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Structural Analysis, Plastid Localization, and Expression of the Biotin Carboxylase Subunit of Acetyl-Coenzyme A Carboxylase from Tobacco[1]

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Acetyl-coenzyme A carboxylase (ACCase, EC 6.4.1.2) catalyzes the synthesis of malonyl-coenzyme A, which is utilized in the plastid for de novo fatty acid synthesis and outside the plastid for a variety of reactions, including the synthesis of very long chain fatty acids and flavonoids. Recent evidence for both multifunctional and multisubunit ACCase isozymes in dicot plants has been obtained. We describe here the isolation of a tobacco (*Nicotiana tabacum* L. cv bright yellow 2 [NT1]) cDNA clone (E3) that encodes a 58.4-kD protein that shares 80% sequence similarity and 65% identity with the *Anabaena* biotin carboxylase subunit of ACCase. Similar to other biotin carboxylase subunits of acetyl-CoA carboxylase, the E3-encoded protein contains a putative ATP-binding motif but lacks a biotin-binding site (methionine-lysine-methionine or methionine-lysine-leucine). The deduced protein sequence contains a putative transit peptide whose function was confirmed by its ability to direct in vitro chloroplast uptake. The subcellular localization of this biotin carboxylase has also been confirmed to be plastidial by western blot analysis of pea (*Pisum sativum*), alfalfa (*Medicago sativa* L.), and castor (*Ricinus communis* L.) plastid preparations. Northern blot analysis indicates that the plastid biotin carboxylase transcripts are expressed at severalfold higher levels in castor seeds than in leaves.

ACCase is a biotinylated enzyme that catalyzes the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate. Malonyl-CoA is an essential substrate for fatty acid biosynthesis in the plastids (Harwood, 1988) and chain elongation in the cytosol (Pollard and Stumpf, 1980). In addition, malonyl-CoA is required in the cytosol for several reactions, including the biosynthesis of flavonoids (Ebel and Hahlbrock, 1977; Ebel et al., 1984). Regulation of the activity of the plastid ACCase is a major determinant of the flux of carbon into fatty acid synthesis in both spinach (Post-Beittenmiller et al., 1991, 1992) and barley and maize leaves (Page et al., 1994).

Two forms of ACCase, termed "prokaryotic" and "eukaryotic," have been isolated and characterized. The prokaryotic form is an MS enzyme composed of dissociable subunits. For example, *Escherichia coli* ACCase is composed of four subunits: the BC, BCCP, and CT α and β subunits (Alix et al., 1989; Kondo et al., 1991; Li and Cronan, 1992a, 1992b). The eukaryotic form of ACCase is an MF polypeptide containing the BC, BCCP, and CT functional domains with a molecular mass of more than 200 kD. The full-length MF-ACCase has been cloned from mammals (Lopez-Casillas et al., 1988; Takai et al., 1988; Ha et al., 1994), yeast (Al-Feel et al., 1992), algae (Roessler and Ohlrogge, 1993), and plants (Gornicki et al., 1994; Roesler et al., 1994; Shorrosh et al., 1994). Also, partial MF-ACCase sequences have been reported from several plants (Ashton et al., 1994; Elborough et al., 1994). To date, all MF-ACCase sequences reported from plants show high sequence identity in their encoded amino acid sequences. Thus, to date, there is no sequence evidence for differences, if any, between plastidial and cytosolic forms of MF-ACCase. Preliminary evidence suggested that the alfalfa (*Medicago sativa*) and Arabidopsis MF-ACCases are localized in the cytosol (Roesler et al., 1994; Shorrosh et al., 1994). However, two isoforms of the MF-ACCase have been characterized from maize based on their chloroplastic and extrachloroplastic localization and their herbicide sensitivity (Egli et al., 1993). Recently, it has been shown that MF-ACCase isolated from young pea (*Pisum sativum*) leaves is localized in the epidermal tissues (Alban et al., 1994). In addition, western blot analysis using biotin antibodies detected MF-ACCase in the total protein extract from both Gramineae and dicot plants, but the MF-ACCase was present only in the chloroplasts of Gramineae plants (Wurtele and Nikolau, 1992; Baldet et al., 1993; Egli et al., 1993; Gornicki and Haselkorn, 1993; Konishi and Sasaki, 1994). Recently, immunological data describing the expression of a pea chloroplast-encoded protein, which shares sequence similarity with the *E. coli* carboxyltransferase β subunit (*acc*D) of ACCase was reported (Sasaki et al., 1993). Antibodies against the pea

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Abbreviations: ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CT, carboxyltransferase; MF, multifunctional; MS, multisubunit.

*acc*D-like protein precipitated the activity of pea chloroplast ACCase with the concomitant precipitation of three polypeptides, including a 35-kD biotin-containing protein (Sasaki et al., 1993). Also, an MS-ACCase enzyme consisting of dissociable subunits with molecular masses ranging from 32 to 79 kD has been partially purified and characterized from the epidermal and mesophyll tissues of pea leaves. One of these subunits, with a molecular mass of 38 kD, was biotinylated (Alban et al., 1994). Western blot analysis of pea chloroplasts localized only one biotin-containing protein with an apparent molecular mass of 35 to 38 kD (Baldet et al., 1993; Sasaki et al., 1993); whereas others have reported multiple biotin-containing proteins in dicot chloroplasts (Wurtele and Nikolau, 1992; Gornicki et al., 1993).

