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Mapping of a resistance gene effective against Karnal bunt pathogen of wheat

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Abstract A set of 130 wheat recombinant inbred lines (RILs) developed from a cross between parents susceptible (WL711) and resistant (HD29) to Karnal bunt (caused by *Tilletia indica*), were screened for 3 years with the pathogen populations prevalent in northern India. When 90 simple sequence repeats (SSRs) and 81 amplified fragment length polymorphism (AFLP) loci were mapped on the RILs, markers on chromosomes 2A, 4B and 7B accounted collectively for about one-third of the variation in the disease reaction. The genomic region of largest effect, identified on the long arm of chromosome 4B, reduced Karnal bunt disease by half in three different experiments and accounted for up to 25% of the phenotypic variation for KB reaction. A closely linked SSR marker, GWM538, may be useful in marker-assisted selection for Karnal bunt resistance in wheat.

Keywords Wheat · Microsatellite · Karnal bunt · QTL · Resistance · *Tilletia indica* · EST-SSR

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Introduction

Karnal bunt (KB), caused by *Tilletia indica* Mitra [Syn. *Neovossia indica* (Mitra) Mundkar], is a disease of wheat (*Triticum aestivum* L.) that was first reported at Karnal in northern India in 1931. The major wheat-growing areas affected by KB include the irrigated, spring wheat-growing areas of northwest India, Pakistan, and northwestern Mexico. Karnal bunt has also been reported in other countries including the U.S.A. (Ykema 1996) and the Republic of South Africa. The southern Great Plains of the U.S. is the only place in the world where Karnal bunt has been detected in a winter wheat-growing area (Fuentes-Dávila, personal communication).

The KB pathogen causes infection on the floral parts, entering into the glumes, rachis and ovary. Infected portions of the kernel are replaced with masses of dark, fishy-smelling, fungal teliospores. Infection varies from traces to invasion of the entire kernel. Wheat with more than 3% bunted kernels is considered unsatisfactory for human consumption (Mehdi et al. 1973). Although yield losses are usually low, international quarantine policies against the disease may restrict the free flow of the global wheat trade (Royer and Rytter 1988).

Conventional approaches for controlling KB include cultural practices such as crop rotation, sowing of disease-free seeds, and adjustment of the time of irrigation to minimize disease infection (Munjal 1974; Singh 1985). Chemical control of the disease is very difficult and is not cost effective. Most of the fungicides tested against teliospores are fungistatic and not fungicidal. The teliospores of *T. indica* have staggered germination and can stay viable for 5–7 years (Singh and Singh 1985).

The most-effective and economical method of disease management is through host plant resistance. The wheat cultivars with sufficient levels of resistance to KB have been observed among Indian, Chinese, and Brazilian wheats (Fuentes-Dávila and Rajaram 1994). A few lines of durum wheat (*Triticum turgidum*), common wheat, *Aegilops*, rye, and barley were also reported to be resis-

tant to Karnal bunt under artificial conditions (Dhaliwal et al.1986; Warham et al. 1986; Warham 1988; Singh and Dhaliwal 1989; Gill and Aujla 1997). Genetic variation for resistance has also been identified in synthetic hexaploid wheats derived from *Triticum turgidum* × *Aeilops tauschii* crosses (Multani et al. 1988; Villareal et al. 1996).

Previous genetic studies on KB resistance in wheat indicated that one to six major genes condition resistance in various wheat germplasms (Morgunov et al. 1994; Fuentes-Dávila et al. 1995; Singh et al. 1995a, b, 1999; Villareal et al. 1995). Six wheat chromosomes (1D, 2A, 3B, 3D, 5D and 7A) were identified as influencing reaction to the pathogen (Gill et al.1993; Singh et al. 1994). Nelson et al. (1998), reported RFLP markers on chromosome arms 3BS and 5AL associated with KB resistance inherited from a synthetic hexaploid wheat.

