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Isolation and Characterization of Delta 15 Desaturase (FAD3) Gene From *Camelina sativa* L.

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Abstract

Introduction: Omega 3 desaturase (fatty acid desaturase 3 or delta 15 desaturase) is an important polyunsaturated fatty acid (PUFA) in oilseeds which plays a great role in converting 18:2 to 18:3. Omega 3 fatty acids have a crucial function in human and plants physiological activity due to presence in cell transmembrane.

Materials and Methods: In the present study, at first the fatty acid desaturase 3 (FAD3) gene was cloned from *Camelina sativa* by cloning via T/A cloning vector (pTG19-T plasmid) and sequenced it. Second, some different bioinformatics software were used to characterize the *Cs*FAD3 gene and its protein.

Results: Sequencing analysis of the *Cs*FAD3 gene showed that this fragment contains 1164 bp and the start and stop codons were ATG and TAA, respectively. The bioinformatics analysis of this gene can provide important information on the gene and protein structure. The alignment of cloned sequence was done with other FAD3 sequences which revealed three conserved histidine boxes. The results based on Neighbor-Joining (NJ) alignment showed that there is a close relationship between the oilseeds from the same family such as Brassica napus and Camelina sativa in relation with their bioinformatics characteristics.

Conclusions: It can be concluded that isolated gene (*Cs*FAD3) can be used to increase the conversion of 18:2 to 18:3 unsaturated fatty acid to improve oilseed quality for human food. It was found that *Cs*FAD3 is a transmembrane protein which can convert ω6 to ω3 fatty acids and may simultaneously act as an ion channel in the endoplasmic reticulum.

Keywords: *Camelina sativa*, Fatty Acid Desaturase 3 (FAD3) Gene, T/A Cloning, Transmembrane Protein

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Introduction

Polyunsaturated fatty acids (PUFAs) are consisted of essential fatty acids such as omega 3 and omega 6 which have a great role in industrial purposes such as lubricants, drying agent and medical purposes.[1-3] Some of the most important fatty acids among omega 3s are alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). ALA needs a desaturase which name is delta 15 desaturase to be converted from linoleic acid.[4,5] According to their localization delta-15 fatty acid desaturases exist in two endoplasmic reticulum (FAD3) or plastid types (FAD7 and FAD8) in plants.[6,7] The structure of desaturase genes provides more information of how their gene structure can explain the signal transduction and its characterization in crop plants. Delta 15 desaturase is a main desaturase that converts 18:2 to 18:3 in non-mammalian cell which then converts to long PUFAs like EPA (20:5n-3) and DHA (22:6n-3) in mammalian cells. *Camelina sativa* (False flax) is one of the most important oilseeds with remarkable oil traits which has attracted many scientists' interests in recent years. *Camelina* has high levels of omega 3, up to 45%, which has made it a valuable resource for genetic studies like omega 3 desaturase gene transformation.

Many efforts have been made to improve the level of ω-3 as well as to apply fatty acid biosynthetic pathways from a diverse range of organisms, including fungi, animals, algae, moss and higher plants such as soybean,[9] *Brassica juncea*,[10] tobacco,[11,12] *Arabidopsis thaliana*,[13] linseed,[14] safflower,[15] and rice.[16] Lack of information about different aspects of bioinformatics characteristic such as transmembrane domain structure, codon usage and protein localization are the reasons of which some of the aspects of the *Cs*FAD3 gene and its protein is isolated, cloned and characterized.

Materials and Methods

Experimental Reagents

The T4 DNA ligase, *Taq* DNA polymerase, DNA restriction enzymes, One Step RT-PCR Master Mix, RNA extraction and DNA gel extraction kits were supplied from the Fermentas Company. The *Escherichia coli* DH5 <alpha> was used in all molecular biological experiments. Plasmid of pTG19 was used for cloning and sequencing.

RNA Isolation

Total RNA was isolated from *Camelina sativa* (DKDH0065) by RNA extraction Ribospin™ Plant kit. Its quality and quantity were analyzed by gel electrophoresis and spectrophotometer, respectively. Total extracted RNA was used as a template for reverse transcription polymerase chain reaction (RT-PCR) to make cDNA. Also, the cDNA was used as a template for PCR reaction for the isolation of *Cs*FAD3 gene.

