The AngFus3 Mitogen-Activated Protein Kinase Controls Hyphal Differentiation and Secondary Metabolism in Aspergillus niger

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Adaptation to a changing environment is essential for the survival and propagation of sessile organisms, such as plants or fungi. Filamentous fungi commonly respond to a worsening of their growth conditions by differentiation of asexually or sexually produced spores. The formation of these specialized cell types is, however, also triggered as part of the general life cycle by hyphal age or density. Spores typically serve for dispersal and, therefore, translocation but can also act as resting states to endure times of scarcity. Eukaryotic differentiation in response to environmental and self-derived signals is commonly mediated by three-tiered mitogen-activated protein (MAP) kinase signaling cascades. Here, we report that the MAP kinase Fus3 of the black mold Aspergillus niger (AngFus3) and its upstream kinase AngSte7 control vegetative spore formation and secondary metabolism. Mutants lacking these kinases are defective in conidiom induction in response to hyphal density but are fully competent in starvation-induced sporulation, indicating that conidiation in A. niger is triggered by various independent signals. In addition, the mutants exhibit an altered profile of volatile metabolites and secrete dark pigments into the growth medium, suggesting a dysregulation of the secondary metabolism. By assigning the AngFus3 MAP kinase pathway to the transduction of a potentially self-derived trigger, this work contributes to the unraveling of the intricate signaling networks controlling fungal differentiation. Moreover, our data further support earlier observations that differentiation and secondary metabolism are tightly linked in filamentous fungi.

Growth and propagation of filamentous fungi typically involve the differentiation of specialized cell types or multicellular structures, such as spores, spore-bearing hyphae, and fruiting bodies, which serve specific biological functions. Differentiation of sexual and vegetative spores, for example, allows the dispersal and, therefore, translocation of otherwise sessile fungi. In addition, spores commonly function as resting structures to endure hostile growth conditions, such as wintertime or droughts. The induction of fungal differentiation usually involves both internal and external signals. For example, conidiation in the red bread mold Neurospora crassa underlies an internal circadian rhythm but is also induced through environmental signals, including light and nutrient deprivation (1, 2). There is growing evidence that fungi also produce a wide range of chemical signals to govern the development and differentiation of individual cells or a mycelial colony within a population (3). It has, for example, long been appreciated that in sexually propagating, heterothallic fungi, cells polarize and direct their growth toward peptide pheromones secreted by the mating partner (4). Cell-to-cell signaling also occurs on the population level or within an individual colony. For example, the primarily unicellular fungus Candida albicans secretes the sesquiterpene alcohol farnesol and the aromatic alcohol tyrosol to regulate morphogenesis in quorum-sensing processes (5, 6). In the filamentous fungus Penicillium cyclopium, the cell density within the mycelial colony determines the accumulation of the diterpene conidiogenone, which above a certain threshold induces conidiation (7). Similarly, in Aspergillus nidulans, FluG/AcoD is essential for the production of a colony-derived, diffusible signaling molecule involved in the induction of conidiation. Disruption of the fluG/AcoD gene results in undifferentiated mycelia with a fluffy appearance (8, 9). Other self-derived fungal signaling molecules involved in the regulation of sporulation and other developmental processes include oxylipins, a group of structurally related long-chain-fatty acid-derived compounds. In A. nidulans, these molecules control differentiation during the life cycle and regulate sexual versus asexual sporulation (10, 11).

Integration of the multitude of external and internal differentiation signals into appropriate cellular responses requires a fine-tuned network of signal transduction cascades. Mitogen-activated protein (MAP) kinase cascades are highly conserved eukaryotic signaling modules that transmit environmental and self-derived signals into the cell. These modules typically consist of three-tiered hierarchical kinases that activate each other consecutively via phosphorylation. The targets of MAP kinases are highly variable and control a plethora of different functions, including transcriptional regulation, organization of the cytoskeleton, metabolism, and cell fusion (12, 13).

The fungal differentiation processes that depend on MAP kinase signaling include sexual and vegetative propagation. For example, in various fungi, including A. nidulans and N. crassa, a MAP kinase module homologous to the pheromone response pathway of Saccharomyces cerevisiae (Fig. 1) is essential for the
formation of fruiting bodies (14–16). In the gray mold Botrytis cinerea, the homologous signaling module is essential for the differentiation of sclerotia and contributes to the control of microconidiation (17). In various plant-parasitic fungi, MAP kinases are pathogenicity factors and are involved in plant surface recognition and the differentiation of infection structures, such as appressoria (18–21).

While these numerous examples illustrate the importance of MAP kinase signaling for fungal development, the role of fungus-derived signals in these differentiation processes is only poorly understood. Recent studies have identified MAP kinases as important mediators of intercellular communication in filamentous fungi. In many ascomycete species, germinating vegetative spores communicate with each other and fuse in order to form functional units, which further develop into the mycelial colony (22). This cellular interaction likely involves a secreted chemical signal (3, 23). In N. crassa and Fusarium oxysporum, the homologous MAP kinases MAK-2 and Fmk1 (homologous to Fus3 of Saccharomyces cerevisiae) are essential for the cellular recognition and communication preceding germling fusion and presumably translate the cellular communication signal into directed growth of the fusion cells (Fig. 1) (24–26).

In this study, we show that the MAK-2/Fus3-homologous MAP kinase and its upstream MAP kinase kinase of Aspergillus niger mediate conidiation in response to cell density or cell age, while both proteins are dispensable for starvation-induced sporulation. Furthermore, the lack of these kinases results in an altered profile of volatile metabolites and piggment secretion, suggesting a dysregulation of the secondary metabolism. Together, our data identify the A. niger Fus3 (AngFus3) MAP kinase as a linker of specific fungal differentiation and secondary metabolite production.

