SURE 静岡大学学術リポジトリ
Shizuoka University REpository

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

メタデータ	言語: English 出版者: 公開日: 2021-09-21 キーワード (Ja): キーワード (En): Ashbya gossypii, MG-132, riboflavin, succinate, dehydrogenase 作成者: Kato, Tatsuya, Yokomori, Ami, Suzuki, Riho, Azegami, Junya, El Enshasy, Hesham A., Park, Enoch Y. メールアドレス: 所属:
URL	http://hdl.handle.net/10297/00028366

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

Tatsuya Kato[1,2,3]*, Ami Yokomori[2], Riho Suzuki[3], Junya Azegami[2], Hesham A. El Enshasy[4,5], Enoch Y. Park[1,2,3]

[1] Green Chemistry Research Division, Research Institute of Green Science and Technology, Shizuoka University, Ohya 836, Suruga-ku, Shizuoka, Japan

[2] Department of Agriculture, Graduate School of Integrated Science and Technology, Shizuoka University, Ohya 836, Suruga-ku, Shizuoka, Japan

[3] Department of Applied Life Science, Faculty of Agriculture, Shizuoka University, Ohya 836, Suruga-ku, Shizuoka, Japan

[4] Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), 81310 UTM, Johor Bahru, Malaysia

[5] City of Scientific Research and Technology Applications, New Borg Al Arab, Alexandria, Egypt.

Running title: Effects of MG-132 on the riboflavin production.

*Corresponding author.

Address: Department of Applied Life Science, Faculty of Agriculture, Shizuoka University, Ohya 836, Suruga-ku, Shizuoka, Japan

Tel. & fax: +81-54-238-4937. E-mail address: kato.tatsuya@shizuoka.ac.jp (Tatsuya Kato)

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

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

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

82

83 ## Materials and methods

84

85 ### Strain cultivation and spore isolation

86 *A. gossypii* ATCC10895 was used as the wild type (WT) strain. The strain was maintained in
87 YD agar medium (1% glucose, 1% yeast extract, and 2% agar, pH 6.8). Grown mycelia were
88 kept at -80 °C with 20% glycerol. Cultures were produced from glycerol stocks, of which 0.3
89 mL was inoculated into 30 mL of liquid YD medium and cultivated for 24 h at 100 rpm. Next,
90 as a pre-culture, 0.3 mL of the culture medium was inoculated in 30 mL of liquid YD medium
91 and cultivated for 24 h at 100 rpm. Then, 0.5 mL of the pre-culture medium was inoculated to
92 50 mL of the liquid YD medium and cultivated for indicated time. MG-132 was dissolved with
93 DMSO at 25 mM as a stock solution and added to culture medium at 25 μM final concentration.
94 As a negative control, DMSO only was added at equal amounts.

95 Spore isolation was carried out according to a protocol reported previously (Tajima *et al.*
96 2009). Mycelia grown in YD agar medium were harvested and suspended with 0.5 ml sterile
97 water followed by the addition of 0.25 mL of 15 mg/mL Zymolyase 40-T (Seikagaku Co.,

98 Tokyo, Japan). After incubation at 37°C for 30 min, the suspension was centrifuged to
99 precipitate spores. The pellet was washed with 0.03% Triton X-100 twice and resuspended with
100 0.03% Triton X-100 containing 15% glycerol.
101

102 **Staining of intracellular reactive oxygen species (ROS)**

103 Mycelia cultivated in YD liquid medium for 1 to 2 days were harvested and washed with sterile
104 water. Intracellular ROS in mycelia (40 mg) was stained with ROS brite 570 (AAT Bioquest,
105 Sunnyvale, CA, USA) for oxidative stress detection. Fluorescence was observed using a
106 fluorescent microscope BX60 (Olympus, Tokyo, Japan). Fluorescent intensity was measured
107 using a filter-based multimode microplate reader (Infinite F200 M; Tecan Ltd., Männedorf,
108 Switzerland).
109

110 **Riboflavin assay**

111 The amount of riboflavin was assayed according to a protocol reported previously (Jeong *et al.*
112 2015). Briefly, 0.8 mL of the culture broth was thoroughly mixed with 0.2 mL of 1 N NaOH.
113 A 0.4 mL aliquot of the resulting solution was neutralized with 1 mL of 0.1 M potassium
114 phosphate (pH 6.0), and the absorbance of the solution at a wavelength of 444 nm was
115 measured. The riboflavin concentration was calculated at an extinction coefficient of $1.04 \times$
116 10^{-2} M^{-1} cm^{-1} (127 mg riboflavin/L at ABS_{444}).
117

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

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

123 (StressMarq Bioscience, Victoria, BC, Canada) for ubiquitinated proteins and 2000-fold diluted
124 serum from rabbit immunized by AgSdh1p (ACR052Wp, 577−590 aa domain) (Eurofins
125 Genomics K.K., Tokyo, Japan) for AgSdh1p. A 10,000-fold diluted goat anti-rabbit IgG
126 horseradish peroxidase (HRP)-linked antibody (Medical & Biological Laboratories, Nagoya,
127 Japan) was used as a secondary antibody. Specific proteins were detected using Immobilon
128 Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA) and Fluor-
129 S Max Multi-Imager (Bio-Rad).
130

131 **Enzyme assay**

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

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

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

148 (pH 7.4) were disrupted by sonication and used as a crude extract without any centrifugation
149 step. Protein concentration was determined by Pierce BCA Protein Assay kit (Thermo Fisher
150 Scientific K.K.).
151 Statistics analysis was carried out using GraphPad Prism 8 (GraphPad Software, San Diego,
152 CA, USA). All data were analyzed for the statistical significance by unpaired Student's *t*-test
153 with two-side test.
154

