Effects of Methylenetetrahydrofolate Dehydrogenase Overexpression on Lipid Synthesis in Mortierella alpina (Postprint)

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Abstract

Mortierella alpina is a filamentous fungus rich in polyunsaturated fatty acids; however, the source of NADPH in its lipid synthesis process remains poorly understood. In this study, using Mortierella alpina (uracil auxotroph) as the parental strain, we investigated the effect of methylenetetrahydrofolate dehydrogenase (MTHFD1) on lipid synthesis in Mortierella alpina. First, the overexpression vector pBIG2-ura5s-MTHFD1 was constructed, and the binary expression vector was transformed into Mortierella alpina CCFM501 using an Agrobacterium tumefaciens-mediated fungal transformation method, followed by selection on SC-CS selection medium plates to obtain the genetically stable MTHFD1-overexpressing strain (MA-MTHFD1). Second, genomic DNA was extracted from the MA-MTHFD1 strain for PCR verification, and in combination with qPCR analysis, it was demonstrated that the MTHFD1 gene was successfully overexpressed in Mortierella alpina. Finally, by analyzing the fatty acid content, NADPH content, and transcription levels of related genes in the NADPH synthesis pathway in MA-MTHFD1, the effect of MTHFD1 overexpression on lipid synthesis was investigated. The results demonstrated that overexpressing the MTHFD1 gene could enhance the lipid synthesis capacity of Mortierella alpina. Compared with the prototrophic Mortierella alpina, the fatty acid content in the MA-MTHFD1 strain increased by 40.13%, NADPH content increased by 26.45%, and the transcription levels of other related genes in the NADPH synthesis pathway, malic enzyme (ME) and isocitrate dehydrogenase (IDH), were also upregulated. These results indicate that the MTHFD1 gene plays a crucial role in the formation of reducing power for lipid synthesis in Mortierella alpina. This study provides a theoretical basis for elucidating the NADPH source in Mortierella alpina and for further investigating the lipid synthesis mechanism, thereby enabling molecular-level modification of its intracellular fatty acid metabolic pathway.

Full Text

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Abstract

Mortierella alpina is a filamentous fungus rich in polyunsaturated fatty acids, yet the sources of NADPH required for its lipid synthesis remain incompletely understood. This study investigated the role of methylenetetrahydrofolate dehydrogenase (MTHFD1) in lipid synthesis in *M. alpina* using a uracil auxotrophic strain as the parent. First, the overexpression vector pBIG2-ura5s-MTHFD1 was constructed and transformed into *M. alpina* CCFM501 via *Agrobacterium tumefaciens*-mediated transformation (ATMT). Transformants were selected on SC-CS plates to obtain a genetically stable MTHFD1-overexpressing strain (MA-MTHFD1). PCR verification of the MA-MTHFD1 genome, combined with qPCR analysis, confirmed successful overexpression of MTHFD1. Finally, fatty acid content, NADPH levels, and transcription of NADPH synthesis pathway genes were analyzed to assess the impact of MTHFD1 overexpression on lipid synthesis. The results demonstrated that MTHFD1 overexpression enhanced lipid synthesis capacity in *M. alpina*. Compared to the prototrophic strain, MA-MTHFD1 exhibited a 40.13% increase in fatty acid content and a 26.45% increase in NADPH content. Additionally, transcription of other NADPH synthesis genes, malic enzyme (ME) and isocitrate dehydrogenase (IDH), was upregulated. These findings indicate that MTHFD1 plays a crucial role in generating reducing power for lipid synthesis in *M. alpina*, providing a theoretical basis for elucidating NADPH sources and engineering fatty acid metabolic pathways at the molecular level.

Keywords: methylenetetrahydrofolate dehydrogenase; *Mortierella alpina*; lipid synthesis; NADPH

Introduction

Mortierella alpina is an oleaginous filamentous fungus that produces various polyunsaturated fatty acids (PUFAs) [1,2]. PUFAs are essential fatty acids with important physiological functions and can be used to prevent and treat chronic diseases [3,4]. Current research has focused on optimizing culture media, strain breeding, and functional validation of enzymes in the lipid synthesis

pathway [5,6], but the underlying mechanisms of lipid synthesis remain poorly understood.

NADPH serves as the reducing power for lipid synthesis [7]. Studies have shown that in *M. alpina*, glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), and malic enzyme (ME) can provide NADPH for lipid synthesis [8-10]. Our laboratory' s whole-genome sequencing analysis revealed a complete folate metabolic pathway in *M. alpina* [11]. In this pathway, 5,10-methylenetetrahydrofolate is oxidized to 5,10-methenyltetrahydrofolate by methylenetetrahydrofolate dehydrogenase (MTHFD1), with concomitant NADPH generation (Figure 1 [Figure 1: see original paper]) [12-14]. Furthermore, Wang et al. [15] reported that folate metabolism inhibitors methotrexate (MTX) and trimethoprim (TMP) impaired the adipogenic differentiation of OP9 mouse bone marrow stromal cells. We therefore hypothesized that MTHFD1 in the folate metabolism pathway of *M. alpina* could also generate NADPH and play an important role in lipid synthesis. This study overexpressed MTHFD1 in *M. alpina* to further elucidate its function in lipid synthesis, providing a theoretical basis for molecular-level modification of fatty acid metabolic pathways.

