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RESEARCH ARTICLE

The utility of NBS-profiling for characterization of yellow rust resistance in an F_6 durum wheat population

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Abstract. Seedling and adult plant (field) resistance to yellow rust in the durum wheat (*Triticum turgidum* ssp. *durum*) cross Kunduru-1149 x Cham-1 was characterized using a functionally-targeted DNA marker system, NBS-profiling. Chi-squared analysis indicated a four gene model conferring seedling yellow rust resistance against *Puccinia striiformis* f. sp. *tritici* isolate WYR85/22 (virulent on *Yr2*, *Yr6*, *Yr7* and *Yr9*). Interval mapping located two QTL for yellow rust resistance on the long arm of chromosome 1B, while Kruskal–Wallis single marker regression identified a number of additional marker loci associated with seedling and/or adult plant, field resistance to yellow rust. These results suggested that much of the yellow rust resistance seen in the field may be due to seedling expressed resistance (R) genes. Characterization of the DNA sequence of three NBS marker loci indicated that all showed significant homology to functionally-characterized R-genes and resistance gene analogues (RGAs), with the greatest homology being NBS-LRR-type R-genes and RGAs from cereal species.

Keywords. disease resistance; durum wheat; NBS-profiling; quantitative trait loci analysis; yellow rust; *Puccinia striiformis*.

Introduction

About half of the agricultural land in Turkey is devoted to wheat production. In a more marginal wheat growing areas, durum wheat is preferred because of its drought tolerance (Göçmen 2001). In these economically poorer regions, the deployment of disease resistance genes is the only economically viable option of disease control. Cultivated durum wheat is an allotetraploid (*Triticum turgidum* ssp. *durum*; AABB, $2n = 28$) species originating from *T. turgidum* ssp. *dicoccoides*, a wild, emmer wheat, following the selection of free-threshing genotypes.

The rust diseases are a major constraint to wheat production in Turkey, as seen in all wheat growing regions of the world (Göçmen 2001; Marris 2009). Among the rust diseases, yellow rust of wheat, also known as stripe rust (*Puccinia striiformis* f. sp. *tritici*) is by far the most important disease causing yield losses in all parts of the country. A significant yellow rust epidemic occurs once or twice every decade, often more frequently (Çetin *et al.* 2000; Göçmen 2001; Chen 2005; Gilbert 2010). Since the year 2000, the central and west regions of Asia (including Turkey) has been plagued by severe epidemics of yellow rust, due to new virulent isolates of the fungus emerging (Ziyaev *et al.* 2011).

It is estimated that most of the wheat genome is non-transcribed, consisting of transposons and duplicated pseudogenes, with ~5% genes often present in clusters (Dilbirligi and Gill 2003; Gill *et al.* 2004; Feuillet and Eversole 2007). The genomic organization of wheat therefore consists of small, gene-rich regions interspersed with large, gene-poor regions mostly made up of repetitive DNA (Dilbirligi *et al.* 2004; Charles *et al.* 2008). DNA marker systems that randomly target the genome therefore stand a high probability of falling within a noncoding region of the wheat genome. Alternative approaches, using known

transcribed sequences to develop DNA markers allows the gene rich regions of the genome to be targeted (van der Linden *et al.* 2004).

The majority of cloned resistance genes (R-genes) encode for proteins with a nucleotide-binding site (NBS) domain and a series of leucine-rich repeat residues (LRR). The NBS domain is composed of a number of motifs which are highly conserved and considered unique to NBS-type R-genes, with the kinase 1a (p-loop), kinase 2 and the putative kinase 3a being the best conserved motifs (Meyers *et al.* 1999; Dilbirligi and Gill 2003; Mun *et al.* 2009). The NBS-LRR-type R-genes, which are present in both monocot and dicot plant species, tend to be present as multigenic families along with resistance gene analogs (RGAs) (Hammond-Kosack and Jones 1997; Meyers *et al.* 1999; Ellis *et al.* 2000; Zhang *et al.* 2007; Miller *et al.* 2008; Ellen *et al.* 2009).

Using the search string 'NBS-LRR' the Chinese Spring RefSeq genome (International wheat genome sequencing consortium *et al.* 2018) and the Svevo, durum wheat RefSeq genome sequences reveal over 3600 and 3100 open reading frames annotated as potential NBS-LRR-type R-genes, respectively. While many of these may not be functional, the co-localization of R-genes and RGAs means that markers that target the conserved NBS-motifs have a higher probability of targeting genomic regions containing functional R-genes (Ayliffe and Lagudah 2004; Dilbirligi *et al.* 2004; Liu and Ekramoddoullah 2007; Maccaferri *et al.* 2019).

NBS-profiling (van der Linden *et al.* 2004) is a PCR-based marker system that screens for polymorphisms associated with conserved NBS-motifs. In potato, 50–90% of NBS-profiling fragments were found to be linked to disease R-loci and RGA sequences (Ayliffe and Lagudah 2004). NBS-profiling therefore provides a functionally-targeted marker system that enables the rapid identification of disease resistance genes, being particularly useful with large, complex plant genomes such as polyploid wheat.

The durum wheat variety Kunduru1149 has long been favoured by Turkish farmers for its suitability to arid conditions, but it lacks yellow rust resistance (Göçmen 2001). At ICARDA in Syria a recombinant inbred lines (RIL) population (F_6) was developed by crossing Kunduru-1149 with Cham-1, a durum variety known for its yellow rust field resistance. This material was developed as part of a breeding program to improve yellow rust resistance in the Kunduru 1149 background (Göçmen *et al.* 2003). The field resistance in this RIL population was previously characterized by Göçmen *et al.* (2003).

The objective of the current study was to characterize the molecular basis of the seedling and adult plant resistance to yellow rust in the Kunduru-1149 x Cham-1 RIL (F_6) durum wheat population using NBS-profiling as a potentially powerful tool for molecular resistance characterization.

Materials and methods

Plant material

The F_6 durum wheat RIL population used in this study was developed at the International Centre for Agricultural Research in the Dry Areas (ICARDA, Aleppo, Syria) by Dr. M. Nachit and was provided by the Central Research Institute for Field Crops (CRIFC; Ankara, Turkey). The population was developed from a cross between the yellow rust resistant durum wheat Cham-1 and the yellow rust susceptible Kunduru-1149 selection (Nachit *et al.* 2001; Göçmen *et al.* 2003). The population, consisting of 150 RILs was previously tested for yellow rust resistance in the field at CRIFC against a *P. striiformis* field population (showing virulence for *Yr2*, *Yr6*, *Yr7* and *Yr9*) (Göçmen *et al.* 2003).

