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Inhibition of the Pathogenicity of *Magnaporthe grisea* by Bromophenols, Isocitrate Lyase Inhibitors, from the Red Alga *Odonthalia corymbifera*

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Magnaporthe grisea is a fungal pathogen of rice that forms appressoria that penetrate the outer cuticle of the rice plant. Data from recent studies indicate that *M. grisea* isocitrate lyase (ICL), a key enzyme in the glyoxylate cycle, is highly expressed during appressorium-mediated plant infection. Bromophenols isolated from the red alga *Odonthalia corymbifera* exhibited potent ICL inhibitory activity and blocked appressoria formation by *M. grisea* in a concentration-dependent manner. In addition, these compounds protected the rice plants from infection by *M. grisea*. Rice plants infected with wild-type *M. grisea* Guy 11 exhibited significantly lower disease severity with bromophenol treatment than without, and the treatment effect was comparable to the behavior of the Δ*icl* knockout mutant I-10. The protective effect of bromophenols and their strong inhibition of appressorium formation on rice plants suggest that ICL inhibitors may be promising candidates for crop protection, particularly to protect rice plants against *M. grisea*.

KEYWORDS: ***Magnaporthe grisea*; appressoria; infection; rice; disease symptom; isocitrate lyase inhibitor; *Odonthalia corymbifera*; bromophenols**

INTRODUCTION

The glyoxylate cycle is a modified form of the tricarboxylic acid cycle; it was initially discovered in microorganisms and plays a fundamental role in nature by providing the means for microorganisms to grow on acetate, ethanol, or fatty acids (*1*). This function has been confirmed by the analysis of mutants of pathogenic microorganisms that lack isocitrate lyase (ICL) and malate synthase (MLS), key enzymes in the glyoxylate cycle (*2*−*6*). The carbon-conserving glyoxylate pathway is present in most prokaryotes, lower eukaryotes, and plants, but it has not been observed in vertebrates (*7*). Several lines of evidence suggest that pathogenic microorganisms use primarily fatty acids, rather than carbohydrates, as carbon substrates during infection. The expression of ICL is up-regulated during infection by macrophages of the pulmonary bacterium *Mycobacterium tuberculosis*, and ICL mutants show virulence attenuation (*3*, *8*). Research on candidiasis in mice has shown that *Candida albicans*, the most serious human pathogenic fungus, requires ICL to be fully virulent (*2*). In plants, recent genetic studies have confirmed that the glyoxylate cycle plays an important role in both gluconeogenesis and anaplerotic processes during germination and postgerminative growth of plant oilseeds (*9*).

Magnaporthe grisea (anamorph *Pyricularia grisea*) is a typical heterothallic ascomycete and the causal agent of rice blast, one of the most destructive diseases of cultivated rice worldwide (*10*, *11*). The fungus infects plants via appressoria, which are specialized structures consisting of dome-shaped cells with enormous turgor that invade and rupture the cuticle of leaves (*12*). Appressorial turgor in *M. grisea* depends on lipid reserves to make glycerol. During conidial germination and appressorium formation in this fungus, lipid bodies are mobilized from the conidium to the germ tube apex (*13*). At the onset of turgor generation, lipid bodies coalesce and are taken up by vacuoles before rapid lipolysis. One consequence of reliance upon lipid metabolism for turgor generation during the prepenetration stage of development may be induction of the glyoxylate cycle to provide a mechanism of generating glucose (*13*). Infection of rice with *M. grisea* leads to the expression of genes involved in the glyoxylate cycle (*4*). In addition, Δ*icl* mutants

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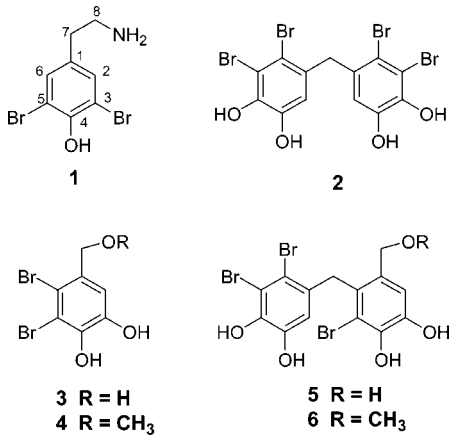

