cAMP regulation of “pathogenic” and “saprophytic” fungal spore germination

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Abstract

We report on the elucidation of two separate pathways of spore germination in a plant pathogenic fungus Colletotrichum gloeosporioides f. sp. aescinomenae. Conidia of the fungus can germinate either from one side or from both sides, depending on external conditions. In shake culture that includes an extract made up from fresh peas, the unicellular conidium divides and one of the two cells develops a germ tube. On a solid surface this germ tube differentiates an appressorium. In rich medium without pea extract, germination is highly similar to Aspergillus spore germination: the conidium swells, forms a single germ tube and then divides and forms a second germ tube. Conidia that germinate in a rich medium do not form appressoria even on a solid surface and are non-pathogenic. In rich medium, cAMP stimulates germination in rich liquid cultures and induces appressoria formation on a hard surface. In pea extract cAMP induces swelling and formation of irregular germ tubes and appressoria. Our results suggest that plant surface signals induce pathogenic-specific spore germination in a cAMP-independent manner. cAMP is required for saprophytic germination and for appressorium formation.

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1. Introduction

Spore germination is a key process common to all fungi. It can be divided into four stages: (i) breaking of spore dormancy, (ii) isotropic swelling, (iii) establishment of cell polarity, and (iv) formation of a germ tube and maintenance of polar growth (d’Enfert, 1997; Wendland, 2001). In the yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe, glucose is sufficient to induce spore germination. The signal is transmitted through Gα/Ras pathways that cause rapid increase in cellular cAMP levels, which in turn activate cPKA catalytic subunits (Hatanaka and Shimoda, 2001; Hreman and Rine, 1997; Xue et al., 1998). The activation of cPKA leads to activation of trehalose degradation and glycerol synthesis that increase the cell osmotic potential (Pahlman et al., 2000).

Conidial germination in the filamentous model species Aspergillus nidulans is also initiated by fermentable sugars. After the isotropic phase, the rounded, unicellular A. nidulans conidium differentiates a single germ tube. Next, the germ tube is separated from the conidium by a septum and a second germ tube develops opposite to the first germ tube (d’Enfert, 1997; Harris, 1999; Wolkow et al., 1996). Similar to yeast spore germination, conidial germination in A. nidulans and Neurospora crassa also involves activation of the cAMP pathway and trehalose breakdown (d’Enfert et al., 1999). However, recent studies reveal substantial differences between S. cerevisiae and A. nidulans in regulation of cAMP levels and in the interaction with other signal cascades (Fillinger et al., 2002).

Spores are the most common fungal infection structures. Upon contact with a putative host they attach to the surface and then germinate. Following spore germination, many plant pathogenic fungi differentiate appressoridia that assist in penetration of the fungus into the host tissues. Unlike A. nidulans that requires an external energy source, spores of plant pathogenic fungi
usually germinate in a water droplet deficient of any nutrients. These spores depend on the limited endogenous resources present within the resting spore for completing all the developmental stages that precede host penetration. These processes, which occur on the host surface, must therefore be highly efficient to ensure successful penetration into the host tissues within a short period of time. Indeed, spores of plant pathogenic fungi respond to plant-specific signals, including chemophysiological characteristics of the plant surface (thigmotropism) and specific plant chemicals such as cuticular waxes, root exudates or plant volatiles (Allen et al., 1991a; Gilbert et al., 1996; Podila et al., 1993; Staples and Hoch, 1997; Tucker and Talbot, 2001). Studies in several plant pathogens and especially in *Magnaporthe grisea* showed that spore germination and appressorium formation are tightly linked and coordinated with each other through several signal pathways. Involvement of the cAMP pathway and of the Pmk1 and Mps1 MAP kinase cascades, have been demonstrated (Dean, 1997; Lee et al., 2003; Thines et al., 2000; Xu, 2000; Xu and Hamer, 1996; Yang and Dickman, 1997). Thus, although the signals that induce spore germination are different, similar signal components govern spore germination in pathogenic and saprophytic fungi.