Yellow rust disease assessment – seedling tests

Seed of each of the 150 RILs and parents were pre-germinated in the dark at 25°C. Three days after sowing, five germinated seeds were transferred into John Innes number-2 compost, in 7x7 cm pots. The plants were grown in a spore-free greenhouse, with a 16/8 hour light/dark cycle supplemented with sodium lighting ($300\mu m\ m^{-2}\ s^{-1}$) at 18°C during the day and 15°C during the night (Boyd and Minchin 2001). The International and European wheat differential sets and the Avocet near-isogenic lines (NILs) for yellow rust (provided by C.R. Wellings, Plant Breeding Institute, Cobbitty, Australia) were included as controls. Fourteen day old seedlings were inoculated with the *P. striiformis* isolate WYR 85/22 (avir/vir 1,3,4,5,8,10,15,17/2,6,7,9). The inoculation procedure was as described by Boyd and Minchin (2001). Disease reactions were scored 14 days after inoculation using a modification of the Plant Breeding Institute, Cobbity, Australia descriptive infection type (IT) scale (table 1). For further analysis, the descriptive IT scale was converted to numerical values, ranked according to the phenotype of the plant resistance response (IT nominal; Rodrigues *et al.* 2004). The greater the resistance response is (i.e. small versus large necrotic areas and small versus large uredia) the lower the numerical value (tables 1 and 2).

Yellow rust disease assessment – field tests

Seed of the 150 RILs and the two parents were grown, in three replicates, in a disease nursery at the CRIFC in Ankara-Yenimahalle in 1999/2000 (Göçmen *et al.* 2003). Yellow rust infection was measured on each RIL using the modified Cobb Scale. With this scale infection severity was evaluated from 1 to 100% and the following nomenclature used for the reaction type (RT); 0: no visible infection; R: Resistant, having necrotic areas with or without small uredia,

Table 1. IT scale used to score yellow rust infection on wheat seedlings and corresponding numerical IT nominal used for genetic mapping.

IT	Disease reaction	IT nominal
0	No visible symptoms	1
;	Small necrotic flecks	1
n;	Necrotic regions greater than 1mm in diameter	2
0^{n}	Necrotic regions greater than 2mm in diameter	3
0^{nn}	Spreading necrotic regions greater than 4mm in diameter	4
1	Small, sporulating uredia surrounded by necrotic tissue	5
2	Moderately sized, sporulating uredia surrounded by necrotic tissue	6
3	Moderately sized, sporulating uredia surrounded only by chlorotic tissue (susceptible reaction)	7
4	Large, sporulating uredia surrounded by green tissue (susceptible reaction)	7
c	Chlorotic tissue associated with uredia	

MR: Moderately Resistant, having small uredia surrounded by necrotic areas, MS: Moderately Susceptible, having medium-sized uredia, no necrosis, but some chlorosis possible, S: Susceptible, having large uredia, no necrosis or chlorosis. The RT was converted to a numerical value using the conversions; R = 0.2, MR = 0.4, MR-MS = 0.6, MS = 0.8, MS-S = 0.9, and S = 1.0. Coefficient of infection (CoI) values were calculated for each RIL by multiplying the reaction intensity score by the reaction type numerical value (table 2) (Göçmen *et al.* 2003).

DNA extraction and marker analysis of yellow rust resistance

The 150 RILs were assessed based on their reaction to *P. striiformis* isolate WYR85/22. This produced four phenotypic categories. A subpopulation of 53 lines were randomly selected which represented the four categories and maintained the same ratio of lines within each category. This subpopulation, along with the parents was used for DNA extraction and marker analysis. DNA was isolated from leaf tissue of uninfected 14 day-old seedlings using a modified protocol that combined the protocols for DNeasy 96 Plant kit and the DNeasy Plant Mini kit (Tufan 2006). To determine chromosomal locations, the parents Kunduru 1149 and Cham-1 were screened with 75 SSR markers, including GWM (Röder *et al.* 1998), BARC (Song *et al.* 2002) and WMC (Edwards *et al.* 1996) wheat SSR markers. The SSR markers were selected, aided by the wheat consensus maps (Röder *et al.* 1998; Elouafi *et al.* 2001 and www.graingenes.org) to represent each chromosome arm of the A and B genomes. Out of 75 SSR markers screened, 33 polymorphic SSR markers were selected, representing at least one marker per chromosome arm.

For NBS-profiling, the protocol of van der Linden *et al.* (2004) was used. Genomic DNA was digested with the restriction enzyme *Mse*I. Selective NBS primers NBS2 (targets the conserved p-loop/kinase-1a motif) and NBS5 (kinase-2 motif), plus a modified NBS2 primer, NBS3 (Calenge *et al.* 2005) were used to amplify the target DNA along with the adapter primer (van der Linden *et al.* 2004). NBS2 and 3 were designed to amplify DNA 5′ of the targeted NBS-motif, while NBS5 amplifies 3′.

Both polymorphic SSR markers and NBS-profiling PCR products were separated on 5% denaturing polyacrylamide gels (19:1 acrylamide/bisacrylamide) and visualized by silver staining (Boyd *et al.* 2002). Polyacrylamide gels were run in 1 x TBE buffer (Seven Biotech Ltd) for 90 to 120 min at 80 Watts. To perform silver staining, polyacrylamide gels were passed through the following steps; fixation for 30 min 10% acetic acid; washed in ddH_2O for 10 min; stained in silver nitrate solution (0.0057N silver nitrate plus 0.15% (v/v) formaldehyde) for 30 min; and placed in developer (0.73M sodium carbonate, 0.15% (v/v) formaldehyde plus 0.015N sodium thiosulphate), chilled to 4°C, until bands were clearly visible. The reaction was then stopped by adding 10% acetic acid to the developer.

The parents and each RIL of the subpopulation were screened with the polymorphic SSR markers and the three NBS primers. For SSR markers polymorphism is detected based on a shift in the size of the bands amplified in each parent. The allele contributed by the susceptible parent, Kunduru-1149 was designated as the ‘a’ allele, while the allele contributed by the resistant parent, Cham-1 was designated as the ‘b’ allele. NBS-profiling bands are dominant, so the RIL will either show a band representing an allele from one of the parents, or no band, as in the other parent. Bands present in the resistant parent were designated ‘c’, the null allele (absence of the band in the susceptible parent) being designated ‘a’. Bands present in the susceptible parent, but absent from the resistant parent were designated ‘d’, the null allele (no band) being designated ‘b’.

