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Assessment of Molecular Diversity and Mapping of Bruchid Tolerance Loci in Green Gram [*Vigna radiata* (L.) Wilczek]

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ABSTRACT

Background: Bruchid poses threat to the greengram production and development of tolerant varieties avoids heavy losses.

Methods: A total of 47 diverse genotypes were screened for bruchid (*Callasobruchus chinensis*) tolerance and available molecular variation at reported bruchid tolerant loci there by the markers associated with tolerance.

Result: ANOVA of pod and seed characters along with bruchid infestation (%) revealed significant variation. Bruchid incidence at 40 days after treatment (DAT) was ranged from 19.67% (OBGG-57) to 99.33% (NRI-SAKTHI-POLISH) with 59.38% mean. When tested for long term tolerance, at 150 DAT, OBGG-57 alone remained as resistant with 28.67% seed infestation. Hence, OBGG-57 can be used as bruchid resistant genotype directly for release/as donor in breeding programmes. Molecular screening targeting reported bruchid tolerant loci revealed high informativeness for DMBSSR125 marker with PIC of 0.90. Association analysis revealed the linkage of DMBSSR125 (EST-SSR) to bruchid tolerance with *p*-value of 0.0378. BLAST analysis of the marker sequence against greengram genome aligns to two Metallothionein (MTs) proteins (vigra. Vradi09g07610, Vradi0111s00070). MTs are small cysteine-rich proteins. A cysteine-rich protein, 'VrCRP' has been reported for wild greengram (TC1966) tolerance towards *Callasobruchus chinensis*. Hence, DMBSSR125 that corresponds to a cystine-rich protein can be further validated through mapping/introgression approaches to confirm association and to use in bruchid resistant breeding programmes.

Key words: Association study, Bruchid tolerance, Diversity, Greengram, Pod and seed traits.

INTRODUCTION

Greengram, scientifically known as *Vigna radiata* (L.) Wilczek, holds significant importance as a pulse crop in Asia, with a consistent increase in its production. This crop is renowned for its high protein content and serves as an abundant source of folic acid and iron. Due to its small genome size of 578 Mb, close genetic relationship to other legumes, short life cycle and self-pollinating nature, it has become a preferred model organism for molecular studies among all legume crops. The versatility and genetic traits of greengram make it an important candidate for further research and development in the field of agriculture.

Bruchid beetles pose a major challenge to greengram production, particularly in stored conditions. These destructive pests, including species like *Callasobruchus chinensis* and *Callasobruchus maculatus*, significantly impact both the quantity and quality of greengram seeds. Infestation starts modestly in the field but escalates during storage as eggs hatch into larvae, leading to rapid population growth. Within a span of two to three months, bruchid beetles can cause complete seed loss, resulting in financial setbacks for farmers who also lose valuable seed for subsequent planting. Effective management strategies are crucial to mitigate the damage caused by these pests and safeguard greengram yields.

The level of resistance towards bruchids in pulses relies on morphological barriers preventing colonization of the seed by bruchid larvae, or on secondary metabolites and other toxic compounds interfering with bruchid growth, development or reproduction. Genetic diversity studies either at phenotypic level or by combining molecular studies employing varied genotypes is pre-requisite for any crop improvement through trait breeding and molecular mapping of targeted traits. Previous reports suggest that some wild mungbean accessions (TC1966, ACC41, V2709, V2802 *etc*.) and cultivated (RS4, RMG11, IC325770, IC333175, IC329039, Sonamung, Dantan, *etc*.) genotypes showed resistance to bruchids (Sarkar *et al*., 2011; Chen *et al*., 2013 and War *et al*., 2017).

Identification of potent molecular markers that associated with bruchid resistance would help overcome

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the menace caused by bruchids both at field level and at storage level. However, the available bruchid tolerant loci and associated markers till now, haven't been validated precisely, to use in the breeding program. Further, in-depth study on reported bruchid tolerance loci is needed to develop tightly linked markers to use in marker assisted breeding for bruchid-resistant greengram varieties. Hence, the current study was aimed to identify the molecular diversity at reported bruchid tolerant loci along with the markers linked with the trait, by screening of diverse greengram genotypes.

MATERIALS AND METHODS

Field experiment was carried out with a panel of 47 diverse greengram genotypes comprising of popularly cultivated varieties, other released varieties and advanced breeding lines procured from different AICRP (All India Co-Ordinate Research Project) Center at Dryland farm, Regional Agricultural Research Station (RARS), ANGRAU, Tirupati during summer, 2019 in a randomized block design with three replications and molecular analysis was carried at Biotechnology Lab, Institute of Frontier Technology (IFT), RARS, Tirupati.

