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NOTE

Molecular Cloning and Expression Analysis of a Delta 6-Fatty Acid Desaturase Gene from *Rhizopus stolonifer* Strain YF6 Which Can Accumulate High Levels of Gamma-Linolenic Acid

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The delta 6-desaturase gene was cloned from *Rhizopus stolonifer*, which could accumulate up to 49% of gamma-linolenic acid (GLA, C18:3 $\Delta^{6,9,12}$) to the total fatty acids. The cloned DNA contains a 1,380 bp open reading frame encoding a protein of 460 amino acids, which showed high similarity to those of fungal delta 6-desaturases with three conserved histidine-rich motifs and HPGG motif. Notably, this deduced sequence had a shorter C-terminus. Results demonstrated that the cDNA sequence exhibited delta 6-desaturase activity by accumulation of about 22.4% of GLA to the total fatty acids in the recombinant *Pichia pastoris* strain GS115.

***Keywords*:** desaturase, *Rhizopus stolonifer*, gamma-linolenic acid

Studies on searching for novel and rich resources of polyunsaturated fatty acids (PUFAs) have proceeded in various fields regarding health and dietary requirements. The most readily available PUFAs sources have been fish oils, animal tissues and microbial cells. Transgenic plants such as tobacco with some desaturase genes derived from other organisms have been reported to produce n-3 and n-6 PUFAs (Vrinten *et al*., 2007). In addition, mutants from filamentous fungi *Mortierella* have also been reported to be rich in unique PUFAs (Sakuradani *et al*., 1999; Hong *et al*., 2002; Sakuradani and Shimizu, 2003). Among them, Gamma linolenic acid (GLA, C18:3, n-6) is of great interest due to their important functions (Sakuradani and Shimizu, 2003; Lu *et al*., 2007, 2009; Flowers and Ntambi, 2008). GLA, an n-6 PUFA, is present in trace amounts in some green leafy vegetables, organ meats and nuts. The most significant sources of GLA are plant seed oils of evening primrose (7-10 g/100 g GLA), blackcurrant (15-20 g/100 g GLA), borrage (18-26 g/100 g GLA) and microbial oils (23-26 g/100 g GLA) (Horrobin, 1992; Gema *et al*., 2002). GLA is further metabolized to dihomogamma linlenic acid (DGLA) which undergoes oxidative metabolism by cyclooxygenases and lipoxygenases to produce anti-inflammatory eicosanoids such as prostaglandins and leukotrienes. GLA and its metabolites have also been identified to affect expression of various genes by regulating the levels of gene products. These gene products play significant roles in immune functions and apoptosis (Horrobin, 1993; Fan and Chapkin, 1998; Kapoor and Huang, 2006; Flowers and Ntambi, 2008).

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GLA is synthesized from linoleic acid (LA, C18:2 $\Delta^{9,12}$), an essential fatty acid of omega-6 series by the action of enzyme delta 6-fatty acid desaturase. Delta 6-fatty acid desaturase gene has been previously cloned and characterized from several fungi, such as *Thamnidium elegans*, *Cunninghamella echinulata*, *Mortierella alpina*, *M. isabellina*, *Mocur rouxii*, *Pythium irregulare*, *Rhizopus arrhizus*, *R. nigricans*, and *R. stolonifer* strain As 3.38 (Laoteng *et al*., 2000; Hong *et al*., 2002; Zhang *et al*., 2004, 2007; Chen *et al*., 2005; Lu *et al*., 2007, 2009; Wang *et al*., 2007; Wan *et al*., 2009). In our previous work, we have isolated an endophytic fungus *R. stolonifer* strain YF6 from GLA producing plant *Oenothera biennis*. Interestingly, this strain could accumulate up to above 49% of GLA to the total fatty acids (data not shown). In this study, we report the cloning of a delta 6-desaturase from *R. stolonifer* strain YF6, and characterization of its ability to direct the synthesis of GLA by heterologous expression in *Pichia pastoris*.

Cloning of the delta 6-fatty acid desaturase gene from *R. stolonifer*

Recently, the delta 6-desaturase genes have been cloned from several fungi including the genus *Rhizopus* (Zhang *et al*., 2004; Lu *et al*., 2007, 2009). Our previous paper also reported that the delta 6-desaturase gene was isolated from oleaginous fungus *C. echinulata* (Wan *et al*., 2009). In this study, to clone the delta 6-fatty acid desaturase full-length cDNA and structural gene, *R. stolonifer* strain YF6, which was an endophytic fungus previously isolated from inner stem of *Oenothera biennis* L., was used. This strain was maintained on YPD plate (10 g yeast extract, 20 g peptone, and 20 g agar per L) at 4°C and regularly transferred every three months. It was grown at 28°C for 3 days in a liquid YPD medium with constant shaking

(200 rpm/min).

