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Identification of Putative Sources of Tolerance to Aphid in Mustard and Generation of Molecular Marker for Use in Crop Improvement

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A B S T R A C T

RAPD (random amplified polymorphic DNA) analysis was used to identify molecular markers linked to putative gene conferring resistance to the mustard aphid (*Lipaphis erysimi* Kalt.). A set of 30 germplasm accessions of *Brassica juncea* were screened with 284 RAPD primers (87 primers were amplified) for analysis of putative source of aphid resistance. These amplified primers were screened with bulked DNA of tolerant and susceptible accessions and from that four polymorphic primers were obtained. These four primers were screened with individual tolerant and susceptible accessions and only one RAPD primer (oligos-193, Genemed 10-mer) with clear cut polymorphism between tolerant and susceptible accessions was obtained. This polymorphic band of 600 bp found in susceptible was eluted from two accessions i.e. IC-491558 and IC-491564. DNA polymorphisms were converted in to SCAR (Sequence Characterized Amplified Region) primers (BJ 01 and BJ 02). These SCAR primers however could not provide unequivocal result since the association between marker and resistance was not always unidirectional. This indicates the need to further refine the marker identified by primer walking procedure.

Introduction

Indian mustard (*Brassica juncea* (L.) Czern.) has been an important crop to India for a long time. It is predominantly cultivated in Rajasthan, Uttar Pradesh, Haryana, Madhya Pradesh, and Gujarat (Shekhawat*et al.*, 2012) and stands at the third position in the list of rape-mustard producing countries.However, India's average yield of mustard is low in comparison to other mustard-growing countries chiefly due to aphid pest causing excessive losses in the yield of the crop (Pandey *et al.* 2013; Rao *et al.* 2014).The yield loss in rapeseed mustard also varies with their germplasms and agro-ecological practices (Ansari, *et al.* 2007).The mustard aphid is considered to be a major limiting factor for successful cultivation of the crop; causing up to 96 per cent yield losses (Singh and Sachan, 1994; Singh and Premchand, 1995; Sharma and Kashyap, 1998; Singh and Sharma, 2002). Though, it can be controlled by the pesticide to some extent but it has

residue left in the soil and oil products, which can cause adverse effect on the environment as well as human health; so, it is of utmost importance to mine for the sources of the genetic resistance against mustard aphid control mustard aphid using genetic resistance. Yadav *et al.,* 2017 studied 240 *B. juncea* accessions for resistance/tolerance against mustard aphid and found 16 out of 240 accessions as resistant, 83 accessions falling under moderately resistant category, 102 accessions as susceptible accessions whereas 39 accessions were found to be highly susceptible. The percentage of highly resistant and tolerant germplasms in different species of Brassica ranged from 5% to 100% in closely related species (Jenson *et al.,* 2002).

Plenty of research has been done in many field crops to explain the molecular mechanism behind the resistance to biotic stresses. A SCAR marker developed using RAPD molecular markers, linked to the *Dn2* gene which confers resistance to the Russian wheat aphid (*Diuraphis noxia* Mordvilko) (Myburg *et al.,* 1997). Monika *et al.,* (2010) used *Mi-1.2* gene which when expressed in tomato plants, contributed to endogenous resistance against nematodes and some hemipteran insects. Similarly RAPD markers has been identified for white rust resistance in an F_1-derived doubled-haploid (DH) population originating from a cross between white rust-susceptible and white rust-resistant breeding lines of *B. juncea* (Prabhu *et al.,* 1998). Molecular marker like RAPD and microsarellites has been efficiently utilized for tagging leaf rust resistance gene *Lr 19* in wheat (Gupta *et al.,* 2006) and blackleg disease resistance gene analogues (RGA) in the B genome of Brassica (Saal *et al.,* 2005). Atri *et al.,* 2012 concluded that *B. juncea* - *B. fruticulosa* introgression set may prove to be a very powerful breeding tool for aphid resistance related QTL/gene discovery and fine mapping of the desired genes/QTLs to facilitate marker assisted transfer of identified gene(s) for mustard aphid resistance in the background of commercial mustard genotypes. With the above background and keeping in view of derth of information with regard to unavailability of SCAR marker in *B. juncea* against mustard aphid an attempt has been made to identify the putative source of aphid resistance employing DNA based molecular markers for the first time.

