composed of two distinct subunits, ACLA (45 kD) and ACLB (65 kD). The holoprotein has a molecular mass of 500 kD, which corresponds to a heterooctomer with an A₄B₄ configuration. ACL activity and the ACLA and ACLB polypeptides are located in the cytosol, consistent with the lack of targeting peptides in the ACLA and ACLB sequences. In the Arabidopsis genome, three genes encode for the ACLA subunit (*ACLA-1*, At1g10670; *ACLA-2*, At1g60810; and *ACLA-3*, At1g09430), and two genes encode the ACLB subunit (*ACLB-1*, At3g06650 and *ACLB-2*, At5g49460). The *ACLA* and *ACLB* mRNAs accumulate in coordinated spatial and temporal patterns during plant development. This complex accumulation pattern is consistent with the predicted physiological needs for cytosolic acetyl-CoA, and is closely coordinated with the accumulation pattern of cytosolic acetyl-CoA carboxylase, an enzyme using cytosolic acetyl-CoA as a substrate. Taken together, these results indicate that ACL, encoded by the *ACLA* and *ACLB* genes of Arabidopsis, generates cytosolic acetyl-CoA. The heteromeric organization of this enzyme is common to green plants (including Chlorophyceae, Marchantimorpha, Bryopsida, Pinaceae, monocotyledons, and eudicots), species of fungi, Glaucophytes, *Chlamydomonas*, and prokaryotes. In contrast, all known animal ACL enzymes have a homomeric structure, indicating that a evolutionary fusion of the *ACLA* and *ACLB* genes probably occurred early in the evolutionary history of this kingdom.

Acetyl-coenzyme A (CoA) is an intermediate metabolite that is juxtaposed between catabolic and anabolic processes. As the entry point for the tricarboxylic acid (TCA) cycle, acetyl-CoA can be considered the gateway in the oxidation of carbon derived from the catabolism of fatty acids, certain amino acids (e.g. Leu, Ile, Lys, and Trp), and carbohydrates. Furthermore, acetyl-CoA is the intermediate precursor for the biosynthesis of a wide variety of phytochemicals. Because membranes are impermeable to CoA derivatives, it can be inferred that acetyl-CoA is generated in at least four distinct metabolic pools representing

the four subcellular compartments where acetyl-CoA metabolism occurs: plastids, mitochondria, peroxisomes, and the cytosol (Fig. 1). Therefore, plants should have distinct acetyl-CoA-generating systems in mitochondria (for the TCA cycle), in plastids (for de novo fatty acid biosynthesis), in peroxisomes (the product of β -oxidation of fatty acids), and in the cytosol (for the biosynthesis of isoprenoids, flavonoids and malonated derivatives, and the elongation of fatty acids). Such compartmentalized and distinct acetyl-CoA-generating systems would enable the organism to precisely regulate the supply of acetyl-CoA to different metabolic pathways.

In addition to the differential subcellular requirements for acetyl-CoA, the requirements for acetyl-CoA vary among different cell types. For example, whereas de novo fatty acid biosynthesis is required for the assembly of membrane lipids, it is also induced for assembly of triacylglycerides at discreet times during the development of embryos, endosperm, and tapetum. In the epidermis of flower petals of many species, anthocyanin pigments (which require acetyl-CoA for their synthesis) accumulate during development to act as visual attractants of insect pollinators. The biosynthesis of anthocyanins is also induced in the leaf epidermis in response to biotic and abiotic stresses (Schmid et al., 1990). In

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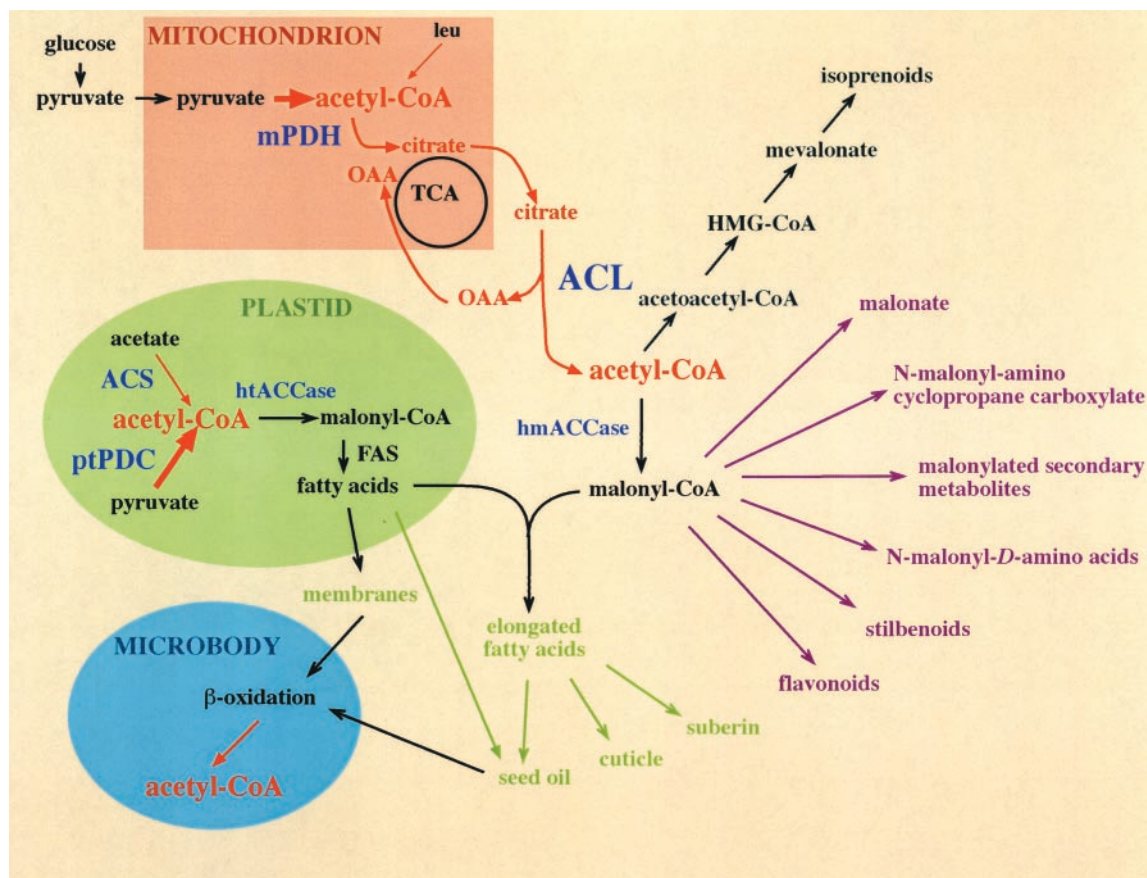


Figure 1. Subcellular compartmentation of acetyl-CoA metabolism in plants. Because acetyl-CoA is impermeable to membranes, it is envisioned that it is generated independently in each compartment where it is required (cytosol, mitochondria, plastids, and peroxisomes). Cytosolic ACL is depicted together with a postulated citrate cycle that would provide citrate from the mitochondria (red). In the cytosol, acetyl-CoA can be carboxylated by acetyl-CoA carboxylase to form malonyl-CoA; alternately, two molecules of acetyl-CoA can undergo condensation to form the isoprenoid-precursor acetoacetyl-CoA. Both of these intermediates can give rise to a wide variety of metabolites. Plastidic pyruvate dehydrogenase complex and acetyl-CoA synthetase contribute to plastidic acetyl-CoA, which can be carboxylated by acetyl-CoA carboxylase and hence converted to fatty acids. In the peroxisomes, acetyl-CoA is generated during the β oxidation of fatty acids.

addition, epidermal cells of aerial portion of plants require the plastidic and cytosolic acetyl-CoA pools for the biosynthesis of the cuticle. Another acetyl-CoA-derived phytochemical is malonic acid, which accumulates to high levels in certain legumes during development and following stress (Arnold and Hill, 1972; Stumpf and Burris, 1981; Li and Copeland, 2000). In maize (*Zea mays*) seedlings, army worm predation induces a 30-fold increase in the accumulation of an acetyl-CoA-derived naphthalene-based sesquiterpene (Shen et al., 2000). Thus, the generation of acetyl-CoA may be tightly regulated in response to developmental and environmental signals.

Despite its metabolic importance, the pathways for the biogenesis of acetyl-CoA in plants are still not well understood. Several possible mechanisms for generating acetyl-CoA in the plastid for fatty acid biosynthesis have been described. However, the relative significance of each of these mechanisms is not yet clear (Mattoo and Modi, 1970; Elias and Givan,

1979; Liedvogel and Stumpf, 1982; Givan, 1983; Kaethner and ap Rees, 1985; Randall et al., 1989; Wurtele et al., 1998; Bao et al., 2000; Ke et al., 2000a). Recent studies indicate that the acetyl-CoA pool required for de novo fatty acid biosynthesis is primarily generated by the plastidic isoform of the pyruvate dehydrogenase complex (Ke et al., 2000a).

Far fewer studies have directly addressed how the cytosolic pool of acetyl-CoA is generated in plants (Kaethner and ap Rees, 1985; Burgess and Thomas, 1986; Wurtele et al., 1998; Rangasamy and Ratledge, 2000). In vertebrates (Stryer, 1988; Sato et al., 2000) and possibly insects (Sutherland and Feyerisen, 1996), de novo fatty acid biosynthesis and cholesterol genesis are cytosolic processes, and cytosolic ATP-citrate lyase (ACL) generates the required acetyl-CoA precursor. ACL catalyzes the ATP- and CoA-dependent cleavage of citrate to form acetyl-CoA and oxaloacetate: citrate + ATP + CoA \rightarrow oxaloacetate + acetyl-CoA + ADP + Pi.

In vertebrates, ACL is a homotetramer of 110-kD subunits, and as a lipogenic enzyme, it is highly regulated by complex transcriptional and posttranslational mechanisms (Sato et al., 2000). ACL activity was first reported in plants in the 1970s (Mattoo and Modi, 1970; Nelson and Rinne, 1975, 1977). However, its role in acetyl-CoA generation has been difficult to assess due to inconsistent findings regarding its subcellular location (Fritsch and Beevers, 1979; Kaethner and ap Rees, 1985; Ratledge et al., 1997; Rangasamy and Ratledge, 2000).

We report the isolation and characterization of *Arabidopsis* cDNAs coding for ACL subunits. These characterizations demonstrate that the plant ACL is structurally distinct from the animal enzyme, consisting of two subunits of 45 and 65 kD, probably in an A₄B₄ stoichiometry. Our results indicate that plant ACL is cytosolic. The complex spatial and temporal accumulation pattern of ACL mRNAs indicates that ACL may supply the acetyl-CoA substrate for the cytosolic acetyl-CoA carboxylase, which generates the malonyl-CoA used for the synthesis of a multitude of compounds, including very long chain fatty acids and flavonoids.