155 **Gene disruption**

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

173 was confirmed by PCR using the primer sets (P1-P2, P3-P4, P5-P6 and P7-P8 primer sets,
174 Table1 and Fig. S1). To isolate homozygous *AgSDH1*Δ strain, spores were isolated from a
175 heterozygous mutant and cultivated in the presence of 300 μg/mL G418.
176

177 **DNA extraction**

178 Mycelia were suspended with 0.5 mL of 1 M sorbitol containing 100 mM EDTA (pH 7.5) and
179 0.2 mL of 10 mg/mL Zymolyase 40-T and incubated with gently stirring. Cells were pelleted
180 by centrifugation, and the pellet was resuspended with 0.5 mL of 50 mM Tris-HCl (pH 7.5)
181 containing 20 mM EDTA and 0.05 mL of 10% sodium dodecyl sulfate (SDS). After incubation
182 at 65°C for 30 min, 0.2 mL of 5 M potassium acetate was added to the suspension followed by
183 incubation of the mixture on ice for 30 min. Then, genomic DNA was precipitated by ethanol
184 and treated with RNase.
185

186 **Results**

187

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

189 To investigate the relationship of riboflavin production with UPS, MG-132, a 26S proteasome
190 inhibitor, was added when *A. gossypii* was cultivated (Fig. 1A). In the presence of 25 μM MG-
191 132, the colony size was decreased and the mycelial color turned cream-colored. In the
192 submerged cultivation, specific riboflavin production at 24 h in the presence of 25 μM MG-
193 132 was reduced by 79% in comparison with the control (in the presence of DMSO only) (Fig.
194 1B).

195 In *S. cerevisiae*, the presence of MG-132 inhibited its proteasome activity when *ise1* (*erg6*)
196 gene, which encodes S-adenosylmethionine:Δ^{24}-methyltransferase involved in ergosterol
197 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

223 malonate , specific riboflavin production in the WT strain after 24 h cultivation was decreased
224 to 78% compared to that without malonate (Fig. 4B). Additionally, specific activity of SDH in
225 the presence of 20 mM malonate was reduced to 63% compared to that without malonate (Fig.
226 4C). These results indicated that the SDH may have some roles in riboflavin production in *A.*
227 *gossypii*.
228

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

230 To investigate the influences of SDH on the riboflavin production in *A. gosypii*, the disruption
231 of *AgSDH1* gene was performed. Several colonies ($AgSDH1^{-/+}$) were isolated after the
232 transformation of kanamycin (geneticin) resistance gene expression cassette. After the spore
233 isolation of the transformant, we also isolated several geneticin-resistant colonies ($AgSDH1^{-/-}$).
234 The gene disruption was confirmed by PCR (Fig. S1). This kanamycin resistance gene
235 expression cassette contains promoter and terminator sequences of the *AgTEF1* gene. By means
236 of primers P3 and P4, which match the 5'- and 3- ends of the cassette, respectively, the cassette
237 was amplified by PCR using genomic DNA of the $AgSDH1^{-/+}$ as a template. This resulted in
238 amplicons of the *AgSDH1* gene and the kanamycin resistance gene cassette (Fig. S1A, B),
239 providing evidence of its heterozygous nature. Additionally, PCR was performed using P1-P2
240 primer set. P1 and P2 have the sequence of *AgSDH1* gene at 5' and 3' end. In this case, *AgSDH1*
241 gene and the kanamycin resistance gene cassette were amplified using the genome DNA of
242 $AgSDH1^{-/+}$ (Fig. S1A and B). However, only *AgSDH1* gene was amplified using the genome
243 DNA of $AgSDH1^{-/-}$, indicating that the homozygous nature of $AgSDH1^{-/-}$ was not confirmed.
244 Therefore, in this study, only $AgSDH1^{-/+}$ was isolated. The riboflavin production in $AgSDH1^{-/+}$
245 produced less amount of riboflavin than WT strain (Fig. 5A) and the specific riboflavin
246 production in $AgSDH1^{-/+}$ was reduced to 63% compared to that inWT strain (Fig. 5B).
247

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

273 that mitochondrial function regulates 26S proteasome assembly and UPS regulates
274 mitochondrial homeostasis (Alsayyah *et al.* 2020; Meul *et al.* 2020). Mitochondrial proteins in
275 the outer and inner membranes and matrix are degraded by UPS following its ubiquitination,
276 and UPS controls mitochondrial functions (Lehmann *et al.* 2016; Shirozu *et al.* 2016; Lavie *et*
277 *al.* 2018; Liao *et al.* 2020). In addition, 3-nitropropionic acid, which is known as an SDH
278 inhibitor, influences the proteasome activity in Huntington's disease model cells (Hunter *et al.*
279 2007). MG-132 increases the aggregation of proteins in the mitochondrial respiratory chain
280 including Complex II, III, IV and V (Rawat *et al.* 2020). In this study, MG-132 decreased the
281 amount of AgSdh1p and SDH activity in *A. gossypii* and increased NADH dehydrogenase
282 activity (Fig. 3). Why and how MG-132 influences the SDH activity has not been elucidated
283 yet, but UPS is clearly implicated in mitochondrial functions and homeostasis in *A. gossypii*.

