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Molecular cloning, expression analysis and growth temperature dependent regulation of a novel oleate desaturase gene (*fad2*) homologue from *Brassica juncea*

Suresha G.S[1, 2*], Rai R.D[1], Santha I.M[1]

[1]Division of Biochemistry, Indian Agricultural Research Institute (IARI), New Delhi, 110012, India
[2]Present address: Scientist (Biochemistry), Sugarcane Breeding Institute (ICAR), Coimbatore, Tamil Nadu-641007, India

***Corresponding author: sureshgiriyapur@gmail.com**

Abstract

In plants the endoplasmic reticulum (ER) associated oleate desaturase (FAD2) is the key enzyme responsible for the production of linoleic acid in non-photosynthetic tissues. In the present study, we isolated a novel gene encoding *fad2* isoform through RT-PCR, designated as *Bjfad2* (Gen Bank Accession No. EF639848) from *Brassica juncea*, important source of edible oil in India. The cDNA sequence of *Bjfad2* was 1,445 bp containing a 1,155 bp open reading frame (ORF) encoding a polypeptide of 384 amino acids. The deduced amino acid sequences of the isolated gene displayed the typical three histidine boxes, characteristic of all membrane bound desaturases and possessed a C-terminal signal for ER retention. Phylogenetic analysis showed *Bjfad2* grouped within the plant housekeeping-type *fad2* genes. Genomic Southern blot analysis was consistent with the presence of atleast two copies of *Bjfad2* gene in *B. juncea* genome. Study of tissue specific expression confirmed that *Bjfad2* was constitutively expressed in both vegetative tissues and developing seeds. Expression of *Bjfad2* gene is developmentally regulated with increased expression in mid-maturation stage [30 days after flowering (DAF)], as compared to early (15 DAF) and late (45 DAF) stages of seed development. Real-Time PCR analysis of *fad*2 expression showed one fold higher under the lower temperature and three fold lower under the higher temperature treatments. Differences in fatty acid contents among the temperature treatments were consistent with the expression data of the gene. Differential expression of the *fad2* gene was observed between high and low erucic acid *B. juncea* genotypes.

Introduction

Polyunsaturated fatty acids (PUFA) of plant lipids such as linoleic and linolenic acids play crucial roles in plant metabolism-as storage compounds mainly in the form of triacylglycerol (TAG), as structural components of membrane lipids, and also as the precursors of signalling 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 affects its properties such as oxidative stability (Marquez-Ruiz et al., 1999) and nutritional value (Cunnane, 2003).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). *Arabidopsis* harbors only a single copy of the *fad2* gene (Accession number-AT3G12120), which is constitutively and abundantly expressed (Beisson et al., 2003). In contrast to *Arabidopsis*, crops, including soybean (*Glycine max*), cotton (*Gossypium hirsutum*), sesame (*Sesamum indicum*), 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; Suresha and Santha, 2008).

Brassica spp are now the second largest oilseed crop after soybean and *B. juncea* (Indian Mustard) is an important source of edible oil in many countries across globe. It is a major oil seed crop of north India and is grown on more than 6 million ha of land mostly under the rainfed conditions during the winter season. *Brassica juncea* oil has 50-60% erucic acid, 10-15% oleic acid, 10-15% linoleic acid and 14-16% linolenic acid. High levels of erucic acid are associated with health problems (Beare et al., 1963). Oleic and linoleic acids are considered to be neutral fatty acids and linolenic acid is undesirable because its three double-bond structure pre-disposes it to oxidation, resulting in off-flavors and reduced shelf life (Galliard, 1980). Therefore, producing rapeseed oil with high oleic acid, low PUFA and very low or zero erucic acid is the primary goal of rapeseed breeding (Burton et al., 2004; Hardin-Fanning, 2008). Oleic acid is the substrate for both linoleic acid and erucic acid biosynthesis. Oleate desaturase is involved in biosynthesis of linoleic acid, whereas fatty acid elongase catalyzes the biosynthesis of erucic acid. A simplified biosynthetic pathway for major fatty acids (modified from Downey and Rakow, 1987) is shown in Fig1. The information available on the regulation of fatty acid biosynthesis in *Brassica* is scanty and it is of great importance to increase our understanding of the regulation of *fad2* gene expression. In this paper, we report the isolation of a novel oleate desaturase gene (*fad2)* from *B.juncea*. Expression studies showed that *fad2* gene is constitutively expressed in all the tissues and developmentally regulated. Possible role of growth temperature on the *fad2* gene expression and fatty acid profile of seed oil and also differential expression of *fad2* gene from high and low erucic acid *B. juncea* genotypes are discussed.

Table 1. Oligo nucleotide sequences used in the isolation and expression analysis of *fad2* gene from *B. juncea.*

Reactions	Primers	Sequences
Isolation of *Bjfad2* gene	BcaF	F 5'- AGAACCAGAGAGATTCATTACC-3'
	BcaR	R 5'- AGACACTAACTTCCAACATCAC-3'
Southern blot analysis and expression analysis of *Bjfad2* gene using RT-PCR and qPCR	QRTF2	F 5'-CCTTCAGCGACTACCAGTG-3'
	QRTR2	R 5'-GTACCACTTGATGTCTGAC-3'
Internal control for all the RT- PCR and qPCR reactions	ActF	F 5'- CTCACGCTATCCTCCGTCTC-3'
	ActR	R 5'-TTCTCCACCGAAGAACTGCT-3'

F-Forward primer, R-Reverse primer

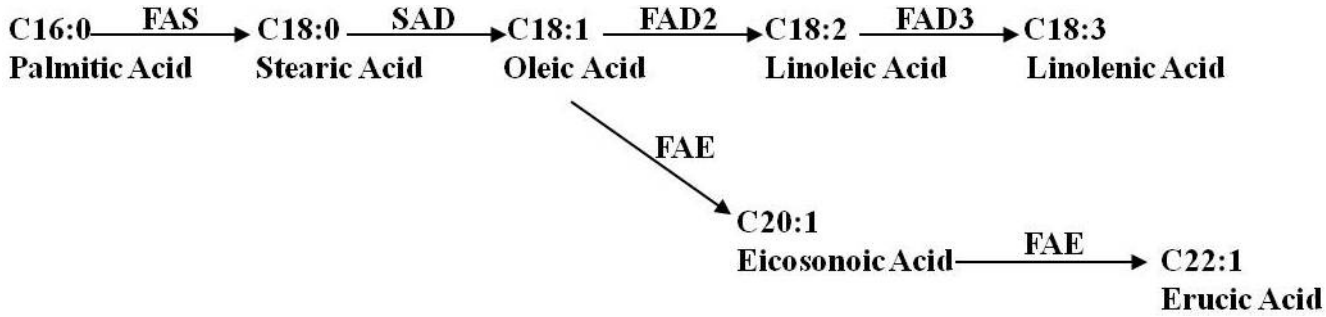

Fig 1. A simplified biosynthetic pathway of poly unsaturated fatty acids (PUFA) in plants.