Per cent seed infestation (PSI -%)

The artificial study of the genotype's response to bruchid pest tolerance/susceptibility was conducted using the following procedure. The pulse beetle, *Callasobruchus chinensis* (*C. chinensis*), which is a major storage pest of greengram and causes damage in both field and storage conditions, was used as the test insect. The culture of *C. chinensis* was obtained from the Department of Entomology, S.V. Agriculture College, Tirupati and was multiplied on locally available greengram seeds. To mass culture the insects, approximately 25 pairs of adult beetles were released into plastic boxes containing 500 g of disinfested greengram seed. The containers were covered with muslin cloth and secured with rubber bands. These containers were left undisturbed under ambient conditions until the progeny emerged. The newly emerged adult beetles (1-2 days old) from the culture after 25-30 days of release were used for the experimental studies. The adult beetles were identified as per the key traits given by Southgate *et al*. (1958).

One hundred healthy, sound and disinfested seeds of each greengram genotype were placed in Petri plate. Those Petri plates were arranged in trays with the free choice test insect *i.e*., the Petri plates were not closed, mouth of the tray was covered with mosquito net and fastened with rubber bands. Ten pairs (10 male + 10 female) of freshly emerged adult pulse beetles were released into each tray for oviposition prior the trays were secured with mosquitoes' net. Each genotype was replicated three times (Supplementary Fig 1). Control treatment was maintained similarly, without releasing bruchids. Seven days after infestation (DAI), all adults were removed and each seed was checked to make sure each had more than two eggs. The samples were examined at 40 DAI to record the number of bruchid holes that had emerged and percentage of seed damage was estimated.

The seed with hole is considered as damaged seed. The percentage of damaged seed was estimated Raghuwanshi *et al*. (2016) using the formula:

Per cent damaged seed =

$$\frac{\text{Number of seeds with bored holes}}{\text{Total number of sedds observed}} \times 100$$

Genotypes were classified into different response groups based on per cent damaged seeds (Chen *et al*., 2007) *viz*., Highly resistance (0-10.0%), Resistance (10.1-35.0%), Moderately resistance (35.1-65.0%), Susceptible (65.1-90.0%) and highly susceptible (>90.0%) groups.

Statistical analysis

The data on pod pubescence, pod wall thickness, test weight, seed length, seed width, seed length to seed weight ratio, seed coat texture and percent seed infestation was subjected for statistical analysis *viz*., mean, range, ANOVA, standard deviation, standard error mean and critical difference using data analysis package of Windows-Microsoft/ Excel v. 2010.

Molecular analysis

Molecular analysis was carried out by using reported markers for bruchid tolerance along with designed candidate gene markers for the reported bruchid tolerant greengram gene, Vr05G03950 (Supplementary Table 1). The genomic DNA of Vr05G03950 was downloaded from LIS (legume information system) database (https://legumeinfo.org/) and gene specific primers were designed using primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) keeping the default parameters.

Association between marker alleles and bruchid tolerance was performed with TASSEL (Bradbury *et al*., 2007). The association between the marker and phenotypic traits was done using both GLM (General linear model) and MLM (Multiple linear model) methods, where markers tested and subpopulation data (Q matrix) was considered as fixed-effect factors, whereas the kinship matrix was considered as a random-effect factor.

RESULTS AND DISCUSSION

The data on pod pubescence, pod wall thickness, test weight, seed length, seed width, seed length to seed weight ratio, seed coat texture and percent seed infestation was subjected for statistical analysis *viz*., mean, range, ANOVA, standard deviation, standard error mean and critical difference using data analysis package of Windows-Microsoft/ Excel v. 2010 (Table 1).

Analysis of variance (ANOVA) study revealed existence of highly significant variation (p=<0.0005) for the entire pod and seed related traits along with the per cent bruchid infestation. Hence, the genotype panel can be employed

for diversity studies and for gene mapping approaches apart from development of mapping populations.

Per cent seed infestation (PSI-%)

