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.

Keywords Riboflavin · *Ashbya gossypii* · Malate synthase · Gene disruptant · Gene targeting disruption · Glyoxylate cycle

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 (FMN) and flavin adenine dinucleotide (FAD). *A. gossypii* has been utilized for industrial riboflavin production, and recently, its entire genome has been completely sequenced and annotated (Dietrich et al. 2004; Hermida 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 overexpression 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

59 role in cell growth on nonfermentable carbon sources such as acetate, ethanol, and fatty
60 acids and in fungal virulence in microorganisms. Dysfunctional mutants of *Candida*
61 *albicans* that lacked isocitrate lyase (ICL1, E.C. 4.1.3.1) or malate synthase (MLS1, E.C.
62 2.3.3.9) in the glyoxylate cycle lost their ability to form pseudohypha and their fungal
63 virulence in mice (Lorenz and Fink 2001). In *Saccharomyces cerevisiae*, disruptants of
64 these genes were unable to utilize carbon sources such as ethanol, acetate, or oleic acid
65 (Fernandez et al. 1992; Hartig et al. 1992; Kunze et al. 2002). The metabolic importance
66 of ICL1 has been well-studied as a key enzyme in riboflavin biosynthesis from oils in *A.*
67 *gossypii* since its activity was positively correlated to the riboflavin yield (Kanamasa et
68 al. 2007; Maeting et al. 1999; Schmidt et al. 1996a).
69 Malate synthase is an acyltransferase that converts glyoxylate and acetyl-CoA to
70 malate. In this reaction, the acetyl residue from acetyl-CoA is transferred to glyoxylate.
71 The malate that is generated is either converted to oxaloacetate for continuous
72 glyoxylate cycle or is used as the initial substrate in gluconeogenesis for conversion to
73 phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (PCK1, E.C. 4.1.1.49).
74 Although MLS1 activity is believed to be necessary for mycelial growth on
75 nonfermentable carbon sources, *A. gossypii* MLS1 has not been functionally identified
76 and characterized in riboflavin biosynthesis from vegetable oils.
77 In this study, the *MLS1* homologue was disrupted and overexpressed in *A.*
78 *gossypii* to facilitate the identification and characterization of the gene product. We
79 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 strains, respectively. *E. coli* DH5α was grown in LB medium (pH 7.5) consisting of 1% (w/v) polypeptone-S (Nihon Pharmaceut. 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 $ZnSO_4{\cdot}7H_2O$, and 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 28ºC. 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 (AGD; 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 (DDBJ; 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

122 in Scheme 1A. The *ACR268C* gene was PCR amplified with 100 ng of *Ag*WT
123 chromosomal DNA as the template using the KOD-Plus DNA polymerase (Toyobo, Co.,
124 Ltd., Osaka, Japan). PCR was carried out using the AgMLS1-Ex-F and AgMLS1-Ex-R
125 primers (Table 1) under the following conditions: 1 cycle at 95ºC for 2 min followed by
126 35 cycles at 95ºC for 30 s, 55ºC for 1 min, and 68ºC for 2 min. The amplified fragment
127 was purified by GFX PCR DNA and the Gel Band Purification Kit (GE Healthcare UK
128 Ltd., Buckinghamshire, England). The *ACR268C* gene was inserted between the *Kpn*I
129 and *Xba*I sites located downstream of the alcohol dehydrogenase promoter (ADH_{pro}) in
130 pAUR123, using a DNA ligation kit (Mighty Mix, TaKaRa Bio Inc.). The resulting
131 plasmid was designated pARM.
132 The kanamycin-resistance gene cassette ($TEF1_{pro}$-Kan^{r}), which contains the
133 kanamycin resistance gene used as a dominant marker for Geneticin resistance in
134 eukaryotes (Jiménez and Davies 1980) under the control of the *A. gossypii* translation
135 elongation factor 1α promoter ($TEF1_{pro}$) and terminator ($TEF1_{ter}$), was amplified using
136 pPKT as the template (Kato and Park 2004) and the AgTEFproH3-F and
137 AgTEFterH3-R primers (Table 1). PCR was carried out using *LA-Taq* Hot Start Version
138 (TaKaRa Bio Inc.) under the following conditions: 1 cycle at 95ºC for 2 min followed
139 by 35 cycles at 95ºC for 30 s, 55ºC for 1 min, and 72ºC for 1.5 min. Final extension was
140 carried out with 1 cycle at 72ºC for 10 min. The amplified fragment was purified as
141 described above and inserted into the *Hin*dIII sites of pARM and pAUR123. The
142 resulting plasmids were designated pAMK and pARK, respectively. pAMK harbored

143 both *TEF1*$_{pro}$-*Kan*^{r} and *ACR268C*, while pARK contained only *TEF1*$_{pro}$-*Kan*^{r}.

144

145 PCR-based gene targeting disruption

146

147 The knockout mutant of the *ACR268C* gene was constructed by PCR-based gene
148 targeting disruption, as described by Wendland et al. (2000). Technical protocol of
149 transformation in *A. gossypii* was performed according to the method described by
150 Monshau et al. (1988) and Kanamasa et al. (2007) with some modifications. The 60-bp
151 homologous sequence of the *ACR268C* gene on both ends of *TEF1*$_{pro}$-*Kan*^{r} was
152 amplified using pPKT as the template, as shown in Scheme 1B, and the Agmls901-F
153 and Agmls1600-R primers (Table 1). PCR amplification was carried out using *LA-Taq*
154 Hot Start Version (TaKaRa Bio Inc.) under the following conditions: 1 cycle at 95°C for
155 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.
156 Final extension was carried out with 1 cycle at 72°C for 10 min. The PCR product was
157 purified as described above.

