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Identification of flanking SSR markers for a major rice gall midge resistance gene *Gm1* and their validation

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Abstract Host-plant resistance is the preferred strategy for management of Asian rice gall midge (*Orseolia oryzae*), a serious pest in many rice-growing countries. The deployment of molecular markers linked to gall midge resistance genes in breeding programmes can accelerate the development of resistant cultivars. In the present study, we have tagged and mapped a dominant gall midge resistance gene, *Gm1*, from the *Oryza sativa* cv. W1263 on chromosome 9, using SSR markers. A progeny-tested F_2 mapping population derived from the cross W1263/TN1 was used for analysis. To map the gene locus, initially a subset of the F_2 mapping population consisting of 20 homozygous resistant and susceptible lines each was screened with 63 parental polymorphic SSR markers. The SSR markers RM316, RM444 and RM219, located on chromosome 9, are linked to *Gm1* at genetic distances of 8.0, 4.9 and 5.9 cM, respectively, and flank the gene locus. Further, gene/marker order was also determined. The utility of the co-segregating SSR markers was tested in a backcross population derived from the cross Swarna/W1263//Swarna, and allelic profiles of these markers were analysed in a set of donor rice genotypes possessing *Gm1* and in a few gall midge-susceptible, elite rice varieties.

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Introduction

Asian rice gall midge (*Orseolia oryzae*) is a dipteran pest in major rice-growing areas of India, Bangladesh, Sri Lanka, Southeast Asia and southern China, causing an estimated annual yield loss of US $550 million in Asia (Herdt 1991). In India, the pest is widely distributed and is considered a significant constraint to rice production (Bentur et al. 2003). Since chemical control of gall midge is often ineffective, breeding of resistant varieties has proved to be a viable and ecologically acceptable approach for management of the pest. More than 20,000 rice accessions have been evaluated for gall midge resistance and, so far, nine genes (*Gm1–Gm9*) conferring resistance have been identified. Of the 59 gall midge-resistant rice varieties developed in India, three sources, Eswarakora (*Gm1*), Siam29 (*Gm2*) and Ptb21 (uncharacterised), have individually contributed resistance in 49 varieties (Bentur et al. 2003).

Existence and emergence of new and virulent biotypes of rice gall midge is a cause for concern, as it has resulted in breakdown of resistance in many of these varieties warranting new breeding strategies to combat the insect menace. Six distinct gall midge biotypes have been characterised in India (Katiyar et al. 2000), and none of the varieties released are resistant to all six biotypes. Broadening the genetic base of resistance through gene pyramiding is one of the strategies envisioned for developing rice cultivars with durable gall midge resistance. Gene pyramiding efforts through conventional breeding strategies based on plant phenotype selection are time consuming, resource intensive and sometimes inconclusive, necessitating the deployment of more efficient and accurate phenotyping.

Molecular markers can support classical breeding in crop plants by saving time and labour in breeding programmes. Seven gall midge resistance genes have been tagged and mapped so far (Nair et al. 1994; Mohan et al. 1997; Katiyar et al. 2001; Sardesai et al. 2002). Though the potentiality of the identified markers in marker-assisted selection (MAS) has been envisioned, their effective use in

real-time segregating populations other than those of the original parents used for their identification is limited. Among the problems encountered in deployment of the markers in large-scale MAS, the most serious are (1) the lack of close linkage between the marker and the gene of interest and (2) dominant inheritance of the RAPD, AFLP and SCAR markers. It is, therefore, necessary to identify tightly linked (preferable <2 cM) and/or flanking co-dominant markers (<5 cM on either side of gene locus) in order to gainfully deploy them in practical MAS.

Among the widely deployed gall midge resistance sources/genes, *Gm1*, a dominant gene, is known to confer durable resistance. This gene was first identified in the land race Eswarakora (Sastry et al. 1975; Chaudhary et al. 1985) and confers resistance to Indian gall midge biotypes 1, 3, 5 and 6 (Bentur et al. 2003). The gene has been extensively used in breeding gall midge-resistant varieties such as Kavya, Samridhi and Mahamaya that sustained resistance over a long period. Here, we report tagging and mapping of *Gm1* in the rice cultivar W1263 with SSR markers and validation of the linked markers in an alternate population.

