Inhibitory effect of aflastatin A on melanin biosynthesis by *Colletotrichum lagenarium*

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The effect of aflastatin A (AsA), a novel inhibitor of aflatoxin production, on melanin biosynthesis of *Colletotrichum lagenarium* was examined. Addition of a low concentration of AsA (0.5 µg ml⁻¹) to the culture medium almost completely inhibited the melanin production of this organism. AsA also inhibited the production of scytalone, an early intermediate of melanin biosynthesis. Melanin production was restored by addition of exogenous scytalone in the presence of AsA, suggesting that the late steps after the synthesis of scytalone were not significantly affected by AsA. This was confirmed by the results from RT-PCR analysis of the expression of genes encoding melanin biosynthetic enzymes (*SCD1*, *THR1*) and a regulatory gene (*CMR1*). By contrast, expression of *PKS1* was severely impaired by AsA, although catalytic activity of a polyketide synthase (*PKS1*) was not inhibited by AsA. These results indicate that AsA inhibits an early step in melanin production, which suppresses the expression of *PKS1*.

Keywords: aflastatin A, *Colletotrichum lagenarium*, melanin, *PKS1*, RT-PCR

INTRODUCTION

Aflastatin A (AsA) was isolated from the mycelia of *Streptomyces* sp. MRI 142 as an inhibitor of aflatoxin production by *Aspergillus parasiticus* (Ono *et al.*, 1997). It is a novel tetramic acid derivative with a long alkyl side chain and shows strong inhibitory activity towards aflatoxin production, essentially without inhibiting the growth of its producer (Sakuda *et al.*, 1996; Ono *et al.*, 1998; Ikeda *et al.*, 2000). Therefore, AsA is a lead compound to protect foods and feeds from aflatoxin contamination without incurring rapid spread of a resistant strain. The molecular mechanism of inhibition of aflatoxin production by AsA has not been verified. Since no accumulation of any biosynthetic intermediates of aflatoxin was detected in the broth of *A. parasiticus* when AsA was added to the culture, AsA may not be an inhibitor of an enzyme involved in the biosynthetic pathway of aflatoxin. This observation prompted us to examine the effect of AsA on the production of a variety

of secondary metabolites by fungi. In these preliminary experiments, we found that AsA can strongly inhibit melanin production by *Colletotrichum lagenarium*.

The melanin of this fungus is a polymer formed from 1,8-dihydroxynaphthalene (1,8-DHN). 1,8-DHN is biosynthesized from five molecules of malonyl-CoA (Fujii *et al.*, 2000) via four biosynthetic intermediates, 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), scytalone, 1,3,8-trihydroxynaphthalene (1,3,8-THN) and vermelone (Fig. 1; Bell & Wheeler, 1986). Several key enzymes and genes involved in the biosynthetic pathway have been clarified. In some fungal pathogens such as *C. lagenarium* or *Magnaporthe grisea*, melanization of appressoria is essential for penetration into the host plant (Kubo & Furusawa, 1991). Fungal conidia germinate and the tips of germ tubes differentiate into appressoria, which synthesize melanin. The melanin mediates the build-up of pressure in the appressorium and this high pressure provides the essential driving force for mechanical penetration into the host plant (Howard & Ferrari, 1989). Since blocking of melanin biosynthesis renders the fungus unable to generate the high pressure required for host leaf penetration by appressoria, melanin is an ideal target for the de-

Abbreviations: AsA, aflastatin A; 1,8-DHN, 1,8-dihydroxynaphthalene; 1,3,6,8-THN, 1,3,6,8-tetrahydroxynaphthalene; 1,3,8-THN, 1,3,8-trihydroxynaphthalene.

Fig. 1. Biosynthesis of fungal melanin.

velopment of an effective drug to protect host plants from infection by pathogens (Kubo & Furusawa, 1991). Inhibitors of enzymes involved in the melanin biosynthetic pathway are actually applied to control rice blast disease (Bell & Wheeler, 1986; Yamaguchi & Kubo, 1992).

In this study, we examined the effect of AsA on melanin production by *C. lagenarium*, which could provide clues to the mode of action of AsA. Our results indicate that AsA inhibits an early step in the biosynthetic pathway of melanin in this fungus.

