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Functional analysis of cis-aconitate decarboxylase and trans-aconitate metabolism in a riboflavin producing filamentous *Ashbya gossypii*

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Abstract

In *Ashbya gossypii*, isocitrate lyase (ICL1) is a very crucial enzyme for riboflavin production. Itaconate, the inhibitor of ICL1, has been used as an antimetabolite for mutagenic studies in *A. gossypii*. It has been reported that itaconate is produced from *cis*-aconitate by *cis*-aconitate decarboxylase (CAD1) in *Aspergillus terreus*. In this study, identification of CAD1 gene and determination of the presence of itaconate in the riboflavin biosynthetic pathway in *A. gossypii* were carried out to confirm itaconate metabolism. Although no CAD1 candidate gene was found and no itaconate production was observed, *cis*- and *trans*-aconitate were detected in the riboflavin production phase. It is known that *trans*-aconitate inhibits aconitase (ACO1) in the tricarboxylic acid cycle. In *A. gossypii*, the transcription level of AGR110Wp, the homolog of *trans*-aconitate 3-methyltransferase (TMT1), was enhanced by almost threefold during riboflavin production than that during its growth phase. TMT1 catalyzes the methylation reaction of *trans*-aconitate in *Saccharomyces cerevisiae*. Thus, these results suggest that the enhancement of the transcription level of this TMT1 homolog decreases the *trans*-aconitate level, which may mitigate the inhibition of ACO1 by oxidative stress in the riboflavin biosynthetic pathway in *A. gossypii*. This is a novel finding in *A. gossypii*, which may open new metabolic engineering ideas for improving riboflavin productivity.

[**Keywords:** Riboflavin; *Ashbya gossypii*; Isocitrate lyase; *cis*-Aconitate decarboxylase; Antimetabolite; *trans*-Aconitate 3-methyltransferase]

INTRODUCTION

Riboflavin (vitamin B_2) is a yellow, water-soluble compound and is an essential growth factor in mammals, plants, and microorganisms, acting as a precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are required for various enzymatic reactions as electron acceptors for oxidoreductases. Riboflavin is used in many kinds of food additives and multivitamin supplements as a colorant and/or nutrient for human health, and a large amount of it is utilized as feed additives for livestock (1). The filamentous fungus *Ashbya gossypii* is used as a riboflavin producer at an industrial scale with vegetable oil as the sole carbon source.

Several studies have predicted the biosynthetic pathway from fatty acids to riboflavin and elucidated the important pathways, which are employed for improving riboflavin productivity. For enzymatic functional analysis, threonine aldolase (2) and alanine:glyocylate aminotransferase (3) have been overexpressed, and the effects of serine hydroxymethyltransferase isoenzyme (4), isocitrate dehydrogenase, isocitrate lyase (5), phosphoribosyl pyrophosphate synthase (6), and malate synthase (7) have been examined. These studies had been based on the metabolic engineering of purine pathway (8). It is believed that glyoxylate cycle has an important role in riboflavin productivity, because it had been identified as the origin of carbon metabolism after β-oxidation. The turnover of glyoxylate cycle could be facilitated by collaboration with tricarboxylic acid (TCA) cycle, because the TCA cycle could supply isocitrate for glyoxylate cycle. Aconitase (ACO1; E.C. 4.2.1.3) catalyzes a reversible reaction from citrate to isocitrate, with *cis*-aconitate as an intermediate. However, ACO1 exhibits functional deficiency in peroxisome because of

53 oxidative stress (9, 10). Till date, there are only a few studies in the literature on the
54 functional identification of the metabolic enzymes in the TCA cycle related to the riboflavin
55 biosynthetic pathway in *A. gossypii*.

56 On the other hand, numerous studies have been carried out to examine the glyoxylate
57 cycle to improve riboflavin productivity in *A. gossypii*. Schmidt et al. (11) demonstrated that
58 the activity of isocitrate lyase (ICL1; E.C. 4.1.3.1), which is one of the specific enzymes in
59 the glyoxylate cycle, is positively correlated with riboflavin productivity in *A. gossypii* using
60 soybean oil as the sole carbon source. It has been reported that the ICL1 catalyzes the
61 cleavage of isocitrate to form succinate and glyoxylate, but is strongly inhibited by
62 itaconate (12). Therefore, itaconate had been used as an antimetabolite for the isolation of
63 mutants producing high amount of riboflavin in conventional mutagenic studies to improve
64 riboflavin productivity (13,14). Moreover, engineering of *A. gossypii* to confer itaconate
65 biosynthetic ability might efficiently improve riboflavin production. However, there are no
66 reports about the corresponding itaconate biosynthetic enzyme in *A. gossypii*, which has not
67 yet been identified. The itaconate biosynthetic pathway was identified by Bonnarme et al.
68 (15) in *Aspergillus terreus,* who concluded that *cis*-aconitate decarboxylase (CAD1; E.C.
69 4.1.1.6) catalyzes the decarboxylation of *cis*-aconitate to itaconate. Subsequently, Dwiarti et
70 al. purified CAD1 from *A. terreus* (16) and Kanamasa et al. succeeded in cloning the gene of
71 CAD1 (17).

