Improved γ-linolenic acid production in *Mucor circinelloides* by homologous overexpressing of delta-12 and delta-6 desaturases

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**Abstract**

**Background:** γ-Linolenic acid (GLA) is important because of its nutritional value and medicinal applications. Although the biosynthetic pathways of some plant and microbial GLA have been deciphered, current understanding of the correlation between desaturases and GLA synthesis in oleaginous fungi is incomplete. In previous work, we found that a large amount of oleic acid (OA) had not been converted to linoleic acid (LA) or GLA in *Mucor circinelloides* CBS 277.49, which may be due to inadequate activities of the delta-12 or delta-6 desaturases, and thus leading to the accumulation of OA and LA. Thus, it is necessary to explore the main contributing factor during the process of GLA biosynthesis in *M. circinelloides*.

**Results:** To enhance GLA production in *M. circinelloides*, homologous overexpression of delta-12 and two delta-6 desaturases (named delta-6-1 and delta-6-2, respectively) were analyzed. When delta-6 desaturase were overexpressed in *M. circinelloides*, up to 43% GLA was produced in the total fatty acids, and the yield of GLA reached 180 mg/l, which were, respectively, 38 and 33% higher than the control strain.

**Conclusion:** These findings revealed that delta-6 desaturase (especially for delta-6-1 desaturase) plays an important role in GLA synthesis by *M. circinelloides*. The strain overexpressing delta-6-1 desaturase may have potential application in microbial GLA production.

**Keywords:** Delta-6 desaturase, Delta-12 desaturase, GLA production, Homologous overexpression, *Mucor circinelloides*

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**Background**

γ-Linolenic acid (GLA, 18:3, delta-6,9,12) is an important n-6 polyunsaturated fatty acid (PUFA) that has many health and medicinal effects for prevention and treatment of inflammatory disorders, diabetes, cardiovascular disorders, cancers, and some other diseases [1–4]. The traditional sources of GLA are from a relatively small number of plant seeds, including evening primrose (*Oenothera biennis* L.), borage (*Borago officinalis* L.) and blackcurrant (*Ribes nigrum* L.) [5–7] where the content of GLA may be up to 22% of the total fatty acids. However, GLA production from plant seeds is easily influenced by region, climate and seasons, resulting in variable quantities and qualities of the oils. Genetically-engineered safflower seeds are now available in which GLA may now be up to 70% of the total fatty acids [8, 9] but, because of the uncertainties surrounding the acceptability of genetically modified (GM) materials as nutraceuticals, there is continued interest in developing microbial oils over conventional sources. Thus, a promising alternative source comes from oleaginous microorganisms with high GLA contents [5, 10–12].
Generally in plant and microbial cells, the biosynthesis of PUFA derives from saturated stearic acid (18:0), which is first converted by delta-9 desaturase to yield oleic acid (OA, 18:1, delta-9). Then, delta-12 desaturase converts OA to linoleic acid (LA, 18:2 delta-9, 12). Subsequently, GLA is synthesized by introducing a double bond into LA using a delta-6 desaturase. Thus, three desaturases, the delta-9, delta-12 and delta-6, accomplish the desaturation processes in GLA synthesis [5, 13, 14]. Genes coding for those desaturases have been cloned from diverse organisms ranging from prokaryotes to higher eukaryotes, and overexpressed in several hosts, including microalgae, yeasts and plants [8, 13–16]. However, few researches on homologous expression of fungal desaturases have been reported.

Mucor circinelloides, a typical oleaginous filamentous fungus, has been widely used to investigate GLA production since the 1980s [12, 17]. Previously, we found that the proportion of OA, LA and GLA in total fatty acids of M. circinelloides were 38, 11, and 19%, respectively [18, 19]. This suggested that a large amount of OA had not been converted to LA or GLA, which may be due to inadequate activities of the delta-12 or delta-6 desaturases, and thus leading to the accumulation of OA and LA. Thus, it is necessary to explore the main contributing factor during the process of GLA biosynthesis in M. circinelloides. In this study, we report the cloning and homologous overexpression of the delta-12 and delta-6 desaturases derived from M. circinelloides itself with the aim of determining the correlation between fatty acid desaturases and GLA biosynthesis in oleaginous fungi.

