Identification and characterization of a novel Δ^6-fatty acid desaturase gene from *Rhizopus arrhizus*

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Abstract A cDNA sequence putatively encoding a Δ^6-fatty acid desaturase was isolated from *Rhizopus arrhizus* using reverse transcription polymerase chain reaction and rapid amplification of cDNA ends methods. Sequence analysis indicated that this cDNA sequence had an open reading frame of 1377 bp encoding 458 amino acids of 52 kDa. The deduced amino acid sequence showed high similarity to those of fungal Δ^6-fatty acid desaturases which comprised the characteristics of membrane-bound desaturases, including three conserved histidine-rich motifs and hydropathy profile. A cytochrome b_5-like domain was observed at the N-terminus. To elucidate the function of this novel putative desaturase, the coding sequence was expressed heterologously in *Saccharomyces cerevisiae* strain INVScI. The result demonstrated that the coding product of the sequence exhibited Δ^6-fatty acid desaturase activity by the accumulation of γ-linolenic acid.

Key words: Δ^6-Fatty acid desaturase gene; γ-Linolenic acid; *Rhizopus arrhizus*; *Saccharomyces cerevisiae*

1. Introduction

Fatty acid desaturases play essential roles in fatty acid metabolism and the maintenance of proper structure and function of biological membranes in living organisms. There are two main types of fatty acid desaturase: the soluble and membrane-bound desaturases, both of which are diiron-oxo enzymes [1]. The soluble desaturases that introduce double bonds into fatty acids esterified to acyl carrier proteins are restricted to higher plants, and they have two conserved histidine-rich motifs [2], while membrane-bound desaturases which distribute widely and introduce double bonds into fatty acids esterified to acyl-CoA and glycerolipids are characterized by three histidine-rich motifs and four transmembrane domains [1,3]. Among the membrane-bound desaturases, microsomal desaturases can be further divided into two groups: carboxyl-directed and methyl-directed desaturases. Carboxyl-directed desaturases, known as 'front-end' desaturases such as Δ^4-, Δ^5- and Δ^6-desaturases [4–6], introduce a new double bond between the existing double bond and carboxy-terminus of the fatty acyl chain. This mode differs from the methyl-directed membrane-bound desaturases such as Δ^{12}-desaturase [7] that introduce a new double bond between the existing double bond and methyl-terminus of the fatty acyl chain. Both carboxyl-directed and methyl-directed desaturases have their characteristic consensus motifs.

With the growing number of desaturase genes isolated from various organisms, the knowledge of the enzymatic reactions and corresponding metabolic pathways is well elucidated. In general, Δ^6-desaturase catalyzes the conversion of linoleic acid (LA, $18{:}2\Delta^{9,12}$) and α-linolenic acid ($18{:}3\Delta^{9,12,15}$) to γ-linolenic acid (GLA, $18{:}3\Delta^{6,9,12}$) and stearidonic acid ($18{:}4\Delta^{6,9,12,15}$) respectively. Subsequently, the resultant fatty acids can be further introduced into the biosynthesis of long-chain polyunsaturated fatty acids (PUFAs) such as arachidonic acid ($20{:}4\Delta^{5,8,11,14}$) and eicosapentaenoic acid ($20{:}5\Delta^{5,8,11,14,17}$) through an alternating series of desaturation and elongation. These fatty acids are important components of cellular structure and function and serve as precursors of physiologically active molecules such as prostaglandins, thromboxanes and leukotrienes [8,9]. GLA has been claimed to play a crucial role in development and prevention of some skin diseases, diabetes, reproductive disorder and others [10,11]. GLA is commonly obtained from plants such as evening primrose, borage and blackcurrant [9]. Attempts have been made to produce GLA from fungi as well [12]. However, it is evident that GLA production from current sources is inadequate for supplying the expanding market due to the significant problems of low productivity, complex and expensive downstream processing and unstable quality [9,13]. Modification of the fatty acid biosynthesis pathways by genetic manipulation to produce desired oil in transgenic microorganisms and oilseed crops, as a possible alternative source, has been investigated, and this led to the isolation of the gene encoding Δ^6-desaturase from various organisms. Nevertheless, only a few of them have been isolated from fungi [14–17] by now, even though fungi represent an important group of PUFA producers.

