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# Mapping and marker-assisted breeding of a gene allelic to the major Asian rice gall midge resistance gene *Gm8*

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**Abstract** Host plant resistance is the preferred management strategy for Asian rice gall midge (*Orseolia oryzae*), a serious pest in many rice-growing countries. Identification of simple sequence repeat (SSR) markers that are tightly linked to pest resistance genes can accelerate development of gene pyramids for durable/multiple resistance. Based on conventional and molecular allelism tests, we report herein that rice genotype Aganni possesses *Gm8* gene, conferring hypersensitive independent (HR– type) resistance to gall midge biotypes GMB1, GMB2, GMB3, GMB4, and GMB4M. The gene *Gm8* was mapped to chromosome 8 within a 400-kbp region, and the SSR markers RM22685 and RM22709 flank the gene closely. Using these closely linked flanking markers, nine other gall midge-resistant genotypes were identified as carrying the same gene *Gm8*. Through marker-assisted selection, *Gm8* has been introgressed into an elite bacterial blight-resistant cultivar, Improved Samba-Mahsuri (IS).

**Keywords** Bacterial blight · Biotype · Hypersensitive reaction · Resistance gene · Rice gall midge (*Orseolia oryzae*) · Simple sequence repeat markers

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## Introduction

The Asian rice gall midge *Orseolia oryzae* (Wood-Mason) (Diptera: Cecidomyiidae) is a serious pest of rice (*Oryza sativa* L.) in India, causing average annual yield loss of about US $80 million (Bentur et al. 2003). Over 70 gall midge-resistant rice varieties have been developed and released for commercial cultivation since 1975 (Rani et al. 2011). Widespread cultivation of some of the resistant varieties carrying a single resistance gene has led to evolution of virulent biotypes of the pest. Further, prevalence of bacterial blight (BB) in some of the gall midge-endemic areas limits the choice of varieties that can be grown by farmers. Such a situation demands new breeding

strategies to meet the challenge of durable and multiple pest resistance.

So far, 11 gall midge resistance genes have been characterized in rice (Himabindu et al. 2010), and seven biotypes of the pest have been reported (Vijayalakshmi et al. 2006). Interestingly, none of the identified genes confers resistance to all the gall midge biotypes, while none of the gall midge biotypes is virulent against all the resistance genes. Linked molecular markers are available for 8 of the 11 gall midge resistance genes (*Gm1*, *Gm2*, *Gm4*, *Gm5*, *Gm6*, *Gm7*, *Gm8*, and *Gm11*), seven of which (except *Gm5*) have been mapped onto the rice genome (Himabindu et al. 2010). The majority of these genes (except *Gm1* and *Gm8*) confer resistance associated with hypersensitive reaction (HR+ type).

Jain et al. (2004) tagged and mapped the *Gm8* gene present in a landrace, Jhitpiti, on the short arm of chromosome 8 with two amplified fragment length polymorphism (AFLP) fragments, AR257 and AS168, linked to the gene at genetic distance of 2 cM. This gene confers HR-independent (HR– type) resistance against gall midge biotypes GMB1, GMB2, GMB3, GMB4, and GMB4M (Bentur et al. 2011). In the present study, we confirmed the presence of *Gm8* in another landrace (Aganni) using both conventional and molecular allelism tests and mapped *Gm8*. Further, other possible sources of this gene were also identified using the flanking markers and the gene was introgressed into an elite *indica* cultivar, Improved Samba-Mahsuri, having BB resistance gene *Xa21* through marker-assisted selection (MAS).

## Materials and methods

### Inheritance of resistance

The gall midge-resistant genotype Aganni and the gall midge-susceptible variety TN1 were used as parents to study inheritance of resistance using $F_2$ ($n = 185$) and $F_{10}$ recombinant inbred line (RIL) ($n = 426$) populations. Conventional allelism test was conducted between Aganni and the gall midge resistance gene differentials, viz. Kavya (*Gm1*), Phalguna (*Gm2*), RP2068-18-3-5 (*gm3*), Abhaya (*Gm4*), ARC5984 (*Gm5*), Dukong #1 (*Gm6*), RP2333-156-8 (*Gm7*), Jhitpiti (*Gm8*), Madhuri L9 (*Gm9*), BG380-2 (*Gm10*), and CR57-MR1523 (*Gm11*).

