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An EST-SSR marker linked with yellow rust resistance in wheat

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Abstract

Expressed sequenced tags containing simple sequence repeats (EST-SSRs) were used to identify molecular markers associated with yellow rust resistance in wheat (*Triticum aestivum* L.). A cross between yellow rust resistant (PI178383) and susceptible (Harmankaya99) wheat genotypes was performed and respective DNA pools from the resistant and susceptible F_2 seedlings were constructed. 78 EST-SSR primers were used for bulked segregant analysis and one EST-SSR marker (*Pk54*), identified as 200 bp fragment, was present in the resistant parent and resistant F_2 hybrids but not in the susceptible ones. 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 the *Pk54* marker, further suggesting that the presence of this marker correlates with yellow rust resistance.

Additional key words: breeding, *Puccinia striiformis* f. sp. *tritici*, *Triticum aestivum*.

Introduction

Yellow rust of wheat caused by *Puccinia striiformis* f. sp. *tritici* presents a serious problem for wheat production in many parts of the world and it is the most damaging to grain yields in cool and moist environments (Singh *et al*. 2000, Akar *et al*. 2007). Characterization of pathogen avirulence and plant disease resistance genes and search for the new resistance sources, all contribute to the development of wheat genotypes with increased disease resistance (Kaur *et al.* 2008). Molecular markers are powerful tools in marker-assisted selection, gene pyramiding and gene cloning of important crop traits especially for disease resistance (Amiri *et al.* 2009). Once the markers for the resistance are identified, they may potentially be used in marker-assisted breeding programmes to develop resistant cultivars. The genomes of all eukaryotes contain a class of di-, tri- or tetra-nucleotide repeats, termed microsatellites or simple sequenced repeats (SSRs) (Kumar *et al.* 2007). SSR markers derived from ESTs are commonly called EST-derived SSRs. Since a putative function based on corresponding ESTs can be deduced for the EST-SSRs, they represent a class of functional markers (Andersen and Lubberstedt 2003). Analysis of ESTs is a simple strategy to study the transcribed parts of genomes, thus rendering even complex and highly redundant genomes like wheat amenable to large-scale analysis. Therefore, if an EST marker is found to be genetically associated with a trait of interest, it may be possible that this could be the gene affecting the trait directly (Chen *et al.* 2001, Thiel *et al.* 2003). These markers have been studied for genetic diversity in wheat (Leigh *et al.* 2003). The occurrence of microsatellites in EST sequences has been reported for different species including barley (Holton *et al.* 2002, Thiel *et al.* 2003), rice (Temnykh *et al*. 2000), durum wheat (Eujayl *et al.* 2002), bread wheat (Kantety *et al.* 2002), rye (Hackauf and Wehling 2002), sugarcane

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Abbreviations: BSA - bulked segregant analysis; EST - expressed sequenced tag; PCR - polymerase chain reaction; SSR - simple sequence repeat.
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(Cordeiro *et al.* 2001), and grape (Scott *et al.* 2000). Thus, the generation of EST-derived SSR markers has become an attractive complement to existing SSR collections for different studies. SSR markers have been described within sugarcane stress related-ESTs and their utilization as markers in a group of modern sugarcane commercial-type genotypes (Da Silva and Solis-Gracia 2006). The transferability of EST-SSR markers was analysed for crown rust in ryegrass (Dracatos *et al.* 2006) and their potential has been used as new molecular tools for introgression, variability and phylogenetic analysis of the *Hordeum chilense* genome (Castillo *et al.* 2008).

In the present study, we report the identification and screening of an EST-SSR marker associated with yellow rust resistance in wheat using bulked segregant analysis (BSA). This kind of result is vital for plant breeding programmes to develop new cultivars with higher resistance.

Materials and methods

A cross between the yellow rust resistant PI178383 and susceptible Harmankaya99 Turkish wheat (*Triticum aestivum* L.) cultivars was made in the wheat breeding program of the Anatolian Agricultural Research Institute (AARI). The resistance of parental cultivars and F_2 generations were tested in the greenhouse by applying uredospores collected from the experimental research sites of the Central Research Institute for Field Crops (CRIFC). Collected spores were dried in silica gel for 8 - 12 h and the urediospores were stored in glass tubes in liquid nitrogen. Inoculum is virulent for *Yr2, Yr6, Yr7, Yr8, Yr9, Yr11, Yr12, Yr17, Yr18, Yr27, and YrA+* and avirulent for *Yr1, Yr5, Yr10, Yr15, Yr24, YrSP, YrCV* genes.

