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# Uniformity Among *Magnaporthe grisea* Isolates on Appressorium Formation by cAMP and Hydrophobicity of Contact Surface

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# cAMP와 표면 소수성에 의한 도열병균의 부착기 형성

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**ABSTRACT : *Magnaporthe grisea*, a causal agent of blast, forms a specialized infection structure, an appressorium, to infect host. Hydrophobicity of contact surface and cAMP have been suggested as a primary environmental signal and a second messenger to trigger and mediate appressorium formation in this fungus, respectively. To generalize these factors in field isolates of *M. grisea*, twenty isolates originated from rice and other gramineous hosts were tested. Seventeen including rice and non-rice isolates formed appressoria on hydrophobic surface, but none of isolates formed appressoria on hydrophilic surface. Eighteen isolates formed appressoria on hydrophilic surface in the presence of IBMX, an inhibitor of phosphodiesterase, except two rice isolates. These results strongly support the hypothesis that appressorium formation by *M. grisea* is induced by hydrophobic hard surface and regulated by the endogenous level of cAMP in the cells. Understanding fungal development is not only of biological interest but provides new targets for novel disease control strategies.**

***Key words* : Appressorium, hydrophobicity, cAMP, signal transduction, *Magnaporthe grisea*.**

*Magnaporthe grisea* (Hebert) Barr is a hermaphroditic ascomycete (3) and its anamorph, *Pyricularia grisea*, is a causal agent of rice blast. Rice blast is one of the most serious diseases in rice growing regions throughout the world. Controlling of this disease has been dependent on the breeding of resistant cultivars, the application of chemical fungicides and cultural practices. However, frequent appearance of new races has shortened the duration of resistant cultivars in the fields (14). Environmental regulations also tend to restrict the extensive use of chemical fungicides. In spite of economic importance of this disease and amenability of the fungus for biochemical and molecular approaches, little is known about the precise mechanisms involved in pathogenesis.

For the successful infection by many plant pathogenic fungi including *M. grisea*, the formation of a specialized infection structure, an appressorium, is required to adhere tightly and to penetrate into the host (4, 6, 17). This unique cellular differentiation is induced by environmental stimuli. Considerable effort has been focused on thigmotropic signal to induce this cell differentiation. The use of artificial surfaces to induce appressorium formation has greatly contributed to elucidate the ultrastructure and cytology of this infection-related morphogenesis (1, 7). In the case of *Uromyces appendiculatus*, artificial ridges which mimic those formed by the guard cells surrounding the stomatal opening are sufficient to induce appressorium formation (5). However, other fungi including *M. grisea* and *Colletotrichum* sp. do not respond to topographic signals for appressorium formation but appear to have other signal recognition systems. Recently, the hydrophobicity of contact surface has been reported as an important signal to induce appressorium formation in *M. grisea*. Germinating conidia formed appressoria on hy-

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drophobic surfaces, whereas they grew vegetatively on hydrophilic surfaces (9, 10). Furthermore, the addition of cyclic adenosine monophosphate (cAMP) to germinating conidia bypassed the requirement of surface hydrophobicity as a primary signal (9). Appressorium-deficient mutants, generated by UV, were complemented to form appressoria by addition of cAMP (12). To understand the function of cAMP in appressorium differentiation of *M. grisea* at the molecular level, a catalytic subunit gene of cAMP dependent protein kinase was cloned (13). Differential hybridization strategy also has been used to clone appressorium specific genes (11).

Understanding of this important infection-related morphogenesis is prerequisite to design the novel strategy to control this disease. Since previous studies on the induction of appressoria used a laboratory strain of *M. grisea*, we intended to generalize the hypothesis of hydrophobicity and cAMP regulation on appressorium formation by using 20 isolates of *M. grisea* originated from rice and several gramineous hosts.

## MATERIALS AND METHODS

**Fungal isolates.** All rice isolates of *M. grisea* were obtained from either Korean Research Institute of Chemical Technology or Seoul National University, and non-rice isolates were obtained from Agricultural Sciences Institute, Rural Development Administration (Table 1). All isolates were maintained as completely dried mycelia on complete medium at −20℃ for permanent stocks. Sporulation was induced on oatmeal or rice polish agar medium as streaking the mycelial debris or conidia.

