Extracellular Glycoprotein(s) Associated with Cellular Differentiation in *Magnaporthe grisea*

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Received 27 December 1993. Accepted 25 May 1994.

During the infection process of *Magnaporthe grisea* on rice plants, the conidial germ tube differentiates into a specific infection structure, an appressorium, for penetration into the host. Formation of appressoria was observed not only on plant leaves of various species including hosts and nonhosts, but also on synthetic solid substrata. We reported that the hardness of the solid surface contacted by conidial germ tubes was an important parameter for differentiation (Xiao *et al.* 1994). Possible cellular factors involved in sensing solid surfaces and signal transduction during these early stages in pathogenesis were investigated. When germinated on a synthetic substratum, germ tubes and appressoria adhered firmly to the contact surface. Scanning electron microscopy showed abundant mucilaginous substances around germ tubes and appressoria. Conidial adhesion and appressorium formation were significantly inhibited by protease, α–mannosidase and α–glucosidase, but not by β–glucosidase, α–galactosidase, lipase, and chitinase. The mucilage disappeared when germinated in the presence of protease, α–mannosidase, and α–glucosidase. Concanavalin A, a lectin binding to α–D-mannose and α–D-glucose, specifically suppressed appressorium formation at concentrations higher than 10 μg/ml, but did not significantly affect conidial germination and adhesion. Mucilaginous materials were also observed around germ tubes and appressoria on various plant leaves. These data suggest that extracellular glycoprotein(s) bind germ tubes and appressoria to a contact surface, and at least a part of those glycoprotein(s) are further involved in sensing and transmission of information about the inductive parameters for cellular differentiation. The same mechanisms are probably involved in pathogenesis *in vivo*.

Additional keywords: Con A, *Pyricularia oryzae*, rice blast.

Many plant pathogens require formation of a specific infection structure termed an appressorium during their pathogenesis (for reviews, see Emmett and Parbery 1975, Hoch and Staples 1987). Appressorium formation is a cellular differentiation that is observed on some synthetic substrata as well as plant surfaces, suggesting that physical and/or chemical mechanisms are involved in the induction. *Magnaporthe grisea* Barr (imperfect stage *Pyricularia oryzae* Cav.) is the pathogen that causes the devastating rice blast disease. Formation of a specific, darkly melanized appressorium is a prerequisite for penetration into the host tissues by this pathogen (Chumley and Valent 1990; Howard and Ferrari 1989; Howard *et al.* 1991; Yamaguchi *et al.* 1982, 1983). Lee and Dean (1993) reported that a hydrophobic surface is sufficient for inducing the cellular differentiation and that cAMP acts as a mediator. However, by using various germination substrata, we found that *M. grisea* conidia formed appressoria equally well on both hydrophobic and hydrophilic surfaces, and that no topographic parameter appeared to be involved in the induction of appressorium formation (Xiao *et al.* 1994). Further, we observed that *M. grisea* conidia formed appressoria only on the surfaces of solid, but not of liquid or soft substrata, leading us to infer that hardness of the substratum is important for cellular differentiation (Xiao *et al.* 1994).

How do the fungi sense the physical parameter of surfaces? The germinated conidia of *M. grisea* were known to be firmly attached to the contact surfaces and highly resistant to physical removal by washing or other means (H. Yaegashi, National Institute of Agro-Enviromental Sciences, Ibaraki 305, Japan, personal communication). Uchiyama *et al.* (1979) observed mucilage or slime located around appressoria. These observations indicate that adhesive substances exist around the germ tubes and appressoria of *M. grisea*, but the physiological roles are not completely understood.

In this paper, we provide evidence that extracellular glycoprotein(s) is involved in surface adhesion of germ tubes and appressoria and acts to mediate appressorium formation by *M. grisea*.

RESULTS

Conidial adhesion and appressorium formation on polycarbonate film.

