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Heterologous Expression of *Mucor rouxii* Δ^{12}-Desaturase Gene in *Saccharomyces cerevisiae*

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In this study we present the cloning and functional characterization of a gene whose product is responsible for Δ^{12}-desaturase activity and is involved in the metabolic pathway of γ-linolenic acid (GLA) synthesis of *Mucor rouxii*. A cDNA encoding for Δ^{12}-desaturase of *M. rouxii* was obtained using the combination of reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification cDNA ends (RACE) techniques. The 1191 bp code for an open reading frame of 396 amino acid residues. The deduced amino acid sequence of the cloned cDNA comprises three conserved histidine regions and two hydrophobic domains and showed similarity with microsomal ω-3 and ω-6 desaturases of plants. Expression of this open reading frame in *Saccharomyces cerevisiae* resulted in the accumulation of linoleic acid (C18:2), suggesting that this gene encodes for a membrane-bound desaturase, Δ^{12}-desaturase, of *M. rouxii* that is functional in yeast.

The growing interest in essential fatty acids for human nutrition has driven the study of mechanisms involving their biosynthesis pathway, particularly the modes of fatty acid desaturation. Essential fatty acids such as GLA can be obtained from various sources, having their origin in a plant or a microorganism. However, the bioavailabity of GLA may differ significantly among these oil sources (1). It is well established that the type of desaturases found in individual organisms controls their composition in fatty acids. The relative ratio of saturated/unsaturated fatty acids has a major role in the maintenance of cell membrane physical properties under a certain condition (2, 3). A number of Zygomycete molds contain high amount of polyunsaturated fatty acids (PUFAs). It has been reported that *Mortierella* sp. produces large amount of long chain PUFAs and 18-carbon fatty acids with the mixture of α and γ isomer of linolenic acids while some *Mucor* sp. synthesize only up to 18-carbon PUFA with γ isomer of linolenic acid (4). Thus, *Mucor* sp. are promising producers of GLA. In addition, these organisms are also potential models to elucidate the mechanisms of fatty acid biosynthesis and desaturation.

M. rouxii is a dimorphic phycomycete with the ability to grow in a yeast-like form as well as in filaments depending on the environmental conditions (5, 6) and it is capable of producing high quantity content of GLA in the lipid fraction (39.7% wt/wt) when grown in fed-batch culture (7). Studies on the biosynthesis of C18 PUFA in the fungus *Mucor* sp. have shown that three membrane-bound enzymes, Δ^9, Δ^{12} and Δ^6-desaturases accomplish the desaturation processes in GLA synthesis (8). Oleic acid is further converted by Δ^{12} and Δ^6-desaturases into linoleic acid and GLA, respectively. Recently, a gene encoding for a Δ^9-desaturase which is responsible for introduction of the first double bond into saturated palmitic and stearic fatty acids, yielding mono-unsaturated palmitoleic and oleic acids, has been cloned from *M. rouxii* and some features of its regulation have been characterized (9). In the present study, we describe the isolation and characterization of Δ^{12}-desaturase gene of *M. rouxii*. The gene function was determined using *S. cerevisiae* as a heterologous host.

MATERIAL AND METHODS

Organisms and growth. *M. rouxii* strain ATCC 24905 was grown as previously described (9). *S. cerevisiae* strain DBY746 (α, his 3-Δ1, leu 2-3, leu 2-112, ura 3-52, trp 1-289) was used as recipient strain and was grown at 30°C in either complex medium (YPD) containing 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose or synthetic minimal medium (SD) containing 0.67% bacto-yeast nitrogen base without amino acids and 2% glucose. Appropriate amino acids, L-tryptophane, L-histidine-HCl and L-leucine, were added at concentration of 20 mg/liter when required. *Escherichia coli* strain DH5α was grown at 37°C in Luria-Bertani medium (LB) supplemented with 100 mg/liter of ampicillin (10).

