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ORIGINAL PAPER

Searching for novel sources of field resistance to Ug99 and Ethiopian stem rust races in durum wheat via association mapping

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Abstract *Puccinia graminis* f. sp. *tritici*, the causative agent of stem rust in wheat, is a devastating disease of durum wheat. While more than 50 stem rust resistance (*Sr*) loci have been identified in wheat, only a few of them have remained effective against Ug99 (TTKSK race) and other durum-specific Ethiopian races. An association mapping (AM) approach based on 183 diverse durum wheat accessions was utilized to identify resistance loci for stem rust response in Ethiopia over four field-evaluation seasons and artificial inoculation with Ug99 and a mixture of durum-specific races. The panel was profiled with simple sequence repeat, Diversity Arrays Technology and sequence-tagged site markers (1,253 in total). The resistance turned out to be oligogenic, with twelve QTL-tagging markers that were significant ($P < 0.05$) across three or four seasons. R^2 values ranged from 1.1 to 11.3 %.Twenty-four additional single-marker/QTL regions were found to be significant over two seasons. The AM results confirmed the role of *Sr13*, previously described in bi-parental mapping studies, and the role of chromosome regions putatively harbouring *Sr9*, *Sr14, Sr17* and *Sr28.* Three minor QTLs were coincident with those reported in hexaploid wheat and five overlapped with those recently reported in the Sebatel × Kristal durum mapping population. Thirteen single-marker/QTL regions were located in chromosome regions where no *Sr* genes/QTLs have been previously reported. The allelic variation identified in this study is readily available and can be exploited for marker-assisted selection, thus providing additional opportunities for a more durable stem rust resistance under field conditions.

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Introduction

Durum wheat (*Triticum durum* Desf.) is an important crop in the Mediterranean Basin, a region accounting for approximately 75 % of global worldwide production (Belaid 2000; Habash et al. 2009). In Sub-Saharan Africa, Ethiopia is the largest wheat-growing country and is considered one of the centers of diversity of tetraploid wheat (Vavilov 1929, 1951). Durum wheat is grown on approximately 40 % of the total wheat area in Ethiopia, with a tendency to increase due to the growing internal demand for pasta products (Badebo et al. 2009). Among the factors that negatively affect durum production and kernel quality, rust diseases play an important role (Singh et al. 2005). Historically, stem rust infections due to *Puccinia graminis* Pers. f. sp. *tritici* have caused severe losses to wheat production (Zwer et al. 1992; McIntosh and Brown1997; Eversmeyer and Kramer 2000; Singh et al. 2011). Until the appearance of Ug99, stem rust control through the use of genetic resistance was considered a remarkable success story worldwide. Although more than 50 stem rust

resistance (*Sr*) loci have been identified in wheat (Singh et al. 2006), including those introgressed from its wild relatives, only a few remain effective against Ug99 or its variants and even fewer are useful against the durum-specific Ethiopian races (Admassu et al. 2009). Susceptibility in some CIMMYT-derived germplasm was first noted in Uganda (Pretorius et al. 2000) and soon after was observed in all germplasm groups. This new race, designated as Ug99 or TTKS (Wanyera et al. 2006), spread to Kenya in 2001 and to Ethiopia in 2003 (Singh et al. 2006). By 2006, TTKS was identified in Sudan and Yemen (http://www.fao.org), and in 2008 its presence was confirmed in Iran (Nazari et al. 2009). Ug99 is projected to spread farther into the major wheat-growing regions of Asia (Singh et al. 2009). In Ethiopia, Ug99 and its variants were added to previously existing races, the latter specifically virulent on durum wheat. Two such races have been characterized as TRTTF and JRCQC with a combined virulence to *Sr9e* and *Sr13*, two genes present in high frequency in the durum wheat germplasm (Olivera et al. 2012). These races are predominant in durum-growing areas of Ethiopia. Effective resistance to them was found in only 5.2 % of a collection of 996 tetraploid genotypes evaluated for field resistance at the Debre Zeit Research Station in Ethiopia in 2009 (Olivera et al. 2010). Therefore, the combination of Ug99 + *Sr13*-virulent Ethiopian races currently poses a major threat to durum wheat production in Ethiopia and represents a potential danger elsewhere, should these virulent races reach distant durum-growing areas such as central India, where conditions are known to be conducive to the epidemic development of this pathogen. Three different races from the TTKS or Ug99 lineage were identified in Kenya, which led to the re-designation of the original race as TTKSK, and the other two races as TTKST (with additional virulence on *Sr24*) (Jin et al. 2008) and TTTSK (with additional virulence on *Sr36*) (Jin et al. 2009). The effectiveness and durability of the genetic resistance approach to control the disease require the availability of many sources of resistance, preferably involving genes that act on adult plant field resistance, to counter the continuing evolution of new virulence in pathogen populations.