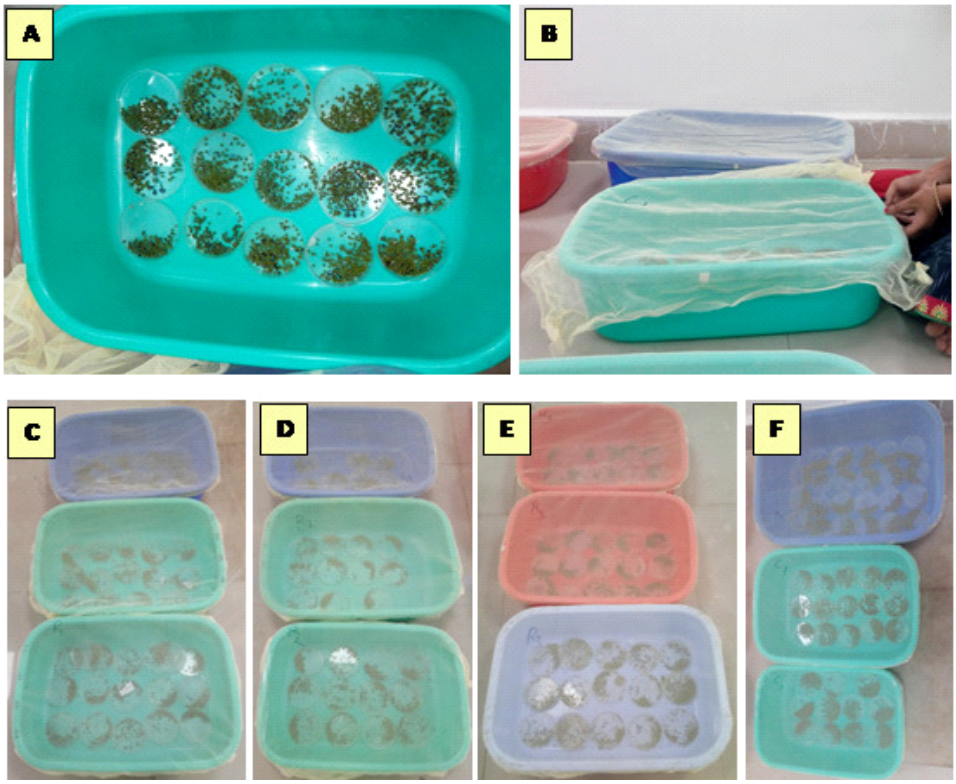
40 days after treatment (artificial release of bruchids), seed damage per cent due to bruchid attack *i.e*., per cent incidence (%) was recorded among the genotypes and were classified into different response groups based on PSI (Chen *et al*., 2007).

The PSI among greengram genotypes used in control treatment (without bruchid release) was ranged from 0 to 5% with the mean of 0.85% where as under the artificial conditions per cent bruchid incidence was ranged from 19.67% to 99.33% with the mean of 59.38% (Table 2). The highest PSI was recorded in the genotype NRI-AMULYA (99.33%), followed by NRI-SAKTHI (POLISH) (93.33%), LGG-577 (92.33%) and hence, these are grouped into highly susceptible class. The genotypes VGG-15-030 (33.33%), NVL-722 (31.67%), COGG-13-19 (30.67%), OBGG-58 (26.42%), MGG-387 (23%) and OBGG-57 (19.67%) were grouped into resistant class (10.1-35.0%). A total of 16 genotypes were sustained under moderately resistant class (35.1- 65.0%) after 40 DAI (days after infestation).

The genotypes that sustained under resistant (10.1-35.0%) and moderately resistant classes were maintained under the treatment condition for another two complete life cycles of bruchid then the PSI was again recorded at 150 days after treatment (Table 3, Fig 1). Among the resistant genotypes, VGG-15-030 (37.67%), NVL-722 (41%), COGG-13-19 (36.33%), OBGG-58 (53%) and MGG-387 (39%) found with an increase of 4.34%, 9.33%, 5.66%, 26% and 16% bruchid incidence, respectively and thus, fall in moderately resistant class after four months of treatment. However, the genotype OBGG-57 showed 28.67% with an increase of 9% incidence and remained in resistant class after three complete life cycles of bruchid *i.e*., after 150 days after treatment. Hence, 'OBGG-57' can be used as bruchid resistant genotype either directly for release or as donor parent in trait-oriented breeding programmes.

Molecular analysis for bruchid resistance

Greengram genotypes were screened at molecular level by employing the reported bruchid tolerant primers along with designed candidate gene specific primers as detailed in material and methods section. Out of 19 primers amplified (Table 4), only one primer namely DMBSSR 125 showed polymorphic alleles *viz*., 240 bp and 310 bp (Fig 2)



(A): Arranging the petriplate with seeds of different genotypes in a plastic tub for the free choice method of artificial bruchid treatment, (B): After releasing the bruchids in to the tub covering with the fine nylon net, (C): R_1 replication of the treatment, (D): R_2 replication of the treatment, (E): R_3 replication of the treatment, (F): Control (C) treatment.

Supplementary Fig 1: Artificial screening of the greengram genotypes for bruchid tolerance/susceptibility reaction.

Supplementary Table 1: List of primers associated with bruchid tolerance used in greengram genotype screening.

