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

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2 Functional analysis of cis-aconitate decarboxylase and

3 trans-aconitate metabolism in a riboflavin producing

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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 B₂) 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:glyoxylate 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

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

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

In the present study, we aimed to identify itaconate and the corresponding itaconate biosynthetic enzyme in the riboflavin biosynthetic pathway in *A. gossypii* during riboflavin 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, (NH₄)₂SO₄ (Wako); 6.3 g, KNO₃ (Wako); and 2.0 g, MgSO₄·7H₂O (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, NH₄NO₃ (Wako); and 1.8 g, MgSO₄·7H₂O. 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 $\mu\text{g/l}$ of Co^{2+} , 5 $\mu\text{g/l}$ of Mn^{2+} , 10 $\mu\text{g/l}$ of Zn^{2+} , and 1 $\mu\text{g/l}$ 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 $\times g$ 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 μ l of the enzyme solution were added to 800 μ l 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 μ l 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 μ mol 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 $\times$ 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 C₁₈ MG; 5.0 µm and 3.0 × 250 mm; Shiseido Co., Ltd., Tokyo, Japan) through a guard column (CAPCELL PAK C₁₈ 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

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

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

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

least >50 $\mu\text{mol/g}$ 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 ($3.9\text{e-}61$, 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 μ M), 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 μ M), 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

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

In the present study, we found a novel antimetabolite, *trans*-aconitate, which is more available than itaconate for improving riboflavin productivity in *A. gossypii*. Nevertheless, 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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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	<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</i> (<i>VPS45</i>)	Protein of the Sec1p/Munc-18 family
<i>AFL076W</i>	9.8	<i>YPL195W</i> (<i>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

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S.cerevisiae   MSTFSASDFNSERYSSSRPSYPSPDFYKMIDEYHDGERKLLVDVGC GPGTATLQMAQELKP 60
A.gossypii    MAAFSDSDYNSTGYSDYRPRYPGTWYSRIKEYHSGARGLVVDVGC GPGTATFQLRENLP 59
               *: : * * * : * * * * * : * * * * * * * * * * * : : * *

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

S.cerevisiae   VECAHWFDFEKFQRSAYANLRKDGTIAIWGYADPIFPDYPEFDDLMIEVPYQGLGPYW 179
A.gossypii    AQC VHWLDWERFQLAAADNLRAGGTLAIWDYTDPSIVGYPELDPLMKEFIYGESH LGPHW 174
               . : * . * * : : : * * : * * * . * * : * * : * * : * * : * * : *

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

S.cerevisiae   ADYVRTWSAYHQWK-QDPKNKDKEDVADWFIKESLRRRPPELSTNTKIEVVWNTFYKLGKR 298
A.gossypii    NSYLSTWSAFHSWRKQDPAG---SIAAKSAFFQFIFSKTSMWSSEVCLTWNSVGILARR 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%)