Materials and methods

Plant material and mapping population

Two populations were used in the present study:

1. A progeny-tested F_2 population consisting of 150 individuals derived from a cross between two indica rice genotypes, W1263 with *Gm1* and TN1 (susceptible to all biotypes of gall midge), was used for identification of linked markers.
2. A progeny-tested BC_1F_2 population consisting of 145 individuals derived from a single heterozygous resistant BC_1F_1 plant of the cross Swarna/W1263//Swarna was used for testing the utility of the linked SSR markers in predicting resistance/susceptibility.

In addition, a set of *Gm1* donors and susceptible lines listed in Table 1 were screened with the linked SSR markers.

Screening for gall midge resistance

Traditional screens for gall midge resistance involve natural infestation of rows of each genotype by gravid females in the field or in boxes under artificial infestation in the greenhouse. In such screens, the insect pressure is declared adequate if galls are seen in all the plants of a susceptible check grown in rows at specified interval. However, it is possible that some false negatives ('escapes') will be obtained if the susceptible check is more easily or preferentially infested compared with the genotypes under test. To overcome this problem, we designed a more rigorous infestation method. The genotype of each F_2 plant was inferred based on the phenotype of its progeny. About 40 15- to 20-day-old F_3 seedlings from each F_2 plant of the cross W1263/TN1, along with the susceptible (TN1) and resistant (W1263) parents, were screened in the greenhouse against Indian biotype 1 of gall midge (avirulent on plants possessing *Gm1* gene). About 25–30 mated females of the insect were released in a 2-l Borosil glass beaker (covered with muslin cloth) containing 15- to 20-day-old seedlings. Two releases of the insect were made at 2-day intervals to maintain a very high selection pressure and to minimize escapes. The seedlings were transferred to 200-ml culture tubes 5 days after the second release and plugged with cotton to maintain a high humidity. From the 15th day after release, when gall formation was apparent in the seedlings of TN1, F_3 seedlings were scored qualitatively either as susceptible or resistant based on gall formation or observation of dead

Table 1 Microsatellite alleles for the linked markers in *Gm1* donors and gall midge-susceptible cultivars

Cultivar	Characteristic features	Amplicon length (bp) for the indicated primers		
		RM316	RM219	RM444
W1263	A gall midge-resistant cultivar developed from the cross Eswarkora/MTU15	170	230	320
Eswarakora	A gall midge-resistant land race from Andhra Pradesh State, India, which has served as the *Gm1* donor for W1263, Mahamaya and Kavya	170	230	320
Mahamaya	A widely grown gall midge-resistant rice variety possessing *Gm1*	160	230	240
Kavya	A fine grain rice variety possessing *Gm1* conferred gall midge resistance. Widely used as donor for gall midge resistance	160	230	185
ARC6605	A land culture exhibiting like resistance *Gm1*	160	203	227
TN1	A semi dwarf rice variety developed from the cross Dee-Geo-Woogen/TYC	160	210	183
Swarna	A widely grown rice variety, susceptible to gall midge	160	186	191
Samba Mahsuri (BPT5204)	A fine grained rice variety widely grown in many parts of India, severely susceptible to gall midge	160	196	240
PR106	A fine grained rice variety susceptible to gall midge	160	196	240
Krishnahamsa	A boro season rice variety susceptible to gall midge	160	220	240

maggots. An F_2 individual was scored as homozygous susceptible if all its progeny exhibited gall formation. On the other hand, if all the progeny of an F_2 individual were devoid of galls and only harboured dead maggots, it was scored as homozygous resistant. Those F_2 lines whose progeny segregated with respect to gall formation were scored as heterozygous resistant. The BC_1F_3 seedlings of the cross Swarna/W1263//Swarna were also screened similarly to identify homozygous and heterozygous BC_1F_2 lines.

