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Genetics of white rust resistance in Indian mustard (*Brassica juncea* L.) and its validation using molecular markers

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Abstract

White rust resistance loci (AcB1-A4.1 and AcB1-A5.1) associated with intron polymorphic (IP) markers i.e. At5g41560 and At2g36360, respectively, were used for validation of P_1, P_2, F_1, F_2, BC_1F_1 and BC_2F_1 generations. The donor parents namely, Bio-YSR and BEC-144 produced desired banding pattern of 430 and 750 bp while recipients *viz*., NRCHB 101 and DRMR-150-35 exhibited different pattern from donors confirming white rust resistance loci 4.1 and 5.1 with marker At5g41560 and At2g36360, respectively. Confirmation of these set of two IP markers in the parents and F_1s lead us to further screening of selected F_2, BC_1F_1 and BC_2F_1 populations. Available data on white rust reaction in different generations under study revealed that single dominant gene is responsible for white rust resistance. Potential of molecular markers in developing white rust resistant genotypes is proved under present study.

Key words: Marker assisted selection, *Brassica juncea*, white rust resistance, marker validation

Introduction

White rust, caused by oomycete pathogen *Albugo candida*, is highly destructive disease of Indian mustard particularly in India. This fungal pathogen results in heavy yield losses by affecting both vegetative and reproductive phases of the plants, particularly in systemic infection (Saharan and Verma 1992; Bisht et al. 1994). Substantial crop damage and yield losses in susceptible cultivars were up to 20-60% with more drastic losses to 90% (Kolte 2002; Khunti et al. 2003; Sachan et al. 2004; Kumar and Kalha. 2005). Although, stable donors for white rust resistance are available but obligate nature of the pathogen limits the availability of inoculum from the infected plants making breeding efforts difficult. Large scale screening of breeding material as well as segregating population with artificial inoculation is also a hard task (Varshney et al. 2004). Direct selection for resistance by the use of tightly linked DNA markers is the only solution to artificial inoculation, which would enable and promote direct transfer of the white rust resistance gene(s) to agronomically superior genetic background without the need for establishment of artificial epiphytotic conditions. Several studies on genetic analysis of white rust resistance have identified resistance loci and molecular markers linked to it (Prabhu et al. 1998; Cheung et al. 1998; Mukherjee et al. 2001; Somers et al. 2002; Varshney et al. 2004). Earlier, Panjabi et al. (2010) mapped two independent loci (AcB1-A4.1 and AcB1- A5.1) with the assistance of PCR based intron polymorphic (IP) markers on linkage groups A4 and A5 in eastern European lines, Heera and Donskaja-IV, respectively. Singh et al. (2015) successfully validated a set of 25 genotypes of Indian mustard and three F_2 populations by using already reported *Arabidopsis*-derived intron polymorphic (IP) markers (At5g41560 and At2g36360), which were highly linked with white rust resistant loci i.e. AcB1-A4.1 and AcB1-A5.1, respectively. These markers can be efficiently used in molecular marker assisted (MAS) breeding for gene pyramiding. Therefore, the present investigation was undertaken to validate the already reported intron polymorphic (IP) markers (At5g41560 and At2g36360) derived from *Arabidopsis*, which were highly linked with AcB1-A4.1 and AcB1-A5.1 loci, respectively.

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Materials and methods

Plant materials

The materials for the present investigation comprised of parents, P_1=NRCHB101 and DRMR150-35, P_2=BEC-144 and BioYSR, their F_1s and F_2 (NRCHB101 x BEC-144, NRCHB101 x BioYSR, DRMR150-35 x BEC-144 and DRMR150-35 x BioYSR) and backcrosses (BC_1 and BC_2). The details of parental genotypes taken for present investigation are given in Table 1. The Indian mustard varieties i.e. NRCHB 101 and DRMR 150-35, susceptible to white rust, were used as recipient parents whereas BEC-144 and BioYSR genotypes resistant to white rust were taken as donors. The details of molecular markers used for validation of rust resistance is given in Table 2.

