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Identification of Molecular Markers for Karnal Bunt Resistance by Using RGAP in Wheat (*Triticum aestivum* L. em. Thell)

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ABSTRACT

Wheat is one of the main staple foods used in many countries after rice. Due to increasing population day by day, it is necessary to increase wheat production at high scale. India has potential to export wheat to other countries due to its surplus production, but its yield and quality is highly affected by many biotic stresses. Karnal bunt is one of them adversely affects quality of wheat. Due to quality measurements, even after more production than our needs, we are unable to trade wheat in other countries due to quarantine regulations. So it is necessary to find molecular markers directly linked to karnal bunt resistance genes for the selection of karnal bunt resistance genotypes at the early stages as these would allow indirect marker assisted selection of karnal bunt resistant genotypes. In the present study, a set of 104 recombinant inbred lines (RILs) developed from a cross between parents H567.71 (resistant) and WH542 (susceptible) to Karnal bunt were screened and used to identify RGAP markers linked with resistance to karnal bunt. In this study, three markers RGAP 7, RGAP 8 and RGAP 9 found to be directly associated with Karnal bunt resistance genes. These markers may be useful in marker assisted selection for karnal bunt resistance in wheat.

Key words: *Karnal bunt, Neovossia indica, Resistance Gene Analog Polymorphism.*

INTRODUCTION

Due to quarantine regulation, Karnal bunt caused by *Neovossia indica* fungus becomes very important disease of wheat [*Triticum aestivum* (L.) em. Thell] worldwide. Karnal bunt caused by *Neovossia indica* (Mitra) Mundkur (*Tilletia indica* Mitra), was first reported from Karnal, India in 1931 that affects wheat production in the Indian subcontinent. It infects the plant at boot leaf stage and then pathogen penetrates individual florets, entering the embryo end of the kernel and proceeds along the ventral crease. The disease can be damaging even at lower incidence as colour and palatability of wheat products is adversely affected.

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This disease has gained bigger dimension in recent years due to its wide spread prevalence in the main wheat growing region of North Western India and causing significant qualitative losses. The worldwide distribution of *N. indica* is limited but numerous wheat importing countries have quarantines against the import of wheat with KB, so the presence of KB in a region seriously affects the international grain trade. In order to maintain the sales of wheat in the foreign market phytosanitary certificates are required indicating that wheat lots are free from karnal bunt. Since the disease is soil, air and seed borne, only a limited success in its control can be achieved through fungicides. The use of resistant varieties is the best and the most economical method for management of Karnal bunt but there is limited variability for resistance to Karnal bunt in cultivated wheat3 and truly resistant varieties are not available[8]. Thus, breeding resistant variety is the only efficient method for combating this disease. Developing karnal bunt resistant wheat cultivars with conventional methods is time consuming and labour-intensive. An alternative to direct selection for karnal bunt resistance, the indirect selection using DNA markers linked to genes conferring karnal bunt resistance is more competent. Resistance gene analog polymorphism (RGAP) has been used to identify many genes associated with disease resistance *eg.*, *Yr9* and *YrZak* genes for resistance to stripe rust in wheat[9]. RGAP markers show promise to be better candidates for locating genomic regions associated with disease resistance when compared to other DNA markers. The objective of this study was to identify RGAP markers for the karnal bunt resistance gene through single marker analysis (SMA). The information of association of KB resistance to functional resistance genes through markers will lead to a better understanding of the plant-pathogen interaction. Molecular markers should be useful in marker-assisted selection to incorporate this gene into commercial cultivars and to combine with other genes to achieve durable resistance.

MATERIAL AND METHOD

The mapping population and evaluation for karnal bunt resistance

The resistant wheat genotype H567.71 obtained from CIMMYT (Mexico) was crossed with susceptible cultivar WH 542 from CCS HAU, Hisar as the female parent, then F9 RILs were developed from this cross through single-seed descent.

