Cloning and characterization of cotton heteromeric acetyl-CoA carboxylase genes*

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Abstract Heteromeric acetyl-coenzyme A (CoA) carboxylase (ACCase) catalyzes the formation of malonyl-CoA from acetyl-CoA. It plays an essential role in fatty acid synthesis in prokaryotes and most of plants. The heteromeric ACCase is composed of four subunits: biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and α- and β-subunits of carboxyltransferase (α- and β-CT). In this study, we cloned five novel genes encoding the subunits of heteromeric ACCase (one BC, BCCP and β-CT, and two α-CTs) from cotton (*Gossypium hirsutum* cv. zhongmian 35) by RACE-PCR. The deduced amino acid sequence of these cDNAs shares high similarity with other reported heteromeric ACCase subunits. The phylogenetic analysis indicated that the different subunits of heteromeric ACCase were grouped in a similar pattern. Southern blot analysis revealed the milti-copy patterns of these heteromeric ACCase genes in cotton genome. Semi-quantitative RT-PCR demonstrated that heteromeric ACCase genes were constitutively expressed in all of the cotton tissues, but the transcripts accumulated at a relatively low level in roots. To our knowledge, this is the first report about characterization of the heteromeric ACCase genes in cotton.

Keywords: heteromeric ACCase, cotton, gene cloning, fatty acid synthesis.

Fatty acids are the fundamental components in membrane and storage lipids in all of the life kingdoms. The acetyl-coenzyme A (CoA) carboxylase (ACCase) catalyzes the first committed step of fatty acid synthesis, the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA[1]. And this reaction is found nearly in all living organisms. To date, two structurally distinct forms of ACCase have been found in nature: homomeric ACCase and heteromeric ACCase[2]. The homomeric ACCase exists in eukaryotes with a single large polypeptide (> 200 kD). And the heteromeric ACCase, consisting of four separate subunits, is usually found in prokaryotes and most plants. Plants, except for the *Poaceae*, have both forms of ACCase. The homomeric ACCase catalyzes the formation of malonyl-CoA in cytosol for the synthesis of flavonoids, storage lipids, sphingolipids and waxes[3]. And the heteromeric ACCase which functions in plastids provides the main sink of malonyl-CoA for *de novo* fatty acids synthesis. So far, the only exception is that the plastids of rapeseed may contain two forms of ACCase simultaneously[4].

The four subunits of heteromeric ACCase can be divided into three functional domains: biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC) and carboxyltransferase (made up of two polypeptides, α-CT and β-CT). Previous results indicated that in the modern plants, β-CT is encoded by the plastid genome[5], and the remaining three subunits are encoded by the nuclear genome[6]. The reaction catalyzed by ACCase can be divided into two half-reactions: (1) Carboxylation of the biotin covalently attached to the BCCP subunit, which is catalyzed by the BC domain, and (2) translocation of the carboxyl group from BCCP subunit to acetyl-CoA to form malonyl-CoA, which is catalyzed by the CT domain[7].

1. BCCP-biotin + HCO_3^- → BCCP-Biotin-CO_2^-
(catalyzed by BC subunit)

2. BCCP-biotin-CO_2^- + acetyl-CoA → BCCP-biotin + malonyl-CoA
(catalyzed by CT subunit)

So far, the heteromeric ACCase genes have been cloned and characterized in many species, such as *E. coli*[8, 9], *Arabidopsis*[10—12], pea[5, 13] and soybean[14]. Previous experiments had proved that AC-

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Case was very important in fatty acid biosynthesis. Over-expression of four genes encoding ACCase subunits in equimolar amount could significantly increase the ACCase activity and result in a 6-fold increase in the fatty acid biosynthesis rate in *E. coli*[15]. And ectopic over-expression of an *Arabidopsis* homomeric ACCase gene, *ACC1*, in plastids of rape seed (*B. napus*) could increase the total oil content of seeds approximately by 5%[16]. Cotton is one of the most important economic crops worldwide, not only for its fiber in textile industry, but also as an important source of edible oil. To date, there is no report about the heteromeric ACCase genes in cotton. In this paper, we report the molecular cloning and primary characterization of cotton heteromeric ACCase genes.