Table 1. A brief description of genotypes used in study

S.No.	Parents	Reaction to white rust	Description	AcB1A4.1/ At5g41560	AcB1A5.1/ At2g36360
1	BEC-144	R	Exotic white rust resistant line.	R	S
2	BIO YSR	R	An indigenous white rust resistant somaclone (INGR No. 04099)	R	R
3	NRCHB101	S	Variety released for late sown conditions of Zone III.	S	S
4	DRMR150-35	S	Variety developed for rain fed conditions of Zone V.	S	S

R-Resistant, S-Susceptible

Crosses between the susceptible parents, NRCHB101 and DRMR150-35 and resistant donors BEC-144 and BioYSR were attempted at the experimental field of ICAR-DRMR, Bharatpur. During *rabi* 2015-16, selected buds were hand emasculated 24 to 48 hours before anthesis, and fresh pollen from the paternal parent was applied to the stigmas of recurrent parent and the flowers were protected with paper bags and labelled. The F_1 plants thus obtained were advanced during *kharif* 2016 (off-season nursery at IARI-regional station, Wellington, Tamil Nadu) and were backcrossed with recurrent parent to obtain BC_1F_1 generations. BC_1F_1 generation plants were raised in *rabi* 2016-17, selected for resistance and were further backcrossed to the corresponding parent of each cross to produce BC_2F_1 generation. The BC_2F_1 generation plants were raised in *rabi* 2017-18 and were further backcrossed for generation advancement. Each generation plants i.e. F_1, BC_1F_1, BC_2F_1 were validated using set of two intron polymorphic molecular markers linked to white rust resistance gene in their corresponding sowing seasons.

Two rows of BC_1F_1 plants along with parents (NRCHB101, DRMR150-35, BEC-144 and BioYSR) and F_2 were sown (*rabi* 2016-17), whereas BC_2F_1 plants were sown in *rabi* 2017-18. The materials was shown in 5 m plot having 5 rows in each plot with a spacing of 30 x 10 cm. Recommended agronomic practices for raising a healthy crop were followed.

Phenotyping in field and statistical analysis

The parents and segregating generations were subjected to artificial inoculation and reactions to white rust were recorded during their corresponding sowing seasons. Plant susceptibility for white rust reaction was rated after two weeks of inoculation. For recording the observations for white rust, the total number of plants taken in each generation is shown in Table 3.

Methods of inoculation

Primary inoculum was prepared by grinding previous year oosporic material and was mixed with the seeds and also applied into the soil at the time of sowing.

Table 2. Details of molecular markers used for validation of white rust resistance genes

S. No.	Locus code	Marker	Oligo sequence (5'→3')	Amplicon size (bp)	References
1	AcB1-A4.1	At5g41560F At5g41560R	GAGGTGGAAGAGTACGGTTGTG CCTCACAATTTCAGTCAACATCGT	~430	Panjabi et al. (2010)
2	AcB1-A5.1	At2g36360F At2g36360R	GCCACCTCCTAGATGTGGTCATA GTCCATCCAGGTGTTTCACG	~750	Panjabi et al. (2010)

Table 3. Segregation pattern for white rust resistance in crosses among susceptible parents and resistant donors

