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basA Regulates Cell Wall Organization and Asexual/Sexual Sporulation Ratio in *Aspergillus nidulans*

Shaojie Li,*[,1] Dapeng Bao,*[,1] Gary Yuen,[†] Steve D. Harris[†] and Ana M. Calvo*[,2]

**Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115 and [†]Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68588-0660*

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ABSTRACT

Sphingolipid C4 hydroxylase catalyzes the conversion of dihydrosphingosine to phytosphingosine. In *Saccharomyces cerevisiae*, Sur2 is essential for sphingolipid C4 hydroxylation activity but not essential for normal growth. Here we demonstrate that the *Aspergillus nidulans* Sur2 homolog BasA is also required for phytosphingosine biosynthesis but is also essential for viability. We previously reported that a point missense mutation in *basA* resulted in aberrant cell wall thickening. Here our data suggest that accumulation of dihydrosphingosine is responsible for this phenotype. In addition, two different mutations in *basA* consistently accelerated the transition from asexual development to sexual development compared to the wild-type strain. The phenotype could be suppressed by exogenous addition of phytosphingosine. Northern analysis suggests that faster sexual development in the *basA* mutant might be due to a higher transcription level of *ppoA* and *steA*, genes demonstrated to coordinate a balance between asexual and sexual development in *A. nidulans*. Consistent with these findings, mutations in the ceramide-synthase-encoding genes *barA* and *lagA* also caused faster transition from asexual to sexual development, supporting the involvement of sphingolipid metabolism in fungal morphogenesis.

SPHINGOLIPIDS are a group of lipids, derived from long chain bases (also known as sphingoid bases), which are *N*-acylated and linked with various polar ligands. Sphingolipids are ubiquitous components of membranes in eukaryotic species, forming, together with sterols, membrane microdomains (also known as lipid rafts). These lipid rafts serve as platforms for signal transduction and membrane trafficking (Simons and Ikonen 1997). In addition, the intermediates in the sphingolipid synthesis pathway are also signaling molecules that mediate cell-to-cell recognition, differentiation, apoptosis, and modulation of the immune response in mammalian cells (Merrill *et al.* 1993; Vaux and Korsmeyer 1999; Warnecke and Heinz 2003; Helms and Zurzolo 2004). Sphingolipid synthesis is essential for cell viability in mammal cells (Hanada *et al.* 2000), in the yeast *Saccharomyces cerevisiae* (Pinto *et al.* 1992), and in the filamentous fungus *Aspergillus nidulans* (Cheng *et al.* 2001).

The budding yeast *S. cerevisiae* has been a model for studies on sphingolipid biosynthesis, thereby permitting the identification of most genes required for sphingolipid synthesis in *A. nidulans* (summarized in Figure 1). *De novo* synthesis of sphingolipids in *S. cerevisiae* starts with the condensation of serine and palmitoyl Coenzyme A (CoA) to yield 3-ketosphinganine, which is catalyzed by a serine palmitoyltransferase (Hanada 2003). The *lcbA* gene is required for this step in *A. nidulans* (Cheng *et al.* 2001). 3-Ketosphinganine is then reduced to form sphingoid base dihydrosphingosine (DHS) in yeast (Beeler *et al.* 1998). The corresponding gene for this step has not been reported in *A. nidulans*. DHS is hydroxylated on the C-4 to form the sphingoid base phytosphingosine (PHS) in yeast and further converted to phytoceramide by the condensation of acyl-CoA with PHS (Merrill 2002). Two functionally nonoverlapping ceramide synthases, BarA and LagA, are responsible for this step in *A. nidulans* (Li *et al.* 2006). Phytoceramides are further converted to inositol phosphorylceramide (IPC) by the addition of myo-inositol phosphate in yeast (Merrill and Wang 1986). The *aurA* gene is required for IPC synthesis in *A. nidulans* (Cheng *et al.* 2001). A number of sphingolipid synthesis inhibitors are well characterized. Among them, myriocin and aureobasidin A (AbA), with inhibitory activity against serine palmitoyltransferase (Ikushiro *et al.* 2004) and IPC synthase (Zhong *et al.* 2000), respectively, are widely used to investigate the physiological functions of sphingolipid synthesis (Cheng *et al.* 2001; Gopee and Sharma 2003; Jahnson *et al.* 2004).

In yeast, phytosphingosine synthesis is catalyzed by Sur2 (identical to SYR2), a sphingolipid C4-hydroxylase that converts dihydrosphingosine to phytosphingosine

[1]These authors contributed equally to this work.

[2]*Corresponding author:* Department of Biological Sciences, 1425 W. Lincoln Highway, Montomery Hall Bldg., Northern Illinois University, DeKalb, IL 60115. E-mail: amcalvo@niu.edu

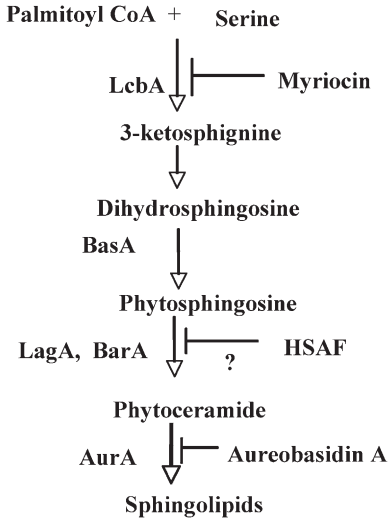

Figure 1.—A simplified scheme for the sphingolipid synthesis pathway in *A. nidulans*. Enzymes and inhibitors for the corresponding reaction steps are listed on the left and right, respectively.

(Grilley *et al.* 1998). Genes encoding Sur2 homologs in *Pichia ciferrii* (Bae *et al.* 2004) and in *Arabidopsis thaliana* (Sperling *et al.* 2001) are also required for DHS C4 hydroxylation, suggesting that the biochemical activity of Sur2 homologs is highly conserved in a wide range of species. Other than yeast, the physiological role of Sur2 homologs has not been investigated in other fungi. Predicted Sur2 homologs are widely found in fungal species but their functions are still unknown in filamentous fungi. Recently, we identified an *A. nidulans* mutant with a missense point mutation (TGG → TGC; W44 → C) in the Sur2 homolog (47% identity over 328 amino acids) encoded by the *basA* gene. This mutant, *basA1*, displays hypersensitivity to an antibiotic [*h*eat *s*table *a*ntifungal *f*actor (HSAF)] produced by the biocontrol agent *Lysobacter enzymogenesis* strain C3 (Li *et al.* 2006). *basA1* also displays other defects, including hyperbranching, cell wall thickening, and growth arrest at restrictive temperature (Li *et al.* 2006). In contrast, deletion of yeast *SUR2* does not cause growth defects (Grilley *et al.* 1998).

Cell wall thickening is a striking phenotypic caused by the *basA1* mutation and other perturbations of ceramide synthesis (Li *et al.* 2006). Similar to our observation in *A. nidulans*, dramatic alterations in cell wall structure were also observed in other fungi in response to defects in sphingolipid metabolism. For example, the cell wall is drastically thicker in *S. cerevisiae* when the paralogous ceramide synthases Lag1 and Lac1 are simultaneously deleted (Barz and Walter 1999). Cell wall thickening was also observed in a *Schizosaccharomyces pombe* temperature-sensitive mutant defective in sphingolipid hydrolytic activity (Feoktistova *et al.* 2001). All these observations suggest that cell wall thickening might be a general response to the disruption of sphingolipid metabolism. However, the mechanism for cell wall thickening in these studies was not investigated.