RESULTS

Plant ACL Is Composed of Two Distinct Subunits

The *Arabidopsis* expressed sequence tag (EST) database was searched with the TBLASTN algorithm (Altschul et al., 1990) using the human ACL sequence as the search query. This search identified two groups of nonoverlapping ESTs that exhibit high sequence similarity to the N-terminal one-third and C-terminal two-thirds of the human ACL protein, respectively (Wurtele et al., 1998). A representative EST from each of these two groups was completely sequenced. One of these, TASG097 (hereafter referred to as *ACLA-1*), shares 60% sequence similarity with the N-terminal portion of the human ACL and is a near full-length cDNA clone. The other clone, VB-VYC01 (hereafter referred to as *ACLB-2*-partial), shares 71% sequence similarity with the C-terminal portion of the human ACL and is a partial cDNA. A 2.2-kb full-length cDNA clone of *ACLB-2*-partial (hereafter referred to as *ACLB-2*) was isolated from a lambda cDNA silique library (made by L.A. Castle and D.W. Meinke, Oklahoma State University), obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH).

To investigate the biochemical function of these cDNAs, and thus test the sequence-based hypothesis that they encode subunits of ACL, each cDNA was expressed in *Escherichia coli*, and the recombinant proteins were used to generate antisera (see supplemental data at <http://molebio.iastate.edu/~mash/Ecoli.html>). Western-blot analyses of SDS-PAGE-fractionated *Arabidopsis* protein extracts revealed that anti-*ACLA-1* serum reacts with a 45-kD

polypeptide, and anti-*ACLB-2* serum reacts with a 65-kD polypeptide (Fig. 2). The molecular weights of these immunologically identified polypeptides are in close agreement with those predicted from the *ACLA-1* (423-residue polypeptide) and *ACLB-2* (608-residue polypeptide) cDNA sequences.

The biochemical function of these two proteins was identified by expressing each cDNA in *Saccharomyces cerevisiae*, an organism without ACL. The *ACLA-1* and *ACLB-2* cDNAs were cloned into the *S. cerevisiae* integrative expression vectors, pYX042 and pYX012, and were integrated individually or in combination at the *leu2* and *ura3* loci of *S. cerevisiae* strain α D273. The integration of each of these transgenes was selected on the basis of Leu and/or uracil auxotrophy, and was confirmed by Southern-blot analyses of DNA isolated from the resulting transgenic strains (see supplemental data at <http://molebio.iastate.edu/~mash/yeast.html>). Western-blot analyses of protein extracts prepared from the recombinant yeast strains confirmed that the *Arabidopsis* transgenes were expressed (Fig. 3, A and B). No immunological reaction was detected in extracts from the parental yeast strain α D273 with anti-*ACLA-1* or anti-*ACLB-2* sera. However, immunological reactions were de-

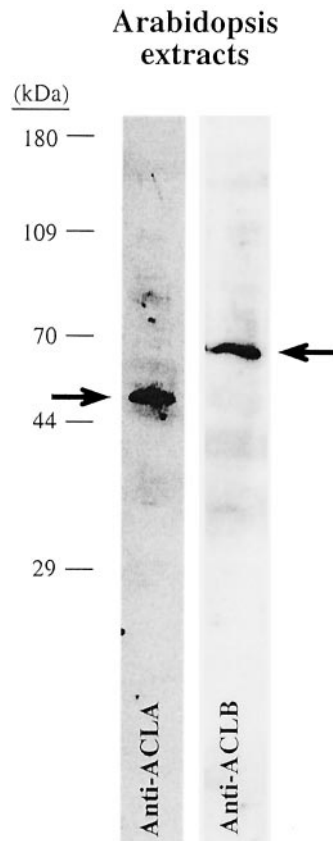


Figure 2. Immunological characterization of the *Arabidopsis* ACL subunits. Western-blot analysis of protein extracts from *Arabidopsis* siliques, probed with anti-*ACLA-1* or anti-*ACLB-2* sera. These antisera react with 45- and 65-kD polypeptides, respectively.

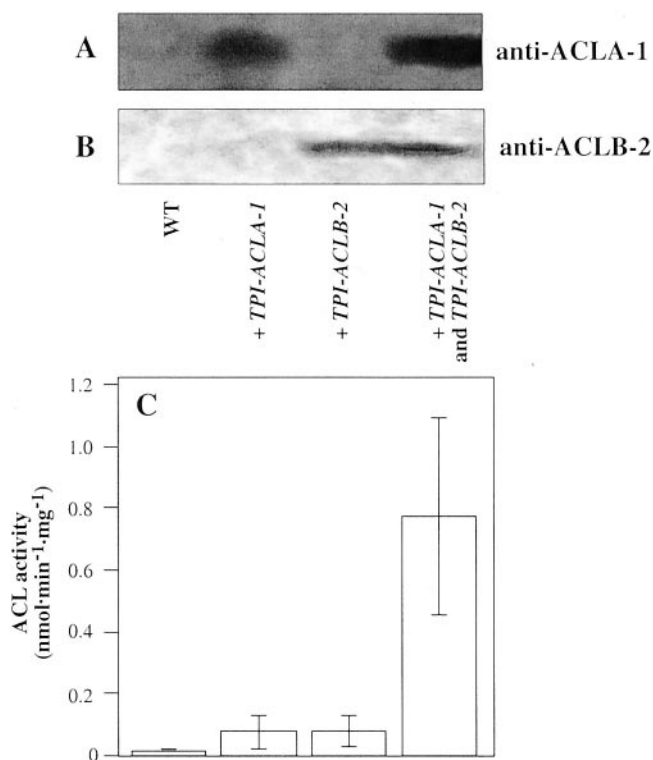


Figure 3. Plant ACL is composed of two subunits. Arabidopsis cDNAs *ACLA-1* and *ACLB-2* were cloned into the yeast expression vectors, pYX042 and pYX012, immediately downstream of the *TPI* promoter. The resulting transgenes were integrated individually or in combination at the *leu2* and *ura3* loci of *S. cerevisiae* strain α D273. A, Western blot of yeast proteins reacted with anti-ACLA-1 serum. B, Western blot of yeast proteins reacted with anti-ACLB-2 serum. C, ACL activity in extracts from parental strain α D273 and its derivatives that carry the indicated transgenes. The data represents the mean and SD of three separate extractions. Only the *S. cerevisiae* strain that carries and expresses both transgenes has ACL activity.

tected at the predicted molecular weights in strains carrying the *ACLA-1* and *ACLB-2* transgenes. Extracts from each of these yeast strains were assayed for ACL activity (Fig. 3C). ACL activity was only detectable in the strain coexpressing the *ACLA-1* and *ACLB-2* transgenes, but not in the parental strain or the two strains expressing these transgenes individually. These results conclusively demonstrate that *ACLA-1* and *ACLB-2* code for two distinct and essential subunits of the Arabidopsis ACL. We termed these subunits ACLA and ACLB, respectively.

Arabidopsis ACL Is a Nondissociable Heteromeric Complex of 500 kD

To ascertain if the ACLA and ACLB subunits are associated in a complex, and to begin to determine the physical nature of this complex, the distribution of ACL activity and ACLA and ACLB subunits were determined after fractionation of plant extracts by nondenaturing gel electrophoresis, anion-exchange chromatography, and gel-filtration chromatography.

Following gel-filtration chromatography of Arabidopsis silique extracts, 64% of the ACL activity applied to the column was recovered in fractions 11 through 19 (Fig. 4A). The ACLA and ACLB subunits cofractionate with ACL activity (Fig. 4B), coeluting as a complex of 500 ± 30 kD. Analogous experiments were also conducted with extracts from pea (*Pisum sativum*) seedlings (data not shown), and again in these experiments, ACL activity and the immunologically identified ACLA and ACLB subunits coeluted, with an M_r of 470 ± 30 kD.

Additional evidence for ACL being a heteromeric complex of ACLA and ACLB subunits was obtained from ion-exchange chromatography experiments with pea shoot extracts. The ACLA and ACLB subunits coelute from a Mono-Q column as a single peak at 110 mM NaCl (data not shown). ACL-containing Arabidopsis extracts were fractionated by nondenaturing PAGE. In each of four repetitions of this experiment, ACLA and ACLB subunits comigrated, indicating that these proteins associate in a single complex (Fig. 4C).

ACL Is a Cytosolic Enzyme

Knowing the subcellular location of ACL is critical to deducing its physiological function. Previous studies of the subcellular location of this enzyme have been inconclusive. An initial study indicated a plastidic location (Fritsch and Beevers, 1979), whereas Kaethner and ap Rees (1985) indicated a cytosolic location; more recent studies indicated a cytosolic and plastidic location (Ratledge et al., 1997; Rangasamy and Ratledge, 2000).

The three ACLA and two ACLB polypeptides encoded in the Arabidopsis genome do not contain an N-terminal organelle-targeting extension sequence relative to the animal ACL sequence. Computational predictions with PSORT (Nakai and Kanehisa, 1992) and TARGETP (Emanuelsson et al., 2000) algorithms are inconclusive. PSORT indicates that the three ACLA proteins are in the cytosol or in peroxisomes; TARGETP predicts that they are not targeted to plastids, mitochondria, or secreted. PSORT predicts a nuclear localization for the two Arabidopsis ACLB gene products, whereas TARGETP predicts a mitochondrial localization. Neither program predicts a plastidic location for any of the ACLA or ACLB polypeptides.