One hundred and four RILs along with their parents evaluated for karnal bunt resistance under artificial inoculation condition. Parents H 567.71 and WH 542 along with their 104 recombinant inbred lines (RILs) were grown in the paired rows each of 2 m length in three replications in randomised block design (RBD) at the experimental area of Department of Genetics and Plant Breeding CCS Haryana Agricultural University, Hisar (India) in the crop seasons of 2011-12. Recommended agronomic practices were followed to raise a good crop.

Data recording and analysis

At maturity, the inoculated spikes were harvested individually and threshed manually. Percent infected grains were recorded. Grains were separated in to different grades by visual observation and Coefficient of infection was calculated out as suggested by Aujla et al.[2]

DNA extraction

Fresh leaves (5 g) from parents and 104 RILs at the seedling stage were grounded in liquid nitrogen. DNA was extracted from the leaf powder using the cetyltrimethylammonium bromide (CTAB) procedure and stored at −20°

C. DNA was quantified using a NanoDrop spectrophotometer and the concentration was adjusted to 50 ng μL^{-1}. The RGAP primers (Table 1) were used to screen the RILs along with their parents for molecular characterization. Resistance gene analog polymorphism (RGAP) primers were used for determination of genetic diversity in wheat genotypes based on karnal bunt reaction data. The PCR reaction was carried out in a reaction volume of 20 µl containing 1.5 ul of 1X PCR (polymerase chain reaction) buffer, 200 µM dNTPs (deoxy ribonucleotide tri phosphate), 0.5 µl of each primer, 1 unit Taq DNA polymerase and 50 ng templates DNA. PCR amplification was carried out. After an initial 5 min denaturation at 95°C, 40 cycles of 1 min at 94°C, 1 min at either 45, 50, 55 or 60°C, 2 min at 72°C were carried out, finalized by a 7 min extension step at 72°C. The success of amplification was checked by electrophoresing the PCR products on a 1% (w/v) agarose gel in 0.5×TBE (Tris- Boric acid – EDTA) buffer. PCR amplified products were viewed under UV light (350 nm) fluorescence and photographs were taken by gel documentation system. For Karnal bunt resistant, individual primer combination was used to amplify genomic DNA of both resistant and susceptible parents. The primers showing polymorphism between parents were used for amplification of genomic DNA of RILs individually[5] for genotyping.

Single marker analysis (Marker-trait association)

The genotypic (A, B and H code) and phenotypic data obtained from 104 RILs population produced from the cross H 567.71 / WH 542 was subjected to single marker analysis. The association between RGAP molecular markers with percentage of infection and coefficient of infection was assessed with simple regression analysis approach using one-factor ANOVA method as implemented in Microsoft Excel (MS office 2007) programme. Magnitude of the marker associated with infection effect was described by the coefficient of determination (% R^2) which represented the fraction of variance explained by the polymorphism of the marker.

RESULTS AND DISCUSSION

Analysis of variance for coefficient of infection and percentage of infection showed significant variations among all the recombinant inbred lines showed in Table 1. The range of coefficient of infection (CI) in the RILs was 0.00 to 14.21 % in the year 2011-12. There was a wide variation among the 104 RILs evaluated for Karnal bunt resistance. The distribution of RILs based on Karnal bunt disease was skewed higher towards the resistant parent H 567.71(Table 2, Figure 1). According to the study of Kumar (2010-11), the range of coefficient of infection (CI) in the susceptible RILs was 0 to 8.28 and 0 to 14.45 in the year 2010 and 2011 respectively. Similarly, the distribution of RILs based on Karnal bunt disease was skewed higher towards the resistant parent (H567.71). In the present study, under molecular diversity analysis total 18 different RGAP primers (Table 3) used for screening of parental genotype and out of total 121 RGAP marker combinations, only 9 were found to be polymorphic (Table 4). A total of 89 unambiguous bands were amplified by the 9 RGAP of which 46 bands were polymorphic (51.68 %) and ranged in size from 150 – 725 bp. Yan *et al.* reported that out of the 379 primer pairs from 48 individual RGA primers that were screened in bulk segregant analyses, 78 pairs (21 %) produced 89 polymorphic bands in wheat for *Yr5* gene showing

