

Original Research Article

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Markers Assisted Selection for Pyramiding of Gallmidge Resistance Genes in Kavya, a Popular Rice Variety

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ABSTRACT

Kavya, a high yielding, gall midge resistant (*Gm1*), Medium Slender (MS) grain type rice variety with 135 days of duration, but is susceptible to biotype 4M prevalent in Warangal condition (Telangana State, India). Considering the susceptibility of Kavya to gallmidge resistance especially for biotype GMB4M, we crossed Kavya with a gallmidge resistant introgression line of Samba Mahsuri (RP-4516-3-6) possessing the two gallmidge resistant genes i.e. *gm3* and *Gm8*. The true F_1 s were selfed and the F_2 plants were subjected to marker-assisted selection (MAS) for *gm3* and *Gm8* genes by using functional/gene linked markers. The 'double' positive F_2 plants were further screened with *Gm1* gene linked marker, since the recurrent itself possessing *Gm1* gene. The 'triple' positive plants (i.e., *Gm1*, *gm3* and *Gm8*) were selfed and their progeny were subjected to MAS for *Gm1*, *gm3* and *Gm8* genes coupled with phenotype based visual selection for agro-morphological characters. At F_5 generation, one improved line (i.e., WGL-1068) possessing gallmidge resistance (*Gm1*, *gm3* and *Gm8* genes), higher yield than Kavya and fine-grain type was identified. The best improved line of Kavya i.e. WGL-1068, after further evaluation for 2-3 seasons will be nominated for multi-location trials under All India Coordinated Rice Improvement Project (AICRIP) for their release to the farming community.

Keywords

Rice, Gall midge resistance, Gene pyramiding, Marker-assisted selection

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Introduction

Rice (*Oryza sativa* L.) is one of the world's most important staple food crops and is a primary source of carbohydrate for more than half of the world's population.

Worldwide, rice is cultivated in 163 million hectares with an annual production of 741 million tonnes (FAO, 2015). India stands first in area with 43.4 million hectares and second

in rice production with 157.2 million tonnes, constituting up to 21% of global rice production (FAO, 2015). However, stability in the rice production could not be sustained as high yielding varieties became susceptible to a wide variety of pests and diseases. Approximately 52% of global rice production is lost annually owing to the damage caused by biotic stress factors, of which 25% is attributed to the attack by insect pests (Yarasi, *et al.*, 2008).

Major insect pests of rice that cause huge economic losses in South Asia are stem borer, brown plant hopper (BPH) and gall midge (GM). Of these, GM alone is responsible for a worldwide damage of more than US\$ 700 million annually (Herdt, 1991). Two species of the rice GM have been identified so far, the Asian rice GM, *Orseolia oryzae* Wood-Mason and the African rice GM, *O. oryzivora*. Both species belong to the family Cecidomyiidae of the order Diptera. The Asian rice GM is a serious pest of rice in South and Southeast Asia. In India, the pest is widely distributed and is considered as a significant constraint to rice production (Bentur *et al.*, 2003). GM incidence has been reported from almost all rice growing states except western Uttar Pradesh, Uttaranchal, Punjab, Haryana and the hilly states of Himachal Pradesh and Jammu and Kashmir.

The insect takes about 2-3 weeks for completion of its life cycle and the young larvae cause maximum damage. The presence of an active first instar larva at the meristem stimulates the formation of a gall and suppresses the development of the growth cone due to which the growing tips are deformed in the shape of a gall and turns into 'onion tip' or 'silver shoot'. The affected tillers do not bear panicle, thus causing significant yield losses.

Chemical control is inefficient due to internal feeding habit of the pest and the prevailing hydrological and edaphological conditions during the wet season. Use of resistant varieties has been the most feasible alternative to manage the pest and several sources of resistance are available in cultivated rice (Bentur *et al.*, 2003). However, extensive cultivation of varieties containing single resistance gene has resulted in frequent breakdown of resistance due to emergence of new virulent biotypes of the insect across many locations in India. While there is a long

term need to deploy new breeding strategies to develop superior rice genotypes having durable resistance to GM across different biotypes, it is also necessary to incorporate specific resistance genes against specific biotype for the most suitable and popular varieties of a particular region.

