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Development of Sequence Tagged Site and Cleaved Amplified Polymorphic Sequence Markers for Wheat Stripe Rust Resistance Gene *Yr5*

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ABSTRACT

The *Yr5* gene confers resistance to all races of the wheat stripe rust pathogen [*Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (*P. s. tritici*)] identified so far in the USA. Cosegregating resistance gene analog polymorphism (RGAP) markers for *Yr5* are available but their use requires skills in polyacrylamide gel electrophoresis and may not be polymorphic across various varieties. To develop better markers to be used in marker-assisted selection for the *Yr5* resistance, sequence tagged site (STS) primers were designed on the basis of the sequences of RGAP markers *Xwgp-18* (AY167598) from the spring wheat (*Triticum aestivum* L.) 'Avocet Susceptible' (AVS) and *Xwgp-17* (AY167597) from the *Yr5* near isogenic line (NIL) in the AVS background carrying the *Yr5* gene from *T. aestivum* subsp. *spelta* (L.) Thell. cv. Album (TSA). Three sets of STS markers (two codominant and one dominant) were developed to amplify a region including a polymorphic 6-base pairs (bp) insertion–deletion (indel). The cosegregation of the STS markers with *Yr5* was confirmed with 114 BC_7F_3 lines developed from the cross between AVS and TSA. The STS markers worked well in five out of 17 non-*Yr5* wheat varieties, but the remaining varieties had a similar size of fragment to the *Yr5* marker. Because the codominant STS markers were based on a 6-bp indel, they could not be separated by agarose gel electrophoresis. Cleaved amplified polymorphic sequence (CAPS) markers were then developed on the basis of a *Dpn*II restriction site that is present in all non-*Yr5* varieties and absent in the *Yr5* NIL. The CAPS markers for the *Yr5* NIL and non-*Yr5* varieties can be separated by agarose gel electrophoresis. The codominant STS markers are easier to score than the original RGAP markers. The CAPS markers are not only easier to score, but also can be used in crosses of an *Yr5* donor with a much wider range of wheat germplasms. These markers should be valuable tools to accelerate the introgression of *Yr5* into commercial cultivars and to combine *Yr5* with other genes for durable resistance to stripe rust.

Molecular markers are useful in developing resistant cultivars and, especially useful in developing cultivars with pyramided resistance genes (Castro et al., 2003). Usefulness of a molecular marker for a disease resistance gene is determined by (i) how close the marker is linked to the gene, (ii) how easy the marker can be identified, and (iii) more importantly, whether the marker is polymorphic between the gene donor and a wide range of the crop genotypes. The RGAP technique (Chen et al., 1998), which utilizes high-resolution electrophoresis and sensitive detection of polymerase chain reaction (PCR) products amplified with primers based on conserved domains of plant resistance genes, has been used to identify molecular markers tightly linked to or cosegregating with disease resistance genes (Toojinda et al., 2000; Shi et al., 2001; Yan et al., 2003). Some of the RGAP markers were used to detect the presence and absence of a resistance gene in various germplasms (Shi et al., 2001). In the present study, we report that RGAP markers cosegregating with a stripe rust resistance gene may not be directly used to incorporate the gene into any germplasm of the crop. However, with further improvement of the RGAP markers by means of the STS and CAPS techniques, the RGAP-STS-CAPS markers should allow rapid incorporation of the resistance gene into a wider range of genotypes.

Stripe rust is one of the most destructive diseases on common wheat and durum wheat (*T. turgidum* L. *var. durum*) worldwide. In the USA, the disease is most destructive in the western states and has become increasingly important in the central USA (Line and Chen, 1996; Chen et al., 2002). Growing resistant cultivars is the preferred method of controlling stripe rust (Line and Chen, 1995). However, rapid changes in the pathogen virulence can circumvent stripe rust resistance in wheat cultivars (Line and Qayoum, 1991; Chen et al., 2003). Resistance gene pyramiding is an effective way to obtain high level and durable resistance. Toward combining other effective genes with *Yr5*, a gene originally derived from TSA (Macer, 1966) and confers resistance to all races identified so far in the USA (X.M. Chen, unpublished data), RGAP markers have been developed for *Yr5* (Yan et al., 2003) and other stripe rust resistance genes (Shi et al., 2001; Chen and Yan, 2002).