Scores from SSR and NBS-profiling marker analyses were entered into JoinMap version 5.0 for Windows (Kyazma; Stam and Ooijen 1995). Linkage groups were determined using a minimum LOD score of 3.0 and a maximum recombination frequency (REC) of 0.45. The recombination values were converted into genetic distances using the Kosambi mapping function. Linkage groups formed in JoinMap, along with phenotypic data and marker scores were entered into the QTL mapping program MapQTL version 5.0 for Windows (Kyazma). MapQTL was used to locate QTLs for resistance to yellow rust in the Kunduru-1149 x Cham-1 subpopulation using the Kruskall–Wallis and interval mapping (IM) functions. Kruskall–Wallis is a nonparametric test in which no assumptions are made about the probability distribution of the quantitative trait (Rodrigues *et al.* 2004). For QTL mapping an imposed significance value of 5% gave an upper LOD significance threshold of 1.7 following a permutation analysis (1000 permutations, MapQTL).

Table 2. Seedling and adult plant, field yellow rust infection phenotypes of parents and 150 RILs of the Kunduru-1149 x Cham-1 cross.

Line	IT	IT Nom	CoI	Line	IT	IT Nom	CoI	Line	IT	IT Nom	CoI	Line	IT	IT Nom	CoI
1[a]	;/0$^{n/c}$ to 3	3.60	10.13	40	3/4	7.00	17.50	79[b]	0[c]	2.10	3.47	118[a]	; to 3	2.20	2.27
2	; to 1	1.60	4.47	41	;	1.00	1.33	80[b]	;	1.00	0.13	119	0$^{n/c}$ to 1/2	3.80	10.25
3[a]	;/0$^{n/c}$ to 3/4	3.70	3.90	42[a]	;/0$^{n/c}$ to 2	2.10	19.25	81	;	1.00	1.47	120[b]	0$^{n/c}$ to 1/2	4.30	16.00
4[a]	;/0$^{n/c}$ to 2	2.20	1.47	43[b]	;	1.00	0.13	82[a]	0$^{n/c}$ to 3/4	4.90	1.80	121	;/0$^{n/c}$ to 1/2	3.30	21.00
5	;	1.00	0.13	44	;	1.00	0.13	83	;	1.00	7.13	122[a]	; to 2/3	3.00	15.33
6	;	1.00	0.00	45[b]	;	1.00	0.27	84	2/3	5.70	13.13	123[b]	;	1.00	0.00
7[b]	;	1.00	0.00	46	0$^{n/c}$ to 2	3.30	21.15	85	;	1.00	10.15	124	1 to 2	4.00	19.42
8[b]	;	1.00	0.00	47[b]	;	1.00	0.13	86[b]	;	1.00	0.20	125[a]	;/0$^{n/c}$ to 3/4	2.80	25.50
9[b]	;	1.00	0.00	48	;/0$^{n/c}$	1.80	5.90	87[a]	; to 3/4	5.10	3.25	126	0$^{n/c}$ to 2	4.30	50.00
10	;	1.00	0.00	49[b]	;/0$^{n/c}$	1.20	0.00	88[b]	;/0$^{n/c}$	1.60	6.00	127[b]	2 to 3	6.50	34.00
11[b]	0$^{n/c}$/1	3.00	6.73	50	;	1.00	0.00	89[b]	;/0$^{n/c}$	2.10	8.53	128[a]	0$^{n/c}$ to 2/3	4.25	17.33
12	;	1.00	4.27	51[b]	0$^{n/c}$ to 1	3.20	17.58	90[a]	; to 2/3	3.80	4.33	129	0$^{n/c}$ to 2	3.70	7.33
13[b]	0$^{n/c}$	1.90	21.00	52	;	1.00	0.27	91	;	1.00	18.48	130[a]	; to 3/4	2.00	0.00
14	1 to 2	4.20	9.75	53[b]	2 to 4	6.30	33.00	92[a]	;/0$^{n/c}$ to 1/2	2.00	3.23	131[b]	2 to 3	5.80	64.67
15	;	1.00	0.00	54	;	1.00	0.13	93	1 to 2	4.50	5.60	132	;	1.00	0.00
16	;	1.00	0.00	55	;	1.00	0.00	94[b]	0$^{n/c}$	2.00	4.33	133	1 to 2	4.50	14.43
17[a]	0$^{n/c}$ to 3/4	3.90	22.30	56	1 to 3	5.20	21.00	95	;	1.00	1.13	134	1 to 2	4.20	22.50
18[b]	;	1.00	0.00	57	;	1.00	1.50	96[b]	;	1.00	0.13	135[b]	3/4	7.00	18.75
19[b]	3/4	7.00	37.50	58	1 to 3	5.10	30.00	97[b]	3/4	7.00	16.68	136	1 to 2	5.50	21.33
20[a]	0$^{n/c}$ to 3	4.20	3.40	59	;	1.00	0.00	98	;	1.00	6.67	137	1 to 2	4.20	6.65
21	0$^{n/c}$ to 1	3.40	24.33	60	;	1.00	0.27	99[b]	;	1.00	0.13	138	;	1.00	3.77
22	3/4	7.00	7.33	61[b]	3/4	7.00	11.40	100[b]	;/0$^{n/c}$	1.30	0.13	139	0$^{n/c}$ to 2	3.60	26.17
23[b]	2 to 3	6.20	11.83	62[b]	;	1.00	0.00	101	;	1.00	0.00	140[b]	3/4	7.00	15.33
24	0$^{n/c}$ to 1	3.10	8.98	63[a]	; to 1/2	3.20	7.00	102	;	1.00	5.20	141[b]	0[c]	2.00	0.00
25	;	1.00	0.30	64	2/3	5.50	8.85	103[b]	;/0$^{n/c}$ to 1	1.80	12.47	142	;/0$^{n/c}$	1.20	0.40
26[b]	;	1.00	0.13	65[a]	; to 1/2	3.30	4.90	104	0$^{n/c}$ to 1	3.00	7.37	143	;	1.00	0.67
27	1/2 to 3	4.70	19.00	66[a]	; to 1/2	1.75	16.13	105	0$^{n/c}$	3.00	2.65	144[a]	0$^{n/c}$ to 3/4	3.60	18.00
28[b]	;	1.00	0.00	67	2 to 3/4	6.10	27.83	106	;	1.00	4.58	145	0$^{n/c}$ to 2/3	5.50	13.08
29[b]	;/0[c]	2.00	0.00	68[b]	;	1.00	0.00	107	0$^{n/c}$ to 2	4.30	4.47	146	;	1.00	0.53
30	3/4	7.00	2.73	69	; to 2	4.00	8.13	108[b]	;	1.00	0.00	147	0$^{n/c}$ to 1	3.40	18.83
31[b]	;/0$^{n/c}$	1.70	3.20	70[b]	; to 2	3.20	7.33	109[b]	;/0$^{n/c}$	1.80	0.73	148[b]	0[c]	2.00	0.00
32	0$^{n/c}$	1.80	16.08	71[b]	2/3	6.30	10.00	110[b]	;	1.00	0.13	149	0[c]	2.00	0.87
33	;	1.00	0.40	72[b]	3/4	7.00	22.50	111[b]	1/2 to 3	5.40	11.05	150	;	1.00	0.40
34[b]	3/4	7.00	10.33	73	;	1.00	0.00	112[b]	; to 1/2	2.20	11.25				
35[b]	;/0$^{n/c}$	2.30	19.10	74	;	1.00	1.60	113	;	1.00	4.00	151[b]	2 to 3	5.30	60.00
36	;/0$^{n/c}$	1.30	0.27	75[b]	1 to 3	5.70	21.92	114	; to 1	1.70	0.40	152[b]	;	1.00	0.00
37[a]	; to 2	2.30	58.00	76	1 to 3	5.70	27.75	115[b]	;	1.00	0.00				
38	;/0$^{n/c}$	1.20	0.13	77[a]	; to 3/4	3.90	14.40	116[b]	;	1.00	1.00				
39[a]	0$^{n/c}$ to ¾	5.10	2.27	78	;	1.00	0.13	117	0$^{n/c}$ to 1/2	3.70	11.47				

