Effects of sirtuins on the riboflavin production in Ashbya gossypii

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Abstract

This study focuses on sirtuins, which catalyze the reaction of NAD^+-dependent protein deacetylase, for riboflavin production in *A. gossypii*. Nicotinamide, a known inhibitor of sirtuin, made the color of *A. gossypii* colonies appear a deeper yellow at 5 mM. *A. gossypii* has 4 sirtuin genes (*AgHST1*, *AgHST2*, *AgHST3*, *AgHST4*) and these were disrupted to investigate the role of sirtuins in riboflavin production in *A. gossypii*. *AgHST1*Δ, *AgHST3* Δ, and *AgHST4* Δ strains were obtained, but *AgHST2* Δ was not. The *AgHST1*Δ and *AgHST3* Δ strains produced approximately 4.3- and 2.9-fold higher amounts of riboflavin than the WT strain. The *AgHST3* Δ strain showed a lower human sirtuin 6 (SIRT6)-like activity than the WT strain and only in the *AgHST3* Δ strain was a higher amount of acetylation of histone H3 K9 and K56 (H3K9ac and H3K56ac) observed compared to the WT strain. These results indicate that AgHst3 is SIRT6-like sirtuin in *A. gossypii* and the activity has an influence on the riboflavin production in *A. gossypii*. In the presence of 5 mM hydroxyurea and 50 μM camptothecin, which causes DNA damage, especially double-strand DNA breaks, the color of the WT strain colonies turned a deeper yellow. Additionally, hydroxyurea significantly led to the production of approximately 1.5 higher amounts of riboflavin and camptothecin also enhanced the riboflavin production even through the significant difference was not detected. Camptothecin tended to increase the amount of H3K56ac, but the amount of H3K56ac was not increased by hydroxyurea treatment. This study revealed that AgHst1 and AgHst3 are involved in the riboflavin production in *A. gossypii* through NAD metabolism and the acetylation of H3, respectively. This new finding is a step toward clarifying the role of sirtuins in riboflavin over-production by *A. gossypii*.

Key points

- Nicotinamide enhanced the riboflavin production in *Ashbya gossypii*.
- Disruption of *AgHST1* or *AgHST3* gene also enhanced the riboflavin production in *Ashbya gossypii*.
- Acetylation of H3K56 led to the enhancement of the riboflavin production in *Ashbya gossypii*.

Keywords *Ashbya gossypii* • Riboflavin • Sirtuin • Histone acetylation • Camptothecin

Introduction

Ashbya gossypii is a natural riboflavin producer and has been utilized for the industrial production of riboflavin (Revuelta et al., 2017; Schwechheimer et al., 2016). To improve efficiency of production, some metabolic engineering has been carried out. Overexpression of *RIB* genes which encode enzymes in the riboflavin biosynthetic pathway enhances riboflavin production (Ledesma-Amaro et al., 2015). Moreover, deregulation of the expression of genes in its purine biosynthetic pathway also improves riboflavin production because guanosine triphosphate (GTP) is one of the precursors of riboflavin (Jimenez et al., 2005; Jimenez et al., 2008; Mateos et al., 2006).

Apart from metabolic engineering, other factors are also involved in riboflavin production by *A. gossypii*. Oxidative stress induced by exposure to H_2O_2 increases riboflavin production and light exposure also increases riboflavin production together with the accumulation of reactive oxygen species (ROS) (Silva et al., 2019; Walther and Wendland, 2012). Previously, we isolated a riboflavin-overproducing mutant by disparity mutagenesis; in this mutant, 33 homozygous and 1377 heterozygous mutations in open reading frames were found (Kato et al., 2020; Park et al., 2011). The genomic analysis of this mutant suggests that oxidative stress and the aging of cells may be involved in riboflavin over-production in this mutant because many mutations in genes involved in mitochondrial function, DNA mismatch repair, and oxidative stress response were found in addition to the increased number of ribosomal RNA gene repeat (Kato et al. 2020). These properties of cells showing compromised mitochondrial function and oxidative stress response are often shown in aged yeast and mammalian cells (Barja, 2019; Breitenbach et al., 2012) and the maintenance of the mitochondria function and oxidative stress response need several flavoproteins (Gudipati et al., 2014). These

suggest that the aging may be connected with the riboflavin over-production in *A. gossypii*.

In *Saccharomyces cerevisiae*, whose genes show both homology and a particular pattern of synteny with more than 90% of *A. gossypii* genes (Dietrich et al., 2004), sirtuin controls aging (Wierman and Smith, 2014). Sirtuin is a member of the NAD^+-dependent protein deacetylase family and is involved in longevity, energy metabolism, and stress responses (Wierman and Smith, 2014). *S. cerevisiae* has 5 sirtuins (Sir2, Hst1−4). Sir2 regulates transcriptional silencing in silent mating cassettes, homothallic mating left (HML) and homothallic mating right (HMR), and telomere length together with Sir3 and Sir4 (Wierman and Smith, 2014). In addition, ribosomal RNA gene repeats are silenced by Sir2 (Saka et al., 2013). This silencing is connected with replicative lifespan (Kaeberlein et al., 1999). Focusing on the relationship of sirtuin with metabolism, Sir2 deacetylates phosphoenolpyruvate carboxykinase (Pck1), leading to its inactivation and the regulation of gluconeogenesis (Casatta et al., 2013; Lin et al., 2009). Other sirtuins also deacetylate non-histone proteins and regulate metabolism and transcriptional silencing (Li et al., 2013; Madsen et al., 2015; Wierman and Smith, 2014).

In this study, the involvement of sirtuins in riboflavin production was investigated in *A. gossipii*. This fungus has four sirtuin genes and each was disrupted to reveal the functions of sirtuin for riboflavin production. This study describes the generation of a new type of riboflavin-overproducing mutant.

Materials and methods

Strains and growth conditions

Ashbya gossypii ATCC10895 was used as the WT strain. MT strain was isolated previously (Park et al., 2011). The fungus was cultivated in YD medium (1% glucose, 1% yeast extract, pH 6.8) and mycelia were kept at −80°C with 20% glycerol. To investigate the color and size of each strain, the glycerol stock was inoculated onto YD agar medium. Mycelia were isolated as 1 cm^2 and put into medium. The additives except for camptothecin were dissolved with sterile water and added to medium at each concentration. Camptothecin was dissolved with methanol and methanol was added as a negative control at the same volume as camptothecin. For the investigation of riboflavin production in each strain, 0.3 mL of the glycerol stock was inoculated into 30 mL of liquid YD medium and cultivated for 24 h at 100 rpm. As a pre-culture, 0.3 mL of the culture medium was inoculated into 30 ml of liquid YD medium and cultivated for 24 h at 100 rpm. Then, 0.5 ml of the pre-culture medium was inoculated into 50 ml of the liquid YD medium and cultivated at 100 rpm.

Spore isolation was carried out according to our previous paper (Tajima et al., 2009). In brief, mycelia were suspended in 0.5 ml sterile water followed by the addition of 0.25 mL of 15 mg/ml Zymolyase 40-T (Seikagaku Co., Tokyo, Japan). After incubation at 37°C for 30 min, spores were pelleted by centrifugation. The pellet was washed with 0.03% Triton X-100 two times and resuspended in 0.03% Triton X-100 containing 15% glycerol.

