Genetic dissection of resistance of two rice cultivars against blast fungus *Magnaporthe oryzae*

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Genetic dissection of resistance of two rice cultivars against blast fungus *Magnaporthe oryzae*

(イネ 2 系統が保有するいもち病抵抗性の遺伝学的解析)

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Contents **Page**

List of indexes

CHAPTER I

GENERAL INTRODUCTION

Plant diseases are caused by biotic or abiotic environmental factors. However, the maximal damage by plant disease is due to attacks by biotic agents called pathogens, such as fungi, bacteria, mycoplasmas, spiroplasmas, viruses, viroids, nematodes, protozoans (Agrios, 1998). Each has a unique mode of pathogenicity. Among those agents, fungi cause most plant diseases and threaten agricultural production worldwide.

Among integrated pest management practices, using resistant cultivars is the most economically viable and practically feasible way of controlling diseases. Resistance of plants to pathogens has been conventionally classified into host and non-host forms. Suppose at least some accessions or cultivars of a plant species are susceptible to a pathogen isolate. In that case, the plant species is called host plant, and resistance shown by other accessions/cultivars is called host resistance. Host resistance is divided into two categories: race-specific type and race-nonspecific type. Race-specific resistance is highly effective but specific to only some pathogen genotypes (race, strains) and known to follow the gene-for-gene concept (Flor, 1956). Race-nonspecific resistance is weak but adequate for the whole pathogen genotype. By contrast, if all accessions/cultivars of a plant species are resistant to a pathogen, the plant species is called non-host, and the resistance involved is called non-host resistance. Heath (1985), suggested that non-host resistance to fungi may be under complex genetic control and often involves various protective factors that may segregate within the species without compromising overall resistance. Mechanisms of host and non-host resistance are different. Host and non-host resistance expression involve many inducible defense responses that pathogen-specific or nonspecific signals can cause. Understanding the type of resistance and

interactions between plants and pathogens is necessary to find the most suitable breeding methods and selection methodology for crop improvement.

Plants use their innate immune system to protect themselves from various pathogenic microorganisms. The plant innate defensive mechanism is composed of two levels: PAMP/MAMP-triggered immunity (PTI) and effector-triggered immunity (ETI; Dodds and Rathjen, 2010; Liu *et al.*, 2013). Plant pathogenic bacteria usually proliferate in extracellular spaces. Most fungi and oomycetes pathogens penetrate plant cell walls by their invasive hyphae but are limited by the plasma membrane. The basal plant defense, PTI, is activated when evolutionarily conserved, cell surface-localized pattern recognition receptors (PRR) recognize molecules released from the pathogens into the extracellular spaces (Medzhitov and Janeway, 1997; Nürnberger and Brunner, 2002). These PRRs are usually characterized by the N-terminal extracellular Leucine-Rich Repeat (LRR) domain and the C-terminal intracellular kinase domains (Dodds and Rathjen, 2010). This basal defense has a comprehensive immune response against pathogens but is significantly less robust. Effector proteins are delivered into the host cell by bacterial pathogens via type-III secretion pilus, whereas fungi and oomycetes use haustoria or other intracellular structures to do so. Pathogen effectors delivered inside host cells frequently suppress PTI. However, these effectors are recognized by corresponding intracellular Nucleotide Binding site Leucine-Rich Repeat (NB-LRR) receptors, which induce ETI (Jones and Dangl, 2006). Usually, ETI culminates in the hypersensitive response (HR), a kind of localized programmed cell death (Dangl *et al.*, 1996; Keen *et al.*, 1993). NB-LRR proteins consist of an N-terminal Toll, interleukin-1 receptor (TIR; Swiderski *et al.*, 2009) or coiled-coil (CC) domain, a central Nucleotide Binding (NB) domain, and a C-terminal Leucine Rich-Repeat (LRR) domain.

According to Flor (1971), gene-for-gene interactions control ETI. Flor (1956) initially identified the genetic basis of plant resistance in the early 1940s. Flor's research on the flax rust pathogen *Melampsora lini* showed that resistance to this fungus is caused by the coexistence of an *R* gene in the host and an *Avirulence* (*Avr*) gene in the fungus. The absence of either the *R* gene or the *Avr* gene results in disease. The hypothesis of gene-for-gene complementarity between hosts and pathogens (Keen, 1990) also supports Flor's idea. A signal transduction cascade that activates plant defenses is triggered by the recognition of the Avr gene product, which is made possible by the plant resistance gene product. The gene-for-gene system is undoubtedly an oversimplification of the phenomenon; however, it has been a helpful starting point for predicting plant-pathogen interactions (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003; Innes, 2004).

Over the past three decades, *R* genes have been cloned from a wide range of plant species (Hammond-Kosack and Parker, 2003). According to the gene-for-gene model, most of the classic NLR genes, *MLA* locus of barley, *Sr50* of wheat, *L6* of flax, *RPP13* and *ZAR1* of Arabidopsis and *Piz/Pizt* of rice, function as singletons that perform sensing and signaling as a single genetic unit. The host's hypersensitive immune response is triggered when these NLRs directly or indirectly recognize effectors (Ade *et al.*, 2007; Baudin *et al.*, 2017; Bernoux *et al.*, 2016; Chen *et al.*, 2017; Ravensdale *et al.*, 2012; Zhou *et al.*, 2006). The functioning of NLR-mediated immunity is more complex than previously believed, with many NLRs requiring the activation of other NLR proteins, according to recent studies (Gabriëls *et al.*, 2007; Roberts *et al.*, 2013).

Most *R* genes identified so far act as singletons (Adachi *et al.*, 2019). A simple hypothesis that plant NLRs self-associate via their N-terminal domains to induce cell death and activate immune signaling might comprehend how singleton NLRs activate immunity upon

effector detection (Bentham *et al.*, 2018; Williams *et al.*, 2014). TIR domains from flax TIR-NLR L6, Arabidopsis TIR-NLRs RPS4, and SUPPRESSOR OF npr1-1 CONSTITUTE 1 (SNC1) self-associate and are adequate to trigger HR cell death in plants. Site-directed mutagenesis of the self-association interfaces hindered TIR-NLR-mediated signaling (Zhang *et al.*, 2017). A CC domain of CC-NLRs may also facilitate immune activation through self-association. The CC domains of MLA10 and the orthologs Sr33 and Sr50 from wheat and rye may self-associate and trigger cell death in the plant. Mutations in these CC domains inhibited self-association and HR activation (Casey *et al.*, 2016, 2016; Maekawa *et al.*, 2011). However, two recent groundbreaking investigations on the structure and function of the ZAR1 CC-NLR provided the mechanistic insight into CC domain function (Wang, Hu, *et al.*, 2019; Wang, Wang, *et al.*, 2019).

Some NLRs are genetically linked and function in pairs, where the sensor NLR which is specialized to detect the pathogen and the helper NLR also referred to as the executor being responsible for initiating immunological signaling upon signal perception from the sensor NLR (Cesari, *et al.*, 2014a). The rice NLR pairs, RGA4/RGA5 (Cesari *et al.*, 2013; Okuyama *et al.*, 2011), Pik1/Pik2 (Ashikawa *et al.*, 2008; Maqbool *et al.*, 2015) and Pii1/Pii2(Fujisaki *et al.*, 2015; Takagi *et al.*, 2013a) and Arabidopsis RRS1/RPS4 (Le Roux *et al.*, 2015; Williams *et al.*, 2014) are well-known examples. A major clade of NLRs in Solanaceae plant species was shown to form an intricate immunoreceptor network. In this network, multiple helper NLRs are required by a large number of sensor NLRs (Wu *et al.*, 2017).

Rice (*Oryza sativa* L.) is a major staple food crop for more than 50% of the world population, which is projected to be 9.1 billion (34% higher than the current rate) by 2050. Rice production must be doubled to meet the rising food requirements of the projected population (FAO, 2009).

Blast disease caused by the filamentous fungus, *Magnaporthe oryzae* (syn. *Pyricularia oryzae*; Couch and Kohn, 2002), is one of the major crop diseases, accounting for approximately 30% of global production loss of rice (Nalley *et al.*, 2016). Soong Ying-shin originally recorded the disease as "rice fever" in China in 1637, and it was subsequently documented as Imochi-byo in Japan in 1704 (Couch *et al.*, 2005). Breeding and deployment of resistant cultivars is the most economical way of controlling the diseases. *Magnaporthe oryzae* is made up of a number of genus-specific subgroups or pathotypes (Kato *et al.*, 2000), including the *Oryzae* isolates that are pathogenic on rice (*Oryzae sativa*), the *Setaria* isolates that are pathogenic on foxtail millet (*Setatia italica*), the *Eleusine* isolates that are pathogenic on finger millet, and the *Triticum* isolates that are pathogenic on wheat (*Triticum aestivum*). This pathogen affects crops at every developmental stage, and symptoms can be seen on leaves and spikes. However, at the moment, rice blast caused by the *Oryzae* isolates are the most economically important disease. All foliar tissues of rice are affected by blast disease, which is widespread throughout at least 85 different countries (Greer and Webster, 2001). Total loss of grains may be caused by infection of the panicle. To combat rice blast, resistant rice cultivars have been deployed. However, the fungus quickly overcomes host resistance, and resistant cultivars frequently lose their efficacy within two to three years (Ou, 1985; Zeigler *et al.*, 1994).

To date, more than 500 quantitative trait loci (QTLs) for blast resistance have been mapped (Ashkani *et al.*, 2014) and 146 of them have been identified as *R* genes (Sahu *et al.*, 2022). Amongst these, 36 *R* genes have been cloned and characterized at the molecular level (Ning *et al.*, 2020; Sahu *et al.*, 2022). Twenty-eight cloned *R* genes, except for *pi21* (Fukuoka *et al.*, 2009), *Pid2* (Chen *et al.*, 2006) and *Ptr* (Zhao *et al.*, 2018), encode Nucleotide Binding site (NB) Leucine-Rich Repeat (LRR) domain-containing proteins (NLR). So far, 24 *AVR* genes of *M. oryzae* have been mapped and 12 of them have been cloned (Wang *et al.*, 2017). In four of the *NLR-AVR* gene pairs that have been characterized, Pita/AVR-Pita (Jia *et al.*, 2000; Orbach

et al., 2000), Pik/AVR-Pik (Ashikawa *et al.*, 2008; Kanzaki *et al.*, 2012, 2; Maqbool *et al.*, 2015; Yoshida *et al.*, 2009; Yuan *et al.*, 2011; Zhai *et al.*, 2010), Pia/AVR-Pia (Okuyama *et al.*, 2011; Ortiz *et al.*, 2017; Yoshida *et al.*, 2009) and PiCO39/AVR1-CO39 (Cesari *et al.*, 2013; Lei *et al.*, 2013; Liu *et al.*, 2021), the NLR and AVR proteins show direct physical interactions. In the Pii/AVR-Pii pair, however, another host protein, OsExo70, is required for their interaction (Fujisaki *et al.*, 2015; Takagi *et al.*, 2013b).

In the current study, we used two Bangladeshi rice cultivars (Shoni and Tupa121-3) from the world rice collection (WRC) reported by Kojima et al. (2005). These cultivars were evaluated in 1997 (Shoni) and 1993 (Tupa121-3) for field resistance against blast fungus in Akita and Niigata respectively. Both the cultivars are reported to show high filed resistance for blast disease (NARO: https://www.gene.affrc.go.jp/databases-plant_search_detail_en.php?jp=70609) but their resistance factors haven't been identified yet (Table1.1). We utilized this data and further tried to dissect disease resistance of these two cultivars. I tried to identify a new resistance gene(s) from each of the cultivars that may be useful for breeding of resistant rice cultivars.

Table 1.1 Three rice cultivars used in the current study

Cultivar	JP No. (Accession No.)	Origin	Resistance to leaf blast	Evaluation (Location, year)	Causal gene
Shoni	54535	BANGLADESH	Very high	(AKITA, 1997)	Unknown
TUPA 121-3	70609	BANGLADESH	Very high	(NIIGATA, 1993)	Unknown
Hitomebore	249515	Japan (IBARAKI)	-	-	-

Source: Genebank Project, NARO, (https://www.gene.affrc.go.jp/databases-plant_search_detail_en.php?jp=70609)

CHAPTER II
IDENTIFICATION AND ISOLATION OF A BLAST RESISTANCE GENE FROM AN AUS RICE CULTIVAR SHONI

2.1. INTRODUCTION

As explained above, resistant rice cultivars are essential to controlling blast disease. The highest levels of resistance are, in general, conferred by one or a few major genes that are efficient only toward the avirulent *M. oryzae* isolates. To date, significant progress has been achieved through the genetic analysis of rice resistance and *M. oryzae* avirulence.

Over the past three decades, *R* genes have been cloned from a wide range of plant species (Hammond-Kosack and Parker, 2003). Some NLRs work as a single genetic unit to sense and signal. When these NLRs directly or indirectly identify effectors, the host's HR is triggered. Some NLRs work in pairs and are genetically related, with the sensor NLR uniquely specialized to detect the pathogen. In the three classic examples of paired *NLR* genes of rice, namely the *Pik* (*Pikp-1* and *Pikp-2*), *Pia* (*RGA4* and *RGA5*), and *Pi5* (*Pi5-1* and *Pi5-2*), also known as *Pii* (*Pii-1* and *Pii-2*), two *NLR* genes are genetically linked in head-to-head orientation (Ashikawa *et al.*, 2008; Lee *et al.*, 2009; Maqbool *et al.*, 2015; Okuyama *et al.*, 2011). One NLR of the paired NLRs (Pikp-1, RGA5, and Pii-2) has a non-canonical domain called the integrated domain (ID), which shares amino acid sequence similarity with domains in the rice protein and possibly functions as a decoy for recognizing AVR (Cesari, *et al.*, 2014a; Kroj *et al.*, 2016; Sarris *et al.*, 2016). These NLRs are called "sensor NLRs" because they detect AVRs, whereas the other NLRs play a role in signaling ("helper NLRs"). In the cases of Pikp-1 and RGA5 sensor NLRs, they have a heavy-metal-associated (HMA) domain as an ID to which their respective AVRs (AVR-PikD, as well as AVR-Pia and AVR1-CO39) bind directly (Białas *et al.*, 2018; Cesari, *et al.*, 2014b).

Rice blast has become a popular model pathosystem to study host-pathogen interactions thanks to recent progress in molecular understanding of the disease (Liu *et al.*, 2010). The allelic series of *R* genes provide a good platform for a deep understanding of molecular mechanisms involved and the genetic basis of the resistance specificity of the *R* genes. The genomic regions harboring allelic series of *R* genes are an excellent resource for comparative study to understand genomic organization and evolution (Zhou *et al.*, 2007). One such loci is *Piz,* which comprises four allelic series: *Piz, Piz-t, Pi9, and Pi2*. These genes recognize a different set of *M. oryzae* isolates. Eight amino acid substitutions within LRR regions of *Pi2* and *Piz-t* resulted in different resistance specificities. The *Pik* locus on rice chromosome 11 is another typical example. At the *Pik* locus, several alleles, including *Pikm* (Ashikawa *et al.*, 2008), *Pik* (Zhai *et al.*, 2010), and *Pikp* (Yuan *et al.*, 2011), have been identified. From the pathogen side, four cognate *AVR-Pik* alleles (*AVR-PikA*, *C*, *D*, and *E*) have been identified and isolated from *M. oryzae* (Yoshida *et al.*, 2009). These *AVR-Pik* alleles are specifically recognized by different *Pik* alleles. For example, *AVR-PikD* is recognized by *Pik*, *Pikm*, and *Pikp*, while both *Pikm* and *Pik* recognize *AVR-PikE*, but *AVR-PikA* is recognized only by *Pik*. *AVR-PikC* is recognized by none of the *Pik* alleles reported so far (Kanzaki *et al.*, 2012). This gene-for-gene relationship is explained by the *NLR-AVR* gene co-evolution model (Kanzaki *et al.*, 2012).

In the current study, we found that *Oryza sativa* subgroup aus cv. Shoni shows resistance against 10 *M. oryzae* strains. By QTL mapping and candidate gene cloning strategy, we identified an allele of the rice blast resistant gene *Pik* and named it *Pikps*. Rice blast resistance evaluation of cloned genes indicated that, like other *Pik* alleles, *Pikps* also consists of two NLR genes (*Pikps-1* and *Pikps-2*). Characterization of *Pikps* showed that its resistance spectrum and race specificity are similar to that of the *Pikp* allele of the *Pik* locus.

2.2 MATERIALS AND METHODS

2.2.1 Plant materials

The japonica-type rice (*O. sativa* subsp. japonica) cultivar Hitomebore and the aus-type rice (*O. sativa* subgroup aus) cultivar Shoni (WRC31), provided by the National Agriculture and Food Research Organization (NARO) World Rice Core Collection (Kojima *et al.*, 2005), were used as parental materials. Hitomebore and Shoni showed different responses to nine *M. oryzae* isolates (Fig 2.1). We crossed these two cultivars and 125 RILs of the F_9 generation were developed by the single seed descent (SSD) method (Fig 2.2; Brim, 1966; Goulden, 1939). All the RILs and the parents were grown in a greenhouse between 26 ℃ and 28 ℃.

2.2.2 Fungal materials

M. oryzae isolates used in this study were obtained from Ministry of Agriculture, Forestry and Fishery (MAFF), Japan. The details of isolates and their race codes are given in Table 2.1. They were maintained on sterilized barley grains under dry conditions at 4 ℃ for long-term storage at Iwate Biotechnology Research Centre Kitakami, as previously described by Hirata *et al.*, (2007). The seed cultures were transferred to a potato dextrose agar slant (PDA, 50mL of potato dextrose broth, 5g of sucrose, and 4.5g of agar powder, Nacalai Tesque, Kyoto, in 250 mL of water) in a test tube and incubated at 25 ℃. Slant cultures of 1 week to 3 months old were used for preparing spore production on Oatmeal Agar (OMA; 16g Oatmeal powder, 2g sucrose, and 8g of agar in 400mL water) medium. Fungi were grown on OMA for 7-10 days at 26 ℃. Hyphae were scraped using a cotton stick and standard distilled water (SDW) and placed under blue light for four days for spore induction. The spores were scraped in SDW using cotton sticks, and spores were counted in a hemocytometer.

2.2.3 Pathogenicity assay

Spore suspension of 1×10^4 conidia ml^{-1} of *M. oryzae* isolate Naga69-150 was spray-inoculated onto the leaves of 14-day-old plants, which were grown in a greenhouse. Then the inoculated plants were kept in the dark condition for 24 hours at 27 ºC with 100% relative humidity (RH) for spore induction and germination. The plants were then transferred to an incubation chamber with a 16-h light/8-h dark photoperiod. Disease severity was observed visually and scored at eight days post inoculation (DPI). A schematic experimental design is shown in Fig 2.3. For punch inoculation, a conidial suspension of 3×10^5 conidia ml^{-1} was punch-inoculated onto a rice leaf blade one month after seed sowing. The inoculated plants were placed in a dew chamber at 27 °C for 24 h in the dark with high RH and transferred to an incubation chamber with a 16-h light/8-h dark photoperiod. We measured the lesion size at 8 DPI with the help of ImageJ software (Schneider *et al.*, 2012).

2.2.4 Genotyping of RILs by whole-genome resequencing

We genotyped each RIL using whole-genome resequencing of the parents and 235 RILs using the Illumina platform. These sequences were trimmed and filtered using Prinseq and FaQCs (Lo and Chain, 2014; Schmied *et al.*, 2018). After that, the Burrows-Wheeler Alignment (BWA) tool was used to align the quality-trimmed short reads against the reference genome (Li and Durbin, 2009). As a reference, we used the genome sequence of OsNipponbare-Reference-IRGSP-1.0 (Kawahara *et al.*, 2013). After mapping, we used samtools (Li *et al.*, 2009) to sort and prepare index files from BAM files. Further, bcftools (Narasimhan *et al.*, 2016) was used to call variants on these BAM files. Finally, we used LB-impute (Fragoso *et al.*, 2016) to impute the variations based on Hitomebore and Shoni genotypes. There are three genotypic groups for biallelic SNPs in our RILs: Hitomebore - Hitomebore, Hitomebore - Shoni, and Shoni - Shoni. A scheme of genotyping of 125 RILs by whole genome sequencing is shown in Fig. 2.4C.

2.2.5 Genome assembly of Shoni

DNA was extracted from leaf tissue using a NucleoBond HMW kit (Takara Bio, Otsu, Japan). DNA sequencing was performed by Oxford Nanopore Technologies (ONT) using the MinION system with a FLO-MIN106 flow cell (ONT). Using Guppy, the base-calling of ONT reads was performed on FAST5 files (Wick *et al.*, 2019). Subsequently, low-quality reads were filtered out, and *de novo* assembly was performed using NECAT software (Chen *et al.*, 2021). To further improve the accuracy of the assembly, Racon (Levitsky, 2004) software was applied twice, and Medaka (https://github.com/nanoporetech/medaka) was used to correct misassembly. One round of consensus correction was performed using BWA and HyPo (https://github.com/kensung-lab/hypo) on Illumina short reads (trimmed paired-end 150–200-bp reads) for the accession (Fig. 2.4A and 2.4B).

2.2.6 GWAS analysis

GWAS analysis was performed using the phenotypic data (Disease severity index scores) of the 125 RILs and genotypic data. We used the R package "GWASpoly" (Rosyara *et al.*, 2016) to identify genomic regions that show significant association with the phenotypic effect. Manhattan and QQ plots were produced using the R package. Then, we selected SNPs with the $-\log_{10}(p)$ values greater than 20 as the representative SNPs for the significant region of the QTL (Fig. 2.6A).

2.2.7 Comparative mapping, dot-plot analysis, gene conservation analysis, and multiple sequence alignment

We performed NECAT (https://github.com/xiaochuanle/NECAT/) assembly of the Shoni genome using Nanopore and Illumina sequence reads to compare it with the Nipponbare reference genome (Table 2.2). Genome-wide dot-plot analysis was performed using D-

GENIES (Cabanettes and Klopp, 2018). Exonerate package Version 2.2.0 (protein2genome; http://www.ebi.ac.uk/~guy/exonerate) was used to discover the genes conserved in the Shoni genome assembly. Multiple sequence alignment was performed using CLUSTAL-W and MAFFT (Katoh and Standley, 2013; Thompson *et al.*, 1994).

2.2.8 RNAi-mediated knock-down of the candidate genes

Two gene knock-down (by RNA interference: RNAi) constructs pANDA-Pi-Shoni for candidate genes were generated by PCR amplifying a specific fragment of complementary DNA (cDNA) of candidate *NLR* genes from Shoni. The sequences were cloned into the Gateway vector pENTR/D-TOPO (Invitrogen, CA, USA) and transferred into the recombination sites of pANDA vector (Miki and Shimamoto, 2004) using LR Clonase (Invitrogen). One of the 59 RILs that carried Pikps, RIL#43, with a sufficient transformation efficiency, was selected as the recipient line. The resulting vectors were introduced into *Agrobacterium tumefaciens* (strain EHA105) and used for *Agrobacterium*-mediated transformation of rice RIL#43 following the method described by Okuyama *et al.,* (2011). Total RNA was extracted from leaves of transgenic plants using an SV Total RNA Isolation System (Promega, WI, USA) and used for quantitative RT-PCR (qRT-PCR). cDNA was synthesized from 500 ng total RNA using a PrimeScript RT Reagent Kit (Takara Bio). qRT-PCR was performed using a StepOne Real-time PCR Instrument (Applied Biosystems, CA, USA) with KAPA SYBR FAST PCR Master Mix (Kapa Biosystems, MA, USA). Melting curve analysis (from 60 to 95 °C) was included at the end of the cycles to ensure the consistency of the amplified products. The comparative Ct (ΔΔCt) method was used to calculate the expression of *Pikps-1* and *Pikps-2* relative to the rice *Actin* gene (LOC_Os03g50885) as an internal control. The data presented are the average and standard deviations from three

experimental replications. The primers used to generate the RNAi construct and for qRT-PCR are listed in Table 2.7.