Cross	Locus tested	Genera-tions	Total plants	Observed		Expected ratio	χ^2	P value
				R	S			
	AcB1A4.1/ AcB1A5.1	P_1	31	0	31	-	-	-
		P_2	28	28	0	-	-	-
NRCHB101(P1) x BEC-144(P2)		F_1	32	32	0	-	-	-
		F_2	100	67	33	3:1	3.4	0.05
F_1 x NRCHB101		BC_1F_1	28	17	11	1:1	1.2	0.1
		BC_2F_1	81	48	34	1:1	2.8	0.05
	AcB1A4.1/ AcB1A5.1	P_1	43	0	43	-	-	-
		P_2	49	49	0	-	-	-
NRCHB101(P1) x BioYSR(P2)		F_1	23	23	0	-	-	-
		F_2	115	78	37	3:1	3.1	0.05
F_1 x NRCHB101		BC_1F_1	48	30	18	1:1	1.50	0.1
		BC_2F_1	62	37	25	1:1	2.4	0.1
	AcB1A4.1/ AcB1A5.1	P_1	29	0	29	-	-	-
		P_2	37	37	0	-	-	-
DRMR150-35(P1) x BEC-144(P2)		F_1	20	20	0	-	-	-
		F_2	168	116	52	3:1	3.2	0.05
F_1 x DRMR150-35		BC_1F_1	36	20	16	1:1	0.6	0.1
		BC_2F_1	51	29	22	1:1	1.0	0.1
	AcB1A4.1/ AcB1A5.1	P_1	36	0	36	-	-	-
		P_2	24	24	0	-	-	-
DRMR150-35(P1) x BioYSR(P2)		F_1	19	19	0	-	-	-
		F_2	110	75	35	3:1	2.7	0.05
F_1 x DRMR150-35		BC_1F_1	40	20	20	1:1	0.00	0.995
		BC_2F_1	77	41	36	1:1	0.4	0.5

White rust pustules from heavily infected fresh leaves served as secondary source of inoculum. From these leaves sporangial suspension was prepared by mixing sterile distilled water and it was adjusted to 10^4 sporangia/ml with the aid of haemocytometer. The sporangial suspension of *Albugo candida* was sprayed directly to the plants at two stages first at 2/3 leaf and second at initiation of flowering, in the evening time. The experimental fields were frequently irrigated to maintain relative humidity and moisture with regular tap water spraying as per the requirement. After spraying, the crop was continuously monitored for appearance of the disease. The white rust disease assessment was done as per the method described by Fox and Williams (1984). The genotypes were evaluated for disease infestation on ventral surface of leaf after 10 days of inoculation or the disease appeared. Scoring of the disease was done by using 0-9 scale (Williams 1985) and the level of disease was classified into six categories: “0” as no symptoms or necrosis, “1” as hypersensitive response, “3” as one or few isolated pustules on the abaxial or the adaxial surface, sometimes chlorosis/necrosis, “5” as moderate pustule density on the abaxial and/or adaxial surface,

sometimes chlorosis or necrosis, "7" as high pustule density on the abaxial surface and moderate density on the adaxial surface and "9" as heavy pustule density on both the abaxial and the adaxial surfaces. The goodness of fit of observed and expected frequency in segregating generations was done by Chi-square ($\chi 2$) test.

DNA extraction and marker analysis

Genomic DNA was isolated from young and healthy leaves using the standard Cetyl Trimethyl Ammonium Bromide (CTAB) protocol (Doyle and Doyle 1990). A set of two earlier reported IP markers derived from *Arabidopsis* that are linked to white rust loci AcB1-A5.1 and AcB1-A4.1, respectively (Panjabi et al. 2010) were used for amplification of parents, F_2, BC_1F_1 and BC_2F_1 populations derived from crosses namely NRCHB 101 x BEC-144, NRCHB 101 x BioYSR, DRMR 150-35 x BEC-144 and DRMR 150-35 x BioYSR.

PCR thermal amplification was carried out in 10 μl reaction volume containing 25 ng of genomic DNA, 1.0 unit Taq DNA polymerase (Dream Taq),10X PCR assay buffer with 1.5 mM $MgCl_2$, 20 ng each primer and 0.2 μld NTPs mix. The volume was made up to 10 μl using nuclease free water. PCR amplification was carried out in the Eppendorf Thermocycler. The PCR cycles consisted of initial denaturation for 5 min at 94°C, cyclic denaturation for 30 s at 94°C, annealing temperature for 40 s at 56-58°C and the primer extension for 30 sat 72°C followed by final extension for 7 min at 72°C. The PCR cycle was repeated 35 times followed by incubation at 4°C. PCR-amplified products were separated electrophoretically on 2.5 % agarose gel containing 0.01% ethidium bromide prepared in 1x TAE buffer (Tris-Acetic acid-EDTA). The amplicon sizes were determined by comparing with 100bp DNA Ladder (Thermo Scientific). The gel was run for 3 h at 80V. After electrophoresis, the amplified DNA fragments were visualized by staining the gel using ethidium bromide and photographed using a gel documentation system (IG/LHR, Syngene, UK).