1 Materials and methods

1.1 Plant materials

Cotton (*Gossypium hirsutum* cv. zhongmian 35) plants were grown in a controlled environment at 25 ℃ with a 16 h light and 8 h dark cycle. Leaves, cotyledons, stems and roots were collected from 2-week-old seedlings. Embryo buds and flowers were prepared from 90-day-old mature cotton plants. Fibers and seeds were harvested at 5 or 40 days post anthesis respectively. All samples for nucleic acid extraction were frozen immediately in liquid nitrogen and stored at −70 ℃. The fresh leaves for chloroplast DNA isolation were harvested from 4-week-old seedlings.

1.2 Preparation of genomic DNA, chloroplast DNA and total RNA

Total genomic DNA was prepared from young leaves as described by Paterson[17]. The chloroplast DNA was isolated based on the sucrose step gradient centrifugation method described previously[18]. Total RNA was extracted from various tissues by PUREScript® RNA purification kit (Gentra, Minneapolis, USA) according to the manufacturer's instruction.

1.3 Molecular cloning of cotton heteromeric ACCase

The RACE system (3′-Full RACE Core Set, Takara, Dalian, China) was applied to isolate the genes encoding heteromeric ACCase subunits according to the product's protocol. We first searched the cotton EST database (National Center for Biotechnology Information BLAST search program) and found a series of cotton ESTs which might encode heteromeric ACCase. Based on these ESTs, we designed gene specific primers and applied nested-PCR to clone their cDNAs. The primers are BC1: 5′-GGGATCAATAAAAACTTGCCTC-3′ and BC2: 5′-CTCCCTGTCTCACTACATTGCTCTG-3′ for *BC*; BP1: 5′-TTGGAACCTCACTTTCTACTATCG-3′ and BP2: 5′-CCTTTTATTTCTTCTCTGTAACTC-3′ for *BCCP*; and CTa1: 5′-GGAGAAATCGAAGTTTGGAGTGTG-3′ and CTa2: 5′-GTGATGGCTTCGATAGCGTGTTC-3′ for α-*CT*. Because the β-CT subunit is encoded by the plastid genome, we isolated the cotton chloroplast DNA and then cloned the most conserved central region of β-*CT* by a pair of primers (CTb1: 5′-GTTTATGGGGGGTAGTATGGGATC-3′ and CTb2: 5′-CGGCAATAATGATATCCCCCAAC-3′). For cloning the remaining regions, we applied the TAIL-PCR[19] by additional four primers (CTb3: 5′-GCAAATGGCTAAAATATCTTCCGCTT-3′, CTb4: 5′-TGCTCCTCCGGAAGCACACACTAG-3′, CTb5: 5′-CTGGTGGAGTGACTGCGAGTTTTG-3′ and CTb6: 5′-GGGTGATTTTCTCGCCTACTACGG-3′). The PCR-amplified products were cloned into pMD18-T vector (Takara, Dalian, China) and then sequenced. The deduced protein sequences were analyzed by Clustal X[20] and the phylogenetic tree was drawn by TreeView[21].

1.4 Southern blot hybridization and semi-quantitative RT-PCR analysis

Genomic DNA (30 μg per sample) was separately digested with *Dra* I, *Eco*R I, *Eco*R V, *Hin*d III and *Xba* I at 37 ℃ overnight, separated on 0.8% agarose gels and transferred onto Hybond-N$^+$ nylon membranes (GE Healthcare, Buckinghamshire, UK). The probes were prepared by amplifying the 5′ region of cotton *BC*, *BCCP* and α-*CT* cDNAs (~1000 bp) and labeled with [α-^{32}P] dCTP (Random Primer DNA Labeling Kit Ver. 2, Takara, Dalian, China). After hybridization, the membrane was washed in the following buffers twice: 2× SSC with 0.1% SDS and 0.5× SSC with 0.1% SDS, at 65 ℃ and exposed to X-ray films (Kodak) at −70 ℃ for 24 h.

