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FULL PAPER

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Accumulation of anthraquinones in the reddish brown-colored polyoxin-resistant mutants of *Cochliobolus heterostrophus*

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Abstract *Cochliobolus heterostrophus Pol2* and *Pol5* mutants are pleiotropic, and each mutant gene is responsible for alterations of both unrelated phenotypes: reddish-brown pigmentation and polyoxin resistance. The three pigments accumulated in these mutants were isolated. Structural characterization by spectroscopic analyses indicated that these three pigments were polyhydroxyanthraquinones: emodin, chrysophanol, and citreorosein. Emodin is known to be an antidote against benzimidazole fungicide, although no antidoting activity against polyoxin was observed.

Key words Anthraquinone · *Bipolaris maydis* · Chrysophanol · *Cochliobolus heterostrophus* · Emodin

Introduction

Cochliobolus heterostrophus (anamorph: *Bipolaris maydis*) is the incitant of southern corn leaf blight. Its anamorph is common, but ascocarps are rarely found in the field, although they easily develop in culture when two compatible isolates are paired under suitable conditions. This organism has been studied genetically by its laboratory resistance against polyoxin. Five genes (*Pol1*, *Pol2*, *Pol3*, *Pol4*, and *Pol5*) responsible for polyoxin resistance were identified and studied for the manner of inheritance (Gafur et al. 1998; Tanaka et al. 2002). In these mutants, *Pol2* and *Pol5* were characteristic because of their reddish-brown colony color. Each of the genes *Pol2* and *Pol5* was responsible for phenotypic alterations of unrelated phenotypes, the reddish-brown pigmentation and polyoxin resistance. In our previous study, the results of the crossing and mutation rate study suggested that each gene was pleiotropic for reddish-brown color and polyoxin resistance (Tanaka et al. 2002). In this article, we describe the results of chemical analyses of accumulated colored substance conferred by the pleiotropic polyoxin resistance gene in these mutants.

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Materials and methods

Strains and culture media

The polyoxin resistance mutants PRE058-301 (*Pol2*) and N14PO1SA1-25 (*Pol5*), wild-type strains HITO7711 and Mashiki2-2, were used in this study. The mutant strains used in this study accumulated reddish pigment in their hyphae and had a reddish-brown colony color in addition to melanin. The results of our previous crossing experiment revealed that each of the genes *Pol2* and *Pol5* was responsible for phenotypic alterations in both the reddish-brown pigmentation and polyoxin resistance and that each of the genes was independent, without linkage between them (Tanaka et al. 2002).

Minimal medium agar (MM agar; $Ca(NO_3)_2 \cdot 4H_2O$ 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, KH_2PO_4 0.4 g, K_2HPO_4 0.03 g, glucose 10 g, and agar 15 g in 1 l of distilled water) was used as a basal medium. The polyoxin used in this study is commercially available Polyoxin AL WP (Kaken Pharm., Tokyo, Japan, 10% active ingredients). It was suspended in 70% ethanol and added to MM agar at appropriate concentrations (MM pol).

General chemical procedures

Thin-layer chromatography (TLC) was carried out on Merck precoated silica gel plates (60 F254; E. Merck,

Darmstadt, FRG, layer thickness, 0.25 mm). High performance liquid chromatography (HPLC) analyses were performed on a Shimadzu LC10A system (dual LC10AS pumps), equipped with a SPD10A UV-visible detector (Shimadzu, Kyoto, Japan) and a Hitachi F-1050 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The UV–visible spectra were recorded in 99.5% ethanol on a Beckman DU-64 spectrophotometer (Beckman, Fullerton, USA). Mass spectrometry (MS) was performed on a Hitachi M-80, operated at 70 eV. ^{1}H-NMR spectra were recorded in DMSO-d_6 at 300 MHz on a Bruker AC300 spectrometer (Bruker, Rheinstetten, FRG).

Extraction and fractionation of pigments

Three liters of 3- to 5-day-old cultures of *Pol2* mutant on MM agar were chopped and extracted with the same volumes of acetone three times. The extracts were combined and concentrated to aqueous phase. The aqueous phase was saturated with NaCl, then was extracted with equal volumes of ethyl acetate three times. The combined ethyl acetate extracts were dehydrated with anhydrous sodium sulfate and concentrated. The ethyl acetate extract (1.4 g) was chromatographed in a silica gel column (Kieselgel 60, 70–230 mesh ASTM; E. Merck, Darmstadt, FRG) by successively eluting with hexane–diethyl ether (10:0, 9:1, 8:2, 6:4, 2:8, and 0:10) mixtures (42 ml each). The pigments were eluted in the following sequence: pigment 1, pigment 2, and pigment 3. Each fraction was monitored by TLC, and the fractions containing the same pigments were combined.