Figure 1. Chemical structures of bromophenols: 3,5-dibromo-4-hydroxyphenylethylamine (**1**); 2,2′,3,3′-tetrabromo-4,4′,5,5′-tetrahydroxydiphenylmethane (**2**); 2,3-dibromo-4,5-dihydroxybenzyl alcohol (**3**); 2,3-dibromo-4,5-dihydroxybenzyl methyl ether (**4**); 2,2′,3-tribromo-3′,4,4′,5-tetrahydroxy-6′-hydroxymethyldiphenylmethane (**5**); 3-bromo-4-(2,3-dibromo-4,5-dihydroxybenzyl)-5-methoxymethylpyrocatechol (**6**).

are less virulent than an isogenic wild-type strain of *M. grisea* and exhibit impaired virulence-associated functions such as germ tube emergence, appressorium development, and cuticle penetration. Therefore, ICL could be a promising target for the control of fungal infection and the development of antifungal agents.

In recent years, many new bromophenols have been isolated from marine red algae of the family Rhodomelaceae. These metabolites possess diverse biological activities such as cytotoxic (*14*−*17*), antimicrobial (*17*, *18*), antioxidant (*19*), α-glucosidase inhibitory (*20*), aldose reductase inhibitory (*21*), feeding deterrent (*22*), and antibacterial (*23*) activities. In the course of searching for secondary metabolites of biological significance from marine organisms, we collected the red alga *Odonthalia corymbifera* (family Rhodomelaceae, order Ceramiales) by scuba diving (5−10 m depth) off the coast of Sokcho, South Korea. A crude extract of this specimen exhibited potent inhibitory activity (IC_{50} = 23 μg/mL) against ICL from *M. grisea*. Six bromophenols were isolated from a methanol extract (**Figure 1**): 3,5-dibromo-4-hydroxyphenylethylamine (*24*); 2,2′,3,3′-tetrabromo-4,4′,5,5′-tetrahydroxydiphenylmethane (*22*); 2,3-dibromo-4,5-dihydroxybenzyl alcohol (*22*); 2,3-dibromo-4,5-dihydroxybenzyl methyl ether (*22*); 2,2′,3-tribromo-3′,4,4′,5-tetrahydroxy-6′-hydroxymethyldiphenylmethane (*25*); and 3-bromo-4-(2,3-dibromo-4,5-dihydroxybenzyl)-5-methoxymethylpyrocatechol (*22*), hereafter referred to as compounds **1**−**6**, respectively. Here, we investigated the potential of these bromophenols as ICL inhibitors of appressorium formation and the pathogenicity of the rice blast fungus *M. grisea*.

MATERIALS AND METHODS

Microorganisms and Growth Conditions. The wild-type rice pathogenic fungus *M. grisea* Guy 11 and its isogenic knockout mutant I-10 (Δ*icl*) were obtained from Dr. N. J. Talbot (University of Exeter, U.K.) (*4*). The strains were grown on oatmeal agar (50 g of oatmeal/L) at 25 °C under fluorescent light to promote conidiation. Conidia were harvested from 14-day-old cultures and suspended in sterile distilled water. The suspension was filtered through glass wool to remove mycelial debris and centrifuged at 2000*g* for 10 min. Conidia were washed and resuspended in sterile water to give a concentration of 1.25×10^5 conidia/mL.

Preparation of ICL and MLS. The preparation of recombinant ICL protein from *M. grisea* Guy 11 was performed as described previously (*26*). In a similar manner, we used cDNA of *M. grisea* Guy 11 as a template to amplify the MLS gene (GenBank accession no. AAN28719). We used the two synthetic primers MS-1 (5′-ATGGCTTCCACA-GAGTCCAT-3′) and MS-2 (5′-GGCCCGCTTCGAAATTGTAA-3′) to perform PCR amplification. The amplified fragment was purified from 0.7% Seakem GTG agarose (FMC Bioproducts, Rockland, ME) gel using Suprec-01 (Takara Shuzo Co., Kyoto, Japan) and ligated into the pBAD/Thio-TOPO vector (Invitrogen, Carlsbad, CA) for TA cloning. The ligation mixture was transformed into *Escherichia coli* TOP10 (Invitrogen), and the transformant was selected on LB agar plates containing ampicillin (50 μg/mL). The construct was confirmed by restriction enzyme digestion. The construct was made by cloning the *Eco*RI fragment into pET32(b) (Novagen, Darmstadt, Germany) and was expressed in *E. coli* BLR (DE3). The recombinant ICL and MLS enzymes were purified using Ni-NTA column chromatography (Qiagen, Hilden, Germany).