IT, seedling infection type score; IT Nom, average seedling infection type nominal from five seedlings; CoI, coefficient of Infection of adult plant, field scores (Göçmen *et al*. 2003); 151, Kunduru-1149; 152, Cham-1.

[a] Lines segregating for seedling yellow rust infection phenotypes.

[b] Lines selected for marker analysis.

[c] Chlorosis also visible.

Statistical analysis of yellow rust resistance

Chi-squared (χ^2) analysis was used to predict the number of genes conferring seedling yellow rust resistance in the Kunduru-1149 x Cham-1 cross against *P. striiformis* isolate WYR85/22. The models for 1 gene (1R:1S), 2 genes (3R:1S), 3 genes (7R:1S), 4 genes (15R:1S) and 5 genes (31R:1S) segregating within a recombinant inbred population were tested. A correlation analysis was carried out comparing the seedling IT nominals to the adult plant CoI values using Genstat (version 7.0 for Windows).

Sequence analysis of polymorphic NBS bands

NBS bands were excised from the parent that donated the band and one line that carried the band. NBS bands were cut from the polyacrylamide gel and left in 100 μL 1X TE overnight at 4°C. The gel slice was heated at 50°C for 10 min and centrifuged at 16,000 rpm for 5 min. The supernatant was re-amplified using the same primers and PCR conditions as used for the exponential PCR step of the NBS-profiling protocol (van der Linden *et al.* 2004; Calenge *et al.* 2005). PCR products were cloned into the pGEM-T Easy vector (Promega) as described by Smith *et al.* (2002). Sixty white recombinant colonies of each cloned band (30 from the parental donor and 30 from the positive RIL) were selected. Plasmids were isolated using the Wizard *Plus* SV Minipreps DNA purification system (Promega). The size of the insert in each recombinant plasmid was confirmed by T7-Sp6 PCR amplification (Smith *et al.* 2002). Single strand confirmation polymorphism (SSCP) analysis (Hayashi 1991) was carried out on plasmid insert DNA to identify a predominant clone type (Smith *et al.* 2002).

The DNA inserts from 12 recombinant plasmids of each cloned NBS band were sequenced using the BigDye (Applied Biosystems) sequencing reaction system with the T7 primer. Sequence reactions were read using an ABI 3700 automated sequencer (Applied Biosystems, USA). All 12 sequences were analysed using the Gap 4 program (version 4.10) (Bonfield *et al.* 1995). DNA sequences obtained from the same cloned NBS band, but displaying less than 95% homology were considered distinct sequences and removed from further analysis. A consensus sequence was constructed for each NBS band from the contig of the remaining sequences. The consensus sequence was *blastx* (nucleotide query – protein database; Altschul *et al.* 1997) searched for homology to other sequences held within the NBCI databases (www.ncbi.nlm.nih.gov).

Results

Genetic assessment of seedling and adult plant (field) yellow rust resistance in the durum wheat Kunduru-1149 x Cham-1 cross.

The 150 RILs of the Kunduru-1149 x Cham-1 population were tested for seedling yellow rust resistance using the *P. striiformis* isolate WYR 85/22 (virulent for *Yr2*, *Yr6*, *Yr7*, *Yr9*). Isolate WYR 85/22 was chosen because its virulence profile represented that of the isolate mixture used in field tests to determine adult plant yellow rust resistance in the Kunduru-1149 x Cham-1 RIL population (Göçmen *et al.* 2003). Ten lines were fully susceptible as seedlings to isolate WYR85/22, having IT3/4, the remaining 140 lines showing some degree of resistance (table 2). Chi-squared analysis gave an optimum fit to an expected ratio of 15R:1S (χ^2 = 0.044, df = 1), which for a homozygous, RIL population indicates a four resistance gene model.

Kunduru-1149, the yellow rust susceptible parent, was not fully susceptible in seedling tests, with 23 of the lines being more susceptible than Kunduru-1149 (table 2). This was not observed in the field tests, where only one line was slightly more susceptible than Kunduru-1149. This would indicate residual seedling resistance in Kunduru-1149 towards isolate WYR85/22. Seedling yellow rust IT nominals were compared to the adult plant CoI values obtained by Göçmen *et al.* (2003) (figure 1). Some of the lines deviated from the predicted linear correlation (r = 0.60), being susceptible at the seedling stage, but resistant to yellow rust in the field, indicating resistance segregating in this cross that was not detected by isolate WYR85/22.

