Plant Physiol. (1997) 115: 1371–1383

Biochemical and Molecular Biological Characterization of *CAC2*, the *Arabidopsis thaliana* Gene Coding for the Biotin Carboxylase Subunit of the Plastidic Acetyl-Coenzyme A Carboxylase[1]

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The biotin carboxylase subunit of the heteromeric chloroplastic acetyl-coenzyme A carboxylase (ACCase) of *Arabidopsis thaliana* is coded by a single gene (*CAC2*), which is interrupted by 15 introns. The cDNA encodes a deduced protein of 537 amino acids with an apparent N-terminal chloroplast-targeting transit peptide. Antibodies generated to a glutathione *S*-transferase-CAC2 fusion protein react solely with a 51-kD polypeptide of Arabidopsis; these antibodies also inhibit ACCase activity in extracts of Arabidopsis. The entire *CAC2* cDNA sequence was expressed in *Escherichia coli* and the resulting recombinant biotin carboxylase was enzymatically active in carboxylating free biotin. The catalytic properties of the recombinant biotin carboxylase indicate that the activity of the heteromeric ACCase may be regulated by light-/dark-induced changes in stromal pH. The *CAC2* gene is maximally expressed in organs and tissues that are actively synthesizing fatty acids for membrane lipids or oil deposition. The observed expression pattern of *CAC2* mirrors that previously reported for the *CAC1* gene (J.-K. Choi, F. Yu, E.S. Wurtele, B.J. Nikolau [1995] Plant Physiol 109: 619–625; J. Ke, J.-K. Choi, M. Smith, H.T. Horner, B.J. Nikolau, E.S. Wurtele [1997] Plant Physiol 113: 357–365), which codes for the biotin carboxyl carrier subunit of the heteromeric ACCase. This coordination is probably partially established by coordinate transcription of the two genes. This hypothesis is consistent with the finding that the *CAC2* and *CAC1* gene promoters share a common set of sequence motifs that may be important in guiding the transcription of these genes.

The biotin-containing enzyme ACCase (acetyl-CoA:carbon dioxide ligase [ADP-forming], EC 6.4.1.2) catalyzes the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. ACCase is the major regulatory point of fatty acid formation in a wide variety of organisms (Vagelos, 1971; Wakil et al., 1983; Hasslacher et al., 1993; Li and Cronan, 1993; Ohlrogge et al., 1993). In plants malonyl-CoA that is generated in plastids has a single known fate, the formation of fatty acids (Ohlrogge et al., 1979; Stumpf, 1987; Harwood, 1988); in contrast, cytosolic malonyl-CoA is not utilized for de novo fatty acid biosynthesis but for the synthesis of a variety of phytochemicals (Conn, 1981; Nikolau, et al., 1984). These include epicuticular waxes, suberin, flavonoids, stilbenoids, a variety of malonylated chemicals, and free malonic acid. Because malonyl-CoA cannot freely move across membrane barriers, it must be formed in the subcellular compartments in which it will be utilized, i.e. the plastid and the cytosol. Hence, ACCases occur in each of these compartments to generate malonyl-CoA.

In most flowering plants, including Arabidopsis, there are two structurally distinct forms of ACCase (Sasaki et al., 1995). The plastidic enzyme is a heteromer composed of four different types of polypeptides organized into three functional proteins: BCC, biotin carboxylase, and carboxyltransferase (Sasaki et al., 1993; Choi et al., 1995; Shorrosh et al., 1995, 1996). The plant heteromeric ACCase is similar in structure to the ACCase found in eubacteria such as *Escherichia coli* (Guchhait et al., 1974; Kondo et al., 1991; Li and Cronan, 1992a, 1992b). In contrast, the plant cytosolic ACCase is a homodimer, similar in structure to the cytosolic ACCase of other eukaryotes, including mammals and yeast (Lopez-Casillas et al., 1988; Walid et al., 1992; Gornicki et al., 1993; Roessler and Ohlrogge, 1993; Roesler et al., 1994; Schulte et al., 1994; Shorrosh et al., 1994; Yanai et al., 1995). An exception to the above is Gramineae, in which both the plastidic and cytosolic ACCases are homodimers (Egli et al., 1993; Gornicki et al., 1994; Konishi et al., 1996).

The heteromeric, plastidic ACCase from plants (Kannangara and Stumpf, 1972; Sasaki et al., 1993; Alban et al., 1994, 1995; Konishi and Sasaki, 1994; Choi et al., 1995; Shorrosh et al., 1995), like that of its bacterial homologs, readily dissociates. This feature has hindered the biochemical characterization of the plant enzyme. In this paper, we report the isolation and characterization of a full-length cDNA and the gene coding for the biotin carboxylase subunit of the heteromeric, chloroplastic ACCase of *Arabidopsis thali-*

[1] This work was supported in part by a grant from the Iowa Soybean Promotion Board to E.S.W. and B.J.N., and by a research award to J.K. from the Iowa State University Molecular, Cellular, and Developmental Biology graduate program. This is journal paper no. J-17,380 of the Iowa Agricultural and Home Economics Experiment Station, Ames (project nos. 2997 and 2913) and was supported by the Hatch Act and State of Iowa funds.

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Abbreviations: ACCase, acetyl-CoA carboxylase; BCC, biotin carboxyl carrier; DAF, days after flowering; EST, expressed sequence tag; GST, glutathione *S*-transferase.

ana. Consistent with the precedent established by *CAC1*, the name of the Arabidopsis gene coding for the BCC subunit of the heteromeric ACCase (Ke et al., 1997), we labeled the biotin carboxylase gene, *CAC2*. The *CAC2* cDNA was expressed in *E. coli* in a catalytically active form and its catalytic properties were characterized. The *CAC2* mRNA was found to accumulate to highest levels in cells that are undergoing rapid growth and/or are in the process of oil deposition. We suggest that the activity of the heteromeric ACCase may be regulated both by mechanisms that control the transcription of the genes coding for its subunits and by metabolic effectors of biotin carboxylase activity.

MATERIALS AND METHODS

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia were germinated in sterile soil and plants were grown at 25°C with constant illumination. The following items were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus): a cDNA library in the vector λZAP II (Stratagene), prepared from poly(A^+) RNA isolated from 3-d-old seedling hypocotyls of *A. thaliana* (L.) Heynh. ecotype Columbia (Kieber et al., 1993); a genomic library in the vector λFIX, prepared from DNA of *A. thaliana* (L.) Heynh. ecotype Landsberg erecta (Voytas et al., 1990); and the cDNA clone 150M20T7 (Newman et al., 1994).

Plasmids

The expression vector pGEX-CAC2 was obtained by cloning the 997-bp *Sal*I fragment from 150M20T7 into the *Sal*I site of pGEX-4T-2 (Pharmacia), such that the cDNA sequence was in-frame with the *GST* gene. pET-CAC2 was obtained by cloning the *Nsp*7524I-*Eco*RI fragment from the full-length *CAC2* cDNA into the *Nde*I/*Eco*RI sites of pET5a using an *Nde*I-*Nsp*7524I adaptor that encoded an S-tag peptide. Proteins containing the S-tag peptide can be detected or purified via their interaction with the S-protein derived from pancreatic RNase A (Richards and Wyckoff, 1971).

