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著者	Sugimoto Takashi, Kato Tatsuya, Park Enoch Y.
journal or publication title	Journal of Bioscience and Bioengineering
volume	117
number	5
page range	563-568
year	2014-05
出版者	Elsevier
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URL	http://hdl.handle.net/10297/7471

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

5 Takashi Sugimoto,¹ Tatsuya Kato,^{1,2} and Enoch Y. Park^{1,2*}

6 ¹*Department of Bioscience, Graduate School of Science and Technology, Shizuoka University,*

7 *836 Ohya, Suruga-Ku, Shizuoka 422-8259, Japan;* ²*Green Chemistry Research Division,*

8 *Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya,*

9 *Suruga-Ku, Shizuoka 422-8259, Japan*

10

* Corresponding author. Tel/Fax: +81-54-238-4887.
E-mail address: acypark@ipc.shizuoka.ac.jp

11 **Abstract**

12 **In *Ashbya gossypii*, isocitrate lyase (ICL1) is a very crucial enzyme for riboflavin**
13 **production. Itaconate, the inhibitor of ICL1, has been used as an antimetabolite for**
14 **mutagenic studies in *A. gossypii*. It has been reported that itaconate is produced from**
15 ***cis*-aconitate by *cis*-aconitate decarboxylase (CAD1) in *Aspergillus terreus*. In this study,**
16 **identification of CAD1 gene and determination of the presence of itaconate in the**
17 **riboflavin biosynthetic pathway in *A. gossypii* were carried out to confirm itaconate**
18 **metabolism. Although no CAD1 candidate gene was found and no itaconate production**
19 **was observed, *cis*- and *trans*-aconitate were detected in the riboflavin production phase.**
20 **It is known that *trans*-aconitate inhibits aconitase (ACO1) in the tricarboxylic acid cycle.**
21 **In *A. gossypii*, the transcription level of AGR110Wp, the homolog of *trans*-aconitate**
22 **3-methyltransferase (TMT1), was enhanced by almost threefold during riboflavin**
23 **production than that during its growth phase. TMT1 catalyzes the methylation reaction**
24 **of *trans*-aconitate in *Saccharomyces cerevisiae*. Thus, these results suggest that the**
25 **enhancement of the transcription level of this TMT1 homolog decreases the**
26 ***trans*-aconitate level, which may mitigate the inhibition of ACO1 by oxidative stress in**
27 **the riboflavin biosynthetic pathway in *A. gossypii*. This is a novel finding in *A. gossypii*,**
28 **which may open new metabolic engineering ideas for improving riboflavin productivity.**

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

31 INTRODUCTION

32 Riboflavin (vitamin B₂) is a yellow, water-soluble compound and is an essential growth
33 factor in mammals, plants, and microorganisms, acting as a precursor of flavin
34 mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are required for
35 various enzymatic reactions as electron acceptors for oxidoreductases. Riboflavin is used in
36 many kinds of food additives and multivitamin supplements as a colorant and/or nutrient for
37 human health, and a large amount of it is utilized as feed additives for livestock (1). The
38 filamentous fungus *Ashbya gossypii* is used as a riboflavin producer at an industrial scale
39 with vegetable oil as the sole carbon source.

40 Several studies have predicted the biosynthetic pathway from fatty acids to riboflavin
41 and elucidated the important pathways, which are employed for improving riboflavin
42 productivity. For enzymatic functional analysis, threonine aldolase (2) and alanine:glyoxylate
43 aminotransferase (3) have been overexpressed, and the effects of serine
44 hydroxymethyltransferase isoenzyme (4), isocitrate dehydrogenase, isocitrate lyase (5),
45 phosphoribosyl pyrophosphate synthase (6), and malate synthase (7) have been examined.
46 These studies had been based on the metabolic engineering of purine pathway (8). It is
47 believed that glyoxylate cycle has an important role in riboflavin productivity, because it had
48 been identified as the origin of carbon metabolism after β -oxidation. The turnover of
49 glyoxylate cycle could be facilitated by collaboration with tricarboxylic acid (TCA) cycle,
50 because the TCA cycle could supply isocitrate for glyoxylate cycle. Aconitase (ACO1; E.C.
51 4.2.1.3) catalyzes a reversible reaction from citrate to isocitrate, with *cis*-aconitate as an
52 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,

