

## A Developmentally Regulated Gene Cluster Involved in Conidial Pigment Biosynthesis in *Aspergillus fumigatus*

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*Aspergillus fumigatus*, a filamentous fungus producing bluish-green conidia, is an important opportunistic pathogen that primarily affects immunocompromised patients. Conidial pigmentation of *A. fumigatus* significantly influences its virulence in a murine model. In the present study, six genes, forming a gene cluster spanning 19 kb, were identified as involved in conidial pigment biosynthesis in *A. fumigatus*. Northern blot analyses showed the six genes to be developmentally regulated and expressed during conidiation. The gene products of *alb1* (for “albino 1”), *arp1* (for “aspergillus reddish-pink 1”), and *arp2* have high similarity to polyketide synthases, scytalone dehydratases, and hydroxynaphthalene reductases, respectively, found in the dihydroxynaphthalene (DHN)-melanin pathway of brown and black fungi. The *abr1* gene (for “aspergillus brown 1”) encodes a putative protein possessing two signatures of multicopper oxidases. The *abr2* gene product has homology to the laccase encoded by the *yA* gene of *Aspergillus nidulans*. The function of *ayg1* (for “aspergillus yellowish-green 1”) remains unknown. Involvement of the six genes in conidial pigmentation was confirmed by the altered conidial color phenotypes that resulted from disruption of each gene in *A. fumigatus*. The presence of a DHN-melanin pathway in *A. fumigatus* was supported by the accumulation of scytalone and flaviolin in the *arp1* deletant, whereas only flaviolin was accumulated in the *arp2* deletants. Scytalone and flaviolin are well-known signature metabolites of the DHN-melanin pathway. Based on DNA sequence similarity, gene disruption results, and biochemical analyses, we conclude that the 19-kb DNA fragment contains a six-gene cluster which is required for conidial pigment biosynthesis in *A. fumigatus*. However, the presence of *abr1*, *abr2*, and *ayg1* in addition to *alb1*, *arp1*, and *arp2* suggests that conidial pigment biosynthesis in *A. fumigatus* is more complex than the known DHN-melanin pathway.

Many fungi produce melanins, which are dark brown and black pigments formed by oxidative polymerization of phenolic compounds. Melanin is known to contribute to the survival and longevity of fungal propagules (40). In addition, it is an important virulence factor for both plant- and animal-pathogenic fungi, e.g., *Magnaporthe grisea*, *Colletotrichum lagenarium*, *Cryptococcus neoformans*, *Exophiala dermatitidis* (*Wangiella dermatitidis*), and *Aspergillus fumigatus* (10, 14, 20, 21, 23, 29, 35, 37, 40). Pigment biosynthesis has been studied mainly in plant-pathogenic fungi, including *Colletotrichum lagenarium*, *M. grisea*, *Verticillium dahliae*, *Cochliobolus miyabeanus*, and *Alternaria alternata* (40). All these brown and black fungi synthesize their pigments through the dihydroxynaphthalene (DHN)-melanin pathway, in which hydroxynaphthalene (HN) and tetralone polyketides are the common intermediates (Fig. 1). DHN-melanin biosynthesis starts with polyketide synthase using acetate as a precursor (Fig. 1). An HN reductase then converts 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone (4, 40). Dehydration of scytalone forms 1,3,8-trihydroxynaphthalene (1,3,8-THN), which is converted to 1,8-DHN after an additional reduction and dehydration step. Finally, oxidative polymerization of 1,8-DHN gives the end product, DHN-melanin. These reduction steps are sensitive to tricyclazole, a fungicide that specifically inhibits HN reductase involved in DHN-melanin biosynthesis (40). To date, molecular genetic studies have identified three different genes in-

involved in the DHN-melanin biosynthetic pathway in brown and black fungi. These genes encode polyketide synthases, HN reductases, and scytalone dehydratases, respectively (7, 14).

Among fungi pathogenic to humans, pigment biosynthesis has been studied in the white yeast *C. neoformans*, the black yeast *E. dermatitidis*, and the filamentous fungus *A. fumigatus*. *C. neoformans* uses a laccase encoded by the *CNLAC1* gene to produce DOPA-melanin in the presence of diphenolic compounds (27), while *E. dermatitidis* produces DHN-melanin constitutively (19). However, the genes involved in the biosynthesis of DHN-melanin in *E. dermatitidis* have yet to be identified. The only other human-pathogenic fungus in which pigment biosynthesis has been studied is *A. fumigatus* (34, 35). This ubiquitous opportunistic pathogen produces conidia bearing bluish green pigment. *A. fumigatus* is a saprophyte that propagates via highly dispersible conidia. The airborne conidia may be inhaled and cause a life-threatening disease, invasive aspergillosis, in patients with prolonged neutropenia (19, 38). *A. fumigatus* is thought to synthesize its conidial pigment by using a pathway similar to that of DHN-melanin because of its observed sensitivity to tricyclazole (34, 41). However, the biosynthesis of this unique bluish green pigment has not been studied in detail. A reddish pink conidial mutant, RP3, was restored to produce bluish green conidia by a cosmid from the *A. fumigatus* genomic library through a complementation screening. This cosmid was rescued from the complemented strain and designated pG1-1 (34). To date, two genes, *alb1* (for “albino 1”) and *arp1* (for “aspergillus reddish pink 1”), involved in conidial pigment biosynthesis in *A. fumigatus* have been mapped on this cosmid (34, 35). The *arp1* gene encodes a putative scytalone dehydratase based on its amino acid sequence similarity to the