158 Spores of *Ag*WT (approximately 1.0×10^{6}) were grown on YD medium for 27 h.
159 The grown mycelia were harvested by filtration, washed with distilled water, and
160 suspended in 50 mM potassium phosphate buffer (pH 6.8) containing 25 mM
161 2-mercaptoethanol. The suspension was incubated at 30°C for 30 min with gentle
162 agitation, and the mycelia were collected by filtration and washed with transformation
163 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

185 detection were carried out with the AlkPhos Direct Labeling and Detection system (GE
186 Healthcare), according to the manufacturer's protocol. Positive signals were detected by
187 Fluor-S/MAX (Bio-Rad).
188 To confirm the transformants carrying pARK or pAMK, 10 μg of total DNA
189 from each transformant was introduced into *E. coli* cells, and the rescued plasmid was
190 confirmed by restriction enzyme mapping.
191
192 Quantification of malate synthase mRNA by real-time quantitative RT-PCR
193
194 *A. gossypii* mycelia grown in production medium in flask for 26 h were harvested by
195 filtration. Resulting mycelia were mixed with 0.3 g of acid washed glass beads (Sigma)
196 and 1ml of ISOGEN (NIPPON GENE Co., Ltd. Tokyo, Japan), and then fractured by
197 vigorous agitation. Complementary DNA was obtained by reverse transcription-PCR
198 using PrimeScript RT-PCR Kit (TaKaRa Bio Inc.) with the extracted total RNA as the
199 template with random 6 mers primer in the elongation condition at 40°C for 1 h. The
200 template RNA was degraded by 10 mg of RNase (Sigma) at 37°C for 30 min.
201 Messenger RNA levels of malate synthase and actin were quantified by using
202 FullVelocity SYBR Green QPCR Master Mix (Agilent Technologies Inc., CA, USA)
203 under the condition of 1 cycle at 95°C for 5 min, 60 cycles at 95°C for 10 sec and 60°C
204 for 30 sec for amplification plot, and 1 cycle at 95°C for 1 min, 55°C for 30 sex, and
205 95°C for 5 min for dissociation plot. QMLS1-F, -R and QACT1-F, -R primers (Table 1)

206 were used for malate synthase and actin, respectively. Actin was used as an internal
207 standard because of its constitutive expression.
208
209 Enzyme assay
210
211 The malate synthase activity was determined according to the method of Dixon and
212 Kornberg (1959). Ten microliters of the enzyme solution was added to 50 mM Tris-HCl
213 (pH 7.5), 5 mM $MgCl_2$, 2 mM sodium glyoxylate, and 50 μM acetyl-CoA (Wako), and
214 the final volume was made up to 1 ml. The specific absorbance of acetyl-CoA was
215 measured at 232 nm. One unit of malate synthase activity was defined as the amount of
216 enzyme required to deacetylate 1 micromole of acetyl-CoA per minute. Nonspecific
217 deacetylated acetyl-CoA was measured in the absence of $MgCl_2$ and sodium glyoxylate.
218 The protein concentration was determined by the Bradford method using a protein assay
219 kit (Bio-Rad) with bovine serum albumin as the standard.
220
221 Analytical methods
222
223 The riboflavin and residual oil concentrations were measured according to the method
224 previously described by Park and Ming (2004). The dry cell weight was measured as
225 follows. The mycelia from the culture broth were filtered using filter paper No. 5A
226 (Advantec, Tokyo, Japan). The mycelia paste was dried overnight in an oven at 100ºC,

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* (*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

248

249 The *ACR268C* gene was disrupted, and 6 Geneticin-resistant colonies of *A. gossypii*
250 were isolated with an efficiency of approximately 20 colony forming units (cfu)/μg of
251 DNA. The *ACR268C* gene disruption was confirmed by genomic PCR using the
252 AgMLS1-V1 and AgMLS1-V2 primers. The presence of the 2.7-kb fragment (lane 2 in
253 Fig. 1A) indicated the introduction of $TEF1_{pro}$-Kan^r into the *ACR268C* gene. This also
254 led to the identification of the Geneticin-resistant colony as an *ACR268C* gene
255 disruptant. Southern blot analysis was carried out to confirm the disruption of the
256 *ACR268C* gene. The 2.6-kb DNA fragment was detected using a chemiluminescent
257 kanamycin-resistance gene probe from the chromosomal DNA that had been
258 double-digested with *Bam*HI and *Xho*I at both ends of the *ACR268C* gene (Fig. 1B).
259 These results demonstrated that the $TEF1_{pro}$-Kan^r gene cassette was integrated into the
260 *ACR268C* gene, and the gene disruptant was designated *Ag*Δmls1.

261 To investigate the phenotypic variations between the *Ag*WT and *Ag*Δmls1
262 strains, both strains were cultured in the production medium. The specific malate
263 synthase activity of *Ag*Δmls1 was less than 5 mU/mg protein, which was one-seventh
264 that of the *Ag*WT strain (Fig. 2A). This indicated that replacement of the +961 to +1599
265 region of the *ACR268C* gene, including the 527^{th} aspirate residue, with $TEF1_{pro}$-Kan^r
266 leads to complete loss of enzyme activity. This resulted in a significant decrease in
267 riboflavin production by the *Ag*Δmls1 strain—approximately 10-fold less than that by
268 the *Ag*WT strain (Fig. 2B). The oil consumption and dry cell weight of *Ag*Δmls1 was

269 half or less than half that of the *Ag*WT strain (Fig. 2C and D).

