Genetic analyses of *Cochliobolus heterostrophus* albino mutant with deficiencies at two loci

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A new class of albino mutant of *Cochliobolus heterostrophus* was isolated. Its colony color was indistinguishable from that of albino mutants previously reported. Application of the melanin intermediate scytalone induced this mutant to pigment slightly, but not completely. Genetic analyses showed that the mutant had two deficient genes. When only one of these genes was deficient, the colony color was indistinguishable from the wild type, whereas deficiency of both genes resulted in the albino phenotype. These deficiencies lie upstream of scytalone biosynthesis. These genes were designated as *Cal1* and *Cal2*.

Key Words——albino mutant; *Cochliobolus heterostrophus*; genetics; melanin.

Melanin is the most widespread of pigments produced by fungi. In *Agaricus bisporus* (Lange) Singer, it is synthesized via γ-glutaminyl hydroxybenzene and its related compounds; In *Aspergillus nidulans* (Eidam) Winter via N-acetyl-6-hydroxytriptophane; and in *Verticillium dahliae* Klebahn via 1,8-dihydroxynaphthalene (DHN; DHN-melanin; reviewed by Bell and Wheeler, 1986). Importance of melanin to fungal survival has been recognized for some time. Studies in recent years have revealed relationship between melanin synthesis in plant pathogenic fungi and their pathogenicity. In *Colletotrichum lagenarium* (Pass.) Ellis et Halsted or *Pyricularia oryzae* Cavara, for example, fungicides prevent penetration of fungi into the plant tissue by inhibiting melanin synthesis in their appressoria (Woloshuk et al., 1980; Kubo et al., 1985). In *Alternaria eichhorniae* Nag Raj et Ponnappa, some phytotoxin was discovered as a shunt product of melanin biosynthesis (Robeson et al., 1984).

Cochliobolus heterostrophus (Drechs.) Drechs. was reported to produce DHN-melanin, the same melanin produced by *V. dahliae*. It was also shown to possess a unique novel gene, *Alb3*, which has not been found in other fungi (Tanaka et al., 1991). The colony color of the mutant is albino, but melanin synthesis is not complemented by application of the melanin intermediate scytalone. DHN-melanin has been studied in a number of fungi including *V. dahliae*, but details of how melanin is controlled or synthesized remain unclear. It is still necessary to find unidentified genes such as *Alb3*.

This report describes the genetic analysis of a melanin-deficient mutant, identifies melanin synthesis-related genes in *C. heterostrophus* that have not hitherto been reported in fungi, and proposes a modified melanin biosynthesis pathway in *C. heterostrophus*.

Materials and Methods

Fungal strains The strains of *C. heterostrophus* used in this study are shown in Table 1. The laboratory stock strain HITO7711 (*MAT1-2*) was used as the wild-type strain in mutagenesis. The laboratory stock strain Mashiki2-2 (*MAT1-1*) was also used as the wild-type strain in crossing experiments. These fungi were stock cultures of the Pesticide Research Institute (PRI), Kyoto University. Determination of mating types of these strains was reported previously (Tanaka et al., 1991).

Mutagenesis and isolation of melanin deficient mutants The mutagen used was ethyl methanesulphonate. Mutagenesis and isolation of mutants were described by Tanaka et al. (1988).

Crossing and ascospore analyses Crossings were made on Sach's agar medium with rice-straw as described by Ueyama and Tsuda (1975). Ascospore analyses were carried out according to the method of Taga et al. (1978). Ascospore-isolates were cultured on complete agar medium (CM; Ca(NO$_3$)$_2$·4H$_2$O 1.5 g, MgSO$_4$·7H$_2$O 0.5 g, KCl 0.5 g, KH$_2$PO$_4$ 0.4 g, K$_2$HPO$_4$ 30 mg, tryptone 1 g, yeast extract 1 g, glucose 10 g, and agar 15 g dissolved in 1 L of distilled water and autoclaved before use), and their genotypes were determined from their colony color or by application of the melanin intermediate, scytalone.

