The Panicle Blast Resistance Mechanism of \textit{qPbm11} in the Rice Cultivar Miyazaki-mochi is Independent from that of \textit{Pb1}

Haruhiko INOUE* and Nagao HAYASHI*

Division of Plant and Microbial Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Japan
*Co-corresponding authors

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 \textit{qPbm11} locus in the blast resistant cultivar Miyazaki-mochi exerts panicle resistance similar to the well-known quantitative panicle resistance gene \textit{Pb1}. \textit{qPbm11} and \textit{Pb1} show similarity in panicle blast resistance, quantitative resistance, and proximity of loci on the rice genome. \textit{Pb1} resistance is dependent on the expression of transcription factor \textit{WRKY45}. To identify the resistance mechanism of the \textit{qPbm11} locus, we downregulated \textit{WRKY45} in the cultivar Miyazaki-mochi through an \textit{Agrobacterium}-mediated transformation and surveyed its panicle resistance. \textit{WRKY45 RNAi} transgenic Miyazaki-mochi plants exhibited similar levels of panicle resistance compared to WT, indicating that the \textit{qPbm11} locus is independent of the \textit{Pb1} resistance pathway. Our results suggested that cloning the genes responsible for \textit{qPbm11}-mediated resistance along with \textit{Pb1} using pyramiding technology could enhance panicle resistance in rice.

Discipline: Agricultural Environment
Additional key words: \textit{Oryza sativa}, \textit{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 \textit{pi21}, \textit{Pi34}, \textit{Pi35}, \textit{Pi39}, and \textit{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 \textit{Pb1} confers partial resistance to rice blast with no fungal race specificity (Hayashi et al. 2010). The cultivars carrying \textit{Pb1} have remained resistant to panicle blast for over 35 years. The cloning of \textit{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 \textit{Pb1} expression (Hayashi et al. 2010). After the heading stage, the \textit{Pb1} cultivar shows very strong resistance to rice blast. The mechanism of \textit{Pb1} resistance is attributed to its interaction with the \textit{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 \textit{WRKY45} protein binds to the \textit{Pb1} coiled-coil domain upon localization to the nucleus, resulting in a \textit{Pb1-WKRY45} complex that is more stable than \textit{WRKY45} against protein degradation (Inoue et al. 2013). \textit{WRKY45} is degraded by a ubiquitin-proteasome system in the nucleus (Matsushita et al. 2012). Transgenic rice plants overexpressing \textit{WRKY45} manifested strong resistance against both leaf blast and panicle blast (Shimono et al. 2012). The mechanism of resistance through \textit{WRKY45} expression was unraveled using the cultivar Kanto209, which has weaker resistance compared with other \textit{Pb1} cultivars. QTL analysis and physiological investigation identified that cultivar Kanto209 was defective in SA

*Co-corresponding authors: e-mail haruhiko@affrc.go.jp, nhayash@affrc.go.jp
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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 (H2O) 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%
The qPbm11 Locus Resistance Mechanism Differs from that of Pb1

The qPbm11 Locus Resistance Mechanism Differs from that of Pb1 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 inoculation of panicle blast was successful (Fig. 2 A). The Miyazaki-mochi WRKY45 RNAi lines #3, #4 and #15 exhibited no significant difference in disease gene 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 disease gene 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.
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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References


Hayashi, N. et al. (2010) Durable panicle blast-resistance gene $Pb1$ encodes an atypical CC-NBS-LRR protein and was generated by acquiring a promoter through local genome duplication. The Plant Journal, 64, 498-510.


Inoue, H. et al. (2017) Panicle blast 1 ($Pb1$) resistance is dependent on at least four QTLs in the rice genome. Rice, 10, 36.


Satake, T. (1972) Circular dense-culture of rice plants in pots, the
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