METHODS

Fungal strains. *C. lagenarium* (Pass.) Ellis and Halsted strain 104-T was used as the wild-type strain. Melanin-deficient mutant 9201Y (Scd⁻), which was obtained by random gene disruption using plasmid pCTUB29 (Kubo *et al.*, 1996), was used as a scytalone producer. These strains were maintained on a potato sucrose agar medium at 26.5 °C and subcultured at about monthly intervals. *Aspergillus oryzae* M-2-3(pTAPSG), which highly expresses the *C. lagenarium* PKS1 gene under the starch-inducible α -amylase promoter (Fujii *et al.*, 1999), was used for *in vitro* assessment of the effect of AsA on polyketide synthase activity.

Chemicals. AsA was extracted from the mycelium of *Streptomyces* sp. MRI 142 and purified as described by Ono *et al.* (1997). Scytalone was prepared by the method of Kubo *et al.* (1983) with some modifications. Briefly, a culture filtrate (potato-sucrose medium) of strain 9201Y was adjusted to pH 5.0 with H₃PO₄ and saturated with NaCl, and extracted with ethyl acetate. The ethyl acetate extract was evaporated *in vacuo* and the oily residue was subjected to silica gel chromatography. The column was washed with n-hexane/ethyl acetate (2:1, v/v), and scytalone was eluted with n-hexane/ethyl acetate (1:1, v/v).

Induction of melanin synthesis. Melanin biosynthesis at the vegetative mycelial growth stage was induced as described previously (Takano *et al.*, 1995). Conidia of *C. lagenarium* 104-T were inoculated into 100 ml of the potato-sucrose liquid

medium containing 0.2% of yeast extract and grown at 26.5 °C in a rotary shaker (120 r.p.m.) for 40 h. For rapid induction of melanin synthesis, the mycelia were separated from the medium by filtration through two layers of sterile cheesecloth, and transferred into 50 ml 1.2 M sucrose solution. The mycelial suspension (2 ml) was added to 2 ml 1.2 M sucrose solution containing AsA (0–2 µg ml⁻¹, final concentration) in a sterile test tube [1.8 cm (i.d.) × 20 cm (length)] and incubated at 26.5 °C in a test tube shaker (250 r.p.m.) for 20 h. AsA was dissolved in DMSO at appropriate concentrations and added to the sucrose solution. Melanin pigment in the culture filtrate was determined by measuring the A₅₈₅ on a spectrophotometer (model FP777; JASCO).

Induction of scytalone synthesis. Scytalone biosynthesis was induced as described above except that strain 9201Y instead of wild-type strain 104-T was used. After induction by incubation in 1.2 M sucrose, the culture filtrate was adjusted to pH 5.0 with H₃PO₄ and scytalone was extracted with ethyl acetate. The amount of scytalone in the ethyl acetate extract was determined semi-quantitatively by TLC (silica gel 60 F₂₅₄; Merck). TLC plates were developed with chloroform/methanol (9:1, v/v) and inspected by short wavelength UV illumination.

Restoration of melanin production by exogenous scytalone in the presence of AsA. Restoration of melanin production by addition of exogenous scytalone was examined in the presence of AsA (1 µg ml⁻¹) using strain 104-T. Scytalone (0.5 or 1.0 mM, final concentration) was added to 1.2 M sucrose, and melanin production was inspected at 20 h after the induction in the same way as described above.

Measurement of polyketide synthase activity. *A. oryzae* M-2-3(pTAPSG) was grown at 30 °C in Czapek–Dox medium containing starch, and a cell lysate was prepared by grinding in liquid nitrogen with a mortar and pestle. The mycelial powder (8 g) was then suspended in 40 ml extraction buffer (50 mM potassium phosphate, pH 7.5, 30% glycerol, 2 mM β -mercaptoethanol, 1 mM EDTA and 0.1 mM benzamidine). Polyclar VT (0.8 g; Gokyou Sangyou) was added to the mixture, and the mixture was stirred on ice for 1 h and filtered through four-layered gauze. The obtained filtrate was centrifuged at 15 000 g for 20 min to remove residual cell debris. Bio-Beads S-X1 (4 g; Bio-Rad) were added to the supernatant, and

the mixture was stirred on ice for 30 min. After removing the beads by filtration, the filtrate was centrifuged at 210000 g for 2 h. The obtained pellet was dissolved in 4 ml of the extraction buffer mentioned above and used as a crude enzyme solution.