Isolation and Characterization of Macromolecules

Arabidopsis protein extracts were centrifuged through Sephadex G25 to remove low-molecular-weight compounds (Nikolau et al., 1984). The CAC2 protein and protein-bound biotin were detected by western analysis of protein extracts after SDS-PAGE. Antigen-antibody complexes and protein-bound biotin were detected with ^{125}I-protein A and ^{125}I-streptavidin (Nikolau et al., 1985), respectively.

Nucleic acids were isolated and manipulated by standard techniques (Sambrook et al., 1989). DNA sequencing was done at the Iowa State University DNA Facility (Ames) on double-stranded DNA templates using a DNA sequencer (model 373A, ABI, Columbia, MD). Both strands of all DNA fragments were sequenced at least twice. All computer-assisted analyses of nucleotide and predicted amino acid sequences were performed with the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Madison, WI).

The Arabidopsis genomic and cDNA libraries were screened by hybridization with the approximately 1-kb cDNA insert from 150M20T7. Approximately 200,000 recombinant phage from each library were grown on Petri plates and replicated to nitrocellulose membranes. The replica filters were incubated at 65°C in hybridization solution (5× SSC, 1× Denhardt's solution, 0.2% [w/v] SDS, 10 mM EDTA, 0.1 mg/mL salmon-sperm DNA, 10% [w/v] dextran sulfate, and 50 mM Tris-HCl, pH 8.0) with a ^{32}P-labeled probe for 12 h. After hybridization, filters were washed at 65°C in 2× SSC and 0.5% (w/v) SDS and subsequently with 0.1× SSC and 0.1% (w/v) SDS.

Recombinant Proteins

The expression of recombinant proteins from pGEX-CAC2 and pET-CAC2 plasmids was undertaken in *Escherichia coli*. Expression was induced with isopropylthio-β-galactoside. The GST-CAC2 fusion protein was purified by agarose-glutathione-affinity chromatography, as described by the manufacturer (Pharmacia; Smith and Johnson, 1989). The mature, full-length CAC2 protein was expressed from pET-CAC2 with an N-terminal S-tag extension. Cells expressing this mature CAC2 recombinant protein were lysed by sonication, and the cell extract was clarified by centrifugation (15,000*g*, 20 min). The supernatant was directly loaded to the S-tag-agarose-affinity column; after extensive washing to remove nonbound proteins, the mature CAC2 protein was eluted with 2 M guanidine thiocyanate. The denatured protein was renatured by dialysis against 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl.

Assays

Biotin carboxylase activity was determined by the method of Guchhait et al. (1974) and as previously adapted for the plant enzyme (Nikolau et al., 1981; Alban et al., 1995). We determined the rate of the biotin-dependent conversion of radioactivity from $NaH^{14}CO_3$ (unstable to CO_2 bubbling) into the carboxy-biotin product (stable to CO_2 bubbling). All assays were carried out in duplicate, and control assays lacked biotin. Values of kinetic constants are averages of three determinations.

ACCase activity was determined as the rate of acetyl-CoA-dependent conversion of radioactivity from $NaH^{14}CO_3$ into an acid-stable product (Wurtele and Nikolau, 1990). Protein concentrations were determined by a Coomassie-binding assay (Bradford, 1976).

Immunological Methods

Antiserum was generated in a female New Zealand White rabbit immunized with purified expressed GST-CAC2 fusion protein emulsified with Freund's Complete Adjuvant. Emulsion containing approximately 300 μg of protein was injected intradermally at multiple sites on the back of the animal. Thirty days after the initial immuniza-

tion, and at 2-week intervals thereafter, the rabbit was given muscular injections of 150 to 200 μg of GST-CAC2 fusion protein emulsified in Freund's incomplete adjuvant. One week after each injection, 2 to 3 mL of blood was withdrawn from the ear of the rabbit, allowed to coagulate, and the serum was collected. Alkaline phosphatase-labeled S-protein was obtained from Novagen (Madison, WI).

In Situ Techniques

In situ hybridization to RNA using paraffin-embedded sections was conducted as described previously (John et al., 1992; Ke et al., 1997). ^{35}S-Labeled RNA probes (sense and antisense) were synthesized from a subclone consisting of the 3′-most 600 nucleotides of the *CAC2* cDNA. Tissue sections affixed to slides were hybridized, coated with nuclear track emulsion (NTB2, Kodak), exposed for 3 d, and developed. Photographs were taken with an Orthoplan microscope (Leitz, Wetzlar, Germany) under bright-field illumination. In situ hybridization results were repeated three times using two sets of plant materials that had been independently processed, all of which gave similar results.

RESULTS

Isolation and Characterization of the Arabidopsis *CAC2* cDNA

The cDNA clone 150M20T7 was identified in the Arabidopsis collection of EST clones (Newman et al., 1994) because it codes for a peptide that shows a high degree of sequence similarity to the biotin carboxylase of *E. coli* and *Anabaena* sp. strain PCC 7120 (Li and Cronan, 1992a, 1992b; Gornicki et al., 1993). This clone was fully sequenced and was found to be a partial copy of the corresponding mRNA. Later analyses showed that it encoded the 3′, 970-bp segment of the full-length cDNA clone. We isolated a full-length cDNA clone by screening a cDNA library made from mRNA isolated from 3-d-old seeding hypocotyls of Arabidopsis (Kieber et al., 1993). Seven independent clones were isolated from approximately 200,000 recombinant bacteriophage. The longest of these, which we call *CAC2*, was 1995 bp in length. Beginning at position 119 of the nucleotide sequence was an "ATG" codon, which initiated a 1614-bp open reading frame, the longest present on this cDNA. Upstream of this ATG were stop codons in all three reading frames. The 3′ untranslated region contained a putative polyadenylation signal, ATAATTTT, which was located 31 nucleotides upstream of the poly(A^+) addition site.

The deduced polypeptide sequence encoded by the largest open reading frame was 537 amino acids long, with a predicted molecular mass of 58 kD and a pI of 7.23 (Fig. 1). The sequence at the N terminus had features of a plastid-targeting sequence (Keegstra et al., 1989) and was about 35% identical to the chloroplast transit peptide of the tobacco (*Nicotiana tabacum* L.) biotin carboxylase (Shorrosh et al., 1995). The sequence homology between CAC2 and biotin carboxylase subunits from prokaryotes, which lack such transit peptides, began immediately following this putative transit peptide, at residue 76.

The deduced CAC2 protein sequence was most similar to the biotin carboxylase subunit of the heteromeric ACCases from a variety of organisms, including tobacco (84% identical), blue-green algae (65% identical), and other eubacteria (about 53% identical; Fig. 1). Lesser, but still significant sequence identities occurred between CAC2 and biotin carboxylase domains of other biotin-containing enzymes. These included a biotin carboxylase subunit of a biotin-containing enzyme of unidentified biochemical function of *Methanococcus jannaschii* (51% identical), propionyl-CoA carboxylase (44% identical), methylcrotonyl-CoA carboxylase (45% identical), pyruvate carboxylase (42% identical), and urea carboxylase (37% identical). The similarity between CAC2 and the biotin carboxylase domain of homomeric ACCases was lower, e.g. CAC2 had 31 and 29% identity to the yeast and Arabidopsis homomeric ACCases, respectively. The sequence similarities between CAC2 and the homologs shown in Figure 1 were particularly high in four domains. These include the ATP-binding domain and a domain that showed sequence similarity to carbamoylphosphate synthetase (Toh et al., 1993).

