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Marker assisted selection for developing superior breeding lines for white rust resistance in Indian mustard (*Brassica juncea*) using IP markers

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Abstract

White rust caused by *Albugo candida* is one of the significant diseases for yield reduction in rapeseed-mustard. Molecular marker technology in combination with the traditional breeding method offers precise selection in crop improvement programme. RH0749 a commercial and susceptible variety of Indian mustard was crossed with resistant donors (Bio-YSR and BEC-144) for improving white rust resistance. BC_1F_1, BC_2F_1 populations were evaluated for various yield attributes and analysed at molecular level to undertake marker-assisted selection (MAS). Intron polymorphism and cleaved amplified polymorphic sequence markers were used for forward selection. Three IP markers in RH0749 × Bio-YSR cross and two IP markers in RH0749 × BEC-144 cross showed polymorphism. Three hundred and forty-eight SSRs were used for background selection and 72 SSR markers were found polymorphic in RH0749 × Bio-YSR cross while in RH0749 × BEC-144 cross, only 43 SSR markers were found polymorphic. The promising progenies (9 in BC_2F_1 for RH0749 × Bio-YSR and 8 in BC_2F_1 for RH0749 × BEC-144 cross) were selected based on molecular, morphological and disease data which can be further used in Indian mustard improvement programme for white rust resistance.

Keywords: *CAPS, Indian mustard, marker assisted selection, IP, simple sequence repeat*

Introduction

Brassica species, an important commodity in agriculture, contains eight distinct species grown around the world across 53 countries. It is an economically significant genus of the *Brassicaceae* family, constituting oil crops (*Brassica napus*, *B. rapa* and *B. juncea*), vegetables (cabbage, cauli-flower) and spices (*B. carinata, B. nigra*) (Zhang *et al.,* 2018). India is the world's third largest producer of vegetable oil yielding edible mustard oil with medicinal value (Panigrahi *et al.,* 2018). In India, rapeseed–mustard occupied around 23.3 % area (6.69 mha) and 26.8 % (10.11 mt) production of total oilseeds in the country during 2020-21 (Choudhary *et al.,* 2023). Oilseeds are vulnerable to a huge number of insect pests and diseases causing yield losses. *Albugo candida*, an obligate parasite comes among the top ten pathogens on the scientific and economic basis (Arora *et al.,* 2019). White rust, caused by *Albugo candida* (Pers. ex Lev.) Kuntze, is most wide-spread and highly destructive disease in *Brassica juncea*. The first report of *A. candida* infection on *Brassicaceae* family was reported by Colmeiro (1867). The plant pathogen affects vegetative as well as reproductive phase of the plant leading to extensive yield losses causing maximum damage because of stag head formation (Meena *et al.*, 2014) causing yield losses of 20-60% (Awasthi *et al.*, 2012). As heavy yield losses are stated to occur due to white rust disease, there is an immediate need to implement a breeding programme for developing white rust resistant Indian mustard. Indian mustard cultivars are vulnerable to white rust, and its germplasm provides access to sources of resistance to the disease. Numerous studies reported that a single dominant gene available in its germplasm controls resistance to white rust (Vignesh *et al.*, 2010). However, if molecular markers that are closely linked to the gene(s) of interest are available, indirect selection can accelerate breeding for white rust resistance (Ashkani *et al.,* 2016). Traditional breeding selection is time-consuming and prone to linkage drag since it is dependent on phenotypic data selection. As a result, using DNA molecular markers is faster, easier, and less costly than traditional breeding operations (Arabzai and Gul, 2021). The traditional breeding methods combined with the marker assisted selection, MAS (Ceballos *et al.,* 2015) provides advanced plant breeding method allowing precise and efficient introgression of essential agronomic trait(s) from

