한국식물병리학회지 14(5) : 413-417(1998)
Korean J. Plant Pathol. 14(5) : 413-417(1998)

Factors Affecting Appressorium Formation in the Rice Blast Fungus *Magnaporthe grisea*

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벼 도열병균의 부착기 형성에 미치는 요인 분석

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ABSTRACT: ***Magnaporthe grisea*, the casual agent of rice blast, requires formation of an appressorium, a dome-shaped and well melanized infection structure, to penetrate its host. Environmental cues that induce appressorium formation include hydrophobicity and hardness of contact surface and chemicals from its host. Artificial surfaces are widely used to induce appressorium formation, but frequencies of appressorium induction are not always consistent. To understand variable induction of appressorium formation in *M. grisea*, several factors were tested on GelBond. High levels of appressorium formation were induced over a wide range of temperature (20~30°C) and pH (4~7). Spore age up to 3-week-old did not significantly affect appressorium formation, but only a few appressoria were formed from 4-week-old spores. NH_4^+, Na^+, and K^+ ions did not affect the ability to form appressoria on GelBond. However, adenosine specifically inhibited appressorium formation. Adenosine inhibition of appressorium formation was restored by exogenous addition of cAMP. Germ tube tips of *M. grisea* maintained the ability to differentiate appressoria by chemical inducers on GelBond at least up to 16 h after conidia germination. These results suggest that environmental factors have little effect on the variable induction of appressorium formation on the artificial surface in *M. grisea*.**

***Key words:* *infection structure, Magnaporthe grisea*, adenosine, cAMP.**

Magnaporthe grisea (Hebert) Barr (anamorph: *Pyricularia grisea*) is a typical heterothallic ascomycete and the causal agent of rice blast, one of the most destructive diseases on rice (*Oryza sativa* L.) worldwide. Successful infection by this fungus involves a series of steps including attachment of spores to the host surface, spore germination, appressorium formation, and penetration by infection peg and hyphae. The appressorium, a dome-shaped and melanized infection structure, is formed from the tip of an emerging germ tube. Turgor pressure in mature appressoria increases as high as 8 Mpa and facilitates penetration of rice leaf cuticle by the infection peg (8).

Appressorium formation in plant pathogenic fungi is induced by environmental and chemical stimuli (16). The germ tube of *Uromyces appendiculatus* is able to recognize the ridge such as those formed by guard cells surrounding stomatal opening for appressorium formation (7). Chemical ions including K^+ also induce appressorium formation in this fungus (6, 17). On the other hand, *Colletotrichum gloeosporioides* isolated from avocado recognizes specific wax components from the host to form appressoria (15).

Much has been learned about the environmental cues and endogenous signaling systems involved in appressorium formation of *M. grisea* over the past few years. Differentiation of appressoria in this fungus is induced by hydrophobicity of contact surface, and chemicals from the plant surface (4, 5, 11, 12). However, others reported that solid surface such as slide glass is sufficient to induce appressorium formation (18, 19). Jellito *et al.* (9) went as far as to state that no external signal beyond surface attachment is required for appressorium formation, but appressorium initiation is dependent on intracellular signaling. For intracellular signaling pathway, cAMP-dependent and mitogen-activated protein kinase pathways have been shown to be responsible for the induction and maturation of appressorium formation in

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M. grisea (10, 14, 20).

Although much research has been conducted on the role of physical factors and cellular signaling systems on appressorium formation in *M. grisea*, little information is available on environmental factors including temperature, pH, spore age, chemical ions, and competency of germ tubes on appressorium formation.

We report here that germ tubes of *M. grisea* maintain the competency to form appressoria at least up to 16 h after conidial germination. Appressorium formation of *M. grisea* was little affected by a number of environmental factors, but was specifically inhibited by adenosine. Adenosine inhibition of appressorium formation was restored by exogenous addition of cAMP.

MATERIALS AND METHODS

Fungal strain and cultural conditions. *Magnaporthe grisea* 70-15 was kindly provided by Dr. A. Ellingboe at University of Wisconsin, Madison, U.S.A., and used throughout the experiments. This isolate was routinely maintained on complete medium. Fungal culture was grown on oatmeal agar (50 g oatmeal per liter) at 22 °C under constant fluorescent light to promote conidiation. Conidia were collected from 10-day-old cultures and washed with distilled water twice.

