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PCR Assays for the *Lr37-Yr17-Sr38* Cluster of Rust Resistance Genes and Their Use to Develop Isogenic Hard Red Spring Wheat Lines

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ABSTRACT

Rust resistance genes *Lr37*, *Sr38*, and *Yr17* are located within a segment of *Triticum ventricosum* (Tausch) Cess. chromosome 2NS translocated to the short arm of bread wheat chromosome 2AS. Characterization of this chromosome segment by 13 restriction fragment length polymorphism (RFLP) markers indicated that the 2NS translocation replaced approximately half of the short arm of chromosome 2A (distal 25–38 centimorgans, cM). The objective of this study was to develop polymerase chain reaction (PCR) assays based on RFLP marker cMWG682 to facilitate the transfer of this cluster of rust resistance genes into commercial wheat (*Triticum aestivum* L.) cultivars. DNA sequence was obtained from the A-, B-, D-, and N-alleles of cMWG682 and was used to design N-allele specific primers. The 2NS fragment amplified by PCR primers cosegregated with the presence of the RFLP-2NS band in all backcross populations. A cleaved amplified polymorphic sequence (CAPS) was used to develop a marker for the 2A-allele. This marker can be used to differentiate homozygous and heterozygous plants carrying the 2NS translocation in the final cycle of backcross introgression or in screenings for homozygous plants in segregating populations. Finally, a third PCR assay was developed by means of TaqMan technology as a high-throughput alternative for selection of the 2NS/2AS translocation in large segregating populations in breeding programs that have access to real time PCR equipment. These molecular markers were used to develop four hard red spring isogenic lines homozygous for the 2NS chromosome segment. One of the isogenic lines, derived from 'Anza,' did not show the expected resistance in spite of the presence of all the RFLP markers for the 2NS chromosome segment. Analysis of F_1 hybrids suggested that a suppressor of the *Lr37* gene is present in Anza. These isogenic lines will provide a valuable tool to test the effects of the large 2NS translocation on quality and agronomic performance.

Diploid species with genomes related to A, B, and D genomes from bread wheat offer a pool of traits that can be used to increase genetic diversity in wheat. The gene pool of cultivated wheat has been heavily exploited and therefore, it has become more difficult to find new resistance genes in hexaploid germplasm. Wild relatives of wheat can be used as sources of disease resistance to supply new genes to counter the continuous evolution of different pathogen populations and to reduce fungicide applications in wheat production. These alien genes can be transferred into cultivated wheat cultivars by the use of the *ph1* gene (Riley and Chapman, 1958) that promotes homeologous chromosome recombination. The *ph1* mutation has been extensively used to incorporate new disease resistance genes in wheat from wild Triticeae species such as *T. monococcum* L., *T. speltoides* (Tausch) Gren., and *T. ventricosum* (McIntosh et al., 1995; Friebe et al., 1996; Dubcovsky et al., 1998).

The *Yr17*, *Lr37*, and *Sr38* rust resistance genes, which confer resistance in wheat against stripe rust (caused by *Puccinia striiformis* West. f. sp. *tritic*i), leaf rust (caused by *Puccinia triticina* Eriks), and stem rust (caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn.), respectively, have been used by breeders in different parts of the world (Dyck and Lukow, 1988; McIntosh et al., 1995; Robert et al., 1999; Seah et al., 2000). These linked resistance genes were initially introgressed in the winter bread wheat 'VPM1' from *Triticum ventricosum* (Maia, 1967) and are located in a 2NS/2AS translocation (Bariana and McIntosh, 1993; McIntosh et al., 1995). Rust races with virulence to *Yr17* and *Lr37* have been identified in different countries (Robert et al., 1999; J. Kolmer unpublished data) but this gene cluster still provides resistance to a wide range of races and is useful in combination with other rust resistance genes.

Molecular markers have been developed to accelerate the transfer of these genes into commercial cultivars. Robert et al. (1999) developed a dominant SCAR (sequence characterized amplified region) marker that was closely, but not completely, linked (0.8 ± 0.7 cM) to *Yr17* and tentatively to *Lr37* and *Sr38*. Seah et al. (2001) developed a PCR marker from the sequence of a nucleotide-binding-site leucine-rich repeat disease resistance gene located in the 2NS chromosome segment. This dominant marker was completely linked to the rust resistance genes *Yr17*, *Lr37*, and *Sr38* in 14 isogenic lines. Unfortunately, primer sequences corresponding to both PCR markers were not provided in the last two publications.

The objective of this study was to develop a set of public PCR assays for an efficient selection of the *Yr17-Lr37-Sr38* cluster of resistance genes in wheat breeding programs. Different types of markers were evaluated including PCR-based markers, CAPS, and TaqMan systems (Livak, 1999). This last system was included in this study to evaluate the TaqMan technology as a high-throughput marker system in polyploid wheat. When marker assisted selection (MAS) is used in a backcrossing strategy, only a few plants are tested with molecular markers in each generation, and the cost of the molecu-

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Abbreviations: BC, backcross; CAPS, cleaved amplified polymorphic sequence; HRS, hard red spring; MAS, marker assisted selection; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism.

lar marker system is not very critical. However, if the objective of a breeding program is to apply a high-throughput MAS in large segregating populations, the cost and automation of the molecular marker become critical. The TaqMan system uses real time fluorogenic detection of PCR products and therefore does not require gels to visualize the PCR products, facilitating automation of the MAS process (Livak, 1999).

A second objective of this research was to develop four hard red spring isogenic lines differing in the presence of the 2NS translocation. These isogenic lines will be useful tools to evaluate the effect of the 2NS/2AS translocation on agronomic performance and bread-making quality. If yield or quality penalties were associated with the 2NS chromosome segment, it would be necessary to reduce this chromosome segment's length by a second round of homeologous recombination. To facilitate this task we developed an RFLP map of the 2NS translocated chromosome segment.

