Cyclic AMP Restores Appressorium Formation Inhibited by Polyamines in *Magnaporthe grisea*

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ABSTRACT

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Magnaporthe grisea, the causal agent of rice blast, forms a dome-shaped melanized infection structure, an appressorium, to infect its host. Environmental cues that induce appressorium formation in this fungus include the hydrophobicity and hardness of the contact surface and chemicals produced by the host. An elevated concentration of intracellular cyclic AMP (cAMP) has been implicated in appressorium differentiation in *M. grisea*. Polyamines (putrescine, spermidine, and spermine) are involved in cell growth and differentiation in a wide range of organisms. To understand the role of polyamines in appressorium dif-

ferentiation in *M. grisea*, intracellular polyamines were quantified, and the effects of polyamines and polyamine biosynthesis inhibitors on conidial germination and appressorium formation were tested. High levels of polyamines were detected in freshly collected spores, but the levels decreased during conidial germination. Spermidine was found to be the major component. Polyamines and polyamine biosynthesis inhibitors did not affect conidial germination, but polyamines specifically impaired appressorium formation. Furthermore, exogenous addition of cAMP restored appressorium formation inhibited by polyamines. These results suggest that polyamines may reduce intracellular cAMP levels in *M. grisea*, leading to the inhibition of appressorium formation.

Magnaporthe grisea (T. T. Hebert) Yaegashi & Udagawa (anamorph: *Pyricularia grisea* (Cooke) Sacc.) is a typical hermaphroditic ascomycete and the causal agent of rice blast, the most destructive disease of rice throughout the rice-growing areas of the world. Controlling this disease has been dependent on breeding resistant cultivars and the application of chemical fungicides. However, the frequent appearance of new races has reduced the effectiveness of resistant cultivars in the field (18). Furthermore, environmental regulations have also restricted the use of chemical fungicides. An understanding of infection mechanisms is a prerequisite for the development of new strategies to control this disease.

M. grisea, like many other plant pathogens, forms a specialized infection structure, an appressorium, to adhere tightly to and then penetrate its host. The differentiation and maturation of appressoria are critical steps for successful infection. Appressorium formation in plant-pathogenic fungi is induced by environmental stimuli including thigmotropic and chemical signals from the host surface (7,20). Environmental cues inducing appressorium formation in *M. grisea* include the hydrophobicity and hardness of the contact surface and chemicals from the plant surface (5,15,32). Intracellular signaling systems involved in appressorium differentiation in this fungus have also been investigated during recent years. Lee and Dean (15) found that appressorium formation was induced on noninductive surfaces by the addition of exogenous cyclic AMP (cAMP). Twenty isolates of *M. grisea* originating from rice and other gramineous plants also formed appressoria on noninductive surfaces in the presence of cAMP (14). Furthermore,

targeted disruption of the catalytic subunit gene (*cpkA*) of cAMP-dependent protein kinase in *M. grisea* abolished the ability to form normal appressoria (16). Recently, Beckerman and Ebbole (3) reported that *MPG1*-disrupted mutants, which are unable to form appressoria, formed appressoria when cAMP was added. These experiments suggest that cAMP plays an important role in the regulation of appressorium formation in *M. grisea*.

Polyamines (putrescine, spermidine, and spermine) are ubiquitous organic cations of low molecular weight that bind to polyanionic macromolecules like DNA, RNA, and phospholipids in cells (8). Putrescine is synthesized from ornithine by ornithine decarboxylase (ODC) (EC 4.1.1.17) or from arginine by arginine decarboxylase (ADC) (EC 4.1.1.19) via agmatine and *N*-carbamoylputrescine. Polyamines are involved in cell growth and development in a wide range of organisms (24,28). In fungi, polyamines have been reported to be important in cell differentiation during spore germination, dimorphic transition, and conidiation (13,17,23,25). High levels of cellular polyamine lead to hyphal growth rather than yeastlike growth in the dimorphic fungus *Mucor racemosus* (9). In *Aspergillus flavus*, a low putrescine/spermidine ratio is important for microcycle conidiation (13). Higher plants and bacteria utilize both the ornithine and the arginine pathways for the synthesis of putrescine, but most fungi are thought to have only the ODC pathway (19,29). Therefore, ODC inhibitors and product analogs of polyamines have been tested for inhibition of mycelial growth and used to control plant diseases caused by fungi (6,12,21,29). Recently, it was reported that polyamine biosynthesis inhibitors impair appressorium formation in *Uromyces viciae-fabae* (22). Furthermore, an inverse relationship between intracellular cAMP and polyamines has been reported in bacteria and fungi (17,31). However, to date no attempts have been made to examine the effects of polyamines and polyamine biosynthesis inhibitors on conidial germination, mycelial growth, and appressorium formation in *M. grisea*.