MATERIALS AND METHODS

A. niger strains, media, and growth conditions. The A. niger strains used in this study are listed in Table 1. Strain AB1.13, which is a derivative of the wild-type strain NRRL 3, was used as the reference strain (27–29). Strains were grown on a synthetic minimal medium consisting of (per 1 liter): 20 ml ASPA + N (350 mM KCl, 550 mM KH₂PO₄, 3.5 M NaNO₃), 2 ml MgSO₄, and 1 ml trace element solution [per liter, 10 g EDTA, 4.4 g ZnSO₄·7H₂O, 1.01 g MnCl₂·4H₂O, 0.315 g CuSO₄·5H₂O, 1 g FeSO₄·H₂O, 0.32 g CoCl₂·6H₂O, 1.47 g CaCl₂·2H₂O, 0.22 g (NH₄)₂MoO₄·4H₂O, pH adjusted to 5.5 with KOH]. The standard medium contained 20 g/liter glucose. In experiments testing the influence of the carbon source concentration, different amounts of glucose were applied. The pH of the final medium was adjusted with HCl to 5.5. If required, supplements and agar (15 g/liter) were added.

Strains were incubated at 37°C if not indicated otherwise.

Construction and complementation of angfus3 and angste7 deletion mutants. The angfus3 and angste7 genes were inactivated in a gene replacement approach using homologous recombination. The replacement cassettes consisted of 1-kb upstream and downstream flanking sequences of the respective open reading frames (ORFs). The angfus3 replacement cassette contained a hygromycin resistance cassette as a selectable marker (30), and the angste7 replacement cassette contained the pyrG gene (31). The replacement cassettes were assembled by yeast recombination cloning as described in reference 32. Both constructs were integrated into strain AB1.13 by protoplast transformation as described previously (33, 34). Protoplasts were embedded into minimal medium containing 0.6% (wt/vol) agar and 1.2 M sorbitol and plated onto solid minimal medium containing agar (1.2% [wt/vol]), sorbitol (1.2 M) and, if required, hygromycin (20 μg/ml). After 3 to 4 days of incubation at 37°C, growing colonies were separated and subsequently purified by single spore isolation. The homologous, single integration of the construct was controlled by using Southern blot analysis (35) and PCR analysis.

The angfus3 complementation plasmid was based on pARAn37 (34). A genomic fragment containing the ORF flanked by its 1-kb upstream and downstream sequences was amplified by PCR. MluI and NdeI restriction sites were introduced into the oligonucleotides that were employed and were used to clone the fragment into the vector. The angste7 complementation plasmid was based on pUB0276, a derivative of pARAn37 carrying the hph resistance cassette instead of the original pyrG marker gene. The angste7 ORF, together with the 1-kb upstream and downstream flanking sequences, was amplified by PCR. Through the oligonucleotides, KpnI and HindIII restriction sites were introduced, which were used to clone the fragment into the vector. The final plasmids were ectopically integrated into the Δangfus3 mutant or the Δangste7 mutant by protoplast transformation as described above.

Construction of angfus3-gfp strains and of strains carrying a potentially dominant activated version of AngSte7. To fuse the angfus3 ORF with the green fluorescent protein gene gfp at the original gene locus, a knock-in strategy was employed. The transformation cassette consisted of 800 bp of the 5′ part of the angfus3 ORF without the stop codon, followed by the gfp sequence, the pyrG selection marker, and 1 kb of the downstream flanking sequence of the angfus3 ORF. The knock-in cassette was assembled by yeast recombinational cloning and integrated into A. niger by protoplast transformation. Transformants were analyzed by PCR and fluorescence microscopy.

To overexpress an angfus3-gfp fusion construct, the angfus3 ORF was amplified by PCR and cloned into plasmid pARAn37-gfp using the restriction enzymes PacI and XbaI. In pARAn37-gfp, expression is controlled by the constitutive pkI (protein kinase A gene) promoter. The final plasmid was ectopically integrated into A. niger by protoplast transformation. Transformants expressing the gfp construct were identified by fluorescence microscopy.

| TABLE 1 Strains used in this study |
|-----------------------------------|
| **Strain** | **Relevant genotype** | **Reference or source** |
| AB1.13 | pyrG<sup>−</sup> | 29 |
| Δfus3 strain | Δfus3<sup>−</sup> Δmak-b<sup>−</sup>:hph | This study |
| Δfus3-c strain | Δmak-b<sup>−</sup>:hph P<sup>−</sup>mak-b<sup>−</sup> T<sup>−</sup>mak-b<sup>−</sup> pyrG<sup>+</sup> | This study |
| Δste7 strain | Δste7<sup>−</sup>:pyrG<sup>+</sup> | This study |
| Δste7-c strain | Δste7<sup>−</sup>:pyrG<sup>+</sup> P<sup>−</sup>ste7<sup>−</sup>T<sup>−</sup>ste7<sup>−</sup> hph | This study |
| fus3-gfp strain | mak-b<sup>−</sup> gfp pyrG<sup>+</sup> | This study |
| pkfus3-gfp strain | p<sup>−</sup>pkfus3<sup>−</sup>-gfp P<sup>−</sup>pkfus3<sup>−</sup>-T<sup>−</sup>pkfus3<sup>−</sup> pyrG<sup>+</sup> | This study |
| ste7Δ3(1) strain | ste7<sup>−</sup> S218D,T222D pyrG<sup>+</sup> | This study |
| ste7Δ1(2) strain | ste7<sup>−</sup> S218D,T222D pyrG<sup>+</sup> | This study |
To construct a potentially permanently activated version of AngSte7, serine 218 and threonine 222 were replaced with glutamic acid by site-directed mutagenesis. The mutated ORF was cloned behind the pki promoter into the expression vector pARAn37. The resulting plasmid was ectopically integrated into the recipient strain, AB1.13.

**Determination of the radial growth rate.** To determine hyphal extension rates, 20 μl of a spore suspension containing $2 \times 10^5$ conidia was spotted on a minimal medium agar plate. After 48 h of incubation at 37°C, the colony diameter was measured every 24 h for at least 7 days. The growth rate was calculated as the mean of at least three independent test assays per strain.