Taken together, the reported data suggest that dicot plastids contain MS-ACCase; however, the number of subunits and their organization is not yet understood. Here we present further evidence for an MS-ACCase in plants. We report the isolation and characterization of a full-length tobacco (*Nicotiana tabacum* L. cv bright yellow 2) cDNA that encodes a BC subunit of MS-ACCase. Also, we present its genomic organization, expression, and evidence for its chloroplastic localization and function.

MATERIALS AND METHODS

Screening Tobacco cDNA Library

A partial castor (*Ricinus communis* L.) cDNA clone (pCRS706) that encodes an amino acid sequence with 75% similarity to and 57% identity with the BC subunit of *Anabaena* ACCase was identified by partially sequencing cDNA clones selected from a developing castor seed cDNA library by differential screening (van de Loo et al., 1995). The coding region of the castor cDNA was amplified by PCR using synthetic primers and then used as a probe to screen a tobacco cDNA library by the plaque hybridization method using GeneScreenPlus (New England Nuclear) membranes as described by Shorrosh and Dixon (1991). The cDNA library was prepared in λZAPII (Stratagene) from poly(A)$^+$ RNA isolated from 3-d-old NT1 tobacco (*Nicotiana tabacum* L. cv bright yellow 2) cells. Prehybridization was at 42°C for 4 h in solution A (5× SSC, 1% [w/v] SDS, 5× Denhardt's solution, 30% [w/v] formamide, 100 μg/mL salmon sperm DNA, and 0.1 M KPO_4, pH 6.8). Hybridization was at 42°C overnight in solution A containing denatured, labeled probe and 10% (w/v) dextran sulfate. Blots were washed twice in 2× SSC, 1% SDS at room temperature and at 42°C prior to autoradiography.

Plasmid Isolation and Sequencing

The pBluescript SK(−) vector containing the tobacco cDNA insert was excised in vivo from the λZAPII using the helper phage R408 as described by the manufacturer (Stratagene). The rescued pBluescript phagemid containing the cloned insert was grown in *Escherichia coli* XL1-Blue cells. Plasmids were prepared as described by Holmes and Qigley (1981), and the cDNA insert was sequenced on both strands using universal and synthetic primers and AmpliTaq DNA polymerase (Boehringer Mannheim) with an Applied Biosystems robotic catalyst and 373A DNA sequencer. Sequence alignments were determined by using the GAP program of the Genetics Computer Group (University of Wisconsin, Madison).

Genomic DNA Isolation and Southern Blot Analysis

High mol wt genomic DNA was isolated from the leaves of Arabidopsis, castor, tobacco, corn, rice, and wheat as described by Maniatis et al. (1982). The DNA (10 μg) was digested with *Eco*RV, *Hin*dIII, *Xba*I, or *Xho*I restriction endonucleases, resolved in a 0.7% (w/v) agarose gel, blotted to a GeneScreenPlus nylon membrane, and hybridized at 55°C in a solution containing denatured, labeled probe generated by PCR using E3 cDNA clone primers JO288 and JO280 (Fig. 1), 1% (w/v) SDS, 50 mM Tris, pH 7.6, 5× Denhardt's solution, 2.5 mM EDTA, 5× SSC, and 100 μg/mL denatured salmon sperm DNA. Prior to autoradiography, blots were washed at 55°C for 20 min in 2× SSC, 0.2% SDS; 1× SSC, 0.2% SDS; and 0.5× SSC, 0.2% SDS.

RNA Isolation and Northern Blot Analysis

Poly(A)$^+$ RNA was prepared from castor leaves and developing seeds using oligo(dT) cellulose as described by Maniatis et al. (1982). mRNA (3 μg) was resolved in 1% agarose gel containing 2.2 M formaldehyde, blotted onto nylon membrane (Hybond N, Amersham), and fixed to the filter by exposure to UV light for 2 min. The blot was prehybridized at 42°C for 4 h in a solution containing 5× SSC, 10× Denhardt's solution, 0.1% SDS, 0.1 M KPO_4, pH 6.8, and 100 μg/mL denatured salmon sperm DNA. Subsequently, the blot was hybridized at 42°C overnight in a solution containing denatured, labeled probe, 5× SSC, 10× Denhardt's solution, 0.1 M KPO_4, pH 6.8, 100 μg/mL denatured salmon sperm DNA, 10% dextran sulfate, and 30% formamide. The blot was washed twice in 2× SSC, 0.5% SDS at room temperature and at 50°C prior to autoradiography.