Developing KB-resistant wheat cultivars with conventional methods is time-consuming and labor-intensive. Progenies resistant to KB are identified by inoculation at the boot stage with spordia of *T. indica*. Reliable production of inoculum requires skilled personnel, and the boot-inoculation method is laborious. Disease expression is highly influenced by environmental conditions. In addition, scoring disease symptoms requires hand harvesting, threshing, and counting of infected and uninfected kernels. Because symptoms are scored on mature grain, resistant plants cannot be used in a crossing program in the same season. Quarantine restrictions present the greatest challenge to the development of resistant wheat cultivars in many parts of the world. In the U.S., testing of lines in inoculated field nurseries is prohibited. Evaluating U.S. winter wheat in inoculated nurseries in Mexico and India requires photoperiod and vernalization requirements that may have to be provided by artificial means. Even with such treatment, many winter wheat lines will not flower early enough to obtain a reliable KB infection. The large amount of money and effort required to conduct these nurseries limits their use for breeding KB-resistant winter wheat cultivars.

An alternative to direct selection for KB resistance is the indirect selection for DNA markers linked to genes conferring KB resistance. DNA markers linked to disease-resistance genes in wheat have been reported, including markers linked to leaf rust (Huang and Gill 2001, Raupp et al. 2001), stripe rust (Singh et al. 2000), common bunt (Demeke et al. 1996) and Fusarium head blight resistance genes (Anderson et al. 2001). Many of the linked markers identified in various studies are restriction fragment length polymorphisms (RFLPs) that are co-dominant and reproducible, but are of limited use in wheat breeding programs since, on average, only 15–20% of the RFLPs are polymorphic between lines of *T. aestivum* (Liu et al. 1990). Microsatellite or simple sequence repeat (SSR) markers, a class of PCR-based markers with tandem repeats of a basic motif of fewer than six base pairs, are easier to use and more polymorphic than RFLP markers (Röder et al. 1998). Identification of PCR-based markers linked to KB resistance offers the prospect of using marker-assisted selection schemes in developing resistant wheat cultivars.

Here we report the identification and tagging, with PCR-based markers, of a gene conferring resistance to *T. indica* in a cross of the highly KB-resistant wheat line HD29, with the KB-susceptible Indian spring wheat WL711.

Materials and methods

Karnal bunt screening

A population of 130 F_8 recombinant inbred lines (RILs) was developed at Punjab Agricultural University, Ludhiana, India, by single-seed descent from a cross between two semi-dwarf spring wheats. WL711, a widely adapted Indian wheat cultivar, is highly susceptible to KB, while HD29, a line developed at the Indian Agricultural Research Institute (IARI), New Delhi, is resistant to a wide range of *T. indica* isolates (Gill et al. 1993).

The RILs were screened for resistance to KB in four different field experiments at Ludhiana in three growing seasons: two experiments in 1996–97, and one each in 1997–98, and 1998–99. In each experiment, the RILs were grown in plots consisting of two rows, 1-m long with a plant spacing of 10 cm. Parents were planted similarly in plots consisting of four rows. In each line, 5 to 15 tillers were inoculated at the boot stage as described by Aujla et al. (1982). Pathogen populations were collected from wheat cultivars (PBW154, TL-1210 and WL711) grown in Punjab, a state in northern India.

At maturity, the inoculated heads were harvested, threshed separately, and the percentage of infected grains in each head recorded. Mean incidence of the disease was determined from five inoculated spikes per entry. Data from the three experiments was analyzed using the CORR and GLM procedures of SAS (SAS 1989) with the different experiments treated as replicates. Effects were considered as random and mean squares were used to estimate the heritability of the disease response in the population.

Molecular analysis

The parents and RILs were grown in the greenhouse, and DNA was isolated from fresh leaves following the method of Riede and Anderson (1996). A total of 522 SSRs were used for parental polymorphism surveys. The collection included, 246 GWM SSRs with dinucleotide repeats (Röder et al. 1998), 65 D-genome specific SSRs with dinucleotide repeats (Pestsova et al. 2001), 141 trinucleotide repeats with prefix 'BARC' (Song et al. 2000) and 70 SSRs derived from the wheat EST data base designated with the prefix KSM (Singh et al. 2000). All BARC and KSM markers were screened on nulli-tetrasomic and ditelosomic lines of Chinese Spring wheat to determine chromosome location. PCR amplification conditions were as described by Röder et al. (1998). PCR products were separated on 2.3% agarose (Metaphor) gels in 0.5 × TRIS borate buffer. Gels were stained with ethidium bromide, visualized with UV light, and photographed. One end of the primer GWM538 was end-labeled with fluorescent dye and amplified fragments were run on an ABI 3700 DNA analyzer (ABI Prism) in a 96-well capillary array. A ROX (6-carboxy-X-rhodamine) labeled internal size standard of 71–227-bp size was run in each capillary along with the DNA samples. Phenogram files were documented and genotypes analyzed with GeneScan 3.0 and Genotyper 2.0 (ABI Prism) software programs.

