High-resolution mapping of GRH6, a gene from Oryza nivara (Sharma et Shastry) conferring resistance to green rice leafhopper (Nephotettix cincticeps Uhler)

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The green rice leafhopper (GRH), Nephotettix cincticeps Uhler, is a major insect pest of cultivated rice, Oryza sativa L., throughout the temperate regions of East Asia. GRH resistance had been reported in the wild species Oryza nivara but genetic basis of GRH resistance in wild rice accession has not been clarified. Here, we found a major QTL, qGRH4.2, on chromosome 4 conferred GRH resistance with 14.1 of the logarithm of odds (LOD) score explaining 67.6% of phenotypic variance in the BC 1F1 population derived from a cross between the susceptible japonica cultivar ‘Taichung 65’ (T65) and O. nivara accession IRGC105715. qGRH4.2 has been identified as GRH6 between the markers RM5414 and C60248 in a BC 3F2 population derived from two BC 1F1 plants resistant to GRH. In a high-resolution mapping, the GRH6 region was delimited between the markers G6-c60k and 7L16f, and corresponded to an 31.2-kbp region of the ‘Nipponbare’ genome. Understanding the genetic basis of GRH resistance will facilitate the use of GRH resistance genes in marker-assisted breeding in rice.

Key Words: rice, wild relative, green rice leafhopper, antibiosis test, high-resolution mapping.

Introduction

The green rice leafhopper (GRH; Nephotettix cincticeps Uhler), a major insect pest of cultivated rice (Oryza sativa L.), is distributed throughout the temperate regions of East Asia (Ghauri 1971, Pathak and Khan 1994). It has been reported that GRH decreases plant vigor and yield by sucking sap from both xylem and phloem of susceptible rice plants in northeastern Japan (Naito 1977, Nirei and Nakazato 1975, Pathak and Khan 1994). GRH is the major vector for viruses such as rice transitory yellowing virus (RTYV), rice waika virus (RWV), and rice yellow dwarf virus (RYDV) (Hirao and Inoue 1978, Inoue 1978, Inoue and Hirao 1981, Nakasuji 1974), which can cause long-term damage in rice cultivation. To reduce the damage caused by GRH, identifying novel genes for resistance to this pest and introducing them into cultivated rice is one of effective approach to achieve sustained resistance without environmental damage.

Molecular markers are powerful tools for identifying gene loci in plants through linkage and QTL analysis. To date, six genes and two QTLs conferring resistance to GRH have been identified and mapped in resistant rice cultivars and wild relatives. The genes GRH1 to GRH4 were identified by mapping GRH resistance in subspecies indica. GRH1 was mapped on chromosome 5 from ‘Pe-bi-hun’, ‘IR24’, and ‘Singwang’ (Kadowaki et al. 2003, Park et al. 2013, Tamura et al. 1999, Yasui and Yoshimura 1999). ‘Lepedumai’ and ‘DV85’ carry the GRH2 and GRH4 genes on chromosomes 11 and 3, respectively, and are highly resistant to GRH (Fukuta et al. 1998, Kadowaki et al. 2003, Yazawa et al. 1998). GRH2 confers resistance to GRH, while GRH4 contributes to the resistance in the presence of GRH2 (Fujita et al. 2010b). GRH3(t) has been mapped on chromosome 6 (Saka et al. 2006). GRH6, from the Surinam cultivar ‘SML17’, has been mapped on the short arm of chromosome 4 (Tamura et al. 2004). The use of exotic germplasm or wild relatives is important for improving the resistance of rice to insect pests because gene resources for insect resistance in cultivated rice are limited (Heinrichs et al. 1985). GRH5 and the QTL qGRH4 from Oryza rufipogon accession W1962 (Fujita et al. 2006), and GRH6-nivara(t) from Oryza nivara accession IRGC105715 (Fujita et al. 2004) have been mapped. Another QTL, qGRH9 from Oryza glaberrima accession IRGC104038 has been located on the long arm of chromosome 9 (Fujita et al. 2010a).
Virulent biotypes of the brown planthopper (BPH; Nilaparvata lugens (Stål)) appeared after the release of modern improved rice cultivars carrying a single major gene for resistance to this insect pest (Tanaka and Matsumura 2000). In East Asia, the BPH resistance genes BPH1 and BPH2 have lost their effectiveness due to new virulent biotypes (Myint et al. 2009, Tanaka and Matsumura 2000). The development of resistance to insecticides (Kiritani 1977, Miyata 1989) and appearance of virulent insects in the presence of resistance genes are important challenges in managing insect populations in the field. In GRH, three different biotypes (1–3) virulent to resistant rice varieties carrying GRH1, GRH2, and GRH3(t), respectively, have been selected by maintenance of GRH population grown on resistant rice variety across successive generations in growth chamber studies (Hirae et al. 2007), demonstrating that single GRH resistance genes are not effective for keeping long-duration resistance of varieties.

