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Flanking SSR markers for allelism test for the Asian rice gall midge (*Orseolia oryzae*) resistance genes

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Abstract Screening of rice germplasm against Asian rice gall midge, *Orseolia oryzae* (Wood-Mason), biotypes in India has led to identification of over 300 resistant rice genotypes. However, only ten resistance genes have been characterized so far. Identification of new genes through classical allelism test is tedious and time consuming. We propose to use closely linked flanking Simple Sequence Repeat (SSR) markers in allelism tests for identification of resistance genes. Of the ten known gall midge resistance genes, eight have been tagged and mapped. The *Gm1* and *Gm2* genes have closely linked flanking markers. Hence SSR markers RM219 and RM444, flanking the gene *Gm1*, and RM317, RM241 along with the SCAR marker F8, flanking the gene *Gm2*, were selected for this study. Tests with one set of 13 genotypes likely to carry *Gm1* and another set of 17 genotypes suspected to contain *Gm2* suggested the presence of the respective allele in all the 13 and 15 genotypes from these sets, respectively. Classical allelism test perfectly matched with the markers test. There were two exceptions involving amplification with RM444 in cultivar Kavya and with RM241 in genotype AE20, suggesting a single recombination which could have resulted in the mismatch. All the three markers in the genotype Bhumansan and the two flanking markers RM317 and F8 in AE20 indicated the absence of the *Gm2* allele. This was validated through a classical test, revealing a segregation ratio of 15 resistant: 1 susceptible F_2 progeny of both the crosses between the *Gm2* source Phalguna and these genotypes. We performed the allelism test with the markers on another set of 56 randomly selected gall midge resistant genotypes to discover possible sources of new resistance genes.

Keywords Allelism test · Gall midge · *Orseolia oryzae* · *Oryza sativa* · Rice · SSR markers

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Introduction

The Asian rice gall midge, *Orseolia oryzae* (Wood-Mason), is one of the most destructive pests of rice in south and south east Asia (Heinrichs 1994). Because the insect feeds internally, chemical control is not very effective. Breeding for resistance has been the main approach for gall midge management. Field and greenhouse evaluation of about 50,000 germplasm accessions has resulted in the identification of over 300 primary sources of resistance (Bentur et al. 2003). Genetic analysis in some of these sources led to the characterization of nine dominant genes and

one recessive gene conferring resistance (Kumar et al. 2005). Utilizing 4–5 of these sources of resistance, over 60 gall midge resistant rice varieties have been developed and released for commercial cultivation in India since 1972 (Bentur et al. 2003). However, 84% of the released resistant varieties derive their resistance from only three genes or sources viz., *Gm1, Gm2* and an unidentified gene in the genotype Ptb21. Extensive cultivation of some of the resistant varieties has led to evolution of virulent biotypes of the pest that are capable of overcoming the resistance. So far, seven distinct biotypes have been characterized based on reaction pattern against four groups of differential rice varieties (Vijaya Lakshmi et al. 2006). This situation has necessitated new breeding strategies to achieve the goal of durable gall midge resistance. Pyramiding of two or more genes in a single cultivar is one such strategy being followed.

To identify a new resistance gene, crosses can be made between the suspected new source and genotypes possessing each of the ten known genes in a classical allelic test. This tedious process is not a practical approach for analyzing large numbers of resistant genotypes. As an alternative, many of these gall midge resistant genotypes have been evaluated against the six common biotypes of the gall midge that are found in India. Based on the gene-for-gene interaction between rice and gall midge, these genotypes have been classified into four differential groups (Bentur et al. 2003). Each of these groups has some member with a known genetic basis of resistance. The task of looking for new genes is now narrowed down to members within a group. Members of one group differ from those of other groups in reaction pattern and hence are likely to contain different gene(s). Even then, the number of crosses to be made to identify a new gene remains high.

Development of molecular markers that are tightly linked to the gene of interest has improved the efficiency of conventional plant breeding (Huang et al. 1997; Hittalmani et al. 2000). Among the several molecular markers available, the SSR markers have several advantages over other markers and hence have received more attention in molecular marker studies (Fjellstrom et al. 2006). SSR markers are reliable, co-dominant, multi-allelic, chromosome-specific and highly informative (Swarup et al. 2006). SSR markers have been used to develop linkage maps in many plant species including major cereals, viz., wheat (Song et al. 2005), rice (Mc Couch et al. 2002), maize (Sharopova et al. 2002), barley (Thiel et al. 2003) and oat (Zhu and Kaeppler 2003). These markers have also been used in several other studies like genetic diversity in different crops (Ellwood et al. 2006), germplasm analysis (Yong et al. 2002), genetic relationships (Zhang et al. 2003), phylogenetic studies (Provan et al. 2001) and others. SSR markers have also been used for tagging and mapping insect resistance genes in rice against brown planthopper (Jena et al. 2006) and gall midge (see below), in wheat against the Hessian fly (Liu et al. 2005) and in maize against pink stem borer (Butron et al. 2005).

Eight of the ten gall midge resistant genes have been tagged using various molecular markers (Nair et al. 1995; Mohan et al. 1997; Katiyar et al. 2000, 2001; Sardesai et al. 2002; Biradar et al. 2004; Jain et al. 2004). Since some of these markers were not useful in other breeding populations, additional closely linked SSR markers were identified (Sundaram et al. 2003). We propose here a novel use of these closely linked flanking SSR markers for allelic tests for gall midge resistance genes. We aimed at two of the major gall midge resistance genes *Gm1* and *Gm2* that have closely linked SSR markers (Sundaram et al. 2003; Biradar et al. 2004). A molecular marker based test was validated with classical allelic test crosses for a total of 18 genotypes. The marker test was extended to an additional set of 56 genotypes with gall midge resistance to identify new gene sources.