Materials and Methods

Plant material

A total of 34 accessions of *B. juncea*(30 accessions with 4 check) (Table-1) were grown at ICAR-NBPGR farm, New Delhi, during *rabi* 2009-10. The experiment was laid out in Randomized Block Design(RBD) with three replication. Tender and young leaf sample were collected for molecular studies.

Scoring and screening of *B. juncea* accessions

The screening was based on the parameters of Aphid Infestation Index (A.I.I.) and aphid population count. The aphid incidence appeared during mid of January and the observations were recorded at an interval of 15 days during the aphid period. The infestation was scored based on the 0-5 grades (Bakhetia and Sandhu, 1973) with slight modifications. The scale comprised (based on 0-5 grades: highly tolerant= 0 to 1, tolerant= 1.1 to 2, moderately tolerant= 2.1 to 3, susceptible= 3.1 to 4, highly susceptible= 4.1 to 5). The A.I.I. was calculated with the following formula

Aphid Infestation Index

$$= \frac{0xa + 1xb + 2xc + 3xd + 4xe + 5xf}{a + b + c + d + e + f}$$

Where a, b, c, d, e, and f are the frequencies of plants falling in each grade from 0-5.

Aphid population

For aphid population count was made at the initiation of inflorescence stage till the pod formation stage. For this purpose five randomly selected plants were tagged and top 10 cm apical twigs of the selected plants were cut and put in polythene bags separately and brought to the lab. The number of aphids per plant was counted under high magnification in the laboratory.

Based on mean aphid infestation index and aphid population, the accessions were grouped as highly tolerant, tolerant, moderately tolerant, susceptible and highly susceptible.

Genomic DNA extraction

Stored leaf samples of *B. juncea* was used for DNA extraction during 2010-11. DNA was extracted from leaf sample of individual plant the the CTAB extraction procedure of Saghai-Maroof *et al.,* (1984), with minor modifications same as of Ruchi Vir *et al.,* 2009. Extracted DNA was quantified using a Nanodrop spectrophotometer (ND1000 spectrophotometer, NanoDrop Technologies, Montchanin, DE) and diluted to 20 ng/µl in 1x T.E. The diluted samples were kept at -20^{0}C (Yamamoto *et al.,* 1989).

RAPD analysis

Eighty seven RAPD primers (oligos-193, Genemed 10-mer) were selected based on good amplification, by using these primers 34 accessions of *B. juncea* were screened for analysis of putative source of aphid resistance. Two separate contrasting DNA bulks were made by pooling equal amount of DNA from susceptible and tolerant accessions and screened with above mentioned amplified RAPD primers.

PCR amplification was performed in a total volume of 25 µl of Taq PCR buffer (10X), dNTPs (20mM), $MgCl_2$ (25mM), Taq DNA polymerase (5U/µl), (Fermentas, LIFE SCIENCE), with optimized conditions for PCR amplification was of an initial denaturation for 6 min at 94°C, followed by 40 cycles each with denaturation for 1 min at 94°C, annealing for 1 min at 35°C and extension for 1 min at 72°C, with final extension of 10 min at 72°C and the reaction was carried in a Bioer XP cycler. Amplified PCR product was separated on 1.8 % agarose gel, stained with Gelred (Life Technologies Pvt. Ltd, 5µl/100ml). Gels were viewed and photographed under medium wavelength UV light. The amplification products were scored across the gel comparing their molecular weights.

SCAR design and analysis

Polymorphic fragments amplified by closely linked RAPD marker from two accessions (IC- 491558 and IC- 491564) were excised from the gel and purified using standard Gel elusion kit (Axygen biosciences, USA). The purified DNA was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The resulting white colonies on X-gal-IPTG-LA plates containing 100g/ml ampicillin were picked, grown in Luria broth and plasmid DNA was isolated using the Sambrook and Russel (2001) method. Plasmid was then purified by treating with RNase A (10 mg/ml) followed by extraction with phenol chloroform. Isolated plasmid was run on 1% agarose gel, and the band size of blue and white colonies were compared, colonies containing band size larger than the control (blue colony) were considered as recombinant colonies (Fig. 3). The cloned fragment was sequenced from both ends using universal primers (T_7 and SP_6) in automated DNA sequencer (Chromous Biotech, Bangalore, India). Based on the sequence information forward and reverse SCAR primers were designed using Oligos 4.0 software (National Biosciences) and were synthesized from