When a drop of conidium suspension was dispersed on the surface of a polycarbonate film, the conidia settled to the contact surface and some of the settled conidia were observed to adhere to the contact surface within 30 min prior to germ tube emergence (Fig. 1). Conidium germination occurred within 1 hr, followed by swellings evolving on the tips of most germ tubes. At approximately 8 hr after beginning incubation, darkly melanized, mature appressoria were observed on almost all of the germ tubes. Germinated conidia with or without form-

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MPMI Vol. 7, No. 5, 1994, pp. 639-644

ing appressoria adhered firmly to the contact surface and were completely resistant to removal by rotating in water for 2 min or even overnight.

Effect of hydrolytic enzymes on conidial adhesion and appressorium formation.

M. grisea conidia were allowed to germinate in the presence of each of the hydrolytic enzymes listed in Table 1 at 500 and 1,000 μg/ml. α-Glucosidase, α-mannosidase, and protease strongly inhibited appressorium formation as well as conidial adhesion. In the presence of these enzymes, germinated conidia failed to adhere to the contact surface and floated in the water droplets. α–Glucosidase, α–mannosidase, and protease inhibited conidial adhesion and appressorium formation in a dose-dependent manner (Fig. 2). α–Mannosidase showed the highest activity against conidial adhesion as well as appressorium formation. Protease suppressed conidial adhesion at relatively higher concentrations (over 500 μg/ml) but inhibited appressorium formation at lower concentrations. Higher concentrations of α–glucosidase were needed (over 500 μg/ml) to block appressorium formation and conidial adhesion.

Neither conidial adhesion nor appressorium formation was significantly affected by β-glucosidase, lipase, α-galatosidase, or chitinase. None of the enzymes affected conidium germination at the treated concentrations (data not shown).

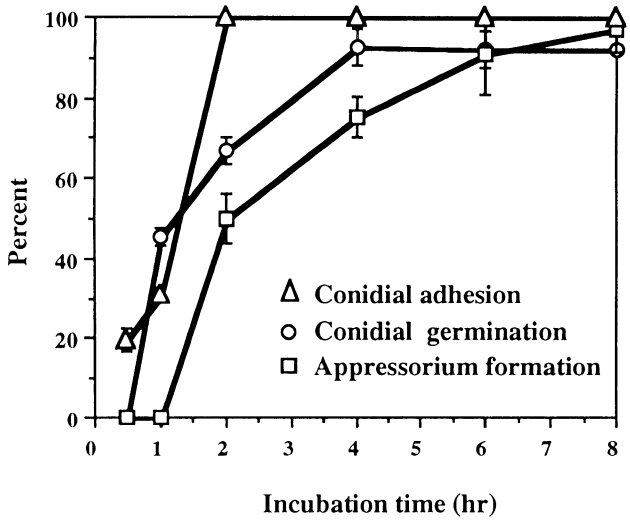

Fig. 1. Time-course of conidial adhesion, germination, and appressorium formation by *Magnaporthe grisea* on polycarbonate film. Percent conidial adhesion was determined by assessing approximately 1,000 conidia by a method as described in Materials and Methods. Percent germination was determined by assessing approximately 500 conidia. Percent appressorium formation was determined by assessing number of appressoria formed per approximately 400 germ tubes. Results are the combined data from two separate experiments with three replicates per treatment.

Effect of lectins on appressorium formation.

Since the above enzymatic studies indicated the presence of glycan components in the adhesive materials, the effects of lectins with different sugar-binding specificity on appressorium formation and conidial adhesion were examined. Of the nine lectins tested at 50 μg/ml (Table 2), only concanavalin A (Con A) and wheat germ agglutinin (WGA) showed a

Table 1. Effects of hydrolytic enzymes on appressorium formation by *Magnaporthe grisea*[a]

	Appressorium formation (%)	
	1,000 μg/ml	500 μg/ml
α-Glucosidase	0	5.3 ± 5.7
α-Mannosidase	0	0
Protease	0	0
β-Glucosidase	79.3 ± 5.6	93.5 ± 10.1
α-Galactosidase	89.6 ± 6.3	97.8 ± 7.5
Lipase	79.3 ± 9.4	88.3 ± 10.3
Chitinase	88.5 ± 5.6	92.5 ± 6.9

[a] Droplets (60 μl) of conidia suspended in each enzyme solution (2×10^4 conidia/ml) were incubated on polycarbonate film for 8–10 hr. Percent appressorium formation was determined as described in Figure 1. Similar levels (>90%) of conidium germination were obtained for each treatment. Results are the combined data from two separated experiments with three replicates per experiment.

