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Expression of alanine:glyoxylate aminotransferase gene from *Saccharomyces cerevisiae* in *Ashbya gossypii*

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Abstract Two plasmids containing an autonomously replicating sequence from *Saccharomyces cerevisiae* were constructed. Using these vectors, the *AGX1* gene encoding alanine:glyoxylate aminotransferase (AGT) from *S. cerevisiae*, which converts glyoxylate into glycine but is not present in *Ashbya gossypii*, was expressed in *A. gossypii*. Geneticin-resistant transformants with the plasmid having the kanamycin resistance gene under the control of the translation elongation factor 1 α (*TEF*) promoter and terminator from *A. gossypii* were obtained with a transformation efficiency of approximately 10–20 transformants per microgram of plasmid DNA. The specific AGT activities of *A. gossypii* pYPKTPAT carrying the *AGX1* gene in glucose- and rapeseed-oil-containing media were 40 and 160 mU mg^{-1} of wet mycelial weight, respectively. The riboflavin concentrations of *A. gossypii* pYPKTPAT carrying *AGX1* gene in glucose- and rapeseed-oil-containing media were 20 and 150 mg l^{-1}, respectively. In the presence of 50 mM glyoxylate, the riboflavin concentration and the specific riboflavin concentration of *A. gossypii* pYPKTPAT were 2- and 1.3-fold those of *A. gossypii* pYPKT without the *AGX1* gene.

Introduction

Riboflavin is widely distributed in microorganisms, plants, and animals and plays an important role because it is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). A daily dose of 0.3–1.8 mg of riboflavin is essential for humans (Cooperman and Lopez 1984; Eggersdorfer et al. 1996) and used as an animal feed supplement in the less pure form. More than 80% of industrial products of riboflavin are used as animal feed additives (Stahmann et al. 2000).

Ashbya gossypii, a filamentous hemiascomycete, is a producer of riboflavin (Demain 1972), and recently its genome sequence has been completed (Dietrich et al. 2004). It was the first microorganism used in industrial-scale riboflavin production and has been used for the biotechnological production of riboflavin. An improvement of its productivity by random mutagenesis and genetic engineering allows the industrial application of *A. gossypii*. The production of riboflavin is now performed using not only *A. gossypii* but also *Candida famata* and *Bacillus subtilis* (Stahmann et al. 2000).

The improvement of riboflavin production using *A. gossypii* has been attempted by genetic manipulation. The transformation method for *A. gossypii* has been established using plasmid DNA carrying an autonomously replicating sequence (ARS) from *Saccharomyces cerevisiae* (Wright and Philippsen 1991). To improve riboflavin productivity, a specific gene of *A. gossypii* is expressed or sometimes disrupted (Monschau et al. 1998; Foster et al. 1999; Schlupen et al. 2003). The ARS elements of *S. cerevisiae* do not work in other fungi (Fingcham 1989), but *A. gossypii* is able to integrate recombinant plasmids without the background nonhomologous recombination (Steiner et al. 1995). These are rapid developments in genetic manipulation of *A. gossypii*. Recently, *A. gossypii* has been transformed by restriction-enzyme-mediated integration (Schlupen et al. 2003).

In this report, two plasmids containing ARS elements from *S. cerevisiae* were constructed, and *A. gossypii* mycelia were transformed using these plasmids. Using this vector, the *AGX1* gene encoding alanine:glyoxylate aminotransferase (AGT) from *S. cerevisiae* (Schlosser et al. 2004), which is not present in *A. gossypii*, was expressed in *A. gossypii*. When *S. cerevisiae*, whose *GLY1* (encodes threonine aldolase), *SHM1*, and *SHM2* (encode serine hydroxymethyltransferase1 and 2, respectively) were disrupted, was cultivated using ethanol as the sole carbon source, this *AGX1* gene was found to be indispensable for

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growth. This indicates that AGT activity may be important for growth using a nonfermentative carbon source. In the case of *A. gossypii*, since the carbon source is vegetable oil, the *AGX1* gene is useful for mycelial growth and riboflavin production. Because AGT converts glyoxylate into glycine, which is a precursor of riboflavin, an overexpression of AGT in *A. gossypii* may enhance riboflavin productivity by the increase in the level of the precursor glycine.

