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PCR Markers for *Triticum speltoides* Leaf Rust Resistance Gene *Lr51* and Their Use to Develop Isogenic Hard Red Spring Wheat Lines

M. Helguera, L. Vanzetti, M. Soria, I. A. Khan, J. Kolmer, and J. Dubcovsky

**ABSTRACT**

New leaf rust resistance genes are needed in wheat (*Triticum aestivum* L.) to provide additional sources of resistance to the highly variable and dynamic leaf rust pathogen *Puccinia triticina* Eriks. Leaf rust resistance gene *Lr51*, located within a segment of *Triticum speltoides* Taush chromosome 1S translocated to the long arm of chromosome 1B of bread wheat, is resistant to the current predominant races of leaf rust in the USA. The objectives of this study were to determine the genetic length of the translocated 1S segment, develop a PCR marker for *Lr51*, and use this marker to generate isogenic lines for this gene. Characterization of two translocation lines (F-7-3 and F-7-12) with 10 molecular markers indicated that F-7-3 has an interstitial *T. speltoides* chromosome segment of 14 to 32 cM long including loci XAg7 and Xmwg710, whereas line F-7-12 has a complex series of translocations among chromosomes of homeologous group 1. On the basis of the DNA sequence of the A, B, D, and S alleles of the XAg7 locus, we designed a cleavage amplified polymorphic sequence (CAPS) marker for the S genome allele. Primers S30-13L and AGA7-759R preferentially amplified the XAg7 1S (819 bp) and 1B alleles (783 bp). These amplification products can be separated in agarose gels after digestion with *PstI* or *BamHI* restriction enzymes. This CAPS marker was validated in a collection of 32 common wheat cultivars and was used to develop three pairs of hard red spring isogenic lines from the donor parent F-7-3. These isogenic lines will be valuable for future assessment of the effect of this chromosome introgression on agronomic performance and end-use quality.

**Leaf Rust** (caused by *Puccinia triticina* Eriks.) is one of the most common and widespread diseases of wheat worldwide; therefore, incorporating genetic resistance to this pathogen into adapted germplasm is a major goal of most wheat breeding programs. In the USA, yield losses due to leaf rust occur annually throughout the soft red winter wheat region of the eastern states, the hard red winter wheat region of the southern and mid Great Plains states, and the northern spring wheat area of the upper Midwest. Yield losses of 10% or more occur when heavy rust infections defoliate flag leaves during grain filling (Chester, 1946).

Utilizing disease resistance genes minimizes the need for the application of costly fungicides, thus reducing environmental contamination risks and decreasing production costs. Approximately 50 leaf rust resistance genes from wheat and wheat relatives have been cataloged (McIntosh et al., 2003), and molecular markers are available for many of them (Procunier et al., 1995; Gold et al., 1999; Helguera et al., 2000; Huang and Gill, 2001; Helguera et al. (2003), http://maswheat.ucdavis.edu; verified 18 November 2004). Unfortunately, many of the race specific genes are no longer effective against new virulent races of this pathogen. Wild relatives of wheat may be a useful source of additional resistance genes to counter balance the continuous evolution of leaf rust populations.

*Triticum speltoides* Taush (2n = 14, S genome) is an attractive source of high levels of resistance to leaf, stem, and stripe rust of wheat (Dvorak, 1977), and leaf rust resistance genes *Lr28*, *Lr35*, *Lr36*, and *Lr47* were derived from this species (McIntosh et al., 2003). Crosses between this species and hexaploid wheat (*T. aestivum* L. 2n = 42, ABD genomes), followed by selection of resistant progeny, frequently resulted in translocations between the *T. speltoides* and common wheat chromosomes since many *speltoides* genotypes have the ability to promote homeologous chromosome pairing when hybridized with wheat (Dvorak, 1977).