Amplification and Cloning of FAD3 Genes in TA Cloning Vector

Appropriate primers were designed regarding Semi-Kozak sequence to increasing the yield of gene production. The *Cs*FAD3 gene isolated from *C. sativa* amplified by PCR using EX *Taq* DNA polymerase and PFU polymerase for strong proofreading and specific primers ZSA: 5' aga ggatcc actagt atg gtt gtt gct atg gac 3′ with a *Bam*HI and *Spe*I restriction sites at the 5′ end (underlined) and CSZS: 5′ tgagctcagcgcttta attgattttagacttgtc3′ with *Sac*I and *Afe*I sites at the 5′ end (underlined) were designed with Vector NTI ver.10. These primers were synthesized based on the reported *Cs*FAD3 gene sequences of *C. sativa* available at NCBI (GenBank accession number: KJ541074.1, KJ541075.1, KJ541076.1).

PCR Program, Transformation and Selection Method

The PCR was run with a 25 µL final volume, using 2.5 mM of each deoxyribonucleotide triphosphate (dNTPs), 10 pmol of each primer, 1.5 mM Mg^{2+} and 2.5 units of *Taq* DNA polymerase enzyme. The thermocycler was programmed, as touch-down method, for one cycle at 95°C for 3 minutes, followed by 5 cycles at 94°C for 1 minute; 44°C for 1.25 minutes; 72°C for 2.25 minutes, again followed by and 30 cycles at 94°C for 1 minute; 57°C for 1.25 minutes; 72°C for 2.25, one cycle at 72°C for 10 minutes and 1 cycle 30°C for 3 minutes as a final extension. The PCR product was extracted by a DNA agarose gel extraction kit (from Gene All), ligated in pTG19 vector and transferred to competent cells of *E. coli* by the heat shock method. The transformed colonies were screened by selecting a medium containing 100 mg/L ampicillin plus isopropyl-D-1-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Colony PCR with specific primers, restriction enzymes and jumping method were done to test white colonies for recombinant plasmids. Plasmid DNA was purified for sequencing using the standard method.[17]

FAD3 Sequences Collection and Sequence Analysis

All oilseed protein sequences of FAD3 were collected from NCBI (http://www.ncbi.nih.gov) and UNIprot (http://uniprot.org/) databases with the FASTA format. The codon usage analysis was calculated with Minitab version 17. The alignments to drawing the dendrograms was made with Mega 7 software.

Three-Dimensional Modeling

The secondary structure prediction and topology prediction of membrane-spanning domain (MEMSAT-SVM Cartoon) was done using PSIPRED version 3.3 (http://bioinf.cs.ucl.ac.uk/psipred/[18]). The TMHMM is a method based on a hidden Markov model Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM[19,20]) used to predict transmembrane helices. Transmembrane regions (motifs) were predicted and annotated using SMART (http://smart.embl-heidelberg.de/) and the NCBI Conserved Domain Database search program. The phylogeny tree based on NJ tree Phylogenetic comparison of FAD3 genes in some oilseed was generated by ClustalW (MEGA 7.0.25).

Results

The *Cs*FAD3 gene fragment was obtained by PCR reaction via specific primers ZSA and CSZS. These primers were designed based on different sequences reported in NCBI gene bank (Figure 1). The *Cs*FAD3 was cloned into the pTG19 plasmid. The positive colonies were confirmed by colony PCR, digestion with restriction enzymes (*Bam*HI and *Sac*I) and sequencing with M13F and T7 standard primers. Sequencing analysis of the *Cs*FAD3 gene showed that this fragment contains 1164 bp and the start and stop codons were ATG and TAA respectively (Figure 2).