Materials and methods

Strain, media, and culture methods

A. gossypii (ATCC10895) was used throughout this study. The cells were maintained in a solid medium containing 1% yeast extract, 0.3% glycine, and 2% agar (pH 6.0) at 30°C. Either 1% glucose or rapeseed oil was used as the sole carbon source. A subculture was carried out once a month. Cells were cultured at 28°C in 200-ml shaking flasks containing 20 ml of the medium on a rotary shaker at 195 rpm. For the isolation of transformants, geneticin (Invitrogen) was added to all media at either 200 or 300 μg ml^{-1}. *Escherichia coli* strains carrying plasmids were grown in Luria–Bertani (LB) medium (1% polypeptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 100 μg ml^{-1} ampicillin.

One thousand spores per milliliter were inoculated onto a 500-ml flask containing 50 ml of the nutrient-rich medium and cultured with shaking at 120 rpm and 20°C for 6 days. Samples were collected every 24 h to determine amino acid consumption.

Construction of plasmids

The scheme of plasmid construction is shown in Fig. 1. A 221-bp-containing terminator region and eight codons at the C-terminus of the translation elongation factor 1 α (*TEF*) gene from *A. gossypii* was amplified and inserted into pUC19 at the *Kpn*I and *Eco*RI sites (pUT). The kanamycin resistance gene was amplified from pENTR (Invitrogen) and inserted into pUT at the *Xba*I and *Kpn*I sites (pKT). A 351-bp region containing the promoter and eight codons at the N-terminus of the *TEF* gene from *A. gossypii* was amplified and inserted into pKT at *Pst*I and *Xba*I sites (pPKT). The kanamycin resistance cas-

