Development of near-isogenic lines and pyramided lines carrying resistance genes to green rice leafhopper (*Nephotettix cincticeps* Uhler) with the Taichung 65 genetic background in rice (*Oryza sativa* L.)

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The green rice leafhopper (GRH), *Nephotettix cincticeps* Uhler, is a serious insect pest of cultivated rice (*Oryza sativa* L.) in temperate East Asia. Six GRH-resistance genes (*Grh1*, *Grh2*, *Grh3*, *Grh4*, *Grh5*, and *Grh6*) and one quantitative trait locus (QTL; *qGRH4*) have been identified. We selected near-isogenic lines (NILs) carrying *Grh1*, *Grh2*, *Grh4*, *Grh5*, *Grh6*, and *qGRH4* with the *japonica* genetic background (Taichung 65 cultivar) by means of marker-assisted selection using new simple sequence repeat markers flanking the GRH-resistance genes and QTL. We also developed three pyramided lines (PYLs; *Grh2/Grh6*-PYL, *Grh4/Grh6*-PYL, and *Grh5/qGRH4*-PYL) using each NIL that carried a GRH-resistance gene or QTL. The NILs, PYLs, and donor parents were evaluated by using an antibiosis test. The resistance of *Grh1*-NIL and *Grh5*-NIL did not differ significantly from those of the donor parents, whereas the resistances of *Grh2*-NIL and *Grh6*-NIL were significantly lower than those of the donor parents. *Grh4*-NIL and *qGRH4*-NIL were highly susceptible. The resistance levels of the pyramided lines for *Grh2* and *Grh6*, *Grh4* and *Grh6*, and *Grh5* and *qGRH4* demonstrated a gene pyramiding effect that significantly increased their resistance. The developed NILs and PYLs should be useful genetic resources for rice improvement and deployment of the resistance genes.

Key Words: green rice leafhopper, marker-assisted selection, near-isogenic line, pyramided line, resistance gene, rice.

Introduction

The green rice leafhopper (GRH), *Nephotettix cincticeps* Uhler, is a major insect pest of cultivated rice and is distributed mostly in the temperate regions of East Asia (Ghauri 1971). The GRH sucks sap from both the xylem and phloem of susceptible rice cultivars, leading to yield losses, particularly in northeastern Japan (Nirei and Nakazato 1975), and in western Japan the insect also damages rice plants by transmitting several viral diseases (Nakasuji and Nomura 1968). Genetic analyses of GRH resistance have been performed by using molecular markers, and six loci for GRH resistance have been identified. The following resistance genes have been mapped: *Grh1* from Pe-bi-hun and IR24, on chromosome 5 (Tamura *et al*. 1999, Yasui and Yoshimura 1999, Kadowaki *et al*. 2003); *Grh3*(t) from Rantaj-emas 2, on chromosome 6 (Saka *et al*. 1997, 2006); and two dominant genes, *Grh2* on chromosome 11 and *Grh4* on chromosome 3, both from Lepedumai and DV85 (Fukuta *et al*. 1998,

Yazawa *et al*. 1998, Kadowaki *et al*. 2003). *Grh5* from *Oryza rufipogon* (W1962) has been mapped on chromosome 8 (Fujita *et al*. 2006), and *Grh6* from the Surinam cultivar SML17 (Tamura *et al*. 2004) and *Grh6-nivara*(t) from *Oryza nivara* IRGC105715 have been mapped on the short arm of chromosome 4 (Fujita *et al*. 2004). In addition to these Mendelian genes, a quantitative trait locus (QTL) with a minor effect derived from *O. rufipogon* acc. W1962 (*qGRH4*) has been identified on the long arm of chromosome 4 (Fujita *et al*. 2006).

Virulent biotypes of insect pests became dominant in brown planthopper (*Nilaparvata lugens* (Stål), BPH) populations after the release of modern improved rice cultivars that carry a single major gene for resistance to this insect pest. The emergence of dominant virulent biotypes that have overcome single-gene host resistance is a serious impediment to insect pest management. For example, two of the genes that confer resistance to BPH (*Bph1* and *bph2*) have completely lost their effectiveness in East Asia because of the dominance of virulent biotypes in the current BPH populations (Tanaka and Matsumura 2000, Myint *et al*. 2009b). In addition, elite varieties that carry other BPH resistance gene (*Bph3* or *bph4*) that have been widely cultivated in

southeastern Asia (Khush 1989) have recently become susceptible. Virulent biotypes of the planthoppers and of leafhoppers have been experimentally identified by continuously rearing of the insects on resistant lines of rice, each of which carried a single major resistance gene (Ketipearachchi *et al*. 1998, Hirae *et al*. 2007). This suggests that natural strains of the insects are also likely to feed on rice plants that possess a single major gene for resistance. The development of pyramided lines (PYLs) that carry multiple genes for resistance would be an effective way to delay the breakdown of GRH resistance (Hirae *et al*. 2007) and BPH resistance (Myint *et al*. 2009a). Deployment of PYLs can therefore be a useful tool because it can increase the durability of resistance against virulent biotypes and may even suppress the occurrence of virulent biotypes.

Simple sequence repeat (SSR) marker loci are widely distributed throughout the genome and can be easily analyzed by using the polymerase chain reaction (PCR; McCouch *et al*. 2002). The use of DNA markers to develop rice PYLs carrying major genes for resistance, including bacterial blight resistance genes (Yoshimura *et al*. 1995), blast resistance genes (Hittalmani *et al*. 2000), and BPH resistance genes (Sharma *et al*. 2004), has already been reported. The development of PYLs carrying genes with epistatic effects or minor QTLs with a weak phenotypic contribution can be difficult because of the difficulty of identifying resistance to the GRH through conventional breeding methods. The availability of DNA markers that are closely linked to resistance genes and QTLs would facilitate the identification of plants carrying multiple genes for resistance.

