Materials and Methods
MATERIALS AND METHODS

3.1. PARENTAL GENOTYPES AND RAISING OF MAPPING POPULATION:

A tall *indica* variety, Ptb 8 and a high yielding dwarf *indica* variety Taichung (Native)1, herein after cited as TN1, were chosen as the parents to raise the mapping population of the present study. The two varieties differ contrastingly with regard to several qualitative and quantitative traits. Ptb 8 is known to be resistant to both the Philippines biotype (*Pb*) and the Bangladesh biotype (*Bb*) of green leafhopper (GLH), while TN1 is universally susceptible (Athwal *et al.* 1971, Siwi and Khush 1977, Rezaul Karim and Pathak 1982, Ghani and Khush 1988). TN1 is more profuse tillering as compared to Ptb 8 and most of the tillers are productive in TN1. The grains of TN1 are heavier than those of Ptb 8. Comparative depiction of the contrasting differences between the two varieties is depicted with regard to their response to GLH infestation, tillering habit and grain characteristics (Fig. 1 a,b,c).

Ten $F_1$ seeds were produced from the cross between TN1 as the female parent and Ptb 8 as the male parent following routine hybridization methods in rice (IRRI 1996). A single $F_1$ seed was selfed to obtain the $F_2$ seeds. Seeds of the two parents originally chosen for crossing were multiplied under controlled selfing. An $F_2$ population of 143 plants forming the mapping population was grown along with 20 plants of each parent under green house conditions at the Directorate of Rice Research (ICAR), Hyderabad.
FIGURE 1 Contd.

(b)
The F₂ plants and the parents were grown individually in earthen pots (20 cm diameter x 30 cm height), filled with a mixture of garden soil, sand and farmyard manure in a proportion of 3:1:1. Recommended cultural practices were followed.

3.2. SCREENING FOR HOST RESISTANCE TO THE GLH:

The insect green leafhopper belongs to the order Homoptera and family Cicadellidae. The adult insect possess conspicuous black spots on center or tips of wings (Shepard et al. 1995). Photograph of an adult GLH and nymph is presented in Fig. 2. The average life span of insect is about 25-26 days. At ideal temperatures (20°C) the eggs laid by female hatch and nymphs come out. The average total nympha period is about 16-19 days. Nymphs moult to adult after 5 instars. Both nymphs and adults feed on plants and cause direct damage by sucking plant cell sap. Consequently susceptible plants have reduced vigor, fewer tillers and a higher percentage of unfilled grains.

Host response to GLH incidence was studied following ‘tiller test’ method of screening (Athwal et al. 1971) wherein a single tiller, with roots intact, from each of the F₂ plants as well as from the standard resistant and susceptible checks planted in pots was screened under green house conditions. In this test TN1 and Vikramarya were used as standard susceptible and resistant checks, respectively. Thirty nymphs at 2ⁿᵈ and 3ⁿᵈ instar stage of the insect were released on the leaf blades of each plant followed by immediate covering with a mylar cage (Fig. 3) with its top covered by muslin cloth to facilitate aeration. Observation on
host response to GLH reaction was recorded when all the plants of the susceptible variety TNI were completely damaged, wilted and dead. A 0-9 scale of the Standard Evaluation System of Rice (IRRI 1996) was used to record the reaction scores for evaluation of the test material for response to the pest (Table 3). The various degrees of host reaction to GLH infestation are depicted in Fig. 4 with the corresponding scores.

Table 3: The reaction scores of rice plants against GLH infestation following Standard Evaluation System for Rice (IRRI 1996)

<table>
<thead>
<tr>
<th>Score</th>
<th>Description of symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No damage</td>
</tr>
<tr>
<td>1</td>
<td>Very slight damage</td>
</tr>
<tr>
<td>3</td>
<td>1st and 2nd leaves show partial yellowing and wilting</td>
</tr>
<tr>
<td>5</td>
<td>1st, 2nd and 3rd leaves show pronounced yellowing and wilting</td>
</tr>
<tr>
<td>7</td>
<td>All the leaves show yellowing and wilting, stem remaining unaffected</td>
</tr>
<tr>
<td>9</td>
<td>Whole plant including stem gets wilted and die.</td>
</tr>
</tbody>
</table>

No plant with score of 0 was observed during the screening. For qualitative evaluation, plants with the score of 1 were taken as resistant, while those with the score of 9 as highly susceptible and the plants with the scores of 3, 5 or 7 as moderately susceptible. The scores of host response to the pest were used for analysis qualitatively as well as quantitatively. Three major yield component traits including total number of tillers, number of effective tillers and 100-grain weight (g)
were evaluated quantitatively following the Standard Evaluation System of Rice (IRRI 1996).