2.3 RESULTS

2.3.1 Characterization of blast resistance in *O. sativa* subgroup aus cv. Shoni

The rice cultivar Shoni (WRC31) belongs to the *O. sativa* subgroup aus and is a member of the world rice collection (WRC) reported by Kojima et al. (2005). Shoni shows resistance to ten rice *M. oryzae* isolates (Fig. 2.1). To identify the resistance gene of Shoni, we used the isolate Naga69-150 (MAFF305471; race code 007.-) as the pathogen. Spray inoculation tests were conducted for the parents (Hitomebore and Shoni) and 125 recombinant inbred lines (RILs) of the F_9 generation (Fig 2.2), which were developed by a cross between them. Disease symptoms of the RILs were observed eight days post inoculation (DPI), and their disease severity index (DI) scores were categorized into the three classes: no symptoms (DI = 0), with resistance similar to Shoni; 0–20% infected leaf area (DI = 1), and over 20% infected leaf area (DI = 2), with susceptibility similar to Hitomebore (Fig. 2.5A). Based on the distribution of the average DI of two technical replications of the 125 RILs, we classified RILs with DI below 0.99 (n = 58) and over 1.0 (n = 67) as resistant and susceptible, respectively (Fig. 2.5B). The observed segregation ratio was tested for goodness of fit to test for a trait controlled by a single locus using chi-square analysis. The expected segregation ratio of a single locus for the RIL population is 1:1 (Liu *et al.,* 2014), and the observed segregation ratio was nearly 1:1 (chi-square, $P = 0.65$), which indicates that Shoni has a single resistance gene for the *M. oryzae* isolate Naga69-150.

2.3.2 Identification of candidate resistance genes by QTL mapping and comparative analysis

QTL analysis was performed using SNP data from whole-genome sequences of 125 RILs and the DI scores of the inoculation assay. We identified a total of 1,580,242 SNPs between the genomes of the two parents, Hitomebore and Shoni. We selected one SNP per 5-kb interval and used 63,551 SNPs for subsequent QTL analysis (Fig. 2.4C). QTL analysis was performed using 125 RILs by the R package GWASpoly (Rosyara *et al.*, 2016) to detect SNPs associated with the observed blast resistance. We identified a single QTL showing statistical significance, i.e., $-\log_{10}(P) > 3.36$, at the end of chromosome 11 (Fig. 2.6A), which was tentatively named *Pi-Shoni*. We focused on the region ($-\log_{10}(P) > 20$) corresponding to the position chr11: 27,490,669 to the terminus of chromosome 11 of the Nipponbare reference genome (Kawahara *et al.*, 2013), within which candidate genes were searched. We found a total of 230 protein-coding genes within this region, 11 of which encode NBS-LRR domain-containing proteins (Fig. 2.6B; Table 2.3). We performed NECAT (https://github.com/xiaochuanle/NECAT/) assembly of the Shoni genome using Nanopore and Illumina sequence reads to compare it with the Nipponbare reference genome (Table 2.2). Genome-wide dot-plot analysis using D-GENIES (Cabanettes and Klopp, 2018) indicated that the *Pi-Shoni* region of Nipponbare had synteny with the bctg00000014 scaffold (Fig. 2.7). The conservation analysis software exonerate (protein2genome; http://www.ebi.ac.uk/~guy/exonerate) showed that 9 out of 11 genes annotated by the Rice Genome Annotation Project (http://rice.uga.edu) (*LOC_Os11g45620*, *LOC_Os11g45750*, *LOC_Os11g45790*, *LOC_Os11g45930*, *LOC_Os11g45980*, *LOC_Os11g46200*, *LOC_Os11g46210*, *LOC_Os11g47447,* and *LOC_Os11g47780*) were conserved in the bctg00000014 scaffold generated from the Shoni genome assembly (Fig. 2.6B; Table 2.3). Two of these genes, *LOC_Os11g46200* and *LOC_Os11g46210*, are homologs of *Pikm-1 and Pikm-2* (Fig. 2.6B), both of which are required for the resistance mediated by the rice blast resistance gene *Pikm* (Ashikawa *et al.*, 2008). We named the homologs of *LOC_Os11g46200* and

LOC_Os11g46210 in Shoni *Pikps-1* and *Pikps-2*, respectively. We considered these two genes as candidates in *Pi-Shoni* for conferring resistance against *M. oryzae* isolate Naga69-150.

2.3.3 RNAi-mediated gene knock-down of *Pikps-1* and *Pikps-2*

To verify the function of the gene *Pikps*, we knocked down the *Pikps-1* and *Pikps-2* genes by the RNA interference (RNAi) method. One of the RILs, RIL#43, carrying *Pikps* and with suitable transformation efficiency, was used for RNAi-mediated gene silencing of *Pikps-1* and *Pikps-2* (Fig. 2.8). To ensure that *Pikps* knock-down is really effective in the change of phenotype, we designed two gene silencing constructs for each gene targeting the coiled-coil (CC) and leucine-rich repeat (LRR) domains (Table 2.7). A total of 76 independent calli were developed after the *Agrobacterium*-mediated transformation of RIL#43 with the four constructs. Of these, 22 and 16 lines were generated with constructs targeting the CC and LRR domains of *Pikps-1*, respectively, and 21 and 17 lines were developed for the CC and LRR domains of *Pikps-2*, respectively. We used another RIL as recipient line, RIL#94 (the information of calli regenerated is given in Table 2.8). To confirm the function of *Pikps-1* and *Pikps-2*, we carried out punch inoculation of *M. oryzae* isolate Naga69-150 onto these transformants. Silencing of either of the genes resulted in the compatible reaction, which indicated that both *Pikps-1* and *Pikps-2* genes are required for *Pikps*-mediated resistance (Fig. 2.9B). Reduction of transcript levels of *Pikps-1* and *Pikps-2* genes was confirmed by qRT-PCR (Fig. 2.9A). This result supports previous data showing that the *Pikm* locus on chromosome 11 requires two *NLR* genes (*Pikm-1* and *Pikm-2*) to manifest disease resistance against rice blast (Ashikawa *et al.*, 2008).

2.3.4 Allelic differentiation between *Pikps* and *Pik* series

Pikps-1 shared 99% amino acid sequence identity with Pikp-1 with a single amino acid change from serine to proline at position 351 in the NBS domain (Fig. 2.10). Furthermore,

Pikps-1 shared 95% amino acid identity with two other Pik alleles, Pik-1 and Pikm-1. On the other hand, Pikps-2 was 100% identical to Pikp-2 and 99% similar to Pik-2 and Pikm-2 (Fig. 2.11 and Table 2.4). Pik-1, Pikp-1, and Pikm-1 proteins are known to act as sensor NLRs that bind corresponding AVR-Pik allele effector protein via the integrated HMA domain, whereas Pik-2, Pikp-2, and Pikm-2 are helper NLRs required for activation of the immune response upon effector recognition. Pikps consists of Pikps-1 sensor NLR with an integrated HMA domain and Pikps-2 helper NLR, which is presumably required for initiating resistance signaling. There were no amino acid differences between Pikp-1 and Pikps-1 within the integrated HMA domain (Fig. 2.10).

2.3.5 Characterization of *Pikps* against known *AVR-Pik* alleles

Previous studies have shown that *Pikp* recognizes *AVR-PikD* but does not recognize *AVR-PikA*, *C,* or *E* (Kanzaki *et al.*, 2012). Since the amino acid sequences of Pikp and Pikps sensor NLR (Pikp-1 and Pikps-1) differ by only one amino acid, it was presumed that *Pikps* recognize *AVR-PikD* and induce resistance in the same manner as *Pikp*. Gene expression of *AVR-PikD* was confirmed in rice leaves infected with *M. oryzae* isolate Naga69-150 (Fig. 2.12B). To determine the recognition specificity of *Pikps* for *AVR-Pik* alleles, we inoculated RIL#43 with two isolates of *M. oryzae* (Sasa2 with either the *AVR-PikD* or the *AVR-PikE* transgene, as developed by Kanzaki *et al.,* (2012). The wild-type Sasa2 isolate does not harbor known *AVR-Pik* alleles (Yoshida *et al.*, 2009). From the inoculation assay, RIL#43 is incompatible to Sasa2 harboring *AVR-PikD* but compatible to Sasa2 as well as Sasa2 carrying *AVR-PikE.* These results suggest that *Pikps* recognize *AVR-PikD* but not *AVR-PikE* (Fig. 2.12A).

2.4 DISCUSSION

In the current study, by QTL mapping and cloning strategy, we identified an allele of the rice blast resistance gene *Pik*, which we named *Pikps*. Like other *Pik* alleles, *Pikps* consisted of two NLR genes (*Pikps-1* and *Pikps-2*). Characterization of *Pikps* indicated that its race specificity is similar to that of *Pikp*. Furthermore, Shoni showed resistance against nine *M. oryzae* isolates apart from our test isolate (Naga69-150) (Fig. 2.1). Based on race code information and association genetics study by Yoshida *et al.*, (2009), five isolates recognize known *R* genes among which none of the isolates carry *AVR-PikD*. This was confirmed by the presence/absence of *AVR-PikD* by PCR amplification and Sanger sequencing of PCR products in case of Sasa2, 85-141 and Ao92-06-2 (Fig. 2.12C) and based on the previous data from Yoshida *et al.,* (2009) and MAFF manual vol. 18 for isolates SL91-48D and 24-22-1-1 (Table 2.6). These results suggest that Shoni harbors another resistance gene(s) against these five isolates. We addressed the conservation of six well-studied NLR genes (*Pia*, *Pib*, *Pii*, *Pit*, *Pita*, *Piz-t*) in the Shoni genome by BLASTP searches using their amino acid sequences, which showed that *Pib* and *Piz-t* are not conserved, while *Pia*, *Pii*, *Pit* and *Pita* are conserved in Shoni with the amino acid identity ranging from 93.5 to 99.8% (Table 2.5). The race code information of these five strains indicates that they do not have *AVR-Pii* or *AVR-Pia* (Table 2.6). Therefore, the resistance of Shoni against the five isolates may be either because of the presence of functional alleles of *Pit* and/or *Pita* or other cloned *R* genes, as described in Sahu *et al.*, (2022), or due to the presence of a novel *R* gene(s). Further studies are needed to dissect these additional *R* genes in Shoni. Since Shoni cultivar might harbor many resistance genes, it could serve as a good donor or *R* genes for pyramiding strategy in future resistance breeding.

Table 2.1 List of *M. oryzae* isolates used in the study

MAFF No.	Designation	Race	Location
101126	0423-1	Race 007	Japan (Miyagi)
238767	85-141	Race 037.3	Japan (unknown)
238997	H98-315-1	Race 107.2	Japan (Aichi)
-	2012-1	Race 007.4	-
101530	Ao92-06-2	Race 337.1	Japan (Aomori)
238984	SL 91-48D	Race 077.1	Japan (unknown)
238993	Ina85-182	Race 103.0	Japan (unknown)
305471	Naga69-150	Race 007	Japan (Nagano)
101519	24-22-1-1	Race 037.1	Japan (Mei)
238993	Ina85-182	Race 103.0	Japan (unknown)

Source: Genebank Pro1ject, NARO, (https://www.gene.affrc.go.jp/databases-plant_search_detail_en.php?jp=70609)

Table 2.2 Summary of genome assembly of the resistance line Shoni. Genome assembly was performed by NECAT software

Number of scaffolds	86
Contig N50	17.5 Mbp
Largest contig	30.4 Mbp
Genome assembly	385.5 Mbp

Table 2.3 Conservation of Nipponbare candidate genes in Shoni draft genome as revealed by exonerate

Gene ID (Query)	Protein length	Chromosome	Position in Nipponbare		Domains	vs Shoni draft sequence (target)	Query range		Target range		Candidate gene ID
			start	end			start	end	start	end	
LOC_Os11g45620.1	771	11	27,603,341	27,609,143	CN	bctg00000014	1	769	12,896,764	12,891,481	*LOC_Os11g45620.1 Shoni*
LOC_Os11g45750.1	1,200	11	27,683,640	27,695,070	CN	bctg00000014	1	1,083	12,958,003	12,953,747	*LOC_Os11g45750.1 Shoni.1*
							231	1,083	12,987,455	12,992,112	*LOC_Os11g45750.1 Shoni.2*
							1	1,083	13,042,783	13,038,776	*LOC_Os11g45750.1 Shoni.3*
							269	1,083	13,098,083	13,092,579	*LOC_Os11g45750.1 Shoni.4*
							211	1,083	13,168,922	13,159,803	*LOC_Os11g45750.1 Shoni.5*
LOC_Os11g45790.1	998	11	27,703,761	27,707,310	CNL	bctg00000014	1	998	12,959,146	12,962,361	*LOC_Os11g45790.1 Shoni.1*
							1	998	12,975,145	12,971,849	*LOC_Os11g45790.1 Shoni.2*
							1	998	13,043,899	13,047,150	*LOC_Os11g45790.1 Shoni.3*
							1	998	13,170,883	13,174,269	*LOC_Os11g45790.1 Shoni.4*
LOC_Os11g45930.1	1,097	11	27,793,777	27,797,821	CNL	bctg00000014	1	1,097	13,043,893	13,047,153	*LOC_Os11g45930.1 Shoni.1*
							1	1,097	13,170,880	13,174,272	*LOC_Os11g45930.1 Shoni.2*
							383	1,097	12,960,313	12,962,364	*LOC_Os11g45930.1 Shoni.3*
							383	1,097	12,973,880	12,971,849	*LOC_Os11g45930.1 Shoni.4*
LOC_Os11g45980.1	852	11	27,820,309	27,824,920	NL	bctg00000014	1	852	13,102,437	13,104,993	*LOC_Os11g45980.1 Shoni*
LOC_Os11g46070.1	481	11	27,884,593	27,890,936	CN	-	-	-	-	-	-
LOC_Os11g46140.1	584	11	27,928,731	27,931,045	CN	-	-	-	-	-	-
LOC_Os11g46200.1	1,125	11	27,978,368	27,983,597	CNL	bctg00000014	5	1,074	13,201,101	13,194,970	***Pikps-1***
LOC_Os11g46210.1	1,044	11	27,984,697	27,989,134	NL	bctg00000014	1	1,044	13,203,620	13,206,846	***Pikps-2***
LOC_Os11g47447.1	384	11	28,653,511	28,655,918	NL	bctg00000014	1	384	13,679,524	13,680,675	*LOC_Os11g47447.1 Shoni*
LOC_Os11g47780.1	1,078	11	28,811,740	28,815,723	CNL	bctg00000014	1	1,073	13,875,463	13,871,961	*LOC_Os11g47780.1 Shoni*

C, coiled-coil domain; N, NBS domain; L, leucine-rich repeat.

Table 2.4. Amino acid sequence identity of Pikps to three Pik alleles

Pi-Shoni	Pik allele	GenBank Acc.	Amino acid identity (%)
Pikps-1	Pikp-1	ADV58352.1	99
(1,142 aa)	Pik-1	ADZ48537.1	95
	Pikm-1	BAG72135.1	95
Pikps-2	Pikp-2	ADV58351.1	100
(1,021 aa)	Pik-2	ADZ48538.1	99
	Pikm-2	BAG72136.1	99

Table 2.5 Amino acid sequence identity of reported rice NLR to its alleles found in the Shoni genome

Gene	GenBank Acc.	% aa identity
Pia	AB604622	99.89
Pib	BAA76281.2	-
Pii	BAN59294.1	95.05
Pit	BAH20861.1	93.50
Pita	AAK00132.1	99.78
Piz-t	ABC73398.1	-

Table 2.6. Avirulence specificity of tested isolates for known *R* genes in rice

		R-gene						
Isolate	Race	*Pik*	*Pikp*	*Pikm*	*Pit*	*Pia*	*Pii*	*Pita*
Naga69-150	007.-	R	Nd	R	Nd	S	S	R
Sasa2	037.1	S	S	S	R	S	S	R
85-141	037.3	S	S	S	R	S	S	R
Ao92-06-2	337.1	S	S	S	R	S	S	S
SL91-48D	077.1	S	S	S	R	S	S	R
24-22-1-1	037.1	S	S	S	R	S	S	R

S, susceptible; R, resistant; Nd, not determined.
The table is adopted from MAFF Microorganism Genetic Resources Manual vol. 18 and Yoshida et al. (2009).

Table 2.7 List of primers used in this study

Gene	Primer name	Primer sequences (5'–3')		Usage
		Forward	Reverse	
Pikps-1	*Pikps-1*-RNAi-CC	GGGAGTGCAATCTTCTGGAG	GCTTCTTCGCTGCTCGAAAT	Construction of the plasmid pANDA-*Pikps-1*-CC
	Pikps-1-RNAi-LRR	CACACACTTGCAGCCTTGTC	CAGAACCTCCCTGCATTTTG	Construction of the plasmid pANDA-*Pikps-1*-LRR
Pikps-2	*Pikps-2*-RNAi-CC	GACATCCAGTACATCAATGACGAG	TAGTTAACCAAACCTCAAGCTCCT	Construction of the plasmid pANDA-*Pikps-2*-CC
	Pikps-2-RNAi-LRR	ATGACTGCACCAAGCAATAAAGTC	GAAGGTATCACCACCCTTTGTTAT	Construction of the plasmid pANDA-*Pikps-2*-LRR
Pikps-1	*Pikps-1* for qPCR	GTAACCGATACACTTAGCAC	GGAATGGACTTTCTGATGAC	for qRT-PCR
Pikps-2	*Pikps-2* for qPCR	ACGGACGATGATCAAAACCCCAG	TGAACCAACAGCTTGAAATC	for qRT-PCR
OsActin	*OsActin* for qPCR	ACCATTGGTGCTGAGCGTTT	CGCAGCTTCCATTCCTATGAA	for qRT-PCR

Table 2.8. Number of calli regenerated from two recipient lines RIL#43 and RIL#93 .

Recipient	Target gene	Target domain	Number of calli
RIL#43	*Pikps-1*	CC	22
		LRR	16
RIL#43	*Pikps-2*	CC	21
		LRR	17
RIL#94	*Pikps-1*	CC	3
		LRR	4
RIL#94	*Pikps-2*	CC	3
		LRR	4

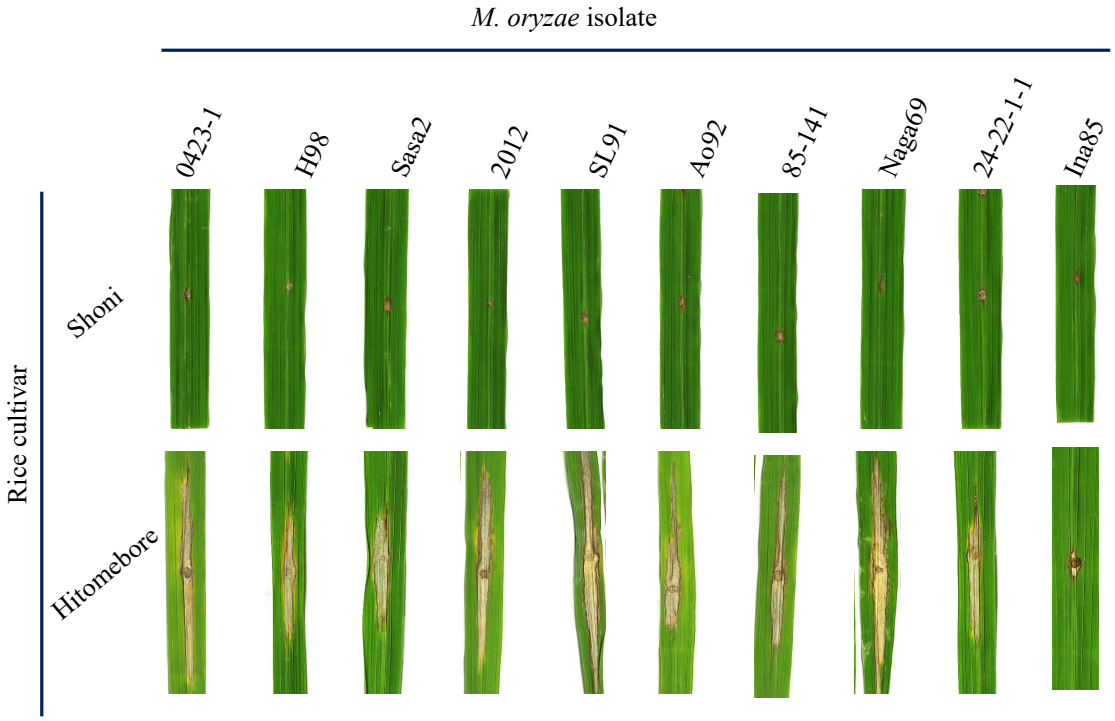


Fig. 2.1 Inoculation results of ten *M. oryzae* isolates to the parental lines (Shoni and Hitomebore). The aus cultivar Shoni showed resistance against the ten different isolates, whereas the *japonica* cultivar Hitomebore showed susceptibility to 9 of them.

