Characterization of a trifunctional fatty acid desaturase from oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast expression system

Hiroshi Kikukawa[1], Eiji Sakuradani[1*], Shigenobu Kishino[1], Si-Bum Park[2], Akinori Ando[3,4], Jun Shima[4], Misa Ochiai[5], Sakayu Shimizu[6] , and Jun Ogawa[1,4]

[1]Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan;

[2]Laboratory of Industrial Microbiology, Graduate School of Agriculture, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan;

[3]Research Unit for Physiological Chemistry, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan;

[4]Research Division of Microbial Sciences, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan;

[5]Institute for Microbial Science, Suntory Business Expert Ltd., 1-1-1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan;

[6]Faculty of Bioenvironmental Science, Kyoto Gakuen University, 1-1 Otani, Nanjo, Sogabe-cho, Kameoka, Kyoto 621-8555, Japan

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***Correspondent footnote:** Phone: +81-75-753-6114
FAX: +81-75-753-6128
e-mail: esakura@kais.kyoto-u.ac.jp (E. Sakuradani)

A ω3-fatty acid desaturase gene (*maw3*) which is involved in biosynthesis of *n*-3 polyunsaturated fatty acids (PUFAs) was previously isolated from *Mortierella alpina* 1S-4. In this report, we investigated the products of MAW3 catalyzing reaction with endogenous and exogenous fatty acids in the yeast transformant. Two unusual fatty acids *de novo* synthesized in the yeast transformant expressing *maw3* gene were identified as *n*-4 hexadecadienoic acid (16:2^{9cis,12cis}) and *n*-1 hexadecatrienoic acid (16:3^{9cis,12cis,15}) by GC-MS and ^{1}H-NMR analyses. In addition to the desaturation activity at the ω3-position for 18- and 20-carbon PUFAs, MAW3 in the yeast transformant inserted a double bond at Δ12-position of endogenous palmitoleic acid (16:1^{9cis}) and further at Δ15-position of the resulting 16:2^{9cis,12cis} to result in the formation of 16:3^{9cis,12cis,15} leading to a bifunctional Δ12/Δ15-desaturase for 16-carbon fatty acids. Moreover, we evaluated the activity of MAW3 in the yeast transformant under different temperatures. The MAW3 did not have desaturation activities in *M. alpina* 1S-4 at 28°C but it had in the yeast transformant for various fatty acids. The MAW3 was demonstrated to be a trifunctional Δ12/Δ15/ω3-desaturase, exhibiting Δ12-desaturation for 16:1^{9cis}, Δ15-desaturation for 16- and 18-carbon fatty acids that had a preexisting *cis*-double bond at Δ12 position, and ω3-desaturation for 20-carbon fatty acids

having that at Δ14-position. It is the first report that the fatty acid desaturase (MAW3) is shown to have Δ12- and Δ15-desaturation activities for a 16-carbon fatty acid, in addition to its major function, ω3-desaturation activity.

Biosynthetic pathways of fatty acids have been determined in many species by identification of their genes encoding fatty acid desaturases (1-4). An oleaginous filamentous fungus, *Mortierella alpina* 1S-4, has been shown to contain Δ5-, Δ6-, Δ9-, Δ12-, and ω3-fatty acid desaturases and four kinds of elongases involved in polyunsaturated fatty acids (PUFAs) biosynthesis (5). In *M. alpina* 1S-4, Δ5-, Δ6-, Δ9-, and Δ12-desaturases exhibit their desaturation activity at a cultural temperature of 28°C, whereas ω3-fatty acid desaturase exhibits its activity only below a cultural temperature of 20°C.

M. alpina 1S-4 is capable of producing triacylglycerols rich in arachidonic acid (ARA; $20{:}4^{5cis,8cis,11cis,14cis}$) with the amount ranging of 3-20 g/L of culture broth and 30-70% in the total fatty acid composition. On the other hand, this fungus accumulates a small amount of eicosapentaenoic acid (EPA; $20{:}5^{5cis,8cis,11cis,14cis,17cis}$) with 0.3 g/L of culture broth and 10% in total fatty acids below a cultural temperature of 20°C (5). We had previously reported isolation of the gene (*maw3*) encoding an ω3-desaturase, involved in biosynthesis of *n*-3 PUFAs, from *M. alpina* 1S-4, and have confirmed that the gene product (MAW3) converts *n*-6 PUFAs into *n*-3 PUFAs (6). MAW3 possesses three histidine rich-cluster motifs containing eight histidine residues that are proposed to form an active-site domain and two hydrophobic

membrane-spanning regions in its deduced amino acid sequence (6,7). Similar to Δ12- and ω3-desaturases found in other organisms, MAW3 has no cytochrome b_5 motif in its structure (6,8,9). Overexpression of *maw3* gene in *M. alpina* 1S-4 through *Agrobacterium tumefaciens*-mediated transformation led to an increase in EPA production contributing to 40% of total fatty acid content (10).

Fatty acid desaturases are known to have five specificities: chemoselectivity (desaturation or hydroxylation), regioselectivity (double bond position), stereoselectivity (*cis* or *trans*), chain length specificity, and acyl carrier specificity (acyl-CoA, acyl-lipid, or acyl-ACP substrate) (11). Three types of regioselectivities have been defined (11). The Δx-desaturases such as Δ9-desaturases insert a first double bond at x carbon atom position away from the carboxyl end. On the other hand, the ωy-desaturases such as ω3-desaturases insert a double bond at position y from the methyl end. The v + z-desaturases require a preexisting double bond as a mark (v) and catalyze the formation of a new double bond z carbon atoms further along the acyl chain, either toward the methyl end (*i.e.* Δ12-desaturase) or toward the carboxyl end (*i.e.* Δ6-desaturase). The aforementioned types of regioselectivity are explained for bifunctional fungal Δ12/ω3-desaturases from *Fusarium moniliforme* (12) and *Aspergillus nidulans* (13). These bifunctional enzymes desaturate oleic acid (OA;

18:1^{9cis}) at Δ12-position to form linoleic acid (LA; 18:2^{9cis,12cis}) and further at the Δ15-position to form α-linolenic acid (ALA; 18:3^{9cis,12cis,15cis}). Additionally, these desaturases also convert *n*-6 PUFAs: LA, γ-linolenic acid (GLA; 18:3^{6cis,9cis,12cis}), dihomo-γ-linolenic acid (DGLA; 20:3^{8cis,11cis,14cis}), and ARA to their corresponding *n*-3 PUFAs with preference of the 18-carbon PUFAs. Other bifunctional Δ12/Δ15-desaturases that catalyze the synthesis of unusual *n*-1 hexadecatrienoic acid (16:3^{9cis,12cis,15}) have been reported in the free-living soil protozoon *Acanthamoeba castellanii* (14), phytopathogenic fungus *Claviceps purpurea* (15), basidiomycete *Coprinus cinereus* (16), and nematode *Caenorhabditis elegans* (17).

