Inactivation of the genes involved in histone H3-lysine 4 methylation abates the biosynthesis of pigment azaphilone in *Monascus purpureus*

Bijinu Balakrishnan¹ · Yoon Ji Lim¹ · Jae-Won Suh¹ · Hyung-Jin Kwon¹

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Abstract Di- and tri-methylation of lysine 4 on histone H3 (H3K4me2 and H3K4me3, respectively) are epigenetic markers of active genes. Complex associated with Set1 (COMPASS) mediates these H3K4 methylations. The involvement of COMPASS activity in secondary metabolite (SM) biosynthesis was first demonstrated with an *Aspergillus nidulans cclA* knockout mutant. The *cclA* knockout induced the transcription of two cryptic SM biosynthetic gene clusters, leading to the production of the cognate SM. *Monascus* spp. are filamentous fungi that have been used for food fermentation in eastern Asia, and the pigment *Monascus* azaphilone (MAz) is their main SM. *Monascus* highly produces MAz, implying that the cognate biosynthetic genes are highly active in transcription. In the present study, we examined how COMPASS activity modulates MAz biosynthesis by inactivating *Monascus purpureus cclA* (*Mp-cclA*) and *swd1* (*Mp-swd1*). For both $\Delta Mp-cclA$ and $\Delta Mp-swd1$, a reduction in MAz production, accompanied by an abated cell growth, was observed. Suppression of MAz production was more effective in an agar culture than in the submerged liquid culture. The fidelity of the $\Delta Mp-swd1$ phenotypes was verified by restoring the WT-like phenotypes in a reversion recombinant mutant, namely, *trpCp: Mp-swd1*, that was generated from the $\Delta Mp-swd1$ mutant. Real-time quantitative Polymerase chain reaction analysis indicated that the transcription of MAz biosynthetic genes was repressed in the $\Delta Mp-swd1$ mutant. This study demonstrated that MAz biosynthesis is under the control of COMPASS activity and that the extent of this regulation is dependent on growth conditions.

Keywords Complex associated with Set1 · Histone H3 lysine 4 methylation · *Monascus purpureus* · Pigment azaphilone · Secondary metabolite biosynthesis

Introduction

In eukaryotic transcriptional control, di- and tri-methylation of lysine 4 on histone H3 (H3K4me2 and H3K4me3, respectively) are epigenetic markers for actively transcribed genes. Complex associated with Set1 (COMPASS) mediates these H3K4 methylations and Set1 is the catalytic methyltransferase [1,2]. It is generally accepted that H3K4me3 marks transcriptional activation. However, COMPASS-mediated repression also occurs as demonstrated in *Saccharomyces cerevisiae* [3]. This repression appears to be mediated by H3K4 tri-methylation at the 3'-end, which elevates antisense transcription. Thus, this COMPASS-mediated repression event can be regarded as consistent with the general idea that H3K4me2/3 results in activation of downstream transcription.

The involvement of COMPASS activity in secondary metabolite (SM) biosynthesis was first demonstrated with an *Aspergillus nidulans cclA* knockout mutant [4]. *CclA* is an ortholog of the yeast Bre2, a component of COMPASS [2]. The *cclA* knockout induced the transcription of two cryptic SM biosynthetic gene clusters, leading to the production of the cognate SM. It was shown that the levels of H3K4me2/3 and H3K9me3 of the SM biosynthetic genes were significantly abated in the *cclA* knockout, while their transcription was elevated. It is confusing that, contrary to H3K4me2/3, H3K9me3 is an epigenetic marker associated with repressed genes [5]. Thus, a correlation between H3K4me2/3 and cognate gene transcription could not be generalized due to the complexity of cellular systems. It is widely accepted that the impact of H3K4me2/3 on transcription is context dependent involving the molecular recognition of H3K4me2/3 and that the related epigenetic control of transcription is the result of a complex interplay between distinctive histone modification markers [6].

Hyung-Jin Kwon (✉)
E-mail: hjink@mju.ac.kr

¹Department of Biological Sciences and Bioinformatics, Myongji University, Yongin-si, Gyeonggi-do, 17058, Republic of Korea

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Although the detailed molecular mechanism is yet to be defined, it is significant that the *cclA* knockout can activate SM production in filamentous fungi [4]. Although the underlying molecular mechanism is largely unclear, biotechnological applications of the *cclA* knockout in fungal SM production have been regarded as significant. Growth retardation, H3K4me2/3 deficiency and increased SM biosynthesis were also demonstrated in the *cclA* knockout mutant of *Aspergillus fumigatus* [7]. Overproduction of several SMs was found to be associated with a dramatic reduction in H3K4 methylation in the *cclA* knockout mutant of *Aspergillus oryzae* [8]. Overproduction of SMs with the *cclA* knockout was also demonstrated in *Pestalotiopsis fici*, an endophytic fungus [9]. However, this apparent simple correlation was not present in a genome-scale investigation. In the *cclA* (*ccl1*) knockout of the *Fusarium* species, the production of many SMs was promoted, although an inhibitory effect was also observed for some SMs [10]. A significant finding in this systemic study is that the levels of H3K4me2/3 at the 5'-ends of SM biosynthetic genes bear no direct correlation with the production level of the cognate SM. It was also shown that the levels of H3K4me2/3 at the 5'-end of some SM biosynthetic genes are enhanced in the *cclA* knockout mutant [10]. The authors proposed that CclA modulates the biosynthesis of the respective SMs through regulation of hidden regulatory factors. This idea might be relevant for the original *cclA* knockout study in *A. nidulans* [4]. Other than *cclA*, another COMPASS component gene, *swd1*, was characterized in terms of the mitotic regulation of *Aspergillus oryzae* [11]. Swd1 is a WD40 repeat domain protein (COG2319) and serves as an organizing factor for COMPASS [2]. The *swd1* gene is convergently arranged with *laeA*, which encodes the global regulatory factor of both SM biosynthesis and filamentous fungal development [12]. The pivotal role of *laeA* in *Monascus azaphilone* (MAz) biosynthesis and *Monascus* development was also demonstrated in *Monascus ruber* [13].