The uredospores (250 000 cm^{-3}) were inoculated in mineral oil (*Soltrol 170*) at two-leaf stage of susceptible cultivars (Michigan Amber, Gerek, Seri 82, Little Club) in greenhouse. Following inoculation, the seedlings were incubated in chambers with humidity of 95 - 100 % at 9 ^{o}C for one day and then transferred to a greenhouse adjusted to 15 - 25 ^{o}C. In greenhouse, two weeks following the inoculation, the infection type was recorded using the 0 - 9 scale (McNeal *et al*. 1971) when the susceptible checks scored 8 or more, treating 0 - 6 as low infection type and 7 - 9 as high infection type.

Leaf tissue samples were collected, frozen in liquid nitrogen, and ground to a powder using the *Retsch MM301* system. Extraction of the DNA was carried out as described by Weining and Langridge (1991). For BSA, 78 EST-SSR primer pairs (Gupta *et al.* 2003) were used to identify markers associated with yellow rust resistance in the F_2 population of PI178383 × Harmankaya99. Aliquots of DNA from thirty resistant and thirty susceptible F_2 plants at seedling stage were combined to produce bulks. PCR reactions for each primer pairs were performed on the extracted genomic DNA from the following groups of wheat plants: *1*) the resistant parent, *2*) the susceptible parent, *3*) the pooled DNA of the most resistant F_2 plants, and *4*) the pooled DNA of the most susceptible F_2 plants. PCR contained 100 ng of genomic DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.25 μM of each primer, and 0.5U Taq polymerase (*MBI Fermentas*, St. Leon-Rot, Germany). Amplification was performed in *Applied Biosystems* (Carlsbad, USA) *Gene Amp PCR System 9700* thermocycler as follows; 3 min at 94 °C, 1 min at 94 °C, 1 min at 50, 55, 60 °C (depending on the annealing temperature), 1 min at 72 °C for 40 cycles with 10 min final extension at 72 °C. Putative polymorphisms among bulks and parents were checked by repeated amplifications and all the individuals contributing to the respective pools were tested separately.

Fluorescent-labelled forward primer, specific for *Pk54*, was synthesized according to *GeXP GenomeLab* (*Beckman*, Coulter, USA) genetic analysis system manufacturer instructions. PCR mixture was prepared as described previously except fluorescent-labelled forward primer was included instead of the unlabelled one. The fluorescent-labelled PCR products (1 mm^3) were mixed with 0.25 mm^3 size standard-400 and the volume was completed to 30 mm^3 with sample loading solution (SLS; *Beckman*). The electrophoretic separation was performed by using *GenomeLab GeXP* and the data was analysed by fragment analysis module of the system. Each experiment was replicated at least three times to verify the reproducibility of the marker.

PCR products obtained by *Pk54* primers were separated on 6 % polyacrylamide sequencing gel. The fragment showing 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,* Madison, USA). Following *Escherichia coli* bacterial transformation and blue-white colony screening, positive white colonies were analysed for the presence of the fragment through PCR reaction performed with T7 *vs*. SP6 universal primers. The recombinant plasmid was harvested by using *Qiagen* (Valencia, USA), *QIAprep Spin Miniprep* kit. Sequence analysis of the fragment was performed on the isolated plasmid with M13-47 sequencing primer using *GenomeLab GeXP* according to the manufacturer's instructions.

Results

The disease score of PI178383 was 0 while that of Harmankaya99 was 8 in greenhouse assays. These assays confirm that the parental genotypes greatly differ in their resistance to yellow rust disease. Infection type of selected resistant (R) F_2 individuals was between 0 - 1, while it was 8 - 9 in susceptible (S) F_2 individuals. For BSA, 30 resistant and 30 susceptible F_2 seedlings were undertaken into investigation.