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

294 In this study, MG-132, which is a 26S proteasome inhibitor, had significant effects on the
295 riboflavin production, the amount of AgSdh1p, which is a flavoprotein in mitochondria and
296 respiratory chain, and SDH activity in *A. gossypii*. Moreover, *A. gossypii AgSDH1*$^{-/+}$ showed
297 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.

Funding

This work was supported by JSPS KAKENHI (Grant-in-Aid for Scientific Research (C) Grant Number JP21K05390).

References

Aguiar, T.Q., Silva, R,, Domingues, L. (2015) *Ashbya gossypii* beyond industrial riboflavin production: A historical perspective and emerging biotechnological applications. *Biotechnol Adv* **33**, 1774−1786.

Alsayyah, C., Ozturk, O., Cavellini, L., Belgareh-Touzé, N., Cohen, M.M. (2020) The regulation of mitochondrial homeostasis by the ubiquitin proteasome system. *Biochim Biophys Acta Bioenerg* **1861**, 148302.

Branco, D.M., Arduino, D.M., Esteves, A.R., Silva, D.F.F., Cardoso, S.M., Oliveira, C.R. (2010) Cross-talk between mitochondria and proteasome in Parkinson's disease pathogenesis. *Front Aging Neurosci* **2**, 17.

Dietrich, F.S., Voegeli, S., Brachat, S., Lerch, A., Gates, K., Steiner, S., Mohr, C., Pöhlmann, R., Luedi, P., Choi, S., Wing, R.A., Flavier, A., Gaffney, T.D., Philippsen, P. (2004) The *Ashbya gossypii* genome as a tool for mapping the ancient *Saccharomyces cerevisiae* genome. *Science* **304**, 304−307.

Fleming, J.A., Lightcap, E.S., Sadis, S., Thoroddsen, V., Bulawa, C.E., Blackman, R.K. (2002) Complementary whole-genome technologies reveal the cellular response to proteasome inhibition by PS-341. *Proc Natl Acad Sci USA* **99**, 1461−1466.

Giancaspero, T.A., Wait, R., Boles, E., Barile, M. (2008) Succinate dehydrogenase flavoprotein subunit expression in *Saccharomyces cerevisiae*--involvement of the mitochondrial FAD transporter, Flx1p. *FEBS J* **275**, 1103−1117.

Giancaspero, T.A., Colella, M., Brizio, C., Difonzo, G., Fiorino, G.M., Leone, P., Brandsch, R., Bonomi, F., Iametti, S., Barile, M. (2015) Remaining challenges in cellular flavin cofactor homeostasis and flavoprotein biogenesis. *Front Chem* **3**, 30.

Gibeaux, R., Politi, A.Z., Philippsen, P., Nédélec, F. (2017) Mechanism of nuclear movements in a multinucleated cell. *Mol Biol Cell* **28**, 645−660.

344 Gladfelter, A.S., Hungerbuehler, A.K., Philippsen, P. (2006) Asynchronous nuclear division
345 cycles in multinucleated cells. *J Cell Biol* **172**, 347−362
346 Gudipati, V., Koch, K., Lienhart, W.D., Macheroux, P. (2014) The flavoproteome of the yeast
347 *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1844**, 535−544.
348 Hunter, J.M., Lesort, M., Johnson, G.V.M. (2007) Ubiquitin-proteasome system alterations in
349 a striatal cell model of Huntington's disease. J Neurosci Res 85, 1774−1788.
350 Kato, T. and Park, E.Y. (2006) Expression of alanine:glyoxylate aminotransferase gene from
351 *Saccharomyces cerevisiae* in *Ashbya gossypii*. *Appl Microbiol Biotechnol* **71**, 46−52.
352 Kim, H.J. and Winge, D.R. (2013) Emerging concepts in the flavinylation of succinate
353 dehydrogenase. *Biochim Biophys Acta* **1827**, 627−636.
354 Kubo, Y, Takagi, H., Nakamori, S. (2000) Effect of gene disruption of succinate dehydrogenase
355 on succinate production in a sake yeast strain. *J Biosci Bioeng* **90**, 619−624.
356 Jiménez, A., Santos, M.A., Pompejus, M., Revuelta, J.L. (2005) Metabolic engineering of the
357 purine pathway for riboflavin production in *Ashbya gossypii*. *Appl Environ Microbiol* **71**,
358 5743−5751.
359 Jing, B.F., He, K., Wang, H.X., Wang, J., Zhou, T., Lan, Y., Hu, M.R., Wei, K.H, Yang, S.C.,
360 Shen, B.F., Zhang. X.M. (2003) Proteomic analysis of ubiquitin-proteasome effects:
361 insight into the function of eukaryotic initiation factor 5A Oncogene **22**, 4819−4830.
362 Lavie, J., Belvalet, H.D., Sonon, S., Ion, A.M., Dumon, E., Melser, S., Lacombe, D., Dupuy,
363 J.W., Lalou, C., Bénard, G. (2018) Ubiquitin-dependent degradation of mitochondrial
364 proteins regulates energy metabolism. *Cell Rep* **23**, 2852−2863.
365 Lehmann, G., Ziv, T., Braten, O., Admon, A., Udasin, R.G., Ciechanover, A. Ubiquitination of
366 specific mitochondrial matrix proteins. (2016) *Biochem Biophys Res Commun* **475**, 13-18.
367 Liao, P.C., Wolken, D.M.A., Serrano, E., Srivastava, P., Pon, L.A. (2020) Mitochondria-
368 associated degradation pathway (MAD) function beyond the outer membrane. *Cell Rep*

32, 107902.