Antidoting effect of polyhydroxyanthraquinone against polyoxin

Polyhydroxyanthraquinones, emodin (purchased from Fluka Chemie, Buchs, Switzerland) and chrysophanol (purchased from Aldrich, Sheboygan, USA) were used for testing the antidoting effect against polyoxin. The polyhydroxyanthraquinon was dissolved in 99.5% ethanol and added to MM pol and MM agar at 5, 50, and 250 μM. The antidoting effect of polyhydroxyanthraquinone against polyoxin was evaluated by measuring changes of colonial growth and the minimum inhibitory concentration (MIC) on MM pol with or without emodin, using the plate dilution method. MIC was determined as the lowest concentration of the fungicide that produced no visible growth of the fungus. Mycelial disks (4.5 mm in diameter) were cut with a sterilized cork borer from the margin of a 1-week-old colony of HITO7711, and each disk was placed upside down on a series of media amended with a polyoxin (100–0.195 μg/ml, 1:2 dilution series). The MIC was estimated 3 days after incubation at 27°C by observing mycelial growth on the medium. Colonial growth in diameter was measured 7 days after inoculation. Each experiment was done in three replicates. Effective concentration of polyoxin for 50% inhibition of colonial growth (EC_{50}) was calculated by the computer software GRD probit (Sakuma 1998).

Results

Isolation of pigments accumulated in the reddish-brown-colored polyoxin-resistant mutants *Po2* and *Pol5*

Ethyl acetate extracts from *Pol2*, *Pol5*, and wild types were analyzed by preparative TLC developed in hexane–diethyl ether-acetic acid, 20:80:1.

The extracts from the wild types gave no visible colored spot (as detected on TLC). However, the extracts from *Pol2* and *Pol5* produced three yellow-to-orange pigment spots (pigment 1, R_F = 0.86; pigment 2, R_F = 0.66; pigment 3, R_F = 0.45). In the extract from the *Pol5* culture, the amounts of pigment 1 and pigment 3 were small. Therefore, we used the *Pol2* culture for chemical analyses of these pigments. The ethyl acetate extract was fractionated and the pigments were isolated according to the scheme described in Materials and methods. All pigments elucidated in this study were yellow in color and soluble in aqueous sodium hydroxide, giving a stable deep-colored solution. The color of the pigment solutions in 5 N NaOH turned lighter when sodium hydrosulfite was added, which is characteristic of anthraquinones or hydroxyquinones (Feigl 1960).

Pigment 1 (1,8-dihydroxy-3-methylanthraquinone; chrysophanol)

The fractions containing pigment 1 were rechromatographed on 1 g silica gel in 9:1 hexane–diethyl ether (Fig. 1a). The yellow eluate was subjected to HPLC under the following conditions: column, silica gel Waters (Milford, USA) RCM 8 × 10/Radial-pak 8SI10μ; mobile phase, 20:1 hexane–ethyl acetate; flow rate 4 ml/min; detected by 286 nm absorbance and 560 nm fluorescent emission (excitation, 430 nm). Further purification was achieved by crystallization from ethanol to give orange leaflets (0.9 mg, melting point 196°C). It gave a pale rose-pink coloration in 3% (w/v) magnesium acetate solution.

UV-Vis (ethanol): λ_{max} (log ε) = 226(4.1), 256(2.8), 278(1.4), 288(1.4), 436(1.2).
MS (70 eV) *m/z* = 254 (M^+, 100%), 225(10%), 197(10%).
^{1}H-NMR (DMSO-d_6): δ, 11.93 (2H, br s, -OH•••O- × 2), 7.81 (1H, t, *J* = 8.1 Hz, 6-H), 7.72 (1H, dd, *J* = 8.1, 1.2 Hz, 5-H), 7.56 (1H, d, *J* = 0.9 Hz, 4-H), 7.39 (1H, dd, *J* = 8.1, 1.2 Hz, 7-H), 7.23 (1H, d, *J* = 0.9 Hz, 2-H), 2.45 (3H, s, 3-Me).

The structure of this compound was also established by comparison with authentic chrysophanol obtained from Aldrich.

