Research Note

Targeted Disruption of a Melanin Biosynthesis Gene Affects Conidial Development and UV Tolerance in the Japanese Pear Pathotype of *Alternaria alternata*

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Structural analysis of the *BRM2* gene involved in melanin biosynthesis of the Japanese pear pathotype of *Alternaria alternata* suggested that this gene encodes 1,3,8-trihydroxynaphthalene reductase. Targeted disruption of the *BRM2* gene did not affect pathogenicity, vegetative growth, or the number of conidia produced. Targeted disruption, however, did reduce conidial size and septal number, suggesting that melanin is associated with conidial development. The conidia of *brm2* mutant transformants were more sensitive to UV light than those of the wild type, demonstrating that melanin confers UV tolerance.

Additional keywords: appressorium, host-specific toxins.

The imperfect fungus *Alternaria alternata* is ubiquitous and generally saprophytic. In addition, *A. alternata* contains seven pathotypes that cause diseases on different plants by producing host-specific toxins (Nishimura and Kohmoto 1983). *A. alternata* produces a dark brown or black pigment, melanin, that accumulates in the cell walls of hyphae and conidia (Tanabe et al. 1995). Many fungi, including *A. alternata*, produce melanin via polyketide biosynthesis (Fig. 1) (Bell and Wheeler 1986). We have previously isolated the gene cluster required for melanin biosynthesis of the Japanese pear pathotype of *A. alternata* (Kimura and Tsuge 1993), which produces the host-specific AK-toxin and causes black spot of Japanese pear (Tanaka 1933; Nakashima et al. 1985). The gene cluster (about 30 kb) contains three genes, *ALM*, *BRM1*, and *BRM2*, that can restore albino (*alm*), light brown (*brm1*) and brown (*brm2*) mutants, respectively (Kimura and Tsuge 1993).

In *A. alternata*, melanin is considered to play an important role in longevity and survival in nature (Rotem 1994). Using melanin-deficient (*Mel*[−]) mutants induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), Tanabe et al. (1995)

showed that melanin is probably not relevant to pathogenicity of the Japanese pear pathotype. In contrast, melanin has been implicated as a pathogenicity factor in *Magnaporthe* and *Colletotrichum* spp. (Woloshuk et al. 1980; Kubo et al. 1982; Wolkow et al. 1983). Studies with melanin biosynthesis inhibitors and *Mel*[−] mutants have shown that appressorium melanization is essential for penetration of host cells by these fungi: melanin mediates pressure build-up in the appressorium, and this pressure provides the essential driving force for mechanical penetration (Howard and Ferrari 1989). While these fungi produce melanized appressoria, *A. alternata* produces colorless appressoria. In contrast, *A. alternata* produces melanized conidia, but conidia of *Magnaporthe* and *Colletotrichum* spp. are colorless. This implies that melanin is likely to have a number of diverse roles in morphogenesis and plant infection in phytopathogenic fungi. In this study, we analyzed the structure of the *A. alternata* *BRM2* gene and the role of melanin in pathogenicity, conidiation, and UV tolerance with *Mel*[−] mutants produced by targeted disruption of the *BRM2* gene.

Based on pigmentation phenotype and accumulation of 2-hydroxyjuglone, a shunt product of 1,3,8-trihydroxynaphthalene (1,3,8-THN), in the *brm2* mutant, *BRM2* was proposed to encode 1,3,8-THN reductase (Fig. 1) (Kimura and Tsuge 1993; Tanabe et al. 1995; Kawamura et al. 1997). The *BRM2* gene was subcloned from pMBEP10 (Kimura and Tsuge 1993) as a 4.2-kb *EcoRI/PstI* fragment in pBluescript KS⁺ (pBBR2), and several overlapping restriction fragments were subcloned from pBBR2 in pBluescript KS⁺. DNA sequencing was carried out on an automated DNA sequencer (Applied Biosystems, Warrington, UK) with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence analysis of the 4.2-kb fragment detected one open reading frame (ORF) of 801 bp (Fig. 2A). The ORF potentially encodes a 267 amino acid protein, after splicing of two introns of 51 and 49 bp (Fig. 2B). Data base comparison demonstrated that the predicted *BRM2* product (*Brm2*) is clearly related to 1,3,8-THN reductases of *Cochliobolus heterostrophus* (*Brn1*, 95% identity; Shimizu et al. 1997) and *C. lagenarium* (*Thr1*, 67% identity; Perpetua et al. 1996), and 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) reductase of *M. grisea* (*ThnR*, 69% identity; Vidal-Cros et al. 1994) (Fig. 2A). The organization of the *BRM2* gene resem-

