J Gen Plant Pathol (2012) 78:30–38
DOI 10.1007/s10327-011-0356-4

FUNGAL DISEASES

Functional analysis of the melanin biosynthesis genes *ALM1* and *BRM2-1* in the tomato pathotype of *Alternaria alternata*

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Received: 3 October 2011 / Accepted: 22 November 2011 / Published online: 17 December 2011

Abstract The tomato pathotype of *Alternaria alternata* (*A. arborescens*) produces the dark brown to black pigment melanin, which accumulates in the cell walls of hyphae and conidia. Melanin has been implicated as a pathogenicity factor in some phytopathogenic fungi. Here, two genes of the tomato pathotype for melanin biosynthesis, *ALM1* and *BRM2-1*, which encode a polyketide synthetase and a 1,3,8-trihydroxynaphthalene (THN) reductase, respectively, have been cloned and disrupted in the pathogen. The gene-disrupted mutants, *alm1* and *brm2-1*, had albino and brown phenotypes, respectively. The wild-type and the mutants caused the same necrotic lesions on the leaves after inoculation with spores. These results suggest that melanin is unlikely to play a direct role in pathogenicity in the tomato pathotype *A. alternata*. Scanning electron microscopy revealed that the conidia of both mutants have much smoother surfaces in comparison to the wild-type. The conidia of those mutants were more sensitive to UV light than those of the wild-type, demonstrating that melanin confers UV tolerance.

Keywords *Alternaria alternata* tomato pathotype · *Alternaria arborescens* · Melanin · Polyketide biosynthetic gene · UV tolerance

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Introduction

Most strains of *Alternaria alternata* (Fr.) Keissler, a species that is distributed all over the world, are generally saprophytic (Rotem 1994). Some strains, however, produce host-specific toxins (HSTs), which are responsible for fungal pathogenicity, and cause severe diseases on host plants (Kohmoto et al. 1995; Thomma 2003). HSTs produced by the tomato pathotype *A. alternata* (synonym *A. alternata* f. sp. *lycopersici*, synonym *A. arborescens*), the causal agent of Alternaria stem canker disease in tomato, are called AAL-toxins, and they lead to severe necrosis on susceptible tomato cultivars (Gilchrist and Grogan 1976; Peever et al. 2004). A major factor in pathogenicity in *A. alternata*–tomato interactions is the production of host-specific AAL toxins that are capable of inducing cell death in susceptible cultivars (Akamatsu et al. 1997; Brandwagt et al. 2000; Yamagishi et al. 2006).

In addition to HST biosynthesis genes that determine fungus-specific pathogenicity, a number of genes involved in the general virulence of fungi have been isolated in many plant pathogens, e.g., genes for melanin biosynthesis, signal transduction, infection structures and cell-wall-degrading

enzymes (Thomma 2003). Among them, melanin biosynthesis has been considered to be an important general virulence factor in many plant pathogens (Bell and Wheeler 1986; Howard and Ferrari 1989).

Many fungi including *A. alternata* produce red, brown or black melanins consisting of phenolic polymers. Most fungal melanins are derived from the precursor molecule 1,8-dihydroxynaphthalene (DHN) and are known as DHN-melanins. The melanin biosynthetic pathway through DHN is termed the polyketide pathway, and it resides mainly in ascomycetes and related deuteromycetes (Bell and Wheeler 1986). In general, melanin is important for the survival and longevity of fungal propagules (Rotem 1994). Fungal melanin accumulates in cell walls of conidia and hyphae and has been reported to confer tolerance to environmental stresses, such as UV radiation, microbial lysis, and defense responses of host plants and animals against fungal infection. The melanization of conidia has also been considered to be an important factor for the saprophytic properties of fungi in fields (Kawamura et al. 1999; Rehnstrom and Free 1996; Wang and Casadevall 1994). Although UV resistance would seem to have no direct association with the host–parasite relationship, melanized strains of fungi are more resistant to UV irradiation than nonmelanized strains. However, melanized spores of any species might be more infectious when aerosolized in sunlight (Wang and Casadevall 1994).

Melanin has been implicated as a pathogenicity factor in *Magnaporthe* and *Colletotrichum* spp. (Kubo et al. 1982; Takano et al. 1995; Wolkow et al. 1983). Studies with melanin biosynthesis inhibitors and melanin-deficient mutants have shown that appressorium melanization is essential for these fungi to penetrate host cells. Melanization is observed during the penetration process in diverse fungal cell types. Fungi such as *Alternaria* and *Cochliobolus* species produce melanized conidia and colorless tiny appressoria. In general, melanization of appressoria of those fungi is not relevant to the penetration process and pathogenicity (Fry et al. 1984; Kawamura et al. 1999; Moriwaki et al. 2004). By contrast, fungi such as *Colletotrichum* and *Magnaporthe* species produce nonmelanized conidia and well-developed appressoria pigmented with melanin (Emmet and Parberry 1975).

