Metabolic engineering of oleaginous fungus *Mortierella alpina* for high production of oleic and linoleic acids

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Abstract

The aim of this work was to study the molecular breeding of oleaginous filamentous *Mortierella alpina* for high production of linoleic (LA) or oleic acid (OA). Heterologous expression of the *Δ12-desaturase* (DS) gene derived from *Coprinopsis cinerea* in the Δ6DS activity-defective mutant of *M. alpina* increased the LA production rate as to total fatty acid to 5 times that in the wild strain. By suppressing the endogenous *Δ6I* gene expression by RNAi in the Δ12DS activity-defective mutant of *M. alpina*, the OA accumulation rate as to total fatty acid reached 68.0%. The production of LA and OA in these transformants reached 1.44 and 2.76 g/L, respectively, on the 5th day. The *Δ6I* transcriptional levels of the RNAi-treated strains were suppressed to 1/10th that in the parent strain. The amount of *Δ6II* RNA in the *Δ6I* RNAi-treated strain increased to 8 times that in the wild strain.

Key words

Mortierella alpina; Oleic acid; Linoleic acid; Fatty acid desaturase; RNAi

1. Introduction

Oleic acid (18:1ω9, OA) accounts for about 80% of total fatty acids in virgin olive oil, and linoleic acid (18:2ω6, LA) for about 65% in safflower oil (Beltrán et al., 2004; Matthaus et al., 2015). OA is the most well-known monounsaturated fatty acid, and is known to cause reductions in cholesterol levels, blood pressure and several human cancers (Kurushima et al., 1995; Ferrara et al., 2000; Menendez and Lupu, 2006). Furthermore, OA is converted into a hydroxyl fatty acid, ricinoleic acid (18:1ω9-OH), which has been used in chemical industry as a raw material, by the fatty acid hydroxylase from such as caster beans or a fungal plant pathogen (van de Loo et al., 1995; Azcan and Demirel, 2008; Meesapyodsuk and Qiu, 2008). LA also been converted from OA by Δ12-desaturase (DS) and cannot be synthesized *de novo* in mammals. LA can be some positional and geometric isomers, conjugated linoleic acid, which induce reductions in carcinogenesis, atherosclerosis, inflammation, diabetes and so on (Belury, 2002; Bergamo et al., 2014; Yang et al., 2015). Among these, OA and LA are in high demand as medical and industrial resources. Although vegetable oils such as virgin olive oil and safflower oil contain OA and LA, those are used as edible. By making selective production of OA or LA by breeding of an oleaginous fungus, it is expected to stably supply fermented microbial oils and to develop new functional lipids using these breeding fungi.

The oleaginous filamentous fungus *Mortierella alpina* is a producer of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4ω6, ARA) and dihomo-γ-linoleic acid (20:3ω6), which are rich in triacylglycerols (Sakuradani et al., 2009b; Sakuradani, 2010). In addition, the lipid productivity of this fungus reaches 600 mg/g of dried mycelia. For these reasons, the fungus has been used as a model

oleaginous microorganism for the biosynthesis and accumulation of lipids, including PUFAs (Kawashima et al., 1995; Sakuradani et al., 1999, 2008, 2013; Kikukawa et al., 2013). The Δ6DS-defective mutant strain *M. alpina* ST66 has a mutation site in the *Δ6I* gene, which results in an amino acid replacement, W314Stop, and accumulates LA with a decrease in ARA productivity (Abe et al., 2005; Sakuradani et al., 2009a). On the other hand, the Δ12DS-defective mutant JT-180 has one point mutation in the *Δ12ds* gene, which causes an amino acid replacement, P166L, and accumulates OA and Mead acid (20:3ω9, MA) with loss of ω6 fatty acids production capacity (Sakuradani et al., 2009a, 2009b). Here, we improved the LA and OA productivity by molecular breeding of *M. alpina* ST66 and JT-180, respectively.

The Δ12DS and Δ6DS from *M. alpina* convert OA to LA and 18:2ω9 by desaturation at Δ12 and Δ6 positions of OA, respectively. It has been reported that the transformed *M. alpina* strain in which Δ12DS was suppressed by RNAi accumulates ω9 and ω6 fatty acids (Takeno et al., 2005). The present study is the first report related with the silencing of *Δ6ds* by RNAi in microorganisms. Several Δ12DSs from various organisms were characterized in yeast (Kikukawa et al., 2013; Sayanova et al., 2006; Yan et al., 2013; Zhou et al., 2011). The *Coprinopsis cinerea* (*Coprinus cinereus*) *Δ12ds* gene was reported to exhibit high Δ12DS activity in *Saccharomyces cerevisiae* (Zhang et al., 2007). In the present stucy, *C. cinerea* Δ12DS (CopΔ12DS) with a high Δ12DS activity was functionally expressed in *M. alpina*, leading to the high production of LA.