Monascus spp. are filamentous fungi utilized for food fermentation, and their main SM is the pigment azaphilone (Az) [14]. MAz is biosynthesized by a nonreducing polyketide pathway, which is associated with a special fatty acid synthase [15,16]. Extensive characterization of MAz biosynthetic genes established the biosynthetic mechanism [17–21], firmly establishing that MAz biosynthesis is the sole factor responsible for pigmentation of *Monascus* under general culture conditions. *Monascus* is highly productive of MAz, implying that the cognate biosynthetic genes are highly active in transcription.

In the present study, we examined how COMPASS activity affects MAz biosynthesis by inactivating *M. purpureus cclA* (*Mp-cclA*) and *swd1* (*Mp-swd1*). For both Δ *Mp-cclA* and Δ *Mp-swd1*, a significant reduction in MAz production, accompanied by an abated cell growth, was observed. The fidelity of Δ *Mp-swd1* genotype was verified by restoring the WT-like phenotypes in the *trpCp:Mp-swd1* recombinant mutant that was generated from the Δ *Mp-swd1* mutant.

Materials and Methods

Strains, culture conditions, and extraction methods

Monascus purpureus KACC (Korean Agricultural Culture Collection) 42430 (DSM 1379) was used in this study. *M. purpureus* and its derivative strains were cultured on Difco (Becton, Dickinson and Company, Sparks, MD, USA) potato dextrose agar (PDA) at 30 °C for 7 days. Agar blocks retrieved from the PDA culture were used to inoculate Gellix (Ventech Bio, Seoul, Republic of Korea) potato dextrose broth (PDB). The submerged liquid cultures, prepared in a 50 mL culture volume in a 250-mL baffled flask, were maintained in a rotary shaker at 28 °C at 250 rpm.

The PDA culture was extracted with the culture volume of methanol and used in measuring ultraviolet (UV)-visible absorption after 20-fold dilution. For the more concentrated sample, the absorption values were collected at 40-fold dilution and converted to correspond to that of 20-fold dilution. UV-visible absorption spectra were collected with a Cary50 spectrophotometer (Varian, Palo Alto, CA, USA). The PDA plate used in this study contained approximately 40 mL of medium in the original preparation. For PDB cultures, one milliliter of culture was spun at ambient temperature in a microcentrifuge tube for 15 min at 12,000 rpm to remove the supernatant. We neglected a minor portion of MAz in the supernatant in this study. Several rounds of brief centrifugation were necessary to remove residual supernatant. The cell mass left in the microcentrifuge tube was dried at 55 °C, and the mass was measured. After measuring the dried mass, the dried cells were submerged in 1 mL methanol to extract MAz. The methanolic extract was used in measuring UV-visible absorption after proper dilution, as noted above. Collection of samples from PDB cultures was performed in triplicate. Mean values are provided with error bars based on the highest and the lowest values.

Mp-cclA inactivation

The primers used in this study are listed in Table 1. Nucleotide sequence information was retrieved from the genome portal of the Department of Energy-Joint Genome Institute (DOE-JGI; <https://genome.jgi.doe.gov/Monpu1/Monpu1.home.html>). Polymerase chain reaction (PCR) for plasmid construction was performed with Herculase II Fusion DNA Polymerase (Agilent, Santa Clara, CA, USA). The *Mp-cclA* inactivation construct was designed to eliminate an 1,479-bp DNA fragment internal to *Mp-cclA* and replace it with neomycin phosphotransferase resistance cassette (*nptII*) that was retrieved from pII99 [22], by using a primer pair *npt-F/npt-cclA-R*. The *nptII* is 2.8 kb long and is composed of *trpC* (*A. nidulans* gene encoding indole-3-glycerol phosphate synthase in tryptophan biosynthesis) promoter, neomycin phosphotransferase, and *trpC* terminator. A 1,836- and 1,960-bp fragments, upstream and downstream to the deletion position within *Mp-cclA*, were amplified by PCR with primer sets *cclA*-up-

Table 1 Primers used in this study. The nucleotide sequences introduced for cloning process are in gray color and italicized