An AFLP kit (Invitrogen Life Technologies) was used with slight modification from the manufacturer's instructions (Vos et al. 1995) to identify additional polymorphic markers. Eight primer-pair combinations (*Eco*RI-AGC/*Mse*I-CAT, *Eco*RI-AGG/*Mse*I-CAA, *Eco*RI-ACT/*Mse*I-CTC, *Eco*RI-ACC/*Mse*I-CTA, *Eco*RI-ACT/*Mse*I-CTG, *Eco*RI-AGG/*Mse*I-CAT, *Eco*RI-AAC/*Mse*I-CAC and *Eco*RI-

ACG/*Mse*I-CAG) from the kit were used on the RIL population. The PCR conditions for pre-amplification and selective amplification were as described by Vos et al. (1995). Seeds of WL711 and HD29 along with the RILs, were scored for the activity of polyphenol oxidase (PPO) as described by Shelton and Park (1993). Phenolics such as PPO are well known antifungal, antibacterial, and antiviral compounds occurring in plants and the total phenol status of wheat has been correlated with host resistance to a variety of diseases, including stem rust (Moerschbacher et al. 1989), KB (Gill et al. 1993) and take-all (Regel et al. 1994). Considering these observations, the polyphenol oxidase activity in the kernel of the parents (HD29 and WL711) and the RIL population was compared. HD29 had a high level of activity and WL711 a low PPO activity. RILs were classified as either high or low.

A total of 207 polymorphic markers were evaluated on the RILs. A linkage map was constructed with the MAPMAKER 2.0 computer program (Lander et al. 1987). In this program, the two-point/group command was used for establishing possible linkage groups with a minimum LOD score of 3.0. The most-likely order of markers in each group was determined by using the 'order', 'compare' and 'ripple' commands. Order information from previously published maps (Röder et al. 1998) was also used. Chromosome-specific SSR markers were used as anchors to integrate AFLP markers and assign linkage groups. Markers with distorted segregation ratios were identified using the chi-square test for fitting a 1:1 ratio. Markers and phenotypic data were analyzed with the computer program QGene v.3.06 (Nelson 1997). Simple interval mapping (Haley and Knott 1992) was used to identify chromosomal regions influencing resistance.

Results

Disease reaction of the RILs

The two parents differed sharply for mean incidence of the disease in two experiments in 1996–97 and one in 1997–98. Disease incidence in the 1998–99 was very low, and the data were not included in this analysis. Mean disease incidence of KB on HD29 and WL711 over the three experiments was 1.0% and 56%, respectively. The range of disease on the RILs was 0–74% (Table 1). The distribution of KB disease incidence on the RILs was skewed toward the resistant parent type for the three experiments (Fig. 1). Levels of disease in the population in the three experiments were significantly correlated ($r = 0.50–0.52$, $P < 0.001$).

Significant variation for disease incidence was observed among the RILs (Table 2). The heritability of KB resistance in this population on an entry mean basis and for individual observation was 0.69 and 0.52 respectively. Given that lines were not randomized in each experiment, the calculated heritability measure may overestimate the proportion of the phenotypic variation accounted for by genetic variation.

