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Bulk Segregant Analysis Using Micro Sattelites for Powdery Mildew Resistance in Mungbean RIL Population Derived from Chinamung × LM-1668

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Abstract

Sixty four F_8 Recombinant inbred lines derived from a cross between Chinamung (highly susceptible to powdery mildew disease) and LM-1668 (highly resistant to Powdery mildew) were analyzed to identify the SSR markers associated with powdery mildew disease resistance. Parental polymorphism was performed with 248 primer pairs of simple sequence repeats (SSR), which revealed 17 polymorphic markers for the parents. In order to detect the markers linked to powdery mildew disease resistance, a strategy of combining the DNA pooling from selected segregants and genotyping was adopted. The association of putative markers identified based on DNA pooling from selected segregant was established by Single Marker Analysis (SMA). The results of SMA revealed that SSR markers, CEDG044 and DMBSSR080 showed significant association with powdery mildew and accounted for 81.5 per cent and 77.5 per cent of the total variation, respectively. The results obtained from the DNA pooling of phenotypic extremes could be a useful strategy to detect the genetic loci with major effects of the complex trait such as disease resistance in mungbean.

Keywords: Bulk segregant analysis, Powdery mildew, Mungbean and SSR markers

Mungbean [*Vigna radiata* (L.) Wilczek], also known as green gram is widely cultivated in south and Southeast Asia. India alone accounts for most of the mungbean harvest worldwide with more than half of the world's total production, with the total cultivable area of about 6 million hectares (Nair *et al.,* 2013), being occupied by mungbean. Apart from its importance as food and cash crop, mungbean has emerged as an important source of proteins, carbohydrates (51%), protein (24% - 26%), minerals (4%), and vitamins (3%) (Karthikeyan *et al.,* 2014). Their roots fix atmospheric nitrogen through symbiosis with nitrogen-fixing Rhizobium bacteria that in turn improves the soil quality (Yaqub *et al.,* 2010).

In the present scenario of the increasing global human population, decreasing arable land, predicted increases of water scarcity, soil salinity, severe diseases, emerging resistance of pests and pathogens to pesticides and climate change pose significant challenges to modern mungbean research. The biotic stresses *viz., yellow mosaic virus, cercospora* leaf spots and powdery mildew diseases cause severe economic losses to mungbean production. Among them powdery mildew disease (PM) is an important fungal disease caused by *Erysiphe polygoni* causing more than 40 per cent of yield loss if there is no prevention or even cause death of the plants if it occurs at the seedling stage apart from degrading the seed quality (Bainade *et al.,* 2014).

Although the disease can be controlled by chemical spraying, the farmers rarely practice such control measures due to the increase in production cost. In addition, the use of pesticides can negatively affect health and environment. Therefore, using resistant varieties is the most desirable means of managing the disease. The study was therefore conducted with the aim of identifying Recombinant inbred lines (RILs) resistant to powdery mildew disease (PM) using bulk segregant analysis

Material and Methods

Plant material and field design: Sixty four mungbean RILs derived from the cross of chinamung×LM-1668 were screened for identification of resistance sources

against natural infection by powdery mildew disease under field conditions at the University of Agricultural Sciences, Bangalore during *rabi* 2018-19. The RILs were planted during mid October and harvested during the first weeks of January. Each test entry was planted in a single row subplot of 1m length in an augmented design with row to row and plant to plant spacing of 45 cm and 10 cm, respectively. Susceptible check (Chinamung) was also planted in each plot along with test entries. All the recommended package of agronomic practices were followed except spraying of plant protection chemicals, which was meant to allow maximum inoculum of powdery mildew. The natural disease incidence was quite severe during the season due to conditions favourable for the development of the disease. Disease intensity on each accession was recorded on 40 days after sowing (DAS), 50 DAS and at the time of harvesting. Powdery mildew was scored on 0-5 scale as recommended by Reddy *et al.* (1994) where as the susceptible check rows exhibited hundred percent infections.

DNA extraction and parental polymorphism study: Genomic DNA was isolated from fresh leaf tissue of 64 F_8 recombinant inbred lines along with both the parents (chinamung and LM-1668) as described by Doyle and Doyle, 1987. The quality and quantity of DNA were estimated using both 0.8 per cent agarose gel electrophoresis and Nano Drop.