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

80 **MATERIALS AND METHODS**

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

88 *A. terreus* TN484-M1 was grown in seed medium (pH 3.0) containing the following (per
89 l): 55.5 g, glucose; 5.2 g, corn steep liquor; 4.1 g, (NH₄)₂SO₄ (Wako); 6.3 g, KNO₃ (Wako);
90 and 2.0 g, MgSO₄·7H₂O (Wako). For itaconic acid production using *A. terreus* TN484-M1,
91 the production medium (pH 2.0) was used, which comprised the following (per l): 140 g,
92 glucose; 2.1 g, corn steep liquor; 2.9 g, NH₄NO₃ (Wako); and 1.8 g, MgSO₄·7H₂O. For DNA
93 microarray analysis, *A. gossypii* was cultured in optimized medium (19) containing the
94 following (per l): 90.5 g, rapeseed oil; 40.3 g, corn steep liquor (Sigma Chem. Co., St. Louis,
95 MO, USA); 36.1 g, yeast extract; 15.0 g, soybean mill (Wako); 2.0, glycine (Wako); 0.2 g,

96 alanine (Wako); 0.47 g, glutamic acid (Wako); 1.5 g, KH₂PO₄ (Wako); and mineral ions (2
97 µg/l of Co²⁺, 5 µg/l of Mn²⁺, 10 µg/l of Zn²⁺, and 1 µg/l of Mg²⁺). The pH of the medium was
98 adjusted to 6.8 using 1M KOH. Cultures of *A. terreus* TN484-M1 and *A. gossypii* strains
99 were grown in 500-ml shake flasks with a working volume of 50 ml in a rotary shaker
100 (TB-25R, Takasaki Scientific Instrument Co., Kawaguchi, Saitama, Japan) at an agitation rate
101 of 220 rpm and 28°C.

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

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

118 buffer (pH 6.2), 1 mM EDTA, and 5 mM dithiothreitol. The mycelia were fractured by
119 sonication for three times at 40 Ω for 30 s with 1-min intervals on ice using an ultrasonicator
120 (Sonics & Materials Inc., Newtown, CT, USA). The resulting homogenate was centrifuged at
121 $14,000 \times g$ for 30 min at 4°C, and the supernatant was used for the enzyme assays.

122 The CAD1 activity was measured according to the method described by Bentley and
123 Thiessen (21) with some modifications. A total of 50 μ l of the enzyme solution were added to
124 800 μ l of 200 mM sodium phosphate buffer (pH 6.2) containing 8 mM (final concentration)
125 *cis*-aconitic acid (Sigma-Aldrich Co., St. Louis, MO, USA), and incubated for 15 min at 30°C.
126 The enzyme reaction was terminated by addition of 50 μ l of 12 N HCl. The intracellular and
127 extracellular itaconate concentrations were measured by HPLC. One unit of enzyme activity
128 was defined as the amount of enzyme required to form 1 μ mol itaconate per minute. The
129 ICL1 activity was measured according to the method described by Schmidt et al. (11). The
130 total protein concentration was evaluated by the Bradford method using a protein assay kit
131 (Bio-Rad) with bovine serum albumin (Sigma-Aldrich) as the standard.

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

140 **Analytical methods** Riboflavin and residual oil concentrations were measured by
141 methods previously described by Park and Ming (22). The dry cell weight was measured as
142 follows. The mycelia from the culture broth were filtered using filter paper No. 5A
143 (Advantec) and dried overnight in an oven at 100°C. Detection of itaconate, *cis*-aconitate,
144 and *trans*-aconitate was performed by using HPLC (LC-8A; Simadzu Co., Kyoto, Japan). A
145 total of 10 µl of appropriately diluted sample were applied to a column (GAPCELL PAK C₁₈
146 MG; 5.0 µm and 3.0 × 250 mm; Shiseido Co., Ltd., Tokyo, Japan) through a guard column
147 (CAPCELL PAK C₁₈ MG; 5.0 µm and 3.0 × 35 mm; Shiseido), both of which were thermally
148 insulated at 45°C (CTO-6A; Simadzu). Each compound was detected by UV detector
149 (SPD-20A; Simadzu) at 210 nm. A mobile phase consisting of 1.2% (v/v) acetonitrile and
150 0.3% (v/v) phosphoric acid was prepared, degassed by sonication for 1 h, and used at a flow
151 rate of 0.5 ml/min. The resulting chromatogram was re-analyzed and modified by CLASS-VP
152 (Shimadzu). The detection limit of itaconate in this system was approximately 0.2 µM.

