Molecular Evidence That the Rate-Limiting Step for the Biosynthesis of Arachidonic Acid in *Mortierella alpina* Is at the Level of an Elongase

Seiki Takeno[a], Eiji Sakuradani[a], Shoichi Murata[a], Misa Inohara-Ochiai[b], Hiroshi Kawashima[c], Toshihiko Ashikari[b], and Sakayu Shimizu[a,*]

[a]Laboratory of Fermentation Physiology and Applied Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan, and Institutes for [b]Advanced Technology and [c]Health Care Science, Suntory Ltd., Osaka 618-0001, Japan

ABSTRACT: The oil-producing fungus *Mortierella alpina* 1S-4 is an industrial strain for arachidonic acid (AA) production. To determine its physiological properties and to clarify the biosynthetic pathways for PUFA, heterologous and homologous gene expression systems were established in this fungus. The first trial was performed with an enhanced green fluorescent protein gene to assess the transformation efficiency for heterologous gene expression. As a result, strong fluorescence was observed in the spores of the obtained transformant, suggesting that the foreign gene was inherited by the spores. The next trial was performed with a homologous PUFA elongase (GLELOp) gene, this enzyme having been reported to catalyze the elongation of GLA (18:3n-6) to dihomo-γ-linolenic acid (20:3n-6), and to be the rate-limiting step of AA production. The FA composition of the transformant was different from that of the host strain: The GLA content was decreased whereas that of AA was increased. These data support the hypothesis that the GLELOp enzyme plays an important role in PUFA synthesis, and may indicate how to control PUFA biosynthesis.

Paper no. L9634 in *Lipids 40,* 25–30 (January 2005).

PUFA can serve as precursors of the eicosanoids of signaling molecules including prostaglandins, thromboxanes, and leukotrienes, and they also play important roles as structural components of membrane phospholipids (1,2). The principal PUFA that act as precursors for eicosanoid synthesis are dihomo-γ-linolenic acid (DGLA, 20:3n-6), arachidonic acid (AA, 20:4n-6), and EPA (20:5n-3). All mammals synthesize eicosanoids, which are involved in regulating inflammatory responses, reproductive function, immune responses, and blood pressure (3). Therefore, studies on PUFA biosynthesis are important in both the medical and pharmaceutical fields.

We found the filamentous fungus, *Mortierella alpina* 1S-4, to be a producer of PUFA-containing lipids (4). This species is unique in its capacity to produce C_{20} PUFA, such as DGLA, AA, and EPA. We have studied FA metabolism in this strain and succeeded in using it for the industrial production of AA (5). Therefore, this fungus is a good model for analyzing a FA desaturation and/or elongation system from both fundamental and applied standpoints. A number of mutants derived from this fungus that show alterations in the FA synthetic pathways have been determined through analyses of the FA composition or accumulation.

Wynn and Ratledge (6) determined that the rate-limiting step for AA production is at the level of the elongation of GLA (18:3n-6) to DGLA (6). They also predicted that enhancement of the elongase activity would lead to an increase in AA production (6). In plants and mammals, it is believed that microsomal FA elongation is a four-step process comprising one condensation step, two reduction steps, and one dehydration step (7). Biochemical studies have provided indirect evidence that the reaction catalyzed by the condensing enzyme of the elongation system is rate-limiting (7–9). This enzyme regulates the substrate specificity in terms of the chain length and the degree of unsaturation of FA. Recently, the elongase responsible for the conversion of GLA to DGLA, designated as GLELO, was identified through expression studies on a *M. alpina* expression sequence tag library in yeast (10,11).

We have established a transformation system for this fungus (12) and have applied it to the study of PUFA production, especially AA. In this study, we succeeded in increasing AA production by *M. alpina* through metabolic engineering, namely, construction of a *M. alpina* strain over-expressing the GLELO gene: (i) As a first step, the enhanced green fluorescent protein (EGFP) gene was expressed in *M. alpina* 1S-4 to assess expression of two genes (*ura5* and *EGFP*), and (ii) GLELO cDNA was cloned and isolated from the strain, and then over-expressed in the same strain, hereby leading to increased AA production. This is the first report of the significant importance of the FA composition in an AA industrial strain, *M. alpina* 1S-4.

