Indian J. Plant Physiol., Vol. 13, No. 4, (N.S.) pp. 334-338 (Oct.-Dec., 2008)

MOLECULAR CLONING OF PARTIAL OLEATE DESATURASE GENE FROM *BRASSICA NAPUS*

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Received on 24 Dec., 2008

SUMMARY

The polyunsaturated fatty acids linoleate and α-linolenate are important membrane components and are the essential fatty acids of human nutrition. The major enzyme responsible for the synthesis of these compounds is the plant oleate desaturase of the endoplasmic reticulum, and its activity is controlled in *Brassica napus* by the fatty acid desaturation 2 (*fad2*) locus. We report the isolation of partial *fad2* gene fragment from *Brassica napus*. A pair of primers were synthesized based on the conserved region of all plant *fad 2* gene sequences available in NCBI database. Using these primers in PCR, 987 bp fragment was amplified from *Brassica napus* genomic DNA. After purification, the fragment was cloned into the pGEMT Easy vector and identified as a part of the gene encoding oleate desaturase. Comparison of the nucleotide sequence revealed similarity with other reported oleate desaturase gene sequences. It can be used to isolate full length gene/cDNA encoding oleate desaturase.

Key words: **Cloning, oleate desaturase, triacylglycerols**

INTRODUCTION

The polyunsaturated fatty acids linoleate (delta, 12-18:2) and α-linolenate (Δ 9, 12, 15-18:3) are synthesized by plants but not by most other higher eukaryotes. Both of these fatty acids are essential components of human nutrition, because in mammals they act as precursors not only of membrane lipids but also of families of signaling molecules including the prostaglandins, thromboxanes, and leukotrienes (Smith and Borgeat 1985). In many higher plants, 18:2 and 18:3 account for more than 70% of the fatty acids in leaf cells and 55 to 70% of the fatty acids in non photosynthetic tissues such as roots (Harwood 1980). In both plants and animals, polyunsaturated acyl structures are considered to be essential membrane components, in part because they are virtually ubiquitous in the membranes of higher eukaryotes. Experimental manipulations designed to reduce membrane polyunsaturation also point to a critical role for these structures in ensuring proper membrane function and organism viability (Hugly and Somerville 1992, Miquel *et al*. 1993). In angiosperms as a whole, the vast majority of polyunsaturated lipid synthesis passes through a single enzyme, the 18:l desaturase of the endoplasmic reticulum. Although a chloroplast 18:l desaturase also operates in leaf cells of at least some plants, it is likely that the endoplasmic reticulum enzyme is quantitatively more important even in these cells (Browse and Somerville 1991, Miquel and Browse 1992). Furthermore, it is responsible for more than 90% of the polyunsaturated fatty acid synthesis in non photosynthetic tissues, such as roots, and in the developing seeds of oil crops, including soybean, sunflower, and canola, in which fatty acids are stored as triacylglycerol oils. Thus, one important function of the endoplasmic reticulum 18:l desaturase is to provide 18:2 and (following further

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desaturation) 18:3 required for the correct assembly of cellular membranes throughout the plant. Just as importantly, the enzyme provides the polyunsaturated fatty acids found in vegetable oils that in turn are the major source of essential fatty acids in most human diets. Mutants of *Arabidopsis* at the *fatty* acid desaturarion 2 *(fad2)* locus are deficient in activity of the endoplasmic reticulum desaturase (Miquel and Browse 1992). Biochemical and genetic studies of these mutants have been important to our understanding of this desaturation step because the enzyme, like most of the plant desaturases, is an integral membrane protein that has been difficult to solubilize and therefore, to investigate by traditional enzymological methods. In the absence of a purified enzyme, genetic techniques can also provide an alternative means to clone the relevant genetic locus. For example, cDNAs corresponding to the *FAD3* gene have been obtained by both map-based cloning (Arondel *et al*. 1992) and by gene tagging (Yadav *et al.* 1993). The homology of these cDNAs to other desaturase genes indicates that they correspond to the structural gene encoding the endoplasmic reticulum 18:2 desaturase. The *FAD3* cDNAs have made possible the cloning of at least three distinct, additional desaturase genes. Each of these contained a 5' peptide sequence with the characteristics of a chloroplast transit peptide and, on this basis, the genes are thought to encode three of the chloroplast desaturases (Iba *et al.* 1993, Yadav *et al*. 1993). In this paper we report the PCR amplification and cloning of a partial gene encoding oleate desaturase from *Brassica napus.*

MATERIALS AND METHODS

Brassica napus seeds were obtained from Indian Agricultural Research Institute, New Delhi.

