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The Panicle Blast Resistance Mechanism of *qPbm11* in the Rice Cultivar Miyazaki-mochi is Independent from that of *Pb1*

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Abstract

Understanding the mechanism of disease resistance is critical for combating rice blast disease that causes the most deleterious effects on rice yield, and for evolving successful blast tolerance breeding programs using blast resistance (R) genes. The *qPbm11* locus in the blast resistant cultivar Miyazaki-mochi exerts panicle resistance similar to the well-known quantitative panicle resistance gene *Pb1*. *qPbm11* and *Pb1* show similarity in panicle blast resistance, quantitative resistance, and proximity of loci on the rice genome. *Pb1* resistance is dependent on the expression of transcription factor *WRKY45*. To identify the resistance mechanism of the *qPbm11* locus, we downregulated *WRKY45* in the cultivar Miyazaki-mochi through an *Agrobacterium*-mediated transformation and surveyed its panicle resistance. *WRKY45 RNAi* transgenic Miyazaki-mochi plants exhibited similar levels of panicle resistance compared to WT, indicating that the *qPbm11* locus is independent of the *Pb1* resistance pathway. Our results suggested that cloning the genes responsible for *qPbm11*-mediated resistance along with *Pb1* using pyramiding technology could enhance panicle resistance in rice.

Discipline: Agricultural Environment
Additional key words: *Oryza sativa*, *Pyricularia oryzae*, panicle blast, QTL, salicylic acid, resistance gene

Introduction

Rice blast caused by pathogenic fungi is one of the many deadly diseases affecting rice plants worldwide. Developing rice cultivars using resistance-affording R genes is a technique widely used in rice breeding programs to combat the disease. In the last two decades, several molecular approaches have been developed for investigating rice blast resistance genes, resulting in the isolation of blast *R* genes in the rice genome.

Resistance conferred by quantitative trait loci (QTL)-mediated genes is more durable for rice blast, but is less understood (Fukuoka et al. 2015). Several quantitative leaf blast resistance genes, including *pi21*, *Pi34*, *Pi35*, *Pi39,* and *Pi63*, were recently investigated (Fukuoka et al. 2009, Nguyen et al. 2006, Terashima et al. 2008, Xu et al. 2014 and Zenbayashi-Sawata et al. 2007). The panicle blast resistance gene *Pb1* confers partial resistance to rice blast with no fungal race specificity (Hayashi et al. 2010). The cultivars carrying *Pb1* have remained resistant to panicle blast for over 35 years. The cloning of *Pb1* indicated a weak resistance in the early developmental stages followed by stronger resistance at the reproductive stage of the plant, varying with the increase in *Pb1* expression (Hayashi et al. 2010). After the heading stage, the *Pb1* cultivar shows very strong resistance to rice blast. The mechanism of *Pb1* resistance is attributed to its interaction with the WRKY45 protein (Inoue et al. 2013), which plays a crucial role in the salicylic acid (SA) pathway in rice immunity (Shimono et al. 2007). The WRKY45 protein binds to the Pb1 coiled-coil domain upon localization to the nucleus, resulting in a Pb1-WKRY45 complex that is more stable than WRKY45 against protein degradation (Inoue et al. 2013). WRKY45 is degraded by a ubiquitin-proteasome system in the nucleus (Matsushita et al. 2012). Transgenic rice plants overexpressing WRKY45 manifested strong resistance against both leaf blast and panicle blast (Shimono et al. 2012). The mechanism of resistance through WRKY45 expression was unraveled using the cultivar Kanto209, which has weaker resistance compared with other *Pb1* cultivars. QTL analysis and physiological investigation identified that cultivar Kanto209 was defective in SA

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pathway and unable to induce WRKY45 expression against rice blast (Inoue et al. 2017).

Miyazaki-mochi is bred at the Miyazaki Agricultural Research Institute and is known as a rice panicle blast resistance cultivar (Uchiyamada et al. 1979). The *qPbm11* locus in rice blast resistant cultivar Miyazaki-mochi displays similarity to the *Pb1* resistance gene in two key respects. First, the *qPbm11* locus is involved in panicle resistance against rice blast just like the *Pb1* gene, which has been shown to control panicle resistance with increased expression during the course of rice plant development (Hayashi et al. 2010). Secondly, the *qPbm11* locus has been placed between markers RM 26890 and RM 27207 of chromosome 11 of the rice genome (Ishihara et al. 2014), and the *Pb1* gene also exists between these markers. Yet, the Miyazaki-mochi QTL cannot be strictly differentiated from the *Pb1* gene. Despite the proximity of the *qPbm11* locus to the *Pb1* gene, the expression of *Pb1* is not detected using RT-PCR analysis in the cultivar Miyazaki-mochi (Ishihara et al. 2014). Understanding whether the same resistance mechanism as the *Pb1* gene exists in the *qPbm11* locus can be useful for disease resistance breeding. Although the *qPbm11* locus differs in sequence from the *Pb1* gene, panicle resistance may be imparted by utilizing the same resistance mechanism. Therefore, we decided to compare disease resistance imparted by the *qPbm11* locus and the *Pb1* gene by creating a knockdown of the *WRKY45* gene in a variety possessing the *qPbm11* locus.

