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Proteasome Inhibitors Affect Appressorium Formation and Pathogenicity of the Rice Blast Fungus, *Magnaporthe oryzae*

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(Received on May 6, 2011; Revised on June 4, 2011; Accepted on June 14, 2011)

Previously, we identified the 20S proteasome α -subunit of *Magnaporthe oryzae* (*M. oryzae*) induced during appressorium formation, and detected an increase in multiple protein ubiquitination during the early appressorium formation process (Kim et al., 2004). In this study, we further attempted to determine whether the proteasome is involved in the appressorium formation of *M. oryzae* both *in vitro* and *in planta*, using proteasome inhibitors. A significant increase in 20S proteasome during fungal germination and appressorium formation was observed using Western blot analysis with 20S proteasome antibody, demonstrating that proteasome-mediated protein degradation was involved in appressorium formation. Pharmacological analysis using proteasome inhibitors, MG-132, proteasome inhibitor I (PI) and proteasome inhibitor II (PII) revealed that germination and appressorium formation were delayed for 4 to 6 h on rice leaf wax-coated plates. Similarly, the treatment of proteasome inhibitors with fungal conidia on the rice leaf surface delayed appressorium formation and host infection processes as well. Additionally, fungal pathogenicity was strongly reduced at 4 days' post-fungal infection. These data indicated that the fungal 20S proteasome might be involved in the pathogenicity of *M. oryzae* by the suppression of germination and appressorium formation.

Keywords : Appressorium formation, *Magnaporthe oryzae*, Pathogenicity, Proteasome inhibitor, 20S proteasome

Rice blast fungus, which is caused by the filamentous fungus *Magnaporthe oryzae*, is the most serious disease in

food-source cultivar rice. *M. oryzae* conidia begin their germination after landing on the hard cell surfaces of the host, given appropriate temperature and humidity conditions. Then, an infection-related structure, the appressorium, differentiates from the germ tube tip within a few hours (Hoch et al., 1987). This dome-shaped cell could accumulate proteins and enzymes, and generate mechanical force to break down the cell wall structure of the host (Howard et al., 1991; Park et al., 2009). This fungal appressorium formation process is the initial stage in host penetration, which is essential for host infection. Previous reports have demonstrated that chemicals or physical signals could interfere with fungal germination and appressorium formation, including cutin monomers, nitrogen or carbon starvation stress, surface hardness, hydrophobicity of the leaf cuticle, and exogenous cAMP (Dickinson et al., 1977; Dick et al., 1979; Gilbert et al., 1996; Jelitto et al., 1994; Lee et al., 1993; Lee et al., 1994; Xiao et al., 1994). cAMP, a signal compound, induces appressorium formation on a non-inductive plate (Lee et al., 1993). To evaluate the molecular mechanisms of fungal appressorium formation, serial analysis of gene expression (SAGE) was carried out to evaluate the formation of *M. oryzae* appressoria triggered by cAMP (Irie et al., 2003). Twenty eight tags were highly induced during the appressorium formation process, including pathogenicity-related genes, subtilisin-like protease, MPG1, and MAS. Later, a proteomics analysis was conducted to identify the differentially-regulated proteins involved in *M. oryzae* appressorium formation (Kim et al., 2004). Changes in protein patterns during rapid progress in germination and appressorium formation were observed under inductive and non-inductive conditions. The major proteins significantly induced in appressorium development were the 20S proteasome family proteins (Kim et al., 2004). The 20S proteasome, a subunit of the 26S proteasome, was initially detected in *Thermoplasma acidophilum* (Dahlmann et al., 1989). The proteasomes are large multi subunit protease complexes, which operate in particular via the ubiquitin-proteasome pathway, and selectively degrade intra-