Results and discussion

Phenotypic evaluation of genotypes

In the present investigation, all parental lines screened for the disease clearly indicated that the donors Bio-YSR and BEC-144 were resistant to local *A. candida* population whereas recipients, NRCHB101 and DRMR 150-35 were susceptible or highly susceptible to white rust disease. Several other workers (Singh et al. 2015; Yadava et al. 2012; Vignesh et al. 2011) have also used the same donors in their studies on genetics of white rust resistance. The F_1s (NRCHB101 x BEC-144, NRCHB101 x BioYSR, DRMR150-35 x BEC-144 and DRMR150-35 x BioYSR) were white rust indicating complete dominance of white rust resistance gene. The segregation pattern of white rust resistance in F_2 population follow 3 resistant (R) :1 susceptible (S) ratio in each cross with χ^2 value of 3.4, 3.1, 3.2 and 2.7, respectively (Table 3). Similar studies on mode of inheritance of white rust resistance using BEC-144 and BioYSR sources of white rust resistance have been reported earlier (Singh et al. 2015; Vignesh et al. 2009, 2011) indicating monogenic nature of white rust resistance gene.

The segregation analysis in backcross generations (BC_1F_1) from the crosses between F_1 and susceptible parent in present study was almost in equal frequencies of 1 resistant: 1 susceptible plant indicating the presence of a single major locus imparting resistance to white rust. Several other researchers also reported monogenic inheritance of white rust resistance (Sachan et al. 2000; Chauhan et al. 2001; Prabhu et al. 1998; Mukherjee et al. 2001; Punjabi et al. 2010; Yadava et al. 2011). It was further confirmed through the segregation of 1 resistant: 1 susceptible while screening the BC_2F_1 generation for white rust resistance in the field Singh et al. (2012) also studied mode of inheritance in four crosses, Krishna x WRR-9801, Pusa Jaikisan x WRR9801, PusaBold x WRR9801 and Jagganth x WRR9801 involving a different set of crosses with different parents. Manjunath et al. (2007) determined similar ratios of resistance : susceptible in F_2 and backcross generations dominant nature of white rust resistance confirming in Indian mustard.

Genotyping for white rust

In present investigation intron polymorphic markers (At5g41560 and At2g36360) linked to white rust resistance loci AcB1-A4.1 and AcB1-A5.1, respectively, (Punjabi et al. 2010) were used for validation of six generations i.e., P_1, P_2, F_1, F_2, BC_1F_1 and BC_2F_1 (Table 2). IP markers used in present study were also used earlier by Singh et al. (2015). They validated these markers in a set of 25 genotypes of Indian Mustard and in three different F_2 populations and indicated that At5g41560 and At2g36360 are genotype-non specific markers that are highly linked to white rust resistance loci AcB1-A4.1 and AcB1-

A5.1, respectively. The donors Bio-YSR and BEC-144 revealed desired banding pattern of 430 bp and 750 bp while recipients *viz*., NRCHB101 and DRMR150-35, exhibited different pattern from donors confirming white rust resistance loci 4.1 (Fig. 1) and 5.1 (Fig. 2) with marker At5g41560 and At2g36360 respectively. Confirmation of these set of two IP markers in all the parents as well as F_1s lead to further screening of selected F_2, BC_1F_1 and BC_2F_1 populations.