Selecting for the resistant phenotypes conferred by major, race-specific loci is relatively straightforward, and initially rewarding though eventually becomes ineffective due to the fast evolution and selection of virulent strains of the pathogen, as seen with Ug99. Although a number of resistance genes have been introgressed into cultivated wheat from wild relatives (Ceoloni et al. 2005; Feuillet et al. 2008), the successful utilization of such materials has often been hampered by the inherent difficulties of operating with alien genomes.

Marker-based approaches allow us to identify genes/quantitative trait loci (QTL) governing plant response to diseases. The effective deployment of stem rust resistance alleles from different sources requires a thorough genetic characterization of the available germplasm. The standard approach is to use biparental mapping populations to relate phenotypic information to genotypic data obtained from molecular markers to determine the number and the chromosomal location of resistance loci (Gupta et al. 1999; Maccaferri et al. 2008; Simons et al. 2011). An alternative to the use of bi-parental mapping is association mapping (AM) or linkage disequilibrium (LD)-based mapping in which genotype-phenotype relationships are explored in germplasm collections or natural populations (Rafalski 2002, 2011; Flint-Garcia et al. 2003). The underlying principle of this approach is that LD tends to be maintained over many generations between loci that are genetically linked. With AM, statistical assessments are made for associations between genotypes based on molecular markers and phenotypes for various traits in reference germplasm sets (Buntjer et al. 2005). Since its first use in plants a decade ago (Thornsberry et al. 2001), AM has been used in many important crops thanks to advances in high-throughput genotyping technologies, increased interest in identifying useful and/or novel alleles, and improvements in statistical methods (Gupta et al. 2005; Yu et al. 2006; Zhu et al. 2008). In both tetraploid and hexaploid wheat, AM has already proven to be an effective strategy to identify marker-trait associations for agronomically valuable traits (Breseghello and Sorrells 2006; Crossa et al. 2007; Maccaferri et al. 2010, 2011a), including resistance to stem rust (Yu et al. 2011), *Stagonospora nodorum* blotch (Tommasini et al. 2007), *Fusarium* head blight (Miedaner et al. 2011) in bread wheat and leaf rust (Maccaferri et al. 2010) and SBMCV (Maccaferri et al. 2011b) in durum wheat.

The objective of this study was to evaluate a panel of durum wheat accessions well-suited for AM studies (Maccaferri et al. 2006, 2010, 2011a, b) to identify genomic regions associated with field-based resistance to the combination of Ug99 with Ethiopian races of stem rust.

Materials and methods

Plant materials

A collection of 183 elite durum genotypes including cultivars released or breeding lines developed in Italy, Morocco, Spain, Syria, Tunisia, Southwestern USA and Mexico was assembled to represent different spring durum germplasm groups. The genotypes included in the AM panel were chosen from a larger pool of 330 accessions obtained from various sources and evaluated in a field trial in 2003 in Cadriano, near Bologna, Italy (Maccaferri et al.

2006). The accessions of this panel were chosen based on their pedigrees and morpho-physiological traits critical to adaptation, such as plant height and heading date. Highly related accessions (e.g. sibs from the same cross, backcross lines, etc.) and/or with excessively large differences in heading date, a feature that could have biased the phenotypic evaluation of traits influenced by flowering time, were excluded. Most of the accessions were semi-dwarf, short- to medium-cycle elite cultivars and breeding lines released from the early 1970s up to the late 1990s. The collection comprises also 'founder genotypes' widely used as parents in breeding programs throughout the Mediterranean Basin and at International CGIAR Centers (CIMMYT and ICARDA). The accessions were assembled for conducting AM studies and are hitherto collectively referred to as the 'AM durum panel'. A detailed phenotypic and molecular characterization of the panel was previously reported in Maccaferri et al. (2006, 2010). Briefly, the panel included accessions belonging to one of five main population subgroups: accessions from ICARDA bred for the dryland areas (subgroup 1), from ICARDA bred for temperate areas (subgroup 2), from the Italian and early 1970s CIMMYT breeding programs (subgroup 3), from CIMMYT in the late 1970s early 1980s (subgroup 4), from CIMMYT in the late 1980s early 1990s (subgroup 5). As compared to the panel of accessions described in Maccaferri et al. (2010), 25 accessions were dropped due to their relatively high relatedness while 19 additional accessions from the CIMMYT breeding programs, mainly classified as belonging to subgroup 5, were added to the panel. Based on their molecular profiles, the accessions clustered into the five subgroups with balanced frequencies.