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cellular proteins in eukaryotic cells (Baumeister et al., 1998). The proteolytic core of this large (2,000 kDa) complex is formed by the 20S proteasome, a barrel-shaped structure shown to be composed of four rings, each containing seven subunits ($\alpha_7\beta_7\beta_7\alpha_7$). The α -subunits make up the two outer rings and the β -subunits comprise the two inner rings of the stack (Lowe et al., 1995). The 26S proteasome is known to perform a critical function in cell cycle progresses in various eukaryotic cells and plants by mediating protein degradations (Kawahara et al., 1992; King et al., 1996; Yanagawa et al., 2002; Kurepa et al., 2009; Skaar et al., 2009). They also involve multiple specific functions including cell signaling, gene regulation, selective elimination of abnormal proteins, programmed cell death, and substrate flux through metabolic pathways (George et al., 1999; Rock et al., 1999). Proteasome catalytic activity may also be influenced by a variety of environmental factors and pathological states (Shah et al., 2001). However, the biological and cellular functions of proteasome in fungal phytopathogens including *M. oryzae* remain poorly understood. During the last several years, importance of massive protein degradation has showed closely relation with fungal infection, especially appressorium formation (Kershaw et al., 2009). Similar results were also described in the infection structure-specific development of other pathogens (Nguyen et al., 2011; Oku et al., 2010; Veneault-Fourrey et al., 2006). However, no information is available of the proteasome mediated protein degradation during fungal and host interaction. In this study, a pharmacological assay was conducted using various proteasome inhibitors to determine whether the 20S proteasome may be involved in appressorium formation and fungal pathogenicity. The delay of fungal germination and appressorium formation was noted in the presence of the proteasome inhibitors MG-132 and PII under both *in vitro* and *in planta* conditions. The pathogenicity of *M. oryzae* was also reduced by those inhibitors. In this study, we suggest for the first time that the 20S proteasome may be involved in appressorium formation and fungal pathogenicity.

Materials and Methods

Proteasome inhibitors. The proteasome inhibitors, MG-132 (benzyloxycarbonyl-leucyl-leucyl-leucinal: Z-LLL-CHO), proteasome Inhibitor I (N-carbobenzoxyl-L- γ -t-butyl lglutamyl-L-alanyl-L-leucinal: Z-Ile-Glu(OtBu)-Ala-Leu-CHO), and proteasome inhibitor II (benzyloxycarbonyl-leucyl-leucyl-phenylalaninal: Z-LLF-CHO) were purchased from Calbiochem (San Diego, CA, USA). These proteasome inhibitors were dissolved in DMSO and small aliquots were stored at -20°C until treatment. Proteasome inhibitors

dissolved in DMSO were applied to the spores at final concentrations of 20 μM or 40 μM . The final concentration of DMSO was 0.5–1% in the *M. oryzae* conidia.

Fungal strains, growth conditions and plant inoculation.

The conidia of the rice blast fungus (race KJ301), which is compatible with rice cv. Jinheung, was used in this study. The conidia were prepared in accordance with the method described by Kim et al. (2004). In brief, the conidia were incubated on rice bran agar medium (25 g of rice bran, 1 g of sucrose and 20 g of agar in 1 liter deionized water, autoclaved) in darkness for 3 days at 28°C , and the aerial mycelia were removed with a sterilized loop and cultured under fluorescent light conditions. Six- or seven-day old conidia were harvested by distilled water containing 0.02% Tween-20, then filtered through Wypall L25 kintowels (Kimberly-Clark, USA) to remove aerial mycelia and cell debris. The conidia were washed twice in distilled water containing 0.02% Tween-20. The conidia were determined using a hemocytometer, and then diluted to $5 \times 10^5/\text{ml}$ with distilled water containing 0.02% Tween-20. The prepared fungal conidia were then spread using a hand sprayer on wax-coated plates and leaves. For inoculation on detached leaves, the conidia were dropped onto the leaf surface.

Rice preparation and treatment. The dehulled seeds of rice (*Oryza sativa* cv. Dongjin) were washed for 5 min with 70% ethanol, followed by a second washing for 30 min in 3% sodium hydrochloride. The seeds were rinsed at least five times with distilled water, followed by 48 h of imbibition in distilled water at 4°C . The imbibed seeds were transferred onto 0.8% phytigel plates and incubated in a light chamber at 28°C . The germinated rice seedlings were transferred into soil mixture, and cultured in a greenhouse. Four- to six-leaf stage rice leaves were used for chemical or fungal treatments. The rice leaves were cut and attached on Grade No. 2 filter paper (Waterman, USA), and placed on moistened glass plates. Droplets of 15 μl of spore suspensions ($1 \times 10^5/\text{ml}$) with 40 μM of inhibitors were applied to inductive plates and the surfaces of detached rice leaves, then incubated for 6 and 8 h at 25°C .

