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Assessment of genetic diversity of wheat genotypes by resistance gene analog-EST markers

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ABSTRACT. Resistance gene analog-expressed sequence tag (RGA-EST)-based markers have been used for variety discrimination and studies of genetic diversity in wheat. Our aim is to increase the competitiveness of public wheat breeding programs through intensive use of modern selection technologies, mainly marker-assisted selection. The genetic diversity of 77 wheat nucleotide binding site (NBS)-containing RGA-ESTs was assessed. Resistant and susceptible bread wheat (*Triticum aestivum*) genotypes were used as sources of DNA for PCR amplifications. In our previous studies, the F_2 individuals derived from the combinations PI178383 x Harmankaya99, Izgi2001 x ES14, and Sonmez2001 x Aytin98 were evaluated for yellow rust resistance at both seedling and adult stages to identify DNA markers. We have now examined the genetic variability among the resistant and susceptible Turkish wheat cultivars for yellow rust disease and the mean genetic distance between the cultivars. The highest similarity was 0.500 between Harmankaya99 and Sonmez2001. The lowest similarity was 0.286 between Aytin98,

PI178383 and Aytin98, ES14. A relatively high level (49.5%) of polymorphism was observed with 77 RGA-EST primers across the six wheat genotypes, despite the fact that all of them were local cultivars from geographically close locations. RGA-EST sequences were compared by BlastX algorithms for amino acid sequences to determine the polymorphic categories among the combinations. BlastX analyses of six RGA-ESTs that gave polymorphic patterns for all combinations were NBS-LRR class RGA, NB-ARC domain containing protein, NBS-type resistance protein RGC5, NBS-LRR-S/TPK stem rust resistance protein, and putative MLA1 proteins, while 38 RGA-EST gave a monomorphic pattern.

Key words: *Triticum*; Biodiversity; RGA-EST; Genetic diversity; Yellow rust

INTRODUCTION

Wheat (*Triticum aestivum* L.) is the most important human food crop, and production has to be increased significantly in the next decades. The allohexaploid wheat genome (2n = 6x = 42) is one of the largest among crop species with a haploid size of 16 billion bp (Bennett and Leitch, 1995), and wheat genetics and genome organization have been extensively studied by molecular markers (Ercan et al., 2010; Akfirat-Senturk et al., 2010; Karakas et al., 2010). Molecular markers have been widely used in genetic analyses, breeding studies and investigations of genetic diversity and the relationship between cultivated species and their wild parents, because they have several advantages as compared with morphological markers, including high polymorphism and independence from effects related to environmental conditions and the physiological stage of the plant (Bertini et al., 2006).

Polymerase chain reaction (PCR)-based molecular markers are easy to use and exhibit a high degree of polymorphism. Microsatellites (SSRs: simple sequence repeats) (Plaschke et al., 1995), restriction fragment length polymorphism (RFLP) (Nagaoka and Ogihara, 1997), amplified fragment length polymorphism (AFLP) (Gulbitti-Onarici et al., 2007), selective amplification of microsatellite polymorphic loci (SAMPL) (Altintas et al., 2008), random amplified polymorphic DNA (RAPD) (Asif et al., 2005), and expressed sequence tag (EST)-derived contigs and singletons (Karakas et al., 2010) have been widely used to characterize genetic diversity in wheat accessions. Besides these marker types, the resistance gene-analog polymorphism (RGAP) approach (Chen et al., 1998), which utilizes high-resolution electrophoresis and sensitive detection of PCR products amplified with primers based on conserved domains of plant resistance genes, has been used to identify molecular markers tightly linked to or co-segregating with disease resistance genes and also genetic diversity (Shi et al., 2001; Yan et al., 2003). Many plant resistance gene analogs (RGA) have been isolated and identified from different plant species. Linkage analysis has shown that these RGAs are distributed throughout the genome and exist in clusters (He et al., 2003). Some RGAs have been demonstrated to be linked with known R genes. Most characterized RGA-encoding proteins containing an LRR (leucine-rich repeat) motif appear to be grouped in clusters and colocalized with a known resistance gene (Geffroy et

al., 1999). Many RGAs containing an LRR motif have been isolated from wheat (Qin et al., 2003). Yellow rust, triggered by the biotrophic fungus *Puccinia striiformis* f. sp. *tritici*, is a fungal disease of considerable importance in cereal production in Turkey and in other temperate cereal-growing areas (Akar et al., 2007). McFadden et al. (2006) analyzed wheat EST sequences separately to identify a representative set of RGA families. Sequences that showed greater than 70% DNA sequence identity over at least 200 bp were considered to be members of the same family, and the 115 wheat ESTs were grouped into 77 RGA families. In this study, these 77 wheat nucleotide binding site (NBS)-containing RGA-ESTs were used to assess genetic diversity among the yellow rust-resistant and -susceptible Turkish wheat cultivars. The objective of the present study was to establish genetic relationships between six wheat accessions and to assess the existing genetic variation and the potential among the accessions to start new breeding programs.