The final DNA concentration was adjusted to 50 ng/µl. Parental polymorphism survey involving 248 SSR markers was carried out. PCR was performed in a 9 µl volume containing 50 ng of template DNA, 0.3 units of Taq DNA polymerase (Bangalore Genei Ltd., Bengaluru, India), 2.5 mM of dNTPs, and 0.2 µM primers in a 1 × PCR Taq buffer containing $MgCl_2$. The amplification was carried out by Eppendorf Mastercycler Germany. PCR conditions included 94°C of 5 min. for initial denaturation followed by 35 cycles each consisting of a denaturation step for 1 min at 94°C, an annealing step for 45 sec at 40°C, an extension step for 1 min at 72°C and the final extension for 10 min at 72°C. Amplified products were separated by 3.0 per cent Metaphor agarose gel electrophoresis at 70 V. The gels were stained with ethidium bromide and visualized on a digital gel documentation and image analysis system (Alpha Innotech, Multimage TM Light, Cabinet Filter Positions - JH Bio Innovation Pvt., Ltd., Bengaluru, India).

Bulk Segregant Analysis and SSR Analysis

BSA was performed using the protocol given by Liu *et al.* (2001). DNA bulks of plants with extreme resistance and those with extreme susceptibility were prepared from phenotyped progenies. This was done by pooling aliquots, containing equivalent amounts of total DNA approximately, 50 ng/µl from each of ten highly resistant and ten highly susceptible plants of the RILs based on phenotypic observations. Polymorphic SSR primers between the parents were used to screen the parents and the two bulked DNA samples. DNA of individual RILs that were included in bulks was also analyzed with co-segregating markers to confirm their linkage to the PM disease resistance. The SSR markers that were polymorphic among the parents and the bulks were used for progeny analysis. DNA of the RILs and parents were analyzed to study co-segregation of these markers.

Data Analysis

The clearly resolved amplicons of SSR were scored manually ashomozygote for the allele for susceptible parent (A), homozygote for the allele for resistant parent (B) in the data sheet. Chi-square (χ^2) test was performed to test the goodness of fit of the F_8 population for the phenotyping and marker data by comparing an observed frequency distribution with an expected one. Marker-trait association was analyzed by simple linear regression method to know the association between the markers and the PM disease score using software Microsoft Office 2007 Excel frequency distribution curve for PM resistance of 64 F_8 RILs were drawn separately using Microsoft Office 2007 Excel.

Results and Discussion

In order to validate the markers associated with PM resistance, RILs derived from a cross between

chinamung x LM-1668 were phenotyped as resistant and susceptible based on the field evaluation by using rating scale. The results revealed a considerable amount of variability among RILs for powdery mildew resistance. Out of 64 F_8 RILs screened for powdery mildew under field condition, 12 RILs were resistant (R0), 20 moderately resistant (R1), 13 moderately susceptible (MS), 8 susceptible (S), 10 were highly susceptible (HS) to powdery mildew while none recorded higher resistance (HR). The varying responses of each RIL screened for powdery mildew in the season are presented in Table 1. The chi-square test (χ^2) was employed for phenotypic analysis which revealed a significant 1:1 segregation ratio of the resistance trait in the F_8 RIL population. This segregation pattern (Table 2) of resistance trait showed that PM resistance and susceptibility is controlled by a single or a group of closely linked genes, thus confirming the earlier findings of Priya *et al.,* 2012 and Reddy, 2009.

Identification of PM Resistance using Bulk Segregant and SSR Analysis

Several strategies have been proposed to identify molecular markers near a gene / QTL of interest with reduced number of plants to be genotyped. The two

TABLE 1

Grouping of 64 F_8 RILs of the cross Chinamung × LM-1668 based on Powdery mildew reaction under field conditions

Scale	Per cent leaf infection	Disease Reaction	Number of RILs	RILs
0	0	HR	0	
1	1 - 5	Resistant (R0)	12	C2-6-1, C2-14-3, C2-17-1, C2-63-2, C2-108-1, C2-115-3,C2-120-4, C2-134-2, C2-237-5, C2-243-3, C2-270-2, C2-H-78-1
2	5.1 - 30	Moderately Resistant (R1)	21	C2-15-1, C2-34-1, C2-72-2, C2-89-1, C2-93-1, C2-106-1, C2-247-3, C2-259-1, C2-J-95-2, C2-I-104-2, C2-J-123-1, C2-J-171-C2-113-2, 3,C2-K-187-1, C2-K-192-2, C2-K-199-1, C2-K-207-1, C2-K-211-4, C2-K-213-1, C2-K-227-1
3	30.1 - 65	Moderately susceptible (MS)	13	C2-65-2, C2-121-1, C2-199-2, C2-236-2, C2-243-3, C2-H-78-1, C2-J-96-3, C2-101-2, C2-K-185-1, C2-K-188-1, C2-193-2, C2-K-212-1, C2-K-252-3
4	65.1 - 90	Susceptible (S)	8	C2-9-1, C2-30-1, C2-82-1, C2-83-1, C2-110-1, C2-117-1, C2-119-1, C2-K-211-1
5	90.1 - 100	Highly susceptible (HS)	10	C2-122-1, C2-I-122-1, C2-I-124-1, C2-J-160-2, C2-J-161-2, C2-J-170-1, C2-J-173-2, C2-K-206-4, C2-K-214-1, C2-K-210-1