Results and Discussion

Cloning of Bjfad2 gene

Two gene specific primers were designed from the comparison of known plant *fad2* gene sequences available in NCBI database (Table 1) and these primers were used in PCR amplification reaction. The analysis of the amplified product on 0.8% agarose gel electrophoresis showed an intact band of 1.45 kb (Fig. 2).The amplified product was found to contain 1,445 nucleotides. The open reading frame (ORF) of isolated *Bjfad2* cDNA sequence codes for putative protein having 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 of 147 bp and 3' UTR of 143 bp (Fig. 3). The sequences flanking the methionine start codon in all the *fad2* genes were highly conserved AAACATGGG**.** However, isolated *Bjfad2* gene in the present study showed AAACATGAG. Replacement of single nucleotide (G with A) results in the change of amino acid sequence (Glycine to Serine) of BjFAD2 protein. BLAST N, BLAST X and BLAST P analysis of isolated cDNA sequence showed 77.69% and 95.09% similarity to already reported *Bjfad2-1* gene (X91139) from *B. juncea* by Singh et al., (1995) both at nucleotide and amino acid level respectively. These results clearly revealed that isolated gene is novel homologue of *fad2* gene from *B .juncea*. Sequence data of *Bjfad2* has been deposited at Gen Bank under accession number EF639848.

Sequence analysis of Bjfad2 gene

The ClustalW analysis of deduced amino acid sequence of *Bjfad2* in comparison with that of already reported *Bjfad2-1* gene (X91139) from *B. juncea* and other plant desaturases from *B. rapa, B. napus, B. carinata* and *Arabidopsis thaliana* confirmed that the isolated *Bjfad2* gene is identical to all the above plant desaturases. However, analysis of deduced amino acid sequences from the two *fad2* genes of *B. juncea* showed that isolated *Bjfad2* gene is different from that of *Bjfad2-1* gene (X91139) isolated by Singh et al., (1995) for 19 amino acids at different positions of the protein (Table 2).This confirms that the *Bjfad2* gene isolated in the present study is a different *fad2* homologue, although most of the sequences in the conserved domains are identical. This may be due to the allelic differences in the *fad2* gene which represent the complexity of tetraploid *Brassica* genome. There were nine histidines among the conserved amino acids in the *Bjfad2* sequence, which have been shown to be essential for desaturase activity (Shanklin et al., 1994). These invariant residues are arranged in three histidine boxes (HEC/NGHH, HFEHH and HVAHH) with conserved spaces between them (Fig. 4); the feature is characteristic of all membrane-bound desaturases. Analysis of *Bjfad2* for functional conserved domain by using CDART (conserved domain retrieval tool) showed putative di-iron ligands in the predicted protein (Fig. 5). Histidine boxes are presumed to comprise the catalytic centre of the enzyme, shown essential for desaturase activity and 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 and these proteins probably use a common reactive center. 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). To elucidate the phylogenetic relationships of the *Bjfad2* with the known *Bjfad2-1* gene (X91139) from *B. juncea* and other plant microsomal (*fad2*) and plastidial (*fad6*) oleate desaturases, all of their deduced amino acid sequences were aligned and N-J tree was constructed (Fig. 6).

Table 2. Comparative analysis of deduced amino acid sequence of *Bjfad2* homologue (EF639848) with the already reported *fad2* gene (X91139) from *B. juncea.* Position and type of amino acids differ between the two *fad2* genes from *B. juncea* are tabulated below.

Sl No	Amino acid position	Name of the Amino acid in *Bjfad2-1* (X91139)	Name of the Amino acid in *Bjfad2* (EF639848)
1	2	Glycine (G)	Serine (S)
2	12	Serine (S)	Proline (P)
3	13	Proline (P)	Serine (S)
4	21	Leucine (L)	Isoleucine (I)
5	62	Valine (V)	Isoleucine (I)
6	84	Valine (V)	Phenyl alanine (F)
7	95	Valine (V)	Cysteine (C)
8	155	Glutamic acid (E)	Glycine (G)
9	193	Tyrosine (Y)	Cysteine (C)
10	194	Tryptopan (W)	Leucine (L)
11	204	Proline (P)	Asparagine (D)
12	205	Glutamic acid (E)	Glycine (G)
13	228	Valine (V)	Isoleucine (I)
14	246	Alanine (A)	Valine (V)
15	255	Leucine (L)	Phenyl alanine (F)
16	265	Alanine (A)	Glycine (G)
17	333	Valine (V)	Alanine (A)
18	343	Asparagine (D)	Glutamic acid (E)
19	352	Tryptopan (W)	Valine (V)

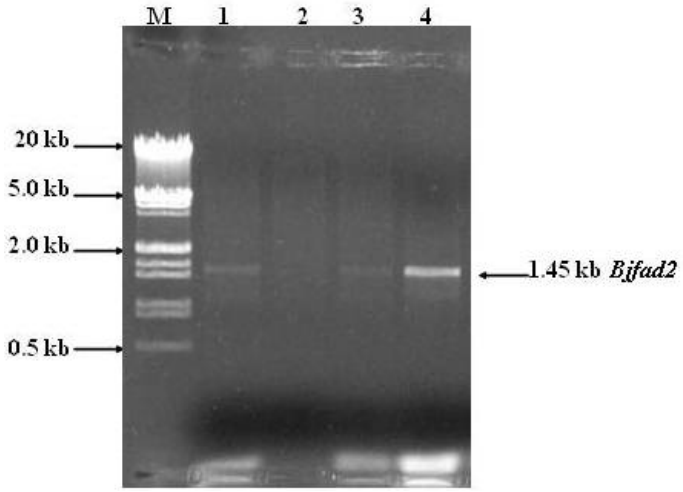

Fig 2. RT-PCR amplification of *Bjfad2* gene from developing seeds of *B. juncea*. Lane M-Marker (*Eco*RI+*Hind*III digested DNA ladder), lane 1-4-different melting temperatures (Tm) for *Bjfad2* gene specific primers (1-51^{0}C, 2-52^{0}C, 3-53^{0}C and 4-54^{0}C). Arrows indicate the molecular size of the marker and gene.

Based on their homology, these plant delta 12 desaturases were classified into three major branches, namely, housekeeping-type *fad2*, *fad6* and seed-type *fad2*. Both the *fad2* genes of *B. juncea* [*Bjfad2-1* (X91139) and *Bjfad2* (EF639848)] were positioned in a subgroup with FAD2 enzymes that exhibit a housekeeping pattern of expression which was similar to the earlier reports in olive (Hernandez et al., 2005) and soybean (Li et al., 2007). However, *Bjfad2* gene isolated from *B. juncea* was more related to *fad2* gene of *B. rapa*, thereby forming a same clade whereas, already known *Bjfad2-1* gene (X91139) from *B. juncea* was shown to be more related with *fad2* gene of *B. Carinata*. This result also confirms that the isolated *Bjfad2* is a different homologue of already known *fad2* gene of *B. juncea.* Unlike the reports by Li et al., (2007), the position of seed-type *fad2* was closer to housekeeping-type *fad2* than that of the *fad6*. 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. In the hydropathy plots of the *Bjfad2* amino acid sequence generated by the method of Kyte and Doolittle (1982), BjFAD2 protein showed five prominent hydrophobic peaks at a position of 55aa-77aa, 82aa-104aa, 119aa-136aa, 176aa-198aa and 224aa-275aa (Fig. 7a). Interestingly, predicted transmembrane domains using TMHMM server showed that already reported *Bjfad2-1*(X91139) have only four transmembrane domains at a position of 55aa-77aa,82aa-104aa, 176aa-198aa, and 253aa-275aa (Fig. 7b), whereas *Bjfad2* gene isolated from present study have five

Table 3. Fatty acid composition of developing seeds from *B. juncea* (cv Pusa Bold) treated at different temperature and incubation periods.

