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ARTICLE

Exploration of allelic diversity reveals a novel *FAD2* (Oleate desaturase) gene in *Brassica juncea*

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Abstract: *Indian mustard (Brassica juncea) is an important contributor towards edible oil supply in India. Traditional Indian mustard varieties contain high proportion of 18C polyunsaturated fatty acids (linoleic and linolenic acids) and large amounts of long-chain monounsaturated FAs, mainly erucic acid, in seeds. Oleate desaturase (FAD2) regulates the composition of 18C PUFAs in cellular membranes and TAG in seed oil. The present study was conducted to gain insight into the allelic diversity of the FAD2 gene in Indian mustard. Analyses of cloned FAD2 genes of three Indian mustard varieties revealed a novel FAD2 gene that has a longer ORF (1167 bp) owing to insertions and several SNPs across its length that distinguish it from the more prevalent native FAD2 gene. Overall, the Indian mustard varieties possess three FAD2 alleles, but there is limited nucleotide diversity among members of each FAD2 type across varieties, suggesting narrow genetic diversity among the varieties examined.*

Keywords: *FAD2; Indian mustard: allelic diversity*

INTRODUCTION

The composition of seed fatty acids (FAs) varies greatly depending on the plant species. Oil derived from vegetable sources is predominantly used for food-based applications, mainly for cooking purposes; therefore, the oxidative stability of the oil is one of the important properties. With the increase in unsaturation or introduction of double bonds in fatty acyl chains, the corresponding FAs become unstable at higher temperatures; therefore, the oxidative stability of oil is a critical consideration. Oilseed species typically contain five most common FAs, 16:0 (palmitic acid), 18:0 (stearic acid), 18:1 (oleic acid), and 18:3 (linolenic acid), in their seed oils; interestingly, these five FAs are also primary constituents of the bipolar lipid membrane of all plant cells (Kumar et al. 2006) and hence are regarded as usual FAs. In addition to being stored in seeds as TAG (triacylglycerol) molecules, FAs and their derivatives are involved as components of the cellular membrane structure and precursors of signaling molecules that are associated with stress response and plant growth (Harwood 1996, Weber 2002). In contrast to the usual FA-forming constituents of seed TAG in most cultivated oilseed species, the seeds of 300 plant species are reported to store FAs with hydroxy, epoxy or other functional groups or conjugated double bonds (Badami and Patil 1980). Interestingly, these FAs are largely excluded from the lipid bilayer (Gurr 1980), as their altered

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chemical structure has the potential to destabilize the fluidic nature of cellular membranes; hence, they are designated novel or unusual FAs.

The majority of polyunsaturated fatty acids (PUFAs) are synthesized through oleate desaturase, also known as fatty acid desaturase-2 (FAD2), which is a membrane-bound enzyme located in microsomes or the endoplasmic reticulum (ER). Moreover, FAD2 is responsible for more than 90% of PUFA synthesis in nonphotosynthetic tissues such as roots and in the developing seeds of oilseed crops, including *Brassica* (Okuley et al. 1994). Oleic acid (18:1) has one double bond, and by the catalytic action of the FAD2 enzyme, it is converted to linoleic acid (18:2) (two double bonds); by another round of desaturation, catalyzed by the FAD3 enzyme, 18:2 converts into linolenic acid (18:3) (three double bonds) (Browse et al. 1998, Park and Kim 2022). Interestingly, these three 18C unsaturated FAs constitute the majority of the total FAs found in the seed oil of many cultivated oilseed species. Among the three 18C unsaturated fatty acids, 18:3 is the least stable oxidatively and thus is utilized for drying oil. On the other hand, 18:1 is suitable for cooking purposes because it has greater oxidative stability at higher temperatures and is thus less prone to metabolizing into trans-fatty acids that cause various coronary diseases (Browse et al. 1998). In addition to the abovementioned usual FAs, the seed oil of Brassica species also contains significant amounts of long-chain monounsaturated FAs, mainly erucic acid (C22:1), which are synthesized by the FAE1 enzyme (Millar and Kunst 1997, Shi et al. 2017). Erucic acid in *Brassica* seed oil is a nutritionally undesirable constituent and is reported to be a health hazard. Vegetable oil from seeds of *Brassica juncea* (Indian mustard) contributes significantly to human consumption in the form of cooking oil in India. Indian mustard (*Brassica juncea*) constitutes the second largest oilseed crop after soybean, and Indian mustard is positioned as an important source of edible oil in the Indian subcontinent (Jat et al. 2019).

