Genetic loci associated with root-knot nematode resistance in rice cv. Ramakrishna

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ABSTRACT

The rice root-knot nematode (RRKN), Meloidogyne graminicola, is a potent nematode pest of rice in upland and direct-sown, medium-land rice ecosystems. During the last decade, its occurrence in epidemic proportions over hundreds of acres in India and the Philippines raised concern. Chemical and cultural control methods are inefficient due to its subterranean habitat, internal feeding and polyphagous nature. The indica rice cv. Ramakrishna (a derivative of TKM6) showed continuous resistance/tolerance against RRKN in our studies. We developed an F$_9$ recombinant inbred mapping population of the cross Annapurna x Ramakrishna to map the RRKN resistant gene(s) and develop molecular markers for use in marker assisted breeding. Three traits, mean no. of galls, eggs and eggs/gram root produced by RRKN 45 days after inoculation, were used to score resistance/susceptibility of the plants in the mapping population. The traits were highly correlated. Analysis of phenotypic and genotypic data suggested, the existence of three quantitative trait loci (QTLs) i.e qMg-1, qMg-3a and qMg-3b for RRKN resistance in rice cv. Ramakrishna. qMg-3a, on chromosome 3, is a major QTL reducing production of eggs by up to 41.10 percent.

Key words: rice, root- knot nematode, resistance, Meloidogyne graminicola, QTLs, recombinant inbreed lines, galls

Rice is grown under three major ecosystems; rain-fed upland, irrigated medium-land and rain-fed lowland. The rain-fed upland constitutes 16% of the total cultivated area in India and 12% of the total area planted to rice on a global basis (Anonymous, 1993). The productivity of upland rice is very low compared to irrigated and rain-fed lowlands. Among the abiotic and biotic stresses that limit production of upland rice, the rice root-knot nematode (RRKN) Meloidogyne graminicola is of considerable importance. RRKN is prominent in both upland and lowland rice ecosystems in Asia (Prot et al., 1994) and is principally a problem in upland rice in West Africa (Plowright and Hunt, 1994; Babatola, 1984).

The second-stage larvae (J2) of the nematode penetrate the roots at the zone of elongation and proliferation (Jena and Rao, 1973) and migrate intercellularly towards the meristem and the region of cell differentiation. In response to signals from the nematode, root cells in the vascular cylinder adjacent to the head of the nematode enlarge to form metabolically active, multinucleate giant cells. These serve as a source of nutrients for the developing endoparasitic form of the nematode. Concurrent swelling and the division of the cortical cells around the nematode, leads to the formation of galls or root-knots on the roots. The above ground symptoms consist of chlorotic plants with leaf bronzing from the tip downward and from the margins inward towards the midrib of the leaf blade. Emerging leaves are crinkled, grain setting is poor and the grains are chaffy (Patnaik and Padhi, 1987). In the upland, these severely affected plants often wither due to non translocation of water by diseased roots. In deepwater rice, these stunted plants lose the ability to elongate fast enough to keep pace with rising flood water. They become detached from their anchor roots and float to the surface. Globally the nematode causes an annual average yield loss of
Nematode control is important in the development of sustainable agriculture. Unfortunately, chemical control is unsuitable on both economic and environmental grounds. The wide host range of the Meloidogyne species that attack rice also places other crops in rotation with rice at significant risk. The most feasible alternative is the use of resistant varieties. Resistance to RRKN is manifested by non-development of feeding sites. Instead of giant cells, a localised region of necrotic cells develops near the head of the invading J2, which fails to establish a feeding site and either dies or leaves the plant roots. It is reported that J2 RRKN penetrate both susceptible and resistant plant roots in equal numbers (Jena and Rao, 1974), but subsequent development is retarded with fewer galls on resistant plant roots. Another effect of host resistance is the interference in the nematode’s ability to reproduce, manifested as a reduced number of egg masses and eggs on the resistant plant (Holbrook & Noe, 1992). No suitable source of genetic resistance or tolerance to RRKN is currently available in cultivated rice, although moderate levels of resistance were recorded in some cultivars, and wild progenitors of rice are known to posses good resistance levels (Sahu et al., 1994; Soriano et al., 1999). Reports of pathotype variability in RRKN also suggest that the development of resistant rice cultivars may be an unsustainable method of RRKN control (Sahu and Chawla, 1986). The present need is to identify the number of resistance genes that might be deployed against RRKN in a suitable breeding strategy. Conventional resistance breeding is very difficult, time consuming, and labour intensive, and the process of screening large numbers of segregating progenies under artificial infestation conditions would likely be one of the most limiting steps in a conventional breeding effort. Therefore, the use of molecular markers in an RRKN resistance breeding strategy is essential.