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	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
5%	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	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
1%	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.

Values are means ± SE of three determinations. T -Treatment (T_1-10^0C, T_2-21^0C & T_3-32^0C), I-Incubation time (24 hr and 72 hr), NS -Non Significant

*Unidentified fatty acid among the fatty acid standards (Caproic acid, Caprylic acid, Lauric aicd, Myristic aicd, Palmitic acid, Oleic acid, Linoleic acid, Linolenic acid, Arachidonic acid and Erucic acid) used in the study.

Table 4. Fatty acid composition of developing seeds from *B. juncea* genotypes having high (Pusa Bold) and low erucic acid (LES-39 and LES 1-27) content.

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	-	-

Values are means ± SE of three determinations. NS -Non Significant

*Unidentified fatty acid among the fatty acid standards (Caproic acid, Caprylic acid, Lauric aicd, Myristic aicd, Palmitic acid, Oleic acid, Linoleic acid, Linolenic acid, Arachidonic acid and Erucic acid) used in the study.

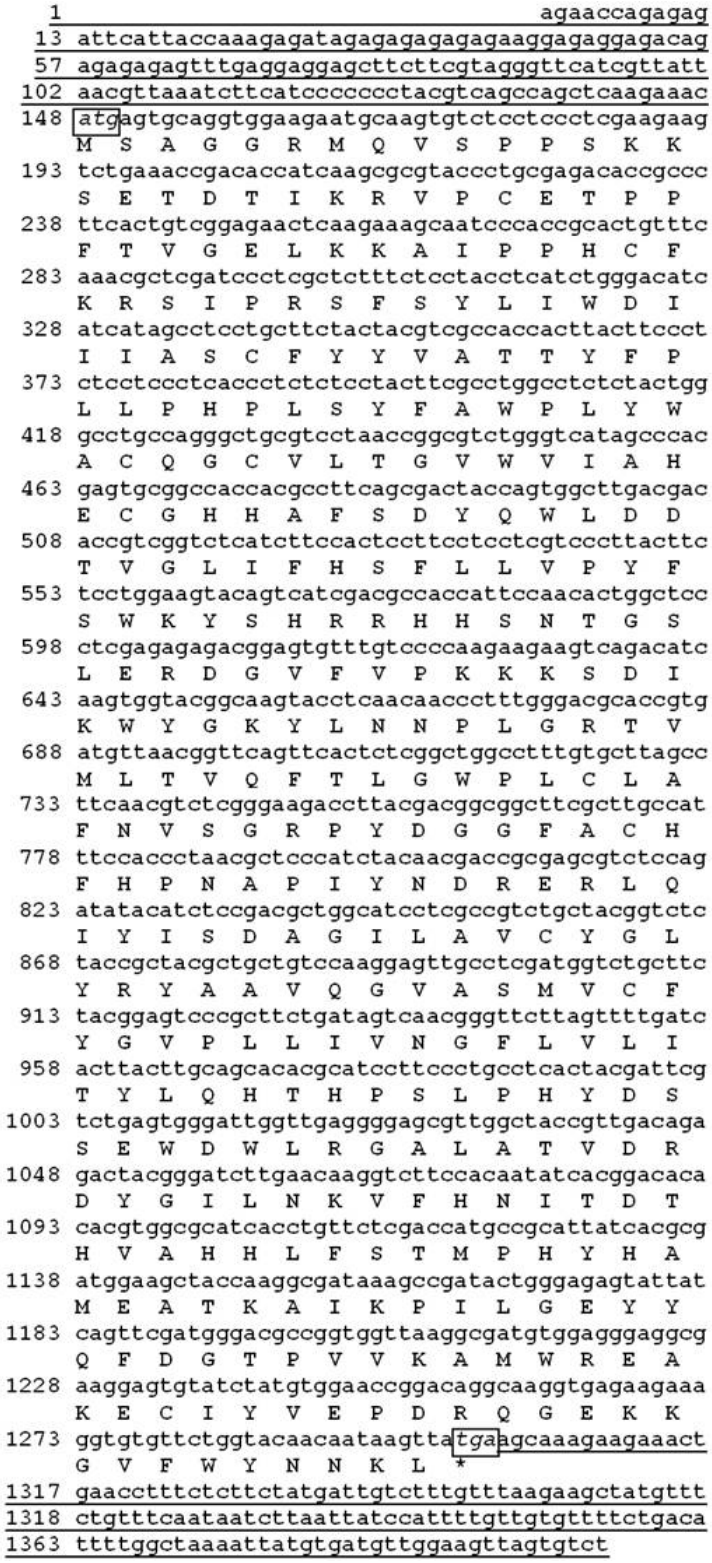

Fig 3. *Bjfad2* gene with corresponding deduced amino acid sequence from *B. juncea.* Start (ATG) and stop (TGA) codons are boxed in italics. 5'UTR and 3'UTR regions are underlined.

transmembrane domains at a position of 55aa-77aa,82aa-104aa,176aa-198aa,224aa-246aa and 253aa-275aa (Fig. 7c) in its protein. This is one of the important observations, where we confirmed that isolated *Bjfad2* is novel and totally a different *fad2* homologue due to the differences in the conserved transmembrane domains between the two *fad2* genes of *B. juncea*. The domains present in the *Bjfad2,* that apparently span the membrane five times are different from all acyl lipid and acyl-CoA desaturases that apparently span the membrane four times, 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; Hernandez et al., 2005). Putative BjFAD2 was also analyzed for its intracellular 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. It showed 95% and 90% identity to putative protein of already reported *Bjfad2-1*(X91139) of *B. juncea* and *Arabidopsis* FAD2 protein for ER localization respectively. It lacked 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 were specific C- terminal amino acids residues (-YNNKL) predicted to function as part of the different putative ER retrieval motifs in FAD2 (Fig. 4), which has been reported to be necessary and sufficient for maintaining localization of the enzymes in the ER (McCartney et al*.,* 2004; Hernandez et al*.,* 2005; Li et al., 2007).

Genomic organization of B. juncea microsomal oleate desaturase gene

The occurrence of *fad2* gene families has been frequently reported in plants (Heppard et al*.,* 1996; Martinez-Rivas et al*.,* 2001). Genomic Southern blot analysis using *Bjfad2* gene specific probe confirmed that there are atleast two copies of *fad2* gene present in *B. juncea*, which was consistent with the tetraploid nature of the *B. juncea* genome. While hybridizing with *Bjfad*2 gene specific probe, two bands with genomic DNA digested with *Eco*RI, three bands each with *Bam*HI and *Pst*I, and single band with *Hin*dIII were observed (Fig. 8b). Based on these studies, we also isolated and cloned full length genomic sequences of three *Bjfad2* gene homologues through sub-genomic library and colony PCR approach (Accession numbers-FJ696650, FJ696651 and FJ696652) (data not shown). But all these three isoforms were found to be different from isolated *Bjfad2* gene in the present study. This confirmed that, there may be more than two *fad2* isoforms exist in *B. juncea* genome. Previous studies of Southern analysis of soybean genomic DNA with *gmfad2-1* and *gmfad2-2* specific sequence fragments indicated atleast two copies of each of these genes in the tetraploid soybean (Heppard et al., 1996). Similar results were reported by Hernandez et al*.* (2005) in *Olea europaea.* Microsomal omega–6 desaturase gene family from *B. napus* was 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 *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 herbacium* and *Gossypium raimondii*) and presumably this is 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).