Amplification and Cloning of FAD3 Genes in TA Cloning Vector

The CsFAD3 gene was isolated by PCR reaction with specific primers CZSF and CZSR (Figure 1). The CsFAD3 was cloned into the pTG19 plasmid. The positive colonies were confirmed by colony PCR then the digestion was done with *Afe*I and *Sac*I restriction enzymes. Sequencing of CsFAD3 was done with M13F and T7 standard primers. Sequencing analysis of the CsFAD3 gene showed that this fragment contains 1164 bp and the start and stop codons had ATG and TTA, respectively. The sequence was submitted in NCBI server (https://www.ncbi.nlm.nih.gov) with gene bank number: MG253926.

Codon Usage and Phylogeny Tree

The cluster analysis of amino acids for codons show that they are classified into two distinct groups: The 4, 5, and 9 genotypes were placed in one group, and genotypes 1, 6, 10, 9, 11, 8, 7, 13, 12 and 14 in the other group. This was further divided into sub-groups as shown in Figure 3. In the first group, oilseed 4 and 5 from the Brassicaceae family and in the second group, oilseed 12 and 14 from the Lamiaceae family showed the highest similarity of codon usage (69.19% and 70.74%, respectively). One notable point is that both first and second groups contain oilseeds which have high unsaturated fatty acids, including chia, *Perilla* and canola. This reveals the point that oilseeds with high contents of unsaturated fatty acids can have different codon preferences. Phylogenic analysis of FAD3 protein sequences shows two main groups

(red line, Figure 4) except for partials. *Linum usitatissimum*, *Ricinus communis* and *Vernicia fordii* were classified in a separate group. This analysis showed the closest resemblance between genotypes 4 and 5 (Figure 3).

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches.

Results show that there is a close relationship between *B. napus, C. sativa* and *A. mustard* in the phylogeny tree (Figure 5). On the other hand, castor bean and sunflower have farthest distance from each other.

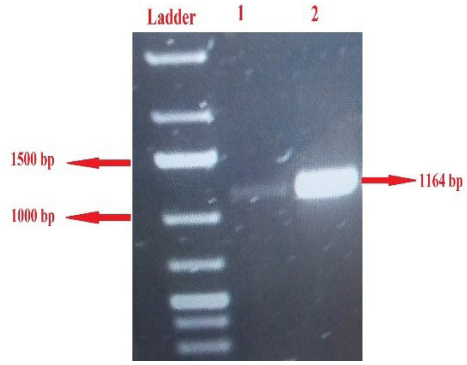

Figure 1. Amplified Fragment With Specific Primers CZSF and CZSR for *Cs*FAD3.

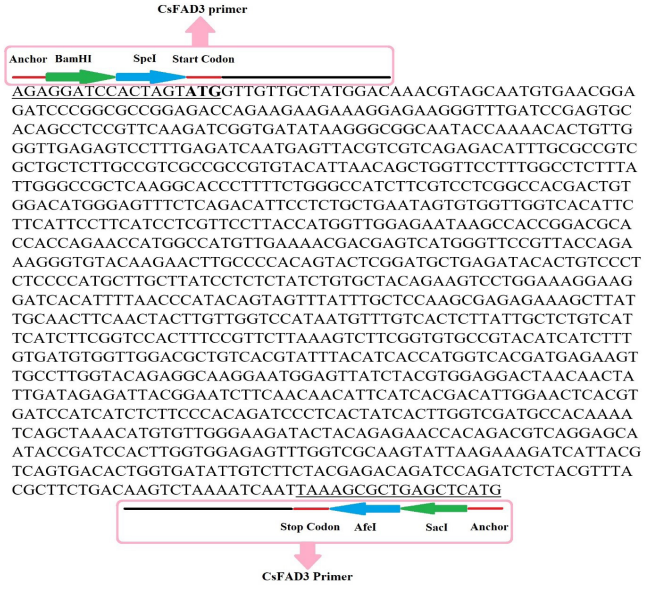

Figure 2. Sequence Analysis of *Cs*FAD3 Gene With Primers Sites, Start and Stop Codons and Restriction Enzyme Sites. Protein sequence of *Cs*Fad3.

Transmembrane Protein Analysis

The *Cs*FAD3 proteins have 6 membrane-spanning domains with C_Terminal= IN/N_Terminal= IN predicted by PSIPRED (Figure 6). Biological process predictions by this software demonstrate that this protein participates in transmembrane transport, including ion and cation transmembrane transport, with high reliability.