Appressorium formation assay. Conidia germination and appressorium formation were measured on the hydrophobic side of GelBond (FMC product, ME, U.S.A.), as previously described (10). Briefly, fifty-microliters of conidia suspension (10^5 conidia/ml) was placed on GelBond, sealed in a moistened box, and incubated at room temperature for 12 h, unless otherwise indicated. The percentages of germinated conidia and germinating conidia induced to form appressoria were determined from direct microscopic examination of at least 100 conidia per replicate in at least 3 experiments with three replicates per treatment. To test the effect of incubation temperature on appressorium formation, 7 different temperature regimes from 5°C to 35°C were established with 5°C intervals. For the effect of spore age on appressorium formation, conidia were harvested from 1, 2, 3, and 4-week-old cultures. Three different buffer systems were prepared for the effect of different pH on appressorium formation; citrate-phosphate buffer for pH 3 to pH 7, Tris-HCl buffer for pH 8, and Glycine-NaOH buffer for pH 9 and 10. The effect of chemicals including NH_4Cl, KCl, and NaCl was evaluated at 10 and 50 mM. Actinomycin D and proflavin were used as transcription inhibitors and cycloheximide was used as a translation inhibitor. To evaluate the competency of germlings to form appressoria, conidial suspension was incubated on hydrophilic side of GelBond followed by the addition of N^6-monobutyryl cAMP and 1,16-hexadecanediol at different time points. Appressorium formation was observed at 24 h after initial incubation.

RESULTS AND DISCUSSION

The use of artificial surfaces to induce appressorium formation in *M. grisea* has greatly contributed to elucidating the ultrastructure and cytology of this infection-related morphogenesis. Artificial surfaces such as GelBond and Teflon are widely used for physiological and molecular biological studies of appressorium formation by this organism. However, appressorium induction on artificial surfaces appears to be highly variable as reported by several different laboratories (5, 9, 10, 19). Therefore, we investigated the effects of several environmental factors to elucidate variable induction of appressorium formation in *M. grisea*.

High frequency of appressorium formation was observed between 20°C to 30°C (Fig. 1). However, appressorium formation decreased at temperatures beyond this range. No appressorium was formed at 35°C or 10°C. Jelitto *et al.* (9) also reported that appressorium formation was reduced above temperature 30°C. This is not surprising since conidia of *M. grisea* are dispersed and infection of rice occurs mainly during night when temperatures are relatively low. Conidia germination is

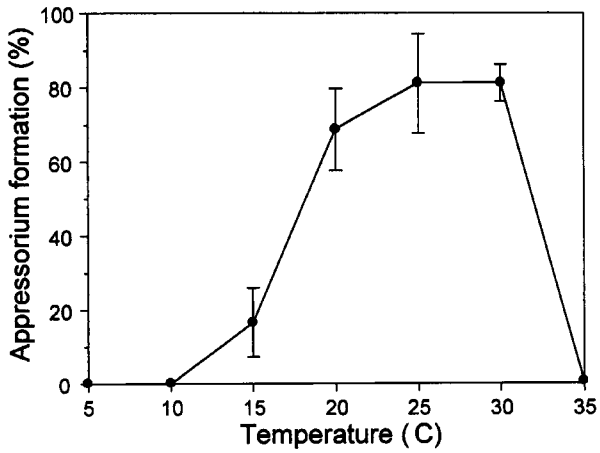

Fig. 1. Appressorium formation of *M. grisea* on hydrophobic side of GelBond at different temperatures. The results represent the means and standard errors of three separate experiments.

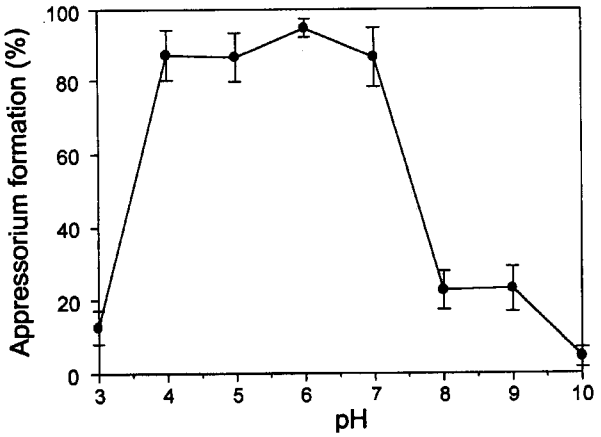

Fig. 2. Appressorium formation of *M. grisea* on hydrophobic side of Gelbond at different pH. The results represent the means and standard errors of three separate experiments.

less sensitive to temperature extremes than appressorium formation as has been shown for other fungi (13).