germplasm to the desired commercial genotypes/ lines. Based on the pattern of bands obtained from the related molecular markers, it is an indirect method of selection for the targeted trait. For the identification of introgressive genes in plant species, molecular markers have been commonly used. Crop improvement is based on identifying desirable genes and superior genotypes in a breeding population that have those genes. The method of selecting desirable genes or characteristics indirectly using morphological, biochemical, or DNA-based/molecular markers is known as MAS. MAS precisely exhibits the desired trait in fewer selection cycles with fewer unintended mistakes in a single genotype. As a result, MAS saves time and money when it comes to improving high-yield crops. It is an excellent tool for increasing agricultural yields and protecting crops from a variety of challenges (Arabzai and Gul, 2021). Using the integrated approach plant breeding with molecular markers, scientists are using various white-rust resistant germplasm/lines which could be used as successful donors for white-rust resistance. BioYSR, BEC-144, BEC-286, EC-399301, JM-1 and JM-2 (Yadava *et al.,* 2012), Donskaja-IV and Heera (Panjabi *et al.,* 2010) are some of the promising donors for white rust resistance. The use of these resistant donors in improving local commercial varieties may lead to the precision deployment of genes/ QTLs associated with white rust resistance in a shorter timeframe with guaranteed results. The present study describes the crossing of a commercial and susceptible variety, RH0749 with resistant donors BioYSR and BEC-144 for improving white rust resistance. Populations BC_1F_1, BC_2F_1 were evaluated for agronomical yield traits and marker-assisted selection was undertaken. IP and CAPS markers were used for foreground selection in the backcross populations of both the crosses (RH0749 × BioYSR and RH0749 × BEC-144). SSRs were used for background selection and disease severity was also screened in these populations. After evaluation of molecular, morphological and disease data, the promising and superior lines were selected from both the populations for use in mustard breeding programme for white rust resistance.

Materials and Methods

Indian mustard variety RH0749, a commercial variety that is vulnerable to white rust, was used to crossed with resistant donors (Bio-YSR and BEC-144) to increase resistance. In order to conduct marker-assisted selection, the BC_1F_1 and BC_2F_1 populations were examined. Seeds of RH0749, BioYSR and BEC-144 genotypes were sown in the year 2015 and 2016, and backcrosses were attempted in off season at Research Station, Wellington, Tamil Nadu. Crosses were attempted to produce F_1, BC_1F_1 and BC_2F_1 generations. During sowing, weather was cool and soil had enough moisture content for proper growth. Young leaves from field grown plants were used for isolating genomic DNA using CTAB method of Saghai-Maroof *et al.* (1984). The quantification of the genomic DNA was observed by recording the absorbance using UV-VIS spectrophotometer at wavelength of 260 nm and 280 nm. The quality was checked by running the DNA samples on 0.8% agarose gel. For validation and foreground selection, IP (Panjabi *et al.,* 2010) and CAPS (Varshney *et al.,* 2004) markers were used while for background selection genomic SSRs were used (Table 1).

PCR Amplification was done in ABI thermocycler and conditions for PCR reaction included 95°C for 3 minutes, 40 cycles of 94°C for 1 minute, T_m for 1 minute, 72°C for 1 minute and final extension at 72°C for 7 minutes before cooling at 4°C. The amplified products were stored at -20°C until use. The amplified products, electrophoresed on agarose gel (1.5%) and electrophoretic pattern of bands was observed. The PCR products from molecular markers study were scored visually for presence or absence of bands. Based on the presence of specific bands unique in parents i.e., RH0749, BioYSR and BEC-144, the markers were selected.

Data analysis

After visualizing the bands on the gel, presence of band was scored as 1 and absence of band as 0 for each locus of the marker for evaluation. For the estimation of genetic distance, similarity using the 0/1 matrix, NTSYS-PC (Numerical Taxonomy Method for Personal Computer; Rohlf, 1990) software with "simqual" subprogram was used. The distance matrix was used by the UPGMA (unweighted pair-group system with arithmetic average) subprogram of the NTSYS-PC package to generate dendrograms.

Screening for white rust under field conditions

The pathogen *Albugo candida*, an obligate parasite that could not be produced in the laboratory so inoculum was prepared. White rust-infected leaves were collected and dipped for 4-5 hours in water, and then water was strained using muslin cloth and collected in spray bottles. In the evening, when the temperature was down and the humidity was high, spraying was carried out to ensure optimal conditions for the growth of spores. For two weeks, spraying was conducted on a routine basis. Plants were then observed for visible symptoms of disease and the severity of the disease was determined according to the method provided by Conn *et al*. (1990). A crossing

