The Melanin Biosynthesis Genes of *Alternaria alternata* Can Restore Pathogenicity of the Melanin-Deficient Mutants of *Magnaporthe grisea*

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The phytopathogenic fungi *Magnaporthe grisea* and *Alternaria alternata* produce melanin via the polyketide biosynthesis, and both fungi form melanized colonies. However, the site of melanin deposition and the role of melanin in pathogenicity differ between these two fungi. *M. grisea* accumulates melanin in appressoria, and their melanization is essential for host penetration. On the other hand, *A. alternata* produces colorless appressoria, and melanin is not relevant to host penetration. We examined whether the melanin biosynthesis genes of *A. alternata* could complement the melanin-deficient mutations of *M. grisea*. Melanin-deficient, nonpathogenic mutants of *M. grisea*, albino (*Alb*⁻), rosy (*Rsy*⁻), and buff (*Buf*⁻), were successfully transformed with a cosmid clone pMBR1 that carries melanin biosynthesis genes *ALM*, *BRM1*, and *BRM2* of *A. alternata*. This transformation restored the melanin synthesis of the *Alb*⁻ and *Buf*⁻ mutants, but not that of the *Rsy*⁻ mutant. The melanin-restored transformants regained mycelial melanization, appressorium melanization, and pathogenicity to rice. Further, transformation of *Alb*⁻ and *Buf*⁻ mutants with subcloned *ALM* and *BRM2* genes, respectively, also produced melanin-restored transformants. These results indicate that the *Alternaria* genes *ALM* and *BRM2* can restore pathogenicity to the mutants *Alb*⁻ and *Buf*⁻, respectively, due to their function during appressorium development in *M. grisea*.

Additional keywords: conidial melanization, conidiation, host-specific toxin, the Japanese pear pathotype of *A. alternata*.

Many fungi produce a distinctive dark brown or black pigment, melanin, via polyketide biosynthesis, the last step of

which is the polymerization of 1,8-dihydroxynaphthalene (DHN) (Fig. 1A) (Bell and Wheeler 1986). The biosynthetic pathway of DHN melanin was first characterized in *Verticillium dahliae* by using melanin-deficient mutants (Bell et al. 1976; Stipanovic and Bell 1976). In general, melanin accumulates in fungal cell walls and has been considered to confer tolerance to environmental stresses, such as UV radiation (Durrell 1964; Zhdanova and Pokhodenko 1974; Wang and Casadevall 1994), microbial lysis (Lockwood 1960; Bloomfield and Alexander 1967; Kuo and Alexander 1967; Hyakumachi et al. 1987), and defense responses of host plants and animals against fungal infection (Aver'yanov et al. 1989; Dzhavakhiya et al. 1990; Jacobson et al. 1995).

The imperfect fungus *Alternaria alternata* is generally a ubiquitous saprophytic fungus. In addition, *A. alternata* contains seven phytopathogenic variants, designated pathotypes, that cause severe diseases on different plants by producing

Fig. 1. Melanin biosynthetic pathway in filamentous fungi and the melanin biosynthesis gene cluster of *Alternaria alternata*. **A**, The steps controlled by the *A. alternata* genes *ALM*, *BRM1*, and *BRM2* are denoted by thick arrows (Kimura and Tsuge 1993). Corresponding genes identified in *Magnaporthe grisea* are shown in parentheses (Chumley and Valent 1990). 1,3,6,8-THN = 1,3,6,8-tetrahydroxynaphthalene; 1,3,8-THN = 1,3,8-trihydroxynaphthalene; DHN = 1,8-dihydroxynaphthalene. **B**, Restriction endonuclease map of the 40-kb region containing the *ALM*, *BRM1*, and *BRM2* genes. Arrows indicate direction of transcription and maximum sizes of the gene transcription units (Kimura and Tsuge 1993). Cosmid pMBR1 and plasmids pALM-1 and pBRM2-1 were used for transformation of melanin-deficient mutants of *M. grisea*. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I.

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host-specific toxins (Nishimura and Kohmoto 1983; Kusaba and Tsuge 1995). *A. alternata* produces DHN melanin, which accumulates in the cell walls of conidia and hyphae (Wheeler 1983; Tanabe et al. 1995). We have previously reported the isolation of the gene cluster involved in melanin biosynthesis of the Japanese pear pathotype of *A. alternata* (Kimura and Tsuge 1993), which causes black spot of Japanese pear by producing host-specific AK-toxin (Tanaka 1933; Nakashima et al. 1985; Otani et al. 1985). A genomic cosmid library of a wild-type strain of this pathogen was screened for the presence of genes required for melanin biosynthesis by introducing DNA of the library into the albino (*Alm*⁻), light brown (*Brm1*⁻), and brown (*Brm2*⁻) mutants (Kimura and Tsuge 1993; Tanabe et al. 1995). A cosmid clone, named pMBR1, was found to restore melanin synthesis to all three mutants, and the genes complementing each of the three mutations were designated *ALM*, *BRM1*, and *BRM2*, respectively (Fig. 1B) (Kimura and Tsuge 1993). These genes are now considered to code for the enzymes controlling the respective steps in the melanin biosynthetic pathway shown in Figure 1 (Kimura and Tsuge 1993; Tanabe et al. 1995).