In this study, we attempted to characterize the MAW3 using the yeast *Saccharomyces cerevisiae* expression system. Two unusual fatty acids synthesized *de novo* in *maw3*-expressing yeast transformant were identified by GC-MS and NMR. The MAW3 in yeast transformant inserted a double bond at Δ12-position and further at Δ15-position for endogenous 16-carbon fatty acids as well as at ω3-position of exogenous 18- and 20-carbon PUFAs. The *M. alpina* 1S-4 ω3-desaturase was found to be a trifunctional Δ12/Δ15/ω3-desaturase that mainly acts as Δ15- and ω3-desaturase, and as a Δ12-desaturase for 18- and 20-carbon PUFAs in addition to 16-carbon fatty acids. The finding is different from other bifunctional desaturases so far reported

(12-17).

MATERIALS AND METHODS

Strains, plasmid, growth media, and culture conditions The *maw3* gene from *M. alpina* 1S-4 (18) was registered in the DDBJ database under the accession number AB182163 (6). *S. cerevisiae* EH1315 (*α trp1*) was used as the recipient strain in transformation experiments (19). Plasmid pYE22m, used as a shuttle vector, carried the ampicillin resistance gene (for selection in *Escherichia coli*) and *TRP1* (for tryptophan prototrophy selection in *S. cerevisiae* EH1315), with an expression of a target gene controlled under the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and the GAP terminator from *S. cerevisiae*.

M. alpina 1S-4 strain was cultured in GY medium (containing 20 g/L of glucose, 10 g/L of yeast extract, pH 6.0) for 5 days at 28°C. Yeast strains were cultured in a yeast synthetic complete medium (SC medium; containing 20 g/L of glucose, 5 g/L of ammonium sulfate, 1.7 g/L of yeast nitrogen base without amino acids and ammonium sulfate, 60 mg/L of isoleucine, 60 mg/L of leucine, 60 mg/L of phenylalanine, 50 mg/L of threonine, 40 mg/L of lysine, 30 mg/L of tyrosine, 20 mg/L of adenine, 20 mg/L of arginine, 20 mg/L of uracil, 20 mg/L of histidine, and 10 mg/L of methionine) for 2 days at 15°C (or at 28°C).

maw3 expression in yeast The full-length *maw3* cDNA was amplified by PCR in

a total reaction volume of 50 μl sample containing < 0.2 μg of plasmid pYMAW3 (6) as template DNA, 0.5 μl of *PrimeSTAR HS* DNA polymerase (Takara Bio, Shiga, Japan), 10 μl of 5 × *PrimeSTAR* buffer (Mg^{2+} plus), 200 μM of each dNTP and 200 pM of each primer: ω3-1F (5'-ATGGCCCCCCCTCACGTTGTCGACGAACA-3') containing an ATG start site (underline) and ω3-3R (5'-TTAATGCTTGTAAAACACTACATCTCC-3') containing a TAA stop site (underline). PCR amplification was performed as follows: initial denaturation at 94°C for 2 min, followed by 25 cycles of 98°C for 10 sec, 55°C for 15 sec, and 72°C for 2 min. The resultant 1212-bp PCR fragment was digested with *Eco*RI and treated with DNA Blunting Kit (Takara Bio) for blunt-end ligation into the yeast expression vector pYE22m to result in the construction of a plasmid, designated as pYE22MAW3, which was further transformed into the *S. cerevisiae* EH1315 strain by means of the lithium acetate-mediated transformation protocol (20). Transformants was selected for tryptophan (Trp) auxotrophy on SC (-Trp) medium.

The transformants were grown at 28°C or 15°C with shaking for 48 h in SC medium. To test for the substrate specificities of MAW3 in yeast, various free fatty acids (FFAs) were added to the culture broth at 0.1 mM concentration after 24 h of cultivation. The yeast transformant with an empty vector was used as the control

strain.

Fatty acid analysis Total fatty acid compositions of the yeast transformants were analyzed as described by Sakuradani et al. (6). Yeast cells after cultivation were harvested by centrifugation and dried at 120°C for 1.5 h. The dried cells were directly transmethylated with 10% methanolic HCl at 55°C for 1.5 h. The resultant fatty acid methyl esters (FAMEs) were extracted with *n*-hexane, concentrated and analyzed by gas chromatography (GC; Shimadzu, Kyoto, Japan). The conversion rate of substrate into the product was calculated as the enzymatic activity of the desaturase [conversion rate (%) = 100 × product/(product + substrate)].

Total lipids were extracted from wet yeast cells using water/chloroform/methanol (2/2.5/2.5, v/v/v) according to the Bligh-Dyer method (21). The extracted lipids were saponified to obtain their FFAs using 0.5 N KOH in 90% MeOH. The unidentified FFAs were separated by HPLC with a LC-20A system (Shimadzu). A Develosil C30-UG-5 column (4.6 × 250 mm, Nomura chemical, Aichi, Japan) was used for FFA separation by HPLC with 80% (v/v) acetonitrile aqueous solution as a mobile phase at a flow rate of 1.0 ml/min. The column oven temperature was maintained at 35°C, and the absorbance was measured by UV/VIS detector at 205 nm. The mixture of FFAs separated by HPLC were transmethylated with methanol/benzene/1% diazomethane

(4/6/1, v/v/v) for 30 min at room temperature for preparation of FAMEs. Through the Discovery Ag-Ion SPE column (SUPELCO, Bellefonte, PA, USA), most of FAMEs in the UK2 fraction except for the UK2 were removed with acetone and the UK2 was eluted with acetonitrile. The 4,4-Dimethyloxazoline (DMOX) derivatives of the UK1 and UK2 obtained through the HPLC purification was prepared and analyzed by GC-MS as described by Yu et al. (22). A GC-MS QP2010 (Shimadzu) with GC-2010 gas chromatograph was used for MS analysis. The SPB^{TM-1} column (0.25 mm I.D. × 30 m, SUPELCO) was used for separation of DMOX derivatives. Initial column temperature was set at 180°C and was raised to 300°C later at a rate of 5°C/min. The apparatus provides the electron impact mode at 70 eV with a source temperature of 250°C. The injection temperature was 250°C. The ^{1}H-NMR and DQF-COSY experiments were performed with a Bruker Advance 500 (500 MHz). Samples were dissolved in chloroform-*d* (Sigma, St. Louis, MO, USA). Chemical shifts were assigned relative to the solvent signal.