The wheat breeding line ‘Neepawa’/6/Triticum speltoides F-7 was selected from the cross T. aestivum cv. Neepawa × *T. speltoides* accession F (Dvorak, 1977). A 3-to-1 segregation ratio of resistant to susceptible plants in the F_{1} population indicated that a single gene determined this source of resistance. Monosomic and dielosomic analysis provided evidence that the resistance gene was transferred to chromosome arm 1BL (41% recombination with centromere) (Dvorak and Knott, 1980). This gene, temporarily named *LrF7*, has been designated *Lr51* (B. McIntosh, personal communication).

In tests for resistance to *P. triticina* race 5, plants homozygous for *Lr51* were highly resistant with hypersensitive flecks, whereas heterozygous plants showed slightly lower levels of resistance with small pustules surrounded by necrosis and chlorosis, indicating incomplete dominance (Dvorak, 1977). The Neepawa*6/Triticum speltoides* F-7 line exhibited very low infection types with the seven leaf rust races tested (Dvorak and Knott, 1980).

In spite of its high levels of resistance to predominant leaf rust races, *Lr51* has not been widely deployed in breeding programs, probably because of negative genetic effects associated with the presence of large *T. speltoides* chromosome segments and/or additional homeologous translocations in other wheat chromosomes. The objectives of this study were to (i) determine the length of the *T. speltoides* translocations in distinct *Lr51*.
lines, (ii) explore the presence of additional structural changes induced by *T. speltoides*, (iii) develop a set of PCR markers for efficient selection of *Lr51* among segregating progeny, and (iv) develop hard red spring (HRS) *Lr51* isogenic lines for use in evaluating the effect of this alien chromosome introgression on agronomic performance and bread-making quality.

**MATERIALS AND METHODS**

Seeds from the two lines derived from Neepawa*^b*/6/*Triticum speltoides* F-7 line (Dvorak, 1977; Dvorak and Knott, 1980), F-7-3, and F-7-12 were kindly provided by Dr. J. Dvorak (University of California, Davis). He also provided seeds of the original recurrent parent Neepawa, Chinese Spring nullisomic-tetrasomic lines 1N1T1B, 1N1B1D, and 1N1D1A (Sears, 1954), *T. speltoides* accessions (#3362, #3343), *T. tauschii* (Cosson) Schmalh. (D genome, accession DV148), and *T. urartu* Tum. (A genome, accession G3221).

Recurrent HRS cultivars Express (WestBred), Kern (University of California), and breeding line UC1037 (University of California) were crossed with the Neepawa*^b*/6/*Triticum speltoides* F-7-3 line. The F1s were backcrossed six times with the respective recurrent parents, and in each generation, two individuals heterozygous for *Lr51* were selected by marker-assisted selection (MAS). Finally, BC2 plants heterozygous for *Lr51* were self-pollinated and homozygous plants were selected among BC3F1 plants using the molecular marker described below. After six backcrosses, selected plants are expected to be more than 99% identical to the recurrent parent.

Finally, a diverse set of 32 wheat cultivars and breeding lines (mainly from USA and Argentina) was analyzed to validate the *Lr51* CAPS marker (Table 1).

**Tests for Resistance to *P. triticina***

Lines homozygous for *Lr51* and sister lines homozygous for the complementary IBL segment without the resistance gene were selected from BC3F1 plants for each of the three recurrent backcross populations (Kern, Express, and UC-1037). The homozygous IBL plants from the BC3F1 populations were preferred as a negative control over the recurrent parent to rule out possible residual heterozygosity in genes affecting the resistance reaction.

Four to six BC3F1 plants from one or two independent homozygous BC3F1 plants per genotype were evaluated for seedling resistance to leaf rust at the Cereal Disease Laboratory (St. Paul, MN), as previously described (Kolmer et al., 2003). Plants were grown in a greenhouse set at 18 to 21°C with 8 h of metal halide supplemental lighting at 400 to 450 μmol m⁻² s⁻¹ at bench level. Seedlings were inoculated with leaf rust races THBJ (avirulence/virulence formula 9, 24, 3ka, 11, 17, 30, 18/1, 2a, 2c, 3, 16, 26, 10) and MCDS (avirulence/virulence formula 1, 3, 26, 10/2a, 2c, 9, 16, 24, 3ka, 11, 30, 18), which are common races in the USA (Kolmer et al., 2003).