Primer name	Forward sequence (5′-3′)	Reverse sequence (3′-5′)
BM202	ATGCGAAAGAGGAACAATCG	CCTTTACCCACACGCCTTC
BMarc 7	GTCTTCTGCAGGTACCATCT	CCAAAGAACTATTGGGATCA
BMarc 10	CGAGCATCGAATACCTTTAC	GGATCACTCTTCCTCTCTTTCTT
BMarc 12	GAAAACGATGCACACAATC	TATGTTGTACCATCGGTTGA
BMarc 14	GGAAGACACATGTGAAGAGG	CTTCCTCTGACTTTCCACTG
BMarc 15	AAGAGGGCTAGGCAAAGTAA	GGCATCGTTGCTTAATTCT
BMarc 17	CTTCTGAGCTGAAGTAAAACG	AGTGAGACAGAAAGGCAAAT
BMarc 20	AGCTGAAGAACGAGGACAT	GAGAAGCACTTGATCGTCAT
BMarc 21	AGGACACATGGACATGGATA	AAGAAGGGGATGAAATGTCT
BMarc 22	GAGTTTTGGGGAGACATTG	ATTTGAGAGCGAAAGATCAC
Br 11 (779)	CTAATAAATCATCTATACGTCTC	ATTGCTATTTAGCGAATAATAGT
CEDG 086	GAGTTTACAACAGATGGGGCTAA	AGGTCTTGATTGACTTTCTGGGT
CEDG 133	GCATACATAATGTGGTGAGATG	GTCTCGTGCCTTTCACAC
CEDG 149	GGCTGAAGGTGATGACAGAAG	GGCACTGGTTTTCTAAGGTTGTTG
CEDG 154	GTCCTTGTTTTCCTCTCCATGG	CATCAGCTGTTCAACACCCTGTG
CEDG 261	GGTCCCAAAATCACCCAG	GGTTCATTTGGAGCACTGAG
DMBSSR125	AAAATGAGTGACAGAGGTGGAAA	ACATGCACATTCTGAACCACAT
DMBSSR130	CAACTGCAAATGAGGTGAAGAT	ATCCAAGAGCATTTGAACTTCC
DMBSSR 151	F:AATGAAGGCTTGTCAAATCCA	TTATTTCACCTTGGCTGGATCA
DMBSSR 158	TGGAAAATTTGCAGCAGTTG	ATTGATGGAGGGCGGAAGTA
DMBSSR 160	GGTGGATCAAATCCATTTTAGG	ACAGATCACATAGCAACCAAACA
MAS-APA-TS1	F1-GCGGGCTCTATTGATCTAAGAAG	AGTTCATTCCATACGGATCTCCT
	F2-GCGGGCAGGGCGACCTATTCATC	
MB-87	TCCCTTGTGGGAGATCCT	CTTTCCCACACTCCTTGC
OPW02a4	CCAAACGAGTCGAGTGAAACT	CAACAACCCTTCCTCTATCTC
PV-atct 001	CAATTAAAACTCAACCAACCCAAATA	TTTCCCGCCATAGAATATGAGA
STSbr1	CAGAAAACAAATCACAAGGC	GTAAGCATTGAAAAAGGGTG
STS Br2	CCACCCTATTCATTCGCTTAC	ACACTTGAATGGCGGACG
Vr 34480	AATTCTTGATTGGTCCACA	AAAAAATTACACCTCGTTCG
Vr2-627	TCGTGTAGTGAATAAAGCCAAA	GAAAGAAGAGAAGAAGGTTTTAG
XM 014649632-m	GGTTGTCTGCCACCAACTTT	TGTTGGCAAAACATGGAAAA
XM 0146449632-g	AGTGGGAGTTGAACAAGGTC	TGAACAGAACTTAAACATCATTGG
Vr05G03950-1*	GGTGCAGGGGTAAGTCACAT	GGCTTTGTTTTGGCGTGTAT
Vr05G03950-2*	GGCGTAGGTGACGGAACTAA	CTGCCCTGCACCAAGTAGAT

*-Candidate gene primers designed using primer 3 software.

Table 1: Mean performance and ANOVA of phenotypic observations recorded on the greengram genotypes under study.