DNA extraction and SSR marker analysis

Total genomic DNA was isolated from the parental lines, F_2 and BC_1F_2 plants, using the method of Dellaporta et al. (1983). In order to map *Gm1* on the rice genome and to identify linked SSR markers, we used a set of 133 SSR markers (data not shown) uniformly spaced across the 12 chromosomes (RM series, Research Genetics, USA). Their map locations, primer sequences and other details are available online at http://gramene.org.ricemicrosat.html. The polymorphism between W1263 and TN1 with respect to these markers was assessed following PCR conditions described by Chen et al. (1997). The PCR products were resolved on 3.5% agarose (US Biochemicals, USA) and/or 12–15% native polyacrylamide gels (Sigma, USA) in 1× TBE buffer, stained with ethidium bromide (0.5 μg/ml) and photographed under UV light (Sambrook and Russell 2001). The size of the amplified fragments was calculated using Alphaease software (Alpha Innotech, USA) with a 50-bp ladder (MBI Fermentas, Lithuania) as size reference standard. Polymorphic SSR markers were used for co-segregation analysis with the trait phenotype, initially in a subset of 40 F_2 individuals of the cross W1263/TN1 (consisting of 20 homozygous resistant and 20 homozygous susceptible lines). This was done in order to quickly identify the tentative chromosomal location of *Gm1* as well as SSR marker(s) co-segregating with the trait phenotype. Once the tentative chromosome location was known, all the SSR markers on that particular chromosome were further analysed in the entire F_2 population.

The utility of the *Gm1* linked SSR markers in predicting the trait phenotype was validated in a progeny-tested BC_1F_2 population of the cross Swarna/W1263//Swarna.

Linkage analysis

Linkage analysis and map construction were performed using MAPMAKER/EXP, version 3 (Lander et al. 1987). Initially, linkage groups were obtained using two-point analysis with a log-likelihood of odds (LOD) score of 4.0 and maximum recombination level of 0.3. This step was implemented by using the 'group' command. Linked markers within the linkage groups were ordered using multipoint analysis with 'compare', 'suggest subset' and 'try' commands. Best order of marker was then confirmed with the 'ripple' command, using a minimum LOD of 4.0. Finally, the map distances were calculated using 'map' command. The map distances were converted into centiMorgans, using the Kosambi (1944) function.

Results

Inheritance of gall midge resistance in rice genotype W1263

Of the 150 F_2 individuals of the cross W1263/TN1, 45 were homozygous resistant (*RR*), 70 heterozygous resistant (*Rr*) and 35 homozygous susceptible (*rr*), confirming single-gene inheritance (χ^2=1.99, P<0.40). Similarly, the 145 BC_1F_2 individuals derived from a single heterozygous resistant BC_1F_1 plant of the cross Swarna/W1263//Swarna