Yan et al. (2003) identified RGAP markers for *Yr5* and constructed a high-resolution map for the locus. Among the 16 RGAP markers, six markers were completely associated with the *Yr5* locus. Two of the six markers cosegregated with the *Yr5* resistance allele and the other four cosegregated with the susceptible allele from AVS. Of the five markers that were cloned and sequenced, *Xwgp-17* (546 bp, GenBank no.: AY167597) and *Xwgp-18* (540 bp, AY167598), which were codominant and completely cosegregated with the *Yr5* locus, had 98% homology to each other and had over 50% homology with many plant resistance genes or resistance gene analogs. These markers had 59% amino acid homology with the NB-ARC domain, a signaling motif shared by plant resistance gene products and regulators

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Abbreviations: AVS, 'Avocet Susceptible'; CAPS, cleaved amplified polymorphic sequence; NIL, near isogenic line; PCR, polymerase chain reaction; RGA, resistance gene analog; RGAP, resistance gene analog polymorphism; STS, sequence tagged site; TSA, *T. aestivum* subsp. *spelta* cv. Album.

of cell death in animals (van der Biezen and Jones, 1998). Although the RGAP markers were reproducible and could be directly used to identify *Yr5*, these markers require careful scoring because the primers also amplify many other fragments. Therefore, more robust and easy to detect markers are needed for *Yr5*. The objective of this study was to develop STS markers on the basis of the previously identified RGAP sequences to facilitate incorporating *Yr5* into diverse wheat germplasm and cultivars.

MATERIALS AND METHODS

Plant Materials and Evaluation for Stripe Rust Resistance

The wheat *Yr5* NIL (*Yr5*/6*AVS) was developed at the Plant Breeding Institute, Sydney, Australia, by backcrossing the *Yr5* donor, TSA, with the recurrent susceptible spring wheat genotype AVS (Wellings and McIntosh, 1998). Three individual plants of the *Yr5* NIL were crossed to AVS to develop the mapping population BC_7:F_3 lines as described by Yan et al. (2003).

Seedlings of parents, progeny, and TSA were grown and tested for stripe rust resistance under controlled greenhouse conditions by the methods described by Chen and Line (1992a, b). Collection of phenotypic data for stripe rust reactions in the parents and the BC_7:F_3 lines was described in the previous study (Yan et al., 2003). Seedling reactions of wheat cultivars and elite breeding lines were evaluated with races PST-17, PST-20, PST-27, PST-29, PST-37, PST-43, PST-45, PST-59, and PST-78 of *P. s. tritici* in the greenhouse over the past 3 yr by the same methods.

DNA Extraction, PCR Amplification, Electrophoresis, and Gel Visualization

The genomic DNA of the parents and 114 BC_7:F_3 lines described by Yan et al. (2003) were used in this study. Genomic DNA was extracted from parents and F_1 progeny from crosses of the *Yr5* NIL with various elite breeding lines using the same procedures. Conditions and procedures for PCR amplification, electrophoresis, and gel visualization were described by Yan et al. (2003).

Cloning and Sequencing

The PCR fragments from elite lines were cloned and sequenced by the procedures described by Yan et al. (2003). The bands subjected to cloning and sequence analysis were excised from a dried polyacrylamide gel after applying a drop of sterile water. An excised band was soaked in 2 μL of H_2O for at least 1 h, and the solution was used as the template DNA for reamplification with the original RGA or STS primers. The reamplification product was used for cloning. Four microliters of the reamplification mixture was used for cloning into vector TOPO TA pCR2.1 (Invitrogen, Carlsbad, CA) following the procedures recommended by the manufacturer. Plasmid DNA from 10 single colonies derived from each cloning reaction was examined in a 1% (w/v) agarose gel to determine the size of the inserted fragment. To obtain more accurate sequences, two or more clones with the expected insert size were sequenced with the ABI 377 sequencer (Applied Biosystems, Foster City, CA).

Table 1. Sequence tagged site (STS) primers for *Yr5* designed on the basis of the sequences of *Xwgp-17* and *Xwgp-18* markers identified using the resistance gene analog polymorphism (RGAP) technique.

