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Importance of malate synthase in the glyoxylate cycle of *Ashbya gossypii* for the efficient production of riboflavin

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Abstract The glyoxylate cycle is an anabolic pathway that is necessary for growth on nonfermentable carbon sources such as vegetable oils and is important for riboflavin production by the filamentous fungus *Ashbya gossypii*. The aim of this study was to identify malate synthase in the glyoxylate cycle of *A. gossypii* and to investigate its importance in riboflavin production from rapeseed oil. The *ACR268C* gene was identified as the malate synthase gene that encoded functional malate synthase in the glyoxylate cycle. The *ACR268C* gene knockout mutant lost malate synthase activity, and its riboflavin production and oil consumption were 10- and 2-fold lower, respectively, than the values of the wild-type strain. In contrast, the *ACR268C* gene-overexpressing strain showed a 1.6-fold increase in the malate synthase activity and 1.7-fold higher riboflavin production than the control strain. These results demonstrate that the malate synthase in the glyoxylate cycle has an important role not only in riboflavin production but also in oil consumption.

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Introduction

The filamentous hemiascomycete *Ashbya gossypii* is a natural producer of riboflavin (Demain 1972). Riboflavin is an important growth factor in higher eukaryotes because it is the precursor of flavocoenzymes such as flavin mononucleotide and flavin adenine dinucleotide. *A. gossypii* has been utilized for industrial riboflavin production, and recently, its entire genome has been completely sequenced and annotated (Dietrich et al. 2004; Hemida et al. 2005). Currently, *A. gossypii* is used in the biorefining of waste vegetable oil. However, when waste oily resources are used as the carbon source, increased riboflavin productivity is required for the process to be economically viable. Therefore, several research groups (Schmidt et al. 1996a; Park et al. 2007) have applied classical mutagenesis and mutant selection techniques using antimetabolites such as itaconate and oxalate for this purpose. Schmidt et al. (1996b) found that itaconate is inhibitory to isocitrate lyase and itaconate-resistant strain is useful to improve riboflavin yield. Thus, metabolic engineering has been currently practiced for improving the riboflavin yield by over-expression and modification of key enzymes, e.g., threonine aldolase (Monschau et al. 1998) and phosphoribosyl pyrophosphate synthase (Jiménez et al. 2005, 2008).

The glyoxylate cycle is a C_4-dicarboxylic acid interconversion pathway, which has been characterized as a "glyoxylate bypass of tricarboxylic acid (TCA) cycle" because the malate dehydrogenase, citrate synthase, and aconitase activities are shared by both cycles (Kornberg and

Madsen 1957). The glyoxylate cycle plays an essential role in cell growth on nonfermentable carbon sources such as acetate, ethanol, and fatty acids and in fungal virulence in microorganisms. Dysfunctional mutants of *Candida albicans* that lacked isocitrate lyase (ICL1, E.C. 4.1.3.1) or malate synthase (MLS1, E.C. 2.3.3.9) in the glyoxylate cycle lost their ability to form pseudohypha and their fungal virulence in mice (Lorenz and Fink 2001). In *Saccharomyces cerevisiae*, disruptants of these genes were unable to utilize carbon sources such as ethanol, acetate, or oleic acid (Fernandez et al. 1992; Hartig et al. 1992; Kunze et al. 2002). The metabolic importance of ICL1 has been well studied as a key enzyme in riboflavin biosynthesis from oils in *A. gossypii* since its activity was positively correlated to the riboflavin yield (Kanamasa et al. 2007; Maeting et al. 1999; Schmidt et al. 1996a).

Malate synthase is an acyltransferase that converts glyoxylate and acetyl-CoA to malate. In this reaction, the acetyl residue from acetyl-CoA is transferred to glyoxylate. The malate that is generated is either converted to oxaloacetate for continuous glyoxylate cycle or is used as the initial substrate in gluconeogenesis for conversion to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PCK1, E.C. 4.1.1.49). Although MLS1 activity is believed to be necessary for mycelial growth on nonfermentable carbon sources, *A. gossypii* MLS1 has not been functionally identified and characterized in riboflavin biosynthesis from vegetable oils.

In this study, the *MLS1* homolog was disrupted and overexpressed in *A. gossypii* to facilitate the identification and characterization of the gene product. We could demonstrate that MLS1 is one of the important key enzymes for the improved production of riboflavin from rapeseed oil. Moreover, supplementation malate into the *A. gossypii* culture was effective in improving riboflavin productivity.

Materials and methods

Strains and growth conditions

A. gossypii ATCC 10895 (*Ag*WT) and *Escherichia coli* DH5α were used as the *A. gossypii* wild-type and DNA manipulation host strain, respectively. *E. coli* DH5α was grown in LB medium (pH 7.5) consisting of 1% (*w*/*v*) polypeptone-S (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 0.5% (*w*/*v*) bacto yeast extract (Becton, Dickinson and Company, NJ, USA), and 0.5% (*w*/*v*) sodium chloride (Wako Pure Chem. Ind., Ltd., Osaka, Japan).