Stem rust response evaluation under field conditions

Field experiments were conducted in Ethiopia at the Debre Zeit Agricultural Research Center (DZARC), located at an altitude of approximately 1,900 m above sea level, with latitude of 8°44′N and longitude of 38°85′E. This Center is a hot spot for wheat-stem rust during the main cropping season (July to November) as well as during the off-season (mid-January to May), if irrigation is provided to ensure proper plant development. DZARC has been identified as an international durum wheat screening site as part of the Borlaug Global Rust Initiative.

The AM durum panel was evaluated during four consecutive growing seasons in 2009 and 2010. In both years, the evaluation was carried out both in the off-season under supplementary irrigation and in the main season under rainfed conditions. The off-season is warmer than the main season and as a result stem rust disease pressure is often higher than in the main season, depending on the moisture availability for disease development. The accessions were evaluated in non-replicated field trials, using an augmented design, with plots consisting of 1-m long twin rows flanked by spreader rows that were sown with a seed mixture of PBW343, Morocco (bread wheat susceptible to Ug99) and Local Red or Arendeto (susceptible durum) accessions in 2:1:1 proportion, respectively. Spreader rows were artificially inoculated with Ug99 (TTKSK race) and a mixture of durum-specific races prevalent in Ethiopia. The Ug99 (TTKSK) stem rust race was isolated and maintained on the variety PBW343 under greenhouse conditions. Race purity was regularly checked on the North American stem rust differential lines. In addition, bulk spores were collected directly from the durum wheat nurseries in the field and temporarily stored at 4 °C after drying. Field inoculation was carried out following the methodology described in Roelfs et al. (1992). Inoculation was carried out on spreader rows starting at stem elongation growth stage and was repeated two to three times at weekly intervals. The cultural practices including fertilizer, weeds and insect control were applied according to the local site recommendations.

Stem rust disease severity was recorded two to three times during the epidemics development using a modified Cobb's scale (Peterson et al. 1948). Disease severity score (DSS) was calculated as the percentage of infected stem area covered by pustules (visually estimated over the whole canopy); at the same time, the major infection type was also recorded (Roelfs et al. 1992). Infection types were categorized into four discrete classes: resistant (R), moderately resistant (MR), moderately susceptible (MS) and susceptible (S). The DSS and the corresponding infection types were used to compute the values of the Coefficients of Infection (Stubbs et al. 1986). For each evaluation season, the terminal disease severity at the soft-dough stage (Zadoks scale, 85; Zadoks et al. 1974), in coincidence with the peak of disease severity, was considered as the most informative disease score and was, therefore, used to carry out the molecular-phenotype association tests.

Molecular profiling

A bulk of 25 seeds from the original pure stock of each accession was germinated and grown in growth chamber at 20 °C. After 2 weeks, seedling leaves were collected, freeze-dried, ground and used for genomic DNA extraction as previously described in Maccaferri et al. (2010). The accessions were profiled with 350 simple sequence repeat loci (SSR), 900 Diversity Arrays Technology (DArT) markers and three additional sequence-tagged site (STS) markers, including those previously reported as markers associated with major stem rust resistance genes (Yu et al. 2010).

SSR and STS markers

Most of the SSR primers used were chosen among the publicly available sets catalogued in the GrainGenes database (http://wheat.pw.usda.gov) as BARC (*barc* marker loci), CFA, CFD and GPW from INRA (*cfa*, *cfd* and *gpw, respectively*), KSUM (*ksum*), WMC (*wmc*) and WMS (*gwm*). An additional subset of private genomic WMS primers from TraitGenetics (supplied by M. Ganal, TraitGenetics, Gatersleben, Germany) were also considered. The SSR loci used to genotype the accessions were preselected for (i) clarity and repeatability of amplicon profiles, (ii) polymorphism level and (iii) even distribution on all the A- and B-genome chromosomes. The choice was carried out based on the results of a survey of SSR primer pairs conducted on a small subset of eight founder accessions and lines used as parents of mapping populations.