Genotype	PP (No/Mic. area)	PWT (mm)	TW (g)	SL (mm)	SW (mm)	SL/SW	SCT	PSI (%)	
								Cont.	40 DAI
CO-6	73.6	0.08	3.58	3.87	2.63	1.47	S	2	67.51
PUSA-9072	63.4	0.09	3.45	4.06	3.25	1.25	S	2	68.33
VGG-05-009	62.6	0.08	3.80	3.80	3.14	1.21	C	0	48.33
TARM-1	71.4	0.10	3.07	3.44	2.74	1.26	C	0	63.67
VBN GG-2	31.0	0.08	3.38	4.14	3.19	1.30	C	0	70.67
COGG 11-02	45.0	0.08	3.95	4.25	3.34	1.27	C	0	74.33
VGG-10-008	51.4	0.08	3.72	4.26	3.00	1.42	C	0	46.42
IGKM-0642	37.4	0.09	3.27	3.97	3.15	1.26	C	1	66.33
PUSA-15161	51.6	0.09	4.35	4.07	3.31	1.23	C	1	67.00
LGG-544	55.0	0.08	3.41	3.97	2.98	1.33	S	0	75.33

Table 1: Continue...

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VGG-15-038	36.6	0.08	4.04	4.11	3.25	1.26	C	0	75.00
GGG-1-1	50.6	0.08	3.75	4.04	3.14	1.29	S	2	69.33
MGG-385	32.6	0.09	3.50	4.23	3.16	1.34	C	2	79.33
IPM-312-9	27.8	0.12	3.63	4.20	3.07	1.37	S	1	78.33
VGG-14-001	43.4	0.08	3.30	3.92	3.33	1.18	S	0	77.00
LGG-595-1	34.6	0.09	3.45	3.86	3.14	1.23	S	2	67.67
IPM-409-4	34.6	0.11	3.37	4.71	3.52	1.34	C	0	61.33
COGG-13-19	35.0	0.06	3.64	4.68	3.45	1.36	C	0	30.67
VGG-15-030	53.2	0.06	3.01	3.43	2.88	1.19	S	0	33.33
MGG-373	45.6	0.07	3.74	3.69	2.76	1.34	C	0	62.67
MGG-385-1	46.2	0.07	3.60	4.03	3.07	1.31	S	0	52.33
VGG15-030-1	60.6	0.09	3.28	3.39	2.92	1.16	S	0	46.00
LGG-607	44.2	0.07	2.96	3.84	3.17	1.21	S	0	51.67
MGG-387	36.8	0.11	3.77	4.00	3.19	1.25	C	0	26.00
VGG-16-036	58.8	0.06	3.65	4.33	2.92	1.48	C	0	49.33
NVL-722	48.8	0.09	3.37	3.89	3.32	1.17	S	0	31.67
COGG-13-39	47.0	0.08	4.31	3.64	2.84	1.28	S	0	37.33
VGG-16-027	26.4	0.10	4.36	4.35	3.44	1.26	C*	0	38.00
VGG-16-055	53.8	0.08	3.96	3.98	3.11	1.28	S*	0	35.67
AGG-35	70.0	0.09	3.31	3.90	2.97	1.31	S	0	38.67
OBGG-56	69.4	0.11	3.24	3.89	3.03	1.28	C	0	41.33
OBGG-57	69.8	0.08	3.32	3.36	2.90	1.16	S	0	19.67
OBGG-58	57.0	0.09	4.32	4.38	3.15	1.39	C	0	26.42
IPM-2-14	34.4	0.08	4.27	4.38	3.22	1.36	C	0	40.67
LGG-594	37.4	0.08	3.41	3.88	2.85	1.36	S	0	37.33
LGG-574	54.0	0.11	3.64	3.70	2.69	1.38	S	1	37.33
PANT MUNG 5	29.0	0.08	5.26	4.57	3.54	1.29	C	0	36.00
LGG-578	68.0	0.09	3.22	3.66	2.88	1.27	S	2	59.33
LGG-460	31.8	0.08	3.39	4.07	3.22	1.26	S	2	80.00
GGG-1-1	44.0	0.08	4.02	4.02	3.32	1.21	S	1	89.00
LGG-577	43.4	0.08	3.63	4.01	3.07	1.31	S	1	92.33
LGG-596	32.2	0.07	3.40	3.78	3.09	1.22	C	2	86.67
LGG-595	29.4	0.07	3.28	4.19	3.08	1.36	S	3	86.33
WGG-42	47.8	0.08	3.55	3.69	2.95	1.25	S	4	87.67
NRI- Amulya	40.6	0.08	3.67	3.89	3.23	1.20	C	5	99.33
NRI- Amulya (Polish)	59.8	0.09	3.34	3.76	3.08	1.22	S	3	88.67
NRI- Sakthi (Polish)	47.8	0.12	3.21	3.77	2.96	1.27	S	3	93.33
Minimum	26.4	0.06	2.96	3.36	2.63	1.16	-	0	19.67
Maximum	73.6	0.12	5.26	4.71	3.54	1.48	-	5	99.33
Mean	47.34	0.09	3.62	3.98	3.10	1.29	-	0.85	59.38
p value	3.44E-16	0.0003	2.24E-43	3.45E-29	1.57E-2	4.3E-10	-	3.44E-17	0.0007
SD	13.26	0.01	0.43	0.31	0.21	0.08	-	1.25	21.62
SEM	1.93	0.00	0.06	0.04	0.03	0.01	-	0.18	3.15
CD	0.28	0.17	0.12	0.08	0.07	0.06	-	1.47	0.36

Note: Pod dehiscence and Pod texture-all the genotypes were observed with non-dehiscent and coarse textured pods.