Enzyme reaction mixture, containing 175 µl 1 mM potassium phosphate buffer, pH 7.2, 25 µl 1 µM malonyl-CoA, 50 µl enzyme solution and 5 µl DMSO with or without AsA (5.0 or 10.0 µg ml⁻¹), was incubated for 1 h at 24 °C. The reaction mixture was heated at 100 °C for 20 min to convert 1,3,6,8-THN to flaviolin and acidified with addition of 50 µl 6 M HCl. Products were extracted with 300 µl ethyl acetate, dried by flushing of nitrogen gas, and dissolved in 100 µl acetonitrile for HPLC analysis. HPLC conditions were as follows: SenshuPak ODS-H-1151 (4.6 × 150 mm; Senshu Kagaku), maintained at 40 °C; mobile phase, linear gradient from 5% CH₃CN in H₂O to 35% CH₃CN in H₂O (each contained 2% acetic acid) over 30 min with detection at 305 nm; flow rate, 0.8 ml min⁻¹; retention time of flaviolin, 21.2 min (Fujii *et al.*, 2000; Funa *et al.*, 1999).

RT-PCR. Melanin biosynthesis was induced as described above in the presence or absence of 1 µg AsA ml⁻¹. Samples were taken periodically and cells were collected by filtration. Cells (50–70 mg) were ground under liquid nitrogen to a fine powder using a mortar and pestle and then transferred to an Eppendorf tube. Total cellular RNA was prepared using the RNeasy Plant Mini Kit (Qiagen) as recommended by the manufacturer. Total RNA (1 µg) was treated with 1 unit DNase I (Gibco-BRL) to remove residual genomic DNA and used for RT-PCR. First-strand cDNA was prepared using random hexamer primers and ReverTra Ace (Toyobo) reverse transcriptase in a final volume of 20 µl. cDNA was used as template in PCR reactions with the following primer sets: *PKS1* 5'-CCGAGCATCCTTGCGAAGACGTTCC-3' and 5'-TGAGTCTCTCCGCCAGCGAACCGAC-3' (amplified fragment size, 398 bp), *SCD1* 5'-TTGCGGTACCAGTGCATGTTGTAGC-3' and 5'-CTCCTACGACTCCAAGGACTGGGAC-3' (324 bp), *THR1* 5'-CCTTTGGCCACGTCAAGGACGTCAC-3' and 5'-TCAACACCCTCGTCGTCGAGCTCAC-3' (348 bp) and *CMR1* 5'-GCATGGAGTGGCGACAAATGGTTG-3' and 5'-TGAAGTTCGGTGATGGCCAGTTGA-3' (260 bp). A housekeeping gene, *G3PDH* 5'-ATCGGTCGTATCGTCTTCCGCAAC-3' and 5'-CGTTGACACCCATCACGTACATGG-3' (369 bp), was used as a control to certify that the same amount of RNA was used in all reactions. The PCR reactions were performed with AmpliTaq Gold DNA polymerase (PE Applied Biosystems) and a buffer provided by the manufacturer in the presence of 200 µM deoxynucleoside triphosphate, 0.5 µM each primer, 1 µl cDNA and 2.5 U enzyme in a final volume of 50 µl. The reactions consisted of 32–40 cycles. The first cycle was for 9 min at 95 °C (for denaturation and enzyme activation) and 30 s at 68 °C (for annealing and extension); the denaturation step was shortened to 30 s in the subsequent cycles. Control reactions without reverse transcriptase gave no signal.

RESULTS

Effect of AsA on melanin biosynthesis of *C. lagenarium*

AsA has antimicrobial activity against some microorganisms other than aflatoxin inhibitory activity. AsA inhibited growth of *C. lagenarium* at the MIC value of 0.1 µg ml⁻¹, which was tested by the agar dilution method using a potato-sucrose agar medium. Therefore,

Fig. 2. Time course of melanin production in the induction medium. Melanin production was induced by transferring non-melanized mycelia of *C. lagenarium* cultured in a potato-sucrose liquid medium containing 0.2% yeast extract to 1.2 M sucrose solution with (●) or without (○) AsA (1.0 µg ml⁻¹). Each value represents the mean of duplicate experiments.