Transformation of *A. gossypii* to disrupt each sirtuin gene

To disrupt each sirtuin gene, the transformation in *A. gossypii* was carried out according to our previous paper (Wendland et al., 2000). In brief, 300 μL of spores were

inoculated into 100 mL of complete medium (2% glucose, 1% polypeptone, 1% yeast extract) and cultivated for approximately 24 h at 100 rpm. After mycelia were collected and washed with sterile water, they were suspended in 40 mL of 50 mM potassium phosphate buffer (pH 7.5) containing 25 mM 2-mercaptoethanol. The mycelia were incubated for 30 min at 28°C at 100 rpm and collected. Mycelia were washed with STM buffer (10 mM Tris-HCl, pH 7.5, 270 mM sucrose, and 1 mM $MgCl_2$) and suspended in 120 μL of the same buffer. DNA (several micrograms) were put into the mycelia and electroporation performed using Gene-Pulser Xcell System (Bio-Rad, Hercules, CA, USA) with settings of 1.5 kV, 500 Ω, and 25 μF using 2 mm pre-chilled cuvettes (Bio-Rad). Mycelia were collected from the cuvette and suspended with 1 ml of full medium. Mycelia were cultivated for 20 min at 100 rpm, followed by inoculation onto TD agar medium. After the plates were incubated at 28°C for 6 h, YD agar medium (0.6% agar) containing 300 μg/ml G418 was overlaid onto the plates. Spores were collected from mycelia grown in the presence of G418 and homozygous gene-disrupted strains were isolated. The gene disruption was confirmed by PCR using each primer set (Table 1).

To disrupt each sirtuin gene, kanamycin resistance gene expression cassette containing 50 bp of the target gene at the 5′ and 3′ ends was prepared by PCR. In this PCR, the forward primer containing 50 bp of 5′ region of the target gene and reverse primer containing 50 bp of 3′ region of the target gene were used (Table 1). As a template DNA, pYPKT vector was used (Kato et al., 2006).

Riboflavin and protein measurement

The concentration of riboflavin produced in *A. gossypii* was measured according to our previous paper (Jeong et al., 2015). In brief, 0.8 mL of the culture broth was picked up

and mycelia were disrupted by sonication. Then, the suspension was centrifuged and supernatant collected. The supernatant contained extracellular and intracellular riboflavin. The supernatant was thoroughly mixed with 0.2 mL of 1 N NaOH. A 0.4 mL aliquot of the solution was neutralized with 1 mL of 0.1 M potassium phosphate (pH 6.0), and its absorbance at 444 nm was measured. The riboflavin concentration was calculated using its extinction coefficient of 1.04×10^{-2} M^{-1} cm^{-1} (127 mg riboflavin/L at ABS_{444}).

Protein concentration of the solution was measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific K. K. Tokyo, Japan)

Sirtuin assay

SIRT6-like activity assay was carried out using CycLex® SIRT6 Deacetylase Fluorometric Assay Kit Ver.2 (MEDICAL ＆ BIOLOGICAL LABORATORIES, Nagoya, Japan). Briefly, 200 mg of mycelia cultivated in liquid YD medium for approximately 24 h were suspended with 3 mL of 10 mM sodium phosphate buffer (pH 5.8) containing 1.2 M magnesium sulfate and 1.6 mg/mL Zymolyase-40T to prepare its protoplasts. The protoplasts were washed twice with 10 mM Tris-HCl (pH 7.5) containing 1M sorbitol. Then, the protoplasts were disrupted with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate). The homogenate was centrifuged and the supernatant used to measure sirtuin activity. For this assay, 8.5 μg of protein in each sample was used.

SDS-PAGE and western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

178 performed using 15% polyacrylamide gel. Gels were stained with coomassie brilliant
179 blue R250 (CBB R250). In the case of western blot, proteins were transferred onto a
180 nitrocellulose membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-
181 Rad) after SDS-PAGE. Blocking were carried out with 5% skimmed milk in Tris-
182 buffered saline containing 0.1% Tween 20 (TBST, pH 7.6). The PVDF membrane was
183 incubated with TBST containing the primary antibody. As a primary antibody, 500-fold-
184 diluted rabbit anti-Histone H3 antibody (GeneTex, Irvine, CA, USA), 2000 fold-diluted
185 rabbit anti-Histone H3K56ac antibody (Active Motif, Carlsbad, CA, USA) and 2000
186 fold-diluted rabbit anti-Histone H3K9ac antibody (Active Motif) were used. After the
187 membrane was washed with TBST three times, the membrane was incubated with
188 15000 fold-diluted goat Anti-IgG (H + L chain) conjugated with horseradish peroxidase
189 (HRP) (MEDICAL ＆ BIOLOGICAL LABORATORIES). Detection was based on the
190 HRP reaction and carried out using Immobilon Western Chemiluminescent HRP
191 Substrate (Merck Millipore Japan, Tokyo, Japan). Protein bands were detected on a
192 Fluor-S/MAX imager (Bio-Rad). Densitometry analysis was performed by Image J (US
193 National Institutes of Health)
194
195 **NAD measurement**
196 The amount of intracellular total NAD was measured using a NAD/NADH Assay Kit-
197 WST (Dojindo, Kumamoto, Japan). The homogenate was prepared by sonication of
198 mycelia grown for 24 h, centrifuged and the supernatant used as the sample.
199
200 **Statistics analysis**
201 Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software, San

Diego, CA, USA). All data were analyzed for statistical significance by unpaired Student's *t*-test with two-side test. Error bars in each figure indicates standard deviation.

RESULTS

Effects of a sirtuin inhibitor on riboflavin production

Sirtuin is a NAD^+-dependent histone deacetylase (class III histone deacetylase) and NAD^+ is converted to nicotinamide (NAM) during the deaceylation of histones. NAM is known as an inhibitor of sirtuins (Avalos et al., 2005). To inhibit sirtuins in *A. gossypii*, NAM was added into the culture medium (Fig. 1A). The addition of 5 or 10 mM NAM enhanced the intensity of the yellow color of *A. gossypii*, but the addition of the same concentration of nicotinic acid (NA) did not change the color of *A. gossypii* mycelia. NAM and NA are precursors of NAD^+ in its salvage pathway (Orlandi et al., 2020) and only NAM changed the mycelial color of *A. gossypii*. In liquid culture, an approximately 5 fold higher amount of riboflavin was detected in the presence of 10 mM NAM (Fig. 1B). These results suggest that the inhibition of sirtuins may lead to the over-production of riboflavin in *A. gossypii* which may be controlled by epigenetic regulation.

Disruption of sirtuin genes in *A. gossypii*

In *A. gossypii*, we found four sirtuin genes (AGOS_AEL013C, AGOS_AGL018C, AGOS_AEL229W, AGOS_AGL118W) using the amino acid sequences of five yeast sirtuins (SIR2, HST1, HST2, HST3, HST4) (Wierman and Smith, 2014). In Table. 1, amino acid sequences of each sirtuin gene in *A. gossypii* and *S. cerevisiae* is shown.

