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CLONING OF ACETYL-CoA CARBOXYLASE GENE FROM *BRASSICA JUNCEA* CV. PUSA BOLD

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SUMMARY

Using degenerate primers, a partial genomic sequence encoding *ACCase* gene was PCR amplified, cloned and analyzed for sequence homology. Using this partial sequence as probe and on screening genomic library of *B. juncea,* two putative positive clones were isolated and confirmed by restriction and Southern analysis. A ~5 kb fragment, identified by Southern blot hybridization was subcloned which showed strong hybridization signal with probe pACC1. This confirmed the cloning of ACCase gene of *B. juncea.*

Key words: ***ACCase* (acetyl-CoA carboxylase), cloning, genomic library screening, PCR amplification, subcloning.**

INTRODUCTION

Acetyl-CoA carboxylase (ACCase, EC.6.4.1.2) is the first enzyme in the lipid biosynthetic pathway of plants, which catalyzes the formation of malonyl-CoA and HCO^-_3 in an ATP dependent two step mechanism (Wood and Barden 1977). ACCase is the key enzyme in the regulation of lipid biosynthesis (Kim *et al.* 1989), as it plays regulatory role in the fatty acid elongation in plants. Two isoforms of ACCase are present in plants. In plastids, ACCase provides malonyl-CoA for fatty acid biosynthesis (Harwood 1988) and in cytosol, malonyl-CoA enroutes for fatty acid elongation, flavonoid biosynthesis (Ebel and Halbrock 1977) and several other pathways (Nikolau *et al*.1984). To have insight and to understand the role of ACCase and its genetic manipulation, it requires the isolation and characterization of the gene encoding it. In the present work, a partial genomic sequence encoding *ACCase* was PCR amplified using specific designed primers and used as a probe to screen the genomic library of *B.juncea*.

MATERIALS AND METHODS

Plant material

Six-seven days old etiolated seedlings of *Brassica juncea* were used for genomic DNA isolation.

Genomic DNA isolation

DNA was isolated from the etiolated seedlings of *B.juncea* by following the cetyl trimethyl ammonium bromide (CTAB) DNA extraction method (Murray and Thomson 1980). DNA was purified by RNase treatment and phenol chloroform - isoamyl alcohol extraction.

PCR amplification

A pair of degenerate primers were designed and custom synthesized from Genetix India Pvt. Ltd. They are:

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Forward primer: 5' CCT GAT GAC GGA TTC ATA CC 3'

Reverse primer: 5' AAA AGG TCA ATC GTG ATG TC 3'

PCR amplification of genomic DNA was done in Minicycler M J Research, following the programme of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, a final extension at 72°C for 10 min.

Cloning of PCR product

The PCR amplified product was purified and cloned in plasmid vector T/A tailed pUC 57 from MBI Fermentas.

DNA sequencing

Sequencing of the double stranded plasmid DNA was performed following the method of Hattori and Sakaki (1986), using a commercially available Reader ™ DNA sequencing kit from MBI Fermentas and automated DNA sequencing facility from South campus, Delhi University.

Sequence analysis

DNA sequence analysis for similarity and protein encoding was done using programme BLASTN (Altschul *et al*. 1997) search and FASTA (Pearson and Lipman 1988) server at internet site http:// www.genome.ad.jp.

Screening of genomic library

A genomic library of *B. juncea,* constructed with partial *Sau*3A digested genomic DNA in *Bam* HI restriction arms of λ EMBL –3 vector available in our lab (Garg *et al*.1999), was screened using PCR amplified and cloned *ACCase* sequence as probe following standard protocols (Sambrook *et al*. 1989).

Restriction analysis of λ–Phage clones

The positive clones identified were picked up and DNA was isolated from the λ phage clones by using miniprep protocol for phage isolation (Sambrook *et al*. 1989) and used for restriction and Southern analysis (Southern 1975)

RESULTS AND DISCUSSION

ACCase is an important regulatory enzyme of plant fatty acid synthesis. It catalyzes the synthesis of malonyl-CoA which is utilized in plastids for fatty acid synthesis and outside the plastids for a variety of reactions like elongation of fatty acid chain, flavanoid biosynthesis and malonation of amino acids (Shorrosh *et al*. 1995). Two isoforms of ACCase, prokaryotic and eukaryotic, have been isolated and characterized in many plant species. The prokaryotic (multisubunit) form exists in plastids while, eukaryotic form is present in the cytoplasm. To gain insight into the gene sequence of *ACCase*, in the present study attempts were made to clone gene that encodes enzyme acetyl-CoA carboxylase from *Brassica juncea*. A two step strategy was followed to isolate and clone the *ACCase* gene (Gould *et al.* 1989). The first step was to generate probe (a partial *ACCase* gene sequence), and the second was screening of genomic library using the probe.