we used a melanin induction system to assess the effect of AsA on melanin production of *C. lagenarium*. In the induction system, melanin biosynthesis can be induced by transferring non-melanized mycelia cultured in a potato-sucrose liquid medium containing 0.2% yeast extract to 1.2 M sucrose solution (Takano *et al.*, 1995). Melanin production started at 10 h after the induction and then increased during the course of the experiment (Fig. 2). When AsA was added to the culture at a concentration of 1.0 µg ml⁻¹, melanin production was almost completely inhibited throughout the cultivation time (Fig. 2). Melanin production at 18 h after the induction was 58.1, 99.2 and 99.6% inhibited by AsA at concentrations of 0.25, 0.5 and 1.0 µg ml⁻¹, respectively.

Effect of AsA on scytalone biosynthesis

Scytalone has been shown to be an early intermediate in melanin biosynthesis by *C. lagenarium* (Kubo *et al.*, 1983). *C. lagenarium* strain 9201Y has a deletion in the *SCD1* gene region and is defective in the conversion of scytalone to 1,3,8-THN (Fig. 1), resulting in accumulation of scytalone in the culture medium (Kubo *et al.*, 1996). We examined the effect of AsA on scytalone biosynthesis using this strain. AsA was added to the induction medium and accumulation of scytalone was analysed by TLC. As shown in Fig. 3, AsA inhibited scytalone production at a concentration of 0.25 µg ml⁻¹.

Restoration of melanin production by exogenous scytalone in the presence of AsA

To test whether AsA blocks the late steps after the synthesis of scytalone in the melanin biosynthetic pathway, we examined restoration of melanin production by adding exogenous scytalone in the presence of AsA. Scytalone (0.5 or 1.0 mM) and AsA (1.0 µg ml⁻¹)

Fig. 3. Inhibition of scytalone production by AsA. Scytalone production by strain 9201Y was induced with or without AsA ($0\text{--}1.0\text{ }\mu\text{g ml}^{-1}$). Scytalone was extracted from the culture medium at 20 h after the induction and analysed by TLC. Lanes: 1, authentic scytalone; 2–5, cultured with 0, 0.25, 0.5 or $1.0\text{ }\mu\text{g ml}^{-1}$ AsA, respectively.

Aflastatin A ($1\text{ }\mu\text{g ml}^{-1}$)...	–	+	+	+
Scytalone (mM)...	–	–	0.5	1.0

Fig. 4. Restoration of melanin production by addition of scytalone. Melanin production was induced with or without AsA ($1.0\text{ }\mu\text{g ml}^{-1}$) or scytalone (0.5 or 1.0 mM). The formation of melanin was observed at 20 h after the induction.

were simultaneously added to the induction medium, and the formation of melanin was examined. As shown in Fig. 4, melanin production was restored by the addition of scytalone, while no visible pigmentation was observed without scytalone. Restoration of melanin production with 1.0 mM scytalone was estimated to be up to 40% of the control experiment without scytalone and AsA from the A_{585} values in the culture filtrate.

Effect of AsA on polyketide synthase activity

Polyketide synthase (encoded by *PKS1*) catalyses the step from five molecules of malonyl-CoA to 1,3,6,8-THN in melanin biosynthesis by *C. lagenarium* (Fig. 1; Takano *et al.*, 1995; Fujii *et al.*, 2000). To test if AsA can inhibit *PKS1* activity, AsA was added to an *in vitro* reaction mixture containing *PKS1*, which was produced by *A. oryzae* M-2-3(pTAPSG) (Fujii *et al.*, 1999), and

malonyl-CoA (Fujii *et al.*, 2000; Funa *et al.*, 1999). After incubation of the reaction mixture, 1,3,6,8-THN produced was converted to its stable oxidation product (flaviolin) by boiling, and the amount of flaviolin was analysed by HPLC. When AsA was added to the enzyme reaction at a concentration of 5.0 or $10.0\text{ }\mu\text{g ml}^{-1}$, flaviolin production was detected at the same level as that of control without AsA in both cases (97 and 98% of control, respectively). This indicated that activity of the enzyme was not affected by AsA even at a concentration of $10\text{ }\mu\text{g ml}^{-1}$.