Our objectives were to develop near-isogenic lines (NILs) and PYLs for GRH resistance by means of marker-assisted selection (MAS), and to facilitate the use of the GRH-resistance genes and QTLs in future rice improvement. First, we developed NILs carrying five of the six GRH-resistance genes (*Grh1*, *Grh2*, *Grh4*, *Grh5*, and *Grh6*) and a minor QTL (*qGRH4*) from four GRH-resistant lines by means of MAS. Subsequently, we used MAS to develop PYLs carrying two GRH-resistance genes or one resistance gene plus the QTL from NILs carrying *Grh2*, *Grh4*, *Grh5*, *Grh6*, and *qGRH4*. Finally, we compared nymph mortality on the NILs with that on each donor parent and compared the nymph mortality on the PYLs with that on each NIL. The results will be useful for rice breeding leading to the deployment of the genes and QTLs that confer resistance to GRH in rice fields.

Materials and Methods

Plant materials

We used Taichung 65 (T65), a *japonica* cultivar, as a recurrent parent for development of the NILs. We used four lines (IR24, DV85, W1962, and IRGC105715) as the donor parents because they had been shown previously to carry genes conferring a high level of GRH resistance. IR24, an *indica* cultivar, carries *Grh1* (Yasui and Yoshimura 1999),

and DV85, an *indica* cultivar from Bangladesh, carries *Grh2* and *Grh4* (Yazawa *et al*. 1998). W1962, a wild accession of *Oryza rufipogon* from China, carries *Grh5* and *qGRH4* (Fujita *et al*. 2006), and IRGC105715, a wild accession of *O. nivara* from Cambodia, carries *Grh6* (Fujita *et al*. 2004). The W1962 accession that we used was obtained from the collection maintained by Japan's National Institute of Genetics (http://www.shigen.nig.ac.jp/rice/oryzabase/nbrpStrains/nig.jsp). The IRGC105715 accession was obtained from the International Rice Genebank Collection of the International Rice Research Institute (IRRI) in the Philippines.

Development of NILs and PYLs

We crossed the four donor lines (IR24, DV85, W1962, and IRGC105715) with the recurrent parent, T65. The F_1 plants were repeatedly backcrossed with T65 to develop the BC_3F_1 (IR24 and IRGC105715), BC_4F_1 (W1962), and BC_6F_1 (DV85) generations, and then several plants were self-pollinated for three to five generations. Individuals with homozygous alleles from the donor parents at the resistance gene or QTL loci were selected from the segregating populations by means of MAS (as described later in the Methods section). NILs carrying GRH-resistance genes or the QTL with the T65 genetic background were also selected from the progeny of the selected individuals by means of MAS.

PYLs for three GRH-resistance genes were developed by using the NILs for the GRH-resistance genes: *Grh2*-NIL (BC_6F_4), *Grh4*-NIL (BC_6F_4), and *Grh6*-NIL (BC_3F_2). These NILs for developing the PYLs were derived from sib lines of the developed NILs. F_1 plants carrying two GRH-resistance genes were self-pollinated, and then individuals with homozygous alleles at the GRH-resistance gene loci were selected from the F_2 populations by using MAS. A PYL carrying both *Grh5* and *qGRH4* was selected from the segregating population (BC_4F_3) derived from a cross between W1962 and T65.

Genotyping using SSR markers

Total DNA of the NILs and PYLs carrying the GRH-resistance genes or the QTL was extracted from freeze-dried leaves of individual plants by using the potassium acetate method (Dellaporta *et al*. 1983). The genotypes of the NILs and PYLs for GRH resistance were then determined by means of PCR amplification using a PCR System-9700 (Perkin Elmer, Waltham, Massachusetts). The 15-μL PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl$_2$, 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 a 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. There was no final extension phase. The PCR products were resolved in 4.0% agarose gels by means of electrophoresis at 200 V for 1 h in 0.5× TBE buffer. The gels were stained with ethidium bromide and photographed under ultraviolet light.

Marker-assisted selection (MAS)

The six NILs and three PYLs for GRH resistance were developed by means of MAS. To select individuals carrying the GRH-resistance genes or QTL, we chose 25 SSR markers near the *Grh1*, *Grh2*, *Grh4*, *Grh5*, *Grh6*, and *qGRH4* loci based on the location of the GRH-resistance genes and the QTL in the linkage map (Table 1). In addition to the 15 SSR markers that have previously been reported (McCouch *et al.* 2002), we developed 10 new SSR markers (*G1-5*, *G2-10*, *ag.17.1.2*, *G4-t4*, *G4-SSR8*, *G4-SSR16*, *G4-SSR21*, *G6-9*, *G6-16*, and *G6-30*) near the resistance genes or QTL based on the Nipponbare genome sequence. We used Primer3 (v. 0.3.0) to develop these new SSR markers (Rozen and Skaletsky 2000). We constructed linkage maps of *Grh1* and *Grh2* by using restriction fragment length polymorphism (RFLP) markers on chromosomes 5 and 11, respectively (Kadowaki *et al.* 2003). On the basis of the linkage map for *Grh1*, we set up *G1-5* and *RM3381*, which showed polymorphism between T65 and IR24, between the RFLP markers *R569* and *R3313* on chromosome 5 (Fig. 1A). On the basis of the linkage map for *Grh2*, we set up *G2-10* and *RM5961*, which showed polymorphism between T65 and DV85, to flank *Grh2* on chromosome 11 (Fig. 1B). We set up *G4-SSR8* and *G4-SSR21*, which showed polymorphism between T65 and DV85, to flank *Grh4* on chromosome 3 (Fig. 1C). We set up *RM502* and *RM3120*, which showed polymorphism between T65 and W1962, to flank *Grh5* on chromosome 8 (Fig. 1D). We set up *RM8213* and *G6-9*, which showed polymorphism between T65 and IRGC105715, to flank *Ghr6* on chromosome 4 (Fig. 1E). Finally, we set up three SSR markers (*RM1155*, *RM6997*, and *RM7051*) that showed polymorphism between T65 and W1962 and flanked *qGRH4* on chromosome 4 (Fig. 1F). The *qGRH4*-NIL had chromosomal segments derived from W1962 only in the region between *RM1155* and *RM7051*. In light of the weak phenotypic effect of *qGRH4*, we could not have constructed the linkage map between these markers and *qGRH4*. The 25 SSR markers near the GRH-resistance genes or QTL were used for MAS when plants with the GRH-resistance genes or QTL were selected from the segregating populations.

