Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 6860–6864, July 1994
Plant Biology

Wheat acetyl-coenzyme A carboxylase: cDNA and protein structure

(amino acid sequence/biotinylation/gene expression)

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ABSTRACT **cDNA fragments encoding part of wheat (*Triticum aestivum*) acetyl-CoA carboxylase (ACC; EC 6.4.1.2) were cloned by PCR using primers based on the alignment of several biotin-dependent carboxylases. A set of overlapping clones encoding the entire wheat ACC was then isolated by using these fragments as probes. The cDNA sequence contains a 2257-amino acid reading frame encoding a 251-kDa polypeptide. The amino acid sequence of the most highly conserved domain, corresponding to the biotin carboxylases of prokaryotes, is 52–55% identical to ACC of yeast, rat, and diatom. Identity with the available C-terminal amino acid sequence of maize ACC is 66%. The biotin attachment site has the typical eukaryotic EVMKM sequence. The cDNA does not encode an obvious chloroplast targeting sequence. Various cDNA fragments hybridize in Northern blots to a 7.9-kb mRNA. Southern analysis with cDNA probes revealed multiple hybridizing fragments in hexaploid wheat DNA. Some of the wheat cDNA probes also hybridize with ACC-specific DNA from other plants, indicating significant conservation among plant ACCs.**

Acetyl-CoA carboxylase [ACC; acetyl-CoA: carbon-dioxide ligase (ADP-forming), EC 6.4.1.2] catalyzes the first committed step in *de novo* fatty acid biosynthesis, the addition of CO_2 to acetyl-CoA to yield malonyl-CoA. There are two types of ACC: prokaryotic ACC, in which the three functional domains—biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyltransferase (CT)—are located on separable subunits (as in *Escherichia coli, Pseudomonas aeruginosa, Anabaena, Synechococcus*, and probably pea chloroplast), and eukaryotic ACC, in which all the domains are located on one large polypeptide (as in rat, chicken, yeast, diatom, and wheat). All these enzymes show significant sequence similarity (1–3). The conservation is especially high in the BC domain and around the biotinylation site. Biotin is attached to the lysine residue of a conserved pentapeptide, E(V/A)MK(M/L).

E. coli ACC consists of a dimer of 49-kDa BC monomers, a dimer of 17-kDa BCCP monomers, and a CT tetramer containing two 33-kDa and two 35-kDa subunits. The primary structures of all of the *E. coli* ACC subunits (4–8), as well as the structures of the BC and BCCP of *Anabaena* 7120 (9), *Synechoccocus* 7942 (L. Phung and R.H., unpublished work), and *P. aeruginosa* (10), are known, based on the gene sequences. The genes encoding the subunits of *E. coli* ACC are called *accA* (CT α subunit), *accB* (BCCP), *accC* (BC), and *accD* (CT β subunit). *accC* and *accB* form one operon, whereas *accA* and *accD* are not linked to each other or to *accCB* (8). In cyanobacteria, *accC* and *accB* are unlinked as well (9).

Yeast, rat, chicken, and human ACCs are cytoplasmic enzymes consisting of 250- to 280-kDa subunits, whereas diatom ACC is most likely a chloroplast enzyme consisting of 230-kDa subunits. Their primary structure has been deduced from cDNA sequences (11–15). In eukaryotes, homologs of the four bacterial genes are fused in order *accC, accB, accD, accA*. Animal ACC activity varies with the rate of fatty acid synthesis or energy requirements in different nutritional, hormonal, and developmental states. In the rat, ACC mRNA is transcribed from different promoters in different tissues and can be regulated by alternative splicing. The rat enzyme activity is also allosterically regulated by a number of metabolites and by reversible phosphorylation (ref. 15 and references therein). The expression of the yeast gene was shown to be coordinated with phospholipid metabolism (16, 17).

Studies on plant ACC are far less advanced. Early attempts at characterization of plant ACC led to the suggestion that it consisted of low molecular weight subunits similar to those of bacteria (18). More recent efforts indicate that the plant enzyme is composed of >200-kDa subunits, similar to the enzyme from other eukaryotes (19–23). Plant ACC (at least one form of it) is located in plastids, the primary site of plant fatty acid synthesis, but the gene must be nuclear because no corresponding sequence has been seen in the complete chloroplast DNA sequences of tobacco, liverwort, or rice. The idea that plant ACC consists of several smaller subunits has been revived recently by the discovery of an *accD* homolog in some chloroplast genomes (24). Indeed, it has been shown that the product of this gene in pea binds two other peptides, one of which is biotinylated. The complex may be a chloroplast isoform of ACC in pea and perhaps some other plants (25).

