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Δ^6-Desaturase of *Mucor rouxii* with High Similarity to Plant Δ^6-Desaturase and Its Heterologous Expression in *Saccharomyces cerevisiae*

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Gamma-linolenic acid (GLA, γ-C18:3) is an essential fatty acid that plays a vital role in biological structures and cellular functions. Based on available sequence information and using polymerase chain reaction (PCR) technique, we cloned from the fungus *Mucor rouxii* the entire coding sequence of a Δ^6-desaturase enzyme, which is responsible for the transformation of linoleic acid into GLA. The deduced amino acid sequence of *M. rouxii* gene showed the highest homology with the plant Δ^6-desaturase. It comprises the characteristics of membrane-bound desaturases, including histidine-rich motifs and hydrophobic regions. A cytochrome b_5-like domain was observed at the N-terminus. In addition to three conserved histidine-rich motifs, we found an additional histidine-rich motif, HKHHSH, downstream of the cytochrome b_5-like domain, which is not present in previously cloned Δ^6-desaturase genes. Heterologous expression of the *M. rouxii* cDNA in *Saccharomyces cerevisiae* resulted in the synthesis and accumulation of GLA. © 2000 Academic Press

Key Words: **Δ^6-desaturase; essential fatty acid; gamma-linolenic acid; *Mucor rouxii; Saccharomyces cerevisiae;* cytochrome b_5.**

Unsaturated fatty acids (UFAs) are required in lipids of biological membranes and storage depots of eukaryotes. Recently, the identification of the therapeutic properties of essential fatty acids has been an active area of research in human nutrition (1). It is becoming increasingly clear that ω-6 and ω-3 fatty acids have critical roles functioning as precursors for the biosynthesis of hormone-like compounds known as eicosanoids, that are important for the proper functionality of the human body (1, 2). Regulation of cell membrane structure and eicosanoids metabolism through dietary supplements may help to maintain good health. In ω-6 fatty acids, γ-linolenic acid (GLA) is a conditionally essential fatty acid due to the possible impairment of the Δ^6-desaturase activity or imbalance intake between ω-6 and ω-3 fatty acids in humans (1, 3, 4). It has been documented that certain organisms, including plants, filamentous fungi and cyanobacteria, produce substantial portions of GLA (5–7). Filamentous fungi, such as zygomycetes, are considered to be potentially an important economical source for the production of GLA (8, 9). However, some fundamental understanding on the general mechanisms related to biosynthesis of polyunsaturated fatty acids (PUFAs) in such organisms must be obtained to achieve higher yield and quality of the desired product.

In fungi, a multi-enzyme complex, which is composed of cytochrome b_5, NADH-dependent cytochrome b_5 reductase and fatty acid desaturase catalyzes PUFAs formation (10). Three desaturases, Δ^9-, Δ^{12}- and Δ^6-desaturases are involved in the introduction of double bonds at the specific positions of fatty acyl chain to form GLA. Genes coding for those desaturases have been cloned from diverse organisms ranging from higher eukaryotes to prokaryotes. In our work, we have focused on the metabolic control of fatty acid biosynthesis and desaturation in *M. rouxii*, which is a phycomycete capable of GLA production (11). This fungus synthesizes predominantly C18 PUFAs without the presence of long chain fatty acids facilitating the downstream process. In addition, *M. rouxii* is also an excellent microorganism used as a simple model for studying, in eukaryotes, metabolic pathways of PUFAs formation. In this regard, our initial task was to iden-