Yr5STS-Primer	Sequence (5′-3′)	Sense (S) or anti-sense (AS)
1	GGGGGGGTGGGGAAGACGAC	S
2	GAGGGCGAGGGGGAGGCC	AS
3	GGGGGGGTGGGGAAGACGACACTC	S
4	GAGGGCGAGGGGGAGGCCAGCAC	AS
5	GGAAGACGACACTCGCTAGAAAAG	S
6	GGAGGCCAGCACACTTCCTTGC	AS
7	GTACAATTCACCTAGAGT	S
8	GCAAGTTTTCTCCCTATT	AS
9	AAAGAATACTTTAATGAA	S
10	CAAACTTATCAGGATTAC	AS
11	GTGTACAATTCACCTAGAGTTAAAG	S
12	CATCACAAACATAAGTCGGCAT	AS
13	CATCACAAACATAAGTTGGCAT	AS
14	ATCACAAACATAAGT	AS
15	GATGTTGCGCATCATATTCAA	S
16	GAGCTTTAGATCACAAACATAAGT	AS

Design STS Primers

The sequences of RGAP markers that were determined in the previous study (Yan et al., 2003) were used to design STS primers. Because markers *Xwgp-17* (GenBank no. AY167597) and *Xwgp-18* (AY167598</exlink>) were codominant, completely cosegregated with the *Yr5* locus, and had resistance gene like sequences (Yan et al., 2003), STS primers were designed on the basis of the sequences of *Xwgp-17* and *Xwgp-18*. Sequences of the STS primers are shown in Table 1 and their positions in the *Xwgp-17*/*Xwgp-18* fragments are shown in Fig. 1. Fragment sizes and codominant or dominant natures of the STS markers produced by 19 primer pairs tested in this study are shown in Table 2.

Developing CAPS Markers

The STS markers were not polymorphic in 10 out of 17 cultivars and advanced breeding lines that did not have the *Yr5* gene and therefore additional polymorphisms were tested. PCR products from wheat genotypes UC896, UC1107, UC1128, UC1358, and RSI 5, which were not polymorphic with the *Yr5* NIL for the STS markers, and UC 1358, which was polymorphic with the *Yr5* NIL, were sequenced and compared with the sequences from the *Yr5* NIL and AVS. A single base pair polymorphism that generates a *Dpn*II restriction site in the DNA of susceptible parents was used to develop a CAPS marker that was tested in a large set of cultivars (Table 3) using primers Yr5STS-9 and Yr5STS-10. Conditions for PCR amplifications were the same as for the STS markers. The reaction mixture for enzymatic digestion contained 17.5 μL of PCR product, 0.5 μL (5 U) of restriction enzyme *Dpn*II (New England Biolabs, Beverly, MA) and 2 μL of 10× buffer for *Dpn*II (New England Biolabs). Samples were incubated at 37°C for 2 h and the digestion products were separated in either 1.5 or 2.8% (w/v) agarose gels.

Data Analyses

The rust reaction phenotype for each of the 114 BC_7:F_3 lines was available from the previous study (Yan et al., 2003). The segregation ratios of stripe rust reactions and STS markers were determined using the Chi-square test (Steel and Torrie, 1980). The association of the rust reaction and molecular markers were determined by comparison and linkage mapping analysis using the Mapmaker program (Lander et al., 1987).