*C. gloeosporioides* f. sp. *aeschnomene* (C. *gloeosporioides*) specifically attacks the legume weed *Aeschynomene virginica*. The fungus produces unicellular, oval conidia (asexual spores) that do not germinate readily in liquid cultures due to self-inhibitory compounds (Hegde and Kolattukudy, 1998). We previously showed that exposure of conidia to pea extract overcomes this inhibition and induces high rate of conidial germination in shake cultures (Robinson and Sharon, 1999). Germination in pea extract is characterized by rapid nuclear and cell division without swelling, and by the formation of a single germ tube after 2.5 h. The other conidial cell does not germinate and it has been unclear whether it remains viable or whether it loses viability and dies. Although not specifically mentioned, this unidirectional spore germination is typically seen in a range of additional plant pathogens.

In this paper we report that different signals activate two separate germination pathways in *C. gloeosporioides*. Unidirectional spore germination that leads to appressorium formation and plant infection occurs only in the presence of stimuli that are common to plant surfaces, such as pea extract and a hard surface. In rich medium and in the absence of plant signals conidia germinate from both sides as in *Aspergillus*, they do not form appressoria and are reduced in virulence. The ungerminating conidial cell remains viable and can be induced to germinate by cAMP. Our results show that although germination and appressorium formation are separate processes, fungal pathogenicity is determined by the type of germination-inducing signals already at the onset of spore germination.

2. Materials and methods

2.1. Fungi and plants

*Colletotrichum gloeosporioides* f. sp. *aeschnomene*

3.1.3 WT and transgenic strains were used. The fungus was maintained on Emerson’s Yps8s (EMS) medium as previously described (Robinson et al., 1998). For spray inoculation experiments we used 12-day-old *Aeschynomene virginica* plants, which are the natural host of the fungus. Droplet inoculation was performed on the first true leaves of *Pisum aestival* cv. White Sugar, which is also susceptible to the fungus. Plants were grown in 10 cm pots in a growth chamber at 25 °C with a 14 h photoperiod.

2.2. Transgenic fungi

*C. gloeosporioides* transgenic isolates expressing cytoplasmic or nuclear GFP were produced. For cytoplasmic GFP expression we used the gGFP plasmid (Maor et al., 1998). For nuclear labeling we generated the plasmid pRS31w. The plasmid pRS31 (Suelmann et al., 1997) was digested with *Kpn I* and *Ban HI* to release the sGFP-StuA cassette. The fragment was ligated into plasmid pC8HS (Turgeon et al., 1993) that was digested with the same enzymes. Transgenic isolates were obtained by electroporation of germinated conidia according to Robinson and Sharon (1999). The transgenic isolates had WT phenotypes and pathogenicity.

2.3. Dyes and staining procedures

FDA and FM4-64 were purchased from Molecular Probes (Eugene, OR, USA). DAPI was obtained from Sigma. Viable cells were stained with FDA (10 μg/ml in phosphate buffer, pH 7.5) or FM4-64 (7 μM in water). DAPI was added to cells after fixation with 0.1 N NaOH for 30 min. Excitation and emission wavelengths for FDA, FM4-64 and DAPI were 493/510 nm, 506/751 nm, and 385/420, respectively.

2.4. Microscopy

Fluorescent and light microscopy were performed with a Zeiss Axioskop 2 epifluorescence microscope, or with an Olympus SZX 12 fluorescent stereoscope equipped with an eGFP filter. Confocal microscopy was performed by a confocal laser scanning microscope (Zeiss, CLSM 510). Digital images were captured with a DVC-1310 color digital camera (DVC, Austin Texas, USA).
2.5. Spore germination

The fungus was grown for 5 days on EMS plates and conidia were harvested by washing the plates with sterile distilled water. After dilution and counting, the conidia were used to inoculate EMS or pea extract (PE) liquid media (Robinson and Sharon, 1999). We used $10^6$ conidia/ml unless otherwise mentioned. Conidia were germinated in shake cultures or on a solid surface. Germination in a shake culture was performed in 250 ml Erlenmeyer flasks containing 50 ml liquid medium. The experiments were conducted in a shaking incubator operating at 190 rpm, at 28°C in the light. Germination on a solid surface was performed on a microscope glass slide, on leaves, or on the surface of a Petri dish.

2.6. cAMP treatments

cAMP was purchased from CalBiochem. Conidia were harvested in water, inoculated into media containing 20 or 25 mM cAMP and then placed under the different germination regimes.

