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Alternaria alternata transcription factor CmrA controls melanization and spore development

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Melanin is a black pigment widely distributed across the kingdoms, from bacterial to human. The filamentous fungus *Alternaria alternata* is a typical 'black fungus', which produces melanin in its hyphal and especially its asexual spore cell walls. Its biosynthesis follows the dihydroxynaphthalene (DHN) pathway with 1,8-DHN as an intermediate. Two genes, encoding a polyketide synthase (*pksA*) and a 1,3,8-trihydroxynaphthalene (THN) reductase (*brm2*), along with a putative transcription factor, CmrA, comprise a small gene cluster. Here we show that CmrA controls the expression of *pksA* and *brm2*, but that it also controls the expression of a scytalone dehydratase encoding gene (*brm1*) located elsewhere in the genome. The regulatory function of CmrA was shown in a reporter assay system. *Al. alternata* CmrA was expressed in the filamentous fungus *Aspergillus nidulans* where it was able to induce the expression of a reporter construct under the control of the putative *pksA* promoter. This suggests direct binding of CmrA to the promoter of *pksA* in the heterologous system. Likewise, silencing of *cmrA* in *Al. alternata* led to white colonies due to the lack of melanin. In addition, hyphal diameter and spore morphology were changed in the mutant and the number of spores reduced. Silencing of *brm2* and inhibition of melanin biosynthesis by tricyclazole largely phenocopied the effects of *cmrA* silencing, suggesting a novel regulatory function of melanin in morphogenetic pathways.

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INTRODUCTION

Melanin is a high-molecular-mass black pigment, which is produced across kingdoms and in general serves protective roles against UV light, reactive oxygen species (ROS) and other environmental stresses. This applies also to filamentous fungi such as *Alternaria alternata*. This mould belongs to the group of 'black fungi', where melanin is very abundant in cell walls especially of the spores (Carzaniga *et al.*, 2002). *Al. alternata* causes large economic losses of food and feed every year, because it not only produces melanin but a large variety of secondary metabolites such as carcinogenic alternariol (Pruß *et al.*, 2014; Saha *et al.*, 2012; Tsuge *et al.*, 2013). As in other organisms, it was shown that melanin-deficient strains are more sensitive to UV light (Kawamura *et al.*, 1999). In addition to the protective function, melanin may play different roles. For instance, in *Magnaporthe grisea*, the causal agent of rice blast disease, melanization of the cell walls of appressoria is required to build up sufficient pressure to penetrate the plant surface (Bechinger *et al.*, 1999; Howard *et al.*, 1991).

The biosynthesis of melanin can follow different biosynthetic routes, among which is the 1,8-dihydroxynaphthalene (DHN) pathway with 1,8-DHN as intermediate (Eisenman & Casadevall, 2012). As in many other fungi, in *Al. alternata* melanin is synthesized via this pathway (Kimura & Tsuge, 1993). Its biosynthesis requires a polyketide synthase (ALM or PksA in *Al. alternata*, Pks18 in *Cochliobolus heterostrophus*), a 1,3,6,8-trihydroxynaphthalene (THN) reductase (Brn2 in *Coc. heterostrophus*), a scytalone dehydratase (Scd1 in *Coc. heterostrophus*, Brm1 in *Al. alternata*), and a 1,3,8-THN reductase (Brn1 in *Coc. heterostrophus* and Brm2 in *Al. alternata*) (Eliahu *et al.*, 2007). The phenolic compound DHN is then probably oxidatively polymerized. In *Al. alternata, brm2* and *pksA* were cloned by complementation of melanin-deficient *Al. alternata* mutants (Kimura & Tsuge, 1993; Tseng *et al.*, 2011). Three classes of mutant of *Al. alternata* pear pathotype were isolated, one albino mutant, one light-brown and a brown one. Using a cosmid library, all three mutants could be complemented with the same cosmid, suggesting that all three genes are located in a 30 kb genomic region (Kimura & Tsuge, 1993). In addition to genes encoding enzymes required for melanin biosynthesis,

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DHN, dihydroxynaphthalene; mCDB, modified Czapek–Dox Broth; ROS, reactive oxygen species; siRNA, small interfering RNA; THN, trihydroxynaphthalene.

The GenBank/EMBL/DDBJ accession number for the *crmA* sequence of ATCC66981 or DSM12633 is KJ939356.

One supplementary figure is available with the online version of this paper.

a regulator for melanin biosynthesis was discovered. It was first described in *Colletotrichum lagenarium* and *M. grisea* in a screen for *M. grisea* insertional mutants with reduced pathogenicity (Tsuji *et al.*, 2000). In *Coc. heterostrophus* the regulator gene, *cmr1* is closely linked to the polyketide synthase and *brn1* (Eliahu *et al.*, 2007).

Here, we studied the role of the Cmr1 homologue, CmrA, in *Al. alternata* and found that it is located between the *pksA* and the *brm2* genes, and that it controls the expression of at least three structural genes for melanin biosynthesis.

METHODS

Culture conditions and harvesting of spores of *Al. alternata*. *Al. alternata* ATCC66981 or DSM12633 cultures were grown on modified Czapek–Dox Broth (mCDB) agar. For RNA analysis, cultures were incubated in liquid mCDB medium for 3 or 7 days at 28 °C. To inhibit melanin biosynthesis, 3 mg tricyclazole was diluted in 100 µl DMSO and mixed with 100 ml mCDB agar to a final concentration of 30 µg ml^{-1} (Lee *et al.*, 2003). *Al. alternata* strains were grown for 3 days on mCDB agar supplemented with tricyclazole and on mCDB with 100 µl DMSO as control.