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

279

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

282

283 The presence of the pARK and pAMK plasmids in the transformant was verified by
284 digesting the extracted plasmid DNA with *Kpn*I and *Xba*I. Electrophoretic analysis
285 demonstrated the presence of bands of size approximately 850 bp and 1.9 kb, which
286 represent the kanamycin-resistance gene and *ACR268C* gene, respectively (lane 3 in Fig.
287 4A). The transformants with *Ag*WT/pARK and *Ag*WT/pAMK were designated *Ag*WP
288 and *Ag*MLS1, respectively.

289 The specific malate synthase activity of *Ag*MLS1 was significantly higher than

290 the activities of the *Ag*WP (Fig. 4B) and *Ag*Δmls1 strains (Fig. 2A), indicating that the
291 *ACR268C* gene encodes malate synthase. When the *Ag*WP and *Ag*MLS1 transformants
292 were cultured in the production medium, the riboflavin production by *Ag*MLS1 was
293 1.7-fold higher than that by *Ag*WP (Fig. 4C), even though the values of oil consumption
294 and dry cell weight were similar to those of *Ag*WP (data not shown).
295 Real-time quantitative RT-PCR revealed that *MLS1* mRNA level of *Ag*MLS1
296 strain was approximately 1.6- and 8-fold higher than those of *Ag*WP and *Ag*Δmls1,
297 respectively (Figs. 5 and 4B). This indicates that the plasmid pAMK expressing *MLS1*
298 functions appropriately.
299
300 Additive effect of glyoxylate and malate on riboflavin production in the *ACR268C*
301 gene-disruptant
302
303 Malate synthase gene disruption led to a significant decrease in riboflavin production,
304 suggesting that continuous glyoxylate cycling and/or gluconeogenesis bypass from
305 malate might be blocked. Glyoxylate and malate are the substrate and product of malate
306 synthase, respectively. Therefore, to investigate the effects of glyoxylate and malate on
307 metabolic flux, the *Ag*WT and *Ag*Δmls1 strains were cultured in glyoxylate- or
308 malate-supplemented production medium. Remarkable phenotypic differences were not
309 observed between the strains upon culture in the 50 mM glyoxylate-supplemented
310 culture (data not shown). However, the 50 mM malate-supplemented cultures of *Ag*WT

311 and *Ag*Δmls1 strains showed higher riboflavin production, oil consumption, and dry cell
312 weight in comparison with the values of the control culture. The riboflavin
313 concentrations of the *Ag*Δmls1 and *Ag*WT strains were more than 2-fold higher than
314 those of the strains cultured without the malate additive (Fig. 6A). In particular, in the
315 culture of the *Ag*Δmls1 strain, 50 g/l of oil was completely consumed (Fig. 6B), which
316 differed drastically from the result obtained in the absence of the malate additive (Fig.
317 2C).
318
319 **Discussion**
320
321 In this study, the *ACR268C* gene was identified to encode malate synthase, and its role
322 in riboflavin biosynthesis in *A. gossypii* was investigated using rapeseed oil as the sole
323 carbon source. We analyzed the amino acid sequence of ACR268Cp and found
324 sequences that were similar to two active sites and the C-terminal peroxisome targeting
325 signal (-SRL) (Gould et al 1988) of *S. cerevisiae* malate synthase. In the *ACR268C*
326 gene-disruptant, the 527th aspartate, which functions as a proton donor, was replaced
327 with the kanamycin-resistance gene, and the disruptant did not exhibit any malate
328 synthase activity (Fig. 2A). This strain also showed decreases in the riboflavin
329 concentration, oil consumption, and dry cell weight. Therefore, malate synthase is
330 important for riboflavin biosynthesis and the assimilation of vegetable oils. This is
331 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 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 level (Figs. 4B-C and 5). Therefore we expect and suggest that adoption of strong expression promoters may

353 increase the riboflavin yield to a better extent in this experiment.

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

369 Using an *MLS1* disruptant and *MLS1*-overexpressing transformant, we
370 demonstrated that malate synthase is one of important key enzymes for improving
371 riboflavin production in *A. gossypii*. However, riboflavin production of *Ag*WP and
372 *Ag*MLS1 strains was half or less than that of the wild-type. It may be due to addition of
373 antibiotics for maintaining subnuclear plasmid, resulting in lower mycelial growth of

374 *Ag*WP strain than that of *Ag*WT strain (data not shown). Kato and Park (2004) showed
375 similar phenomenon with 2 μm origin plasmid transformants as well as yeast
376 autonomously replicating sequence (*ARS1*) contained in pARK and pAMK, both of
377 which were functioned in *A. gossypii* as replication origins (Wright and Philippsen
378 1991). Therefore, to further improve riboflavin production, it is necessary to
379 overexpress important key genes in the *A. gossypii* genome. Jiménez et al. (2008) have
380 succeeded in chromosomal integration by gene-targeting recombination using a specific
381 gene cassette with tandem placement of a drug-resistant gene and riboflavin
382 production-positive gene expression cassettes. In the near future, chromosomal
383 integration by malate synthase recombination will be useful for improving the riboflavin
384 yield of *A. gossypii*.
385
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389
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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] Underlines indicate restriction enzyme site.

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

475 **Figure legends**

476 **Scheme 1** (A) Flow diagram of plasmid construction. Black arrows indicate *TEF1*
477 promoter-kanamycin-resistance gene-*TEF1* terminator ($TEF1_{pro}$-Kan^r). Diagonal
478 squares denote the *ACR268C* (*MLS1*) gene, which was amplified using the
479 AgMLS1-Ex-F and –R primers. (B) Disruption of the *ACR268C* gene in the *A.*
480 *gossypii* wild-type strain. The asterisk and diagonal box represent the 527th codon
481 encoding the aspartate residue, which acts as a proton donor, and a 60-bp
482 homologous region of the *ACR268C* gene, respectively.