resistance for stripe rust. In few studies, single marker analysis (SMA) was used to study marker-trait association, and to detect quantitative trait loci for grain weight[1,4,10]. Using single marker analysis, three markers RGAP 7, RGAP 8 and RGAP 9 were found to be associated with coefficient of infection and explained up to 11.5, 7.9 and 7.5 per cent of the phenotypic variance (Table 5). RGAP 7, RGAP 8 and RGAP 9 were identified to be associated with coefficient of infection of Karnal bunt with phenotypic variation range from 7.5 to 11.5 per cent. It means that RGAP 7, RGAP 8 and RGAP 9 sequences were directly associated with Karnal bunt resistance genes. Similarly, based on single marker analysis some markers were found to be common associated with different traits. Marker *Xgwm573-7A* was associated with days to heading and days to anthesis, marker *Xgwm234-5B* was associated with days to maturity and chlorophyll fluorescence, marker *Xwmc695-7A* was associated with days to maturity, tillers/plant and 1000-grain weight and *Xgwm369-3A* was associated with 1000-grain weight, coleoptile length and chlorophyll content The markers explained between 3.70 and 18.00 per cent of the phenotypic variation in drought condition. A total of 15 putative SSRs were recorded to be linked to the traits by single marker analysis ranging from 1 for effective tiller/plant, plant height and chlorophyll fluorescence to 3 for coleoptile length[3].

The present study combining phenotypic traits and molecular data paves way for developing Karnal bunt resistant high yielding wheat genotypes. Development of such wheat varieties is priority for plant breeders because of exports which have positive impact on Indian economy. From the present studies it could be concluded that evaluation of germplasm is an important step in plant breeding so that the genotypes having inherent ability to perform better for different traits can be selected. Karnal bunt is an important disease which require the simultaneous attention of pathologists and the breeder. The use of molecular markers is essential for improving the efficiency of traditional plant breeding by facilitating indirect selection through markers linked to genes of interest because these markers are not influenced by the environment and can be scored at all stages of plant growth. In the present study, three RGAP markers found to be directly linked with KB disease, so after validation of these markers the resistance gene can be identified in any genotype with more authenticity and can be exploited as and when required. However, the knowledge of amplification profile of the marker is essential so that a particular gene can be followed through the product it amplifies.

Table 1: Analysis of variance for Percent Infection and Coefficient of Infection in 104 RILs derived from wheat cross H 567.71 / WH 542

Source of variation	Degree of freedom	Mean squares	
		Percent infection	Coefficient of infection (%)
		2011-12	2011-12
Genotypes	103	219.12**	40.34**
Error	206	0.55	0.08

Table 2: Mean performance of 104 RILs derived from wheat cross H 567.71 / WH 542 for Percent Infection (PI) and Coefficient of Infection (CI) during the year 2011-12*

