Gene Cloning and Functional Analysis of a Second Δ6-Fatty Acid Desaturase from an Arachidonic Acid-producing Mortierella Fungus

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We demonstrated that Mortierella alpina 1S-4 has two Δ6-desaturases, which are involved in the desaturation of linoleic acid to γ-linolenic acid. For one of the two Δ6-desaturases, designated as Δ6I, gene cloning and its heterologous expression in a fungus, Aspergillus oryzae, has previously been reported. In addition, we indicated in this paper that there is an isozyme of the two Δ6-desaturases, designated as Δ6II, in M. alpina 1S-4. The predicted amino acid sequences of the Mortierella Δ6-desaturases were similar to those of ones from other organisms, i.e. borage and Caenorhabditis elegans, and had a cytochrome b₅-like domain at the N-terminus, being different from the yeast Δ9-desaturase, which has the corresponding domain at the C-terminus. The full-length Δ6II cDNA was expressed in A. oryzae, resulting in the accumulation of γ-linolenic acid (which was not detected in the control Aspergillus) up to 37% of the total fatty acids. The analysis of real-time quantitative PCR (RTQ-PCR) showed that the quantity of Δ6II RNA was 2.4-, 9-, and 17-fold higher than that of Δ6II RNA on 2, 3, and 4 days in M. alpina 1S-4, respectively. M. alpina 1S-4 is the first fungus to be confirmed to have two functional Δ6-desaturase genes.

Key words: Mortierella alpina; Δ6-fatty acid desaturase; cloning; polyunsaturated fatty acid; γ-linolenic acid

Polyunsaturated fatty acids (PUFAs) play important roles as structural components of membrane phospholipids, and as precursors of the eicosanoids of signaling molecules including prostaglandins, thromboxanes, and leukotrienes.1,2) The principal PUFAs are dihomo-γ-linolenic acid and arachidonic acid, n-6 PUFAs, and eicosapentaenoic acid, an n-3 PUSA. All mammals synthesize eicosanoids that are involved in the inflammatory response, reproductive function, immune response, and regulation of blood pressure.3) Therefore, studies on PUFAs are important in both the medical and pharmaceutical fields.

Previously, we screened for microorganisms capable of accumulating lipids containing PUFAs,4) and found a filamentous fungus, Mortierella alpina 1S-4, belonging to the Zygomycetes. This strain is unique in that it produces the n-6 PUFAs. We have studied the fatty acid metabolism in this strain and succeeded in its application to the industrial production of arachidonic acid.5) In this strain, most PUFAs are present in triacylglycerols as storage oils, while some are present in phospholipids as structural components of membranes. Therefore, we used this fungus for analyzing some genes encoding the enzymes concerned with PUFA biosynthesis.

γ-Linolenic acid (GLA; Δ6,9,12-18:3) is an intermediate in the metabolic pathway for (n-6) essential fatty acids from linoleic acid to arachidonic acid in mammals. GLA is also the direct precursor of dihomo-γ-linolenic acid, which is a precursor of anti-inflammatory 1-series eicosanoids. Dietary supplementation with GLA is reported to be effective in treating a number of conditions (e.g., atopic eczema, diabetic neuropathy, viral infections, and cancer).6,7) Although GLA is regarded as being useful as an ingredient of functional foods and pharmaceutical products, GLA is a rare fatty acid, because it is only synthesized in limited numbers of higher plants [e.g., borage (Borago officinalis L.)] and evening primrose (Oenothera biennis L.), fungi, and cyanobacteria.

From various organisms, several kinds of fatty acid desaturase genes have been cloned, genes encoding desaturase isoymes being found in some cases. For example, Δ9-desaturase isoymes were found in a nematode worm (Caenorhabditis elegans) and Mortierella fungi.8,9) On the other hand, two functional Δ5-fatty acid desaturases were reported to have substrate specificities in the cellular slime mould Dictyostelium discoideum.9) Δ6-Desaturase genes have been cloned from various organisms: borage,8,10) a nematode worm (C. elegans),11) Synechocystis,12) the moss Physcomitrella patens,13) rat,14) M. alpina,15) and man.16) However, there has been no report that

Abbreviations: GLA, γ-linolenic acid; PUFA, polyunsaturated fatty acid

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two Δ6-desaturases function in the biosynthesis of PUFAs in an organism.