By comparing the available sequence of *ACCase* in the GenBank forward and reverse primers were designed from the conserved region and got custom synthesized. They were used for amplifying a genomic sequence from *B. juncea* genomic DNA using PCR technique. The PCR amplified product was separated on agarose gel and was found to have a size of 0.5 kb. The amplified product was gel eluted and cloned in pUC 57 using the T/A cloning system and *E. coli* DH5-α competent cells. Recombinants were selected on X-gal / IPTG plates and plasmid isolated from recombinants, and analyzed on agarose gel after restriction with *Bam* HI and *Xba* I. Fig.1a shows the agarose gel electrophoresis pattern of PCR amplified as well as cloned product. The clone having the 0.5 kb insert was further subjected to DNA sequence analysis. The insert was also confirmed by Southern hybridization using PCR amplicon as probe (Fig.1b). Sequencing of the clone was carried out using vector specific primers by manual sequencing as well as by automated sequencing facility available at Delhi University (South Campus).

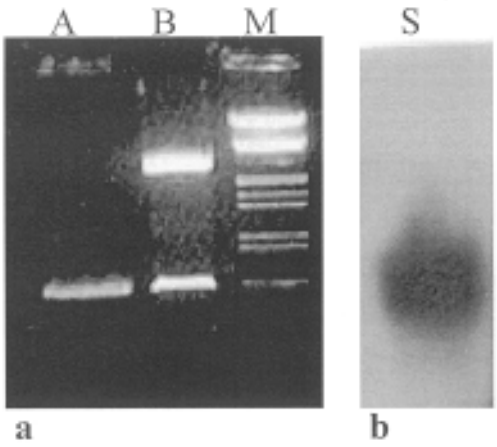

Fig.1a. Agarose gel (1.2%) showing PCR amplified fragment with specific primers. (Lane A) , *Bam* HI & *Xba* I restricted pUC57 clone with insert (Lane B) and Marker (Lane M).

b. Autoradiogram showing Southern blot hybridization of cloned insert (lane-S)

The nucleotide sequence of 498 bp with the predicted exons and introns is shown in Fig. 2. Similarity search using BLASTN showed 95% similarity with *ACCase* gene of *B.napus* and 98% similarity with *B. napus* strain Darmor *bzh* acetyl-CoA carboxylase (*ACC*) gene. On BLASTX search analysis, sequence showed 100% similarity with *B. napus ACCase*. These observations confirm the amplification and cloning of partial *ACCase* gene sequence of *B. juncea*. The *ACCase* gene sequence on comparison with cDNA sequence of *B. napus* and consensus splicing sequences found to have 4 exons and 3 introns. The exons have precise splicing sequences AG and GT on their ends. First and last exons are not complete. The sizes of exons vary from 37 nucleotides to 127, while size of introns vary from70 to 90 nucleotides.

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ctgatgacgg attcataccc accggcggta aagtacaggt    40
aatatatagc tgcggaatgc aaaagttcaa agtcttttaa    80
ctgagaattg tgtggggta acactcgtat gaacttgcaa   120
caggagttga gttttaaaag caagccaaat gtgtgggcct   160
acttctctgt caaggtagac atatctatat atgctctgct   200
atattatgtg tttggctatg tgactatggc ttaaatttta   240
caaccatctt tttcttacag cctggtggag gcatccacga   280
gttctcagat tcccaatttg gtgagttaaa taaaatatgt   320
gctactaggt cagcttgagt ttaccttgtt gaaactttct   360
ttaaccttct gttcaggcca tgtttttgca tttggggaat   400
ccagagcctt ggcaatagca aatatggtcc ttgggcttaa   440
agaaattcaa attcgtggag aaattaggac aaacgttgac   480
tacacgattg acctttt                           500
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Fig.2. Nucleotide sequence of PCR amplified fragment of *ACCase* gene of *B. juncea*. Bold letters are representing exons and normal letters are showing intronic regions. The sequence is submitted to EMBL GenBank with Accession No. AJ564012

This sequence of *ACCase* of *B. juncea* was submitted to EMBL GenBank and the Accession no. given is AJ564012. The gene sequence isolated is a partial gene sequence of *ACCase* and showed homology with region 2016 to 2505 of *ACCase* gene of *B. napus*.