Genomic DNA of strain YF6 was isolated by using Plant/Fungi DNA Isolation kit (Norgen Biotek Corporation, Canada). Based on the nucleotide sequence of the reported delta 6-desaturase gene from *R. stolonifer* strain R31.6, two specific primers, D6DRF (5'-atgagtacattagatcgtcaat) and D6DRR (5'-ttaaa acgacttttgcttaa) were designed. PCR amplification was carried out using genomic DNA as template. The obtained fragment was cloned into the pMD 18-T vector (TaKaRa Bio, China) and sequenced (Invitogen, China). Total RNA was extracted from the powder by using TRIzol reagent (Invitrogen). Then the mRNA was extracted from the total RNA by using an Oligotex mRNA Mini kit (QIAGEN, Germany). First-strand cDNA was synthesized with the first-strand cDNA Synthesis kit (Promega, USA) and was used as a template for reverse transcription polymerase chain reaction (RT-PCR). The forward primer and the reverse primer D6DRF and D6DRR were also used. The amplified product of expected length was subcloned into pMD 18-T vector and then sequenced (Invitogen). Sequence alignment and phylogenetic analysis were performed using the software DNAMAN (version 4.0) and CLUSTAL X (Version 2.0). The cDNA sequence of delta 6-desaturase gene from *R. stolonifer* YF6 has been deposited at GenBank database under the accession number DQ291156.

Sequence analysis revealed that the structural gene was 1,556 bp in length and it contained three introns. These introns were flanked by the typical (GT-AG) intron splice site and were AT-rich (data not shown). The cDNA sequence contained an ORF of 1,380 bp, designated as *D6DR*, encoding 460 amino acid residues with an estimated molecular mass of 53 kDa. The deduced amino acid sequence of *D6DR* was compared with those desaturases from other organisms. Results showed that this sequence had 88% identity to the delta 6-fatty acid desaturase from *R. stolonifer* As3.38, 76% identity to that from *R. oryzae*, 79% identity to that from *T. elegans*, and 56% identity to that from *C. echinulata*. The comparison of the deduced amino acids of *D6DR* with other fungal delta 6-fatty acid desaturases revealed that four conserved histidine-rich motifs at amino acid positions 48, 210, 352, and 391 (Fig. 1). Hydrophobic regions which known to all membrane-bound desaturases are also found in this cDNA sequence (data not shown). In addition, a 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 *D6DR* (Fig. 1) (Sayanova and Smith, 1997; Ranong *et al*., 2006). Interestingly, the C-terminus of *D6DR* was obviously shorter than other delta 6-desaturases (Fig. 1), which suggested that this 24-bp C-terminus might not be essential to the activity of delta 6-desaturase. All these results indicated that full-length of cDNA and structural gene sequences of delta 6-fatty acid desaturase was isolated from *R. stolonifer* strain YF6.

Functional analysis of delta 6-fatty acid desaturase from *R. stolonifer*

To further demonstrate the function of this putative delta 6-fatty acid desaturase, *D6DR* was inserted between *Not*I and *Cpo*I sites of the expression vector pHBM906 (preserved in our laboratory) to create plasmid pHBM954. Sequence of the product was verified. The resulting vector was linearized by *Sal*I 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℃ for 3 days.

Heterologous expression of *D6DR* was induced under tran-

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D6DR      MSTLDRQSIFTIKELESISQRIHDGDEEAMKFIIIDKKVYDVTEFIEDHPGGAQVLLTHVGKDASDVFHAMHPESAYEVL
RnD6D     MSTLDRQSIFTIKELESISQRIHDGDEEAMKFIIIDKKVYDVTEFIEDHPGGAQVLLTHVGKDASDVFHAMHPESAYEVL
RAD6D     MSTsDRQSvFTlKELElInQkhrDGDksAMKFIIIDrKVYDVTEFlEDHPGGAQVLLTHVGKDASDVFHAMHPESAYEiL
TED6      MSTLDRQSIFTIKELESISQRIHDGDEEAMKFIIIDKmVYDVTEFIEDHPGGAQVLLTHVGKDASDVFHAMrPESAYEVL
D6DM      ..msgqtrvFkrsEvsdslkayqaGDknAdKFlIvDnKVYDiTdFIaDHPGGAQVisTHiGKDASDVFHAMHPESAYElL

