Original Research Article

Molecular ~~confirmation~~ verification of gall midge resistance in ~~the phenotypically promising~~ rice cultures ~~by~~ using SSR markers

ABSTRACT

The experiment was conducted during *Rabi* 2023-24 at Regional Agricultural Research Station, Warangal. The Asian gall midge *Orseolia oryzae* is one of the major pests of rice which is causing significant economic loss to the crop. ~~30~~ Thirty rice cultures along with parents resistant checks (Aganni and RMSGM3) and susceptible check (TN-1) were screened for the presence of three gall midge resistance genes namely, *gm3, Gm4* and *Gm8* by using SSR markers like gm3del3, LRR-del and PRP, respectively. Out of 30 rice cultures five rice cultures namely, WGL-1940, WGL-1942, WGL-1963, WGL-1956 and MIL-12 were observed to be triple positives by possessing all the three gall midge resistant genes (i,e.*gm3, Gm4* and *Gm8*), While three rice cultures namely, WGL-1778, WGL-1800 and BM-71 were observed to posses *gm3* and *Gm8* gall midge resistance genes and 14 rice cultures namely, WGL-1781, WGL-2000, WGL-2003. WGL-1941, WGL-1949, WGL-1960, WGL-2038, WGL-1145, INRC-3021, WGL-1124, Aganni, WGL1127, WGL-2039 and WGL-2040 were observed to posses *Gm4* and *Gm8* genes. These promising rice cultures resistant to rice gall midge can be used as donors in breeding programmes as a source of gall midge resistance or may be released as varieties, if found to have acceptable phenotypic traits.

Comment [Ma1]: What meant by Rabi? Could you translate to English language to make it clear for all

Key Words: Rice, gall midge, Molecular Markers,

INTRODUCTION

Rice (*Oryza sativa*) is a stable food for more than half of the world population, especially in Asia. Approximately 52% of global rice production is annually lost due to the damage caused by biotic stress factors, of which 25% is attributed to the attack by insect pests (Yarasi *et al*., 2008). Among the various insects pests of rice that cause economic losses in south Asia, the rice gall midge ranks third after stem borers and plant hoppers (Bentur *et al*., 2016). The Asian rice gall midge, *Orseola oryzae* (Wood-Mason) (Diptera: cecidomyiidae). Is widely spread in Asia, causing significant yield losses In India. gall midge damage causes an average annual yield loss of about 0.8% of the total production accounting to US $ 80 million (Krishnaiah. 2004). The larva of gall midge feeds on the apical meristem causing the formation of tubular gall called as silver shoot. Galls occurs generally during the tillering stage. Early gall midge infestation results in profuse tillering and stunting but these tillers do not bear panicles resulting in yield losses (Bentur *et al*., 1987). The best way to manage the pest is the cultivation of resistant varieties. Till date there are seven gall midge biotypes (GMB1, GMB2, GMB3, GMB4, GMB5, GMB6 and GMB4M) were reported (Vijaya Lakshmi *et al*., 2006) and 12 gall midge resistance genes (designated as *Gm1* to *gm*12) have been identified from different sources (Himabindu *et al*., 2010:Leelagud *et al*., 2020), .among them 10 genes, namely *Gm1, Gm2, gm3, Gm4, Gm5, Gm6, Gm7, Gm8, Gm11* and *gm12* were tagged and mapped successfully (Leelagud *et al*., 2020). The marker gm3del3 designed for the candidate gene NB-ARC for *gm3* gene which is located on the chromosome 4 completely co segregated with the trait in the mapping population of 300F 10 RILs (Sama *et al.,* 2014). It was identified that a LRR gene as a candidate gene for *Gm4* based on physical location, structural diversity, co segregation and functional validation also revealed LRR-del as a functional marker which can be used for detecting *Gm4* as this marker produces amplified fragments at 600 bp in TN-1 (susceptible check) and 350 bp in Abhaya (resistance check) (Divya *et al*., 2014). Further in-silico analysis were made by Yasala *et al.,* (2012) revealing functional gene loci and later attempts narrowed down the search with a candidate gene coding for proline rich protein in the genomic region of *Gm8*, and was further validated and the marker PRP was used for identification of several genotypes with *Gm8* gene (Divya *et al*., 2015). These three gene based