So far 11 GM resistance genes (*Gm1*, *Gm2*, *Gm3*, *Gm4*, *Gm5*, *Gm6*, *Gm7*, *Gm8*, *Gm9*, *Gm10* and *Gm11*) and seven biotypes (GMB1 through GMB6 and GMB4M) of GM have been identified (Vijayalakshmi *et al.*, 2006 and Himabindu *et al.*, 2010). Of these, eight genes *viz.*, *Gm1*, *Gm2*, *Gm4*, *Gm5*, *Gm6*, *Gm7*, *Gm8* and *Gm11* have linked markers, and seven of these with the exception of *Gm5* have been mapped onto different rice chromosomes (Yasala *et al.*, 2012).

Out of eight genes, '*Gm4*' confer resistance against GM biotypes 1,2,3,4 and 4M (Vijayalakshmi *et al.*, 2006 and Dutta *et al.*, 2014). With the availability of gene linked molecular markers or functional markers, it is possible to track gall midge resistance genes precisely in each segregating generation. In the states of Andhra Pradesh and Telangana, GM incidence is mainly in the Telangana and coastal regions of Andhra Pradesh. At Warangal, Ragolu and Jagtial regions biotypes GMB4M, GMB4 and GMB3 have been reported to occur. It has been reported that the resistance genes, '*gm3*', '*Gm4*' and '*Gm8*' confer resistance against GM biotypes 1,2,3,4 and 4M (Vijayalakshmi *et al.*, 2006, Bentur *et al.*, 2009 and Dutta *et al.*, 2014).

Considering the susceptibility of Kavaya to gallmidge resistance especially for biotype 4M, we have through the present study improved Kavaya for gallmidge resistance through marker-assisted selection coupled with pedigree based breeding strategy and phenotype based selection for agro-morphological traits.

Materials and Methods

Plant Material

An introgression line of Samba Mahsuri (i.e. RP-4516-3-6) possessing *gm3* and *Gm8* genes in homozygous condition was used as the donor parent for gall midge resistance genes. Kavya, a high yielding, gall midge resistant (*Gm1*), Medium Slender (MS) grain type rice variety with 135 days of duration released in the year 1991 and is derived from the cross between WGL-27120/WGL17672 and Mayuri and Surekha was used as a recurrent parent. In addition to these, Taichung Native 1 (TN1) was used as a susceptible check while screening the improved lines for gall midge resistance.

Crossing scheme

RP-4516-3-6 was used as the male parent and crossed with Kavya. The F₁s were screened with PCR based molecular markers linked to the target genes for selection of plants possessing the resistance allele of *Gm1*, *gm3* and *Gm8* genes in heterozygous condition. The selected F₁ plants were selfed to generate F₂, which were then screened with the gene linked markers to identify the plants which are homozygous for *Gm1*, *gm3* and *Gm8* genes. The homozygous F₂ plants were then selfed to generate F₃, F₄ and F₅ generations and at each generation the improved lines were selected based on high gall midge resistance, fine-grain type (i.e. medium-slender grain type) and yield through phenotype based selection coupled with marker assisted selection for gall midge resistance.

Screening for gall midge resistance

For Phenotypic screening of Gallmidge resistance, advanced lines along with parents and susceptible check (TN1) were raised under field conditions. All the recommended

agronomic practices for rice cultivation were followed except application of any insecticide throughout the crop growth during Kharif, 2016. Symptoms on plants were scored on 30 and 50 days after transplanting based on percent of silver shoot damage. Test entries with nil damage and up to 5% silver shoot damage were considered as resistant while others were grouped as susceptible (Vijaya Lakshmi *et al.*, 2006). Scoring was done as per Standard Evaluation System (SES) (IRRI, 1988).

Marker assisted selection for Gall midge resistance

DNA was isolated from the parents and progenies by following the protocol of Zheng, *et al.*, (1995). The PCR based markers RM219, *gm3del3* and RM22685 were used to confirm the presence of the resistant allele of *Gm1*, *gm3* and *Gm8* genes in the F₁ generation and subsequent generations.

The PCR mixture contained 50 ng template DNA, 5 pmoles of each primer, 0.05 mM dNTPs, 1x PCR buffer (10 mM Tris, pH 8.4, 50 mM KCl, 1.8 mM MgCl₂ and 0.01 mg/ml gelatin) and 1 unit of Taq DNA polymerase (Fermentas, Lithuania) in a reaction volume of 10 μ l. Template DNA was initially denatured at 94 °C for 5 min followed by 35 cycles of PCR amplification with the following parameters: a 30-s denaturation at 94°C, a 30-s annealing at 55°C and 1 min of primer extension at 72°C.