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bles that of the *C. heterostrophus Brn1* gene. These two genes have the same size exons and the same location of two introns (Fig. 2B). We now propose that the *A. alternata BRM2* gene encodes 1,3,8-THN reductase.

The targeting vector pGDB2-2 was used for disruption of the *BRM2* gene. A 0.8-kb fragment internal to the *BRM2* ORF was cloned in the transformation vector pSH75 (Kimura and Tsuge 1993) to produce pGDB2-2 (Fig. 3A). The wild-type strain 15A was transformed with pGDB2-2 by the method previously described (Tsuge et al. 1990). Transformation yielded 49 out of 133 hygromycin B-resistant transformants, of which colonies on potato dextrose agar (PDA) were brown similar to those of the MNNG-induced *brm2* mutant.

The integration of the targeting vector in Mel⁻ transformants was analyzed by polymerase chain reaction (PCR) (Fig. 3). The primer combination G1/G2 produced the expected 1.2-kb DNA from total DNA of the wild-type strain and the melanin-producing (Mel⁺) transformants (BR2C-1 to BR2C-5) (Fig. 3B). All Mel⁻ transformants (BR2D-1 to BR2D-5), however, had no amplified DNA, suggesting that *BRM2* had been disrupted by homologous integration of pGDB2-2 (Fig. 3B). To confirm the *BRM2* disruption, primer combinations G1/V1

and V2/G2 were used for detection of the junctions between the recipient *BRM2* region and integrated pGDB2-2. With these primer combinations, PCR failed to produce DNA fragments in the wild-type strain (Fig. 3C and D). In contrast, primer combination G1/V1 produced an expected 1.1-kb DNA in all Mel⁻ transformants (Fig. 3C); V2/G2 produced an expected 1.0-kb DNA in four Mel⁻ transformants (BR2D-1, BR2D-2, BR2D-3, and BR2D-5) and a fragment of about 3.2-kb DNA in the remaining transformant (BR2D-4) (Fig. 3D). The homologous integration of pGDB2-2 in BR2D-4 was probably accompanied by recombination.

Fig. 3. Targeted disruption of *BRM2* in *Alternaria alternata*. **A**, Structure of the *BRM2* locus before and after homologous integration of the targeted disruption vector pGDB2-2. Arrowheads denote orientation and location of oligonucleotide primers used in polymerase chain reaction (PCR) experiments. A 0.8-kb fragment internal to the *BRM2* open reading frame (ORF) was amplified from pBBR2 by PCR with primers GD1 (5'-AACTCGAGCAAGGTCGCCGTGTTA-3') and GD2 (5'-AAGCATGCAGTCACCGTCTTGAGAAG-3'). GD1 and GD2 included *Xho*I and *Sph*I sites (underlined), respectively, flanking the *BRM2* sequences. PCR product was digested with *Xho*I and *Sph*I, and then cloned in the transformation vector pSH75 (Kimura and Tsuge 1993) to produce pGDB2-2. Primers G1 (5'-TCTTCGTCTTGACCGTGGAA-3'), G2 (5'-ACATGCTAGCTCATGACTGC-3'), V1 (5'-TACCCAGAATGCACAGGTAC-3'), and V2 (5'-GATTTAGGTGACACTATAG-3') were used to verify homologous integration of pGDB2-2 in Mel⁻ transformants. Abbreviations: E, *Eco*RI; P, *Pst*I; S, *Sph*I; X, *Xho*I. **B–D**, PCR analysis of *BRM2* disruption with primer combinations (**B**) G1/G2, (**C**) G1/V1, and (**D**) V2/G2. W, wild-type strain 15A; BR2D, Mel⁻ transformants; BR2C, Mel⁺ transformants.