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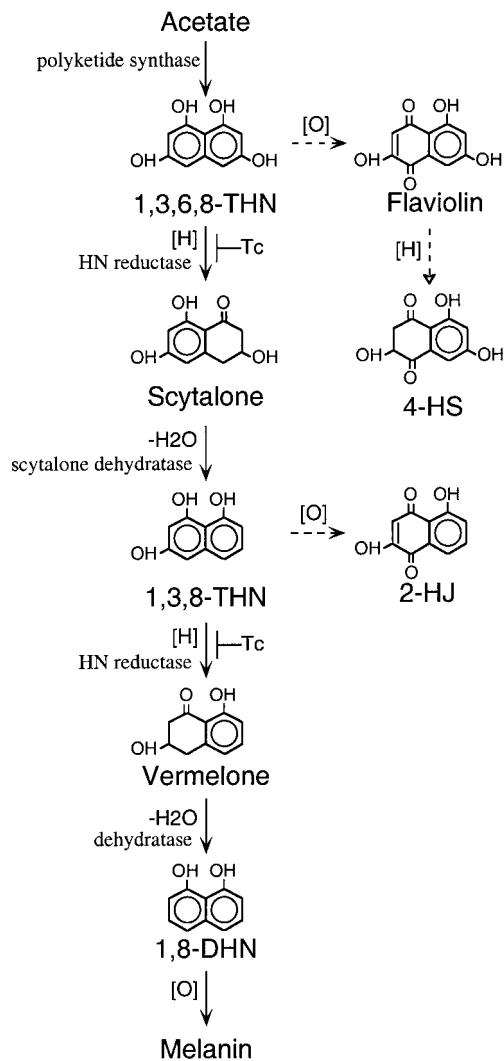


FIG. 1. Biosynthetic pathway of melanin in brown and black fungi. The solid arrows indicate the main melanin pathway, and the dashed arrows indicate branching pathways from the melanin pathway. The hollow arrowhead indicates that the conversion requires more than one step. The reduction steps are indicated by [H] and the oxidation steps are indicated by [O]. Dehydration steps are labeled  $-H_2O$ . A reduction step which can be inhibited by the fungicide tricyclazole is indicated with Tc.

scytalone dehydratases identified from plant-pathogenic fungi (34). Disruption of *arp1* results in the production of reddish pink conidia. The disruption also significantly increases the ability of the human complement component C3 to bind to these conidia (34). Deposition of C3 on conidia is an important step for efficient phagocytosis of inhaled conidia. The *alb1* gene product has high similarity to polyketide synthases from various fungi (35). Disruption of the *alb1* gene results in an albino conidial phenotype and significantly reduced virulence in a murine model (35). Thus, conidial pigment biosynthesis in *A. fumigatus* appears to be an important virulence factor in the establishment of infection.

The three genes involved in the DHN-melanin biosynthetic pathway identified in brown and black fungi are dispersed in the genome of *Colletotrichum lagenarium*, while they are organized as a cluster in *Alternaria alternata* (14). Here, we report the identification of a six-gene cluster involved in conidial

pigmentation in *A. fumigatus*. Biochemical analysis of the scytalone dehydratase and HN reductase gene deletants indicates that *A. fumigatus* utilizes a pathway similar to the DHN-melanin pathway for biosynthesis of its conidial pigment.

## MATERIALS AND METHODS

**Strains and media.** *A. fumigatus* B-5233, which produces bluish green conidia, was isolated from a patient with a fatal invasive aspergillosis. Strain B-5233/RGD4-2 is an *arp1* gene deletant of B-5233 (34). Strains B-5233/RGD3-1 and B-5233/RGD10-1 are the *arp2* gene deletants of B-5233. Both the *arp1* and *arp2* gene deletants produce reddish pink conidia.

Aspergillus minimal medium contained 1% glucose, 10 mM NaNO<sub>3</sub>, and trace elements (13). Malt extract medium contained 2% glucose, 2% malt extract, and 0.1% peptone. All cultures were grown at 37°C. Asparagine-sucrose agar (ASA) medium, which is identical to the alkaline medium (TM medium) previously described (43), was used to culture *A. fumigatus* for the detection of flaviolin and 2-hydroxyjuglone (2-HJ) accumulation (35). Modified ASA medium, which was used for tricyclazole inhibition and scytalone feeding experiments, contained 30 µg of tricyclazole per ml, 1 mM scytalone, or both, as previously described (35).

**Preparation and analysis of nucleic acids.** Total DNA and RNA were isolated from *Aspergillus* cultures as described previously (32). Cultures grown in Aspergillus minimal medium were harvested for total RNA preparation at 0 and 14 h after induction of conidiation, as described by Parta et al. (25). DNA sequencing was done with a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, Ohio) and an ABI automatic DNA sequencing system (Perkin-Elmer, Foster City, Calif.). Purified DNA fragments were recovered with the GeneClean II kit (Bio 101, Vista, Calif.). DNA cloning and Southern blot analyses were done by standard methods (30). Hybond-N nylon membranes (Amersham, Arlington Heights, Ill.) were used for blot analysis. DNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (Amersham) by using a Prime It kit (Stratagene, La Jolla, Calif.). The Genetics Computer Group program Gap (9), with the default parameters, was used to analyze the similarity and identity between proteins.

**Plasmids.** Cosmid pG1-1, a cosmid containing the vector pCosHX and a 42.5-kb genomic DNA of *A. fumigatus*, was obtained via plasmid rescue from a complemented conidial color mutant, RP3/G1-1 (34).

To disrupt the *arp2* gene, pRGD10 (see Fig. 3A) was constructed as follows. pBC KS+ (Stratagene) was digested with *SacI* and *SacII* and then self-ligated to eliminate the *SacI* site, producing pBCK558. The 11-kb *HindIII* DNA fragment of pG1-1 was cloned into the *HindIII* site of pCosHX to produce pGH10 (34). The 7-kb *EcoRV* DNA fragment from pGH10 was cloned into the *SmaI-EcoRV* sites of pBCK558. The resulting construct was digested with *AvrII* and *XbaI*, polished with T4 DNA polymerase, and self-ligated to yield pRGAS40. The 0.6-kb *SacI-SfiI* DNA fragment of pRGAS40 was then replaced with the 2.8-kb *SacI-HindIII* (blunt-ended) DNA fragment of pAN7-1 containing the hygromycin B phosphotransferase gene (*hph*) (28) to give the gene disruption construct pRGD10 (see Fig. 3A). The disruption construct pRGD3 was identical to pRGD10, except that the *hph* gene was inserted in the opposite orientation. A similar strategy was used to disrupt *abr1*, *abr2*, and *ayg1* in B-5233, by using disruption constructs pRGD24, pRGD17, and pRGD15S1, respectively. All of the gene deletants were confirmed by Southern blot analysis (data not shown).