In Vitro Transcription and Translation

Based on the primary amino acid sequence of E3 (Fig. 1) and its alignment with *Anabaena* BC (Fig. 2), we suspected that the E3 cDNA encodes a chloroplastic protein of which the first 68 amino acids represent a transit peptide (see "Results"). As a negative control for chloroplast uptake experiments, the cDNA sequence of the E3 clone that encodes the suspected mature E3 protein but lacks the first 68 amino acids and has the Cys residue at position 69 replaced with Met (Fig. 1) was amplified by PCR using primers JO288 (contains engineered *Sac*I and *Nde*I sites) and JO280 (contains engineered *Xba*I site) (Fig. 1). The amplified sequence was designated "-TE3" and was subcloned into the *Sac*I/*Xba*I sites of pBluescript II KS(+). Both E3 and -TE3 clones were digested with *Avr*II (Fig. 1). Also, as a positive control, plasmid pCALACP (Savage and Post-Beittenmiller, 1994) containing the spinach ACP-I (spinach ACP isoform I) was sequentially digested with *Eco*RI and

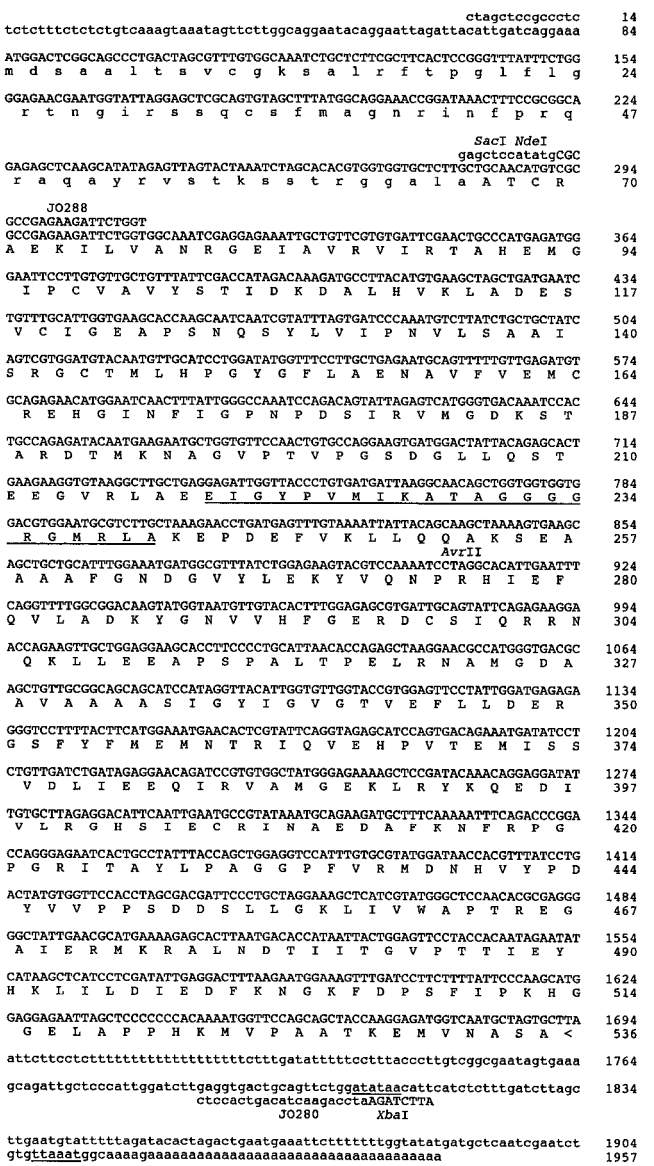

Figure 1. The nucleotide sequence and the deduced amino acid sequence of the tobacco E3 cDNA clone. The 5′ and 3′ untranslated nucleotide regions are indicated by lowercase letters. The location of primers JO288 and JO280 used to prepare probes and subclones are indicated. The *Avr*II restriction site used to generate truncated transcripts is indicated. The peptide in lowercase letters is rich with Ser, Thr, Arg, and hydrophobic amino acids, does not align with *Anabaena* BC (Fig. 2), and is proposed to be part of a chloroplast transit peptide. The putative polyadenylation signal in the nucleotide sequence and a putative ATP-binding site in the amino acid sequence are underlined.

*Bam*HI. Subsequently, capped RNA transcripts were synthesized using 5 μg of the linearized clones and the MAXIscript in vitro transcription kit (Ambion, Austin, TX) as described by the manufacturer but with undiluted GTP (10 mM stock) and 40 units of both RNAsin and RNA polymerases (Boehringer). After 1 h, an additional 40 units of the polymerases were added, and the reaction was incubated for a further 1 h at 37°C. Linearized E3 and ACP-I cDNAs were transcribed with T3 RNA polymerase and linearized -TE3 cDNA was transcribed with T7 RNA polymerase. The DNA template was digested with 20 units of DNase/RNase-Free (Boehringer), and the RNA was precipitated sequentially at −20°C in 3.75 M ammonium acetate, 67% (v/v) ethanol, and then in 56 mM Hepes-KOH, pH 7.0/67% (v/v) ethanol. The final RNA pellet was resuspended in 50 μL of diethyl pyrocarbonate-treated water, and 3 μL were checked for degradation on a 6% polyacrylamide urea sequencing gel. The RNA (3 μL) was diluted with 6 μL of water, heated for 10 min at 70°C, stored on ice, and translated in rabbit reticulocyte lysate (Promega) in a total volume of 50 μL at 30°C for 2 h using L-[^{35}S]Met (1200 Ci mmol^{-1}) (New England Nuclear) and 40 units of RNAsin (Boehringer).