The *CAC2* cDNA Codes for the Biotin Carboxylase Subunit of the Heteromeric ACCase of Arabidopsis

To confirm directly that *CAC2* cDNA codes for the biotin carboxylase subunit of the heteromeric, plastid-localized ACCase, and to characterize the biochemical properties of the CAC2 protein, we expressed it in *E. coli* (Fig. 2). An expressed GST-CAC2 fusion protein was used to generate antiserum (Fig. 2A). The resulting antiserum was used on western blots to identify the CAC2 protein in Arabidopsis leaf extracts (Fig. 2B). This anti-GST-CAC2 serum, but not the control preimmune serum, reacted solely with a 51-kD polypeptide, which was similar in size to the mature CAC2 protein predicted from the cDNA sequence, and to that of the tobacco biotin carboxylase (Shorrosh et al., 1995). The anti-GST-CAC2 serum specifically inhibited ACCase activity in extracts from Arabidopsis (Fig. 3). This immunoinhibition was not complete; rather, the antiserum inhibited about 80% of the activity found in extracts. These results indicate that the *CAC2* cDNA codes for the biotin carboxylase subunit of the heteromeric ACCase. Approximately 20% of the ACCase was resistant to immunoinhibition and this was probably due to the activity of the immunologically distinct homomeric ACCase.

The mature CAC2 protein was expressed with an N-terminal S-tag-extension from a pET5a-derivative plasmid. The expressed 51-kD CAC2 protein was identified immunologically in extracts of *E. coli* with anti-GST-CAC2 serum (Fig. 2D, lane 1) and with alkaline phosphatase-labeled S-protein (Fig. 2E, lane 1). The expressed CAC2 protein was mostly soluble, the majority being recovered in the 15,000*g* supernatant of sonicated *E. coli* extracts. This protein was purified by affinity chromatography, as described in "Materials and Methods." The purification of the expressed CAC2 protein was monitored immunologically (Fig. 2, D and E). Some of the protein underwent partial

Figure 1. The amino acid sequence of the CAC2 protein compared with sequences of other biotin carboxylase domains. The CAC2 sequence (bc.at) was deduced from the nucleotide sequence of the corresponding cDNA, which has been deposited in the GenBank database and assigned accession no. U90879. The sequences of the following biotin carboxylase structural domains are compared: tobacco (bc.tob; Shorrosh et al., 1995); *Anabaena* (bc-anb; Gornicki et al., 1993); *Synechococcus* PCC7942 (bc.syn; GenBank accession no. U59234); *M. jannaschii* (bc.mth; Bult et al., 1996); *E. coli* (bc.col; Kondo et al., 1991; Li and Cronan, 1992a); residues 1 to 520 of the α-subunit of human propionyl-CoA carboxylase (pcc.hum; Lamhonwah et al., 1986); residues 1 to 500 of methylcrotonyl-CoA carboxylase from Arabidopsis (mcc.at; Weaver et al., 1995); residues 1 to 550 of human pyruvate carboxylase (pyc.hum; Wexler et al., 1994); residues 551 to 1100 of yeast urea carboxylase (uc.yst; Genbauffe and Cooper, 1991); residues 1 to 600 of yeast ACCase (acc.yst; Walid et al., 1992; Hasslacher et al., 1993); and residues 1 to 600 of Arabidopsis homomeric ACCase (acc.at; Roesler et al., 1994; Yanai et al., 1995). Residues that are identical in six sequences, including the CAC2 sequence, are shaded black; conservative substitutions are shaded gray. Gaps introduced to maximize alignments are indicated by periods. Four highly conserved subdomains are identifiable: the ATP-binding site (residues 223–242), the biotin carboxylation site with similarity to carbamoyl phosphate synthetase (residues 344–391), BC-1 (residues 75–120), and BC-2 (residues 278–316). The functional significance of the BC-1 and BC-2 subdomains are unclear.

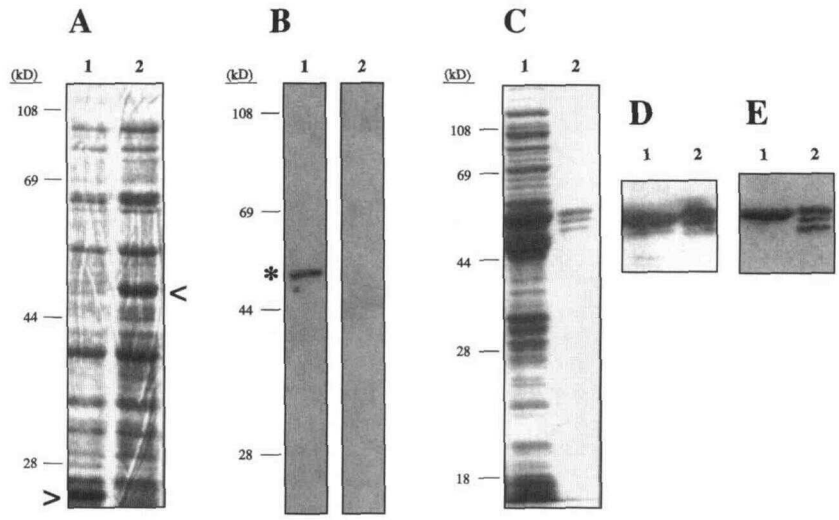

Figure 2. A, Expression of a GST-CAC2 fusion protein from the vector pGEX-CAC2. Extracts from *E. coli* harboring pGEX-4T-2 (lane 1) and pGEX-CAC2 (lane 2) were subjected to SDS-PAGE and stained with Coomassie brilliant blue. The positions of the nonrecombinant GST protein (>) and the recombinant GST-CAC2 protein (<) are indicated. Each lane contained 30 μg of protein. B, An aliquot of Arabidopsis leaf extract (50 μg of protein) was subjected to SDS-PAGE and western-blot analysis using either anti-GST-CAC2 serum (lane 1) or preimmune control serum (lane 2). The 51-kD CAC2 protein is marked with an asterisk (*). C, The mature CAC2 protein was expressed in *E. coli* from the vector pET-CAC2, as described in "Materials and Methods." SDS-PAGE gel stained with Coomassie brilliant blue of the 15,000*g* supernatant of an extract from *E. coli* harboring pET-CAC2 (30 μg of protein; lane 1) and the affinity-purified mature CAC2 protein (0.5 μg of protein; lane 2). D, Gel identical to the one shown in C subjected to western-blot analysis using anti-GST-CAC2 serum. E, Gel identical to one shown in C subjected to western-blot analysis using alkaline phosphatase-labeled S-protein.

proteolytic clipping during purification so that the purified preparation contained, in addition to the intact CAC2 protein, two slightly smaller polypeptides.

The purified mature CAC2 protein was tested for its ability to catalyze the carboxylation of free biotin using an assay originally developed to characterize the biotin carboxylase of *E. coli* (Guchhait et al., 1974) and subsequently used on the plant enzyme (Nikolau et al., 1981; Alban et al., 1995). These experiments showed that the expressed CAC2 protein could catalyze the carboxylation of free biotin. The formation of [^{14}C]carboxy-biotin was linear with incubation time, up to 30 min, and with increasing enzyme concentration (data not shown). This reaction was dependent on the presence of biotin, ATP, HCO_3^-, and Mg^{2+} (Figs. 4 and 5). Hence, these data clearly and unequivocally prove that CAC2 is the biotin carboxylase subunit of the heteromeric ACCase.

