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# Molecular cloning and *in silico* analysis of novel oleate desaturase gene homologues from *Brassica juncea* through sub-genomic library approach

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**Abstract**

The oleate desaturase represents a diverse gene family in plants and is responsible for conversion of oleic acid (18:1) to linoleic acid (18:2). Using the sequence information of *Bjfad2* (EF639848) gene that was isolated and characterized earlier from *Brassica juncea*, three additional novel *fad2* gene homologues designated as *Bjfad2a* (FJ696650), *Bjfad2b* (FJ696651) and *Bjfad2c* (FJ696652) were isolated from sub-genomic library of *B. juncea*. Sequence analysis of these genes revealed the sequence size of 2526 bp (*Bjfad2a*), 2326 bp (*Bjfad2b*) & 2325 bp (*Bjfad2c*), an open reading frame of 1155 bp that encodes a single polypeptide of 384 amino acids. Sequence comparison of isolated *Bjfad2* gene sequences shared 99% (*Bjfad2a*), 96% (*Bjfad2b*) and 95% (*Bjfad2c*) sequence similarity to reported *B. juncea fad2* gene (EF639848). The deduced amino acids of the these gene homologues displayed the typical three histidine boxes, a characteristic of all membrane bound desaturases along with five membrane spanning domains that are different from known plant desaturases. The signature amino acid sequence 'YNNKL' was found to be present at the N terminus of the protein, which is necessary and sufficient for ER localization of enzyme. Neighbor-Joining tree generated from the genomic structures alignment grouped them within the cluster of *fad2* gene(s) of *Brassicacea* and are distinct from *fad2* gene sequences from sesame (42%), soybean (38%) and sunflower (19%). Comparison of the *Bjfad2* genomic structures with other plant *fad2* genes revealed the presence of single large intron in their 5' UTR region which is evolutionarily conserved. However, the length of the intron varies across the plant species. Variation in the restriction sites of all three *Bjfad2* genes and its further confirmation through restriction analysis showed that the isolated genes are different homologues but code for the same enzyme, oleate desaturase. These observations confirm that atleast four copies of *fad2* gene exist which is consistent with the tetraploid nature of *B. juncea* genome.

**Keywords:** Fad2; clones; sequence analysis; restriction enzymes; genomic structures.
**Abbreviations:** FAD-fatty acid desaturase; ER-endoplasmic reticulum; ACP-acyl carrier protein; PC-phospho choline; CTAB-cetyl trimethylammonium bromide; PUFA-poly unsaturated fatty acids; ORF-open reading frame; CDS-coding DNA sequence, kb-kilo base; bp-base pair (s); kDa-kilo Dalton; UTR-untranslated region.

## Introduction

Biosynthesis of polyunsaturated fatty acids (PUFA) in plants is a three step sequential process. Stearoyl-ACP desaturase, or Δ9-desaturase, catalyses the first desaturation of stearic acid (18:0) to oleic acid (18:1). Oleoyl-PC desaturase, or Δ12-desaturase, catalyses the second desaturation of oleic acid (18:1) to linoleic acid (18:2); and linoleoyl-PC desaturase, or Δ15-desaturase, catalyses the third desaturation of linoleic acid (18:2) to linolenic acid (18:3). The oleate desaturase (FAD2; EC1.3.1.35; 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine delta 12-desaturase) is the principal enzyme in the fatty acid biosynthetic pathway of plants which catalyses the conversion oleic acid to linoleic acid through insertion of a double bond at Δ12 position of oleic acid (Shanklin and Cahoon, 1998). This enzyme is an integral membrane protein that needs l-acyl-2-oleoyl-sn-glycero-3-phosphocholine as substrate and requires NADH, NADH:Cyt $b_5$ oxidoreductase, Cyt $b_5$, and oxygen for activity (Smith et al., 1990; Kearns et al., 1991). This pathway is regulated at utilization of oleic acid pool in the cell which is a precursor for synthesis of both linoleic acid and erucic acid in the seed oils.

Much of the interests 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 oleate desaturase/omega-6 desaturase that converts oleate to linoleate in oilseed crops. The availability of the gene encoding oleate 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 on the genetic regulation of the lipid desaturation with regard to membrane properties and synthesis of storage lipids in plants. The genes for endoplasmic reticulum and plastid-derived Δ12 FADs have been characterized and reported from various plant species. These reports established the fact that oleate desaturase exist as complex gene families in plant genomes. On the contrary, *Arabidopsis* harbours only a single copy of the *fad2* gene (AT3G12120), which is constitutively

**Table 1.** Comparative analysis of deduced amino acid sequences of *Bjfad2* homologues (*Bjfad2a*, *Bjfad2b* & *Bjfad2c*) with the already isolated *fad2* genes, *Bjfad2* (EF639848) and *Bjfad2-1*(X91139) from *B. juncea*. Position and type of amino acids differ from the each *fad2* gene homologues are tabulated below.