2.7. Plant assay

*Aeschynomene virginica* plants were sprayed to runoff with 0.1% Tween 20 conidial suspension containing

![Fig. 1. Characteristics of *C. gloeosporioides* conidial germination. (A) Germination in pea extract. Conidia of a transgenic isolate expressing nuclear GFP were inoculated into PE medium and incubated with shaking at 190 rpm. All images are from a confocal microscope and are a combination of light and fluorescent spectra except for Ab, which shows only the fluorescent spectrum. Time points: a, resting conidia; b, 0.5 h; c, 1 h; d, 1.5 h; e, 2.5 h; and f, 3 h. (B) Viability stains of conidia germinated in PE. Conidia were stained with either FM4-64 (a, c, and e) or with FDA (b, d, and f). (a,b) Negative controls of FM4-64 at 4°C, and FDA at 55°C, (c,d) positive staining of viable resting conidia, and (e,f) staining of conidia 4 h after germination in PE. Both cells are stained with both dyes indicating that they are viable. (g) DAPI staining after 3 h showing that both cells contain an intact nucleus. (C,D) Germination in EMS. Conidia were incubated in EMS with shaking at 190 rpm. (C) Differential interference contrast microscopy, (D) fluorescent microscopy after staining with DAPI. Time points: (C) a, resting conidia; b, 4 h; c, 6 h; d, 9 h; e, 12 h; and f, 15 h. (D) a, resting conidia; b, 4 h; c, 9 h; and d, 13 h. (E) Germination of conidia on different surfaces. Conidia were harvested from plates in water and placed on: a, *A. virginica* leaf; b, corn leaf; and c, microscope glass slide. Scale bars represent 10 μm in all pictures.
5 × 10⁴ conidia/ml. Sprayed plants were closed in plastic cylinders and placed in a growth chamber at 25 °C. After 24 h, the cover was removed and the plants were maintained until the end of the experiment in the growth chamber. Pea leaves were drop inoculated by placing 5 μl droplets of conidial suspension containing 5 × 10⁵ conidia/ml on the leaf surface. The leaves were placed in a Petri dish with moisture and the plates were maintained under the same conditions as described for the spray-inoculated plants. Pathogenicity was determined by the rate of plant mortality and fresh weight (whole plants) or by microscopic examination and scoring of necrosis in the drop-inoculated leaves.

3. Results

3.1. Germination in PE shake-culture

When submerged in pea extract (PE) conidia of C. gloeosporioides undergo rapid nuclear and cell division (1 h), after which a single germ tube develops. We further characterized this process using a transgenic strain expressing nucleus-localized GFP (Fig. 1A), and by staining the cells with viable dyes (Fig. 1B). Nuclear migration was observed less than 30 min after submerged conidia in PE shake cultures, and nuclear division was noted after less than 1 h. Next, the two nuclei moved to opposite poles and a septum formed in the middle of the cell (Fig. 1A, d). A germ tube started to emerge from one side after about 2.5 h. The nucleus close to the germ tube divided, one of the resulting nuclei migrated into the germ tube and a septum was formed between the conidial cell and the elongating germ tube (Fig. 1A, f). Viability and nuclear staining showed that the un-germinating cell remained viable and contained an intact nucleus during the entire process (Fig. 1B, g).

3.2. Germination in EMS shake culture

Different germination morphology was observed when conidia were germinated in EMS. Conidia underwent swelling after 5–7 h, and then formed a single germ tube (Fig. 1C, d). After emergence of the germ tube a septum formed inside the conidium and then a second germ tube emerged from the other side. The whole process lasted 12–15 h, depending on conidial age. The morphological changes, nuclear division and the time course resembled the major events that take place during A. nidulans spore germination (Momany and Taylor, 2000; Wolkow et al., 1996). Germination in additional media, e.g., EMS without yeast extract or glucose rich media had similar characteristics (not shown). Thus, two different signals, PE and a free carbon source, induce two highly dissimilar styles of conidial germination.

To test how the different signals affect each other, we placed conidia in PE for variable periods of time, then washed the conidia, moved them into EMS, and scored the percent of germination 2.5 h after introduction into PE. As little as five minutes in PE were sufficient to induce 30% germination after 2.5 h, all from one side (Fig. 2). Germination rates after 2.5 h increased with increasing exposure time to PE. Thus, even a short exposure to PE was sufficient to induce a high rate of unidirectional germination and the early PE signal was superior to the EMS signal. The conidia that did not germinate by 2.5 h underwent swelling and germinated from both sides after longer periods of time (not shown). When submerged in PE + EMS germination was mainly from one side, even when the medium contained only 10% PE. These results suggest that the PE signal is epistatic to the EMS signal.

