Heteroexpression of *Rhizopus arrhizus* Δ^6-Fatty Acid Desaturase Gene in *Pichia pastoris*

少根根霉 Δ^6-脂肪酸脱氢酶基因在毕赤酵母中的表达

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摘 要 Δ^6-脂肪酸脱氢酶是一种膜整合蛋白,也是多不饱和脂肪酸合成途径中的限速酶。在前期工作中,通过 RT-PCR 和 RACE 技术,从少根根霉 NK300037 中克隆到一个潜在编码 Δ^6-脂肪酸脱氢酶的序列,序列和功能分析结果表明该序列具有一个长度为 1377bp、编码由 458 个氨基酸组成、大小为 52kD 的新的 Δ^6-脂肪酸脱氢酶基因。把少根根霉 Δ^6-脂肪酸脱氢酶基因(*RAD6*)亚克隆到表达载体 pPIC3.5K,构建重组表达载体 pPICRAD6,并转化到毕赤酵母菌株 GS115 进行表达。提取酵母细胞总脂肪酸和进行甲酯化,经气相色谱和气相色谱-质谱连用分析表明,目的基因的编码产物能将 C16:1、C17:1、C18:1、亚油酸和 α-亚麻酸在 Δ6 和 7 位间特异性脱氢而引入一个新的双键,生成更高不饱和的脂肪酸,该催化反应没有链长特异性,只有键位特异性。此外,按 Kozak 序列特点,改变目的基因转译起始密码子周边序列结构,并把改变后序列导入毕赤酵母 GS115 中进行功能表达分析,结果表明在毕赤酵母中这种改变同样能提高目的基因的表达水平。综合所有分析结果表明,巴斯德毕赤酵母更适合用来综合分析 Δ^6-脂肪酸脱氢酶基因的功能。

关键词 少根根霉,Δ^6-脂肪酸脱氢酶基因,γ-亚麻酸,巴斯德毕赤酵母,表达

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Abstract Δ^6-fatty acid desaturase is a membrane-bound enzyme, which is rate-limiting for the biosynthesis of polyunsaturated fatty acids. A cDNA sequence putatively encoding a Δ^6-fatty acid desaturase was isolated from *Rhizopus arrhizus* NK300037 using RT-PCR and RACE methods in our previous work. Sequence and function analysis indicated that this sequence was a novel Δ^6-fatty acid desaturase gene which had an open reading frame of 1377bp coding 458 amino acids of 52kD. The methylotrophic yeast *Pichia pastoris*, has been developed into a highly successful system for the production of a variety of heterologous proteins during the past 20 years. In this work, the *Rhizopus arrhizus* Δ^6-fatty acid desaturase gene (*RAD6*) was subcloned into expression vector pPIC3.5K to generate a recombinant plasmid pPICRAD6, which was subsequently transformed into *Pichia pastoris* strain GS115 for heterologous expression by electroporation method. Total fatty acids were extracted from the induced

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cells and methylated. The resultant fatty acid methyl esters (FAME) were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). Fatty acids analysis showed that the coding product introduced a new double bond at Δ6 position of appropriate fatty acid substrates including C16∶1, C17∶1, C18∶1, linoleic acid and α-linolenic acid without chain length specificity of fatty acids. Furthermore, modification of sequence flanking AUG codon of this Δ^6-fatty acid desaturase gene increased the expression of target gene in *P. pastoris*. All of these results suggest that *P. pastoris* is an optimal expression system of Δ6-fatty acid desaturase gene.

Key words *Rhizopus arrhizus*, Δ^6-fatty acid desaturase gene, γ-linolenic acid, *Pichia pastoris*, expression