| Sl No | Amino acid position | BJFAD2-1 (X91139) | BJFAD2 (EF639848) | BJFAD2A (FJ696650) | BJFAD2B (FJ696651) | BJFAD2C (FJ696652) |
|---|---|---|---|---|---|---|
| 1 | 2 | Glycine(G) | Serine (S)* | Glycine(G) | Glycine(G) | Glycine(G) |
| 2 | 12 | Serine (S)* | Proline (P) | Proline (P) | Proline (P) | Proline (P) |
| 3 | 13 | Proline (P)* | Serine (S) | Serine (S) | Serine (S) | Serine (S) |
| 4 | 20 | Threonine (T) | Threonine (T) | Threonine (T) | Threonine (T) | Threonine (T) |
| 5 | 21 | Leucine (L)* | Isoleucine (I) | Isoleucine (I) | Isoleucine (I) | Isoleucine (I) |
| 6 | 62 | Valine (V)* | Isoleucine (I) | Isoleucine (I) | Isoleucine (I) | Isoleucine (I) |
| 7 | 84 | Valine (V)* | Phenyl alanine (F) | Phenyl alanine (F) | Phenyl alanine (F) | Phenyl alanine (F) |
| 8 | 95 | Valine (V)* | Cysteine (C) | Cysteine (C) | Cysteine (C) | Cysteine (C) |
| 9 | 155 | Glutamic acid (E) | Glycine (G)* | Glutamic acid (E) | Glutamic acid (E) | Glutamic acid (E) |
| 10 | 193 | Tyrosine (Y) | Cysteine (C)* | Tyrosine (Y) | Tyrosine (Y) | Tyrosine (Y) |
| 11 | 194 | Tryptopan (W)* | Leucine (L) | Leucine (L) | Leucine (L) | Leucine (L) |
| 12 | 204 | Proline (P)* | Aspartic acid (D) | Aspartic acid (D) | Aspartic acid (D) | Aspartic acid (D) |
| 13 | 205 | Glutamic acid (E)* | Glycine (G) | Glycine (G) | Glycine (G) | Glycine (G) |
| 14 | 224 | Leucine (L) | Leucine (L) | Leucine (L) | Leucine (L) | Proline (P)* |
| 15 | 228 | Valine (V)* | Isoleucine (I) | Isoleucine (I) | Isoleucine (I) | Isoleucine (I) |
| 16 | 246 | Alanine (A)* | Valine (V) | Valine (V) | Valine (V) | Valine (V) |
| 17 | 255 | Leucine (L)* | Phenyl alanine (F) | Phenyl alanine (F) | Phenyl alanine (F) | Phenyl alanine (F) |
| 18 | 265 | Alanine (A)* | Glycine (G) | Glycine (G) | Glycine (G) | Glycine (G) |
| 19 | 301 | Aspartic acid (D) | Aspartic acid (D) | Aspartic acid (D) | Aspartic acid (D) | Asparagine (N)* |
| 20 | 333 | Valine (V)* | Alanine (A) | Alanine (A) | Alanine (A) | Alanine (A) |
| 21 | 343 | Aspartic acid (D)* | Glutamic acid (E) | Glutamic acid (E) | Glutamic acid (E) | Glutamic acid (E) |
| 22 | 352 | Tryptopan (W)* | Valine (V) | Valine (V) | Valine (V) | Valine (V) |

*Indicate the type of amino acid differ from the each *fad2* gene homologues.

and abundantly expressed (Beisson et al., 2003). Agriculturally important crops like soybean (*Glycine max*), cotton (*Gossypium hirsutum*), sesame (*Sesamum indicum*), corn (*Zea mays*) and canola (*Brassica napus*), contains 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; Suresha and Santha, 2008). Based on EST survey, atleast seven members of *fad2* gene were identified in four regions of soybean genome and expression analysis revealed that atleast three *fad2*-like genes are expressed in soybean seeds and one house keeping gene in most of the plant tissues (Tang et al., 2005; Scheffler et al., 1997). Two forms of *fad2* genes, *Oepfad2-1* and *Oepfad2-2* have been reported in olive (Hernandez et al., 2005), while three different forms, *Hafad2-1*, *Hafad2-2*, and *Hafad2-3*, have been isolated from sunflower (Martinez-Rivas et al., 2001). Expression of two copies of *fad2* gene was identified in developing seeds of flax (Fofana et al., 2006). More recently, a novel *fad2 gene* isoform from *B. juncea* encoding a polypeptide of 384 amino acids was isolated and characterized (Suresha et al., 2012). The deduced amino acid sequences of this gene displayed the typical three histidine boxes; characteristic of all membrane bound desaturases and possessed a C-terminal signal for ER retention. Based on genomic Southern blot analysis, more than two copies of *fad2* gene were predicted representing the allelic diversity in the tetraploid *B. juncea* genome. *Bjfad2* gene was constitutively expressed in all the tissues and is developmentally regulated. Up regulation of *Bjfad2* gene expression under low growth temperature resulted in high PUFA content of *B. juncea* seeds. Differential expression of *Bjfad2* gene from high and low erucic acid *B. juncea* genotypes was also reported (Suresha et al., 2012).

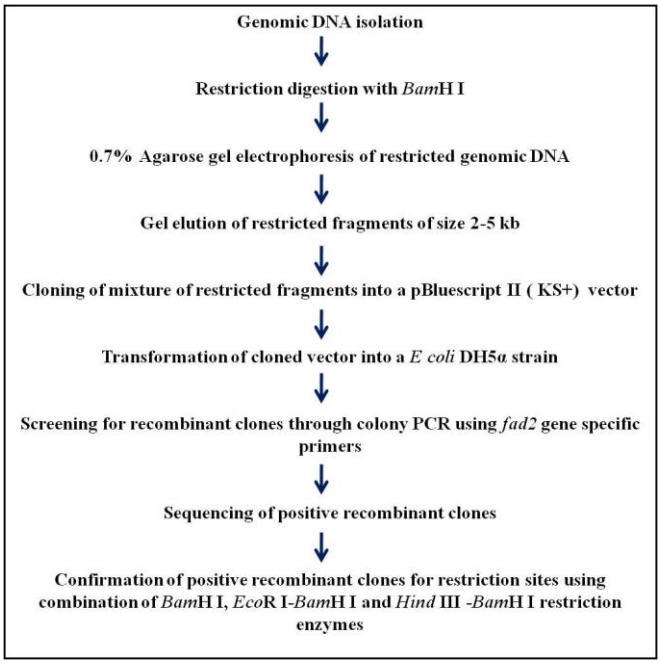


**Fig 1.** Flow chart showing the sequential of methods used for identification of *Bjfad2* homologues from *Brassica juncea* genome.

In continuation of the previous work and utilizing the sequence information of already identified *Bjfad2* gene, in this article, we report the isolation of three novel *fad2* gene homologues isolated from *B. juncea* sub-genomic library.

Restriction enzymes digestion and sequence analysis of the three genomic clones confirmed that, all these clones are different homologues of *fad2* gene coding for oleate desaturase enzyme and could be a novel target for genetic manipulation to alter the fatty acid composition of *B. juncea* seed oils.