2. Materials and methods

2.1. Culture conditions

Δ6 Desaturation-defective mutant ST66 and Δ12 desaturation-defective mutant JT-180 used for this study were derived from wild strain *M. alpina* 1S-4 (Abe et al., 2005; Sakuradani et al., 2009a, 2009b). All the *M. alpina* strains, including the uracil auxotroph, were precultured at 28°C on Potato Dextrose Agar medium (Difco, USA), inoculated into GY medium consisting of 2% (w/v) glucose and 1% yeast extract (Difco, USA), and cultivated at 28°C, for analyzing fatty acid composition and extracting total RNA (Yamada et al., 1987). Czapek-Dox medium consisting of 3% sucrose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$, 0.005% uracil, 2% agar, pH 6.0, was used for the sporulation of the fungi. SC medium was used as a uracil-free synthetic medium, and contained 1.7 g of Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco, USA), 5 g of $(NH_4)_2SO_4$, 20 g of glucose, 20 mg of adenine, 30 mg of tyrosine, 1 mg of methionine, 2 mg of arginine, 2 mg of histidine, 4 mg of lysine, 4 mg of tryptophan, 5 mg of threonine, 6 mg of isoleucine, 6 mg of leucine, 6 mg of phenylalanine, and 18 g agar per liter adjusted to pH 5.5. *Escherichia coli* DH5α cells were used for DNA manipulation and cultivated at 37°C.

2.2. Isolation of uracil auxotrophs of *M. alpina* ST66 and JT-180

The uracil auxotroph *M. alpina* ST66 and JT-180 strains were obtained as described by Ando et al. (2009) with slight modification. ST66 and JT-180 were incubated on Czapek-Dox agar medium at 28°C for two weeks, and then allowed to sporulate at 10°C for two weeks or more. Spores of these strains were harvested from the surface of

Czapek-Dox agar, followed by filtering of the suspension through Miracloth (Calbiochem, USA), and about 2.0×10^7 spores were spread on GY medium containing 0.1% 5-fluoroorotic acid, 0.005% uracil and 1.8% agar at 28°C. Then, the 5-fluoroorotic acid resistance strains were isolated as uracil auxotrophs. The subsequent experiments were carried out according to the flowchart in Fig. 1.

2.3. Complimentary DNA synthesis of *M. alpina* mutants

Total RNA was isolated using ISOGEN (Nippon Gene, Japan) according to the manufacturer's protocol. After treatment with RNase-free DNase (TaKaRa, Japan), mRNA was reverse transcribed using a PrimeScript RT Regent Kit (TaKaRa, Japan) according to the manufacturer's instructions, and the complimentary DNA liberally used for constructing of the *Δ6I* silencing cassette and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) analysis.

2.4. Construction of transformation vectors for *M. alpina* uracil auxotrophs

LA is synthesized from OA by Δ12 desaturation, and converted to GLA by Δ6 desaturation. The Δ6DS-defective mutant strain *M. alpina* ST66 accumulates LA and OA with a decrease in GLA and ARA (Abe et al., 2005; Sakuradani et al., 2009a). In order to accumulate LA, the *Δ12-desaturase* gene was overexpressed in ST66 strain. The *C. cinereus Δ12ds* gene (*CopΔ12ds*; accession no. AB269266), which had been reported to exhibit high Δ12DS activity in *S. cerevisiae* (Zhang et al., 2007), was synthesized with optimized codon usage to reflect the codon of *M. alpina* (obtained from

the Kazusa database; http://www.kazusa.or.jp/codon/). The binary vector pBIG3CopΔ12DS was constructed by inserting the modified *CopΔ12ds* gene into the expression cassette with a *SSA2* promoter and a *SdhB* terminator of plasmid pBIG35ZhGUSm (Okuda et al., 2014), which harbored a *ura5* expression cassette for breaking uracil auxotroph and a bacterial kanamycin resistance *NPTIII* gene (Fig. 2a).

