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Molecular Confirmation for Gall Midge (Biotype 3) Resistance in Phenotypically Resistant Rice Genotypes Using Functional Markers

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Authors' contributions

This work was carried out in collaboration among all authors. Authors AK, JS and SP designed the study and wrote the protocol. Authors YH, BE and RSK prepared the manuscript, formatted it according to the journal's requirements, managed the literature searches and wrote the manuscript. Authors BS, SO and CNR supported the field evaluation for gall midge, contributed to manuscript corrections. All authors read and approved the final manuscript.

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ABSTRACT

This study performed molecular screening of rice genotypes, which showed no gall infestation in field conditions at the Regional Agricultural Research Station (RARS), Jagtial, during *rabi*, 2021-22. using functional markers such as “gm3del3” for the *gm3* gene, “LRR del” for the *Gm4* gene, and “PRP” for the *Gm8* gene, six genotypes were analyzed. WGL-1145 contained all three resistance genes (*gm3*, *Gm4*, and *Gm8*). Additionally, three genotypes namely WGL-1147, WGL-1127, and Kakai had the *Gm4* and *Gm8* genes, while RP-5332-54-11-8-2-13 had only *Gm8* and IR72476-B-P-9-3-1-1 possessed only *gm3*. These findings contribute to developing new rice varieties with resistance to gall midge, which can improve crop yields and food security in affected regions. Some promising rice varieties may be used as donors in breeding programs aimed at creating pyramided lines with durable gall midge resistance.

Keywords: Rice; Gall midge resistance; functional markers.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is a staple for approximately 40% of the global population, cultivated over 164 million hectares with an annual production of 756.7 million tonnes FAOSTAT. (2022). Asia dominates production and consumption, with India leading in area (45 million hectares) and ranking second in production (178.3 million tonnes), contributing 23.5% of the global rice supply FAOSTAT. (2022). Despite significant yields, rice cultivation faces challenges due to pest and disease susceptibility, which hampers productivity. Biotic stress factors account for nearly half of global rice production losses, with insect pests responsible for approximately 25% of these damages (Yarasi et al., 2008). Among the major pests, the stem borer, brown plant hopper, and gall midge are particularly destructive. The gall midge alone causes global damage exceeding US$700 million annually (Herdt 1991).

The rice gall midge comprises two species: the Asian rice gall midge (*Orseolia oryzae*) and the African rice gall midge (*Orseolia oryzivora*). The Asian rice gall midge is particularly problematic, inflicting annual yield losses valued at over US$700 million (Herdt 1991, Venkanna et al., 2018, Hari et al., 2022). In India, this pest contributes to an average annual yield loss of about 4.77 lakh tonnes, equivalent to 0.8% of total production and worth approximately US$80 million (Bentur et al., 2003). The Asian rice gall midge affects various South and Southeast Asian countries, including Bangladesh, China, India, Indonesia, Myanmar, Sri Lanka, and Vietnam, and is ranked as the third most significant pest in India, following the stem borer and plant hopper (Bentur et al., 1992).

Chemical control methods for gall midge are largely ineffective and environmentally damaging. Thus, cultivating resistant rice varieties offers a more sustainable solution (Hari et al., 2022). While several gall midge resistant varieties have been developed and released in India, the widespread use of varieties with single resistance genes has led to the emergence of new virulent biotypes and resistance breakdown (Bentur et al., 2003). To address this issue, the pyramiding of multiple resistance genes not previously deployed and mechanistically diverse has been proposed (Cohen et al., 2004). To date, eleven major resistance genes (*Gm1-Gm11*) have been identified with nine of these mapped to rice chromosomes (Yasala et al, 2012). Among these, four genes- *Gm2*, *gm3*, *Gm4,* and *Gm8* have been functionally validated (Khush 1997, Sama et al., 2014, Divya et al., 2018). Marker-assisted breeding has facilitated the introgression of *gm3*, *Gm4,* and *Gm8* genes into elite rice varieties (Balachiranjeevi et al., 2015, Venkanna et al., 2018, Hari et al., 2022).

2. MATERIALS AND METHODS

Molecular analysis was conducted on six rice genotypes that exhibited 'nil' gall infestation, as detailed in Table 1. DNA was isolated from these six genotypes grown under field conditions at the Regional Agricultural Research Station, Jagtial, during the *rabi,* 2021-22 following the protocol described by (Dodiya et al., 2024). To determine the allelic status of the *gm3, Gm4,* and *Gm8* genes, functional markers were employed as listed in Table 2. The PCR-based analysis was performed with 1 U of Taq DNA polymerase (Fermentas, Lithuania), 1X PCR buffer (Genei, India), and a 10-µl reaction volume, using a thermal profile of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes. Gel

Table 1. Genotypes used for molecular screening and their phenotypic reaction for Gall midge resistance

