Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 4323–4327, May 1994
Plant Biology

Molecular cloning, characterization, and elicitation of acetyl-CoA carboxylase from alfalfa

BASIL S. SHORROSH*†‡, RICHARD A. DIXON†, AND JOHN B. OHLROGGE*

*Department of Botany and Plant Pathology, Plant Biology Building, Michigan State University, East Lansing, MI 48824-1312; and †Plant Biology Division, The Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, OK 73402

Communicated by Paul K. Stumpf, December 20, 1993 (received for review October 13, 1993)

ABSTRACT **Acetyl-CoA carboxylase [ACCase; acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2] catalyzes the ATP-dependent carboxylation of acetyl CoA to produce malonyl CoA. In plants, malonyl CoA is needed for plastid localized fatty acid biosynthesis and for a variety of pathways in the cytoplasm including flavonoid biosynthesis. We have determined the full nucleotide sequence of an ACCase from alfalfa, which appears to represent a cytoplasmic isozyme. Partial cDNAs were isolated from a cDNA library of suspension culture cells that had been elicited for isoflavonoid phytoalexin synthesis. The full-length sequence was obtained by primer extension and amplification of the cDNA with synthetic primers. The sequence codes for a protein of 2257 amino acids with a calculated M_r of 252,039. The biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase domains, respectively, show approximately 72%, 50%, and 65% sequence similarity to those of animal, diatom, and yeast ACCase sequences. ACCase enzyme activity and transcripts are induced severalfold upon addition of yeast or fungal elicitors to alfalfa cell cultures.**

Acetyl-CoA carboxylase [ACCase; acetyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.2], a biotin-containing enzyme, catalyzes the first step in fatty acid biosynthesis via ATP-dependent carboxylation of acetyl CoA to form malonyl CoA. This reaction has been implicated as a key regulatory step in *de novo* fatty acid biosynthesis in the plastids of plant leaves (1–3) and in the cytosol of animal cells (4).

A single ACCase polypeptide has been purified from several higher plants, algae, and animals. However, two ACCase isozymes differing in their sensitivity toward the herbicides haloxyfop and sethoxydim have recently been identified in maize (5). In addition to providing malonyl CoA for plastid fatty acid synthesis, additional ACCase isozymes may be needed in the cytosol to supply malonyl CoA for the synthesis of very long chain fatty acids (6), flavonoids (7, 8), isoflavonoid phytoalexins (9), and stilbenes (10), and in the inactivation of the ethylene precursor 1-aminocyclopropane 1-carboxylate (11). ACCase activity is induced in UV-illuminated parsley cultures (7) and elicitor-treated soybean cultures (8) at the onset of accumulation of flavonoids and isoflavonoid phytoalexins, respectively. The increased ACCase activity in these cultures is presumed due to a higher demand for malonyl CoA for the chalcone synthase reaction.

ACCase has been cloned from *Escherichia coli* (12–15), chicken (16), rat (17), yeast (18), and the diatom *Cyclotella cryptica* (19). Recently, two subunits of *Anabaena* sp. ACCase have been cloned (20). The full-length sequence of a higher plant ACCase has not previously been reported. The *E. coli* ACCase is composed of four separate proteins: the biotin carboxyl carrier protein (BCCP), the biotin carboxylase (BC), and two subunits of carboxyltransferase (CT). In contrast, mammalian and yeast ACCases contain the BCCP, BC, and CT as functional domains within a single polypeptide with an apparent mass of 200–250 kDa. In plants, both multisubunit (21–23) and multifunctional polypeptide forms of ACCase (3) have been reported.

We report here the isolation of alfalfa ACCase cDNAs covering the complete ACCase open reading frame, which exhibits 60–63% sequence similarity and 40–42% sequence identity at the amino acid level to chicken, rat, yeast, and *C. cryptica* ACCases. We also show that treatment of alfalfa cell suspension cultures with fungal or yeast elicitors results in substantial induction of ACCase activity and transcript levels. Based on the absence of a transit peptide in the sequence and the induction of expression by elicitors, we suggest that the alfalfa ACCase clone codes for a cytoplasmic isozyme.§

MATERIALS AND METHODS

Growth and Elicitation of Alfalfa Cell Cultures. Cell suspension cultures of alfalfa (*Medicago sativa* L.) cv. Apollo were initiated and maintained as described elsewhere (24). Dark-grown cells were treated at 5 days after subculture with elicitor preparations from the cell walls of *Colletotrichum lindemuthianum* (24) or yeast (25) at final concentrations of 50-μg glucose equivalents per ml of medium.

