

Development of a molecular marker for rust resistance genes Sr39 and Lr35 in wheat breeding lines

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Polymorphic DNA bands were identified between a near iso-isogenic line of wheat carrying both stem (Sr39) and leaf (Lr35) rust resistance genes and the recurrent line Thatcher (Tc) which lacks these genes. Both resistance genes are located on a translocated chromosomal segment derived from *Aegilops speltoides* and thus are genetically linked. The primers used to generate polymorphic bands were 3'-anchored inter-simple sequence repeat primers which identified genomic microsatellites with a repeated motif of 3 nucleotides in length. The primers were used singly to amplify genomic segments which were flanked by inversely orientated, closely spaced, identical microsatellite sequences. One of the polymorphic bands, a 900 base pair band, was completely linked to the Sr39 and Lr35 rust resistance genes in the segregating population used in this study. After cloning and sequencing this polymorphic band, the inter-simple sequence repeat marker was converted to a sequence characterized amplified region marker by designing primer sets which amplify a single, easily resolved band from DNA of plants with Sr39/Lr35 genes. This marker is present in six wheat lines carrying the Sr39 and Lr35 genes on the translocated chromosome segment from *Ae. speltoides*. The marker has facilitated efforts to breed Canada Prairie Spring and Canada Western Extra Strong lines with these rust resistance genes.

The wheat stem rust pathogen, *Puccinia graminis* Pers. f. sp. tritici Erik. & Henn., can potentially devastate wheat (*Triticum aestivum* L. and *T. turgidum* L.) crops. Resistant cultivars have long been depended upon to control disease epidemics. All spring wheat cultivars recommended for western Canada are resistant to the prevalent races of stem rust and this resistance has been effective since the last epidemic in 1954 (Peterson 1958). However, the potential for new virulent pathotypes to arise makes it important to continue to breed new stem rust resistance genes into commercial wheat cultivars.

The stem rust resistance gene Sr39 was transferred to the hexaploid wheat cultivar Thatcher(Tc) from the wild wheat relative *Aegilops speltoides* L. (Kerber and Dyck 1990). Thatcher carries stem rust resistance genes Sr5, Sr9g, Sr12, and Sr16 (McIntosh et al. 1995). Gene Sr39 is on a translocated chromosome segment, putatively 2S#2, from *A. speltoides* (Friebe et al. 1996). A Tc/Sr39 line demonstrated highly resistant infection type reactions in the seedling stage to approximately 1200 isolates of *P. graminis* f. sp. tritici (D. E. Harder, unpublished). This gene has not yet been used in wheat breeding. Also associated with the translocated Sr39 resistance is the adult plant leaf rust (*P. recondita* Rob. ex Desm. f. sp. tritici Eriks.) resistance gene Lr35. Lr35 is highly effective against North American populations of *P. recondita*

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f. sp. tritici (Kolmer 1997). Both the Sr39 and Lr35 genes are located on the alien translocated segment and co-segregate in crosses with a recombination value of 3.0 between them (Kerber and Dyck 1990). Genetic tests established that both resistance genes are on chromosome 2B of wheat.

Diploid progenitors of durum and bread wheats (eg. *T. monococcum* L., *Aegilops speltoides* Tausch, and *A. squarrosa* L.) have been exploited as sources of new resistance genes, although genetic determination of rust resistance can be complicated by difficulties in obtaining appropriate isolates for testing purposes. Moreover, while pathologists consider multigenic resistance to be more durable (Roelfs 1988), a single gene of interest in a complex background of other resistance genes may be difficult to detect through traditional phenotypic analysis. Hence, specific genetic markers can be a valuable tool to allow gene pyramiding in marker assisted selection (MAS) breeding programs. Moreover, several resistance genes can be tracked simultaneously by testing for the presence of multiple molecular markers (multiplexing), and the markers can be used at an early developmental stage (seed/seedling). In wheat, comparisons between near-isogenic lines (NILs) and their recurrent parents have been used to identify molecular markers linked to pathogen resistance genes (Schachermayr et al. 1994, Autrique et al. 1995, Procnier et al. 1995). This study was undertaken to identify a molecular marker for the Lr35/Sr39 resistance genes because of their potential value as rust resistance sources and the specific marker would facilitate the transfer of these genes to elite wheat lines.