Whole-genome survey of the genetic background of the NILs and PYLs

We analyzed the developed NILs and PYLs by using SSR markers scattered throughout the rice chromosomes to survey their genetic backgrounds as well as those of the targeted genes and QTL. In this analysis, we used SSR markers

Table 1. The 25 simple sequence repeat markers used for marker-assisted selection of five genes and one QTL for resistance to the green rice leafhopper, *Nephotettix cincticeps* Uhler

Marker	Resistance gene tagged	Nipponbare BAC or PAC	Forward primer sequence (5′→3′)	Reverse primer sequence (5′→3′)	Predicted size (bp)[b]
G1-5	*Grh1*	AC135422	GGTTTTGACGACGATTGC	ACGCCCACACGTTACGTACT	110
RM289[a]	*Grh1*	AC132487	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAAAG	86
RM3381[a]	*Grh1*	AC132495	ACGAACGCGAGCTGACAGAGG	AATAGCTGCCAGCAACTGCAACG	92
RM3437	*Grh1*	AC136221	AACCACCTAGGTTTCTCCCC	TAGCAACGAGGTTATTGGGC	172
RM5349[a]	*Grh2*	AC134925	AGGGCATGCTTACATCCAAC	CATTTGCTTCTATGCCCCAG	123
RM5961	*Grh2*	AC108223	GTATGCTCCTCCTCACCTGC	ACATGCGACGTGATGTGAAC	129
G2-10[a]	*Grh2*	AC104844	CACCATGGAGTCCCATTGA	GGGTCAAGTCGGGGATAGTA	145
ag.17.1.2[a]	*Grh4*	AC125784	CCTCCTCCTCTACACTGCTTTCC	CTTGGGTAGCTTTTCCGTTG	169
G4-SSR8	*Grh4*	AC082644	CCACTCAGCCAACTACTAGAAC	TGGTCCTTACATGTGTCTGTCC	116
G4-SSR16[a]	*Grh4*	AC084022	GAGTCGGATTCGGCCTACTG	ACTCCGACTCGGTCTAAATGG	100
G4-SSR21	*Grh4*	AC092075	GTCGAGCTCTATTGCTGGTG	GCAATGGAGCACTGTAGGTC	140
G4-t4	*Grh4*	AC097279	GCCGTGAACAACCAGAAGAT	GGACTCATGACCAATTAATCCA	230
RM1615[a]	*Grh5*	AP003857	GAAGAGGAGTTATTAGGTAG	GTTTTTACCTTTAAAATTTA	122
RM502[a]	*Grh5*	AP005529	GCGATCGATGGCTACGAC	ACAACCCAACAAGAAGGACG	265
RM3754	*Grh5*	AP004592	TCGTAGGTGGGGCTAACAAG	CACCCTCTTCTTCCTCCGAC	92
RM3761	*Grh5*	AP003914	CCTCAACAATAGCACCACCC	CTGCAAGTCTGCAAGCACAG	177
RM6845[a]	*Grh5*	AP003912	GTGACGGCAAGAGGAAGAAG	GTTCGACAGGAACGCCAC	143
RM3120	*Grh5*	AP003928	ATCGATGGAAGCTCTTTGCC	GGATGTACAAGAGCTTAGGAGC	120
G6-30	*Grh6*	AL606654	GTGGGTGCTGTTAGCTCAAGT	CTGAATCCAGCTGCTCTCACT	116
RM8213[a]	*Grh6*	AL662959	AGCCCAGTGATACAAAGATG	GCGAGGAGATACCAAGAAAG	211
G6-9	*Grh6*	AL663013	GCCGTGGAGAAGAAGCTGTA	CCTAGACCTCATCCCCTTCC	140
G6-16[a]	*Grh6*	AL662994	GCGGTGAGATAGATGGATGG	CCTCGTAAACCCTGTCCTCA	224
RM1155	*qGRH4*	AL606453	AGGGAGTGTGGCAACTATGC	GGGAGGAGTGAGAAGGGATC	148
RM6997[a]	*qGRH4*	AL606632	CAACGCGGCAGTAAATTTGC	GGCCTTGTCAGTCTACATGC	154
RM7051[a]	*qGRH4*	AL627350	CTCGATGAGCTTGGCGTC	TTCAGTGTTCATCGCCTCTG	176

[a] The marker was most closely linked to and encompassed the marker for detection of the GRH resistance gene or QTL in the surveyed genetic background.

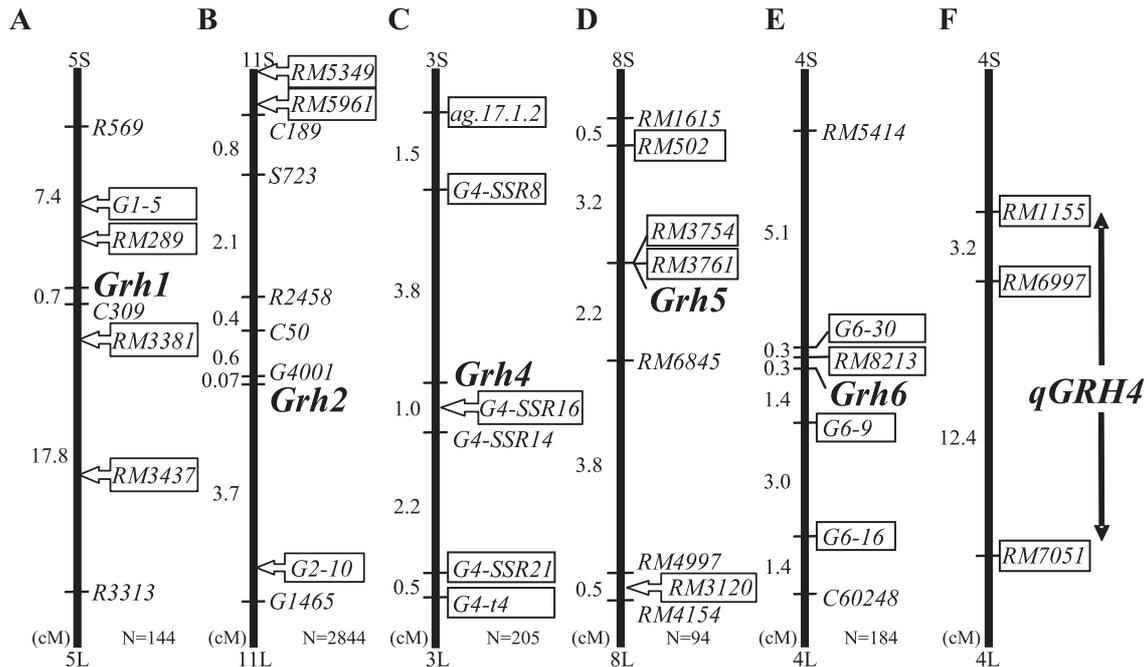[b] The PCR product size was estimated on the basis of the Nipponbare genome sequence.

