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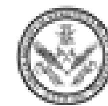

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RESEARCH ARTICLE

Validation of molecular markers for the identification of resistant sources against white rust disease of rapeseed mustard caused by *Albugo candida*

Pooja Upadhyay, A. K. Tewari[1*], Usha Pant[2], Prashant Vikram, Nitesh Singh and H. Rajashekara[3]

Abstract

White rust or white blister disease caused by oomycete fungi, [*Albugo candida* (Persoon) Roussal] is one of the major devastating fungal diseases of rapeseed (*Brassica napus*) mustard. The continuous emergence of new pathogenic races is responsible for the breaking down of the resistance in already existing resistant cultivars. So, for finding new resistant sources against the pathogen, molecular markers have become new tools. In the present study, validation of 29 simple sequence repeat (SSR) markers was done to know the presence or absence of resistance (R) genes against selected *Brassica* genotypes using PCR analysis. Marker trait association analysis and multiple regression analysis indicated a significant association of six markers, namely, At5g41560, Ni2BO3, BrgMS329, MB5, BRMS-017 and NI-F02a with the immune, highly resistant and moderately resistant *Brassica* cultivars. These molecular markers may be utilized for the identification of resistant genotypes against *A. candida* pathogens in rapeseed mustard.

Keywords: White rust, molecular makers, resistance, association analysis, *Albugo candida*.

Introduction

Oilseed crops are an important group of crops that are grown for their oil-rich seeds, which are used for a variety of purposes, including cooking, biodiesel production, and industrial applications (Chaganti et al. 2021; Ahmad et al. 2021). India is one of the major producers of oilseeds in the world, with a diverse range of crops being cultivated across the country. Among these, rapeseed (*Brassica napus*) and mustard (*Brassica juncea*) are some of the most important oilseed crops grown in India and are members of the Brassicaceae family. They are also used as a source of animal feed and as a cover crop to improve soil health (Sharma et al. 2018).

According to data from the Ministry of Agriculture and Farmers' Welfare, the total area under oilseed cultivation in India during 2021-2022 was around 26.4 mha, with a total production of around 36.7 mt (https://agricoop.nic.in/Documents/annual-report-2021-22, pdf). Rapeseed and mustard are predominantly grown in the northern and central regions of India a source of income for millions of farmers. Rajasthan alone accounts for around 40% of the total area and production of rapeseed and mustard in India, followed by Uttar Pradesh, Haryana, and Madhya Pradesh. In 2021-2022, the total area under rapeseed and mustard cultivation in India was around 7.23 mha, with a total production of around 9.6 mt (https://agricoop.nic.in/Documents/annual-report-2021-22.pdf). The average yield of these crops in India is around 1,327 kg/ha, which is lower than the global average of 1,762 kg/ha (Kalia et al. 2021). In addition, the by-products of oilseed processing, such as

Faculty of Agricultural Science, SGT University, Gurugram 122 505, Haryana, India.

[1]Department of Plant Pathology, GBPUA&T, Pantnagar 263 145, Uttarakhand, India.

[2]Department of Genetics and Plant Breeding, GBPUA&T, Pantnagar 263 145, Uttarakhand, India.

[3]ICAR-Directorate of Cashew Research, Puttur 574 202, Karnataka, India.

***Corresponding Author:** A. K. Tewari, Department of Plant Pathology, GBPUA&T, Pantnagar, 263 145, Uttarakhand, India, E-Mail:anandtewari9@gmail.com

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oil cake and meal, are used as animal feed (Chmielewska et al. 2021).

These crops are highly susceptible to a range of biotic and abiotic stresses, which can significantly reduce their yield and quality. Among the various biotic stresses, white rust caused by [*Albugo candida* (Persoon) Roussal] is one of the most important diseases that affect the productivity of these crops due to its destructive nature, wide distribution and ability to cause grain yield losses (Dev et al. 2020). Among different management practices available, breeding for resistant genotypes/cultivars is one of the most eco-friendly, economical, effective and best alternative methods for the management of white rust disease (Kalpana et al. 2019). White rust pathogen is emerging as a vital experimental model for exploring the molecular basis of disease resistance, host-pathogen interaction, pathogen virulence, and defense suppression phenomenon (Links et al. 2011; Nirwan et al. 2022). The resistance against white rust in *Brassica* crops is governed by a single dominant gene in *B. juncea* against pathogen race2, while *B.rapa* controls resistance against race2A, while three dominant genes in *B.napus* against race7(Punjabi et al. 2010; Pushpa et al. 2016). In this context, molecular markers have become a fundamental tool for crop breeding and improvement programmes because of their abundance, technical ease, detection at any developmental stage of the plant and not being affected by environmental factors.

However, the availability of molecular markers tightly linked to the gene(s) of interest would enable direct selection at the seedling stage without the need for the creation of artificial epiphytotic conditions. Several studies utilizing RFLP and RAPD molecular markers and cleaved amplified polymorphic sequence (CAPS) markers for mapping white rust resistance gene(s) in *B. juncea* have been conducted in the past (Somers et al. 2002; Varshney et al. 2004), but their suitability for mapping and MAS has not been much useful. Contrastingly, simple sequence repeats (SSR) or microsatellite markers are considered excellent as they are most suitable and efficient for the studies of population genetics and MAS facilitating the indirect selection of the linked gene or genes in breeding populations without the need for disease screening. The present study was carried out to validate already reported SSR markers against selected *Brassica* cultivars for the identification of white rust-resistant traits within a very short time and with more accuracy.