RESULTS

Functional analysis of the maw3 gene product in the yeast transformant To

characterize the *maw3* gene product from *M. alpina* 1S-4 by using a yeast expression system, the yeast transformant with pYE22MAW3 containing the *maw3* cDNA was obtained. The yeast transformants were grown in SC medium, and their total fatty acid compositions were analyzed by GC (Fig. 1A and 1B). The *maw3*-expressing yeast transformant accumulated two unusual fatty acids, named UK1 and UK2 respectively, which were not detected in the control strain. The UK1 was expected to be $16{:}2^{9cis,12cis}$, because the retention time of the UK1 was consistent with that of an authentic standard (Fig. 1C).

Each FFA of UK1 and UK2 was purified by HPLC equipped with a Develosil C30-UG-5 column, followed by the further purification through Discovery Ag-Ion SPE column for UK2. The positions of the double bonds in UK1 and UK2 were determined by GC-MS analysis of their DMOX derivatives (Fig. 2). MS analysis of UK1 and UK2 DMOX derivatives in Fig. 2 revealed molecular ions of *m/z* 305 and 303, and the gaps of 26 atomic mass unit (amu) between *m/z* 196 and 222, and *m/z* 236 and 262, indicating double bond at the Δ9- and Δ12-positions in their structures, respectively. The gap of 41 amu between *m/z* 262 and 303 in the mass spectrum of UK2 (Fig. 2B) suggested a double bond at the Δ14- or Δ15-position. To determine a position of a double bond at methyl terminal of UK2 completely, the FAME of UK2 showing 95%

purity on GC chromatogram was applied for ^{1}H-NMR analysis (Fig. 3) and DQF-COSY analysis (FIGURE S1). ^{1}H-NMR δ_H ($CDCl_3$) spectral data of UK2: 5.82 (1H, *tt*, -CH_2-C*H*=CH_2, H-15), 5.38 (4H, *m*, -CH_2-C*H*=CH-, H-9, H-10, H-12, H-13), 5.02 (2H, *d*, *J*= 10.1, 17.0 Hz, -CH_2-CH=C*H*$_2$, H-16), 3.67 (3H, *s*, -C(=O)-O-C*H*$_3$), 2.83 (2H, *dd*, *J*= 6.07, 6.07 Hz, =CH-C*H*$_2$-CH=, H-14), 2.79 (2H, *dd*, *J*= 6.07, 6.07 Hz, =CH-C*H*$_2$-CH=, H-11), 2.30 (2H, *t*, *J*= 7.28 Hz, -C(=O)-C*H*$_2$-CH_2-, H-2), 2.05 (2H, *dt*, *J*= 6.78, 6.78 Hz, -CH_2-C*H*$_2$-CH=, H-8), 1.679 (2H, *tt*, *J*= 7.33, 7.41 Hz, -CH_2-C*H*$_2$-CH_2-, H-3), and 1.31 (8H, *m*, -CH_2-C*H*$_2$-CH_2-, H-4, H-5, H-6, H-7). The absorption peaks at δ 5.82 (-CH_2-C*H*=CH_2, H-15) and 5.02 (-CH_2-CH=C*H*$_2$, H-16) in Fig. 3 revealed a double bond at the Δ15-position in the structure of UK2. From these analyses, UK1 and UK2 were identified as 16:2^{9cis,12cis} and 16:3^{9cis,12cis,15}, respectively.

As shown in Table 1, the fatty acid composition of the *maw3*-expressing yeast transformant was different from that of the control strain. The 16:2^{9cis,12cis} and 16:3^{9cis,12cis,15} comprised 0.8% and 4.0% of the total fatty acid content, respectively, which were not detected in the control strain. The percentage of 16:1^{9cis} was decreased slightly in the yeast transformant, compared to that in the control strain. Neither LA nor ALA was detected in both yeast strains, suggesting that MAW3 in the transformant prefers a 16-carbon fatty acid like 16:1^{9cis} to an 18-carbon fatty acid like OA.

Catalytic properties of MAW3 gene product analyzed in the transformant To investigate the substrate preference of MAW3, the yeast transformants were grown in SC medium containing 0.1 mM of exogenous FFA substrates such as 16:2^{9cis,12cis}, 18:1^{9cis} (OA), crepenynic acid (9*cis*-octadecen-12-ynoic acid; 18:1^{9cis,12yn}), octadecadienoic acid isomers [18:2^{6cis,9cis}, 18:2^{9cis,12cis} (LA), 18:2^{9cis,11trans}, 18:2^{9trans,12cis}, 18:2^{9trans,12trans}, and 18:2^{10trans,12cis}], 18:3^{6cis,9cis,12cis} (GLA), 20:2^{9cis,12cis}, 20:3^{8cis,11cis,14cis} (DGLA), and 20:4^{5cis,8cis,11cis,14cis} (ARA). Conversions of the substrates by the *maw3*-expressing yeast transformant were carried out during cultivation at 15°C or 28°C (Table 2). *M. alpina* 1S-4 has no ω3-desaturation activity at 28°C cultivation, whereas the yeast transformant exhibited the activity for the substrates even at 28°C. The MAW3 in the yeast transformant desaturated the following PUFAs: 16:2^{9cis,12cis}, 18:2^{9cis,12cis} (LA), 18:3^{6cis,9cis,12cis} (GLA), 20:3^{8cis,11cis,14cis} (DGLA), and 20:4^{5cis,8cis,11cis,14cis} (ARA) to 16:3^{9cis,12cis,15}, 18:3^{9cis,12cis,15cis} (ALA), stearidonic aicd (SDA; 18:4^{6cis,9cis,12cis,15cis}), eicosatetraenoic acid (ETA; 20:4^{8cis,11cis,14cis,17cis}), and 20:5^{5cis,8cis,11cis,14cis,17cis} (EPA). Putative Δ15-desaturation products of 18:2^{9cis,12yn,15cis} (9*cis*,15*cis*-octadecadien-12-ynoic acid), 18:3^{9trans,12cis,15cis}, and 18:3^{10trans,12cis,15cis} from 18:1^{9cis,12yn}, 18:2^{9trans,12cis}, 18:2^{10trans,12cis}, respectively, were also observed on GC chromatograms. The *n*-6 PUFAs (LA, GLA, DGLA, and ARA) were more effectively

