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FUNCTIONAL ANALYSIS OF OLEATE DESATURASE GENE EXPRESSION FROM *BRASSICA JUNCEA*

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Functional analysis of oleate desaturase gene expression from *Brassica juncea*

By

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This is to certify that the thesis entitled, **"Functional analysis of oleate desaturase gene expression from *Brassica juncea*"** submitted to the Faculty of Post-Graduate School, Indian Agricultural Research Institute, New Delhi by **Suresha G.S**, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Biochemistry**, embodies the of *bona fide* research work carried out by him under my guidance and supervision and that no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation have been duly acknowledged.

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INTRODUCTION

Oilseed crops and their products are the second most important commodity in world today. New global markets are opening up opportunities for novel products produced by oilseed crops which can be either edible or non edible. The fatty acids have been extensively exploited for industrial uses in products such as lubricants, plasticizers, soaps and surfactants. In fact, approximately 10% of vegetable oils produced in the world are used for non-food purposes (Topfer *et al* ., 1995).The alcohol esters of some plant oils may find future use as a diesel fuel substitute which in addition to being less polluting will not need expensive refining methods (Goddijin *et al*., 1995). In view of rich heritage of knowledge in the chemical industry of the properties and chemical potential of fatty acids and their derivatives, oilseed crops can be seen as efficient, low polluting chemical factories that harness energy from sunlight and transform it into a variety of chemical structures with a multitude of non-food uses (Ohlrogge, 1994).

Recently there has been a trend away from animal derived fats, for medical or dietary reasons, putting more emphasis on improving the quality of vegetable oils which account for 30% of the calories in human diet. The modification of plant oils to reduce saturated fatty acid content (Grayburn *et al*, 1992) is highly desirable nutritionally as it would aid in reducing cholesterol levels. On the other hand, about half of human consumption of vegetable oils is in the form of the liquid vegetable oils. The creation of an alternative to vegetable oil hydrogenation is desirable because of several reasons including the conversion of much of the naturally occurring *cis*-double bonds to undesired *trans*-configuration during hydrogenation (Kinney, 1996).

Plant lipids contain polyunsaturated fatty acids, mainly linoleic and α-linolenic acids, which play crucial roles in plant metabolism as storage compounds mainly in the form of triacylglycerols (TAG), as structural components of membrane lipids, and as precursors of signaling molecules involved in plant development and stress response (Ohlrogge and Browse, 1995; Weber, 2002). Linoleic acid, together with oleic acid, is a major fatty acid in vegetable oils and its content greatly affects

technological properties such as their oxidative stability (Marquez-Ruiz *et al*., 1999) and nutritional characteristics (Cunnane, 2003).

The genetic engineering for the fatty acid composition of plant lipids has opened a large field of academic research and provides an enormous potential for applications. Using suitable genes and promoter it is now possible to redesign the fatty acid profile of either membranes or of plant storage lipids.

In the recent past, most of the genes encoding proteins involved in *de-novo* fatty acid biosynthesis have been cloned and characterized from a wide spectrum of plants (Topfer and Martini, 1994). The enzyme omega-6 desaturase encoded by *fad-2* gene plays a major role in controlling the conversion of oleic acid to linoleic acid within storage lipids during seed development. Large proportions of linoleic acid and linolenic acid are oxidized under air and contribute to the development of rancid oils. Much of the interest in altering the fatty acid composition of oilseed crops are centered on reducing the levels of PUFA contributing to its oxidative instability. Increasing the precursor monounsaturated fatty acid (oleic acid) shall thus produce oils high in monounsaturates, which will be more stable at high temperature and will have an improved nutritional value. The increase in the oleic acid content can potentially be achieved by reducing the activity of membrane bound enzyme, microsomal omega-6 desaturase which converts oleate to linoleate by adding the second double bond at n-6 in the developing seeds of oilseed crops. The availability of the gene encoding omega-6 desaturase will permit the manipulation of tissue fatty acid composition. In addition, characterization of the corresponding genomic sequence including the promoter region will help in the studies of the genetic regulation of the lipid desaturation with regard to membrane properties and to the synthesis of storage lipids in plants.

Thus, there is an urgent need to exploit our native plants as source of these genes and use them for manipulation of lipid metabolism in crops of major importance. *Brassica juncea* being the major oilseed crop in India, has been chosen for the present study with the following main objectives.

(a) To isolate oleate desaturase gene from *Brassica juncea.*

(b) To study the developmental expression of oleate desaturase gene in different stages of seed development.

(c) To study the effect of temperature on the expression of oleate desaturase gene.

(d) To study the differential expression of oleate desaturase gene in different lines of *Brassica* differing in fatty acid content.

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MATERIALS AND METHODS

Plant Materials

Mature seeds were collected from *Brassica juncea* cv. Pusa Bold grown in the farm of Indian Agricultural Research Institute, New Delhi. Seeds washed with 0.1% mercuric chloride, were spread on a germination paper and were allowed to germinate in dark. Watering of the seeds was done daily for a week and these etiolated seedlings were used for DNA isolation.

For the isolation of cDNA sequence of oleate desaturase gene and other expression studies, RNA was isolated from the developing seeds of *Brassica juncea* cv. Pusa Bold grown in glasshouse under standard conditions (flowers have been tagged on the day of anthesis) in National Phytotron Facilities, IARI, New Delhi.

For the study of differential expression of oleate desaturase gene, RNA was isolated from developing seeds of 3 different genotypes of *Brassica juncea* having high erucic acid (Pusa Bold) and zero erucic acid content (LES 1-27 and LES -39) grown in IARI farm by Division of Genetics.

Chemicals

The molecular biology grade chemicals used were from Sigma, USA and Amresco, USA were used for the present study. Other chemicals were of Analytical or Molecular biology grade from Merck, Qualigens, Himedia, Glaxo or SRL.

Restriction enzymes, PCR kit, T/A cloning kit, miniprep DNA purification kit, random primer labeling kit, gel elution kit and Real - Time PCR kit were obtained from Promega, Qiagen and MBI Fermentas. Indu X-ray films, developer and fixer were from Hindustan Photofilm Manufacturing Co. Ltd.

Radiolabelled biomolecules were obtained from BRIT of Bhabha Atomic Research Centre, Mumbai. The primers for PCR were custom synthesized from Integrated DNA Technologies, Inc, USA.

Total RNA from *Brassica juncea* was isolated using TRI-Reagent®, Sigma. For synthesis of cDNA from total RNA, RevertAid™ H Minus First strand cDNA Synthesis Kit from Fermentas was used.

DNA sequencing was done manually by the Reader™ DNA sequencing kit from Fermentas and by making use of the automated DNA sequencing facility at University of Delhi, South Campus.

Sterilization Procedures

All the tips, microcentrifuge tubes and culture media were autoclaved at 15 psi for 15 minutes. The reagent solutions and double distilled water were used after autoclaving while the heat labile solutions such as antibiotics (eg. Ampicillin), IPTG etc were filter sterilized.

The glasswares for RNA isolation were first washed thoroughly in 0.1% SDS, dipped in 0.1% Diethyl pyrocarbonate (DEPC) for few hours and baked at 180^0C for overnight. The microtips and microcentrifuge tubes were dipped in 0.1% DEPC solution overnight, dried and autoclaved. The sterile water used for RNA isolation was also treated with 0.1% DEPC before autoclaving.

Table 3: Oligonucleotide sequences used in the isolation and expression analysis of *fad2* gene from *Brassica juncea.*

Primers were designed manually by using conserved sequences of *fad2* genes from different *Brassica* sps available in NCBI database according to the instructions given by Sambrook *et al* (1989).

Primers	**Sequences**
SGS F	5’- GCTCCTTCTCCTACCTCATC-3’
SGS R	5’- TACCAGAACACACCTTTCTTC-3’
BcaF	5’- AGAACCAGAGAGATTCATTACC-3’
BcaR	5’- AGACACTAACTTCCAACATCAC-3’

QRTF-2	5’- CCTTCAGCGACTACCAGTG-3’
QRTR-2	5’- CGTACCACTTGATGTCTGAC-3’
act F	5’- CTCACGCTATCCTCCGTCTC-3’
act R	5’-TTCTCCACCGAAGAACTGCT-3’

Bioinformatic tools used for sequence analyses

Nucleotide sequence of both genomic and cDNA clones and the deduced amino acid sequence were identified by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/blast/). Restriction map of *fad2* genomic sequence was derived by using NEB cutter ver 2.0 (tools.neb.com/NEB cutter2/index.php). Transmembrane regions were predicted by TMHMM server ver.2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Prediction of subcellular localization of the deduced amino acids was conducted by using the PSORT (http://www.psort.nibb.ac.jp/form.html) and TargetP (http://www.cbs.dtu.dtu/services/TargetP/) algorithm. Multiple amino acid alignments were performed with clustalw using default parameters. A phylogenetic tree was constructed using the neighbor-joining method and protdist algorithm in the PHYLIP package (version 3.63).Analysis of amino acid composition, ORF and hydrophobicity profile was done by using BIOEDIT version 7.0.9.1 (www.mbio.ncsu.edu/BioEdit/biodit.html). Functional conserved domain of putative protein was determined by using CDART retrieval tool from NCBI (www.ncbi.nlm.nih.gov/structure/lexington/lexington.cgi).

Plant DNA Isolation

Etiolated seedlings of *Brassica juncea* cv. Pusa Bold was used for genomic DNA isolation following the CTAB method of Webb and Knapp (1990).

Solutions

DNA Extraction buffer (DEB)

1.4 M NaCl

2.0% CTAB

100 mM EDTA

0.2% β-mercaptoethanol (added just before use)

Chloroform:isoamyl alcohol mixture (24:1 v/v)

3 M Sodium acetate, pH 5.2

TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA)

Isopropanol

RNase A (10mg/ml) (10mg RNase A/ml in 10mM Tris-Cl, pH 7.5 and 15 mM NaCl)

Phenol saturated with TE (pH 8.0)

Protocol

In a 35ml centrifuge tube aliquoted 16ml of the DNA extraction buffer (DEB) and to that 40µl of β-mercaptoethanol was added. The tubes were incubated at 65^0C for an hour in a water bath. Four grams of the plant sample was crushed in liquid Nitrogen to a fine powder and was transferred to the pre-incubated DEB kept at 65^0C for one and a half hour with intermittent mixing by inverting the tubes. An equal volume of chloroform:isoamyl alcohol was added and mixed well by inverting the tubes. It was then centrifuged at 10,000 x g for 10 minutes at 4^0C in Sorval 5C centrifuge using SS34 rotor and the upper aqueous phase was transferred into a corex tube. To this added an equal volume of chilled isopropanol and kept at room temperature for sometime. DNA threads started appearing in the tube which was spooled out and pelleted down by centrifuging at 10,000 x g for 10 minutes. The DNA pellet was washed with 70% ethanol, dried for sometime at room temperature to get rid of traces of ethanol and dissolved in TE buffer.

CsCl/Ethidium Bromide Equilibrium Ultracentrifugation

In order to purify the DNA, it was subjected to CsCl/Ethidium Bromide Equilibrium Ultracentrifugation which yields high quality DNA free of most contaminants. For this, DNA pellet was resuspended in 4ml TE buffer. To this added 4.4 g CsCl, dissolved and then added 0.4 ml of 10 mg/ml ethidium bromide. This solution was then transferred to a 5ml quick-seal ultracentrifuge tube and centrifuged

for 14 hours or more at 350,000 x g, and 20^0C. The DNA band was recovered by piercing the tube using a needle at the bottom of the band and by sucking into a syringe. In order to remove the ethidium bromide, the DNA solution was equilibrated with several volumes of saturated iso-butanol. Finally the genomic DNA was diluted three times using TE, precipitated using 2 volumes of absolute alcohol by keeping at-20^0C for an hour and pelleted by centrifuging. The pellet was washed with 70% ethanol, dried and dissolved in an appropriate volume of TE.

PCR amplification

In order to amplify a partial genomic sequence of *fad2* gene from *Brassica juncea*, PCR was carried out using *Brassica juncea* genomic DNA as template using SGSF and SGSR primers (Table 3). For PCR amplification, the following reagents were added in a 0.2 ml PCR tube.

Template DNA	50 ng
10x PCR buffer	2.5 µl
25 mM $MgCl_2$	2.5 µl
2mM dNTP	1.0 µl
Forward primer	100 pmoles
Reverse primer	100 pmoles
Taq DNA Polymerase	2 Units
Deionized sterile water up to 25 µl	

Partial amplification of *fad2* gene partial genomic sequence by PCR reaction was carried out in a thermal cycler (Eppendorf) following a programme of initial

denaturation at 95^0 C for two minute followed by 35 cycles of denaturation at 94^0C for 45 sec, annealing at 55^0C for 30 sec and extension at 72^0C for 90 sec and a final extension at 72^0C for ten minutes after the completion of the cycles. To check the amplification, an aliquot of the PCR reaction mixture was run on a 1.0 % (w/v) agarose gel containing 0.5 μg/ml ethidium bromide.

Preparation of 1.0% agarose gel

Solutions

10x TBE (pH 8.0)

Tris base - 108 g

Boric acid - 55 g

0.5M EDTA, pH 8.0 - 40 ml

Water to 1 litre

6x loading dye

Glycerol - 5 ml

10x TBE - 1 ml

Bromophenol blue - 1 ml

(Saturated)

Xylene cyanol 10% - 1 ml

Mixed well and autoclaved.

0.3 g of agarose was weighed and added to 30 ml of 1x TBE buffer (pH. 8) and was allowed to boil well until no crystals of agarose remained. Then the solution was allowed to get cooled to around 50^0C. 1.5 μl of 0.5 μg/ml ethidium bromide was added to the solution and poured on to a gel casting tray assembled with a comb. When the gel was solidified completely, comb was removed carefully taking care not to break the gel after putting 1x TBE over the gel.

1x TBE buffer was used as the running buffer also. Electrophoresis was carried out at 5V/cm (Sambrook *et al*., 1989) and a 6x loading dye was used to load the samples. The PCR product was analyzed on the agarose gel along with λ *Hin*dIII + *Eco*RI as molecular size marker.

Recovery of PCR fragments from agarose gel

After electrophoresis the gel was photographed and the required fragment was cut out, eluted using QIAquick® gel extraction kit from Qiagen according to the manufacturer's protocol.

Protocol for gel elution of DNA using QIAquick® gel extraction kit

This protocol is designed to purify DNA of 70 bp to 10 kb from standard agarose gels in TBE or TAE buffer. After electrophoresis and photography of the gel, the DNA fragment was excised from the gel using a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose. The gel was weighed and 3 volumes of Buffer QG was added to 1 volume of the gel. 100 mg gel weight was taken as 100 µl. Until the gel slice has completely dissolved, the sample was incubated at 50^0C for 10 min. After the gel slice has dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed well. The sample was then added to a QIAquick® column placed in a 2 ml collection tube and centrifuged for 1 min at maximum speed to allow the DNA to get bound to the column. The flow through was discarded and 0.75 ml of Buffer PE was used to wash the column by centrifuging for 1 min. The column was centrifuged again for an additional 1 min after discarding the flow-through and was then placed in a new 1.5 ml microcentrifuge tube. Finally to elute the DNA, added 50µl buffer EB (10 mM Tris-Cl, pH 8.5) and centrifuged the column for 1 min.

Cloning of the PCR product

PCR amplified fragment was cloned into the pGEMT Easy vector (Fig.1). The ligation reaction was set up as follows:

Eluted PCR product - 6µl

5x ligation buffer - 2µl

T4 DNA ligase enzyme (5U/µl) - 1µl

vector - 1µl

The reaction mix was incubated at 4^0C for overnight.

Competent cell preparation

Competent cells of the *E. coli* strain DH5α were prepared by calcium chloride method as described by Ausubel *et al*. (1999). A single colony of *E. coli* DH5α from a freshly streaked LB agar plate was inoculated into 10 ml LB medium and allowed to grow overnight at 37^0C with shaking at 200 rpm speed. Subcultured 5 ml of the overnight grown culture into 500 ml of LB medium contained in a 2L flask. Grown at 37^0C with shaking to an OD_{590} of ~0.4 - 0.5. Aliquoted the culture into sterile prechilled centrifuge tubes and kept on ice for 5 to 10 min. Pelleted the cells by centrifuging at 2500 x g for 5 min at 4^0C. Resuspended the pellet in 100 ml ice cold 0.1 M $MgCl_2$ solution ($1/5^{th}$ of the original culture volume) and transferred the cell suspension to two 50 ml tubes. The cells were incubated on ice for 5 min and centrifuged at 2500 x g for 5 min at 4^0C. The pellet obtained was washed with ice cold 0.1 M $CaCl_2$ and again centrifuged at the same speed for 5 min. The supernatant was discarded and the pellet was suspended in 7 ml of ice cold 0.1 M $CaCl_2$ and incubated it overnight at 4^0C. 3 ml of ice cold 50% glycerol + 50mM $CaCl_2$ mixture was added to each tube and mixed gently. 100µl of the cells were then aliquoted into prechilled microcentrifuge tubes, quick freezed in liquid nitrogen and stored them at -80^0C.

Transformation

Recombinant plasmids were transformed using the competent cells of *E. coli* DH5α as host and LB as the growth medium. The tubes containing the ligation mixture were briefly centrifuged and placed on ice. One tube of 100µl of competent cell was also thawed by keeping in ice. 10µl of ligation mixture was pipetted on to the thawed competent cells and mixed gently by tapping and was kept in ice for an hour with intermittent tapping. A heat shock was given to the tube for around 60 - 90 seconds in a 42^0C water bath. The tube was then immediately placed in ice for 2 min. 400µl LB medium was added to the tube and the cells were allowed to grow for 1 - 11/2 hours at 37^0C with shaking. Spreaded 250µl of the culture separately into two LB Amp/X-Gal/IPTG plates [LB with 15 g/L agar, autoclaved for 20 min. at 15 psi, cooled to ~55^0C and added Ampicillin, X-Gal (40mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside dissolved in dimethylformamide) and IPTG each to a final

concentration of 100μg/ml, 40μg/ml and 0.1 M respectively. When the liquid was absorbed, the plates were kept inside the incubator maintained at 37^0C overnight. The next day plates were shifted to 4^0C to allow proper colour development. White colonies were selected against the blue ones as recombinants and analysis was done by isolating plasmids from them and checking their restriction digestion.

Plasmid Isolation

Solutions

RESUSPENSION BUFFER

50mM Tris-Cl, pH 8.0

10mM EDTA, pH 8.0

2μg RNase A

ALKALINE SDS SOLUTION

1% SDS

0.2 N NaOH

NEUTRALISING SOLUTION

Potassium acetate, 5 M, pH 5.2

The plasmid DNA was isolated from host *E. coli* DH5α by the alkaline lysis minipreparation method (Ahn *et al*., 2000). The white colonies obtained after transformation were inoculated in 2-5 ml of LB medium containing Ampicillin to a final concentration of 100μg/ml and grown at 37^0C overnight with shaking. This culture was transferred to a 2ml microcentrifuge tube and centrifuged at ~10,000 rpm at 4^0C. The supernatant was discarded and to the pellet added100μl resuspension buffer and vortexed well until the pellet was completely resuspended. To this added 100μl of alkaline SDS, mixed gently by inverting the tube several times and kept on ice for 5 min. Added 120μl of Potassium acetate solution (neutralization solution) to neutralize the lysate, mixed gently and incubated for 5 min in ice. Centrifuged at 12,000 rpm for 10 min until the cell debris precipitated and a clear supernatant was

obtained. The supernatant was carefully transferred to a new tube and to this added 0.6 volume of isopropanol to facilitate precipitation of DNA. The solution was again centrifuged at 10,000 rpm for 10 min to obtain the DNA as a pellet. The pellet was washed with 70% ethanol to remove the salts and was air dried and the dried pellet was dissolved in 30µl of TE buffer. An aliquot of the DNA solution was checked on a 0.8% agarose gel by electrophoresis.

Restriction of recombinant plasmid

The recombinant plasmid isolated was restricted with a proper restriction enzyme to check the presence of the insert. A 30µl reaction mixture was set up as follows

DNA (1-2 µg) - 5µl

10x restriction enzyme buffer - 3µl,

Enzyme (10units/µl) - 1µl

sterile water to a final volume - 20µl

The reaction mixture was incubated at 37^0C for 3 hrs and analyzed on 0.8% agarose gel.

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 T3 were used for this.

Southern analysis of genomic DNA

Restriction of plant DNA

Genomic DNA from *B. juncea* cv Pusa Bold was restricted with different restriction enzymes [50µl DNA (5µg), 10µl of 10x restriction enzyme buffer, 3µl enzyme (30 units), made up to 100µl reaction mixture with nuclease free water]. Digestion mixture was incubated at 37^0C overnight. Once the restriction was completed the DNA samples were loaded in a 0.8% agarose gel containing ethidium

bromide along with a molecular weight marker and the electrophoresis was carried out at 5V/cm. The electrophoresis was stopped when the bromophenol blue tracking dye reached ¾ of the gel. The gel was viewed under UV transilluminator and photographed.

Treatment of the gel for Southern hybridization

Solutions

DENATURING SOLUTION

1.5M NaCl and 0.5M NaOH

NEUTRALISING SOLUTION

1M Tris-Cl, pH 8.0 and 1.5M NaCl

20x SSC

3M NaCl and 0.3M Sodium citrate

Procedure

After photography of the gel, it was dipped and shaken in 200 ml of 0.25 M HCl for 10- 15 min at room temperature in a glass baking dish until the bromophenol blue barely turned yellow. The HCl was decanted and gel was rinsed with distilled water for 1min and the DNA was denatured by soaking the gel in several volumes of denaturation solution for 1 hr with constant shaking. After decanting the denaturation solution and rinsing the gel again in distilled water for 1min, it was soaked in several volumes of neutralizing solution for 1 hr at room temperature with constant shaking.

Southern blotting

A piece of Whatman 3 MM filter paper was wrapped around a glass plate and placed it inside a large baking dish. The dish was filled with 20 x SSC almost to the top. And the air bubbles in the 3 MM paper were removed with a glass rod. The gel was inverted so that its original underside became uppermost and placed it on the wet 3 MM paper. Air bubbles between the gel and paper, if any were removed. A piece of nylon membrane was cut about 1-2 mm larger than the gel and was dipped in sterile water (for >20 min) and then in 20 x SSC for 5- 10 min. This wet nylon membrane

was placed on top of the gel and air bubbles in between were removed. Two pieces of Whatman 3MM paper were cut to exactly the same size as the gel and were wet in 20 x SSC; placed on top of the nylon membrane followed by 6 cm stack of dry paper towels. A glass plate was placed on top of the stack and above it, a weight of 500 g. To prevent short circuiting of fluid between the paper towels and 3MM paper under the gel, the gel was surrounded with a water tight border of Saran wrap. The transfer of DNA was allowed to proceed for overnight.

The blot was disassembled in the reverse order and using a soft pencil, the slots on the membrane were clearly labeled. The filter was also marked to define its orientation relative to the gel. The membrane was then soaked in 10x SSC at room temperature for 5-10 min and was placed on a 3MM paper to air dry. It was then baked at 80^0C for 1 hr in an oven and sealed and stored in the dark until use. This Southern blot was allowed to hybridize with a radioactively labeled probe.

Radioactive labeling of probe by Hexalabel™ DNA labeling kit

The recombinant plasmid carrying the insert of *fad2* partial gene fragment from the above experiment was digested with an appropriate restriction enzyme to release the insert. The reaction was set up by adding 5µl of DNA (1-2µg), 4µl 10 x restriction enzyme buffer, 1µl restriction enzyme (5U/µl) and 10µl sterile water. The reaction mixture was incubated at 37^0C for 3 hrs and separated on a 0.8% agarose gel. The gel portion carrying the insert alone was cut and eluted using gel elution kit.

Hexalabel™ DNA labeling kit from Fermentas was used to label the eluted insert DNA. This method produces uniformly labeled radioactive DNA of high specific activity. 100ng of the DNA template was mixed with hexanucleotide in 5 x reaction buffer and made up the volume to 40µl with nuclease free water. The mix was vortexed and incubated in a boiling water bath for 5- 10 min, cooled it on ice and spun down quickly. Based on the choice of labeled triphosphate (dATP or dCTP), Mix A or Mix C were used respectively. In the same tube added the following reagents: 3µl Mix A (or Mix C), 6µl of [α- ^{32}P]-dATP or [α-^{32}P]-dCTP] and 1µl of Klenow fragment, exo$^-$(5u) and incubated for 10 min at 37^0C. Then added 4 µl of dNTP Mix and

incubated for 5 min at 37^0C. The reaction was stopped by adding 1µl of 0.5M EDTA, pH 8.0. The labeled DNA was used directly for hybridization.

Hybridization and washing of the membrane

Sodium phosphate buffer (pH 7.2) was used for hybridization of the membrane. A total of 40 ml hybridization solution was made by mixing 20 ml of 0.5M phosphate buffer, pH 7.2, 14 ml of 20% SDS and 6 ml of sterile water. Hybridization solution was used at the rate of 1ml/ cm^2 of the membrane. The well dried membrane was dipped in an appropriate volume of pre-hybridization solution (which is hybridization solution without probe) in a hybridization tube at 65^0C for an hour. After one hour incubation, probe was added to the solution and kept overnight at 65^0C gently rotating the tube.

Washing the membrane and autoradiography

After completion of hybridization, the membrane was removed from the bag and washed briefly in 2x SSC and 0.1% SDS. The membrane was kept in a fresh bag and poured 250 ml/ 100 cm^2 2 x SSC + 0.1% SDS buffer; sealed and agitated vigorously for 5 min at room temperature and this was repeated three times. Another solution containing 0.1 x SSC + 0.1% SDS has been added (250ml/100cm^2) and agitated vigorously for 15 min at 50^0C which was repeated thrice. The membrane was removed from the bag and kept in between Saran wrap. The remaining fluid, if any, was removed by pressing with tissue paper and exposed to X-ray film. The film was developed after appropriate number of days using X-ray developer and fixer.

Long PCR for amplification of full length genomic sequence of *fad2* gene

In order to amplify full length genomic sequence, long PCR was done by using BCaF and BCaR primers (Table 3) with QIAGEN^R LongRange PCR kit according to manufacturer's instructions which is given below.

Component	Volume in each reaction	Final concentration
LongRange PCR buffer with Mg^{2+},10x	5 µl	1x; 2.5 mM Mg^{2+}

dNTP mix (10mM each)	2.5 µl	500µM of each dNTP
5x Q solution	10 µl	1x
Forward primer	1µl	100 p mol
Reverse primer	1µl	100 p mol
Long Range PCR enzyme mix	0.4µl	2 units per 50µl reaction
Template DNA	2µl	0.5µg
Nuclease water	28.10µl	

PCR reaction was carried out with a programme of initial denaturation at 95^0 C for two minute followed by 35 cycles of denaturation at 94^0C for 45 sec, annealing at 56^0C for 30 sec and extension at 72^0C for 3 minutes and a final extension at 72^0C for ten minutes after the completion of the cycles. To check the amplification, a PCR reaction mixture was run on a 0.8 % (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. After electrophoresis the gel was photographed and the required fragment was cut out, eluted and cloned in pGEM-T Easy vector system of Promega according to manufacturer's protocol (Transformation was done as mentioned earlier).

RNA isolation

For RNA isolation, all the reagents were prepared using 0.1% Diethyl pyrocarbonate (DEPC) treated water or treated directly with 0.1% DEPC. These were then autoclaved at 15 psi pressure for 30 min. All the plastic wares and glass wares were kept dipped in 0.1% DEPC treated water for 12 hr and either baked at 180^0C for a minimum of 6 hours in the case of glass wares or autoclaved at 15 psi for 30 min in the case of plastic wares. Hand gloves were worn throughout the operations as a precaution against RNases.

For the isolation of cDNA sequence and expression studies on *fad2* gene, RNA was isolated from developing seeds using TRI-Reagent® from Sigma. This is a reagent for the preparation of good quality RNA free from DNA.

100 mg developing seeds for every experiment were frozen and ground in liquid N_2 and homogenized in 1ml of TRI-reagent®. The homogenate was stored at room temperature for 5 min to permit dissociation of nucleoprotein complexes. 0.2ml of chloroform was added per ml of TRI-reagent, covered the sample tightly and shaken vigorously for 15 seconds. The mixture was stored at room temperature for 2-15 min and centrifuged at 12,000 x g for 15 min at 4^0C. The mixture then separated into a lower phenol-chloroform phase, interphase and the colourless upper aqueous phase. RNA remains in the aqueous phase which was transferred to a fresh tube and was precipitated by the addition of isopropanol (0.5ml of isopropanol per ml of TRI-reagent®). After adding isopropanol, the mixture was stored at room temperature for 5-10 min and centrifuged at 12,000 x g for 8 min at 4^0C. The supernatant was removed, washed the pellet with 75% ethanol by centrifuging at 7,500 x g for 5 min and finally dissolved the pellet in an appropriate volume of DEPC water after air drying.

An aliquot of the sample was also analyzed spectrophotometricaly at 260 and 280nm and the ratio of OD 260/280nm was determined. From the absorbance value at 260nm, the concentration was calculated by using conversion factor of 40 (40µg/ml=1 OD).The ratio of OD 260/280 was found to be 1.8.

In order to avoid DNA contamination RNA was treated with RNAse free DNAse.

RNA gel electrophoresis

In order to know the integrity of RNA, 1.2% agarose gel electrophoresis was carried out containing 0.5µg/ml ethidium bromide and 20mM GTC (Guanidium thiocyanate) to remove secondary structures, if any, in the RNA (Gowda *et al*., 1995). RNA dissolved in DEPC water was mixed with an equal volume of RNA loading dye and the sample was heated at 65^0C for 5 min and snap cooled by keeping in ice. The sample was run on the gel at 5V/cm to check the quality as well as quantity of RNA and an appropriate amount of RNA was used for doing Reverse Transcription reaction (RT) using gene specific primers.

RT PCR

Isolation of cDNA sequence of *fad2* from developing seeds of *Brassica juncea*.

In order isolate corresponding cDNA sequence of *fad2*, RT-PCR was carried out by using BCaF and BCaR primers (Table 3) with RevertAid^{TM} H Minus first cDNA synthesis kit of Fermentas according to manufacturer's protocol which is given below.

Synthesis of first strand cDNA

Following reaction mixture was prepared in 0.2ml tube on ice

Template RNA (1µg)	------	10µl
Oligo $(dT)_{18}$ primer (0.5 µg/ µl)	------	1µl
DEPC-treated water	------	to 12 µl

Mixed gently and centrifuged briefly

Incubated the mixture at 70^0C for 5 min, chilled on ice and centrifuged briefly

Placed the tube on ice and added the following components

5x reaction buffer	-------- 4 µl
Ribolock^{TM} ribonuclease inhibitor (20U / µl)	-----1 µl
10 mM dNTP mix	-----2 µl

Mixed gently and centrifuged briefly

Incubated at 37^0C for 5 min

Added RevertAid^{TM} H Minus M-MuLV RT (200U/µl) ----- 1 µl

Final volume -----20 µl

Incubated the mixture at 42^0C for 60 min

Stopped the reaction by heating at 70^0C for 10 min and chilled on ice

PCR amplification

cDNA which was prepared by above protocol was used to amplify *fad2* cDNA sequence using Bca F and BcaR primers

Fermentas PCR master mix was used according to manufacturer's protocol which is given below.

Component of sample	Volume	Final concentration
PCR master mix (2x)	25 µl	1x
Forward primer	1µl	100 p mol
Reverse primer	1µl	100 p mol
cDNA	2µl	0.5µg
Nuclease free water	to 50µl	

PCR reaction was carried out with a programme of initial denaturation at 95^0 C for two minute followed by 35 cycles of denaturation at 94^0C for 45 sec, annealing at 56^0C for 30 sec and extension at 72^0C for 90 sec and a final extension at 72^0C for ten minutes after the completion of the cycles. To check the amplification, an aliquot of PCR reaction mixture was run on a 1.0 % (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. After electrophoresis the gel was photographed and the required fragment was cut out, eluted and cloned in pGEMT Easy vector system of Promega according to manufacturer's protocol (Transformation was done as mentioned earlier).

Expression studies of *fad2* gene from developing seeds of *Brassica juncea.*

Developmental expression

For the study of developmental expression of *fad2* gene, RNA was isolated as described earlier from 3 different stages of seed development namely 15 DAF (Early), 30 DAF (Mid) and 45 DAF (Late), RT-PCR was performed for all 3 stage samples by using QRTF2 and QRTR2 primers (Table 3) as described earlier.

Temperature dependent expression

In order to study the effect of temperature on *fad2* gene expression in developing seeds of *Brassica juncea* ,plants were given prolonged heat shock in growth chambers at 3 different temperatures namely 10^0C (low), 21^0C (normal) and 32^0C (high) for 24 hrs to 72hrs.The light/dark cycle was 12/12hrs. Seeds were collected from all 3 treatments after 24hrs and 72 hrs. RNA was islolated from each treatment as described earlier and RT-PCR was done to know the expression of *fad2* gene under different temperatures.