A preliminary screening of the recurrent parents and the *Lr51* donor line indicated that race THBJ was virulent on Express but avirulent on *Lr51* donor line. Race MCDS was virulent on Kern and UC1037 and was avirulent on the *Lr51* donor line. Seedlings were inoculated at 7 to 8 d after planting, when the primary leaves were fully extended. For each race, an oil-spore mixture was atomized onto the seedling plants. The plants were allowed to dry, and were then placed in a dew chamber for 18 h, with no light. After incubation, the plants were placed in a greenhouse set at 18 to 21°C with supplemental metal halide lighting. Infection types were scored 12 d after inoculation by the scale in Long and Kolmer (1989).

**DNA Extraction and RFLP Procedures**

A large-scale DNA isolation procedure (Dvorak et al., 1988) was used for the restriction fragment length polymorphism analysis (RFLP) and to optimize the amplification conditions for PCR markers. A fast, small-scale DNA isolation procedure more appropriate for MAS (Wining and Langridge, 1991) was used to test BC and BC3F1 populations. Procedures for Southern blots and hybridization were as described previously (Dubcovsky et al., 1994). A total of 10 low copy probes previously mapped on chromosome 1A* from *T. monococcum* (Dubcovsky et al., 1996) were used to characterize the *T. speltoides* segment carrying the leaf rust resistance gene *Lr51* (Fig. 1).

**Cloning and Sequence Analysis**

Genomic DNAs from Neepawa, Neepawa*^b*/6/*Triticum speltoides* F-7-3 and F-7-12 lines, Chinese Spring nullisomic-tetrasomic lines of group 1 chromosomes, *T. speltoides* accessions S3343, and S3362, *T. tauschii* accession DV148 and *T. urartu* accession G3221 were used as template for PCR-amplification using primers AGA7-342F and AGA7-759-R (Table 2). Purified PCR products were cloned into pCR4-TOPO vector (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s protocols. Recombinant clones were purified and sequenced using an ABI377 automatic sequencer. Program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; verified

<table>
<thead>
<tr>
<th>Table 1. Origin and accession numbers of hexaploid cultivars and breeding lines used to validate the CAPS marker for <em>Lr51</em>.</th>
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<tbody>
<tr>
<td><strong>Name</strong></td>
</tr>
<tr>
<td><strong>Buck Mantanial’</strong></td>
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<tr>
<td><strong>Coop. Millan’</strong></td>
</tr>
<tr>
<td><strong>Klein Pegaso’</strong></td>
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<tr>
<td><strong>Klein Rendidor’</strong></td>
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<tr>
<td><strong>Prointa Gaucho’</strong></td>
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<tr>
<td><strong>Prointa Granar’</strong></td>
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<tr>
<td><strong>Prointa Millenium’</strong></td>
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<td><strong>Prointa Oasis’</strong></td>
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<tr>
<td><strong>Avocet’</strong></td>
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<td><strong>Sunfield’</strong></td>
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<tr>
<td><strong>Columbus’</strong></td>
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<tr>
<td><strong>Manitou’</strong></td>
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<tr>
<td><strong>Neepawa’</strong></td>
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<tr>
<td><strong>Chinese Spring’</strong></td>
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<tr>
<td><strong>Fugini Komugi’</strong></td>
</tr>
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<td><strong>Pavon 76’</strong></td>
</tr>
</tbody>
</table>

\(^\d\) Accession numbers were obtained from http://www.ars-grin.gov/npgs/acc/acc_queries.html; verified 18 November 2004.