Aspergillus nidulans was grown on supplemented minimal medium (MM) and standard strain construction procedures were used (Hill & Kafer, 2001). Expression of tagged genes under control of the *alcA* promoter was regulated by the carbon source; repression on glucose, derepression on glycerol, and induction on threonine or ethanol (Waring *et al.*, 1989).

Protoplast transformation of *Al. alternata* and of *As. nidulans*. *Al. alternata* spores were harvested from a mCDB culture plate and inoculated in 300 ml Richard's Liquid Medium (sucrose 20 g l^{-1}, KNO_3 10 g l^{-1}, KH_2PO_4 5 g l^{-1}, $MgSO_4.7H_2O$ 2.5 g l^{-1}, yeast extract 1 g l^{-1}) for 19–24 h at 30 °C and 120 r.p.m. Mycelium was harvested by filtering, washed with 0.7 M NaCl and digested in Kitalase (Wako Chemicals) suspension (60 mg in 6 ml 0.7 M NaCl) for 2–3 h under shaking at 110 r.p.m. and 30 °C. Protoplast quality and quantity were checked microscopically. Protoplasts were separated by filtering through two layers of Miracloth and washed with 0.7 M NaCl by centrifugation in a Hettich Universal 320R microfuge at 2430 r.p.m. at room temperature for 10 min, followed by a second washing step with 10 ml ice-cold 0.7 M NaCl, centrifugation again at 2430 r.p.m. at 4 °C for 10 min. A second washing step with 10 ml STC [1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris/HCl (pH 8.0)] and another centrifugation step (2400 r.p.m., 4 °C, 10 min) followed. The pellet was resuspended in 70 µl STC and protoplasts were placed on ice and 5–10 µg DNA were added to the protoplast suspension followed by incubation on ice for 10 min. Cells were heat-shocked for 2–10 min at 42 °C and further incubated on ice for 10 min. Next, 800 µl of 40 % PEG in STC was added to the suspension and the cells were incubated for 15 min at room temperature. The suspension was then mixed with regeneration medium [1 M sucrose, 0.5 % caseic acids, (w/v) 0.5 % (w/v) yeast extract] and warmed to 42 °C. Cultures were incubated at 28 °C for 24 h and then overlaid with 25 ml regeneration medium supplemented with hygromycin B (100 mg ml^{-1}). Plates were incubated for up to 10 days (Cho *et al.*, 2006; Pruß *et al.*, 2014). Standard methods were used for plasmid constructions. All plasmids used in this study are listed in Table 1 and all oligonucleotides listed in Table 2.

Transgenic *As. nidulans* strains were generated by protoplast transformation as described previously (Yelton *et al.*, 1984). *Al. alternata* and *As. nidulans* strains are characterized in Table 3.

Expression analysis. All samples were harvested from mycelium grown on mCDB liquid culture for 3 or 7 days in the dark at 28 °C. Fresh mycelium was frozen in liquid nitrogen and ground to a powder in a mortar. Total RNA was extracted from mycelia by using the RNeasy Plant mini-kit (Qiagen or VWR), following the manufacturer's instructions and purified by treatment with DNase I. RNA was diluted to 50 ng μl^{-1} and used as a template for quantitative reverse transcriptase (RT)-PCR, which was performed on a Bio-Rad iCycler MyIQ using the SensiFAST SYBR and the Fluorescein kit (Bioline). For each sample, three technical replicates were performed.

Laccase assay. *As. nidulans* colonies were picked on solid medium (pH 4) containing 1 mM ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] and different carbon sources. Glucose represses the *alcA* promoter, whereas glycerol derepresses and threonine induces the promoter. We used 2 % (w/v) glucose, glycerol or threonine as carbon source. Glucose (0.2 %, w/v) was added in addition to media containing glycerol or threonine, to allow better growth of the colonies. For the laccase assay mycelium was grown in liquid medium containing glucose to repress the *alcA* promoter. After 16 h of incubation at 28 °C and 120 r.p.m., mycelium was washed with sterile H_2O and transferred into a medium containing threonine to induce the promoter. After 4 h of incubation, laccase activity was measured at regular intervals. For the assay, 100 µl of the supernatant was mixed with 500 µl 100 mM sodium acetate buffer (pH 3.5), 200 µl 5 mM ABTS and 200 µl H_2O. As negative control we used 500 µl 100 mM sodium acetate buffer (pH 3.5), 200 µl 5 mM ABTS, 200 µl H_2O and 100 µl medium. Absorption was measured after 1 h of incubation at 420 nm in a Pharmacia LKB - Ultrospec III spectrophotometer (Mander *et al.*, 2006).

RESULTS

Identification of a *cmr1* orthologue close to the polyketide synthase gene involved in melanin biosynthesis

Among the central enzymes for the synthesis of fungal secondary metabolites are the polyketide synthases. The

Table 1. Plasmids used in this study

Name	Genotype	Source
pJET1.2	Blunt-end PCR fragments cloning vector	Fermentas
pSilent	Silencing vector	Nakayashiki *et al.* (2005)
pMT-OvE	Vector for overexpression in *Aspergillus nidulans* using the *alcA* promoter	Toews *et al.* (2004)
pAH16	pMT-OvE with $pksA^{899\ bp\ 5'UTR}$::*lccC, argB*	This study
pKS16	pJET1.2 with *alcA*(p)::*cmrA*	This study
pRD37	pSilent::*trpC*(p)::*cmr1* sense::IT::*cmr1* antisense::*trpC(t), hph*	This study
pMW06	pSilent::*trpC*(p)::*brm2* sense::IT::*brm2* antisense::*trpC(t), nptII*	This study