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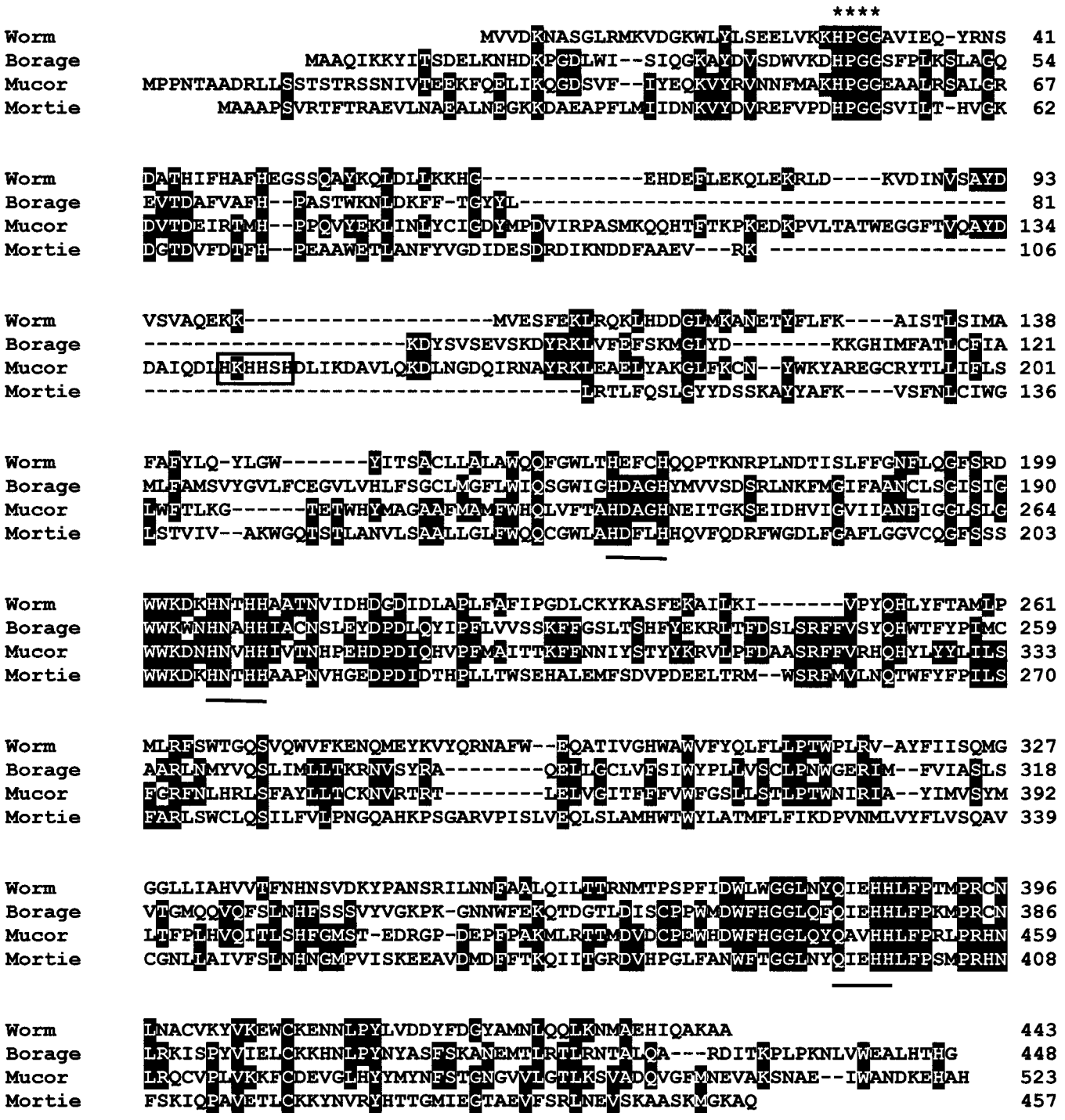

FIG. 1. Sequence alignment of the deduced amino acids of the *M. rouxii* Δ^6-desaturase (Mucor) with the Δ^6-desaturases of *C. elegans* (Worm), *B. officinalis* (Borage) and *M. alpina* (Mortie). Black backgrounds indicate identity of amino acid residues. The three conserved histidine-rich motifs are underlined. The unusual histidine-rich motif found in Δ^6-desaturase of *M. rouxii* is marked by a box. The cytochorme b_5-like domain is indicated by asterisks.

tify *Mucor* genes associated with the synthesis of PUFAs as genetic tools for a comprehensive study. Recently, we have cloned and characterized the Δ^9- and Δ^{12}-desaturase genes from *M. rouxii* which are responsible for the formation of mono- and di-ene fatty acids (12, 13). Some of the features of the regulation of Δ^9-desaturase gene expression in this fungus have been characterized (12). In this study, the Δ^6-desaturase gene of *M. rouxii* coding for a key enzyme which is involved in the transformation of linoleic acid into GLA