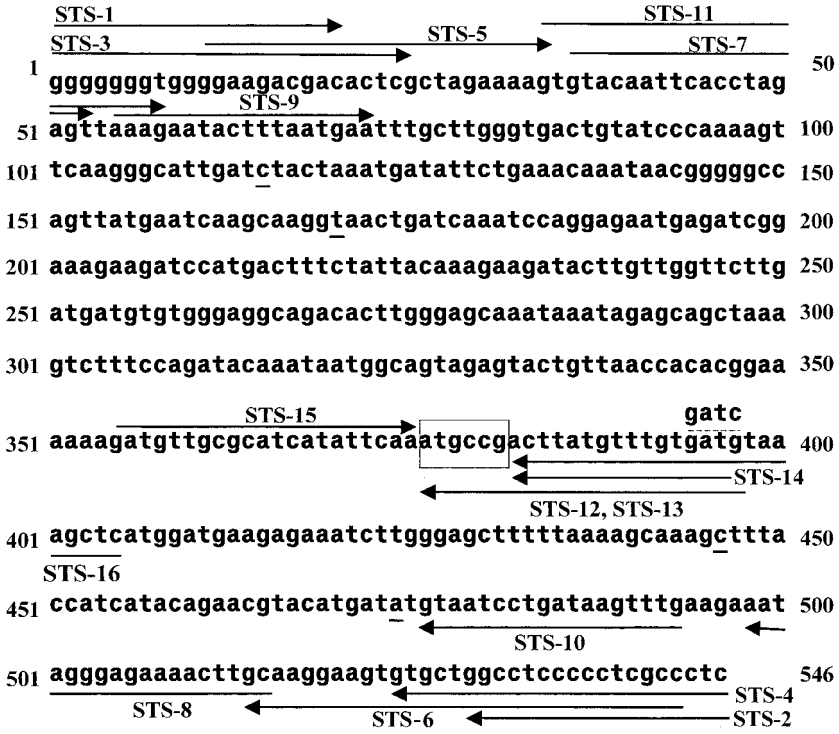

Fig. 1. The sequences of the resistance gene analog polymorphism (RGAP) marker *Xwgp-17* and *Xwgp-18* and the positions of sequence tagged site (STS) primers. The six base pairs in the box were deleted and the underlined base pairs were substituted in *Xwgp-18*. The sequences of primer Yr5STS-12 and Yr5STS-13 are identical except for the 17th base pair based on different clones that were differed at that base position. The *Dpn*II site (gatc) used for the CAPS is shown at the bp 394 to 397.

RESULTS

STS Marker Development

Sixteen STS primers were designed on the basis of the sequences of *Xwgp-17* and *Xwgp-18* markers previously identified by the RGAP technique (Table 1). Twenty-one combinations of these primers were used to amplify genomic DNA from the *Yr5* NIL, AVS, and the resistant and susceptible BC_7:F_3 bulks. Nineteen primer pairs (5/6, 7/8, 9/10, 11/13, 5/8, 5/10, 5/12, 5/13, 5/16, 7/12, 7/13, 7/16, 9/12, 9/13, 9/16, 11/8, 11/12, 11/14, 15/8) produced the expected polymorphic bands in the bulked segregant analysis. Six primer pairs (5/12, 5/13, 7/12, 7/13, 9/12, and 11/12) produced dominant markers and 13 produced codominant bands (Table 2). The amplification product from the *Yr5* NIL was 6 bp larger than the AVS product, and this difference could be seen in denaturing polyacrylamide gel electrophoresis but was difficult to detect in agarose gels.

Table 2. Sequence tagged site (STS) markers for *Yr5* produced by primers designed based on DNA sequences of the resistance gene analog polymorphic (RGAP) markers *Xwgp-17* and *Xwgp-18*.

STS marker(s)	*Yr5* STS primers		Size	Dominant or codominant
	Sense	Antisense	bp	
Yr5STS-5/6	5	6	533/527	Codominant
Yr5STS-7/8	7	8	478/472	Codominant
Yr5STS-9/10	9	10	439/433	Codominant
Yr5STS-11/13	11	13	364/358	Codominant
Yr5STS-5/8	5	8	505/499	Codominant
Yr5STS-5/10	5	10	483/477	Codominant
Yr5STS-5/12	5	12	387	Dominant
Yr5STS-5/13	5	13	387	Dominant
Yr5STS-5/16	5	16	395/386	Codominant
Yr5STS-7/12	7	12	362	Dominant
Yr5STS-7/13	7	13	362	Dominant
Yr5STS-7/16	7	16	370/364	Codominant
Yr5STS-9/12	9	12	343	Dominant
Yr5STS-9/13	9	13	343/337	Codominant
Yr5STS-9/16	9	16	351/345	Codominant
Yr5STS-11/8	11	8	482/476	Codominant
Yr5STS-11/12	11	12	364	Dominant
Yr5STS-11/14	11	14	363/357	Codominant
Yr5STS-15/8	15	8	161/155	Codominant