BSA relies strongly on unequivocal scoring of resistant and susceptible plants as false readings can occur when unsuccesful plant is scored as resistant. For this reason, microsatellite primers were initially used to generate polymorphic bands for the F_2 bulks and parents DNAs. All parents and bulks were screened with 78 EST-SSR primer pairs and of these, 3 primer pairs (*Pk10, Pk12, Pk30*) gave no amplification. In total, 75 EST-SSR primer pairs were tested at different annealing temperatures (50 - 60 ^oC). 54 out of 75 primer pairs (69.23 %) amplified monomorphic fragments in PI178383 and Harmankaya99. 21 primer pairs (26.92 %) produced polymorphic amplification products in parents. In this group, only *Pk54* amplified a DNA fragment of approximately 200 bp that was present both in the resistant parent and resistant bulk but not in the susceptible ones. Yellow rust resistance was confirmed by the amplification of polymorphic fragment in thirty resistant F_2 individuals with the *Pk54* primer pair. The 200 bp fragment, was present in 27 out of 30 individuals in the resistant bulk (Fig. 1*A*), and was absent in 26 out of 30 individuals in the susceptible bulk (Fig. 1*B*). It was significant that the 3 F_2 plants (Nos. 18, 27, 28) in the resistant bulk did not generate the 200 bp fragment, and the 4 F_2 plants (Nos. 2, 9, 15, 19) in the sensitive bulk generated the 200 bp fragment (Fig. 1). The reason may be that the recombination between the marker and the resistance locus has occured.

New generation flourescence-based capillary electrophoresis system was used for the verification of the *Pk54* marker based on the fragment sizes. Fig. 2 shows the fragment profile of resistant parent with six peaks labelled as 118.48, 166.53, 176.87, 191.21, 202.09 and 204.69 bp. Fig. 2 also indicates the susceptible parent with four peaks at 118.51, 166.40, 176.90 and 192.65 bp.

DNA fragments (200 bp), identified on the 3 % agarose gel, were separated as 202.9 and 204.69 bp on capillary electrophoresis which verified the presence