Liuzzi, V.C., Giancaspero, T.A., Gianazza, E., Banfi, C., Barile, M., Giorgi, C.D. (2012) Silencing of FAD synthase gene in *Caenorhabditis elegans* upsets protein homeostasis and impacts on complex behavioral patterns. *Biochim Biophys Acta* **1820**, 521–531.

Maharjan, S., Oku, M., Tsuda, M., Hoseki, J., Sakai, Y. (2014) Mitochondrial impairment triggers cytosolic oxidative stress and cell death following proteasome inhibitions. *Sci Rep* **4**, 5896.

Martínez-Reyes, I., Chandel, N.S. (2020) Mitochondrial TCA cycle metabolites control physiology and disease. Nat Commun 11, 102.

Meul, T., Berschneider, K., Schmitt, S., Mayr, C.H., Mattner, L.F., Schiller, H.B., Yazgili, A.S., Wang, X,. Lukas, C., Schlesser, C., Prehn, C., Adamski, J., Graf, E., Schwarzmayr, T., Perocchi, F., Kukat, A., Trifunovic, A., Kremer, L., Prokisch, H., Popper, B., von Toerne, C., Hauck, S.M., Zischka, H., Meiners, S. (2020) Mitochondrial regulation of the 26S proteasome. *Cell Rep* **32**, 108059.

Monschau, N., Sahm, H., Stahmann, K.P. (1998) Threonine aldolase overexpression plus threonine supplementation enhanced riboflavin production in *Ashbya gossypii*. *Appl Environ Microbiol* **64**, 4283–4290.

Munakata, H. (1993) Fundamental and clinical studies of chemosensitivity test performed by measuring specific activity of succinate dehydrogenase in subrenal capsule assay — Influence of host immune response and clinical correlation—. *Jpn J Oral Maxillofac Surg* **39**, 313–321.

Ravanelli, S., den Brave, F., Hoppe, T. (2020) Mitochondrial quality control governed by ubiquitin. *Front Cell Dev Biol* **8**, 270.

Rawat, S., Anusha, V., Jha, M., Sreedurgalakshmi, K., Raychaudhuri, S. (2019) Aggregation of respiratory complex subunits marks the onset of proteotoxicity in proteasome inhibited

394 cells. J Mol Biol 431, 996–1015.

395 Riemer, J., Appenzeller-Herzog, C., Johansson, L., Bodenmiller, B., Hartmann-Petersen, R.,
396 Ellgaard, L. (2009) A luminal flavoprotein in endoplasmic reticulum-associated
397 degradation. *Proc Natl Acad Sci USA* **106**, 14831–14836.

398 Revuelta, J.L., Ledesma-Amaro, R., Lozano-Martinez, P., Díaz-Fernández, D., Buey, R.M.,
399 Jiménez, A. (2017) Bioproduction of riboflavin: a bright yellow history. *J Ind Microbiol*
400 *Biotechnol* **44**, 659−665.

401 Robinson, K.M. and Lemire, B.D. (1996) Covalent attachment of FAD to the yeast succinate
402 dehydrogenase flavoprotein requires import into mitochondria, presequence removal, and
403 folding. *J Biol Chem* **271**, 4055–4060.

404 Ross, J.M., Olson, L., Coppotelli, G. (2015) Mitochondrial and ubiquitin proteasome system
405 dysfunction in ageing and disease: Two sides of the same coin? *Int J Mol Sci* **16**, 19458-
406 19476.

407 Sato, K., Nishina, Y., Shiga, K. (1996) In vitro refolding and unfolding of subunits of electron-
408 transferring flavoprotein: characterization of the folding intermediates and the effects of
409 FAD and AMP on the folding reaction. *J Biochem* **129**, 276–285.

410 Schlüpen, C., Santos, M.A., Weber, U., de Graaf, A., Revuelta, J.L., Stahmann, K.P. (2003)
411 Disruption of the SHM2 gene, encoding one of two serine hydroxymethyltransferase
412 isoenzymes, reduces the flux from glycine to serine in *Ashbya gossypii*. *Biochem J* **369**,
413 263–273.

414 Schwechheimer, S.K., Park, E.Y., Revuelta, J.L., Becker, J., Wittmann, C. (2016)
415 Biotechnology of riboflavin. *Appl Microbiol Biotechnol* **100**, 2107−2119.

416 Shirozu, R., Yashiroda, H., Murata, S. (2015) Proteasome impairment induces recovery of
417 mitochondrial membrane potential and an alternative pathway of mitochondrial fusion.
418 *Mol Cell Biol* **36**, 347−362.