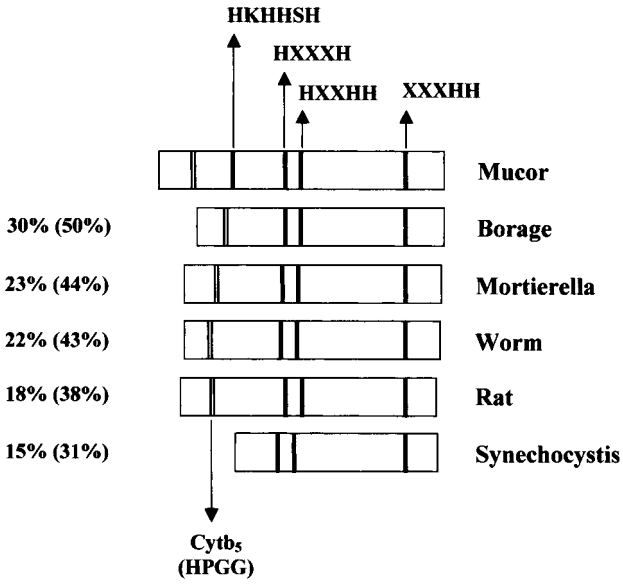

FIG. 2. Percentage of amino acid identity (similarity) of the Δ^6-desaturase of *M. rouxii* (Mucor) to *B. officinalis* (Borage), *M. alpina* (Mortierella), *C. elegans* (Worm), rat and *Synechocystis*. Single vertical lines and double vertical lines represent the relative locations of histidine-rich motifs and cytochrome b_5 domain (cyt b_5), respectively.

has been identified and its functional expression was assessed in the yeast, *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Strains and growth conditions. *M. rouxii* ATCC 24905 was grown as previously described (12). The recipient strain used for functional analysis of the cloned *Mucor* gene was *S. cerevisiae* DBY746 (α, his 3-Δ1, leu2-3, leu2-112, ura 3-52, trp 1-289) 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 a concentration of 20 mg/litre when needed.

Probe construction. The available sequence information in the database at the National Center for Biotechnology Information (NCBI) and reverse transcriptase-polymerase chain reaction (RT-PCR) approach were employed to construct a homologous probe for the Δ^6-desaturase gene of *M. rouxii*. Total RNA of *M. rouxii* was extracted as previously described (12). Template for PCR was generated by reverse transcription of *M. rouxii* total RNA using Super-Script II RNase H$^-$ (GIBCO BRL) and Oligo $(dT)_{15}$ primer. The PCR was done using degenerate primers designed from the conserved amino acid regions of Δ^6-desaturases of several organisms such as the fungus (*Mortierella alpina*) (14, 15), plant (*Borago officinalis*) (16) and nematode (*Caenorhabditis elegans*) (17). The forward and reverse primers were 5′-TGGTGGAA(A/G) (G/T) (A/G)GAAICA(C/T)AA(C/T) (A/G)CICA(C/T)CA-3′ and 5′-GTIGG(G/A)AAIA(G/A) (G/A)TG(G/A)TG(T/C)TC-3′ corresponding to the amino acid sequences WWK(D/W) (K/N)HN(A/T)HH) and EHHLFPT, respectively. The amplified product was subcloned into pGEM-T easy (Promega) and then sequenced.

Cloning of M. rouxii Δ^6-desaturase genomic gene. To clone the full-length of *M. rouxii* Δ^6-desaturase gene, the homologous probe carrying the partial Δ^6-desaturase cDNA was used for screening the genomic library of *M. rouxii* using plaque hybridization technique (18). Southern blot of the positive recombinant clone was done to localize the Δ^6-desaturase gene. The restricted DNA fragment containing the gene was subcloned into pGEM7Z+. Nucleotide sequence was determined subsequently using the dideoxy chain termination method on both strands (19).

Cloning of M. rouxii Δ^6-desaturase cDNA and plasmid construction for its functional analysis. To identify the presence of introns and to analyze the function of the cloned gene of *M. rouxii*, the cDNA encoding for the putative Δ^6-desaturase was cloned by RT-PCR using a pair of specific primers, 5′-CGCGGATCCATGCCCCCAAATACTGCG-3′ (forward primer) and 5′-GCCGAATTCCTAATGAGCGTGTTCTTTATC-3′ (reverse primer), which correspond to the coding regions of the initiation and termination of the *M. rouxii* genomic gene, respectively. The *Bam*HI and *Eco*RI restriction sites were added at the 5′ end of the forward and reverse primers, respectively, to facilitate further manipulation. The PCR product was subcloned into the vector pYES2 (Invitrogen) downstream of the GAL1 promoter to generate a recombinant plasmid designated pMG87. Its sequence was determined and compared to the genomic sequence. The sequences of *M. rouxii* Δ^6-desaturase gene and cDNA have been assigned the GenBank Accession Nos. AF290983 and AF296076, respectively.

