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Melanin Biosynthesis in the Maize Pathogen *Cochliobolus heterostrophus* Depends on Two Mitogen-Activated Protein Kinases, Chk1 and Mps1, and the Transcription Factor Cmr1▽†

Noa Eliahu,[1] Aeid Igbaria,[1] Mark S. Rose,[2] Benjamin A. Horwitz,[1] and Sophie Lev[1]*

Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel,[1] and Biopharma, Syngenta Biotechnology, Inc., 3054 Cornwallis Rd., Research Triangle Park, North Carolina 27709[2]

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The maize pathogen *Cochliobolus heterostrophus* requires two mitogen-activated protein kinases (MAPKs), Chk1 and Mps1, to produce normal pigmentation. Young colonies of *mps1* and *chk1* deletion mutants have a white and autolytic appearance, which was partially rescued by a hyperosmotic environment. We isolated the transcription factor Cmr1, an ortholog of *Colletotrichum lagenarium* Cmr1 and *Magnaporthe grisea* Pig1, which regulates melanin biosynthesis in *C. heterostrophus*. Deletion of *CMR1* in *C. heterostrophus* resulted in mutants that lacked dark pigmentation and acquired an orange-pink color. In *cmr1* deletion strains the expression of putative scytalone dehydratase (*SCD1*) and hydroxynaphthalene reductase (*BRN1* and *BRN2*) genes involved in melanin biosynthesis was undetectable, whereas expression of *PKS18*, encoding a polyketide synthase, was only moderately reduced. In *chk1* and *mps1* mutants expression of *PKS18*, *SCD1*, *BRN1*, *BRN2*, and the transcription factor *CMR1* itself was very low in young colonies, slightly up-regulated in aging colonies, and significantly induced in hyperosmotic conditions, compared to invariably high expression in the wild type. These findings indicate that two MAPKs, Chk1 and Mps1, affect Cmr1 at the transcriptional level and this influence is partially overridden in stress conditions including aging culture and hyperosmotic environment. Surprisingly, we found that the *CMR1* gene was transcribed in both sense and antisense directions, apparently producing mRNA as well as a long noncoding RNA transcript. Expression of the antisense *CMR1* was also Chk1 and Mps1 dependent. Analysis of chromosomal location of the melanin biosynthesis genes in *C. heterostrophus* resulted in identification of a small gene cluster comprising *BRN1*, *CMR1*, and *PKS18*. Since expression of all three genes depends on Chk1 and Mps1 MAPKs, we suggest their possible epigenetic regulation.

Fungal pathogens perceive and respond to a variety of environmental signals as well as molecules from the host plant, triggering morphogenetic changes. Mitogen-activated protein kinases (MAPKs) are ubiquitous and evolutionarily conserved enzymes connecting cell surface to intracellular regulatory targets activating various morphogenetic changes. The budding yeast *Saccharomyces cerevisiae* has five MAPKs, encoded by *FUS3*, *KSS1*, *HOG1*, *SLT2*, and *SMK1*. These MAPK cascades regulate fungal growth and differentiation processes such as pheromone response, pseudohyphal growth, osmoadaptation, cell wall integrity, and ascospore formation. Fus3/Kss1 orthologs in filamentous fungi are involved in appressorium formation and pathogenicity in general but have diverse functions in hyphal growth, sexual and asexual reproduction, and conidial germination (4, 18, 22, 24, 25, 27, 29, 39, 41). For example, in the rice blast fungus *Magnaporthe grisea* Pmk1 is required for appressorium formation and invasive growth on the host, whereas in the anthracnose fungus *Colletotrichum lagenarium*, Cmk1 is also required for conidiation and conidial germination (29, 39).

Slt2 homologs in filamentous fungi function to maintain cell wall integrity and are essential for the formation of functional appressoria and for virulence (6, 13, 19, 21, 40). *mps1* deletion mutants of *M. grisea* are sensitive to cell wall-digesting enzymes, have reduced conidiation and fertility, form nonfunctional appressoria, and are unable to infect plants (40). *C. lagenarium maf1* mutants completely lack appressoria and have reduced conidiation and pathogenicity (13). Mg*slt2* mutants of the dothideomycete *Mycosphaerella graminicola* are sensitive to glucanase and several fungicides and are unable to grow invasively on the plant. Unlike other fungi, Mg*slt2* mutants form unmelanized colonies with short aerial hyphae. Mutant mycelium is defective in polarized growth of the tip cells and undergoes progressive autolysis (19).

It can be concluded that in filamentous fungi MAPKs of two classes, Fus3/Kss1 and Slt2, often control the same cellular functions but are not redundant. For example, *C. lagenarium* Maf1 controls the early differentiation stage of appressorium formation, whereas Cmk1 is involved in the maturation of appressoria (19).

Cochliobolus heterostrophus, the cause of Southern corn leaf blight, is a filamentous necrotrophic ascomycete. Numerous eyespot-like lesions on the leaves are typical of this disease. In the presence of moisture, spores of the pathogen adhere to a leaf or other surface, germinate, and develop small appressoria, unlike the large melanized appressoria formed by *M. grisea* and by *Colletotrichum* species (3). *C. heterostrophus* appressoria

* Corresponding author. Mailing address: Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel. Phone: 972-48293544. Fax: 972-48225153. E-mail: sophia@tx.technion.ac.il.

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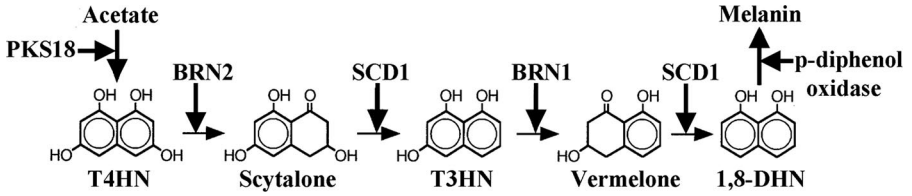

FIG. 1. Schematic representation of the fungal DHN-melanin biosynthesis pathway. PKS18 (polyketide synthase), BRN2 (T4HN reductase), BRN1 (T3HN reductase), SCD1 (scytalone dehydratase), and *p*-diphenol oxidase are enzymes presumably involved in the indicated biosynthetic steps.

are not considered essential for penetration, which occurs directly or through stomata (20).