Primer name	Sequence (5'→3')
cclA-up-F	<i>CCATGATTACGAATTCATGACAGAGGACACGATGTG</i>
cclA -up-R	<i>GTTTAGAGGTAATCCGAGAAGACCTCGGATTCAGA</i>
npt-F	GGATTACCTCTAAACAAGTG
npt-cclA-R	<i>GGATAGCAGAGCCACGAATTCATGCCAGTTGTTCC</i>
cclA-dw-F	GTGGCTCTGCTATCCTGAAG
cclA-dw-R	<i>TACCGAGCTCGAATTCACCATGGAACATATGTGCTCA</i>
I-WD-up-F	<i>CCATGATTACGAATTCAGGAGAAGGTTTGGCGTTGC</i>
I-WD-up-R	<i>GCTCGACGTATTTCAATGCTGTGATGAATTCGCCTG</i>
Hyg- F	TGAAATACGTCGAGCCTGCT
hyg -WD-R	<i>ACTGCGTTTACGCATCCTGTGCATTCTGGGTAAAC</i>
I-WD-dw-F	ATGCGTAAACGCAGTCCCGG
I-WD-dw-R	<i>TACCGAGCTCGAATTCCTTATGGTTCTCCCGTCCAC</i>
WD-ups-F	<i>TACCGAGCTCGAATTCGTGATAACAGTACGAGCTGA</i>
WD-ups-R	TGCTGTGGTGAATGGTCA
OV-WD-F	<i>CCATTCACCACAGCA</i> GGATTACCTCTAAACAAGTG
OV-WD-R	GTAGCTGTTAGTCAAGCTGC
WD-dws-F	<i>TTGACTAACAGCTACGCAGTAATTCCTTGATGA</i>
WD-dws-R	<i>CCATGATTACGAATTCCTGTCCTTGAATCCATCTA</i>
PgpA-F	<i>GAATTC</i> ACTGAAATACGTCGAGCCTG
PgpA-R	<i>AGATCT</i> GTAGCTGTTAGTCAAGCTGC
WDup-R	<i>AACTGGCATGAATTC</i> GTGCTGTGATGAATTCGCCTG
Ptpc-F	GAATTCATGCCAGTTGTTCC
Ptpc-R	<i>GAGAGATAAATTCATATCGATGCTTGGGTAGAATA</i>
WD-F	ATGAATTTATCTCTCATTGA
WD-R	<i>ACGTAAAGTGGATCCTCATCGCCGTCGTTATTCT</i>
Ttpc-F	GGATCCACTTAACGTTACTG
Ttpc-R	<i>AACTGGCATGAATTC</i> GGATTACCTCTAAACAAGTG
npt-F(sacI)	<i>ATTGAGCTCGAATTCATGCCAGTTGTTCC</i>
npt-R(sacI)	<i>ATTGAGCTCGGATTACCTCTAAACAAGTG</i>
wd-dw-F	<i>CCGGGGATCCTCTAGATGCGTAAACGCAGTCCCGG</i>
wd-dw-R	<i>GCAGGTCGACTCTAGTTATGGTTCTCCCGTCCAC</i>
swd1-check-F	AAGGGCTGGATCAACATCATC
swd1-check-R	GCCGTCAGAATTCATCCACT
RT-actin-F	ATTCTACAACGAAGTCCG
RT-actin-R	TCAGGGAGTTCATAGGAC
RT-mppR1-F	GAACAGAGAAAACACGACA
RT-mppR2-R	GACTCGTTAGCAACATGCCA
RT-MpPKS5-F	TGGTCAGTCGGATATTGCGC
RT-MpPKS5-R	ATTGACCTGGCCAGTCGTGAA

F/-R and cclA-dw-F/-R, respectively. These two PCR fragments and *nptII* were cloned to the *EcoRI* site of pCambia1300 by using In-Fusion cloning method, generating pI-cclA. The pI-cclA was used to generate the *Mp-cclA* inactivation mutant of $\Delta Mp-cclA::nptII$ in *M. purpureus* wild-type (WT). A neomycin-resistant phenotype was used to select transformants in *Agrobacterium*-mediated transformation that was performed as previously described [15].

***Mp-swd1* inactivation**

The *Mp-swd1* inactivation construct was designed to eliminate a 444-bp DNA fragment internal to *Mp-swd1* and replace it with the hygromycin resistance cassette (*hyg*). The *hyg* cassette was amplified from pUR5750 [23], by using the primer pair of *hyg-F*/*hyg-WD-R*. A 1,521- and 1,452-bp DNA fragments, upstream and downstream to the deletion position of *Mp-swd1*, were amplified by PCR with primer pairs I-WD-up-F/-R and I-WD-dw-F/-R,

respectively. These two PCR fragments and the 3.9-kb of *hyg* were cloned into the *EcoRI* site of pCambia1300 by using In-Fusion cloning method to generate pI-WD. The pI-WD was used to generate the *Mp-swd1* inactivation mutant of $\Delta Mp-swd1::hyg$ in WT. A hygromycin-resistant phenotype was used to select transformants in *Agrobacterium*-mediated transformation.

***Mp-swd1* expression under an artificial promoter**

We aimed to activate *Mp-swd1* expression by inserting *gpdA* (*A. nidulans* gene encoding glyceraldehyde-3-phosphate dehydrogenase) promoter (*gpdAp*) as previously described [24]. There is the gene encoding DNA-dependent RNA polymerase II subunit RPB1 next to *Mp-swd1*, being divergently arranged. A 2.0-kb DNA fragment upstream to *Mp-swd1* was amplified with a primer pair WD-ups-F and WD-ups-R. The 2.0 kb downstream fragment was amplified by a primer pair WD-dws-F and WD-dws-R; this downstream fragment includes the intact *Mp-swd1* plus its 102-bp upstream and 226-bp downstream regions. The 2.1-kb *gpdAp* fragment was connected first with *nptII* for a convenience of cloning process. The *gpdAp* fragment was amplified with a primer pair of PgpD-A-F/-R from pUR5750, cloned in T-vector; the PCR amplification was done by using Herculase II fusion polymerase and was then treated with Ex Taq polymerase (Takara Korea Biomedica, Seoul, Korea) for A-tailing. From the resulting construct whose insert direction was confirmed by nucleotide sequencing, *gpdAp* was retrieved as an *EcoRI*-*BglII* fragment and cloned into pII99. In this *nptII*-*gpdAp* construct, *nptII* and *gpdAp* are divergently arranged. From this pII99 derivative harboring *nptII*-*gpdAp*, the 4.9-kb *nptII*-*gpdAp* region was amplified by using primer pair of OV-WD-F/-R. This fragment was connected with the upstream and downstream fragments (respective to the 5'-untranslated region of *Mp-swd1*) into the *EcoRI* site of pCambia1300 by using In-Fusion cloning method to generate pWD-OV. pWD-OV was introduced into *M. purpureus* to generate the *Mp-swd1* expression recombinant of *gpdAp*:*Mp-swd1*. A neomycin-resistant phenotype was used to select transformants and a hygromycin-sensitive phenotype was used to identify the desired gene replacement mutant.