Chumley and Valent (1990) isolated three classes of melanin-deficient mutants of the rice blast pathogen *M. grisea*, albino (*Alb*⁻), rosy (*Rsy*⁻), and buff (*Buf*⁻), and showed that the three mutant phenotypes are due to defects of single genes, designated *ALB*, *RSY*, and *BUF*, at unlinked loci. Based on comparison of pigmentation phenotypes of mutants, *ALM*, *BRM1*, and *BRM2* of *A. alternata* can be considered to have functions identical with those of *ALB*, *RSY*, and *BUF* of *M. grisea*, respectively, in melanin biosynthesis (Fig. 1A) (Chumley and Valent 1990; Kimura and Tsuge 1993).

Melanin-deficient mutants and the parent strain of the Japanese pear pathotype of *A. alternata* were found to develop approximately the same numbers of similar-size lesions on susceptible pear leaves in laboratory tests, indicating that the ability to produce melanin is probably not relevant to the pathogenicity of this pathogen (Tanabe et al. 1995). In contrast, melanin production is known to be an essential factor for pathogenicity in *M. grisea* (Woloshuk et al. 1980; Chumley and Valent 1990). Both *M. grisea* and *A. alternata* produce melanin during vegetative growth, resulting in melanized colonies, but the melanization sites and the roles of melanin seem to differ. The *Alternaria* spp. produce darkly melanized conidia, while the *Magnaporthe* conidia are colorless. Conidial melanization in *A. alternata* is considered to play an important role in longevity and survival of conidia in nature (Rotem 1994). On the other hand, appressoria, differentiated forms at the tips of germ tubes with a spherical or dome shape, of *M. grisea* are darkly melanized, while the *Alternaria* appressoria are colorless. Many pathogenic fungi penetrate host cells by elaborating infection hypha from appressoria (Staples and Hoch 1987; Mendgen and Deising 1993). Appressorium melanization of *M. grisea* is implicated as an essential step for the successful penetration of the host cell (Woloshuk et al. 1983; Howard and Ferrari 1989; Chumley and Valent 1990; Howard et al. 1991). Howard and Ferrari (1989) provided convincing evidence that DHN melanin mediates the buildup of hydrostatic pressure in the appressorium and that this high pressure provides the essential driving force for a mechanical penetration component. Similar roles of melanin have been reported in *Colletotrichum lagenarium*

(Kubo et al. 1982) and *C. lindemuthianum* (Wolkow et al. 1983). These facts suggest a diversity of roles for melanin and the regulation mechanisms of melanin biosynthesis genes in morphogenesis and pathogenicity among fungi.

We are interested in the similarity and diversity of the regulation mechanisms and roles of melanin genes in the morphogenesis, pathogenicity, and fitness among phytopathogenic fungi. In this study, we isolated three classes of melanin-deficient mutant of *M. grisea* and introduced the *Alternaria* melanin synthesis genes into the *M. grisea* mutants. The transformants were examined for melanin production during vegetative growth and morphogenesis, and also for pathogenicity, to determine whether the introduced melanin synthesis genes of *A. alternata* functionally displace the deficient genes of *M. grisea*.

RESULTS

Homology of the melanin biosynthesis genes between *A. alternata* and *M. grisea*.

Melanin biosynthetic pathways of *A. alternata* and *M. grisea* are considered to be identical (Woloshuk et al. 1980; Wheeler 1983; Chumley and Valent 1990; Tanabe et al. 1995). To deduce the nucleotide sequence homology in the melanin biosynthesis genes between *A. alternata* and *M. grisea*, DNA of *M. grisea* strains was hybridized with the *A. alternata* melanin genes. Southern blots of total cellular DNA from five strains of *M. grisea*, digested with *Eco*RI, were probed with the *ALM*, *BRM1*, and *BRM2* sequences of *A. alternata*. The wash of hybridized blots was conducted under relatively low stringency condition with 2× SSPE containing 0.1% sodium dodecyl sulfate at 50°C. These probes did not hybridize (data not shown), suggesting that the degree of nucleotide sequence homology between melanin biosynthesis genes is relatively low for these two fungi.

Isolation of melanin-deficient mutants of *M. grisea*.