Table 1: Molecular markers used for DNA polymorphism and foreground selection

Primer	Original Primer name	Forward Primer (5"!3')	Reverse Primer (5"!3')
CAPS	OPB061000	GCTCACTTCAGCAGGGGAAGGC	GGAACCGAACAGACAGACATGAGTTG
IP1	At5g41560	TCAACAACTTGTCATTGTCTCTCAG	GCGTCACAGTTCTCTATGAGCTG
IP2	At5g41940	CATGGCATATATCAGGAGACTGAG	GCCTCCATTGAGTTCCATCC
IP3	At5g36950	CTCAGCTGATTTTGAATTTCCG	CATTTGCAGCCACAGGTATCAT
IP4	At5g37580	CCCAACTCGTGACCGTCTCTT	CCATCCTCCTCCTTGACAGTTG
IP5	At5g40670	GTGTTAGCCATTGGAACTGAATG	GACCACCAGAGTTCCCAGA
IP6	At5g40390	CGTTCGTCAACGTGGCACTAAG	TCTGTAACTGTTGGGATTCTCTGG
IP7	At5g40200	GCTGCTGTTTCCGTTGGTCT	GATCGTGAATCCATCACCACC
IP8	At5g40950	TGGCCGTTCCTACTTGGAGT	CGACACTGTCTCCGCAGGT
IP9	At5g41360	AGAATGGTCTTGTCGCCCATAA	TTAAGATGTGCAAACCGCTCAC
IP10	At2g45790	GCCACCTCCTAGATGTGGTCATA	GTCCATCCAGGTGTTTCACG
IP11	At2g36360	AAACTTCGCCGGTCGAAGAC	GAGTCTCGAAGTCGCCGTTAAC
IP12	At2g34510	TGATTACCAAGGAGCAAGAGATGC	CACAGATGCACTAGGCTCAGATT
IP13	At2g32920	CCTCACAATTTCAGTCAACATCGT	GAGGTGGAAGAGTACGGTTGTG

programme was started for introducing white rust resistance in a commercial cultivar RH0749 of Indian mustard using marker assisted selection approach. Both donor genotypes were used and F_1, BC_1 F_1, BC_2F_1 populations were used for molecular and agronomic evaluation (Table 2 and 3). The populations were grown at the research farm of CCS Haryana Agricultural University Hisar, during *Rabi* seasons, 2015 and 2016. The agronomic yield traits were recorded at the maturity of the crop. Data were analyzed with OPSTAT developed by Sheoran *et al.* (1998).

Results and Discussion

Agronomic traits

The data on various agronomic traits for both the crosses RH0749 × BioYSR and RH0749 × BEC-144 in BC_1F_1, BC_2F_1

Table 2: Agronomic traits in RH0749 × Bio-YSR cross for BC_1F_1 and BC_2F_1 populations in Indian mustard

Agronomic traits	RH0749 (Mean±SE)	BioYSR (Mean±SE)	BC_1F_1 population (Mean±SE)	Range	BC_2F_1 population (Mean±SE)	Range
Plant height (cm)	180±0.38	189±0.68	183±0.67	171-200	180±0.77	164-200
Primary branches/plant	5.2±0.20	5.8±0.35	6.8±0.54	5-9	6.2±0.32	5-7
Secondary branches /plant	12.6±0.65	11.2±1.11	15.4±1.01	9-21	15.9±1.12	5-19
Main shoot length (cm)	77±0.80	59±1.20	67±0.98	51-78	74±0.89	63-86
Siliqua on main shoot	45±1.83	31±1.18	48±0.69	42-54	51±0.94	38-55
Seeds/siliqua	13.8±0.29	10.8±0.66	14.1±0.32	12.6-15.8	13.92±0.45	10.2-16.4
Days to maturity	141±0.07	143±0.09	144±0.04	143-144	142±0.04	141-142
1000-seed weight (g)	5.6±0.40	2.7±0.39	5.8±0.21	5.2-6.8	6.2±0.36	4.8-8.1
Seed yield/plant (g)	21.3±0.72	15.5±0.94	25.4±0.72	18.2-30.77	23.1±1.04	13.9-29.6

Table 3: Agronomic traits in RH0749 × BEC-144 cross for BC_1F_1 and BC_2F_1 populations in Indian mustard

