



## Virulence adaptation in a rice leafhopper: Exposure to ineffective genes compromises pyramided resistance

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### ARTICLE INFO

#### Keywords:

Brown planthopper  
Crop improvement  
Integrated pest management  
Marker-assisted breeding  
Philippines  
Resistance durability  
Resistance management

### ABSTRACT

Pyramiding resistance genes is predicted to increase the durability of resistant rice varieties against phloem-feeding herbivores. We examined responses by the green leafhopper, *Nephotettix virescens* (Hemiptera: Cicadellidae), to near-isogenic rice lines with zero, one and two resistance genes. The recurrent parent (T65) and monogenic lines (*GRH2*-NIL and *GRH4*-NIL) with genes for resistance to the green rice leafhopper, *Nephotettix cincticeps* (Hemiptera: Cicadellidae), were susceptible to the green leafhopper, but the pyramided line (*GRH2/GRH4*-PYL) was highly resistant to the green leafhopper. We selected green leafhoppers, *N. virescens*, from five sites in the Philippines for over 20 generations on each of the four lines. Populations selected on *GRH2/GRH4*-PYL gained partial virulence (feeding and development equal to that on T65) to the pyramided line within 10 generations and complete virulence (egg-laying equal to that on T65) within 20 generations. After 20 generations of rearing on the susceptible monogenic lines, green leafhoppers were also capable of developing and laying eggs on *GRH2/GRH4*-PYL. Furthermore, green leafhoppers reared on the susceptible *GRH4*-NIL for 20 generations showed equal preferences for T65 and *GRH2/GRH4*-PYL in choice bioassays. Our results indicate that previous long-term exposure to ineffective genes (including unperceived resistance genes) could dramatically reduce the durability of pyramided resistance. We suggest that informed crop management and deployment strategies should be developed to accompany rice lines with pyramided resistance and avoid the build-up of virulent herbivore populations.

### 1. Introduction

Crop improvement increasingly relies on advanced molecular techniques to accelerate breeding pipelines by targeting specific traits of interest and avoiding undesirable trade-offs (Varshney et al., 2005; Wang et al., 2005; Collard and Mackill, 2008). A major objective of rice improvement has been to use marker-assisted selection (MAS) to increase resistance against a range of insect herbivores. In recent years, a number of research papers have described new anti-herbivore resistance genes/loci, identified useful genetic markers to support breeding programs, or developed advanced breeding lines with enhanced resistance to insect herbivores (Fujita et al., 2013; Bentur et al.,

2016; Hu et al., 2016). Rice leafhoppers (Hemiptera: Cicadellidae) and planthoppers (Hemiptera: Delphacidae) are among the principal targets of molecular breeding for resistance in rice (Fujita et al., 2013; Horgan, 2018). Leafhoppers occur throughout tropical rice growing regions. They occasionally cause mechanical damage to rice plants, including ‘hopperburn’ (the drying and wilting of rice plants in large patches) and are vectors of rice diseases (i.e., tungro viruses transmitted by *Nephotettix virescens* [Distant] and *Recilia dorsalis* Motschulsky, and rice dwarf disease and transitory yellowing disease transmitted by *Nephotettix cincticeps* [Uhler]) (Azzam and Chancellor, 2002; Asano et al., 2015).

A range of genes for resistance to rice leafhoppers has been identified (Fujita et al., 2013). However, leafhopper virulence adaptation (the

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selection of populations able to feed and develop on resistant hosts) is often rapid and many genes are only locally effective (Sato and Sogawa, 1981; Heinrichs and Rapusas, 1985; Dahal et al., 1997). Virulence adaptation can be partial or complete (Heinrichs and Rapusas, 1985; Dahal et al., 1997; Vu et al., 2014). Partial adaptation in leafhoppers can occur in as little as 5–6 generations of selection (Vu et al., 2014). Pyramiding resistance genes (i.e., combining two or more genes in a single line) has been proposed to reduce rates of virulence adaptation in insect herbivores (Wang et al., 2016; Fan et al., 2017). Several authors have described protocols associated with pyramiding resistance genes in rice and verified that the resulting resistance is stronger than that from monogenic rice lines (Fujita et al., 2010; Wang et al., 2016; Fan et al., 2017). However, there is still little information to assess pyramiding as a strategy to prolong field resistance (Horgan, 2018). Indeed, without informed deployment strategies, pyramiding resistance could result in more rapid losses of resistance genes than if the genes had been sequentially deployed in monogenic lines (Cheng, 1985; Nemoto and Yokoo, 1994; Horgan, 2018).

Zhao et al. (2005) used a series of replicated mesocosms to examine adaptation by the diamondback moth, *Plutella xylostella* (Linnaeus), to pyramided Cry1Ac and Cry1C genes in *Bt*-transgenic broccoli. Their results indicated that the concurrent deployment of *Bt* genes in monogenic and pyramided lines can dramatically reduce the durability of pyramided resistance. In mesocosms with monogenic and pyramided resistant lines, the moth populations first overcame the monogenic resistance (either Cry1Ac or Cry1C) before sequentially adapting to the remaining effective gene in the pyramided line. In this example of *Bt* transgenic broccoli, moth populations adapted to detoxify two independently functioning toxins. However, the potential effects of concurrently deploying conventionally bred monogenic and pyramided resistance crops are more difficult to predict. This is because conventional resistance can depend on networks of interacting genes (Fujita et al., 2013), with the same resistance genes often producing different effects depending on the genetic background of the host plant (Heinrichs and Rapusas, 1985; Cohen et al., 1997; Alam and Cohen, 1998; Peñalver Cruz et al., 2011). Furthermore, pyramiding resistance loci in rice can result in strong resistance even where each of the loci are ineffective in monogenic lines (e.g., *GRH2* and *GRH4*: Vu et al., 2014; *BPH25* and *BPH26*: Srinivasan et al., 2015). Deploying susceptible varieties that possess ineffective and potentially unperceived resistance genes could therefore threaten the utility of pyramiding certain gene combinations.