D6DR      NNYFVGDVQETVVTEKSSSAQFAV.........EMRQLRDQLKKEGYFHSSKLFYAYKVLSTLAICIAGLSPLYAYGRTST
RnD6D     NNYFVGDVQETVVTEKSSSAQFAV.........EMRQLRDQLKKEGYFHSSKLFYAYKVLSTLAICIAGLSlLYAYGRTST
RAD6D     NNYFVGDVkdahVkEtp.SAQFAs.........EMRQLRDQLKKEGYFHSSKayYvYKVLSTLAlCaAGLtlLYAYGhTST
TED6      NNYFVGDVQETVVTEKSSSAQFAV.........EMRQLRDQLKKEGYFHSSKLFYAYKVLSTLAICIAGLSlLYAYGvlpl
D6DM      aNcyVGDlaadhagvqgelvngvhkkskafadEMRsLRerLetEGaFngSvpFYiYKVvSTLAIgatGLamLYygGhsts

D6DR      LAVVASAITVGIFWQQCGWLAHDFGHHQCFEDRTWNDVLVVFLGNFCQGFSLSWWKNKHNTHHASTNVHGQDPDIDTAPV
RnD6D     LAVVASAITVGIFWQQCGWLAHDFGHHQCFEDRTWNDVLVVFLGNFCQGFSLSWWKNKHNTHHASTNVHGQDPDIDTAPV
RAD6D     LAVVASAIiVGIFWQQCGWLAHDFGHHQCFEDRsWNDVLVVFLGNFCQGFSLSWWKNKHNTHHASTNVHGhDPDIDTAPV
TED6      wlsshllllvsFgnsvvgwltisdiinasktalgtmfLVVFLGNFCQGFSLSWWKdKHNvHHASTNVHGQDPDIDTAPV
D6DM      vvlaAavvvgl.FWQQCGWLAHDFGHHQaFaDhTvNDVmiaFLGgFCQGFSLSWWKNKHNTHHASTNVHGhDPDIDTAPV

D6DR      LLWDEYASAAYYASLDQEPTMV.SRFLAEQVLPHQTRYFFFILAFARLSWALQSLSYSFKKESINKSRQLNLFERVCIVG
RnD6D     LLWDEYASAAYYASLDQEPTMV.SRFLAEQVLPHQTRYFFFILAFARLSWALQSLSYSFKKESINKSRQLNLFERVCIVG
RAD6D     LLWDEYASAAYYASLDeEPTMi.SRFLAEsVLPHQTRYyFFvLgFARLSWAiQSLlYSFKqgaINKShQLNLFERfClVs
TED6      LLWDEYASAAYYASLDQEPTMV.SRFLAEQVLPHQTRYFFFILAFARLSWALQSLSYSFKKESINKSRQLNLFERVCIVG
D6DM      LLWDEfAtAnfYgnLegqkdsafSRFiAEhVLPyQTRYyFFvLgFARLSWAiQSLqYSFtvgtlNKSktLNLFERtmlVs

D6DR      HWALSAFCIYSWCSNVYHMVLFFLVSQATTGYTLALVFALNHNGMPVITEEKAESMEFFEIQVITGRDVTLSPLGDWFMG
RnD6D     HWALfAFCIYSWCSNVYHMVLFFLVSQATTGYTLALVFALNHNGMPVITEEKAESMEFFEIQVITGRDVTLSPLGDWFMG
RAD6D     HWtLftyCtlaWCSNVYHMiLFFLVSQATTGYTLALVFALNHNGMPVITEEKAESMEFFEIQVITGRDVTLSPLGDWFMG
TED6      HWALfAFCIYSWCSNVYHMVLFFLVSQATTGYTLALVFALNHNGMPVITEEKAESMEFFEIQVITGRDVTLSPLGDWFMG
D6DM      HWiLfttwtllfinswtnMVmFFvVSQATTGYaLALVFAmNHsGMPVlTqEqAqkMEFyEIQVvTaRDVTLgaLGDWFcG