Figure 1 The reaction of MTHFD1 in *M. alpina*

Materials and Methods

1.1 Materials and Culture Media

Mortierella alpina ATCC 32222 was purchased from the American Type Culture Collection. The uracil auxotrophic strain CCFM 501, *Escherichia coli* DH5 , the genetic manipulation vector pBIG2-ura5s-ITs, and *Agrobacterium tumefaciens* CCFM 834 were obtained from our laboratory' s culture collection. Trizol (Invitrogen, USA), PrimeScript RT reagent kit (TaKaRa, Japan), Taq DNA polymerase (Cwbiotech, China), KOD plus high-fidelity DNA polymerase (TOYOBO, Japan), restriction endonucleases (NEB, USA), T4 DNA ligase (Promega, USA), iTaq Universal SYBR Green Supermix (Bio-Rad, USA), PCR product purification kit, plasmid extraction kit, gel extraction kit (Fermentas, USA), Biospin Fungus Genomic DNA Extraction Kit (BioFlux), and other analytical grade reagents were used.

Culture media compositions are listed in Table 1 .

Table 1 Culture media used in this study

Medium (1 L)	Composition
LB liquid medium	10 g tryptone, 10 g NaCl, 5 g yeast extract (add 20 g agar for solid medium)

Medium (1 L)	Composition
SOC recovery medium	20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 3.6 g glucose, 0.95 g MgCl , 0.186 g KCl
YEP liquid medium	10 g yeast extract, 10 g tryptone, 5 g NaCl (add 20 g agar for solid medium)
MM liquid medium	1.74 g K HPO , 1.37 g KH PO , 0.078 g CaCl , 0.0025 g FeSO · 7H O, 0.146 g NaCl, 0.49 g MgSO · 7H O, 0.53 g (NH) SO , 7.8 g MES, 1.8 g glucose, 5 g glycerol, pH 6.8
IM liquid medium	MM liquid medium + 0.0392 g acetosyringone (AS)
SC solid medium	20 g glucose, 5 g yeast nitrogen base without amino acids and ammonium sulfate, 1.7 g (NH) SO , 60 mg isoleucine, 60 mg phenylalanine, 60 mg leucine, 50 mg threonine, 40 mg lysine, 30 mg tyrosine, 20 mg arginine, 20 mg adenine, 20 mg histidine, 10 mg methionine, 20 g agar, pH 6.8
SC-CS solid medium	SC solid medium + 0.1 g spectinomycin and 0.1 g cefotaxime sodium
Broth liquid medium	20 g glucose, 5 g yeast extract, 1 g K HPO , 0.25 g MgSO · 7H O, 10 g KNO , pH 6.0
GY solid medium	20 g glucose, 10 g yeast extract, 2 g KNO , 1 g NaH PO , 3 g MgSO · 7H O, 20 g agar, pH 6.8
GY-U medium	GY solid medium + 0.1 g uracil

1.2 Instruments and Equipment

PCR thermal cycler (ABI 9700, USA), GC-2000 gas chromatograph and UV-2450 spectrophotometer (Shimadzu, Japan), QSC-12T nitrogen evaporator (Quandao, Shanghai), gel imaging system (UVP CDS-8000, USA), NanoDrop ND-1000 microspectrophotometer (USA), electroporator (Eppendorf, Germany), freeze dryer (LABCONCO, USA), and real-time PCR system (Bio-Rad, USA).

1.3 Experimental Methods

1.3.1 Culture of *M. alpina* *M. alpina* was cultured in Broth fermentation medium for 7 days, after which mycelia were harvested. A portion was lyophilized for dry weight measurement and fatty acid analysis, while the remainder was stored at -80°C for subsequent RNA extraction, gene transcription analysis, NADPH quantification, and fatty acid content determination [16].