GRH6-nivara(t) has been found on the short arm of chromosome 4 of the resistant accession IRGC105715 of the wild rice O. nivara by a chromosome substitution approach in the genetic background of the susceptible O. sativa ssp. japonica cultivar ‘Taichung 65’ (T65) (Fujita et al. 2004). A near-isogenic line (NIL) carrying GRH6-nivara(t) has been developed (Fujita et al. 2010b); however, possibility has not been tested that other genes/QTLs on chromosomal regions are associated to antibiosis in IRGC105715 by genome-wise approach and the candidate gene for Grh6-nivara(t) (hereafter GRH6) have not been determined. Thus, the objectives of this study were to determine the genetic basis of GRH resistance in IRGC105715 through genetic analysis of backcross-derived progenies. QTL analysis of GRH resistance was conducted in a BC1F1 population, and the major QTL, GRH6, was mapped using SSR markers on the short arm of chromosome 4. To define the candidate gene(s) for GRH6, a high-resolution map was constructed using a large population segregating at GRH6.

**Materials and Methods**

**Plant materials**

A BC1F1 population was developed from a cross between the susceptible cultivar T65 and a wild accession of O. nivara (IRGC105715) from Cambodia that was provided by Genetic resources center in International Rice Research Institute (Fig. 1). The BC1F1 population was evaluated for resistance to GRH and subsequently used for QTL analysis. Several BC1F1 plants were repeatedly backcrossed with T65 as a recurrent parent to develop the BC3F1 generation. Among 54 BC1F1 plants, 2 resistant plants were selected via the antibiosis test, genotyped with molecular markers covering the whole genome, and self-pollinated. The BC3F1 population (185 plants) was used to map GRH resistance gene. For high-resolution mapping of GRH6, 20 clearly resistant BC3F1 plants heterozygous at the marker loci flanking GRH6 were self-pollinated to develop the BC3F2 generation. A high-resolution linkage mapping population (6834 BC3F3 individuals) segregating for GRH6 was screened for recombination events between flanking SSR markers to delimit the candidate region of GRH6.

**Evaluation of GRH resistance**

GRH resistance was evaluated as in Kishino and Ando (1978). The GRH population was collected in Fukuoka Prefecture in 1991 and was maintained by continuously rearing the insects on seedlings of the susceptible japonica cultivar ‘Nipponbare’. Insects were kept at 25°C ± 1°C and 16 h light:8 h dark photoperiod. In the antibiosis test, a 7–10-day-old seedling was infested with 7–10 first-instar nymphs in a test tube. GRH resistance was scored as follows: 0% to 30% nymph mortality, susceptible; 30% to 70%, moderately resistant; 70%–100%, resistant.

**Genotyping with SSR markers**

Total DNA of the BC1F1 plants and BC3F2 populations was extracted from freeze-dried leaves using the CTAB method (Murray and Thompson 1980). For high-resolution mapping, crude total genomic DNA of recombinant plants in BC3F3 population was extracted from fresh leaves as follows. Leaves were collected in 96-deep-well plates containing 100 μl NaOH (0.25 M) per well, and were crushed using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) with two 2-mm stainless beads. At 2 min after crushing, 400 μl Tris-HCl (100 mM, pH 7.5) was added to each well and the plates were centrifuged at 540 × g for 10 min. Supernatants were diluted 1:20 and used as a template for PCR.

PCR amplification was performed in a PCR System-9700 (Perkin Elmer, Waltham, MA, USA). The 15-μl PCR
mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 200 μM of each dNTP, 0.2 μM primer, 1 unit of Taq polymerase (TaKaRa Bio, Shiga, Japan), and 5 to 10 μg/ml of genomic DNA as a template. The thermal cycler was programmed for the first denaturation step of 5 min at 95°C, followed by 35 cycles each of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. No final extension phase was used. The PCR products were resolved by electrophoresis in 4.0% agarose gels at 4.7 V/cm for 1 h in 0.5× TBE buffer. The gels were stained with ethidium bromide and photographed under ultraviolet light.