**Conidial germination rate.** To determine the germination rate, spores from 4-day-old cultures were harvested. A suspension of these spores was spread onto minimal medium agar plates. After 6 and 8 h of incubation at 37°C, agar blocks were cut out of the plates and analyzed by light microscopy. At least 100 conidia and germ cells of each sample were counted. The germination rate was calculated as the mean value from at least three independent test assays.

**Analysis of conidiophore formation.** To investigate conidiophore development, we adapted a method described previously (36). In brief, minimal medium agar plates were covered with 20 ml of a suspension of liquid minimal medium and 2$\times10^6$ conidia after inoculation. All images showed mycelium from the colony center to the colony growth front. From these images, development stages were assigned to certain time points by combining the measurement of the images with the growth rate.

**Synchronization of growth conditions.** To synchronize conidiophore development, we adapted a method described previously (36). In brief, minimal medium agar plates were covered with 20 ml of a suspension of liquid minimal medium and $2 \times 10^6$ conidia. After 20 h of static liquid incubation at 37°C, the liquid medium was removed, thereby exposing the so-far-undifferentiated mycelium that had developed at the agar surface to the air. Cultures were further cultivated at 37°C for defined time periods.

**Quantification of spore formation.** To quantify conidiation of radially growing colonies, the entire colony was transferred to a Falcon tube containing 5 ml of water. After thorough vortexing, the suspension was filtered through eight layers of cheesecloth, and the conidia were counted in a Thoma cell counting chamber. The number of spores was normalized to the size of the colony, which was determined before harvesting.

The conidiation of synchronized mycelia was quantified using flow cytometry analysis. Colony pieces measuring 19.6 mm² were punched out and the conidia were suspended in 1.25 ml water. Samples were analyzed together with a reference containing a defined number of particles in a fluorescence-activated cell sorting (FACS) apparatus. Spore numbers were calculated as conidia per cm².

**Glucose concentration measurements.** To determine the glucose concentrations of culture filtrates, samples of 200 μl were taken from static liquid cultures just below the mycelium mat. The samples were diluted to an estimated range of 2.5 to 50 mM glucose and analyzed as previously described (37).

**Bright-field and fluorescence microscopy.** Samples were observed on a Zeiss Axioshot 2 microscope equipped with Nomarski optics using a Zeiss Plan Neofluar 100×/1.30 oil immersion objective (product code ZEISS-440481). As a light source for fluorescence excitation, an Osram mercury short arc bulb (HBO 100 W/2) was used. Images were captured with a PCO Pixelfly camera that was controlled by a modified version of 4-D microscopy software (four-dimensional [4-D] microscopy is threedimensional imaging over time) (38) programmed by Christian Hennig and Ralf Schnabel.

To obtain overview images of entire colonies or larger regions of a colony, samples were analyzed with a Leica M60 stereomicroscope equipped with a Leica DFC295 camera. Fluorescence images were obtained with a Leica MZ205 FA stereomicroscope.

**SEM.** Scanning electron microscopy (SEM) was performed as described previously (39). Conidiophores and conidia of fully sporulated, 6-day-old colonies grown on minimal medium agar plates were transferred with a cotton swab to sticky pin stubs. The samples were covered with gold under vacuum and subsequently analyzed with a Zeiss DSM 982 electron microscope.

**Southern blot analysis.** Mutants were analyzed by Southern blot analysis for correct integration of the knockout cassette and the absence of additional heterologously integrated fragments. Southern blotting and hybridization were performed as described in reference 40. Genomic DNA of selected transformants and the recipient strain was digested with EcoRI (for analysis of the Δfus3 mutant) or HindIII (for analysis of the Δste7 mutant). The entire gene knockout cassettes were used as probes.

**Analysis of pigment production.** To investigate pigment secretion of the mutant strains, 10$^6$ spores/ml were added to 5 ml liquid minimal medium containing glucose in concentrations varying from 0.0125 to 3.2 M as a carbon source. The test tube cultures were incubated at 37°C in total darkness. After 20 and 50 days, the cultures were photographed. At the end of the cultivation, the mycelial dry mass was determined. All assays were conducted as independent triplicates.

**Analysis of volatile secondary metabolites.** For analysis of volatile secondary metabolites, the wild-type A. niger strain AB1.13 or the A. niger Δfus3 mutant was grown on agar plates containing synthetic minimal medium and incubated at 37°C until fully grown out in the plates. The volatiles were collected on charcoal filters by use of a closed-loop stripping apparatus (CLSA), a specialized apparatus that used a circulating air stream to trap volatiles emitted by the agar plate cultures on charcoal filters (41, 42). After a collection time of 24 h, the filters were extracted with CH$_2$Cl$_2$ (30 μl, analytical grade) and the extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 7890B GC fitted with an HP-5 fused-silica capillary column (30-m length, 0.25-mm inner diameter, 0.25-μm film; Agilent) that was connected to an Agilent 5977A mass spectrometer. The GC conditions were as follows: inlet pressure, 77.1 kPa; He at 23.3 ml min$^{-1}$; injection volume, 1.5 μl; transfer line, 300°C; electron energy, 70 eV; splitless operation mode with 60-s valve time; carrier gas, He at 1.2 ml min$^{-1}$. The GC was programmed as follows: 5 min at 50°C and then increasing at 5°C min$^{-1}$ to 320°C.

**RESULTS**

**Identification of angfus3 and angste7 of Aspergillus niger.** Via a BLAST analysis (43), the gene An08g10670 of A. niger was identified as the homolog of FUS3 of S. cerevisiae. According to the common gene nomenclature in aspergilli, the gene was named angfus3. The open reading frame (ORF) of angfus3 comprises 1,216 bp and contains three introns of 55, 49, and 47 bp. The sizes and positions of the introns were confirmed by sequencing of full-length cDNA. The encoded protein possesses 61% identity and 78% similarity to Fus3 of S. cerevisiae.