According to Barr et al. (2000), marker validation is a process in which marker is tested for its efficacy in assessment of phenotype in different genetic backgrounds and in new populations. Varshney et al. (2004) developed and validated a more tightly linked marker for the white rust resistance gene by AFLP and cleaved amplified polymorphic sequence (CAPS) marker. They validated CAPS markers in two different F_2 populations derived from the crosses Varuna × BEC-144 and Varuna × BEC-286 and established the utility of molecular markers in marker assisted selection.

In a similar study on marker validation for white rust, RAPD markers for white rust resistance in an F_1-derived doubled-haploid (DH) population were identified, while white rust resistance linked markers (WR2 and WR3) which are efficient in identification of presence or absence of the resistance gene were developed by Prabhu et al. (1998) which can be utilized in breeding for white rust resistance in *B. juncea*. The F_2 populatins showed normal Mendelian segregation ratio of 3 (resistant):1(susceptible) for white rust resistance loci AcB1-A4.1 and AcB1-A5.1 (Table 3). Four BC_1F_1 populations, were genotyped for white rust resistance using IP markers namely, At5g41560 and At2g36360. Out of these, individuals developed from the crosses

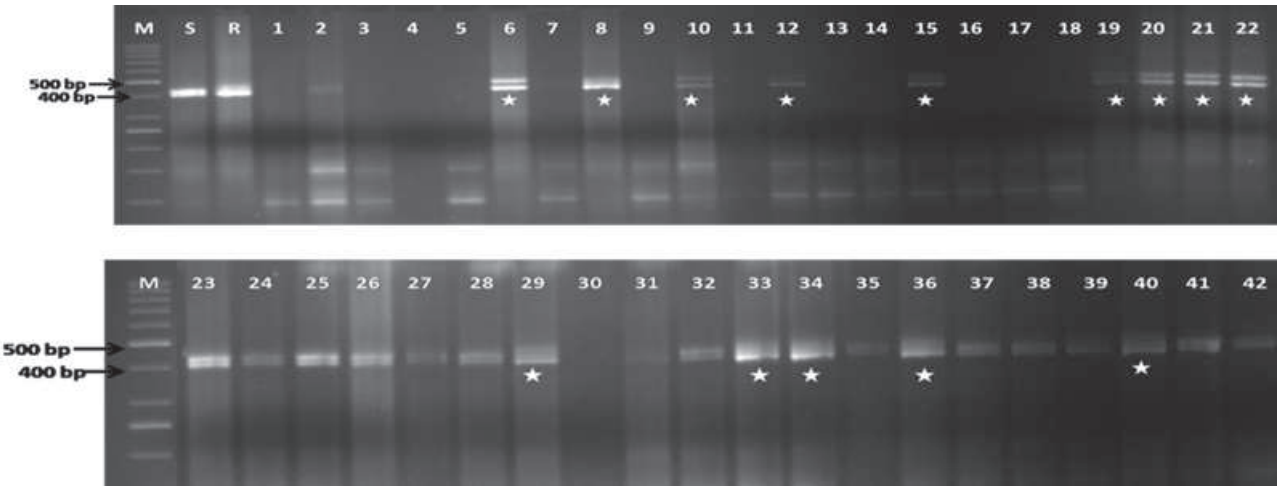

Fig. 1. Agarose gel showing validation of markers associated with white rust At5g41560 (AcB1-A4.1) loci, with an expected product size 430bp. M-50bp ladder, S (Susceptible)-DRMR150-35 (Recipient parent) and NRCHB101 (Recipient parent), R (Resistant)-BEC-144 (Donor) and BioYSR (Donor), 1-6 BC_1F_1 (NRCHB-101xBioYSR), 7-12 BC_1F_1 (NRCHB-101xBEC-144), 13-17 BC_1F_1 (DRMR150-35xBioYSR), 18-22 BC_1F_1 (DRMR150-35xBEC-144), 23-27 BC_2F_1 (NRCHB-101xBioYSR), 28-32 BC_2F_1 (NRCHB-101xBEC-144), 33-37 BC_2F_1 (DRMR150-35xBioYSR), 38-42 BC_2F_1 (DRMR150-35xBEC-144). *indicates resistant to white rust