Extraction and Assay of ACCase Activity. Alfalfa cell suspension cultures (1 g) were homogenized with a Polytron on ice in 2 ml of 100 mM Mes, pH 6.5/2 mM dithiothreitol. The supernatant was brought to 45% saturation with solid $(NH_4)_2SO_4$ and left on ice overnight. The precipitated protein was resuspended in 400 μl of extraction buffer and 30 μl of the extract was used immediately for ACCase assay (based on the formation of acid-stable radioactive malonyl CoA from $H^{14}CO_3$ and acetyl CoA) as described elsewhere (21). Assays lacking acetyl CoA were included for each cell extract as a control. Protein content was determined in duplicate by the Bradford assay (26) using bovine serum albumin as a standard.

Construction and Screening of an Alfalfa Genomic Library. Genomic DNA was isolated from alfalfa leaves (cv. Apollo), partially digested with *Bam*HI, filled in with dGTP and dATP, and ligated to Lambda FIX II/*Xho* I filled in with dATP and dGTP as described by the supplier (Stratagene). The ligated product was packaged with a Gigapack II Gold kit and amplified in the host strain PLK17 (Stratagene). The library was plated with the host P2PLK17 and screened with the 3′ACC probe by plaque hybridization at 42°C as described (27). Positive plaques were amplified in PLK17 and purified as described elsewhere (28). A region of the genomic

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ACCase, acetyl CoA carboxylase; BCCP, biotin carboxyl carrier protein; BC, biotin carboxylase; CT, carboxyltransferase.

‡Present address: Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824-1312.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L25042).

DNA insert was amplified by PCR using universal primer $(CAU)_4T3$, which anneals to the Lambda FIX II arm, and primer 180 (described below). The amplified genomic DNA was annealed to the pAMP1 vector and sequenced as described below.

Screening of an Alfalfa cDNA Library. A cDNA library was constructed in Lambda ZAP II starting with poly(A) RNA isolated from alfalfa cell suspension cultures treated with fungal elicitor for 2, 3, and 4 h. A cDNA fragment (585 bp) encoding a putative alfalfa ACCase was identified by its sequence (see *Results*). This fragment was used to screen the library as described (27). To find additional cDNAs overlapping the 5′ end of characterized ACCase cDNAs, probes corresponding to 200–300 bp at the 5′ ends of the identified cDNAs were generated by PCR (27) and used to further screen the cDNA library.

5′ Rapid Amplification of cDNA Ends (RACE) PCR for Isolation of Sequences Corresponding to the 5′ End of the ACCase Transcript. *First-strand cDNA synthesis by primer extension.* Total RNA (10 μg) was isolated from cell suspension cultures treated with yeast elicitor for 5 h and mixed with 200 ng of 30-mer primer corresponding to the 5′ end of clone T1 (Fig. 1). The RNA/primer mixture was denatured at 65°C, annealed at 42°C, and reverse-transcribed in a total vol of 20 μl using M-MLV (BRL) at 42°C in the presence of 10% dimethyl sulfoxide. The RNA was hydrolyzed with 2 μl of 2 M NaOH at 65°C for 1 h and then neutralized with 2 μl of 2 M HCl.

Poly(A) tailing and second-strand cDNA synthesis. Four of the first-strand cDNA synthesis reaction mixtures were pooled and the cDNA was purified into 50 μl of H_2O using a GeneClean II kit (Bio 101). The cDNA (50 μl) was tailed at its 3′ end with poly(A) in a final vol of 100 μl using 75 units of terminal deoxynucleotidyltransferase (BRL) at 37°C. The second strand was synthesized with 2.5 units of *Taq* DNA polymerase in a total vol of 100 μl containing 50 μl of poly(A)-tailed cDNA, 200 μM each dNTP, 1 μM anchor primer 134 (AAGCTTCTGCAGGAGCTCTTTTTTTTT-TTTTTTTT), and 3 mM $MgCl_2$. The mixture was incubated at 95°C for 3 min, 25°C for 5 min, 30°C for 10 min, 40°C for 15 min, and 72°C for 30 min.

Nested primers used for amplification. The second-strand cDNA reaction mixture (10 μl) was amplified at 50°C annealing temperature with 20-mer primers that included at their 5′ end the base sequence CAUCAUCAUCAU or CUACUACUACUA to facilitate cloning into the pAMP1 vector. The first PCR product was generated by primers 135 (based on primer 134 above) and 136 (corresponding to the 5′ end of clone T1). The second amplification was with primers 141 (corresponding to the 5′ end of the cDNA amplified with primers 135 and 136) and 142 (designed to encode the peptide EGGGGKG with codon usage based on the earlier characterized alfalfa ACCase cDNA sequences). The third amplification was with primers 146 (corresponding to the 5′ end of the cDNA amplified with primers 141 and 142) and 147 (designed to encode the peptide KVLIANNG). The peptides EGGGGKG and KVLIANNG are conserved between rat, chicken, and yeast ACCases. The fourth amplification used primer 209 (based on genomic ACCase sequences) and 180 (corresponding to the 5′ end of the cDNA amplified with primers 146 and 147) (Fig. 1).