### New sources of *Gm8*

A set of 20 gall midge-resistant genotypes selected based on similar resistance spectrum across gall midge biotypes (Bentur et al. 2003) along with Aganni and the gall midge-susceptible check TN1 were used to detect the presence of *Gm8* using the flanking SSR markers (details in Table 1).

### Greenhouse evaluation for gall midge resistance

TN1, Aganni, and $F_2$ plants and $F_{10}$ RILs of the cross TN1/Aganni were evaluated under greenhouse conditions for resistance against GMB4 following a standardized screening procedure (Vijayalakshmi et al. 2006). Insect damage was recorded 20 days after adult release, when the susceptible checks showed fully exerted galls. A test was considered to be valid only when >90 % of the susceptible check TN1 plants showed damage. Resistant plants were dissected to confirm the presence of dead maggots. Test lines recording ≤10 % plant damage were rated as resistant, while those with >80 % plant damage were rated as susceptible. RILs which segregated with >10 but <80 % plant damage were scored as heterozygous ($n = 14$) and excluded from analysis. $F_2$ plants that did not show either galls or dead maggots were labeled as escapes and were not considered in the total; such plants were less than 2 %.

### DNA extraction and PCR

Total genomic DNA was isolated from leaf tissue of the test plant through the modified method of Zheng et al. (1995) and then used for polymerase chain reaction (PCR) amplification following the protocol of Chen et al. (1997). A set of 432 SSR markers uniformly spread across the 12 chromosomes of rice (RM series; Research Genetics, USA) was used for noting polymorphic markers between the parents. The map locations, primer sequences, and other details of these markers are available online at http://www.gramene.org. The PCR products were resolved on 3.5 % agarose gel (US Biochemicals, USA) in 0.5× Tris/borate/ethylenediamine tetraacetic acid (TBE) buffer, stained with ethidium bromide (0.5 µg/ml), and photographed under ultraviolet (UV) light. The size of the amplified fragments was calculated using Alphaease software (Alpha Innotech, USA) with 100-bp ladder (MBI

**Table 1** Amplification of gene-specific alleles with linked SSR markers in the selected set of gall midge-resistant rice genotypes

| Group | Genotype | Reaction against the biotypes | Presence of gene-specific amplicon with marker | | | | |
|---|---|---|---|---|---|---|---|
| | | | RM23956 (*Gm1*, 600 bp)[a] | RM17480 (*Gm2*, 280 bp)[b] | RM547 (*Gm4*, 182 bp)[c] | RM22709 (*Gm8*, 170 bp)[d] | RM28706 (*Gm11*, 240 bp)[e] |
| 1 | AC169 | R–R–R–R–S–S–R | – | – | – | + | – |
| | AC355 | | – | – | – | + | – |
| | AC630 | | – | – | – | – | – |
| | AC710 | | – | – | – | – | – |
| | AC1224-2 | | – | – | – | + | – |
| | AC3283 | | – | – | – | + | – |
| | Aganni | | – | – | – | + | – |
| | ARC5378 | | – | – | – | – | – |
| | ARC15831 | | – | – | – | + | – |
| | INRC202 | | – | – | – | + | – |
| | INRC1997 | | – | – | + | – | – |
| | INRC3021 | | – | – | – | + | – |
| | INRC17494 | | – | – | + | + | – |
| | MNP762 | | – | – | + | + | – |
| | Nagrasal | | – | – | – | – | – |
| | NHTA8 | | – | – | + | – | – |
| | T1477 | | – | – | – | – | – |
| | Jhitpiti | | – | – | – | + | – |
| | Vellathilcheera | | – | – | + | – | – |
| | INRC17459 | | – | – | – | – | + |
| 2 | Sakthi | R–R–R–R–S–S–S | – | – | – | – | + |
| | T10 | | – | – | – | – | + |
| 3 | TN1 | S–S–S–S–S–S–S–S | – | – | – | – | – |