To directly determine the subcellular location of ACL, pea organelles were fractionated by differential centrifugation. This fractionation resulted in the isolation of two fractions that were enriched in chloroplasts, and mitochondria plus peroxisomes, plus the 12,000g supernatant fraction, which should contain the cytosol and the contents of broken organelles. The chloroplasts, mitochondria, and peroxisomes were further purified by Percoll-density gradient centrifugation. The integrity and purity of each frac-

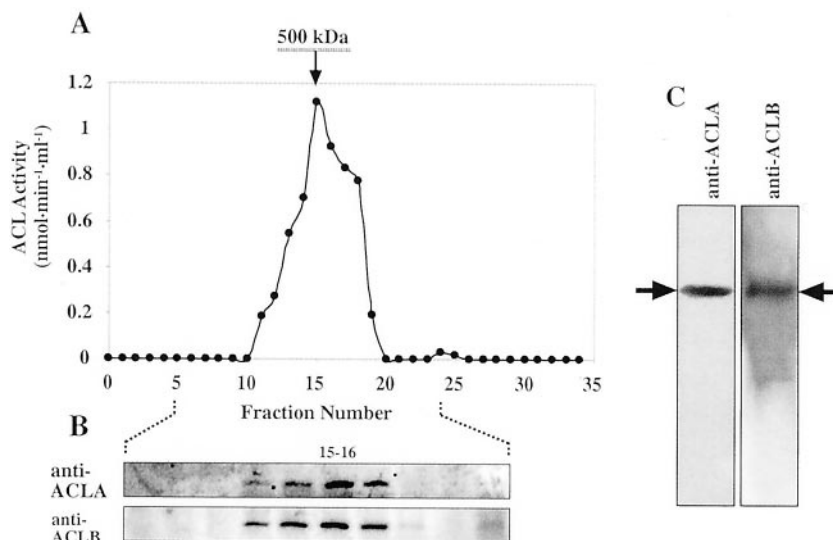


Figure 4. Arabidopsis ACL is a heteromeric complex. Protein extract from Arabidopsis siliques was subjected to gel-filtration chromatography on a Superdex 200 column. Individual fractions were collected and assayed for ACL activity, which elutes at a volume corresponding to a M_r of 500 kDa (A), and SDS-PAGE/western blots: ACLA subunit (B, top) and ACLB subunit (B, bottom). ACL activity elution profile corresponds closely to the elution profile of the ACLA and ACLB subunits. C, Arabidopsis seedling extract (100 μ g of protein), fractionated by nondenaturing gel electrophoresis and subjected to western-blot analysis. Blots were probed for ACLA (left) or ACLB (right). Consistent with ACLA and ACLB being organized in a protein complex, the ACLA and ACLB subunits migrate to the same position on the native gel.

tion was ascertained by assaying each fraction with a series of organelle-specific marker enzymes (assayed enzymatically or immunologically; Fig. 5). To be specific, NADP-GAPDH and the BCCP1 subunit of the chloroplastic acetyl-CoA carboxylase (Ke et al., 1997) were used as markers for the chloroplasts; cytochrome *c* oxidase and MCC-A, a mitochondrial matrix protein (Baldet et al., 1992; Weaver et al., 1995), were used as markers for the mitochondria; HPR and catalase were used as markers for peroxisomes; and PEP carboxylase was used as the marker for the cytosol.

As judged by the specific activities of each of the organelle-specific enzymes and the distribution of immunologically detected protein markers, the three Percoll-purified organelle fractions are highly enriched in chloroplasts, mitochondria, and peroxisomes (Fig. 5). The low level of PEP carboxylase activity in the mitochondrial and peroxisomal pellet indicates a low level of cytosolic contamination, and the Percoll-purified chloroplast, mitochondrion, and peroxisome fractions had no detectable cytosolic contamination (Fig. 5A). In addition, the Percoll-purified chloroplast fraction appears to be nearly devoid of mitochondrial and peroxisomal contamination based on the absence of cytochrome *c* oxidase and HPR activities (Fig. 5A), MCC-A (Fig. 5B), and the low level of catalase (Fig. 5B). Based on the recoveries of HPR and cytochrome *c* oxidase activities, MCC-A, and catalase, the Percoll-purified peroxisomes and mitochondria are cross-contaminated; however, these fractions are free from chloroplastic contamination as judged by the absence of NADP-GAPDH

activity (Fig. 5A) and BCCP1 (Fig. 5B). As expected, the 12,000g supernatant fraction contains the cytosol (as indicated by PEP carboxylase activity), as well as enzymes from broken chloroplasts, mitochondria, and peroxisomes.

The specific activity of ACL (Fig. 5A) and ACLA and ACLB subunits (Fig. 5B) among these subcellular fractions closely mirrors that of the cytosolic marker, PEP carboxylase. ACLA subunit, ACLB subunit, and ACL and PEP carboxylase activities are detected only in the crude extract and in the 12,000g supernatant. ACL activity or ACLA and ACLB subunits are undetectable in the Percoll-purified organelle fractions. These experimental data are consistent with the hypothesis that ACL is a cytosolic enzyme.

A Complex Coordinated *ACLA* and *ACLB* mRNA Accumulation Pattern Is Coincident with the Accumulation of the Acetyl-CoA Carboxylase (*ACC*) mRNA

Insights into the potential functions of cytosolic acetyl-CoA generation can be obtained from evaluating the spatial and temporal pattern of pattern of ACL expression. Northern-blot hybridizations were conducted to examine the temporal pattern of *ACLA* and *ACLB* mRNA accumulation in leaves, flower buds, and during silique development. Peak accumulation of these two mRNAs occurs at the youngest stages of silique development (1–2 d after flowering [DAF]) with a level of accumulation comparable with that in flower buds (Fig. 6). By about 4 DAF, when the siliques have ceased expanding, the accumulation

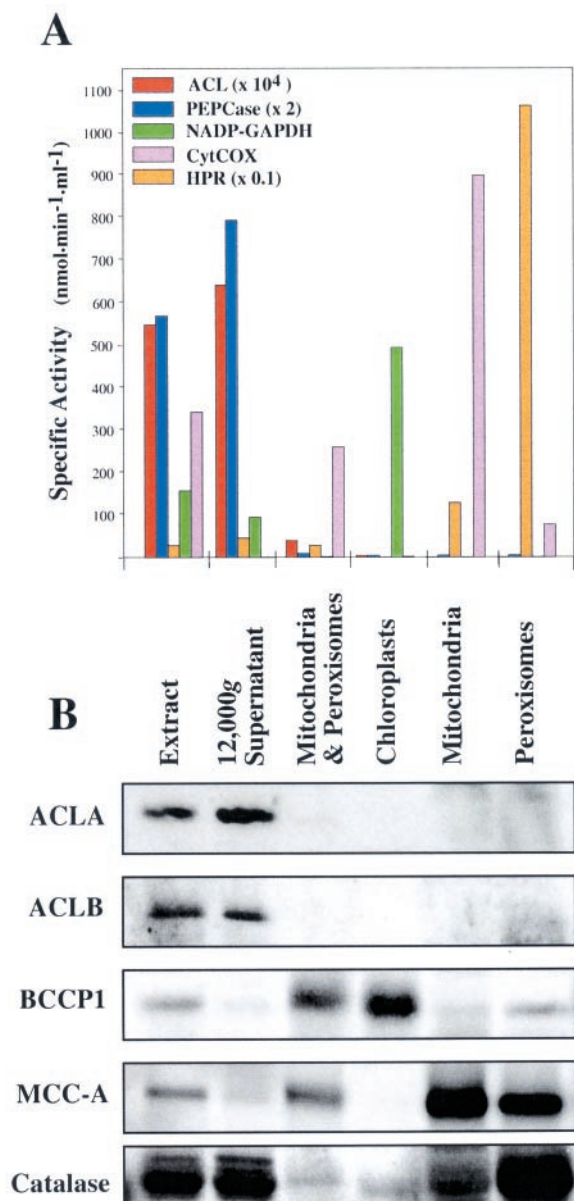


Figure 5. ACL is a cytosolic enzyme. Chloroplasts, mitochondria, and peroxisomes were purified from pea seedling extract by a combination of differential centrifugation and Percoll-density gradient centrifugation. The specific activities of ACL, NADP-dependent glyceraldehyde 3-P dehydrogenase (NADP-GAPDH), cytochrome *c* oxidase, hydroxypyruvate reductase (HPR), and phosphoenolpyruvate (PEP) carboxylase were determined (A). Aliquots from each fraction, containing 50 μ g of protein, were subjected to western-blot analysis for immunological detection of ACLA, ACLB, BCCP1 subunit of the chloroplastic acetyl-CoA carboxylase, the A subunit of methylcrotonyl-CoA carboxylase (MCC-A), and catalase (B).

of the *ACLA* and *ACLB* mRNAs steadily declines, and by 8 to 9 DAF, accumulation is about 5% of peak levels. Throughout this development, the accumulation patterns of *ACLA* and *ACLB* mRNAs are closely coordinated.

In situ hybridizations revealed a far more complex pattern of *ACL* mRNA accumulation: The *ACL* mR-

NAs accumulate transiently in discrete cell types at specific developmental stages (Fig. 7). In expanding leaves, the *ACLA* (Fig. 7A) and *ACLB* (Fig. 7E) mRNAs accumulate preferentially in trichomes and epidermal cells. In flower buds at stage 6 of development, the accumulation of these mRNAs is highly concentrated in tapetal cells (Fig. 7, B and F). By stage 10 of flower development, accumulation is less discreet but is concentrated in the epidermal cells of growing petals and ovaries (Fig. 7, C and G). In young siliques, when the embryos are approximately at the 4-cell stage (1 DAF), the *ACLA* and *ACLB* mRNAs are highly concentrated in the inner integument of the ovules (Fig. 7, D and H). The accumulation in the inner integument is transient and occurs just prior to testal deposition (Fig. 7, D and M). In all analyses, the *ACL-B* mRNA accumulation pattern is indistinguishable from that of *ACLA* (Fig. 7, A–H). In addition, this pattern of accumulation of *ACL* mRNAs is almost indistinguishable from that of the homomeric acetyl-CoA carboxylase mRNA (Fig. 7, I–L). The spatial distribution of the *ACLA* and *ACLB* mRNAs changes within the tissues of the silique during its development. Whereas these mRNAs are initially concentrated in the inner integument of the ovules at 1 DAF (Fig. 7, D and H), they disappear from this tissue by 3 DAF and begin to accumulate within the developing embryo, which is now at the globular stage of development (Fig. 7M). At 5 DAF (Fig. 7N) and 7 DAF (Fig. 7O), when the embryos are undergoing rapid growth and initiation of oil accumulation occurs (heart and torpedo stages), there is maximal accumulation of these mRNAs within the embryos. (Only the *ACLA in situ* hybridization data is shown for Fig. 7 M–AA; the *ACLB* data are virtually identical.) Later in silique development, at 9 DAF (Fig. 7P), when the embryos are reaching maturity, the accumulation of these two mRNAs decline, and they are no longer detectable in mature embryos at 12 DAF (Fig. 7Q).