Transmembrane helices predicted using TMHMM software (Figure 7) and web-based SMART tool confirmed the existence of a minimum of three transmembrane domains in *Cs*FAD3.

Discussion

According to the findings of the present study, there is a close relationship between the family of oilseeds in phylogeny tree of protein sequences and phylogeny tree of gene sequences. Most transmembrane proteins transport specific material through the biological membrane by changing their conformation in a way to move a substance. This means that studying their role in membrane of oilseed especially in *C. sativa* is extremely important. Findings predicted that three transmembrane domains exist for CsFAD3 which have an important role in the biological process of its membrane. Isolation of FAD3 from *C. sativa* was performed by subcloning

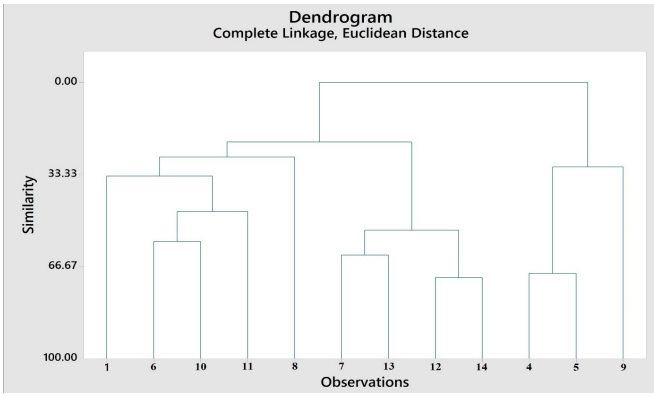

Figure 3. Cluster Dendrogram of Codon Usage of FAD3 Protein Sequences: 1. Safflower, 4. Rapeseed, 5. Camelina (our sequence), 6. Soybean, 7. Olive, 8. Flax, 9. Castor bean, 10. Tung tree, 11. Upland cotton, 12. Chia, 13. Hemp, 14. Perilla.

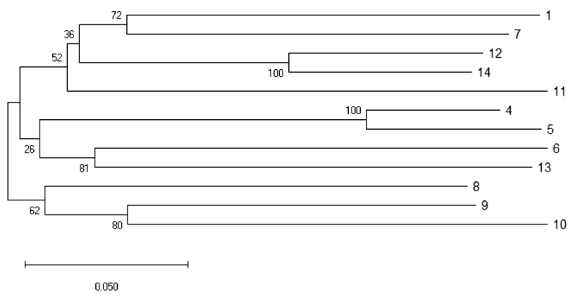

Figure 4. Cluster Analysis of FAD3 Sequences of the Studied Oilseeds. The phylogenetic tree was generated by ClustalW (MEGA 7.0.25 and The Neighbor-Joining (NJ) method). Protein sequences of FAD3 were: 1. Safflower, 4. Rapeseed, 5. Camelina (our sequence), 6. Soybean, 7. Olive, 8. Flax, 9. Castor bean, 10. Tung tree, 11. Upland cotton, 12. Chia, 13. Hemp, 14. Perilla.

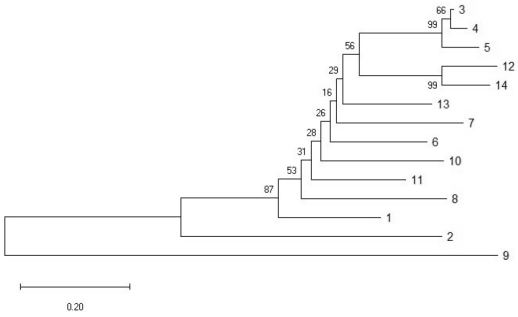

Figure 5. Phylogeny Tree of Gene Sequences of All Oilseed: 1. Safflower, 2. Sunflower 3. Abyssinian mustard 4. Rapeseed, 5. Camelina (our sequence), 6. Soybean, 7. Olive, 8. Flax, 9. Castor bean, 10. Tung tree, 11. Upland cotton, 12. Chia, 13. Hemp, 14. Perilla.

the gene into pTG19-T vector for sequencing. The result of sequencing showed that the cDNA of CsFAD3 had a 1164 bp Open Reading Frame (ORF). Then, the isolated sequence was submitted in the NCBI database with the accession number of: MG253926.