In the present study, we use a series of near-isogenic rice lines with zero, one and two genes/loci for resistance against leafhoppers. The lines were developed by MAS using donor varieties with known resistance against the green rice leafhopper, *N. cincticeps*. Although the improved lines (with one or two resistance loci) can be moderately or strongly resistant to *N. cincticeps* (Fujita et al., 2010), only the pyramided line is resistant to the closely related green leafhopper, *N. virescens* (Vu et al., 2014). This herbivore-plant system provided us with an opportunity to examine aspects of virulence adaptation to pyramided lines and to test whether monogenic plants with ineffective genes could accelerate adaptation by leafhoppers to pyramided lines with the same genes. We therefore (a) describe virulence adaptation to the pyramided line using replicated green leafhopper colonies, and (b) determine whether exposure to monogenic lines predisposes populations to virulence against pyramided lines with the same resistance gene(s). We discuss our results in the light of sustainable deployment of pyramided lines to increase the durability of field resistance and to preserve rare resistance genes.

## 2. Materials and methods

### 2.1. Plant materials

We used monogenic near-isogenic-lines (NILs) carrying either the

*GRH2* or *GRH4* gene loci (henceforth *GRH2*-NIL and *GRH4*-NIL, respectively) and a pyramided line carrying both genes together (henceforth *GRH2/GRH4*-PYL [we use 'PYL' to indicate a near-isogenic line with pyramided,  $\geq 2$  resistance genes]) in our experiments with *N. virescens*. The monogenic and pyramided near-isogenic lines were originally developed using marker assisted selection. The resistance genes on either locus (*GRH2* or *GRH4*) have not yet been cloned.

The two genes, *GRH2* and *GRH4*, were first identified from DV85 using the green rice leafhopper, *N. cincticeps* during plant phenotyping (Fujita et al., 2010, 2013). Previous studies demonstrated that *GRH2* in monogenic NILs produced resistance against *N. cincticeps*, but that the PYL (containing both genes) developed using T65 as a recurrent parent, had notably higher resistance (Fujita et al., 2013; Asano et al., 2015).

DV85 and T65 were obtained from the Germplasm Bank at the International Rice Research Institute (IRRI) in the Philippines. The resistant lines we used were BC<sub>6</sub>F<sub>5</sub> generations selected using Simple Sequence Repeat markers associated with the target loci during repeated backcrossing of the donor variety DV85 and the recurrent parent T65 (Fujita et al., 2010). Seed of the NILs was bulked-up in a screen-house at IRRI during the dry-season when temperatures were coolest.

In all experiments, rice plants were grown in size-zero terracotta pots (5 × 2.5 cm: Height × Radius [H × R]) filled with paddy soil. The pots were held in flooded metal trays to maintain soil at saturation.

### 2.2. Green leafhopper colonies

In this study, we used a range of greenhouse colonies derived from five initial *N. virescens* populations (Fig. S1). One colony (henceforth, 'Los Baños') was initiated in 2008 using wild-caught individuals from Los Baños, Laguna Province in Southern Luzon, the Philippines. Four further colonies were initiated in 2010 using *N. virescens* collected at four locations (Batangas, Quezon, Rizal and San Pablo - Laguna) in Southern Luzon. These sites were each separated by distances of between 10 and 30 Km (See Fig. S1).

Colonies were initiated with ca. 500 adults collected from rice fields at the sites and placed on the susceptible rice variety TN1 ( $\geq 30$ -day old rice plants) in wire mesh cages of 120 × 60 × 60 cm, Height × Width × Length [H × W × L] under greenhouse conditions (temperatures ranged from 25 to 37 °C, 12D:12N photoperiod). During the first two generations of rearing, the colonies were synchronized (such that key life-stages were available at any one time across all five colonies). During these initial generations, the colonies were also monitored for possible transmission of rice viruses. Based on the health of feeding plants (these did not yellow or show other symptoms of virus), we assumed that the leafhoppers did not transmit tungro virus.

After two or three generations, a series of bioassays was conducted to determine population reactions to the test rice lines. The methods and results of these bioassays are presented in the supplementary information (Tables S1 and S2). Bioassays indicated that each of the populations was largely virulent against rice lines with the *GRH2* or *GRH4* gene loci (monogenic), but that all populations were avirulent against rice lines with both genes together. The Los Baños and Rizal populations had delayed nymph development on a rice line with the *GRH4* gene compared to the other three populations, but nymph survival and weight gain on this line were not significantly different from the other populations (full results are available in Table S2).

### 2.3. Multi-generation selection and monitoring of leafhopper colonies

After two or three generations on TN1, the five populations were each divided into four parts and placed in separate cages with either T65, *GRH2*-NIL, or *GRH4*-NIL (ca 200 adult pairs) or with *GRH2/GRH4*-PYL (ca 500 pairs). The larger number of pairs on the pyramided line was to overcome initial high mortality of adults and low rates of oviposition (Table S2). For the purposes of this paper, the rice lines on which colonies were selected are referred to as 'natal hosts'. Plants used

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