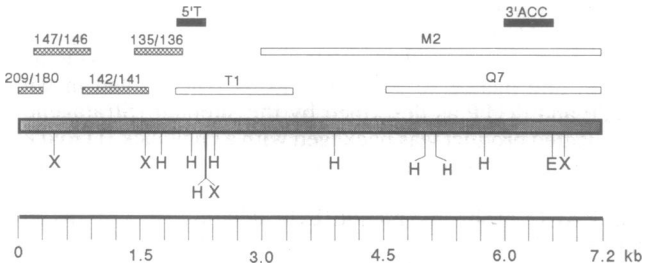

FIG. 1. Schematic representation of alfalfa ACCase sequences. Nucleotide sequence position (in kb) and partial restriction map sites are indicated. Regions used as probes for Southern and Northern blot analysis are indicated by solid bars (5′T, 3′ACC). cDNA clones are represented by open bars. PCR-generated cDNAs are represented by hatched bars. Numbers above each hatched bar represent the pair of primers used to generate the PCR product. H, *Hin*dIII; X, *Xba* I; E, *Eco*RV.

Isolation and Sequencing of DNA Templates. Plasmids isolated from the alfalfa cDNA library were subjected to double-strand sequencing of both strands using dideoxynucleotide chain termination (29) with T7 DNA polymerase (United States Biochemical). Each PCR-generated clone was isolated from three independent colonies and was fully sequenced on both strands using universal and synthetic primers (30) and *Taq* DNA polymerase with an ABI robotic catalyst and model 373A DNA sequencer. In some cases, the PCR product was directly subjected to sequence analysis. The alignment was determined by using the GAP program of Genetics Computer Group (University of Wisconsin, Madison).

Northern and Southern Blot Analysis. Total RNA (15 μg) was isolated from alfalfa cell suspension cultures (31), separated on 1% agarose containing 2.2 M formaldehyde, and blotted onto nitrocellulose membranes. The blot was hybridized at 42°C in the presence of 50% formamide as described elsewhere (32). RNA blots were tested for equal loading by probing with alfalfa 25S rRNA or G1 cDNA probes (32, 33) (data not shown). High molecular weight genomic DNA was isolated from plant leaves (34), digested with restriction endonucleases, electrophoresed in 0.7% agarose, and blotted onto a GeneScreen*Plus* nylon membrane with 0.4 M NaOH. Blots were hybridized and washed at 65°C or 55°C as described elsewhere (32).

RESULTS

Isolation of Alfalfa ACCase cDNA Clones. An alfalfa cDNA clone designated G1 (32), isolated by screening an alfalfa cDNA library with a human protein disulfide isomerase probe, was found to be fused through its poly(A) tail to an unrelated 585-bp cDNA fragment. This fragment (3′ACC) (Fig. 1) encoded a polypeptide with 40% amino acid sequence identity and 77% sequence similarity to the chicken ACCase in their overlapping regions. Rescreening of the alfalfa cDNA library (see *Materials and Methods*) led to the isolation of three clones: Q7 (2567 bp), M2 (4200 bp), and T1 (1467 bp) (Fig. 1). When aligned, the three clones covered 5.3 kb of the ACCase coding region and their overlapping regions were 100% identical. The cDNA library lacked clones containing the 5′ coding region of the putative alfalfa ACCase. This region of the ACCase sequence was therefore obtained by the 5′ RACE PCR method. This led to the isolation of four additional clones designated 135/136 (625 bp), 141/142 (835 bp), 146/147 (700 bp), and 209/180 (351 bp) (Fig. 1).

Primary Sequence of Alfalfa ACCase. Alignment of all the above alfalfa ACCase cDNA clones yielded a sequence of 7194 bp with 69 and 353 bp of 5′ and 3′ untranslated regions, respectively. The 3′ untranslated region contained two potential polyadenylylation signals (AATAA) starting at positions 7113 and 7124 (data not shown). Of 7 cDNA clones sequenced at the 3′ untranslated ends, 1 was polyadenylylated at position 7171, 4 at position 7175, and 2 after addition of the sequence TTTTAT to position 7175.