The best primer combinations for separating the resistant and susceptible alleles at the *Yr5* locus were primer pairs 7/8, 9/10, 5/12, 11/13, 7/16, 11/8, 9/16, 11/12, and 15/8 because of their high specificity. Figure 2 shows two examples of these codominant bands differentiated by the 6-bp indel. The original primers S2 (5′-GGI GGIGTIGGIAAIACIAC-3′) and AS3 (5′-IAGIGCIA GIGGIAGICC-3′) that were used for amplifying the *Xwgp-17* and *Xwgp-18* RGAP markers amplified numerous monomorphic bands besides the polymorphic ones (Yan et al., 2003). These additional bands were not amplified by the STS markers and therefore, the STS markers were much easier to score than the previous RGAP markers.

Cosegregation Analysis of STS Markers with the *Yr5* Resistance

Among the 114 BC_7:F_3 lines that were used in this study, 21 were homozygous resistant, 58 were heterozygous, and 35 were homozygous susceptible, which fit a 1:2:1 ratio ($P = 0.18$). The results indicated that a single gene was present in the *Yr5* line.

Table 3. Wheat cultivars and lines used to validate the CAPS markers for the *Yr5* locus, their origin, reactions to races of *Puccinia striiformis* f. sp. *tritici*, and presence (+) or absence (+) of the CAPS markers produced by *Dpn*II digestion of fragments amplified with the Yr5STS-9 and Yr5STS-10 primers

Cultivar or breeding line	Identification number	Origin	Reaction to PST races of *P. striiformis* f. sp. *tritici*†									Presence of the CAPS markers	
			17	20	27	29	37	43	45	59	78	89 bp	182/188 bp
The*Yr5* NIL	WG00004	Australia	R	R	R	R	R	R	R	R	R	+	–
Avocet S	PI 464644	Australia	S	S	S	S	S	S	S	S	S	–	182
Avalon	PI 446910	England	R	R	R	R						–	+
Brooks												–	+
Cavallier												–	+
Columbus	PI 496258	Canada	R				S	R	R			–	+
Glupro												–	+
Madsen	PI 511673	WA, USA	R	R	R	R	S	R	R	R	R	–	+
Manitou	CItr 13775	Canada	R	R	R	R						–	+
Neepawa	CItr 15073	Canada		S	S	S						–	188
North Dakota												–	188
RSI 5		CA, USA								S	S	–	188
Tadinia	PI 494096	CA, USA										–	+
Kern	UC1036	CA, USA					R			S	S	–	182
UC896		CA, USA					R			S	S	–	188
UC1037		CA, USA				R	R		R	S	S	–	182
UC1041		CA, USA					R		R		R	–	182
UC1107		CA, USA				R	R		R	R	S	–	188
UC1110		CA, USA										–	182
UC1128		CA, USA										–	188
UC1358		CA, USA										–	188
Yecora Rojo	CItr 17414	CA, USA				R	R		R		S	–	188
Yolo	CItr 17961	CA, USA										–	+
Chinese Spring	CItr 14108	China	S	S	S	S	S	S	S	S	S	+	188
Hyak	PI 511674	WA, USA	R	R	R	R	R	R	S	R	R	+	188
Sunfield												+	182 & 188
LEN	CItr17790	ND, USA										+	182 & 188

† The reactions of the cultivars or breeding lines to the specific races were based on seedling tests in the greenhouse conditions. Yolo was resistant in the fields near Pullman but susceptible near Mt. Vernon, WA. LEN was susceptible in the fields near Pullman but resistant near Mt. Vernon. *R* = resistant, S = susceptible, and blank indicates no data.

Two codominant STS primer pairs (Yr5STS-7/8 and Yr5STS-9/10, Table 1) and one dominant pair (Yr5STS-5/12, Table 1) were selected to determine their association with the *Yr5* locus. As expected, all the markers produced by these STS primers completely cosegregated with the resistance phenotype of the 114 BC_7:F_3 lines. The 21 homozygous resistant lines showed only the larger fragment, the 35 homozygous susceptible lines showed only the lower fragment, and the 58 heterozygous lines for *Yr5* were also heterozygous for the two codominant STS markers (Fig. 3). When tested with the primer pair Yr5STS-5 and Yr5STS-12, which produced a dominant marker for the resistant *Yr5* allele, the 21 homozygous resistant and 58 heterozygous lines produced the specific amplification product and the 35 homozygous susceptible lines didn't have the specific product. The marker bands in the homozygous resistant lines were stronger than those in heterozygous lines.