TABLE 2

Chi-square test for segregation of resistance and susceptibility in F_8 populations during *rabi* season, 2018/19 revealing nature of inheritance to powdery mildew disease

F_8 Generation	Total plants	Powdery mildew				Ratio S:R	χ^2	*P* value
		Observed		Expected				
		R	S	R	S			
Chinamung×LM1664	64	34	30	32	32	1:1	0.125	3.85

main strategies are selective genotyping and bulk segregant analysis (BSA). Selective genotyping is relatively a low-cost approach to detect QTL with large effects by genotyping individuals from the two tails of the phenotypic distribution. Bulk segregant analysis, a gene tagging technique (Collard *et al.,* 2005), has been successfully used to identify markers associated with disease resistance (Akinbo *et al.,* 2007; Priya *et al.,* 2013 and Yi *et al.,* 2013). Several studies have been conducted to identify resistant sources of powdery mildew in mungbean (Zhang *et al.,* 2008; Kasethanan *et al.,* 2010 and Chankaew *et al.,* 2013). In this study, an attempt was made to identify markers linked to resistance to powdery mildew disease using bulk segregant analysis.

Using the BSA method, two bulks having distinct and often contrasting phenotypes for the trait of interest are generated from a segregating population from a single cross. Seventeen polymorphic markers between the parents were used for screening of parents Chinamung, LM-1668, resistant bulk (RB) and susceptible bulk (SB) along with the RIL populations used to obtain the respective bulks. Two markers CEDG044 and DMBSSR088 clearly distinguished the susceptible bulks from resistant bulks (Fig. 1 and 2). Bainade *et al*. (2014) reported that out of 54 primers used to screen greengram lines, only one

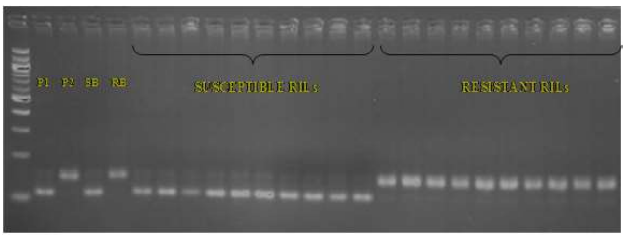

Fig.1 : Co-segregation of the SSR marker CEDG044 showing clear polymorphism in parents, bulks abd RILs that made up the bulks.

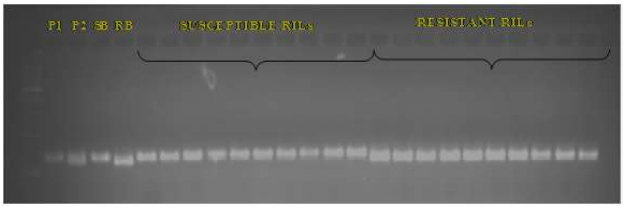

Fig. 2 : Co-segregation of the SSR marker DMBSSR080 showing clear polymorphism in parents, bulks abd RILs that made up the bulks.

produced specific fragment in both the bulks and was found polymorphic between the parents as well as resistant and susceptible bulk, indicating its possible linkage in BSA. The 64 RILs were therefore genotyped with these two primers to as certain / understand their possible association with powdery mildew resistance. Segregation pattern with marker CEDG044 recorded a resistant allele in 34 RILs; susceptible allele of recipient was amplified in 30 RILs. Similarly for marker DMBSSR08033 RILs showing donor allele while 30 RILs of recipient allele. Genetic analysis with chi-square test indicated goodness of fit to the expected ratio of 1:1 for co-dominant marker indicating the association of CEDG044 and DMBSSR088 with PM resistant gene in the present RIL population. To determine the strength of association between the putative markers and the respective phenotypes, linear regression analysis was carried out using marker genotype as groups. The simple regression analysis between phenotypic data of PM resistance and the genotypic data of SSR markers CEDG044 and DMBSSR088 indicated that these markers are significantly linked with PM resistance (Table 3). Similar results have been reported by Karthikeyan *et al.,* 2014; Sudha (2009) and Anushya (2009). Such lines which are showing stable resistant response to the powdery mildew can be screened for yield related traits and should be recommended for the development of highly resistant breeding lines for powdery mildew disease with high yielding ability in green gram (Priya *et al.,* 2013; Pool Sawat, 2017). Several similar studies have been conducted in different crops using BSA. Gupta and Gupta (2013) used 31 markers that were found to be polymorphic between the parents in mungbean to study genes linked to mungbean yellow mosaic virus