Similarly, An11g10690 was identified as the homolog of STE7, encoding the upstream MAP kinase kinase of Fus3 of S. cerevisiae. We named the A. niger gene angste7. The predicted ORF of the A. niger gene consists of 1,816 bp, containing three introns of 55, 49, and 47 bp. The sizes and positions of the introns were confirmed by sequencing of full-length cDNA. The encoded protein shows 43% identity and 62% similarity to Ste7 of S. cerevisiae.

**Construction of angfus3 and angste7 knockout mutants.** We predicted that if AngFus3 and AngSte7 are part of a common signaling cascade, their loss would result in similar growth or developmental defects. To test this hypothesis and to analyze the function of this MAP kinase module for the growth and differentiation of A. niger, individual knockout mutants were generated for both genes by using a gene replacement strategy. In the refer-
ence strain AB1.13, the angfus3 ORF was replaced with a hygromycin resistance cassette, and in an independent approach, the angste7 ORF of AB1.13 was replaced with a functional copy of the pyrG gene, thereby complementing the uracil auxotrophy of the recipient strain (see Fig. S1 in the supplemental material). The primary transformants obtained by these approaches were tested by PCR analysis for homologous integration of the replacement cassettes (data not shown). Positive isolates were further analyzed by Southern blot analysis in order to ensure the absence of additional, unwanted heterologous integrations of the transformation fragments (see Fig. S1). Finally, homokaryotic strains were isolated from the primary transformants via single-spore isolation, and their homokaryotic stage was confirmed by PCR analysis (data not shown). As a result, an angfus3 mutant strain, denoted as the Δfus3 strain, and an angste7 mutant strain, denoted as the Δste7 mutant, were obtained. In order to ensure that potential phenotypic defects of these strains were solely caused by the gene knockouts, a copy of angfus3 or angste7, including a 1-kb region upstream from the start codon and a 1-kb region downstream from the stop codon, was reintroduced into the respective mutant. These tester strains for complementation were denoted as the Δfus3-c strain and the Δste7-c strain.

**Conidiation is affected in the Δfus3 and Δste7 mutants.** When grown on solid minimal medium, the most noticeable phenotype of both kinase mutants was a significant delay in the onset of conidiation formation. After 48 h of cultivation, colonies of the wild-type strain and both complementation tester isolates exhibited a characteristic blackening of the colony center, indicative of conidiation formation. In contrast, the Δfus3 and Δste7 strains remained cream colored, suggesting that no conidia were formed (Fig. 2A). After 72 h, both mutants had initiated conidium formation, which became more apparent at 96 h after inoculation (Fig. 2A). Quantification of conidia formed by individual, entire colonies at the different time points supported the notion of a conidiation delay and revealed that overall sporulation was significantly reduced in the kinase mutants (Fig. 2B). In parallel with the onset of conidiation, the colony underside of the two gene knockout mutants became pigmented, an observation not made for the wild type or the complemented mutant strains (Fig. 2A).

To test whether the delay in conidiation is caused by a general growth defect of the mutant colonies, the linear hyphal growth rates of the different strains were compared. Both knockout mutants grew slightly more slowly than the wild type, but the defect appeared not to be strong enough to explain the reduced sporulation (Fig. 2C). Therefore, the conidiation defect is likely not just a secondary effect of generally delayed, poor growth. Since the mutants carry a sporulation defect, we also tested the quality of the conidia formed. However, no differences in their viability, surface structure, or surface hydrophobicity were observed (see Fig. S2 in the supplemental material). The germination of the mutant spores was only slightly delayed compared to that of the wild type. After 6 h of incubation at 37°C, 83% ± 3% (mean ± standard deviation) of AB1.13 spores had germinated, while only 16% ± 9% of the Δfus3 strain and 10% ± 4% of the Δste7 strain had done so. However, after 8 h of incubation, both mutants had caught up (85% ± 3% for the Δfus3 strain and 84% ± 5% for the Δste7 strain). Taken together, these data indicate that the loss of either angfus3 or angste7 causes a comparable phenotype, suggesting that AngSte7 and AngFus3 function together in one signaling cascade that controls the onset of conidiation and quantity of spores produced but that they are dispensable for the process of conidium formation itself.

In order to quantify the delay in conidiation, we determined the average distance between the borders of the growing colonies and the first dense fronts of conidiophores within radially growing wild-type and mutant colonies. Repeated growth measurements indicated that each colony grows at a constant rate. This spatial distance could therefore be used to determine the temporal onset...
of conidiation (see Materials and Methods). In the wild type, the first aerial hyphae appeared after 7h (Fig. 3). After 14 h, the first conidiophores were detected, and after 19 h, numerous mature conidiophores producing rich conidial chains were present. In contrast, in the Δfus3 and Δste7 mutants, the formation of aerial hyphae was delayed by about 3 h and conidiophore development by about 5 h. These differences were maintained during further colony development, and the spore formation in the mutants did not catch up with that in the wild-type colonies (data not shown). Together, these observations suggest that signaling events controlling the onset of conidiophore and spore formation are affected by deficiencies in the AngFus3 MAP kinase signaling cascade.

The sporulation defect in the Δfus3 and Δste7 strains is nutrient dependent. Earlier studies indicated that sporulation in the aspergilli is influenced by nutrient availability and is induced during starvation (44, 45). To test the contribution of a nutrient signal, we compared the growth of the mutants and the wild-type reference strain on minimal medium containing different glucose concentrations, ranging from 12.5 mM to 3.2 M. The radial growth of the strains tested varied depending on the amount of available sugar. However, for each individual condition, the wild type and the two mutants showed comparable hyphal extension rates, supporting the earlier notion that the loss of the kinases does not result in significant general growth defects. While the radial growth rates were comparable on medium containing concentrations of up to 0.2 M sugar, hyphal extension accelerated on plates containing 0.8 M glucose. At the higher sugar concentration of 1.6 M, the growth of all strains was slightly reduced, and growth became highly restricted on 3.2 M glucose (Fig. 4). This defect was likely due to osmotic stress. Sporulation of the mutant strains occurred as described above on medium containing up to 0.1 M glucose, was reduced on 0.4 M sugar, and was absent at higher concentrations of the carbon source (Fig. 4). The comparable wild-type colonies, however, exhibited robust and rich conidiation on medium with up to 1.6 M glucose. The suppression of conidiation in the mutant was apparent at a glucose concentration that otherwise supported radial colony extension well (1.6 M), suggesting that it was not caused by an inability of the mutants to properly cope with osmotic stress. To further exclude the possibility of an osmotic stress effect, we tested the mutants’ growth and conidiation on medium with different osmotic concentrations using glucose or NaCl as osmolytes. While the above-de-
scribed sporulation defects were reproduced at 0.4 osmol/liter and 0.8 osmol/liter glucose, no significant effects of identical osmolarities of NaCl on spore formation were observed (see Fig. S3 in the supplemental material). In summary, these data indicate that at low carbon source concentrations, the induction of conidiation in the Δfus3 and Δste7 strains is delayed, while sporulation is completely inhibited on carbon source-rich medium.