We have previously characterized the *C. heterostrophus* MAPK gene *CHK1*, homologous to the yeast *FUS3*/*KSS1* (18). *chk1* deletion mutants are female sterile, lack conidia, and do not form appressoria on an inductive surface, and their virulence is severely reduced. Colonies of *chk1* mutants, white and sometimes autolytic, become darkly pigmented during prolonged growth. Recently, we have isolated a *C. heterostrophus* homolog of yeast *SLT2* designated *MPS1* (A. Igbaria et al., unpublished data). Interestingly, white colony appearance and formation of autolytic regions were even more pronounced in Δ*mps1* mutants than in Δ*chk1* mutants. The aim of this study was to establish how two MAPKs, Chk1 and Mps1, control mycelial melanization.

Wild-type colonies of *C. heterostrophus* undergo melanization and produce abundant conidia when grown under continuous illumination. The melanization is even more pronounced in colonies exposed to hyperosmotic stress. Melanin is a dark pigment tissue considered to protect the fungus from environmental stresses such as UV irradiation (8, 26). Albino strains of *C. heterostrophus* cannot survive in the field, but under laboratory conditions they cause lesions similar to those of the wild type on the host plant (5). *C. heterostrophus* produces 1,8-dihydroxynaphthalene (1,8-DHN)–melanin, as do *Bipolaris oryzae*, *Colletotrichum lagenarium*, *Magnaporthe grisea*, *Alternaria alternata*, and many other fungi (31, 32). DHN-melanin biosynthesis starts with a polyketide synthase (PKS) using acetyl coenzyme A or malonyl coenzyme A as a precursor (Fig. 1). The PKS produces 1,3,6,8-tetrahydroxynaphthalene (T4HN), which is reduced by hydroxynaphthalene reductase to form scytalone. Dehydration of scytalone by scytalone dehydratase forms 1,3,8-trihydroxynaphthalene (T3HN). T3HN reductase converts T3HN to vermelone, which is further dehydrated to 1,8-DHN. Subsequent steps are thought to involve a dimerization of 1,8-DHN molecules, followed by polymerization catalyzed by *p*-diphenol oxidase (16, 32). The *BRN1* gene encoding T3HN reductase has been isolated in *C. heterostrophus* (28). Genes involved in melanin biosynthesis, including the polyketide synthase (*PKS1*), T3HN reductase (*THR1*), and scytalone dehydratase (*SCD1*) genes, have been isolated in *B. oryzae*, a rice pathogen closely related to *C. heterostrophus*, and in *C. lagenarium* (10, 11, 23, 30).

C. lagenarium and *M. grisea* produce melanin during appressorium formation, on hyperosmotic media, and in the late stationary stage of mycelial growth. Orthologous transcription factors Cmr1 and Pig1 control melanin biosynthesis genes in *C. lagenarium* and *M. grisea*, respectively (34). The expression of transcription factor *CMR1* itself was shown to be up-regulated during melanin production. Cmr1 regulates the expression of *THR1* and *SCD1* genes in vegetative hyphae upon induction of melanization, but during formation of appressoria the expression of *THR1* and *SCD1* is *CMR1* independent (34).

In this study, we determined the influence of two MAPKs of *C. heterostrophus*, Chk1 and Mps1, on the expression of melanin biosynthesis genes through the *C. heterostrophus* Cmr1 ortholog.

MATERIALS AND METHODS

Fungal strains and growth media. Wild-type (C4 and C5) and *chk1* and *mps1* deletion strains of *Cochliobolus heterostrophus* and standard growth conditions have been described elsewhere (17, 18, 38; Igbaria et al., unpublished). CMX medium was prepared in the same way as standard complete medium (CM), except that glucose was replaced with the same amount of xylose. For hygromycin B (50 μg/ml; Calbiochem) and nourseothricin (120 μg/ml; Werner BioAgents, Jena, Germany) selection, CM and CMX were prepared without salts.

***CMR1* replacement constructs and transformant verification.** The vector for deletion of *CMR1* was assembled from the following fragments: 827-bp 5′ flanking sequence (amplified with primers d-SacI-s and d-XhoI-a), 2.1-kb hygromycin resistance cassette, 623-bp 3′ flanking sequence amplified with primers d-NheI-s and BglII-a, and pBluescript cloning vector (Stratagene) (Table 1; see Fig. S1A in the supplemental material). For transformation, the resulting vector was amplified by PCR with primers d-SacI-s and d-BglII-a using BIO-X-ACT Long High Fidelity DNA polymerase (Bioline; BIO-21049). Polyethylene glycol-mediated transformation into fungal protoplasts was performed as described previously (35, 36).

Genomic DNA was isolated from the fungal mycelium using the Extract-N-Amp Plant PCR Kit (Sigma; XNAP2). The deletion of *CMR1* in hygromycin-resistant colonies was confirmed by PCR amplification crossing the junction points of the vector with genomic DNA formed following a homologous recombination event. Primer pairs used for PCR were 5′o and ptrp, 3′o and ttrp (or ttrpe), and s and a (or ctrl-s and V-a) (see Fig. S1A, B, and D in the supplemental material; Table 1).

CMR1p-green fluorescent protein (GFP) vector was assembled by ligation of the following DNA fragments: 1.1-kb *CMR1* promoter (amplified with primers p-XhoI-s and p-BamHI-a), *GFP* amplified with GFP-BamHI-s and GFP-SpeI-a, *CMR1* 3′ untranslated region/terminator, nourseothricin resistance cassette including the *Streptomyces noursei* nourseothricin acetyltransferase-encoding gene (GenBank accession number X73149), 818-bp 3′ flanking sequence amplified using primers 3′-BglII-s and 3′-SacI-a, and pBluescript vector (see Fig. 7A; Table 1). For transformation, CMR1p-GFP vector was linearized.