Preparation of rice wax-coated plates. The surface wax of rice leaves was extracted by submersion in organic solvent (petroleum ether:chloroform:benzene = 3:1:0.1) for 30 min. The wax solution was evaporated under a rotary evaporator. Plates in hydrophobic inductive condition were coated with the rice leaf extracts at a $1.2 \mu\text{g}/\text{cm}^2$ wax concentration. The wax coated plates were rinsed with methanol and sterilized water, then air-dried. Plates in hydrophilic non-inductive condition were cleaned up for 1

h using cleaning solution (0.1 M potassium dichromate/0.1 N H₂SO₄ solution), followed by several changes and washings with sterilized distilled water.

Western blot analysis. Total protein (20 µg) was extracted with Mg/NP-40 extraction buffer as previously described (Kim et al., 2004). The total proteins were separated by 12% SDS-PAGE, and then transferred to a PVDF membrane (Millipore, Massachusetts, USA) using a semidry electrophoretic apparatus (Hoefer, Holliston, MA). The blotted membrane was blocked for 4 h at room temperature in 1 × TTBS buffer (50 mM Tris-HCl, pH 8.2, 0.1% v/v Tween 20, and 150 mM NaCl) with 7% (w/v) skimmed milk (BD Bio, USA), and incubated for 2 h after the addition of primary antibody (diluted to 1:10,000). The membranes were sequentially incubated with anti-ubiquitin or anti-20S proteasome α-subunits from *Methanosarcina thermophila* (Calbiochem, CA, USA), then washed 3 times for 15 min each with 1 × TTBS. A secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase diluted 1:10,000 in 1 × TTBS was used for immunodetection. Signals were detected via ECL (Perkin Elmer Life Sciences, Boston, MA).

Southern blot analysis. Same amount of genomic DNA (10 µg) from rice blast fungus was digested with *Eco*RI, *Eco*RV, *Kpn*I and *Xho*I, respectively. Digested DNA was separated by electrophoresis on a 0.8% (w/v) agarose gel, and blotted onto a nylon membrane (Zeta Probe, BIORAD, USA). The blotted membrane was hybridized with [α -³²P]dCTP labeled 20S proteasome full length probe. The hybridized membrane was incubated at 65 °C overnight and washed with 2 × SSC with 0.1% (w/v) SDS at room temperature for 15 min, and three times in 0.2 × SSC with 0.1% (w/v) SDS at 65 °C for 15 min. The membrane was exposed to an X-ray film after 24 h keeping at -80 °C.

Detection of fungal germination and appressorium formation on rice leaves. The infected leaves were fixed with ethanol:acetic acid (2:1), and stained with 1% v/v acid fuchsin-lactophenol solution (20% (v/v) phenol, 20% (v/v) lactic acid, 40% (v/v) glycerine) for 1 min. To observe infected mycelium and callose formation via fluorescence microscopy, the leaves were preserved in 1 N KOH. The KOH-preserved specimens were briefly autoclaved, rinsed with 0.067 M K₂HPO₄ at pH 9.0, and stained with 0.05% aniline blue dye in 0.067 M K₂HPO₄ at pH 9.0. Three biological replicate assays were conducted for proteasome inhibitors and infection foci were analyzed after 3 days of incubation in a humidity chamber.

Results and Discussion

Expression of proteasome in *M. oryzae* during fungal germination and appressorium formation. Proteasome is a conserved protein in all plants, microbes, and animals. Here, we first compared the homology of the deduced amino acid sequences of the 20S proteasome from *M. oryzae* with sequences from other species. Highly conserved domains were detected in all sequences (Fig. 1). The *M. oryzae* 20S proteasome evidenced 58.6%, 58%, 54.7%, and 91.3% homology with mouse, human, *Arabidopsis*, and *Neurospora*, respectively. It was also found to be highly homologous with *Neurospora*, indicating that the *M. oryzae* 20S proteasome is tightly conserved with the fungi kingdom. The copy number of the 20S proteasome in *M. oryzae* was confirmed via Southern blot analysis, resulting in at least two copies of 20S proteasome in the fungal chromosome (Supplementary Fig. 1).