The possibility of different ACC isoforms, one present in plastids and another in the cytoplasm, is still open. The rationale behind the search for a cytoplasmic ACC isoform is the requirement for malonyl-CoA in this cellular compartment, where it is used in fatty acid elongation and synthesis of secondary metabolites. Indeed, two isoforms were found in maize, both consisting of >200-kDa subunits but differing in size, herbicide sensitivity, and immunological properties. The major form was found to be located in mesophyll chloroplasts. It is also the major ACC in the endosperm and in embryos (22). Wheat ACC subunits of 220 kDa were also detected associated with leaf chloroplasts and in wheat germ (21). Partial cDNA sequences of maize and wheat ACC have been reported recently (26, 27). In this paper we report the cloning and complete sequence of cDNA encoding wheat (*Triticum aestivum*) eukaryotic-type ACC.§

MATERIALS AND METHODS

PCR Amplification. Degenerate PCR primers were based on the alignment of amino acid sequences of the following proteins (GenBank accession numbers in parentheses): rat (J03808) and chicken (J03541) ACCs; *E. coli* (M80458,

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

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U10187).

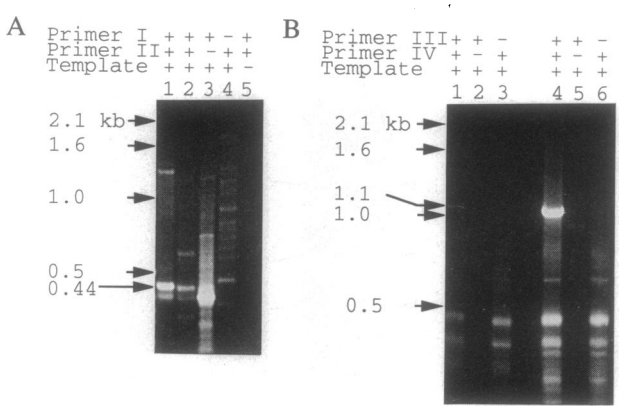

FIG. 1. PCR amplification of wheat ACC-specific cDNA encoding a part of the BC domain (*A*) and the interval between the BC domain and the biotinylation site (*B*). Wheat single-stranded cDNA (*A*, lanes 2–5, and *B*, lanes 1–3) or *Anabaena* 7120 DNA (*A*, lane 1) was used as template. The 1.1-kb product of the first round PCR (*B*, lane 1) was purified and used as a template for reamplification (*B*, lanes 4–6). Nucleotide sequences of primers were as follows: primer I, 5′-TCGAATTCGTNATNATHAARGC-3′ [underlined sequence encoding amino acids V(M/I)IKA (N-terminal to C-terminal)]; primer II, 5′-GCTCTAGAGKRTGYTCNACYTG-3′ [P(H/T)EVQ, C-terminal to N-terminal]; primer III, 5′-TCGAATTCWNCATYT-TCATNRC-3′ [XMKM(V/A), C-terminal to N-terminal]; primer IV, 5′-GCTCTAGAATACTATTTCCTG-3′ (EYYEL, N-terminal to C-terminal). (In nucleotide sequences N = G, A, T, or C; Y = T or C; R = G or A; H = A; H = A, C, or T; W = A or T.)

M79446, X14825, M32214), *Anabaena* 7120 (L14862, L14863), and *Synechococcus* 7942 (L. Phung and R.H., unpublished work) BCs and BCCPs; rat (M22631) and human (X14608) propionyl-CoA carboxylase α subunit; yeast (J03889) pyruvate carboxylase; *Propionibacterium shermanii* (M11738) transcarboxylase 1.3S subunit; and *Klebsiella pneumonia* (J03885) oxaloacetate decarboxylase α subunit. Each primer (Fig. 1) consisted of a 14-nucleotide specific sequence based on the amino acid sequence and a 6- or 8-nucleotide extension at the 5′ end.