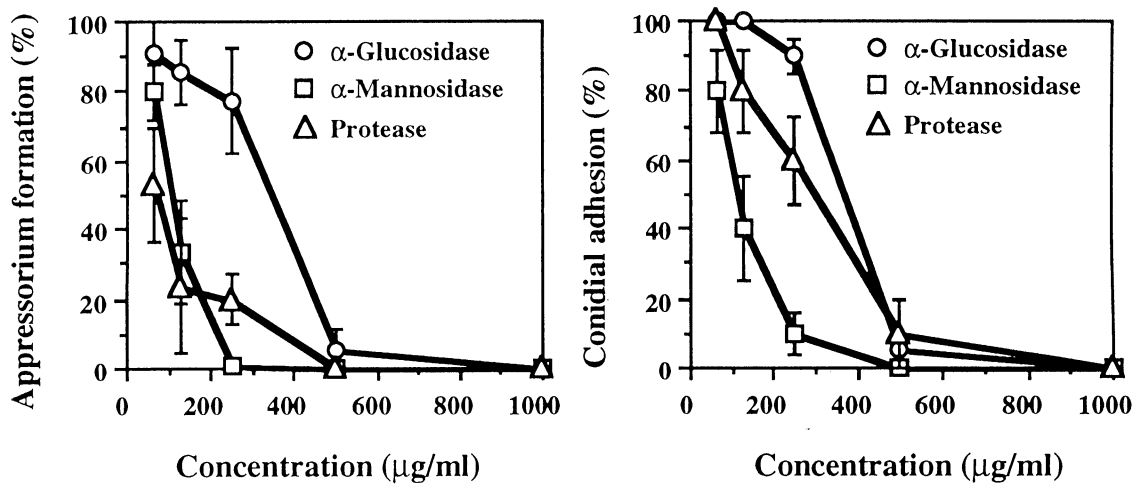

Fig. 2. Effect of α-glucosidase, α-mannosidase, and protease on appressorium formation and conidial adhesion by *Magnaporthe grisea*. Conidia suspended in each enzymatic solutions (2×10^4 conidia/ml) were incubated on polycarbonate film for 8–10 hr. Percentage of appressorium formation (**A**) and conidial adhesion (**B**) was determined as described in Figure 1. Similar levels (>90%) of conidium germination were obtained for each treatment. Results are the combined data from two separated experiments with three replicates per treatment.

significant inhibitory effect on appressorium formation. Con A strongly inhibited appressorium formation at concentrations higher than 10 µg/ml in a dose-dependent manner, while WGA exhibited the inhibitory effect at concentrations of more than 30 µg/ml, giving an incomplete inhibition (around 30%) up to 1,000 µg/ml (Fig. 3). In the presence of Con A or WGA, no significant effect was observed on either conidium germination or adhesion of the germ tubes to the substratum. Ricin (RCA60) and *Ulex europeus*-II (UEA–II) partially suppressed appressorium formation only at a high concentration (500 µg/ml). The other lectins affected neither appressorium formation nor conidial adhesion.

Addition of FITC-conjugated Con A to germinating conidia yielded strong labeling around the germ tubes, swelling tips of germ tubes and maturing appressoria (Fig. 4). Similar labeling by WGA was observed (data not shown).