Pigment 2 (1,3,8-trihydroxy-6-methylanthraquinone; emodin)

The fractions containing pigment 2 were rechromatographed on 1 g silica gel in 3:1 hexane–diethyl ether (Fig. 1b). The eluate was purified by HPLC under the same con-

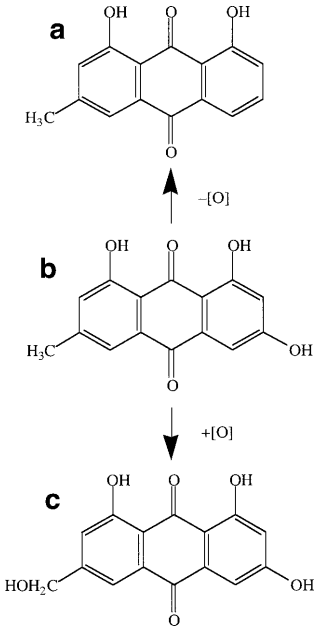

Fig. 1. Chemical structures of the anthraquinones isolated from the reddish-brown-colored polyoxin-resistant mutants: **a** chrysophanol; **b** emodin, **c** citreorosein

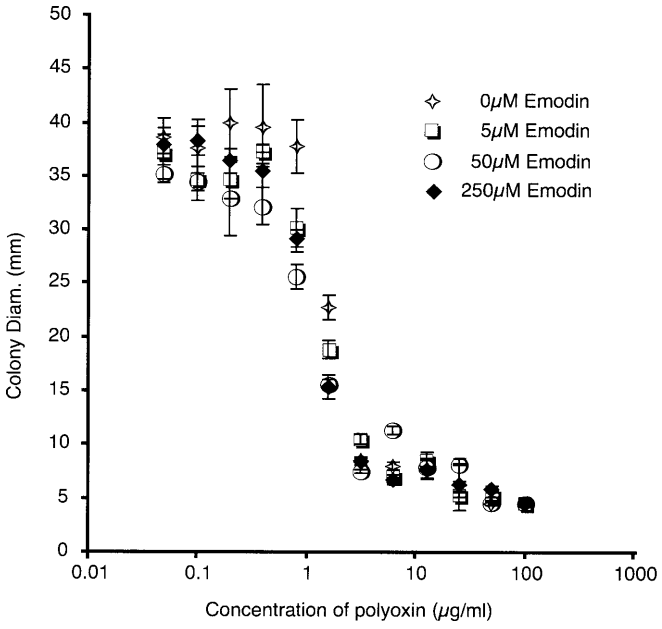

Fig. 2. Effect of emodin on colony growth of *Cochliobolus heterostrophus* HITO7711 on minimal medium agar (MM agar) amended with various concentrations of polyoxin

ditions as those already described, except for the composition of the mobile phase (3:1 hexane–diethyl ether). Pigment 2 was also recrystallized from ethanol, giving orange needles (2.4 mg, melting point 257°C). It gave an orange-red coloration in 3% (w/v) magnesium acetate solution.

UV-Vis (ethanol): $\lambda_{max}(\log\varepsilon)$ = 222(4.6), 252(4.3), 265(4.3), 289(4.3), 437(4.1).

MS (70 eV) *m/z* = 270 (M^+, 100%), 213(14%), 169(14%).

^{1}H-NMR (DMSO-d_6): δ, 12.05 (1H, s, -OH•••O-), 11.98 (1H, s, -OH•••O-), 7.44 (1H, d, *J* = 0.9 Hz, 5-H), 7.13 (1H, d, *J* = 0.9 Hz, 7-H), 7.08 (1H, d, *J* = 2.4 Hz, 4-H), 6.57 (1H, d, *J* = 2.4 Hz, 2-H), 2.40 (3H, s, 6-Me).

The structure of this compound was also established by comparison with authentic emodin obtained from Fulka Chemie.

Pigment 3 (1,3,8-trihydroxy-6-hydroxymethylanthraquinone; citreorosein)

The fractions containing pigment 3 were subjected to HPLC (mobile phase, 3:2 hexane–diethyl ether) (Fig. 1c). The yellow-colored elution after HPLC still contained a small amounts of colorless impurities when monitored by TLC. The solution was orange red in 3% (w/v) magnesium acetate.

UV-Vis (ethanol): λ_{max} = 207, 221, 253, 268, 290, 435, 448sh.

MS (70 eV) *m/z* = 286 (M^+, 100%), 257(92%), 215(21%).

Structure elucidation by NMR spectroscopy was hampered by the insufficient amount of sample. However, MS spectra indicated that this compound had a molecular weight larger than that of emodin by 16 mass units, or one oxygen atom. The fragment ion of $M^+ - 29$ (CHO) strongly suggested the presence of a hydroxymethyl group on the phenyl ring in the molecule. Pigment 3 was thus identified as citreorosein.