Material and methods

Plant material

The rice varieties, W1263 and Phalguna, possessing the major dominant genes *Gm1* and *Gm2*, respectively, along with two sets of diverse genotypes from differential groups I and II were selected for study based on their reaction pattern against six known gall midge biotypes (Bentur et al. 2003). Thirteen diverse genotypes likely to carry *Gm1* gene from differential group I, and 17 genotypes likely to carry *Gm2* gene from differential group II, were selected. The genotypes in these two groups, though of diverse

geographic origin, showed a similar reaction pattern against W1263 and Phalguna, respectively (Table 1). TN1, Swarna and BPT 5204 with no gall midge resistance gene were included as the susceptible checks. An additional 56 genotypes with gall midge resistance from the DRR germplasm bank were randomly selected for validation of the study.

DNA extraction and PCR

DNA was isolated from leaf tissue of selected genotypes through the method of Zheng et al. (1991) and used for PCR following the protocol of Chen et al. (1997). Linked SSR markers used for PCR amplification of genomic DNA were RM444 and RM219 for *Gm1* which flank the gene locus on chromosome 9 (Biradar et al. 2004) and RM241, RM317 (Sundaram et al. 2003) for *Gm2* on chromosome 4. In addition to these SSR markers, a SCAR marker F8 closely linked to the gall midge resistance gene *Gm2* (Nair et al. 1995) and which flanks the gene along with RM241 and RM317 was also used in this study. Primer sequence information for the above SSR markers is available at http://www.gramene.org while that for F8 marker is given by Nair et al. (1995). The size of the amplified fragments was calculated using Alphaease software (Alpha Innotech, USA) with λ/*Hin*dIII + *Eco*R1 double digest and 100-bp ladder (MBI Fermentas, Lithuania) as size reference standards. The exact physical positions of the closely linked markers for *Gm1* or *Gm2* were determined through BLAST search using bioedit software against the *indica* sequence database for Chromosome 9 and 4 (http://rice.genomics.org.cn/rice/index2.jsp).

Classical allelism test

Results obtained by the molecular approach were verified with a classical approach. Reaction of the selected genotypes against gall midge biotype 1 was reconfirmed in the greenhouse. Crosses were made between W1263 and Assamchudi to test the allelic status of the gene in the latter. Since the other 12 genotypes from the first set have known descent from the donor sources such as W1263 or Eswarakora carrying the *Gm1* gene, no crosses were made between these genotypes and W1263. Separate crosses were made between Phalguna and 17 genotypes of the second set. One or two F_1 plants were tested for gall midge resistance while 5–10 F_1 plants were grown to collect F_2 seeds. Seeds were collected separately from each plant.

Greenhouse evaluation for gall midge reaction

The selected genotypes and F_2 plants were evaluated in a greenhouse for the reaction against gall midge biotype 1 following a standardized screening procedure (Bentur and Kalode 1996). This involved sowing of pre-germinated seeds in rows 5 cm apart with 10–15 plants per row, in plastic trays (60 × 30 × 30 cm) filled with 8 cm of puddled soil. The soil was enriched with fertilizers in the ratio of 2:1:1 of N:P:K,

Table 1 Diverse genotypes selected for the study as based on reaction pattern observed against the six gall midge biotypes

Group[a]	Genotype	Gene	Reaction pattern[a]	Linked markers
I	W1263	*Gm1*	R-S-R-S-R-R	RM444 and RM219
I	Samridhi, Kavya, MDU3, Rajendradhan 202, Asha, Usha, Lalat, Kakatiya, Pothana, RPW 9-4-85, RPW 9-6-12, RD4, Assamchudi	Suspected to carry *Gm1*	R-S-R-S-R-R	RM444 and RM219
II	Phalguna	*Gm2*	R-R-S-S-R-S	RM 317, RM241 and F8
II	ARC11704, ARC14766, ARC15570, ARC5823, ARC5913, ARC5939, AE 20, ARC 5988, ARC 6221-1, ARC 6632, ARC 7255, MNP 709, MNP 85, ORSJR18A, Phodum, Ritiang, Bhumansan	Suspected to carry *Gm2*	R-R-S-S-R-S	RM 317, RM241, and, F8
IV	TN1, Swarna, BPT5204	None	S-S-S-S-S-S	None

[a] Based on reaction observed against the gall midge biotypes 1–6, in that order, in multi-location evaluation (Bentur et al. 2003); R-Resistant, S-Susceptible

approximating doses of 120, 60 and 60 kg ha^{-1}. One row each of the standard resistant (Phalguna/Kavya) and susceptible (TN1) check genotypes were sown in each test tray. When the seedlings were 10–12 days old, they were exposed to about 25 female and 10 male adults on each of two consecutive days by placing the tray in a nylon mesh cage and releasing newly emerged adults. For following 2 days the tray was placed in a humid chamber with relative humidity (RH) > 90% and a temperature of 25 ± 5°C, for egg incubation and maggot establishment. Subsequently, these trays were maintained at room temperature in a growth chamber of a greenhouse with the annual range of temperature of 25 ± 10°C and 60 ± 10% RH. Insect damage was recorded 20 days after adult release, when the susceptible checks showed fully extended galls. A test was considered valid only when >90% of the susceptible check TN1 plants showed damage symptoms. Test genotypes recording nil or less than 10% plant damage were rated as resistant while those with a higher level of damage were rated as susceptible. Plant damage in F_2 populations was recorded on a single plant basis. Plants with galls were rated as susceptible while the rest were dissected to confirm the presence of dead maggots and/or expression of hypersensitive reaction (HR) for rating as resistant. Plants that did not show these features were labeled as escapes and were not considered under the total of test plants. Escape plants did not exceed 10% of the total in any of the experiments. Plant damage data from F_2 plants were analyzed with the χ^2 test of goodness of fit (Gomez and Gomez 1984).

Results

Marker test

Primers for the marker RM444 in W1263 amplified five bands in the range of 290–320 bp, while in the susceptible cultivar TN1 it amplified a clear single band of ~183 bp (Fig. 1a). However, in the other two susceptible checks viz., Swarna and BPT5204 it amplified slightly different alleles of ~210 and ~216 bp, respectively. RM219 primers amplified polymorphic fragments of ~230 bp in W1263 and ~210 bp in TN1 (Fig. 1b). In the other two susceptible checks Swarna and BPT 5204 these markers amplified fragments of ~196 and ~220 bp.