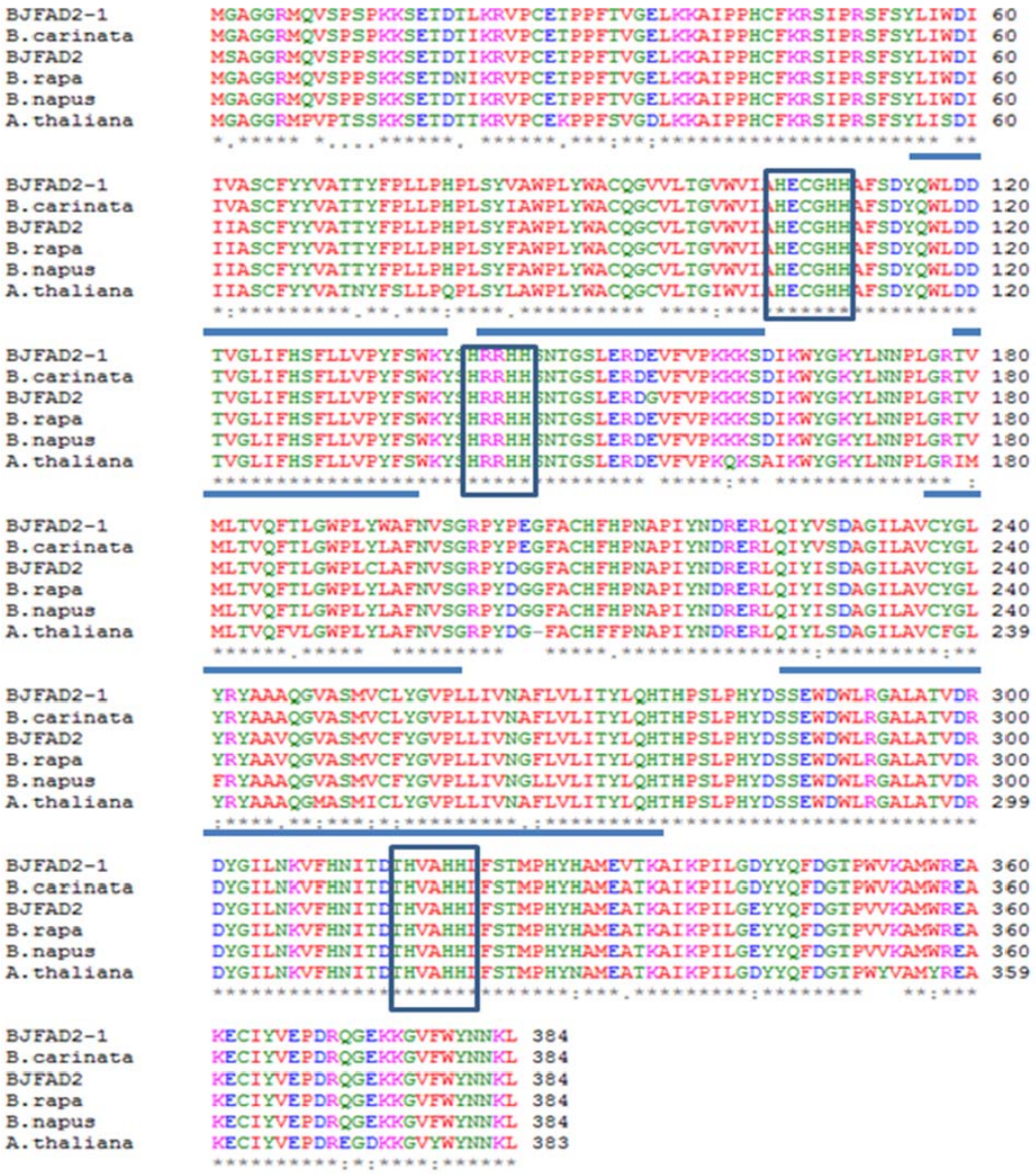

Fig 4. Deduced amino acid sequence alignment of *Bjfad2* gene with already known *B. juncea fad2* (X91139) designated in the figure as BJFAD2-1, *B. rapa* (AJ459107), *B. napus* (AF243045), *B. carinata* (AF124360) and *Arabidopsis thaliana* (L26296) FAD2 homologues. Boxes represent three histidine motifs (HXXXHH, HXXHH and HXXHH); five hydrophobic regions are underlined.

Developmental and tissue specific expression of fad2 gene

Expression of *fad2* gene was induced in early stages of seed development (15 DAF), peaked in mid maturation stage (30 DAF) and declined thereafter, as seeds matured (45 DAF) (Fig. 9a). In conjunction with the previous reports from soybean, cotton and linseed, the expression leve1 of the *fad2-2* gene was higher than that of the *fad2-1* 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 thereafter 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 (Heppard et al., 1996; Tiwari et al., 2004; Liu et al., 1999; Fofana et al., 2006). The expression pattern observed in different tissues confirmed that the isolated *Bjfad 2* gene is novel and constitutively expressed in both, vegetative tissues and developing seeds, but the level of expression varied with the tissues where highest transcript expression was observed in developing seeds as compared to other tissues (Fig. 9b). There was no information available on the expression pattern of the already reported *Bjfad2-1* gene **(**X91139) in different tissues. Viewed with the previously reported results (Heppard et al., 1996; Tang et al., 2005), there were four different *fad2* encoding microsomal oleate deasaturases in soybean: *fad2-1* seemed to be expressed primarily in developing seeds, while *fad2-2* expressed in both vegetative tissues and developing seeds. Hence, *fad2-1* and *fad2-2* genes not only differ in structure but also have distinct expression patterns. The difference in developmental and tissue-specific expression of the two genes probably suggest a non-overlapping role for the genes

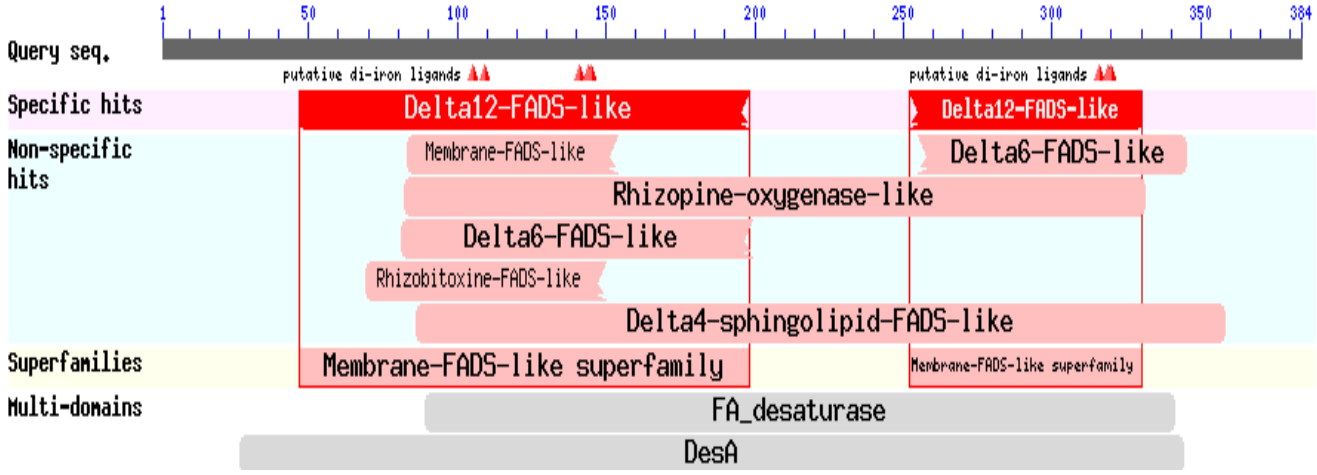

Fig 5. Functional conserved domain of putative BjFAD2 protein showing multi domain nature of all fatty acid desaturases. CDART (conserved domain retrieval tool) was used to derive conserved domain provided by NCBI, USA.

in desaturation of membrane and storage lipids in developing seeds and vegetative tissues (Heppard et al., 1996).