MATERIALS AND METHODS

Plant Materials

Nine lines were used in the molecular characterization of the translocated 2NS segment. These lines included the original *T. ventricosum* accession #10 ($2n = 28$, genomes DN) used to transfer the 2NS chromosome segment to bread wheat (Maia, 1967) and *T. tauschii* (Cosson) Schmalh. (D genome, KU#2073) and *T. uniaristatum* (Vis.) Richter (N genome, PI276996), the diploid donors of the D and N genomes to *T. ventricosum* (Dubcovsky and Dvorak, 1994). They also included the hexaploid line VPM1 ($2n = 42$) that carries the original 2NS translocation and the lines used to develop it (Maia, 1967), *T. persicum* (Percival) Vavilov ex Zhukovsky ($2n = 28$) and 'Marne' ($2n = 42$). Finally, two winter wheats carrying the 2NS translocation (cultivars Madsen and Hyak) and two hard red spring wheats without the translocation [cultivar Yecora Rojo and breeding line UC1041 (Yecora Rojo/'Tadinia')] were included for comparison.

A diverse set of wheat cultivars was analyzed to validate the CAPS marker for the 2AS allele. This set included the cultivars Attila, Avocet, Brooks, Cavalier, Chinese Spring, Columbus, Cuyama, Klasic, Len, Marne, Opata, Pavon, Red Egyptian, RSI5, Sunfield, Tadinia, Yecora Rojo, and Yolo. Chinese Spring nullisomic–tetrasomic lines were used to assign amplified bands to homeologous chromosomes (Sears, 1954).

The 2NS chromosome segment was introgressed into hard red spring (HRS) wheat cultivars Yecora Rojo, Anza, Express (from Western Plant Breeders), and University of California breeding line UC1041 by marker assisted backcrossing (BC). Each cultivar was crossed with Madsen, and the four F_1s were backcrossed with the respective recurrent parents. In each of the six BC generations, two individuals heterozygous for the 2NS translocation were selected by MAS and used in crosses with the corresponding recurrent parent. Finally, BC_6 plants heterozygous for the 2NS translocation were self-pollinated and homozygous 2NS plants were selected from BC_6F_2 plants by means of molecular markers. These selected BC_6F_2 plants are expected to be more than 99% identical to the recurrent parent and homozygous for the *Lr37-Yr17-Sr38* resistance genes.

Tests for Resistance to *Puccinia triticina*

Lines homozygous for the 2NS segment and for the complementary 2AS segment were selected from the BC_6F_2 plants for each of the four recurrent parents. The 2AS homozygous plants selected from the BC_6F_2 were used instead of the original recurrent parent to rule out any possible residual heterozygosity in genes affecting the resistance reaction after six generations of backcrossing.

At least four BC_6F_3 plants from two different lines per genotype were evaluated for resistance to leaf rust. Plants were raised in a growth cabinet at 18°C for 16 h with florescent and incandescent lighting for day conditions and at 15°C for 8 h with no lights for night conditions. The plants were inoculated at flag leaf stage with *P. triticina* virulence phenotype THBJ (Long and Kolmer, 1989). Inoculated plants were incubated in a dew chamber with no lighting for 18 h. After inoculation and incubation, the plants were placed on a greenhouse bench with overhead lighting. Rust infection types were evaluated 14 d after inoculation. Infection type scoring was according to Long and Kolmer (1989). Leaf rust isolate THBJ was selected because of its virulence to the recurrent parents and avirulence to *Lr37* (Kolmer, 1997).

DNA Extraction and RFLP Procedures

Two different plant DNA extraction procedures were used in this study. The first one, a large-scale and high-quality DNA isolation procedure (Dvorak et al., 1988) was used for the RFLP analysis and to optimize the amplification conditions for the PCR markers. The second one, a rapid and small-scale DNA isolation procedure more appropriate for MAS (Wining and Langridge, 1991), was used to test the reproducibility of the PCR markers. Procedures for Southern blots and hybridization were as described before (Dubcovsky et al., 1994). A total of 13 low copy probes previously mapped on chromosome arm $2A^mS$ from *T. monococcum* (Dubcovsky et al., 1996) or 2AS from wheat (Devos et al., 1993) were used to characterize the *T. ventricosum* segment carrying rust resistance genes *Lr37*, *Yr17*, and *Sr38* (Fig. 1A). Probe VRGA1D, provided by Dr. E. Lagudah, is a nucleotide-binding-site leucine-rich repeat disease resistance gene previously found to be linked to the 2NS translocation (Seah et al., 2000).

Selection of the RFLP Probe to be Converted into a PCR Marker

The 2NS chromosome segment is not expected to recombine with the 2AS chromosome segment in the presence of the *Ph1* gene and therefore, any RFLP marker detected within the 2NS translocation could be potentially useful for conversion to a PCR marker. The criteria used to select the *Xcmwg682* locus were the single copy nature of this probe and the presence of well separated RFLP fragments for the A, B, D, and N genomes. Another positive aspect of the *Xcmwg682* locus was its distal location. Although, the *Lr37*, *Yr17*, and *Sr38* resistance genes have not been mapped within the 2NS chromosome segment, other clusters of resistance genes have been observed in distal regions of different wheat chromosomes (McIntosh et al., 1995).

The sequence of the barley (*Hordeum vulgare* L.) clone cMWG682 was similar to other sequences from oat (*Avena sativa* L.) (AA231913), rice (*Oryza sativa* L.) (C24814), and wheat (BI750635). These sequences were significantly similar ($E = 1e^{-18}$) to *Arabidopsis* putative membrane related protein *CP5* (NC_003070).