Melanin synthesis inhibitor Chlobenthiazone and tricyclazole were used as inhibitors for melanization. Chlobenthiazone was dissolved in acetone 40 mg/ml, and tricyclazole in ethanol 5 mg/ml. They were mixed into media at suitable concentrations.

Results

Isolation and characterization of albino mutants The colony color of the wild-type HITO7711 is dark green.

Table 1. Fungal strains used in this study.

Strain	Colony color	Genotype	Origin
HITO7711	Dark green	Wild-type	Laboratory stock strain
Mashiki2-2	Dark green	Wild-type	Laboratory stock strain
803-18	White	Cal1 Cal2	Mutant of HITO7711
818-3	Brown	Brn1	Mutant of HITO7711
823-1	Salmon	Sal1	Mutant of HITO7711
SA1R14	Brown	Brn1	Progeny of 818-3 × Mashiki2-2
SA1AS1-2	Brown	Brn1	Progeny of 818-3 × Mashiki2-2
A7	White	Cal1 Cal2	Progeny of 803-18 × Mashiki2-2
SC1AS1-2	White	Cal1 Cal2	Progeny of A7 × Mashiki2-2
SC1AS2-1	Dark green	Cal1	Progeny of A7 × Mashiki2-2
SC1AS2-3	Dark green	Cal2	Progeny of A7 × Mashiki2-2

After mutagenesis, 12 albino isolates were obtained. One of them, strain 803-18, exhibited a colony color on CM resembling that of our previously reported albino mutants, *Alb1* and *Alb3* (Tanaka et al., 1991). The reaction on application of scytalone was different: the *Alb1* mutant expressed dark green coloration, which was like the colony color of the wild type, but the *Alb3* mutant did not; and isolate 803-18 showed slight pigmentation, of much lower intensity than that of the *Alb1* mutant.

Genetic analyses of the mutant Crossing between the albino mutant 803-18 and the wild-type strain Mashiki2-2, yielded 64 offspring. Of these, 50 were dark green, the remaining 14 were white, indicating that the segregation ratio of dark green (wild type; WT) : white (mutant type; MT) is 3 : 1. The characters of the white offsprings were determined using the same strategy outlined above, and were the same as those of the parental strain of 803-18. Some of the white offsprings were back-crossed to Mashiki2-2. Again, the segregation ratio WT : MT was 3 : 1.

Results of crossing between dark green offspring obtained from A7 (one of white offsprings of 803-18 × Mashiki2-2) × Mashiki2-2 are shown in Table 2. Allthough both SC1AS2-1 and SC1AS2-3 were the same dark green as the wild type, white offsprings were obtained, and the segregation ratio of WT to MT was 3 to 1. The crossing of SC1AS2-3 × A7 gave the segragation ra-

Table 2. Results of crosses between dark green offspring obtained from A7 and Mashiki2-2.

Strains tested	Number of offspring		
	MT[a]	WT[a]	χ^2 [b]
SC1AS2-1 × SC1AS2-3	5	19	0.056[c]
SC1AS1-5 × SC1AS3-6	25	44	4.06[d]
SC1AS3-4 × SC1AS1-1	0	16	*

a) Abbreviations: MT, mutant type; WT, wild type.
b) χ^2 values were calculated on the null hypothesis that the segregation ratio was 1 : 3 and corrected for Yate's continuity. The probarbility (P) of obtaining the χ^2 value shown by chance is shown as follows: c) $P>0.05$; d) $0.05>P>0.01$. * apparent departure from 1 : 3.

Table 3. Result of the cross SC1AS1-2 (*Cal1 Cal2*) × 823-1 (*Sal1*).

Colony color	Number of offspring[a]
Dark green	14
Salmon	15
White*	5
White**	6

a) χ^2(3 : 3 : 1 : 1)=0.12. The probability of obtaining the χ^2 value is more than 0.05. Reaction of the white offspring by co-inoculation with *Sal1* mutant was similar to *Cal1 Cal2* mutant*, or unlike *Cal1 Cal2*, which showed no change of colony color**.