OA is converted to LA and 18:2ω9 by Δ12 and Δ6 desaturation, respectively. The Δ12DS-defective mutant strain *M. alpina* JT-180 produces 18:2ω9 which is an intermediate on the MA biosynthetic pathway, with a loss of LA (Abe et al., 2005; Sakuradani et al., 2009a, 2009b). In order to accumulate OA, the *Δ6I* gene silencing was performed in JT-180. There are two Δ6DS-encoding genes, the transcription level of *Δ6I* (accession no. AB070557) being 17-fold higher than that of *Δ6II* (AB070556), in *M. alpina* (Sakuradani et al., 1999; Sakuradani and Shimuzu, 2003). We constructed a *Δ6I* repression vector, pBIG3Δ6Ii, which has a short hairpin (sh) RNA expression cassette based on antisense orientation nucleotides 1374–712, and sense nucleotides 836–1360 of the *Δ6I* cDNA were ligated in the inverted orientation under the control of the *SSA2* promoter and *SdhB* terminator just like in pBIG3CopΔ12DS (Fig. 2b). The sense nucleotide of *Δ6I* cDNA was amplified by PCR using primers Δ6Ii-shortF and Δ6Ii-R (Table 1), and was cut with restriction enzymes, *Spe*I and *Bgl*II, for insertion upstream of the *SdhB* terminator. Additionally, the antisense nucleotide was amplified with Δ6Ii-longF and Δ6Ii-R, cut with *Spe*I and *Xho*I, and then inserted into upstream of the sense nucleotide.

2.5. Transformation of *M. alpina* using the ATMT method

The *Agrobacterium tumefaciens*-mediated transformation (ATMT) method used for transcription of *M. alpina* uracil auxotroph strains was a described previously (Okuda et al. 2014) with slight modification. The compositions of the LB-Mg agar plate, Minimal Medium (MM), Induction Medium (IM), and Synthetic Complete (SC) agar media were described by Ando et al. (2009). *A. tumefaciens* C58C1 was transformed with the binary vector via electroporation, and transformants of it were isolated on LB-Mg agar plates supplemented with kanamycin (20 μg/ml), ampicillin (50 μg/ml), and rifampicin (50 μg/ml). *A. tumefaciens* transformants were cultivated in 100 ml of MM supplemented with kanamycin (20 μg/ml) and ampicillin (50 μg/ml) at 28°C for 48 h with shaking (120 rpm). Bacterial cells were harvested by centrifugation at 8,000 × *g*, washed once with fresh IM, and then resuspended 1 ml of fresh IM. After pre-incubation for 4 h at 28°C with shaking (120 rpm), all of the suspension was mixed with an equal volume of a spore suspension (approximately 10^8 spores/ml) of the *M. alpina* uracil auxotroph strain, and then spread on membranes (Whatman #50 Hardened Circles; Whatman International Ltd, UK) kept on co-cultivation medium (IM with 1.5% agar) and incubated at 23°C for four days. After co-cultivation, the membranes were transferred to SC plates that contained 0.03% Nile blue A (Sigma-Aldrich, Japan) to inhibit bacterial growth. After three days incubation at 28°C, hyphae arising from visible fungal colonies were transferred to fresh SC plates to obtain candidates, and the resulting uracil auxotroph break strains were picked up as transformants.

2.6. Fatty acid analysis

All the strains were inoculated into 10 ml of GY medium in a thick test tube and then

cultured at 28°C with reciprocal shaking at 300 rpm. For selection of transformants, total fatty acids from mycelia cultivated for 5 days were analyzed. For measurement of time course of fatty acid production, the selected transformants were cultivated 3, 5, 7 or 10 days. The transmethylation of microbial lipids were performed on the conditions described at the previous report (Yamada et al., 1987). The mycelia were harvested by suction filtration and dried at 120°C. The dried cells were transmethylated with 10% methanolic HCl and dichloromethane at 55°C for 2 h. The resultant fatty acid methylesters were extracted with *n*-hexane, concentrated and then analyzed by gas chromatography as described previously (Okuda et al., 2015). Δ6 and Δ12DS activity was expressed as the 'desaturation index', which is the ratio of the amount of the substrate of the desaturase to that of the product and further metabolites.

2.7. Transcription level analysis of *Δ6I* and *Δ6II*

For determination of the transcription levels of the *Δ6I*, *Δ6II* and *GADPH* genes, real time RT-PCR amplification was carried out. The amplification was performed using gene-specific primers (Table 1), a Roche LightCycler (Roche Diagnostics, Japan), and KOD SYBR qPCR Mix (TOYOBO, Japan) according to the manufacturer's instructions. When comparison among different strains was performed, the total amount of cDNA was normalized as to the amount of glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) as the endogenous reference gene. Data were analyzed by one-way repeared-measures ANOVA, and significant differences between the groups were determined by means of Tukey's HSD test ($P < 0.01$ or 0.05) using R version 3.1.1.