MATERIALS AND METHODS

The plant material consisted of rice varieties Annapurna and Ramakrishna and 107 recombinant inbred lines (RILs). Annapurna, a drought tolerant upland rice variety, is highly susceptible to the RRKN while Ramakrishna is resistant (Bose et al., 1998). The RIL population consisted of 107 F2 families derived from independent F1 seeds of a single F1 plant of the cross Annapurna × Ramakrishna.

The RRKN population used throughout the experiment was obtained from the experimental farm of the Central Rice Research Institute. Galled roots were cut into pieces and mixed with autoclaved soil and sand (1:1) in zinc trays and seeds of the susceptible cv. Annapurna were sown. At the time of development of egg masses, the plants were cut and the soil disturbed. Ten days after, fresh Annapurna seeds were sown and the culture was maintained for subsequent use.

The RILs were screened for number of galls and eggs (Garcia et al., 1996) under artificial infection conditions. Five seeds each of Annapurna, Ramakrishna and 107 F2 RILs were sown in 5" diameter plastic pots containing 500 gm autoclaved sand & soil (1:1). After one week the plants were thinned to one. Ten-day-old plants were inoculated with 500 freshly hatched second stage juveniles (J2) of RRKN by exposing the root surface. The plants were watered to keep the soil in field moisture condition. We used a completely randomized design with five replications (Taylor and Sasser, 1978; Sahu and Chawla, 1988). Forty-five days after inoculation (normally two complete RRKN life cycles) the plants were uprooted and washed free of soil. After counting the total number of galls under a binocular microscope, the roots were blotted dry and weighed. Roots were then briefly blended in a blender and the suspension passed through a 200-mesh sieve.
followed by a 500-mesh sieve. Finally the aliquot remaining in the 500-mesh sieve was collected in water and the total numbers of eggs were counted (Hussey and Barker, 1973). Lines showing fewer than 10% galls and eggs in comparison to the susceptible parent were scored as resistant (Bridge and Page, 1980). All the experiments were carried out in the green house in the Division of Crop Protection, Central Rice Research Institute, Cuttack, Odisha, India at an optimum temperature range of 25°C – 30°C.

Four-hundred-thirteen microsatellite loci (www.gramene.org) distributed over twelve rice chromosomes at an interval of ~10 cM were screened for identification of polymorphism between parents. Further, individual F₉ RILs were genotyped with the polymorphic markers.

A molecular linkage map of the marker loci was constructed using MAPMAKER/EXP Version 3.0 (Lander et al., 1987). The Kosambi mapping function was used to construct the genetic linkage map with minimum LOD score 3 and maximum recombination frequency 0.4. The marker order within a linkage group was determined by “first order” and “ripple” command of MAPMAKER.

The frequency distribution of F₉ RILs for the resistant traits was plotted using the MS EXCEL program. One-way analysis of variance (ANOVA) was conducted using PROC GLM in SAS (SAS institute, 1990) to test the differences between averages of traits. Broad-sense heritability was computed by one way ANOVA (factor, genotype) from the estimates of genetic ($\hat{\sigma}^2_G$) and residual ($\hat{\sigma}^2_e$) variances derived from the expected mean squares as $h^2 = \hat{\sigma}^2_G / (\hat{\sigma}^2_G + \hat{\sigma}^2_e / k)$, where $k$ is the number of replications. The Pearson’s correlation coefficients among the traits were estimated using Minitab 15 English statistical software.

