



Assessing wheat (*Triticum aestivum*) genotypes for “Yr” resistance genes using conserved regions and simple-sequence motifs

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ABSTRACT. Analysis of DNA sequence variation among genotypes is useful for differentiation of wheat accessions, selection strategies and genetic development of crop plants. We screened molecular markers for yellow rust resistance genes (*Yr7*, *Yr9*, *Yr15*, *Yr18*, *Yr26*, and *YrH52*), which are in the gene-rich regions of wheat chromosomes 1B, 2B, and 7D, to investigate DNA sequence differences and repeat motifs and numbers between wheat cultivars resistant (Izgi2001, Sonmez2001, PI178383) and susceptible (Aytin98, ES14, Harmankaya99) to yellow rust. The F₂ individuals derived from the crosses were evaluated for yellow rust resistance at both the seedling and adult stages to identify DNA markers genetically linked to yellow rust resistance. The most repeated motif was found to be GA and the least repeated motif TAGA among the cultivars. When we examined DNA sequence differences (insertion, deletion and single nucleotide changes), the molecular markers *Xgwm526* (*Yr7*) and *Xgwm273* (*YrH52*) were found to have the most conserved regions and *Yr15* (*Xgwm413*) the least conserved regions among the cultivars. This DNA sequence information can be

used for selection of suitable parents, creating mapping populations and developing molecular markers associated with yellow rust resistance in plant breeding programs.

Key words: DNA sequence; Gene-rich region; Molecular marker; Resistance; Yellow rust

INTRODUCTION

Yellow rust caused by *Puccinia striiformis* f.sp. *tritici*, is one of the most devastating diseases of wheat (*Triticum aestivum* L.) throughout the world. The use of fungicides to control disease has some drawbacks such as cost of the chemicals, limited shelf life, negative effects on the environment, and acquired resistance of the pathogen to applied chemicals. Therefore, growing genetically resistant cultivars has been the main mechanism and the most economical method to control the disease.

Environmental effects on expression of quantitative resistance make selection and pyramiding of R-genes and quantitative trait loci (QTLs) for rust resistance difficult, but pyramiding can be greatly aided by the use of molecular markers closely linked to the gene/QTL. These markers can be readily identified using a rapid, high-through-put screening procedure as part of the breeding program, an approach referred to as marker-assisted selection (MAS) (McDowell and Woffenden, 2003). The genomic abundance of microsatellites and their ability to be associated with many phenotypes, make this class of molecular markers a powerful tool for diverse application in plant genetics (Maia et al., 2009).

Microsatellites show a much higher level of polymorphism and informativeness in hexaploid bread wheat than any other marker system (Roder et al., 1998). The efficiency of microsatellite marker development depends on the abundance of repeats in the target species and the ease with which these repeats can be developed into informative markers. The wheat genome is extremely large (16×10^9 bp/1C) and contains much repetitive DNA (more than 80%) (Bennett and Smith, 1976). At many microsatellite loci, a high frequency of variation in the number of repeat units has been observed (Tautz et al., 1986). This makes microsatellites an abundant source of highly polymorphic and codominant genetic markers. Microsatellites are hot spots for genome mutation and variation (Weber, 1990; Ellegren, 2004). The variability seen in microsatellites is primarily due to sequence length polymorphisms caused by variable numbers of tandem repeats (Ellegren, 2000, 2004; Neff and Gross, 2001). Based on genomic library screening, it was observed that, in plants, $(AG/TC)_n$ is more frequent than $(AC/TG)_n$, (Lagercrantz et al., 1993), whereas in humans $(AC/TG)_n$ repeats are the most common (Weber and May, 1989). Akkaya et al. (1992) indicated that $(AT)_n$ and $(ATT)_n$ microsatellite repeat motifs were the most frequent in soybean. Similarly, Morgante and Olivieri (1993) confirmed that AT repeat motif was the most common in plants, shown in many studies they have done.

In this study, DNA sequence analysis was performed with primers derived from microsatellite markers linked to *Yr* genes in gene-rich regions of wheat chromosomes. The most conserved regions and single nucleotide polymorphisms were detected, and sequence analyses were also carried out. As an extremely conservative sequence in most plant introns, the CACGC sequence greatly increases the probability of a full match between primers and templates in the

PCR reaction. In surveying conserved regions, single nucleotide polymorphisms are potent (Shaheen et al., 2010). The property of SNP markers is to discriminate alleles at the level of individual organisms (Nakitandwe et al., 2007).

