Plastid-localized acetyl-CoA carboxylase of bread wheat is encoded by a single gene on each of the three ancestral chromosome sets

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ABSTRACT 5'-End fragments of two genes encoding plastid-localized acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) of wheat (Triticum aestivum) were cloned and sequenced. The sequences of the two genes, Acc-1,1 and Acc-1,2, are 89% identical. Their exon sequences are 98% identical. The amino acid sequence of the biotin carboxylase domain encoded by Acc-1,1 and Acc-1,2 is 93% identical with the maize plastid ACCase but only 80–84% identical with the cytosolic ACCases from other plants and from wheat. Four overlapping fragments of cDNA covering the entire coding region were cloned by PCR and sequenced. The wheat plastid ACCase ORF contains 2,311 amino acids with a predicted molecular mass of 255 kDa. A putative transit peptide is present at the N terminus. Comparison of the genomic and cDNA sequences revealed introns at conserved sites found in the genes of other plant multifunctional ACCases, including two introns absent from the wheat cytosolic ACCase genes. Transcription start sites of the plastid ACCase genes were estimated from the longest cDNA clones obtained by 5'-RACE (rapid amplification of cDNA ends). The untranslated leader sequence encoded by the Acc-1 genes is at least 130–170 nucleotides long and is interrupted by an intron. Southern analysis indicates the presence of only one copy of the gene in each ancestral chromosome set. The gene maps near the telomere on the short arm of chromosomes 2A, 2B, and 2D. Identification of three different cDNAs, two corresponding to genes Acc-1,1 and Acc-1,2, indicates that all three genes are transcriptionally active.

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyzes the first committed step in de novo fatty acid biosynthesis. It also provides malonyl-CoA for the synthesis of very long chain fatty acids and a variety of important secondary metabolites, and for malonylation. Plants have two forms of ACCase (reviewed in ref. 1). One, located in plastids, the primary site of plant fatty acid synthesis, can be either a high molecular weight multifunctional enzyme (e.g., wheat and maize) or a prokaryotic-type multisubunit enzyme (e.g., pea, soybean, tobacco, and Arabidopsis thaliana). The cytosolic plant ACCase is of the multifunctional eukaryotic type. In addition, a recent report suggests that plastids of at least some plants—e.g., Brassica napus—contain both prokaryotic- and eukaryotic-type enzymes (2).

In Gramineae, genes for both cytosolic and plastid multifunctional ACCases are nuclear—e.g., in maize (3) and in wheat (4, 5). In other plants, subunits of plastid ACCase are encoded in the nuclear DNA, with the exception of the β subunit of carboxyltransferase, which is encoded in the chloroplast genome by a homolog of the Escherichia coli accD gene (1, 6). Plastid ACCase synthesized in the cytoplasm is imported into the plastid. A chloroplast targeting signal is present at the N terminus of the plastid ACCase from maize (3) and B. napus (2). There is some experimental evidence suggesting that, in plants, ACCase activity controls metabolic flux through the fatty acid biosynthetic pathway and therefore may serve as an important regulation point of plant metabolism (reviewed in ref. 7). For example, ectopic expression of A. thaliana cytosolic ACCase in B. napus targeted to plastids alters fatty acid composition and seed oil content (8). The molecular mechanism of this regulation is unknown. Multiple differentially regulated genes may be involved in differential tissue and/or developmental expression of ACCase activity. Many plant enzymes are encoded by gene families (9). Two genes arranged in a tandem repeat encode multifunctional ACCase in Arabidopsis (10). Our earlier results suggest that at least two isozymes of the cytosolic ACCase are encoded by each of the three ancestral chromosome sets in hexaploid wheat (5).

In this paper we describe the cloning of full-length cDNA and parts of the corresponding genes encoding plastid ACCase. We also report the results of chromosome mapping of the plastid ACCase genes in wheat.