Reaction against gall midge biotypes GMB1, GMB2, GMB3, GMB4, GMB5, GMB6, and GMB4M, respectively. "R" and "S" indicate resistant and susceptible, respectively

"+" indicates presence of the resistance linked allele for the marker; "−" indicates absence of the resistance linked allele

[a] Biradar et al. (2004)

[b] Sundaram (2008)

[c] Himabindu (2009)

[d] present study

[e] Himabindu et al. (2010)

Fermentas, Lithuania) as size reference standard. The exact physical positions of the linked markers were determined through basic local alignment search tool (BLAST) search using BioEdit software against the *indica* sequence database (http://rice.genomics.org.cn/rice/index2.jsp). Polymorphic SSR markers ($n = 82$) were used for linkage analysis with the trait phenotype, initially in a subset of $F_{10}$ RILs (consisting of 27 resistant and 27 susceptible lines). Once a tentative chromosome location was detected, all the polymorphic SSR markers on that particular chromosome were used for genotyping the entire 412 $F_{10}$ RILs. Molecular allelism test (Himabindu et al. 2007) was performed using the linked SSR markers for the genes *Gm1*, *Gm2*, *Gm4*, *Gm8*, and *Gm11* to detect the corresponding allele in Aganni and 20 gall midge-resistant genotypes. The corresponding gene differentials (Kavya, Phalguna, Abhaya, Jhitpiti, and CR57-MR1523, respectively) and the susceptible parent TN1 were also included in the test. Linkage analysis and map

construction were performed using MAPMAKER/EXP version 3 (Lander et al. 1987).

### Introgression of *Gm8* gene into elite *indica* cultivar Improved Samba-Mahsuri

A cross was made between Improved Samba-Mahsuri carrying *Xa21* gene (recurrent parent) and Aganni (*Gm8* donor), and $F_1$ plants were genotyped using polymorphic SSR markers to confirm heterozygosity. $F_1$ plants were backcrossed with the recurrent parent. A single $BC_1F_1$ plant with *Gm8* and *Xa21* genes was again backcrossed to generate $BC_2F_1$. A single $BC_2F_1$ plant was selfed to generate $BC_2F_2$ plants. Out of several $BC_2F_2$ plants tested, four plants that were double homozygous for these two genes were identified. $F_3$ progeny from these plants were phenotyped for resistance to BB and gall midge.

### Screening for BB resistance

The virulent isolate of the BB pathogen, DX-020, collected from fields of DRR-Hyderabad, was used to screen the gene-introgressed lines for resistance under both greenhouse and field conditions as per Kauffman et al. (1973). Top 5–10 leaves at maximum tillering stage (45–55 days after transplanting) were clip-inoculated with $10^9$ cfu/ml bacterial suspension. Disease reaction was scored on 14th day after inoculation on plant basis as resistant (average lesion length <4 cm), moderately resistant (4–6 cm), moderately susceptible (7–9 cm), or susceptible (>9 cm) as per International Rice Research Institute standard evaluation system (IRRI-SES) (Anonymous 2002).

## Results

### Inheritance of resistance and allelism

Of the 185 $F_2$ plants of the TN1/Aganni cross tested, 140 were resistant and 45 were susceptible (Table 2). The segregation data fitted with the ratio of 3 resistant (R):1 susceptible (S), as expected from the involvement of a single dominant gene controlling this resistance ($\chi^2 = 0.044$; $P = 0.83$). Of the 426 $F_{10}$ RILs from this cross, 210 lines were resistant, 202 were susceptible, and 14 were heterozygous. Thus, the $F_{10}$ families segregated in 1R:1S ratio ($\chi^2 = 0.83$; $P = 0.36$), confirming the involvement of a single dominant gene.