Table 2. Oligonucleotides used in this study

Name	Sequence (5′–3′)
cmr1_XhoI_fw	CTCGAGAATGCGACAAGAAGTTTCCTTG
cmr1_HindIII_rv	AAGCTTGGCTCTCATTTTCCGGTTG
cmr1_KpnI_fw	GGTACCAATGCGACAAGAAGTTTCCTTG
cmr1_BglII_rev	AGATCTGGCTCTCATTTTCCGGTTG
RNAi_Brm2_se_fw	CTATCTCGAGGCCGTGGTATCGGAAAGGC
RNAi_Brm2_se_re	CTAAAGCTTCGATAGCCATGCACCTGG
RNAi_Brm2_as_fw	CTGGTACCGCCGTGGTATCGGAAAGGC
RNAi_Brm2_as_re	CTAGATCTCGATAGCCATGCACCTGG
pksA_RT_fwd	GATTGCCATCGTCGGTATG
pksA_RT_rev	GGCTCATCGATGAAGCAAC
Cyt-P450_RT_fwd	CATTTCGTCCAGCTTGCAC
Cyt-P450_RT_rev	CTGTGACAGCACCATGAAG
Pc_RT_fwd	CATTGACCACGTCCATAGC
Pc_RT_rev	GAAGTCTCTACATGGCGAAC
MT_RT_fwd	CTTGCGCATTACGCCAATG
MT_RT_rev	GAACACAATCAATGCCTCCC
pksA(p)_XbaI_fwd	TGCTTTCTAGAGCATGAAAAGAAATCCTTCACC
pksA(p)_BsiWI_rev	TGCTTCGTACGCAGCATACATATACTCTGGCG
Brm2_RT_f	CCGTGGTATCGGAAAGGC
Brm2_RT_r	GAAGTGGGCAACAACGTCAT
Brm1_RT_f	CAATGGTAGCGATTCGAAAATC
Brm1_RT_r	GTGCGCGGACGTTGACAT
cmr1_RT_fwd	GAA ATG TCA CCT GCG CAA AC
cmr1_RT_rev	GTCTTGGGCTGCGATAATG
H2B_RT_fwd	CTCTGGCGACAAGAAGAAG
H2B_RT_rev	TTGACGAAAGAGTTGAGAATG
β-tub_RT-fwd	GTTGAGAACTCAGACGAGAC
β -tub_RT_rev	CATGTTGACGGCCAACTTC

genome of *Al. alternata* encodes at least ten of these multidomain enzymes, one of which is required for melanin biosynthesis (Kimura & Tsuge, 1993; Saha *et al.*, 2012). The *pks* gene (*ALM*, *pksA*) is located in a genomic region (33 kb) in which the 1,3,8-THN reductase gene (*brm2*) is located (Fig. 1a, b). Originally, a second open reading frame was indicated within this region and the gene was named *brm1* (Kimura & Tsuge, 1993). It was thought that it encodes the scytalone dehydratase. However, in a more recent paper it was shown that *pksA* and *brm2* are located close together in the genome, but that the scytalone dehydratase, *brm1* is located elsewhere (Tseng *et al.*, 2011). However, the third gene located in the cluster was not analyzed and is described here. The gene encodes a putative transcription factor containing two Cys_2His_2 and one $Zn(II)_2Cys_6$ DNA-binding motifs. Comparison of the genomic DNA to a cDNA sequence revealed the presence of three introns (100, 84 and 56 bp). The derived protein

Table 3. Strains used in this study

Name	Genotype	Source
Aspergillus nidulans		
RMS011	*pabaA1*; Δ*argB::trpC*Δ*B*; *veA1*	Stringer *et al.* (1991)
SKS07	RMS011 with pAH16	This study
SKS08	RMS011 with pAH16 and pKS16	This study
SAH27	RMS011 with pAH16	This study
SAH29	RMS011 with pAH16 and pKS16	This study
Alternaria alternata		
ATCC66981	Wild-type	ATCC, UK
DSM12633	Wild-type	DSM, Germany
SRD6	DSM12633 with pRD37	This study
SMW1	ATCC66981 with pMW6	This study