Polyunsaturated fatty acids (PUFAs) are required for normal cellular function, involved in roles of ranging from maintaining membrane fluidity to acting as signal molecules[1,2], and this have attracted considerable interest as pharmaceutical and nutrient components[2,3]. The PUFAs can been classified into three groups, *n*-3, *n*-6 and *n*-9, according to the position (*n*) of the double bond nearest to the methyl end of the fatty acid. Linoleic acid (LA; 18∶2Δ^9, 12 *n*-6) and α-linolenic acid (ALA; 18∶3$\Delta^{9,12,15}$ *n*-3) are essential fatty acids because mammals lack the ability to synthesize them. The two fatty acids can be converted into γ-linolenic acid (GLA; 18∶3$\Delta^{6,9,12}$ *n*-6) and octadecatetraenoic acid (OTA, 18∶4$\Delta^{6,9,12,15}$ *n*-3) respectively by the catalysis of Δ^6-fatty acid desaturase. Subsequently, the resultant fatty acids can be further introduced into the biosythesis of long-chain polyunsaturated fatty acids (LC-PUFAs) such as arachidonic acid (AA, 20∶4$\Delta^{5,8,11,14}$ *n*-6) and eicosapentaenoic acid (EPA, 20∶5$\Delta^{5,8,11,14,17}$ *n*-3) by an alternating series of desaturation and elongation through *n*-6 or *n*-3 pathway. These fatty acids are not only important components of cellular structure and function but also precursors of physiologically active molecules such as prostaglandins, thromboxanes and leukotrienes[1,3]. It is well known that Δ^6-fatty acid desaturase introduces a double bond between the existing double bond and carboxyl terminus at the Δ6 position of fatty acyl chain for LA and ALA. Except for the above-mentioned LA and ALA, recent studies have shown that Δ^6-fatty acid desaturase can also act on the substrates including hexadecanoic acid (C16∶1Δ^9 *n*-9), oleic acid (C18∶1Δ^9 *n*-9) and tetracosapentaenoic acid (C24∶5$\Delta^{9,12,15,17,21}$ *n*-3)[4]. These results demonstrate that Δ^6-fatty acid desaturase has position specificity of fatty acid substrates other than fatty acyl chain-length specificity. Unfortunately, the structural information on Δ^6-fatty acid desaturase is scarce because of the technical limitations in obtaining large quantities of purified protein and the intrinsic difficulties in obtaining crystals from Δ^6-fatty acid desaturase.

GLA has been claimed to play a crucial role in development and prevention of some skin diseases, diabetes, reproductive disorder and others[5]. GLA is commonly obtained from plant source such as evening primrose, borage and black-current[6]. Attempts have been also made to produce GLA from microorganisms including bacteria, fungi and microalgae[3,6,7]. However, it is evident that GLA production from current sources is inadequate for supplying the expanding market due to the low productivity, complex and expensive downstream process and unstable quality[1,8]. Modification of the fatty acid biosynthesis pathways by genetic manipulation to produce desired oil in transgenic microorganisms and oilseed crops, as a putatively alternative source, has been searched. A gene of Δ^6-fatty acid desaturase and appropriate expression system for functional analysis are two of prerequisites for the application.

In our previous work, a novel Δ^6-fatty acid desaturase gene (*RAD6*, GenBank Accession No. AY320288) was isolated and characterized from *Rhizopus arrhizus*[9]. In this communication, we report the heterologous expression of this gene in *P. pastoris* strain GS115 with the aim to establish a expression system for the function analysis of Δ^6-fatty acid desaturase gene, providing primary basis for the studies on PUFAs biosynthesis, structural and functional analysis of Δ^6-fatty acid desaturase and the future application of gene engineering about GLA production.

1 MATERIALS AND METHODS

1.1 strains and plasmids

P. pastoris strain GS115 (his$^-$. mut$^+$) and vector pPIC3.5K, used for the expression, were purchased from Invitrogen. *P. pastoris* manipulations were performed according to the manufacturer's instructions except that indicated otherwise in the text. pGEM-TRAD6, a recombinant of pGEM-T (Promaga, Madison, WI, USA) and *R. arrhizus* Δ^6-fatty acid desaturase gene, was stored in our lab.

1.2 Plasmids construction and DNA sequencing

Two specific primers, 5′-CTCGGATCCT CAATATGAGTACATCAGATCGTC-3′ and 5′-TAGCGGCCGCTTAAAATGACTTTTTGCTC-3′ that correspond to the nucleotide sequences of start and stop codons (in bold) of *RAD*6, respectively, were used to obtain the full-length coding sequence. The 5′ ends of forward primer and reverse primer contain a *Bam*HⅠ and *Not*Ⅰ restrictive sites (underlined) respectively to facilitate subsequent manipulation. In addition, a forward primer 5′-TAGGATCCACCATGGGTACATCAGATCGTC-3′ and the above- mentioned reverse primer were used to obtain another full-length coding sequence *RAD*6-1 by modifying the sequence compositions around the start codon of *RAD*6 to CCACCATGG, which is generally a consensus sequence in eukaryotes at the first translation AUG codon[10,11]. Recombinant plasmid pGEM-TRAD6 was used as the template for PCR amplification. These primers (0.2μmol/L) were run on the Biometra™ T-gradient thermal cycler using a program of 1 min at 94℃, 1min at 60℃ and 2 min at 72℃ for 35 cycles, followed by the extension at 72℃ for 10 min. The amplified products of expected length (~ 1.4kp) were digested and subcloned into *Bam*HⅠ-*Not*Ⅰ site of the expression vector pPIC3.5K to generate two plasmids designated as pPICRAD6 and pPICRAD6-1, which were confirmed by restriction analysis and DNA sequencing (TaKaRa Bio. (Dalian) Co., LTD, China).