A reversion of $\Delta Mp-swd1$ into *trpCp*:*Mp-swd1*

The main purpose to generate *trpCp*:*Mp-swd1* from $\Delta Mp-swd1::hyg$ is to validate that the phenotypes of $\Delta Mp-swd1$ originated from the loss of *Mp-swd1*. It was expected that the observed phenotypes of $\Delta Mp-swd1$ was largely reverted in *trpCp*:*Mp-swd1*, being comparable to WT. We used *trpCp*, instead of *gpdAp* that was used in generating the *gpdAp*:*Mp-swd1* recombinant, and the *trpCp*:*Mp-swd1* construct was introduced into $\Delta Mp-swd1$. The upstream fragment used in this procedure was the same as that used in the *Mp-swd1* inactivation construct pI-WD. This 2.0-kb upstream fragment was amplified with a primer pair WD-ups-F and WDups-R. The 1.3-kb promoter and 0.7-kb terminator regions of *trpC* were amplified from pII99 with

primer pairs Ptrpc-F/-R and Ttrpc-F/-R, respectively. As described above, the original source of *trpCp* and the *trpC* terminator region is *A. nidulans*. The intact *Mp-swd1* was amplified with a primer pair WD-F and WD-R. This 1.7-kb *Mp-swd1* DNA starts from its ATG start codon to its TGA termination codon. The four segments of the upstream region, *trpCp*, *Mp-swd1*, and *trpC* terminator fragments were combined by overlapping PCR and cloned into the *EcoRI* site of pCambia1300 by In-Fusion cloning method, yielding pR-WD1. The 2.8-kb *nptII* was amplified from pII99 with a primer pair npt(sacI)-F and npt(sacI)-R, and cloned into the *SacI* site of pR-WD1 to yield pR-WD2. The *SacI* site is a part of the polycloning site of pCambia1300 in pR-WD1. The 2.0-kb downstream fragment was the same as that used in the *Mp-swd1* inactivation construct pI-WD. This fragment was amplified with a primer pair wd-dw-F and wd-dw-R and cloned into the *XbaI* site of pR-WD2. End sequencing of the cloning products was used to select the construct with the proper direction of the downstream segment. The final construct was named pR-WD. pR-WD was introduced into $\Delta Mp-swd1$ ($\Delta Mp-swd1::hyg$) to eliminate *hyg* and replace it with *trpCp*:*Mp-swd1*. A neomycin-resistant phenotype was used to select transformants. A hygromycin-sensitive phenotype was used to identify the desired gene replacement mutant.

Total RNA isolation and real-time quantitative PCR (RT-qPCR)

For total RNA isolation, PDB culture was harvested at 3 days after the culture initiation. The cell was collected by filtration under reduced pressure and ground in a mortar with aid of liquid nitrogen. The ground cell from approximately 10 mL culture was subjected to total RNA isolation with Direct-zol RNA miniprep kit (Zymo research, Irvine, CA, USA) according to the manufacturer's manual. Approximately 0.3 μ g each of total RNA was used for the first strand complementary DNA (cDNA) synthesis with the reverse (complementary) primer specific to target genes by using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with inclusion of RNaseOUT (Invitrogen) following the manufacturer's manual. The primers are listed in Table 1 and their names start with RT-; RT-actin-F/R, RT-mppR1-F/R, and RT-MpPKS5-F/R for actin gene, *mppR1*, and *MpPKS5*, respectively. The details of primer design were previously described [15] and the target fragments are 250 bp long for all three genes. General PCR amplification using the cDNA samples was performed with Enzynomics (Daejeon, KOREA) 2 $\times$ TOPsimple premix-HOT with the annealing temperature of 60 $^{\circ}$ C. RT-qPCR analysis was carried out using SolGent (Daejeon, KOREA) 2 $\times$ Real-time PCR smart Mix with EvaGreen dye (SolGent) with the annealing temperature of 60 $^{\circ}$ C. Thermocycling and fluorescence detection were performed using a Stratagene Mx3000p RT-qPCR machine with Mx3000p software v2.02 (Stratagene). qPCRs were performed in triplicate for each cDNA sample. The threshold amplification cycles for *mppR1* and *MpPKS5* were normalized with respective to the values of actin gene (ΔC_T) and differences in the normalized