**Appressorium formation.** Appressorium formation on different substrata was described in the previous report (9). Briefly, fifty-microliter drops ($10^4$ conidia/ml) were placed on test materials, sealed in a moistened box, and incubated at room temperature for 16~24 hrs, unless otherwise indicated. The percentage of appressorium formation was obtained by direct microscopic observation of at least 100 germinated conidia per replicate. Experiments were conducted at least twice with three replicates.

**Table 1.** Appressorium formation on hydrophilic and hydrophobic sides of Gelbond and onion surface, and pathogenicity of *Magnaporthe grisea* isolates originated from rice and other gramineous hosts

| Isolates | Appressorium formation (%) | | | Ds[a] (%) | Origin |
|---|---|---|---|---|---|
| | H-philic[b] | H-phobic | Onion | | |
| MG01 | 0 | 0 | 0 | 0 | *Oryza sativa* |
| MG02 | 0 | 0 | 0 | 0 | *Oryza sativa* |
| MG03 | 0 | 21.3± 9.5 | 98.5± 2.4 | 46 | *Oryza sativa* |
| MG04 | 0 | 18.7± 8.1 | 91.7± 2.1 | 44 | *Oryza sativa* |
| MG05 | 0 | 20.0± 8.0 | 93.8± 3.0 | 0 | *Oryza sativa* |
| MG06 | 0 | 6.7± 7.0 | 94.1± 1.9 | 32 | *Oryza sativa* |
| MG07 | 0 | 90.0± 3.5 | 96.6± 1.8 | 55 | *Oryza sativa* |
| MG08 | 0 | 3.3± 1.2 | 90.5± 3.5 | 45 | *Oryza sativa* |
| MG14 | 0 | 52.7± 5.5 | 95.3± 2.6 | 1 | *Oryza sativa* |
| MG15 | 0 | 18.7± 4.2 | 79.8± 5.3 | 26 | *Oryza sativa* |
| MG16 | 0 | 74.7± 8.1 | 92.8± 3.4 | 31 | *Oryza sativa* |
| MG17 | 0 | 90.0± 5.3 | 94.5± 3.1 | 0 | *Oryza sativa* |
| MG19 | 0 | 15.3± 1.2 | 92.2± 5.8 | 27 | *Oryza sativa* |
| MG20 | 0 | 33.3± 6.4 | 93.4± 3.2 | 7 | *Oryza sativa* |
| MG101 | 0 | 76.0± 5.2 | 98.0± 1.2 | 0 | *Seratia italica* |
| MG105 | 0 | 92.0± 2.7 | 94.2± 1.7 | 0 | *Seratia italica* |
| MG102 | 0 | 70.0± 4.3 | 81.8± 4.6 | 60 | *Eleusine coracana* |
| MG103 | 0 | 87.0± 4.9 | 95.4± 1.2 | 53 | *Festuca elatior* |
| MG106 | 0 | 0 | 0 | 0 | *Phleum pararense* |
| MG107 | 0 | 45.0± 8.7 | 98.2± 1.8 | 0 | *Panicum miliaceum* |

[a] Disease severity : percent of diseased leaf area.
[b] Gelbond film (FMC, Rockland, ME, USA).

**Pathogenicity test.** Spores of each isolate were harvested from 10-day-old colonies on oatmeal or rice polish agar plates. Concentration of spore suspension was adjusted to approximately $1\times10^5$/ml and 5 ml of spore suspension per pot was sprayed to 3~4 leaf stages of rice cultivar 'Nakdong' with an atomizer. The inoculated plants were placed into a dew chamber in darkness at 25℃ for 24 hrs and then transferred to growth chamber at 25℃ for 4~5 days.