markers have shown a high degree of confidence in detecting the presence of genes in mapping populations (Dutta *et al,* 2014). With the availability of gene linked molecular markers it is possible to identify the gall midge resistance genes precisely. So the present study was aimed for molecular ~~confirmation~~ verification of gall midge resistance genes in the rice cultures.

MATERIALS AND METHORDS

Molecular ~~confirmation~~ verifivation of phenotypically promising gall midge resistant rice cultures (Table 1) for presence of the 3 gall midge resistant genes (viz., *gm3*, *Gm4* and *Gm8*) by using linked markers or functional markers like Gm3del3, Gm4-LRR and PRP, respectively, was carried out at Biotechnology Laboratory, RARS, Warangal during *Rabi,* 2023-24.

Plant genomic DNA was extracted from leaf tissue of the test rice cultures and susceptible check TN-1 (Table 1), ~~through the method of~~using CTAB (Zheng et al., 1995). The purity and concentration of isolated genomic DNA was estimated by running 0.8% agarose gel electrophoresis technique and through the Nanodrop method. The reaction volume of the samples was made upto 11 µl that consisted of 3 µl of DNA template,5 µl of PCR master mix (consisting of dNTPs, Taq DNA polymerase, MgCl 2 and 10X PCR buffer), 0.5 µl of each forward and reverse primer and diluted with 2 µl of Double Distilled Water. Amplification was done in a programmable thermo cycler that was programmed with. The initial denaturation was done at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds. Annealing was carried out at 55°C for 30 seconds. T~~t~~he first elongation was carried at 72°C for 1 minute and a final elongation at 72°C for 7 minutes.

Three markers Gm3del3 (Sama *et al*,2014) LRR-del (Divya *et al* ., 2015) and PRP (Divya *et al,*2013) were used for the identification of 3 gall midge resistance genes *viz, gm3, Gm4* and *Gm8* respectively. PCR reactions are conducted separately for detecting each of these genes by using their respective markers.

Amplified PCR products were subjected to agarose gel electrophoresis and were visualized in a UV gel documentation system.

Table 1 : Molecular confirmation of rice cultures for the presences of *gm3*, *Gm4* and *Gm8* genes for gall midge resistance.

S.No.	Name of the rice culture	Genotyping results			Remarks
		gm3	***Gm4***	***Gm8***	
1	WGL-1778	aa	rr	RR	Double positives for *gm3* and *Gm8* genes
2	WGL-1781	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
3	WGL-1782	AA	RR	rr	Positive for *Gm4* gene
4	WGL-1800	aa	rr	RR	Double positives for *gm3* and *Gm8* genes
5	WGL-1989	AA	rr	rr	Negatives for three gall midge resistance genes
6	WGL-1990	AA	rr	rr	Negatives for three gall midge resistance genes
7	WGL-2000	AA	RR	RR	Double positives for *Gm4* and *Gm8*

Comment [Ma2]: Is it the same with Gm4-LRR or similar with it?

Comment [Ma3]: 1.Take it to the result and discussion part
2. Write the description of each abbreviated (resistance level: homozygous recessive and homozygous dominant) in the table as footnote to make clear for readers.