**Construction of a linkage map and QTL analysis**

A genetic map for the BC₁F₁ population was constructed using a total of 106 SSR markers distributed across the 12 rice chromosomes. The resulting map spanned 1563 cM, with an average interval of 15.6 cM between markers. Linkage of loci and map distances were determined using MAPMAKER/Exp 3.0 (Lander et al. 1987). The data on nymph mortality and the genotype data of the BC₁F₁ population were used together for QTL analysis. Composite interval mapping was conducted using Windows QTL Cartographer V2.0 (Wang et al. 2004). The threshold value of the LOD score for QTL detection was calculated by conducting 1000 permutation tests and was 2.6 at a significance level of 0.05.

**Whole-genome survey**

The genotypes of BC₁F₁ plants were analyzed using 1148 SSR markers scattered throughout the 12 rice chromosomes (McCouch et al. 2002). A total of 520 SSR markers polymorphic between T65 and IRGC105715 were used for a whole-genome survey of the selected BC₁F₁ plants with qGRH4.2. The genotypes of the selected BC₁F₁ plant were detected by using bulked DNA of BC₁F₂ plants. The genotype of each line was graphically displayed according to the concept proposed by Young and Tanksley (1989). Physical distances between markers were calculated on the basis of the Nipponbare genome sequence in RAP-DB (http://rapdb.dna.affrc.go.jp/).

**Mapping of the GRH6 region**

The BC₁F₂ population was used for linkage analysis of the GRH6 and SSR marker loci. The GRH6 genotype of each individual was determined from its phenotype and the genotype of each SSR marker. GRH6 genotypes were confirmed by an F₃ progeny test. Detailed linkage analysis was conducted using additional DNA markers (RM5414, RM8213, G6-5, G6-9, G6-16, G6-30, and C60248) on chromosome 4 (Table 1, Fujita et al. 2010b). G6-5 were designed on the basis of the Nipponbare genome sequence using Primer3 (v. 3.0) (Rozen and Skaltsky 2000). The RM5414 and RM8213 were as described in McCouch et al. (2002).

**Construction of a high-resolution linkage map of GRH6**

The BC₁F₂ population (6834 plants) was used to construct a high-resolution map of the GRH6. Preliminary screening for recombinant plants was performed by using the SSR markers G6-5 and G6-16, which flank the GRH6 region. Plants with recombination events close to GRH6 were then selected with the SSR markers G6-c60k, G6-c70k, and 7L16f. To determine the precise location of the GRH6, RM8213, 7L16f, G6-c60k, G6-c70k, and 7L16f were used (Table 1). The progenies of recombinants were grown to select homozygous recombinants in the region of interest for evaluation in the antibiosis test.

**Results**

**Detection of QTLs for GRH resistance**

IRGC105715 showed 100% GRH nymph mortality, whereas T65 did not show any nymph mortality. The nymph mortalities of the BC₁F₁ population were distributed continuously from 0% to 100% (Fig. 2), suggesting that IRGC105715 has several QTL controlling GRH resistance. To understand the genetic basis of GRH resistance in IRGC105715, we performed QTL analysis using the BC₁F₁ population. A single major QTL (LOD = 14.1) for GRH resistance was identified on the short arm of chromosome 4 by simple interval mapping. This QTL, designated qGRH4.2, explained 67.6% of the phenotypic variation, and was located within a 13.4 cM interval flanked by RM8213 and RM5414.
Fig. 2. Frequency distribution of GRH nymph mortality after feeding on plants of the BC1F1 population derived from a cross between T65 (recurrent parent) and IRGC105715.

Table 2. Quantitative trait loci (QTL) affecting resistance to green rice leafhopper in the BC1F1 population derived from a cross between ‘Taichung 65’ and IRGC105715.

| QTL   | Chromosome | Marker interval       | Peak LOD score | PEV (%) | Additive effect |
|-------|------------|-----------------------|----------------|---------|----------------|
| qGRH4.2 | 4         | RM8213–RM5687         | 14.1           | 67.6    | -53.5          |
| qGRH7  | 7         | RM5405–RM3691         | 1.9            | 4.8     | -11.9          |
| qGRH12 | 12        | RM1261–RM1246         | 2.1            | 5.1     | -12.5          |

\* Critical threshold value of the LOD score was equivalent to 3.5 at an experiment-wise significance level of 0.05.
\* Percentage of explained phenotypic variation.
\* The value indicates the effect of the Taichung 65 allele.

and RM5687. The IRGC105715 allele at qGRH4.2 increased resistance to GRH. No other significant QTLs were detected. By composite interval mapping, we also detected a QTL on chromosome 7 (qGRH7; LOD score: 1.9) and on chromosome 12 (qGRH12; LOD score: 2.1; Table 2). qGRH7 and qGRH12 explained only 4.8% and 5.1% of phenotypic variation, respectively.

