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RESEARCH ARTICLE

Molecular Profiling of Erucic Acid Biosynthesizing *FAE1* Gene of Indian Mustard Varieties

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Abstract

Indian mustard is a major contributor to the edible oil supply in India. Traditional Indian mustard varieties contain very high proportion of 18C polyunsaturated fatty acids and large amounts of long-chain (20C and more) monounsaturated fatty acids, mainly erucic acid. The composition of fatty acids in oil determines its end usage. Erucic acid, though an important feedstock for industrial applications, is an undesirable property for cooking oil due to its potential health hazard. Erucic acid is synthesized by condensing enzyme fatty acid elongase 1 (FAE1). The present study aimed to gain insight into the molecular profile of FAE1 gene in Indian mustard varieties. The detailed characterization of a full-length coding sequence of FAE1 gene revealed a near absence of uniqueness among haplotypes of paralogous in Indian mustard varieties. The absence of variation among haplotypes in FAE1 paralogous in the studied Indian mustard varieties plausibly indicates the use of germplasm/varieties having a narrow genetic base towards the development of such varieties.

Keywords: FAE1, Indian mustard, Molecular profiles, Erucic acid.

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Introduction

The five fatty acids, palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3), considered "usual" fatty acids, are the most widely occurring fatty acids in seeds of higher plants. Interestingly, the same fatty acids constitute a structural component of all cellular membranes. Their synthesis and accumulation in seed tissues is a relatively simple modification of the general pathways of fatty acid and glycerolipid synthesis in plants (Voelker and Kinney, 2001). While seed triacylglycerol (TAG) vary widely in fatty acid composition among species, 18-carbon unsaturated and polyunsaturated fatty acids generally predominate. To date more than 300 naturally occurring fatty acids with various functional groups such as hydroxy, epoxy or double bonds in unusual positions have been reported in seed oils of various plant species (Badami and Patil, 1980). Due to their special functional groups or double bond position, they have many industrial end-uses, such as components of plasticizers, adhesives, paints or as a precursor of nylon and composite materials (Jaworski and Cahoon, 2003). Since their chemical structure deviates significantly from usual fatty acids, they are termed "unusual" fatty acids and are largely excluded from polar lipids and cell membranes (Millar and Kunst, 1997). Very long chain fatty acids (VLCFA), with 20 to 30Cs, are mostly required for the synthesis of wax that forms a protective covering of epidermal cells of plants also qualify as unusual fatty acids.

The seed oil of *Brassica* species, in addition to the above-mentioned "usual" fatty acids, also contains significant amounts of VLCFA, mainly erucic acid (C22:1), catalyzed by FAE1 enzyme (Millar

and Kunst, 1997), is stored primarily in storage TAG molecule of seeds. Though erucic acid serves as a useful industrial feedstock for the manufacturing of adhesives, plasticizers, lubricants, etc. (Millar and Kunst, 1997 and references therein), nutritionally, it is an undesirable fatty acid as it poses a health hazard (Metzger and Bornscheuer, 2006).

The biosynthesis of erucic acid (22:1) takes place by the endoplasmic reticulum resident fatty acid elongase enzyme complex and the major rate-limiting enzyme is 3-ketoacyl-CoA synthase (KCS), also known as fatty acid elongase 1(FAE1) which catalyzes initial condensation step (Millar and Kunst, 1997). *FAE1* gene has been cloned and characterized in many brassica species. In the present study, *FAE1* gene of Indian mustard varieties was examined to explore allelic diversity, if any, among themselves.

Materials and Methods

DNA Extraction and PCR Condition

Indian mustard varieties [Basanti (IC 305113), Pusa Jagannath (IC 427782) Pusa Agrani (IC 73187), Coral-432(IC 574232), JM-2 (IC 471177), Kranti (IC 113116), Krishna (IC 113117) and Laxmi (IC 305061)] were selected for the present study. The seeds of above mustard varieties, procured from NBPGR Gene-Bank, were allowed to germinate on wet filter paper placed inside petri dishes. The genomic DNA was isolated from 10 days old seedlings according to 2xCTAB protocol (Lukowitz *et al*., 2000). The polymerase chain reaction (PCR) condition for amplification was as follows: initial denaturation at 94°C for 5 minutes; 30 cycles at 30 seconds at 94°C, 30 seconds, at 56°C and 1.5 minutes at 72°C and final extension at 72°C for 10 minutes. The primers of *FAE1* (F- 5'ATGACGTCCATTAACGTAAAG3'; R- 5' TTAGGACCGACCGTTTTGGAC 3') were designed based on published sequence information of *FAE1* gene of *Brassica*. For PCR, the reaction volume of 20 µL included 1U Ex-*Taq* DNA polymerase (Takara), 1x PCR buffer (Takara), 0.5 µM primers (IDT), 0.2 mM of dNTP mix (Takara) and 1-µL of dissolved DNA as template. The total volume was adjusted with nuclease-free water (Gibco). After PCR, the amplified products were electrophoresed on 1% native agarose gel.