In addition, sphingolipids and their intermediates serve as secondary messengers mediating differentiation in mammal cells (Schwarz *et al.* 1995). The role of sphingolipids or their intermediates in fungal differentiation has not been described until now. Differentiation from vegetative hyphal growth into asexual or sexual spores is essential for fungal dissemination, survivability, and pathogenecity for many filamentous fungal species (Adams *et al.* 1998; Deising *et al.* 2000; D'Souza and Heitman 2001; Calvo *et al.* 2002). *A. nidulans* has been a useful model system for studies of both asexual and sexual differentiation in filamentous fungi (Adams *et al.* 1998; Calvo *et al.* 2002). Morphological differentiation is triggered by specific environmental factors such as light, air, and nutrients. For example, light suppresses sexual sporulation and promotes asexual sporulation (Mooney and Yager 1990; Yager 1992). Responding to environmental cues, the expression of genes required for sexual or asexual development is activated through complex signal transduction networks. Several developmental genes have been identified. For example, *brlA*, which encodes a zinc (Zn)-finger protein that plays an essential role in the initiation of asexual sporulation (Adams *et al.* 1988; Mirabito *et al.* 1989), or the regulatory gene s*teA*, which encodes a homeodomain-C2/H2-Zn+2 finger transcription factor required for development of ascogenous tissue and cleistothecia (Vallim *et al.* 2000). The delicate balance between asexual and sexual differentiation is regulated by multiple mechanisms. The *veA* gene is a major regulator controlling the asexual/sexual ratio (Yager 1992; Kim *et al.* 2002; Kato *et al.* 2003). Deletion of *veA* completely suppresses sexual development and promotes asexual sporulation (Kim *et al.* 2002). In addition, lipogenic signal molecules, known as *p*recocious *s*exual *i*nducers (psi factors), also play an important role in governing asexual and sexual differentiation (Champe *et al.* 1987; Champe and el-Zayat 1989; Mazur *et al.* 1991). Psi factors are composed of hydroxylated oleic (18:1) and linoleic (18:2) moieties called psiβ and psiα, respectively (Calvo *et al.* 2001). The position of the hydroxyl groups on the fatty acid backbone further defines the psi compounds as psiB (8′-hydroxy-), psiC (5′,8′-dihydroxy-), and psiA with a lactone ring at the 5′ position of psiC (Mazur *et al.* 1991). psiBα and psiCα promote sexual development and suppress asexual development (Champe and el-Zayat 1989) while psiAα has the opposite effect (Champe *et al.* 1987). PpoA, a putative fatty acid dioxygenase required for biosynthesis of psiBα, is known to be required for sexual development. On the other hand, overexpression of *ppoA* reduced asexual sporulation and increased sexual sporulation (Tsitsigiannis *et al.* 2004a). Psi factors communicate with transcriptional factors regulating fungal differentiation. For example, expression of *brlA* was elevated in

TABLE 1

Strain list

Strain	Pertinent genotype	Source
A28	*pabaA, biA; veA1*	FGSC[a]
GR5	*pyrG89; wA3; pyroA4; veA1*	FGSC[a]
Ree1	*pyroA4; veA1*	Stinnett *et al.* (2006)
A773	*pyrG89; wA3; pyroA4; veA1*	FGSC[a]
8-145	*pabaA, biA; basA1, veA1*	Li *et al.* (2006)
TSA2	*pabaA, biA, pyrG89; basA::pyrG; basA1, veA1*	Li *et al.* (2006)
FGSC4	*veA*$^{+}$	FGCS[a]
UV13	*pabaA6; biA1; barA1, veA1*	Li *et al.* (2006)
UV13p	*pyrG89; wA3; pyroA4; barA1, veA1*	This study
ASL5	*pyrG89; wA3; pyroA4; alcA(p)::basA::pyr-4, veA1*	This study
ASL6	*pyrG89; wA3; pyroA4; alcA(p)::basA::pyr-4, veA1*	This study
ASL10	*alcA(p)::lagA::pyr-4; wA3; pyrG89; veA1*	Li *et al.* (2006)

[a] Fungal Genetic Stock Center, Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas 66160-7420.

the *ppoA* deletion mutant while *ppoA* expression was reduced in the *brlA* deletion mutant (Tsitsigiannis *et al.* 2004a). *ppoA* is also regulated by *veA*. Expression of *ppoA* was completely suppressed in the *veA* deletion mutant (Tsitsigiannis *et al.* 2004b).

In this study, we demonstrate that BasA, essential for hyphal growth in *A. nidulans*, is required for phytosphingosine synthesis. Our results here indicate that accumulation of DHS might be the cause of the cell wall thickening in the *basA1* mutant. Furthermore, we found that BasA has a role in *A. nidulans* morphogenesis, regulating the transition from asexual and to sexual development via its effects on the expression of the oxilipin gene *ppoA* and *steA*.

MATERIALS AND METHODS

Fungal strains and media: The strains used in this study are described in Table 1. Media used for growing *A. nidulans* include minimal medium MNV (1% glucose, nitrate salts, vitamins, trace elements, pH 6.5), MNVTF (0.1 M threonine, 0.2% fructose, nitrate salts, vitamins, trace elements, and vitamins, pH 6.5), MAG (2% malt extract, 2% glucose, 0.2% peptone, trace elements, and vitamins), YGV (2% dextrose, 0.5% yeast extracts, and vitamins), and YGT (2% dextrose, 0.5% yeast extracts, and 1 ml/liter trace elements). Trace elements, vitamins, and nitrate salts are described in the Appendix to Käfer (1977). Media were solidified using 1.5% agar. Uridine (5 mM) and uracil (10 mM) were added as needed. Strains were grown at 28° unless otherwise indicated. For temperature-sensitive (Ts) strains, permissive temperature was 28° and restrictive temperature was 42°.

Culture chemical treatments and preparation of samples for microscopy studies: Phytosphingosine (Avanti Polar Lipids, Alabaster, AL), C2 phytoceramide (Sigma-Aldrich, St. Louis), Aba (Takara, Madison, WI), and myriocin (Sigma-Aldrich) were dissolved in methanol at 1 mg/ml, 10 mM, 1 mg/ml, and 80 mg/ml, respectively, and stored at −20°. For chemical treatment, chemicals were added into media with 0.05% Tergitol NP-40 (Sigma-Aldrich). For treatments in liquid media, conidiospores (~10^4 conidia/ml) were incubated in YGV statically at 28° for 12 hr, allowing them to fully germinated. Coverslips attached with germlings were then transferred to YGV supplemented with chemicals and incubated at 42° for the indicated time. Germlings were fixed and stained with Calcofluor before microscopic observation (as described in Harris *et al.* 1994).