The coding sequence of 6771 bp contains a methionine residue, which initiates the longest open reading frame of 2257 amino acids encoding a protein of M_r 252,039 (Fig. 2). This methionine residue has a nucleotide context similar to the Kozak initiator methionine consensus sequence (35) and

is preceded by a cDNA sequence rich with stop codons in all three reading frames. Based on sequence alignment with other known ACCases, the putative alfalfa ACCase protein contains three functional domains arranged, starting from the N-terminal end, in the following order: BC, BCCP, and CT (Fig. 2). A putative biotin binding site at a lysine residue flanked by two methionine residues starts at position 712 in the alfalfa ACCase protein (Fig. 2). The two proline residues present 27 and 35 residues upstream of the putative biotin

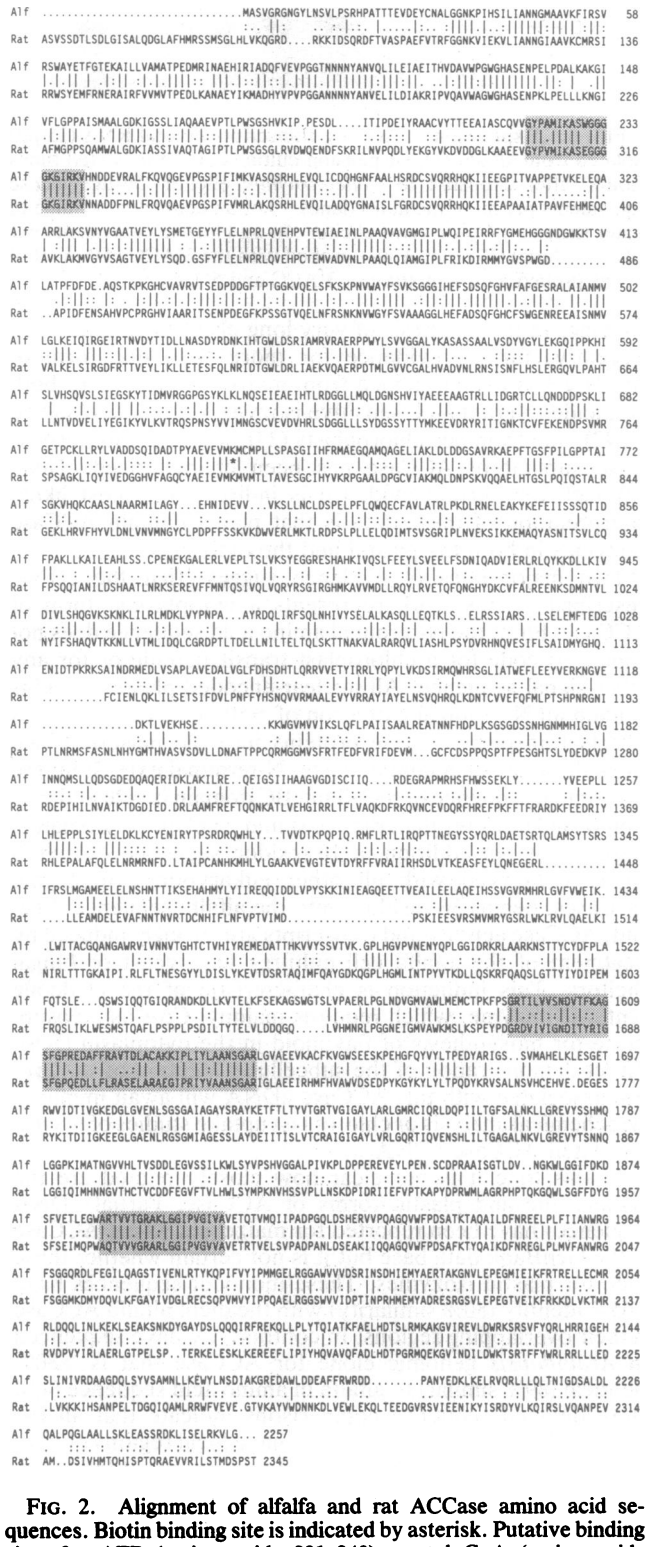

FIG. 2. Alignment of alfalfa and rat ACCase amino acid sequences. Biotin binding site is indicated by asterisk. Putative binding sites for ATP (amino acids 221–240), acetyl CoA (amino acids 1884–1903), and carboxybiotin (amino acids 1594–1643) are shaded.