3. Results and discussion

3.1. Fatty acid analysis of the ST66 transformants

The ST66 transformants, named SC12 strains, having a plasmid of pBIG3CopΔ12DS, which were constructed by means of the ATMT method, showed high level LA accumulation of over 40% of the total fatty acid with a reduction of OA (Table 2). The Δ12DS activity was defined as the Δ12DS index, i.e., the ratio of OA (18:1ω9) to LA (18:2ω6) + 18:3ω6 + 20:3ω6 + ARA (20:4ω6) in total fatty acids. Therefore, a low Δ12DS index value means that OA as a substrate was effectively converted to the following ω6 fatty acids through Δ12 desaturation. The SC12#129 strain showed the lowest Δ12DS index value among the mutants, and accumulated lipids comprising 49.5% LA as to total fatty acids, which is equivalent to five-times of the level in the wild strain. These results suggested that the LA biosynthetic pathway in the SC12 strains was enhanced by overexpression of the *CopΔ12ds* gene. *Lactobacillus* genus is known to convert LA to conjugated LA (CLA), which is an isomer of LA via 10-hydroxy-12-octadecenoic acid, with the myosin-cross-reactive antigen (Ogawa et al. 2001; Takeuchi et al. 2016), and to suppress carcinogenesis, atherosclerosis, inflammation, diabetes and so on (Belury, 2002; Bergamo et al., 2014; Yang et al., 2015). The SC12 strains as producers of LA might be prominent hosts for production of LA derivatives such as CLA.

M. alpina has a gene encoding ω3DS that converts LA to α-linoleic acid (18:3ω3, ALA) (Sakuradani et al., 2005; Okuda et al., 2015). The ω3DS in *M. alpina* shows the activity only under low temperature conditions. On the other hand, CopΔ12DS from *C.*

cinerea shows not only Δ12DS but also Δ15DS activity in *S. cerevisiae* cultivated at 28ºC (Zhang et al., 2007). Thus, the ALA of the SC12 strains was considered to be synthesized through Δ15DS activity of CopΔ12DS (Table 2), and was not converted to 18:4ω3, 20:4ω3, and eicosapentaenoic acid (20:5ω3, EPA) because of its Δ6DS activity-deficiency. Transformants having two or more *CopΔ12ds* genes are expected to exhibit high LA and ALA productivities.

3.2. Fatty acid analysis of the JT-180 transformants

The Δ6DS activity of the JT-180 mutant was defined as the Δ6DS index, i.e., the ratio of OA (18:1ω9) to 18:2ω9 + 20:2ω9 + MA (20:3ω9) in total fatty acids, since OA is converted to 18:2ω9 through Δ6 desaturation and the generated 18:2ω9 is finally converted to MA in JT-180 (Sakuradani et al., 2002). The low activity of Δ6DS with RNAi led to a high Δ6DS index value. The Δ6DS index value of strain JT-6Ii#12 was the highest among the transformants, and the ratio of OA in total fatty acids reached 67.6% (Table 3). This finding suggested that the MA biosynthetic pathway in JT-6Ii was suppressed by RNAi of the *Δ6I* gene. The present study is the first report related with the silencing of *Δ6ds* by RNAi in microorganisms. Ricinoleic acid rich in castor oil is converted to sebacic acid by chemical conversion and becomes a raw material of nylon (Azcan and Demirel, 2008). In microorganisms, it is known that phytopathogenic fungus *Claviceps purpurea* produces ricinoleic acid (Meesapyodsuk and Qiu, 2008), but high production of ricinoleic acid by microorganisms has not been realized. The OA-producing JT-6Ii strains obtained in this study are expected to realize production of ricinoleic acid with conversion of accumulated OA by expressing their heterologous

oleate 12-hydroxylase (FAH) genes.

3.3. Time course of OA and LA production by transformants

Strains SC12#129 and JT-6Ii#12 showed the highest fatty acid productivities, 3.32 and 3.85 g/L, on the 5th day, respectively (Table 4). However, their production and dry cell weights decreased after the 7th day. These findings suggested that the fatty acid accumulation of these transformants in GY batch culture was maximal between the 5th and 7th days. *Aspergillus oryzae* transformants, enhanced expression of the fatty acid synthase gene, produced only 1.23 g/l of triacylglycerol at 5th day (Tamano et al., 2013). The overexpression of *Δ12ds* genes derived from *M. alpina* and *Fusarium verticillioides* in oleaginous yeast *Rhodosporidium toruloides* led to LA production (1.3 g/l) at 5th day (Wang et al., 2016). On the other hand, *M. alpina* 1S-4 produces 20 g/L of fatty acid as triacylglycerols in a 10-kl fermentor containing 5% soy flour, 1.8% glucose, and 0.1% soybean oil on the 6th day (Higashiyama et al., 2002). Therefore, the continuous cultivation of the transformants is expected to lead to much higher production of total fatty acids, OA, and LA.