EXPERIMENTAL PROCEDURES

Enzymes and chemicals. Restriction enzymes and other DNA-modifying enzymes were obtained from Takara Bio Inc. (Shiga, Japan) and New England BioLabs (Beverly, MA). All other chemicals were of the highest purity commercially available.

Strains, media, and growth conditions. Mortierella alpina 1S-4 *ura5*$^-$ (13) was maintained on Potato Dextrose Agar

*To whom correspondence should be addressed.
E-mail: sim@kais.kyoto-u.ac.jp

Abbreviations: AA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; EGFP, enhanced green fluorescent protein; GLELO, elongase responsible for conversion of GLA to DGLA.

medium (Difco, Detroit, MI) containing 5-fluoroorotic acid (0.5 mg/mL) and uracil (0.05 mg/mL). GY medium containing glucose and yeast extract was used for FA composition analyses. The composition of the GY medium depended on the kind of assay (see below). The compositions of the Czapek–Dox and SC media were given in a previous paper (13). For sporulation of the *ura5*$^-$ strain, uracil was added to the Czapek–Dox medium at 0.05 mg/mL. SC medium was used as the uracil-free synthetic medium for cultivation of the *ura5*$^-$ strains and the transformants. This synthetic medium was also used to maintain the transformants. Fungal strains were cultivated at 28°C with reciprocal shaking (120 strokes/min).

Preparation of genomic DNA and construction of a cDNA library. Preparation of genomic DNA of the *M. alpina* strain was performed according to the method described previously (14). Purification of mRNA and cDNA synthesis were also performed according to the methods described previously (15,16).

Isolation of GLELO cDNA of M. alpina 1S-4 and its expression in Aspergillus oryzae. Two primers were synthesized for amplification of the *M. alpina* 1S-4 GLELO cDNA by PCR with the following primers, designed on the basis of the nucleotide sequence of GLELOp of *M. alpina* ATCC 32221 (10): a sense primer, 5′-ATGGAGTCGATTGCGCAATTCCT-3′, and an antisense primer, 5′-TTACTGCAACTTCCTTGCCTTCTCCTT-3′. These primers were used in a Biometra T Gradient thermal cycler (Biometra GmbH, Göttingen, Germany) with a program of 1 min at 94°C, 1 min at 52.5°C, and 2 min at 72°C, for 35 cycles, followed by extension for 10 min at 72°C. The amplified 1.0-kb PCR product was cloned into the pT7Blue T-Vector (Novagen, Madison, WI) to construct pT7-GLELO, and then used to transform *Escherichia coli* DH5α. This clone was determined to encode a protein similar to GLELOp of *M. alpina* ATCC 32221 by DNA sequencing, as described below. Transformation of *A. oryzae* was performed by the method described by Gomi *et al*. (17) and Iimura *et al.* (18). A shuttle vector, pNGA142 (19), with the GLELO cDNA insert was used as the transformation vector for *A. oryzae*. Stable transformants were isolated by repeated sporulation on Czapek–Dox medium plates.

Construction of transformation vectors for the M. alpina 1S-4 ura5$^-$ *strain.* Transformation vector pD4 (20), which was developed for the transformation of *M. alpina*, was kindly supplied by Prof. David B. Archer (University of Nottingham, United Kingdom). pD4 originally contained an expression unit composed of the histone H4.1 promoter, the modified *hpt* gene, and the *N*-(5′-phosphoribosyl)anthranilate isomerase (*trpC*) transcription terminator. The region from the promoter to the terminator was amplified using a forward primer, HisProFX (5′-TACGAATTCAAGCGAAAGAGAGATTATGAA-3′), and a reverse primer, TrpCRX (5′-GAAGAATTCCCTCTAAACAAGTGTACCTGT-3′), with pD4 as a template. The two primers contained an *Eco*RI restriction site (underlined). A modified pBluescript® II SK+ (Stratagene, La Jolla, CA) was prepared by deletion of its *Bam*HI site for convenient manipulation in further experiments. The approximately 2.7-kb PCR product was digested with *Eco*RI, followed by ligation into the modified pBluescript® II SK+ digested with *Eco*RI, and designated as pBlues-hpt.