Results and discussion
Construction of delta-12 and delta-6 desaturase genes overexpressing strains
Based on the genomic data of M. circinelloides CBS 277.49, we found one delta-12 desaturase gene, named D12 (JGI accession number ID155268, 1351 bp), and two delta-6 (delta-6-1 and delta-6-2) desaturase genes, named D61 (JGI accession number ID37214, 1738 bp) and D62 (JGI accession number ID105367, 1575 bp). To determine if the delta-12 and delta-6 desaturases were involved in fatty acid accumulation, overexpressing strains of these genes were generated. The genes, D12, D61 and D62, were cloned from the genome of M. circinelloides and subsequently inserted into the expression vector, pMAT1552, that contains the strong zrt1 promoter, the pyrG gene as a selectable marker and flanking sequences corresponding to regions surrounding the carotenogenic carRP gene to allow its chromosomal integration by homologous recombination (Fig. 1a). The D12, D61 and D62 overexpressing plasmids, pMAT1552-D12, pMAT1552-D61 and pMAT1552-D62, were transformed into the recipient strain MU402 and transformation and selection of the colonies were carried out as described by [20]. Three overexpressing and control transformants were selected at random and then confirmed by PCR analysis. Amplification was carried out using a primer pair (P3-F/R) that amplified the desaturase gene and 1000 bp sequences of the plasmid pMAT1552. Thus, the corresponding D12 (2736 bp), D61 (2351 bp) and D62 (2575 bp) gene fragments were amplified and, as expected, resulted in transformants genomes, whereas no desaturase gene fragment was amplified in the control strain (Mc-1552 carrying the vector pMAT1552) and wild-type strain (M. circinelloides CBS 277.49) as shown in Fig. 1b. The PCR amplification results suggest that the D12, D61 and D62 genes had been respectively integrated into the genome of the fungus in the transformants.

Overexpressing strains were grown in complete medium for 3–4 days in 1 l baffled flasks with 200 ml K&R medium. Compared with the control strain, Mc-1552, Mc-D12 and Mc-D61 had a similar biomass (Fig. 2A) and lipid content (Fig. 2B), suggesting that the knockout of CarRP gene and overexpression of delta-12 or delta-6-1 desaturase did not significantly affect the growth or lipid synthesis of the strains. However, Mc-D62 showed a large decrease in biomass and lipid content (Fig. 2). This indicated that the overexpression of delta-6-2 desaturase has an impact on growth and fatty acid synthesis. The possible explanation is that transformation procedure is stressful for the cells and this may cause mutations. In addition, based on the best growth and lipid production of different transformants, the strains (Mc-D12-3, Mc-D61-1, Mc-D62-2, Mc-1552-3) were selected for further analysis. The code number of the transformant is no longer marked below.

Expression levels of delta-12 and delta-6 desaturase genes in the overexpressing strains
Real-time quantitative PCR were carried out to analyze the mRNA level of D12, D61 and D62 in the selected overexpression strains at 24, 48 and 72 h of growth in 2 l fermenter with K&R medium (Fig. 3). The mRNA expression level of D12, D61 and D62 was increased, respectively, by 8-, 7- and 3.6-fold at 24 h in overexpressing strains compared to the control, although it decreased with the incubation time. The fact that D12, D61 and D62 mRNA were maintained at elevated levels throughout the whole culture time confirmed that D12, D61 and D62 were overexpressed in the recombinant strains.

Lipid accumulation in the delta-12 and delta-6 desaturase overexpressing strains
Growth and lipid accumulation in D12, D61 and D62 overexpressing strains were compared to the control
strain over 72 h growth in a 2 l fermenter. Overall, the growth patterns of different strains were similar (Fig. 4a): the mycelia grew rapidly during the first 48 h after which the growth entered into stationary phase, and then after 72 h, growth ceased. Except for Mc-D62, other overexpressing strains, Mc-D12 and Mc-D61, had a similar amount of biomass compared with the control strain, Mc-1552. Manipulation of the expression of delta-12 and delta-6 desaturase genes had no significantly effect on the total lipid content (Fig. 4b), whereas it had an obvious influence on the fatty acid composition (Table 1).