3.4. Measurement of *Δ6I* and *Δ6II* transcriptional levels

The *Δ6I* and *Δ6II* expression in JT-180 markedly increased to 3.6- and 10.1-times ($P < 0.01$) the levels in the wild strain, respectively (Fig. 3). These findings supported the previous data showing that the Δ6DS activity of JT-180 was higher than that of the wild strain (Sakuradani et al., 2002). Transformants, JT-6Ii#12 and WT-6Ii, harboring the

Δ6Ii cassette showed *Δ6I* transcriptional levels that were 28.2- ($P < 0.01$) and 19.8-times ($P < 0.05$) lower than that of each host strain (JT-180 and the wild strain), respectively. In contrast, the *Δ6II* transcriptional levels in these transformants were 7.9- and 8.2-times ($P < 0.01$) higher than that in the wild strain. However, there was no significant difference in *Δ6II* transcription between JT-6Ii#12 and JT-180. These findings suggested that *Δ6II* transcription was triggered by a deficiency of Δ6DSI activity, and that the maximum *Δ6II* level was about 10-times that in the wild strain.

In *M. alpina* 1S-4, it was reported that the *Δ6I* gene is expressed more than 17-times higher than the *Δ6II* one (Sakuradani and Shimizu, 2003). This study indicated that RNAi of *Δ6I* suppressed the *Δ6I* level by one-fifth, and that the *Δ6II* transcription was induced by the *Δ6I* suppression (Fig. 3). However, the 10-times amount of *Δ6II* level gave no adequate MA production (Table 3). These findings indicated that Δ6I plays a major function in Δ6 desaturation in *M. alpina*. When Δ6I activity is deficient, Δ6II might sufficiently compensate for the Δ6 desaturation. Similarly, it has been reported that when the mutation occurs in *Δ9I* in the Δ9 desaturation-defective mutants, the amount of *Δ9II* RNA increases in such mutants (Abe et al., 2006).

4. Conclusions

Transformants that are excellent in either LA or OA productivity were obtained by molecular breeding of oleaginous filamentous fungus *M. alpina*. SC12#129 and JT-6Ii#12 accumulated 49.5% LA and 68.0% OA, respectively. The amounts of fatty acids produced by SC12#129 and JT-6Ii#12 were maximum (3.32 and 3.85 g/l) at the 5th day

in batch culture, respectively. In the RNAi transformant, the expression level of *Δ6I* was suppressed to 1/5 or less that in the wild strain and, in contrast, the expression of *Δ6II* increased. These transformants are expected not only to be LA- and OA-producing strains, but also hosts for production of subsequent fatty acid derivatives.

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Figure Captions

Fig. 1 Experimental flowchart in this study.

Fig. 2 Maps of binary vectors used for the *M. alpina* transformation. For overexpression of CopΔ12DS in the ST66 strain (a), and for RNAi of endogenous *Δ6I* in JT-180 strain (b). RB, right border; LB, left border.

Fig. 3 Relative quantities of the transcripts of the *Δ6I* and *Δ6II* genes in strains cultivated for 5 days. Error bars show the SD for 3 repetitions. The asterisks indicate significantly differences between the strains on the same gene (*$P < 0.05$, **$P < 0.01$, n.s. indicates not significant), as estimated by means of Turkey's HSD test following one-way ANOVA.