In *Aspergillus fumigatus*, mutation in the *PKSP* (*ALB1*) gene led to morphological changes of the conidia (Pihet et al. 2009). SEM observations showed that these pigmentless mutants produced smooth-walled conidia, whereas the conidia of *A. fumigatus* typically have a rough surface. These results indicate that melanin is a structural component of the conidial wall that is required for the correct assembly of the cell wall layers and expression at the conidial surface of adhesions and that it plays an indirect role in virulence of the fungus (Pihet et al. 2009). In addition, melanin is likely to have a number of diverse roles in morphogenesis in plant and animal pathogenic fungi.

In the Japanese pear pathotype *A. alternata*, which produces AK-toxin and causes black spots on these pears, melanin was determined to be irrelevant to pathogenicity using melanin-deficient mutants induced by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (Tanabe et al. 1995). The gene cluster (ca. 30 kb) required for melanin biosynthesis by the pathogen contains three genes, *ALM*, *BRM1*, and *BRM2*, which can restore melanin synthesis in the albino (*alm*), light brown (*brm1*) and brown (*brm2*) mutants, respectively (Kimura and Tsuge 1993). The structural analysis of the *BRM2* gene, which encodes 1,3,8-trihydroxynaphthalene (THN) reductase, and the functional analysis of the gene by targeted disruption have also been reported (Kawamura et al. 1999).

To investigate the role of melanin in the general pathogenicity of the tomato pathotype *A. alternata* (*A. arborescens*), we have cloned and characterized the *ALM1* gene, which encodes a polyketide synthetase, from the pathogen. This is the first report of the isolation and functional analysis for the melanin PKS gene in *A. alternata*. In addition, we analyzed the role of melanin in pathogenicity, conidiation, conidial surface structures and UV tolerance with melanin-deficient mutants produced by targeted disruption of the *ALM1* and *BRM2-1* genes.

Materials and methods

Fungal strains and culture conditions

Strain As-27 of the tomato pathotype of *A. alternata* (synonym *A. alternata* f. sp. *lycopersici*, synonym *A. arborescens*) (Siler and Gilchrist 1983) and transformants derived from this isolate were used in this study. All isolates were maintained on potato dextrose agar (PDA) (Difco, Detroit, MI, USA) slants or as 20% (v/v) glycerol mycelial fragments at −80°C. The isolates were cultured on V8 juice agar medium for conidia production or in potato dextrose broth (PDB) for genomic DNA preparation.

Assays for pathogenicity and toxin production

Pathogenicity of the wild-type and transformant strains of *A. alternata* were assessed as described previously (Akamatsu et al. 1997; Yamagishi et al. 2006). The production of AAL-toxin was examined in cultures grown on autoclaved rice with a slight modification of a method described previously (Yamagishi et al. 2006). In summary 0.5 g of rice medium was incubated for 2 weeks after inoculation, then mixed with 2 ml of 50% (v/v) acetonitrile in water. The extract was filtered, and 0.8 ml of the filtrate

was mixed with 4 ml of water and then applied to a pre-conditioned Sep-pak C-18 cartridge (Waters, Milford, MA, USA). The cartridge was washed with 2 ml of 15% (v/v) acetonitrile in water, and the toxin was eluted with 2 ml of 70% (v/v) acetonitrile in water. The final extracts were stored at −20°C until use. The AAL-toxin T_A was quantified using HPLC and pre-column derivatization of the toxin with *o*-phthalaldehyde as described previously (Kodama et al. 1995; Yamagishi et al. 2006).

Polymerase chain reaction (PCR) analysis

For the extraction of DNA, fungi were grown in 50 ml of potato sucrose broth in 100-ml Erlenmeyer flasks at 25°C for 2 days on an orbital shaker (120 rpm). The resulting mycelia were ground in liquid nitrogen using a mortar and pestle. Total genomic DNA was extracted from the mycelia as described previously (Akamatsu et al. 1997). All PCR primers used in this study are shown in Table 1. PCR was performed in a Thermal Cycler Dice TP650 (Takara Bio, Ohtsu, Japan) or a MyCycler 170-9703JA (Bio-Rad Laboratories, Hercules, CA, USA) with an initial step of 5 min at 95°C; followed by 30 cycles of 15 s at 95°C, 15 s at 59°C, and 30 s at 72°C; and a final extension of 5 min at 72°C.