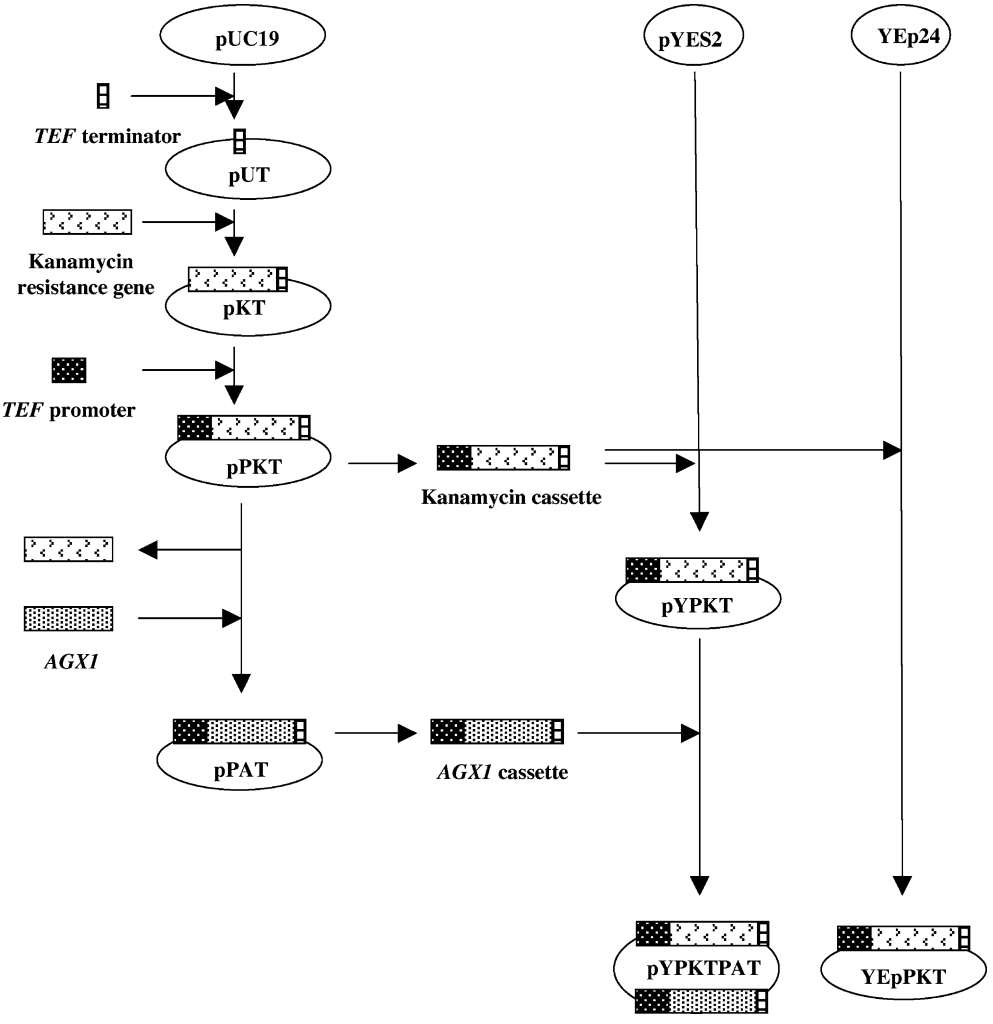

Fig. 1 Scheme of plasmid construction

sette, consisting of the promoter and terminator of the *TEF* and kanamycin resistance genes, was amplified. pYES2 (Invitrogen) was digested at the *Hin*dIII site and filled up with the Klenow fragment. The amplified kanamycin-resistant cassette was inserted into the pYES2. This constructed plasmid was designated as pYPKT. The other plasmid, YEpPKT, was constructed by inserting the *Pvu*II and *Sph*I fragments of pYPKT, including the kanamycin resistance cassette, into YEp24 at the *Pvu*II and *Sph*I sites.

The *AGX1* gene from *S. cerevisiae* was amplified and inserted into pPKT at the *Xba*I–*Kpn*I site, where the kanamycin resistance gene was removed (pPAT). The *AGX1* cassette, consisting of the promoter and terminator of the *TEF* gene, and the *AGX*1 gene were amplified. pYPKT was digested with the *Bam*HI and filled up with the Klenow fragment. The amplified *AGX1* cassette was inserted into the pYPKT. This constructed plasmid was designated as pYPKTPAT. All PCR primers used in this experiment are shown in Table 1.

Transformation of *A. gossypii* and total DNA extraction

A. gossypii was transformed by the method reported by Monschau et al. (1998). Total DNA was extracted according to the yeast DNA miniprep method (Rose et al. 1990).

Cell extraction

A. gossypii mycelia were harvested by filtration and then washed with distilled water. The mycelia were suspended in 0.1 M potassium phosphate buffer (pH 8.0), and sonicated for 2 min (four 30-s bursts at 1-min intervals) on ice at 40 W using a sonicator (VC-130PB, Sonics, Newtown, CT, USA). The crude extract was centrifuged at 20,000×*g* for 5 min, and the supernatant was used as the enzyme solution.

Southern hybridization

Southern hybridization was performed with the PCR-amplified kanamycin resistance gene labeled using the ECL direct nucleic acid labeling and detection system (Amersham Biosciences Corp.) as probe.

Table 1 Primers

Kan-F[a]	GCTCTAGAATGAGCCATATTCAACGGGAAACG
Kan-R[a]	GGGGTACCTTAGAAAAACTCATCGAGCATC
AgPro-F[b]	AACTGCAGACATGGAGGCCCAGAATACCC
AgPro-R[b]	GCTCTAGAAACGTGAGTCTTTTCCTTACC
AgTer-F[c]	GGGGTACCCAAAAGGCTGGTAAGAAATAG
AgTer-R[c]	CGGAATTCTTTCTGCGCACTTAACTTCGC

[a]For the amplification of kanamycin resistance gene
[b]For the amplification of *TEF* promoter from *A. gossypii*
[c]For the amplification of *TEF* terminator from *A. gossypii*

Analytical methods

For riboflavin measurement, 0.8 ml of culture broth was mixed well with 0.2 ml of 1 N NaOH. A 0.4-ml aliquot of the mixture was mixed well with 1 ml of 0.1 M potassium phosphate buffer (pH 6.8). Absorbance was then measured at 444 nm (Lim et al. 2003). Riboflavin concentration was calculated using an extinction coefficient of 1.04×10^{-2} M^{-1} cm^{-1} (127 mg riboflavin l^{-1} at ABS_{444}).

Alanine:glyoxylate aminotransferase activity was measured according to the method reported by Takada and Noguchi (1985). The reaction mixture contained 40 mM L-alanine, 5 mM glyoxylate, 40 mM pyridoxal-5′-phosphate, 0.1 M potassium phosphate buffer (pH 8.0), and enzyme solution in a total volume of 0.5 ml. Pyruvate produced by the enzyme reaction was detected with a pyruvate detection kit (F-kit pyruvate, Roche Diagnostics K.K., Tokyo, Japan). One unit of enzyme activity is defined as the amount of enzyme capable of catalyzing the production of 1 μmol pyruvate per minute.

