Journal of Microbiology (2013) Vol. 51, No. 6, pp. 807–813
DOI 10.1007/s12275-013-3144-3


# Isolation and Functional Characterization of a Delta 6-Desaturase Gene from the Pike Eel (*Muraenesox cinereus*)

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(Received March 11, 2013 / Accepted July 12, 2013)

**Stearidonic acid (STA; 18:4n-3) and γ-linolenic acid (GLA; 18:3n-6) are significant intermediates in the biosynthetic pathway for the very-long-chain polyunsaturated fatty acids of eicosapentaenoic acid (EPA; 20:5n-3) and arachidonic acid (ARA; 20:4n-6), respectively. To develop a sustainable system for the production of dietary polyunsaturated fatty acids, we focused on the action of the enzyme delta 6-desaturase (D6DES) on the essential acids, linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3). A 1,335-bp full-length cDNA encoding D6DES (McD6DES) was cloned from *Muraenesox cinereus* using degenerate PCR and RACE-PCR methods. To investigate the enzymatic activity of McD6DES in the production of n-6 and n-3 fatty acids, a recombinant plasmid expressing *McD6DES* (pYES-McD6DES) was transformed into and expressed in *Saccharomyces cerevisiae*. The exogenously expressed McD6DES produced GLA and STA at conversion rates of 14.2% and 45.9%, respectively, from the exogenous LA and ALA substrates. These results indicate that McD6DES is essentially a delta 6-desaturase involved in very-long-chain polyunsaturated fatty acid synthesis.**

***Keywords***: delta 6-desaturase, γ-linolenic acid, *Muraenesox cinereus*, *Saccharomyces cerevisiae*, stearidonic acid

## Introduction

With the recent worldwide increases in several human health disorders, including obesity, hypertension, and heart problems, there has been increasing interest in the study of foods that contain functional components or substances that act on human physiology, not only to improve health but to prevent disease (Forman *et al*., 1997; Damude and Kinney, 2008; Valenzuela, 2009). Fish and fish oils are the main dietary sources of very-long-chain polyunsaturated fatty acids (VLC-PUFAs). The therapeutic and preventive benefits of dietary VLC-PUFAs for cardiovascular diseases and rheumatoid arthritis are well documented (Metcalf *et al*., 2008). Most evidence suggests that these benefits are derived from the VLC-PUFAs eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Wang *et al*., 1990; Ackman and McLeod, 1998).

VLC-PUFAs are fatty acids of 20 or more carbon molecules in length, with three or more methylene-interrupted double bonds in the *cis* position. These fatty acids can be grouped into two main families, the omega-6 (or n-6) and omega-3 (or n-3) families, depending on the position of the first double bond proximal to the methyl end of the fatty acid. In most vertebrates, the biosynthesis of VLC-PUFAs involves the sequential desaturation and elongation of the precursor PUFAs, linoleic acid (LA; 18:2n-6) and α-linolenic acid (ALA; 18:3n-3) (Fig. 1). The synthesis of arachidonic acid (ARA; 20:4n-6) is achieved by delta 6-desaturation (EC 1.14.19.3) (Horrobin, 1993; Nakamura and Nara, 2004) of LA to produce γ-linolenic acid (GLA; 18:3n-6), which is elongated to dihomo γ-linoleic acid (20:3n-6), with subsequent delta 5-desaturation (Cook and McMaster, 2004). The synthesis of EPA (20:5n-3) from ALA requires the same enzymes and pathway as the synthesis of ARA, but DHA (22:6n-3) synthesis requires two further elongation steps: a second delta 6-desaturation and a peroxisomal chain-shortening step (Sprecher, 2000).

Because the human body cannot synthesize VLC-PUFAs *de novo* in adequate quantities, dietary intake of these compounds is important. Moreover, there is growing concern regarding the sustainability of global fish stocks because marine fish stocks are in severe decline after decades of overfishing. Some industrial processes exploit these efficient VLC-PUFA producers as factories for the sustainable production of these compounds (Ratledge, 2004), although the culture of nonadapted nonnative organisms can prove problematic and costly. Alternatively, the heterologous production of VLC-PUFAs in dedicated hosts is seen as a potential solution. Many reports have described the isolation of desaturases from a variety of organisms and their successful expression in several hosts, including *Saccharomyces cerevisiae* and plants (Spychalla *et al*., 1997; Sakuradani *et al*., 1999; Abbadi *et al*., 2004; Oura and Kajiwara, 2004; Domergue *et al*., 2005; Napier and Sayanova, 2005; Robert, 2006), confirming the availability of genetic technology with which to