1.3.2 Construction of Binary Expression Vector Total RNA was extracted from *M. alpina* using Trizol (Invitrogen) and reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa) to obtain cDNA. Based on the MTHFD1 gene sequence from *M. alpina* ATCC32222, primers were designed (Table 2) with KpnI and SacI restriction sites (underlined). The MTHFD1 gene was amplified from cDNA using KOD plus high-fidelity polymerase under the following conditions: 95°C for 30 s, 55°C for 30 s, 68°C for 1.5 min (30 cycles), and 68°C for 5 min. PCR products and digested fragments were purified using a PCR purification kit. The purified gene fragment was ligated to vector pBIG2-ura5s-ITs using T4 ligase at 4°C, then electroporated into *E. coli* DH5 . Positive transformants were screened by PCR and confirmed by sequencing using primers HisproF1 and TrpCR1 (Table 2), yielding the binary expression vector pBIG2-ura5s-MTHFD1. This plasmid was subsequently electroporated into *A. tumefaciens* CCFM 834.

Table 2 Primers used in this study

Primer	Sequence (5' →3')
MTHFD1-F	CGGGGTACCGCATGCCTGTGGCATATCAGAG
MTHFD1-R	CGAGCTCTTACATGATCTTGGTCATCGC
HisproF1	CACACACAAACCTCTCTCCCACT
TrpCR1	CAAATGAACGTATCTTATCGAGATCC
MTHFD1 (qPCR)	CGGCTACGCAAGGACAT
MTHFD1 (qPCR)	GCCACCATCGGGTTATTC
6PGD (qPCR)	AAGTTGCCTGTCCGCCATC
6PGD (qPCR)	TAGTGCCAGCCGTTCTCCTT
G6PD (qPCR)	CGTATGCTGGGTCTGGTTAGG
G6PD (qPCR)	AGAAGGCTAGGTCTCCCGATG
ME (qPCR)	CCTTGCAGGACCGTAACGAGA
ME (qPCR)	CCTGGAGCGACGATAAATGGA
IDH (qPCR)	CTCGTCCCTGGGTGGACAG
IDH (qPCR)	CCATCAGCGGGCGTAAAA
18S rDNA	CGTACTACCGATTGAATGGCTTAG
18S rDNA	CCTACGGAAACCTTGTTACGACT

Note: Underlined sequences indicate restriction enzyme sites.

1.3.3 *Agrobacterium tumefaciens*-Mediated Transformation of *M. alpina* CCFM501 ATMT was performed as previously described [16]. Briefly, *A. tumefaciens* CCFM 834 harboring pBIG2-ura5s-MTHFD1 was streaked on YEP solid medium containing 100 g/mL rifampicin and 100 g/mL kanamycin, then incubated inverted at 30°C for 1-2 days. A single colony was inoculated into 20 mL YEP liquid medium with antibiotics and cultured at 30°C, 200 rpm for 1-2 days. Then 200 L was transferred to MM liquid medium and cultured at 30°C, 200 rpm for 1-2 days, followed by transfer to 20 mL IM liquid medium to adjust OD to ~0.3. The culture was incubated at 30°C, 200 rpm until OD reached ~0.8. Spores of uracil-auxotrophic *M. alpina* CCFM 501 grown on GY-U slants for >1 month were harvested using 4-5 mL Broth-U medium, counted with a hemocytometer, and adjusted to 10 spores/ L. Equal volumes of *A. tumefaciens* culture and spore suspension were mixed, spread onto IM solid medium overlaid with sterile cellophane, and incubated at 16°C for 2 days followed by 23°C for 1 day. The cellophane was then transferred to SC-CS plates and incubated at 16°C for 2 days and then 23°C. Emerging colonies were transferred to fresh SC-CS plates using fine-tipped forceps and screened three times.

Genomic DNA from the stable transformant MA-MTHFD1 was extracted using the Biospin Fungus Genomic DNA Extraction Kit and verified by PCR using primers HisproF1 and TrpCR1 (Table 2) specific for the promoter and terminator.

1.3.4 Fatty Acid Extraction and Analysis Fatty acids were extracted and analyzed by GC-MS according to the method of Bligh and Dyer [17].

1.3.5 NADPH Extraction and Quantification NADPH was extracted using the NADP/NADPH Quantification Colorimetric Kit. Approximately 0.1 g of lyophilized mycelia was ground in liquid nitrogen, suspended in 1 mL extraction buffer, and centrifuged at 12,000 g for 10 min. The supernatant was transferred to a 1.5 mL tube and processed according to the manufacturer' s protocol.

1.3.6 RT-qPCR Analysis of Gene Transcription Total RNA from recombinant strains was reverse-transcribed using the PrimeScript RT reagent kit. RT-qPCR was performed on a CFX96 Real-Time PCR System using iTaq Universal SYBR Green Supermix [18]. The reaction system is shown in Table 3 . Cycling conditions were: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 55°C for 10 s. 18S rDNA served as the housekeeping gene. Relative gene expression was calculated using the 2^(-ΔΔCt) method, where ΔΔCt = ΔCt(sample) - ΔCt(control). Primer sequences are listed in Table 2.