72 In the present study, we aimed to identify itaconate and the corresponding itaconate
73 biosynthetic enzyme in the riboflavin biosynthetic pathway in *A. gossypii* during riboflavin
74 production using vegetable oil as the sole carbon source. Measurements of CAD1 activity,

intra- and extracellular itaconate, and intracellular accumulation of *trans*-aconitate were carried out. Based on our investigation, the activities of CAD1 and ACO1 as well as accumulation of itaconate and *trans*-aconitate during the riboflavin biosynthetic pathway in *A. gossypii* were discussed. The findings of this study are novel and may be important for improving riboflavin productivity in *A. gossypii*.

MATERIALS AND METHODS

Cell lines and culture conditions *A. gossypii* (ATCC 10895, Manassas, VA, USA) was used as the riboflavin producer, and *A. terreus* TN484-M1, an itaconate overproducing strain (18), was used as the positive control. *A. gossypii* was grown in YD medium (pH 6.8) containing 1% (w/v) yeast extract (Oriental Yeast Co., Ltd., Tokyo, Japan) and 1% (w/v) glucose (Wako Pure Chem. Ind. Ltd., Osaka, Japan) for growth, and in YR medium (pH 6.8) containing 1% (w/v) yeast extract and 1% (w/v) rapeseed oil (Wako) for riboflavin production.

A. terreus TN484-M1 was grown in seed medium (pH 3.0) containing the following (per l): 55.5 g, glucose; 5.2 g, corn steep liquor; 4.1 g, $(NH_4)_2SO_4$ (Wako); 6.3 g, KNO_3 (Wako); and 2.0 g, $MgSO_4 \cdot 7H_2O$ (Wako). For itaconic acid production using *A. terreus* TN484-M1, the production medium (pH 2.0) was used, which comprised the following (per l): 140 g, glucose; 2.1 g, corn steep liquor; 2.9 g, NH_4NO_3 (Wako); and 1.8 g, $MgSO_4 \cdot 7H_2O$. For DNA microarray analysis, *A. gossypii* was cultured in optimized medium (19) containing the following (per l): 90.5 g, rapeseed oil; 40.3 g, corn steep liquor (Sigma Chem. Co., St. Louis, MO, USA); 36.1 g, yeast extract; 15.0 g, soybean mill (Wako); 2.0, glycine (Wako); 0.2 g,

alanine (Wako); 0.47 g, glutamic acid (Wako); 1.5 g, KH_2PO_4 (Wako); and mineral ions (2 μg/l of Co^{2+}, 5 μg/l of Mn^{2+}, 10 μg/l of Zn^{2+}, and 1 μg/l of Mg^{2+}). The pH of the medium was adjusted to 6.8 using 1M KOH. Cultures of *A. terreus* TN484-M1 and *A. gossypii* strains were grown in 500-ml shake flasks with a working volume of 50 ml in a rotary shaker (TB-25R, Takasaki Scientific Instrument Co., Kawaguchi, Saitama, Japan) at an agitation rate of 220 rpm and 28°C.

Amino acid sequence homology search BRENDA (http://www.brenda-enzymes.org/) and BLAST in Saccharomyces genome database (SGD; http://www.yeastgenome.org/cgi-bin/blast-fungal.pl) were used for the acquisition of amino acid sequences and fungal homology search including *A. gossypii*, respectively. For the identification of CAD1 candidates of *A. gossypii*, the amino acid sequence from *A. terreus* TN484-M1 (DNA Data Bank of Japan accession number; AB326105) (17) was used as the query for BLAST. The candidate proteins were annotated by Ashbya Genome Database (AGD; http://agd.vital-it.ch/index.html) and described in the study by Gattiker et al. (20). Sequence analysis by multi-alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Enzyme assays A crude enzyme solution was prepared as follows. The mycelia of *A. gossypii* and *A. terreus* TN484-M1 strains grown in the respective media were harvested by filtration through filter paper No. 5A (Advantec, Tokyo, Japan). The filtrates were used for the measurement of extracellular itaconate by using high performance liquid chromatography (HPLC), as described in the Analytical Methods Section. Each of the collected wet mycelia sample was suspended in 200 μl of cooled buffer comprising 200 mM sodium phosphate

buffer (pH 6.2), 1 mM EDTA, and 5 mM dithiothreitol. The mycelia were fractured by sonication for three times at 40 Ω for 30 s with 1-min intervals on ice using an ultrasonicator (Sonics & Materials Inc., Newtown, CT, USA). The resulting homogenate was centrifuged at 14,000 × *g* for 30 min at 4°C, and the supernatant was used for the enzyme assays.

The CAD1 activity was measured according to the method described by Bentley and Thiessen (21) with some modifications. A total of 50 μl of the enzyme solution were added to 800 μl of 200 mM sodium phosphate buffer (pH 6.2) containing 8 mM (final concentration) *cis*-aconitic acid (Sigma-Aldrich Co., St. Louis, MO, USA), and incubated for 15 min at 30°C. The enzyme reaction was terminated by addition of 50 μl of 12 N HCl. The intracellular and extracellular itaconate concentrations were measured by HPLC. One unit of enzyme activity was defined as the amount of enzyme required to form 1 μmol itaconate per minute. The ICL1 activity was measured according to the method described by Schmidt et al. (11). The total protein concentration was evaluated by the Bradford method using a protein assay kit (Bio-Rad) with bovine serum albumin (Sigma-Aldrich) as the standard.

