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Production of Gamma-Linolenic Acid in *Pichia pastoris* by Expression of a Delta-6 Desaturase Gene from *Cunninghamella echinulata*

Wan, Xia, Yinbo Zhang, Ping Wang, Fenghong Huang, Hong Chen, and Mulan Jiang*

Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, 430062 Wuhan, P. R.China

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Gamma-linolenic acid (GLA, C18:3 $\Delta^{6,9,12}$) is synthesized by a delta-6 fatty acid desaturase using linoleic acid (LA, C18:2 $\Delta^{9,12}$) as a substrate. To enable the production of GLA in the conventional yeast *Pichia pastoris*, we have isolated a cDNA encoding the delta-6 fatty acid desaturase from *Cunninghamella echinulata* MIAN6 and confirmed its function by heterogeneous expression in *P. pastoris*. Sequence analysis indicated that this cDNA sequence has an open reading frame of 1,404 bp, which encodes a 52 kDa peptide of 468 amino acids. This sequence has 64% identity to the previously reported delta-6 fatty acid desaturase from *Rhizopus oryzae*. The polypeptide has a cytochrome *b5* domain at the N-terminus including the HPGG motif in the heme-binding region, as reported for other delta-6 fatty acid desaturases. In addition, this enzyme differs from other desaturases by the presence of three possible N-linked glycosylation sites. Analysis of the fatty acid composition demonstrated the accumulation of GLA to the level of 3.1% of the total fatty acids. Notably, the amounts of ginkgolic acid (C17:1) and palmitic acid (C16:0) were increased from 1.3% to 29.6% and from 15% to 33%, respectively. These results reveal that the modification of the fatty acid biosynthetic pathway by genetic manipulation in order to produce specific polyunsaturated fatty acids in *P. pastoris* is a promising technique.

Keywords: Gamma-linolenic acid, delta-6 fatty acid desaturase, *Cunninghamella echinulata*, *Pichia pastoris*

Gamma-linolenic acid (GLA, C18:3$\Delta^{6,9,12}$), a polyunsaturated fatty acid of the n-6 series, is an intermediate in the biosynthesis of hormone-like eicosanoids [12]. GLA is currently derived from fish, several plants, and microorganisms. It is widely used as a dietary supplement and for treatment of various medical conditions. Many studies have shown the beneficial impact of GLA on human health [4, 14]. Consequently, there is an increasing demand for GLA-rich oils for applications in pharmaceutical, nutraceutical, cosmetic, and animal feed industrial sectors. Currently, GLA-rich oils are largely supplied from several oilseed plants including evening primrose, borage, and black-currant [3]. There is growing interest in fungal oils or microbial oils, as a result of many advantages over conventional sources, through fermentation and recombinant DNA technologies. Concerns have been raised about the economic viability of oil production from microbial sources. Therefore, an alternative source comes from oleaginous microorganisms with high GLA accumulation [1, 5, 19].

The desaturation takes place at the sn-1 position of the glycerol moiety of monogalactosyl diacylglycerol and digalactosyl diacylglycerol. The first double bond is introduced at the Δ^9 position of stearic acid (18:0) to yield oleic acid (18:1). The second double bond is then introduced at the Δ^{12} position of 18:1, yielding linoleic acid (LA, 18:2). Subsequently, the third double bond is added at the Δ^6 position of LA to yield GLA. Three different desaturation enzymes, delta-9, delta-12, and delta-6 desaturases, are involved in these steps [8]. GLA is synthesized by the introduction of a double bond into linoleic acid using a membrane-bound enzyme, delta-6 desaturase [13]. Similar to the ortholog desaturase enzymes, this desaturase contains three histidine-rich motifs, two long transmembrane stretches, and an N-terminal cytochrome b_5 domain. Most of the reported delta-6-desaturases contain a cytochrome b_5 domain, which is fused to the N-terminus and serves as an electron donor [22]. Oleaginous fungus *Cunninghamella echinulata* is considered as a potential GLA producer [1, 5, 6, 7, 9], and *C. echinulata* MIAN6 has also been reported to produce high levels of GLA [15]. In addition, the growth conditions, including temperature, pH, and medium compositions, have been well studied for this strain to produce GLA [23, 26]. However, there is no report on the

*Corresponding author
Phone: +86-27-86838791; Fax: +86-27-86822291;
E-mail: mljiang@oilcrops.cn

gene involved in GLA formation in this genus. In this study, we first report the cloning of the delta-6 desaturase from oleaginous fungus *C. echinulata*, and characterization of its ability to direct the synthesis of GLA from LA when heterologously expressed in *Pichia pastoris*.