binding site in alfalfa ACCase (Fig. 2) may be involved in the formation of a hinge region for the movement of carboxybiotin (36). Putative acetyl CoA and carboxybiotin binding sites in the alfalfa ACCase have 80% and 60% sequence identity to the corresponding regions in the rat ACCase (Figs. 2 and 3). Also, a putative ATP-binding site in the alfalfa ACCase matches the consensus sequences Gly-Xaa-Gly-Xaa-Xaa-Gly or Gly-Xaa-Xaa-Gly-Xaa-Gly shared by nucleotide binding proteins (37, 38) and has 86% identity with the corresponding region in the rat ACCase (Figs. 2 and 3). The sequence Glu-Leu-Asn-Pro-Arg located at positions 354–358 in the BC region of alfalfa ACCase (Fig. 2) is similar to the consensus sequence Glu-Met-Asn-Pro-Arg, which was proposed to be a candidate for the catalytic site of biotin-dependent carboxylases and carbamoyl phosphate synthetases (39).

Genomic Organization of ACCase. Two bands with different hybridization intensities were detected on probing alfalfa genomic DNA with the 3′ACC cDNA (Fig. 1) at high-stringency hybridization and washing conditions (65°C) (Fig. 4*A*). Southern blot analysis using mixed alfalfa 3′ACC and 5′T cDNA probes (Fig. 1) detected, at stringent hybridization and washing conditions (55°C), only one band in *Arabidopsis* (Fig. 4*C*), approximately two bands in rice and wheat (Fig. 4*B*), and three or four bands in pea, peanut, soybean, and tobacco (data not shown).

Elicitation of ACCase. The specific activity of ACCase in alfalfa suspension cells increased in response to treatment with yeast elicitor, reaching a maximum 4- to 5-fold increase between 8 and 12 h postelicitation (Fig. 5). The average specific activities of control and induced cells between 8 and 12 h postelicitation were 5.3 and 23.4 nmol of acetyl CoA per mg of protein per min, respectively.

ACCase transcripts were not detected in unelicited alfalfa cell suspension cultures. However, transcripts were significantly induced within 2 h of exposure to fungal or yeast elicitor (Fig. 6). ACCase transcripts reached maximum levels at 6 h and declined rapidly by 8 h after treatment with the fungal elicitor (Fig. 6*A*). In contrast, transcript levels remained elevated beyond 8 h in yeast elicitor-treated cultures (Fig. 6*B*).

DISCUSSION

Possible Role for Multiple ACCase Enzymes in Plants. In most organisms, the major role of ACCase is production of malonyl CoA for fatty acid synthesis. In plants, the biochem-

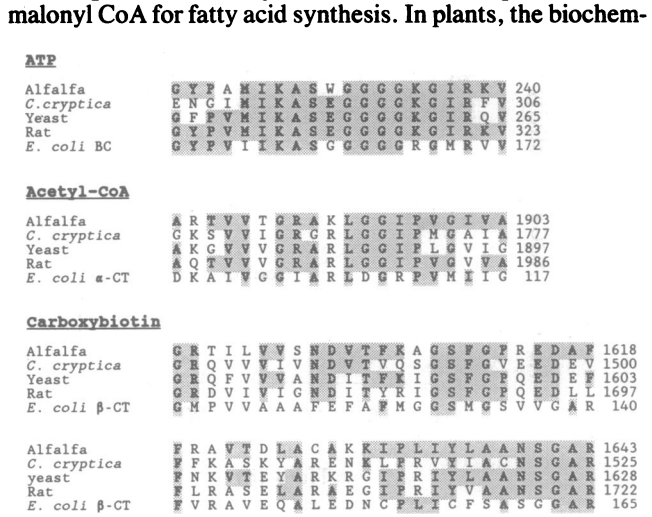

FIG. 3. Amino acid sequence alignments of putative ATP, acetyl CoA, and carboxybiotin binding sites from several ACCases. Residues identical to the putative alfalfa sequence are shaded. α-CT, α subunit of CT; β-CT, β subunit of CT.