Agronomic traits	RH0749 (Mean±SE)	BEC-144 (Mean±SE)	BC_1F_1 population (Mean±SE)	Range	BC_2F_1 population (Mean±SE)	Range
Plant height (cm)	180±0.38	233±3.61	181±1.66	144-207	176±1.13	153-198
Primary branches/plant	5.2±0.20	9.8±0.69	5.5±0.41	4-7	5.46±0.49	4-7
Secondary branches /plant	12.6±0.65	17±1.93	12±1.28	6-20	12.5±0.72	9-18
Main shoot length (cm)	77±0.80	41±0.81	73±1.63	51-94	79±0.93	58-93
Siliqua on main shoot	45±1.83	44±0.88	53±1.7	34-70	50±0.76	36-60
Seeds/siliqua	13.8±0.29	10.2±0.53	13.54±0.18	10.4-15.4	12.92±0.85	5.6-17.8
Days to maturity	141±0.07	140±0.05	140±0.04	139-140	139±0.04	138-139
1000-seed weight (g)	5.6±0.40	1.6±0.22	4.7±0.23	3.7-5.3	5.8±0.38	4-7.3
Seed yield/plant (g)	21.28±0.72	13.08±1.58	16.23±1.58	8.12-27.85	17.87±1.30	11.76-32.53

populations along with the parents were recorded (Table 2 and 3). In cross RH0749 × BioYSR, the mean plant height of RH0749 was 180 cm and 189 cm of BioYSR while in BC_1F_1 and BC_2F_1 population it was 183 cm and 181 cm. The mean value of no. of primary branches/ plant was found to be 6.8 cm in BC_1F_1 and 6.2 cm in BC_2F_1 population. The mean value of main shoot length was 67 cm and 74 cm in BC_1F_1 and BC_2F_1 population. In cross RH0749 × BEC-144, the mean plant height of RH0749 was 180 cm and 233 cm of BEC-144 while in BC_1F_1 and BC_2F_1 populations it was 181 cm and 176 cm. The mean value of no. of primary branches/ plant was found to be 5.5 cm in BC_1F_1 and 5.46 cm in BC_2F_1 population. The mean value of main shoot length was 73 cm in BC_1F_1 and 79 cm in BC_2F_1 populations.

MAS in BC_1F_1 and BC_2F_1 generation in crosses RH0749 × BioYSR and RH0749 × BEC-144

In the present study, RH0749, was selected as recipient parent as it is bold seeded high yielding variety developed by CCSHAU, Hisar by crossing RH 781 with RH 7846. It has long siliquae with higher number of seeds. BioYSR and BEC-144 were taken as donors as they were resistant to white rust. BioYSR is developed by NRCPB, New Delhi by crossing Clipper/BH75/BK0019 while BEC-144 is an exotic collection from Poland. Molecular markers reported to be linked with white rust resistance i.e. IP markers by Panjabi *et al.* (2010) and CAPS marker by Varshney *et al* (2004) were validated in the parents of the two populations and these were used for foreground selection. Background selection was undertaken for recovery of recurrent parent i.e. RH0749. IP and CAPS markers tagged for white rust resistance were validated in F_1s of crosses RH0749 × BioYSR and RH0749 × BEC-144. In cross RH0749 × BioYSR, out of 13 IP markers, three IP markers [At5g40670, At5g40390, At2g36360] were polymorphic while in cross RH0749 × BEC-144, two IP markers viz. At5g41940 and At5g4020 were polymorphic. CAPS marker (restriction enzyme *MboI*) was not amplified in any of the parents in crosses RH0749 × BioYSR and RH0749 x BEC-144. F_1 plants were selfed to generate F_2 in cross RH0749 × BioYSR, and selected plants were back-crossed with the recurrent parent, RH0749, to generate BC_1F_1. DNA from BC_1F_1 plants was isolated and used for MAS. BC_1F_1 generation genotyping was performed. Sixteen of the 57 BC_1F_1 plants assessed were positive for the validated markers (Fig.1). The positive plants were advanced by backcrossing with RH0749. DNA from the BC_2F_1 plants was isolated and used for marker-assisted selection. Of the 28 BC_2F_1 plants, 9 (B-6, B-8, B-13, B-14, B-16, B-18, B-22, B-25 and B-28) were found to be positive for the validated markers.