Fig. 1 Gene inactivation of *Mp-cclA* resulted in suppression of growth and MAZ production in *M. purpureus*. (A) Genetic organization of wild-type (*WT*) *M. purpureus*, the insert of *pI-cclA* (the *Mp-cclA* inactivation plasmid), and the $\Delta Mp-cclA$ mutant (*Mp-cclA::nptII*). In *WT* and the $\Delta Mp-cclA$ mutant, *Mp-cclA* and two flanking genes (designated with the protein identification number) are shown as arrows. The sizes of the PCR products with the primer pair of I-WD-up-F and I-WD-dw-R are shown in kb above the corresponding arrow. (B) Analytical PCR results of total DNA from the $\Delta Mp-cclA$ mutant (lane 1) and a random insertion transformant (lane 2) with the primer pair of I-WD-up-F and I-WD-dw-R. Lane M indicates a DNA molecular weight marker, with sizes of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 kb (from bottom to top). Relevant marker fragments are highlighted with the bars and the size in kb. The estimated sizes of the PCR products are provided at the arrows on the right side. (C) Approximately 3 cm² blocks of PDA cultures were placed on new PDA media and maintained for 7 days. (D) UV-visible spectra of the methanolic cell extracts. The *WT* and $\Delta Mp-cclA$ strains are represented by filled and unfilled circles, respectively

cycle values between *WT* and the recombinant strains ($\Delta\Delta C_T$) was used to calculate the mRNA levels relative to that of *WT* ($2^{-\Delta\Delta C_T}$).

Results and Discussion

Inactivation of *Mp-cclA* abated MAZ production

The protein identification number of the *Mp-cclA* product is 114895, and the sequence position is at scaffold 17:217733-220137 (+) in the *M. purpureus* genome portal of the DOE-JGI. The *Mp-cclA* gene was inactivated by inserting the *nptII* cassette (Fig. 1A). After the transformation with the inactivation plasmid *pI-cclA*, neomycin resistant colonies were subjected to PCR analysis with the primer pairs *cclA*-up-F and *cclA*-dw-R. In this PCR analysis, *WT* and $\Delta Mp-cclA$ were expected to yield 5.3-kb and 6.6-kb fragments, respectively (Fig. 1A). Random insertion transformants were predicted to yield both fragments. Through PCR analysis, we identified an expected $\Delta Mp-cclA$ genotype (lane 1) and a random insertion transformant (lane 2) from the neomycin-resistant colonies (Fig. 1B).

On a PDA plate, the $\Delta Mp-cclA$ mutant appeared inferior to *WT*

Table 2 Growth and MAZ content of *WT* and the $\Delta Mp-cclA$ mutant in PDB cultures at 7 days. Growth and MAZ content were measured by weighing dried cells in microcentrifuge tubes and through visible absorption of methanolic dried cell extracts, respectively. Absorbance values at three wavelengths of 390, 470, and 500 nm are provided

	WT	$\Delta Mp-cclA$
*DCW (mg/mL)	7.5±0.6	4.9±2.5
A ₃₉₀	0.77±0.03	0.42±0.04
A ₄₇₀	0.93±0.10	0.39±0.03
A ₅₀₀	1.04±0.07	0.73±0.13

*dried cell weight

in both growth and pigmentation (Fig. 1C). Spores were spread on PDA, and the resulting agar cultures were extracted to measure the UV-visible absorption (Fig. 1D). The results indicated that the MAZ production was significantly abated in the $\Delta Mp-cclA$ mutant. In PDB-submerged cultures, both growth and MAZ production were similarly reduced in the $\Delta Mp-cclA$ mutant when compared to *WT* (Table 2). Although microbial cell growth could not be correlated to the production of secondary metabolites, our findings regarding the $\Delta Mp-cclA$ mutant made it difficult to deny that

Fig. 2 Preparation of genetic recombinant strains related to *Mp-swd1*. Gene inactivation schemes (A, C, and E) and analytical PCR results (B, D, F, and G) for the generation of the Δ *Mp-swd1* mutant (A and B), the *gpdAp:Mp-swd1* recombinant (C and D), and the Δ *Mp-swd1* reversion recombinant of *trpCp:Mp-swd1* (E, F, and G). (B, D, F, and G) Details of the picture presentation are the same as Fig. 1B, and the respective primer pairs are described in the text

growth retardation was simply reflective of a reduction in MAZ production. Thus, we selected another component of COMPASS for the subsequent gene inactivation study. For this extended study, in addition to the gene inactivation mutant, we prepared overexpression and genetic reversion strains.

Inactivation of *Mp-swd1* abated MAZ production

To elucidate how COMPASS activity contributes to MAZ production, we further inactivated *Mp-swd1*, another genetic constituent of COMPASS. The ortholog of this gene (locus AN0808) was previously characterized in *A. nidulans* in the study of mitotic control [11]. The protein identification number of the *Mp-swd1* product is 503758, and the sequence position is at scaffold 184:8984-11083 (–) in the *M. purpureus* genome portal of the DOE-JGI. *Mp-swd1* was inactivated by inserting the *hyg* cassette (Fig. 2A). After the transformation with the inactivation plasmid pI-WD, hygromycin-resistant colonies were subjected to PCR

analysis with the primer pairs of I-WD-up-F and I-WD-dw-R. In this PCR analysis, WT and Δ *Mp-swd1* were expected to yield 3.4-kb and 6.9-kb fragments, respectively (Fig. 2A). Through PCR analysis, we identified the Δ *Mp-swd1* genotype (lane 2), in which the 3.4 kb-WT band (lane 1) was absent and replaced with a 6.9-kb band (Fig. 2B). For the *Mp-swd1* inactivation study, we also generated an *Mp-swd1* overexpression recombinant (*gpdAp:Mp-swd1*) strain with the gene replacement plasmid pWD-OV (Fig. 2C). This genetic recombination was aimed to constitutively drive the expression of *Mp-swd1* with the promoter of *A. nidulans*, *gpdA*. Integrating the insert of pWD-OV into the WT genome, by homologous recombination, was expected to place *gpdAp* upstream of *Mp-swd1*. The *nptII* cassette was included as a selection marker of this genetic recombination. PCR analysis with the primer pair WD-ups-F and WD-dws-R was used to identify the *gpdAp:Mp-swd1* recombinant. This PCR analysis was expected to yield 4.0- and 8.9-kb bands for WT and *gpdAp:Mp-swd1*, respectively (Fig.