1.3 Transformation of *P. pastoris* GS115

P. pastoris strain GS115 was transformed with 10μg of *Sac*Ⅰ- linearized recombinant plasmid by electroporation with MicroPulser Electroporation Apparatus (Bio-Red, Hercules, USA). Transformants were screened for their ability to grow for 72 h on histidine-deficient MDS plates containing 1.34% YNB, 4×10^{-5}% Biotin, 1% Dextrose and 1mol/L D-Sorbitol. The multi-copy intergrants were selected on YPD plates containing various concentrations of G418. In general, transformants growing on plates containing 5mg/mL G418 were selected for *RAD*6 expression.

1.4 Analysis of genomic DNA

For the verification of gene integrated into the Pichia genome and the determination of the phenotype, genomic DNA of a number of transformants was isolated according to the method of Lee *et al*[12]. Colonies from the YPD-G418 plates were picked up to grow in 5 mL minimal medium overnight for genomic DNA extraction. PCR amplifications were carried out with 5 μL of genomic DNA and primers corresponding to 5′and 3′region of the *AOX*1 gene (forward primer: 5′-GACTGGTTCCAATTGACAAGC-3′ and reverse primer: 5′-GCAAATGGCATTCTGACATCC-3′ with the followed program: 1 min at 94℃, 1min at 58℃, and 2 min at 72℃ for 30 cycles, followed by the extension at 72℃ for 10 min.

1.5 Induced expression in *P. pastoris*

Colonies growing on plates containing 5 mg/mL G418 were used to inoculate 5 mL buffered complex medium containing glycerol (BMGY, Invitrogen) and were grown overnight at 30℃. This 5 mL cultures was used to inoculate 100 mL of BMGY for 16 ~ 18h until the log phase growth (A_{600nm} = 2 ~ 6). Cells were harvested, washed and resuspended in 100 mL BMGY. The induced expression was carried out for further 72h at 20℃ in BMMY (BMGY supplemented with methanol to a final concentration of 0.5%). Yeast cells were harvested by centrifugation and washed by sterile distilled water for three times. The cells were dried and ground with mortar into a fine powder.

1.6 Fatty acid analysis

Cellular fatty acids were extracted by incubating 100mg yeast powder in 5mL of 5% KOH in methanol for saponification at 70°C for 5h. Adjusted pH to 2.0 with HCl, the fatty acid was then subjected to methyl-esterification with 4mL of 14% boron trifluoride in methanol at 70°C for 1.5h. Subsequently, Fatty acid methyl esters (FAMEs) were extracted with hexane after addition of saturated sodium chloride solution. FAMEs were analyzed by gas chromatography (GC, GC-9A; Shimadzu, Kyoto, Japan) Qualitative analysis of FAMEs was performed by GC-mass spectrometry (GC-MS) using a HP G1800A GCD SYSTEM (Hewllett-packard, Palo Alto, CA, USA). Both analysis were carried out with the same polar capillary column (HP-5, 30m × 0.25mm internal diameter, 0.25mm internal film thickness). The mass spectrum of new peak was compared with that of standard for identification of fatty acid.

1.7 Southern blotting

Southern blotting was performed using the genomic DNA extracted from the yeasts with highest-level production. Genomic DNA was digested with *Bam*HⅠ and size-fractionated on a 0.8% agarose gel, and then transferred to a Hybond-N nylon membrane according to Sambrook *et al*[16]. The DNA was cross-linked to membrane by Hoefer UVC500 ultraviolet crosslinker (Pharmacia, San Francisco, CA,

USA) and dried. Prehybridization was carried out at 50°C with DIG Easy Hyb Buffer for 1 h, and hybridization was carried out overnight at 50°C with the same solution containing the digoxigenin-labeled full-length *RAD*6 as probe. The followed labeling of *RAD*6-specific probe and detection were carried out with the DIG DNA Labeling and Detection Kit (Roche, Beijing, China) according to the manufacturer' instruction.