The $F_1$ plants derived from all the crosses of Aganni were resistant to GMB4. $F_2$ plants of the crosses with Kavya, Phalguna, ARC5984, Dukong #1, RP2333-

**Table 2** Segregation of GMB4 resistance in $F_2$ and $F_{10}$ populations of different crosses involving Aganni

| Cross combination | Generation | Plants tested | Number of plants | | Segregation ratio tested | $\chi^2$ | Probability (*P*) |
|---|---|---|---|---|---|---|---|
| | | | R plants | S plants | | | |
| TN1/Aganni | $F_2$ | 185 | 140 | 45 | 3R:1S | 0.044 | 0.83 |
| TN1/Aganni | $F_{10}$ | 426 | 210 | 202[a] | 1R:1S | 0.83 | 0.36 |
| Aganni/Kavya (*Gm1*) | $F_2$ | 99 | 70 | 29 | 3R:1S | 0.97 | 0.32 |
| Aganni/Phalguna (*Gm2*) | $F_2$ | 110 | 85 | 25 | 3R:1S | 0.30 | 0.58 |
| Aganni/RP2068-18-3-5 (*gm3*) | $F_2$ | 266 | 220 | 46 | 13R:3S | 0.37 | 0.54 |
| Aganni/Abhaya (*Gm4*) | $F_2$ | 179 | 169 | 10 | 15R:1S | 0.14 | 0.70 |
| Aganni/ARC 5984 (*Gm5*) | $F_2$ | 158 | 119 | 39 | 3R:1S | 0.008 | 0.92 |
| Aganni/Dukong (*Gm6*) | $F_2$ | 80 | 57 | 23 | 3R:1S | 0.6 | 0.43 |
| Aganni/RP2333-156-8 (*Gm7*) | $F_2$ | 133 | 103 | 30 | 3R:1S | 0.42 | 0.51 |
| Aganni/Jhitpiti (*Gm8*) | $F_2$ | 117 | 117 | 0 | – | – | – |
| Aganni/Madhuri L 9 (*Gm9*) | $F_2$ | 69 | 54 | 15 | 3R:1S | 0.39 | 0.53 |
| Aganni/BG 380-2 (*Gm10*) | $F_2$ | 180 | 134 | 46 | 3R:1S | 0.02 | 0.86 |
| Aganni/CR57-MR 1523 (*Gm11*) | $F_2$ | 98 | 89 | 9 | 15R:1S | 1.67 | 0.19 |

"R" and "S" indicate resistant and susceptible, respectively

[a] 14 RILs displayed intermediate (10–80 %) level of plant damage and were considered heterozygous

156-8, Madhuri L9, or BG 380-2 segregated in the ratio 3R:1S (Table 2). $F_2$ plants derived from the cross of Aganni with RP2068-18-3-5 segregated in the ratio 13R:3S, while $F_2$ plants of the crosses of Aganni with Abhaya or CR57-MR1523 segregated in the ratio 15R:1S. It should be noted that $F_2$ plants from the cross of Aganni with Jhitpiti did not show segregation for resistance; all $F_2$ plants were resistant. Thus, these results suggest that the gall midge resistance in Aganni is conferred by a single dominant gene, nonallelic to *Gm1*, *Gm2*, *gm3*, *Gm4*, *Gm5*, *Gm6*, *Gm7*, *Gm9*, *Gm10*, and *Gm11*, but allelic to *Gm8*.