Eurofins, Germany (Table 3). PCR amplification was performed in a total volume of 25 µl of Taq PCR buffer (10X), dNTPs (20mM), $MgCl_2$ (25mM), Taq DNA polymerase (5U/µl), (Fermentas, LIFE SCIENCE), SCAR primer (1 µM) and template DNA (20ng/µl). PCR amplifications programme consisted of an initial denaturation for 6 min at 94°C, followed by 35 cycles each with denaturation for 1 min at 94°C, annealing for 1 min at 54°C and extension for 1 min at 72°C, with final extension of 10 min at 72°C. Amplification products were separated on 1.5 agarose gel, stained with Gelred from Life Technologies Pvt. Ltd (5µl/100ml), visualised under UV light and photographed using gel documentation system (Syngene, Germany).

Results and Discussion

Thirty accessions were screened under condition of natural infestation of mustard aphid in the field conditions, in randomized block design with three replications each were screened for the resistance/tolerance to aphid. Eleven accessions were found to be under the tolerant category (with 9 being tolerant & 2 highly tolerant), whereas 6 accessions were of susceptible category (three susceptible & highly susceptible), remaining 13 accessions showed a reaction of moderately tolerance, when compared with tolerant and susceptible check.

The strain of *B. juncea* has tough inflorescence twigs with loosely packed flowers buds, which offer a less suitable site for colonization of aphid (Rai and Sehgal, 1975). Generally, *B. juncea* strains are also moderately resistant to *L. erysimi* infestation as compared to brown and yellow sarson (Singh *et al.*, 1982; Rohilla *et al.*, 1993; Bakhetia *et al.*, 2002). The differential behaviour of germplasm/accessions ranged from highly susceptible to highly tolerant (Subhash *et al.*, 2013).

Fig.1 Four RAPD Primers found Polymorphic with Separate Bulked DNA of Tolerant and Susceptible Accessions

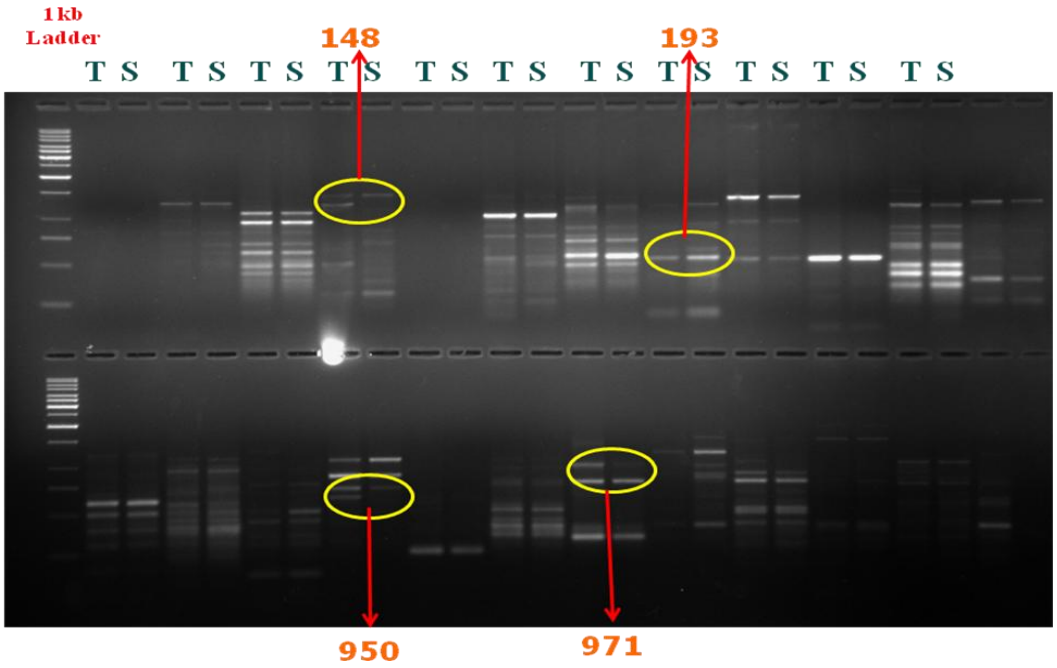

Fig.2 RAPD marker oligos-193 (Genemed 10-mer) shows an unique band of 600 bp in susceptible accessions which was absent in tolerant accessions