When compared with the control strain, Mc-1552, OA (18:1) decreased by 10% while LA (18:2) increased by 41% in Mc-D12 (Table 1), suggesting that the overexpression of delta-12 desaturase leads to the conversion of more OA to LA which should then become the substrate for GLA synthesis. However, the content of GLA in Mc-D12 decreased rather than increasing compared to the control, which suggests that delta-12 desaturase is not the rate-limiting step in GLA biosynthesis. Kelder et al. [21] has found that the n-6 series of fatty acids in rat L cells showed a downward trend upon overexpression of Mortierella alpina delta-12 desaturase which is similar to what was seen in this study. The reason for this result was likely that the increase in the membrane fluidity with the increase of LA content by delta-12
desaturase expression might cause the suppression of delta-6 desaturase.

As opposed to the overexpression of delta-12 desaturase, compared to the control strain Mc-1552, the GLA content of Mc-D61 and Mc-D62 were, respectively, increased by 38 and 19% whilst the other fatty acids essentially remained unchanged thereby indicating that delta-6 desaturase probably plays an important role in GLA synthesis. However, the increment of GLA increase for Mc-D62 was about half of that for Mc-D61, and with a lower biomass and total lipid content. This indicated that the delta-6-2 desaturase may play a supplementing role for delta-6-1 desaturase in the synthesis of GLA. In addition, the D61 overexpressing strain, Mc-D61, finally reached 180 mg GLA/l, which was 33% greater than that of the control strain Mc-1552. Notably, the proportion of GLA in total lipid content for Mc-D61 reached 43%, was much higher than that (18–19%) in the strain of M. circinelloides used for the commercial production of GLA in the 1980s [12]. This indicates that the strain Mc-D61 may have great industrial prospects in future if the cell total lipid content can be improved.

Surprisingly in our study, the effects of these two delta-6 fatty acid desaturases were quite different. The two delta-6 desaturases shared only 22.8% homology and their phylogenetic positions were far apart from each other, which is in accordance with the delta-6 genes in M. circinelloides HUT1121 [22]. Similarly, a low degree of homology particularly for fungal delta-6 desaturases is evident when comparing the genes from Mortierella [23] and Mucor [24]. Although the sequence of delta-6-2 desaturase from M. circinelloides was close to the delta-6 desaturase of the high GLA-producing borage (B. officinalis), it is not efficient in GLA synthesis in M. circinelloides. The physiological functions of delta-6-2 desaturase need further investigation.

**Co-expression of delta-12 and delta-6 desaturase genes**

To further explore the combined effect of delta-12 and delta-6 desaturases on GLA production, co-expression of D12 and D61 were constructed in M. circinelloides. The D12 gene was inserted into the expression vector pLEU4 containing LeuA gene and then transformed into the recipient strain MU402 with the plasmid, pMAT-D61,
overexpressing D61 gene. Finally, a recombinant strain, Mc-D1261, co-expressing D12 and D61 was obtained. The mRNA expression levels of D12 and D61 were significantly increased by 8- and 6.5-fold in the co-expressing strain Mc-D1261 compared to the control strain Mc-1552, which was similar with the result of its single overexpression.

Growth and lipid accumulation in the co-expression strain, Mc-D1261, was examined in a 2 l fermenter using K&R medium at 72 h (Fig. 5A). Mc-D1261 had a slightly decrease in biomass compared with the control strain Mc-1552 but no significant change in its total lipid content. Compared with the control strain Mc-1552, the OA content of Mc-D1261 was decreased by 12%, whereas the LA and GLA content of Mc-D1261 were increased by 26.5 and 15.5%, respectively. Unexpectedly, the increase of GLA by co-expression of D12 and D61 was lower than that achieved by overexpression of the single D61. The reason for this might be that delta-12 desaturase is not rate-limiting in LA production and also, as same as the overexpression of single D12, the expression of delta-12 desaturase may have restrained the expression of delta-6 desaturase due to the changes of membrane fluidity. Further study for the introduction of a mutation that cancels the repression of delta-12 desaturase would be needed to solve this problem.