Materials and Methods

Plant material. RL6082 is a *T. aestivum* cv. Thatcher(Tc) near-isogenic line carrying the partially dominant genes Sr39 and Lr35, for resistance to stem rust and leaf rust, respectively. The NIL was derived from an amphiploid of *Ae. speltoides* x *T. monococcum* L. by direct crossing and backcrosses (Friebe et al. 1996; Kerber and Dyck 1990). For this project, RL6082 was crossed to Tc. The resulting F1 population was selfed and an F2 population was generated from which leaf tissue was harvested for genomic DNA extraction. The F2 plants were grown to maturity and selfed; infection types from F3 families homozygous for resistance or susceptibility, or segregating for resistance to stem rust were used to deduce F2 genotypes. For each of the 22 F2 plants, at least twenty seedling F3 plants were inoculated with an isolate of *P. graminis* f. sp. tritici, race TPM (Sr39, Roelfs and Martens 1988) that was avirulent on RL6082, but virulent on Tc. Infection types were scored 14 days after inoculation. Also, the F3 families were inoculated with an epidemic mix of *P. recondita* f. sp. tritici (i.e. a mix of isolates prevalent in western Canada) to verify the presence of the Lr35 resistance gene. Space constraints limited testing to 12 adult plants of each of the 22 F3 families.

Six wheat lines originating from the *Ae. speltoides* x *T. monococcum* amphiploid and carrying Sr39 and Lr35 were tested for the presence of the marker. Leaf tissue was tested from the lines: RL5711 and RL5910 were obtained from Dr. E. R. Kerber (Cereal Research Centre, Winnipeg, MB); p8810 from Dr. R. Knox (Semi-arid Prairie Agriculture Research Centre, Swift Current, SK); and segregants of RL6082 x BW148, RL6082 x BW173 and RL6082 x MqK from Dr. J. A. Kolmer (Cereal Research Centre, Winnipeg, MB).

DNA isolation, PCR conditions, and gel electrophoresis.

Genomic DNAs from freeze-dried wheat leaf tissue from Thatcher, RL6082, and each of the F2 segregating RL6082 x Tc plants were extracted according to the CTAB method of Silva and Procnier (1994), except that tissue was pulverized by vigorous shaking with glass beads and sand. Individual homozygous resistant and susceptible F2 plant DNA samples were initially screened for polymorphic bands. The F2 segregating population was used to determine the polymorphic band's inheritance pattern and linkage to the resistance Sr39/Lr35 genes. The inter-simple sequence repeat (ISSR) primers (Set #9) used for screening were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, B.C., Canada. These are complementary to genomic microsatellite loci with variable two base pair anchors at their 3' end. A single band represents two, closely spaced, random, amplified microsatellites with an intervening sequence. The 'hot start' polymerase chain reaction (PCR) technique was used as previously described (Silva and Procnier 1994) with an annealing temperature of 48°C and 120 ng of DNA. Primer reaction products were resolved by gel electrophoresis carried out in 8% polyacrylamide (PAGE) gels for 1200 volt hours, followed by silver staining (Silver Sequence Kit, Promega), or in 1.4% agarose gels stained by ethidium bromide.

Diagnostic fragment cloning and sequencing.

The UBC degenerate ISSR primer 836 (5'-AGA GAG AGA GAG AGA GYA; Y = C,T) yielded a 900 base pair (bp) band that was tightly linked to the Sr39 gene (Table 1), present in resistant plants and absent in the Tc parent and other susceptible F2 plants. The polymorphic band was excised from an agarose gel and soaked in 100 µl of double distilled water overnight. The DNA solution was filtered through an Ultrafree-MC filter unit (Millipore Corp., Bedford, MA). The DNA was then amplified with UBC 836 primer as previously described, except for the addition of a final one hour extension step (Li and Guy 1996). The final product was TA ligated into the pCRII vector (Invitrogen, San Diego, CA). Clones having a 900 bp insert were selected for dideoxy sequencing (PBI, Saskatoon, SK, Canada).