Transformation vectors pDura5GFP and pDura5GLELO were constructed by modification of pDura5 developed as a transformation vector of the *M. alpina* 1S-4 *ura5*$^-$ strain (12). pDura5GFP is a vector for the expression system of the enhanced green fluorescent protein (EGFP) gene. An EGFP gene was amplified using a forward primer, GFPforward (5′-TCGCCACCATGGTGAGCAAG-3′), and a reverse primer, GFPreverse (5′-CGCGGATCCTTTACTTGTA-3′), designed on the basis of the sequence of the EGFP gene on the pEGFP vector (Clontech Laboratories Inc., Palo Alto, CA) at an annealing temperature of 58°C with the pEGFP vector as a template. The GFPforward and GFP reverse primers contained *Nco*I and *Bam*HI restriction sites, respectively (underlined). The approximately 700-bp fragment digested with *Nco*I and *Bam*HI was ligated to pBlues-hpt digested with the same enzymes to remove the pre-existing *hpt* gene, resulting in the construction of a vector designated as pBlues-GFP. The EGFP gene expression unit obtained from pBlues-GFP on digestion with *Eco*RI was ligated to the *Eco*RI site of pDura5, resulting in the construction of pDura5GFP (Fig. 1).

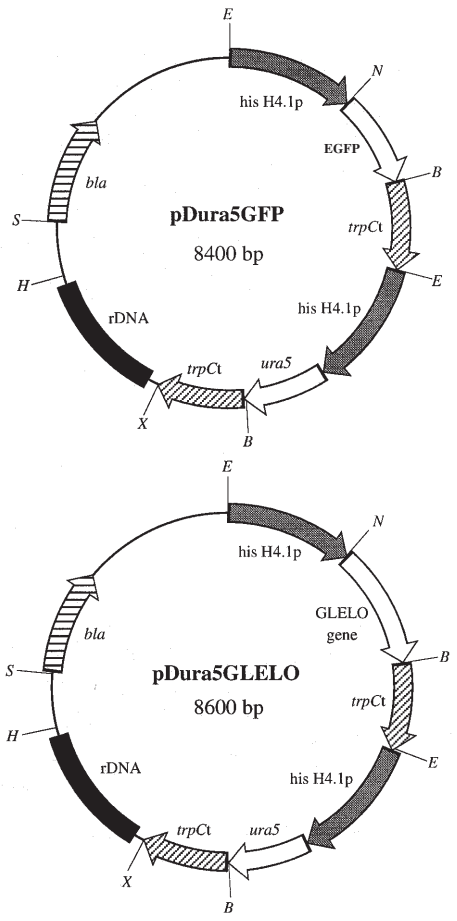

FIG. 1. Two transformation vectors for *Mortierella alpina* 1S-4 *ura5*$^-$ mutant. The details of each gene and the method for constructing these vectors are given in the Experimental Procedures section. Restriction sites: *E, Eco*RI; *N, Nco*I; *B, Bam*HI; *X, Xba*I; *H, Hin*dIII; *S, Ssp*I.

pDura5GLELO is a vector for expression of the GLELO gene. *Mortierella alpina* 1S-4 GLELO cDNA was amplified using a forward primer, GLELOF (5′-CACCATGGAGTC-GATTGCGC-3′), and a reverse primer, GLELOR (5′-GT-GGATCCTTACTGCAACTTCCTTGCCTT-3′), at an annealing temperature of 54°C with the 1S-4 cDNA library as a template. The GLELOF and GLELOR primers contained *Nco*I and *Bam*HI sites, respectively (underlined). The approximately 1.0-kb PCR product was ligated to the pT7Blue T-Vector to construct pT7-GLELO. By following the same strategy as for the construction of pDura5GFP, pDura5GLELO was constructed (Fig. 1).

Transformation of the M. alpina 1S-4 ura5⁻ strain. Transformation of the *M. alpina* 1S-4 *ura5⁻* strain with pDura5GFP or pDura5GLELO was performed with a PDS-1000/He Particle Delivery System (Bio-Rad Laboratories Inc., Hercules, CA) as described previously (12).

Isolation of stable transformants and checking of transformation by PCR were performed by the methods described previously (12).

FA analyses. The *A. oryzae* recombinant transformed with the *M. alpina* 1S-4 GLELOp cDNA was inoculated into a test tube containing 5 mL GY medium containing 2% glucose and 1% yeast extract, and then cultivated at 28°C with reciprocal shaking (120 strokes/min) for 3–4 d. The spores or mycelia of *M. alpina* 1S-4 recombinants transformed with pDura5GLELO and the host cells were inoculated into 20-mL Erlenmeyer flasks containing 4 mL of GY medium containing 2 or 5% glucose, 1% yeast extract, and 0.05 mg/mL of uracil. The culture was performed at 28°C with reciprocal shaking (120 strokes/min) for the desired period. FA analysis was performed basically as described in the previous paper (21).