A final extension was done at 72°C for 7 min. The PCR amplified products of *gm3del3* was electrophoretically resolved on a 1.5 % Seakem LE® agarose gel (Lonza, USA), while the amplicons of RM219 and RM22685 were resolved on a 3.5 % Seakem LE® Agarose gels containing 0.5 mg/ml of ethidium bromide in 0.5x TBE buffer and visualized under UV.

Evaluation of agro morphological characters

Thirty-day-old seedlings of the selected improved lines along with parents were transplanted in the main field at a spacing of 20 x 15 cm. Standard agronomic practices were followed to raise a healthy crop, which were evaluated during the wet season (July–November) in 2016. Data was recorded for the agronomic traits, *viz.*, days to 50% flowering (DFF), mean plant height (cm), number of grains per panicle, number of productive panicles per plant, panicle length (cm), grain yield per plant (gm), 1000 seed weight (g) and grain type.

In order to assess the effect of gallmidge on yield, the improved lines along with Kavya were grown following a standard package of practices, in 10 m² plots in the experimental farm of the Regional Agricultural Research Station during the wet season (*Khariif*) of 2016 at a spacing of 20 x 15 cm. The experiment was conducted in three replications.

Results and Discussion

Marker-assisted selection for gall midge resistance

The F₁s generated from the cross, RP-4516-3-6/Kavya were screened using the *Gm1*, *gm3* and *Gm8* gene linked markers *viz.*, RM219, gm3del3 and RM22685 respectively to identify 'true'F₁s showing heterozygous amplification pattern. A total of 12 'positive' F₁s were identified and these positive F₁s were selfed to generate F₂ plants. Out of 696 F₂ plants, a total of 378 were identified to be positive for *Gm1*, a total of 362 were identified to be positive for *gm3*, 293 were positive for *Gm8* and 36 were identified to be triple positives for *Gm1*, *gm3* and *Gm8* genes in homozygous condition by using the functional/gene-linked markers. These were

then advanced from F₂ to F₅ generations by following pedigree based method. At F₅ generation we identified one improved line namely WGL-1068 (Figure 1) displayed high level gall midge resistance (Table 1) on par with donor parent and high yield (Table 2) as compared to the original recurrent parent. The above said gene pyramided line was showing durable resistance to gallmidge against many biotypes prevalent Warangal conditions of Telangana State, India.

Phenotypic screening of improved lines for gallmidge resistance

In the present study, field level screening was employed for phenotypic evaluation of the advanced lines for GM resistance at RARS, Warangal. Field screening of the lines for GM incidence at this location was appropriate as Warangal is one among the hot spot locations of India for gall midge incidence. Towards the same, TN1 was used as susceptible check, WGL-1068 along with recurrent parents Kavya and donor parent RP-4516-3-6 were screened for GM reaction during *Khariif*, 2016. As expected the donor genotype (RP-4516-3-6) showed high resistance (score '0') (Table 1) whereas recipient genotype (Kavya) showed resistance (score '7') (Table 1) while the check (TN1) showed high susceptibility (score '9') (Table 1). The improved line (namely WGL-1068) displayed high resistance with (score '0') (Table 1).

Yield and agronomic performance of improved parental lines

The improved lines of Kavya along with parents were evaluated for grain yield during the wet season 2016 as explained in materials and methods. The recipient parent, Kavya recorded an overall mean grain yield of 5312 kg/ha, donor parent recorded 4803 kg/ha, while the improved line of kavya (WGL-1068) exhibited grain yields on par with Kavya

(6106 kg/ha) with marginal differences (Table 2). However no variation was observed in terms of number of grains per panicle, number of productive panicles per plant, panicle length (cm), grain yield per plant (gm), 1000 seed weight (g) when compared to Kavya (Table 2).