Materials and methods

Experimental material used in the study

Brassica genotypes used for the validation of markers were selected on the basis of the host-pathogen interaction study. A set of 44 diverse *A. candida* isolates were collected from 17 states of India and their description is given in Table 1 and Fig. 1. These were used in cross inoculation of 32 *Brassica* genotypes (Table 2) belonging to 12 different *Brassica* species planted under field conditions. About 29 Simple Sequence Repeat (SSR) markers, specific to white rust resistance were taken for validation based on the presence or absence of white rust resistance genes in selected different *Brassica* cultivars by using PCR technique. The collected samples were in the form of diseased leaf specimens from 8 *Brassica* genotypes. The purified samples were preserved at different temperatures in a deep freezer (-20°C) for further studies. Seeds of these cultivars were collected from the Department of Genetics and Plant Breeding, South Campus, New Delhi, Govind Ballabh Pant University of Agriculture & Technology, Pantnagar and different AICRP Rapeseed Mustard centers in India.

Pathogen inoculation

Fresh sporangial suspension of each pathogen isolate was prepared with a single white rust pustule collected by a sterile scalpel or blade in a glass vial containing 1-2 ml sterilized distilled cold water. The suspension was mixed properly and sporangial concentration in the suspension was maintained at about 2.5×10^5 zoosporangia per ml by using a hemocytometer. This suspension was kept overnight at 10°C for the release of zoospores. Seedlings of each *Brassica* cultivar were cross-inoculated with each pathogen suspension at the true leaf stage (12-15 DAS) with 10µl spore suspension by using a micropipette and ensured even inoculation of all the seedlings. After inoculation, plants were transferred to a plant propagator box, which

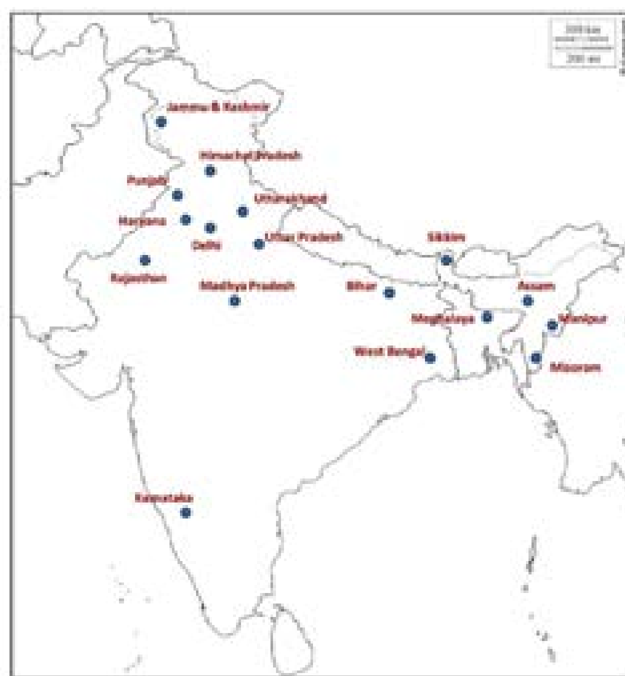

Fig. 1. Collection and isolation of *A. candida* isolates from different states of India

Table 1. List of purified *A. candida* isolates collected from diverse places of India

S. No.	Name of the state	Name of the isolate	Brassica species	Place of collection	Latitude & Longitude
1	Uttarakhand	Ac- *Bj*-Pnt	*B. juncea*	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
2		Ac-*B.np*-Pnt	*B. napus*	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
3		Ac-*B.ngr*-Pnt	*B. nigra*	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
4		Ac- B.rai-Pnt	Banarasi rai	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
5		Ac-Y.srs-Pnt	*B. rapavar.Yellow Sarson*	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
6		Ac-bioysr-Pnt	*B. juncea* (Bioysr)	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
7		Ac-*B.rug*-Pnt	*B. rugosa*	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
8		Ac-*Bj*-Kran-Pnt	*B. juncea* (Kranti)	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
9		Ac-O.rai-Pnt	Ornamental Rai	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
10		Ac-*Cir.arvs*-Pnt	*Circium arvense*	Pantnagar	29° 02′ 60» N 79° 30′ 59» E
11	Uttar Pradesh	Ac-*Bj*-Knp	*B. juncea*	Kanpur	26°26′59.6»N 80°19′54.8»E
12		Ac-*Bj*-Merut	*B. juncea*	Meerut	28° 59′ 4″ N 77° 42′ 21″ E
13		Ac-*Bj*-Faiz	*B. juncea*	Faizabad	26° 46′ 12″ N 82° 9′ 0.00″ E
14	New Delhi	Ac-*Bj*- Iari	*B. juncea* (Varuna)	IARI	28° 37′ 55″ N 77° 8′ 19″ E
15		Ac-*Bj*-Pbhr- Iari	*B. juncea* (Pusa Bahar)	IARI	28° 37′ 55″ N 77° 8′ 19″ E
16		Ac-bcs-Iari	*B. carinata* (BCS-1)	IARI	28° 37′ 55″ N 77° 8′ 19″ E
17		Ac-*Bj*-Uds	*B. juncea*	UDSC	28° 38′ 41″ N 77° 13′ 0″ E
18	Haryana	Ac-*Bj*-Hisr	*B. juncea*	Hisar	29° 9′ 6.69″ N 75° 43′ 16″ E
19		Ac-*Bj*-Sirs	*B. juncea*	Sirsa	29° 32′ 11″ N 75° 1′ 31″ E
20	Rajasthan	Ac-*Bj*-Alwr	*B. juncea*	Alwar	27° 33′ 39″ N 76° 37′ 30″ E
21		Ac-*Bj*-Bhart	*B. juncea*	Bharatpur	27° 13′ 1″ N 77° 29′ 22″ E
22		Ac-*Bj*-Sriganga	*B. juncea*	Sri Ganganagar	29° 90′ 94″ N 73° 88′ 00″ E
23		Ac-*Bj*-Kotpt	*B. juncea*	Kotputli	27° 13′ 1″ N 77° 29′ 22″ E
24	Punjab	Ac-*Bj*-Ludh	*B. juncea*	Ludhiana	30° 42′ 37″ N 76° 11′ 56″ E
25		Ac-*Bj*-Abhr	*B. juncea*	Abhor	30° 08′ 40″ N 74° 11′ 43″ E

Contd. ....