Poly(A)$^+$ RNA from 8-day-old plants (*T. aestivum* cv. Era), prepared as described (28), was used for the synthesis of the first strand of cDNA with random hexamers as primers for avian myeloblastosis virus reverse transcriptase (29). Reverse transcriptase was inactivated by incubation at 90°C, and low molecular weight material was removed by filtration. All components of the PCR (Perkin–Elmer/Cetus kit), except the *Taq* DNA polymerase, were incubated for 3–5 min at 95°C. The PCR was initiated by the addition of polymerase. Conditions were optimized by amplification of the BC gene from *Anabaena* 7120. Amplification was for 45 cycles, each consisting of 1 min at 95°C, 1 min at 42–46°C, and 2 min at 72°C. The $MgCl_2$ concentration was 1.5 mM. Both the reactions using *Anabaena* DNA and those using the single-stranded wheat cDNA as template yielded the expected 440-bp products. The wheat product was separated by electrophoresis in low-melting-point agarose and reamplified by using the same primers and a piece of the agarose slice as a source of the template. That product, also 440 bp, was cloned into the Invitrogen vector pCR1000 by their A·T-tail method and sequenced.

In eukaryotic ACCs, the BCCP domain is located about 300 amino acids downstream from the end of the BC domain. Therefore, it was possible to amplify the cDNA encoding that interval between the two domains by using a primer corresponding to the C-terminal end of the BC domain (Fig. 1, primer IV based on the wheat cDNA sequence obtained as described above) and a primer corresponding to the conserved biotinylation site (Fig. 1, primer III). The expected 1.1-kb product of the first low-yield PCR with primers III and IV was separated by electrophoresis in low-melting-point agarose, reamplified by another round of PCR, and then cloned into the Invitrogen vector pCRII and sequenced. The PCR conditions were the same as those described above.

Isolation and Analysis of ACC cDNA Clones. A wheat cDNA library (*T. aestivum* cv. Tam 107, Hard Red Winter, 13-day light-grown seedlings) was purchased from Clontech. This λgt11 library was prepared by using both oligo(dT) and random primers. Colony Screen*Plus* (DuPont) membrane was used according to the manufacturer's protocol (hybridization at 65°C in 1 M NaCl/10% dextran sulfate). The library was first screened with the 1.1-kb PCR-amplified fragment of ACC-specific cDNA (above). Fragments of clones 39-1, 45-1, and 24-3 were used in subsequent rounds of screening. In each case, $\approx 2.5 \times 10^6$ plaques were tested. λ clones containing ACC-specific cDNA fragments were purified, and *Eco*RI fragments of the longest cDNA inserts were subcloned into pBluescript SK (Stratagene) for further analysis and sequencing. A subset of the clones (Fig. 2) was sequenced on both strands by the dideoxy chain-termination method with Sequenase (United States Biochemical) or using the Perkin–Elmer/Applied Biosystems Taq DyeDeoxy Terminator cycle sequencing kit and an Applied Biosystems 373A DNA sequencer.

RNA and DNA. Total RNA from 10-day-old wheat plants was prepared as described (29). RNA was separated in a glyoxal denaturating gel (30). GeneScreen*Plus* (DuPont) blots were hybridized in 1 M NaCl/10% dextran sulfate at 65°C (wheat RNA and DNA) or 58–60°C (soybean and canola DNA). All cloning, DNA manipulation, and gel electrophoresis were as described (30).

