A Target-Site-Specific Screening System for Antifungal Compounds on Appressorium Formation in *Magnaporthe grisea*

Hong-Sik Oh and Yong-Hwan Lee

School of Agricultural Biotechnology and Research Center for New Bio-Materials in Agriculture, Seoul National University, Suwon 441-744, Korea.

Accepted for publication 24 May 2000.

ABSTRACT

Oh, H.-S., and Lee, Y.-H. 2000. A target-site-specific screening system for antifungal compounds on appressorium formation in *Magnaporthe grisea*. *Phytopathology* 90:1162-1168.

Chemical fungicides are a major method of control for plant diseases in spite of potential negative effects on the environment and the appearance of resistant strains. Development of new chemical fungicides has been largely dependent upon *in vivo* efficacy tests in the greenhouse or in fields, which is in contrast to target-oriented *in vitro* screening systems widely used in the pharmaceutical field. To establish a target-site-specific screening system for antifungal compounds, specific inhibition on appressorium formation of the rice blast fungus *Magnaporthe grisea* was employed. For many plant-pathogenic fungi, including *M. grisea*, appressorium formation is an essential step to infect host plants. Among 1,000 culture filtrates of members of the class Actinomycetes and fungi, five (A5005, A5008, A5314, A5387, and A5397) from the class Actinomycetes showed differential inhibitory effects on appres-

sorium formation of *M. grisea* in a dosage-dependent manner. Three (A5005, A5314, and A5387) of these were further fractionated into ethyl acetate and water fractions. The ethyl acetate fraction of A5005 and both the ethyl acetate and water fractions from A5314 and A5387 inhibited appressorium formation, while conidial germination remained little affected. Inhibition of appressorium formation by the ethyl acetate or water fraction was reversed by the exogenous addition of cyclic AMP. Significantly reduced numbers of conidia with appressoria were observed on rice leaves in the presence of culture filtrates. Furthermore, these culture filtrates also exhibited significant disease control of rice blast in the greenhouse. This rapid and target-oriented screening system could be adopted to screen candidate compounds for rice blast control and could be applicable for other appressorium-forming, plant-pathogenic fungi.

Additional keywords: high-throughput screening, infection structure, signal transduction pathways.

Control of plant diseases has depended primarily upon the application of chemical fungicides, despite potentially toxic effects on humans, wildlife, and the environment (7,15). The advent of new pathogen races that are resistant to fungicides has been another problem to plant pathologists and agrochemists (6,25). Therefore, new fungicides that are safe to humans and wildlife and have benign environmental profiles, low use rates, and high specificity to target organisms are needed to ensure stable crop production (15).

Generally, to develop a new fungicide, thousands of chemicals must be biologically screened, often taking several years (11). Collections of synthetic or natural compounds and metabolites of microorganisms are generally the sources of new fungicides. To prove antifungal activity, *in vitro* assays such as inhibition of mycelial growth and spore germination and *in vivo* efficacy tests in the greenhouse and agronomic fields are performed (11,14). Among the thousands of candidates, most chemicals are discarded during biological screening. Although *in vitro* screening has been an effective method to screen a massive number of candidate compounds and reduces time and cost, the results of *in vitro* tests have not always been consistent and correlated with those of field tests. *In vivo* assays provide accurate means of predicting likely field activity of candidate molecules as well as their chemical properties such as uptake by plants, metabolism and translocation in plants, and physical properties including persistency and stability against light and humidity (15). To screen a large number of compounds in this manner, however, requires enormous facilities and many trained personnel and is costly.

In the pharmaceutical field, however, automated and target-site-specific strategies for biological screening have been widely adopted for new drug development (5,21,27). These are based on the microtiter plate format and allow screening of 10,000 or more compounds per week. In contrast, traditional screens only permit throughputs of about 10,000 compounds per annum (11,15). Cell-based assays for antifungal activity in which target organisms can be used are also widespread (8,14). Although less suitable for predicting field activity than *in vivo* or cell-based bioassays, screening of antifungal compounds at the biochemical level against isolated enzymes offers the greatest degree of specificity (14,15). Despite limitations in our understanding of the basic biochemistry of pathogenesis by plant-pathogenic fungi, application of technical innovations in the pharmaceutical field provide opportunities for the development of more efficient screening methods in the agrochemical area. Recent biochemical and molecular biological studies of plant-microbe interactions have revealed new insight into pathogenesis and offer potential target sites for new fungicide development (18,22,23,28).