Construction of the *alcA(p)::basA* strain: A 635-bp fragment starting from the predicted initiation codon of *basA* was amplified with the primers Kpn0640 (ACTGGTACCATGGC TACAAACACAACTTTG; *Kpn*I site underlined) and Pac0640R (ACC TTAATTAAGCACGACACAGATACCCCAGGTGAAC; *Pac*I site underlined). The amplified fragment was cloned into pMCB17apx (Efimov 2003) using the *Kpn*I and *Pac*I cloning sites. This construct was transformed in wild-type strain GR5. Homologous integration of this construct generates a single full-length copy of *basA* regulated by *alcA*(p), plus a truncated version (51% of the encoding region was truncated off) controlled by the native promoter. Accordingly, transformants were propagated under *alcA*(p)-inducing conditions (0.1 M threonine and 0.2% fructose) and then grown under repressing conditions (1% glucose) to characterize the mutant phenotypes (Oakley and Osmani 1993).

Studies of sexual and asexual development: To calculate the production of conidiospores, Hülle cells, and ascospores, a plug (12.5 mm^2) of fungal growth on agar surface was harvested with a borer and homogenized in 0.2 ml distilled water. Conidiospores, Hülle cells, and ascospores were counted with a Bright-Line hemacytomer (Hausser Scientific, Horsham, PA) under a light microscope. The mean of conidiospores, Hülle cells, and ascospores per square millimeter from three replicate growth plugs was used to represent the asexual or sexual reproductive levels on each plate. Three independent experiments were conducted.

mRNA studies: Mycelium was harvested at various developmental stages (as indicated in each case) and lyophilized. RNA was exacted using Trizol as described by the supplier (Invitrogen, San Diego). Approximately 20 μg of total RNA was used for RNA blot analysis. Probes for *steA* and *brlA* were generated according to our previous description (Kato *et al.* 2003). Other probe templates were made by PCR amplifying from *A. nidulans* genomic DNA with the following primer pairs: for *fksA*, 5′-AGGAATTGACCACCGACA-3′ and 5′-GG AATCCAGGGTGAGCA′; for *chsB*, 5′-AGCGTGACGTTGATG GA-3′ and 5′-ACCAGGCAACACACTGA-3′; for *ppoA*, 5′-CCTG GTGTTGTTGTGGAA-3′ and 5′-CTGGGAGACCACTATCCA-3′; and for *basA*, 5′-CCTTCGCTTGTTGATGGA-3′ and 5′-TGAA AGCTGCCAGAGACA-3′.

RESULTS

BasA is required for phytosphingosine synthesis and essential for hyphal growth: To identify the possible antifungal mechanism of a dihydromaltophilin-like antibiotic HSAF produced by *L. enzymogenes* strain C3 (a bacterial strain used for biocontrol), 1156 temperature-sensitive mutants were generated by 4-nitroquinoline l-oxide (Harris *et al.* 1994). One mutant, 8-145, displayed hypersensitivity to HSAF. Genetic analysis revealed that HSAF hypersensitivity and the Ts growth were caused by

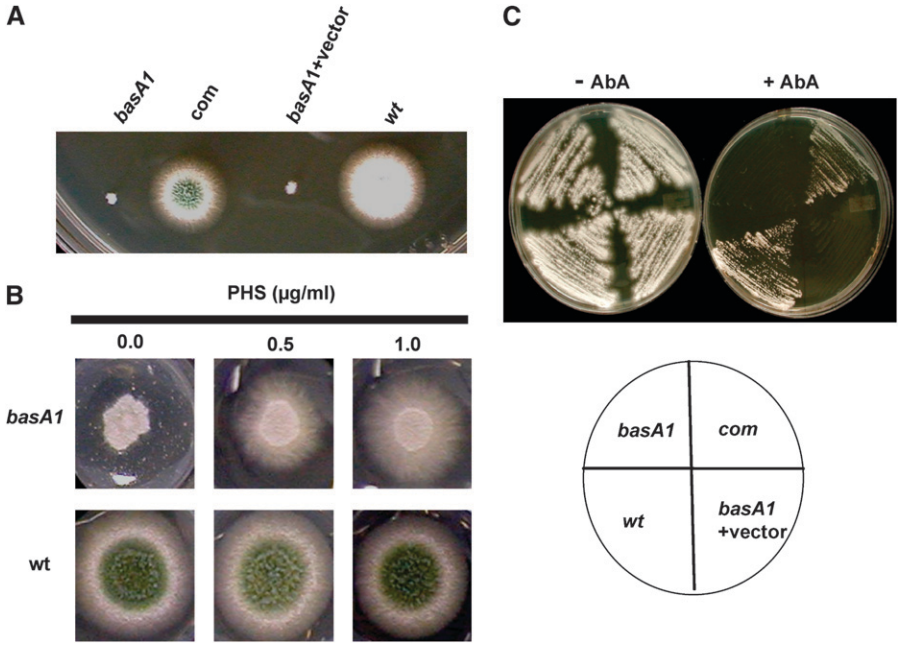

FIGURE 2.—Functional characterization of *basA*. (A) Complementation of *basA1* mutant. *basA1* mutant, a complemented strain cotransformed with a *basA* PCR product, and plasmid pRG3-AMA1, a *basA1* strain transformed with vector pRG3-AMA1 and wild-type strain (GR5), were grown at 42° for 3 days. (B) Restoration of wild-type growth of *basA1* mutant with exogenous phytosphingosine on solid medium. Wild-type strain A28 or *basA1* mutant conidiophores were inoculated on MAG with PHS at the indicated dosages and incubated at 42° for 3 days. (C) Sensitivity of *basA1* mutant to AbA. Strains were streaked onto MAG plates with or without 0.25 μg/ml AbA and incubated at 28° for 3 days.

a single recessive mutation. The corresponding gene and mutation were annotated as *basA* and *basA1*, respectively. Sequence analysis of the *basA1* allele showed that it is caused by a missense point mutation (TGG → TGC; W44C). Complementation of the *basA1* mutation with the wild-type allele resulted in normal growth (Figure 2A). BLAST searches revealed that BasA is 47% identical to the yeast SUR2 dihydrosphingosine hydroxylase over 328 amino acids (LI *et al.* 2006).

Dihydrosphingosine hydroxylase is required for PHS synthesis. To determine whether BasA is functionally similar to Sur2, we assessed colony growth of the *basA1* mutant on agar medium with various concentrations of PHS. Our results show that addition of PHS dramatically improves the growth of the *basA1* strain at restrictive temperature, whereas the concentrations used (0.5–1.0 μg/ml) have no effect on wild-type growth (Figure 2B). Furthermore, the *basA1* mutant displayed hypersensitivity to the inositol phosphorylceramide synthase inhibitor AbA at permissive temperature (Figure 2C). These data, together with the hypersensitivity of the *basA1* mutant to the putative ceramide synthesis inhibitor HSAF (LI *et al.* 2006), strongly suggest that BasA is required for sphingolipid synthesis and is functionally equivalent to its homolog gene Sur2.