Fig. 1

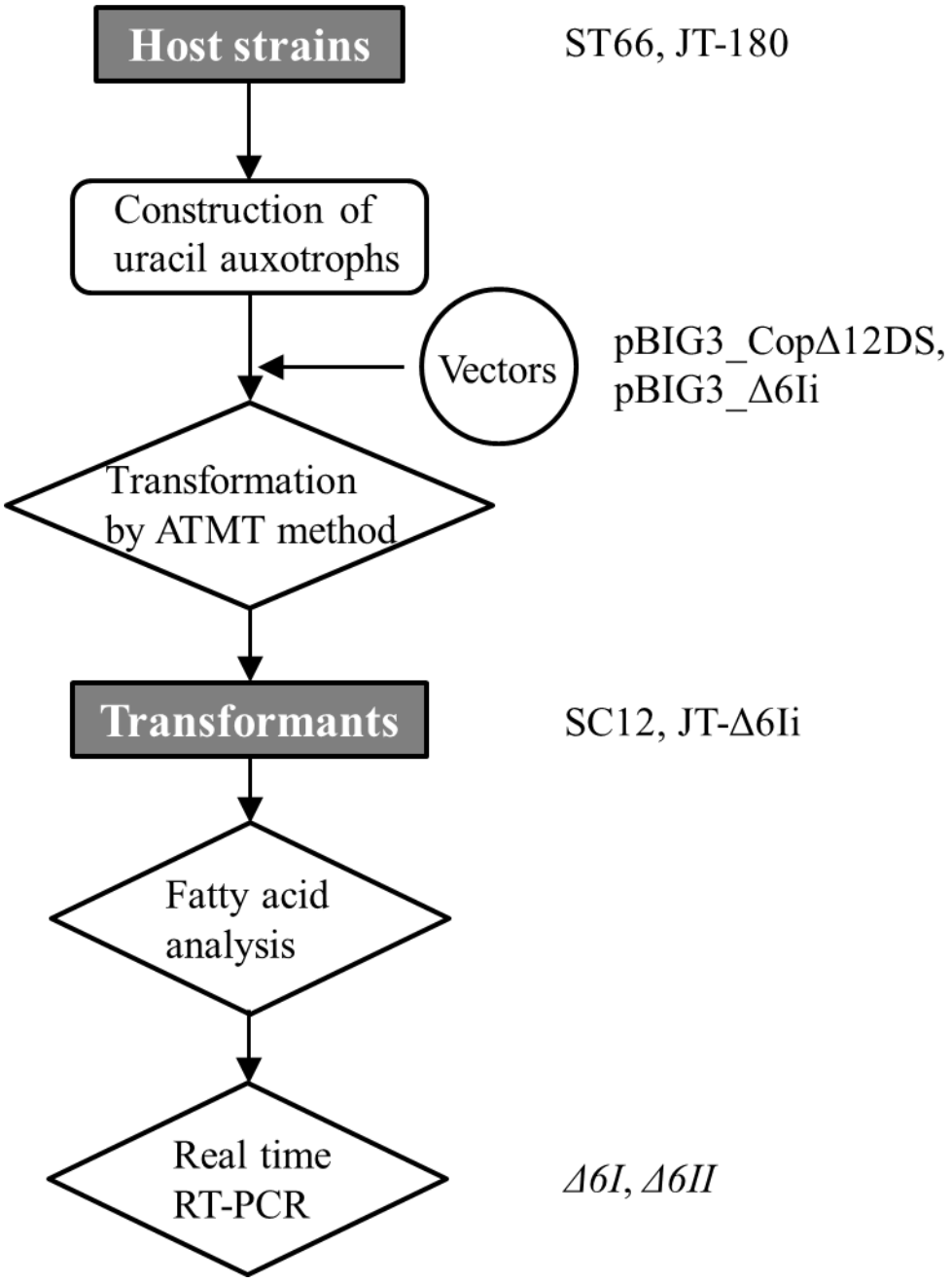
Host strains
ST66, JT-180
Construction of uracil auxotrophs
Vectors
pBIG3_CopΔ12DS, pBIG3_Δ6Ii
Transformation by ATMT method
Transformants
SC12, JT-Δ6Ii
Fatty acid analysis
Real time RT-PCR
Δ6I, Δ6II

Fig. 2

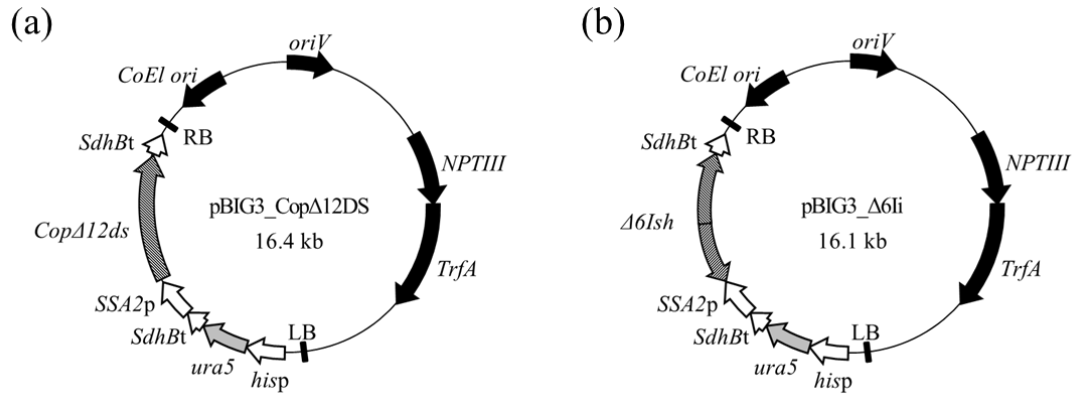
(a)
oriV
CoEl ori
RB
SdhBt
NPTIII
pBIG3_CopΔ12DS
16.4 kb
CopΔ12ds
TrfA
SSA2p
SdhBt
LB
ura5
hisp
(b)
oriV
CoEl ori
RB
SdhBt
NPTIII
pBIG3_Δ6Ii
16.1 kb
Δ6Ish
TrfA
SSA2p
SdhBt
LB
ura5
hisp