3.3. Germination on a solid surface

On leaves of A. virginica, conidia germinated as in pea extract, only from one side (Fig. 1C, d). Over 30% of the germ tubes differentiated appressoria, in contrast to germination in PE shake cultures in which appressoria were almost never observed. Conidia germinated in the same way and produced appressoria on the leaf surface of non-host plants (Fig. 1E, b). To test if the signal was plant specific we germinated conidia in EMS on a glass slide. Under these conditions germination still occurred only from one side, but the germ tubes kept elongating and did not form an appressorium (Fig. 1E, c). On a Petri dish, (a hydrophobic surface), germination was mainly from one side and approximately 90% of conidia

![Fig. 2. Short exposure of conidia to pea extract is sufficient to induce “pathogenic” germination. Conidia were harvested from plates and then suspended in PE for various periods of time. At each time point conidia were collected by centrifugation, washed three times with water and transferred to EMS. Germination rate was always scored 2.5 h after suspending the conidia in PE. For example, after 5 min in PE conidia were washed, incubated in EMS for 145 min and then the germination rate was scored. All the conidia that germinated after 2.5 h germinated as in PE at all time points. At each time point we counted 250 conidia. Germination rate was calculated as percent of germination rate in PE after 2.5 h.](image-url)
formed appressoria (Fig. 4A, f). Thus, a hard surface was sufficient to induce unidirectional germination even without PE, but a hard surface + PE or a hydrophobic surface was necessary for appressoria formation. These results show that at least three different signals affect conidial germination: PE, rich carbon source, and a hard surface. Each of the PE and hard surface signals is sufficient to induce unidirectional germination, while appressoria formation occurs only on a hard surface and requires, in addition to a hard surface, hydrophobicity or PE.

3.4. Effect of germination style on conidial pathogenicity

The two styles of conidial germination suggested that they may have different roles and may be important under different conditions. Since on plants conidia germinated from one side while in rich medium from two sides, we hypothesized that only the unidirectional germination may be associated with plant pathogenesis. To test this hypothesis we germinated conidia in PE and in EMS up to the stages of germ tube formation and swelling, respectively, and then used these conidia to inoculate plants. When germinated in PE, conidia were as pathogenic to A. virgínico as the ungerminated control treatment, whereas pathogenicity of conidia that were pre-incubated in EMS was drastically reduced compared to the other two treatments (Fig. 3A). We noted that some of the EMS-treated conidia produced new conidia on the plant surface (not shown). These conidia germinated only from one side and produced delayed symptoms. Indeed, the differences in pathogenicity of the PE and EMS-treated conidia were more distinct four days than six days post inoculation. Microscopic observations of pea inoculated leaves revealed that the control and PE-germinated conidia penetrated the plants and only very little mycelium could be detected on the plant surface after 24 h. Necroses developed under the penetration sites after 36–48 h (Fig. 3B). In contrast, when conidia were pre-incubated in EMS, the mycelium developed on the plant surface without plant penetration, and the leaf tissue underneath the mycelium remained intact. These results confirmed the association of unidirectional germination with plant pathogenesis. We therefore coined the term “pathogenic germination” to describe this type of spore germination while the bi-directional mode was termed “saprophytic germination.”