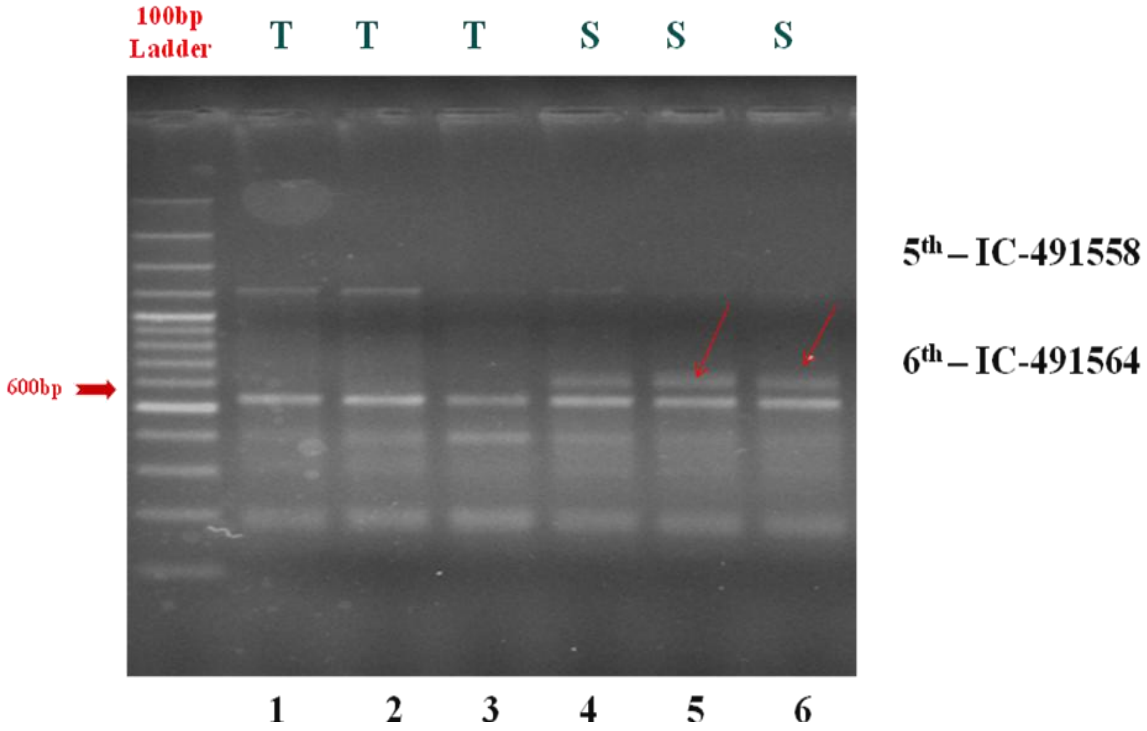

Fig.3 Confirmation of the transformed clones by comparing plasmid of blue and white colonies