Fig. 3

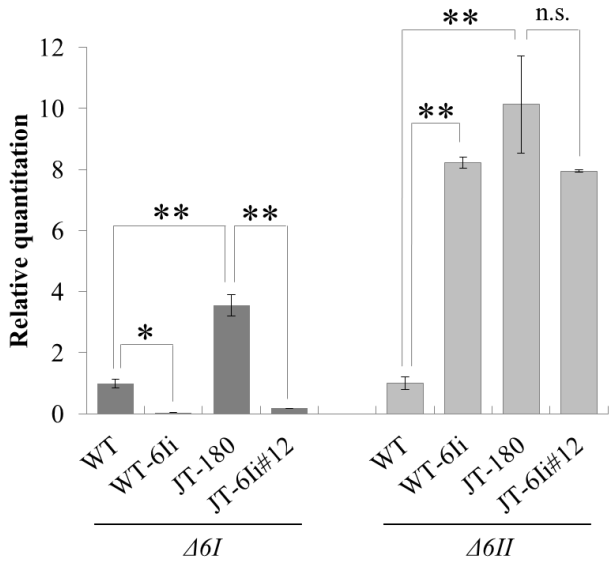
Relative quantitation
12
10
8
6
4
2
0
**
*
**
**
**
n.s.
WT
WT-6Ii
JT-180
JT-6Ii#12
WT
WT-6Ii
JT-180
JT-6Ii#12
Δ6I
Δ6II

Table 1 Primers used in this study.

Primer	Sequence (5'-3') [a]	Intended use
Δ6Ii-shortF	TGTACTAGTGCCTCCAGTCGACTATG	RNAi construct of *Δ6I*
Δ6Ii-longF	TCGTCTCGAGATGTTCTCGGATGTTC	RNAi construct of *Δ6I*
Δ6Ii-R	GTTTACTAGTCCTTGCAGATCTTGGAGGC	RNAi construct of *Δ6I*
Δ6I-qF	GATGTTCACCCTGGTCTGTTT	qPCR of *Δ6I*
Δ6I-qR	GTGGTATGGTATCGGACACCGTA	qPCR of *Δ6I*
Δ6II-qF	GGTGCATCCAGTCGATTCTT	qPCR of *Δ6II*
Δ6II-qR	ATGTTGACGGGATCCTTGATGA	qPCR of *Δ6II*
GAPDH-qF	CGGTGTCTTCACCACCATTG	qPCR of *GAPDH*
GAPDH-qR	GTGCACGAGGCGTTGGAAA	qPCR of *GAPDH*

[a] The underlined sequences show the synthesized restriction enzyme site.

Table 2 Fatty acid compositions of *M. alpina* ST66 as the host strain, the derived transformants, SC12 strains, and the wild strain.

Strain	Fatty acid composition (%) [a]									Δ12DS index [b]
	16:0	18:0	18:1ω9	18:2ω6	18:3ω3	18:3ω6	20:3ω6	20:4ω6	Others	
ST66 (host)	9.2	5.3	37.3	34.4	— [c]	3.0	0.8	4.8	5.2	0.87
SC12#01	17.2	7.8	18.7	43.8	1.2	1.3	0.2	2.2	7.6	0.39
SC12#17	11.4	5.8	28.0	43.3	0.3	2.8	0.3	3.4	4.8	0.56
SC12#33	15.5	7.2	22.8	41.8	0.5	2.1	0.4	3.4	6.3	0.48
SC12#71	15.8	7.1	18.0	45.7	0.9	2.1	0.4	3.5	6.4	0.35
SC12#129	12.1	6.6	18.3	49.5	0.6	2.4	0.3	3.5	6.6	0.33
Wild strain	19.3	8.1	11.3	10.3	—	2.9	4.3	31.8	11.9	0.23

[a] Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω6, linoleic acid; 18:3ω3, α-linolenic acid; 18:3ω6, γ-linolenic acid; 20:3ω6, dihomo-γ-linolenic acid; 20:4ω6, arachidonic acid.