26	Madhya Pradesh	Ac-*Bj*-varu-Mor	*B. juncea* (Varuna)	Morena	26° 30′ 0.0″ N 78° 0′ 0.0″ E
27		Ac.*Bj*-mya.Mor	*B. juncea* (Maya)	Morena	26° 30′ 0.0″ N 78° 0′ 0.0″ E
28		Ac-*Bj*-vard-Mor	*B. juncea* (Vardan)	Morena	26° 30′ 0.0″ N 78° 0′ 0.0″ E
29		Ac-*Bj*-pb-Mor	*B. juncea* (Pusa Bold)	Morena	26° 30′ 0.0″ N 78° 0′ 0.0″ E
30	Himachal Pradesh	Ac-*Bj*-Simr	*B. juncea*	Sirmaur	30° 38′ 24″N 77° 26′ 24″E
31		Ac-*Bj*-Ponts	*B. juncea*	Ponta Sahib	30° 26′16″N 77°37′ 26″E
32		Ac-*Bj*-Kngr	*B. juncea*	Kangra	32° 5′ 59″ N 76° 16′ 8″ E
33	Jammu & Kashmir	Ac-*Bj*-Chata	*B. juncea*	Chatha	32° 43′ 58″ N 74° 51′ 51″ E
34	Karnataka	Ac-*Bj*-Kanach	*B. juncea*	Kannachalli	12° 52′ 15″N 76° 47′ 18″E
35		Ac-*Bj*-Dharw	*B. juncea*	Dharwad	12° 72′ 15″N 79° 39′ 13″E
36		Ac-*Bj*-Bang	*B. juncea*	Bangaluru	12°97′ 16″ N 77° 59′46″ E
37	Bihar	Ac-*Bj*-Samsti	*B. juncea*	Samastipur	25°51 39″N 85°46′56″E-
38	West Bengal	Ac-*Bj*-Kolk	*B. juncea*	Kolkata	22° 34′ 21″ N 88° 21′ 50″ E
39		Ac-Amr-Kolk	Amaranthus	Kolkata	22° 34′ 21″ N 88° 21′ 50″ E
40	Mizoram	Ac-*Bj*-Aizwl	*B. juncea*	Aizawal	23° 73′ 07″ N 92° 71′ 73″ E
41	Assam	Ac-*Bj*-Jorht	*B. juncea*	Jorhat	26° 45′ 21″ N 94° 12′ 34″ E
42	Meghalaya	Ac-*Bj*-Umi	*B. juncea*	Umiam	25° 39′ 11″ N 91° 53′ 3.4″ E
43	Manipur	Ac-*Bj*-Imphl	*B. juncea*	Imphal	24° 48′ 50″ N 93° 57″ 1″ E
44	Sikkim	Ac- *Bj*-Mrchk	*B. juncea*	Marchak	27° 28′ 97″ N 88° 58′ 87″ E

was kept in a dark place for 72 hours, with relative humidity (RH) of more than 90% and a temperature of 18 ± 2℃. After three days, plants were taken out and kept in a controlled environment of temperature (18 ± 2℃) and RH (more than 80%) in a glasshouse for further observation.

Observations on per cent disease index

Per cent disease index in each host against each isolate was recorded one week after the appearance of the disease at the true leaf stage using 0-6 rating scale as suggested by Conn et al. (1990).

The per cent disease index was calculated by using following formula:

$$\text{Disease index (\%)} = \frac{\text{Sum of all numerical ratings}}{\text{Number of leaves examined x Maximum rating}} \times 100$$

Rating scale	Leaf area (%) covered by the pustules	Disease reaction (DR)
0	No symptom	Immune (I)
1	0-5	Highly resistant (HR)
2	5-10	Resistant (R)
3	10-20	Moderately resistant (MR)
4	20-35	Moderately susceptible (MS)
5	35-50	Susceptible (S)
6	More than 50	Highly susceptible (HS)

Table 2. List of Brassica genotypes used for host pathogen interaction study

S. No.	Brassica spp./cultivar	S. No.	Brassica spp./cultivar	S. No.	Brassica spp./cultivar
1	*B. juncea* cv. Varuna	12	*B. nigra* 2782	23	*B. juncea* cv. EC 399301
2	*B. juncea* cv. Pusa Bold	13	*B. nigra* cv. Sangam	24	*B. juncea* cv. Kranti
3	*B. juncea* cv. EC 399313	14	*B. juncea* cv. Cutlass	25	*B. rapavar, brown sarson* cv. Pusa Kalyani
4	*B. juncea* cv. Heera	15	Eureca sativa	26	*B. rapa* var. *toria* cv. TL 15
5	*B. juncea* cv. Donskaja	16	*B. carinata* cv. DLSC 1	27	*B. rapa* var. *toria* cv. Bhawani
6	*B. napus* cv. Wester	17	*B. rapa* var. brown sarson cv. BSH 1	28	*B. rapa* var. *toria* cv. PT 303
7	*B. rapa* cv. Torch	18	Sinapis alba	29	*B. napus* cv. GSL 1
8	*B. rapa* cv. Tobin	19	*B. juncea* cv. NRCDR 515	30	*B. carinata* cv. Kiran
9	*B. rapa* cv. Candle	20	*B. juncea* cv. RH 30	31	*Raphanus sativus* (Local cultivar)
10	*B. juncea* cv. RL 1359	21	*B. rapa* var. Yellow Sarson cv. Ragini	32	*B. olerecea* (Local cultivar)
11	*B. rapa* var. Yellow sarson YSPB 24	22	*B. juncea* cv. Bioysr		