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We demonstrate here that polyamines, including putrescine, spermidine, and spermine, specifically inhibit appressorium formation in *M. grisea*. Furthermore, the inhibition of appressorium formation by polyamines is overcome by the addition of exogenous cAMP.

MATERIALS AND METHODS

Fungal strain and cultural condition. *M. grisea* MG07 was used throughout the experiments and was routinely maintained on complete medium. Fungal cultures were grown on oatmeal agar (50 g of oatmeal per liter) at 21°C under fluorescent light to promote conidiation. Conidia were collected from 10-day-old cultures and washed twice with distilled water.

Conidial germination, mycelial growth, and appressorium formation. Conidial germination and appressorium formation were measured on GelBond film (FMC BioProducts, Rockland, Maine) as previously described (15). Briefly, a 50-μl drop of a conidial suspension (10⁴ conidia per milliliter) was placed on GelBond film, sealed in a moistened box, and incubated at room temperature for 20 h, unless otherwise indicated. The percentages of germinated and germinating conidia induced to form appressoria were determined from direct microscopic examination of at least 100 conidia per replicate in at least four experiments, with three replicates per treatment. Mycelial disks (5 mm in diameter) from actively growing margins of colonies of *M. grisea* MG07 on complete medium were transferred to complete medium containing chemicals and incubated at 24°C for 5 days. Each experiment, with three replicates, was repeated at least twice. Putrescine, spermidine, spermine, methylglyoxal bis-(guanyldihydrazone) (MGBG), N⁶-monobutyl cAMP, and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma (St. Louis, Mo.). Difluoromethylornithine (DFMO) was kindly provided by C. K. Seoung (Chungnam National University, Taejeon, Korea).

Extraction and analysis of polyamines. Potato-dextrose broth was inoculated with a conidial suspension and incubated at 25°C for 1, 2, 4, 8, and 24 h. Germinating conidia were harvested by filtration, washed with distilled H₂O, and ground in ice-cold 5% perchloric acid. The homogenates were placed in an ice bath for 1 h and centrifuged at 26,000 × g for 15 min. The supernatant (perchloric acid-soluble) fraction was used for analysis of free polyamines (4). A 500-μl portion of the sample extract was added to 1 ml of 2N NaOH, mixed with 10 μl of benzoyl chloride, and vortexed for 10 s. Then 2 ml of water saturated with NaCl was added. Benzoylated polyamines were extracted in 2 ml of diethyl ether and centrifuged at 5,000 × g for 10 min. A 1-ml portion

of the ether fraction was evaporated to dryness and dissolved in 100 μl of methanol. Polyamines were analyzed with a Shimadzu LC-6A high-performance liquid chromatograph; a Zorbax octadecylsilane column (4.6 mm × 15 cm; particle size 5 μm) (Dupont Co., Kyoto, Japan) with a mobile phase of 60% aqueous methanol, a flow rate of 1 ml/min, and a detection wavelength of 254 nm was used. The calculation of the polyamine concentration in the extracts was based on the average area counts of each of the three standard polyamines.

RESULTS

Effect of polyamines and polyamine biosynthesis inhibitors on conidial germination, appressorium formation, and mycelial growth. Appressorium formation in *M. grisea* was inhibited by 1 to 5 mM putrescine, spermidine, and spermine in a dose-dependent manner (Table 1). Spermine was the most potent inhibitor, followed by putrescine and spermidine. However, appressorium formation was not affected by the polyamine biosynthesis inhibitors DFMO and MGBG. Appressoria formed in the presence of these inhibitors were well melanized and indistinguishable

TABLE 1. Effect of putrescine, spermidine, spermine, difluoromethylornithine (DFMO), and methylglyoxal bis-(guanyldihydrazone) (MGBG) on conidial germination and appressorium formation in *Magnaporthe grisea*^a