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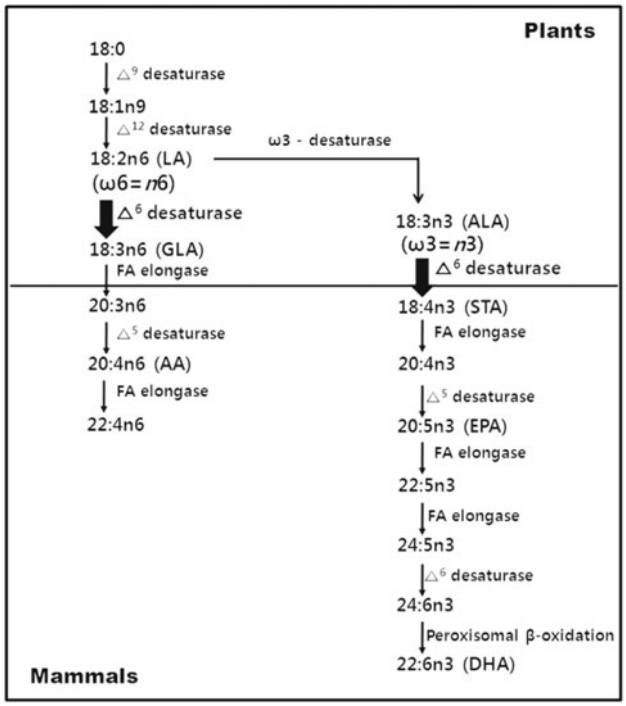


**Fig. 1. Proposed pathways of polyunsaturated fatty acid (PUFA) biosynthesis from α-linolenic acid (ALA, C18:3) and linoleic acid (LA, C18:2).** The desaturase involved in this work is indicated in the pathway with big bold arrow. Solid lines represent the steps that have been shown to occur in fishes.

metabolically engineer the synthesis of these important fatty acids. Nevertheless, the isolation of more-effective recombinant enzymes to generate the desired fatty acids in oilseed crops is still required. Because plant seed oils are currently used as an alternative source of VLC-PUFAs in the human diet, genetic modification of the fatty acid biosynthetic pathways in oilseed crops to produce the desired marine PUFA compounds could provide a viable source of these important fatty acids in the future.

*Muraenesox cinereus* is a functional food-fish species that contains high levels of n-3 fatty acids (EPA 20:5n-3 and DHA 22:6n-3) (Soccol and Oetterer, 2003). In the present study, we explored alternative sources of PUFAs for the manipulation of storage oils in plant seeds by isolating a delta 6-desaturase gene (*McD6DES*) from the pike eel, *M. cinereus*. We expressed the McD6DES protein and identified its function in *S. cerevisiae*. Yeast transformed with *McD6DES* successfully produced STA and GLA from ALA and LA, respectively (Fig. 1). These results provide evidence that McD6DES encodes a delta 6-desaturase and suggest that McD6DES is another candidate desaturase for use in the future technological production of PUFAs in heterologous expression systems, such as yeast and oilseed crops.

## Materials and Methods

### Fish materials

Liver tissue samples from the pike eel (*M. cinereus*), which contain high levels of PUFAs, were obtained from Jeju Island, Korea. These tissues were frozen in liquid nitrogen and stored at -80°C until RNA extraction.

### Isolation of the full-length delta 6-desaturase cDNA from *M. cinereus*

The delta 6-desaturase cDNA was isolated by PCR amplification from the cDNA derived from the total liver RNA of *M. cinereus* using TRIZol® Reagent (Invitrogen, USA). 5′- and 3′-rapid amplification of cDNA ends (RACE) was performed with the BD SMART™ RACE cDNA Amplification Kit and the BD Advantage 2 PCR Kit (Clontech, USA). Degenerate primers were designed for the initial cDNA isolation based on conserved motifs in the putative *D6DES* sequences of *Danio rerio* (AF309556), *Oncorhynchus masou* (AB070444), and *Salmo salar* (AY458652). The forward primer (primer set A, Table 1) targeted the conserved region encoding NHRHFQHH (Fig. 2A) and the reverse primer (primer set A, Table 1) targeted the conserved region encoding LNFQIEHH (Fig. 2A). Following the isolation of the cDNA fragment flanked by Uni*D6D*-F and Uni*D6D*-R, specific primers were designed for use in 5′-RACE and 3′-RACE PCR (primer set B, Table 1). Each RACE product was cloned and sequenced, and from this information, additional primers were designed to amplify the full-length cDNA of this gene (primer set C, Table 1).