Validation of molecular markers

Isolation of high molecular weight genomic DNA is a prerequisite for molecular analysis. CTAB procedure described by Doyle and Doyle (1990) was used for the isolation of genomic DNA from the leaf of selected *Brassica* cultivars with required minor modifications. DNA polymerase chain reaction procedure described by Choi et al. (2006) was used and the following concentrations of PCR components were used. The sequence of 29 SSR primers obtained from the Department of Genetics and Plant Breeding, GBPUA&T used in the study is given in supplementary Table S1. The PCR amplified product was run along with 100 bp ladder (HiMedia) on 2.5% agarose gel using 1X TAE buffer at 80V for 2 hours and stained with ethidium bromide dye (4 µL/100 mL) (Supplementary Tables S2 and S3). The gel image was documented on the gel documentation system.

Data analysis

On the basis of gel pictures and banding profile of different primers, 0-1 scoring was done manually for each primer for the presence (1) and absence (0) of the band. On the basis of data scoring, a range of bands, polymorphic and monomorphic bands were identified. Data was analyzed by two methods, one by comparative analysis of amplified bands and another by using Multiple Regression analysis (MRA) for finding a statistical association between genotypic data and phenotypic data by using software SPSS version 2.04.

Results

The disease response of 44 *A. candida* isolates on 32 *Brassica* genotypes was recorded and the percent disease index (PDI) was calculated. On the basis of PDI, these genotypes were divided into 6 different categories/groups based on resistance and susceptibility. Among those, Varuna showed a maximum percent disease index ranging from 22.8 to 51.1% followed by Kranti (26.70–45.90%), RH 30 (28.60–45.60%), RL 1359 (22.6–40.2%) and considered as highly susceptible genotypes. Pusa Bold (15.5–38.85%), EC 399301 (13.80–37.30%), Bhawani (16.2–33.5%), TL 15 (14.8–33.5%), Pusa Kalyani (17.6–32.8%), YSPB 24 (17.4–30.5%), PT 303 (12.8–30.2%), EC 399313 (11.8-27.6%) considered as susceptible genotypes. Cutlass (13.7–22.8%), Bn2782 (12.67–23.4%), BSH 1 (11.5–17.9%), Torch (6.9–19.6%), Tobin (4.8–17.9%), Ragini (8.9–18.4%), Heera (6.3–16.6%), Bioysr (9.40–16.30%) considered as moderately resistant. Candle (5.6–13.6%), *Sinapis alba* (4.9–11.8%), Sangam (4.9–11.3%), *E. sativa* (3.5–6.7%) are considered as resistant genotypes against the disease. Donskaja (2.2–4.6%) *B. olerecea* (3.7–4.01%) are considered highly resistant. NRCDR 515, Wester, GSL 1, Kiran, DLSC 1, *Raphanus sativus* showing 0.00% disease index are considered immune or free from disease against all the *A. candida* isolates**.** Out of 6 groups, eight *Brassica* cultivars were selected on the basis of different level of resistance viz., highly susceptible (Varuna), susceptible (Pusa Bold), moderately resistant (Cutlass) all of *B. juncea*; resistant (Candle, *B. rapa*), highly resistant (Donskaja, *B. juncea*) and immune (NRCDR 515, *B. juncea*), Wester (*B. napus*), DLSC1 (*B. carinata*) for the validation of SSR molecular markers for white rust resistance.

Validation of SSR markers for white rust resistance in selected 8 Brassica genotypes

Out of 29 SSR markers used, 24 showed DNA amplification, while 5 (BRMS-006, BRMS-033, Ni3C05, NI02-D08a and sORA43) did not amplify any band after the PCR reaction

(Plate 1). An association between SSR markers and selected *Brassica* cultivars was done to identify suitable markers for the presence of resistance gene against *A. candida* by using two methods, one as comparative analysis of marker-genotype association and the other as multiple regression analysis (MRA) for statistical association between marker and genotypes.

Comparative analysis of DNA bands for finding molecular marker-genotypes association

For comparative analysis of association, amplified DNA bands for each marker were analyzed (Table 3). A maximum number of amplified DNA bands (34) were obtained in marker BrgMS70, followed by NA10-C01c and SSR Ni2-A07 with 27 positive amplifications, while the least number of allele amplifications (5 bands) was obtained in BrgMS329 and BrgMS1237, followed by At5g41940 and At5g41560 (7 bands). Out of 24 markers, 21 markers showed 100% polymorphism, while 3 markers viz. SSR Ni2-A07, BN25A and BrgMS70 showed monomorphic bands with 96.29, 90.00 and 97.05%, respectively. Polymorphism information content (PIC) value was calculated for each marker, which ranged from 0.046 to 0.968. The maximum PIC value (0.968) was observed in NA10-C01c, followed by BrgMS70 (0.906), while the minimum PIC value was observed in SSR Ni2-A07 (0.046), followed by BrgMS329 (0.078). Hence, markers with high PIC values could be highly useful in studying polymorphism in white rust resistance in diverse *Brassica* genotypes and in marker-assisted selection for white rust resistance for effective disease management.

