Molecular Cloning and Characterization of Expression Patterns of a Plastid ω-3 Fatty Acid Desaturase cDNA from *Perilla frutescens*

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Received February 14, 2001

An ω-3 fatty acid desaturase gene which is involved in *de novo* synthesis of -linolenate was isolated from cDNA library of *Perilla frutescens*. A cDNA library was constructed with mRNA extracted from perilla seeds of 12 DAF. The cDNA clone consisting of 1317-bp open reading frame encoding 438 amino acids with a relative MW of 50 kDa, was isolated and showed 65-83% similarities to other known genes. This cDNA is deduced to encode a plastidal ω-3 fatty acid desaturase based on the fact that it has higher homology to plastidal ones than to microsomal ones and its N-terminal sequence shares several characteristics of transit peptides of chloroplast proteins. Southern blot analysis of genomic DNA indicated that more than one gene or alleles for ω-3 fatty acid desaturase are present in the genome of perilla. Northern blot analysis showed that the ω-3 fatty acid desaturase gene is mainly revealed in early developing seeds and has different expression patterns depending on tissue types compared to the microsomal ones.

Key words: *ω-3 fatty acid desaturase, α-linolenate, Perilla frutescens.*

Biosynthetic pathways of fatty acids and glycerolipids have been a major issues in lipid metabolism due to the abundance of lipid in most cells. Two distinct pathways are found glycerolipid biosynthesis. One is mediated by plastid via prokaryotic pathway and the other by ER to enter eukaryotic pathway.[3,23] Evidence from *Arabidopsis* mutants suggested that lipid exchange between the ER and chloroplast is reversible to some extent.[2,16]

Biochemical approaches of the desaturases have proven to be difficult due to the membrane localization. But, current understanding on the mechanism and regulation of the desaturases has progressed considerably through studies involving *Arabidopsis* mutants. Four loci (originally called *fad*A, *fad*B, *fad*C, and *fad*D but now renamed *fad*4, *fad*5, *fad*6, and *fad*7, respectively) deficient in chloroplast desaturation and two loci (*fad2* and *fad3*) with defects in the ER desaturation pathway have been reported.[3] Until now, seven types of *fad* genes were cloned in plants: *fad2* [21] and *fad3*[1] genes via T-DNA tagging and map-based chromosome walking, *fad6*[9], *fad7* [10,27] and *fad8* [6] genes were all cloned based on their homology to *fad2* and *fad3*.

It has been known that fatty acid composition affects the nutritional quality and oxidative stability in fats and oils.[13] Indeed, a major challenge in modifying the composition of plant storage oil involved the changing of the degree of fatty acid desaturation.[26] Molecular gene transfer techniques have been used to reduce the content of stearic acid in canola and tobacco through antisense repression[11] and the introduction of mammalian desaturase gene.[7]

Polyunsaturated fatty acids, linoleate (18 : 2-Δ 9,12) and α-linolenate (18 : 3-Δ 9,12,15) produced by plants, but not by other higher eukaryotes, are essential components in human diets. Among these, α-linolenic acid has been known to be important in plant physiology such as cold resistance[6,12] and wound response.[18] But interestingly, one group recently showed that lower level of trienoic fatty acid enables to acclimatation to high temperature with transgenic plant which was silenced the chloroplast omega-3 fatty acid desaturase by suppression.[17]

Perilla, abundant in ω-3 fatty acid (especially, α-linolenate), has been consumed in Northeast Asia from ancient times. Biosynthesis of α-linolenate is mediated by ω-3 fatty acid desaturase via sequential insertion of double bonds into the derivatives of stearic acid (18 : 0). In this paper, we report on the isolation and identification of plastidal ω-3 fatty acid desaturase cDNA from perilla seeds and on its expression profiles in various tissues as well as during seed maturation.