153

RESULTS

154 **Homology search of *A. gossypii* CAD1 with the amino acid sequence of *A. terreus***

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

160 **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-mediated 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

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

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

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

208 **DISCUSSION**

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

217 As a novel finding, *trans*-aconitate was detected, whose intracellular accumulation was
218 more than that of the *cis* form during riboflavin production in *A. gossypii* (Fig. 2). As
219 *trans*-aconitate strongly inhibits ACO1, which is one of the key enzymes in the TCA cycle as
220 well as in riboflavin biosynthesis, ACO1 in *A. gossypii* may have been inhibited by
221 intracellular *trans*-aconitate (Fig. 5). It has been speculated that the intracellular
222 *trans*-aconitate might be generated spontaneously from the *cis* form because of its instability
223 (28). With regard to *trans*-aconitate metabolism, two enzymes are known to react with
224 *trans*-aconitate as the substrate. One is aconitate delta-isomerase (E.C. 5.3.3.7) found in some
225 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

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

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347 **Figure legends**

348 FIG. 1. HPLC chromatograms for *A. gossypii* and *A. terreus* cultured for 4 days (A) and the
349 specific activities of CAD1 (B) and ICL1 (C) in *A. terreus* and *A. gossypii*. Numbers 1, 2, and
350 3 in (A) denote *cis*-aconitate acid, *trans*-aconitate, and itaconate, respectively. Open
351 rhombuses and closed circles in (B) denote the specific CAD1 activity in *A. terreus* and *A.*
352 *gossypii*, respectively.