MATERIAL AND METHODS

Plant materials and DNA extraction

Six homozygous bread wheat genotypes (three yellow rust-resistant cultivars: PI178383, Izgi2001, Sonmez2001, and three yellow rust-susceptible cultivars: Harmankaya99, ES14, Aytin98) were obtained from the Anatolian Agricultural Research Institute, Eskişehir, Turkey. Leaves from resistant and susceptible plants were used for total genomic DNA extraction using the miniprep method of Weining and Landridge (1991) modified by Song and Henry (1995).

Disease assay

The resistance of cultivars was tested in the greenhouse by applying uredospores. 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 (Ercan et al., 2010; Akfirat-Senturk et al., 2010). These assays confirm that the genotypes differ greatly in their resistance to yellow rust disease.

Analysis of wheat RGA-ESTs

RGA-ESTs from two divergent NBS regions of wheat sequences of the NBS-LRR class were chosen from the NCBI web site (http://www.ncbi.nlm.nih.gov) according to McFadden et al. (2006). These RGA-EST sequences were further processed for vector contamination at the http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html web site and undesired vector fragments extracted from sequence lists. RGA-ESTs of *T. aestivum* were then subjected to Primer Premier 5.0 and Primer 3.0 programs for PCR primer designing (Table 1). A total of 77 RGA-EST-derived primers were screened against six wheat genotypes to assess genetic diversity (Figure 1). They were also queried using the BlastX algorithm of the Basic Alignment Search Tool (Altschul et al., 1990) to determine functional annotation of polymorphic categories among wheat genotypes.

Table 1. RGA-EST primers used for genomic amplifications.