The seed FA composition of Indian mustard primarily includes erucic acid (50-60%), linolenic acid (14-16%), linoleic acid (10-15%), oleic acid (10-15%) and palmitic acid (8-10%) (Suresha et al. 2012). In Indian mustard (*Brassica juncea*) varieties, the seed oleic acid content ranges from 14-15% in Pusa Agrani (Anand et al. 2010) and 12-13% in Pusa Jagannath and 18-19% in Basanti (Chauhan and Kumar 2011). However, *Brassica juncea* seed oil, which is significantly rich in oleic acid (18:1), preferably in the range of 70-80%, along with an optimal balance of linoleic (18:2) and linolenic (18:3) acids and a near-zero level of erucic acid (22:1), will be the most desirable cooking oil commodity due to its balanced nutritional composition (Dar et al. 2017). Moreover, oils derived from vegetable sources rich in oleic acid (18:1) are helpful for reducing cholesterol, suppressing tumor formation, and protecting against inflammatory diseases (Yamaki et al. 2005). The human body is unable to synthesize the essential PUFAs linoleic (18:2) and linolenic acids (18:3) (Guan et al. 2012), and these two 18C PUFAs serve as precursors for long-chain PUFAs, such as EPA, DHA and ARA, which are important for maintaining a healthy body (Damude et al. 2006). There are several reports in which a reduction in PUFA levels in *Brassica juncea* was accomplished through genetic engineering techniques. For example, in the Australian *Brassica juncea* variety, cosuppression of *fad2* resulted in an increase in oleic acid (18:1) content and simultaneous decreases in linoleic (18:2) and linolenic (18:3) acid levels (Stoutjesdijk et al. 2000). Efforts were also made to achieve desirable levels of 18C polyunsaturated fatty acids (FAs), namely, linoleic (18:2) and linolenic (18:3) acids, by antisensing the *fad2* gene driven by a seed-specific promoter (napin) in the zero-erucic Indian mustard variety (Sivaraman et al. 2004). Similarly, breeding successes have been achieved in developing nearly zero (less than 2%) erucic acid (EA) lines through introgression of the *fae1* allele from the ZEM1 mustard variety (Australian near zero erucic acid *B. juncea* variety) in cultivated Indian mustard varieties (Yadava et al. 2014).

FAD2 is a regulatory gene that controls the synthesis and thus overall composition of downstream synthesized 18C PUFAs, viz. 18:2 and 18:3. The two 18C PUFAs, 18:2 (linoleic acid) and 18:3 (linolenic acid), constitute essential components of our diet, as humans lack the enzymes required for their biosynthesis (Guan et al. 2012). However, the increased number of double bonds in fatty acyl chains renders them susceptible to oxidation, particularly at high temperatures, and leads to the transformation of fatty acyl chains into trans fats (Browse et al. 1998). Therefore, increased consumption of unsaturated fatty acids with trans double bonds enhances susceptibility to cardiovascular disease and metabolic syndrome in humans (Hirata et al. 2021). Natural variability of low 18:2 and 18:3 levels has not been reported in Indian mustard (*Brassica juncea*) (Sivaraman et al. 2004). In the present study, *FAD2* genes were cloned from three Indian mustard varieties to aid in understanding the molecular nature of their alleles.

MATERIAL AND METHODS

The genomic DNA of three released Indian mustard varieties (Basanti, Pusa Jagannath, and Pusa Agrani) was isolated from developing seedlings according to the 2xCTAB protocol (Lukowitz et al. 2000). The PCR (polymerase chain reaction) conditions for amplification were as follows: initial denaturation at 94 °C for 5 min; 30 cycles at 94 °C, 56 °C and 72 °C; and a final extension at 72 °C for 10 min. The primers (F-5' ATGGGTGCAGGTGGAAGAATG 3'; R- 5' TCATAACTTATTGTTGTACCAG 3') were designed based on published sequence information of the *FAD2* gene (NCBI accession numbers FJ696650 and EF639848) of oilseed *Brassica juncea*. The reaction mixture (20 µL) for PCR included 1 U of Ex *Taq* DNA polymerase (Takara, USA), 1x PCR buffer (Takara, USA), 0.5mM primers (IDT), 0.2 mM dNTP mixture (Takara, USA) and 1 µL of dissolved DNA as a template. The final volume was adjusted with nuclease-free water (Invitrogen, USA). Following PCR, the amplified products were electrophoresed on a 1% native agarose gel. The fragments of the expected size (~1.1 kb) were eluted from the gel by an extraction kit (Zymo Research, USA). The gel-purified DNA fragment was finally dissolved in the requisite volume of nuclease-free water and stored in a freezer until use.