**Mapping of the GRH resistance gene**

For confirming the detailed location of qGRH4.2, a BC3F1 population segregating at qGRH4.2 was developed through advanced backcrossing with T65 as recurrent parent and marker-assisted selection (MAS). Among a total of the 54 BC3F1 plants, 2 plants showing resistance to GRH were eventually selected. These two plants were analyzed using SSR markers to detect introgressed chromosomal segments derived from IRGC105715 in the T65 genetic background. Introgressed chromosome segments derived from IRGC105715 were found on chromosomes 1, 3, 4, 7, and 8; the segment on chromosome 4 included qGRH4.2 (Fig. 1).

Nymph mortality of the BC3F2 population showed discrete bimodal segregation (0%–25% for 45 plants and 56%–100% for 140 plants; Fig. 3). The segregation ratio fit a 3:1 ratio ($\chi^2 = 0.05$), indicating that a single dominant gene controlled GRH resistance in the BC3F2 population. We designated this gene GRH6 (green rice leafhopper resistance 6 from O. nivara), since Grh6 was previously used to designate a GRH resistance gene from a Surinam variety ‘SML17’ on the short arm of chromosome 4 (Tamura et al. 2004). The BC3F2 population was subsequently analyzed for GRH resistance using SSR markers near the GRH6 region. Genotypes at GRH6 of the resistant BC3F2 individuals were determined by progeny test in BC3F3 lines to distinguish the heterozygotes and the homozygotes for the IRGC105715 allele at GRH6. Consequently, genotypes as GRH6 completely co-segregated with the genotypes at SSR marker RM8213 on chromosome 4. Linkage analysis demonstrated that the map distances between RM8213 and G6-9, RM8213 and GRH6, and GRH6 and G6-9 were 1.7 cM, 0.3 cM, and 1.4 cM, respectively. Finally, GRH6 was located between RM8213 and G6-9 (Fig. 4A).

**High-resolution mapping of GRH6**

Using flanking SSR markers G6-5 and G6-9, we screened a large segregating population (6834 BC3F3 plants) for recombinant individuals. A total of 235 recombinants between these two markers were obtained. Among them, we developed self-pollinated BC3F4 progenies lines derived from 8 BC3F3 plants carrying recombinant genotypes between SSR markers G6-c60k and sts4, and 14 BC3F3 plants carrying recombinant genotypes between SSR markers 7L16f and sts1 to evaluate homozygous recombinants in each region for genetic dissection of GRH6 (Fig. 4B). We evaluated nymph mortality in the 4 homozygous recombinants
According to sequence annotation at rice annotation project database (RAP-DB, https://rapdb.dna.affrc.go.jp/index.html), four genes, $\text{Os04g0165700}$, $\text{Os04g0165801}$, $\text{Os04g0165900}$, and $\text{Os04g0166000}$, are predicted as cysteine synthase, hypothetical protein, conserved hypothetical protein and conserved hypothetical protein in the 31.2-kbp target region in 'Nipponbare' reference sequence (Table 3). To compare functions of the predicted genes with other database (MSU Osa1 Release 7 Annotation, http://rice.plantbiology.msu.edu/index.shtml), $\text{Os04g0165700}$ is corresponding to $\text{LOC_Os04g08350}$ that predicted as cysteine synthase, while $\text{Os04g0165801}$ was not predicted. The $\text{Os04g0165900}$ and $\text{Os04g0166000}$ are coincided with $\text{LOC_Os04g08370}$ and $\text{LOC_Os04g08390}$ those were predicted as Leucine Rich Repeat (LRR) family protein. Additionally, $\text{Os04g0165900}$ and $\text{Os04g0166000}$ are predicted carrying LRR domain superfamily, through InterPro (https://www.ebi.ac.uk/interpro/).
More than 70 loci conferring resistance to insect pests have been mapped on rice chromosomes: 38 loci for BPH, 14 for white-backed planthopper, 14 for green leafhopper, and 8 for GRH (Fujita et al. 2013). Among the 38 loci for resistance to BPH, only 4 have had their causal genes cloned. BPH14 on chromosome 3 (from an accession of *Oryza officinalis*) was cloned by Du et al. (2009). BPH17 on chromosome 4 (from *O. sativa* ssp. indica ‘Rathu Heenati’) was cloned by Liu et al. (2015). BPH26 on chromosome 12 (from *O. sativa* ssp. indica ‘ADR52’) was cloned by Tamura et al. (2014). A different allele of BPH26, BPH18, was subsequently cloned from an accession of *Oryza australiensis* (Ji et al. 2016). BPH29 from an accession of *O. rufipogon* was cloned by Wang et al. (2015). There were no reports for cloning genes for resistance to the other three pest species. In this study, a total of 12 lines homozygous in the GRH6 region were selected and characterized for resistance to GRH. Six of them (Line 8-5, 13-2, 6-2, 7-5, 18-4, and 19-8) were susceptible with low nymph mortality. The other six lines (Line 4-3, 11-3, 14-1, 15-5, 1-9, and 3-3) were resistant with high nymph mortality. The distinct differences in nymph mortality between resistant and susceptible lines made it possible to delimit the candidate region of GRH6.