Accession number	Primer	Sequence (5'-3')	Accession number	Primer	Sequence (5'-3')
BE400250	1-BE400250	F- 5' CAT CTT ATT gTT TgT CCC T 3' R- 5' gAg TAT gAT TTA TCC TTg g 3'	CA742849	40-CA742849	F- 5' CAg gTg TCg TAg gTg AAT g 3' R- 5' TgT ggA TAg CAg Agg gAT T 3'
BE405711	2-BE405711	F- 5' CAT TTC gCA TAC TTT TTg A 3' R- 5' TTC TgT gAT TTT TAC TCC C 3'	CA499328	41-CA499328	F- 5' TgA AAg TgA AAA ggA ggg C 3' R- 5' CCA AAg ACA AAT gTg AgA A 3'
BE498831	3-BE498831	F- 5' AgA CAA gAT TAg Tgg AAC g 3' R- 5' AAA gAA AgA TAg CAT Agg T 3'	CA692539	53-CA692539	F- 5' Cgg TTg ATg AAT gAC ACg AA 3' R- 5' CTg ACA CTg AAg CAg ggA gC 3'
BE499177	4-BE499177	F- 5' ATC AgT CAC CgT gTC TCA 3' R- 5' TTC TTT CTC gCT ATA TTT 3'	BJ246024	58-BJ246024	F- 5' TCT ACA CAA CAg AAg ggC A 3' R- 5' gAA ggT TTA ggA CAC ggA C 3'
BE500158	5-BE500158	F- 5' AAC AgA TgA gAA ACC AAA g 3' R- 5' ATC AAC gAC AAC AAA ATA C 3'	BJ245188	59-BJ245188	F- 5' AAA TCC TCC gAA gAg AAC A 3' R- 5' TTA CAA TCg CAg CCA ACA A 3'
BF482358	6-BF482358	F- 5' gAg AAA CCC gCA gCg AgA g 3' R- 5' TTT gTg AAC ATA gCC ATT A 3'	CA501245	60-CA501245	F- 5' ATA gCg gAA ggC TTT gTC 3' R- 5' CgA TgT TCg gTg gTA gTA 3'
BJ255861	7-BJ255861	F- 5' ggA ATC ggC AAg ACC ACT C 3' R- 5' CAC ATC ATC CAg CAC CAg g 3'	BM134773	61-BM134773	F- 5' AgA TTT TgC TAT gCT TTC AC 3' R- 5' TTA CTC ATT TTC TTT gCT gC 3'
BJ258770	8-BJ258770	F- 5' TTg TCg ggC TTg gTg gTC TA 3' R- 5' TgC TgT TTg CTT CAT TAC TT 3'	BQ752781	62-BQ752781	F- 5' gTg gAT AgC AgA Agg ATT 3' R- 5' ACT TgA TTA gAT ggA ACA 3'
BJ269157	9-BJ269157	F- 5' gCT TCA TCT ACC TCC CAA CA 3' R- 5' CCC CCA CAC TCA TCC TCA TA 3'	BE591190	63-BE591190	F- 5' AAT CAA TgA ggA Agg AAT gg 3' R- 5' gTA ACg ggA gAg AAA ACA gC 3'
BJ270203	10-BJ270203	F- 5' ggC AgA gCA gTC CAg Cag 3' R- 5' TCg TAA TAA CAC gCA AAT 3'	CD910548	64-CD910548	F- 5' AAg Tgg gTT gCT gAg ggT TT 3' R- 5' TgC ggC AgT TCT TCT AgT gT 3'
BJ272146	11-BJ272146	F- 5' ggA gCg gAC AgT gAg ACA T 3' R- 5' ggC AAg AAg CAg gAg CAT A 3'	BE517538	65-BE517538	F- 5' ATg gAT TgC TgA Agg gTT 3' R- 5' gAg TgA TCT ggC ATg ggA 3'
BJ286558	12-BJ286558	F- 5' ACC ATA Agg AAA AAC ACC A 3' R- 5' CTC AAC AAA ggC AAg ACT A 3'	CA497657	66-CA497657	F- 5' CCg AAg gCA AgT AAA AAg g 3' R- 5' AAg AgC CCg AAC TgC gAC A 3'
BJ295708	13-BJ295708	F- 5' TTT AgC ACA TCA ACC TCg 3' R- 5' gAT TCT CTC AAA AAC gCC 3'	BQ744464	67-BQ744464	F- 5' ggg ATC TTC CAC ACC ATT 3' R- 5' gCT TTC CgA TTT CAC CAg 3'
BJ300496	14-BJ300496	F- 5' CTA TTg TTg gAT TTg gTg gg 3' R- 5' CgA TAC ggg TTg TCg TgA TT 3'	CA617198	68-CA617198	F- 5' gTC CAC ATg ggg TgA AgT AA 3' R- 5' AAg TCC AAC AAC gCg AgA gA 3'
BQ239089	15-BQ239089	F- 5' CAA CCC TTC TCA AAA CAT T 3' R- 5' TCA TTC AAg CAC TCC ATC C 3'	BM134978	70-BM134978	F- 5' CAg AAA CTg AAg AgA TgC T 3' R- 5' TAT TgA CAg ATg ACg gAC A 3'
BQ241493	16-BQ241493	F- 5' CCg TgC CCT CAg TTC AAT 3' R- 5' CAT ACg TAg CTg CCC TCC 3'	BU099584	71-BU099584	F- 5' ggC TCT CTC TgT CTT CTg 3' R- 5' gCT TTA TTC TgT gCA TCA 3'
BQ246913	17-BQ246913	F- 5' gAA CAg CgA AAg Tgg gAg gA 3' R- 5' TgT AgA AgA Cgg CgC AgA Ag 3'	CA736742	72-CA736742	F- 5' TTC TCC CCT CTg TCA AC 3' R- 5' AgC CAT gCA TCT TCT AC 3'
BQ579469	18-BQ579469	F- 5' ggT CTg gAA CTC AAT gAT gC 3' R- 5' CAA ggC TgT TTT Tgg CTg gT 3'	CA740494	73-CA740494	F- 5' gAg Cgg ggA gCA ATT CTT CT 3' R- 5' ATC CTT CAg CgA CCC ACA TA 3'
BQ753146	19-BQ753146	F- 5' AAg ACC TgT gTg TTT ggA T 3' R- 5' CAg CTT TgT gTA CTT gCA T 3'	CA724373	74-CA724373	F- 5' ggg CTg CCA TAT CAT CTC AA 3' R- 5' ACC TCC gTC CAA gTT CCT Cg 3'
BQ802253	20-BQ802253	F- 5' ATC TAT CAC ATC gAg CCC C 3' R- 5' gAC ATC CCA TCA TAC TgC C 3'	CA498317	75-CA498317	F- 5' TAC TTC CgC AAA TgC CCA g 3' R- 5' TTC CAg TTT CCA AAC ACC g 3'
BQ802688	21-BQ802688	F- 5' CTA TTA gTT gTg CTT TTC C 3' R- 5' TTC ACC TAT CCA TTg TTT A 3'	CA696482	76-CA696482	F- 5' ATg ACC TTC CAg ACg ACC T 3' R- 5' TCC gCT TTC CAT CCA CTT A 3'

Continued on next page

Table 1. Continued.