Genetic marker analysis of seedling and adult plant (field) yellow rust resistance in the durum wheat Kunduru-1149 x Cham-1 cross

Within the Kunduru-1149 x Cham-1 RIL population 22 lines (14.6%) were segregating for seedling yellow rust resistance, indicating residual heterogeneity within those lines (table 2). These lines were therefore omitted from the marker analysis. To concentrate the marker analysis to those regions of the wheat genome having a higher probability of containing functional R-genes, NBS-profiling was applied (van der Linden *et al.* 2004). To optimize the NBS-profiling system, and to test its utility in a cross population, the marker analysis was carried out in a randomly selected, 53 RIL subpopulation of the Kunduru-1149 x Cham-1 cross (figure 2). However, to ensure optimal genetic variation within this subpopulation all disease phenotypes were equally represented, comparable to the original population.

Three NBS primers; NBS2, NBS3 and NBS5 were screened against Kunduru-1149 and Cham-1 using the restriction enzyme, *Mse*I (van der Linden *et al.* 2004; Calenge *et al.* 2005). NBS-profiling produces dominant markers, with bands being present in one parent and absent in the other. Polymorphic bands, present in Kunduru-1149 and Cham-1 were scored. In total, 14 polymorphic NBS bands were selected for mapping in the subpopulation. Five bands originated from NBS2 (3.8% polymorphism), six from NBS3 (5.0% polymorphism) and three from NBS5

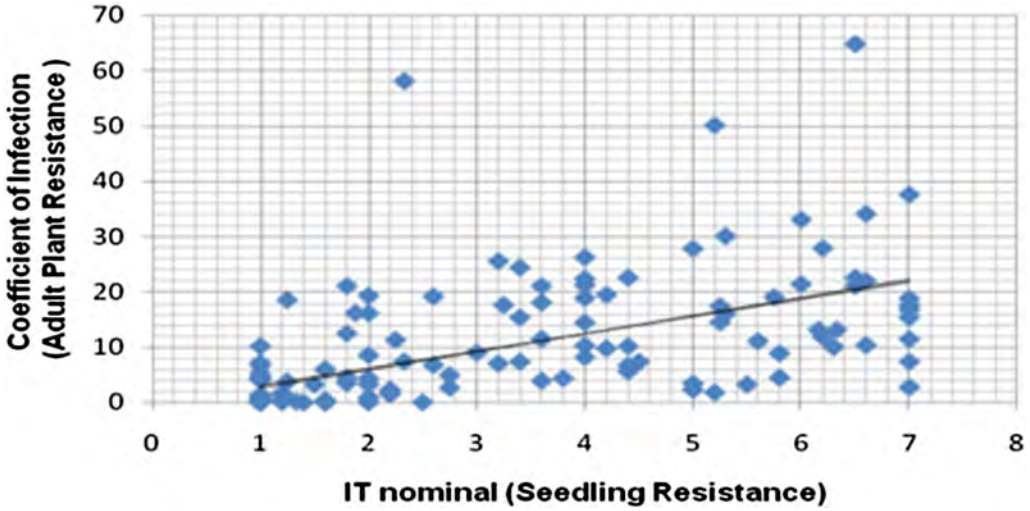

Figure 1. Correlation between seedling IT nominal scores and adult plant, coefficient of infection values of yellow rust infection in the Kunduru-1149 x Cham-1 population. The linear correlation has an r-value = 0.60.

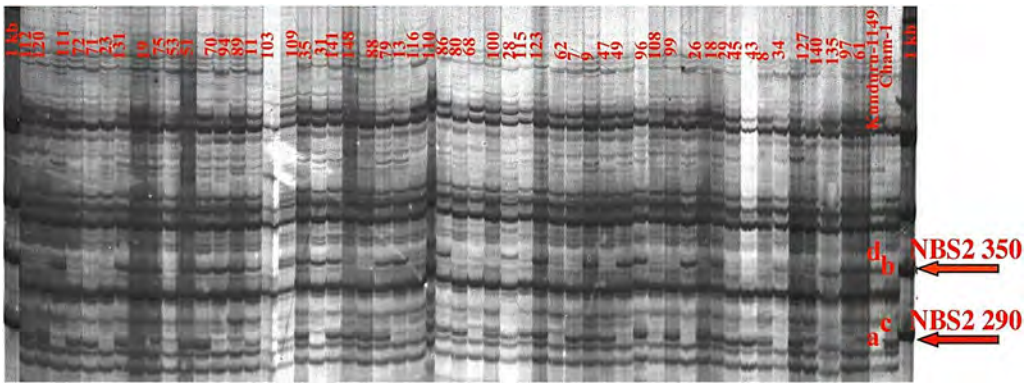

Figure 2. NBS2-*Mse1* primer profile of the 53 RILs and parents of the Kunduru-1149 x Cham-1 cross. The two polymorphic bands, NBS2-290 and NBS2-350 are highlighted.

(2.5% polymorphism), all in combination with *MseI*. NBS-*MseI* band profile's ranged in size from 100 to 800 bps (figure 2).

A and B genome SSR markers were used to identify the chromosomal locations of yellow rust resistance loci identified within the cross. Seventy-five SSR markers were screened against the parents Kunduru-1149 and Cham-1, of which 39 were polymorphic (52%). Thirty-three of these polymorphic markers were successfully screened in the subpopulation, providing an SSR marker locus for nearly all chromosome arms. Six linkage groups were formed using a mapping LOD threshold of 3.00 (figure 3). Linkage group 1: The four NBS marker loci, NBS2-350, NBS2-750, NBS3-350 and NBS3-800 formed a single linkage group. Linkage group 2: three SSR markers (BARC188, GWM268 and GWM153) assigned to the long arm of chromosome 1B amplified loci linked to the NBS locus NBS3-290. Linkage group 3: loci of the SSRs GWM297 (7BS) and GWM333 (7BL) linked with the NBS marker locus NBS2-290. Linkage group 4: the SSR GWM526 (2BL) identified a locus linked with NBS locus NBS2-325. Linkage group 5 consisted of SSR loci on chromosome 3A, *Xgwm376* (3AS) and *Xgwm391* (3AL). Linkage group 6: the two 1BL SSRs, BARC181 and WMC156 (Somers *et al.* 2004), while remaining unlinked to the 1BL SSR marker loci forming Linkage group 2, did link together, forming Linkage group 6.