Table 1. Effect of emodin and chrysophanol for EC_{50} and MIC of *Cochliobolus heterostrophus* HITO7711 against polyoxin

Concentration (μM)	EC_{50}(μg/ml)	LL	UL	MIC(μg/ml)
Emodin				
0	1.58	0.88	2.76	12.5
5	1.40	0.80	2.46	12.5
50	1.67	0.97	2.80	12.5
250	1.29	0.73	2.26	12.5
Chrysophanol				
0	1.31	0.71	2.30	12.5
5	1.57	0.88	2.75	12.5
50	1.43	0.82	2.31	12.5
250	1.62	0.80	2.93	12.5

EC_{50}, effective concentration of polyoxin for 50% inhibition of colony growth; LL, lower limit of 95% confidence interval for the EC_{50}; UL, upper limit of 95% confidence interval for the EC_{50}; MIC, minimum inhibitory concentration

Antidoting effect of emodin against polyoxin

The antidotal properties of emodin and chrysophanol against polyoxin were assayed by measuring changes of MIC and EC_{50} of a wild type in the presence of emodin or chrysophanol. The MIC and calculated EC_{50} in each test were not significantly different (Table 1). Figure 2 shows the effect of exogenous emodin on the colony growth of the wild-type HITO7711 on MM with various concentration of polyoxin. In this experiment, growth of the mycelia of the wild type was not inhibited by any concentration of emodin. Antidoting effects of emodin and chrysophanol against polyoxin were not observed.

Discussion

Cochliobolus heterostrophus Pol2 and *Pol5* mutants accumulated emodin, chrysophanol, and citreorosein in the cultures. These three anthraquinones are structurally related. Emodin is considered to be biosynthesized via octaketide, as a key precursor in the fungal polyhydroxyanthraquinone biosynthetic pathway (Torssell 1997). Chrysophanol and citreorosein would be synthesized by deoxygenation and oxidation of emodin, respectively (Anderson et al. 1990; Müller et al. 1998). These anthraquinones are rather widely observed in fungi (Turner 1971; Turner and Aldridge 1983). However, in *Cochliobolus* spp., there is no previous report of the production of emodin, chrysophanol, or citreorosein. Other types of polyhydroxyanthraquinones, cynodontin (1,4,5,8-tetrahydroxy-2-methylanthraquinone) and helminthosporin (1,4,8-trihydroxy-6-methylanthraquinone), have been reported from *C. sativus*, *C. miyabeanus, C. victoriae*, and *C. cynodontis* (Engström et al. 1993; Johnson and White 1969; Pringle 1958; Raistrick et al. 1933). No emodin or other anthraquinone was detected in our strains of *C. heterostrophus* wild type.

Cochliobolus spp. have an ability to produce emodin. In *C. sativus*, *C. miyabeanus, C. victoriae*, and *C. cynodontis*, emodin would be converted into cynodontin and helminthosporin. In the case of wild-type strains of *C. heterostrophus*, production of anthraquinones is likely to be genetically controlled and to be suppressed under our experimental conditions, although *Cochliobolus* spp. essentially have the ability to produce anthraquinones. It is also likely that anthraquinones are actually biosynthesized but are immediately metabolized to colorless compounds, e.g., benzophenone (Curtis et al. 1970). In the former case, the mutant genes *Pol2* and *Pol5* would be involved in control of polyketide synthase for anthraquinones. In the latter case, mutant genes may encode enzymes that can convert anthraquinones to benzophenones, or may be involved in the regulation of these enzymes.

Anthraquinones are known to exhibit a variety of biological activities, as in the case of antibiotics (Fujikawa et al. 1953), mutagens (Brown and Dietrich 1979), and inhibitors of protein tyrosine kinase regulating cell division and tumorigenesis (Chang and Geahlen 1992). In addition to these biological effects, emodin has an antidoting activity against benzimidazole fungicides (Tahara et al. 1993), and an inhibitory activity for uridine and tymidine incorporation (Anke et al. 1980). Polyoxin is a fungicide having nucleosidepeptide structure with uracil or its derivatives (Isono et al. 1969). We assumed that emodin and related polyhydroxyanthraquinones would be also antidotes against polyoxin. However, neither exogeneous emodin nor chrysophanol showed an antidoting effect against polyoxin in our experiment. This result indicates that polyoxin resistance in these mutants was not due to an accumulation of anthraquinones in the cultures. The resistance mechanism in these mutants is still unclear.

Studies on the genetically deficient steps in *Pol2* and *Pol5* are currently in progress. Further elucidation of the molecular genetic and biochemical nature of *Pol2* and *Pol5* should lead to better understanding of the resistance mechanism of these pleitropic mutants.

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