3.5. Effect of cAMP on spore germination and appressoria formation

In A. nidulans, cAMP is activated early on and induces spore germination and swelling. We therefore decided to test if early external cAMP application will affect pathogenic germination. Conidia were grown in PE shake cultures amended with 25 mM cAMP and the germination phenotypes were determined after 2.5 and 8 h. In PE + cAMP only 30% of the conidia germinated from one side after 2.5 h as opposed to the 90% germination in PE without cAMP. The other 70% started to swell, similar to germination in EMS. After 8 h some conidia germinated from both sides, as in EMS, but there was a range of additional germination phenotypes as illustrated in Fig. 4B. The most common phenotypes included germination from two cells at the same time, formation of a germ tube on one side and an appressorium on the other side, and formation of two germ tubes from the same cell. Only a few regular appressoria formed normally, at the end of a short germ tube, while the rest of them included a range of uncommon phenotypes (Fig. 4A). These changes occurred only in PE medium. Addition of cAMP to EMS did not affect the germination phenotype, but it enhanced germination; conidia swelled earlier and formed a single or even two germ tubes after less than 8 h. Results of additional experiments showed that the saprophytic germination is activated by cAMP, while different, cAMP-independent signal pathways probably regulate pathogenic germination (see Table 1 and Fig. 4 for complete phenotypes and effects of cAMP). cAMP was found important also for appressoria formation. Normally appressoria were formed only on a hard hydrophobic surface or when germinated on a glass slide in PE medium. Formation of appressoria was enhanced by cAMP under additional conditions such as EMS on glass. This result suggested that cAMP might also cause a change in conidial pathogenicity.

3.6. Effect of cAMP on conidial pathogenicity

We tested the effect of external cAMP on conidial pathogenicity after pre-incubation in PE and in EMS. As shown earlier, there was a clear difference in the response of conidia to cAMP in PE and in EMS. In PE, cAMP induced swelling after 2.5 h, and formation of irregular germination and appressoria after 8 h (Table 1, Fig. 4). In EMS, cAMP enhanced swelling and bi-directional germination in shake cultures, and appressoria formation on a glass slide. Two different cAMP treatments were therefore used for PE and EMS germinated conidia. In PE, the purpose was to test pathogenicity of conidia that have been induced to swell and formed abnormal germination and appressoria. cAMP was therefore added to the PE incubation medium and the conidia were allowed to germinate for 2.5 h. This treatment results in enhanced spore swelling and some 30% of unidirectional germination (Table 1). After incubation the conidia were washed with water several times to remove the medium and any remnants of cAMP. In EMS, the purpose was to test pathogenicity of conidia that were germinated under saprophytic conditions...
Fig. 3. Plant infection by conidia that were germinated in PE or EMS. Conidia of a transgenic isolate expressing GFP were harvested from plates and incubated for 2.5 h in PE or for 5 h in EMS. (A) Conidia were sprayed on A. virginica plants and pictures were taken after 6 days. (a) Ungerminated conidia, (b) PE-germinated conidia, (c) EMS-germinated conidia, and (d) uninfected control. The average fresh weight of 6 plants ± SD is shown under each treatment. (B) Conidia were drop inoculated onto detached pea leaves and pictures were taken after 24 h. Lower part fluorescent, upper part visible light micrographs. Pictures (a–f) were taken with a microscope, pictures (g,h) were taken with a fluorescent stereoscope. (a,b) Ungerminated conidia, (c,d,h) PE-germinated conidia, (e,f,g) EMS-germinated conidia. Magnification is similar in panels (a–f). Scale bars are: a, 40 μm; g, 100; and h, 60 μm.

Fig. 4. Germination and appressoria phenotypes induced by cAMP. (A) a–e, Various appressoria phenotypes induced by addition of cAMP to PE or EMS. f, Germination and appressorium formation on a Petri dish without cAMP. (B) Various germination phenotypes induced by addition of cAMP to PE or EMS. Bars represent 10 μm in all pictures.

Fig. 5. Effect of cAMP on conidial pathogenicity. Conidia were harvested from plates and incubated for 2.5 h in PE or for 6 h in EMS. cAMP was added to PE before inoculation such that the conidia were incubated in PE + cAMP during the entire 2.5 h. In EMS cAMP was added after the incubation, before plant inoculation. Conidia were sprayed on A. virginica plants and pictures were taken after 24 h. Six plants were used for each treatment. All plants within a treatment showed identical results. Top without cAMP, bottom with 20 mM cAMP. (A) PE-germinated conidia; (B) EMS-germinated conidia; and (C) ungerminated conidia.
Table 1
Summary of the germination and appressoria phenotypes with and without cAMP