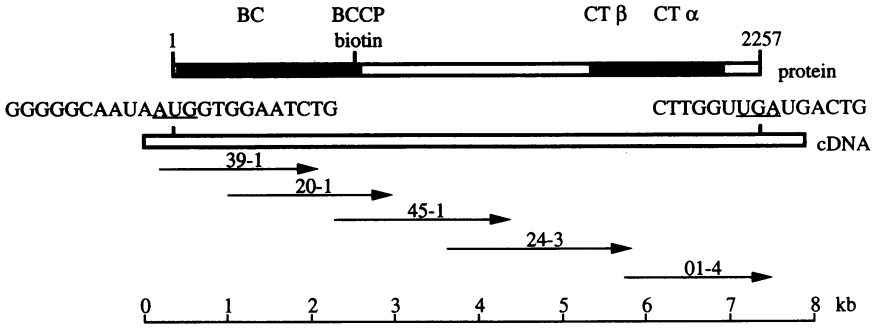

FIG. 2. Structure of wheat ACC deduced from cDNA sequence. The most conserved parts of the protein are marked in black. They include the core of the BC domain, a motif around the biotinylation site, and two conserved motifs (α and β) identified previously in the α and β subunits of *E. coli* CT (8). Parts of the protein that are well conserved among eukaryotic ACCs are shown in dark gray and the least conserved parts in white. The amino acid alignment and amino acid identities used to draw this diagram are that of Fig. 5. A set of overlapping cDNA fragments that were sequenced, with their relative orientation, is shown by arrows. Nucleotide sequence around the translation start and stop codons is also shown.

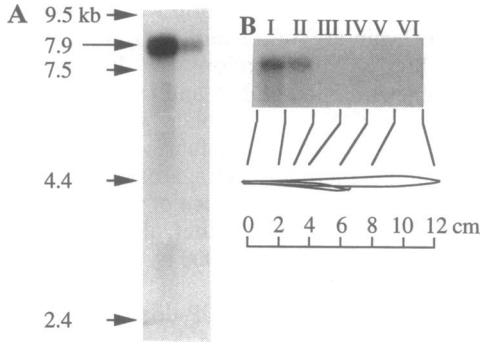

FIG. 3. Northern analysis of ACC-specific mRNA in whole wheat leaves (*A*) and in various leaf sectors (*B*). In *A*, 40 and 4 μg of total RNA were used to prepare the Northern blot. In *B*, 15 μg of total RNA from 10-day old plants was used in each lane. Results of hybridization with the 24-3 cDNA probe are shown. Positions of molecular size markers are indicated by arrows.

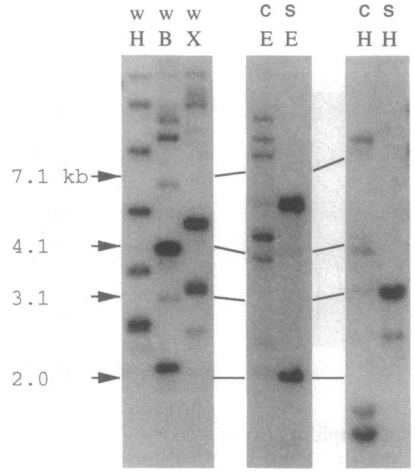

FIG. 4. Southern analysis of wheat (w), canola (c), and soybean (s) DNA with wheat ACC cDNA fragment 24-3 (Fig. 2) as a hybridization probe. Conditions used for canola and soybean hybridization were less stringent than those used for wheat. H, *Hin*dIII; B, *Bam*HI; X, *Xba* I; E, *Eco*RI. Eight to 10 μg of DNA was run in each lane.

RESULTS

PCR Cloning of the Wheat ACC cDNA Fragments. A 440-bp cDNA fragment encoding a part of the BC domain of wheat ACC and a 1.1-kb cDNA fragment encoding the interval between the BC domain and the conserved biotinylation site were amplified (Fig. 1). These fragments were cloned and sequenced. In fact, three different 1.1-kb products, corresponding to closely related sequences that differ from each