UNDER PEER REVIEW

					genes
8	WGL-2001	AA	rr	rr	Negatives for three gall midge resistance genes
9	WGL-2003	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
10	WGL-2004	AA	RR	rr	Positive for *Gm4* gene
11	WGL-1940	aa	RR	RR	Triple positives for *gm3,Gm4* and *Gm8* genes
12	WGL-1941	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
13	WGL-1942	aa	RR	RR	Triple positives for *gm3,Gm4* and *Gm8* genes
14	WGL-1943	AA	rr	rr	Negatives for three gall midge resistance genes
15	WGL-1944	AA	rr	rr	Negatives for three gall midge resistance genes
16	WGL-1947	aa	rr	rr	Positive for *gm3* gene
17	WGL-1949	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
18	WGL-1956	aa	RR	RR	Triple positives for *gm3,Gm4* and *Gm8* genes
19	WGL-1957	AA	rr	rr	Negative for three gall midge resistance genes
20	WGL-1959	AA	rr	rr	Negative for three gall midge resistance genes
21	WGL-1960	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
22	WGL-1962	AA	rr	RR	Positive for *Gm8* gene
23	WGL-1963	aa	RR	RR	Triple positives for *gm3,Gm4* and *Gm8* genes
24	WGL-1964	aa	rr	rr	Positive for *gm3* gene
25	WGL-1969	AA	rr	rr	Negatives for three gall midge resistance genes
26	WGL-2038	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes

UNDER PEER REVIEW

27	WGL-2041	AA	rr	rr	Negatives for three gall midge resistance genes
28	WGL-1865	AA	rr	rr	Negatives for three gall midge resistance genes
29	WGL-1126	AA	RR	rr	Positive for *Gm4* gene
30	WGL-1145	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
31	INRC-3021	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
32	WGL-1121	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
33	BM-71	aa	rr	RR	Double positives for *gm3* and *Gm8* genes
34	MIL-13	AA	rr	rr	Negatives for three gall midge resistance genes
35	Aganni	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
36	TN-1	AA	rr	rr	Negatives for three gall midge resistance genes
37	MIL-12	aa	RR	RR	Triple positives for *gm3,Gm4* and *Gm8* genes
38	WGL-1121	AA	rr	rr	Negatives for three gall midge resistance genes
39	WGL-1127	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
40	WGL-2039	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
41	WGL-2040	AA	RR	RR	Double positives for *Gm4* and *Gm8* genes
	RMSGM3	aa	RR	RR	Triple positives for *gm3*,*Gm4* and *Gm8* genes

UNDER PEER REVIEW

	TN-1	AA	rr	rr	Negatives for three gall midge resistance genes

Table 2: Details of molecular markers used in the study.

S.No.	Name of the Gene	Name of the Marker	Sequence of Marker	Resistant allele(bp)	Susceptible allele(bp)	Reference
1	*gm3*	gm3del3	F-5'CTGCCAGAGATGGGCCTTCCA3' R5'CGTACAAATTCCTGTACCACTC3	250	550	Sama *et al.*(2014)
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.*(1013)

UNDER PEER REVIEW

RESULTS AND DISCUSSION

Molecular confirmation of gall midge resistance was carried out for 30 rice cultures using functional markers or gene linked markers to know the presence or absence of gall midge resistance genes. ~~For the c~~Control of rice gall midge, development of resistant rice varieties using marker assisted selection can be sustainable and cost-effective approach (Datta *et al*., 2014).

PCR analysis.

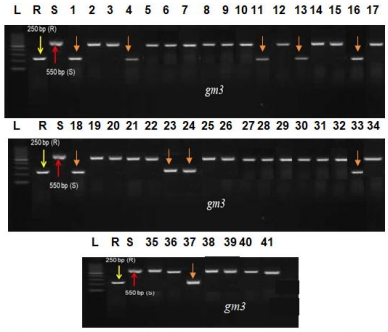
1. Molecular confirmation for presence of *gm3* gene by using Gm3 del 3 functional marker.

Out of 30 rice cultures 11 rice cultures *viz.* WGL-1778, WGL-1800, WGL-1940, WGL-1942, WGL-1947, WGL-1956, WGL-1963, WGL-1964, BM-71 and MIL-12, were observed to be positives for *gm3* when screened with Gm3 del3 functional marker (Table 1) (Figure 1).