Accession number	Primer	Sequence (5'-3')	Accession number	Primer	Sequence (5'-3')
BQ803195	22-BQ803195	F- 5' ACT gCg ggg CTT TTg TCT C 3' R- 5' gCT TCT TAg gTg gTg ggg A 3'	BJ213107	77-BJ213107	F- 5' TTA AgC TCC TgT TTg Tgg 3' R- 5' TTg CCC TTC TgT TgT ATg 3'
BQ901277	23-BQ901277	F- 5' gAg CTg CTC CAC ATC CAA AT 3' R- 5' CCA CAC TTg TgA Agg CTg Tg 3'	BJ316279	78-BJ316279	F- 5' CTg AAA AgA TgT ggT gg 3' R- 5' TTT Agg TgT ggA ggT Ag 3'
BU100242	24-BU100242	F- 5' ATA TCC CAA AAA CCT AAC A 3' R- 5' AgC AAC TCA CCA TAC TCA A 3'	BJ277253	79-BJ277253	F- 5' AgA AgA CAC AgC AgA AgA gT 3' R- 5' TTC AAC ATC CCC AAT AAA AC 3'
CA500988	25-CA500988	F- 5' ACg ACA CCA TTC AAC gCC T 3' R- 5' TCC CgA ACC TCC AAA ACC A 3'	BQ743300	80-BQ743300	F- 5' AAg TCg TgC TTC CTC TAC 3' R- 5' AgT CTC CCT TAT gTC CAg 3'
CA600403	26-CA600403	F- 5' Agg TgA AgT CgT gCT TAT gg 3' R- 5' TTC TgA TgT CCg AgT TTT gg 3'	BF484437	81-BF484437	F- 5' CAg gAg gTg gTT AgC ggA 3' R- 5' ATg gAT ggA AAg gTg gCA 3'
CA606728	27-CA606728	F- 5' AgA TgA gTg ggA gAg ggT 3' R- 5' CAg gTC TTg gAg ATg ggT 3'	BJ207304	82-BJ207304	F- 5' TAT TTT TgC TCT ATT Tgg g 3' R- 5' gTC TTC ACC TCT gTg CTg T 3'
CA610289	28-CA610289	F- 5' gTC AgT Cgg AgA ggT ggg A 3' R- 5' AgT ATC ggg AgT ggA AAA T 3'	CA744411	83-CA744411	F- 5' CgA gTg Tgg TTA TCC CTT g 3' R- 5' gCT CCT CTg CTA TCT gTg C 3'
CA652512	29-CA652512	F- 5' gAT AgC ACT ACg CAC ATT 3' R- 5' CCA TCA ACC ATC CAA CAg 3'	BJ276947	84-BJ276947	F- 5' AAC CTA TTC CCT CCA TTC 3' R- 5' TCA TTT CTT CAA CTT CCA 3'
CA662188	30-CA662188	F- 5' AAA CTC TTT CCC ACA TCT 3' R- 5' gTg CCT ggA CAA TAA CCT 3'	BJ225910	85-BJ225910	F- 5' TTg CTT gAT TTA gCg gAT A 3' R- 5' CAg ACT CTT gAg gAC TTT C 3'
CA662651	31-CA662651	F- 5' gAC CTT CCC CAT CAC CTA 3' R- 5' TTC CCg TTC CAA ACC CAg 3'	CA727476	86-CA727476	F- 5' gCA gTg TAg ggA AAT CgT g 3' R- 5' AAg TCA TTg gAg TTg ggA g 3'
CA676926	32-CA676926	F- 5' TgC CAT TTC ATA CAC ACg 3' R- 5' AgA gCA ACA gTC CCC CAg 3'	BE405507	87-BE405507	F- 5' ATg CTT AgT gTT gTC TCC A 3' R- 5' TAA TCC ATC ACA TTT CCT T 3'
CA679534	33-CA679534	F- 5' ATg gAC ATC CTC CTT CAA 3' R- 5' TCA TCC CAA Tgg TTA gTT 3'	BE418533	126-BE418533	F- 5' ACA ggC AAA TgA ACg ACg 3' R- 5' TgT gAA ATg CTC TCT AAg 3'
CA681703	34-CA681703	F- 5' CCC ATA AAA ACA CAA TCT 3' R- 5' gAC gAC AAC Agg ACA 3'	BF199788	127-BF199788	F- 5' ATC gCC AAC CAA ATC AAT g 3' R- 5' TgT TTT TCC CAA CCC ACC A 3'
CA725884	35-CA725884	F- 5' CTT TgC CgA TTT gAg ACA 3' R- 5' Agg ATT gAg ggA TgC TTC 3'	BF473313	128-BF473313	F- 5' CTg gAA AAg CAC AgT TgA 3' R- 5' gTg gAT gAT ggA AgA AgA 3'
CA726158	36-CA726158	F- 5' TAA TCC gAC CAA AAA CAg gC 3' R- 5' CCA AgA ggT gAA ACC AAA gA 3'	BE445244	129-BE445244	F- 5' TAA ggC ggT TAT gAA gCA 3' R- 5' TCg gAA ggA CTg Agg AAA 3'
CA733486	37-CA733486	F- 5' CAT CAC CAA gCC AAA AgC A 3' R- 5' CTg gAg AAg TAA gAC CCg A 3'	BE605005	130-BE605005	F- 5' ATA AAg gAT ggg ATg gAA 3' R- 5' CTT gAg ggA ATC TgA gCA 3'
CA741642	38-CA741642	F- 5' AgA gAA TCA gCA gAC AAg gC 3' R- 5' AAA CAT CAT CCA gCA CgA gC 3'	BE426789	131-BE426789	F- 5' AAT gTT ggg TgT CAg TTT CC 3' R- 5' AgC ATg TCC ATC CAg gTT TC 3'
CA742788	39-CA742788	F- 5' TCT CCT CAT CTT CCT TAg CA 3' R- 5' ATC ACA gTC TCg CAg TCA TT 3'			