The seedling and adult plant, field yellow rust infection phenotypes were analysed separately using the Kruskal–Wallis and IM functions in MapQTL (version 5.0 for Windows). Two significant QTL associated with yellow rust resistance (LOD values above the LOD threshold of 1.7) were identified on the long arm of chromosome 1B using the seedling yellow rust data set (figure 4). The QTL of biggest effect, explaining 25% (LOD=2.11) of the phenotypic variation segregating in the Kunduru-1149 x Cham-1 cross lay on Linkage group 2, between loci *Xgwm153* and NBS3-290. This QTL was given the designation *Qyr.jic-1B.1*. The second QTL, explaining 19% (LOD=2.19) of the phenotypic variation, was associated with the SSR loci *Xwmc156* and *Xbarc181* (linkage group 6), and was given the designation *Qyr.jic-1B.2*. Both QTL were derived from the yellow rust resistant parent, Cham-1.

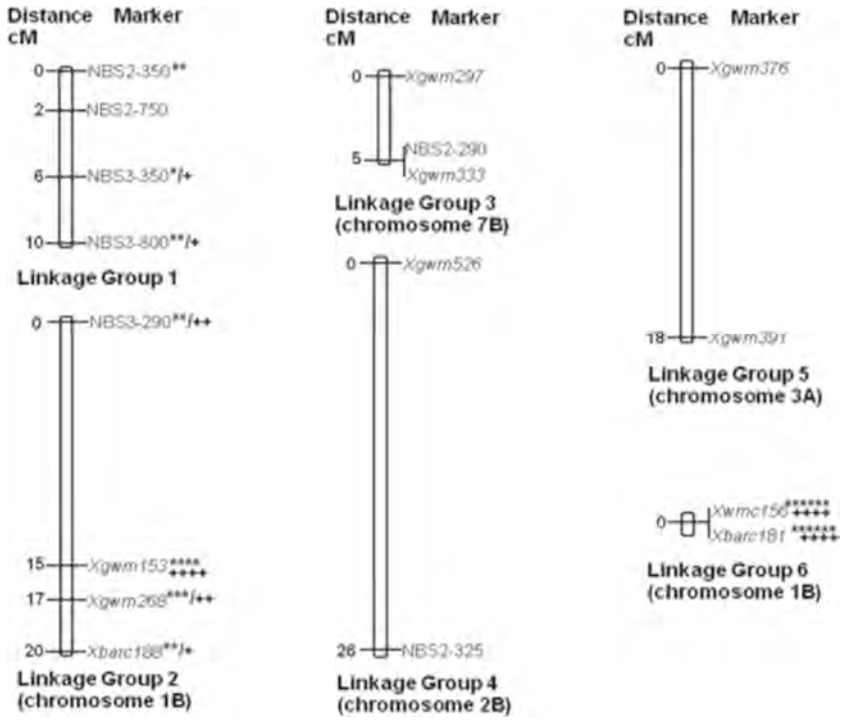

Figure 3. Linkage groups identified in the Kunduru-1149 x Cham-1 cross. Marker loci showing significant associations with yellow rust resistance following a Kruskal–Wallis analyses (MapQTL, version 5.0 for Windows; Van Ooijen *et al.* 2004) of the seedling (asterisk; *) and adult plant, field (cross; +) yellow rust data sets are shown at $P < 0.1$*, 0.05**, 0.01***, 0.005****, 0.001***** and 0.0005******.

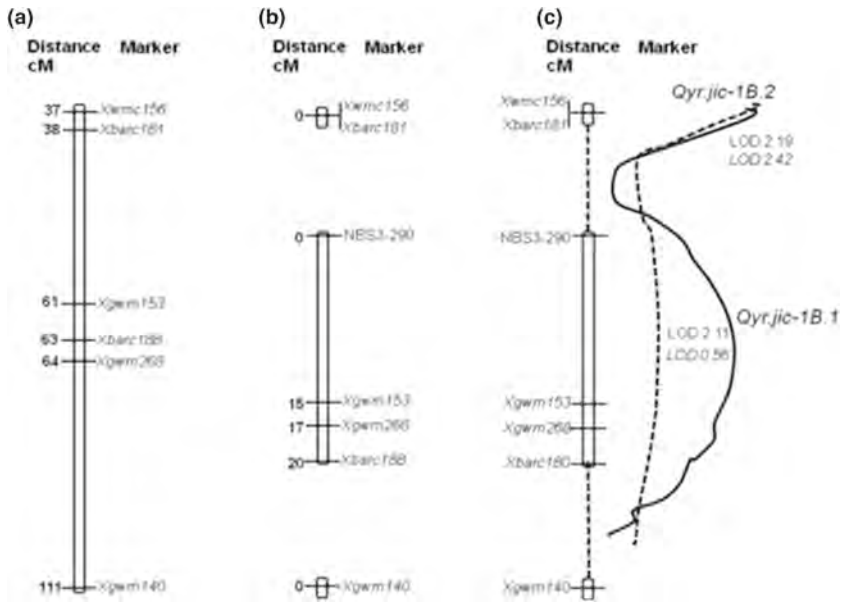

Figure 4. (a) The wheat consensus map of Somers *et al.* (2004) was used to align, (b) linkage groups 2 and 6, and the unlinked marker locus, *Xgwm140* on the long arm of chromosome 1B. (c) QTL for yellow rust resistance found on the long arm of chromosome 1B in the Kunduru-1149 x Cham-1 cross. Two significant QTL, *Qyr.jic-1B.1* and *Qyr.jic-1B.2*, were identified by IM (MapQTL, version 5 for Windows) using the seedling yellow rust data set (solid line). However, only *Qyr.jic-1B.2* was significant with the adult plant, field yellow rust data set (broken line). The LOD values associated with each significant QTL peak are given for the seedling yellow rust data set (plain text) and the adult plant, field yellow rust data set (italics).

With the adult plant, field yellow rust data set a significant QTL (*Qyr.jic-1B.2*) was also found associated with loci *Xwmc156* and *Xbarc181* (LOD=2.42), accounting for 20.8% of the phenotypic variation (figure 4). However, *Qyr.jic-1B.1*, located between loci *Xgwm153* and NBS3-290, was not detected by IM using the adult plant, field data set,

Table 3. The nucleotide and predicted protein sequences of three cloned NBS loci. The p-loop motif of the NBS domain is marked in bold.