Materials and methods

1. Salicylic acid treatment

The 21-day-old leaves of plants were cut into 1-cm fragments, which were then submerged in mock (H_2O) and 1mM SA solution including 0.01% Silwet L-77. After five hours of incubation, the plants were wiped with a paper towel and then kept in liquid nitrogen until RNA extraction.

2. RNA analysis

Total RNA was isolated from rice tissue using Trizol (Invitrogen, USA). For quantitative RT-PCR, total RNA was treated with DNA remover to remove contaminating genomic DNA. cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo, Japan). To determine *OsWRKY45* expression, quantitative RT-PCRs were run on a Thermal Cycler Dice TP800 system (Takara Bio, Japan) as shown previously (Hayashi et al. 2010) using primers WRKY45 Fw; 5′-CGGGTAAAACGATCGAAAGA-3′ and WRKY45 Rv; 5′-TTTCGAAAGCGGAAGAACAG-3′. *Rice ubiquitin1* (Rubq1; AK121590) was used as an internal standard.

3. Generation of rice transformants

A *WRKY45*-kd construct (Shimono et al. 2007) was introduced into rice cultivar Miyazaki-mochi by *Agrobacterium tumefaciens* (strain EHA101)-mediated transformation (Hashizume et al. 1999).

4. Evaluation of panicle blast resistance

Forty young seedlings were transplanted in a circular pattern in 3.5-L plastic pots (20 plants per pot) (Satake 1972) and were grown in the glasshouse room of the NIAS and used for inoculation within 10 days from the day when the neck of the panicle emerged. Spores of Ina86-137 (Race 007.0), a blast fungus pathogenic to Miyazaki-mochi and Bikei 22, were suspended in 0.01% Tween 20 and sprayed with 15 ml per pot at a concentration of 8 to 10 x 10^4 spores/ml, with the plants being kept in a dew chamber for 20 h at 24.5°C and grown in a glasshouse. The proportions of diseased grains per panicle were examined about three to four weeks after inoculation (Hayashi et al. 2010). Cultivar Bikei 22, which was very weak to panicle blast, was used as a susceptibility check of the blast fungus inoculation test.

5. Statistical analysis

Panicle blast resistance was analyzed by using Excel add-in software (http://www.oms-publ.co.jp/4steps04/index.html). Statistical analysis using the Tukey-Kramer test was conducted for real-time quantitative PCR. A steel test was used to analyze significant differences between Miyazaki-mochi and Miyazaki-mochi *WRKY45* RNAi transformants.

Results and discussion

The activation mechanism of R genes has not been elucidated to a great extent and not much focus is placed on quantitative resistance. In Koshihikari Aichi SBL, a variety of *Pb1* resistant cultivar, a resistant pathway was deduced by knocking down the *WRKY45* gene (Inoue et al. 2013). Thus, we expressed *WRKY45* RNAi fragments in Miyazaki-mochi through *Agrobacterium* transformation. Eight lines of transgenic plants were generated and three lines that had high quantity seeds were used for further experiments. To check the efficiency of the RNAi vector, these three lines and WT Miyazaki-mochi plants were incubated with or without SA, as SA induces *WRKY45* transcription in rice (Shimono et al. 2007). The relative expressions of *WRKY45* in the Miyazaki-mochi *WRKY45* RNAi lines were 25.7% (#3), 16.3% (#4) and 34.3%

