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Identification and Molecular Characterization of Methionine Sulfoxide Reductase B Gene in Rice Blast Fungus, *Magnaporthe oryzae*

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Magnaporthe oryzae, a major cause of rice blast, is one of the most destructive plant fungal pathogens. Secretion of reactive oxygen species (ROS) during the infection phase of plant pathogenic fungus plays a key role in the defense mechanism of a plant. ROS causes oxidative damage and functional modification to the proteins in a pathogenic fungus. Methionine, especially, is a major target of ROS, which oxidizes it to methionine sulfoxide. To survive from the attack of ROS, plant pathogenic fungus has antioxidative systems - one example would be methionine sulfoxide reductase B (*MSRB*), which reverses the oxidative alteration of methionine to methionine sulfoxide. In the present study, identification and molecular characterization of the *MSRB* gene in *M. oryzae* KJ201 were investigated. The *MSRB* gene was amplified by PCR from the *M. oryzae* KJ201 genomic DNA. The copy number of *MSRB* in the genome of *M. oryzae* KJ201 was identified by Southern blot analysis, which revealed that the gene exists as a single copy. To study the molecular function of an *MSRB* gene, the expression level of the *MSRB* gene was assayed with hydrogen peroxide treatment by Northern blot analysis and RT-PCR. The expression of the *MSRB* gene was increased by treatment of hydrogen peroxide, without significant correlation to hydrogen peroxide concentrations. These results indicate that the *MSRB* gene in *M. oryzae* KJ201 could contribute to protection against plant defense compounds such as ROS and offer a novel strategy for the control of rice blast.

Key words : *Magnaporthe oryzae*, methionine sulfoxide reductase B, hydorogen peroxide.

Introduction

A very large number of potential plant pathogens exist in nature. However, individual plant species are susceptible to only a limited number of pathogens because of the presence of effective general defense mechanisms. Secretion of reactive oxygen species (ROS) during infection of plant pathogenic fungus plays a key role in defense mechanism of plant [10,14]. ROS induce oxidative damage and functional modification to proteins of pathogenic fungus. Especially, methionine is one of the major targets of ROS, where it is oxidized to methionine sulfoxide. To survive from the attack of ROS, plant pathogenic fungus has antioxidative system such as methionine sulfoxide reductase B (*MSRB*) [6,8,17,21]. *MSRB* can repair specifically oxidative alteration of methionine to methionine sulfoxide [1,2,5, 11,12]. There is not much information on functional characterization of *MSRB* in plant pathogenic fungi. Functional analysis of the *MSRB* is required to understand the biology of fungal plant pathogens in the plant-pathogen interaction and provide new control strategy.

Rice blast, caused by *Magnaporthe oryzae*, is one of the most destructive diseases in cultivated rice [15], which feeds one-half of the world's population [7,18]. The disease is capable of destroying enough rice to feed 60 million people every year [22]. *M. oryzae* has evolved the ability to penetrate and subsequently colonize its host plants [3]. Due to its genetic and molecular tractability, this fungus has served as an important model organism in the studies aimed at understanding the biology of fungal plant pathogens [19,20]. Over the past decades, significant efforts have been made to elucidate the molecular controls of rice blast. These efforts include the breeding of resistant cultivars and the use of fungicides. However, the effectiveness of these methods is limited because of the frequent emergence of *M. oryzae* isolates that are able to overcome resistant varieties or fungicides or because of the environmental and safety concerns on the use of fungicides.

The entire *M. oryzae* genome has been sequenced [4], and functional genomics approaches have led to the identification of hundreds of genes involved in its pathogenesis [9]. A number of *M. oryzae* genes that are responsible for the pathogenicity have been identified based on their ex-

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pression patterns or homologies to known or suspected pathogenicity genes in other organisms [4]. Especially, it is speculated that *MSRB* gene in the *M. oryzae* could contribute to protection against plant defense compounds such as ROS.