To distinguish the nutrient influence from possible effects caused by hyphal age and density, we turned to the analysis of synchronized cultures. Solid medium was covered with a suspension of liquid medium and spores of the various strains. After 24 h of incubation, when an undifferentiated mycelium had formed, the liquid was removed. The subsequent exposure to air induced the formation of conidiophores and conidia evenly over the culture’s surface. Since the mutants’ growth is generally not significantly affected in liquid medium, the hyphal age and density are comparable to those of wild-type cultures and should therefore not influence further development. After an additional 24, 48, and 72 h, the samples were assayed for their macroscopic and microscopic appearance and the number of spores formed.

After 24 h, the development of the wild-type and the mutant cultures was comparable for the individual growth conditions. On medium containing up to 0.2 M glucose, the darkening of the colony surfaces was indicative of sporulation (see Fig. S4 in the supplemental material). These data indicate that at this time point, both the mutants and the wild type are generally competent to differentiate conidiophores. At higher sugar concentrations, the sporulation of all strains appeared to be reduced, and it was only observed at 1.6 M or 3.2 M glucose (Fig. 5A). After 48 h, the mutants and the wild type still exhibited comparable development on medium with up to 0.1 M glucose. On medium containing a larger amount of sugar, however, the wild type showed increased sporulation, while conidium formation appeared suppressed in the mutants. At 3.2 M glucose, sporulation was fully absent in the mutant strains, while the wild type reached its highest levels of sporulation (Fig. 5B; see also Fig. S4). After 72 h, the observed trends had stabilized (Fig. 5C; see also Fig. S4). In summary, under low-glucose conditions, both the wild type and the mutants induced sporulation after short times of cultivation, suggesting an induction due to starvation. In contrast, significant differences between the wild type and the mutants were observed at high concentrations of glucose. On rich medium, the wild type sporulated after prolonged incubation times, while the sporulation of the angfus3 and angste7 mutants was highly reduced and delayed.

To test whether the sugar concentration needed to drop below a certain threshold before sporulation would be induced in the mutant strains, we analyzed standing liquid cultures, in which the fungus forms a floating mycelium on the surface of the medium. Cultures with an initial glucose concentration of 200 mM or 400 mM were compared. After 2, 3, 4, 5, and 6 days of incubation, the number of spores and the concentration of glucose directly below the mycelial mat were determined. Both the Δfus3 strain and the Δste7 strain induced sporulation independent of the time of incubation but dependent on the sugar concentration. Conidiation took place only after the glucose concentration was reduced to 110 to 150 mM (see Fig. S5 in the supplemental material). Since this induction was independent of the age of the culture, we hypothesize that this sugar concentration is not high enough to fully support growth on the colony surface due to the diffusion restrictions of the mycelial mat. As a consequence, starvation reactions might take place at the colony surface.

Based on the data presented here, we hypothesize that at least two alternative triggers induce conidiation in A. niger. One of these signals is starvation, which is received by the hyphae independent of AngFus3. While A. niger does not differentiate in liquid culture at high nutrient concentrations, it is well known that conidiation takes place under nutrient-limiting conditions. Based on our hypothesis, we expected that this type of sporulation would still be induced in the Δfus3 and Δste7 strains. When tested, this expectation was fully met (Fig. 5D).
To further investigate the role of the AngFus3 MAP kinase pathway in the induction of sporulation, we set out to analyze the effect of an artificial activation of this signaling cascade. It has been shown that replacement of the phosphorylated serine or threonine residues of MAP kinase kinases by negatively charged residues can mimic phosphorylation (46). By using a BLAST analysis, serine 218 and threonine 222 were identified as the main phosphorylation sites in STE7. Both residues were exchanged for glutamic acid residues by site-directed mutagenesis. The mutated construct was transformed into the recipient strain AB1.13, and two independent transformants (denoted the ste7da-1 strain and the ste7da-2 strain) were further analyzed. The number of conidia produced by both strains was significantly reduced compared to the number produced by the wild type (Fig. 6). Analysis of the spatial distribution of conidiophores within the growing colony, however, surprisingly revealed a significantly reduced distance between the mycelial growth front and the first appearance of mature conidiophores (Fig. 6). Unfortunately, due to technical reasons, we were unable to test whether the expression of these constructs indeed result in a hyperactivation of the downstream MAP kinase AngFus3 (see Discussion). Taken together, these observations suggest that a potential hyperactivation of the AngFus3 cascade results in premature conidiophore formation. However, proper activity of this pathway seems to be essential for yielding normal spore counts.