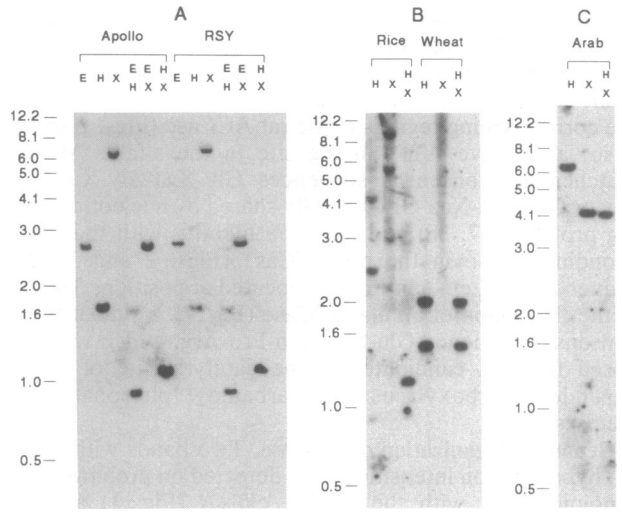

FIG. 4. Genomic organization of ACCase in alfalfa cv. Apollo and Regen SY (RSY), rice, wheat, and *Arabidopsis* (Arab). Genomic DNA (10 μg) was digested with *Eco*RV (E), *Hin*dIII (H), and *Xba* I (X), or mixtures of these enzymes, resolved on a 0.7% agarose gel, and blotted to GeneScreen*Plus*. Blot A was probed with the 3′ACC cDNA. Blots B and C were probed with a mixture of the 3′ACC and 5′T cDNAs. Numbers on left are kb.

ical fate of malonyl CoA is considerably more diverse. ACCase activity has been reported in plastid preparations from numerous species and it is likely that malonyl CoA is used almost exclusively for *de novo* fatty acid synthesis in this organelle (3). Outside the plastid, malonyl CoA enters into several pathways (7–9, 11, 40), including the elongation of fatty acids (6). Production of malonyl CoA for these diverse roles could be accomplished by multiple ACCase isozymes in different subcellular compartments or, alternatively, by a single enzyme accompanied by transport of malonyl CoA or malonate across membranes.

Early observations on fatty acid elongation in oilseeds strongly implicated the participation of multiple ACCase

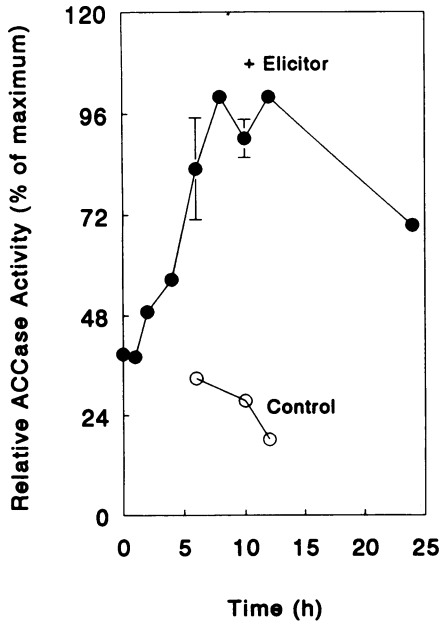

FIG. 5. Elicitation of ACCase activity in alfalfa cell suspension cultures in response to yeast elicitor (50-μg glucose equivalents per ml of medium) (+ elicitor) or an equivalent volume of water (control). Bars represent the spread of values from duplicate elicitor-treated cultures.

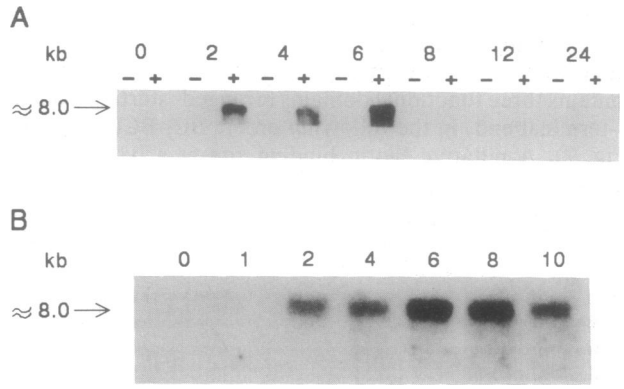

FIG. 6. Expression of ACCase transcripts in total RNA (15 μg) isolated from alfalfa cell suspension cultures at the indicated times (hours) after exposure to *Colletotrichum* elicitor (50-μg glucose equivalents per ml of medium) (lanes +) or an equivalent volume of water (lanes −) (*A*) or yeast elicitor (50-μg glucose equivalents per ml of medium) (*B*). Blot A was probed with the 3′ACC cDNA. Blot B was probed with a mixture of 3′ACC and 5′T cDNAs.

enzymes in the synthesis of very long chain fatty acids (41). Recently, further evidence for more than one form of ACCase has been obtained from maize leaves, where both herbicide-tolerant and -sensitive isozymes could be resolved (5, 42), and from pea chloroplasts (23), where a transcarboxylase subunit of ACCase has been characterized. Thus, although the current evidence is indirect, the most likely interpretation of these data is that plants contain ACCase isozymes in both the plastid and the cytosol.