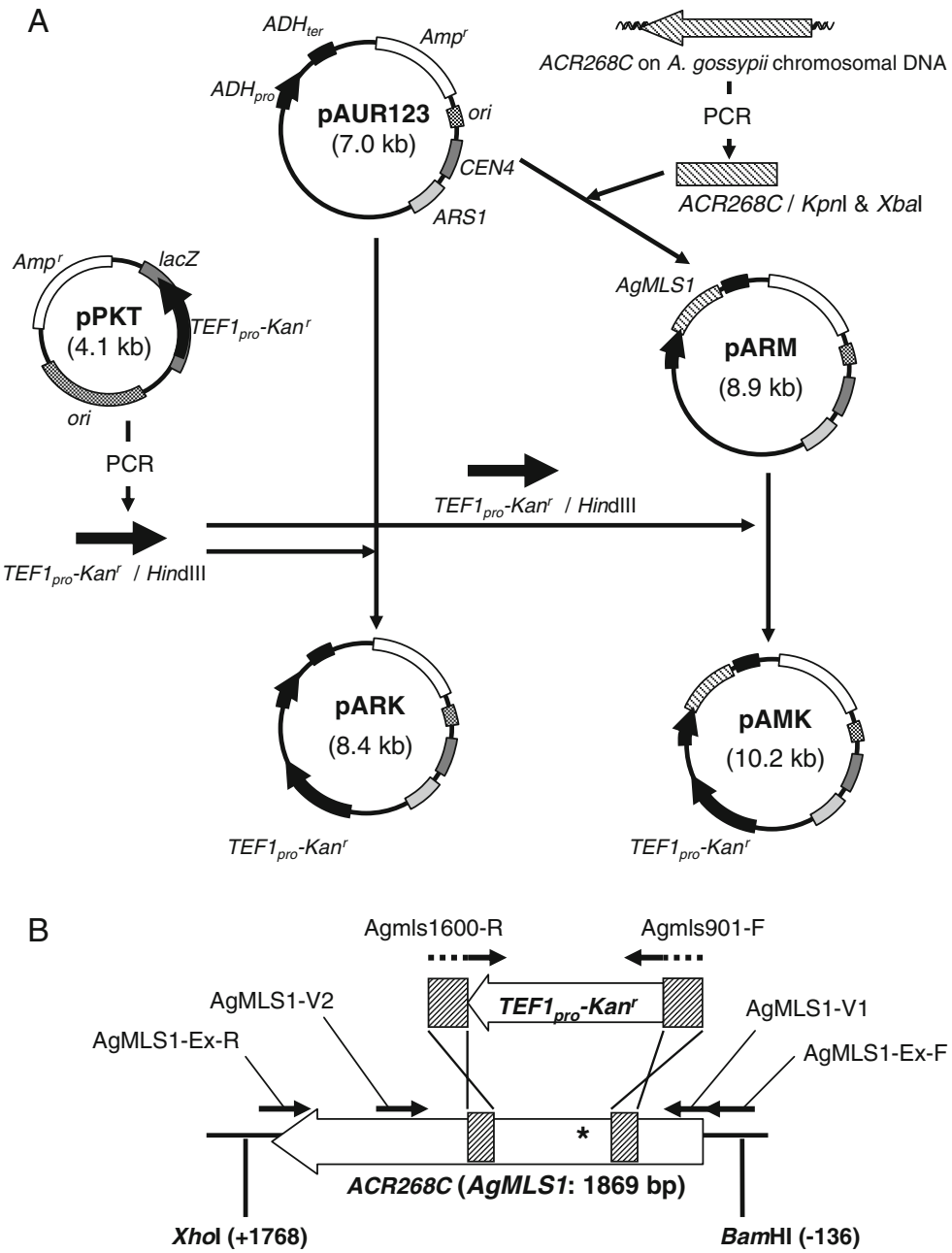
The media used for *A. gossypii* culture were as follows: YD medium (pH 6.8) containing 1% (*w*/*v*) yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan) and 1% (*w*/*v*) glucose; YR medium (pH 6.8) containing 1% (*w*/*v*) yeast extract and 1% (*w*/*v*) rapeseed oil; seed medium for riboflavin production (per liter) consisting of 30 g corn steep liquor (Wako), 9 g yeast extract, and 15 g rapeseed oil (pH 6.8); production medium (per liter) containing 60 g corn steep liquor, 30 g gelatin (Wako), 1.5 g KH_2PO_4, 1.5 g glycine, 4.4 mg $CoCl_2$, 17.9 mg $MnCl_2{\cdot}4H_2O$, 44.2 mg $ZnSO_4{\cdot}7H_2O$, 10.3 mg $MgSO_4{\cdot}7H_2O$, and 50 g rapeseed oil (pH 6.8). Cultures were performed in 500-ml shaker flasks with a working volume of 50 ml of each medium. The cultures were incubated on a rotary shaker (Bio Shaker; Takasaki Scientific Instrument Co.) at 220 rpm and 28°C. For selective growth of the transformants, Geneticin (Wako) was added to the cultures to a final concentration of 200 μg/ml.

Homology search of *A. gossypii* malate synthase using BLAST

The amino acid sequence of *A. gossypii* malate synthase was obtained from the Ashbya Genome Database (http://agd.vital-it.ch/index.html) described by Hemida et al. (2005). The amino acid sequence of the malate synthase from *S. cerevisiae* that was identified by Hartig et al. (1992) was used as the query sequence for cross-species BLAST homology searching in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The acquired FASTA format amino acid sequences of several species, including *A. gossypii*, were multialigned by ClustalX (Larkin et al. 2007) and modified by GeneDog.

Plasmid constructions

DNA was manipulated using standard procedures (Sambrook and Russell 2001). The control plasmid (pARK) and expression plasmid with the *ACR268C* gene in *A. gossypii* (pAMK) were constructed using pAUR123 (TaKaRa Bio Inc., Shiga, Japan), as shown in Scheme 1A. The *ACR268C* gene was polymerase chain reaction (PCR)-amplified with 100 ng of *Ag*WT chromosomal DNA as the template using the KOD-Plus DNA polymerase (Toyobo, Co., Ltd., Osaka, Japan). PCR was carried out using the AgMLS1-Ex-F and AgMLS1-Ex-R primers (Table 1) under the following conditions: 1 cycle at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, 55°C for 1 min, and 68°C for 2 min. The amplified fragment was purified by GFX PCR DNA and the Gel Band Purification Kit (GE Healthcare UK Ltd., Buckinghamshire, England, UK). The *ACR268C* gene was inserted between the *Kpn*I and *Xba*I sites located downstream of the alcohol dehydrogenase promoter (ADH_{pro}) in pAUR123, using a DNA ligation kit (Mighty Mix, TaKaRa Bio Inc.). The resulting plasmid was designated pARM.



