

SNP-Based Improvement of a Microsatellite Marker Associated with Karnal Bunt Resistance in Wheat

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ABSTRACT

Marker-assisted selection (MAS) has become the technology of choice for introgressing important traits with indistinct phenotypes into agronomically elite cultivars. Karnal bunt (KB, causal agent *Tilletia indica* Mitra) is an economically important fungal pathogen of wheat (*Triticum aestivum* L.) which has caused economic losses in the USA since it was first reported in 1996. To protect U.S. wheat from this emerging disease and the losses incurred from export quarantines, genetic sources of resistance are needed by breeders to improve U.S. germplasm. Resistance to KB is difficult to score phenotypically, making MAS an ideal choice for deploying this trait into U.S. wheat. Here we describe the conversion of a codominant microsatellite marker, *Xgwm538*, associated with a quantitative trait locus (QTL) for KB resistance into a single nucleotide polymorphism (SNP) based marker. The SNP marker was developed to improve gel-based resolution and amplification consistency. The *gwm538* primers amplify three fragments in the KB resistant line HD29: 137-, 147-, and a 152-bp fragment that maps to the long arm of chromosome 4B and is linked to the KB QTL. By cloning and sequencing all three fragments, we were able to exploit a SNP and design a new primer to selectively amplify the 152-bp fragment of interest (*gwm538snp*). Amplification consistency is improved with *gwm538snp* since the amplification of competing nontarget fragments is eliminated, and ambiguity is reduced since heterozygous plants are easily identified among backcross progeny.

MICROSATELLITE-based markers are commonly used to rapidly survey the genome of cereal crops for association with known genes. In recent years, the development of high-density microsatellite-based genetic linkage maps for wheat has provided genome-wide marker availability (Somers et al., 2004). High polymorphism detection levels, high throughput capability, and low cost compared to other marker systems enhance the utility of microsatellites for mapping genes associated with agronomically important traits and for MAS strategies (reviewed by Dubcovsky, 2004). MAS is especially valuable when selecting for traits that are difficult to screen for, such as resistance to Karnal bunt disease of wheat (<http://maswheat.ucdavis.edu>; URL verified 21 March 2006; reviewed by Dubcovsky, 2004; Dekkers and Hospital, 2002). KB resistance is identified in the

adult plant stage by determining disease incidence as a percentage of bunted kernels following harvest (Singh et al., 2003). Field screening is labor intensive, expensive, and the disease is highly influenced by environmental factors. KB was discovered in the USA in 1996 and poses a serious threat to the wheat export industry because of international quarantine regulations (Ykema et al., 1996). Development of resistant cultivars is an important preemptive measure to prevent the spread of this emerging disease. Since *T. indica* is a quarantined pathogen in the USA, MAS is the only feasible option for incorporating KB resistance into U.S. wheat germplasm.

Singh et al. (2003) reported the association of microsatellite marker *Xgwm538* with a QTL for KB resistance on the long arm of chromosome 4B, that accounted for 18.3% of disease variation (P value = 0.00) on average in a recombinant inbred line (RIL) mapping population. *Xgwm538* was predicted to be valuable for use in MAS to incorporate KB resistance genes into elite germplasm; however, direct implementation of *Xgwm538* into MAS programs has been difficult because of spurious amplification of target sequences and the masking of the target allele by a paralogous fragment resulting in an inability to identify heterozygotes among segregating progeny. We have found that these problems are not unique to *gwm538*, and others also have reported difficulties with microsatellite marker resolution (Bryan et al., 1995; Hourihan et al., 2001). Here we described the development of a single nucleotide polymorphism (SNP) based marker from *Xgwm538*. The SNP strategy was used in a similar fashion as described by Somers et al. (2003) where locus-specific sequence differences were used to distinguish paralogous and homoeologous sequences. Our objective was to selectively amplify the allele associated KB resistance and produce by MAS a marker that has direct utility for cultivar enhancement.

MATERIALS AND METHODS

Plant Material and Genomic DNA Isolation

The following plant materials were provided by the Wheat Genetics Resource Center at Kansas State University: Chinese Spring (CS), CS derived nullisomic-tetrasomic (NT) lines (N4AT4D, N4BT4D, and N4DT4B; Sears, 1954, 1966), the International Triticeae Mapping Initiative (ITMI) mapping population (TA4040; Nelson et al., 1995) and the KB RIL mapping population HD29(KB resistant) \times WL711(KB susceptible) (TA4325, Singh et al., 2003). One hundred thirty $F_{2:8}$ RILs were scored for disease incidence for 3 yr at the Punjab Agricultural University, Punjab, India (Singh et al., 2003). Seedling leaf tissue was harvested from all 130 RILs at the three-leaf stage for genomic DNA isolation by a CTAB DNA extraction protocol (Hulburt and Bennetzen, 1991).

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PCR Conditions and Fragment Separation

PCR was performed with the Promega *Taq* DNA polymerase kit (Promega, Madison, WI). A 25- μ L reaction was used containing 50 ng of template DNA, 1 \times concentration reaction buffer, 0.1 mM dNTPs, 1.25 mM MgCl₂, 4 pmol of each primer and 1.25 units of *Taq* DNA Polymerase. MJ Research (Waltham, MA) PTC-200 thermal cycler conditions were as follows: initial denaturing step at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. Primer sequences for gwm538 were obtained from Röder et al. (1998): gwm538F 5'-GCATTCGGGTGAACCC-3' and gwm538R 5'-GTTGCATGTATACGTTAAGCGG-3'. gwm538snpF1 was designed by extending the original forward primer by five nucleotides on the 3' end (5'-GCATTCGGGTGAACCATCAT-3'). Ten microliters of each reaction was run on 2.3% (w/v) agarose gels in 0.5 \times TBE for 3 h at 5 V per centimeter of gel length. Gels were stained with a solution of ethidium bromide in water (0.5 μ g/mL) for 30 min on a rotary platform shaker and destained for 5 min with water before visualization with a UV light box.