Two major lines of evidence suggest that the cDNA described in this work represents a cytoplasmic form of ACCase. First, the N-terminal amino acid sequence does not appear to code for a chloroplast transit peptide or for any other signal sequence. All nuclear-encoded plastid proteins contain an N-terminal extension of ≈50 amino acids, which directs transport of these proteins into the plastid. When aligned, rather than containing an N-terminal extension, the N-terminal sequence of the alfalfa BC domain is ≈20 and ≈80 amino acids shorter than the yeast and chicken BC domains, respectively. In addition, a 16-residue sequence, which is highly conserved with all other eukaryotic cytoplasmic ACCases, begins 36 residues into the alfalfa sequence. Second, ACCase activity and transcripts are strongly induced by treatment of the alfalfa cell cultures with yeast or fungal elicitors. This induction is characteristic of the chalcone synthase enzyme, which requires malonyl CoA as a cosubstrate for biosynthesis of flavonoid in the cytoplasm (7–9). Direct demonstration of the subcellular location of the protein associated with this ACCase cDNA will await immunocytochemical analysis or chloroplast uptake studies of *in vitro* translated ACCase cDNA.

At this time it is not clear how closely related are the cytoplasmic and plastid forms of ACCase. Partial cDNA sequences have been reported for maize and wheat ACCase in the GenBank data base but it is not certain whether these code for plastid isozymes. These monocot sequences have ≈65% identity (80% similarity) with the dicot alfalfa ACCase in their overlapping regions. In contrast, we have sequenced an *Arabidopsis* genomic clone for ACCase that is >80% identical (89% similar) to alfalfa in amino acid sequence (43). More detailed sequence comparisons indicate that all the available plant ACCase sequences have local regions of very high identity and therefore heterologous probes at low stringency may cross-hybridize between them. For this reason, hybridization strategies to determine tissue or developmental expression patterns may need to account for potential contributions from a mixture of ACCase mRNA species.

Genomic Organization. The detection of two bands in Southern blots with different hybridization intensities suggests that ACCase in alfalfa is encoded by at least two related genes. Southern blot analysis of alfalfa ACCase-related genes in other plants suggests that *Arabidopsis*, rice, and wheat may have one or two corresponding genes in their genome. At this time it is not possible to determine whether genes coding for both cytoplasmic and plastidial forms of ACCase would be detected by the alfalfa probe in these blots. Since the nucleotide sequences of >71 independently isolated alfalfa ACCase cDNAs were identical in their overlapping regions, both in the coding and in the untranslated regions, it is likely that all characterized cDNAs isolated from the elicitor-induced alfalfa library correspond to one gene.

Elicitor Induction of ACCase Transcripts and Activity. The elicitor-induced increase in ACCase transcript levels (Fig. 6) precedes the increase in enzymatic activity (Fig. 5), and this timing correlates with the induction kinetics of transcripts/activities of the series of enzymes involved in the synthesis of the isoflavonoid phytoalexin medicarpin from phenylalanine (9). It is possible that most of the undetectable ACCase message in unelicited, control alfalfa cell cultures is associated with the plastidial form, which may not cross-hybridize with the cytosolic ACCase probe (Fig. 6).

Elicitation of isoflavonoid phytoalexin biosynthesis in legume cell suspension cultures requires three molecules of malonyl CoA as cosubstrate with 4-coumaroyl CoA in the chalcone synthase reaction (9). Thus, ACCase is coinduced with chalcone synthase in elicited alfalfa and soybean (8) cell suspensions, suggesting that insufficient malonyl CoA is produced under normal conditions to satisfy the requirements for both flavonoid synthesis and fatty acid elongation. ACCase is also induced by UV irradiation in parsley cells, associated with the accumulation of UV-protective flavonoid compounds (7); in this species, fungal elicitation does not involve ACCase induction (42) as the parsley furanocoumarin phytoalexins do not require malonyl CoA for their synthesis. It is not yet known whether elicitation and the concomitant induction of ACCase has quantitive or qualitative effects on fatty acid elongation. The present work opens up the possibility of investigating regulation of channeling of malonyl CoA into primary and secondary metabolism by genetic manipulation of ACCase levels.

We thank Karen Dalkin for construction of the alfalfa cDNA library, Nancy Paiva for providing some of the elicited cell suspension cultures, Gansan Gowri for assistance in constructing the alfalfa genomic library, and Yves Poirier and Dave Shintani for critical reading of the manuscript.