MATERIALS AND METHODS

Isolation and Analysis of ACCase Genomic Clones. A λ EMBL3 wheat genomic library (Triticum aestivum, var. Hard Red Winter Tam 107, 13-day light grown seedlings, CLONTECH) was screened as described before (5) with a 420-bp cDNA probe (ucg1). Thirty-nine positives were found among ~5 × 10⁶ plaques tested. Probe ucg1 was PCR-cloned by using primers based on the cDNA sequence available from GenBank (Z23038). Single-stranded cDNA was prepared as described before (4), using a gene-specific primer (CCTCCGAGTTTTCGCTCTG). A DNA fragment amplified by PCR with primers specific to cDNA sequence (TTTCCCTTGCGTATCATCA and TATTCTAGGGCCTATGAG) was cloned into the Invitrogen vector pCRII and sequenced. DNA from the partially purified A pools was prepared by the DEAE-cellulose method (11) and analyzed by PCR using a gene-specific downstream primer (AGCAATTGCTTGGGCTGTCTTAGT) and λ EMBL3 specific primers flanking the BamHI cloning site.

Abbreviation: ACCase, acetyl-CoA carboxylase; RACE, rapid amplification of cDNA ends; NT, nullisomic-tetrasomic.

The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF029895, AF029896, and AF029897).

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(right arm primer, CAGCGCACTAGTGTCACACGAAAG or left arm primer, CATGGTGTCGACTTATGCCCC). The Expand Long Template PCR System (Boehringer Mannheim) was used according to the manufacturer's protocol. The PCR products were analyzed on Southern blots with probe ucg1. Five large hybridizing fragments corresponding to the genomic clones extending upstream of the target site for probe ucg1 were identified. A 6-kb fragment from one of these clones was PCR-cloned into vector pCRII by using the left arm primer and the gene-specific primer and was sequenced to verify its identity. A 1.3-kb Sau–XbaI 5'-end fragment of this clone was then used as a probe to rescreen the library. Two different clones, 274 and 325, containing ACCase-specific sequences hybridizing to this probe but not to ucg1 were found, purified, and mapped. SauI restriction fragments of these genomic clones were subcloned into pGEM and sequenced. Sequenced regions of the two clones and an approximate position of the probe ucg1 are shown in Fig. 1. The identity of the 5'-end portion of the gene present in genomic clone 274 was verified by PCR using primers GCCGAGCGGACGAGGGCGCTGATC and CGGCCCAATGCACAACTGATCG and wheat (cultivar Tam 107) genomic DNA as template. PCR products were cloned into the vector pCR2.1 (Invitrogen) and sequenced. Analysis of mRNA by Rapid Amplification of cDNA Ends (RACE). A set of nine cDNA fragments corresponding to mRNA 5'-ends was prepared by T/A cloning of 5'-RACE products into the vector pCR2.1 (Invitrogen) and sequenced. Total RNA from a sector of 15-day-old wheat plants (cultivar Tam 107) containing leaf meristem was prepared as described in ref. 12. A GIBCO BRL 5'-RACE kit was used according to the manufacturer’s protocol. The first strand of cDNA was prepared by using a gene-specific primer (GGCGGACGAGGGCGCTGATC and CGGCCCAATGCACAACTGATCG) and wheat (cultivar Tam 107) genomic DNA as template. PCR products were cloned into the vector pCR2.1 (Invitrogen) and sequenced. Cloning of Four Overlapping cDNA Fragments Containing the Entire Plastid ACCase Coding Sequence. Single-stranded cDNA prepared as described (4) was used as template for PCR. The following primers were used for cDNA synthesis and PCR, respectively: fragment 1, GACTGTTAGGCCAGCTCAGTGC; GAAGACTGGAACGCCGCTCGTTTG, and GCAACTT-GAUCTCAAGATGTCGAC; fragment 2, GCCGAAAGACATGTTGGTGAGTTGCG and GCTGCTGTAGAAGCTCATT; and fragment 3, TATACGGACGGGTTGACTGAGG, AGGATCCAGATGACCCCTAGCC and GCTTGGTTCAGCCGGAATAGTATCCT, ACTTGCCATACGTTGACATAAGG. The four sets of primers were designed on the basis of available genomic clones 274 and 325 described above and cDNA sequences (GenBank ZZ3038) and used to amplify distinct overlapping cDNA fragments such that the 5'-end of fragment 1 included the ACCase translation start codon and the 3'-end of fragment 4 included the stop codon. The resulting products were cloned into pCR2.1 and sequenced. A single clone was analyzed for each of fragments 1 and 4, and the three different clones were sequenced and used for fragments 2 and 3 by sequencing. The sequences of the three clones of fragment 3, which was about 4 kb in size, differed by 2 nucleotides when compared in pairs. Because of this low degree of variation, we assumed that the differences were due to PCR errors and that the three clones originated from the same transcript.