From these sequences, AGOS_AEL013C, AGOS_AGL018C, AGOS_AEL229W and AGOS_AGL118W genes were named as *AgHST1*, *AgHST2*, *AgHST3*, and *AgHST4*, respectively (Table 2). Then, disruption of each *A. gossypii* sirtuin gene was carried out by homologous recombination using a kanamycin (geneticin) resistant gene expression cassette (Fig. S1A) (Sugimoto et al; Wendland et al., 2000). In the case of *AgHST1*, *AgHST3*, and *AgHST4* genes, some homozygous geneticin-resistant colonies were grown. However, no homozygous geneticin-resistant colonies were observed when the *AgHST2* gene was disrupted. To confirm each gene disruption, PCR was carried out using each primer set (Table 1) (Fig. S1A and B). The sizes of *AgHST1*, *AgHST3*, and *AgHST4* genes are 1680, 1509, and 1245 bp, respectively. In each gene-disrupted strain, each sirtuin gene was replaced with a kanamycin resistant gene expression cassette (1.5 kbp).

Riboflavin production of each gene-disrupted mutant

Colonies of *AgHST1*Δ and *AgHST3*Δ strains showed a deep yellow color compared to the WT strain, suggesting these strains produced a higher amount of riboflavin than the WT strain (Fig. 2A). The growth of *AgHST1*Δ and *AgHST3*Δ strains was slower on YD agar plates than the WT strain (Fig. 3B). *AgHST1*Δ and *AgHST3*Δ strains produced approximately 4.3- and 2.9-fold higher amounts of riboflavin at 72 h than the WT strain (Fig. 3C). Additionally, the *AgHST4*Δ strain also produced a slightly higher (1.3 fold) amount of riboflavin than the WT strain. The number of spores produced in each gene-disrupted mutant was almost the same as that of WT strain.

Properties of each gene-disrupted mutant

Sirtuin assay was also performed to confirm the gene disruption. We used the SIRT6 assay kit in this experiment because Hst3 and Hst4 have human SIRT6-like activity which catalyzes the acetylation of histone H3 lysine 56 (H3K56) (Bosch-Presegué and Vaquero, 2015; Wierman and Smith, 2014). SIRT6-like activities in *AgHST3*Δ and *AgHST4*Δ strains were reduced to 65% and 75%, respectively, compared to that in the WT strain, but the *AgHST1*Δ strain showed almost the same specific SIRT6 activity as the WT strain (Fig. 3A). This result indicates that AgHst3 and AgHst4 have SIRT6-like deacetylase activity and AgHst1 has other sirtuin deacetylase activity. The specific SIRT6 activity in the MT strain, which was isolated as a riboflavin -overproducing mutant previously (Park et al., 2011), was also lower than that in the WT strain. MT has a heterozygous missense mutation (I70T) in the *AgHST3* gene (Kato et al., 2020). This mutation may cause the lower SIRT6-like deacetylase activity of the MT strain. In *S. cerevisiae*, Hst3 and Hst4 catalyzed the deacetylation of H3K56ac to maintain genome integrity (Wierman and Smith, 2014). Additionally, the deacetylation of H3K9ac is catalyzed by SIRT6 in mammalian cells (Bosch-Presegué and Vaquero, 2015). We investigated the amount of H3K56ac and H3K9ac using specific antibodies in each gene-disrupted strain (Fig. 3B). In *AgHST3*Δ strain, increased amount of both H3K9ac and H3K56ac were detected in *AgHST3*Δ strain compared to the WT strain, as well as in MT strain. This result indicates that AgHst3 catalyzes the deacetylation of both H3K56ac and H3K9ac and further suggests its disruption may lead to improved riboflavin production by increasing the amount of both H3K56ac and H3K9ac.

In *S. cerevisiae*, Hst1 controls the amount of intracellular NAD^+ regulating expression of the *BNA2* gene encoding indoleamine 2,3-dioxygenase, which catalyzes the first reaction in de novo NAD^+ biosynthesis from tryptophan (Bedalov et al., 2003).

We measured the total intracellular amount of NAD (NAD^+ and NADH) in each strain. The *AgHST1*Δ strain had approximately a 1.3-fold higher amount of total NAD than the WT strain, but *AgHST3*Δ strain had almost the same amount as WT strain (Fig. 3C). On the other hand, the total amount of NAD in *AgHST4*Δ was reduced to 47% compared to that in the WT strain. This result indicates that the disruption of the *AgHST1* gene led to the increase of total intracellular NAD.

Riboflavin production in the presence of hydroxyurea and camptothecin

*AgHST3*Δ strain had higher amount of both H3K9ac and H3K56ac than WT strain, but other gene-disrupted strain did not (Fig. 3B). **I**n *S. cerevisiae*, the disruption of *HST3* and *HST4* genes causes the hyperacetylation of H3K56 and the accumulation of spontaneous DNA damage (Celic et al., 2008). In mammalian cells, hydroxyurea, which inhibits ribonucleotide reductase, leading to stalled replication forks, the collapse of the forks and double-strand DNA breaks, also increases the amount of H3K56ac (Petermann et al., 2010; Singh and Xu, 2016; Yuan et al., 2009). In yeasts, sirtuin gene-disrupted mutants showed sensitivity to hydroxyurea (Konada et al., 2018; Simoneau et al., 2015). To investigate the involvement of DNA damage in riboflavin production in *A. gossypii*, it was cultivated in the presence of hydroxyurea. In solid medium, colonies of *A. gossypii* showed a more yellowish color following cultivation for a week in the presence of 5 mM hydroxyurea, compared to the control (0 mM), even though growth in the presence of 5 mM hydroxyurea was slower (Fig. 4A). In liquid medium, addition of 5 mM hydroxyurea allowed *A. gossypii* to produce approximately a 1.5-fold higher amount of riboflavin than the control (0 mM) (Fig. 4B). Along with hydroxyurea, camptothecin known as a topoisomerase I inhibitor that causes double-strand DNA

298 breaks increases the amount of H3K56ac in yeast and mammalian cells (Matsumoto et
299 al., 2005; Yuan et al., 2009). In the presence of 50 μM of camptothecin, the color of the
300 mycelia also showed a deeper yellow, indicating that camptothecin induces the
301 production of riboflavin in *A. gossypii* (Fig. 4C). Additionally, in YD liquid medium, 50
302 μM camptothecin enhanced riboflavin production by 1.4-fold even through the
303 difference was not statistically significant ($p = 0.054$) (Fig. 4D). Acetylation of H3K56
304 tended to be increased by 50 μM camptothecin, suggesting that DNA double-strand
305 breaks may enhance riboflavin production in *A. gossypii* (Fig. 4E). However, 5 mM
306 hydroxyurea did not increase the acetylation of H3K56, suggesting that hydroxyurea
307 enhances riboflavin production in *A. gossypii* by unknown mechanism. The mycelia
308 grown in the presence of hydroxyurea showed pale yellow color when N-acetyl-L-
309 cysteine, which is a precursor of intracellular cysteine and glutathione and known as an
310 anti-oxidant, was added (Fig. 4F) (Sun, 2010). These results suggest that the generation
311 of reactive oxygen species (ROS) may enhance the riboflavin production in *A. gossypii*
312 by hydroxyurea instead of DNA double-strand breaks. However, the quantification of
313 ROS in the mycelia using some specific dyes was not successful (Data not shown).
314