Enzyme Activity Assay. ICL enzyme activity was determined using Dixon and Kornberg's method (*27*). A 1-mL aliquot of the reaction mixture contained 20 mM sodium phosphate buffer (pH 7.0), 1.27 mM *threo*-DL-(+)-isocitrate, 3.75 mM $MgCl_2$, 4.1 mM phenylhydrazine, and 2.5 μg/mL purified ICL (*28*). The reaction was performed at 37 °C for 30 min. MLS enzyme activity was determined using Nakata and Selitrennikoff's method (*29*). The reaction mixture (100 μL) contained 50 mM sodium phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 10 mM glyoxylate, 400 μM AcCoA, and 2.5 μg/mL purified MLS. After the mixture had been incubated at 37 °C for 30 min, we terminated the reactions by adding 100 μL of 6.4 M guanidine hydrochloride. We then added 100 μL of 400 μM 5,5′-dithiobis(2-nitrobenzoic acid). After the mixture had been incubated for 10 min at room temperature, we measured the absorbance at 412 nm. We used 3-nitropropionate and bromopyruvate as positive controls for ICL and MLS, respectively.

Extraction and Isolation of ICL Inhibitors. The red alga *O. corymbifera* was collected off the coast of Sokcho, South Korea, in April 2006 and identified by Dr. Rae-Sun Kang of the Korea Ocean Research and Development Institute, South Korea. A voucher specimen was deposited at the Institute's Marine Resources Research Department. The freshly collected alga was immediately frozen using dry ice and kept at −25 °C until chemical investigation. We extracted air-dried *O. corymbifera* (271 g) with methanol (1.0 L × 2) and CH_2Cl_2 (1.0 L × 1) at room temperature. After removal of the solvent under reduced pressure, the residue (26.1 g) was suspended in water and successively partitioned with dichloromethane and *n*-BuOH. The *n*-BuOH fraction (7.05 g) was separated by ODS vacuum flash chromatography using a stepwise gradient of water/acetone. To yield compound **1** (13.3 mg), the 65% aqueous acetone fraction (1.93 g) was subjected to HPLC on a C_{18} reversed-phase ODS gel. For compounds **3** (288 mg) and **5** (73.8 mg), we separated the 50% aqueous acetone fraction (1.51 g) using reversed-phase HPLC (YMC-ODS-A column, 60% aqueous CH_3CN). For compounds **4** (140.4 mg) and **6** (108.6 mg), we purified the 30% aqueous acetone fraction (700 mg) using C_{18} reversed-phase preparative HPLC with 35% aqueous MeOH as a mobile phase. The dichloromethane layer (3.03 g) was repartitioned between 15% aqueous methanol and *n*-hexane. The aqueous MeOH layer (2.25 g) was dried and subjected to chromatography on a silica gel, eluting with a gradient-increasing MeOH (0−20%) in CH_2Cl_2. For compound **2** (20 mg), we purified a portion (124 mg) of the fraction eluted with 10% MeOH in CH_2Cl_2 (780 mg) using reversed-phase HPLC (YMC-ODS-A column, 50% aqueous CH_3CN). The spectra of ^1H and ^{13}C NMR were recorded on a Varian Unity 500 spectrometer. Proton and carbon NMR spectra were measured at 500 and 125 MHz, respectively. Mass spectra were obtained using a JEOL JMS-HX 110 mass spectrometer.