Mutant characterization. To compare formation of appressoria by the mutants and that by the wild type, water-suspended conidia were placed in drops on the surface of a 9-cm polystyrene petri dish (Miniplast, Ein Shemer, Israel). The dishes were incubated for 3 to 5 h at 30°C and then studied under an inverted microscope (Olympus). For virulence tests, sweet maize plants (*Zea mays* hybrid 'Grand Jubilee', purchased locally) were grown for about 2 weeks at 22 to 24°C. Intact leaves were inoculated with conidial suspension in 0.05% Tween 80, and the plants were sealed in moist plastic bags and incubated for 2 days at 30°C under continuous light. For inoculation of detached leaves, they were placed on 1% agar plates just before solidification. Mycelial mats (1 × 1 mm) were placed on the agar near the leaves. The dishes were sealed and incubated under continuous illumination at 30°C for 2 days.

TABLE 1. Primers used in this study

Gene or vector	First primer		Second primer	
	Name	Sequence[a]	Name	Sequence[a]
Actin, RT-PCR	Act1-s	TTCTCCACCACTGCCGAGCG	Act1-a	GCGGTGAACGATGGAGGGAC
Actin, RT-PCR	Act2-s	AATGGTTCGGGTATGTGCAAGG	Act2-a	GGGACCGCTCTCGTCGTACTC
SCD1, RT-PCR	Scd1-s	TGGGAGGCGATGCCAGCGGAT	Scd1-a	GCTCGCGGCCCTCTGCAAACA
BRN1, RT-PCR	Brn1-s	TGGGTGGCCGCATTATCCTCA	Brn1-a	GAAGCAGACGACGCGGGCAAT
BRN2, RT-PCR	Brn2-s	CATTGCCGCTGGTCTTCTCGG	Brn2-a	AAGCCACAACCCTCGCAACAT
CMR1, RT-PCR, transformant verification	ctrl-s	GCAATCATCGCCGCCCAAGA	V-a	aacaggtaccGACGGCGGGCGAACATCCAG
CMR1, transformant verification and intron analysis	s	GCCAATCCAATCTGCCCCAT	a	CAATGCGAGAGGACAGCGAAA
CMR1 deletion vector, 5′ flank	d-SacI-s	tacgagcTCCCTGCCATCGCTGAGTCTT	d-XhoI-a	aggctcgAGGGGTTGTTGGTGATGGCTG
CMR1 deletion vector, 3′ flank	d-NheI-s	tatgctAGCGGGCGTCTTCGGCGTTG	d-BglII-a	catagatCTGCCAAAGACAATCAACACTGG
CMR1 genomic sequence outside 5′ and 3′ flanks	5′o	CCGCACGCACCTCCACCTCG	3′o	GCAAGAAGAGGAGGATGGATGG
Hyg^r cassette, promoter and terminator	ptrp	GGTCGTTCACTTACCTTGCTTG	ttrp ttrpe	GGTGTTCAGGATCTCGATAAG GTGAATGCTCCGTAACACCCAATAC
CMR1p-GFP vector, GFP RT-PCR	GFP-BamHI-s	gaagGATCCCATGGTGAGCAAGGGC	GFP-SpeI-a	tgaactagtCTTGTACAGCTCGTCCATGCCGT
CMR1p-GFP vector, *CMR1* promoter	p-XhoI-s	caactcgAGCACCGGGCAGGACAGGACT	p-BamHI-a	caaggATCCGGCTCGAATGTCTACTGCTC
CMR1p-GFP vector, 3′ flank	3′-BglII-s	taccatagatCTCCTCGTTTGTTCATTCGCCC	3′-SacI-a	taggagcTCAAGTGGGCGGGTGGTTGTA
Mating type-specific gene *MAT1-1*	MAT1-1s	GTCGTCGATGGTGATGAAAGAAA	MAT1-1a	CCGCACTGGAGCTCAAATGGT
Mating type-specific gene *MAT1-2*	MAT1-2s	GTTGCATCTCCGTCTGCGCCA	MAT1-2a	GGCTGCAAGGATGACTGGCAT

[a] The lowercase letters represent sequence with no homology to template DNA, whereas homologous regions are shown in uppercase.

Crosses between fungal strains C4 and C5 were set up according to the method in reference 17. The plates were incubated in darkness at 25°C for 23 to 24 days, and then the ascospores were isolated. The mating type of the progeny was determined by PCR using primer pairs MAT1-1s–MAT1-1a and MAT1-2s–MAT1-2a (Table 1) corresponding to the genes *MAT1-1* and *MAT1-2*, respectively.

For the UV sensitivity test, water-suspended conidia and mycelial fragments were incubated on glass slides until conidia became firmly attached to the glass surface and started to germinate (45 min). Then the slides were washed with water to eliminate unattached mycelium and unviable conidia. The slides were irradiated at 254 nm at energy levels of 20 to 70 mJ/cm^2 in a UV cross-linker and incubated in aluminum-foil-wrapped petri dishes overnight until examination.

RNA isolation and reverse transcription-PCR (RT-PCR). RNA was isolated from mycelia ground in liquid nitrogen followed by extraction with TRI reagent (Molecular Research Center) using the manufacturer's protocol. Routinely, 2 μg RNA was treated with RQ DNase (Promega; M610A) and then used for cDNA synthesis with oligo(dT)$_{17}$N primer (protocol supplied with Moloney murine leukemia virus reverse transcriptase; Promega; M170A). To synthesize cDNA with a sequence-specific primer, the primer was added to a final concentration of 2 μM, along with 0.2 μM of act2-a primer. Semiquantitative PCR was performed with Ready-To-Use mix (Larova; PCR-0540). We performed a series of PCRs for each primer pair to establish the optimal cycle number, which was then used in the actual experiment (Table 1). Each PCR was done at least twice, and data shown are representative of two independent experiments.

Identification of melanin biosynthesis gene clusters. The *Magnaporthe grisea* genome sequence was obtained from the PEDANT database (http://pedant.gsf.de/cgi-bin/wwwfly.pl?Set=Magnaporthe_grisea_BI&Page=index). The *Alternaria brassicicola* complete genome was downloaded from http://genome.wustl.edu/pub/organism/Fungi/Alternaria_brassicicola/assembly/draft/Alternaria_brassicicola-1.0/. For the analysis in Fig. 8, sequences from four contigs (1.244 to 1.247) were combined. *A. brassicicola* melanin biosynthesis genes were identified by similarity to other fungal species. GenBank accession numbers of the annotated genes are listed in Table 2.