Heterologous expression of the M. rouxii cDNA in S. cerevisiae and fatty acid analysis. The pMG87 plasmid harbouring the putative Δ^6-desaturase cDNA and pYES2 plasmid were transformed into *S. cerevisiae* by the poly-ethyleneglycol method (20). The recombinant yeasts were selected on a uracil-deficient medium. Expression of the *M. rouxii* gene was induced under transcriptional control of GAL1 promoter. Cultures were grown to logarithmic phase at 30°C in SD medium supplemented with 0.5 mM linoleic acid, C18:2 (Sigma). Subsequently, cells were centrifuged and then transferred into SD medium containing 2% galactose. Cultures were then grown for 24 h at 25°C. Cells were harvested by centrifugation and washed with distilled water. The wet cells were used directly to determine fatty acid composition as previously described (12). Fatty acids were identified by comparing their retention times to those of fatty acid methyl ester standards (Sigma). The areas of chromatographic peaks were calculated for relative amounts of fatty acid.

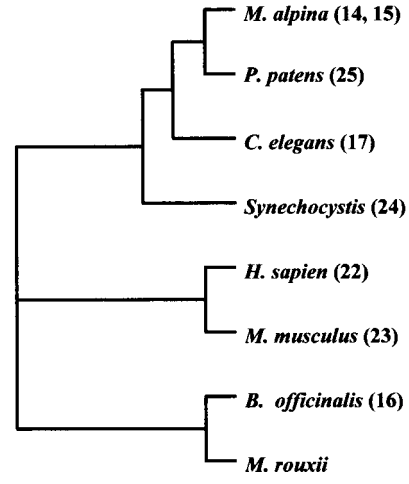

FIG. 3. Phylogenetic analysis of Δ^6-desaturase from various organisms. Dendogram was generated using the CLUSTAL-X and TreeView programs. Numbers in parentheses indicate the references for the sequences.