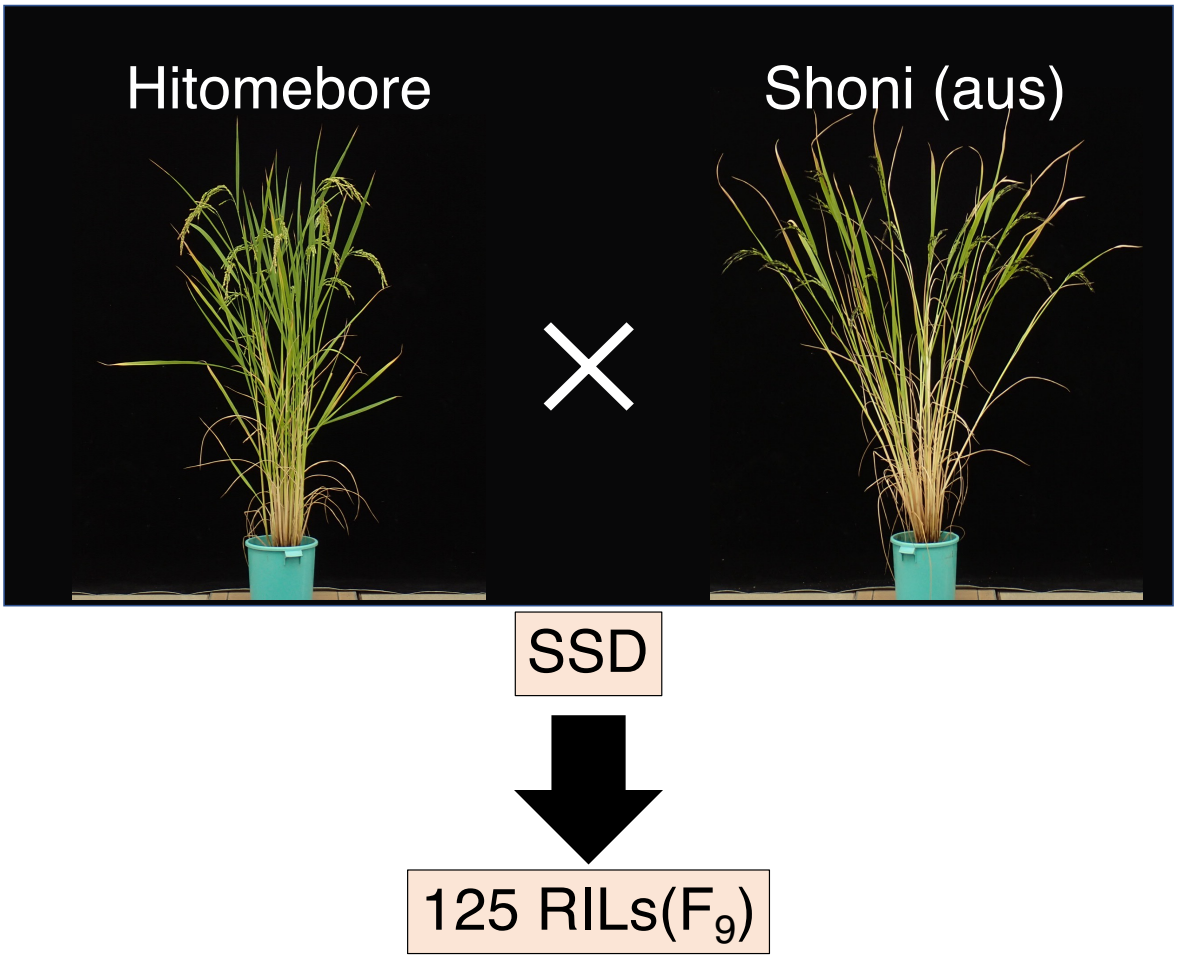


Fig. 2.2 A scheme of development of Recombinant Inbred Lines (RILs) from a cross between Hitomebore and Shoni by Single Seed Descent (SSD) method

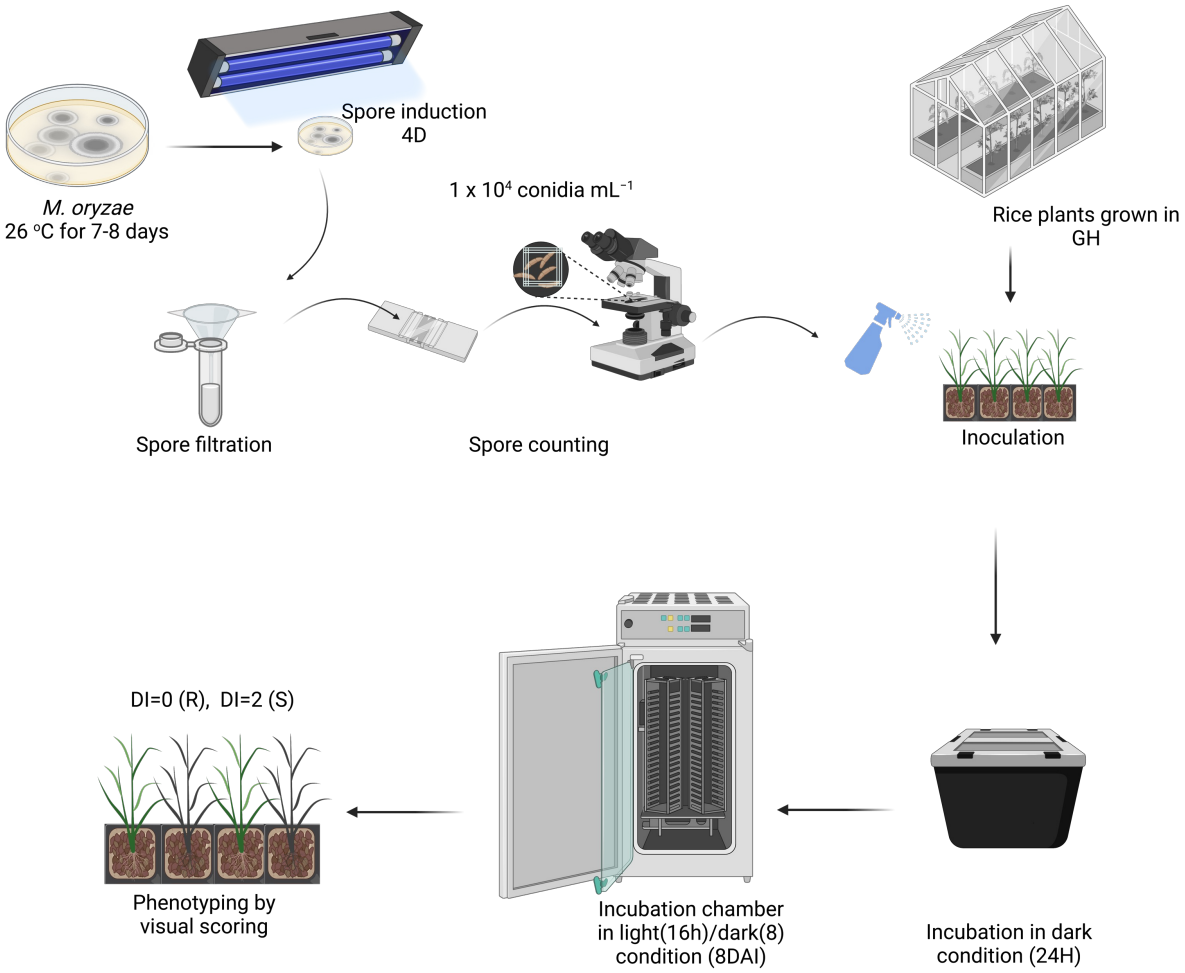


Fig. 2.3 Schematic representation of experimental design.

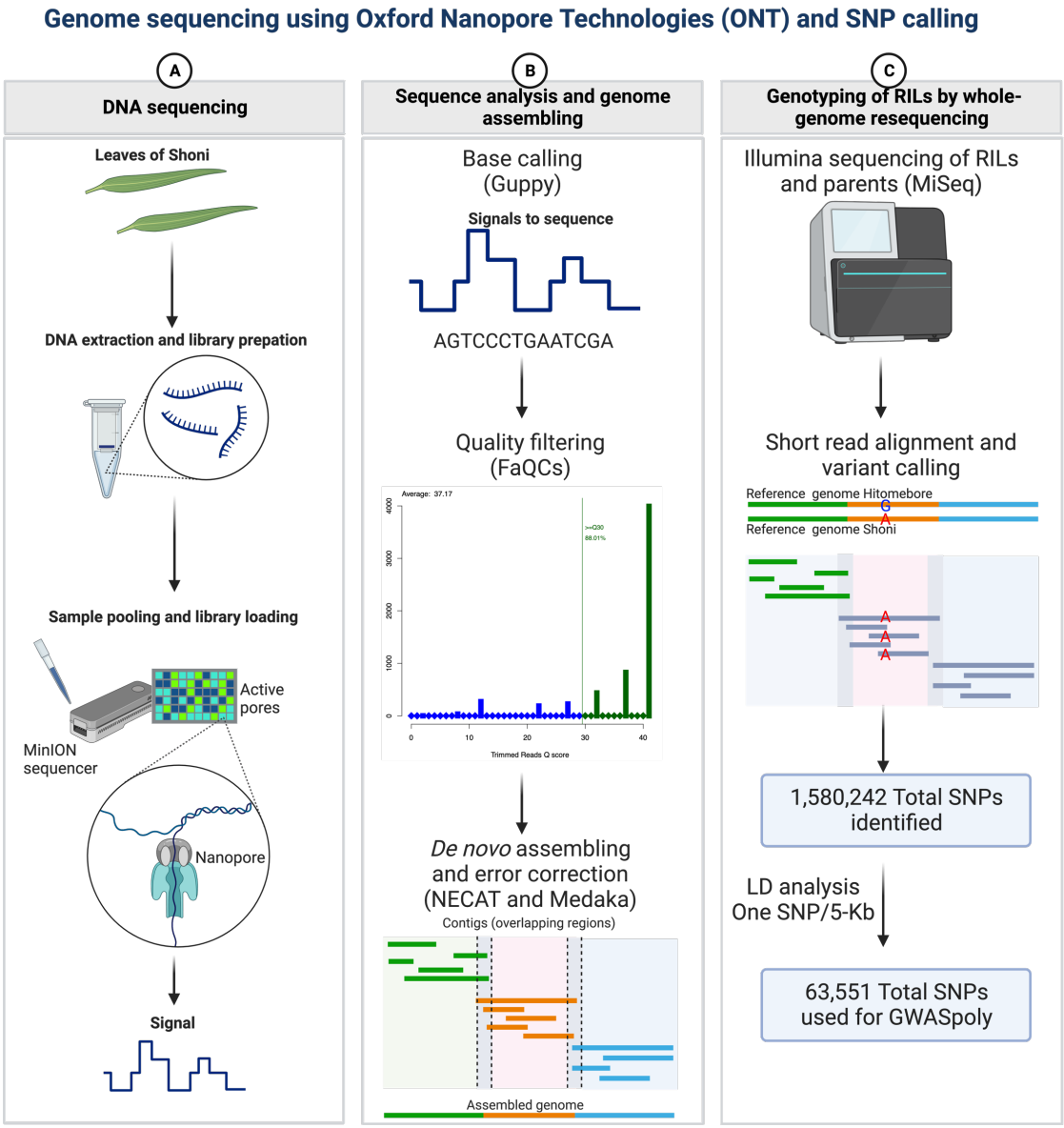


Fig. 2.4 Workflow of *de novo* assembly of Shoni reference genome and genotyping of 125 RILs by whole genome sequencing. A) DNA extraction, library preparation and sequencing using MinION sequencer. B) Sequence analysis and *de novo* assembling of reference genome. C) Genotyping of recombinant inbred lines and SNP calling.

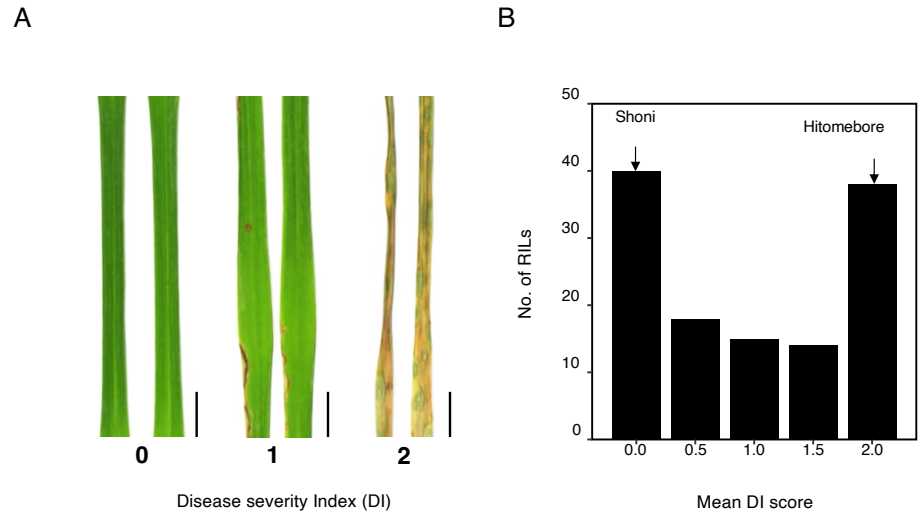


Fig. 2.5. Resistance assay of 125 RILs against Naga69-150 isolate of *Magnaporthe oryzae*.
A) Disease severity Index (DI) employed in evaluating phenotypes of RILs after spray inoculation of the fungus. DI=0; no symptom, DI=1; 0 to 20 % infected leaf area, DI=2; over 20% infected leaf area. Scale bar, 0.5 cm. B) Frequency distribution of the DI for 125 RILs derived from a cross between Hitomebore and Shoni. Arrows indicate approximate value obtained for the parental (Hitomebore and Shoni) lines. DI score of each RIL is represented by the average value of two technical replications.

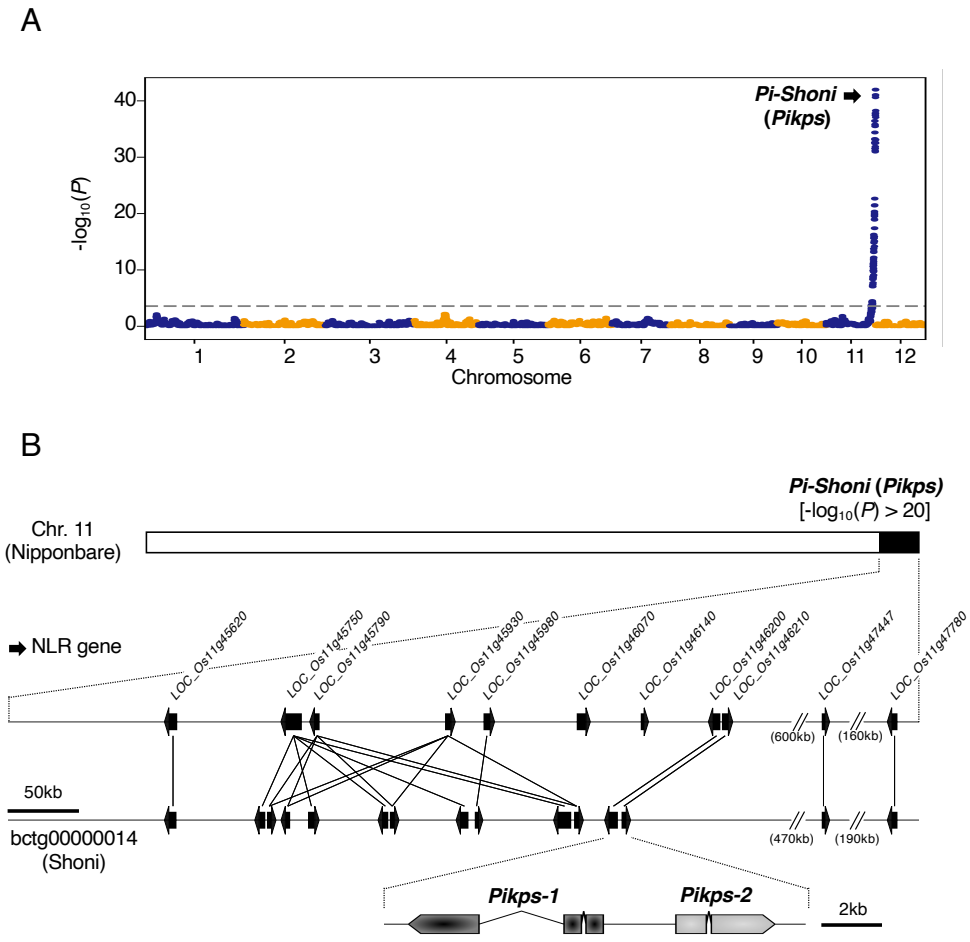


Fig. 2.6. Identification of *Pi-Shoni* (*Pikps*) conferring resistance on Shoni against Naga69-150.
A) QTL analysis of DI scores obtained from the 125 RILs. The dashed line indicates the significance threshold ($-\log_{10}(P) > 3.36$). B) Comparative genomic mapping of the 11 NLR genes in Nipponbare within scaffold bctg00000014 of the Shoni genome assembly. The black arrows indicate NLR genes. *Pikps-1* and *Pikps-2* correspond to *LOC_Os11g46200* and *LOC_Os11g46210*, respectively.

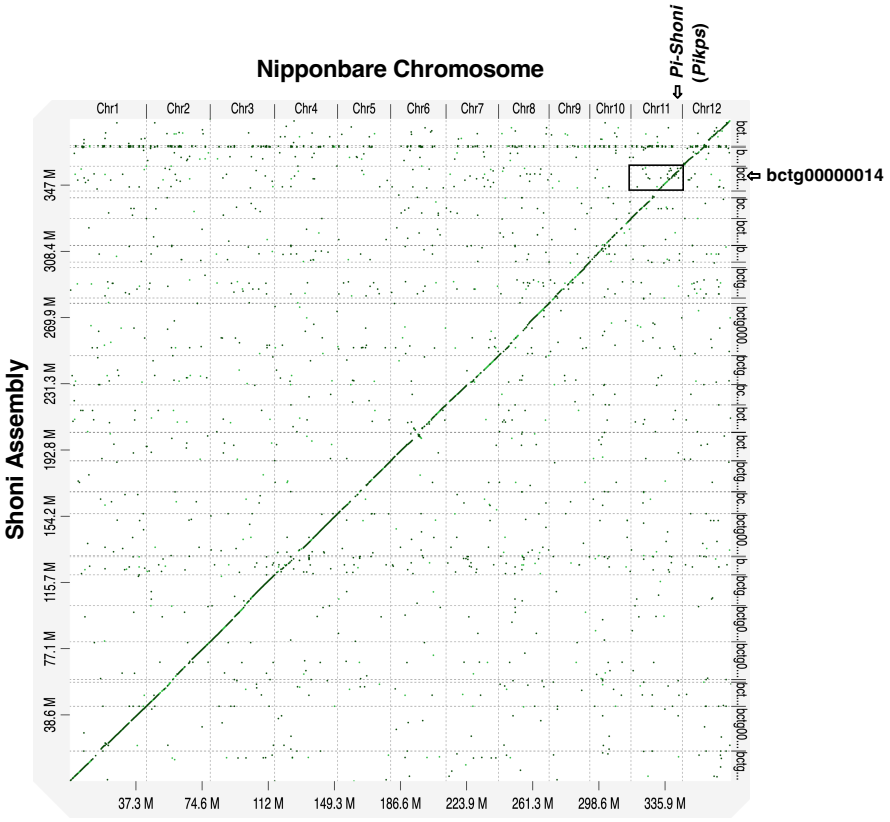


Fig. 2.7 Dot plot analysis of the assembly scaffolds of Shoni and Nipponbare genomes by D-GENIES (https://dgenies.toulouse.inra.fr/). The *Pi-Shoni* region of Nipponbare had synteny with the bctg00000014 scaffold.

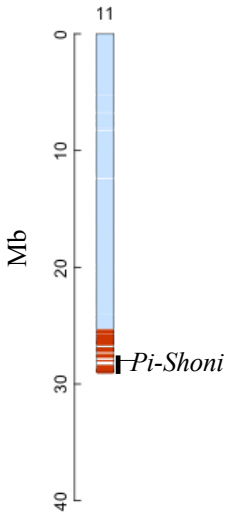


Fig. 2.8. Graphical representation of the genetic architecture of chromosome 11 in RIL#43. Red indicates the genotype of Shoni, blue indicates the genotype of Hitomebore, and white indicates missing genotype.

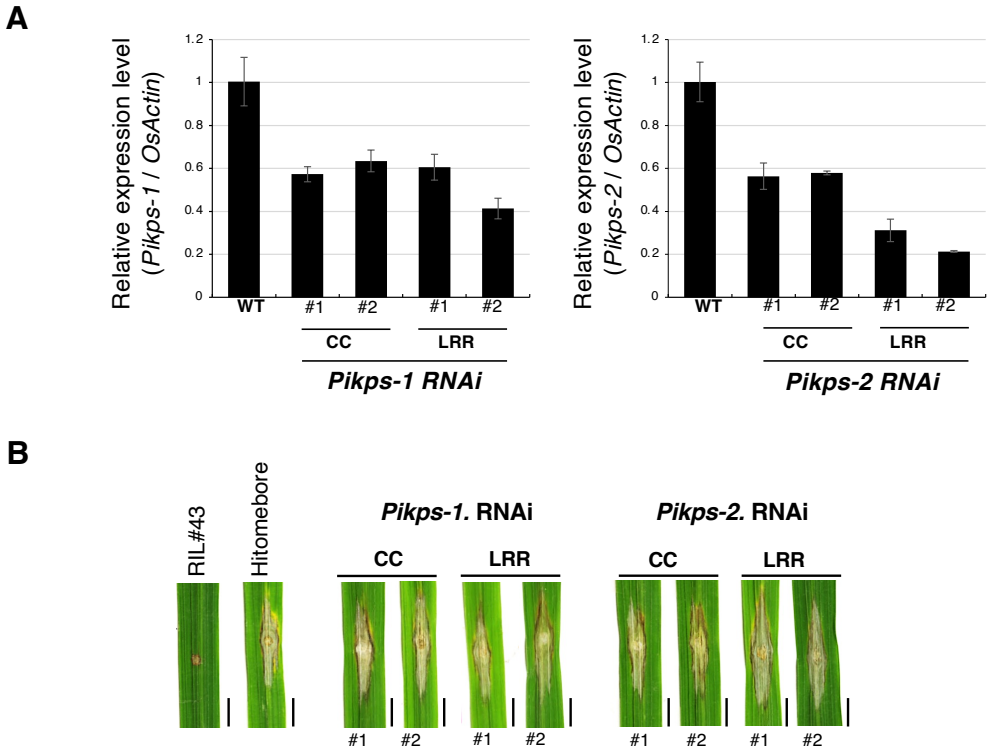


Fig. 2.9. The *Pikps-1* and *Pikps-2* genes of the cultivar Shoni are responsible for its resistance against Naga69-150.
A) Results of gene silencing of *Pikps-1* and *Pikps-2* in the line RIL#43. Numbers below the leaves indicate the callus number. Scale bar, 0.5 cm. B) Reaction of rice cultivars Hitomebore (*Pikp*$^{-}$, *Pikm*$^{-}$), RIL#43 (*Pikps*), K60 (*Pikp*) and Kanto51 (*Pikm*) against two *AVR-Pik* alleles, *AVR-PikD* and *AVR-PikE*. The photographs were taken 10 days after inoculation. WT; wild type Sasa2 isolate of *M. oryzae*. Scale bar, 0.5 cm

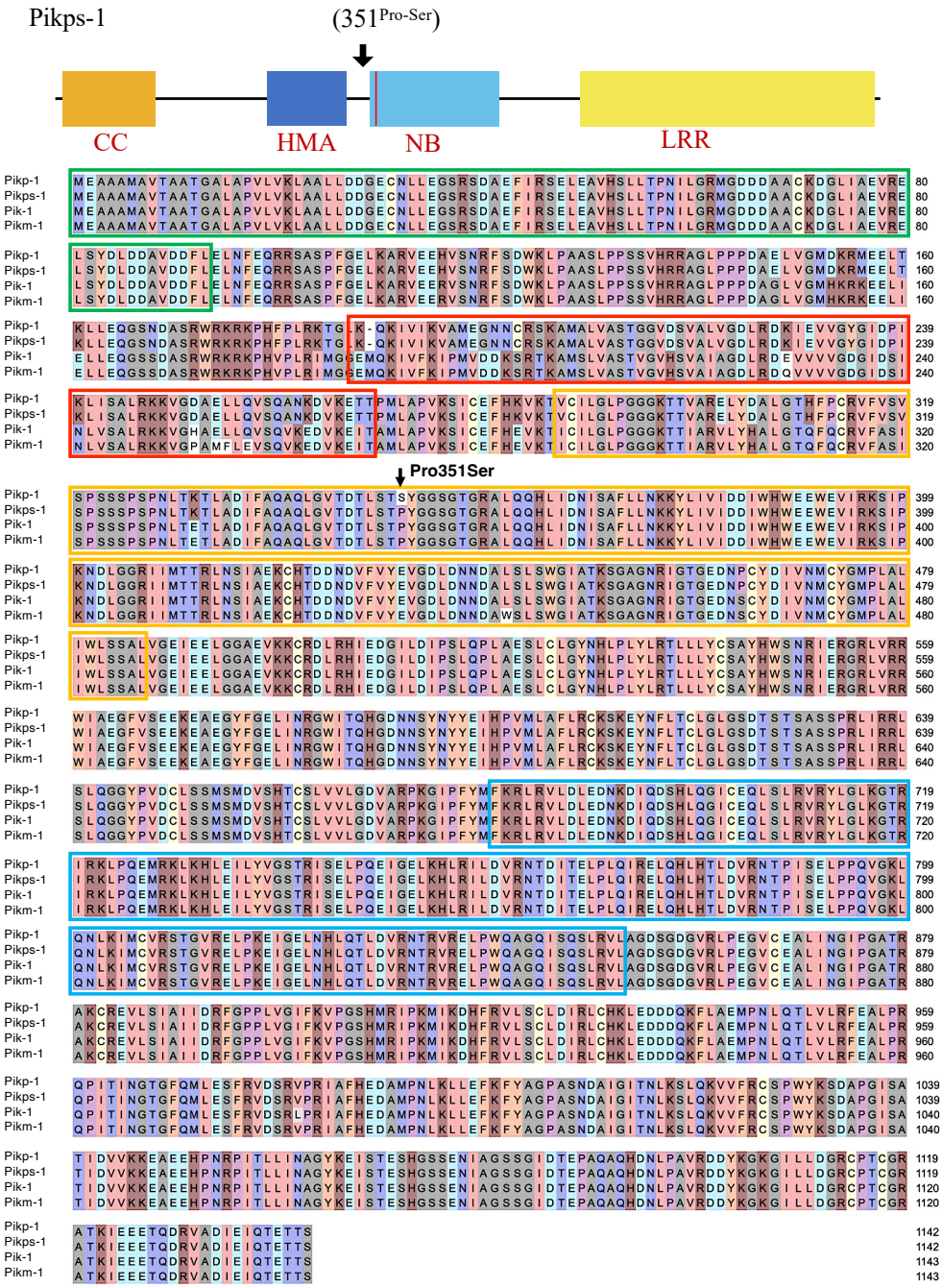


Fig. 2.10. Amino acid sequence alignment of the sensor NLRs of the *Pik* locus. Green box represents coiled-coil domain, red box represents integrated domain (HMA), orange box represents NBS domain and blue box indicates LRR region. The site of amino acid substitution between Pikps-1 and Pikp-1 (Pro351Ser) is shown by a black arrow.