315 ## Discussion

316 Sirtuins are known as NAD^+-dependent histone deacetylases involved in metabolism,
317 DNA repair, and aging (Fiorino et al., 2014). In particular, yeast Sir2 has been identified
318 as a longevity factor (Wierman and Smith, 2014). We previously reported that a
319 riboflavin-overproducing mutant isolated by disparity mutagenesis has approximately
320 1400 homo- and heterozygous mutations in protein-encoding regions and exhibits the
321 features of aging (Kato et al., 2020). In this study, to reveal the relationship of aging

with riboflavin production in *A. gossypii*, we focused on sirtuins. Four sirtuin genes (*AgHST1*, *AgHST2*, *AgHST3*, *AgHST4*) were found in *A. gossypii* and these genes were individually disrupted. *AgHST1*Δ, *AgHST3*Δ, *AgHST4*Δ strains were isolated, but *AgHST2*Δ strain was not. *AgHST1*Δ and *AgHST3*Δ strains produced a higher amount of riboflavin in liquid YD medium than the WT strain, but the *AgHST4*Δ strain produced almost the same amount of riboflavin as the WT strain (Fig. 2C). In the case of the *AgHST1*Δ strain, the total intracellular amount of NAD was increased compared to the WT strain and other gene-disrupted strains (Fig. 3C). On the other hand, *AgHST3*Δ strain had lower SIRT6-like deacetylase activity than the WT strain and the other gene-disrupted strains (Fig. 3A). This indicates that the disruption of *AgHST1* and *AgHST3* genes increased riboflavin production via different routes. In the *AgHST3*Δ strain, the amount of H3K9ac and H3K56ac was increased by the reduction of SIRT6-like deacetylase activity as well as in the riboflavin-overproducing MT strain (Fig. 3B). However, in *AgHST4*Δ strain, lower SIRT6-like deacetylase activity was detected compared to in WT strain, but no increase of the amount of H3K9ac and H3K56ac was observed. Additionally, little enhancement of the riboflavin production was observed on the contrary to *AgHST3*Δ strain (Fig. 2C). These results indicate that the acetylation of H3K9 and H3K56 is involved in the riboflavin over-production in *AgHST3*Δ strain showing the deeper yellow color compared to the WT strain (Fig 2A).

H3K56ac is a post-translational modification of histone H3 responsive to DNA damage. Increase of H3K56ac is observed when DNA double-strand breaks (and ultraviolet radiation) induce DNA damage that occurs in yeast and mammalian cells Celic et al., 2008; Matsumoto et al., 2005; Miller et al., 2010; Petermann et al., 2010; Singh and Xu, 2016; Yuan et al., 2009; Zhu et al., 2015). In addition to acetylation of

346 H3K56, the acetylation of H3K9 is also involved in double-strand DNA breaks in
347 fission yeast and mammalian cells (Bosch-Presegué and Vaquero, 2015; Yamada et al.,
348 2013). Camptothecin, which causes DNA double-strand breaks, also induced riboflavin
349 production in *A. gossypii* (Fig. 4C). A previous report described that riboflavin-
350 overproducing mutants are sensitive to photo-induced DNA damage (Silva et al., 2019).
351 These results suggest that DNA double-strand breaks and the acetylation of H3K56 may
352 be important factors inducing riboflavin production in *A. gossypii*. Regarding
353 camptothecin, *A. gossypii* is less sensitive than *S. cerevisiae* which cannot grow
354 normally in the presence of 50 μM camptothecin (Puddu et al., 2017). This result
355 suggests that riboflavin production in *A. gosyypii* may be involved in its resistance to
356 camptothecin.
357 The addition of N-acetyl-L-cysteine led to the loss of the yellow color of mycelia in
358 the presence of hydroxyurea, which induced the riboflavin production (Fig. 4F).
359 Hydroxyurea also generates ROS and activate Yap and Arf regulons, which regulate
360 redox and iron homeostasis, in *S. cerevisiae* (Dubacq et al., 2006; Singh and Xu, 2016).
361 Hydroxyurea also induces DNA double-strand breaks, but H3K56ac was not increased
362 in *A. gossypii* by hydroxyurea in this study even though the riboflavin production was
363 enhanced by hydroxyurea (Fig. 4A, B and E). The enhancement of the riboflavin
364 production may not be caused by DNA double-strand breaks, but by ROS produced by
365 hydroxyurea. It was reported previously that ROS is involved in the riboflavin
366 production in A. gossypii (Silva et al., 2018). ROS is also one of important factors for
367 the riboflavin production in *A. gossypii*.
368 Based on the identity of the amino acid sequences, AgHst1 is a homolog of yeast
369 Sir2 and Hst1 (Table 2), but *A. gossypii* has only a single type of sirtuin, AgHst1. As

well as yeast Hst1, AgHst1 controls the amount of intracellular NAD (Fig. 3C). NAD^+ biosynthesis is regulated by purine metabolism and ATP synthesis in yeast and Bas1, a Myb-related transcription factor, upregulates de novo NAD^+ and purine biosynthesis (Pinson et al., 2019; Zhang et al., 1998). In *A. gossypii*, the disruption of the *AgBAS1* gene leads to enhanced riboflavin production even though the gene disruption promotes adenine-auxotrophy (Mateos et al., 2006). The purine biosynthesis pathway is important for riboflavin production in *A. gossypii* because guanosine triphosphate (GTP) is a precursor of riboflavin (Jimenez et al., 2005; Jimenez et al., 2008). In addition, NAD metabolism is regulated in human cells by epigenetic control (Anderson et al., 2017; Etchegaray and Mostoslavsky, 2016). Therefore, NAD and purine biosynthesis may be connected with riboflavin production in *A. gossypii*.

This study revealed first that two sirtuins, AgHst1 and AgHst3, control riboflavin production in *A. gossypii* via two routes, the acetylation of H3 and the enhancement of NAD biosynthesis. This finding leads to the elucidation of the mechanism of the riboflavin production in *A. gossypii* and the generation of new riboflavin-overproducing mutants of *A. gossypii*.

SUPPLEMENTAL MATERIAL

Supplemental file (Fig. S1)

Authors contribution TK, HAE and EYP conceived and designed this research and the experiments. JA and MK performed all experiments. TK, HAE and EYP wrote this manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

Ethical approval The authors declare that no human participants or animals were used for the purpose of this study.

Data availability

All data analyzed in this study are shown in this published article including its supplementary information files.