Materials and Methods

Fatty acid analysis. Extraction and purification of seed lipids were done as described previously.[14] Fatty acid methyl

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Abbreviations: DAF, days after flowering; DES, desaturase; ER, endoplasmic reticulum; FAD, fatty acid desaturation; PCR, polymerase chain reaction.

esters were subjected to a Hewlett Packard 5890 series II using a capillary column (HP-20 M, 0.2 mm × 25 m, Hewlett Packard), with pentadecanoic acid as an internal standard. The column was kept at 175°C, the injector at 210°C, and the flame ionization detector at 210°C.

Nucleic acid manipulation. Total RNA was prepared according to the RNAzol™ B method (Biotecx), and poly(A)$^+$ RNA was isolated through two cycles of oligo(dT)-cellulose chromatography (Pharmacia). Total RNA was fractionated on 1.2% agarose gel containing formaldehyde and blotted onto nylon membranes.[24] RNA blot hybridization was carried out overnight in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 0.1% SDS, 50 mM NaH_2PO4 (pH 7.0) and 100 μg/ml denatured salmon sperm DNA at 42°C. They were washed with 1 × SSC/0.1% SDS at room temperature, and then with 0.5 × SSC/0.1% SDS at 42°C. Genomic DNA of perilla leaf was isolated via CTAB method[22], digested with various restriction enzymes, electrophoresed on 0.9% agarose gel, and transferred onto nylon membranes. DNA blots were hybridized overnight in 5 × SSC, 5 × Denhardt's solution, 0.5% SDS and 100 μg/ml of denatured salmon sperm DNA at 65°C. They were washed in 1 × SSC at room temperature, and then in 0.5 × SSC and 0.1% SDS at 65°C. Both DNA and RNA blots were hybridized with a ^{32}P- radiolabeled 1.1-kb fragment of ω-3 fatty acid desaturase cDNA as a probe.

PCR conditions and primers. We used cDNA library as template to obtain a probe for screening of ω-3 fatty acid desaturase. Three degenerate primers were designed on the basis of the amino acid sequences which are highly conserved among the known plant linoleate desaturases: Despl (1st upstream primer), 5'-GAG AAT A(T/C)AG C(T)CA T(C)C(A)G G(A)AC A(T)CA C(T)C-3'; Desp2 (downstream primer), 5'-A(G)TA A(G)CT CCA T(C)TC CTT G(C/T)CC T(A)CT(G) GTA-3'; Desp3 (2nd upstream primer), 5'-TTG GC(T)C AC(T)G AC(T)T GTG GA(T)C AT-3'. The PCR reaction was performed using a thermal cycle program of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, and an extension of 10 min at 72°C. The PCR products of 540 bp was obtained and subcloned into the PCR 2.1 vector (Invitrogen) for sequencing.

Construction and screening of cDNA library. A cDNA library was constructed with poly(A)$^+$ RNA isolated from the developing perilla seed harvested at 12 day after flowering (DAF). Preparation of double-strand cDNA and cloning into λ ZAP vector were performed according to the manufacturer's instruction (Stratagene). The cDNA library was screened with a ^{32}P-labeled 540-bp PCR fragment under the same hybridization condition as described in DNA blot analysis. Filters were washed with 2 × SSC at room temperature and then with 0.5 × SSC/ 0.1% SDS at 42°C. Several positive plagues were isolated and *in vivo* excised into plasmids for further characterization.

DNA sequencing. The nucleotide sequence was determined through dideoxynucleotide chain-termination method[25] with Sequenase version 2.0 (USB). To isolate a full length cDNA clone, a plasmid containing the longest insert was fully sequenced. The sequence of the cDNA was deposited in the GenBank database under the accession number of U59477.

Results and Discussion

Fatty acid composition in developing perilla seeds. Total fatty acid content of young developing seeds (7 DAF) was 0.5% of dry weight, and increased up to 21.8% of dry weight as maturation proceeded (Table 1). As mentioned by Min *et al.*[15], perilla contained large amounts of unsaturated fatty acids (oleate, linoleate, and linolenate) and saturated fatty acids (palmitate and stearate) in seeds. The contents of saturated fatty acids decreased, while that of unsaturated fatty acids increased during seed maturation. Linolenic acid (18 : 3) increased significantly up to 63% of total fatty acid and became the major component of unsaturated fatty acids in mature seeds (29 DAF). cDNA library constructed at 12 DAF, when linolenic acid (18 : 3) level in seeds increased significantly compared to that of linoleic acid (18 : 2), could be useful for isolating ω-3 fatty acid desaturase gene from perilla.