The purified DNA fragment was subsequently cloned and inserted into the PCR2.1 vector (Invitrogen, USA) according to the protocol of the Topo T/A cloning kit (Invitrogen, USA). The positive clones, obtained after verification by PCR using both gene-specific and vector-borne primers, were subsequently subjected to plasmid isolation via a kit (Zymo Research, USA). The plasmids with inserts were subjected to bidirectional sequencing. Sequence analyses were carried out using vector NTI software (Invitrogen, USA) and the BLAST service of NCBI.

RESULTS AND DISCUSSION

Fatty acid desaturase (FAD) enzymes are resident enzymes of both plastids and the endoplasmic reticulum. FADs not only regulate seed FA composition but also helps in maintaining the bipolar nature of lipidic cellular membranes (Browse et al. 1998). In addition, the degree of FA desaturation, mediated by FADs, is also associated with diverse abiotic stress responses and stress adaptation (Upchurch 2008, Singer et al. 2016, Xue et al. 2018). FADs are also associated with maintaining phytohormone levels in plants. Modulation of PUFA content in hypomorphic *fad2* mutants affects plant growth and development through the abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) pathways (Kachroo et al. 2003, Regente et al. 2008). Linolenic acid (18:3) is the precursor of jasmonate, which was corroborated by the fact that spraying either jasmonate or 18:3 on floral buds of triple mutants of *Arabidopsis* (*fad3-2 fad7-2 fad8*) resulted in fertility (Stintzi and Browse 2000). Among the FADs, FAD2 synthesizes >90% of the total PUFAs in nonphotosynthetic tissues, such as seeds and roots (Miquel and Browse 1992). In seed storage, TAG and polyunsaturated fatty acids (PUFAs) are synthesized from phosphatidylcholine (PC), a membrane lipid (He et al. 2020). The desaturation of stearic acid (C18:0) to oleic acid (C18:1) is catalyzed by soluble stearoyl-ACP desaturase (SAD) housed inside the stroma of plastids. Once synthesized in plastids, oleic acid (18:1) is then transported out for further desaturation in the ER or microsomes. 18:1 undergoes the first round of desaturation by FAD2, after which it is converted to 18:2, and by another round of desaturation by FAD3, 18:2 is converted to 18:3 (Browse et al. 1998, Park and Kim 2022) (Figure 1). The *FAD2* gene harbors a single intron positioned at the 5'-untranslated region (UTR), which is evolutionarily conserved (Dar et al. 2017, Cao et al. 2021). The sequence variation of the cis-acting intron of this gene has been attributed to the primary regulation of desaturation by *FAD*2 in *Arabidopsis* (Menard et al. 2017).

In the present study, we explored the allelic nature of the *FAD2* gene, which encodes an oleate desaturase/delta-12 desaturase or ω-6 desaturase enzyme, in three Indian mustard varieties (Basanti, Pusa Jagannath, and Pusa Agrani). *Brassica juncea* (AABB) is an amphidiploid derived via natural interspecific hybridization between *Brassica rapa* (AA, n=10) and *Brassica nigra* (BB, n=8) (Kang et al. 2021). Two earlier studies conducted independently (Singh et al. 1995, Suresha et al. 2012) reported the occurrence of the 1155 bp long *FAD2* gene in *Brassica juncea*. However, comparisons of the *FAD2* cDNA sequence of B. jun-2 (EF639848) (Suresha et al. 2012) with that of the B. jun-1 (X91139) (Singh et al. 1995) gene of *B. juncea* revealed 77.69% and 95.09% similarity at both the nucleotide and amino acid levels, respectively. In the present investigation, two homologs of the *FAD2* gene with 1155 bp long ORFs were identified in the Indian mustard variety (Pusa Jagannath). Based on the similarities of the 1155 bp long *FAD2* gene sequence identified in the present study with those of the *FAD2* genes of *Brassica rapa* (AA) and *Brassica nigra* (BB), the two homologs were designated *FAD2-A* and *FAD2-B,* respectively. Taken together, the above results point toward the natural occurrence of two homologs