More than 80% of germinating conidia formed appressoria in the range of pH 4 to pH 7. The highest frequency of appressorium formation was observed at pH 6. However, appressorium formation was dramatically decreased above pH 8 (Fig. 2). Jelitto *et al.* (9) also reported that germ tubes formed most appressoria in the pH range 5.0 to 6.0. Our results indicate that appressorium formation by germlings of *M. grisea* occurs more readily in acidic rather than in slightly alkali conditions.

To investigate the effect of spore age on appressorium differentiation, 1, 2, 3, and 4 week old conidia were harvested from oatmeal plates. Conidia from 1, 2, and 3-week-old oatmeal plates formed appressoria at high frequencies, but those from 4-week-old formed only a few appressoria (data not shown). This result indicates that spore age has little effect on appressorium formation. Although there are several methods to sporulate *M. grisea*, all methods, up to our knowledge, take less than 3 weeks to harvest conidia.

Chemical ions including NH_4^+, Na^+, and K^+ did not show any significant effect on appressorium formation in *M. grisea*. However, adenosine inhibited appressorium formation at 1 mM while conidia germination remained unaffected. Addition of cAMP restored appressorium formation inhibited by adenosine to a high level (Table 1, Fig. 3). These results suggest that adenosine inhibition of appressorium formation is in the upstream where cAMP functions. In *Dictyostelium discoideum*, adenosine inhibits cAMP-induced response during development by inhibiting the binding of cAMP to its

Table 1. The effect of adenosine on appressorium formation in *Magnaporthe grisea*[a]

Treatments	Appressorium formation (%)
H_2O	98.0±1.0
Adenosine, 0.1 mM	65.0±16.5
Adenosine, 1 mM	2.7±2.5
Adenosine, 1 mM+N^6-monobutyryl cAMP, 10 mM	83.7±2.5

[a]: Appressorium formation was induced on hydrophobic surface of the Gelbond. Conidia germination was not affected by treatments of adenosine.

cell surface receptor (2). However, a cell surface receptor of cAMP has not been identified in *M. grisea*. Further investigation is required to elucidate the specific effect of adenosine on appressorium formation in *M. grisea*.

Appressorium formation was reduced to 70% in the presence of 0.1 ppm of cycloheximide, an inhibitor of protein synthesis, whereas conidia germination remained unaffected. However, appressorium formation and conidia germination were completely inhibited at 1 ppm (3.55 mM). This suggests that new protein synthesis is required for appressorium formation in *M. grisea*. Two transcription inhibitors, actinomycin D and proflavine, were also evaluated. However, the effect of these inhibitors on appressorium formation was not conclusive.

Previously it has been documented that cAMP and 1, 16-hexadecanediol induce appressorium formation by *M. grisea* on non-inductive surfaces (4, 10). To examine the competency of germinating conidia to differentiate appressoria, N^6-monobutyryl cAMP or 1,16-hexadecanediol was added into germinating conidia on a non-inductive surface. Addition of either compound to germlings on the non-inductive surface, hydrophilic side of GelBond, up to 16 h even after initial incubation, resulted in high levels of appressorium formation (Table 2). This suggests that germ tube tips of *M. grisea* maintain their competency to differentiate into appressoria for at least 16 hours after conidial germination. This also implies that new gene expression is required to form appressoria, because pretranscribed mRNA for appressoria formation is unlikely to be maintained after several rounds of cell division following conidial germination. However, data from transcription inhibitors were inconclusive. Maintaining competency to form appressoria seems to be different when inhibitors for appressorium formation are present. Inhibition of appressorium formation by *M. grisea* in the presence of yeast extract

