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Effects of a proteasome inhibitor on the riboflavin production in *Ashbya gossypii*

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Abstract

Aims: Effects of a proteasome inhibitor, MG-132, on the riboflavin production in *Ashbya gossypii* were investigated to elucidate the relationship of the riboflavin production with flavoprotein homeostasis.

Methods and results: The addition of MG-132 to the liquid medium reduced the specific riboflavin production by 79% in *A. gossypii* at 25 μM after 24 h. Addition of the inhibitor also caused the accumulation of reactive oxygen species and ubiquitinated proteins. These results indicated that MG-132 works in *A. gossypii* without any genetic engineering and reduces riboflavin production. In the presence of 25 μM MG-132, specific NADH dehydrogenase activity was increased by 1.4-fold compared to DMSO, but specific succinate dehydrogenase activity was decreased to 52% compared to DMSO. Additionally, the amount of AgSdh1p (ACR052Wp) was also reduced. Specific riboflavin production was reduced to 22% when 20 mM malonate, a succinate dehydrogenase (SDH) inhibitor, was added to the culture medium. The riboflavin production in heterozygous *AgSDH1* gene-disrupted mutant (*AgSDH1*$^{-/+}$) was reduced to 63% compared to that in wild type.

Conclusions: MG-132 suppresses the riboflavin production and SDH activity in *A. gossypii*. SDH is one of the flavoproteins involved in the riboflavin production in *A. gossypii*.

Significance and Impact of the Study: This study shows that MG-132 has a negative influence on the riboflavin production and SDH activity in *A. gossypii* and leads to the elucidation of the connection of the riboflavin production with flavoproteins.

Keywords *Ashbya gossypii*, Riboflavin, MG-132, Succinate dehydrogenase

Introduction

Ashbya gossypii is a natural riboflavin producer and has been utilized for industrial riboflavin production due to its high productivity (Schwechheimer *et al.* 2016; Revuelta *et al.* 2017). This fungus is also regarded as a model organism because most genes in *A. gossypii* are syntenic to those in budded yeast, *Saccharomyces cerevisiae* (Dietrich *et al.* 2004). Therefore, *A. gossypii* is an industrially and scientifically important organism.

The metabolic pathway of riboflavin biosynthesis in *A. gossypii* has been investigated and engineered for its overproduction. The purine biosynthetic pathway is one of the target pathways for its overproduction in *A. gossypii* to supply its precursor, GTP (Revuelta *et al.* 2017). The expression of mutated phosphoribosyl pyrophosphate amidotransferase, which is no longer regulated by the feedback inhibition of GTP and ATP, enhanced riboflavin production by 10-fold in *A. gossypii* with the addition of GTP and ATP (Jiménez *et al.* 2005). Glycine is also known as a precursor of riboflavin and enters the purine biosynthetic pathway. Glycine metabolism is also involved in riboflavin production in *A. gossypii* (Monschau *et al.* 1998; Schlüpen *et al.* 2003). To improve riboflavin productivity in *A. gossypii*, pathways to supply precursors have been engineered.

Riboflavin is a precursor of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), which are co-factors of flavoproteins. Some flavoproteins need FAD for folding and cellular localization (Robinson and Lemire 1996; Sato *et al.* 1996). FAD synthase, which catalyzes the synthesis of FAD, controls flavin homeostasis and flavoprotein biogenesis (Giancaspero *et al.* 2015; Liuzzi *et al.* 2012). This result indicates that flavin metabolism is important for functions of flavoproteins in cells. Most flavoproteins work in mitochondria and participate in redox homeostasis and redox reaction (Gudipati *et al.* 2014), but, regarding protein homeostasis, some flavoproteins are also related to the ubiquitin-proteasome system (UPS) (Lavie *et al.* 2018; Reimer *et al.* 2009; Sollner and Macheroux 2009). Moreover, UPS

maintains mitochondrial homeostasis (Sulkshane *et al.* 2020). UPS is connected with mitochondrial function including the mitochondrial respiratory chain and flavoproteins, even though mitochondria perform ubiquitin-dependent quality control (Martínez-Reyes and Chandel 2020; Ravanelli *et al.* 2020; Rawat *et al.* 2019).

However, the riboflavin production in *A. gossypii* has not been investigated in view of protein homeostasis yet and this study aimed at elucidating the relationship of riboflavin production and flavoprotein homeostasis. Here, the effects of the 26S proteasome inhibitor MG-132 on riboflavin production was investigated to elucidate the relationship of riboflavin production and flavoprotein in *A. gossypii*.

Materials and methods

Strain cultivation and spore isolation

A. gossypii ATCC10895 was used as the wild type (WT) strain. The strain was maintained in YD agar medium (1% glucose, 1% yeast extract, and 2% agar, pH 6.8). Grown mycelia were kept at -80 °C with 20% glycerol. Cultures were produced from glycerol stocks, of which 0.3 mL was inoculated into 30 mL of liquid YD medium and cultivated for 24 h at 100 rpm. Next, as a pre-culture, 0.3 mL of the culture medium was inoculated in 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 to 50 mL of the liquid YD medium and cultivated for indicated time. MG-132 was dissolved with DMSO at 25 mM as a stock solution and added to culture medium at 25 μM final concentration. As a negative control, DMSO only was added at equal amounts.

Spore isolation was carried out according to a protocol reported previously (Tajima *et al.* 2009). Mycelia grown in YD agar medium were harvested and suspended with 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, the suspension was centrifuged to precipitate spores. The pellet was washed with 0.03% Triton X-100 twice and resuspended with 0.03% Triton X-100 containing 15% glycerol.