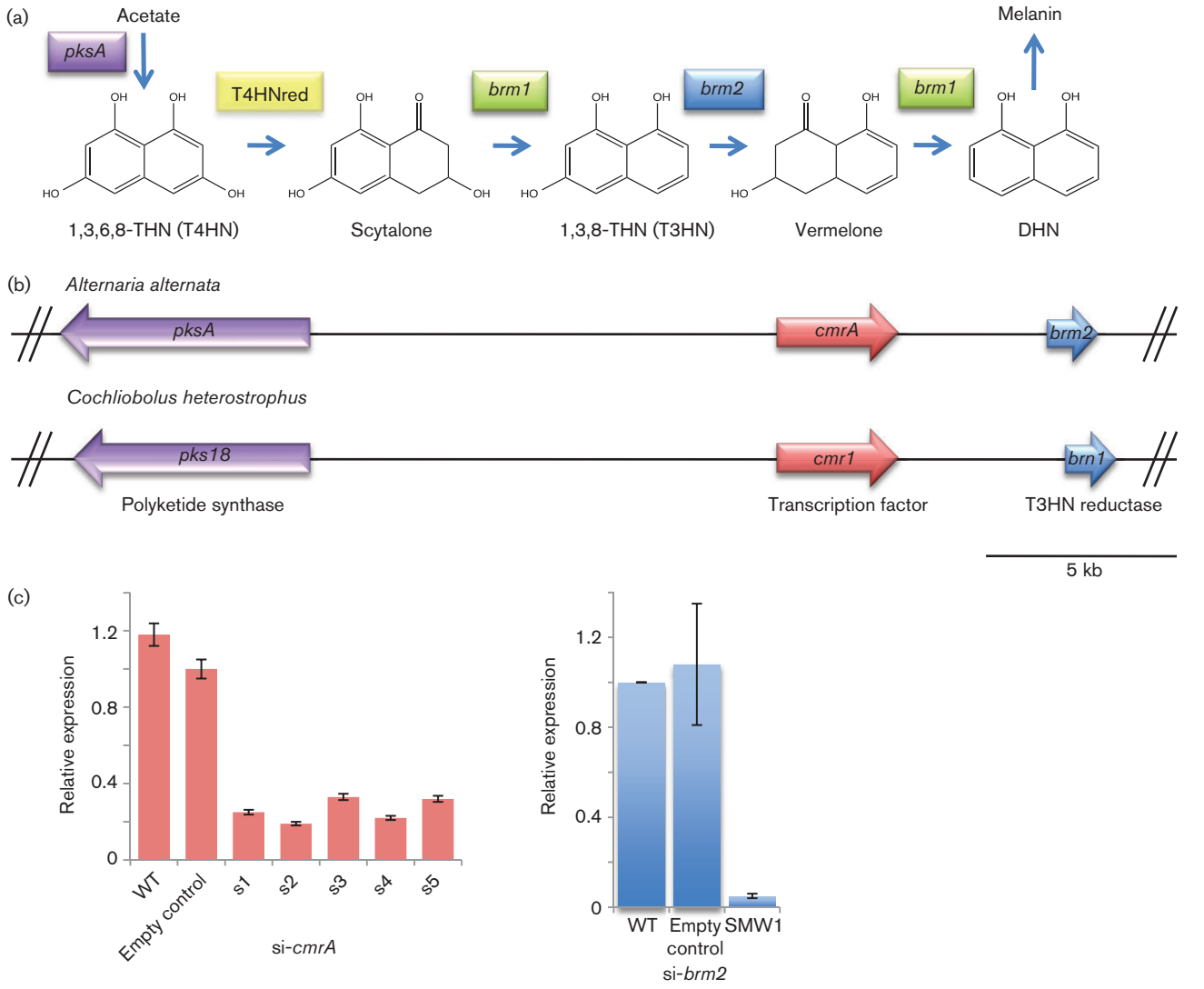

Fig. 1. Analysis of genes involved in melanin biosynthesis. (a) Schematic of the biosynthetic pathway and the role of three genes, *alm* (*pksA*), *brm1* and *brm2*. The T4HN (1,3,6,8-THN; trihydroxynaphthalene) reductase has not yet been characterized. In *Cochliobolus heterostrophus* the gene is named *brn2* (Eliahu *et al.*, 2007). The genome of *Alternaria alternata* encodes a protein with more than 80 % similarity to Brn2. DHN, dihydroxynaphthalene; T3HN, 1,3,8-THN. (b) Schematic of the gene cluster in *Al. alternata* and *Coc. heterostrophus* (Eliahu *et al.*, 2007). Two genes involved in melanin biosynthesis, *brm1* and the putative T4HN reductase gene, are not located in the melanin gene cluster. The *cmrA* sequence was deposited under the accession number BankIt1732484 KJ939356. (c) Analysis of the expression of *cmrA* and *brm2* in mycelium of wild-type and five knockdown strains (s1–s5). Strains were grown for 3 days in modified Czapek–Dox Broth medium before mycelium was harvested and RNA isolated as described in the text. In a control strain, the empty vector was transformed and the expression of *cmrA* and *brm2* studied. RT-PCR was performed as described in Methods with the β-tubulin gene as standard. The mean ± SD of three technical replicates are shown.

consists of 1010 aa and a calculated molecular mass of 112.6 kDa. The sequence was deposited at the GenBank database (accession number KJ939356). Because the protein shares 63 % identical aa with the first identified regulator of this type, Cmr1 from *Coc. heterostrophus* and 46 % with Pig1 from *M. grisea*, we named the gene *cmrA*. It is 95 % identical to the *Al. brassicicola* Amr1 protein. The same gene arrangement was described before for *Coc. heterostrophus* and is also found in *Al. brassicicola* (Eliahu *et al.*, 2007). The alignment of the sequences is shown in Fig. S1 (available in the online Supplementary Material).

Functional analysis of *cmrA* and *brm2*

Because CmrA and its orthologues in *Magnaporthe oryzae* and *Al. brassicicola* control melanin biosynthesis, we anticipated that CmrA plays a similar role in *Al. alternata*.

To analyse such a function we lowered the transcript level by small interfering RNA (siRNA). An intron-free region (598 bp) of the gene was amplified with the forward primer cmr1_XhoI_fw, containing a *Xho*I site, and the reverse primer cmr1_HindIII_rv, containing a *Hin*dIII site, to amplify the sense fragment. The same region was amplified by the primers cmr1_KpnI_fw and cmr1_BglII_rev to get the antisense construct. Both PCR fragments were double digested with appropriate enzymes and then ligated consecutively at the multiple cloning site of the pSilent-1 vector (Nakayashiki *et al.*, 2005), the sense fragment in front of the intron, which forms a hairpin loop, and the antisense fragment behind this intron. *Al. alternata* DSM12633 was transformed with the plasmid containing a hygromycin B resistance gene as the dominant marker. Five transformants appeared whitish at the colony level in comparison with the dark-green colony colour of wild-type. In addition to the colour change, the colony size was reduced to 80–90 % of the diameter of wild-type colonies. Determination of the *cmrA* transcript level revealed 80 % reduction in comparison with wild-type (Fig. 1c).