DNA microarray analysis Briefly, the harvested mycelia of *A. gossypii* were frozen at −80°C and used for RNA extraction. The RNA extraction and its quality check were performed by Nihon Gene Research Lab. Inc. (Sendai, Japan). Custom arrays of *A. gossypii* were manufactured by Roche NimbleGen Inc. (Tokyo, Japan) using 4 × 72K (4 plex) format. Each slide contained 4726 ORFs of *A. gossypii*. Double-stranded cDNAs were synthesized using oligo-dT and labeled with Cy3 dye. The labeled cDNAs were hybridized to custom arrays, and the data were extracted by NimbleGem software and analyzed by DNASTAR ArrayStar v3.0 (Madison, WI, USA).

Analytical methods Riboflavin and residual oil concentrations were measured by methods previously described by Park and Ming (22). The dry cell weight was measured as follows. The mycelia from the culture broth were filtered using filter paper No. 5A (Advantec) and dried overnight in an oven at 100°C. Detection of itaconate, *cis*-aconitate, and *trans*-aconitate was performed by using HPLC (LC-8A; Simadzu Co., Kyoto, Japan). A total of 10 μl of appropriately diluted sample were applied to a column (GAPCELL PAK C_{18} MG; 5.0 μm and 3.0 × 250 mm; Shiseido Co., Ltd., Tokyo, Japan) through a guard column (CAPCELL PAK C_{18} MG; 5.0 μm and 3.0 × 35 mm; Shiseido), both of which were thermally insulated at 45°C (CTO-6A; Simadzu). Each compound was detected by UV detector (SPD-20A; Simadzu) at 210 nm. A mobile phase consisting of 1.2% (v/v) acetonitrile and 0.3% (v/v) phosphoric acid was prepared, degassed by sonication for 1 h, and used at a flow rate of 0.5 ml/min. The resulting chromatogram was re-analyzed and modified by CLASS-VP (Shimadzu). The detection limit of itaconate in this system was approximately 0.2 μM.

RESULTS

Homology search of *A. gossypii* CAD1 with the amino acid sequence of *A. terreus* CAD1 In the BLAST homology search against *A. terreus* CAD1 amino acid sequence (17), five proteins of *A. gossypii* were hit as the CAD1 candidates (Table 1). However, multi-alignment using ClustalX 2 (23) revealed that all the five candidates had low homologies and no conserved region against *A. terreus* CAD1 (data not shown). These results indicated that *A. gossypii* has no or lesser ability to synthesize itaconic acid by using CAD1.

CAD1 activity of *A. gossypii* To confirm that *A. gossypii* has no or lesser ability to

161 synthesize itaconic acid using CAD1, its CAD1 activity was measured. A few previous
162 investigations (13,17) had revealed that a mobile phase with higher polarity (than that
163 employed in the present study) was necessary to achieve a definite independent peak of
164 itaconate on HPLC analysis. The chromatograph of 4-day *A. terreus* culture showed
165 independent itaconate peak (Peak 3 of Fig. 1A) at a retention time of 14.8 min, which was
166 reproducible in the loading itaconate standard. Furthermore, *cis*-aconitic acid (Peak 1 of Fig.
167 1A) and *trans*-aconitic acid (Peak 2 of Fig. 1A) were detected in both *A. gossypii* and *A.*
168 *terreus*; however, the itaconate peak (Peak 3 of Fig. 1A) was not observed for *A. gossypii*, but
169 was noted for *A. terreus*. Moreover, the specific CAD1 activity of *A. terreus* TN484-M1
170 increased up to 0.25 mU/mg until 4 days of culture, but that of *A. gossypii* was not detected
171 (Fig. 1B). On the other hand, the ICL1 activity was detected in *A. gossypii* during the cell
172 growth phase (Fig. 1C). These results suggested that the CAD1-mediating biosynthetic
173 pathway from *cis*-aconitate to itaconate is absent in riboflavin biosynthesis in *A. gossypii*.

174 **Intra- and extracellular itaconate in *A. gossypii*** To confirm whether *A. gossypii*
175 synthesizes itaconate in the riboflavin production phase or not, intracellular itaconate
176 concentration was measured. The intracellular itaconate peak at 14.8 min was not detected in
177 the chromatograms of *A. gossypii* cultured for 1–4 days (Peak 3 of Fig. 2A–D). In addition,
178 both extra- and intracellular itaconate were also not detected (data not shown). Hence, these
179 results indicated that *A. gossypii* does not have the ability to synthesize itaconate in the
180 riboflavin biosynthetic pathway.

181 **Novel prediction of *trans*-aconitate metabolism in *A. gossypii*** In *A. gossypii,*
182 *trans*-aconitate (Peak 2 of Fig. 2A–D) tended to get accumulated intracellularly, measuring at

least >50 μmol/g dry cell weight during the whole culture periods. This indicated that *A. gossypii* could possibly carry out *trans*-aconitate metabolism by using *trans*-aconitate methyltransferase (TMT1; E. C.2.1.1.145), identified in *Saccharomyces cerevisiae,* which catalyzes the monomethyl esterification of *trans*-aconitate and related compounds (24, 25). The genetic homology and a particular pattern of synteny of TMT1 with the *A. gossypii* homolog, AGR110Wp, were found to be > 90% (26) (Fig. 3). Comparison of the amino acid sequences of both TMT1 and AGR110Wp exhibited low error value, indicating that the *A. gossypii* homolog might function similar to TMT1 (3.9e-61, 42% identity and 59% similarity; Fig. 3). Gawron and Jones reported that *trans*-aconitate strongly inhibits ACO1 (K_i; 25 μM) (27). During riboflavin production in *A. gossypii,* ACO1 might be inhibited by intracellular accumulation of *trans*-aconitate. A product of this TMT1 enzymatic reaction has been reported to mitigate ACO1 inhibition by approximately one-seventh times, when compared with *trans*-aconitate (24). These results suggested that TMT1 may be involved in riboflavin production in *A. gossypii*.