Staining of intracellular reactive oxygen species (ROS)

Mycelia cultivated in YD liquid medium for 1 to 2 days were harvested and washed with sterile water. Intracellular ROS in mycelia (40 mg) was stained with ROS brite 570 (AAT Bioquest, Sunnyvale, CA, USA) for oxidative stress detection. Fluorescence was observed using a fluorescent microscope BX60 (Olympus, Tokyo, Japan). Fluorescent intensity was measured using a filter-based multimode microplate reader (Infinite F200 M; Tecan Ltd., Männedorf, Switzerland).

Riboflavin assay

The amount of riboflavin was assayed according to a protocol reported previously (Jeong *et al.* 2015). Briefly, 0.8 mL of the culture broth was thoroughly mixed with 0.2 mL of 1 N NaOH. A 0.4 mL aliquot of the resulting solution was neutralized with 1 mL of 0.1 M potassium phosphate (pH 6.0), and the absorbance of the solution at a wavelength of 444 nm was measured. The riboflavin concentration was calculated at an extinction coefficient of 1.04×10^{-2} M^{-1} cm^{-1} (127 mg riboflavin/L at ABS_{444}).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

SDS-PAGE was carried out to separate proteins using 12% acrylamide. Proteins were transferred to a polyvinylidene fluoride membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After blocking with 5% skim milk in TBS-Tween 20 (pH 7.6), the membrane was incubated with a 500-fold diluted rabbit ubiquitin polyclonal antibody

(StressMarq Bioscience, Victoria, BC, Canada) for ubiquitinated proteins and 2000-fold diluted serum from rabbit immunized by AgSdh1p (ACR052Wp, 577−590 aa domain) (Eurofins Genomics K.K., Tokyo, Japan) for AgSdh1p. A 10,000-fold diluted goat anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (Medical & Biological Laboratories, Nagoya, Japan) was used as a secondary antibody. Specific proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA) and Fluor-S Max Multi-Imager (Bio-Rad).

Enzyme assay

Succinate dehydrogenase activity was measured as described previously (Munakata 1996). Briefly, the reaction mixture (0.49 mL) containing 0.1% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 50 mM sodium succinate in phosphate-buffered saline (PBS; pH 7.4) was incubated at 30°C as pre-incubation, and 0.01 mL of the crude extract was added into the reaction mixture to start the reaction. After 10 min, 1 mL acetone was added to the reaction mixture and stirred completely. After centrifugation, absorbance in the acetone fraction was measured at 570 nm.

NADH dehydrogenase activity was also measured as described previously (Velázquez and Pardo 2001). Briefly, the reaction mixture (990 µL) containing 0.05 mM 2,6-dichloroindophenol sodium (DCIP), 0.1 mM NADH, 200 mM KCl, and 1 mM EDTA in 0.1 M potassium phosphate (pH 6.0) was pre-incubated at room temperature. To start the reaction, 10 µL of the crude extract were added to the reaction mixture. The slope of the absorbance at 600 nm was measured and calculated using the extinction coefficient of reduced DCIP (1.9×10^4 M^{-1} cm^{-1}).

In each enzymatic assay, one unit of the activity was defined as the amount of enzyme required to produce 1 µmol reduced electron receptor in 1 min. Mycelia suspended with PBS

(pH 7.4) were disrupted by sonication and used as a crude extract without any centrifugation step. Protein concentration was determined by Pierce BCA Protein Assay kit (Thermo Fisher Scientific K.K.).

Statistics analysis was carried out using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). All data were analyzed for the statistical significance by unpaired Student's *t*-test with two-side test.

Gene disruption

The kanamycin resistance gene expression cassette containing 50 bp *AgSDH1* gene at the 5′ and 3′ ends was amplified by polymerase chain reaction (PCR) using the primer set (P3 and P4) in Table 1 and Fig. S1 and pYPKT vector (Kato and Park, 2006). The amplified DNA fragment (several micrograms) was transformed into *A. gossypii* using a protocol described previously (Wendland *et al.* 2000). In brief, 300 µL of *A. gossypii* spores was cultivated in 100 mL of YD liquid medium inoculated with spores for 17–24 h at 100 rpm, and mycelia were harvested by filtration. After mycelia were washed with sterile water, mycelia were suspended with phosphate buffer containing 25 mM 2-mercaptoethanol. The suspension was incubated for 30 min at 28°C with shaking followed by harvesting mycelia by filtration. Mycelia were washed with STM buffer (10 mM Tris-HCl, pH 7.5, 270 mM sucrose, and 1 mM $MgCl_2$) and suspended with 120 µL of STM buffer. Several micrograms of amplified DNA were added into the mycelial suspension, and electroporation was performed using Gene-Pulser Xcell System (Bio-Rad, Hercules, CA, USA) under the condition of 1.5 kV, 500 Ω, and 25 µF using 2 mm pre-chilled cuvettes (Bio-Rad). Mycelia were resuspended in YD liquid medium and incubated at 28°C for 1 h with shaking. The suspension was inoculated on YD agar plate and incubated at 28°C for 6 h followed by overlaying the YD agar medium (0.6% agar) containing 300 µg/mL G418 (geneticin). Colonies (heterozygous) were isolated, and disruption of the *AgSDH1* gene

was confirmed by PCR using the primer sets (P1-P2, P3-P4, P5-P6 and P7-P8 primer sets, Table1 and Fig. S1). To isolate homozygous *AgSDH1*Δ strain, spores were isolated from a heterozygous mutant and cultivated in the presence of 300 μg/mL G418.

DNA extraction

Mycelia were suspended with 0.5 mL of 1 M sorbitol containing 100 mM EDTA (pH 7.5) and 0.2 mL of 10 mg/mL Zymolyase 40-T and incubated with gently stirring. Cells were pelleted by centrifugation, and the pellet was resuspended with 0.5 mL of 50 mM Tris-HCl (pH 7.5) containing 20 mM EDTA and 0.05 mL of 10% sodium dodecyl sulfate (SDS). After incubation at 65°C for 30 min, 0.2 mL of 5 M potassium acetate was added to the suspension followed by incubation of the mixture on ice for 30 min. Then, genomic DNA was precipitated by ethanol and treated with RNase.