Conidia of *M. grisea* strain Ken60-19, which produces dark gray or black colonies due to melanin accumulation, were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Three types of phenotypic mutants of this strain were selected, based on colony color on potato sucrose agar (PSA) medium: albino (*Alb*⁻), rosy (*Rsy*⁻), and buff (*Buf*⁻). Three *Alb*⁻, one *Rsy*⁻, and three *Buf*⁻ mutants were detected from about 1,200 colonies derived from MNNG-treated conidia. Among these seven mutants, three, designated MNK91-1 (*Alb*⁻), MNK91-2 (*Rsy*⁻), and MNK91-3 (*Buf*⁻), were used in this study (Table 1). Appressoria of MNK91-1 and MNK91-2 were hyaline, and those of MNK91-3 were lightly pigmented (Table 1). The

Table 1. Melanin-deficient mutants of *Magnaporthe grisea*

Strain	Phenotype ^a	Mycelial color ^b	Appressorium pigmentation ^c	Pathogenicity ^d
Ken60-19	Wild type	Black	Dark	+
MNK91-1	<i>Alb</i> ⁻	Albino	Hyaline	-
MNK91-2	<i>Rsy</i> ⁻	Rosy	Hyaline	-
MNK91-3	<i>Buf</i> ⁻	Buff	Light	-

^a Phenotype nomenclature followed that of Chumley and Valent (1990).

^b Mycelial color was observed on potato sucrose agar.

^c Appressorium pigmentation was observed on Teflon membranes.

^d Pathogenicity was assayed by spray inoculation of conidial suspension on rice seedlings of compatible cultivar Aichi-Asahi. +, pathogenic; -, nonpathogenic.

MNK91-1 and MNK91-2 were clearly distinguished, because MNK91-2 was verified to accumulate scytalone in the culture (about 40 µg/ml).

The pathogenicity of these mutants was examined by spray inoculations of conidial suspensions on rice seedlings of compatible cultivar Aichi-Asahi. The parent strain caused many spreading lesions on leaves, but melanin-deficient mutants caused no lesions (Table 1). These results were consistent with the data reported previously (Woloshuk et al. 1980, 1983; Chumley and Valent 1990).

Transformation of the *Magnaporthe* melanin-deficient mutants with the *Alternaria* melanin genes.

The melanin-deficient mutants of *M. grisea* were transformed with the cosmid pMBR1 containing the *A. alternata* genes *ALM*, *BRM1*, and *BRM2*, to examine whether these *A. alternata* melanin genes could complement the melanin-deficient mutations of *M. grisea*. The successful transformants were confirmed by hygromycin B resistance (Hyg^r), and melanin restoration of these transformants was examined based on colony melanization on PSA plate. About 32 and 50% of Hyg^r transformants from the Alb⁻ mutant MNK91-1 and the Buf⁻ mutant MNK91-3, respectively, formed melanized colonies on PSA (Table 2). Colony melanization of the transformants tended to be delayed and slightly lighter than that of the wild-type strain. In contrast, none of 60 Hyg^r transformants of the Rsy⁻ mutant MNK91-2 regained melanin production (Table 2). These results suggest that the *A. alternata* genes *ALM* and *BRM2* can complement the *M. grisea* *alb* and *buf* mutations, respectively, but that the *BRM1* gene cannot complement the *rsy* mutation.

Further, each *ALM* and *BRM2* gene region in the *Alternaria* cosmid pMBR1 was subcloned into the transformation vector pSH75, and subclones, named pALM-1 and pBRM2-1, were introduced into Alb⁻ and Buf⁻ mutants of *M. grisea*, respectively. Among the Hyg^r transformants, melanin-restored transformants appeared at frequency rates of 10.8 and 24.5% from Alb⁻ and Buf⁻ mutants, respectively (Table 2).

Hybridization and expression analyses of the *A. alternata* melanin genes in *M. grisea* transformants.

The melanin-restored transformants from Alb⁻ and Buf⁻ mutants with the *A. alternata* cosmid pMBR1 were designated TAR and TBR series, respectively. The integrations of the *Alternaria* genes into the chromosome of the transformants were confirmed by Southern blot analysis. Total DNA, extracted from

the parent strain Ken60-19, the Alb⁻ mutant MNK91-1, and five TAR series transformants, was digested with *Eco*RI or *Sal*I, and the blots were hybridized with the *ALM* probe. The probe hybridized to DNA of all the melanin-restored transformants, but not to Ken60-19 or MNK91-1 DNA (Fig. 2A). TAR series transformants shared a 9.4-kb *Eco*RI fragment and a 6.2-kb *Sal*I fragment, showing the presence of the full-length *ALM* gene in the transformant genome. Total DNA, extracted from the strain Ken60-19, the Buf⁻ mutant MNK91-3, and five TBR series transformants, was digested with *Eco*RI or *Pst*I, and the blots were hybridized with the *BRM2* probe. The probe hybridized to DNA of all the transformants, but not to Ken60-19 and MNK91-3 DNA (Fig. 2B). An 8.5-kb *Eco*RI fragment and a 4.5-kb *Pst*I fragment were detected from all TBR series transformants, and the presence of the full-length *BRM2* gene in the transformant genome was also identified.