In this study, identification and molecular characterization of *MSRB* gene in the *M. oryzae* KJ201 were investigated. *MSRB* gene was amplified by PCR from the *M. oryzae* KJ201 genomic DNA. The copy number of *MSRB* in the genome of *M. oryzae* KJ201 was identified by Southern blot analysis. To study molecular function of *MSRB* gene, expression level of *MSRB* gene was assayed under the condition of different hydrogen peroxide concentrations and exposure times by Northern blot analysis and RT-PCR. The result shows that *MSRB* gene in the *M. oryzae* KJ201 was likely involved in fungal pathogenesis along with the function in anti-oxidation.

Materials and Methods

Fungal strain and culture condition

Magnaporthe oryzae KJ201 strain derived from the Fungal Plant Pathology Lab (FPPL) in Seoul National University. KJ-201 strain was maintained on V8 Juice agar media (4% V8 juice and 1.5% agar, pH 7.0) with constant fluorescent lighting at 25±1°C for conidiation. Mycelia for DNA isolation were prepared by culturing in CM liquid media (7.5 g of yeast, 7.5 g of casein acid hydrolysate, and 10 g of sucrose per liter) with shaking at 25°C in the dark for 3 or 4 days.

Genomic DNA isolation, polymerase chain reaction (PCR), and Southern blot analysis

Genomic DNA of the *M. oryzae* KJ201 was prepared by using a modification of the rapid isolation procedure developed by M. G. Murray, M. G., and W. F. Thomson [13]. Two degenerate primers were designed on the highly conserved genome sequences of the *M. oryzae* strain 70-15, and PCR was performed with the *M. oryzae* KJ201 genomic DNA. The primers are as follows: *MSRB*-F and *MSRB*-R (Table 1). Each primer was used alone or in combinations of forward and reverse pairs. PCR was performed with Taq polymerase (Takara-Korea Biomedical, Inc., Seoul, Korea) using the following cycling parameters: 3 min at 94°C, followed by 30 cycles (1 min at 94°C, 30 sec min at 55°C, and 1 min at 72°C), with a final extension of 72°C for 10 min. The PCR product was gel isolated using a Gel Extraction Kit (General Bio System).

Table1. Primers used for PCR amplification of *MSRB* gene in the genome of *M. oryzae* KJ201

	Primer names	Sequences
Probe	*MSRB*-F	5′-AAG AGC AAT TCC GCA TCT TG-3′
	MSRB-R	5′-ATC CTC GGA CGA AAA CTT GA-3′
RT-PCR	*MSRB*RT-F	5′-AAG AGC AAT TCC GCA TCT TG-3′
	*MSRB*RT-R	5′-ATC CTC GGA CGA AAA CTT GA-3′
	β-tubulin-F	5′-TCA CTG TTC CCG AGT TGA CC- 3′
	β-tubulin-R	5′-GGC CTC AGT GAA CTC CAT CT-3′

The copy number of *MSRB* in the genome of *M. oryzae* KJ201 was identified by Southern blot analysis. DNA was digested with restriction enzymes (*Bam*HⅠ, *Hin*dⅢ, *Pst*Ⅰ, and *Sal*Ⅰ) at 37°C for 5 hr, separated in 0.7% agarose gel and transferred to Hybond N^+ membrane (Amersham Bioscience) according to the standard methods [16] including 0.2 N HCl, Denaturation buffer (1.5 M NaCl, 0.5 M NaOH, Neutralization buffer (1 M Tris-HCl, 1.5 M NaCl), and 2X SSC (0.3 M NaCl, 0.03 M Sodium Citrate, adjust pH 7.0 with NaOH). Hybridization was done at 65°C in Amersham Rapid-hyb buffer (GE Healthcare, UK). The probes were made from PCR product gel extracts radiolabeled with [α-^{32}P]dCTP using a Random Prime Labelling System (Amersham, Rediprime Ⅱ, GE Healthcare, UK). Washings were performed twice at 65°C in wash1 buffer (2X SSC, 0.5% SDS.) and wash2 buffer (0.5X SSC, 0.1% SDS) in series.