Results

Effects of MG-132 on the physiology of *A. gossypii*

To investigate the relationship of riboflavin production with UPS, MG-132, a 26S proteasome inhibitor, was added when *A. gossypii* was cultivated (Fig. 1A). In the presence of 25 μM MG-132, the colony size was decreased and the mycelial color turned cream-colored. In the submerged cultivation, specific riboflavin production at 24 h in the presence of 25 μM MG-132 was reduced by 79% in comparison with the control (in the presence of DMSO only) (Fig. 1B).

In *S. cerevisiae*, the presence of MG-132 inhibited its proteasome activity when *ise1* (*erg6*) gene, which encodes S-adenosylmethionine:Δ^{24}-methyltransferase involved in ergosterol biosynthesis, or the *pdr5* gene, which encodes a major drug efflux pump, were disrupted (Lee

198 and Goldberg 1996; Fleming *et al.* 2002). To further assess the effects of MG-132 on the
199 physiology of *A. gossypii*, the accumulation of ubiquitinated proteins and ROS in *A. gossypii*
200 in the presence of MG-132 was investigated (Fig. 2). More ubiquitinated proteins in the
201 presence of 25 μM MG-132 were detected by western blot using rabbit ubiquitin polyclonal
202 antibody than those in the presence of DMSO only (Fig. 2A). In mammalian cells, MG-132
203 treatment enhanced ROS production (Maharjan *et al.* 2014). In *A. gossypii*, ROS accumulation
204 was observed in the presence of 25 μM MG-132 compared to the negative control (DMSO
205 addition) (Fig. 2B). These results indicated that MG-132 normally works to inhibit the
206 proteasome activity in *A. gossypii* without any gene manipulation when it is added to the culture
207 medium.
208
209 **Effects of MG-132 on enzymatic activities in *A. gossypii***
210 In this study, MG-132 reduced the riboflavin production in *A. gossypii*. Therefore, enzymatic
211 activities of flavoproteins in mitochondria were investigated. In this study, the activities of
212 NADH dehydrogenase (NDH) and succinate dehydrogenase (SDH) were measured. In *S.*
213 *cerevisiae*, NDH activity comes from Ndi1p, Nde1p and Nde2p, which are flavoproteins. *A.*
214 *gossypii* also has AgNdi1p (AFR447Cp) and AgNde1p (ADR262Cp). SDH is also a
215 flavoprotein and composed of 4 subunits AgSdhp1−4p (ACR052Wp, ACL065Cp, AFR207Cp
216 and AGL137Wp). FAD is covalently attached to SDH1 subunit (Kim and Winge 2013).
217 Specific NDH activity was increased to 40% compared to DMSO, but specific SDH activity
218 was decreased to 52% compared to DMSO (Fig. 3A and B). Additionally, the amount of
219 AgSdh1p, which is a subunit of SDH in *A. gossypii*, was reduced in the presence of MG-132
220 (Fig. 3C). To confirm the effect of SDH on riboflavin production, a SDH inhibitor, malonate,
221 was added to the culture medium (Fig. 4). In the agar medium, fading of the yellow color was
222 observed in a concentration-dependent manner (Fig. 4A). In the liquid medium with 20 mM

malonate , specific riboflavin production in the WT strain after 24 h cultivation was decreased to 78% compared to that without malonate (Fig. 4B). Additionally, specific activity of SDH in the presence of 20 mM malonate was reduced to 63% compared to that without malonate (Fig. 4C). These results indicated that the SDH may have some roles in riboflavin production in *A. gossypii*.

Disruption of *AgSDH1* gene in *A. gossypii*

To investigate the influences of SDH on the riboflavin production in *A. gosypii*, the disruption of *AgSDH1* gene was performed. Several colonies ($AgSDH1^{-/+}$) were isolated after the transformation of kanamycin (geneticin) resistance gene expression cassette. After the spore isolation of the transformant, we also isolated several geneticin-resistant colonies ($AgSDH1^{-/-}$). The gene disruption was confirmed by PCR (Fig. S1). This kanamycin resistance gene expression cassette contains promoter and terminator sequences of the *AgTEF1* gene. By means of primers P3 and P4, which match the 5'- and 3- ends of the cassette, respectively, the cassette was amplified by PCR using genomic DNA of the $AgSDH1^{-/+}$ as a template. This resulted in amplicons of the *AgSDH1* gene and the kanamycin resistance gene cassette (Fig. S1A, B), providing evidence of its heterozygous nature. Additionally, PCR was performed using P1-P2 primer set. P1 and P2 have the sequence of *AgSDH1* gene at 5' and 3' end. In this case, *AgSDH1* gene and the kanamycin resistance gene cassette were amplified using the genome DNA of $AgSDH1^{-/+}$ (Fig. S1A and B). However, only *AgSDH1* gene was amplified using the genome DNA of $AgSDH1^{-/-}$, indicating that the homozygous nature of $AgSDH1^{-/-}$ was not confirmed. Therefore, in this study, only $AgSDH1^{-/+}$ was isolated. The riboflavin production in $AgSDH1^{-/+}$ produced less amount of riboflavin than WT strain (Fig. 5A) and the specific riboflavin production in $AgSDH1^{-/+}$ was reduced to 63% compared to that inWT strain (Fig. 5B).