Amplification of genomic DNA in the set I genotypes by primers for the marker RM444 showed the W1263 (*Gm1*)-specific pattern of bands, W allele (290–320 bp), in all the selected genotypes except in Kavya (Fig. 1a). The RM444 primers amplified a single band of ~185 bp in Kavya. RM219 primers amplified the W allele (~230 bp) linked to resistance in all the genotypes tested (Fig. 1b). These results suggested the presence of *Gm1* gene in all the 13 genotypes of this set.

Amplification of the *Gm2* gene linked marker RM241 revealed a band of ~182 bp in Phalguna

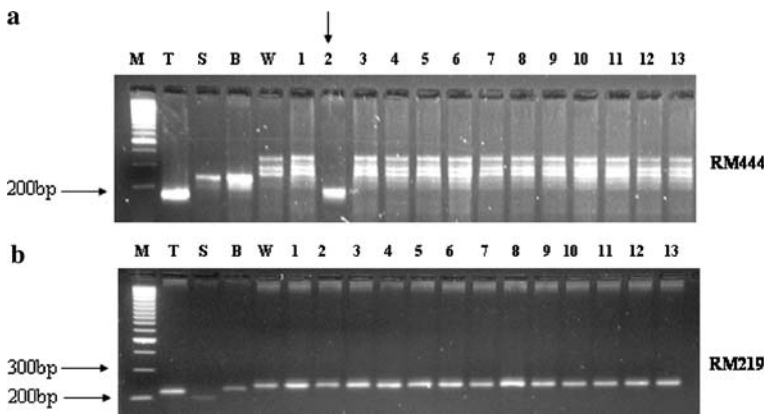

Fig. 1 Amplification pattern of *Gm1* linked SSR markers RM444 and RM219 in 13 selected genotypes of set 1. (**a**). RM444, (**b**). RM219, M—Mol. Weight marker (100 bp DNA ladder), T—TN1, S—Swarna, B—BPT 5204, W—W1263. (1–13) lanes—test genotypes: 1. Samridhi, 2. Kavya, 3. MDU3, 4. Rajendradhan 202, 5. Asha, 6. Usha, 7. Lalat, 8. Kakatiya, 9. Pothana, 10. RPW 9-4-85, 11. RPW 9-6-12, 12. RD4, 13. Assamchudi

(P allele) and a band of ~170 bp in all the susceptible checks (Fig. 2a). Primers for RM317 amplified a band of ~170 bp in Phalguna (P allele) and a band of ~180 bp in the susceptible checks (Fig. 2b). Primers for marker F8 amplified a larger segment of 1.7 kb in Phalguna (P allele) and 2 kb in the susceptible checks (Fig. 2c). Further, the RM241 primers amplified the P allele in all the test genotypes of set II, except in Bhumansan (Fig. 2a). RM317 and F8 primers also amplified the respective P alleles in all the test genotypes of this set, except in Bhumansan and AE20 (Fig. 2b and c). These results suggested presence of the *Gm2* gene in 15 of the 17 test genotypes of this set. The test also suggested the absence of the *Gm2* gene in Bhumansan and AE20.

Set I genotypes were also tested with *Gm2*-linked SSR markers and set II genotypes were tested with *Gm1*-linked SSR markers (Table 2). In the first set, RM317 primers amplified other alleles (OA), neither specific to W allele nor those noted for the susceptible checks, in six genotypes. The rest of the genotypes showed a null allele (NA) with no amplification of any fragment. The RM241 primers amplified OA in eight and NA in five genotypes. In Set II, primers for both the *Gm1* linked SSR markers, RM219 and RM444, amplified OA allele in all the 17 genotypes. This suggested the absence of the target gene in the non-corresponding set of genotypes.

Classical test

Reactions of the genotypes from set I against gall midge biotype 1 confirmed resistance in all the genotypes selected for the analysis (Table 3). A small percentage of susceptible plants (between 10% and 20%) was noted against some of the differentials in the tests conducted in 2005 but was not observed in the retesting done in 2006. Reactions of genotypes from set II against biotype 1 were unambiguous (Table 4) and confirmed the earlier results.

All the 114 F_2 plants derived from the cross W1263 × Assamchudi showed resistance and did not segregate (Table 5). This strongly suggested that the unknown gene in Assamchudi was allelic to *Gm1*,