Magnaporthe grisea (T. T. Herbert) Yaegashi & Udagawa is the causal agent of rice blast, the most destructive disease of rice throughout the rice-growing areas of the world. *M. grisea* forms a specialized infection structure, an appressorium, to adhere tightly to and then penetrate its host by accumulated turgor pressure (2, 12). The formation and maturation of appressoria are critical steps for successful infection by this fungus. Hydrophobicity of the contact surface and chemicals from the host plant have been reported as environmental cues for this fungus to form appressoria (10,19). During the last several years, intracellular signaling systems for appressorium formation have been extensively studied in *M. grisea*. Lee and Dean (18) reported that the exogenous addition of cyclic AMP (cAMP) induces appressorium formation on noninductive surfaces. Subsequent studies identified and characterized the genes

Corresponding author: Y.-H. Lee; E-mail address: yonglee@plaza.snu.ac.kr

Publication no. P-2000-0726-01R

© 2000 The American Phytopathological Society

responsible for the signal transduction pathways involved in appressorium formation in this fungus. Targeted disruption of the catalytic subunit gene (*cpkA*) of cAMP-dependent protein kinase and deletion of the adenylate cyclase gene (*MAC1*) effectively inhibited normal appressorium formation and function (3,23). Furthermore, other proteins including MPG1 (a hydrophobin) (1,16,26), MACB (B-subunit of GTP-binding protein) (22), and Mps1 (mitogen-activated protein kinase) (28,29) have been shown to be involved in surface sensing and the signaling pathways for appressorium formation. Recently, pharmacological evidence suggests that a calcium/calmodulin signaling system is also involved in appressorium formation (17). These components of the signaling pathways for appressorium formation represent potent targets for novel fungicide development to control rice blast.

In this study, a rapid high-throughput and target-oriented screening system for screening antifungal compounds against appressorium formation by *M. grisea* was established. Comparative studies with in vivo tests were also conducted to demonstrate the reliability of this screening system. Selected compounds identified by the novel screening system in this study effectively controlled rice blast in in vivo tests. These results suggest that the target-site-specific screening strategy could be used to screen candidate compounds for new fungicide development to control rice blast.

MATERIALS AND METHODS

Fungal strains. *M. grisea* strain 70-15 was provided by A. Ellingboe, University of Wisconsin, Madison, and used throughout the experiments. *M. grisea* strain KJ201, a Korean field strain, was used to confirm the efficacy of selected culture filtrates and for in vivo tests. Fungal cultures were grown on oatmeal agar (OMA; 50 g of oatmeal per liter) at 25°C under continuous fluorescent light to promote conidiation. Conidia were harvested from 7- to 10-day-old cultures.

Methanol mixtures and fractions of culture filtrates from members of the class Actinomycetes and fungi. All methanol mixtures and fractions of culture filtrates from 600 members of the class Actinomycetes and 400 fungi, which were isolated from soil, were provided by S. U. Kim, Korean Research Institute of Bioscience and Biotechnology, Taejeon, Korea. Briefly, each isolate was grown in E-medium (2% starch, 0.4% Bacto-Soytone [Difco Laboratories, Detroit], 0.3% Pharma media [Southern Cotton Oil Company, Memphis, TN], 0.2% polypeptone, 0.1% yeast extract, 0.1% K_2HPO_4 , 0.05% $MgSO_4$, 0.2% NaCl, and 0.3% $CaCO_3$) at 26°C for 4 days with constant shaking (150 rpm). Each culture filtrate of members of the class Actinomycetes or fungi was mixed with an equal volume of methanol for 30 min, and bacterial cells or fungal mycelia were removed by centrifugation. To trace the effective chemical components, culture filtrates of selected members of the class Actinomycetes were further fractionated by the addition of an equal volume of ethyl acetate and separated into ethyl acetate and water fractions. Methanol mixtures and fractions were tested for their inhibitory effect on conidial germination and appressorium formation of *M. grisea*.

Conidial germination and appressorium formation. Conidia of *M. grisea* were harvested in sterile distilled water and passed through four layers of cheesecloth. Thirty-six microliters of conidial suspension (3×10^4 conidia per milliliter) and 4 μ l of methanol mixtures or fractions of culture filtrates from members of the class Actinomycetes or fungi were placed on the hydrophobic side of GelBond (FMC BioProducts, Rockland, ME) in a moistened plastic box. After 24 h of incubation at 25°C, inhibitory effects of methanol mixtures and fractions of culture filtrate from members of the class Actinomycetes or fungi on conidial germination and appressorium formation were determined. The percentages of germinated conidia and of germinating conidia induced to form appressorium were determined by microscopic examination for at least 100 conidia per replicate in at least three experiments with

three replicates per test material. To reveal possible sites of inhibition on appressorium formation, complementation tests were performed by the addition of known effector chemicals for appressorium formation in this fungus. cAMP, 1,16-hexadecanediol (a cutin monomer), or 3-isobutyl-1-methylxanthine (IBMX; an inhibitor of phosphodiesterase) was added in a final concentration of 10 mM, 1 μ M, or 2.5 mM, respectively. All experiments consisted of three replicates per treatment and were carried out independently at least three times.