Results

Construction of two plasmids that autonomously replicate in *A. gossypii*

Wright and Philippsen (1991) reported that plasmids containing ARS, 2-micron plasmid replication origin (2μ ARS) and chromosomal replication origin (ARS1) from *S. cerevisiae*, are widely distributed in *A. gossypii* and autonomously replicate. Moreover, YEp24-based pAG-100

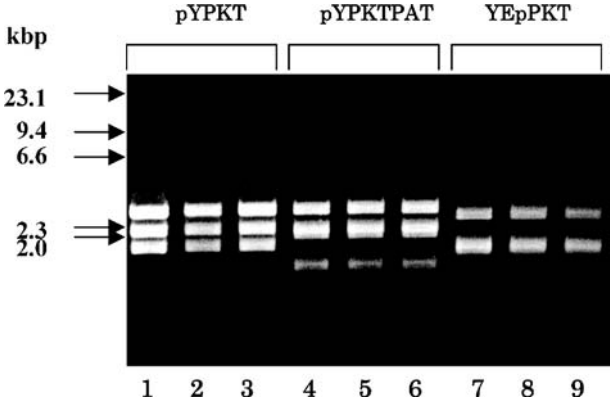

Fig. 2 DNA patterns of plasmids from *E. coli* pYPKT, pYPKTPAT, and YEpPKT transformants. Plasmids were isolated from ampicillin-resistant *E. coli* JM109 transformed with total uncleaved DNA of *A. gossypii* transformants, *A. gossypii* pYPKT, *A. gossypii* pYPKTPAT, and *A. gossypii* YEpPKT. Plasmids obtained from *E. coli* transformed with *A. gossypii* pYPKT1-1 and 1-2 DNA (*lanes 2* and *3*) and *A. gossypii* pYPKTPAT1-1 and 1-2 DNA (*lanes 5* and *6*) *A. gossypii* YEpPKT1-1, 1-2 DNA (*lanes 8* and *9*) were cleaved by *Bam*HI and *Pst*I and analyzed on a 0.8% agarose gel. *Lanes 1*, *4*, and *7* denote pYPKT, pYPKTPAT, and YEpPKT, respectively

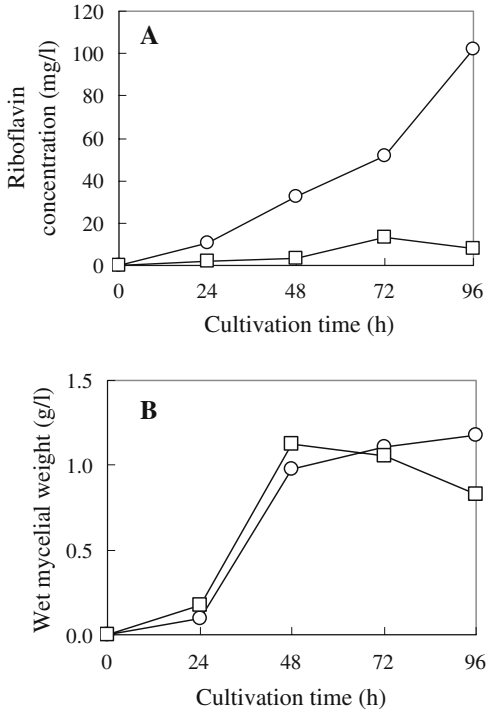

Fig. 3 Riboflavin concentrations (**a**) and wet mycelial weights (**b**) in the culture of *A. gossypii* pYPKT1-1 (*circles*) and *A. gossypii* YEpPKT1-1 (*squares*), using rapeseed oil as the sole carbon source

(Steiner and Philippsen 1994) containing the kanamycin resistance gene under the control of the *TEF* promoter conferred G-418 (geneticin) resistance on *A. gossypii*. In this report, the kanamycin cassette, consisting of kanamycin resistance gene under the control of the *TEF* promoter and terminator, was constructed and inserted into pYES2, which was designed for the inducible expression of the recombinant protein in *S. cerevisiae* and designated as pYPKT. The kanamycin resistance cassette was also inserted into YEp24 and the resulting plasmid designated as YEpPKT, which is similar to pAG-100.