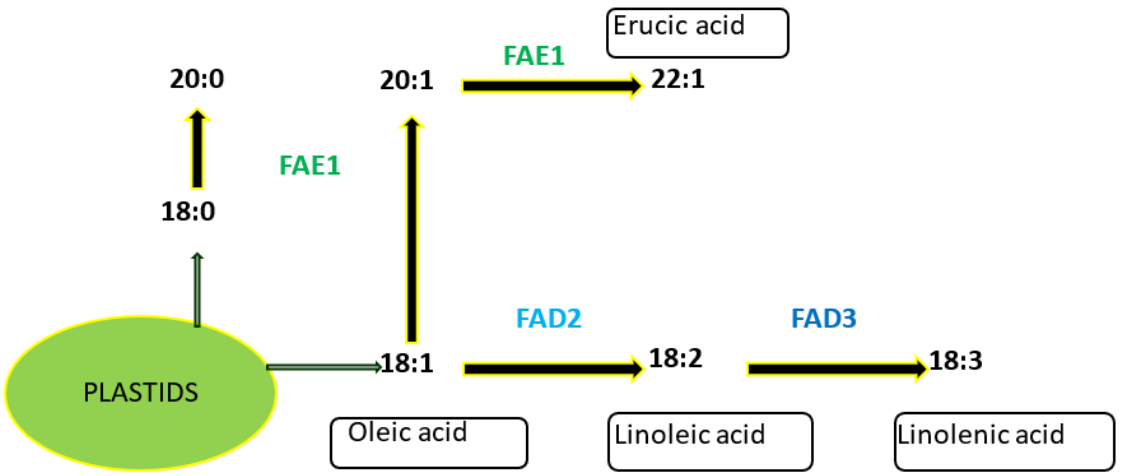

Figure 1. Simplified scheme of seed fatty acid biosynthesis in *Brassica species.*

of the *FAD2* gene, which is consistent with its origin from both the diploids *B. rapa* and *B. nigra* (Figure 2). In the Indian mustard variety Pusa Jagannath, two homologs of *FAD2,* i.e., *FAD2*-A and *FAD2*-B, were obtained; however, in the other two varieties (Basanti and Agrani), only one homolog, i.e., *FAD2*-A, was isolated (Figure 2).

Brassica napus (AACC) is also an amphidiploid species, but unlike *Brassica juncea*, it originates from spontaneous hybridization of *Brassica rapa* (AA) and *Brassica oleracea* (CC) (Nagaharu and Nagaharu 1935). It was estimated that the *Brassica napus* genome contains four FAD2 genes (Scheffler et al. 1997). Later, four homologs of the FAD2 gene were cloned (Yang et al. 2012) and grouped into two clusters. The two homologs of each cluster were ~97% identical at the nucleotide level, but the nucleotide identity between homologs of the two clusters was less than 90% (Lee et al. 2013). Like in *Brassica napus*, we also identified two molecularly different FAD2 genes (FAD2-A and -B) in the Pusa Jagannath variety of *Brassica juncea* (Indian mustard) that were 92% identical at the nucleotide level. However, we could not isolate the other homologs of each of the two divergent FAD2 gene types (A/B) in the three varieties. Sequence analysis of fewer clones could explain this lack of recovery, as we were unsuccessful in isolating the FAD2-A gene type in two varieties. Alternatively, the other homolog of each FAD2 type could be ~100% identical at the nucleotide level because of the low polymorphism level among Indian mustard varieties, which has been attributed to their narrow genetic base (Chauhan et al. 2011).

However, interestingly, an altogether novel type of *FAD2* gene with a 1167 bp long ORF was found in all three varieties. We designated the novel *FAD2* gene *FAD2-N*. A comparison of the FAD2-N sequence among the three varieties revealed 8 SNPs in Basanti compared to the other two varieties (Pusa Jagannath and Pusa Agrani), but only three SNPs positioned at 368, 797, and 901 changed the amino acid composition to D123G, I266T and T301A, respectively. Among the two SNPs located at 96 and 242 in Pusa Jagannath, only one exhibited change in the amino acid H81R compared to the other two varieties. In Pusa Agrani, there was a lone SNP at 381, but this did not change the amino acid composition. When the FAD2-N sequences of three varieties were used as queries to explore the genome of Varuna, an oleiferous variety of *Brassica juncea* (Paritosh et al. 2021) in the NCBI database, > 99% (on Chr. B1) nucleotide identity was detected. Interestingly, after searching against the genome of Tumida (http://brassicadb.cn/), a vegetable variety of *Brassica juncea* (Yang et al. 2016), the nucleotide identity was also found to be ~ >99% (on Chr. 07). Sequence comparison of *FAD2-N* with *FAD2* genes of oilseed *Brassica* species revealed the presence of three insertions in *FAD2-N* differing in nucleotide numbers (boxed) (Figure 2). In addition to having longer ORFs owing to insertions, *FAD2-N* also presented several SNPs across its length, which distinguishes it from the *FAD2-A* and *FAD2-B* of Indian mustard varieties and other *Brassica* species. Additionally, several pseudogenes of *FAD2* were also identified in the varieties examined; these included internal stop codons that encode nonfunctional proteins (data not shown).