Table 3 Real-time PCR system (20 L)

Component	Volume
Power SYBR® Green PCR Master Mix	10 L
Forward/Reverse primers	1 L each

Results

2.1 Construction of Overexpression Vector

Primers were designed based on the MTHFD1 gene sequence (Table 2) to amplify a 1200 bp fragment (Figure 2 [Figure 2: see original paper]). The purified PCR product was ligated into pBIG2-ura5s-ITs to generate pBIG2-ura5s-MTHFD1 (Figure 3 [Figure 3: see original paper]). Transformants were verified by PCR, yielding two bands corresponding to the ura5 (818 bp) and MTHFD1 (1364 bp, including 164 bp from the vector) expression cassettes (Figure 2). After sequencing confirmation, pBIG2-ura5s-MTHFD1 was electroporated into *A. tumefaciens* CCFM 834.

Figure 2 PCR cloning products of MTHFD1 (1), PCR identification of recombinant plasmid pBIG2-ura5s-MTHFD1 (2), and PCR identification of recombinant strain MA-MTHFD1 (3)

Figure 3 Schematic diagram of binary expression vector pBIG2-ura5s-MTHFD1 construction

2.2 Screening and Identification of Overexpression Strain

ATMT was used to transform *M. alpina* CCFM 501 with *A. tumefaciens* CCFM 834 harboring the binary vector. Stable overexpression strain MA-MTHFD1 was selected on SC-CS plates and maintained on GY slants. Genomic PCR verification using primers HisproF1 and TrpCR1 (Table 2) yielded two bands corresponding to ura5 (818 bp) and MTHFD1 (1364 bp) cassettes (Figure 3), confirming successful integration. qPCR analysis showed that MTHFD1 transcription increased by 88.98% compared to the prototrophic strain (Figure 4 [Figure 4: see original paper]), demonstrating successful overexpression.

Figure 4 Effects of MTHFD1 overexpression on transcript levels of NADPH-generating genes

2.3 Dry Cell Weight Analysis of Overexpression Strain

After 7 days in Broth medium, dry cell weights of the wild-type and MA-MTHFD1 strains were 8.54 g/L and 8.89 g/L, respectively (Figure 5 [Figure 5: see original paper]), with no significant difference. This indicates that MTHFD1 overexpression does not affect biomass accumulation or growth.

Figure 5 Effects of MTHFD1 overexpression on dry cell weight

2.4 Fatty Acid Content Analysis of Overexpression Strain

Fatty acid composition was determined as described in section 1.3.4 (Table 4 and Figure 6 [Figure 6: see original paper]). Total fatty acid content increased by 40.13% in MA-MTHFD1 compared to the wild type (Figure 6). Notably, arachidonic acid content increased by 91.12%, indicating that MTHFD1 overexpression enhances fatty acid unsaturation.

Table 4 Fatty acid content in *M. alpina*

Fatty acid composition (mg/mg DCW)	Total fatty acids (mg/mg DCW)	C16:0	C18:0	C18:1	C18:2	C18:3	C20:3	C20:4
M. alpina	0.178±	0.116±	0.305±	0.063±	0.036±	0.037±	0.228±	0.128±
MA-MTHFD1	0.137±	0.100±	0.081±	0.048±	0.039±	0.435±	0.350±	0.491±

Figure 6 Effects of MTHFD1 overexpression on relative fatty acid composition

2.5 NADPH Content Analysis of Overexpression Strain

NADPH content increased from 0.0379 mg/g to 0.0479 mg/g in MA-MTHFD1 (Figure 7 [Figure 7: see original paper]), representing a 26.38% increase. This demonstrates that MTHFD1 overexpression enhances NADPH synthesis capacity.

Figure 7 Effects of MTHFD1 overexpression on NADPH content

2.6 Transcription Analysis of NADPH Synthesis-Related Genes

RT-qPCR was performed to investigate how MTHFD1 overexpression affects the lipid synthesis pathway. As shown in Figure 4, while 6PGD and G6PD transcription decreased by 39.66% and 36.89%, respectively, ME and IDH transcription increased by 38.44% and 43.25% compared to the wild type. This suggests that MTHFD1 plays an important regulatory role in NADPH production for lipid synthesis.

Discussion

In summary, to investigate the relationship between MTHFD1 overexpression and lipid synthesis in *M. alpina*, we constructed an overexpression vector and successfully obtained the recombinant strain MA-MTHFD1. The results demonstrate that MTHFD1 plays a significant role in lipid synthesis. Compared to the wild type, MA-MTHFD1 showed substantial increases in total fatty acid content, fatty acid unsaturation, and NADPH levels. Moreover, MTHFD1 overexpression upregulated transcription of ME and IDH in the NADPH synthesis pathway, enhancing the supply of reducing power for lipid synthesis.

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Note: Figure translations are in progress. See original paper for figures.