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

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

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

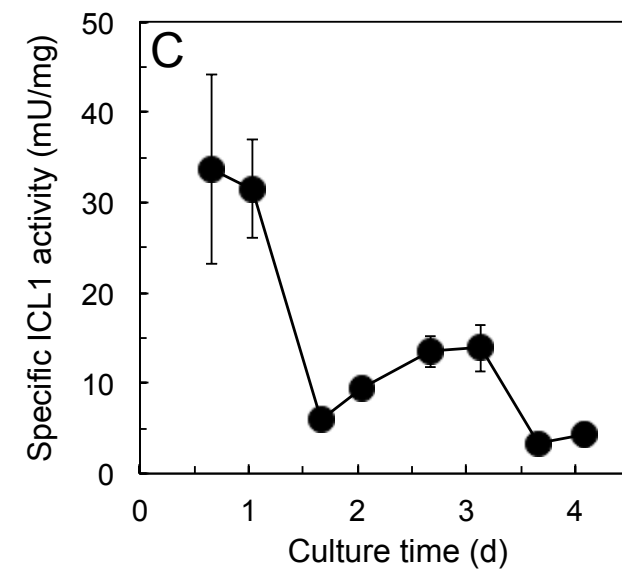
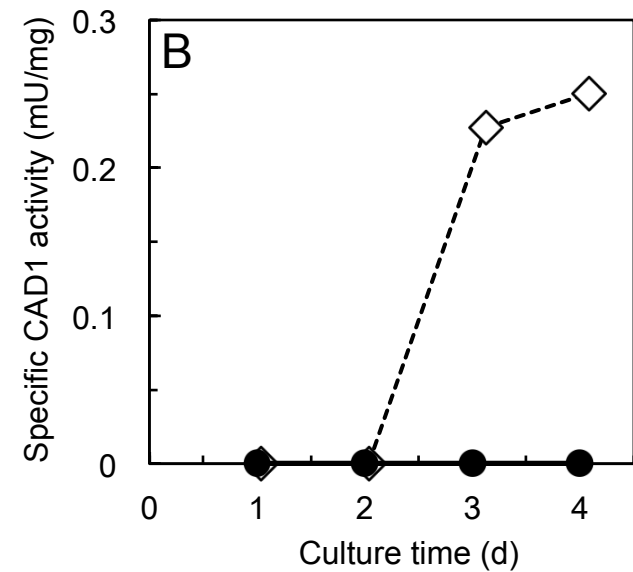
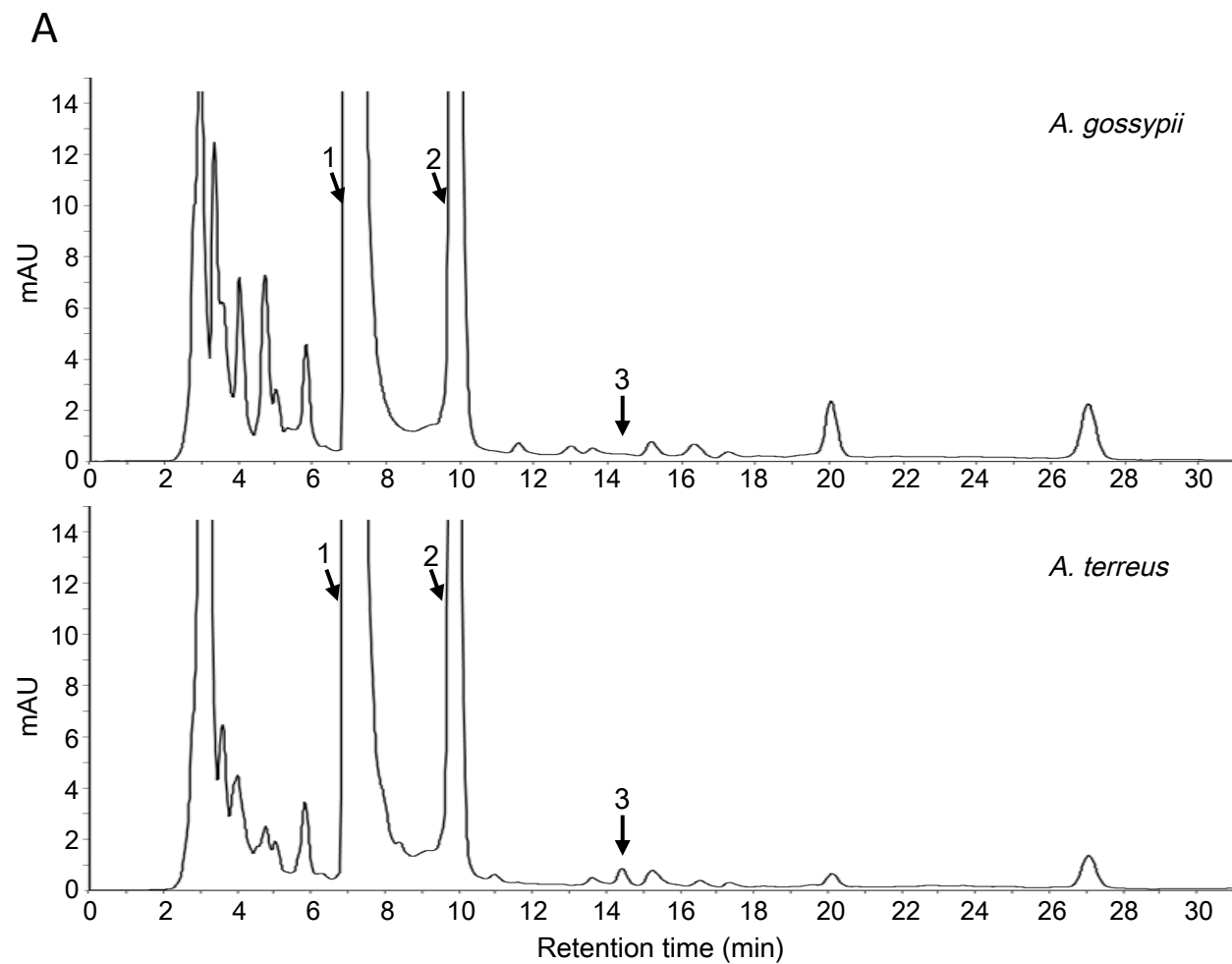
368 FIG 5. Predicted partial metabolic pathway of *trans*-aconitate and its inhibition during
369 riboflavin synthesis in *A. gossypii*. ACO1, CAD1, ICL1, and TMT1 denote aconitase,
370 *cis*-aconitate decarboxylase, isocitrate lyase, and *trans*-aconitate 3-methyltransferase,
371 respectively.