419 Smith, E.H., Janknecht, R., Maher III, L.J. (2007) Succinate inhibition of alpha-ketoglutarate-
420 dependent enzymes in a yeast model of paraganglioma. *Hum Mol Genet* **16**, 3136−3148.
421 Sollner, S. and Macheroux, P. (2009) New roles of flavoproteins in molecular cell biology: an
422 unexpected role for quinone reductases as regulators of proteasomal degradation. *FEBS J*
423 **276**, 4313−4324.
424 Sulkshane, P., Duek, I., Ram, J., Thakur, A., Reis, N., Ziv, T., Glickman, M.H. (2020) Inhibition
425 of proteasome reveals basal mitochondrial ubiquitination. *J Proteomics* **229**, 103949.
426 Tajima, S., Itoh, Y., Sugimoto, T., Kato, T., Park, E.Y. (2009) Increased riboflavin production
427 from activated bleaching earth by a mutant strain of *Ashbya gossypii*. *J Biosci Bioeng* **108**,
428 325−329.
429 Tang, J., Sun, F., Deng, X.J., Ma, Y.L., Li, K.Y., Tang, Y., Chen, G.J. (2019) Ubiquitination
430 status does not affect Vps34 degradation. *Genes Dis* **7**, 401−407.
431 Tanner Jr., F.W., Vojnovich, C., van Lanen, J.M. (1949) Factors affecting riboflavin production
432 by *Ashbya gossypii*. *J Bacteriol* **58**, 737-745.
433 Udhayabanu, T., Manole, A., Rajeshwari, M., Varalakshmi, P., Houlden, H., Ashokkumar, B.
434 (2017) Riboflavin responsive mitochondrial dysfunction in neurodegenerative diseases. *J*
435 *Clin Med* **6**, 52.
436 Wang, D., Xu, Q., Yuan, Q., Jia, M., Niu, H., Liu, X., Zhang, J., Young, C.Y.f., Yuan, H. (2019)
437 Proteasome inhibition boosts autophagic degradation of ubiquitinated-AGR2 and
438 enhances the antitumor efficiency of bevacizumab. Oncogene **38**, 3458−3474.
439 Wendland, J., Ayad-Durieux, Y., Knechtle, P., Rebischung, C., Philippsen, P. (2000) PCR-based
440 gene targeting in the filamentous fungus *Ashbya gossypii*. Gene **242**, 381−391.
441 Xu, Y., Fang, S., Zhang, E., Pu, T., Cao, R., Fu, Q., Li, F., Chen, S., Sun, K., Rang, Xu. (2017)
442 A 3 base pair deletion in TBX1 leads to reduced protein expression and transcriptional
443 activity. *Sci Rep* **7**, 44165.

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

448 ## Figure legends

449 **Figure 1** Riboflavin production in the presence of MG-132. (A) Growth and riboflavin
450 production of *A. gossypii* on YD agar plates in the presence of 25 μM MG-132 after 6
451 days of cultivation. (B) Specific riboflavin production in *A. gossypii* cultivated in the YD
452 liquid medium with DMSO or 25 μM MG-132 for 24 h. Data were analyzed for the
453 statistical significance by unpaired Student's *t*-test with two-side test. *$p < 0.05$, (n =
454 3).

455

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

464

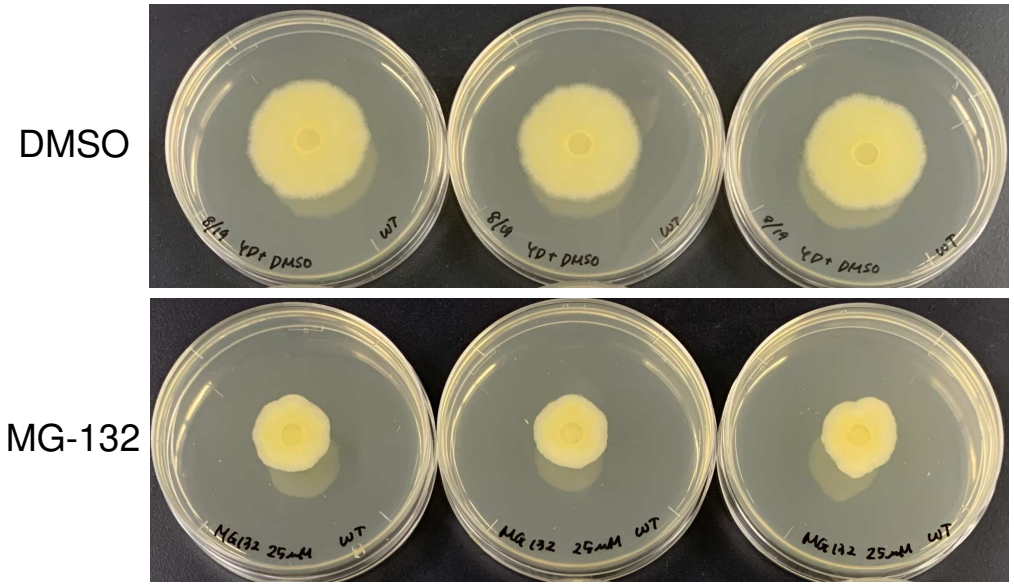
465 **Figure 3** Effects of MG-132 on NDH and SDH in *A. gossypii*. (A) Specific NDH activity
466 in the presence of DMSO or 25 μM MG-132 in the WT strain after 24 h cultivation.
467 NDH activity was measured using DCIP as an electron receptor. Data were analyzed
468 for the statistical significance by unpaired Student's *t*-test with two-side test. *$p < 0.05$,
469 (n = 3). (B) Specific SDH activity in the presence of DMSO or 25 μM MG-132 in the
470 WT strain after 24 h cultivation. SDH activity was measured using MTT as an electron
471 receptor. Data were analyzed for the statistical significance by unpaired Student's *t*-
472 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

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

Figure 1, Kato et al.

(A)



(B)

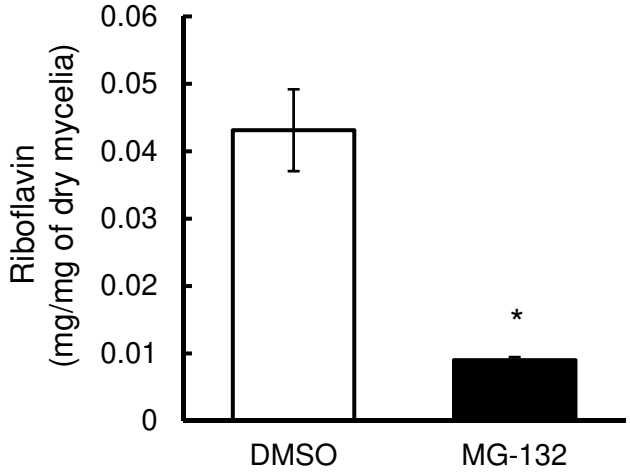


Figure 2, Kato et al.