The translation of the novel *FAD2-N* ORF yielded 388 polypeptides. It is premature to draw conclusions about the functional nature of these proteins in plants, as further study is needed. Interestingly, the novel *FAD2*-N proteins of all three varieties were 99.8% identical at the nucleic acid (NA) level, while their translated protein showed 98.7% identity at the amino acid level, suggesting little structural or functional differences among them. Among the *FAD2-B* alleles of the three varieties, 23 SNPs were found in Pusa Agrani compared to Basanti and Pusa Jagannath. On the other hand, a single SNP (C596T) was found in Basanti as compared to Pusa Jagannath. Interestingly, except for one change in amino acid (S199L) in Basanti, the translated proteins of the three varieties were nearly identical. For the *FAD2-B* allele (nigra type), all three varieties shared 97.7% identity at the NA level and 99.7% identity at the amino acid level. Such a high level of amino acid identity suggested little or no difference in the functionality of the proteins. However, when the *FAD2-B* allele (nigra type) was compared to the *FAD2*-A allele (rapa type), both exhibited 90.8% identity at the NA level and 95.6% identity at the amino acid level, indicating allelic divergence. Overall, Indian mustard possess three different *FAD2* alleles but limited nucleotide diversity among members of the *FAD2-B* type (B and N), indicating narrow genetic diversity among the varieties examined. Interestingly, among oilseed *Brassica* species, the *FAD2*-A/-B ORF is 1155 bp long in *Brassica napus*, *Brassica juncea*, *Brassica carinata*, and *Brassica rapa*. However, it is 1152 bp long in *Brassica nigra* and 1158 bp long in *Sinapis alba*. *Arabidopsis*, a close relative of *Brassica*, harbors a single *FAD2* gene that is 1152 bp long, similar to that of *Brassica nigra*. Intriguingly, the amphidiploid *Brassica juncea* acquired a 1155 bp long ORF