Temperature dependent regulation of Bjfad2 gene expression and seed fatty acid content

Fatty acid composition of lipids varies with the environmental temperature and level of unsaturation of membrane, as well as seed-storage lipids, has been shown to be inversely correlated with the growth temperature (Rennie and Tanner, 1989; Heppard et al., 1996). With a view to find out whether the increase of PUFA levels in seeds at the low temperature was related to the enhanced *fad*2 gene expression, we determined the level of transcripts by RT-PCR and Real-Time PCR analysis. Results from the RT-PCR revealed that the expression of *Bjfad2* gene increased at lower temperature treatments with incubation time as compared to the higher temperature treatments where transcript level of *fad*2 gene was drastically reduced (Fig. 9c). Real-Time PCR analysis confirmed that transcript level of *Bjfad2* gene increased by one fold at lower temperature and decreased by three fold at higher temperature treatment, as compared to those at normal temperature regime (Fig. 10a & 10b). Fatty acid composition of seeds of the plants grown at different temperatures was analysed by gas chromatography (Table 3). Plants incubated at lower temperature (10^{o}C) for a period of 24 hr showed significant increase in linoleic acid content, while with increased incubation period for 72 hr, significant decrease in oleic acid and increase in both linoleic and linolenic acids content was observed. Although plants grown at normal (21^{0}C) and higher (32^{0}C) temperature regimes showed significant increase in linoleic acid content, no reduction in oleic acid content was observed with increased incubation period to 72 hr. Interestingly, plants grown at lower (10^{0}C) and normal (21^{0}C) temperatures showed significant reduction in erucic acid content with increased incubation period from 24 to 72 hr, whereas at higher temperature (32^{0}C), showed reverse trend with significant increase in erucic acid content of seeds with incubation period. From the present study, it is confirmed that although there were significant differences in fatty acid composition among all the treatments, in general, the proportion of desaturation was very high at lower temperature as compared to higher temperature treatments revealing the high expression of fatty acid desaturases (FADs). Decreased proportion of erucic acid content of seeds at lower temperature has indicated the high expression of FADs as compared to fatty acid elongases (FAEs) (Table 3 & Fig.10c). Studies from various crops have also shown that the fatty acid composition is greatly influenced by growth temperature and unsaturation level increases in response to low growth temperature (Badea and Basu, 2009). 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 may confer greater membrane fluidity, which may allow bacteria and plants to maintain membrane function under lower growth temperature conditions (Neidleman, 1987; Thompson,1993). As previously described by Chang et al., (2001), a 10^{0}C exposure of the chilling sensitive Mungbean (*Vigna radiata*) protected it from injuries caused when plants were exposed to 4^{0}C. A recent study on *fad*2-3 from soybean (Li et al., 2007) also indicated increase in di-unsaturated fatty acid composition under cold stress due to post-transcriptional/post-translational modifications on the enzyme rather than increase in mRNA levels (Heppard et al., 1996; Falcone et al., 2004; Tang et al., 2005). But in cotton, *fad*2-3 and *fad*2-4 mRNA levels increased as temperatures were lowered far below the lowest germination permissive temperature (Liu et al., 1999).

Differential expression of fad2 gene

Differential expression of *fad2* gene in developing seeds of *B. juncea* varying in erucic acid content was analyzed using Real-Time PCR. Results from this study showed that expression of *Bjfad2* gene was two and four fold higher in LES-39 and LES 1-27 (low erucic acid lines) respectively, compared to a high erucic acid line (Pusa Bold) (Fig. 11a & 11b). These results suggested that expression of *fad2* gene depends on availability of oleic acid, which serve as the substrate for both linoleic acid and erucic acid biosynthesis. High erucic acid lines showed lower expression of *fad2* gene due to the diversion of oleic acid pools to erucic acid synthesis by the activity of FAE enzyme. It can be speculated that the expression of *fad*2 and *fae* genes are inter-regulated depending on the availability and source of oleic acid pool in the cell but detailed study is required to confirm. Oleic acid content in high erucic acid genotype (Pusa Bold) was found to be significantly low (15.94%) as compared to low erucic

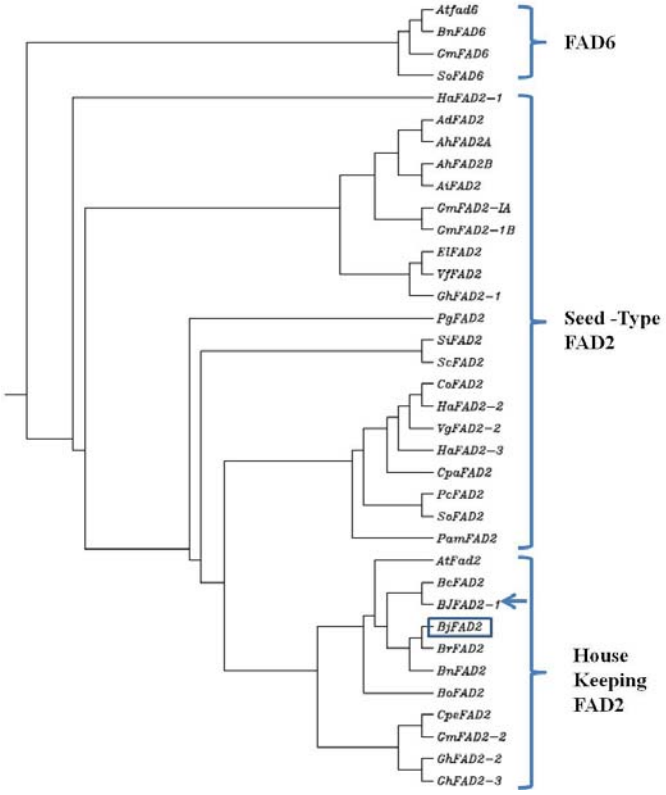

Fig 6. Phylogenetic relationships between deduced amino acid sequences from BjFAD2 and other plant microsomal (FAD2) or plastidial (FAD6) oleate desaturases. Position of the *B. juncea* FAD2 genes (BJFAD2-1, X91139; BjFAD2, EF639848) is marked with arrow and box respectively. The enzymes and Gen Bank 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 Neighbor-Joining algorithm.