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567 **Table 1** Used primers

	Sequence (5' to 3')
*AgHST1*Δ-F	ATGTCTGAGAGCGCAAGTATGCTCCAGGGCAGCAA ACGAGGAACGGATTCAACTGCAGACATGGAGGCCC AGAATACCC
*AgHST1*Δ-R	CTAGGGCGCCTGAACTTGGCGCTGCCCTGTATCTTTA GCCTGTTCCTTTACGGAATTCTTTCTGCGCACTTAAC TTCGC
*AgHST2*Δ-F	ATGGATCCTCAAGAACTTGCTAGCATCAACAAGGTT GCCAAGTATATCAAAACTGCAGACATGGAGGCCCAG AATACCC
*AgHST2*Δ-R	TCAATCATCAGAATTCTCCCTGCTGAGATCCAACTTAA GGATCTTCTCGGCGGAATTCTTTCTGCGCACTTAACT TCGC
*AgHST3*Δ-F	ATGCCGAGTTTAATACAGAGCCAAGAATCGTTTGATG AAGAGCTGCCGAGAACTGCAGACATGGAGGCCCAGA ATACCC
*AgHST3*Δ-R	TTACGGCTCGTCAACATCAGTTGGTACTTCATCGAAAT CCATGCTCTGGCCGGAATTCTTTCTGCGCACTTAACTT CGC
*AgHST4*Δ-F	ATGTCCGCTACACGCCACGCAGCGGGGCAACCACGA GTGCAGCAAGCGGCAACTGCAGACATGGAGGCCCA GAATACCC
*AgHST4*Δ-R	CTAATTGAATGAAGGCGCTAGTGTAGTGAGGTCCT GGCAGTCGCCAACAACGGAATTCTTTCTGCGCAC TTAACTTCGC
AgHST1-F	ATGTCTGAGAGCGCAAGTAT
AgHST1-R	CTAGGGCGCCTGAACTTGGC
AgHST3-F	ATGCCGAGTTTAATACAGAG
AgHST3-R	TTACGGCTCGTCAACATCAG
AgHST4-F	ATGTCCGCTACACGCCACGC
AgHST4-R	CTAATTGAATGAAGGCGCTA

568

569

570

571 **Table 2** Identity of amino acid sequences between sirtuins in *S. cerevisiae* and *A.*

572 *gossypii*.

	Sir2	Hst1	Hst2	Hst3	Hst4
AgHst1	**56%**	**55%**	32%	28%	26%
AgHst2	35%	32%	**52%**	24%	23%
AgHst3	30%	31%	25%	**56%**	33%
AgHst4	24%	25%	25%	34%	**53%**

573

574

Figure legends

Fig 1 Growth and mycelial color of *A.gossypii* with each additive. (**A**) Effect of NAM and NA on the growth and mycelial color of *A. gossypii*. *A. gossypii* was cultivated in YD agar medium with each concentration of NAM or NA for 3 days. (**B**) Riboflavin production of *A. gossypii* in YD liquid medium with each concentration of NAM after 1 day cultivation. Significant difference was indicated by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, n = 3).

Fig 2 Growth and riboflavin production in the gene-disrupted mutants. (**A**) Colonies of each gene-disrupted mutant on YD agar plates. Each strain was cultivated for 3 days. (**B**) Size of each colony on YD agar plates. Each strain was cultivated for 3 days (n = 3). (**C**) Riboflavin production in each gene-disrupted mutant. Each mutant was cultivated in the YD liquid medium and the riboflavin concentration was measured according to the Materials and methods (n = 3). In each figure, significant difference was indicated by asterisks ($^{*}p < 0.05$, $^{***}p < 0.001$,) and MT indicates the riboflavin over-producing mutant isolated previously (Park et al., 2011).

Fig 3 Properties of each gene-disrupted mutant. (**A**) SIRT6-like activity in each gene-disrupted mutant and MT strain cultivated for 1 day. For this assay using CycLex SIRT6 Deacetylase Fluorometric Assay Kit Ver.2, 8.5 μg of proteins in each protoplast homogenate was used (n = 3). (**B**) The amount of H3K56ac and H3K9ac in each gene-disrupted mutant cultivated for 1 day. For SDS-PAGE, 80 μg of proteins in the protoplast homogenate was used, followed by western blot. H3, H3K56ac and H3K9ac

were detected using anti-H3 antibody, anti-H3K56ac and anti-H3K9ac antibodies, respectively, according to the Materials and methods. (**C**) Total NAD amount in each gene-disrupted mutant. Total NAD amount was measured using NAD/NADH Assay Kit-WST (n = 3). In each figure, significant difference was indicated by asterisks ($^{*}p < 0.05$) and MT indicates the riboflavin over-producing mutant isolated previously (Park et al., 2011).

Fig 4 Effects of hydroxyurea and camptothecin on the growth and the riboflavin production in *A. gossypii*. (**A**) Growth on the YD agar medium in the presence of hydroxyurea. *A. gossypii* was cultivated for a week (**B**) Riboflavin production in the YD liquid medium with 5 mM hydroxyurea. WT strain was cultivated in the YD liquid medium with 5 mM hydroxyurea for 2 days and the riboflavin concentration and the dry mycelial weight were measured (n = 3). (**C**) Growth on the YD agar medium in the presence of camptothecin. As a control, methanol was added to the YD agar medium at the same volume (5%) as 50 μM of camptothecin. *A. gossypii* was cultivated for a week (**D**) Riboflavin production in the YD liquid medium with 50 μM camptothecin. WT strain was cultivated in the YD liquid medium with 50 μM camptothecin for 2 days and the riboflavin concentration and the dry mycelial weight were measured (n = 7). As a control, methanol was added to the YD agar medium at the same volume (5%) as 50 μM of camptothecin. The significant difference is not detected ($p = 0.054$). **(E)** Acetylation of H3K56 in the presence of 5 mM hydroxyurea or 50 μM camptothecin. For SDS-PAGE, 80 μg of proteins in the protoplast homogenate was used and western blot was carried out by the same method as Fig 3B. In the case of camptothecin, methanol was added to the YD agar medium at the same volume (5%) as 50 μM of

623 camptothecin as a control (0 mM). Densitometry analysis was carried out using Image J
624 (n = 3) (**F**) Growth of *A. gossypii* in the presence of hydroxyurea and N-acetyl-L-
625 cysteine. Each concentration of N-acetyl-L-cysteine was added to the YD agar medium
626 with 5 mM hydroxyurea and *A. gossypii* was grown for 4 days.
627

Fig. 1 Kato et al.