Cloning and Sequence Analyses

The genomic fragment of *FAE1*, which included complete coding sequences (CDS), was amplified from genomic DNA. An extraction kit eluted the expected size fragment from gel (Zymo Research). The gel-purified fragment was finally dissolved in the requisite volume of nuclease-free water. The purified product was subsequently cloned in PCR2.1 vector (Invitrogen) according to manufacturer protocol. The positive clones, obtained after colony PCR, were subjected to plasmid isolation by using kit (Zymo Research). The expected size (~1.5 kb) of cloned *FAE1* gene fragments was further verified by PCR using vector-born primers. The purified plasmids were then subjected to bi-directional sequencing (Sanger method). Sequence analyses was carried out on VectorNTI software (Invitrogen) and NCBI blast service.

Results and Discussion

Although *de novo* fatty acid synthesis occurs inside plastids in the plant kingdom, desaturation and elongation predominantly occur on endoplasmic resident enzymes encoded by *FAD2*, *FAD3* and *FAE1* genes (Figure 1). VLCFA, such as erucic acid (22:1) and eicosenoic acid (20:1), being regarded as unusual fatty acids and thus stored primarily in seed TAG as the ectopic expression of *FAE1* resulted in severely altered morphology of plant due to incorporation of VLCFA in polar lipids of membranes (Millar *et al*., 1998).

Brassica juncea (AABB) is amphidiploid and composed of genomes of both *B. rapa* (AA) and *B. nigra* (BB). Accordingly, two paralogous of *FAE1* gene were identified in *B. juncea* having 1521 base pairs (bp) long coding sequence (CDS) (Gupta *et al*., 2004). *B. napus*, another amphidiploid oilseed species of *Brassica* also reported to contain two *FAE1* paralogous belonging to A and C (*B. oleracea*) genome, respectively having 1521 bp long CDS sharing ~98% identity at nucleotide level (Wang *et al*., 2010). Both *B. juncea* and *B. napus* contain very high levels of erucic acid in seeds. Erucic acid (22:1), is synthesized by β-Ketoacyl CoA synthase encoded by *FAE1* gene (Millar and Kunst, 1997) (Figure 1).

In the present study, *FAE1* gene fragments from Indian mustard varieties were cloned to study the polymorphism in Indian mustard varieties. The sequence analyses of full-length coding sequences (CDS) of *FAE1* gene revealed the presence of two paralogues (*FAE1-A* and *FAE1-B*) in both Basanti (1) and Pusa Jagannath (8) varieties of Indian mustard. It was based on the nucleic acid alignment of full-length CDS of *FAE1* paralogous of Indian mustard varieties of the present study with that of a published sequence of *FAE1* of *B. rapa* and *B. juncea* cv. Varuna, the two paralogous were named *FAE1-A* and *FAE1-B,* respectively.

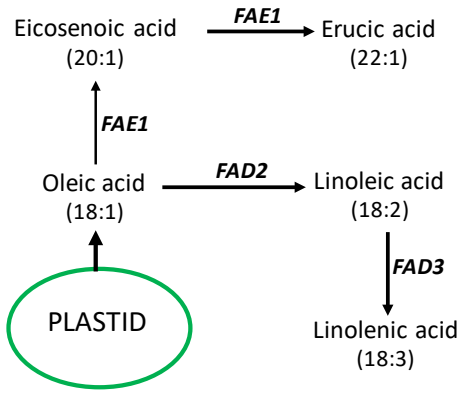

Figure 1: Simplified outline of fatty acid biosynthesis pathway in *Brassica* oilseed. (FAE1 = Fatty acid elongase1; FAD2 = Oleate desaturase, FAD3 = Linoleate desaturase)

Figure 2: A representative nucleic acids alignment of *FAE1* genes of Indian mustard (*B. juncea*) varieties. The NCBI accession number of Indian mustard varieties for *FAE1-A* were [JM-2 (KP074961), Coral-432 (KP074959), Laxmi (KP074957), Pusa Jagannath (KP074955), Kranti (KP074953), Krishna (KP074951) and Basanti (KP074949)] and for *FAE1-B* [Pusa Agrani (KP074963), Basanti (KP074950), Laxmi (KP074958), JM-2 (KP074962), Kranti (KP07954), Krishna (KP074952), Coral-432 (KP074960) and Pusa Jagannath (KP074956)]. The published *FAE1* sequences of *B. rapa* (AF490461) and *B. juncea* cv. Varuna (E1-AJ558197 and E2-AJ558198) were used for alignment.