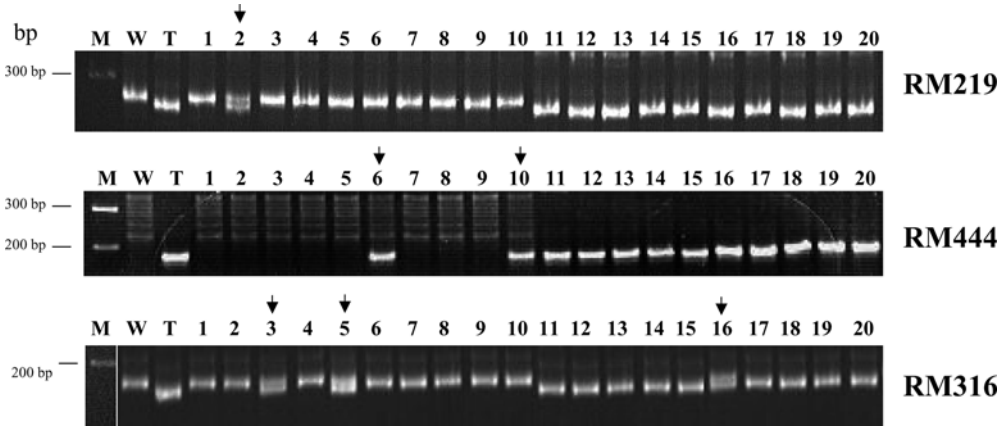

Fig. 1 Co-segregation analysis of *Gm1* with the linked SSR markers. PCR was performed with the markers RM219, RM444 and RM316, using DNA isolated from 20 plants representing the F_2 generation of the cross W1263/TN1. The samples were resolved on 12% polyacrylamide-TBE gel for the markers RM219, RM444 and on 15% polyacrylamide-TBE gel for the marker RM316 and stained with ethidium bromide. *M* Molecular-weight marker (100-bp ladder), *W* W1263 (resistant parent), *T* TN1 (susceptible parent). *Lanes1–10* Homozygous resistant F_2 plants, *11–20* homozygous susceptible F_2 plants. *Numbers left of the lanes* represent molecular weights. *Arrows above lane numbers* indicate recombinants

showed segregation of 42 *RR*, 74 *Rr* and 29 *rr* (χ^2=2.473, P<0.25), confirming that gall midge resistance in W1263 is controlled by a single dominant gene.

Tagging and mapping *Gm1*

Of the 133 SSR markers used for parental polymorphism survey, 63 showed polymorphism between the parental lines W1263 and TN1 (data not shown). The markers RM316, RM444 and RM219, located on chromosome 9, co-segregated with the trait phenotype in the subset mapping population (Fig. 1). The marker RM316 amplified polymorphic fragments of 170 bp in W1263 and 160 bp in TN1. Similarly, RM219 amplified polymorphic segments of 230 bp in W1263 and 210 bp in TN1. RM444 amplified a clear band of 183 bp in TN1 but amplified several bands in W1263; the latter were more diffuse on polyacrylamide gels than on agarose gels. When this analysis was extended to 110 of 150 F_2 individuals, MAPMAKER analysis indicated that SSR markers RM316, RM444 and RM219 are located at a genetic distance of 8, 4.9 and 5.9 cM, respectively, from *Gm1*. A genetic map with a total length of 13 cM, consisting of these three SSR loci and the gene *Gm1* was constructed (Fig. 2).

The utility of the identified SSR markers to predict resistance to gall midge was analysed in a progeny-tested BC_1F_2 population derived from the cross Swarna/W1263//Swarna. RM219, RM444 and RM316 showed polymorphism between the parents (Swarna and W1263) and predicted the presence of the trait phenotype in 136, 138 and 132 out of 145 BC_1F_2 individuals, respectively.

Amplification pattern of the linked SSR markers in a set of rice genotypes

To examine whether the linked markers would be of use in resistance-breeding programmes, DNA samples from a set of five putative gall midge resistance donors and five high-priority susceptible recipients were amplified by PCR using markers RM219, RM316 and RM444 (Fig. 3). Four donors known to possess *Gm1*—W1263, Eswarakora, Kavya and Mahamaya (Bentur et al. 2003)—showed a band of 230 bp with marker RM219, while the remaining six genotypes amplified bands between 186 bp and 210 bp. With respect to the marker RM444, the cultivars W1263 and Eswarakora gave a similar, indistinct pattern of bands in the range of 290–320 bp, while the resistant cultivars Mahamaya and Kavya amplified alleles of 240 bp and 185 bp, respectively. The land race ARC6605, carrying an uncharacterised gene(s) for gall midge resistance, yielded a band of 227 bp when amplified with RM444, and the susceptible cultivars (TN1, Swarna, Samba Mahsuri, PR106 and Krishnahamsa) amplified alleles of between 183 bp and 240 bp. The marker RM316 amplified an allele of 170 bp in two of the resistant cultivars (W1263 and Eswarakora), while the remaining eight genotypes yielded bands of ~160 bp.