MATERIALS AND METHODS

Organisms and Growth Conditions

C. echinulate strain MIAN6 was kindly provided by Professor Zhang from Huazhong University of Science and Technology (Wuhan, China). This strain was maintained on potato dextrose agar (PDA) medium at 4°C and regularly transferred every three months. It was grown at 28°C for 3 days in a liquid PDA medium with constant shaking (240 rpm/min). The amount of GLA from *C. echinulate* is about 15% of total fatty acids. *Escherichia coli* strain XL10-Gold (Stratagene) was cultured at 37°C in Luria–Bertani (LB) medium supplemented with 100 mg/l of ampicillin. *P. pastoris* strain GS115 (his^-, mut^+) was purchased from Invitrogen.

Cloning of the Delta-6 Desaturase Gene

Mycelia were harvested by filtration and washed with phosphate-buffered saline buffer. The dried mass was frozen in liquid nitrogen, and then ground with a mortar and pestle into a fine powder. Total RNA was extracted from the powder by using TRIzol reagent (Invitrogen, Beijing, China). Then, the mRNA was extracted from the total RNA by using the Oligotex mRNA Mini Kit (Qiagen). First-strand cDNA was synthesized with the first-strand cDNA Synthesis Kit (Promega, WI, U.S.A.) and was used as a template for reverse transcription polymerase chain reaction (RT–PCR) amplification with degenerate primers designed according to available sequence information for other delta-6 fatty acid desaturases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The forward primer and the reverse primer were designated as D6MDF (5'-AARCAYAAYACNCAYCAYGC) and D6DMR (5'-TG YTCRATYTGRTARTTCAAWCC), corresponding to the conserved histidine-rich motifs KHNTHHA and GGLNYQIEHH, respectively. The amplified product of expected length about 600 bp was subcloned into the pMD 18-T vector (TaKaRa Bio, Dalian, China) and then sequenced (Invitogen).

The full-length 5' and 3' ends of the cDNA were obtained by the method of rapid amplification of cDNA ends (RACE) using the SMART1 RACE cDNA Amplification Kit (BD-Clontech, CA, U.S.A.). Gene-specific primers D6DM1 (5'-GCGCGTCTGGTAGGGGAGCAC) and D6DM2 (5'-TGGCCTGGCTGACGACAAAGAAC) were designed to amplify the 5'-end of cDNA, and D6DM3 (5'-GGCCATCCAATC GCTCCAATACTC) and D6DM4 (5'-GCCCGTCCTCACCCAAGA ACAG-3') were designed to amplify the 3'-end of the cDNA. All PCR fragments were subcloned into the pMD 18-T vector and the nucleotide sequences were subsequently determined. Sequence alignment and phylogenetic analysis were performed using the software DNAMAN (version 4.0) and CLUSTAL X (version 2.0).

Heterologous Expression of the Delta-6 Desaturase Gene in *P. pastoris*

Based on the sequences of 5' and 3' ends, two gene-specific primers, 5'-GTCATGTCAGGGCAAACTCGAG-3' (forward primer) and 5'-GGCCATCATCTAAAACATCTTTTGAGAG-3' (reverse primer), were designed to amplify full-length cDNA by the RACE program. To facilitate preparation of the yeast expression construct, the 5' ends of the forward primer and the reverse primer contained a NotI and a CpoI restriction enzyme site (underlined), respectively. The amplified product was inserted between the NotI and CpoI sites of the expression vector pHBM906 (preserved in our laboratory) to create plasmid pHBM605. The sequence of the product was verified. The resulting vector was linearized by SalI and electroporated into *P. pastoris* GS115 (his^-) host cells. Transformants were selected by plating on synthetic minimal medium agar lacking histidine and grown at 28°C for 3 days.