converted into *n*-3 PUFAs (ALA, SDA, ETA, and EPA) at a low temperature (15°C) cultivation of *M. alpina* 1S-4 than at 28°C temperature. In addition, conversion rates for 16:2^{9cis,12cis} and crepenynic acid were observed at similar or higher levels at 28°C as compared to the ones at 15°C. Neither Δ15-desaturation nor ω3-desaturation products were detected for 20:2^{9cis,12cis} in the yeast transformant.

The Δ12-desaturation catalyzed by MAW3 was observed only for 16:1^{9cis}, and not for 18:1^{9cis} and 18:2^{6cis,9cis}. The high conversion rate with more than 60% was shown for 16:2^{9cis,12cis} to 16:3^{9cis,12cis,15} by Δ15-desaturation of MAW3 in the yeast transformant. Furthermore, the ω3-desaturation of MAW3 was observed for 18- and 20-carbon PUFAs having a preexisting 12*cis*-double bond or 12-triple bond and a preexisting 14*cis*-double bond, respectively.

DISCUSSION

The fungus *M. alpina* 1S-4 not only produces a great number of *n*-6 PUFAs rich in ARA, but also accumulates *n*-3 PUFAs such as EPA below a cultural temperature of 20°C (23). The *n*-3 PUFAs in *M. alpina* 1S-4 *in vivo* are expected to be synthesized from *n*-6 PUFAs through ω3-desaturation. The *maw3* gene was previously isolated

and characterized as to its function of *n*-6 PUFAs conversion to *n*-3 PUFAs in *M. alpina* 1S-4 (6).

In this study, the *maw3*-expressing yeast transformant was found to accumulate two newly synthesized fatty acids, $16:2^{9cis,12cis}$ and $16:3^{9cis,12cis,15}$. Thus far, we had no idea that the MAW3 had Δ12-deasturase activity as an alternative function. The accumulation of $16:2^{9cis,12cis}$ and $16:3^{9cis,12cis,15}$ suggested that the yeast transformant had both Δ12-desaturation activity with a conversion rate of 10.2% (calculated as 100 × [16:2 + 16:3]/[16:1 + 16:2 + 16:3]) for endogenous $16:1^{9cis}$, and Δ15-desaturation one at ω1-position, not ω3, with a conversion rate of 83.0% (calculated as 100 × [16:3]/[16:2 + 16:3]) for the resultant $16:2^{9cis,12cis}$. Once the $16:2^{9cis,12cis}$ was formed from $16:1^{9cis}$ by Δ12-deasturation, $16:2^{9cis,12cis}$ was demonstrated to be rapidly converted to $16:3^{9cis,12cis,15}$.

The analysis of substrate specificities (Table 2) showed that the MAW3 not only had ω3-desaturation activity for 18- and 20-carbon PUFAs, but also demonstrated bifunctional Δ12/Δ15-desaturation activities for 16-carbon fatty acids. The Δ12-, Δ15-, or ω3-desaturation activity of MAW3 requires a preexisting double bond at Δ9-, Δ12-, or ω6-position, respectively. Hence, MAW3 is classified as v + 3 desaturase (v indicating the position of the preexisting double bond) which inserted a new double

bond 3 carbon atoms further along the acyl chain toward the methyl end.

Compared to other bifunctional Δ12/Δ15-desaturases [amoeba *A. castellanii* (14); basidiomycete *C. cinereus* (16); nematode *C. elegans* (17)], MAW3 in the yeast transformant exhibited Δ12-desaturation for $16{:}1^{9cis}$, not $18{:}1^{9cis}$, and in addition to that, also exhibited Δ15-desaturation for $16{:}2^{9cis,12cis}$ with a high conversion rate (83.0%). The previously reported bifunctional Δ12/Δ15-desaturases mainly act as a Δ12-desaturase, except for the fungal ω3-desaturases described by Damude et al. (12) and Hoffmann et al. (13), however do not possess Δ12-desaturation activity for the 16-carbon fatty acid. The ratios of ω3/ω6 fatty acids (calculated as [16:3 + 18:3]/[16:2 + 18:2]) in yeast transformant were calculated as follows: 5.0 [MAW3, described here], 21 [ω3-desaturase from *F. moniliforme* (12)], 0.0080 [Δ12-desaturase from *F. moniliforme* (12)], 2.1 [ω3-desaturase from *A. nidulans* (13)], 0.48 [Δ12-desaturase from *A. castellanii* (14)], 0.042 [Δ12-desaturase from *C. cinereus* (16)], and 0.090 [Δ12-desaturase from *C. elegans* (17)] respectively based on the information derived from each report. The bifunctional Δ12/Δ15-desaturases from *F. moniliforme* and that has a high ratio of 21, mainly work as a ω3-desaturase, which prefers the 18-carbon PUFAs as substrates and not the 16-carbon fatty acids. On the other hand, the bifunctional Δ12/Δ15-desaturase from *C. purpurea* (15) introduces a double bond for

both 16- and 18-carbon fatty acids, demonstrating increased activity of Δ12-desaturation as compared to Δ15-desaturation.