A B C D E F

5S 11S 3S 8S 4S 4S

A:
R569
7.4 G1-5
RM289
Grh1
0.7 C309
RM3381
17.8 RM3437
R3313
(cM) N=144
5L

B:
RM5349
RM5961
C189
0.8
S723
2.1
R2458
0.4 C50
0.6 G4001
0.07 Grh2
3.7
G2-10
G1465
(cM) N=2844
11L

C:
ag.17.1.2
1.5
G4-SSR8
3.8
Grh4
1.0 G4-SSR16
G4-SSR14
2.2
G4-SSR21
0.5 G4-t4
(cM) N=205
3L

D:
RM1615
0.5 RM502
3.2
RM3754
RM3761
2.2 Grh5
RM6845
3.8
RM4997
0.5 RM3120
RM4154
(cM) N=94
8L

E:
RM5414
5.1
G6-30
0.3 RM8213
0.3 Grh6
1.4 G6-9
3.0
G6-16
1.4
C60248
(cM) N=184
4L

F:
RM1155
3.2
RM6997
qGRH4
12.4
RM7051
(cM)
4L

Fig. 1. Map positions of *Grh1*, *Grh2*, *Grh4*, *Grh5*, *Grh6*, and *qGRH4* and the corresponding marker loci on each chromosome. (A) *Grh1* on the short arm of chromosome 5 (Kadowaki *et al*. 2003); (B) *Grh2* on the long arm of chromosome 11 (Kadowaki *et al*. 2003); (C) *Grh4* on the short arm of chromosome 3; (D) *Grh5* on the long arm of chromosome 8 (Fujita *et al*. 2006); (E) *Grh6* on the short arm of chromosome 4 (Fujita *et al*. 2004); and (F) *qGRH4* on the long arm of chromosome 4 (Fujita *et al*. 2006). Rectangles show the SSR markers used for marker-assisted selection; rectangles with an arrow show the position of the SSR markers based on the Nipponbare sequence.

described by McCouch *et al*. (2002). We detected the substituted regions of *Grh1*-NIL by using 117 SSR markers that were polymorphic between T65 and IR24. We surveyed the substituted regions of *Grh2*-NIL, *Grh4*-NIL, *Grh2/Grh6*-PYL, and *Grh4/Grh6*-PYL by using 122 SSR markers that were polymorphic between T65 and DV85. We identified the substituted regions of *Grh5*-NIL and *qGRH4*-NIL by using 88 SSR markers that were polymorphic between T65 and W1962. We detected the substituted regions of *Grh6*-NIL, *Grh2/Grh6*-PYL, and *Grh4/Grh6*-PYL by using 96 SSR markers that were polymorphic between T65 and IRGC105715. By using the genotype data for these NILs and PYLs, we graphically displayed the genotypes of each line according to the concept of graphical genotypes proposed by Young and Tanksley (1989).

Evaluation of GRH resistance

The GRH population was collected in Fukuoka Prefecture in 1991 and was subsequently maintained by continuously rearing the insects on seedlings of the Nipponbare *japonica* cultivar. Insects were kept at 25°C ± 1°C and 16 h light: 8 h dark. We modified the antibiosis test described by Kishino and Ando (1978) for use in our study. In this test, seedlings at the second-leaf stage were infested with 7 to 10 first- or second-instar nymphs in test tubes, 10 days after sowing. Nymph mortality was then calculated at 3 days after infestation. Additionally, the nymph mortality on *qGRH4*-NIL was calculated at 7 days after infestation. Seedlings of

the NILs and PYLs carrying the GRH-resistance genes or the QTL were used for the antibiosis tests. These experiments were conducted at least 9 replicates, but tests of *Grh2/Grh6*-PYL and *Grh4/Grh6*-PYL were repeated at least 26 replicates. Differences between means were statistically analyzed by using the Tukey-Kramer test following analysis of variance, with significance at $P < 0.01$.

Results

Development of NILs

We developed six NILs for GRH resistance and clarified their genetic backgrounds (Table 2 and Fig. 2). *Grh1*-NIL was confirmed by the flanking markers of *Grh1* (*RM289* and *RM3381*), which encompass *Grh1* on chromosome 5 (Fig. 2A). The size of the substituted segment was estimated as 17.6 to 27.9 Mbp on the basis of the loci of two SSR markers (*RM3419* and *RM6841*). The other substituted segment was detected between *RM3513* and *RM2334* on chromosome 3, with a size of 1.6 to 5.0 Mbp. *Grh2*-NIL was confirmed by the flanking marker *G2-10*, which is tightly linked to *Grh2* on chromosome 11 (Fig. 2B), and by the antibiosis test (see later in the Results section for the results of this test). The size of the substituted segment around *Grh2* was less than 9.3 Mbp. There were no other substituted segments in the T65 genetic background. *Grh4*-NIL was confirmed by the flanking markers of *Grh4* (*ag.17.1.2* and *G4-SSR16*), which encompass *Grh4* on chromosome 3 (Fig. 2C). The

Table 2. Near-isogenic lines (NILs) and pyramided lines (PYLs) developed for green rice leafhopper resistance genes (*Grh1*, *Grh2*, *Grh4*, *Grh5*, and *Grh6*) and a resistance QTL (*qGRH4*)