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

491 **Fig. 2** Time course of malate synthase activity (A), riboflavin concentration (B),
492 residual oil concentration (C), and dry cell weight (D) in cultures of the *Ag*WT
493 (closed rhombus) and *Ag*Δmls1 (opened triangle) strains. Both cultures were carried
494 out in triplicates, and the average data and standard deviations are shown.

495 **Fig. 3** Morphological changes in the *ACR268C* (*MLS1*) gene disruptant and *Ag*WT
496 (A-C) and *Ag*Δmls1 (a-c) strains. The mycelia are shown at 3 d (A and a), 5 d (B

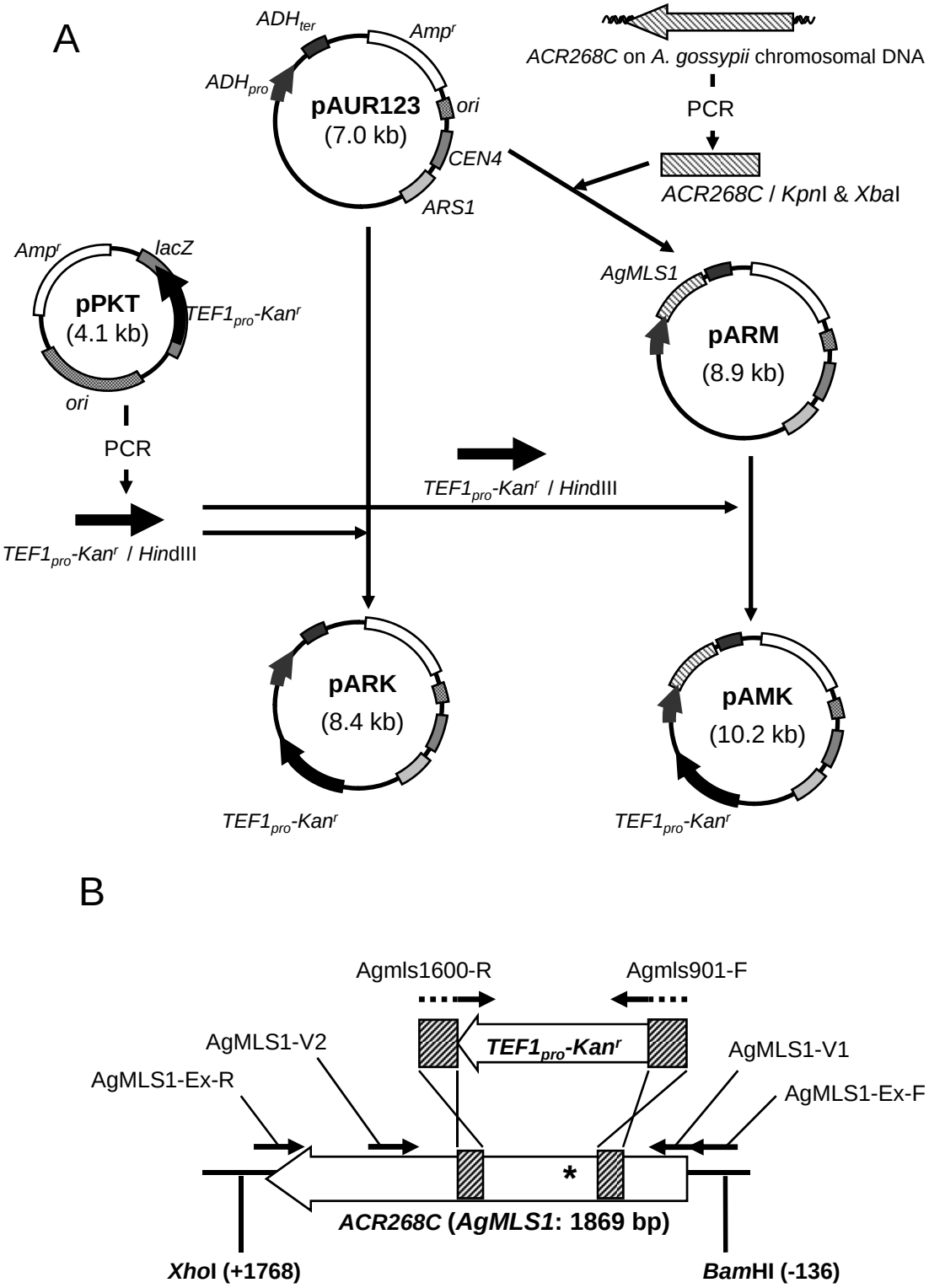
and b), and 7 d (C and c) of culture. HM, O, FM, R, CR, and S denote hypertrophic mycelia, residual oil droplet, filiform mycelia, riboflavin, crystallized riboflavin, and spore, respectively. Bars indicate a 10-μm scale.

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 (opened 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.

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).

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.