Scheme 1 **A** Flow diagram of plasmid construction. *Black arrows* indicate *TEF1* promoter–kanamycin-resistance gene–*TEF1* terminator (*TEF1*$_{pro}$-*Kan*^{r}). Diagonal squares denote the *ACR268C* (*MLS1*) gene, which was amplified using the AgMLS1-Ex-F and AgMLS1-Ex-R primers. **B** Disruption of the *ACR268C* gene in the *A. gossypii* wild-type strain. The *asterisk* and *diagonal box* represent the 527th codon encoding the aspartate residue, which acts as a proton donor, and a 60-bp homologous region of the *ACR268C* gene, respectively

The kanamycin-resistance gene cassette (*TEF1*$_{pro}$-*Kan*^{r}), which contains the kanamycin-resistance gene used as a dominant marker for Geneticin resistance in eukaryotes (Jiménez and Davies 1980) under the control of the *A. gossypii* translation elongation factor 1α promoter (*TEF1*$_{pro}$) and terminator (*TEF1*$_{ter}$), was amplified using pPKT as the template (Kato and Park 2004) and the AgTEFproH3-F and AgTEFterH3-R primers (Table 1). PCR was carried out using *LA-Taq* Hot Start Version (TaKaRa Bio Inc.) under the following conditions: 1 cycle at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min. Final extension was carried out with 1 cycle at 72°C for 10 min. The amplified fragment was purified as described above and inserted into the *Hin*dIII sites of pARM and pAUR123. The resulting plasmids were designated pAMK and pARK, respectively. pAMK harbored both *TEF1*$_{pro}$-*Kan*^{r} and *ACR268C*, while pARK contained only *TEF1*$_{pro}$-*Kan*^{r}.

PCR-based gene targeting disruption

The knockout mutant of the *ACR268C* gene was constructed by PCR-based gene targeting disruption, as described by Wendland et al. (2000). Technical protocol

Table 1 Primers list

Name	Nucleotide sequence (5′→3′)
AgMLS1-Ex-F	TGGGGTACCCCATGAATCACTGTCCTGA[a]
AgMLS1-Ex-R	AGCTCTAGAGCTCAGAGACGCGACTT[a]
AgTEFproH3-F	TGTAAGCTTGACATGGAGGCCCAGAATACCC[a]
AgTEFproH3-R	ACGAAGCTTCTTTCTGCGCACTTAACTTCGC[a]
Agmls901-F	ctggaggccaagctgtggaacgacattttcaacgtggcgcaggacta catcggcatgcgcCTGCAGACATGGAGGCCCAGAATAC[b]
Agmls1600-R	ctcgccagtgtccgcgagcttgacgcggtggcggacccactggtgaag ctggcagcgcgaGAATTCTTTCTGCGCACTTAACTTCGCA[b]
AgMLS1-V1	AAGCAGCGCAATGGTAGCAGTCAG
AgMLS1-V2	GTAATCTCCGGCTCGAGGTACTTAGC
QACT1-F	ACGGTGTTACCCACGTTGTTCC
QACT1-R	TCATATCTCTGCCGGCCAAGTC
QMLS1-F	TTCTTCCACAACGCGGCTAAGCTA
QMLS1-R	ATGTAGTCCTGCGCCACGTTGAAA

[a] Underlined data indicate restriction enzyme site

[b] Lower case letters indicate 60 bp of homologous sequence derived from *ACR268C* (*MLS1*) gene

of transformation in *A. gossypii* was performed according to the method described by Monschau et al. (1998) and Kanamasa et al. (2007) with some modifications. The 60-bp homologous sequence of the *ACR268C* gene on both ends of $TEF1_{pro}$-Kan^r was amplified using pPKT as the template, as shown in Scheme 1A, and the Agmls901-F and Agmls1600-R primers (Table 1). PCR amplification was carried out using *LA-Taq* Hot Start Version (TaKaRa Bio Inc.) under the following conditions: 1 cycle at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min 42 s. Final extension was carried out with 1 cycle at 72°C for 10 min. The PCR product was purified as described above.

Spores of *Ag*WT (approximately 1.0×10^6) were grown on YD medium for 27 h. The grown mycelia were harvested by filtration, washed with distilled water, and suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 25 mM 2-mercaptoethanol. The suspension was incubated at 30°C for 30 min with gentle agitation, and the mycelia were collected by filtration and washed with transformation buffer consisting of 270 mM sucrose, 10 mM Tris–HCl (pH 7.5), and 1 mM $MgCl_2$. The mycelia were finally resuspended in cooled transformation buffer, and 350 μl of the mycelial suspension was mixed with 300 ng of the above-purified gene disruption cassette (Scheme 1B). The cassette was introduced into the mycelium by electroporation in a Gene Pulser Xcell system (Bio-Rad Lab. Inc., Hercules, CA, USA) at 1.5 kV/cm, 400 Ω, and 25 μF using 2-mm pre-chilled electrocuvettes (Bio-Rad). The postelectroporated mycelia were incubated on a YD plate to regenerate the mycelia at 30°C for 6 h. Subsequently, the mycelia were covered with 20 ml YD medium containing 0.6% agar and 300 μg/ml Geneticin for isolating the transformants.

Confirmation of transformants

The disruption of the *ACR268C* gene was confirmed by PCR using *Ex-Taq* Hot Start Version (TaKaRa Bio Inc.) with the AgMLS1-V1 and AgMLS1-V2 primers (Table 1) and 200 ng chromosomal DNA from the transformant. The PCR conditions were as follows: 1 cycle at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 3 min. Final extension was carried out by 1 cycle at 72°C for 10 min. For Southern blotting analysis of the mutant, 100 μg of the chromosomal DNA was simultaneously digested with *Bam*HI and *Xho*I, and the products were separated by 0.9% (*w*/*v*) agarose gel electrophoresis. The DNA fragments were transferred onto Hybond-N^+ (GE Healthcare). A DNA fragment of the kanamycin-resistance gene was used as the hybridization probe. Preparation of the labeled probe and chemiluminescent detection were carried out with the AlkPhos Direct Labeling and Detection system (GE Healthcare), according to the manufacturer's protocol. Positive signals were detected by Fluor-S/MAX (Bio-Rad).

To confirm the transformants carrying pARK or pAMK, 10 μg of total DNA from each transformant was introduced into *E. coli* cells, and the rescued plasmid was confirmed by restriction enzyme mapping.