Frequency of appearance of melanin-restored transformants from MNK91-1 or MNK91-3 was relatively low (Table 2). When MNK91-1 was transformed with pALM-1, only 10.8% of

Fig. 2. Southern blot analysis of the melanin-restored transformants of *Magnaporthe grisea*. Total DNA (1 µg/lane) was digested with a restriction enzyme, electrophoresed in 0.8% agarose gel, transferred to nylon membranes, and probed. **A**, DNA from wild-type strain Ken60-19 (lane 1), the Alb⁻ mutant MNK91-1 (lane 2) and the melanin-restored transformants TAR-1 to TAR-5 (lanes 3 to 7) was probed with the 9.4-kb *Eco*RI fragment of pMBE1 containing the *ALM* sequence. **B**, DNA from wild-type strain Ken60-19 (lane 1), the Buf⁻ mutant MNK91-3 (lane 2) and the melanin-restored transformants TBR-1 to TBR-5 (lanes 3 to 7) was probed with the 4.2-kb *Eco*RI-*Pst*I fragment of pMBEP10 containing the *BRM2* sequence. The sizes (in kilobases) of marker DNA fragments (*Hind*III-digested λ DNA) are indicated on the left.

Table 2. Genetic complementation of the melanin-deficient mutations of *Magnaporthe grisea* with the melanin biosynthesis genes of *Alternaria alternata*

Strain	Transforming DNA ^a	Transformants (no.) ^b		Mel ⁺ transformants (%)
		Mel ⁺	Mel ⁻	
MNK91-1 (Alb ⁻)	pMBR1	13	28	31.7
	pALM-1	4	33	10.8
MNK91-2 (Rsy ⁻)	pMBR1	0	60	0.0
MNK91-3 (Buf ⁻)	pMBR1	20	20	50.0
	pBRM2-1	23	71	24.5

^a See Figure 1B.

^b Each value represents the number of Mel⁺ (melanin-restored) and Mel⁻ (melanin-unrestored) transformants.

transformants restored the melanin production (Table 2). Southern blot analysis of integration manner of transforming DNA in the melanin-unrestored pALM-1 transformants indicated that most of these transformants did not contain the full length of the *ALM* gene, probably resulting from double-strand breaks or recombination within the gene sequence during its integration into the recipient chromosomes (data not shown).

Expression of the *Alternaria* genes in the *Magnaporthe* melanin-restored transformants was confirmed by RNA blot analysis with total RNA from melanized mycelia of TAR-1, TAR-2, TBR-1, and TBR-2, grown on oatmeal sucrose agar. As a positive control, total RNA of *A. alternata* strain 15A was extracted from mycelia grown on the same plates. A single hybridizing band of about 7.2 kb was detected in total RNA from TAR-1, TAR-2, and 15A with the *ALM* probe (Fig. 3A). The *BRM2* probe hybridized to a single band of about 0.9 kb in total RNA from TBR-1, TBR-2, and 15A (Fig. 3B). These transcript sizes were identical to those of *ALM* and *BRM2* determined previously for *A. alternata* (Kimura and Tsuge 1993). The hybridization signals for the *M. grisea* transformants, however, were weaker than those for *A. alternata* RNA. The *ALM* transcript levels in TAR-1 and TAR-2 were less than 50% of that in *A. alternata*, and the *BRM2* transcript levels in TBR-1 and TBR-2 were less than 30% of that in *A. alternata*. Total RNA of wild-type strain Ken60-19 of *M. grisea* did not contain any bands hybridized with these probes (data not shown).

Morphogenesis and pathogenicity of *M. grisea* transformants.

The morphogenesis and pathogenicity of each three transformants of TAR and TBR series were determined. Conidial suspensions of these transformants were placed on Teflon membranes to observe spore germination, appressorium formation, and appressorium pigmentation. TAR and TBR series transformants formed melanized appressoria, while the melanin-deficient mutants MNK91-1 and MNK91-3 formed hyaline and lightly pigmented appressoria, respectively (Fig. 4A). Appressorium melanization of transformants, however, was slightly lighter than that of the wild-type strain. There were no significant differences in spore germination and appressorium formation rates among the wild-type strain, melanin-deficient mutants, and melanin-restored transformants (data not shown). These results indicate that the *A. alternata* genes *ALM* and *BRM2* are functional and can complement the deficiency of melanization during appressorium development as well as during vegetative growth in *M. grisea*.

Since the appressorium pigmentation in *M. grisea* is known to be essential for host penetration, pathogenicity of the melanin-restored transformants was examined by using both spray and drop inoculation of conidial suspensions. Conidial suspensions were sprayed on rice seedlings of compatible cultivar Aichi-Asahi. Melanin-deficient mutants did not cause any lesions on rice leaves, but melanin-restored TAR and TBR transformants caused many spreading lesions on leaves similar to wild-type strain (Fig. 4B). To confirm the origin of the lesion development, conidiation from the lesions caused by transformants was induced on 2% water agar, and the Hyg^r and appressorium pigmentation of the resulting conidia were examined. All single-isolated conidia from the lesions grew well and formed melanized colonies on PSA containing hygromycin B. When conidia formed on a lesion were inoculated on Teflon membranes, they

germinated, and pigmentation of appressoria was observed. These results confirm that the lesions were caused by the melanin-restored transformants.