Molecular genetic analysis

Polymorphism survey

A survey of polymorphism between WL711 and HD29 was done using SSR and AFLP markers. Primer pairs for dinucleotide markers isolated from a wheat genomic library (designated 'GWM' by Röder et al. 1998) revealed the highest (28%) level of polymorphism between the resistant and susceptible parents. Approximately 10% polymorphism was detected with both the dinucleotide SSR markers isolated from *Ae. tauschii* (Pestova et al. 2001) and wheat SSRs having trinucleotide repeats. Simple sequence repeat markers designed from the wheat EST database were the least polymorphic between HD29 and WL711. The percent polymorphism obtained with B-genome-specific GWM-SSR markers was 31.7 compared to 27.0 and 16.7 for A- and D-genome-specific markers, respectively. In a survey of eight AFLP primer combinations, the number of visible amplified DNA fragments in each combination ranged from 46 to 80 of which 4 to 14 were polymorphic between the parents. Over all eight-primer combinations tested, 81 of 521 bands (16%) were polymorphic between the parents. The polymorphism level for each combination ranged from 9.4 to 29.8%.

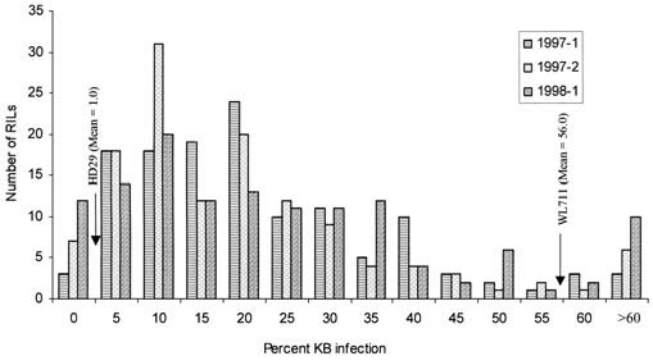

Fig. 1 Histogram showing mean percentage of Karnal bunt incidence (experiments 1997-1, 1997-2, 1998-1) on 130 RILs developed from the cross of susceptible (WL711) and resistant (HD29) wheat lines

Table 1 Mean and range of disease incidence of recombinant inbred lines in the HD29 × WL711 population evaluated for Karnal bunt disease in inoculated field experiments

% Kernels infected			
Experiment	Mean	Range	# of Lines
1997-1	14.8	0–53.0	129
1997-2	13.1	0–67.2	128
1998-1	15.2	0–73.7	129

Table 2 ANOVA table of Karnal bunt disease in recombinant inbred lines in the HD29 × WL711 population evaluated in inoculated field experiments

Source	*df*	MS	F-ratio	*P*
Replications	2	148.36	1.69	0.19
RILs	131	357.98	4.07	<0.001
Error	254	87.98		
Total	385			

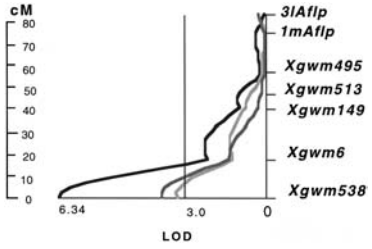

Fig. 2 Interval map of chromosome arm 4BL of wheat for response to artificial inoculation with Karnal bunt pathogen populations in recombinant inbred lines derived from the cross of WL711 × HD29. The distal part of chromosome is toward the bottom and the upper part is toward the centromere on the map. Maximum LOD score is shown to the left of the base of the map. The *dark contour* represents experiment 1997-1 and *gray and light gray* for the experiments 1997-2 and 1998-1, respectively