Heterologous expression of D6DM was induced under transcriptional control of the yeast AOX1 promoter. Selected colonies were grown on BMGY medium (Invitrogen) at 28°C overnight. Then, 5-ml cultures were used to inoculate 100 ml of BMGY medium for 16–18 h until the log-phase growth (A_{600}=2–6). Cells were harvested, washed, and resuspended in 100 ml of BMGY. The induced expression was carried out for a further 72-h incubation at 20°C in BMMY medium (Invitrogen). Subsequently, cells were harvested by centrifugation, and washed three times with sterile distilled water. Then, cells were dried as described before.

Fatty Acid Analysis

Fatty acid compositions of total lipid from yeast cultivated under different growth conditions were determined by modification of the direct transmethylation method [16, 18, 27]. Dried yeasts were crushed, and samples were transmethylated with 5% HCl in methanol at 80°C for 1 h. Fatty acid methyl esters were analyzed by gas chromatography (GC, Agilent) and a HP-INNOWax column (30 m by 320 mm inner diameter, purchased from HP Company). The areas of chromatographic peaks were calculated for relative amounts of fatty acid methyl esters.

Sequence Data

The cDNA sequence of the delta-6 desaturase gene from *C. echinulata* MIAN6 has been deposited in the GenBank database under the accession number DQ177498.

RESULTS AND DISCUSSION

Cloning of Delta-6 Fatty Acid Desaturase Gene from *C. echinulata*

Fungal lipids represent a potential source of edible fat. In particular, oleaginous moulds, having the ability to accumulate large amounts of lipids, are thought as alternative sources for the production of polyunsaturated fatty acids [19, 24]. Among them, GLA is of great interest owing to its important functions [3, 4, 12, 13]. Although various examples of GLA production have been described in *C. echinulata*, there is no report on genes encoding delta-6 fatty acid desaturases that may be involved in GLA production [1, 4, 5, 7, 9, 15, 23, 26]. Recently, delta-6 desaturase genes have been cloned from other fungi, such as *Mortierella alpine*, *Mucor rouxii*, *Thamnidium elegans*, and *Pythium irregulare* [2, 11, 16, 18, 25]. In this study, two conserved

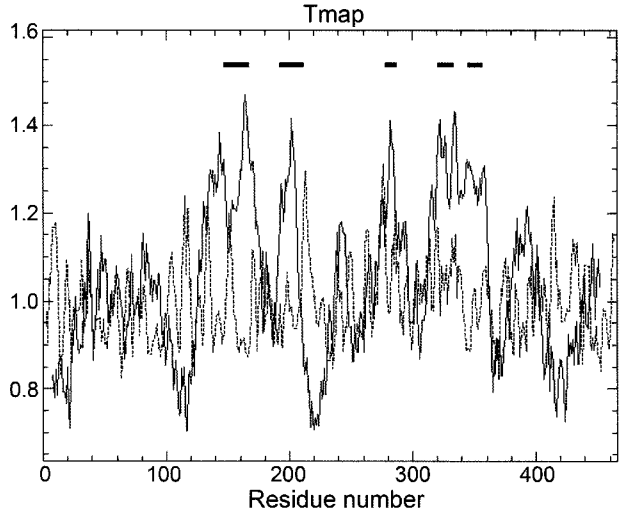

Fig. 1. Hydropathy profiles of the delta-6 fatty acid desaturase of *C. echinulata* MIAN6.