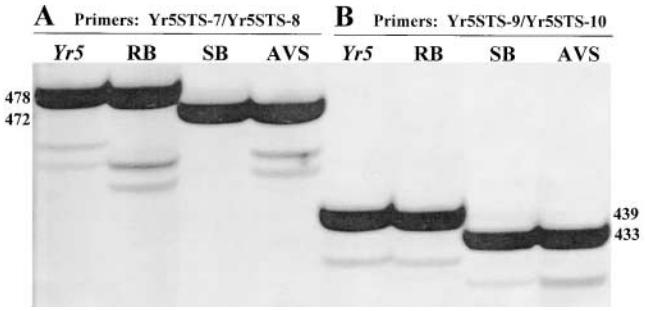

Fig. 2. Silver stained polyacrylamide gel showing polymorphic bands associated with the *Yr5* resistance. A**: Codominant STS markers amplified with primers Yr5STS-7 (5′-GTACAATTCACCTAG AGT-3′) and Yr5STS-8 (5′-GCAAGTTTTCTCCCTATT-3′). The higher band (478 bp) was present in the resistant parent (the *Yr5* NIL) and the resistant BC_7:F_3 bulk and the lower band (472 bp) was present in the susceptible BC_7:F_3 bulk and the susceptible parent (AVS for 'Avocet Susceptible').** B**: Codominant STS markers amplified with primers Yr5STS-9 (5′-AAAGAATACTTTAAT GAA-3′) and Yr5STS-10 (5′-CAAACTTATCAGGATTAC-3′). The higher band (439 bp) was present in the resistant parent (the *Yr5* NIL) and the resistant BC_7:F_3 bulk and the lower band (433 bp) was present in the susceptible BC_7:F_3 bulk and the susceptible parent (AVS).**

Validation of the STS Markers in Diverse Wheat Germplasms

The codominant STS markers amplified with primer pairs Yr5STS-7/8 and Yr5STS-9/10 were tested in a set

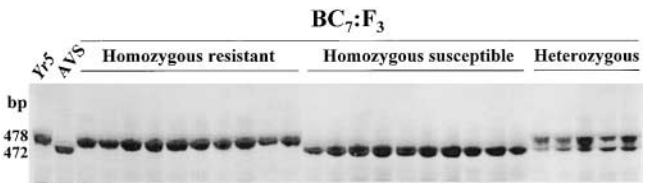

Fig. 3. Silver stained polyacrylamide gel showing association of the codominant markers amplified with STS primers Yr5STS-7 (5′-GTA CAATTCACCTAGAGT-3′) and Yr5STS-8 (5′-GCAAGTTTTC TCCCTATT-3′) with the *Yr5* locus. The higher band (478 bp) was present in the resistant parent (the *Yr5* NIL), the homozygous resistant BC_7:F_3 lines, and the heterozygous BC_7:F_3 lines. The lower band (472 bp) was present in the susceptible parent (AVS), the homozygous susceptible BC_7:F_3 lines, and the heterozygous BC_7:F_3 lines. Phenotypic reactions of the parents and the progeny were evaluated with races PST-29 and PST-43 of *Puccinia striiformis* f. sp. *tritici*.

of 24 wheat genotypes including cultivars grown in California and the Pacific Northwest and advanced breeding lines from the UC Davis wheat-breeding program that do not have the *Yr5* gene (Table 3). Of the 17 non-*Yr5* lines tested for presence or absence of the 6-bp indel, five showed the presence of the 6-bp shorter fragment characteristic of the susceptible variety AVS, 10 had the same size fragment as the *Yr5* NIL, and two were not clear at this time. These results indicated that the Yr5STS-7/8 and Yr5STS-9/10 markers could be used for marker-assisted selection in crosses with some wheat cultivars, but could not be used for crosses involving these nonpolymorphic cultivars.