Because of the notable accumulation of several PUFAs, 13 all M. alpina 1S-4 desaturases are expected to have high activity as to fatty acid desaturation. M. alpina, as a useful-lipid producing fungus, has attracted attention as to the biosynthesis of PUFAs, and the genes encoding Δ9-, Δ12-, Δ6-, and Δ5-fatty acid desaturases and a fatty acid elongase have been cloned. 15,18–21) The cloning of various desaturase genes from M. alpina 1S-4 would certainly help us understand the regulation of the production of PUFAs, leading to the specific production of PUFAs in vivo or in vitro. Although one gene encoding M. alpina 1S-4 Δ6-desaturase was previously reported, we report here the isolation and characterization of two Δ6-desaturase cDNAs from M. alpina 1S-4, and their expression in Aspergillus oryzae, as a heterologous host. This study is the first on the cloning of two Δ6-desaturase genes from microorganisms including fungi, and on the expression of the gene in an Aspergillus fungus, leading to the selective production of useful lipids and the modification of fatty acid compositions.

Materials and Methods

Enzymes and chemicals. Restriction endonucleases and other DNA-modifying enzymes were obtained from Takara Shuzo Co., Ltd. (Tokyo, Japan), and Toyobo Co., Ltd. (Osaka, Japan). [α-32P]dCTP (110 TBq/mmol) was from Amersham Japan (Tokyo). n-Heptadecanoic acid was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were of the highest purity commercially available.

Filamentous fungal strains and growth media. For the cloning of the Δ6-desaturase genes, M. alpina 1S-4 3 was used. A. oryzae AON-1013 (niaD-), used as the recipient strain in transformation experiments. 22) pNGA142 is a shuttle vector carrying the ampilin resistance gene (for selection in Escherichia coli) and the niaD gene of A. oryzae (for nitrate prototrophy selection in Aspergillus). 23) This plasmid also carries the promoter region of the glucoamylase gene (glaA), and the terminator region of the α-glucosidase gene (agdA) of A. oryzae flanked by unique HindIII and SpeI cloning sites.

M. alpina 1S-4 was cultured in GY medium containing Czapek-Dox medium [consisting of 2% glucose, 0.5% potassium dihydrogen phosphate, 0.1% sodium nitrate, 0.05% magnesium sulfate heptahydrate, and iron (II) sulfate heptahydrate (pH 6.0)].

Fatty acid analysis. The A. oryzae recombinant containing the M. alpina Δ6-desaturase cDNA was inoculated into a 20-ml Erlenmeyer flask containing 4 ml of DP medium, and then the culture was grown at 30°C with reciprocal shaking (120 strokes/min). Fatty acid analysis was done as described basically in the previous report. 20) The mycelial cells were
harvested by filtration, washed with distilled water, and then dried at 100°C. The dried cells were directly transmethyalted with 10% methanolic HCl at 50°C for 3 h, and the resultant fatty acid methyl esters were extracted with n-hexane, concentrated, and then analyzed by gas chromatography. n-Heptadecanoic acid was added before transmethylation, as an internal standard.

Preparation of total RNA and first strand cDNA. Total RNA was extracted from M. alpina 1S-4 grown in GY medium at 28°C for 2 to 4 days using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. The first strand cDNA was synthesized from 0.5-μg of total RNA in a 20-μl reaction mix using First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. A 10-fold dilution of the first-strand cDNA reaction mixture was used as a template for real-time quantitative PCR (RTQ-PCR).