amino acid sequences (KHNTHHA and GGLNYQIEHH) have been found in these fungal desaturases by alignment. According to these sequences, two degenerate primers were designed and used for RT–PCR. A 576-bp DNA fragment was amplified from *C. echinulata*. This deduced amino acid sequence showed 71% identity to the delta-6 fatty acid desaturase from *Rhizopus oryzae*, 67% identity to that from *R. stolonifer*, and 66% identity to that from *Thamnidium elegans*. This result indicated that a partial gene sequence encoding delta-6 fatty acid desaturase was isolated from *C. echinulata*. In order to obtain the full length of this gene, RACE was chosen to amplify the 5' and 3' ends of this partial gene. A 694-bp fragment at the 5' end of the gene and a 345-bp fragment at the 3' end were obtained by the method of 5'- and 3'-RACE, respectively. The nucleotides of both products shared identical sequences overlapping on flanking regions of the cloned 5' and 3' ends of the RT–PCR DNA fragment, suggesting that these fragments were part of the same gene. Sequence analysis revealed that the cDNA sequence contained an ORF of 1,404 bp, designated as *D6DM*, encoding 468 amino acid residues with an estimated molecular mass of 52 kDa. An ATG translation initiation codon was identified in the 51-bp region of the 5' terminus (Fig. 2). The coding region was flanked by a 51-bp 5'-untranslated region and a 162-bp 3'-untranslated region, which contains a putative polyadenylation site, AATAAA, at 29 bp upstream of the poly(A) tail.

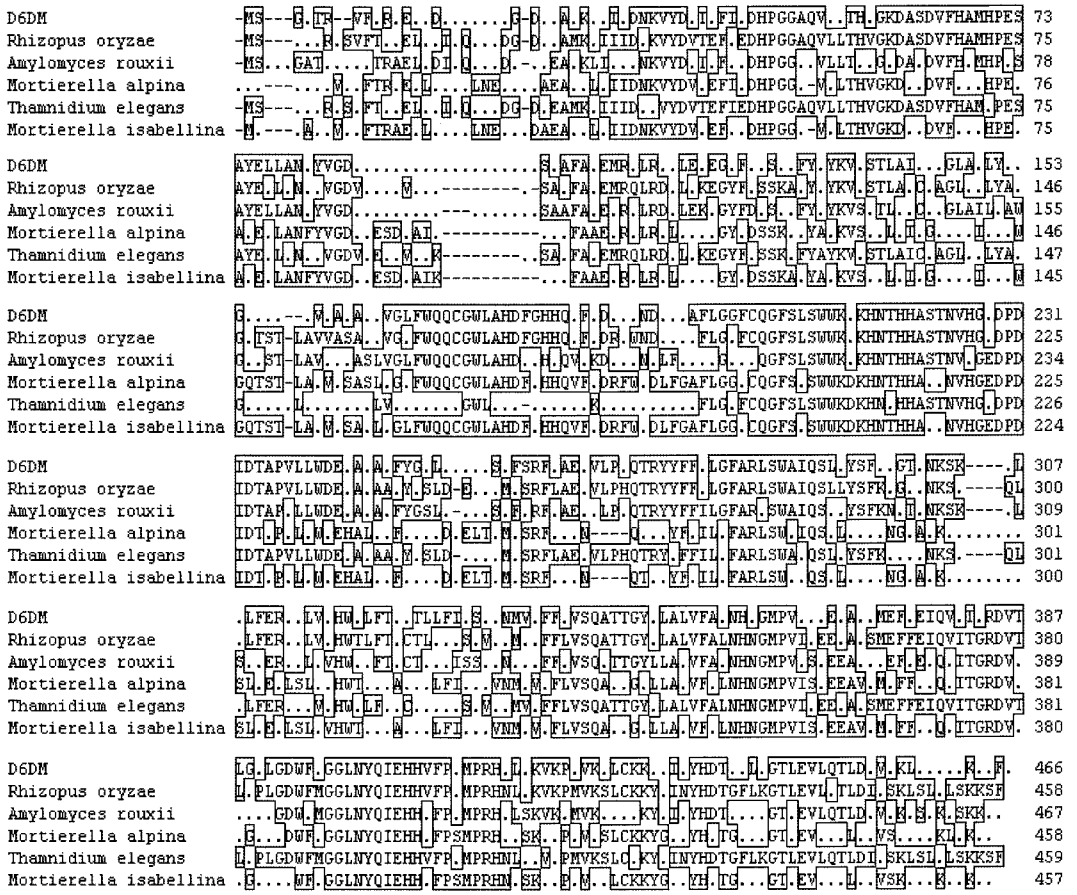

Fig. 2. Alignment of the deduced amino acid sequences of six delta-6 fatty acid desaturases.