As described in Maccaferri et al. (2008), a unique thermo-cycling protocol was used for all primer sets and SSR profiles of the accessions were obtained using the automated LI-COR 4200 IR2 System (LiCor, Lincoln, NE, USA). Genotyping was performed for most SSR markers using the M13-labelled primers and amplification protocol (Schuelke 2000). Alleles were scored using founder genotypes as an allele reference set. Most markers produced only one band assigned to a unique wheat chromosome in previous mapping studies. For SSR primer pairs amplifying two or more loci, each locus was independently scored and assigned to the respective linkage group based on either the score of the parental lines or the LD with adjacent markers.

DArT markers

In addition to SSR and STS markers, the panel was profiled with DArT markers. DArT markers were generated by Triticarte Pty. Ltd. (Canberra, Australia; http://www.triticarte.com.au), a whole-genome profiling service company, as described by Akbari et al. (2006). The durum wheat *Pst*I/*Taq*I array v 2.0, containing 7600 single DArT clones obtained as described in Mantovani et al. (2008), was used for genotyping the panel. The locus designation used by Triticarte Pty. Ltd. was adopted ('wPt', 'rPt' and 'tPt' loci corresponding to wheat, rye and triticale clones, respectively), and alleles at polymorphic loci were scored as hybridization positive (1) or negative (0).

Construction of the consensus map

The majority of the SSR markers considered herein had previously been mapped in five intra-specific durum recombinant inbred line (RIL)-based linkage maps, whose genotypic data were used to obtain a consensus durum wheat-specific linkage map. Four mapping populations, i.e. Kofa × Svevo (KS RIL population, Maccaferri et al. 2008), Colosseo × Lloyd (CL RIL, Mantovani et al. 2008), Meridiano × Claudio (MC RIL, Maccaferri et al. 2011a, b) and Simeto × Levante (SL RIL, Maccaferri et al. unpublished) were developed by DiSTA in collaboration with Produttori Sementi Bologna SpA (Argelato, BO, Italy). For the fifth linkage map, obtained from the cross Kofa × UC1113 (KU RIL population, Zhang et al. 2008), the genotypic data were downloaded from the GrainGenes web database.

The consensus linkage map was obtained from the five data-sets using the Carthagene v.4.0 software (de Givry et al. 2005). Merging was performed with the *dsmergen* command, after checking for marker order consistency across maps, so that for each marker pair a single recombination rate was estimated based on all available meioses. A framework-mapping method was applied. Non-framework markers were incorporated in the framework map by building a complete map using the framework map as a fixed order. The marker order and inter-marker genetic distances from the consensus map were used to report the LD and association results.

The consensus map included a total of 2,036 markers (mostly SSR and DArT markers) and it is reported as Supplemental Table 1.

Association mapping

To avoid LD inflation effects and to reduce the risk of false-positive marker-trait associations (Myles et al. 2009), data points for rare alleles (those with frequencies of 0.10 or less) were considered as missing data. Data points showing residual allelic heterogeneity within accession were also considered as missing data.

In total, the genotypic score of the AM durum panel genotypes was available for 1,211 markers suitable for association mapping (minor allele frequency >0.10), including SSR, DArT and STS markers

Among those, the 320 SSR, 3 STS and 538 DArT markers that could be projected onto the consensus linkage map were retained for marker-phenotype association tests (861 markers), while the others with undefined map position were not considered for further analyses.

Genetic structure and linkage disequilibrium analysis

Prior knowledge suggested the presence of significant population structure in the panel. To decrease the false-positive rate, this structure was accounted for in the association test models. The genetic structure of the panel was investigated with a combination of model- and distance-based analyses. Model-based population structure using a

selection of 96 highly informative and evenly spread SSRs was assessed using the program STRUCTURE v. 2 (Pritchard et al. 2000). STRUCTURE parameter settings were: linkage model, allele frequencies correlated, burn-in length 100,000 and 100,000 MCMC repetitions. An optimum number of five hypothetical subgroups were chosen to obtain the Q matrix of membership coefficients of each accession to all subgroups (for details see Maccaferri et al. 2011a, b). In the distance-based analysis, pairwise genetic similarity values (GS_{ij}) were calculated for all possible pairs of accessions using the simple matching coefficient for multi-state markers: a co-ancestry K (kinship) matrix was thus obtained for SSRs (for details see Maccaferri et al. 2010). Similarly, the kinship matrix was also calculated for DArT markers, separately.