Developing CAPS Markers for *Yr5*

DNAs from five of the nonpolymorphic lines (UC896, UC1107, UC1128, UC1358, and RSI 5) were amplified with the Yr5STS-9/10 primer pair and sequenced. As expected from the electrophoresis results, the DNA sequences from these lines revealed the absence of the 6-bp indel that differentiated the *Yr5* NIL from AVS (Yan et al., 2003).

Comparison of these DNA sequences with the sequence from the *Yr5* NIL showed the presence of a single base pair polymorphism that determines an additional *Dpn*II restriction site (GATC/GATG in Fig. 1) in AVS and the non-*Yr5* genotypes compared to the *Yr5* NIL.

Digestion of the Yr5STS-9/10 PCR products with the *Dpn*II restriction enzyme resulted in five restriction fragments in the *Yr5* NIL (289, 63, 56, 20, and 12 bp), and four restriction fragments (182, 102, 20, and 12 bp) in AVS and other non-*Yr5* genotypes. The differences between the 289-bp fragment in the resistant lines and the 182-bp fragment in the susceptible lines were easy to visualize in agarose gels.

As expected, the *Dpn*II polymorphism cosegregated with the resistance phenotype in the 114 BC_7:F_3 lines (Fig. 4). Of the 26 non-*Yr5* cultivars or elite breeding lines tested with the CAPS markers (Table 3), 22 had the additional *Dpn*II restriction (182- or 188-bp fragments) and did not have the 289-bp fragment. However, four cultivars, Chinese Spring, Hyak, Sunfield, and LEN, had a fragment(s) with the same mobility as the 289-bp fragment and a fragment with the same mobility as the 182- or 188-bp fragment resembling a heterozygous genotype. The results indicate that the codominant CAPS markers could be used to incorporate *Yr5* into an extended set of cultivars or breeding lines, but that a preliminary test for polymorphism is required before starting the marker assisted selection process in crosses of the *Yr5* donor with cultivars that were not tested in this study.

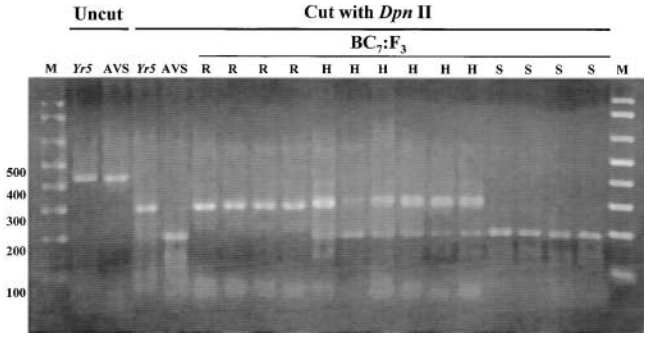

Fig. 4. Ethidium bromide stained 1.5% (w/v) agrose gel showing undigested fragments of the *Yr5* NIL and AVS and *Dpn*II-digested fragments of the *Yr5* NIL, AVS, and homozygous resistant (R), heterozygous (H), and homozygous susceptible (S) BC_7:F_3 lines amplified with the STS primers, Yr5STS-9 (5′-AAAGAATACTTTA ATGAA-3′) and Yr5STS-10 (5′-CAAACTTATCAGGATTAC-3′). *M* stands for 1-kp plus DNA size marker (Gibco BRL, Rockville, MD).

DISCUSSION

The resistance gene analog (RGA) approach that utilizes conserved domains of plant resistance genes increases the probability of identifying markers associated with resistance genes (Kanazin et al., 1996; Leister et al., 1996; Yu et al., 1996). Chen et al. (1998) improved the efficiency of the RGA method by separating PCR fragments amplified with RGA primers using polyacrylamide gel electrophoresis and visualizing the bands using silver staining. The technique was referred to as resistance gene analog polymorphism (RGAP) (Shi et al., 2001). Using the RGAP technique, Yan et al. (2003) developed the markers *Xwgp-17* and *Xwgp-18* that cosegregated with the *Yr5* locus in a population of 202 BC_7:F_3 lines. Absence of recombination between two codominant markers in an F_2 population indicates that a 95% confidence interval for the distance between the STS marker and *Yr5* is +/− 0.7 cM (Hanson, 1959). This genetic interval in wheat can represent between 400 and 2800 kb of DNA sequence on the basis of previous estimates of the ratios between genetic and physical distance in wheat (Lagudah et al., 2001). Therefore, even though the STS markers have very high homology with more than 700 protein sequences for cloned plant resistance genes (Yan et al., 2003), the demonstration that the RGAP or STS marker is part of the *Yr5* gene would require high-density mapping populations and positional cloning strategies that are beyond the scope of this study.