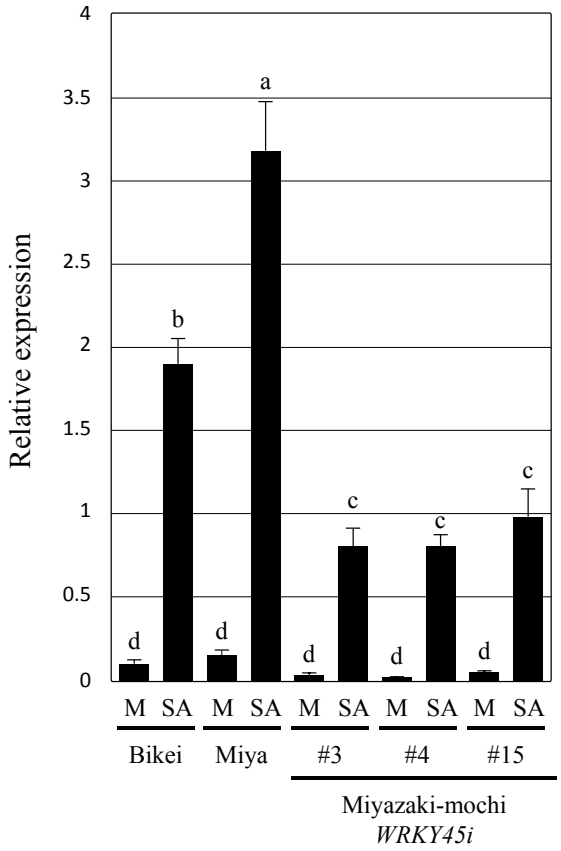

Fig. 1. q-RT-PCR analysis of *WRKY45* expression in Miyazaki-mochi WRKY45 RNAi lines
Leaves after mock (M) or salicylic acid treatment for 5 hours (SA) were used for expression analyses. Y-axis indicates mRNA expression relative to that of *Rubq1*. Average values with SD are shown. Different letters indicate significant differences among the different lines ($P < 0.01$ one-way ANOVA, $P < 0.01$ Tukey-Kramer). Experiments were repeated twice with similar results being obtained.

(#15) compared to Miyazaki-mochi in the mock treatment condition (Fig. 1). In SA treatment, the ratios were 25.3% (#3), 26.0% (#4) and 31.3% (#15) (Fig. 1). These results indicated that the *WRKY45* RNAi vector successfully transformed and decreased the expression of *WRKY45* in all three lines.

To check the differences in blast resistance between Miyazaki-mochi and its *WRKY45* RNAi lines, we inoculated the panicles of rice plants with rice blast fungus and analyzed their blast susceptibility. We sowed the seeds in April 2017 and by mid-May we transferred 20 plants to a 3.5-L plastic pot in a glasshouse. From the end of July to early August, the lines entered the heading stage. In order to test the rice plant within 10 days after the heads came out, the spore suspension (8×10^4 spores/mL) of rice blast was spray-inoculated on different days according to the heading day of each line. A survey was conducted 21 days after inoculation with the blast. The percentage of diseased grains was 15.7% for Bikei 22 because it is a weak cultivar for panicle blast, indicating that the inoculation of panicle blast was successful (Fig. 2 A). The Miyazaki-mochi *WRKY45* RNAi lines #3, #4 and #15 exhibited no significant difference in diseased grain ratio compared to the Miyazaki-mochi cultivar (Steel test method of nonparametric multiple comparison test) (Fig. 2 A). For the second experiment in 2018 (Fig. 2 B), we sowed the seeds in February and by late-February we transferred the seeds to the pots. From the end of April to early May, the lines entered the heading stage. A blast test was then conducted as in the previous experiments. The percentage of diseased grains of Bikei 22 as a positive control was 11.7%, and there was no difference in the diseased grain ratio (Steel test) between Miyazaki-mochi and its *WRKY45* RNAi lines (Fig. 2 B). Thus, the *WRKY45* expression did not affect panicle resistance of Miyazaki-mochi *qPbm11*, suggesting that the resistance of *qPbm11* was independent of that of *Pb1*.

Little is known about the molecular mechanism behind the resistance against *Pyricularia oryzae* imparted by quantitative resistance genes in rice. *WRKY45* knockdown decreased *Pb1* resistance in Koshihikari Aichi SBL (Inoue et al. 2013). *WRKY45* expression is critical for *Pb1*-mediated rice blast resistance as indicated by the muted resistance displayed by cultivar Kanto209 (Inoue et al. 2017). The *WRKY45* knockdown analysis in the present study suggests that the *qPbm11* locus makes use of a different pathway for panicle blast resistance compared to that of *Pb1*. However, we cannot exclude the possibility that WRKY45 knockout analysis may alter the dependence of Miyazaki-mochi resistance as the slight WRKY45 expression continues to work in the RNAi plants, and its major resistance gene remains unidentified. The isolation of genes responsible for *qPbm11* resistance in the future would be helpful to further understand the resistance mechanism to rice blast disease. The *qPbm11* locus possibly activates an unknown defense mechanism(s) against rice blast. There may be many mechanisms other than the Pb1-WRKY45 pathway by which tolerance such as in Miyazaki-mochi is imparted.