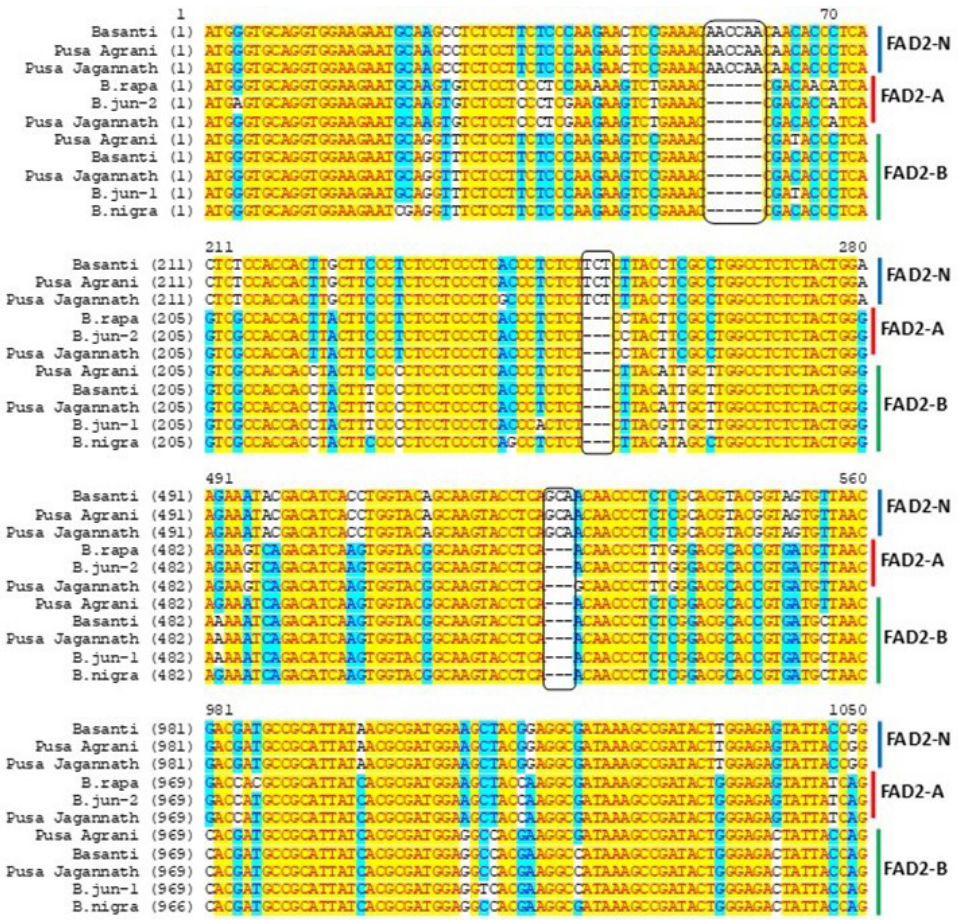

Figure 2. Regions of nucleic acid alignment of *FAD2* genes of *Brassica juncea* varieties (Basanti, Pusa Jagannath, and Pusa Agrani) showing major differences. The published *FAD2* gene sequences of *B. juncea* [B. jun-1 (X91139) and B. jun-2 (EF639848)], *B. nigra* (HM138369) and *B. rapa* (JN859550) were used as references. [The NCBI accession number of Indian mustard in this study. Basanti (*FAD2-N*)- MN585114, Pusa Agrani (*FAD2-N*)- MN585115, Pusa Jagannath (*FAD2-N*)- MN585116, Pusa Jagannath (*FAD2-A*)- MN585117, Basanti (*FAD2-B*)- MN585119, Pusa Agrani (*FAD2-B*)- MN585118 and Pusa Jagannath (*FAD2-B*)- MN585120].

of the FAD2 gene even though one of the progenitors, *Brassica nigra*, possessed a 1152 bp long ORF of the *FAD2* gene.

Alignment of translated proteins of *FAD2* genes, including the novel gene (*FAD2-N*), which was cloned in the present study, revealed the presence of three conserved histidine boxes (red box) similar to those present in other *Brassica* species (Figure 3). Additionally, the FAD2 protein has the canonical amino acid sequence YNNKL at the 3' end, which is essential for microsome/endoplasmic reticulum (ER) retention. The conserved histidine motifs are iron binding sites in the cytoplasmic domain of the FAD2 protein and are important for the interaction between Cytochrome b5 and ER desaturases (Okuley et al. 1994, Shanklin and Cahoon 1998). Unlike chloroplasts, where ferredoxin supplies electrons to desaturases, in microsomes, cytochrome b5 is the direct source of electrons for desaturation reactions (Kumar et al. 2006, Kumar et al. 2012). Interestingly, stearoyl CoA desaturase, located in plastids, is a soluble FAD that contains two conserved histidine boxes, in contrast to membrane-bound FADs (FAD2 and FAD3), which reside in the ER and contain three conserved histidine boxes (Xue et al. 2018). Furthermore, the translated FAD2 protein of *B. nigra* is having one amino acid less and *Sinapis alba* is having one additional amino acid in contrast to 384 amino acid long FAD2 proteins