Real time PCR analysis in all temperature treatments was carried out by using QuantiTect^R SYBR^R Green PCR kit from Qiagen according to manufacturer's protocol. The instrument used was from Strategene.

cDNA was prepared from all temperature treatments as described earlier. Real time PCR was performed using following protocol.

Thaw 2x QuantiTect SYBR Green PCR Master Mix, cDNA primers and RNAse free water.

Mixed the individual solutions.

Prepare a master mix:

Component	Volume	Final concentration
2x QuantiTect SYBR Green PCR Master Mix	25 µl	1x
Forward primer	2µl	0.2µM
Reverse primer	2µl	0.2 µM
cDNA	2µl	0.5µg
RNAse free water	to 50 µl	

Real-time cycle conditions:

Step	**Time**	**Temperature**
1. PCR Initial activation step	10 min	95^0C
3-step cycling		
Denaturation	30 sec	95^0C
Annealing	1 min	57^0C
Extension	30 sec	72^0C
Cycle number - 40 cycles		

Fatty acid analysis in temperature treatments:

Condition for analysis of Fatty acids

Fatty acids were analyzed by Perkin Elmer Claurus 500, a gas liquid chromatograph fitted with megabore column packed with stationary phase (30 meter long and 0.53mμ, packed with OV-101, polymer of methyl silicone)and Flame ionization detector (FID). It was analysed under the following conditions.

Column temperature 150^oC-270^0C

Injector Temperature $250\ ^oC$

Detector temperature $250\ ^oC$

Measured the retention time and identified fatty acids by comparing with retention time of standard methyl esters. Esters appeared in the order of increasing number of carbon atoms and for increasing unsaturation for the same number of carbon atoms. In the computerized automated gas chromatographs, area under each peak was calculated automatically. After computing total peak area for the sample, calculated percent area under each peak that would give percent of respective fatty acid.

Procedure

Preparation of Methyl esters:

Fatty acid methyl esters were prepared as per the method described by Vasudev *et al*., (2008).

20 mg (1-2 drops) of each oil sample was taken in a separate tube, with three replicates of each sample.

To each tube 5 ml of methanol was added, followed by two drops of conc. H_2SO_4. These tubes containing oil:methanol:acid mixture were incubated in a water bath, at 65^{o}C, for an hour. The tubes were cooled and 2 ml of hexane was added in each tube. The tubes were shaken well, and allowed to stand till the hexane layer separated out. Methyl esters of fatty acids obtained from oil by Methanol and Conc. H_2SO_4, were removed into the hexane layer in a new vial. A pinch of anhydrous sodium sulphate was added to each vial so as to remove any moisture present in the sample.

Fatty acid analysis

1 µl of the hexane layer containing methyl ester was injected into pre-conditioned gas chromatograph. The individual fatty acids were identified by their relative retention times and comparing with known standards. Percent fatty acid composition was determined by measuring area under each peak.

Differential expression

Differential expression of *fad2* gene was studied through Real-Time PCR in 3 *Brassica juncea* genotypes having high erucic acid (Pusa Bold) and low erucic acid content (LES -39 and LES 1- 27). LES -39 is also called Pusa Karishma having 0.3% erucic acid content and LES 1- 27 is also called as Pusa Mustard-21 having zero erucic acid content developed by Division of Genetics IARI. Seeds of all three genotypes were collected from IARI farm during mid stage (30 DAF) of seed development and RNA was isolated as described earlier. Real Time PCR analysis was carried out in order to study the differential expression of *fad2* gene, and fatty acid analysis in corresponding genotypes was done as described earlier.

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REVIEW OF LITERATURE

Manipulation of biosynthetic pathways in transgenic plants offers a number of exciting opportunities for plant molecular biologists to redesign plant metabolism for production of specific higher value products (Ohlrogge, 1994; Goddiin and Pen, 1995; Knauf, 1995; Yuan and Knauf, 1997). This includes plant based raw materials for human nutrition as well as renewable sources for industrial purposes (Topfer and Martini, 1994). One of the major objectives of modern plant biotechnology is to manipulate the amount and quality of seed storage lipids (Knutzon *et al*., 1992; Voelkar *et al*., 1992; Cahoon *et al*., 1992; Murphy, 1992). Many plants accumulate large amounts of storage lipids upto 45% of dry weight, in the form of triacylglycerol in their fruits or seeds (Somerville and Browse, 1991). The oil derived from plant sources account for the bulk of the total world production of oils and fats. Many plant lipids are utilized in both the food and non-food industries, whereas, a few are used only for non-food benefits.

In the oils of the six major oils crops (soybean, oil palm, rapeseed, sunflower, cotton seed and groundnut) which account for 84% of worldwide vegetable oil production (Topfer *et al*., 1995), the fatty acid composition of the triacylglycerols is limited to C_{16} to C_{22} acyl chains with upto three double bonds. Although these fatty acids can be instrumental in the production of relatively wide variety of technical products, the value of the fatty acids is generally increased by the presence of certain functional groups that expand the applicability of the fatty acids as starting materials for industrial synthesis (Kishore and Somerville, 1993). Higher plants produce at least 210 different kinds of fatty acids, many of which are likely to have industrial non-food uses of higher value than edible fatty acids (Kishore and Somerville, 1993) but majority of them are not available for economic uses because they occur in non-crop plants.

Oils and Nutrition

Vegetable oils play a major role in human nutrition because of their high energy content and account for ~ 30% of the calories in the human diet. Ninety percent of the vegetable oils produced is used for human consumption predominantly

in margarines, shortenings, salad oils and frying oils (Topfer *et al.*, 1995). The recent trend away from animal-derived fats for medical or dietary reasons has put even more emphasis on improving the quality of vegetable oils. It is also well known that mammals require polyunsaturated fatty acids with the $\Delta^{9, 12}$ double bond configuration for good health. Such 'essential fatty acids' are normally thought of as synonymous with the plant acids, linoleate and α-linolenic. Because the animal Δ^{6}-desaturase may often be limiting for the conversion of linoleate into the principal mammalian polyunsaturated, arachidonate, there has also been recent interest in plant production of γ-linoleate (Harwood, 1996).

The words "reduced saturates" immediately bring to mind the health issues associated with the consumption of edible oils (Kinney, 1996). Because vegetable oils generally contain far less saturated fatty acids than the 40-50% found in animal fats, the replacement of animal fats by vegetable oils is considered to be beneficial in reducing cholesterol levels. However, most vegetable oils still contain 10-20% saturated fatty acids (Ohlrogge, 1994). There is considerable anxiety surrounding fat consumption and plasma cholesterol level. The relationship between coronary heart disease and diet fat intake is believed to be clear to some expert groups but not to others (Clandinin *et al.*, 1997). Lauric and myristic acid apparently result in greater hypercholesterolemia than palmitic acid or stearic acid (Clandinin *et al.*, 1997). Dietary supplementation with omega-3 fatty acid rich fish oil has been found to inhibit atherosclerosis in both animal models and humans (Sugano *et al*., 1997).

As in all other organisms, fatty acids in plants are the major structural components of membrane phospholipids and triacylglycerol storage oils. The relative quantities of the various saturated and polyunsaturated fatty acids (PUFAs) are the major factors influencing the quality of plant oils. For example, oils high in oleic acid (18:1) and low in PUFAs appear to have improved nutritional benefits to human and animal consumption and increased stability (Liu and White, 1992).

Oleic acid is a monounsaturated fatty acid and has the function of reducing the content of low-density lipoprotein (LDL) in human bodies, without lowering the content of high-density lipoprotein (HDL). However, linoleic acid is unhealthy

polyunsaturated fatty acid since it can decrease both LDL and HDL in human bodies (Shen, 1998).

The studies made by Clandinin *et al*. (1997) suggest that consumption of omega-3 fatty acids, particularly 20:5, ω-3 and 22:6, ω-3, at the reported physiological level of intake seems to affect the partitioning or metabolic preferences of the LDL particle for specific fatty acids. This led them to speculate that some of the antiatherogenic effect of dietary omega-3 fatty acids may occur through this mechanism. Others have suggested that the beneficial cardiovascular effects of consumption of a salmon diet high in omega-3 fatty acids was attributable to lowering of the level of more dense HDL_3 and elevation of the larger, less dense HDL_2 (Clandinin *et al*., 1997). Reducing the saturate content of vegetable oils thus improves its nutritional qualities, as has been shown in a recent report by Reddy and Thomas, (1996). They expressed the cyanobacterial Δ^6 desaturase gene in tobacco which introduces a third double bond into linoleic acid to produce γ-linolenic acid (GALA). GALA is found mainly in fish oils and is thought to help alleviate hypercholesterolemia.

The polyunsaturated fatty acids linoleate (delta 9, 12-18:2) and α-linolenate (delta 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).

Another nutritional concern related to edible oils is the effect of *trans* fatty acids. These are predominantly the *trans* isomers of oleic acids which are not normally found in plant oils but are produced when the oil is hydrogenated (Ohlrogge, 1994; Kinney, 1996). The *trans* fatty acids are not metabolically equivalent to *cis*-isomers and their consumption appear to adversely affect serum lipids (Clandinin *et al*., 1997) and has been regarded as a possible risk factor for coronary heart disease (Kinney, 1996).

Most of the vegetable oils produced for commercial food applications are hydrogenated to increase its oxidative stability during storage or frying or to make the

oil solid for edible spreads and margarines. The high levels of polyunsaturated fatty acids reduce the oxidative stability of oils and thereby affect the flavour and quality of the oil (Thormann *et al.*, 1996; Byrum *et al.*, 1997). These above concerns may be best overcome by the use of desaturase gene to suppress the endogenous genes. In one such effort, a omega-6 desaturase gene from soybean which inserts a second double bond into oleic acid was used to suppress the endogenous soybean gene (Kinney, 1996). This resulted in almost complete elimination of polyunsaturated fatty acids in soybean oil. Similarly, Calgene has reported the suppression of an endogenous Δ^9 desaturase to produce high stearate canola oil for possible use in margarines and other solid fat applications (Knutzon *et al.*, 1992). Both these oils are free from *trans* fatty acids and should provide significant health benefits once they are widely available.

In soybean, linoleic acid content has been shown to increase by 10% by antisense expression of linoleate (ω-3) desaturase (Topfer *et al.*, 1995). Thus this is also an enzyme of choice to decrease polyunsaturated fatty acid content of oils to improve their oxidative stability. On the other hand, unsaturation level can be improved by transforming plants with additional membrane bound desaturases (oleate desaturase and linoleate desaturase).

Industrial Perspective

Currently about 10% of the vegetable oils produced are used in non-food applications such as lubricants, hydraulic oil, oleochemicals for coatings plasticizers, soaps and detergents (Somerville and Browse, 1991; Topfer *et al.*, 1995).

The alcohol esters of some plant oils may find future use as a diesel fuel substitute in urban settings where the desire for reduced emissions of sulfur and aromatic pollutants may offset the increased costs of these oils relative to those of petroleum (Somerville and Browse, 1991). In contrast to fossil oils, seed derived oleochemicals do not need expensive refining methods (some of which generate toxic waste), as the final products may be harvested directly from the seed (Goddiin and Pen, 1995).

In addition to providing food, oilseed crops can also be seen as efficient, low polluting chemical factories that are able to harness energy from sunlight and transform it into a variety of valuable chemical structures with a multitude of non-food

uses (Ohlrogge, 1994). The availability of structural and biosynthetic genes for specific biosynthetic pathways and the development of downstream processing technology will determine the progress in this area (Murphy, 1992; Goddiin and Pen, 1995).

Application of recombinant-DNA technology to seed oil biochemistry has allowed the introduction of novel enzymatic activities for the production of 'designer oils'. Such oils not only include modified edible oils with improved nutritional or technological characteristics but may also encompass novel compounds (e.g. petroselinic acid) whose main use is in chemical manufacture (Murphy, 1992; Topfer *et al.*, 1995; Harwood, 1996). Because oil of different fatty acid composition is required for various applications, it is essential to have a complete understanding of the regulation of triacylglycerol biosynthesis and to have the genes available that are required to engineer the process (Somerville and Browse, 1991). Tissue specific promoter elements from genes encoding seed storage proteins can be used to direct gene expression to the desired storage tissue, thus avoiding possible deleterious effects that could result from expression of the transgene throughout the plant (Murphy, 1992; Topfer *et al.*, 1995).

New chemical reactions have implications for biofuels and replacement of petro-chemicals.

Scientists at the U.S. Department of Energy's (DOE) Brookhaven National Laboratory to discover a fundamental shift in an enzyme's function that could help expand the toolbox for engineering biofuels and other plant-based oil products. "Placing double bonds in different positions allows us to change the structure of the fatty acids to make products with different potential applications," explained Brookhaven biochemist John Shanklin, who led the research. The ultimate goal to engineering designer plant oils to be used as biofuels and/or raw materials to reduce the use of petroleum. To try to change the position of a double bond, the Brookhaven team modified a desaturase enzyme, changing three of the 363 amino acids in its protein sequence. But when they tested the modified enzyme and looked for the expected product with its altered double-bond position, it wasn't there. Instead of producing a shift in double-bond position, the enzyme modification had yielded three

completely new products, two variations of a hydroxylated product called an allylic alcohol and a fatty acid containing two double bonds (Whittle *et al*., 2008).

From their experiment Whittle *et al*. (2008) reported that plants contain thousands of fatty acid structures, many of which arise by the action of membrane-bound desaturases and desaturase-like enzymes. The details of ‘‘unusual’’ e.g., hydroxyl or conjugated, fatty acid formation remain elusive, because the enzymes involved await structural characterization. However, soluble plant acyl-ACP (acyl carrier protein) desaturases have been studied in far greater detail but typically only catalyze desaturation (dehydrogenation) reactions. They described a mutant of the castor acyl-ACP desaturase (T117R/G188L/D280K) that converts stearoyl-ACP into the allylic alcohol *trans*-isomer (*E*)-10-18:1-9-OH via a cis isomer (*Z*)-9-18:1 intermediate. The use of regiospecifically deuterated substrates shows that the conversion of (*Z*)-9-18:1 substrate to (*E*)-10-18:1-9-OH product proceeds via hydrogen abstraction at C-11 and highly regioselective hydroxylation (>97%) at C-9. O^{18}-labeling studies show that the hydroxyl oxygen in the reaction product is exclusively derived from molecular oxygen. The mutant enzyme converts (*E*)-9-18:1-ACP into two major products, (*Z*)-10-18:1-9-OH and the conjugated linolenic acid isomer, (*E*)-9-(*Z*)-11-18:2. The observed product profiles can be rationalized by differences in substrate binding as dictated by the curvature of substrate channel at the active site. This three amino acid substitutions, remote from the diiron active site, expand the range of reaction outcomes to mimic some of those associated with the membrane-bound desaturase family underscores the latent potential of O_2-dependent nonheme diiron enzymes to mediate a diversity of functionalization chemistry. In summary, this study contributes detailed mechanistic insights into factors that govern the highly selective production of unusual fatty acids (Whittle *et al*., 2008).

Biological role of plant lipids

Lipids are an essential constituent of all plant cells. The vegetative cells of plants contain ~ 5 to 10% lipid by dry weight and almost all of this weight is found in the membranes. Epidermal cells produce cuticular lipids that coat the surface of plants, providing the crucial hydrophobic barrier that prevents water loss and also forming a protection against pathogens and other environmental stresses. One of the remarkable

characteristics of the plant cell membranes is that they have unusually high content of lipids containing trienoic fatty acids such as α–linolenic (18:3) and hexadecatrienoic (16:3) acids (Somerville and Browse, 1991). The membrane lipid composition has been widely postulated to be an important factor in plant processes such as tolerance to extreme temperatures, the efficiency of photosynthetic function and organellar morphology (Gibson *et al*., 1994b). In addition, membrane lipids are the precursors of various signal molecules such as phosphoinositides and jasmonic acid which are produced by environmentally stimulated enzymatic alteration of membrane components.

Fatty acid desaturases play important roles in controlling the physical properties of membranes and in the synthesis of signal molecules such as prostaglandins and pheromones. Most desaturases are membrane proteins that have been recalcitrant to characterization by conventional biochemical methods. Only one enzyme of this class has been characterized from animals or fungi. In this context, plants have proved to be useful sources of experimental materials. Substantial progress has been made in characterizing and manipulating nine classes of desaturases that control the fatty acid composition of both plant membranes and plant storage lipids, which account for 30% of the calories in the human diet (*S*omerville and Browse, 1996).

Plant lipid metabolism in brief

The lipid biosynthesis takes place at two main sites. The plastids and the endoplasmic reticulum (ER), but the recent characterization of several mitochondrial acyl tranferases suggests that the mitochondrion is at least partially autonomous with respect to lipid synthesis (Inui *et al*., 1985; Somerville and Browse, 1991).

In the plant cells, *de novo* fatty acids biosynthesis occurs exclusively via a dissociable multisubunit prokaryotic-like fatty acid synthetase (Type II) located in stroma of plastids (Ohlrogge, 1982; Ohlrogge *et al*., 1991; Slabas and Fawcet, 1992). The first committed step in fatty acids biosynthesis in the plastid is the formation of malonyl CoA from acetyl CoA and HCO_3 in an ATP-dependent reaction catalyzed by acetyl CoA carboxylase. Individual enzymes constituting the type II fatty acid synthetase consecutively add two carbon units derived from malonyl CoA to a

growing acyl chain that is bound to the small cofactor, acyl carrier protein (ACP) (Topfer *et al*., 1995). In a series of seven condensation cycles a C_{16} acyl thioester, palmitoyl-ACP is formed which in most lipid accumulating plant tissues is elongated to $C_{18:0}$ (stearoyl-ACP). The subsequent desaturation is catalyzed by a soluble $\Delta 9$ stearoyl-ACP desaturase present in the stroma of plastids and converts most of the stearoyl-ACP to oleoyl ACP (Topfer *et al*., 1995; Ohlrogge and Jaworksi, 1997). The primary fatty acids formed (palmitic, stearic and oleic acids) are then released from the ACP by the action of thioesterase. These acyl residues are then either used directly by plastidic pathway for membrane lipid synthesis or exported to cytosol by carnitine pathway as coenzyme A esters (Slabas and Fawcet, 1992). Further desaturation or modification of fatty acids (eg. Polyunsaturation, epoxides, hydroxides, conjugates, very long chain fatty acids, wax) requires the lipid linked forms both in plastids and in cytosol.

The synthesis of glycerolipids in plants takes place almost exclusively in the plastids and the ER. However, the relative amount of glycerolipid synthesis in these two cellular compartments may vary in different tissues or in different plant species. The lipids synthesized in the plastid have a C_{16} fatty acid on the Sn-2 position, whereas in ER they have a C_{15} fatty acids on the Sn-2 position (Roughan and Slack, 1982; Browse and Somerville, 1991; Somerville and Browse, 1991; Ohlrogge and Browse, 1995). This is because of the acyl group specificity of the acyl transferases in the various organelles. This molecular pedigree permits the measurement of the relative flux of fatty acids through the two cycles.

In *Arabidopsis*, about half of the fatty acids remain in the chloroplast and are made into chloroplast specific lipids by a series of reactions that are collectively termed the ‘prokaryotic pathway’ (Browse *et al.*, 1986; Ohlrogge and Browse, 1995; Roughan and Slack, 1982). The other half are exported from the chloroplast and are converted to lipid in the ER by a parallel pathway termed the ‘eukaryotic pathway’. Curiously, a substantial proportion of the lipid synthesized in the endoplasmic reticulum may be reimported into the chloroplast.

The presence of this polymorphism was recognized 20 years ago in the observation that some angiosperms (designated “16:3 plants”) have substantial

quantities of 16:3 fatty acids in their leaf lipids whereas other (18:3 plants") do not (Somerville and Browse, 1991). Since lipids containing 16:3 fatty acids are only produced by the prokaryotic pathway, the amount of lipids with 16:3 fatty acids reflects the relative flux through the two pathways. In 18:3 plants (such as pea, barley, wheat and maize) the chloroplast is almost entirely dependent on imported lipids. By contrast, in 16:3 plants (e.g. many green algae, *Arabidopsis*, spinach and tobacco) the chloroplast is almost entirely autonomous with respect to membrane lipid synthesis (Somerville and Browse, 1991).

An abbreviated schematic diagram of the two pathways of glycerolipid synthesis was described for the first time in *Arabidopsis* leaves (Somerville and Browse, 1991; Ohlrogge and Browse, 1995). Both pathways are initiated by the synthesis of phosphatidic acid (PA). In the chloroplast PA is converted to phosphatidyl glycerol (PG), monogalactosyl diacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulphoquinovosyl diacylglycerol (SQDG). In the eukaryotic pathway, the principle glycerolipids are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI) and phosphatidyl serine (PS) (Ohlrogge and Browse, 1995).

Desaturases

Desaturases are the enzymes that introduce double bonds into fatty acids. They are broadly of three types. Acyl-CoA desaturases introduce double bond into fatty acids bounds to coenzyme A. These enzymes are bound to the endoplasmic reticulum in animals, yeast and fungal cells. Acyl-ACP desaturases introduce double bonds into fatty acids that are bound to ACP. They are present in the stroma of plant plastids. Acyl-lipid desaturases introduce double bonds into fatty acids that have been esterfied to glycerolipids and they are bound to the endoplasmic reticulum/chloroplast membrane in plant cells and the thylakoid membrane in cyanobacterial cells (Murata and Wada, 1995). This last type of desaturases are the most efficient regulators of unsaturation of membrane lipids in response to changes in temperature.

The acyl lipid desaturases can be further classified into sub-groups according to their electron donors. One subgroup, present in the ER of plant cells, use cytochrome b_5 as the electron donor (Iba *et al.*, 1993). The other present in the

chloroplasts of plant cells and in cyanobacterial cells uses ferredoxin as the electron donor (Schmidt and Heinz, 1990). A unique characteristics of the acyl-lipid desaturases is that they recognize, by an unknown mechanism, exact positions within various carbon chains at which double bonds are to be specifically introduced (Murata and Wada, 1995).

The omega-3 desaturase is an acyl-lipid desaturase, which acts on fatty acids having double bonds at the Δ^9 and Δ^{12} positions but not on those without a double bond at the Δ^{12} position (Sakamoto *et al*., 1994; Murata and Wada, 1995). Predominantly ω-3 desaturases catalyze the desaturation of hexadecadienoic (16:2) and linoleic (18:2) acids (ω-3 designation refers to position of the double bond from the methyl end of fatty acid).

All acyl-lipid desaturases are integral membrane proteins. Therefore, their purification and characterization has not been possible by conventional techniques of solubilization of integral membrane proteins due to the loss of their activity. However, substantial information concerning the substrate specificity and regulation of these desaturases has been obtained by an alternative approach of molecular-genetic analysis of *Arabidopsis* mutants each one deficient in a specific desaturation step (Lemieux *et al*., 1990; Somerville and Browse, 1991; Browse, *et al.*, 1993; McConn *et al.*, 1994).

A comprehensive collection of mutants of *Arabidopsis* that have defects in one of eight desaturases, which are distinguished by substrate specificity, cellular location or mode of regulation (Table 1) was made by analysing lipid samples from leaves or seeds of heavily mutagenized populations of plants for altered fatty acid composition by gas chromatography (Somerville and Browse, 1991). The loci defined by four of these classes were originally called *fad* A, *fad* B, C and *fad* D (for Fatty Acid Desaturation), but these have now been renamed as *fad*4, *fad* 5, *fad* 6 and *fad* 7, respectively. Mutations in two loci *fad* 2 and *fad* 3 primarily affect desaturation of the extrachloroplast lipids, whereas mutations in the remaining five loci *fad* 4, *fad* 5 *fad* 6, *fad* 7 and *fad* 8 affect chloroplast lipid desaturation (Table 1) (Ohlrogge and Browse, 1995). Out of these loci, three namely *fad* 3, *fad* 7 and *fad* 8 result in the defects in omega-3 desaturation.

TABLE 1 – PROPERTIES OF FATTY ACID DESATURASES FROM *ARABIDOPSIS*

Gene	Old name[a]	Substrate	Location	Precursor	Product	Electron donor
fab2	–	acyl-ACP	CP	18:0	18:1(9*c*)	Fd
fad2	–	phospholipid	ER	18:1(9*c*)	18:2(9*c*,12*c*)	b_5
fad3	–	phospholipid	ER	18:2(9*c*,12*c*)	18:3(9*c*,12*c*,15*c*)	b_5
fad4	*fadA*	PtdGro	CP	16:0	16:1(3*t*)	Fd
fad5	*fadB*	GL, SL	CP	16:0	16:1(7*c*)	Fd
fad6	*fadC*	GL, SL, PtdGro	CP	16:1(7*c*) 18:1(9*c*)	16:2(7*c*,10*c*) 18:2(9*c*,12*c*)	Fd
fad7	*fadD*	GL, SL, PtdGro	CP	16:2(7*c*,10*c*) 18:2(9*c*,12*c*)	16:3(7*c*,10*c*,13*c*) 18:3(9*c*,12*c*,15*c*)	Fd
fad8	–	GL, SL, PtdGro	CP	16:2(7*c*,10*c*) 18:2(9*c*,12*c*)	16:3(7*c*,10*c*,13*c*) 18:3(9*c*,12*c*,15*c*)	Fd

[a]Alternative gene symbols used in articles published prior to change in *Arabidopsis* gene nomenclature.
Abbreviations: ACP, acyl-carrier protein; b_5, cytochrome b_5; *c*, *cis*-olefinic; CP, chloroplast; ER, endoplasmic reticulum; Fd, ferredoxin; GL, galactolipid; PtdGro, phosphatidylglycerol; SL, sulpholipid; *t*, *trans*-olefinic.

(**Source**: Somerville and Browse, 1996)

Somerville and Browse, (1991) isolated a range of mutations affecting at least 12 of the steps in glycerolipid metabolism. Most of the mutations which caused the loss or reduction in the amount of an unsaturated fatty acid namely *fad* 4, *fad* 5, *fad* 6, *fad* 7, *fad* 2 and *fad* 3, were defective in the desaturation of lipid-linked fatty acids.

Analysis of the effects of these *fad* mutations on the acyl composition of the various lipids confirmed the metabolic labeling studies indicating that except for the Δ^9 double bond in C_{18} fatty acids, double bonds are introduced into fatty acids esterified to lipids, rather than to acyl-CoA or acyl-ACP. The defects caused by each of these mutations are summarized in the Table 1. Including the stearoyl-ACP desaturase gene for which there is no known mutant, there are at least eight genes that control the activity of specific desaturases in the leaf (Harwood, 1996).

The biochemical and genetic characterization of *fad 3* mutant was later done by Browse *et al*., (1993) which indicated that the *fad* 3 locus represents the structural gene for an ER 18:2 desaturase. The studies with the *fad* 7 mutant indicated the presence of a cold-induced desaturase that is not expressed at normal growth

temperature (Iba *et al*., 1993). It was found that the independent *fad* 7 mutants had wild-type fatty acid compositions at low temperature but were deficient in trienoic fatty acids at growth temperatures above 20^{o}C. This new mutation termed *fad 8* was later characterized by McConn *et al*., (1994). They identified the *fad 8* locus encoding a chloroplast localized 16:2/18:2 desaturase but that is induced by low temperature.

All of the *fad* mutations have been genetically mapped and form the basis for attempts to isolate the corresponding genes by methods such as transposon tagging or chromosome walking from flanking RFLP sites (Lemieux *et al*., 1990; Somerville and Browse, 1991). The identity of all these genes has been confirmed by using the cloned genes to complement the corresponding mutations in transgenic *Arabidopsis* plants. The *fad* 3 locus encodes the endoplasmic reticulum-localized desaturase and the *fad* 7 and *fad* 8 loci encode the chloroplast localized enzyme species. The analysis of the effects of all these mutations has provided several insights into the regulation of membrane lipid desaturation (Somerville and Browse, 1991). From an examination of F_1 hybrids from a cross of the wild type with the *fad* mutants, it was observed that for the *fad A, fad D* and *fad 3* mutations, the heterozygotes had fatty acid compositions almost exactly intermediate between those of the parents pointing towards the gene-dosage effect of these genes. By contrast, in the *fad B, fad C* and *fad 2* mutants, the heterozygotes more closely resemble the wild type, indicating that a single functional gene resulted in adequate levels of activity. These observations suggest that the activity of the terminal enzyme in each pathway is determined by the amount of gene expression, whereas the activity of the intermediate steps in each pathway appears to be regulated by the activity of the particular enzyme (Somerville and Browse, 1991).

Isolation, cloning and functional expression of oleate desaturase genes

Higher plants express one or more microsomal oleic acid desaturase (FAD2; EC1.3.1.35) isoforms which catalyze the insertion of a double bond between carbons 12 and 13 of oleic acid at both the sn-1 and sn-2 positions of phosphatidylcholine (Shanklin and Cahoon, 1998).Various approaches have been used for the isolation of microsomal and plastidial omega-6 desaturase gene from a varitety of sources (summarized in Table 2).

One of the most important enzymes for the production of polyunsaturates in plants is the oleate desaturase (1-acyl-2-oleoyl-sn-glycero-3-phosphocholine delta 12-desaturase) of the ER. This enzyme is thought to be an integral membrane protein that accepts l-acyl-2-oleoyl-sn-glycero-3-phosphocholine a substrate and requires NADH, NADH:Cyt b_5, oxidoreductase, Cyt b_5, and oxygen for activity (Smith *et al*., 1990; Kearns *et al*., 1991). *Arabidopsis* harbors only a single copy of the *fad2* gene (AT3G12120), which is constitutively and abundantly expressed (Beisson *et al*., 2003). In contrast to *Arabidopsis*, crops, including soybean (*Glycine max*), cotton (*Gossypium hirsutum*), sesame, corn (*Zea mays*),and canola (*Brassica napus*), express at least one additional *FAD2* gene(s), which is tightly regulated during seed development (Okuley *et al*., 1994; Heppard *et al*., 1996; Jin *et al*., 2001; Pirtle *et al*., 2001; Kinney *et al*., 2002).

The genes for ER and plastid-derived Delta 12 FADs have been characterized from some plant species. Several different microsomal oleate desaturase (FAD2) genes may exist, depending on the particular plant. For instance, there is only one *fad2* gene existing in *Arabidopsis* (Okuley *et al*., 1994), and two different *fad2* genes in olive have been identified, one having seed specificity and constitutively expressed microsomal oleate desaturases (Hernandez *et al*., 2005). Three different FAD2 genes have been identified from both cotton and sunflower, with one expressed specifically in seed and the other two expressed in all tissues tested (Liu *et al*., 1999; Martinez-Rivas *et al*., 2001; Pirtle *et al*., 2001).

Okuley *et al.* (1994) studied the identification of a *fad2* allele in a population of *Arabidopsis* in which mutations have been generated by T-DNA insertion (Feldmann, 1991) and the subsequent cloning and characterization of the wild-type *fad2* gene.

Hitz *et al.* (1994) studied the cloning of a higher plant plastid omega-6 fatty acid desatuarase cDNA. They used the oligomers based on aminoacids conserved between known plant omega-3 and cyanobacterium omega-6 fatty acids desaturases to screen an *Arabidopsis* cDNA library for related sequences. They identified a clone encoding a novel desaturase like polypeptide which was used to isolate homologs from *Glycine max* and *Brassica napus*. *Synechococcus* transformed with a chimeric

gene that contains a prokaryotic promoter fused to the rapeseed cDNA encoding all but the first 73 amino acids partially converted its oleic acid to linoleic acid, and the 16:1(9C,12) fatty acid was converted primarily to 16:2(9C,12) *in vivo*. Thus, the plant omega-6 desaturase, which utilizes 16:1(7C) in plants, can utilize 16:1(9C) in the cyanobacterium. The plastid and cytosolic homologs of plant omega-6 desaturases are much more distantly related than those of omega-3 desaturases.

Heppard *et al.* (1996) reported the isolation of two different cDNA sequences, *fad2-1* and *fad2-2,* encoding microsoma1 omega-6 desaturase in soybean. The cDNA sequences of *fad2-1* and *fad2-2* have significant homology with the *Arabidopsis fad2* gene (Okuley *et al.*, 1994). The two soybean sequences share 73% identity at the deduced amino acid sequence level. The functional identity of the two sequences was confirmed by genetic complementation of *Arabidopsis fad2-1* mutation. The *fad2-1* gene is specifically induced during seed development when the rate of storage lipid synthesis is at a maximum. In contrast, the *fad2-2* gene was constitutively expressed in both vegetative tissues and throughout seed development, although the highest expression level was found in leaf tissues.