In an effort to determine whether the 20S proteasome is involved in the fungal germination and appressorium

Fig. 1. Sequence conservancy between *M. oryzae* and other species. Amino acid sequences of 20S proteasome from different species were obtained from the National Center for Biotechnology Information (NCBI) on line database (<http://www.ncbi.nlm.nih.gov/>) with the accession number of XM_368091 (*Magnaporthe oryzae*, *M. oryzae*), NP_002782 (Mouse), P21243 (Human), P21243 (Yeast), AAC32054 (*Arabidopsis thaliana*, *A. thaliana*), and EAA28671 (*Neurospora Crassa*, *N. Crassa*). Alignment between the amino acid sequences was carried out using the BioEdit sequencing alignment Editor Program (v7.0.5), and the homology analyses were carried out using Vector NTI 7.0 software.

Fig. 2. Time-course accumulation of 20S proteasome during fungal germination and appressorium formation processes. (A) Western blots were prepared from total proteins obtained from fungal conidia during germination and appressorium formation in samples cultured on hydrophilic surfaces, and blotted with the anti-20S proteasome α -subunit. (B) SDS-PAGE was conducted using Coomassie Brilliant Blue.

formation processes, we analyzed the protein expression level of the 20S proteasome in a time-dependent manner. Western blot analysis was employed to detect the accumulation of 20S proteasome using 20S proteasome monoclonal antibody at 0, 2, 4, 6, 8, 12 h after germination or appressorium formation. Germinated and appressorium-formed conidial protein extracts were separated via SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) (Fig. 2). A significant increase of 20S proteasome was detected via Western blot analysis, revealing that the 20S proteasome may be related closely with the fungal germination and appressorium formation processes (Fig. 2). Double bands with molecular weights of 21 and 24 kDa may indicate different 20S proteasome subunits. Similarly, results were detected in the previous proteomic analysis of *M. oryzae* during appressorium formation. Two subunits of the 20S proteasome, designated $\alpha 4$ and $\alpha 6$, were increased during the appressorium formation process; the results showed that the $\alpha 4$ and $\alpha 6$ subunits evidenced different molecular weights on SDS-PAGE; this result was consistent with our previous results (Kim et al., 2004).

Protein ubiquitination and effect of proteasome inhibitors during appressorium formation. The protein degradation induced by the proteasome was mediated by the multiple ubiquitination of ubiquitin proteins (Clague et al., 2010). Therefore, we evaluated the protein ubiquitination occurring during appressorium formation using ubiquitin-specific antibody. An increase of multiple bands with different molecular weights was detected, suggesting a rapid protein ubiquitination was occurred during appressorium formation. Even more, some detected bands showed mono-/bi-ubiquitination rather than poly-ubiquitination, implying that

Fig. 3. Detection of protein ubiquitination during appressorium formation. The same amount of total proteins was extracted from *M. oryzae* at 0, 2, 4, 6, 8 and 12 hr after appressorium formation, and separated on 12.5% SDS-PAGE. Arrows indicated the ubiquitinated protein patterns.

the protein modification by ubiquitination may be as important as proteasome pathway mediated degradation during fungal appressorium formation (Fig. 3).