Vector construction

The PCR approach (Kuwayama et al. 2002; Nayak et al. 2006; Ninomiya et al. 2004) for constructing the gene disruption vectors is outlined in Fig. 1. To construct the *BRM2-1* disruption vector, we PCR-amplified two DNA fragments from the genomic DNA of As-27. A 501-bp fragment (left-side arm of the vector) and a 560-bp fragment (right-side arm of the vector) internal to the *BRM2-1* ORF were amplified using primers BRM2AF/BRM2AR and BRM2BF/BRM2BR, respectively. These two amplified fragments were digested with *Sfi*I. The hygromycin B phosphotransferase gene (*hph*) from plasmid p71*sfi* was amplified using primers hphF and hphR, which have an *Sfi*I site at both ends. The ligated fragment was finally amplified with primers BRM2AF/BRM2BR to obtain the disruption vector, and transformation with this fragment led to the replacement of *BRM2-1* with *hph* (Fig. 1). The *ALM1* replacement vector was similarly constructed by the fusion PCR method (Kuwayama et al. 2002), as shown in Fig. 1. A 560-bp fragment (left-side arm of the vector) and a 600-bp fragment (right-side arm of the vector) internal to the *ALM1* ORF were amplified by PCR from the genomic DNA of As-27, using primers ALMAF/ALMAR and ALMBF/ALMBR, respectively. The primer set was designed for the deletion of the *ALM1* internal sequence, which contains an acyl carrier protein (ACP) domain (also known as a phosphopantetheine attachment site [PP] domain). The *hph* marker cassette was amplified by PCR from p*71sfi* with primers fushphF/fushphR. Those PCR fragments were mixed, and the final fusion product was amplified using nested primers ALMAF/ALMBR. The final fused products were purified with a QIAquick Kit (Qiagen, Tokyo, Japan) before transforming the *A. alternata* tomato pathotype As-27.

Table 1 Oligonucleotide primers used in this study

Primers	Sequences (5′–3′)
BRM2AF	tcctgccgaaccctgagaga
BRM2AR-sfira	cacggcctatatggccaccggtatcccaacatccat
BRM2BF-sfira	gtgggccacgcaggccagccagtagacatcgcccgt
BRM2BR	gctggccgctagcaaggtaa
HphF	gacgtctgtcgagaagtttc
HphR	gtattgaccgattccttgcg
BRM2inF	cgtggtatcggaaaggcgat
BRM2inR	ttgggtacacccttggcctg
BRM2homoF	cggattgcgtccggagttca
HphhomoR	caatagctttgggacgatgcaag
ALMAF	ccacagctccacttctcagggcacttcatg
ALMAR	atcaggtcgatgctagcatcgatgatgaggacgaagaggt
ALMBF	atgcgagtgctaccagatgtcgatagccttgacagaggtg
ALMBR	ctgcccaagttgagcctatccttgagaagt
ALMhomoF	ccatccaccgacgaggtaag
fushphF	gatgctagcatcgacctgatttacactttatgcttccg
fushphR	acatctggtagcactcgcatcttcgctattacgcca
ALMinF	gagcacaagaactcaccatc
ALMinR	cactgtacgctgaccaggcc

Transformation and screening

Fungal protoplasts were prepared using the described method (Akamatsu et al. 1997) with modifications. Protoplasts (80 µl, 1.25×10^8 protoplasts/ml) were transformed with the disruption vectors by methods described previously (Akamatsu et al. 1997). Three different pairs of primers were used to identify the *ALM1*- or *BRM2-1*-deleted mutants from the hygromycin B-resistant colonies. First, a pair of primers for the *hph* cassette was used to verify the insertion of the vectors. Then, primer pairs ALMinF/ALMinR or BRM2inF/BRM2inR were used to verify the insert. Primer pairs ALMhomoF/HphhomoR or BRM2homoF/HphhomoR were used to examine integration of the *hph* cassette by a double-crossover homologous recombination event at the *ALM1* and *BRM2-1* loci, respectively. Putative disruptants yielding the expected diagnostic amplification fragments (Fig. 1) were purified by single-spore isolation.