The objectives of this study were to analyze repeat motifs and numbers and to determine DNA sequence differences among the wheat cultivars in terms of molecular markers showing a genetic link to *Yr* genes (*Yr7*, *Yr9*, *Yr15*, *Yr18*, *Yr26*, *YrH52*) in gene-rich regions of wheat chromosomes 1B, 2B, 7D.

MATERIAL AND METHODS

Plant material

Six homozygous bread wheat genotype (*Triticum aestivum* L. cvs. PI178383, Izgi2001 and Sonmez2001-known yellow rust resistant cultivars, and *Triticum aestivum* L. cvs. Harmankaya99, ES14 and Aytin98-known yellow rust susceptible cultivars) obtained from Anatolian Agricultural Research Institute, Eskisehir, Turkey was used as plant material. The resistance of parental cultivars and F₂ generations were tested in the greenhouse by applying uredospores. Two weeks following the inoculation, the infection type was recorded using the 0-9 scale (McNeal et al., 1971), treating 0-6 as low infection type and 7-9 as high infection type. The disease score of PI178383, Izgi2001, and Sonmez2001 was 0 while that of Harmankaya99, ES14, and Aytin98 was 8 in greenhouse assays. These assays confirm that the parental genotypes greatly differ in their resistance to yellow rust disease.

Analysis of molecular markers linked to *Yr* genes in gene-rich regions

Leaves from resistant and susceptible plants were used for total genomic DNA extraction using the method of Weining and Landridge (1991), modified by Song and Henry (1995). Bread wheat genotypes were screened by molecular markers (*Xgwm526*, *Xgwm582*, *Xgwm413*, *Xgwm295*, *Xgwm18*, *Xgwm11* and *Xgwm273*) located in gene-rich regions of wheat linked to *Yr* genes (*Yr7*, *Yr9*, *Yr15*, *Yr18*, *Yr26*, *Yr26* and *YrH52*) (Table 1). DNA amplifications were carried out in 25- μ L reaction mixtures, each containing 1X Taq Buffer (MBI Fermentas, Germany), 0.4 mM forward primer, 0.4 mM reverse primer, 0.2 mM dNTPs (MBI Fermentas, Germany), 2.5 mM MgCl₂ (MBI Fermentas, Germany), 0.625 U/ μ L Taq polymerase (MBI Fermentas EP0402, Germany) and 100 ng template DNA. The following PCR profile was used in an Eppendorf Mastercycler Gradient machine; initial denaturation at 94°C for 3 min; 40/45 cycles at 94°C for 1 min, 50°C/55°C for 1 min, 72°C for 2 min; and a final extension at 72°C for 10 min. The amplification products were resolved on a 2% agarose gel. To determine the size of PCR products, a 100-bp DNA ladder marker (MBI Fermentas SM0323, Germany) and 50-bp DNA ladder marker (MBI Fermentas SM0371, Germany) were used.

Sequence analysis of the DNA fragments

PCR products obtained by *Xgwm526* (*Yr7*), *Xgwm582* (*Yr9*), *Xgwm413* (*Yr15*),

Xgwm295 (*Yr18*), *Xgwm11* (*Yr26*), *Xgwm18* (*Yr26*), and *Xgwm273* (*YrH52*) were separated on a 6% polyacrylamide sequencing gel. The fragment showing a polymorphic profile for the yellow rust resistant genotypes was cut out from the gel and cloned into PCR cloning vector (pGEMT-Easy Vector Systems, Promega). Following *E. coli* bacterial transformation and blue-white colony screening, positive white colonies were analyzed for the presence of the fragment by PCR with T7 vs SP6 universal primers. The recombinant plasmid was harvested using the Qiagen, QIAprep Spin Miniprep kit. Sequence analysis of the fragment was performed on the isolated plasmid with M13-47 sequencing primer using GenomeLab GeXP Genetic Analysis System (Beckman Coulter) according to manufacturer instructions.

Table 1. Primer sequences and T_a values which belonging to the markers associated with *Yr* genes at the gene-rich region of wheat chromosome that used in the study.