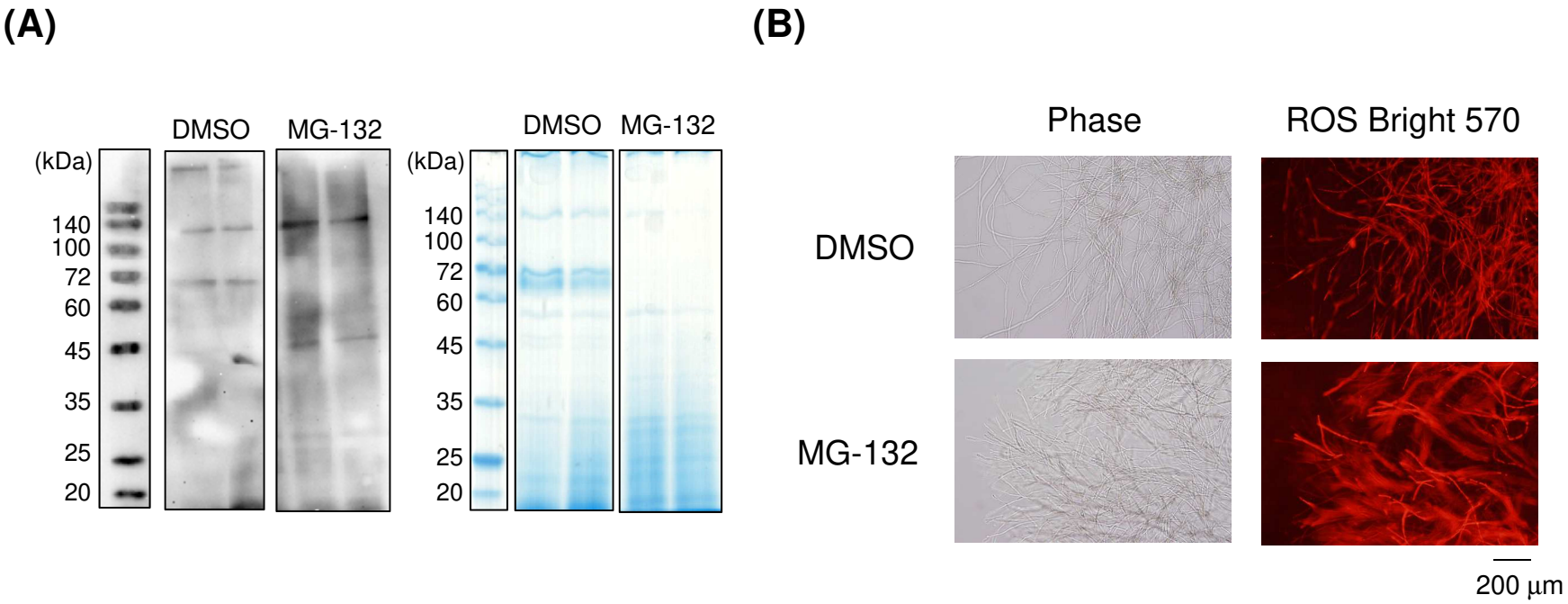


Figure 3, Kato et al.