2 RESULTS

2.1 Construction of expression plasmids pPICRAD6 and pPICRAD6-1

The PCR products amplified with the two pairs of primers combination, *RAD*6 and *RAD*6-1, were introduced into plasmid pPIC3.5K (Fig. 1) between 5′ AOX1 and 3′ AOX1 (TT) to generate two recombinant plasmids pPICRAD6 and pPICRAD6-1, which were subsequently confirmed by restriction analysis and DNA sequencing.

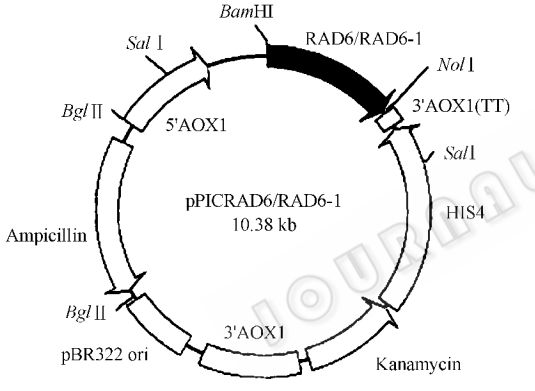

Fig. 1 Construction of expression plasmid pPICRAD6/RAD6-1
5′*AOX*1: promoter fragment; TT: transcription termination; *HIS*4 ORF: a selection marker; *RAD*6/*RAD*6-1 gene was inserted between 5′*AOX*1 and 3′*AOX*1 (TT).

2.2 PCR analysis of *P. pastoris* transformants

Plasmids pPICRAD6, pPICRAD6-1 and pPIC3.5K were linearized by *Sac* Ⅰ, respectively and electroporated into the host *P. pastoris* GS115 (his^- mut^+). Thirty-four His^+ Mut^+ recombinants for pPICRAD6 or pPICRAD6-1 were identified by PCR from 50 His^+ transformants screened on MDS plates. The PCR amplification results showed that positive recombinants produced the *AOX*1 gene (2.2 kb) and a 1.5 kb product, the control yeast transformed with plasmid pPIC3.5K produced the *AOX*1 gene (2.2 kb) and a 220 product. However, the negative control only produced the *AOX*1 gene (2.2 kb) (Fig.2). These results demonstrated

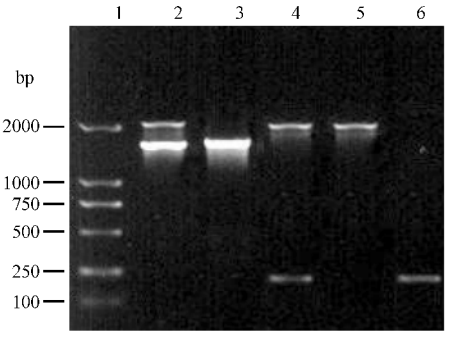

Fig. 2 PCR analysis of *P. pastoris* integrants
1: DNA Marker DL2000; 2: *P. pastoris* transformed with pPICRAD6; 3: Mut^s; 4: *P. pastoris* transformed with pPIC3.5K; 5: *P. pastoris*; 6: pPIC3.5K.

that *RAD*6/*RAD*6-1 was stably integrated into the yeast chromosome.

2.3 Induced expression of the *R. arrhizus* Δ^6-fatty acid desaturase gene

Heterologous expression was under transcriptional control of the *AOX*1 promoter. Cells were grown in BMGY medium as described in Materials and Methods, and expression was induced for 72h by supplement of methanol as sole carbon source. GC analysis results of the FAMEs from the induced cells revealed that five novel fatty acid peaks were detected in the yeasts transformed with pPICRAD6, which were absent in the yeasts containing control vector pPIC3.5K (Fig. 3). Among them, two main peaks showed the retention time of 11.8 min and 13.2 min, similar to those of GLA and OTA methyl ester standards, respectively (Fig.3). Gas chromatography-mass spectrometry (GC-MS) analysis of this fatty acid methyl derivatives demonstrated that they were GLA and OTA methyl esters (Fig.4C, D). The mass peak m/z = 292 and m/z = 290 indicated the molecular mass of the methyl-derivative GLA and OTA, and the fragmentation patterns were identical to that of the standards, respectively. The other three novel peaks were identified to be C16 : $2\Delta^{6,9}$, C17 : $2\Delta^{6,9}$ and C18 : $2\Delta^{6,9}$ by their retention time and mass spectra (Fig.4 A, B, E), which were Δ6 desaturated products from C16 : $1\Delta^9$, C17 : $1\Delta^9$ and C18 : $1\Delta^9$. These results demonstrate that Δ^6-fatty acid desaturase have position specificity of fatty acid substrates other than chain-length specificity of fatty acyl chains. Furthermore, according to the percentage of novel fatty acid products, this enzyme showed preference for C18 fatty acid substrates, especially for LA and OTA (Table.1).