TABLE 3

Association of molecular markers with powdery mildew resistance by single marker analysis

SSR Marker	MSS	F-Value	P-Value	R^2 (%)
CEDG044	56233.609 ***	273.764 ***	<0.001	81.5
DMBSSR080	93.81869 ***	130.99 ***	<0.001	77.5

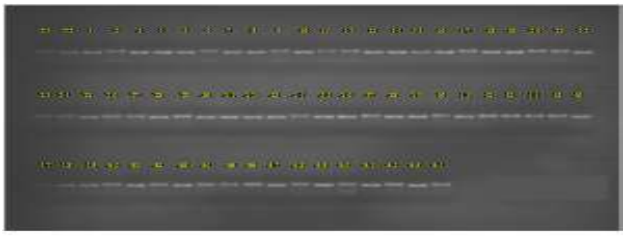

Fig. 3 : Genotyping of the RILs with CDG044

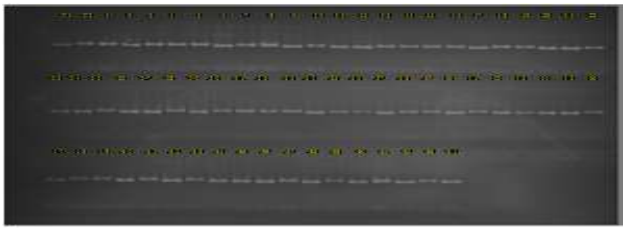

Fig. 4 : Genotyping of the RILs with DMBSSR080

P1:Chinamung,	P2: LM1668,	1: C2-6-1,	2: C2-9-1,	3: C2-14-3,
4: C2-15-1,	5: C2-17-1,	6: C2-30-1,	7: C2-34-1,	8: C2-63-2,
9: C2-65-2,	10: C2-72-2,	11: C2-82-1,	12: C2-83-1,	13: C2-89-1,
14: C2-93-1,	15:C2-106-1,	16: C2-108-1,	17: C2-110-1,	18: C2-113-2,
19:C2-115-3,	20:C2-117-1,	21:C2-119-1,	22: C2-120-4,	23: C2-121-1,
24: C2-122-1,	25:C2-134-2,	26: C2-199-2,	27: C2-236-2,	28: C2-237-5,
29: C2-238-3,	30: C2-243-3,	31: C2-247-3,	32: C2-259-1,	33: C2-270-2,
34: C2-H-78-1,	35: C2-H-80-1,	36:C2-J-95-2,	37:C2-J-96-3,	38: C2-J-101-2,
39: C2-J-104-2,	40: C2-J-122-1,	41: C2-J-123-1,	42: C2-J-124-1,	43:C2-J-160-2,
44:C2-J-161-2,	45: C2-J-170-1,	46: C2-J-171-3,	47:C2-J-173-2,	48: C2-K-185-1,
49: C2-K-187-1,	50:C2-K-188-1,	51: C2-K-192-2,	52:C2-K-193-2,	53:C2-K-199-1,
54: C2-K-200-3,	55: C2-K-206-4,	56: C2-K-207-1,	57:C2-K-209-3,	58: C2-K-210-1,
59: C2-K-211-4,	60: C2-K-212,	61: C2-k-213-1,	62:C2-K-214-1,	63: C2-K-227,
64: C2-K-252-4				

resistance. The marker CEDG 180 marker was found to be linked with resistance gene by the bulk segregant analysis. Shoba *et al.* (2012) identified the SSR marker PM384100 allele for late leaf spot disease resistance in groundnuts by bulked segregant analysis. The identified SSR marker PM 384100 was able to distinguish the resistant and susceptible bulks and individuals for late leaf spot disease in groundnut.

The association of these two markers with powdery mildew resistance can be employed for selection of powdery mildew resistant genotypes in mungbean. This is because once powdery mildew disease resistance-responsible gene (or genes) have been identified and further characterized is not only potentially useful for the development of durable powdery mildew-resistant cultivars but also is likely to be of value for the improvement of other related cultivated leguminous plants.

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