Treatment	Concentration (mM)	Conidial germination (% ± SD)	Appressorium formation (% ± SD)
Putrescine	5	88.7 ± 2.5	1.7 ± 1.5
	1	92.3 ± 2.5	17.0 ± 5.3
Spermidine	5	90.7 ± 2.1	5.0 ± 1.0
	1	92.3 ± 4.0	79.3 ± 4.5
Spermine	5	91.3 ± 3.1	1.7 ± 1.5
	1	94.7 ± 1.5	8.0 ± 2.6
DFMO	5	94.3 ± 1.2	94.3 ± 1.5
	1	92.7 ± 3.2	93.7 ± 2.1
MGBG	5	96.0 ± 1.5	84.3 ± 5.7
	1	95.5 ± 1.0	91.3 ± 5.5
Untreated		96.3 ± 1.5	93.3 ± 1.5

^a A conidial suspension (10⁴ conidia per milliliter) containing polyamine, DFMO, or MGBG was placed on the hydrophobic side of GelBond film and incubated in a moistened box at 25°C. Appressorium formation was determined as described in Materials and Methods.

Fig. 1. Effect of polyamines, applied at different times after conidial germination, on appressorium formation in *Magnaporthe grisea*. The lengths of the bars indicate the duration of treatment with polyamines (added to a final concentration of 5 mM). The frequency of appressorium formation was measured 24 h after incubation on the hydrophobic side of GelBond film. Results are the average of four independent experiments, each with three replicates. The data for water indicate the number of appressoria that had formed at the time of polyamine addition.

from those formed in the untreated control. To investigate the inhibition window of polyamines on appressorium formation, putrescine, spermidine, and spermine were added to conidial suspensions on a hydrophobic surface at different times. Appressorium formation was completely blocked by the addition of a polyamine at the start of incubation or 2 h after the start of incubation. The number of appressoria formed in cultures to which a polyamine was added 4, 6, 8, or 10 h after the start of incubation was similar to the number present at the time the chemical was added (Fig. 1). In other words, no additional appressoria developed after the polyamines were added. Conidial germination of *M. grisea* was not affected by treatment with polyamines at concentrations up to 5 mM (Table 1). The three polyamines did not affect mycelial growth at 0.1 mM, but mycelial growth was significantly reduced at 10 mM (data not shown). Spermine was the most effective inhibitor of mycelial growth of *M. grisea*, followed by spermidine and putrescine. MGBG strongly inhibited mycelial growth at 10 mM.

Effect of cAMP on inhibition of appressorium formation by polyamines. The inhibition of appressorium formation by putrescine, spermidine, or spermine on the hydrophobic surface of Gel-Bond film was reversed by treatments with 10 mM N⁶-monobutyl cAMP or 2.5 mM IBMX (Table 2). N⁶-monobutyl cAMP was much more effective than IBMX. IBMX did not restore appressorium formation inhibited by spermidine or spermine, although 45% of germings formed appressoria in the presence of putrescine and IBMX. Appressoria induced by N⁶-monobutyl cAMP in the presence of polyamines were well melanized and indistinguishable from those formed in the untreated control (Fig. 2). However, appressoria induced by IBMX in the presence of polyamine were small and irregularly shaped (data not shown).

1,16-Hexadecanediol also induces appressorium formation in *M. grisea* on a noninductive surface (5). This chemical also restored appressorium formation in *M. grisea* to a high level after it had been inhibited by polyamines (data not shown).

Polyamine concentration during conidial germination. Freshly collected conidia of *M. grisea* contained a high level of polyamines. The level of polyamines in the fungal cells decreased during conidial germination (Fig. 3). Spermidine was the major polyamine component in *M. grisea*, followed by spermine. Only trace amounts of putrescine were detected. Levels of spermidine decreased during conidial germination, whereas those of putrescine and spermine remained unchanged.

TABLE 2. Effect of N⁶-monobutyl cyclic AMP (mb cAMP) and 3-isobutyl-1-methylxanthine (IBMX) on the inhibition of appressorium formation in *Magnaporthe grisea* by polyamines on a hydrophobic surface^a

Treatment	Appressorium formation (% ± SD)
Putrescine	1.7 ± 1.5
Putrescine + mb cAMP	86.0 ± 2.6
Putrescine + IBMX	44.7 ± 4.0
Spermidine	5.0 ± 1.0
Spermidine + mb cAMP	87.3 ± 1.5
Spermidine + IBMX	10.3 ± 4.5
Spermine	1.7 ± 1.5
Spermine + mb cAMP	91.0 ± 2.6
Spermine + IBMX	2.3 ± 2.5
Untreated	88.7 ± 5.5

^a A conidial suspension (10⁴ conidia per milliliter) containing polyamine, polyamine plus mb cAMP, or polyamine plus IBMX was placed on the hydrophobic side of GelBond film and incubated in a moistened box at 25°C. Appressorium formation was determined as described in Materials and Methods. Polyamines, mb cAMP, and IBMX were added to final concentrations of 5, 10, and 2.5 mM, respectively.