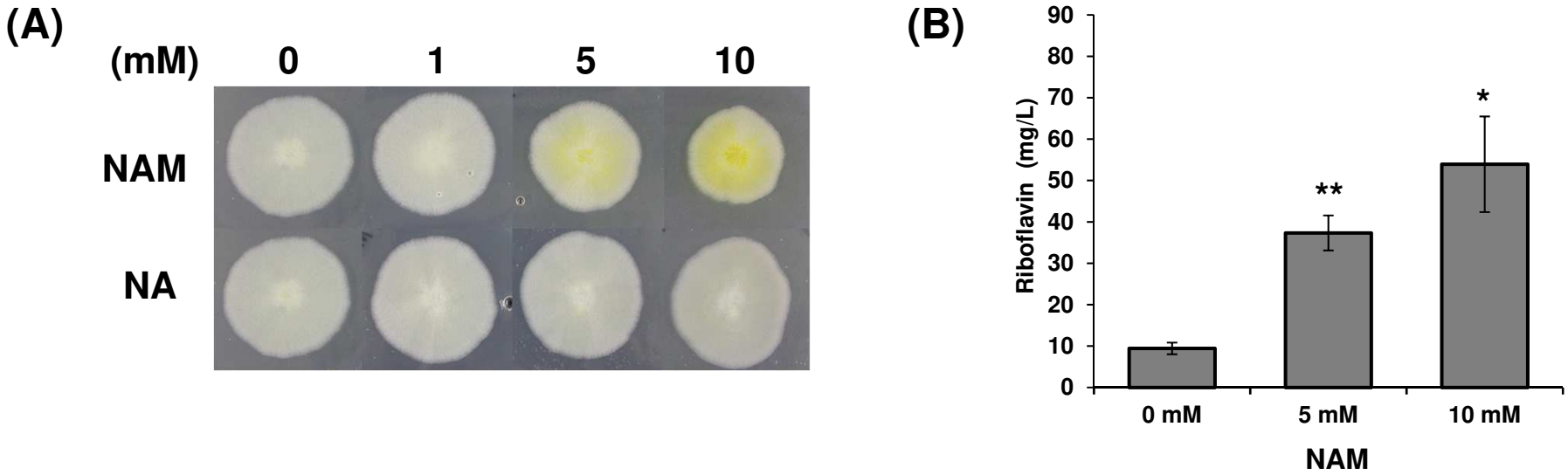
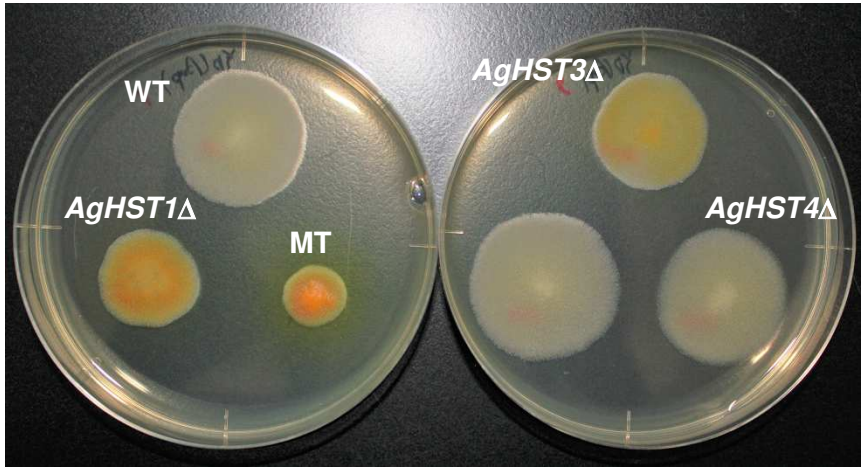


Fig. 2 Kato et al.

(A)



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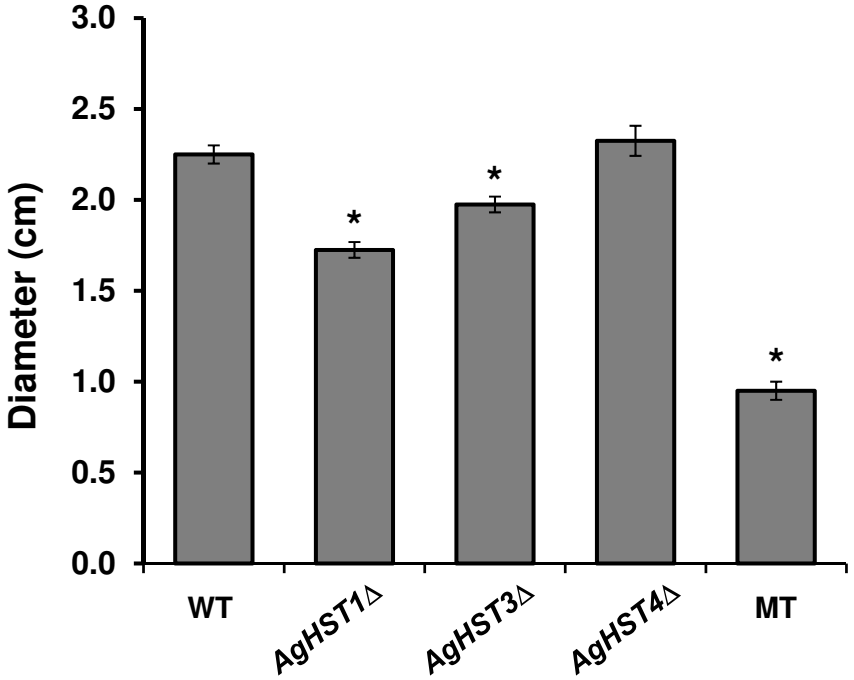

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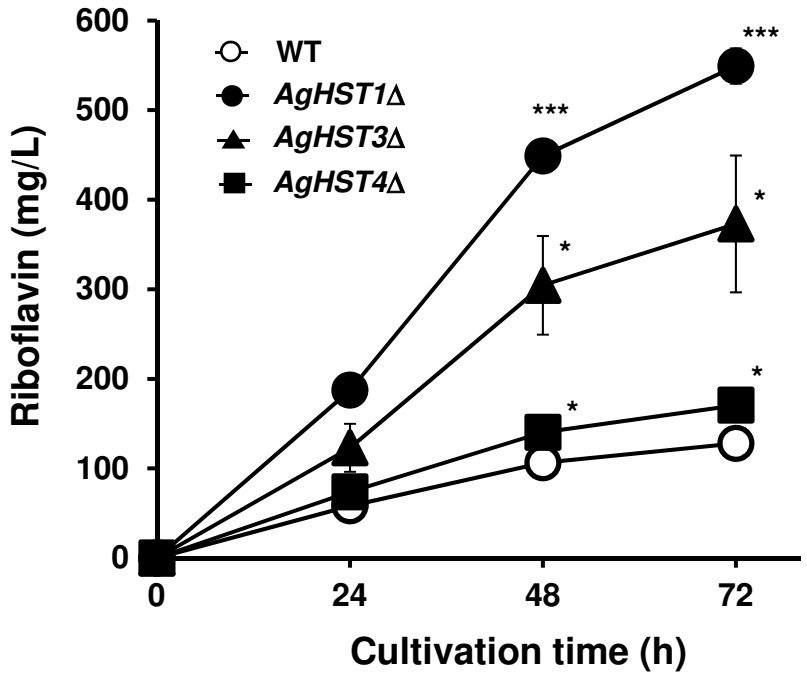