Line	Origin	Genotype	Genetic background	Generation
Grh1-NIL	IR24 (*O. sativa*)	*Grh1/Grh1*	Taichung 65	BC$_3$F$_4$
Grh2-NIL	DV85 (*O. sativa*)	*Grh2/Grh2*	Taichung 65	BC$_6$F$_5$
Grh4-NIL	DV85 (*O. sativa*)	*Grh4/Grh4*	Taichung 65	BC$_6$F$_5$
Grh5-NIL	W1962 (*O. rufipogon*)	*Grh5/Grh5*	Taichung 65	BC$_4$F$_3$
Grh6-NIL	IRGC105715 (*O. nivara*)	*Grh6/Grh6*	Taichung 65	BC$_3$F$_4$
qGRH4-NIL	W1962 (*O. rufipogon*)	*qGRH4/qGRH4*	Taichung 65	BC$_4$F$_3$
Grh2/Grh6-PYL	*Grh2*-NIL, *Grh6*-NIL	*Grh2/Grh2*; *Grh6/Grh6*	Taichung 65	F$_3$(BC$_6$F$_4$ / BC$_3$F$_2$)^a
Grh4/Grh6-PYL	*Grh4*-NIL, *Grh6*-NIL	*Grh4/Grh4*; *Grh6/Grh6*	Taichung 65	F$_3$(BC$_6$F$_4$ / BC$_3$F$_2$)^a
Grh5/qGRH4-PYL	W1962 (*O. rufipogon*)	*Grh5/Grh5*; *qGRH4/qGRH4*	Taichung 65	BC$_4$F$_3$

^{a} The generations of the PYLs shown in parentheses indicate the generations of the NILs that were used as the female and male parents, respectively.

size of the substituted segment around *Grh4* was estimated as 0.6 to 10.3 Mbp on the basis of the SSR marker loci *ag.17.1.2* and *G4-SSR16*. The other substituted segments were detected at *RM3505* on chromosome 2 (with a size of less than 5.1 Mbp) and at *RM3533* on chromosome 9 (with a size of less than 7.9 Mbp). *Grh5*-NIL was confirmed by the flanking markers of *Grh5* (*RM502* and *RM6845*), which encompass *Grh5* on chromosome 8 (Fig. 2D). The size of the substituted segment around *Grh5* was estimated as 4.3 to 9.5 Mbp on the basis of the SSR marker loci *RM6976* and *RM3155*. The other substituted segment was detected at *RM6840* on chromosome 1, with a size of less than 8.3 Mbp. *Grh6*-NIL was confirmed by the flanking markers of *Grh6* (*RM8213* and *G6-16*), which encompass *Grh6* on chromosome 4 (Fig. 2E). The size of the substituted segment around *Grh6* was estimated as 11.8 to 19.9 Mbp on the basis of the SSR marker loci *RM5414* and *RM7279*. There were three other substituted segments, at loci heterozygous for *RM5336* and *RM5552* on chromosome 1 (with a size of 0.8 to 7.5 Mbp), at *RM6111* on chromosome 7 (with a size of less than 3.5 Mbp), and at a locus heterozygous for *RM6356* on chromosome 8 (with a size of less than 3.8 Mbp). *qGRH4*-NIL was confirmed by the flanking markers (*RM6997* and *RM7051*), which are linked to *qGRH4* on chromosome 4 (Fig. 2F) and by the antibiosis test (see later in the Results section for the results of this test). The size of the substituted segment around *qGRH4* was estimated as 3.4 to 10.9 Mbp on the basis of the SSR marker loci *RM6997* and *RM7051*. There were two other substituted segments, at *RM7451* on chromosome 2 (with a size of less than 5.2 Mbp) and at *RM6707* on chromosome 9 (with a size of less than 2.5 Mbp).

Development of the PYLs

We developed three PYLs for two GRH-resistance genes and analyzed their genetic backgrounds by using SSR markers evenly distributed across the 12 chromosomes (Table 2 and Fig. 2). *Grh2/Grh6*-PYL was confirmed by the flanking markers *RM5349* and *G2-10* for *Grh2* and *RM8213* and *G6-16* for *Grh6* (Fig. 2G). The sizes of the substituted segments around *Grh2* and *Grh6* were estimated as 4.4 to 17.9 Mbp

and 9.4 to 19.9 Mbp, respectively. Four other substituted segments were detected, at *RM6324* and *RM5552* on chromosome 1 (with a size of 1.8 to 4.8 Mbp), at a locus heterozygous for *RM1031* on chromosome 6 (with a size of less than 5.3 Mbp), at *RM6356* on chromosome 8 (with a size of less than 1.8 Mbp), and at *RM6707* on chromosome 9 (with a size of less than 2.2 Mbp). *Grh4/Grh6*-PYL was confirmed by the flanking markers *ag.17.1.2* and *G4-t4* for *Grh4* and *RM8213* and *G6-16* for *Grh6* (Fig. 2H). The substituted segments around *Grh4* and *Grh6* were estimated as having sizes of 9.3 to 17.2 Mbp and 9.4 to 19.9 Mbp, respectively. There was only one substituted segment, at *RM3533* on chromosome 9, with a size of less than 7.9 Mbp. Among the substituted segments other than the targeted genes of the PYLs (four chromosomal segments for *Grh2/Grh6*-PYL and one chromosomal segment for *Grh4/Grh6*-PYL), the origin of chromosomal segments could not be determined by using our SSR markers because the DNA bands of several SSR markers in DV85 were similar to those of IRGC105715. *Grh5/qGRH4*-PYL was confirmed by the flanking markers *RM1615* and *RM6845* for *Grh5* and *RM6997* and *RM7051* for *qGRH4* (Fig. 2I). The substituted segments around *Grh5* and *qGRH4* were estimated as having sizes of 1.6 to 2.3 Mbp and 3.4 to 10.9 Mbp, respectively. There was only one substituted segment other than the targeted gene or QTL; it occurred at *RM7451* on chromosome 2 and had a size of less than 5.2 Mbp.