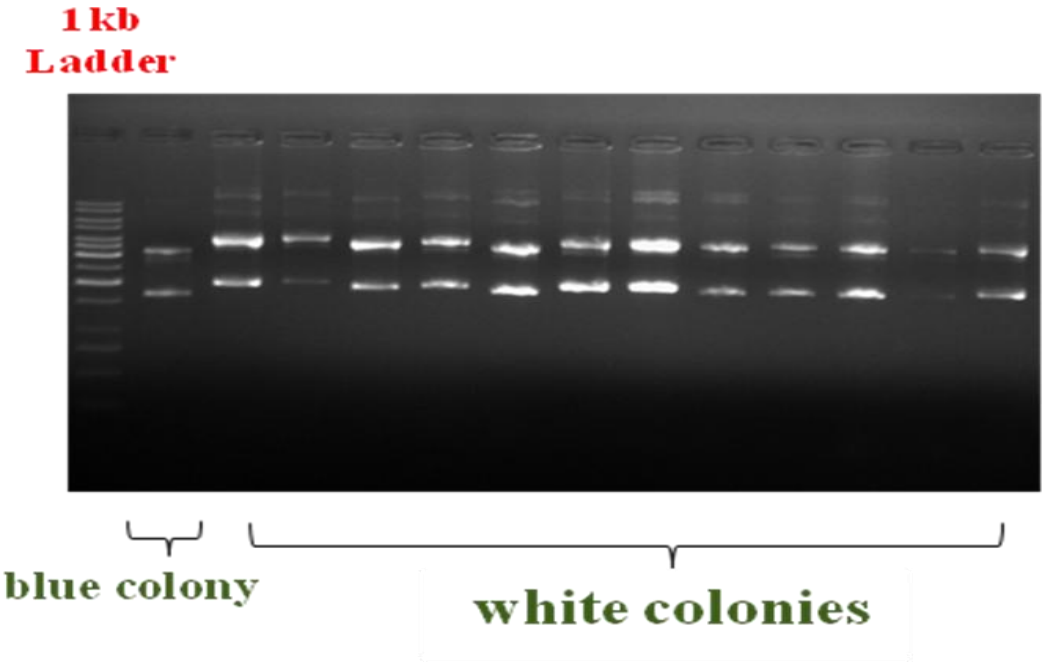

Table.1 Thirty four accessions of *B. juncea* with their reaction to mustard aphid under field conditions

S.No	Accession	Reaction (A)
C1	PJ Kisan	T
1	IC 326353	HT
2	IC 426349	MT
3	IC 491063	MT
4	IC 491260	MT
5	IC 491385	MT
6	IC 491386	T
7	IC 491414	HT
8	IC 491416	T
9	IC 491470	MT
10	IC 491472	MT
C2	Rajat	MT
11	IC 491473	T
12	IC 491482	MT
13	IC 491488	T
14	IC 491490	S
15	IC 491512	T
16	IC 491515	MT
17	IC 491517	MT
18	IC 491521	T
19	IC 491534	T
20	IC 491541	MT
C3	RH 30	HS
21	IC 491542	T
22	IC 491543	HS
23	IC 491548	MT
24	IC 491554	MT
25	IC 491557	T
26	IC 491558	HS
27	IC 491562	MT
28	IC 491564	S
29	IC 491565	MT
30	IC 491566	S
C4	Varuna	HS

A: Reaction of B. juncea accession to aphid based on visual observation; HS: Highly Susceptible, S: Susceptible, MT: Moderately Tolerant, T: Tolerant, HT: Highly Tolerant