When conidial suspensions were directly placed on wounded and unwounded leaves, melanin-deficient mutants caused lesions only on wounded leaves (Fig. 4C). In contrast, wild-type strain and the melanin-restored transformants caused lesions on unwounded leaves as well as wounded leaves (Fig. 4C). These results strongly suggest that the melanin-restored transformants regained penetration ability, subsequently with restoration of appressorium pigmentation due to the function of the transformed *Alternaria* genes. However, TAR and TBR tended to cause smaller-sized lesions than the wild-type strain.

DISCUSSION

Melanin acts as a pathogenicity factor in the rice blast fungus *M. grisea*. Melanin-deficient mutants or wild-type strain treated with a melanin biosynthesis inhibitor cannot penetrate the host plant (Woloshuk et al. 1980, 1983; Chumley and Valent 1990). The critical role of melanin is considered to be due to the differential permeability of the melanin layer to water and solute allowing high turgor pressure in the protoplasm to build up and force a specialized penetration peg through the outer plant surface into the plant tissue (Howard

Fig. 3. Detection of mRNA of the *Alternaria alternata* genes in the melanin-restored transformants of *Magnaporthe grisea*. Total RNA (20 µg/lane) was electrophoresed in a 1.0% agarose gel containing 2.2 M formaldehyde and 0.5 µg of acridine orange per ml, transferred to nylon membranes, and probed. **A**, Total RNA from *A. alternata* strain 15A (lane 1) and the *M. grisea* melanin-restored transformants TAR-1 and TAR-2 (lanes 2 and 3) was probed with the 9.4-kb *Eco*RI fragment of pMBE1 containing the *ALM* sequence. **B**, Total RNA from *A. alternata* strain 15A (lane 1) and the *M. grisea* melanin-restored transformants TBR-1 and TBR-2 (lanes 2 and 3) was probed with the 4.2-kb *Eco*RI-*Pst*I fragment of pMBEP10 containing the *BRM2* sequence. The 28S rRNA stained with acridine orange in the electrophoresed gel is also shown.

and Ferrari 1989; Howard et al. 1991). In contrast, *A. alternata* produces colorless, tiny appressoria, and melanin is not directly involved in pathogenicity (Tanabe et al. 1995). Although both fungi produce melanin, resulting in melanized colony formation, the role and deposition sites of melanin seem to differ. This study attempted to analyze the similarities and the diversity in melanin biosynthesis, especially the functions of melanin genes, of *A. alternata* and *M. grisea*.

We first examined nucleotide homology of the melanin biosynthesis genes between these two fungi by Southern blot analysis with the *Alternaria* gene probes. The analysis could not detect the *Alternaria* gene homologues in DNA of *M. grisea* strains. However, similar analysis with several other fungal species detected the homologous DNA from *Cochliobolus* and *Colletotrichum* spp. (C. Kawamura, N. Kimura, and T. Tsuge, unpublished data), which are also known to produce DHN melanin (Bell and Wheeler 1986). Further comparisons of direct nucleotide sequences will probably clarify the phylogeny of the melanin biosynthesis genes among fungi.

Fig. 4. Appressorium pigmentation and pathogenicity of the melanin-restored transformants of *Magnaporthe grisea*. 1, wild-type strain Ken60-19; 2, Alb⁻ mutant MNK91-1; 3, melanin-restored transformant TAR-1 from MNK91-1; 4, Buf⁻ mutant MNK91-3; 5, melanin-restored transformant TBR-1 from MNK91-3. **A**, Conidial suspensions were placed on Teflon membranes and incubated at 25°C for 20 h. **B**, Conidial suspensions were sprayed onto five-leaf-stage rice seedlings of the compatible cultivar Aichi-Asahi. Plants were incubated at 25°C for 5 days. **C**, Conidial suspensions were placed on wounded (W) or unwounded (UW) leaves and incubated at 25°C for 4 days. Sixth leaves were detached from eight-leaf-stage seedling and used for inoculation.

Although Southern blot analysis could not detect the *Alternaria* gene homologues from *M. grisea* strains, the roles of the *ALM*, *BRM1*, and *BRM2* genes of *A. alternata* are considered to be the same as *ALB*, *RSY*, and *BUF* genes, respectively, of *M. grisea* (Chumley and Valent 1990; Kimura and Tsuge 1993; Tanabe et al. 1995). To compare the function of melanin biosynthesis genes in these two fungi, we introduced the *A. alternata* genes into the melanin-deficient mutants of *M. grisea* and characterized the phenotypes of the transformants in detail.