### Construction of recombinant plasmid

The full-length *McD6DES* cDNA was reamplified with primer set D (Table 1) before *Hin*dIII and *Xho*I digestion, gel purification with the Qiagen Purification Kit (Qiagen, USA), and ligation into the corresponding sites of pYES2 to gen-

**Table 1. Primers used for amplifying Mc*D6D* cDNA and ORF.** The data include sequences and annealing temperatures (Ta) for primer pairs.

| Primer set | Primer name | Sequence (5′→3′) | Ta |
|---|---|---|---|
| A | Uni*D6D*-F | C CAT CGK CAC TTC CAG CAY CAC | 65°C |
| | Uni*D6D*-R | ATG RTG YTC RAT CTG RAA GTT GAG | |
| B | Mc*D6D* 3' RACE | AAC CAT CGG CAT TTC CAG CAC CAT GCA AAA CCC AA | 64°C |
| | Mc*D6D* 5' RACE | CAC TGG CTG GGT CTT TCC TAG CAC AAA GGT GTG GA | |
| C | Mc*D6D* Full Length F | ATG GGG GGC GGA GGT CAA CAG ACG GAG TC | 62°C |
| | Mc*D6D* Full Length R | TTA TTT ATG GAG ATA CGC ATC CAG CCA CAG | |
| D | Mc*D6D Hin*dIII F | CCC AAG CTT ACC ATG GGG GGC GGA GGT CAA | 68°C |
| | Mc*D6D Xho*I R | CCG CTC GAG TTA TTT ATG GAG ATA CGC ATC CAG CC | |

Restriction enzyme sites are underlined.