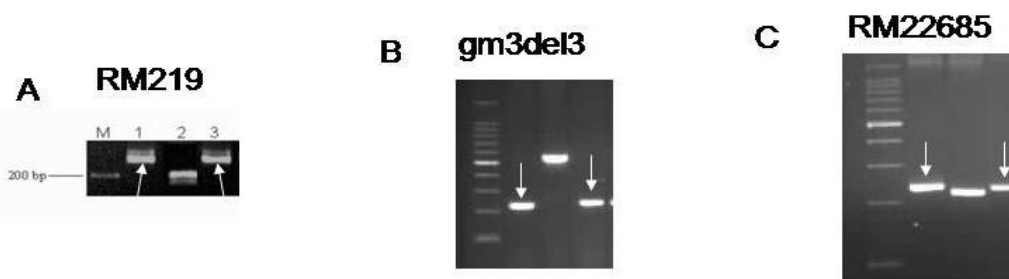
The effect of gallmidge on yield of Kavya and improved lines was assessed as described in Materials and Methods. No significant difference was observed in the yield between Kavya and improved lines under gallmidge free conditions. However, under conditions of gallmidge infection, there was a 24 % reduction in yield of Kavya while yield reduction was negligible in the improved Kavya lines.

The present study was carried out with the objective to improve the gallmidge resistance of Kavya especially for biotype 4M through marker-assisted pedigree based selection coupled with phenotypic selection. In order to achieve these objectives, an introgression line

of Samba Mahsuri (RP-4516-3-6) which possesses high gallmidge resistance (due to presence of GM resistance genes, *gm3* and *Gm8*) was used as a donor parent.

Gene pyramiding is a very useful approach to maximize utilization of existing gene resources. Genes leading to different races or biotypes being resistant to a disease or insect pest can be effectively pyramided using tightly linked molecular markers to develop lines with multi-race or multi-biotype resistances (Sundaram, *et al.*, 2008; Rajapurohit, *et al.*, 2010; Divya, *et al.*, 2015 and Pradhan, *et al.*, 2015). These multi-race or multi-biotype resistant genotypes proved to be more durable than any single-race or single-biotype resistance. Several researchers have reported the successful application of MAS in rice for targeted improvement of varieties and hybrid rice parental lines (Sundaram *et al.*, 2009; Basavaraj *et al.*, 2010; Rajpurohit *et al.*, 2010; Hari *et al.*, 2011; 2013; Khanna *et al.*, 2015; Ellur *et al.*, 2015; Pradhan *et al.*, 2015 and Bhaskar *et al.*, 2015).

Figure 1: Foreground selection of improved line of Kavya along with donor and recurrent parents by using gene linked/Functional markers for gall midge resistance



A-Foreground selection of improved line of Kavya for *Gm1* gene, by using PCR based gene linked marker RM219. M - Molecular weight marker (100 bp ladder), the lane numbers indicates 1-Kavya (donor parent), 2-TN1 (negative check) and 3-Improved Kavya line i.e. WGL-1068, respectively. Arrow indicates plants positive for *Gm1*.

B-Foreground selection of improved line of Kavya for *gm3* gene, by using PCR based Functional marker *gm3del3*. M-Molecular weight marker (100 bp ladder), the lane numbers 1-RP-4516-3-6 (donor parent), 2-TN1 (negative check) and 3- Improved kavya line i.e. WGL-1068, respectively. Arrow indicates plants positive for *gm3*.

C-Foreground selection of improved lines of Kavya for *Gm8* gene, by using PCR based gene linked marker RM22685. M - Molecular weight marker (100 bp ladder), the lane numbers indicates 1-RP-4516-3-6 (donor parent), 2-TN1 (negative check) and 3-Improved kavya lines i.e. WGL-1068, respectively. Arrow indicates plants positive for *Gm8*.

Table.1 Phenotypic screening of F₅ lines for gall midge resistance during *Kharif*, 2016 at RARS, Warangal

Date of sowing: 15-07-2016

Date of planting: 10-08-2016

S. No.	Entry No.	30 DAT		50 DAT	
		% Damage on Hill basis	% Galls on tiller basis	% Damage on Hill basis	% Galls on tiller basis
1	WGL-1068 (Improved kavya line)	0	0.0	0	0.0
2	RP-4516-3 (donor parent)	0	0.00	0	0.00
3	Kavya (susceptible parent)	50	6.99	70	15.27
4	TN-1 (Control)	100	23.00	100	22.80

Table.2 Yield and agro-morphological characters of improved lines of Kavya along with parents during *Kharif*, 2016