\(^\d\) NA = not available.
RESULTS AND DISCUSSION

Infection Types to Puccinia triticina

The presence of Lr51 in F-7-3 improved resistance to leaf rust isolates BBDL, MCDS, MDRL, MBRL, SBDB, TFGQ, and THBJ relative to the recurrent parent Neepawa (data not shown). Seedlings of the recurrent parent Express had high infection types with race THBJ, whereas Kern and UC1037 had high infection types with race MCDS (Table 3). All the plants homozygous for the T. speltoides chromosome segment carrying the Lr51 gene derived from backcrosses with Express, UC1037, and Kern, were homogeneous for very low infection types (hypersensitive flecks), similar to the Neepawa®6/Triticum speltoides F-7-3 line, the 1S/6/7 translocation donor. As expected, all sister line plants lacking the T. speltoides segment were homozygous susceptible, with high infection types similar to those observed in the original parental lines (Table 3). These results demonstrate that the Lr51 gene was successfully transferred into these three recurrent parents by MAS and that Lr51 was effective in the three genetic backgrounds tested in this study.

Molecular Characterization of the T. speltoides Chromosome Segment

Molecular characterization of translocation lines Neepawa F-7-3 and F-7-12 with 10 molecular markers revealed that different chromosome rearrangements exist in these lines (Fig. 1). Neepawa F-7-3 had RFLP not present in the recurrent parent Neepawa for loci XAga7 and Xmwg710 (Fig. 1). The corresponding 1B fragments for these two loci were missing, confirming that the T. speltoides segment was translocated to wheat chromosome 1B (Dvorak and Knott, 1980). The other eight molecular markers from the long arm of homeologous group 1 did not detect polymorphism between Neepawa speltoides and Neepawa F-7-3. The T. speltoides translocation does not include the high-molecular weight glutenin Glu-B1 locus, which has a significant impact on bread-making quality.

Since the original T. speltoides F7 accession was not available, it was not possible to determine if the absence of polymorphisms in the markers flanking XAga7 and Xmwg710 was originated in the absence of the T. speltoides segment or in the lack of polymorphism between Neepawa and Neepawa F-7-3. The T. speltoides translocation does not include the high-molecular weight glutenin Glu-B1 locus, which has a significant impact on bread-making quality.

For the CAPS markers, 10 μL of the PCR amplification products were digested with restriction enzymes Psrl or BamHI (Promega) by adding 5 units of enzyme to the PCR product. Samples were separated by electrophoresis in 2% agarose gel and visualized by means of ethidium bromide and UV light.

Table 2. Primer used for (1) initial cloning of the XAga7 A, B, D and S genome alleles and (2) B and S allele specific CAPS primers.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>PCR conditions</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>AGA7-342F</td>
<td>GCT TCA ACA GTG GCA TCA ACA AG</td>
</tr>
<tr>
<td>AGA7-759R</td>
<td>TGG CTG CTC AGA AAA CTG GAC C</td>
<td>40 cycles; 3 mM MgCl₂</td>
</tr>
<tr>
<td>2</td>
<td>Sb-1L</td>
<td>GCA TCA ACA AGA TAT TCG TTA TGA CC</td>
</tr>
<tr>
<td>AGA7-759R</td>
<td>TGG CTG CTC AGA AAA CTG GAC C</td>
<td>40 cycles; 1.5 mM MgCl₂</td>
</tr>
</tbody>
</table>

† Each cycle includes a 45 s denaturation step at 94°C, a 45 s annealing step at 60°C or 52°C, and a 60 s extension step at 72°C. Both cycles include a final extension step at 72°C for 10 min.
only one restriction enzyme (J. Dvorak, personal communication). Since none of the four restriction enzymes used in this study (EcoRI, BamHI, HindIII, and XbaI) detected polymorphisms for the markers flanking Xmwg710, we assumed this indicated that the T. speltoides segment did not extend to those flanking markers. On the basis of this assumption, the minimum and maximum estimates for the genetic length of the interstitial 1S chromosome segment were 14 cM (XAg7 and Xmwg710) and 32 cM (Xmwg984 and XksuE11), respectively (Fig. 1). These genetic distances were based on a T. monococcum map (Dubcovsky et al., 1996) that included all of the markers used in this study.