The comparison of the deduced amino acids of D6DM with other fungal delta-6 fatty acid desaturases revealed four conserved histidine-rich motifs at amino acid positions 46, 215, 358, and 397 (Fig. 2). Hydrophobic regions known in all membrane-bound desaturases are also found in this cDNA sequence [21] (Fig. 1). In addition, the cytochrome b_5-like domain HPGG reported in the cytochrome b_5 superfamily, which is required as an electron donor for fatty acid desaturation, was observed at the N-terminus of D6DM [20, 22]. D6DM showed a high identity to delta-6 fatty acid desaturases of *R. oryzae* (64%), *Amylomyces rouxii* (58%), *T. elegans* (56%), *M. alpine* (46%), and *M. isabellina* (45%). We also noticed that the cytochrome b_5-like domain and the three histidine-rich motif areas of D6DM are the most conserved regions compared with other desaturases. These results suggested that the obtained cDNA encodes for a putative delta-6 fatty acid desaturase that is responsible for the synthesis of GLA in the *C. echinulata* MIAN6.

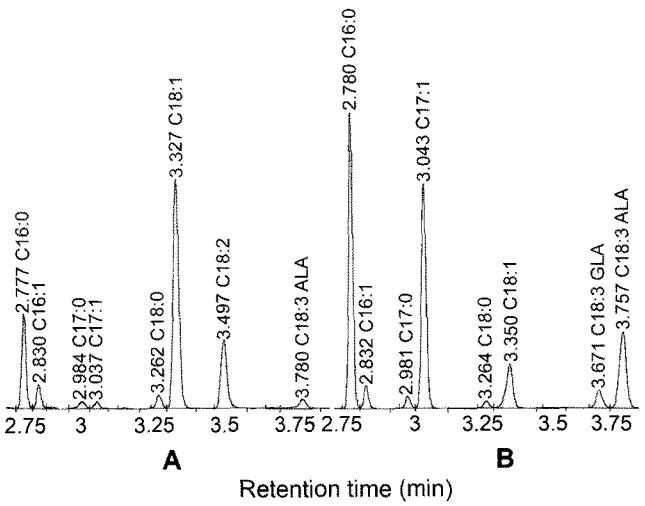

Fig. 3. Identification of fatty acid compositions in *P. pastoris* GS115 harboring pHBM906 (**A**) and pHBM605 (**B**) by gas chromatography analysis.

Functional Analysis of Delta-6 Fatty Acid Desaturase from *C. echinulata*.

To further demonstrate the function of this putative delta-6 fatty acid desaturase, *D6DM* was subcloned into pHBM906 under the control of the inducible AOX1 promoter. The recombinant plasmid pHBM605 harboring *D6DM* was then transformed into *P. pastoris* GS115 for heterologous expression. Expression of the D6DM was induced by supplementation of methanol as the sole carbon source, and the cells were grown for another 72 h after induction. One novel fatty acid peak corresponding to the GLA methyl ester standard was detected in GC analysis of FAME from the recombinant yeast harboring pHBM605 (Fig. 3). This peak was absent in the yeast harboring the empty vector as control. The percentage of this new fatty acid was 3.03% of the total fatty acids (Table 1). Notably, the amount of ginkgolic acid (C17:1) and palmitic acid (16:0) was incredibly increased (Table 1), which suggested that delta-6 fatty acid desaturase may play a role in some other fatty acid formation. This observation awaits further investigations. This is the first report that delta-6 fatty acid desaturase expression could increase the amount of ginkgolic acid and palmitic acid accumulation in addition to producing GLA formation. It was reported that the delta-6 fatty acid desaturase gene from *T. elegans* was also expressed in *P. pastoris*; however, two novel fatty acids, GLA and C18:2$\Delta^{6,9}$, were both detected by GC analysis [25]. It was also reported that the expression of the delta-6 fatty acid desaturase of *M. rouxii* in *Saccharomyces cerevisiae* can introduce a double bond into C15, C16, C17, and C18 fatty acid substrates at the delta-6 position of the fatty acyl chains, suggesting that delta-6 fatty acid desaturase may have no fatty acyl chain-length specificity but only position specificity [17]. We also observed that the amount of ALA increased when delta-6 desaturase was expressed in *P. pastoris*. We deduced that delta-6 desaturase does not directly catalyze the formation of ALA, whereas the whole flux of fatty acids changed when it expressed in *P. pastoris*. This might be the real reason for ALA accumulation. It has been reported that delta-12 desaturase, delta-6 desaturase, and delta-6 elongase, which were proven to be irrelevant to the ALA accumulation, increased the amount of ALA when they were introduced into *S. cerevisiae* [10]. Taken together, this result strongly supported that *D6DM* from *C. echinulata*, encoding a delta-6 fatty acid desaturase, is responsible for GLA accumulation in *P. pastoris*.