Genomic DNA isolation: Etiolated seedlings of *Brassica napus* were used for plant DNA isolation following the CTAB method of DNA isolation (Webb and Knapp, 1990). Four grams of plant sample was crushed in liquid nitrogen and transferred to 16 ml DNA extraction buffer preincubated at 62^0C (10ml 1M Tris-Cl pH 8.0, 4ml 0.5 M EDTA pH 8.0, 35ml 4M Nacl, 20 ml 10% CTAB, 31 ml H_2O 40 µl β- mercaptaoethanol) and left at 62^0C for 1 1/2 hr. An equal volume of chloroform:isoamylalcohol (24:1) was added, centrifuged at 10,000 x g for 10 min. To the supernatant equal volume of isopropanol was added, centrifuged and the pellet was washed with 70% ethanol, air dried and dissolved in TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA).

Designing of gene specific primers: Available nucleotide sequences of oleate desaturase from GenBank database were aligned to find conserved stretches. From the conserved regions of *Brassica compestris fad2* (SGS F) forward primer (5'-GCTCCTTCTCCTACC TCATC-3', Tm ~52^0C) and reverse primer (SGS R) (5'-TACCAGAACACACCTTTCTTC-3', Tm~ 54^0C) were designed and got custom synthesized (Sigma, USA).

PCR amplification and purification of PCR product: For PCR amplification 2.5 µl 10x PCR buffer, 2.5ul 0.1 mM dNTP mix,100 pmol of forward and reverse primers,1.25 U Taq DNA polymerase,50 ng template DNA were added and volume made to 25 µl and kept in a thermal cycler following a programme of initial denatuartion of 94^0C for 4 min, followed by 35 cycles of denaturation at 94^0C for 45 seconds, annealing at 52^0C for 30 seconds, extension of 72^0 C for 1.5 min and final extension at 72^0C for 10 min after completion of the cycles. The PCR product was electrophoresed on a 1% agarose gel and amplified product was gel eluted using QIAquick® gel extraction kit from Qiagen according to the manufacturer's protocol.

Cloning and sequencing of PCR amplified fragment: PCR amplified fragment was cloned into T overhangs of pGEMT Easy vector from Promega (USA) by ligating using 3 µl PCR fragment, 1 µl pGEMT Easy vector, 5 µl 2x ligation buffer and 1 µl T4 DNA ligase at 14^0C overnight and transforming the ligation mix into competent *E.coli* (DH5α) cells.

Sequencing of the insert DNA fragment: Sequencing of the insert DNA fragment was done by automated DNA sequencing facility available at Delhi University South campus. The universal primers T7 and SP6 were used for this.

RESULTS AND DISCUSSION

The gene specific primers SGS F and SGS R were used in PCR and electrophoresis of the PCR product in a 1% EtBr stained agarose gel showed a single, prominent band at ~0.98 kb (Fig.1).This amplified DNA

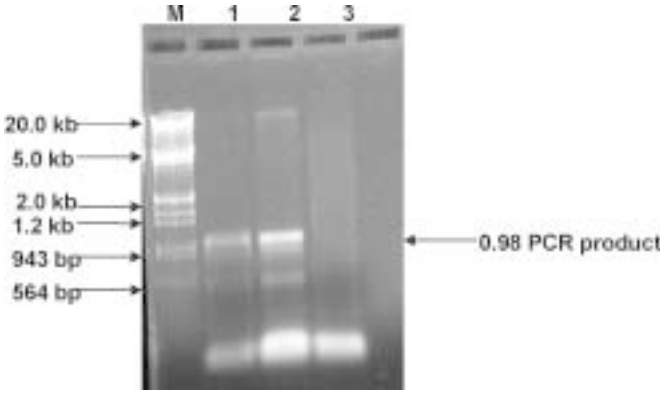

Fig. 1. 1.0 % agarose gel electrophoresis showing 0.98 kb amplicon obtained after PCR amplification from *Brassica napus* genomic DNA using *fad2* gene specific primers. M-marker (λ *Eco*RI + *Hin*dIII), lanes: 1, 2, 3 at different Tm (Gradient temperature)

fragment was gel eluted, ligated and transformed in *E.coli*. The recombinant clones were selected by blue/ white screening. Plasmids from recombinant clones were isolated, digested with *Eco*RI and was separated on 1.0% agarose gel along with λ DNA cut with *Hin*dIII + *Eco*RI marker (Fig. 2). Restriction digestion of recombinant

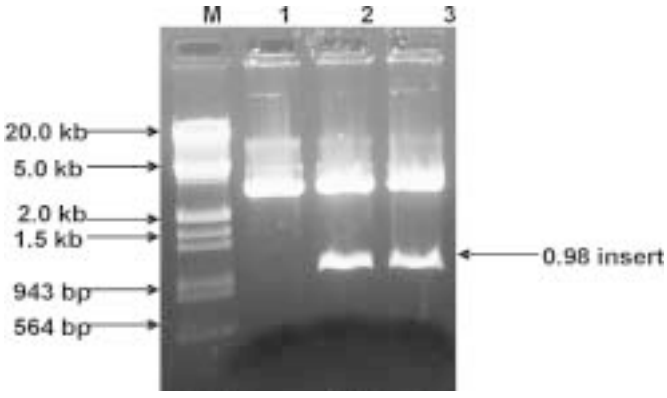

Fig. 2. EtBr stained 1.0% agarose gel showing the *Eco*RI restriction of the recombinant clone containing partial *fad2* gene. M-marker (λ *Eco*RI + *Hin*dIII), lanes: 1, 2, 3- different clones.