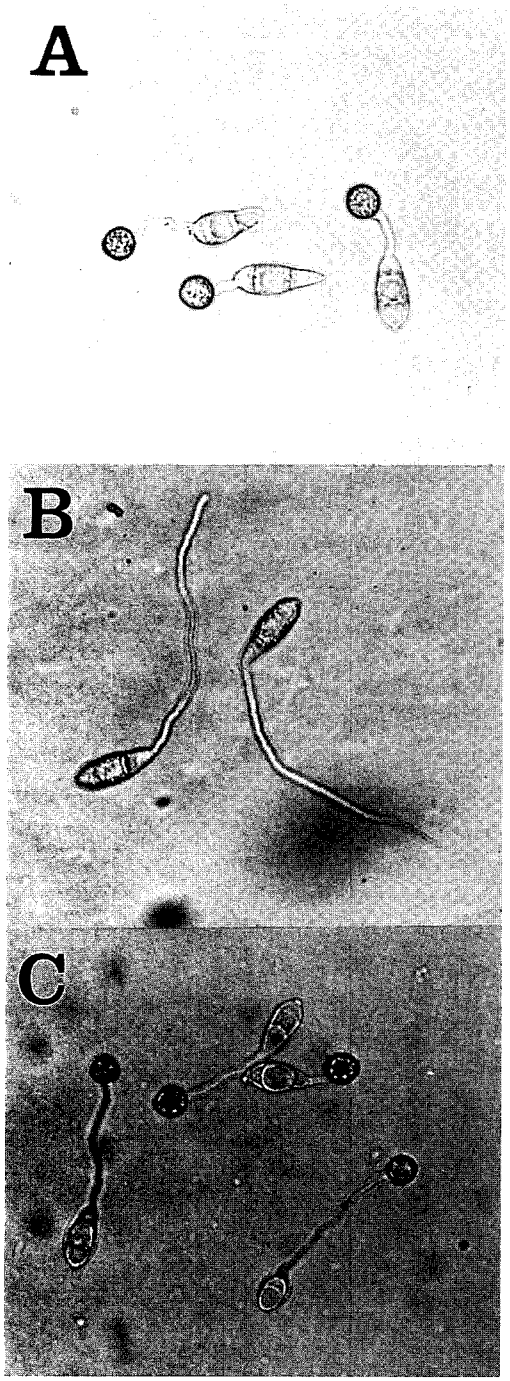

Fig. 3. Adenosine inhibits appressorium formation of *M. grisea*. A; Conidia germinated in the absence of adenosine, B; Conidia germinated in the presence of 1 mM adenosine, C; Conidia germinated in the presence of 1 mM adenosine and 10 mM N^6-monobutyryl cAMP.

was recovered only when cAMP was added short after germination (1). Likewise, appressorium formation inhibited by polyamines was restored when cAMP was added shortly after germination (3). These results indicate

Table 2. Effect of chemical inducers for appressorium formation in *Magnaporthe grisea* on a non-inductive surface

Hours incubated prior to inducer treatments[a]	Appressorium formation (Percent±S.D.)	
	N^6-monobutyryl cAMP[b]	1,16-hexadecanediol
0	86.7±1.5[c]	84.0±4.6
2	83.0±1.7	84.7±4.0
4	68.0±6.1	80.7±3.1
8	75.0±4.6	62.0±3.0
16	83.0±7.6	71.0±3.6

[a]: Distilled water was replaced with chemical inducers at designated times.
[b]: Final concentrations of N^6-monobutyryl cAMP and 1,16-hexadecanediol were 10 mM and 0.1 mM, respectively.
[c]: The frequency of appressorium formation was measured at 24 h after initial incubation.

that competency of germ tube tips to differentiate into appressoria in *M. grisea* may be inactivated in the presence of certain compound.

We demonstrate here that germ tube tips of *M. grisea* maintain their competency to induce appressorium formation at least up to 16 h after conidial germination in the absence of interfering compounds. Also adenosine specifically inhibits appressorium formation and cAMP restores this inhibition. Other factors including pH, incubation temperature, spore age, and chemical ions exhibited little effect on appressorium formation. These results suggest that environmental factors have little effect on the variable induction of appressorium formation on the artificial surface in *M. grisea*.

요 약

벼 도열병균인 *Magnaporthe grisea*는 기주 식물을 침입하기 위해 부착기라 불리는 특수한 침입구조를 형성해야 한다. 부착기 형성을 유도하는 요인들로는 접촉표면의 소수성과 식물체 표면의 cutin monomer 등과 같은 화학물질들이 알려져 있다. 이러한 부착기 형성은 식물체 표면에서 뿐만 아니라 인공표면 위에서도 유도되는데 형성율은 실험실에 따라 많은 차이를 보이고 있다. 부착기 형성에 영향을 미칠 수 있는 변이 요인을 규명하기 위해 온도, pH 및 여러 이온들의 영향을 살펴보았다. 온도 및 pH는 부착기 형성율에 큰 영향을 미치지 않았고, 3주된 분생포자도 높은 부착기 형성율을 나타내었다. 실험한 대부분들의 이온들은 큰 영향을 미치지 않았으나 adenosine은 부착기 형성을 특이적으로 억제하였다. 이러한 억제는 cAMP에 의해 회복되어 작용점이 cAMP의 작용점 보다 아래에 위치함을 제시하였다. 또한 분생포

자가 발아한지 16시간 이후에도 유도물질에 의해 부착기가 형성되어 부착기 형성능은 오랜 시간 동안 유지됨이 밝혀졌다.

ACKNOWLEDGMENTS

This work was supported by Seoul National University POSCO Research Fund and Korean Science and Engineering Foundation through the Research Center for New Bio-Materials in Agriculture at Seoul National University.

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(Received July 17, 1998)