## RESULTS

**Appressorium formation by *M. grisea* on hydrophobic and hydrophilic surfaces.** Twenty isolates of *M. grisea* isolated from rice and other gramineous hosts were tested for appressorium formation on hydrophobic and hydrophilic inert surfaces and onion surface. Seventeen isolates formed appressoria at high levels on onion surface, but three (MG01, MG 02, and MG106) did not form any appressoria. The little difference is observed among the isolates at the level of appressorium formation on onion surface. None of isolates were induced to form appressoria on hydrophilic side of Gelbond. However, all seventeen isolates which formed appressoria on onion surface formed appressoria on the hydrophobic side of Gelbond. The levels of appressorium formation on Gelbond greatly varied from 3% to 92% among isolates tested. Isolates from rice were more variable than those from other hosts at the level of appressorium formation on hydrophobic Gelbond surface. Fig. 1 and 2 illustrate the typical appressorium formation on hydrophobic and hydrophilic surfaces of Gelbond by rice and non-rice isolates. All appressoria formed on hydrophobic side of Gelbond and onion surface were well melanized and were indistinguishable each other.

**Pathogenicity.** Nine rice isolates, out of 14, were pathogenic on rice cultivar Nakdong. Two isolates each from finger millet (*Eleusine coracana*) and tall fescue (*Festuca elatior*) were pathogenic to rice, whereas other non-rice isolates were non-pathogenic. Three isolates (MG01, MG02, and MG106) which did not form any appressoria on onion surface were non-pathogenic. Several rice isolates which formed appressoria on hydrophobic side of Gelbond and onion surface at high level were non-pathogenic to rice (Table 1).

**Appressorium formation by *M. grisea* on non-induc-**

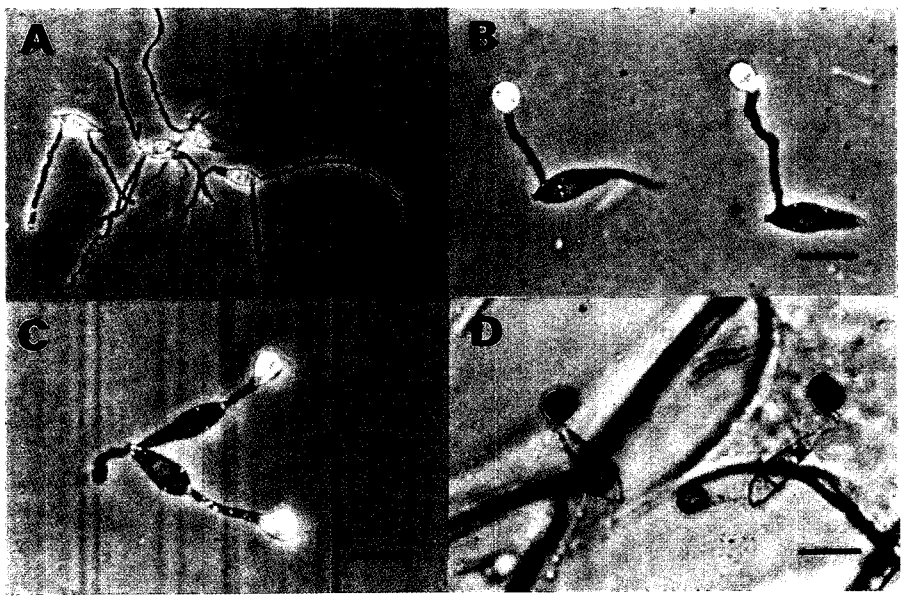


**Fig. 1.** Phase contrast (A, B, C) and light (D) microscopic observations of appressorium formation by *M. grisea* MG07 on different surfaces. A : On hydrophilic side of Gelbond, B : On hydrophobic side of Gelbond, C : On hydrophilic side of Gelbond in the presence of IBMX, 2.5 mM, D : On onion surface. Bars indicate 50 mm for pannel A, and 25 mm for B, C, and D.