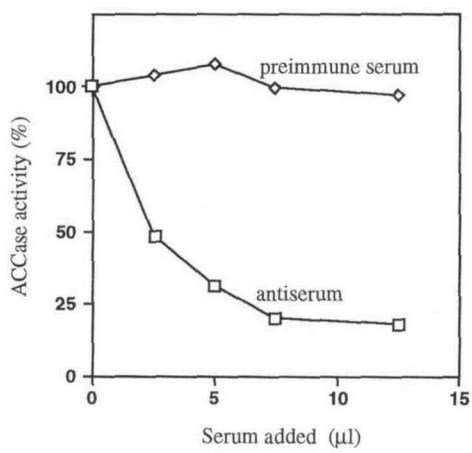

Figure 3. Immunoinhibition of ACCase activity by anti-GST-CAC2 serum. Increasing amounts of either preimmune or anti-GST-CAC2 serum were added to an Arabidopsis leaf extract. After a 1-h incubation on ice, residual ACCase activity was determined. ACCase-specific activity in the extract without added serum was 13.5 nmol min^{-1} mg^{-1}.

Having established that the CAC2 protein was active in catalyzing the biotin carboxylase reaction, we monitored the purification of the CAC2 protein by assaying for this activity. The purified CAC2 protein had a specific activity of about 15 nmol min^{-1} mg^{-1}, which represented a 28-fold purification from the initial extract. In addition, the purified fraction contained about 20 to 30% of the biotin carboxylase activity found in the initial extract. The majority of the biotin carboxylase activity that was lost during purification occurred during the final purification procedure, i.e. the S-tag affinity-chromatography step. Most of this loss was due to lack of recovery of the CAC2 protein from the affinity matrix, judging from the western analyses of the purification fractions. However, the final specific activity was considerably lower than that obtained with the biotin carboxylase purified from pea (500 nmol min^{-1} mg^{-1} protein; *Pisum sativum* L.). This difference may have been due to the fact that renaturation after affinity chromatography was incomplete, or it may represent the fact that the purified pea biotin carboxylase preparation was a complex with the BCC subunit, which activates the enzy-

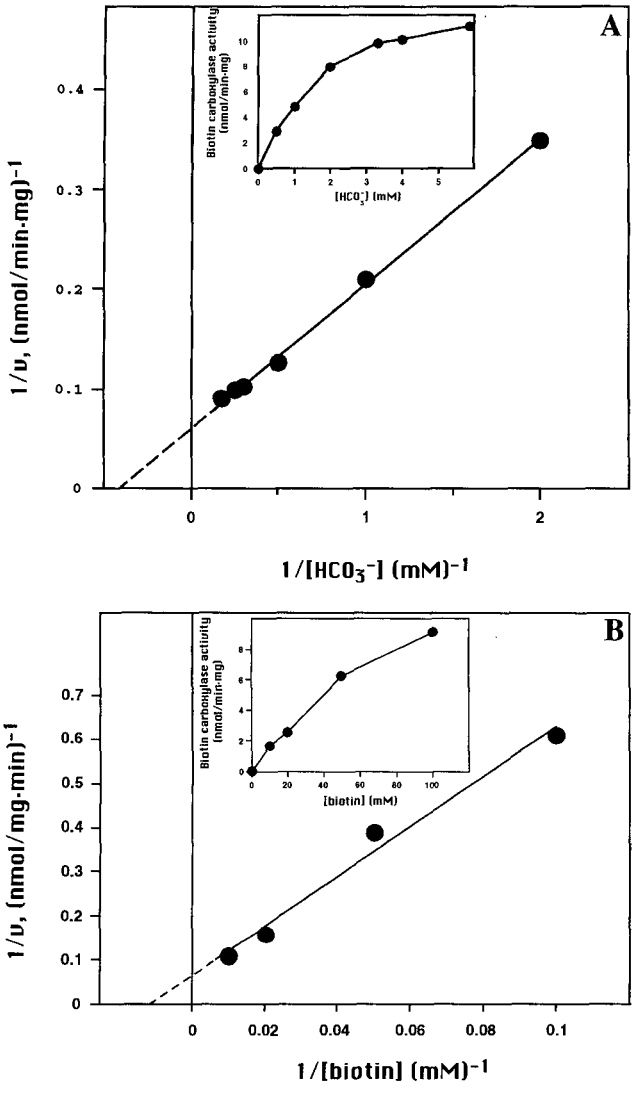

Figure 4. Lineweaver-Burk analysis of the dependence of biotin carboxylase activity on substrate concentration. Unless otherwise stated, the concentrations of the assay components were 50 mM biotin, 5 mM $NaHCO_3$, 1 mM ATP, and 0.5 mM $MgCl_2$. A, The purified recombinant biotin carboxylase was assayed in the presence of the indicated concentrations of $NaHCO_3$. The inset shows the effect of changing $NaHCO_3$ concentration on enzyme activity. B, The purified recombinant biotin carboxylase was assayed in the presence of the indicated concentrations of biotin. The inset shows the effect of changing biotin concentration on enzyme activity.

matic activity. Indeed, such activation of biotin carboxylase has been reported for the *E. coli* enzyme (Guchhait et al., 1974).

Analysis of the Catalytic Properties of the Recombinant Biotin Carboxylase

The catalytic properties of the recombinant biotin carboxylase enzyme were characterized by determining the effect of increasing the concentration of each substrate on enzyme activity, while the concentrations of the other substrates were maintained at a constant concentration. In addition, the effect of changing pH on enzyme activity was determined. We determined the K_m and V_{max} for each substrate and the pH optimum for catalysis (the results of a typical experiment are shown in Figs. 4 and 5). The values of V_{max} obtained from Lineweaver-Burk analyses of data in which biotin or bicarbonate concentrations were altered are 15.7 ± 0.6 and 16.3 ± 0.9 nmol min^{-1} mg^{-1} protein, respectively (Fig. 4). The K_m values for these two substrates are 2.3 ± 0.1 and 88 ± 4 mM, respectively.

Biotin carboxylase activity has an absolute requirement for Mg^{2+}. When activity was assayed in the presence of 0.1 mM ATP, activity was undetectable unless Mg^{2+} was added to the assay (Fig. 5A). Increasing the Mg^{2+} concentration resulted in a hyperbolic increase in biotin carboxylase; maximal activity was observed when the Mg^{2+}/ATP molar ratio was 1. Increasing the concentration of Mg^{2+} above that of ATP resulted in inhibition of activity; when the Mg^{2+} concentration was 1 mM, biotin carboxylase activity was inhibited to 40% of maximal activity. When the Mg^{2+} concentration in the assay was maintained at 0.4 mM, increasing the ATP concentration up to 0.1 mM resulted in a hyperbolic increase in biotin carboxylase activity (Fig. 5B). Further increases in ATP concentration had little effect on activity. The results obtained from these two experiments indicate that biotin carboxylase utilizes the Mg/ATP complex as the substrate and that free Mg^{2+} is an inhibitor of the enzyme.

Biotin carboxylase activity showed a narrow pH optimum, with activity being relatively unaffected between pH 8.3 and 8.9 and maximal activity occurring at pH 8.6 (Fig. 5C). Beyond this pH range, activity was drastically affected, particularly on the acidic side of the optimum; at pH 7.3, activity was 2% of optimum.