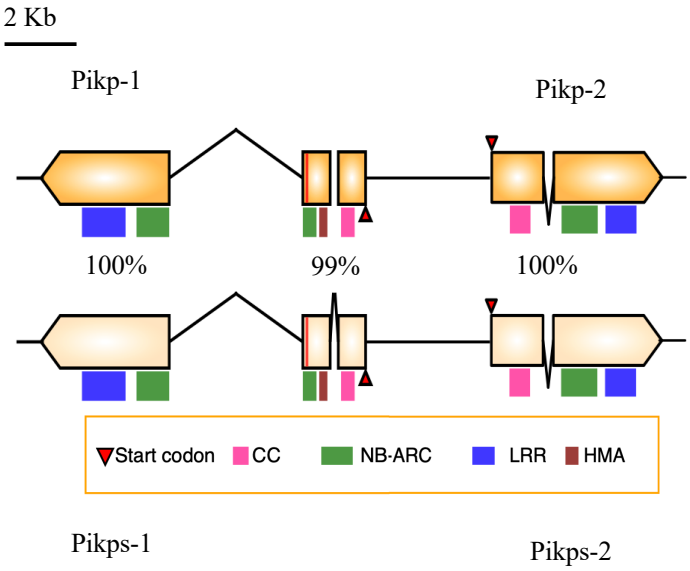


Fig. 2.11. Domainwise structural comparison of Pikp and Pikps amino acid sequences. The site of amino acid substitution between Pikps-1 and Pikp-1 (Pro351Ser) is shown by a red line in the NBS domain.

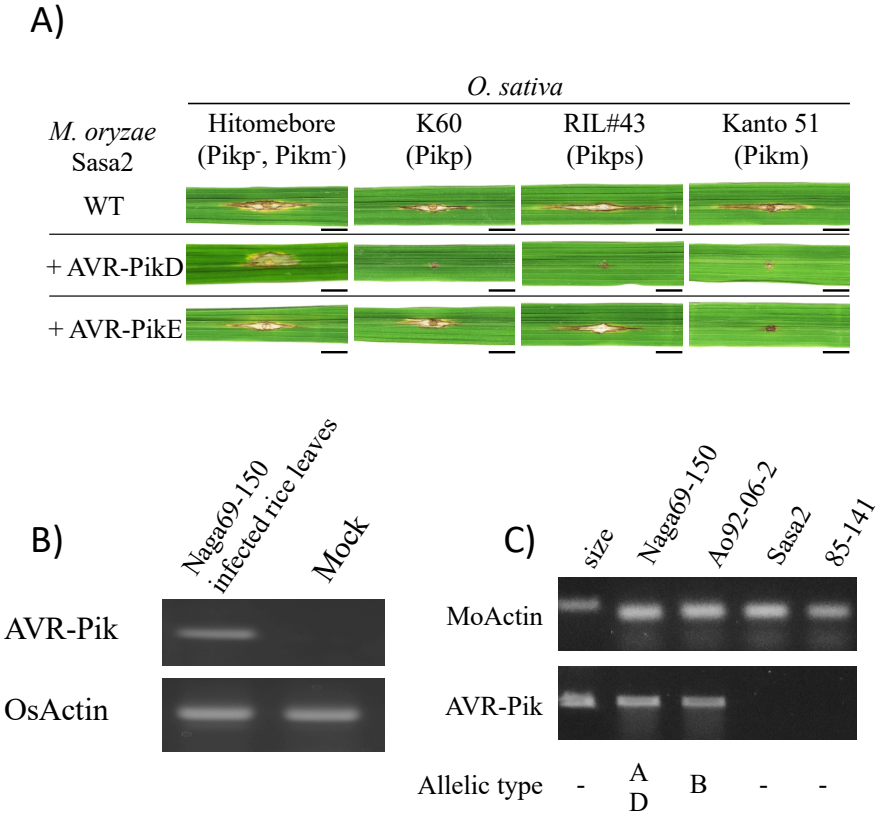


Fig. 2.12. Race specificity test of *Pikps* and confirmation of *AVR-PikD*. A) Reaction of rice cultivars Hitomebore (Pikp^{-}, Pikm^{-}), RIL#43 (Pikps), K60 (Pikp) and Kanto51 (Pikm) against two AVR-Pik alleles, AVR-PikD and AVR-PikE. The photographs were taken 10 days after inoculation. WT; wild type Sasa2 isolate of *M. oryzae*. Scale bar, 0.5 cm. B) RT-PCR confirmation of expression of *AVR-Pik* in leaves of Shoni infected with the Naga69-150 isolate. RNA was extracted from infected leaves 48 hours post inoculation (hpi). Mock, RNA from water-inoculated leaf samples at 48 hpi. The expression of *AVR-PikD* was confirmed using Sanger sequencing and a BLASTN search in NCBI. C) Confirmation of the presence/absence of *AVR-Pik* alleles in *M. oryzae* isolates tested by genomic PCR amplification using an *AVR-Pik* primer pair (F, TCGCCTTCCCATTTTTA; R, GCCCATGCATTATCTTAT). Upper panel represents the actin gene as an internal control. Lower panel represents the presence/absence of *AVR-Pik* alleles. Allelic types were confirmed using Sanger sequencing and a BLASTN search in NCBI.

CHAPTER III

IDENTIFICATION AND ISOLATION OF A BLAST RESISTANCE GENE FROM AN INDICA RICE CULTIVAR TUPA121-3

3.1 Introduction

In the current study, we found that *Oryza sativa* subgroup indica cv. Tupa121-3 shows resistance against thirteen *M. oryzae* strains. We aimed to identify *R* gene of Tupa121-3 against the isolate 85-141 of *M. oryzae* pathogen.

3.2 Materials and methods

3.2.1 Plant materials

The japonica-type rice (*O. sativa* subsp. japonica) cultivar Hitomebore and the indica-type rice (*O. sativa* subgroup indica) cultivar Tupa121-3 (WRC32), provided by the National Agriculture and Food Research Organization World Rice Core Collection (Kojima *et al.*, 2005), were used as parental materials. The resistance reaction of Tupa121-3 to 13 *M. oryzae* isolates panel seems to be strong (Table 3.1). We crossed Tupa121-3 to the cultivar Hitomebore which is highly susceptible to most Japanese *M. oryzae* isolates and obtained F_2 (Figure 3.1). Each F_2 progeny was established as a line and advanced to the F_9 generation by a single-seed descent (SSD) method to generate 150 RILs (Brim, 1966; Goulden, 1939).

3.2.2 Fungal materials

Magnaporthe oryzae isolates used in this study were obtained from MAFF, Japan. Details of the isolates and their race codes are given in Table 3.1. They were maintained on sterilized Whatman filter paper under dry conditions at 4 °C for long-term storage at Iwate Biotechnology Research Centre Kitakami, as described in Molinari and Talbot, (2022). The

seed cultures were transferred to a potato dextrose agar slant (PDA, 50mL of potato dextrose broth, 5g of sucrose, and 4.5g of agar powder, Nacalai Tesque, Kyoto, in 250 mL of water) in a test tube and incubated at 25 ℃. Slant cultures of 1 week to 3 months old were used for preparing spore production on Oatmeal Agar (OMA; 16g Oatmeal powder, 2g sucrose, and 8g of agar in 400mL water) medium. Fungi were grown on OMA for 7-10 days at 26 ℃. Hyphae were scraped using a cotton stick and standard distilled water (SDW) and placed under blue light for four days for spore induction. The spores were scraped in SDW using cotton sticks, and spores were counted in a hemocytometer.

3.2.3 Pathogenicity assay

Five individuals from each RIL and parents were sown in a 9 × 9 cm pot and grown in a greenhouse up to the fifth leaf stage before the inoculation assay. Spore suspension of 3×10^5 conidia ml^{-1} of *M. oryzae* isolate 85-141 was punch-inoculated onto a rice leaf blade one month after seed sowing. The infected plants were kept in the dark dew chamber at 27 °C for 24 hours with high RH. The inoculated plants were then transferred to an incubation chamber with a 16-h light/8-h dark photoperiod. We measured the lesion size at 8 DPI with the help of ImageJ software. Three independent trials were conducted in August 2018, June 2019, and October 2019. Workflow of pathogenicity assay is shown in Fig. 3.2.

3.2.4 Genome assembly and reference guiding of the draft genome

As explained in our previous study, DNA extraction and *de novo* genome assembly were performed. Statistical summary of genome assembly of Tupa121-3 is given in Table 3.2. To further align the contigs into chromosomes, we used RaGOO, a reference-guided contig ordering and orienting tool which takes advantage of the speed and sensitivity of Minimap2 to build chromosome-scale assemblies (Alonge *et al.*, 2019). We used publicly available Nipponbare reference genome version IRGSP-1.0 to align the scaffolds onto chromosomes

(Sasaki and Burr, 2000). Reference scaffolding of *de no* assembled Tupa121-3 reference genome is shown in Fig. 3.3. We also made *de novo* assembly of the reference genome of the susceptible parent Hitomebore following the same method.

3.2.5 Bulked DNA whole genome sequencing of RILs and QTL seq analysis

The DNeasy Plant Mini Kit was used to extract DNA from 100 mg fresh rice leaves (QIAGEN Sciences). As previously reported by Abe *et al.,* (2012) bulked DNA samples were generated by mixing an equal amount of DNA from each individual. The Illumina sequencing library was constructed from 5 µg of DNA sample and sequenced for 76 cycles using an Illumina Genome Analyzer IIx as reported by Abe *et al.*, (2012). Short reads with a phred quality score of 30 or above were omitted from the subsequent analysis. QTL-seq analysis was performed as described by Takagi *et al.* (2013a). The RILs selected for constituting the R and S bulks are shown in the Fig. 3.4 (Appendix 1 and Appendix 2).

3.2.6 Fine mapping population development

A heterozygous individual from RIL#143 was grown in the field in 2020. Seeds were harvested after maturation and dried in a drier at 55 ℃ for seven days to break seed dormancy (Shiratsuchi *et al.*, 2017) and to attain less than 14% moisture content. The seeds were grown in a 296-well tray filled with nutrient-rich soil, as described by Ashkani *et al.*, (2014). The plants were regularly watered and monitored for pest and disease infestation. The seeds were harvested from each individual separately in a small plastic bag and repeated the dormancy breaking and drying procedure until the F_4 stage (Ashkani *et al.*, 2014). The selection of RILs and fine mapping population development is shown in Fig. 3.8.

3.2.7 Molecular marker designing

All the markers used for fine mapping are given in Table 3.4. Sequence alignments were performed between Hitomebore and Tupa121-3 to identify putative CAPs and InDel markers to differentiate them. The CAPS markers were designed using CAPs designer tool in Sol Genomics (Fernandez-Pozo *et al.*, 2015). InDel markers and Sanger sequence-based markers were designed with the help of Premier3 v.0.4.0 (Untergasser *et al.*, 2012), the PCR product of which was visualized using 1.5-2% agarose gel electrophoresis. Sanger sequencing of the PCR products was performed according to the Sanger Sequencing Kit (Thermo Fisher Scientific). Detailed information on type of markers and primers sequences is given in Table 3.4.

3.2.8 Gene prediction and RGA detection

To predict genes of the Tupa121-3 parent, the leaves were inoculated with 85-141 and sampled for RNA-seq at 24 and 48 hpi. A conidial suspension (3×10^5 conidia/mL) of *M. oryzae* isolate 85-141 was spot inoculated onto leaf blades of Tupa121-3 rice plants. The infected plants were placed in a dark dew room at 27 °C and their leaves were collected at 24 and 48 hpi. Inoculated leaf spots were collected through a punch hole and instantly frozen in liquid nitrogen prior to storing at 80 °C. Total RNA was extracted from inoculated leaf samples using an SV Total RNA Isolation System (Promega, WI, USA). For RNA-Seq, 2 μg of total RNA was used to construct cDNA libraries using a TruSeq RNA Sample Prep Kit v. 2 (Illumina, CA, USA). The libraries were subjected to 75 cycles of SE sequencing on the NextSeq 500 platform. The sequence reads were filtered by quality using PRINSEQ (Schmieder and Edwards, 2011). RNA-seq reads were aligned and mapped to the reference genome using Hisat2 (Pertea *et al.*, 2016). Genes were predicted, and transcripts were compiled with the help of StringTie (Pertea *et al.*, 2016). Resistant gene analogs were detected with the help of RGAugury software (Fig. 3.10; Li *et al.*, 2016).

3.2.9 Multiple sequence alignment and gene structure comparison

Multiple sequence alignment of protein sequences was performed using CLUSTAL-W and MAFFT (Katoh and Standley, 2013; Thompson *et al.*, 1994). The structural and domain architecture was searched using the CDD search option of NCBI (Marchler-Bauer *et al.*, 2007).

3.2.10 CRISPR/Cas9-mediated gene knock-out of NLR-1 and NLR-2 candidate genes

Rice knockout mutants of NLR-1 and NLR-2 were generated using the CRISPR-Cas9 system developed by Mikami *et al.*, (2015). Sense and antisense target sequences were designed using the web-based service CRISPRdirect (crispr.dbcls.jp), which were annealed, and cloned into the pU6::ccdB:: gRNA cloning vector following digestion with BbsI. The target sequence with the OsU6 promoter was cloned into the pZH::gYSA:: MMCas9 vector following digestion with AscI and PacI. The resulting vectors (pZH::gYSA::MMCas9-NLR-1 and -NLR-2) were introduced into *Agrobacterium tumefaciens* (strain EHA105) and used for *A. tumefaciens*–mediated transformation of the rice line RIL143#2 following the method of Okuyama *et al.* (2011). The resulting regenerated T_0 plants were sequenced, and mutation type was confirmed using primers listed in Table 3.5.

3.3 Results

3.3.1 Resistance characterization of RILs and parents

The rice cultivar Tupa121-3 (WRC32) belongs to the *O. sativa* subgroup indica and is a member of the World Rice Collection (WRC) as reported by Kojima *et al.* (2005). Tupa121-3 shows resistance to 14 rice *M. oryzae* isolates (Table 3.1). To identify the resistance gene of Tupa121-3, we used *M. oryzae* isolate 85-141 (MAFF238767; race code 037.3) as the pathogen. Punch inoculation tests were conducted for the parents (Hitomebore and Tupa121-3) and 150 recombinant inbred lines (RILs) of the F_9 generation derived from a cross between the two

parents. Disease symptoms of the RILs were measured eight days post inoculation (DPI) with the help of ImageJ software, and their degree of disease resistance was categorized into three classes: resistant (lesion area <9.99 mm^2), resistance similar to Tupa121-3; intermediate (lesion area 10> and <19.99 mm^2), and susceptible (lesion area >20 mm^2), with susceptibility similar to Hitomebore (Fig. 3.4). Based on the distribution of the average lesion area of five individuals from each RIL, a bar plot was generated. Three independent assays were conducted over two years; August 2018, June 2019, and October 2019. In the August 2018 assay, 100 RILs were assayed, and in the other two assays, all 150 RILs were studied. Based on the frequency distribution of resistance among all three assays, the observed segregation ratio between highly resistant and highly susceptible was nearly 4:1 and the frequency distribution was not bimodal, suggesting that multiple loci are involved in the resistance. The expected segregation ratio of a single locus for the RIL population is 1:1 (Liu *et al.*, 2014), but the observed segregation ratio was nearly 4:1, which indicates that Tupa121-3 may have more than one resistance gene for the *M. oryzae* isolate 85-141. The lesion area sizes scored across three independent assays are given in Appendix 3.

3.3.2 QTL-seq analysis and identification of the QTL *Pi-Tupa*

We applied QTL-seq analysis to detect QTLs involved in the resistance of the rice cultivar Tupa121-3 against the fungal pathogen *M. oryzae* isolate 85-141. Using the 150 RILs, we carried out inoculation assay to assess the resistance of the progeny. We selected 25 RILs consistently showing high resistance (lesion area <9.99 mm^2) as Resistant (R-) progeny and another set of 25 RILs consistently showing high susceptibility (lesion area <9.99 mm^2) as Susceptible (S-) progeny. The genomic DNA of R-progeny was bulked in an equal ratio to generate R-bulk DNA, and that of S-progeny was bulked to generate S-bulk DNA. Each DNA bulk was subjected to whole-genome resequencing using an Illumina GAIIx sequencer. The

same method was applied to all three replications independently. The reads obtained were aligned to the reference sequence of the Tupa121-3 cultivar using BWA software (Li and Durbin, 2009). The SNP index was calculated for each SNP and plotted against their genomic positions as described by Takagi *et al.* (2013) (Fig. 3.5). We found a significant QTL from the August 2018 assay on chromosome 05, while in June 2019 assay, we found three significant QTLs; the same major QTL on chromosome 05 and two minor QTLs, one each on chromosome 06 and chromosome 10. The QTL-seq of the October 2019 assay identified two significant QTLs; one on chromosome 05 and one on chromosome 07. Among all the QTLs identified, the QTL on chromosome 05 was consistent amongst the assays. I named the QTL on chromosome 05 as *Pi-Tupa* (Fig. 3.5). I further focused on the QTL *Pi-Tupa*, which spanned a very wide region in August 2018 assay but about 3 Mbp in the case of the other two assays. Information on QTLs identified in this study is given in Table 3.6. I superimposed the 99% confidence region of *Pi-Tupa* QTL from all three assays. The superimposed 99% confidence region of *Pi-Tupa* spanned from 23.8 Mbp to 26.2 Mbp of chromosome 5 (Fig. 3.5, 3.6).

3.3.3 Fine mapping of *Pi-Tupa*.

Careful observation of inoculation assay results of all the RILs allowed us to identify a line RIL#143, which showed segregation in the *Pi-Tupa*-containing genomic region. I designed a CAPs marker BK4 around the peak of the QTL *Pi-Tupa* to detect DNA polymorphisms between the two parents Tupa121-3 and Hitomebore. The result of punch inoculation assay of 34 progeny derived from selfing of RIL#143 showed a perfect correlation with the marker genotype of BK4. The individuals that showed Tupa121-3-type BK4 marker showed highly resistant phenotype, and the individuals with the Hitomebore-type BK4 marker showed highly susceptible phenotype but the BK4-marker heterozygotes exhibited intermediate phenotype (Fig. 3.7). These results suggest that the resistant gene of interest could be a semi-dominant.

Previous reports have shown that semi-dominant genes are sometimes involved in defense mechanism (Du *et al.*, 2019; Tang *et al.*, 2011; Yu *et al.*, 2018). To do the fine mapping of the gene, a heterozygous individual identified by the BK4 marker was selected. A total of 1170 seeds derived from selfing of this heterozygous individual were harvested, and DNA was extracted from each individual grown. Two InDel markers, BK1 and BK2, were designed at the extreme ends of *Pi-Tupa* QTL, and recombinants were identified (Fig. 3.8A).

A total of 420 recombinants were recovered from the F_2 population and advanced to the F_3 generation. Since the phenotype of the heterozygous individuals is uncategorizable between completely resistant and completely susceptible, an attempt was made to identify homozygous recombinants. Since the number of homozygous individuals identified was few, they were advanced to the F_4 generation to recover a sufficient number of seeds (Fig. 3.8B). Multiple homozygous individuals were assayed, and the average lesion area was used for fine mapping. A series of InDel, CAPs, and Sanger sequencing markers were introduced in the candidate region of *Pi-Tupa* (Fig. 3.9ABC). Genotypes of the recombinants and the phenotypes (R or S), average lesion area, and the number of individuals assayed are given in Fig. 3.9D . Based on fine mapping, the recombinant 371 with Tupa121-3 type allele at the marker position BK16 was resistant. Likewise, the recombinant 274 with Tupa121-3 type allele at the marker position BK22 was resistant. These two recombinants limited the candidate gene to 127.9 kb interval between two markers BK15 and BK21 (Fig. 3.9C).

3.3.4 Gene prediction and candidate gene search

To determine the candidate genes within the *Pi-Tupa* region, we performed RNA-seq of Tupa121-3 leaves inoculated with *M. oryzae* isolate 85-141. The RNA-seq reads were mapped to the Tupa121-3 reference genome, revealing 38,552 genes expressed from Tupa121-3 genome. We identified a total of 1,578 resistance gene analogs (RGAs) using "RGAugury"

(Li *et al.*, 2016), which predicted putative genes encoding NLRs, receptor-like kinases (RLKs), and receptor-like proteins (RLPs) (Fig. 3.10; Table 3.3). From the gtf file generated, we found seven candidate genes expressed within the 127.9 kb interval of *Pi-Tupa* (Table 3.6). Three of the seven candidate genes were related to disease resistance; two genes encode NLRs (NLR1 and NLR2), and another encodes receptor-like kinase (RLK). Since most rice resistance genes against *M. oryzae* reported to date are NLRs, we focused on the two NLRs (NLR1 and NLR2) for further gene validation. We compared the amino acid sequences of NLR1 and NLR2 with their respective orthologs of the Nipponbare reference genome (Fig. 3.12). The amino acid comparison revealed that NLR1 of Tupa121-3 is 301 amino acids shorter than its ortholog in Nippobare but has all the three domains required for functioning as a resistance protein. NLR2, on the other hand, has insertion of 106 amino acids. Domain-wise amino acid sequence similarity between Tupa121-3 and Nipponbare is given in Fig. 3.12.

3.3.5 CRISPR/Cas9 mediated knockout of candidate genes *NLR1* and *NLR2*

To validate the function of *NLR1* and *NLR2* in the Tupa121-3 background, we used the seeds of completely resistant individuals from RIL#143 for generating CRISPR/Cas9 mediated gene knockout mutants following the method described in Shimizu *et al.* (2022). We designed three sgRNA primers targeting three positions in each gene. Table 3.5 lists the primers used to create sgRNA constructs. In the *NLR1* knockout experiment, seven T_0 mutants with a guanine base deletion were generated by *NLR1* target 2 (Fig. 3.13A). In the *NLR2* knockout experiment, three T_0 mutants were created, each having an insertion, deletion, and point mutation (Fig. 3.13B). These mutants were challenged with *M. oryzae* isolate 85-141. Based on the phenotypic evaluation, the T_0 individuals heterozygous for *NLR2* gene knockout showed susceptible phenotype (Fig. 3.13B).

In contrast, the the T_0 plants heterozygous for *NLR1* knockout showed a resistant phenotype. Based on these results, we hypothesize that *NLR2* is presumably the candidate gene of *Pi-tupa* (Fig. 3.13B).