Cloning and sequence analysis of a perilla ω-3 fatty acid desaturase cDNA. To clone the perilla ω-3 fatty acid desaturase sequence we used three degenerate PCR primers designed on the basis of the conserved regions of known desaturase sequences. The nested PCR with degenerate primers using cDNA library as template yielded a 540 bp cDNA fragment (Fig. 1). The 540-bp PCR product was subcloned into TA PCR cloning vector (Invitrogen) and sequenced. The DNA sequence of the perilla PCR product was approximately 84.8% identical to the corresponding regions of the *Sesame*

Table 1. Fatty acid composition of total lipids in developing perilla seeds.

DAFa	Total fattyb acid (mg/g)	Composition of fatty acid (weight % of total fatty acid)				
		Palmitate (16 : 0)	Stearate (18 : 0)	Oleate (18 : 1)	Linoleate (18 : 2)	Linolenate (18 : 3)
7	5.04	27.01	2.19	2.48	26.11	42.20
12	10.42	19.20	0.91	9.50	10.80	59.53
19	62.86	9.45	1.45	9.94	19.55	59.61
29	217.70	6.25	0.68	14.95	13.54	64.59

The values represent an average of the values from the three separate analyses.
[a]Days after flowering.
[b]The sum of each fatty acid mainly detected in Perilla seeds (mg fatty acid/g dry weight).

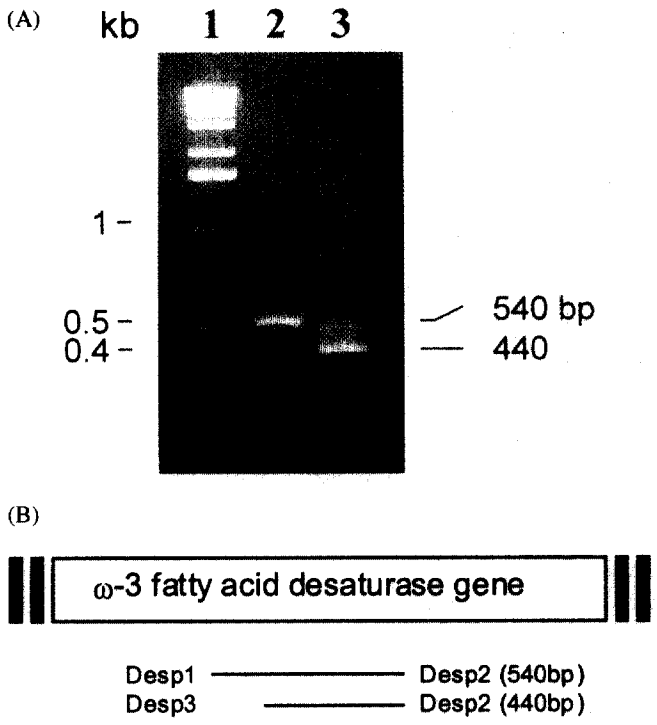

Fig. 1. PCR amplification of ω-3 fatty acid desaturase gene fragments. (A) Electrophoresis patterns of PCR products separated on 1% agarose gel. Lane 1, 1 kb ladder; lane 2, PCR product using primers Desp1 and Desp2; lane 3, PCR product using primer Desp3 and Desp2. (B) Brief scheme of PCR strategy to obtain ω-3 fatty acid desaturase gene fragments.