MG-132, proteasome inhibitor I (PI) and proteasome inhibitor II (PII) have been shown to be effective proteasome inhibitors in both fungi and mammals (Genin et al., 2010; Nandi et al., 2006). The proteasome inhibition activity during *M. oryzae* germination and appressorium formation was first confirmed. The *M. oryzae* spore suspensions treated with 40 μ M inhibitors were incubated on the hydrophobic surfaces of glass plates coated by rice leaf wax. The fungal morphologies were observed at 6 h and 8 h after incubation. Without inhibitor treatment, *M. oryzae* spores form the germ tube and the appressorium begins to differentiate at 6 h, and mature appressoria were detected at 8 hr (Fig. 4A). The application of the proteasome inhibitors MG-312, PI, and PII delayed the appressorium formation of *M. oryzae* on the wax-coated plates. Only the germ tube was detected at 6 h after treatment with those inhibitors, but no appressorium formation was observed to begin (Fig. 4A). At 8 h, mature appressorium was detected in the PI-treated fungus, but treatment with MG-132 and PII still suppressed appressorium formation (Fig. 4A). We further evaluated the inhibitory effects of MG-132 and PII at concentrations of 20 μ M and 40 μ M on conidial germination and appressorium formation (Fig. 4B, C). Without inhibitors, 60% of spores were germinated at 2 h, whereas more than 90% were germinated at 4 hr (Fig. 4B). After treatment with 20 μ M or 40 μ M MG-132, half the numbers of fungus were germinated at 4 h, which represented a 2 h delay compared with the non-treated samples. After treatment with 20 μ M and 40 μ M proteasome inhibitor PII, the 50% germination rate was delayed by 5 h and 6 h, respectively

Fig. 4. The effect of proteasome inhibitors on *M. oryzae* spore germination and appressorium formation on rice leaf surface wax-coated glass plates. (A) Micrographs of conidia after 6 h and 8 h of incubation with 40 μ M proteasome inhibitors MG-132, PI, and PII. AP, appressorium; G, germ tube; S, spore. (B) Average rates of conidial germination and (C) appressorium formation in control (DMSO) and proteasome inhibitor treatment. At least 100 spores were counted per replicate. Data are the mean of three replications. MG-20, 20 μ M MG-132; MG-40, 40 μ M MG-132; PII-20, 20 μ M PII; PII-40, 40 μ M PII. Approximately 200 spores of each replicate were scored for germination or appressorium formation.

(Fig. 4B). The effects of MG-132 and PII on appressorium formation were then confirmed herein (Fig. 4C). No significant differences were detected with MG-132 treatment at concentrations of 20 μ M and 40 μ M until 12 hr. Half of the appressorium formation was detected at 8 h with MG-132 treatment. The proteasome inhibitor PII suppressed 70% of fungal appressorium formation at 8 h at a concentration of 20 μ M, whereas 40 μ M of PII effected an 85% suppression of appressorium formation (Fig. 4C). These results showed that PII has the strongest inhibition activity, and the inhibition activity occurs in a dosage-dependent fashion. MG-132 at 20 μ M exhibits efficient inhibition activity against germination and appressorium formation. The pro-

teasome inhibitor PII strongly suppressed appressorium formation. This inhibitor is unique, in its properties shared with the chymotrysin-like proteases, among the serine proteases in its ability to cleave proteins next to hydrophobic side chains (Marian et al., 1997). Among these treatments, serine protease inhibitors such as aprotinin, AEBSF, and PMSF exerted inhibitory effects on appressorium formation as well (data not shown). However, aspartic proteinase, pepstatin A and the cysteine protease inhibitor, leupeptin did not show evidence inhibitory effects against appressorium formation. This result indicates that specific proteolytic processes of serine protease may perform an important function in appressorium formation.

Pharmacological assay of appressorium formation on rice leaves. To evaluate the effects of those proteasome inhibitors on fungal infection *in planta*, we inoculated the *M. oryzae* spore on detached rice leaves supplemented with these proteasome inhibitors. Lactophenol staining demonstrated that *M. oryzae* conidia germinated on leaves treated with DMSO and all inhibitors at 6 h post-inoculation, but mature appressoria appeared only in the control leaves (Fig. 5A). Treatment with PI inhibitor suppressed appressorium formations for 6 h, and MG132 and PII delayed appressorium formation for more than 8 h (Fig. 5A). The fungal infections were detected at 48 h post-inoculation with droplets of a *M. oryzae* conidial suspension of 1×10^5 /ml. The application of DMSO had no effect, and PI exerts a limited effect on fungal infection. However, the proteasome inhibitors, MG-132 and PII, strongly suppressed fungal