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.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Amp^r
ADH$_{pro}$
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ori
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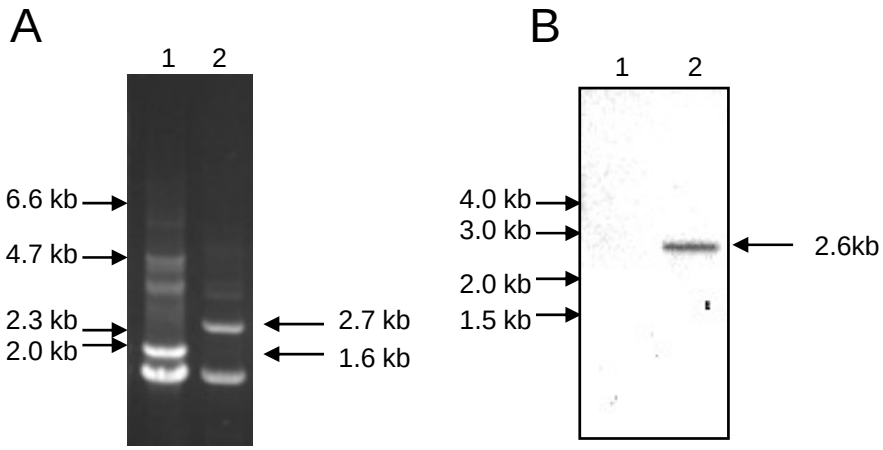
ARS1
ACR268C on A. gossypii chromosomal DNA
PCR
ACR268C / KpnI & XbaI
Amp^r
lacZ
pPKT
(4.1 kb)
TEF1$_{pro}$-Kan^r
ori
PCR
TEF1$_{pro}$-Kan^r / HindIII