TA Cloning and Plasmid Preparations

CS derived NT lines N4BT4D and N4DT4B were used to amplify and clone PCR products of gwm538. Excised bands were purified with the QIAGEN MinElute Gel Extraction Kit, reagents and protocol (QIAGEN, Valencia, CA). PCR products were cloned with the TOPO TA Cloning kit, reagents and protocol (Invitrogen, Carlsbad, CA). Amplified products were ligated directly into plasmid vector pCR 2.1-TOPO and transformed into TOP10 One Shot chemically competent *E. coli* cells (Invitrogen). Plasmids were prepared for sequencing with the QIAprep Spin Miniprep Kit, reagents, and protocol (QIAGEN, Valencia, CA).

Sequencing and Sequence Analysis

Purified plasmids were sequenced directly with primers M13 reverse and T7 by the Kansas State University DNA Sequencing Facility (Manhattan, KS). Three independent clones were sequenced from both directions for each PCR fragment. Vector sequence was trimmed manually and alignment of sequences was performed with AssemblyLIGN version 1.0.9c (Oxford Molecular Ltd., Madison, WI).

RESULTS

Two polymorphic alleles of 152 and 171 bp in the resistant (HD29) and susceptible (WL711) parent, respectively, were amplified by gwm538. Two additional monomorphic fragments (137 and 147 bp) also were amplified, and the 147-bp fragment masked the 152-bp fragment in HD29 (Fig. 1). In Lane 2, HD29 appears to have only two fragments, whereas WL711 (Lane 3) clearly has three fragments. What appears as a single upper band in HD29 is actually both the 152- and 147-bp fragments, that can only be resolved on denaturing polyacrylamide sequencing gels (not shown). Furthermore, the monomorphic fragments compete for amplification in the PCR reaction resulting in inconsistent amplification of the target sequence (not shown).

Using the Chinese Spring (CS) derived nullisomic-tetrasomic lines for group 4 chromosomes, the three fragments amplified by gwm538 were physically mapped, cloned, and sequenced. Use of NT lines allowed easy separation of fragments on 2.3% agarose gels and excision of bands without cross contamination. Two fragments (174 and 147 bp) were located on chromosome 4B and the 137-bp fragment mapped to chromosome 4D (Fig. 1). The same three fragments (174, 147, and 137 bp) were amplified in the nullisomic 4A line (N4AT4D; Lane 15) as in its "parent" line CS (Lane 14), indicating that none of these fragments were located on chromosome 4A. Only the 137-bp fragment was amplified in the nullisomic 4B line (N4BT4D; Lane 16), physically mapping the two uppermost fragments to chromosome 4B. For nullisomic 4D, (N4DT4B; Lane 17) the 137-bp fragment was absent placing it on chromosome 4D.

Alignment of sequence data of the 174-, 147-, and 137-bp fragments amplified by gwm538 in the CS NT lines revealed four SNPs in the regions flanking the SSR (Fig. 2, in bold) from which fragment specific primers were made. Only one SNP was unique to the 174-bp fragment of interest and was used for development of the selective marker (Fig. 2, Position 22). By extending the gwm538F primer an additional five bases on the 3'

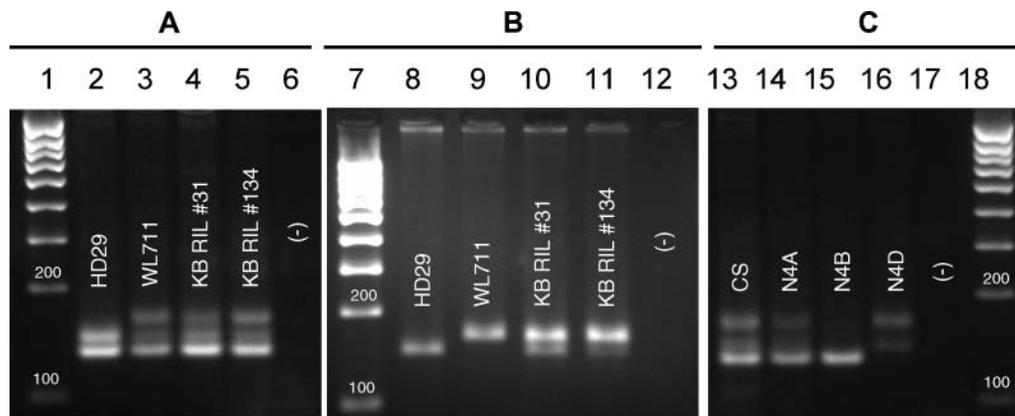


Fig. 1. Panel A; Lanes 1– 6. Fragments generated when the microsatellite gwm538 associated with Karnal bunt resistance was used to evaluate genotypes HD29 (resistant parent), WL711 (susceptible parent) and KB RILs #31 and #134 (heterozygotes). Panel B; Lanes 7– 12. Fragment profiles for HD29(R), WL711(S) and KB RILs #31 and #134 (H) generated with the SNP marker. Panel C; Lanes 13– 18. Physical mapping of microsatellite gwm538 fragments in Chinese Spring (control), N4AT4D (nullisomic for 4A), N4BT4D (nullisomic for 4B) and N4DT4B (nullisomic for 4D). Size standard HyperLadder IV (BioLigne, Randolph, MA), with 100- and 200-bp markers indicated in Lanes 1, 7, and 13. Negative (no template DNA) controls in Lanes 6, 12, and 18.

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