Isolation of Plastids from Leaves and Seeds

Chloroplasts were isolated from pea (*Pisum sativum* cv little marvel [Burpee]) shoots and alfalfa (*Medicago sativa* L. cv Apollo) shoots (grown hydroponically) by using a Percoll gradient as described by Cline et al. (1985). Chloroplasts were resuspended in 1 mL of import buffer (330 mM sorbitol, 50 mM Hepes-KOH, pH 7.8). Resuspended pea chloroplasts (500 μL) were fractionated into soluble and insoluble fractions by freeze-thawing in liquid nitrogen

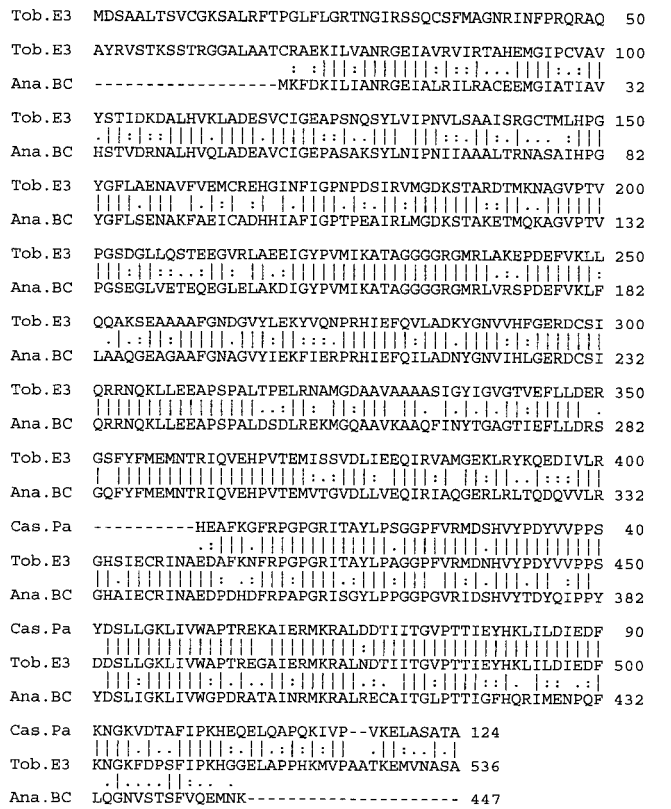

Figure 2. Amino acid sequence alignment of tobacco E3 (Tob.E3) with *Anabaena* BC (Ana.BC), and a partial castor cDNA-encoded protein (Cas.Pa). Identical and conserved amino acid substitutions are indicated by "|" and "." or ":", respectively.

three times (with vortexing) and then centrifuging at 14,000*g*. The pellet was resuspended in 500 μL of import buffer. Plastids were isolated from castor endosperm as described by Miernyk (1985). The final pellet was resuspended in 30 mL of import buffer and then pelleted through 10 and 20% (v/v) Percoll cushions as described by Borchert et al. (1989). Protein content was determined by the Bradford assay (Bradford, 1976) using BSA as a standard.

Chloroplast Uptake Reactions

Pea chloroplasts were isolated from 11- to 13-d-old pea shoots using a Percoll gradient as described by Cline et al. (1985). The import reaction, 200 μL total volume, was carried out at 27°C for 30 min and contained 10 mM Met, 3 mM Mg-ATP, 40 μL of translation reaction (see above), 0.5 μg/μL Chl, and import buffer (350 mM sorbitol, 50 mM Hepes-KOH, pH 7.8). The reaction was terminated with 0.2 mg/mL thermolysin for 30 min on ice. Proteolysis was stopped with 10 mM EDTA, and the intact chloroplasts were recovered by centrifugation in an Eppendorf tube at 4,200*g* for 6 min through 1 mL of a 40% (v/v) Percoll cushion made in import buffer and 5 mM EDTA. The pellet was resuspended in 1 mL of import buffer, 5 mM EDTA and pelleted at 3,000*g* for 3 min. The pellet was resuspended in 80 μL of 1 mM $MgCl_2$, 10 mM Hepes-KOH, pH 7.8, freeze-thawed in liquid nitrogen three times, and centrifuged at 15,000*g* for 15 min. The supernatant was boiled in SDS sample buffer and separated in 15% (w/v) SDS-PAGE. The gel was fixed in 10% (v/v) acetic acid, 40% (v/v) ethanol, vacuum dried, and exposed to x-ray film (Kodak XAR) for 3 to 6 d.