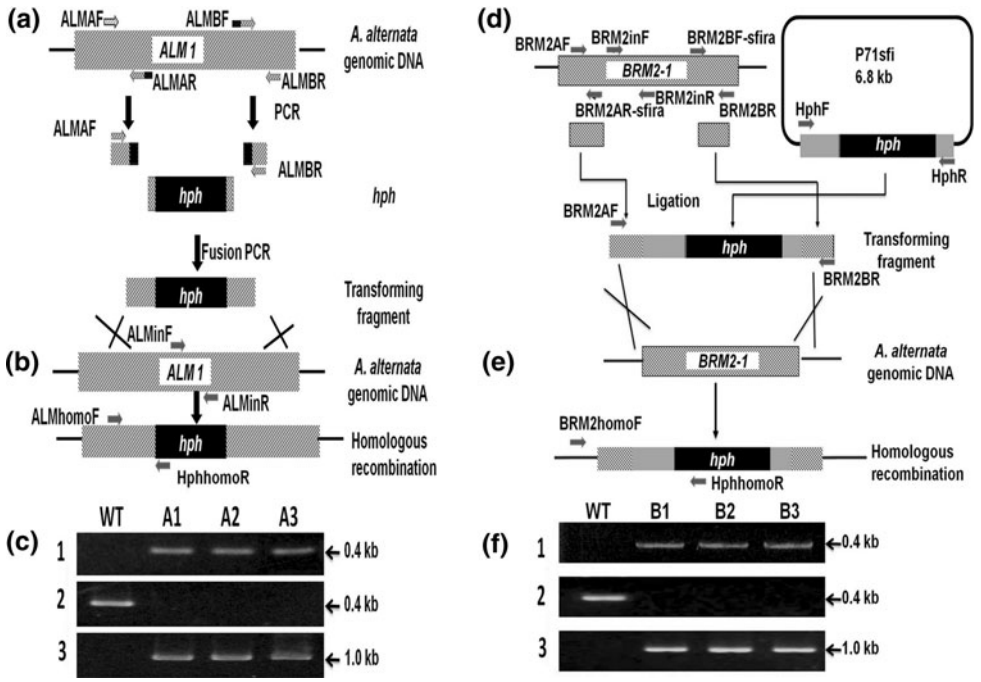

Fig. 1 Deletion strategies for *ALM1* and *BRM2-1* genes in genome of tomato pathotype *Alternaria alternata*. **a** Fusion PCR was used to construct the *ALM1* replacement vector. All PCR primers used in this figure are listed in Table 1. **b** *ALM1* locus before and after replacement of *ALM1* with marker gene *hph*. **c** PCR analysis of gene replacement events in *ALM1* in wild-type strain As-27 (WT) and three *ALM1* disruptants (A1 to A3) using primer pairs HphF/HphRc-1, ALMinF/ALMinRc-2, and ALMhomoF/HphhomoRc-3. **d** Construction of *BRM2-1* gene replacement vector by PCR. **e** *BRM2-1* locus before and after replacement of *BRM2-1* with marker gene *hph*. **f** PCR analysis of gene replacement events in *BRM2-1* in wild-type strain As-27 (WT) and three *BRM2-1* disruptants (B1 to B3) using primer pairs HphF/HphRf-1, BRM2inF/BRM2inR f-2, andBRM2homoF/HphhomoRf-3

Conidia formation

The wild-type and mutant strains were grown on V8 juice agar plates at 25°C for 14 days, followed by placing the plates under a BLB light (FL20S, 20 W, Toshiba, Osaka, Japan) for 2 days after removing the aerial mycelia with a sterilized brush. Conidia that had formed on the plates were brushed off, washed three times and then suspended in sterilized water as described previously (Yamagishi et al. 2006).

UV irradiation

To study the UV tolerance of the mutants, we dropped 20 µl of a conidial suspension (1.0×10^6 conidia/ml) from the wild-type or mutants onto a cellophane membrane (Wako Pure Chemical, Osaka, Japan) on a glass slide, followed by exposure with UV light (254 nm) using a GS Gene Linker UV chamber (Bio-Rad). The UV strength was set at 600 W/cm^2 on the membranes, and the conidia were irradiated for 0–180 s. After incubation at 25°C for 24 h, the viability of 200 conidia was evaluated by assessing germination with microscopy after 1% (w/v) aniline blue staining. The experiment was repeated three times in each treatment. Colony growth of the strains was examined in Petri dishes containing 20 ml of PDA. Mycelial blocks of 7-day-old cultures on PDA (2 mm in diameter) were transferred to new PDA plates followed by exposure to UV light under the same conditions already described and incubation for 1 week at 25°C. The colony diameter was measured daily during incubation. The mean colony diameter was calculated from three independent experiments.

Scanning electron microscopy (SEM) with an ionic liquid (IL)

The IL used in this study, 1-ethyl-3-methyl-imidazolium tetrafluoroborate (EMI-BF4) (Tokyo Chemical Industry, Tokyo, Japan) (Jiménez and Bermúdez 2009), was purified before use. EMI-BF4 was mixed with pure water prior to use and vortexed vigorously for 1 min. The mixture was then pre-warmed at 40°C in a block heater to decrease its viscosity. Conidia from the wild-type and the mutants were fixed with 2.5% (v/v) glutaraldehyde for 2 h, washed with pure water, and then mounted on electrical conductive tape (Nisshin EM, Tokyo, Japan) that was placed on an aluminum sample stub for SEM. Fifty microliters of the

IL-water mixture was dropped onto the mounted conidia and left for 1 min at room temperature. Excess IL was absorbed onto a laboratory tissue. Conductivity staining, dehydration, drying, and coating were not done before examination with a Hitachi S3400N SEM (Hitachi High-Technologies, Tokyo, Japan) in high-vacuum mode (<1 Pa) with a working distance of 6 mm.