Inhibition test of mycelial growth. Mycelial disks (5 mm in diameter) from actively growing margins of colonies of *M. grisea* on potato dextrose agar (PDA) were transferred to the center of new PDA plates. Three paper disks (8 mm in diameter) were placed 2 cm from agar blocks, and 40 μ l of methanol mixture at a fivefold dilution was added every 24 h until mycelial margins in control plates reached the paper disks. The experiment was repeated three times with three replicates.

Assay of appressorium formation on detached rice leaves. Rice leaves were cut and attached to slide glass with cellophane tape and placed in a moistened petri dish. Twenty microliters of spore suspension (1.0×10^4 conidia per milliliter) with Tween 80 (250 ppm) containing a 100-fold dilution of methanol mixtures of culture filtrates was placed on the leaves and incubated for 16 h at 25°C. To determine the number of conidia and appressoria, a modified whole leaf staining and clearing method was used (24). Briefly, fragments of rice leaves containing an inoculated site were cut and fixed with lactophenol for 16 h. After fixation, dechlorophyllization was carried out twice in the solution of lactophenol and ethanol (1:1, vol/vol) for 2 h at 95°C. After staining in aniline blue staining solution (0.01% aniline blue in a mixture of equal volume of ethanol, lactic acid, and phenol) at 25°C for at least 24 h, the numbers of conidia and appressoria were determined by microscopic observation. This experiment was repeated three times with three replicates.

Control of rice blast in the greenhouse. Rice plants (cv. Nipponbare) were grown in commercial potting soil (Boonong, Kyongbuk, Korea) in plastic pots (5 cm in diameter) for 4 weeks in the greenhouse. Selected methanol mixtures of culture filtrates from A5005, A5314, and A5387 were sprayed on rice plants at the fifth-leaf stage and air-dried. After 24 h, a conidial suspension of *M. grisea* (5×10^5 conidia per milliliter) with Tween 80 (250 ppm) was sprayed on the treated rice plants, placed in a dew chamber (25°C, 100% relative humidity) for 24 h, and then moved into the greenhouse. Disease severity of nine plants per treatment was evaluated 7 days after inoculation. Disease severity was determined using a scale of 0 (no symptoms) to 10 (more than 50% of whole leaves were necrotic) according to the method developed by the International Rice Research Institute (13). The experiment was repeated three times and conducted once with *M. grisea* strain KJ201.

RESULTS

Effect of culture filtrates on conidial germination, appressorium formation, and mycelial growth. Methanol mixtures of culture filtrates from 600 members of the class Actinomycetes and 400 fungi were used for screening inhibition of conidial germination and appressorium formation in *M. grisea*. From these mixtures, 16 exhibited inhibition on appressorium formation. Five (A5005, A5008, A5314, A5387, and A5397) of them were highly effective and inhibited appressorium formation in a dosage-dependent manner (Fig. 1; Table 1). Inhibition of appressorium formation was observed at a 100-fold dilution of A5005, A5008, and A5397, and at a 200-fold dilution of A5314 and A5387. Conidial germination was little affected by 100- or 200-fold dilutions but was completely inhibited at a 10-fold dilution of all methanol mixtures tested (Table 1).

To evaluate the possible mode of action on the inhibition of appressorium formation by culture filtrates, cAMP, 1,16-hexade-

canediol, or IBMX was added at 10 mM, 1 μ M, or 2.5 mM, respectively. Inhibition of appressorium formation by A5005 was counteracted by cAMP (Fig. 1), and inhibition by A5397 was overcome by 1,16-hexadecanediol. Both cAMP and 1,16-hexadecanediol enhanced the appressorium formation inhibited by A5008. None of the effector chemicals tested was capable of restoring appressorium formation inhibited by A5314 and A5387 (data not shown). Based on their inhibitory effect on appressorium formation and mode of action, three culture filtrates (A5005, A5314, and A5387) were selected for further analysis. These three culture filtrates did not inhibit mycelial growth at concentrations up to a fivefold dilution.