AgMLS1
pARM
(8.9 kb)
TEF1$_{pro}$-Kan^r / HindIII
pARK
(8.4 kb)
TEF1$_{pro}$-Kan^r
pAMK
(10.2 kb)
TEF1$_{pro}$-Kan^r
B
Agmls1600-R
Agmls901-F
AgMLS1-V2
TEF1$_{pro}$-Kan^r
AgMLS1-V1
AgMLS1-Ex-R
AgMLS1-Ex-F
*
ACR268C (AgMLS1: 1869 bp)
XhoI (+1768)
BamHI (-136)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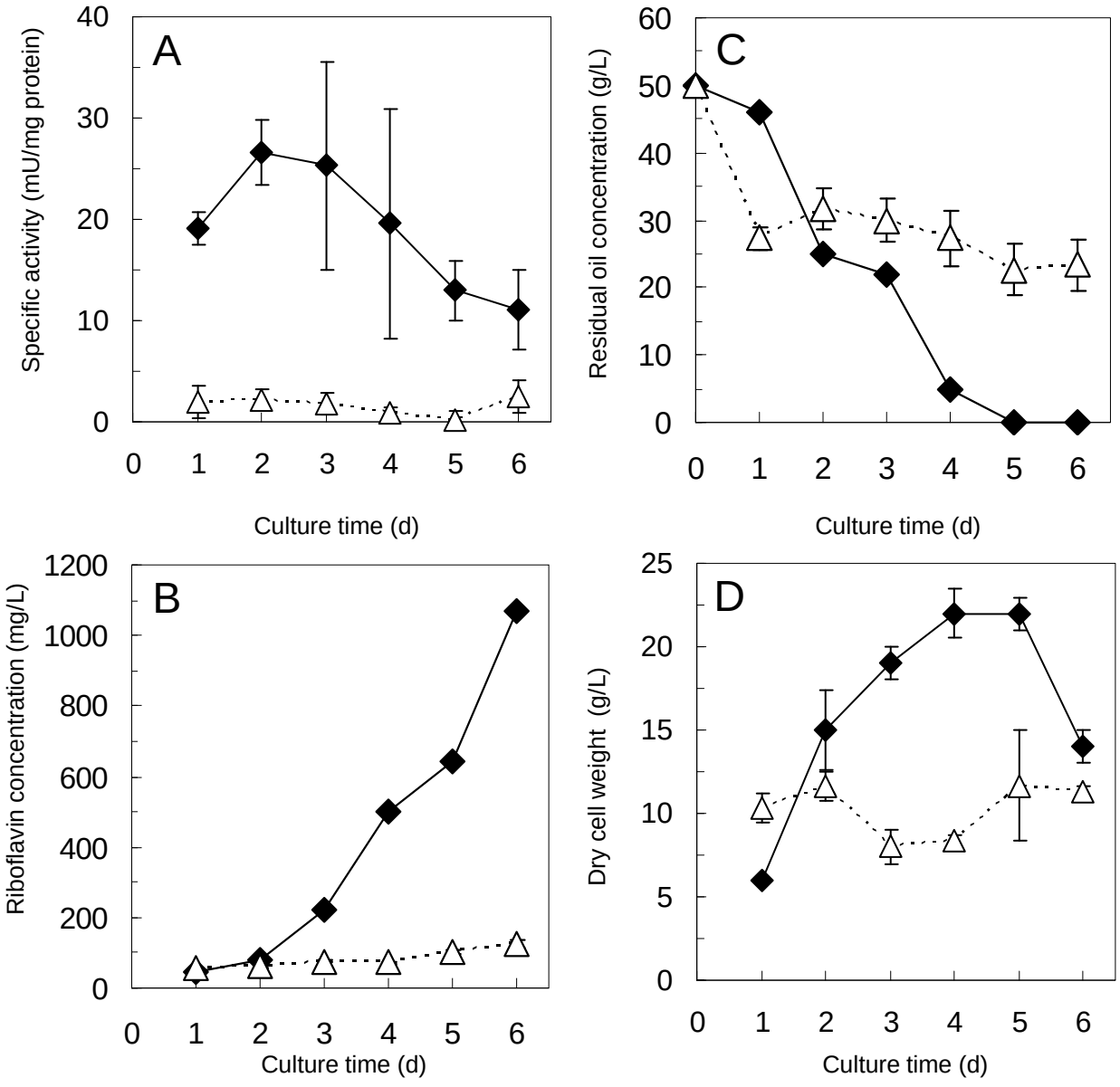
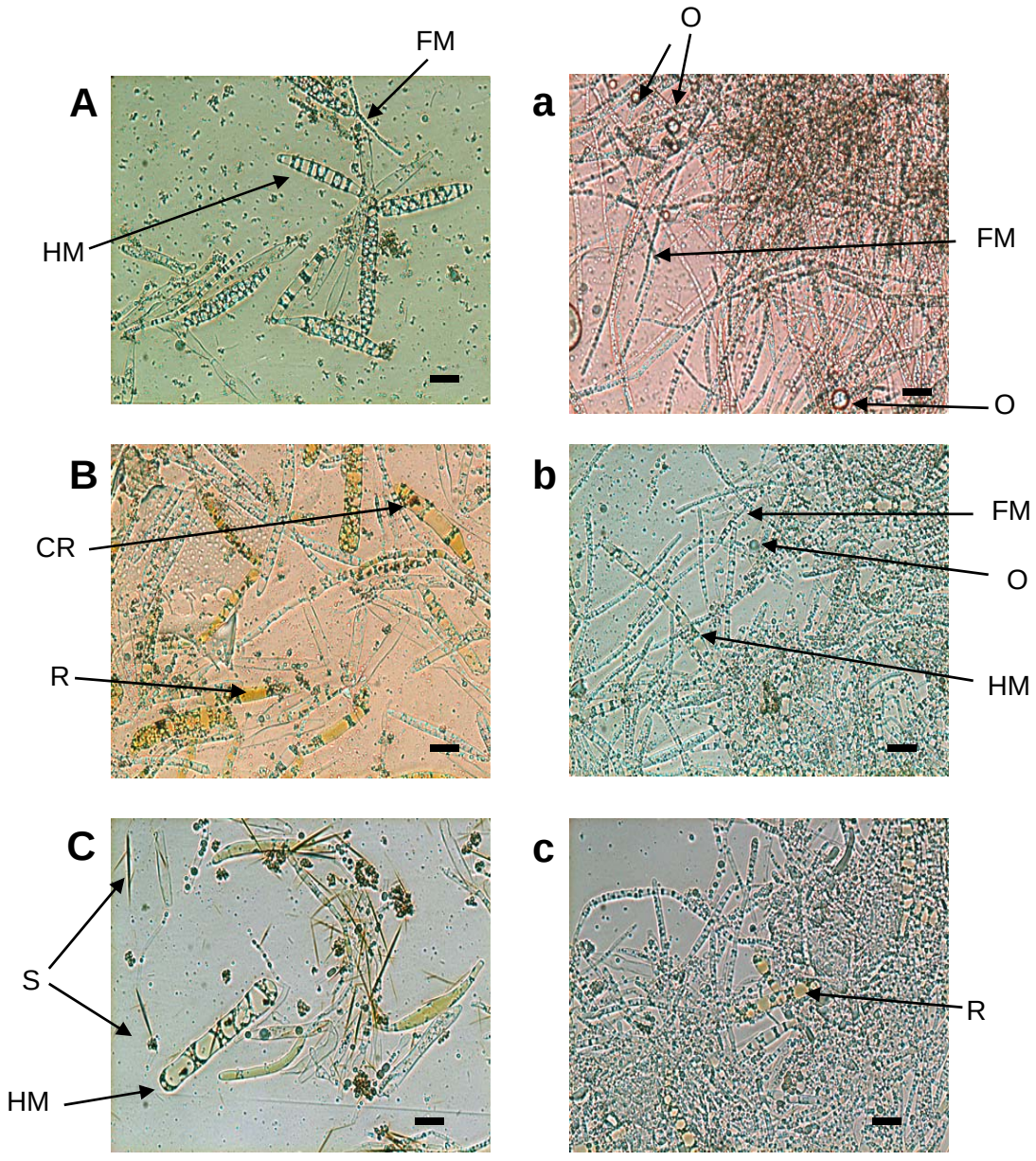
1.5 kb
2.6kb