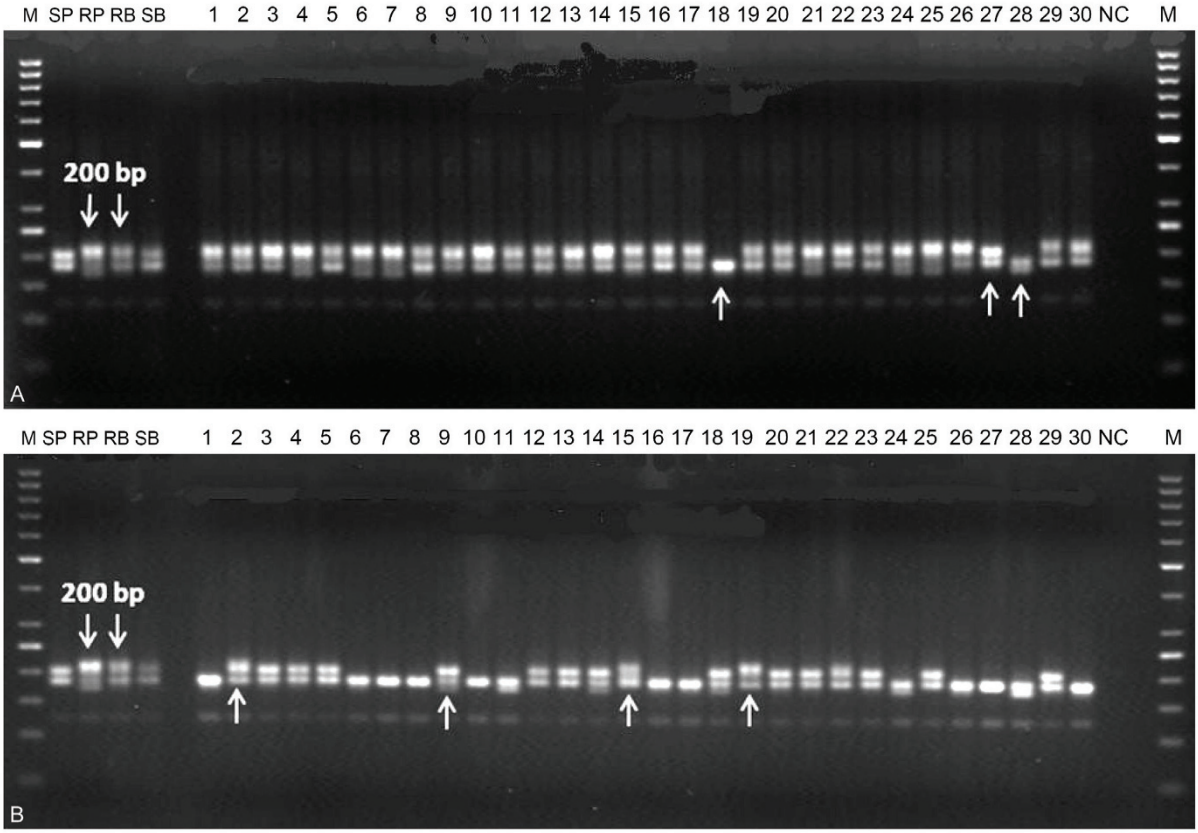

Fig. 1. Amplification products of microsatellite PCR obtained by PK54 primer pair in genomic DNA of the parents and resistant (*A*) and susceptible (*B*) F_2 hybrids at seedling stage (1 to 30). M - 50 bp DNA ladder, SP - susceptible parent (Harmankaya99), RP - resistant parent (PI178383), RB - resistant bulk, SB - susceptible bulk, NC - negative control.

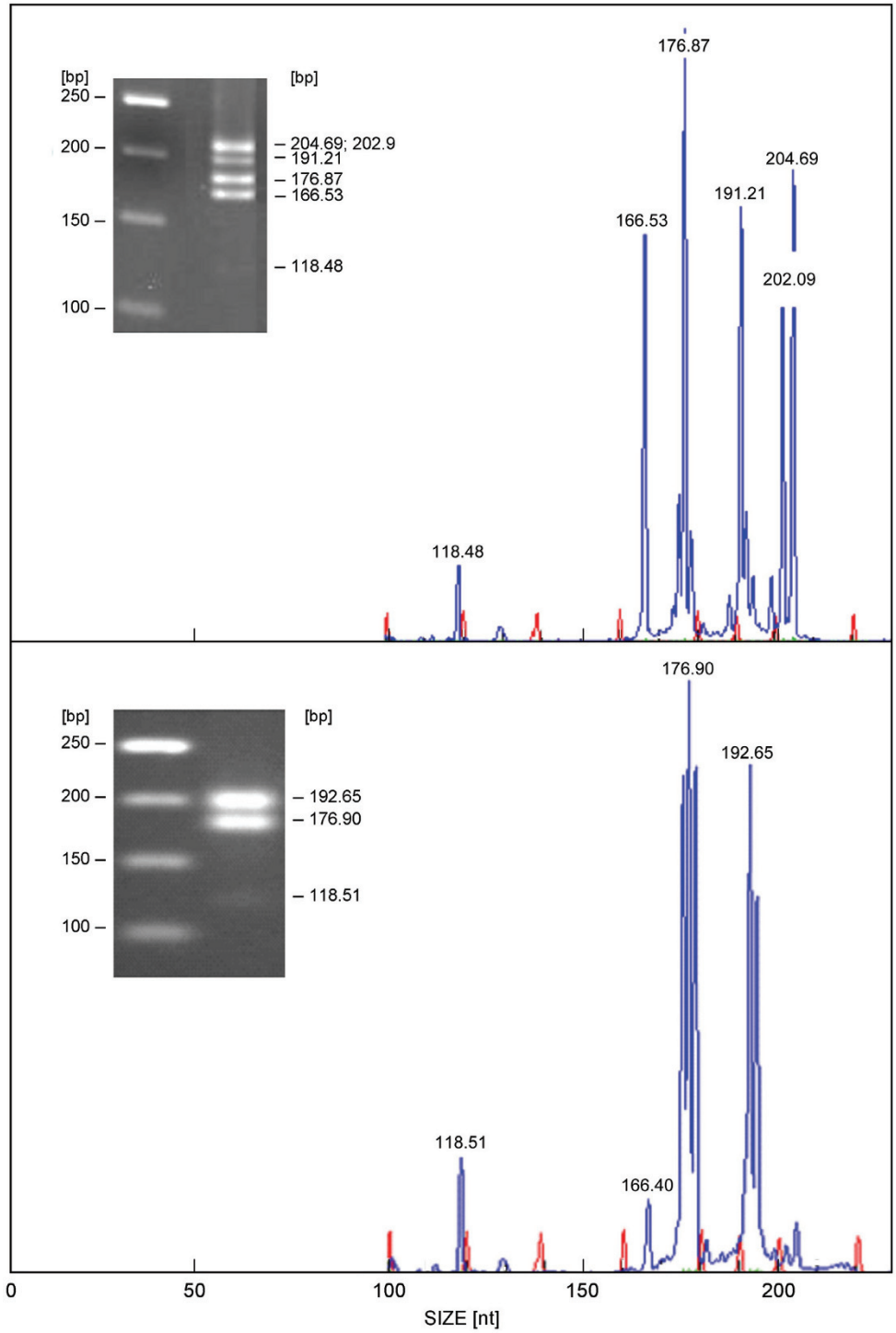

Fig. 2. Fragment analysis of *Pk54* by florescence-based capillary electrophoresis in resistant PI178383 (*above*) and susceptible Harmankaya99 (*below*) parents.