3.4 Discussion

3.4.1 The leaf blast-resistant phenotypes in the RILs population were relatively stable in various trials.

A Bangladeshi cultivar Tupa121-3 exhibited high leaf blast resistance (NARO, https://www.gene.affrc.go.jp/databases-plant_search_detail_ en.php?jp=70609). In this study, Tupa121-3 showed high leaf blast resistance to 13 *M. oryzae* isolates (Table 3.1). The frequency distributions of resistance scores among RILs in the three tested seasons were not bimodal, which indicates that the leaf blast-resistant in the RILs population involves multiple loci (Wang *et al.*, 2016). Also, the resistance in RILs were relatively stable in various trials.

Four leaf blast resistance QTLs, *Pi-Tupa*, *qPi-Tupa6–1*, *qPi-Tupa7–1*, and *qPi-Tupa10–1,* from Tupa121-3 were detected by inoculation assay with 85-141isolate (Table 3.7). The *Pi-Tupa* QTL on chromosome 05 consistently detected in three trials, indicating its major contribution to the resistance phenotype. Up to date, only three QTLs conferring blast resistance, *Pi26(t)* (Sallaud *et al.*, 2003), *Pi23* (Ahn *et al.*, 1997), and *Pi-10(t)* (Naqvi *et al.*, 1995) have been identified on chromosome 05. None of the genes have been cloned or verified the function. The *Pi-Tupa* identified in this study is not located in the same region as the previously identified three QTLs and could be a novel leaf blast-resistant gene. The minor QTLs, *qPi-Tupa6–1, qPi-Tupa7–1*, and *qPi-Tupa10–1,* were detected in only one trial in each case, which indicates that environmental factors may greatly influence these minor QTLs.

3.4.2 Seven candidate genes were predicted in the *Pi-Tupa* region

With the fine mapping populations, *Pi-Tupa* was mapped in an interval of 127.9 kb region between the markers BK15 and BK21 (Fig. 3.9C), and seven expressed resistance-related genes were predicted in the interval (Table 3.6). Among these seven candidate genes, NAM05.12949.5 and NAM05.12945.1 encode NLRs and named *NLR1* and *NLR2*, respectively. The predicted gene NAM05.12944.1 encodes DUF26-containing receptor-like kinases named as RLK. Among the cloned 36 resistant genes from rice, 28 genes encode NBS-LRR-containing proteins. The exceptions include pi21 encoding for proline rich protein (Fukuoka *et al.*, 2009), Pid2 encoding for a β-lectin receptor kinase (Chen *et al.*, 2006; CHEN *et al.*, 2010) and *Ptr* encoding for an atypical protein (Zhao *et al.*, 2018). The assay results of heterozygous knockout mutants of *NLR2* gene indicated that NLR2 may be the candidate gene of *Pi-Tupa* (Fig. 3.13B).

This result conforms to the semi-dominant nature of Pi-Tupa (Fig. 3.7). It has been previously reported that semi-dominant genes are involved in the defense mechanism (Du *et al.*, 2019; Tang *et al.*, 2011; Yu *et al.*, 2018). Zhou *et al.* (2017), reported that mutation of *LIL1* gene created a semi-dominant allele, and the *LIL1* mutants displayed increased expression of defense-related genes and enhanced resistance to rice blast fungus. We hypothesize that the gene identified in this study may be a novel gene on chromosome 05. In our future research, we will validate the function of NLR2 using homozygous knockout lines and also by a complementation experiment.

Table 3.1 Reaction of Tupa121-3 cultivar to 13 Japanese *M. oryzae* isolates

Sl. No.	MAFF No.	Race	Designation	Location	Tupa121-3	Hitomobore
1	101523	077.1	Sasamori121	Japan (Yamagata)	R	S
2	238767	037.3	85-141	Japan (unknown)	R	S
3	238976	047.0	83R-131B	Japan (unknown)	R	S
4	238977	047.0	85-259	Japan (unknown)	R	S
5	238978	047.0	85-260	Japan (unknown)	R	S
6	238984	077.1	SL 91-48D	Japan (unknown)	R	S
7	238985	077.1	Ine91-10	Japan (unknown)	R	S
8	238997	107.2	H98-315	Japan (Aichi)	R	S
9	305471	007.-	Naga69-150	Japan (Nagano)	R	S
10	238768	037.3	R115A-19	Japan (unknown)	R	S
11	-	007.4	2403	Japan (unknown)	R	S
12	-	037.1	Sasa2	Japan (unknown)	R	S
13	-	007.4	2012-1	Japan (unknown)	R	S

R; Resistant, S; Susceptible

Source: Genebank Pro1ject, NARO, (https://www.gene.affrc.go.jp/databases-plant_search_detail_en.php?jp=70609)

Table 3.2 Summary of genome assembly of the resistance line Tupa121-3. Genome assembly was performed by NECAT software

No. of contigs	36
Contig N50 (Mbp)	18.5
Largest contig (Mbp)	44.2
Genome assembly (Mbp)	386.9

Table 3.3 The total resistance gene analogs detected in the Tupa121-3 cultivar by RGAugury software.

Sl. No	Class of genes	Number of genes
1	NBS	80
2	CNL	124
3	CN	62
4	TN	1
5	NL	161
6	TX	1
7	RNL	1
8	RLP	111
9	RLK	906
10	TM-CC	137
11	RPW8	3
	Total	1587

Table 3.4 Primers used for fine mapping of the QTL, *Pi-Tupa*.

No	Primer	Forwar primer sequence	Reverse primer sequence	Type	Enzyme
1	BK1	AAACAACTCGCATGGTGCAC	AATCCCAATAGACGGCCAGC	InDel	-
2	BK3	TTTTTGTAAGCTAGTGTAAAAGT	TCTCAATGGCTGTACATTCCTC	InDel	-
3	BK5	ATAATATGCAATACCTTGGATCC	AGCTTGAGCTTCCGGAGATT	CAPs	DraI
4	BK7	TCCTTGCTATTTTTGGCTACAAG	CGCGCTTGGACGCCATGAGGAAC	Sanger sequenceing	-
5	BK8	ACCTGGACCCGGAGTACTACATG	GGCTGATCTCCACGTCGCTGTAG	Sanger sequenceing	-
6	BK9	GACGATGATCAGGAATAAACCAG	AGACATGCACGCAAACAATTAC	Sanger sequenceing	-
7	BK10	CACAAGGTACTGGTGCCATGAATG	ACTTGGGTCGCTCTCAGCCAAATG	Sanger sequenceing	-
8	BK11	CCCCTCGTCCATGATGTCTATG	GAGAGCTGATTGTCCACACGAC	Sanger sequenceing	-
9	BK19	GGGTCAGTTTTCCCTTGGCA	GGACGCAAATAGTGGTGGCT	InDel	-
10	BK20	CGAGGAAAAGATAGGACTCGGT	AGGTGGTAGCTCTAGTCTGTGT	InDel	-
11	BK21	TGACAAGCTGGTTAGGTGCC	TGGACTTCGTGCGGAACATA	InDel	-
12	BK22	AGGACATCCTCTCGAGGGAT	AGTTTCAGTTTGCGTGCGTC	InDel	-
13	BK23	AGTTGACTGAGCAGCTTGGC	CTGTCCCAGGGCTTGCATAA	InDel	-
14	BK4	GAAGCGATGTTTTGCGTTTTGCT	TTCACCTGCCTCTTCTGCACTAT	CAPs	HindII
15	BK18	GCGGGGGACCTGCTGTTCGTGTC	GCGAATGTCTCGTCGTCGCTGTG	Sanger sequenceing	-
16	BK17	AGCATCCTCACCTGCTGTTC	CCGCTCCTCTGCATCTTCAA	InDel	-
17	BK16	GGAAGGAGTTTTCGGACACG	GAGTCAGAAAGTTGCAAGTTGC	InDel	-
18	BK15	CTCTTCTCACGGTACTCCCC	AGTCAAGAGGAGACCATAACCA	InDel	-
19	BK14	TGCATGAGGGTCAGAGGGTT	ACCTCCTCTTCTTCAAGGCG	InDel	-
20	BK13	ATCCATGGCGATGAGAAATCT	CTAGCCCTTCCCAAGCCAAA	InDel	-
21	BK12	CTGGTGAACGTGTACCCCTACTTC	AGCAGCTGAAAGAAAGAAATGTG	Sanger sequenceing	-
22	BK6	CAGGGACGCGATCGAAAGTA	AATGGGGCACGAGAAGGAAG	InDel	-
23	BK2	AAACAACCACTTTACACACAGCA	TTGCTCGATCAAAAGTCGAA	InDel	-

Table 3.5 List of primers used to design sgRNA constructs

Target gene	Primer	Forward primer sequence	Reverse primer sequence
NLR1	NAM05.12949.5_C9-1	gttgTGCTGAGGCTCGGCAACTTA	aaacTAAGTTGCCGAGCCTCAGCA
NLR1	NAM05.12949.5_C9-2	gttgTCCATGGTACTGCTCATATC	aaacGATATGAGCAGTACCATGGA
NLR1	NAM05.12949.5_C9-3	gttgAGTACTTCCAGTTACGGGTG	aaacCACCCGTAACTGGAAGTACT
NLR2	NAM05.12945.1_C9-1F	gttgTGTGCTGTCCGCGTTCATGC	aaacGCATGAACGCGGACAGCACA
NLR2	NAM05.12945.1_C9-2F	gttgGGATTGAGGGGAAGATCGAT	aaacATCGATCTTCCCCTCAATCC
NLR2	NAM05.12945.1_C9-3F	gttgAAGCAGTCTGTCGACAATTC	aaacGAATTGTCGACAGACTGCTT

Table 3.6 List of seven candidate genes flanked by markers BK15 and BK21.

Sl. No	Candidate	Nipponbare locus ID	FPKM values	Function in Nipponbare annotation project
1	NAM05.12950.1	LOC_Os05g41220	18.553658	SNF1-related protein kinase regulatory subunit beta-1, putative, expressed
2	NAM05.12948.2	LOC_Os05g41240	13.741819	Myb-like DNA-binding domain containing
3	NAM05.12945.1	LOC_Os05g41310.1	0.212365	disease resistance RPP13-like protein 1, putative, expressed. NLR2
4	NAM05.12949.5	LOC_Os05g41290.1	0.288749	protein|disease resistance RPP13-like protein 1. NLR1
5	NAM05.12944.1	LOC_Os05g41370	1.381647	TKL_IRAK_DUF26-la.1 - DUF26 kinases have homology to DUF26 containing loci, expressed RLK
6	NAM05.12943.1	LOC_Os05g41380	1.237443	expressed protein
7	NAM05.12937.1	LOC_Os05g41510	21.256019	SH2 motif, putative, expressed

Table 3.7 Four QTLs identified by QTL-seq analysis of three experimental data

Sl. No.	QTL	Chromosome	August 2018	June 2019	June 2019
			99% Genomic position (mbp)		
1	Pi-Tupa	05	21.4-27.4	23.8-26.2	23.0-26.8
2	qPi-Tupa6–1	06	-	1.8-18.5	-
3	qPi-Tupa7–1	07	-	-	15.4-25.1
4	qPi-Tupa10–1	10	-	6.6-12.1	-

Fig. 3.1. Development of materials required for the study

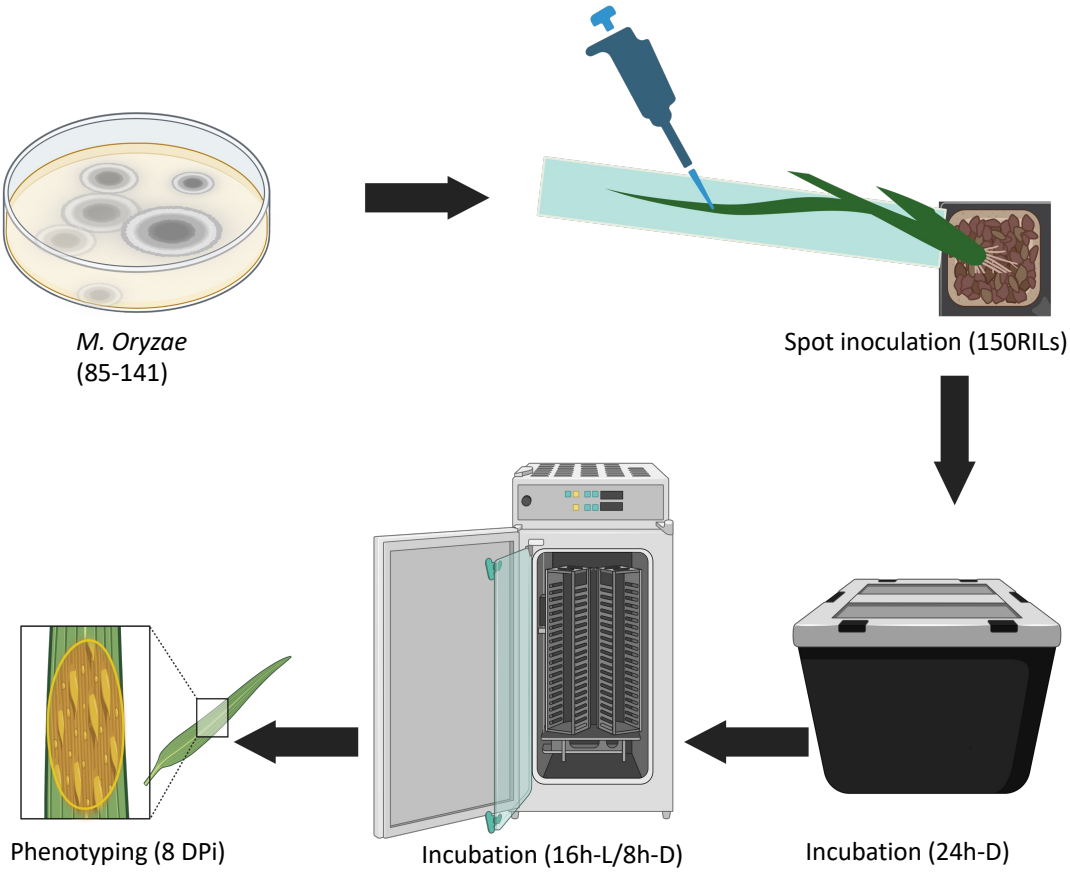


Fig. 3.2 Experimental procedures of the pathogenicity assay

Reference
***Oryza sativa japonica*; "Nipponbare"**
(International Rice Genome Sequencing Project. 2005. Nature)

Contigs
"Tupa121-3 reference genome"
(Present study)

RaGOO: fast and accurate reference-guided scaffolding of draft genomes

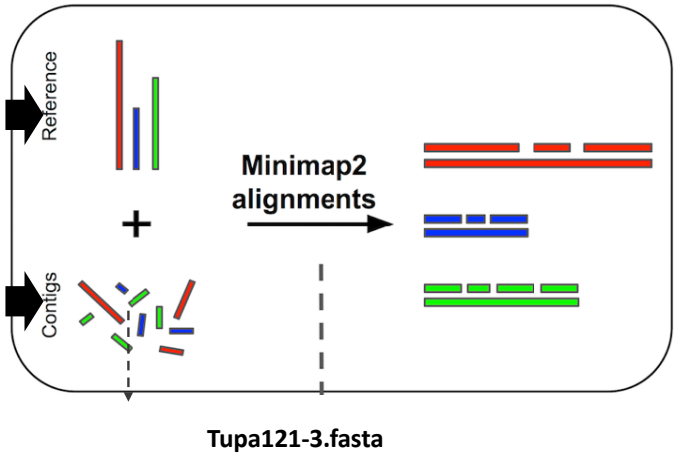


Fig. 3.3. Chromosome scale reference scaffolding of *de novo* assembled Tupa121-3 genome.

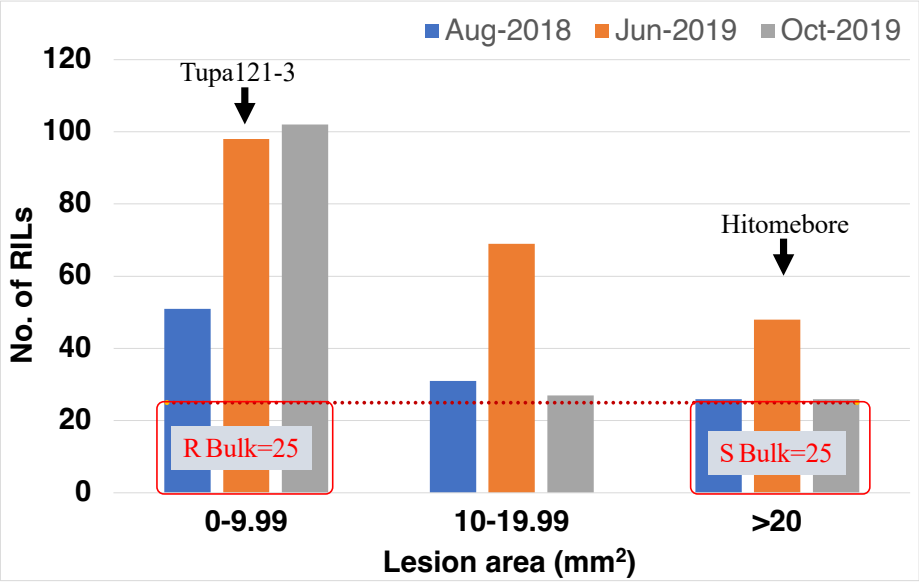


Fig. 3.4 Resistance assay of 150 RILs against 85-141 isolate of *Magnaporthe oryzae*. Frequency distribution of resistance for 125 RILs derived from a cross between Hitomebore and Tupa121-3. Arrows indicate lesion obtained for the parental (Hitomebore and Shoni) lines. Blue bars indicate experiment conducted in August 2018 (n=100), orange bars represent experiment conducted in June-2019 (n=150), grey bars represent experiment conducted in October-2019 (n=150). 25 individuals within red boxes were selected for generating resistant bulk and susceptible bulk.

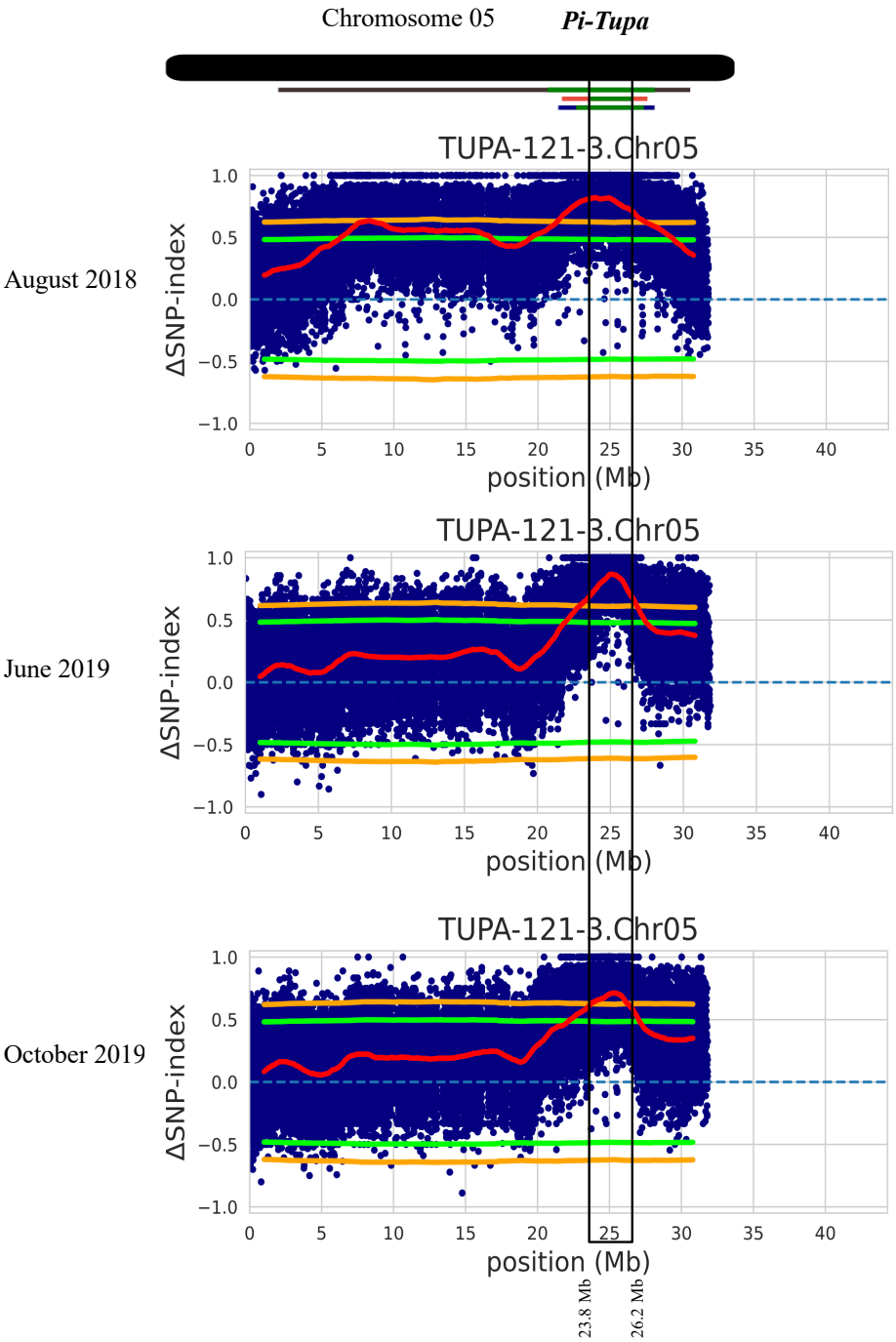


Fig. 3.5. Identification of a QTL for blast resistance using QTL-seq. X-axis; genomic position, y-axis; SNP-index, Orange line indicate 99% confidence threshold line, green line depict 95% confidence threshold line and red line indicate mean SNP-index. Blue dot represent SNP.

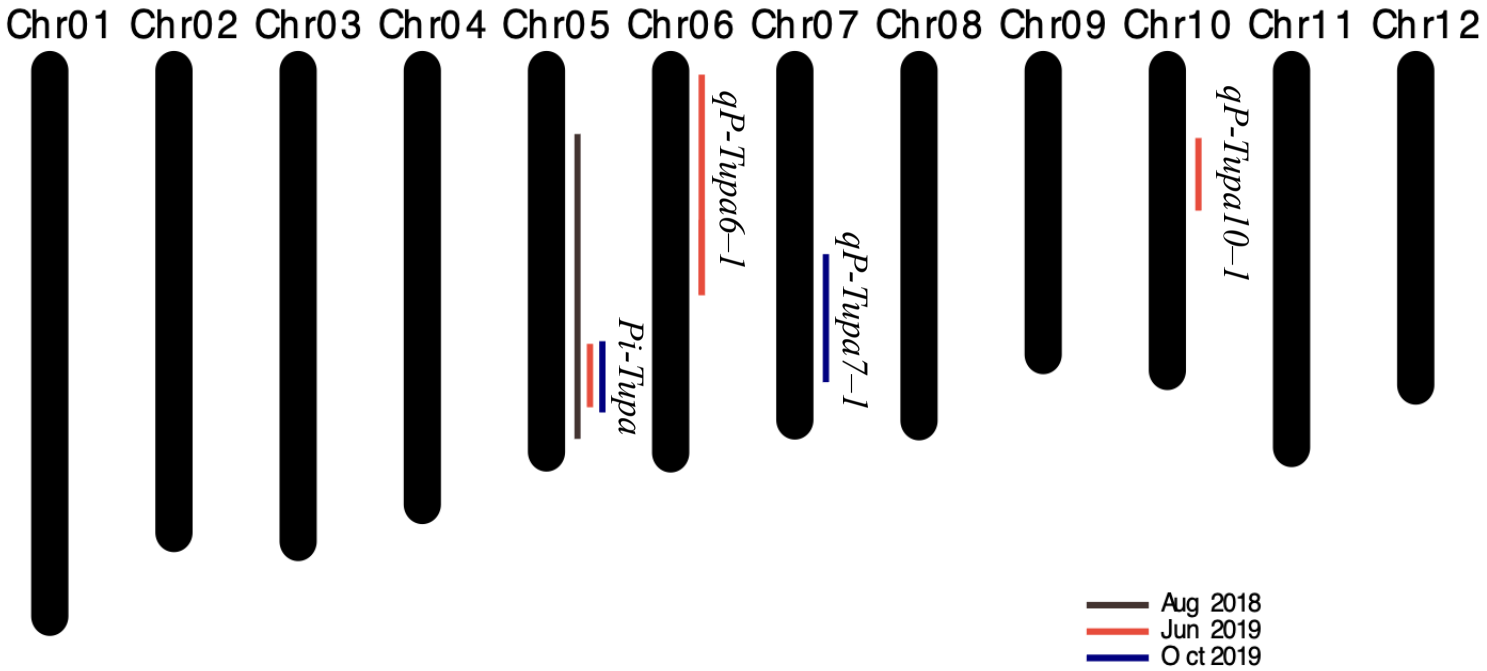


Fig. 3.6. Identification of leaf blast resistance QTLs in Tupa121-3 by QTL-seq analysis. Four QTLs were detected by QTL-seq analysis of RIL population derived from cross between Tupa121-3 and Hitomebore. Grey line indicate QTL identified by August 2018 phenotyping data. Red lines indicate QTLs identified by June 2019 phenotyping data. Blue line indicate QTLs identified by October 2019 phenotyping data.