The association between molecular markers and amplified alleles was analyzed for each *Brassica* genotype. For each marker, the presence (+) and absence (-) of amplified bands for each Brassica genotype were recorded (Table 4) and arranged in 0–1 binary matrix for the presence (1) and absence (0) of bands for analyzing obtained data. On this basis, markers were differentiated into different categories and the possible association of a particular marker with disease resistance was analyzed.

According to this, 07 markers viz. BRMS-006, MB5, BRMS-030, Ni2B03, Ni3C05, BrgMS329 and BrgMS1237 showed amplification in highly susceptible (Varuna) and susceptible (Pusa bold) genotypes while 08 markers viz. At5g41940, At5g41560, O110B11, BRMS-017, BN6A3, NI02-D08a, rgMS710 and NI03-H07a did not show any band in highly susceptible genotypes. Two markers viz. At5g41940 and BrgMS710 showed bands in resistant (Candle) and highly resistant (Donskaja) genotypes, 02 markers viz. At5g41560 and Ni2B03 showed bands in highly resistant (Donskaja) and Immune genotypes (NRCDR 515, DLSC1, Wester). Marker BrgMS329 showed only a single band in one immune (Wester) genotype, while 05 markers viz. SSR Ni2-A07, NA10-C01c, BrgMS339, BN25A and BrgMS70 showed bands in all the Brassica genotypes. Three markers viz. BrgMS787, O110B11 and NA10-C01c showed bands in moderately resistant, resistant, highly resistant, immune and susceptible genotypes, so these markers were not taken into consideration for association with resistant genes. Among all the markers, 03 markers viz. At5g41560, Ni2B03and BrgMS329 were found in association with only highly resistant and immune genotypes, so these markers can be further utilized to identify white rust resistant genotype or identify the locus particular to white rust resistant in any Brassica genotype. Besides these 03 other markers, viz. MB5, BRMS-017 and NI-F02a were associated with moderately resistant, highly resistant and immune genotypes without any association with highly susceptible or susceptible genotypes. Hence, they can also be possible markers to identify white rust-resistant genotypes. The rest of the markers did not show any significant association with white rust-resistant traits (Table 5). Cluster analysis of the designed binary matrix for marker-genotype association was done using Jaccard's similarity coefficient with software PAST version 4.03 and a dendrogram was obtained to differentiate the markers (Fig. 2). The comparative analysis of marker-genotype association and cluster analysis showed 06 markers viz. At5g41560, Ni2B03, BrgMS329, MB5, BRMS-017 and NI-F02a are associated with the immune, highly

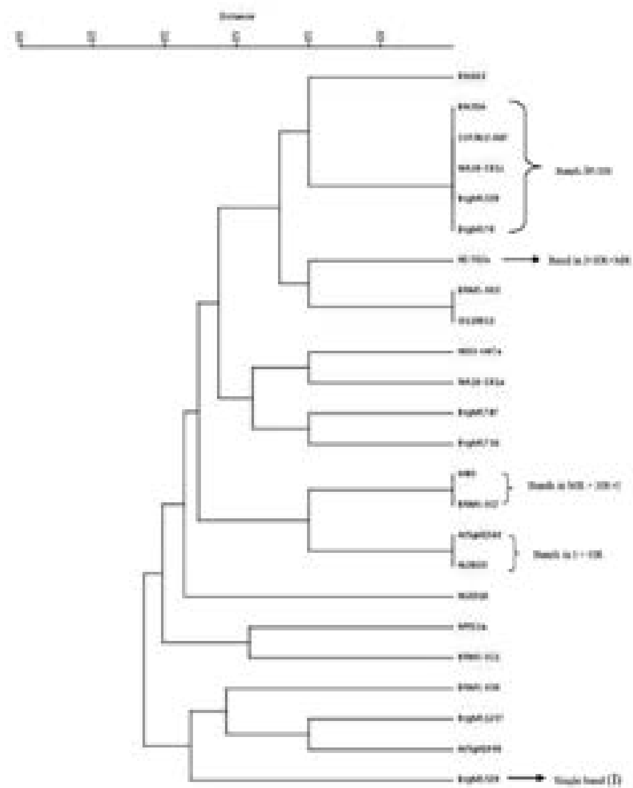

Fig 2. Cluster analysis of SSR markers for association between markers and selected *Brassica* genotypes

Table 3. Summary of molecular markers profile

S. No.	Marker	Total no. of bands	Polymorphic bands	Monomorphic bands	Percent Polymorphism	PIC value	Range of bands (bp)
1	At5g41940	7	7	0	100.00	0.171	240-600
2	At5g41560	7	7	0	100.00	0.359	300-1000
3	SSR Ni2-A07	27	26	1	96.29	0.046	180-1100
4	BRMS-003	21	21	0	100.00	0.890	190-1200
5	O110B11	25	25	0	100.00	0.937	600-1120
6	MB5	11	11	0	100.00	0.703	600-700
7	NA10-C01c	27	27	0	100.00	0.968	180-1190
8	BRMS-017	14	14	0	100.00	0.625	450-1080
9	BRMS-030	14	14	0	100.00	0.359	270-1100
10	BN6A3	17	17	0	100.00	0.859	240-600
11	Ni2B03	12	12	0	100.00	0.312	280-1110
12	MR52a	11	11	0	100.00	0.296	300-990
13	BRMS-011	20	20	0	100.00	0.890	510-1040
14	Ni2D10	17	17	0	100.00	0.421	190-1140
15	BrgMS710	15	15	0	100.00	0.546	140-1100
16	NI03-H07a	8	8	0	100.00	0.406	200-1100
17	BrgMS339	8	8	0	100.00	0.781	230-240
18	BrgMS329	5	5	0	100.00	0.078	190-650
19	BrgMS1237	5	5	0	100.00	0.109	250-600
20	BrgMS787	12	12	0	100.00	0.437	200-480
21	NA10-C01a	15	15	0	100.00	0.734	200-1150
22	BN25A	10	9	1	90.00	0.843	200-800
23	BrgMS70	34	33	1	97.05	0.906	200-1200
24	NI-F02a	12	12	0	100.00	0.843	150-210

resistant and moderately resistant *Brassica* genotypes. So, these markers can be utilized for the identification of resistant genes or resistant genotypes against the white rust pathogen.