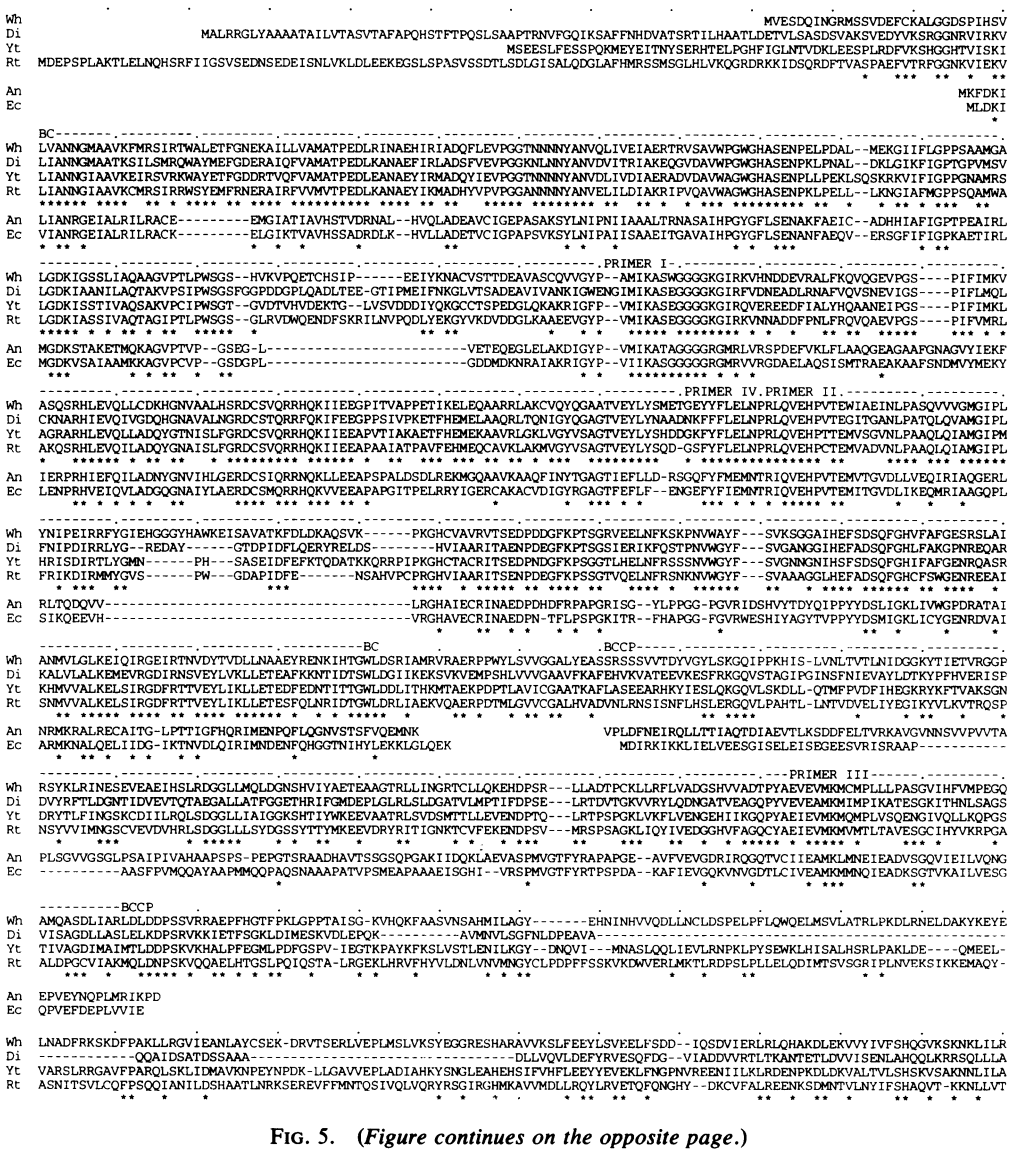

FIG. 5. (*Figure continues on the opposite page.*)