RESULTS

Identification and structural analysis of the *C. heterostrophus CMR1* gene. We searched the genome of *C. heterostrophus* with *C. lagenarium* Cmr1 and *M. grisea* Pig1 amino acid sequences to identify their homolog in *C. heterostrophus*. The gene with the best score showed significant homology to both *CMR1* and *PIG1* over a large part of the coding sequence. The *C. heterostrophus CMR1* coding region is interrupted by three introns. The deduced Cmr1 protein is 1,014 amino acids long and shares 69% and 70% similarity with Cmr1 of *C. lagenarium* and Pig1 of *M. grisea*, respectively. The predicted *C. heterostrophus* Cmr1 protein has two Cys_2His_2 zinc finger domains and one $Zn(II)_2Cys_6$ binuclear cluster domain close to its N terminus, similar to Pig1 and *C. lagenarium* Cmr1 (Fig. 2). Interestingly, all three proteins are highly similar at their N termini and their C-terminal halves, but a region of low similarity separates these conserved regions.

Deletion of the *CMR1* gene. We designed a vector to delete the *CMR1* gene by double-crossover integration of the hygromycin resistance cassette (see Fig. S1A in the supplemental material). All emerging transformants lacked the typical gray-green color of the wild-type *Cochliobolus* when grown on plates. We verified the *CMR1* deletion by PCR using primers homologous to the hygromycin resistance cassette and genomic sequence outside the flank regions (see Fig. S1B in the supplemental material). Thus, only transformants in which the *CMR1* coding region was replaced with the hygromycin resistance cassette would provide the correct template. The deletion of the *CMR1* coding region in the transformants was confirmed using two internal primers. Notably, even in the first days of hygromycin selection on the transformation plates, when new transformants emerge as heterokaryons, complete loss of the black pigment was observed. We can speculate that production of melanin requires some critical quantity of *CMR1* transcripts. Reduction in this amount leads to complete loss (at least visibly) of melanin production.

Characterization of the mutants. Wild-type *Cochliobolus* acquires a gray-green color when grown on plates, along with the onset of conidiation. Colonies of the *cmr1* deletion mutants developed a light orange-pink color during maturation (Fig. 3A). This color was different from the near-white of *mps1* and *chk1* mutants, and characteristic autolytic-appearing areas of MAPK mutants were absent in the Δ*cmr1* strain. The conidiation of mutant colonies was significantly decreased compared to the wild type. To ensure that conidiation of mutants was

TABLE 2. GenBank accession numbers

Organism[a]	Gene	Description	GenBank accession no.
Cochliobolus heterostrophus	*CMR1*	Transcription factor containing two Cys_2His_2 zinc finger and $Zn(II)_2Cys_6$ binuclear cluster domains	DQ902714
	BRN1	T3HN reductase	AB001564
	BRN2	T4HN reductase	EF060260
	SCD1	Scytalone dehydratase	EF060261
	PKS18	Polyketide synthase	AY495659
	ACT1	Actin	AY748990
	MAT1-1	Mating-type gene	X68399
	MAT1-2	Mating-type gene	X68398
Aspergillus fumigatus	*ABR1*	Multicopper oxidase	AF116901
	ABR2	Laccase	AF104823
	AYG1	Encodes DUF1100 family protein of unknown function	AF116902
	ARP1	Scytalone dehydratase	U95042
	ARP2	T4HN reductase	AF099736
	ALB1	Polyketide synthase	AF025541
Magnaporthe grisea	*PIG1*	*CMR1* homolog, Cys_2His_2 zinc finger and $Zn(II)_2Cys_6$ binuclear cluster-containing transcription factor	AF230811
		T4HN reductase	AF290182
		Hypothetical protein-encoding gene	XM_367292
		Hypothetical GAL4-like transcription factor containing Zn_2Cys_6 binuclear cluster domain	XM_367293
		Polyketide synthase	XM_367294
		Multicopper oxidase	XM_367295

[a] *Alternaria brassicicola* melanin biosynthesis genes have not been annotated.

not due to residual wild-type nuclei, we isolated single spores from the mutant fungus and plated them separately. These single spores developed into orange-pink colonies which also produced a small number of conidia. These conidia germinated and formed appressoria on a glass surface undistinguishable from those of the wild type. Inoculation of intact maize plants and detached leaves with Δ*cmr1* strain conidial suspension and mycelial mats, respectively, resulted in disease symptoms similar to those caused by the wild-type strain.

We made a cross between Δ*cmr1* mutants (mating type *MAT1-2*) and wild-type C5 (*MAT1-1*). Black pseudothecia were formed from the wild-type side of maize leaf, and light brown pseudothecia were formed on the side of the mutant (Fig. 3B). Both types of fruiting bodies contained viable ascospores. The progeny of the cross had a wild-type or mutant appearance identical to the parents, and hygromycin resistance segregated together with the pigment deficiency trait. Out of the six pigment-deficient progeny strains, four belonged to the mating type *MAT1-2* (as their Δ*cmr1* parent) and two were of mating type *MAT1-1* (see Fig. S1C in the supplemental material). No traces of *CMR1* DNA were detected in the mutants, as verified by PCR (see Fig. S1D in the supplemental material). Mutant progeny of the Δ*cmr1* cross with the wild type were used in subsequent gene expression studies. When crossed with one another, Δ*cmr1* strains were able to form only light brown pseudothecia (Fig. 3B).

Since melanin is known to protect fungal cells from UV irradiation, we compared the sensitivities of the *cmr1* mutant and the wild type to UV light at different intensities. Conidia germinating on a glass surface were UV irradiated, and hyphal growth was observed after overnight incubation in darkness. Irradiation with 40 mJ/cm^2 caused complete inhibition of growth in *cmr1* mutants, whereas wild-type germ tubes continued to elongate even after 60 mJ/cm^2 (Fig. 3C).