Conidial Germination and Appressorium Formation Assays. The formation of appressoria in germinating conidia was monitored on the hydrophobic surface of GelBond (FMC Bioproducts). GelBond sheets were washed in sterile water for 30 min. Conidial suspension (40 μL) was added to the hydrophobic side of the GelBond sheet. Conidia were allowed to attach to the surface for 1 h before sterile water (0.96 mL) was carefully added. For ICL inhibitor addition, the halisulfate **1** dissolved in dimethyl sulfoxide (DMSO) (10 mg/mL) was added to a final solvent concentration of 0.5%. The GelBond sheets were then sealed in a moistened box and incubated at 25 °C for 12 h. The

Table 1. Effect of Bromophenols on Virulence-Associated Functions in *M. grisea* Guy 11[a]

	IC_{50}, μM		conidial germination at 20 μM, %		appressorium formation at 20 μM, %	
compd	MLS	ICL	Guy 11	Δ*icl* I-10	Guy 11	Δ*icl* I-10
1	>678.0	116.1 ± 7.3	98.1 ± 0.9	98.3 ± 1.1	98.4 ± 2.2	7.1 ± 0.3
2	>365.1	2.0 ± 0.1	69.7 ± 4.8	70.2 ± 2.8	1.2 ± 0.1	0.0 ± 0.0
3	>671.3	92.6 ± 5.8	97.2 ± 2.0	96.4 ± 3.1	94.1 ± 1.2	4.6 ± 0.1
4	>641.1	125.6 ± 8.6	98.2 ± 0.8	97.7 ± 1.3	93.4 ± 0.9	5.3 ± 0.2
5	>400.8	2.8 ± 0.2	97.5 ± 2.3	95.8 ± 2.2	9.2 ± 1.3	1.0 ± 0.1
6	>389.9	2.1 ± 0.1	97.6 ± 1.3	96.1 ± 2.3	1.6 ± 0.2	0.0 ± 0.0
3-NP	>1680.7	92.4 ± 4.6	98.0 ± 2.2	98.6 ± 0.8	87.5 ± 4.1	3.0 ± 0.2
BP	142.8 ± 7.5	>1197.6	98.0 ± 2.2	98.0 ± 2.2	90.7 ± 3.6	3.5 ± 0.2

[a] The percentages of germinated conidia and conidia-formed appressorium after incubation on GelBond sheet for 12 h at 25 °C were determined by direct microscopic examination (n = 300). Values are presented as mean ± SE of three separate replicates. 3-Nitropropionate (3-NP) and bromopyruvate (BP) inhibitors of ICL and MLS, respectively, were used as positive controls.

percentage of germinated conidia forming appressoria was determined by performing a direct microscopic examination of at least 100 spores per replicate in at least three experiments with three replicates per treatment.

Fungal Growth Inhibition Assay. The growth of *M. grisea* was monitored in a minimal growth medium (*30*) with glucose or sodium acetate as the carbon source. Each stock solution of test compounds was diluted with growth medium to prepare the prescribed concentrations prior to use. Conidia (10^5 conidia/mL) were incubated for 7 days at 28 °C in a minimal growth medium containing variable concentrations of test compound, and the minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound at which no growth was observable.

Plant Infections. Conidial suspension (1×10^4 conidia/mL, containing 0.5% DMSO) was sprayed evenly onto susceptible rice seedlings (*Oryza sativa* L. cv. Nakdong) in the third and fourth leaf stages (*31*). In a separate experiment, rice seedlings were infected with conidial suspension (1×10^4 conidia/mL) containing 2, 4, 8, and 16 μM compounds **2** and **6** (final DMSO concentration of 0.5%) by spray inoculation. Inoculated plants were placed in a dew chamber at 25 °C for 24 h in the dark and then transferred to a growth chamber at 25 °C for 8 days with a 16 h photoperiod. We recorded the percent DLA and the proportion of DLA based on lesion types to permit an accurate evaluation of the virulence of the pathogenic fungi. We calculated disease severity using Ordonez's modified equation (*31*):

$$\text{disease severity (\%)} = \Sigma\{[\text{proportion of DLA based on lesion type (\%)}] \times [\text{lesion type (i)}]\}/(100 \times 3) \quad (1)$$

In eq 1 a value of 1 is a resistant-type lesion (pinpoint or larger pinhead), 2 is an intermediate-type lesion (round to slightly elongated, necrotic gray spot with a brown margin), and 3 is a susceptible-type lesion (elliptical lesion with a grayish center and brown margin). Disease severity was calculated from three separate experiments.