Production of Antiserum

The cDNA region of the castor pCRS706 clone encoding 124 amino acids (Fig. 2) was amplified by PCR to include *Nde*I (encodes the initiator Met) and *Bam*HI sites at the 5′ and the 3′ ends of the amplified cDNA. The amplified cDNA was subcloned into the *Nde*I/*Bam*HI sites of the pET15b expression vector and then used to transform BL21 (DE3) cells (Novagen, Madison, WI). Subsequently, the castor E3-like protein was induced in BL21 (DE3) cells with 1 mM isopropyl-β-D-thiogalacto-pyranoside dioxane free and then purified by the pET His-Tag system (Novagen). The purified protein was dialyzed against PBS (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) buffer, and antiserum was obtained by immunizing a female New Zealand White rabbit.

Immunoblot Analysis

For immunoblot analysis, proteins were extracted as follows. Alfalfa leaf proteins were extracted in solution B (100 mM Mes, pH 6.5, 2 mM DTT, 0.4 mM PMSF, and 5 μg/mL antipain, pepstatin, leupeptin, ε-aminocaproic acid, and aprotinin). Percoll gradient-purified alfalfa and pea plastids were resuspended in import buffer (330 mM sorbitol, 50 mM Hepes-KOH, pH 7.8). Castor plastids, pelleted through 10 and 20% (v/v) Percoll cushions, were resuspended in 50 mM Tris, pH 8.0, 150 mM NaCl. Castor endosperm proteins were extracted in 50 mM Tris, pH 6.5, 1 mM EDTA. Protein solutions were mixed with SDS sample buffer, resolved by SDS-PAGE as described by Laemmli (1970), and then transferred to nitrocellulose, using the Pharmacia Novablot system, in 25 mM Tris, pH 8.3, 192 mM Gly, 20% (v/v) methanol. Transferred protein was reversibly stained with Ponceau S and blocked in Tris-buffered saline (10 mM Tris, 0.9% [w/v] NaCl) containing 2.5% (w/v) BSA. Blotted protein was detected with either preimmune or immune serum (1:500) in Tris-buffered saline containing 0.05% (v/v) Tween 20 and 1% (w/v) BSA and visualized with alkaline phosphatase-conjugated anti-(rabbit IgG).

RESULTS

Library Screening and Sequence Analysis

cDNA clones were selected from a developing castor seed cDNA library by differential screening with developing-seed and leaf total cDNA probes. Clones expressed at low levels in leaves and low or moderate levels in developing seeds were partially sequenced (van de Loo et al., 1995). cDNA clone pCRS706 was found to encode a protein with 75% sequence similarity and 57% identity with the *Anabaena* BC subunit of ACCase (Gornicki et al., 1993). To obtain a full-length tobacco cDNA clone, a ^{32}P-labeled coding region of the castor cDNA was used as a probe to screen a tobacco cDNA library constructed from mRNA isolated from 3-d-old NT1 tobacco cells. Of 200,000 plaques screened, 7 positives were identified, and the cDNA insert was recovered in pBluescript SK(−). All positive cDNAs were partially sequenced at their 5′ and 3′ ends and were found to be identical.

The longest cDNA insert (1957 bp), in clone E3, was subjected to full-length sequence analysis in both strands. The 5′ untranslated region (84 bp) contains a stop codon in frame with the initiator Met. The 3′ untranslated region (265 bp) contains two putative polyadenylation signals, ATATAA and TTAAAT, starting at positions 1107 and 1908, respectively. Early polyadenylation after position 1894 (Fig. 1) was identified in some clones. E3 encodes an open reading frame of 536 amino acids with a calculated mol wt of 58,394 and a pI of 6.69. The first 68 amino acids of the E3-encoded protein do not align with *Anabaena* BC (Fig. 2) and are rich in Ser, Thr, Arg, and hydrophobic amino acids, which is characteristic of chloroplast transit peptides. The E3-encoded protein shares high sequence similarity with prokaryotic BCs and other carboxylases (Fig. 1). The E3-encoded protein contains a putative ATP-binding motif (amino acid residues 219–240; Fig. 1), similar to that of other carboxylases. As observed for other prokaryotic BC subunits of ACCase, the E3-encoded protein lacks a biotin-binding motif (Met-Lys-Met or Met-Lys-Leu) (Fig. 1), which, however, is present in other carboxylases not associated with ACCase.