Semi-quantitative RT-PCR analysis was carried out according to the manufacturer's instructions (Re-

verse Transcription System, Promega, Madison, WI, USA). Three pairs of primers were designed for semi-quantitative RT-PCR to amplify cDNA of *BC* transcripts (5′-TCCCTGTCTCACTACATTGCTCT-3′ and 5′-TCCTGCTTCCCACCATAAAGGT-3′), *BCCP* transcripts (5′-CCAACAGCAGAAGCAAAGGATG-3′ and 5′-GCAACTCCACAATACCTCGTGA-3′) and α-*CT* transcripts (5′-CCATTGGTTTGATTGTTGAGGGTG-3′ and 5′-AGGTTTGTTTCCTGCCATTGCT-3′). In order to normalize the template amount of different samples, we designed two primers (UBQ7-1: 5′-AGGCATTCCACCTGACCAAC-3′ and UBQ7-2: 5′-GCTTGACCTTCTTCTTCTTGTGC-3′) to amplify *Ubiquitin7*, a house-keeping gene, as the internal control. The PCR products were separated by electrophoresis on 1.2% agarose gels.

2 Results and discussion

2.1 Isolation and sequence characterization of cotton heteromeric ACCase genes

Table 1 summarizes the general information of the five cloned genes which are designated as *GhBC1*, *GhBCCP1*, *GhCTα1*, *GhCTα2* and *GhCTβ*. The entire nucleotide sequences of the five novel genes have been deposited in GenBank with the accession numbers assigned in Table 1. In most higher plants, the BC, BCCP and α-CT are encoded by the nuclear genome, translated in cytoplasm and then imported into the plastid[22]. Sequence analysis indicated that all of the deduced GhBC1, GhBCCP1, GhCTα1 and GhCTα2 amino acid sequences had a typical Met-Ala sequence at their N terminal ends. In addition, they were rich in hydroxylated amino acids (Ser and Thr), and contained several small hydrophobic amino acids (Ala and Val) and positively charged residues (Arg and Lys). These are typical of many transit sequences[23]. So we further analyzed the protein sequences of the five genes by ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/). The analysis results confirmed the presence of chloroplast transit peptides (cTP) in GhBC1, GhBCCP1, GhCTα1 and GhCTα2 subunits, but not in GhCTβ subunit (Table 1). The cleavage sites of potential cTPs were also predicted by the Server. The results indicated that the cotton heteromeric ACCase had the similar cTP length with those from soybean[14].

Table 1. Features of cotton heteromeric ACCases

Gene	ACCase Subunit	Accession No.	cDNA length (bp)	Coding region (bp)	Predicted cTP[a] length	Deduced protein MM[b] (kD)		Calculated pI	
						Precursor	Mature	Precursor	Mature
GhBCCP1	BCCP	EF555556	1246	843	62	29.6	22.9	8.86	4.62
GhBC1	BC	EF534181	1867	1602	53	58.6	52.8	7.71	6.52
GhCTα1	α-CT	EF564625	2883	2277	49	84.9	80.0	8.32	7.53
GhCTα2		EF564626	2808	2280	50	85.0	80.0	8.57	7.79
GhCTβ	β-CT	EF564627	1728	1488	na[c]	na	56.1	na	4.40

[a] cTP, chloroplast transit peptides; [b] MM, molecular mass; [c] na, not applicable

The catalytic regions of heteromeric ACCase subunits were denoted by NCBI (http://www.ncbi.nlm.nih.gov/) (Fig. 1). The functional domain was located in the C terminal region of GhBC1, GhBCCP1 and GhCTβ subunits, but near the N terminal region of GhCTα1 and GhCTα2 subunits. The GhBC1 subunit also contains an ATP binding domain in its central region. A highly conserved sequence motif, AMKLM, was identified in the C terminal region of GhBCCP1 subunit and the biotin was attached to the lysine residue of this motif (Fig. 1). While this research was undertaking, the chloroplast genome of *G. hirsutum* (cv. coker 310 FR) was completely sequenced[24]. By comparison, we found that the *GhCTβ* was nearly the same as the counterpart gene in the reported chloroplast genome (Data not shown).

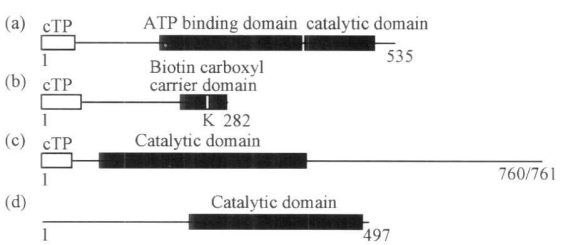

Fig. 1. Schematic diagrams of cotton heteromeric ACCase subunits. (a) GhBC1; (b) GhBCCP1; (c) GhCTα1 and GhCTα2; (d) GhCTβ.