acid genotypes (LES-39 and LES 1-27) having 38.47% and 36.75% respectively. This may be due to the low expression of *fad2* gene in Pusa Bold resulting in significantly increased accumulation of erucic acid (25.48%). Linoleic acid content in LES-39 (34.64%) and LES 1-27 (33.81%) lines was also found to be significantly high as compared to Pusa Bold (21.31%) whereas erucic acid content in these genotypes was 0.94% and 0.87% respectively, which was significantly very low as compared to Pusa Bold (Table 4 & Fig. 11c). These results when viewed in conjunction with the differential expression analysis of *Bjfad*2 gene through Real-Time PCR confirm that expression of *fad*2 gene varies among the high and low erucic *B. juncea* genotypes. However, detailed investigations on regulation of *fad*2 and *fae* genes in high and low erucic *B. juncea* genotypes are needed. Expression of *fad*2 and *fae* genes depends on the oleic acid pools in the cell thereby controlling the regulation of desaturation and elongation reactions in the seed oils. Previous studies have also shown that expression of *fae* gene can be increased by suppressing the *fad*2 gene expression. Over-expression of the *Crambe abyssinica fae* gene in *B. carinata* resulted in a substantial increase in the proportion of erucic acid in seeds compared to the wild type control (Mietkiewska et al., 2007). By altering the level of *fad*2 gene expression using antisense and co-suppression approaches, it was 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; Mietiewska et al., 2008).

Materials and methods

Plant material and growth conditions

Brassica juncea (cv Pusa Bold) was grown at 21/21^{0}C with light/dark cycle 12/12 hr in glasshouse under standard conditions (flowers were tagged on the day of anthesis) at National Phytotron Facilities, IARI, New Delhi. Developing seeds were harvested at 15, 30 and 45 DAF, chilled in liquid nitrogen and stored at -70^{0}C.

RNA extraction and RT-PCR reaction

For all the experiments in the present study, RNA was isolated using TRI-Reagent® from Sigma (USA), as described by the manufacturer. RNA concentration was determined spectrophotometrically and verified by ethidium bromide staining of agarose gel. In order to avoid DNA contamination, RNA was treated with RNAse free DNAse. One micro gram of RNA was used as template for the first strand cDNA sythesis using first strand cDNA synthesis system from Fermentas Inc. (USA) and oligo (dT) as primer according to manufacturer's protocol. All the PCR experiments were carried out using PCR mastermix with 0.5 µg of cDNA as template.

Cloning and sequencing of oleate desaturase gene

Two gene specific primers, BcaF/BcaR (Table 1) were designed from the known plant *fad2* nucleotide sequences available in the NCBI database (Accession numbers-AJ459108 and HM189213). The primers for PCR were custom synthesized by Integrated DNA Technologies Inc, USA.

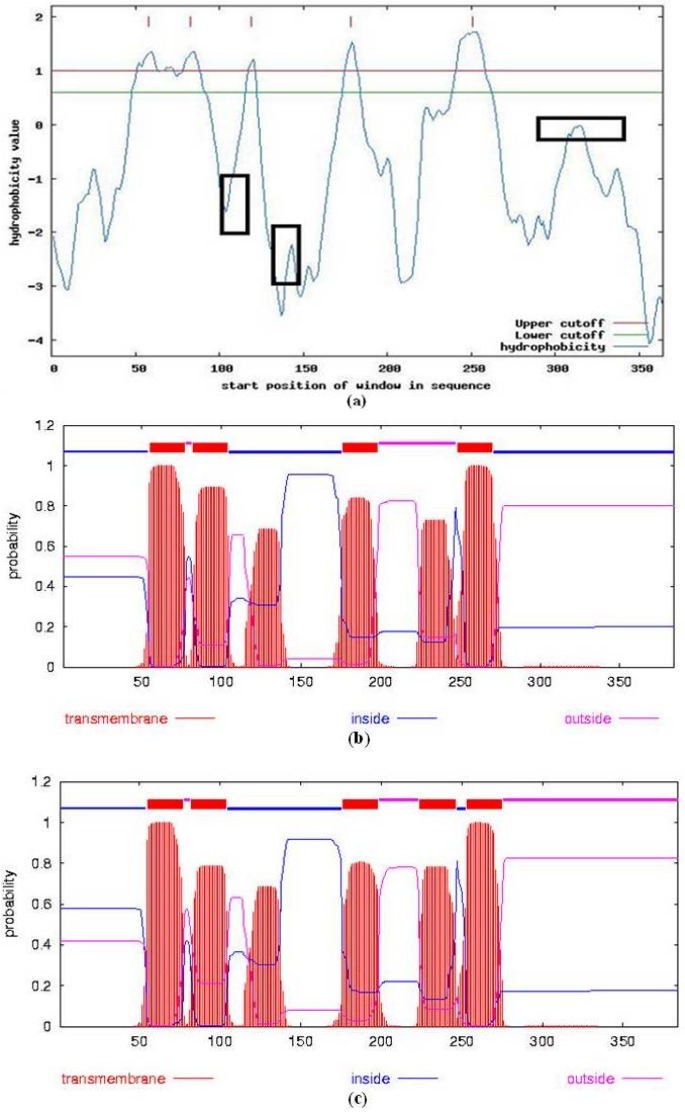

Fig 7. (a) Hydropathy plot of *Bjfad2*.Open boxes represent positions of conserved Histidine motifs. (b) Predicted transmebrane domains in the putative proteins of already reported *Bjfad2-1* gene (X91139) and (c) isolated *Bjfad2* gene in the present study from *B. juncea*. Red colour boxes represent the putative transmembrane domains. Blue and Pink colour lines indicate the inner and outer membranes of the cell respectively.

This pair of primers together with an aliquot of cDNA (0.5 µg) synthesized with RNA isolated from 30 DAF seeds were used in standard PCR amplification protocol with Taq DNA polymerase (Fermentas Inc. USA). Amplified product was then sub cloned into the pGEMT Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* (strain DH5α) cells. DH5α cells transformed with recombinant plasmids were selected based on antibiotic resistance as well as α-complementation method. Ampicillin resistant putative recombinants were selected for further analysis. Plasmids were isolated from the confirmed colonies and restriction analysis was carried out by using *Eco*RI restriction enzyme flanking the cloning site of the vector pGEMT Easy to confirm the presence of cloned insert cDNA. Cloned insert cDNA in the pGEMT Easy vector was sequenced by dideoxy chain termination method (Sanger et al., 1977) using T7 and SP6 primers making use of the automated DNA sequencing

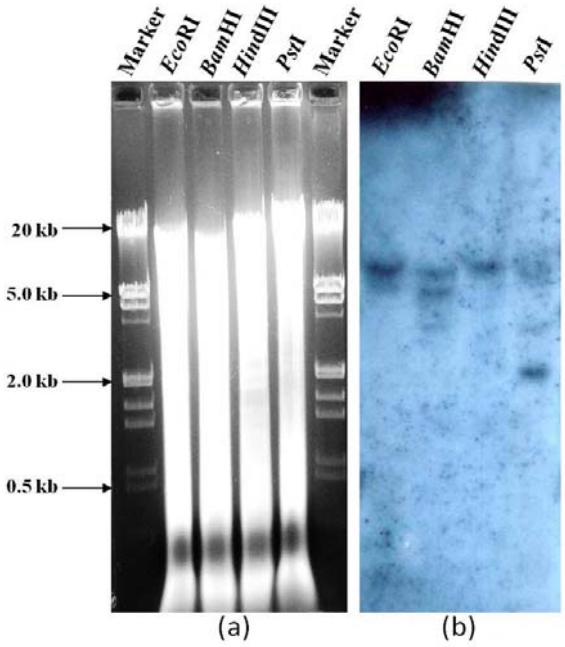

Fig 8. Southern blot analysis. (a) Restriction pattern of *B. juncea* genomic DNA (5µg) digested with *Eco*RI, *Bam*HI, *Hin*dIII and *Pst*I restriction enzymes and hybridized with *Bjfad2* gene specific probe. Left arrows indicate molecular size of the marker (*Eco*RI+*Hin*dIII digested DNA ladder). (b) Pattern of genome organization of *Bjfad2* gene in *B. juncea.*

facility at the University of Delhi, South Campus, India.