Rhizopus arrhizus is phylogenetically related to *Mucor* sp. and also synthesizes PUFAs only up to C18, especially the γ isomer of linolenic acid. Identifying the genes associated with the synthesis of GLA will contribute to characterizing the desaturase genes in *R. arrhizus*, establishing a simple model for studying metabolic pathways of PUFAs in eukaryotes and providing the primary basis for the future application of gene engineering to GLA production. In this study, we describe the identification and characterization of the Δ^6-desaturase gene encoding an enzyme involved in the GLA biosynthesis in *R. xarrhizus*.

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Abbreviations: LA, linoleic acid; GLA, γ-linolenic acid; PUFAs, polyunsaturated fatty acids; FAME, fatty acid methyl esters

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2. Materials and methods

2.1. Organisms and growth conditions

R. arrhizus strain NK030037 was grown at 28°C for 3 days in a liquid medium containing 2% glucose, 1% bacto-yeast extract, 0.2% KH_2PO_4 and 0.1% $MgSO_4$, with pH adjusted to 6.0. *Saccharomyces cerevisiae* strain INVScl was used as recipient in transformation experiments and was grown at 30°C in complex medium (YPD) containing 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose.

2.2. Total RNA preparation

Mycelia were harvested by filtration and washed with phosphate-buffered saline buffer. The dried mass was frozen in liquid nitrogen, ground with mortar and pestle into a fine powder. Total RNA was extracted from the powder according to the method of Chomczynski and Sacchi [18] based on guanidinium thiocyanate and stored at −70°C for future use.

2.3. Cloning of partial R. arrhizus Δ^6-desaturase cDNA

First-strand cDNA was synthesized with the first-strand cDNA Synthesis kit (Promega, Madison, WI, USA) and was used as a template for reverse transcription polymerase chain reaction (RT-PCR) amplification with degenerate primers designated according to available sequence information of Δ^6-desaturase of *Mortierella alpina* [14], *Mucor rouxii* [15], and *Pythium irregulare* [16] at the National Center for Biotechnology Information (NCBI). The forward primer and the reverse primer were 5′-TGGTGGAAGRAYAAICAYAACIYK-CAYCA-3′ and 5′-GGGAAYARRTGRTGCTCRATCTG-3′ corresponding to the conserved histidine-rich motifs II and III WWKD-KHNTHH and QIEHHLFP, respectively. These primers (0.2 μM) were run on a Biometra™ T-gradient thermal cycler using a program of 1 min at 94°C, 1 min at 46°C and 2 min at 72°C for five cycles, followed by 1 min at 94°C, 1 min at 58°C and 1.5 min at 72°C for 30 cycles, finally extended at 72°C for 10 min. The amplified product of expected length (~600 bp) was subcloned into pGEM-T vector (Promega) and then sequenced (TaKaRa Bio, Dalian, China).

2.4. Cloning of full-length cDNA

To obtain the full-length sequence of *R. arrhizus* Δ^6-desaturase cDNA, the method of rapid amplification of cDNA ends (RACE) was adopted to determine the nucleotide sequences of 3′ and 5′ ends. Amplifications were carried out with the SMART™ RACE cDNA Amplification Kit (BD-Clontech, Palo Alto, CA, USA) using the gene-specific primers 5′-GCCATCCAATCCTTACTCTA-CTC-3′ (3′RACE) and 5′-TGTGGGTTCTTCATCCAGTGAGG-CA-3′ (5′RACE) designed from the nucleotide sequences of RT-PCR DNA fragments, following the manufacturer's instruction with the cycling parameters: 1 min at 95°C, followed by 30 s at 95°C and 3 min at 68°C for 30 cycles, finally extended at 68°C for 3 min. All PCR fragments were subcloned into pGEM-T vector and nucleotide sequences were determined subsequently. Based on the information of 5′ and 3′ end sequences, two gene-specific primers (forward primer: 5′-ATCTTCTTTTCTTTCACTATTTTCAAT-3′, reverse primer: 5′-GTTGAACTAAAAGTGATTATTTGATC-3′) were designed to amplify full-length cDNA by the RACE program. The resultant cDNA sequence has been deposited in the GenBank database and assigned the accession number AY320288.