**Transformation of *A. fumigatus*.** Protoplasts of *A. fumigatus* were prepared with mureinase (Amersham) as previously described (34) and transformed by the standard polyethylene glycol transformation method (44). Transformants were selected on Aspergillus minimal medium supplemented with 200 µg of hygromycin B per ml.

**Tricyclazole inhibition assay.** *A. fumigatus* strains were point inoculated on agar media consisting of malt extract, malt extract plus 1% ethanol, or malt extract plus 1% ethanol and tricyclazole (8 µg/ml). Conidiated cultures were photographed after 3 days of incubation at 37°C.

**TLC analysis.** Technical-grade tricyclazole (5-methyl-1,2,4-triazole[3,4-*b*] benzothiazole) was obtained from Eli Lilly Research Laboratories (Greenfield, Ind.). Flaviolin and 2-HJ were synthesized as previously described (3, 42). Scytalone and 4-hydroxyscytalone were purified from the *Brm-1* mutant strain of *Verticillium dahliae* (3).

ASA medium was inoculated with  $5 \times 10^5$  conidia and incubated at 24°C for 8 days as previously described (35). Then culture extracts were analyzed for the presence of flaviolin, 2-HJ, scytalone, and 4-hydroxyscytalone (4-HS) by ethyl acetate extraction and thin-layer chromatography (TLC) procedures as described previously (35, 41).

**Nucleotide sequence accession numbers.** The sequence data have been submitted to the GenBank database under accession no. AF025541 (*alb1*), U95042 (*arp1*), AF099736 (*arp2*), AF116901 (*abr1*), AF116902 (*ayg1*), and AF104823 (*abr2*).

## RESULTS

**Cloning and disruption of *arp2*.** Previous analysis of the *arp1* gene led to the discovery that the DNA adjacent to *arp1* encodes a 1.1-kb transcript designed ORF2, which is detected

	1					50
<i>C. lagenarium</i>	MPGVTSQSAG	SKYDAIPGPL	GLASA..SLM	GKVALVTGAG	RGIGRENAME	
<i>M. grisea</i>	MPAVTQPRGE	SKYDAIPGPL	GPQSA..SLE	GKVALVTGAG	RGIGRENAME	
<i>A. fumigatus</i>	.....	.....M	VNTCTYLPLS	GKVALVTGGG	RGIGAGIALE	
Consensus	-----	-----	-----L-	GKVALVTG-G	RGIG---A-E	
	51					100
<i>C. lagenarium</i>	LGRRGAKVIV	NYANSAETAETAE	EVVQAIKKSG	SDAASIKANV	SDVDQIVKMF	
<i>M. grisea</i>	LGRRGCKVIV	NYANSTESAE	EVVAAIKKNG	SDAACVKANV	GVVEDIVRMF	
<i>A. fumigatus</i>	LARRGASVAI	NYGHSAKSAQ	EVVEAIQAIG	RQAVAIQADL	TCVPNIESLI	
Consensus	L-RRG--V--	NY--S---A-	EVV-AI---G	--A----A--	--V--I----	
	101					150
<i>C. lagenarium</i>	GEAKQIWGRL	DIVCSNSGVV	SFGHVKDVTP	EEFDRVFAIN	TRGQFFVARE	
<i>M. grisea</i>	EEAVKIFGKL	DIVCSNSGVV	SFGHVKDVTP	EEFDRVFTIN	TRGQFFVARE	
<i>A. fumigatus</i>	QEVVRHFGRL	DIVVSNSGME	KFKPLEETTL	EDFNEVFNLN	TRAQMFVARY	
Consensus	-E-----G-L	DIV-SNSG--	-F-----T-	E-F--VF--N	TR-Q-FVAR-	
	151					200
<i>C. lagenarium</i>	AYKHLEVGGR	LILMGSITGQ	AKGVPKHAVY	SGSKGTIETF	VRCMAIDFGD	
<i>M. grisea</i>	AYKHLEIGGR	LILMGSITGQ	AKAVPKHAVY	SGSKGAIETF	ARCMAIDMAD	
<i>A. fumigatus</i>	AYDHIQPGGR	VILMSSIAA.	GLGVPGHALY	AGSKAAIEGF	TRCLAADFGR	
Consensus	AY-HL--GGR	-ILM-SI---	---VP-HA-Y	-GSKG-IE-F	-RC-A-D---	
	201					250
<i>C. lagenarium</i>	KKITVNAVAP	GGIKTDMYRD	VCREYIPNGG	ELDDEGVDEF	AA.GWSPMHR	
<i>M. grisea</i>	KKITVNVVAP	GGIKTDMYHA	VCREYIPNGE	NLSNEEVDEY	AASAWSPLHR	
<i>A. fumigatus</i>	KGCTVNAIAP	AGVKSDMWRE	NAWRYAPGCD	KSSSLEEIET	ALASGSPLKR	
Consensus	K--TVN--AP	-G-K-DM---	----Y-P---	-----E-	A----SP--R	
	251					293
<i>C. lagenarium</i>	VGLPIDIARV	VCFLASQDGE	W.....	.....	...	
<i>M. grisea</i>	VGLPIDIARV	VCFLASNDGG	WVTGKVIDID	GGACM.....	...	
<i>A. fumigatus</i>	CGVPEDIGKV	VSFLASPAE	WVNGEFFSPP	PCKGPLPGGD	LEC	
Consensus	-G-P-DI--V	V-FLAS-D--	W-----	-----	---	

FIG. 2. Amino acid alignment of HN reductases. The amino acid sequence comparison between Arp2p and HN reductases of *Magnaporthe grisea* (GenBank NCBI ID 1127197) and *Colletotrichum lagenarium* (GenBank accession no. D86079) is shown (18). The alignment was performed with the Genetics Computer Group program Pileup, using the default parameters (9). Dots represent gaps which were introduced during alignment. Dashes indicate the nonconsensus residues among three proteins.

only in the conidiating stage (34). Here, we sequenced the genomic and cDNA clones corresponding to the 1.1-kb transcript. The cDNA contains an 819-nucleotide open reading frame (ORF) which encodes a putative protein of 273 amino acids. No introns are present within *orf2*. A BLAST search with the deduced amino acid sequence revealed that the putative protein has 46% identity and 67% similarity to the HN reductase from *Magnaporthe grisea* (37) and 48% identity and 70% similarity to the HN reductase from *Colletotrichum lagenarium* (26) (Fig. 2).