RNA isolation, Northern blot analysis, and Reverse transcription-polymerase chain reaction (RT-PCR)

Expression of *MSRB* gene in *M. oryzae* KJ201 was identified by Northern blot analysis and RT-PCR. For mRNA induction experiments, pre-cultures were grown in 200 ml of CM medium at 25°C and 100 rpm for 3 days. Various concentrations (0, 1, 10, and 100 ppm) of hydrogen peroxide were added and mycelium was harvested on gauze filters and washed with sterile water. Total RNA was isolated by the ilustra RNAspin mini RNA lsolation kit (GE Healthcare).

For Northern blot analysis, equal amounts of total RNA (20 μg) were separated by a 1% agarose-formaldehyde gel electrophoresis, transferred by capillary blotting to a Hybond N+ charged nylon membrane(Amersham International plc., England), and fixed to the membrane by UV cross-linking. For hybridization, *MSRB* PCR product probe (1 μg) was ^{32}P-labeled using DNA-labelling beads and purified through a Sephadex column. For all northern blotting, the *MSRB* PCR product was used as the probe. Probes were denatured be-

fore bring added to the hybridization solution. Pre-hybridization (3 hr) and hybridization (15 hr) were carried out at 45°C in denatured sperm DNA. Following hybridization, membranes were washed two times at 45°C in wash1 buffer (2X SSC, 0.1% SDS) and wash2 buffer (0.5X SSC, 0.1% SDS) and then exposed to X-ray film at -20°C for 5 days.

For RT-PCR, total RNA was isolated. Concentration of RNA was determined with spectrophotometer and concentration and integrity were further checked by agarose gel electrophoresis. Residual DNA, if any, was eliminated from the samples by using DNase I (RNase-free, Takara Bio Inc). cDNA was synthesized from the RNA using the PrimeScript™ Reverse transcriptase (Takara Bio Inc) using oligo dT primer as a primer at 42°C for 60 min. And then, PCR is performed with this single cDNA synthetic as a template. The primers were as follows: *MSRB*RT-F and *MSRB*RT-R (Table 1). The amplification reaction conditions were as follows: initial denaturation at 95°C for 6 min; 30 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min 30 sec, and a final extension at 70°C for 10 min. PCR products were separated on 1% agarose gels and stained with ethidium bromide.

Results and Discussion

Identification of *MSRB* gene in the genome of the *M. oryzae* KJ201

M. oryzae, the causal agent of rice blast, is one of the most destructive fungal pathogens throughout the world [3,7,15,18]. Plant pathogenic fungus may act as virulence factors if they provide protection against plant defense compounds during disease development [10,14]. ROS is one of the powerful plant defense compounds, which causes oxidative damage and functional modification to proteins of pathogenic fungus. Among the amino acids, methionine is the most susceptible to oxidation by almost all forms of ROS. This malformation can be repaired specifically by *MSRB* [8,17]. *M. oryzae* is the first plant pathogenic fungus to be fully sequenced, and functional genomic approaches have led to the identification of hundreds of genes involved in its pathogenesis [4,9]. However, there is not much information on functional characterization of the *M. oryzae* genes, especially concerning *MSRB*. In this study, we hypothesized that *MSRB* gene in the *M. oryzae* could contribute to protection against plant defense compounds such as ROS and offer novel strategy for the control of rice blast.

To confirm molecular characterization of *MSRB* gene of the *M. oryzae* KJ201, which is the Korean field strain, *MSRB* gene was amplified by PCR analysis. *MSRB* gene of *M. oryzae* was annotated from the *M. oryzae* genome sequence from strain 70-15, which is the international standard laboratory strain, using bioinformatics tools. Primers were designed from the genome sequences of the *M. oryzae* strain 70-15, and PCR was performed with the *M. oryzae* strain KJ201 genomic DNA. As shown in Fig. 1, the 350 bp fragment from *MSRB* gene in the *M. oryzae* strain KJ201 was identified by PCR. The amplified fragment was sequenced to confirm the originality. Amplified *MSRB* gene fragment was used as a probe for Southern and Northern blot analysis.