Preparation of quantification standard. The PCR-amplified products, with the primers I and III for Δ6I and II and IV for Δ6II were ligated into pBluescript II SK + /EcoRV, which resulted in pBMLDI and pBMLDII were 4,356 bp and 4,359 bp, respectively. The copy number of the Δ6I and the Δ6II genes was measured by assuming that, based on the molecular weight of the 4.3-kb-sized pBMLDI and pBMLDII, 1 pg of plasmids equals 4.2 × 10⁶ copies. To prepare the standard curve of RTQ-PCR, 3, 0.3, 0.03, 0.003, and 0.0003 pg of purified pBMLDI and pBMLDII was used in identical PCR reactions.

RTQ-PCR with TaqMan Probe and the LightCycler. The primers used for amplifying the 122-bp gene fragment of Δ6I with the primers FI and RI and the 119-bp of Δ6II with the primers FII and RI, and the internal TaqMan probes are shown in Table 1. At the 5’ end, the TaqMan probes were labeled with 6-carboxy-fluorescein (FAM), and the 3’ end was labeled with 6-carboxy-tetramethyl-rhodamine (TAMRA). RTQ-PCR using a TaqMan probe was done in a LightCycler (Roche Diagnostics GmbH) by using the LightCycler-FastStart DNA Master Hybridization Probe Kit (Roche Diagnostics GmbH). The 20-μl reaction mixture contained a 1 × concentration of LightCycler-FastStart DNA Master Hybridization probes, 3 mM MgCl₂, a 900 nM concentration of the primers, a 250 nm concentration of probe, and 5 μl of the template. Amplification involved one cycle at 95°C for 10 min for initial denaturation and then 40 cycles of 95°C for 10 s with a 1°C/s increment followed by annealing and extension at 60°C for 30 s. Channel F1 was used for data acquisition, the gain of F1 was set at 2, and the F1 (FAM)/1 ratio was used for normalization of fluorescent data.

Nucleotide sequence accession numbers. The following sequences have been submitted to the DDBJ database: Δ6I cDNA and genomic genes (AB020032 and AB070557), and Δ6II cDNA and genomic genes from M. alpina 1S-4 (AB070555 and AB070556).

Results

Cloning of the two types of M. alpina Δ6-desaturase cDNAs

Using the ³²P-labeled fragment as a probe, two types of M. alpina Δ6-desaturase cDNAs were isolated from the λgt10 cDNA library. Each EcoRI fragment derived from the positive λgt10 clones was subcloned into pBluescript II, and then sequenced. We have already reported one Δ6-desaturase gene designated as Δ6I, and the other is deduced to be an isozyme of the Δ6-desaturase designated as Δ6II, as reported in this paper. This EcoRI fragment of Δ6II contains an open reading frame, which should encode a protein of 458 amino acids with a molecular mass of 51,989.

Comparison of the Δ6-desaturase sequence of M. alpina 1S-4 with those of other organisms

The deduced amino acid sequences of the Mortierella Δ6-desaturase cDNAs were compared with other Δ6-fatty acid desaturase sequences (Fig. 1). Although Δ6II (458 amino acid residues) had one more residue at the N-terminal than Δ6I (457...
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Fig. 1. Comparison of the Deduced Amino-acid Sequences of Δ6-Desaturases from Mortierella, Borage, C. elegans and Synechocystis.

Identical amino acid residues with Morfd6II are highlighted. The three regions containing histidine residues (His boxes I, II, and III) and the cytochrome b$_5$ motif are overlined. Abbreviations: Morfd6I and Morfd6II, two Mortierella Δ6-desaturases; Borfd6, borage Δ6-desaturase; Caefd6, C. elegans Δ6-desaturase; Synfd6, Synechocystis Δ6-desaturase.

amino acid residues), the whole amino acid sequences of the two Mortierella Δ6-desaturases were extremely similar, 92%, with each other. The Δ6II of M. alpina 1S-4 and the Δ6-desaturase of borage$^{10}$ have 30.5% sequence identity, and the M. alpina 1S-4 and C. elegans$^{11}$ ones 34.2%. In particular, three histidine-cluster motifs,$^{28}$ which comprise the consensus sequence of fatty acid desaturases, are well conserved in the two Δ6-desaturases of M. alpina 1S-4.