2.5. Plasmid construction and yeast transformation

Two specific primers, 5′-CACGGTACC**ATG**AGTACATCAGA-AGTACATCAGATCGTCAATCAG-3′ and 5′-CTCGCATGC**TT**A-AATGACTTTTTGCTCAATTGC-3′ which correspond to the nucleotide sequences of start and stop codons (in bold face) of *R. arrhizus* Δ^6-desaturase gene (RAD6), respectively, were used to obtain the full-length coding region. The 5′ ends of the forward primer and the reverse primer contain a *Kpn*I and an *Sph*I restriction site (underlined) respectively to facilitate subsequent manipulation. The amplified cDNA was digested and subcloned into the *Kpn*I-*Sph*I site of the expression vector pYES2.0 (Invitrogen, Beijing, China) to generate a plasmid designated pYRAD6. *S. cerevisiae* was transformed with pYRAD6 and pYES2.0 using the lithium acetate method [19]. Transformants were selected by plating on synthetic minimal medium agar lacking uracil (SC-Ura) and grown at 30°C for 2–3 days.

2.6. Heterologous expression in S. cerevisiae

Heterologous expression of RAD6 was induced under transcriptional control of the yeast GAL1 promoter. Yeast cultures were grown to logarithmic phase at 30°C in synthetic minimal medium (SC-Ura) containing 2% raffinose, 0.67% yeast nitrogen, 0.1% tergitol NP-40 (sigma), supplemented with 0.5 mM linoleic acid (Sigma). The cells were induced by the addition of 2% galactose and cultivated for a further 48 h at 20°C. Subsequently, cells were harvested by centrifugation, and washed three times with sterile distilled water. The cells were dried and ground with a mortar and pestle into a fine powder.

2.7. Fatty acid analysis

Cellular fatty acid was extracted by incubating 100 mg yeast powder in 5 ml 5% KOH in methanol for saponification at 70°C for 5 h. After the pH was adjusted to 2.0 with HCl, the fatty acid was subjected to methyl-esterification with 4 ml 14% boron trifluoride in methanol at 70°C for 1.5 h. Subsequently, fatty acid methyl esters (FAME) were extracted with hexane after addition of saturated sodium chloride solution. FAME were analyzed by gas chromatography (GC; GC-9A, Shimadzu, Kyoto, Japan) and identified by the comparison of their peaks with that of standards (Sigma). Lauric acid (C12:0) methyl ester was use as internal standard for quantitative analysis of fatty acids. Qualitative analysis of FAME was performed by GC-mass spectrometry (GC-MS) using a HP G1800A GCD system (Hewlett-Packard, Palo Alto, CA, USA). Both analyses were carried out with the same polar capillary column (HP, 5.30 m×0.25 mm internal diameter, 0.25 mm internal film thickness). The mass spectrum of a new peak was compared with that of the standard for identification of fatty acid.

3. Results and discussion

3.1. Isolation of R. arrhizus Δ^6-fatty acid desaturase gene

Several genes encoding Δ^6-desaturase have been identified from fungi. The presence of Δ^6-desaturase isozymes in *Mucor circinelloides* is reported by Michinaka et al. [17]. Two conserved amino acid sequences, the second histidine-rich motif (WWKDKHNTHH) and the third histidine-rich motif (QIEHHLFP), found in these fungal desaturases by alignment were used to design two degenerate oligonucleotide primers for RT-PCR. A 593 bp DNA fragment was amplified from *R. arrhizus*. The deduced amino acid sequence of the amplified fragment showed high identity to *M. circinelloides* (63% identity), *M. alpina* (50%), *P. irregulare* (38%) and *Caenorhabditis elegans* (37%) Δ^6-desaturase, which indicated that a partial gene sequence encoding a novel putative Δ^6-desaturase was isolated from *R. arrhizus*. To clone the full-length Δ^6-desaturase cDNA, the RACE method was used to amplify the 5′ and 3′ ends of this gene. 781 bp of 5′ RACE and 614 bp of 3′ RACE were amplified and sequences were determined. The nucleotide sequences of both products from RACE experiments shared an identical 141 bp and 367 bp sequence overlap on flanking regions of the 5′ and 3′ ends of the RT-PCR DNA fragment, suggesting that these fragments are portions of the same gene. Therefore, 1482 bp full-length cDNA sequence information was obtained and confirmed by cloning and sequencing with the primers derived from the 5′ and 3′ ends. Sequence analysis revealed that the cDNA sequence contained an open reading frame of 1377 bp, designated RAD6, encoding 458 amino acid residues with an estimated molecular mass of 52.3 kDa. An ATG translation initiation codon was identified in the sequence of the 5′ terminus (28–30 bp), and a TAA termination codon was found 1375 nucleotides downstream of the initiation site. The coding region was flanked by a 27 bp 5′ untranslated region of the mRNA, along with a full 78 bp 3′ untranslated region with the characteristics of two putative polyadenylation sites, AATAAA