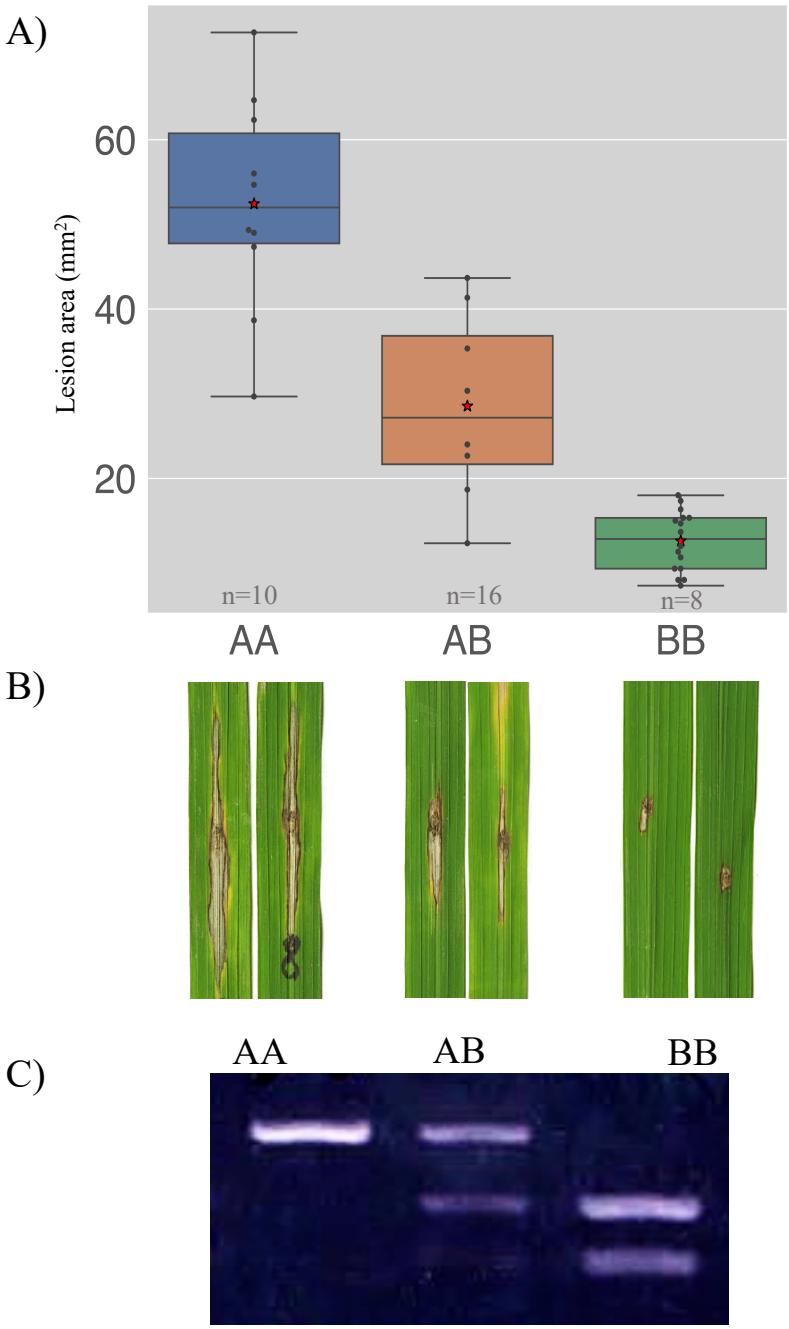


Fig. 3.7. Assay results of 34 individuals from segregating RIL#143 at QTL *Pi-Tupa*. A) Boxplot representing 34 individuals genotype and phenotype correlation. Blue box; lesion area of Hitomebore type individuals, green box; lesion area of individuals with Tupa121-3 type genotype, orange box; lesion area of heterozygous individuals. B) Phenotype of representative individuals from segregating RIL#143. C) Genotype of representative individuals from segregating RIL#143 at BK4 CAPs marker. AA ; Hitomebore, AB ; Heterozygote, BB ; Tupa121-3.

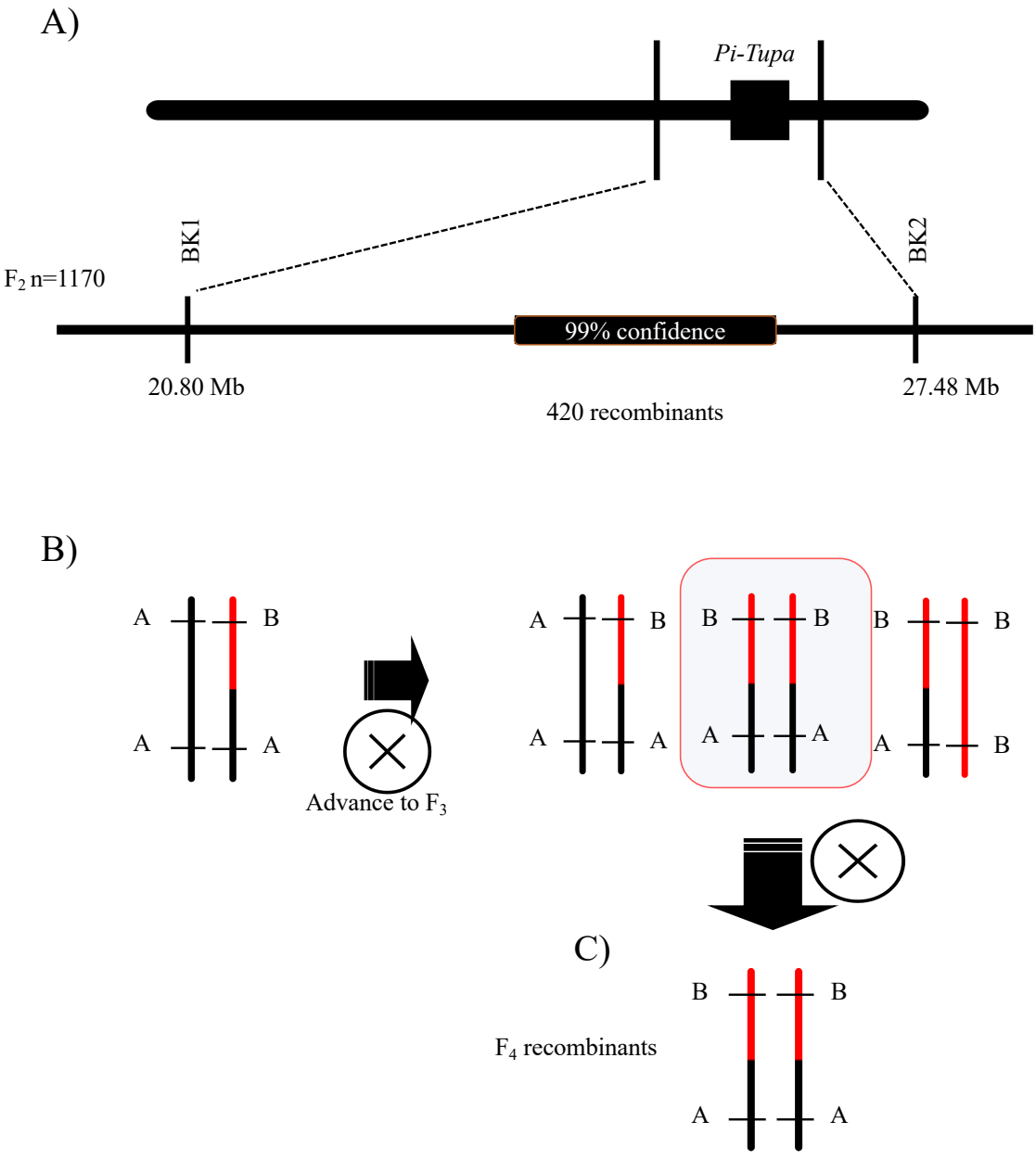
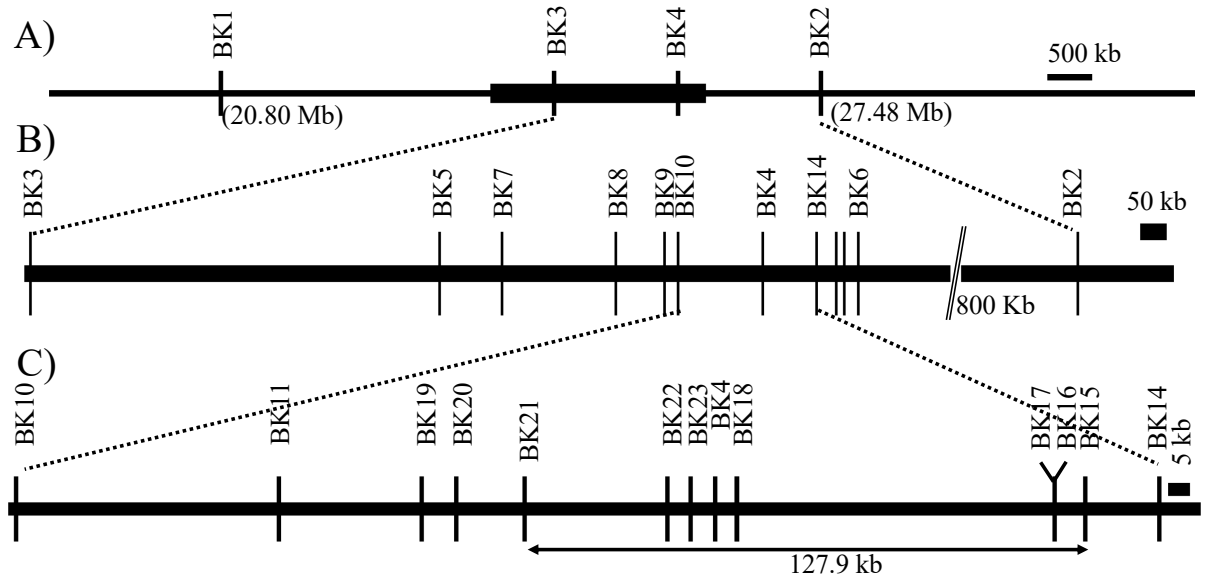


Fig. 3.8. Fine mapping population development. A) Expansion of QTL *Pi-Tupa* showing the two markers covering outside of QTL region. B) Genotypic representation of recombinants identified. Representative recombinants selected for advancing to next generation. C) Representative homozygous recombinants used for assay.



D)

Line	BK3	BK5	BK7	BK8	BK9	BK10	BK11	BK19	BK20	BK21	BK22	BK23	BK4	BK18	BK17	BK16	BK15	BK14	BK13	BK12	BK6	BK2	Phenotype	Lesion area (mm2)	No. individuals
438	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	H	R	4.88	27
868	T	T	T	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	S	43.83	7
638	T	T	T	T	T	T	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	S	38.25	10
371	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	H	H	H	H	H	H	R	8.33	17
274	H	H	H	H	H	H	H	H	H	H	T	T	T	T	T	T	T	T	T	T	T	T	R	6.94	14
426	H	H	H	H	H	H	H	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	R	13.75	4
1156	T	T	T	T	T	T	T	T	H	H	H	H	H	H	H	H	H	H	H	H	H	H	S	56.40	7

Tupa genotype
Hitomebore genotype

Fig. 3.9. Fine mapping of Pi-Tupa in Tupa121-3. A) *Pi-Tupa* was located between BK1 and BK2. 420 recombinants were screened between BK1 and BK2. B) Seven recombinants were screened from BK3 , and *Pi-Tupa* was located between markers BK2 and BK3. C) Two recombinants were screened, *Pi-Tupa* was finally flanked by markers BK14 and BK21 in the region of 127.9 kb. d) Genotype of seven recombinants screened by BK2 and BK3 markers. Red box Hitomebore genotype, blue box Tupa121-3 genotype. Right side of D) represents phenotype, mean lesion area and no of individuals screened. R; resistant, S; susceptible

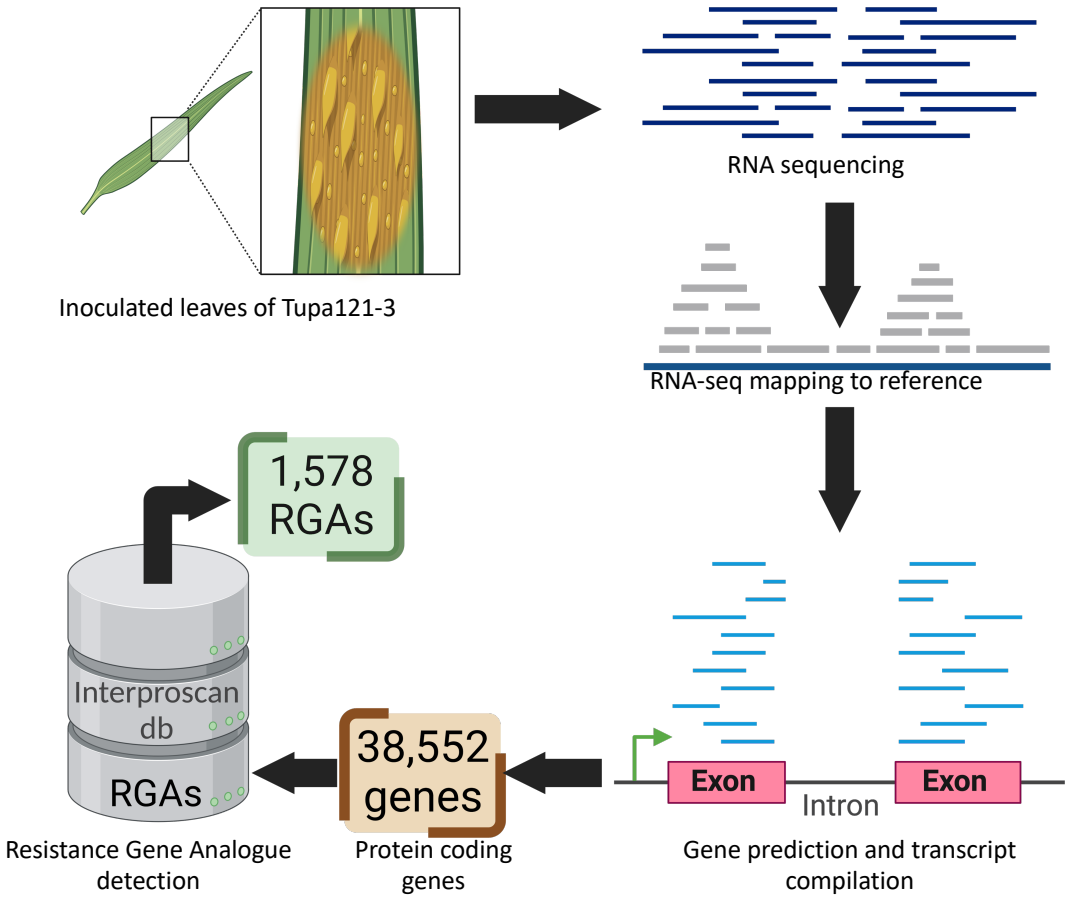


Fig. 3.10. Flowchart of gene prediction from Tupa121-3 parent.

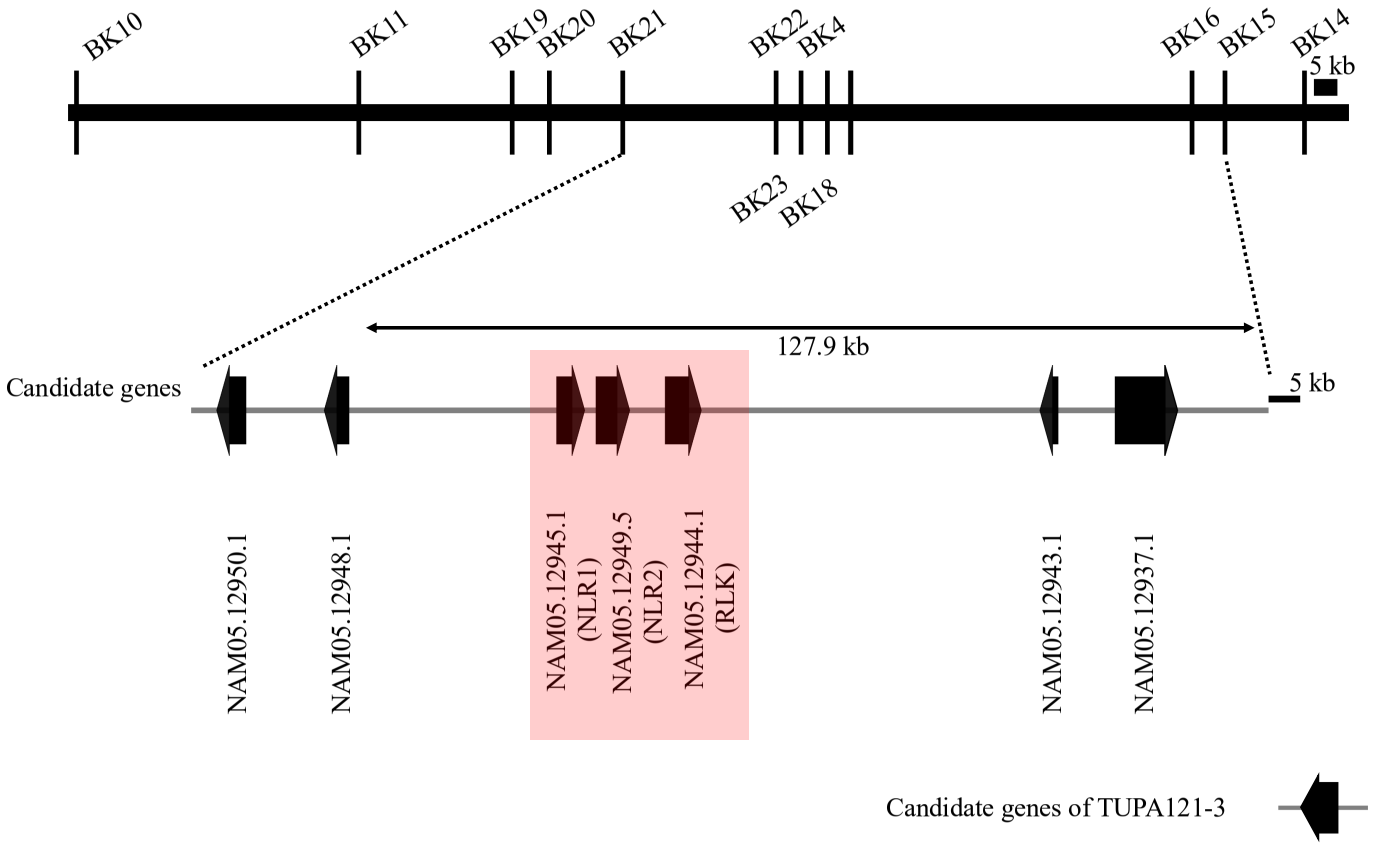


Fig. 3.11 Expressed candidate genes within the 127.9 kb of *Pi-Tupa*. Seven candidate genes were expressed within the region flanked by markers BK14 and BK21. Arrow represents the orientation of the candidate gens. Candidate highlighted by red box are the genes related with the disease resistance from the Nipponbare annotation project. Labels in the parenthesis are the names given after checking the function in Nipponbare annotation project.

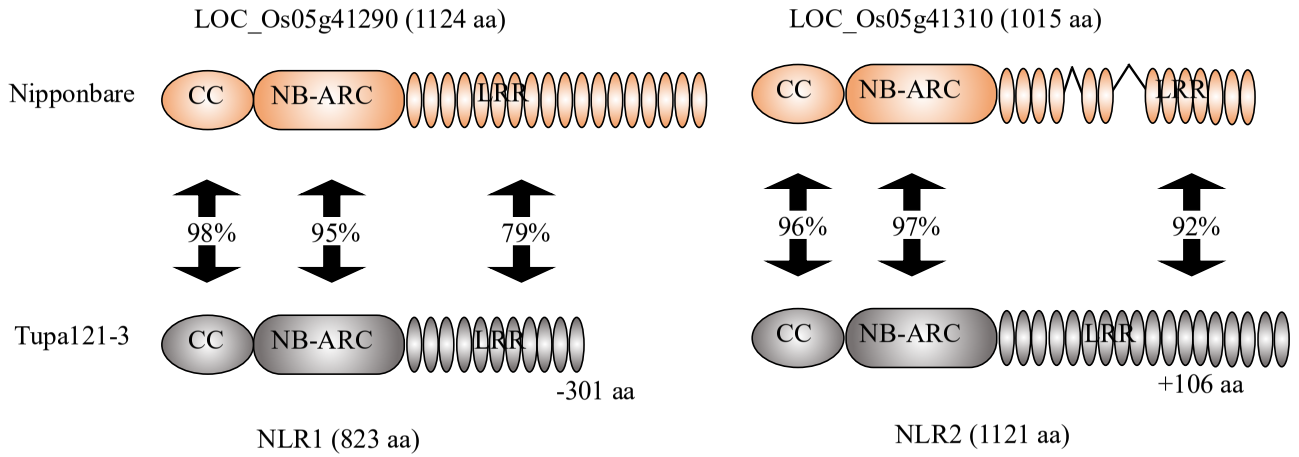
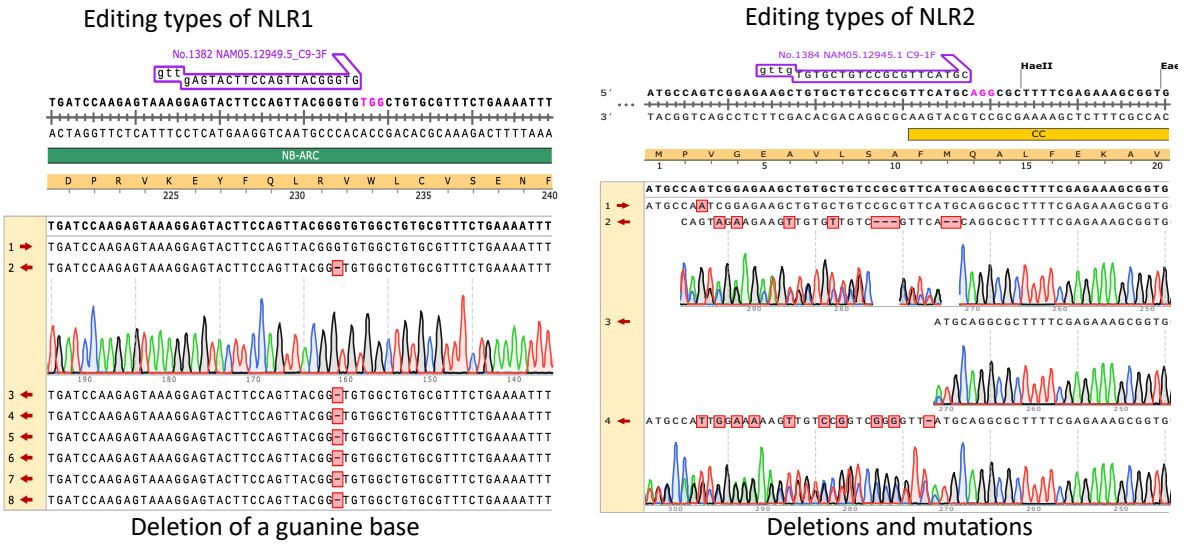


Fig. 3.12. Domainwise structural comparison of NLR1 and NLR2 in Tupa121-3 with their Nipponbare orthologs based on amino acid sequences. CC; coiled coil domain, NB-ARC; Nucleotide binding-ARC domain, LRR; Leucine rice repeat receptor region. Percentage indicate percent similarity between Tupa121-3 and Nipponbare orthologs within each domain.