Table 2. Effect of lectins on appressorium formation by *Magnaporthe grisea*[a]

Lectins	Sugar-binding specificity	Appressorium formation (%) 500 µg/ml	50 µg/ml
Concanavalin A	α-D-Man, α-D-glc	11.1 ± 5.2	13.7 ± 3.7
Lentil	α-D-Man, α-D-glc	84.6 ± 10.5	79.8 ± 6.3
Pea	α-Man, α-glc	89.8 ± 9.2	89.5 ± 8.1
Peanut	β-D-Gal(1,3)D-galNAc	95.1 ± 10.2	90.6 ± 9.2
Ricin (RCA60)	β-D-Gal, D-galNAc	36.3 ± 11.6	94.9 ± 9.7
Soybean	D-GalNAc	97.0 ± 8.5	96.6 ± 9.5
UEA-I	α-L-Fuc	95.6 ± 6.2	97.1 ± 4.6
UEA-II	D-GlcNAc	42.5 ± 8.9	94.8 ± 10.5
Wheat germ	D-GlcNAc	35.6 ± 11.5	39.6 ± 12.3

[a] Droplets (60 µl) of conidium suspensions (2 × 10^4 conidia/ml) with lectin were incubated on polycarbonate film for 8–10 hr. Percent appressorium formation was determined as described in Figure 1. Similar levels (>90%) of conidium germination were obtained for each treatment. Results are the combined data from two separated experiments with three replicates per experiment.

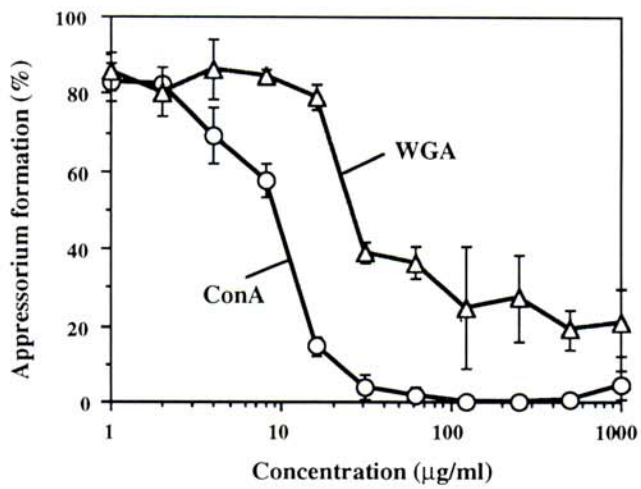

Fig. 3. Effect of concanavalin A and wheat germ agglutinin on appressorium formation by *Magnaporthe grisea*. Conidium suspensions (2 × 10^4 conidia/ml) with each lectin were incubated on polycarbonate film for 8–10 hr. Percentage of appressorium formation was determined as described in Figure 1. Similar levels (>90%) of conidium germination were obtained for each treatment. Results are the combined data from two separated experiments with three replicates per treatment.

To further examine if Con A inhibited appressorium formation by binding to mannose or glucose residues of the glycan components in the adhesive materials, we added different sugars to the conidium suspension and looked for blockage of Con A inhibition of appressorium formation (Table 3). Methyl-α-D-mannoside and methyl-α-D-glucoside were the most effective competitors for Con A inhibition of appressorium formation. Other compounds, such as D-mannose and *N*-acetyl-D-glucosamine, blocked Con A inhibition of appressorium formation, but were less active.

Scanning electron microscopic observation of conidium adhesion and appressorium formation.

Conidia allowed to germinate on the surface of polycarbonate film either in distilled water or in the presence of hy-

