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A novel Δ^{12}-fatty acid desaturase gene from methylotrophic yeast *Pichia pastoris* GS115★

D. SH. Wei, M. CH. Li, X. X. Zhang, H. Zhou and L. J. Xing✉

Tianjin Key Laboratory of Microbial Functional Genomics, Department of Microbiology, Nankai University, Tianjin 300071, PR China; ✉e-mail: Xinglaijun@126.com

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The methylotrophic yeast *Pichia pastoris* GS115, a widely used strain in production of various heterologous proteins, especially membrane-bound enzymes, can also produce linoleic and linolenic acids, which indicates the existence of membrane-bound Δ^{12} and Δ^{15}-fatty acid desaturases. This paper describes the cloning and functional characterization of a novel Δ^{12}-fatty acid desaturase gene from this methylotrophic yeast. The open reading frame of the gene (named Pp-FAD12) is 1263 bp in size and encodes a 420-amino-acid peptide. The deduced Pp-FAD12 protein shows high identity (50–67%) with Δ^{12}-fatty acid desaturases from other fungi. It also shows a high identity (57%) with Δ^{15}-fatty acid desaturase (named Sk-FAD15) from *Saccharomyces kluyveri*. Expression of Pp-FAD12 in polyunsaturated fatty acids non-producing yeast *Saccharomyces cerevisiae* demonstrated that its product converted oleic acid (18 : 1) to linoleic acid (18 : 2). This result suggests that Pp-FAD12 encodes a novel Δ^{12}-fatty acid desaturase in *P. pastoris* GS115. This is the first report about the cloning and functional characterization of Δ^{12}-fatty acid desaturase gene in methylotrophic yeast.

Keywords: *Pichia pastoris*, fatty acid desaturase gene, linoleic acid, *Saccharomyces cerevisiae*

INTRODUCTION

The metabolisms of *P. pastoris* relating to the utilization of methanol as a carbon resource has been extensively investigated, because *Pichia pastoris* GS115 has been widely used for high level expression of heterologous proteins, especially membrane-bound enzymes (Cregg *et al.*, 2000) that are very important both in applied and in academic field, taking advantage of its very strong and specific inducible AOX promoter (Ellis *et al.*, 1985). Relatively little is known about its fatty acid desaturation mechanisms. Analyzing its fatty acid composition with Gas Chromatography (GC) indicated that apart from the ordinary monounsaturated fatty acids (MUFAs) such as 16 : 1, 18 : 1 commonly seen in *Saccharomyces cerevisiae*, *P. pastoris* GS115, like higher plants, also produces several polyunsaturated fatty acids (PUFAs) including linoleic (18 : 2) and linolenic acid (18 : 3). These substances are considered to be precursors of highly valued substances such as eicosapentaenoic acid and docosahexaenoic acid in various fields, including medicine and nutrition. But, unlike *Hansenula polymorpha* and *P. augusta*, *P. pastoris* GS115 also produce 17:1 which is rarely detected in other yeasts. Also, 16:2 was not detected with GC analysis in *P. pastoris* GS115. However, the phylogenetically related yeasts such as *H. polymorpha* and *P. augusta* can produce 16:2 (Leu *et al.*, 2000) which is also produced by *S. kluyveri*, one of the PUFAs-producing yeast in which Δ^{12}-fatty acid desaturase is responsible for converting oleic acid to linoleic acid and C16:1 to C16:2 (Kyoko *et al.*, 2004). There is no Δ^{12}-fatty acid desaturase sequence information from methylotrophic yeast available that can be used for analyzing this difference. The third interesting characteristic is that although *P. pastoris* strain GS115 contains linolenic acid, no Δ^{15}-fatty acid desaturase

★Accession numbers: The accession number of the new amino-acid sequence of Δ^{12}-fatty acid desaturase reported in the manuscript is AAX20125.

Abbreviations: FAD, fatty acid desaturase; FAME, fatty acid methyl esters; GC, gas chromatography; GSP, gene specific primer; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

gene commonly responsible for production of α-linolenic acid has been cloned from methylotrophic yeasts until now. All these characteristics plus the available genetic tools for *P. pastoris* GS115 make it a suitable model for investigating the fatty acid desaturation mechanism.