**BPH14** and **BPH26** encode a coiled-coil-nucleotide-binding-site–leucine-rich repeat (CC–NBS–LRR) proteins (Du et al. 2009, Tamura et al. 2014). **BPH17** is a cluster of three genes encoding plasma membrane–localized lecithin receptor kinases (Liu et al. 2015). The protein encoded by **BPH29** contains a B3 DNA-binding domain (Wang et al. 2015). In this study, four candidate genes for GRH6 were detected through constructing a high-resolution linkage map. Through InterPro, two candidate genes, Os04g0165900 and Os04g0166000, are predicted carrying LRR domain superfamily. The LRR domain related to produce a structure for the formation of protein–protein interaction and proteins contain of LRR are associated with various biological process such as disease resistance, insect resistance, and immune response in plant (Ooijen et al. 2008). Therefore, these genes are a strong candidate for GRH6. To reveal the mechanism of GRH resistance, cloning of **GRH6** will be necessary in a future study.

The identified resistance genes to insect pests such as BPH have been transferred into elite varieties by MAS without the need to evaluate resistance each generation. MAS is a powerful tool for developing elite varieties with resistance genes and for pyramiding multiple resistance genes (Jairin et al. 2009, Yara et al. 2010). Occasionally, there is linkage drag around the introduced resistance gene, resulting in co-introduction of genes for unfavorable traits such as waxy starch in endosperm, late heading, or hybrid sterility. Our high-resolution linkage map of **GRH6** will provide detailed marker information needed to introduce **GRH6** into elite varieties without linkage drag.

**Discussion**

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Epistatic effects of pyramiding among resistance genes for GRH have been reported for several combinations of genes from the same donor parent (Fujita et al. 2013). **GRH2** and **GRH4** from ‘DV85’ are dominant genes, and the resistance level of **GRH2/GRH4** pyramided lines (PYLs) is significantly higher than those of **GRH2-NIL** and **GRH4-NIL** (Fujita et al. 2006). An epistatic effect of pyramiding of Grh5 and qGRH4 in Grh5/qGRH4-PYL derived from the *O. rufipogon* accession W1962 has been observed (Fujita et al. 2010). These results suggest that the development of PYLs for resistance genes from the same donor parent can increase resistance to almost the same level as that of the donor parent itself. In our study, two minor QTLs for GRH resistance, qGRH7 and qGRH12, were detected on chromosomes 7 and 12, respectively, using a BC1F1 population. The level of GRH resistance of IRGC105715 was higher than that of **GRH6-NIL**, suggesting that IRGC105715 has additional minor QTLs for GRH resistance. However, it is not known whether the minor QTLs would confer GRH resistance as a single gene. Development of NILs for these minor QTLs would be needed to evaluate their effects on resistance. The epistatic effect of pyramiding **GRH6** and minor QTLs will be examined in a future study.

**Author Contribution Statement**

DF and HY designed the research. DF, CNP, and YY performed the research. DF and HY developed the plant materials. AY provided advice on the experiments. DF, CNP, YY and HY wrote the paper.

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