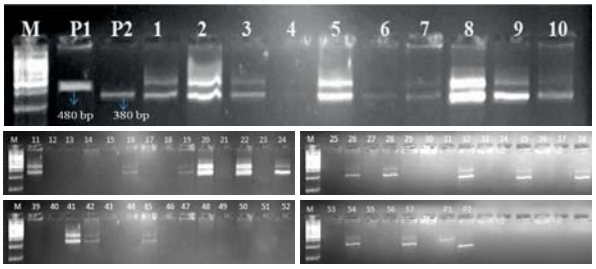

Fig. 1: Electrophoretic pattern of PCR amplified fragments using IP5 (At5g40670) marker in parents and BC_1F_1 population of RH0749 × BioYSR cross (M:100 bp ladder; P1: RH0749; P2: BioYSR;1-57 plants: BC_1F_1 population)

In cross RH0749 × BEC-144, of the 32 plants in BC_1F_1 (Fig. 2), 12 plants were positive for the two polymorphic markers. For the BC_2F_1 population, these were genotyped and the positive plants were backcrossed with RH0749. The BC_2F_1 population was screened for the validated markers. Of the 26 plants screened, 8 (B-1, B-2, B-8, B-13, B-16, B-18, B-20 and B-26) were positive for both validated markers.

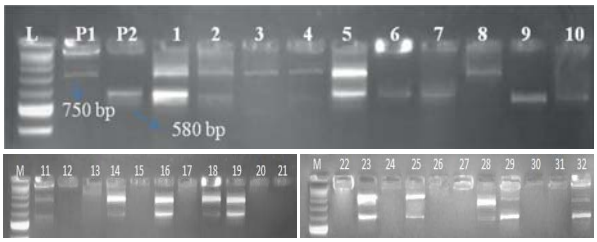

Fig. 2: Electrophoretic pattern of PCR amplified fragments using IP2-At5g41940 marker in parents and BC_1F_1 population of RH0749 × BEC-144 cross (L, M: 100 bp ladder; P1: RH0749; P2: BEC-144;1-32 plants: BC_1F_1 population)

Cluster analysis was performed and a dendrogram was created for polymorphic background markers for generations BC_1F_1 and BC_2F_1 (Fig. 3 and 4). Three hundred and forty-eight SSRs were used for background selection out of which, 72 were polymorphic in cross RH0749 × BioYSR while 43 were polymorphic in cross RH0749 × BEC-144.

In cross RH0749 × BioYSR, BC_1F_1 generation five plants were found to be similar to RH0749 (B-2, B-11, B-35, B-28 and B-5) while in BC_2F_1 generation five plants (B-22, B-32, B-16, B-19 and B-6) were found to be similar to BioYSR. In cross RH0749 × BEC-144, BC_1F_1 generation five plants were found to be similar to RH0749 (B-5, B-22, B-18, B-28 and B-16) while in BC_2F_1 generation four plants were found to be similar to BEC-144 (B-13, B-14, B-25 and B-8). Background selection was done using 338 SSRs and in cross RH0749 × BioYSR, 72 SSR markers were found to be polymorphic while in cross RH0749 × BEC-144, 43 markers were polymorphic.

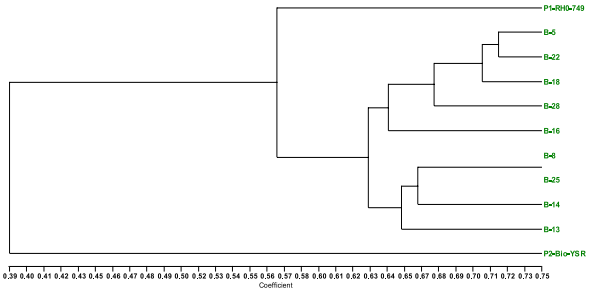

Fig.3: Dendrogram (NTSYS-PC) displaying diversity among nine positive BC_2F_1 lines and parental genotypes based on molecular data of 72 polymorphic SSR loci for RH0749 × BioYSR cross

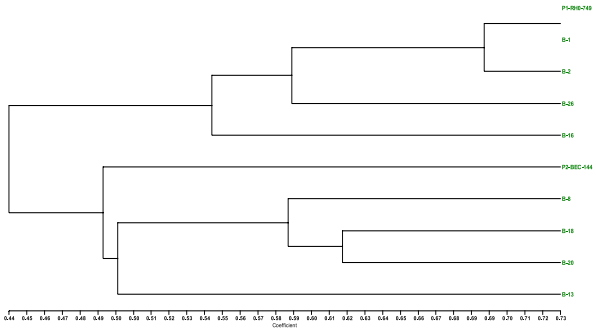

Fig.4: Dendrogram (NTSYS-PC) displaying diversity among eight positive BC_2F_1 lines and parental genotypes based on molecular data at 43 polymorphic SSR loci RH074 9 × BEC-144 cross