In order to determine whether the phenotypic changes caused by the silencing of *cmrA* were due to the reduction of the melanin content or to other regulatory functions of CmrA, we aimed to prevent melanin formation without disturbing the *cmrA* function. To achieve this, the *brm2* gene was downregulated using the same approach as *cmrA*. The *brm2* gene is predicted to contain two introns (51 and 49 bp) and encodes a protein of 268 aa. For the siRNA construct, a 477 bp fragment of an intron-free region was amplified with the primers RNAi_brm2_se_fw with a *Xho*I restriction site and RNAi_brm2_se_re with a *Hin*dIII restriction site for the sense fragment, and RNAi_brm2_as_fw with a *Kpn*I restriction site and RNAi_brm2_as_re with a BglII restriction site for the antisense fragment. The fragments, as well as the pSilent vector, were double digested with the corresponding enzymes and then ligated. The sense fragment was ligated in front of the hairpin loop and the antisense fragment behind. The marker in the pSilent vector was changed from the hygromycin B resistance gene to the geneticin resistance gene (*nptII*). This final vector construct was then transformed in *Al. alternata* wild-type ATCC66981. The wild-type obtained from the ATCC strain collection was used in this experiment. The phenotype compared to the wild-type obtained from the DSM strain collection was identical. Several transformants appeared as brownish colonies. Four transformants were checked by real-time RT-PCR. One of these showed a reduction in the expression of *bmr2* of 95 % (Fig. 1c).

Both knockdown strains (si-*cmrA* and si-*brm2*) looked alike and were subsequently analysed in parallel (Fig. 2). Given that the dark colour of the colony is mainly due to the black spores produced by the mycelium, we anticipated that spores would be colourless instead of dark pigmented. Indeed the spores looked pale and not black. However, in addition the spore morphology was different in the mutants compared with wild-type. The multicellular spores in *Al. alternata* contain transversal and longitudinal septa. Whereas in wild-type an average of 5.3 compartments per spore were counted, in both mutant strains the number of compartments was reduced to 3.5 (si-*cmrA*) and 3.1 (si-*brm2*), respectively (Fig. 2b, c). This phenotype was described before for the *brm2* mutant (Kawamura *et al.*, 1999). Furthermore, the number of spores was reduced in both mutant strains. For wild-type we counted 1.15×10^6 spores per 0.25 cm^2, whereas in the *cmrA* mutant strains (RNAi-*cmrA*) only 9.2×10^4 spores per 0.25 cm^2 were counted (Fig. 2d). The effect was less obvious in the *brm2* mutant where the spore number reached 3.5×10^5. In the previous study, this slight reduction of the spore number was not obvious (Kawamura *et al.*, 1999). The fact that silencing of *brm2* caused the same effects as silencing of *cmrA* suggested that the morphological changes were due to the absence of melanin. To further substantiate our hypothesis, melanin biosynthesis was inhibited by tricyclazole. Indeed the colour of the colony was identical to the colour of the *brm2* knockdown strain, and the effects on spore septation and the number of spores were similar to the values determined for the *brm2* and *cmrA* knockdown strains (Fig. 2a–d).

Because the colony diameter was affected in both mutant strains, hyphal morphology was also analysed. We measured hyphal diameters in 10 μm increments starting 5 μm behind the tip of the hypha. Mutant hyphae were about 3 μm in diameter, whereas wild-type hyphae measured only about 2 μm at the tip (Fig. 2e). The diameter of the hyphae of the si-*cmrA* strain varied between 3.2 and 7.4 μm and the hyphae of the si-*brm2* mutant varied between 3.7 and 5.2 μm.

Melanin has been reported to play a role in protecting hyphae from ROS (Jacobson *et al.*, 1995). To test whether reduction of the melanin level caused any changes in the sensitivity against H_2O_2, strains were grown on medium containing 0 to 6 mM H_2O_2. The wild-type reached a colony diameter of 3.4 cm without H_2O_2 in the medium, and the colony size decreased with increasing concentrations of H_2O_2. At the final concentration of 6 mM H_2O_2 the colony was only 1.5 cm in diameter. After the same incubation time both mutants produced smaller colonies. At 6 mM H_2O_2 the si-*cmrA* strain did not grow any further (Fig. 3).

CmrA controls the expression of *pksA*, *brm1* and *brm2*

Downregulation of *cmrA* caused a similar phenotype as downregulation of *brm2*. This result suggested that CmrA is indeed involved in the regulation of the expression of the genes encoding the melanin biosynthetic enzymes. To confirm this hypothesis, the expression of *pksA* and *brm2* were studied in both the si-*cmrA* and si-*brm2* strains and compared to wild-type. In addition, the expression of *brm1*, encoding the scytalone dehydratase and not being located in the melanin gene cluster, was analysed. As a control, we studied the expression of several other genes,