To summarize, we first isolated a delta-6 desaturase gene involved in GLA accumulation in oleaginous fungus *C. echinulata*. In addition, this gene has been successfully

Table 1. Fatty acid compositions (%) of total lipid from yeast transformants harboring the control plasmid pHBM906 and the recombinant plasmid pHBM605. Each assay was repeated three times.

P. pastoris harboring plasmids	Fatty acid composition (% of total fatty acid)								
	16:0	16:1	17:0	17:1	18:0	18:1	18:2	18:3GLA	18:3ALA
pHBM906	15.0	4.1	1.6	1.3	2.7	49.6	16.2	0	2.9
pHBM605	33.0	2.8	1.6	29.6	1.0	8.0	0.1	3.1	14.3

expressed in *P. pastoris* and makes it accumulate GLA. This work would be helpful for further investigation on the polyunsaturated fatty acids metabolic pathways in *C. echinulata*. Furthermore, these results may prove advantageous in production of GLA by recombinant yeast.

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References

1. Chen, H. and C. Chang. 1996. Production of gamma-linolenic acid by the fungus *Cunninghamella echinulata* CCRC31840. *Biotechol. Prog.* **12:** 338–341.
2. Chen, R., S. Tsuda, K. Matsui, M. Fukuchi-Mizutani, M. Ochiai, S. Shimizu, *et al.* 2005. Production of gamma-linolenic acid in *Lotus japonicus* and *Vigna angularis* by expression of the delta-6 fatty acid desaturase gene isolated from *Mortierella alpina*. *Plant Sci.* **169:** 599–605.
3. Dyer, J. M., S. Stymne, A. G. Green, and A. S. Carlsson. 2008. High-value oils from plants. *Plant J.* **54:** 640–655.
4. Fan, Y. Y. and R. S. Chapkin. 1998. Importance of dietary gamma-linolenic acid in human health and nutrition. *J. Nutr.* **128:** 1411–1414.
5. Fakas, S., M. Certik, S. Papanikolaou, G. Aggelis, M. Komaitis, and M. Galiotou-Panayotou. 2008. Gamma-linolenic acid production by *Cunninghamella echinulata* growing on complex organic nitrogen sources. *Bioresour. Technol.* **99:** 5986–5990.
6. Fakas, S., S. Papanikolaou, M. Galiotou-Panayotou, M. Komaitis, and G. Aggelis. 2008. Organic nitrogen of tomato waste hydrolysate enhances glucose uptake and lipid accumulation in *Cunninghamella echinulata*. *J. Appl. Microbiol.* **105:** 1062–1070.
7. Fakas, S., S. Papanikolaou, M. Galiotou-Panayotou, M. Komaitis, and G. Aggelis. 2006. Lipids of *Cunninghamella echinulata* with emphasis to gamma-linolenic acid distribution among lipid classes. *Appl. Microbiol. Biotechnol.* **73:** 676–683.
8. Flowers, M. T. and J. M. Ntambi. 2008. Role of stearoyl-coenzyme A desaturase in regulating lipid metabolism. *Curr. Opin. Lipidol.* **19:** 248–256.
9. Gema, H., A. Kavadia, D. Dimou, V. Tsagou, M. Komaitis, and G. Aggelis. 2002. Production of gamma-linolenic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel. *Appl. Microbiol. Biotechnol.* **58:** 303–307.
10. Hisashi, Y., I. Hitoshi, K. Yasushi, K. Kazuyoshi, A. Tsunehiro, O. Kazuhisa, and U Hiroshi. 2007. Heterologous production of dihomo-γ-linolenic acid in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **73:** 6965–6971.
11. Hong, H., N. Datla, D. W. Reed, P. S. Covello, S. L. Mackenzie, and X. Qiu. 2002. High-level production of gamma-linolenic acid in *Brassica juncea* using a delta-6 desaturase from *Pythium irregulare*. *Plant Physiol.* **129:** 354–362.