Expression pattern of *TMT1* in *A. gossypii* during riboflavin production To confirm the presence of TMT1 in the riboflavin biosynthetic pathway in *A. gossypii*, transcription analysis was carried out. The samples were harvested at each significant point: growth phase (S1), beginning of riboflavin production phase (S2), and riboflavin production phase (S3), which were distinguished by time-dependent changes in the residual oil concentration, dry cell weight, and riboflavin concentration (Fig. 4A). The expression level of *TMT1* showed an increase in the riboflavin production phase (threefold higher than that in S1), whereas both the levels of *ACT1* (actin gene) and *ACO1* synchronously decreased or

remained the same (Fig. 4B). This result indicated that *trans*-aconitate metabolism and/or mitigation of ACO1 inhibition by TMT1 could have been activated during riboflavin production in *A. gossypii*.

DISCUSSION

Itaconic acid is one of the strong inhibitors of ICL1 activity (K_i: 170 μM), which is negatively correlated with riboflavin productivity in *A. gossypii* (11) and is used as an effective antimetabolite to acquire mutants producing high amount of riboflavin for improving riboflavin productivity (13,14). In the present study, we tried to confirm the presence of intra- and extracellular itaconate in *A. gossypii* during the riboflavin production phase, and found no significant peak of the compound in HPLC analysis. Considering the detection limit (0.2 μM), it was concluded that ICL1 was not inhibited by itaconate in *A. gossypii* during riboflavin production, although *A. gossypii* could synthesize the inhibitor.

As a novel finding, *trans*-aconitate was detected, whose intracellular accumulation was more than that of the *cis* form during riboflavin production in *A. gossypii* (Fig. 2). As *trans*-aconitate strongly inhibits ACO1, which is one of the key enzymes in the TCA cycle as well as in riboflavin biosynthesis, ACO1 in *A. gossypii* may have been inhibited by intracellular *trans*-aconitate (Fig. 5). It has been speculated that the intracellular *trans*-aconitate might be generated spontaneously from the *cis* form because of its instability (28). With regard to *trans*-aconitate metabolism, two enzymes are known to react with *trans*-aconitate as the substrate. One is aconitate delta-isomerase (E.C. 5.3.3.7) found in some bacteria (*Pseudomonas* sp.) (29), but not reported in yeasts, which interactively converts

226 *trans*-aconitate to *cis*-aconitate. The other is TMT1, described earlier (annotated as
227 AGR110Wp in *A. gossypii*; Fig. 3), which catalyzes the methylation reaction of
228 *trans*-aconitate. Transcription analysis of the *TMT1* gene revealed that the expression of
229 *TMT1* was enhanced by almost threefold in the course of riboflavin production phase in *A.*
230 *gossypii*, when compared with that in the growth phase. On the other hand, transcription of
231 *ACT1* (actin) and *ACO1* (aconitase) was not enhanced during the riboflavin production phase.
232 This suggested that the product of TMT1 (AGR110Wp) reaction may have strongly mitigated
233 ACO1 inhibition in *A. gossypii*. From this finding, it could be hypothesized that during
234 riboflavin production, the decrease in intracellular *trans*-aconitate causes alleviation of ACO1
235 inhibition and improvement in riboflavin production (Fig. 5 and Table 2). This hypothesis is
236 supported by the study by Cai et al. (24), who demonstrated that TMT1 activity markedly
237 increased during the diauxic metabolic transition in *S. cerevisiae*. As sporulation of *A.*
238 *gossypii* could be initiated after production of a certain amount of riboflavin (30), TMT1
239 homolog expression in *A. gossypii* might induce alteration in the metabolism from its growth
240 cycle to riboflavin production and sporulation cycles. Moreover, the expression of TMT1 in *A.*
241 *gossypii* may also contribute to activation of amino acid synthetic pathway, because TMT1
242 has been reported to catalyze a novel biosynthetic branch reaction of the leucine biosynthetic
243 pathway (31). Nevertheless, to prove the role of TMT1 in *A. gossypii*, it is necessary to
244 accurately determine the function of AGR110Wp.

245 In the present study, we found a novel antimetabolite, *trans*-aconitate, which is more
246 available than itaconate for improving riboflavin productivity in *A. gossypii*. Nevertheless,
247 metabolomics analysis and DNA microarray technique are needed to acquire further

information on the targeting genes and/or important metabolic pathway for improving riboflavin productivity in *A. gossypii* through metabolic optimization using genetic and chromosomal engineering.

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Figure legends

FIG. 1. HPLC chromatograms for *A. gossypii* and *A. terreus* cultured for 4 days (A) and the specific activities of CAD1 (B) and ICL1 (C) in *A. terreus* and *A. gossypii*. Numbers 1, 2, and 3 in (A) denote *cis*-aconitate acid, *trans*-aconitate, and itaconate, respectively. Open rhombuses and closed circles in (B) denote the specific CAD1 activity in *A. terreus* and *A. gossypii*, respectively.

FIG. 2. HPLC chromatograms for *A. gossypii* cultured in YR medium for 1 day (A), 2 days (B), 3 days (C), and 4 days (D). Numbers 1, 2, and 3 in (A) denote *cis*-aconitate, *trans*-aconitate, and itaconate, respectively.