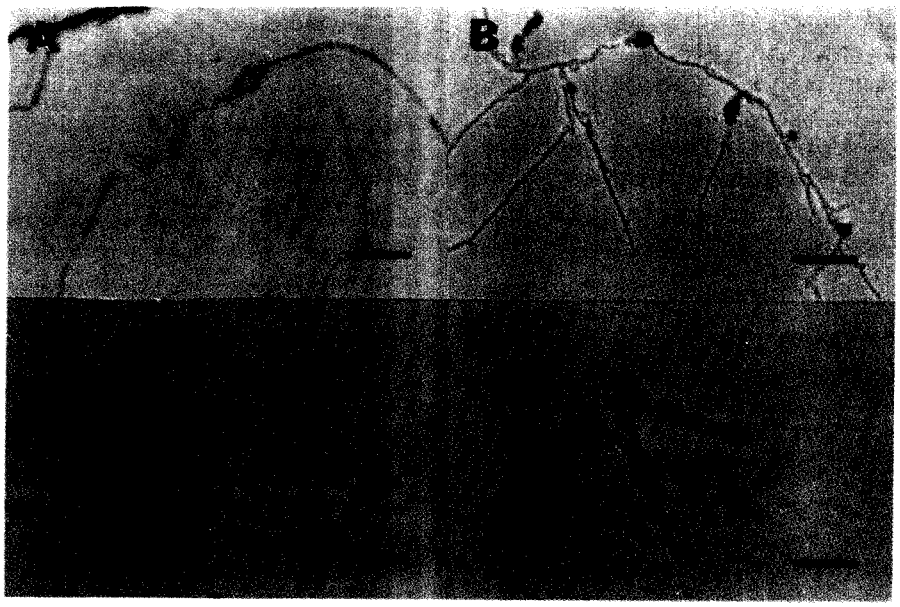

**Fig. 2.** Appressorium formation by two weed isolates of *M. grisea* on hydrophilic side of Gelbond in the absence (A and B) or presence (C and D) of IBMX, 2.5 mM. A and B are MG102 and MG103, respectively. Bars indicate 50 mm.

**Table 2.** Appressorium formation by *Magnaporthe grisea* in the presence of cAMP, Monobutyryl cAMP, or IBMX on non-inductive surface

| Isolates | Appressorium formation (%) | | |
|---|---|---|---|
| | cAMP 10 mM | MB cAMP[a] 1 mM | IBMX[b] 2.5 mM |
| MG01 | 0 | 0 | 0 |
| MG02 | 0 | 0 | 0 |
| MG03 | 3.3± 1.5 | 4.7± 2.1 | 84.7± 3.5 |
| MG04 | 3.7± 1.2 | 0 | 90.7± 2.1 |
| MG05 | 36.7± 4.7 | * 38.3± 6.0 | 82.0± 3.6 |
| MG06 | 64.5± 7.5 | 51.4± 8.8 | 77.2± 6.3 |
| MG07 | 67.4± 8.1 | 49.5± 8.3 | 81.9± 4.7 |
| MG08 | 26.9± 7.4 | 29.2± 5.7 | 62.5± 5.9 |
| MG14 | 14.2± 2.6 | 6.7± 2.1 | 88.7± 3.8 |
| MG15 | 75.5± 4.7 | 65.1± 8.8 | 86.4± 4.3 |
| MG16 | 22.8± 8.5 | 13.2± 7.3 | 41.4± 7.2 |
| MG17 | 69.6± 6.2 | 51.7± 5.9 | 91.3± 3.1 |
| MG19 | 63.2± 8.6 | 40.3± 7.7 | 79.3± 4.8 |
| MG20 | 72.4± 4.5 | 40.8± 6.9 | 86.1± 6.5 |
| MG101 | 30.8± 2.0 | 0 | 88.5± 6.7 |
| MG105 | 42.1± 9.5 | 5.7± 3.2 | 84.9± 4.0 |
| MG102 | 69.0± 7.5 | 0 | 84.4± 6.9 |
| MG103 | 16.8± 6.4 | 0 | 82.7± 1.7 |
| MG106 | 0 | 0 | 43.0± 7.8 |
| MG107 | 16.0± 9.5 | 15.7± 6.8 | 92.4± 2.5 |

[a] N6-monobutyryl cAMP.
[b] 3-isobutyl-1-methylxanthine.