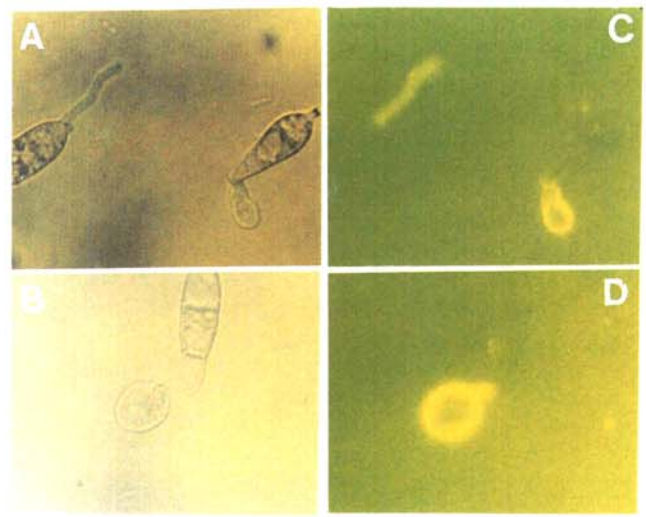

Fig. 4. Light and fluorescent micrographs of germinated conidia of *Magnaporthe grisea* labeled with FITC-concanavalin A. Conidia were allowed to germinate and form appressoria in distilled water on polycarbonate film and then labeled with FITC-concanavalin A for 30 minutes. **A** and **B,** Germinating conidia prior to or after appressorium formation; **C** and **D,** Fluorescence around the germ tubes and appressoria corresponding to **A** and **B**, respectively.

Table 3. Compounds competing with concanavalin A for inhibiting appressorium formation by *Magnaporthe grisea*[a]

	Appressorium formation (%)
N-Acetyl-D-glucosamine	56.4 ± 8.9
Fucose	14.8 ± 3.3
Galactose	9.3 ± 1.4
D-Glucose	18.5 ± 1.7
D-Mannose	43.9 ± 2.5
Methyl-α-D-glucoside	84.7 ± 9.4
Methyl-β-D-glucoside	3.4 ± 2.1
Methyl-α-D-mannoside	92.1 ± 5.7
Control	
Con A alone	12.4 ± 3.5
Distilled water	96.8 ± 2.7

[a] Droplets (60 µl) of conidia suspension (2 × 10^4 conidia/ml) with Con A (100 µg/ml) and each sugar (25 mM) were incubated on polycarbonate film for 8–10 hr. Percent appressorium formation was determined as described in Figure 1. No effect on appressorium formation was found for each sugar applied in the absence of Con A. Values are the means ± SD of three replicates. The experiment was repeated with similar results.

drolytic enzymes or lectins were fixed and observed using scanning electron microscopy. When germinated in distilled water, mucilaginous substances were observed around germ tubes and appressoria, which appeared to mediate the attachment of germ tubes and appressoria to the contact surface (Fig. 5A). Physical removal of germinated conidia by sonicating (Ultrasonic disrupter model UR-200P, Tomy Seiko, Tokyo, Japan) for 2 min left traces of the germ tubes and appressoria. Electron microscopic observation clearly indicated the presence of mucilaginous substances around the traces (Fig. 5B).

Conidia germinated in the presence of α–glucosidase, α–mannosidase, or protease at 500 μg/ml were difficult to examine microscopically since the germinated conidia did not attach to the contact surface. Of the conidia observed, mucilaginous substances were not present around the germ tubes (Fig. 6). However, in the case of germination in the presence of Con A (250 μg/ml), germ tubes adhered to the contact surface and abundant mucilage-like materials were observed around them (Fig. 7A and B). Extensive elongation of the germ tubes was observed in the absence of appressorial differentiation.

Appressorium formation on plant leaves.

M. grisea conidia were allowed to germinate on leaves of rice, wheat, citrus, pepper, and soybean. As measured with a contact angle meter, rice and wheat leaves were highly hydrophobic, citrus and pepper leaves were highly hydrophilic, and soybean leaves had intermediate levels of hydrophobicity (Xiao *et al.* 1994). All plant leaves were highly inductive for conidium germination and yielded a high frequency of appressorium formation (more than 85%) after 10 hr of incubation.