The sequence similarity of the putative protein to the HN reductases involved in the DHN-melanin biosynthesis suggested that this ORF is involved in pigment biosynthesis. Gene disruption was performed to substantiate the potential involve-

ment of the gene in conidial pigmentation. A gene disruption construct of this ORF, pRGD10, was made by replacing a 0.6-kb coding region of this gene with a 2.8-kb DNA fragment containing the *hph* gene, which is used as a selection marker for transformation (Fig. 3A). Plasmid pRGD3 was a similar disruption construct, except that the *hph* gene was in the opposite orientation. Several transformants of the wild-type strain B-5233 with these plasmids produced reddish pink conidia. These transformants were analyzed by Southern blot hybridization to confirm the disruption of the gene. The pRGD10 DNA probe hybridized to a 7.0-kb DNA segment of B-5233 (Fig. 3B, panel I). In contrast, the transformants generated a 9.2-kb hybridizing signal which resulted from the loss of the 0.6-kb *SacI-SfiI* fragment from the gene and the gain of

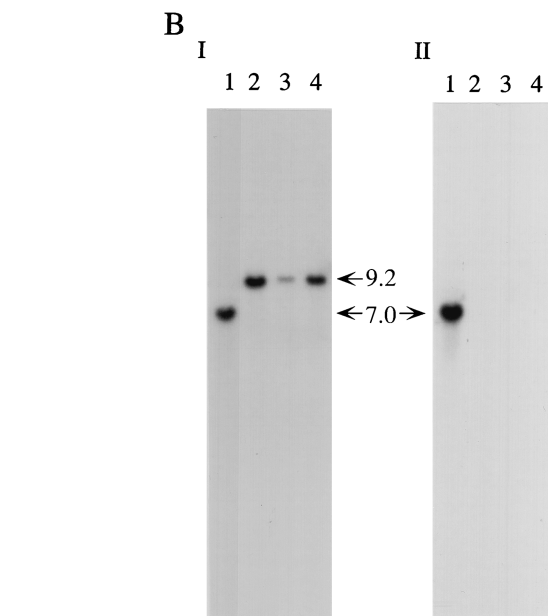
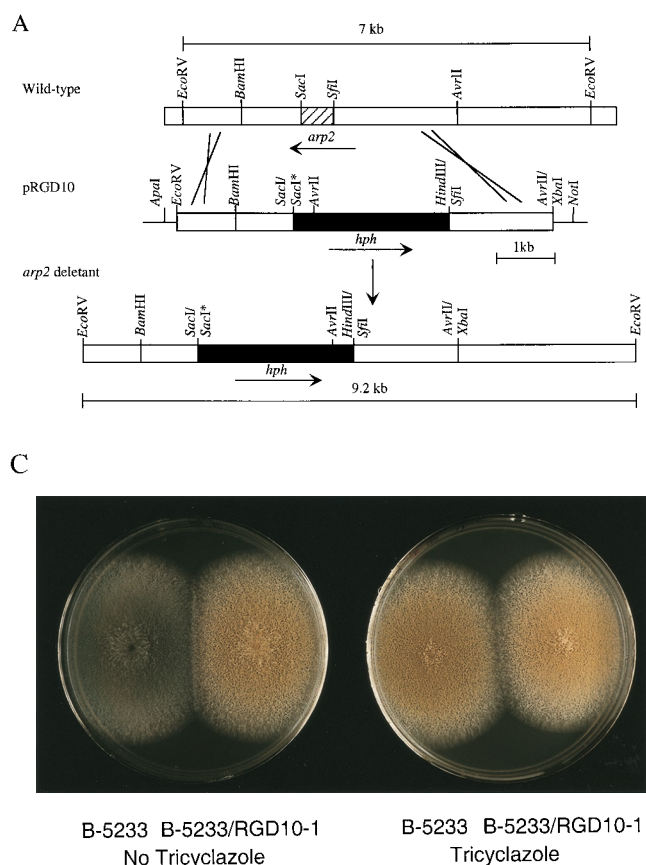


FIG. 3. *arp2* gene disruption in B-5233. (A) Diagram of a double-crossover event via homologous recombination. Open boxes represent DNA from *A. fumigatus*. Solid boxes represent the *hph* gene. Asterisks indicate *SacI* sites that were destroyed during vector construction. (B) Southern blot analysis of B-5233 and the *arp2* deletants. Total DNA was digested with *EcoRV*. The blot was analyzed with pRGD10 (I) or a 0.6-kb *SacI-SfiI* DNA fragment (hatched box in Fig. 3A) as a probe (II). Lanes: 1, B-5233; 2, B-5233/RGD10-1; 3, 5233/RGD10-2; 4, 5233/RGD10-3. The sizes of hybridized DNA fragments are indicated by arrows. (C) Conidial color phenotype of B-5233 and B-5233/RGD10-1. Cultures were grown on *Aspergillus* minimal medium for 3 days with (right) or without (left) tricyclazole.

a 2.8-kb fragment from *hph* (Fig. 3B, panel I). These results indicate that pRGD10 was integrated into the homologous locus in these transformants. Deletion of the wild-type copy of the gene in these transformants was confirmed by using the 0.6-kb *SacI-SfiI* DNA fragment as a probe. There was no detectable signal in these transformants, in contrast to the 7.0-kb signal in B-5233 (Fig. 3B, panel II). Thus, we concluded that the reddish pink conidial color phenotype resulted from the replacement of the wild-type copy with a disrupted copy by homologous recombination through a double crossover. A similar hybridization pattern was also observed in the reddish pink conidial color transformants which received pRGD3 (data not shown). Since disruption of this gene results in mutants producing reddish pink conidia, we designated the gene *arp2* (for "aspergillus reddish pink 2").

The conidial color of both the *arp1* and *arp2* deletants is reddish pink. However, the color of the *arp2* deletants appeared darker than that of the *arp1* deletant. Interestingly, when the wild-type strain was grown on *Aspergillus* minimal medium containing 8  $\mu$ g of tricyclazole per ml, it produced reddish pink conidia similar to those of the *arp2* deletants (Fig. 3C). Tricyclazole is a fungicide which specifically inhibits the HN reductases involved in DHN-melanin biosynthesis in brown and black fungi (33, 40). Thus, disruption of *arp2* revealed a phenotype similar to that obtained by growth in the presence of this specific reductase inhibitor. Collectively, the data indicated that *arp2* encodes an HN reductase involved in conidial pigment biosynthesis.