RESULTS

Structural Analysis of Bromophenols. Compound **1** was isolated as an amorphous solid. Fast atom bombardment mass spectroscopy exhibited a characteristic molecular ion peak cluster at *m*/*z* 292:294:296 $[M + H]^+$ with a ratio of 1:2:1, suggesting the presence of two bromine atoms. High-resolution molecular spectroscopy and ^{13}C nuclear magnetic resonance (NMR) spectrometry revealed the molecular composition to be $C_8H_9Br_2NO$. The ^1H NMR spectrum showed a singlet at δ 7.61 and two triplets at δ 3.25 and 2.96, which indicated the presence of a proton in a phenolic ring system and an ethylene moiety. The ^{13}C NMR spectrum of the compound displayed six carbon signals for tetrasubstituted symmetric benzene (δ 147.0, 138.1, 134.4, 119.3) and ethylene (δ 41.0, 32.4) rings. In the heteronuclear multiple-bond correlation (HMBC) spectrum, the presence of a strong correlation of H-2 to C-4 and C-7 confirmed the relative position of the proton in the aromatic ring system. Accordingly, we determined compound **1** to be 3,5-dibromo-4-hydroxyphenylethylamine, which we isolated for the first time as a natural product, but which had been previously used to produce other synthetic intermediates (*24*). Compounds **4** and **6** were previously isolated from a methanol extract of red algae and may also be artifacts of the extraction procedure because we obtained these compounds only via the methanol extraction process (*20*, *25*, *32*). We readily identified the known natural compounds **2**, **3**, and **5** by spectroscopy and by comparing them to published data (*22*, *25*).

Inhibition of ICL Activity. To confirm the existence of glyoxylate cycle enzyme inhibitors, we evaluated the effects of bromophenols on ICL and MLS by comparing the concentrations of compounds that cause an approximate 50% reduction (IC_{50}) in enzyme activity. We tested 3-nitropropionate and bromopyruvate inhibitors of ICL and MLS, respectively, as positive controls (*5*, *8*, *33*). The 3-nitropropionate and bromopyruvate showed specific inhibitory activities against ICL and MLS with IC_{50} values of 92.4 ± 4.6 and 142.8 ± 7.5 μM, respectively (**Table 1**). Bromophenols had no inhibitory effect on MLS activity, but did inhibit ICL activity. Among the compounds tested, the ICL inhibitory activities (IC_{50}) of compounds **2** (IC_{50} = 2.0 ± 0.1 μM), **5** (IC_{50} = 2.8 ± 0.2 μM), and **6** (IC_{50} = 2.1 ± 0.1 μM) were 46, 33, and 44 times stronger, respectively, than that of 3-nitropropionate. Bromophenols are therefore effective inhibitors of *M. grisea* ICL activity. Compounds **2**, **5**, and **6** also showed stronger ICL inhibitory activity than did simple brominated phenols such as compounds **1**, **3**, and **4**. Debromination of compounds **1**−**6** did not display any inhibitory effect upon ICL activity at the highest concentration tested (100 μM) (data not shown). Taken together, these data indicate that the diphenylmethane skeleton and bromine moiety of bromophenols are essential for potent inhibition of ICL activity.

Reduction of Appressorium Formation. To determine whether the inhibition of ICL activity by bromophenols was correlated with reduced appressorium formation in *M. grisea*, we evaluated the effect of bromophenols on conidial germination and appressorium formation in both the Δ*icl* mutant I-10 and the wild-type Guy 11 strain. In a preliminary test, the rates of conidial germination were very similar in both strains 12 h after incubation (>98%). The formation of appressoria, as reported in previous work (*4*), was markedly lower in the I-10 strain [10.2 ± 1.7% (n = 300) of germ tubes within 12 h of germination] than in the Guy 11 strain [99.2 ± 0.4% (n = 300)]. On the basis of these data, we evaluated the inhibitory activity of bromophenols against conidial germination and appressorium formation in *M. grisea* (**Table 1**). In the presence of 20 μM