DISCUSSION

In these experiments, we show that polyamines, including putrescine, spermidine, and spermine, specifically inhibit appressorium formation in *M. grisea*. Significantly, conidial germination is not affected by these polyamines or by the polyamine synthesis inhibitors DFMO and MGBG, at concentrations of 5 mM. These inhibitors did not affect appressorium formation. In contrast, appressorium formation in *Uromyces viciae-fabae* is inhibited by DFMO, while conidial germination remains unaffected (22). In *M. grisea*, the lack of an observable effect of DFMO suggests that putrescine and the other polyamines are not required for conidial germination and appressorium formation or that *M. grisea* contains the ADC pathway. Most fungi contain only the ODC pathway, but ADC activity has been detected in *Ceratocystis minor* and *Verticillium dahliae* (11). An alternative explanation is that this concentration of DFMO is not effective in *M. grisea*. Several fungi, including *Septoria tritici* and *Ustilago maydis*, have been reported to be insensitive to DFMO (27, 29), whereas many others, such as *Phytophthora infestans* and *Sclerotium rolfsii*, are highly sensitive (2, 26, 30).

Freshly collected conidia contained high levels of polyamines, which decreased during conidial germination. Our results differ somewhat from other observations. In *Mucor racemosus*, low

Fig. 2. Restoration of appressorium formation inhibited by polyamines in *Magnaporthe grisea*. **A** and **B**, Conidia germinated on the hydrophilic and hydrophobic sides of GelBond film, respectively. **C**, **E**, and **G**, Inhibition of appressorium formation in the presence of putrescine, spermidine, and spermine, respectively, on the hydrophobic side of GelBond film. **D**, **F**, and **H**, Appressorium formation restored by cyclic AMP after inhibition by putrescine, spermidine, and spermine, respectively, on the hydrophobic side of GelBond film.

levels of polyamines were detected in spores, and high ODC activity was detected during early stages of germination (9). Spermidine was the most prevalent polyamine in *M. grisea*. Similar results have been observed in several other fungi, such as *Phytophthora infestans* and *Pythium ultimum* (30). However, in *Aspergillus flavus* putrescine was present at the highest concentration, followed by spermidine and spermine (13). During microcyclic spore differentiation in *A. flavus*, the level of putrescine dropped. However, the putrescine/spermidine ratio increased in *M. grisea* during conidial germination, because of a decrease in the level of spermidine.

Intracellular cAMP is an important modulator of appressorium formation in *M. grisea* (15,16). The level of cAMP in the cells is regulated by two enzymes, adenylate cyclase and phosphodiesterase, for synthesis and degradation, respectively. N⁶-monobutyl cAMP restored appressorium formation inhibited by polyamines at high frequencies (Table 2 and Fig. 2). This indicates that polyamine inhibition of appressorium formation in this fungus is upstream of where cAMP functions and suggests that polyamines may decrease intracellular cAMP levels. Because IBMX was unable to restore appressorium formation inhibited by polyamines, the inhibition of phosphodiesterase activity may not be sufficient to allow the accumulation of effective levels of cAMP in the presence of polyamines. Therefore, polyamine inhibition of appressorium formation may be due to the suppression of adenylate cyclase. It has been documented that polyamines depress cAMP synthesis by inhibiting adenylate cyclase in other organisms (1,17). Also, ODC activity has been shown to be negatively regulated by cAMP in both eukaryotic and prokaryotic cells (10,31). Alternatively, polyamines may directly or indirectly increase the requirement for cAMP by causing other cellular effects. In this case, the normal activity of adenylate cyclase might be insufficient to induce appressorium formation. The mechanism by which polyamines mediate cAMP metabolism in *M. grisea* remains to be elucidated.

Although the precise mechanisms involved in the inhibition of appressorium formation by polyamines are not clear, our data suggest that polyamines exert these effects through cAMP in *M. grisea*. Understanding the specific inhibition of appressorium differentiation is not only of interest in cell biology but also important in providing direction for the development of novel strategies to control rice blast disease.

Fig. 3. Polyamine analysis by high-performance liquid chromatography during germination of conidia of *Magnaporthe grisea*. Polyamines were extracted from freshly collected conidia and germinating conidia 1, 2, 4, 8, and 24 h after inoculation of potato-dextrose broth. The polyamines include putrescine, spermidine, and spermine.

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