Cloning and Sequence Analysis

The following strategy was used to convert RFLP marker cMWG682 into a PCR marker. First, 15 μg of genomic DNA from Madsen (homozygous 2NS) and Yecora Rojo were di-

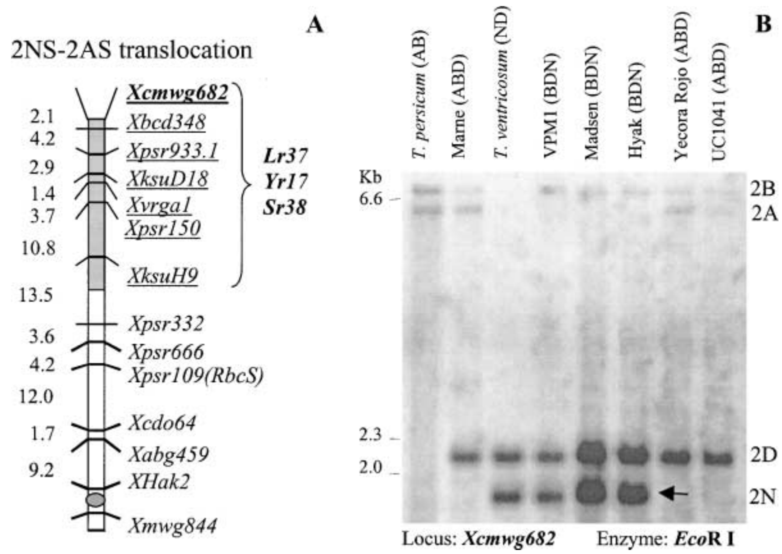

Fig. 1. A. Inferred location of the translocation point between chromosome 2NS from *T. ventricosum* (gray rectangle, underlined loci) and chromosome 2A from *T. aestivum* (white rectangle). The gray circle indicates the position of the centromere. Distances in centimorgans were based on the $2A^mS$ map of *T. monococcum* (Dubcovsky et al., 1996). B. Southern blot hybridization of cMWG682 clone with *Eco*RI digested genomic DNAs. Letters in parentheses indicate the genomic constitution at the *Xcmwg682* locus. λ *Hin*dIII was used as a molecular size standard. An arrow indicates the 2NS-specific fragment.

gested with *Eco*RI and separated by electrophoresis in a 1% (w/v) low melting point (LMP) agarose gel including molecular marker λ-*Hin*dIII. Agarose blocks from the approximate location of the 2A, 2B, 2D, and 2N cMWG682-RFLP fragments were excised from the ethidium bromide strained gel (Fig. 1B). DNAs were purified from the LMP agarose blocks with the Wizard PCR Preps DNA Purification kit (Promega, Madison, WI) and used as template for PCR-amplification using primers URIC and LOAT (Table 1). These two primers were designed on the basis of conserved regions of the oat and rice sequences. Yecora Rojo DNA was used as the source for the *Xcmwg682* alleles from chromosomes 2A, 2B, and 2D, and Madsen as the DNA source of the allele from chromosome 2N. Purified PCR products were cloned into pCR 2.1 vector (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Recombinant clones were purified and sequenced with an ABI377 automatic sequencer. Sequences were aligned by the computing program Clustal W 1.8 (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html; verified 11 April 2003). Fig. 2 was constructed by means of Boxshade (http://www.ch.embnet.org/software/BOX_form.html; verified 11 April 2003). Identity values for best-fit alignments were calculated by BLASTN 2.1.2. (Altschul et al., 1997). Programs OLIGO ver. 4.0 and Primerfinder (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi; verified 11 April 2003) were used for primer design.

PCR Procedures

The PCR reactions were performed with PerkinElmer (Shelton, CT) GeneAmp PCR system 7700. PCR reactions used 120 ng of wheat genomic DNA and final concentrations of 1× *Taq* polymerase buffer (Promega), 1.0 U *Taq* DNA polymerase (Promega), 1.5 m*M* $MgCl_2$, 0.2 μ*M* of each primer, and 200 μ*M* of each dNTP. Final volume for PCR reactions was 25 μL. Primer names, function, sequences and PCR cycling conditions are summarized in Table 1. Following amplification with CAPS primers, 5 μL of the PCR reaction were digested with restriction enzyme *Dpn*II by adding 5 U of enzyme to the PCR product. Samples were separated by electrophoresis in 2% (w/v) agarose gel and visualized by means of ethidium bromide and UV light.

TaqMan Procedures

Reactions were performed with two systems with different pairs of primers but with the same probe W2NS-55T. Primer and probe sequences are listed in Table 1. Probe W2NS-55T included a single point mutation at base pair (bp) 17 that differentiated the 2NS allele from the 2A, 2B, and 2D alleles (Fig. 2). The probe was labeled with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) at the 5′ end and the quencher 6-carboxy-*N,N,N′,N′*-tetramethyl-rhodamine (TAMRA) at the

Table 1. Primer names, sequences and cycling conditions used in identifying markers associated with the 2NS translocation in wheat.