Quantification of malate synthase messenger RNA by real-time quantitative reverse transcriptase-polymerase chain reaction

A. gossypii mycelia grown in production medium in flask for 26 h were harvested by filtration. Resulting mycelia were mixed with 0.3 g of acid washed glass beads (Sigma) and 1 ml of ISOGEN (NIPPON GENE Co., Ltd. Tokyo, Japan), and then fractured by vigorous agitation. Comple-

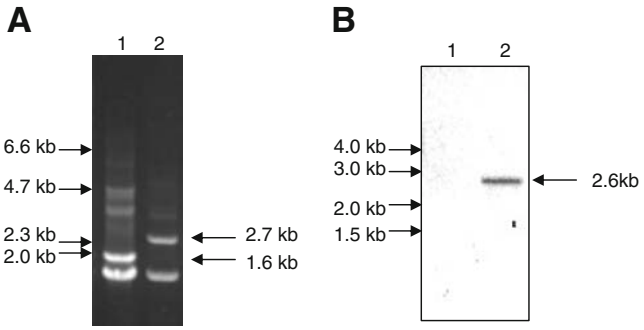


Fig. 1 Confirmation of the *ACR268C* gene-targeted disruptant by PCR and Southern blot analysis. **a** The PCR products were amplified from chromosomal DNA using the AgMLS1-V1 and AgMLS1-V2 primers (Scheme 1B). The *up* and *down arrows* indicate fragments of sizes 2.7 and 1.6 kb, respectively. **b** In the Southern blot analysis, chromosomal DNA was digested simultaneously with *Bam*HI and *Xho*I. The kanamycin-resistance gene, which is absent in the *Ag*WT chromosomal DNA, was used as the probe. *Lanes 1 and 2* in **a** and **b** denote *Ag*WT and *Ag*Δmls1 (*ACR268C* gene-disruptant), respectively

mentary DNA was obtained by reverse transcription-PCR using PrimeScript RT-PCR Kit (TaKaRa Bio Inc.) with the extracted total RNA as the template with random 6-mers primer in the elongation condition at 40°C for 1 h. The template RNA was degraded by 10 mg of RNase (Sigma) at 37°C for 30 min. Messenger RNA levels of malate synthase and actin were quantified by using FullVelocity SYBR Green QPCR Master Mix (Agilent Technologies Inc., CA, USA) under the condition of 1 cycle at 95°C for 5 min, 60 cycles at 95°C for 10 s, and 60°C for 30 s for the amplification plot, and 1 cycle at 95°C for 1 min, 55°C for 30 s, and 95°C for 5 min for the dissociation plot. QMLS1-F, QMLS1-R and QACT1-F, QACT1-R primers (Table 1) were used for malate synthase and actin, respectively. Actin was used as an internal standard because of its constitutive expression.

Enzyme assay

The malate synthase activity was determined according to the method of Dixon and Kornberg (1959). Ten microliters of the enzyme solution was added to 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM sodium glyoxylate, and 50 μM acetyl-CoA (Wako), and the final volume was made up to 1 ml. The specific absorbance of acetyl-CoA was measured at 232 nm. One unit of malate synthase activity was defined as the amount of enzyme required to

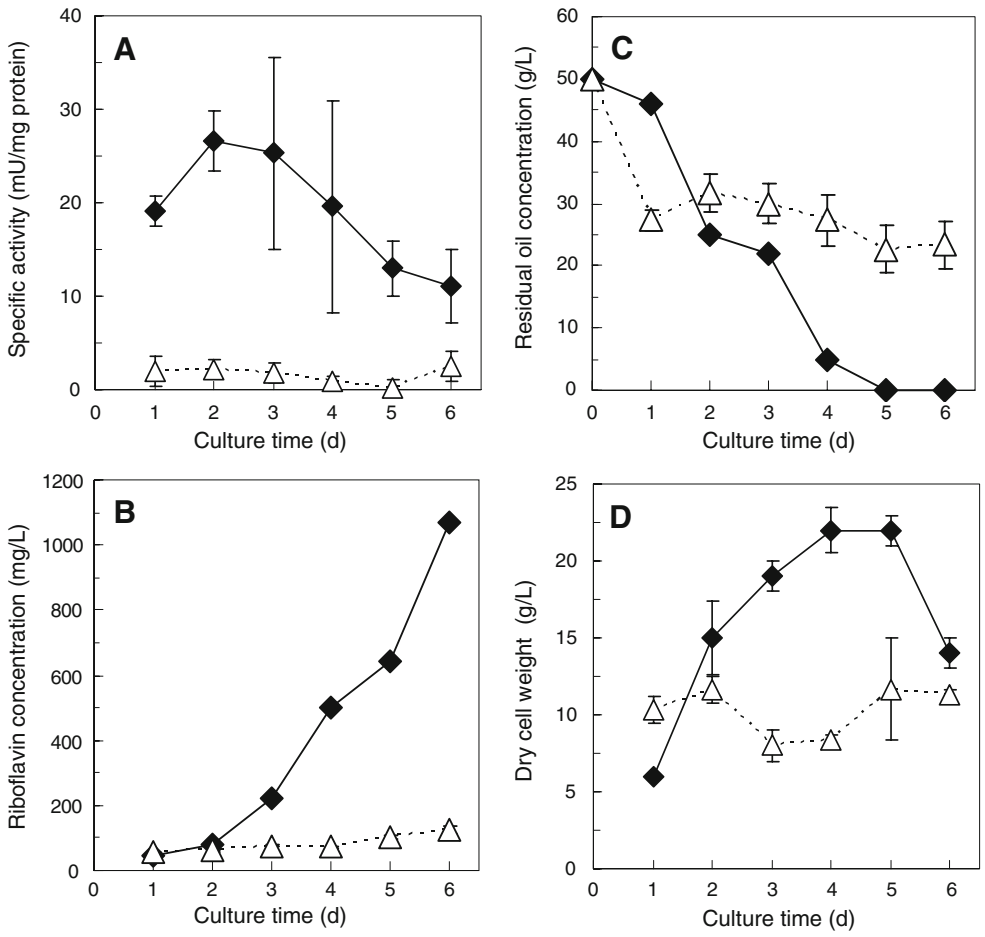


Fig. 2 Time course of malate synthase activity (**a**), riboflavin concentration (**b**), residual oil concentration (**c**), and dry cell weight (**d**) in cultures of the *Ag*WT (*closed rhombus*) and *Ag*Δmls1 (*open triangle*) strains. Both cultures were carried out in triplicates, and the average data and standard deviations are shown