Liu *et al.* (1999) isolated a cDNA (*ghfad2-1*) encoding a seed specific microsomal omega-6 desaturase from cotton (*Gossypium hirsutum* L cv deltapine-16) embryo cDNA library. Southern blot analysis using the coding region and 3' untranslated region of *ghfad2-1* revealed that microsomal omega-6 desaturase is encoded by a small multigene family. They are atleast two copies of *ghfad2-1* in two tetraploid cotton species (*G.hirsutum* and *G.barbedense* L) and at least one copy in diploid cotton species (*G.herbaceum* L., *G.raimondii* Ulbrich and *G.robinsonii* Mueller).

Two cDNA sequences coding for microsomal oleoyl-PC desaturases, *ahfad2A* and *ahfad2B,* have been isolated by screening cDNA library constructed from the developing peanut seeds with normal oleate phenotype using an *Arabidopsis fad2* cDNA as a probe. The study showed that multiple genes coding for microsomal oleoyl-PC desaturases are present in peanut due to cultivated peanut is an allo tetraploid (Jung *et al.,* 2000a).

Hernandez *et al.* (2005) isolated two different cDNA sequences, designated *Oepfad2-1* and *Oepfad2-2*, encoding two microsomal oleate desaturases (FAD2) have been isolated from olive (*Olea europaea* cv. Picual) using a PCR approach. Genomic Southern blot analysis is consistent with the presence of at least two copies of each *Oepfad2* gene in the olive genome. Functional expression of the *fad2* cDNAs in yeast confirmed that they encode microsomal oleate desaturases. *Oepfad2-1* transcript was strongly detected in very young seeds and in leaves, showing low levels in mesocarps, while the transcript of the *Oepfad2-2* gene was moderately expressed in developing seeds, ripening mesocarp and leaves. These expression data suggest differential functions for the two olive microsomal oleate desaturase genes, with *fad2-1* possibly responsible for the desaturation of reserve lipids in the young seed, while *fad2-2* may be mainly involved in storage lipid desaturation in the mature seeds and the mesocarp

Tang *et al.* (2005) studied the post transcriptional mechanisms that regulate the activity of oleate desaturase enzymes in soybean. The soybean genome possesses two seed-specific isoforms of *fad2*, designated *fad2-1A* and *fad2-1B*, which differ at only 24 amino acid residues. Expression studies in yeast revealed that the *fad2-1A* isoform is more unstable than *fad2-1B*, particularly when cultures were maintained at elevated growth temperatures. Analysis of chimeric *fad2-1* constructs led to the identification of two domains that appear to be important in mediating the temperature-dependent instability of the *fad2-1A* isoform. The enhanced degradation of *fad2-1A* at high growth temperatures was partially abrogated by treating the cultures with the 26S proteasome-specific inhibitor MG132, and by expressing the f*ad2-1A* cDNA in yeast strains devoid of certain ubiquitin-conjugating activities, suggesting a role for ubiquitination and the 26S proteasome in protein turnover. In addition, phosphorylation state-specific antipeptide antibodies demonstrated that the Serine-185 of *fad2-1* sequences is phosphorylated during soybean seed development. Expression studies of phosphopeptide mimic mutations in yeast suggest that phosphorylation may down regulate enzyme activity. Collectively, the results show that post-translational regulatory mechanisms are likely to play an important role in modulating *fad2-1* enzyme activities.

Yin and Cui (2006) studied the identification and molecular phylogenetic relationships of delta 12 fatty acid desaturase in *Arachis*. On the basis of the conservative sequence of delta12-fatty acid desaturase, they isolated a cDNA encoding the enzyme from different genotypes of *Arachis*. The cDNA consisted of 1140 bp and encoded a polypeptide of 379 amino acid resides with a calculated molecular weight of 42 KD. The predicted amino acid sequence had three histidine conserved regions sharing integral-protease specificity. Hydrophobicity analysis showed that the sequence of the encoded amino acids had two hydrophobic structures sharing the characteristics of membrane-anchored protein, which totally crossed the membrane four times. These analyses revealed that the obtained sequence was delta12-fatty acid desaturase. Phylogenetic-tree analysis demonstrated that the found *delta* 12-fatty acid desaturase sequences in *Arachis* was conserved and was highly homologous to the delta12-fatty acid desaturase sequences from other species.

The omega-6 fatty acid desaturase (*fad2*) gene family in soybean (*Glycine max* (L.) Merr.) consists of at least five members in four regions of the genome and are responsible for the conversion of oleic acid to linoleic acid. Schlueter *et al.* (2007) reported the identification of two new omega-6 fatty acid desaturase (*fad2*) gene copies from soybean expressed sequence tags (ESTs). Four bacterial artificial chromosomes (BACs) containing five *fad2* genes were sequenced to investigate structural and functional conservation between duplicate loci. Sequence comparisons show that the soybean genome is a mosaic, with some duplicate regions retaining high sequence conservation in both genic and intergenic regions, while others have only the *fad2* genes in common. Genetic mapping using SSRs from within the BAC sequences showed that two BACs with high sequence homology mapped to linkage groups I and O; these groups share syntenic markers. Another BAC mapped to linkage group L. The fourth BAC could not be mapped.

Li *et al.* (2007) isolated a novel gene encoding *fad2* isoform, designated as *fad2-3* from soybean. The deduced amino acid sequence of the *fad2-3* displayed the typical three histidine boxes characteristic of all membrane-bound desaturases, and possessed a C-terminal signal for ER retention. Yeast cells transformed with a plasmid construct containing the *fad2-3* coding region accumulated a considerable amount of

linoleic acid (18:2), normally not present in wild-type yeast cells, suggesting that the isolated gene encodes a functional FAD2 enzyme. They also studied the constitutive expression of *fad2-3* gene in both vegetative tissues and developing seeds of soybean through semiquantitative PCR and tissue specific expression of *fad-1A* and *fad2-1B* genes in developing seeds of soybean.

Intron mediated enhancement of gene expression

Introns not only have important functions in the generation of new proteins, via exon shuffling or alternative splicing, but they also have a role in determining the expression level or the spatial and developmental expression of genes (Long *et al*., 1995; Bolle *et al.,* 1996; Deyholos and Sieburth, 2000; Maniatis and Tasic, 2002).Intron mediated enhancement of gene expression (IME) was initially observed in the first intron of the maize *adh1* gene and it has subsequently been observed for many plant genes (Rose and Beliakoff, 2000; Clancy and Haanah, 2002).In particular *Arabidopsis* polyubiquitin transcription elongation factor EF-1A and rice β-tubulin isotype 16 (*ostub 16*) genes are known to harbor an intron within the 5´-untranslated region (UTR) that increases the expression of the reporter gene by 2.5 to 1000 fold relative to the intron less controls (Curie *et al*., 1993; Norris *et al.,* 1993; Morello *et al.,* 2002). The occurrence of introns within the 5'-UTR of *fad2* genes appears to have been evolutionarily conserved (Okuley *et al*., 1994; Verwort *et al.,* 2000; Liu *et al.,* 2001; Pirtle *et al.,* 2001).The question arises as to whether or not the *fad 2* introns are involved in the enhancement of gene expression and /or in the spatial and developmental expression of their respective genes.

FAD2-1 is highly expressed and seed-specific and, therefore, is probably the main contributor of the polyunsaturated fatty acids in the seed oil of cultivated cottons (Liu *et al*., 1999). In addition to the three histidine boxes that are typical of all membrane-bound desaturases, the *ghfad2-1* gene contains a stretch of six contiguous glycine residues in the C-terminus of the open reading frame. Moreover, comparisons of genomic and cDNA clones encoding the *ghfad2-1* gene revealed a single large intron of 1133 bp in the 5' untranslated region (UTR) located 9 bp upstream from the putative translation start site (Liu *et al.*, 1997). Preliminary examination of the *fad2-1* gene from five species (*G. arboreum, G. barbadense, G. hirsutum, G. raimondii,* and

G. robinsonii) revealed that the size and position of the intron were conserved. Sequence comparisons also suggested that the *fad2-1* intron may be evolving at a quick enough rate for inferring evolutionary relationships among recently diverged lineages and, in this regard, could be particularly useful for elucidating evolutionary pathways among the 17 *Gossypium* species indigenous to Australia, a group whose evolutionary history remains unresolved (Seelanan *et al.*, 1999).

Liu *et al.* (2000) studied the evolution of the *fad2-1* fatty acid desatuarse 5´ UTR intron and the molecular systematics of *Gossypium* (Malvacea). The *fad2-1* microsomal omega-6 desaturase gene contains a large intron 1133 bp in the 5´ untranslated region that may participate in gene regulation and, in *Gossypium,* is evolving at an evolutionary rate useful for elucidating recently diverged lineages. *Fad2-1* is single copy in diploid *Gossypium* species, and two orthologs are present in the allotetraploid species. Among the diploid species, the D-genome *fad2-1* introns have accumulated substitutions 1.4–1.8 times faster than the A-genome introns. In the tetraploids,the difference between the D-subgenome introns and their A-subgenome orthologs is even greater. The substitution rate of the intron in the D-genome diploid *G. gossypioides* more closely approximates that of the A-genome than other D-genome species, highlighting its unique evolutionary history. However, phylogenetic analyses support *G. raimondii* as the closest living relative of the D-subgenome donor. The Australian K-genome species diverged 8–16 million years ago into two clades. One clade comprises the sporadically distributed, erect to suberect coastal species; a second clade comprises the more widely spread, prostrate, inland species. A comparison of published gene trees to the *fad2-1* intron topology suggests that *G. bickii* arose from an early divergence, but it carries a *G. australe*-like rDNA captured via a previously undetected hybridization event.

Kim *et al.* (2006) studied the seed specific expression of sesame microsomal oleic acid desaturase controlled by combinatorial properties between negative *cis*-regulatory elements in the se *fad 2* promoter and enhancer in the 5' UTR intron. They have characterized transcriptional regulatory mechanisms controlling seed specific *fad2* expression in sesame (*Sesamum indicum*). Promoter analysis of the sesame *fad2* gene (*sefad2*) using the β – glucoronidase GUS reporter system demonstrated that the

-660 to -180 promoter region functions as a negative *cis* element in the seed specific expression of the *sefad2* gene. Sesame and *Arabidopsis fad 2* genes harbor one large intron within their 5'-untranslated region. These introns conferred up to 100 fold enhancement of GUS expression in transgenic *Arabidopsis* tissues compared with intron less controls. Prerequisite *cis* elements for the *sefad2* intron mediated enhancement of gene expression and the promoter like activity of *sefad2* intron have been identified. *SeFAD2* intron transcripts were induced by abscisic acid (ABA) in developing sesame seeds, and the -660 to -548 and -179 to -53 regions in the *Sefad2* promoter were implicated in ABA responsive signaling. These observations indicate that an intron mediated regulatory mechanism is involved in controlling not only the seed specific expression of the *Sefad2* gene but also the expression of plant *fad2* genes which are essential for the polyunsaturated fatty acids.

Expression studies of oleate desaturase genes

Developemental expression

Heppard *et al.* (1996) studied the tissue specific and developmental expression of soybean microsomal omega-6 desaturase genes. To determine the relevance of two microsomal omega-6 desaturase genes in controlling the levels of polyunsaturated fatty acids in developing seeds and other tissues, they measured the steady-state transcript levels of the two genes by northern blot analysis. Both the *fad2*-1 and *fad2-2* genes were expressed in developing seeds. The expression leve1 of the *fad2-2* gene was higher than that of the *fad2-2* gene at the earlier stage (6-10 DAF) of seed development when large amounts of membrane lipids are synthesized. However, the transcript of the *fad2-1* gene rapidly increased during embryo development, peaked in the mid-maturation stages (19-21 DAF), and then gradually declined as seeds matured further. Thus, the timing of *fad2-1* gene expression coincided with that of fatty acid biosynthesis and oil deposition in developing seeds. Whereas the transcript for the *fad2-1* gene increased significantly during the embryo development, the expression level of the *fad2-2* gene appeared to increase only slightly during embryo development. The *fad2-2* gene was also expressed in vegetative tissues, including leaves, stems, and roots, with the highest level in leaves.

Liu *et al.* (1999) studied the expression of *ghfad2-1* in embryo at different stages of seed development by northern blot analysis. They observed that expression of *ghfad2-1* was induced during early stage of embryo development, peaked at 30 DAA and the high expression is maintained until 36 DAA and then it declined to significantly lower amounts in embryos near maturity (45 DAA).

Jung *et al.* (2000a) studied the steady-state transcript levels of the two microsomal oleoyl-PC desaturases, *ahfad2A* and *ahfad2B* genes using Northern hybridization analysis from three different developmental stages, from leaves and cotyledons obtained from seedlings of a normal oleate peanut variety (AT108). The steady-state transcript level was highest in developing seeds at stage 2, lower in stages 1 and 3, and considerably lower in the cotyledons of seedlings and in the leaf. Studies with other oilseed crops such as sunflower and soybean have also shown that oleoyl-PC desaturase mRNA accumulation peaks in the developing seeds at mid-maturation (Heppard *et al.,* 1996; Hongtrakul *et al.,* 1998) when high level accumulation of oleic acid (Powell *et al*., 1990) and oil deposition (Pattee *et al.*, 1974) occur. Oleoyl-ACP, the product of stearoyl-ACP desaturase, either serves as the precursor for synthesis of plastid lipids (prokaryotic pathway), or is hydrolyzed to free fatty acid which is exported as oleoyl-CoA into the cytoplasm for polyunsaturated lipid synthesis in the endoplasmic reticulum (eukaryotic pathway; Ohlrogge and Browse, 1995). The oleoyl group in oleoyl-CoA is transferred to 2-lyso-phosphotidyl choline (2-lyso-PC) by acyltransferase, generating oleoyl-PC, which is considered to be the substrate for the microsomal oleoyl-PC desaturases (Ohlrogge and Browse, 1995). Thus, in the peanut seed, the patterns of accumulation of stearoyl- ACP desaturase and oleoyl-PC desaturase mRNAs during seed development appear to correlate, respectively, with the onset of each desaturation step in fatty acid modification (Jung *et al.,* 2000a).

Tiwari *et al.* (2004) studied the developmental expression of microsomal omega–6 desaturase gene (*fad2-1*) in soybean seeds. They have isolated *fad2-1* gene which encodes microsomal omega–6 desaturase which plays a major role in controlling the conversion of oleic acid to linoleic acid within storage lipids during seed development. Northen blot analysis using the labeled amplicon as probe indicated that the *fad2 -1* expression was induced during early stages of embryo development

and peaked in the mid maturation stages. Tissue specificity of *fad 2-1* transcripts was confirmed by the complete absence of *fad 2-1* transcripts in the vegetative tissues of leaf, stem and root. Developmental profile of fatty acids showed increase levels of linoleic acid during the mid maturation stages which coincided with the expression of *fad2-2* during same period.

Temperature dependent expression

The composition of saturated and unsaturated fatty acids of both membrane and storage lipids also varies depending on environmental temperature. There is a general inverse relationship between polyunsaturation of fatty acids and growth temperature; polyunsaturated fatty acids increase with decreasing temperature in membrane as well as seed storage lipids (Neidleman, 1987; Rennie and Tanner, 1989; Thompson, 1993). It has long been recognized that growth temperature affects plant lipid metabolism (Harwood *et al.,* 1994). In general, reduced growth temperature increases membrane lipid unsaturation (Harwood *et al.,* 1994; Murata and Wada, 1995) and it is thought that it contributes to the maintenance of membrane fluidity at low temperatures. It was suggested that the unsaturation level in leaf lipids is determined by the activation of the desaturase enzymes at low temperature and their inactivation at high temperature (Williams *et al.,* 1992).

Plant response to cold stress is a complex process involving several membrane, cytoskeletal, and cytosolic elements responsible for cold perception, signal transmission, and, finally, development of cold stress resistance through the up-regulation of cold-related genes (Jonak *et al*., 1996; Murata and Los, 1997; Jaglo-Ottosen *et al*.,1998; Johnson and Cornell, 1999; Knight, 2000; Abdrakhamanova *et al.*, 2003). As with any adaptation process, resistance to cold stress results from an interplay of several pathways dependent on hormonal regulation as well as on other environmental and physiological factors (Fowler and Limin, 2004; Li *et al.*, 2004; Ludwig *et al.*,2005; Vandenbussche *et al.*, 2003). Cold stress can be differentially defined depending on the plant species. Chilling, non-freezing temperatures may lead to the acclimation of cold-tolerant species, including the development of resistance under freezing temperatures. Such temperatures, however, may result in damage of cold sensitive plants affecting growth and yield. In addition, anti-freezing proteins

induced in cold-resistant plants under cold temperatures may exist in cold-sensitive plants with the same catalytic activity. However, their induction in the latter occurs in response to pathogens instead of cold (Kirsch *et al*., 1997; Gobel *et al*., 2001).The study of low temperature adaptation mechanisms of the cold-tolerant plant *Arabidopsis thaliana* are of great interest for other cold-tolerant species such as cereals. However, little is known about the mechanisms of cold perception by cold-sensitive plants and the possibility of existence of cold-acclimation processes in such species.

Several low-18:3 and/or high-18:1 soybean mutants have been isolated (Ohlrogge *et al*., 1991; Kinney, 1994), but many of these mutants were temperature sensitive so that the 18:3 content of these plants increased when they were grown at lower temperatures. This might be due to the presence of the second locus of the microsomal omega-3 desaturase gene, expression of which is induced by cold temperature (Kinney, 1994). A cold-inducible plastid omega-3 desaturase gene *(fad7)* has been isolated from *Arabidopsis* (Gibson *et al*., 1994a). Expression of the cold-inducible desaturase gene may allow plants to make more trienoic fatty acid of the membrane lipids, which is required for plants grown at low temperatures. Similar to the low-18:3 mutants, the high-18:l soybean mutant A5 is also temperature sensitive (Rennie and Tanner, 1989), indicating potential regulation of omega-6 desaturase genes by growth temperature. In *Arabidopsis*, microsomal omega-6 desaturase is encoded by a single *fad2* gene, and expression of this gene is not regulated by low growth temperature (Okuley *et al*., 1994).

Heppard *et al*. (1996) reported that the elevated PUFA levels in leaves grown at low temperature were not due to enhanced expression of *fad2-2* in soybean. To test whether the increase of PUFA levels in leaf by low temperature is related to enhanced *fad2-3* expression, they determined the level of transcripts by semi-quantitative RT-PCR analysis. The transcript level of *fad2-3* gene was relatively constant in leaves at different growth temperatures, and the low grown temperature did not result in enhanced expression of *fad2-3*. Interestingly, the expression of *fad2-2* was lower than that of *fad2-3* at different growth temperatures, and the transcript of *fad2-2* was not detected at high growth temperature ($32^0/28^0$C).These results further confirmed the

hypothesis that the increase level of PUFAs at low temperature is likely the result of translation and posttranslational regulation, such as altered desaturase enzyme activity (Cheesbrough, 1989), rather than transcriptionally induced or enhanced expression of the characterized *fad2* genes in soybean.

The growth temperature modifies the relative contents of oleate and linoleate in the seed lipids, thus suggesting a temperature regulation of the *FAD2* activity. In the developing seeds of sunflower the *in-vivo* oleate desaturation increased dramatically as growth temperature declined (Garces *et al.,* 1992; Sarmiento *et al.,* 1998). Moreover, in sunflower seeds incubated for short time periods both *FAD2* activity and the percentage of linoleate were increased when the seeds were transferred to a lower temperature. However the percentage of linoleate remained unchanged after shifting to a higher temperature, inspite of the fact that the enzyme activity was only partially inhibited (Sarmiento *et al.,* 1998).

The temperature and oxygen regulation of the microsomal oleate desaturase (*FAD2*, EC1.3.1.35) activity has been studied in developing sunflower (*Helianthus annus* L) seeds (Martinnez-rivas *et al.,* 2003).In plants cultivated in growth chambers, the linoleic acid content in the seed lipids increased along the 25/15^{0}C (day/night) cycle, except during the first hours of the warm period, where it decreased significantly. In contrast, FAD2 activity decreased notably at the beginning of the warm period, showing a small and continuous increase during the rest of the cycle. The temperature effect on the linoleic acid content and the FAD2 activity was also investigated using peeled seeds and detahced achens subjected to temperature changes. In peeled seeds a change of temperature from 10 to 30^{0}C brought about a significant decrease of FAD2 activity. On the contrary, when the temperature shifted from 30 to 10^{0}C, FAD2 activity only increased slightly. Unlike peeled seeds, detached achenes showed a fast and dramatic increase or decrease in the level of FAD2 activity in response to a temperature change from 30 to 10^{0}C, respectively.

In the *Synechocystis* sp. PCC 6803 the levels of the mRNAs transcribed from the genes that encode the delta 6, delta 12, and omega 3 desaturases increased about 10-fold, but at different rates, upon a decrease in temperature from 34^{0}C to 22^{0}C, whereas the level of the mRNA for the delta 9 desaturase remained constant (Los *et*

al., 1997). FAD2 desaturases present particular interest since their modifying ability under cold-stress responses has been attributed solely to post-transcriptional/posttranslational modifications rather than an increase in their mRNA levels (Tang *et al.*, 2005). With the exception of FAD8 and FAD7 omega 3 desaturases (Berberich *et al.*,1998; Matsuda *et al.*, 2005), which are induced under low temperatures, the induction of other desaturases under stress responses has not been demonstrated in higher plants (Falcone *et al*., 2004). A microarray analysis has also suggested induction in delta 9 desaturase under cold stress in *A. thaliana* (Kreps *et al*., 2002).

Garcia *et al.* (2004) studied the oxygen-independent temperature regulation of three sunflower microsomal oleate desaturase (FAD2) isoforms using *Saccharomyces cerevisiae* cells expressing each *fad2* gene. Yeast cells transformed with the *fad2-1* gene showed the highest percentage of dienoic acids when they were grown at 10-15° C. In contrast, the maximal level of dienoic acids for *S. cerevisiae* cells expressing the *fad2-2* and *fad2-3* genes was obtained at 30 and 35 °C, respectively. Temperature shifts in the phase of exponential growth, from 30 to 15 °C or from 15 to 30 °C, produced changes in the final percentage of dienoic acids, mostly in yeast cells transformed with the *fad2-1* gene, to reach the content corresponding to the new temperature. Low temperature (15°C) increased the amount of neutral lipids in all transformed yeast cells, mainly because it favored triacylglycerol accumulation. In addition, the *FAD2*-expressing yeast cells showed a higher polar lipid content than those transformed with the empty vector. Dienoic acids were present in all lipids, although high temperature (30 °C) favored their accumulation in neutral lipids. As the main conclusion, the low thermal stability observed for the major and seed specific isoform (*fad2-1*) is the key factor controlling the direct temperature regulation in sunflower seeds.

Schlueter *et al.* (2007) studied the effect of temperature on the expression of soybean *fad2* genes. Reverse transcriptase polymerase chain reaction (RT–PCR) analysis of the five *fad2* genes showed that the *fad2-2B* and *fad2-2C* copies were the best candidates for temperature-dependent expression changes in developing pod tissue. Semiquantitative RT-PCR confirmed these results, with *fad2-2C* showing

upward of an eight fold increase in expression in developing pods grown in cooler conditions relative to those grown in warm conditions. The implications of these results suggest a candidate gene for controlling the levels of linoleic acid in developing pods grown in cooler climates.

Kargiotidou *et al.* (2008) observed the induction of microsomal delta 12 fatty acid desaturases at mRNA level under cold stress in plants. Quantitative PCR showed that though both delta 12 omega 6 fatty acid desaturase genes *fad2-3* and *fad2-4* identified in cotton are induced under cold stress, *fad2-4* induction is significantly higher than *fad2-3*. The induction of both isoforms was light regulated, in contrast a third isoform *fad2-2* was not affected by cold or light. Stress tolerance and light regulatory elements were identified in the predicted promoters of both *fad2-3* and *fad2-4* genes. Di-unsaturated fatty acid species rapidly increased in the microsomal fraction isolated from cotton leaves, following cold stress. Expression analysis patterns were correlated with the observed increase in both total and microsomal fatty acid unsaturation levels suggesting the direct role of the FAD2 genes in membrane adaptation to cold stress.

Differential expression

Plant oils rich in oleate are considered superior products compared to oils rich in polyunsaturated fatty acids. Studies with high oleate mutant varieties in oilseed crops, such as sunflower, soybean and rapeseed, have also demonstrated that microsomal oleoyl-PC desaturase activity is lost in developing seeds in these mutants (Martin and Rinne, 1986; Garces and Mancha, 1991; Lee and Guerra, 1994). In contrast to peanut, the high oleate trait in sunflower is due to a dominant mutation, but the high oleate character is confined to the seed, as in the case of the high oleate peanut line (Martinez-Rivas *et al*., 1998). Furthermore, recent studies have reported that the steady-state level of oleoyl-PC desaturase gene transcripts is dramatically reduced in high oleate sunflower seeds (Kabbaj *et al.,* 1996; Hongtrakul *et al*., 1998; Martinez-Rivas *et al*., 1998). In addition, antisense repression of the microsomal oleoyl-PC desaturase in transgenic rapeseed resulted in an increase in oleic acid concentrations of up to 83% (reviewed in Topfer *et al.*, 1995). Co-suppression of this

gene has been successfully used to generate soybean and rapeseed with high oleic acid levels (Hitz *et al*., 1995; Kinney, 1998; Kinney and Knowlton, 1998).

Jung *et al.* (2000b) studied the differential expression of *ahfad2A* and *ahfadB* genes in both normal and high oleate peanut seeds. The results from this study indicated that *ahfad2A* is expressed in both normal and high oleate peanut seeds, but the steady state level of the *ahfad2B* transcript is severely reduced in the high oleate peanut varieties. These data suggested that the reduction in *ahfad2B* transcript level in the developing seeds is correlated with the high oleate trait.

The prevailing hypothesis on the biosynthesis of erucic acid in developing seeds is that oleic acid, produced in the plastid, is activated to oleoyl-coenzyme A (CoA) for malonyl-CoA-dependent elongation to erucic acid in the cytosol. Several *in vivo*-labeling experiments were designed to probe and extend this hypothesis. To examine whether newly synthesized oleic acid is directly elongated to erucic acid in developing seeds of *Brassica rapa* L., embryos were labeled with [^{14}C]acetate, and the ratio of radioactivity of carbon atoms C-5 to C-22 (*de novo* fatty acid synthesis portion) to carbon atoms C-1 to C-4 (elongated portion) of erucic acid was monitored with time. If newly synthesized 18:1 (oleate) immediately becomes a substrate for elongation to erucic acid, this ratio would be expected to remain constant with incubation time. However, if erucic acid is produced from a pool of preexisting oleic acid, the ratio of ^{14}C in the 4 elongation carbons to ^{14}C in the methyl-terminal 18 carbons would be expected to decrease with time. This labeling ratio decreased with time and, therefore, suggests the existence of an intermediate pool of 18:1, which contributes at least part of the oleoyl precursor for the production of erucic acid. The addition of 2-[{3-chloro-5-(triflurомethyl)-2-pyridinyl}oxyphenoxy] propanoic acid, which inhibits the homodimeric acetyl-CoA carboxylase, severely inhibited the synthesis of [14C]erucic acid, indicating that essentially all malonyl-CoA for elongation of 18:1 to erucate was produced by homodimeric acetyl-CoA carboxylase. Both light and 2-[{3-chloro-5-(triflurомethyl)-2-pyridinyl}oxyphenoxy]-propanoic acid increased the accumulation of [^{14}C]18:1 and the parallel accumulation of [^{14}C] phosphatidylcholine (Bao *et al*., 1998).

The fatty acid elongase (often designated FAE or β-(or 3-) ketoacyl-CoA synthase] is a condensing enzyme and is the first component of the elongation complex involved in synthesis of erucic acid (22:1) in seeds of garden nasturtium (*Tropaeolum majus)*. Using a degenerate primers approach, a cDNA of a putative embryo FAE was obtained showing high homology to known plant elongases. This cDNA contains a 1,512-bp open reading frame that encodes a protein of 504 amino acids. A genomic clone of the nasturtium FAE was isolated and sequence analyses indicated the absence of introns. Northern hybridization showed the expression of this nasturtium FAE gene to be restricted to the embryo. Southern hybridization revealed the nasturtium β-ketoacyl-CoA synthase to be encoded by a small multigene family. To establish the function of the elongase homolog, the cDNA was introduced into two different heterologous chromosomal backgrounds (*Arabidopsis* and tobacco [*Nicotiana tabacum*]) under the control of a seed-specific (napin) promoter and the tandem 35S promoter, respectively. Seed-specific expression resulted in up to an 8-fold increase in erucic acid proportions in *Arabidopsis* seed oil, while constitutive expression in transgenic tobacco tissue resulted in increased proportions of very long chain saturated fatty acids. These results indicate that the nasturtium FAE gene encodes a condensing enzyme involved in the biosynthesis of very long chain fatty acids, utilizing monounsaturated and saturated acyl substrates (Mietkiewska *et al.*, 2004).

The 3´-UTR of the *fad2* gene from *Brassica carinata* was cloned by PCR and used to prepare an intron-spliced hairpin RNA (ihpRNA) construct. Compared to that of the wild type (WT) background, this construct, when expressed in *B. carinata*, resulted in a high degree of *fad2* gene silencing accompanied by strong increases of up to 16 and 10% in oleic acid and erucic acid proportions, respectively. The increase in 18:1 was accompanied by a concomitant proportional reduction in 18:2. A second construct containing ihpRNA targeted to the endogenous *fad2* gene in addition to the heterologous *Crambe abyssinica* FAE gene under the control of seed specific napin promoter, was used to transform *B. carinata*. This approach resulted in an even greater increase in erucic acid proportions, by up to 16% in T1 segregating seeds as compared to that of the WT control. To our knowledge, this is currently the highest accumulation

of erucic acid achieved in *B. carinata* seeds using transgenic approaches, making it an increasingly-attractive alternative to high erucic *B. napus* cultivars as an industrial oil crop (Mietkiewska *et al*., 2008)

A zero erucic acid (C22:1) line of *Brassica juncea* (VH486), adapted to the agronomic conditions of Northern India, has been modified for its fatty acid composition in the seed oil with antisense constructs using the sequence of *fad2* gene of *B. rapa*. The full-length *B. rapa fad2* cDNA sequence was determined by 5' and 3'RACE of a partial sequence available in the EST database. Construct pASfad2.1 contained 315 to 1251 bp and construct pASfad2.2 contained 1 to 1251 bp fragment of the *fad2* gene, both in antisense orientation, driven by a truncated napin promoter. Analysis of the levels of linoleic acid (C18:2) in the BC1 seeds of single-copy transgenics showed that the construct pASfad2.2 gave better suppression of the *fad2* gene as compared to the construct pASfad2.1. The BC1 transgenic seeds containing the pASfad2.2 construct segregated into two distinct classes of C18:2 >20% (putative null homozygotes) and C18:2 <20% (putative heterozygotes) in a 1:1 ratio, while the T1 seeds segregated into three classes, C18:2 >20%, C18:2 between 12% and 20%) and C18:2 <12% (putative homozygotes) in a 1:2:1 ratio. Putative homozygous T1 seeds (C18:2 <12% analyzed by the half-seed method) of four of the transgenic lines were grown to establish T2 homozygous lines. These had ca. 73% C18:1 and 8 to 9% each of C18:2 and C18:3 (α-linolenic acid) fractions in comparison to ca. 53% C18:1, 24% C18:2 and 16% C18:3 in the parental line VH486 (Sivaraman *et al*., 2004).

SUMMARY AND CONCLUSIONS

The present study was carried out to isolate, characterize and to analyze the expression of oleate desaturase gene with a view to use it in future for the manipulation of lipid metabolism in oilseed crops and to allow the studies for its regulation.

- As a first step of the study, a partial genomic and cDNA fragment was isolated by genomic PCR and RT-PCR and cloned in pGEMT Easy vector. Sequence analysis of these clones showed high similarity to the known sequences of delta -12 FADs from *Brassica* sequences. Both genomic and cDNA fragments were 100% identical and showed that isolated genomic fragment does not contain any intron. This partial genomic fragment was used as probe in Southern hybridization.
- Southern blot analysis using partial *fad 2* genomic sequence as probe confirmed that there are atleast 2 copies of *fad2* gene present in the *Brassica juncea* genome which is consistent with the tetraploid nature of the *Brassica juncea* genome.
- Full length genomic and cDNA sequences of *fad2* gene have been isolated by using PCR approach from *Brassica juncea*. Analysis of genomic structure of *fad2* gene revealed that isolated genomic sequence (2526bp) contains 1228 bp 5' untranslated region which is having a large single intron of 1081 bp and 143 bp 3' untranslated region and a CDS of 1155 bp. BLASTN analysis of isolated genomic sequence showed 98% similarity to *Brassica rapa fad2* sequence.
- Isolation and analysis of cDNA sequence (1445 bp) corresponding to genomic sequence (2526bp) revealed that isolated cDNA sequence exactly corresponded with the genomic sequence containing 5'UTR of 147 bp, CDS of 1155 bp and 3'UTR of 143 bp. BLASTN, BLASTX and BLASTP analysis of isolated cDNA sequence showed 98% homology with *Brassica compestris fad2* (Acc No AJ 459108) gene both at nucleotide and amino acid levels.
- Comparision of the *fad2* genomic structures from *Brassica juncea*, *Brassica campestris*, *Sesamum indicum, Arabidopsis thaliana,* Cotton, Soybean and Rice revealed that, *fad2* genomic sequence contains single large intron in their 5' UTR

region which is evolutionarily conserved, although length of the intron varies across the species.