Function	Name	Sequence (5′–3′)	PCR conditions†
Conserved for initial cloning	URIC	GGT CGC CCT GGC TTG CAC CT	35 cycles. Annealing T: 55°
	LOAT	GCT CTC AGC TTC ACA GAC TG	Extension: 72° 7 min
2NS specific	VENTRIUP	AGG GGC TAC TGA CCA AGG CT	30 cycles. Annealing T: 65°
	LN2	TGC AGC TAC AGC AGT ATG TAC ACA AAA	Extension 72° 7 min
2AS CAPS	URIC	GGT CGC CCT GGC TTG CAC CT	38 cycles. Annealing T: 64°
	LN2	TGC AGC TAC AGC AGT ATG TAC ACA AAA	Extension: 72° 7 min
TaqMan System 1	2NS-11F	GCT ACT GAC CAA GGC TCT CGT GT	2′ 50°C, 10′ 95°C, 40 cycles of
	2NS-113R	GTG CAT GGC ATC GCG AT	15″ 95°C and 60″ 60°C
TaqMan System 2	VENTRIUP	AGG GGC TAC TGA CCA AGG CT	2′ 50°C, 10′ 95°C, 40 cycles of
	LN2	TGC AGC TAC AGC AGT ATG TAC ACA AAA	15″ 95°C and 60″ 60°C
TaqMan probe‡	W2NS-55T	TGT TTG GTT CCT ATC TCC TTC CTG GTC CTG	

† Each cycle includes a denaturation step at 94°C for 45 s, an annealing step at the indicated temperature for 30 s, and an extension step at 72°C for 60 s.
‡ The underlined C in the TaqMan probe indicates the SNP.

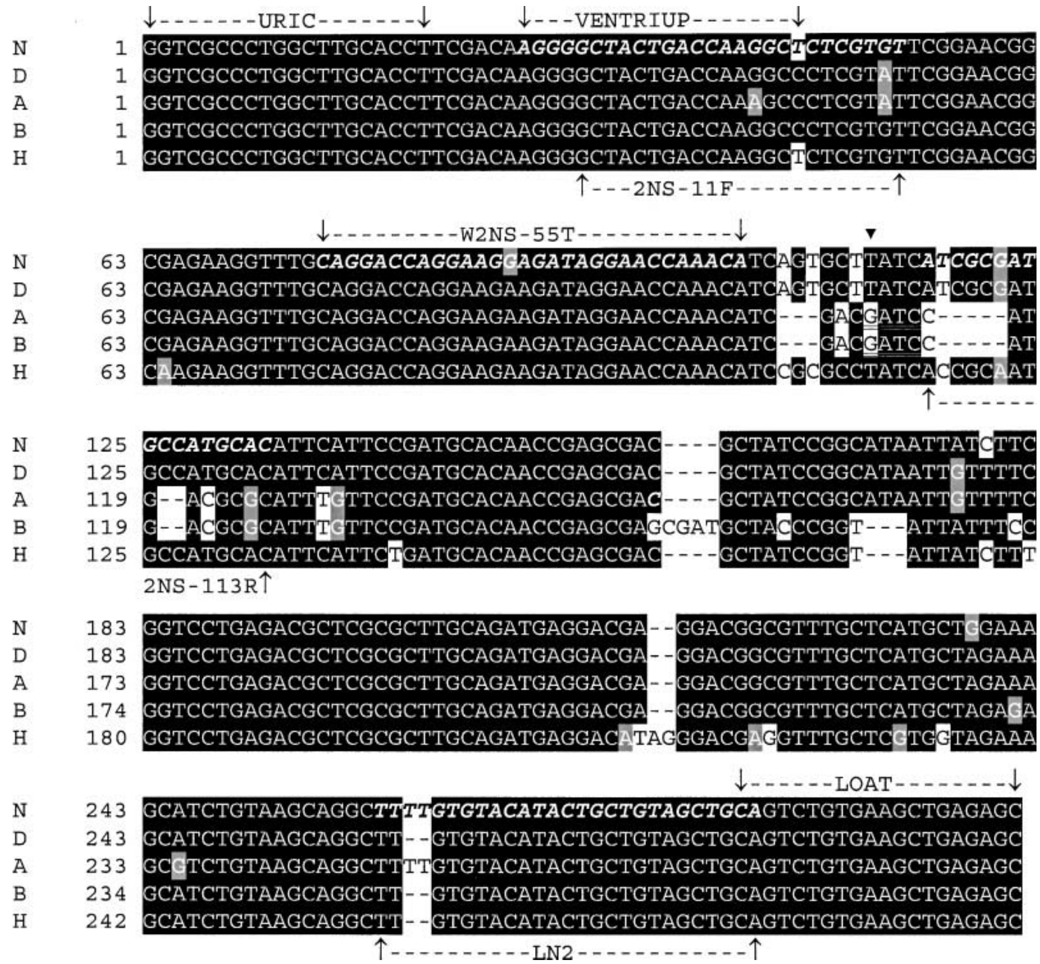

Fig. 2. ClustalW alignment of nucleotide sequences from different genomes. H: sequence from barley cDNA cMWG682; N: sequences from genomic clones obtained from *T. ventricosum*; A, B, and D: sequences from the three genomes of *T. aestivum*. Identities are indicated with black boxes and gaps introduced to optimize alignment are indicated with dashes. Location of PCR primers URIC, LOAT, VENTRIUP, LN2, 2NS-11F, 2NS-113R, and SNP probe W2NS-55T are in bold-italics in the genome that was used to design the primers. The polymorphic *Dpn*II restriction site (A and B genomes) used to develop the CAPS marker is underlined and indicated by an arrowhead.

3′ end. When the target sequence is being amplified, the 5′ nuclease activity of the *Taq* DNA polymerase cleaves the fluorogenic probe included into the PCR reaction. The physical separation of the fluorescent reporter dye from the quencher causes an increase in its fluorescence intensity that is detected in "real time" by the quantitative PCR equipment. System 1 used primers 2NS-11L- 2NS-113R and System 2 used primers VENTRIUP- LN2 (Table 1).