Discussion

MG-132, which is a 26S proteasome inhibitor, prevents the degradation of proteins and prolongs the lifetime of proteins in cells. Therefore, in most cases, the addition of MG-132 increases the amount of proteins in cells (Tang *et al.* 2019; Xu *et al.* 2017). On the contrary, it has been also reported that proteasome inhibitors reduce the amount of some proteins in cells (Jing *et al.*, 2003; Wang *et al.* 2019). Lavie *et al.* reported that inhibition of succinate dehydrogenase subunit A (SDHA) degradation by UPS promotes oxygen consumption and produces more ATP in mammalian cells (Lavie *et al.*, 2018). However, in this study, the activity and amount of SDH was decreased in the presence of MG-132 (Fig. 3B and C). The riboflavin production was also reduced by MG-132 (Fig. 1). SDH inhibitor experiment and *AgSDH1* gene disruption experiment support that SDH is connected with the riboflavin production in *A. gossypii*. SDH are members of the mitochondrial respiratory chain complexes as Complex II. It was reported that the riboflavin production in *A. gossypii* needs aerobic condition when the carbon source is glucose (Aguiar *et al.* 2015; Tanner Jr. *et al.* 1949). In this study, *AgSDH1*$^{-/-}$ was not isolated during the respiratory growth on glucose, indicating that the respiratory chain is indispensable to the growth in *A. gossypii*. These results also support the connection of SDH with the riboflavin production in *A. gossypii*.

SDH is a flavoprotein and has FAD attached to AgSdh1p subunit covalently (Kim and Winge 2013). Riboflavin is a precursor of FAD. In yeast, the mitochondrial FAD transporter Flx1p maintains the function of SDH flavoprotein subunit Sdh1p (Giancaspero *et al.* 2008). Additionally, riboflavin supplementation supports mitochondrial function in neurodegenerative diseases (Udhayabanu *et al.* 2017). Mitochondrial functions are clearly related to flavin metabolism. SDH may be important enzymes in mitochondria for the riboflavin production in *A. gossypii*. It has recently been reported that mitochondrial function and biogenesis are connected to UPS in diseases and aging (Branco *et al.* 2010; Ross *et al.* 2015). It was reported

that mitochondrial function regulates 26S proteasome assembly and UPS regulates mitochondrial homeostasis (Alsayyah *et al.* 2020; Meul *et al.* 2020). Mitochondrial proteins in the outer and inner membranes and matrix are degraded by UPS following its ubiquitination, and UPS controls mitochondrial functions (Lehmann *et al.* 2016; Shirozu *et al.* 2016; Lavie *et al.* 2018; Liao *et al.* 2020). In addition, 3-nitropropionic acid, which is known as an SDH inhibitor, influences the proteasome activity in Huntington's disease model cells (Hunter *et al.* 2007). MG-132 increases the aggregation of proteins in the mitochondrial respiratory chain including Complex II, III, IV and V (Rawat *et al.* 2020). In this study, MG-132 decreased the amount of AgSdh1p and SDH activity in *A. gossypii* and increased NADH dehydrogenase activity (Fig. 3). Why and how MG-132 influences the SDH activity has not been elucidated yet, but UPS is clearly implicated in mitochondrial functions and homeostasis in *A. gossypii*.

In this study, *AgSDH1*$^{-/-}$ was not isolated. *S. cerevisiae* has SDH1 homologue, SDH1b (SDH9/YJL045W). *sdh1*Δ strain can grow on the glucose-based medium (Smith et al., 2007). However, the double-disrupted mutant (*sdh1* and *sdh1b* gene-disrupted) can not grow on the glucose-based medium (Kubo *et al.* 2000). In *A. gossypii*, *sdh1b* gene is not found and *A. gossypii* has only single sdh1 gene, *AgSDH1*. Therefore, these suggest that the disruption of *AgSDH1* gene leads to no growth of *A. gossypii* on glucose-based medium. Growth rates of WT strain and *AgSDH1*$^{-/+}$ were almost the same because the sizes of both colonies are the same. *A. gossypii* is a multinucleate fungus and a cell has multiple nuclei (Gibeaux *et al.* 2017; Gladfelter *et al.* 2006). A cell of the heterozygous strain has genomes of both WT strain and *AgSDH1*Δ. Therefore, we suppose that phenotype is not shown clearly.

In this study, MG-132, which is a 26S proteasome inhibitor, had significant effects on the riboflavin production, the amount of AgSdh1p, which is a flavoprotein in mitochondria and respiratory chain, and SDH activity in *A. gossypii*. Moreover, *A. gossypii AgSDH1*$^{-/+}$ showed the reduced the riboflavin production. This, this study shows that the riboflavin production in

A. gossypii is connected with SDH. Based on this study, the detailed mechanism to overproduce riboflavin in *A. gossypii* in view of flavoprotein homeostasis and mitochondrial function will be revealed.

Conflicts of interests

None declared.

Authors contribution

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

Data Availability Statement

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

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444

445

446 **Table 1.** Primers used in this study

	Sequence (5’ to 3’)
P1	ATGCTTTCTGTTACTCGAACCGCTCCTGGCGTGCGTACGCGGGCGCTCAG
P2	TCAGTAAGCACGTACGGTTGGAGGCACTGGTGGGCACTCGGTCTCATCGA
P3	ATGCTTTCTGTTACTCGAACCGCTCCTGGCGTGCGTACGCGGGCGCTCAGACTGCAGACATGGAGGCCCAGAATACCC
P4	TCAGTAAGCACGTACGGTTGGAGGCACTGGTGGGCACTCGGTCTCATCGACGGAATTCTTTCTGCGCACTTAACTTCGC
P5	AGCGTCCGCGCGCTAACCTT
P6	GCTCAGCGAAGCTCACCAAG
P7	ATGAGCCATATTCAACGGGAAACG
P8	TTAGAAAAACTCATCGAGCATC

447

Figure legends

Figure 1 Riboflavin production in the presence of MG-132. (A) Growth and riboflavin production of *A. gossypii* on YD agar plates in the presence of 25 μM MG-132 after 6 days of cultivation. (B) Specific riboflavin production in *A. gossypii* cultivated in the YD liquid medium with DMSO or 25 μM MG-132 for 24 h. Data were analyzed for the statistical significance by unpaired Student's *t*-test with two-side test. *$p < 0.05$, (n = 3).