Identification of the melanin biosynthesis genes and their expression. The genome of *C. heterostrophus* is unusually rich in polyketide synthase-encoding genes (15). *C. heterostrophus*

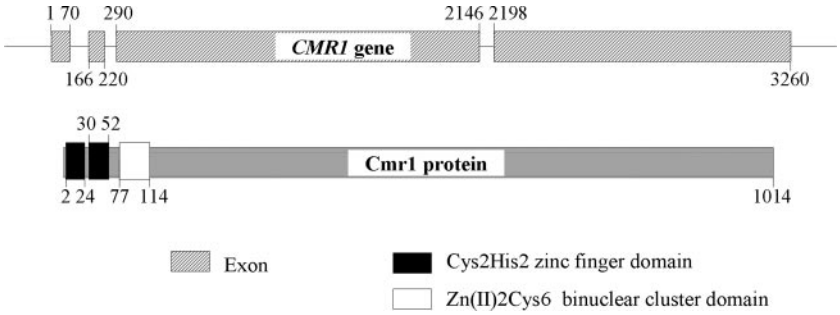

FIG. 2. *CMR1* gene features and domain architecture of the protein product.

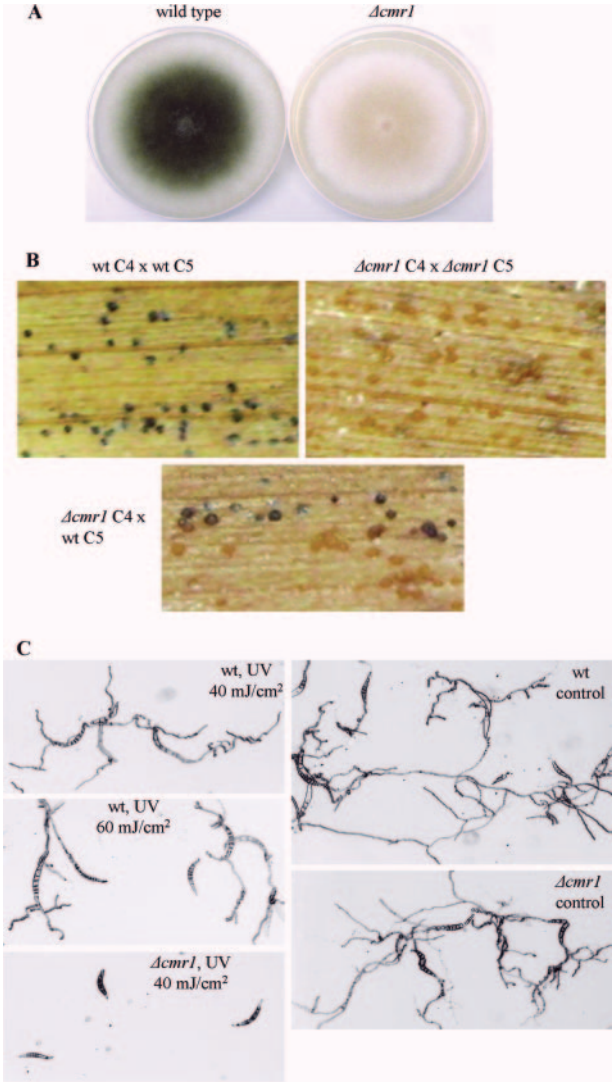

FIG. 3. Characterization of the *cmr1* mutant phenotype. (A) Colonies of wild type and Δ*cmr1* mutant; (B) Δ*cmr1* mutants form light brown pseudothecia when crossed with one another and with the wild-type strain; (C) growth inhibition of mutant and wild-type strains as a result of UV irradiation. Wild-type and mutant conidia were allowed to attach to the slides (45 min); mycelial debris and conidia that remained unattached were washed off. Following UV irradiation germ tubes of the mutant stopped further growth. The energy of irradiation is indicated. wt, wild type.

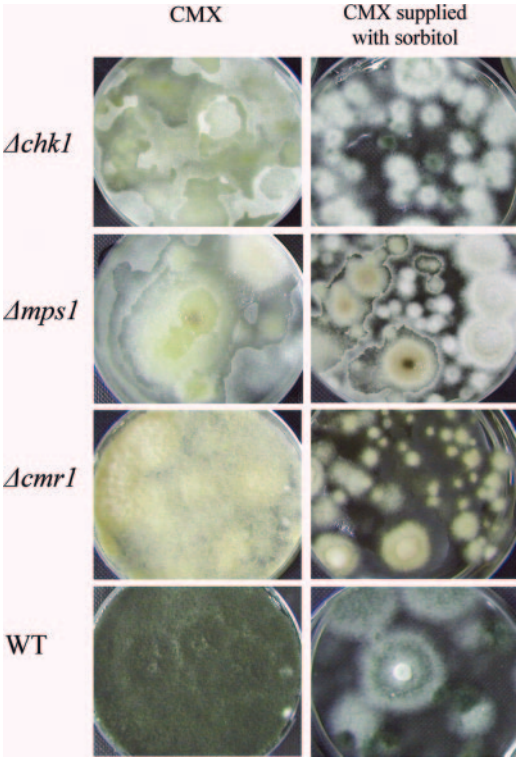

FIG. 4. Pigmentation and autolytic phenotypes. Five-day-old stationary liquid cultures of Δ*cmr1*, Δ*chk1*, and Δ*mps1* mutants and the wild-type strain (WT) were grown in complete medium with xylose (CMX) with or without addition of 1.5 M sorbitol.

PKS18 has 96% similarity to the *PKS1* gene of *B. oryzae*, which was shown to be involved in melanin biosynthesis (23).

We searched the *C. heterostrophus* genome for hydroxynaphthalene reductases with the *BRN1* (T3HN reductase) gene sequence. As a result of this search we identified another hydroxynaphthalene reductase, designated *BRN2*, which showed a significant degree of homology to the fungal T4HN reductases. Another genome search was performed with the *SCD1* gene of *B. oryzae*. As result of this search we identified a *C. heterostrophus* gene, also designated *SCD1*, which had 99% similarity with *SCD1* of *B. oryzae* (GenBank accession numbers are given in Table 2).