Multiple regression analysis for association between genotype and phenotype

Another method of association analysis is multiple regression analysis (MRA) for finding an association between genotypic data and phenotypic data. For calculating genotypic data, amplified DNA bands were scored on the basis of presence (1) or absence (0) of bands and a binary matrix was created using molecular markers. For phenotypic data, the percent disease index of Brassica cultivars recorded during glasshouse studies was used and multiple regression analysis (MRA) was done.

Association analysis between molecular data (as independent variables) and phenotypic data (as dependent variables) was performed using the stepwise "backward" method of "linear regression analysis" in software SPSS version 2.04 to identify informative markers associated with phenotypic traits of disease resistance. This association analysis, based on a regression model, revealed the variation in the dependent variable as a linear function of the set of independent variables. Multiple correlation coefficient (R) and R^2 were calculated which depicted the proportion of phenotypic variation associated with molecular markers. *P* value (Regression value) was calculated for each marker to test the significance of the marker. Markers that showed significant regression values ($p < 0.05$) were considered as significantly associated with the phenotypic trait and proposed to be suitable markers for identifying resistance Brassica genotypes.

The multiple regression analysis revealed that out of 24 SSR markers, 06 markers viz. At5g41560, MB5, BRMS-017, Ni2B03, BrgMS329 and NI-F02a were found to be significantly associated with a phenotypic trait of disease resistance with *p-value* of 0.001, 0.015, 0.015, 0.001, 0.037 and 0.014,

Table 4. Presence (+) or absence (-) of amplified bands on selected *Brassica* genotypes

S. No.	Marker	Brassica genotype							
		Varuna	Pusa Bold	DLSC 1	NRCDR515	Cutlass	Candle	Donskaja	Wester
1	At5g41940	-	-	-	+	-	+	+	-
2	At5g41560	-	-	+	+	-	-	+	+
3	SSR Ni2-A07	+	+	+	+	+	+	+	+
4	BRMS-003	-	+	+	+	+	+	+	+
5	O110B11	-	+	+	+	+	+	+	+
6	MB5	-	-	+	+	+	-	+	+
7	NA10-C01c	+	+	+	+	+	+	+	+
8	BRMS-017	-	-	+	+	+	-	+	+
9	BRMS-030	-	-	+	-	-	+	-	-
10	BN6A3	+	+	+	+	-	+	+	+
11	Ni2B03	-	-	+	+	-	-	+	+
12	MR52a	+	+	-	+	-	-	+	-
13	BRMS-011	+	+	-	+	+	+	+	-
14	Ni2D10	-	+	+	+	+	+	-	-
15	BrgMS710	-	+	+	-	+	+	+	+
16	NI03-H07a	-	+	-	-	-	+	+	+
17	BrgMS339	+	+	+	+	+	+	+	+
18	BrgMS329	-	-	-	-	-	-	-	+
19	BrgMS1237	-	-	-	-	-	+	+	-
20	BrgMS787	-	+	+	-	+	+	+	+
21	aloNA10-C01a	-	+	+	-	-	+	+	+
22	BN25A	+	+	+	+	+	+	+	+
23	BrgMS70	+	+	+	+	+	+	+	+
24	NI-F02a	-	-	+	+	+	+	+	+

respectively. These significant markers viz. At5g41560, Ni2B03 with 86.6%, BrgMS329 with 75.4%, NI-F02a with 65.9% and MB5, BRMS-017 with 65.8% accounted higher proportion of phenotypic variation (R^2) (Table 6).

Discussion

The present study revealed that above mentioned 6 molecular markers viz., At5g41560, MB5, BRMS-017, Ni2B03, BrgMS329 and NI-F02a are quite associated with a phenotypic trait of disease resistance in the studied *Brassica* cultivars. Hence, these markers can be utilized in marker-assisted selection for identifying white rust-resistant genotypes in rapeseed mustard as molecular markers that are associated with the resistant genes. Besides this, the high polymorphic information content (PIC) value of these SSR markers makes them highly suitable for diversity analysis among *Brassica* genotypes, which would be an important component of finding resistant sources against the pathogen. Besides this, a marker-trait association was done by comparative analysis and multiple regression techniques for the identification of resistant traits containing *Brassica* genotype. MRA was based on an association of a marker with the phenotypic trait of disease resistance, which revealed a test of the significance of the parameters of multiple linear regression equations. The coefficient of determination (R^2) obtained during the study indicates the proportion of the variability of a dependent variable and that can be explained by a linear function of independent variables, which will be helpful in association analysis for molecular resistance markers. The methods of associations used in the present study provide an easy and reliable analysis for the identification of cultivars with desirable traits at the early stages of breeding programs (Kar et al. 2008**).** The MRA technique can be conveniently used for oilseed crops and is a quick approach for establishing marker–trait association by avoiding the need to map the populations.