Chromosome Localization. Nullisomic–tetrasomic (NT) lines of Chinese Spring wheat (13), where nullisomy for a specific chromosome is compensated by two extra copies of a homoeologue, were used to assign ACCase gene fragments to individual chromosomes. NT 2A and NT 4B plants were identified cytologically, as these stocks are maintained as monosomic–tetrasomic lines. Ditesosomes (Dt) of Chinese Spring (14) were used for arm location of ACCase gene fragments. Dt lines 2AL, 4AS, 5AS, 2BS, 4BL, 5BS, and 5DS were cytologically identified in the parent stock of dihetero- or ditelosomic lines. Probes were hybridized to DNA from NT and Dt stocks, and the resulting autoradiograms were scored visually to identify fragments absent in any of the stocks. When a fragment was absent in a particular NT stock, we inferred it to be located on the chromosome in the nullisomic condition. The concomitant presence of a double-dose fragment in the stocks tetrasomic for a particular chromosome was used as additional evidence for the correct localization of the genes. In the analysis of Dt's, a fragment absent in a stock indicated its presence on the opposing arm of that chromosome. An ACCase gene was placed on the genetic linkage map of an Aegilops tauschii F2 mapping population that consisted of 60 F2 progeny derived from the cross of Ae. tauschii accessions TA1691, var. meyeri, and TA1704, var. typica (15, 16). Ae. tauschii Coss. [syn. Aegilops squarrosa L., syn. Triticum tauschii (Coss.) Schmal., 2n = 14, DD] is the D genome progenitor of common bread wheat (T. aestivum L. em. Thell., 2n = 6x = 42, AABBDD). The ACCase gene was placed on the genetic linkage map created by the International Triticeae Mapping Initiative. A mapping population consisting of 114 recombinant inbred lines derived from a cross between a synthetic hexaploid wheat, W-7984, and the wheat variety Opata 85 were used in that study as described (17). For the experiments described in this paper, 60 recombinant inbred lines were used. Isolation of DNA and Southern analysis were done as described (18). Linkage analysis was done using MAPMAKER version 2.0 (19) and the Kosambi mapping function (20). Probe ucg1 was used in all chromosome mapping experiments. DNA manipulations and gel electrophoresis were performed as described (21). DNA was sequenced by the Uni-
versity of Chicago Cancer Center Sequencing Facility. Sequence alignments were created using CLUSTAL W (22).

RESULTS

A collection of clones containing fragments of wheat nuclear genes encoding a putative plastid ACCase was obtained by screening a wheat genomic library with a CDNA probe, ucg1, targeted to the 3' end of the ACCase ORF. The probe was prepared by PCR with primers based on a CDNA sequence encoding a wheat ACCase (23) whose sequence was significantly different from that of wheat cytosolic ACCase (4). An end fragment of one of the genomic clones extending about 6 kb upstream from the target site of ucg1 was then used to rescreen the library. Two genomic clones, 274 and 325, extending toward the 5' end of the gene and long enough to include the beginning of the ACCase ORF as well as the promoter region, were sequenced. The two λ clones represent two different but very closely related genes, Acc-1,1 and Acc-1,2. The overall sequence identity of the two 5-kb fragments (Fig. 1) is 89%. Their predicted exon and coding sequences are 98% and 99.4% identical, respectively. The corresponding 619-amino acid ORFs encoded by Acc-1,1 and Acc-1,2 differ by three amino acids (99.5% identity). The predicted amino acid sequence of the biotin carboxylase domain deduced from the exon sequence is 94% identical with the maize plastid ACCase but only 80–84% identical with cytosolic ACCases from other plants and wheat (Table 1). This comparison suggested that the two genomic clones encode plastid ACCase isozymes. The amino acid sequence of the ACCase encoded by Acc-1,1 and Acc-1,2, when compared with the wheat cytosolic ACCase, revealed a 100-amino acid extension at the N terminus, corresponding to a putative plastid transit peptide. Comparison of the N-terminal amino acid sequence of the Acc-1,1 and Acc-1,2 gene products with the maize plastid ACCase (3) strongly supports this conclusion (Fig. 2). Similarity with the transit peptide of the maize plastid ACCase isozymes, the amino acid identity being the beginning of the biotin carboxylase domain. One of the mature ACCase (processing site) has not yet been established for any of the plastid ACCases. An amino acid identity with the T. aestivum plastid ACCase; #, an amino acid identical in all the sequences. Sequence accession numbers are listed in Table 1.