As the first step, we isolated three classes of melanin-deficient mutants of *M. grisea* as recipients for transformation with the *Alternaria* genes. Based on pigmentation phenotypes of vegetatively growing colonies, these mutants were supposed to result from the mutations of *ALB*, *RSY*, or *BUF* genes reported by Chumley and Valent (1990). Pathogenicity assays of these mutants confirmed the previous reports that these mutations cause the loss of pathogenicity to the host plant, subsequent to the lack of appressorium pigmentation (Woloshuk et al. 1980, 1983; Chumley and Valent 1990).

As the next step, we transformed these *Magnaporthe* mutants with the *Alternaria* cosmid pMBR1 containing *ALM*, *BRM1*, and *BRM2* genes, and evaluated melanin restoration of transformants based on colony pigmentation. The melanin restoration was frequently observed in the transformants from Alb⁻ and Buf⁻ mutants. These melanin-restored transformants carried full-length copies of respective genes in their genome; transcripts in vegetatively growing mycelia were also detected. Furthermore, melanin production of Alb⁻ and Buf⁻ mutants was also restored by transformation with the *ALM* and *BRM2* subclones, respectively. These results indicate that the *Alternaria* genes *ALM* and *BRM2* can be expressed in *M. grisea* during vegetative growth, and that their functions in DHN melanin synthesis are identical to those of the *Magnaporthe* genes *ALB* and *BUF*, respectively.

In contrast, no melanin-restored transformants were obtained from the Rsy⁻ mutant. The Rsy⁻ mutant used in this study accumulated scytalone, an intermediate of melanin biosynthesis, suggesting that this mutant lacks scytalone dehydratase activity to convert scytalone to 1,3,8-trihydroxynaphthalene (Bell et al. 1976; Chumley and Valent 1990). The Brm1⁻ mutants of *A. alternata*, which were induced by both MNNG mutagenesis and transformation-mediated gene targeting, also accumulated scytalone in their cultures (Shiotani and Tsuge 1995; Tanabe et al. 1995). These facts suggest that two genes are involved in the step to convert scytalone to 1,3,8-trihydroxynaphthalene. The cDNA (740 bases) encoding scytalone dehydratase of *M. grisea* was sequenced and verified to encode the full-length (172 amino acids) scytalone dehydratase protein (Lundqvist et al. 1993). However, we detected a *BRM1* transcript of about 4.0 kb (Kimura and Tsuge 1993), indicating that the sizes of the *RSY* and *BRM1* transcripts are significantly different. The structure and function of these two genes might differ, and remain to be clarified.

The sites of melanin deposition in *M. grisea* and *A. alternata* are different. Conidia of *M. grisea* are colorless, and their appressoria are darkly melanized. On the other hand, the *Alternaria* spp. produce darkly melanized conidia, but their appressoria are colorless. Thus, we had much interest in whether the *Alternaria* genes can restore appressorium melanization in the *M. grisea* transformants. Our data presented here show that the melanin-restored transformants produced melanized

appressoria like wild-type strains. More interestingly, all these transformants regained the ability to penetrate rice leaves of a compatible cultivar. These results strongly suggest that the *Alternaria* genes can be functional for appressorium melanization and also for restoration of pathogenicity in *M. grisea*. Recently, Takano et al. (1996) reported that the *A. alternata* *ALM* gene also complemented the albino *pkS1* mutation of *C. lagenarium*. The *Colletotrichum* *PKS1* gene and the *Alternaria* *ALM* gene are both involved in polyketide synthesis in the melanin biosynthetic pathway (Kubo et al. 1991; Kimura and Tsuge 1993; Takano et al. 1995; Tanabe et al. 1995). The *ALM* gene transformants of *C. lagenarium* *pkS1*⁻ mutant regained mycelial melanization, appressorium melanization, and pathogenicity (Takano et al. 1996). This result also confirms the potential ability of the *Alternaria* melanin genes to substitute for the melanin genes of other fungal species.

Expression of the melanin biosynthesis genes of other fungi in *A. alternata* should also be determined. Recently, several melanin synthesis genes have been isolated from *M. grisea* and *C. lagenarium*: putative *RSY* and *BUF* genes of *M. grisea* (Lundqvist et al. 1993; Vidal-Cros et al. 1994) and *PKS1*, *SCD1*, and *THR1* genes of *C. lagenarium* (Takano et al. 1995; Kubo et al. 1996a; Perpetua et al. 1996). *A. alternata* produces darkly melanized conidia, while conidia of both *M. grisea* and *C. lagenarium* are colorless. Indeed, melanin-deficient mutations in *A. alternata* were found to induce less conidiation (Y. Fujita, C. Kawamura, and T. Tsuge, unpublished data). Thus, it will be important to observe not only mycelial melanization but also conidial melanization of *A. alternata* transformants with the *Magnaporthe* or *Colletotrichum* genes in future.