The original RGAP degenerate primers S2 and AS3 (Kanazin et al., 1996) showed the polymorphic bands completely linked to *Yr5* but they also amplify many other monomorphic bands that can complicate the scoring of the *Yr5* specific bands. Moreover, the competition of these bands with the *Yr5* markers for the primers can result in weak bands that can be difficult to score. Therefore, more locus specific markers are preferable.

The *Dpn*II CAPS marker developed from the STS markers solved most of the problems of the STS markers. It produces a codominant marker that can be visualized in agarose gels and is polymorphic in a wider range of germplasms than the 6-bp indel. This CAPS marker showed polymorphism with the *Yr5* NIL in 22 out of the 26 tested non-*Yr5* wheat lines. However, the presence of the 289-bp fragment in four cultivars (Chinese Spring, Hyak, Sunfield, and LEN) that could not be differentiated by means of the CAPS marker indicates that a

preliminary test for polymorphism is required before using any of these PCR markers.

In addition to the 289-bp band, Chinese Spring and Hyak had the 188-bp band while Sunfield and LEN had both 182- and 188-bp bands when the fragments amplified with primers Yr5STS-9 and Yr5STS-10 were digested with *Dpn*II (data not shown). More likely, one of the PCR fragment amplified in these cultivars is from a locus different from *Yr5*. In the previous study, Yan et al. (2003) reported that the sequence of the fragment amplified by the *Xwgp-17* marker was 97% identical in DNA sequence with a resistance gene like fragment (GenBank no. AJ249949) from *Aegilops ventricosa* Tausch that does not have the 6-bp deletion. The 289-bp fragment in Hyak could be from *A. ventricosa* because it had *A. ventricosa* in the pedigree for resistance to stripe rust and eyespot foot rot, caused by *Psedocercosporella herpotrichoides* (Fron) Deighton (teleomorph *Tapesia yallundae* Wallwork & Spooner), (Allan et al., 1990). It is not clear why Chinese Spring, Sunfield, and LEN had the 289-bp fragment. This result indicates that the designed primers could amplify additional resistance gene-like sequences located in other regions of the genome. Further studies would be required to determine sequences and sources of the amplification products in these four cultivars.

Multiple alleles in the marker locus make it impossible to use the original RGAP and STS markers in crosses of the *Yr5* donor with any non-*Yr5* genotype. However, the multiple alleles provide an opportunity to use the CAPS approach to separate the fragment of the *Yr5* NIL and TSA from fragments of non-*Yr5* genotypes. At least four haplotypes have been detected in the small marker region completely linked to the *Yr5* locus. The first one is the 433-bp fragment in AVS, Kern, UC1037, UC1041, and UC1110 that combines the presence of the 6-bp deletion with the presence of the additional *Dpn*II site (5/17). The second one is the 439-bp fragment in Neepawa, ND, RSI 5, UC896, UC1107, UC1128, UC1358, and Yecora Rojo that combines of the absence of the deletion and the presence of the additional *Dpn*II site (8/17). The third one is the 439-bp fragment in Hyak and Chinese Spring that combines absence of the deletion and absence of the additional *Dpn*II site but does not provide the *Yr5* resistance (2/17). The fourth one is the 439-bp fragment in the *Yr5* NIL that does not have the deletion and the additional *Dpn*II site. In addition, the haplotypes of the fragments in Sunfield and LEN (2/17) are not clear, and the remaining nine cultivars in Table 3 were not determined.

The cosegregating markers developed with the RGAP, STS, and CAPS techniques and the sequence diversity at the marker locus will be useful in research toward cloning of alleles at the *Yr5* locus and understanding mechanisms of disease resistance. Meanwhile, the tightly linked STS and CPAS markers developed from the RGAP markers presented in this study would be useful tools to accelerate the deployment of *Yr5* in a wide range of commercial cultivars and to combine with other genes for high-level and durable resistance.

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