- The complete ORF of Bjfad2 corresponds to a 384 amino acid polypeptide (MW– 44.112 kDa) with a pI-8.62 and containing highest % of hydrophobic amino acids showing membrane bound nature of fatty acid desaturases and hydrophobic structures sharing important characteristics of membrane anchored protein.
- Analysis of *Bj*FAD2 for functional conserved domain by using CDART conserved retrieval tool showed that predicted protein is having putative di iron ligands which forms reactive center by binding with histidine ligands required for catalysis.
- ClustalW alignment of deduced amino acid sequences of the BjFAD2 protein together with the other plant fatty acid desaturases like *Brassica campestris*, B*rassica carinata, Brassica napus, Arabidopsis thaliana, Arachis hypogea, Gossypium hirsutum, Sesamum indicum, Helianthus annus* and *Glycine max* showed that all these membrane-bound desaturases contain the three conserved histidine clusters [HXXXH,HXXHH and HXXHH] which have been shown essential for desaturase activity-acting as potential ligands for non-heme iron atoms.
- Phylogenetic analysis of deduced amino acid sequences of *Bj*FAD2 with other plant microsomal (FAD2) and plastidial (FAD6) oleate desaturases revealed that a BjFAD2 was positioned in a subgroup with FAD2 enzymes that exhibit a house keeping pattern of expression.
- Hydrophobic profile of BjFAD2 protein using BIOEDIT tool showed five prominent hydrophobic peaks at position 55aa-77aa, 82aa-104aa, 119aa-136aa, 176aa-198aa and 224aa-275aa.These peaks were considered likely to be potential candidates for transmembrane domains.
- Prediction of transmembrane helices by using TMHMM server 2.0 showed that BjFAD2 protein is having 5 transmembrane helices that span the membrane 5 times with a portion of the protein, including N and C termini.
- Prediction of protein for its intra cellular localization using PSORT and SIGNAL P software showed that the predicted protein was found to be non secretory in nature and and it lacks any N- terminal transit peptide and also C- terminal motifs (-**KDEL** or **KXKXX**) that would be required for the plastid targeting and there were specific C-

terminal amino acids residues (**-YNNKL)** predicted to function as part of the different putative ER retrieval motifs in FAD2.

- Study of developmental expression of *fad2* gene from developing seeds of *Brassica juncea* through RT-PCR revealed that expression of *fad2* gene was induced in early stages of seed development (15 DAF), peaked in mid maturation stage (30 DAF) and declined as seeds matured (45 DAF).
- Study of temperature dependent expression of *fad2* gene through RT-PCR revealed that expression of *fad2* gene increased under lower temperature treatments with incubation time as compared to the higher temperature treatments where transcript level of *fad2* gene was drastically reduced.
- Effect of temperature on *fad2* gene expression through Real-Time PCR showed that expression of *fad2* gene was increased one fold under lower temperature (10^0C) and decreased three fold in higher temperature treatment (32^0C) as compared to control temperature treatments (21^0C).
- Fatty acid analysis in temperature treatments by gas chromatography showed that there were significant differences in linoleic and linolenic acid content between the treatments with incubation time although these results did not coincide with the results of RT-PCR and Real-Time PCR since short time exposure of plant to different temperatures was not sufficient for alteration in fatty acid composition but *fad2* gene expression was varied among the treatments which induces fatty acid biosynthesis at later stages of seed development.
- Study of differential expression of *fad2* gene in different genotypes of *Brassica* having high erucic acid content (Pusa Bold) and low erucic acid content (LES-39 and LES 1-27) through Real - Time PCR showed that expression of *fad2* gene was two fold higher in LES -39 and 4 fold higher in LES 1-27 as compared to Pusa Bold.
- Fatty acid analysis in high erucic acid (Pusa Bold) and low erucic acid (LES -39 and LES 1-27) lines by gas chromatography revealed that there were significant differences in oleic acid, linoleic acid and erucic content among all genotypes. Oleic acid in high erucic acid genotype like Pusa Bold was found to be significantly low (15.94%) as compared to low erucic acid genotypes (LES-39 and LES 1-27) having 38.47% and 36.75% respectively. In case of low erucic acid genotypes (LES-39 and

LES 1-27) oleic acid was found to be significantly high and erucic acid content was found to be 0.94% and 0.87% in LES-39 and LES 1-27, respectively which was significantly very low as compared to Pusa Bold which was having 25.48% of erucic acid content.

CONCLUSIONS

Thus, in the present study it has been possible to isolate full length genomic and cDNA sequence of *Brassica juncea fad2* gene and to functionally analyze the oleate desaturase gene expression under different conditions. As the omega-6 desaturase is an integral membrane protein, it is difficult to characterize this enzyme by the conventional means of isolating the protein and studying its characteristics. Thus, the sequence based analysis of the deduced protein sequence of *B. juncea fad2* gene isolated in the present study was done and its structural and functional properties were predicted. The isolation of respective promoter region of the gene which is present upstream to the 5'UTR intron will allow the study of genetic regulation of this gene and to study the role of 5'UTR intron in the enhancement of *fad2* gene expression. Consequently, with the aim of improving the quality of vegetable oils, it is of great importance to increase our understanding of the regulation of seed specific *fad2* gene expression.

Table 2: cDNAs and genes for oleate desaturase that have been cloned from higher plants and other sources.

Plant	cDNA/Gene	Approach	Reference
ENDOPLASMIC RETICULUM ORIGIN			
Arabidopsis thaliana	cDNA	T-DNA tagging	Okuley *et al,(1994)*
Arachis hypogea	cDNA	-	*AF030319
	Gene	Heterologous probe	Jung *et al,(2000)*
Brassica carinata	cDNA	Heterologous probe	Amrilla and Taylor, (1999)
Brassica rapa	cDNA	-	*AJ459108
	Gene	-	*AJ459107
Brassica napus	cDNA	-	*AF243045
Glycine max			
Fad2-1A	cDNA	Heterologous probe	Heppard *et al,(1996)*
Fad2-1B	Gene	-	*AB188251
Fad2-2	cDNA	Heterologous probe	Heppard *et al,(1996)*
Gossypium hirsutum			
Fad2-1	cDNA	Heterologous probe	Liu *et al,(1999)*
Fad2-2	cDNA	Heterologous probe	Liu *et al,(1999)*
Fad2-3	Gene	Chromosome walking	Pirtle *et al,(2001)*
Helianthus annus			
Ha fad 2-1	cDNA	PCR	
Ha fad 2-2	cDNA	PCR	Martinez-Rivas *et al,(2001)*
Ha fad 2-3	cDNA	PCR	
Sesamum indicum	Gene/cDNA	PCR	Kim *et al,(2001)*
Olea europaea cv picus			
Oepfad2-1	cDNA	PCR	Hernadez *et al,(2005)*
Oepfad2	cDNA		
PLASTIDIAL ORIGIN			
Arabidopsis thaliana	cDNA	Heterologous probe	Falcone *et al,(1994)*
Brassica napus	cDNA	Heterologous probe	Hitz *et al,(1994)*
Glycine max	cDNA	Heterologous probe	Hitz *et al,(1994)*
Spinacea oleracea	cDNA	PCR	Schimidt *et al,(1994)*

***accession number**

Table 4: Fatty acid composition of triacylglycerols from seeds of *Brassica juncea* cv Pusa Bold treated at different temperatures for different time intervals.

Treatments		Fatty acid content (%)											
		Unidentified	Palmitic acid (16:0)	unidentified	Oleic acid (18:1)	Linoleic acid (18:2)	Linolenic acid (18:3)	Arachidonic acid (20:4)	unidentified	unidentified	Erucic acid (22:1)	unidentified	unidentified
T_1 24hr		1.54±0.12	10.76±0.11	0.00±0.00	21.15±0.22	31.84±0.30	14.33±0.01	6.53±0.02	0.00±0.00	0.00±0.00	10.17±0.03	3.17±0.23	0.00±0.00
T_1 72 hr		3.45±0.09	15.49±0.19	4.15±0.04	5.57±0.17	17.09±0.12	38.5±0.16	2.26±0.04	0.00±0.00	0.00±0.00	4.78±0.05	8.35±0.08	0.00±0.00
T_2 24 hr		0.00±0.00	9.25±0.28	0.00±0.00	20.45±0.26	30.00±0.46	12.44±0.35	8.51±0.24	0.00±0.00	0.00±0.00	13.56±0.17	6.66±0.09	0.00±0.00
T_2 72 hr		0.00±0.00	10.40±0.15	0.00±0.00	22.83±0.08	30.74±0.13	11.14±0.04	6.41±0.07	2.64±0.12	2.07±0.00	9.32±0.20	4.50±0.01	0.00±0.00
T_3 24 hr		5.04±0.03	13.83±0.02	2.79±0.10	10.09±0.29	23.92±0.45	30.62±0.20	0.00±0.00	0.00±0.00	0.00±0.10	5.52±0.15	6.33±0.06	2.42±0.22
T_3 72 hr		2.31±0.10	11.76±0.12	2.84±0.20	13.83±0.20	21.49±0.27	22.33±0.20	4.20±0.04	0.00±0.00	0.00±0.00	10.94±0.41	8.39±0.11	2.05±0.14
SEM ±	T	0.056	0.12	0.06	0.15	0.22	0.14	0.07	0.03	0.02	0.15	0.08	0.07
	I	0.045	0.09	0.05	0.12	0.18	0.11	0.06	0.02	0.02	0.12	0.07	0.06
	TXI	0.079	0.17	0.09	0.21	0.32	0.19	0.10	0.05	0.04	0.21	0.12	0.11
CD AT 5%	T	0.172	0.37	0.20	0.47	0.70	0.43	0.23	0.10	0.09	0.46	0.26	0.24
	I	0.141	0.30	0.16	0.38	0.57	0.35	0.19	0.08	0.07	0.38	0.21	N.S.
	TXI	0.244	0.52	0.28	0.67	0.99	0.61	0.33	0.15	0.12	0.65	0.37	N.S.
CD AT 1%	T	0.24	0.51	0.25	0.64	0.94	0.60	0.30	0.12	0.08	0.64	0.34	1.03
	I	0.19	0.38	0.21	0.51	0.77	0.47	0.25	0.08	0.08	0.51	0.30	N.S.
	TXI	0.34	0.73	0.38	0.90	1.38	0.57	0.43	0.21	0.17	0.90	0.51	N.S.

The values are the average ± SD of three determinations. T – Treatment (T_1-10^0C, T_2-21^0C & T_3-32^0C), I-Incubation time, NS -Non Significant

Table 5: Fatty acid composition of triacylglycerols from seeds of *Brassica juncea* having high erucic acid (Pusa Bold) and low erucic acid (LES-39 and LES 1-27) varieties having different fatty acid composition.

Treatments	Fatty acid content (%)											
	Unidentified	Palmitic acid (16:0)	Oleic acid (18:1)	Linoleic acid (18:2)	Linolenic acid (18:3)	Arachidonic acid (20:4)	unidentified	Erucic acid (22:1)	unidentified	unidentified	unidentified	unidentified
Pusa Bold	0.00±0.00	8.23±0.12	15.94±0.24	21.31±0.04	7.71±0.09	6.15±0.26	1.06±0.25	25.48±0.27	2.46±0.20	6.25±0.06	3.42±0.34	2.12±0.41
LES-39	0.27±0.02	8.57±0.33	38.47±0.02	34.64±0.18	13.47±0.04	1.42±0.01	0.00±0.00	0.94±0.02	0.32±0.01	1.82±0.11	0.00±0.00	0.00±0.00
LES1-27	2.47±0.06	8.22±0.07	36.75±0.40	33.81±0.22	8.23±0.23	2.58±0.17	0.00±0.00	0.87±0.01	2.92±0.02	2.83±0.16	0.00±0.00	0.00±0.00
SEM±	0.04	0.21	0.27	0.17	0.14	0.18	-	0.15	0.11	0.12	-	-
CD AT 5%	0.15	N.S.	0.96	0.60	0.52	0.64	-	0.56	0.41	0.43	-	-
CD AT 1%	0.17	N.S.	1.16	0.73	0.60	0.77	-	0.64	0.47	0.51	-	-

The values are the average ± SD of three determinations. NS -Non Significant

ABBREVIATIONS

PUFA-Poly Unsaturated Fatty Acids

LDL – Low Density Lipoprotein

HDL- High Density Lipoprotein

GALA-Gamma Linolenic Acid

ACP-Acyl Carrier Protein

ER-Endoplasmic Reticulum

PA-Phosphatidic Acid

PG-Phosphatidyl Glycerol

MGDG-Mono Galactosyl Diacyl Glycerol

DGDG-Digalactosyl Diacyl Glycerol

SQDG-Sulpho Quinosyl Diacyl Glycerol

PC-Phosphatidyl Choline

PE-Phosphatidyl Ethanolamine

PI-Phosphatidyl Inositol

PS-Phosphatidyl Serine

NADH-Nicotinamide Adenine Dinucleotide (Reduced form)

TAG- Triacylglycerol

BAC-Bacterial Artificial Chromosome

DTT-Dithiothreitol

IME-Intron Mediated Enhancement

UTR-Untranslated Region

GUS-Glucoronidase

ABA-Abscisic Acid

DAF-Days After Flowering

DAA-Days After Anthesis

kDa- kilo Daltons

FAD-Fatty Acid Desaturase

RT -Reverse Transcriptase

FAE-Fatty Acid Elongase

IPTG-Isopropyl-β-D-thiogalactopyranoside

DEPC-Diethyl Pyro Carbonate

NCBI-National Centre of Biotechnology Information

CTAB-Cetyl trimethyl ammonium bromide

EDTA-Ethylene diamine tetra acetic acid

LB-Luria Broth

SDS-Sodium Dodecyl Sulfate

UV-Ultra Violet

SSC-Saline Sodium Citrate

dNTP-Deoxy Nucleotide Tri Phosphate

OD-Optical Density

GTC-Guanidium Thio Cyanate

FID-Flame Ionization Detector

BLAST-Basic Local Alignment Search Tool

CDS-Coding DNA Sequence

EMBL-European Molecular Biology Laboratory

VLUFA-Very Long Chain Unsaturated Fatty Acids

X-GAL- 5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside

FUNCTIONAL ANALYSIS OF OLEATE DESATURASE GENE EXPRESSION FROM *BRASSICA JUNCEA*

ABSTRACT

Higher plants express one or more microsomal oleic acid desaturase (FAD2; EC1.3.1.35) isoforms which catalyze the insertion of a double bond between carbons 12 and 13 of oleic acid at both the sn-1 and sn-2 positions of phosphatidylcholine. In the present study, full length genomic and cDNA sequences of *fad2* gene have been isolated by using PCR approach from *Brassica juncea*. Analysis of genomic structure of *fad2* gene revealed that isolated genomic sequence (2526bp) contains 1238 bp 5' untranslated region which is having a large single intron of 1081 bp, 143 bp 3' untranslated region, and a CDS of 1155 bp. Analysis of cDNA sequence (1445 bp) corresponding to genomic sequence (2526bp) revealed that isolated cDNA sequence exactly corresponded with the genomic sequence containing 5'UTR of 147 bp, CDS of 1155 bp and 3'UTR of 143 bp. Southern blot analysis using partial *fad 2* genomic sequence as probe confirmed that there are atleast 2 copies of *fad2* gene present in the *Brassica juncea* genome. ClustalW alignment of deduced amino acid sequences of the BjFAD2 protein together with the other plant fatty acid desaturases showed that all these membrane-bound desaturases contained the three conserved histidine clusters [HXXXH,HXXHH and HXXHH] that have been shown essential for desaturase activity-acting as potential ligands for non-heme iron atoms. Phylogenetic analysis of deduced amino acid sequences of *Bj*FAD2 with other plant microsomal (FAD2) and plastidial (FAD6) oleate desaturases showed that a BjFAD2 was positioned in a subgroup with FAD2 enzymes that exhibit a house keeping pattern of expression. Study of developmental expression of *fad2* gene from developing seeds of *Brassica juncea* through RT-PCR revealed that expression of *fad2* gene was induced in early stages of seed development (15 DAF), peaked in mid maturation stage (30 DAF) and declined as seeds matured (45 DAF). Study of temperature dependent expression of *fad2* gene through RT-PCR revealed that expression of *fad2* gene increased under lower temperature treatments with incubation time as compared to the higher

temperature treatments where transcript level of *fad2* gene was drastically reduced. Effect of temperature on *fad2* gene expression through Real-Time PCR showed that expression of *fad2* gene was one fold increased under lower temperature (10^0C) and three fold decreased in higher temperature treatment (32^0C) as compared to normal temperature treatments (21^0C).Fatty acid analysis in temperature treatments by gas chromatography showed that there were significant differences in linoleic and linolenic acid content between the treatments with incubation time. Study of differential expression of *fad2* gene in different lines of *Brassica* having high erucic acid content (Pusa Bold) and low erucic acid genotypes (LES-39 and LES 1- 27) through Real - Time PCR showed that expression of *fad2* gene was two fold increased in LES-39 and 4 fold high in LES 1-27 as compared to Pusa Bold. Fatty acid analysis in high erucic acid (Pusa Bold) and low erucic acid (LES-39 and LES 1- 27) lines by gas chromatography revealed that there were significant differences in oleic acid, linoleic acid and erucic content between all three genotypes. Oleic acid in high erucic acid line like Pusa Bold was found to be significantly low (15.94%) as compared to low erucic acid genotypes (LES-39 and LES 1-27) having 38.47% and 36.75% of oleic acid and 0.94% and 0.87% of erucic acid respecticely which was significantly low as compared to Pusa Bold having 25.48% of erucic acid content.

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Place: New Delhi **(Suresha G.S.)**

DISCUSSION

Lipids are the major structural components of all cellular membranes of living organisms. Oil-producing crop plants also synthesize and store energy in the form of TAGs, which are composed of a glycerol backbone molecule esterified by three saturated and/or unsaturated fatty acyl groups. The relative composition of saturated and unsaturated fatty acids in seed TAGs is one of the major factors influencing the quality of edible oils. Oils high in 18:l and low in polyunsaturated fatty acids appear to have improved nutritional benefits and increased stability (Liu and White, 1992; Yadav, 1995). Therefore, the food industry has a major commercial interest in understanding how to regulate desaturation of fatty acids within storage lipids in oil seed crops.

Plant lipids contain polyunsaturated fatty acids, mainly linoleic and α-linolenic acids, which play crucial roles in plant metabolism as storage compounds mainly in the form of triacylglycerols (TAG), as structural components of membrane lipids, and as precursors of signaling molecules involved in plant development and stress response (Ohlrogge and Browse, 1995; Weber, 2002). Linoleic acid, together with oleic acid, is a major fatty acid in vegetable oils and its content greatly affects technological properties such as their oxidative stability (Ma´rquez-Ruı´z *et al*., 1999) and nutritional characteristics (Cunnane, 2003). In higher plants, the fatty acid biosynthesis is catalyzed in the plastid by a type II (dissociable) fatty acid synthase, leading primarily to the synthesis of palmitoyl-ACP and stearoyl-ACP by successive additions of two carbon atoms from acetyl-CoA (Harwood, 1996). Still in the plastid, most of the stearoyl-ACP is desaturated to oleoyl-ACP by the soluble stearoyl-ACP desaturase. Oleic acid, which is the main product of the plastidial fatty acid synthesis, is largely activated to oleoyl-CoA and exported to the cytosol, where it is incorporated into glycerolipids and can be further desaturated to linoleic acid by the microsomal oleate desaturase (FAD2). This enzyme is located in the endoplasmic reticulum (ER), uses phospholipids as acylsubstrates, and NADH, NADH-cytochrome b_5 reductase and cytochrome b_5 as electron donors (Shanklin and Cahoon, 1998).

In the present study, both genomic and cDNA sequences of *fad2* gene have been isolated by using PCR approach from *Brassica juncea*. Analysis of genomic structure of *fad2* gene revealed that isolated genomic sequence (2526 bp) contains 1228 bp 5' untranslated region which is having a large single intron of 1081 bp, 143 bp 3' untranslated region, and CDS of 1155 bp (Fig. 17). BLASTN analysis of isolated genomic sequence showed 98% similarity to *Brassica rapa* sequence (Acc.No AJ459107) (Figure 14). Similar studies have been reported in number of plant species (Table 2) like *Arachis hypogea* (Jung *et al.,* 2000), *Glycine max* (AB188251), *Gossypium hirsutum* (Pirtle *et al.*, 2001) and S*esamum indicum* (Kim *et al*., 2006).

Comparision of the *fad2* genomic structures from *Brassica juncea, Brassica campestris*, S*esamum indicum, Arabidopsis thaliana,* Cotton, Soybean and Rice revealed that, *fad2* genomic sequence contains single large intron in their 5' UTR region which is evolutionarily conserved, although length of the intron varies across the species (Fig. 18). Evolution of 5' UTR intron and its role on expression of *fad2* gene has been studied in *Sesamum indicum* (Kim *et al*., 2006) and *Gossypium* (Liu *et al.,* 2001; Okuley *et al*., 1994; Voewort *et al.,* 2000; Pirtle *et al.,* 2001). Kim *et al.* (2006) isolated the *SeFAD2* genomic clones and the *At*FAD2 promoter region containing the intron with the aim of studying the intron-mediated regulatory mechanism involved in controlling not only the seed-specific expression of the *SeFAD2* gene but also the expression of plant *FAD2* genes. The *cis*-elements in the *SeFAD2* promoter required for seed-specific expression and the effects of the *SeFAD2* and *AtFAD2* introns on gene expression were assessed in transgenic *Arabidopsis* plants using the β-glucuronidase (GUS) reporter system. The sequences of the *SeFAD2* intron, which are essential for the IME and the promoter-like activity of the *SeFAD2* intron, were also identified. An investigation of the factors responsible for the control of *SeFAD2* gene expression during seed development revealed that ABA was clearly involved in the regulation of *SeFAD2* expression. ABA-responsive elements were also analyzed in the *SeFAD2* promoter.

A positive effect of introns on gene expression has been observed for many plant genes. Expression of reporter genes under the control of the maize *Adh1*, *Sh1*, *Bx1*, or *Act* promoter is increased up to several hundred fold by the inclusion of an

intron (Callis *et al*., 1987; Maas *et al.,* 1991; Oard *et al.,* 1989; Vasil *et al.*, 1989). Similarly, *Arabidopsis thaliana* genes encoding polyubiquitin (Norris *et al.,* 1993), transcription factor EF-1a (Currie *et al.*, 1991, 1993) all have an intron in the 5' UTR region that increases the expression of reporter gene fusions 2.5- to 1000-fold relative to intron-less controls. This enhancement of gene expression has been ascribed to intron splicing (Gidekel *et al*., 1997).

Southern blot analysis using partial *fad 2* genomic sequence as probe confirmed that there are atleast 2 copies of *fad2* gene present in the *Brassica juncea* which is consistent with the tetraploid nature of the *Brassica juncea* genome (Fig. 5). Previous studies of Southern analysis of soybean genomic DNA with *gmFAD2-1* and *gmFAD2-2* specific sequence fragments indicated that there are at least two copies of each of genes in soybean genome which is also a tetraploid species (Heppard *et al.,* 1996). Similar results have been reported by Hernandez *et al*. (2005) in *Olea europaea* cv. Picual. Microsomal omega–6 desaturase gene family from *Brasica napus* has been estimated to contain 4-6 gene copies per haploid genome and they originated from gene duplications or triplications in its progenitor species prior to the formation of *Brassica napus* (Scheffler *et al.,* 1997). In cotton *ghFAD2-1* appears to be a single copy gene in each of the two diploid species (*G. herbacium*) and *G. raimondii*) and presumably this is also true in the progenitor species of the allotetraploid cotton and the combination of them make up the two copies in tetraploid cotton (*G. barbedense* and *G. hirsutum*) (Liu *et al*., 1999).

Isolation and analysis of cDNA sequence (1445 bp) corresponding to genomic sequence (2526bp) revealed that isolated cDNA sequence exactly corresponded with the genomic sequence containing 5'UTR of 147 bp, CDS of 1155 bp and 3'UTR of 143 bp (Fig. 16 & Fig. 17). BLASTN, BLASTX and BLASTP analysis of isolated cDNA sequence showed 98% homology with *Brassica compestris fad2* (Acc No AJ 459108) gene both at nucleotide and amino acid levels (Fig.19, Fig, 20 and Fig. 21). Similar studies have been reported in number of plants using different approaches (Table 2). A locus corresponding to microsomal omega-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), *Brassica napus* (Scheffler *et al*.,1997), *Brassica carinata* (Marilla and Taylor, 1999), *Gossypium hirsutum* (Liu *et al.*, 1999), *Helianthus annus* (Martinez-Rivas *et al.*, 2001), *Sesamum indicum* (Kim *et al.*, 2006) and *Olea europaea* cv Picus (Hernadez *et al*., 2005).

The complete ORF corresponds to a 384 amino acid polypeptide (MW– 44.112 kDa) and a pI-8.62 (Fig. 22 & Fig. 23A) and containing highest % of hydrophobic amino acids (Fig. 23A & Fig. 23B) showing membrane bound nature of fatty acid desaturases and hydrophobic structures sharing important characteristics of membrane anchored protein.

Analysis of *Bjfad2* for functional conserved domain by using CDART conserved retrieval tool showed that predicted protein is having putative di iron ligands (Fig. 24) which forms reactive center by binding with histidine ligands required for catalysis (Shanklin *et al.,* 1997).

ClustalW alignment of deduced amino acid sequences of the BjFAD2 protein together with the other plant fatty acid desaturases like *Brassica campestris*, B*rassica carinata*, *Brassica napus*, *Arabidopsis thaliana*, *Arachis hypogea*, *Gossypium hirsutum*, *Sesamum indicum*, *Helianthus annus* and *Glycine max* showed that all these membrane-bound desaturases contained the three conserved histidine clusters [HXXXH,HXXHH and HXXHH] (Fig. 25) that have been shown essential for desaturase activity-acting as potential ligands for non-heme iron atoms (Shanklin *et al*., 1994; Shanklin and Cahoon, 1998). A group of enzymes including desaturases, hydroxylases, and epoxygenases found in animals, fungi, plants, and bacteria catalyze diverse reactions. These proteins probably use a common reactive center, and these histidine-rich motifs are thought to form part of the di iron center where oxygen activation and substrate oxidation occur (Shanklin *et al*., 1997). A single histidine mutation in three conserved histidine motifs has been found to cause the loss of Spirulina-delta 6 desaturase activity (Kurdrid *et al*., 2005).

For doing the phylogenetic analysis of BjFAD2, its deduced amino acid sequence was aligned with other homologous delta-12 desaturase sequences and an N-J tree was constructed (Fig. 26). As reported previously (Hernandez *et al*., 2005; Li *et al.*, 2007), these plant delta-12 desaturase sequences were classified into three major

branches, termed Housekeeping-type FAD2, FAD6 and Seed-type FAD2. BjFAD2 was positioned in a subgroup with FAD2 enzymes that exhibit a housekeeping pattern of expression (Fig. 25). Unlike reports by Li *et al*., (2007), the position of seed type FAD2 was closer to Housekeeping-type FAD2 than that of the FAD6 (Fig. 26). These observations supported the hypothesis that diverged FAD2 enzymes with the same functionality, but not forming the same clade, may arise independently several times during evolution, and the evolutionary process of Housekeeping-type FAD2, FAD6 and Seed-type FAD2 genes might be different from each other (Dyer *et al*., 2002; Banilas *et al*., 2005). In addition, the observed relative high level of divergence within the Housekeeping-type FAD2 group implied that certain genes might have evolved differently, although more data are required to further confirm this observation.

Hydropathy plot of the BjFAD2 amino acid sequence was generated by the method of Kyte and Doolittle (1982). Five different hydrophobic regions were found (Fig. 27). Hernandez *et al.*, (2005) generated the hydropathy plots for two FAD2 amino acid sequences from *Olea europaea* cv Picus. They found 4 hydrophobic regions where the two flanking hydrophobic domains were long enough to span the membrane twice and corresponded to the predicted membrane spanning domains in desaturase integral membrane protein models. In contrast, the other two are too short, thus they may be single-pass monolayer segments (Shanklin *et al*., 1994). The three conserved histidine boxes were located in hydrophilic regions, and according to this topological model, all of them are exposed to the cytoplasmic side (Los and Murata, 1998). Similar results were also found in soybean (Li *et al.*, 2007).

Prediction of transmembrane helices by using TMHMM server 2.0 showed that BjFAD2 protein is having 5 transmembrane helices that span the membrane 5 times with a portion of the protein, including N and C termini (Fig. 28) which is different from all acyl lipid and acyl-CoA desaturases that apparently span the membrane four time, with a portion of the protein, including N- and C- termini and active site histidine boxes, exposed on the cytosolic side of the membrane (Shanklin *et al*., 1994).

Prediction of signal peptide and protein localization signal was determined by using SIGNALP and PSORT prediction software. The predicted protein was found to be non secretary in nature (Fig. 29) and it lacks any N terminal transit peptide and also

C terminal motifs (-**KDEL** or **KXKXX**) that would be required for the plastid targeting (Jackson *et al.,* 1990).There was an specific C- terminal amino acids residues (**-YNNKL)** predicted to function as part of the different putative ER retrieval motifs in FAD2 (Fig. 25), which has been reported to be necessary and sufficient for maintaining localization of the enzymes in the ER (McCartney *et al.*, 2004). Similar results have been reported in *Olea europaea* cv Picus (Hernandez *et al.*, 2005) and *Glycine max* (Li *et al*., 2007).

Study of developmental expression of *fad2* gene from developing seeds of *Brassica juncea* through RT-PCR revealed that expression of *fad2* gene was induced in early stages of seed development (15 DAF), peaked in mid maturation stage (30 DAF) and declined as seeds matured (45 DAF) (Fig. 32). These results corresponded with the previously reported in soybean (Heppard *et al.,* 1996), Cotton (Liu *et al.,* 1999,) Groundnut (Jung *et al.,* 2000), and Soybean (Tiwari *et al*., 2004).

Study of temperature dependent expression of *fad2* gene through RT-PCR revealed that expression of *fad2* gene increased under lower temperature treatments with incubation time as compared to the higher temperature treatments where transcript level of *fad2* gene was drastically reduced (Fig. 33).

Frequently, Real-Time polymerase chain reaction is combined with reverse transcription polymerase reaction to quantify low abundance messenger RNA (mRNA), enabling a researcher to quantify relative gene expression at a particular time, or in a particular cell or tissue time. Development of PCR technology that uses fluorophores to measure DNA amplification in real time allows researchers to bypass the extensive optimization associated with normal RT PCR. In Real-Time PCR the amplified product is measured at the end of each cycle. These data can be analyzed by computer software to calculate relative gene expression between several samples, or mRNA copy number based on a standard curve.

DNA binding dye binds to all double stranded DNA in PCR reaction, causing fluorescence of the dye. An increase in DNA product during PCR therefore, leads to an increase in florescence intensity and measured at each cycle thus allowing DNA concentration to be quantified. However, double stranded DNA binding dyes such as

SYBR Green will bind to all dsDNA PCR products including nonspecific pcr products (primer dimer).

Relative concentrations of DNA present during the exponential phase of the reaction are determined by plotting fluorescence against cycle number on a logarithmic scale. A threshold for detection of fluorescence above background is determined. The cycle at which the fluorescence from a sample crosses the threshold is called the cycle threshold (Ct). Since the quantity of DNA doubles every cycle during the exponential phase relative amounts of DNA can be calculated (Nolan *et al.,* 2006).

Effect of temperature on *fad2* gene expression was studied through Real-Time PCR and the results from this study coincided with the RT-PCR results showing that expression of *fad2* gene was one fold increased under lower temperature and three fold decreased in higher temperature treatment as compared to normal temperature treatments (Fig. 34). Similar results have been reported in Sunflower (Garces *et al.,* 1992; Sarmiento *et al.,* 1998; Martinnez-rivas *et al.,* 2003; Garcia-diaz *et al.,* 2002; Garcia *et al.,* 2004), Soybean (Sclueter *et al* ., 2007) and Cotton (Karigiotidou *et al.,* 2008).