The linkage relationships of the melanin biosynthesis genes also vary among these fungi. Although the *A. alternata* genes *ALM*, *BRM1*, and *BRM2* reside within an approximately 30-kb region in the fungal chromosome (Kimura and Tsuge 1993), the *M. grisea* genes *ALB*, *RSY*, and *BUF* are unlinked (Chumley and Valent 1990). Kubo et al. (1996b) reported that the *C. lagenarium* genes *PKS1*, *SCD1*, and *THR1* apparently are not closely linked. Such differences in linkage relationships of the melanin biosynthesis genes among these fungi suggest the complex processes of acquisition and establishment of melanin biosynthesis in fungi.

We are now analyzing the period and site of expression of the melanin biosynthesis genes in *A. alternata* during the infection process. The promoter region of the *Alternaria* melanin genes will be also analyzed by using the reporter gene constructs fused with the melanin gene promoter to observe the promoter activity in situ. To study molecular evolution of melanin biosynthesis in fungi, future work will be directed at identification and comparison of the *cis*-acting elements of the melanin genes among fungi and at detection of the *trans*-acting regulators. Recently, some genes involved in infection have been cloned from *M. grisea* (Talbot et al. 1993; Sweigard et al. 1995; Dean et al. 1996). Among them, the *MPG1* gene encoding a fungal hydrophobin was characterized as having an important role in appressorium formation (Talbot et al. 1993; 1996). Lau and Hamer (1996) definitively identified two genes, designated *NPR1* and *NPR2*, encoding putative *trans*-acting regulators that induce *MPG1* expression during appressorium development. The relationship between these genes and melanin synthesis genes during infection-related differentiation will be another important study in future.

MATERIALS AND METHODS

Fungal strains.

The rice-infecting strains of *M. grisea*, Ken60-19 (race 037), Ina83R-25A (race 037), Ina85-182 (race 003), Ina91-02 (race 001), and TH68-126 (race 033), were used in Southern blot analysis. Strain Ken60-19 was used as the parent strain for isolation of melanin-deficient mutants. Fungi were maintained on PSA.

Conidiation.

Fungi were grown on oatmeal sucrose agar (5% oatmeal, 1.5% sucrose, 1.5% agar) plates at 25°C for 14 days, followed by placing the plates under BLB lamps (FL20S, 20W, Toshiba, Osaka, Japan) for 4 days after removing the aerial mycelia with a sterilized brush (Xiao et al. 1994). Conidia formed were brushed off, suspended in sterilized water, and used for experiments.

Isolation of the *M. grisea* mutants.

Melanin-deficient mutants were isolated by mutagenesis of conidia of strain Ken60-19 with MNNG. Conidia were suspended in 40 µM MNNG and incubated at 25°C for 4 h with shaking (70 rpm; Bio-Shaker BR-30LF, Taitec, Saitama, Japan). Under these conditions, about 5% of conidia survived. The treated conidia were collected by centrifugation at 700 × g for 5 min and plated on PSA. After incubation at 25°C for 2 to 3 days, growing colonies were transferred to a new PSA, and then their mycelial pigmentation was observed. Scytalone, an intermediate in the direct pathway to melanin, was extracted from the mutant culture on PSA by the method of Bell et al. (1976) and analyzed by high-performance liquid chromatography as described previously (Tanabe et al. 1995). Genetic and phenotypic nomenclature of *M. grisea* is that of Chumley and Valent (1990).

Bacterial strains, cosmids, and plasmids.

Escherichia coli HB101 and AG1 were used for cloning and propagation of plasmids and cosmids, respectively.

A cosmid clone pMBR1 was used for transformation of the melanin-deficient mutants of *M. grisea* (Fig. 1B). pMBR1 has been isolated from a cosmid library of the wild-type strain 15A of the Japanese pear pathotype of *A. alternata* (Kimura and Tsuge 1993). It carries an approximately 40-kb fragment of the fungal genomic DNA in which three genes, *ALM*, *BRM1*, and *BRM2*, involved in melanin biosynthesis are located (Fig. 1B) (Kimura and Tsuge 1993).

Subclones pMBE1, pMBE8, and pMBEP10, containing *ALM*, *BRM1*, and *BRM2* sequences, respectively, were used as hybridization probes (Kimura and Tsuge 1993). The 9.4-kb *EcoRI* insert of pMBE1 and the 4.2-kb *EcoRI*-*PstI* insert of pMBEP10 were purified by electroelution and blunted with T4 DNA polymerase. Each of these fragments was introduced into the *EcoRV* site of fungal integrative transformation vector pSH75 to make pALM-1 and pBRM2-1 (Fig. 1B) (Kimura and Tsuge 1993). The plasmid pSH75 carries the *hph* (hygromycin B phosphotransferase) gene as a selectable marker (Gritz and Davies 1983; Cullen et al. 1987; Kimura and Tsuge 1993). In pALM-1 and pBRM2-1, transcriptional directions of the melanin genes are the same as that of the *hph* gene.