2C). In the *gpdAp:Mp-swd1* recombinant (lane 2), the 4.0-kb WT band (lane 1) was absent and replaced with an 8.9-kb band (Fig. 2D).

We also generated a reversion recombinant (*trpCp:Mp-swd1*) of $\Delta Mp-swd1$ to verify its phenotype. Integration of the insert of pR-WD into the $\Delta Mp-swd1$ genome, by homologous recombination, was expected to restore *Mp-swd1* under the control of *trpCp*. PCR analysis with the primer pairs I-WD-up-F and I-WD-dw-R of the $\Delta Mp-swd1$ mutant and its reversion recombinant (*trpCp:Mp-swd1*) was expected to yield 6.9- and 9.5-kb fragments (Fig. 2E). In the PCR analysis of the *trpCp:Mp-swd1* recombinant, the 6.9-kb WT band (lane 1) was replaced by a large band approaching 10 kb in size (lane 2) (Fig. 2F). The expected 9.5-kb band seemed too large to be reliably analyzed under these electrophoresis conditions. Furthermore, smearing bands were also found in the PCR sample of the *trpCp:Mp-swd1* recombinant, and our PCR methods could not solve this problem. Thus, we designed another primer set, namely, *swd1-check-F/-R*, to amplify the internal region of *Mp-swd1*. This primer set was designed to amplify the

0.8-kb band within *Mp-swd1*, flanking the *hyg* insertion point in the $\Delta Mp-swd1$ mutant ($\Delta Mp-swd1::hyg$). The subsequent PCR analysis amplified a 4.0-kb band from $\Delta Mp-swd1$ (lane 1) while yielding a 0.8-kb band in both of the *trpCp:Mp-swd1* recombinant (lane 2) and WT (lane 3) (Fig. 2G). This finding supports the successful restoration of *Mp-swd1* in the *trpCp:Mp-swd1* recombinant.

The $\Delta Mp-swd1$ mutant showed growth retardation on PDA, while the growth of the *gpdAp:Mp-swd1* and *trpCp:Mp-swd1* recombinants were found to be comparable to that of WT (Fig. 3A). Pigmentation of the $\Delta Mp-swd1$ mutant was found to be inferior to the other three strains. When spores were spread on PDA, weak pigmentation of the $\Delta Mp-swd1$ mutant was visually observed (Fig. 3B). UV-visible absorption measurements of the PDA culture extracts verified that MAZ production was severely impaired in the $\Delta Mp-swd1$ mutant (Fig. 3C). Notably, the MAZ level of the reversion recombinant of *trpCp:Mp-swd1* was found to be similar to that of the WT, indicating that the impairment of MAZ production in the $\Delta Mp-swd1$ mutant resulted from the loss of *Mp-swd1* and, presumably, a loss of the COMPASS activity.

Fig. 3 Gene inactivation of *Mp-swd1* resulted in suppression of growth and MAZ production in *M. purpureus*, and these suppressions were reverted in the $\Delta Mp-swd1$ reversion recombinant of *trpCp:Mp-swd1*. The WT, $\Delta Mp-swd1$, *gpdAp:Mp-swd1*, and *trpCp:Mp-swd1* strains are represented by black-filled circles, unfilled circles, gray-filled rectangles, and black-filled triangles, respectively, in graphs. To avoid overlapping, each symbol at the same time point is slightly shifted in terms of the X-axis. Approximately 3 cm² blocks of PDA cultures (A) or approximately 10⁶ spores (B) were placed on PDA media and maintained for 7 days. (C) UV-visible spectra of the methanolic extract of PDA cultures. Time course experiment of PDB cultures for growth (D; DCW, dried cell weight) and MAZ production (E) represented by absorbance values at 470 nm. (F) UV-visible spectra of the methanolic cell extracts of PDB cultures at 7 days

In the PDB culture, we determined growth and MAz production in a time-dependent manner. The growth of the $\Delta Mp\text{-}swd1$ mutant was also limited, as was that of the $\Delta Mp\text{-}cclA$ mutant (Fig. 3D). Unlike the PDA cultures, growth of the $gpdAp\text{:}Mp\text{-}swd1$ recombinant was as limited as that of the $\Delta Mp\text{-}swd1$ mutant, while the $trpCp\text{:}Mp\text{-}swd1$ revertant showed a relatively moderate growth reduction compared to WT. MAz production, as demonstrated with an absorbance at 470 nm, was severely impaired in the $\Delta Mp\text{-}swd1$ mutant, while no significant difference was found in the $trpCp\text{:}Mp\text{-}swd1$ revertant when compared to WT (Fig. 3E and F). The $gpdAp\text{:}Mp\text{-}swd1$ recombinant produced MAz at a moderately reduced level, although its growth was suppressed as much as that of the $\Delta Mp\text{-}swd1$ mutant. This finding supports the involvement of $Mp\text{-}swd1$ in the control of MAz biosynthesis and suggests that deterred expression of $Mp\text{-}swd1$, as seen in the $gpdAp\text{:}Mp\text{-}swd1$ recombinant, negatively affects growth. The expression of MAz biosynthetic genes is proposed to be controlled by the COMPASS activity, specifically H3K4 methylation.