The PCR reactions for the TaqMan assays contained 400 n*M* of each primer, 80 n*M* of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, CA). PCR mastermix contained 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 5 m*M* $MgCl_2$, 2.5 m*M* deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 200 ng of DNA in a final volume of 25 μL. The samples were placed in 96 well plates and amplified in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 60°C.

Twenty-four BC_6F_2 plants previously classified by RFLP as heterozygous or homozygous for the 2AS or 2NS chromosome segments were evaluated with both TaqMan systems to determine the variability of the tests. Linearized values were calculated as $2^{(40\text{-CT})}$, where CT is the threshold cycle. One-way analyses of variance were used to test the significance of the differences among genotypes in linearized values. The SAS program version 8.0 (SAS Institute Inc., 2001) was used for the statistical analysis.

RESULTS AND DISCUSSION

Infection Types to *Puccinia triticina*

Adult plants of the susceptible control 'Thatcher' and recurrent BC parents Yecora Rojo, Anza, Express, and UC1041 had high infection types with the leaf rust phenotype THBJ (Table 2). The Thatcher line with *Lr37* (2NS) had a low infection type, as did the isogenic lines derived from Yecora Rojo, Express, and UC1041 carrying the 2NS translocation (Table 2). These results demonstrated that the *Lr37* gene was successfully transferred to these three recurrent parents.

An unexpected result was observed in the Anza isogenic BC_6F_3 plants homozygous for the 2NS chromosome segment. All the plants had high infection types similar to those observed in the parent Anza (Table 2). Presence of the 2NS chromosome segment in the suscep-

Table 2. Infection types produced in BC_6F_2 lines with different genetic backgrounds when tested against *Puccinia triticina* virulence phenotype THBJ.

	THBJ Infection type†	
Genetic backgrounds‡	**2NS segment absent**	**2NS segment present**
Thatcher	4	;2^-‡
Anza	33^+	2^+3^+
Yecora Rojo	4	;2^+
Express	2^+3^+	;2^+
UC 1041	2^+3^+	;1^+2^+

† 0 = Immune,; = necrotic flecks, 1 = necrotic flecks and small uredinia, 2 = small uredinia with chlorosis, 3 = moderate size pustules without chlorosis of necrosis, 4 = Large uredinia without necrosis or chlorosis. Symbols "–" and "+" denote smaller or larger uredinia.
‡ (Thatcher^8/VPM) Thatcher-Lr37 RL6081.

tible plants was reconfirmed with RFLP markers cMWG682, BCD348, VRGA1D, PSR933, and KSUH9 (Fig. 1A).

One possible explanation for this lack of resistance is the presence of a recombination event between *Lr37* and the available RFLP markers for the 2NS *T. ventricosum* translocation. Although no recombination was observed among the currently available RFLP markers, it is not possible to rule out the possibility of a recombination event in a region outside the one covered by current markers. However, that recombination event should have happened in the F_1 generation because all the BC_1F_2 plants from the progeny of a 2NS/2AS BC_1 plant were also susceptible.

An alternative explanation is the presence of an interaction between *Lr37* and a gene(s) in Anza that suppresses the expression of *Lr37*. Suppressors of leaf and stem rust resistance genes have been previously reported in wheat (Kerber and Green, 1980; Dyck, 1982; Nelson et al., 1997). To test this hypothesis the homozygous-2NS susceptible line derived from Anza was crossed with a susceptible Yecora Rojo parental line, and a normal Anza without the 2NS translocation was crossed with the homozygous-2NS resistant Yecora Rojo. If recombination between the RFLP marker and *Lr37* occurred in the 2NS segment originally transferred to Anza, then the F_1 of Anza-2NS × Yecora Rojo-2AS would be susceptible and the F_1 of Anza-2AS × Yecora Rojo-2NS would be resistant. The 18 F_1 inoculated plants from each of the two crosses showed similar high infection types as the recurrent parent Anza (Table 2), suggesting that a suppressor gene is present in Anza.

Molecular Characterization of the *T. ventricosum* Chromosome Segment

The position and length of the distal *T. ventricosum* chromosome segment translocated to chromosome 2AS was characterized with 13 RFLP probes that were previously mapped in the short arm of chromosome $2A^mS$ (Dubcovsky et al., 1996; Santa-María et al., 1997) or 2AS (Devos et al., 1993) (Fig. 1A). Loci *Xcmwg682, Xbcd348, XksuD18, Xvrga1, Xpsr150,* and *XksuH9* were included in the 2NS chromosome segment on the basis of the observed polymorphisms between 2AS and 2NS RFLPs. One example is presented for the *Xmwg682* locus (Fig. 1B). This probe showed a restriction fragment of similar mobility in *T. ventricosum* accession #10 and in the hexaploid wheats VPM1, Madsen, and Hyak that are known to carry the 2NS translocation. The *Xmwg682* 2NS fragment is absent in Marne, Yecora Rojo, and breeding line UC1041 that do not carry the 2NS translocation (Fig. 1B). RFLP markers *Xpsr150,* and *Xvrga1* were shown before to be part of the 2NS translocation present in VPM1 (Seah et al., 2000, Robert et al., 1999).

The most proximal marker included in the 2NS chromosome segment is *XksuH9* (Fig. 1A). This multicopy probe was previously mapped on chromosomes $2A^m$, $4A^m$, $5A^m$, and $7A^m$ in *T. monococcum* (Dubcovsky et al., 1996). In spite of the complex hybridization pattern of the KSUH9 probe, one *Hin*dIII restriction fragment was observed in Anza, Express, Yecora Rojo and UC1041 but was absent in the respective isogenic homozygous lines carrying the 2NS translocation. This result indicates that the *XksuH9* locus on chromosome two is tightly linked with the other markers previously assigned to the 2NS segment, and that most likely the complete 2NS segment was transmitted without recombination into the four isogenic lines.