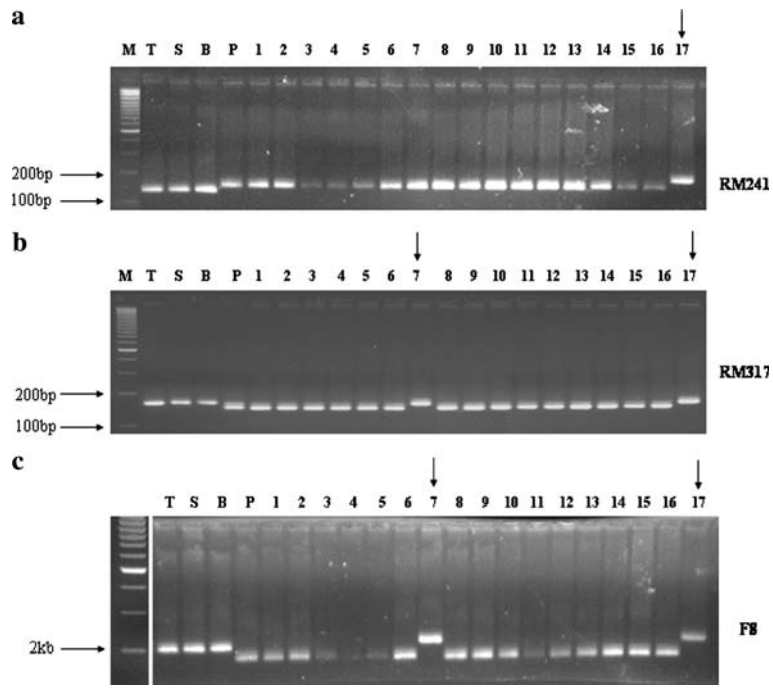

Fig. 2 Amplification pattern of *Gm2* linked SSR markers RM241, RM317 and F8 in 17 selected genotypes of set 2. (**a**). RM241, (**b**). RM317, (**c**). F8. M—Mol. weight marker (100 bp DNA ladder), T—TN1, S—Swarna, B—BPT 5204, P—Phalguna. (1–17) lanes—test genotypes: 1. ARC11704, 2. ARC14766, 3. ARC15570, 4. ARC5823, 5. ARC5913, 6. ARC5939, 7. AE20, 8. ARC5988, 9. ARC6221-1, 10. ARC6632, 11. ARC7255, 12. MNP709, 13. MNP85, 14. ORSJR18A, 15. Phodum, 16. Ritiang, 17. Bhumansan

Table 2 Amplification pattern of genotypes in set I and set II with the selected SSR markers

S. No.	Genotype	RM219	RM444	RM317	RM241
1	W1263	W	W	OA	OA
2	Phalguna	OA	OA	P	P
3	Samridhi	W	W	OA	OA
4	Kavya	W	OA	OA	OA
5	MDU3	W	W	OA	OA
6	Rajendradhan 202	W	W	OA	OA
7	Asha	W	W	NA	NA
8	Usha	W	W	NA	NA
9	Lalat	W	W	NA	NA
10	Kakatiya	W	W	NA	OA
11	Pothana	W	W	OA	OA
12	RPW 9-4-85	W	W	NA	NA
13	RPW 9-6-12	W	W	OA	OA
14	RD4	W	W	NA	OA
15	Assamchudi	W	W	NA	NA
16	ARC 11704	OA	OA	P	P
17	ARC 14766	OA	OA	P	P
18	ARC 15570	OA	OA	P	P
19	ARC 5823	OA	OA	P	P
20	ARC 5913	OA	OA	P	P
21	ARC 5939	OA	OA	P	P
22	AE 20	OA	OA	OA	OA
23	ARC 5988	OA	OA	P	P
24	ARC 6221-1	OA	OA	P	P
25	ARC 6632	OA	OA	P	P
26	ARC 7255	OA	OA	P	P
27	MNP 709	OA	OA	P	P
28	MNP 85	OA	OA	P	P
29	ORS JR 18A	OA	OA	P	P
30	Phodum	OA	OA	P	P
31	Ritiang	OA	OA	P	P
32	Bhumansan	OA	OA	OA	OA

NA—null allele, OA—other allele, P—Phalguna allele, W—W1263 allele

present in W1263. Of the 17 crosses between Phalguna and the test genotypes from set II, F_2 progeny from 14 crosses recorded no segregation for resistance. Cases involving one or two susceptible plants of more than 100 plants tested were considered to be non-segregating population since this ratio did not fit in any normal ratios. However in the case of the Phalguna × ARC 5988 cross one out of 62 plants tested showed susceptibility and the observed ratio was in agreement with a 63:1 ratio (χ^2 = 0.0003, P = 0.974), suggesting involvement of three genes. Two of the crosses viz., Phalguna × AE20 and Phalguna × Bhumansan, showed an F_2 segregation ratio of 167 resistant: 5 susceptible and 93 resistant: 6 susceptible, respectively. These ratios did not differ significantly from the expected ratio of 15 resistant: 1 susceptible (χ^2 = 3.28, P = 0.07; χ^2 = 0.00645, P = 0.937, respectively). Thus it was evident that the gene conferring gall midge resistance in AE20 and Bhumansan was non-allelic to *Gm2*.

Table 3 Reaction of selected genotypes suspected to carry *Gm1* gene against gall midge biotype 1 in greenhouse during 2 years

S. No.	Rice genotype	Rating	2005		2006	
			DP	TP	DP	TP
1	Samridhi	R	0	42	0	20
2	Kavya	R	0	27	0	22
3	MDU3	R	0	10	0	12
4	Rajendradhan 202	R	0	10	1	22
5	Asha	R	3	28	0	17
6	Usha	R	5	30	0	15
7	Lalat	R	1	40	0	20
8	Kakatiya	R	5	39	1	25
9	Pothana	R	6	47	0	24
10	RPW 9-4-85	R	0	40	0	16
11	RPW 9-6-12	R	2	29	1	12
12	RD4	R	2	24	0	12
13	Assamchudi	R	0	18	0	12
Checks						
14	TN1	S	33	33	21	21
15	Swarna	S	19	19	16	16
16	BPT 5204	S	26	26	20	20
17	W1263	R	0	31	0	18

R—resistant, S—susceptible, DP—No. of damaged plants; TP—No. of total plants tested

Evaluation of resistant rice germplasm

Among the 56 randomly selected gall midge resistant rice genotypes tested for the presence of *Gm1* and *Gm2*, nine showed the presence of the W allele with both the flanking markers RM444 and RM219 while six others recorded this allele with the marker RM219 only (Table 6). The presence of the P allele in eight accessions was detected with all the three markers RM241, RM317 and F8. In addition, five more accessions showed the presence of the P allele with the two flanking markers RM317 and F8. One of the accessions, INRC1421, recorded the presence of both the W and P alleles. Primers for these markers amplified OA in five of the accessions, not specific to *Gm1*, *Gm2* or TN1, with all five markers suggesting the absence of both the *Gm1* and *Gm2* alleles. In addition, 17 more accessions did not amplify either the W or P alleles with these markers.