Discussion

Genetic analysis of gall midge resistance involving donor parent W1263 showed that it has a dominant gene, confirming the earlier reports of *Gm1* of Shastry et al. (1972), Sastry et al. (1984), Chaudhary et al. (1985) and Reddy et al. (1997). The present study has established that

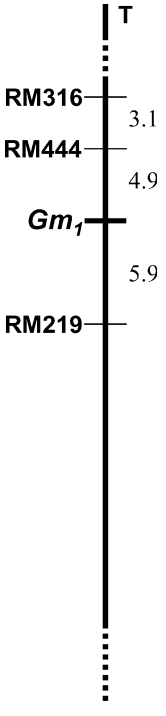

Fig. 2 Linkage map of a portion of chromosome 9 in the vicinity of the *Gm1* gene. Positions are shown for the markers RM316, RM444 and RM219 relative to *Gm1*, with map distances in centiMorgans. *T* Telomere

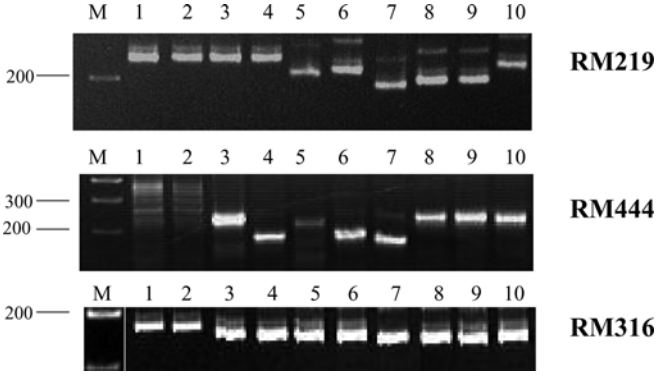

Fig. 3 Determination of microsatellite marker allele size (base pairs) in rice cultivars with and without the *Gm1* resistance allele. DNA isolated from a set of four known *Gm1* donors (*lane 1* W1263, *lane 2* Eswarakora, *lane 3* Mahamaya, *lane 4* Kavya), a land race, ARC6605 [exhibiting *Gm1*-like resistance (*lane no. 5*)] and a set of five gall midge-susceptible but popular rice cultivars (*lane 6* Swarna, *lane 7* TN1, *lane 8* Samba Mahsuri, *lane 9* PR106, *lane 10* Krishnahamsa) were used for PCR amplification with *Gm1*-linked microsatellite markers RM219, RM444 and RM316, and resolved using 15% polyacrylamide-TBE gel. *M* Molecular-weight marker (100 bp ladder). *Numbers left of the lanes* represent molecular weight in base pairs

the SSR markers RM316, RM444 and RM219, are linked to the *Gm1* locus.

Progeny testing of the F_3 generation coupled with a stringent phenotyping procedure as described in Materials and methods ensured correct identification of homozygotes and heterozygotes in F_2 with no escapes. The choice of the F_2 mapping population for tagging qualitatively inherited gene(s) is considered to be useful, as it saves time and resources in tagging and mapping of major genes compared to RILs or NILs (Michelmore et al. 1991; Haley et al. 1994). Initially we used 520 RAPD primers (Operon, USA) for co-segregation analysis in the F_2s, (data not shown), from which only one marker ($OPB02_{480}$) exhibited co-segregation with the resistance phenotype. We did not use the marker $OPB02_{480}$ for further analyses since it was not close enough for use in practical MAS (linkage distance of >12 cM, data not shown), was dominant in its inheritance and did not show polymorphism among many of the parental lines studied. Xu and Mackill (1996) have reported on limitations of RAPDs in mapping a major submergence gene locus on chromosome 9.

The use of homozygous segregants of F_2 as a subset mapping population facilitated quicker identification of linked SSR markers and the tentative chromosomal location of *Gm1*. Once the tentative chromosomal location was identified, all the SSR markers of that chromosome could be tested for co-segregation with the trait phenotype in the entire mapping population.