Table 1. Vegetative growth and conidiation of *brm2* mutants of *Alternaria alternata*

Strain ^v	Mycelial growth ^w (mm)	No. of conidia ^x (× 10 ⁴ /cm ²)	Conidium size ^y (μm)		No. of septa/conidium ^y	
			Width	Length	Transverse	Longitudinal + oblique
15A	58.0 ± 1.4 ab ^z	9.6 ± 4.4 a	10.8 a	22.6 c	2.7 c	2.1 a
BR2C-1	54.8 ± 2.2 c	8.5 ± 3.0 a	10.1 a	25.2 ab	3.4 ab	1.9 a
BR2C-2	56.0 ± 1.4 bc	15.5 ± 6.5 a	10.7 a	26.6 a	3.5 a	1.9 a
BR2C-3	58.0 ± 1.4 ab	14.0 ± 8.0 a	10.8 a	25.5 ab	3.2 ab	2.1 a
BR2C-4	58.5 ± 1.3 ab	15.2 ± 7.7 a	10.2 a	24.0 bc	3.1 bc	1.8 a
BR2D-1	59.4 ± 0.6 a	5.3 ± 4.1 a	8.3 b	17.6 d	2.1 d	0.7 b
BR2D-2	57.0 ± 1.2 bc	4.7 ± 4.9 a	8.8 b	19.5 d	2.2 d	0.7 b
BR2D-3	56.3 ± 1.9 bc	2.1 ± 1.8 b	9.0 b	19.0 d	2.3 d	0.7 b
BR2D-5	54.8 ± 3.3 c	10.0 ± 7.1 a	8.1 c	17.7 d	2.0 d	0.6 b

^v 15A, wild type; BR2C-1 to BR2C-4, Mel⁺ transformants; BR2D-1 to BR2D-5, *brm2* mutant transformants.

^w Strains were grown on potato dextrose agar at 25°C for 4 days, and colony diameter was measured. Each value represents mean ± SD from four experiments.

^x Strains were grown in 30 ml of potato dextrose broth in 100-ml Erlenmeyer flasks at 25°C for 10 days. The mycelial mats were harvested and subjected to induction of conidiation as previously described (Hayashi et al. 1990). Conidia were collected in water and counted. Each value represents mean ± SD from four experiments.

^y Each value represents the average of 100 conidia.

^z Values followed by different letters are significantly different among strains at the *P* < 0.01 level according to Fisher's protected least significant difference test.

To assess the effect of *BRM2* disruption on pathogenicity, conidial suspensions (about 2×10^5 conidia per ml) of the wild-type strain and *brm2* mutant transformants were spray inoculated on detached leaves of susceptible pear cultivar Nijisseiki. Within 24 h after inoculation, the wild-type and *brm2* mutants caused necrotic lesions. The number and size of these lesions were almost the same on leaves inoculated with wild type and each of the transformants (data not shown). This result was consistent with our previous observation in the MNNG-induced Mel⁻ mutants of *A. alternata* (Tanabe et al. 1995).

Similar results were reported in *C. heterostrophus*, in which Mel⁻ strains exhibited the same degree of pathogenicity on corn as the wild type in growth-chamber or greenhouse experiments (Fry et al. 1984; Guillén et al. 1994). Thus, melanin is unlikely to play a direct role in pathogenicity and virulence in these fungi. While these two fungi directly penetrate into the host cells through appressoria, their appressoria are colorless, in contrast to those of *Magnaporthe* and *Colletotrichum* spp. Thus, the mechanisms of appressorial penetration of host cells in these two fungi are probably different from those in *Magnaporthe* and *Colletotrichum* spp.