**Results and Discussion**

***Isolation and cloning of fad2 genes***

Sub-genomic library followed by screening through colony PCR was carried out for isolation of *fad2* gene homologues from the *B. juncea* genome. For this, *BamH* I restricted sub-genomic library of *B. juncea* was constructed. Various steps of isolation are given as flow chart in Fig. 1. Using specific primers of *Bjfad2* gene reported earlier by our group, three *fad2* gene homologues designated as *Bjfad2a, Bjfad2b & Bjfad2c* were identified from *B. juncea* sub-genomic library respectively. A total of 458 recombinant clones screened, three clones showed positive for *fad2* gene in the colony PCR (Fig. 2). These three positive recombinant clones were sequenced through primer walking and size of the clones varied; 2526 bp (*Bjfad2a*), 2326 bp (*Bjfad2b*) and 2325 bp (*Bjfad2c*) respectively. These sequences have been deposited at Gen-Bank under the accession numbers FJ696650 (*Bjfad2a*), FJ696651 (*Bjfad2b*) and FJ696652 (*Bjfad2c*) respectively**.** These gene sequences were annotated for the presence of exons, introns, coding DNA sequences (CDS)/ORF and 5'& 3' untranslated regions (UTR). The open reading frame (ORF) of *Bjfad2* gene homologues containing 1155 bp, code for putative protein of 384 amino acids, corresponding to a calculated molecular mass of 44 kDa and pI of 8.62. This ORF was flanked by unique 5' UTR and 3' UTR regions (Fig.3, 4 & 5). The size of 5' UTR region was 1228 bp, 1151 bp and 1150 bp for *Bjfad2a*, *Bjfad2b* and *Bjfad2c* homologues, respectively. The size of the 3'UTR was 143 bp in *Bjfad2a* and 20 bp in both *Bjfad2b* and *Bjfad2c* respectively. All the three homologues contain 2 exonic sequences in which Exon I is of 143 bp in *Bjfad2a* and 2 bp in both *Bjfad2b* & *Bjfad2c* whereas, Exon II is of 1302 bp in *Bjfad2a*, 1179 bp in both *Bjfad2b* and *Bjfad2c* homologues respectively. A single large intronic sequence of size 1081 bp, 1075 bp and 1074 bp is present in between two exonic sequences of *Bjfad2a, Bjfad2b* and *Bjfad2c*, respectively (Fig. 3, 4 & 5). The sequences flanking the methionine start codon in all the *fad2* gene homologues were highly conserved AAACATGGG**.** However, these sequences were differing with the already reported *fad2* gene (Fig. 3, 4 & 5). Replacement of single nucleotide (A with G) results in the change of amino acid sequence (Serine to Glycine) of BJFAD2 protein.

The isolated genes showed similarity of 96-98% at nucleotide level with *B. rapa* (HM189213.1) and *B. compestris* (AJ459108.2) genes reported earlier. The amino acid sequence of isolated three *Bjfad2* homologues showed 99% (*Bjfad2a*), 96% (*Bjfad2b*) and 95% (*Bjfad2c*) similarity to already reported *B. juncea fad2* gene (EF639848) (Table 1). *Bjfad2a and Bjfad2b* differ with *Bjfad2c* by two amino acids, proline and asparagine at the position of 224 and 301, respectively. However, all these three homologues differed with already known *fad2* genes of *B. juncea* by three (*Bjfad2*, EF639848) and 16 amino acids (*Bjfad2-1*, X91139) (Table 1). This confirms that the isolated *Bjfad2* gene homologues are different, although most of the sequences in the conserved domains are identical.

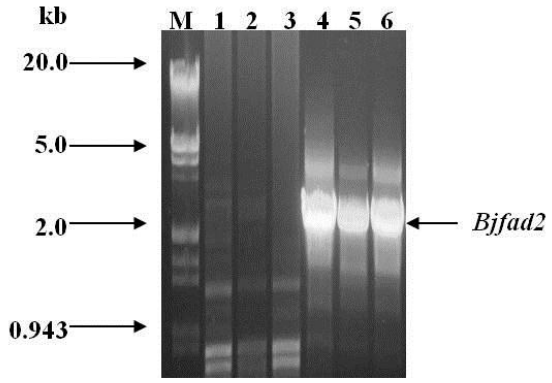


**Fig 2.** PCR amplification of *Bjfad2* gene homologues from *B. juncea* sub-genomic library. Genomic DNA of *B. juncea* was restricted with *Bam*HI restriction enzyme and the resulting fragments of 2-5 kb size were cloned in to pBluescript II KS (+) vector and transformed to *E. coli* DH5α strain. Recombinant clones were screened for the presence *Bjfad2* gene homologues through colony PCR. Lane M-Marker (*Eco*RI+*Hind*III digested DNA ladder); lane 1, 2 & 3- non recombinant clones showed no amplification; lane 4, 5 & 6- showing amplification of *Bjfad2a*, *Bjfad2b* and *Bjfad2c* gene homologues respectively. Arrows indicate the molecular size of the marker and genes.

This may be due to the allelic differences in the *fad2* gene which represent the complexity of tetraploid *Brassica* genome.

***Sequence analysis of Bjfad2 homologues***

The deduced amino acid sequences of *Bjfad2* gene homologues showed typical features including the presence of three histidine boxes, HECGH, HRRHH, and HVAHH, a characteristic feature of all membrane-bound desaturases (Shanklin et al., 1994) (Fig. 6). These histidine boxes are present in all reported membrane-bound desaturases and are essential for acquiring Fe ions and forming the catalytic pocket at the interface of membrane and cytoplasm after anchoring to the membrane by virtue of specialized transmembrane regions (Shanklin et al., 1997). Moreover, a group of different enzymes consisting of desaturases, hydroxylases, and epoxygenases reported ubiquitously from animals, fungi, plants and bacteria that catalyze diverse reactions, use the similar histidine-rich motifs to form the di-iron center of activity. To predict whether any signal or transit peptide is present in the N-terminal region of predicted BjFAD2 proteins and to determine its cellular localization, the algorithms for amino acid sequence analysis (http://www.psort.nibb.ac.jp/form.html) and (http://www.cbs.dtu.dk/services/TargetP/) were employed (Emanuelsson et al., 2000, 2007). Any identification for probable localization of this protein in chloroplast, mitochondria, or secretory pathway could not be recognized ruling out the possibility of identified *Bjfad2* homologues to be a plastidial counterpart and that it is not targeted to the photosynthetic apparatus. On the contrary, all these predicted proteins contains 'YNNKL' motif at the C terminus of the protein (Fig. 6) which is necessary and sufficient for ER localization of enzyme (McCartney, et al., 2004), indicating that *Bjfad2* gene homologues encode a microsomal enzyme. The hydropathy profiles of the deduced amino acid sequence of *Bjfad2a*,