Table 1 Fatty acid compositions(wt %)of total lipid from yeast transformants containing pPIC3.5K, pPICRAD6 and pPICRAD6-1.

Transgenic yeasts	Fatty acid composition(%)												
	16 :0	16 :1 Δ^9	16 :2 $\Delta^{6,9}$	17 :0	17 :1 Δ^9	17 :2 $\Delta^{6,9}$	18 :0	18 :1 Δ^9	18 :2 $\Delta^{6,9}$	18 :2 LA	18 :3 GLA	18 :3 ALA	18 :4 OTA
pPIC3.5K	7.7	2.5	0	3.8	8.5	0	2.47	46.7	0	22.2	0	6.47	0
pPICRAD6	9.83	2.5	0.53	2.0	3.6	0.8	2.90	50.8	0.9	14.7	6.2	2.4	3.5
pPICRAD6-1	9.5	2.3	0.8	2.2	4.0	1.3	2.6	47.8	1.9	9.8	10.7	2.0	4.7

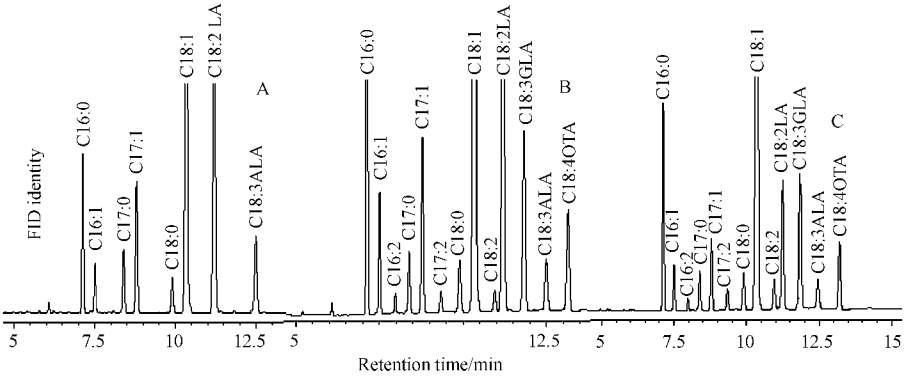

Fig. 3 Identification of novel peaks in *P. pastoris* GS115 transformed with pPIC3.5K, pPICRAD6 and pPICRAD6-1 by GC analysis. (A) *P. pastoris* GS115 transformed with pPIC3.5K; (B) *P. pastoris* GS115 transformed with pPICRAD6; (C) *P. pastoris* GS115 transformed with pPICRAD6-1.

Similarly, same results were detected in the yeasts transformed with pPICRAD6-1 (Fig. 3). As expected, the percentage of novel peaks to the total fatty acids was increased. In particular, the percentage of GLA and OTA was increased from 6.2% and 3.5% in GS115/pPICRAD6 to 10.7% and 4.7% in GS115/pPICRAD6-1 (Table 1), The results suggested that modification of sequence flanking AUG codon of *R. arrhizus* Δ^6-fatty acid desaturase gene not only maintained the specificity of Δ^6-fatty acid desaturase but also increased the expression of the target gene.

2.4 Southern blot analysis

Isolates with the highest-level production were selected for southern blot analysis of genomic DNA. Hybridization bands were detected in both hybridization patterns of GS115/ pPICRAD6 and GS115/ pPICRAD6-1. One or two bands were observed in the transformants (Fig. 5), which indicated that some transgenic strains might have more than one copy of the vector integrated into its genome. No positive signal was detected in that of yeasts transformed pPIC3.5K. However, no significant increase of target product was detected in yeasts with the increase in copy number of integrated recombinant DNA, inconsistent with the observations that the expression level of products was linked to an increase in copy number of integrated recombinant DNA[14], which possibly have relation with the characteristics of the expressing product as a membrane-bound enzyme.