The *Xpsr666* locus was not included in the translocated 2NS chromosome segment. PSR666 is a single copy probe that hybridizes with only two *Bgl*II restriction fragments in the original *T. ventricosum* used for the introgression of the 2NS segment. The larger of the two restriction fragments observed in *T. ventricosum* showed identical mobility to a restriction fragment observed in diploid *T. uniaristaum* (N genome) and the smaller restriction fragment showed identical mobility to a restriction fragment observed in *T. tauschii* (D genome). The N genome fragment was not observed in the hexaploid Madsen that carries the 2NS translocation, demonstrating that the *Xpsr666* locus is outside the 2NS translocation.

The results for the presence of *Xpsr332* outside the 2NS translocation were less conclusive because the PSR322 probe hybridized with multiple restriction fragments. It was not possible to assign many of the PSR322 restriction fragments to the D or N genome because of polymorphisms between *T. ventricosum* and the available diploid accessions of *T. uniaristaum* and *T. tauschii*. However, two lines of evidence suggest that *Xpsr332* is outside the 2NS translocation. First, no polymorphic fragment was detected with four restriction enzymes between the isogenic lines with and without the 2NS segment. In addition, no *T. ventricosum* restriction fragment was simultaneously present in the hexaploid cultivars that included the 2NS segment and absent in the other cultivars.

Genetic distances in the A genomes of *T. monococcum* and *T. aestivum* are very similar (Dubcovsky et al., 1995). On the basis of the $2A^mS$ map of *T. monococcum* (Dubcovsky et al., 1996) the genetic length of the 2AS segment replaced in the 2NS/2AS translocation can be estimated to be between 25 cM (*Xcmwg682-XksuH9*) and 38 cM (*Xcmwg682-Xpsr332*). In barley, the *Xcmwg682* locus was mapped 3 cM from the telomeric locus *Tel2S* on chromosome arm 2HS (Kilian et al., 1999), suggesting that an additional 3 cM may be added to the previous estimates of the genetic length of the translocated segment.

In spite of the large genetic length estimated for the translocated segment, no recombination was detected between *Xcmwg682* and *XksuH9* in the four isogenic lines during the six generations of backcrossing. This is an expected result because recombination between the 2A and 2N chromosomes is prevented in the presence of the *Ph1* gene. Therefore, a single molecular marker located within the 2NS segment should be sufficient to monitor the transfer of all the genes present within this chromosome segment.

Development of PCR Markers for the *Xcmwg682* Locus

Sequence Analyses

Recombinant clones corresponding to the A, B, D, and N genome were obtained from PCR amplification products of size-selected *Eco*RI-digested genomic DNAs (see Materials and Methods). Sequences amplified from these genomic DNAs were almost identical in length to the sequence of barley cDNA MWG682 suggesting that no introns were present within the PCR amplified region.

Sequence identity among clones from the different genomes varied from 89 to 97%. As expected, the most divergent sequence was the barley cMWG682 cDNA, that differed from the other four wheat sequences by 13 specific point mutations and one insertion–deletion event (indel) (89–93% identity). Percent identity values among the three wheat genomes (A vs. B 95%, A vs. D 95%, and B vs. D 92%) were similar to those reported for other wheat genes (A vs. B 96%, A vs. D 93%, and B vs. D 93%) (Bryan et al., 1999). The N genome sequence was more similar to the sequence from the D genome (97%) than to the sequences of the A (94%) and B (92%) genomes.

Comparison of the four *Triticum* sequences for genome specific changes showed four specific point mutations and one indel in the B genome sequence, two specific point mutations in the N genome sequence (three if barley sequence is excluded), two specific point mutations in the A genome, and no specific mutations in the D genome (Fig. 2). These specific mutations were exploited to develop genome specific PCR markers.

PCR Marker Specific for the N Genome

The pair of primers VENTRIUP-LN2 was designed to amplify preferentially the N-allele of *Xcmwg682*. The 3′ end from the left primer VENTRIUP was designed to match a point mutation at position 46 that differentiates the N-allele from the other three wheat alleles (Fig. 2). To reinforce the N-allele specificity of this set of primers, the 3′ end of the right primer LN2 was designed to match the TT indel at positions 261-262 that is specific for the N and A-alleles (Fig. 2). These two primers are expected to amplify a 259-bp fragment in plants carrying the 2NS translocation.

The VENTRIUP-LN2 primers were tested on 57 individuals from the backcrossing populations from Yecora Rojo, Anza, UC 1041, and Express previously tested with RFLP marker cMWG682. After adjusting the PCR conditions (Table 1), the 259-bp PCR amplification product was observed only in plants carrying one or two doses of the 2NS translocation as previously determined by the RFLP marker (Fig. 3).

The 259-bp PCR product from primers VENTRIUP-LN2 was a dominant marker and therefore, was not suitable to differentiate heterozygous from homozygous 2NS individuals. A marker to differentiate these two classes is a desirable tool for selection of 2NS homozygous plants in F_2 segregating populations or after self-pollination of the heterozygous BC plants from the last cycle of a backcrossing program. One way to differentiate the homozygous 2NS plants from the heterozygous is by the absence of the 2A-allele in the homozygous 2NS plants. A CAPS marker for the 2A-allele was developed to complement the 2N-allele specific primers.

Cleaved Amplified Polymorphic Sequence Marker

Primers URIC and LN2 (Table 1) were designed to amplify preferentially the N and A-alleles of *Xcmwg682*. These two alleles could then be differentiated by a *Dpn*II restriction site at position 110 that is present in the A and B genomes and is absent in the D and N genomes (Fig. 4). The URIC primer is not genome-specific, but the 3′ end of the LN2 primer was selected to match the TT indel that differentiates the A and N alleles from the two other alleles (Fig. 2).