**Mapping the gene cluster associated with conidiation.** Because the genes *alb1*, *arp1*, and *arp2* are adjacent to each other, the possibility exists that other genes involved in conidial pig-

ment biosynthesis are located in close proximity. The structure and sequences of pG1-1 were further analyzed. No DNA rearrangement had occurred in pG1-1 during its cloning process, indicated by the same restriction enzyme digestion patterns of pG1-1 and the genomic DNA of B-5233 (Southern blot data not shown). Northern blot analysis was used to detect the transcripts that might be encoded by pG1-1. Eight DNA fragments, a, b, c, d, e, f, g, and h, spanning a 15.5-kb region of pG1-1, were used as DNA probes to analyze RNA from conidiating and nonconidiating cultures (Fig. 4). Six transcripts hybridizing to the DNA probes used were found to be expressed in 14-h conidiating cultures but not in nonconidiating cultures (Fig. 4C and D). Therefore, these six transcripts ranging from 0.8 to 7 kb in size are associated with conidiation. Similar Northern analysis was carried out with probes of the 4-kb *HindIII* DNA fragment located adjacent to *alb1* and the 5.5-kb *NotI-HindIII* DNA fragment adjacent to fragment g (Fig. 4A to C). The 4-kb *HindIII* DNA probe detected two transcripts expressed in both conidiating and nonconidiating cultures, while the 5.5-kb *NotI-HindIII* DNA probe did not detect any transcript at either developmental stage (data not shown). The difference in gene expression patterns suggested that only the 19-kb region of pG1-1 (Fig. 4B) encodes transcripts which are associated with conidiation.

**Characterization of the conidiation-associated gene cluster.** The cDNAs corresponding to these conidiation-associated transcripts were isolated by using DNA fragments g (6-kb *HindIII* fragment) and c (1.6-kb *HindIII-BamHI* fragment) as probes, respectively. The sequences of these cDNA clones and the 19-kb region of pG1-1 were determined. The corresponding ORFs were designated *abr1*, *abr2*, and *ayg1* (Fig. 4B).

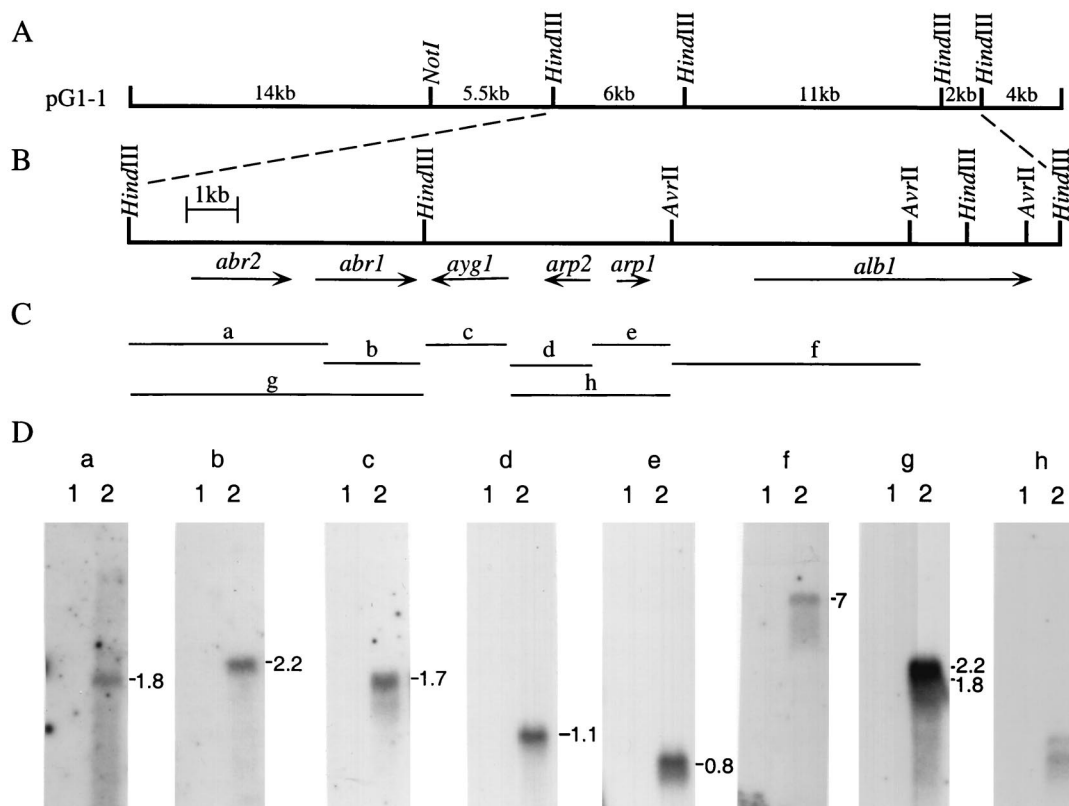


FIG. 4. Gene cluster for conidial pigment biosynthesis in *A. fumigatus*. (A) Physical map of pG1-1. (B) Map of transcripts associated with conidiation. Arrows indicate the direction of transcripts. All of the six transcripts are found within a 19-kb DNA fragment. (C) Probes used for Northern analyses. The DNA fragments used as probes are labeled a, b, c, d, e, f, g, and h. (D) Northern blot analyses of gene expression at two different developmental stages. The sizes of the transcripts are labeled on the right of the blot in kilobases. Total RNA from B-5233 was isolated from mycelia 0 h (lane 1) or 14 h (lane 2) after induction of conidiation. A 12- $\mu$ g portion of total RNA was fractionated on the 1% formaldehyde-agarose gel. The sizes of the hybridizing fragments are indicated at the side. rRNA stained with ethidium bromide was used as a control for the quantity and quality of the RNA preparation (data not shown).