Table 5. Differentiation of markers on the basis of banding patterns on selected *Brassica* genotypes

S. No.	Position of bands in Brassica genotypes	Associated markers
1	Bands in HS and S genotypes	SSR Ni2-A07, NA10-C01c, BN6A3, BRMS-011, BrgMS339, BN25A, BrgMS70, BrgMS787
2	No bands in HS and S genotypes	At5g41940, At5g41560, MB5, BRMS-030, Ni2B03, BrgMS329, BrgMS1237, NI-F02a
3	Bands in MR and HR genotypes	At5g41940, BrgMS1237
4	Bands in immune and HR genotypes	At5g41560, Ni2B03
5	Bands in all MR, HR and immune genotypes	NI-F02a
6	Bands in some MR, HR and Immune genotypes	MB5, BRMS-017
7	Band in immune (Single band)	BrgMS329
8	Bands in all the genotypes	SSR Ni2-A07, NA10-C01c, BrgMS339, BN25A, BrgMS70

Major advantage of using stepwise MRA is that it can allow the detection of QTL that varies across a wide spectrum of biodiversity rather than just between two planned parental genotypes and it requires less time, labor and financial resources compared to the linkage-based QTL identification **(**Ruan et al. 2009; Arora et al. 2019**).** Earlier, this regression analysis technique has been used for qualitative trait analysis such as morphological traits in cotton (Shen et al. 2007; Zeng et al. 2009), wheat **(**Ma et al. 2005**)**, maize **(**Song et al. 2004**)**, *Morus laevigata* (Chatterjee et al. 2004), tea (Mishra and Sen-Mandi 2004) and pomegranate (Basaki et al. 2011), Cherry fruit shape, color etc. for the selection of high grading fruits (Khadivikhub 2014) but the application of this methodology for the identification of disease resistant trait is a novel approach for future discoveries as well. Thus, validation of molecular markers by MRA association analysis opens doors for the selection of potential molecular markers for the identification and development of resistant cultivars against the white rust pathogen in less time with more efficacy.

Many earlier workers have also worked on the validation and identification of molecular markers against the white rust pathogen. The molecular marker At5g41560, which was found to be highly associated with the white rust-resistant trait in the present studies, was also reported earlier (Panjabi et al. 2010; Arora et al. 2019; Singh et al. 2020). They reported that marker At5g41560, derived from *Arabidopsis thaliana* was highly associated with resistance loci in Heera and Donskaja IV cultivars against *A. candida* race 2V in East European lines. Similarly, Singh et al. (2015) validated SSR markers in a set of 25 genotypes of *B. juncea* and confirmed two markers viz., At5g41560 and At2g36360, which were closely linked to white rust resistance loci AcB1-A4.1 and AcB1-A5.1, respectively. Vignesh et al. (2009) studied the polymorphism of STMS markers in different *Brassica* genotypes for breeding resistant cultivars. Singh et al. (2024) conducted a parental polymorphism survey using 315 microsatellites (SSR) markers and confirmed introgression of the white rust resistance gene using *Arabidopsis*-derived IP markers that are linked to the white rust loci AcB1-A5.1 (At2g36360) in Donskaja and BioYSR and AcB1- A4.1 (At5g41560) for BEC-144. Further, they advocated that these lines have good potential for future release and their utilization for precision in accelerating the development of new disease-resistant varieties of Indian mustard. Earlier

Table 6. SSR markers associated with disease resistance using MRA

S. No.	Marker	R	R^2	p-value
1	At5g41940	0.341	0.117	0.408
2	At5g41560	0.932	0.866	0.001*
3	SSR Ni2-A07			NA
4	BRMS-003	0.605	0.367	0.112
5	O110B11	0.605	0.367	0.112
6	MB5	0.811	0.658	0.015*
7	NA10-C01c			NA
8	BRMS-017	0.811	0.658	0.015*
9	BRMS-030	0.204	0.042	0.627
10	BN6A3	0.221	0.049	0.599
11	Ni2B03	0.932	0.868	0.001*
12	MR52a	0.290	0.084	0.486
13	BRMS-011	0.599	0.359	0.117
14	Ni2D10	0.057	0.003	0.892
15	BrgMS710	0.213	0.045	0.613
16	NI03-H07a	0.071	0.005	0.867
17	BrgMS339			NA
18	BrgMS329	0.892	0.754	0.037*
19	BrgMS1237	0.132	0.017	0.755
20	BrgMS787	0.503	0.253	0.204
21	NA10-C01a	0.341	0.116	0.408
22	BN25A			NA
23	BrgMS70			NA
24	NI-F02a	0.812	0.659	0.014*

* Significant association between genotype and phenotype as $p < 0.05$
NA = Not applicable

studies conducted on validation of SSR markers for disease resistance against white rust (Vinu et al. 2013; Sharma et al. 2018; Chandana et al. 2020; Singh et al. 2024) supported the present findings. Besides, effector molecules such as CCG, which are polymorphic in nature and show the presence or absence of variability among *A. candida* races (Furzer et al. 2021) can be effective in decision-making with respect to specific R genes (Redkar et al. 2021; Castel et al. 2021; Furzer et al. 2021). The monogenic resistance was found to be effective against a specific race of *A. candida* and resistant lines obtained can be utilized in breeding programs against the prevalent race in a particular region or gene pyramiding to develop lines against multiple pathogenic races (Singh et al. 2021ab).