The secondary metabolism appears to be affected in the mutants. In the above-described experiments, we frequently noticed that the medium of older Δfus3 and Δste7 strain cultures turned dark, suggesting the secretion of a pigment, while a similar effect was not observed for wild-type cultures. To investigate this effect in more detail, standing liquid cultures of the wild-type strain and the mutants containing different glucose concentrations (12.5 mM to 1.6 M) were analyzed. After 20 days of incubation, mycelial mats had formed in each culture tube. In all wild-type cultures, independent of the glucose concentration, sporulation had taken place and the medium exhibited no visible pigmentation. In the mutants, sporulation and pigment formation were mostly absent at high glucose concentrations (1.6 and 3.2 M), whereas at low glucose concentrations up to 0.4 M, the culture medium had turned dark (Fig. 7). Interestingly, at 0.8 M glucose, the mutants had formed spores but the medium had remained clear, suggesting that pigment production starts after conidium formation has been induced. This notion is supported by the observation that after 30 days of further incubation, pigments had also formed in the cultures with an initial glucose concentration of 0.4 M. At the same time, condiation had been initiated in the cultures with originally even higher glucose concentrations, while the medium was still clear (Fig. 7). To test the variability of these results, the experiment was repeated two times independently. Each time, comparable observations were made (data not shown). Taken together, these data indicate that in the Δfus3 and Δste7 strains, regulation of pigment production is indirectly or directly disturbed and is initiated when nutrients become limited.

In addition to the altered pigment production, we noticed that mutant cultures on solid medium exhibited a characteristic earthy smell, which was less obvious for wild-type cultures. The differences between the volatiles released by wild-type and Δfus3 strain cultures were investigated by use of a closed-loop stripping apparatus (CLSA) (41). Two representative chromatograms for the wild-type and the mutant strain and the structures of the compounds identified are shown in Fig. 8.

The extracts of both the wild-type and the mutant cultures contained the typical fungal volatile oct-1-en-3-one (compound 1) (Fig. 8) and a series of terpenoids, including the musty odor compound 2-methylisoborneol (compound 2), (8S,9R,10S)-8,10-dimethyl-1-octalin (compound 3), and traces of the earthy odorant geosmin (compound 4). Further compounds identified in headspace extracts from both the wild-type and the mutant were the sesquiterpenes β-ylangene (compound 5), β-copaene (compound 6), 6,11-epoxyisodaturane (compound 7), germacrene D (compound 9), and [1(10)E,5E]-germacradien-11-ol (compound 10). All of these compounds were found in larger amounts in the mutant’s extracts than in the extracts from the wild type. Furthermore, the mutant’s extracts contained an additional compound that was not emitted by the wild-type strain. Its mass spectrum (see Fig. S6A in the supplemental material) was similar to the mass spectrum of a compound in our mass spectral library, (6S,10S)-6,10-dimethylbicyclo[4.4.0]dec-1-en-3-one (compound 11).
pound 11) (see Fig. S6B), which is an oxidation product of the octalin derivative compound 3 arising by allylic oxidation (see Fig. S7A) (47). Therefore, we assumed that the volatile observed in the headspace extracts from the mutant is a similar oxidation product of compound 3, arising by an alternative allylic oxidation (Fig. S7B), since the most activated carbons for oxidative transformations are those in the allylic positions. These assumptions resulted in the suggested structure of (1S,6S,10S)-6,10-dimethylbicyclo[4.4.0]dec-2-en-4-one (compound 8), which was corroborated by careful interpretation of the mass spectrum of the emitted volatile in the light of plausible fragmentation mechanisms (see Fig. S7C). Correct interpretation of the fragment ion formation (48) was demonstrated by a feeding experiment with [6-13C]mevalonolactone (49) that resulted in the incorporation of the label into methyl groups of both compound 3 and its oxidation product compound 8. All fragment ions shifted to increased m/z ratios as expected, thus corroborating the fragmentation mechanisms and structural assignment for compound 8 (see Fig. S7C).

AngFus3 accumulates in conidiophores. Studies in various model organisms have revealed that the activity of MAP kinases is strongly correlated with their subcellular spatial dynamics. To investigate such a potential correlation for AngFus3, we constructed fusion constructs of this kinase and the green fluorescent protein (GFP). In a first approach, the angfus3 ORF was fused at its 3′ end with gfp. The construct was overexpressed under the constitutive pki (protein kinase A gene) promoter in the Δfus3 mutant. In the resulting strains, the wild-type phenotype was fully restored, indicating full functionality of the fusion construct (data not shown). Fluorescence microscopy of spores, germlings, mature hyphae, and conidiophores revealed a cytoplasmic signal and significant accumulation of the fusion construct in nuclei (Fig. 9). To gain a better understanding of the natural expression levels, we tagged angfus3 at the original gene locus with gfp via a knock-in strategy. The growth and development of the resulting mutant were fully comparable to those of the wild type (Fig. 2). Interestingly, we detected no GFP signal in spores, germlings, mature hyphae, or undifferentiated mycelia from liquid cultures (Fig. 9A, C, and E), suggesting that angfus3 is only very weakly expressed at these developmental stages. In contrast, a clear GFP signal was visible in the heads of developing conidiophores. However, no signal was present in the stalks (Fig. 9G to I). Because of the structural complexity of the sporophore heads, we were unable to assign the signal to a specific subcellular localization, such as the nucleus. However, these observations strongly support the role of AngFus3 in conidiation of A. niger.

Based on the observed phenotype of the Δfus3 mutant, we hypothesized that AngFus3 is involved in density-induced sporulation but is dispensable for conidium formation induced by nutrient limitation. To further investigate this question, we compared the induction of AngFus3-GFP in conidiophores formed on radially growing cultures on rich and poor medium. Consistent with the mutant phenotype and the earlier observation, a clear GFP signal was detected in the conidiophore vesicles on the rich medium, while these structures formed under starvation conditions exhibited no fluorescence (Fig. 10). Together, these observations...
further support the hypothesis that AngFus3 is specifically involved in hyphal-density-induced conidiation but is dispensable for starvation-triggered spore formation.