Results

Cloning and targeted disruption of *ALM1* and *BRM2-1* in the tomato pathotype of *A. alternata*

The genes encoding a PKS (*ALM1*) (GenBank accession AB665444) and a 1,3,8-THN reductase (*BRM2-1*) (GenBank accession AB665445) for melanin biosynthesis in the tomato pathotype of *A. alternata* (*A. arborescens*) were determined by analyzing the draft sequence of the As-27 strain. The full-length As-27 *ALM1* and *BRM2-1* genes were 6393 and 804 bp, respectively. *ALM1* had 98% identity at the nucleotide level and 98% at the deduced amino acid level with the melanin PKS gene of *A. alternata* BCRC30501 (GenBank accession HM486910) (Tseng et al. 2011). *BRM2-1* had 98% identity at the nucleotide level and 99% at the deduced amino acid level with the 1,3,8-THN reductase gene (*BRM2*) of the Japanese pear pathotype *A. alternata* (GenBank accession AB015743) (Kawamura et al. 1999). The predicted amino acid sequence of *ALM1* had a high degree of homology to the type I PKS genes for melanin biosynthesis such as *C. lagenarium PKS1* (Takano et al. 1995), and it contained four potential catalytic modules: beta-ketoacyl synthase (KS), acyltransferase (AT), ACP and thioesterase (TE).

To examine the role of *ALM1* and *BRM2-1* on morphology, growth, pathogenicity and toxin production of the tomato pathotype, we examined the effects on the pathogen of deleting these genes using transformation-mediated gene disruption. Two targeting vectors containing partial fragments of *ALM1* or *BRM2-1* were constructed to disrupt the genes by homologous recombination (Fig. 1). Transformation of the tomato pathotype As-27 protoplasts with the *ALM1*- and *BRM2-1*-disruption vectors resulted in 24 and 27 colonies, respectively, which were able to grow on PDA plates containing hygromycin B. Three white (A1 to A3) and brown mutants (B1 to B3) were selected to examine homologous integration by PCR screening. Primer set HphF/HphR produced the expected 0.4-kb band for all of the mutants (Fig. 1c1, f1), and primer sets ALMinF/ALMinR and BRM2inF/BRM2inR did not amplify any fragments from the mutants (Fig. 1c2, f2), suggesting that *ALM1* and *BLM2-1* were deleted by homologous integration of each disruption vector. To confirm the *ALM1* and *BRM2-1* disruption, we used primer combinations ALMhomoF/HphhomoR and BRM2homoF/HphhomoR to detect the junctions between the recipient *ALM1* and *BRM2-1* regions and the integrated vectors, respectively (Fig. 1b, e). With these primer combinations, PCR failed to produce DNA fragments for the wild-type strain. By contrast, primer combinations ALMhomoF/HphhomoR and BRM2homoF/HphhomoR produced the expected 1.0-kb band in all mutants (Fig. 1c3, f3). The deletion of *ALM1* and *BRM2-1* in the As-27 strain through homologous integration of the each disruption vector was confirmed in the albino (A1–A3) and brown (B1–B3) mutants. One of each mutant type (A1 and B1) was selected for further work.

Phenotypic characterization of *ALM1*- and *BRM2-1*-targeted strains

The effects of the *ALM1* and *BRM2-1* disruptions on conidiation and vegetative growth were examined. Agar blocks of colonies grown on PDA plates were transferred onto V8 juice agar and grown at 25°C for 14 days. Then

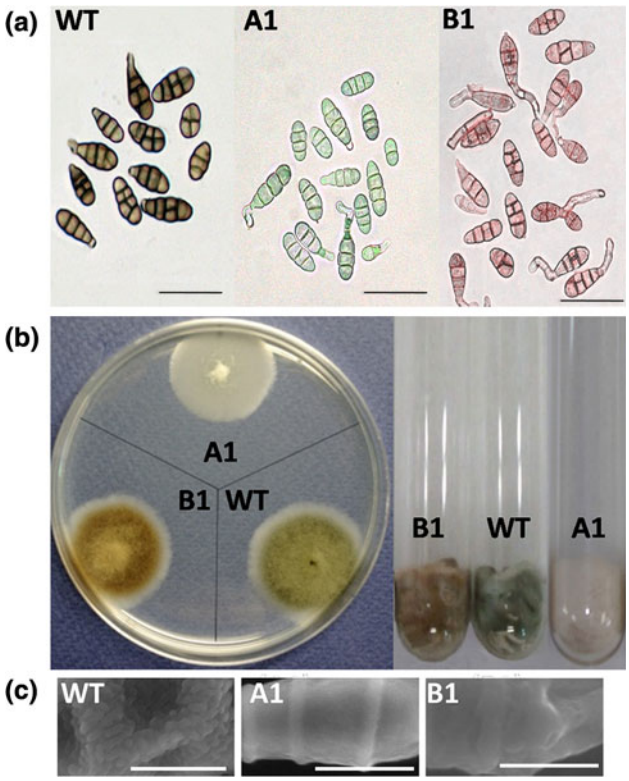

Fig. 2 Melanin production and morphology of *Alternaria alternata* As-27 wild-type (WT) and *ALM1*-disrupted (A1) and *BRM2-1*-disrupted (B1) mutants. **a** Light micrographs of conidia. *Bars* 20 μm. **b** Melanin production on PDA plate (*left*) and on rice media after 5 days of culture. **c** Scanning electron micrographs of conidial surface. *Bars* 5 μm