Expression of the *CAC2* Gene in Arabidopsis

The expression of the *CAC2* gene was investigated by monitoring the accumulation of the biotin carboxylase protein and the *CAC2* mRNA. These experiments showed that the expression of the *CAC2* gene is developmentally regulated. The biotin carboxylase protein and *CAC2* mRNA accumulated to maximal levels in expanding young leaves of 16-d-old plants (Fig. 6, A and B, lanes 1) and bolting shoots of 45-d-old plants (Fig. 6, A and B, lanes 2). In contrast, both the biotin carboxylase protein and *CAC2* mRNA were barely detectable in fully expanded leaves of 45-d-old plants (Fig. 6, A and B, lanes 3).

To obtain a more detailed characterization of *CAC2* expression, we examined the spatial distribution of the *CAC2* mRNA by in situ hybridization (Fig. 7). Siliques at 7 DAF have ceased their expansion and contain embryos at the torpedo stage of development; these embryos are rapidly accumulating seed oil. In such siliques, the *CAC2* mRNA accumulated to highest levels within the embryo; much less *CAC2* mRNA was detectable in other tissues of the silique (Fig. 7A). In contrast, the *CAC2* mRNA was evenly distributed among all of the cells of an expanding leaf from a 9-d-old plant (Fig. 7, B and C).

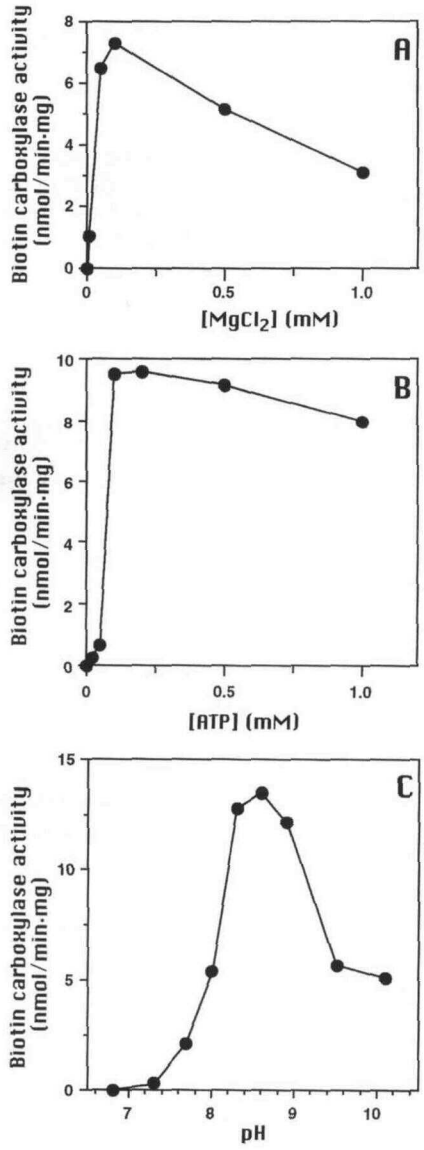

Figure 5. Effect of changing Mg^{2+}, ATP, and pH on biotin carboxylase activity. Unless otherwise stated, the concentrations of the assay components were 50 mM biotin, 5 mM $NaHCO_3$, 1 mM ATP, and 0.5 mM $MgCl_2$. A, The purified recombinant biotin carboxylase was assayed at the indicated concentrations of $MgCl_2$, while ATP concentration was maintained at 0.4 mM. B, The purified recombinant biotin carboxylase was assayed at the indicated concentrations of ATP, while $MgCl_2$ concentration was maintained at 0.1 mM. C, The purified recombinant biotin carboxylase was assayed at the indicated pH, using Tris-bis-propane-HCl as the buffer.

Isolation and Characterization of the *CAC2* Gene

Arabidopsis DNA was digested with individual restriction endonucleases and subjected to electrophoresis and Southern-blot analysis using the *CAC2* cDNA insert as a probe. With the exception of the *Eco*RI digest (a site that occurs within the *CAC2* gene), the *CAC2* probe hybridized to a single DNA fragment (Fig. 6C). In very-low-stringency hybridization and wash conditions, additional weakly hybridizing bands were observed; these probably represent genes for other biotin-containing enzymes that show low-sequence similarity with the *CAC2* cDNA. These observations indicate that biotin carboxylase is probably encoded by a single nuclear gene.

An Arabidopsis ecotype Landsberg erecta genomic library (Voytas et al., 1990) was screened by hybridization with the *CAC2* cDNA clone. Four hybridizing genomic clones were isolated. The 6-kb *Sal*I fragment that is common to all of these clones and that hybridizes to the *CAC2* cDNA was subcloned into pBluescript SK(+). Sequencing of this fragment established that it contained the entire *CAC2* gene (4265 bp) and 1296 bp upstream and 490 bp downstream of the coding region (Fig. 8). The *CAC2* gene contained 15 introns, which were between 75 nucleotides (intron 2) and 364 nucleotides (intron 6) in length. The resulting 16 exons ranged from 49 nucleotides (exon 14) to 343 nucleotides (exon 2) in length. The 3′ end of the transcribed region of the gene was at position 5345 (numbered relative to the adenosine of the first ATG codon). The ATP-binding site spanned exons 5 and 6. The sequences at the intron-exon junctions were similar to other plant genes (Brown, 1989; Ghislain et al., 1994); in the *CAC2* gene, the preferred sequences are 5′-exon-GT-intron-AG-exon-3′.

The exact 5′ end of the transcribed region of the *CAC2* gene has not been determined, although it must be at or slightly upstream of −119, the 5′ end of the longest *CAC2*

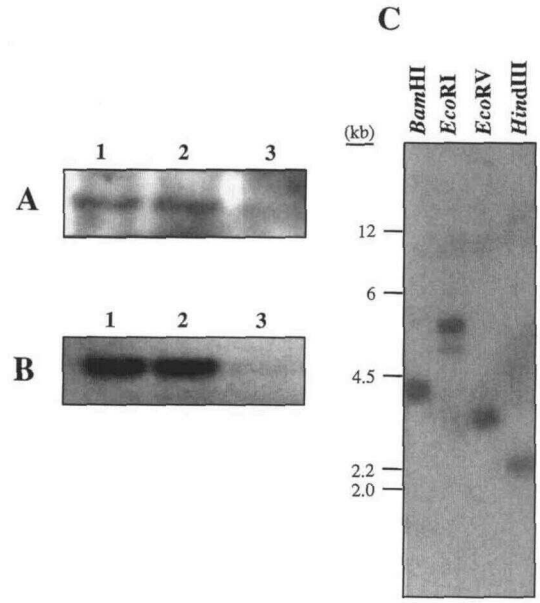

Figure 6. Expression and detection of the *CAC2* gene in Arabidopsis. A, Western-blot analysis of protein extracts isolated from rosette leaves of 16-d-old plants (lane 1), bolting shoots of 45-d-old plants (lane 2), and rosette leaves of 45-d-old plants (lane 3). The biotin carboxylase protein was detected immunologically with anti-GST-CAC2 serum. Each lane contained 50 μg of protein. B, Northern-blot analysis of RNA isolated from rosette leaves of 16-d-old plants (lane 1), bolting shoots of 45-d-old plants (lane 2), and rosette leaves of 45-d-old plants (lane 3). The *CAC2* mRNA was detected by probing the blots with the *CAC2* cDNA. Each lane contained 10 μg of RNA. C, Southern-blot analysis of Arabidopsis DNA digested with *Bam*HI, *Eco*RI, *Eco*RV, and *Hin*dIII and probed with the *CAC2* cDNA.