12. Horrobin, D. F. 1992. Nutritional and medical importance of gamma-linolenic acid. *Prog. Lipid Res.* **31:** 163–194.
13. Horrobin, D. F. 1993. Fatty acid metabolism in health and disease: The role of delta-6 desaturase. *Am. J. Clin. Nutr.* **57:** 732–736.
14. Kapoor, R. and Y. S. Huang. 2006. Gamma linolenic acid: An anti-inflammatory omega-6 fatty acid. *Curr. Pharm. Biotechnol.* **7:** 531–534.
15. Liang, S., X. Zhang, and H. Wang. 2004. Study on metabolic law of gamma-linolenic acid produced by *Cunninghamella echinulata*. *Biotechnol.* (*Chinese*) **14:** 48–50.
16. Laoteng, K., R. Mannontarat, M. Tanticharoen, and S. Cheevadhanarak. 2000. Delta-6 desaturase of *Mucor rouxii* with high similarity to plant delta-6 desaturase and its heterologous expression in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **279:** 17–22.
17. Ranong, S. N., K. Laoteng, P. Kittakoop, M. Tanticharoen, and S. Cheevadhanarak. 2006. Targeted mutagenesis of a fatty acid D6-desaturase from *Mucor rouxii*: Role of amino acid residues adjacent to histidine-rich motif II. *Biochem. Biophys. Res. Commun.* **339:** 1029–1034.
18. Sakuradani, E. and S. Shimizu. 2003. Gene cloning and functional analysis of a second delta-6 fatty acid desaturase from an arachidonic acid-producing *Mortierella* fungus. *Biosci. Biotechnol. Biochem.* **67:** 704–711.
19. Sakuradani, E., M. Kobayashi, and S. Shimizu. 1999. Delta-6 fatty acid desaturase from an arachidonic acid-producing *Mortierella* fungus: Gene cloning and its heterologous expression in a fungus, *Aspergillus*. *Gene* **238:** 445–453.
20. Sayanova, O. and M. A. Smith. 1997. Expression of a borage desaturase cDNA containing an N-terminal cytochrome *b5* domain results in the accumulation of high levels of delta-6 desaturated fatty acid in transgenic tobacco. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 4211–4216.
21. Shanklin, J., E. Whittle, and B. G. Fox. 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-coA desaturase, and are conserved in alkane hydroxylase and xylase monooxygenase. *Biochemistry* **33:** 12787–12794.
22. Sperling, P., H. Schmidt, and E. Heinz. 1995. A cytochrome-*b5*-containing fusion protein similar to plant acyl lipid desaturase. *Eur. J. Biochem.* **232:** 798–805.
23. Tao, J., L. Zhao, X. Zhang, S. Zhang, H. Wang, and Y. Gu. 2007. Growth law of gamma-linolenic acid production by *Cunninghamella echinulata*. *J. U.S. China Med. Sci.* **4:** 55–60.
24. Vrinten, P., G. Wu, M. Truksa, and X. Qiu. 2007. Production of polyunsaturated fatty acids in transgenic plants. *Biotechnol. Genet. Eng. Rev.* **24:** 263–279.
25. Wang, D., M. Li, D. Wei, Y. Cai, Y. Zhuang, and L. Xing. 2007. Identification and functional characterization of the delta-6 fatty acid desaturase gene from *Thamnidium elegans*. *J. Eukaryot. Microbiol.* **54:** 110–117.
26. Wang, H., Z. Deng, S. Zhou, and X. Zhang. 2007. Using potato starch wastewater to produce GLA with *Cunninghamella echinulata*. *China Oils Fats* (*Chinese*) **32:** 49–51.
27. Zhang, Q., M. Li, H. Ma, Y. Sun, and L. Xing. 2004. Identification and characterization of a novel delta-6 fatty acid desaturase gene from *Rhizopus arrhizus*. *FEBS Lett.* **556:** 81–85.