Fatty acid analysis in temperature treatments by gas chromatography showed that there was significant differences in linoleic and linolenic acid content between the treatments with incubation time (Table 4 & Fig. 35) although these results were not coincided with the results of RT-PCR and Real-Time PCR since short time exposure of plant to different temperatures was not sufficient for alteration in fatty acid composition but *fad2* gene expression varied among the treatments which may induce fatty acid biosynthesis at later stages of seed development.

The fatty acid composition is greatly influenced by growth temperature; the fatty acid polyunsaturation level increases in response to low growth temperature. This appears to be a general phenomenon for both prokaryotic and eukaryotic organisms (Neidleman, 1987). Unsaturated fatty acids have lower melting points than their saturated counterparts and thus may confer greater membrane fluidity, which may allow bacteria and plants to maintain membrane function under lower growth temperature conditions (Neidleman, 1987; Thompson, 1993). In cyanobacteria,

expression of the omega-6 desaturase gene *(desA)* is markedly enhanced by low growth temperature (Los *et al.*, 1993). Introduction of *desA* from a chilling-resistant cyanobacterium, *Synechocystis* PCC6803, into a chilling-sensitive cyanobacterium, *Anacystis*, increased the tolerance of the recipient to low temperature (Wada *et al.*, 1990). This provided direct evidence for the contribution of the unsaturation of fatty acids to low-temperature tolerance in cyanobacteria.

It is assumed that temperatures that induce an increase in the mRNA levels of FAD2-3 and FAD2-4 could be correlated to acclimation temperatures. As previously described (Chang *et al.*, 2001), a 10^0 C exposure of the chilling sensitive *Vigna radiata* (Mungbean) protected it from injuries caused when plants were exposed to 4^0 C. However, this was not the case for *G. hirsutum*. Acclimation experiments performed at a temperature of 10^0C, together with the lipid analysis data, suggested that, although the increase in mRNA levels may result in an increase in lipid unsaturation, no acclimation was achieved. A possible explanation could be that the 10^0C temperature causes damaging effects, the results of which cannot be overcome by the increase in lipid unsaturation. It is also possible that the increase in di-unsaturated fatty acid species at 10^0C could occur in order to protect the species from the direct 10^0C effect. The results indicated that the longer the exposure to low temperatures the higher was the plant death rate (Karigiotidou *et al.*, 2008). It was however, clear that light played an important role in plant survival ability since plants achieved much higher survival rates when exposed to cold temperatures under dark conditions (Rikin *et al.*, 1993). By contrast, although no increase in FAD2 occurs at 20^0C, increasing lipid unsaturation as observed by Rikin *et al.* (1993) may be achieved by post-transcriptional/translational modifications of FAD2 or other desaturase isoforms leading to the observed increase in the acclimation ability of the plant.

Study of differential expression of *fad2* gene in different genotypes of *Brassica* having high erucic acid (Pusa Bold) and low erucic acid content (LES-39 and LES 1-27) through Real-Time PCR showed that expression of *fad2* gene increased two fold in LES-39 and 4 fold in LES 1-27 as compared to Pusa Bold (Fig. 36). Fatty acid analysis in these lines by gas chromatography revealed that there were significant differences in oleic acid, linoleic acid and erucic content between all three genotypes.

Oleic acid content in high erucic acid genotype (Pusa bold) was significantly low as compared to low erucic acid genotypes (LES-39 and LES 1-27) (Table 5 & Fig. 37). This may be due to the high expression of *fae* gene in Pusa Bold as compared to *fad2* whose expression was significantly high in low erucic acid lines since % erucic acid in these genotypes was found to be significantly very low as compared to Pusa Bold.

Oleic acid is precursor for linoleic acid and for VLUFA (very long chain unsaturated fatty acids) like erucic acid. So the plants which are having high erucic acid in their seed oil have less expression of *fad2* gene and vice varsa.

The *Arabidopsis FAE1* gene encoding a fatty acid condensing enzyme, which synthesizes the long-chain fatty acids (C20 and C22) in seeds, has recently been isolated (James *et al.*, 1995) and has been shown to specifically express in developing seeds. This finding is in agreement with the observed phenotype of the *fael* mutation, which is only expressed in the seeds. Hence, some genes encoding fatty acid biosynthetic enzymes are regulated by a seed developmental program. These genes appear to play a major role in determining the composition of storage lipids in developing seeds. The timing of *FAD2-1* gene expression appears to be earlier than that of seed storage protein genes (Goldberg *et al*., 1989). Similarly, the *Brassica napus* ACP gene is also expressed earlier than the seed storage genes encoding napin and cruciferin (Kridl e*t al.*, 1993). Therefore, regulation of genes encoding fatty acid biosynthetic enzymes and seed storage proteins may be different. Comparison of the *FAD2-1* gene with seed storage protein genes revealed no similarity in their promoter regions (G.-H. Miao, unpublished observation). It will be interesting to determine whether *FAD2-1*, *FAE1*, and other fatty acid biosynthetic genes highly expressed in seeds share any common structural features in their regulatory elements.

Over-expression of the *Crambe abyssinica* FAE gene in *Brassica carinata* resulted in a substantial increase in the proportion of erucic acid in seeds compared to the wild type control (Mietkiewska *et al.,* 2007).The synthesis of erucic acid in transgenic *B. carinata* plants was probably, in part, limited by the smaller microsomal pool of oleoyl moieties (7–8%) available for elongation. As pointed out previously by Bao *et al.,* (1998) and subsequently by Jadhav *et al.,* (2005) the flux of 18:1 through distinct intermediate lipid pools before elongation might be a factor that limits the

availability of 18:1 for elongation. The oleate desaturase, FAD2, is one of the crucial enzymes for the production of polyunsaturated fatty acids in plants (Okuley *et al.,* 1994). By altering the level of FAD2 gene expression using antisense and cosuppression approaches, it is possible to increase the pool of 18:1 available for elongation to enhance production of erucic acid in *B. carinata* seeds (Jadhav *et al.*, 2005 and Mietiewska *et al.,* 2008). However, the antisense and cosuppression strategies have variable and unpredictable effectiveness and require the production of large populations of transgenic plants to obtain a reasonable number of lines showing sufficient levels of target gene suppression (Liu *et al.*, 2002). Mietkiewska *et al.*, (2008) observed that hairpin-RNA mediated silencing of endogenous FAD2 gene combined with heterologous expression of *Crambe abyssinica* FAE gene causes an increase in the level of erucic acid in transgenic *Brassica carinata* seeds. They used a partial 3'UTR of the seed-specific *B. carinata* FAD2 gene to prepare an intron-spliced hpRNA construct to silence the seed FAD2 gene and consequently, to increase the pool of oleic acid available for elongation and how an increased pool of oleic acid can contribute to a dramatic increase in the content of erucic acid in *Brassica* seeds, particularly when combined with heterologous *C. abyssinica F*AE expression.

Improvement of the nutritional value of the seed oil through genetic engineering is a major thrust area of research in plant biotechnology (Przybylski and Mag, 2002). Many of the genes involved in the fatty acid biosynthetic pathway have been characterized and isolated. The enzyme delta 12 desaturase is known to be responsible for the conversion of the C18:1 to C18:2 which is further converted to C18:3 by the enzyme delta 15 desaturase (Miquel and Browse, 1992; Browse *et al.*, 1993). The microsomal delta 12 desaturase gene coding for the enzyme fatty acid desaturase 2 (FAD2) is primarily responsible for more than 90% of the PUFA in non-photosynthetic tissues, such as roots and developing seeds of oilseed crops (Miquel and Browse, 1992). There have been a number of reports wherein the introduction of seed-specific antisense and sense *fad2* constructs have led to a reduction in the PUFA levels in oilseed crops (Töpfer *et al*., 1995, Stoutjesdijk *et al*., 2000 and Liu *et al*., 2002). In rapeseed (*B. napus*) transgenic lines have been developed with as high as 85% oleic acid and PUFA fraction reduced to 4-5% in their seed oils using co-

suppression and antisense technologies (Kinney, 1994). A preliminary report by Stoutjesdijk *et al.,* (2000), on increasing the oleic acid fraction in *B. juncea* (Australian variety) using co-suppression of *fad2* reported an increase in the C18:1 fraction and a concomitant reduction in the C18:2 and C18:3 (PUFA) fractions. Shivaraman *et al.,* (2004) used the antisense methodology for the suppression of *fad*2 expression in order to develop low PUFA lines in the '0' erucic line, VH486 of *B. juncea*. Two antisense constructs were made from different regions of the *fad2* gene isolated from *B.rapa*, one of the diploid progenitors of the allotetraploid *B. juncea* (Morinaga, 1934). They reported the development and characterization of transgenic lines with high oleic acid fraction (ca. 73%) and lower levels of polyunsaturated fractions as compared to VH486 using the antisense constructs.

In recent years, genes have been cloned for all the major enzymes that control fatty acid biosynthesis in oilseeds, including the delta 9 and delta 12-desaturases that determine the relative proportions of C18 saturated, monounsaturated and polyunsaturated fatty acids. Furthermore, methods of posttranscriptional gene silencing (PTGS) have been developed that enable the expression of these genes to be precisely down regulated during oil synthesis in the developing seed, without affecting their expression in other parts of the plant. PTGS can be invoked to modify seed oil fatty acid composition by seeds pecifically expressing a DNA sequence that is complementary to the whole or part of the appropriate target fatty acid biosynthesis gene. The genetic modifications were achieved by seed-specific silencing of the two genes encoding the key fatty acid desaturase enzymes determining the fatty acid composition of seed oils, namely stearoyl-ACP delta 9-desaturase and oleoyl-PC delta 12-desaturase.Several candidate genes for these enzymes were first cloned from a cotton seed cDNA library based on their expected homology to the already sequenced delta 9-desaturase gene from castor bean and the delta 12- desaturase gene from *Arabidopsis thaliana*. Analysis of expression patterns for the candidate sequences revealed the particular genes that were responsible for the activity of these enzymes in the developing seed, namely the detlta 9-desaturase gene *ghSAD-1* (Liu *et al.,* 1996) and the delta 12-desaturase gene *ghFAD2-1* (*Liu et al.,* 1999). Liu *et al., (*2002) reported the hpRNA-mediated PTGS in cotton to down-regulate key fatty acid

desaturase genes and develop nutritionally-improved high-oleic (HO) and high-stearic (HS) cottonseed oils (CSOs). Silencing of the *ghFAD2-1* delta 12-desaturase gene raised oleic acid content from 13% to 78% and silencing of the *ghSAD-1* delta 9-desaturase gene substantially increased stearic acid from the normal level of 2% to as high as 40%. Additionally, palmitic acid was significantly lowered from 26% to 15% in both HO and HS lines. Intercrossing the HS and HO lines resulted in a wide range of unique intermediate combinations of palmitic, stearic, oleic and linoleic contents. The oxidative stability, flavor characteristics and physical properties of these novel CSOs are currently being evaluated by food technologists.

Gene technology has provided plant researchers with powerful new tools for manipulating the composition of plant products. In oilseeds, this has significantly extended the capability to achieve major alterations in the relative proportions of the fatty acids present in the oil, for the purposes of improving nutritional value without compromising on functionality. Oilseeds that are rich in stearic or oleic acid will provide commercial cooking fats and oils with the required stability and performance and enable industry to move away from the use of oils with high contents of palmitic acid and *trans*-fatty acids. The wide-scale introduction of these improved oils has the potential to deliver substantial public health benefits through lowering of serum LDL-cholesterol levels and consequent reduced incidence of cardiovascular disease. Such nutritionally-enhanced oils will be some of the first food products to be genetically modified specifically to provide consumer benefits and are considered likely to have greater consumer appeal than foods derived from initial GM crops that were engineered for predominantly agricultural benefits.

Modifying the fatty acid composition of *Brassica* seed oil to increase its value as nutritional or as industrial oil has been a major objective in *Brassica* breeding programmes world wide. As an alternative to the conventional breeding approaches, genetic engineering opened a large field of academic research and provides an enormous potential applications for altering fatty acid composition to develop a designer oilseed crops.

As omega-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.

Thus, in the present study the genomic and cDNA sequences of *fad2* have been isolated and expression analysis of *fad2* gene has been studied from *Brasssica juncea*. Intron present in 5'UTR region is evolutionarily conserved and predicted to act as enhancer on *fad2* gene expression. So there is a need to isolate promoter specific for the *fad2* gene and to study the transcriptional regulatory mechanism of *cis* regulatory elements in *fad2* promoter and enhancers in the 5'UTR intron. Intron-mediated regulatory mechanism was found to be essential in controlling expression of the plant *fad2* genes, which play a pivotal role in the synthesis of polyunsaturated fatty acids (Kim *et al.,* 2006). For the development of new crop cultivars through the application of plant biotechnological methodology, one critical factor is the availability of suitable promoters that are able to provide strong expression of genes within a specific tissue. So the present study provides an easy way to isolate and characterize promoter specific for *fad2* gene from *Brassica juncea* and to study its regulatory role along with 5' UTR intron on the expression of gene.

Since oleic acid is the precursor for both linoleic acid and erucic acid biosynthesis in the seed oil, it is essential to study further the inter-regulation of both *fad2* and *fae* genes expression during seed development.

EXPERIMENTAL RESULTS

In plants, the endoplasmic reticulum (ER)-associated oleate desatuarse (FAD 2) is the key enzyme responsible for the production of linoleic acid in non-photosynthetic tissues. In the present study an attempt has been made to isolate, clone oleate desaturase gene from *Brassica juncea* and to analyze the expression of gene under different conditions. This has been done by isolating genomic and cDNA fragments of *Brassica juncea fad2* gene encoding omega-6 desaturase and sequence characterization by using various bioinformatics tools which are available in public domain. Expression analysis of *fad2* gene was studied by using RT- PCR and Real-Time PCR. The results obtained are described below.

Isolation of partial genomic and cDNA fragments by genomic PCR and RT-PCR.

Partial genomic and cDNA fragments were amplified by genomic and RT-PCR by using *fad2* gene specific primers, SGS F and SGS R as given in Table 3. Template DNA for genomic PCR and RNA for RT-PCR was isolated as mentioned in Materials and Methods. Analysis of amplified product for both genomic PCR and RT-PCR on 1% agarose gel electrophoresis showed an intact band of 0.98 kb (Fig. 2A & Fig. 2B). The amplified products from both the reactions were eluted from the gel and ligated into pGEMT Easy vector facilitating T/A cloning and transformed into competent cells of *E coli* DH5α strain. The recombinant clones were selected by blue/white screening. Plasmids from recombinant clones were isolated, digested with *Eco*RI and separated on 1.0% agarose gel along with λ DNA cut with *Hin*dIII + *Eco*RI marker (Fig. 3). Restriction digestion of recombinant plasmid released an insert of 0.98 kb as shown in Fig. 3. Inserts from both genomic and cDNA clones(one each) were sequenced by automatic DNA sequencing facility in Delhi university, South Campus and the size of both genomic and cDNA fragments were found to be 987bp (Fig. 4A & Fig. 4B). Sequence analysis of these clones showed high similarity to the known sequences of delta -12 *fads* from *Brassica*. Both genomic and cDNA fragments were 100% identical and showed that isolated genomic fragment does not contain any intron (Fig. 4A & Fig. 4B). This partial genomic fragment was used as probe in Southern hybridization.

Southern blot analysis:

In order to carry out Southern hybridization, 5 µg of genomic DNA was digested with 4 restriction enzymes namely, *Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I (Fig. 5) whose restriction sites were not present inside partial genomic fragment sequence which was used as probe, after labeling with (α - ^{32}p) dCTP. The results from this study showed 2 bands each with genomic DNA digested with *Bam*HI and *Pst*I when hybridizing with partial *fad2* gene fragment as probe. *Eco*RI, *Bam*HI and *Hin*dIII digested genomic DNA showed one common band of high molecular weight (Fig. 6).This may due to the incomplete digestion of genomic DNA. These results suggested that there were at least two copies of *fad2* gene in the *Brassica juncea*, which is consistent with the tetraploid nature of the *Brassica juncea* genome.

Isolation and cloning of full length cDNA sequence.

Full length cDNA sequence of *fad2* gene from *Brassica juncea* was isolated through RT-PCR by using BcaF and BcaR primers (Table 3). RNA was isolated from developing seeds of *Brassica juncea* (Fig. 7) and RT-PCR was carried out as mentioned in Materials and Methods. The analysis of amplified product on 0.8% agarose gel electrophoresis showed an intact band of 1.45 kb (Fig. 8). The amplified product was eluted from the gel and ligated to pGEMT Easy vector facilitating T/A cloning and transformed into competent cells of *E coli* DH5α strain. The screening of recombinant clones was done by restriction digestion of plasmids isolated from white colonies and separated on 0.8% agarose gel electrophoresis along with λ *Hin*dIII + *Eco*RI marker. *Eco*RI digestion of recombinant plasmid released an insert size of 1.45 kb and is shown in Fig. 9. One of the recombinant clones was sequenced from automatic sequencing facility in Delhi University, South Campus and shown to have sequence of 1445 nucleotides (Fig. 10).

Isolation and cloning of full length genomic sequence of *fad2* from *Brassica juncea*.

In order to isolate full length genomic sequence of *fad2* gene, corresponding to cDNA sequence, BcaF and BcaR primers were used (Table 3). Long PCR kit was used to amplify the full length genomic sequence according to manufacturer's instructions

as mentioned in Materials and Methods. Amplified product was analyzed on 0.8% agarose gel electrophoresis (Fig. 11).An about 2.5 kb of amplified product was observed and was eluted from the gel, ligated into T overhang arms of pGEMT Easy vector. Ligated product was transformed into competent cells of *E coli* DH5α strain. The recombinants were screened by *Eco*RI restriction digestion of plasmid isolated from white colonies (Fig. 12).One of the positive clones was sequenced from automatic sequencing facility in South campus, Delhi University .The sequencing results showed the size of amplified product to be 2526 bp (Fig. 13).

Homology search of *Bjfad2* genomic sequence was done using advanced BLASTN search tool developed by NCBI, USA (Altschul *et al*., 1990) (Fig. 14).It showed maximum homology of 98% with genomic sequence of *Brassica rapa* (AJ459108).

Restriction map analysis by using NEB cutter software (Fig. 15) revealed that *Bjfad2* sequence does not contain restriction sites for the most commonly used enzymes like *Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I which are frequently used in molecular biology.

By comparing cDNA sequence of *Bjfad2* with genomic sequence revealed that it contains 2 exonic sequences which are exactly identical in both cDNA and genomic sequences (Fig. 16 & Fig. 17). Exon I is having length of 143 bp, and Exon II is of 1302 bp (Fig. 16 & Fig. 17). A single large intronic sequence of size 1081 bp is present in between two exonic sequences in *Bjfad2* genomic sequence which is completely absent in cDNA sequence (Fig. 16). The gene sequence was further analyzed for 5'UTR, CDS and 3'UTR region. 5' UTR region in cDNA sequence of *Bjfad2* consists of Exon I and 4 bp from Exon II having total size of 147 bp (Fig. 16). The size of CDS region of cDNA sequence was 1155 bp, starting from 148 bp to 1302 bp. The 3' UTR region is having size of 143 bp starting from 1303 to 1445 bp (Fig. 16). In case of *Bjfad2* genomic sequence, from the first base of Exon I to 1228 base position of Exon II was classified as 5' UTR region comprising of 143 bp of Exon I, 1081 bp of intronic sequence and 4 bp of Exon II sequence. The size of CDS is 1155 bp starting from 1229 bp to 2383 bp. The length of 3'UTR is 143 bp which is from 2384 to 2526 bp (Fig. 17).

Role of intron in enhancement of *fad2* gene expression.

Analysis of *Bjfad2* gene sequence showed that a single large intron of size 1081 bp is present in 5' UTR region of the gene (Fig. 17). Comparison of *fad2* genomic structures from *Brassica juncea*, Sesame, *Arabidopsis*, Cotton, Soybean and Rice revealed that single intron present in 5' UTR region of *fad2* gene is evolutionarily conserved. Although the length of the intron varies across the species, it is highly conserved and involved not only in the enhancement of *fad2* gene expression but also in the change of tissue specificity and of seed specific gene expression into constitutive expression (Fig. 18).

***In silico* analysis of *Bjfad2* cDNA sequence by using various bioinformatics tools which are available in databases.**

BLASTN, BLASTX and BLASTP analysis showed that isolated cDNA sequence is maximum identical (98%) to *Brassica rapa fad2* both at nucleotide and amino acid level *(*Fig. 19, Fig. 20 & Fig. 21).

Isolated cDNA sequence was submitted to the EMBL nucleotide sequence database using WEBIN, the EMBL online sequence submission tool at the URL:http://www.ebi.ac.uk and bears the Accession number EF639848.

Open Reading Frame (ORF) of isolated *Bjfad2* cDNA sequence codes for putative protein having 384 amino acids and size of 44 kDa with start codon present at 148 bp position and stop codon at 1302 bp position (Fig. 22).Theoretical pI of predicted protein was 8.62 (Fig. 23A)

Amino acid composition

Analysis of amino acid composition of predicted protein showed that it comprises highest % of hydrophobic amino acids like Leucine (8.6%), Valine (7.3%), Glycine (6.8%), Alanine (6.2%) and Isoleucine (5.5%) showing the membrane bound nature of fatty acid desaturases and hydrophobic structures sharing important characteristics of membrane anchored protein. The total number of negatively charged residues (Asp + Glu) are 30 and total number of positively charged residues (Arg + Lys) are 36 (Fig. 23A & Fig. 23B).

Functional conserved domain search

Isolated *Bjfad2* cDNA sequence was further analyzed for functional conserved domain by using CDART conserved retrieval tool provided by NCBI. The results from this study showed that predicted protein was having putative di iron ligands which are required for catalysis of fatty acid desaturases and was showing multi domain nature of all fatty acid desaturases (Fig. 24).

Multiple clustalW analysis of *Bj fad2*

ClustalW analysis of deduced amino acid sequence of *Brassica juncea* fatty acid desaturase with other plant desaturases like *Brassica campestris*, *Brassica carinata*, *Brassica napus*, *Arabidopsis*, *Arachis*, *Gossypium*, *Sesame*. *Helianthus annus and Glycine max* confirmed that all these membrane bound desaturases contained three histidine clusters (HXXXH, HXXHH and HXXHH) that have been shown essential for desaturase activity (Fig. 25).

Phylogenetic analysis

To elucidate the phylogenetic relationships of the *Bjfad2* with other plant microsomal (*fad2*) and plastidial (*fad6*) oleate desaturases, all their deduced amino acid sequences were aligned and N-J tree was constructed (Fig. 26). Based on their homology these plant delta 12 desaturases were classified into three major branches termed house keeping type *fad2*, *fad6* and seed type *fad2*. *Bjfad2* was positioned in a subgroup with FAD2 enzymes that exhibit a house keeping pattern of expression.

Hydrophobicity profile

BjFAD2 protein was characterized for its hydrophobicity using hydrophobic scale provided by Kyte and Doolittle (1982) available in BIOEDIT tool. BjFAD2 protein showed five prominent hydrophobic peaks at a position of 55aa-77aa, 82aa-104aa, 119aa-136aa, 176aa-198aa and 224aa-275aa (Fig. 27). These peaks were considered likely to be potential candidate for transmembrane domains.

Prediction of transmembrane helices

BJFAD2 was characterized for the presence of trransmembrane helices and the location of intervening loop using TMHMM server 2.0 (http://www.cbs.dtu.dr/services/TMHMM/). It has been predicted to have 5

transmembrane helices. These five membrane spanning domains that apparently span the membrane 5 times with a portion of the protein, including N and C termini (Fig. 28).

Prediction of protein localization

Fatty acid desaturase 2 from *Brassica juncea* was analyzed for its intra cellular localization using SIGNAL P and PSORT prediction software. The predicted protein was found to be non secretary in nature and does not contain either chloroplast or mitochondrial signal peptide sequence (Fig. 29). It showed 95 % and 90 % identity to endoplasmic reticulum localized *Brassica* fatty acid desaturase 2 protein and *Arabidopsis* ER localized FAD2 protein respectively (Fig. 30).

Expression analysis of *fad2* gene

Study of developmental expression of *fad2* gene

In order to study the developmental expression of the *fad2* gene, the transcript level was examined by RT-PCR using *fad2* gene specific QRTF2 and QRTR2 primers (Table 3).Total RNA was isolated from developing seeds at 3 different stages (15 ,30 and 45 DAF)(Fig. 31). RT-PCR was carried out for all 3 stages as mentioned in Material and Methods. The results from this study indicated that the expression of *fad2* gene was induced during early stages of seed development i.e. 15 DAF (Fig. 32). The transcript levels of *fad2* gene rapidly increased and peaked at mid maturation stages i.e. 30 DAF. However, expression of *fad2* gene gradually declined as seeds matured i.e. (45 DAF) (Fig. 32).Thus the timing of *fad2* gene expression induces fatty acid biosynthesis and oil deposition in later stages of seed development.

Effect of temperature on *fad2* gene expression

Temperature dependent expression of *fad2* gene was studied though RT PCR and Real-Time PCR. *Brassica juncea* plants were grown in growth chambers at 3 different temperatures namely 10^0C (low), 21^oC (normal) and 32^0C (high) for 24 hrs to 72 hrs with the light/dark cycle of 12/12hrs.

RNA was isolated from the seeds of all treatments during mid stage of seed development (30DAF) and RT-PCR was carried out. Results from this study showed that expression of *fad2* gene was gradually increasing from normal temperature treatments to lower temperature treatments with incubation time 24 hrs and 72 hrs.

Transcript levels of *fad2* gene in plants grown at 32^0C for 72 hrs was drastically reduced as compared to plants grown at same temperature for 24 hrs as well as other temperature treatments (Fig. 33).

In order to confirm the results of RT-PCR, Real-Time PCR analysis was done to study the effect of temperature on *fad2* gene expression.0.5 µg cDNA from 72hr incubated treatments (which was prepared for RT PCR as discussed earlier) was used to carry out Real-Time PCR using QRTF2-QRTR2 as *fad2* gene specific primers and actf-actr *β-actin* gene specific primers as internal control (Table 3). The protocol has been already mentioned in Materials and Methods. The results from this study showed that, the expression of *fad2* gene is one fold higher in lower temperature treatment (10^0C) and more than three fold less in higher temperature treatment (32^0C) as compared to normal temperature treatment 21^0C (Fig. 34).These results were corresponded with the RT-PCR results which were shown earlier.

Fatty acid analysis at different temperature treatments

Fatty acids content in different temperature treatments was analyzed as mentioned in Materials and Methods and the results have been shown in Table 4 & Fig. 35. Oleic acid content in plants grown at 10^0C for 24 hr was 21.16% and there was significant increase in linoleic acid content to 31.84% (Table 4).This may due to the high activity of FAD2 enzyme under low temperature. Plants which are incubated at 10^0 C for 72 hrs had 5.57% of oleic acid and 17.09% of linoleic acid (Table 4). But linolenic acid content was significantly increased to 37.54%.This may be due to the high activity of FAD3 enzyme which is coded by *fad7* gene and low activity FAD2 enzyme.

Plants grown at normal temperature (21^0C) had 20.45% (T2 24hr) and 22.84% (T2 72hrs) of oleic acid respectively and there was significant increase in linoleic acid content to 30.0% (T2 24hrs) and 30.75% (T2 72hrs) due the activity of FAD2 enzyme (Table 4).

Under higher temperature treatments, oleic acid content was found to be 9.92% (T3 24 hr) and 13.84% (T3 72hrs) and linoleic acid content was 23.92% (T3 24hrs) and 21.50% (T3 72 hrs) which were significantly less as compared to a low temperature and normal temperature treatments. But linolenic acid content was

significantly increased to 30.63% (T3 24 hr) and 22.33% (T3 72 hr) as compared to linoleic acid content in both the treatments (Table 4).

Fatty acid content in different temperature treatments were not much coinciding with the results of RT-PCR and Real-Time PCR although linoleic acid and linolenic acid contents were significantly more in lower and normal temperature treatments as compared to higher temperature treatments (Table 4 & Fig. 35). This may be due to the fact that short time (24 & 72hrs) exposure of plants to different temperatures is not sufficient to alter the fatty acid content, although gene expression altered at different temperatures which induce fatty acid biosynthesis at later stages of seed development.

Study of Differential expression of *fad2* gene

Oleic acid is precursor for linoleic acid and for VLUFA (very long chain unsaturated fatty acids) like erucic acid .So the plants which are having high erucic acid in their seed oil may have less expression of *fad2* gene and vice varsa.

Differential expression of *fad2* gene was studied through Real-Time PCR. *Brassica juncea* lines having high erucic acid content (Pusa Bold) and low erucic acid lines (LES–39 and LES 1-27) were taken for this study. Seeds from all 3 lines were collected during mid maturation stage of seed development (30 DAF). RNA was isolated and first strand cDNA was synthesized as mentioned in Material and Methods. Real- Time PCR was carried out in all *Brassica* lines using 0.5 µg of cDNA from each sample .The results from this study showed that expression of *fad2* gene was 2 fold high in LES-39 and 4 fold high in LES 1-27 as compared to high erucic acid line (Pusa Bold) (Fig. 36). There was a difference in *fad2* gene expression between the two low erucic acid genotypes although both are having zero erucic acid genotypes. This may be due to differences in fatty acid elongase (FAE) gene expression which codes for elongase enzyme that catalyzes the conversion of oleic acid to erucic acid, and also there may be difference in expression of *fad2* gene between these two lines. These results confirmed that expression of *fad2* gene depends on availability of oleic acid which is substrate for both linoleic acid and erucic acid biosynthesis. Hence high erucic acid lines have less expression of *fad2* gene which

may be due to the diversion of oleic acid pools to erucic acid synthesis by the activity of fatty acid elongase enzyme. It can be speculated that expression of *fad2* gene and *fae* gene are interregulated depending on the availability and source of oleic acid pool in the cell.

Fatty acid analysis in different *Brassica juncea* genotypes

In order to analyze the fatty acid content in these genotypes, seeds from each line were collected during mid maturation stage (30 DAF) and fatty acid content was determined as described in Materials and Methods. The results from this study showed that oleic acid pools in high erucic acid line like Pusa Bold was found to be significantly low (15.94%) as compared to low erucic acid genotypes (LES-39 and LES 1-27) having 38.47% and 36.75% respectively (Table 5). This may due to the high expression of fatty acid elongase gene in Pusa Bold since % erucic acid content was found to be significantly high (25.48%) as compared to linoleic acid (21.34%) (Table 5).These results showed that expression of *fad2* gene was significantly less in Pusa Bold due to the high activity of fatty acid elongase.

In case of low erucic acid lines (LES-39 and LES 1-27) oleic acid pools were found to be significantly high (Table 5 & Fig. 37). Due to this there was a competition between fatty acid desaturase and elongase enzyme for their activities. Linoleic acid content in these lines were found to be significantly very high (34.64% and 33.81%) as compared to Pusa Bold (Table 5).This may be due to the high expression of *fad2* gene in these lines as compared to *fae* gene since erucic acid content was found to be 0.94% and 0.87% in LES -39 and LES 1-27, respectively which was significantly very low as compared to Pusa Bold which was having 25.48% of erucic acid content (Table 5).These results corresponded with the results from differential expression analysis of *fad2* gene through Real-Time PCR and also confirmed that expression of *fad2* gene and *fae* gene differs and interregulated in *Brassica* lines having different fatty acid content.

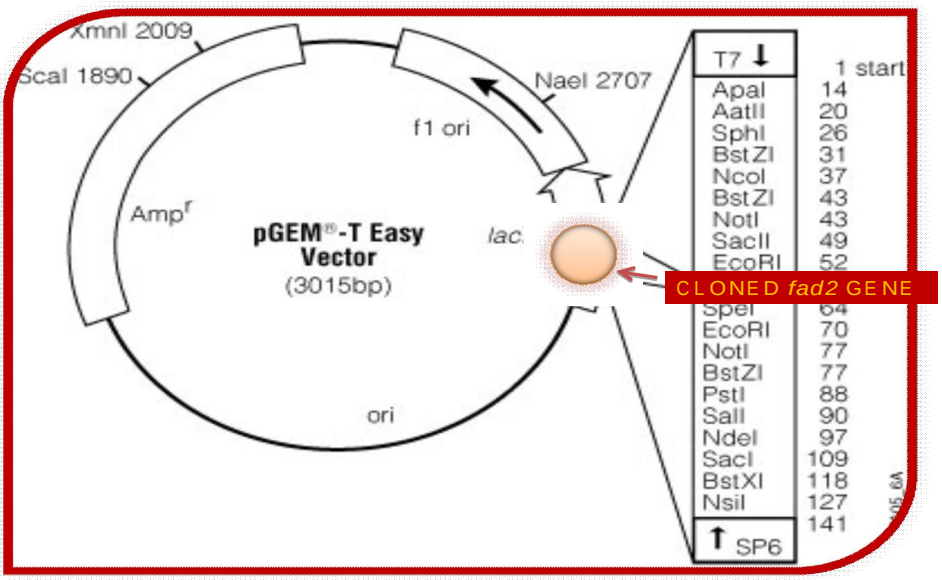

Figure 1: pGEMT Easy vector used for cloning of both genomic and cDNA sequence of *fad2* gene isolated from *Brassica juncea*.