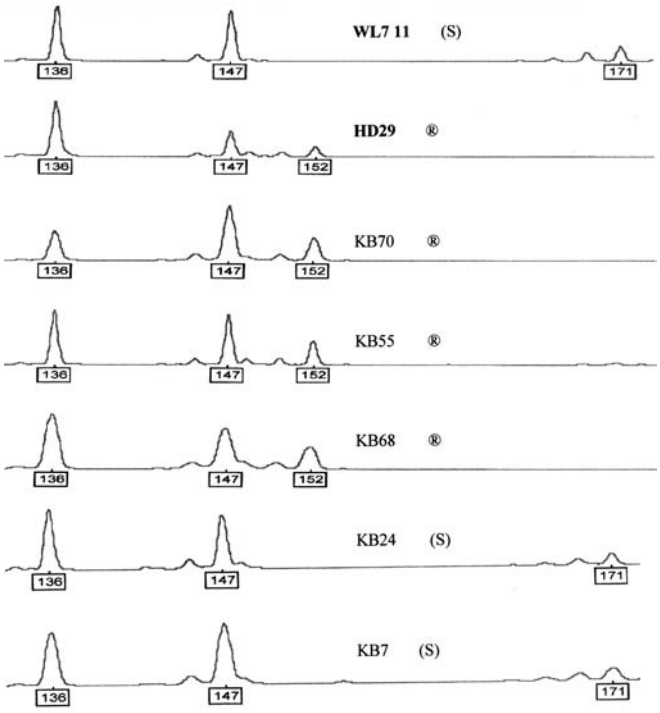

Fig. 3 GeneScan analysis illustrating segregation of the marker *Xgwm538* in RILs. One end of the primer pair was labeled with fluorescent dye and amplified fragments were run on an ABI 3700 DNA analyzer; peaks represent fragments and sizes in base pairs were calculated from internal standards. Fragments specific to resistant (*R*) and susceptible (*S*) parents are 152 and 171 bp, respectively. Parents are indicated in *bold*

Mapping

A total of 90 SSR, 116 AFLP loci, and PPO activity, were evaluated on the 130 RILs. Three SSR markers and 35 of the AFLP markers deviated significantly from the expected 1:1 segregation ratio and were not used for mapping. Of 68 GWM SSR markers 22 (32%) having dinucleotide repeat units behaved as dominant markers, only two (1.4%) SSR markers having trinucleotide repeats were dominant. Two-point linkage analysis of the 171 scored fragments yielded 30 linkage groups containing 125 loci. Twenty one groups (99 loci) were assigned to individual chromosomes of wheat, with five chromosome maps (3B, 5A, 6B, 7A and 7B) represented by more than one linkage subgroup. Our mapping results gave approximately 60% coverage based on the established wheat map (Röder et al. 1998). The orders of markers in linkage groups generally agreed with those in the published map.

Genetic analysis of disease

Interval analysis indicated that only one genomic region on chromosome arm 4BL near SSR marker *Xgwm538* exerted a strong and consistent influence on the disease (Fig. 2). The SSR primer pair GWM538 amplified a 152-bp DNA fragment in the resistant parent HD29 and a 171-bp fragment in the susceptible parent WL711 (Fig. 3). This marker accounted for 19.6%, 9.1% and 10.7% of the phenotypic variation in the population in experiments 1997-1, 1997-2 and 1998-1, respectively (Table 3). The region accounted for 18.3% of disease variation over all three experiments. Recombinant inbred lines with the HD29 allele at the *Xgwm538* locus had a mean KB incidence of 10.3%, 9.6% and 11.1% in experiments 1997-1, 1997-2 and 1998-1, respectively, whereas

Table 3 DNA markers associated with Karnal bunt resistance in wheat, their chromosome location, and percent variation explained for KB disease incidence

Locus	Location[a]	$R^2 \times 100$[c]			
		1997-1[b]	1997-2	1998-1	Mean
Xaflp2b	2A	–	5.6 (0.012)	5.3 (0.01)	4.2 (0.03)
Xgwm538[c]	4B	19.6 (<0.01)	9.1(<0.01)	10.7 (<0.01)	18.3 (0.00)
Xgwm6	4B	5.9 (<0.01)	–	3.4 (0.04)	6.0 (<0.01)
Xppo	NP	9.3 (<0.01)	5.3 (0.01)	–	7.0 (<0.01)
Xgwm46	7B	6.7 (0.05)	4.8 (0.02)	–	5.7 (0.00)

[a] Chromosome location of the markers was based on the genetic map. NP means that the locus could not be localized to a chromosome

[b] The year of the study followed by the experiment number. Mean = the grand mean over all experiments

[c] Phenotypic variation percent explained for with each locus. *P* values are given in brackets. Data not shown for *P*-values > 0.05

RILs with the WL711 allele had means of 20.6%, 17.6% and 21.2%.

The PPO locus, which was unlinked, was of second importance, accounting for 6–9% of the variation for all the experiments. Together with *Xgwm538*, PPO accounted for 15–25% of the disease variation. Markers from chromosomes 2A and 7B also showed minor ($R^2 < 0.06$) effects (Table 3). A multiple-regression model including the most-significant markers over all experiments, accounted for 30% of the variation in the KB response.