Name of primer	Linked <i>Yr</i> gene	Forward primer (5'-3')	Reverse primer (5'-3')	T_a (°C)
XGWM526	<i>Yr7</i>	CAATAGTTCGTGAGAGCTGCG	CCAACCCAAATACACATTCTCA	55°C
XGWM582	<i>Yr9</i>	AAGCACTACGAAAATATGAC	TCTTAAGGGGTGTTATCATA	50°C
XGWM413	<i>Yr15</i>	TGCTTGTCTAGATTGCTTGGG	GATCGTCTCGTCCTTGGA	54°C
XGWM295	<i>Yr18</i>	GTGAAGCAGACCCACAACAC	GACGGCTGCGACGTAGAG	55°C
XGWM11	<i>Yr26</i>	GGATAGTCAGACAATCTTGTG	GTGAATTGTGTCTTGTATGCTCC	50°C
XGWM18	<i>Yr26</i>	TGGCGCCATGATTGCATTATCTC	GGTTGCTGAAGAACCCTATTAGG	50°C
XGWM273	<i>YrH52</i>	ATTGGACGGACAGATGCTTT	AGCAGTGAGGAAGGGGATC	55°C

T_a = annealing temperature.

RESULTS

Simple-sequence motifs among wheat genotypes

Seven SSR markers genetically linked to *Yr* genes were characterized in six bread wheat cultivars and amplified PCR products successfully. Of the SSRs analyzed, 50% of the primer pairs amplified monomorphic [XGWM582 (150 bp), XGWM295 (250 bp), XGWM18 (200 bp)] and rest of the primer pairs amplified polymorphic [XGWM526 (140-160 bp), XGWM413 (95-100-110 bp), XGWM11 (200 bp) and XGWM273 (170-180-190-200 bp)] PCR products. Multiple discrete PCR products were observed among di- and tetranucleotide SSR markers. There were more dinucleotide repeats than tetranucleotide repeats in the six bread wheat genotypes used in this study.

Among the dinucleotide repeat classes of microsatellites, the most abundant repeat motifs were GA, TC, and CA. Based on the results of DNA sequence analysis, the repeated motifs and the numbers were evaluated for each SSR marker. After evaluation, 2 (140 and 160 bp), 3 (95, 100 and 110 bp) and 4 (170, 180, 190, and 200 bp) different allele profiles were observed for TC, AG and GA motifs, respectively (Figure 1).

GA was significantly more common than the other dinucleotide motifs. It was observed that the most GA motifs are in Aytin98 and the least GA motifs are in Harmankaya99, among the genotypes used in study. The distribution of other repeat motifs was also calculated. For example, the CA motif is the most common in PI178383 and the least in Sonmez2001-Aytin98, the TC motif is the most frequent in Izgi2001 and the least in PI178383 and Harmankaya99. In addition, the most repeated motifs were found in