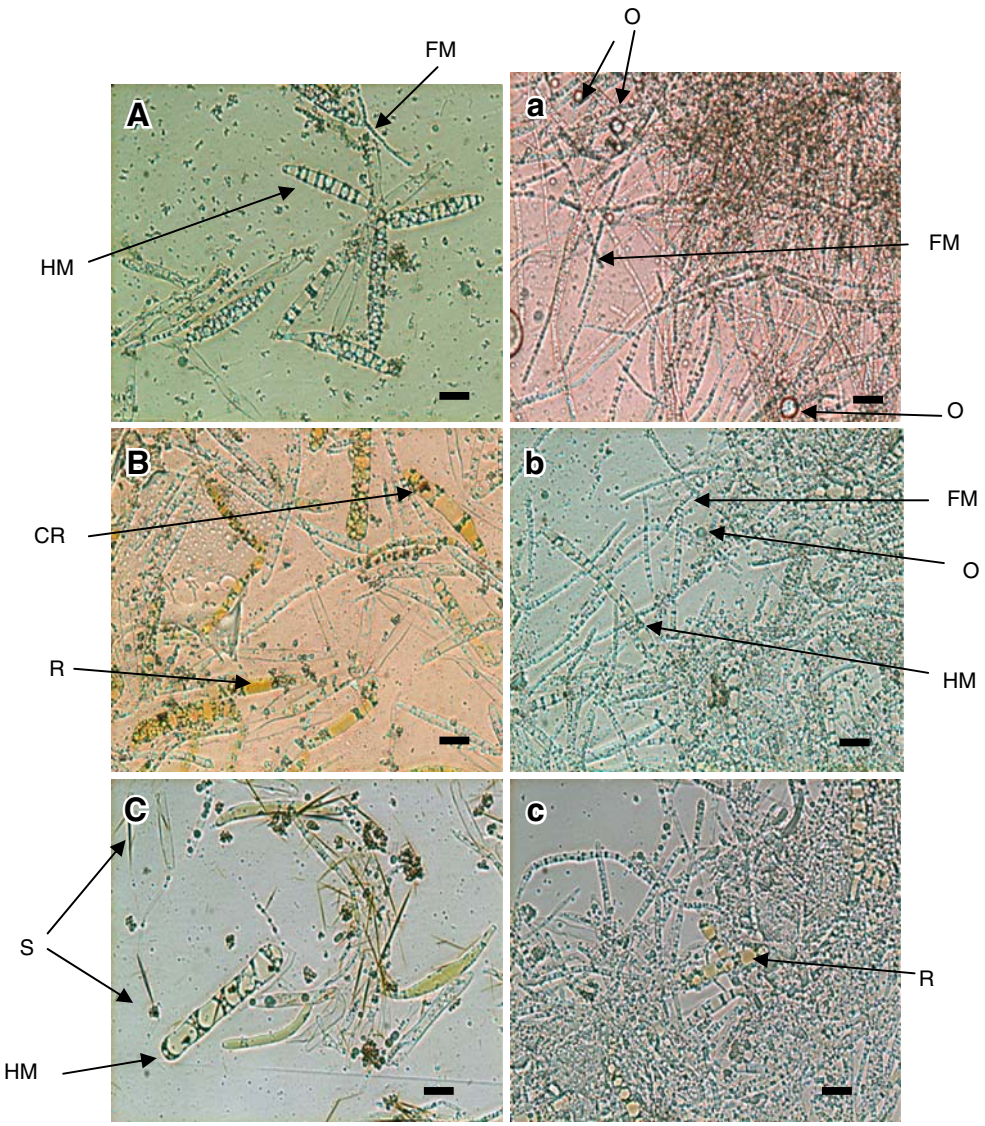


Fig. 3 Morphological changes in the *ACR268C* (*MLS1*) gene disruptant and *Ag*WT (*A–C*) and *Ag*Δmls1 (*a–c*) strains. The mycelia are shown at 3 days (*A* and *a*), 5 days (*B* and *b*), and 7 days (*C* and *c*) of culture. *HM* hypertrophic mycelia, *O* residual oil droplet, *FM* filiform mycelia, *R* riboflavin, *CR* crystallized riboflavin, *S* spore. *Bars* indicate a 10-μm scale

deacetylate 1 μmol of acetyl-CoA per minute. Nonspecific deacetylated acetyl-CoA was measured in the absence of $MgCl_2$ and sodium glyoxylate. The protein concentration was determined by the Bradford method using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

Analytical methods

The riboflavin and residual oil concentrations were measured according to the method previously described by Park and Ming (2004). The dry cell weight was measured as follows. The mycelia from the culture broth were filtered using filter paper No. 5A (Advantec, Tokyo, Japan). The mycelia paste was dried overnight in an oven at 100°C, and the dry cell weight was measured.

Results

Multiple alignment of amino acid sequences of *A. gossypii* ACR268Cp

When multiple alignment analysis of amino acid sequences of the proteins of yeasts and fungi was carried out, several conserved regions and similar peroxisomal-targeting sequences were observed in the C-terminal region; these were designated SRL and SKL. The malate synthase of *S. cerevisiae* has a signal sequence and is believed to be transported into the peroxisome (Kunze et al. 2002). The amino acid sequence of ACR268Cp showed 73% identity to the malate synthase from *S. cerevisiae* (*Sc*MLS1), 60% identity to the enzyme from *Aspergillus niger* (*An*MLS1), and 57% identity to the enzymes from *C. albicans*