Table 1. Comparison of the amino acid sequence of wheat plastid ACCase deduced from cDNA sequence (pew, accession number AF029895) with those deduced from gene sequence (Acc-1,1, Acc-1,2) and with sequences of some other biotin-dependent carboxylases.

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<th>Species</th>
<th>Location</th>
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A dash indicates sequence not available or not found as one colinear contig in the prokaryotic-type carboxylases. Sequence alignments were created by using CLUSTAL W (22).

of the insert found in clone 325 by PCR on genomic DNA were unsuccessful (not shown). Verification of the 5’ untranslated portion of each gene is important because many clones isolated from the genomic library contain unrelated fragments of genomic DNA. λ clone 325 contains such a ligation artifact, in which an unrelated DNA is fused at a Sau3A site to the 5’ end of the fragment of gene Acc-1,2 shown in Fig. 1. The 5’-end sequence of clone 274 resembles retrotransposon sequences (Fig. 1), suggesting that this part of clone 274 contains intergenic DNA.

Four overlapping CDNA fragments covering the entire coding region of the wheat plastid ACCase were cloned by PCR and sequenced. Within the overlapping sequences (total length 600 nucleotides) these CDNAs are 99% identical. The corresponding amino acid sequences are 95.5% identical. The

![Table 1](image)
comparing composite cDNA (pcw, Fig. 1) is 6,993 nucleotides long and encodes a 2,311-amino acid peptide with a calculated molecular size of 255 kDa. This peptide includes the plastid transit peptide described above. The DNA and the corresponding amino acid sequence of the putative plastid ACCase is 80% identical to the corresponding sequences deduced for genes Acc-1,1 and Acc-1,2. The results of amino acid sequence comparisons for the biotin carboxylase domain and the full-length wheat plastid ACCase are summarized in Table 1.

Our cloning and sequencing experiments revealed three different transcriptionally active genes encoding the putative plastid ACCase in hexaploid T. aestivum. Southern analysis of genomic DNA from diploid Ae. tauschii, the progenitor of the D genome of T. aestivum, strongly indicates the presence of a single-copy gene (Fig. 3a). Only one hybridizing band was observed for DNA digested with each of four different restriction enzymes. This observation is true for both Ae. tauschii accession TA1691, var. meyeri, and Ae. tauschii accession TA1704, var. typica, despite significant polymorphism evident from the Southern analysis (Fig. 3a). Three of the four restriction enzymes yield differently sized hybridizing fragments for the two accessions. This conclusion is in agreement with the result of Southern analysis of genomic DNA from hexaploid wheat digested with five different enzymes (Fig. 3b). One to three hybridizing bands are observed, and their intensities reflect gene dosage. The small HindIII fragment can be explained by the presence of a HindIII site within the cDNA probe (ucg1) used in all experiments described above.