PP- Pod pubescence/microscopic area (No.); PWT- Pod wall thickness (Measured with vernier caliper in mm); TW- Test weight/Seed weight; SL- Length of seed; SW- Width of seed; SL/SW- SL to SW ratio; SCT- Seed coat texture (coarse or smooth texture); PSI- Per cent seed infestation.

*C- Coarse and *S- Smooth; Cont.- Control, DAI- Days after infestation.

Table 2: Per cent Seed Infestation (PSI) in greengram genotypes under study.

Genotype	PSI %	
	Control	40 Days after infestation
CO-6	2	67.51
PUSA - 9072	2	68.33
VGG - 05 - 009	0	48.33
TARM-1	0	63.67
VBN GG-2	0	70.67
COGG 11-02	0	74.33
VGG-10-008	0	46.42
IGKM-0642	1	66.33
PUSA-15161	1	67.00
LGG-544	0	75.33
VGG-15-038	0	75.00
GGG-1-1	2	69.33
MGG-385	2	79.33
IPM-312-9	1	78.33
VGG-14-001	0	77.00
LGG-595-1	2	67.67
IPM-409-4	0	61.33
COGG-13-19	0	30.67
VGG-15-030	0	33.33
MGG-373	0	62.67
MGG-385-1	0	52.33
VGG15-030-1	0	46.00
LGG-607	0	51.67
MFG-387	0	26.00
VGG-16-036	0	49.33
NVL-722	0	31.67
COGG-13-39	0	37.33
VGG-16-027	0	38.00
VGG-16-055	0	35.67
AGG-35	0	38.67
OBGG-56	0	41.33
OBGG-57	0	19.67
OBGG-58	0	26.42
IPM-2-14	0	40.67
LGG-594	0	37.33
LGG-574	1	37.33
PANT MUNG 5	0	36.00
LGG-578	2	59.33
LGG-460	2	80.00
GGG-1-1	1	89.00
LGG-577	1	92.33
LGG-596	2	86.67
LGG-595	3	86.33
WGG-42	4	87.67
NRI- Amulya	5	99.33
NRI- Amulya (Polish)	3	88.67
NRI- Sakthi (Polish)	3	93.33

among the genotypes under study with 5.3% of polymorphism, which denotes the existence of very low polymorphism at the targeted bruchid tolerant loci.

Association study of DMBSSR 125

Association analysis employing MLM approach revealed that the marker DMBSSR 125 was linked to the greengram bruchid tolerance at p-value of 0.0378 and with R^2 value of 8.60 (Table 5). However, GLM model couldn't reveal significant association. The marker-trait association can be confirmed either by employing larger and diverse population or through linkage mapping approaches. The primer that is having an R^2 value of about 15 can be termed as strongly associated marker to the trait. Hence, DMBSSR 125 with a moderate R^2 value of 8.60 needs to be re-validated employing large populations either through linkage or association mapping approaches.

The marker DMBSSR 125 is an EST-SSR marker. BLAST analysis of the marker sequence against greengram genome (https://legumeinfo.org/) aligns to a functional gene that belongs to Metallothionein (MTs) group of proteins on Chromosome 9 (vigra. Vradi09g07610) and to a scaffold with ID, Vradi0111s00070 (Table 6).

Table 3: Bruchid infestation levels among resistant and moderately resistant greengram genotypes at 40 DAI and 150 DAI.

Genotype	40 DAI*	150 DAI*
Resistant (10.1-35%) genotypes		
OBGG -57	19.7	28.67
MGG-387	23.0	39.00
OBGG-58	26.4	53.00
COGG-13-19	30.7	36.33
NVL-722	31.7	41.00
VGG-15-030	33.3	37.67
Moderate resistant (35.1-65%) genotypes		
VGG-16-027	35.7	39.67
PANT MUNG 5	36.0	43.33
COGG-13-39	37.3	47.00
LGG-594	37.3	49.00
LGG-574	37.3	57.00
VGG-16-027	38.0	55.00
AGG-35	38.7	47.67
IPM-2-14	40.7	52.00
OBGG-56	41.3	56.00
VGG15-030-1	46.0	55.00
VGG-10-008	46.4	59.33
VGG-05-009	48.3	63.67
VGG-16-036	49.3	53.33
MGG-385-1	52.3	58.33
LGG-607	51.7	75.33*
LGG-578	59.3	67.67*

DAI*- Days after infestation.