2.2 Phylogenetic analysis and amino acid sequence similarity of cotton heteromeric ACCase

The heteromeric ACCase is found in bacteria and most plants (except for the *Poaceae*), but not in animals and fungi. Furthermore, the locations of the four genes encoding the heteromeric ACCase subunits are not the same within different species. In bacteria, the four subunits are encoded by the same one circular genome. And in modern plants, only the β-*CT* remains in the circular bacterial type plastid genome, the other three subunits are encoded by the nuclear genome. However, in some primitive plants, both of the genes encoding the CT subunits (α-CT and β-CT), sometimes even the BCCP subunit are encoded by the plastid genome[25]. All of these findings suggest a complex and continuous evolutionary pattern of the different heteromeric ACCase subunits.

The phylogenetic analysis was performed using the reported heteromeric ACCase subunits from the modern plants (*G. hirsutum*, *A. thaliana*, *G. max*, *B. napus*, *N. tabacum*, *M. truncatula*, *P. sativum*, *L. japonicus* and *S. tuberosum*), bacteria (*E. coli* and *D. acidovorans*) and algaes (*S. elongatus* and *T. erythraeum*). As shown in Fig. 2, all the subunits from the modern plants are grouped together. And the proteins from bacteria and algaes are also grouped into two separate subgroups respectively, suggesting that the four subunits diverged with the similar pattern. The phylogenetic analysis clearly indicated the co-evolution pattern of the four heteromeric ACCase subunits. It also appeared that cotton heteromeric ACCase is closely related to soybean in the plant heteromeric ACCase subgroup.