Characterization of transformants

A. gossypii was transformed with pYPKT and YEpPKT, and the transformants were obtained with an efficiency of 10–20 transformants per microgram of plasmid DNA in each plasmid. Primary transformants, which seemed to be heterokaryotic, were isolated, and then primary spores were isolated from the primary transformants. Homokaryotic transformants were isolated from the spores of primary transformants. To confirm the integration of plasmids into genome DNA, total DNA was extracted and then analyzed with Southern blotting using kanamycin resistance gene as a probe. No signal was detected from 10 μg total DNA of each transformant, suggesting that plasmids were not integrated into genome DNA in each transformant. To confirm whether these plasmids were widely distributed in *A. gossypii*, *E. coli* JM109 was transformed with its uncleaved total DNA. The plasmids from *E. coli* transformants were analyzed by treatment with *Bam*HI–*Pst*I using an agarose gel electrophoresis (Fig. 2). The cleavage pattern of each plasmid was identical to the corresponding control plasmid, pYPKT or YEpPKT. On the other hand, *A. gossypii* containing either pYPKT or YEpPKT was unstable in the medium containing rapeseed oil; its stability was 3–5%. These results indicate that each plasmid might replicate autonomously in *A. gossypii*, but their stability was low. This low stability might cause the failure of detection in Southern blotting.

Two transformants, *A. gossypii* pYPKT1-1 and *A. gossypii* YEpPKT1-1, were cultivated in the medium containing either glucose or rapeseed oil as the sole carbon source. Wet mycelium weight and the production of riboflavin were compared. In the glucose-containing medium, no differences in cell growth and riboflavin concentration between the two strains were observed (data not shown). However, in the rapeseed-oil-containing medium, the production of riboflavin by *A. gossypii* YEpPKT1-1 decreased markedly regardless of similarity in mycelial growth to that of *A. gossypii* pYPKT1-1 (Fig. 3). The YEpPKT1-1 strain produced a lower level of riboflavin in the rape-

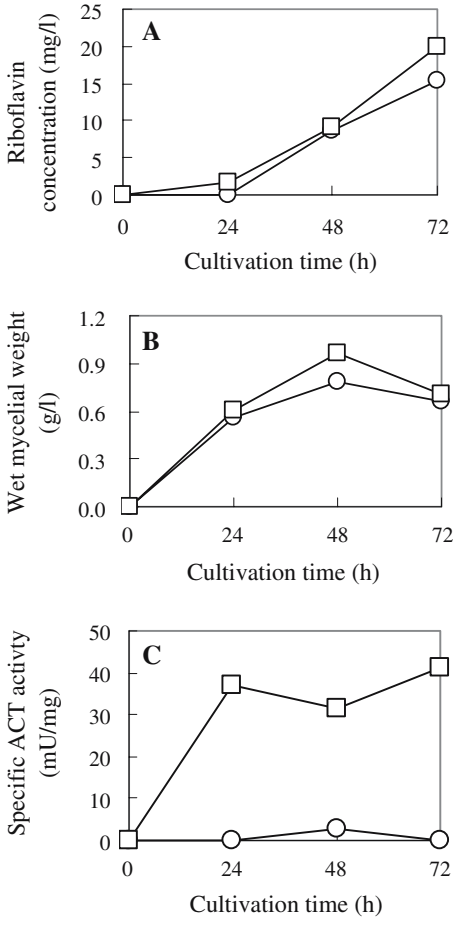

Fig. 4 Riboflavin concentrations (**a**), wet mycelial weights (**b**), and specific AGT activity (**c**) in the culture of *A. gossypii* pYPKT1-1 (*circles*) and *A. gossypii* pYPKTPAT1-1 (*squares*), using glucose as the sole carbon source

seed-oil-containing medium than in the glucose-containing medium.