Upon seed germination, the accumulation of the two *ACL* mRNAs is induced, but their spatial distri-

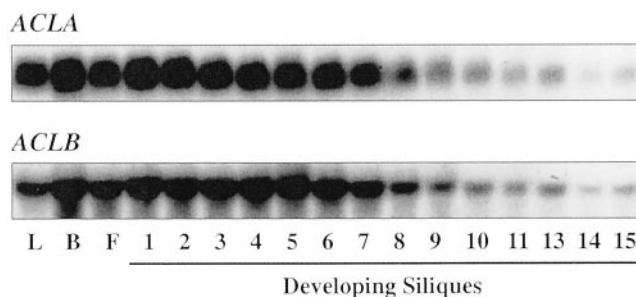


Figure 6. Temporal changes in accumulation of *ACLA* and *ACLB* mRNAs in Arabidopsis. RNA (20 μ g lane⁻¹) was hybridized with *ACLA-1* and *ACLB-2* ³²P-labeled antisense RNA probes. RNA was isolated from expanding leaves (L), flower buds (B), flowers (F), and developing siliques at the indicated DAF. The data presented in this figure were gathered from a single experiment; near identical data were obtained in two replicates.

bution within the seedling is tissue specific. At 2 d after imbibition, the *ACL* mRNAs are concentrated in the vascular bundles, the apical meristem (Fig. 7, S and T), and the epidermis of the seedling cotyledon, stem, and root (Fig. 7, R, T, and U). Most dramatic is the immense accumulation of these mRNAs in the root tip (Fig. 7V shows seedlings 4 d after imbibition).

Tissues within a number of organs show a dramatic but transient accumulation of the *ACL* mRNAs. For example, within the anthers, high levels of *ACLA* and *ACLB* mRNAs accumulate for 1 d in the tapetal cells when the flower buds are at stage 10 of their development (Fig. 7, compare B with C). Another such example is the stigma, where the *ACLA* and *ACLB* mRNAs are highly abundant when the flower is at stage 12 of development (Fig. 7W), but decrease about 1 d later when the flowers open (Fig. 7X).

Young vascular bundles of a number of organs accumulate high levels of the *ACLA* and *ACLB* mRNAs. These include expanding leaves (Fig. 7E), cotyledons (Fig. 7R), roots (Fig. 7, U and V), pedicel of flowers (Fig. 7Z), and the 2-DAF siliques (Fig. 7AA). *ACLA* and *ACLB* mRNAs accumulate in discreet tissues of the flower receptacle, namely, the nectaries and transiently in the newly forming abscission zones of petal and sepals in stage 12 flowers (Fig. 7Y).

Organization and Structure of the Arabidopsis *ACL* Genes

The three *ACLA* genes in the Arabidopsis genome are located on chromosome 1, and we labeled them *ACLA-1*, *ACLA-2*, and *ACLA-3*. These genes are positioned at approximately 3.5, 21.6, and 3.0 Mb of the chromosome 1 sequence, respectively. The *ACLA* subunit that has been the focus of the characterizations presented herein represents the product from the *ACLA-1* locus. With the exception of the 5'- and 3'-untranslated regions (UTRs), the mRNAs predicted to be derived from the *ACLA-2* and *ACLA-3* loci share 89% and 73% sequence identity with the *ACLA-1* mRNA, respectively. These similarities at the nucleotide sequence level correspond to 95% and 81% identities at the level of amino acid sequence. Because the 5'-UTRs of the *ACLA-2* and *ACLA-3* genes are as yet undefined, it is not clear if the single intron present in the 5'-UTR region of the *ACLA-1* gene is conserved among all the genes. The protein coding regions of the *ACLA-2* and *ACLA-3* genes are interrupted by 11 introns, whereas in the *ACLA-1* gene, 10 introns interrupt the protein coding region; the terminal intron of the *ACLA-2* and *ACLA-3* genes is absent from the *ACLA-1* gene. All introns within the *ACLA-2* and *ACLA-3* gene coding sequences are placed at identical positions relative to the amino acid sequences of the *ACLA-2* and *ACLA-3* proteins. This conservation of intron positions also extends to the 10 *ACLA-1* introns.

The two *ACLB* genes, *ACLB-1* and *ACLB-2*, are located at position 2.0 Mb of chromosome 3 and 20.1

Mb of chromosome 5, respectively. The *ACLB* subunit that has been the focus of the characterizations herein is encoded by the *ACLB-2* locus. The protein coding regions of these genes are interrupted by 15 and 14 introns, respectively. The first 14 introns of the *ACLB-2* gene are identically positioned in the *ACLB-1* gene, relative to the amino acid sequence of the respective protein products. Because the 5'-UTR of the *ACLB-1* gene is still undefined, it is not clear if the intron located at the 5' end of the *ACLB-2* gene is conserved in the *ACLB-1* gene. The terminal intron in the *ACLB-1* gene is absent from the *ACLB-2* gene. Despite this difference, the protein-coding region of the mRNA predicted from the *ACLB-1* locus shares 89% identity with the *ACLB-2* mRNA. However, the 5'- and 3'-UTRs of the two mRNAs are very divergent. The *ACLB-1* and *ACLB-2* proteins are 97% identical.

Phylogenetic Distribution and Structure of *ACL* Genes

ACL genes are present in a range of eukaryotes, including molds, fungi, plants, protists, and animals, as well as one prokaryote, a green sulfur bacterium (Fig. 8; additional sequences are presented at <http://molebio.iastate.edu/~mash/alignment.html>). Mammalian *ACL* is a homotetramer with a subunit of about 1,100 amino acids (Elshourbagy et al., 1990, 1992). We deduced from sequenced genomes that *ACL* also has a homomeric structure in a primitive chordate (*Ciona intestinalis*), fruit fly (*Drosophila melanogaster*), and *Caenorhabditis elegans*. In contrast, plant *ACL*, typified by the Arabidopsis enzyme, is heteromeric, consisting of *ACLA* and *ACLB* subunits. Based upon sequence similarities to genomic and EST sequences, *ACL* from monocot and dicot angiosperms, a Bryopsida, the liverwort (*Marchantia* spp.), and the green alga, *Chlorella vulgaris*, have a heteromeric structure similar to that of Arabidopsis. Photosynthetic protists, including *Chlamydomonas reinhardtii* and the Glaucophyte, *Cyanophora paradoxa*, have a similar heteromeric structure. Ascomycota, including the yeast, *Schizosaccharomyces pombe*, and the filamentous fungi, *Sordaria macrospora*, contain *ACLA* and *ACLB* genes (Nowrousian et al., 2000). However, our search of GenBank for orthologs of the *ACLA* and *ACLB* polypeptides indicates that *S. cerevisiae* does not contain these genes, consistent with the absence of *ACL* activity in this species (Kohlhaw and Tan-Wilson, 1977). *S. cerevisiae* generates acetyl-CoA for fatty acid synthesis via acetyl-CoA synthetase (van den Berg et al., 1996). To date, only a single prokaryotic *ACL*-like sequence has been reported, from the photosynthetic green sulfur bacterium *Chlorobium limicola* (Kanao et al., 2001). As with plants, algae, and fungi, *C. limicola* has separate *ACLA* and *ACLB* subunits.

Alignment of *ACL* sequences reveals a high degree of similarity among the proteins from different organisms. With the exception of several discreet re-

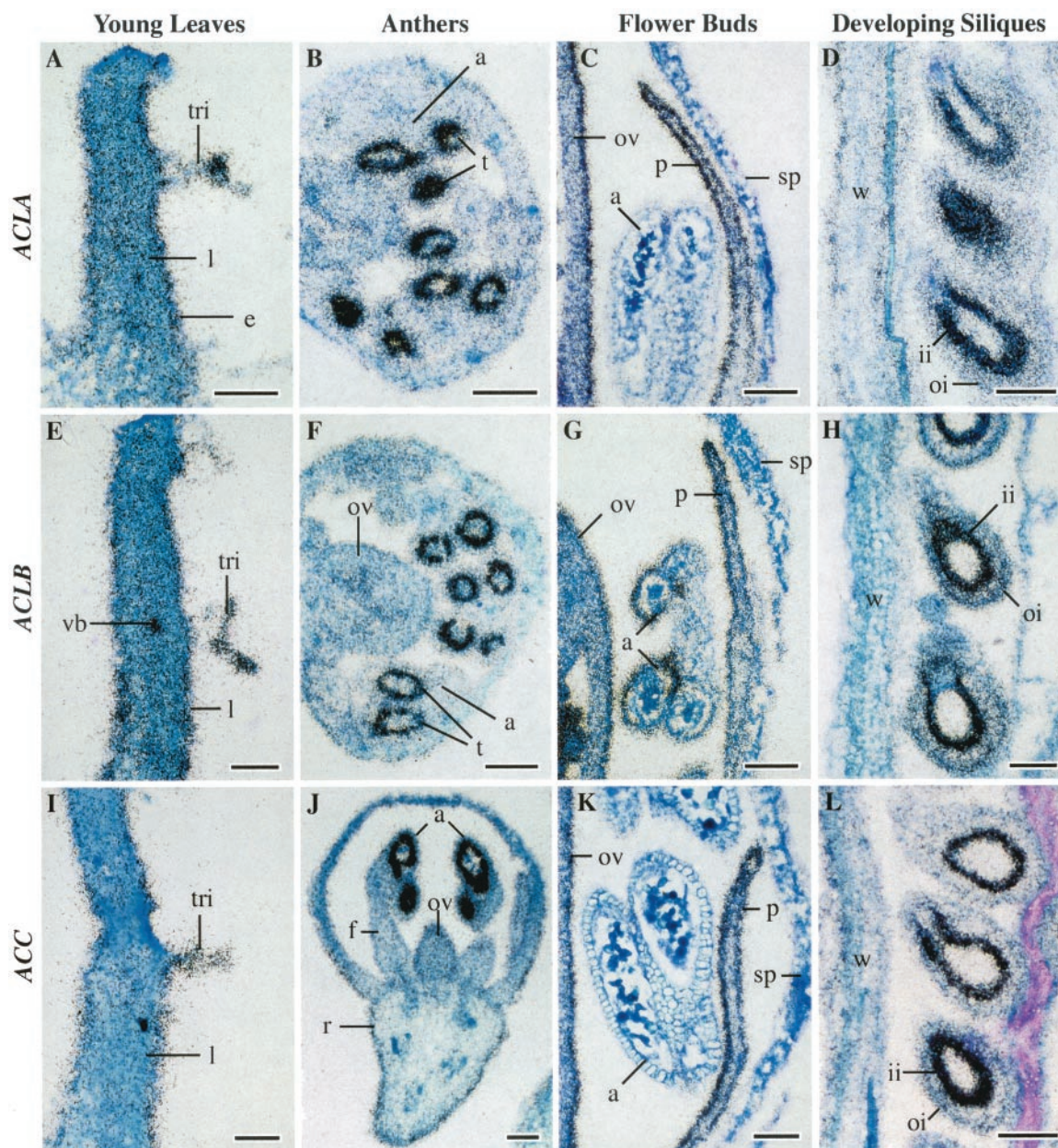


Figure 7 (Figure continues on next page.)