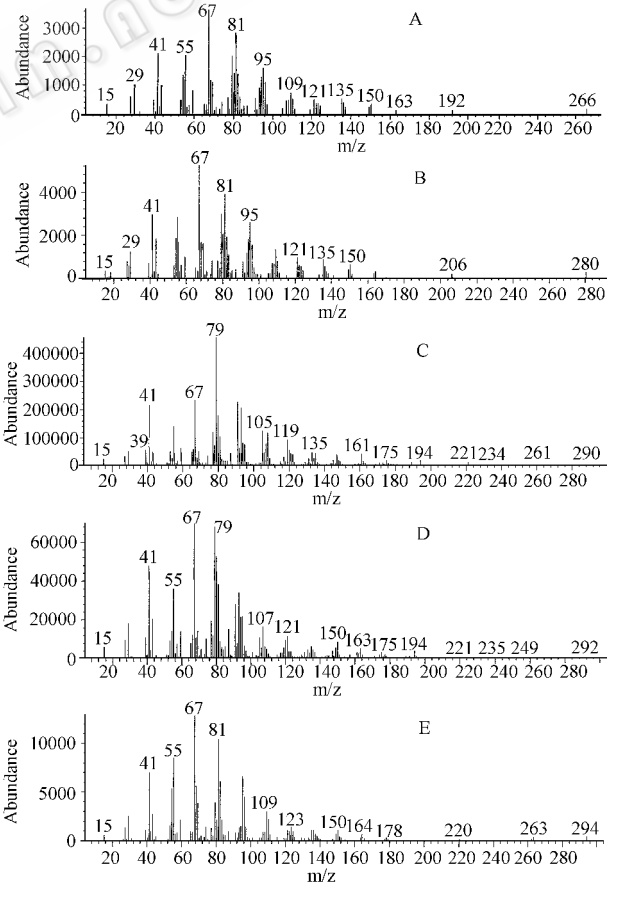

Fig. 4 GC-MS analysis of the novel peaks identified in *P. pastoris* GS115 transformed with pPICRAD6 and pPICRAD6-1
A: C16 :2$\Delta^{6,9}$; B: C17 :2$\Delta^{6,9}$; C: C18 :2$\Delta^{6,9}$; D: GLA; E: C18 :4$\Delta^{6,9,12,15}$.

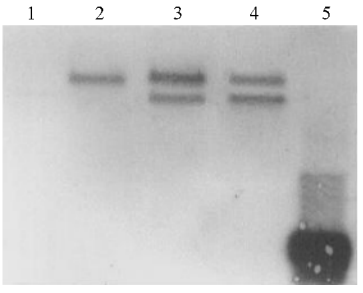

Fig. 5 Southern blot analysis of genomic DNA isolated from *P. pastoris* GS115 transformants

1: negative control; 2: yeast transformants intergrated with one copy; 3 ~ 4: yeast transformants intergrated with two copies; 5: positive control.

3 DISCUSSIONS

At present a range of heterologous expression systems are available to meet the need of various proteins useful for fundamental structure/function analysis and biotechnological and pharmaceutical purposes. For several centuries, *S. cerevisiae*, as a GRAS organism, has been used in the production of food, alcoholic beverages and pharmaceutical industry. *S. cerevisiae* is also susceptible to genetic manipulation and analysis, which has been even further facilitated by the availability of the complete genome sequence of *S. cerevisiae* published in 1996[15]. However, the initial limitations including oft-times instability of the engineering strains, undesired overglycosylation, relatively low yields and the lack of strong promoters have been relieved during the past decade by a number of expression systems based on the alternative yeasts[16]. *S. cerevisiae* contains a Δ^9-fatty acid desaturase capable of producing monounsaturated palmitoleic and oleic acid, but does not carry out futher desaturation[17]. *S. cerevisiae* is generally used as an expression system for the heterologous expression of Δ^6-fatty acid desaturase genes from various organisms[4]. However, *S. cerevisiae* is, unfortunately, not optimal for researches on fatty acid metabolism in eukaryotic organisms because the capability of this yeast to synthesize fatty acids is limited. *S. cerevisiae* can not produce polyunsaturated fatty acids such as C18 :2 and C18 :3, whereas most other yeasts, such as *Candida*, *Pichia*, and *Rhodotorula* species, like animals and plants, are able to synthesize them[18]. This leads to other limitations for the comprehensive study of catalytic specificity of Δ^6-fatty acid desaturase in *S. cerevisiae* and future application of GLA production. A more suitable expression host/system is imperative.