The development of resistant cultivars is an effective method of controlling the rice blast disease. Quantitative blast resistance genes offer durable, non-race specific and stable resistance to rice. QTL combinations (pyramiding) of the locus control the different response mechanisms to *P. oryzae* (Fukuoka et al. 2015). Yasuda et al. (2015) suggested that a combination of genes having different resistance mechanisms will confer strong resistance effects. The pyramiding of *Pb1* and *qPbm11* is expected to enhance the quantitative resistance to panicle rice blast,

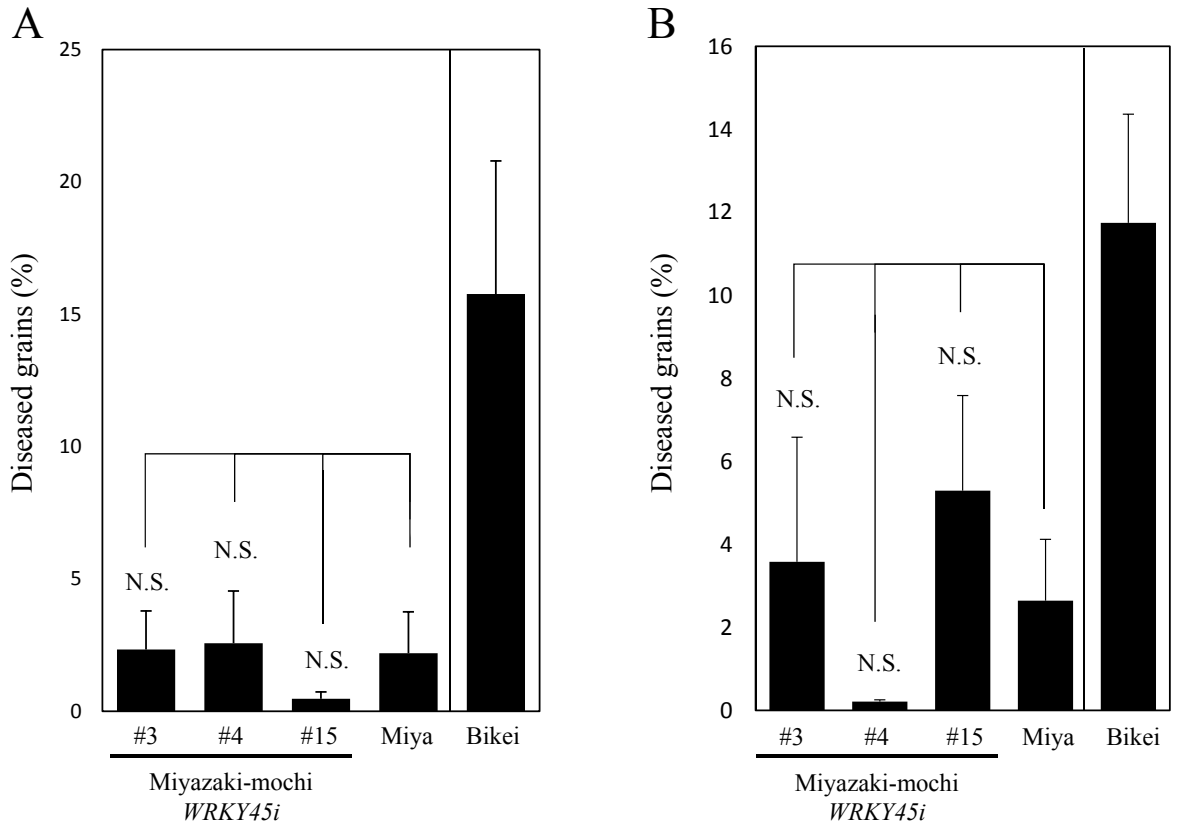

Fig. 2. Panicle blast resistance tests of Miyazaki-mochi and its WRKY45 RNAi lines

Miyazaki-mochi rice lines transformed with *WRKY45 RNAi* vector were tested for blast resistance at full heading stage. This experiment was conducted once in 2017 (Fig. 2 A) and twice in 2018 (one of which is shown in Fig. 2 B), with similar results being obtained in all experiments. Bikei 22 (Bikei) (n=26, 23), Miyazaki-mochi (Miya) (n=28, 37), and Miyazaki-mochi *WRKY45* RNAi lines #3 (n=35, 33), #4 (n=35, 37) and #15 (n=35, 39) were used for the experiments (first number in the pair was from 2017 and the second was from 2018). Y-axis represents diseased grain percentage of the rice blast. Average values with standard error are shown. N.S.: not significant

leading to defense stability and sustainability. Since *Pb1* and *qPbm11* were expected to be very close to each other in the rice genome, more specific markers to select the rice cultivar with both genes will be required based on the genomic sequence through next-generation sequencing.

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