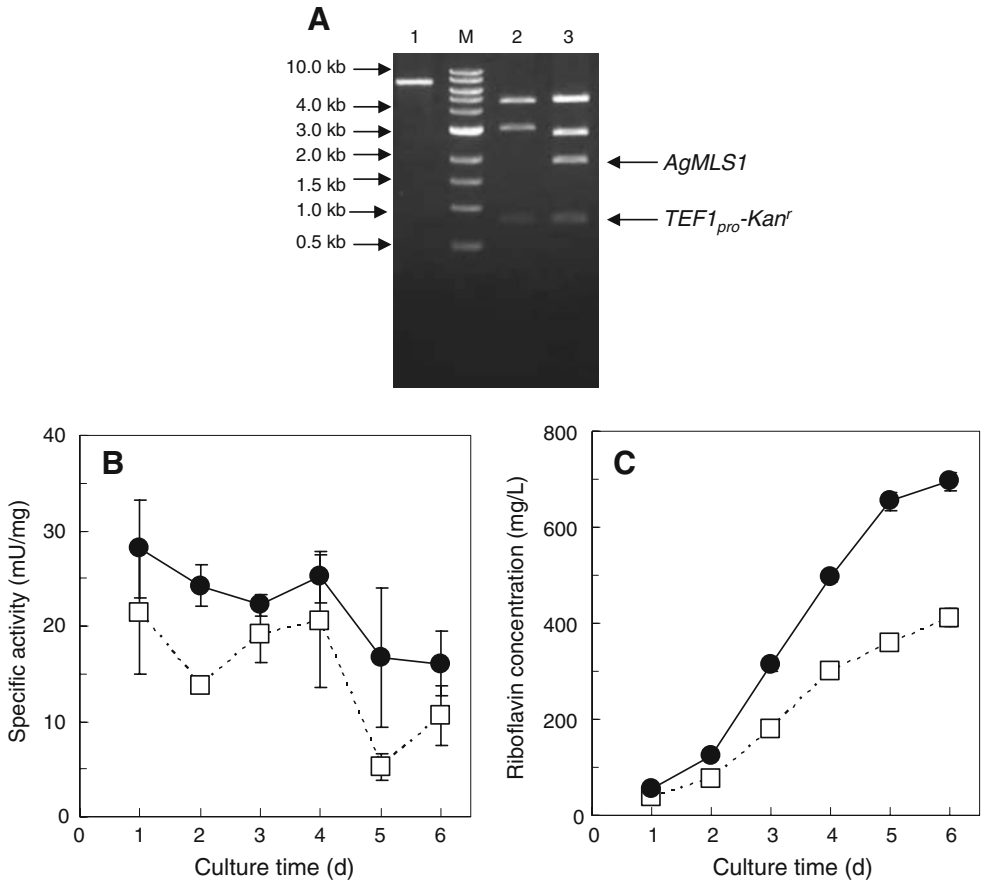


Fig. 4 Confirmation of constructed plasmids (**a**) and time course of malate synthase activity (**b**) and riboflavin concentration (**c**) in cultures of the *Ag*WP (*open square*) and *Ag*MLS1 (*closed circle*) strains. **a** The plasmids were digested with *Kpn*I and *Xba*I. *M*, 1-kb ladder; *lane 1*, pAUR123; *lane 2*, pARK; and *lane 3*, pAMK. Each of the constructed plasmids pARK and pAMK was prepared from the total DNA extracted from the *Ag*WP and *Ag*MLS1 strains, respectively. Each culture was carried out in triplicates, and the average data and standard deviation are shown

(*Ca*MLS1) and *Neurospora crassa* (*Nc*MLS1). Characterization of the features based on the *Sc*MLS1 amino acid sequence obtained by UniProt (http://www.pir.uniprot.org/) suggested that ACR268Cp had two active sites, i.e., a proton acceptor and donor on the 247th arginine residue and 527th aspartate residue, respectively.

ACR268C gene disruption and its phenotype

The *ACR268C* gene was disrupted, and six Geneticin-resistant colonies of *A. gossypii* were isolated with an efficiency of approximately 20 colony-forming units (cfu)/μg of DNA. The *ACR268C* gene disruption was confirmed by genomic PCR using the AgMLS1-V1 and AgMLS1-V2 primers. The presence of the 2.7-kb fragment (lane 2 in Fig. 1a) indicated the introduction of $TEF1_{pro}$-Kan^r into the *ACR268C* gene. This also led to the identification of the Geneticin-resistant colony as an *ACR268C* gene disruptant. Southern blot analysis was carried out to confirm the disruption of the *ACR268C* gene. The 2.6-kb DNA fragment was detected using a chemiluminescent kanamycin-resistance gene probe from the chromosomal DNA that had been double-digested with *Bam*HI and *Xho*I at both ends of the *ACR268C* gene (Fig. 1b). These results demonstrated that the $TEF1_{pro}$-Kan^r gene cassette was integrated into the *ACR268C* gene, and the gene disruptant was designated *Ag*Δmls1.

To investigate the phenotypic variations between the *Ag*WT and *Ag*Δmls1 strains, both strains were cultured in

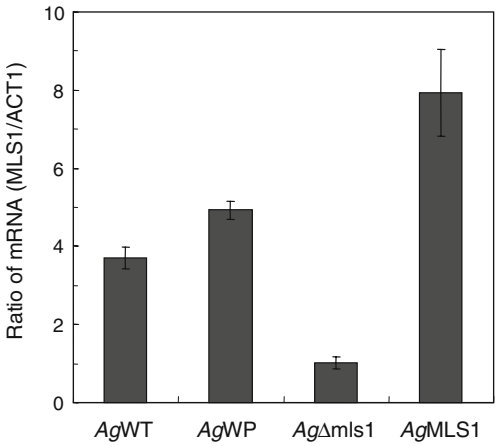


Fig. 5 Analysis of intracellular transcriptional level using quantitative real-time PCR. Quantification of *MLS1* mRNA was represented by ratio of *MLS1* and *ACT1*. Total RNA was extracted from 26-h grown mycelia (n=4)

the production medium. The specific malate synthase activity of *Ag*Δmls1 was less than 5 mU/mg protein, which was one-seventh that of the *Ag*WT strain (Fig. 2a). This indicated that replacement of the +961 to +1599 region of the *ACR268C* gene, including the 527th aspirate residue, with $TEF1_{pro}$-Kan^r leads to complete loss of enzyme activity. This resulted in a significant decrease in riboflavin production by the *Ag*Δmls1 strain—approximately 10-fold less than that by the *Ag*WT strain (Fig. 2b). The oil consumption and dry cell weight of *Ag*Δmls1 were half or less than half that of the *Ag*WT strain (Fig. 2c, d).