In the present study, we present the cloning and characterization of the Δ^{12}-fatty acid desaturase gene from *P. pastoris*. Functional characterization of this Δ^{12}-fatty acid desaturase gene in the PUFAs-nonproducing yeast *S. cerevisiae* and its phylogenetic relationship with other organisms are discussed.

MATERIALS AND METHODS

Organisms and growth conditions. *P. pastoris* strain GS115 (*his- mut*$^+$) was purchased from Invitrogen and grown at 28°C for 2 days in liquid medium YPD containing 2% glucose, 2% peptone, 1% yeast extract. *S. cerevisae* strain INVScI (*His*$^-$, *Leu*$^-$, *Trp*$^-$, *Ura*$^-$ was used as a recipient strain in transformation experiments and grown at 30°C in complex medium containing 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose. *Escherichia coli* strain DH5α (genotype: F$^-$, ϕ80d*lacZ*ΔM15, Δ(*lacZ*YA-argF) U169, *deoR*, *recA*1, *endA*1, *hsdR*17(r^{K-}m^{K+}), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1) was grown at 37°C in Luria–Bertani medium (LB) supplemented with 100 mg/l of ampicillin.

Total RNA preparation. Yeast cells were harvested by centrifugation and washed three times with phosphate buffer. The extraction of total RNA was done according to the method of Chomczynski and Sacchi (1987). RNA was stored at –70°C for future use.

DNA manipulation. Restriction endonucleases and other DNA-modifying enzymes were obtained from TaKaRa Bio, Dalian, China Co. Ltd. All recombinant DNA procedures were done according to standard methods (Sambrook *et al.*, 1989). DNA sequencing was done by the dideoxy chain termination method using T7 sequence as sequencing primer and version 2.0 DNA sequencing kit.

PCR-based cloning of Δ^{12} desaturase gene. Two degenerate sense primers (SP1, SP2) and one antisense primer (ASP1) (Table 1) were selected to clone the conserved region of this kind of desaturase from genomic DNA prepared as previously described (Lee, 1992) with a touch-down PCR program (Don *et al.*, 1991): 94°C 5 min for denaturation, the annealing temperature was decreased 2°C every two cycles from 65°C to 45°C. At this temperature, 20 cycles were carried out, followed by extension for 10 min at 72°C. Full-length cDNA was obtained by the method of SMART rapid amplification of cDNA ends (RACE) 5′-RACE System (BD Clontech, Palo Alto, CA, USA) and 3′-RACE method as described by Frohman (1990). All PCR fragments were subcloned into pGEM-T vector (Promega, Madison, WI, USA) and transformed into *E. coli* strain DH5α. Sequence analysis was done with DNAMAN software (version 4.0, Lynnon BioSoft, Quebec, Canada).

Plasmid construction and yeast transformation. The ORF of Δ^{12}-fatty acid desaturase from *P. pastoris* was amplified by RT-PCR using two specific primers GSP5 and GSP6 (Table 1) corresponding to the nucleotide sequence of start and stop codon (in boldface) of Δ^{12}-fatty acid desaturase gene, respectively. 5′-end of the two primers contained *Bam*HI and *Xba*I restriction sites underlined respectively facilitate subsequent manipulation. PCR product was digested and subcloned into the expression vector pYES2.0 (Invitrogen, Beijing, China) to generate a recombinant plasmid designated pYPP-FAD12. *S. cerevisiae* was transformed with pYPP-FAD12 and empty pYES2.0 using electroporation (Hinnen *et al.*, 1978). Transformants were selected by plating on complex synthetic minimal medium agar lacking uracil (SC-Ura) and grown at 30°C for 2–3 days.