FIG 3. Multi-alignment of amino acid sequences of TMT1 in *S. cerevisiae* and its homolog, AGR110Wp, in *A. gossypii*.

FIG 4. Riboflavin production (A) and expression level of *ACT1*, *ACO1*, and *TMT1* (B) in *A. gossypii* in each culture period. (A) Concentrations of residual oil, dry cell weight, and riboflavin during the culture period of 1–7 days. S1, S2, and S3 denote mycelial sampling times in the growth phase, beginning of riboflavin production phase, and riboflavin production phase, respectively. Arrows indicate mycelial sampling points (S1–S3) for DNA microarray analysis. Open squares, closed triangles, and closed circles in (A) denote residual oil concentration, dry cell weight, and riboflavin concentration, respectively. (B) Relative expression level of *ACT1*, *ACO1*, and *TMT1* in each culture period. Relative gene expression ratio in (B) was calculated based on the gene expression level in S1. *ACT1* was used as internal standard of gene expression.

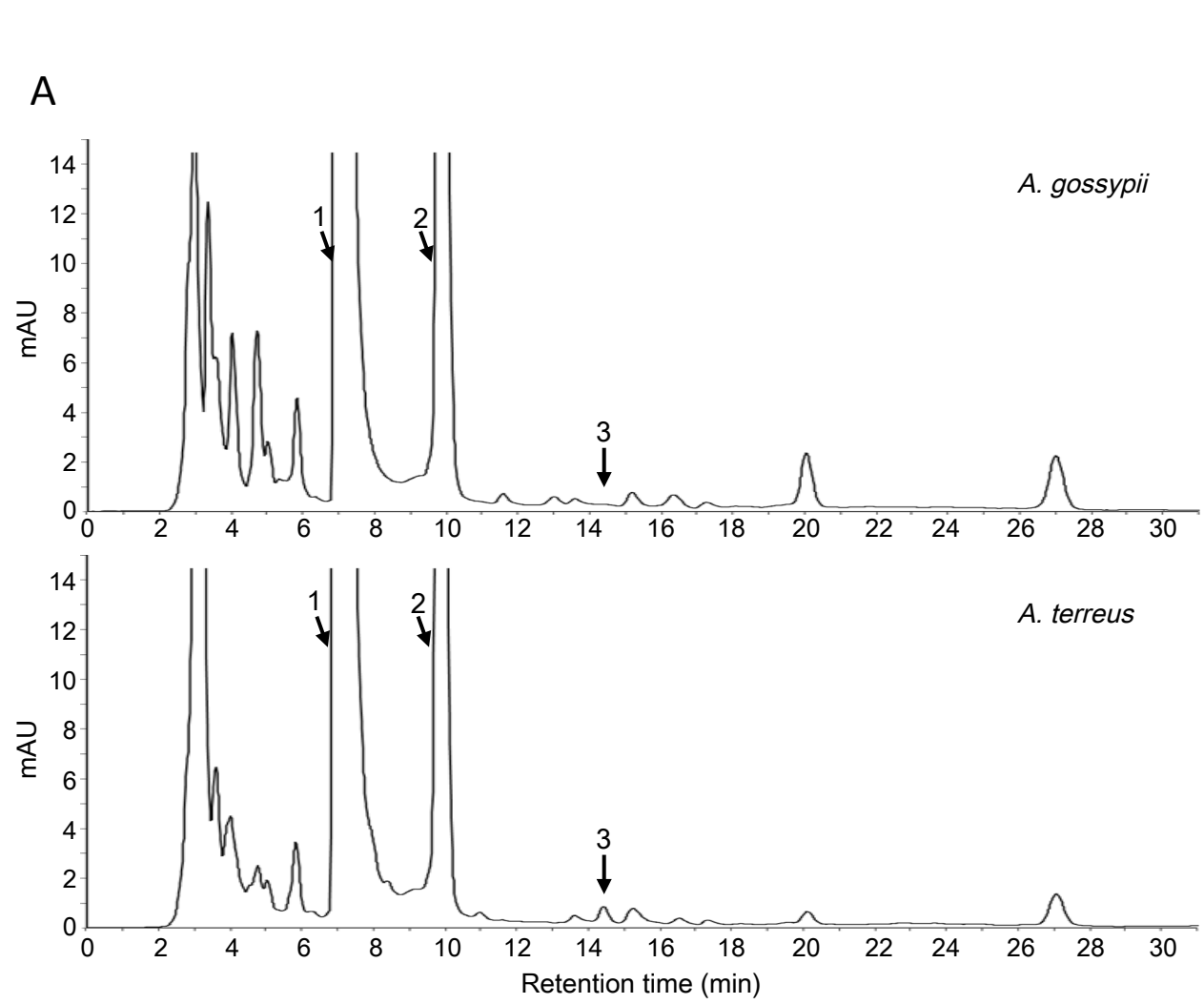
368 FIG 5. Predicted partial metabolic pathway of *trans*-aconitate and its inhibition during
369 riboflavin synthesis in *A. gossypii*. ACO1, CAD1, ICL1, and TMT1 denote aconitase,
370 *cis*-aconitate decarboxylase, isocitrate lyase, and *trans*-aconitate 3-methyltransferase,
371 respectively.