Fungal transformation.

Protoplasts of *M. grisea* were prepared as described by Parsons et al. (1987), with an enzyme solution containing 10 mg of Novozym 234 (Novo Nordisk, Bagsvaerd, Denmark) per ml in 1 M sorbitol. Protoplasts were suspended in STC (1.2 M sorbitol, 25 mM Tris-HCl, pH 7.5, 25 mM CaCl₂) at 3×10^8 per ml, and 200- μ l portions were treated with cosmid DNA (25 μ g) or plasmid DNA (10 μ g) according to the method of Leung et al. (1990). Following transformation, the protoplasts were collected by centrifugation at $950 \times g$ for 5 min. The pellet was suspended in 5 ml of complete medium (CM) (Leung et al. 1990) containing 1.2 M sorbitol and plated directly onto regeneration agar medium (CM containing 1.2 M sorbitol and 2% agar). Plates were incubated at 25°C for 3 days and then overlaid with regeneration agar medium containing 150 μ g of hygromycin B (Wako Pure Chemicals, Osaka, Japan) per ml. Colonies that grew out of the overlay were transferred to PSA containing 150 μ g of hygromycin B per ml. Mycelial pigmentation was observed after incubation at 25°C for 7 days.

Assays for infection-related morphogenesis and pathogenicity.

For observation of conidial germination and appressorium formation, droplets (10 μ l) of conidial suspension (10^4 conidia/ml) were placed on Teflon membranes (Flonchemical, Osaka, Japan) and left in a humid chamber at 25°C for 20 h. The frequencies of germination and appressorium formation were determined by counting at least 600 conidia, and the appressorium pigmentation was observed.

Pathogenicity assays were carried out with rice seedlings of cultivar Aichi-Asahi, which is compatible to strain Ken60-19. Two methods, spray inoculation and drop inoculation, were employed for pathogenicity assays. For spray inoculation, five-leaf-stage seedlings were used. Pots containing 10 rice seedlings were each sprayed with 3 ml of a conidial suspension (10^5 conidia/ml) supplemented with 0.01% Tween 20. After incubation at 25°C for 5 days, lesions on leaves were observed. Tissues of the leaves infected with transformants were excised and placed on 2% water agar to induce conidiation. The infecting pathogen was reisolated by single-conidial isolation from infected lesions and assayed for Hyg^r on PSA plates containing 150 μ g of hygromycin B per ml and appressorium pigmentation on Teflon membrane.

For drop inoculation, sixth leaves were detached from eight-leaf-stage seedlings and used. Conidial suspension (20 μ l, 10^5 conidia/ml) was placed on the underside of leaves. Inoculation was also done on the wounded leaves. Detached leaves were wounded slightly by picking the surface of leaves, and conidial suspension was placed on the picked portion. Inoculated leaves were incubated in a humid chamber at 25°C for 4 days, and lesion development was observed.

Nucleic acid extraction.

For extraction of DNA, fungi were grown in 50 ml of potato sucrose broth in 100-ml Erlenmeyer flasks at 25°C for 3 days on an orbital shaker (120 rpm; Bio-Shaker BR-30LF, Taitec, Saitama, Japan). The resulting mycelia were ground in liquid nitrogen in a mortar with a pestle. Total cellular DNA was prepared from the powdered mycelia as described previously (Adachi et al. 1993).

For extraction of RNA, fungi were grown on oatmeal sucrose agar at 25°C for 5 days. The resulting colony was recovered and ground in liquid nitrogen in a mortar with a pestle. Total cellular RNA was extracted from the powdered mycelia as described by Timberlake (1986).

Plasmid DNA and cosmid DNA were isolated from *E. coli* cells by the alkaline lysis method (Sambrook et al. 1989).

Nucleic acid hybridization.

Restriction enzyme digestion of DNA and fractionation in agarose gels were performed by standard methods (Sambrook et al. 1989). RNA was electrophoretically fractionated in formaldehyde-agarose gels (Sambrook et al. 1989). Fractionated DNA and RNA were transferred to Hybond N⁺ nylon membranes (Amersham, Arlington Heights, IL) and subjected to hybridization according to the manufacturer's recommendations. To detect genomic DNA of *M. grisea* homologous to the *A. alternata* melanin genes, hybridized blots were washed in 2 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄ [pH 7.7], and 1 mM EDTA) plus 0.1% sodium dodecyl sulfate at 50°C. In other cases, hybridized blots were washed at 65°C; the final wash was done in 0.1 $\times$ SSPE plus 0.1% sodium dodecyl sulfate.

DNA probes were labeled with [α -³²P]dCTP (ICN Biochemicals, Costa Mesa, CA) by the random-primer method (Feinberg and Vogelstein 1983).

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