3.3. GENETIC ANALYSIS OF THE TARGET TRAITS:

Chi-square ($\chi^2$) test for goodness of fit was applied to determine the mode of inheritance of resistance to GLH.

In respect of the quantitatively evaluated yield components the customary statistical parameters used for measuring central tendency and dispersion, such as mean, range, standard deviation, coefficient of variation, skewness, kurtosis and quartile ratio were computed.

3.4. MOLECULAR MARKER ANALYSIS:

Molecular marker analysis involved three precise steps, viz., identification of primers detecting polymorphism between the parents, screening of primers detecting polymorphism between two bulks made on the basis of the only one qualitative target trait, GLH resistance as the anchor character and finally using primers screened at parental and bulked level for analysis of the $F_2$ population.

3.4.1. DNA extraction, purification and quantification:

Marker analysis was done by using genomic DNA extracted from leaf tissues (Reddy 1995). Young leaves were cut into pieces and ground in liquid nitrogen. The concoction so obtained was suspended in 20 ml of an extraction buffer [100 mM Tris.HCl, pH 8.0; 0.20 mM EDTA, pH 8.0; 500 mM NaCl; 2% SDS; 1% poly vinyl pyrolidine] for 30-60 min at
65°C. One-fourth volume of 5 M potassium acetate (pH 7.6) was added to it, followed by uniform mixing. An equal volume (25 ml) of chloroform: isoamyl alcohol (24:1) was added to it, followed by slow shaking for 15 min. This suspension was centrifuged at 3000 rpm (1884.7 xg), 4°C for 30 min. An equal volume of prechilled isopropanol was added to the supernatant, mixed uniformly and stored at -20°C for 2 hr. The DNA spool was hooked out by a sterile Pasteur capillary pipette and was washed in 70% ethanol by centrifugation at 10000 rpm (10975 xg) for 5 min at 4°C. The DNA pellet was dried overnight at room temperature and was dissolved in Tris-EDTA buffer (10 mM Tris; 1 mM EDTA, pH 8.0). The DNA solution treated with RNase A (10 mg/ml) and incubated at 37°C for 1 hr was added with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), mixed well by centrifugation at 10000 rpm (10975 xg) at 4°C for 5 min. The supernatant was collected and added with two volumes of prechilled absolute alcohol, centrifuged at 10000 rpm (10975 xg) at 4°C for 1 min followed by pellet washing with 70% ethanol. The pellet was dried overnight at room temperature and resuspended in 250 μl of Tris-EDTA buffer and stored at −20°C until further use.

DNA concentration was measured by using a UV absorbance spectrophotometer (Beckman DU 650, USA) with TE buffer (pH 8.0) as the blank. Purity of DNA was checked by electrophoresis in an agarose (0.8%) gel with ethidium bromide staining.
3.4.2. Polymerase chain reaction:

A set of 141 random decamer primers (Operon Technologies Inc., USA) was used for polymerase chain reaction (PCR) of the DNA samples as per the procedure described by Williams et al. (1990).

Amplification reactions were performed in a Gene Amp PCR system 9700 (PE Applied Biosystems, Switzerland) using a reaction volume of 25 µl containing 100 mM Tris.HCl (pH 9.00); 500 mM KCl; 2 mM MgCl₂; 0.1% gelatin; 100 mM each of the dNTPs; 5 pM of primer; 1U of Taq polymerase (Genei Bangalore, India) and 50 ng of template DNA. Initial denaturation of DNA was done at 94°C for 5 min. A program of 45 cycles each of 2 min at 94°C, 1 min at 36°C and 2 min at 72°C followed by final elongation at 72°C for 5 min was used for amplification.

3.4.3. Electrophoretic analysis of PCR products:

The PCR products were electrophoresed in 8 mm thick, 20 cm × 20 cm, 1.5% Agarose slab gels in a horizontal electrophoresis set (Amersham Pharmacia Biotech, EPS 600, USA) using TBE buffer (0.045 M Tris–Borate, 0.001 EDTA) at constant 60 V for about 7 hr. A gel loading buffer (6x: 0.25% bromophenol blue and 40% w/v sucrose) was used as the tracking dye. A lane of λ DNA/EcoR1-HindIII double digest (Genei Bangalore, India) was used as the molecular weight (MW) marker. Gels were stained in 0.05% ethidium bromide solution for 15 min followed by destaining for 30 min. Gels were documented in an
Image Master VDS (Amersham Pharmacia Biotech, USA) and the photographs were obtained. The molecular weights of the polymorphic bands were obtained from Image Master VDS software 3.0 program (Amersham Pharmacia Biotech, USA).