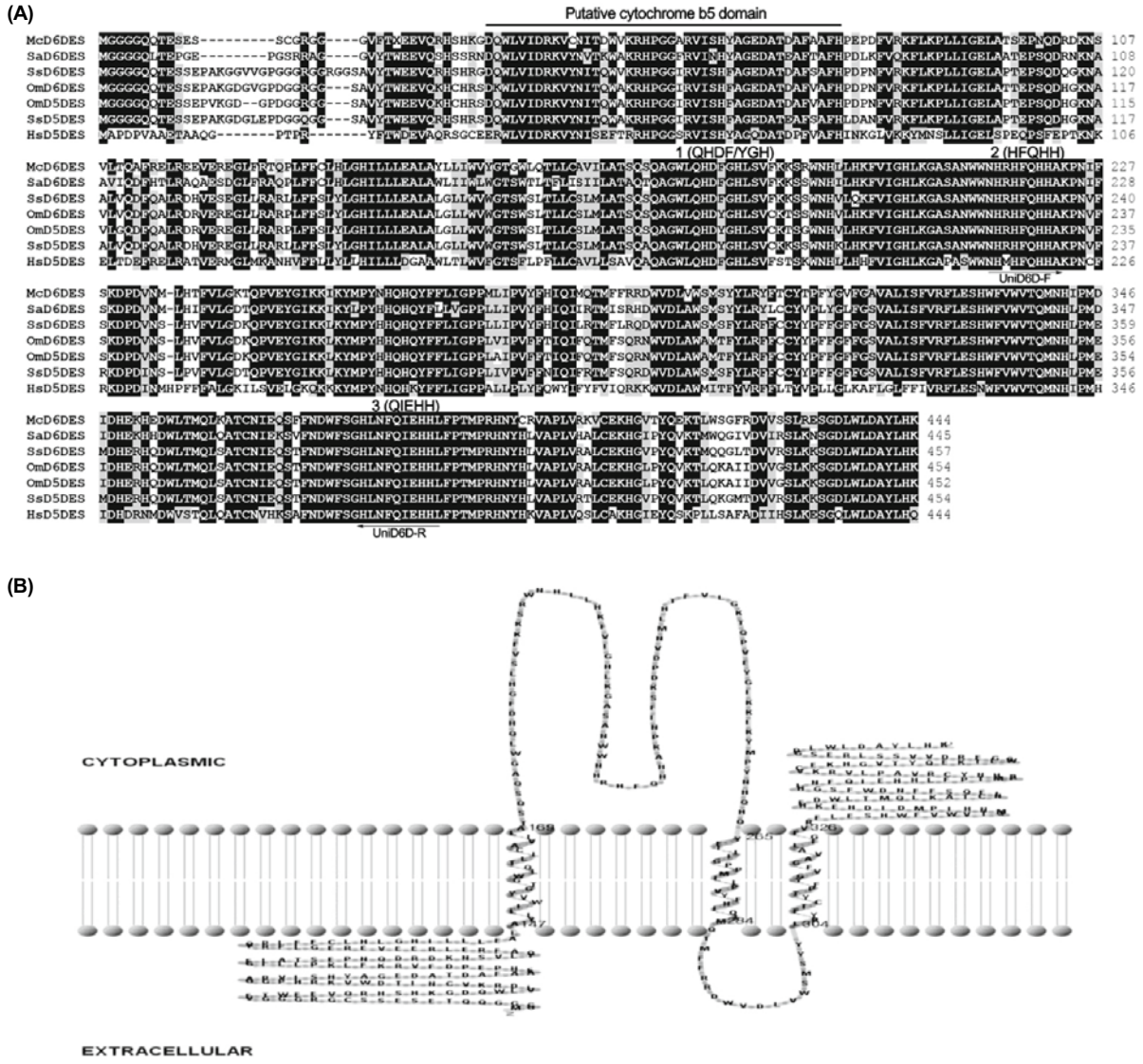


**Fig. 2. Alignment of the deduced amino acid sequences and topology model of McD6DES.** (A) The putative cytochrome-$b_5$-like domain is underlined. Three putative histidine-rich regions are indicated also (1, 2, and 3). The two arrows indicate the binding locations of the degenerate primers of forward and reverse. Sequence identities between *Muraenesox cinereus* and other species are black boxed with white letters. McD6DES (Pike eel, *Muraenesox cinereus,* HQ727979), SaD6DES (gilthead seabream, *Sparus aurata* delta 6-desaturase, AAL17639), SsD6DES (Atlantic Salmon, *Salmo salar* delta 6-desaturase, NP_001165752), OmD6DES (Rainbow trout, *Oncorhynchus mykiss* delta 6-desaturase, NP_001117759), OmD5DES (*Oncorhynchus masou* delta 6-desaturase, ABU87822), SsD5DES (Atlantic Salmon, *Salmo salar* delta 5-desaturase, NP_001117014), and HsD5DES (Human, *Homo sapiens* delta 5-desaturase, AF29378). (B) Topology model of *M. cinereus* delta 6-desaturase based on prediction using TMHMM and TMRPres2D. Three transmembrane helixes were shown to exist at position 147–169, 265–284, and 304–326 of the amino acid residues.

erate pYES-McD6DES.

### Functional characterization of McD6DES in yeast

The pYES-McD6DES and pYES2 vectors were introduced into *S. cerevisiae* INVSc1 using the Yeast Transformation Kit (YEAST1; Sigma, USA), according to the manufacturer's instructions. The transformants were selected on minimal SD Base (Clontech) agar plates containing 2% glucose and lacking uracil. The selected transformants were then grown overnight at 28°C in minimal SD Base broth lacking uracil and supplemented with 2% glucose. The cultured cells were diluted to an optical density ($OD_{600}$) of 0.4 in minimal medium (SD) containing 2% galactose and 0.1% Tergitol®-Nonidet P-40 (Sigma), with or without 0.5 mM substrate fatty acid, either LA or ALA. The cells were then incubated at 20°C for three days and at 15°C for a further three days, as described by Qiu *et al.* (2001). The cells were harvested by centrifugation at ~4,000×g for 5 min and the cell pellets were washed twice with water, freeze-dried, and stored at -80°C until the fatty acid analysis was performed.