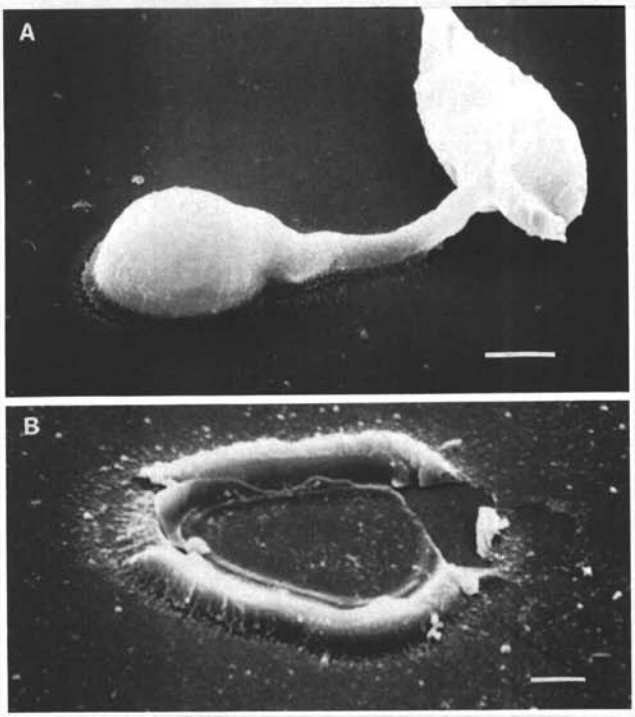

Fig. 5. Scanning electron micrographs of appressoria of *Magnaporthe grisea* formed on polycarbonate film. **A,** Conidium with appressorium after 10 hr of incubation, showing mucilage around the germ tube and appressorium; the bar indicates 4 μm. **B,** Mucilagious material remained after removal of appressorium by sonication; the bar indicates 2 μm.

When observed by scanning electron microscopy, mucilaginous substances were also found around germ tubes and appressoria on all of the plant surfaces examined, as was observed on polycarbonate film.

DISCUSSION

Cellular differentiation of the darkly melanized appressorium by *M. grisea*, which is prerequisite for pathogenicity, has been studied extensively. Surface hydrophobicity (Lee and Dean, 1993) and hardness (Xiao *et al.* 1994) have been suggested as important factors in triggering the differentiation. These studies clearly demonstrated that thigmotropic mechanisms are involved in inducing the cellular differentiation, although the details await further examination.

The ability of a fungus to react to a thigmotropic stimulus requires contact between the fungus and the stimulating surface (Epstein *et al.* 1985, 1987). Mucilage was observed around the germ tubes as well as the appressoria (Uchiyama *et al.* 1979; Xiao *et al.* 1994). Our results presented here indicate that fungal extracellular glycoprotein(s) is involved in the adhesion. Addition of protease, α–mannosidase, and α–glucosidase to the germination drops strongly inhibited conidial adhesion and appressorium formation, but without

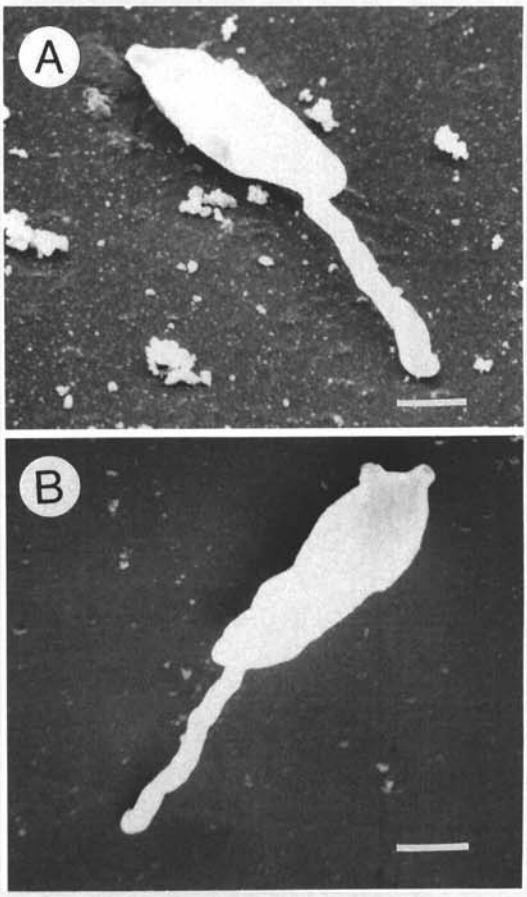

Fig. 6. Scanning electron micrographs of *Magnaporthe grisea* conidium germination on polycarbonate film in the presence of hydrolytic enzymes. Conidia suspended in each enzyme solution were incubated for 10 hr. **A,** Conidia germinated in the presence of α-mannosidase (500 μg/ml); **B,** Conidia germinated in the presence of protease (500 μg/ml). Each bar indicates 5 μm.