Wild-type *C. heterostrophus* and *cmr1*, *mps1*, and *chk1* deletion mutants were grown for 3 to 5 days in stationary liquid culture (complete medium, CMX). Pigment deficiency and autolytic appearance of the Δ*chk1* and Δ*mps1* mutants were very pronounced under these conditions (Fig. 4). Partial restoration of pigmentation and decrease of the autolytic phenotype (especially in the Δ*chk1* mutant) were observed in cultures supplemented with 1.5 M sorbitol (hyperosmotic stress). We tested the expression of *PKS18*, *BRN1*, *BRN2*, and *SCD1* in all cultures. In the wild-type strain the expression of *PKS18*, *BRN1*, *BRN2*, and *SCD1* was high in 3-day-old stationary culture, as well as in 5-day-old cultures with or without sorbitol. In *cmr1* deletion strains the expression of *BRN1*, *BRN2*, and *SCD1* was undetectable under all growth conditions tested, indicating that these genes absolutely require Cmr1 for their expression (Fig. 5A). Expression of *PKS18* was only moderately reduced in 3-day-old Δ*cmr1* culture compared to the wild type. Aging and hyperosmotic stress caused further decrease in *PKS18* expression in both Δ*cmr1* and wild-type strains (Fig. 5B).

In MAPK mutants the expression of *BRN1*, *BRN2*, and *SCD1* was undetectable (very low for *PKS18*) in 3-day-old cultures, barely detectable in 5-day-old CMX cultures, and significantly induced in the presence of sorbitol (Fig. 5A and B). These data indicate that both MAPKs, Chk1 and Mps1, are required for the expression of the melanin biosynthesis genes, but this dependence is overridden by the hyperosmotic stress. Nevertheless, even under conditions of hyperosmotic stress the expression of *SCD1*, *BRN1*, and *BRN2* was decreased in

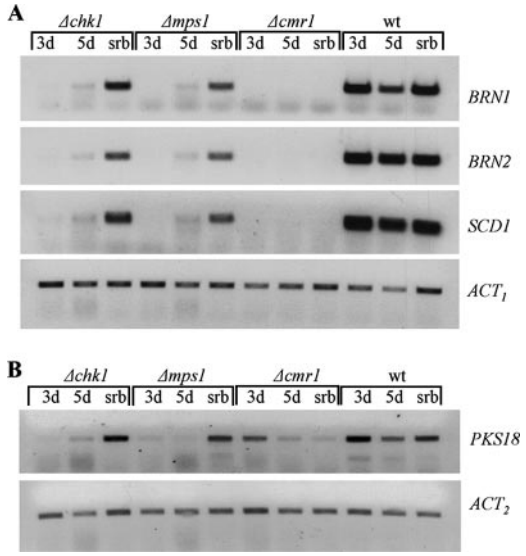

FIG. 5. RT-PCR analysis of expression of the melanin biosynthesis genes *BRN1*, *BRN2*, and *SCD1* (A) and *PKS18* (B) in Δ*cmr1*, Δ*chk1*, and Δ*mps1* mutants and the wild-type strain (wt). The stationary liquid cultures were grown for 3 and 5 days in CMX (3d and 5d, respectively) or 5 days in CMX supplemented with 1.5 M sorbitol (srb). Expression of the actin-encoding gene (*ACT*) indicates relative RNA quantities in each sample. ACT_1 and ACT_2 indicate different primer pairs used for PCR (Table 1).

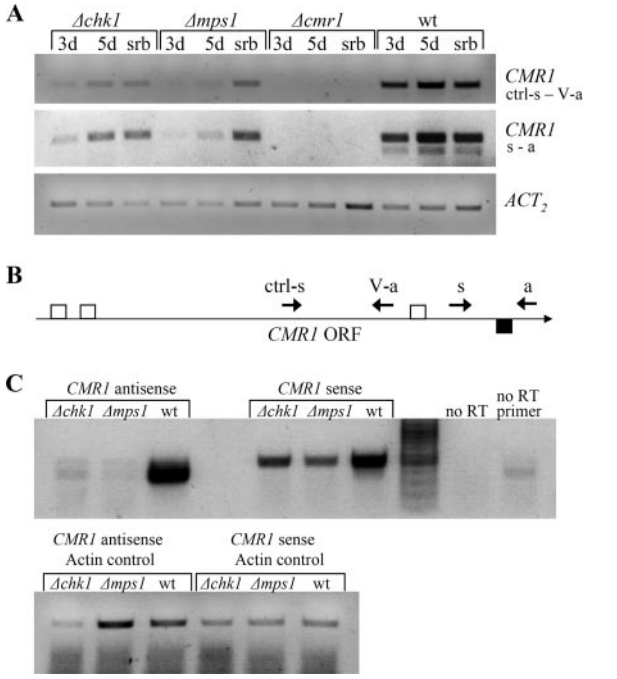

FIG. 6. Expression of sense and antisense *CMR1*. (A) Semiquantitative RT-PCR analysis of *CMR1* gene expression in wild type and Δ*chk1* and Δ*mpk1* mutants grown as described in the Fig. 5 legend. Polyadenylated mRNA was primed with standard oligo(dT) primer. Two primer pairs were used for the subsequent PCR: ctrl-s and V-a, and s and a (Table 1). Expression of the actin-encoding gene (ACT_2) indicates relative RNA quantities in each sample. (B) Schematic representation of the primer and intron positions as related to the *CMR1* open reading frame (ORF). Small arrows indicate primers used for RT and subsequent PCR. Open squares denote introns in the sense *CMR1* transcript, whereas the filled square indicates an intron in the antisense *CMR1*. (C) Analysis of sense and antisense *CMR1* transcript levels by directional cDNA synthesis. RNA used for RT-PCR was prepared from wild type and Δ*chk1* and Δ*mps1* mutants grown for 3 days in CMX, as described in the Fig. 5 legend. To examine antisense *CMR1* expression, cDNA was synthesized with *CMR1* primer s and actin primer act2-a to serve as an internal control. For sense *CMR1* expression, RT was performed with *CMR1* primer a and actin primer act2-a. Primers s and a (and act2-s and act2-a for actin) were used for the subsequent PCRs. Note that for detection of the antisense *CMR1* 34 PCR cycles were required, compared to 28 cycles for the sense transcript. wt, wild type.