In contrast to the occurrence of two FAE1 paralogues in Basanti and Pusa Jagannath, in Pusa Agrani (19), only one paralogue, i.e., FAE1-B, was found. However, to verify our non-occurrence of other paralogue in Pusa Agrani, a similar study was extended to five more Indian mustard varieties (Coral-432, JM-2, Kranti, Krishna and Laxmi). From analyses, it was found that each of these five varieties contained two paralogues of *FAE1,* unlike Pusa Agrani thus concluding that Indian mustard possess two paralogues of *FAE1*. The finding of only one paralogue in Pusa Agrani might be due to the analysis of sequences with fewer clones.

The *FAE1* paralogues in all varieties examined had 1521 bp long CDS with no intron. The paralogues' similarity in each variety was ~ 96% at the nucleotide level. However, haplotypes of each paralogous were nearly ~100% identical at the nucleotide level across the Indian mustard varieties examined (Figure 2). The generated *FAE1* gene sequences of above varieties have been submitted to NCBI (KP074949-KP074963). The absence of variation in *FAE1* paralogous in the studied Indian mustard varieties largely indicates the exploitation of narrow genetic base germplasm/varieties towards the development of such varieties.

Both *FAE1* paralogues encode 506 amino acids long polypeptides in all the eight Indian mustard varieties studied. However, in an earlier study, the two *FAE1* paralogous of Pusa Bold, a variety of Indian mustard, were reported to encode 509 and 510 amino acids long polypeptide, respectively (Yadav *et al*., 2003). Like Indian mustard, both the *FAE1* paralogous of *B. napus* also encode 506 amino acids long polypeptide (Wang *et al*., 2010), a report consistent with our present finding. Moreover, the genetic study with low erucic acid (LEA) Indian mustard variety have clearly demonstrated the additive and differential contribution of *FAE1* paralogous towards the accumulation of erucic acid in seed (Saini *et al*., 2019).

When coding sequence (CDS) of *B. napus* (AACC) *FAE1-A* (EU543282) was aligned with *FAE1-A* paralogues of Indian mustard varieties (JM-2, Coral-432, Laxmi, Pusa Jagannath, Kranti, Krishna and Basanti), they all shared ~ >99% identity at the nucleotide level. Such a high degree of conservation of *FAE1* gene originating from *B. rapa* (AA) genome in both the amphidiploids i.e. *B. napus* (AACC) and *B. juncea* (AABB), seemingly explain the evolutionary advantage for retaining large amounts of erucic acid in seed. However, CDS of *FAE1-B* paralogous of the above-mentioned Indian mustard varieties were only ~95% identical with *B. napus FAE1-A* (EU543282).

The seed oil of *B. juncea* oil contains 10 to 15% oleic acid, 10 to 15% linoleic acid and 14 to 16% linolenic acid and 50 to 60% erucic acid (Suresha *et al*., 2012). However, oil is significantly rich in oleic acid (18:1), preferably in the range of 70 to 80%, along with the optimal ratio of linoleic (18:2) to linolenic (18:3) acids and almost zero level of erucic acid (22:1) will be most desirable cooking oil commodity due to its balanced nutritional fatty acid composition.

Conclusion

The result of *FAE1* paralogues study in Indian mustard varieties clearly indicates narrow genetic differences among themselves. This further suggests the plausible reason for the near absence of significant differences in erucic acid content in the seeds of mustard varieties examined. It is also important to note that *FAE1* is a key biosynthetic gene for seed erucic acid accumulation. Therefore, the generation of null or hypomorphic alleles of both *FAE1* paralogous and/or associated structural changes/disruption in the upstream promoter region offers some of the approaches for diversifying the genetic base of Indian mustard varieties. Such measures may ensure the breeding of cooking-compatible, healthy vegetable oil that contains elevated levels of oleic acid and near absence of erucic and eicosenoic acid in seeds.

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