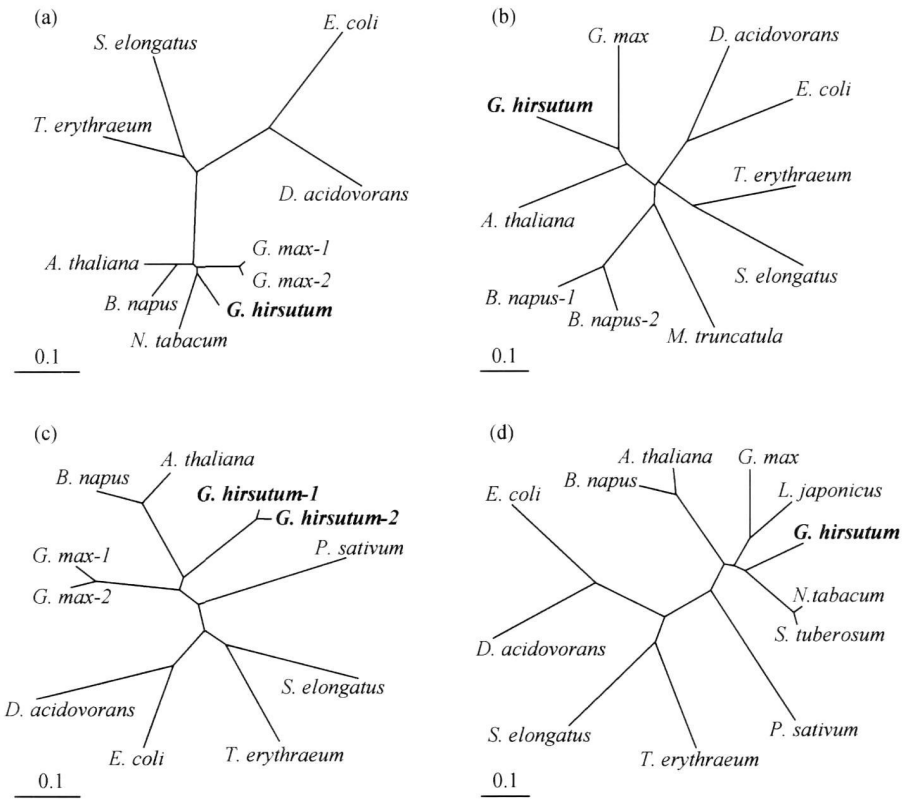

Fig. 2. Phylogenetic analysis of heteromeric ACCase subunits from bacteria, algeas and plants. (a) Phylogenetic analysis of BC subunit from *E. coli* (M79446), *D. acidovorans* (ZP_01578982), *S. elongates* (YP_477622), *T. erythraeum* (YP_723239), *A. thaliana* (U90879), *G. max* (AF007100, AF068249), *B. napus* (AAK60339), *N. tabacum* (L38260) and *G. hirsutum* (EF534181, the present study). (b) Phylogenetic analysis of BCCP subunit from *E. coli* (M32214), *D. acidovorans* (ZP_01578983), *S. elongates* (AAB82026), *T. erythraeum* (YP_721112), *A. thaliana* (U62029), *G. max* (U40666), *B. napus* (X90730, X90731), *M. trunacatula* (ABE89480) and *G. hirsutum* (EF555556, the present study). (c) Phylogenetic analysis of α-CT subunit from *E. coli* (M96394), *D. acidovorans* (EAV72378), *S. elongates* (ZP_01469470), *T. erythraeum* (ABG50351), *A. thaliana* (AF056970), *G. max* (U34392, U40979), *B. napus* (AAS46759), *P. sativum* (AB029556) and *G. hirsutum* (EF564625, EF564626, the present study). (d) Phylogenetic analysis of β-CT subunit from *E. coli* (D90862), *D. acidovorans* (ZP_01577507), *S. elongates* (ZP_01468249), *T. erythraeum* (ABG51873), *A. thaliana* (AF056971), *G. max* (U26948), *L. japonicus* (AP002983), *S. tuberosum*, (AF069288), *B. napus* (Z50868), *P. sativum* (X15268), *N. tabacum* (NC_001879) and *G. hirsutum* (EF564627, the present study).

In addition, there are significant similarities among the ACCase subunits from different, even very divergent species. The overall amino acid sequence similarity of cotton heteromeric ACCase subunits with other species is summarized in Table 2. The GhBC1 is the most conserved among these subunits, showing very similar sizes and high identity with *A. thaliana* (85.1%), *G. max* (85.8%), *E. coli* (43.8%) and *S. elongatus* (55.3%). The BCCP subunits have the variable protein length and low similarity compared with others. But the biotinylation motif (Fig. 1) is very conservative and could be easily identified among all of the reported BCCP subunits. Previous experimental results indicated that plant BCCPs were readily biotinylated by *E. coli*[14], suggesting the importance of the overall structure for its proper function[26,27]. Similarly, although the plant β-CTs are somewhat larger than bacterial and algae proteins, the β-CTs are also much conserved in their functional regions. The notable exception is the α-CT subunit. The α-CT from different species aligned well over the catalytic region, but modern plant α-CT was more than twice the size of the bacterial and algae proteins (Data not shown). This is because that the modern plant proteins have a large extra segment in its C-terminal region. This segment is poorly conserved among plant α-CT subunits and is of unknown function. It often contains repeated sequences and the experimental results showed that the C-terminal segment could be deleted without loss of activity[28].

Table 2. Homologies (% identical amino acids) with other heteromeric ACCases subunits

Subunit	*E. coli*	*S. elongatus*	*A. thaliana*	*G. max*
GhBC1	43.8	55.3	85.1	85.8
GhBCCP1	24.1	22.3	53.5	56.3
GhCTα1	19.5	21.1	63.5	62.5
GhCTα2	19.4	21.7	64.0	62.5
GhCTβ	22.6	21.5	66.3	61.4

GenBank accession number: BC subunit (*E. coli*, M79446; *S. elongatus*, YP_477622; *A. thaliana*, U90879; *G. max*, AF007100), BCCP subunit (*E. coli*, M32214; *S. elongatus*, AAB82026; *A. thaliana*, U62029; *G. max*, U40666), α-CT subunit (*E. coli*, M96394; *S. elongatus*, ZP_01469470; *A. thaliana*, AF056970; *G. max*, U34392) and β-CT subunit (*E. coli*, D90862; *S. elongatus*, ZP_01468249; *A. thaliana*, AF056971; *G. max*, U26948).