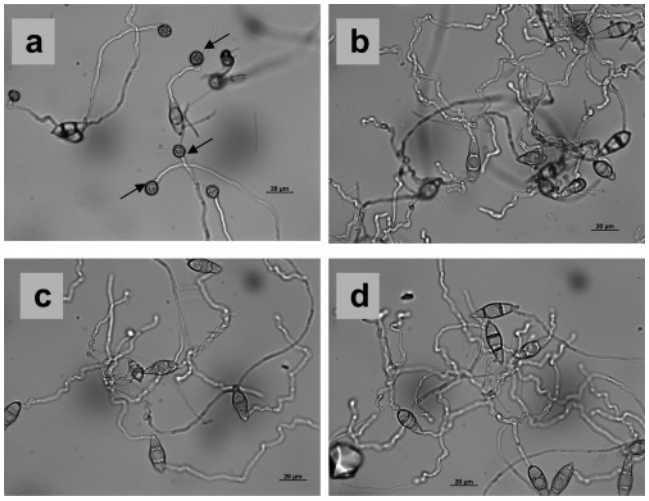

Figure 2. Chemical inhibition of isocitrate lyase blocks appressorium formation by *M. grisea*: growth and appressorium formation by *M. grisea* (**a**) wild-type Guy 11 and (**b**) Δ*icl* mutant I-10 without inhibitor. Wild-type Guy 11 was treated with 20 μM (**c**) compound **2** (11.1 μg/mL) and (**d**) compound **6** (10.3 μg/mL). Conidial suspensions (1.25×10^5 conidia/mL) with different concentrations of test compounds were inoculated into the hydrophobic side of the GelBond film, sealed in a moistened box, and incubated at 25 °C for 12 h. Appressoria are the spherical cells indicated by arrows.

bromophenols, a high level of conidial germination (>95%) was induced by all compounds except compound **2** in both Guy 11 and I-10 on the hydrophobic surface of GelBond sheet after 12 h of incubation at 25 °C. We observed no effects on conidial germination or mycelial growth for either strain of *M. grisea* with ICL inhibitors (**Figure 2**). In contrast, appressorium formation in *M. grisea* Guy 11 was strongly inhibited (>90%) when conidia were germinated in the presence of 20 μM compounds **2**, **5**, and **6**. Compound **2** was also inhibitory, to a lesser extent, for conidial germination of the strain (30% inhibition at 20 μM). Therefore, compound **2** has weak antifungal activity.

Inhibition of C_2 Substrate Use. To determine whether ICL inhibitors affected C_2 substrate use, we cultured *M. grisea* strains in medium containing either glucose or sodium acetate as the sole carbon source. We inoculated *M. grisea* to a minimal growth medium containing variable concentrations of bromophenols and evaluated inhibition based on the minimum inhibitory concentration (MIC). Both wild-type Guy 11 and the Δ*icl* mutant I-10 grew normally on glucose. However, I-10 failed to grow in acetate, whereas Guy 11 grew normally (data not shown; *4*). Among the compounds tested (**Table 2**), compounds **2**, **5**, and **6** showed a weak or no inhibitory effect for Guy 11 grown in glucose, but showed a potent inhibitory effect for Guy 11 grown in acetate. These results indicate that *M. grisea* ICL is involved in the proliferation of the fungus on C_2 substrates.

Protection of Plant Infections. To determine whether the inhibition of appressorium formation by ICL inhibitors was correlated with reduced pathogenicity, we performed infection assays to evaluate the pathogenicity of *M. grisea* strains on the susceptible rice cultivar Nakdong (*31*). Rice plants inoculated with the wild-type strain Guy 11 developed typical diamond-shaped and gray-centered lesions on the leaves, but the Δ*icl* mutant I-10 showed a significant reduction in pathogenicity (**Figure 3**). In contrast, disease protection was evident in rice plants with Guy 11 treated with 16 μM compounds **2** and **6** (**Figure 3**). We calculated disease severity on the basis of

Table 2. Effect of Bromophenols on *M. grisea* Guy 11 Grown in Glucose or Acetate as Sole Carbon Source[a]

	MIC, μM	
compd	glucose	acetate
1	84.6	42.3
2	182.4	5.9
3	167.8	41.9
4	>641.1	160.2
5	>400.8	25.0
6	>389.8	12.1
3-NP	>1680.8	420.2

[a] Conidia (10^5 conidia/mL) were incubated for 7 days at 28 °C in a minimal growth medium containing variable concentrations of test compound, and the MIC was defined as the lowest concentration of compound at which no growth was observable. 3-NP, 3-nitropropionate.