Discussion

The rice variety W1263 carries the dominant gene *Gm1* (Chaudhary et al. 1985) and Phalguna possesses the dominant gene *Gm2* (Mohan et al. 1994). Genotypes with these genes have been grouped into two differential groups I and II, respectively, based on their reaction pattern against the six gall midge biotypes (Bentur et al. 2003). *Gm1* confers resistance against gall midge biotypes 1, 3, 5 and 6 while *Gm2* confers resistance against biotypes 1, 2 and 5. Moreover, resistance by *Gm2* involves expression of a HR while that by *Gm1* does not involve HR (Bentur and Kalode 1996). Each of these genes has been deployed in about one third of the resistant rice varieties developed so far in India. Virulence against *Gm1* in the biotype 2 pest population at Cuttack in Orissa state, India and virulence against *Gm2* gene in the biotype 3 population at Ranchi in Bihar state, India were recorded even prior to the release of resistant rice varieties (Kalode and Bentur 1989). Subsequent to extensive cultivation of resistant rice varieties, virulence against these genes has been reported from different pest endemic regions of the country, including the combined virulence against the *Gm1* and *Gm2* genes in biotype 4 (Bentur et al. 1987). Thus there is a need for identifying new genes to replace those currently used in resistant rice varieties.

Table 4 Reaction of the selected genotypes suspected to carry *Gm2* gene against gall midge biotype 1 in greenhouse during 2 years

S. No.	Rice genotype	Rating	2005		2006	
			DP	TP	DP	TP
1	ARC 11704	R	0	10	0	19
2	ARC 14766	R	0	11	0	19
3	ARC 15570	R	0	10	0	12
4	ARC 5823	R	0	12	0	14
5	ARC 5913	R	0	10	0	18
6	ARC 5939	R	0	10	0	25
7	AE 20	R	0	10	0	23
8	ARC 5988	R	0	10	0	23
9	ARC 6221-1	R	0	12	0	13
10	ARC 6632	R	0	11	0	17
11	ARC 7255	R	0	10	0	11
12	MNP 709	R	0	12	0	19
13	MNP 85	R	0	10	0	16
14	ORS JR 18A	R	0	12	0	24
15	Phodum	R	0	10	0	18
16	Ritiang	R	0	10	0	30
17	Bhumansan	R	0	14	0	13
Checks						
18	TN1	S	10	12	26	26
19	Swarna	S	10	10	10	10
20	BPT 5204	S	14	16	24	24
21	Phalguna	R	0	12	0	14

R—Resistant, S—Susceptible, DP—No. of damaged plants, TP—No. of total plants tested

Because there is the potential for the rapid development of new biotypes in response to the release of varieties with single gene resistance (Vijaya Lakshmi et al. 2006), gene pyramiding to incorporate two or more genes should be considered for durable gall midge resistance. A simple test to detect the presence of *Gm1* and *Gm2* in the large pool of gall midge resistant genotypes will greatly reduce the effort required to identify new genes. Random selection of genotypes and classical allelic tests have led to the characterization of 10 resistance genes so far (Kumar et al. 2005). None of these genes confers resistance against all the known gall midge biotypes. Thus it is necessary to quickly identify new genes useful for durable gall midge resistance.

The informative content of the SSR markers, their ease of handling through PCR, and reports of linked markers for gall midge resistance genes prompted us to conceive an alternative approach for detecting alleles using these markers. Flanking SSR markers for *Gm1* and closely linked markers for *Gm2* and well characterized genotypes under group I and II guided us to select the first 30 genotypes for testing this approach. Marker-based tests with the set I of genotypes did not show any deviation in amplification except in the case of Kavya. This result was also observed in our earlier study (Biradar et al. 2004). All but one of the genotypes from this set are known derivatives from the crosses involving the *Gm1* donor parent Eswarakora (Venkataswamy 1996) or its derivative W1263. However, diversity in these genotypes has been brought about by background selection made at different rice breeding stations across different countries. Recombination between the gene and the markers appeared to be rare, except in the case of Kavya, despite the fact that RM219 and

Table 5 Reaction of F_2 plants derived from the crosses made between different rice genotypes against rice gall midge biotype 1 in greenhouse

S. No.	Cross	TP	R	S	Segregation ratio tested	$\chi 2$ (P=)
1	(W1263 × Assamchudi)	114	114	0	All resistant	–
2	(Phalguna × AE 20)	172	167	5	15:1	3.28, (0.07)
3	(Phalguna × ARC 11704)	135	134	1	All resistant	
4	(Phalguna × ARC 14766)	155	155	0	All resistant	
5	(Phalguna × ARC 15570)	198	198	0	All resistant	–
6	(Phalguna × ARC 5823)	167	166	1	All resistant	
7	(Phalguna × ARC 5913)	123	123	0	All resistant	–
8	(Phalguna × ARC 5939)	149	148	1	All resistant	–
9	(Phalguna × ARC 5988)	62	61	1	63:1	0.0003, (0.974)
10	(Phalguna × ARC 6221-1)	154	154	0	All resistant	–
11	(Phalguna × ARC 6632)	188	186	2	All resistant	
12	(Phalguna × ARC 7255)	187	187	0	All resistant	–
13	(Phalguna × MNP 709)	114	114	0	All resistant	–
14	(Phalguna × MNP 85)	193	192	1	All resistant	
15	(Phalguna × ORSJR 18A)	18	18	0	All resistant	–
16	(Phalguna × Phodum)	89	89	0	All resistant	–
17	(Phalguna × Ritiang)	215	215	0	All resistant	–
18	(Phalguna × Bhumansan)	99	93	6	15:1	0.00645, (0.937)
Checks						
21	Phalguna	186	186	0	–	–
22	TN1	193	0	193	–	–

TP—Total plants tested, R—Number resistant, S—Number susceptible

RM444 are located at physical locations of ~7.1 Mb (5.9 cM away with reference to the gene) and ~5.4 Mb (4.9 cM), respectively, on either side of the gene. As suggested by Biradar et al. (2004), though the recombination probabilities are 0.06 and 0.05 with the individual markers, the combined probability of double recombination would be very low (0.003) and the confidence level of detecting the gene would be high. Proximity of the gene locus to the centromere could be the reason for this. The remaining genotype in the set, Assamchudi, is a land race from eastern India and is remote from southern India, where the donor parent W1263 was developed. The presence of *Gm1* gene in these two land races was detected by the marker test and confirmed by the classical allelism test.