NBS3-290 (Genbank accession number: DQ978778.1)

```
gtttactcgattctcaacccgaaagtatagatcccataaggtctttagtaaaattgccgagg
   L  L  D  S  Q  P  E  S  I  D  P  I  R  S  L  V  K  L  P  R
aagttatagagatagaggggagatcaggcacatcaaagaactacatatttattggagta
 K  L  -  R  -  R  G  R  S  G  T  S  K  N  Y  I  F  I  G  V
ctatagtccaacccattagaaatgagcatgcagaggttgataggcagcgatgaggacctt
 L  -  S  N  P  L  E  M  S  M  Q  R  L  I  G  S  D  E  D  L
gtcggaattggtgaaaacaggggcaagttgaccgaatggctgatcaccgatgaaaaagaa
 V  G  I  G  E  N  R  G  K  L  T  E  W  L  I  T  D  E  K  E
accacagtgattacagtttctggcatgggcggcttgggcaaaacaac
 T  T  V  I  T  V  S  G  M  G  G  L  G  K  T
```

NBS2-290 (Genbank accession number: DQ978777.1)

```
gtttactcgattctcaacccgaaagtatagatcccataaggtctttagtaaaattgccgagg
   L  L  D  S  Q  P  E  S  I  D  P  I  R  S  L  V  K  L  P  R
aagttatagagatagaggggagatcaggcacatcaaagaactacatatttattggagta
 K  L  -  R  -  R  G  R  S  G  T  S  K  N  Y  I  F  I  G  V
ctatagtccaacccattagaaatgagcatgcagaggttgataggcagcgatgaggacctt
 L  -  S  N  P  L  E  M  S  M  Q  R  L  I  G  S  D  E  D  L
gtcggaattggtgaaaacaggggcaagttgaccgaatggctgatcaccgatgaaaaagaa
 V  G  I  G  E  N  R  G  K  L  T  E  W  L  I  T  D  E  K  E
accacagtgattacagtttctggtatgggcggcttaggcaaaacaac
 T  T  V  I  T  V  S  G  M  G  G  L  G  K  T
```

NBS2-350 (Genbank accession number: DQ978779.1)

```
gtttactcgattctcaacccgaaagtatagatcccataagctgaaggaccgccatcggatt
  F  T  R  F  S  T  R  K  Y  R  S  H  K  L  K  D  R  H  R  I
gccagccaaattcgtgatctcaaagcaagagtagaagaggtgagcaacaggaacacacgc
 A  S  Q  I  R  D  L  K  A  R  V  E  E  V  S  N  R  N  T  R
tacaacttgatcaccgtcgatgcctccagtagcattgatgaggtgaattcctacacggaa
 Y  N  L  I  T  V  D  A  S  S  S  I  D  E  V  N  S  Y  T  E
gatattcgcaaccactcagctagcaacattgatgaggcagagcttgtgggctttgctaag
 D  I  R  N  H  S  A  S  N  I  D  E  A  E  L  V  G  F  A  K
gctaaacaagagctgattgagatggtggatgtcaactccagagatggtctttgcaagatg
 A  K  Q  E  L  I  E  M  V  D  V  N  S  R  D  G  L  C  K  M
atatttctcgttggtatgggcggcttaggcaaaacaac
 I  F  L  V  G  M  G  G  L  G  K  T
```

although a significant association was found with these marker loci in the Kruskal–Wallis analysis (figure 3). In addition, the Kruskal–Wallis analysis identified significant marker loci associations on linkage groups 1 with both the seedling and adult plant yellow rust phenotype data sets (figure 3).

Sequence analysis of NBS markers

Three NBS loci, NBS3-290, NBS2-350 and NBS2-290, were selected for sequencing. NBS3-290 (linkage group 2-1BL) was associated with both seedling and adult plant, field yellow rust resistance (figures 3 and 4), NBS2-350 (linkage group 1) was only associated with seedling yellow rust resistance (figure 3), while NBS2-290 (linkage group 3; figure 3) showed no association with yellow rust resistance in the Kunduru-1149 x Cham-1 cross against the *P. striiformis* isolates used in the seedling and field yellow rust assessments.

Although the cloned NBS bands all produced PCR fragments of the expected size, SSCP analysis indicated the presence of multiple DNA sequences, a single dominant clone not being distinguishable. Therefore, for each isolated NBS band six clones from the donating parent and six clones from a RIL containing the band were sequenced. For each NBS band a consensus sequence was obtained using Gap 4 (version 4.10). For NBS2-290 and NBS2-350, 11 DNA sequences all formed one contig. With NBS3-290 eight of ten DNA sequences formed one contig (table 3). NBS2-290 and NBS3-290 produced the same DNA sequence, although linkage analysis placed these two NBS bands on different linkage groups, Linkage group 3 (chromosome 7B) and 2 (chromosome 1B), respectively.

Table 4. Blastx search of cloned NBS loci DNA sequences.

NBS Marker	Length of query search (bp)	Homology to	Species	E value	Pos. (%)	GenBank accession no.
NBS2-290/ NBS3-290	289	NBS-LRR type disease resistance protein	*Zea mays*	$7e^{-10}$	62%	NP_001105809.1
		NBS-LRR disease resistance protein homologue	*Hordeum vulgare*	$3e^{-09}$	76%	CAD45034.1
		NBS-LRR type resistance protein	*Saccharum arundinaceum*	$1e^{-07}$	67%	ACD70325.1
		RPR1	*Oryza sativa*	$2e^{-06}$	65%	NP_001067557.1
		Hv1LRR	*Hordeum vulgare*	$1e^{-05}$	57%	BAD73414.1
		LRR14	*Triticum aestivum*	$4e^{-03}$	47%	AAK20742.1
NBS2-350	339	NBS-LRR disease resistance protein homologue	*Hordeum vulgare*	$3e^{-38}$	79%	CAD45030.1
		Hypothetical protein SORBIDRAFT_02g001150	*Sorghum bicolor*	$2e^{-31}$	68%	XP_002459239.1
		Pi2	*Oryza sativa*	$8e^{-29}$	62%	ABC94598.1
		Disease resistance protein RPM1, putative	*Ricinus communis*	$2e^{-10}$	46%	XP_002532244.1

Pos (%), percentage of similar amino acids between query and target sequences.