[b] Δ12DS index, ratio of 18:1ω9/(18:2ω6 + 18:3ω6 + 20:3ω6 + 20:4ω6) in total mycelial fatty acids.

[c] "—", not detected.

Table 3 Fatty acid compositions of *M. alpina* JT-180 as the host strain, the derived transformants, JT-6Ii strains, and the wild strain.

Strain	Fatty acid composition (%) [a]								Δ6DS index [c]
	16:0	18:0	18:1ω9	18:2ω9	20:2ω9	20:3ω9	ω6 fatty acids [b]	Others	
JT-180 (host)	8.8	8.9	51.5	8.2	1.6	6.9	— [d]	14.0	3.08
JT-6Ii#02	9.0	8.0	66.2	3.6	0.8	2.3	—	10.1	9.84
JT-6Ii#11	9.4	6.2	68.0	4.5	0.7	1.3	—	10.0	10.39
JT-6Ii#12	7.0	12.0	67.6	0.7	0.7	0.6	—	11.4	33.77
JT-6Ii#14	8.8	8.0	59.8	8.6	1.5	4.3	—	8.9	4.14
Wild strain	19.3	8.1	11.3	—	—	—	49.3	11.9	N.C. [e]

[a] Abbreviations: 16:0, palmitic acid; 18:0, stearic acid; 18:1ω9, oleic acid; 18:2ω9, ω9 octadecadienoic acid; 20:2ω9, ω9 eicosadienoic acid; 20:3ω9, Mead acid.

[b] ω6 fatty acids, sum of 18:2ω6 (linoleic acid), 18:3ω6 (γ-linolenic acid), 20:3ω6 (dihomo-γ-linolenic acid), and 20:4ω6 (arachidonic

acid).

[c] Δ6DS index, ratio of 18:1ω9/(18:2ω9 + 20:2ω9 + 20:3ω9) in total mycelial fatty acids.

[d] "—", not detected.

[e] N.C., not calculated.

Table 4 Effect of cultivation time on fatty acid productivity and growth of *M. alpina* transformants, SC12#129 and JT-6Ii#12, in GY medium.

Strain	Cultivation time (days)	Fatty acid composition (%) [a]										Total fatty acid (g/L)	Dry cell weight (g/L)
		18:1ω9	18:2ω9	20:1ω9	20:2ω9	20:3ω9	18:2ω6	18:3ω6	20:3ω6	20:4ω6	Others		
SC12#129	3	14.9	— [b]	—	—	—	55.3	2.1	—	2.5	25.2	0.43	2.42
	5	14.3	0.3	0.6	0.6	—	43.3	1.1	0.3	1.8	37.6	3.32	10.29
	7	11.0	0.3	0.7	0.7	—	46.4	0.9	0.1	2.3	37.5	2.92	9.38
	10	12.9	—	0.4	0.9	—	47.3	1.5	0.3	2.1	34.5	1.90	7.63
JT-6Ii#12	3	81.8	4.3	0.1	1.8	1.6	—	—	—	—	10.3	0.66	2.45
	5	71.6	—	0.8	3.0	1.4	—	—	—	—	23.2	3.85	7.82
	7	69.5	1.6	1.0	3.6	1.8	—	—	—	—	22.5	2.68	11.15
	10	67.7	2.2	1.5	5.2	3.3	—	—	—	—	20.2	2.42	9.53

[a] Abbreviations: 18:1ω9, oleic acid; 18:2ω9, ω9 octadecadienoic acid; 20:2ω9, ω9 eicosadienoic acid; 20:3ω9, Mead acid; 18:2ω6, linoleic acid; 18:3ω6, γ-linolenic acid; 20:3ω6, dihomo-γ-linolenic acid; 20:4ω6, arachidonic acid.

[b] "—", not detected.

Highlights

1. Transformants that are excellent in either linoleic or oleic acid productivity were obtained by molecular breeding of oleaginous filamentous fungus *M. alpina*.
2. The production of linoleic and oleic acids in these transformants reached 3.32 and 3.85 g/L, respectively, on the 5th day.
3. The amount of *Δ6II* RNA in the *Δ6I* RNAi-treated strain increased to 8 times that in the wild strain.