**DISCUSSION**

**Sporulation is induced in response to different triggers.** Our findings that the Δfus3 and Δste7 mutants exhibit overall almost identical developmental defects suggests that, in *A. niger*, both kinases function within one signaling pathway. Conservation of this MAP kinase signaling pathway is well established and has been shown for various fungi, including *N. crassa* (24), *A. nidulans* (14), and various pathogenic species, such as *F. oxysporum* (reviewed in reference 50). The phenotypes of the respective mutants vary to some extent between the different species. For example, in *N. crassa*, the growth rate of the Δmak-2 mutant is significantly reduced (51), while we did not observe a significant growth defect in the Δfus3 or the Δste7 mutant of *A. niger*. Therefore, signaling through this pathway appears to have different contributions to the various fungal life styles. However, defects in the regulation of vegetative spore formation appear to be a common theme. In *N. crassa*, mutants of the mak-2 MAP kinase cascade exhibit overall reduced conidiation; however, the sporulation pattern within the colony is altered and appears to be derepressed. As a result, an even conidial layer is formed on the colony surface, while in the wild type, spores are typically formed at the colony’s periphery (24). This phenotype was even name giving for the respective MAP kinase kinase kinase NRC-1, standing for nonrepressible conidiation (16). In *A. nidulans*, defects in sporulation appear to not only include the temporal and spatial regulation of conidiation but also the formation of the spore-bearing structures, which exhibit an altered and deficient morphology in the mpkB mutant (15). In contrast, we observed no defects in the process of spore formation itself in the Δfus3 and Δste7 mutants of *A. niger*. However, regulatory processes resulting in the induction of conidiation are clearly affected in these strains.

Based on the observed mutant phenotypes, we hypothesize that at least two substantially different triggers of conidiation exist in *A. niger*. On medium containing low carbon source concentrations, the wild type and the mutants initiate sporulation at time points when no conidia are formed on a medium rich in sugar. This suggests that the onset of starvation induces sporulation.

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**FIG 8 Volatiles released by wild-type and Δfus3 mutant agar plate cultures.** (A) Total ion chromatogram of a CLSA headspace extract from the wild-type strain AB1.13. (B) Total ion chromatogram of a headspace extract from the mutant *A. niger* Δfus3 strain. (C) Structures of compounds identified. For direct comparability of compound quantities, chromatogram scaling in panels A and B is the same. Peak numbers in panels A and B refer to compound numbers in panel C. Compounds originating from the medium or contaminants such as plasticizers are not shown.
Conidium formation in response to nutrient limitation appears to be common and has been described for various fungi, including the model organisms *A. nidulans* and *N. crassa* (44, 45). Interestingly, conidiation in response to nutrient limitation was not affected in the /H9004 fus3 and /H9004 ste7 mutants of *A. niger*, suggesting that the signals triggering the induction are not relayed through this MAP kinase cascade. Consistent with this notion, starvation-induced conidiation in liquid culture also took place in the mutant strains.

On medium containing higher sugar concentrations, wild-type strains sporulated abundantly after 2 days, while the mutants never reached higher spore levels than those observed on low-sugar medium. Together, these data suggest that at these later time points, a different trigger of sporulation is active in the wild type that cannot be processed or is not produced by the two kinase mutants. It is well established for other fungal species that signals produced by the mycelial colony can induce sporulation in a hyphal-density-dependent manner. In *A. nidulans*, mutations in the

![FIG 9](image_url) Localization of AngFus3-GFP fusion constructs. (A, C, E, G, I) fus3-gfp strain expressing fus3-gfp from the original gene locus. (B, D, F) Ppk1-fus3-gfp strain overexpressing an ectopically integrated copy of fus3-gfp. (A, B) Ungerminated conidia. (C, D) Germlings after 6 h of incubation. Inset shows nuclei stained by 4',6-diamidino-2-phenylindole (DAPI). (E, F) Hyphae after 24 h of incubation. (G) Mycelium with conidiophores imaged by light microscopy (left) and GFP (center) and merged image (right). (H) Conidiophore imaged by light microscopy (left) and GFP (right). (I) Side view of colony margin imaged by light microscopy (top) and GFP (bottom). Size bars, 10 μm (A to F and H) and 1 mm (G and I).
fluG gene result in fluffy strains lacking conidiation. This phenotype is suppressed when the mutant grows in close proximity to the wild type, suggesting that fluG is involved in the production of a diffusible conidiation signal (8). Similar to the Δfus3 and Δste7 mutants of A. niger, fluG mutants of A. nidulans are not affected in sporulation in response to starvation. It is therefore a consistent hypothesis that the AngFus3 signaling cascade in A. niger is relaying a colony-derived, hyphal-density-dependent signal similar to the fluG-based trigger of A. nidulans. This hypothesis is supported by the observation that the introduction of a potentially permanently active variant of AngSte7 results in the onset of conidiation closer to the colony’s periphery. In the wild type, conidiation was absent from this region, probably because of an insufficient hyphal density. Similarly, the overexpression of fluG in A. nidulans resulted in sporulation under conditions that usually did not support conidiation (8). Unfortunately, we were unable to ultimately prove that AngFus3 is truly hyperactivated by the AngSte7 variant, since the melanin formed by sporulating cultures hampered all attempts at Western blot analysis. In samples from liquid cultures, which were not sporulating and therefore were not producing the pigment, we could not detect AngFus3 by Western blot analysis. Consistent with this finding, we did not detect AngFus3-GFP fusion constructs expressed under the control of the native promoter in hyphae grown in liquid culture, suggesting that the ang-fus3 gene is only poorly expressed in liquid culture.

Following the GFP signal in the AngFus3 reporter strains provided additional evidence that the functions of AngFus3 are restricted to age- or density-dependent induction of conidiation rather than being part of a general sporulation mechanism. While GFP signals were very prominent in conidiophores formed on rich medium, no fluorescence was detected in starvation-induced spore-bearing structures. It is likely that the AngFus3 MAP kinase module is involved in the transduction of a self-derived signal measuring hyphal density, such as the fluG-based signal of A. nidulans or conidiogenone of P. cyclopium. Interestingly, the AngFus3 homologs Fus3 of S. cerevisiae and MAK-2 of N. crassa are also involved in cell-to-cell communication via fungus-derived signals; Fus3 in yeast mating and MAK-2 in hyphal fusion. Together, these observations suggest that this MAP kinase pathway possesses a conserved function in cell-to-cell signaling, while the signals and their biological functions have diverged.