Results with *Gm2*-linked SSR and SCAR markers suggested the presence of this gene in 15 of the 17 genotypes of the set II. The test suggested the absence of *Gm2* in AE20 and Bhumansan with respect to markers RM317 and F8. A classical test confirmed the non-allelic nature of the resistance gene in AE20 and Bhumansan with respect to *Gm2* in Phalguna. However, primers for RM241 amplified P allele in AE20. This marker is at a physical location of ~25 Mb (16.8 cM) while RM317 is at ~28 Mb (5.6 cM) and lie on the same side of the gene, whereas F8 is located at ~30 Mb (7.4 cM) on the other side of the gene locus (Sundaram et al. 2003). Thus the recombination would have occurred between the markers RM317 and RM241 in AE20. The presence of a dominant gene non-allelic to *Gm2* in Bhumansan was also reported earlier (Reddy et al. 1997). Genotypes in set II, unlike in set I, represented a diverse pool of land races with no known pedigree. But all the genotypes represented the *indica* subgroup and most of these are landraces from the north-eastern region of India. Hence despite being members of the same differential group II, AE20 and Bhumansan are likely to carry new genes. Another variation noted between the marker test and the classical test was that of ARC 5988. While the

Table 6 Screening of randomly selected gall midge resistant rice genotypes for presence of *Gm1* or *Gm2* allele with flanking markers

S. No.	Variety/Accession	Allele amplified with the marker[a]				
		RM219	RM444	RM241	RM317	F8
1	ARC 6616	W	W	P	P	OA
2	DIVYA	W	W	P	OA	OA
3	T 2005	W	W	P	OA	OA
4	ARC 15159	W	W	OA	OA	OA
5	INRC 1421	W	W	P	P	P
6	INRC 1997	W	W	P	OA	P
7	INRC 7948	W	W	P	OA	P
8	W 1253-2	W	W	T	OA	OA
9	INRC 2324	W	W	P	NA	OA
10	ARC 10817	W	OA	T	P	OA
11	ARC 11331	W	T	T	P	OA
12	CALROSE 76	W	T	T	OA	OA
13	INRC 7773	W	T	OA	OA	OA
14	INRC 2995	W	T	T	OA	P
15	INRC 2326	W	T	OA	P	P
16	INRC 17465	OA	OA	P	P	P
17	ARC 5848	OA	OA	P	P	P
18	INRC 1711	OA	T	P	P	P
19	INRC 542	T	T	P	P	P
20	CR 94-CRRP 51	T	T	P	P	P
21	ARC 13564	T	T	P	P	P
22	T 1477	T	T	P	P	P
23	ARC 5833	OA	OA	T	P	P
24	PARAKULAM	OA	OA	T	P	P
25	ARC 5780	OA	T	T	P	P
26	ARC 18596	OA	T	T	P	P
27	MORANGKAYAN	OA	T	OA	NA	P
28	ARC 10659	T	T	P	P	OA
29	ARC 14549	OA	OA	OA	OA	OA
30	INRC 3021	OA	OA	OA	OA	OA
31	Aganni	OA	OA	OA	OA	OA
32	ARC15831	OA	OA	OA	OA	OA
33	MR1523	OA	OA	OA	OA	OA
34	INRC 5073	OA	OA	OA	OA	NA
35	ARC 18601	T	OA	NA	NA	OA
36	INRC 2980	OA	OA	T	NA	OA
37	ARC 6605	T	NA	OA	OA	OA
38	ARC 10360	T	T	OA	OA	OA
39	INRC 7040	T	T	OA	OA	OA
40	INRC 8867	OA	OA	T	OA	OA
41	INRC 8843	OA	OA	T	OA	OA
42	710	OA	T	T	OA	OA

Table 6 continued

S. No.	Variety/Accession	Allele amplified with the marker[a]				
		RM219	RM444	RM241	RM317	F8
43	INRC 4598	OA	T	T	OA	OA
44	INRC 8403	T	T	T	OA	OA
45	ARC 14787	OA	OA	OA	OA	OA
46	ARC 15151	OA	T	OA	T	OA
47	ARC 10377	T	OA	OA	T	T
48	INRC 3038	T	T	OA	T	OA
49	ARC 6157	T	T	T	T	OA
50	S 2204	T	T	T	T	OA
51	IET 9405 (OR 437)	T	T	P	OA	T
52	INRC 541	OA	OA	P	T	P
53	INRC 1590	OA	T	P	P	OA
54	INRC 1662	OA	T	P	P	OA
55	INRC 1485	OA	OA	P	P	OA
56	INRC 8383	OA	T	P	OA	OA
Checks						
59	TN1	T	T	T	T	T
60	Swarna	OA	OA	T	T	T
61	BPT5204	OA	OA	T	T	T
62	W1263	W	W	OA	OA	OA
63	Phalguna	OA	OA	P	P	P

[a] T—TN1 allele, W—W1263 allele, P—Phalguna allele, OA—other allele, NA—null Allele

marker test suggested the presence of *Gm2* in this genotype, the F_2 population from the cross Phalguna × ARC 5988 had a segregation ratio of 63 resistant:1 susceptible plants, suggesting involvement of three genes. However, this needs further confirmation in view of the small population ($n = 62$) of F_2 plants tested.