On investigation of substrate specificities, the characteristic feature of MAW3 was shown to have both the Δ12/Δ15-desaturation activity for the 16-carbon fatty acids and ω3-desaturation activity for 18- and 20-carbon PUFAs, indicating its substrate specificities that differed from the other bifunctional Δ12/Δ15-desaturases (12-17). It is of interest to note that, the *maw3*-expressing yeast transformant exhibited the ω3-desaturation activity on cultivation at 28°C, whereas *M. alpina* 1S-4 had no such ω3-deaturation activity under similar culture conditions. This may imply the possibility of some regulation in the translation process and/or localization of MAW3 in *M. alpina* 1S-4 *in vivo*. The localization of MAW3 might be detected in cells of *M. alpina* 1S-4 by means of labeling techniques. We expect that the elucidation of the mechanism for the difference in MAW3 activity (depending on the culture temperature) may contribute to the accumulation of basic information on membrane-bound desaturases and to efficient production of n-3 PUFAs by *M. alpina* breeding.

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References

1. **Napier, J. A., Michaelson, L. V.:** Genomic and functional characterization of polyunsaturated fatty acid biosynthesis in *Caenorhabditis elegans*, Lipids, **36:**761-766 (2001).
2. **Uttaro, A. D.:** Biosynthesis of polyunsaturated fatty acids in lower eukaryotes, IUBMB *Life*, 58:563-571 (2006).
3. **Sakuradani, E., Ando, A., Ogawa, J., Shimizu, S.:** Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding, Appl. Microbiol. Biotechnol., 84:1-10 (2009).
4. **Pereira, S. L., Leonard, A. E., Mukerji, P.:** Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes, Prostaglandins Leukot. Essent. Fatty Acids, **68:**97-106 (2003).
5. **Sakuradani, E.:** Advances in the Production of Various Polyunsaturated Fatty

Acids through Oleaginous Fungus *Mortierella alpina* Breeding, Biosci. Biotechnol. Biochem., **74:** 908-917 (2010).

6. **Sakuradani, E., Abe, T., Iguchi, K., and Shimizu, S.:** A novel fungal ω3-desaturase with wide substrate specificity from arachidonic acid-producing *Mortierella alpina* 1S-4, Appl. Microbiol. Biotechnol., **66:** 648-654 (2005).

7. **Los, D. A., Murata, N.:** Structure and expression of fatty acid desaturases, Biochim. Biophys. Acta., **1394:**3–15 (1998).

8. **Petrini G. A., Altabe S. G., Uttaro A. D.:** *Trypanosoma brucei* oleate desaturase may use a cytochrome *b5*-like domain in another desaturase as an electron donor, Eur. J. Biochem., **271:**1079-1086 (2004).

9. **Yazawa, H., Iwahashi, H., Kamisaka, Y., Kimura, K., Uemura, H.:** Improvement of polyunsaturated fatty acids synthesis by the coexpression of *CYB5* with desaturase genes in *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol., **87:**2185-2193 (2010).

10. **Ando, A., Sumida, Y., Negoro, H., Suroto, D. A., Ogawa, J., Sakuradani, E., and Shimizu, S.:** Establishment of *Agrobacterium tumefaciens*-mediated transformation of an oleaginous fungus, *Mortierella alpina* 1S-4, and its application for eicosapentaenoic acid producer breeding, Appl. Environ. Microbiol., **75:** 5529-5535

(2009).

11. **Meesapyodsuk, D., Reed, D. W., Savile, C. K., Buist, P. H., Ambrose, S. J., and Covello, P. S.:** Characterization of the Regiochemistry and Cryptoregiochemistry of a *Caenorhabditis elegans* Fatty Acid Desaturase (FAT-1) Expressed in *Saccharomyces cerevisiae*, Biochemistry, **39:** 11948–11954 (2000).

12. **Damude, H. G., Zhang, H., Farrall, L., Ripp, K. G., Tomb, J. F., Hollerbach, D., and Yadav, N. S.:** Identification of bifunctional Δ12/ω3 fatty acid desaturases for improving the ratio of ω3 to ω6 fatty acids in microbes and plants, Proc. Natl. Acad. Sci. U S A, **103:** 9446-9451 (2006).

13. **Hoffmann, M., Hornung, E., Busch, S., Kassner, N., Ternes, P., Braus, G. H., and Feussner, I.:** A Small Membrane-peripheral Region Close to the Active Center Determines Regioselectivity of Membrane-bound Fatty Acid Desaturases from *Aspergillus nidulans*, J. Biol. Chem., **282:** 26666–26674 (2007).

14. **Sayanova, O., Haslam, R., Guschina, I., Lloyd, D., Christie, W. W., Harwood, J. L., and Napier, J. A.:** A bifunctional Δ12,Δ15-desaturase from *Acanthamoeba castellanii* directs the synthesis of highly unusual *n*-1 series unsaturated fatty acids, J. Biol. Chem., **281:** 36533-36541 (2006).

15. **Meesapyodsuk, D., Reed, D. W., Covello, P. S., and Qiu, X.:** Primary Structure,

Regioselectivity, and Evolution of the Membrane-bound Fatty Acid Desaturases of *Claviceps purpurea*, J. Biol. Chem., **282:** 20191–20199 (2007).

16. **Zhang, S., Sakuradani, E., Ito, K., and Shimizu, S.:** Identification of a novel bifunctional Δ12/Δ15 fatty acid desaturase from a basidiomycete, *Coprinus cinereus* TD#822-2, FEBS Lett., **581:** 315-319 (2007).

17. **Zhou, X. R., Green, A. G., and Singh, S. P.:** *Caenorhabditis elegans* Δ12-desaturase FAT-2 is a bifunctional desaturase able to desaturate a diverse range of fatty acid substrates at the Δ12 and Δ15 positions, J. Biol. Chem., **286:** 43644-43650 (2011).

18. **Yamada, H., Shimizu, S., and Shinmen, Y.:** Production of arachidonic acid by *Mortierella elongata* 1S-5, Agric. Biol. Chem., **51:** 785-790 (1987).

19. **Ashikari, T., Kiuchi-Goto, N., Tanaka, Y., Shibano, Y., Adachi, T., and Yoshizumi, H.:** High expression and efficient secretion of *Rhizopus oryzae* glucoamylase in the yeast *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol., **30:** 515-520 (1989).

20. **Ito, H., Fukuda, Y., Murata, K., and Kimura, A.:** Transformation of intact cells treated with alkali cations, J. Bacteriol., **153:**163-168 (1983).