Bioinformatics tools used for sequence analyses

Nucleotide sequence of cDNA clone and the corresponding amino acid sequence was deduced by the NCBI BLAST program.Transmembrane regions were predicted by TMHMM server ver.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/) algorithm. Multiple sequence alignment was performed with Clustalw using default parameters. A phylogenetic tree was constructed using the neighbour-joining method and protdist algorithm in the PHYLIP package (version 3.63). Analysis of 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).

Genomic Southern blot analysis

Brassica juncea genomic DNA (5µg) was digested with restriction enzymes (*Eco*RI, *Hin*dIII, *Bam*HI *and Pst*I) (Fermentas Inc.(USA) and electrophoresed through a 0.7% agarose gel. The gel was soaked in 0.25 M HCl for 10 min, in 0.5 M NaOH and 1.5 M NaCl for 1 hr, in 1.0 M Tris HCl (pH 8.0) and 1.5 M NaCl for 1 hr and finally blotted on to a nylon membrane and probed with (α-$P^{(32)}$) dCTP labelled *Bjfad2* gene specific DNA fragment. Radio labelled biomolecules was obtained from BRIT of Bhabha Atomic Research Centre,

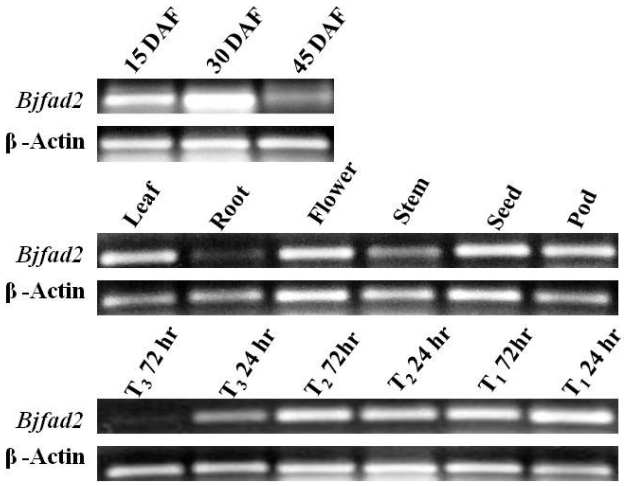

Fig 9. Expression profile of *Bjfad2* from *B.juncea* by RT-PCR. (a) Transcript expression of *Bjfad2* in three developing stages of *B. juncea* seeds (15, 30 and 45 DAF). (b) *Bjfad2* transcript level analysis from different tissues like leaf, root, flower, stem, seed and pod for spatial expression; flower, seed and pods were sampled at 30 DAF. (c) Growth temperature effects on expression of *fad2* at varying incubation times in developing seeds of *B. juncea*. Total RNA was extracted from indicated treatments (T_1-10^0C, T_2-21^0C and T_3-32^0C). Samples were collected after 24 hr and 72 hr incubation time. β-actin was used as a reference gene for all the experiments.

(India). The *Bjfad2* gene specific probe with a size of 160 bp was obtained by PCR amplification with the QRTF2/QRTR2 primers (Table 1). Hybridization was performed in 0.5 M Na_2HPO_4 (pH 7.2), 1mM Na_2EDTA and 7% SDS overnight at 65 ^{0}C.The filter was washed twice in 40 mM Na_2HPO_4 (pH 7.2), 1mM Na_2EDTA and 1% SDS for 5 min at 50 ^{0}C (Sambrook et al.,1989).

Expression analysis by RT- PCR and qPCR

RNA was isolated from seeds collected 15 DAF, 30 DAF, 45 DAF and different tissues like leaf, root, flower, stem, seed and pod. RT-PCR was performed for all the treatments as described earlier to study the developmental and tissue specific expression of *Bjfad2*. To study the effect of temperature on *fad2* gene expression in developing seeds of *B. juncea,* plants were given prolonged heat shock in growth chambers at three different temperatures, namely 10^0C (low), 21^0C (normal) and 32^0C (high) for 24-72 hr with light/dark cycle of 12/12 hr in National Phytotron Facilities, Indian Agricultural research institute (IARI), New Delhi. Seeds were collected from all three treatments after 24 and 72 hr. RNA was isolated from each treatment as described earlier and RT-PCR was performed. Real-Time PCR was performed only in treatments which were incubated for 72 hr. For the study of differential expression of *Bjfad2* gene, RNA was isolated from developing seeds (30 DAF) of different *B. juncea* genotypes having high (Pusa Bold) and low (LES 1-27 and LES -39) erucic acid content grown at IARI farm by Division of Genetics. Real -Time PCR was performed for all the treatments. qRT-PCR analysis was performed using QuantiTect^R SYBR^R Green PCR kit from Qiagen (USA) and the Stratagene Mx3000P thermocycler according to manufacturer's protocol. PCR was performed with 0.5 µg of cDNA synthesized from all the treatments using