Figure 2 Effects of MG-132 on *A. gossypii*. (A) Detection of ubiquitinated proteins in the presence of 25 μM MG-132 by western blot. Each mycelium cultivated in the liquid culture was collected at 24 h. Ubiquitinated proteins were detected using rabbit ubiquitin polyclonal antibody. For western blotting, 50 μg proteins in the presence of DMSO or MG-132 were subjected to SDS-PAGE. CBB staining shows band patterns and amount of 50 μg of loaded proteins in each lane. (B) ROS production in *A. gossypii* in liquid YD medium in the presence of 25 μM MG-132 after 24 h cultivation. ROS was stained with ROS brite 570.

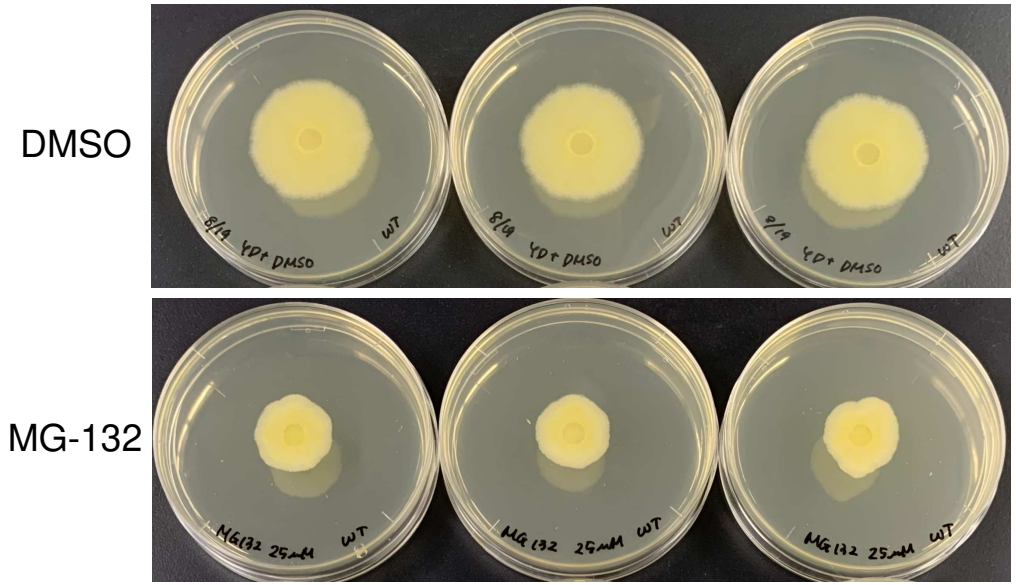
Figure 3 Effects of MG-132 on NDH and SDH in *A. gossypii*. (A) Specific NDH activity in the presence of DMSO or 25 μM MG-132 in the WT strain after 24 h cultivation. NDH activity was measured using DCIP as an electron receptor. Data were analyzed for the statistical significance by unpaired Student's *t*-test with two-side test. *$p < 0.05$, (n = 3). (B) Specific SDH activity in the presence of DMSO or 25 μM MG-132 in the WT strain after 24 h cultivation. SDH activity was measured using MTT as an electron receptor. Data were analyzed for the statistical significance by unpaired Student's *t*-test with two-side test *$p < 0.05$, (n = 3). (C) Western blot analysis of AgSDH1 in the

473 presence of DMSO or 25 μM MG-132 in the WT strain after 24 h cultivation. Estimated
474 molecular weight of AgSDH1 is 69 kDa. We used 50, 100 and 250 μg for the SDS-
475 PAGE and western blot.
476
477 **Figure 4** Effect malonate on the riboflavin production. (A) Growth of *A. gossypii* on the
478 liquid medium in the presence of malonate. WT strain was cultivated for 6 days on the
479 YD agar medium. (B) Specific riboflavin production in *A. gossypii* cultivated in the YD
480 liquid medium with 20 mM malonate for 24 h. Data were analyzed for the statistical
481 significance by unpaired Student's *t*-test with two-side test. *$p < 0.05$, (n = 3). (C)
482 Specific SDH activity in *A. gossypii* cultivated in the YD liquid medium with 20 mM
483 malonate for 24 h. Data were analyzed for the statistical significance by unpaired
484 Student's *t*-test with two-side test *$p < 0.05$, (n = 4).
485
486 **Figure 5** Riboflavin production and SDH activity in *AgSDH1*$^{-/+}$. (A) Mycelial color of
487 WT and *AgSDH1*$^{-/+}$ on the YD agar medium. Both strains were cultivated for 96 h on
488 the medium. (B) Specific riboflavin production in WT and *AgSDH1*$^{-/+}$. Each strain was
489 cultivated in YD medium for 24 h. Data were analyzed for the statistical significance
490 by unpaired Student's *t*-test with two-side test. *$p < 0.05$, (n = 3).
491
492 **Figure S1** Disruption of *AgSDH1* gene. (A) Scheme of the gene disruption. The
493 disruption of *AgSDH1* gene was achieved by homologous recombination between
494 *AgSDH1* gene and PCR product containing 50 bp of 5' and 3' *AgSDH1* gene and
495 kanamycin (geneticin) resistance gene expression cassette. The detailed method was
496 described in Materials and methods. (B) PCR to confirm the disruption of *AgSDH1*
497 gene. Using both P1-P2 and P3-P4 primer sets, 1488 bp of DNA fragment was