Conclusion
Delta-12 and delta-6 fatty acid desaturases were overexpressed by homologous recombination in the oleaginous fungus M. circinelloides CBS 277.49 to enhance the production of GLA. Delta-6 desaturase overexpressing M. circinelloides generated in this study had up to 43% GLA in the final lipid with a final yield of 180 mg GLA/l. These values are, respectively, 38 and 33% higher than those in the control strain. Thus, we can conclude that delta-6 desaturase (especially for delta-6-1 desaturase) plays an important role in GLA synthesis by M. circinelloides. The D61 overexpressing strain, Mc-D61, may have potential application in microbial GLA production.

Methods
Strains, plasmids and culture conditions
Mucor circinelloides CBS 277.49 was used as the source of delta-12 desaturase gene, D12, and two delta-6 desaturase genes, D61 and D62. The leucine and uracil double auxotroph strain, MU402 [25], was used as the recipient strain in transformation experiments to overexpress the desaturase genes. Escherichia coli strain Top 10 was used for all cloning experiments and grown in lysogeny broth at 37 °C. Plasmids pMAT1552 and pLEU4 [26] were used as the cloning and expression vectors. Cultures were grown at 26 °C in YPG [27]. Media were supplemented with leucine (20 μg/ml) or uridine (200 μg/ml) when required. The pH was adjusted to 4.5 and 3 for mycelia and colonial growth, respectively.

The recombinant strains Mc-D12 (D12 overexpression strain), Mc-D61 (D61 overexpression strain), Mc-D62 (D62 overexpression strain), Mc-D1261 (D12 and D61 coexpression strain), and Mc-1552 (strain carrying the vector pMAT1552) as the control were initially cultivated in 500 ml flasks containing 100 ml K&R medium [28] for 24 h at 30 °C with shaking at 150 rpm and then inoculated at 10% (v/v) into a 2 l fermenter (BioFlo/CelliGen115, New Brunswick Scientific, Edison, NJ, USA) containing 1.5 l K&R medium modified to contain 80 g glucose/l. Fermenters were held at 30 °C, stirred at 500 rpm, with aeration at 0.5 vvm. The pH was maintained at 4.5 by automatic addition of 1 M NaOH or 1 M
HCl, and medium was supplied with leucine (20 μg/ml) or uracil (200 μg/ml) when required.

**Plasmids construction**

The vector, pMAT1552, which was used for construction of *D12, D61* and *D62* overexpression plasmids, contains only a *XhoI* restriction site between its promoter and homology arm *CarRP* sequence. To facilitate subsequent cloning screening, a *CarRP* sequence was amplified by using primers Car-F/R (Additional file 1: Table S1), with another *XbaI* restriction site inserted between the *XhoI* and *SacII* sites of original *CarRP* fragment. The amplification product was isolated and ligated into the vector pMD19T-simple to generate an intermediate vector pMD19T-*CarRP*.

The genes, *D12, D61* and *D62*, were isolated by PCR amplification from the genome of *M. circinelloides* CBS 277.49 with separate primers D12-F/R, D61-F/R and D62-F/R (Additional file 1: Table S1) that introduced the *XhoI* and *XbaI* restriction sites, respectively, at the 5’- and 3’-ends of desaturase genes. The products were digested by *XhoI* and *XbaI* and ligated into the similarly digested intermediate vector pMD19T-*CarRP*. The ligation mixture was used to transform chemically competent *E. coli* Top 10 cells. The plasmids isolated from these transformants were verified by restriction analysis and the gene sequences were confirmed by DNA sequencing. The plasmids with the correct sequences of *D12, D61* and *D62* were named pMD19T-*CarRP*-D12, pMD19T-*CarRP*-D61 and pMD19T-*CarRP*-D62, respectively. Because *D12* and *D61* genes contain one internal *XhoI* site, site-directed mutagenesis was performed to eliminate these sites while keeping the encoded amino acid unchanged. The resulting plasmids were digested with *XhoI* and *EcoRI* and ligated into the similarly digested expression vector pMAT1552. The ligation mixture was used to transform chemically competent *E. coli* Top 10 cells. The plasmids isolated from these transformants were verified by restriction analysis and the gene sequences were confirmed again by DNA sequencing.