Whole genome scanning was done to identify and map QTLs using the software QTL Cartographer V2.5 (Zhang et al., 2010). Composite interval mapping (CIM) was employed by selecting the default model with a window size of 10 cM. A LOD threshold of e^0.3 was used to declare a QTL as significant.

RESULTS AND DISCUSSION
The parent cultivar showed significant differences ($P < 0.001$) in the susceptibility to RRKN, with Annapurna having a greater number of galls than Ramakrishna (Table 1). The number of galls ranged from 8.3-9.3 on susceptible parent Annapurna and from 3.4-4.6 on the resistant parent Ramakrishna, with a mean of 8.86 ± 0.37 and 4.07 ± 0.51, respectively. The gall number in the RILs ranged from 2.23-8.96 with a mean of 5.27 ± 1.64. Nearly one-quarter (23.36%) of the RILs had fewer galls than the resistant parent, suggesting a high degree of transgressive segregation towards resistance.

The number of eggs collected from Annapurna ranged from 153.2-187.6 with a mean of 168.5 ± 14.4. The number of eggs collected from Ramakrishna ranged from 17.3-60 with a mean of 39.3 ± 20.4. The number of eggs collected from the RILs ranged from 16.12-163.83 with a mean of 88.92. Transgressive segregation towards resistance was again in evidence as 7.47% of the RILs had fewer eggs than the resistant parent. The number of eggs gram root⁻¹ in Annapurna ranged from 156.4-210.9 with a mean of 181.8 ± 20.5. On Ramakrishna, the number of eggs gram root⁻¹ ranged from 46.3-144.3 with a mean of 91 ± 37.06. The number of eggs gram root⁻¹ ranged from 47.09-233.75 with a mean of 134.42 ± 39.58 on the RILs. Again, a significant number (11.21%) of the RILs had fewer eggs gram root⁻¹ than the resistant parent. All the traits fit a normal distribution (skewness < 1). Estimates of heritability for numbers of galls, eggs and eggs gram root⁻¹ were relatively high (83%, 83.4% and 83.3%.

### Table 1. Number of galls, eggs and eggs gram root⁻¹ plant⁻¹ and broad-sense heritability ($h^2$) in the mapping population and the parental lines infected with Meloidogyne graminicola

<table>
<thead>
<tr>
<th>Traits</th>
<th>galls plant⁻¹</th>
<th>eggs plant⁻¹</th>
<th>eggs gram root⁻¹ plant⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± range</td>
<td>mean ± range</td>
<td>mean ± range</td>
</tr>
<tr>
<td>Ramakrishna</td>
<td>4.07 ±0.51</td>
<td>39.3±20.4</td>
<td>91 ± 37.06</td>
</tr>
<tr>
<td>Annapurana</td>
<td>8.86 ±0.37</td>
<td>168.5±14.4</td>
<td>181.8±20.5</td>
</tr>
<tr>
<td>F₉ RILs</td>
<td>5.27±1.64</td>
<td>88.92±38.8</td>
<td>134.42±39.58</td>
</tr>
<tr>
<td>$h^2$</td>
<td>83%</td>
<td>83.4%</td>
<td>83.3%</td>
</tr>
</tbody>
</table>

Root-knot nematode resistance in rice  Mamta Jena et. al
respectively). The correlations between galls and eggs, galls and eggs gram root$^{-1}$ and eggs and eggs gram root$^{-1}$ were 0.939, 0.764 and 0.804, respectively.

Of the 413 loci screened for polymorphism between Annapurna and Ramakrishna, 46 (11%) were polymorphic. All 107 $F_9$ RILs were genotyped for these 46 markers. On average, the RILs had achieved homozygosity for more than 98% of these SSR loci (P > 0.05). The frequency distribution showed that 52% of loci skewed towards Annapurna and 48% skewed towards Ramakrishna. Although the segregation of some of the markers was distorted, these markers were not eliminated from the linkage analysis because they did not show extreme segregation distortion. MAPMAKER eliminated 16 markers as unlinked and constructed the linkage map with 30 markers distributed over nine linkage groups on seven rice chromosomes.