Fig.4 Amplification of SCAR marker with Tolerant and Susceptible accessions

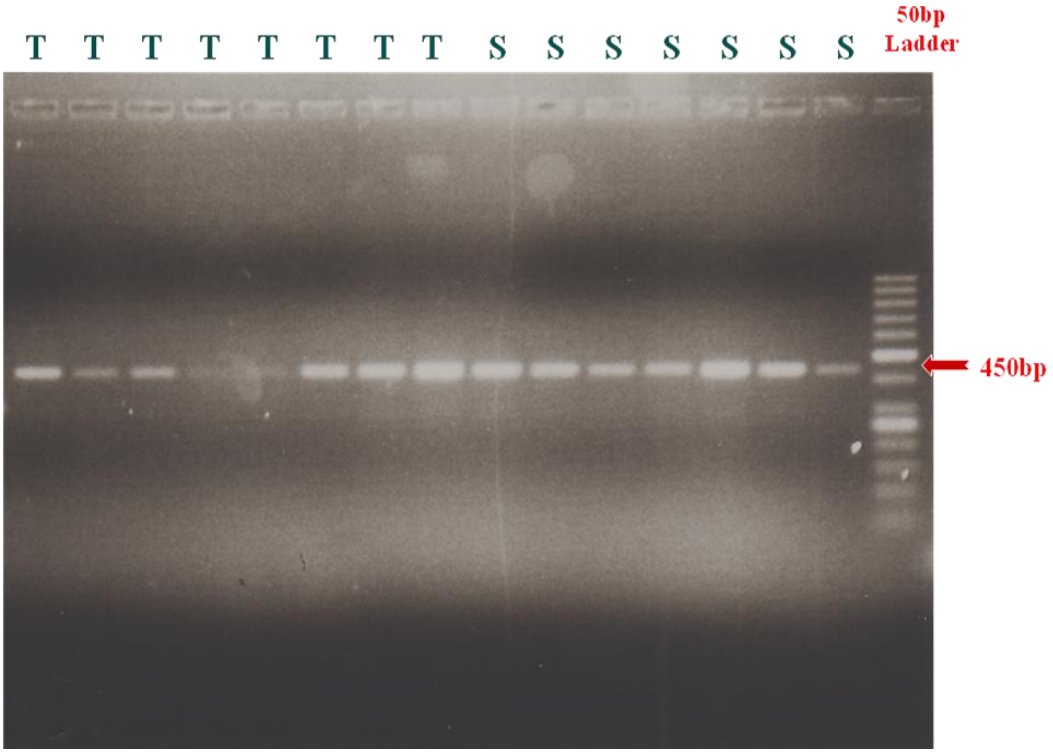

Table.2 GENEMED'S 10- MER Oligos Concentration 0.5 OD (16.5 µg)

S. No.	Oligos NO	Sequences
1	148	AGAGAGACAG
2	193	CTATAGGCAG
3	950	CCAGCCTCAT
4	971	AGGCTGACTT

Table.3 SCAR primer sequences

Primer ID	Primer Sequence	Annealing Temperature	Amplicon size (bp)
BJ01	F: 5'-TATAGGCTATTTACTCCTGGG R:5'-ATCACCTGGGAACTTGTCCC	54.6	396
BJ102	F:5'-TGAGCGATAAAGGACATGGG R:5'-GAGTTAAGCAGTAACGGGAGG	52.2	445

Scoring and screening of *B. juncea* accessions

Scale	Aphid reaction	Aphid populations
0.1-1.0	Highly tolerant	≤ 20 mean no .of aphids/ 10 cm inflorescence
1.1-2.0	Tolerant	20-50 mean no .of aphids/ 10 cm inflorescence
2.1-3.0	Moderately tolerant	51-100 mean no .of aphids/ 10 cm inflorescence
3.1-4.0	Susceptible	101-150 mean no .of aphids/ 10 cm inflorescence
4.1-5.0	Highly susceptible	>150 mean no .of aphids/ 10 cm inflorescence

Genomic DNA from 11 tolerant accessions (referred to as tolerant/resistant bulk) and 6 susceptible accessions (referred to as susceptible bulk) were pooled separately. A total of 284 RAPD primers were used to screen the DNA of 4 accessions (2 tolerant and 2 susceptible) to find out the possible rate of amplification. The 87 primers showed quality amplification and were selected for further screening of tolerant and susceptible bulks. Amongst them only 4 RAPD primers showed polymorphism on tolerant and susceptible bulks. The results formed the basis to hypothesize the concept of putative sources of resistance backed by polymorphic RAPD markers. Further 4 polymorphic markers thus obtained were subjected to screen three randomly selected individual tolerant and susceptible accessions. Interestingly only single primer (oligos-193, CTATAGGCAG) showed clear cut polymorphism among the two contrasts. Specific locus was present in susceptible accessions (amplifying a band of 600 bp) and absent in the tolerant accessions.

This locus was isolated, cloned and sequenced; RAPD marker oligos-193 (Genemed 10-mer) was converted in to SCAR markers BJ-01and BJ-02. The locus specific markers for single band amplification are expected to provide ease of selection and reproducibility across laboratories. In the above study, the SCAR markers were ambiguous for distinguishing the tolerant and susceptible accessions as these primers gave similar band size of 450 bp with both the tolerant and susceptible accessions (Fig.4). Failure of SCAR markers to produce polymorphism either due to original RAPD polymorphisms was caused by mismatches in nucleotides in the priming sites as reported by Paran and Michelmore (1993) or due to the crossing over between the gene controlling the trait and marker.

Sufficient variation for tolerant and susceptible accessions were found in replicated trial, corresponding variation was also found at DNA level with RAPD analysis, but results could not validate with designed SCAR marker successfully, due to crossing over between the gene and marker or mismatches in nucleotides in the priming sites. Further study can be conducted to develop cleaved amplified polymorphic sequence (CAPS) by restriction digestion using RFLP analysis and fine mapping of this particular locus by developing mapping populations or genome walking technique. If

we could tag this susceptible gene in *B. juncea* accessions, it would be very useful to find resistant sources using marker assisted selections (MAS).

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