The mycelial morphology of *Ag*Δmls1 differed from that of *Ag*WT. In the *Ag*WT strain, as the culture progressed, the mycelia transformed into hypertrophic cells (HM in Fig. 3). In contrast, the *Ag*Δmls1 cells maintained their morphology as thin filamentous mycelia (FM in Fig. 3) from the beginning, i.e., when the culture was initiated. Oil droplets in the *Ag*Δmls1 strain remained even after a culture time of 6 days, indicating the presence of residual oil (O in Fig. 3). Fewer riboflavin-accumulating yellowish mycelia (R in Fig. 3) were observed in *Ag*Δmls1 than in *Ag*WT. Crystallized riboflavin (CR in Fig. 3) was observed in hypertrophic mycelia of *Ag*WT but not in those of the *Ag*Δmls1 strain.

Riboflavin production, malate synthase activity, and the transcriptional level in the malate synthase-overexpressing transformant

The presence of the pARK and pAMK plasmids in the transformant was verified by digesting the extracted plasmid DNA with *Kpn*I and *Xba*I. Electrophoretic analysis demonstrated the presence of bands of size approximately 850 and 1.9 kb, which represent the kanamycin-resistance gene and *ACR268C* gene, respectively (lane 3 in Fig. 4a). The transformants with *Ag*WT/pARK and *Ag*WT/pAMK were designated *Ag*WP and *Ag*MLS1, respectively.

The specific malate synthase activity of *Ag*MLS1 was significantly higher than the activities of the *Ag*WP (Fig. 4b) and *Ag*Δmls1 strains (Fig. 2a), indicating that the *ACR268C* gene encodes malate synthase. When the *Ag*WP and *Ag*MLS1 transformants were cultured in the production medium, the riboflavin production by *Ag*MLS1 was 1.7-fold higher than that by *Ag*WP (Fig. 4c), even though the values of oil consumption and dry cell weight were similar to those of *Ag*WP (data not shown).

Real-time quantitative RT-PCR revealed that *MLS1* messenger RNA (mRNA) level of *Ag*MLS1 strain was approximately 1.6- and 8-fold higher than those of *Ag*WP and *Ag*Δmls1, respectively (Figs. 4b and 5). This indicates that the plasmid pAMK expressing *MLS1* functions appropriately.

Additive effect of glyoxylate and malate on riboflavin production in the *ACR268C* gene disruptant

Malate synthase gene disruption led to a significant decrease in riboflavin production, suggesting that continuous glyoxylate cycling and/or gluconeogenesis bypass from malate might be blocked. Glyoxylate and malate are the substrate and product of malate synthase, respectively. Therefore, to investigate the effects of glyoxylate and malate on metabolic flux, the *Ag*WT and *Ag*Δmls1 strains were cultured in glyoxylate- or malate-supplemented production medium. Remarkable phenotypic differences were not observed between the strains upon culture in the 50-mM glyoxylate-supplemented culture (data not shown). However, the 50-mM malate-supplemented cultures of *Ag*WT and *Ag*Δmls1 strains showed higher riboflavin production, oil consumption, and dry cell weight in comparison with the values of the control culture. The riboflavin concentrations of the *Ag*Δmls1 and *Ag*WT

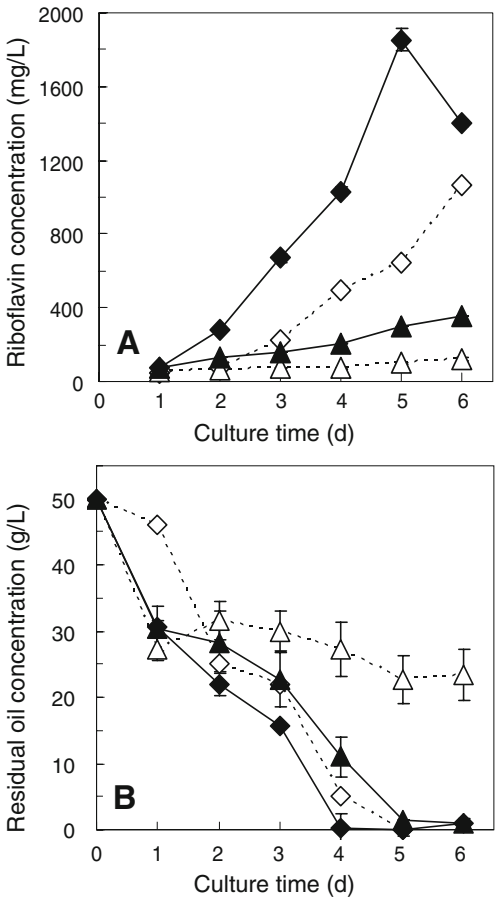


Fig. 6 Riboflavin production (**a**) and residual oil concentration (**b**) in cultures of *Ag*WT (*rhombus*) and *Ag*Δmls1 (*triangle*) strains with (*closed symbols*) or without (*open symbols*) 50 mM malate. Each culture was carried out in triplicate, and the average data and standard deviations are shown

strains were more than 2-fold higher than those of the strains cultured without the malate additive (Fig. 6a). In particular, in the culture of the *Ag*Δmls1 strain, 50 g/l of oil was completely consumed (Fig. 6b), which differed drastically from the result obtained in the absence of the malate additive (Fig. 2c).