the plates were placed under BLB lamps. The wild-type tomato pathotype strain produces melanin, a black pigment, which accumulates in the cell walls of hyphae and conidia. Two phenotypic classes of color mutants, an albino mutant (*alm1* mutant) and a brown mutant (*brm2-1* mutant), were obtained by transformation (Fig. 2). The *alm* mutant A1 produced colorless spores, while the *brm2-1* mutant B1 produced brown spores (Fig. 2a). The disrupted mutants produced shorter and narrower conidia and very few with longitudinal septa (Fig. 2a). Conidium length/width (μm) of the wild-type, A1 and B1 strains was 29.2 ± 4.4/10.2 ± 0.7, 25.5 ± 4.2/8.0 ± 0.3 and 27.0 ± 3.2/9.9 ± 0.6, respectively. Conidial yield and vegetative growth rate did not differ significantly between the wild-type strain and the *alm1* and *brm2-1* mutants (data not shown).

The surface structures of conidia of the wild-type and mutant strains were examined with IL-SEM. The surface of the wild-type conidia was rough and ornamented with many protrusions. Conidia of the *alm1* mutant, however, had a smooth surface and lacked ornamentation. Those of the *brm2-1* mutant had less ornamentation than the wild-type (Fig. 2c). These results indicate that the melanin produced by the pathogen affects not only conidial size and shape but also the surface structure of conidia.

Pathogenicity and AAL-toxin production of *ALM1*- and *BRM2-1*-targeted strains

The pathogenicities of the mutants were tested by spraying conidia of each strain onto young detached leaves of susceptible tomato cv. Aichi-first. The wild-type and the *alm1* and *brm2-1* mutants caused necrotic lesions within 3 days after inoculation. The number and size of the lesions were nearly the same on all leaves (Fig. 3a).

When the strains were cultured on rice medium to compare their ability to produce AAL-toxin, HPLC quantification of AAL-toxin T_A revealed that the wild-type and the mutant strains did not differ (data not shown). In addition, in a leaf necrosis bioassay with the susceptible tomato plants to determine production of AAL-toxin in the culture, the *alm1* and *brm2-1* mutants were equivalent to the wild-type strain (Fig. 3b).

UV tolerance of *ALM1*- and *BRM2-1*-targeted strains

After conidial suspensions of the wild-type and mutant strains on the cellophane membranes were exposed to UV irradiation for 0–180 s and then incubated for 24 h at 25°C, conidia from the *alm1* and *brm2-1* mutants were more sensitive to UV irradiation than those from the wild-type strain (Fig. 4a). Conidia from the *alm1* mutant A1 completely lost the ability to germinate after exposure to UV

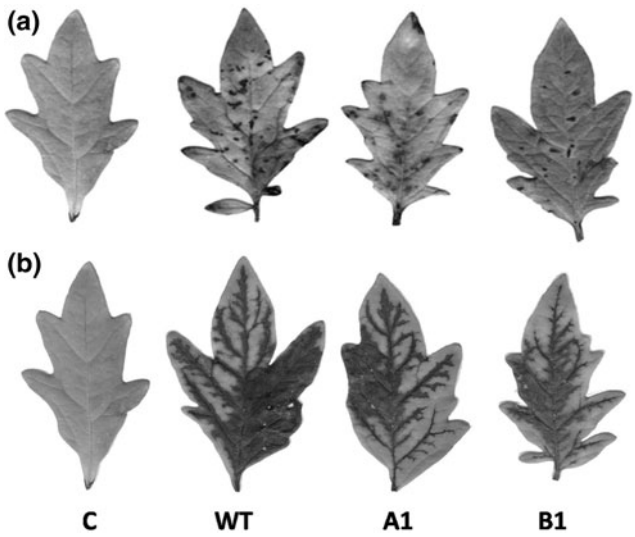

Fig. 3 Pathogenicity test (**a**) and tomato leaf necrosis bioassay for AAL-toxin (**b**) after 3 days at 25°C. **a** Leaves were inoculated with a spore suspension (10^5 conidia/ml) of *Alternaria alternata* As-27 wild-type (WT) or with *ALM1*-disrupted (A1) or *BRM2-1*-disrupted (B1) mutants. *C* is the DW control treatment. **b** AAL-toxin production in leaves of susceptible tomato cultivar Aichi-first that were treated with culture filtrates of WT, A1 or B1

irradiation for 110 s. By contrast, the germination rates were 15% in the *brm2-1* mutant and 22% in the wild-type (Fig. 4a). The *brm2-1* mutant B1 completely lost the ability to germinate after exposure to UV irradiation for 120 s. UV irradiation reduced the colony radial growth rate of the *alm1* mutant after exposures up to 180 s (Fig. 4b). However, the *brm2-1* mutant and the wild-type strains were more resistant to UV irradiation. These results indicate that melanin confers UV tolerance when it is densely deposited on the conidial surface.