An alternative hypothesis explaining the mutant phenotypes is an inability of the kinase-deficient isolates to produce the postulated self-derived conidiation trigger. However, when we culti-

FIG 10 AngFus3-GFP accumulates in conidiophores formed on glucose-rich medium but not in those formed on glucose-poor medium. Side view of fus3-gfp colonies growing on high-glucose (A) and low-glucose (B) medium. The growing colony margin is on the left side, the differentiating interior of the colony on the right side. The rectangle in each image on the left marks the region shown in higher magnification on the right. Arrows point to developing conidiophores. Size bar, 500 μm.
vated the mutant in close proximity to wild-type cultures or exposed them to medium obtained from sporulating wild-type cultures, no changes in the mutant phenotype were observed (data not shown), suggesting that the signal transduction rather than the signal production is defective in these strains.

The role of the AngFus3 signaling cascade in secondary metabolism. Both the Δfus3 and the Δste7 strain of A. niger secrete a dark pigment into the culture medium. This observation indicates an effect on the regulation of secondary metabolism. A similar observation had been made for *Aspergillus fumigatus* mutants affected in the cell wall integrity MAP kinase cascade. In the presence of tyrosine, these mutants produced elevated levels of a dark pigment, which was identified as pyomelanin (52). Melanin production is considered a defense response of fungi against different physical or chemical stresses (53). Consistent with this notion, pyomelanin production in *A. fumigatus* is induced by cell wall stress. The observed pigment secretion in the Δfus3 and Δste7 mutants of *A. niger* might therefore represent a reaction to stress caused by a generally altered physiology of the mutant mycelia. However, more direct links between signaling by Fus3/MAK-2-homologous kinases and secondary metabolism have been observed. For example, the ΔmpkB mutant of *N. nidulans* produces reduced amounts of sterigmatocystin, and the expression levels of genes involved in terrequinone A and penicillin are reduced (ΔmpkB mutant of *N. nidulans*). In the Δfus3 and Δste7 strains of *A. niger*, pigment production correlates temporally and spatially with conidiation. It appears briefly after the start of starvation-induced conidiation in standing liquid cultures. During growth on solid medium, the pigment appears first at the colony center, following the path of conidium induction. An elegant study by Bayram and coworkers unraveled the potential role of MAP kinase signaling in the coordination of differentiation and secondary metabolite production in *N. nidulans* (14). The AngFus3-homologous kinase AnFus3 (Fus3 of *A. nidulans*) physically interacts with LaeA and components of the velvet complex, a major regulator involved in the coordination of development and secondary metabolism. In addition, VeA has been shown to be a direct target of phosphorylation through AnFus3, which might promote VeA’s association with VelB and LaeA, resulting in altered expression of secondary metabolism genes (14). At the same time, AnFus3 interacts in the nucleus with the transcription factor AnSte12, a major regulator of fungal differentiation. For example, AnSte12 controls sexual development in *A. nidulans*, while the homologous protein in *N. crassa* is also involved in the control of proper conidium formation (14, 51, 56). The observed phenotype of the Δfus3 and Δste7 strains of *A. niger* suggests the presence of a conserved signaling network that includes the AngFus3 signaling cascade, AngSte12, the velvet complex, and LaeA.

A striking phenotype that is further indicative of a role of AngFus3 signaling in secondary metabolite production is the earthy smell of the Δfus3 mutant strain. The homomonoterpenoid compound 2 (Fig. 8) is a drinking water contaminant and widespread bacterial metabolite that was isolated from various streptomycetes, cyanobacteria, and myxobacteria (57–59). Reports from fungi are comparatively rare, but compound 2 has been previously reported from *A. niger* and *Penicillium aurantiogriseum* (60). While the genes and enzymes for the biosynthesis of compound 2 in bacteria are well described (61, 62) and its biosynthesis has been thoroughly studied in feeding experiments (59, 63), the corresponding fungal genes and enzymes still await identification. Similarly, the degraded sesquiterpenoid compound 4 was first isolated from a streptomyces (64) and later also reported from cyanobacteria and myxobacteria (41, 65). Its biosynthesis was also studied in detail by feeding experiments with isotopically labeled precursors (66), followed by identification of the geosmin synthase (67). This enzyme is composed of two domains. The N-terminal domain was shown to catalyze the conversion of the sesquiterpene precursor farnesyl diphosphate into the intermediate compound 10 and the side product compound 9, while the C-terminal domain converts intermediate compound 10 via compound 3 to compound 4. Interestingly, all these intermediates and side products are also observed in the *A. niger* headspace extracts. Compounds 5 to 7 have also previously been described as side products of geosmin biosynthesis (68). While compound 4 is usually the main product of bacterial geosmin syntheses, this compound was only observed as a trace component in the *A. niger* headspace extracts, accompanying the octalin compound 3 as the major product. Future identification of a fungal geosmin synthase will be a first important step toward an understanding of the differences between bacterial and fungal biosynthesis of compound 4 and its closely related metabolites. In the work presented here, we could show that the terpene cyclase’s main product, compound 3, in *A. niger* is oxidized to a compound for which we have suggested the structure of compound 8 based on interpretation of its mass spectrum. The unidentified oxidase for its formation might be under the suppressive control of the AngFus3 MAP kinase cascade.

Conclusion. The data presented in this study provide evidence that vegetative sporulation in *A. niger* is controlled by various triggers and at least two independent signaling pathways. The AngFus3 MAP kinase cascade appears to mediate hyphal-density-dependent sporulation but is dispensable for the induction of conidiation by starvation. The absence of this signaling module also results in a dysregulated secondary metabolism, indicated by anomalous pigment secretion and an altered profile of volatile compounds. Together, these observations illustrate the tight connections between fungal differentiation and secondary metabolism and further our understanding of the intricate signaling networks controlling fungal growth and development.

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