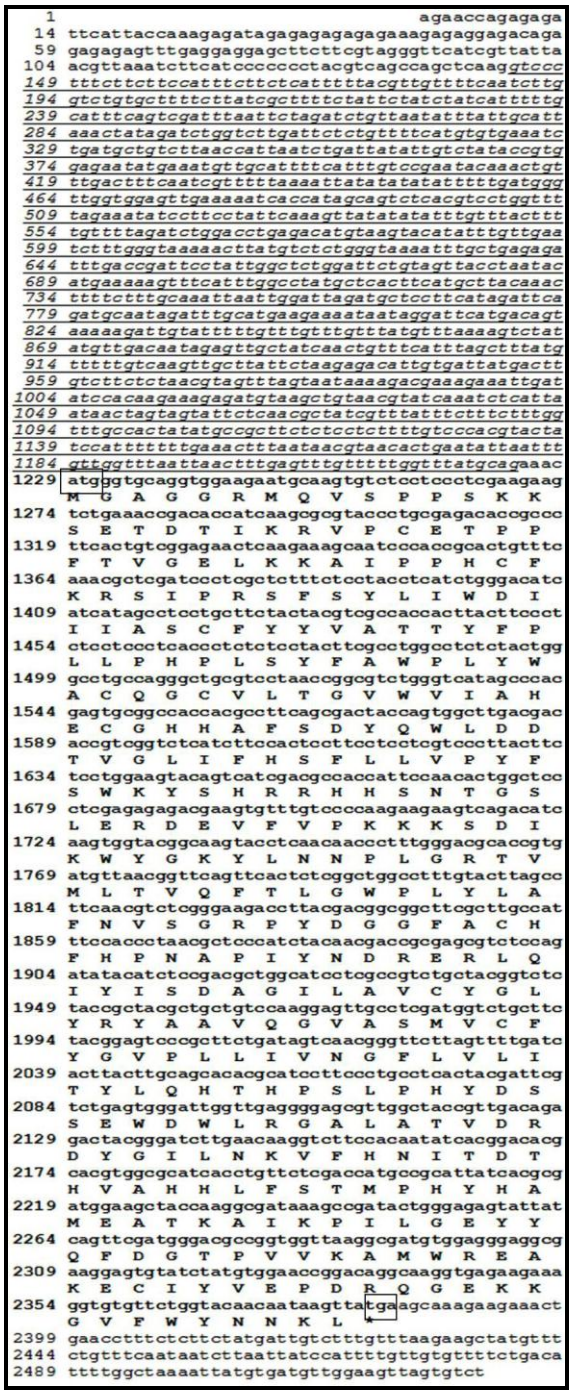

**Fig 3.** *Bjfad2*a showing 5'UTR, 3'UTR and ORF/CDS with corresponding deduced amino acid sequences from *B. juncea*. Start (ATG) and stop (TGA) codons are boxed and 5'UTR intron is underlined in italics.

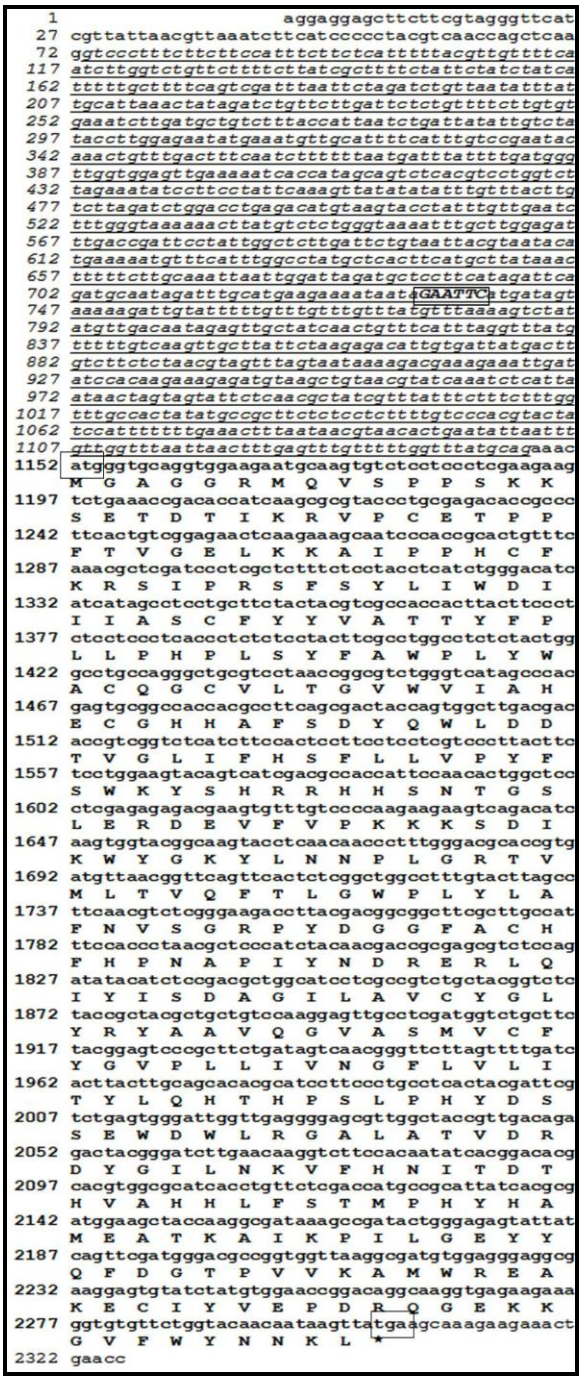

**Fig 4.** *Bjfad2*b showing 5'UTR, 3'UTR and ORF/CDS with corresponding deduced amino acid sequences from *B. juncea*. Start (ATG) and stop (TGA) codons are boxed and 5'UTR intron is underlined in italics. Presence of *Eco*R I restriction site (GAATTC) is shown in box.