Spatial distribution of *ACLA* and *ACLB* mRNAs in Arabidopsis. Histological tissue sections were hybridized with antisense or sense (control) *ACLA-1*, *ACLB-2*, or *ACC1*, ^{35}S -labeled RNA probes. Slides are stained with toluidine blue to visualize the tissue. Black spots are silver grains reflecting the location of *ACLA*, *ACLB*, or *ACC1* mRNAs. Sense controls, which were conducted for each type of section, had negligible background (not shown). Hybridizations were repeated three times with similar results. *ACLA*, *ACLB*, and *ACC1* mRNAs coaccumulate in particular cell types during development. Accumulation is high in the epidermis and trichomes of expanding leaves (A, E, and I), the tapetal cells of anthers of stage 10 flowers (B, F, and J), epidermal cells of growing organs (petals and ovaries) of flowers stage 11 (C, G, and K), and inner integuments of ovules the day preceding testal (seed coat) deposition (D, H, and L). Hybridization to *ACLA* mRNA only is shown in M through AA; results with *ACLB* and *ACC1* are similar. Ovules of siliques at 3 DAF (M), 5 DAF (N), 7 DAF (O), 9 DAF (P), and 12 DAF (Q). Seedlings 1 d after imbibition (R), 2 d after imbibition (S and T), 3 d after imbibition (U), and 4 d after imbibition (V). Ovary of flower stage 12 (W) and stage 13 (X); nectaries and abscission zones of petal and sepals of flower stage 12 (Y). Pedicel of flower stage 13 (Z). Upper one-third of silique 2 DAF (AA). a, Anther; ap, apical meristem; ce, curled embryo; cot, cotyledon; f, filament; ge, globular embryo; he, heart embryo; ii, inner integument of ovule; l, leaf; me, mature embryo; n, nectary; o, ovule; oi, outer integument of ovule; ov, ovary; p, petal; pa, petal abscission zone; r, receptacle; ro, root; rtp, root tip; sa, sepal abscission zone; sp, sepal; stg, stigma; t, tapetum; te, torpedo embryo; tri, trichome; vb, vascular bundle; w, silique wall. Bars = 50 μm in A through O, Q, T through W, and Y through AA; bars = 150 μm in P, R, and X; and bars = 25 μm in S.

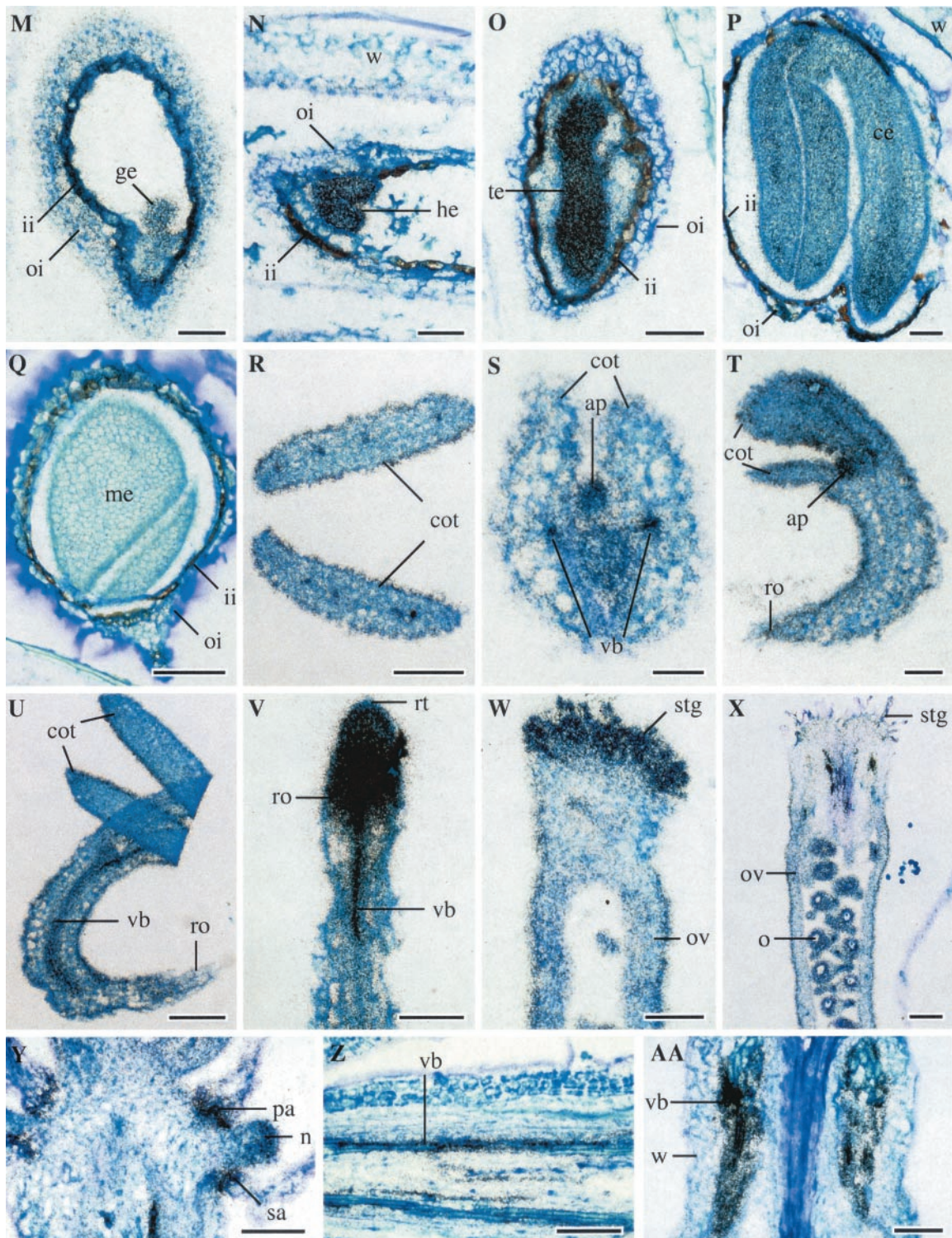


Figure 7. (Figure continued from preceding page.)

gions of the ACL polypeptides, this sequence conservation is evenly distributed throughout the sequence of these proteins. The most notable exception is the approximately 60-amino acid "spacer" region in animal ACL (residues 427–486 of the human ACL), which corresponds to the segment between the Ara-

bidopsis ACLA and ACLB sequences, and is absent from Arabidopsis and other heteromeric ACLs. This spacer segment is highly divergent, but still recognizable as a homolog, among the animal ACLs. Another notable divergence among these sequences is the insertion of 30 to 35 residues in the middle of the

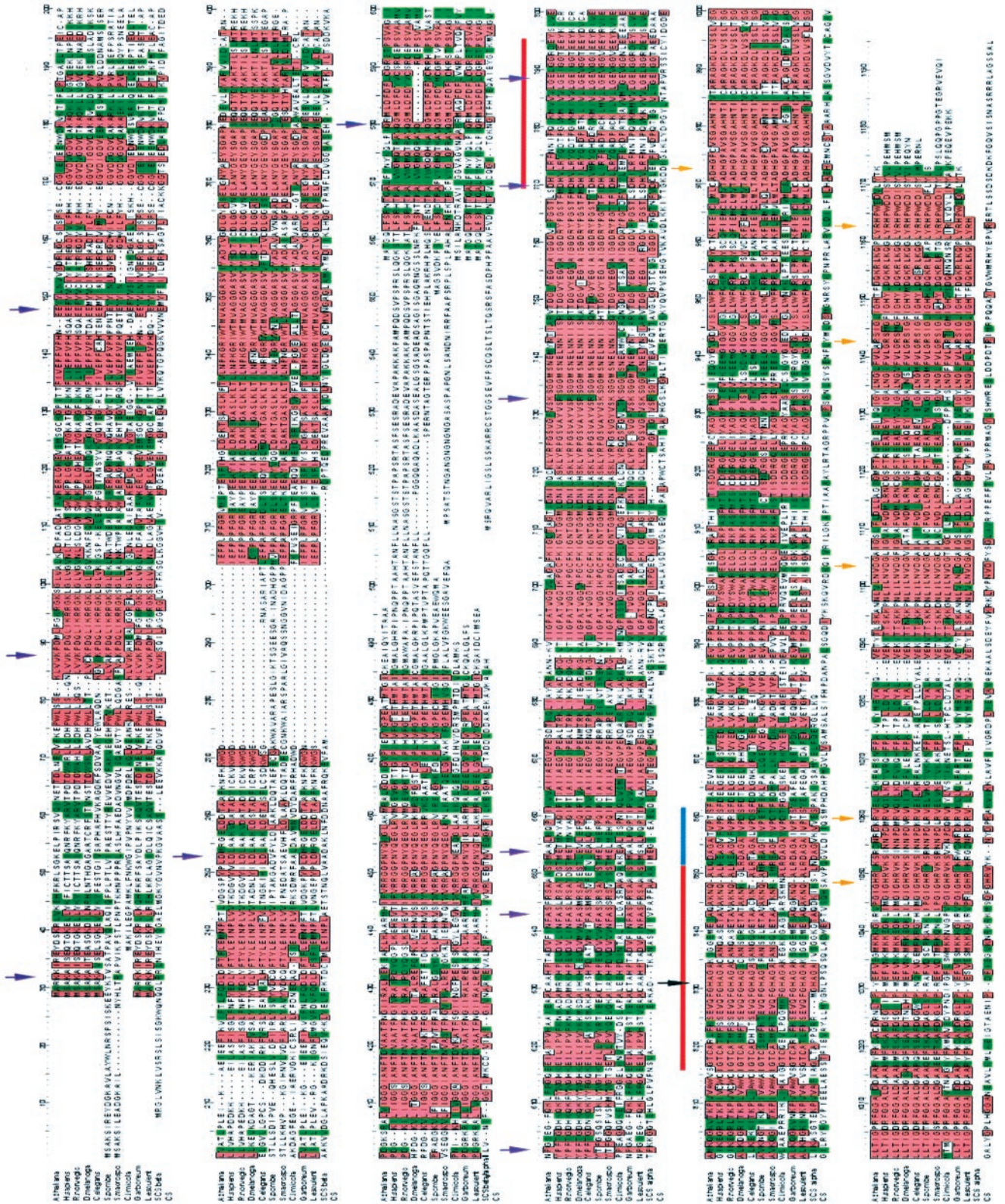


Figure 8. (Legend appears on next page.)