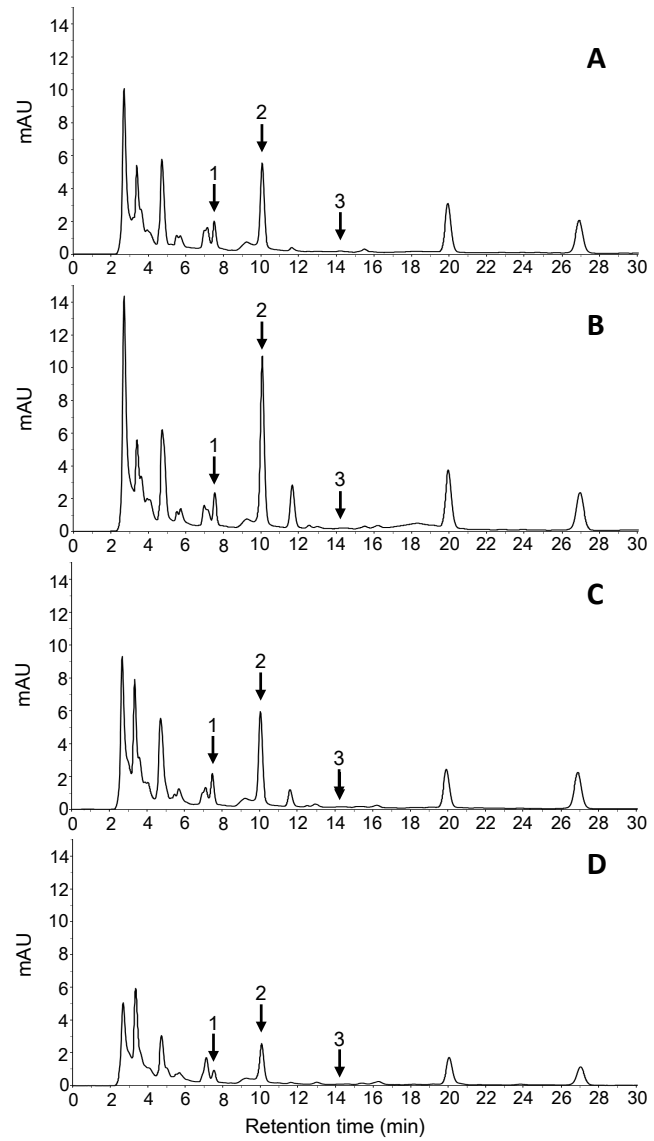
TABLE 1. List of candidate *CAD1* genes in *A. gossypii*

Systematic name	Error value	<i>Saccharomyces cerevisiae</i> systematic name	<i>Saccharomyces cerevisiae</i> functional description
<i>ADR209W</i>	2.0	<i>YOR066W</i>	YOR066W; Protein of unknown function
		<i>YKR077W</i>	YKR077W; Hypothetical protein
<i>AGL243W</i>	4.4	<i>YDR249C</i>	Hypothetical protein
<i>AFL053W</i>	7.5	<i>YGL095C (VPS45)</i>	Protein of the Sec1p/Munc-18 family
<i>AFL076W</i>	9.8	<i>YPL195W (APL5)</i>	Delta adaptin-like subunit of the clathrin associated protein complex (AP-3)
<i>ACL086C</i>	9.8	<i>YNL091W</i>	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	<i>trans</i> -Aconitate	Riboflavin
Growth phase	low	high	high	low
Riboflavin production phase	high	Low or remaining	low	high






```

S.cerevisiae      MSTFSASDFNSERYSSSRPSYPSPDFYKMIDEYHDGERKLLVDVGC GPGTATLQMAQELKP 60
A.gossypii       MAAFSDSDYNSTGYSDYRPRYPGTWYSRIKEYHSGARGLVVDVGC GPGTATFQLRENL-P 59
*::* **:* ** . ** ** . :* . *.**.* * * *:*****:* ::* *

S.cerevisiae      FEQIIGSDLSATMIKTA-EVIKEGSPD TYKNVSFKISSSDDFKFLGADSVDRKQKIDMITA 119
A.gossypii       FKR VVGVDVSTAMVERARQTAREKVVTDAGRVEFV VSTADNFSFLNG-----AKADMITA 114
*::::* *:::***: * .: *          .** *:***:*.***.   * *****

S.cerevisiae      VECAHWFDFEFQRSAYANLRKDGTIAIWGYADPIFPDYPEFDDLMIEVVPYKQGLGPYW 179
A.gossypii       AQC VHWLDWERFQLAAADNLRAGGT LAIW DYTDP SIVGYPELDPLMKEFIYGESH LGPHW 174
.:*.**:*:*:*:* *  *** .**:* **:* ** : .***:* ** *. **:. ***:*

S.cerevisiae      EQPGRSRLRNMLKDSHLDPELFHDIQVSYFCAEDVDRDKVKLHQHTKKPLLIRKQVTLVEF 239
A.gossypii       EQPGTRMLKQLLRNLHFDEKLFDDI---YVCRESTDDALRG TAG-RTPLHVEKTM TLEQM 230
****  *::::*: *:* :*.**  *. *.. * ::      :.* :.* :.* ::

S.cerevisiae      ADYVRTWSAYHQWK-QDPKNKDKEDVADWFIKESLRRRPELSTNTKIEVWNTFYKLGKR 298
A.gossypii       NSYLSTWSAFHSWRKQDPAG---SIAAKSAFFQFIFSKTSM SWSSEVCLTWN SVGILARR 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%)