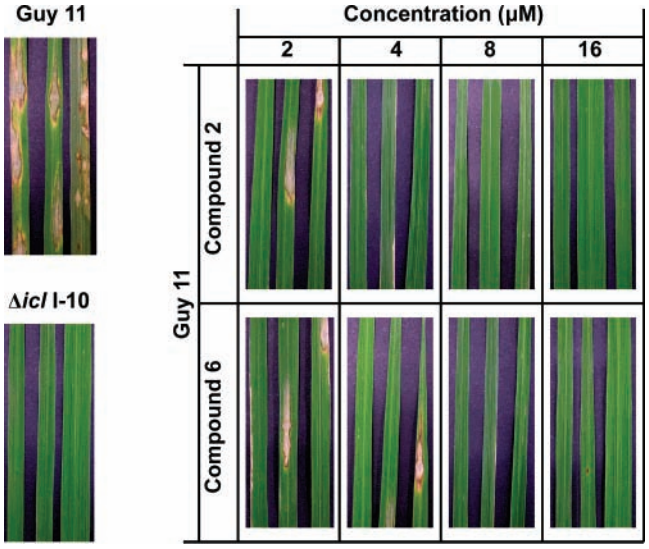

Figure 3. Effect of bromophenols on rice blast disease symptoms produced by *M. grisea*: infection of rice plants by wild-type Guy 11 (untreated), Δ*icl* mutant I-10 (untreated), and wild-type Guy 11 treated with 2, 4, 8, and 16 μM compounds **2** and **6**. Eight-day-old conidia (10^4 conidia/mL) with and without test compounds were spray-inoculated onto the leaves of rice plants (*Oryza sativa* L. cv. Nakdong) in the third and fourth leaf stages. Disease symptoms were assessed 8 days postinoculation.

diseased leaf area (DLA) and lesion type (see Materials and Methods). Eight days after inoculation, the disease severity was evident in Guy 11 (11.19 ± 2.31%; **Figure 4**). A significant reduction in disease severity on individual leaves was evident in Guy 11 treated with bromophenols. The average reduction in disease severity was >99% in Guy 11 treated with 16 μM compounds **2** and **6**. It is important to note that the onset and magnitude of the inhibition of disease severity in Guy 11 treated with compounds **2** and **6** were comparable to those of untreated Δ*icl* I-10 (disease severity = 0.08 ± 0.07%; **Figure 4**).

DISCUSSION

The fungal mutants in glyoxylate cycle enzymes such as ICL (*2*, *4*) and MLS (*6*) show a reduction in pathogenicity. These observations indicate the potential widespread use of the glyoxylate cycle during host infection by diverse pathogenic fungi. As a new target for drug development, the glyoxylate cycle is attractive because it appears to be absent in mammalian cells. However, there have been only a few reports in the literature describing inhibitors of glyoxylate cycle enzymes (*5*,

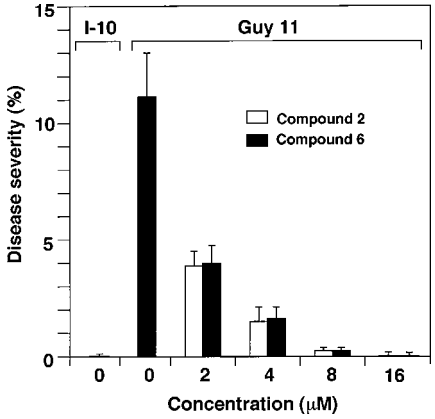

Figure 4. Effect of bromophenols on *M. grisea* pathogenicity. Eight-day-old conidia (10^4 conidia/mL) of wild-type Guy 11 and Δ*icl* mutant I-10 (Δ*icl*) with and without bromophenols (compounds **2** and **6**) were spray-inoculated onto the leaves of rice plants (*Oryza sativa* L. cv. Nakdong) in the third and fourth leaf stages. Disease severity (percent) was measured 8 days postinoculation and calculated using Ordonez's equation, modified as described under Materials and Methods. Data are the mean ± standard deviation of three separate experiments ($n = 30$ diseased leaves in each compound concentration).