Table 4. Result of cross SC1AS1-2(*Cal1 Cal2*) × 818-3 (*Brn1*).

Colony color	Number of offspring[a]
Dark green	27
Brown	18
White	34

a) χ^2(3 : 2 : 3)=0.74. The probability of obtaining the χ^2 value is more than 0.05.

Table 5. Result of cross SC1AS2-1(*Cal1*) × SA4R14 (*Brn1*).

Colony color	Number of offspring[a]
Dark green	75
Brown	62
White	31

a) χ^2(2 : 1 : 1)=12.5. The probability (P) of obtaining the χ^2 value is $0.01>P$.

Table 6. Result of cross SC1AS2-3 (*Cal2*) × SA4AS1-2 (*Brn1*).

Colony color	Number of offspring[a]
Dark green	23
Brown	23

a) χ^2(1 : 1)=0.00. The probability of obtaining the χ^2 value is more than 0.05.

tio of 1 WT to 1 MT. Consequently, phenotypes of the mutant 803-18 and the MT offsprings obtained from these crossings had mutations at two genes. These genes were designated as *Cal1* and *Cal 2*: SC1AS2-1 has *Cal1* and SC1AS2-3 has *Cal2*.

Crossing between SC1AS1-2(*Cal1 Cal2*) and 823-1(*Sal1*) was performed, and the results are shown in Table 3. In this cross, recombinant *Sal1 Cal1 Cal2* was obtained. This recombinant showed a white colony, and the result suggests epistasis of *Cal1 Cal2* to *Sal1*. Co-inoculation of *Cal1 Cal2* and *Sal1* showed that *Cal1 Cal2* was epistatic to *Sal1*. Table 4 shows the results of the cross between SC1AS1-2 and *Brn1* mutant 818-3. If albinism caused by *Cal1* and *Cal2* is epistatic to *Brn1*, and *Brn1*, *Cal1*, and *Cal2* have no linkage to one another, the segragation ratio of albino : wild type : brown should be 2 : 3 : 3. However, the results obtained here were far from this hypothesis. Therefore, SC1AS2-1(*Cal1*) and SC1AS2-3(*Cal2*) were crossed with the *Brn1* mutants for further analyses. The cross of *Cal1* mutant × *Brn1* mutant gave albino offsprings (Table 5), but the results did not so in the cross of *Cal2* × *Brn1* did not (Table 6).

Melanin synthesis inhibitor tests Wild type, *Cal1* mutant, and *Cal2* mutant were inoculated onto media containing melanin synthesis inhibitors, triscyclazole and chlobenthiazone. Tricyclazole inhibited melanization at 1.25 μg/ml, and at higher concentrations of over 400 μg/ml, it stopped the growth of *C. heterostrophus*. Chlobenthiazone inhibited melanization at 5 μg/ml, and it inhibited growth at 80 μg/ml. When tricyclazole was added into the media at 160 μg/ml, and chlobenthiazone at 30 μg/ml, the pigmentation of each strain was different. While the wild type and *Cal2* mutant showed brown pig-

mentation, the *Cal1* mutant did not pigment but showed an albino colony (Fig. 1).

Discussion

In this study, we isolated a new type of albino mutant. Various albino mutants have already been reported in *C. hetrostrophus*, but this mutant was different in certain features. Its albinism was the result of conjunction of deficiencies in two genes. When either one of these genes is deficient, the phenotype resembles that of the wild type; however, when both genes are deficient, albinism appears. Albino mutants have also been isolated in *Alternaria alternata* (Fr.) Keissler, *C. lagenarium, P. oryzae*, and other species (Woloshuk et al., 1980; Kubo et al., 1985; Tanabe et al., 1988). These mutants have all been considered to have a single deficient gene. Enzymatic activities in some steps of melanization were recognized, including scytalone dehydration and DHN oxidation (Tanaka et al., 1992), but these were not studied in the step of scytalone synthesis, because it was not clear how scytalone was biosynthesized in *C. heterostrophus*. Moreover, the albino mutant we isolated in this study is different from any albino mutant reported before (Tanaka et al., 1991). We designated the mutated genes *Cal1* and *Cal2* from components of albinism. In this mutant, melanin biosynthesis was stopped upstream of scytalone, an intermediate of naphthalenediol melanin considered to be synthesized from acetate via the pentaketo acid, tetrahydroxynaphthalene (4HN; Bell and Wheeler, 1986). *Cal1* and *Cal2* function in one such pathway.