Therefore, including *alb1*, *arp1*, and *arp2*, a total of six ORFs were clustered in the 19-kb genomic fragment. Comparison of the cDNA sequences with the 19-kb genomic sequences revealed the genomic arrangement of these ORFs in the cluster (Fig. 4B). The *alb1*, *arp1*, *abr1*, and *abr2* genes were transcribed in the opposite direction to *arp2* and *ayg1* (Fig. 4B). Within this arrangement, *abr1* and *ayg1* were convergently transcribed whereas *arp1* and *arp2* are divergently transcribed. These ORFs are closely spaced: for instance, *abr1* was separated from *ayg1* by 427 nucleotides. The largest intergenic distance, 1,101 nucleotides, was found between *arp1* and *alb1* (Fig. 4B).

Further sequence analysis revealed that *abr1* encodes a pu-

tative protein (Abr1p) of 664 amino acids. A BLAST search with Abr1p amino acid sequences revealed that it possesses two multicopper oxidase signatures and has 34% identity and 43% similarity to FET3, an iron multicopper oxidase from *Candida albicans* (GenBank accession no. Y09329) (Table 1). Two introns, of 47 and 52 nucleotides, were present in the *abr1* gene. A putative protein of 587 amino acids (Abr2p) was encoded by *abr2*. Abr2p had 41% identity and 65% similarity to a laccase encoded by *yA* of *A. nidulans* (2). Six introns were observed in the *abr2* gene, and the sizes of these introns ranged from 43 to 59 nucleotides. All of these introns were located close to the 5' end of *abr2*. The *ayg1* gene encoded a putative

TABLE 1. Analysis of genes involved in conidial pigmentation in *A. fumigatus*

Gene	Putative activity	Size of transcripts (kb)	Size of deduced protein (amino acids)	% Identity <sup>a</sup>	Reference protein
<i>alb1</i>	Polyketide synthase	7.0	2,146	66	wA (24)
<i>arp1</i>	Scytalone dehydratase	0.8	168	55	SCD1 (17)
<i>arp2</i>	HN reductase	1.1	273	49	THR1 (26)
<i>abr1</i>	Multicopper oxidase	2.2	664	34	FET3 <sup>b</sup>
<i>ayg1</i>	Unknown	1.7	406	NA <sup>c</sup>	NA
<i>abr2</i>	Laccase	1.8	587	41	<i>yA</i> (2)

<sup>a</sup> Percent identity of each putative protein to the reference protein is based on analysis with the Genetics Computer Group Gap program and the default parameter.

<sup>b</sup> GenBank accession no. Y09329.

<sup>c</sup> NA, not applicable.

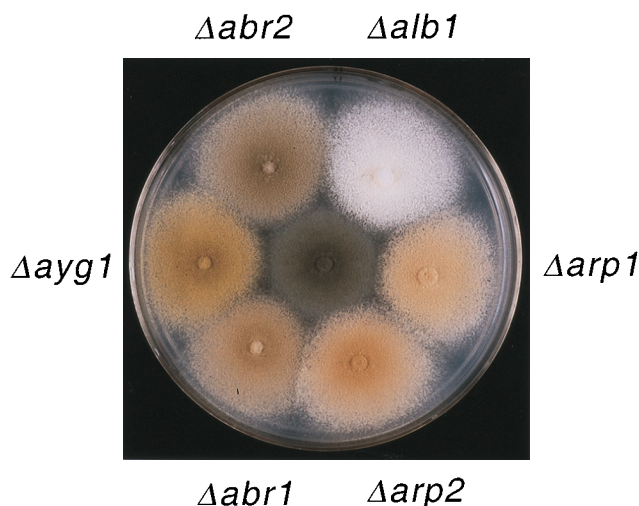


FIG. 5. Sporulating cultures of the *A. fumigatus* wild-type strain and six single-gene deletants. The wild-type strain, producing bluish green conidia, is at the center of the plate. Each gene deletant is designated by the name of the deleted gene.

protein (Ayg1p) of 406 amino acids. Four introns were present in the *ayg1* gene, and the sizes of these introns ranged from 45 to 56 nucleotides. A BLAST search with either nucleotide or amino acid sequences of *ayg1* did not identify any homolog.

Disruption of each *abr1*, *abr2*, and *ayg1* in *A. fumigatus* resulted in an alteration of the conidial color phenotype. Both *abr1* and *abr2* single-gene deletants produced brown conidia, while the *ayg1* deletant produced yellowish green conidia (Fig. 5). It is of interest that the *ayg1* deletant produced yellow conidia at an early growth stage. However, as the cultures aged, the yellow conidial color gradually converted to green, which was distinct from the bluish green color of wild-type conidia.

The presence of these three genes in addition to *alb1*, *arp1*, and *arp2* suggested that conidial pigment biosynthesis in *A. fumigatus* is more complex than the known DHN-melanin pathway in brown and black fungi.

#### Biochemical evidence for a melanin pathway in *A. fumigatus*.

To confirm that the *arp1* and *arp2* putative proteins (Arp1p and Arp2p) are homologs of scytalone dehydratase and HN reductase respectively, extracts obtained from cultures grown on ASA medium were analyzed by TLC for pigment biosynthetic intermediates. Based on the melanin studies that have been carried out with brown and black fungi, blockage of the 1,3,6,8-THN reduction step results in the accumulation of flaviolin (Fig. 1). Blockage of the scytalone dehydration step results in accumulation of stable intermediates or branch products, i.e., scytalone, flaviolin, and 4-HS (Fig. 1). TLC analysis showed that B-5233 did not produce any detectable flaviolin (Fig. 6A, panel I, lane 1). In contrast, the *arp2* deletants, B-5233/pRGD3-1 and B-5233/RGD10-1, produced flaviolin abundantly (lanes 3 and 4). When B-5233 was grown on ASA medium containing 30  $\mu\text{g}$  of tricyclazole per ml, it also produced flaviolin abundantly (lane 2).