Disease severity

The disease severity in susceptible parent, RH0749 was found to be 30.8 % while the donor parents, BioYSR and BEC-144 (resistant in nature) showed complete resistance (0 %) showing vertical resistance and monogenic nature of the white rust resistance gene. The donors (BioYSR and BEC-144) for white rust resistance used in the present investigation are also reported by several other workers (Singh *et al.*, 2020; Singh *et al.*, 2015; Yadava *et al.*, 2012; Vignesh *et al.*, 2011). The study also revealed that the resistance in the indigenous resistant source BioYSR is monogenic. As a result, backcross breeding may readily transfer the main gene regulating white rust resistance to the well-adapted, high-yielding but vulnerable genotypes. By diversifying resistant sources and gene pyramiding, the existence of a monogenic resistance gene in the indigenous source BioYSR is highly beneficial in breeding for long lasting resistance (Vignesh *et al.*, 2011).

In the present analysis, foreground markers available in the public domain linked to white rust resistance were used. Three markers (At5g40670, At5g40390 and At2g36360) were validated for RH0749 x BioYSR cross, out of 13 IP markers, two markers (At5g41940 and At5g40200) were validated for RH0749 x BEC-144. However, CAPS marker was not amplified in each of the parents. Singh *et al.* (2015) have also used the markers which we have used in the present work in marker-assisted pyramiding for white rust resistance. Twenty-five genotypes of Indian mustard and three separate F2 populations were evaluated to research disease reaction at two stages: cotyledonary and true leaf level for two years. They found that in both phases, BioYSR and Donskaja-IV showed a resistant response. IP markers such as At5g41560 and At2g36360 for white rust resistance were validated. They concluded that these two markers are not peculiar to the genotype and have unique loci which can be used for the incorporation of white rust resistant loci in marker-assisted breeding programmes. Singh *et al.* (2020) utilized IP markers viz, At5g41560 and At2g36360 for validation and screening of white rust resistance in parents, F_1, F_2 and Backcross generations (BC_1F_1 and BC_2F_1). They have also used BioYSR and BEC-144 as donor parents and NRCHB101 and DRMR-150-35 as recipient. After validation and screening in parents and generations, results were same as were found in the present work that only one dominant gene is responsible for white rust resistance.

By cluster analysis, we have found that some of the plants in BC_2F_1 generation in both the crosses were found similar to the recipient parent showing that although receiving the donor gene, their genome content was mostly of the recipient parent which itself defines the use of marker assisted selection. For background selection, 348 SSR markers were used in the present research. In the BC_2F_1 population of RH0749xBioYSR cross, 72.7 percent of genome recovery was observed using 72 polymorphic SSR markers, while in RH0749xBEC-144 cross, only 50 percent of the genome was recovered with 43 polymorphic SSR markers. The lower percentage of RH0749xBEC-144 cross genome recovery may be due to the use of fewer history selection markers. One explanation may be that these markers were present on the chromosome at a distance of more than 100 cM (Cheng *et al.* 2017).

Conclusion

The study supports that validated IP markers can be used for white rust resistance for marker-assisted breeding. The promising plants (9 in BC_2F_1 for RH0749 × BioYSR for markers At5g40670, At5g40390, At2g36360 and 8 in BC_2F_1 for markers RH0749 × BEC-144 for markers At5g41940 and At5g40200) were selected based on genetic, morphological and disease data and can be

further used in the white rust resistance *Brassica juncea* breeding programme. The present study specifically reports that validated white rust resistance markers can be further used for improving Indian mustard. In the backcrossing programme using MAS, the promising plants in the two-cross BC_2F_1 generation can be further used to produce new enhanced versions of RH0749. The improved variant can also be used in other commercial Indian mustard and gene pyramid cultivars to add white rust resistance. The superior lines developed in this study are further utilized in other Indian mustard breeding programmes.

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