and AAATAA, located at 40 bp and 22 bp upstream of the poly(A) tail [20].

3.2. Sequence comparison of Δ^6-fatty acid desaturases from fungi

The comparison of the deduced amino acid sequence of RAD6 with the fungal Δ^6-desaturases given above by alignment in Fig. 1 revealed three conserved histidine-rich motifs (amino acid positions 173–177, 210–214, and 395–399) and hydrophobic regions known to all membrane-bound desaturases [21]. A cytochrome b_5-like domain HPGG for the cytochrome b_5 superfamily that is required for fatty acid desaturation as electron donor was observed at the N-terminus [2,22,23]. Alignment of those homologous sequences indicated that the homology occurs mainly in the cytochrome b_5-like domain and in the three conserved histidine-rich motif areas. RAD6 showed a high amino acid sequence identity to Δ^6-desaturases of *M. circinelloides* (56.5% identity), *M. alpina* (47.9%), and *P. irregulare* (34.5%), but a relatively low identity to that of *M. rouxii* (25.6%) which showed a higher identity to plant desaturases instead [15]. Phylogenetic analysis also demonstrated the homology between RAD6 and Δ^6-desaturase from various organisms (Fig. 2). RAD6 clustered with the main group of Δ^6-desaturases from fungi, *C. elegans* [24] and moss [25–27], which are distinguished from Δ^6-desaturases from higher plants [28,29] and animals [30–34]. Furthermore, the hydropathy profile of amino acid sequences revealed the presence of two membrane-spanning domains similar to fungal desaturase that are characteristics of membrane-bound desaturases (Fig. 3). All of the above suggested that the novel sequence encoded a putative Δ^6-desaturase involved in the synthesis of GLA in *R. arrhizus*.

Similar to all the Δ^6-desaturase except that of *Synechocystis* sp. PCC 6803 [35], the sequence of the third conserved histidine-rich motif started with a glutamine Q instead of a histidine H, which is a conserved feature of all 'front-end' desaturases, Δ^8-acyl-lipid desaturase and Δ^8-sphingolipid desaturase [36,37]. But some amino acid residues in these motifs are different in some organisms [5,36,37]. Residue leucine L in the third motif QIEHHLF is conserved in all Δ^6-desaturases but is replaced by the residual valine V^{400} in *R. arrhizus* Δ^6-desaturase. Site-directed mutations of the borage Δ^6-desaturase gene have shown that the replacement of L by other residues reduced the activity of the enzyme, but did not alter