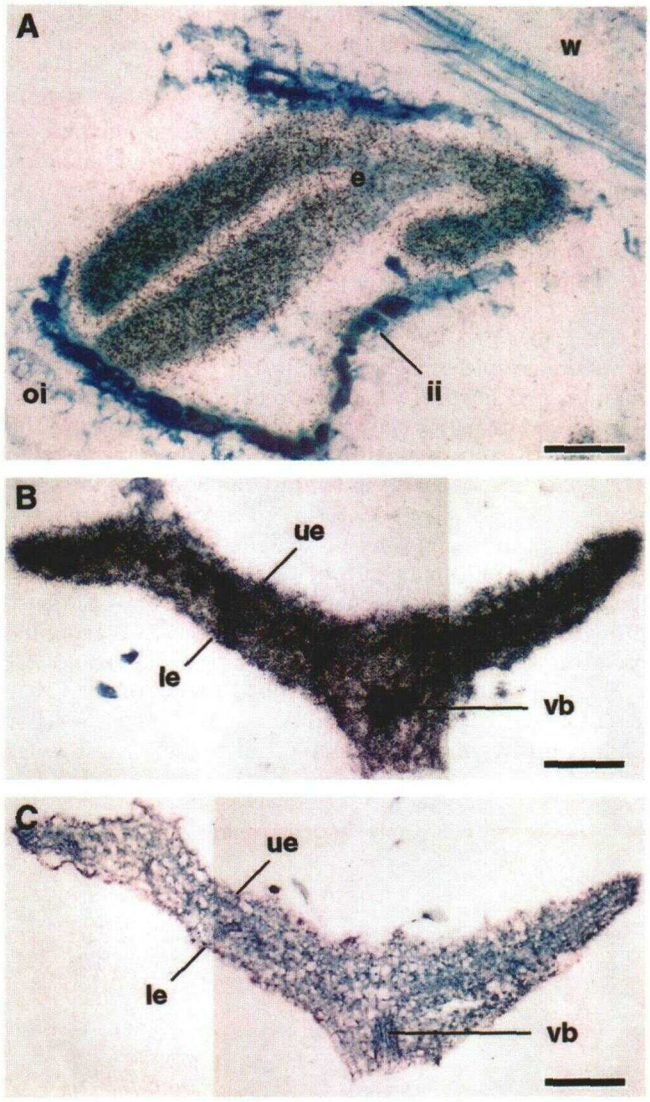

Figure 7. Spatial distribution of the *CAC2* mRNA among tissues of expanding leaves and siliques. In situ hybridization analyses of the *CAC2* mRNA on microscopic sections using ^{35}S-labeled antisense (A and B) and sense (C) RNA probes (see "Materials and Methods"). A, Embryo within a developing silique at 7 DAF. Bar = 43 μm. B, Expanding leaf from a 9-d-old plant. Bar = 135 μm. C, Expanding leaf from a 9-d-old plant (control). Sections were stained with toluidine blue. Bar = 135 μm. w, Silique wall; ii, inner integument of ovule; oi, outer integument of ovule; e, embryo; ue, upper epidermis; le, lower epidermis; and vb, vascular bundle.

cDNA we sequenced. A putative TATA box with the sequence TATAA was located beginning at nucleotide −248. Since TATA boxes usually occur between −40 and −20 of the transcription start site, the 5′ end of exon 1 may be located between −220 and −200. Upstream of the coding region, the *CAC2* gene contained several sequence motifs that have been shown to be important in regulating the transcription of plant genes in response to environmental and developmental signals. These include the I-box and GT-1-box, which have been implicated in light-stimulated transcription of genes (Terzaghi and Cashmore, 1995), the G-box for ABA stimulation (Guiltinan et al., 1990; Salinas et al., 1992), the E-box for seed-specific expression (Kawagoe and Murai, 1992), and the GARE-boxes, which are implicated in GA stimulation of gene expression (Lanahen et al., 1992; Rogers and Rogers, 1992).

Last, the sequence 5′ of the coding region contained extensive nested duplications. The most pronounced is a direct repeat of the segment from −47 to −559, which was repeated with 80% sequence identity between positions −870 and −1410 (repeats R-1 and R-2, Fig. 8). Within these two direct repeats were a number of short sequence repeats: the pentanucleotide GCGTT and the dinucleotide CT, which were found in up to 10 repetitions. In addition, R-2 contained a 45-nucleotide identical tandem duplication beginning at position −1137; a very similar sequence occurred in R-1 beginning at position −224. The functional significance and evolutionary origins of these duplications are unclear.

DISCUSSION

In plants de novo fatty acid biosynthesis from acetate occurs in plastids. In most flowering plants the first committed reaction of this pathway is catalyzed by the heteromeric ACCase. This enzyme has only recently been identified and thus its quaternary structure and regulation is still not fully defined. The enzyme consists of four subunits, three of which are nuclear-encoded and one is plastid-encoded. The characterization of the heteromeric ACCase is complicated by the fact that it undergoes dissociation during isolation. We have taken a molecular biological approach to investigate the structure and regulation of this enzyme. Previously, we reported the cloning and characterization of the cDNA (Choi et al., 1995) and gene (Ke et al., 1997) coding for the BCC subunit of the heteromeric ACCase of Arabidopsis. In this manuscript we report the isolation and characterization of the protein, cDNA, and gene coding for the biotin carboxylase subunit of the Arabidopsis enzyme.

Primary Structure of the Biotin Carboxylase Subunit

The catalytic carboxylation of acetyl-CoA to form malonyl-CoA occurs in two steps: (a) the ATP-dependent carboxylation of the enzyme-bound biotin prosthetic group, and (b) the transfer of the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA. Biotin carboxylase catalyzes the first of these two half-reactions. We determined that the Arabidopsis *CAC2* cDNA codes for biotin carboxylase by three criteria: (a) sequence similarity to biotin carboxylase subunits of heteromeric ACCases from tobacco, cyanobacteria, and eubacteria; (b) immunoinhibition of ACCase activity by antibodies directed against the GST-CAC2 fusion protein; and (c) direct demonstration that the expressed CAC2 protein can catalyze the in vitro carboxylation of biotin.