The development of resistant cultivars against *Albugo candida* is a tedious task due to the obligate nature of the pathogen. The expression of white rust disease requires specific conditions that are difficult to create under natural field conditions. If required, conditions are maintained under glasshouse conditions even though it does not support large-scale screening of *Brassica* genotypes for resistance. Thus, the identification of molecular markers associated with disease-resistant traits can be a suitable, efficient and less time-consuming method for indirect selection of the resistant Brassica genotypes even at the seedling stage of the plant without creating artificial epiphytotic conditions (Chand et al. 2022). The investigation of molecular markers associated with the resistant trait can provide clues for the identification of promising resistant genotypes against the pathogen by marker-assisted selection. These markers can be used for the elite selection of resistant cultivars, particularly when no other genetic information like linkage maps and quantitative trait loci are available. So, the application of these SSR markers would be interesting for an effective MAS and QTL breeding program as well as for the cost-effective, eco-friendly management of white rust disease in rapeseed mustard.

Supplementary material

Supplementary Tables S1 to S3 are provided and can be accessed at www.isgpb.org

Authors' contribution

Conceptualization of research (AKT); Designing of the experiments (VP); Contribution of experimental materials (AKT); Execution of field/lab experiments and data collection (PU); Analysis of data and interpretation (PU); Preparation of the manuscript (PV, NS, HR).

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Supplementary Table S1. List of SSR primers used for PCR profiling

S. No.	Primer name	Forward primer	Reverse primer	Tm^{0}C
1	At5g41940	TGGCCGTTCCTACTTGGAGT	CGACACTGTCTCCGCAGGT	55
2	At5g41560	CCTCACAATTTCAGTCAACATCGT	GAGGTGGAAGAGTACGGTTGTG	55
3	BRMS-006	TGGTGGCTTGAGATTAGTTC	ACTCGAAGCCTAATGAAAAG	60
4	SSR Ni2-A07	GGAACCCAACAAGTGAGTCC	AGAGCTTGAGACACATAACACC	60
5	BRMS-003	ACGAATTGAATTGGACAGAG	CAGATGGGAGTCAAGTCAAC	54
6	O110B11	AAAATGTGAGGCTGTTTGGG	TTTCGCAGCAGTAAACATGG	53
7	MB5	AACATCTTTTTGCGTGATAT	AATAGCATTGAAGCCTTAC	55
8	NA10-C01c	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC	54
9	BRMS-017	GAAAGGGAAGCTTCATATC	CTGGAAAGCATACACTTTGG	54
10	BRMS-030	TCAGCCTACCAACGAGTCATAA	AAGGTCTCATACGATGGGAGTG	52
11	BN6A3	GCTACCCACTCATGTCCTCTG	CCAAGCTTATCGAATCTCAGCTA	54
12	BRMS-033	GCGGAAACGAACACTCCTCCCATGT	CCTCCTTGTGCTTTCCCTGGATACG	51
13	Ni2B03	ACTTCTTGCCCTCCTCACC	AAATACTCACTGCAATACCCAGG	52
14	MR52a	TCGACATGGATTCTACCAA	GAACTTGCAAGCTGCAATTA	55
15	BRMS-011	GAACGCGCAACAACAAATAGTG	CGCGTCACAATCGTAGAGAATC	54
16	Ni2D10	GATGCCCCAAATCTGTTACG	CAATTCGTGAAAAATAGCCG	53
17	Ni3C05	TTTCGTGCTTTGGTGTGAAG	TCCCCAAATCGAACCATAAG	54
18	NI02-D08a	TTTAGGGAAAGCGAATCTGG	ACAACAACCATGTCTTCCG	54
19	BrgMS710	ATCATCATCGTCTTCCTCTTCC	CTGCCCAAAATAAACACAGTCA	55
20	NI03-H07a	GCTGTGATTTTAGTGCACCG	AGCCGTTGATGGAATTTTTG	53
21	BrgMS339	CTACCTGAAGATGACCCAGACG	GCATACAAACCTCCGTCCTAAGC	53
22	BrgMS329	TCATCATCATAGCTTTCGCTTC	AAAACCTCCTCCTCCTCCTC	51
23	BrgMS1237	ATCAAAAGATGCAGGGAGAGAG	GTCCTCAATGGATTACACATGC	51
24	BrgMS787	CCATCTCAGCTCTATCTACCAAAA	TCAAAACACCGAGTAAACTGGA	55
25	NA10-C01a	TTTTGTCCCACTGGGTTTTC	GGAAACTAGGGTTTTCCCTTC	54
26	BN25A	CACGTGGTATGTTGGTATTGGG	TGATTCTCCTCCGACGCATGC	53
27	sORA43	GCGCGTGTGGGATCAGAA	CTTCTCCACCGTCGATCG	53
28	BrgMS70	TACAATGAAGATGTGATCCCGA	CGTGCGTGAGCTTATCAATACA	52
29	NI-F02a	TGCAACGAAAAAGGATCAGC	TGCTAATTGAGCAATAGTGATTCC	54

Supplementary Table S2. PCR reaction mixture for SSR primer

S. No.	Components	Final conc.	Single tube (µL)
1.	DNA template	40 ng/µL	1.0
2.	10X Hi Buffer S (With 17.5 mM $MgCl_2$)	1X	2.5
3.	Taq polymerase (1 unit/µL)	0.76 unit	0.25
4.	dNTPs (2.5 mM each)	200 mM	2.0
5.	Forward primer	20 ng/µL	0.5
6.	Reverse primer	20 ng/µL	0.5
7.	DdW		18.25
	Total		25.0

Supplementary Table S3. Thermal cycling program for SSR primers

Steps	Details	
Initial denaturation	94°C for 5 minutes	
Denaturation	94°C for 1 minute	
Primer annealing	As per given in Table 5	39 cycles
Primer extension	72°C for 2 minutes	
Final extension	72°C for 10 minutes	
Incubation	4°C for infinity to hold the sample	