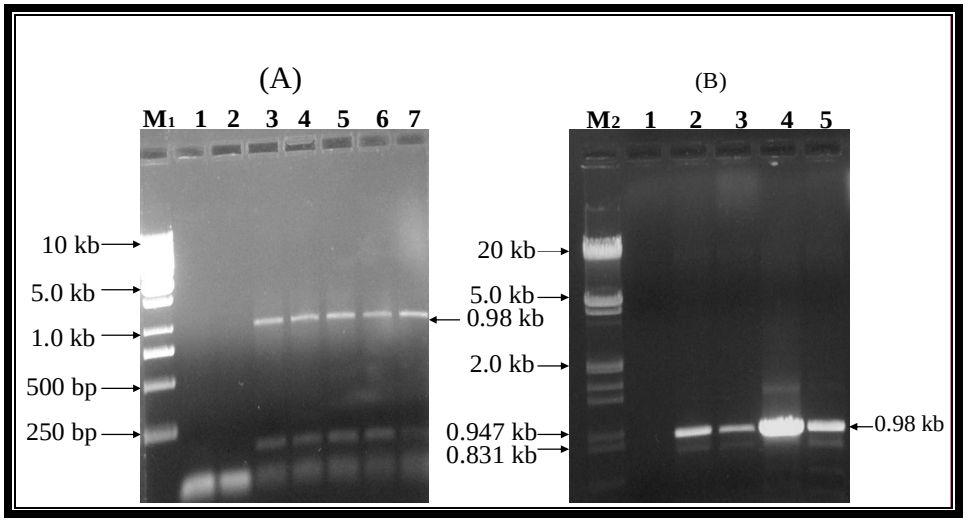

Figure 2: PCR amplification of partial (A) genomic and (B) cDNA fragment separated on 1.0% agarose gel along with molecular size markers (M1) 1kb ladder and (M2) λ *Hin*dIII & *Eco*RI , lanes 1, 2, 3, 4, 5, 6, 7- (given gradient temperatures) used in PCR.

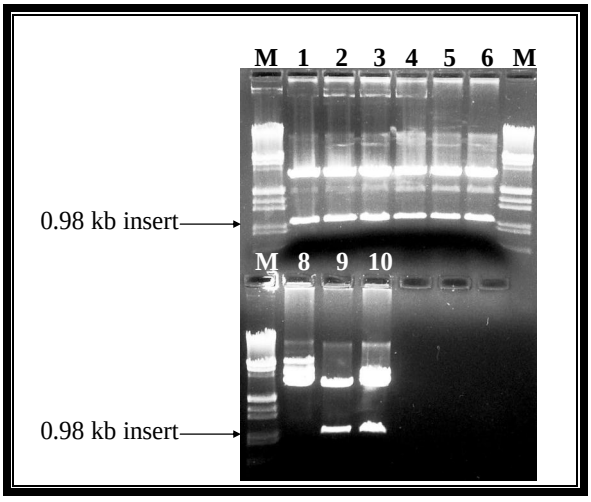

Figure 3: Restriction of genomic and cDNA recombinant clones separated on 1.0% agarose gel along with molecular size markers (λ *Hin*dIII & *Eco*RI), M– marker, lanes 1, 2, 3, 4 & 5 – genomic clones and lanes 6, 8, 9 & 10 - cDNA clones.

5’GCTCCTTCTCCTACCTCATCTGGGACATCATCATAGCCTCCTGCTTCTACTA
CGTCGCCACCACTTACTTCCCTCTCCTCCCTCACCCTCTCTCCTACTTCGCCT
GGCCTCTCTACTGGGCCTGCCAGGGCTGCGTCCTAACCGGCGTCTGGGTCAT
AGCCCACGAGTGCGGCCACCACGCCTTCAGCGACTACCAGTGGCTTGACGAC
ACCGTCGGTCTCATCTTCCACTCCTTCCTCCTCGTCCCTTACTTCTCCTGGAA
GTACAGTCATCGACGCCACCATTCCAACACTGGCTCCCTCGAGAGAGACGAA
GTGTTTGTCCCCAAGAAGAAGTCAGACATCAAGTGGTACGGCAAGTACCTCA
ACAACCCTTTGGGACGCACCGTGATGTTAACGGTTCAGTTCACTCTCGGCTG
GCCTTTGTACTTAGCCTTCAACGTCTCGGGAAGACCTTACGACGGCGGCTTC
GCTTGCCATTTCCACCCTAACGCTCCCATCTACAACGACCGCGAGCGTCTCC
AGATATACATCTCCGACGCTGGCATCCTCGCCGTCTGCTACGGTCTCTACCG
CTACGCTGCTGTCCAAGGAGTTGCCTCGATGGTCTGCTTCTACGGAGTCCCA
CTTCTGATAGTCAACGGGTTCTTAGTTTTGATCACTTACTTGCAGCACACGCA
TCCTTCCCTGCCTCACTACGATTCGTCTGAGTGGGATTGGTTGAGGGGAGCG
TTGGCTACCGTTGACAGAGACTACGGGATCTTGAACAAGGTCTTCCACAATA
TCACGGACACGCACGTGGCGCATCACCTGTTCTCGACCATGCCGCATTATCA
CGCGATGGAAGCTACCAAGGCGATAAAGCCGATACTGGGAGAGTATTATCAG
TTCGATGGGACGCCGGTGGTTAAGGCGATGTGGAGGGAGGCGAAGGAGTGT
ATCTATGTGGAACCGGACAGGCAAGGTGAGAAGAAAGGTGTGTTCTGGTA 3’

Figure 4A: Nucleotide sequence of partial genomic sequence isolated from *Brassica juncea*. It has a total of 987 nucleotides.

5’GCTCCTTCTCCTACCTCATCTGGGACATCATCATAGCCTCCTGCTTCTACTA
CGTCGCCACCACTTACTTCCCTCTCCTCCCTCGCCCTCTCTCCTACTTCGCCT
GGCCTCTCTACTGGGCCTGCCAGGGCTGCGTCCTAACCGGCGTCTGGGTCAT
AGCCCACGAGTGCGGCCACCACGCCTTCAGCGACTACCAGTGGCTTGACGAC
ACCGTCGGTCTCATCTTCCACTCCTTCCTCCTCGTCCCTTACTTCTCCTGGAA
GTACAGTCATCGACGCCACCATTCCAACACTGGCTCCCTCGAGAGAGACGAA
GTGTTTGTCCCCAAGAAGAAGTCAGACATCAAGTGGTACGGCAAGTACCTCA
ACAACCCTTTGGGACGCACCGTGATGTTAACGGTTCAGTTCACTCTCGGCTG
GCCTTTGTACTTAGCCTTCAACGTCTCGGGAAGACCTTACGACGGCGGCTTC
GCTTGCCATTTCCACCCTAACGCTCCCATCTACAACGACCGCGAGCGTCTCC
AGATATACATCTCCGACGCTGGCATCCTCGCCGTCTGCTACGGTCTCTACCG
CTACGCTGCTGTCCAAGGAGTTGCCTCGATGGTCTGCTTCTACGGAGTCCCG
CTTCTGATAGTCAACGGGTTCTTAGTTTTGATCACTTACTTGCAGCACACGCA
TCCTTCCCTGCCTCACTACGATTCGTCTGAGTGGGATTGGTTGAGGGGAGCG
TTGGCTACCGTTGACAGAGACTACGGGATCTTGAACAAGGTCTTCCACAATA
TCACGGACACGCACGTGGCGCATCACCTGTTCTCGACCATGCCGCATTATCA
CGCGATGGAAGCTACCAAGGCGATAAAGCCGATACTGGGAGAGTATTATCAG
TTCGATGGGACGCCGGTGGTTAAGGCGATGTGGAGGGAGGCGAAGGAGTGT
ATCTATGTGGAACCGGACAGGCAAGGTGAGAAGAAAGGTGTGTTCTGGTA 3’

Figure 4B: Nucleotide sequence of partial cDNA sequence isolated from *Brassica juncea*. It has a total of 987 nucleotides.

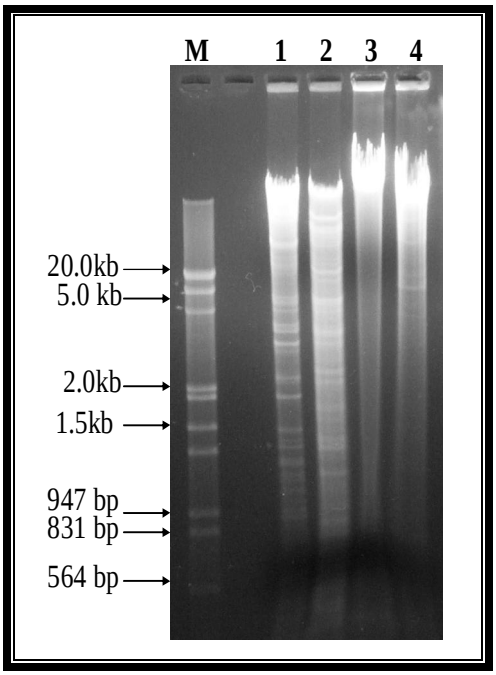

Figure 5: Electrophoretic pattern of genomic DNA isolated from *Brassica juncea* restricted with 1-*Eco*RI, 2-*Bam*HI, 3-*Hin*dIII and 4-*Pst*I separated on 0.8% agarose gel.

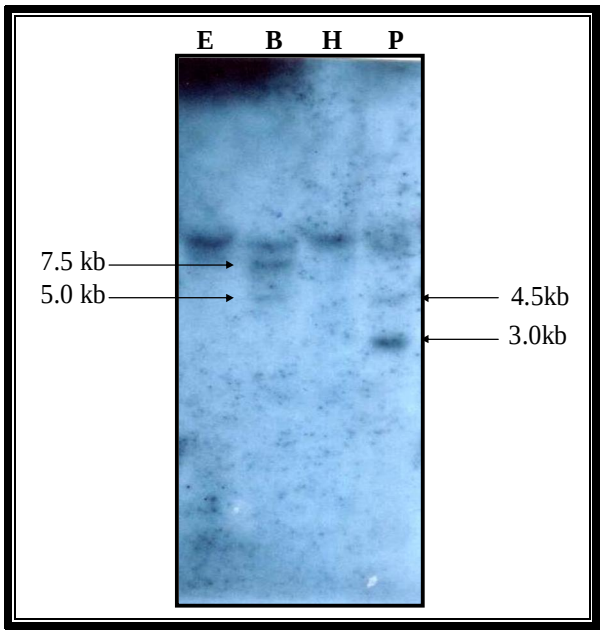

Figure 6: Southern hybridization pattern of restricted genomic DNA of *Brassica juncea* with α ^{32}P-dCTP labeled partial genomic DNA fragment *Bjfad2* as a probe.

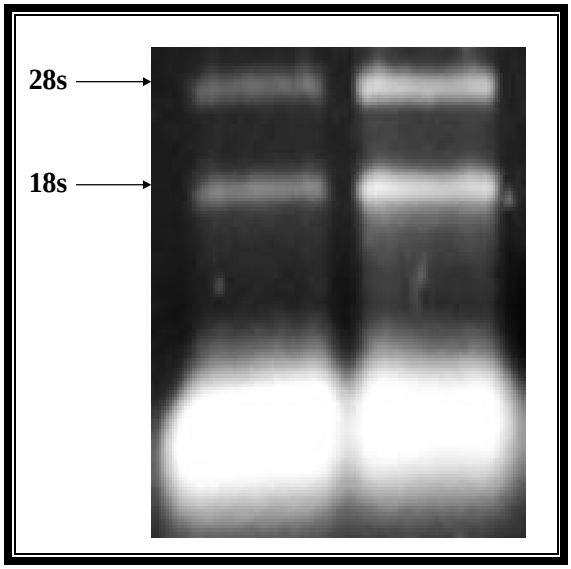

Figure 7: 1.2% agarose gel electrophoresis of RNA isolated from developing seeds of *Brassica juncea*.

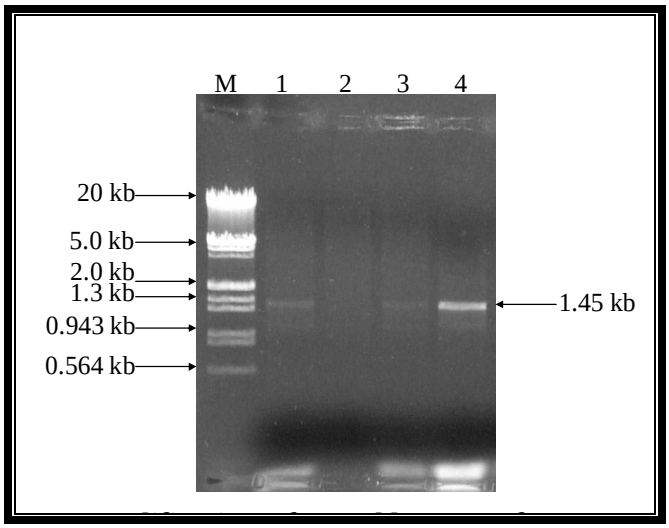

Figure 8: PCR amplification of 1.45 kb cDNA fragment isolated from *Brassica juncea* using *fad2* gene specific primers and separated on 0.8% agarose gel along with molecular size markers (λ *Hin*dIII & *Eco*RI), M-Marker, lanes at different Tm: 1-51^{0}C, 2-52^{0}C, 3-53^{0}C and 4-54^{0}C.

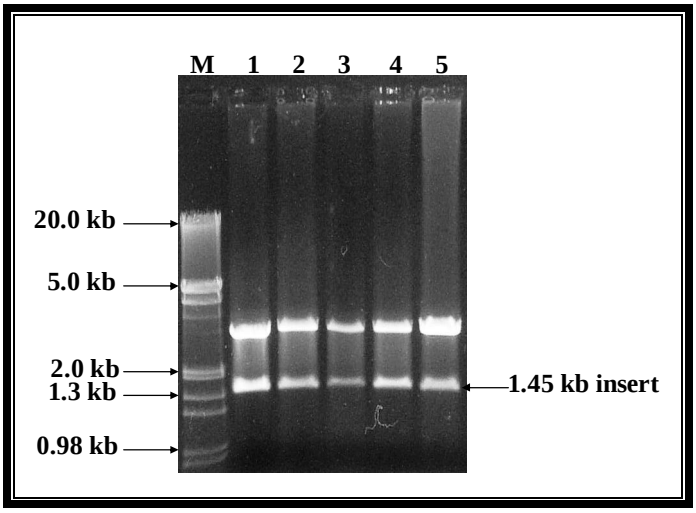

Figure 9: *Eco*RI restriction digestion of 1.45 kb cDNA fragment recombinant clones separated on 0.8% agarose gel along with molecular size markers (λ *Hin*dIII & *Eco*RI), M-marker, 1, 2, 3, 4 & 5–recombinant clones.

AGAACCAGAG AGATTCATTA CCAAAGAGAT AGAGAGAGAG AGAAGGAGAG GAGACAGAGA
GAGAGTTTGA GGAGGAGCTT CTTCGTAGGG TTCATCGTTA TTAACGTTAA ATCTTCATCC
CCCCCTACGT CAGCCAGCTC AAGAAACATG AGTGCAGGTG GAAGAATGCA AGTGTCTCCT
CCCTCGAAGA AGTCTGAAAC CGACACCATC AAGCGCGTAC CCTGCGAGAC ACCGCCCTTC
ACTGTCGGAG AACTCAAGAA AGCAATCCCA CCGCACTGTT TCAAACGCTC GATCCCTCGC
TCTTTCTCCT ACCTCATCTG GGACATCATC ATAGCCTCCT GCTTCTACTA CGTCGCCACC
ACTTACTTCC CTCTCCTCCC TCACCCTCTC TCCTACTTCG CCTGGCCTCT CTACTGGGCC
TGCCAGGGCT GCGTCCTAAC CGGCGTCTGG GTCATAGCCC ACGAGTGCGG CCACCACGCC
TTCAGCGACT ACCAGTGGCT TGACGACACC GTCGGTCTCA TCTTCCACTC CTTCCTCCTC
GTCCCTTACT TCTCCTGGAA GTACAGTCAT CGACGCCACC ATTCCAACAC TGGCTCCCTC
GAGAGAGACG GAGTGTTTGT CCCCAAGAAG AAGTCAGACA TCAAGTGGTA CGGCAAGTAC
CTCAACAACC CTTTGGGACG CACCGTGATG TTAACGGTTC AGTTCACTCT CGGCTGGCCT
TTGTGCTTAG CCTTCAACGT CTCGGGAAGA CCTTACGACG GCGGCTTCGC TTGCCATTTC
CACCCTAACG CTCCCATCTA CAACGACCGC GAGCGTCTCC AGATATACAT CTCCGACGCT
GGCATCCTCG CCGTCTGCTA CGGTCTCTAC CGCTACGCTG CTGTCCAAGG AGTTGCCTCG
ATGGTCTGCT TCTACGGAGT CCCGCTTCTG ATAGTCAACG GGTTCTTAGT TTTGATCACT
TACTTGCAGC ACACGCATCC TTCCCTGCCT CACTACGATT CGTCTGAGTG GGATTGGTTG
AGGGGAGCGT TGGCTACCGT TGACAGAGAC TACGGGATCT TGAACAAGGT CTTCCACAAT
ATCACGGACA CACACGTGGC GCATCACCTG TTCTCGACCA TGCCGCATTA TCACGCGATG
GAAGCTACCA AGGCGATAAA GCCGATACTG GGAGAGTATT ATCAGTTCGA TGGGACGCCG
GTGGTTAAGG CGATGTGGAG GGAGGCGAAG GAGTGTATCT ATGTGGAACC GGACAGGCAA
GGTGAGAAGA AAGGTGTGTT CTGGTACAAC AATAAGTTAT GAAGCAAAGA AGAAACTGAA
CCTTTCTCTT CTATGATTGT CTTTGTTTAA GAAGCTATGT TTCTGTTTCA ATAATCTTAA
TTATCCATTT TGTTGTGTTT TCTGACATTT TGGCTAAAAT TATGTGATGT TGGAAGTTAG
TGTCT

Figure 10: Nucleotide sequences of *Bjfad2* cDNA sequence isolated from *Brassica juncea* and sequenced using Di-deoxy method of Sanger's. It has total of 1445 nucleotides. The 5'& 3' UTR region is shown in red.

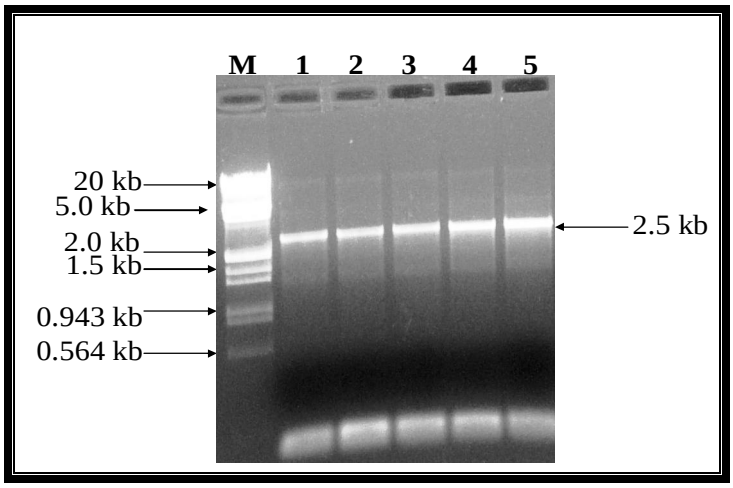

Figure 11: PCR amplification of 2.5 kb genomic DNA fragment isolated from *Brassica juncea* using *fad2* gene specific primers and separated on 0.8% agarose gel along with molecular size markers (λ *Hin*dIII & *Eco*RI), M-Marker, lanes at different Tm :1-51^{0}C, 2-52^{0}C, 3-53^{0}C, 4-54^{0}C and 5-55^{0}C.

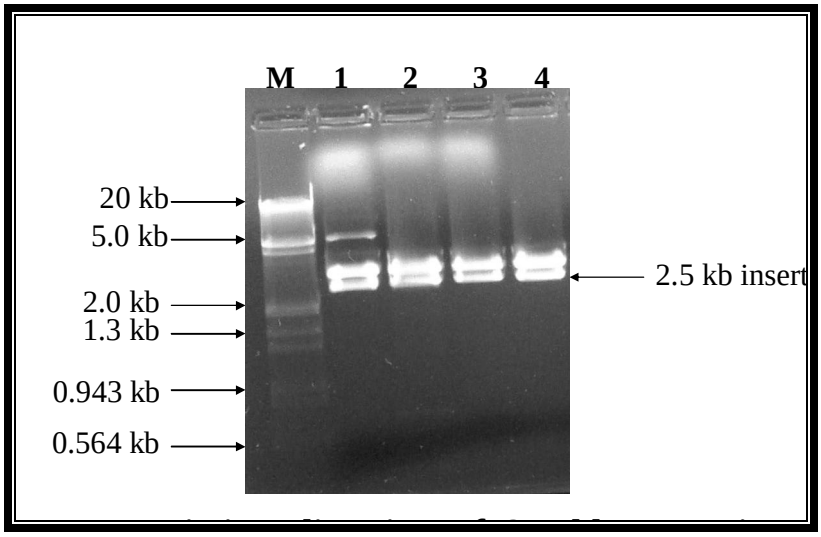

Figure 12: *Eco*RI Restriction digestion of 2.5 kb genomic DNA fragment recombinant clones separated on 0.8% agarose gel along with molecular size markers (λ *Hin*dIII & *Eco*RI), M-Marker, 1, 2, 3 & 4-recombinant clones.

5’AGAACCAGAGAGATTCATTACCAAAGAGATAGAGAGAGAGAGAAAGAGAGGAGACAGAGAGAGAGTTTGAGGAGGAGCTTCTTCG
TAGGGTTCATCGTTATTAACGTTAAATCTTCATCCCCCCCTACGTCAGCCAGCTCAAGGTCCCTTTCTTCTTCCATTTCTTCTCATTTT
TACGTTGTTTTCAATCTTGGTCTGTGCTTTTCTTATCGCTTTTCTATTCTATCTATCATTTTTGCATTTCAGTCGATTTAATTCTAGATC
TGTTAATATTTATTGCATTAAACTATAGATCTGGTCTTGATTCTCTGTTTTCATGTGTGAAATCTGATGCTGTCTTAACCATTAATCTG
ATTATATTGTCTATACCGTGGAGAATATGAAATGTTGCATTTTCATTTGTCCGAATACAAACTGTTTGACTTTCAATCGTTTTTAAAAT
TATATATATATTTTTGATGGGTTGGTGGAGTTGAAAAATCACCATAGCAGTCTCACGTCCTGGTTTTAGAAATATCCTTCCTATTCAAA
GTTATATATATTTGTTTACTTTTGTTTTAGATCTGGACCTGAGACATGTAAGTACATATTTGTTGAATCTTTGGGTAAAAACTTATGTC
TCTGGGTAAAATTTGCTGAGAGATTTGACCGATTCCTATTGGCTCTGGATTCTGTAGTTACCTAATACATGAAAAAGTTTCATTTGGC
CTATGCTCACTTCATGCTTACAAACTTTTCTTTGCAAATTAATTGGATTAGATGCTCCTTCATAGATTCAGATGCAATAGATTTGCATG
AAGAAAATAATAGGATTCATGACAGTAAAAAGATTGTATTTTTGTTTGTTTGTTTATGTTTAAAAGTCTATATGTTGACAATAGAGTTG
CTATCAACTGTTTCATTTAGCTTTATGTTTTTGTCAAGTTGCTTATTCTAAGAGACATTGTGATTATGACTTGTCTTCTCTAACGTAGTT
TAGTAATAAAAGACGAAAGAAATTGATATCCACAAGAAAGAGATGTAAGCTGTAACGTATCAAATCTCATTAATAACTAGTAGTATTC
TCAACGCTATCGTTTATTTCTTTCTTTGGTTTGCCACTATATGCCGCTTCTCTCCTCTTTTGTCCCACGTACTATCCATTTTTTTGAAAC
TTTAATAACGTAACACTGAATATTAATTTGTTGGTTTAATTAACTTTGAGTCTTTGCTTTTGGTTTATGCAGAAACATGGGTGCAGGTG
GAAGAATGCAAGTGTCTCCTCCCTCCAAAAAGTCTGAAACCGACAACATCAAGCGCGTACCCTGCGAGACACCGCCCTTCACTGTCG
GAGAACTCAAGAAAGCAATCCCACCGCACTGTTTCAAACGCTCGATCCCTCGCTCTTTCTCCTACCTCATCTGGGACATCATCATAGC
CTCCTGCTTCTACTACGTCGCCACCACTTACTTCCCTCTCCTCCCTCACCCTCTCTCCTACTTCGCCTGGCCTCTCTACTGGGCCTGCC
AAGGCTGCGTCCTAACCGGCGTCTGGGTCATAGCCCACGAGTGCGGCCACCACGCCTTCAGCGACTACCAGTGGCTGGACGACACC
GTCGGCCTCATCTTCCACTCCTTCCTCCTCGTCCCTTACTTCTCCTGGAAGTACAGTCATCGACGCCACCATTCCAACACTGGCTCCC
TCGAGAGAGACGAAGTGTTTGTCCCCAAGAAGAAGTCAGACATCAAGTGGTACGGCAAGTACCTCAACAACCCTTTGGGACGCACCG
TGATGTTAACGGTTCAGTTCACTCTCGGCTGGCCTTTGTACTTAGCCTTCAACGTCTCGGGAAGACCTTACGACGGCGGCTTCGCTTG
CCATTTCCACCCTAACGCTCCCATCTACAACGACCGCGAGCGTCTCCAGATATACATCTCCGACGCTGGCATCCTCGCCGTCTGCTAC
GGTCTCTACCGCTACGCTGCTGTCCAAGGAGTTGCCTCGATGGTCTGCTTCTACGGAGTCCCGCTTCTGATAGTCAACGGGTTCTTA
GTTTTGATCACTTACTTGCAGCACACGCATCCTTCCCTGCCTCACTACGATTCGTCTGAGTGGGATTGGTTGAGGGGAGCGTTGGCTA
CCGTTGACAGAGACTACGGGATCTTGAACAAGGTCTTCCACAATATCACGGACACGCACGTGGCGCATCACCTGTTCTCGACCATGC
CGCATTATCACGCGATGGAAGCTACCAAGGCGATAAAGCCGATACTGGGAGAGTATTATCAGTTCGATGGGACGCCGGTGGTTAAGG
CGATGTGGAGGGAGGCGAAGGAGTGTATCTATGTGGAACCGGACAGGCAAGGTGAGAAGAAAGGTGTGTTCTGGTACAACAATAAG
TTATGAAGCAAAGAAGAAACTGAACCTTTCTCTTCTATGATTGTCTTTGTTTAAGAAGCTATGTTTCTGTTTCAATAATCTTAATTATC
CATTTTGTTGTGTTTTCTGACATTTTGGCTAAAATTATGTGATGTTGGAAGTTAGTGTCT 3’

Figure 13: Nucleotide sequence of *Bjfad2* genomic sequence isolated from *Brassica juncea* and sequenced using Di-deoxy method of Sanger’s. It has a total of 2526 nucleotides.

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AJ459108.2	Brassica campestris fad2 gene for fatty acid desaturase 2, exons 1-2	4349	4349	100%	0.0	97%
EF639848.1	Brassica juncea fatty acid desaturase 2 (fad2) mRNA, complete cds	2353	2613	57%	0.0	99%
AJ459107.1	Brassica campestris mRNA for fatty acid desaturase 2 (fad2 gene)	2337	2596	57%	0.0	99%
AY577313.1	Brassica napus delta-12 oleate desaturase (FAD2) mRNA, complete cds	2039	2039	45%	0.0	98%
AF243045.1	Brassica napus delta-12 oleate desaturase mRNA, complete cds	1962	1962	45%	0.0	97%

Figure 14: BLASTN homology search result of *Bjfad2* genomic sequence from *Brassica juncea*

Figure 15: Restriction map of *Bifad2* genomic sequence isolated from *Brassica juncea* using NEB CUTTER software.

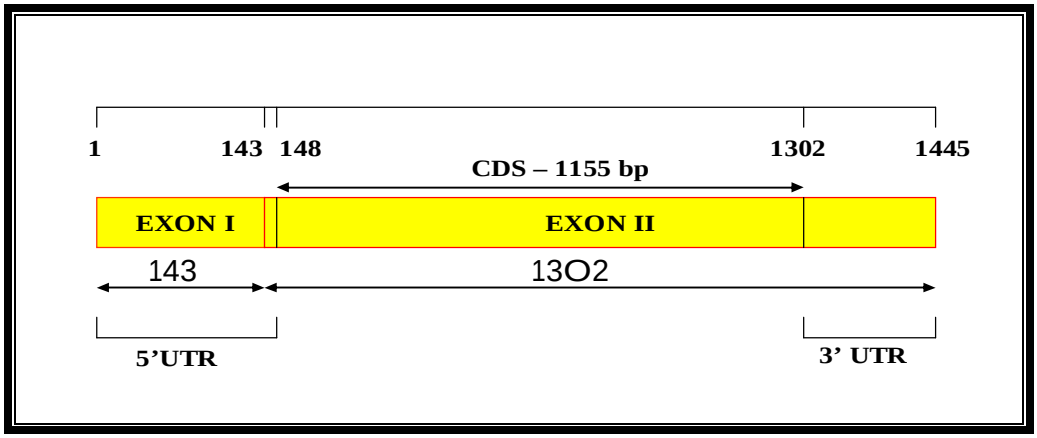

Figure 16: Structure of *Bjfad2* cDNA sequence isolated from *Brasica juncea.*

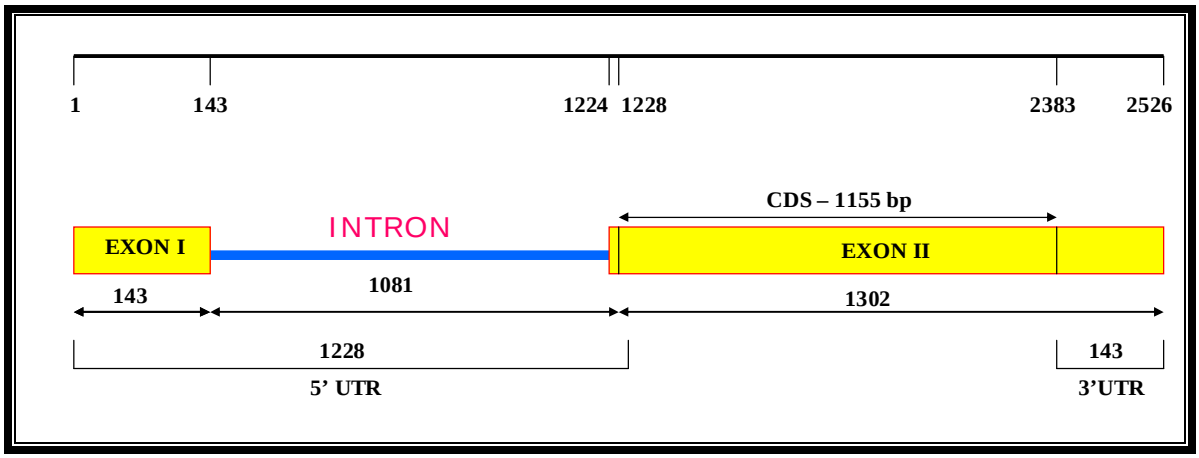

Figure 17: Genomic structure of *Bjfad2* isolated from *Brasica juncea.*

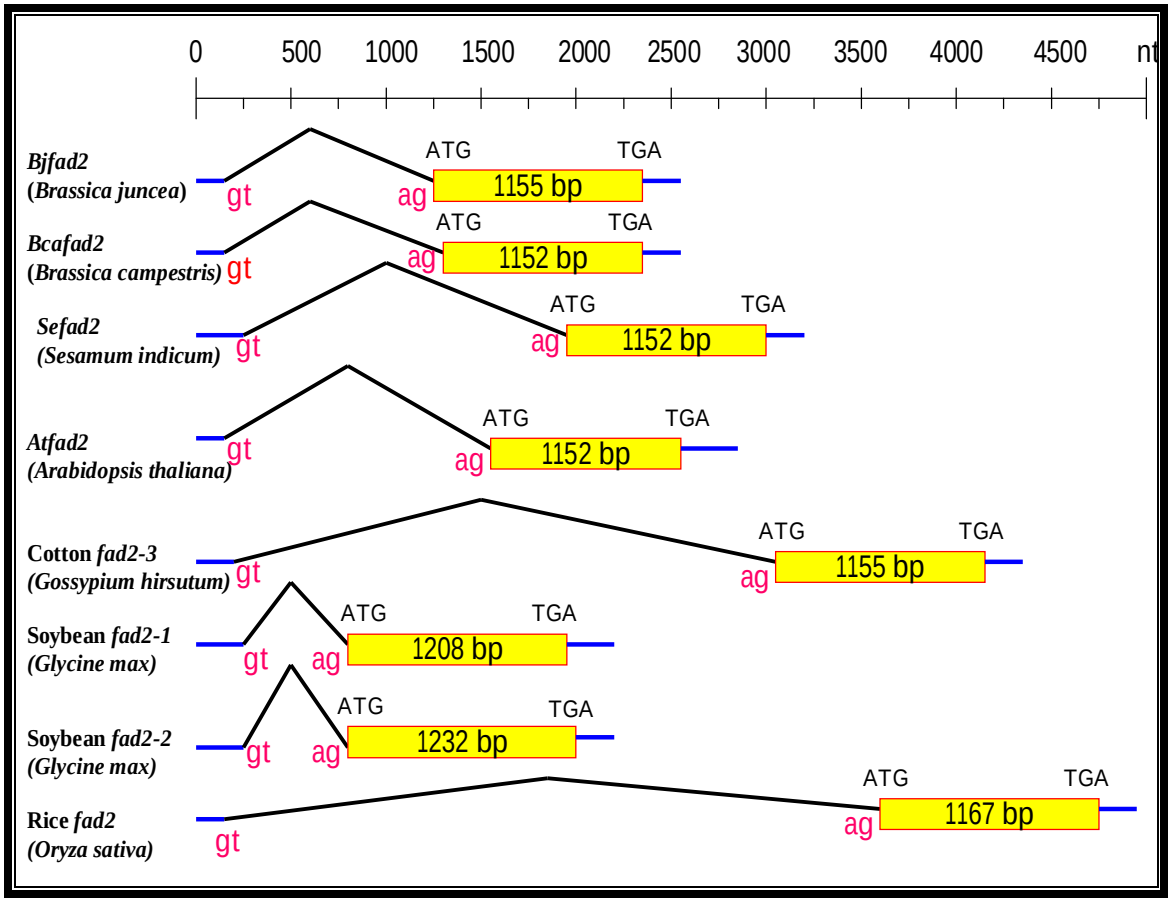

Figure 18: Comparison of the *fad2* genomic structures from *Brassica*, Sesame, *Arabidopsis*, Cotton, Soybean and Rice.