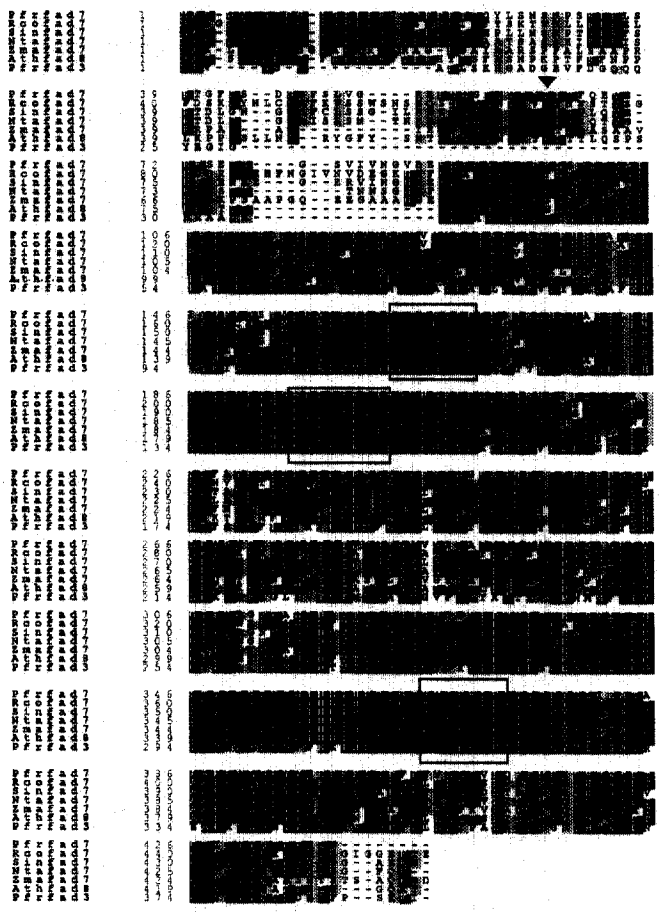

Fig. 2. Comparison of the amino acid sequences of perilla ω-3 fatty acid desaturase with other plant homologs. Multiple sequence alignment was performed by Clustal method using the BioEdit program (Hall, 1999). Identical residues in at least three of the sequences are highlighted and similar residues are shown with gray background. The arrowhead indicates the putative cleavage site of the transit peptide. Highly conserved histidine rich regions that can serve as binding sites for iron are boxed. The identities of the proteins are as follows: Pfrfad7 (P. frutescens FAD7, U59477); Rcofad7 (R. Communis FAD7, L25897); Sinfad7 (S. indicum FAD7, U25817); Ntafad7 (N. tabacum FAD7, D79979); Zmafad7 (Z. mays, FAD7, D63954); Athfad8 (A. thaliana FAD8, P48622); Pfrfad3 (P. frutescens FAD3, AF047039)

fad7 gene (data not shown). Using the PCR product as a probe, we screened a cDNA library constructed from perilla developing seeds, through which several strongly hybridizing plaques were recovered. The restriction enzyme mapping and partial DNA sequence analysis indicated that most of these positive clones represented the same class of DNA sequence. The longest clone consisting of 1797-bp was chosen for complete nucleotide sequence analysis and was revealed to be encoding a predicted polypeptide of 438 amino acids residues with the molecular mass of 50 kDa (Fig. 2).

The deduced amino acid sequence of the cDNA has N-terminal extensions, which consisted of several transit peptide characteristics of the nuclear-encoded plastid proteins. As mentioned by Von Heijne and Nishikawa[28], these include a high content of hydroxylated residues, low of acidic residues (net positively charged), and the N-terminal dipeptide Met-Ala. Although chloroplast transit peptides (cTPs, usually consisting of 35-75 a.a residues) do not contain a conserved homology block near the processing site, they are known to fit a loosely defined cleavage site (the consensus sequence, [Val/Ile]-X-[Ala/Cys]-Ala).[5] The amino acid sequence of the cDNA contained consensus sequence of Val-Ser-Ala-Pro for transit peptide processing site (Fig. 2).

The fatty acid desaturase is a general class of enzyme that contain iron which is not a component of heme prosthetic group[8], and the iron is known to be ligated to histidine rich region as a form of diiron cluster.[20] Membrane-bound desaturase has known to have three highly conserved histidine boxes (HXXHH) spacing the distance between the first and second histidine boxes of about 30 amino acids.[21] Perilla ω-3 fatty acid desaturase also has the histidine boxes (HDCGHGSF, residues 163 to 170; WRISHRTHH, residues 195 to 203; HVIHHLFP, residues 366 to 373). These boxes suggest that they can serve as binding sites for iron of ferredoxin that plays a role as an electron donor in the desaturation process.