1. Post-Beittenmiller, D., Jaworski, J. G. & Ohlrogge, J. B. (1991) *J. Biol. Chem.* **266,** 1858–1865.
2. Post-Beittenmiller, D., Roughan, G. & Ohlrogge, J. B. (1992) *Plant Physiol.* **100,** 923–930.
3. Harwood, J. L. (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39,** 101–138.
4. Kim, K.-H., Lopez-Casillas, F. & Bai, D.-H. (1989) *FASEB J.* **3,** 2250–2256.
5. Egli, M. A., Gengenbach, B. G., Gonwald, J. W., Somers, D. A. & Wyse, D. L. (1993) *Plant Physiol.* **101,** 499–506.
6. Pollard, M. & Stumpf, P. (1980) *Plant Physiol.* **66,** 649–655.
7. Ebel, J. & Hahlbrock, K. (1977) *Eur. J. Biochem.* **75,** 201–209.
8. Ebel, J., Schmidt, W. E. & Loyal, R. (1984) *Arch. Biochem. Biophys.* **232,** 240–248.
9. Dixon, R. A. & Harrison, M. J. (1990) *Adv. Genet.* **28,** 165–234.
10. Lanz, T., Tropf, S., Marner, F.-J., Schroder, J. & Shroder, G. (1991) *J. Biol. Chem.* **266,** 9971–9976.
11. Kionka, C. & Amrhein, N. (1984) *Planta* **162,** 226–235.
12. Li, S.-J. & Cronan, Jr., J. E. (1992) *J. Biol. Chem.* **267,** 855–863.
13. Li, S.-J. & Cronan, Jr., J. E. (1992) *J. Biol. Chem.* **267,** 16841–16847.
14. Kondo, H., Shiratsuchi, K., Yoshimoto, T., Masuda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M. & Tanabe, T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9730–9733.
15. Alix, J.-H. (1989) *DNA* **8,** 779–789.
16. Takai, T., Yokoyama, C., Wada, K. & Tanabe, T. (1988) *J. Biol. Chem.* **263,** 2651–2657.
17. Lopez-Casillas, F., Bai, D.-H., Luo, X., Kong, I.-S., Hermodson, M. A. & Kim, K.-H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5784–5788.
18. Al-Feel, W., Chirala, S. S. & Wakil, S. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4534–4538.
19. Roessler, P. G. & Ohlrogge, J. B. (1993) *J. Biol. Chem.* **268,** 19254–19259.
20. Gornicki, P., Scappino, L. A. & Haselkorn, R. (1993) *J. Bacteriol.* **175,** 5268–5272.
21. Nikolau, B. J. & Hawke, J. C. (1984) *Arch. Biochem. Biophys.* **228,** 86–96.
22. Kannangara, C. G. & Stumpf, P. K. (1972) *Arch. Biochem. Biophys.* **152,** 83–91.
23. Sasaki, Y., Hakamada, K., Suama, Y., Nagano, Y., Furusawa, I. & Matsuno, R. (1993) *J. Biol. Chem.* **268,** 25118–25123.
24. Dalkin, K., Edwards, R., Edington, B. & Dixon, R. A. (1990) *Plant Physiol.* **92,** 440–446.
25. Schumacher, H. M., Gundlach, H., Fiedlar, F. & Zenk, M. H. (1987) *Plant Cell Rep.* **6,** 410–413.
26. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–257.
27. Shorrosh, B. S. & Dixon, R. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10941–10945.
28. Millar, S. J., Dempsey, D. L. & Dickinson, P. D. (1992) *BioTechniques* **13,** 554–562.
29. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **83,** 8073–8077.
30. Sanchez-Pescador, R. & Urdea, M. S. (1984) *DNA* **3,** 339–343.
31. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
32. Shorrosh, B. S. & Dixon, R. A. (1992) *Plant J.* **2,** 51–58.
33. Shorrosh, B. S. & Dixon, R. A. (1992) *Plant Mol. Biol.* **18,** 1189–1190.
34. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
35. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8132.
36. Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Haase, F. C. & Wood, H. T. (1988) *J. Biol. Chem.* **263,** 6461–6464.
37. Sternberg, M. J. E. & Taylor, W. R. (1984) *FEBS Lett.* **175,** 387–392.
38. Moller, W. & Amons, R. (1985) *FEBS Lett.* **186,** 1–7.
39. Konodo, H., Shiratsuchi, K., Yoshimoto, T., Masuda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M. & Tanabe, T. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 9730–9733.
40. Liu, Y., Hoffman, N. E. & Yang, S. F. (1983) *Planta* **158,** 437–441.
41. Ohlrogge, J. B., Pollard, M. R. & Stumpf, P. K. (1978) *Lipid* **13,** 203–210.
42. Howard, J. L. & Ridley, S. M. (1990) *FEBS Lett.* **261,** 261–264.
43. Roesler, K. R., Shorrosh, B. S. & Ohlrogge, J. B. (1994) *Plant Physiol.*, in press.