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max Ident
AJ459107.1	Brassica campestris mRNA for fatty acid desaturase 2 (fad2 gene)	2532	2532	100%	0.0	98%
AJ459108.2	Brassica campestris fad2 gene for fatty acid desaturase 2, exons 1-2	2281	2535	100%	0.0	98%
AY577313.1	Brassica napus delta-12 oleate desaturase (FAD2) mRNA, complete cds	1995	1995	79%	0.0	97%
AF243045.1	Brassica napus delta-12 oleate desaturase mRNA, complete cds	1951	1951	79%	0.0	97%
EF371480.1	Brassica napus cultivar Xiang You 15 mutant delta-12 oleate desaturase (FAD2) gene, complete sequence	1760	1760	79%	0.0	94%
AY592975.1	Brassica napus delta-12 oleate desaturase mRNA, complete cds	1724	1724	80%	0.0	93%
AF042841.1	Brassica rapa delta-12 desaturase (fad2) gene, partial cds	1642	1642	64%	0.0	98%
AF124360.2	Brassica carinata delta-12 desaturase (FAD2) mRNA, complete cds	1613	1613	79%	0.0	91%
DQ518280.1	Brassica nigra isolate B.nig1 delta-12 desaturase gene, partial cds	1435	1435	54%	0.0	99%
NM_112047.3	Arabidopsis thaliana FAD2 (FATTY ACID DESATURASE 2); delta12-fatty acid dehydrogenase (FAD2) mRNA, complete cds	1391	1391	98%	0.0	84%
DQ518278.1	Brassica napus isolate B.nap3 delta-12 desaturase gene, partial cds	1391	1391	54%	0.0	98%

```
>  emb|AJ459107.1|BCA459107  Brassica campestris mRNA for fatty acid
desaturase 2 (fad2 gene)
Length=1465

 Score = 2532 bits (1371),  Expect = 0.0
 Identities = 1422/1447 (98%), Gaps = 2/1447 (0%)
 Strand=Plus/Plus

Query1 AGAACCagagagattcattaccaaagagatagagagagagagaaggagaggagac-agag  59
       |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||| ||||
Sbjct1 AGAACCAGAGAGATTCATTACCAAAGAGATAGAGAGAGAGAGAAAGAGAGGAGACTAGAG  60

Query60agagagtttgaggaggagCTTCTTCGTAGGGTTCATCGTTATTAACGTTAAATCTTCATc 119
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct61AGAGAGTTTGAGGAGGAGCTTCTTCGTAGGGTTCATCGTTATTAACGTTAAATCTTCATC 120

Query120ccccccTACGTCAGCCAGCTCAAGAAACATGAGTGCAGGTGGAAGAATGCAAGTGTCTCC179
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Sbjct121CCCCCCTACGTCAGCCAGCTCAAGAAACATGGGTGCAGGTGGAAGAATGCAAGTGTCTCC180

Query180TCCCTCGAAGAAGTCTGAAACCGACACCATCAAGCGCGTACCCTGCGAGACACCGCCCTT239
         |||||| || ||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct181TCCCTCCAAAAAGTCTGAAACCGACAACATCAAGCGCGTACCCTGCGAGACACCGCCCTT240
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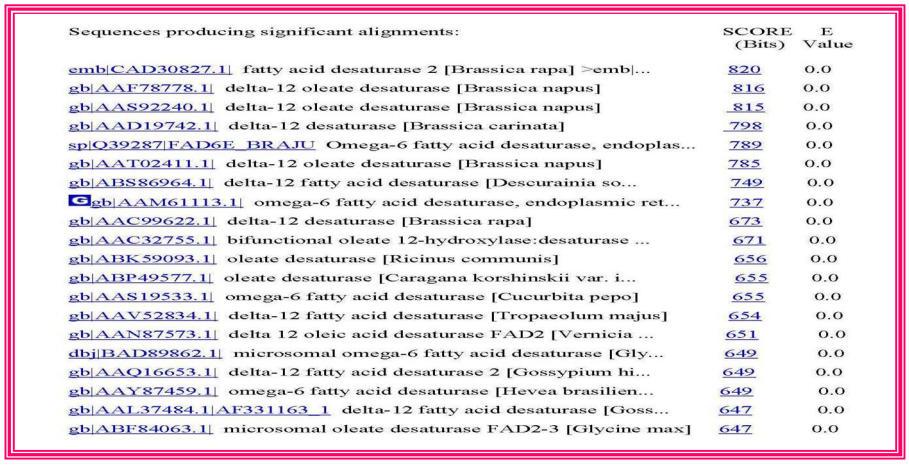
Figure 19: BLASTN homology search result of *Bjfad2* isolated from *Brassica juncea.*



Sequences producing significant alignments:	SCORE (Bits)	E Value
emb|CAD30827.1| fatty acid desaturase 2 [Brassica rapa] >emb|...	820	0.0
gb|AAF78778.1| delta-12 oleate desaturase [Brassica napus]	816	0.0
gb|AAS92240.1| delta-12 oleate desaturase [Brassica napus]	815	0.0
gb|AAD19742.1| delta-12 desaturase [Brassica carinata]	798	0.0
sp|Q39287|FAD6E_BRAJU Omega-6 fatty acid desaturase, endoplas...	789	0.0
gb|AAT02411.1| delta-12 oleate desaturase [Brassica napus]	785	0.0
gb|ABS86964.1| delta-12 fatty acid desaturase [Descurainia so...	749	0.0
gb|AAM61113.1| omega-6 fatty acid desaturase, endoplasmic ret...	737	0.0
gb|AAC99622.1| delta-12 desaturase [Brassica rapa]	673	0.0
gb|AAC32755.1| bifunctional oleate 12-hydroxylase:desaturase ...	671	0.0
gb|ABK59093.1| oleate desaturase [Ricinus communis]	656	0.0
gb|ABP49577.1| oleate desaturase [Caragana korshinskii var. i...	655	0.0
gb|AAS19533.1| omega-6 fatty acid desaturase [Cucurbita pepo]	655	0.0
gb|AAV52834.1| delta-12 fatty acid desaturase [Tropaeolum majus]	654	0.0
gb|AAN87573.1| delta 12 oleic acid desaturase FAD2 [Vernicia ...	651	0.0
dbj|BAD89862.1| microsomal omega-6 fatty acid desaturase [Gly...	649	0.0
gb|AAQ16653.1| delta-12 fatty acid desaturase 2 [Gossypium hi...	649	0.0
gb|AAY87459.1| omega-6 fatty acid desaturase [Hevea brasilien...	649	0.0
gb|AAL37484.1|AF331163_1 delta-12 fatty acid desaturase [Goss...	647	0.0
gb|ABF84063.1| microsomal oleate desaturase FAD2-3 [Glycine max]	647	0.0

```
>  emb|CAD30827.1|  fatty acid desaturase 2 [Brassica rapa]
 emb|CAG26981.1|  fatty acid desaturase 2 [Brassica rapa]
Length=384

 Score = 820 bits (2118), Expect = 0.0
 Identities = 380/384 (98%), Positives = 380/384 (98%), Gaps = 0/384
(0%)
 Frame = +1

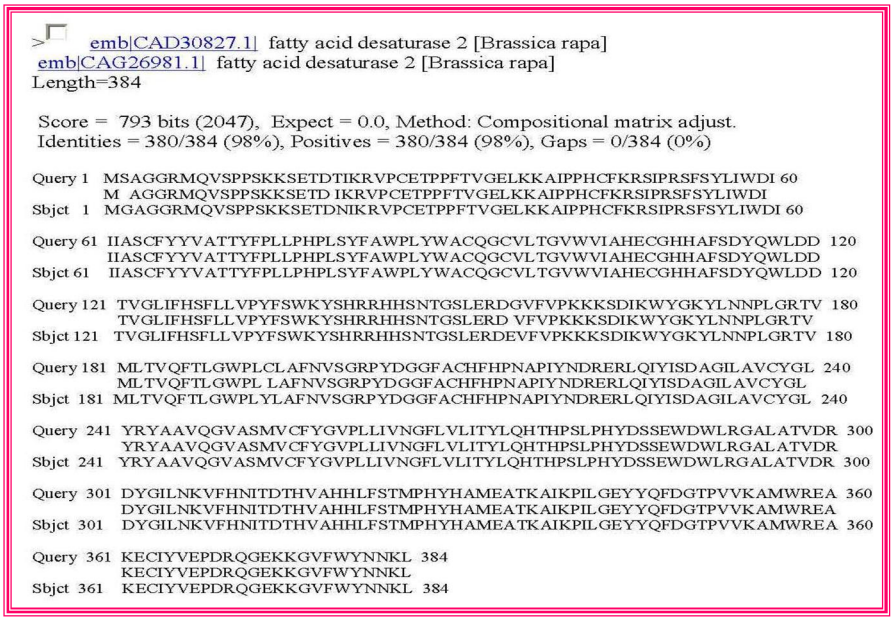
Query 148   MSAGGRMQVSPPSKKSETDTIKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDI  327
             M AGGRMQVSPPSKKSETD IKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDI
Sbjct 1       MGAGGRMQVSPPSKKSETDNIKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDI  60

Query 328   IIASCFYYVATTYFPLLPHPLSYFAWPLYWACQGCVLTGVWVIAHECGHHAFSDYQWLDD  507
             IIASCFYYVATTYFPLLPHPLSYFAWPLYWACQGCVLTGVWVIAHECGHHAFSDYQWLDD
```

Figure 20: BLASTX homology search result of *Bjfad2* isolated from *Brassica juncea.*

Sequences producing significant alignments:	Score (Bits)	E Value
gb|ABR27357.1| fatty acid desaturase 2 [Brassica juncea]	802	0.0
emb|CAD30827.1| fatty acid desaturase 2 [Brassica rapa] >emb|...	793	0.0
gb|AAF78778.1| delta-12 oleate desaturase [Brassica napus]	790	0.0
gb|AAS92240.1| delta-12 oleate desaturase [Brassica napus]	786	0.0
gb|AAD19742.1| delta-12 desaturase [Brassica carinata]	770	0.0
sp|Q39287|FAD6E_BRAJU Omega-6 fatty acid desaturase, endoplas...	762	0.0
gb|AAT02411.1| delta-12 oleate desaturase [Brassica napus]	757	0.0
gb|ABS86964.1| delta-12 fatty acid desaturase [Descurainia so...	726	0.0
gb|AAM61113.1| omega-6 fatty acid desaturase, endoplasmic ret...	717	0.0
gb|AAC32755.1| bifunctional oleate 12-hydroxylase:desaturase ...	651	0.0
gb|AAC99622.1| delta-12 desaturase [Brassica rapa]	645	0.0
gb|ABK59093.1| oleate desaturase [Ricinus communis]	638	0.0
gb|AAS19533.1| omega-6 fatty acid desaturase [Cucurbita pepo]	636	0.0
gb|ABP49577.1| oleate desaturase [Caragana korshinskii var. i...	635	2e-180
gb|AAQ16653.1| delta-12 fatty acid desaturase 2 [Gossypium hi...	633	7e-180
dbj|BAD89862.1| microsomal omega-6 fatty acid desaturase [Gly...	632	2e-179
gb|AAL37484.1|AF331163_1 delta-12 fatty acid desaturase [Goss...	631	3e-179
gb|AAY87459.1| omega-6 fatty acid desaturase [Hevea brasilien...	631	4e-179
gb|ABF84063.1| microsomal oleate desaturase FAD2-3 [Glycine max]	628	2e-178
gb|ABQ01458.1| oleate 12-hydroxylase [Physaria lindheimeri]	625	2e-177
sp|P48631|FD6E2_SOYBN Omega-6 fatty acid desaturase, endoplas...	624	3e-177
gb|ABC41578.1| endoplasmic reticulum 18:1 desaturase [Populus...	623	9e-177
gb|ABA41034.1| delta12-fatty acid desaturase [Jatropha curcas]	623	1e-176
gb|AAV52834.1| delta-12 fatty acid desaturase [Tropaeolum majus]	622	2e-176
gb|AAN87573.1| delta 12 oleic acid desaturase FAD2 [Vernicia ...	621	3e-176
emb|CAA71199.1| omega-6 desaturase [Gossypium hirsutum]	619	1e-175
emb|CAQ23392.1| unnamed protein product [Vitis vinifera]	619	1e-175
gb|ABB05230.1| delta 12 desaturase [Linum usitatissimum]	619	2e-175
gb|ABL86147.1| delta-12 oleic acid desaturase [Vernicia montana]	615	2e-174
emb|CAN71892.1| hypothetical protein [Vitis vinifera]	615	3e-174
gb|AAW63041.1| microsomal delta-12 oleate desaturase [Olea eu...	614	4e-174
gb|AAT72296.2| microsomal omega-6-desaturase [Nicotiana tabacum]	613	6e-174

Figure 21(A): BLASTP homology search result of *Bjfad2* cDNA sequence isolated from *Brassica juncea.*



>emb|CAD30827.1| fatty acid desaturase 2 [Brassica rapa]
emb|CAG26981.1| fatty acid desaturase 2 [Brassica rapa]
Length=384

Score = 793 bits (2047), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 380/384 (98%), Positives = 380/384 (98%), Gaps = 0/384 (0%)

```
Query 1   MSAGGRMQVSPPSKKSETDTIKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDI 60
          M AGGRMQVSPPSKKSETD IKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDI
Sbjct 1   MGAGGRMQVSPPSKKSETDNIKRVPCETPPFTVGELKKAIPPHCFKRSIPRSFSYLIWDI 60

Query 61  IIASCFYYVATTYFPLLPHPLSYFAWPLYWACQGCVLTGVWVIAHECGHHAFSDYQWLDD 120
          IIASCFYYVATTYFPLLPHPLSYFAWPLYWACQGCVLTGVWVIAHECGHHAFSDYQWLDD
Sbjct 61  IIASCFYYVATTYFPLLPHPLSYFAWPLYWACQGCVLTGVWVIAHECGHHAFSDYQWLDD 120

Query 121 TVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERDGVFVPKKKSDIKWYGKYLNNPLGRTV 180
          TVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERD VFVPKKKSDIKWYGKYLNNPLGRTV
Sbjct 121 TVGLIFHSFLLVPYFSWKYSHRRHHSNTGSLERDEVFVPKKKSDIKWYGKYLNNPLGRTV 180

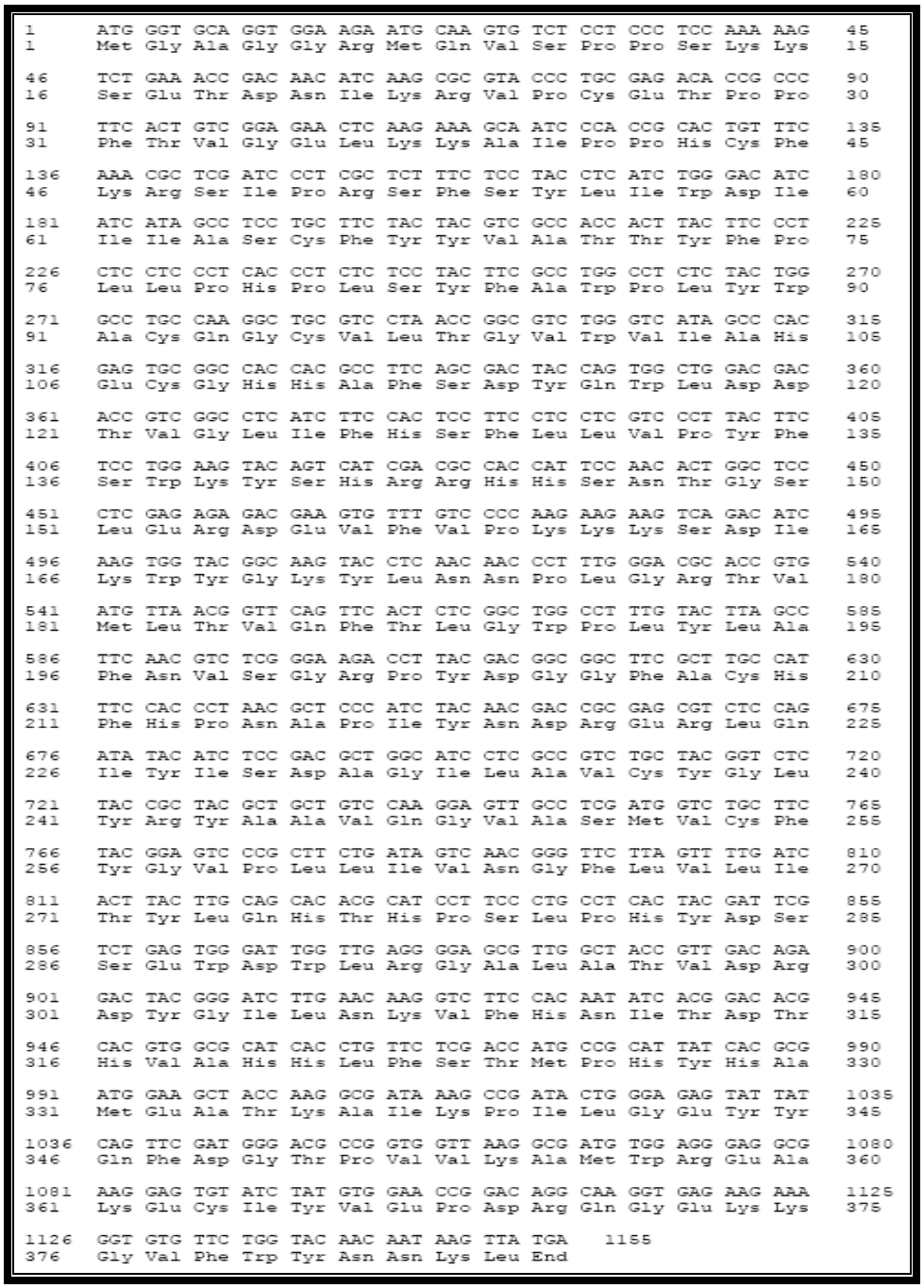
Query 181 MLTVQFTLGWPLCLAFNVSGRPYDGGFACHFHPNAPIYNDRERLQIYISDAGILAVCYGL 240
          MLTVQFTLGWPL LAFNVSGRPYDGGFACHFHPNAPIYNDRERLQIYISDAGILAVCYGL
Sbjct 181 MLTVQFTLGWPLYLAFNVSGRPYDGGFACHFHPNAPIYNDRERLQIYISDAGILAVCYGL 240

Query 241 YRYAAVQGVASMVCFYGVPLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRGALATVDR 300
          YRYAAVQGVASMVCFYGVPLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRGALATVDR
Sbjct 241 YRYAAVQGVASMVCFYGVPLLIVNGFLVLITYLQHTHPSLPHYDSSEWDWLRGALATVDR 300

Query 301 DYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYQFDGTPVVKAMWREA 360
          DYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYQFDGTPVVKAMWREA
Sbjct 301 DYGILNKVFHNITDTHVAHHLFSTMPHYHAMEATKAIKPILGEYYQFDGTPVVKAMWREA 360

Query 361 KECIYVEPDRQGEKKGVFWYNNKL 384
          KECIYVEPDRQGEKKGVFWYNNKL
Sbjct 361 KECIYVEPDRQGEKKGVFWYNNKL 384
```

Figure 21(B): BLASTP similarities search result of *Bjfad2* with *Brassica rapa* sequence.



```
1      ATG GGT GCA GGT GGA AGA ATG CAA GTG TCT CCT CCC TCC AAA AAG   45
1      Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys   15

46     TCT GAA ACC GAC AAC ATC AAG CGC GTA CCC TGC GAG ACA CCG CCC   90
16     Ser Glu Thr Asp Asn Ile Lys Arg Val Pro Cys Glu Thr Pro Pro   30

91     TTC ACT GTC GGA GAA CTC AAG AAA GCA ATC CCA CCG CAC TGT TTC   135
31     Phe Thr Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe   45

136    AAA CGC TCG ATC CCT CGC TCT TTC TCC TAC CTC ATC TGG GAC ATC   180
46     Lys Arg Ser Ile Pro Arg Ser Phe Ser Tyr Leu Ile Trp Asp Ile   60

181    ATC ATA GCC TCC TGC TTC TAC TAC GTC GCC ACC ACT TAC TTC CCT   225
61     Ile Ile Ala Ser Cys Phe Tyr Tyr Val Ala Thr Thr Tyr Phe Pro   75

226    CTC CTC CCT CAC CCT CTC TCC TAC TTC GCC TGG CCT CTC TAC TGG   270
76     Leu Leu Pro His Pro Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp   90

271    GCC TGC CAA GGC TGC GTC CTA ACC GGC GTC TGG GTC ATA GCC CAC   315
91     Ala Cys Gln Gly Cys Val Leu Thr Gly Val Trp Val Ile Ala His   105

316    GAG TGC GGC CAC CAC GCC TTC AGC GAC TAC CAG TGG CTG GAC GAC   360
106    Glu Cys Gly His His Ala Phe Ser Asp Tyr Gln Trp Leu Asp Asp   120

361    ACC GTC GGC CTC ATC TTC CAC TCC TTC CTC CTC GTC CCT TAC TTC   405
121    Thr Val Gly Leu Ile Phe His Ser Phe Leu Leu Val Pro Tyr Phe   135

406    TCC TGG AAG TAC AGT CAT CGA CGC CAC CAT TCC AAC ACT GGC TCC   450
136    Ser Trp Lys Tyr Ser His Arg Arg His His Ser Asn Thr Gly Ser   150

451    CTC GAG AGA GAC GAA GTG TTT GTC CCC AAG AAG AAG TCA GAC ATC   495
151    Leu Glu Arg Asp Glu Val Phe Val Pro Lys Lys Lys Ser Asp Ile   165

496    AAG TGG TAC GGC AAG TAC CTC AAC AAC CCT TTG GGA CGC ACC GTG   540
166    Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu Gly Arg Thr Val   180

541    ATG TTA ACG GTT CAG TTC ACT CTC GGC TGG CCT TTG TAC TTA GCC   585
181    Met Leu Thr Val Gln Phe Thr Leu Gly Trp Pro Leu Tyr Leu Ala   195

586    TTC AAC GTC TCG GGA AGA CCT TAC GAC GGC GGC TTC GCT TGC CAT   630
196    Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Gly Phe Ala Cys His   210

631    TTC CAC CCT AAC GCT CCC ATC TAC AAC GAC CGC GAG CGT CTC CAG   675
211    Phe His Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu Gln   225

676    ATA TAC ATC TCC GAC GCT GGC ATC CTC GCC GTC TGC TAC GGT CTC   720
226    Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu   240

721    TAC CGC TAC GCT GCT GTC CAA GGA GTT GCC TCG ATG GTC TGC TTC   765
241    Tyr Arg Tyr Ala Ala Val Gln Gly Val Ala Ser Met Val Cys Phe   255

766    TAC GGA GTC CCG CTT CTG ATA GTC AAC GGG TTC TTA GTT TTG ATC   810
256    Tyr Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile   270

811    ACT TAC TTG CAG CAC ACG CAT CCT TCC CTG CCT CAC TAC GAT TCG   855
271    Thr Tyr Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser   285

856    TCT GAG TGG GAT TGG TTG AGG GGA GCG TTG GCT ACC GTT GAC AGA   900
286    Ser Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg   300

901    GAC TAC GGG ATC TTG AAC AAG GTC TTC CAC AAT ATC ACG GAC ACG   945
301    Asp Tyr Gly Ile Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr   315

946    CAC GTG GCG CAT CAC CTG TTC TCG ACC ATG CCG CAT TAT CAC GCG   990
316    His Val Ala His His Leu Phe Ser Thr Met Pro His Tyr His Ala   330

991    ATG GAA GCT ACC AAG GCG ATA AAG CCG ATA CTG GGA GAG TAT TAT   1035
331    Met Glu Ala Thr Lys Ala Ile Lys Pro Ile Leu Gly Glu Tyr Tyr   345

1036   CAG TTC GAT GGG ACG CCG GTG GTT AAG GCG ATG TGG AGG GAG GCG   1080
346    Gln Phe Asp Gly Thr Pro Val Val Lys Ala Met Trp Arg Glu Ala   360

1081   AAG GAG TGT ATC TAT GTG GAA CCG GAC AGG CAA GGT GAG AAG AAA   1125
361    Lys Glu Cys Ile Tyr Val Glu Pro Asp Arg Gln Gly Glu Lys Lys   375