Discussion

Melanin is thought to play important roles in development, longevity and stress tolerance in fungi. It is also an essential factor for pathogenicity in many plant and animal pathogens, such as *M. grisea* (Howard et al. 1991), *C. lagenarium* (Kubo et al. 1982) and *A. fumigatus* (Pihet et al. 2009). In these plant pathogenic fungi, melanization of the appressorium is needed for the fungus to be able to penetrate host tissues; melanin mediates the build-up of pressure in the appressorium, providing the essential driving force for mechanical penetration (Howard and Ferrari 1989). Therefore, a melanized appressorium is critical for the pathogenicity of *M. grisea* and *C. lagenarium* against host plants. By contrast, *A. alternata* produces tiny colorless appressoria, and in a study with MNNG mutants, melanin was discovered to have no direct involvement in the pathogenicity of the Japanese pear pathotype

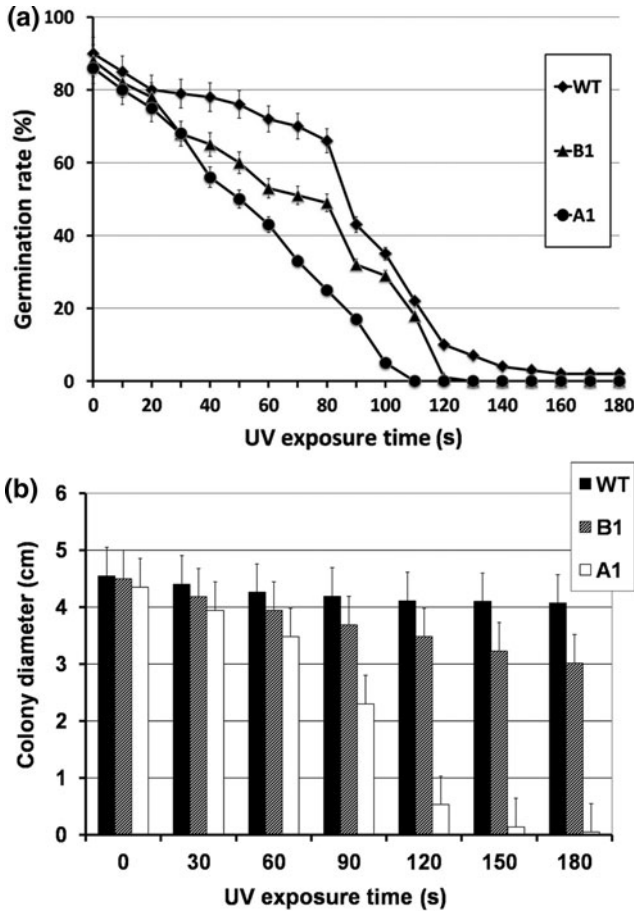

Fig. 4 Effect of UV radiation (0–180 s, 254 nm, 600 W/cm^2) on mean conidial germination (**a**) and hyphal growth (**b**) of *Alternaria alternata* As-27 wild-type (WT) and *ALM1*-disrupted (A1) and *BRM2-1*-disrupted (B1) mutants. Three independent experiments were run for (**a**) and for (**b**). **a** Mean germination rate for 200 conidia in conidial suspensions on cellophane membranes on a glass slide at 25°C for 24 h after treatment. **b** Colony diameter at 25°C on PDA at 1 week after treatment. Mycelial blocks of 7-day-old culture on PDA were transferred to new PDA plates then exposed to UV light

A. alternata (Tanabe et al. 1995). Melanin-deficient mutants of *C. heterostrophus* also had the same degree of pathogenicity on corn as the wild-type in growth-chamber experiments (Fry et al. 1984) but melanized wild-type strains are more resistant than mutant strains to UV irradiation (Dixon et al. 1991; Kawamura et al. 1999; Wang and Casadevall 1994).