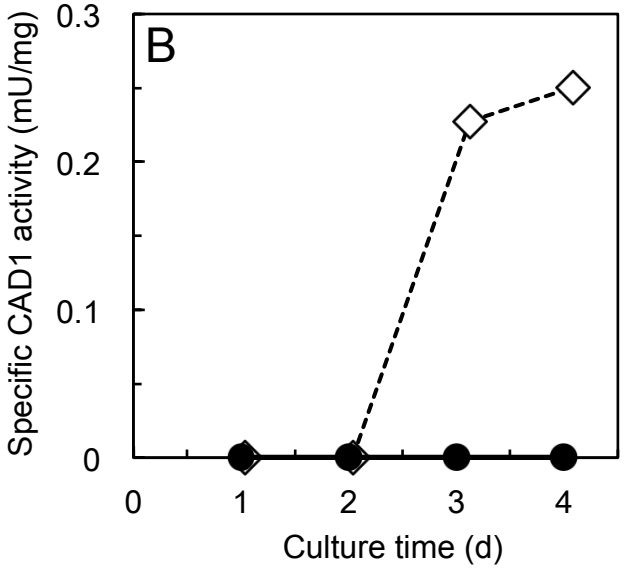
TABLE 1. List of candidate *CAD1* genes in *A. gossypii*

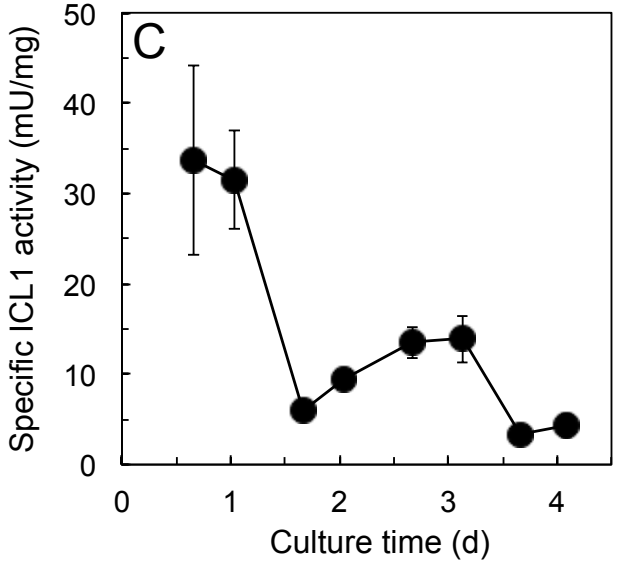
Systematic name	Error value	*Saccharomyces cerevisiae* systematic name	*Saccharomyces cerevisiae* functional description
ADR209W	2.0	*YOR066W*	YOR066W; Protein of unknown function
		YKR077W	YKR077W; Hypothetical protein
AGL243W	4.4	*YDR249C*	Hypothetical protein
AFL053W	7.5	*YGL095C* (*VPS45*)	Protein of the Sec1p/Munc-18 family
AFL076W	9.8	*YPL195W* (*APL5*)	Delta adaptin-like subunit of the clathrin associated protein complex (AP-3)
ACL086C	9.8	*YNL091W*	Protein of unknown function

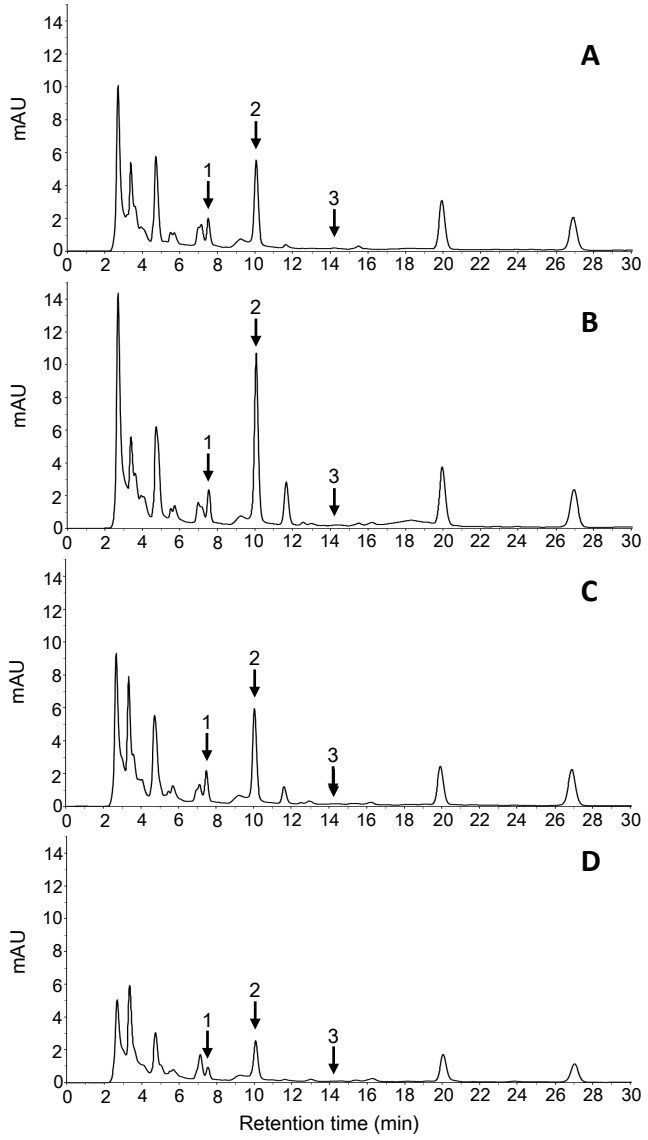
TABLE 2. Predicted production level of *trans*-aconitase and riboflavin in *A. gossypii* based on expression level of TMT1 and ACO1