The primary sequence of biotin carboxylase structural domains of all known biotin-containing enzymes shows high conservation, particularly in four distinct subdomains. The biochemical function of two of these are iden-

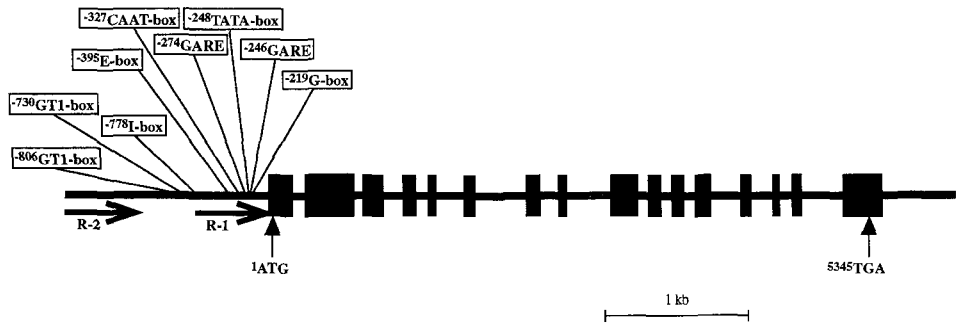

Figure 8. Schematic representation of the structure of the *CAC2* gene of Arabidopsis. The nucleotide sequence of an approximately 6-kb *Sall* genomic fragment containing the *CAC2* gene was determined. Exons are represented by shaded boxes, and noncoding regions (introns and 5′ and 3′ untranscribed segments) are represented by solid lines. Positions of the translational start codon (^{1}ATG), stop codon (^{5345}TGA), and sequence motifs upstream of the first exon, which may be important in the regulation of the transcription of the *CAC2* gene, are indicated (Gasser and Laemli, 1986; Guiltinan et al., 1990; Kawagoe and Murai, 1992; Terzaghi and Cashmore, 1995). The major direct duplications (R-1 and R-2) in the promoter are indicated by bold arrows. Nucleotides are numbered relative to the translational start codon and are shown as superscripts. The sequence of the *CAC2* gene has been deposited in GenBank (accession no. U91414).

tifiable via sequence comparisons, the ATP-binding site, and a domain that is similar to carbamoyl-phosphate synthetase (Toh et al., 1993). This latter domain is probably involved in the synthesis of the product of biotin carboxylase, carboxy-biotin, a reaction mechanistically related to the synthesis of carbamoyl phosphate. The biochemical functions of the other two domains (BC-1 and BC-2) are unclear.

These sequence comparisons reveal the potential evolutionary origin of the higher plant biotin carboxylase. Figure 9 presents an unrooted phylogenetic tree showing the relationship between biotin carboxylase structural domains of biotin-containing enzymes. The higher plant biotin carboxylases occur in a clade that contains all other known heteromeric ACCases (cyanobacterial and eubacterial). In addition, this clade contains an archaeal biotin carboxylase (from *Methanococcus jannaschii*). The biochemical function of this latter biotin carboxylase is as yet unknown; it is unlikely to be a subunit of a heteromeric ACCase, because there is no evidence that archaea synthesize fatty acids or have genes that code for the other subunits of an ACCase. This clade, which contains the biotin carboxylase of heteromeric ACCases, is most distant from the clade that contains the biotin carboxylase domain of homomeric ACCases, which are present in the cytosol of plants and other eukaryotes. We noted a similar phylogenetic relationship between the BCC domains of the homomeric and heteromeric ACCases (Choi et al., 1995). Hence, sequence comparisons of both BCC and biotin carboxylase domains indicate that the homomeric and heteromeric ACCases have distinct evolutionary origins.

These sequence comparisons group the biotin carboxylase domains into clades that have similar domain organizations. The CAC2-containing clade has enzymes in which the biotin carboxylase domain occurs as a separate subunit. In other clades the biotin carboxylase domain is fused with the BCC domain or with the BCC plus carboxyltransferase domains. Because similar domain organizations occur in distant clades, we suggest that fusions of structural domains have occurred repeatedly during the evolution of biotin-containing enzymes.

Biochemical Characterization of Biotin Carboxylase

The biochemical properties of the Arabidopsis biotin carboxylase were determined with the recombinant enzyme produced by expressing the *CAC2* cDNA in *E. coli*. During affinity purification the expressed protein was denatured and subsequently renatured by dialysis. This renatured protein was catalytically active in carboxylating free biotin. Hence, as with the *E. coli* enzyme (Guchhait et al., 1974), the plant biotin carboxylase can catalyze the first half of the ACCase reaction independently of the other subunits of the enzyme (i.e. the BCC and carboxyltransferase subunits). This finding contrasts with the pea leaf ACCase, in which biotin carboxylase activity was lost upon separation from the BCC subunit (Alban et al., 1995). One explanation for this difference is that the biotin carboxylase subunit in the preparations of Alban et al. (1995) was proteolytically degraded during isolation; they reported a biotin carboxylase subunit of about 32 kD, whereas the molecular mass of this subunit is predicted to be 51 kD.

Alban et al. (1995) characterized the catalytic properties of the biotin carboxylase reaction using a preparation from pea that contained both the BCC subunit and a degraded biotin carboxylase subunit in a complex. We have characterized the catalytic properties of the expressed recombinant biotin carboxylase of Arabidopsis. The apparent K_m values we obtained for two of the substrates of the enzyme, bicarbonate and biotin, are similar to those obtained with both the *E. coli* biotin carboxylase (Guchhait et al., 1974) and the pea biotin carboxylase-BCC complex (Alban et al., 1995). The K_m values we obtained are similar to those obtained by Alban et al. (1995), indicating that binding of bicarbonate and biotin to the biotin carboxylase subunit is not affected by the presence of the BCC subunit.

Additional characterizations of the recombinant biotin carboxylase indicate that, as with most other biotin-