### Table 1 The fatty acid composition in DELTA-12 and DELTA-6 desaturase genes overexpressing strains

| Strains     | Time (h) | Fatty acid composition (relative %, w/w)* | 16:0   | 18:0   | 18:1   | 18:2 (LA) | 18:3 (GLA) |
|-------------|----------|------------------------------------------|--------|--------|--------|-----------|------------|
| Mc-1552     | 24       | 23.1 ± 0.5                               | 4.0 ± 2.0 | 23.2 ± 8.7 | 17.4 ± 5.5 | 30.0 ± 7.1 |
|             | 36       | 23.8 ± 0.5                               | 3.6 ± 0.1 | 26.2 ± 0.1 | 14.8 ± 1.6 | 31.0 ± 1.2 |
|             | 48       | 21.1 ± 0.2                               | 2.7 ± 1.3 | 28.3 ± 2.6 | 14.5 ± 1.3 | 31.1 ± 2.6 |
|             | 60       | 18.2 ± 0.9                               | 2.2 ± 0.6 | 24.6 ± 0.2 | 14.3 ± 1.8 | 31.2 ± 0.1 |
|             | 72       | 17.1 ± 0.6                               | 2.0 ± 0.5 | 24.0 ± 3.6 | 14.3 ± 3.2 | 31.2 ± 0.4 |
| Mc-D12      | 24       | 23.0 ± 8.4                               | 3.0 ± 0.9 | 12.1 ± 0.8 | 24.0 ± 1.4 | 28.2 ± 1.7 |
|             | 36       | 21.5 ± 3.1                               | 2.7 ± 0.1 | 20.6 ± 3.0 | 21.5 ± 1.8 | 28.1 ± 2.3 |
|             | 48       | 20.4 ± 1.0                               | 2.2 ± 0.3 | 22.0 ± 2.0 | 20.8 ± 1.2 | 28.6 ± 2.7 |
|             | 60       | 19.1 ± 0.1                               | 1.8 ± 0.1 | 22.4 ± 0.3 | 20.6 ± 0.1 | 28.6 ± 0.4 |
|             | 72       | 17.7 ± 0.2                               | 1.6 ± 0.3 | 21.8 ± 1.3 | 20.1 ± 2.1 | 29.0 ± 0.7 |
| Mc-D61      | 24       | 22.1 ± 0.2                               | ND      | 16.5 ± 0.1 | 17.5 ± 0.1 | 41.9 ± 0.4 |
|             | 36       | 18.0 ± 0.1                               | ND      | 22.2 ± 0.1 | 12.3 ± 0.0 | 42.2 ± 0.1 |
|             | 48       | 16.8 ± 0.2                               | 1.4 ± 0.0 | 22.1 ± 0.2 | 13.3 ± 0.1 | 42.6 ± 0.0 |
|             | 60       | 16.2 ± 0.1                               | 1.3 ± 0.3 | 22.1 ± 0.2 | 14.4 ± 0.2 | 42.8 ± 0.2 |
|             | 72       | 14.9 ± 0.1                               | ND      | 22.1 ± 0.2 | 14.2 ± 0.1 | 43.0 ± 0.6 |
| Mc-D62      | 24       | 22.5 ± 0.1                               | 4.2 ± 0.0 | 19.3 ± 0.1 | 16.6 ± 0.1 | 36.6 ± 0.2 |
|             | 36       | 22.0 ± 0.5                               | 3.6 ± 0.3 | 21.5 ± 0.2 | 15.4 ± 0.1 | 36.5 ± 0.1 |
|             | 48       | 21.3 ± 0.1                               | 3.2 ± 0.1 | 22.1 ± 0.2 | 14.4 ± 0.1 | 37.0 ± 0.1 |
|             | 60       | 20.2 ± 0.2                               | 3.0 ± 0.1 | 22.4 ± 0.0 | 13.6 ± 0.0 | 37.0 ± 0.1 |
|             | 72       | 18.0 ± 0.1                               | 2.7 ± 0.3 | 22.8 ± 0.2 | 13.3 ± 0.1 | 37.1 ± 0.6 |

ND not detected

* Strains were cultured in a 2 l fermenter with the 1.5 l Kendrick medium during 72 h. The fatty acid composition were displayed at different point times. The values are means ± standard deviations of two independent experiments.
The plasmids with the correct sequences of D12, D61, and D62 were named pMAT-D12, pMAT-D61, and pMAT-D62, respectively. Transformation was carried out by electroporation-mediated procedure as described previously [29].