Hydrophobicity plots of the Mortierella Δ6-desaturases (data not shown) indicate that two conserved hydrophobic regions, which are assumed to form two membrane-spanning domains in the endoplasmic reticulum,$^{29}$ are present. In addition, a computer-aided search of protein sequence databases found 30.8% identity over the N-terminal 78 amino acid residues of the Mortierella Δ6II with microsomal cytochrome b$_5$ from rat.$^{29}$ These findings suggest that this domain in the desaturase plays the role of a unique electron transport system, binding a heme group, and therefore it belongs to the cytochrome b$_5$ superfamily.

Comparison of the genomic genes of the two Δ6-desaturases from M. alpina 1S-4

To isolate the two Δ6-desaturase genomic genes from M. alpina 1S-4, forward primers containing a start codon (ATG) sequence and reverse primers containing a stop codon sequence for amplification of the genes encoding the two Δ6-desaturases were designed, respectively. Using these primers and the M. alpina genome as a template, the Δ6I and Δ6II genomic genes, which consist of 2,207 bp and 1,743 bp, respectively, were cloned. Figure 2 shows that each clone has two introns with different lengths and that they show no sequence similarity to each other. The [A+T] percentages of the Δ6I and the Δ6II introns were 50.6% and 56.0%, which were higher than 45.2% and 46.3% of the Δ6I and Δ6II encoding regions, respectively. All introns conformed to the GT-AG rule with regard to RNA splicing. Three parts of exons from the two Δ6-desaturase genomic genes showed significant similarity, 82%, to each other. Genomic Southern analysis with the two Δ6-desaturase genes of M. alpina 1S-4 as probes suggested that two Δ6-desaturase genes surely existed in this strain (data not shown).

Expression of the Mortierella Δ6-desaturase cDNAs in A. oryzae

To examine the expression of the Mortierella Δ6-desaturase genes in A. oryzae, the fatty acid compositions of these transformants were analyzed. The fatty acid composition of a selected transformant,
MLDII, found by gas-chromatography analysis, was different from that of the control strain involving only the pNGA142 vector without any insert DNA (Table 2). Two novel peaks in the chromatogram of fatty acid methyl esters from the transformant had identical retention times to the fatty-acid-methyl-ester standard of GLA and 18:4ω3. Similarly, a plasmid containing the Δ6I cDNA, designated as pMLDI, was constructed, and then the Δ6I transformant, MLDI, was selected. The courses of growth and changes in GLA content were analyzed. Representative changes with time in the mycelial mass and the concentration of fatty acids are shown in Fig. 3. Both MLDI and MLDII grew at the same rate, their dry mycelial masses reaching a peak after about 3 days of cultivation. In MLDI, the GLA content reached 0.105 mg/ml of culture medium at 3 days and then decreased gradually, whereas in MLDII it remained constant throughout the experimental period, reaching 0.121 mg/ml of culture medium at 6 days. The mycelial fatty acid compositions of MLDI and MLDII, the GLA contents of which reached the maximum, are shown in Table 2. The GLA content of MLDI reached 37.2% of the total fatty acid composition at 7 days of cultivation, whereas that of MLDI reached 30% at 3 days. The GLA and the 18:4ω3 in MLDI and MLDII were assumed to be converted from linoleic acid and α-linolenic acid on desaturation at the Δ6-position, respectively.

**Measurement of Δ6I and Δ6II RNA**

The measurement of Δ6I and Δ6II RNA copy numbers in *M. alpina* 1S-4 was done with a LightCycler system under the optimized conditions described in Materials and Methods. A 10-fold dilution series of purified pBMLDI or pBMLDII ranging from $3 \times 10^{-3}$ to 3 pg/μl was measured by RTQ-PCR with TaqMan probe. The RTQ-PCR was done with the final concentration of the primers from 500 to 900 nm and of MgCl2 from 2 to 5 mm. As a result, we selected

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**Table 2. Fatty Acid Compositions of Aspergillus Transformants**