Effect of fractions from culture filtrates on conidial germination and appressorium formation. To begin to identify effective chemical components, culture filtrates were fractionated with ethyl acetate into water-soluble and water-insoluble components. Inhibitory effect on appressorium formation was observed in the ethyl acetate fraction from A5005. However, both fractions from

A5314 and A5387 preferentially inhibited appressorium formation, while conidial germination remained unaffected up to a fivefold dilution (Table 2). The exogenous addition of cAMP restored appressorium formation inhibited by all fractions from A5005, A5314, and A5387. However, the addition of IBMX completely blocked conidial germination in the ethyl acetate fraction of A5005. No significant restoration of appressorium formation was observed in the ethyl acetate fraction of A5314 by IBMX. However, appressorium formation inhibited by other fractions was restored by IBMX. The addition of 1,16-hexadecanediol recovered the ability to form appressoria inhibited by both fractions of A5387. No significant effect by 1,16-hexadecanediol was observed in other fractions, except conidial germination was inhibited in the ethyl acetate fraction of A5005 (Table 3).

Effect of culture filtrates of members of the class Actinomycetes on appressorium formation on detached rice leaves. To evaluate the effect of culture filtrates on appressorium formation

Fig. 1. Inhibition and restoration of appressorium formation by *Magnaporthe grisea*. Appressorium formation was inhibited by the addition of a methanol mixture of **A**, A5005; **B**, A5314; or **C**, A5387 at a 100-fold dilution on the hydrophobic side of GelBond. **D**, Inhibition of appressorium formation by A5005 was restored by the addition of cyclic AMP. **E**, On the hydrophobic side of GelBond, darkly pigmented appressoria were formed in the presence of 10% methanol. **F**, No appressoria were formed on the hydrophilic side of GelBond.

TABLE 1. Effects of culture filtrates from selected members of the class Actinomycetes on conidial germination and appressorium formation by *Magnaporthe grisea* strain 70-15^x

Culture filtrates	Dilutions ^y (%) ± standard deviation)	Conidial germination (%) ± standard deviation)	Appressorium formation (%) ± standard deviation)
A5005	10	0.0 ± 0.0 g ^z	0.0 ± 0.0 f
	100	71.0 ± 4.4 d	4.3 ± 2.5 ef
	200	83.7 ± 1.5 bc	71.0 ± 6.6 bc
	400	92.7 ± 2.1 ab	83.3 ± 11.2 a-c
	1,000	97.0 ± 1.0 a	94.7 ± 1.5 a
A5008	10	0.0 ± 0.0 g	0.0 ± 0.0 f
	100	90.7 ± 4.5 ab	67.0 ± 7.5 c
	200	90.7 ± 1.2 ab	90.3 ± 4.7 a
	400	92.7 ± 1.2 ab	90.3 ± 4.7 a
A5314	10	0.0 ± 0.0 g	0.0 ± 0.0 f
	100	68.3 ± 4.0 d	0.0 ± 0.0 f
	200	87.5 ± 10.2 a-c	41.8 ± 35.7 d
	400	89.7 ± 3.8 a-c	67.7 ± 21.9 c
	1,000	94.3 ± 3.1 a	94.0 ± 1.0 a
A5387	10	0.0 ± 0.0 g	0.0 ± 0.0 f
	100	47.7 ± 8.6 f	0.0 ± 0.0 f
	200	60.0 ± 13.9 e	20.3 ± 17.2 e
	400	96.3 ± 1.5 a	88.0 ± 2.0 ab
	1,000	93.3 ± 2.5 a	92.3 ± 0.6 a
A5397	10	0.0 ± 0.0 g	0.0 ± 0.0 f
	100	81.3 ± 10.7 c	39.3 ± 11.0 d
	200	90.3 ± 2.1 ab	94.0 ± 2.6 a
	400	92.0 ± 1.0 ab	94.3 ± 1.5 a
Distilled water		93.7 ± 1.5 a	94.7 ± 1.5 a
Methanol (10%)		92.3 ± 3.2 ab	95.0 ± 2.0 a

^x Thirty-six microliters of conidial suspension (3×10^4 conidia per milliliter) with 4 µl of methanol mixtures of culture filtrates from members of the class Actinomycetes was dropped on the hydrophobic side of GelBond, placed in a moistened plastic box, and incubated as described in Materials and Methods.

^y All methanol mixtures of culture filtrates were diluted with absolute methanol.

^z Values in the column that are followed by the same letter are not significantly different ($P < 0.05$), as determined by Duncan's multiple range test.