8, *33*), due, in part, to the fact that the importance of the glyoxylate cycle as a new target has only recently been acknowledged.

This work shows the potential for using ICL inhibitors to address the pathogenicity of the rice blast fungus *M. grisea*. Bromophenols isolated from *O. corymbifera* (Rhodomelaceae) exhibited potent ICL enzyme inhibitory activity. Among the red algae, members of the Rhodomelaceae are rich sources of bromophenols of several structural types and with various biological activities (*14*, *17*, *18*). The ecological role of brominated phenols is not yet clear. However, they may play a role in chemical defense and deterrence (*34*). Most, but not all, brominated metabolites are palatable to predators and are not effective antibacterial agents (*35*). Some structurally identical bromophenols are used by industry as flame retardants and fungicides (*36*). Appressorium formation by *M. grisea* was strongly inhibited when conidia were germinated in the presence of 20 μM bromophenol, whereas conidial germination and hyphal growth remained unaffected (**Table 1**; **Figure 2**). The high degree of appressorium inhibition, together with the protection observed upon applying bromophenols to rice plants, suggests that ICL plays a critical role in the pathogenicity of *M. grisea*.

The initial stages of plant infection, prior to glucose acquisition from plant tissue, may be when lipid metabolism is particularly important to the proliferation of *M. grisea*. Data from recent studies indicate that the glyoxylate cycle is stimulated during conidial germination and appressorium formation in *M. grisea* (*4*). Of significance is the fact that ICL is also highly expressed at this time. The deletion of the ICL gene in *M. grisea* delays appressorium maturation and turgor generation and may contribute to the reduction and delay in disease symptom expression in response to Δ*icl* mutants. The Δ*icl* mutant I-10 retains the capacity to cause disease because it appears to be unaffected in its ability to invade and proliferate normally in plant tissue (*4*). In the present study, this strain also produced rice blast symptoms and pathogenicity, although the disease severity was very low (**Figure 4**). These results probably reflect the possibility that fatty acid breakdown continues to occur in the Δ*icl* mutants despite a block in the glyoxylate cycle. Recently, the existence of carnitine acetyl transferase in *M. grisea* and its involvement in lipid metabolism and appressorium function have recently been demonstrated (*37*, *38*). The cooperative interaction between the metabolism associated with the peroxisomes and the carnitine-mediated transport is essential for appressorium function during *M. grisea* infection. Wang et al. (*4*) reported that the Δ*icl* I-10 mutant of *M. grisea* showed attenuated pathogenicity but did retain its pathogenicity. In that paper the disrupted mutant showed good lesion comparable to that of wild type after 120 h of inoculation. The data seem quite different in our data showing almost complete loss of virulence as shown in **Figures 3** and **4**. Therefore, in the next step, it is important to investigate whether ICL inhibitors are involved in full virulence loss or partial attenuation.

The strategy for survival during appressorium-mediated infection in a nutrient-free environment entails a metabolic shift in the fungus carbon source to C_2 substrates generated by the β-oxidation of fatty acids (*12*, *13*). Under these conditions, glycolysis decreases and the glyoxylate shunt is significantly up-regulated to enable anaplerotic maintenance of the TCA cycle and assimilation of carbon via gluconeogenesis. As shown in **Table 2**, fungal growth and survival tests indicated that the ICL inhibitors, especially bromophenol compounds **2** and **6**, had weak or no inhibitory effects on *M. grisea* Guy 11 grown in glucose, but were potently inhibitory to Guy 11 grown in acetate. It has also been reported that the Δ*icl* mutant I-10 failed to grow in acetate, whereas the wild-type Guy 11 grew normally (*4*). Taken together, we conclude that *M. grisea* ICL is involved in the proliferation of the fungus on C_2 compounds or after lipid metabolism.

In conclusion, bromophenols have potential as ICL inhibitors that reduce the pathogenicity of the rice blast fungus. The biological evidence indicates that ICL plays a critical role in appressorium formation and plant infection in *M. grisea*. The high ICL inhibitory potency, together with the protection observed upon application of bromophenols to rice plants, suggests that ICL inhibitors may be promising candidates for crop protection, particularly to protect rice plants against *M. grisea*.

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