In yeast, sphingolipid synthesis is required for survival, but *SUR2* is not essential for viability (HAAK *et al.* 1997). To determine whether BasA is essential for growth (as opposed to a mere requirement for growth only at high temperatures), we generated a conditional disruption mutant following the strategy detailed in Figure 3A and in LI *et al.* (2006) in which full-length *basA* was controlled by an alcohol-dependent promoter while an additional *basA* copy with a 49% truncated coding region was controlled by the natural promoter (Figure 3A). When grown on minimal medium with *alcA*(p)-inducing carbon sources such as 100 mM threonine, 1% glycerol, or 2% ethanol, these strains grew as well as wild-type controls and displayed normal hyphal morphology (Figure 3B). However, on repressing medium, the wild-type colony size was similar to that on inducing medium, whereas the *alcA*(p)::*basA* mutant strain fails to form visible colonies even at permissive temperature, as in the case of the *basA1* mutant. These results, together with the fact that the *basA1* growth phenotype could be remediated by complementation with the wild-type allele (Figure 2A), indicated that the colony growth defect was due to repression of *basA* expression. Conidiospores from the *alcA*(p)::*basA* disruption mutant inoculated on the agar surface of repressing medium exhibited excessive swelling and formed short, fat germ tubes (Figure 3B), similar to *basA1* germlings grown at restrictive temperature (LI *et al.* 2006). These observations demonstrate that BasA is crucial for viability in *A. nidulans*.

Balanced sphingolipid synthesis is required for normal cell wall organization: The *basA1* mutant displayed aberrant deposition of cell wall materials at restrictive temperature (LI *et al.* 2006). To test whether cell wall thickening is a general response to the disruption of sphingolipid synthesis, we examined cell walls of wild-type hyphae grown in the presence of myriocin or AbA. AbA blocks the synthesis of complex sphingolipids, but does not prevent the accumulation of their precursor sphingoid bases and ceramides. Exposure of wild-type hyphae to AbA caused the accumulation of cell wall material (Figure 4A). In contrast, myriocin, which blocks the synthesis of all sphingolipids, including sphingoid bases and ceramide, did not trigger the formation of cell wall thickenings (Figure 4A).

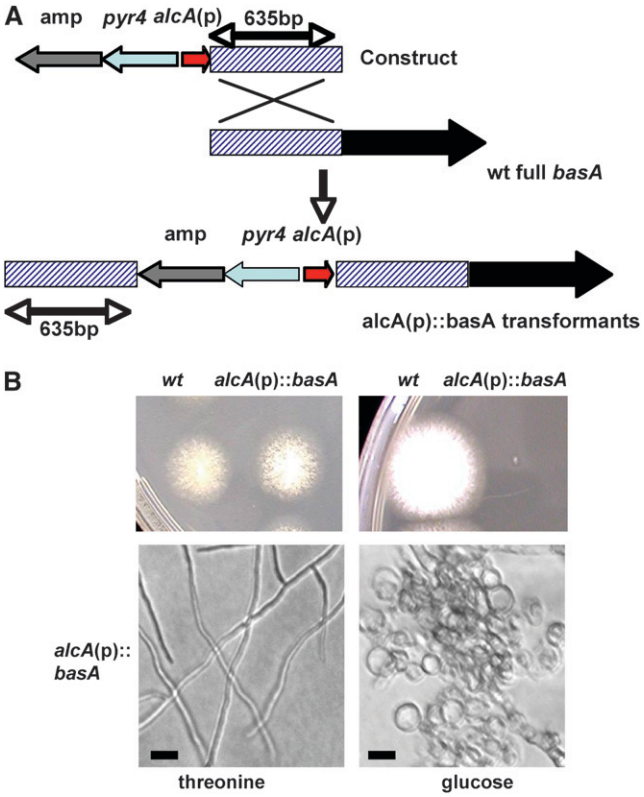

FIGURE 3.—Essential role of *basA* in growth. (A). Schematic of the generation of *alcA*(p)-controlled strains. (B) Phenotypic characterization of the *alcA*(p)::*basA* strain. Wild-type strain A28 and *alcA* (p)::*basA* strain ASL6 conidiospores were inoculated on *alcA*(p)-inducing medium MNVTF (threonine) or repressing medium MNV (glucose) and incubated at 28° for 3 days. Microscopic images were captured under a light microscope with a ×10 objective. Bar, 10 μm.

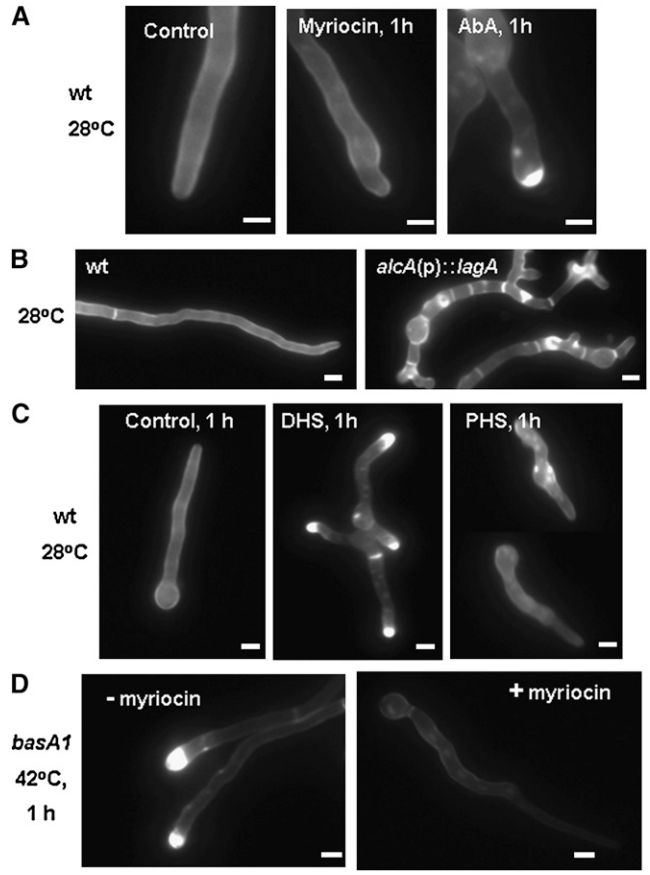

FIGURE 4.—Effects of sphingolipid synthesis disruption on cell wall structure. (A and C) Wild-type strain A28 conidia were germinated at 28° for 12 hr and then shifted to a drug-free YGV (control) or to YGV supplemented with 0.25 μg/ml AbA, 20 μg/ml of myriocin, 1 μg/ml dihydrosphingosine, or 1 μg/ml phytosphingosine and incubated for an additional 1 hr. (B) Wild-type strain A28 and *alcA*(p)::*lagA* strain ASL10 conidia were grown in *alcA*(p)-repressing medium YGV at 42° for 13 hr. (D) *basA1* mutant conidiospores were germinated in YGV at 28° for 12 hr and shifted to fresh YGV with or without 20 μg/ml of myriocin for an additional 1 hr of incubation at 42°. Hyphae were fixed and stained with Calcofluor. Bar, 3 μm.

Therefore, the accumulation of sphingoid bases rather than the depletion of sphingolipids is most likely responsible for the aberrant accumulation of cell wall material in *basA1* mutants.