Estimating LD between markers assess whether markers segregate independently or not. The program TASSEL, v. 2.1 (www. maizegenetics.net, Yu et al. 2006) was used to estimate the LD parameters D' and r^2 values as a function of the corresponding inter-marker distances and the comparison-wise significance was computed with 10,000 permutations. The r^2 LD value was estimated for intra-chromosomal loci and related to genetic distances between loci (cM). If, within a chromosome region, all pairs of adjacent loci were in LD, this region was referred to as an LD block (Stich et al. 2005).

Marker-phenotype association analysis

Genome-wide scans for AM of loci governing stem rust resistance were conducted using the coefficient of infection (CI) as reference phenotypic data. Prior to the AM analysis, homogeneity of experimental variance across experiments was verified through the Bartlett's test. AM analysis was conducted using the TASSEL program, ver. 2.1. The 320 SSRs, 3 STSs and the 538 DArT markers were tested for significance of marker-trait associations under: (1) the fixed general linear model (GLM) including the Q population structure results as covariates (Q GLM), (2) the mixed linear model (MLM) including the Q population structure results plus the K kinship matrix (Q + K MLM).

For GLM analysis, besides the marker-wise association probability values, the experiment-wise association significance probability was obtained based on a permutation test implemented in TASSEL (10,000 permutations in total). The experiment-wise test provides a much more severe threshold for significance as compared to the marker-wise test (Bradbury et al. 2007, 2011). In the MLM analysis, experiment-wise significance was inspected using the false discovery rate (FDR) approach according to Storey and Tibshirani (2003) and implemented in *Qvalue* program.

Multiple adjacent co-segregating significant markers were assigned to a unique QTL region upon satisfaction of the following conditions: less than 20 cM of inter-marker genetic distance, presence of significant and strong LD among markers (possibly with r^2 values ≥ 0.6), consistency of the marker allelic effects in sign (Massman et al. 2011).

Chromosome regions repeatedly associated with stem rust response in two or more seasons and to the combined response across seasons were considered as putative QTLs, regardless of whether the experiment-wise significance threshold was reached. For each putative QTL, the marker with the strongest association to stem rust response was considered as the main QTL-tagging marker. Results on the allelic distribution and effects were examined for the QTL-tagging markers only. Linear regression was used to investigate the fit of the accessions' haplotypes at the main QTLs (significant over three to four seasons) to the corresponding phenotypic responses (CIs averaged across seasons). Based on the results of the GLM and MLM tests, the non-rare alleles at the QTL-tagging markers that were significant over three or four seasons were classified as beneficial, intermediate or deleterious and the cumulative numbers of beneficial and deleterious alleles were counted for each accession. The accessions' disease response averaged across the four seasons was regressed on the cumulative numbers of both beneficial and deleterious alleles. Significance of the regression was estimated with an F test.

Results

Response to stem rust

Stem rust infection was high in all four testing seasons, allowing for clear and unambiguous scoring of field reaction. The mean CI values of the panel accessions ranged from 33.6 for DZm-2010 to 49.3 for DZo-2010. In both years, the off-season experiment showed a disease pressure significantly ($P \leq 0.01$) higher than that recorded in the main season (Table 1). In all seasons, a broad and continuous variation within the panel was noted, from close-to-immune, highly resistant reactions to highly susceptible ones, as indicated by the disease response ranges reported in Table 1 and by the CI frequency distribution in each season and across seasons (reported as supplemental Figure 1).

The analysis of variance for stem rust reaction showed highly significant differences ($P \leq 0.0001$) among accessions and seasons (results not reported); the accession × season interaction was also significant ($P \leq 0.01$).

The heritability coefficient of stem rust response, calculated across seasons using the data from the non-replicated experiments, was equal to 0.80 while the coefficient of variation reached 26.1 %. The Pearson correlation

Table 1 Descriptive statistics for field stem rust response (reported as Coefficient of Infection) of the 183 elite durum wheat accessions evaluated in four growing seasons in Ethiopia

Season[a]	CI		
	Mean	Min	Max
DZo-2009	42.2	0.2	80.0
DZm-2009	36.9	0.0	80.0
DZo-2010	49.3	0.0	90.0
DZm-2010	33.6	7.9	68.2
Mean	40.5	3.5	72.0

[a] Stem rust response evaluation carried out in Debre Zeit (DZ) Agricultural Research Center; DZo-2009: off-season field trial evaluation carried out in 2009 (January to May); DZm-2009: main season evaluation in 2009 (July to November); DZo-2010: off-season evaluation in 2010; DZm-2010: main season evaluation in 2010

coefficients between the stem rust responses recorded in the four seasons (data not reported) were always highly significant ($P \leq 0.001$), with values ranging from 0.40 (DZo-2010 *vs*. DZm-2010) to 0.58 (DZm-2009 *vs*. DZo-2010).