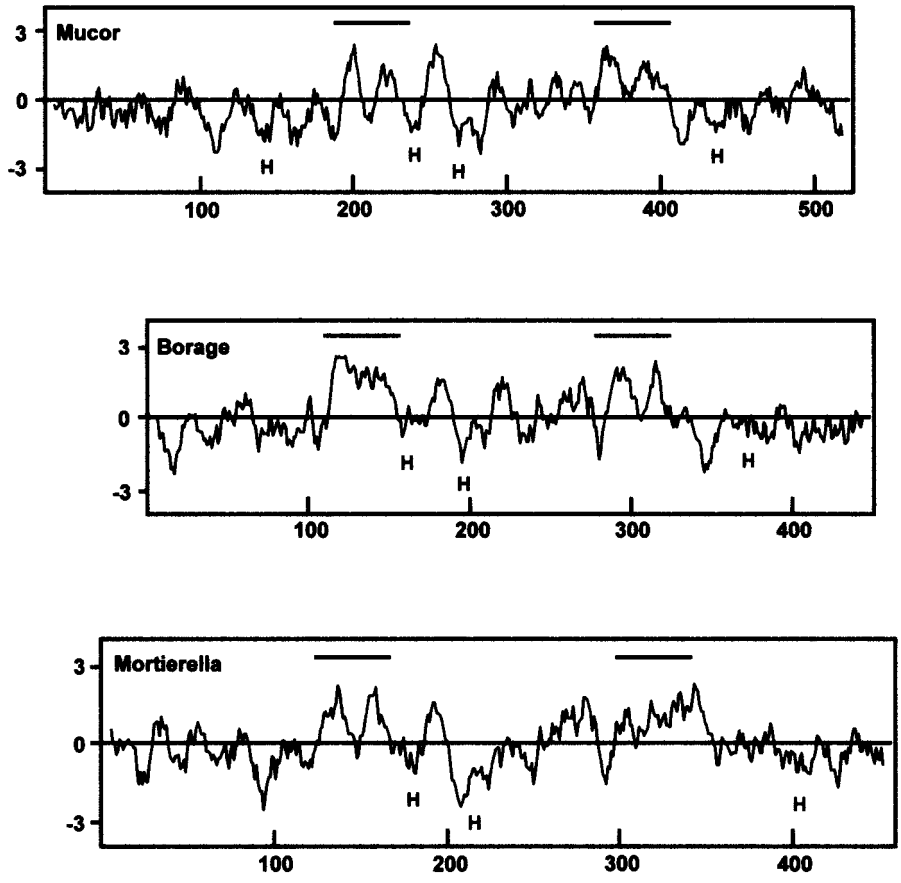

FIG. 4. Hydropathy profile of Δ^6-desaturase of *M. rouxii* (Mucor), *B. officinalis* (Borage) and *M. alpina* (Mortierella). The plots were analyzed using the method of Kyte–Doolittle [31]. Bars indicate two hydrophobic domains. H letters represent the histidine-rich motifs.

RESULTS AND DISCUSSION

Identification and Characterization of Δ^6-Desaturase Gene of M. rouxii

In the present study, we identified the gene responsible for Δ^6-desaturation from *M. rouxii*. Based on the conserved histidine-rich motifs II and III reported in Δ^6-desaturase of other organisms, a 570-bp DNA fragment coding for an incomplete Δ^6-desaturase gene of *M. rouxii* was obtained by RT-PCR technique. One positive recombinant clone was selected from the screening of the genomic library of *M. rouxii* using the partial gene as a probe. Southern blot analysis of the positive clone revealed that a 5-kb *Bam*HI-*Eco*RI fragment contained the full-length gene. The *Bam*HI-*Eco*RI fragment was subcloned into pGEM7Z+ and then sequenced. Analysis of the nucleotide sequence showed an open reading frame coding for a protein of 523 amino acids with a calculated molecular mass of 60,625 Da, with no introns. The absence of introns was confirmed by comparison of the full-length cDNA isolated by RT-PCR and the genomic sequence of the gene. The deduced amino acid sequence revealed high homology with previously cloned Δ^6-desaturases of other organisms, as shown in Fig. 1. The percentages of amino acid similarity and identity of the cloned gene from *M. rouxii* to other Δ^6-desaturase genes are shown in Fig. 2. Surprisingly, it has higher similarity with plant (borage) than its own counterpart (*M. alpina*). It has been demonstrated that borage produces C18 fatty acids containing double bonds at the 6, 9, and 12 positions that are similar to *M. rouxii* whereas *Mortierella* produces up to C20 fatty acids (15, 16). Moreover, biochemical study on GLA biosynthesis in microsomal membrane of *Mucor circinelloides* and oil seed membrane of borage showed similarity of the Δ^6-desaturation with respect to substrate utilization or the reaction type (21). Taken together, it seems likely that the Δ^6-desaturation of *M. rouxii* is more closely related to that of borage corresponding to the result of phylogenetic analysis of various Δ^6-desaturases as shown in Fig. 3. Notably, the length of deduced amino acids of *Mucor* gene was longer than the Δ^6-desaturases of many organisms including *M. alpina* (14, 15), human (22), rat (23), *Synechocystis* (24) and borage (16). However, its length is closely similar to Δ^6-desaturase of mosses, *Physcomitrella patens* (525 amino acid residues) and *Ceratodon purpureus* (520 amino acid residues) which contain the putative N-terminal extensions with unknown function (25, 26).

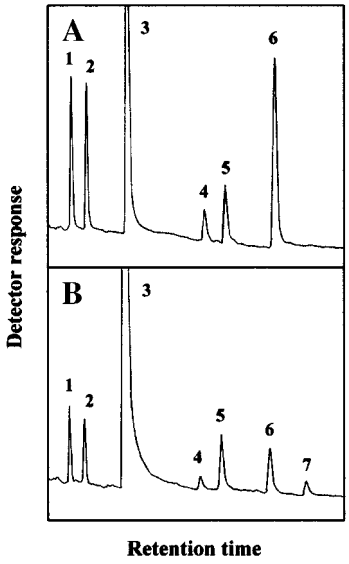

FIG. 5. Chromatograms of fatty acid methyl esters from the yeast transformed with plasmid pYES2 (A) or pMG87 (B). Linoleic acid was added into the growth medium as substrate for Δ^6-desaturase. Peaks 1, C16:0; 2, C16:1(Δ9); 3, C17:0 (internal standard); 4, C18:0; 5, C18:1(Δ9); 6, C18:2(Δ9, 12); 7, C18:3(Δ6, 9, 12).