Nucleotide sequence accession numbers. The nucleotide sequences of the GLELO genomic gene cloned from *M. alpina* 1S-4 have been assigned DDBJ accession no. AB193123.

TABLE 1
FA Compositions of *Aspergillus* Transformant MG-5[a]

	mol% of FA in the total FA of the cells[b]			
		Control strain		MG-5
FA	Control strain	(+ GLA)[c]	MG-5	(+ GLA)
16:0	17.4	9.9	14.0	11.8
18:0	5.8	2.7	5.2	4.1
18:1n-9	14.8	5.5	11.7	7.6
18:2n-6	56.8	30.0	63.8	43.4
GLA	—[d]	50.6	—	17.5
18:3n-3	5.1	1.4	1.4	1.5
20:2n-6	—	—	4.0	2.9
DGLA	—	—	—	11.2

[a]*A. oryzae* MG-5 strain, which was transformed with *Mortierella alpina* 1S-4 GLELO cDNA, and the control strain without the GLELO gene were grown at 28°C for 3–4 d in 5 mL of GY medium with reciprocal shaking (120 strokes/min).
[b]These values are the means of triplicate experiments.
[c]0.2% GLA methyl ester was added to the culture broth.
[d]—, not detected; DGLA, dihomo-γ-linolenic acid.

RESULTS AND DISCUSSION

Transformation of the M. alpina 1S-4 ura5⁻ strain with pDura5GFP. The first trial involved expression of the EGFP gene in the *M. alpina* 1S-4 *ura5⁻* strain using a homologous *ura5* gene-containing vector. This would directly demonstrate that a two-gene expression system is feasible, and that heterologous gene expression in *M. alpina*, which will lead to further applications, has been achieved. The EGFP gene was placed under the control of the same promoter and terminator as previously used for expression of the homologous *ura5* gene (12). On the basis of this idea, an EGFP-expression vector, pDura5GFP, was constructed and used for transformation of the *ura5⁻* strain. Transformation was successfully performed, and six stable transformants were selected from the 45 isolated. All the stable ones were determined to be transformed through vector insertion into the rDNA locus (data not shown). When both host cells (*ura5⁻* strain) and ones of the transformants were cultivated in various media and observed under a fluorescence microscope, strong fluorescence was detected, mainly in the outer part of the cells. To remove this fluorescence, protoplasts were prepared, but the resulting cells still had a strong fluorescence. Although these transformants seemed to show strong fluorescence in their cytoplasm, the observation could not be thought to be sufficient evidence that the EGFP gene was truly expressed.

How the self-fluorescence of this fungus is produced remains unclear, as it was impossible to detect the fluorescence from the EGFP protein precisely in the mycelial cells. On the other hand, when spores were observed under the fluorescence microscope, a strong fluorescence was detected only in those of the transformants (data not shown). This finding implies that the foreign gene could be inherited by both the daughter cells and spores and indicates that the EGFP and *ura5* genes were both successfully expressed in this fungus. Hence, we expected that the same strategies could be used for expression of genes involved in PUFA syntheses, leading to elucidation of FA metabolism and improvement of PUFA production.

Isolation and heterologous expression of the GLELO gene of M. alpina 1S-4. The gene encoding GLELOp, which catalyzes elongation of GLA to DGLA, has previously been cloned from *M. alpina* ATCC 32221 and characterized (10). To perform homologous expression of the GLELO gene, the GLELO cDNA of *M. alpina* 1S-4 was isolated. As a result, a fragment containing an open reading frame with a length of 957 bp, starting with an ATG codon and ending with a TAA codon, was isolated. The gene was suggested to encode a protein consisting of 318 amino acids with a M.W. of 37,000 (data not shown). A computer-aided homology search of the amino acid sequences of other proteins in a database revealed that the deduced amino acid sequence of the resultant gene exhibits 95.9% identity with that of the GLELOp of *M. alpina* ATCC 32221, and 33.5 and 32.5% identity with those of elongation of very long chain FA protein 2 (ELO2) of mouse and human, respectively (22,23). The amino acid sequence of GLELOp showed several regions of identity, including a common histidine box motif, with ELO2. A