A
Specific activity (mU/mg protein)
Culture time (d)
B
Riboflavin concentration (mg/L)
Culture time (d)
C
Residual oil concentration (g/L)
Culture time (d)
D
Dry cell weight (g/L)
Culture time (d)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FM
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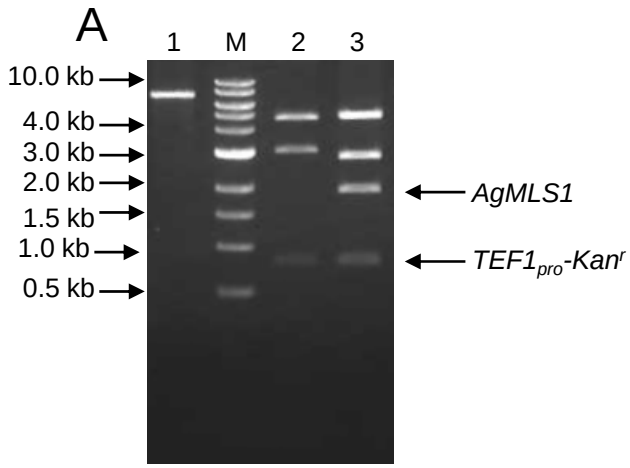
B
CR
R
b
FM
O
HM
C
S
HM
c
R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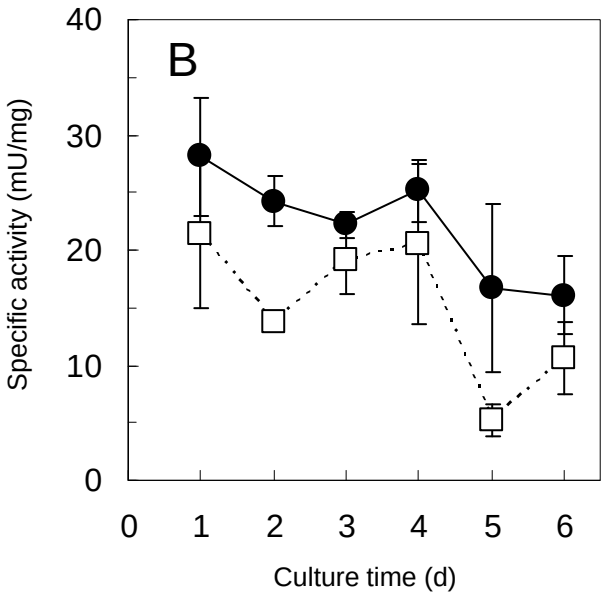
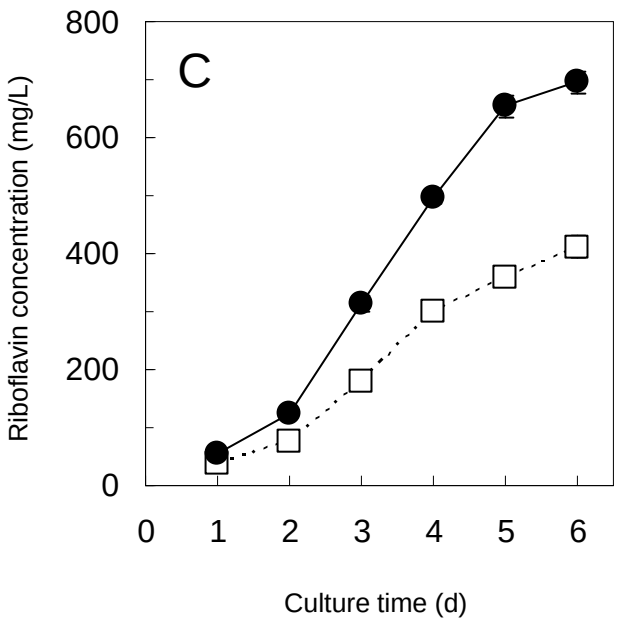
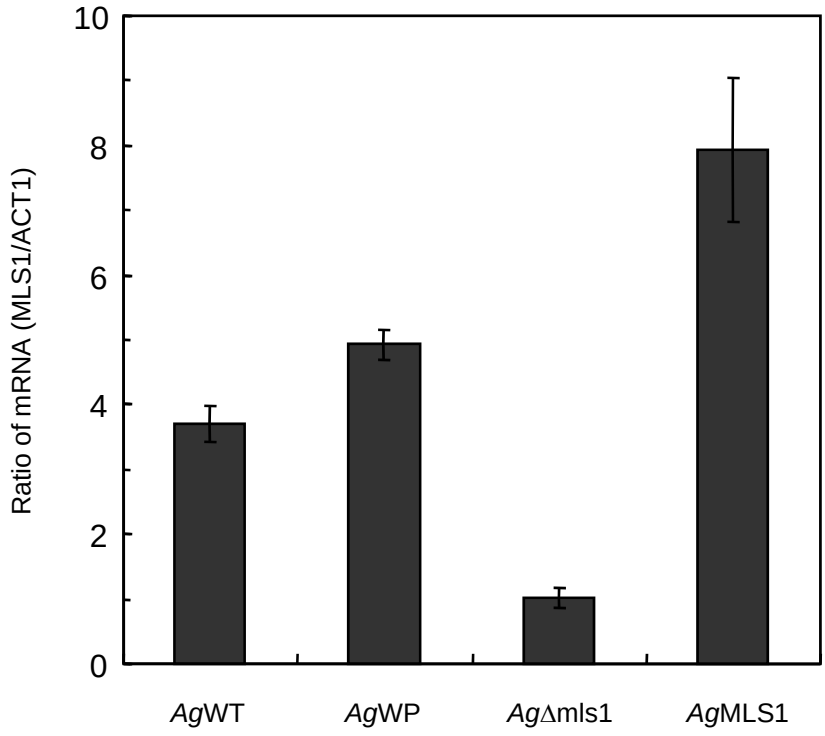
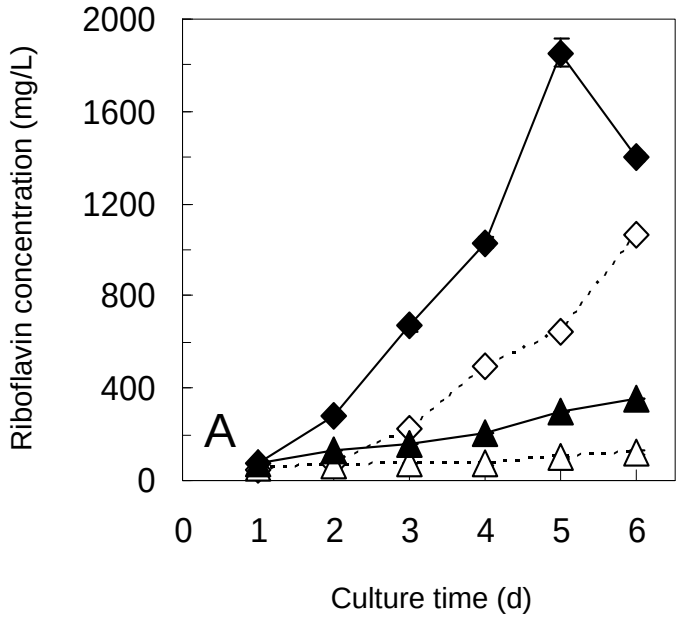
AgMLS1
TEF1pro-Kanr