To achieve the co-expression of D12 and D61, another D12 gene expression plasmid was constructed using the vector pLEU4. The D12 gene was isolated by PCR amplification from the plasmid pMAT-D12 and then cloned into the expression vector pLEU4 with primers D12'-F/R (Additional file 1: Table S1). The amplification product was digested by Kas I and Sal I and ligated into the similarly digested expression vector pLEU4. The ligation mixture was used to transform chemically competent E. coli Top 10 cells. The plasmids isolated from these transformants were verified by restriction analysis and the gene sequences were confirmed by DNA sequencing. The plasmids with the correct sequences of D12 were named pLEU-D12. Then, the plasmids pLEU-D12 and pMAT-D61 were simultaneously transformed into competent MU402 cells.

**Gene expression and RT-qPCR analysis**

D12, D61 and D62 overexpressing transformants were cultivated in YNB for 3 days before extracting the genomic DNA. The mycelia was harvested by suction filtration and washed twice with sterile water. The genomic DNA was extracted by following the protocols of the DNA Quick Plant System (Tiangen). PCR amplification was used to verify whether D12, D61 or D62 had integrated into the genome of M. circinelloides with the universal primers P3-F/R (Additional file 1: Table S1).

For reverse transcription-quantitative PCR (RT-qPCR) analysis, strains were grown in a 2 l fermenter with K&R medium, and the mycelium was harvested at 24, 48, and 72 h. Total RNA of M. circinelloides was extracted with Trizol after grinding under liquid N2 and reverse-transcribed using the Prime ScriptRT reagent kit (Takara) according to the manufacturer’s instructions. RT-qPCR was performed using primers (Additional file 1: Table S1) on CFX Connect Real-Time System (Bio-Rad) with iTaq Universal SYBR Green PCR Supermix (Bio-Rad) according to the manufacturer’s instruction. The mRNA expression level was normalized to levels of 18S rRNA mRNA, and the results were expressed as relative expression levels. The data were quantified by the method of $2^{-\Delta\Delta Ct}$.

**Determination of cell dry weight and fatty acid analysis**

Biomass was harvested on a weighed filter paper by filtration through a Buchner funnel under reduced pressure and washed three times with distilled water, frozen overnight at $-80^\circ$C and then freeze-dried. The weight of the biomass was determined gravimetrically.

Biomass was collected by filtration and dried by lyophilizer. 20 mg dry weight was taken for cell fatty acids extraction. Pentadecanoic acid (15:0) was added into the freeze-dried cells as an internal standard. The total fatty acids were extracted with chloroform/methanol (2:1, v/v) and methylated with 10% (v/v) methanolic HCl at 60 °C for 3 h. The resultant fatty acid methyl esters were extracted with n-hexane and were analyzed by GC equipped a 30 m × 0.32 mm DB-Waxetr column with 0.25 µm film thickness. The program was as follows: 120 °C for 3 min, ramp to 200 °C at 5 °C per min, ramp to 220 °C at 4 °C per min, hold 2 min.

**Statistical analysis**

A statistical analysis was carried out using SPSS 16.0 for Windows (SPSS Inc. Chicago, IL). The mean values and the standard error of the mean were calculated from the data obtained from three independent experiments. The
differences between the means of the test were evaluated by Student’s t test, and P < 0.05 was considered as signifi-
cantly different.

Additional file

Additional file 1: Table S1. Primer sequences used in this study.

Abbreviations

GLA: γ-linolenic acid; PUFA: polyunsaturated fatty acid; GM: genetically modified;
OA: oleic acid; LA: linoleic acid; GC: gas chromatography.

Authors’ contributions

YZ and XL carried out the experiments and drafted the manuscript. HZ, VG, YS
and CR participated in the experimental design and reviewed the manuscript.
YS and CR conceived the study and reviewed the final manuscript. All authors
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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published
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