Table 4: Monomorphic/Polymorphic primers identified from the screening of 47 greengram genotypes.

Marker name	Annealing temperature (°C)	Monomorphic (M)/Polymorphic (P)
BM202	57,58	M
BMarc 15	54	M
CEDG 086	54, 56	M
CEDG 133	54, 56, 59	M
CEDG 149	51, 56, 59	M
CEDG 154	51, 57	M
CEDG 261	54, 56, 59	M
DMBSSR125	59	P
DMBSSR130	59	M
DMBSSR 151	57	M
DMBSSR 158	57	M
DMBSSR 160	54, 56	M
MB-87	57	M
STS Br2	57	M
Vr05G03950-1*	59	M
Vr05G03950-2*	57	M
Vr2-627	57.5	M
XM 014649632-m	51,57	M
XM 0146449632-g	51,57	M

*-Candidate gene primers designed using primer 3 software, for the greengram gene Vr05g03950.

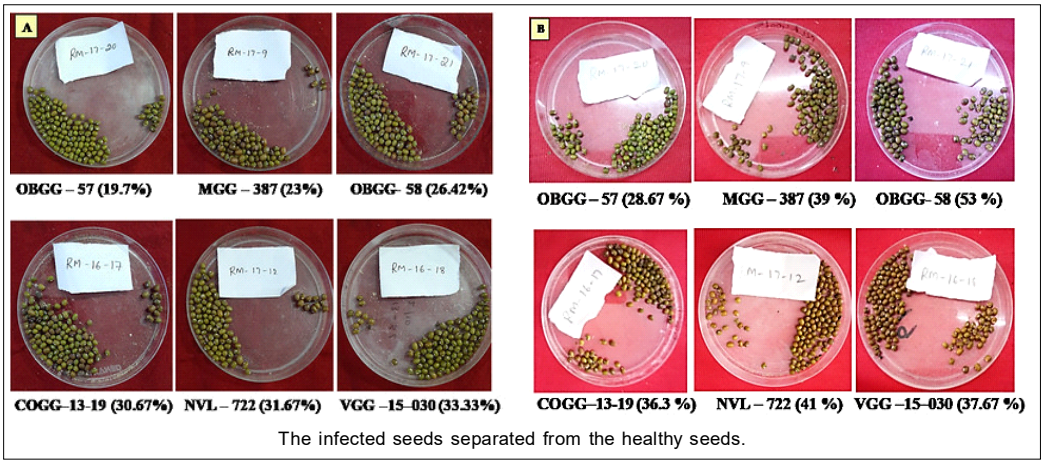
Table 5: Association analysis of DMBSSR 125 with per cent bruchid incidence.

Trait/Marker	Chr	R^2	p_Marker	Model
DMBSSR 125	9	8.60	0.0642	GLM
			0.0378	MLM

Chr- Chromosome; p-_Marker, p value of the marker; GLM- General linear model; MLM- Multiple linear model.

Table 6: BLAST analysis of DMBSSR 125 marker sequence against grengram genome.

Name	Chromosome	Start (bp)	End (bp)	Gene family	Description
vigra.Vradi09g07610	vigra.Vr09	12705898	12709685	legfed_v1_ 0.L_1TW5K1	Metallothionein type 1 [*Glycine max*]; IPR000347 (Metallothionein, family 15, plant); GO:0046872 (metal ion binding)
vigra.Vradi0111s00070	vigra. scaffold_111	596750	597650	legfed_v1_ 0.L_1TW5K1	Metallothionein 2A; IPR000347 (Metallothionein, family 15, plant); GO:0046872 (metal ion binding)



The infected seeds separated from the healthy seeds.

Fig 1: Bruchid resistant genotypes identified through artificial screening at (A) 40 days after treatment and (B) 150 days after treatment.