Comparison of nymph mortality between NILs and donor parents

We compared nymph mortality on the NILs for GRH resistance with those of the donor parents (Fig. 3). The nymph mortality on the donor parents was 95% for IR24, 100% for DV85, 94% for W1962, and 98% for IRGC105715. Nymph mortality on the NILs was 86% for *Grh1*-NIL, 75% for *Grh2*-NIL, 4% for *Grh4*-NIL, 87% for *Grh5*-NIL, 61% for *Grh6*-NIL, and 2% for *qGRH4*-NIL. Although nymph mortality for *qGRH4*-NIL was only 2% at 3 days after infestation, *qGRH4*-NIL showed moderate resistance at 7 days after infestation, with a nymph mortality of 40%, versus
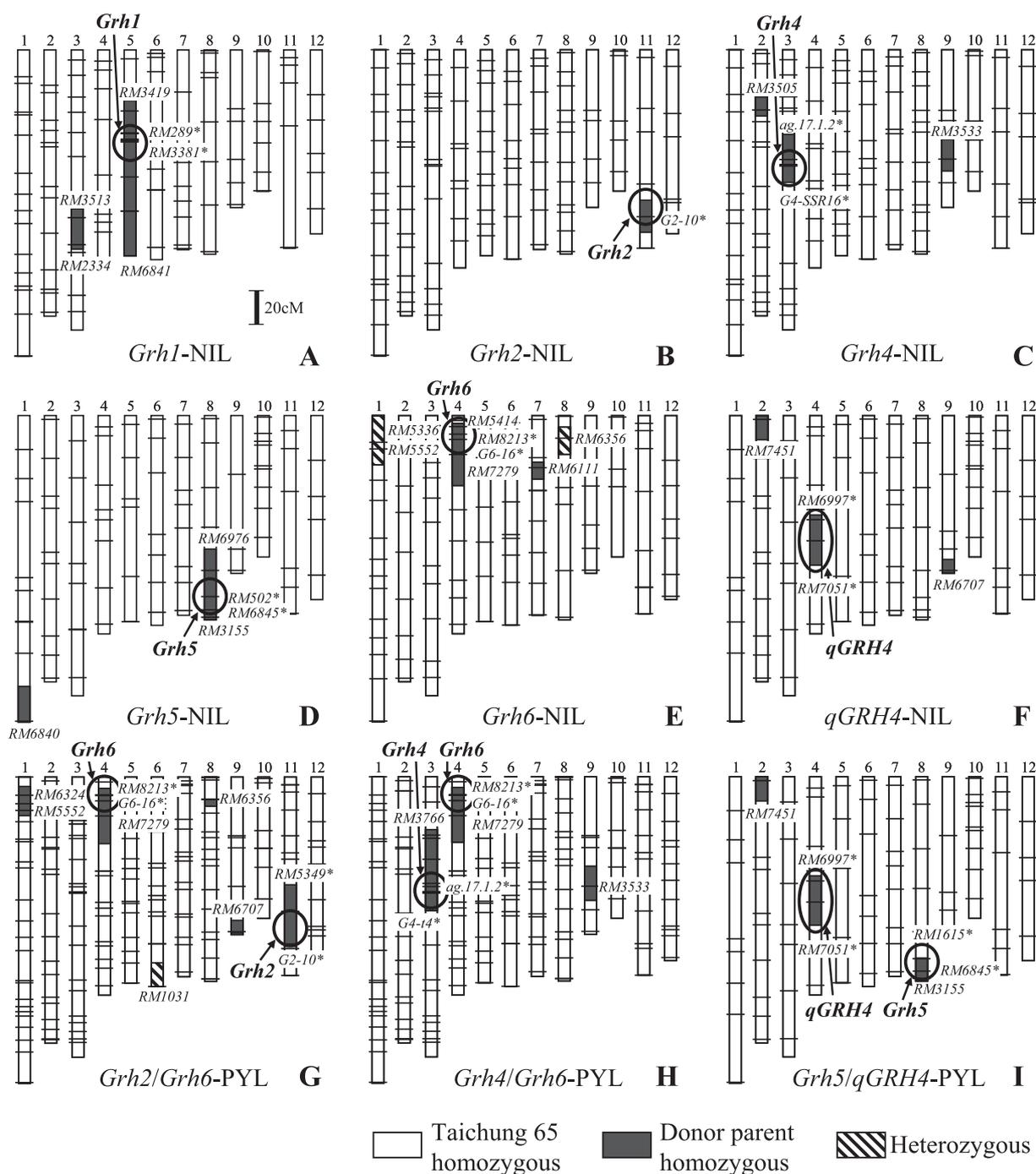
Fig. 2. Graphical genotypes of the NILs and PYLs for the GRH-resistance genes and QTL: (A) *Grh1*-NIL, (B) *Grh2*-NIL, (C) *Grh4*-NIL, (D) *Grh5*-NIL, (E) *Grh6*-NIL, (F) *qGRH4*-NIL, (G) *Grh2*/*Grh6*-PYL, (H) *Grh4*/*Grh6*-PYL, and (I) *Grh5*/*qGRH4*-PYL. The 12 bars indicate the 12 chromosomes of rice. The horizontal lines on the bars show the locations of the polymorphic SSR markers used in the whole-genome survey. The markers labeled with asterisks were used to confirm the resistance genes in the background survey. The circles on the bars represent the positions of the GRH-resistance genes or QTL.

14% for T65. The resistance of *Grh1*-NIL did not differ significantly from that of IR24, although nymph mortality on *Grh1*-NIL was 9% lower (Fig. 3A). *Grh5*-NIL showed resistance equivalent to that of W1962 (no significant difference), although nymph mortality was 7% lower on *Grh5*-NIL (Fig. 3B). On the other hand, *Grh2*-NIL showed