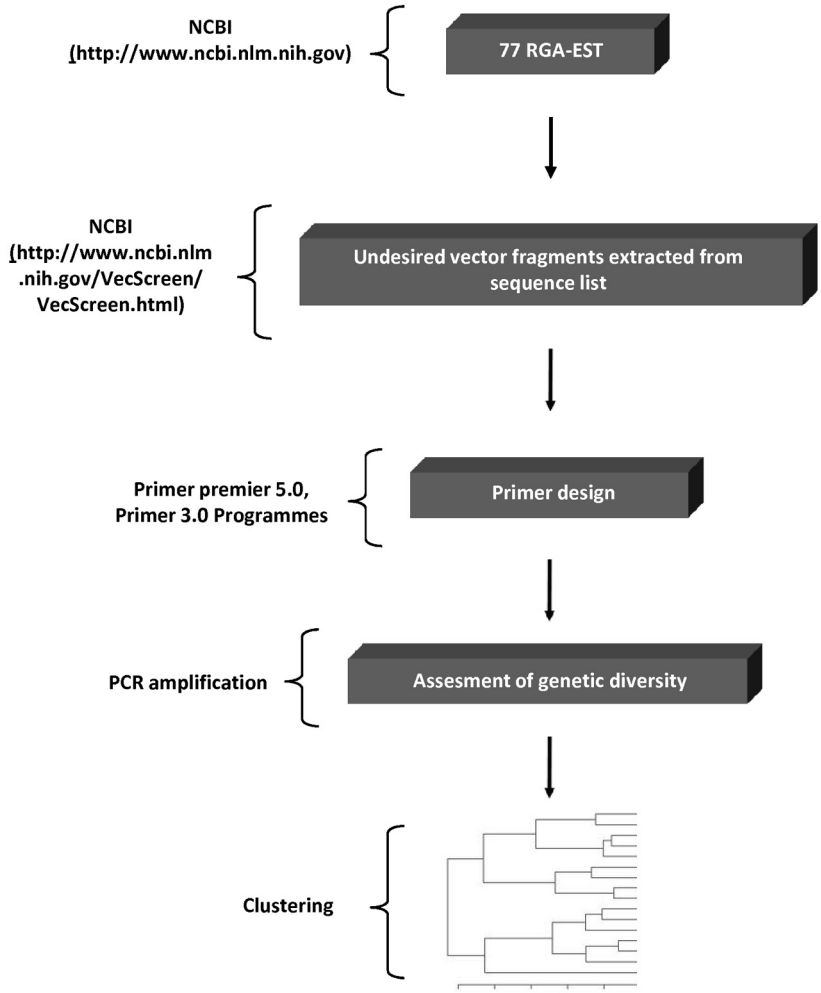


Figure 1. Schematic overview summarizing the strategy for using resistance gene analog-expressed sequence tags (RGA-ESTs) for assessment of genetic diversity.

PCR amplification conditions

Genomic DNA amplifications with the sense and antisense primers designed from RGA-ESTs specific for *T. aestivum* were performed using a PTC-100 MJ thermocycler (MJ Research, Watertown, MA, USA) in a 25 µL reaction volume; each reaction contained 1X Taq buffer (MBI Fermentas, Germany), 2.5 mM $MgCl_2$ (MBI Fermentas), 0.2 mM dNTP (MBI Fermentas), 400 nM forward primer, 400 nM reverse primer (800 nmol for RGA primers) and 0.625 U/µL Taq polymerase (MBI Fermentas) and 100 ng genomic DNA. The thermal cycling parameters were 3 min at 94°C (initial denaturation), 37 cycles of 1 min at 94°C, 1 min at 50-59°C (depending on annealing temperature) and 1 min at 72°C, followed by a final extension

at 72°C for 10 min. PCR amplification products were separated by electrophoresis on 2% TAE agarose gels and stained with ethidium bromide for visualization.

Assessment of genetic similarity

Each RGA-EST band was scored as present (1) or absent (0) for the different cultivars and the data were entered into a binary matrix as discrete variables ('1' for presence and '0' for absence of a homologous fragment). Only distinct, reproducible, well-resolved fragments were scored, and the data were analyzed using the MVSP 3.1 (Multivariate Statistical Package) program (Kovach, 1999). The MVSP software package version 3.1 was used to calculate Jaccard's (1908) similarity coefficients. Using these coefficients a dendrogram was constructed by the neighbor-joining algorithm.