significant effect on germination or growth rate. Since exposure to the above enzymes did not result in observable cellular damage or inhibition of germination or growth, we suggest that the proteolytic and glycolytic enzymes degraded extracellular proteoglycans but did not interfere with the metabolic processes required for the production of adhesive materials. The non-adhesive conidia treated with protease, α–mannosidase, and α–glucosidase apparently lost the mucilage located around germ tubes, as was indicated by scanning electron microscopic observation.

From the enzymatic studies where adhesive materials appeared to consist of mannan and glucan, lectins with sugar-binding specificity were expected to affect conidial adhesion and appressorium formation. Of the nine lectins tested, only Con A and WGA significantly suppressed appressorium formation. In the presence of Con A and WGA, germ tubes adhered to the substratum surface with no difference from those germinated in distilled water, in spite of the inability to form appressoria. Con A exhibited the strong activity which was dose dependent. In the scanning electron microscope, conidia suppressed in appressorium formation by Con A were observed to have extensive growth of germ tubes with abundant mucilage-like materials bound to the germ tubes. These materials might be a complex composed of lectin and the glycoprotein. Treatment of germinating conidia with FITC-conjugated Con A yielded strong, specific labeling around germ tubes and appressoria. Further, Con A inhibition of appressorium formation was blocked by the potential Con A competitors, methyl-α-D-mannoside, methyl-α-D-glucoside, and D-manose. From these results, we infer that Con A suppressed appressorium formation by binding to the adhesive glycoprotein(s), specifically blocking the sensing and transmission of information about the inductive parameters. Similarly, mucilainous substances were observed around germ tubes and appressoria on plant leaves. These observations indicate that a similar binding, sensing, and signal transmission mechanism, as inferred from studies using synthetic substratum, might also function *in vivo*. Studies are in progress to identify genes expressed specifically during these early stages of the infection process.

Hamer *et al.* (1989) reported that conidium tip mucilage, expelled specifically from the *M. grisea* conidial apex before germ tube emergence, is involved in the attachment of conidia to substratum surfaces. The early adhesion was blocked by Con A which was shown to bind to conidium tip mucilage. In our assay system on polycarbonate film, we also observed that Con A inhibited conidial adhesion prior to germination, but found no significant effect of Con A on germling adhesion after conidium germination (data not shown). These different effects may suggest a different level of adhesion existing before and after germination; attachment to the contact surface by germ tubes may be firmer than that by conidium tips.

MATERIALS AND METHODS

Bioassay.

Magnaporthe grisea Cav. isolate P2, a rice-pathogenic isolate, was used throughout this study. The fungus was grown on oatmeal agar medium (consisting of 50 g of oatmeal, 20 g of sucrose, and 15 g of agar in 1 L of water; oatmeal was boiled for 30–40 min, and only the filtered juice was added to the medium) at 27° C for 2 wk, followed by placing the plates under BLB lamps (FL20S, 20 W, Toshiba Co., Ltd.) for sporulation after removing the aerial mycelia with a sterilized brush. Conidia formed were brushed off after 3 more days of incubation, and suspended in sterilized water and then collected by centrifugation.

For germination and appressorium formation, droplets (30–60 µl) of conidium suspensions ($1–2 \times 10^4$ conidia/ml) in distilled water or added with hydrolytic enzymes or lectins were placed on the surface of polycarbonate film, which were then kept in a closed plastic chamber at 25° C with high humidity for an appropriate time. Polycarbonate films (1 mm in thickness) were obtained from Mitsubishi Gas Chemistry Company, Japan. Conidium germination and appressorium formation were evaluated using a light microscope.

Conidial adhesion.