Expression of *AGX1* gene from *S. cerevisiae* in *A. gossypii*

To enhance the supply of glycine, which is known to be a riboflavin precursor, using rapeseed oil as the sole carbon source, the heterologous expression of *AGX1* in *A. gossypii* was attempted. The *TEF* promoter and terminator were linked to the *AGX1*, and then the *AGX1* cassette was constructed. Then, *AGX1* cassette was inserted into pYPKT, and pYPKTPAT was constructed. *A. gossypii* was transformed with pYPKTPAT, and homokaryotic transformants were isolated. An isolated transformant (*A. gossypii* pYPKTPAT1-1) was cultivated in either the glucose-containing or the rapeseed-oil-containing medium in the presence of geneticin. The growth of the pYPKTPAT1-1 strain in both media was similar to that of the pYPKT1-1 strain carrying the vector only. The pYPKTPAT1-1 strain exhibited 30–40 mU mg^{-1} specific AGT activity in the glucose-containing medium from 24 to 72 h of cultivation (Fig. 4c). However, the pYPKT1-1 strain showed a low activity. It indicates that pYPKTPAT works in *A. gossypii*. In the rapeseed-oil-containing medium, the specific AGT activity reached 160 mU mg^{-1} after 24 h of cultivation, but it decreased to 33.8 mU mg^{-1} after 48 h of cultivation (Fig. 5c). Approximately 1.3- and 1.4-fold increases in riboflavin concentration were detected in the cultivation of *A. gossypii* pYPKTPAT1-1 using glucose and rapeseed oil as the sole carbon sources, respectively (Figs. 4 and 5).

Addition of glyoxylate

A. gossypii pYPKT and *A. gossypii* pYPKTPAT1-1 were cultivated in the glucose-containing medium with 50 mM glyoxylate, which is a substrate of AGT. Riboflavin production was investigated after 3 days of cultivation. The growth rate of the pYPKTPAT strain was approximately 1.7-fold higher than that of pYPKT strain in the presence of glyoxylate (data not shown). Moreover, the riboflavin concentration and specific riboflavin concentration of the pYPKTPAT1-1 strain were approximately 2- and 1.3-fold higher than those of the pYPKT strain, respectively (Fig. 6). The effect of *AGX1* expression was observed clearly in the presence of glyoxylate. When 50 mM glyoxylate and 50 mM alanine were added, a difference in riboflavin production between the pYPKT and pYPKTPAT strains was not observed (data not shown).

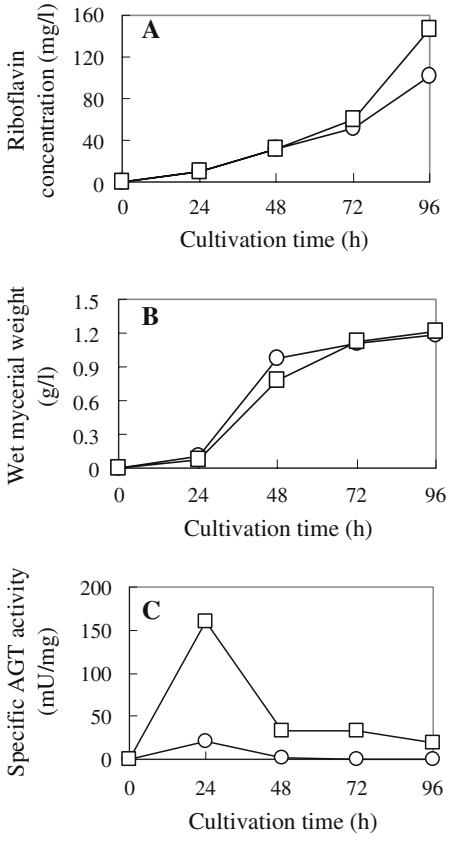

Fig. 5 Riboflavin concentrations (**a**), wet mycelial weights (**b**), and specific AGT activity (**c**) in the culture of *A. gossypii* pYPKT1-1 (*circle*) and *A. gossypii* pYPKTPAT1-1 (*square*), using rapeseed oil as the sole carbon source

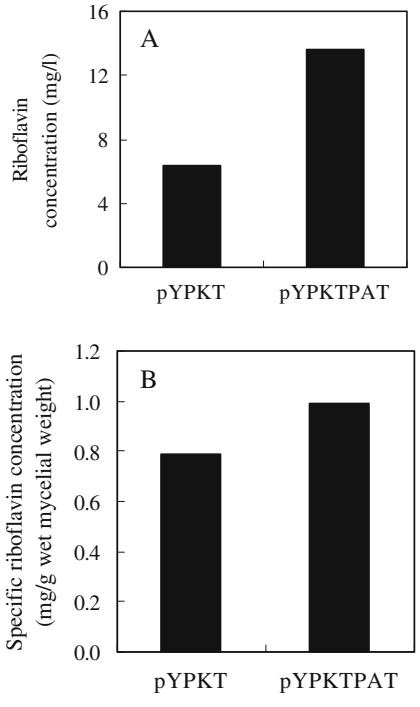

Fig. 6 Riboflavin concentrations (**a**) and specific riboflavin concentrations (**b**) in the culture of *A. gossypii* pYPKT1-1 and *A. gossypii* pYPKTPAT 1-1 in the presence of 50 mM glyoxylate. Riboflavin concentration was measured after 3 days of culture. pYPKT1-1 and pYPKTPAT1-1 denote *A. gossypii* pYPKT1-1 and *A. gossypii* pYPKTPAT1-1 strains, respectively