Chemical or genetic disruption of ceramide synthase activity also causes cell wall thickening in *A. nidulans* (*i.e.*, the *alcA*(p)::*lagA* incubated under repressing conditions; Figure 4B). Because this effect could not be remediated by supplementation with C2-ceramide (10 μM/ml; data not shown), it is presumably caused by the accumulation of upstream intermediate(s) such as the sphingoid bases. To identify which intermediate is able to induce cell wall thickening, we treated wild-type hyphae with either PHS or DHS. DHS treatment (1.0 μg/ml) caused the formation of thick cell wall at hyphal tips in 89% of germlings ($n = 143$) within 1 hr (Figure 4C). Prolonged treatment (4 hr) resulted in the formation of multiple thick cell wall patches distributed throughout the hyphae (supplemental Figure 1 at http://www.genetics.org/supplemental/). At the same dosage, PHS did not cause the accumulation of cell wall material (Figure 4C). Although thickened cell wall patches were ultimately observed in germlings subjected to prolonged treatment with PHS, the effects were milder than those induced by DHS in terms of the intensity of Calcofluor staining (supplemental Figure 1 at http://www.genetics.org/supplemental/). Therefore, accumulation of DHS is likely responsible for cell wall thickening in response to *basA1* mutation and other perturbations of sphingolipid synthesis. In support of this possibility, exposure of the *basA1* mutant to 20 μg/ml myriocin at 42° largely suppressed the accumulation of cell wall material (Figure 4D).

A. nidulans has five chitin synthase genes, among which *chsB* is essential for normal hyphal growth (BORGIA *et al.* 1996). Our Northern analysis showed that *chsB* transcript accumulation was higher at 28° than after the heat treatment (42°) in both wild type and the *basA1* mutant.

chsB expression was similar in the *basA1* mutant and in the wild-type strain (supplemental Figure 2 at http://www.genetics.org/supplemental/). The transcription of other chitin synthesis genes, including *chsA*, *chsC*, and *chsD*, was also examined. However, the transcriptional levels of these genes were too low to make a comparison between wild type and mutant (data not shown). Accumulation of *fksA*, the only glucan synthase gene in *A. nidulans*, was also higher at 28° than at 42° in both wild type and the *basA1* mutant (supplemental Figure 2 at http://www.genetics.org/supplemental/). Most of the mannoprotein-encoding genes remain uncharacterized in *A. nidulans*, with the exception of *mnpA*. However, the *mnpA* mutant did not show any phenotypic differences with respect to wild type (Jeong *et al.* 2003). For this reason, we did not examine the transcriptional response of these genes in the *basA1* mutation background.

Normal developmental pattern requires an intact phytosphingosine synthesis: In cultures growing in the dark, sexual sporulation is the predominant mode of reproductive development in strains with a wild-type *veA*+ genetic background, while conidiation dominates in strains with a *veA1* mutation (encoding a truncated VeA protein missing the first 36 amino acids) (Käfer 1965; Kim *et al.* 2002). Interestingly, the *basA1* strain in the *veA1* background displayed increased sexual reproduction in the dark. At permissive temperature, when conidia were inoculated at the center of the plates and incubated in the dark, the control strain A28 and a complemented *basA1* mutant produced a high number of conidiophores on YGT media during a 7-day incubation period. In contrast, the *basA1* mutant developed sexually under the same experimental conditions (Figure 5). The golden nursing tissue composed of Hülle cells surrounding the cleistothecial primordia could be seen under the microscope (Figure 5, E and H), as well as a higher ascospore production in older cultures (Figure 5J) . Similar observations were also made with the *alcA*(A)::*basA* mutant growing on *alcA*(p)-repressing solid medium. When mycelium grown in liquid *alcA*(p)-inducible medium was transferred onto repressing solid medium, the yield of conidia was 76% lower than that of the wild type. In contrast, the production of Hülle cells in the *alcA*(A)::*basA* mutant was 39-fold higher than that of the wild type (supplemental Figure 3 at http://www.genetics.org/supplemental/). This induction of sexual development observed in *alcA*(A)::*basA* resulted in high production of ascospores. Supplementation of PHS restored a wild-type sporulation pattern to the *basA1* mutant in a concentration-dependent manner. As shown in Figure 6, sexual development in the *basA1* mutant was suppressed and conidiation was restored around wells where 2–4 μg of PHS was added.

Expression of *basA* correlates with Aspergillus development: Mutation of *basA* altered the sporulation pattern. Does this suggest that asexual development requires high levels of *basA* expression compared to those required for sexual development? To test this hypothesis, we compared transcription levels of *basA* in mycelia, as well as during asexual development and sexual development, using a *veA*+ wild-type strain that facilitates the induction of either asexual or sexual sporulation (Käfer 1965). In a shift experiment where the mycelium was initially grown in liquid medium and then transferred onto solid medium, conidiation was induced 12–24 hr after the shift in cultures exposed to light, whereas sexual development was induced after 20 hr of incubation in the dark. Northern blot analysis showed that *basA* was expressed in all developmental stages (Figure 7). However, the highest transcript levels were observed mostly at the early stages of asexual or sexual development. For example, during asexual development, *basA* transcript levels were highest during conidiophore formation (12 hr) and then decreased after that time (12–24 hr). During sexual development, *basA* transcripts reached the highest levels when Hülle cells started to form (after 20 hr of sexual induction) and then gradually decreased to a level similar to that of mycelial growth. Accumulation of *brlA* transcripts coincided with the highest level of expression of *basA* during asexual development, at 12 hr, and continued to accumulate overtime. No obvious differences in *basA* transcription levels between asexual and sexual development stages were observed. These data suggest that high levels of *basA* expression are correlated with the initiation of both asexual and sexual development.

***basA* is required for normal transcription pattern of *ppoA* and *steA*:** To investigate whether *basA* interacts with known regulatory factors directing the morphological development of the fungus, the transcription levels of *ppoA* and *steA* were compared between the *basA1* mutant and both the wild-type and complemented strain. When mycelium grown in liquid medium was transferred onto solid medium and incubated under conditions that induce sexual development, asexual and sexual sporulation were observed in both wild type and *basA1* mutant. However, sexual sporulation was more abundant than asexual sporulation in the *basA1* mutant compared to the control (data not shown). Correlated with these observations, transcript levels of *ppoA* and *steA* were increased in the *basA1* mutant compared to the wild-type and complementation strain after induction of sexual development by reducing aeration and dark conditions (Figure 8). These observations suggest that the effects of the *basA1* mutation on sexual development could be caused by increased expression of *ppoA*, leading to the activation of *steA*.