TABLE 2. Effects of fractions of Actinomycetes culture filtrates on conidial germination and appressorium formation by *Magnaporthe grisea* strain 70-15^w

Fractions of culture filtrates ^x	Dilutions ^y (%) ± standard deviation)	Conidial germination (%) ± standard deviation)	Appressorium formation (%) ± standard deviation)
A5005			
	Ethyl acetate		
	10	0.0 ± 0.0 e ^z	0.0 ± 0.0 e
	100	19.3 ± 14.5 c	1.7 ± 1.5 e
	150	69.3 ± 2.5 b	13.0 ± 5.0 de
Water	10	11.7 ± 2.5 d	0.0 ± 0.0 e
	100	97.3 ± 1.2 a	93.3 ± 3.5 a
A5314			
	Ethyl acetate		
	5	95.3 ± 1.5 a	23.0 ± 19.3 cd
	10	95.0 ± 3.0 a	31.7 ± 12.7 bc
Water	5	96.0 ± 1.7 a	7.4 ± 6.4 e
	10	99.0 ± 0.0 a	2.3 ± 2.1 e
A5387			
	Ethyl acetate		
	5	95.7 ± 1.5 a	21.1 ± 9.7 cd
	10	96.7 ± 1.5 a	39.3 ± 4.5 b
Water	5	90.7 ± 1.2 a	13.0 ± 2.0 de
	10	92.3 ± 5.4 a	39.8 ± 14.0 b
Distilled water		98.7 ± 0.6 a	95.0 ± 1.0 a
Methanol (10%)		98.0 ± 1.0 a	94.7 ± 0.6 a

^w Thirty-six microliters of conidial suspension (3×10^4 conidia per milliliter) with 4 µl of fractions of culture filtrates from members of the class Actinomycetes was dropped on the hydrophobic side of GelBond, placed in a moistened plastic box, and incubated as described in Materials and Methods.

^x Ethyl acetate was evaporated and aliquots were dissolved in an equal volume of methanol.

^y All fractions were diluted with absolute methanol.

^z Values in the column that are followed by the same letter are not significantly different ($P < 0.05$), as determined by Duncan's multiple range test.

on rice leaves, drops of conidial suspension along with culture filtrate were placed onto detached rice leaves. Methanol mixtures (A5005, A5314, and A5387) at a 100-fold dilution reduced the number of attached conidia and appressoria on rice leaves. Due to technical difficulties in measuring the numbers of conidia and appressoria on rice leaves by microscopic observation, rice leaves were first dechlorophyllized and fungal cells stained. This process might reduce the numbers of attached conidia and appressoria because of the vigorous washing steps during the dechlorophyllization and staining processes. The most dramatic reductions in the numbers of conidia and appressoria were observed in the treatment of A5005 culture filtrate, followed by A5314 and A5387 (Fig. 2).

Control of rice blast in the greenhouse. To evaluate the correlation between the antifungal activity on appressorium formation on an artificial surface and the control efficacy of rice blast, in vivo assays were conducted with these culture filtrates from A5005, A5314, and A5387 in the greenhouse. Spraying these filtrates on rice did not cause any obvious phytotoxic symptoms when examined for up to 14 days. Control plants treated with a 10% methanol solution followed 24 h later by inoculation resulted in typical blast symptoms and disease severity was scored 7 (more than 10 typical blast lesions per leaf), at 7 days after inoculation. Treatment of plants with methanol mixtures of A5005 or A5314 at 24 h before inoculation resulted in a significant reduction in the

TABLE 3. Complementation of appressorium formation inhibited by fractions of Actinomycetes culture filtrates by the addition of cyclic AMP, 3-isobutyl-1-methylxanthine (IBMX), or 1,16-hexadecanediol^w

Fractions of culture filtrates ^x	Treatments ^y	Conidial germination (%) ± standard deviation)	Appressorium formation (%) ± standard deviation)
A5005			
	Ethyl acetate		
	Control	76.0 ± 4.0 e ^z	52.7 ± 8.1 d-f
	cAMP	85.0 ± 6.2 d	80.0 ± 3.0 bc
	Diol	33.0 ± 14.2 f	60.0 ± 4.6 d
	IBMX	0.7 ± 0.6 g	0.0 ± 0.0 h
A5314			
	Ethyl acetate		
	Control	97.7 ± 1.5 a	54.3 ± 9.3 de
	cAMP	95.0 ± 1.7 a	79.7 ± 9.3 bc
	Diol	97.3 ± 2.1 a	51.7 ± 16.5 d-f
	IBMX	89.7 ± 3.5b cd	43.0 ± 9.5 ef
Water			
	Control	91.0 ± 1.7 a-d	7.3 ± 7.6 h
	cAMP	98.0 ± 0.0 a	90.0 ± 8.7 ab
	Diol	96.0 ± 1.0 ab	11.0 ± 4.4 h
	IBMX	88.7 ± 4.0 cd	31.3 ± 2.1 g
A5387			
	Ethyl acetate		
	Control	95.3 ± 0.6 a-c	50.7 ± 4.0 d-f
	cAMP	94.0 ± 6.1 a-c	89.7 ± 2.3 ab
	Diol	96.0 ± 2.6 ab	86.3 ± 3.2 ab
	IBMX	96.3 ± 1.5 ab	73.0 ± 6.1 c
Water			
	Control	98.3 ± 0.6 a	41.3 ± 1.2 fg
	cAMP	95.3 ± 3.1 a-c	86.3 ± 2.5 ab
	Diol	98.3 ± 0.6 a	85.7 ± 5.5 ab
	IBMX	96.0 ± 1.7 ab	58.0 ± 3.6 d
Distilled water		97.0 ± 1.0 a	94.7 ± 2.1 a
Methanol (10%) + ethanol (10%)		97.7 ± 0.6 a	94.0 ± 3.5 a