The partial *ACCase* gene was used as a probe for screening the genomic library of *B. juncea*. After tertiary screening, two putative clones namely pKA1 and pKA2 which showed hybridization were identified and phage DNA was isolated from these clones. The DNA was restricted with *Sal*I to take out the insert and analyzed on 0.8% agarose gel which showed an insert of 18 kb. Restriction pattern with S*al*I, *Sal*I + *Bam*HI, *Sal*I+*Eco*RI and *Sal*I+*Pst*I showed that both the clones were similar (Fig 3a). This is because the genomic library is an amplified one and may have more than one copy of the clone. On Southern blotting and hybridization of restricted fragments with pACC1 probe (Fig. 3b), a fragment of ~5 kb in

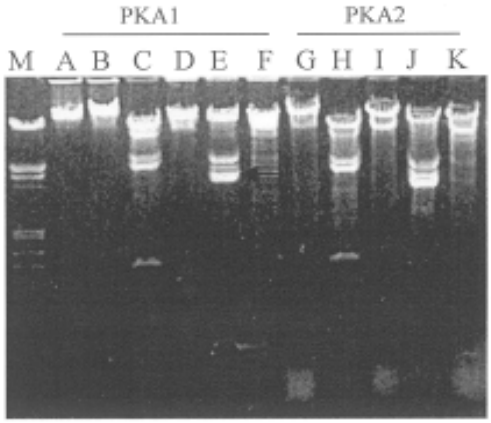

Fig.3a. Agarose gel (0.8%) electrophoresis of pKA1 and pKA2 phage DNA restricted with *Sal*I, *Bam*HI, *Eco*RI and *Pst*I. Marker (lane-M), uncut PKA1(lane- A), cut with *Sal*I (lanes- B,DG&I),cut with *Sal*I and *Bam*HI(lanes-C&H), cut with *Sal*I and *Eco*RI (lanes-E&J) and cut with *Sal*I and *Pst*I (lanes- F &K)

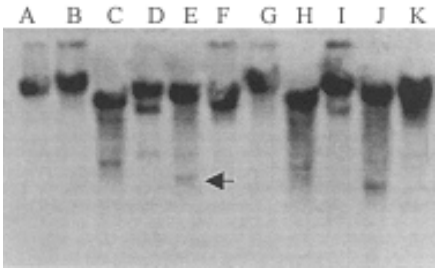

Fig.3b. Autoradiogram showing Southern blot hydridization of PKA1 and PKA2

*Sal*I+*Eco*RI cut lane showed strong hybridization. This fragment was gel eluted and subcloned in order to isolate the complete gene. Restricted plasmid DNA of the subclone with *Eco*RI, showed a ~5 kb fragment on 0.8% agarose gel (Fig. 4a). This subclone was further confirmed by Southern hybridization with pACC1 probe. Hybridization result showed the fragment to be positive (Fig. 4b).End sequencing and homology search showed it

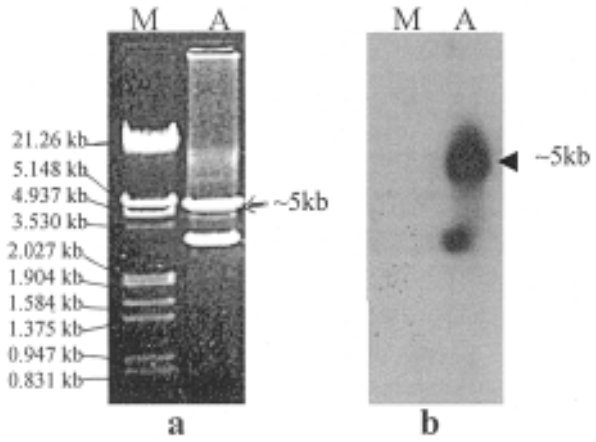

Fig.4a. Agarose gel (0.8%) showing a ~5kb fragment subcloned in SK^+ phagemid vector (lane-A) and Lambda phage DNA cut with *Eco*RI and *Hind*III as marker (lane-M) and
b. Autoradiogram showing hybridization pattern of Fig. 4a.

to carry ACCase gene. Complete sequencing and analysis of this subclone will reveal if it carries the complete sequence of the *ACCase* gene from *B. juncea* and also what isoform does it code. The *ACCase* gene can be used for further genetic manipulation.

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