amplified by PCR when the homologous recombination was achieved. W and Δ indicate WT strain and *AgSDH1*$^{-/+}$, respectively. The asterisk indicates the strain from spores isolated from *AgSDH1*$^{-/+}$. Only *AgSDH1* gene was amplified using P1-P2 primer set and genome DNA of this strain, indicating that this strain was not homozygous *AgSDH1*$^{-/-}$. Finally, we were not able to isolate *AgSDH1*$^{-/-}$. (C) PCR to confirm the disruption of *AgSDH1* gene using P5-P6 and P7-P8 primer sets. P5-P6 primer set allowed to amplify 2041 bp of DNA fragment in WT strain, but both 2041 bp and 1528 bp of DNA fragment in *AgSDH1*$^{-/+}$. P7-P8 primer set allowed to amplify 810 bp of DNA fragment in *AgSDH1*$^{-/+}$, but no DNA fragment in WT strain. W and Δ indicate WT strain and *AgSDH1*$^{-/+}$, respectively.

Figure 1, Kato et al.

(A)



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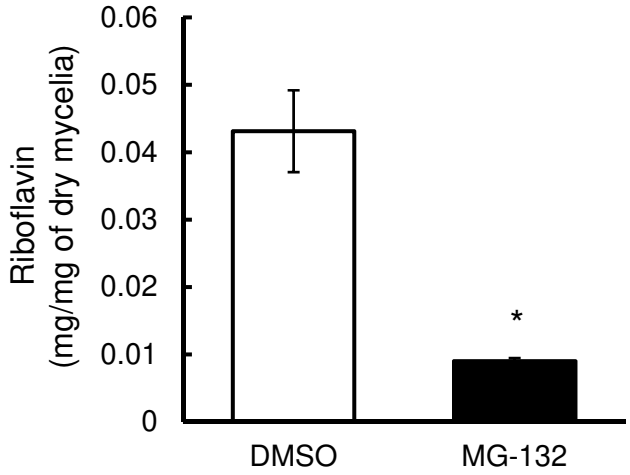


Figure 2, Kato et al.

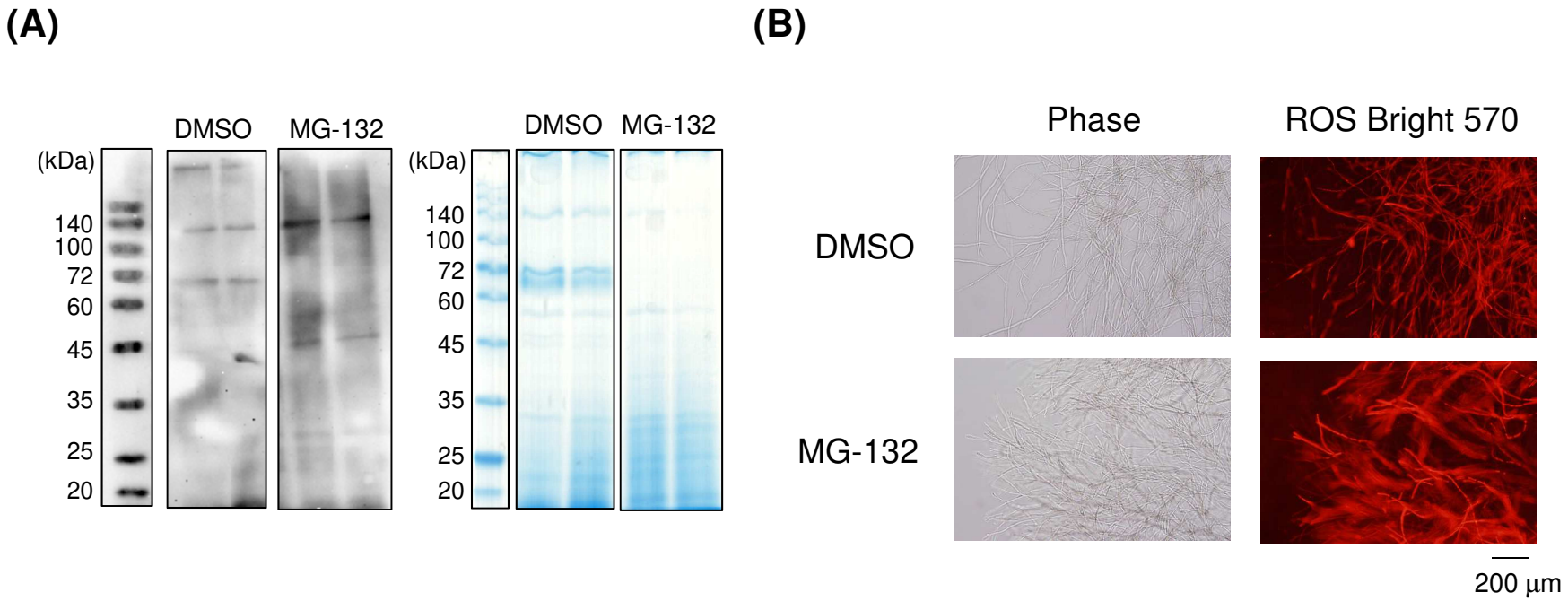


Figure 3, Kato et al.