Fig. 5. *In planta* pathogenicity analysis of *M. oryzae* treated with proteasome inhibitors. (A) Fungal appressorium formation detected by lactophenol staining at 6 hr and 8 hr after inoculation. (B) Effects of proteasome inhibitors on the suppression of fungal infections at 48 h after inoculation. (–), negative control, leaf without chemical treatment; (+), leaf treated with indicated chemicals. (C, D) Aniline blue fluorescent staining of appressorium and hyphae growing inside leaf cells at 48 h (top). A close-up view of fungal infection patterns (bottom).

infection on the detached leaves (Fig. 5B). Less and none spread hyphae were detected after treatment with MG-132 and PII, respectively (Fig. 5B). It has been reported that callose deposition, which was detected by aniline blue and is induced by wounding stress or pathogen infection, was detected among the fungal infection patterns (Yim et al., 1998). The invading hyphae with serious branch spread were detected in infected leaves at 48 h with DMSO or PI treatment (Fig. 5C, D). The minimal spreading area and limited infection were demonstrated along with treatment of the MG-132 and PII inhibitors, respectively (Fig. 5D). Collectively, our results show that the proteasome inhibitors MG-132 and PII could reduce the fungal pathogenicity, demonstrating that the proteasome may be associated with fungal pathogenicity.

Proteasome inhibitor PII suppresses fungal pathogenicity *in planta*. The proteasome inhibitor PII exerted a profound suppressive effect against appressorium formation in *M. oryzae* on inductive glass plates and detached rice leaves. Therefore, we speculated that PII may suppress fungal pathogenicity *in planta*. Three biological replicate assays were conducted and infection foci were analyzed after 4 days of incubation in a humidity chamber. Without PII treatment, the leaves were heavily infected by *M. oryzae* (Fig. 6A). After spreading with 40 μ M PII and fungal spores, fewer infected foci appeared on the leaves after 4 days (Fig. 6A). PII suppressed 70% of fungal-infected foci relative to the non-treated tissues. An average of 35 infected foci were detected on control leaves, and less than 10 infected foci were detected on the PII-treated tissues (Fig. 4B). The expression level of the rice defense marker gene, PBZ1, was confirmed after treatment with proteasome inhibitor. No significant difference was observed by Northern blot analysis, suggesting that those inhibitors have limited effect on the host side (data not shown). These results imply that the biochemical PII may have some

ability to suppress *M. oryzae* pathogenicity via the inhibition of proteasome activity, but not by the activation plant immunity responses.

Conclusions

In this study, we determined that the treatment of proteasome inhibitors have the ability to suppress conidial germination, appressorium formation, and fungal pathogenicity under both *in vitro* and *in planta* conditions. This inhibition may be caused as the result of a defect in appressorium-mediated penetration, and may lead to the reduction or delay of fungal penetration. Ubiquitin genes are associated frequently with protein turnover via the ubiquitin proteasome pathway. *M. oryzae* is likely to be very metabolically active during the infection process, and thus these genes might play important roles; multiple genes are known to participate in appressorium formation and pathogenesis. The proteasome inhibitor PII exhibiting serine protease evidenced the strongest inhibition ability, and reduced the disease symptoms of *M. oryzae* on rice leaves (Fig. 5, 6). Overall, this study is, to the best of our knowledge, the first to demonstrate that the 20S proteasome may prove essential for appressorium formation and infectious growth in *M. oryzae*, and may help in the search for novel targets for fungal pathogenicity. However, the specific roles of the 20S proteasome in the appressorium formation of *M. oryzae* remain to be clearly elucidated.

Acknowledgments

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0005370), by a grant from the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center, PJ008021), Rural Development Administration, Republic of Korea. Y. Wang and J. Wu were Supported by Scholarships from the BK21 Program.

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Fig. 6. Protection of rice leaves against *M. oryzae* by proteasome inhibitor II at 4 days after inoculation. (A) Rice leaf blast symptoms on control and PII-treated leaves were observed. (B) Average infection foci numbers on control and PII-treated leaves. All experiments were determined in at least three replications.

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