![Genetic linkage map of markers, distances shown in centimorgans (cM).](Fig. 1)

Table 2. Identification of QTLs for three RRKN traits: number of galls, eggs and eggs gram root$^{-1}$ using composite interval mapping (QTL cartographer software version 2.5)

<table>
<thead>
<tr>
<th>Traits</th>
<th>Chromosome no.</th>
<th>Maximum LOD position(cM)</th>
<th>Marker interval</th>
<th>LOD</th>
<th>Additive</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galls</td>
<td>1</td>
<td>20.1</td>
<td>RM428-RM490</td>
<td>3.67</td>
<td>-0.6424</td>
<td>14.51</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50.9</td>
<td>RM251-RM490</td>
<td>2.83</td>
<td>-0.7948</td>
<td>23.10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50.9</td>
<td>RM251-RM490</td>
<td>2.51</td>
<td>-0.5611</td>
<td>11.02</td>
</tr>
<tr>
<td>Eggs</td>
<td>1</td>
<td>20.1</td>
<td>RM428-RM490</td>
<td>3.43</td>
<td>-14.6599</td>
<td>13.62</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>22.0</td>
<td>RM251-RM490</td>
<td>5.03</td>
<td>-24.8849</td>
<td>41.10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>49.9</td>
<td>RM251-RM490</td>
<td>4.87</td>
<td>-17.7600</td>
<td>19.89</td>
</tr>
<tr>
<td>Eggs gram root$^{-1}$</td>
<td>1</td>
<td>23.1</td>
<td>RM428-RM490</td>
<td>2.9</td>
<td>-14.8160</td>
<td>13.54</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>26.0</td>
<td>RM251-RM490</td>
<td>3.7</td>
<td>-20.0998</td>
<td>25.40</td>
</tr>
</tbody>
</table>

LOD: log$_{10}$ (Probability of linkage/probability of no linkage); $R^2$= proportion of variation explained by the QTL
Composite interval mapping detected three QTLs on the short arm of chromosome 1 and five on the short arm of chromosome 3 controlling the number of galls, eggs and eggs/gram root (Table 2, Fig. 2). Three QTLs for the three traits were identified on chromosome 1 in the marker interval RM428-RM490 having LOD scores 3.67, 3.43 and 2.9 explaining 14.51, 13.62 and 13.54% of the phenotypic variation respectively. Similarly, three QTLs controlling all three traits were identified on chromosome 3 in the marker interval RM231-RM7 explaining 23.10, 41.10 and 25.40% of phenotypic variation with LOD scores of 2.83, 5.03 and 3.7 respectively. Two QTLs for galls and eggs were also detected on chromosome 3 between the marker interval RM232- RM251 having LOD scores of 2.51 and 4.87 explaining 11.02 and 19.89% of the phenotypic variation.

Root-knot nematodes are potent pests of arable agriculture, horticulture and plantation crops, annually causing approximately 5% of total crop loss worldwide (Sasser et al., 1985). The highly polyphagous nature of the pest, insufficient accessibility of applied pesticide to the pest, which completes most of its life cycle inside the root, and associated environmental hazards have made management of root-knot nematodes difficult. Use of host resistance has been considered the most feasible alternative to manage the nematode. Unfortunately, the genomes of cultivated plants usually lack true resistance, and studies examining the genetics

**Chromosome 1**

**Chromosome 3**

Fig. 3. QTL regions identified on chromosome 1 and 3 for root-knot nematode resistance based on the three RRKN traits: number of galls, eggs and eggs/gram root.
of resistance are rare. This is probably due to the lack of resistance resources and the time consuming and labour required to screen large numbers of segregating lines. The advent of molecular markers has made it possible and easier to identify root-knot nematodes resistance in plants and to assist in breeding for nematode resistance. The trait linked marker genotypes can serve as phenotype predictors, thus laborious greenhouse screening for nematode resistance can be delayed until later stages of line development.