The same cDNA probe (ucg1) specific for the Acc-1 genes encoding plastid ACCase hybridized to homologous group 2 chromosomes in the Chinese Spring NT stocks (Fig. 4a). The three hybridizing HindIII fragments of increasing size correspond to ACCase genes present on chromosomes 2D, 2A, and 2B, respectively. Genetic mapping of hexaploid wheat by using a W-7984 × Opata 85 recombinant inbred population as well as genetic mapping of diploid wheat progenitor Ae. tauschii by using the TA1691 × TA1704 F2 population (data not shown) place the plastid ACCase gene on the short arm of 2A and 2D at a logarithm of odds (LOD) > 3.0 and LOD > 2.0, respectively (Fig. 4b). In the latter case, the gene mapped to the distal tip of the short arm of chromosome 2D. Physical mapping using deletion lines (data not shown) placed the gene near the tip of the short arm of group 2 chromosomes (Fig. 4b). The apparently different position of the gene on the map of chromosome 2A (Fig. 4b) reflects inherent differences between genetic and physical maps. The results of our mapping are consistent and the differences can be reconciled when positions of other common markers are considered.

DISCUSSION

Understanding how a key step of de novo fatty acid biosynthesis and other pathways for which ACCase provides carbon units works is of prime importance. Many cell processes such as membrane biogenesis, deposition of triacylglycerols as storage material, and biosynthesis of very long chain fatty acids, flavonoids, and stilbenes depend on ACCase activity. The requirement of flavonols for pollen germination and tube growth has been documented in maize (27), and other flavonoids provide protection against UV light (28, 29). Some plants produce flavonoid or stilbene phytoalexins, and transcription of cytosolic ACCase genes is induced by fungal elicitor (30).

We described a series of genomic and cDNA clones encoding wheat plastid ACCase. This ACCase isozyme is predicted to be 2,311 amino acids long and to have a sequence most similar to that of the plastid ACCase in maize (3). The amino acid sequences of the two carboxylases are 80% identical. Identity of the amino acid sequence of wheat plastid and cytosolic ACCase (4) is only 67% (Table 1). The first 100-amino acid domain encoded by the Acc-1 genes includes a putative plastid targeting signal. ACCase functional domains in the plastid isozyme (Fig. 1) are arranged in the same order.
as found previously for the wheat cytosolic and other multifunctional ACCases (4).

Identification of six different cDNA and genomic sequences led to the suggestion that at least two isozymes of the cytosolic ACCase are encoded by each of the three ancestral chromosome sets in hexaploid wheat (5). Two ACCase genes arranged in a tandem array were found in *A. thaliana* (10). The products of two different ACCase genes have been identified in human and rat (31, 32). In yeast, on the contrary, ACCase encoded by a single gene provides malonyl-CoA for both de novo fatty acid biosynthesis and fatty acid elongation (33). The presence of multiple cytosolic ACCase genes may reflect the need for differential expression of the enzyme in response to different environmental or developmental cues. These results prompted our investigation of the plastid ACCase gene copy number in wheat. We found that each chromosome set of the allohexaploid genome of *T. aestivum* contains only one copy of the gene.

Plastid ACCase genes were located on maize chromosomes 2 and 10 (34). Comparative mapping experiments have demon-

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**Fig. 4.** Chromosome mapping of wheat plastid ACCase genes. (a) Southern analysis of Chinese Spring NT lines. DNA was digested with *Hind*III. (b) Genetic maps of chromosome 2A and 2D, and consensus physical map of wheat chromosome 2. Position of *Acc-1* gene was revealed by hybridization with cDNA probe *ucg1*. Names of markers, distances in centimorgans (genetic maps), and distances of the deletion breakpoints of various deletion lines from the centromere as fraction of the arm length (physical map) are shown. Lines connecting the maps indicate positions of markers in common, including *Xucg1(ACCp)* marked with the dashed line, and the position of the centromere marked on the genetic maps with a solid oval symbol. The chromosome 2A map, obtained from the GrainGenes database (http://wheat.pw.usda.gov/graingenes.htm), was published previously (36).
strated that regions of conserved synteny exist between the short arms of wheat group 2 chromosomes and maize chromosomes 2, 7, and 10 (35, 36). This synteny suggests that the plastid ACCase genes in Gramineae have a common ancestor. The putative duplication of the maize genome would account for the two genes mapping to different chromosomes.

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