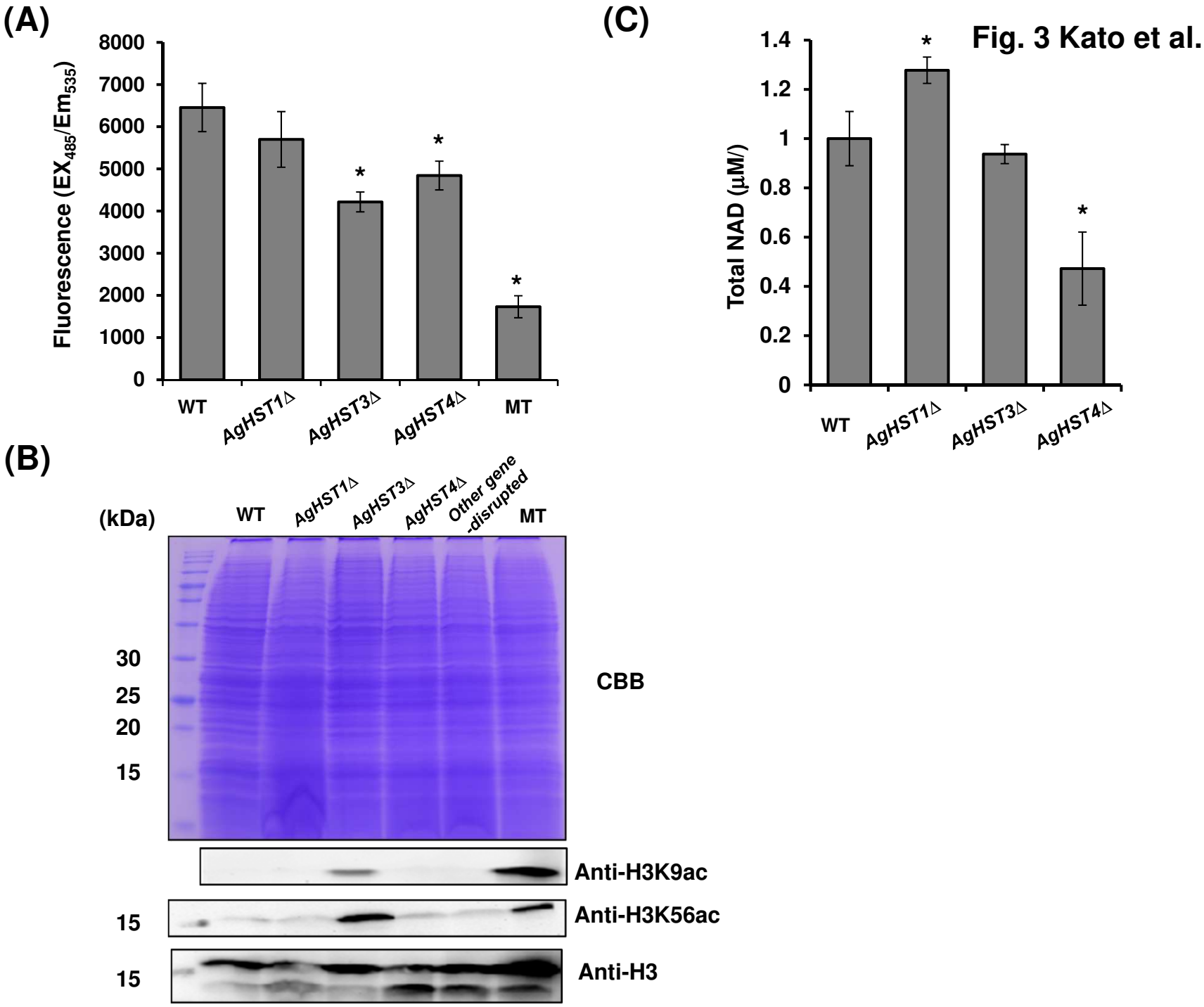

(A)
Fluorescence (EX485/Em535)
8000
7000
6000
5000
4000
3000
2000
1000
0
WT
AgHST1Δ
AgHST3Δ
AgHST4Δ
MT
(B)
(kDa)
WT
AgHST1Δ
AgHST3Δ
AgHST4Δ
Other gene -disrupted
MT
30
25
20
15
CBB
Anti-H3K9ac
15
Anti-H3K56ac
15
Anti-H3
(C)
Total NAD (μM)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT
AgHST1Δ
AgHST3Δ
AgHST4Δ
Fig. 3 Kato et al.

Fig. 4 Kato et al.