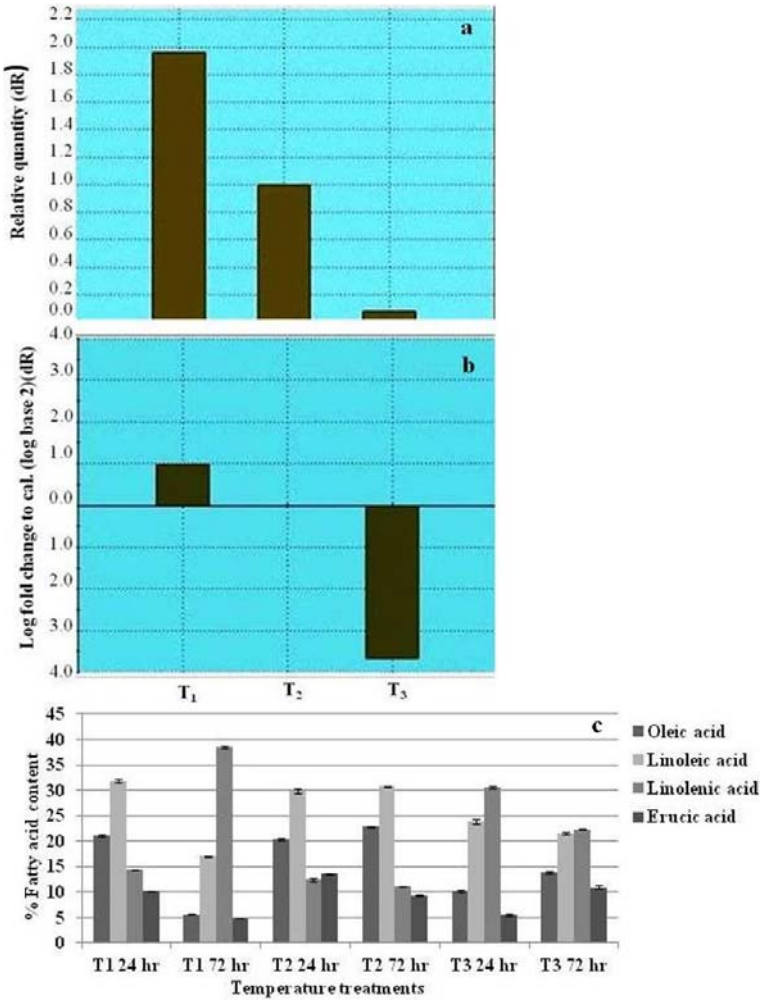

Fig 10. Real-Time PCR analysis of *Bjfad2* gene expression under different temperature. Plants were incubated in growth chamber at different temperatures (T_1-10^0C, T_2-21^0C and T_3-32^0C) for 72hrs. (a) Relative quantity graph. (b) Log fold change of expression of *Bjfad2* among the treatments. (c) Changes in fatty acid content (oleic acid, linoleic acid, linolenic acid and erucic acid) in developing seeds of *B. juncea* at different temperatures (T_1-10^0C, T_2-21^0C and T_3-32^0C) with different incubation time (24 hr and 72 hr). Error bars indicate ±SEM (standard error of mean) from three independent experiments.

QRTF2/QRTR2 gene specific primers. Real-Time PCR conditions were standardized with an initial activation step at 95^0C for 10 min, followed by 40 cycles, each comprising 30s denaturation at 95^0C, one min annealing at 57^0C and 30s extension at 72^0C, with final extension at 72^0C for 30s. For all the RT-PCR and Real-Time PCR reactions, *Arabidopsis* β-actin (ActF/ActR) gene was used as internal control.

Fatty acid analysis

Fatty acid analysis was performed by using Perkin Elmer Claurus 500, a gas liquid chromatography fitted with mega bore column packed with stationary phase (30 meter long and 0.53 mµ, packed with OV-101, a polymer of methyl silicone) and Flame ionization detector (FID). It was analysed under the column temperature of 150^0C-270^0C, injector and.

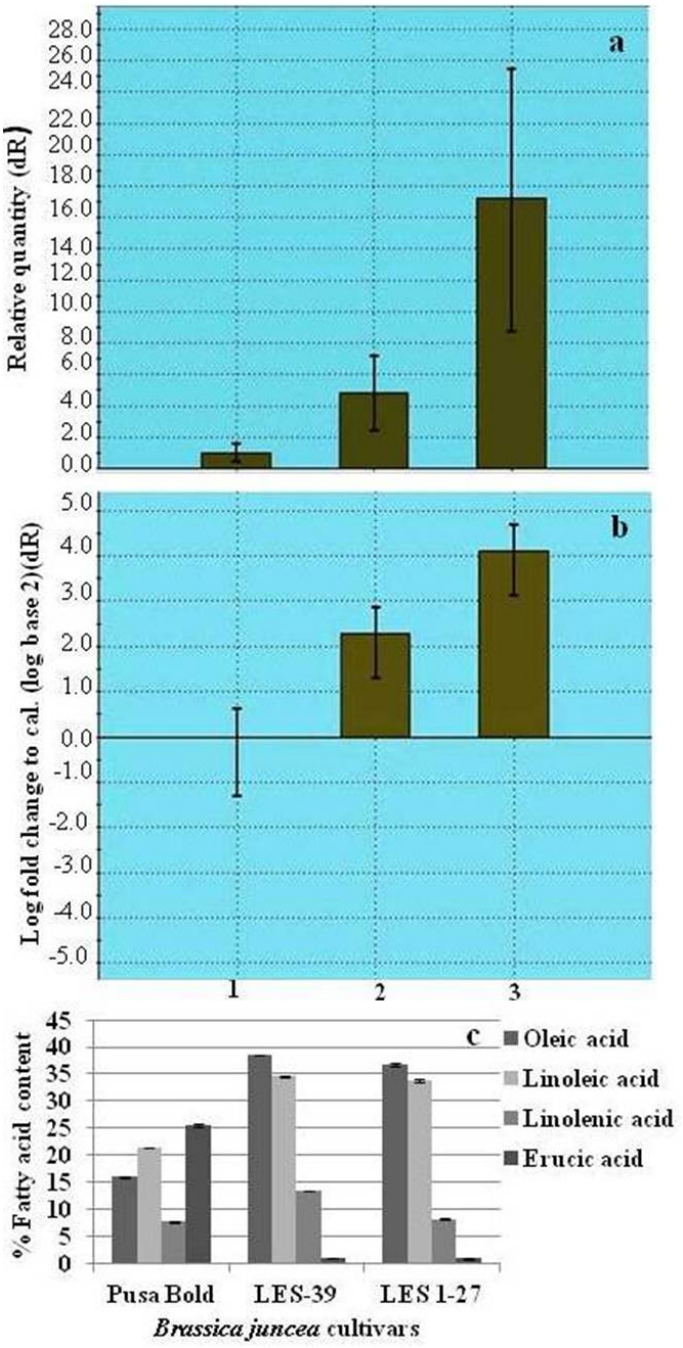

Fig 11. Differential expression of *Bjfad2* gene in high and low erucic acid genotypes of *B. juncea.* (1-Pusa Bold, 2-LES-39 and 3-LES 1-27). (a) Relative quantity graph (b) Log fold change of *Bjfad2* expression among the genotypes. (c) Fatty acid content (oleic acid, linoleic acid, linolenic acid and erucic acid) of different *B. juncea* genotypes having high (Pusa Bold) and low (LES-39 and LES 1-27) erucic acid content. Error bars indicate ±SEM from three independent experiments.

detector temperature of 250 ^{0}C. Fatty acid methyl esters were prepared as per the method described by Vasudev et al. (2008). One microlitre 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 comparison with known standards. Percent fatty acid composition was determined by measuring the area under each peak; the values were computed as means from three independent experiments

Statistical analysis

Data of percent fatty acid content in all the experiments were analyzed using two-way analysis of variance (ANOVA). The differences within and between the treatments at different temperatures and also between high and low erucic acid genotypes were analyzed using Duncan's multiple range test (DMRT) at 5% and 1% level.

Conclusions

As Oleate desaturases regulate the membrane properties and the synthesis of storage lipids, the study of molecular and biochemical regulation of this enzyme is important in *Brassica.* The present investigation of *fad2* gene expression from *B. juncea* has opened up new insights for studying the role of *fad2* gene in the improvement of *B. juncea* oil quality. Novel *fad2* gene homologue reported in the present study is constitutively expressed in all the tissues and developmentally regulated during seed oil synthesis. More than two copies of *fad2* gene are predicted representing the allelic diversity in the tetraploid *B. juncea* genome. Up regulation of the gene at lower growth temperatures resulting in higher contents of PUFA in the developing *B. juncea* seeds has been clearly demonstrated. Differential expression of *fad2* gene in low and high erucic acid genotypes has shown the importance of *fad2* gene in regulation of desaturation and elongation reactions of fatty acid biosynthesis during seed oil formation. However, further studies on mechanisms controlling the *Bjfad2* gene expression are needed to understand clearly the regulation of fatty acid biosynthesis in *Brassica* seed oils.

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