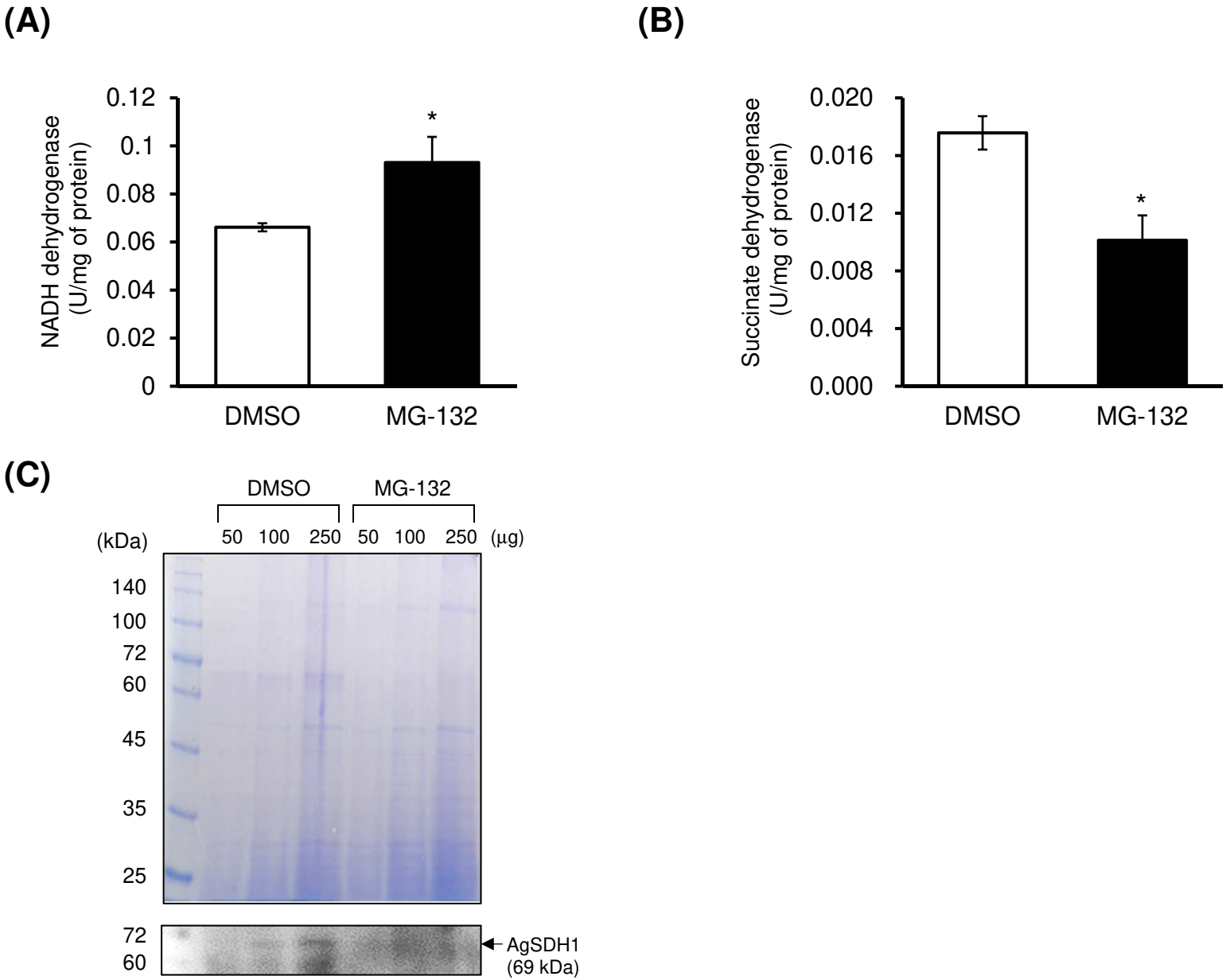
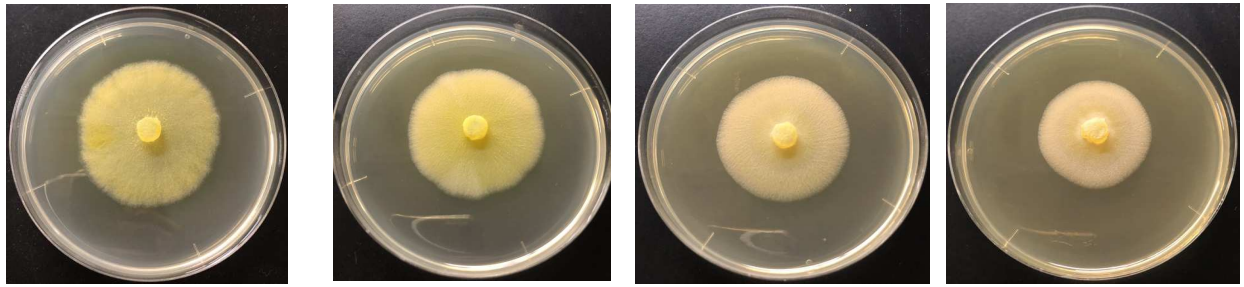


Figure 4, Kato et al.

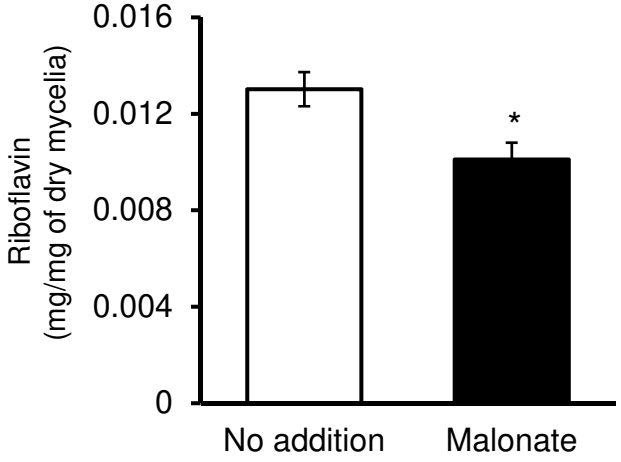
(A)

Malonate (mM)

0 20 50 100



(B)



(C)

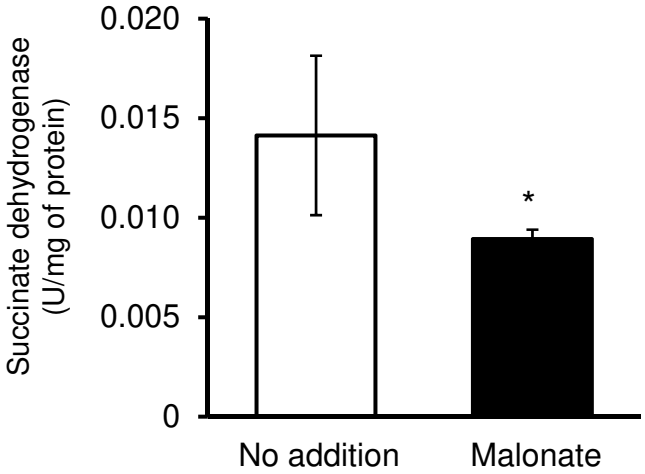
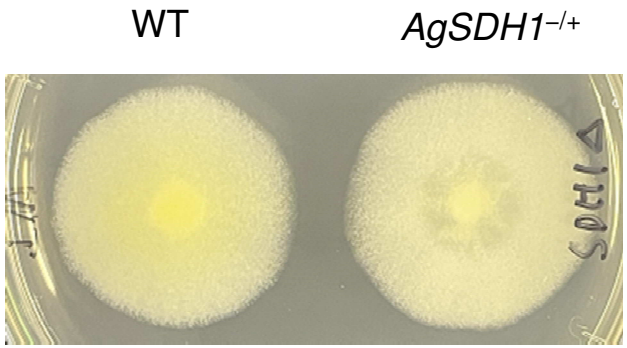


Figure 5, Kato et al.