Tricyclazole did not have an apparent effect on the *arp2* deletants, and culture extracts of these strains appeared identical to those of tricyclazole-treated B-5233. Scytalone, 4-HS, and 2-HJ, which are stable intermediates and branch products of the DHN-melanin pathway, were not detected in the culture extracts. These data suggested that Arp2p is a tricyclazole-sensitive HN reductase which converts 1,3,6,8-THN to scytal-

one. Culture extracts of the *arp1* deletant (B-5233/RGD4-2) (34) were also analyzed by TLC. High levels of scytalone and flaviolin and a detectable amount of 4-HS were present in the *arp1* deletant but not in B-5233 (Fig. 6A, panel II, lanes 1 and 3). The identities of scytalone, flaviolin, and 4-HS were confirmed by mass spectrometry (data not shown). Accumulation of these compounds indicated that the *arp1* deletant was incapable of converting scytalone to 1,3,8-THN. This agrees with the notion that Arp1p is a scytalone dehydratase. Tricyclazole treatment prevented accumulation of scytalone and 4-HS by the *arp1* deletant (lane 4). This indicates that Arp2p is upstream of Arp1p in the pathway.

The accumulation of scytalone and flaviolin by the *arp1* deletant and flaviolin by the *arp2* deletants clearly confirmed the presence of both a scytalone dehydratase and an HN reductase in the conidial pigment biosynthetic pathway of *A. fumigatus*. This strongly supported the notion that *A. fumigatus* utilizes a pathway similar to the DHN-melanin pathway for conidial pigment biosynthesis.

**Two reductases involved in the melanin biosynthesis in *A. fumigatus*.** There is no general assertion that the same HN reductase catalyzes the two reduction steps in the DHN-melanin pathway in all DHN-melanin producing fungi, i.e., 1,3,6,8-THN to scytalone and 1,3,8-THN to vermelone (7, 26, 36). To delineate whether this is the case in *A. fumigatus*, we analyzed the accumulated intermediates and branch products in the *arp2* deletants which were grown on ASA medium and ASA medium supplemented with scytalone or with scytalone and tricyclazole. Detection of scytalone and 4-HS in the *arp1* deletant but not in the *arp2* deletants indicated that Arp2p is required for production of both compounds (Fig. 1 and 6A). The branch product 2-HJ, derived from 1,3,8-THN due to blockage of the second reduction step in the pathway, was not detected in the *arp2* deletants grown on medium without scytalone (Fig. 6B, lanes 1 and 4). A very small amount of 2-HJ was accumulated by the *arp2* deletants grown on ASA medium containing only scytalone (lane 2 and 5). In contrast, 2-HJ was accumulated abundantly in the *arp2* deletants grown on ASA medium containing both scytalone and tricyclazole (lanes 3 and 6). This indicated the presence of another tricyclazole-sensitive HN reductase for metabolizing 1,3,8-THN, which is derived from the exogenous scytalone in *A. fumigatus*.

## DISCUSSION

A developmentally regulated six-gene cluster involved in conidial pigment biosynthesis in *A. fumigatus* was identified and characterized. DNA sequence, Northern blot, and biochemical analyses indicated that *A. fumigatus* synthesizes its conidial pigment through a pathway similar to the DHN-melanin pathway found in many brown and black fungi. To date, this is the largest gene cluster ever reported to be involved in fungal pigment biosynthesis. The six genes have been designated *alb1*, *arp2*, *arp1*, *abr1*, *abr2*, and *ayg1*. According to the deduced amino acid sequence data, the *alb1*, *arp1*, and *arp2* gene products are homologs of polyketide synthase, scytalone dehydratase, and HN reductase, respectively, which are found in the brown and black fungi. Identification of these three genes suggests the presence of a similar DHN-melanin pathway in *A. fumigatus*. The notion of the presence of this melanin biosynthetic pathway in *A. fumigatus* was strengthened by detection of the accumulated intermediates characteristic of the pathway in the wild-type strain treated with tricyclazole and in the strains in which these three genes were individually disrupted. Furthermore, the altered conidial color in each single-

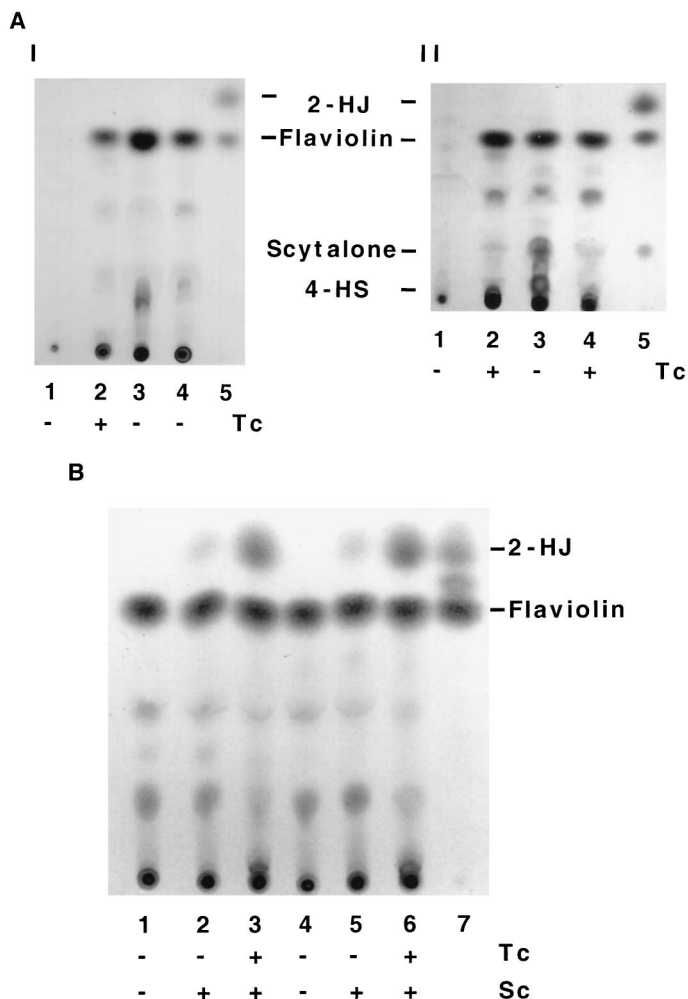


FIG. 6. Biochemical analysis of accumulated intermediates and branch products of the DHN-melanin biosynthetic pathway of *A. fumigatus*. (A) (I) TLC analysis. Lanes: 1 and 2, wild-type strain B-5233; 3 and 4, *arp2* deletants B-5233/RGD3-1 and B-5233/RGD10-1; 5, standards. (II) Lanes: 1 and 2, B-5233; 3 and 4, *arp1* deletant B-5233/RGD4-2; 5, standards. (B) Scytalone feeding experiment. Lanes: 1 to 3, B-5233/RGD3-1; 4 to 6, B-5233/RGD10-1; 7, standards. The strains were grown on ASA medium for 6 days. Sc,  $10^{-3}$  M scytalone in ASA medium; Tc, 30  $\mu$ g of tricyclazole per ml in ASA medium.

gene-disrupted strain indicates that the gene cluster is indeed involved in conidial pigment biosynthesis in *A. fumigatus*.