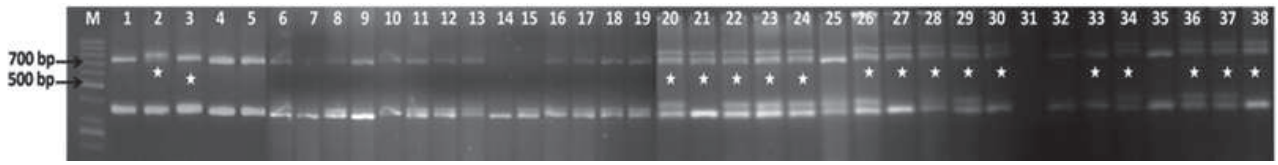

Fig. 2. Agarose gel showing validation of markers associated with white rust At2g36360 (AcB1-A5.1) loci, with amplified product size of 750 bp M-50bp ladder. 1. BEC-144 (Donor) 2. Donskaja (Control) 3. BioYSR (Donor) 4. DRMR150-35 (Recipient parent) 5. NRCHB101 (Recipient parent), 6-8 BC_1F_1 (NRCHB-101xBEC-144), 9-11 BC_1F_1 (DRMR150-35xBEC-144), 12-15 BC_2F_1 (NRCHB-101xBEC-144), 16-19 BC_2F_1 (DRMR150-35xBEC-144), 20-23 BC_1F_1 (NRCHB-101xBioYSR), 24-28 BC_1F_1 (DRMR150-35xBioYSR), 29-33 BC_2F_1 (NRCHB-101xBioYSR), 34-38 BC_2F_1 (DRMR150-35xBioYSR)

NRCHB101 x BioYSR and DRMR150-35 x BioYSR exhibited heterozygous banding pattern with both the white rust resistance loci (AcB1-A4.1 and AcB1-A5.1) linked to IP markers. Whereas, BC_1F_1 populations developed from the crosses DRMR-150-35 x BEC-144 and NRCHB-101 x BEC-144, exhibited heterozygous banding pattern with white rust resistance loci AcB1-A4.1 linked to IP marker At5g41560. All the plants that were heterozygous for white rust resistance linked markers were backcrossed with susceptible parents to generate BC_2F_1 plants. Further, a total of 271 plants of BC_2F_1 population were analyzed with the white rust linked markers for presence of resistance allele in a heterozygous condition. The results indicated that individuals of the BC_2F_1 population had the alleles linked with these two IP markers showing resistance against white rust. The amplification patterns of two polymorphic markers At5g41560 and At2g36360 linked with white rust resistant genes in BC_1F_1 and BC_2F_1 population along with donor and recipient parents are shown in Figs. 1 and 2. Phenotypic data for disease reaction of resistance and susceptibility to white rust also segregated in 1(resistant):1(susceptible) ratio in the BC_2F_1 population. This indicates that single dominant gene controls white rust resistance. These results are in agreement with the findings of Vignesh et al (2009). The plants exhibiting heterozygotic banding pattern for white rust resistance gene were selected for further backcrossing with recurrent parent for generation advancement. These findings have potential use in marker-assisted selection (MAS) to develop Indian mustard cultivars with white rust resistance genes. Further due to high-selection accuracy of these markers for resistant plant sources, they can be used in MAS of the resistant genotypes.

Author's contribution

Conceptualization of research (VVS); Designing of the experiments (VVS, PM); Execution of field/lab experiments and data collection (VVS, MD, NG, Balbeer, MLM, PS, PKR); Analysis of data and interpretation (VVS, PM, MD); Preparation of manuscript (VVS, MD, NG, PS).

Declaration

The authors declare no conflict of interest.

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