ACLA proteins of *Sordaria macrospora*, *S. pombe* (shown in Fig. 8), *Aspergillus nidulans*, *Pneumocystis carinii*, and *C. vulgaris* (see supplemental data at <http://molebio.iastate.edu/~mash/alignment.html>). The *Caenorhabditis elegans* ACL contains a 10-residue insertion at this position relative to other ACL proteins (Fig. 8). The N-termini of the *S. macrospora* and *S. pombe* ACLA proteins extend past other ACL proteins, and these extensions are highly divergent from each other (Fig. 8 and <http://molebio.iastate.edu/~mash/alignment.html>). It is interesting that the N terminus of the ACLB protein of *S. macrospora* is also extended relative to the other ACLB proteins, and this extension has a low similarity to the spacer region in the animal ACL.

Domains within the ACLA and ACLB subunits share significant sequence similarity with the α - and β -subunits of succinyl-CoA synthetase (SCS), and a domain of citrate synthase (CS; Fig. 8). For example, the N-terminal one-half of the Arabidopsis ACLA protein shares 26% sequence identity with the β -subunit of Arabidopsis SCS. The N- and C-terminal domains of Arabidopsis ACLB exhibit 26% and 30% sequence identity with the α -subunit of SCS and a 155-residue domain of CS, respectively.

The Arabidopsis ACLB domain homologous to CS contains the CS-signature motif. This motif includes the sequence, -GIGHRIK- (residues 485–491), which encompasses the CS active site His residue (His488), and additional residues (His413, Arg499, Asp539, Arg578, and Arg598 in *ACLB-2*) that are essential for activity and binding of oxaloacetate by CS (Karpusas et al., 1990). With the exception of *C. intestinalis* ACL, which does not have the His residue corresponding to His413 of Arabidopsis, all of these residues are absolutely conserved among all ACLs.

The phylogenetic trees shown in Figure 9 are based upon the sequence similarities between the SCS and CS homologous domains of ACL. All ACLs partition on separate branches from those that contain the α and β subunits of SCS, and CS. Of the ACL sequences available, the *C. limicola* ACLA and ACLB show the highest conservation with SCS and CS. Arabidopsis ACLA-1 and ACLA-3, as well as ACLB-1 and B-2, appear to have duplicated recently, however, ACLA-2 and ACLA-1/A-3 may have diverged earlier but prior to the time of the division between monocotyledon and eudicot lineages.

DISCUSSION

The occurrence of ACL in plants was suggested over 30 years ago by the detection of its enzymatic activity in plant extracts (Mattoo and Modi, 1970; Nelson and Rinne, 1975, 1977). More recently, EST cDNA clones that share sequence similarity with the animal ACL have further supported this supposition (Wurtele et al., 1998; Suh et al., 2001). The data presented herein formally establish the occurrence of ACL in plants. Our characterization of the Arabidopsis ACL implicate ACL in plants as part of the "citrate shuttle," a mechanism for moving a portion of the mitochondrial pool of acetyl-CoA to the cytosol. In vertebrates, this shuttle generates the acetyl-CoA precursor needed for lipogenesis and cholesterologenesis (Stryer, 1988).

In plant cells, the cytosolic pool of acetyl-CoA is required to support the biosynthesis of a wide range of biomolecules that are important for the growth, development, and protection of plants (Fig. 1). These biomolecules include oils containing very long chain fatty acids, waxes (Pollard and Stumpf, 1980; Bao et al., 1998), flavonoids and stilbenoids (Hrazdina et al., 1978; Preisig-Muller et al., 1997), malonic acid (Stumpf and Burris, 1981), isoprenoids such as essential oils, sterols, sesquiterpenes, and polyprenols (Demetzos et al., 1994; Menhard and Zenk, 1999; Eisenreich et al., 2001), some of the cellular Cys (Rotte and Leustek, 2000; Dominguez-Solis et al., 2001), a subset of the glucosinolates (Graser et al., 2000), malonyl derivatives including *D*-amino acids, 1-aminocyclopropane carboxylic acid (the precursor of ethylene), and xenobiotics such as pesticides (Hohl and Barz, 1995), and, in transgenic plants, bioplastics based on derivatives of polyhydroxybutyrate (Poirier, 2001).

The data presented here indicate that in plants, as typified by Arabidopsis, ACL is cytosolic, and as such would contribute to the generation of the cytosolic pool of acetyl-CoA. This is in agreement with Kaethner and ap Rees (1985). Whether ACL is the sole source of the cytosolic acetyl-CoA pool is still to be determined. For example, carnitine acyltransferase activities have been reported in mitochondrial and plastidic membranes, and it has been speculated that they may function to transport acetyl-CoA or

Figure 8. Comparisons of the primary structure of ACL. Comparisons of the amino acid sequences of the Arabidopsis ACLA and ACLB proteins with ACL, SCS, and CS from other organisms used ClustalW (Thompson et al., 1994). The degree of similarity in sequences is color-coded as follows: similar residues, green; identical residues, salmon. Conserved motifs include the ACL-SCS family signature 3 (PROSITE accession no. PS01217; residues 270–294 of Arabidopsis ACLA); the ACL-SCS family active site with His phosphorylated by ATP (black arrow; PROSITE accession no. PS00399; residues 259–275 of Arabidopsis ACLB); and the Gly-rich ACL-SCS family signature 1 (PROSITE accession no. PS01216; residues 174–203 of Arabidopsis ACLB). These motifs partially encompass the putative ATP-binding site (red bar), and a potential CoA-binding site (blue bar). Other conserved residues across ACL and SCS are Lys 3, Lys 58, Glu 116, and Asp 213 of Arabidopsis ACLA, conserved in the ATP-grasp domains (Fraser et al., 1999; Sanchez et al., 2000), and Gln 24, Pro 46, Ala 86, Arg 175, Asp 212, and Glu 232 of ACLB shown to be critical in the active site in SCS (Wolodko et al., 1994; Fraser et al., 1999; purple arrows). Ala 97 in Arabidopsis ACLB is the plant nonconservative substitution for otherwise conserved Glu. Residues forming the oxaloacetate-binding site in CS (Karpusas et al., 1990) and conserved in ACL are indicated by yellow arrows.

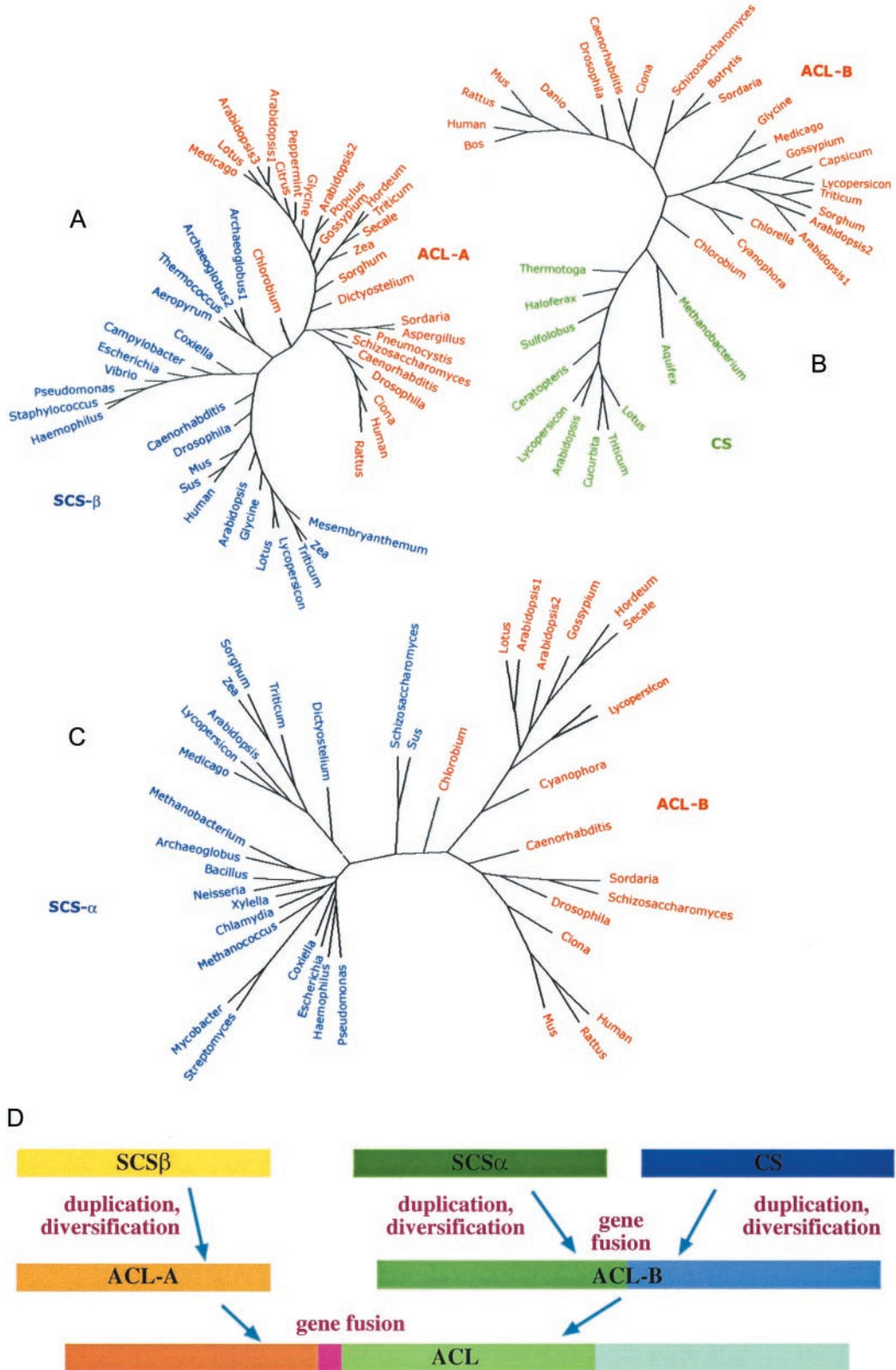


Figure 9. Phylogenetic relationships between ACLA and ACLB and homologous domains in SCS and CS. A through C, Phylogenetic trees were constructed from aligned sequences using maximum likelihood and parsimony with 100 bootstrap resampling methods of the PHYLIP 3.573 software package (Felsenstein, 1989). A, ACLA compared with SCS-β. B, ACLB compared with SCS-α. C, ACLB compared with CS. D, Scheme representing the possible evolutionary history of ACL.

other acyl-CoAs across these organellar membranes (Wood et al., 1983; Burgess and Thomas, 1986; Masterson et al., 1990; Schwabedissen-Gerbling and Gerhardt, 1995; Masterson and Wood, 2000). Most of these studies were undertaken with the goal of determining whether the mitochondrial acetyl-CoA pool can be translocated to the chloroplast as acetyl-carnitine, rather than to establish the export of acetyl-CoA from mitochondria into the cytosol. Furthermore, Roughan et al. (1993) question whether carnitine acetyltransferases exist in plants. In addition, TBLASTN searches of the Arabidopsis genome sequence fail to identify any carnitine acyltransferase-like genes. However, the Arabidopsis genome does contain two genes (At1g79900 and At5g46800) whose products share sequence similarity with carnitine acyltransferases, a protein that would be required to shuttle acyl carnitines across membranes. The products of these two genes are predicted by PSORT to be possibly peroxisomal and by TARGETP to not be in plastids or mitochondria. Thus, to date, ACL appears to be the only substantiated mechanism for generating the cytosolic pool of acetyl-CoA in plant cells. Further indication that the ACL-derived acetyl-CoA pool is critical to plants is derived from the analysis of transgenic plants that have reduced ACL accumulation due to the expression of an ACLA antisense RNA; such plants show aberrant growth and development (Fatland et al., 2000).