A)



B)

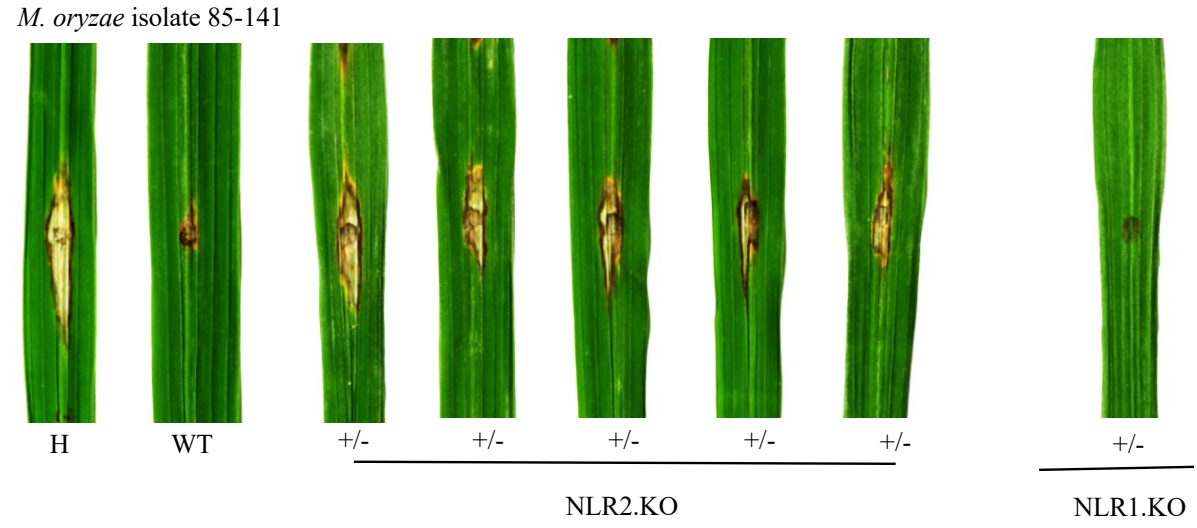


Fig. 3.13. Validation of *Pi-Tupa* candidate genes NLR1 and NLR2 by CRISP/Cas9 experiment. A) Genome editing types of NLR1 and NLR2. Purple boxes indicate the sgRNA target of sense strand. Red highlighted sequence represent PAM sequence. Guanine base deletion in NLR1 is represented by red hyphen. 1 in both the case is Hitomebore aligned to WT type Tupa121-3 sequence. 2,3,4,5,6,7,8 are the mutant types. Mutations are highlighted with red bases. B) Punch inoculation assay results of T_0 genome edited lines of NLR1 and NLR2 genes. +/- represent heterozygous type of edited lines. H; Hitomebore, WT; Wild type of unedited line carrying Pi-Tupa.

CHAPTER IV

SUMMARY

In this study, we attempted to dissect the resistance of two Bangladeshi cultivars, Shoni and Tupa121-3, obtained from the National Agriculture and Food Research Organization World Rice Core Collection (Kojima *et al.*, 2005). According to the data from NARO, the cultivars Shoni and Tupa121-3 show high field resistance to blast disease caused by Japanese *M. oryzae* isolates. We used these two cultivars as pollen parents and crossed to a founder parent Hitomebore which is a highly blast susceptible cultivar against the majority of the *M. oryzae* isolates. Two different RIL populations were developed following the single-seed descent method. These two populations were challenged with two different *M. oryzae* isolates and we tried to identify resistance genes involved.

In the first study, we identified a new allele, *Pikps,* from the aus-type cultivar Shoni. The cultivar Shoni shows resistance against 10 *M. oryzae* strains. By a QTL mapping and candidate gene cloning strategy, we identified an allele of the rice blast-resistant gene *Pik* and named it *Pikps*. Rice blast resistance evaluation of cloned genes indicated that, like other *Pik* alleles, *Pikps* also consists of two NLR genes (*Pikps-1* and *Pikps-2*). Characterization of *Pikps* showed that its resistance spectrum and race specificity are similar to that of the *Pikp* allele of the Pik locus. Furthermore, Shoni showed resistance against five isolates and based on gene conservation analysis it was found that resistance of Shoni to five isolates may be either because of the presence of functional alleles of *Pit* and/or *Pita* or other cloned *R* genes, as described in Sahu *et al.*, (2022), or due to the presence of novel *R* genes. Further study is needed to dissect these five isolates' corresponding *R genes* in Shoni. Since the Shoni cultivar might harbor many resistance genes, it could serve as a good source of genes for deployment/pyramiding strategy to enhance resistance breeding.

In our second study, Tupa121-3 showed resistance against 13 *M. oryzae* strains. By QTL-seq analysis, we found a significant QTL on chromosome 05 and named it *Pi-Tupa*. With a fine mapping approach, *Pi-tupa* was finally mapped in an interval of 127.9 kb region between markers BK15 and BK21. There were seven candidate resistance-related genes predicted and expressed in the interval of Tupa121-3 genome. Among them, NAM05.12949.5 and NAM05.12945.1 encoded a nucleotide binding leucine rice repeat domain-containing proteins and named them *NLR1* and *NLR2*, respectively, and NAM05.12944.1 encoded DUF26 containing receptor-like kinases named as *RLK*. CRISPR/Cas9 mediated gene knockout of *NLR1* and *NLR2* revealed that *NLR2* is presumably involved in the resistance of Tupa121-3 against 85-141 *M. oryzae* isolate. In our future research, we will validate the function of *NLR2* through inoculation assay of homozygous knockout mutants and complementation assay. We are currently attempting isolation of the corresponding AVR gene. Once *R* gene and *AVR* gene pair is identified, molecular interaction between them will give a clear picture of recognition mechanism. Also, the *R* gene of Tupa121-3 would be useful to confer resistance in japonica-type rice.

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References

Abe, A., Kosugi, S., Yoshida, Kentaro, Natsume, S., Takagi, H., Kanzaki, H., et al. (2012) *Genome sequencing reveals agronomically important loci in rice using MutMap*. *Nat Biotechnol*, **30**, 174–178.

Adachi, H., Derevnina, L., and Kamoun, S. (2019) *NLR singletons, pairs, and networks: evolution, assembly, and regulation of the intracellular immunoreceptor circuitry of plants*. *Current Opinion in Plant Biology*, **50**, 121–131.

Ade, J., DeYoung, B.J., Golstein, C., and Innes, R.W. (2007) *Indirect activation of a plant nucleotide binding site–leucine-rich repeat protein by a bacterial protease*. *Proc National Acad Sci*, **104**, 2531–2536.

Agrios, G.N. (1998) *Plant Pathology*. Acadenic Press.

Ahn, S., Kim, Y., Hong, H., Choi, H., Moon, H., Han, S., and Mccouch, S. (1997) *Mapping of genes conferring resistance to Korean isolates of rice blast fungus using DNA markers*. *Korean Journal of Breeding (Korea Republic)*.

Alonge, M., Soyk, S., Ramakrishnan, S., Wang, X., Goodwin, S., Sedlazeck, F.J., et al. (2019) *RaGOO: fast and accurate reference-guided scaffolding of draft genomes*. *Genome Biol*, **20**, 224.

Ashikawa, I., Hayashi, N., Yamane, H., Kanamori, H., Wu, J., Matsumoto, T., et al. (2008) *Two Adjacent Nucleotide-Binding Site–Leucine-Rich Repeat Class Genes Are Required to Confer Pikm-Specific Rice Blast Resistance*. *Genetics*, **180**, 2267–2276.

Ashkani, S., Rafii, M.Y., Shabanimofrad, M., Ghasemzadeh, A., Ravanfar, S.A., and Latif, M.A. (2014) *Molecular progress on the mapping and cloning of functional genes for blast disease in rice (Oryza sativa L.): current status and future considerations*. *Critical Reviews in Biotechnology*, **36**, 353–367.

Baudin, M., Hassan, J.A., Schreiber, K.J., and Lewis, J.D. (2017) *Analysis of the ZAR1 Immune Complex Reveals Determinants for Immunity and Molecular Interactions*. *Plant Physiology*, **174**, 2038–2053.

Bentham, A.R., Zdrzałek, R., Concepcion, J.C.D. la, and Banfield, M.J. (2018) *Uncoiling CNLs: Structure/Function Approaches to Understanding CC Domain Function in Plant NLRs*. *Plant and Cell Physiology*, **59**, 2398–2408.

Bernoux, M., Burdett, H., Williams, S.J., Zhang, X., Chen, C., Newell, K., et al. (2016) *Comparative Analysis of the Flax Immune Receptors L6 and L7 Suggests an Equilibrium-Based Switch Activation Model*. *The Plant Cell*, **28**, 146–159.

Białas, A., Zess, E.K., Concepcion, J.C.D. la, Franceschetti, M., Pennington, H.G., Yoshida, K., et al. (2018) *Lessons in Effector and NLR Biology of Plant-Microbe Systems*. *Mol Plant-microbe Interactions*, **31**, 34–45.

Brim, C.A. (1966) *A Modified Pedigree Method of Selection in Soybeans1*. *Crop Science*, **6**, cropsci1966.0011183X000600020041x.

Cabanettes, F. and Klopp, C. (2018) *D-GENIES: dot plot large genomes in an interactive, efficient and simple way*. *PeerJ*, **6**, e4958.

Casey, L.W., Lavrencic, P., Bentham, A.R., Cesari, S., Ericsson, D.J., Croll, T., et al. (2016) *The CC domain structure from the wheat stem rust resistance protein Sr33 challenges paradigms for dimerization in plant NLR proteins*. *Proc National Acad Sci*, **113**, 12856–12861.

Cesari, S., Bernoux, M., Moncuquet, P., Kroj, T., and Dodds, P. (2014) *A novel conserved mechanism for plant NLR protein pairs: the 'integrated decoy' hypothesis*. *Frontiers in Plant Science*, **5**.

Cesari, S., Kanzaki, H., Fujiwara, T., Bernoux, M., Chalvon, V., Kawano, Y., et al. (2014) *The NB-LRR proteins RGA4 and RGA5 interact functionally and physically to confer disease resistance*. *Embo J*, **33**, 1941–1959.

Cesari, S., Thilliez, G., Ribot, C., Chalvon, V., Michel, C., Jauneau, A., et al. (2013) *The Rice Resistance Protein Pair RGA4/RGA5 Recognizes the Magnaporthe oryzae Effectors AVR-Pia and AVR1-CO39 by Direct Binding*. *The Plant Cell Online*, **25**, 1463–1481.

CHEN, D., CHEN, X., MA, B., WANG, Y., ZHU, L., and LI, S. (2010) *Genetic Transformation of Rice with Pi-d2 Gene Enhances Resistance to Rice Blast Fungus Magnaporthe oryzae*. *Rice Science*, **17**, 19–27.

Chen, J., Upadhyaya, N.M., Ortiz, D., Sperschneider, J., Li, F., Bouton, C., et al. (2017) *Loss of AvrSr50 by somatic exchange in stem rust leads to virulence for Sr50 resistance in wheat*. *Science*, **358**, 1607–1610.

Chen, X., Shang, J., Chen, D., Lei, C., Zou, Y., Zhai, W., et al. (2006) *AB-lectin receptor kinase gene conferring rice blast resistance*. *The Plant Journal*, **46**, 794–804.

Chen, Y., Nie, F., Xie, S.-Q., Zheng, Y.-F., Dai, Q., Bray, T., et al. (2021) *Efficient assembly of nanopore reads via highly accurate and intact error correction*. *Nature Communications*, **12**, 60.

Couch, B.C., Fudal, I., Lebrun, M.-H., Tharreau, D., Valent, B., van Kim, P., et al. (2005) *Origins of Host-Specific Populations of the Blast Pathogen Magnaporthe oryzae in Crop Domestication With Subsequent Expansion of Pandemic Clones on Rice and Weeds of Rice*. *Genetics*, **170**, 613–630.

Couch, B.C. and Kohn, L.M. (2002) *A multilocus gene genealogy concordant with host preference indicates segregation of a new species, Magnaporthe oryzae, from M. grisea*. *Mycologia*, **94**, 683–693.

Dangl, J.L., Dietrich, R.A., and Richberg, M.H. (1996) *Death Don't Have No Mercy: Cell Death Programs in Plant-Microbe Interactions. Plant Cell*, **8**, 1793–1807.

Dangl, J.L. and Jones, J.D.G. (2001) *Plant pathogens and integrated defence responses to infection*. *Nature*, **411**, 826–833.

Dodds, P.N. and Rathjen, J.P. (2010) *Plant immunity: towards an integrated view of plant–pathogen interactions*. *Nat Rev Genet*, **11**, 539–548.

Du, D., Liu, M., Xing, Y., Chen, X., Zhang, Y., Zhu, M., et al. (2019) *Semi-dominant mutation in the cysteine-rich receptor-like kinase gene, ALS1, conducts constitutive defence response in rice*. *Plant Biology*, **21**, 25–34.

FAO's Director-General on How to Feed the World in 2050 (2009) *Population and Development Review*, **35**, 837–839.

Fernandez-Pozo, N., Menda, N., Edwards, J.D., Saha, S., Tecle, I.Y., Strickler, S.R., et al. (2015) *The Sol Genomics Network (SGN)—from genotype to phenotype to breeding*. *Nucleic Acids Research*, **43**, D1036–D1041.

Flor, H.H. (1971) *Current Status of the Gene-For-Gene Concept*. *Annual Review of Phytopathology*, **9**, 275–296.

Flor, H.H. (1956) *The Complementary Genic Systems in Flax and Flax Rust**Joint contribution from the Field Crops Research Branch, Agricultural Research Service, United States Department of Agriculture and the North Dakota Agricultural Experiment Station. Advances in Genetics*, **8**, 29–54.

Fragoso, C.A., Heffelfinger, C., Zhao, H., and Dellaporta, S.L. (2016) *Imputing Genotypes in Biallelic Populations from Low-Coverage Sequence Data*. *Genetics*, **202**, 487–495.

Fujisaki, K., Abe, Y., Ito, A., Saitoh, H., Yoshida, K., Kanzaki, H., et al. (2015) *Rice Exo70 interacts with a fungal effector, AVR-Pii, and is required for AVR-Pii-triggered immunity*. *The Plant Journal*, **83**, 875–887.

Fukuoka, S., Saka, N., Koga, H., Ono, K., Shimizu, T., Ebana, K., et al. (2009) *Loss of Function of a Proline-Containing Protein Confers Durable Disease Resistance in Rice*. *Science*, **325**, 998–1001.

Gabriëls, S.H.E.J., Vossen, J.H., Ekengren, S.K., Ooijen, G. van, Abd-El-Haliem, A.M., Berg, G.C.M. van den, et al. (2007) *An NB-LRR protein required for HR signalling mediated by both extra- and intracellular resistance proteins*. *The Plant Journal*, **50**, 14–28.

Goulden, C. (1939) *Problems in plant selection.-Burnett, RC (ed.): Proceedings of the 7th International Genetic Congress*.

Greer, C.A. and Webster, R.K. (2001) *Occurrence, Distribution, Epidemiology, Cultivar Reaction, and Management of Rice Blast Disease in California*. *Plant Disease*, **85**, 1096–1102.

Hammond-Kosack, K.E. and Parker, J.E. (2003) *Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding*. *Current Opinion in Biotechnology*, **14**, 177–193.

Heath, M.C. (1985) *Implications of nonhost resistance for understanding host-parasite interactions*. 2–2.

Hirata, K., Kusaba, M., Chuma, I., Osue, J., Nakayashiki, H., Mayama, S., and Tosa, Y. (2007) *Speciation in Pyricularia inferred from multilocus phylogenetic analysis*. *Mycological Research*, **111**, 799–808.

Innes, R.W. (2004) *Guarding the Goods. New Insights into the Central Alarm System of Plants*. *Plant Physiology*, **135**, 695–701.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000) *Direct interaction of resistance gene and avirulence gene products confers rice blast resistance*. *EMBO J*, **19**, 4004–4014.

Jones, J.D.G. and Dangl, J.L. (2006) *The plant immune system*. *Nature*, **444**, 323–329.

Kanzaki, H., Yoshida, K., Saitoh, H., Fujisaki, K., Hirabuchi, A., Alaux, L., et al. (2012) *Arms race co-evolution of Magnaporthe oryzae AVR-Pik and rice Pik genes driven by their physical interactions*. *Plant J*, **72**, 894–907.

Kato, H., Yamamoto, M., Yamaguchi-ozaki, T., Kadouchi, H., Iwamoto, Y., Nakayashiki, H., et al. (2000) *Pathogenicity, Mating Ability and DNA Restriction Fragment Length Polymorphisms of Pyricularia Populations Isolated from Gramineae, Bambusideae and Zingiberaceae Plants*. *Journal of General Plant Pathology*, **66**, 30–47.

Katoh, K. and Standley, D.M. (2013) *MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability*. *Molecular Biology and Evolution*, **30**, 772–780.

Kawahara, Y., Bastide, M. de la, Hamilton, J.P., Kanamori, H., McCombie, W.R., Ouyang, S., et al. (2013) *Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data*. *Rice*, **6**, 4.

Keen, N.T. (1990) *Gene-For-Gene Complementarity in Plant-Pathogen Interactions*. *Annual Review of Genetics*, **24**, 447–463.

Keen, N.T., Bent, A., and Staskawicz, B. (1993) *Plant disease resistance genes: interactions with pathogens and their improved utilization to control plant diseases.* In: , pp. 65–88. New York: Wiley-Liss Inc.

Kojima, Y., Ebana, K., Fukuoka, S., Nagamine, T., and Kawase, M. (2005) *Development of an RFLP-based Rice Diversity Research Set of Germplasm*. *Breeding Sci*, **55**, 431–440.

Kroj, T., Chanclud, E., Michel-Romiti, C., Grand, X., and Morel, J. (2016) *Integration of decoy domains derived from protein targets of pathogen effectors into plant immune receptors is widespread*. *New Phytologist*, **210**, 618–626.

Le Roux, C., Huet, G., Jauneau, A., Camborde, L., Trémousaygue, D., Kraut, A., et al. (2015) *A Receptor Pair with an Integrated Decoy Converts Pathogen Disabling of Transcription Factors to Immunity*. *Cell*, **161**, 1074–1088.

Lee, S.-K., Song, M.-Y., Seo, Y.-S., Kim, H.-K., Ko, S., Cao, P.-J., et al. (2009) *Rice Pi5-Mediated Resistance to Magnaporthe oryzae Requires the Presence of Two Coiled-Coil–Nucleotide-Binding–Leucine-Rich Repeat Genes*. *Genetics*, **181**, 1627–1638.

Lei, C., Hao, K., Yang, Y., Ma, J., Wang, S., Wang, J., et al. (2013) *Identification and fine mapping of two blast resistance genes in rice cultivar 93-11*. *The Crop Journal*, **1**, 2–14.

Levitsky, V.G. (2004) *RECON: a program for prediction of nucleosome formation potential*. *Nucleic Acids Research*, **32**, W346–W349.

Li, H. and Durbin, R. (2009) *Fast and accurate short read alignment with Burrows–Wheeler transform*. *Bioinformatics*, **25**, 1754–1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009) *The Sequence Alignment/Map format and SAMtools*. *Bioinformatics*, **25**, 2078–2079.

Li, P., Quan, X., Jia, G., Xiao, J., Cloutier, S., and You, F.M. (2016) *RGAugury: a pipeline for genome-wide prediction of resistance gene analogs (RGAs) in plants*. *Bmc Genomics*, **17**, 852.

Liu, J., WANG, X., MITCHELL, T., HU, Y., LIU, X., DAI, L., and WANG, G. (2010) *Recent progress and understanding of the molecular mechanisms of the rice–Magnaporthe oryzae interaction*. *Mol Plant Pathol*, **11**, 419–427.

Liu, W., Liu, J., Ning, Y., Ding, B., Wang, X., Wang, Z., and Wang, G.-L. (2013) *Recent Progress in Understanding PAMP- and Effector-Triggered Immunity against the Rice Blast Fungus Magnaporthe oryzae*. *Mol Plant*, **6**, 605–620.

Liu, W.-Y., Kang, J.-H., Jeong, H.-S., Choi, H.-J., Yang, H.-B., Kim, K.-T., et al. (2014) *Combined use of bulked segregant analysis and microarrays reveals SNP markers pinpointing a major QTL for resistance to Phytophthora capsici in pepper*. *Theoretical and Applied Genetics*, **127**, 2503–2513.

Liu, Y., Zhang, X., Yuan, G., Wang, D., Zheng, Y., Ma, M., et al. (2021) *A designer rice NLR immune receptor confers resistance to the rice blast fungus carrying noncorresponding avirulence effectors*. *Proc National Acad Sci*, **118**, e2110751118.

Lo, C.-C. and Chain, P.S.G. (2014) *Rapid evaluation and quality control of next generation sequencing data with FaQCs*. *BMC Bioinformatics*, **15**, 366.

Maekawa, T., Cheng, W., Spiridon, L.N., Töller, A., Lukasik, E., Saijo, Y., et al. (2011) *Coiled-coil domain-dependent homodimerization of intracellular barley immune receptors defines a minimal functional module for triggering cell death*. *Cell Host Microbe*, **9**, 187–199.

Maqbool, A., Saitoh, H., Franceschetti, M., Stevenson, C., Uemura, A., Kanzaki, H., et al. (2015) *Structural basis of pathogen recognition by an integrated HMA domain in a plant NLR immune receptor*. *eLife*, **4**, e08709.

Marchler-Bauer, A., Anderson, J.B., Derbyshire, M.K., DeWeese-Scott, C., Gonzales, N.R., Gwadz, M., et al. (2007) *CDD: a conserved domain database for interactive domain family analysis*. *Nucleic Acids Research*, **35**, D237–D240.

Medzhitov, R. and Janeway, C.A. (1997) *Innate Immunity: The Virtues of a Nonclonal System of Recognition*. *Cell*, **91**, 295–298.

Mikami, M., Toki, S., and Endo, M. (2015) *Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice*. *Plant Cell Rep*, **34**, 1807–1815.

Miki, D. and Shimamoto, K. (2004) *Simple RNAi Vectors for Stable and Transient Suppression of Gene Function in Rice*. *Plant and Cell Physiology*, **45**, 490–495.