Discussion

Ashbya gossypii is a filamentous hemiascomycete and its genome was completely sequenced and annotated. More than 90% of *A. gossypii* genes show both homology and a particular pattern of synteny with *S. cerevisiae* (Dietrich et al. 2004). Moreover, genome-wide transcription analysis was performed using a massive parallel signature sequence analysis (Karos et al. 2004).

In this study, the expression vectors in *A. gossypii* were constructed, and heterologous gene expression was performed. The kanamycin resistance gene linked to the *TEF* promoter and terminator from *A. gossypii* was inserted into YEp24 and pYES2, which contain 2μ ARS from *S. cerevisiae*, and YEpPKT and pYPKTPAT were constructed, respectively. When the transformant with YEpPKT was cultivated in the oil-containing medium, riboflavin production decreased markedly compared with the transformant with pYPKT. Several gene expression vectors based on the YEp24 vector have been constructed to date, whereas this decrease in riboflavin production has not been reported to the best of our knowledge. The reason the riboflavin production of *A. gossypii* YEpPKT decreased markedly was not clear. Therefore, the pYPKT-based vector was used as an alternative hereafter.

The *AGX1* gene linked to the *TEF* promoter and terminator was inserted into pYPKT to construct pYPKTPAT. The specific AGT activity of *A. gossypii* pYPKTPAT1-1, the transformant with pYPKTPAT, was approximately 10-fold higher than that of pYPKT1-1. *A. gossypii*, to which the plasmid containing *GLY1* (threonine aldolase gene) of *A. gossypii* under the control of the *TEF* promoter and terminator showed a 10-fold higher specific activity of threonine aldolase than wild-type *A. gossypii*, using glucose as the sole carbon source (Monschau et al. 1998). The *AGX1* gene encodes AGT, which converts glyoxylate into glycine, which is a precursor during de novo purine biosynthesis and has a riboflavin-yield-enhancing effect in *A. gossypii*. Kaplan and Demain (1970) reported that the glycine was not associated with mycelial growth but only the riboflavin formation, suggesting that the glycine may be a limiting precursor in riboflavin overproduction. Therefore, the expression of the *AGX1* gene in *A. gossypii* was expected to enhance riboflavin production. However, the riboflavin production of *A. gossypii* pYPKTPAT1-1 was only 1.3- to 1.6-fold higher than that of *A. gossypii* pYPKT1-1, the control strain. The failure to achieve a high riboflavin production is considered to be due to the expression of the *AGX1* gene in cytosol, where the substrate of AGT, glyoxylate, which was formed by isocitrate lyase in peroxisomes in *A. gossypii*, is not present. This was observed in the pYPKTPAT strain cultured in the glyoxylate-containing medium. When glyoxylate was added to the medium, a higher growth rate of the pYPKTPAT strain compared with the pYPKT strain was observed. However, riboflavin production was not enhanced markedly even with the addition of both glyoxylate and alanine. Moreover, in the case of the rapeseed-oil-containing medium with 50 mM glyoxylate, both *A. gossypii* strains harboring pYPKT and pYPKTPAT1-1 exhibited unstable growth. This means that a sufficient amount of glyoxylate exists in peroxisomes of the *A. gossypii* strain, and the addition of glyoxylate may make the growth of *A. gossypii* worse.

Wild-type *A. gossypii* (ATCC10895) produced 185 mg l^{-1} riboflavin in rapeseed-oil-containing medium (data not shown). However, *A. gossypii* harboring pYPKT1-1 produced a smaller amount of riboflavin (Fig. 5), indicating that retention of plasmid might be a burden to *A. gossypii*. If the pYPKTPAT plasmid is integrated into genome DNA of *A. gossypii*, the riboflavin production may be improved.

In this study, heterologous gene expression was achieved and a high specific AGT activity was observed, but a contribution of AGT to the riboflavin improvement was not as high as our expectations. However, the method used in this study will be useful for metabolic engineering of *A. gossypii* in the near future.

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