Nevertheless, this N-terminal extension present in the mosses was not found in the cloned gene of *M. rouxii*. In this regard, the structure of the gene was characterized. It revealed the presence of histidine-box motifs and hydrophobic regions known to be characteristics of membrane-bound desaturases (27). Moreover, the consensus heme-binding motif (HPGG) designated as cytochrome b_5-like domain present in many Δ^6-desaturases was also found at the N-terminus as shown in Fig. 1.

Previously, we have identified the genes involved in fatty acid desaturation, Δ^9-, and Δ^{12}-desaturase genes in *M. rouxii*. Like many organisms, the cytochrome b_5-like domain was found at the C-terminus of Δ^9-desaturase and at the N-terminus of Δ^6-desaturase of *M. rouxii* whereas this domain was not present in the *M. rouxii* Δ^{12}-desaturase. So far, there is no evidence to explain convincingly the presence of the fusion cytochrome b_5-desaturase, although this tethering has been proposed to have an essential role in transferring electrons during the desaturation process (28, 29). Therefore, these enzymes represent novel members of the cytochrome b_5 superfamily (30).

Interestingly, in addition to conserved histidine-rich motifs I, II and III corresponding to the sequences HXXXH, HXXHH and QXXHH, respectively, we found in *Mucor* Δ^6-desaturase gene an unusual histidine-rich motif (HKHHSH, positions 141–146) downstream of the cytochrome b_5 domain, which has not been reported previously in other Δ^6-desaturase genes. The additional histidine-rich motif is located between amino acid positions 96–155. Alignment of amino acid residues of Δ^6-desaturases indicated a stretch of the 60 amino acid residues (positions 96–155) of the *Mucor* gene, which is absent in the others (Fig. 1). Like other membrane-bound desaturases, the hydropathy plot of *M. rouxii* Δ^6-desaturase revealed the existence of two hydrophobic regions that are critical features of membrane-anchored proteins, as shown in Fig. 4. The first hydrophobic region is located between the new and unusual histidine-rich motif and canonical histidine-rich motif I, while the second hydrophobic region is located between the histidine-rich motifs II and III. It has been reported that the histidine-rich motifs, required for enzymatic activity as iron ligands, reside on the cytoplasmic side of the endoplasmic reticulum (27). Thus it is possible that the additional histidine-rich domain might play some essential catalytic role in Δ^6-desaturation in this fungus.

Functional Analysis of the M. rouxii Δ^6-Desaturase

The presence of the conserved domains reveals that the cloned gene of *M. rouxii* codes for a putative membrane-bound Δ^6-desaturase. Its function was confirmed by heterologous expression in *S. cerevisiae* supplemented with linoleic acid as substrate, which is not normally produced by this yeast. Figure 5 shows the fatty acid profile of the yeast cells transformed with pYES2 and pMG87 plasmids. A novel peak with a retention time identical to that of the authentic GLA was identified in the cells carrying pMG87. However, this peak was absent when the yeast containing pMG87 was not supplemented with linoleic acid (data not shown). The fatty acid composition of the cells transformed with pYES2 and pMG87 is given in Table 1. GLA in the yeast transformants harboring pMG87 plasmid was about 7.1% of total fatty acids. These results demonstrate conclusively that the cloned gene from *M. rouxii* is responsible for Δ^6-desaturation involved in GLA formation. In summary, we have identified and functionally characterized the Δ^6-desaturase gene coding for an enzyme catalyzing GLA biosynthesis in *M. rouxii*. This is the first report to identify the Δ^6-desaturase containing four histidine boxes with an un-

TABLE 1

Relative Fatty Acid Composition from the Yeast Transformants Containing pYES2 and pMG87

Fatty acid	pYES2 (mol%)	pMG87 (mol%)
C16:0	21.5	19.9
C16:1 (Δ9)	12.5	16.6
C18:0	5.7	5.2
C18:1 (Δ9)	19.6	27.3
C18:2 (Δ9, 12)	40.8	23.9
γ-C18:3 (Δ6, 9, 12)	—	7.1

Note. Values represent the average of three independent transformants. The fatty acid composition of each sample was analyzed twice. Cells were grown in the presence of 0.5 mM linoleic acid.

usual histidine box. For its functional characteristics in the yeast and high similarity to higher plants, *M. rouxii* Δ^6-desaturase gene may be used to generate transgenic plants or microorganisms with the potential for exploitation in GLA production.

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