	Expression level		Predicted production level	
	TMT1	ACO1	*trans*-Aconitate	Riboflavin
Growth phase	low	high	high	low
Riboflavin production phase	high	Low or remaining	low	high


A
A. gossypii
A. terreus
1
2
3
mAU
Retention time (min)

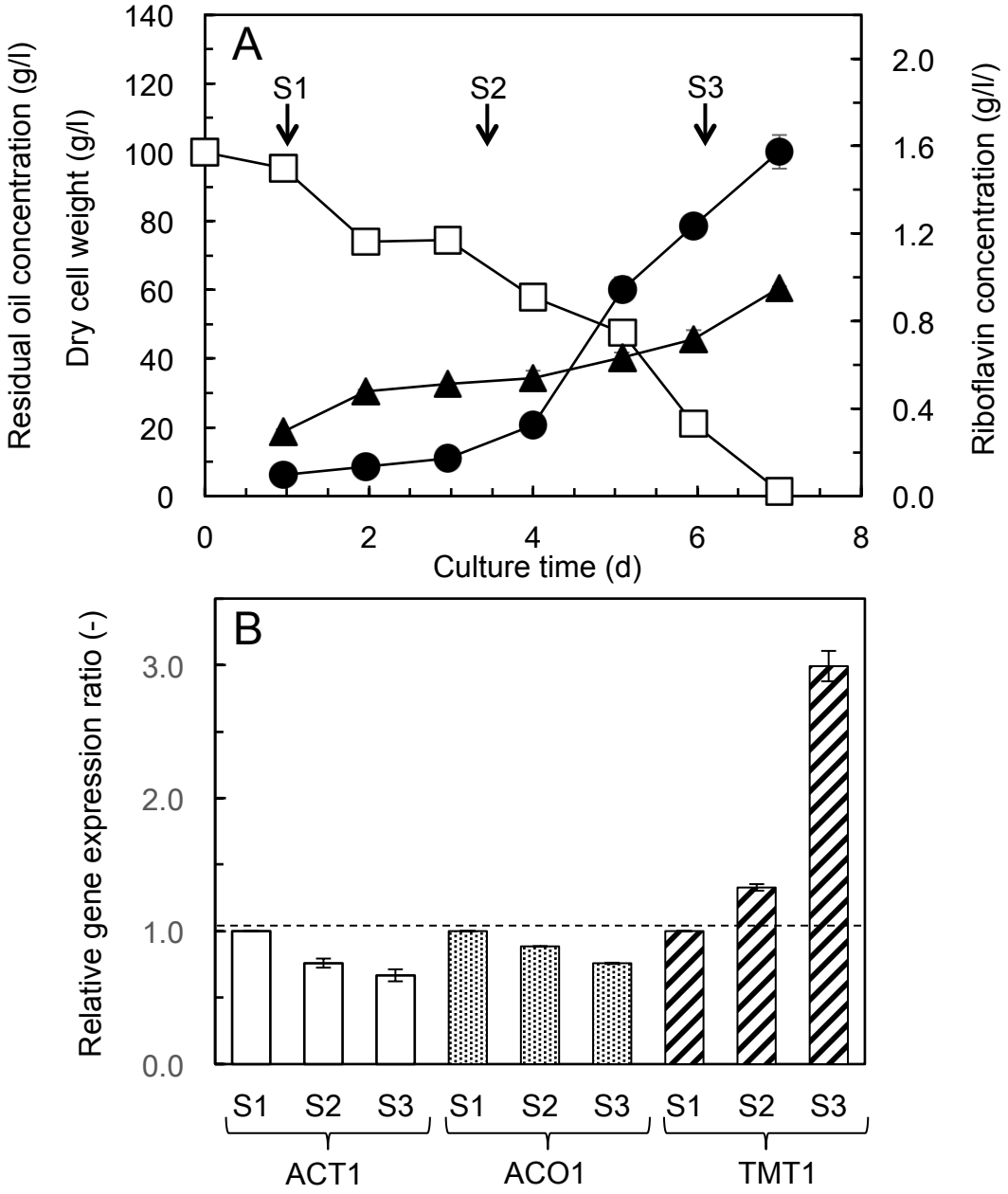

B
Specific CAD1 activity (mU/mg)
Culture time (d)