^w Four microliters of cyclic AMP, 1,16-hexadecanediol, or IBMX was added to 32 µl of conidial suspensions with 4 µl of fractions of culture filtrates from members of the class Actinomycetes. After 24 h of incubation at 25°C in a moistened plastic box, percentages of conidial germination and appressorium formation were determined as described in Materials and Methods.

^x Ethyl acetate was evaporated and aliquots were dissolved in an equal volume of methanol.

^y Control means fractions of culture filtrates of members of the class Actinomycetes only and Diol are 1,16-hexadecanediol. All fractions were treated at a 10-fold dilution, except the ethyl acetate fraction of A5005 that was treated at a 150-fold dilution.

^z Values in the column that are followed by the same letter are not significantly different ($P < 0.05$), as determined by Duncan's multiple range test.

number of lesions. However, A5387 had no effect on controlling rice blast in the greenhouse. Treatment using tricyclazole (Beam) resulted in almost complete control of rice blast (Fig. 3). Similar results were also obtained using *M. grisea* strain KJ201 (data not shown).

DISCUSSION

A novel target-oriented and cell-based screening system for antifungal compounds on appressorium formation by *M. grisea* was established. Candidate chemicals were added to conidial suspensions of *M. grisea* placed on the hydrophobic side of GelBond, a substratum that induces appressorium formation, and their activities that preferentially inhibit appressorium formation, not conidial germination, were assessed. Determination of inhibitory effects on appressorium formation could be done in a single day and other effects, such as inhibition of conidial germination and melanization, could be determined at the same time. Moreover, selected compounds evaluated under this system yielded results that paralleled in vivo tests. This cell-based screening system has many advantages. It requires only a small amount of space, is rapid, and although not automated, can be used to screen at least 3,000 to 5,000 compounds using very small amounts per week.

To establish this target-oriented and cell-based screening system, 1,000 methanol mixtures of culture filtrates of members of the class Actinomycetes and fungi were screened for differential inhibitory effect on appressorium formation in *M. grisea*. Comparative studies using detached leaves and in vivo tests were also conducted to demonstrate the reliability of this screening system. Among these treatments, methanol mixtures of Actinomycetes A5005, A5314, and A5387 showed the greatest inhibitory effects

on appressorium formation by *M. grisea*. The inhibitory effect on appressorium formation was dosage-dependent, which suggested a specific mode of action.

In the detached-leaf assay, greatly reduced numbers of conidia and appressoria were observed when the culture filtrates of A5005 and A5314 were applied. This is consistent with the results of the in vitro screening. The greater the inhibition of appressorium formation in the in vitro screening system, the fewer numbers of attached conidia and appressoria were found using a detached-leaf assay. Due to the vigorous nature of the dechlorophyllization process of rice leaves and staining procedure of fungal cells, conidia without appressoria might be shaken off the rice leaves. In the greenhouse test, disease severity of rice blast by methanol mixtures of A5005 and A5314 were also significantly reduced. However, the extract from A5387 did not exhibit disease control efficacy, although it had a differential inhibitory effect on appressorium formation on GelBond. It may be possible that the active component in A5387 was volatile or was unstable and, thus, at the time of inoculation, 24 h later, biological activity had been lost. This may also apply to A5005 and A5314, reducing their activity. Other explanations are possible, particularly for A5387. The addition of 1,16-hexadecanediol completely recovered appressorium formation inhibited by the ethyl acetate or water fraction of A5387, while no restoration of appressorium formation was observed in fractions of A5005 and A5314. Chemical components present in rice leaves, such as cutin monomers including 1,16-hexadecanediol, may interfere with the inhibitory effect of A5387. This speculation is supported somewhat by the fact that more conidia and appressoria were observed in detached rice leaves in the treatment of A5387 compared with A5005 and A5314. The precise biochemical mechanisms remain to be elucidated. However, it does suggest