The deduced amino acid sequence of the cDNA showed a significant homology (65 to 83% overall similarities) to other known polypeptides of plant ω-3 fatty acid desaturases (Fig. 2). Amino acid sequence identities between the perilla polypeptide and other plastid ω-3 fatty acid desaturases (*fad7*)

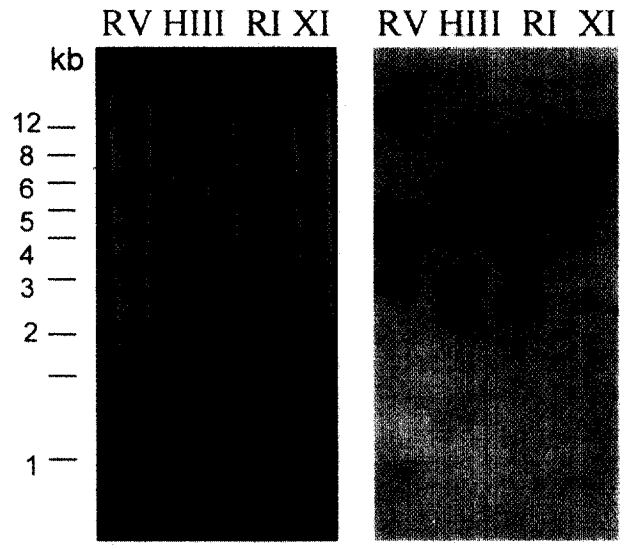

Fig. 3. Southern blot analysis of perilla genomic DNA. The Genomic DNA (20 μg) extracted from young leaves of perilla was digested with EcoRV (RV), HindIII (HIII), EcoRI (RI), XbaI (XI) and hybridized with the EcoRI- and XhoI- digested 1.1 kb fragment of ω-3 fatty acid desaturase cDNA. The DNA size marker is indicated in kb on the left side.

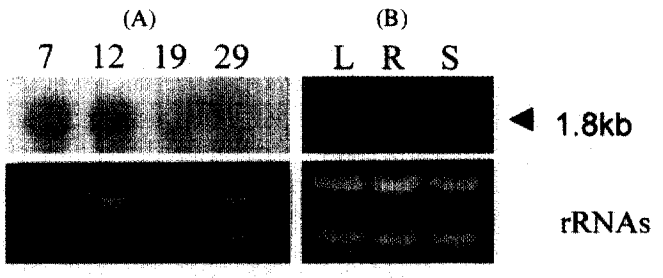

Fig. 4. Northern blot analysis of developing perilla seeds harvested at 7, 12, 19, and 29 DAF (A) and tissue-specific expression of perilla ω-3 fatty acid desaturase gene (B). (A) Total RNA (10 μg) were separated on 1.2% formaldehyde/agarose gels, transferred onto a nylon membrane, and hybridized with a ^{32}P-labeled 1.1 kb fragment of perilla ω-3 fatty acid desaturase cDNA. (B) Total RNA (10 μg) isolated from leaves (L), roots (R), and developing seeds (S) were separated on formaldehyde/agarose gels, and subjected to gel blot analysis using the Pfrfad 7 cDNA fragment as a probe. DAF, days after flowering.

were 77(sesame), 76(tobacco), 68 (castor) and 64% (maize). On the other hands alignment with the amino acid sequences of *fad8* and *fad3* showed 68% (*Arabidopsis*, perilla) identity, indicating that this cloned cDNA is closer to the plastid w-3 fatty acid desaturase gene.