Inducible expression of the Δ^{12}-fatty acid desaturase gene. The putative Δ^{12}-fatty acid desaturase gene was heterologously expressed in yeast, which was induced under the transcriptional control of GAL1 promoter. Yeast cultures were grown to logarithmic phase at 30°C in synthetic minimal medium containing 2% galactose, 0.67% yeast nitrogen. Subsequently, cells were harvested by centrifugation followed by washing the cells three times in sterile

Table 1. Primers used in cloning and characterization of Δ^{12}-fatty acid desaturase gene in this study

Primers	Sequence (5′ to 3′)	Target amino-acid sequence or usage
SP1	CA(TC)GA(AG)TG(TC)GGCA(TC)CA(CA)G	HECGHQ, for partial DNA cloning
SP2	AA(AG)CA(TC)CA(TC)AA(AG)GC(AGT)AC	KHHKATG, for partial DNA cloning
ASP1	(AG)TG(AG)TGIGCIAC(GA)TGIGT	THVI/AHH, for partial DNA cloning
GSP1	GGCGTTTCTC TTTTCCAAG	Cloning of 5′-end of cDNA
GSP2	TGACCTGGGTATCTTGGCACAG	Cloning of 3′-end of cDNA
GSP3	AGGAGATTACTGTTAATAAAAGTAGGGTAC	Cloning of full length cDNA, sense primer
GSP4	AAAATTTAAACATAACTTAATCTAC	Cloning of full length cDNA, antisense primer
GSP5	CC***GGACC*ATG**TCTGCCGTCACAGTTACAG	Cloning of ORF, sense primer, containing *Bam*HI restriction site
GSP6	GGC***TTCTAGAC*TA**TTTCTCACCGGTTC-3	Cloning of ORF, antisense primer, containing *Xba*I restriction site

water. The cells were dried and ground into a fine powder for determination of fatty acid composition by gas chromatography and for gas chromatography-mass spectrometry (GC-MS) analysis.

Fatty acid analysis. Total fatty acid was extracted from the cells by treating 100 mg of yeast powder with 5 ml 5% KOH in methanol for saponification at 70ºC for 5 h. The pH of the product was adjusted to 2.0 with HCl (6 N) before the fatty acid was methyl-esterified with 4 ml of 14% boron trifluoride in methanol at 70º for 1.5 h. Then, fatty acid methyl esters (FAME) were solubilized with hexane after addition of saturated sodium chloride solution. FAME were analyzed by gas chromatography (GC; GC-9A, Shimadzu, Kyoto, Japan) and identified by comparing their peaks with those of standards (Sigma). Heptadecanoic acid (C17:0) methyl ester (Sigma) was used as an internal standard for quantitative analysis of fatty acids. Qualitative analysis of FAME was performed by GC-MS using an HP G1800A GCD system (Hewlett–Packard, Palo Alto, CA, USA). All analysis was carried out with the same polar capillary column (HP, 5.30 mU, 0.25 mm internal diameter, 1 m long). The mass spectrum of novel peaks was compared with those of the standards for identification of the fatty acids.

Phylogenetic relationships analysis among Δ^{12}-fatty acid desaturase genes. The Δ^{12}-fatty acid desaturase amino-acid sequences used in this study were retrieved from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) (Table 2). Sequences were selected to cover organisms containing Δ^{12}-fatty acid desaturase genes that have been functional identified and avoiding 'redundancy'. Amino-acid sequences were aligned using the CLUSTAL X v. 1.81 programs (Thompson *et al.*, 1997). The final alignment was further refined after excluding the poorly conserved regions at the protein ends, and consisted of 400 positions spanning three histidine-rich boxes. The alignment was used to generate a preliminary neighbor-joining unrooted tree. The neighbor-joining (Saitou & Nei, 1987) analysis was performed with the MEGA v.2.1 software package (Kumar *et al.*, 2001) using Poisson–corrected distances and ignoring deletions in pairwise comparisons. Bootstrap (Felsenstein, 1985) with 500 replicates was performed to establish the confidence limit of the tree branches. Parsimony procedures were carried out using the PHYLIP v. 3.2 (Phylogeny Inference Package) software (Felsenstein, 1989). Bootstrap was performed in 100 replicates on the alignment file, and the resulting file was fed to the PROTPARS protein parsimony utility under the multiple data set option. The consensus tree was generated by the CONSENSE program with the majority rule option, and bootstrap values were assigned to each node.