Molinari, C. and Talbot, N.J. (2022) *A Basic Guide to the Growth and Manipulation of the Blast Fungus, Magnaporthe oryzae*. *Curr Protoc*, **2**, e523.

Nalley, L., Tsiboe, F., Durand-Morat, A., Shew, A., and Thoma, G. (2016) *Economic and Environmental Impact of Rice Blast Pathogen (Magnaporthe oryzae) Alleviation in the United States*. *Plos One*, **11**, e0167295.

Naqvi, N.I., Bonman, J.M., Mackill, D.J., Nelson, R.J., and Chattoo, B.B. (1995) *Identification of RAPD markers linked to a major blast resistance gene in rice*. *Molecular Breeding*, **1**, 341–348.

Narasimhan, V., Danecek, P., Scally, A., Xue, Y., Tyler-Smith, C., and Durbin, R. (2016) *BCFtools/RoH: a hidden Markov model approach for detecting autozygosity from next-generation sequencing data*. *Bioinformatics*, **32**, 1749–1751.

Ning, X., Yunyu, W., and Aihong, L. (2020) *Strategy for Use of Rice Blast Resistance Genes in Rice Molecular Breeding*. *Rice Science*, **27**, 263–277.

Nürnberger, T. and Brunner, F. (2002) *Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns*. *Curr Opin Plant Biol*, **5**, 318–324.

Okuyama, Y., Kanzaki, H., Abe, A., Yoshida, K., Tamiru, M., Saitoh, H., et al. (2011) *A multifaceted genomics approach allows the isolation of the rice Pia-blast resistance gene consisting of two adjacent NBS-LRR protein genes*. *The Plant Journal*, **66**, 467–479.

Orbach, M.J., Farrall, L., Sweigard, J.A., Chumley, F.G., and Valent, B. (2000) *A Telomeric Avirulence Gene Determines Efficacy for the Rice Blast Resistance Gene Pi-ta*. *Plant Cell*, **12**, 2019–2032.

Ortiz, D., Guillen, K. de, Cesari, S., Chalvon, V., Gracy, J., Padilla, A., and Kroj, T. (2017) *Recognition of the Magnaporthe oryzae Effector AVR-Pia by the Decoy Domain of the Rice NLR Immune Receptor RGA5*. *Plant Cell*, **29**, 156–168.

Ou, S. (1985) *Rice Diseases, Commonwealth Agricultural Bureau*. **62**.

Pertea, M., Kim, D., Pertea, G.M., Leek, J.T., and Salzberg, S.L. (2016) *Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown*. *Nature Protocols*, **11**, 1650–1667.

Ravensdale, M., Bernoux, M., Ve, T., Kobe, B., Thrall, P.H., Ellis, J.G., and Dodds, P.N. (2012) *Intramolecular Interaction Influences Binding of the Flax L5 and L6 Resistance Proteins to their AvrL567 Ligands*. *Plos Pathog*, **8**, e1003004.

Roberts, M., Tang, S., Stallmann, A., Dangl, J.L., and Bonardi, V. (2013) *Genetic Requirements for Signaling from an Autoactive Plant NB-LRR Intracellular Innate Immune Receptor*. *PLOS Genetics*, **9**, e1003465.

Rosyara, U.R., Jong, W.S.D., Douches, D.S., and Endelman, J.B. (2016) *Software for Genome-Wide Association Studies in Autopolyploids and Its Application to Potato*. *The Plant Genome*, **9**.

Sahu, P., Sao, R., Choudhary, D., Thada, A., Kumar, V., Mondal, S., et al. (2022) *Advancement in the Breeding, Biotechnological and Genomic Tools towards Development of Durable Genetic Resistance against the Rice Blast Disease*. *Plants*, **11**, null.

Sallaud, C., Lorieux, M., Roumen, E., Tharreau, D., Berruyer, R., Svestasrani, P., et al. (2003) *Identification of five new blast resistance genes in the highly blast-resistant rice variety IR64 using a QTL mapping strategy*. *Theor Appl Genet*, **106**, 1532–1532.

Sarris, P.F., Cevik, V., Dagdas, G., Jones, J.D.G., and Krasileva, K.V. (2016) *Comparative analysis of plant immune receptor architectures uncovers host proteins likely targeted by pathogens*. *BMC Biology*, **14**, 8.

Sasaki, T. and Burr, B. (2000) *International Rice Genome Sequencing Project: the effort to completely sequence the rice genome*. *Curr Opin Plant Biol*, **3**, 138–142.

Schmied, W.H., Tnimov, Z., Uttamapinant, C., Rae, C.D., Fried, S.D., and Chin, J.W. (2018) *Controlling orthogonal ribosome subunit interactions enables evolution of new function*. *Nature*, **564**, 444–448.

Schmieder, R. and Edwards, R. (2011) *Quality control and preprocessing of metagenomic datasets*. *Bioinformatics*, **27**, 863–864.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012) *NIH Image to ImageJ: 25 years of image analysis*. *Nature methods*, **9**, 671–675.

Shimizu, M., Hirabuchi, A., Sugihara, Y., Abe, A., Takeda, T., Kobayashi, M., et al. (2022) *A genetically linked pair of NLR immune receptors shows contrasting patterns of evolution*. *Proceedings of the National Academy of Sciences*, **119**, e2116896119.

Shiratsuchi, H., Ohdaira, Y., Yamaguchi, H., and Fukuda, A. (2017) *Breaking the dormancy of rice seeds with various dormancy levels using steam and high temperature treatments in a steam nursery cabinet*. *Plant Production Science*, **20**, 183–192.

Swiderski, M.R., Birker, D., and Jones, J.D.G. (2009) *The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction*. *Mol Plant Microbe Interact*, **22**, 157–165.

Takagi, H., Uemura, A., Yaegashi, H., Tamiru, M., Abe, A., Mitsuoka, C., et al. (2013a) *MutMap-Gap: whole-genome resequencing of mutant F2 progeny bulk combined with de novo assembly of gap regions identifies the rice blast resistance gene Pii*. *New Phytol*, **200**, 276–283.

Takagi, H., Uemura, A., Yaegashi, H., Tamiru, M., Abe, A., Mitsuoka, C., et al. (2013b) *MutMap-Gap: whole-genome resequencing of mutant F2 progeny bulk combined with*

de novo assembly of gap regions identifies the rice blast resistance gene Pii. *New Phytologist*, **200**, 276–283.

Tang, J., Zhu, X., Wang, Y., Liu, L., Xu, B., Li, F., et al. (2011) *Semi-dominant mutations in the CC-NB-LRR-type R gene, NLS1, lead to constitutive activation of defense responses in rice*. *The Plant Journal*, **66**, 996–1007.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice*. *Nucleic Acids Research*, **22**, 4673–4680.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., and Rozen, S.G. (2012) *Primer3—new capabilities and interfaces*. *Nucleic Acids Research*, **40**, e115–e115.

Wang, B., Ebbole, D.J., and Wang, Z. (2017) *The arms race between Magnaporthe oryzae and rice: Diversity and interaction of Avr and R genes*. *Journal of Integrative Agriculture*, **16**, 2746–2760.

Wang, Jizong, Hu, M., Wang, Jia, Qi, J., Han, Z., Wang, G., et al. (2019) *Reconstitution and structure of a plant NLR resistosome conferring immunity*. *Science*, **364**.

Wang, Jizong, Wang, Jia, Hu, M., Wu, S., Qi, J., Wang, G., et al. (2019) *Ligand-triggered allosteric ADP release primes a plant NLR complex*. *Science*, **364**.

Wang, R., Fang, N., Guan, C., He, W., Bao, Y., and Zhang, H. (2016) *Characterization and Fine Mapping of a Blast Resistant Gene Pi-jnw1 from the japonica Rice Landrace Jiangnanwan*. *PLoS ONE*, **11**, e0169417.

Wick, R.R., Judd, L.M., and Holt, K.E. (2019) *Performance of neural network basecalling tools for Oxford Nanopore sequencing*. **20**, 129.

Williams, S.J., Sohn, K.H., Wan, L., Bernoux, M., Sarris, P.F., Segonzac, C., et al. (2014) *Structural Basis for Assembly and Function of a Heterodimeric Plant Immune Receptor*. *Science*, **344**, 299–303.

Wu, Y., Chen, Y., Pan, C., Xiao, N., Yu, L., Li, Y., et al. (2017) *Development and Evaluation of Near-Isogenic Lines with Different Blast Resistance Alleles at the Piz Locus in japonica Rice from the Lower Region of the Yangtze River, China*. *Plant Disease*, **101**, 1283–1291.

Yoshida, Kentaro, Saitoh, H., Fujisawa, S., Kanzaki, H., Matsumura, H., Yoshida, Kakoto, et al. (2009) *Association Genetics Reveals Three Novel Avirulence Genes from the Rice Blast Fungal Pathogen* Magnaporthe oryzae. *The Plant Cell*, **21**, 1573–1591.

Yu, Z., Dong, L., Jiang, Z., Yi, K., Zhang, J., Zhang, Z., et al. (2018) *A semi-dominant mutation in a CC-NB-LRR-type protein leads to a short-root phenotype in rice*. *Rice*, **11**, 54.

Yuan, B., Zhai, C., Wang, W., Zeng, X., Xu, X., Hu, H., et al. (2011) *The Pik-p resistance to Magnaporthe oryzae in rice is mediated by a pair of closely linked CC-NBS-LRR genes*. *Theoretical and Applied Genetics*, **122**, 1017–1028.

Zeigler, R.S., Leong, S.A., and Teng, P.S. (1994) *Rice blast disease*. Int. Rice Res. Inst.

Zhai, C., Lin, F., Dong, Z., He, X., Yuan, B., Zeng, X., et al. (2010) *The isolation and characterization of Pik, a rice blast resistance gene which emerged after rice domestication*. *New Phytologist*, **189**, 321–334.

Zhang, X., Bernoux, M., Bentham, A.R., Newman, T.E., Ve, T., Casey, L.W., et al. (2017) *Multiple functional self-association interfaces in plant TIR domains*. *Proceedings of the National Academy of Sciences*, **114**, E2046–E2052.

Zhao, H., Wang, X., Jia, Y., Minkenberg, B., Wheatley, M., Fan, J., et al. (2018) *The rice blast resistance gene Ptr encodes an atypical protein required for broad-spectrum disease resistance*. *Nature Communications*, **9**, 2039.

Zhou, B., Dolan, M., Sakai, H., and Wang, G.-L. (2007) *The Genomic Dynamics and Evolutionary Mechanism of the Pi2/9 Locus in Rice*. *Molecular Plant-Microbe Interactions*®, **20**, 63–71.

Zhou, B., Qu, S., Liu, G., Dolan, M., Sakai, H., Lu, G., et al. (2006) *The Eight Amino-Acid Differences Within Three Leucine-Rich Repeats Between Pi2 and Piz-t Resistance Proteins Determine the Resistance Specificity to Magnaporthe grisea*. *Mol Plant-microbe Interactions*, **19**, 1216–1228.

Zhou, Q., Zhang, Z., Liu, T., Gao, B., and Xiong, X. (2017) *Identification and Map-Based Cloning of the Light-Induced Lesion Mimic Mutant 1 (LIL1) Gene in Rice*. *Frontiers in Plant Science*, **8**, 2122.

Appendix 1. RILs used to generate resistant bulks for QTL-seq analysis

RILs selected to constitute R-Bulk based on August 2018 phenotypic data					**RILs selected to constitute R-Bulk based on June 2019 phenotypic data**					**RILs selected to constitute R-Bulk based on October 2019 phenotypic data**				
		Lesion area (mm^2)					Lesion area (mm^2)					Lesion area (mm^2)		
Sl. No	R RILs	Aug-18	Jun-19	Oct-19	Sl. No2	R RILs3	Aug-184	Jun-195	Oct-196	Sl. No8	R RILs9	Aug-1810	Jun-1911	Oct-1912
1	N05_091	3.00	6.5	3.67	1	N05_066	6.00	4.25	3.33	1	N05_043	5.25	4.75	3.00
2	N05_082	3.00	7.5	5.33	2	N05_056	7.50	4.25	-	2	N05_066	6.00	4.25	3.33
3	N05_034	3.25	7.5	5.33	3	N05_093	7.00	4.5	4.00	3	N05_111		4.75	3.33
4	N05_083	3.50	6.5	4.33	5	N05_043	5.25	4.75	3.00	5	N05_148		5.75	3.67
5	N05_077	4.20	8	4.33	6	N05_111		4.75	3.33	6	N05_091	3.00	6.5	3.67
6	N05_055	4.25	7	-	7	N05_150		5	4.33	7	N05_093	7.00	4.5	4.00
7	N05_084	4.40	6.75	5.33	10	N05_123		5.5	4.00	9	N05_123		5.5	4.00
8	N05_008	4.40	5.5	5.67	11	N05_122		5.5	4.33	10	N05_063	8.25	5.75	4.00
9	N05_018	4.60	6.33	7.00	12	N05_008	4.40	5.5	5.67	11	N05_052	9.60	6.5	4.00
10	N05_015	4.60	6.75	4.00	13	N05_042	4.80	5.5	-	12	N05_015	4.60	6.75	4.00
11	N05_003	4.75	9.75	9.75	14	N05_148		5.75	3.67	13	N05_132		7.5	4.00
12	N05_042	4.80	5.5	-	15	N05_063	8.25	5.75	4.00	14	N05_150		5	4.33
13	N05_079	5.00	5.75	4.67	16	N05_079	5.00	5.75	4.67	15	N05_122		5.5	4.33
14	N05_043	5.25	4.75	3.00	19	N05_121		6	6.00	16	N05_083	3.50	6.5	4.33
15	N05_080	5.25	6.75	5.00	20	N05_120		6	6	17	N05_077	4.20	8	4.33
16	N05_001	5.25	9	3.33	21	N05_018	4.60	6.33	7.00	18	N05_116		8	4.33
17	N05_012	5.67	8	-	22	N05_091	3.00	6.5	3.67	20	N05_079	5.00	5.75	4.67
18	N05_066	6.00	4.25	3.33	23	N05_052	9.60	6.5	4.00	22	N05_080	5.25	6.75	5.00
19	N05_049	6.25	7.25	-	24	N05_083	3.50	6.5	4.33	23	N05_084	4.40	6.75	5.33
20	N05_094	6.40	7.75	7.00	25	N05_015	4.60	6.75	4.00	24	N05_082	3.00	7.5	5.33
21	N05_067	7.00	6.75	-	26	N05_080	5.25	6.75	5.00	25	N05_034	3.25	7.5	5.33
22	N05_093	7.00	4.5	4.00	27	N05_084	4.40	6.75	5.33	26	N05_008	4.40	5.5	5.67
23	N05_056	7.50	4.25	-	28	N05_067	7.00	6.75	-	27	N05_121		6	6.00
24	N05_063	8.25	5.75	4.00	29	N05_131		7	6.67	28	N05_120		6	6
25	N05_054	8.50	7.5	9.00	30	N05_055	4.25	7.00	-	29	N05_139		7.25	6.00

Appendix 2. RILs used to generate susceptible bulks for QTL-seq analysis

RILs selected to constitute S-Bulk based on August 2018 phenotypic data					RILs selected to constitute S-Bulk based on June 2019 phenotypic data					RILs selected to constitute S-Bulk based on October 2019 phenotypic data				
		Lesion area (mm^2)					Lesion area (mm^2)					Lesion area (mm^2)		
Sl. No	**S RILs**	**Aug-18**	**Jun-19**	**Oct-19**	**Sl. No**	**S RILs**	**Aug-184**	**Jun-195**	**Oct-196**	**Sl. No**	**S RILs**	**Aug-1810**	**Jun-1911**	**Oct-1912**
1	N05_099	62.20	44.25	33.33	1	N05_070	52.80	70.25	64.67	1	N05_070	52.80	70.25	64.67
2	N05_070	52.80	70.25	64.67	2	N05_074	51.60	70.25	56.33	2	N05_100	27.00	49.25	57.33
3	N05_074	51.60	70.25	56.33	3	N05_027	34.80	63.75		3	N05_074	51.60	70.25	56.33
4	N05_021	49	49		4	N05_046	33.00	60.75	23.00	4	N05_086	17.40	20	54.33
5	N05_045	42.25	55.75		5	N05_045	42.25	55.75		5	N05_142		19.25	38.50
6	N05_088	35.75	35.75	8.00	6	N05_087	16.75	54.25		6	N05_031	23.00	39.25	37.00
7	N05_027	34.80	63.75		7	N05_100	27.00	49.25	57.33	7	N05_072	12.00	6.75	35.67
8	N05_046	33.00	60.75	23.00	8	N05_136		49	13.50	8	N05_099	62.20	44.25	33.33
9	N05_100	27.00	49.25	57.33	9	N05_021	49	49		9	N05_096	15.40	13.25	26.33
10	N05_085	23.50	21.25		10	N05_109		46.6		10	N05_065	13.25	28	25.00
11	N05_031	23.00	39.25	37.00	11	N05_068	22.67	45.75		11	N05_013	20.25	13.5	24.00
12	N05_014	23.00	24.25		12	N05_099	62.20	44.25	33.33	12	N05_046	33.00	60.75	23.00
13	N05_071	22.80	12.33	12.33	13	N05_023	16.50	43.75		13	N05_038	20.20	30.25	22.33
14	N05_068	22.67	45.75		14	N05_031	23.00	39.25	37.00	14	N05_149		17.5	21.67
15	N05_089	22.00	25.5	13.67	15	N05_044	15.50	38.25	13.50	15	N05_019	15.40	10	20.33
16	N05_073	21.50	34.75	12.67	16	N05_088	35.75	35.75	8.00	16	N05_037	10.60	12.75	17.00
17	N05_076	20.75	18.5	8.00	17	N05_073	21.50	34.75	12.67	17	N05_114		22.25	15.00
18	N05_013	20.25	13.5	24.00	18	N05_038	20.20	30.25	22.33	18	N05_025	15.75	13.5	14.00
19	N05_038	20.20	30.25	22.33	19	N05_103		28.4	7.33	19	N05_089	22.00	25.5	13.67
20	N05_086	17.40	20	54.33	20	N05_065	13.25	28	25.00	20	N05_136		49	13.50
21	N05_087	16.75	54.25		21	N05_089	22.00	25.5	13.67	21	N05_044	15.50	38.25	13.50
22	N05_023	16.50	43.75		22	N05_039	13.40	25.25		22	N05_104		23.5	13.33
23	N05_025	15.75	13.5	14.00	23	N05_014	23.00	24.25		23	N05_135		16.75	13.00
24	N05_044	15.50	38.25	13.50	24	N05_104		23.5	13.33	24	N05_118		15.25	13.00
25	N05_096	15.40	13.25	26.33	25	N05_133		22.8	7.33	25	N05_073	21.50	34.75	12.67

Appendix 3. Lesion sizes of recombinant inbred lines in three independent inoculation trails.

RIL #	August 2018	June 2019	October 2019	RIL #	August 20183	June 20194	October 20195	RIL #	August 20188	June 20199	October 201910
N05_001	5.25	8	3.33	N05_051	10.75	11	-	N05_101	-	7	-
N05_002	6.25	11	-	N05_052	9.60	6	4.00	N05_102	-	18	-
N05_003	4.75	10	3.67	N05_053	6.33	14	9.00	N05_104	-	22	-
N05_004	5.50	10	8.67	N05_054	8.50	8	9.00	N05_105	-	7	5.67
N05_005	6.25	9	5.33	N05_055	4.25	7	-	N05_106	-	9	3.67
N05_006	9.50	12	9.67	N05_056	7.50	4	-	N05_107	-	11	-
N05_007	9.00	9	6.67	N05_057	13.25	10	-	N05_108	-	8	6.67
N05_008	4.40	6	5.67	N05_058	13.25		-	N05_109	-	36	-
N05_009	3.00	12	-	N05_059	10.00	10	-	N05_110	-	15	-
N05_010	3.75	13	6.67	N05_060	6.50	11	-	N05_111	-	5	3.33
N05_011	16.50	10	3.67	N05_061	8.25	14	-	N05_112	-	13	7.67
N05_012	5.67	8	-	N05_062	11.50	10	-	N05_113	-	8	7.33
N05_013	20.25	14	-	N05_063	8.25	5	4.00	N05_114	-	21	-
N05_014	23.00	25	-	N05_064	13.00	8	5.33	N05_115	-	14	-
N05_015	4.60	7	4.00	N05_065	13.25	28	-	N05_116	-	7	4.33
N05_016	9.75	6	7.33	N05_066	6.00	4	3.33	N05_117	-	9	-
N05_017	9.00	10	6.33	N05_067	7.00	7	-	N05_118	-	14	-
N05_018	4.60	6	7.00	N05_068	22.67	42	-	N05_119	-	11	6.33
N05_019	15.40	10	-	N05_069	12.25	19	-	N05_120	-	6	6.33
N05_020	11.25	13	-	N05_070	52.80	70	-	N05_121	-	6	6.00
N05_021	7.25	45	-	N05_071	22.80	6	-	N05_122	-	6	4.33
N05_022	4.25	9	5.67	N05_072	12.00	9	-	N05_123	-	5	4.00
N05_023	16.50	37	4.00	N05_073	21.50	32	-	N05_125	-	4	-
N05_024	6.00	15	5.33	N05_074	51.60	74	-	N05_126	-	18	-
N05_025	15.75	14	-	N05_075	11.50	11	-	N05_127	-	5	4.67

Continued

N05_026	9.00	9	9.00	N05_076	20.75	17	8.00	N05_128	-	7	-
N05_027	34.80	39	-	N05_077	4.20	7	4.33	N05_129	-	31	-
N05_028	5.25	63	5.67	N05_078	11.00	11	-	N05_130	-	11	7.00
N05_029	8.80	10	-	N05_079	5.00	6	4.67	N05_131	-	7	6.67
N05_030	14.60	9	-	N05_080	5.25	7	5.00	N05_132	-	7	4.00
N05_031	23.00	39	-	N05_081	13.20	6	6.67	N05_133	-	10	7.33
N05_032	10.00	11	-	N05_082	3.00	7	5.33	N05_134	-	7	6.50
N05_033	4.75	12	9.33	N05_083	3.50	6	4.33	N05_135	-	16	-
N05_034	3.25	8	5.33	N05_084	4.40	10	5.33	N05_136	-	7	-
N05_035	8.00	13	8.33	N05_085	23.50	20	-	N05_137	-	8	6.00
N05_036	12.00	18	5.67	N05_086	17.40	19	-	N05_138	-	8	-
N05_037	10.60	13	-	N05_087	16.75	51	-	N05_139	-	7	6.00
N05_038	20.20	34	-	N05_088	35.75	10	8.00	N05_140	-	10	3.50
N05_039	13.40	26	-	N05_089	22.00	25	-	N05_141	-	14	-
N05_040	13.67	6	8.00	N05_090	10.40	7	4.33	N05_142	-	16	-
N05_041	10.50	16	-	N05_091	3.00	7	3.67	N05_143	-	29	-
N05_042	4.80	5	-	N05_092	8.50	8	-	N05_144	-	6	4.67
N05_043	5.25	5	3.00	N05_093	7.00	5	4.00	N05_145	-	8	5.67
N05_044	15.50	37	-	N05_094	6.40	7	7.00	N05_146	-	5	4.00
N05_045	42.25	59	-	N05_095	3.25	13	-	N05_147	-	12	4.67
N05_046	33.00	55	-	N05_096	15.40	12	-	N05_148	-	6	3.67
N05_047	12.00	10	2.67	N05_097	8.80	5	7.67	N05_149	-	17	-
N05_048	8.60	10	8.33	N05_098	9.40	17	-	N05_150	-	5	4.33
N05_049	6.25	7	-	N05_099	62.20	43	-				
N05_050	10.75	13	4.33	N05_100	27.00	49	-				