RESULTS

RGA-EST polymorphism and clustering

Primers designed from RGA-EST sequences are useful for PCR-based discrimination between genotypes where differences between resistance and susceptibility are due to the presence of functional and nonfunctional R-gene homologues. A total of 77 primers were used to characterize the genetic diversity of six wheat genotypes, and 38 RGA-EST primers were polymorphic between susceptible and resistant wheat combinations. These combinations were created by crossing yellow rust tolerant (PI178383, Izgi2001 and Sonmez2001) and susceptible (Harmankaya99, ES14 and Aytin98) parents, respectively, in the wheat breeding program of the Anatolian Agricultural Research Institute. Interestingly, 6 RGA-EST primers (3-BE498831, 6-BF482358, 19-BQ753146, 34-CA681703, 35-CA725884, and 37-CA733486) of 38 gave polymorphic pattern for all combinations. The BlastX homolog of the sequences, which are the source for primer designing, was related to the NBS regions of wheat sequences. The rest of the 38 RGA-EST primers were monomorphic and only one of the RGA-EST primers (84-BJ276947) gave no amplifications in all genotypes (Figure 2). Pairwise similarity within groups, obtained by MVSP 3.1, varied from 0 to 0.500 and is summarized in Table 2. The highest similarity was 0.500 between Harmankaya99 and Sonmez2001. The lowest similarity was 0.286 between Aytin98, PI178383 and Aytin98, ES14. The dendrogram produced two main clusters, the first included the wheat cultivar Aytin98 and Izgi2001, the second main cluster was divided into two subclusters. The first subcluster comprised only PI178383. The second subcluster was also divided into two subclusters. One of them included only ES14, and the other one included Sonmez2001 and Harmankaya99. Similarity index (Jaccard's coefficient) of the tested cultivars resulting in a dendrogram presented in Figure 3, demonstrates the ability of RGA-EST to detect large amounts of genetic diversity in genotypes with expected narrow genetic pool.

A total of 77 wheat NBS-containing RGA-ESTs were compared by BlastX algorithms in the NCBI for amino acid sequences. BlastX analysis of these sequences (BE498831, BF482358, BQ753146, CA681703, CA725884, and CA733486), gave a polymorphic pattern for all combinations, and they were NBS-LRR class RGA, NB-ARC domain containing protein, NBS-type resistance protein RGC5, NBS-LRR-S/TPK stem rust resistance protein, and putative MLA1 proteins (Figure 4), while 38 RGA-EST primers produced a monomorphic

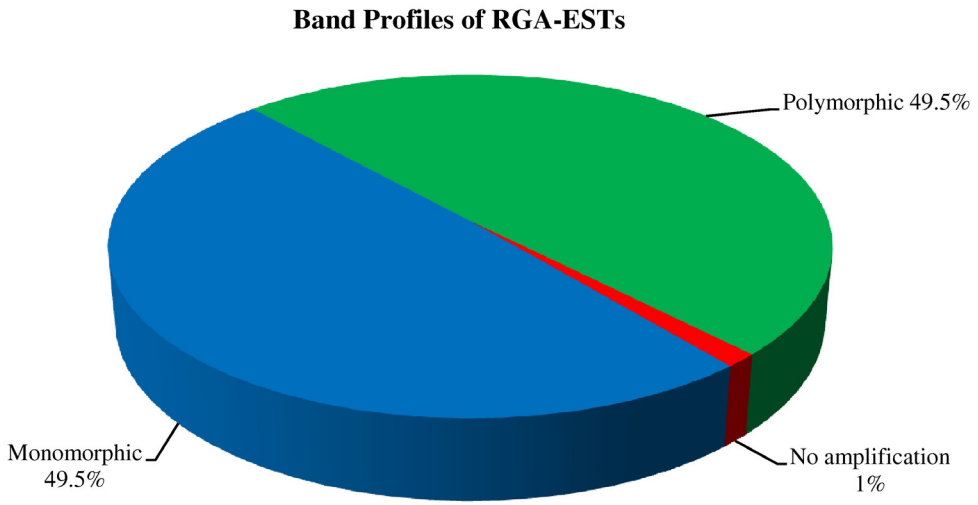


Figure 2. Schematic representation for the band profiles of resistance gene analog-expressed sequence tags (RGA-ESTs).

Table 2. Similarity index (Jaccard's coefficient) between cultivars of *Triticum aestivum*.

Pop. ID	PI178383	Izgi2001	Sonmez2001	Harmankaya99	ES14	Aytin98
PI178383	****					
Izgi2001	0.292†	****				
Sonmez2001	0.344†	0.373†	****			
Harmankaya99	0.393†	0.379†	0.500†	****		
ES14	0.393†	0.333†	0.410†	0.417†	****	
Aytin98	0.286†	0.426†	0.435†	0.333†	0.286†	****

† = genetic similarity.