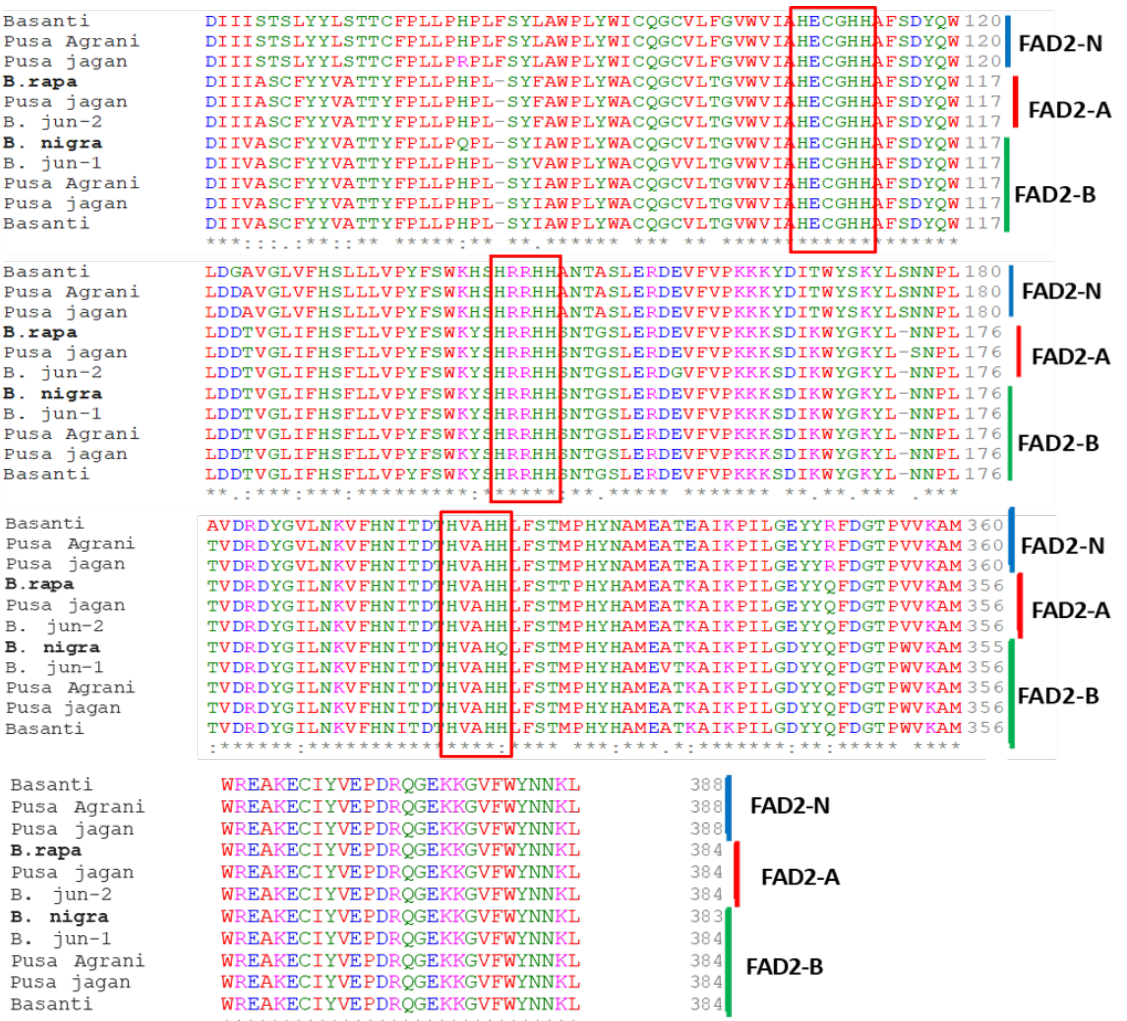

Figure 3. Alignment of FAD2 proteins (selected regions) of *Brassica juncea* varieties (Basanti, Pusa Jagannath and Pusa Agrani). The published FAD2 protein sequences of *B. juncea* [B. jun-1 (CAA62578) and B. jun-2 (ABR27357)], *B. nigra* (ADJ58018) and *B. rapa* (AFC41105) were used as references. The NCBI accession numbers of Indian mustard in this study include the FAD2-N proteins of Basanti (QGW48095), Pusa Agrani (QGW48096), and Pusa Jagannath (QGW48097); the FAD2-A protein of Pusa Jagannath (QGW48098); and the FAD2-B proteins of Basanti (QGW48100), Pusa Agrani (QGW48099) and Pusa Jagannath (QGW48101).

of four other *Brassica* species viz. *B. napus*, *B. juncea*, *B. rapa* and *B. carinata* respectively. There are several reports regarding genetic diversity among genotypes/accessions/varieties of Indian mustards. Many such reports have utilized SSR markers owing to their low cost, codominant nature, wide genome coverage and ease of experimentation (Sharma et al. 2020). However, it is interesting to note that the low polymorphism level among Indian mustard varieties has been attributed to their narrow genetic base (Chauhan et al. 2011). Therefore, the near absence of nucleotide diversity among members of each *FAD2* gene/allele type across varieties in the present study is not surprising. Additionally, since it encodes a vital enzyme (oleate desaturase) for plant growth and development, it is plausibly protected from random changes owing to selection pressure.

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