Recently some other key genes such as GDP-L-galactose phosphorylase and GME were subcloned with the same pTG19-T vector.[21,22] Li et al[23] put one partial FAD3 and FAD1 genes with 272 bp and 252 bp respectively in the expression RNAi vector G2 (pBinGlyBar1-FAD3-FAE1) to decrease the production of high content of polyunsaturated especially 18:3 fatty acid. So, simultaneously down regulating Fad3/FAD1 may lead to improving the *Camelina* for biofuel production.[24] As shown in the results, there are three histidine conserved boxes in CsFAD3 which is confirmed by other related research about oilseed histidine conserved boxes.[25-27] The pressure on the translated codons to use some codons rather than others is effective for special protein expression.[28] Changes in the patterns of codon usage may lead to changes in the amount of fatty acids in oilseeds. Genotypes that have large differences in codon usage may lead to significant differences in the production of fatty acids with the same treatments. This can be attributed to the pattern of using similar nucleotide codons in these oilseeds. Soltani et al[29] confirmed that direct relationships exist between preferred codons and divisions of plant families. These unique coding strategies might challenge molecular evolution.[30]

Conclusions

This study shows that a close similarity exists between the members of the oilseeds families like Brassicaceae family including *B. napus* and C. sativa in relation with their bioinformatics characteristics both for the phylogeny tree and codon usage cluster. Having six proteins membrane-spanning domain show the close relation between membrane and the protein. Based on analyses CsFAD3 with high yield promoter in effective construct might be used to increase conversion of 18:2 to 18:3 unsaturated fatty acid to improve oilseed quality for human diet. It was found that CsFAD3 is a transmembrane protein that can convert ω6 to ω3 fatty acids and may simultaneously act as an ion channel in the endoplasmic reticulum. It can be conclude that isolated CsFAD3 is a key

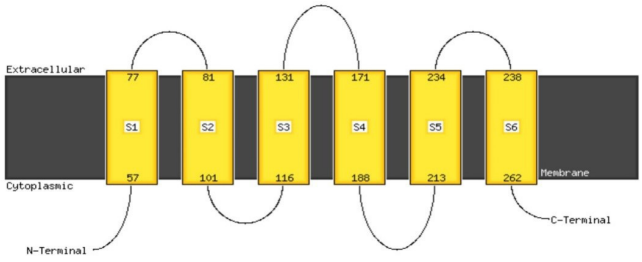

Figure 6. Proteins Membrane-Spanning Domain With Direction of the Ends for CsFAD3.

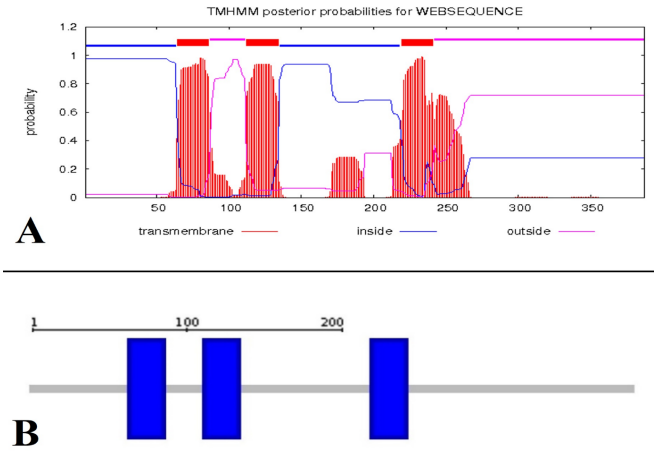

Figure 7. A: TMHMM Result for CsFAd3, B: SMART prediction for CsFAD3.

desaturase membrane enzyme necessary for converting 18:2 to 18:3 which can be used to express in different plants to improve oil quality in oilseeds.

Authors' Contributions

All authors contributed equally to this research.

Conflict of Interest Disclosures

The authors declare they have no conflicts of interest.

Acknowledgments

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