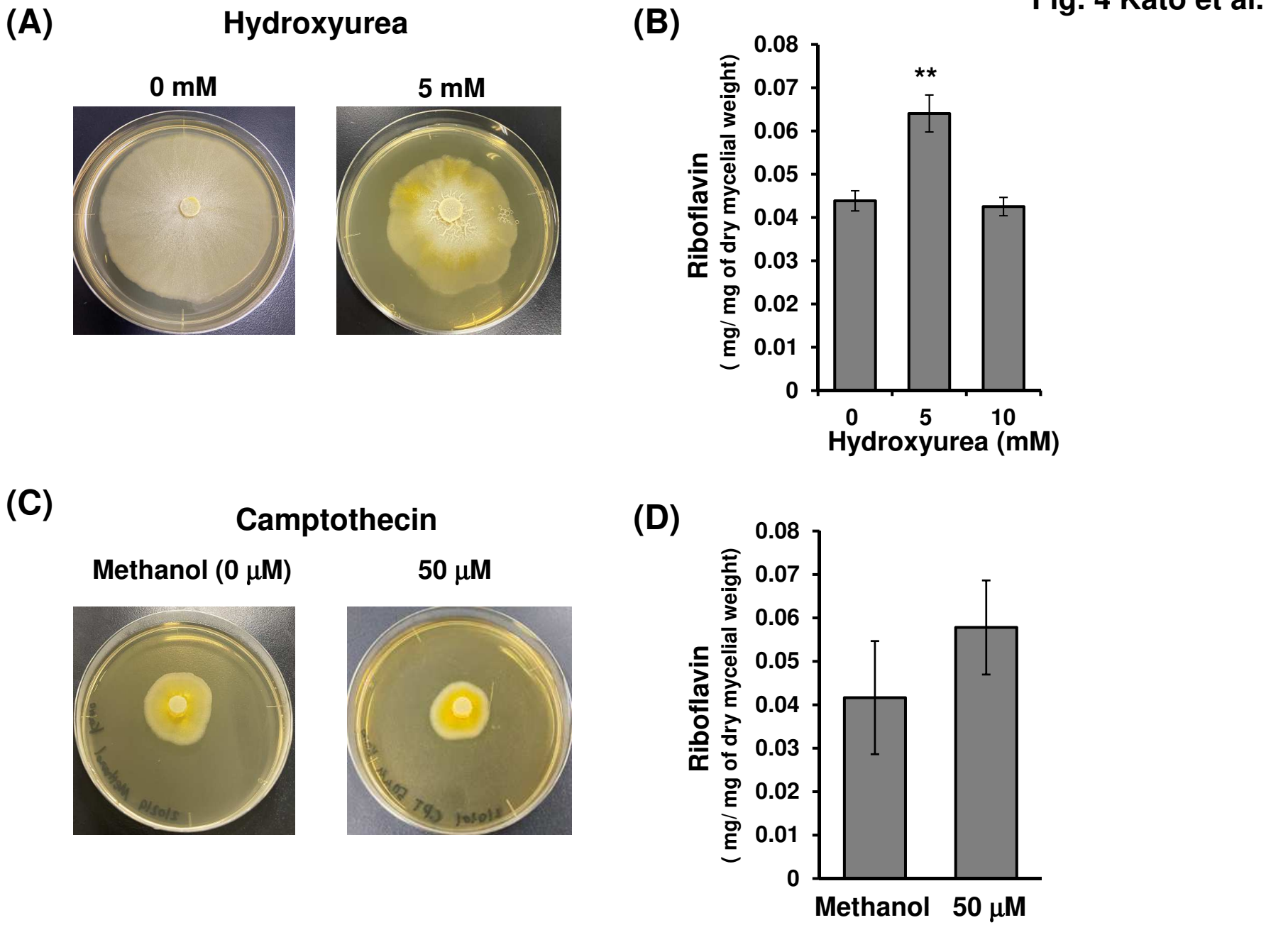
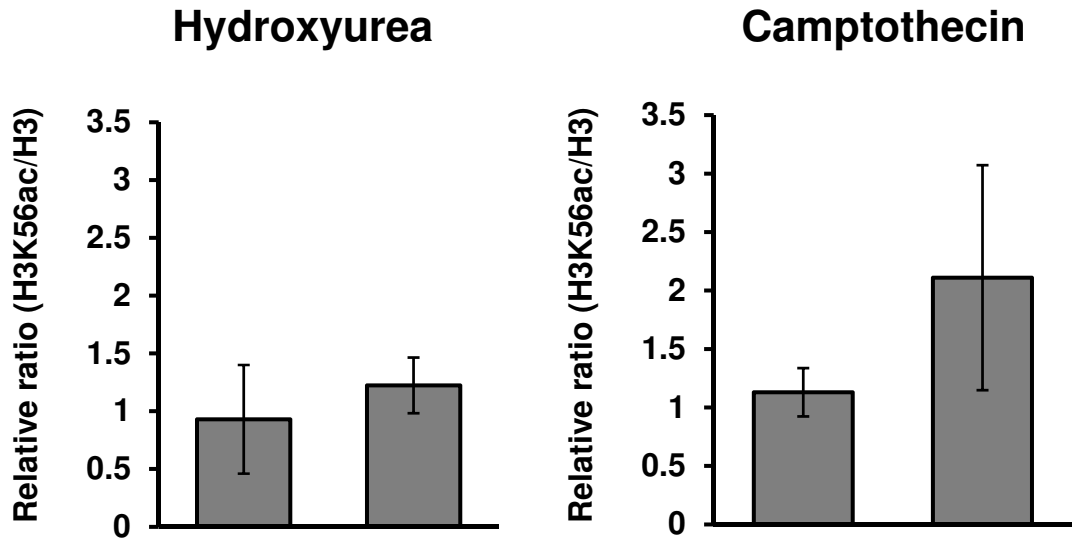
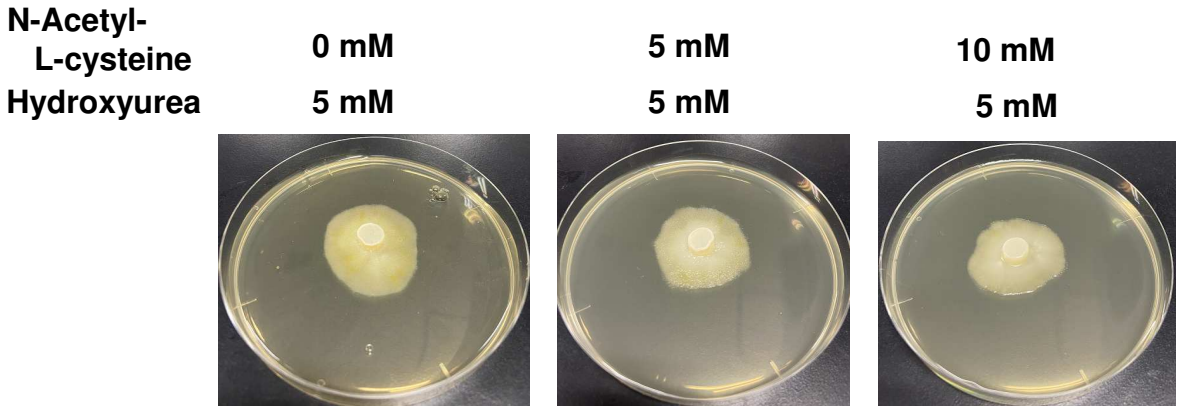


Fig. 4 Kato et al.

(E)



(F)



Effects of sirtuins on the riboflavin production in *Ashbya gossypii*

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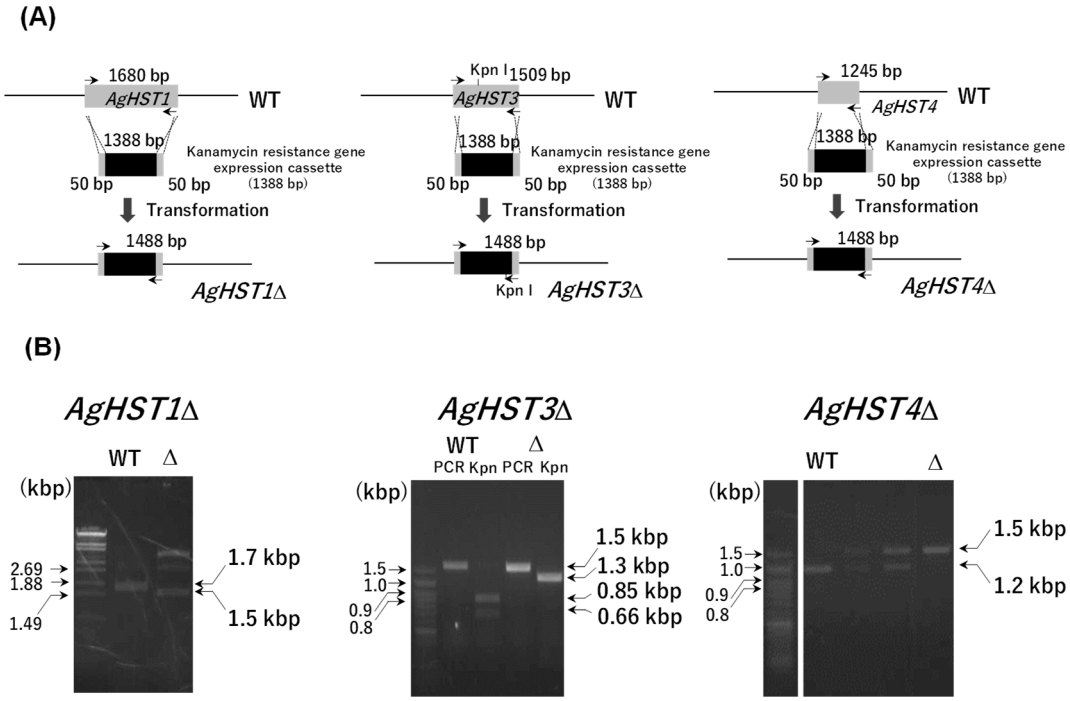

Fig S1 Disruption of each gene (*AgHST1*, *AgHST3* and *AgHST4*) in *A. gossypii*. (**A**) Illustration of the gene disruption using the kanamycin (geneticin) resistant gene expression cassette harboring the 50 bp of the homologous sequences to each gene at both ends. The kanamycin (geneticin) resistant gene expression was prepared by PCR and transformed to *A. gossypii* by electroporation. The gene disruption was achieved by the homologous recombination of the cassette to the target gene. (**B**) Confirmation of the gene disruption of each gene by PCR. PCR was performed using primer sets which produce the full-length of each gene. The kanamycin (geneticin) resistant gene expression cassette and 100 bp of the target gene (1.5 kbp) was amplified by this PCR when the target gene was disrupted.