21. **Bligh, E. G., and Dyer, W. J.:** A rapid method of total lipid extraction and

purification, Can. J. biochem physiol., **37:** 911-917 (1959).

22. **Yu, Q. T., Liu, B. N., Zhang, J. Y., and Huang, Z. H.:** Locationof amrthyl branchings in fatty acids: Fatty acids in uropygial secretion of Shanghai duck by GC-MS of 4,4-dimethyloxazoline derivatives, Lipids, **8:** 33-44 (1988).

23. **Shimizu, S., Kawashima, H., Shinmen, Y., Akimoto, K., and Yamada, H.:** Production of eicosapentaenoic acid by *Mortierella* fungi, J. Am. Oil. Chem. Soc., **65:** 1455-1459 (1988).

Figure legends

FIG. 1. GC chromatograms of FAMEs prepared from total lipids of the control strain (pYE22m) (A), the *maw3*-expressing yeast transformant (pYE22MAW3) (B), and the FAMEs of $16{:}2^{9cis,12cis}$ (C).

FIG. 2. Mass spectra of DMOX derivatives of (A) UK1 and (B) UK2 obtained from the yeast transformant (pYE22MAW3). The specific mass fragments of authentic $16{:}2^{9cis,12cis}$ were *m/z* 113, 126, 140, 154, 168, 182, 196, 208, 222, 236, 248, 262, 276, 276, 290, and 305.

FIG. 3. ^{1}H-NMR analysis of the UK2 methyl ester.

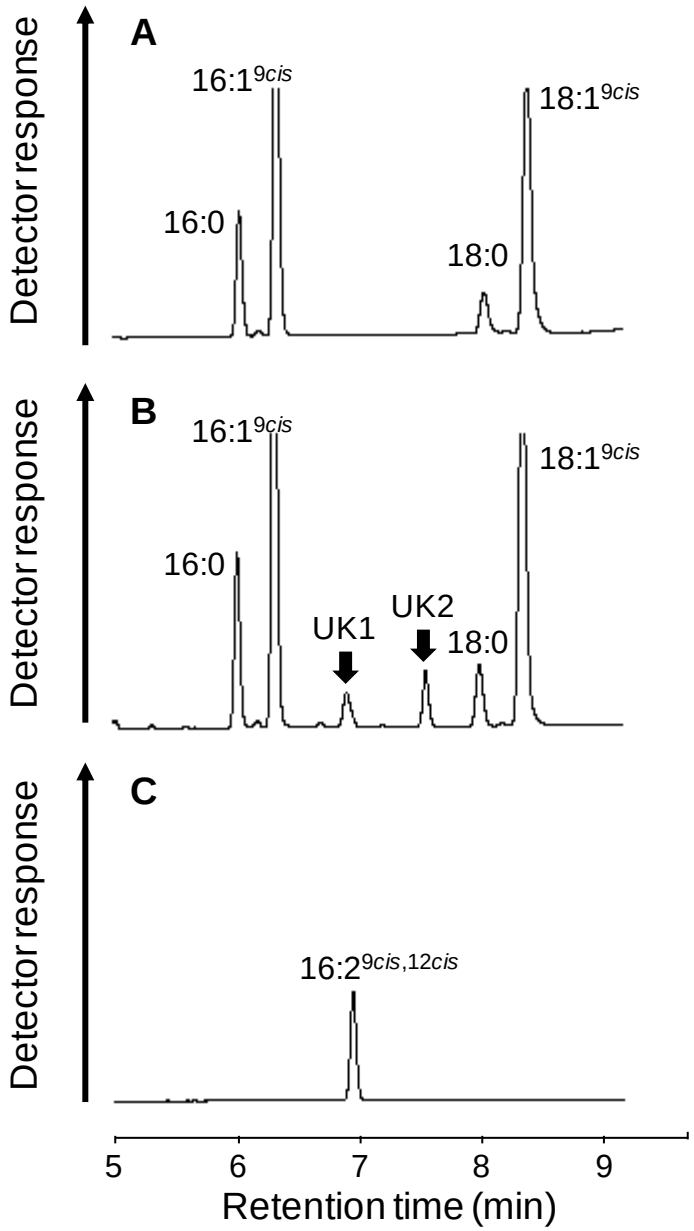

FIG.1. Kikukawa et al.