<table>
<thead>
<tr>
<th>Shake cultures</th>
<th>EMS</th>
<th>No cAMP</th>
<th>+25 mM cAMP</th>
<th>PE</th>
<th>No cAMP</th>
<th>+25 mM cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 h</td>
<td></td>
<td>No change</td>
<td>Conidia start swelling</td>
<td>80% germination from one side after cell division, &gt;1% form appressoria</td>
<td>80% germination from one side after cell division, &gt;1% form appressoria</td>
<td>80% germination from one side after cell division, &gt;1% form appressoria</td>
</tr>
<tr>
<td>8 h</td>
<td></td>
<td>Swelling</td>
<td>30% germination from one or both sides, &gt;1% form a single appressorium</td>
<td>Hyphae formation, one cell does not germinate, no appressoria</td>
<td>90% germination from one side after cell division, 1% form appressoria</td>
<td>&gt;90% one side germination, 30% form normal appressoria</td>
</tr>
</tbody>
</table>

Glass slide

| 2.5 h          |     | 80% germination from one side after cell division, >1% form appressoria | 80% germination from one side after cell division, >1% form appressoria | 90% germination from one side after cell division, >1% form appressoria | 90% germination from one side after cell division, >1% form appressoria |
| 8 h            |     | 80% germination from one side, >10% of various phenotypes, >1% form appressoria | 80% germination from one side, >10% of various phenotypes, >1% form appressoria | >90% one side germination, 30% form normal appressoria | 90% germination from one side, 30% form normal appressoria, 5% abnormal appressoria |

The various appressoria and germination phenotypes are shown in Fig. 4.

(From both sides) and then induced to form appressoria on the leaf by cAMP application. Therefore, the conidia were first germinated for 5 h without cAMP until they reached the stage of swelling, and then they were collected, washed with water, and cAMP was added just before plant inoculation. Conidia from both treatments, as well as ungerminated conidia were inoculated on plants and symptoms were recorded. Addition of cAMP to PE had no effect on conidial pathogenicity. No symptoms were observed on plants inoculated with the PE or PE + cAMP germinated conidia after 24 h (Fig. 5), and normal disease symptoms and plant mortality developed several days later (see Fig. 3 for disease symptoms). In contrast, the addition of cAMP to EMS-germinated conidia caused abnormal disease development. Normally there are no symptoms until 48 h. After 48–72 h small anthracnose lesions appear on the leaves, these lesions develop into larger necroses that spread to the stem and eventually kill the plants after 5–7 days. Addition of cAMP to conidia that were germinated in EMS caused early symptoms on the infected leaves (Fig. 5). Brown necroses developed already 24 h after spraying the plants and after 48 h the leaves died. The addition of cAMP to resting conidia caused a similar phenomenon while cAMP alone had no observable effect on the plants. However, the disease caused by the EMS + cAMP treatment was restricted only to the leaves that were directly inoculated with conidia. These leaves developed the early necroses and died within 48 h, but the disease was restricted to these leaves and it did not spread to new leaves or to the stem (not shown). These results suggest that although conidial penetration was enhanced by cAMP, other processes that are required for full pathogenesis may be mis-regulated in the EMS-germinated spores.

4. Discussion

Colletotrichum gloeosporioides f. sp. aescynomene is a facultative, hemibiotrophic plant pathogen: it can grow as a saprotroph on dead organic matter, or as a pathogen by infecting the legume weed A. virginica. Similar to many other plant pathogens, conidia germinate upon contact with the plant and then form appressoria that penetrate the plant cuticle. During the first 24–36 h following plant penetration the fungus develops intramural or biotrophic hyphae. Minimal secretion of hydrolytic enzymes is essential at this stage to avoid provoking the plant defense system (Mendgen and Hahn, 2002; Perfect et al., 2000). A necrotrophic phase follows during which massive hydrolytic enzymes are secreted causing tissue maceration and cell death. The situation is completely different when the fungus grows as a saprotroph. Under these conditions there is plentiful external energy and nutrients available to the germinating conidium, and therefore germination under saprophytic conditions does not depend on the conidium reserves and there is also no need for appressoria. Instead, the breakdown of large organic molecules may require massive secretion of hydrolytic enzymes.

In this study we showed that C. gloeosporioides uses two different germination strategies for pathogenic and saprophytic development. The pathogenic germination
is triggered by chemical and physical plant surface signals and is characterized by rapid cell division and formation of a single germ tube 2.5 h after induction. The saprophytic germination has the \textit{A. nidulans} characteristics; it is induced by fermentable sugars and consists of spore swelling before germ tube formation. Two germ tubes are eventually formed and the process takes between 8 and 15 h. Only the unidirectional type of germination results in plant infection.