plasmid released an insert of 0.98 kb and is shown in (Fig. 2). One of those positive clones was sequenced by automatic DNA sequencing facility in Delhi university, South Campus. The complete insert was 987 bp in length (Fig. 3) and homology search with BLASTN

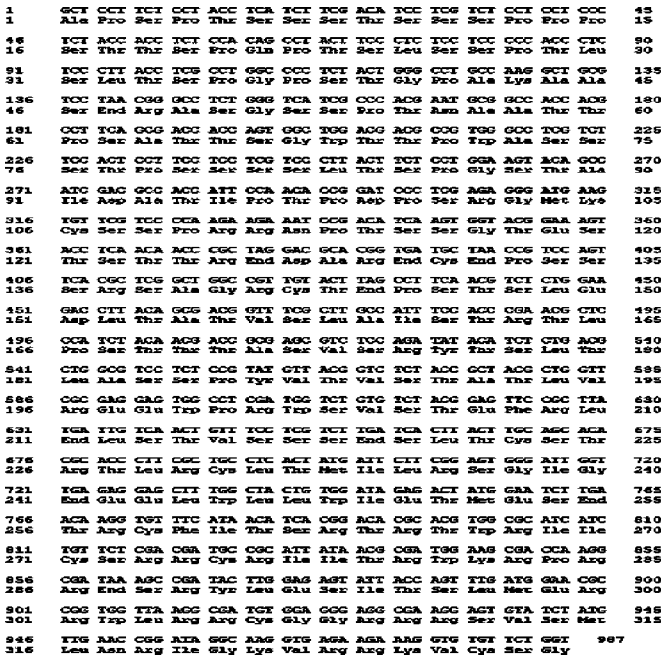

Fig. 3. Nucleotide (upper) and deduced amino acid (lower lane) sequence of *Brassica napus* partial *fad2* gene sequence. Numerals on the right represent nucleic acid and amino acid residue number.

server developed by NCBI, USA (Altschul *et al.* 1990) showed that it has maximum homology 95% with *Brassica napus* mutant *fad2* and also with other *fad2* sequences of different plant species (Fig. 4). Similar studies have been reported in number of plants using different approaches. A locus corresponding to microsomal ω-6 desaturase structural gene was successfully isolated for the first time from *Arabidopsis thaliana* by T- DNA tagging (Okuley *et al.* 1994). Since then cDNAs coding for this gene have also been isolated from Soybean (Heppard *et al.* 1996), *Brasica napus* (Scheffler *et al.* 1997), *Brassica carinata* (Marilla and Taylor 1999), *Gossypium hirsutum* (Liu *et al.* 1999), *Helianthus anus* (Martinez-Rivas *et al.* 2001), *Sesamum indicum* (Kim *et al.* 2006) and *Olea europaea* cv Picus (Hernadez *et al.* 2005).

Sequences producing significant alignments

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF 371480.1	*Brassica napus* cultivar Xiang You 15 mutant delta-12 oleate desaturase (FAD2) gene, complete sequence	1554	1554	100%	0.0	95%
AY 592975.1	*Brassica napus* delta-12 oleate desaturase mRNA, complete cds	1548	1548	100%	0.0	94%
AJ 459108.2	*Brassica campestris* fad2 gene for fatty acid desaturase 2, exons 1-2	1277	1277	100%	0.0	90%
AJ 459107.1	*Brassica campestris* mRNA for fatty acid desaturase 2 (fad2 gene)	1277	1277	100%	0.0	90%
AF 243045.1	*Brassica napus* delta-12 oleate desaturase mRNA, complete cds	1271	1271	100%	0.0	89%
AY 577313.1	*Brassica napus* delta-12 oleate desaturase (FAD2) mRNA, complete cds	1260	1260	100%	0.0	89%
EF 639848.1	*Brassica juncea* fatty acid desaturase 2 (fad2) mRNA, complete cds	1249	1249	100%	0.0	89%

Fig. 4. BLASTN homology search analysis of isolated partial *fad2* gene from *Brassica napus*.

As ω-6 desaturases regulate the membrane properties and the synthesis of storage lipids, the study of genetic regulation of these enzymes is important and needs the isolation and characterization of their cDNA and genomic sequences and to analyze the expression as the first step. This partial *fad2* gene from the present work can be used as homologous/heterologous probe to isolate the full length gene along with promoter from various plants. This will aid in understanding the gene structure and regulation of *fad 2* gene may result in altered oil content to improve the quality of vegetable oils.

The present study provides an easy way to isolate and characterize promoter specific for *fad2* gene from *Brassica napus* and to study its regulatory role along with 5' UTR intron on the expression of gene.

ACKNOWLEDGEMENTS

The financial support to the first author from IARI and CSIR in the form of JRF is thankfully acknowledged.

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