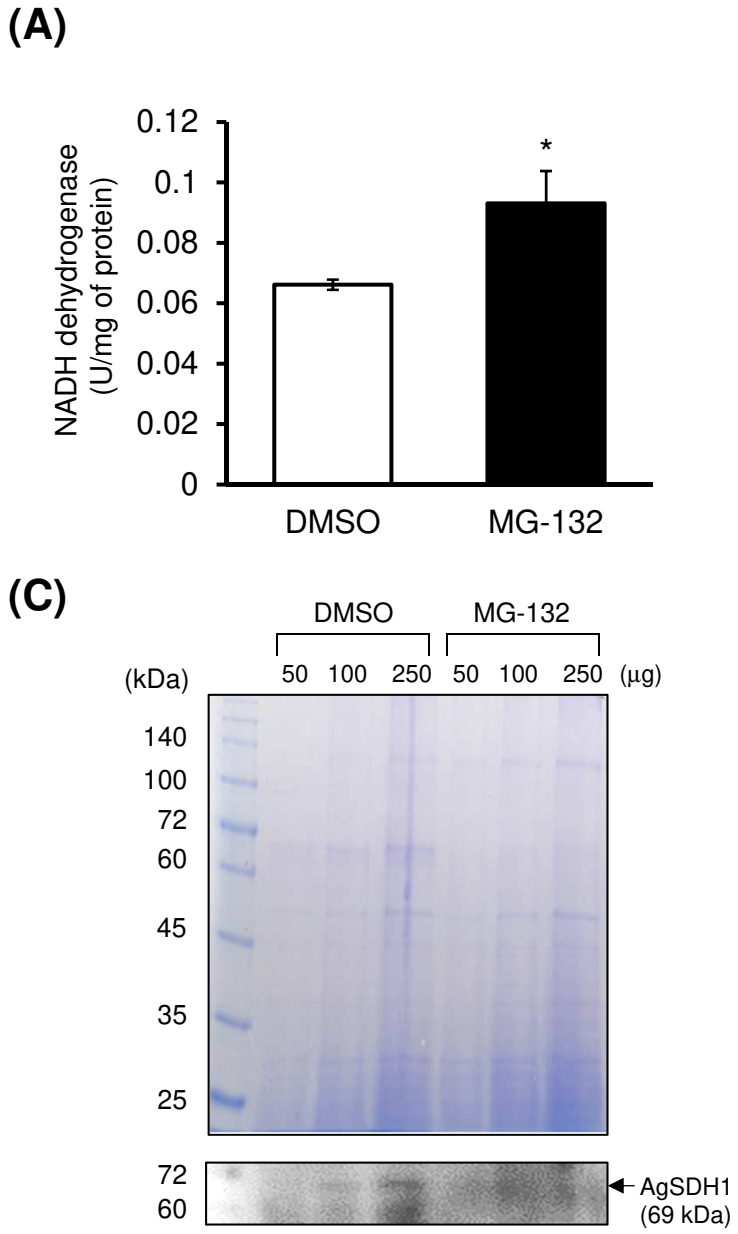


Figure 4, Kato et al.

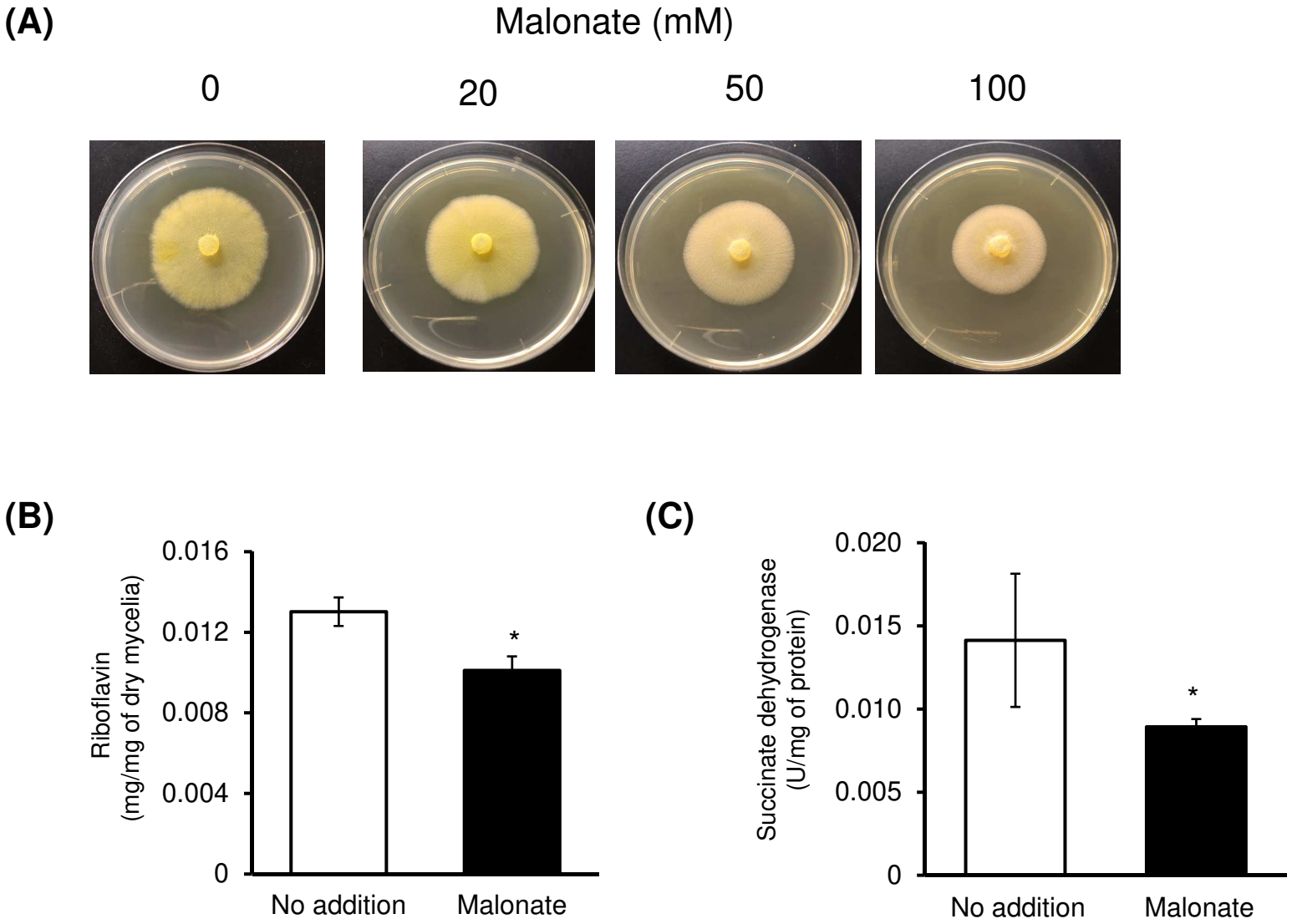
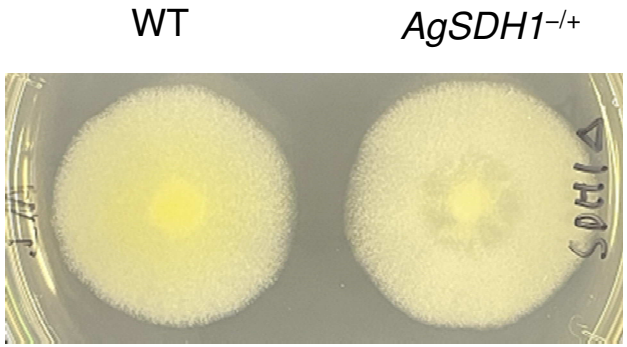