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<tr>
<th>Fatty acid</th>
<th>Fatty acid composition (mol%)</th>
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<tbody>
<tr>
<td></td>
<td>Control strain</td>
</tr>
<tr>
<td>16:0</td>
<td>17.2</td>
</tr>
<tr>
<td>18:0</td>
<td>2.4</td>
</tr>
<tr>
<td>18:1ω9</td>
<td>10.8</td>
</tr>
<tr>
<td>18:2ω6</td>
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</tr>
<tr>
<td>18:3ω6</td>
<td>N.D.</td>
</tr>
<tr>
<td>18:3ω3</td>
<td>3.7</td>
</tr>
<tr>
<td>18:4ω3</td>
<td>N.D.</td>
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</table>

a The control strain and MLDI were grown at 30°C for 3 days in DP medium, and MLDII for 7 days.
b These values are the means of duplicate experiments.
c 16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω6, γ-linolenic acid; 18:3ω3, α-linolenic acid; 18:4ω3, Δ6,9,12,15-octadecatetraenoic acid.
d N.D., not detected.
the final concentration of 900 mM of the primers and 3 mM of MgCl₂. The slope and the error of the standard curves were −3.445 and 0.0424 for the measurement of Δ6I RNA copy numbers and −3.508 and 0.0215 for Δ6II, respectively, which are defined by the LightCycler Operator’s Manual. The total RNA samples were purified twice from the mycelia cultivated for 2, 3, or 4 days under the conditions described in Materials and Methods, and the Δ6I and the Δ6II RNA copy numbers were measured in triplicate by RTQ-PCR (Table 3). The copy numbers of Δ6I RNA increased 1.6-fold on the 3rd day and 1.4-fold on the 4th day compared with that on the 2nd day, whereas the copy numbers of Δ6II RNA fell 2.3-fold on the 3rd day and 5.3-fold on the 4th day. The ratio of the copy numbers of Δ6I RNA / the copy numbers of Δ6II RNA on the 2nd, 3rd, and 4th days was 2.4, 9, and 17. These results indicated that the quantity of Δ6I RNA in M. alpina 1S-4 was kept constant on the 2nd to 4th days, whereas that of Δ6II RNA further declined with the lapse of time.

Discussion

The industrial strain M. alpina 1S-4 accumulates unsaturated C-18 and C-20 fatty acids in its membrane and lipid bodies. In this strain, linoleic acid can be successively desaturated to GLA but not to α-linolenic acid. In the biosynthesis of PUFAs including dihomo-γ-linolenic acid and arachidonic acid, Δ6-desaturation is the first principal step for the synthesis of n-6 fatty acids. Here, we isolated two types of Δ6-desaturase cDNAs from M. alpina 1S-4 using a degenerate PCR approach based on the amino acid sequence motifs conserved in other microsomal fatty acid desaturases. This is the first report to our knowledge of the cloning of two genes encoding Δ6-fatty acid desaturases from a fungus.

Some organisms are known to have fatty acid desaturase isozymes. In higher plants, Δ12- and Δ9-desaturases generally exist in both the endoplasmic reticulum and plastids, which show a little identity to each other. Dictyostelium has two Δ5-desaturases with different substrate specificities. Several Mortierella strains are reported to have two types of Δ9-desaturases. The gene encoding one Δ9-desaturase was transcriptionally active in six M. alpina strains tested, whereas the transcript from the other gene could only be detected in one of the six M. alpina strains. In M. alpina 1S-4, we found genes encoding some isozymes of enzymes involved in PUFA biosynthesis, i.e. NADH-cytochrome b₅ reductase, Δ9-desaturase, and Δ6-desaturase. Although the transcription of two isozymes, NADH-cytochrome b₅ reductase and Δ9-desaturase, is not detected by Northern analysis, a series of such enzymes may have high activity in cells or play a preliminary function facilitating PUFA biosynthesis.