Clustering of functionally related genes, a common feature in prokaryotes, was not recognized in filamentous fungi until 10 years ago (11). Gene clusters discovered in fungi have been mainly divided into two groups, those responsible for low-molecular-weight nutrient utilization and those responsible for secondary-metabolite production (14). Gene clusters of nutrient utilization pathways observed in filamentous fungi include nitrate assimilation and proline utilization in *A. nidulans* (1, 8, 12). Clusters of genes involved in secondary-metabolite biosynthesis include those producing mycotoxins and melanin. Mycotoxin-producing gene clusters include those of the aflatoxin pathway in *A. flavus* (5) and the sterigmatocystin pathway in *A. nidulans* (6). For melanin biosynthesis, the three known genes involved in conidial pigment biosynthesis are clustered in *Alternaria alternata* (15). However, the genes involved in DHN-melanin biosynthesis are not always clustered. In *Colletotrichum lagenarium*, for example, the three genes are dispersed in the genome (14). Furthermore, the *wA* and *yA* genes involved in conidial pigment biosynthesis in a green-spored fungus, *A. nidulans*, also are not linked.

Gene clustering is thought to be beneficial for gene regulation (14). Functionally associated genes work coordinately as a team, and so coregulation of these genes is an advantage for living organisms. This is especially true with developmentally regulated genes. Clustering of genes allows regulatory elements to be shared between genes, i.e., divergent promoters such as *GAL1-GAL10* in yeast and *niiA-niaD* in *Aspergillus* spp. (1, 12, 39). In the pigment biosynthesis gene cluster in *A. fumigatus*, *arp1* and *arp2* are divergently transcribed genes. Their intergenic regions are very small, only 464 nucleotides. It is likely that these two genes share *cis*-acting elements for regulation of their expression. Indeed, Arp1p acts on the products produced by Arp2p. Regulator genes were observed in the gene clusters for nutrient and mycotoxin biosynthesis. However, we have yet to identify the regulator genes for the pigment biosynthetic gene cluster in *A. fumigatus*. Alternatively, gene clustering places genes in the same chromosomal region and may allow gene regulation through modulation of chromatin structure.

Despite minor genetic differences, the DHN-melanin pathways in many brown and black fungi require only polyketide synthase, HN reductase, and scytalone dehydratase to synthe-

size DHN, which forms DHN-melanin through oxidative polymerization (40). It has been suggested that the HN reductase catalyzes the conversion of 1,3,6,8-THN to scytalone as well as the conversion of 1,3,8-THN to vermeline in *M. grisea* (36). Also, scytalone dehydratase was suggested to catalyze two different dehydration steps in the DHN-melanin biosynthetic pathway in *M. grisea*: the conversion of scytalone to 1,3,8-THN and the conversion of vermeline to 1,8-DHN (23). These three enzymes are conserved among the known DHN-melanin pathways in fungi (15–18, 26, 31, 36, 40). The presence of homologs of the three genes in *A. fumigatus* suggests a similarity of melanin biosynthesis in *A. fumigatus* to that of brown and black fungi. However, the final pigments produced by *A. fumigatus* are bluish green. Although *A. fumigatus* and brown-to-black fungi have similarities in their melanin biosynthetic pathways, their melanin pathways must have diverged at steps prior to the final melanin products. Discovery of three additional genes, *abr1*, *abr2*, and *ayg1*, associated with conidial pigmentation supports this hypothesis. Since *abr1* and *abr2* have signature characteristics of oxidases, their existence indicates the presence of oxidation steps in the biosynthetic pathway. A laccase was suggested to polymerize and oxidize DHN into melanin based on previous biochemical studies (40). However, the gene encoding the putative oxidase has not yet been identified in brown and black fungi. Since *Abr2p* has sequence similarity to laccases, it is possible that the *Abr2p* is involved in oxidation steps. A BLAST search with *ayg1* sequences did not identify any homolog; thus, the function of *ayg1* remains unknown. Further genetic and biochemical characterization of the mutant strains will help us to uncover the role of *ayg1* in conidial pigment biosynthesis.

It has been suggested that one reductase is responsible for both reduction steps in the DHN-melanin pathway in *Magnaporthe grisea* (36). However, a second reductase may also be present in the DHN-melanin pathway of other brown and black fungi, as suggested for *Colletotrichum lagenarium* (26). Our TLC analysis of *arp2* deletants, however, detected the accumulation of flaviolin, a well-known branch product of the DHN-melanin pathway, but not of 2-HJ. Supplementing the *arp2* deletant with scytalone and tricyclazole resulted in abundant accumulation of 2-HJ. This suggests the presence of a second reductase involved in conidial pigment biosynthesis of *A. fumigatus*. However, this putative reductase in *A. fumigatus* was not found in the six-gene cluster. These identified six genes can be used for further investigation of pigment biosynthetic pathway in other fungi.

Disruption of *alb1* significantly reduces the virulence of *A. fumigatus* in mice (22, 35). Furthermore, disruption of either *alb1* or *arp1* greatly increased the ability of complement component C3 to bind to *A. fumigatus* conidia (34, 35), a process important for phagocytosis of the inhaled conidia. These effects may be due to alteration of the conidial surface structure or loss of certain intermediates or branch products of the melanin pathway. However, preliminary studies with the murine model showed that the *arp1* deletant but not the *arp2* deletant had significantly reduced virulence relative to the wild-type strain (unpublished data). This suggests that accumulated metabolites may be more important than the conidial pigment itself in influencing the virulence of *A. fumigatus*. Identification of this six-gene cluster will facilitate further investigation of the mechanisms involved in the pathogenesis of *A. fumigatus*. Availability of these color mutants with disruptions in these genes will allow us to identify accumulated intermediates or products. In addition, it will help us to further delineate the melanin biochemical pathway.

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