### Fatty acid analysis

Yeast cells were lyophilized in a 50 ml tube. Five milliliters of MeOH:$CHCl_3$ (2:1, v/v) was added to the lyophilized

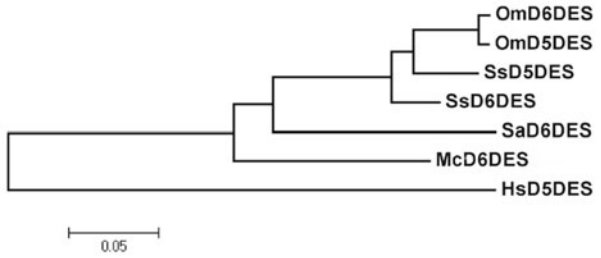


**Fig. 3. Phylogenetic tree of McD6DES and delta 6- and delta 5-desaturases from other species.** Sequence alignments and phylogenetic tree construction were performed using CLUSTAL W. The horizontal branch length is proportional to the amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. McD6DES (*Muraenesox cinereus*, HQ727979), SaD6DES (*Sparus aurata* delta 6-desaturase, AAL17639), SsD6DES (*Salmo salar* delta 6-desaturase, NP_001165752), OmD6DES (*Oncorhynchus mykiss* delta 6-desaturase, NP_001117759), OmD5DES (*Oncorhynchus masou* delta 6-desaturase, ABU87822), SsD5DES (*Salmo salar* delta 5-desaturase, NP_001117014), and HsD5DES (*Homo sapiens* delta 5-desaturase, AF29378).

samples, together with 1 mg of pentadecanoic acid (in 5 ml of MeOH) as the internal standard. Fatty acid methyl esters (FAMEs) were prepared using a lipid extraction method and were analyzed by gas chromatography (GC), as described in our previous report (Kim *et al*., 2011). The FAMEs were identified by reference to well-characterized commercial standard peaks (Sigma) based on their GC retention times and were quantified using computer software. All analyses were performed in triplicate and replicated three times. A methanolic base (Sigma-Aldrich Canada Ltd., Canada) and FAME standards (Sigma) were used as the reference standards.

## Results

### Identification and characterization of McD6DES from *M. cinereus*

A PCR-based cloning approach was used to identify the *M. cinereus* cDNA encoding delta 6-desaturase. Degenerate primers were designed to target the second conserved histidine box (forward primer; Fig. 2A) and the third histidine box (reverse primer; Fig. 2A), which had been identified in putative D6DES proteins from *Danio rerio*, *Oncorhynchus masou*, and *Salmo salar*. The Uni*D6D*-F and Uni*D6D*-R primers (primer set A, Table 1; Fig. 2A) were then used to amplify the corresponding cDNA from *M. cinereus*. The PCR products revealed a DNA band of 510 bp, which encoded a protein with a significant level of homology to the putative D6DES polypeptides of several fish species. Using partial cDNA sequences, 5′- and 3′-RACE primers were designed to amplify both ends of the putative *D6DES* fragment. The sequences of the resulting 5′- and 3′-RACE products were used, in turn, to design primers to amplify the 5′ and 3′ extremities of the sequence (primer set C, Table 1) and to obtain the full-length *McD6DES* cDNA. The open reading frame of *McD6DES* was found to be 1,335 base pairs. The mRNA and corresponding nucleotide sequences of this gene have been deposited in NCBI GenBank under accession number HQ727979 (Fig. 2A).

*McD6DES* encodes a polypeptide of 444 amino acid residues, with a predicted MW of 52.1 kDa. A BlastP search of the NCBI database revealed that the amino acid sequence of the McD6DES protein showed greatest identity to those of delta 6-desaturases from other species. McD6DES showed 77%, 76%, and 75% identity to delta 6-desaturases of the gilt-head bream (*Sparus aurata*), Atlantic salmon (*Salmo salar*), and rainbow trout (*Oncorhynchus mykiss*), respectively, and 76%, 75%, and 61% identity to a delta 5-fatty acid desaturase from *Salmo salar*, *Oncorhynchus masou*, and *Homo sapiens,* respectively. Alignment of McD6DES with the delta 6- and delta 5-desaturases of different species indicated that it shares the highly conserved sequences in the N-terminal cytochrome-$b_5$-like domain, the three histidine boxes (Fig. 2A), and the three transmembrane helices (Fig. 2B).

A phylogenetic analysis also demonstrated considerable homology between McD6DES and the delta 6- and delta 5-desaturases from various organisms. The deduced amino acid sequence of McD6DES clustered with all other delta 6- and delta 5-desaturases of fish, in a cluster that was distinct from the HsD5DES enzyme that processes delta 5-desaturase (Fig. 3). These results strongly suggest that the *McD6DES* gene encodes a putative delta 6-desaturase involved in the synthesis of GLA and STA in *M. cinereus*.