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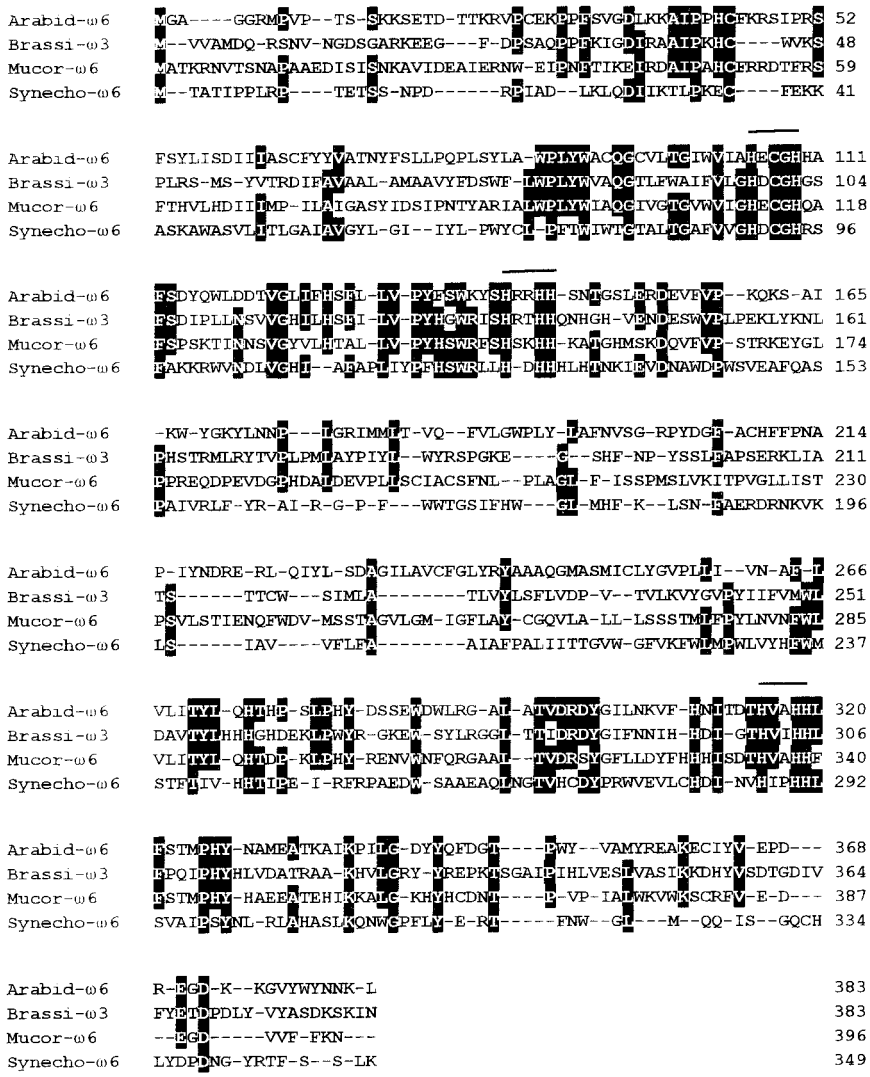

FIG. 1. Comparison of deduced amino acids of Δ^{12}-desaturase cDNA of *M. rouxii* (Mucor-*ω*6), *ω*-3 desaturase of *A. thaliana* (Arabid-*ω*6), *B. napus* (Brassi-*ω*3), and Δ^{12}-desaturase of *Synechosistis* (Synecho-*ω*6). Black backgrounds indicate identity of amino acid residues. The three conserved histidine motifs are indicated by bars.

DNA manipulations. All recombinant DNA procedures were done according to standard methods (10). DNA sequencing was performed by the dideoxy chain termination method (11) using T7 Sequenase, version 2.0 DNA sequencing kit (Amersham, Buckinghamshire, UK). Restriction enzymes were from Boheringer Mannheim (Mannheim, Germany).