*Bjfad2b* and *Bjfad2c* were generated using TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) tools. This revealed five clusters of strong hydrophobic regions at positions 55aa-79aa, 84aa-104aa, 120aa-140aa, 179aa-199aa and 252aa-272aa which was different from the already reported *Bjfad2* genes (EF639848 & X91139) in positions of hydrophobic domains (Fig. 7a & 7b). These clusters are the putative membrane spanning helices common to most of the membrane-bound desaturases and represent well-conserved domains among all three *Bjfad2* gene homologues (Fig 7a & 7b). Interestingly, predicted transmembrane domains using TMHMM server showed that *Bjfad2a* and *Bjfad2b* have five transmembrane domains at positions 55aa-77aa, 82aa-104aa, 176aa-198aa, 224aa-246aa and 253aa-275aa (Fig. 8a & 8b) in its protein, which was similar to the *fad2* homologues (EF639848) isolated previously (Suresha et al., 2012). However, *Bjfad2c* showed only four transmembrane domains at positions 55aa-77aa, 82aa-104aa, 176aa-198aa, and 253aa-275aa (Fig. 8c) similar to the *Bjfad2* isolated by Singh et al., (1995). These

observations confirmed that isolated *Bjfad2* gene homologues are different and predicted to code for FAD2 enzymes having different temporal and spatial expression although function of the enzymes is the same. Further, the dendrogram generated from the genomic structures of isolated *Bjfad2* gene homologues with already known *fad2* gene sequences has revealed that *Bjfad2a* grouped with *fad2* gene of *B. compestris* (95%) whereas *Bjfad2b* and *Bjfad2c* grouped together in the same cluster. However, all three isolated homologues were more related to *fad2* genes of *Brassicacea* family [*B. napus* (95%); *B. nigra* (60%) and *Arabidopsis* (55%)] while distinct from *fad2* genes of sesame (42%), soybean (38%) and sunflower (19%) (Fig. 9). Comparison of the *fad2* genomic structures from *B. juncea*, *B. campestris*, *Sesamum indicum*, *Arabidopsis thaliana*, *Gossypium hirsutum*, *Glycine max* and *Oryza sativa* revealed that *fad2* genomic sequences contain single large intron in their 5' UTR region which is evolutionarily conserved, although the length of the intron varies across the plant species (Fig. 10). Evolution of 5' UTR intron and its role in expression of *fad2* gene have 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). The sequences of the *SeFAD2* intron, were assessed and identified for intron mediated enhancement (IME) of *Sefad2* gene expression and the promoter-like activity (Kim et al., 2006). Expression of reporter genes under the control of the maize *Adh1*, *Sh1*, *Bx1*, or *Act* promoter was increased up to several hundred folds by the inclusion of an intron (Callis et al., 1987; Maas et al., 1991; Oard et al., 1989; Vasil et al., 1989). Genes encoding polyubiquitin and translation elongation factor EF-1 alpha factor EF-1α proteins from *Arabidopsis thaliana* have an intron in the 5' UTR region that increases the expression of reporter gene fusions 2.5-1000-fold relative to intron-less controls (Norris et al., 1993; Currie et al., 1991 & 1993). This enhancement of gene expression has been ascribed to intron splicing (Gidekel et al., 1997). Similar to these studies, the intron present in the 5' UTR region of isolated *Bjfad2* homologues would be a novel target for studying its role in regulation of *fad2* gene expression in *B. juncea*.

***Variation in the restriction enzyme cleavage sites and its confirmation through in vitro restriction analysis***

To study the differences in the isolated gene sequences for restriction enzyme cleavage sites, restriction analysis of isolated three *Bjfad2* sequences was carried out using NEB cutter software. Restriction map generated in this analysis is depicted in Fig. 11. The clone *Bjfad2a* does not have any cleavage sites for both *Eco*R I and *Hind* III restriction enzymes (Fig 11a). The clone *Bjfad2b* has *Eco*R I cleavage site (GAATTC) at the position of 733 bp (Fig. 11b) whereas *Bjfad2c* has *Hind* III (AAGCTT) cleavage site at the position of 532 bp along with the *Eco*R I cleavage site in its gene sequence (Fig. 11c). In order to confirm this, restriction analysis of all the three clones using the restriction enzymes combinations of *Bam*H I, *Eco*R I-*Bam*H I and *Hind* III-*Bam*H I were carried out. Results from this study revealed that all these clones showed variations in restriction patterns where *Bjfad2a* with a single band of insert size 2.5kb, *Bjfad2b* with two bands of insert size 1.59 kb and 0.73 kb and *Bjfad2c* with three bands of insert size 1.59 kb, 0.53 kb and 0.20 kb along with the vector band. The band of 0.20 kb in *Bjfad2c* was not visible due to the small size of the fragment and low concentration agarose gel electrophoresis (Fig 12). Earlier studies on genomic organization of *Bjfad2* gene through southern blot analysis had confirmed that atleast two copies

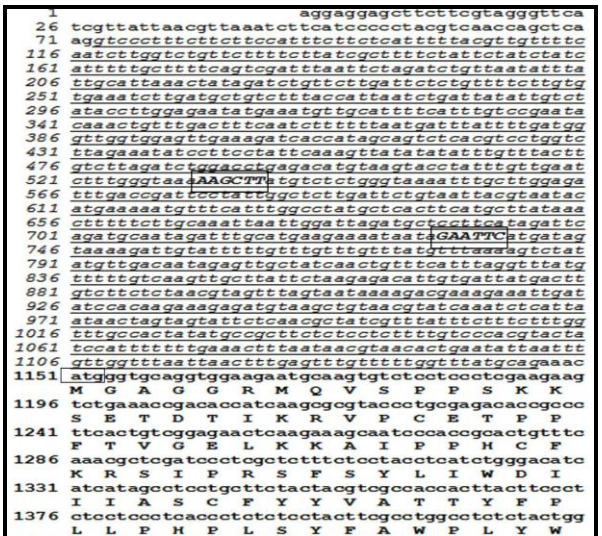

**Fig 5.** *Bjfad2c* showing 5'UTR, 3'UTR and ORF/CDS with corresponding deduced amino acid sequences from *B. juncea*. Start (ATG) and stop (TGA) codons are boxed and 5'UTR intron is underlined in italics. Presence of *Eco*R I (GAATTC) and *Hin*d III (AAGTTC) restriction sites are showed in boxes.

of *fad2* gene are present in *B. juncea*, which was consistent with the tetraploid nature of the *B. juncea* genome (Suresha et al., 2012). Similar results were reported in *Olea europaea* and *Glycine max* (Hernandez et al., 2005; Heppard et al., 1996). Also, microsomal omega-6 desaturase gene family from *B. napus* was reported 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 *B. napus* (Scheffler et al., 1997). In cotton, *ghfad2-1* appears to be a single copy gene in each of the two diploid species (*Gossypium herbaceum* and *Gossypium raimondii*) and presumably this was also true in the progenitor species of the allotetraploid cotton and the combination of them makes up the two copies in tetraploid cotton (*Gossypium barbedense* and *Gossypium hirsutum*) (Liu et al.,1999). In conjunction with the previous studies on copy number of *fad2* gene in various plants, we confirmed that isolated three *Bjfad2* genes appear to be homologues of already reported *fad2* genes of *B. juncea*. Further investigations on functional characterization