Chloroplast Uptake Experiment

To confirm that the N-terminal region of the E3 protein could serve as a chloroplast-targeting sequence, chloroplast uptake experiments were performed. Three in vitro translated proteins (E3–274, -TE3–274, and ACP-I) were tested for uptake by pea or spinach chloroplasts. E3–274 (29 kD) is a truncated E3 protein encoding the first 274 amino acids. As a negative control, -TE3–274 (22 kD) was constructed as a further truncated E3–274 protein that lacks the 68-amino acid putative transit peptide and replaces the Cys residue at position 69 with an initiator Met. We used a truncated form of E3 protein for chloroplast import for the following two reasons: (a) The translated full-length E3 protein (about 58 kD) and the suspected mature -TE3 protein (E3 protein lacking the first 68 amino acids; about 51 kD) were obscured in SDS-PAGE by the high levels of Rubisco protein that ran at 50 to 55 kD on SDS-PAGE. (b) Some endogenous chloroplast protein was radiolabeled in the uptake experiment even in the presence of excess unlabeled Met (see "Materials and Methods"). These radiolabeled chloroplast proteins had similar mobility to the suspected mature -TE3 protein and thus complicated interpretation of the data. Use of truncated forms of E3 protein (E3–274 and -TE3–274) simplified data interpretation because their mobility was not obscured by Rubisco or the endogenously radiolabeled chloroplast proteins. Also, because E3–274 and -TE3–274 transcripts were smaller than E3, the efficiency of in vitro transcription and translation increased. Spinach ACP-I, whose uptake into chloroplasts has been well characterized, was used as a positive control for the uptake experiments.

The apparent molecular masses of E3–274-, -TE3–274-, and ACP-I-translated proteins by SDS-PAGE analysis are 30, 23, and 20 kD, respectively (Fig. 3, lanes 1, 3, and 5). The presence of a second translation product for -TE3–274, with an apparent molecular mass of 19 kD, is probably due to initiation of translation at the next Met residue at position 93 (Fig. 1). The import of E3–274- and ACP-I-translated proteins into pea or spinach chloroplasts was verified by the presence of proteins with apparent molecular masses of 22 and 14 kD, respectively (Fig. 3, lanes 2 and 6). In contrast, -TE3–274-translated protein was not taken up by pea or spinach chloroplasts, as indicated by the absence of radiolabeled protein from the isolated chloroplasts (Fig. 3, lane 4). The processed E3–274 protein was slightly smaller than the -TE3–274 protein, suggesting that E3 protein may contain a transit peptide that is longer than the first 68 amino acids in E3 protein.

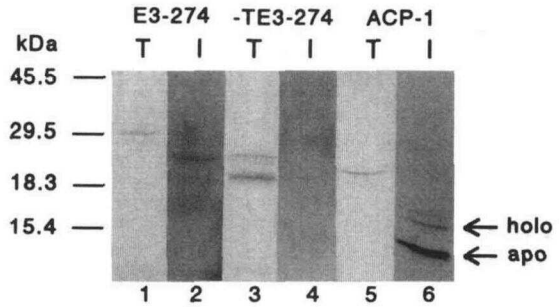

Figure 3. Chloroplast uptake experiment. Plasmids were linearized with the appropriate restriction endonucleases and in vitro transcribed and translated. The [^{35}S]Met-labeled protein was imported into pea chloroplasts. The import reaction was terminated with thermolysin treatment. The chloroplasts were re-isolated, and the imported protein was resolved in 12.5% (w/v) SDS-PAGE and autoradiographed for 3 d. In vitro translated E3–274 (truncated tobacco E3 protein encoding the first 274 amino acids), -TE3–274 (E3–274 protein lacking the first 68 amino acids), and ACP-I (spinach ACP isoform I) proteins were loaded in the specified "T" lanes. Imported E3–274, -TE3–274, and ACP-I proteins were loaded in the specified "I" lanes. Molecular mass markers are indicated in kD. holo, holo ACP-1; apo, apo ACP-1.

Immunoblot Analysis and Subcellular Localization of E3 Protein in Different Plants

Antibodies were raised to the 124 amino acids encoded by the C-terminal region of the putative BC cDNA from castor (Fig. 2). Proteins isolated from alfalfa, pea, and castor were analyzed by immunoblots using preimmune and immune sera (Fig. 4). Both the preimmune and immune sera detected a protein band with a molecular mass of about 40 kD in the pea (lanes 9–11) and alfalfa (lanes 5–8) leaves and chloroplasts. In addition to the 40-kD protein band, immune serum detected a stronger band of about 47 kD in the pea chloroplasts (lane 9) and alfalfa leaves (lane 8). The mobility of the 47-kD protein band appears to be distorted by the high level of Rubisco protein, which migrates at about 50 to 55 kD. When pea chloroplasts were fractionated by freeze-thawing, the 47-kD protein was detected in the soluble fraction (lane 10) and was absent from the insoluble fraction (lane 11). The preimmune serum detected a protein band with a molecular mass of about 53 kD in the plastids of castor seeds (lane 2) but not in the endosperm (lane 1). This is probably because the 53-kD protein is at a higher concentration in the plastid preparation than in the total endosperm protein extract. The immune serum detected a protein band with a molecular mass of about 50 kD in castor seeds in both the endosperm (lane 3) and the plastids (lane 4) and detected the 53-kD protein only in the plastids (lane 4). Thus, the E3-like protein has an apparent molecular mass of about 47 kD in the chloroplasts of pea and alfalfa leaves (because of its distorted mobility) and about 50 kD in the plastids of castor seeds. The E3-encoded protein has a calculated molecular mass of about 58 kD, and the chloroplast uptake experiment (see above) suggested that E3 has a transit peptide with molecular mass of about 8 kD. Thus, the expected mature E3 protein is about 50 kD, which coincides with the apparent molecular mass of E3 in the plastids of castor seeds as determined by immunoblot analysis (Fig. 4).