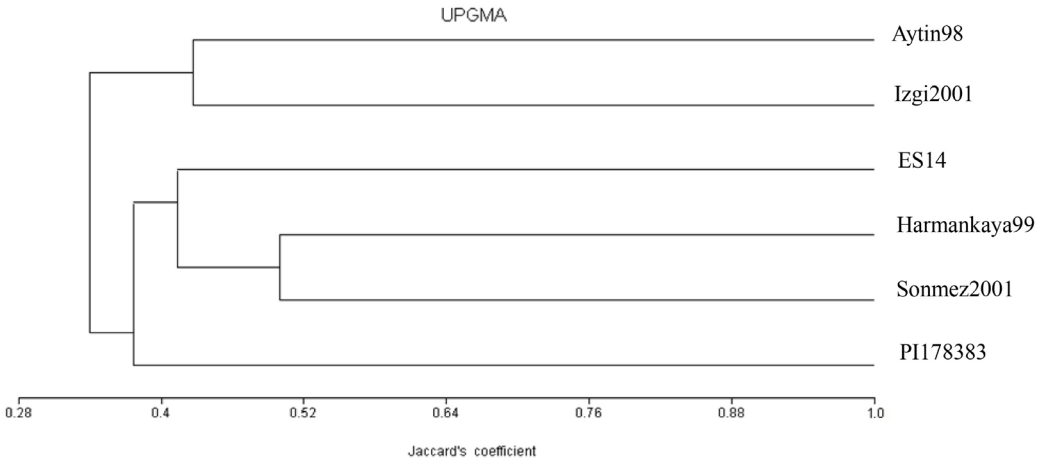
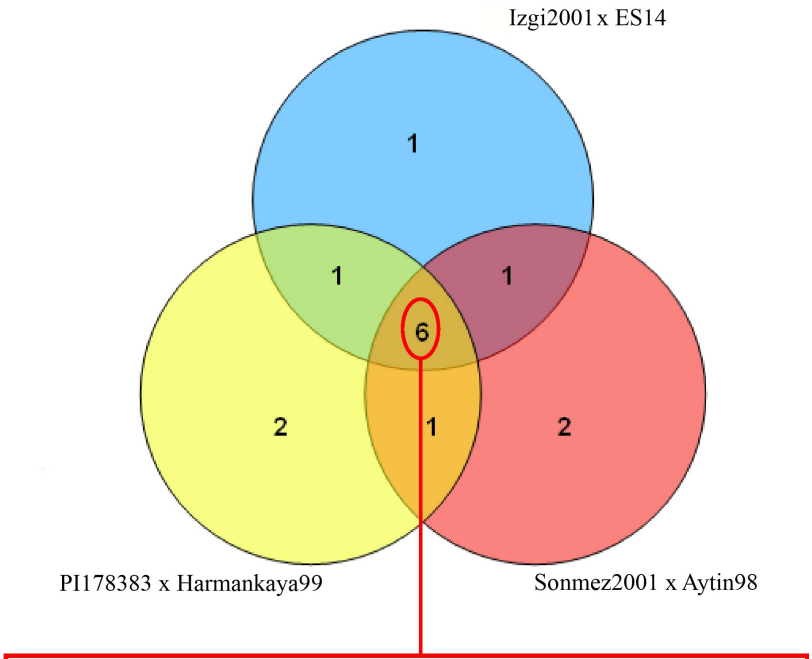


Figure 3. Genetic similarity relationships based on Jaccard's similarity coefficients after cluster analysis of bread wheat (*Triticum aestivum* L.) accessions from Turkey using RGA-EST markers.

pattern, and also the BlastX searches revealed that the blast hit homolog of the RGA-EST sequences match various organisms, such as *Oryza*, *Hordeum*, *Sorghum* (Figure 5).



RGA-accession number	Matched protein	Organism	Accession number	E-value
BE498831	NBS-LRR class RGA	*Aegilops tauschii*	AAM69850.1	6e-55
BF482358	NB-ARC domain containing protein	*Oryza sativa*	ABA92221.2	3e-45
BQ753146	NBS-type resistance protein RGC5	*Musa acuminata*	ACF21695.1	2e-54
CA681703	NBS-LRR-S/TPK stem rust resistance protein	*Hordeum vulgare*	ACH69774.1	3e-28
CA725884	putative MLA1	*Oryza sativa*	BAD28289.1	1e-19
CA733486	NB-ARC domain containing protein	*Oryza sativa*	ABA92221.2	8e-20

Figure 4. BlastX homologs of resistance gene analog-expressed sequence tag (RGA-EST) sequences, which gave polymorphic patterns for all combinations.

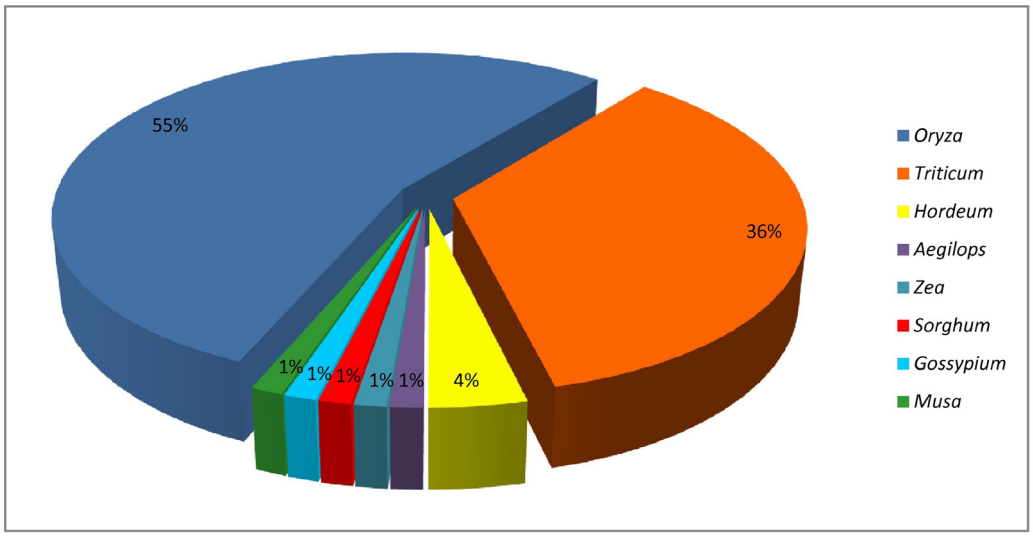


Figure 5. Matched organisms of RGA-EST sequences based on BlastX analyses.