Cloning of Δ^{12}-desaturase cDNA from M. rouxii. Reverse transcription-polymerase chain reaction (RT-PCR) was used for partial Δ^{12}-desaturase cDNA amplification. Mycelia of *M. rouxii* were harvested and used for RNA preparation as previously described (9). Total RNA was reverse transcribed into first-strand cDNA using oligo$(dT)_{17}$ primer and SuperScript II RNase H^- from GIBCO BRL (Paisley, UK). The first-strand cDNA was used as template for PCR using degenerated primers, 5′-GCGAATTC(A/G)TIGGTCA(T/C)-GA(T/C)TG(T/C)GGICA-3′ (forward) and 5′-GCGAATTCATIT(G/T)IGG(A/G)AAIA(A/G)(A/G)TG(A/G)TG-3′ (reverse), which contain inosine (stand by I) and an *Eco*RI site (underline) at 5′ end to facilitate subsequent manipulation. These primers were designed on the conserved histidine boxes of microsomal *ω*-3 desaturase genes of *Glycine max* (12), *Arabidopsis thaliana* (13), *Brassica napus* (12), corresponding to the amino acid sequences GHDCGH and HHLFP, respectively. Nucleotide sequences of 5′ and 3′ ends of the Δ^{12}-desaturase cDNA were amplified by the method of rapid amplification of cDNA ends (RACE). 5′ RACE System (GIBCO BRL, Paisley, UK) was used for amplification of the 5′ cDNA, following by the manufacturers' instruction and using the gene specific primers (GSP), 5′-GAAGTAGTCGAGGAGGAAGC-3′ and 5′-TGATAGTC-TTTGAAGGGCTG-3′, designed from the nucleotide sequences of RT-PCR DNA fragment. Amplification of 3′ end was performed by method of 3′ RACE as described by Innis *et al.* (14). The primers for 3′ RACE were oligo$(dT)_{17}$ adapter primer (for first strand cDNA synthesis), a 5′-CTGTTGATCGTTCTTATGGC-3′ (forward primer) and an adapter primer 5′-GAGGACTCGAGCTCAAGC-3′ (reverse primer). All PCR fragments were subcloned into pGEM-T easy vector (Promaga, Madison, WI) and transformed into *E. coli.* Subsequently, nucleotide sequences were determined. Analysis of the sequences was done with GENETYXE-WIN Version 3.1. The sequence of Δ^{12}-desaturase cDNA has been deposited in GenBank database and assigned the Accession No. AF161219.

Plasmid construction and yeast transformation. The complete Δ^{12}-desaturase cDNA from *M. rouxii* was amplified by RT-PCR using

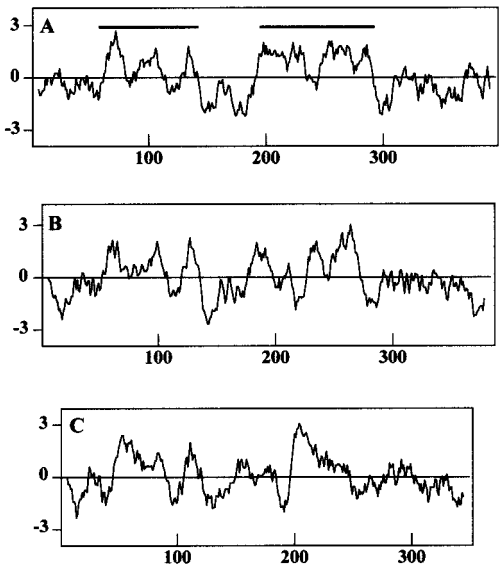

FIG. 2. Hydropathy plot of Δ^{12}-desaturase of *M. rouxii* (A), *A. thaliana* (B), and *Synechocystis sp* PCC6714 (C). Hydropathy profiles were analyzed using a Kyte–Doolittle scale (31). Numbers on the *x*-axis are amino acid residues. Two hydrophobic domains are indicated by bars.

two specific primers, 5′-CGCGGATCC**ATG**GCAACCAAGAGAAACG-3′ and 5′-GCCGAATTC**TTA**GTTCTTAAAGAAGAC-3′ which correspond to the nucleotide sequences of start and stop codons (in boldface) of the Δ^{12}-desaturase coding region, respectively. The 5′ end of both primers containing a *Bam*HI and *Eco*RI sites, respectively, were underlined to facilitate subsequent manipulation. The amplified cDNA was digested and subcloned into *Bam*HI-*Eco*RI site of the expression vector pYES2 (Invitrogen, Netherlands) to generate a plasmid designated pYCD12. *S. cerevisiae* was transformed with pYCD12 and pYES2 using alkali cation yeast transformation kit (BIO 101, Vista, CA) according to manufacturer's instruction. Transformants were selected by plating on SD agar lacking uracil and grown at 30°C for 3–4 days.