The marker-based allelism test was done to detect the presence of the *Gm1* and *Gm2* genes in set III of 56 randomly selected gall midge resistant rice genotypes. Interestingly, of the 15 accessions that are likely to contain *Gm1* allele based on the marker test, Calrose 76 had displayed reaction pattern specific to the differential group I and the resistance was not accompanied with HR (Bentur et al. 2003), thus supporting the likely presence of this gene. Likewise, of the 13 accessions positive in *Gm2* test, ARC 13564 had displayed a reaction pattern specific to group II. The absence of *Gm1* in ARC6605 as suggested by the marker test was further supported by our earlier observation that this genotype differed from W1263 in expressing HR + type reaction and thus was likely to carry a gene other than *Gm1* (Bentur et al. 2003). The marker test detected the presence of both *Gm1* and *Gm2* in the genotype INRC 1421. In contrast, the test with Lalat, derived from parents W1263 carrying *Gm1* and Vikram carrying *Gm2*, showed the presence of only *Gm1* (Table 2). Significantly, 22 accessions inclusive of MR1523, Aganni, INRC 3021, ARC 15831 and ARC 14549 did not amplify either *Gm1* or *Gm2* specific alleles with these markers. These accessions are likely to contain other genes conferring gall midge resistance. Our earlier studies on reaction pattern and nature of gall midge resistance had also suggested unique features of these five gall midge resistant genotypes. The presence of a new gene, *Gm11* (*t*), conferring resistance in MR1523 against gall midge biotype 4 but not against the new biotype 4 M, has been reported recently (Suneetha et al. 2006). Thus the use of SSR markers for allelism tests can assist in new gene discovery.

The use of allele specific markers for detection and characterization of R genes in disease-resistant

genotypes is currently being explored. Davierwala et al. (2001) surveyed rice genotypes conferring resistance to bacterial blight using STMS, STS and SSR markers linked to three major resistance genes (*xa5*, *xa13* and *Xa21*) for the presence of these genes. Two SSR markers closely linked to *xa5* gene used in the study were further used to screen Near Isogenic Lines (NILs) for the presence or absence of the gene. Kim et al. (2004) used STS and RFLP markers linked to brown planthopper resistance gene *Bph1* to classify the source of resistance in rice cultivars. Fjellstrom et al. (2006) have suggested the use of SSR markers closely linked to the blast resistance *Pi-z* gene locus for detection of this gene in a broad array of rice germplasm. Yu et al. (2006) used SSR markers linked to *Fusarium* head blight resistance quantitative trait loci (QTLs) in a wheat line Sumai 3 to detect these alleles in other wheat lines. However, in these studies the presence of the gene was not cross-checked through classical allelism tests. Though the markers used in the present study are located more than 1 cM from the target gene, new markers with closer linkage are being identified that will further improve the precision of the test.

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References

Bentur JS, Kalode MB (1996) Hypersensitive reaction and induced resistance in rice against Asian rice gall midge *Orseolia oryzae*. Entomol Exp Appl 78:77–81

Bentur JS, Srinivasan TE, Kalode MB (1987) Occurrence of a virulent gall midge (GM) *Orseolia oryzae* Wood-Mason biotype (?) in Andhra Pradesh, India. Int Rice Res Newslett 12(3):33–34

Bentur JS, Pasalu IC, Sarma NP, Prasada Rao U, Mishra B (2003) Gall midge resistance in rice. DRR research paper series 01/2003. Directorate of Rice Research, Hyderabad, 20 pp

Biradar SK, Sundaram RM, Thirumurugan T, Bentur JS, Amudhan S, Shenoy VV, Mishra B, Bennett J, Sarma NP (2004) Identification of flanking SSR markers for a major rice gall midge resistance gene *Gm1*and their validation. Theor Appl Genet 109:1468–1473

Butron A, Tarrio R, Revilla P, Ordas A, Malvar RA (2005) Molecular changes in the maize composite EPS12 during selection for resistance to pink stem borer. Theor Appl Genet 110:1044–1051

Chaudhary BP, Srivastava PS, Shrivastava MN, Khush GS (1985) Inheritance of resistance to gall midge in some cultivars of rice. In: Rice genetics, International Rice Research Institute, Los Banos, pp 523–528

Chen X, Temnykh S, Xu Y, Cho YG, McCouch SR (1997) Development of a microsatellite frame work map providing genome wide coverage in rice (*Oryza sativa* L.). Theor Appl Genet 95:553–567

Davierwala AP, Reddy APK, Lagu MD, Ranjekar PK, Gupta VS (2001) Marker assisted selection of bacterial blight resistance genes in rice. Biochem Genet 39:261–278

Ellwood SR, D'Souza NK, Kampheris LG, Burgess TI, Nair RM, Oliver RP (2006) SSR analysis of the *Medicago trumcatula* SARDI core collection reveals substantial diversity and unusual genotype dispersal throughout the Mediterranean basin. Theor Appl Genet 112:977–983

Fjellstrom R, McClung AM, Shank RA (2006) SSR markers closely linked to the *Pi-z* locus are useful for selection of blast resistance in a broad array of rice germplasm. Mol Breed 17:149–157

Gomez KA, Gomez AA (1984) Statistical procedures for agricultural research, 2nd edn. John Wiley & Sons, Singapore, 680 pp

Heinrichs EA (1994) Biology and management of rice insects. Wiley Eastern Limited, New Delhi, 779 pp

Huang N, Angeles ER, Domingo J, Magpantay G, Singh S, Zhang G, Kumaravadivel N, Bennett J, Khush GS (1997) Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. Theor Appl Genet 95:313–320

Hittalmani S, Parco A, Mew TV, Zeigler RS, Huang N (2000) Fine mapping and DNA marker-assisted pyramiding of the three major genes for blast resistance in rice. Theor Appl Genet 100:1121–1128

Jain A, Ariyadasa R, Kuamr A, Srivastava MN, Mohan M, Nair S (2004) Tagging and mapping of a rice gall midge resistance gene, *Gm8*, and development of SCARs for use in marker-aided selection and gene pyramiding. Theor Appl Genet 109:1377–1384

Jena KK, Jeung JU, Lee JH, Choi HC, Brar DS (2006) High-resolution mapping of a new brown planthopper (BPH) resistance gene, *Bph18(t)*, and marker-assisted selection for BPH resistance in rice (*Oryza sativa* L.). Theor Appl Genet 112:288–297