Ceramide synthase genes *barA* and *lagA* are required for normal sporulation pattern: The data above suggest that insufficient PHS synthesis might induce sexual development. To see whether PHS is the sole sphingolipid intermediate involved in Aspergillus development, we also examined the role of the ceramide synthases

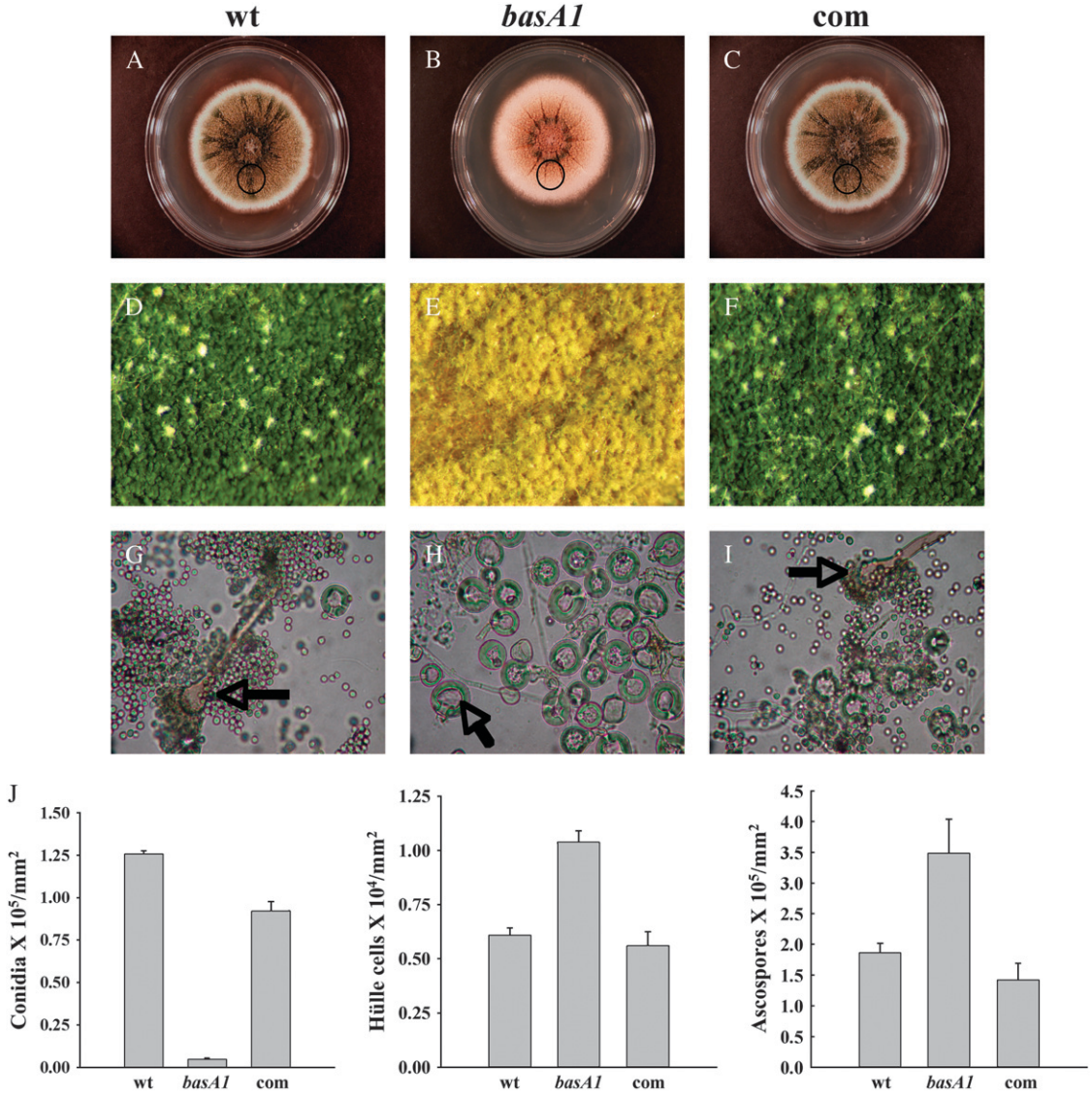

FIGURE 5.—Comparison of asexual and sexual differentiation between wild type and the *basA1* mutant. Conidiospores of A28 (wt), *basA1* mutant, or complementation strain (com) were inoculated at the center of YGT plates and incubated at 28° in constant dark for 7 days. (A–C) Images of full colonies. (D–F) Images of growth in the area 3.5 cm away from the inoculation origin (circled area in A–C) under a dissecting microscope with a ×6 objective. Conidiophores (in G and I) and Hülle nursing cells (in H) are labeled with arrows, respectively. (G–I) Samples were collected, crushed, and fixed. Images in G–I were captured under a light microscope with a ×40 objective. (J) Quantitative comparison of conidiospore, Hülle cell, and ascospore production among wild type, *basA1* mutant, and complementation strain. Quantitative comparison of ascospore production among wild-type, *basA1* mutant, and complementation strain was done 10 days after inoculation. Samples were harvested from circled regions in A–C.

BarA and LagA. In the *barA1* mutant, in which a truncated nonfunctional BarA protein was expressed (LI *et al.* 2006), the sporulation pattern is similar to that of the wild-type strain when the plates were not sealed with parafilm (data not shown). However, if the plates were sealed (sexual-inducing conditions), the yield of conidia was 76% less than that of wild type, while sexual fruiting structures were more abundant than in the wild-type strain and the yield of Hülle cells was 8.3-fold higher than wild type (Figure 9). Although there was notable induction of the initial steps of sexual development in the *barA1*, maturation of cleistothecial primordia was delayed in this case and ascospores were first seen after 14 days of incubation (data not known). Similarly, when the *alcA*(p)::*lagA* strain was grown on repressing media (corresponding to a disruption phenotype), conidial production was 66% lower than in the control strain, while Hülle cell production was 10.9-fold higher compared to the control strain. As in the case of *barA1*, but different from that of *basA1*, maturation of cleistothecia was also decreased (data not shown). Under these conditions, conidial production in the *alcA*(p)::*lagA* strain was higher than in the *alcA*(p)::*basA* strain and Hülle cell production was lower.

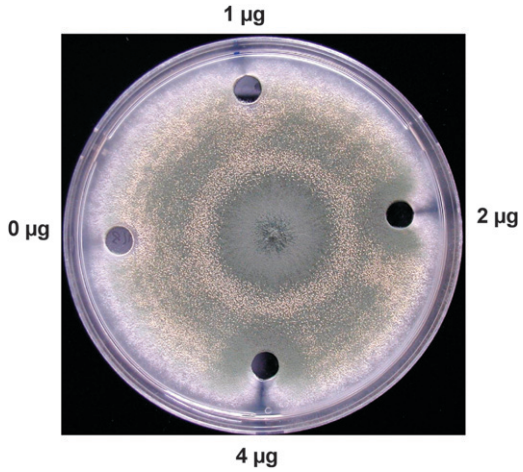

FIGURE 6.—Supplementation of PHS suppresses sexual sporulation in the *basA1* mutant. Conidiospores were inoculated at the center of the MAG plates, and wells (7-mm diameter) were made 3 cm away from the inoculation point. A total of 50 μl of YGV with the indicated doses of PHS was added to the wells.

These data indicate that mutations in *basA*, *barA*, and *lagA* affect *A. nidulans* asexual/sexual developmental balance, although *barA* and *lagA* seemed also to be critical for proper maturation of the forming cleistothecia. Taken together, these observations suggest that, in addition to PHS, other sphingolipid intermediates are also important for Aspergillus development. Alternatively, alteration of the sporulation pattern could be a general response to the disruption of sphingolipid synthesis.