RILs	PI	CI	RILs	PI	CI
1	0.00	0.00	**54**	23.42	9.45
2	0.00	0.00	**55**	10.09	3.44
3	0.00	0.00	**56**	32.63	14.21
4	1.33	0.83	**57**	12.60	3.74
5	12.67	5.63	**58**	10.11	3.93
6	0.00	0.00	**59**	0.00	0.00
7	30.92	11.34	**60**	22.37	7.57
8	2.51	1.41	**61**	14.91	6.58
9	9.09	5.57	**62**	1.61	0.60
10	11.21	6.03	**63**	0.00	0.00
11	5.43	1.63	**64**	0.70	0.18
12	1.88	0.47	**65**	0.00	0.00
13	0.78	0.58	**66**	0.00	0.00
14	0.00	0.00	**67**	0.00	0.00
15	0.00	0.00	**68**	0.00	0.00
16	0.00	0.00	**69**	0.00	0.00
17	22.11	12.67	**70**	0.00	0.00
18	0.00	0.00	**71**	0.00	0.00
19	0.00	0.00	**72**	10.88	4.42
20	5.39	1.45	**73**	0.00	0.00
21	2.77	0.69	**74**	1.16	0.58
22	3.12	1.30	**75**	0.00	0.00
23	11.59	5.43	**76**	20.59	5.71
24	12.26	4.95	**77**	0.00	0.00
25	0.00	0.00	**78**	0.00	0.00
26	4.06	1.01	**79**	0.00	0.00
27	0.00	0.00	**80**	0.64	0.16
28	0.00	0.00	**81**	13.16	6.58
29	0.00	0.00	**82**	3.10	1.36
30	1.61	0.67	**83**	13.01	5.08
31	2.27	1.51	**84**	0.00	0.00
32	1.75	0.87	**85**	0.00	0.00
33	10.71	5.58	**86**	18.25	7.66
34	14.28	5.26	**87**	2.97	1.73
35	14.89	6.73	**88**	0.00	0.00
36	0.00	0.00	**89**	3.10	1.16
37	0.00	0.00	**90**	3.53	1.76
38	0.00	0.00	**91**	0.00	0.00
39	0.00	0.00	**92**	16.35	7.21
40	0.00	0.00	**93**	3.79	1.70
41	0.00	0.00	**94**	5.76	2.70
42	37.14	13.80	**95**	10.68	3.64
43	0.00	0.00	**96**	13.38	5.11
44	0.00	0.00	**97**	3.57	1.34
45	0.00	0.00	**98**	0.00	0.00
46	0.00	0.00	**99**	17.65	7.14
47	0.00	0.00	**100**	12.75	4.41
48	0.00	0.00	**101**	3.42	1.71
49	0.69	0.17	**102**	11.52	4.85
50	18.32	7.32	**103**	10.38	4.48
51	0.00	0.00	**104**	7.94	3.37
52	0.00	0.00	**WH 542**	36.96	14.31
53	17.24	6.89	**H 567.71**	0.00	0.00