Although the different germination styles were demonstrated only in \textit{C. gloeosporioides}, they seem to be common to many other plant pathogens; examination of various publications shows that many other plant pathogenic fungi also germinate on the host only from one side without swelling. Moreover, work in other fungi such as \textit{M. grisea} and \textit{Colletotrichum graminearum}, showed that while these fungi normally form a single germ tube, they are capable of germinating from both sides under some conditions (Chaky et al., 2001; Thines et al., 2000). The work with \textit{M. grisea} signal transduction mutants is of a special interest since some of these mutants have germination defects such as longer germ tubes or formation of multiple appressoria (Adachi and Hamer, 1998; Gilbert et al., 1996; Mitchell and Dean, 1995). These data suggest that separate pathogenic and saprophytic germination pathways are a common strategy in plant pathogenic fungi. Studies in additional plant pathogenic species are necessary to verify the generality of this phenomenon as well as to elucidate the unique characteristics in each species.

PE induces pathogenic germination in liquid cultures but without appressoria. Appressoria are formed only on a hard surface with PE, or on a hard hydrophobic surface. In \textit{M. grisea}, appressoria formation requires functional Pmk1 MAP kinase and is induced by cAMP; pmk1 deletion mutants and various cAMP pathway mutants do not form appressoria, while external addition of cAMP rescues some of the mutations and enhances appressoria formation in the WT (Adachi and Hamer, 1998; Lee et al., 2003; Thines et al., 2000). Treatment of \textit{C. gloeosporioides} conidia with cAMP changed the style of germination and appressoria formation (see Fig. 4 and Table 1 for the various phenotypes). Most significantly, cAMP induced swelling of a large fraction of the conidia in PE (first stage of saprophytic development) and enhanced swelling and germination in EMS. It also induced appressoria formation under conditions which they do not normally form, such as in a PE shake culture, on a hydrophilic surface, or in EMS on plants. The germination and appressoria formation in the presence of cAMP were not always regular and included many uncommon phenotypes (Fig. 4, Table 1). Application of cAMP to PE 2 h after the beginning of incubation resulted in much larger percentage (40%) of regular appressoria. Taken together our results and the works on germination signal transduction pathways in other species suggest that activation of the cAMP pathway is involved in the early stages of saprophytic germination. Cell wall and membrane modifications that are regulated by the cell wall modifying Mpk1/Sl12 MAP kinase pathway might be required at a later stage to allow the following morphogenetic changes (Wendland, 2001). In contrast, the early stages of pathogenic germination are not mediated by cAMP but by other, cAMP independent pathway(s). This is inferred from the disruption of the pathogenic germination by cAMP application. Instead, we suggest that the first events of pathogenic germination are cell division and cell wall modifications. This hypothesis is also supported by the fact that a \textit{C. gloeosporioides} cell wall defected mutant exhibits saprophytic germination in PE (data not shown).

Two MAP kinase modules, homologues of the \textit{S. cerevisiae} Slt2 and Fus3/Kss1 pathways are involved in the regulation of a series of developmental processes in filamentous fungi including conidiation, germination, sexual reproduction, appressoria formation, and pathogenicity (Tudzynski and Sharon, 2003; Xu, 2000). Some functions may be under regulation of either one of these MAP kinases. For example, conidiation and female sterility are regulated in \textit{M. grisea} by the Slt2 pathway.
homologue MPSI (Xu et al., 1998), while in Cochliobolus heterostrophus these processes are regulated by the FUS3/KSS1 homologue CHK1 (Lev et al., 1999). Only the FUS3/KSS1 homologues regulate appressorium formation in a wide range of plant pathogens (Tudzynski and Sharon, 2003; Xu, 2000). C. gloeosporioides forms appressoria only on a hard hydrophobic surface, or on a hard surface in the presence of PE. This suggests that pathogenic germination and appressorium formation are under control of different signal pathways, which may involve the two MAP kinase cascades. A model illustrating our current understanding of the different germination pathways is presented in Fig. 6. According to this model, the PE signal activates cell wall modification and cell division in a process that does not require cAMP. Appressorium formation is induced by surface signals and requires cAMP as well as additional pathways. Under saprophytic conditions the order is reversed: the cAMP pathway is activated first and is followed by cell wall modifications.

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