Genomic Organization and Expression of E3

Genomic DNA digested with restriction endonucleases was analyzed by probing southern blots with the tobacco E3-coding region at moderate hybridization stringency and washing conditions. The probe detected approximately one

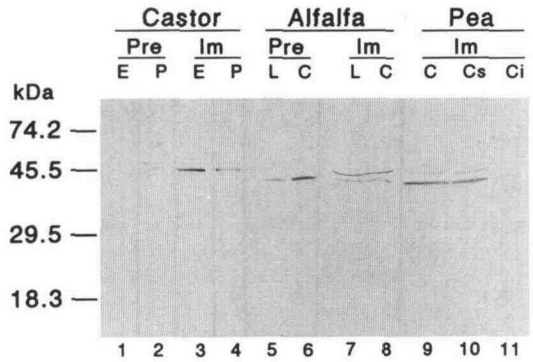

Figure 4. Western blot analysis. Protein was resolved by 15% (w/v) SDS-PAGE and then transferred to a nitrocellulose membrane. Blots were treated with either E3 preimmune serum (Pre) or with E3 immune serum (Im) as indicated. Castor protein (100 μg) was isolated from the endosperm (E) and plastids (P). Alfalfa protein (100 μg) was isolated from leaves (L) and chloroplasts (C). Pea protein (100 μg) was isolated from chloroplasts (C) and fractionated into soluble (Cs) and insoluble (Ci) fractions. Molecular mass markers are indicated in kD.

gene in Arabidopsis, two genes in castor, and three to four genes in tobacco. In contrast, under these conditions, the probe did not hybridize to genomic DNA isolated from corn, rice, or wheat (Fig. 5). These results support the suggestion of Konishi and Sasaki (1994) that the MS-ACCase does not occur in plastids of Gramineae. However, the absence of cross-hybridization could indicate that E3-like DNA in the Gramineae is sufficiently different that it does not cross-hybridize with the tobacco E3 cDNA. The

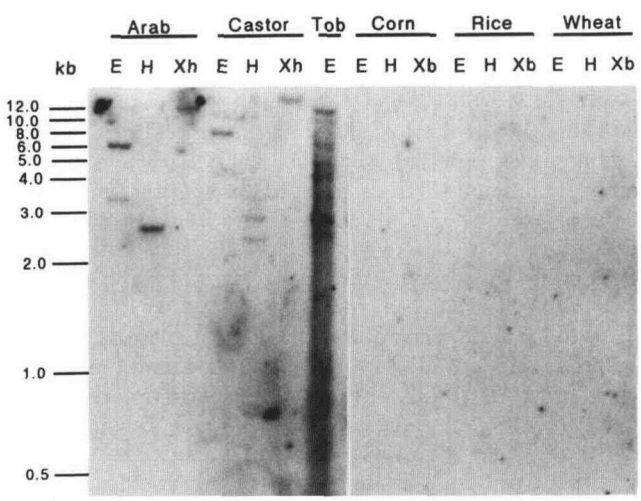

Figure 5. Genomic organization of tobacco E3 DNA. High mol wt genomic DNA isolated from Arabidopsis (Arab), castor, tobacco (Tob), corn, rice, and wheat (10 μg) was digested with *Eco*RV (E), *Hin*dIII (H), *Xba*I (Xb), or *Xho*I (Xh) restriction endonucleases, resolved in an 0.7% (w/v) agarose gel, blotted to a nylon membrane, and hybridized at 55°C, in an aqueous solution, with tobacco E3 cDNA probe. Molecular size markers are indicated in kb. The shown blot was exposed to x-ray film overnight. However, exposure of the blot to x-ray film for 7 d did not reveal any hybridization bands in the Gramineae lanes.

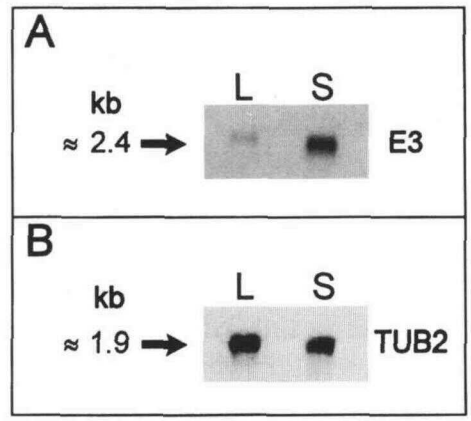

Figure 6. Tissue-specific expression of E3 message. Poly(A)$^+$ RNA (3 μg) was prepared from castor leaves (L) and developing seeds (S), resolved in 1% (w/v) agarose gel containing 2.2 M formaldehyde, blotted to a nylon membrane, and hybridized at 42°C, in 30% formamide solution, with the castor E3-like (E3) cDNA probe (A) or with the β-tubulin 2 (TUB2) gene from *C. graminicola* (Panaccione and Hanau, 1990) (B). Molecular size markers are indicated in kb. Blot A was exposed to x-ray film for 2 h. No other bands appeared with longer exposure.

castor E3-like cDNA probe was used in northern blot analysis of poly(A)$^+$ RNA isolated from developing seeds and leaves of castor plants. A single hybridizing band of about 2.4 kb was detected in both tissues. Expression of E3-related sequences was approximately 10 times higher in the seeds than the leaves (Fig. 6A). As a control for RNA loading, the same blot was re-probed with β-tubulin 2 gene from *Colletotrichum graminicola*, and approximately the same expression level of this constitutive gene was observed in seeds and leaves (Fig. 6B).