### Topology prediction

Topology predictions for the pike eel delta 6-desaturase were made at TMHMM Server v. 2.0, based on the TMHMM 2.0 algorithm (Moller *et al*., 2001) with the default option (http://www.cbs.dtu.dk/services/TMHMM/). The transmem-

**Table 2. Comparison of the deduced amino acids for the transmembrane helixes (TMHs) and orientation in McD6DES and other delta 6-desaturase and delta 5-desaturase**

| Name | Amino acid sites of predicted TMHs[a] | Orientation[b] | Evidence |
|---|---|---|---|
| McD6DES | 147-169, 265-284, and 304-326 | $N_{out}$-$C_{in}$ | |
| SaD6DES | 148-170, 267-289, and 304-326 | $N_{out}$-$C_{in}$ | |
| SsD6DES | 145-167, 278-297, and 317-339 | Nout-$C_{in}$ | |
| OmD6DES | 142-164, 275-297, and 312-334 | Nout-$C_{in}$ | TMHMM Server v. 2.0 |
| OmD5DES | 140-162, 273-295, and 310-332 | Nout-$C_{in}$ | |
| SsD5DES | 142-164, 275-297, and 312-334 | Nout-$C_{in}$ | |
| HsD5DES | 131-153, 266-288, and 301-323 | $N_{in}$-$C_{out}$ | |

[a] The numbers indicate amino acid positions in Fig. 2A.
[b] $N_{out}$-$C_{in}$ orientation is with an extracytoplasmic N-terminus and a cytoplasmic C-terminus