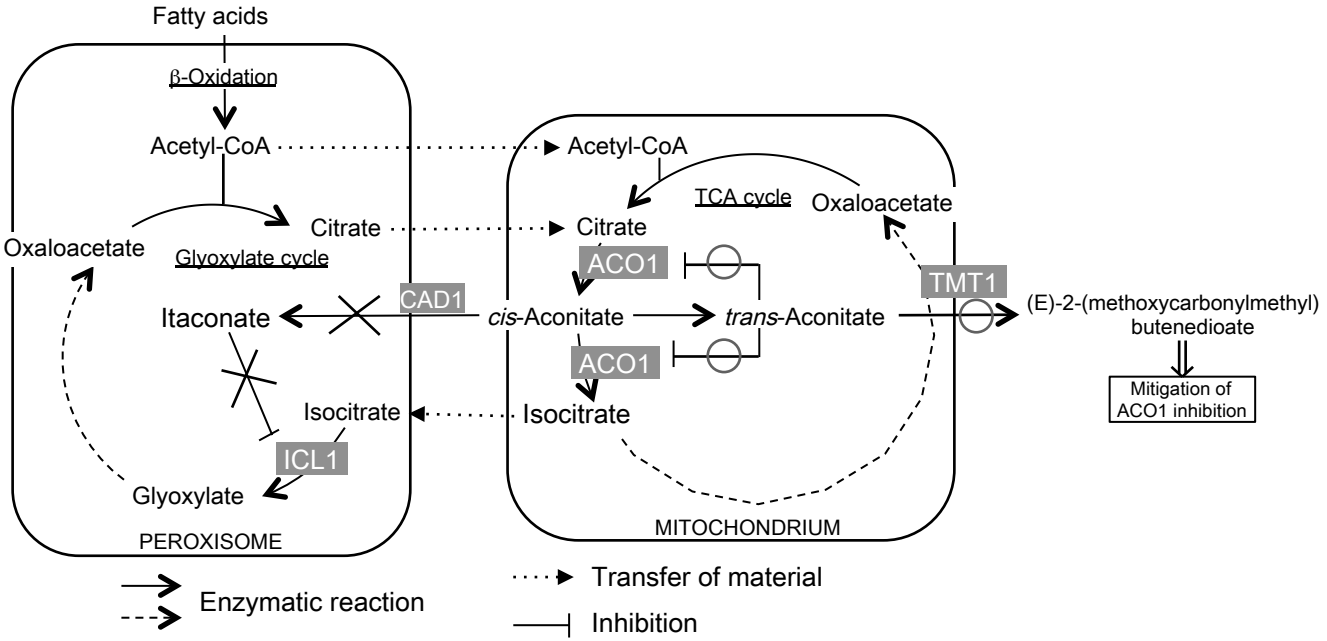

C
Specific ICL1 activity (mU/mg)
Culture time (d)


A
B
C
D
mAU
1
2
3
Retention time (min)

```
S.cerevisiae      MSTFSASDFNSERYSSSRPSYPSDFYKMIDEYHDGERKLLVDVGCGPGTATLQMAQELKP 60
A.gossypii        MAAFSDSDYNSTGYSDYRPRYPGTWYSRIKEYHSGARGLVVDVGCGPGTATFQLRENL-P 59
                  *::** **:**  **. ** **. :*. *.***.* * *:***********:*: ::* *

S.cerevisiae      FEQIIGSDLSATMIKTA-EVIKEGSPDTYKNVSFKISSSDDFKFLGADSVDKQKIDMITA 119
A.gossypii        FKRVVGVDVSTAMVERARQTAREKVVTDAGRVEFVVSTADNFSFLNG-----AKADMITA 114
                  *::::* *:*::*:: * :. :*       .*.* :*::*:*.**..      * *****

S.cerevisiae      VECAHWFDFEKFQRSAYANLRKDGTIAIWGYADPIFPDYPEFDDLMIEVPYGKQGLGPYW 179
A.gossypii        AQCVHWLDWERFQLAAADNLRAGGTLAIWDYTDPSIVGYPELDPLMKEFIYGESHLGPHW 174
                  .:*.**:*:*:** :*  *** .**:***.*:** : .***:* ** *. **:. ***:*

S.cerevisiae      EQPGRSRLRNMLKDSHLDPELFHDIQVSYFCAEDVRDKVKLHQHTKKPLLIRKQVTLVEF 239
A.gossypii        EQPGTRMLKQLLRNLHFDEKLFDDI---YVCRESTDDALRGTAG-RTPLHVEKTMTLEQM 230
                  ****   *:::*:: *:* :**.**   *.* *.. * ::     :.** :.* :** ::

S.cerevisiae      ADYVRTWSAYHQWK-QDPKNKDKEDVADWFIKESLRRRPELSTNTKIEVVWNTFYKLGKR 298
A.gossypii        NSYLSTWSAFHSWRKQDPAG---SIAAKSAFFQFIFSKTSMSWSSEVCLTWNSVGILARR 287
                   .*: ****:*.*: *** .   . .*.  : : :  :..:* .::: :.**:.  *.:*

S.cerevisiae      V 299
A.gossypii        R 288
```

Length = 300
Score = 219.4 bits (609), Expect = 3.9e-61, P = 3.9e-61
Identities = 126/300 (42%), Positives = 177/300 (59%), Gaps = 15/300 (5%)


A
S1
S2
S3
Residual oil concentration (g/l)
Dry cell weight (g/l)
Riboflavin concentration (g/l/)
Culture time (d)
0
20
40
60
80
100
120
140
0.0
0.4
0.8
1.2
1.6
2.0
0
2
4
6
8
B
Relative gene expression ratio (-)
0.0
1.0
2.0
3.0
S1 S2 S3 S1 S2 S3 S1 S2 S3
ACT1
ACO1
TMT1


Fatty acids
β-Oxidation
Acetyl-CoA
Oxaloacetate
Citrate
Glyoxylate cycle
Itaconate
Isocitrate
ICL1
Glyoxylate
PEROXISOME
CAD1
Acetyl-CoA
TCA cycle
Oxaloacetate
Citrate
ACO1
cis-Aconitate
trans-Aconitate
ACO1
Isocitrate
MITOCHONDRIUM
TMT1
(E)-2-(methoxycarbonylmethyl) butenedioate
Mitigation of ACO1 inhibition
Enzymatic reaction
Transfer of material
Inhibition