DISCUSSION

In this study, we demonstrated that the *A. nidulans* Sur2 homolog BasA is also required for the synthesis of PHS by supplementation studies. The functional similarity of Sur2 homologs in a broad range of species such as *S. cerevisiae* (HAAK *et al.* 1997), *P. ciferrii* (BAE *et al.* 2004), *A. nidulans* (this study), and even in plants, as described in *A. thaliana* (SPERLING *et al.* 2001), strongly suggest that this protein group has a conserved enzymatic activity in various eukaryotic systems.

In *S. cerevisiae*, the BasA homolog Sur2 is essential for C4 hydroxylation on sphingoid bases, but is not required for growth (HAAK *et al.* 1997; GRILLEY *et al.* 1998). Therefore, C4 hydroxylation of sphingoid bases is not essential for growth in yeast, which can survive by utilizing dihydrosphongosine as a substrate to form dihydroceramide and sphingolipids (HAAK *et al.* 1997). By contrast, in the model filamentous fungus *A. nidulans*, we have demonstrated that the synthesis of phytosphingosine is essential for hyphal growth. Furthermore, the growth defect of the *basA1* mutant is at least partially due to insufficient supply of substrate for sphingolipid synthesis. Therefore, sphingolipids that have undergone

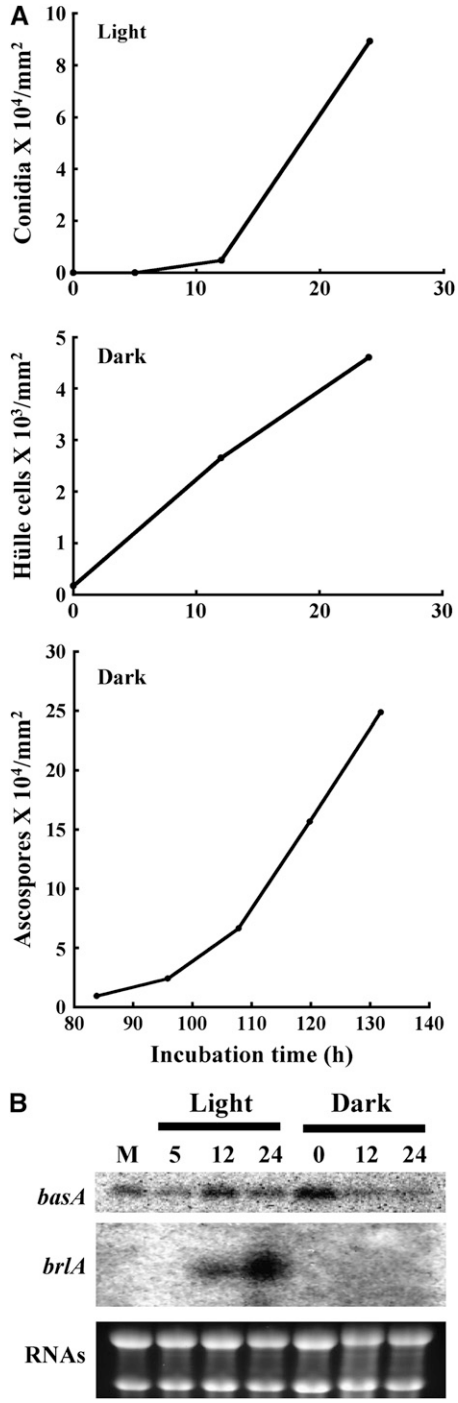

FIGURE 7.—Correlation of *basA* transcription with fungal development. Conidiospores from wild-type strain FGSC4 were germinated in YGV liquid medium at 28° for 18 hr. Mycelium was harvested and then transferred onto YGV solid medium. Asexual development was induced by exposing plates to constant light, and sexual development was induced by sealing plates with parafilm and incubating in the dark for 20 hr. Samples were taken at various time points during asexual development (5–24 hr after shifting to solid medium) and sexual development (0–24 hr after 20 hr sexual induction) for RNA analysis. (A) Quantitative analysis of conidia, Hülle cells, and ascospores after induction of asexual and sexual development, respectively. (B) Northern analysis of *basA* transcripts. rRNAs stained with ethidium bromide were shown as an indicator for equal loading of RNA.

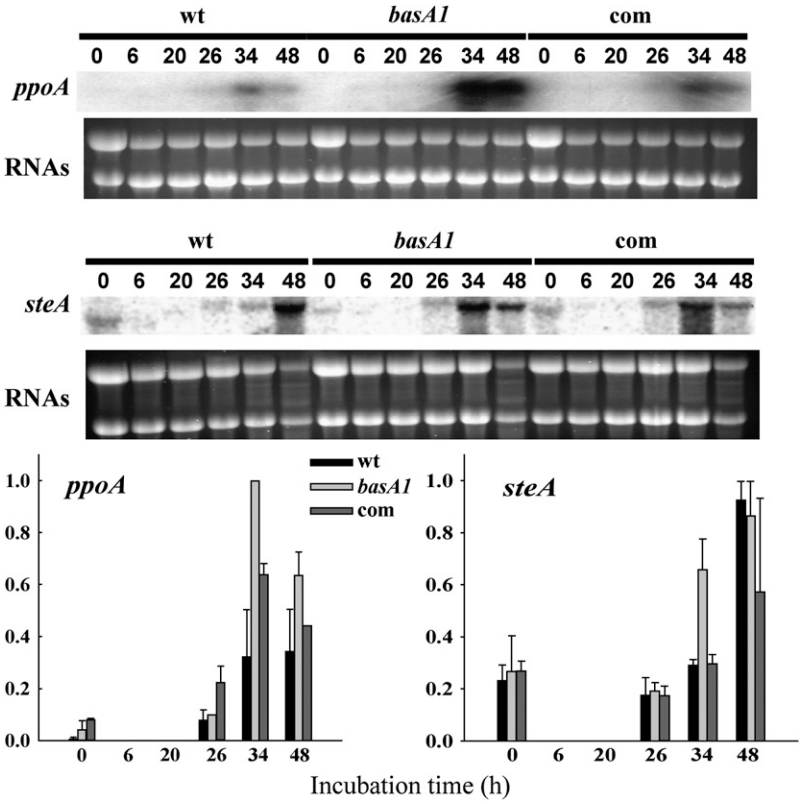

FIGURE 8.—Effects of the *basA* mutation on the transcription of developmental genes in *A. nidulans*. The wild-type strain A28, the *basA1* mutant 8-145, and the complementation strain were cultured in shaken conditions in YGT liquid medium for 20 hr and then shifted onto solid YGT medium. At that time, plates were sealed with parafilm and incubated at 28° in the dark to induce sexual development for 20 hr. Total RNA corresponding to wild type and *basA1* strains was isolated at the time of the shift ($t = 0$), at 6 and 20 hr (during sexual development induction), and at 26, 34, and 48 hr after the shift (6, 14, and 28 hr after sexual development induction, respectively). Transcriptional levels of *ppoA* and *steA* were examined by Northern analysis. rRNAs stained with ethidium bromide are shown to indicate RNA loading. The experiment was repeated twice with similar results (average values are shown). Relative transcript levels were quantified by densitometry using Scion Image Beta 4.0.2.