Translocations in F-7-12 were more complex than in F-7-3. Although T. speltoides bands for the XAg7 and Xmwg710 loci were found in both lines, the missing bands from the B genome extended to loci Xmwg984 and XksuG34 in F-7-12 (Fig. 1). In addition, D genome fragments were missing for all of the markers. B genome fragments showed double hybridization intensity for markers distal to the XAg7 locus, and A genome fragments showed double hybridization intensity for markers proximal to Xmwg710. The double intensity restriction fragments suggest the presence of duplicated chromosome segments. A translocation of the T. speltoides segment and its distal 1B chromosome segment to chromosome 1A, followed by a second translocation distal to XGlu-1 to chromosome 1D would explain the absence of D genome loci at both sides of T. speltoides segment and the duplicated 1A chromosome segment in the proximal region and for chromosome 1B in the distal region (Fig. 1).

The presence of complex translocations among the three homeologous group 1 chromosomes in F-7-12 could explain the limited use of this line in breeding programs. The chromosome duplications would generate multivalents during meiosis, reducing fertility and generating off type plants. In addition the large deletion in chromosome 1D may have a negative effect on agronomic or end-use quality characteristics. On the basis of these results, we decided to introgress Lr51 from the Neepawa F-7-3 line, which does not have the complex homeologous translocations found in F-7-12.

### Selection of the RFLP Probe to Be Converted into a PCR Marker

Of the two RFLP loci detected within the T. speltoides chromosome segment, XAg7 has a more simple hybridization pattern compared with Xmwg710 and, therefore, was selected for conversion into a PCR marker. The XAg7 locus was detected by probe AGA7, a 1798-bp cDNA coding for the large subunit of the wheat endosperm ADP-glucose pyrophosphorylase gene (AGP2, GenBank # X14350.1, Olive et al., 1989). AGP2 homeologous genes are located on the long arms of chromosomes 1A, 1B and 1D, approximately 80 cM away from the centromere (Ainsworth et al., 1995).

The interstitial T. speltoides chromosome segment is not expected to recombine with the 1BL chromosome segment in the presence of the Ph gene and the absence of the rest of the T. speltoides chromosomes (Dvorak, 1977). Therefore, a single marker located within this segment should be sufficient to transfer the Lr51 gene into breeding lines. We tested the homozygous BC3F2 lines from the three recurrent parents with markers for both XAg7 and Xmwg710 loci and found no recombination among these markers. This locus during the six generations of backcrossing and one generation of self-pollination in any of the five BC3F2 homozygous Lr51 lines tested (Table 3). This is equivalent to detecting complete linkage in a backcross or double haploid segregating population of 40 plants.

### Sequence Analysis of the XAg7 locus

Two PCR fragments of approximately 790 and 830 bp were amplified with primers AGA7-342F and AGA7-759R (Table 2) from Neepawa, but only the 830-bp
Fig. 2. Best-fit alignment of partial nucleotide sequences from wheat clones from the A (pNF7-17), D (pNB-25), S (pNF7-16), and B (pNee-2) genomes. Gaps were introduced to maximize nucleotide alignment and are indicated with dashes. Locations of PCR primers AGA7-342F and S30-13L are indicated with arrows and sequences from the primers are italicized. BamHI and PstI restriction sites used to develop the CAPS marker are underlined with letters in gray color. Exons are numbered from the first transcribed exon and indicated above the sequences.

The 790-bp amplification product was cloned from Neepawa and Chinese Spring (CS) (B genome) and the 830-bp band was cloned from Neepawa-F-7-3 (expected mixture of A, D, and S products) and from the three diploid species (separate A, D, and S products) and the three diploid species. The 790-bp B-genome clones (795 bp based on sequence) were almost identical, and only the sequence from the Neepawa B-genome clone (pNee-2, GenBank AY589012) is presented in Fig. 2.