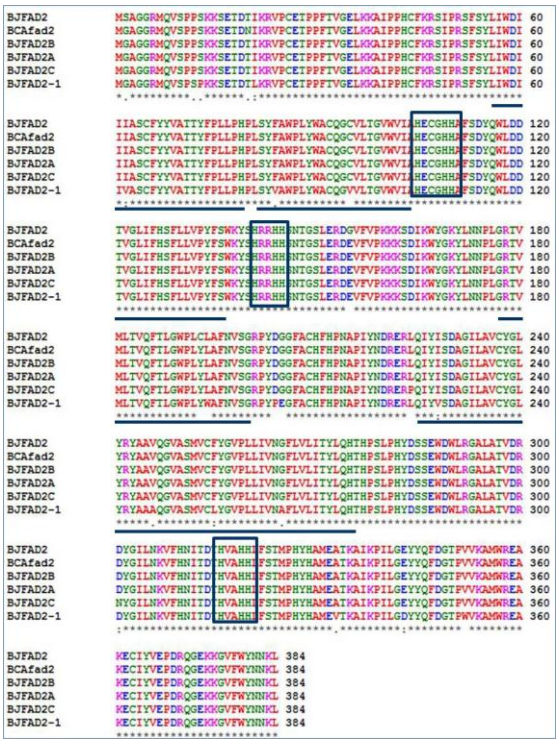


**Fig 6.** Deduced amino acid sequences alignment of *Bjfad2* gene homologues (*Bjfad2a*, *Bjfad2b* and *Bjfad2c*) with already known *fad2* genes of *B. juncea*, *Bjfad2* (EF639848), *Bjfad2-1*(X91139) and *B. compestris* (AJ459107). Boxes represent three histidine motifs (HXXXHH, HXXHH and HXXHH); five hydrophobic regions are underlined.

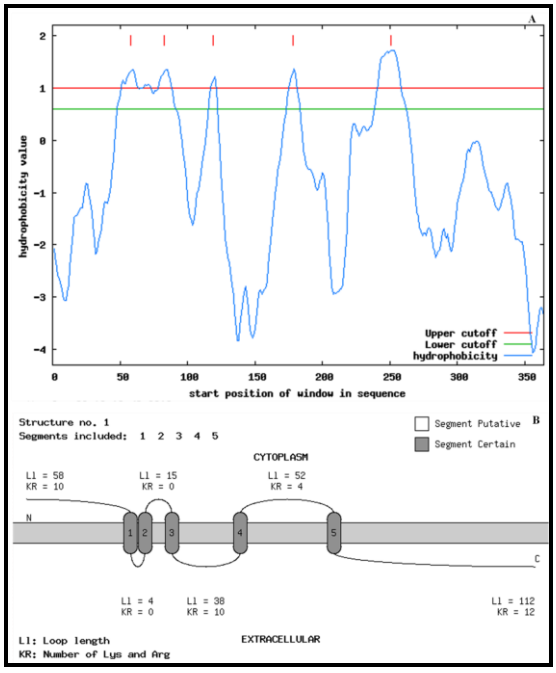


**Fig 7.** Hydropathy plot of *Bjfad2* gene homologues derived using TopPred software. (A) Five candidate membrane spanning segments at the position of 59aa-79aa, 84aa-104aa, 120aa-140aa, 179aa-199aa and 252aa-272aa. (B) Topological structure of corresponding membrane spanning segments represented by 1-5 along with cytoplasmic and extracellular loops in the putative proteins of *Bjfad2a*, *Bjfad2b* and *Bjfad2c* homologues from *B. juncea*.

of these gene homologues are needed in order to understand their role in the regulation of fatty acid biosynthesis in *B. juncea* seed oil.

## Materials and methods

### *Plant material*

Seeds of *B. juncea* variety Pusa Bold were obtained from *Brassica* breeding project, Division of Genetics, Indian Agricultural Research Institute (IARI), New Delhi. Seeds were surface sterilized with 0.1% mercuric chloride for 2-3 minutes and washed 3-5 times with sterile distilled water. The surface sterilized seeds were grown on germination paper in dark. Watering of the seeds was done daily for a week and etiolated seedlings were used for DNA isolation.

### *Genomic DNA isolation and sub-genomic library construction*

Total genomic DNA was extracted from etiolated seedling (1g), using CTAB method as described by Murray and Thompson (1980) with minor modifications. In a 35 ml centrifuge tube aliquoted 16 ml of the DNA extraction buffer (DEB) and to that 40 µl of β-mercaptoethanol was added. The tubes were incubated at $65^0$C for an hour in a water bath. One gram of plant sample was crushed in liquid Nitrogen to a fine powder and was transferred to the pre-incubated DEB kept at $65^0$C for one and half hour with intermittent gentle mixing by inverting the tubes. An equal volume of chloroform: isoamyl alcohol was added and centrifuged at 10,000 x g for 10 minutes at $4^0$C. The resulting upper aqueous phase was transferred into a corex tube and equal volume of chilled isopropanol was added. DNA threads started appearing in the tube were spooled out and pelleted down by centrifuging at 10,000 x g for 10 minutes. The DNA pellet was washed with 70% ethanol, dried at room temperature to remove ethanol traces and dissolved in TE buffer. DNA concentration was determined spectrophotometrically and was verified by ethidium bromide staining of agarose gel. Genomic DNA (5 µg) was restricted with *Bam*H I restriction enzyme (Fermentas Inc.USA) for overnight at $37^0$C. Restricted DNA was separated on 0.8% agarose gel electrophoresis and size selected DNA (2 to 5 kb) fragments were cut out and eluted using QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. Mixtures of restricted fragments were cloned into *Bam*H I arms of pBluescript II KS (+) vector using $T_4$ DNA ligase (Fermentas Inc. USA) and transformed into competent *Ecoli* (DH5α) cells. DH5α cells transformed with recombinant plasmids were selected based on antibiotic resistance as well as α-complementation method (Sambrook et al., 1989). Ampicillin resistant putative recombinants were selected for further analysis.