Southern blot analysis of *MSRB*

The copy number of *MSRB* in the genome of *M. oryzae* KJ201 was identified by Southern blot analysis. The 350 bp fragment of *MSRB* gene was used as a probe in Southern blot analysis of genomic DNA digested with restriction enzymes, *Bam*H Ⅰ, *Hin*dⅢ, *Pst* Ⅰ, and *Sal* Ⅰ. As shown in Fig. 2, single band was detected in most digested DNA. This result indicates that the *M. oryzae MSRB* gene is presenting in a single copy in the genome. In the lane of *Pst* I digestion, two bands showed up since the probe fragment has restriction site for the enzyme.

Expression of *MSRB* gene after treatment with hydrogen peroxide

Secretion of ROS during infection of plant pathogenic

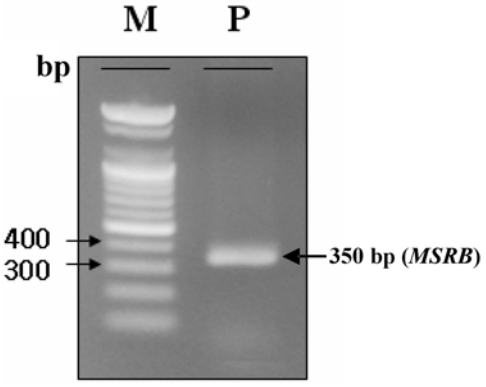

Fig. 1. Amplified *MSRB* gene fragment from the genome of *M. oryzae* KJ201 by PCR analysis. The 350 bp fragment of *MSRB* gene from the KJ201 strain was identified in the lane P by PCR analysis. M, molecular marker of 100 bp ladder; P: PCR product of *MSRB* gene.

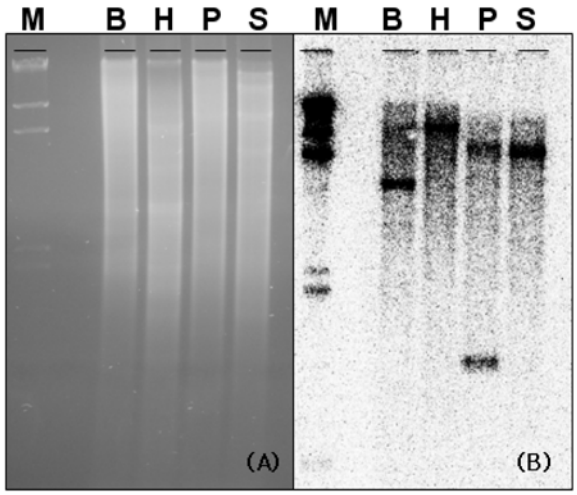

Fig. 2. The copy number of *MSRB* in the genome of *M. oryzae* KJ201. The copy number of *MSRB* in the genome of *M. oryzae* KJ201 was identified by Southern blot analysis. Genomic DNA from the KJ201 was digested with *Bam*H Ⅰ, *Hin*dⅢ, *Pst* Ⅰ, and *Sal* Ⅰ. (A) DNA agarose Gel and (B) X-ray film image. M, molecular marker of lambda DNA digested with *Hin*dIII; B, *Bam*HI; H, *Hin*dⅢ; P, *Pst* Ⅰ; S, *Sal* Ⅰ.

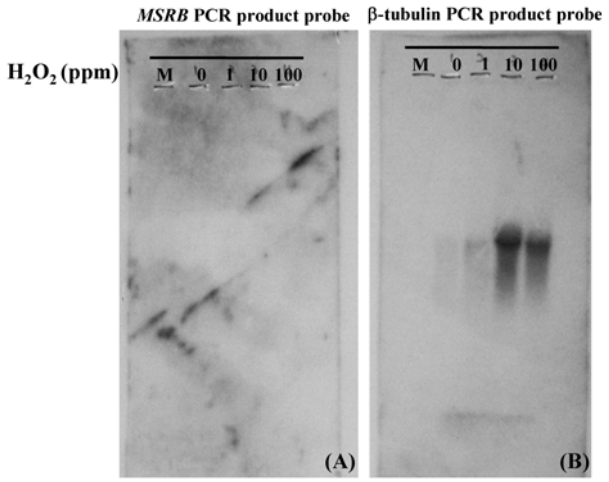

Fig. 3. Northern blot analysis of *MSRB* expression. Expression of *MSRB*(A) and β-tubulin (B, positive control) was shown. PCR product probes were used. The concentration (ppm) of hydrgen peroxide is indicated for each lane.