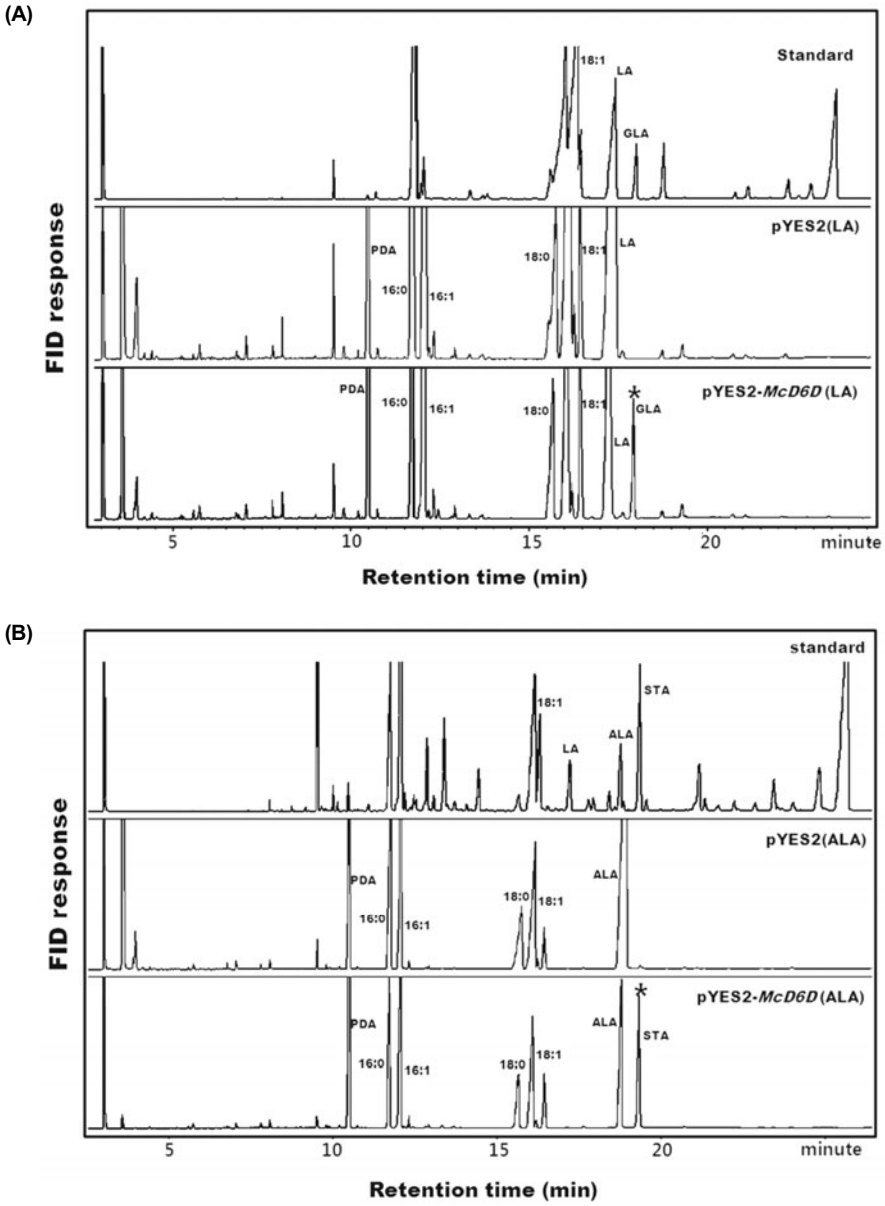


**Fig. 4. Fatty acid profiles of GC analysis from transgenic *S. cerevisiae* INVSc1.** (A) Profiles of the fatty acids in transformed yeast expressing pYES2-McD6DES and empty vector pYES2 as a control in the presence of LA substrate. (B) Profiles of the fatty acids in same transformed yeast mentioned above in the presence of ALA substrate. The new peaks appeared in transformants are marked with asterisks in the profile of fatty acids compared with standard fatty acids.

brane protein structure was drawn with TMRPres2D (Spyropoulos *et al*., 2004) (Fig. 2B). All of the predicted open reading frames shown in Table 2 encode membrane proteins with three transmembrane helices. Most of these proteins have been predicted to have an $N_{out}$-$C_{in}$ orientation (that is, an extracytoplasmic N-terminus and a cytoplasmic C-terminus), except HsD5DES (Table 2).

### Functional analysis of *M. cinereus* delta 6-fatty acid desaturase

The expression of *McD6DES* was induced by the addition of galactose as the carbon source to the yeast growth medium, which was also supplemented with LA or ALA. A subsequent fatty acid analysis indicated that the exogenous LA or ALA had been incorporated into the lipids of the yeast transformants carrying pYES2-McD6DES. Gas chromatographic analysis revealed novel peaks in samples from yeast transformed with pYES2-McD6DES, which corresponded to GLA (C18:$3^{\Delta6,9,12}$) and STA (C18:$4^{\Delta6,9,12,15}$) (Figs. 4A and 4B). These peaks were absent from samples from the control yeast carrying the empty vector. These results indicate that *McD6DES* encodes a delta 6-fatty acid desaturase that can successfully convert incorporated LA or ALA to GLA or STA, respectively. As shown in Fig. 4A, the fatty acid profile of the McD6DES-expressing yeast cells grown in medium supplemented with LA (C18:$2^{\Delta9,12}$) had a distinct extra fatty acid peak compared with the profile of the pYES2-expressing control cells. This peak showed a retention time identical to that of the GLA (C18:$3^{\Delta6,9,12}$) standard in the upper panel (Fig. 4A). This newly synthesized GLA in the transformed yeast cells represented 3.4% of the total fatty acid content (Table 3), indicating that the exogenous delta 6-desaturase efficiently converted LA to GLA

**Table 3. Fatty acid compositions (% w/w) of the total lipid contents of yeast transformants harboring the control plasmid, pYES2 and the recombinant plasmid, pYES2–McD6DES**

| Fatty acid | pYES2 | | pYES2–*McD6DES* | |
|---|---|---|---|---|
| | +LA | +ALA | +LA | +ALA |
| 16:0 | 9.6 ± 0.3 | 13.2 ± 0.2 | 15.0 ± 0.3 | 16.0 ± 0.2 |
| 16:1 | 27.0 ± 0.2 | 13.8 ± 0.2 | 30.1 ± 0.2 | 24.8 ± 0.3 |
| 18:0 | 6.4 ± 0.2 | 2.2 ± 0.1 | 6.2 ± 0.1 | 8.0 ± 0.1 |
| 18:1n-9 | 21.2 ± 0.3 | 13.5 ± 0.3 | 19.6 ± 0.2 | 17.3 ± 0.2 |
| 18:1n-7 | 3.5 ± 0.3 | 0.3 ± 0.2 | 5.3 ± 0.2 | 0.6 ± 0.2 |
| 18:2n-6 (LA) | 32.4 ± 0.2 | ND | 20.5 ± 0.1 | ND |
| 18:3n-3 (ALA) | ND | 57.1 ± 0.2 | ND | 18.1 ± 0.2 |
| 18:3n-6 (GLA) | ND | ND | 3.4 ± 0.1 | ND |
| 18:4n-3 (STA) | ND | ND | ND | 15.3 ± 0.2 |
| Delta 6-desaturation[a] | | | 14.2 | 45.9 |

Each value is the Mean ± SD from three independent experiments.
ND, not detected.
+LA and +ALA, exogenously supplied fatty acids.
[a] The percentage of delta 6-desaturation was calculated using the following formula: [product area/ (product area + substrate area) × 100].