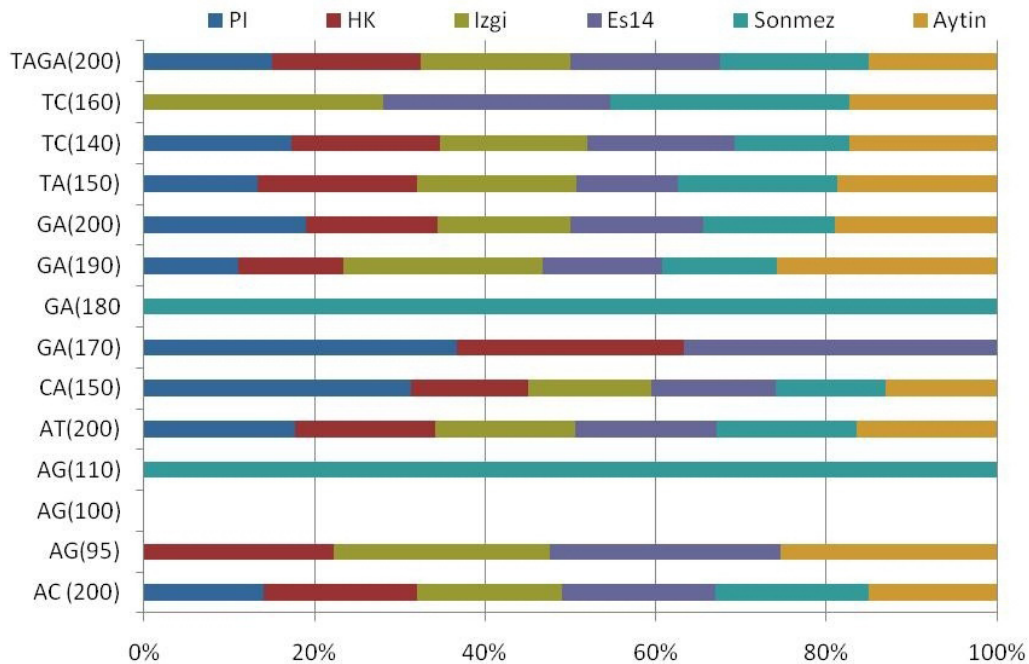


Figure 1. Mean tandem repeat number of microsatellite motifs for 6 different wheat's genomic DNA.

Sonmez2001 but the least in Harmankaya99 in terms of total number of repeated motifs (Table 2).

Frequencies of different types of microsatellite repeat motifs according to seven microsatellite primers derived from yellow rust gene specific markers located in 1BS (*Yr15-Xgwm413*, *Yr9-Xgwm582*, *Yr26-Xgwm11*, *Yr26-Xgwm18* and *YrH52-Xgwm273*), 2B (*Yr7-Xgwm526*) and 7D (*Yr18-Xgwm295*) were also calculated for 6 wheat cultivars (Figure 2).

According to these results, GA was the most and TAGA was the least found.

Conserved regions and sequence differences among wheat genotypes

DNA sequence analysis of PCR products was performed for each molecular marker in order to identify conserved regions and single nucleotide differences. According to ClustalW analysis of DNA sequences, the most single nucleotide polymorphism was obtained with *Xgwm413* (*Yr15*) marker and the least single nucleotide polymorphism was obtained with *Xgwm273* (*YrH52*) marker (Table 2). All insertions, deletions and single nucleotide changes were taken into consideration and it was detected that *Xgwm526* (160 bp) and *Xgwm273* (170-180-190 bp) have the most conserved regions molecular markers among cultivars used in the study.

Table 2. Distribution of determined repeat motifs and numbers for each marker associated with *Yr*-genes of wheat genotypes according to the results of DNA sequencing.