Induction of Δ^{12}-desaturase gene expression and fatty acid analysis. Expression of *M. rouxii* Δ^{12}-desaturase gene was induced under transcriptional control of the yeast *GAL1* promoter. Yeast cultures were grown to logarithmic phase at 30°C with aeration in YPG medium. Subsequently, cells were recovered by centrifugation followed by washing the cells in sterile saline prior to transferring into SD medium containing 2% galactose to induce *GAL1* promoter activity (15). Cultures were then grown aerobically to saturation for further 24 h, subsequently cells were harvested by centrifugation and washed by sterile distilled water. Cell pellets were lyophilized and used for determination of fatty acid composition by gas chromatography (GC) as previously described (9).

RESULTS AND DISCUSSION

Linoleic acid is classified to an essential fatty acid group for human since mammals lack the gene coding for Δ^{12}-desaturase enzyme (4, 16). A partial Δ^{12}-desaturase cDNA of *M. rouxii* was cloned by RT-PCR using degenerated primers which were designed based on the presence of histidine residue motifs characteristic of membrane-bound desaturases (12, 13, 17, 18, 19). A DNA fragment approximately 700 bp long amplified by RT-PCR showed amino acid sequences similar to the ω-6 and ω-3 desaturase genes of other organisms (12, 13, 20, 21). To clone the full length Δ^{12}-desaturase cDNA, 5′ and 3′ ends of the gene were amplified by RACE method. 385 bp of 5′ RACE and 365 bp of 3′ RACE were obtained and sequences determined. The nucleotide sequences of both products from RACE experiments share an identical sequence overlap on flanking regions of 5′ and 3′ end of RT-PCR DNA fragment suggesting that these fragments are portions of the same gene. The full length cDNA contains an open reading frame (ORF) of 1,911 bp encoding for 396 amino acid residues with an estimated molecular mass of 45 kDa. The ORF of this gene exhibits amino acid sequence homology with other desaturases (Fig. 1). It shows 34% identity (62% similarity) with microsomal ω-6 desaturase of *Arabidopsis thaliana* (22) 31% identity (58% similarity) with microsomal ω-3 desaturase of *B. napus* (12) and 16% identity (42% similarity) with *desA* of *Synechocystis* (23). This result reveals that the ORF has more homology with ω-6 desaturase than ω-3 desaturase of plants although we designed primers for the RT-PCR based on plant ω-3 desaturases. Nevertheless, it also shares common characteristics to known membrane-bound desaturases (9, 12, 13, 17, 18, 23). *M. rouxii* ORF comprises three histidine-cluster motifs that are conserved in other membrane-bound desaturases. Moreover, the hydropathy profile of amino acid sequences of this protein shows two hydrophobic domains similar to plant and cyanobacteria Δ^{12}-desaturases (22, 23) which have membrane spanning

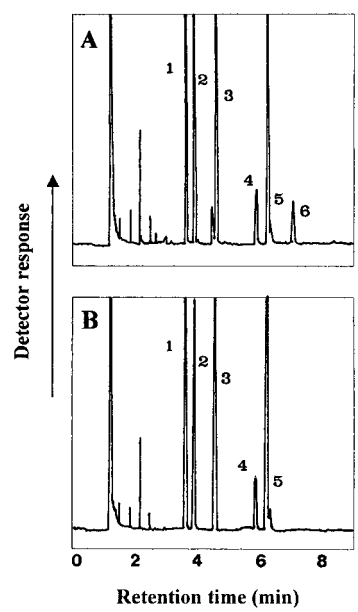

FIG. 3. Gas chromatograms of total fatty acid from the induced yeast cells containing pYES2 (A) and pYCD12 (B). Peaks 1, C16:0; 2, C16:1(Δ-9); 3, C17:0 (internal standard); 4, C18:0; 5, C18:1(Δ-9); 6, C18:2 (Δ-9,12).