The gm3del3 marker was designed based on sequence polymorphism of NB- ARC genes (Sama *et al.,* 2014). It exhibits an allele size of 250 bp for the resistant parent and 550 bp for the susceptible parent (Dutta *et al.,* 2014).

Earlier, Sama *et al.,* (2014) used gm3del3 as a functional marker for introgression of *gm3* gene into the genetic background of the elite bacterial blight resistant cultivar Improved Samba Masuri. Venkanna *et al.,* (2018) employed the gm3del3 marker to screen pyramided lines to determine the presence of the *gm3* gene. Hari *et al.,* (2022) used gm3del3 marker to screen the rice varieties.

Figure 1: Molecular confirmation of the rice cultures for the presence of *gm3* gall midge resistance gene by using gm3del3 functional marker.



The lane numbers (1-42) shown on the top of gel indicates, list of rice cultures used for molecular analysis (Table-1). **L= DNA Ladder (100bp); R=Resistant Check (RMSGM3); S= Susceptible Check (TN1) and arrow mark indicates positive for gm3 gene.**

2. Molecular conformation for presence of *Gm4* gene by using LRR-del functional marker.

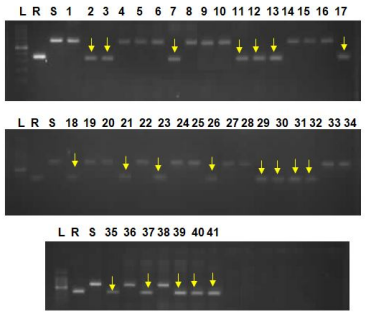
Out of 30 rice cultures 21 rice cultures *viz.* WGL-1781, WGL-1782, WGL-2000, WGL-2004, WGL-1940, WGL-1941, WGL-1942, WGL-1949, WGL-1956, WGL-1960, WGL-1963, WGL-2038, WGL-1126, WGL-1145, INRC-3021, WGL-1124, Aganni, MIL-12, WGL-1127, WGL-2039 and WGL-2040 were observed to be positive for *Gm4* gene when screened with LRR-del functional marker (Table 1) (Figure 2).

The functional marker LRR-del was developed for the identification of *Gm4* gene The allele size of LRR-del functional marker is 350 bp in resistant parent and 600 bp in susceptible parent (Divya *et al.,* 2015).

Similarly Abhiash Kumar *et al.,* (2017) used the marker to screen inter-crossing F4 cultures that carried the *Gm4* gene.

Comment [Ma4]: It is better to change confirmation to verification. This is because your material is well known for their phenotypic resistance. Hence, you are using molecular markers to verify presence or absence of the resistant gene

Figure 2: Molecular confirmation of the rice cultures for the presence of *Gm4* gene by using LRRdel functional marker.



The lane numbers (1-42) shown on the top of gel indicates, list of rice cultures used for molecular analysis (Table 1). **L= DNA Ladder (100bp); R=Resistant Check (RMSGM3); S= Susceptible Check (TN1) and arrow mark indicates positive for Gm4 gene.**

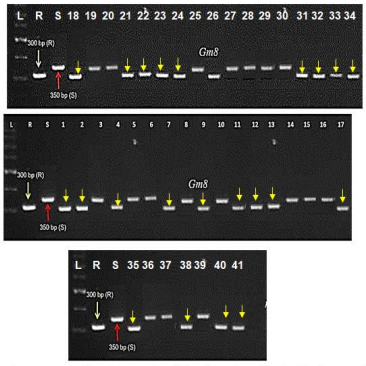
3. Molecular conformation for presence of *Gm8* gene by using PRP functional marker.

Out of 30 rice cultures 23 rice cultures *viz,* WGL-1778, WGL-1781, WGL-1800, WGL-2000, WGL-2003, WGL-1940, WGL-1941, WGL-1942, WGL-1949, WGL-1956, WGL-1960, WGL-1962, WGL-1963, WGL-2038, WGL-1145, INRC-3021, WGL-1124, BM-71, MIL-12, WGL-1127, WGL-2039, WGL-2040 and Aganni were observed to be positive for *Gm8* gene when screened with PRP functional marker (Table 1) (Figure 3)..