SCAR primer design and analysis. From the polymorphic band DNA sequence, two forward (F) and two reverse (R) 17 to 20-mer oligonucleotide primers were designed, such that the original 3'-end of the ISSR primer was now posi-

tioned at the centre of the sequence characterized amplified region (SCAR) primer (Guo et al., 1997). This included 10 to 12 bases from the 3'-end of the original ISSR primer, plus the next 6 to 10 bases of the genomic sequence (Table 2). Primer synthesis was carried out by BRL Life Technologies, Burlington, ON, Canada. Amplification of genomic DNA was done using all combinations of forward and reverse primers at various annealing temperatures in order to retain the original polymorphism between the resistant and susceptible lines. The 50 μ L reaction volume contained 1X PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, plus 1.5 mM MgCl₂), 250 μ M of each dNTP, 125 ng of each primer, 40-60 ng genomic DNA and 2.5 U Taq DNA polymerase. Following an initial denaturation of 3 min at 94 °C, the PCR was carried out for 35 cycles of 1 min at 94°C, annealing of 1 min at 60 °C and extension of 2 min at 72 °C. A final extension of 10 min at 72 °C was done. Electrophoresis in 1.4% agarose gels and ethidium bromide staining was used to resolve the amplification product.

Results and Discussion

One hundred ISSR primers were screened to identify polymorphic bands between the homozygous stem rust resistant

and susceptible F₂ plants. Those primers which generated polymorphic bands were then used to amplify DNA extracted from all plants of the F₂ segregating population in order to establish linkage of a polymorphic band with the Sr39/Lr35 resistance genes. One primer (UBC 836) amplified a 900 bp polymorphic band identified on both agarose and silver-stained PAGE gels which was tightly linked to the resistance of stem rust isolate TPM (Sr39) in the segregating population (Table 1). Testing of the F₃ population with *P. recondita* f. sp. *tritici* confirmed that leaf rust resistance (Lr35) was also linked with the presence of the marker fragment in this F₂ population (data not shown). This frequency of finding a marker was comparable to other studies using random amplified polymorphic DNA (RAPD) molecular markers with NIL lines having an alien translocated segment (Schachermayr et al. 1994, Procnier et al. 1995, Dedryver et al. 1996).

The 900 bp polymorphic band was cloned, sequenced, and a number of forward and reverse primers were designed from the band's sequence (Table 2). Twelve plants from the RL6082 x Tc F₂ segregating population were used for testing of pairwise primer combinations at different temperatures. One primer set, Sr39F2/R3, allowed a more robust PCR reaction and eliminated the multiple band pattern found in ISSR primer

Table 1. Segregation for resistance to *Puccinia graminis* f. sp. *tritici* (Sr39) in F₃ families, their F₂ genotypes, and the association of a 900 bp polymorphic band with the segregating families from RL6082/Thatcher cross.

Segregation for resistance		F ₂ genotypes	Presence (+) or absence (O) of 900 bp band in F ₂ DNA samples	
Reaction	No. families		No. F ₂ plants	Band
Resistant	6	RR	6	+
Segregating	12	Rr	12	+
Susceptible	4	rr	4	O
X ²	0.545		0.545	
P (0.05)	<0.10		<0.10	

Table 2. SCAR primers designed using ISSR 836 primer sequence and the specific 900 bp polymorphic band sequence.

primer	sequence*
ISSR(F1)	<u>AGA GAG AGA GAG AGA GTA</u>
Sr39 F2	<u>AGA GAG AGT</u> AGA AGA GCT GC
Sr39 F3	<u>GAG AGA GAG TAG AAG AGC</u>
ISSR(R1)	<u>AGA GAG AGA GAG AGA GCA</u>
Sr39 R2	<u>AGA GAG AGC ATC CAC GA</u>
Sr39 R3	<u>AGA GAG AGA GCA TCC ACC</u>

* The underlined portion of each sequence (5' - 3') represents the original ISSR primer(#836) sequence.

reactions. This SCAR primer pair amplified a single 900 bp band from DNA of the resistant plants, but no amplification occurred with DNA from susceptible plants (Figure 1). Additionally, this SCAR marker was present in the six other wheat lines carry Sr39/ Lr35 resistance genes on the translocation from *Ae. speltoides* (Figure 2).

The SCAR marker was expressed as a dominant phenotype, i.e. occurring in plants homozygous or heterozygous for the resistance gene. The marker was specific to DNA of the *Ae. speltoides* chromosome segment, in which both of the rust resistance genes are located. The apparent tight genetic linkage between the marker and both resistance genes is probably due to the suppression of recombination between

the wheat and alien DNA. Kerber and Dyck (1990) reported greatly reduced pairing between wheat chromosome 2B and the corresponding *Ae. speltoides* segment, and Jia et al. (1996) demonstrated no recombination between wheat chromosome 6B and a large 6S *Ae. speltoides* chromosomal segment.