Amplification of wheat genomic DNA from heterozygous 2AS/2NS plants with CAPS primers URIC and LN2 amplified two fragments of 285 bp (N-allele) and 275 bp (A-allele) (Fig. 2 and Fig. 4). Although the size difference between these two fragments was small, they could be clearly separated in polyacrylamide gels as well as in 4% (w/v) Metaphore (Cambrex Corporation, East Rutherford, NJ) agarose gels (data not shown). Digestion of the PCR products with restriction enzyme *Dpn*II facilitated the differentiation between these two bands. The undigested 285-bp band corresponded to the N genome PCR product and the 166- and 109-bp fragments corresponded to the A genome digested PCR product (Fig. 4).

Plants homozygous for the 2A-allele showed only the two fragments (166 + 109 bp) corresponding to *Dpn*II digested products (Fig. 4, lanes 7, 8). Although the *Dpn*II restriction site was also absent from the D-allele sequence, the URIC-LN2 primers produced no amplification of the D-allele. The absence of the 285-bp fragment in the *Dpn*II-digested PCR products from the nullitetrasomic lines carrying chromosome 2D and lacking chromosomes 2A and 2B (Fig. 4, lanes 2 and 3) confirmed the absence of D genome amplification.

Plants homozygous for the 2N-allele showed only the undigested 285-bp fragment (Fig. 4, lanes 5 and 6). The absence of

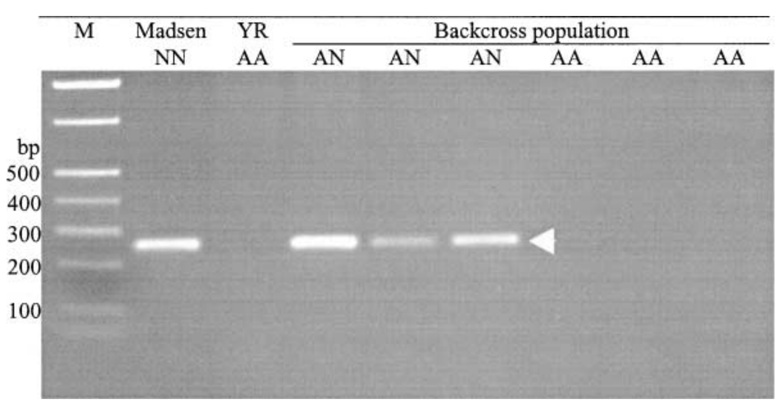

Fig. 3. PCR amplification with 2NS specific primers VENTRIUP and LN2. Genomic DNAs were extracted from Madsen and Yecora Rojo (YR), and from six backcross individual plants. Letters "AA," "AN," and "NN" indicate absence, heterozygosity, or two doses of the 2NS chromosome translocation respectively. A white arrowhead indicates the 2NS specific 262-bp PCR amplification product. "M" indicates the size molecular marker.

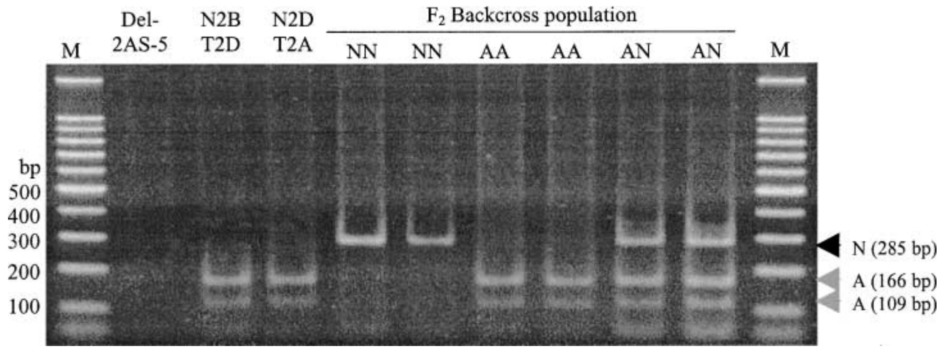

Fig. 4. CAPS marker for the A and N genomes. PCR fragments were amplified with primers URIC– LN2 followed by *Dpn*II digestion. "M" indicates the molecular markers (100-bp ladder, Promega). Del-2AS5 is a deletion line lacking the distal 22% of the 2AS arm and the targeted *Xcmwg682* locus; N2BT2D and N2DT2A are Chinese Spring nullisomic-tetrasomic lines. The remaining six lanes are individuals from an F_2 backcross population. Letters "A," "N," and "B" indicate A, N, and B genomes, respectively. The black arrowhead indicates the 2N genome PCR amplification product (285 bp). The gray arrowheads indicate *Dpn*II digested fragments (166 and 109 bp) from the A genome.

166- + 109-bp fragments in the *Dpn*II-digested PCR products from plants homozygous for the 2NS translocation (Fig. 4, lanes 5 and 6) or the 2AS-5 deletion (Fig. 4, lane 2) confirmed the absence of B genome amplification. However, in nullitetrasomic line N2AT2B where both preferential amplification targets from the A and N genomes are absent, and where four doses of the allele from chromosomes 2B are present, URIC and LN2 primers occasionally amplified a faint band from the B genome (data not shown). The selection of homozygous 2NS plants was the main objective for the development of the URIC-LN2 primers, but since they were very robust in all the tests, they were routinely used in our MAS backcrossing programs to select also for heterozygous 2NS/2AS plants (Fig. 4, lanes 9 and 10).