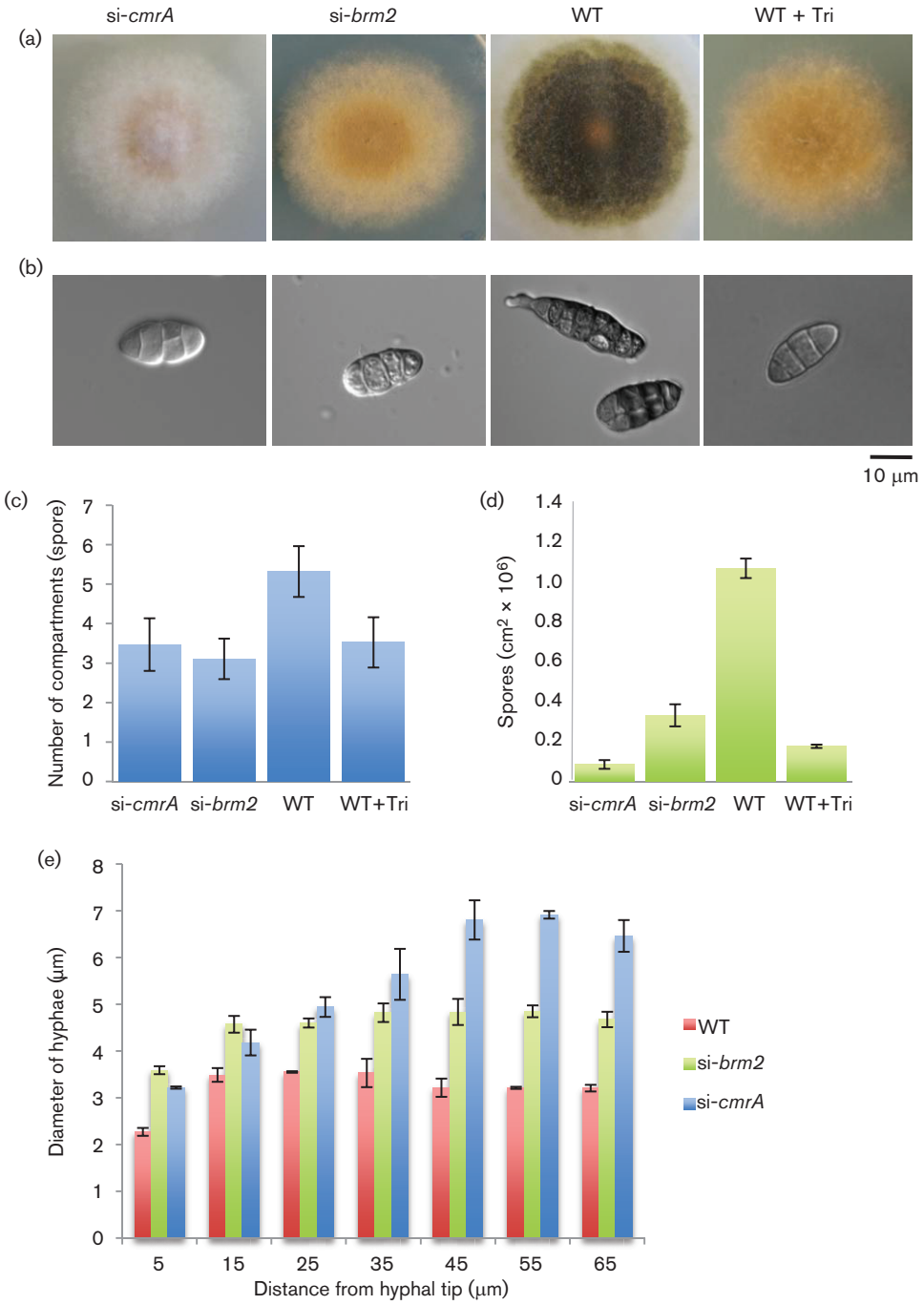

Fig. 2. Analysis of the phenotypes of si-*cmrA* and si-*brm2* and the effect of melanin biosynthesis inhibition. (a) Phenotype on modified Czapek–Dox Broth (mCDB) medium or on mCDB medium supplemented with 30 mg l^{-1} tricyclazole [WT + Tri (WT, wild-type)]. Colonies were grown for 3 days. (b) Microscopy pictures of *Al. alternata* spores in wild-type, the knockdown strains and the strain where melanin biosynthesis was inhibited. Bar, 10 µm. (c) The number of compartments per spore was counted in wild-type, the two knockdown strains and tricyclazole-inhibited wild-type. Fifty spores were analysed for each strain. (d) Number of spores of the strains indicated. Spores were counted in three independent experiments in a 0.25 cm^2 area of the colony using a Neubauer chamber. (e) Hyphal diameter in wild-type and in si-*cmrA* and si-*brm2* strains. Hyphae were grown for 20 h in liquid mCDB medium. Diameters were measured in 10 µm increments starting at the tip in five hyphae for each strain. (c–e) The mean ± SD is shown.

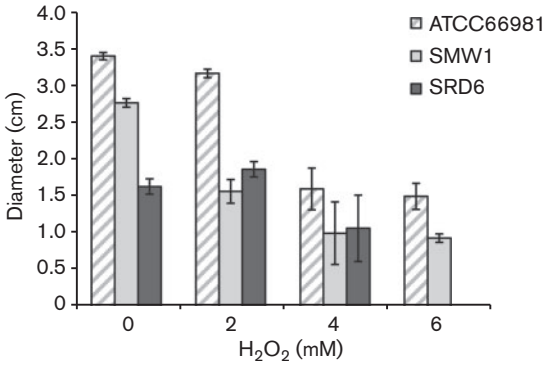

Fig. 3. Sensitivity test of wild-type (WT) and the *brm2* and the *cmrA* knockdown strains towards H_2O_2. Colonies were grown on medium containing different concentrations of H_2O_2 and colony sizes were measured after 3 days of incubation. The mean ± SD of three colonies is shown. ATCC66981 (*Al. alternate* wild-type); SRD6 (DSM12633 *Al. alternata* wild-type with pRD37); SMW1 (ATCC66981 wild-type with pMW6).

located close to pksA and brm2 in the genome (Saha *et al.*, 2012). These were a gene encoding a putative cytochrome P450 enzyme (homology to Saccharomyces cerevisiae Erg11) (Martel *et al.*, 2010), an *O*-methyl-transferase gene (*S. cerevisiae* Emg1) (Liu & Thiele, 2001), and a gene encoding a putative peroxisomal carrier protein (homology to *As. nidulans* AntA) (Hynes *et al.*, 2008) (Fig. 4). The functions of the corresponding homologues in *S. cerevisiae* and *As. nidulans* suggest roles in ergosterol biosynthesis, in 18S rRNA formation and ATP transport across the peroxisomal membrane, and make it unlikely therefore that they are involved in melanin biosynthesis. In the si-*cmrA* strain only the expression of *pksA*, *brm1* and *brm2* was reduced, suggesting an activating function for CmrA. The other genes were not affected.