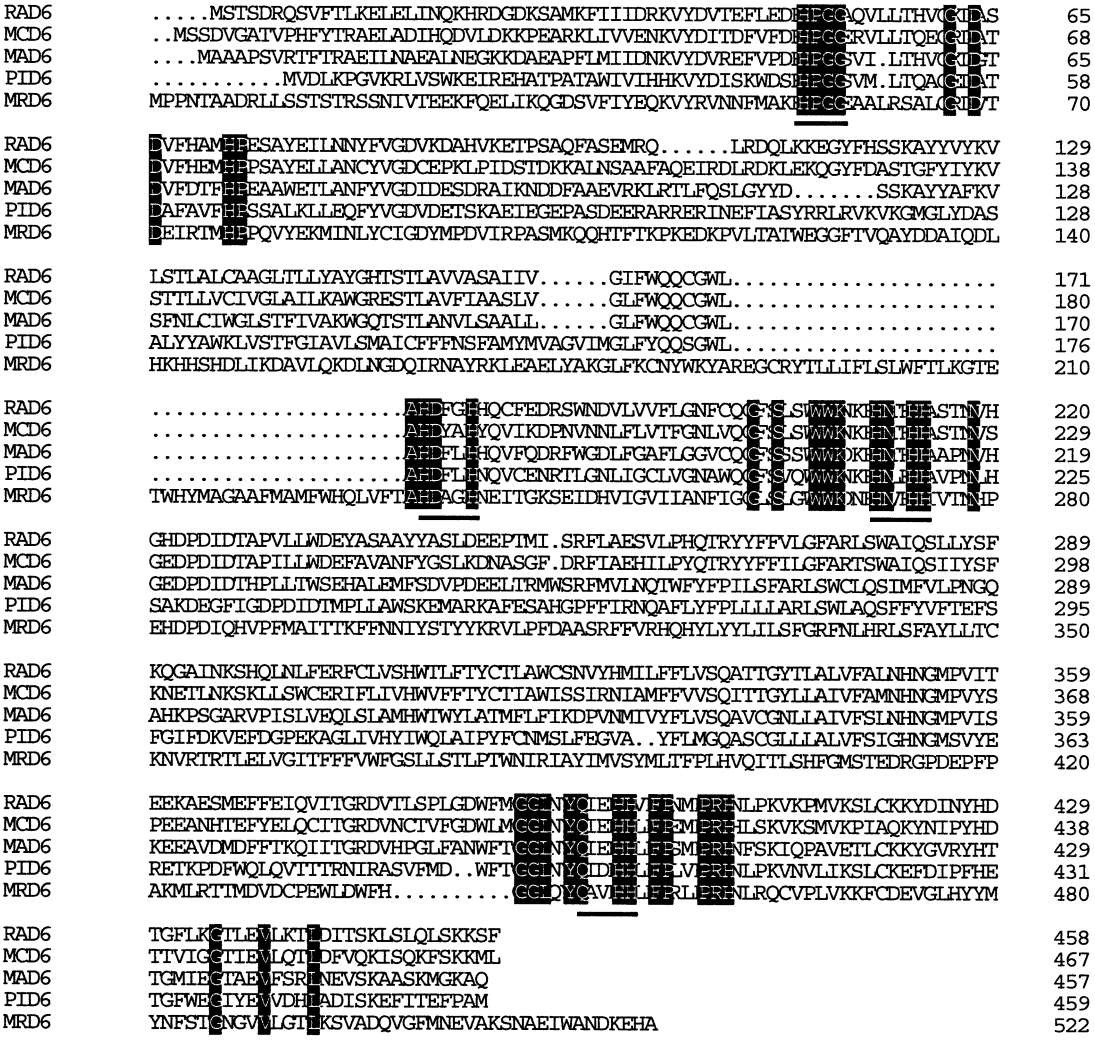

Fig. 1. Sequence alignment of deduced amino acids of the *R. arrhizus* Δ^6-desaturase (RAD6) with the Δ^6-desaturase of *M. circinelloides* (MCD6), *M. alpina* (MAD6), *P. irregulare* (PID6) and *M. rouxii* (MRD6). Black background indicates identity of amino acid residues. The three conserved histidine-rich motifs and a cytochrome b_5-like domain are underlined.