MAPK mutants compared to the wild type. It appears that hyperosmotic stress signaling may bypass the MAPKs but not Cmr1 for the activation of melanin biosynthesis genes.

Coordinated expression of the sense and antisense *CMR1* transcripts. We tested the expression of the *CMR1* gene in wild-type and *chk1* and *mps1* deletion strains using two different primer pairs. Surprisingly, the expression of *CMR1* was in accordance with the expression of its target genes: it was reduced in 3-day-old cultures of MAPK mutants compared to the wild type and up-regulated in the aging cultures and hyperosmotic medium (Fig. 6A).

We noticed that RT-PCR with primer pair s and a repeatedly produced two products in the wild-type strain, even at the highest annealing temperature. The main product was considerably more abundant than the other, shorter one (Fig. 6A). Sequencing of the shorter PCR product revealed the presence of an intron with reverse and complement splice sequences. This finding implies that *CMR1* is transcribed in both the sense and antisense directions. To test this possibility, we performed directional *CMR1* cDNA synthesis using different primers for RT: ctrl-s and s (antisense transcript), and a (sense transcript) (Fig. 6B). Antisense actin primer was added to each cDNA synthesis reaction to serve as an internal standard to indicate RNA quantity. Resulting cDNA was amplified by PCR using primers s and a for *CMR1* and act2-s and act2-a for actin. Antisense *CMR1* transcript was detected in the wild-type strain but was almost undetectable in the MAPK deletion strains (Fig. 6C). Note the different sizes of the product obtained from the sense and antisense transcripts: in the antisense, but not the sense, transcript an intron between primers s and a is spliced (Fig. 6B and C). The overall antisense *CMR1* expression level was significantly lower than that of the sense *CMR1*. A BlastP search with putative protein products of the antisense *CMR1* transcript produced no significant hits, suggesting that the antisense *CMR1* is a noncoding transcript.

Activity of *CMR1* promoter in absence of Cmr1 protein. Since Cmr1 is a transcription factor, it is possible that it participates in the regulation of its own expression. We therefore replaced the *CMR1* coding sequence with *GFP* to report *CMR1* promoter activity (Fig. 7A). A CMR1p-GFP reporter strain was crossed with the *mps1* deletion mutant to obtain Δ*mps1*/CMR1p-GFP progeny. If Cmr1 is involved in its own regulation, we would expect the same low expression of *GFP* in both strains, CMR1p-GFP and Δ*mps1*/CMR1p-GFP. On the other hand, in the case of regulation of *CMR1* via another factor (MAPK dependent), we would expect decreased expression of *GFP* in a Δ*mps1*/CMR1p-GFP strain compared to CMR1p-GFP. Our results show that the *GFP* transcript accu-

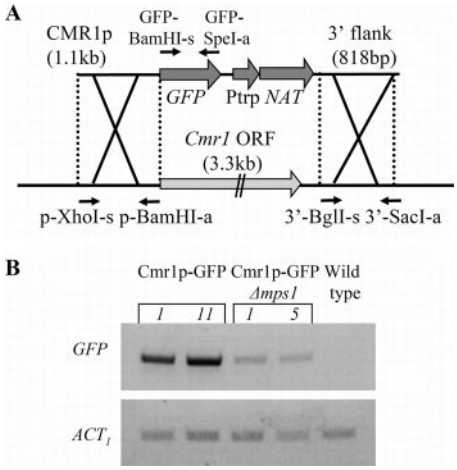

FIG. 7. Construction of CMR1p-GFP reporter strain and analysis of *CMR1* promoter activity in the absence of Cmr1 protein. (A) CMR1p-GFP transformation vector. Abbreviations: Ptrp, constitutive promoter; *NAT*, nourseothricin acetyltransferase-encoding gene; p-XhoI-s, p-BamHI-a, 3′-BglI-s, 3′-SacI-a, GFP-BamHI-s, and GFP-SpeI-a, primers used for vector construction. (B) RT-PCR analysis of *GFP* reporter expression in parental CMR1p-GFP strain and in *mps1*-deleted progeny. Expression of the actin-encoding gene indicates relative RNA quantities in each sample. Numbers 1, 11, and 5 represent different transformants.

mulated in the absence of the *CMR1* coding sequence and to lower levels in the absence of *MPS1* (Fig. 7B). This result implies that the decreased expression of *CMR1* in Δ*mps1* seen in Fig. 6A cannot be explained by decreased levels of the Cmr1 protein. Thus, if *CMR1* transcription is activated by the Mps1 pathway, a factor other than Cmr1 must be involved.

Melanin biosynthesis genes are clustered in several fungal species. In fungi, genes encoding proteins related to the same developmental function or metabolic process are sometimes clustered. It was found that in *Aspergillus fumigatus* and *Alternaria alternata* some of the melanin biosynthesis genes are located in close groups (12, 33). We therefore investigated the chromosomal location of the *C. heterostrophus* melanin biosynthesis genes, compared to other fungal species. Genes *BRN1*, *PKS18*, and *CMR1* of *C. heterostrophus* are located together on a 30-kb chromosomal fragment, and their mutual orientation is the same as in *Alternaria brassicicola*, a close relative of *C. heterostrophus* (Fig. 8). In *Aspergillus fumigatus* and *Magnaporthe grisea* melanin biosynthesis gene clusters also include multicopper oxidases which are probably required for the final 1,4-DHN polymerization step. In general, the most complete cluster among the species tested was found in *A. fumigatus*. This cluster might be the closest to some original ancestral melanin biosynthesis gene cluster (it is also the shortest cluster with the maximal number of genes), which apparently underwent significant shuffling during evolution, losing some of the genes in the process. Interestingly, the *A. fumigatus* cluster does not include a transcriptional regulator, unlike *M. grisea*, *A. brassicicola*, and *C. heterostrophus*.

DISCUSSION

One of the most prominent features in *chk1* and *mps1* MAPK mutants is loss of pigmentation. In this study we established that, at least in part, this loss of pigmentation is the result of underexpression of transcription factor *CMR1* and its target genes involved in melanin biosynthesis.