of two markers possessing alleles with 2.6 bp size differences. The sequence analysis of 204 bp fragment was carried out in resistant parent genotypes by using fluorescence-based capillary electrophoresis and 17 repeats of TTG sequence [$(TTG)_{17}$] were observed.

The linkage between the *Pk54* locus and yellow rust resistance was also investigated in 108 wheat genotypes differing in yellow rust resistance (35 resistant and 73 susceptible) supplied by AARI and CRIFC. These materials were screened and 200 bp fragment was observed in 68 % of resistant genotypes while it was not present in any susceptible genotypes.

Discussion

Identification of yellow rust resistance genes and breeding of resistant cultivars are effective approach for minimizing wheat losses due to this disease (Li *et al.* 2006). Identification of markers linked to the trait of interest has been based on the screening of a relatively large number of individuals in the population. BSA was originally developed to overcome this difficulty, because comparing bulk samples is easier than evaluating many individuals in different populations (Sweeney and Danneberger 1994). Greatest efficiency is achieved by selection as early as possible in the breeding process (especially F_2 or F_3). A number of useful marker-trait associations have been reported for wheat, namely powdery mildew resistance (Chantret *et al.* 2000, Zhu *et al.* 2005, Liu *et al.* 2008), karnal bunt resistance (Kumar *et al*. 2007), leaf rust resistance (Gupta *et al.* 2006), septoria resistance (Adhikari *et al.* 2004), *Mycosphaerella* resistance (Adhikari *et al.* 2003) and water stress tolerance (Altinkut and Gozukirmizi 2001, Altinkut *et al.* 2003). We used yellow rust disease scoring data that can be relatively easily quantified for preparing the yellow rust resistant and susceptible bulks in BSA in this study. In repeated amplifications, the presence of 200 bp EST-SSR marker may significantly enhance the selection of wheat genotypes for yellow rust resistance. Compared with genomic SSRs derived from enriched genomic DNA libraries (g-SSRs), EST-SSRs give better profiles (Holton *et al.* 2002, Gupta *et al.* 2003) and Zhang *et al.* (2005) demonstrated that common wheat EST-SSRs showed a high level of transferability to close and wild relatives of wheat because they are mainly derived from conserved coding regions. As genetic markers, EST-SSRs have been evaluated in several studies and tend to be considerably less polymorphic than those from genomic DNA for rice (Cho *et al.* 2000), sugarcane (Cordeiro *et al.* 2001), barley (Thiel *et al.* 2003), or durum wheat (Eujayl *et al.* 2002). Eujayl *et al*. (2002) showed that EST-SSR primers produce high quality markers, but expose a lower level of polymorphism (25 %) compared with the genomic SSR markers (53 %) in durum wheat.

Most designated yellow rust resistance genes are expressed at seedling growth stages and is usually effective throughout the life of the host. We reported the detection of a 200 bp EST-SSR marker, linked to the seedling plant resistance. An F_2 population from a cross between PI178383 and Harmankaya99 was visually assessed for seedling infection type in greenhouse. Similar to our work, Khlestkina *et al*. (2007) identified *Xgwm533* as a diagnostic marker for disease resistance against yellow rust in spring wheat. The 117 bp allele of *Xgwm533* was found in about 35 % of the cultivars analysed, however, none of them possessed the expected disease resistance. Thus, the utilisation of *Xgwm533* as a diagnostic marker seems to be restricted to certain gene pools. In contrast to this, 200 bp allel of *Pk54* marker was found in 68 % of wheat genotypes analysed in our work and it could be valid for more diverse germplasm. The marker identified in this study, verified in other wheat genotypes, would allow implementation of marker-assisted selection. We expect that this marker may be used for large scale yellow rust resistance screening of segregating populations.

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