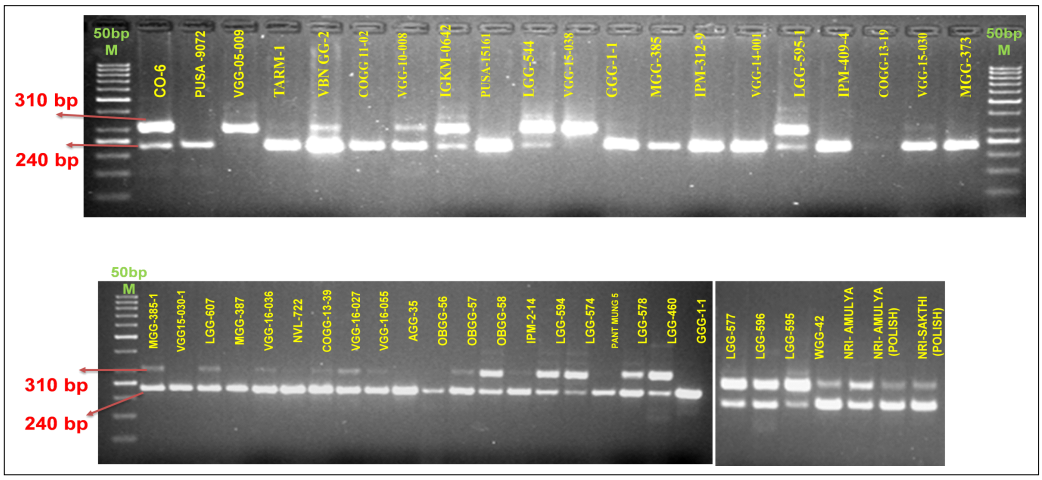


Fig 2: Gel images showing polymorphic alleles of DMBSSR 125 (240 bp and 310 bp) in the greengram genotypes.

Metallothionein (MTs) proteins are reported to be as small cysteine-rich, heavy metal-binding proteins that participate in many of protective stress responses (Nedecky *et al*., 2013). Interestingly, a cysteine rich protein, 'VrCRP' that confers bruchid tolerance has been isolated from seed coat of wild greengram (*Vigna radiata* var. *sublobata*) genotype TC1966. The genotype showed resistance to *Callasobruchus chinensis* through VrCRP's insecticidal activity. Greengram seeds that contain 0.2% VrCRP completely hinder the bruchid larval development (Chen *et al*., 2022).

The DMBSSR 125 marker is an ESTSSR that corresponds to a cystine-rich protein which reported to confer bruchid resistance can be further validated either through mapping or by introgression approaches to confirm its trait association and thereby to use in bruchid resistant breeding programmes.

Host plant resistance involving antibiosis, antixenosis and tolerance mechanisms are preferred in controlling bruchids. It is reported that in the cultivated greengram accessions V2709 and V2802, resistance to *Callasobruchus chinensis* is caused by secondary metabolites which leads to antibiosis in the cotyledons (Talekar and Lin, 1992).

Vignatic acid A, an alkaloid has been isolated from bruchid (*C. chinensis*) resistant wild accession of greengram *viz*., TC1966 (Sugawara *et al*., 1996). In addition, a peptide compound 'GIF-5' toxic to the bruchids has been identified from the source, TC1966 (Kaga *et al*., 2000). Accumulation of cyanogenic glycosides and phytic acids, in greengram seeds during seed maturation to drying plays an important role in defence against bruchids (Lattanzio *et al*., 2005). Bruchid resistance in the genotypes TC1966, VC6089A and a RIL 59 (recombinant inbred line) has been assigned to the presence of a resistant-specific protein, gag/pol polyprotein and aspartic proteinase (Lin *et al*., 2016). Recently, it has been fine mapped by Chen *et al.* (2022) to a candidate gene on chr 5, namely Vradi05g03810 (encoding a probable resistance-specific protein) that possess a 69-bp sequence deletion in the coding region of bruchid-susceptible lines (VC2778A, VC 1973A) when compared to the resistant lines, TC1966, VC6089A.

In the era of omics, identification of probable/potential candidate genes responsible for bruchid tolerance in the genome of a crop spp. can be precisely done. Thus, by employing the knowledge of known/orthologous genes, identification of functional variants that govern targeted trait(s) through re-sequencing/mapping is an immense need especially in the orphan crops like pulses, wherein it can overcome the problem of low molecular variability aroused while using random markers.

CONCLUSION

From the study it can be concluded that gene-based markers are helpful in unraveling the marker-trait association and landing onto a candidate gene than genome wide spanned SSR markers. The DMBSSR 125 marker loci can be used in development of bruchid tolerant varieties with prior validation as described. Further, the two candidate genes vigra. Vradi09g07610 and Vradi0111s00070 can be targeted to reveal the useful variations.

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Author contribution

Srividhya Akkareddy, Devaki Kayam and L. Prasanthi planned the experiments. Y. Soujanya executed the experiments and recorded the phenotypic data and genotypic data. Srividhya Akkareddy and L. Prasanthi analyzed the data. L. Prasanthi, Devaki Kayam and Srividhya Akkareddy provided the required material and facility for laboratory screening. Y. Soujanya, B. Sukrutha and Srividhya Akkareddy wrote the manuscript. All authors read and approved the final manuscript.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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