Constitutive expression of $Mp\text{-}swd1$ under the control of $gpdAp$ failed to enhance MAz production. It is also noteworthy that the $\Delta Mp\text{-}swd1$ mutant yielded a considerable level of MAz in the PDB submerged culture while displaying negligible MAz production in the PDA solid culture (Fig. 3C and F). This finding implied that COMPASS-mediated epigenetic regulation works in an on-and-off-switch manner to control MAz biosynthesis when grown on PDA while playing a tuning role for MAz production in PDB culture.

***Mp-sw*1 is involved in the transcriptional control of MAz biosynthetic genes**

A specific reduction in MAz production in the $\Delta Mp\text{-}swd1$ mutant implied that the transcription of MAz biosynthetic genes was repressed with a loss of COMPASS activity. To support this idea, we examined the mRNA levels of two representative MAz biosynthetic genes, *mppR1* and *MpPKS5*. *MpPKS5* encodes nonreducing polyketide synthase for MAz backbone synthesis, and *mppR1* is the pathway-specific transcription activator for MAz biosynthetic genes including *MpPKS5* [15].

We employed PDB culture, instead of PDA culture, for total RNA isolation, although MAz production was dramatically impaired in the $\Delta Mp\text{-}swd1$ mutant on PDA (Fig. 3C). The reason was that isolating total RNA from the agar culture was not sufficient for cDNA experiments. Simple cDNA PCR failed to dissect a difference in the intensity of amplified fragments (Fig. 4A), which led us to perform RT-qPCR analysis. RT-qPCR analysis indicated that the transcriptional levels of *mppR1* and *MpPKS5* were repressed in the $\Delta Mp\text{-}swd1$ mutant compared to WT, while the calculated mRNA levels were comparable between WT and the *trpCp\text{:}Mp\text{-}swd1* reversion strain (Fig. 4B). It was questionable whether the difference in *MpPKS5* mRNA levels between WT and the *gpdAp\text{:}Mp\text{-}swd1* recombinant was significant. This finding suggests that the COMPASS activity controls the transcription of MAz

Fig. 4 Inactivation of *Mp-sw*1 repressed MAz biosynthetic genes of *mppR1* and *MpPKS5*. (A) PCR analyses used 0.1 μ L of reverse transcriptase reaction samples and were amplified in 30 cycles. *M* indicates the DNA marker, which includes fragments of 100 bp increments from 100-bp fragment. The 500-bp fragment is highlighted with a bar at the left. Lanes 1, 2, 3 and 4 represent the WT, $\Delta Mp\text{-}swd1$, *gpdAp\text{:}Mp\text{-}swd1* and *trpCp\text{:}Mp\text{-}swd1* strains, respectively. (B) RT-qPCR analysis with cDNA of the WT, $\Delta Mp\text{-}swd1$, *gpdAp\text{:}Mp\text{-}swd1* and *trpCp\text{:}Mp\text{-}swd1* strains. Standard deviations are provided as error bars. The calculation method for relative mRNA level is described in *Materials and methods*

biosynthetic genes, probably by mediating H3K4me2/3. However, we have no evidence for the direct involvement of this histone methylation in controlling the expression of MAz biosynthetic genes. As implied in the *cclA* study in *Aspergillus nidulans* [4], expression of an unknown regulator might be the control point of COMPASS activity, and modulation of MAz biosynthetic genes might be controlled by this hypothetical regulator.

We expected MAz overproduction with *Mp-cclA* inactivation but instead found a reduction in the MAz level. The subsequent genetic characterization study of *Mp-sw*1 supported that COMPASS activity controls MAz biosynthesis. If we consider high productivity of MAz and active expression of the biosynthetic genes, suppression of MAz production due to the loss of COMPASS seems reasonable and consistent with a generalized role of COMPASS activity, the marker of active genes. MAz biosynthesis can thus be an excellent model to investigate epigenetic contributions to the control of SM biosynthesis [12]. Inactivation of *cclA* is useful in inducing silent or weakly expressed genes [4,7], but this ability seems irrelevant for SM that is already actively produced. We also performed a preliminary investigation into whether new SM was induced in the $\Delta Mp\text{-}swd1$ mutant, but failed to find any relevant SM peak in the ultraviolet detection of liquid chromatographic separation. Perhaps a more thorough chemical investigation is necessary to identify new SM in the $\Delta Mp\text{-}swd1$ mutant. It also seems possible

that SM biosynthesis is globally repressed in *M. purpureus* when COMPASS activity is lost, at least under the culture conditions tested. Although many aspects of the control of MAZ biosynthesis by the COMPASS activity are yet veiled, our study clearly demonstrated that inactivation of COMPASS activity should not be generalized to enhance fungal SM biosynthesis and that epigenetic modulation might not be a hallmark for fungal SM overproduction.