Table 2. List of organisms and membrane desaturase protein sequences analyzed in this study

Organism	Accession No.	Label
Pichia pastoris	AAX20125	d12Pichia
Saccharomyces kluyveri	BAD08375	d12Saccharomyces
Aspergillus parasiticus	AAP23194	d12Aspergillus
Cryptococcus curvatus	AAU12575	d12Cryptococcus
Mortierella alpine	AAF08684	d12Mortierella
Helianthus annuus	AAX19388	d12Helianthus
Arabidopsis thaliana	AAG51042	d12Arabidopsis
Brassica napus	AAS92240	d12Brassica
Lentinula edodes	BAD51484	d12Lentinula
Sesamum indicum	AAF80560	d12Sesamum
Chlorella vulgaris	BAB78716	d12Chlorella
Mucor rouxii	AAM97924	d12Mucor
Nicotiana tabacum	AAT72296	d12Nicotiana
Glycine max	BAD89861	d12Glycine
Synechococcus sp	AAF21445	d12Synechococcus
Cyanobacteria bacterium	YP_479096	d12Cyanobacteria
Rhizopus oryzae	AAV52631	d12Rhizopus

RESULTS

Isolation of *P. pastoris* Δ^{12}-fatty acid desaturase gene

Three conserved amino-acid sequences found in previously reported Δ^{12}-fatty acid desaturase genes from several fungi were used as the basis for designing three degenerate primers for PCR. A fragment of 681 bp was first generated using primers SP1 and ASP1 corresponding to the conserved histidine-box 1 and 3 motif (Fig. 1), respectively. Semi-nested PCR with primers SP2 (corresponding to conserved histidine-box 2) and ASP1 were used to further validate the result of the first ground PCR. It indeed generated a shorter sequence (not shown). Sequence analysis showed that the longer fragment encodes 227 amino acids. No intron was found. The partial amino-acid sequence has 70% identity with the *S. kluyveri* Δ^{12}-fatty acid desaturase gene and 68% identity with that from *Candida albicans*. These results indicated that a fragment of a putative Δ^{12}-fatty acid desaturase gene was isolated from *P. pastoris*. Nucleic acid sequence information was then used to design gene specific primers GSP1 and GSP2 (Table1) for 5′ and 3′ RACE. A 641 bp fragment of 5′-RACE and a 531 bp fragment of 3′-RACE were amplified and sequences were determined. Nucleotides of both products shared identical sequence overlap on the flanking region of the cloned 5′- and 3′-end of the partial DNA fragment, suggesting that these fragments are portions of the same gene. Two gene specific primers (GSP3 and GSP4) corresponding to