The expression data suggested that CmrA controls at least the expression of *pksA*, *brm1* and *brm2*. In order to test whether CmrA directly controls and is sufficient for the expression of these genes, a reporter assay was performed. To this end CmrA was expressed in *As. nidulans* under the control of the *alcA* promoter. Along with the *cmrA* plasmid the reporter construct, consisting of the *pksA* putative promoter (899 bp upstream of the ATG start codon) fused to the *As. nidulans* laccase gene *lccC*, was cotransformed (Fig. 5). Laccase was established as a reporter in *As. nidulans* because the activity can easily be followed through the colorimetric change of ABTS after oxidation. Electrons are transferred to oxygen (Mander *et al.*, 2006). From 80 transformants 20 were chosen for the analysis on medium containing ABTS. Two transformants developed a blue colour when grown on medium containing glycerol or threonine as inducers for the *alcA* promoter and ABTS as substrate for the laccase enzyme. The relatively low frequency of positive strains can be explained by the co-transformation of the two plasmids. Strains with either plasmid alone cannot express laccase activity. In order to demonstrate the strict dependence of laccase activity on the inducing conditions, strains were first grown in liquid medium under repressing conditions (glucose) and then transferred to the induction medium. Eight hours after the transfer, laccase activity was detectable (Fig. 5).

DISCUSSION

Melanin confers resistance of many fungi to several environmental factors and is thus an important compound for survival under different conditions. In some cases, such as *Magnaporthe oryzae* or *Aspergillus fumigatus*, melanin has been shown to be an important virulence factor (Heinekamp *et al.*, 2012; Liu & Nizet, 2009). In *Al. alternata*, melanin biosynthesis has been studied at the biochemical level and through the analysis of melanin-deficient mutants (Kimura

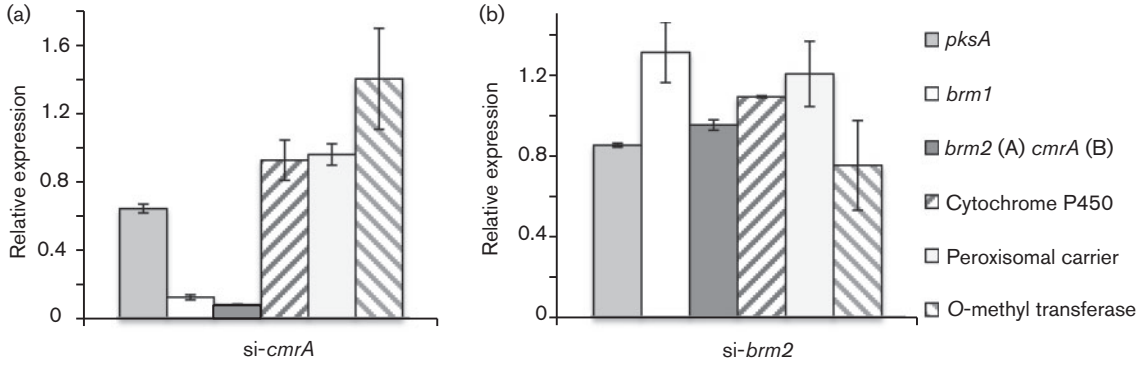

Fig. 4. Expression analysis in the si-*cmrA* and si-*brm2* strains. Expression pattern of melanin biosynthesis genes and control genes in the si-*cmrA* strain (a) and the si-*brm2* strain (b) compared to wild-type. Mycelium was incubated in liquid culture for 7 days in the dark and then harvested. RNA was extracted as described in the text. For quantitative RT-PCR, histone2B was used as standard. The mean ± SD of three biological and two technical replicates is displayed.