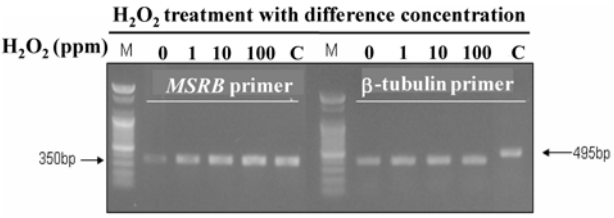

Fig. 4. RT-PCR analysis on the expression of *MSRB*. Expression of *MSRB* and β-tubulin under the condition of different hydrogen peroxide concentrations (0, 1, 10, and 100 ppm). The right lanes (C, DNA control) of each gene show the PCR product from genomic DNA, which confirm the RNA quality of no DNA contamination for RT-PCR. M, 100 bp ladder marker.

fungus plays a key role in defense mechanism of plant. Plant ROS causes oxidative damage and functional modification to proteins of pathogenic fungus. In this study, expression of *MSRB* was investigated using hydrogen peroxide. Hydrogen peroxide is used as a representative of the plant defense compounds such as ROS, which effectively induces oxidative stress in most of organisms. After treatment of various hydrogen peroxide concentrations (0, 1, 10, and 100 ppm) for 2 hr, Northern blot analysis showed no signal for the *MSRB* expression. On the other hand, β-tubulin expression, the positive control, showed significant signal (Fig. 3).

To identify molecular function of *MSRB* gene in the *M. oryzae*, expression level of *MSRB* gene was estimated under the condition of different hydrogen peroxide concentrations (0, 1, 10, and 100 ppm) by RT-PCR. The β-tubulin expression in *M. oryzae* was designed for positive control. In some cases, RT-PCR has a problem with genomic DNA contamination during isolation of total RNA. Our RT-PCR result of β-tubulin clearly showed single band amplified without genomic DNA contamination. As shown in Fig. 4, *MSRB* and β-tubulin amplification with genomic DNA as templates resulted fragments at 350 bp and 495 bp, respectively. The expression of *MSRB* gene was induced by treatment of hydrogen peroxide, not showing a significant correlation with hydrogen peroxide concentrations. These results indicate that *MSRB* gene in the *M. oryzae* KJ201 could contribute to protection against plant defense compounds such as ROS and offer novel strategy for the control of rice blast.

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초록 : 벼도열병균에서의 methionine sulfoxide reductase B 유전자의 분자적 특성

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벼도열병균은 벼의 주요 병해인 벼도열병의 원인균이다. 식물병원균의 침입 시 식물체로부터 발생하는 ROS는 식물의 방어기작으로 중요하며, 특히 아미노산의 하나인 methionine은 ROS에 의해 산화되어 methionine sulfoxide로 변화될 수 있다. 식물병원균은 식물체로 부터의 ROS에 의한 산화반응을 회피하기 위해 methionine sulfoxide reductase B (MSRB)와 같은 항산화 효소를 가지는데 본 연구에서는 벼도열병균에서의 MSRB 유전자를 동정하고 분자적 특성을 살펴보았다. MSRB 유전자는 벼도열병균의 게놈 상에 단일 유전자로 존재하며 과산화수소 처리에 의해 유전자발현이 다소 증가하는 경향을 보였다. 이러한 결과로 MSRB 유전자는 벼도열병균의 항산화 기작에 관여할 가능성이 높다고 판단된다.