Discussion

In this study, the *ACR268C* gene was identified to encode malate synthase, and its role in riboflavin biosynthesis in *A. gossypii* was investigated using rapeseed oil as the sole carbon source. We analyzed the amino acid sequence of ACR268Cp and found sequences that were similar to two active sites and the C-terminal peroxisome-targeting signal (−SRL; Gould et al 1988) of *S. cerevisiae* malate synthase. In the *ACR268C* gene-disruptant, the 527th aspartate, which functions as a proton donor, was replaced with the kanamycin-resistance gene, and the disruptant did not exhibit any malate synthase activity (Fig. 2a). This strain also showed decreases in the riboflavin concentration, oil consumption, and dry cell weight. Therefore, malate synthase is important for riboflavin biosynthesis and the assimilation of vegetable oils. This is similar to the functioning of isocitrate lyase (Schmidt et al. 1996b). However, Kanamasa et al. reported that an isocitrate lyase gene-disrupted mutant lost the ability to produce riboflavin but grew well in the production medium (Kanamasa et al. 2007). This suggests that malate synthase may have an important role not only in riboflavin production but also in the maintenance of hyphal growth and turnover of carbon assimilated from nonfermentable carbon sources under aerobic conditions. With regard to mycelial morphology, hypertrophic mycelia were hardly observed in the disruptant in comparison with the wild-type strain, even during the late culture period. Empirically, hypertrophic mycelia are predominant in riboflavin producing *A. gossypii* throughout the late culture period. This suggests that the stagnation of the glyoxylate cycle results in retardation of both cell growth and riboflavin biosynthesis because the supply of both malate and oxaloacetate may be limited. Malate and oxaloacetate are substrates for glyoxylate cycle turnover, malate/aspartate shuttle (Schmitt and Edwards 1983), and gluconeogenesis (Fig. 7). These processes are necessary for the biosynthesis of the sugar phosphate (such as ribulose-5-phosphate) and purine nucleotide (such as guanosine triphosphate) for the riboflavin scaffold (Stahmann et al. 2000).

As for malate synthase-overexpressing strain, even though the expression promoter and/or replication origin was adopted relatively at low mRNA level, as compared to those of *TEF1* promoter and 2-μm origin, the riboflavin concentration of the *MLS1*-overexpressing strain was 1.7-fold higher than that of the wild-type. This is due to an increase in the malate synthase activity and the mRNA

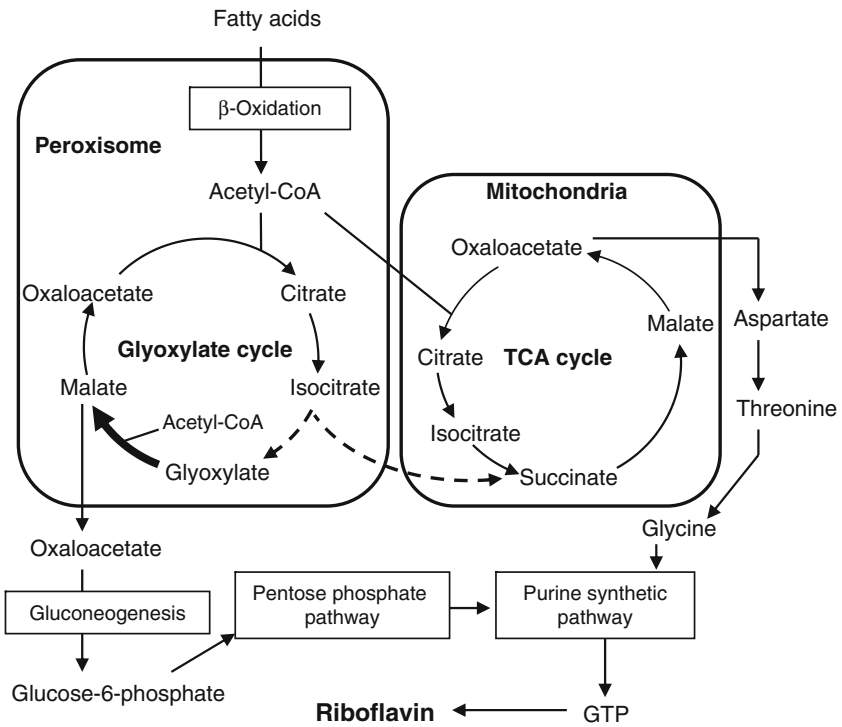


Fig. 7 Putative metabolic pathway for riboflavin biosynthesis in *A. gossypii*. The pathways in which malate synthase and isocitrate lyase are involved are indicated by the *thick solid line* and *dotted line*, respectively

level (Figs. 4b, c and 5). Therefore, we expect and suggest that adoption of strong expression promoters may increase the riboflavin yield to a better extent in this experiment.

MLS1 disruptant strain did not respond to a malate-supplemented culture. Although malate addition led to an increase in oil consumption, riboflavin production was not restored significantly in the disruptant, unlike the wild-type strain. It is probable that ACR268Cp may have another function distinct from malate synthase activity. On the other hand, a glyoxylate (substrate of malate synthase)-supplemented culture did not have any effect on riboflavin production, oil consumption, and cell growth in both the *A. gossypii* wild-type and disruptant strains, in spite of the presence of excess substrate (data not shown). This suggests that the efficiency of acetyl-CoA turnover is a limiting factor for malate synthase activity in glyoxylate additive culture, and that malate can be a driving force for effective turnover of glyoxylate cycle, gluconeogenesis, and TCA cycle (Fig. 7). Therefore, efficient turnover and/or excess supplementation of key metabolites such as malate around log-phase on mycelial growth may be important for effective riboflavin production of *A. gossypii*. An effective oil consumption due to improved mycelia lipase activity (Stahmann et al. 1997) or fortification of metabolic activity on β-oxidation may be necessary for improving riboflavin productivity.

Using an *MLS1* disruptant and *MLS1*-overexpressing transformant, we demonstrated that malate synthase is one of the important key enzymes for improving riboflavin production in *A. gossypii*. However, riboflavin production of *Ag*WP and *Ag*MLS1 strains was half or less than that of the wild-type. It may be due to addition of antibiotics for maintaining subnuclear plasmid, resulting in lower mycelial growth of *Ag*WP strain than that of *Ag*WT strain (data not shown). Kato and Park (2004) showed similar phenomenon with 2-μm origin plasmid transformants as well as yeast autonomously replicating sequence (*ARS1*) contained in pARK and pAMK, both of which were functioned in *A. gossypii* as replication origins (Wright and Philippsen 1991). Therefore, to further improve riboflavin production, it is necessary to overexpress important key genes in the *A. gossypii* genome. Jiménez et al. (2008) have succeeded in chromosomal integration by gene-targeting recombination using a specific gene cassette with tandem placement of a drug-resistant gene and riboflavin production-positive gene expression cassettes. In the near future, chromosomal integration by malate synthase recombination will be useful for improving the riboflavin yield of *A. gossypii*.

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