Fig. 2. Inhibition of appressorium formation of *Magnaporthe grisea* by methanol mixtures of culture filtrates of members of the class Actinomycetes on rice leaves. **A**, Treatment of a methanol mixture of A5005 significantly reduced the numbers of attached conidia and appressoria on rice leaves. Methanol mixtures of **B**, A5314 and **C**, A5387 inhibited appressoria formation specifically. **D**, Conidia were germinated and formed well-pigmented appressoria in the control (10% methanol). Methanol mixtures were treated at 100-fold dilutions.

that the effect of the rice plant must be considered when developing more efficient in vitro screening methods on artificial surfaces. Although the disease control efficacy by these culture filtrates was much less than the commercial fungicide tricyclazole, the fact that selected compounds exhibited control efficacy of rice blast in vivo proved the reliability of the cell-based screening system developed in this study.

A5005 and A5314 controlled disease, although they did not inhibit mycelial growth of *M. grisea*. These results also suggest that this system could be used to screen previously discarded compounds that had no inhibitory effects on mycelial growth. Tricyclazole, which inhibits melanin biosynthesis in appressoria, and probenazole and saccharin, which regulate host resistance to infection by *M. grisea*, are good points of reference (20). Furthermore, chemicals that strongly inhibit conidial germination and mycelial growth tend to have broad spectrum of activity and high relative toxicity (6,9). In these experiments, particular methanol mixtures were able to specifically inhibit appressorium formation without inhibitory effects on other metabolic pathways. This specific inhibitory effect is likely to be advantageous for developing new fungicides with low toxicity on the environment.

Inhibition of appressorium formation by fractions of these culture filtrates was nullified by the addition of cAMP or 1,16-hexadecanediol (cutin monomer), known effector chemicals for appressorium formation, suggesting target specificity. Both cAMP and 1,16-hexadecanediol induce appressorium formation on non-inductive surfaces in this fungus (10,18). These results suggest that the inhibition mechanism(s) by these compounds is upstream of the sites of action of cAMP or 1,16-hexadecanediol. IBMX is also an effector chemical through the inhibition of phosphodiesterase, an enzyme responsible for degradation of cAMP in the cells, in this fungus (18). However, no obvious effects of IBMX were observed in restoring appressorium formation inhibited by test compounds in these experiments. A similar phenomenon has been observed for IBMX and polyamines. Appressorium formation inhibited by polyamines was restored by the addition of cAMP but not IBMX (4).

Together with the identification of suitable and novel biochemical target sites, cell-based screening with specific target sites would be indispensable for saving time and money. The screening system developed in this study will be a useful tool for screening

antifungal compounds for appressorium-forming, plant-pathogenic fungi and provide novel concepts to agrochemists for the development of new fungicides.