Hydropathy profile of the perilla polypeptide sequence revealed that it contains three membrane-spanning regions similar to other known desaturases (data not shown). Putative active site histidines are located in the hydrophilic regions of the protein. This suggests that the exposure of the active sites would facilitate interaction with ferredoxin on cytoplasmic surface of the membrane. Phylogenetic analysis based on Clustal alignment algorithm (Winstar version program) showed that the earliest divergence in ancestral relationships is present between the group of microsomal ω-3 fatty acid desaturase and that of plastid ω-3 fatty acid desaturase (data not shown). The perilla ω-3 fatty acid desaturase was more closely related to the plastidal ones than to the microsomal ones in phylogenetic tree. Based on several lines of evidences, it is assumed that perilla cDNA, designated *Pfrfad7*, encodes plastid ω-3 fatty acid desaturase.

Gene family for ω-3 fatty acid desaturase in perilla. Genomic DNA digested with several restriction enzymes was hybridized with ^{32}P-labeled 1.1 kb fragment of ω-3 fatty acid desaturase cDNA to determine the ω-3 fatty acid desaturase gene dosage and the existence of *fad7*-like genes. Several fragments were hybridized with the probe in each restriction enzyme digested lane (Fig. 3). One band and Two predominant band appeared in *Eco*RV and *Eco*RI digests, respectively, indicating that more than one gene or allele and gene family might exist for ω-3 fatty acid desaturase in perilla. This result indirectly coincided with the fact that three types of ω-3 fatty acid desaturase genes have been isolated from higher plants upto now, but further investigation is required to understand the organization of the genes.

Expression patterns of *Pfrfad7* gene in developing seed stages and tissue types. To know the expression of *Pfrfad7* gene during seed development, northern blot analysis was performed using total RNA isolated from perilla seed at different developmental stages (7, 12, 19, and 29 DAF). RNA transcript of 1.8 kb, nearly corresponding to the full length of *Pfrfad7* clone, was highly detected in the early developing seeds (7 and 12 DAF), but, thereafter the gene expression level decreased during seed maturation (Fig. 4). mRNA could be detected in leaves and seeds but not in roots. This result was in agreement with expression patterns of *Arabidopsis* plastid ω-3 fatty acid desaturase[27]. Recently, microsomal ω-3 fatty acid desaturase encoded by *fad3* has been cloned from perilla developing seeds and this cDNA was only expressed in seeds[4]. To determine the pattern of accumulation of the *Pfrfad3*, northern blot analysis was performed using total RNA isolated from perilla seed at different developmental stages (7, 12, 19, and 29 DAF). *Pfrfad 3* gene highly expressed in 12, 19, 29 DAF seed maturation stages (data not shown). We, therefore suggest a model to explain the relationship between the changes in ω-3 fatty acid composition during seed development and the ω-3 fatty acid desaturase gene expression. Our suggestion is that *fad7* gene product is involved in the changes in ω-3 fatty acid composition in perilla seeds of early developing stage (7, 12 DAF), whereas, *fad3* gene may be expressed throughout the seed development except during the very early developing stage and plays a major role in accumulating α-linolenate during the perilla seed maturation. This speculation well- correlates with the changes in fatty acid composition of perilla the seeds during seed development (Table 1). Unfortunately, *fad8* known to be expressed in response to cold stress,[6] has not yet been

cloned in perilla.

Fad7 gene has been known to be expressed preferentially in the chlorophyllous tissues of the unwounded plants. However, one group recently showed that this gene can be activated through wounding via jasmonic acid-independent or dependent signaling pathway, and revealed that specific regions in *fad7* promoter are required for wound-activated expression in tissue types.[19] Interestingly, another group showed that lower trienoic fatty acids resulted in a better tolerance of high temperature than the wild type plants through studies on transgenic plants which were suppressed by chloroplast ω-3 fatty acid desaturase gene.[17] This result is noteworthy in that trienoic fatty acids has been known to be involved in cold tolerance until now. Further experiments will be required to elucidate the exact role and regulation of ω-3 fatty acid desaturase gene responding to external stress in perilla.

Acknowledgments. We thank Dr. Jeong-Bong Kim for technical assistance in GC analysis. We also thank Drs. Shin-Woo Lee and Seon-Kap Hwang for critical reading of the manuscript. This work was supported by a research grant from the Rural Development Administration (RDA).

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