The genetics of resistance in rice to RRKN is poorly understood. Previously, very few reports existed, which suggested the involvement of both qualitative and quantitative genes in RRKN resistance in rice (Swain et al., 1991, Shrestha et al., 2007). During screening of rice germplasm we identified an indica cv. Ramakrishna which had shown consistent resistance against the Cuttack, Odisha, India population of RRKN. The 107 F$_2$ RILs developed by crossing Ramakrishna with the highly susceptible cv. Annapurna was used to identify genetic loci associated with RRKN resistance in Ramakrishna with respect to number of galls, eggs and eggs gram root$^{-1}$ produced by the nematodes after completion of two life cycles inside the roots (Starr et al., 1985; Luzzi et al., 1987). High heritability of the traits suggested that a genetic component conditioning resistance in the population existed and that the phenotype data were suitable for QTL mapping. The high correlation among the traits indicates that a single plant trait could be affecting three different measures of resistance in the nematode. An inability to feed (starvation) for example would probably affect the number of galls, the number of eggs, and eggs per root mass. All QTLs could be contributing to the inability of the nematode to attack the plant and properly feed. The low frequency of polymorphism among the SSRs tested is probably due to the fact that both the parents belong to indica rice. The largest numbers of polymorphisms obtained were on chromosomes 1, 2 and 3. No polymorphisms were detected on chromosome 10, and low numbers of polymorphic markers were also found on chromosomes 11 and 12. Ten markers showed distortion for 1:1 ratio which may arise due to environmental error, population size or population type (Song et al., 2005).

In RIL populations, segregation distortion is probably related to artificial sampling and selection for many generations (Xu et al., 1997). Association between segregation distortion markers and QTLs or genes was also detected by Kintzios et al., (1994). In general, segregation distortion will not produce more false positive QTLs, nor will it have a significant impact on the estimation of QTL position and effect. If the distortion is not extremely serious, the effect from the distortion can be ignored in the mapping population (Wang et al., 1997). Phenytophory and genotyping of 107 F$_2$ RILs led to the identification of eight putative QTLs for the three traits examined: galls, eggs and eggs gram root$^{-1}$ (Table 2). Three QTLs having low LOD scores were considered authentic because they are in the same marker interval as other QTLs. Three QTLs, one on chromosome 1 between marker interval RM428-RM490 and two on chromosome 3 between marker intervals RM231-RM7 and RM232-RM251 were considered effective with negative effect on the trait value. The QTL on chromosome 3, flanked by markers RM231-RM7, is a major QTL contributing 41.10% to phenotypic variation and associated with resistance to all the three traits. The difference in the QTL position at maximum LOD score for number galls and number of eggs gram root$^{-1}$ may be an artefact caused by environmental error.

We designated the three QTLs as qMg-1, qMg-3a and qMg-3b (Fig. 2) following Mc Couch et al. (1997). These three QTLs are pleotropic in nature. Multifunctional and pleotropic genes have also been reported to be involved in insect and disease resistance in crop plants such as Mi-1 gene that confers resistance in tomato to three species of root-knot nematodes (M. arenaria, M. incognita, and M. javanica), aphids (Kaloshian et al., 1997; Kaloshian et al., 2000) as well as to whiteflies (Bemisia tabaci and B. tabaci biotype B) (Miller et al., 1998; Nombela et al., 2003).

Shrestha et al., (2007) reported six low effect QTLs for RRKN resistance in indica rice cv. Bala on chromosome 1, 2, 6, 7, 9 and 11. The QTL reported on long arm of chromosome 1 was near centromere whereas the qMg-1 is on short arm of chromosome 1. Two QTLs, qMg-3a and qMg-3b for RRKN located on chromosome 3 were not reported earlier. The data presented here provides clear evidence, that RRKN resistance is polygenic as reported earlier in rice. The QTLs detected in the present study should be further validated through fine mapping and candidate gene
analysis to make use of them in marker assisted breeding program to improve RRKN resistance of rice cultivars.

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