TABLE 2
Comparison of FA Compositions of *M. alpina* 1S-4 Transformants[a]

Incubation period (d)	FA	mol% of FA in the total FA of the cells[b]		
		Host cell	Transformant #3	Transfomant #8
4	16:0	18.5	16.8	19.7
	18:0	5.7	5.4	7.0
	18:1n-9	28.2	27.8	27.3
	18:2n-6	9.9	10.2	9.0
	GLA	7.7	9.2	6.7
	DGLA	5.8	6.4	6.7
	AA	19.8	20.9	20.5
10	16:0	19.7	19.9	20.0
	18:0	6.4	7.12	6.5
	18:1n-9	30.6	24.9	32.1
	18:2n-6	8.7	8.5	8.4
	GLA	5.6	3.8	5.6
	DGLA	5.9	6.2	4.5
	AA	18.1	25.6	19.1

[a]Two *M. alpina* 1S-4 recombinants transformed with pDura5GLELO, #3 and #8, and host cells were grown at 28°C for 4 or 10 d in 4 mL of GY medium containing uracil under reciprocal shaking (120 strokes/min).
[b]These values are the means of triplicate experiments. AA, arachidonic acid; for other abbreviations see Table 1.

hydropathy plot (Kyte and Doolittle) of GLELOp indicated that this protein is hydrophobic in nature and contains several presumptive transmembrane domains (data not shown). Furthermore, functional analyses were also performed on gene expression in *A. oryzae* (Table 1). The *Mortierella alpina* 1S-4 GLELO cDNA was ligated to fungal expression vector pNGA142. In the resultant plasmid, designated as pGEGA10, expression of the cloned GLELOp gene was controlled by a glucoamylase promoter of *A. oryzae*. The FA composition of the selected *A. oryzae* transformant, MG-5, was different from that of the control strain without the GLELO gene: The new peak in the chromatogram of FAME from the transformant cultured in GY medium for 4 d exhibited a retention time identical to the FAME standard of 20:2n-6, which is produced through an elongase reaction on endogenous 18:2n-6. In the presence of exogenous GLA, a new peak of DGLA was observed only for the transformant, indicating conversion of GLA to DGLA at the rate of 39%, whereas the rate of conversion of endogenous 18:2n-6 to 20:2n-6 was 5.9%, and 18:1n-9 and 18:3n-3 were not converted to the corresponding 20-carbon PUFA at all. Another experiment indicated that exogenous 18:1n-12 was converted to 20:1n-12 with a conversion rate of 2.1% (data not shown). The GLELOp of *M. alpina* ATCC 32221 showed the same characteristics as that of *M. alpina* 1S-4 (10,11). Thus, the cDNA was determined to be the gene encoding GLELOp throughout this analysis.

It is generally believed that the elongation activity involves four distinct subunit enzymes: a condensing enzyme, two reductases, and a dehydrase. It is unlikely that one protein exhibits all four activities (11). Therefore, the elongase activity of GLELOp in the *Aspergillus* transformants is likely to arise from interaction with other endogenous components of *Aspergillus* elongase.

Transformation of M. alpina 1S-4 with pDura5GLELO. A transformation vector, pDura5GLELO (Fig. 1), was constructed and used for transformation of the *ura5*$^-$ strain to over-express the GLELO gene. Out of the 36 transformants obtained, 9 were determined to be stable transformants with pDura5GLELO (data not shown). To investigate their properties, two transformants (#3 and #8) were cultivated in GY medium, containing 5% glucose, 1% yeast extract, and 0.05 mg/mL of uracil, for 4 or 10 d to investigate their FA compositions (Table 2). It was verified that complementation of *ura5* genes does not influence the FA composition of the host cells (data not shown). Transformant #8 showed the same FA composition as that of the host cells, regardless of the cultivation period. On the other hand, transformant #3 exhibited a different FA composition from that of the host cells: GLA, which serves as a substrate for GLELOp, was decreased and AA was increased. For further investigation, time course experiments were performed with transformant #3 and the host cells (Fig. 2). The accumulation of AA continued after 4 d in both strains, regardless of the culture conditions. With 5% glucose in the medium, AA content remained low in comparison with that with 2% glucose. Although this observation was in accordance with results previously reported, the AA productivity (mg/mL of culture broth) was proportional to the glucose concentration (data not shown) (4). In the case of 2% glucose (Figs. 2A and B), the AA content increased linearly after 4 d in the transformant but not in the host cells. Our previous report also stated that significant AA accumulation was observed in the stationary phase (5,24), and this phase is thought to correspond to the period after 4 d in the present experiment.