D6DR      GLNYQIEHHVFPNMPRHNLPTVKPMVKSLCQKYDINYHDTGFLKGTLEVLQTLDIT                    447
RnD6D     GLNYQIEHHVFPNMPRHNLPTVKPMVKSLCQKYDINYHDTGFLKGTLEVLQTLDITsklslqlskksf      459
RAD6D     GLNYQIEHHVFPNMPRHNLPkVKPMVKSLCkKYDINYHDTGFLKGTLEVLkTLDITsklslqlskksf      458
TED6      GLNYQIEHHVFPNMPRHNLPTVmPMVKSLCQKYDINYHDTGFLKGTLEVLQTLDITsklslqlskksf      459
D6DM      GLNYQIEHHVFPdMPRHyLPkVKPqVKaLCkKhnIlYHDTsaLrGTLEVLQTLDvvqklcasslkrcfr      466
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Fig. 1. Sequence alignment of deduced amino acids of delta 6-desaturase from fungus *R. stolonifer* YF6 (*D6DR*) with those of *R. stolonifer* R31.6 (*RnD6D*), *R. oryzae* NK030037 (*RAD6D*), *T. elegans* (*TED6*), and *C. echinulata* (*D6DM*). A cytochrom b5-like domain and three conserved histidine-rich motifs are underlined.

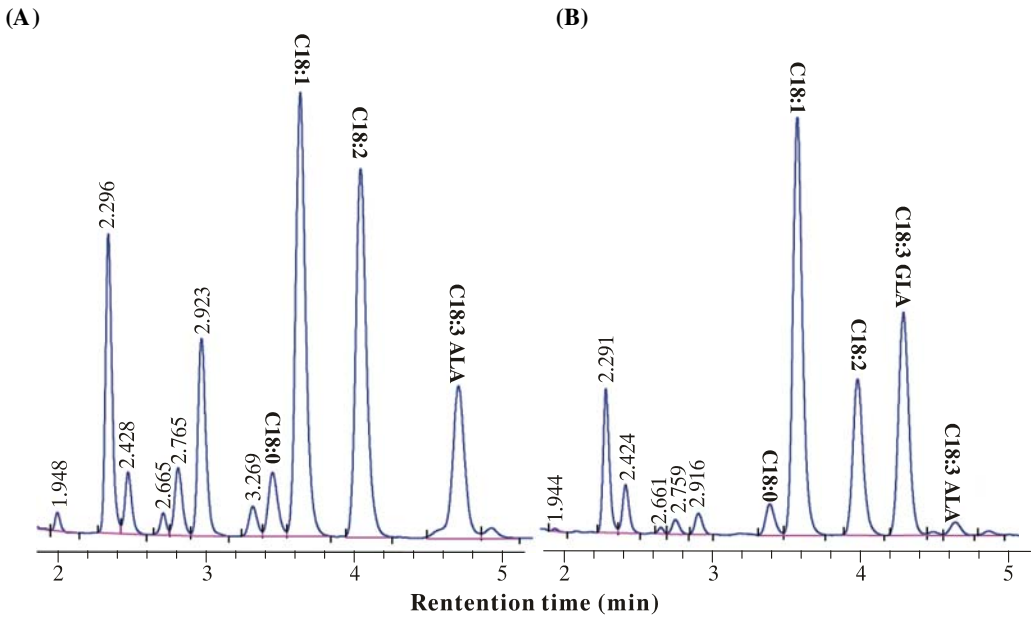

Fig. 2. Identification of fatty acid compositions in *P. pastoris* GS115 harboring pHBM 906 (A) and pHBM 954 (B).

scriptional control of the yeast *AOX1* promoter. Selected colonies were grown on BMGY medium (Invitrogen) at 28°C overnight. Then 5 ml cultures was 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. Expression of the *D6DR* was induced by supplementation of methanol as the sole carbon source, and the cells were grown for another 72 h at 20°C in BMMY medium (Invitrogen). Subsequently, cells were harvested by centrifugation, and washed three times with sterile distilled water.

Fatty acid compositions of total lipid from mycelia cultivated under different growth conditions were determined by modification of direct trans-methylation method (Wan *et al*., 2009). Dried mycelia were crushed, samples were trans-methylated with 5% HCl in methanol at 80°C for 1 h. Fatty acid methyl esters (FAME) were analyzed by gas chromatography (Agilent, USA) 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. One novel fatty acid peaks corresponding to the GLA methyl ester standard was detected in GC analysis of FAME from the recombinant yeast harboring pHBM954 (Fig. 2). This peak was absent in the yeast harboring the empty vector pHBM906 as control. The percentage of this new fatty acid was 22.4% to the total fatty acids (Table 1). We noticed that the delta 6 fatty acid desaturase gene from *R. stolonifer* strain As3.38 was also expressed in *Saccharomyces cerevisiae*, however, the percentage of GLA from recombinant strain was 12.25% to the total fatty acids (Zhang *et al*., 2004). In addition, the amount of alpha linolenic acid (ALA, C18:3$\Delta^{9,12,15}$), an n-3 polyunsaturated fatty acid, was incredibly decreased (Table 1). LA is the precusor for both ALA and GLA formation. Delta 15-fatty acid desaturase has been demonstrated to be the key enzyme responsible for ALA synthesis (Ratledge, 2004). Therefore, we speculate that overexpression of delta 6-desaturase gene in *P. pastoris* might inhibit the expression of delta 15-desaturase gene in this strain. This speculation awaits further investigations. In summary, this result strongly supported that *D6DR* from *R. stolonifer* YF6 encoding a delta 6-fatty acid desaturase which is responsible for GLA accumulation in *P. pastoris*.