C4 hydroxylation are presumably required for essential biological functions in *A. nidulans*. This might reflect an inability of the fungus to use dihydrosphingosine as substrate to form ceramide and sphingolipids.

Cell wall thickening has been previously observed in three different fungal species with defects in sphingolipid metabolism (BARZ and WALTER 1999; FEOKTISTOVA *et al.* 2001; LI *et al.* 2006), suggesting a possible role for sphingolipids in cell wall construction. Here, we provide additional evidence supporting such a role by a systemic study of the cell wall response to the disruption of sphingolipid synthesis. Our results strongly suggest that the cell wall thickening caused by various perturbations of sphingolipid synthesis is due to the accumulation of sphingoid bases such as DHS or PHS. PHS *in vitro* is able to activate the yeast protein kinases Pkh1 and Pkh2

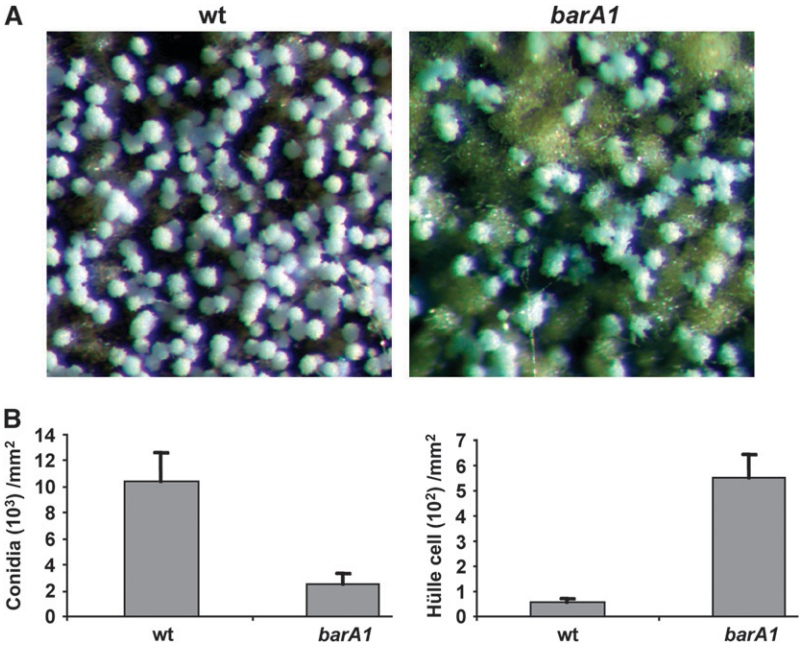

FIGURE 9.—Role of BarA in sporulation. Conidiospores from wild-type strain A773 and the *barA1* mutant UV13p were inoculated at the center of the MAG plates. Plates were sealed with parafilm and incubated at 28° in the dark for 7 days. (A) Image of the colony surface 1 cm away from the growth front. The image was captured with a Spot Insight Color camera on a Leica MZ75 dissecting microscope with a ×5 objective. (B) Quantitative comparison of conidial and Hülle cell production in the imaged area.

(Friant *et al.* 2001; Liu *et al.* 2005). The well-characterized downstream substrate of the Pkh kinases is the protein kinase Pkc1, which is an essential component of the cell wall integrity pathway (de Nobel *et al.* 2000; Levin 2005). In yeast, the expression of ~20 genes involved in cell wall synthesis is upregulated by the PKC1–MAP signaling pathway (Jung and Levin 1999). The entire Pkc1–MAP kinase signaling pathway is conserved in filamentous fungi, including *A. nidulans* (de Nobel *et al.* 2000). Notably, MpkA, the *A. nidulans* homolog of the terminal MAP kinase Mpk1, is required for cell wall integrity (Bussink and Osmani 1999). This information suggests that the sphingoid-base-dependent signal transduction pathway may also be present in *A. nidulans*. Therefore, the increased deposition of cell wall materials in response to the disruption of sphingolipid synthesis might reflect the activation of a sphingoid-base-dependent signaling pathway. Potential targets of this pathway in *A. nidulans* may include the genes involved in chitin synthesis (*i.e.*, *chsB*) or the glucan synthase encoded by the *fksA* gene. However, the expression of these genes was not elevated in the *basA1* mutant. This suggests that the downstream targets of the sphingoid-base-regulated cell wall integrity pathway might vary in different fungal species or that other additional mechanism(s) might trigger cell wall thickening in the *basA1* mutant. PHS is the only identified sphingolipid intermediate that is involved in cell wall synthesis. The role of DHS in cell wall synthesis or deposition was not investigated. However, we found that DHS induced cell wall thickening more quickly than PHS, suggesting that DHS is likely a more effective signaling sphingoid base compared to PHS in regulating cell wall construction in *A. nidulans*. The pathway involved in the activation of cell wall synthesis by DHS will be the subject of future research.

In fungi, sphingolipids and their intermediates regulate multiple physiological processes, including the responses to heat (Dickson 1998; Jenkins 2003) and the loss of cell wall integrity (Friant *et al.* 2001), as well as hyphal polarity (Cheng *et al.* 2001; Li *et al.* 2006). Here, we present the first evidence that sphingolipids regulate the normal developmental pattern in fungal species. The consistent developmental phenotypes caused by mutations affecting the synthesis of either phytosphingosine or ceramide indicate that the normal formation of sphingolipids is required for proper fungal differentiation. Transcription of *basA* was highest during the initiation stage of both conidiation and sexual development, suggesting that a burst of PHS or sphingolipid synthesis may be required for the initiation of both asexual and sexual sporulation. However, mutations of *basA* or the ceramide synthase genes decreased asexual sporulation but enhanced initiation of sexual development, suggesting that initiation of asexual sporulation might require higher levels of sphingolipid synthesis compared to those needed for sexual sporulation.

Although the transcription of the Aspergillus developmental gene *brlA* was not affected in the *basA1* mutant, transcription of the oxilipin gene, *ppoA*, and the sexual development transcription factor *steA* were earlier and slightly elevated compared to wild type. Overexpression of *ppoA* has been demonstrated to increase the ratio of sexual sporulation to asexual sporulation (Tsitsigiannis *et al.* 2004a). Therefore the enhancement in sexual development and reduction of asexual development observed in the *basA1* mutant might be a consequence of the increase in *ppoA* transcript levels that would lead to activation of sexual developmental genes such as *steA* and the consequent increase in sexual development. Considering that sphingolipids and psi factor synthesis share the same upstream precursor substrate, acetyl-CoA, and share an overlapping pathway corresponding to fatty acid synthesis, communication or interaction might exist between these two biosynthetic pathways. In this case, it is also possible that PHS or other sphingolipid intermediates might affect *ppoA* transcription indirectly through interactions between the sphingolipid and the psi factor biosynthetic pathways.

In conclusion, in this study we have demonstrated the role of BasA in hyphal growth and showed that sphingolipid synthesis is required for normal cell wall organization. Furthermore, our study revealed for the first time in fungi that sphingolipid synthesis also regulates the complex balance between sexual and asexual morphological differentiation in the model filamentous fungus *A. nidulans*.

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