A Blastx analysis produced hits to phenotypically identified NBS-LRR-type R-genes and to DNA sequences predicted to be NBS-LRR-type R-genes, i.e. RGAs (table 4). Those hits with the greatest amino acid similarity were to R-genes and RGAs from cereal species. The NBS2-350 sequence showed 62% amino acid similarity to the cloned rice blast resistance gene *Pi2* (Zhou *et al.* 2006). NBS2-290 and NBS3-290 produced the same *blastx* hits, showing 65% amino acid similarity to the rice R-gene *RPR1* (Sakamoto *et al.* 1999), 57 % similarity to the barley Hv1LRR sequence (Feuillet and Keller 1999) and 47% similarity to a wheat candidate R-gene for leaf rust resistance, LRR14 (Feuillet *et al.* 2001). NBS2-350 and NBS2/3-290 hits to cereal RGAs showed as much as 79% and 76% similarity, respectively.

Discussion

Genetic characterization of seedling yellow rust resistance in the Kunduru-1149 x Cham-1 cross identified four genes conferring seedling resistance to the *P. striiformis* isolate WYR85/22 (vir *Yr2*, *Yr6*, *Yr7*, *Yr9*). At least one resistance gene was derived from Kunduru-1149, the yellow rust susceptible durum wheat variety, as seen by transgressive segregation of seedling yellow rust resistance in the Kunduru-1149 x Cham-1 RIL population. The location of three of these yellow rust R-genes were identified, using a relatively small number of DNA markers, of which two were associated with NBS marker loci. Analysis of the adult plant, field yellow rust phenotypes identified the same three genetic locations detected with the seedling data set, i.e. on linkage groups 1, 2 (chromosome 1B) and 6 (also chromosome 1B). However, as the *P. striiformis* isolates used in the field tests in Turkey were not available to carryout seedling tests, no definitive conclusions can be made about the nature of the field resistance, which may also be expressed at the seedling growth stage.

A study by Vales *et al.* (2005) indicated that QTL could be identified in populations as small as 50 individuals if the genetic contribution to the phenotype was sufficiently large. IM carried out in a subpopulation of the Kunduru-1149 x Cham-1 RILs was able to identify two QTL located on the long arm of chromosome 1B which contributed to seedling and adult plant yellow rust resistance. Although increasing the population size may identify QTL of small effect, when selecting for disease resistance within a wheat breeding program it is the QTL/genes of large effect that the plant breeder is mostly interested in.

Kruskal–Wallis single marker regression analysis identified the genomic regions containing *Qyr.jic-1B.2* and *Qyr.jic-1B.1* with both the seedling and adult plant, field data sets. However, IM did not locate *Qyr.jic-1B.1* using the adult plant, field data set. Examination of the RILs carrying the Cham-1 allele at locus *Xgwm153* (*Qyr.jic-1B.1* – peak marker locus) confirmed a significant association with both seedling and adult plant, field yellow rust resistance, the mean seedling IT nominal and the adult plant CoI scores being 1.87 and 5.139, respectively. This would indicate the power of the Kruskal–Wallis analysis, particularly when dealing with a small population size.

A number of seedling expressed yellow rust resistance genes have been assigned to chromosome 1B, including *Yr10*, *Yr15*, *Yr21*, *Yr24* and *Yr26* (Wellings *et al.* 2012; Waqar *et al.* 2018). The adult plant resistance *Yr29* has been mapped to the telomeric end of 1BL (Williams *et al.* 2003; Rosewarne *et al.* 2006), while QTL for yellow rust adult plant resistance have been located on 1B in cultivars express (Lin and Chen 2009), Brigadier (Jagger *et al.* 2011) and Camp Remy (Mallard *et al.* 2005).

NBS-profiling (van der Linden *et al.* 2004) has been used in a number of studies, including genetic mapping in apple and lettuce (Calenge *et al.* 2005; Syed *et al.* 2006), and genetic diversity studies in hexaploid (Sayar-Turet *et al.* 2011) and durum wheat (Mantovani *et al.* 2006). The number of polymorphic loci identified between the durum wheat varieties Kunduru-1149 and Cham-1 was relatively low compared with other studies. In apple, 52 polymorphic NBS-bands were identified using three NBS primer-restriction enzyme combinations (Calenge *et al.* 2005). This gave a polymorphism level of 14.8% (52/350), somewhat higher than the 3.8% (14/368) obtained in this study. Four NBS primers, with two restriction enzymes generated 78 polymorphic NBS marker loci in a collection of hexaploid wheat genotypes (78/880) giving a level of polymorphism detection of 8.86% (Sayar-Turet *et al.* 2011). The low level of polymorphism found between wheat varieties Kunduru-1149 and Cham-1 may reflect a closer genetic relationship between these two durum wheat varieties.

Of the 14 polymorphic NBS-bands identified between Kunduru-1149 and Cham-1 four associated, to some degree, with yellow rust resistance. In addition to NBS3-290, which mapped to linkage group 2 (1B), four NBS marker loci formed a single linkage group (linkage group 1), indicating a region of the genome rich in NBS sequences. In apple, 58% of NBS marker loci mapped close to known genes for resistance to scab and mildew (Calenge *et al.* 2005). While in the wild potato species *Solanum demissum* NBS-profiling identified three NBS fragments linked to candidate R-genes for late blight resistance (Zhang *et al.* 2014). Therefore, it may prove valuable to test the Kunduru-1149 x Cham-1 population for resistance to other pathogens and pests to determine whether the regions containing NBS loci associate with resistance to other diseases.

The three NBS bands cloned in this study all showed high homology to functionally characterized R-genes and RGAs, and in particular R-genes and RGAs from cereal species. NBS marker loci generated by NBS-profiling in potato, tomato, barley and lettuce showed similarity to known R-genes and RGAs in 50–90% of cases, depending on the NBS primer and restriction enzyme used (van der Linden *et al.* 2004). Therefore, well in excess of 50% of the NBS marker loci generated by NBS-profiling should locate to regions of the genome containing potential R-genes.

The large genome size of many crop species, in particular wheat, often makes full scale, genome wide mapping projects prohibitive in terms of time, resources and costs. In this study, using relatively few markers and a small population, significant QTL for yellow rust resistance have been identified. Some 29% of the NBS marker loci identified as polymorphic between the durum wheat varieties Kunduru-1149 and Cham-1 showed a significant association with seedling and/or adult plant, field resistance for yellow rust. These results, along with those reported in apple, (Calenge *et al.* 2005), indicate that NBS-profiling can provide a valuable marker tool for the identification of R-genes in crop species.

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