Taken together, we first cloned a delta 6-desaturase cDNA from an endophytic fungus *R. stolonifer* strain YF6, which could produce high levels of GLA. In addition, this gene has been successfully expressed in *P. pastoris* and makes it accumulate up to 22.4% of GLA to the total fatty acids. This work would be helpful for further investigation on the PUFAs metabolic pathways in *R. stolonifer*. Furthermore, these results may prove advantageous in production of GLA by recombinant yeast.

Table 1. Fatty acid compositions (%) of total lipid from yeast transformants harboring the control plasmid pHBM906 and the recombinant plasmid pHBM954. Each assay was repeated three times.

P. pastoris harboring plasmids	Fatty acid composition (% of total fatty acid)				
	18:0	18:1	18:2	18:3GLA	18:3ALA
pHBM906	3.7	26.6	23.8	0	11.5
pHBM954	2.7	38.5	15.5	22.4	1.3

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References

Chen, R., S. Tsuda., K. Matsui, M. Fukuchi-Mizutani, M. Ochiai, S. Shimizu, E. Sakuradani, and *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.

Fan, Y.Y. and R.S. Chapkin. 1998. Importance of dietary gamma-linolenic acid in human health and nutrition. *J. Nutr*. 128, 1411-1414.

Flowers, M.T. and J.M. Ntambi. 2008. Role of stearoyl-coenzyme a desaturase in regulating lipid metabolism. *Curr. Opin. Lipidol*. 19, 248-256.

Gema, H., A. Kavadia, D. Dimou, V. Tsagou, M. Komaitis, and G. Aggelis. 2002. Production of gamma-linolenic acid by *Cunninghamella echinulata* culticated on glucose and orange peel. *Appl. Microbiol. Biotechnol*. 58, 303-307.

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 delta6 desaturase from *Pythium irregulare*. *Plant Physiol*. 129, 354-362.

Horrobin, D.F. 1992. Nutritional and medical importance of gamma-linolenic acid. *Prog. Lipid. Res*. 31, 163-194.

Horrobin, D.F. 1993. Fatty acid metabolism in health and disease: the role of delta 6-desaturase. *Am. J. Clin. Nutr*. 57, 732-736.

Kapoor, R. and Y.S. Huang. 2006 Gamma linolenic acid: an anti-inflammatory omega-6 fatty acid. *Curr. Pharm. Biotechnol*. 7, 531-534.

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.

Lu, H., Y.R. Chai, X.K. Zhang, T.G. Lei, and J.N. Li. 2007. Cloning and expression of a delta 6-fatty acid desaturase gene from *Rhizopus stolonifer* in *Saccharomyces cervisiae*. *Wei. Sheng. Wu. Xue. Bao*. 47, 59-63.

Lu, H., J.N. Li, Y.R. Chai, and X.K. Zhang. 2009. Identification and characterization of a novel Δ6-fatty acid desaturase gene from *Rhizopus nigrican*s. *Mol. Biol. Rep*. 36, 2291-2297.

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.

Ratledge, C. 2004. Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochim*. 86, 807-815.

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.

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.

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. USA* 94, 4211-4216.

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.

Wan, X., Y.B. Zhang, P. Wang, and M.L. Jiang. 2009. Production of gamma-linolenic acid in *Pichia pastoris* by expression of a delta-6 desaturase gene from *Cunninghamella echinulata*. *J. Microbiol. Biotechnol*. 19, 1098-1102.

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.

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.

Zhang, X., M. Li, D. Wei, L. Wang, X. Chen, and L. Xing. 2007. Disruption of the fatty acid delta6-desaturase gene in the oil-producing fungus *Mortierella isabellina* by homologous recombination. *Curr. Microbiol*. 55, 128-134.