S. No.	Name of the Genotype	Silver Shoot (SS) %		Reaction
		30 DAT	50 DAT	
1.	IR72476-B-P-9-3-1-1	2.14	0.65	R
2.	RP-5332-54-11-8-2-13	1.44	0.52	R
3.	Kakai	0.00	0.00	HR
4.	WGL-1145	0.00	0.00	HR
5.	WGL-1147	0.00	0.00	HR
6.	WGL-1127	0.00	0.00	HR
7.	Agani (Resistant Check)	0.00	0.00	HR
8.	TN 1 (Susceptible Check)	9.25	79.09	HS

HR-Highly resistant, R-Resistant, HS- Highly susceptible, DAT-days after transplanting IRRI (2013)

Table 2. Details of functional markers used for molecular analysis

S. No.	Name of the Gene	Name of the Marker	Sequence of Marker	Resistant allele (bp)	Susceptible allele (bp)	References
1	*gm3*	gm3del3	F-5'CTGCCAGAGATGGGCCTTCCA3' R-5'CGTACAAATTCCTGTACCACTC3'	250	550	Sama et al., (2024)
2	*Gm4*	LRR-del	F-5'GTGGATCGAGAGAAGACAAG3' R-5'CTTGAGGACGATATTCAAGC3'	350	600	Divya et al., (2015)
3	*Gm8*	PRP	F-5'TCATGTTGTGCAGATCAACC3' R-5'AGCCATATGAAAACCACCAA3'	300	350	Divya et al., (2013)

electrophoresis was used to resolve amplified products, with *gm3* and *Gm4* genes analyzed on a 1.2% Seakem LE® agarose gel (Lonza, USA), while the amplified products of *Gm8* gene was analyzed on a 3.5% Seakem LE® agarose gel containing 0.5 mg/ml of ethidium bromide in 0.5X TBE buffer, visualized under UV light.

3. RESULTS AND DISCUSSION

Molecular screening was conducted on six rice genotypes that exhibited 'nil' gall infestation in field screenings. For control of gall midge, development of resistant rice varieties using marker assisted selection can be a sustainable and cost-effective approach (Dutta et al., 2014, Krishnakumar and Kumaravadivel 2018, Thippeswamy et al., 2014, Subburaj et al., 2023). Gene pyramiding with two or additional active genes in a single variety may lead to strong gall midge resistance rice varieties. Nowadays, the use of molecular markers for improvement of gene pyramids in desired combination is being monitored in different rice cultivars and presently using DNA markers for selection of resistant plants for gene pyramiding has been accepted as an established tool (Sundaram et al., 2008, Dutta et al., 2014). In the present investigation, among the molecular markers, we used functional markers, because they are developed from functional gene motifs and therefore, have complete linkage to the desired allele (Andersen and Lübberstedt 2003). Due to the complete linkage of an functional markers with the target gene and the absence of recombination between the marker and the gene, the loss of information and the false selection in Marker Assisted Breeding can be prevented (Ingvardsen et al., 2008). Functional markers can reduce linkage drag, particularly in foreground selection by genotyping a smaller population size (Bagge and Lübberstedt 2008).

Functional Marker gm3del3 for *gm3* Gene: The gm3del3 functional marker was utilized to detect the presence of the *gm3* gene, showing different amplification patterns in resistant and susceptible sources. The gm3del3 marker was designed based on sequence polymorphisms in the NB-ARC gene, which is a candidate gene for resistance (Sama et al., 2014). This functional marker showed an amplicon size of 550 bp for the susceptible allele and 250 bp for the resistant allele (Sama et al., 2014). Among the six genotypes screened, two genotypes namely IR72476-B-P-9-3-1-1 and WGL-1145 were observed to be positives for the *gm3* gene (Fig. 1). These findings are consistent with the results reported by (Dutta et al., 2014, Sama et al., 2014). Recent validation through RT-PCR studies (Divya et al., 2015) has confirmed the efficacy of this marker. Additionally, employed the gm3del3 marker to screen gene pyramided lines containing *Gm1, gm3* and *Gm8* for the presence of the *gm3* gene (Venkanna et al., 2018).

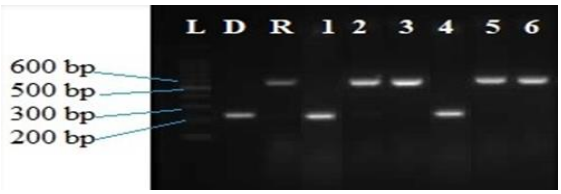

Fig. 1. PCR Amplification pattern of six rice genotypes with *gm3del3* functional marker, screening for presence of *gm3* gene

Fig. 1 Legends: L- 100 bp ladder, D- RP2068 (donor parent for g*m3* gene), R-TN1 (susceptible parent for *gm3* gene). The lane numbers 1-6 written on top of the gel indicates list of genotypes used for molecular screening given in Table 1.