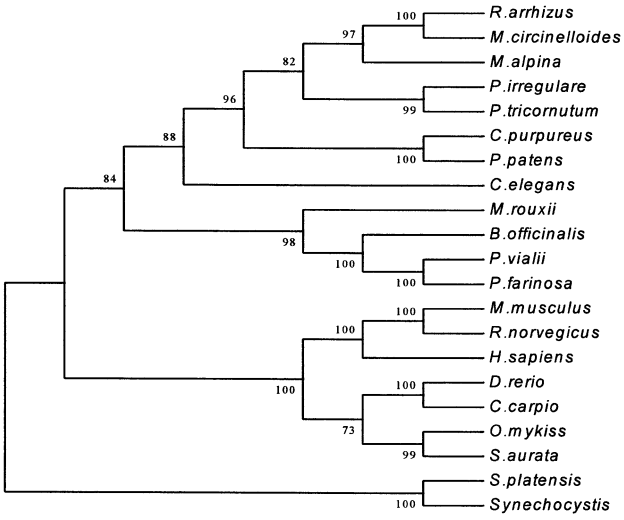

Fig. 2. Phylogenetic relationship between Δ^6-desaturases from various organisms. Sequence alignment and phylogenetic tree construction were done by CLUSTAL X v.1.81 and MEGA v.2.1. *Mucor circinelloides* (BAB69055), *Mortierella alpina* (AF110510), *Pythium irregulare* (AF419296), *Phaeodactylum tricornutum* (AY082393), *Ceratodon purpureus* (AJ250735), *Physcomitrella patens* (AJ222980), *Caenorhabditis elegans* (AF031477), *Cyprinus carpio* (AF309557), *Mus musculus* (AF126798), *Rattus norvegicus* (NM_031344, *Homo sapiens* (AF084559), *Sparus aurata* (AY055749), *Danio rerio* (AF309556), *Oncorhynchus mykiss* (AF301910), *Mucor rouxii* (AF296076), *Borago officinalis* (U79010), *Primula vialii* (AY234127), *Primula farinosa* (AY234125), *Spirulina platensis* (X87094), *Synechocystis* sp. (L11421).

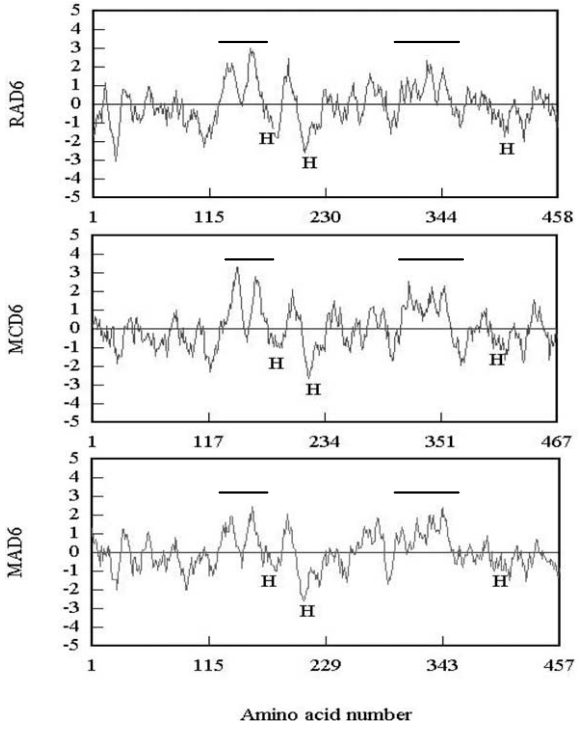

Fig. 3. Hydropathy profile of Δ^6-desaturase of *R. arrhizus* (RAD6), *M. circinelloides* (MCD6), and *M. alpina* (MAD6). The plots were analyzed using the Kyte–Doolittle methods [40]. Bars indicate the two hydrophobic domains.

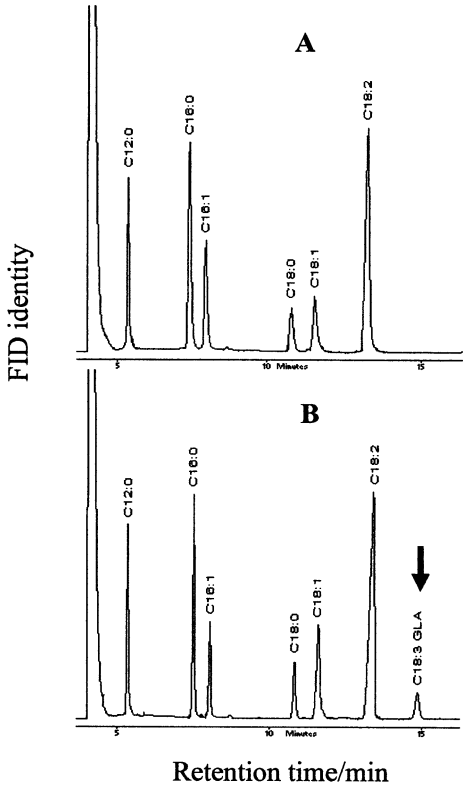

Fig. 4. Identification of GLA in transgenic *S. cerevisiae* by GC analysis with lauric acid (C12:0) as the internal standard. A: *S. cerevisiae* transformed with control vector pYES2.0. B: *S. cerevisiae* transformed with recombinant plasmid pYRAD6. The arrowhead indicates the novel peak of GLA.

the specificity [38]. The influence of this replacement on the expression level of *R. arrhizus* Δ^6-desaturases gene in yeast should be further investigated.