The SCAR marker will allow detection of Sr39 stem rust resistance, as well as Lr35 leaf rust resistance and potentially other traits which are linked to the translocation. Quality tests have shown that the translocation segment does not contribute any deleterious effects for milling and baking characteristics (Kerber and Dyck 1990). The Sr39 SCAR primer

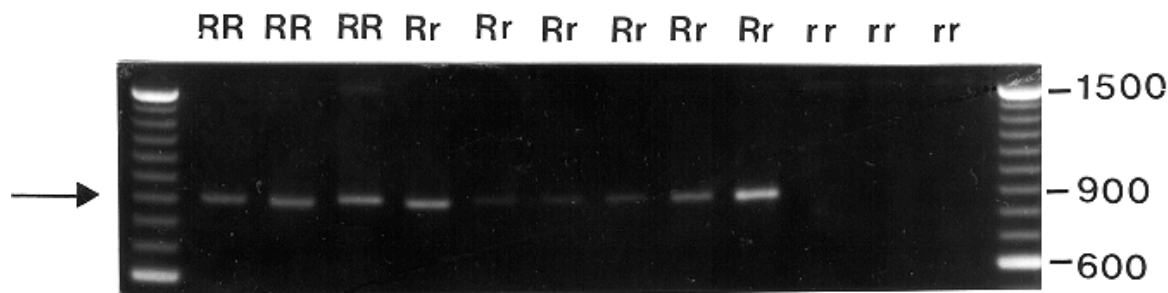


Figure 1. SCAR marker screening of DNA from selected F2 segregants of RL6082/Thatcher cross. RR, homozygous resistant, Rr, heterozygous resistant, rr, susceptible. Far left and right lanes show a 100 base pair marker (BRL Life Technologies). Amplified DNA is resolved by agarose gel (1.4%) electrophoresis and ethidium bromide staining. The fragment of 900 base pairs (bp), indicated by the arrow, was found only in individuals homozygous (RR) or heterozygous (Rr) for the Sr39 resistance gene.

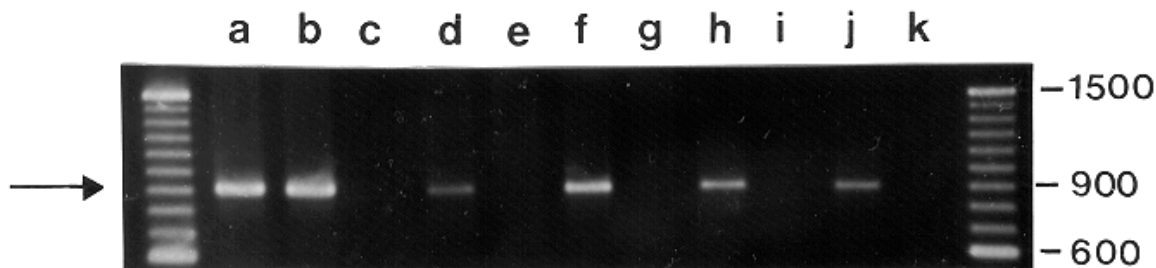


Figure 2. Ethidium bromide-stained 1.4% agarose gel shows SCAR marker presence in several diverse wheat lines bred for Sr39/Lr35 resistance. Far left and right lanes show a 100 base pair marker. Lane a, RL5711 (resistant(R) with Sr39 and Lr35); b, RL5910 (R); c, Marquis K (MqK, susceptible, (S)); d and e, segregants of RL6082 x BW148 (R and S respectively; leaf rust tested); f and g, segregants of RL6082 x BW173 (R and S respectively; leaf rust tested); h, p8810 (R); i, BL31 (S); j and k, segregants of RL6082 x MqK (R and S respectively; stem and leaf rust tested).

has been used successfully in multiplex experiments (i.e. simultaneously using SCAR primers to different rust resistance genes). The marker has already provided a valuable tool for the detection of the Lr35 rust resistance gene. Using SCAR marker assisted selection, the Lr35/Sr39 genes have been successfully backcrossed into Canada Prairie Spring (CPS, Dr. Ron Knox, personal communication) and Canada Western Extra Strong (CWES, Dr. Gavin Humphreys) classes of wheat.

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