The final content of AA in the transformant was higher than that in the host cells. Hence, over-expression of the GLELO gene clearly occurred in this lipogenic phase rather than in the growth phases. With 5% glucose (Figs. 2C and 2D), differences in the FA profile appeared clearly: The AA content of transformant #3 increased more than that of the host cells after 5 d. The GLA content of the transformant cells remained lower than that of the host cells, especially after 4 d. The DGLA content increased at the beginning of cultivation and then decreased concomitantly with the increase in the AA content. The AA content of transformant #3 was 1.4-fold higher than that of the host cells at the end point. The significance of this increase can be appreciated from our previous efforts to enhance the productivity, which did not achieve such significant change. This major increase must therefore have resulted from the enzyme playing an important role in AA biosynthesis. In addition, the analysis of real-time quantitative PCR showed that the quantity of GLELO RNA in transformant #3 was 7.4-fold higher than that of the host strain on 4 d (data not shown). Therefore, the results obtained for stable transformant #3 must be directly due to GLELO gene expression.

In conclusion, GLELOp catalyzes the conversion of GLA to DGLA and serves as the rate-limiting step in AA production. As predicted by Wynn and Ratledge (6), AA productivity can be increased through enhancement of the enzyme activity. This is the first report that genetic manipulation led to an increase in AA production by an industrial oleaginous strain, *M. alpina* 1S-4.