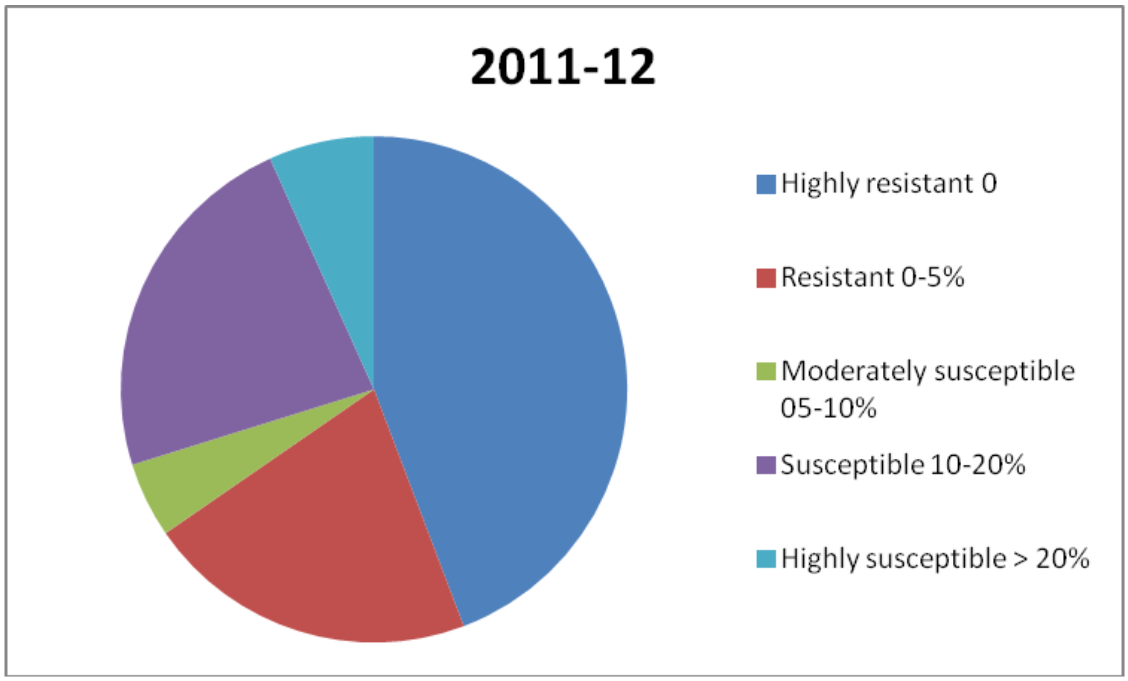

Fig. 1: Categorization of 104 RILs on the basis of percent of infection during 2011-12

Table 3: Sequences of RGAP primers used in the present study

Sr. No.	RGA	Primer Sequence (5'–3')	Gene	Domain
1	S2	GGIGGIGTIGGIAAIACIAC	*N, Rps2*	P-loop
2	AS3	IAGIGCIAGIGGIAGICC	*N, Rps2*	LRR
3	RLRR Rev	ACACTGGTCCATGAGGTT	*Rps2*	LRR
4	LM638	GGIGGIGTIGGIAAIACIAC	*L6, N, Rps2*	P-loop
5	Pto kin1IN	AAGTGGAACAAGGTTACG	*Pto*	Kinase
6	Pto kin2IN	GATGCACCACCAGGGGG	*Pto*	Kinase
7	XLRR Rev	CCCATAGACCGGACTGTT	*Xa21*	LRR
8	Pto kin1	GCATTGGAACAAGGTGAA	*Pto*	Kinase
9	Pto kin2	AGGGGGACCACCACGTAG	*Pto*	Kinase
10	AS3-INV	CCIGAIGGIGAICGIG	*N, Rps2*	LRR
11	CLRR-INV2	TCTTCAGCTATCTGC	*Cf9*	LRR
12	Xa1LR-F	CTCACTCTCCTGAGAAAATTAC	*Xa1*	LRR
13	Pto kin4	AGTGTCTTGTAGGGTATC	*Pto*	Kinase
14	Cre3LR-R	CAGGAGCCAAAAATACGTAAG	*Cre3*	LRR
15	RLK-Rev	TCYGGYGCRATRTANCCNGGITGICC	*LrK10*	Kinase
16	Xa1NBS-F	GGCAATGGAGGGATAGG	*Xa1*	NBS
17	RLK-For	GAYGTNAARCCIGARAA	*LrK10*	Kinase
18	NLRR-INV1	TTGTCAGGCCAGATACCC	*N*	LRR

Table 4: List of RGAP primers showing polymorphism

Sr. No.	Name of marker	Polymorphic primer combination (Forward/Reverse)	Annealing temperature (°C)	Size of band (bp)
1	RGAP 1	Pto kin1IN/Pto kin2IN	55.0	250-650
2	RGAP 2	Cre3LR-R/Pto kin2	58.6	225-600
3	RGAP 3	Xa1LR-F/Pto kin4	54.5	200-675
4	RGAP 4	XLRR Rev/Pto kin1	53.2	200-700
5	RGAP 5	CLRR-INV2/Pto kin1	49.0	180-650
6	RGAP 6	RLRR Rev/Pto kin4	52.3	250-650
7	RGAP 7	Cre3LR-R/Xa1NBS-F	58.3	150-600
8	RGAP 8	Pto kin2/NLRR-INV1	58.0	180-580
9	RGAP 9	Xa1NBS-F/Pto kin4	53.5	225-650

Table 5: Association of Coefficient of infection and RGAP markers detected by single marker analysis (SMA) using RILs population of cross H 567.71 / WH 542

Sr. No.	Markers	R^2 (%)
1.	RGAP 7	11.5*
2	RGAP 8	7.9*
3.	RGAP 9	7.5*

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