Functional marker LRR del for *Gm4* Gene: The LRR del functional marker was used to detect the presence of the *Gm4* gene, exhibiting distinct amplification patterns in resistant and susceptible sources. Specifically, this marker amplified a band size of 350 bp in the *Gm4* resistant source Abhaya and 600 bp in the susceptible source TN1 (Venkanna et al., 2018). Out of six genotypes screened, four genotypes namely Kakai, WGL-1145, WGL-1147 and WGL-1127 were observed to be positive for the *Gm4* gene (Fig. 2). These results are consistent with the findings of (Divya et al., 2014, Dutta et al., 2014). Earlier, the LRR-del marker was employed by (Divya et al., 2018) to screen intercrossed F_4 (ICF_4) lines carrying *Gm4, Gm8* and *Xa21* genes and by (Kalpana 2015) to screen backcross derived lines for the presence of *Gm4*.

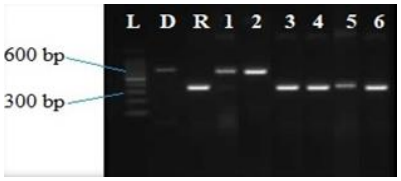

Fig. 2. PCR Amplification pattern of six rice genotypes with *LRR-Del* functional marker, screening for presence of G*m4* gene

Fig. 2 Legends: L- 100 bp ladder, D- TN1 (susceptible parent), R-Abhaya (donor parent for *Gm4* gene). The lane numbers 1-6 written on top of the gel indicates list of genotypes used for molecular screening given in Table 1.

PRP-del functional marker for *Gm8* Gene: The PRP marker, which encodes a Proline Rich Protein (Divya et al., 2013), was used to confirm the presence of the *Gm8* resistance gene in these test entries. PRP marker showed distinct amplification patterns in resistant and susceptible sources. Specifically, this marker amplified a band size of 300 bp in the *Gm8* resistant source Aganni and 350 bp in the susceptible source TN1. Out of six genotypes, five genotypes namely RP-5332-54-11-8-2-13, Kakai, WGL-1145, WGL-1147 and WGL-1127 were observed to be positive for the *Gm8* gene (Fig. 3). These findings are consistent with those reported by (Divya et al., 2013, Dutta et al., 2014).

Recent validation through RT-PCR studies has confirmed the functionality of this gene (Divya et al., 2015). Additionally, the PRP marker has been used by (Kumar et al., 2017) to screen intercrossed F_4 (ICF_4) lines carrying *Gm4, Gm8* and *Xa21* genes for the presence of *Gm8*, by (Kalpana 2015) to screen backcross-derived lines with *Gm4, Gm8*, and *Xa21* genes for *Gm8*, and by Venkanna et al. (2018) to screen gene pyramided lines with *Gm1, gm3*, and *Gm8* for the presence of *Gm8*.

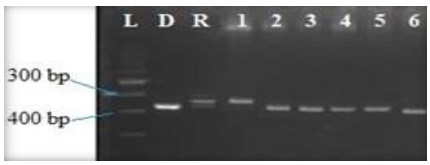

Fig. 3. PCR Amplification pattern of six rice genotypes with *PRP-del functional* marker, screening for presence of *Gm8* gene

Fig. 3 Legends: L-100 bp ladder, D- Aganni (donor parent) R- TN1 (susceptible parent for *Gm8* gene). The lane numbers 1-6 written on top of the gel indicates list of genotypes used for molecular screening given in Table 1.

Earlier, our published results (Hari et al., 2022) indicated that WGL-1145 and WGL-1147 possessed single Gall midge resistance gene *Gm4*. However, further phenotypic and genotypic screening of these two rice genotypes indicated that, WGL-1145 in addition *Gm4* it also possesses other two Gall midge resistance genes i.e. *gm3* and *Gm8*, while WGL-1147 was found to possess other Gall midge resistance gene i.e. *Gm8*.

Based on the current study it can be concluded that, Out of six rice genotypes, WGL-1145 possessed all three resistance genes (i.e. *gm3, Gm4* and *Gm8*) and three genotypes namely WGL-1147, WGL-1127 and Kakai possessing *Gm4* and *Gm8* genes, while RP-5332-54-11-8-2-13 possessed only *Gm8* gene and IR72476-B-P-9-3-1-1 possessed only *gm3* gene. These results will aid in the creation of new rice varieties resistant to gall midge, enhancing crop yields and food security in impacted areas. Some of the promising rice cultures may be utilized as donors in conventional breeding programs for development of pyramided lines with durable gall midge resistance.

4. CONCLUSION

The study investigates the molecular screening of rice genotypes resistant to gall midge infestations, conducted at the Regional Agricultural Research Station (RARS) during the rabi, 2021-22. Using functional markers for the *gm3*, *Gm4,* and *Gm8* resistance genes, six phenotypically resistant genotypes were analyzed. Notably, WGL-1145 was found to possess all three resistance genes, while others showed varying combinations of the genes. The findings underscore the potential for developing new rice varieties with enhanced gall midge resistance, contributing to improved crop yields and food security. The research emphasizes the role of marker-assisted breeding in creating durable resistance through gene pyramiding.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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