### *Colony PCR and gene sequencing*

Gene specific primers, (F-5' AGGAGCTTCTTCGTAG-GGTTC 3' and R-5' ATTGTTGTACCAGAACACAC 3') were designed from the known *Bjfad2* gene (EF639848) and colony PCR was performed from the recombinant clones. A typical PCR amplification protocol with Taq DNA polymerase (Fermentas Inc. USA) consists of initial denaturation at $94^0$C for 4 min, denaturation at $94^0$C for 1 min, annealing at $55^0$C for 45 sec, extension at $72^0$C for 2 min 30 sec for 35 cycles followed by final extension of $72^0$C for 10 min. Positive recombinants were sequenced

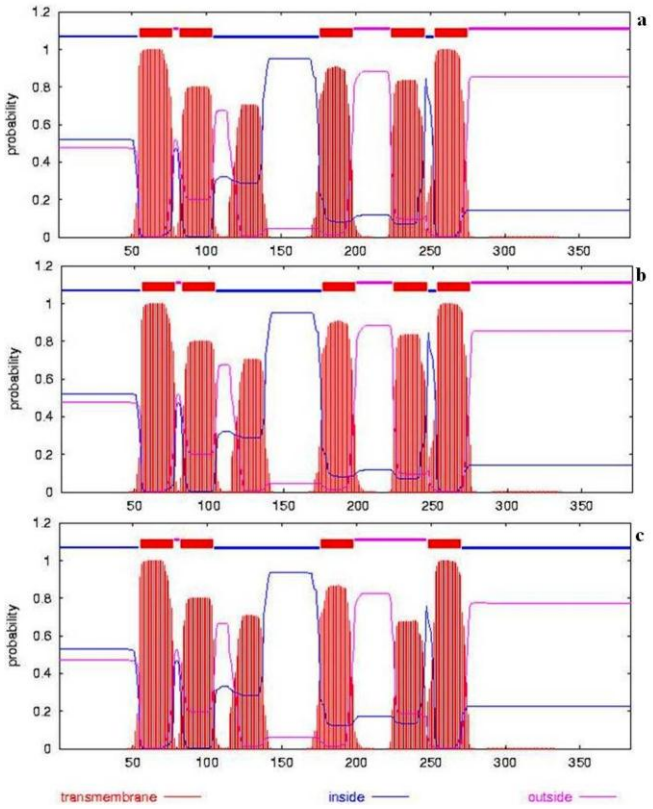


**Fig 8.** Predicted transmembrane domains in the putative proteins of *Bjfad2* gene homologues from *B. juncea* using TMHMM software. (a). *Bjfad2a* and (b).*Bjfad2b* showing the five transmembrane domains at a position of 55aa-77aa, 82aa-104aa, 176aa-198aa, 224aa-246aa and 253aa-275aa and (c). *Bjfad2c* gene homologue showing four transmembrane domains at position of 55aa-77aa, 82aa-104aa, 176aa-198aa, and 253aa-275aa. Red colour boxes represent the putative transmembrane domains. Blue and Pink colour lines indicate the inner and outer membranes of the cell respectively.

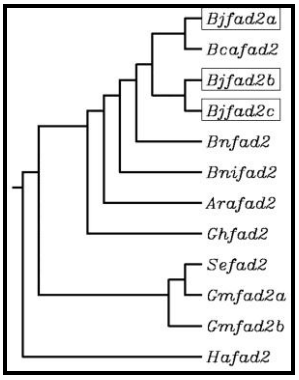


**Fig 9.** Phylogenetic relationships between genomic structures of *Bjfad2* gene homologues and other plant *fad2* genes. Positions of the *B. juncea fad2* gene homologues (*Bjfad2a*, FJ696650, *Bjfad2b*, FJ696651 and *Bjfad2c*, FJ696652) are shown in boxes. The gene sequences and Gen-Bank accession numbers used for the analysis are: *Brassica campestris* (*Bcafad2*, AJ459108), *B. napus* (*Bnfad2*, FJ952144), *B. nigra* (*Bnifad2*, HM138369), *Arabidopsis thaliana* (*Atfad2*, AC069473), *Gossypium hirsutum* (*Ghfad2*, AF331163), *Sesamum indicum* (*Sefad2*, AY770501), *Glycine max* (*Gmfad2a*, AB188251; *Gmfad2b*, AB188253) and *Helianthus annuus* (*Hafad2*, DQ075691).

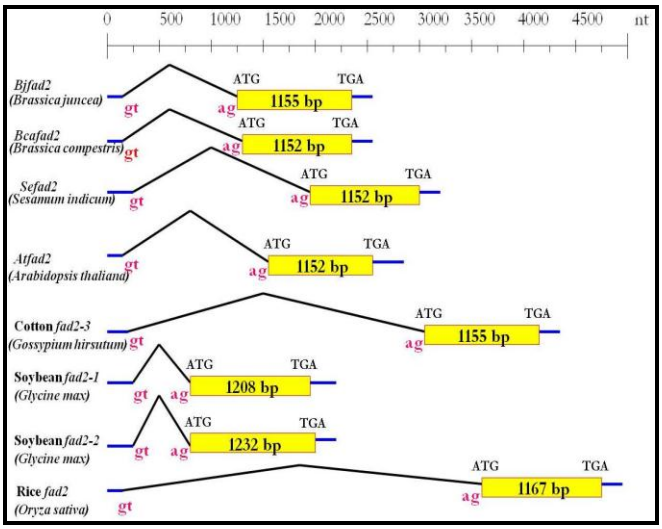


**Fig 10.** Comparison of the *fad2* genomic structures of *B.juncea (Bjfad2a, Bjfad2b & Bjfad2c)*, *Sesamum indicum* (*Sefad2*), *Arabidopsis thaliana (Atfad2)*, *Gossypium hirsutum* (*Ghfad2*), *Glycine max* (*Gmfad2-1*, *Gmfad2-2*) and *Oryza sativa* (Rice *fad2*). 5'UTR region intron of all the *fad2* genes are indicated with 'gt' as start and 'ag' as end nucleotides. ORF/CDS region is marked with ATG to TGA as start and stop codons respectively.

according to dideoxy chain termination method (Sanger et al., 1977) from both 5' and 3' ends atleast three times through primer walking by making use of the automated DNA sequencing facility at University of Delhi, South Campus, (India).

***In silico analysis***

Oleate desaturase gene sequences were identified by the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). The open reading frame of *Bjfad2* gene homologues was predicted using online NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/). Multiple sequence alignment was performed using ClustalW with default parameters. Hydropathy plots were derived by using TopPred (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) (Kyte and Doolittle, 1982; Von Heijne, 1992) and transmembrane regions were predicted by the TMHMM server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Prediction of sub-cellular 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/) algorithms. Phylogenetic tree was constructed using the neighbour-joining method and protdist algorithm in the PHYLIP package (version 3.63) from the genomic structures of isolated *Bjfad2* clones and other plant desaturases retrieved from the Gen-Bank database.