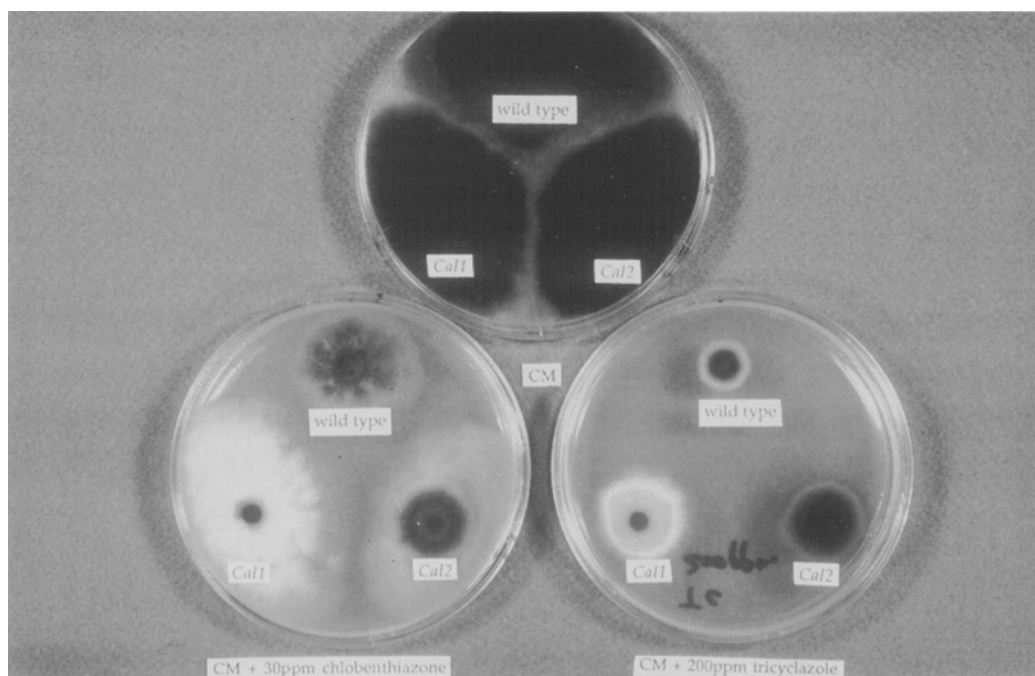The expected ratio of albino offspring from the cross

Fig. 1. Coloration of *Cal1* and *Cal2* mutants on the medium containing tricyclazole (200 μg/ml) and chlobenthiazone (30 μg/ml).

between *Brn1* and *Cal1 Cal2* is 1/4 based on the hypothesis that both *Brn1 Cal1* and *Brn1 Cal2* are the same as, or very difficult to distinguish from, *Brn1*. In this hypothesis, the ratio WT : brown : albino = 3 : 3 : 2 was expected, but the χ^2 value derived from the cross of *Cal1 Cal2 × Brn1* was 14.8, far from the hypothesis. These results suggested that either *Brn1 Cal1* or *Brn1 Cal2* is albino. Crossings between *Brn1* and *Cal1* or *Cal2* were conducted to confirm which genotype shows the albino phenotype, *Brn1 Cal1* or *Brn1 Cal2*. Cossing between *Brn1* and *Cal2* gave WT and brown offspring in a ratio of 1 : 1, whereas *Brn1* and *Cal1* gave 31 albino, 62 brown, and 75 WT (Table 5). These results suggest that melanin synthesis may stop upstream of scytalone when *Cal1* and *Brn1* are both present in *C. heterostrophus*, that *Brn1* and *Cal1* have some linkage to each other, and that the rate of recombinant production is approximately 32.5-43.5%. The location of these genes must be studied in much more detail.