Effects of AsA on the expression of genes responsible for melanin biosynthesis

The effect of AsA on the expression of three genes encoding melanin biosynthetic enzymes, *PKS1*, *SCD1* and *THR1* (Takano *et al.*, 1995; Kubo *et al.*, 1996; Perpetua *et al.*, 1996), and a regulatory gene (*CMR1*) was examined. Expression of *G3PDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as a control. *C. lagenarium* was cultured in the induction medium with or without AsA ($1.0\text{ }\mu\text{g ml}^{-1}$) and samples were taken periodically. Total RNA was prepared and used for RT-PCR analysis. At 0 time, transcription of these melanin-related genes was not strongly activated (Fig. 5). After induction without AsA, expression of these genes was activated and maintained at high levels during the incubation for 8–20 h. When AsA was added to the culture, the expression of *PKS1* was severely impaired at all incubation times. Expression of all other genes was unaffected by this treatment.

DISCUSSION

To examine the effect of AsA on melanin biosynthesis by *C. lagenarium*, we used a replacement culture technique for induction of melanin biosynthesis since growth of the fungus was strongly inhibited by AsA. This strong growth inhibition by AsA was not observed against the aflatoxigenic fungus *A. parasiticus* (Ono *et al.*, 1997). Growth inhibition may affect fungal physiology, which may lead to altered secondary metabolism. In this study, however, a specific inhibition of melanin production in *C. lagenarium* by AsA could be observed using the induction system.

In the present experiments, AsA inhibited scytalone production, and addition of exogenous scytalone was able to restore melanin production under conditions where scytalone production was completely inhibited by AsA. This suggests that AsA targets a step before the synthesis of scytalone in the melanin biosynthetic pathway. This conclusion is supported by the fact that expression of *SCD1*, *THR1* and *CMR1* was not impaired by AsA. *SCD1* catalyses the biosynthetic steps from scytalone to 1,3,8-THN and vermeline to 1,8-DHN, and *THR1* catalyses the step from 1,3,8-THN to vermeline (Fig. 1). *CMR1* encodes a regulatory protein which positively regulates the transcription of *SCD1* and *THR1* in the melanin induction system in *C. lagenarium*.

Fig. 5. Expression of melanin biosynthetic genes. Total RNA was extracted from fungal mycelia incubated for 0, 8, 10, 12, 14, 16 or 20 h after the induction of melanin production with or without AsA ($1.0 \mu\text{g ml}^{-1}$). Expression of *PKS1*, *SCD1*, *THR1*, *CMR1* and *G3PDH* was analysed by RT-PCR.

(Tsuji *et al.*, 2000). There are two biosynthetic enzymes before the synthesis of scytalone, namely *PKS1* and a reductase catalysing the step from 1,3,6,8-THN to scytalone (Fig. 1). AsA did not inhibit *PKS1* activity and may not inhibit the reductase since no accumulation of 1,3,6,8-THN, which was analysed as flaviolin, was observed in the culture filtrate cultured with AsA (data not shown). However, expression of *PKS1* was severely impaired by AsA. This reduction of *PKS1* expression may be very important for AsA's inhibitory effect on melanin production. It is not clear if the expression of *PKS1* is strongly regulated by *CMR1* similarly to the case of *SCD1* and *THR1* (Tsuji *et al.*, 2000). The expression pattern of the four genes caused by the addition of AsA may suggest that the regulatory system of the expression of *PKS1* is different from that of *SCD1* and *THR1*. Our results may suggest that AsA inhibits an early regulatory step prior to the expression of *PKS1* in melanin production or alters *PKS1* expression directly. Expression of *PKS1* was drastically inhibited by AsA, but it was not completely inhibited (Fig. 5). It is not clear if this effect of AsA on *PKS1* expression is enough for complete inhibition of melanin production. It may possibly be presumed that AsA also impairs other parts in the pathway of melanin production, for example, expression of a gene encoding the reductase or production of malonyl-CoA.

Since it has not been clarified how expression of *PKS1* is regulated, AsA may be useful as a probe to investigate the molecular mechanism of the regulatory system. Knowledge of this mechanism could be very important for developing new effective inhibitors of melanin production. It is not clear whether AsA has a common target in melanin production by *C. lagenarium* and aflatoxin production by *A. parasiticus*. We are now examining the effects of AsA on the biosynthetic pathways of aflatoxin and other fungal polyketides.

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