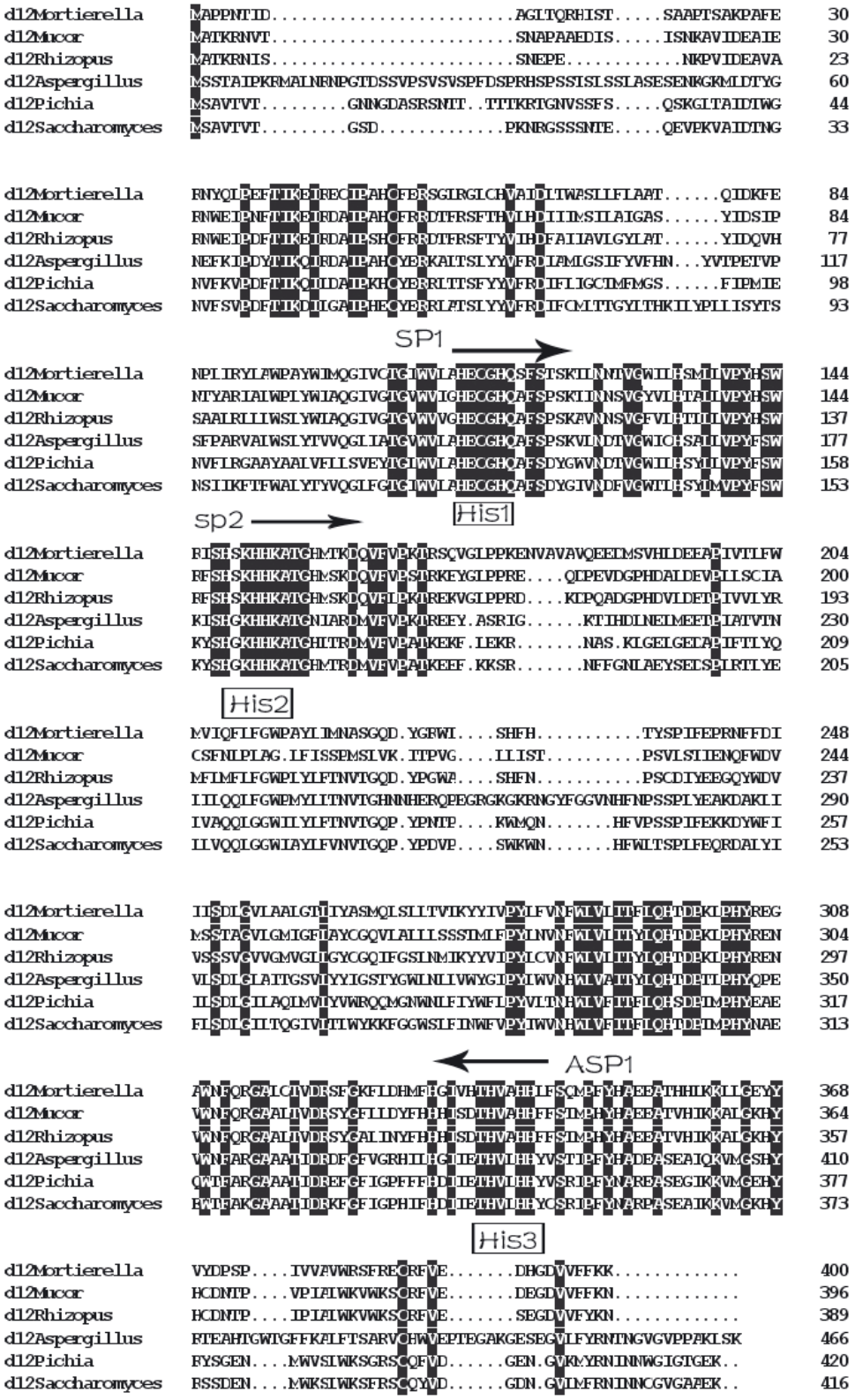

Figure 1. Comparison of the deduced amino-acid sequences of the *P. pastoris* d12 desaturase with other fungal d12 desaturases.

The amino-acid sequences of d12 desaturases from *P. pastoris* (d12Pichia), *Aspergillus parasiticus* (d12Aspergillus), *Mortierella alpina* (d12Mortierella), *Saccharomyces kluyveri* (d12Saccharomyces), *Rhizopus arrhizus* (d12Rhizopus) and *Mucor rouxii* (d12Mucor) are used. Sequences were aligned using the FASTA algorithm. The positions of the three histidine boxes are indicated by boxes numbered His 1, 2, and 3. The arrows indicate the amino-acid sequences used in the design of the primers SP1, SP2 and ASP1. Identical amino acids were shaded with black and similar amino acid with gray.

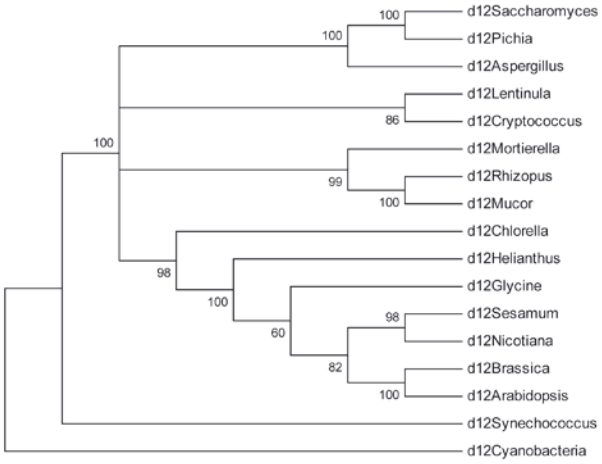

Figure 2. Phylogenetic relationship among Δ^{12}-fatty acid desaturases from various organisms.
Sequence alignment and phylogenetic tree were done using CLUSTAL X v. 1.81 and MEGA v. 2.1.

the 5′- and 3′-end of the full-length cDNA were used to clone the full-length cDNA.