In *C. heterostrophus* melanin was considered to be synthesized via pentaketide, scytalone, trihydroxynaphthalene (THN), vermelone, and DHN (Tanaka et al., 1991). In *V. dahliae*, *chem-2* and *chem-4* mutants have deficiencies in the conversions of 4HN to scytalone and THN to vermelone (Bell and Wheeler, 1986). These steps are both related to reduction. Their report shows

that *V. dahliae* has 4HN as precursor of scytalone. On the other hand, previous studies on melanin biosynthesis in *C. heterostrophus* have not referred to whether it produces 4HN or not, because among melanin-deficient mutants of this fungus, Sal1 and Brn1, *Sal1* is epistatic to *Brn1* (Tanaka et al., 1991), and no mutant which accumulates 4HN has been isolated. As we isolated a *Cal1 Cal2* mutant, it was suggested that *C. hetrostrophus* produces 4HN as an intermediary metabolite of melanin. Therefore, in *C. heterostrophus*, there may be two different pathways from pentaketide to scytalone.

Both tricyclazole and chlobenthiazone inhibit melanin synthesis in the reduction step by inhibiting the reductase activity (Bell and Wheeler, 1986). When these compounds were applied to WT and MT strains of *C. heterostrophus*, the *Cal1* mutant showed a white colony, while WT and the *Cal2* mutant showed a WT colony. This suggests that *Cal1* was affected by these melanin inhibitors and that it encodes a reduction-related function.

Figure 2 shows the scheme of melanin biosynthesis suggested by this study. It includes two pathways, one in which pentaketides circularized directly to scytalone, and one in which pentaketide is circularized to 4HN, then reduced to scytalone. Sankawa et al. (1977) showed that pentaketide would be circularized to both 4HN and scytalone. Thus, there may be several pathways from pentaketide to scytalone in fungi, including *Cochliobolus*, in which melanin is synthesized from DHN. Further study on melanin intermediates and shunt products should lead to better understanding of melanin biosynthesis in *C. heterostrophus*.
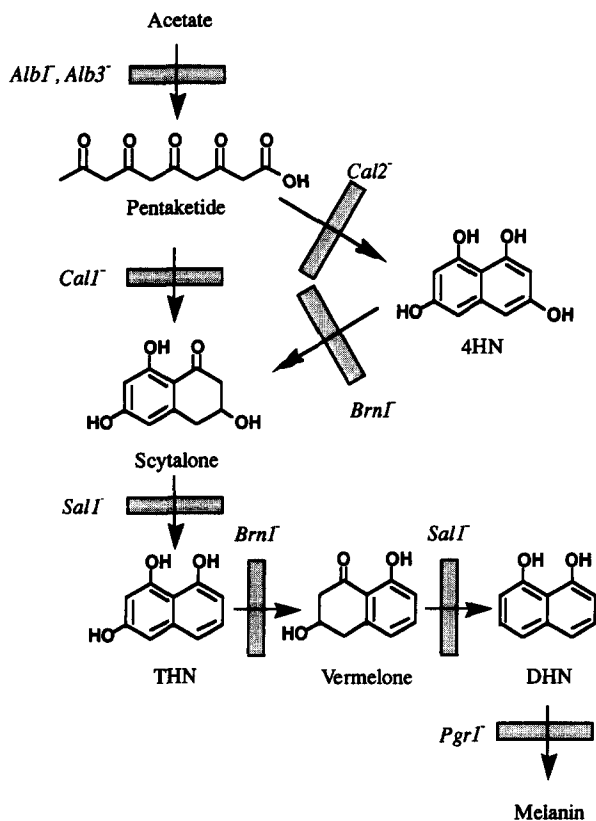

Fig. 2. Putative pathway of melanin biosynthesis in *C. heterostrophus*.
Italic symbols and hatched lines indicate the suggested genetic-deficient steps for the respective mutant genes.

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