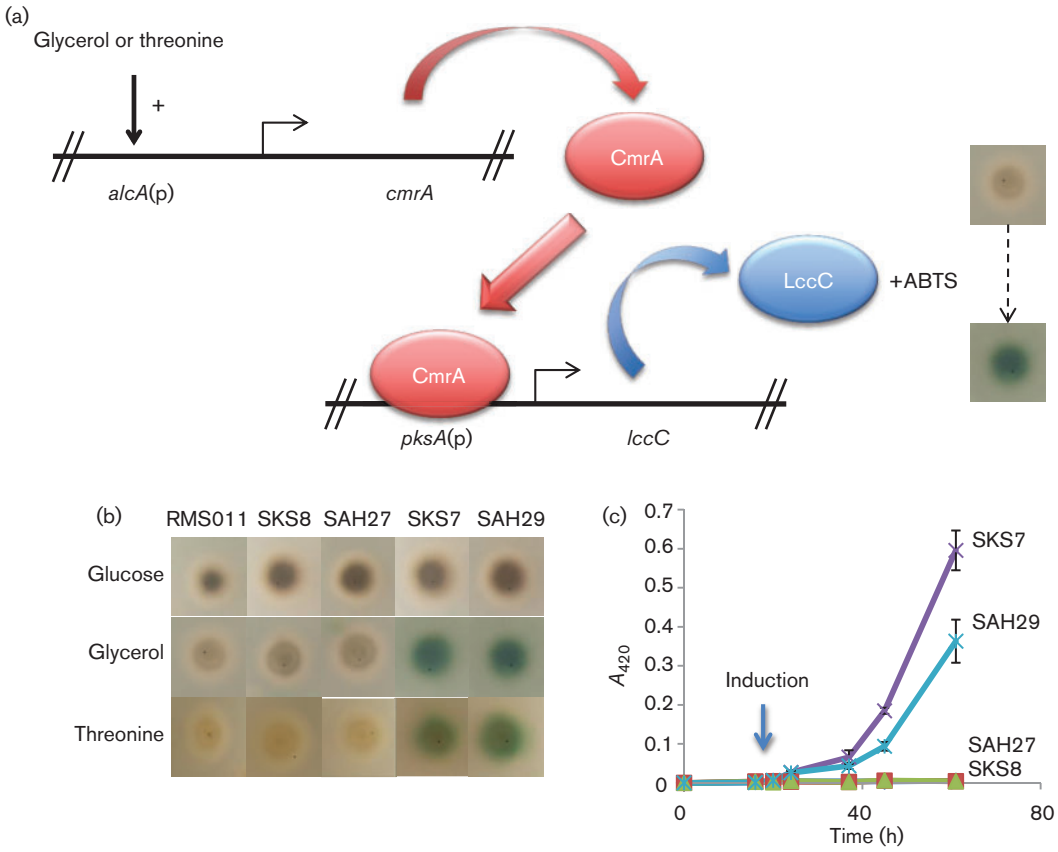

Fig. 5. Reporter gene assay in *Aspergillus nidulans*. (a) Schematic of the assay. *cmrA* expression under the *alcA* promoter [*alcA*(p)] is induced by the addition of 2 % threonine to the medium. If CmrA binds to the *pksA* promoter, *lccC* is expressed and the enzyme oxidizes ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)]. The medium turns blue (Mander *et al.*, 2006). (b) Strains were tested under repressing and inducing conditions. Expression of genes under the control of the *alcA* promoter was regulated by the carbon source; repression on glucose, derepression on glycerol and induction on threonine. The presence of the plasmids was checked by PCR. (c) Measurement of laccase activity over time. Strains were grown under repressing (glucose) conditions for 16 h, washed and then incubated under inducing (threonine) conditions. After 8 h in medium containing threonine, laccase activity was measured in strains SKS7 and SAH29 (containing both plasmids pAH16 and pKS16) but not in wild-type (RMS011) or in the control strains (SKS8, SAH27). Laccase activity was determined in three independent cultures. The mean ± SD is displayed.

& Tsuge, 1993). Two genes required for melanin biosynthesis, *alm* (*pksA*) and *brm2*, were identified and are organized in a gene cluster (Kimura & Tsuge, 1993). In addition, the scytalone dehydratase gene (*brm1*) has been identified elsewhere in the genome and characterized (Tseng *et al.*, 2011). In this paper we describe the role of the regulator CmrA, located between *pksA* and *brm2* in the melanin gene cluster and show that it is required for the expression of at least *brm1*, *brm2* and the polyketide synthase gene, *pksA*. The regulator was first discovered in *Col. lagenarium* and *M. grisea* in a screen for *M. grisea* insertional mutants with reduced pathogenicity (Tsuji *et al.*, 2000). In *Col. lagenarium*, hyphae and appressoria are melanized but Cmr1 appears to control the expression of the structural genes *SCD1* (encodes the scytalone dehydratase) and *THR1* (encodes 1,3,8-THN reductase) in hyphae but not in appressoria. We found that *brm2* (*THR1*), *brm1* (scytalone dehytratase) and *pksA* are positively regulated by CmrA, although *pksA* was less affected by the lack of CmrA. Our data are in agreement with genome-wide expression studies in *Al. brassicicola* where *brm2* (and *brm1*) appear in the list of genes regulated by Amr1, the Cmr1 transcription factor homologue (Cho *et al.*, 2012). The polyketide synthase does not appear in this list, probably reflecting only a weak effect of Amr1 on its expression. Likewise, in *Coc. heterostrophu* polyketide synthase gene *pks18* expression was only slightly affected by the lack of Cmr1 (Eliahu *et al.*, 2007).

In this paper we hence show that the *Al. alternata* regulator CmrA controls melanin biosynthesis genes, but the absence of the protein also affects spore morphology and spore formation. This raises the question of how the two processes could be linked. One explanation for the

observed developmental phenotypes in the *cmrA* knock-down strain could be that CmrA does not only regulate genes involved in melanin biosynthesis but also other genes. Indeed, transcriptional profiling of a corresponding mutant in *Al. brassicicola* revealed that the transcription factor controls more than 260 genes (Cho *et al.*, 2012). Most of these differentially expressed genes have a metabolic function, and hints regarding the regulation of development are not obvious from the gene list. Of course it has to be considered that differential expression in these experiments was studied at a late stage of cabbage infection. Thus it may be unlikely that the developmental genes, required for spore formation, would be expressed under those conditions. Hence, genome-wide expression analyses in *Al. alternata* at appropriate time points and under appropriate conditions are required to further characterize the function of CmrA, and to understand its role in developmental regulation.

Another explanation for the developmental phenotype may be a connection between melanin and ROS. One function of melanin across kingdoms is a protective role against UV light and ROS. Given that the concentration of ROS can trigger developmental decisions (Aguirre *et al.*, 2005), the lack of melanin could alter the intracellular concentration of ROS in such a way that development is disturbed. The fact that silencing of *cmrA* has a more drastic effect than silencing of *brm2* could be due to the complete lack of several intermediates of melanin biosynthesis, given that in the mutant also the *pksA* gene is downregulated. The hypothesis was further confirmed by inhibiting melanin biosynthesis through tricyclazole. Indeed, colonies appeared brownish, the number of spores was largely reduced and septation in the spores was altered. Further experiments are required to fully understand the role of melanin and ROS and the interplay with CmrA functions.

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