Sequence analysis indicated that the full-length cDNA contains an open reading frame of 1263 bp encoding 420 amino-acid residues with an estimated molecular mass of 48.4 kDa. The coding region was flanked by a 84 bp 5′ untranslated region of the mRNA along with a full 46 bp 3′ untranslated region with the characteristic of a putative polyadenylation site, AAATA, located 18 bp upstream of the poly(A) tail. The resultant cDNA sequence and the deduced amino-acid sequence have been submitted into the GenBank database and assigned the Accession No. AAX20125.

Sequence comparison of Δ^{12}-fatty acid desaturase genes from filamentous fungi, yeast and plants

Alignment of the deduced amino-acid sequence from Pp-FAD12 (designated d12Pichia) with other fungal Δ^{12}-fatty acid desaturases identified functionally showed that it exhibited 64% identity with *S. kluyveri* and 41–43% with filamentous fungi Δ^{12}-fatty acid desaturase. The predicted Δ^{12}-fatty acid desaturase also contained three conserved histidine-rich motifs (Fig. 1), and two hydrophobic regions found in all membrane-bound desaturases (Tocher *et al.*, 1998). The homology occurs mainly in three conserved histidine-rich motifs and their surrounding sequences. These results suggested that this gene encodes a putative Δ^{12}-fatty acid desaturase involved in the synthesis of linoleic acid in *P. pastoris*. Although it showed the highest identity with *S. kluyveri,* the substrate difference between the two Δ^{12}-fatty acid desaturases remains to be elucidated. A phylogenetic tree of the amino-acid sequence of *P. pastoris* Δ^{12}-fatty acid desaturase with all the functionally identified Δ^{12}-fatty acid desaturases was generated. When displayed on an unrooted NJ tree, the highest degree of relatedness between d12Saccharomyces and d12Pichia was evident in the phylogram. Surprisingly, d12Pichia was more related to d12Aspergillus than to d12Cryptococcus. This tree illustrates the distinct evolutionary path of Δ^{12}-fatty acid desaturase genes among plants, filamentous fungi and yeast (Fig. 2). The topology of parsimony-based tree (unpublished) agreed well with the phylogenetic tree derived using NJ procedure.

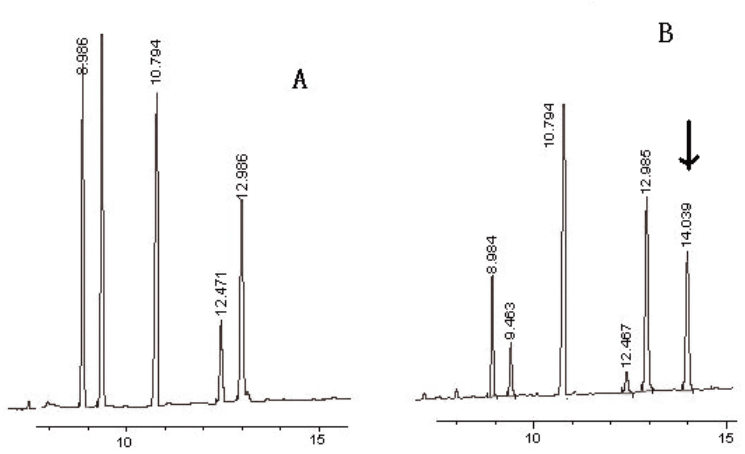

Figure 3. GC Analysis of the fatty acid composition of total lipids from *S. cerevisiae* transformants with heptadecanoic acid (C17:0) as the internal standard.
(A) *S. cerevisiae* transformed with control vector pYES2.0. (B) *S. cerevisiae* transformed with recombinant plasmid pPp-FAD12. The arrow indicates the novel peak of linoleic acid which has identical retention time with linoleic acid standard.