The amino acid sequences of M. alpina 1S-4 Δ6-desaturases have similarity to those of borage, C. elegans, and Synechocystis, although the sequence similarity between the last enzyme and the Mortierella one is weak but significant, particularly around the three histidine-cluster motifs that are located at corresponding positions in each sequence. Two M. alpina 1S-4 Δ6-desaturases have HPGG cytochrome b₅ motifs at the N-terminus and three conserved histidine boxes, a histidine residue in the third box being replaced by a glutamine one. These features are consistent with the structural ones of a Δ6-desaturase. They show significant identity to each other and the genomic genes contain two introns at almost the same nucleotide positions from the start codon. This implies that the two genes had the same origin and resulted from respective evolution.

In general, it is difficult to express a filamentous fungal gene encoding a membrane-bounded protein in a bacterium. An attempt was made to express the M. alpina 1S-4 Δ6-desaturase genes in A. oryzae as a suitable host. Expression of the genes led to the accumulation of GLA in the transformants. The higher GLA content of the Δ6II transformant may be produced by the higher Δ6-desaturation activity in the Δ6II transformant. The substrate specificities of the two Δ6-desaturases in yeast transformants (Saccharomyces cerevisiae) were examined by use of 16:0, 16:1(Δ9), 17:0, 18:0, 18:1(Δ9), 18:2(Δ9,12), and 18:3(Δ9,12,15) (data not shown). Both transformants were able to convert 16:1(Δ9), 18:2(Δ9,12), and 18:3(Δ9,12,15) into 16:2(Δ6,9), 18:3(Δ6,9,12), and 18:4(Δ6,9,12,15), respectively. 18:2(Δ9,12) among them was most efficiently converted into 18:3(Δ6,9,12) in the yeast transformants. In contrast to Aspergillus transformants, the accumulation of GLA in both yeast transformants required 18:2(Δ9,12) added exogenously as a substrate.

The analysis of RTQ-PCR showed that the quantity of Δ6I RNA was 2.4-, 9-, and 17-fold higher than that of Δ6II RNA on 2, 3, and 4 days in M. alpina 1S-4, respectively. This indicates that Δ6I mainly functions in Δ6-desaturation of PUFA biosynthesis in M. alpina 1S-4, while both the Δ6I and the Δ6II genes are transcribed. We think that Δ6-desaturation activity through 2 to 4 days was kept constant by considering that a sum of the Δ6I and the Δ6II RNA copy numbers is 1.6 × 10⁶, 2.0 × 10⁶, and 1.6 × 10⁷ on

<table>
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<tr>
<th>Gene type</th>
<th>RNA concentration (copy number /μg of total RNA)</th>
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<tr>
<td></td>
<td>2 Days</td>
</tr>
<tr>
<td>Δ6I</td>
<td>1.1 × 10⁷</td>
</tr>
<tr>
<td>Δ6II</td>
<td>4.6 × 10⁶</td>
</tr>
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</table>

a The data presented are derived from three independent experiments.
2, 3, and 4 days, respectively. The two types of $\Delta_6$-desaturases were assumed to function in PUFA biosynthesis in M. alpina 1S-4. The mutant Mut49 derived from M. alpina 1S-4 accumulated a non-methylene-interrupted fatty acid, 20:3$\Delta_5$($\Delta_5$,11,14), due to a defect of $\Delta_6$-desaturation.\(5\) Mut49 was also able to produce arachidonic acid through the n-6 route by means of incomplete loss of $\Delta_6$-desaturation. A complete deficiency of $\Delta_6$-desaturation would cause higher accumulation of 20:3$\Delta_5$ without arachidonic acid in the mutant. These findings may be explained by that two types of $\Delta_6$-desaturases exist in Mut49 and that one $\Delta_6$-desaturase can compensate for $\Delta_6$-desaturation.

Expression of M. alpina 1S-4 $\Delta_6$-desaturation led to the production of an oil containing 37% GLA in total fatty acids endogenously in A. oryzae. It may be possible to modify the fatty acid composition and to produce specific lipids with fungi.

Acknowledgments

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References

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