The two subunits of plant ACL, ACLA and ACLB, cofractionate with ACL activity during purification, indicating that they are in a complex held together by strong noncovalent interactions. Because the Arabidopsis ACL holoprotein is about 500 kD and because the ACLA (45 kD) and ACLB (65 kD) subunits correspond to the N-terminal and C-terminal portions, respectively, of the animal ACL, which is a homotetramer, we suggest that the plant ACL is a heterooc-tomer with an A_4B_4 configuration.

Our findings contrast with those of Rangasamy and Ratledge (2000) who identified a plant ACL based on immunocrossreaction with rat ACL antibodies. They postulated that ACL is plastidic, and is composed of subunits in the range of 100 to 120 kD. The difference between the two studies could be explained by the presence of two forms of ACL in plants: a cytosolic isozyme, characterized in this manuscript, and a plastidic isozyme, reported by Rangasamy and Ratledge (2000) and postulated to be important for acetyl-CoA-generation for synthesis of fatty acids destined for seed oil (Ratledge et al., 1997). However, the Arabidopsis genome does not contain any plastid-targeted ACL-like genes, or ACL-like genes coding for 100- to 120-kD proteins, and furthermore, ACL activity consistently cofractionated with the ACLA (45 kD) and ACLB (65 kD) polypeptides, indicating that a distinct plastidic form of ACL probably does not exist.

The intricate spatial and temporal patterns of ACLA and ACLB mRNA accumulation are indistinguishable from each other throughout the development of siliques, flowers, and seedlings, indicating a coordinate regulation of the accumulation of these two mRNAs. The dynamic changes in distribution of the ACL mRNAs probably reflect changes in the metabolic demands for cytosolic acetyl-CoA. The pattern of ACLA and ACLB accumulation is nearly indistinguishable from that of cytosolic ACCase mRNA, but is diverse from that of the plastidic ACCase, pyruvate dehydrogenase, and acetyl-CoA synthetase mRNAs (Choi et al., 1995; Ke et al., 1997, 2000a, 2000b). These results are consistent with the supposition that ACL generates a cytosolic pool of acetyl-CoA, which can be carboxylated by the cytosolic ACCase to form malonyl-CoA in the cytosol.

ACL mRNAs accumulate in distinct cell types at specific times in development when phytochemicals requiring cytosolic acetyl-CoA are being rapidly synthesized. For example, the peak in accumulation of the ACL mRNAs in developing embryos occurs at 7 DAF (i.e. the curled cotyledon stage) slightly preceding the maximal rate of oil accumulation in the embryo (Bowman, 1994). Arabidopsis seed oil contains gadoleic acid (C20) and erucic acid (C22; James and Dooner, 1991), which are synthesized by the elongation of plastid-exported oleic acid (C18) using cytosol-derived malonyl-CoA (Pollard and Stumpf, 1980; Bao et al., 1998). Hence, we surmise that this peak in ACL mRNA accumulation (which also coincides with peak cytosolic ACCase expression) is for expanding the supply of cytosolic acetyl-CoA to support the biosynthesis of gadoleic and erucic acids.

Likewise, ACL mRNAs (and cytosolic ACCase mRNA) accumulate preferentially in the epidermal cells of many organs (leaves, ovaries, petals, sepals, seedling roots, and cotyledons) at a stage of development when cuticular wax and/or flavonoid synthesis is occurring; cytosolic acetyl-CoA is carboxylated by cytosolic ACCase and is used by fatty acid elongases (to produce cuticular wax components) or by chalcone synthase (to produce flavonoids).

In addition, the ACL mRNAs (and cytosolic ACCase mRNA) accumulate in the cells of the inner integument of the developing seed immediately prior to deposition of the testa by this cell layer and may reflect requirements for cytosolic acetyl-CoA and malonyl-CoA for the biosynthesis of testal phlobaphens, a proanthocyanin-derived polymer (Stafford, 1995). In a similar manner, the transient accumulation of ACL and cytosolic ACCase mRNAs in tapetal cells coincident with microsporogenesis may provide for the biosynthesis of elongated fatty acids required for the deposition of the sporopollenin on the pollen by the tapetal cells.

Other studies indicate that ACL expression responds to environmental stresses. In sweet potato (*Ipomoea batatas*), ACL activity increases coincident

with the synthesis of the acetyl-CoA-derived sesquiterpene phytopathogen, ipomeamarone (Takeuchi et al., 1981). In transgenic maize cells, an ACL-like mRNA is one of many mRNAs induced by ectopic expression of the transcription factors *C1/R* and *P*, which induce flavonoid synthesis (Bruce et al., 2000). Fungal infection of peppers (*Capsicum annuum*) induces the accumulation of an ACLB homolog (Suh et al., 2001), possibly associated with the production of the sesquiterpene phytoalexin, capsidiol (Back et al., 1998).

ACL catalyzes the reverse of the CS-catalyzed reaction, and is a member of a thiokinase superfamily, along with SCS, acetyl-CoA synthetase, and malate thiokinase (Sanchez et al., 2000). This functional similarity is translated to conservation in the primary structure of these proteins. The thiokinase superfamily of enzymes catalyzes the transfer of a CoA group to or from an organic acid using a nucleotide triphosphate and a divalent cation as cosubstrates. SCS, like ACL, catalyzes the phosphorylation of an acyl substrate, and the subsequent attack of CoA on the resultant acyl phosphate results in the formation of acyl-CoA (Elshourbagy et al., 1992). Hence, these enzymes share conserved ATP- and CoA-binding domains. Likewise, the oxaloacetic acid-binding motif and the active site motif of CS are conserved in the ACL sequences.

Perhaps the most notable feature of the phylogenetic distribution of ACL is its absence from many prokaryotic organisms. Of the 57 eubacteria and 13 archaea whose genomes have been completely sequenced (as of March 2002), only *C. limicola* (Kanao et al., 2001) has genes identifiable as ACL. *C. limicola* is a representative of an ancient photosynthetic bacterial group and contains the reductive TCA cycle (Fuchs et al., 1980; Ivanovsky et al., 1980; Wahlund and Tabita, 1997; Kanao et al., 2001). The reductive TCA cycle can be envisioned as the TCA cycle operating in reverse direction, and is thus a mechanism for CO₂ fixation. In this cycle, ACL functions to harvest the fixed carbon as acetyl-CoA, and regenerates the oxaloacetate required to continue the cycle (Evans et al., 1966; Beh et al., 1993). The reductive TCA cycle and ACL activity have been reported in a phylogenetically diverse group of extremophiles, including anaerobically grown *Desulfobacter hydrogophilus* (Schauder et al., 1987), the thermophilic bacteria, *Hydrogenobacter thermophilus* and *Aquifex pyrophilus* (Shiba et al., 1985; Beh et al., 1993; Suzuki et al., 2001), and the thermophilic archaeon *Thermoproteus neutrophilus* (Beh et al., 1993). Except for *C. limicola*, no ACL sequences are yet available from these organisms. Thus, the metabolic positioning of ACL may have changed during the course of evolution. ACL initially served to make carbon fixed from CO₂ available for general metabolism. As the atmosphere became oxygen rich and organisms developed respiratory capabilities, ACL assumed the function of

tapping into the TCA cycle for carbon derived from catabolic reactions.

Based upon sequence conservation between ACL and SCS subunits, as well as sequence conservation between ACL and CS, ACL may have arisen from the evolutionary fusion and subsequent adaptation of domains from SCS and CS (Sanchez et al., 2000). The ancestral ACLA protein appears to have arisen via the divergence of the SCS β -subunit (Fig. 9, A and D). Because all ACLA sequences are similar to each other throughout their entire sequences, these proteins probably share this common evolutionary origin. Likewise, ACLB proteins may have a common origin via the fusion and divergence of CS and the SCS α -subunit (Fig. 9, B–D). The intermediate character of ACLA and ACLB manifested in the positioning of *C. limicola* between the major branches of ACL and SCS/CS leads us to surmise that the *C. limicola* ACLA and ACLB proteins are evolutionary closest to the ancestral ACL. Furthermore, based on the fact that ACL genes occur in eukaryotes and the prokaryote, *C. limicola*, these ancestral events probably occurred prior to the evolution of eukaryotes.

The heteromeric ACL structure (i.e. ACLA and ACLB subunits) occurs in *C. limicola*, fungi, protists, and plants, but in animals, ACL has a homomeric structure, indicating that the homomeric ACL probably represents the derived condition (Fig. 9D). Because all known animal ACLs are homomeric, such an ACLA/ACLB fusion must have occurred early in the evolution of this kingdom. This is consistent with the considerable divergence among the animal ACL "spacer" sequences, particularly between the vertebrates and other animal lineages. This divergence also implies that little selective pressure exists for the conservation of the "spacer," thus it may have little functional importance. The possible evolutionary significance of the similarity between the extended N terminus of the ACLB protein of *S. macrospora* and the animal spacer region remains an unresolved and interesting question.

MATERIALS AND METHODS

Materials

Arabidopsis (ecotype Columbia) was grown under constant illumination as described by Ke et al. (1997), except for seedlings for in situ hybridization, which were grown sterily on moist filter paper in petri plates at 25°C, under constant illumination. Other materials collected included leaves between one-third and two-thirds of the final size from 17-d-old plants ("expanding leaves"), flower buds staged according to Bowman (1994), siliques at different stages of development (Ke et al., 2000a, 2000b), and inflorescence stems of 4-week-old plants.

Pea (*Pisum sativum*) seeds were planted in a sterile mixture of 30% (v/v) black soil, 30% (v/v) peat moss, and 40% (v/v) Perlite in a 50- × 30- × 6-cm flats; plants were grown in a greenhouse at 22°C to 25°C under a cycle of 16 h of illumination and 8 h of darkness and were fertilized weekly with a solution of 20:10:20 (N:P:K) fertilizer.