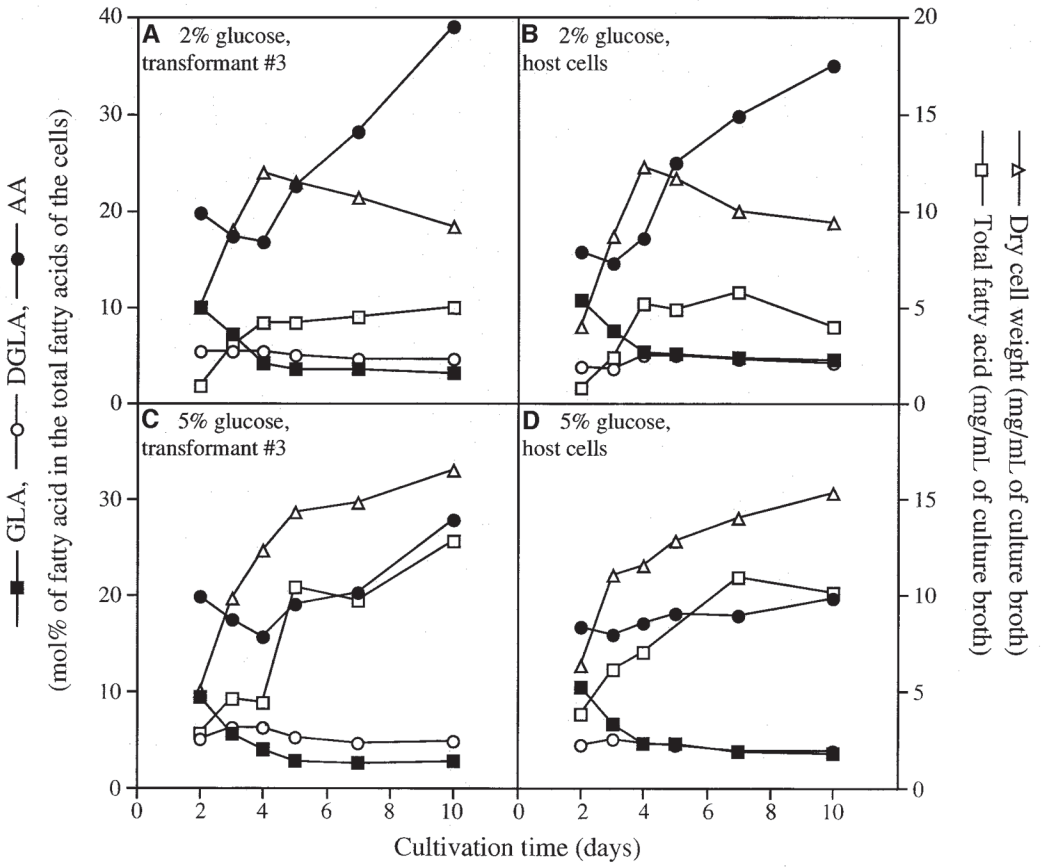

FIG. 2. Time courses of changes in the mycelial FA content in the GLELO-over-expressing *M. alpina* 1S-4 transformant and the host cells. All cultivations were started by inoculation of the respective spores and performed with reciprocal shaking (120 strokes/min) at 28°C. The culture conditions were as follows: (A, B) GY medium containing 2% glucose. (C, D) GY medium containing 5% glucose. All media also contained 1% yeast extract and 0.05 mg/mL uracil. The strains used were as follows: (A, C) transformant; (B, D) host cells. FA analysis was performed with total biomass obtained from each cultivation. The values are the means of triplicate experiments. DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid.

ACKNOWLEDGMENTS

We wish to thank Professor David B Archer (School of Life and Environmental Sciences, University of Nottingham, United Kingdom) for providing *Mortierella* transformation vector pD4. This work was supported in part by the New Energy and Industrial Technology Development Organization (NEDO), and a Grant-in-Aid for Scientific Research (No. 15658024 for S.S.) from the Ministry of Education, Science, Sports, and Culture, Japan.

REFERENCES

1. Needleman, P., Turk, J., Jakschik, B.A., Morrison, A.R., and Lefkowith, J.B. (1986) Arachidonic Acid Metabolism, *Annu. Rev. Biochem. 55*, 69–102.
2. Smith, W.L., and Borgeat, P. (1985) The Eicosanoids: Prostaglandins, Thromboxanes, Leukotrienes, and Hydroxy-eicosaenoic Acids, in *Biochemistry of Lipids and Membranes* (Vance, D.E., and Vance, J.E., eds.), pp. 325–360, Benjamin/Cummings, Menlo Park, CA.
3. Horrobin, D.F. (1992) Nutritional and Medical Importance of γ-Linolenic Acid, *Prog. Lipid Res. 31*, 163–194.
4. Yamada, H., Shimizu, S., and Shinmen, Y. (1987) Production of Arachidonic Acid by *Mortierella elongata* 1S-5, *Agric. Biol. Chem. 51*, 785–790.
5. Shimizu, S., Ogawa, J., Kataoka, M., and Kobayashi, M. (1997) Screening of Novel Microbial Enzymes for the Production of Biologically and Chemically Useful Compounds, in *Advances in Biochemical Engineering/Biotechnology* (Schepter, T., ed.), Vol. 58, pp. 45–87, Springer-Verlag, Berlin.
6. Wynn, J.P., and Ratledge, C. (2000) Evidence That the Rate-Limiting Step for the Biosynthesis of Arachidonic Acid in *Mortierella alpina* Is at the Level of the 18:3 to 20:3 Elongase, *Microbiology 146*, 2325–2331.
7. Cinti, D.L., Cook, L., Nagi, M.N., and Suneja, S.K. (1992) The Fatty Acid Chain Elongation System of Mammalian Endoplasmic Reticulum, *Prog. Lipid Res. 31*, 1–51.
8. Bernert, J.T., Jr. , and Sprecher, H. (1977) An Analysis of Partial Reactions in the Overall Chain Elongation of Saturated and Unsaturated Fatty Acids by Rat Liver Microsomes, *J. Biol. Chem. 252*, 6736–6744.
9. Nugteren, D.H. (1965) The Enzymic Chain Elongation of Fatty Acids by Rat-Liver Microsomes, *Biochim. Biophys. Acta 106*, 280–290.
10. Parker-Barnes, J.M., Das, T., Bobik, E., Leonard, A.E., Thurmond, J.M., Chaung, L.T., Huang, Y.S., and Mukerji, P. (2000) Identification and Characterization of an Enzyme Involved in