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References

- Shilatifard A (2012) The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. *Annu Rev Biochem* 81: 65–95
- Hsu PL, Li H, Lau HT, Leonen C, Dhall A, Ong SE, Chatterjee C, Zheng N (2018) Crystal Structure of the COMPASS H3K4 Methyltransferase Catalytic Module. *Cell*. doi:10.1016/j.cell.2018.06.038
- Margaritis T, Oreal V, Brabers N, Maestroni L, Vitaliano-Prunier A, Benschop JJ, van Hooff S, van Leenen D, Dargemont C, Géli V, Holstege FC (2012) Two distinct repressive mechanisms for histone 3 lysine 4 methylation through promoting 3'-end antisense transcription. *PLoS Genet* 8: e1002952. doi:10.1371/journal.pgen.1002952
- Bok JW, Chiang YM, Szewczyk E, Reyes-Dominguez Y, Davidson AD, Sanchez JF, Lo HC, Watanabe K, Strauss J, Oakley BR, Wang CC, Keller NP (2009) Chromatin-level regulation of biosynthetic gene clusters. *Nat Chem Biol* 5: 462–464
- Black JC, Van Rechem C, Whetstine JR (2012) Histone lysine methylation dynamics: establishment, regulation, and biological impact. *Mol Cell* 48: 491–507
- Zhang T, Cooper S, Brockdorff N (2015) The interplay of histone modifications-writers that read. *EMBO Rep* 16: 1467–1481
- Palmer JM, Bok JW, Lee S, Dagenais TR, Andes DR, Kontoyiannis DP, Keller NP (2013) Loss of CclA, required for histone 3 lysine 4 methylation, decreases growth but increases secondary metabolite production in *Aspergillus fumigatus*. *PeerJ* 1: e4. doi:10.7717/peerj.4
- Shinohara Y, Kawatani M, Futamura Y, Osada H, Koyama Y (2016) An overproduction of astellolides induced by genetic disruption of chromatin-remodeling factors in *Aspergillus oryzae*. *J Antibiot* 69: 4–8
- Wu G, Zhou H, Zhang P, Wang X, Li W, Zhang W, Liu X, Liu HW, Keller NP, An Z, Yin WB (2016) Polyketide production of pestaloficiols and macrodiolide ficiolides revealed by manipulations of epigenetic regulators in an endophytic fungus. *Org Lett* 18: 1832–1835
- Studt L, Janevska S, Arndt B, Boedi S, Sulyok M, Humpf HU, Tudzynski B, Strauss J (2017) Lack of the COMPASS component Ccl1 reduces H3K4 trimethylation levels and affects transcription of secondary metabolite genes in two plant-pathogenic *Fusarium* species. *Front Microbiol* 7: 2144. doi:10.3389/fmicb.2016.02144
- Govindaraghavan M, Anglin SL, Osmani AH, Osmani SA (2014) The Set1/COMPASS histone H3 methyltransferase helps regulate mitosis with the CDK1 and NIMA mitotic kinases in *Aspergillus nidulans*. *Genetics* 197: 1225–1236
- Bayram Ö, Braus GH (2011) Coordination of secondary metabolism and development in fungi: the velvet family of regulatory proteins. *FEMS Microbiol Rev* 36: 1–24
- Liu Q, Cai L, Shao Y, Zhou Y, Li M, Wang X, Chen F (2016) Inactivation of the global regulator LaeA in *Monascus ruber* results in a species-dependent response in sporulation and secondary metabolism. *Fungal Biol* 120: 297–305
- Chen W, He Y, Zhou Y, Shao Y, Feng Y, Li M, Chen F (2015) Edible filamentous fungi from the species *Monascus*: early traditional fermentations, modern molecular biology, and future genomics. *Compr Rev Food Sci Food Saf* 14: 555–567
- Balakrishnan B, Karki S, Chiu SH, Kim HJ, Suh JW, Nam B, Yoon YM, Chen CC, Kwon HJ (2013) Genetic localization and in vivo characterization of a *Monascus azaphilone* pigment biosynthetic gene cluster. *Appl Microbiol Biotechnol* 97: 6337–6345
- Balakrishnan B, Kim HJ, Suh JW, Chen CC, Liu KH, Park SH, Kwon HJ (2014) *Monascus azaphilone* pigment biosynthesis employs a dedicated fatty acid synthase for short chain fatty acyl moieties. *J Kor Soc Appl Biol Chem* 57: 191–196
- Balakrishnan B, Chen CC, Pan TM, Kwon HJ (2014) Mpp7 controls regioselective Knoevenagel condensation during the biosynthesis of *Monascus azaphilone* pigments. *Tetrahedron Lett* 55: 1640–1643
- Bijinu B, Suh JW, Park SH, Kwon HJ (2014) Delineating *Monascus azaphilone* pigment biosynthesis: oxidoreductive modifications determine the ring cyclization pattern in azaphilone biosynthesis. *RSC Adv* 4: 59405–59408
- Balakrishnan B, Park SH, Kwon HJ (2017) A reductase gene *mppE* controls yellow component production in azaphilone polyketide pathway of *Monascus*. *Biotechnol Lett* 39: 163–169
- Balakrishnan B, Park SH, Kwon HJ (2017) Inactivation of the oxidase gene *mppG* results in the selective loss of orange azaphilone pigments in *Monascus purpureus*. *Appl Biol Chem* 60: 437–446
- Balarishnan B, Lim YJ, Hwang SH, Lee DW, Park SH, Kwon HJ (2017) Selective production of red azaphilone pigments in a *Monascus purpureus mppDEG* deletion mutant. *J Appl Biol Chem* 60: 249–256
- Namiki F, Matsunaga M, Okuda M, Inoue I, Nishi K, Fujita Y, Tsuge T (2001) Mutation of an arginine biosynthesis gene causes reduced pathogenicity in *Fusarium oxysporum* f. sp. *melonis*. *Mol. Plant-Microbe Interact* 14: 580–584
- de Groot MJ, Bundock P, Hooykaas PJ, Beijersbergen AG (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nat Biotechnol* 16: 839–842
- Suh JW, Balakrishnan B, Lim YJ, Lee DW, Choi JJ, Park SH, Kwon HJ (2018) Production of a hypothetical polyene substance by activating a cryptic fungal PKS-NRPS hybrid gene in *Monascus purpureus*. *J Appl Biol Chem* 61: 83–91