Functional analysis of *P. pastoris* Δ^{12}-fatty acid desaturase

In order to identify the function of the putative Pp-FAD12 protein, we examined its expression in *S. cerevisiae*. The OFR of this cloned cDNA was first subcloned into the yeast expression vector pYES2.0. The resultant plasmid pYPp-FAD12 was then transformed into *S. cerevisiae* strain INVScl and expressed under the control of the inducible GAL1 promoter. Transformants containing pYPp-FAD12 plasmid were used to analyze the fatty acid composition by GC analysis. The result revealed a novel fatty acid peak in the chromatogram of FAME from these transformants which had an identical retention time with the standard of linoleic acid, and was absent in the yeast containing the empty vector pYES2.0 (Fig. 3).

Table 3. Fatty acid compositions wt percentage of total lipid from yeast transformants pYES2.0 and pYPPD12.

Transformants	Fatty acid composition				
	C16:0	C16:1	C18:0	C18:1	C18:2
YES2.0	27.5	26.3	9.5	37	–
p Pp-FAD12	19.4	9.2	4.2	35.2	30.5

GC-MS analysis of this fatty acid methyl derivative demonstrated that its mass peak was at m/z = 294 which was identical to the C18:2 methyl esters standard (not shown). The fragmentation pattern was also identical to the C18:2 (delta9,12) methyl esters (not shown) standard which demonstrated that Pp-FAD12 indeed encodes a Δ^{12}-fatty acid desaturase that converts oleic acid to linoleic acid. The occurrence of the novel peak led to a decrease in the level of oleic acid and palmic acid. The percentage of this new fatty acid was 30.5% of total fatty acids (Table 3) which is much higher than that of our previous report about the expression level of *R. arrhizus* Δ^{12}-fatty acid desaturase (Ra-FAD12). Analyzing the codon usage of the two genes indicated that the codon usage in Pp-FAD12 apparently prefers those codons whose third position is G or C. Whereas the codon usage in Ra-FAD12 apparently prefers those codons whose third position is A or U, which may result in the formation of several AT-rich signals for transcription termination (Mignone *et al.,* 2002). This may lead to unwanted termination and will prevent the formation of the mature protein.

DISCUSSION

In this short paper, we first describe the cloning and characterization of a new Δ^{12}-fatty acid desaturase gene from the methylotrophic yeast *P. pastoris* GS115. Although no novel property has been detected in this desaturase, the sequence information can be used to construct a transgenic strain of the baker's yeast *S. cerevisiae* for production of PUFAs. In addition, with the lack of three-dimensional information due to the poor expression level and difficulties in crystallizing this kind of membrane-bound desaturases, there is little information on the structure–function relationships of such enzymes. Until now, nearly all information on the structure–function relationships comes from comparing different but nearly identical fatty acid desaturase sequences (Abe *et al.,* 2005; Na-Ranong *et al.,* 2006). Our result will provide additional information useful for analyzing the structure-function relationship. In addition, although promising results have been reported for the expression of multidrug resistance transporters (Lerner-Marmarosh *et al.,* 1999; Cai *et al.,* 2002; Urbatsch *et al.,* 2003), several G-protein-coupled receptors (Sarramegna *et al.,* 2001; Schiller *et al.,* 2001), monoamine oxidase (Li *et al.,* 2002), a peptide transporter (Theis *et al.,* 2001), and a fatty-acid biosynthetic enzyme (Zhu *et al.,* 1997), there is still no report about successful over-expression of fatty acid desaturase genes in *P. pastoris*. Several obstacles need to be overcome first, among which is the codon usage bias which reduced the expression level of the heterologous gene. Cloning fatty acid desaturase genes from *P. pastoris* and then expressing them under a strong and specific promoter may be not a commonly used method for other membrane protein, but it may be an alternative method to circumvent the difficulties in production of fatty acid desaturases.

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