significantly (25%) lower resistance than DV85 (Fig. 3C). Similarly, *Grh6*-NIL showed significantly (37%) lower resistance than IRGC105715 (Fig. 3D). Thus, the resistance levels of *Grh1*-NIL and *Grh5*-NIL were equivalent to those of the donor parents (IR24 and W1962, respectively), although somewhat lower nymph mortality was observed on
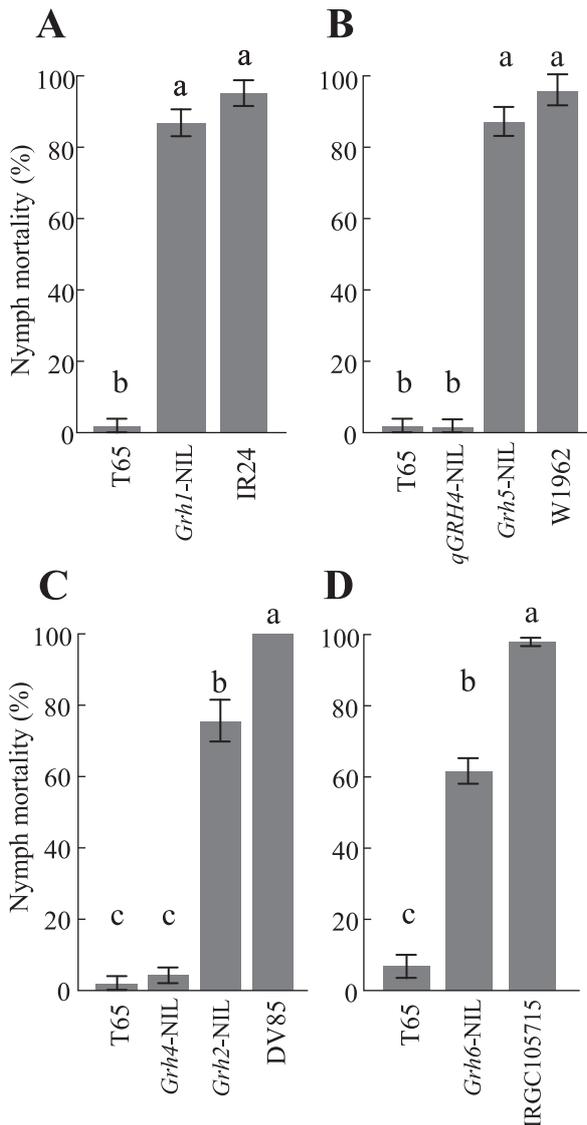
A

B

C

D

Fig. 3. Comparison of GRH nymph mortality between the resistant donor parents and each NIL carrying *Grh1*, *Grh2*, *Grh4*, *Grh5*, *Grh6*, and *qGRH4* at 3 days after infestation. Error bars indicate standard errors. Means labeled with different letters differ significantly ($P < 0.01$, Tukey-Kramer test).

the NILs. The resistance levels of *Grh2*-NIL and *Grh6*-NIL were significantly lower than those of the donor parents (DV85 and IRGC105715, respectively). In contrast to these results, *qGRH4*-NIL and *Grh4*-NIL were highly and significantly susceptible to the GRH (Fig. 3B and 3C).

Comparison of GRH nymph mortality between NILs and PYLs

We compared the nymph mortality levels on *Grh2/Grh6*-PYL, *Grh4/Grh6*-PYL, and *Grh5/qGRH4*-PYL with those on the NILs that each carried a single gene or QTL for resistance to the GRH. The nymph mortality levels on these PYLs were 96.4% for *Grh2/Grh6*-PYL, 74.7% for *Grh4/Grh6*-PYL, and 99% for *Grh5/qGRH4*-PYL (Fig. 4). The

nymph mortality on *Grh2/Grh6*-PYL was 21.0% higher than that of *Grh2*-NIL (75.4%), and was 35.1% higher than that of *Grh6*-NIL (61.3%) (Fig. 4A). The resistance level of *Grh2/Grh6*-PYL was significantly higher than that of *Grh2*-NIL and *Grh6*-NIL, each of which carries a single gene for resistance to the GRH. Similarly, the nymph mortality on *Grh4/Grh6*-PYL was 13.4% higher than that on *Grh6*-NIL (61.3%) and 70.6% higher than that on *Grh4*-NIL (4.1%) (Fig. 4B). The resistance level of *Grh4/Grh6*-PYL was significantly higher than those of *Grh4*-NIL and *Grh6*-NIL. The nymph mortality on *Grh5/qGRH4*-PYL, which was derived from the same donor parent (W1962), was 12% higher than that of *Grh5*-NIL (87%) and 97% higher than that of *qGRH4*-NIL (2%) (Fig. 4C). The resistance level of *Grh5/qGRH4*-PYL was significantly higher than those of *Grh5*-NIL and *qGRH4*-NIL. The effects of pyramiding between *Grh2* and *Grh6*, between *Grh4* and *Grh6*, and between *Grh5* and *qGRH4* significantly increased GRH resistance.

Discussion

We used continuous backcrossing and MAS to develop six NILs and three PYLs that carried GRH-resistance genes or the resistance QTL. Without the utilization of molecular markers, it would have been impossible to develop PYLs through conventional breeding methods because of segregation of the multiple genes or the QTL. Recently, DNA markers that were closely linked to resistance genes or a resistance QTL have been found to be useful for the identification of plants carrying multiple genes. For example, PYLs for resistance genes for bacterial blight (Yoshimura *et al.* 1995, Sanchez *et al.* 2000, Singh *et al.* 2001), blast (Hittalmani *et al.* 2000), and BPH (Sharma *et al.* 2004) have been developed by using DNA markers. MAS made it possible to develop NILs carrying *Grh4* and *qGRH4*, with a weak phenotypic contribution, although the development of the NILs would have been difficult through conventional breeding methods because of their silent resistance phenotypes. Using these NILs for GRH resistance, we eventually developed three PYLs (*Grh2/Grh6*-PYL, *Grh4/Grh6*-PYL, and *Grh5/qGRH4*-PYL) by using MAS. The developed NILs and PYLs will be valuable genetic materials in future genetic studies, including studies of the characteristic of gene effects, the development of molecular markers tightly linked to GRH-resistance genes, studies of gene expression, studies of gene isolation, and the field deployment of the resistance genes.