Tagging and mapping of the *Gm8* gene in Aganni

Of the 432 SSR markers used for parental polymorphism survey, 82 showed polymorphism (data not shown). Of these, 52 markers located on all the chromosomes except chromosome 8 did not show any pattern of cosegregation with the trait phenotype in the selected R and S subset population. Three of the markers, RM22674, RM22685, and RM22709 on chromosome 8, displayed close linkage with the trait phenotype ($\chi^2 = 15.18$, $P = 0.0001$; $\chi^2 = 8.61$, $P = 0.003$; $\chi^2 = 5.76$, $P = 0.016$, respectively) in the R ($n = 27$) and S ($n = 27$) subset population. However, other 27 polymorphic markers on chromosome 8 did not show linkage with the trait.

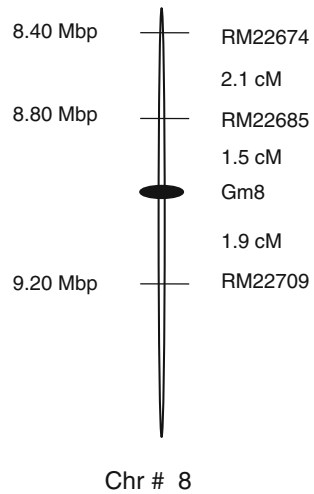


**Fig. 1** Linkage map of *Gm8* with the SSR markers RM22674, RM22685, and RM22709 on chromosome 8. Genetic distance between the markers and the gene in cM indicated on the *right*, and physical position in Mbp of the marker indicated on the *left*

Marker–trait cosegregation in the 412 $F_{10}$ RIL population indicated SSR markers RM22679, RM22685, and RM22709 to be located at genetic distances of 2.1, 1.5, and 1.9 cM, respectively; RM22685 and RM22709 were located on either side of the gene (Fig. 1).

Molecular allelism test

Table 1 shows the absence of *Gm1*, *Gm2*, *Gm4*, and *Gm11* gene-specific amplification in Aganni and Jhitpiti. Thus, the gene conferring resistance to GMB4 in Aganni is, most likely, nonallelic to the genes *Gm1*, *Gm2*, *Gm4*, and *Gm11* but allelic to *Gm8*. Tests with 20 selected gall midge-resistant genotypes showed presence of *Gm4*, *Gm8*, *Gm11* or *Gm4* + *Gm8* in three, seven, three, and two genotypes, respectively (Table 1). Five genotypes showed nonspecific banding for all the gene-specific markers. These are likely to carry other resistance genes.

Multiple resistance for gall midge and BB through gene pyramiding

The marker-assisted selection analysis indicated that $BC_2F_2$ plants 6, 7, 10, and 15 had *Gm8* and *Xa21* genes in homozygous state. $F_3$ progeny of these plants also showed resistance to BB isolate DX-020 and gall midge biotype GMB4 (Fig. 2; Table 3).

## Discussion

Host plant resistance is the ideal approach for pest management in rice. Prevalence of pest complex and rapid evolution of virulent strains and biotypes restrict the scope of cultivation of pest-resistant cultivars. Superimposed upon these are the farmers' choice of varieties, which may vary from region to region and rice ecologies. This is one main reason why, despite the over 950 rice varieties released for cultivation in India during the past six decades, fewer than a dozen varieties occupy 1 million ha or more. These so-called megavarieties are widely adaptable and easily managed by farmers, despite these being susceptible to one or more major pests. Hence, the change in breeding strategy is to improve the megavarieties by reinforcing them with multiple pest resistance. Availability of linked markers for resistance genes against major