EST cDNA clones, TASG097 (GenBank accession no. Z18045), VBVC01 (GenBank accession nos. Z18045 and Z25661), and 60C1T7 (GenBank accession no. T14234) were obtained from the Arabidopsis Biological Resource Center.

In Situ Hybridization

In situ hybridization was carried out as described previously (John et al., 1992; Wang et al., 1995; Ke et al., 1997, 2000a, 2000b). Sense and antisense ³⁵S-labeled RNA probes were transcribed from the *ACLA-1*, *ACLB-2*-partial, and a *SalI-NotI* subclone of 60C1T7 (*ACCI*) cDNAs (Ke et al., 1997). In situ hybridizations were repeated three times using two sets of plant materials that had been independently processed, and they all gave similar results. For all hybridizations, successive sections were hybridized in parallel with sense (negative-control) and antisense RNA probes. In all instances, sense RNA controls showed no or negligible hybridization.

Isolation and Manipulation of Nucleic Acids

Standard procedures were used for manipulation of nucleic acids (Sambrook et al., 1989). Both strands of all DNA fragments were sequenced. RNA was isolated by a phenol/SDS method (Ke et al., 2000a, 2000b). ³²P-labeled RNAs transcribed from the *ACLA-1* and *ACLB-2*-partial cDNA clones were used for hybridization probes (Ke et al., 2000a). The radioactivity was quantified with a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Protein Expression and Generation of Antisera

Recombinant proteins from *ACLA-1* (His and S tagged) and *ACLB-2*-partial (nontagged) were produced in *Escherichia coli* using the pET30 and pET17 expression systems, respectively (Novagen, Madison, WI). Recombinant proteins were recovered in inclusion bodies, purified by preparative SDS-PAGE, and each was used to generate antiserum (Ke et al., 1997).

Immunological Analysis of Proteins

Protein extracts were subjected to electrophoresis in denaturing (Laemmli, 1970) or nondenaturing conditions (Hedrick and Smith, 1968; Lambin and Fine, 1979). *ACLA-1* and *ACLB-2* antiserum were used at a 1:500 dilutions for immunodetection of proteins (Ke et al., 1997). Biotin-containing polypeptides were detected by ¹²⁵I-streptavidin (Nikolau et al., 1985).

Expression of Arabidopsis ACL Polypeptides in Yeast (*Saccharomyces cerevisiae*)

The isolated full-length cDNAs of *ACLA-1* and *ACLB-2* were subcloned into yeast expression vectors pYX042 and pYX012, respectively, and were used to generate yeast strains carrying the integrated *TPI-ACLA-1* or *TPI-ACLB-2* transgenes. Recombinant yeast strains were cultured under conditions to express the respective proteins (Sherman, 1991). Cell pellets were frozen in aliquots in liquid nitrogen. For each ACL assay, 50 to 100 μ L of pellet was thawed on ice, resuspended in 100 μ L of extraction buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM dithiothreitol [DTT], 1.5% [w/v] preswollen polyvinylpyrrolidone, 1 mM phenylmethylsulfonyl fluoride, and 1 mM *p*-aminobenzamidine, pH 8.0), and homogenized for 30 s in an Eppendorf tube. The slurry was frozen in liquid nitrogen and then pulverized until finely ground, thawed, and centrifuged at 12,000g for 5 min at 4°C. The resulting supernatant was desalted through Sephadex G-50 columns (100 μ L extract 0.5 mL⁻¹ bed volume) using an Elution buffer (50 mM NaH₂PO₄, 1 mM MgCl₂, 0.1 mM EDTA, and 1 mM DTT, pH 7.2). Each desalted extract was adjusted to 10% (v/v) glycerol, centrifuged, and assayed immediately for ACL activity.

Spectrophotometric Assay of ACL Activity

ACL activity was determined with a spectrophotometric assay similar to that of Kaethner and ap Rees (1985). The assay detects the ACL-catalyzed generation of oxaloacetate by coupling its appearance to the oxidation of NADH catalyzed by malate dehydrogenase. The oxidation of NADH was monitored by the change in A_{340} , and ACL activity was calculated using the extinction coefficient of NADH (6.22 mm⁻¹ cm⁻¹). Plant extracts not immediately assayed for ACL activity were desalted by passage through Sephadex G-25 (to reduce NADH oxidation in the absence of CoA), adjusted to 10% (v/v) glycerol, and stored frozen in liquid nitrogen. Storage in 10% (v/v) glycerol at 4°C or storage without glycerol in liquid nitrogen resulted in a loss of about two-thirds of the original activity in 7 d. Storage at 4°C

without glycerol resulted in nearly a complete loss of activity in 4 d. The ACL assay is dependent on the ratio of ATP:citrate:Mg²⁺, presumably because ATP and citrate chelate Mg²⁺, and Mg²⁺:ATP is the actual substrate. K_m for citrate is 1.7 mM; K_m for ATP is 1.6 mM. The optimized ACL assay was conducted in a total volume of 0.5 to 1 mL, containing 30 to 250 μ L of extract, 200 mM Tris-HCl, pH 8.4, 20 mM MgCl₂, 1 mM DTT, 10 mM ATP, 10 mM citrate, 0.2 mM CoA, 6 units of malate dehydrogenase, and 0.1 mM NADH. ACL activity is unaffected by up to 600 mM NaCl. NADH oxidation was determined in the absence of CoA, and the ACL reaction was initiated by addition of CoA.

Enzyme Assays

Cytochrome *c* oxidase was assayed according to Anderson and Roberts (1998), HPR was assayed according to Schwitzguebel and Siegenthaler (1984), and NADP-GAPDH and PEP carboxylase were assayed according to Kaethner and ap Rees (1985).

Extraction of Plant Proteins

Protein extracts were prepared and desalted using Sephadex G-25 columns with Elution buffer and were adjusted to 10% (v/v) glycerol (Wurtele et al., 1985).

Chromatography

Gel-filtration and ion-exchange chromatography was conducted with a chromatography system (Biosys 510; Beckman Instruments, Fullerton, CA). For gel-filtration chromatography, a Superdex 200 HR 30/10 column (Amersham Biosciences, Piscataway, NJ) was loaded with 0.25 mL of enzyme extract, eluted at a flow rate of 0.25 mL min⁻¹ with 50 mM NaH₂PO₄, pH 7.2, 0.1 mM EDTA, 1 mM MgCl₂, 1 mM DTT, 100 mM NaCl, and 10% (v/v) glycerol, and 0.25-mL fractions were collected. Three fraction profiles were collected and pooled. A portion of each pooled fraction was assayed for ACL activity and the remainder was subjected to SDS-PAGE and immunoblot analysis.

Ion-exchange chromatography was conducted with a Mono Q HR 5/5 anion-exchange column (Amersham Biosciences) equilibrated with 20 mM HEPES-NaOH, pH 7.5, 1 mM MgCl₂, 1 mM DTT, and 10% (v/v) glycerol, and operated at a flow rate of 0.5 mL min⁻¹. The column was eluted with a 10-mL, 0 to 0.35 M NaCl concentration gradient, followed by a 5-mL, 0.35 to 1.0 M NaCl concentration gradient. Eluant was monitored with a conductivity detector. One-milliliter fractions were collected, the A_{280} was determined, and aliquots were subjected to SDS-PAGE and immunoblot analysis and assayed for ACL activity.

Subcellular Fractionation

Organelles were isolated from 10- to 11-d-old pea shoots using a Percoll gradient-based procedure (Anderson et al., 1998). Seedlings were placed in darkness late in the afternoon of the day prior to the isolation to ensure depletion of starch. Chilled plant material (50 g) was homogenized using a mortar and pestle with 100 mL of 0.33 M sorbitol, 10 mM NaP₂O₇, 5 mM MgCl₂, and 2 mM ascorbate, pH 6.5, and filtered through four layers of cheesecloth. The filtrate was centrifuged at 1,500g for 3 min. Each 1,500g pellet was resuspended in 2 mL of resuspension buffer (0.33 M sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, and 50 mM HEPES, pH 7.6) to yield a crude chloroplast fraction. Chloroplasts were further purified on discontinuous Percoll gradients. Three milliliters of the crude chloroplast fraction was dispensed in each of two 15-mL Corex tubes, each containing 3 mL of 70% (v/v) Percoll and 6 mL of 40% (v/v) Percoll in resuspension buffer, and centrifuged at 2,500g for 12 min. Intact chloroplasts formed a band at the Percoll concentration interface. Intact chloroplasts were washed twice in resuspension buffer with centrifugation at 1,500g for 3 min, and were resuspended in 2 mL of resuspension buffer to yield the Percoll-purified chloroplasts.

The 1,500g supernatant was centrifuged at 12,000g for 10 min and the resulting pellet was resuspended in 1 mL of resuspension buffer, yielding a mitochondria plus peroxisome fraction. Mitochondria and peroxisomes were further purified by Percoll density gradient as detailed by Anderson et al. (1998). The 12,000g supernatant was also retained and analyzed.

Fractions were assayed for ACL activity and activities of organelle marker enzymes. For ACL assays, samples were sonicated 20 s mL⁻¹ at 60% output power, and they were desalted by Sephadex G-25 with 50 mM NaH₂PO₄, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, pH 7.2, and 10% (v/v) glycerol. ACL activity was not assayed in the purified mitochondria and peroxisomes. Samples were also subjected to SDS-PAGE and immunoblot analysis with anti-catalase (Kunze et al., 1988), anti-ACLA, anti-ACLB, and streptavidin (Nikolau et al., 1985). Assays and blots were repeated twice with similar results.

Sequence Analysis

The sequences for phylogenetic analysis were obtained from the GenBank database containing amino acid sequences determined from ESTs, overlapping EST fragments, and putative sequences inferred from DNA (see supplemental data at <http://molebio.iastate.edu/~mash/alignment.html>). Amino acid sequences were aligned with ClustalW (Thompson et al., 1994) in BioEdit package (Hall, 1999). Phylogenetic trees were constructed from aligned sequences using maximum likelihood and parsimony with 100 bootstrap resampling methods of the PHYLIP 3.573 software package (Felsenstein, 1989). The domains used for the comparisons are: ACLA compared with SCS- β (residues 257–364 in *Arabidopsis* ACLA), ACLB compared with SCS- α (residues 187–284 in *Arabidopsis* ACLB), and ACLB compared with CS (residues 419–575 in *Arabidopsis* ACLB; see Fig. 8 to identify location of the domains).

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