(A)



(B)

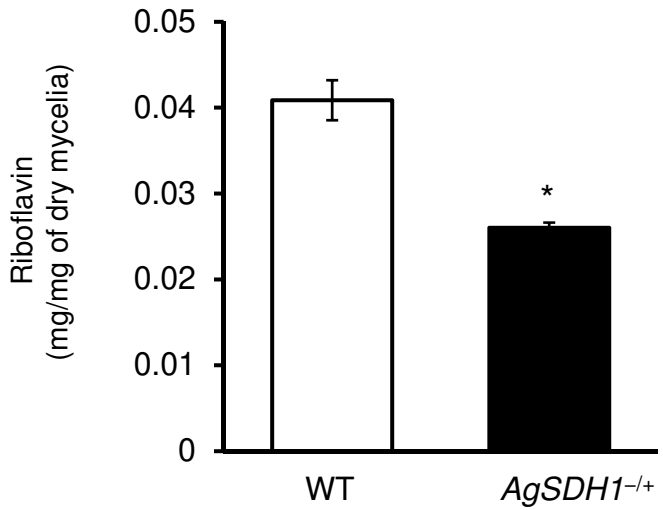
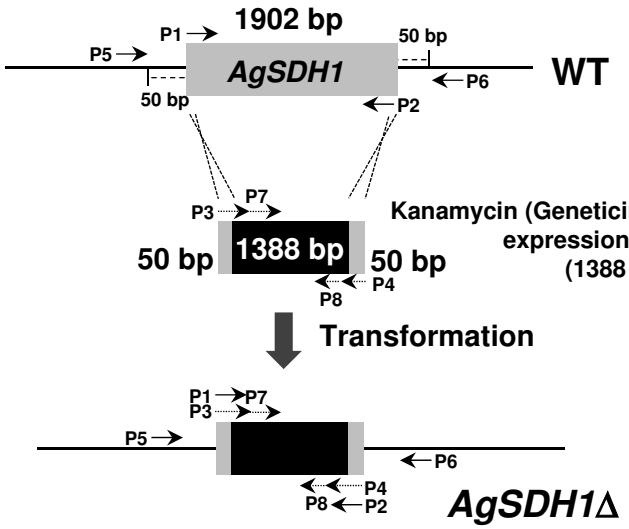


Figure S1, Kato et al.

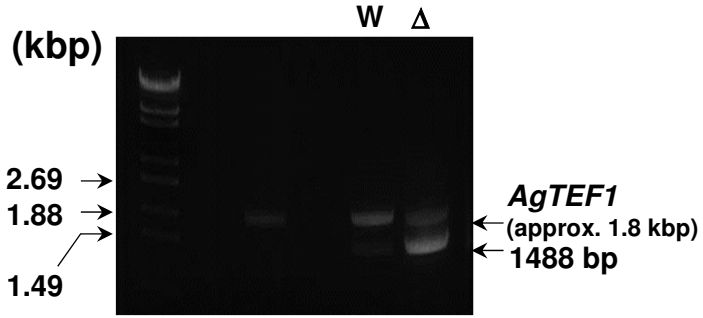
(A)



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)



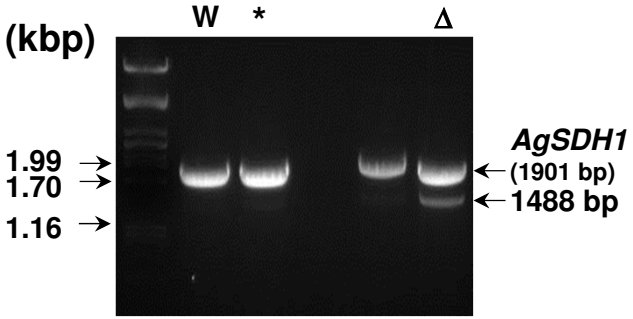
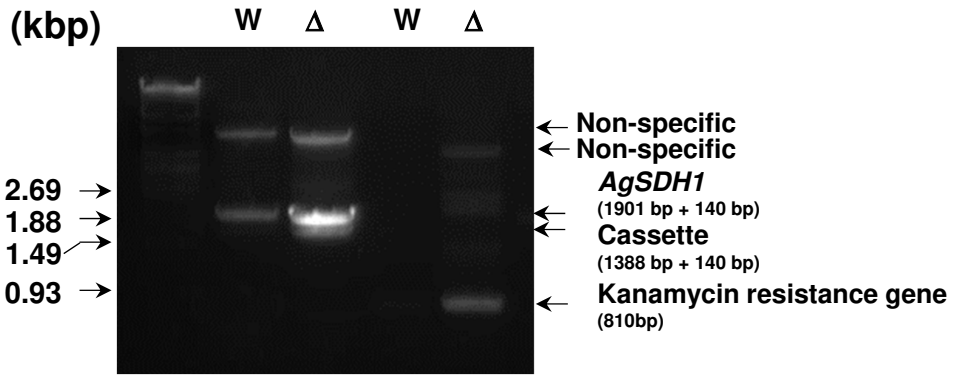


Figure S1, Kato et al.

(C)