3.4.4. Identification of primers detecting polymorphism between parents:

A set of 141 random decamer primers was used to identify the primers detecting polymorphism between the parents of the mapping population. For this purpose, DNA samples from 12 plants each of the two parents were used for PCR analysis.

3.4.5. Screening of polymorphic primers at bulked segregant level:

A set of 110 primers detecting polymorphism at parent level was screened by bulked segregant analysis (BSA) (Michelmore et al. 1991). Resistance to GLH, as a qualitative trait was used as the criterion to prepare the two bulks. Equal amount of DNA from 12 resistant F2 plants (score 1) and from 12 highly susceptible F2 plants (score 9) were pooled separately to make the two bulks. Twenty-four primers detecting polymorphism between these two bulks were screened. These primers were tested twice more for selecting reproducible primers leading to identification of six such primers.
3.4.6. Marker analysis in the mapping population:

DNA samples from 143 F$_2$ plants and those from the two parents and the two bulks as described above were used for PCR analysis with the six reproducible primers to detect random amplified polymorphic DNA (RAPD) markers (Williams et al. 1990). A list of six primers with their base sequences is furnished in Table 4.

3.5. LINKAGE ANALYSIS AND CONSTRUCTION OF MOLECULAR MAP:

3.5.1. Genotyping of F$_2$ individuals:

Segregation of the eight loci produced by the six primers and resistance to GLH in the 143 F$_2$ plants was tested by Chi-square goodness of fit. As RAPD produced only dominant bands, F$_2$ individuals with amplified fragment were genotyped as D for homozygous and heterozygous dominant genotype of A gene of the female parent, TN1 or C for homozygous and heterozygous dominant genotype of B gene of the male parent, Ptb 8. Individuals without amplified fragment were genotyped as A (while amplified fragment absent in the female parent, AA) or B (while amplified fragment absent in the male parent, BB). For genotyping reaction to GLH, resistant, highly susceptible and moderately susceptible individuals were genotyped as B, A and H respectively.
3.5.2. Construction of a linkage map:

Linkage analysis of eight RAPD marker loci and one trait locus was done by using MAPMAKER/EXP v3.0 program (Lincoln et al. 1992a). The markers were grouped initially at the minimum LOD (likelihood of odds) threshold of 3.0 and a maximum recombination fraction (rf) of 0.4. A single linkage map with six loci was framed initially while three loci remained unlinked. The best order of the linkage group was obtained by 'three point analysis' and finally by using the 'compare' command of the program. Another locus was added to the framework map using the 'try' command. The linkage group with seven loci thus obtained was finally framed and its order was checked with the 'ripple' command. The map distances were expressed in centi-Morgan (cM) using the Kosambi (1944) function.

3.5.3. Detection of quantitative trait loci:

The framework map with seven loci was used for interval mapping (Lander and Botstein 1989) taking recombination fraction (rf) as the unit for detection of putative QTLs by employing the MAPMAKER/QTL 1.1 program (Lincoln et al. 1992b). A threshold LOD of 2.4 as suggested by various workers in rice mapping (Fukuta et al. 1996, Li et al. 1996, Price and Thomos 1997) was used for declaring the presence of any putative QTL. The precise position of a QTL peak was obtained by the 'map' command. The confidence interval for a QTL was obtained from rescanning the map with a threshold LOD derived by deducting 1.0 from the LOD of the QTL detected by 'map' command.
except for any QTL with LOD value below 3.0. To check the presence of more than one QTL for a character, the map was rescanned with a threshold LOD derived by adding 2.0 to the original LOD of the fixed QTL. While testing the three genetic models, dominant, recessive and additive, a model with no more than 1.0 LOD difference from the free model was declared as valid.

Table 4: A list of six Operon primers used for RAPD analysis in rice using an F2 population derived from the TN1/Ptb 8 cross

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Molecular weight</th>
<th>p moles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>OPA19</td>
<td>CAAACGTCGG</td>
<td>3028</td>
<td>4988</td>
</tr>
<tr>
<td>2</td>
<td>OPF1</td>
<td>ACGGATCCTG</td>
<td>3019</td>
<td>5300</td>
</tr>
<tr>
<td>3</td>
<td>OPF2</td>
<td>GAGGATCCCT</td>
<td>3019</td>
<td>5300</td>
</tr>
<tr>
<td>4</td>
<td>OPH11</td>
<td>CTTCGCAGT</td>
<td>2970</td>
<td>5918</td>
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<tr>
<td>5</td>
<td>OPI10</td>
<td>ACAACGCAGG</td>
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<tr>
<td>6</td>
<td>OPJ18</td>
<td>TGGTCGCAGA</td>
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