Figure 5, Kato et al.

(A)



(B)

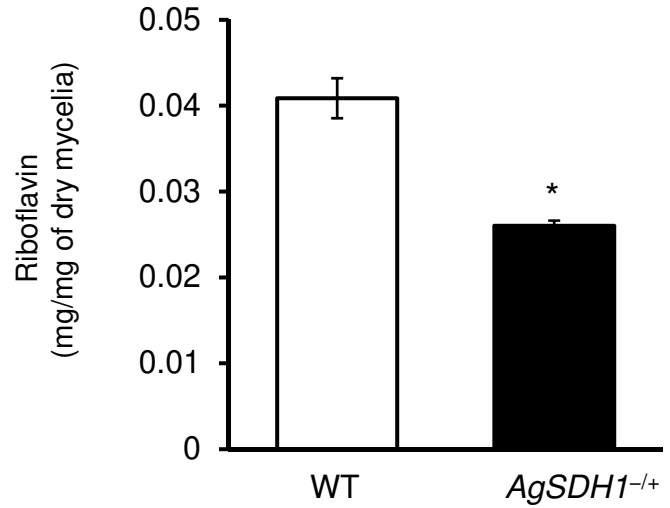
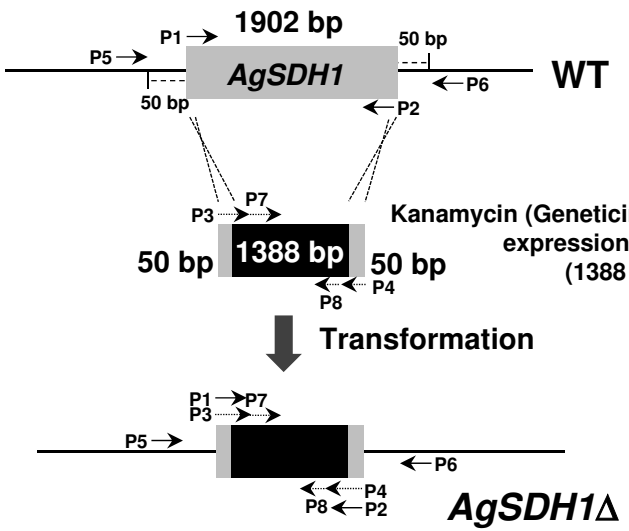


Figure S1, Kato et al.

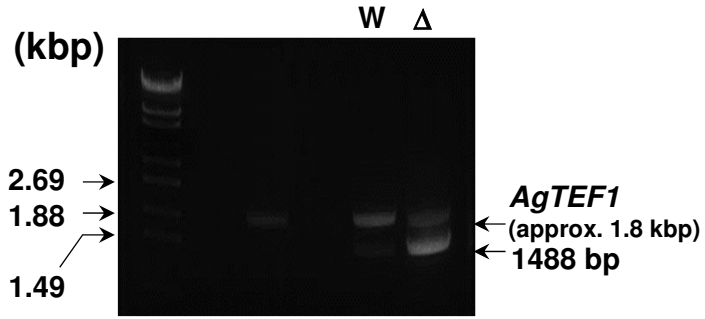
(A)



P1-P2: 1902 bp
P3-P4: No amplification (only *AgTEF1* gene)
P5-P6: 2041 bp
P7-P8: No amplification

P1-P2: 1488 bp
P3-P4: 1488 bp (+ *AgTEF1* gene)
P5-P6: 1528 bp
P7-P8: 810 bp

(B)



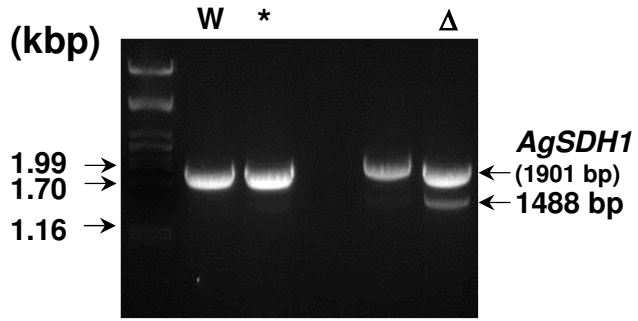


Figure S1, Kato et al.

(C)