We observed that the markers RM316, RM444 and RM219, located on chromosome 9, exhibited co-segregation with the trait phenotype with linkage distances of 8, 4.9 and 5.9 cM, respectively, from *Gm1*. The order of these SSR loci (in the vicinity of *Gm1*) agreed very well with the published SSR map of chromosome 9 (Temnykh et al. 2000). The markers RM316, RM444 and RM219 may not be individually close enough for effective use in MAS for *Gm1*. However, the fact that RM316 and RM444 lie on one side of the gene and RM219 is located on the other side of *Gm1* locus (i.e. flanking nature of the SSR markers) helps in accurate prediction of the presence of *Gm1* with less than 1% error. If the selection accuracy (calculated based on recombination frequencies observed in F_2 population) while using RM316, RM219 and RM444 individually to predict *Gm1* is 92.4, 94.5 and 95.8%, respectively, then based on the product rule of probability, the combined use of the markers RM316 and RM219 or RM219 and RM444 enhances the selection efficiency to 99.64% and 99.77%, respectively. Thus, in breeding programmes the marker combinations RM219 and RM316 and/or RM219 and RM444 with respect to these three flanking markers could be used for indirect selection of *Gm1*. These markers would identify most resistant plants, although some resistant plants with recombination between the flanking markers and the resistance gene would be eliminated. For most breeding applications this would not be a limitation since all the 'false positives' (susceptible plants) would be eliminated in indirect selection using these flanking markers. In order to test the efficacy of such flanking markers in predicting the presence of *Gm1* in a different genetic background, we used a progeny-tested BC_1F_2 population derived from the cross Swarna/W1263//Swarna and observed that the flanking marker combinations RM316-RM219 and RM219-RM444 accurately predicted resistant/susceptible individuals with no false positives. Of the two combinations, the combination RM219-RM444 appears to be better in MAS because both RM219 and RM444 exhibit polymorphism between many resistant donors carrying *Gm1* and susceptible cultivars (see Fig. 3).

Another interesting observation noted while phenotyping was a positive correlation between gall midge resistance locus *Gm1* in W1263 and a morphological trait—brown furrows on the seed hull. More than 70% of the F_3 and BC_1F_3 seeds that were phenotypically resistant also had brown furrows on the hull. Earlier studies by Sastry et al. (1975, 1984) also indicated such a correlation between the gall midge resistance locus *Gm1* and the trait brown-hull colour (controlled by the gene *I-Bf*) in W1263, with a crossover value of 35% between these two traits. Nagao and Takahashi (1963) included the *I-Bf* gene in group V of a linkage map (*indica* group), which was later assigned to a position near the centromeric region of chromosome 9 in the revised linkage map of rice. These observations further confirm our inference that the *Gm1* gene is located on the short arm of the rice chromosome 9.

The present study highlights an important problem frequently encountered in gene mapping studies. None of the SSR markers (available in Cornell SSR map 2001 and IRMI SSR map 2003) in the vicinity of RM219 and RM444 were observed to be polymorphic between the parental lines W1293, TN1 and/or Swarna and hence, a high-resolution linkage map could not be constructed for the gene. Xu et al. (2000, 2004) have also reported lack of sufficient polymorphic SSR markers on chromosome 9 (on the same region to which *Gm1* has been mapped) in their studies on mapping of a major submergence tolerance gene *Sub1*. With the availability of the complete rice genome sequence, the microsatellite repeats in the genomic region of chromosome 9 spanning *Gm1* can be studied in detail, and this may ultimately lead towards identification and development of very tightly linked SSR markers for the gene. The mapping information from the present study provides a starting point for fine mapping of the *Gm1* locus and map-based cloning of the gene to clarify its molecular structure and function.

With the identification of microsatellite markers linked to *Gm1* in this study, all the major gall midge resistance genes have tags, paving the way for gene pyramiding of two or more genes into elite rice cultivars for durable resistance. However, a more crucial issue is which gene or combination of genes would be effective to ensure the durability of resistance. If experience is any indication, pest populations have acquired virulence against the individual major genes contributing resistance in varieties cultivated extensively for the last two decades. For example, the resistance conferred by *Gm2* in the variety Phalguna broke down due to the emergence of a new

biotype in the year 1987 (Bentur et al. 2003). However, the resistance in varieties with *Gm1* (for e.g. Mahamaya and Samridhi) is lasting longer (Bentur et al. 2003). In view of these, the present study of tagging and mapping *Gm1* assumes significance wherein *Gm1* can be deployed in gene pyramiding programmes. We are currently using these flanking markers in a backcross breeding programme for introgression of *Gm1* into high yielding, popular rice cultivars Swarna and Samba Mahsuri.

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