Design: RBD Plot size: Gross: 12.00 m² Net: 10.83 m² Date of sowing: 15.07. 2016
 Replications: 4 Spacing: 20 X 15 cm Date of planting: 10.08.2016
 Entries: 7 (5+2) Fertilizer: NPK 120:60:40 kg/ha

Entry#	Grain yield (kg/ha)	Days to 50% flowering	Ear bearing tillers/m ²	Plant height (cm)	Panicle length (cm)	No. of grains / panicle	1000 grain wt (g)	Silver shoots/ m ²	White ears/ m ²	Grain type
WGL -1068	6106	91	391	113.1	24.8	221	22.9	20	4	LS
Kavya	5312	104	344	100.4	24.1	220	20.5	7	5	MS
RP-4516-3	4803	98	321	115.6	22.4	174	18.2	3	1	MS
NDR 359 ©	5413	107	367	142.9	29.1	158	29.4	23	0	LB
WGL-32100 ©	5315	107	374	114.3	23.5	242	14.8	14	2	MS

The linked markers i.e. RM219, gm3del3 and RM22685 were used for the screening for presence of four gallmidge resistance genes i.e. *Gm1*, *gm3* and *Gm8*, respectively. Divya, *et al.*, (2015) have confirmed IF₂ (Inter crossed F₂'s) involving donors (Kavya and Abhaya) and recipient parent (B277-SM2113) for presence of *Gm4* gene using functional marker LRR-del, *xa13* gene using functional marker *xa13* promoter and *Xa21* gene with tightly linked pTA248. Hence, the markers used for the foreground analysis in the present study were appropriate to confirm the presence of the resistance genes. We also

followed the similar strategy, involving only foreground selection for presence of target traits followed by selfing and pedigree-based selection up to F₅ generation, following phenotype based selection for agronomic traits, grain-type and plant stature. This enabled us to introgress gallmidge resistance from the donor parent.

Phenotypic screening along with genotyping is very important for success of MAS. There are several instances when phenotypic screening can be strategically combined with MAS. In the first instance, 'combined MAS'

coined by Moreau *et al.*, (2004) may have advantages over phenotypic screening or MAS alone in order to maximize genetic gain (Lande and Thompson 1990). Simulation studies indicate that this approach is more efficient than phenotypic screening alone, especially when large population sizes are used and trait heritability is low (Hospital, *et al.*, 1997). Bohn, *et al.*, (2001) investigated the prospect of MAS for improving insect resistance in tropical maize and found that MAS alone was less efficient than conventional phenotypic selection. However, there was a slight increase in relative efficiency when MAS and phenotypic screening were combined. Selection of superior plants carrying the desired resistance genes along with good agronomic traits is very important for the success of MAS.

In the present study, the developed advanced generation lines were screened for the presence of genes conferring the resistance to the target traits *i.e.*, GM resistance during *Kharif*, 2016. Further to identify superior lines, agro-morphological traits were also recorded for these lines. The level of gallmidge resistance in the donor genotype (RP-4516-3-6) showed high resistance (score '0') whereas recipient genotype (Kavya) showed resistance (score '7') while the check (TN1) showed high susceptibility (score '9'). The three improved line (namely WGL-1068) displayed high resistance with (score '0'), which were scored under IRRI-SES scale as highly resistant. Though, in the majority of the studies, advanced lines had been screened in the glass house for resistance against this pest (Nanda *et al.*, 2010; Sama *et al.*, 2014 and Divya *et al.*, 2015), there are reports where in the lines had been screened in field (Mohan *et al.*, 1997; Vijayalakshmi *et al.*, 2006) against gall midge.

In the improved line of Kavya, we did not notice any apparent yield penalty associated

with presence of the resistance genes, *Gm1*, *gm3* and *Gm8*. interestingly, under conditions of gallmidge infection in the field, a significant yield reduction (~ 24 %) was observed in Kavya, while the yield reduction was insignificant with respect to improved Kavya line. This indicates that cultivation of the gallmidge resistant, improved line would be of great advantage in gallmidge endemic areas.

The best improved line of Kavya *i.e.* WGL-1068, after further evaluation for 2-3 seasons will be nominated for multi-location trials under All India Coordinated Rice Improvement Project (AICRIP) for their release to the farming community.

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