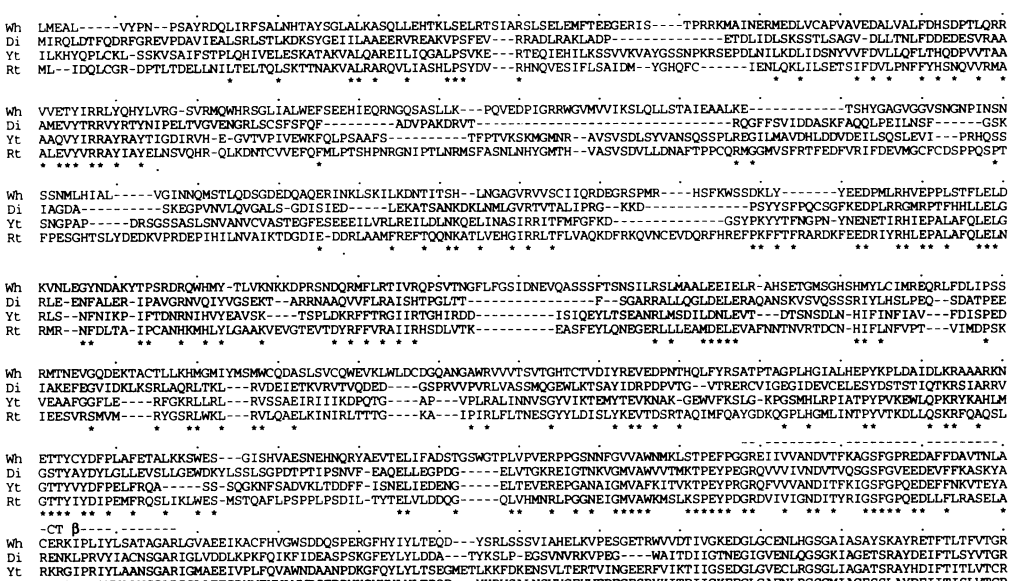

FIG. 5. Amino acid sequence alignment of wheat [Wh (this work)], diatom [Di (14)], yeast [Yt (11)] and rat [Rt (12)] ACCs and *Anabaena* [An (9)] and *E. coli* [Ec (4–7)] BC and BCCP subunits of ACC. Stars mark amino acids that are identical in at least three out of four or at least five out of six compared sequences. The most conserved parts of the BC, BCCP, and CT (β and α) domains are overlined. These are shown on the diagram of Fig. 2 in black. Sequences of the *E. coli* CT subunits are not included in this alignment because of rather low overall identity with eukaryotic ACCs. The conserved sequences of CT were identified previously by Li and Cronan (8). Target sequences for the PCR primers are also marked.

other by 1.5%, were identified. The three products most likely represent transcription products of three different genes, the minimum number expected for hexaploid wheat. The amino acid sequence of the polypeptide predicted from the cDNA sequence of the two overlapping PCR-amplified fragments of wheat cDNA (total length of 1473 nucleotides) was compared with the amino acid sequences of other ACCs. The most significant identities were with the ACC of rat, chicken, and yeast (>50%). Less extensive identity (but still almost 30%) was evident with the BC subunits of bacteria and the BC domains of other enzymes such as pyruvate carboxylase of yeast and propionyl-CoA carboxylase of rat (data not shown). These two DNA fragments were used to screen a wheat cDNA library.

Isolation and Sequence Analysis of Wheat ACC cDNAs. A set of overlapping cDNA clones covering the entire ACC coding sequence was isolated and a subset of these clones was sequenced (Fig. 2). The nucleotide sequence within overlapped regions of clones 39-1, 20-1, and 45-1 differs at 1.1% of the nucleotides within the total of 2.3 kb of the overlaps. The sequence within the overlap of clones 45-1 and 24-3 is identical. The sequence encodes a 2257-amino acid protein with a calculated molecular mass of 251 kDa. In wheat germ the active ACC has an apparent molecular mass of ≈500 kDa and the individual polypeptides have an apparent molecular mass (measured by SDS/PAGE) of about 220 kDa (21). The 220-kDa protein was also present in both total leaf protein and protein from intact chloroplasts. In fact, it was the major biotinylated polypeptide in the chloroplast protein. The cDNAs (total length, 7.4 kb) include 158 bp of the 5′ untranslated sequence and 427 bp of the 3′ untranslated sequence.

Northern Analysis of ACC mRNA. Northern blots with total RNA from 10- to 14-day-old wheat leaves were probed with cDNA fragments (the 1.1-kb PCR-amplified fragment and parts of clones 20-1, 24-3, and 01-4). In each case the only hybridizing mRNA species was 7.9 kb in size (Fig. 3*A*). This result shows clearly that all the cDNA clones correspond to mRNA of large, eukaryotic ACC and that there are no other closely related biotin-dependent carboxylases, consisting of small subunits that are encoded by smaller mRNAs, in wheat.

Northern analysis of total RNA prepared from different sectors of 10-day-old wheat seedlings indicated very high steady-state levels of ACC-specific mRNA in cells of leaf sectors I and II near the basal meristem (Fig. 3*B*). The ACC mRNA level was significantly higher in sectors I and II than in sectors III–VI (Fig. 3*B*). This cannot be explained by dilution of specific mRNA by increased levels of total RNA in

older cells. Based on published results (31), the increase in total RNA between sectors I and VI is expected to be only about 2-fold.