(14.2%, Table 3). McD6DES also showed stronger catalytic activity for ALA (C18:3$^{\Delta9,12,15}$), converting ALA to STA (C18:4$^{\Delta6,9,12,15}$) (Fig. 4B). STA constituted about 15.3% of the total fatty acid content in the transformants and the substrate conversion from ALA to STA was as high as 45.9% (Table 3).

These results confirm that McD6DES is a delta 6-fatty acid desaturase that can introduce a double bond at position 6 in C18:2$^{\Delta9,12}$ and C18:3$^{\Delta9,12,15}$ substrates, thereby producing the delta 6-desaturated fatty acids GLA (C18:3$^{\Delta6,9,12}$) and STA (C18:4$^{\Delta6,9,12,15}$), respectively. Our data also indicate that McD6DES can reconstitute both the n-6 and n-3 pathways in a heterologous system.

## Discussion

Delta 6-desaturase of fatty acids is the key enzyme for the biosynthesis of STA and GLA in the biosynthetic pathway of VLC-PUFAs. STA and GLA are unusual fatty acids found only in a few species of the plant kingdom, predominantly members of the family Boraginaceae (Sayanova *et al.*, 1997). In this study, we isolated and characterized a delta 6-desaturase gene (*McD6DES*) from the pike eel (*M. cinereus*) to find a sustainable system for the production of health-beneficial PUFAs in oil crops.

McD6DES shows strong conservation of the three histidine-rich domains and the cytochrome-$b_5$-like domain found in the delta 6-desaturases of other fish species (Fig. 2A). Its three transmembrane helices are shown in Fig. 2B. These fish delta 6-desaturases show a remarkable level of amino acid sequence similarity, but have distinct enzymatic activities. McD6DES showed significant activity in a heterologous yeast expression system, which was much stronger than the delta 6-desaturase activities of the carp, rainbow trout, and sea bream (but not the turbot) (Table 4). It is noteworthy that the conversion of ALA to STA by McD6DES was extremely efficient. The n-3 fatty acid biosynthetic activity of this enzyme (45.9% conversion of ALA to STA) was much stronger than its n-6 fatty acid biosynthetic activity (14.2% conversion of LA to GLA) (Table 3).

STA is a metabolic intermediate in the biosynthetic pathway of EPA and DHA, and is generated by the delta 6-desaturation of ALA (Fig. 1). Interestingly, McD6DES has the capacity to synthesize n-3 delta 6-desaturated fatty acids. Although McD6DES acts predominantly in the production of n-3 STA, it can also synthesize n-6 GLA. The cosynthesis of n-3 and n-6 fatty acids by McD6DES could be used to produce PUFAs in metabolically engineered plant systems in the future.

**Table 4. Conversion efficiency (%) of McD6DES against other fishes D6DES in transgenic *S. cerevisiae***

| Fish Species | Activity and conversion rate | | Ref. |
|---|---|---|---|
| | Delta 6-desaturation | | |
| | LA → GLA | ALA → STA | |
| Pike eel | 14.2 | 45.9 | This Work |
| Black sea bream | 26.3 | 35.6 | Kim *et al.* (2011) |
| Common carp | 1.5 | 7.0 | Zheng *et al.* (2004) |
| Rainbow trout | 3.6 | 31.5 | Zheng *et al.* (2004) |
| Gilthead sea bream | 12.2 | 23.1 | Zheng *et al.* (2004) |
| Turbot | 31.2 | 59.5 | Zheng *et al.* (2004) |

## Acknowledgements

We thank Dr. Byungwook Lee (Korean Bioinformation Center, Korea) for help with the computational analysis. This work was supported by grants from the National Academy of Agricultural Science (PJ006745), the Rural Development Administration, Republic of Korea.

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