3.3. *Functional analysis of the* R. arrhizus *Δ^6-fatty acid desaturase*

To elucidate the function specificity of this putative desaturase, RAD6 was subcloned into pYES2.0 under the control of the inducible GLA1 promoter. The recombinant plasmid pYRAD6 was then transformed into *S. cerevisiae* INVScl for heterologous expression. The transformed yeast cells were induced with 2% galactose, supplemented with LA as substrate. Fatty acid analysis indicated the exogenous LA was incorporated into the lipid of the transformed yeast (Fig. 4). GC analysis of FAME revealed that a novel fatty acid peak corresponding to GLA methyl ester standard was detected in the yeast transformed with pYRAD6, which was absent in the yeast containing empty vector pYES2.0. The percentage of this new fatty acid was 4.53% of total fatty acids (Table 1). GC-MS analysis of this fatty acid methyl derivative demon-

Table 1
Fatty acid compositions (wt%) of total lipid from yeast transformants containing pYES2.0 and pYRAD6

Transformant	Relative fatty acid composition					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
pYES2.0	22.21	12.59	7.71	10.09	47.52	0
pYRAD6	20.67	8.73	7.52	13.11	44.37	4.53

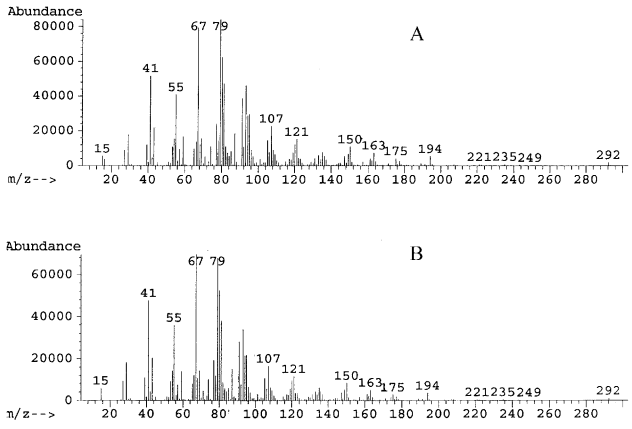

Fig. 5. GC-MS analysis of the novel peak identified in *S. cerevisiae* transformed with pYRAD6. A: *S. cerevisiae* transformed with PYRAD6. B: GLA standard.

strated that the novel peak was GLA methyl ester (Fig. 5). The mass peak $m/z = 292$ indicated the molecular mass of the methyl derivative GLA, and the fragmentation pattern was identical to that of the standard. These results showed that pYRAD6 encoded a Δ^6-fatty acid desaturase. The expressed enzyme converted the incorporated LA to GLA specifically. Moreover, the regulation of desaturase gene expression has been investigated in various organisms. Temperature is one of several factors that influence the gene expression [3,39]. In our study, we also observed that the GLA content showed a tendency to increase with a decrease of cultivating temperature from 30°C to 20°C (3.1% at 30°C). These results suggested that RAD6 was likely to play a role for yeast cells in acclimatizing to low temperature, which could attribute to an improvement of membrane fluidity by increasing the degree of desaturation of cellular fatty acids.

In summary, the role of PUFAs in pathophysiology has attracted great interest in recent years. Comprehensive research into fatty acid biosynthesis and metabolism is required. As a prerequisite, the genes and the gene products involved in the above-mentioned processes need to be identified and characterized. In this study, we have isolated and functionally characterized the gene encoding a Δ^6-fatty acid desaturase involved in GLA biosynthesis in *R. arrhizus*. And this work should be useful for further research of e.g. PUFA metabolic pathways and gene engineering about GLA production.

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