**tive surface in the presence of cAMP, $N_6$ monobutyryl cAMP, or IBMX (3-isobutyl-1-methylxanthine).** All isolates formed appressoria on onion surface were induced to form appressoria on hydrophilic side of Gelbond in the presence of cAMP, monobutyryl cAMP, or IBMX. The levels of appressorium formation were variable among isolates tested. IBMX treatment induced the highest frequency of appressorium formation in all isolates (Table 2). Fig. 1 and 2 show the induction of appressorium formation on hydrophilic surface in the presence of IBMX by rice and non-rice isolates, respectively. Two isolates, MG01 and MG02, did not form any appressoria in the presence of any chemical tested, whereas isolate MG106 formed appressoria in the presence of IBMX. However, malformed germ-tube tips were observed from isolates MG01 and MG02 when treated with cAMP, monobutyryl cAMP or IBMX (Unpublished data). Three weed isolates did not respond to monobutyryl cAMP for the induction of appressorium formation.

## DISCUSSION

Plant pathogenic fungi have evolved highly adaptive infection-related morphogenesis to infect plants. These cellular differentiations are initiated when fu-

ngi recognize the environmental signals and physiological processes are mediated through the second messenger pathways. Although the mechanisms involved in recognition and adaptive response to stimuli are fundamentally similar in all eucaryotes investigated (8, 16), relatively little is known about the differentiation of appressoria in many plant pathogenic fungi. However, hydrophobicity of contact surface has been reported as an important environmental signal to induce appressorium formation in *M. grisea* (10). Cyclic AMP also has played an important role in transmitting the environmental signal in the cell as second messenger in *M. grisea* (9).

Recently a hypothesis was formulated to explain appressorium formation by *M. grisea*. The interaction between cell-wall matrix protein of the emerging germ tube and a hydrophobic surface results in the accumulation of cAMP in the fungus. Elevated levels of cAMP in turn trigger transductive pathways resulting in new gene expression leading to infection structure formation (2). In this report, we describe that hydrophobicity of contact surface and cAMP regulation on appressorium formation could be generalized to isolates of *M. grisea* originated from rice and non-rice gramineous hosts. These results will provide further clues to elucidate the mechanisms involved in appressorium differentiation and pathogenesis.

Twelve out of 14 rice isolates formed appressoria on hydrophobic surface of Gelbond, but the levels of differentiation were variable from 3% to 92%. This difference is believed to be difference in sensitivity of signal perception. This interpretation is supported by the fact that all isolates formed appressoria at high level on onion surface. Onion surface is much more hydrophobic than the hydrophobic side of Gelbond. However, we can not completely exclude the possibility of involvement of other chemical substances from onion surface. Fatty acid components from wax layer of plant surface also induced appressorium formation on non-inductive surface at nano-molar level (R.A. Dean: personal communication). Five out of six non-rice isolates formed appressoria at relatively high level both hydrophobic surface of Gelbond and onion surface.

Appressorium formation was induced on non-inductive surface (hydrophilic) in the presence of cAMP, N6-monobutyryl cAMP, or IBMX. IBMX was the most effective and followed by cAMP and monobutyryl cAMP. Endogenous level of cAMP is tightly regulated in the cells by two enzymes, adenylate cyclase and phosphodiesterase for synthesis and degradation, respectively (8). IBMX inhibits phosphodiesterase and accumulates the endogenous level of cAMP (15). This result confirms previous report that high level of endogenous cAMP is responsible for appressorium differentiation in *M. grisea*.

Three isolates, one from timothy (*Phleum paratense*) and two from rice, did not form any appressoria on any surface and with any treatment tested. These isolates were absolutely non-pathogenic to rice cultivar Nakdong. Isolates from finger millet and tall fescue were pathogenic to rice, whereas isolates from Italian and common millets were not. This is not common that weed isolates of *M. grisea* are pathogenic to rice. There are several reports that rice isolates were pathogenic to other gramineous hosts, but grass isolates were not pathogenic to rice (18, 19). This result awaits further investigation on host range of rice and non-rice isolates of this fungus.