2.3 Southern blot and expression pattern of heteromeric ACCase

In *Arabidopsis*, the *BC*, *α-CT* and *β-CT* exist as single-copy genes and the *BCCP* exists as a two-copy gene[29]. But in soybean, there are several genes that encode each subunit of heteromeric ACCase[14]. Because *G. hirsutum* is an allotertraploid with two distinct subgenomes (A and D) from two ancestors[30], we supposed that there would be several genes encoding each subunit of heteromeric ACCase in the genome of *G. hirsutum*. To determine the approximate gene numbers of each subunit in the cotton genome, the Southern blot analysis was performed with *Dra* I-, *Eco*R I-, *Eco*R V-, *Hin*d III- and *Xba* I-digested cotton genomic DNA hybridized with radioactively labeled probes generated from each gene's partial coding region. As shown in Fig. 3, the complex hybridization patterns in each lane indicate that the cotton genome may have several gene copies of each subunit. Consistent with this result, two cDNAs encoding the same α-CT subunit were successfully cloned, which were very similar in the coding region but different only in UTR.

To investigate the expression pattern of each gene in various tissues under normal growth conditions, total RNA extracted from the roots, stems, true leaves, cotyledons, flowers, seeds, embryo buds and fibers were subjected to semi-quantitative RT-PCR assay using primers specific for each gene. All of the RT-PCR experiments were repeated twice. As shown in Fig. 4, the heteromeric ACCase genes were constitutively expressed in all the tissues of cotton, indicating the indispensable roles played by heteromeric ACCase in cotton growth and metabolism. However, their expressions were at a relatively low level in roots, suggesting that the root's fatty acid synthesis was relatively inactive. In addition, the heteromeric ACCase transcripts were abundant especially in flowers, true leaves and fibers, indicating the active fatty acids synthesis in these tissues. The cotton seeds synthesize a large amount of fatty acids during its development. But our results showed that the ACCase transcripts in seeds were relatively low compared with those of flowers, true leaves and fibers. The reason might be that the seed samples used in this experiment were at 40 days post anthesis and the fatty acid synthesis rate might be slow in the stage. These results confirmed that the heteromeric ACCase is the vital protein in all tissues.

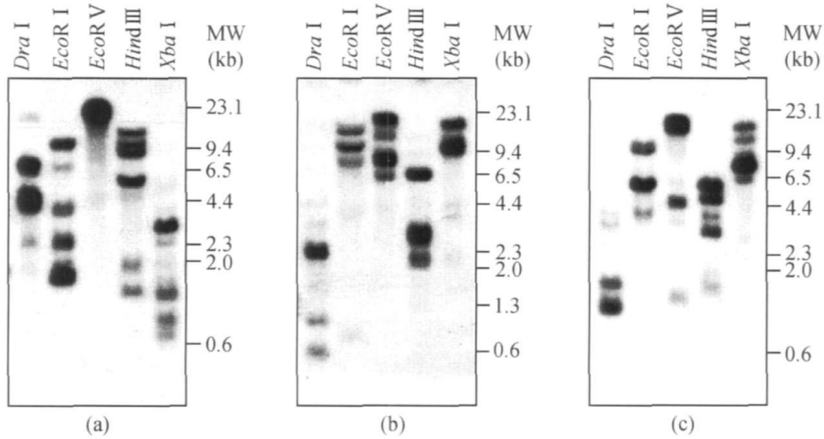

Fig. 3. Southern blot analysis of cotton *BC* (a), *BCCP* (b) and α-*CT* (c).

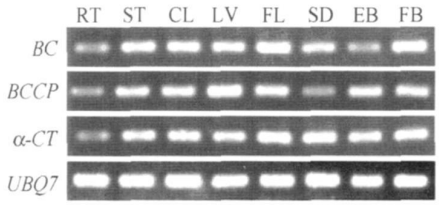

Fig. 4. Expression profiles of *BC*, *BCCP* and α-*CT* in various organs. Total RNA was isolated from roots (RT), stems (ST), true leaves (LV), cotyledons (CL), flowers (FL), seeds (SD), embryo buds (EB) and fibers (FB) of cotton. The *UBQ7*, the abbreviation of *Ubiquitin7*, was used as the internal control.

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