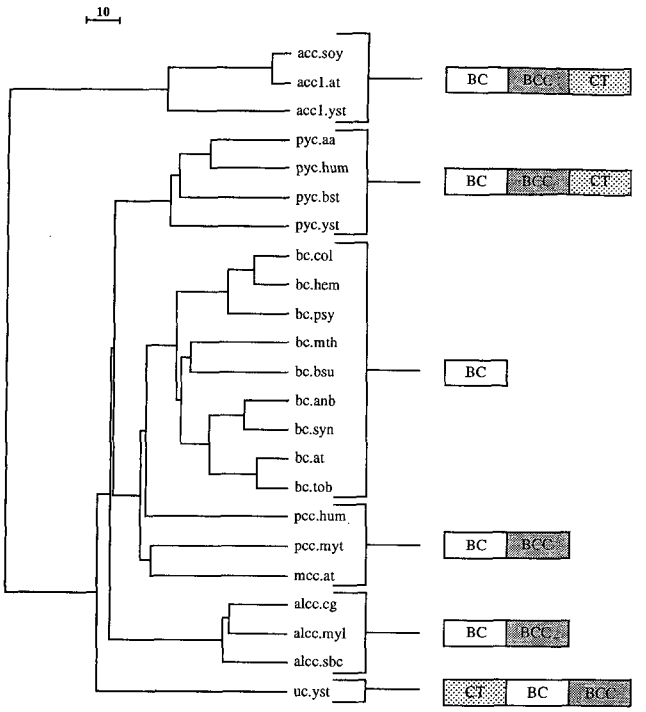

Figure 9. Phylogenetic and structural relationships between biotin carboxylase structural domains. The single most parsimonious unrooted tree obtained with the DISTANCES and GROWTREE functions of the Genetics Computer Group sequence analysis package is shown. In this phylogram sequences with the least divergence have the shortest branches. Distances are estimated as the number of substitutions per 100 amino acid residues. On the right is a schematic representation of the domain organization of the polypeptide that contains the biotin carboxylase structural domain: biotin carboxylase (BC), BCC, and carboxyltransferase (CT). Biotin carboxylase structural domains are compared from: biotin carboxylases from *E. coli* (bc.col; Kondo et al., 1991; Li and Cronan, 1992a), *Hemophilus influenzae* (bc.hem; Fleischmann et al., 1995), *Pseudomonas aeruginosa* (bc.psy; Best and Knauf, 1993), *M. jannaschii* (bc.mth; Bult et al., 1996), *Bacillus subtilis* (bc.bsu; Marini et al., 1995), *Anabaena* (bc-anb; Gornicki et al., 1993), *Synechococcus* PCC7942 (bc.syn; GenBank accession no. U59234), Arabidopsis (bc.at; this paper), tobacco (bc.tob; Shorrosh et al., 1995); residues 1 to 600 of the homomeric ACCases from soybean (acc.soy; Anderson et al., 1995), Arabidopsis (acc.at; Roesler et al., 1994; Yanai et al., 1995), and yeast ACCase (acc.yst; Walid et al., 1992; Hasslacher et al., 1993); residues 1 to 550 of pyruvate carboxylases from *Aedes aegypti* (pyc.aa; GenBank accession no. L36530), humans (pyr.hum; Wexler et al., 1994), and *Bacillus stearothermophilus* (pyc.bst; GenBank accession no. D83706); residues 1 to 500 of the α-subunit of propionyl-CoA carboxylases from humans (pcc.hum; Lamhonwah et al., 1986) and *Mycobacterium tuberculosis* (pcc.myt; Norman et al., 1994); residues 1 to 500 of methylcrotonyl-CoA carboxylase from Arabidopsis (mcc.a; Weaver et al., 1995); residues 1 to 500 of acyl-CoA carboxylases from *Corynebacterium glutamicum* (alcc.cg; Jager et al., 1996), *M. leprae* (alcc.myl; Norman et al., 1994), and *Saccharopolyspora erythraea* (alcc.sbc; Donadio et al., 1996); and residues 551 to 1100 of yeast urea carboxylase (uc.yst; Genbauffe and Cooper, 1991).

containing enzymes (Diez et al., 1994), biotin carboxylase has an absolute requirement for Mg^{2+}, using Mg-ATP as a substrate. However, in marked contrast to the pea biotin carboxylase-BCC complex, which was inhibited by free ATP (Alban et al., 1995), the Arabidopsis biotin carboxylase was inhibited by free Mg^{2+}. This difference may be due to the fact that the pea enzyme preparation was a complex of biotin carboxylase and BCC and/or that the pea preparation had a highly degraded biotin carboxylase subunit. Biotin carboxylase activity of Arabidopsis and pea (Alban et al., 1995) was markedly affected by pH, with optimum activity occurring at pH 8.6 and undetectable activity at pH 7.0.

The catalytic properties of biotin carboxylase may provide a mechanistic explanation for the light dependency of fatty acid biosynthesis in chloroplasts. Changes in the chloroplastic acetyl-CoA and malonyl-CoA pool sizes during light/dark transitions are consistent with fatty acid biosynthesis being regulated by modulations in ACCase activity (Post-Beittenmilleret al., 1992). The stromal pH changes from 8.2 to 7.0 in the transition from light to dark. Our kinetic characterizations indicate that such a decrease in pH would cause a dramatic decrease in biotin carboxylase activity. Hence, these studies of biotin carboxylase reinforce and extend an earlier suggestion (Nikolau and Hawke, 1984) that changes in stromal pH would cause alterations in ACCase activity (mediated by changes in biotin carboxylase), which makes de novo fatty acid biosynthesis light dependent.

In addition, light/dark-induced changes in the stromal concentration of ATP, ADP, and Mg^{2+} have been suggested to affect ACCase activity (Eastwell and Stumpf, 1983; Nikolau and Hawke, 1984). Although in vitro biotin carboxylase activity is modulated by changes in ATP and Mg^{2+} concentrations (Fig. 5), these effects occur in the range of 0.1 to 1 mM, below the range of the stromal concentrations of these effectors (1–3 mM). However, recent findings indicate that the chloroplastic ACCase along with acetyl-CoA synthetase and the fatty acid synthase enzymes may be in a multienzyme complex (Roughan and Ohlrogge, 1996). This complex appears to sequester and recycle a small pool of ATP/ADP nucleotides it requires for catalysis. Hence, the actual concentration of ATP and ADP at the active site of biotin carboxylase is unknown. Therefore, the effect of changing ATP and Mg^{2+} concentrations (in the range of 0.1–1 mM) on biotin carboxylase activity may be physiologically significant in controlling ACCase activity and hence fatty acid synthesis.

Expression of the *CAC2* Gene in Arabidopsis

The pattern of *CAC2* gene expression mirrors that previously observed for the *CAC1* gene encoding the BCC subunit of the heteromeric ACCase (Choi et al., 1995; Ke et al., 1997). Furthermore, accumulation of the *CAC1* and *CAC2* gene products is maximal in cells that are actively synthesizing fatty acids. These tissues include young expanding rosette leaves, bolting shoots, and the embryos within 7-DAF siliques. The former two are each actively synthesizing fatty acids for the deposition of membrane lipids needed for growth, and the embryos are rapidly depositing seed oils. In contrast, *CAC2* and *CAC1* mRNAs were barely detectable in mature rosette leaves and nonembryonic tissues of 7-DAF siliques, which are not undergoing growth or oil deposition. These data indicate that developmentally

induced changes in the activity of the heteromeric ACCase are at least partially controlled by mechanisms that coordinately regulate the accumulation of individual subunit mRNAs.

Structure of the *CAC2* Gene

Southern-blot analyses of Arabidopsis DNA probed with the *CAC2* cDNA indicate that the biotin carboxylase subunit is encoded by a single-copy gene. This conclusion was substantiated by the fact that screening of 15 genomic equivalents of an Arabidopsis genomic library resulted in the isolation of four overlapping genomic clones that contain the *CAC2* gene. Comparison of the sequence of a 6-kb *SalI* fragment that contains the entire *CAC2* gene with the *CAC2* cDNA identified the 16 exons that constitute the *CAC2*-coding region. The nucleotide sequence of these exons correspond to the nucleotide sequence of the *CAC2* cDNA, with the exception of a 2-bp deletion in the 5′ untranslated region and G to T and T to G substitutions at positions 1549 and 1588 of the cDNA sequence. All of these differences are silent and do not affect the biotin carboxylase amino acid sequences. These differences represent polymorphisms between the Columbia (source of the *CAC2* cDNA clone) and Landsberg erecta (source of the *CAC2* gene clone) ecotype genomes.

The *CAC2* promoter contains sequence motifs that have been shown to be significant in the transcriptional regulation of other genes. Four of these motifs are also present in the promoter of the *CAC1* gene that codes for the BCC subunit of the heteromeric ACCase: the GT-1-box, the I-box, and the G-box, which are important in light-regulated transcription (Terzaghi and Cashmore, 1995), and the E-box, which along with the G-box is important in specifying high rates of seed-specific transcription (Guiltinan et al., 1990; Kawagoe and Murai, 1992). Although the function of these motifs need to be experimentally confirmed, the fact that the promoters of genes coding for two ACCase subunits have a common set of putative regulatory motifs suggests that these motifs may have a functional role in controlling and potentially coordinating the transcription of these two genes. Indeed, coordination of the genes coding for the ACCase subunits would enable the organism to conservatively produce the appropriate stoichiometry of each of the ACCase subunits. The evidence we have to date is consistent with such coordination being at least partially controlled at the level of gene transcription.

ACKNOWLEDGMENT

We thank the Arabidopsis Biological Research Center for the genomic and cDNA libraries of Arabidopsis and the EST clone 150M20T7.

Received April 16, 1997; accepted August 25, 1997.

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