Based on the results of appressorium formation and pathogenicity test, largely three categories of pathogen groups were identified. First, isolates were induced to form appressoria at high level on hydrophobic inert surface and plant surface, and on the hydrophilic surface in the presence of cAMP, monobutyryl cAMP, or IBMX. They are also pathogenic to rice. Second, isolates have the same characteristics on appressorium formation as the first group, but they are not pathogenic. Third, isolates do not form any appressoria at any circumstance and they are not pathogenic to rice.

The consequence of appressorium differentiation by *M. grisea* could be divided into three large steps; recognition of the environmental signals, appressorium differentiation, and penetration and disease initiation into host. The second group of isolates is defective in the downstream of appressorium differentiation. They form appressoria but are not pathogenic. The penetration into host by *M. grisea* is dependent on the hydrostatic pressure generated from well melanized appressoria rather than any enzymatic degradation of host surface (7). Most isolates categorized into the second group in this experiment formed well melanized appressoria. This im-

plies that they penetrate the host surface by infection hyphae but could not develop further. This also suggests that the formation of appressoria does not count for disease development although it is the prerequisite for successful disease development. In case of the third group, however, isolates are defective in the upstream where they perceive the signal to induce appressorium formation. The fact that they did not form any appressorium with cAMP, monobutyryl cAMP, or IBMX indicates that they are also defective in the downstream where cAMP functions for appressorium formation.

Recently Lee *et al.* (12) reported that several appressorium-defective mutants of *M. grisea* on inductive surface, generated by UV, were complemented to induce appressorium formation in the presence of cAMP. This seemed to be defective in upstream where cAMP functions for appressorium formation. Recent genetic analyses of mutants indicate that these mutants were defective at single locus, although the location of each locus is not known (R.A. Dean; personal communication, Chun *et al.*, unpublished data).

Developmental fate of germ tube is determined by the two signals in *M. grisea*; infection signal for appressorium differentiation and growth signal for vegetative growth. The infection signal seems to override the growth signal, because the germ tube differentiates into appressorium on inductive surface in the presence of growth signal, including nutrients (9). This fact infers that a series of mutants observed in this experiment may be detrimental process to the pathogen.

Hydrophobicity of contact surface now clearly responsible on the induction of appressorium formation by *M. grisea* as primary environmental signal. Furthermore, the involvement of cAMP in the appressorium formation by *M. grisea* could be generalized to rice field isolates and non-rice isolates. The precise mechanisms involved in this appressorium differentiation are being unraveled in *M. grisea* by biochemical and molecular genetic analyses and await further investigations.

## 요 약

도열병균인 *Magnaporthe grisea*는 기주 식물을 침입하기 위하여 부착기라는 특수한 구조를 형성한다. 기주식물 표면의 소수성이 이 구조를 형성하도록 유도하는 외부환경 정보라고 생각되며, 세포내에서 cAMP가 second messenger로 부착기 형성에 관여한다고 알려져 있다. 이러한 요인들을 일반화시키기 위하여 벼와 다른 화본과 기주로부터 분리한 20개의 균주에 대해 실험하였다. 벼와 다른 기주로부터 분리한 17개의 균주가 소수성 표면에서 부착기를 형성한 반면 모든 균주가 친수성 표면에서는 부착기를 형성하지 못하였다. 전체 20균주 중 18균주는 친수성 표면에서 phosphodiesterase의 inhibitor인 IBMX를 처리했을 때 모두 부착기를 형성하였다. IBMX 처리시 부착기를 형성하지 못한 2균주는 모든 처리조건에서 부착기를 형성하지 못하였다. 이러한 결과는 도열병균의 부착기 형성에 관여하는 표면의 소수성과 세포내 cAMP 작용에 대한 가설을 일반화 시켰다. 이렇게 병원균의 세포 분화 기작을 규명하는 연구는 생물학적 이해 뿐만 아니라 새로운 병 방제 수단을 찾는데 크게 기여하리라 생각된다.

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