Adhesion of germinated conidia to substrata was determined by the following method. Polycarbonate slips containing germinated conidia were immersed in a distilled water pool and then rotated on a shaker at 50 rpm for 2 min. Percentage of adhesion was assessed by the numbers of retained conidia counted by microscopy in comparison with those incubated in distilled water without agitation. Almost 100% conidial adhesion was observed for the conidia allowed to germinate and form appressoria in distilled water on polycarbonate films.

Enzymes.

α–Glucosidase, β–glucosidase, α–mannosidase, lipase, and protease were obtained from Sigma Chemical Company. α–Galactosidase and chitinase GODO were purchased from Seikagaku Corporation, Tokyo. α–Mannosidase was desalted with an Amicon (Danvers, MA) Centricon ultrafiltration unit prior to use.

Effect of enzymes on appressorium formation was tested in distilled water as well as in the following buffer solution; 10 mM MES at pH 5 for α–mannosidase and β–glucosidase, 10 mM MES at pH 7 for α–glucosidase, protease and the other enzymes. Similar effects were observed in distilled water and

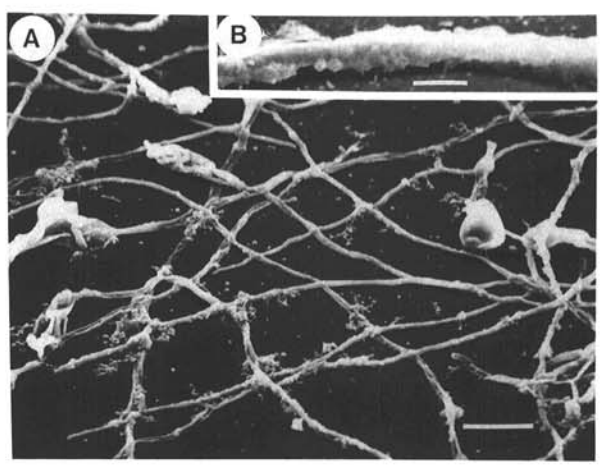

Fig. 7. Scanning electron micrographs of *Magnaporthe grisea* conidium germination on polycarbonate film in the presence of concanavalin A. Conidia suspended in lectin solutions were incubated for 10 hr. **A,** Conidia germinated in the presence of concanavalin A (250 µg/ml); the bar indicates 24 µm. **B,** An enlarged picture showing mucilage-like materials around the germ tubes; the bar indicates 2 µm.

in the buffer solutions, but conidium germination and appressorium formation occurred most frequently in distilled water. To eliminate the buffer's interference in the assay system, most of the experiments were performed using enzyme solutions freshly prepared in distilled water.

Lectins.

The following salt-free lectins were purchased from Seikagaku Corporation, Tokyo, Japan: concanavalin A (Con A, 3 × cryst.), lentil lectin, pea lectin, peanut lectin, ricin (RCA60), soybean lectin, *Ulex europeus*-I (UEA I), *Ulex europeus*-II (UEA II), wheat germ agglutinin (WGA), lectin kit I-FITC consisting of FITC-conjugated Con A, lentil, peanut, UEA I, WGA, etc., and lectin kit II-FITC consisting of FITC-conjugated soybean lectin, RCA60, UEA–II, etc. The effect of lectins on conidium germination, adhesion, and appressorium formation was observed using lectin solutions freshly prepared in distilled water.

Scanning electron microscopic observation of appressorium formation.

Conidia allowed to germinate and form appressoria on polycarbonate films or plant leaves were fixed as previously described (Xiao *et al.* 1994). Conidium germination and appressorium formation were examined with a scanning electron microscope (Hitachi S-2350, Hitachi, LTD, Japan).

ACKNOWLEDGMENTS

We are grateful to A. Takatsuki, T. Watanabe, and S. Sekido, at RIKEN, for their valuable discussions. Heartful thanks are given to T. Arie for his technical advice in scanning electron microscope observation and valuable discussions. This research was supported in part by a grant for "Biodesign Research Program" from The Institute of Physical and Chemical Research (RIKEN).

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