Linked <i>Yr</i> Genes	Genotypes	Numbers of repeat motifs													
		AC		AG		AT		CA		GA		TA		TC	
		200 bp	95 bp	100 bp	110 bp	200 bp	150 bp	170 bp	180 bp	190 bp	200 bp	150 bp	140 bp	160 bp	200 bp
<i>Yr7</i> (XGWM526)	PI178383	-	-	-	-	-	-	-	-	-	-	-	13	-	-
	HK99	-	-	-	-	-	-	-	-	-	-	-	13	-	-
	Izgi2001	-	-	-	-	-	-	-	-	-	-	-	13	21	-
	ESI4	-	-	-	-	-	-	-	-	-	-	-	13	20	-
<i>Yr15</i> (XGWM413)	Sonmez2001	-	-	-	-	-	-	-	-	-	-	-	10	21	-
	Aytun98	-	-	-	-	-	-	-	-	-	-	-	13	13	-
	PI178383	-	0	-	-	-	-	-	-	-	-	-	-	-	-
	HK99	-	14	-	-	-	-	-	-	-	-	-	-	-	-
	Izgi2001	-	16	-	-	-	-	-	-	-	-	-	-	-	-
	ESI4	-	17	-	22	-	-	-	-	-	-	-	-	-	-
<i>Yr9</i> (XGWM582)	Sonmez2001	-	16	-	-	-	-	-	-	-	-	-	-	-	-
	Aytun98	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PI178383	-	-	-	-	-	25	-	-	-	-	6	-	-	-
	HK99	-	-	-	-	-	-	-	-	-	10	10	-	-	-
<i>Yr26</i> (XGWM18)	Izgi2001	-	-	-	-	-	-	-	-	-	-	10	-	-	-
	ESI4	-	-	-	-	-	-	-	-	-	5	-	-	-	-
	Sonmez2001	-	-	-	-	-	-	-	-	-	10	-	-	-	-
	Aytun98	-	-	-	-	-	-	-	-	-	10	-	-	-	-
	PI178383	-	-	-	-	-	16	-	-	-	4	-	-	-	-
	HK99	-	-	-	-	-	18	-	-	-	4	-	-	-	-
<i>Yr26</i> (XGWM11)	Izgi2001	-	-	-	-	-	19	-	-	-	4	-	-	-	-
	ESI4	-	-	-	-	-	19	-	-	-	4	-	-	-	-
	Sonmez2001	-	-	-	-	-	17	-	-	-	4	-	-	-	-
	Aytun98	-	-	-	-	-	17	-	-	-	4	-	-	-	-
	PI178383	14	-	-	-	14	-	-	-	-	-	-	-	-	-
	HK99	18	-	-	-	13	-	-	-	-	-	-	-	-	-
<i>Yr18</i> (XGWM295)	Izgi2001	17	-	-	-	13	-	-	-	-	-	-	-	-	-
	ESI4	18	-	-	-	13	-	-	-	-	-	-	-	-	-
	Sonmez2001	18	-	-	-	13	-	-	-	-	-	-	-	-	-
	Aytun98	15	-	-	-	13	-	-	-	-	-	-	-	-	-
	PI178383	-	-	-	-	-	-	-	-	19	-	-	-	-	-
	HK99	-	-	-	-	-	-	-	-	21	-	-	-	-	-
<i>YrH52</i> (XGWM273)	Izgi2001	-	-	-	-	-	-	-	-	22	-	-	-	-	-
	ESI4	-	-	-	-	-	-	-	-	24	-	-	-	-	-
	Sonmez2001	-	-	-	-	-	-	-	-	23	-	-	-	-	-
	Aytun98	-	-	-	-	-	-	-	-	21	-	-	-	-	6
	PI178383	-	-	-	-	-	-	-	-	-	11	-	-	-	7
	HK99	-	-	-	-	-	-	-	-	18	9	-	-	-	7
<i>YrH52</i> (XGWM273)	Izgi2001	-	-	-	-	-	-	-	-	-	9	-	-	-	7
	ESI4	-	-	-	-	-	-	-	-	-	9	-	-	-	7
	Sonmez2001	-	-	-	-	-	-	-	-	-	9	-	-	-	7
	Aytun98	-	-	-	-	-	-	-	21	23	11	-	-	-	6

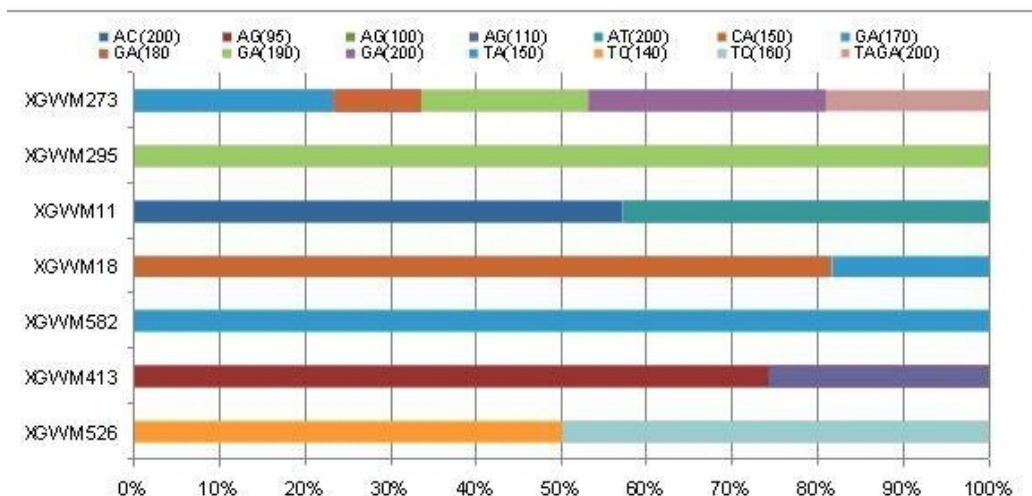


Figure 2. Frequencies of different types of microsatellite repeat motifs according to seven microsatellite primers specific to gene-rich region in 6 different wheat cultivars.