DISCUSSION

The mapped RGAs provide potentially powerful tools for the development of markers for resistance traits, and for the cloning of NBS-LRR-type resistance genes. This could include genes for broad-spectrum, qualitative disease resistance, because recent studies in different plant species (Wisser et al., 2005; McIntyre et al., 2005) found that in some instances there was a significant association between RGA genes and quantitative resistance traits. In previous studies, several RGAP markers were coincident with resistance to different diseases (Chen et al., 1998, 1999). Shi et al. (2001) identified 16 RGAP markers for the Yr9 gene resistance to wheat stripe rust, and they determined the presence or absence of the Yr9 gene in cultivars that have been postulated to have Yr9. Similar to these studies, resistant and susceptible wheat genotypes used in this study have already been used for the development of molecular markers for yellow rust resistance by our research group. Along this line, Temel et al. (2008) investigated the sequence differences of yellow rust resistance gene "Yr10" in seven winter-type bread wheat genotypes, and data mining proved that there have been single nucleotide changes especially in the second exon of Yr10. The sequences most similar to the first exon of Harmankaya99, Izgi2001 and Sonmez2001 are AF509535 (*Aegilops tauschii* NBS-LRR-like gene), AF509534 (*A. tauschii* NBS-LRR-like gene sequence) and AF509534, respectively. In another study from our group, Akfirat-Senturk et al. (2010) used bulk segregant analysis to identify molecular markers associated with yellow rust disease resistance in Izgi2001 x ES14 cross. This analysis showed that 81% of the wheat genotypes known to be yellow rust resistant had the SSR marker (*Xgwm382*). Similar to this, one EST-SSR marker (*Pk54*) has been identified in a PI178383 x Harmankaya99 cross. It was present in the resistant parent and resistant F_2 hybrids but not in the susceptible ones. 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, showed the presence of *Pk54* (Ercan et al., 2010), further suggesting that the presence of these markers correlates with yellow rust resistance in diverse wheat germplasms. Based on the studies mentioned above, RGA-ESTs were used in order to identify genetic diversity between parents and to search for new possible new crosses by using these 6 genotypes for Turkish wheat breeding programs in the frame of this study.

The amount and distribution of genetic diversity within a species must be known if scientific approaches to its conservation and exploitation are to be developed. Methods employing DNA-based markers are currently used to study diversity at the nucleotide level. Among these, PCR-based methods such as RAPD (Williams et al., 1990), AFLP (Vos et al., 1995), and microsatellites (Akkaya et al., 1992) have proven to be useful in many plant species. All of these PCR-based markers have been generated without concern for their function. Given the large size of the wheat genome, these markers mostly reflect variation at non-coding DNA regions. The growing information in databases on plant gene sequences makes it possible to develop universal molecular tools directed at particular targets, i.e., either specific genes or specific genome regions containing clusters of genes with known function. Sequence comparisons among disease resistance genes from different plants have revealed remarkable similarities in their general structure and in the conservation of specific domains that participate in protein-protein interactions and signal transduction (Staskawicz et al., 1995). PCR primers based on conserved peptide motifs have been used to amplify RGA sequences in a large number of plant species (Feuillet et al., 1997; Michelmore, 2000; Pan et al., 2000). It has been reported in different species that about 50% of the products amplified with primers based on motifs of the NBS domain of several R-genes cannot be considered RGAs (Collins et al., 1998; Fourmann et al., 2001). However, in our study, the BlastX analysis of wheat RGA sequences showed that all wheat RGA sequences were related to R genes. Sicard et al. (1999) explored resistance-gene diversity in cultivated and wild populations of *Lactuca* using two molecular markers derived from LRR domains, and a microsatellite also present in the main resistance gene cluster in lettuce. These three markers produced similarly high levels of diversity and estimates correlated across populations. Several other studies have reported polymorphism in self-pollinating plants, including rice (22%) (Maheswaran et al., 1997), sugar beet (50%) (Schondelmaier et al., 1996) and wild barley (76%) (Pakniyat et al., 1997).

Our results indicate that EST-derived RGA primers are good tools for assessing genetic diversity in wheat cultivars. A relatively high level of polymorphism (49.5% of loci were polymorphic) was observed with 77 RGA primers across the six wheat genotypes, despite the fact that all of them were local cultivars from geographically close locations. In conclusion, RGA-EST sequences can be used to identify suitable parents in population studies designed to detect genes related to disease resistance.

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