A cosmid clone harboring a gene cluster (ca. 30 kb) containing three genes, *ALM*, *BRM1*, and *BRM2*, which can restore the albino (*alm*), light brown (*brm1*) and brown (*brm2*) mutants, respectively, was isolated in the Japanese pear pathotype of *A. alternata* (Kimura and Tsuge 1993). Cloning and functional analysis by targeted disruption revealed the importance of the *BRM2* gene in the Japanese pear pathotype (Kawamura et al. 1999). In this study, we isolated the *ALM*-homologous PKS gene *ALM1*, which is involved in melanin biosynthesis, and the *BRM2*-homologous *BRM2-1* gene from the tomato pathotype *A. alternata* and analyzed their functions by gene targeting. This is the first report of the isolation and functional analysis of the melanin PKS gene in *A. alternata* plant pathogens. The structural analysis of *BRM2-1* indicated that the gene has high homology with *BRM2* from the Japanese pear pathotype and that it encodes the 1,3,8-THN reductase for melanin biosynthesis. The deduced amino acid sequence of *ALM1* had a high degree of homology with the type I PKS genes for melanin biosynthesis, including *Bipolaris oryze PKS1* (Moriwaki et al. 2004), *C. lagenarium PKS1* (Takano et al. 1995) and *A. fumigatus alb1*(*pksP*) (Pihet et al. 2009), and potential catalytic modules such as KS, AT, AC and TE.

To investigate the involvement of these melanin biosynthetic genes on the pathogenicity of the tomato pathotype, we inoculated susceptible tomato leaves with conidial suspensions of the wild-type strain and *alm1* and *brm2-1* mutants. The lesions were almost the same on leaves inoculated with the wild-type and each of the transformants, consistent with previous observations in the MNNG-induced melanin-deficient mutants and in the *brm2* mutant of the Japanese pear pathotype *A. alternata* (Tanabe et al. 1995; Kawamura et al. 1999). The results of the *ALM1* and *BRM2-1* disruptions indicate that melanin is not relevant to the pathogenicity and virulence of the toxin-dependent necrotrophic pathogen *A. alternata*. On the other hand, melanin is considered to be an essential factor for pathogenicity in *M. grisea* (Howard et al. 1991) and *C. lagenarium* (Kubo et al. 1982). These two pathogens and *A. alternata* similarly produce melanin during vegetative growth and accumulate melanin in growing mycelia. On the other hand, conidia of *A. alternata* contain melanin and have a dark brown or black color, while *M. grisea* and *C. lagenarium* have nonmelanized, colorless conidia (Emmet and Parberry 1975). By contrast, appressoria of *A. alternata* and *M. grisea*/*C. lagenarium* are colorless and melanized, respectively. Appressorium melanization of *M. grisea*/*C. lagenarium* is a critical step for the successful penetration of host tissues by these pathogens because it generates turgor pressure (Howard et al. 1991). *Alternaria alternata* plant pathogens might not require this step mediated by melanization of appressoria to penetrate host cells.

Melanin accumulation in fungal cell walls has been thought to confer tolerance to environmental stresses such as UV radiation (Akamatsu et al. 2010; Kawamura et al. 1999; Moriwaki et al. 2004; Wang and Casadevall 1994). When melanized wild-type and nonmelanized *alm1* and *brm2-1* mutants were exposed to UV light, the conidia and mycelia of the mutants were more sensitive to UV irradiation than those of the wild-type. UV tolerance of the fungi is dependent on the degree of melanization because the

alm1 mutant is more sensitive than the *brm2-1* mutant. These results demonstrate that melanin plays a role in protection from UV irradiation when it is densely deposited on the conidial surface by reducing the transmission of UV light through the conidial wall. In addition to the UV tolerance, Lockwood (1960) reported that melanin confers resistance to *Alternaria* mycelia against microbial lysis in soil, and Jacobson et al. (1995) reported the antioxidant function of melanin in *A. alternata*. Therefore, melanin in *A. alternata* might play an important role in fitness, survival and pathogenicity. This speculation is supported by results in *C. heterostrophus*; the albino strains do not induce disease under field conditions even though the albino strains and the wild-type cause similar diseases under laboratory conditions (Fry et al. 1984).

Mutations in the *ALM1* and *BRM2-1* genes also led to morphological changes in the conidia. IL-SEM studies of the mutants revealed considerable changes in the organization of the conidial surface structures, with the loss of the outermost ornamentations normally seen on the conidial surface in the wild-type strain. In the animal pathogen *A. fumigatus*, similar changes in the conidial wall were observed in melanin-mutant strains (Pihet et al. 2009). However, the structural changes were considered to affect the virulence of the pathogen.

The results of this study clearly demonstrated that in *A. alternata*, melanin does not play a critical role in the pathogenicity of the pathogen under laboratory conditions. However, the roles of melanin in the fitness, survival and pathogenicity of *A. alternata* in the field remain unknown.

Acknowledgments We thank R.P. Oliver for providing pAN7-1, T.L. Peever and D.G. Gilchrist for providing *Alternaria* strains, and K. Takao for technical assistance. This work was supported by Grant-in-Aid for Scientific Research (S) (19108001), (A) (23248007) and (B) (23380025) from the Japanese Society for Promotion of Sciences, and Global COE Program "Advanced Utilization of Fungus/Mushroom Resources for Sustainable Society in Harmony with Nature", MEXT, Japan.

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