DISCUSSION

Repeat type is one of the important factors that have been found to affect the stability of microsatellites (Schlotterer, 2000; Ellegren, 2004). In the present study, we compared the percentage of repeat motifs in six bread wheat genotypes. Our study compared the polymorphism rate of di- and tetranucleotide microsatellites. The results indicate the dinucleotide microsatellites have the highest rate of polymorphism, while the tetranucleotide microsatellites are the least polymorphic. It has also been reported that the base composition of the repeat motifs may play a role in the variations of microsatellites. (GT) $_n$ and (GA) $_n$ motifs were found to repeat respectively every 704 kb and 440 kb in the wheat genome (Roder et al., 1995). In addition, the number of GA repeat motif is more than the number of GT repeat motifs in wheat (Plaschke et al., 1995), barley (Struss and Plieske, 1998), rice (Wu and Tanksley, 1993), apple (Guilford et al., 1997) and *Brassica napus* (Lagercrantz et al., 1993; Szevc-McFadden et al., 1996). Because there are many GA and GT repeat motifs in wheat, eight wheat varieties have been scanned with designed primers using these motifs (Koike et al., 2006). GA was also shown to be the most abundant motif in grasses (Temnykh et al., 2001; Kantety et al., 2002; Nicot et al., 2004; Parida et al., 2006). It was revealed that the GA repeat motif has the highest polymorphism rate (approx. 74%), whereas the GT repeat motif has the lowest (about 15%). Similarly, our results indicate that there are differences in the polymorphism rate among the three dinucleotide motifs (GA, TC, and CA) and that the GA motif was the most abundant repeat motifs in all wheat genotypes.

Stepien et al. (2007) determined the repeat motif and number of *Xgwm18* marker as (CA) $_{17}$ GA(TA) $_4$ in 53 Polish wheat cultivars. In our study, we found the repeat motif and number of the *Xgwm18* marker to be (CA) $_{16}$ (TA) $_4$, (CA) $_{17}$ (TA) $_4$, (CA) $_{18}$ (TA) $_4$ and (CA) $_{19}$ (TA) $_4$ in PI178383, Sonmez2001-Aytin98, Harmanakaya99 and Izgi2001-ES14, respectively. Nevertheless, no found GA repeat motif was obtained by screening any genotypes with this marker.

As a result (i) motifs and the numbers of repeat regions (ii) single-nucleotide differences (iii) and the most protected areas were detected, which belonged to the sequences obtained from DNA sequence analysis with primers for the markers associated with *Yr* genes in the gene-rich regions of wheat chromosomes, and the original differences were thereby detected among the cultivars studied. By designing new primers based on these original differences, new markers can be obtained, and these markers can be used in screening populations that show segregation in terms of yellow rust disease. Resistant and susceptible wheat genotypes used in this work, have already been used by our research group for the development of molecular markers for yellow rust resistance. In this frame, Temel et al. (2008) investigated the sequence differences of yellow rust resistance gene “*Yr10*” in seven winter-type bread wheat genotypes, and the results obtained from this work indicate that (1) *Yr10* gene sequence is present in all of these varieties (2) the divergence between the varieties is raised from the variations in the second exon, and (3) the first exon is more conserved than the second one in all genotypes. Sequence variations could affect gene expression and could weaken yellow rust resistance. In another work by our group, Akfirat et al. (2010) used bulk segregant analysis (BSA) to identify molecular markers associated with yellow rust disease resistance in a Izgi2001 x ES14 cross. This analysis showed that 81% of the wheat genotypes known to be yellow rust resistant had the SSR marker *Xgwm382*. Similarly, one EST-SSR marker (*Pk54*) has been identified in a PI178383 x Harmankaya99 cross. A total of 108 wheat genotypes differing in yellow rust resistance were screened with *Pk54*, and 68% of the wheat genotypes known to be yellow rust resistant had *Pk54* (Ercan et al., 2010), further suggesting that the presence of these markers correlate with yellow rust resistance in diverse wheat germplasm.

Based on the above mentioned studies, the characterization of SSR markers regarding the repeat numbers, type of motifs and sequence differences could have important implications for molecular marker-assisted selection strategies and pyramiding of the yellow rust resistant genes in wheat breeding programs.

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