To test the potential of the CAPS marker on a wider range of wheat cultivars, the presence of the *Dpn*II restriction site was tested in a diverse set of 18 cultivars (see Materials and Methods for a complete list). After digestion with *Dpn*II, the 285-bp undigested fragment was not observed in any of these cultivars indicating that the *Dpn*II restriction site was conserved in the A genome from all the genotypes.

SNP (Single Nucleotide Polymorphism) TaqMan Assay

Fig. 5 shows the amplification signal obtained from 24 individuals with known AA, AN, and NN genotypes by means of TaqMan System 1 and 2. No differences were observed in the signal between the two DNA extraction methods used in these experiments (see Materials and Methods) and the two data sets were merged. The analysis of variance for the amplification signal obtained with Systems 1 and 2 showed significant differences among genotypes ($P = 0.02$ and $P = 0.002$, respectively). In both systems, the response was linear as evidenced by a significant linear response ($P = 0.0008$ and $P = 0.006$, respectively) and a nonsignificant quadratic response ($P = 0.43$ and $P = 0.48$, respectively).

Although both systems provided discrimination between groups of plants carrying different doses of the A and N alleles, System 2 provided a complete discrimination, with no amplification signal in DNA from plants without the N allele. Therefore, System 2 is a better tool to select individual plants carrying the N allele in breeding programs that have access to quantitative PCR equipment.

The cost of development and optimization of a TaqMan system is higher than the cost to develop a simple PCR or CAPS marker. In addition, more complex and expensive equipment is required and the marker is dominant. However, if the same marker is used repeatedly to select thousands of plants, the cost per data point drops dramatically. The fluorogenic detection of the PCR amplification products eliminates the agarose or polyacrylamide gels, reducing labor and waste disposal costs. Finally, the possibility to automate data collection further reduces the relative costs of the TaqMan systems.

CONCLUSIONS

Molecular characterization of the 2NS *T. ventricosum* translocation showed the presence of a relatively large 2NS chromosome segment (25–38 cM). Therefore, it is important to determine the effect of this translocation on agronomic and quality characteristics. The presence

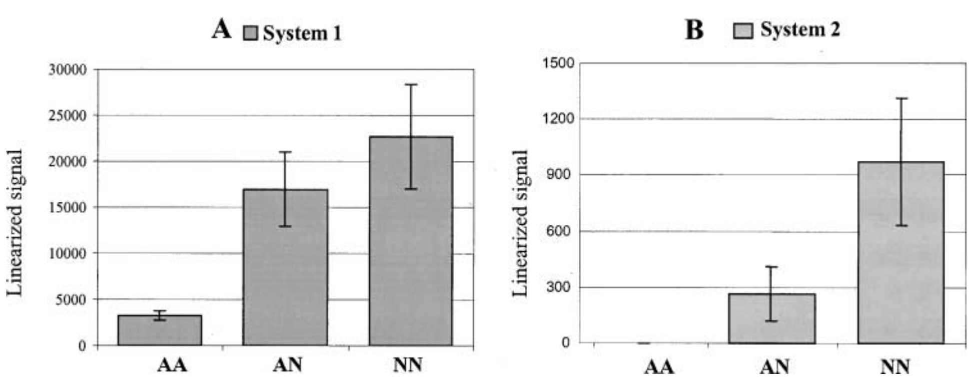

Fig. 5. Mean values of fluorescence (TaqMan signal) obtained from AA, AN, NN genotype groups using TaqMan systems 1 (A) and 2 (B). Standard errors of the means are also indicated. System 2 produced complete discrimination.

of the 2NS *T. ventricosum* translocation in successful cultivars like Madsen suggests that substantial yield penalties should not be expected. Dyck and Lukow (1988) did not find significant yield differences between isogenic lines of Thatcher and Thatcher*8/VPM1 carrying the *Lr37* gene in two experiments performed under rust free conditions. In the same study, these authors reported a significant increase in protein content and a significant decrease in mixing strength associated with the presence of the *Lr37* gene. The four isogenic lines developed in this study will be useful tools to quantify the effect of this chromosome segment on yield and breadmaking quality in different genetic backgrounds. If a significant negative effect on yield or quality is detected, a second round of homeologous recombination in the presence of the *ph1* mutation could be used to reduce the length of the translocated 2NS chromosome segment.

Although three of the isogenic lines carrying the molecular marker for the 2NS segment have shown the presence of an active *Lr37* resistance gene, one exception was observed in the isogenic lines derived from Anza. The presence of a putative suppressor for *Lr37* in this cultivar suggests that it would be advantageous to test F_1 plants by inoculation with the appropriate rust races before the initiation of a marker assisted backcrossing program for this gene. Robert et al. (2000) found that 2% of their lines had conflicting results between the expected presence of the resistance genes on the basis of their molecular marker for the 2NS segment and the resistance tests for *Yr17,* suggesting the possibility of a certain level of recombination between 2AS and 2NS chromosome segments. Therefore, a final challenge to the pathogen should be always part of the validation process.

The combination of an N specific marker and an A specific CAPS marker provides more flexibility to manipulate these genes in breeding programs than previously reported PCR markers. In addition, the development of a TaqMan assay facilitates the implementation of high throughput selection strategies in wheat breeding programs that have access to quantitative PCR equipment. These markers may be particularly useful in the case of *Lr37* because this is an adult plant resistance gene and the availability of molecular markers can accelerate the selection process by testing plants at the seedling stage or even testing half seeds.

Resistance genes present in the 2NS translocation are currently being pyramided in our laboratory with leaf rust resistance genes *Lr47* (Helguera et al., 2000) and *Lr51* (previously *LrF-7*, McIntosh et al., 1995).

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