B
Specific activity (mU/mg)
0
10
20
30
40
0
1
2
3
4
5
6
Culture time (d)


C
Riboflavin concentration (mg/L)
0
200
400
600
800
0
1
2
3
4
5
6
Culture time (d)


10
8
6
4
2
0
Ratio of mRNA (MLS1/ACT1)
AgWT
AgWP
AgΔmls1
AgMLS1


Riboflavin concentration (mg/L)
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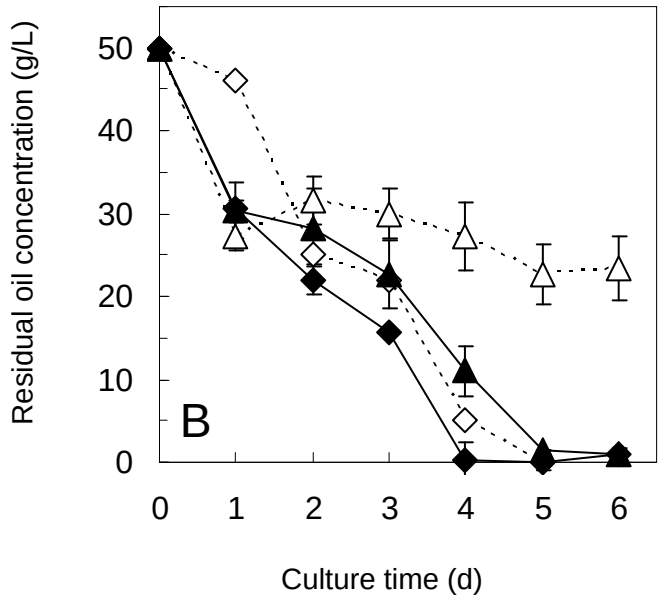
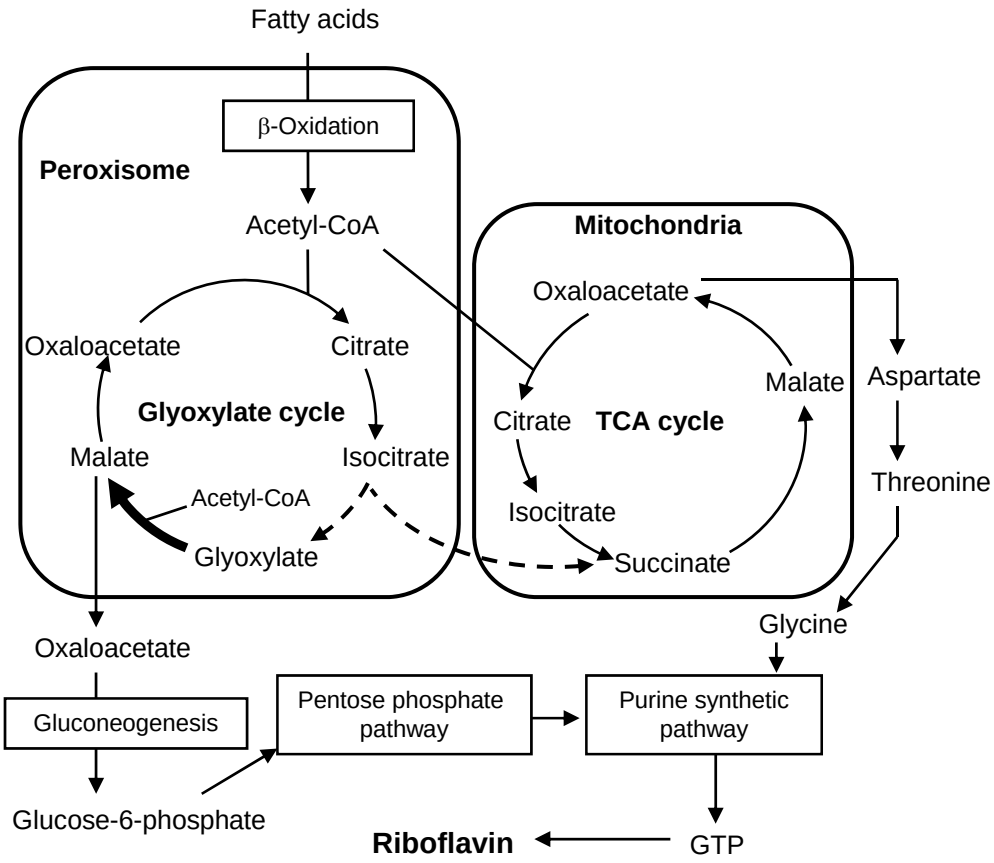
1600
1200
800
400
0
A
0
1
2
3
4
5
6
Culture time (d)


Residual oil concentration (g/L)
50
40
30
20
10
0
B
0
1
2
3
4
5
6
Culture time (d)


Fatty acids
β-Oxidation
Peroxisome
Acetyl-CoA
Mitochondria
Oxaloacetate
Citrate
Glyoxylate cycle
Malate
Isocitrate
Acetyl-CoA
Glyoxylate
Oxaloacetate
Citrate
TCA cycle
Malate
Isocitrate
Succinate
Aspartate
Threonine
Glycine
Oxaloacetate
Gluconeogenesis
Pentose phosphate pathway
Purine synthetic pathway
Glucose-6-phosphate
Riboflavin
GTP