All cell division occurs in the basal meristem, and cells in other sectors are in various stages of development. Differences between these young cells and the mature cells at the tip of the leaf include cell size, number of chloroplasts, and amount of total RNA and protein per cell (31). Expression of some genes is correlated with the cell age (e.g., ref. 32). It is not surprising that the level of ACC-specific mRNA is highest in dividing cells. The burst of ACC mRNA synthesis is necessary to supply enough ACC to meet the demand for malonyl-CoA. The levels of ACC mRNA decrease significantly in older cells, where the demand is much lower. The same differences in the level of ACC specific mRNA between cells in different sectors were found in plants grown in the dark and in plants illuminated for 1 day at the end of the dark period.

Southern Analysis of Plant DNA. Hybridization of wheat total DNA digests with wheat ACC cDNA probes under stringent conditions revealed multiple bands (Fig. 4). This was expected due to the hexaploid nature of wheat (*T. aestivum*). Some of the wheat cDNA probes also hybridized with ACC-specific DNA from other plants (Fig. 4). The specificity of this hybridization was demonstrated by sequencing several fragments of canola genomic DNA isolated from a library by using wheat cDNA probe 20-1 (Fig. 2) and by Northern blot of total canola RNA using one of the canola genomic clones as a probe (data not shown). The Northern analysis revealed a large ACC-specific message in canola RNA that was similar in size to that found in wheat.

DISCUSSION

We have cloned and sequenced a set of overlapping cDNA clones encoding eukaryotic-type ACC from wheat. The predicted polypeptide is 2257 amino acids long and has a calculated molecular mass of 251 kDa. The putative translation start codon was assigned to the first methionine of the open reading frame. An in-frame stop codon is present 21 nucleotides upstream from this AUG codon. The nucleotide sequence around this AUG fits quite well with the consensus for a monocot translation initiation site derived from the sequence of 93 genes (33), except for U at position +5 of the consensus, which was found in only 3 of the 93 sequences:

								−3		+1		+4		
Wheat ACC mRNA	G	G	G	G	G	C	A	A	U	A	A	U	G	G U
Monocot consensus	a	g	c	g	g	c	a	a G	C	c	A	U	G	G C

The ACC mRNA stop codon UGA is also the most frequently used stop codon found in monocot genes, and the surrounding sequence fits the consensus quite well (33).

A comparison of the wheat ACC amino acid sequence with other ACCs shows significant sequence conservation among all carboxylases. The sequence of the polypeptide predicted from the cDNA described above was compared with the amino acid sequences of other ACCs (Fig. 5). The most significant identities are with the ACC of rat, diatom, and yeast (about 40%). Less extensive similarities are evident with subunits of bacterial ACCs. The amino acid sequence of the most highly conserved domain, corresponding to the BCs of prokaryotes, is 52–55% identical to the ACC of yeast, chicken, rat, and diatom and 27% identical to the BCs of *E. coli* and *Anabaena* 7120. The biotin attachment site has the EVMKM sequence. Several conserved amino acids found in the CT domains identified by Li and Cronan (8) are also present in the wheat sequence. Identity with the available partial sequence of maize ACC, about 1300 C-terminal amino acids (27), and wheat ACC, about 500 C-terminal amino acids (26), is 66% and 72%, respectively. These sequences include the least conserved parts of the ACC polypeptide.

None of the four conserved motifs containing serine residues which correspond to phosphorylation sites in rat, chicken, and human ACCs (15) is present at a similar position in the wheat polypeptide.

The wheat cDNA does not encode an obvious chloroplast targeting sequence unless this is an extremely short peptide. There are only 12 amino acids preceding the first conserved amino acid found in all eukaryotic ACCs (a serine residue; Fig. 5). The conserved core of the BC domain begins about 20 amino acids further downstream. The apparent lack of a transit peptide poses the question of whether and how the ACC described in this paper is transported into chloroplasts. It was shown recently that the large ACC polypeptide purifies with chloroplasts of wheat and maize (21, 22). No obvious chloroplast transit peptide between the ER signal peptide and the mature protein was found in diatom ACC either (14).

We are grateful to Gayle Lamppa for advice and for providing wheat poly(A)$^+$ mRNA and to Elizabeth Koshy for technical assistance. This work was supported by a grant from the U.S. Department of Agriculture through the Midwest Plant Biotechnology Consortium and by CIBA-Geigy and Pioneer Hi-Bred International.

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