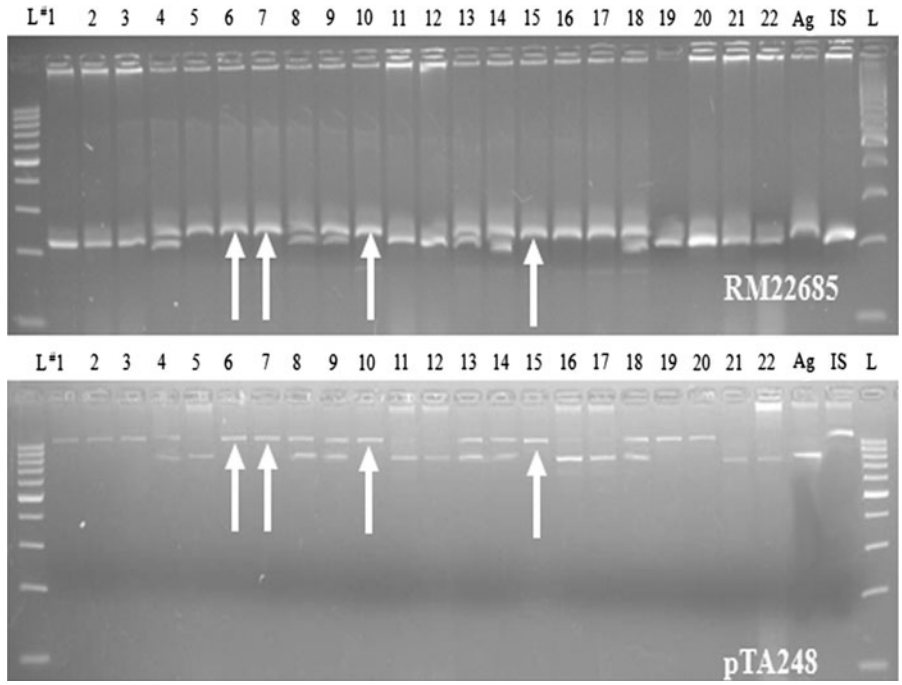


**Fig. 2** MAS of $BC_2F_2$ plants for presence of *Gm8* and *Xa21* genes in homozygous state using gene linked markers. Plants 6, 7, 10, and 15 were selected. *Top panel* for selection of *Gm8* gene using the marker RM22685; *lower panel* for selection of *Xa21* gene using the marker pTA248. Lane order: L, #1–22, Ag and IS indicate 100-bp molecular ladder, $BC_2F_2$ plants 1–22, Aganni and Improved Samba-Mahsuri, respectively

pests such as BB and blast pathogens, gall midge, and brown plant hopper has facilitated this approach. Two of the improved varieties developed through MAS (Improved Pusa Basmati-1 and Improved Samba-Mahsuri) have already been released for cultivation (Sundaram et al. 2008).

Breeding for gall midge resistance has been one of the most successful stories of modern crop improvement. The immune level of resistance conferred by major genes against this important pest makes use of insecticides against it redundant. However, rapid evolution of virulent biotypes against the resistant rice varieties carrying a single major gene during the 1980s and thereafter (Bentur et al. 1987) has called for a rethink of the breeding approach. So far, 11 resistance genes in the plant (Himabindu et al. 2010) and seven biotypes of the pest have been reported (Vijayalakshmi et al. 2006). Characterization of these genes in terms of resistance spectrum (Himabindu 2009) suggested combination of at least two genes to provide broad spectrum of resistance. Monitoring of virulence in pest populations over time (Bentur et al. 2008) revealed that selection pressure exerted by different genes on the pest population will not be uniform and the durability of the deployed resistance genes differs from each other. Initial studies on the nature of resistance conferred by different genes indicated the existence of two distinct mechanisms. Most of the genes confer resistance accompanied with HR (HR+ type), while resistance conferred by the two genes *Gm1* and *Gm8* is independent of HR (HR– type). Gene expression studies carried out recently suggest involvement of typical pest-induced phenyl propanoid-mediated resistance in the rice variety Suraksha carrying *Gm11* gene possessing HR+ resistance, while the genes of this pathway are not modulated in the variety Kavya possessing *Gm1* gene providing HR– type resistance (Rawat et al. 2010, 2012). Thus, it is imperative that informed choices be made in selecting genes for durable gall midge resistance.