TABLE 1

Fatty Acid Composition of Total Lipid from Yeast Transformants Containing pYES2 and pYCD12

	Relation fatty acid composition (mol %)				
Transformants	C16:0	C16:1	C18:0	C18:1	C18:2
pYES2	21.6	46.2	5.6	26.6	—
pYCD12	23.6	37.6	6.9	8.8	22.7

Note. Values represent the averages of three independent transformants. The fatty acid composition of each sample was analyzed twice.

domains (Fig. 2). Each conserved histidine-region is located in the hydrophilic portion that may be located on the cytoplasmic surface of the membrane. These features coincide with a model of the topology of membrane-bound desaturases (24). More recently, a Δ^{12}-desaturase gene has been cloned from a fungus, *Mortierella alpina* (25). We found that the gene of *M. rouxii* also has high homology with the Δ^{12}-desaturase of *M. alpina*. Therefore, we propose that this gene encodes for a putative Δ^{12}-desaturase of *M. rouxii*. Jackson *et al.* have reported that the Δ^{12}-desaturase of the fungus *Mucor circinelloides* introduces double bonds into oleate which is esterifed to glycerolipid (8). The homology of *M. rouxii* Δ^{12}-desaturase with enzymes from plant ω-6 and ω-3 desaturases and cyanobacteria Δ^{12}-desaturase might support the hypothesis that the cloned gene belongs to a member of acyllipid desaturase group.

Cytochrome b_5-domains have been discovered in either the C- or N-terminus of several membrane-bound desaturases such as Δ^9-desaturase of *S. cerevisiae* (26) and *M. rouxii* (9), Δ^6-desaturase of *Borago officinalis* (17), *Caenorhabditis elegans* (27) and rat (28) and sphingolipid desaturase of *A. thaliana* (29). However, this domain is absent in the Δ^{12}-desaturase gene of *M. rouxii* as well as in Δ^{12}-desaturase of plant and *M. alpina* (25). Nevertheless, it has been reported that cytochrome b_5 is required as an electron donor for the desaturation in microsomal membranes of fungi (8).

To study the function of the putative Δ^{12}-desaturase gene of *M. rouxii*, the 396 amino acids of coding region were amplified and subcloned into the yeast expression vector, pYES2, downstream of the galactose inducible *GAL1* promotor. Control (pYES2) and recombinant (pYCD12) plasmids were introduced into yeast and the expression was induced by the addition of 2% galactose. Fatty acid compositions of the cultures transformed with pYES2 and pYCD12 were analyzed as shown in Fig. 3 and Table 1. The GC analysis revealed that a novel dienoic fatty acid (C18:2, Δ-9,12) was found in pYCD12 transformants (22.6% in total lipid) but could not be detected in the control. The reduced amount of oleic acid in pYCD12 transformants was caused by the conversion of oleic into linoleic acid. Thus, the accumulation of linoleic acid in the transformed yeast indicates that the *M. rouxii* cDNA encodes for a protein with Δ^{12}-desaturase activity. This function was accomplished in yeast using its component of redox system, NADH-dependent cytochrome b_5 reductase and cytochrome b_5.

The regulation of genes involved in desaturation pathway has been investigated in various organisms. The expression of fatty acid desaturase genes depends on several factors such as age of culture, species and interacting environment (2, 9, 30, 31). We found that expression of *M. rouxii* Δ^9-desaturase gene was affected by low temperature and cell growth (9). It would be of interest to determine whether the regulation of gene expression of Δ^{12}-desaturase relates to that of Δ^9-desaturase gene in *M. rouxii*. In conclusion, we have identified and functionally characterized the Δ^{12}-desaturase gene encoding an enzyme in GLA synthesis pathway of *M. rouxii*.

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