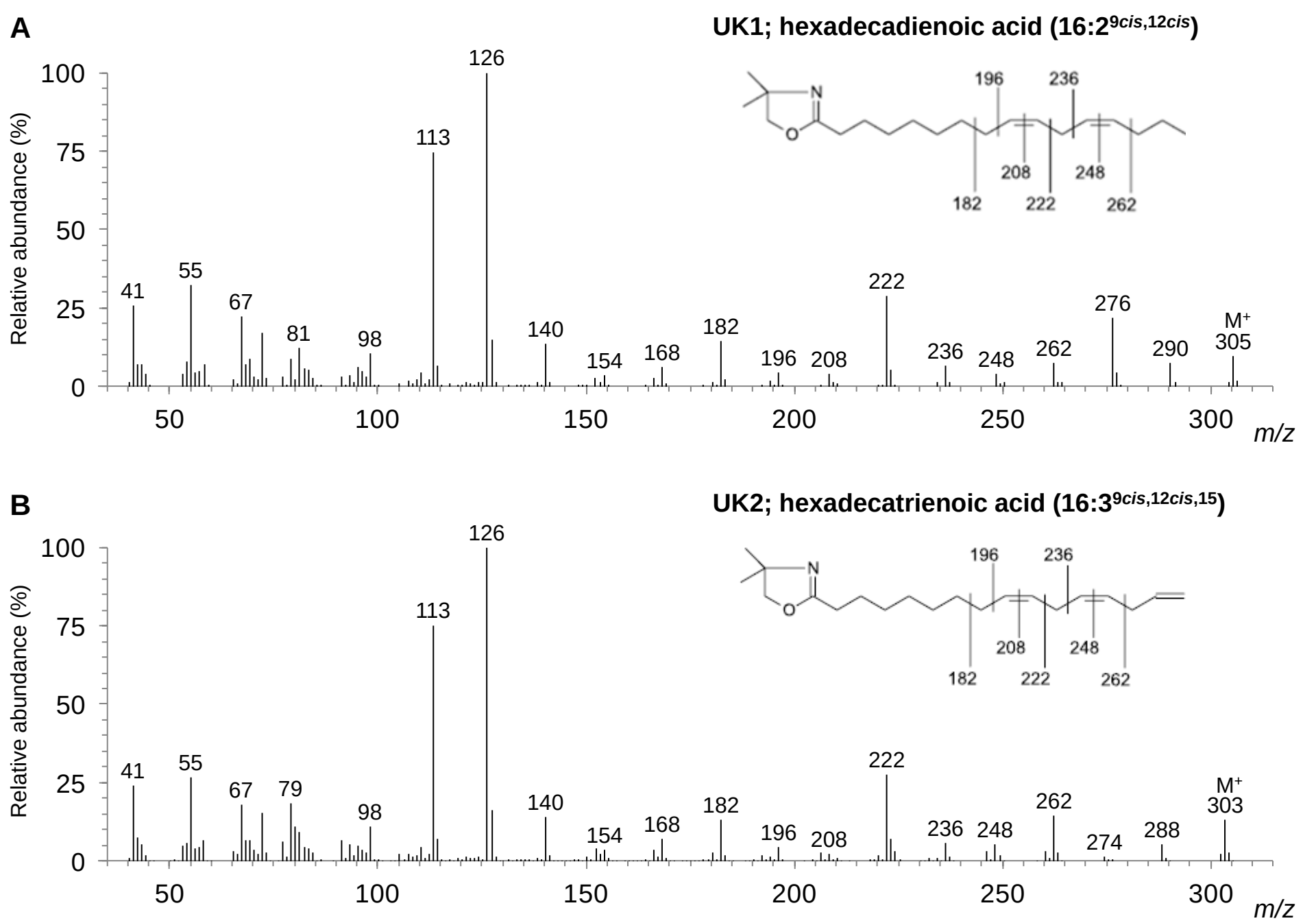

FIG.2. Kikukawa et al.

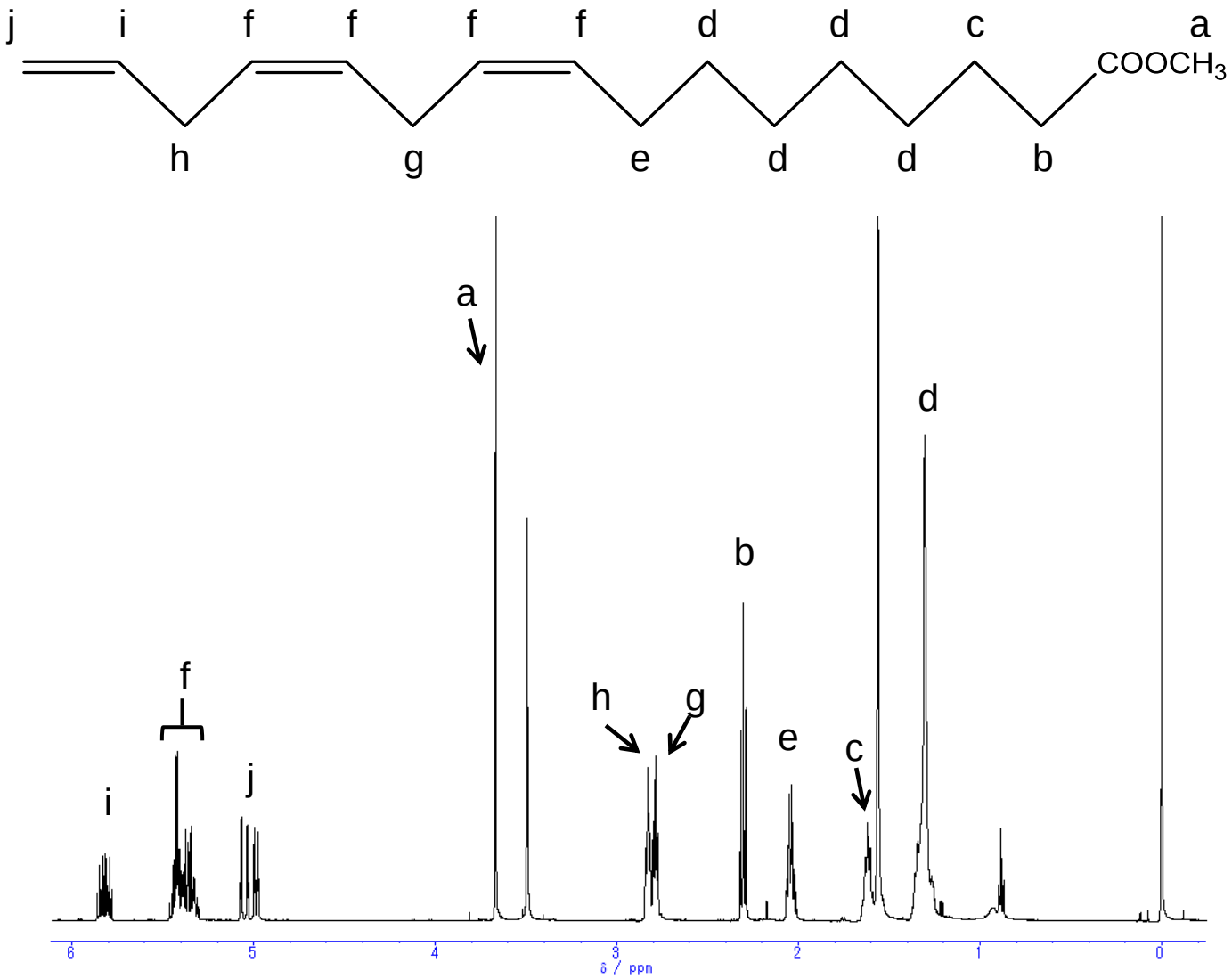

FIG. 3. Kikukawa et al.