The PRP functional marker encoding a Proline Rich Protein was being developed to identify the presence of *Gm8* gene (Divya *et al*., 2013). The allele size of the PRP marker is 300 bp in the resistant parent and 350 bp in the susceptible parent (Dutta *et al .,* 2014).

Similar to this study earlier Dutta *et al.,* (2014) used this marker to detect the presence of *Gm8* gene with high degree of success. Hari *et al.,* (2022) screened the rice varieties for the presence of *Gm8* using the PRP marker.

Figure 3: Molecular confirmation of the rice cultures for the presence of *Gm8* gene by using PRP functional marker.



The lane numbers (1-42) shown on the top of gel indicates, list of rice cultures used for molecular analysis (Table 1). **L= DNA Ladder (100bp); R=Resistant Check (RMSGM3); S= Susceptible Check (TN1) and arrow mark indicates positive for Gm8 gene.**

CONCLUSION

Molecular conformation for gall midge resistance was carried out for 30 rice cultures along with parents, resistant checks (Aganni and RMSGM3) and susceptible check TN-1 using functional markers to identify the presence or absence of gall midge resistance genes. Out of 30 rice cultures, 5 rice cultures *viz.* WGL-1940, WGL-1942, WGL-1956, WGL-1963 and MIL-12 were observed to be triple positive *i.e.* possessing *gm3, Gm4* and *Gm8 g*all midge resistance genes, 3 rice cultures *viz.* WGL-1778, WGL-1800 and BM-71 were observed to be double positive by possessing *gm3* and *Gm8* gall midge resistance genes.14 rice cultures *viz.* WGL-1781, WGL-2000, WGL-2003, WGL-1941, WGL-1949, WGL-1960, WGL-2038, WGL-1145, INRC-3021, WGL-1124, Aganni, WGL-1127, WGL-2039 and WGL-2040 were observed to be double positive by possessing *Gm4* and *Gm8* gall midge resistance genes, and markers like gm3del3 for gm3 gene, LRR-del for *Gm4* gene and PRP for *Gm8* gene to identify the presence or absence of gall midge resistance genes. These promising rice cultures resistant to rice gall midge can be used as donors in breeding programmes as a source of gall midge resistance are can be released as varieties, if found to have acceptable phenotypic traits.

Comment [Ma5]: Rewrite or paraphrase it

REFERENCES

1. Abilash Kumar, V., Balachiranjeevi, C.H., Bhaskar Naik, S., Rekha, G., Rambabu, R., Harika,G., Pranathi, K., Hajira, S.K., Anila, M.,Kousik, M and Kale, R. 2017. Marker-assisted pyramiding of bacterial blight and gall midge resistance genes into-1005, the resorter line of popular rice hybrid DRRH-3. *Molecular Breeding.* 37(7):1-14

2. Bentur, J.S., Rawat, N., Divya, D., Sinha, D.K., Agarrwal, R., Atray, I and Nair,. S. 2016. Rice gall midge interaction: Battle of survival *Journal of Insect Physiology.* 84:40-49.

3. Bentur, J.S., Srinivasan, T.E. and Kalode, M.B.1987.Occurrence of a virulent rice gall midge (GM)*Orseolia oryzae* Wood-Mason biotype in Andhra Pradesh, India *International Rice Research Newsletter.* 12:33-34..

4. Divya, D., Bentur, J.S. and Nair, S. 2013. Identification of putative candidate gene (s) for gall midge resistance *Gm8* gene in Aganni Rice. In: Abstracts: *National Symposium on Innovative Approaches to Crop Improvement and Adaptation: Meeting Challenges of Climate Change*, 22-24 February. UAS, Bangalore. p. 75.