C. heterostrophus produces melanin of the DHN type. It is likely that the first enzyme in the biosynthesis pathway is Pks18, based on its close homology to the Pks1 of *B. oryzae* (23). The *C. heterostrophus* genome includes tetra- and trihydroxynaphthalene reductases, *BRN2* and *BRN1*, respectively, and scytalone dehydratase, *SCD1*. The last step in melanin biosynthesis is the polymerization of 1,8-DHN. In *C. heterostro-*

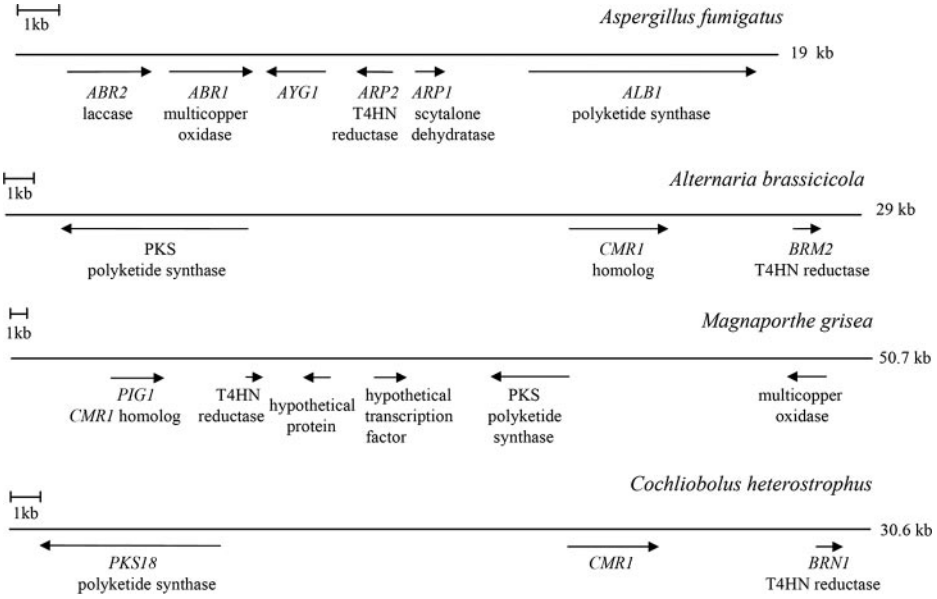

FIG. 8. Organization of the melanin biosynthesis gene clusters in *Aspergillus fumigatus*, *Alternaria brassicicola*, *Magnaporthe grisea*, and *Cochliobolus heterostrophus*. The accession numbers of the indicated genes are listed in Table 2.

phus this step is catalyzed by *p*-diphenol oxidase, which has not been identified yet (32).

Cmr1 regulates transcription of *BRN1*, *BRN2*, and *SCD1* genes, whereas the expression of *PKS18* is only partially Cmr1 dependent. It is possible that upon accumulation of melanin biosynthesis intermediates in Δ*cmr1* mutants, feedback inhibition of *PKS18* occurs. Another signaling pathway may participate in *PKS18* regulation: in *Aspergillus fumigatus* the cyclic AMP/protein kinase A pathway regulates the expression of polyketide synthase, *PKSP* (2).

Mutants with mutations in *cmr1* have a light orange-pink color, different from the white of the MAPK mutants. The different color may result from the accumulation of different melanin biosynthesis intermediates by *cmr1* and MAPK mutants. We can hypothesize that in the absence of Cmr1 Pks18 activity is reduced, but not completely lost, and the melanin biosynthesis pathway is blocked after the Pks18 step, resulting in accumulation of T4HN and its derivative flaviolin. In MAPK mutants all genes of the melanin biosynthesis pathway, including *PKS18*, are coordinately down-regulated, leading to an overall decrease in melanin biosynthesis. Another possibility is that MAPKs control production of an additional reddish-colored secondary metabolite. In melanin-deficient *cmr1* mutants the production of reddish pigment is normal, whereas MAPK mutants are deficient in both melanin and the reddish pigment.

The color of the *chk1* and *mps1* mutants darkened slightly during prolonged growth and was significantly darker in hyperosmotic medium. Correspondingly, the expression of the melanin biosynthesis genes was up-regulated under these conditions. Interestingly, the color of the *cmr1* mutant changed to deep orange in hyperosmotic medium, but the expression of melanin biosynthesis genes was undetectable in this mutant under all conditions tested. We suggest that one of the stress-activated signaling pathways bypasses Chk1 and Mps1, but not Cmr1, to activate expression of melanin biosynthesis genes. This stress-activated pathway does not include the *C. heterostrophus HOG1* homolog because in the absence of *HOG1* the pigmentation significantly increases, indicating that *HOG1* has rather an inhibitory effect on the expression of melanin biosynthesis genes (1; Igbaria et al., unpublished).

We have noticed that sometimes *chk1* mutants formed dark-centered colonies with wide white margins on complete medium with xylose even in the absence of any stress condition. The expression of *CMR1* and its downstream target genes was completely restored in these dark regions. Melanin biosynthesis genes are clustered in several fungal species, including *A. fumigatus*, *M. grisea*, and *A. brassicicola* and, as we established, in *C. heterostrophus* (Fig. 8). It is possible that MAPKs control the epigenetic state of chromatin through regulators like LaeA of *Aspergillus* spp. (9), and restoration of expression of melanin biosynthesis genes in *chk1* mutants is caused by gradual heterochromatin-euchromatin transition in response to a stress-related signal, rather than by direct transcriptional activation. Further evidence to support this hypothesis comes from the identification of the probably noncoding *CMR1* antisense transcript, which was almost undetectable in *chk1* and *mps1* deletion mutants. Notably, the ratio between *CMR1* expression in wild type and in *chk1* (*mps1*) deletion mutants is much greater for the antisense transcript than for the sense *CMR1*. The phenomenon of antisense transcription was extensively studied in mammalian genomes (37). In fungi, the *Neurospora crassa FRQ* gene is transcribed in sense and antisense directions, but only sense transcript codes for a protein product (14). Interestingly, coordinate regulation of the sense and antisense transcripts is common in the mammalian transcriptome, arguing against a model where the antisense transcripts silence the cognate sense RNA (7).

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