Discussion

The identification, mapping and tagging of KB-resistance genes in wheat is important for developing resistant wheat cultivars because of quarantine restrictions on the pathogen and the large effect of the environment on disease development. Most sources of genetic resistance to KB traced to China, India and Brazil (Gill et al. 1993; Fuentes- Dávila et al. 1995; Singh et al. 1995b). Wheat line HD29 is potentially an important source of genetic resistance to KB providing resistance to diverse isolates of *T. indica* (Gill et al. 1993). A true-breeding mapping population (RILs) was developed from the cross of WL711 × HD29, because KB screening over a number of replications and years to obtain reliable phenotypic data is required in order to establish reliable marker/trait associations. A 1998–99 experiment (data not shown) in which little disease occurred because of warm temperatures during flowering, illustrates well the large influence of environment on KB disease development even in inoculated nurseries. The distribution of KB disease severity on the RILs was skewed toward that of the resistant-parent in three experiments, suggesting the segregation of multiple genes with dominant or complementary gene action in wheat line HD29. This is consistent with the results of previous studies (Morgunov et al. 1994; Fuentes- Dávila et al. 1995). The heritability of 0.69 on entry mean basis for KB resistance in this population is consistent with other findings showing that resistance to KB in wheat is highly heritable and under relatively simple genetic control (Fuentes- Dávila et al. 1995; Nelson et al. 1998). Identification of RILs with lower and higher disease incidence than HD29 and WL711 respectively, suggests that WL711 probably has minor genes for KB resistance.

The level of polymorphism observed in wheat using microsatellite or SSR markers is greater than that of RFLPs markers, which makes it more-rapid and economical to map genes of interest. Approximately 80% of the SSR markers tested by Röder et al. (1998) were polymorphic between the *T. turgidum-Ae tauschii* synthetic hexaploid × *T. aestivum* parents of the ITMI mapping population using capillary electrophoresis. Twenty eight percent of the dinucleotide SSR markers from wheat were polymorphic between the spring wheat parents of our mapping population using an agarose-gel system with a lower power of resolution. The level of SSR polymorphism within common wheat is similar to that reported by Bryan et al. (1997). If a large number of SSR markers are developed for wheat, the observed rate of intraspecific allelic variation should be sufficient to construct genetic maps of adequate density to allow the detection of QTLs/genes with a large effect.

The level of polymorphism of trinucleotide SSR markers between wheat lines HD29 and WL711 was less than that observed with SSRs having dinucleotide repeat units. SSR markers developed from wheat ESTs were the least polymorphic between HD29 and WL711, regardless of the length of the repeat unit. This lack of polymorphism may result from the conserved nature of coding sequences and the limited diversity in the cultivated wheats from which these ESTs were derived. Consistent with previous reports (Bryan et al. 1997; Röder et al. 1998), the B genome yielded more polymorphic markers than the A or D genomes, with a striking deficit of D-genome specific markers. The D genome is known to be the least polymorphic in wheat (Cadalen et al. 1997), resulting in poorer map coverage than the other genomes, irrespective of the marker type used. The deficit of polymorphic D-genome markers in our population may account for our inability to map the PPO locus, which was associated with KB resistance. A locus influencing the level of PPO in grain was mapped on chromosome 6D (Li et al. 1999).

Our finding of only one region of chromosome 4BL with a consistent influence on the KB reaction, when segregation data and previous studies suggest that resistance is controlled by multiple genes, may be a consequence of incomplete coverage of the map by the markers used in this study. This is the first report of a QTL for resistance to KB on chromosome 4B. Additional QTLs with minor effects were detected on chromosomes 2A and 7B, each of which explained 3.0–7.3% of the phenotypic variation. Resistance was previously reported on 2A (Gill et al. 1993); this may be the same locus.

Our results, together with the above reports, suggest that several genes/QTLs for KB resistance reside in diverse wheat accessions and have the potential for pyramiding in desirable genotypes using marker-assisted selection to obtain an appropriate level of resistance to KB in wheat. Because RFLP markers in a polyploid crop like wheat are relatively unsuitable for marker-assisted breeding, additional PCR-based markers should be developed to make marker-assisted selection for KB effective. Additional approaches using functional genomics and ESTs may also be applied to identify markers closely linked with KB-resistance genes.

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