ACKNOWLEDGMENTS

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

LITERATURE CITED

1. Beckerman, J. L., and Ebbole, D. J. 1996. MPG1, a gene encoding a fungal hydrophobin of *Magnaporthe grisea*, is involved in surface recognition. *Mol. Plant-Microbe Interact.* 9:450-456.
2. Bourett, T. M., and Howard, R. J. 1990. *In vitro* development of penetration structures in the rice blast fungus *Magnaporthe grisea*. *Can. J. Bot.* 68:329-342.
3. Choi, W. B., and Dean, R. A. 1997. The adenylate cyclase gene *MAC1* of *Magnaporthe grisea* controls appressorium formation and other aspects of growth and development. *Plant Cell* 9:1973-1983.
4. Choi, W.-B., Kang, S.-H., Lee, Y.-W., and Lee, Y.-H. 1998. Cyclic AMP restores appressorium formation inhibited by polyamines in *Magnaporthe grisea*. *Phytopathology* 88:58-62.
5. Corbely, M. J., Cherington, V., Traxler, P. M., Lydon, N. B., and Roberts, T. M. 1996. A strategy for screening anti-tumor drugs utilizing oncogenes encoded in retroviral vectors. *Int. J. Cancer* 66:753-759.
6. Delp, C. J. 1980. Coping with resistance to plant disease control agents. *Plant Dis.* 64:652-657.
7. de Waard, M. A., Georgopoulos, S. G., Hollomon, D. W., Ishii, H., Leroux, P., Ragsdale, N. N., and Schwinn, F. J. 1993. Chemical control of plant diseases: Problems and prospects. *Annu. Rev. Phytopathol.* 31: 403-421.
8. Frost, D. J., Brandt, K. D., Cugier, D., and Goldman, R. 1995. A whole-cell *Candida albicans* assay for the detection of inhibitors towards fungal cell wall synthesis and assembly. *J. Antibiot.* 48:306-310.
9. Fuchs, A., Davidse, L. C., de Waard, M. A., and de Wit, P. J. G. M. 1983. Contemplations and speculations on novel approaches in the control of fungal plant diseases. *Pestic. Sci.* 14:272-293.
10. Gilbert, R. D., Johnson, A. M., and Dean, R. A. 1996. Chemical signals responsible for appressorium formation in the rice blast fungus *Magnaporthe grisea*. *Physiol. Mol. Plant Pathol.* 48:335-346.
11. Goosey, M. W. 1992. Towards the rational design of crop protection agents. *Pestic. Sci.* 34:313-320.
12. Howard, R. J., Ferrari, M. A., Roach, D. H., and Money, N. P. 1991. Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl. Acad. Sci. U.S.A.* 88:11281-11284.
13. International Rice Research Institute. 1988. Standard Evaluation System for Rice, 3rd ed. International Rice Testing Program, International Rice Research Institute, Los Baños, the Philippines.
14. Khun, P. J. 1989. The discovery and development of fungicide—Does biochemistry have a role? *Pestic. Sci.* 25:123-135.
15. Knight, S. C., Anthony, V. M., Brady, A. M., Greenland, A. J., Heaney, S. P., Murray, D. C., Powell, K. A., Schulz, M. A., Spinks, C. A., Worthington, P. A., and Youle, D. 1997. Rationale and perspectives on the development of fungicides. *Annu. Rev. Phytopathol.* 35:349-372.
16. Lau, G., and Hamer, J. E. 1996. Regulatory genes controlling MPG1 expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 8:771-781.
17. Lee, S.-C., and Lee, Y.-H. 1998. Calcium/calmodulin-dependent signaling for appressorium formation in the plant pathogenic fungus *Magnaporthe grisea*. *Mol. Cells* 8:698-704.
18. Lee, Y.-H., and Dean, R. A. 1993. cAMP regulates infection structure formation in the plant pathogenic fungus *Magnaporthe grisea*. *Plant Cell* 5:693-700.
19. Lee, Y.-H., and Dean, R. A. 1994. Hydrophobicity of contact surface induces appressorium formation in *Magnaporthe grisea*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 115:71-76.
20. Leroux, P. 1996. Recent developments in the mode of action of fungicides. *Pestic. Sci.* 47:191-197.
21. Levitzki, A., and Gazit, A. 1995. Tyrosine kinase inhibition: An approach to drug development. *Science* 267:1782-1788.
22. Liu, S., and Dean, R. A. 1997. G protein α subunit genes control growth, development, and pathogenicity of *Magnaporthe grisea*. *Mol. Plant-Microbe Interact.* 10:1075-1086.
23. Mitchell, T. K., and Dean, R. A. 1995. The cAMP-dependent protein kinase catalytic subunit is required for appressorium formation and pathogenesis by the rice blast pathogen *Magnaporthe grisea*. *Plant Cell*

Fig 3. Control of rice blast by culture filtrates of members of the class Actinomycetes. Methanol mixtures of culture filtrates of members of the class Actinomycetes were sprayed on rice plants at the fifth-leaf stage and air-dried. Inoculation with a conidial suspension of *Magnaporthe grisea* was conducted as described in Materials and Methods. The same letters above bars are not significantly different at $P = 0.05$, using the Duncan's multiple range test.

- 7:1869-1878.
24. Peng, Y.-L., Shishiyama, J., and Yamamoto, M. 1986. A whole-leaf staining and clearing procedure for analyzing cytological aspects of interaction between rice plant and rice blast fungus. *Ann. Phytopathol. Soc. Jpn.* 52:801-808.
 25. Staub, T. 1991. Fungicide resistance: Practical experience with antiresistance strategies and the role of integrated use. *Annu. Rev. Phytopathol.* 29:421-442.
 26. Talbot, L. J., Ebbole, D. J., and Hamer, J. E. 1993. Identification and characterization of *MPGI*, a gene involved in pathogenicity from the rice blast pathogen *Magnaporthe grisea*. *Plant Cell* 5:1575-1590.
 27. Tuite, M. F. 1992. Antifungal drug development: The identification of new targets. *Biotechnology* 10:235-239.
 28. Xu, J.-R., and Hamer, J. E. 1996. MAP kinase and cAMP signaling regulate infection structure formation and pathogenic growth in the rice blast fungus *Magnaporthe grisea*. *Genes Dev.* 10:2696-2706.
 29. Xu, J.-R., Staiger, C. J., and Hamer, J. E. 1998. Inactivation of the mitogen-activated protein kinase Mps1 from the rice blast fungus prevent penetration of host cells but allows activation of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 95:12713-12718.