During the past 20 years, the methyltrophic yeast *Pichia pastoris* has been proven to offer significant advantages over the traditional baker's yeast for the high level production of certain proteins. The increasing popularity of this particular expression system can be attributed to several factors: (1) the simplicity of techniques needed for the molecular genetic manipunation of *P. pastoris* and their similarity to those of *S. cerevisiae*; (2) the ability of *P. pastoris* to production foreign proteins at higher level, either intracellularly or extracellularly; (3) the capability of performing many eukaryotic post-translational modifications, such as glycosylation, disulfide bond formation and proteolytic processing[19]. As a membrane-bound enzyme, the structural information of Δ^6-fatty acid desaturase is scarce because of the technical limitations in obtaining large quantities of purified protein and the intrinsic difficulties in obtaining enzyme crystals. With the above-mentioned advantages, *P. pastoris* might be contributed to meet such aims. Moreover, *P. pastoris* was firstly and successfully utilized for the expression of *Mortierella alpine* Δ^6-fatty acid desaturase gene in our previous work[20].

In general, Δ^6-fatty acid desaturase catalyse the conversion of LA and ALA to GLA and OTA in *n*-3 and *n*-6 pathways. Recent studies reported the same rat Δ^6-fatty acid desaturase can introduce a double bond into C16, C18 and C24 fatty acid substrates at their Δ6 position of fatty acyl chains, reinforcing that Δ^6-fatty acid desaturase may have no fatty acyl chain-length specificity but position specificity[4,5]. The primary products of fatty acid biosythesis in *P. pastoris* are 16-, 17-and 18-carbaon compounds. Among them, C16 :1Δ^9, C17 :1Δ^9, C18 :1Δ^9, LA and ALA are endogenously putative substrates for Δ^6-fatty acid desaturase (Table 1), providing an optimal model to detect the catalytic specificity of the enzyme for the fatty acyl chain length or the introduced double bond position, together with its preference for fatty acid substrates. In this work, fatty acid analysis reconfirmed that Δ^6-fatty acid desaturase equally introduced a double bond into C16 :1Δ^9, C17 :1Δ^9, C18 :1Δ^9, LA and ALA at their Δ6 position of fatty acyl chains without chain-length specificity. It is so far the first report about the conversion of C17 :1Δ^9 to C17 :2$\Delta^{6,9}$ catalyzed by the same Δ^6-fatty acid desaturase. Furthermore, according to the percentage of novel fatty acid products and the ratio of GLA/LA and OTA/ALA, this enzyme showed preference for C18 fatty acid substrates, especially for ALA, and followed by LA, which is consistent with our observation in previous work[21].

More than two decades ago Kozak[10,11] proposed the scanning model of translation initiation. In most eukaryotic mRNA, it is thought that the initiation of translation at the first AUG codon encountered by the 40S ribosomal submit as it scans along the 3′ mRNA from 5′ m7Gcap. Sequences flanking the AUG initiation codon are not random. In contrast, they fit a consensus sequence CCACCAugG (Kozak sequence), of which the most conserved nucleotides in animals, plants and fungi are the purine (R), usually A, at position -3 and G at position +4. The sequence context of the first AUG codon modulates the efficiency to halt the scanning 40S ribosomal subunit and increase the translation. The influence of modification of nucleotide composition flanking initiation codon on genes expression has been first testified in animals, which was also demonstrated by the expression of chimeric genes in plants cells and *S. cerevisiae*[22,23]. In this work, this modification was also proved to improve the expression of *R. arrhizus* Δ^6-fatty acid desaturase gene in *P. pastoris*. However, the most favourable nucleotide composition of the sequence flanking the first AUG codon for the heteologous gene expression varies in animals, plants and fungi.

Moreover, the percentage of synthesized GLA in *P. pastoris* transformed with *RAD*6 and *RAD*6-1 was higher than that in *S. cerevisiae* transformants with the same genes[9,23]. Based on these foregoing results, *Pichia pastoris*, as a expression system, is optimal for Δ^6-fatty acid desaturase gene in contrast to *S. cerevisiae*. Due to the intrinsic characteristics of Δ^6-fatty acid desaturase as a membrane-bound enzyme, attempts are being carried out to detect the expression of *RAD*6 and *RAD*6-1 in *P. pastoris* at protein level.

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