the Elongation of n-6 and n-3 Polyunsaturated Fatty Acids, *Proc. Natl. Acad. Sci. USA 97*, 8284–8289.
11. Das, T., Thurmond, J.M., Bobik, E., Leonard, A.E., Parker-Barnes, J.M., Huang, Y.S., and Mukerji, P. (2000) Polyunsaturated Fatty Acid-Specific Elongation Enzymes, *Biochem. Soc. Trans*. *28*, 658–660.
12. Takeno, S., Sakuradani, E., Murata, S., Inohara-Ochiai, M., Kawashima, H., Ashikari, T., and Shimizu, S. (2004) Establishment of an Overall Transformation System for an Oil-Producing Filamentous Fungus, *Mortierella alpina* 1S-4, *Appl. Microbiol. Biotechnol*. *65*, 419–425.
13. Takeno, S., Sakuradani, E., Murata, S., Inohara-Ochiai, M., Kawashima, H., Ashikari, T., and Shimizu, S. (2004) Cloning and Sequencing of the *ura3* and *ura5* Genes, and Isolation and Characterization of Uracil Auxotrophs of the Fungus *Mortierella alpina* 1S-4, *Biosci. Biotechnol. Biochem*. *68*, 277–285.
14. Malardier, L., Daboussi, M.J., Julien, J., Roussel, F., Scazzocchio, C., and Brygoo, Y. (1989) Cloning of the Nitrate Reductase Gene (*niaD*) of *Aspergillus nidulans* and Its Use for Transformation of *Fusarium oxysporum*, *Gene 78*, 147–156.
15. Chomczynski, P., and Sacchi, N. (1987) Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate–Phenol–Chloroform Extraction, *Anal. Biochem*. *162*, 156–159.
16. Sakuradani, E., Kobayashi, M., and Shimizu, S. (1999) Δ9-Fatty Acid Desaturase from Arachidonic Acid-Producing Fungus. Unique Gene Sequence and Its Heterologous Expression in a Fungus, *Aspergillus*, *Eur. J. Biochem*. *260*, 208–216.
17. Gomi, K., Iimura, Y., and Hara, S. (1987) Integrative Transformation of *Aspergillus oryzae* with a Plasmid Containing the *Aspergillus nidulans argB* Gene, *Agric. Biol. Chem*. *51*, 2549–2555.
18. Iimura, Y., Gomi, K., Uzu, H., and Hara, S. (1987) Transformation of *Aspergillus oryzae* Through Plasmid-Mediated Complementation of the Methionine-Auxotrophic Mutation, *Agric. Biol. Chem*. *51*, 323–328.
19. Sakuradani, E., Kobayashi, M., and Shimizu, S. (1999) Identification of an NADH-Cytochrome b_5 Reductase Gene from an Arachidonic Acid-Producing Fungus, *Mortierella alpina* 1S-4, by Sequencing of the Encoding cDNA and Heterologous Expression in a Fungus, *Aspergillus oryzae*, *Appl. Environ. Microbiol*. *65*, 3873–3879.
20. Mackenzie, D.A., Wongwathanarat, P., Carter, A.T., and Archer, D.B. (2000) Isolation and Use of a Homologous Histone H4 Promoter and a Ribosomal DNA Region in a Transformation Vector for the Oil-Producing Fungus *Mortierella alpina*, *Appl. Environ. Microbiol*. *66*, 4655–4661.
21. Sakuradani, E., Kobayashi, M., Ashikari, T., and Shimizu, S. (1999) Identification of Δ12-Fatty Acid Desaturase from Arachidonic Acid-Producing *Mortierella* Fungus by Heterologous Expression in the Yeast *Saccharomyces cerevisiae* and the Fungus *Aspergillus oryzae*, *Eur. J. Biochem*. *261*, 812–820.
22. Tvrdik, P., Westerberg, R., Silve, S., Asadi, A., Jakobsson, A., Cannon, B., Loison, G., and Jacobsson, A. (2000) Role of a New Mammalian Gene Family in the Biosynthesis of Very Long Chain Fatty Acids and Sphingolipids, *J. Cell Biol*. *149*, 707–717.
23. Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R., Wakamatsu, A., Hayashi, K., Sato, H., Nagai, K., *et al.* (2004) Complete Sequencing and Characterization of 21,243 Full-Length Human cDNAs, *Nat. Genet*. *36*, 40–45.
24. Shimizu, S., Kawashima, H., Akimoto, K., Shinmen, Y., and Yamada, H. (1989) Microbial Conversion of an Oil Containing α-Linolenic Acid to an Oil Containing Eicosapentaenoic Acid, *J. Am. Oil Chem. Soc*. *66*, 342–347.

[Received October 18, 2004; accepted January 7, 2005]