The effects of the *BRM2* disruption on vegetative growth and conidiation were tested. Agar blocks (6 mm in diameter) from colonies grown on PDA were transferred onto PDA and grown at 25°C for 4 days. Colony diameters were not significantly different for the wild-type strain, Mel⁺ transformants, and Mel⁻ transformants (Table 1). The *BRM2* disruption did not obviously affect the number of conidia, but did affect conidial pigmentation (Table 1 and Fig. 4A). Conidia of *brm2* mutants were slightly pigmented, while wild-type conidia were dark brown or black (Fig. 4A). Electron microscopic observation showed that electron-dense materials accumulated less on the conidial surface of the *brm2* mutant than on that of the wild-type strain (Fig. 4B and C). *BRM2* disruption reduced conidium size. Conidium width and length of *brm2* mutants were significantly smaller than those of the wild type and Mel⁺ transformants (Table 1). The numbers of transverse, longitudinal, and oblique septa of conidia were also reduced in *brm2* mutants (Table 1), suggesting that melanin is involved in conidial development. Electron microscopic observation of *Alternaria* spp. conidia showed that melanin accumulates not only on the conidial surface but also in septal partitions (Campbell 1969; Park et al. 1990; Tanabe et al. 1995). It was

reported that tricyclazole, a known inhibitor of 1,3,8-THN reductase and 1,3,6,8-THN reductase, inhibited normal development of sclerotia in *Botrytis cinerea* (Zeun and Buchenauer 1985) and *Sclerotinia sclerotiorum* (Buchenauer et al. 1985). Albino mutants of *C. carbonum* and *Ophiostoma piliferum* were observed to produce fewer or no well-developed perithecia (Leonard 1972; Zimmerman et al. 1995). The characterization of melanin function and melanin gene expression during conidiation is of great importance for the study of the conidiation mechanisms of *A. alternata*.

To test the UV tolerance of *BRM2*-disrupted transformants, conidial suspensions were spread on 2% agar plates and irradiated with UV light (GL15, 253.7 nm, 15W, Toshiba, Osaka,

Fig. 5. Reduced tolerance to UV in *BRM2*-targeted transformants. Conidial suspensions (about 5,000 conidia) were spread on 2% agar plates (9 cm in diameter) and irradiated with a UV lamp (253.7 nm, 600 W per cm²). After incubation at 25°C for 24 h, germination of 200 conidia were observed. Each value represents the mean from three experiments. The standard deviation is indicated by the error bar. 15A, wild-type strain; BR2C-1 to BR2C-3, Mel⁺ transformants; BR2D-1 to BR2D-3, *brm2* mutant transformants.

Fig. 4. A, Light micrograph of conidia of wild-type strain 15A (W) and *BRM2*-disrupted transformant BR2D-1 (M). Bar marker represents 20 μ m. B and C, Electron micrographs of conidial surfaces of (B) 15A and (C) BR2D-1. Conidium sections were prepared as described by Park et al. (1990). Specimens were observed in an HU-12A type electron microscope (Hitachi, Tokyo). Bar markers represent 1 μ m.

Japan) placed 10 cm above (600 W per cm²) for 20 to 180 s. After incubation at 25°C for 24 h, the viability of conidia was evaluated by assessing germination (Fig. 5). Conidia of *brm2* mutants were more sensitive to UV irradiation than those of the wild type and Mel⁺ transformants (Fig. 5). Conidia of *brm2* mutants completely lost the ability to germinate after UV irradiation for 120 s. However, germination rates were 57.3% in wild type and 45.4 to 56.3% in each of the Mel⁺ transformants after this irradiation. These results demonstrate that melanin confers UV tolerance when it is densely deposited on the conidial surface.

All *Alternaria* pathogens display a high capacity for survival that assists disease development in harsh habitats (Rotem 1994). Lockwood (1960) demonstrated that melanin confers resistance of *Alternaria* mycelia to microbial lysis in soil. Jacobson et al. (1995) reported the antioxidant function of melanin in *A. alternata*: melanin confers redox buffering properties. In *C. heterostrophus*, the albino strains and wild type cause similar disease symptoms under laboratory conditions. However, the albino strains cause no disease under field conditions, showing the importance of melanin for survival or fitness (Fry et al. 1984). We suppose that the significance of melanin in *A. alternata* is similar to that of *C. heterostrophus*, because no Mel⁻ isolates have been obtained from naturally occurring lesions on host plants found in the field. Mel⁻ mutants are not likely to survive in the field.

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