*Gm8* gene identified in the landrace Jhitpiti (Jain et al. 2004) also confers HR– type resistance and, unlike *Gm1* gene, has a wider spectrum of resistance, covering five of the seven biotypes (Biradar et al. 2004). However, the landrace itself was unsuitable for breeding purposes, and the markers identified were not close enough to be used in MAS. Aganni as another source of *Gm8* is a short statured, profuse tillering, and medium flowering duration variety suitable for breeding. Our confirmation of the presence of *Gm8* and development of more closely linked markers provide a better alternative for breeders. Presence of this gene in yet another nine accessions of germplasm widen this choice. Molecular allelism test also revealed new

**Table 3** Phenotyping of $BC_2F_3$ lines of the cross Improved Samba-Mahsuri (IS)/Aganni against gall midge biotype GMB4 and BB isolate DX-020 in greenhouse

| Gall midge biotype GMB4 | | | BB isolate DX-020 | |
|---|---|---|---|---|
| Line[a] | Plant damage (%) | Rating | Average lesion length (cm) | Rating |
| 1 | 100 | S | 2.2 | R |
| 2 | 100 | S | 2.1 | R |
| 3 | 100 | S | 2.4 | R |
| 4 | 65 | S | 4.9 | MR |
| 5 | 0 | R | 10 | S |
| 6 | 0 | R | 2.2 | R |
| 7 | 0 | R | 1.9 | R |
| 8 | 55 | S | 6 | MR |
| 9 | 55 | S | 5.1 | MR |
| 10 | 0 | R | 2.4 | R |
| 11 | 100 | S | 11.8 | S |
| 12 | 100 | S | 10.7 | S |
| 13 | 75 | S | 6.5 | MS |
| 14 | 75 | S | 7.1 | MS |
| 15 | 0 | R | 2.3 | R |
| 16 | 0 | R | 13 | S |
| 17 | 0 | R | 10 | S |
| 18 | 40 | S | 7.5 | MS |
| 19 | 90 | S | 3 | R |
| 20 | 90 | S | 2.7 | R |
| 21 | 100 | S | 8.8 | MS |
| 22 | 100 | S | 8.8 | MS |
| IS | 100 | S | 2.1 | R |
| Aganni | 0 | R | 13 | S |

[a] The number of total plants was 18–20 per line

sources of *Gm4*, *Gm11* gene and some with combination of *Gm4* and *Gm8* genes. With linked markers for these genes, pyramiding is only a step away. Combination of *Gm4* and *Gm8* is ideal, since the former provides HR+ type resistance while the latter gives HR– type. Both genes are undeployed, and hence the pest population will not be widely exposed to these genes. Another candidate for effective pyramiding is *gm3* reported from the breeding line RP2068-18-3-5. Linked markers are being developed (Sama 2011), and after fine-mapping, insights can be gained about the functional aspects of the recessive resistance gene.

Our proof of concept of pyramiding BB resistance gene *Xa21* with *Gm8* for multiple resistance is the first such attempt, though combination of BB and blast resistance genes in the same elite background has also been done (Ratnamadhavi et al. 2010). Our goal is to combine BB, blast, and gall midge resistance in several popular elite megavarieties. Sundaram et al. (2008) developed a high-yielding, fine-grain-type rice variety possessing BB resistance (named Improved Samba-Mahsuri) through MAS, and the variety is being cultivated in many areas across India by rice farmers. Some of these areas are also infested by gall midge, and it was desired that the variety be introgressed with a major gall midge resistance gene. Towards this effort in the present study, we introgressed *Gm8* into the genetic background of Improved Samba-Mahsuri through marker-assisted backcross breeding using the closely linked SSR markers flanking *Gm8* developed in this study and the sequence-tagged site (STS) marker pTA248 (Ronald et al. 1992), which is closely linked to the BB resistance gene *Xa21*. Lines at $BC_2F_2$ generation possessing both *Gm8* and *Xa21* in homozygous condition have been identified, and their resistance

against gall midge and BB has been confirmed phenotypically under greenhouse conditions in their progeny. Some of the promising multiple pest-resistant lines are being advanced for multilocation testing for yield, quality, and biotic stress tolerance.

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