Kalode MB, Bentur JS (1989) Characterization of Indian biotypes of the rice gall midge *Orseolia oryzae* (Wood-Mason) (Diptera: Cecidomyiidae). Insect Sci Applic 10:219–224

Katiyar SK, Verulkar SB, Adsul G, Dhundre M, Chandel G, Bennett J (2000) Molecular markers for gall midge resistance genes in rice: stage set for MAS and map based gene cloning. In: Abstracts: IV rice genetics symposium, 22–27 October 2000. International Rice Research Institute, Los Banos, Philippines, p 81

Katiyar SK, Tan Y, Huang B, Chandel G, Xu Y, Zhang Y, Xie Z, Bennett J (2001) Molecular mapping of gene *Gm6(t)* which confers resistance against four biotypes of Asian rice gall midge in China. Theor Appl Genet 103: 953–961

Kim S, UnSang Y, JaeKeun S (2004) Classification of cultivars resistant to brown planthopper using DNA markers in rice. Korean J Breed 36(5):290–294

Kumar A, Jain A, Sahu RK, Shrisvastava MN, Nair S, Mohan M (2005) Genetic analysis of resistance genes for the rice gall midge in two rice genotypes. Crop Sci 45:1631–1635

Liu XM, Fritz AK, Reese JC, Wilde GE, Gill BS, Chen MS (2005) *H9*, *H10*, and *H11* compose a cluster of Hessian fly-resistance genes in the distal gene-rich region of wheat chromosome 1AS. Theor Appl Genet 110:1473–1480

Mc Couch SR, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kano I, Yano M, Fjellstrom R, De Clerk G, Schneider D, Cartinhour S, Ware D, Stein L (2002) Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Res 9:199–207

Mohan M, Nair S, Bentur JS, Prasada Rao U, Bennett J (1994) RFLP and RAPD mapping of rice *Gm2* gene that confers resistance to biotype 1 of gall midge (*Orseolia oryzae*). Theor Appl Genet 87:782–788

Mohan M, Sathyanarayanan PV, Kumar A, Shrivastava MN, Nair S (1997) Molecular mapping of a resistance-specific PCR-based marker linked to a gall midge resistance gene (*Gm4t*) in rice. Theor Appl Genet 95:777–782

Nair S, Bentur JS, Prasada Rao U, Mohan M (1995) DNA markers tightly linked to a gall midge resistance gene (*Gm2*) are potentially useful for marker-aided selection in rice breeding. Theor Appl Genet 91:68–73

Provan J, Powell W, Hollingsworth PM (2001) Chloroplast microsatellites: new tools for studies in plant ecology and evolution. Trend Ecol Evol 16:142–147

Reddy VA, Siddiq EA, Prasad Rao U, Bentur JS (1997) Genetics of resistance to rice gall midge (*Orseolia oryzae*). Indian J Genet 57:361–372

Sardesai N, Kumar A, Rajyashri KR, Nair S, Mohan M (2002) Identification and mapping of an AFLP marker linked to *Gm7*, a gall midge resistance gene and its conversion to a scar marker for its utility in marker aided selection in rice. Theor Appl Genet 105:691–698

Sharopova N, McMullin MD, Schultz L, Schroeder S, Sanchez VH, Davis G, Coe EH (2002) Development and mapping of SSR markers for maize. Plant Mol Biol 48:463–481

Song QJ, Shi JR, Singh S, Fickus EW, Costa JM, Lewis J, Gill BS, Ward R, Cregan PB (2005) Development and mapping of microsatellite (SSR) markers in wheat. Theor Appl Genet 110:550–560

Sundaram RM, Bentur JS, Sarma NP (2003) Molecular tagging and mapping of rice gall midge resistance gene *Gm1* and validation of markers for gall midge resistance gene *Gm2*. In: Abstracts 4th Gall midge mini-network meeting. Directorate of Rice Research, Hyderabad, 1–4 December 2003

Suneetha K, Vijaya Lakshmi P, Rama Mohan Rao P, Pallavi Sinha, Pranitha J, Bentur JS (2006) A new gene in rice variety 'MR1523' conferring resistance to gall midge biotype 4 in India. In: Abstract presented at 26th Intl Rice Res Confer, New Delhi, 9–13 October 2006

Swarup K, Parida K, Kumar A, Dalal V, Singh NK, Mohapatra T (2006) Unigene derived microsatellite markers for the cereal genomes. Theor Appl Genet 112:808–807

Thiel T, Michalek W, Varshney RK, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor Appl Genet 106:411–422

Venkataswamy T (1996) Association between hairiness and resistance to gall midge (*Pachydiplosis oryzae*) in rice. Andhra Agric J 13:149

Vijaya Lakshmi P, Amudhan S, Hima Bindu K, Cheralu C, Bentur JS (2006) A new biotype of the Asian rice gall midge, *Orseolia oryzae* (Diptera: Cecidomyiidae), characterized from the Warangal population in Andhra Pradesh, India. Int J Tropic Insect Sci 26:207–211

Yong Z, Yang Kai Y, Cheema AA, Jin WY (2002) Evaluation of germplasm using SSR markers of functional genes in rice. Agric Sci China 1:477–480

Yu JB, Bai GH, Cai SB, Ban T (2006) Marker-assisted characterization of Asian wheat lines for resistance to Fusarium head blight. Theor Appl Genet 113:308–320

Zhang XY, Li CW, Wang LF, Wang HM, You GX, Dong YS (2003) An estimation of the minimum number of SSR alleles needed to reveal genetic relationships in wheat varieties. I. Information from large-scale planted varieties and cornerstone breeding parents in Chinese wheat improvement and production. Theor Appl Genet 106:112–117

Zheng KL, Shen B, Qian HR (1991) DNA polymorphism generated by arbitrary primed PCR in rice. Rice Genet Newslett 8:134–136

Zhu S, Kaeppler HF (2003) A genetic linkage map for hexaploid, cultivated oat (*Avena sativa* L.) based on an intraspecific cross 'Ogle/MAM17-5'. Theor Appl Genet 107:26–35