Table 1. Fatty acid compositions of the yeast transformants

Fatty acid	Fatty acid composition (%)	
	Control strain (pYE22m)	*maw3* transformant (pYE22MAW3)
16:0	12.4	11.9
16:1^{9cis}	45.4	40.2
16:2^{9cis,12cis}	N.D.[a]	0.8
16:3^{9cis,12cis,15}	N.D.	4.0
18:0	5.7	5.2
18:1^{9cis}	34.6	32.8

[a] N.D., not detected

Table 2. Conversion of various fatty acids by the *maw3*-expressing yeast transformant[a]

Substrate	Product	Conversion rate (%)[b]	
		15°C	28°C
Endogenous fatty acid			
16:1^{9cis}	16:2^{9cis,12cis}	10.2 ± 0.2	N.D. [c]
16:2^{9cis,12cis}	16:3^{9cis,12cis,15}	83.0 ± 0.5	N.D.
Exogenous fatty acid			
16:2^{9cis,12cis}	16:3^{9cis,12cis,15}	68.8 ± 0.6	60.3 ± 0.6
18:1^{9cis} (OA)	N.D.	-	-
18:1^{9cis,12yn}	18:2^{9cis,12yn,15cis} d	11.0 ± 0.4	19.5 ± 1.5
18:2^{6cis,9cis}	N.D.	-	-
18:2^{9cis,12cis} (LA)	18:3^{9cis,12cis,15cis} (ALA)	65.2 ± 0.6	24.4 ± 0.4
18:2^{9cis,11trans}	N.D.	-	-
18:2^{9trans,12cis}	18:3^{9trans,12cis,15cis} d	63.8 ± 0.2	40.0 ± 3.7
18:2^{9trans,12trans}	N.D.	-	-
18:2^{10trans,12cis}	18:3^{10trans,12cis,15cis} d	41.3 ± 0.2	18.1 ± 0.5

18:3^{6cis,9cis,12cis} (GLA)	18:4^{6cis,9cis,12cis,15cis} (SDA)	74.8 ± 0.1	32.6 ± 0.7
20:2^{9cis,12cis}	N.D.	-	-
20:3^{8cis,11cis,14cis} (DGLA)	20:4^{8cis,11cis,14cis,17cis} (ETA)	57.7 ± 1.1	6.7 ± 0.3
20:4^{5cis,8cis,11cis,14cis} (ARA)	20:5^{5cis,8cis,11cis,14cis,17cis} (EPA)	52.5 ± 2.6	8.7 ± 0.7

[a] All exogenous free fatty acid substrates were added at 0.1 mM. All the values are for three independent samples (mean ± SD)

[b] Conversion rate (%) = 100 × [product]/[product + substrate]

[c] N.D., not detected

[d] Putative product

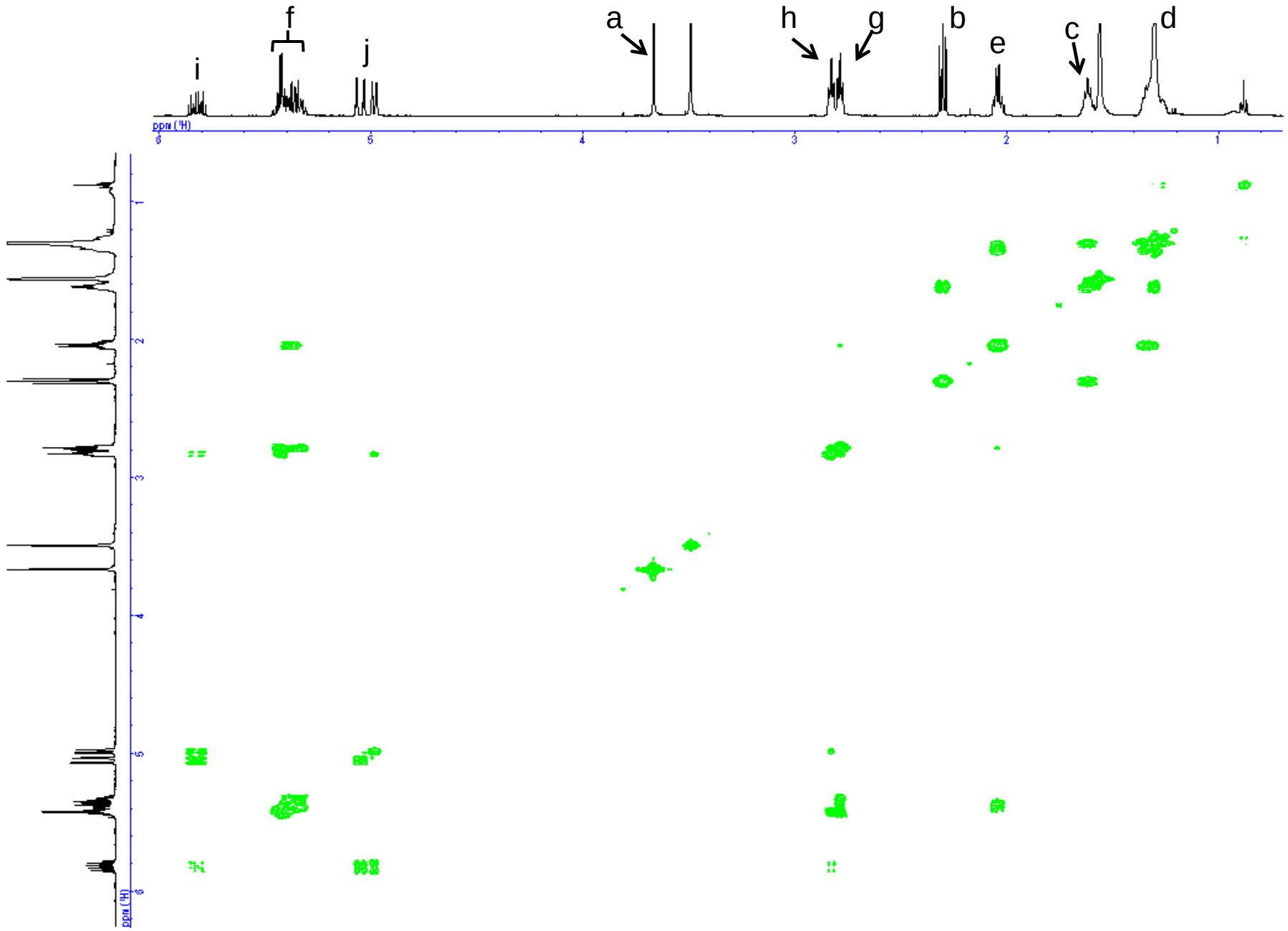

FIGURE S1. DQF-COSY analysis of the UK2 methyl ester.