The sequences from CS 830-bp clone pNB-25 (GenBank AY589010) and pT-41 from T. tauschii were identical and represented the XAgag71D allele (Fig. 2). Two 830-bp clones from Neepawa-F-7-3, pNF16 (GenBank AY589011) and pNF17 (GenBank AY589009) were assigned to the other two genomes. The sequence from clone pNF16 showed higher identity with the sequence from T. urartu clone pU35 (97%) than with those from T. speltoides clones pS29 and pS30 (95%) indicating that it was from the A genome. The sequence from clone pNF17 showed the highest identity values with the T. speltoides clones (97.5%) indicating that it was a mixture of products from the XAgag7-A and XAgag7-D loci. The 830-bp fragment also was detected in PCR amplifications from DNAs of diploid T. urartu, T. tauschii, and T. speltoides accessions S3343 and S3362.

The Development of a Cleavage Amplified Polymorphic Sequence Marker

Primers S30-13L and AGA7-759R (Table 2) amplified preferentially alleles from the S (pNF7-17) and B (pNee-2) genomes, respectively (Fig. 2). The AGA7-759R primer is not genome-specific, but the 3' end of the S30-13L primer was selected to match a cytosine (position 38, Fig. 2) that differentiates the S and B genome alleles from the A (pNF7-17) and D (pNB-25) genome alleles. An additional polymorphism, a thymine...
at position 32, differentiates the S allele from the alleles in the other three genomes. The presence of this difference alone did not interfere with the amplification of the B genome allele, probably because of the internal position of the polymorphisms within the primer. PCR amplification of nullitetrasomic DNAs with these primers produced a 783-bp fragment (B-allele) in lines N1AT1B and N1DT1A but no PCR product in N1BT1D, demonstrating that these primers do not amplify the A or D genome alleles under the PCR conditions used in this study (Fig. 3, Lanes 1, 2, and 3).

The amplification products from primers S30-13L/AGA7-759R from the B (783 bp) and S (819 bp) alleles can be separated by polyacrylamide gels (14%, w/v). These two amplification products also can be separated in agarose gels (2%) after digestion with PstI or BamHI (Fig. 3 and 4). The S genome amplification product has a unique PstI restriction site at position 434 (Fig. 2) that divides the 819-bp fragment into two fragments of similar size (397 and 422 bp, Fig. 3). The B genome amplification product has a unique BamHI restriction site at position 111 that cuts the 783-bp fragment into two 672- and 111-bp fragments (Fig. 4). To validate this CAPS marker in a wide range of wheat cultivars, we evaluated the presence of the diagnostic BamHI and PstI restriction sites in a diverse set of 32 cultivars and breeding lines (Table 1). All of the cultivated wheats from Table 3 showed the 783-bp fragment, which was digested by BamHI into the expected 672- and 111-bp fragments (Fig. 5). None of these amplification products was digested by PstI. These data suggested that the Lr51 CAPS marker presented here will be useful in a wide range of cultivars.

**Development of Leaf Rust Resistant Germplasm using Molecular Markers**

Leaf rust resistance has been particularly short-lived in wheat cultivars with single seedling resistance genes. Wheat cultivars with combinations of effective resistance genes should provide resistance for a longer period of time to an increased number of races than single leaf rust resistance genes.

The development of molecular markers for Lr51 will facilitate combining this gene with additional leaf rust resistance genes. We are currently combining Lr51 with Lr47 and Lr37 before releasing it in a commercial cultivar. This is now possible because molecular markers are available for all three genes (Dubcovsky et al., 1998; Helguera et al., 2000, 2003). We have developed isogenic lines for Lr51, Lr47, and Lr37 in Express, Kern, and UC1037 backgrounds and are currently intercrossing the UC1037 lines to develop a single line with all three genes. An alternative strategy that can be used to extend the useful life of Lr51 is combining this gene with the slow rusting genes Lr34 and Lr46, for which molecular markers also are now available (Schnurbusch et al., 2004; Suenaga et al., 2003; William et al., 2003).
If future comparisons of the *Lr51* isogenic lines reveal the presence of negative effects on yield or end-use quality associated with the introgression of the *T. speltoides* segment, the molecular map presented here can be used to select shorter alien chromosome segments through a second round of homeologous recombination.

**ACKNOWLEDGMENTS**

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