1126   GGT GTG TTC TGG TAC AAC AAT AAG TTA TGA   1155
376    Gly Val Phe Trp Tyr Asn Asn Lys Leu End
```

Figure 22: Open Reading Frame (ORF) of sequenced *Bjfad2* along with corresponding amino acids. It has ORF length of 384 aa with start codon present at 148 bp position and stop codon at 1302 bp position.

Number of amino acids: 384

Molecular weight: 44112.8

Theoretical pI: 8.62

Amino acid composition: CSV format

Ala	(A)	24	6.2%
Arg	(R)	16	4.2%
Asn	(N)	11	2.9%
Asp	(D)	17	4.4%
Cys	(C)	11	2.9%
Gln	(Q)	9	2.3%
Glu	(E)	13	3.4%
Gly	(G)	26	6.8%
His	(H)	20	5.2%
Ile	(I)	21	5.5%
Leu	(L)	33	8.6%
Lys	(K)	20	5.2%
Met	(M)	7	1.8%
Phe	(F)	21	5.5%
Pro	(P)	26	6.8%
Ser	(S)	23	6.0%
Thr	(T)	20	5.2%
Trp	(W)	12	3.1%
Tyr	(Y)	26	6.8%
Val	(V)	28	7.3%
Pyl	(O)	0	0.0%
Sec	(U)	0	0.0%
	(B)	0	0.0%
	(Z)	0	0.0%
	(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 30

Total number of positively charged residues (Arg + Lys): 36

Figure 23A: Amino acid composition of predicted BjFAD2 protein determined using BIOEDIT tool.

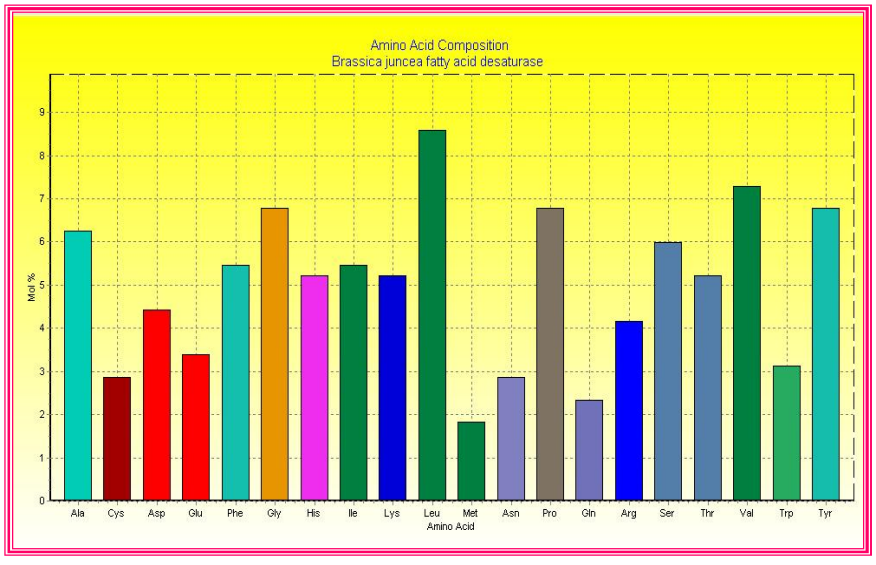


Figure 23B: Graphical representation of amino acid composition in predicted BjFAD2 protein, using BIOEDIT tool.

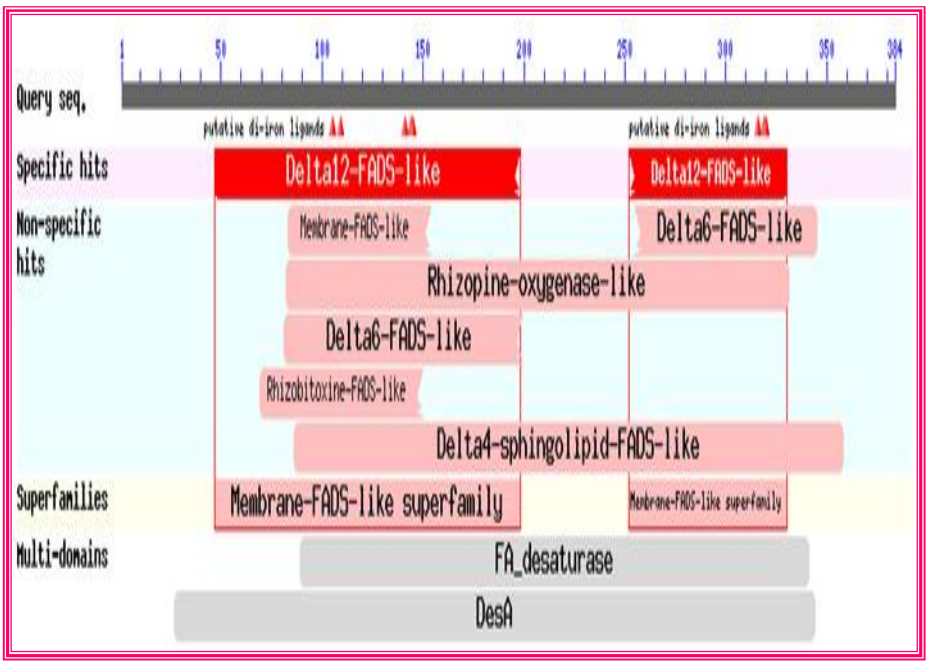
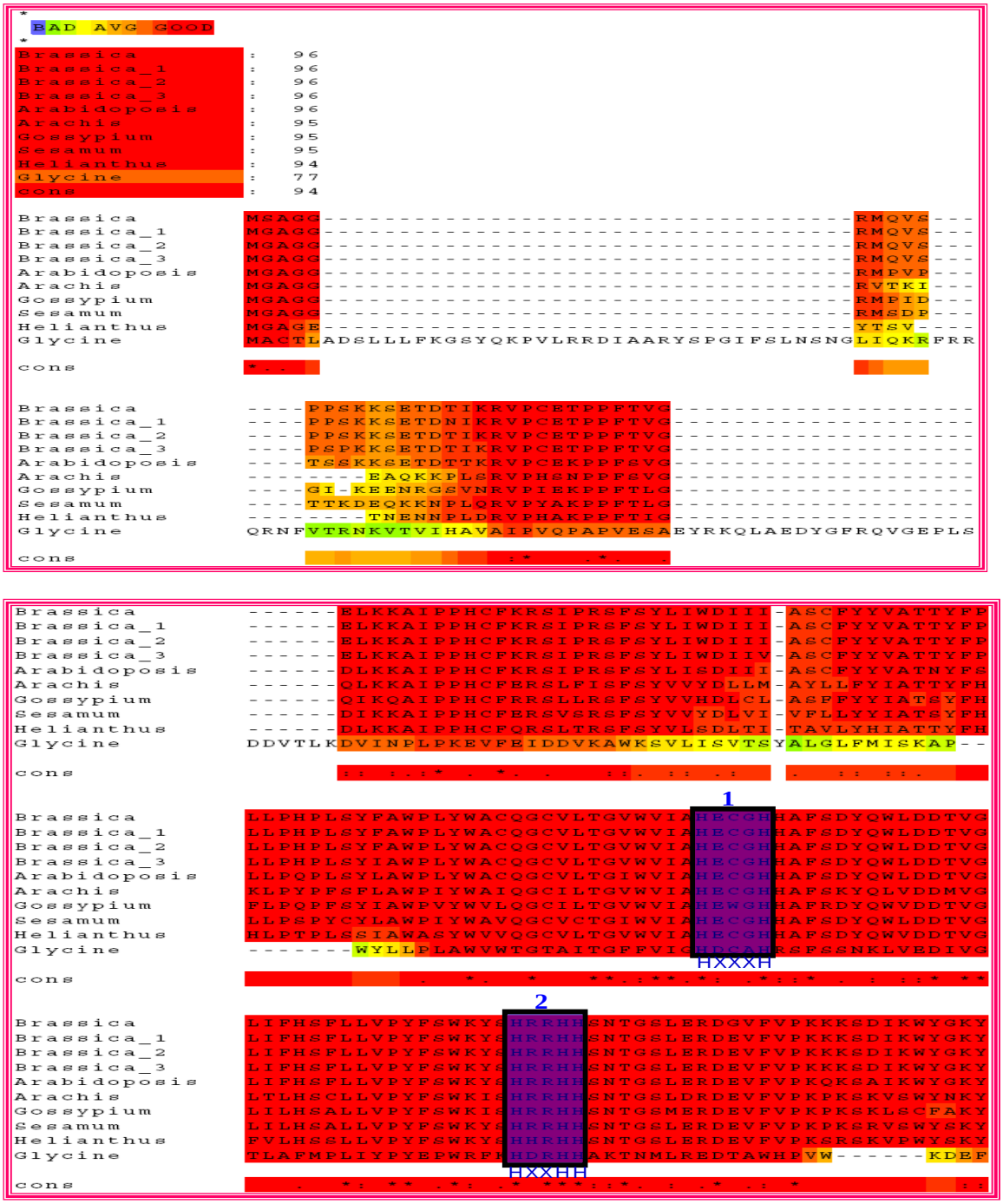


Figure 24: Functional conserved domain of putative BjFAD2 protein showing multi domain nature of all fatty acid desaturases. CDART (conserved domain retrieval tool) was used to derive conserved domain provided by NCBI, USA.


BAD AVG GOOD
Brassica
Brassica_1
Brassica_2
Brassica_3
Arabidopsis
Arachis
Gossypium
Sesamum
Helianthus
Glycine
cons
1
HXXXH
2
HXXHH

Cont... Fig. 25

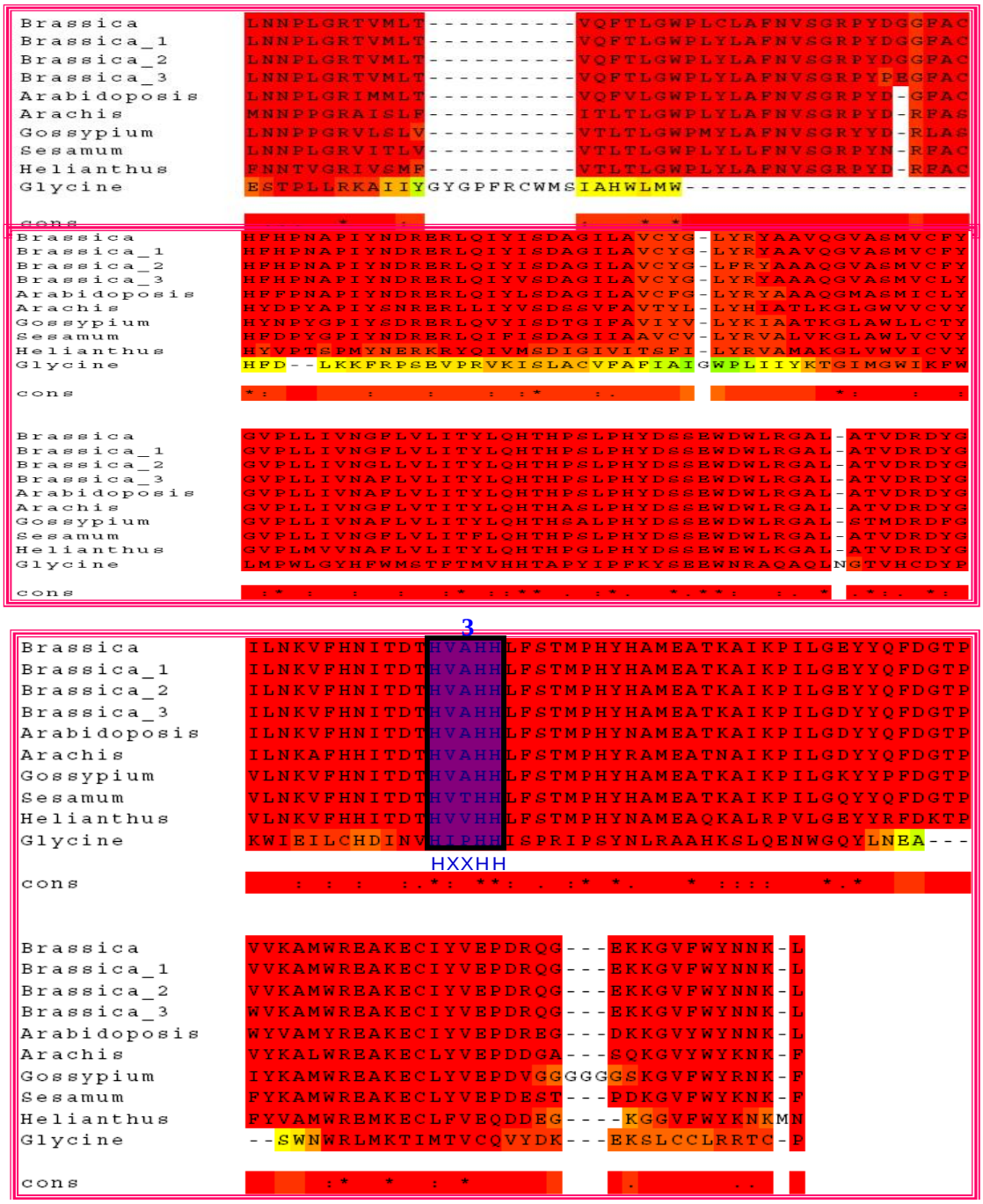


Figure 25: Alignment of the deduced amino acid sequence of putative BjFAD2 protein with those of other FAD2 proteins (*Brassica campestris; Brassica carinata; Brassica napus; Arabidopsis thaliana; Arachis hypogea; Gossypium Hirsutum; sesamum indica; Helianthus anus; Glycine max)* using clustalw software.(Conserved histidine domains are represented by 1-HXXXH, 2-HXXHH and 3-HXXHH).

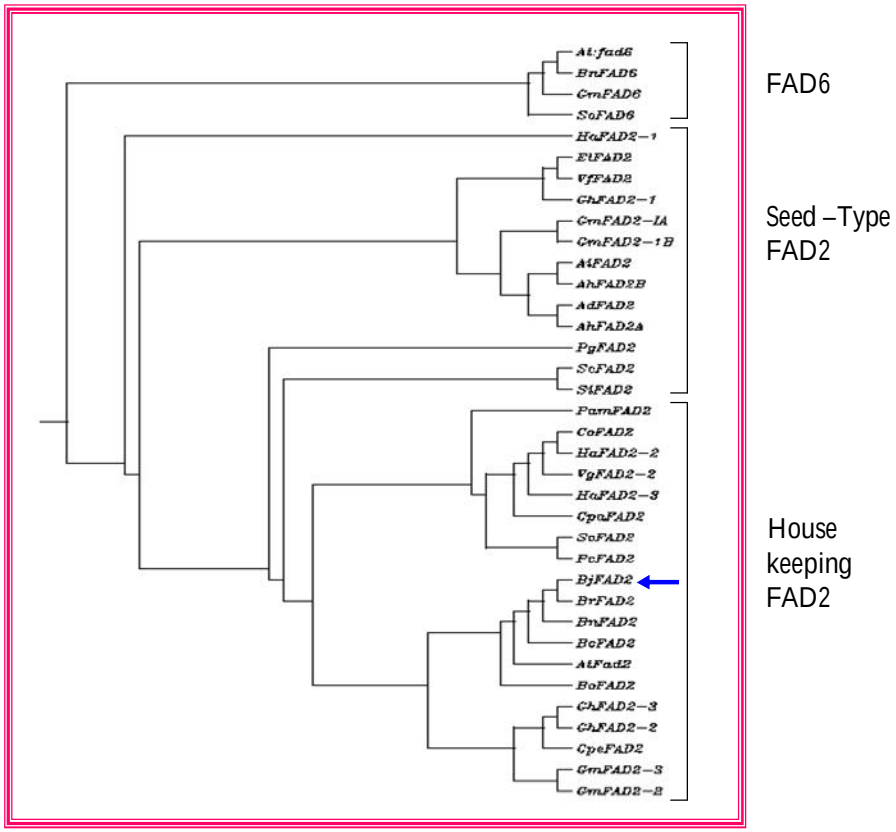


Figure 26: Phylogenetic relationships between deduced amino acid sequences from BjFAD2 and other plantmicrosomal (FAD2) or plastidial (FAD6) oleate desaturases. Position of the *Brassica juncea* FAD2 is marked with arrow. The enzymes and GenBank accession numbers used for the analysis are: *Arabidopsis thaliana* (AtFAD2, L26296; AtFAD6, U09503), *Arachis duranensis* (AdFAD2, AF272951), *Arachis hypogaea* (AhFAD2A, AF030319; AhFAD2B, AF272950), *Arachis ipaensis* (AiFAD2,AF272952), *Borago officinalis* (BoFAD2, AF074324), *Brassica carinata* (BcFAD2, AF124360), *Brassica napus* (BnFAD2, AF243045; BnFAD6, L29214), *Brassica rapa* (BrFAD2, AJ459107), *Calendula officinalis* (CoFAD2, AF343065), *Crepis palestina* (CpaFAD2, Y16284), *Cucurbita pepo* (CpeFAD2, AY525163), *Euphorbia lagascae* (ElFAD2, AY486148), *Glycine max* (GmFAD2-1A, L43920; *GmFAD2-1B*, AB188251; GmFAD2-2, L43921; GmFAD6, L29215),*Gossypium hirsutum* (GhFAD2-1, X97016; GhFAD2-2, Y10112; GhFAD2-3, AF331163), *Helianthus annuus* (HaFAD2-1,AF251842; HaFAD2-2, AF251843; HaFAD2-3, AF251844), *Persea americana* (PamFAD2, AY057406), *Petroselinum crispum* (PcFAD2, U86072), *Punica granatum* (PgFAD2, AJ437139), *Sesamum indicum* (SiFAD2, AF192486), *Solanum commersonii* (ScFAD2, X92847), *Spinacia oleracea* (SoFAD2, AB094415; SoFAD6, X78311), *Vernicia fordii* (VfFAD2, AF525535), *Vernonia galamensis* (VgFAD2-2, AF188264). The tree was constructed by using the Neighbor-Joining algorithm.

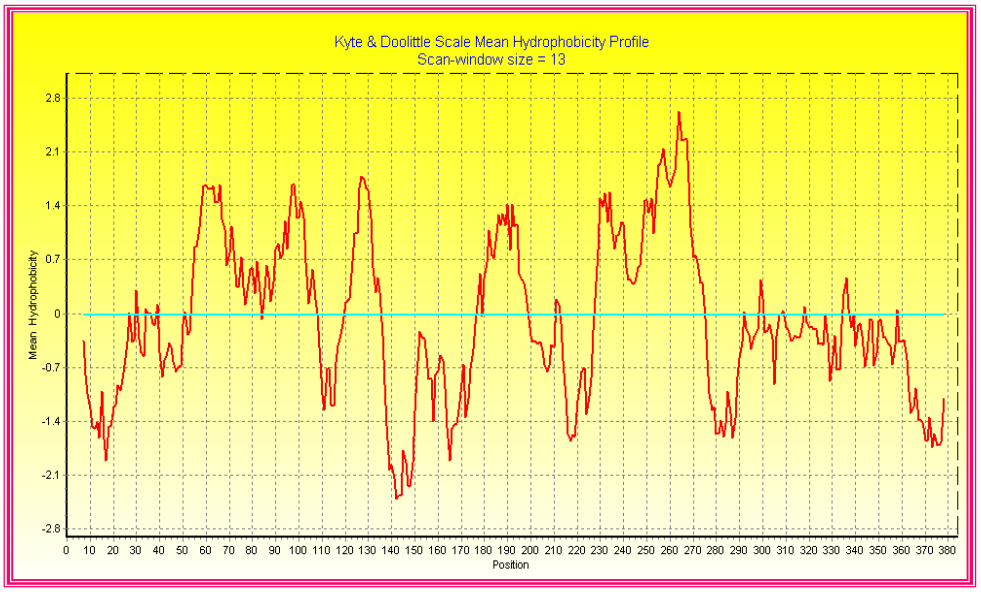


Figure 27: Hydrophobicity profile of *Bj*FAD2 protein using Kyte & Doolittle scale.

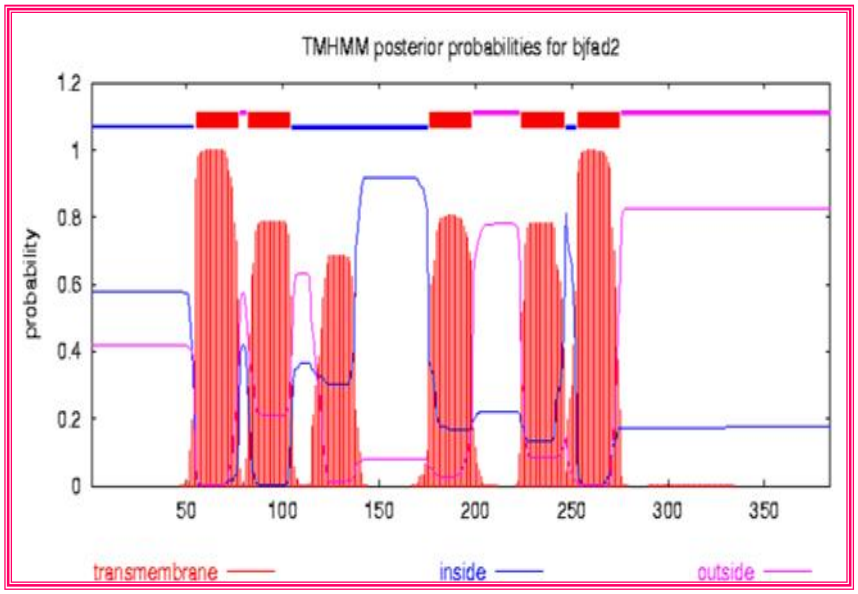


Figure 28: Prediction of transmembrane helices in case of putative *Bj*FAD2 protein using TMHMM 2.0 server.

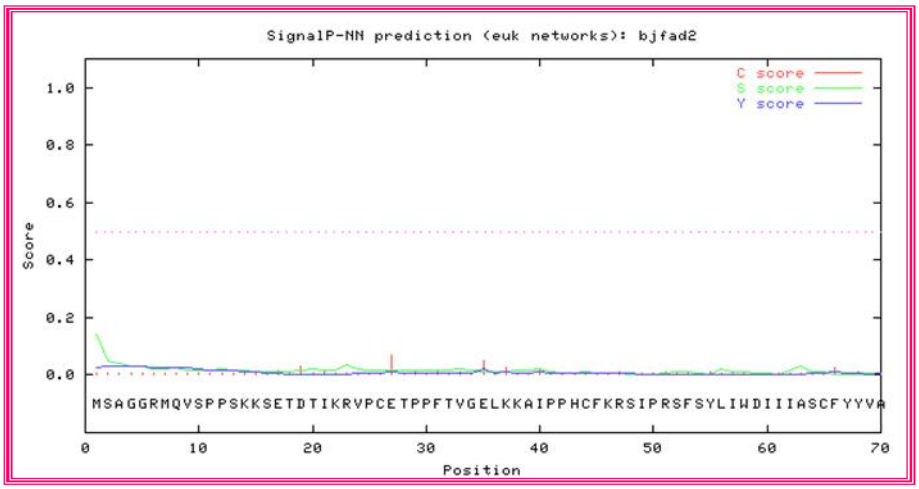


Figure 29: Prediction of signal peptide sequence from putative BjFAD2 protein using Signal P prediction software.

bjfad2 WoLFPSORT prediction chlo: 9.0, E.R.: 4.0

PSORT features and traditional PSORTII prediction

14 Nearest Neighbors

id	site	distance	identity	comments
FD6E_BRAJU	E.R.	70.4	95%	[Uniprot] SWISS-PROT45:Endoplasmic reticulum.
FD6E_ARATH	E.R.	136.1	90%	[Uniprot] SWISS-PROT45:Endoplasmic reticulum.
ISPF_CATRO	chlo	388.3	13%	[Uniprot] SWISS-PROT45:Chloroplast.
F16P_BRANA	chlo	392.7	12%	[Uniprot] SWISS-PROT45:Chloroplast stroma.
FD62_SOYBN	E.R.	393.5	78%	[Uniprot] SWISS-PROT45:Endoplasmic reticulum.
F16P_PEA	chlo	411.6	11%	[Uniprot] SWISS-PROT45:Chloroplast stroma.
CY11_SOLTU	mito	415.7	15%	[Uniprot] SWISS-PROT45:Mitochondrial intermembrane space.
F16P_SOYBN	chlo	417.9	12%	[Uniprot] SWISS-PROT45:Chloroplast.
CHMO_SPIOL	chlo	424.5	12%	[Uniprot] SWISS-PROT45:Chloroplast stroma.
CASS_RICCO	chlo	434.8	17%	[Uniprot] SWISS-PROT45:Chloroplast.

Figure 30: TargetP analysis of BjFAD2 protein for its intracellular localization.

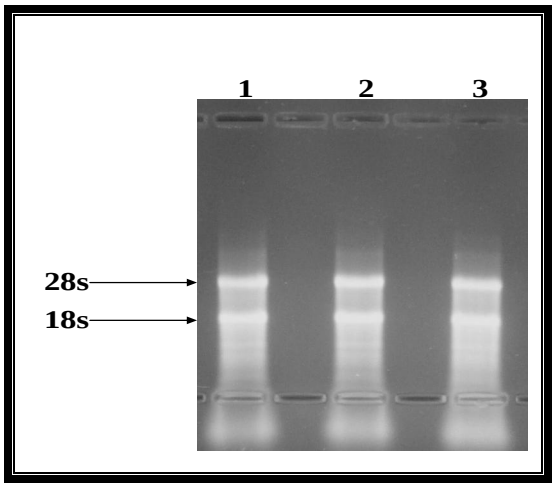


Figure 31: 1.2% agarose gel electrophoresis of RNA from developing seeds of *Brassica juncea* cv Pusa Bold. Lanes: 1-15 days after flowering (DAF), 2-30 DAF and 3–45 DAF.

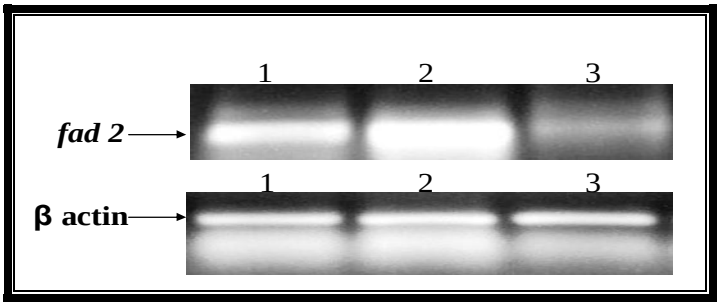


Figure 32: Study of developmental expression of *fad2* gene from developing seeds of *Brassica juncea* cv Pusa Bold using RT-PCR. Lanes: 1-15 days after flowering (DAF), 2- 30 DAF and 3 - 45 DAF.

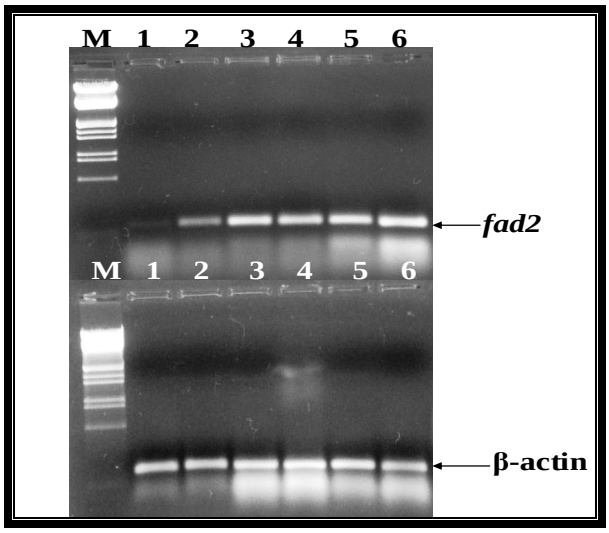


Figure 33: Study of *fad2* gene expression under different temperatures by RT PCR from developing seeds of *Brassica juncea* cv Pusa Bold. M-marker, lanes: 1-72 hrs incubation at 32^0C, 2-24 hrs incubation at 32^0C, 3 - 72 hrs incubation at 21^0C, 4– 24 hrs incubation at 21^0C, 5 -72 hrs incubation at 10^0C and 6–24 hrs incubation at 10^0C.

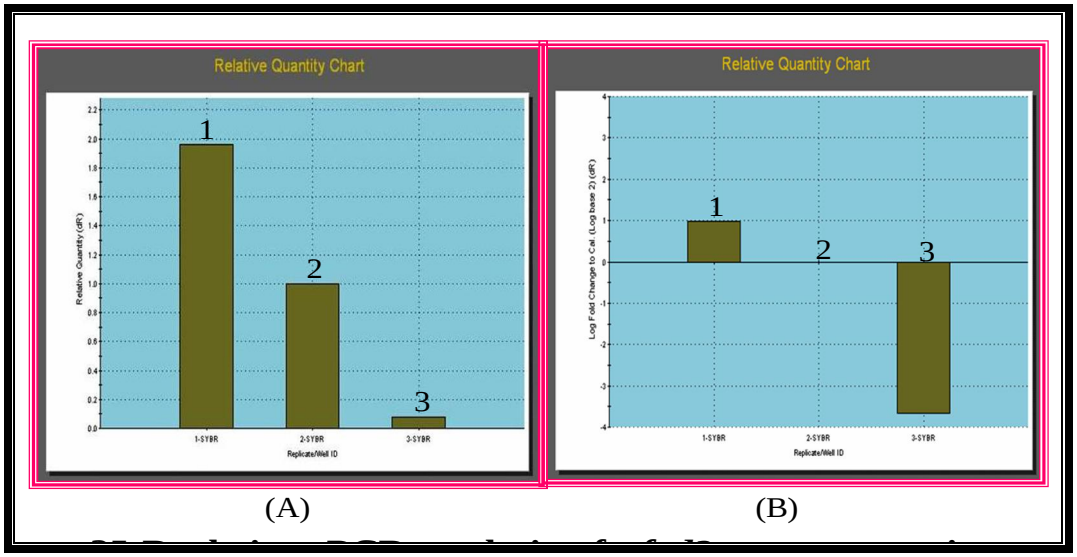


Figure 34: Real time PCR analysis of *fad2* gene expression under different temperature treatments from developing seeds of *Brassica juncea* cv Pusa Bold. (A) Relative quantity graph and (B) Log fold change graph. 1-72 hrs incubation at 10^0 C, 2-72 hrs incubation at 21^0C and 3 -72 hrs incubation at 32^0C.

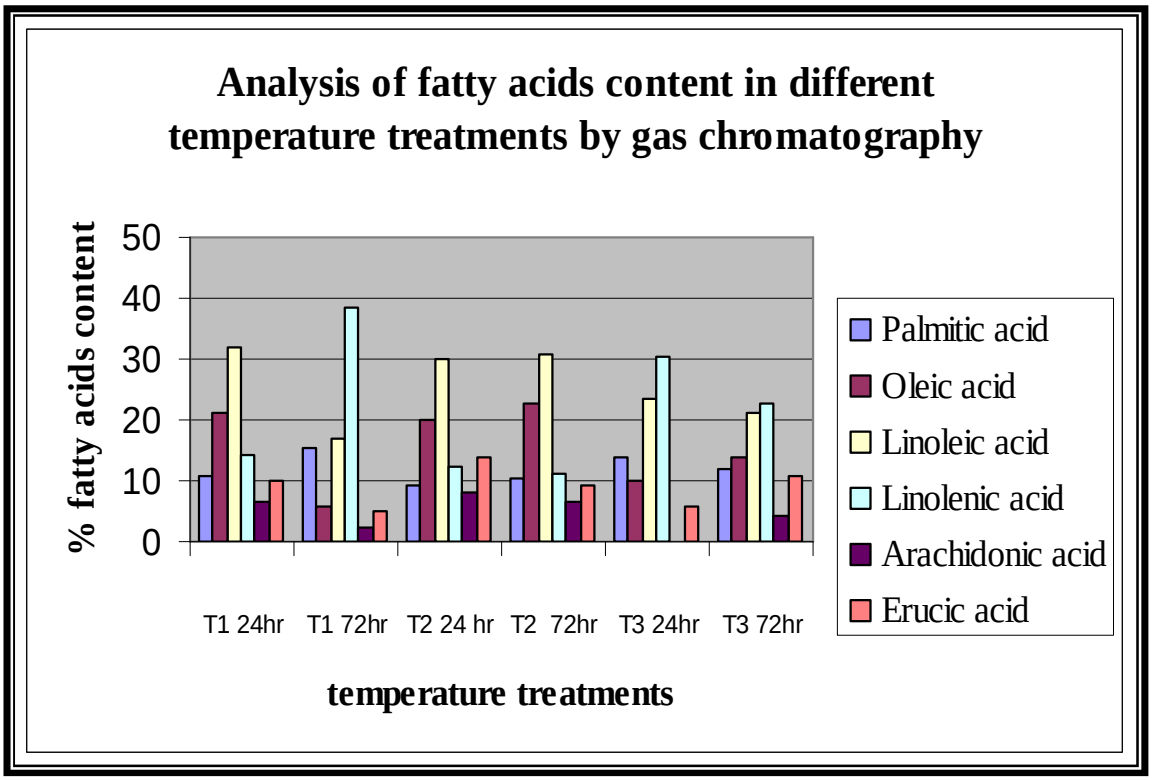


Figure 35: Graphical representation of fatty acid content in different temperature treatments determined using gas chromatography.

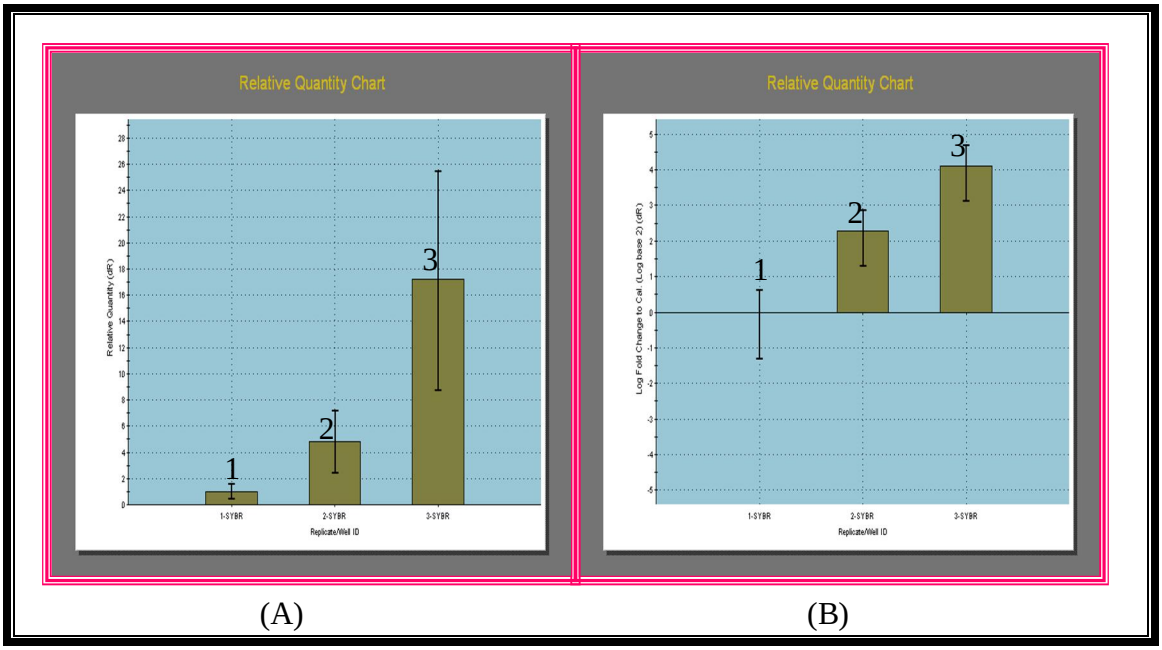


Figure 36: Study of differential expression of *fad2* gene in *Brassica* juncea varieties having different fatty acid composition through Real-time PCR. (A)- Relative quantity graph and (B) Log fold change graph. 1–Pusa Bold, 2-LES-39 and 3–LES 1- 27.

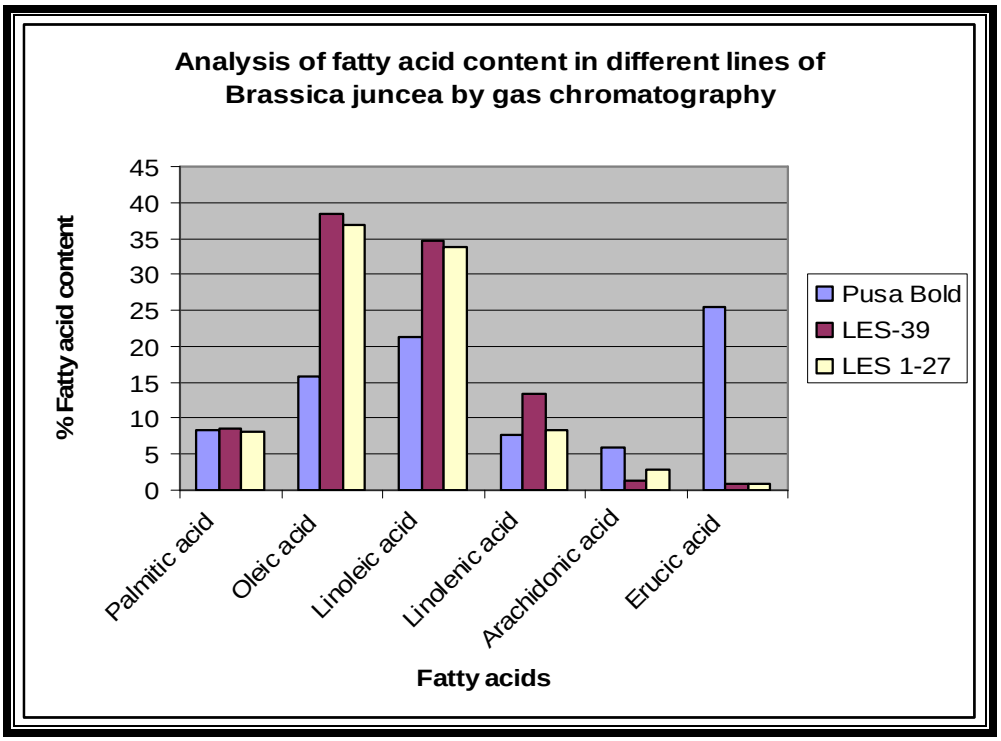


Figure 37: Graphical representation of fatty acid content in different *Brassica* juncea varieties having different fatty acid composition determined using gas chromatography.