***Restriction analysis***

Restriction maps for all the sequences were developed using NEB cutter software (http://tools.neb.com/NEBcutter2/). To confirm the presence of *Eco*R I, *Bam*H I and *Hin*d III internal restriction sites within the gene sequences, all the clones were

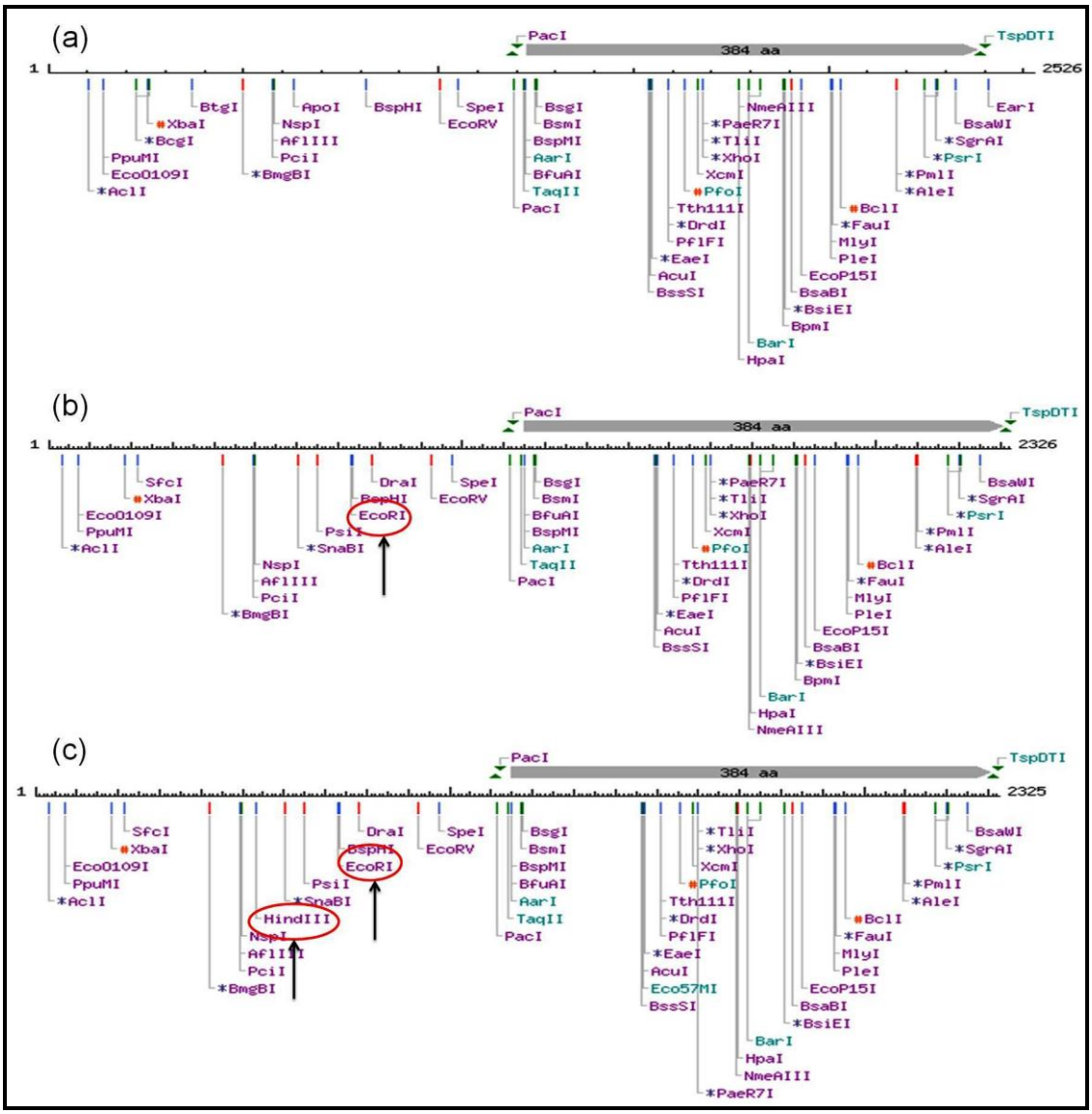


**Fig 11.** Restriction map of *Bjfad2* gene homologues (a) *Bjfad2a*, (b) *Bjfad2b* and (c) *Bjfad2c* using NEB cutter software. Position of *Eco*R I and *Hin*d III internal restriction sites are marked red in circles.

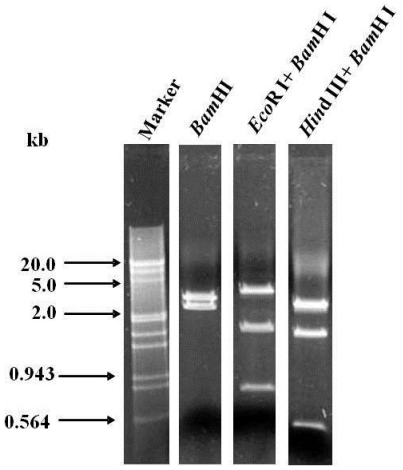


**Fig 12.** *In vitro* restriction pattern of *Bjfad2* clones. Lanes showing-Marker (*Eco*RI+*Hind*III digested DNA ladder); *Bjfad2a* clone restricted with *Bam*H I restriction enzyme alone; *Bjfad2b* and *Bjfad2c* clones restricted with the combination of *Eco*RI & *Bam*H I and *Hin*d III & *Bam*H I restriction enzymes, respectively.

restricted with the restriction enzymes combinations of *Bam*H I, *Eco*R I-*Bam*H I and *Bam*H I-*Hin*d III.

**Conclusion**

In the present study, we have identified three *Bjfad2* gene homologues from *B. juncea*, which code for oleate desaturase, a principal enzyme in biosynthesis of PUFA in the seed oil. The evidences from the structural features of gene sequences, deduced protein analysis and restriction analysis of isolated genomic clones strongly suggest them to be homologues of microsomal Δ12 desaturase and hence denoted as *Bjfad2a*, *Bjfad2b* and *Bjfad2c*. The existence of these gene homologues indicates genic complexity of desaturase genes in *B. juncea* as already evident in this class of microsomal desaturases of other plants.

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