5. Divya, D., Himabindu, K., Nair., S. and Bentur. J.S. 2014. Cloning of a gene encoding LRR protein and its validation as candidate gall midge resistance gene, *Gm4* in rice.*Euphytica.* 203(1):185-195

6. Dutta, S.S., Divya, D., Durga Rani, Ch., Dayakar Reddy, T., Visalakshmi, V., Cheralu,. C., Ibohal Singh, K. and Bentur, J.S. 2014. Characterization of gall midge resistant rice genotypes using resistance gene specific markers. *Journal of Experimental Biology and Agricultural Sciences.* 2(4):440-446.

7. Hari, Y., Malathi, S., Shravan Kumar, R., Lavanya, B., Pranitha, B., Venkanna, V., Rukmini Devi, K., Sathish Chandra, B., Rajendra Prasad, K., Nagabhushanam, U., Raghu Ram Reddy, P., Uma Reddy, R and Sasthree, T. 2022. Phenotypic and Genotypic screening of Warangal rice varieties against Gall Midge (*Orseolia oryzae*). *Environment and Ecology*. 40(3A):1288-1294.

8. Himabindu, K., Suneetha, K., Sama, V.S.A.K and Bentur, J.S. 2010. A new rice gall midge resistance gene in the breeding line CR57-MR1523, mapping with flanking marker and development of NILs, *Euphytica.* 174:179-187

9. Krishnaiah, K., 2004. Rice gall midge *Orseolia oryzae*-an overview. In new approaches to gall midge resistance in rice, Bennett, J., Bentur, J.S., Pasalu, I.C and Krishnaiah, K. (eds.). *Proc, the International workshop*, 22-24 November 1998, Hyderabad, India. Los Banos, Philippines. P.195.

10. Leelagud, P., Kongsila, S., Vejchasarsam, P., Darwell, K., Thansenee, Y., Suthangjai, A., Uparang,C., Kawichai, R., Yajai, P., Boonsa-Nga, K and Chamarer, K., V. 2020. Genetic diversity of Asian rice gall midge based on mtCOI gene sequence and identification of a novel resistance locus *Gm12* in rice cultivar MN 62M. *Molecular Biology Reports.* 47(6):4273-4283.

11. Sama, V.S.A.K., Nidhi Rawath., Sundaram, R.M., Himabindu, K., Bhaskar.S.N., Viraktamath, B.C.and Bentur, J.S. 2014. A putative candidate for the recessive gall midge gene *gm3* in Rice identified and validated. *Theoretical and applied genetics.* 127:113-124.

12. Venkanna, V., Hari, Y., Rukminidevi, K., Satish, C.B., Raju, J., Malathi, S and Raghu, R.R.P. 2018. Markers Assisted selection for pyramiding of gall midge resistance genes in Kavya, a popular rice variety. *International Journal of Current Microbiology and Applied Sciences*. 7(4)2319-7706.

UNDER PEER REVIEW

13. Vijaya Lakshmi, P., Amudhan, S., Himabindu, K., Cheralu, C and Bentur, J.S. 2006.A new biotype of the rice gall midge *Orseolia oryzae* (Diptera:Cecidomyiidae) characterized from the Warangal population in Andhra Pradesh, India. *International Journal of Tropical Insect Science*.26(3):207-211.

14. Yarasi, B., Sadumpati, V., Immanni, C.P., Vudem, D.R., Khareedu, V.R. 2008. Transgenic rice Expressing *Allium sativum* leaf agglutinin (ASAL) exhibits high level resistance against major sap sucking pests. *Plant biology*. 8:102-115.

15. Yasala, A.K., Rawat, N., Sama, V. S. A. K., Himabindu, K., Sundaram, R.M. and Bentur, J. S. 2012. In silico analysis for gene content in rice genomic regions mapped for the gall midge resistance genes. *Plant Omics Journal* 5:405-413.

16. Zheng, K., Subudhi, P.K., Domingo, J., Maopanty, G. and Huang, N. 1995. Rapid DNA isolation for marker assisted selection in rice breeding. *Rice Genetics Newsletter* 12:255-258.

UNDER PEER REVIEW