DISCUSSION

Malonyl-CoA is required for several biochemical reactions, which occur in both the chloroplast and the cytosol. Early observations of fatty acid elongation in oilseeds implicated the participation of multiple ACCase enzymes in the synthesis of very long chain fatty acids (Ohlrogge et al., 1978). These data correlate with the characterization of two MF-ACCase isozymes in maize leaves based on their herbicide sensitivity and chloroplastic and extrachloroplastic localization (Egli et al., 1993) and with the characterization of MF-ACCase and MS-ACCase in pea leaves (Alban et al., 1994; Konishi and Sasaki, 1994). The MF-ACCase was detected in the epidermal tissues of young pea leaves, whereas the MS-ACCase was localized in the chloroplast of mesophyll cells and in the epidermal tissues. Recently, we cloned an extrachloroplastic MF-ACCase from alfalfa and Arabidopsis (Roesler et al., 1994; Shorrosh et al., 1994).

The picture that has now emerged is that many plants contain both MS- and MF-ACCase enzymes. Although the structure of the MF-ACCase enzyme has been characterized from several plants, the protein composition of the MS-ACCase is still under investigation. Early work by Kannangara and Stumpf (1972) indicated that the BC, BCCP, and CT components of spinach ACCase could be

separated, and others have also suggested that plant ACCase could be dissociated into subunits (Nikolau and Hawke, 1984). Recent immunological data suggested that the MS-ACCase from pea chloroplast is composed of at least three polypeptides of 87, 35 (biotinylated protein), and 91 kD (Sasaki et al., 1993). The 87-kD protein is proposed to be the CT subunit and is encoded in the chloroplast genome of pea and tobacco, but it is absent from wheat and is present in a truncated form in rice. In addition, purification of the MS-ACCase enzyme from pea suggested a structure of at least four dissociable polypeptides with molecular masses ranging from 32 to 79 kD (Alban et al., 1994).

The work described in the present study provides additional information concerning the subunit organization of the plastid MS-ACCase. Based on the following, we concluded that a 50-kD BC subunit of the tobacco MS-ACCase is encoded by clone E3: (a) The tobacco E3 protein has high sequence identity with the prokaryotic BC subunit of ACCase, which ranges from 71 to 80% similarity and 52 to 65% identity. (b) Tobacco E3-encoded protein and prokaryotic BCs do not have the biotin-binding site Met-Lys-Met or Met-Lys-Leu, in contrast to eukaryotic carboxylases such as pyruvate carboxylase, propionyl-CoA carboxylase, ACCase, and methylcrotonyl-CoA carboxylase (in which BC is part of BCCP). However, like all carboxylases, the E3-encoded protein contains a putative ATP-binding motif. (c) In vitro uptake experiments and localization by cell fractionation and immunoblot analysis indicate that E3 protein contains a cleavable transit peptide and that the mature 50-kD protein is localized in the chloroplast. (d) Higher expression of E3 transcripts in castor seeds versus leaves corresponds to the higher level of fatty acid biosynthesis in the seeds. (e) The hybridization of tobacco E3 cDNA to genomic DNA only from dicot plants corresponds to the occurrence of the CT subunit of MS-ACCase in the chloroplast genome of tobacco and pea, to its absence from wheat, and to its presence only in truncated form in rice. (f) Preliminary data (not shown) from size-fractionated castor endosperm and pea chloroplast proteins by Sephacryl S-300 gel permeation chromatography showed an exact co-elution of ACCase activity with E3 protein and with a biotin-containing protein with a molecular mass of about 35 kD. A 35- to 38-kD biotin-binding protein is the major if not the only biotin protein in pea plastids and has been implicated to be the BCCP subunit of MS-ACCase in pea chloroplasts (Alban et al., 1994; Konishi and Sasaki, 1994).

To date, the MS-ACCase has been characterized in the chloroplast of dicots and the MF-ACCase in the chloroplast of Gramineae plants. In addition, both graminaceous and dicot plants contain extrachloroplastic MF-ACCase. Gel filtration analysis suggests that the mass of the dicot plastid MS-ACCase complex is approximately 700 kD (Sasaki et al., 1993; Alban et al., 1994). Based on available data, this complex is now believed to include a 85-kD carboxyltransferase subunit (Sasaki et al.,1993), a 50-kD BC (this work), and a 35- to 38-kD BCCP. However, further work is still needed to decipher how these and other potential subunits are organized to produce an active ACCase complex.

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