We used 25 SSR markers that were tightly linked to the five genes and one QTL that conferred resistance to the GRH for MAS and confirmed the presence of the resistance genes in our genetic background survey (Table 1 and Fig. 1). These flanking SSR markers facilitate the selection of resistant genotypes through MAS and the development of plant materials. MAS combined with advanced backcrossing with the recurrent parent can facilitate the transfer of GRH-resistance genes. The SSR markers that are tightly linked to
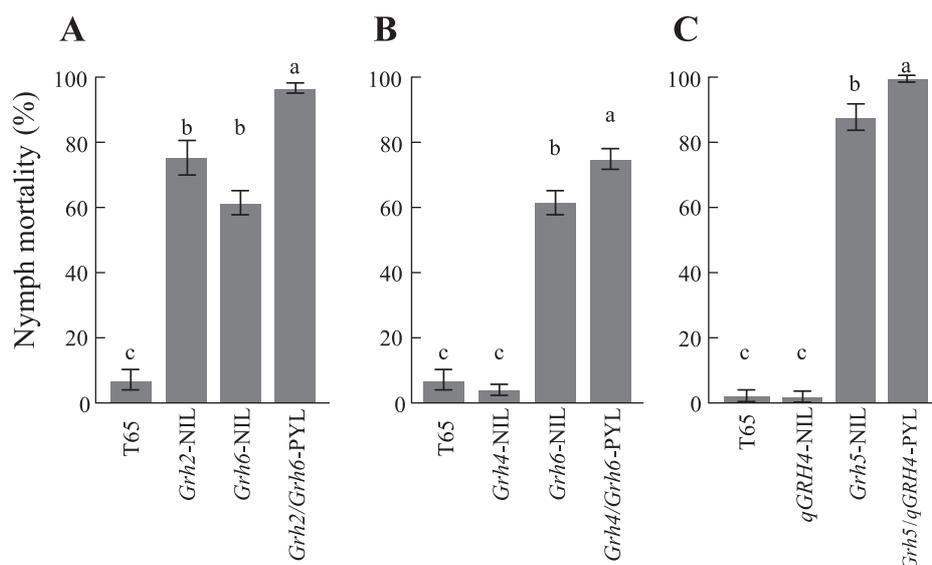
Fig. 4. Comparison of GRH nymph mortality between the NILs and the PYLs carrying *Grh2*, *Grh4*, *Grh5*, *Grh6*, and *qGRH4* at 3 days after infestation. Error bars indicate standard errors. Means labeled with different letters differ significantly (*P* < 0.01, Tukey-Kramer test).

GRH resistance are therefore useful for future improvement of resistance to GRH in rice breeding programs.

Wild species have more sources of resistance than do landraces to insect pests such as BPH, the whitebacked planthopper (*Sogatella furcifera* Horváth), the green leafhopper (*Nephotettix virescens* Distant), and the zigzag leafhopper (*Nephotettix malayanus* Ishihara et Kawase) (Heinrichs *et al*. 1985). In a previous genetic study, the BPH-resistant lines derived from *Oryza officinalis* were identified as having multiple genes resistance to the BPH (Huang *et al*. 2001). W1962, a wild accession of *O. rufipogon*, was also identified as having *Grh5* and a minor QTL conferring resistance to the GRH (Fujita *et al*. 2006). These facts suggested that several wild species with resistance to insects might carry multiple genes for resistance. In our study, the level of GRH resistance of IRGC105715, a wild accession of *O. nivara*, was significantly higher than that of *Grh6*-NIL (Fig. 3D). This suggests that IRGC105715 has additional QTLs for GRH resistance. Further study of the minor QTLs in IRGC105715 should be conducted in the future to identify these genetic resources.

The epistatic effects of pyramiding among resistance genes for bacterial blight have been reported using several gene combinations. A PYL carrying two resistance genes for bacterial blight (*Xa4* and *xa5*) was more resistant than either of the parental lines (Yoshimura *et al*. 1995). Similarly, pyramiding of *xa5* and *xa13*, *xa5* and *Xa21*, and *xa13* and *Xa21* provided a higher level of resistance than the sum of the individual gene effects (Singh *et al*. 2001). *Grh2* and *Grh4* have been reported to be dominant genes for GRH resistance and to exhibit complementary expression (Fukuta *et al*. 1998, Yazawa *et al*. 1998). The resistance level of *Grh2*/*Grh4*-PYL was significantly higher than that of *Grh2*-NIL alone, and *Grh4*-NIL showed susceptibility to GRH (Fujita

et al. 2006). In our study, *Grh2*/*Grh6*-PYL and *Grh4*/*Grh6*-PYL derived from DV85 and IRGC105715 were developed as PYLs that each carried new gene combinations for GRH resistance that had never been previously reported. In addition, we observed epistatic effects of pyramiding for *Grh2* and *Grh6*, *Grh4* and *Grh6*, and *Grh5* and *qGRH4*. These results suggest that the development of PYLs for GRH-resistance genes in rice can enhance the level of host plant resistance. However, we did not investigate the relationship between GRH resistance at the seedling stage and resistance in the field. The developed NILs and PYLs will be useful materials for field evaluation, because these lines shared a uniform genetic background (T65). These materials will therefore provide information on the effects of the GRH-resistance genes in the field without exhibiting any critical difference in agronomic traits such as growth rate. In a future study, it will be necessary to evaluate the GRH resistance of the PYLs in the field as a function of factors such as the density of the GRH and the number of hatched eggs to prove that the PYLs will provide useful levels of host plant resistance.

Virulent insect pests often appear after the release of modern improved cultivars of rice that carry a single major gene for resistance to these pests. Virulent biotypes of GRH have been produced under experimental selection by continuous rearing of GRH on resistant lines, each of which carried a single major gene (*Grh1*, *Grh2*, and *Grh3*) for resistance to the GRH (Hirae *et al*. 2007). In contrast, a biotype that is virulent against *Grh2*/*Grh4*-PYL has not yet been experimentally established despite continuous rearing on the pyramided line (Hirae *et al*. 2007). Thus, *Grh2*/*Grh4*-PYL may play an important role in providing durable resistance against rice leafhoppers. This also suggests that pyramiding of more than two resistance genes or QTLs, as in

Grh2/*Grh6*-PYL and *Grh4*/*Grh6*-PYL, will provide a useful approach to increase the durability of host plant resistance. However, it will be important to obtain information on the ability of the GRH to adapt to the developed PYLs so that breeders can continue to increase the durability of host plant resistance. The PYLs carrying resistance genes or a QTL against the GRH that we developed, with a uniform T65 genetic background, should be useful genetic resources for increasing the durability of host plant resistance in future rice improvement efforts. As noted above, additional studies will be required before deployment of the GRH-resistance genes in rice fields.

Acknowledgments

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