Based on the distribution of the stem rust responses averaged over the four seasons (Table 2), about 5 % of the accessions (nine in total) were highly resistant (mean DSS < 10 %) and 19 % (36 accessions) were categorized as moderately resistant (mean DSS comprised between 10 and 30 %). In addition, 11 accessions (i.e. 6 %) were classified as susceptible or highly susceptible (DSS equal to or higher than 70 %); their number increased to 51 (i.e. 30 % of accessions) when considering the single DZo-2010 season that was characterized by an infection level significantly higher than that reached in the other three seasons.

Relationship between population structure and response to stem rust

The genetic relationships among the accessions were investigated using both a genetic similarity and a model-based Bayesian clustering method and the results have been reported elsewhere (Maccaferri et al. 2006, 2011a, b). Both methods pointed out that the minimum and optimum number of hypothetical well-distinct subgroups present in the panel was equal to five. It was shown that the five subgroups corresponded to clearly distinct breeding lineages: (1) the ICARDA germplasm bred for the dryland areas (subgroup S1); (2) the ICARDA germplasm bred for the temperate areas (subgroup S2); 3) the Italian and early 1970s CIMMYT germplasm (subgroup S3); 4) the late 1970s CIMMYT germplasm, widely adapted to Mediterranean conditions (subgroup S4); 5) the late 1980s to early 1990s CIMMYT germplasm, with increased yield potential (subgroup S5). Based on the molecular assignment of each accession to the subgroup with the highest Bayesian probability, the five subgroups included 11, 55, 26, 56 and 35 accessions, respectively. The membership coefficient to each of the five subgroups, averaged over all the accessions, was equal to 0.09, 0.29, 0.14, 0.29 and 0.19 from S1 to S5, respectively (supplemental Table 2). The differences for stem rust response among the five subgroups were highly significant ($P \leq 0.0001$, results not reported), with the differences among subgroups explaining 15.5 % of the total variance. Although differences among subgroups were significant, the within-group component of variance prevailed, accounting for 53.2 % of the total variation. The effect of population structure on the stem rust response was also investigated by means of regression analysis. Using data of each season separately, a modest population structure effect was detected for the DZo-2009 and DZm-2010 seasons, with R^2 values of 8.9 and 7.7 %, respectively, while a greater influence was detected for DZm-2009 and DZo-2010, with R^2 values of 14.7 and 20.8 %, respectively. The mean and range for stem rust response values (CIs) of each of the five subgroups are reported in Table 3. These values clearly show that all five subgroups included accessions with a wide range of responses, from highly resistant to highly susceptible, thus indicating that all subgroups are equally informative and

Table 2 Frequency distribution of stem rust responses averaged over four growing seasons in Ethiopia for the 183 elite durum wheat accessions included in the association mapping durum panel

Season	Stem rust response[a]					
	(DSS < 10 %)	(DSS 10-20 %)	(DSS 30 %)	(DSS 40 %)	(DSS 50-60 %)	(DSS 70-100 %)
DZo-2009	0.06 (10)[b]	0.06 (10)	0.12 (20)	0.26 (43)	0.45 (75)	0.05 (8)
DZm-2009	0.11 (16)	0.12 (17)	0.19 (27)	0.18 (26)	0.28 (40)	0.13 (19)
DZo-2010	0.05 (9)	0.06 (10)	0.10 (17)	0.16 (27)	0.34 (58)	0.30 (51)
DZm-2010	0.15 (22)	0.10 (14)	0.17 (25)	0.22 (31)	0.30 (43)	0.06 (8)
Mean	0.05 (9)	0.09 (17)	0.10 (19)	0.18 (33)	0.51 (94)	0.06 (11)

[a] Classification of response based on the Disease Severity Score (DSS) as reported in Singh et al. (2009)

[b] Frequencies values; values within brackets report the actual accession numbers

Table 3 Mean and range of stem rust response (reported as Coefficient of Infection) in the five main germplasm subgroups of the association mapping durum wheat panel

Environment	Subgroup 1 (S1) ICARDA drylands (11)[a]			Subgroup 2 (S2) ICARDA temperate (55)			Subgroup 3 (S3) Italian and early 1970s CIMMYT (26)			Subgroup 4 (S4) late 1970s CIMMYT (56)			Subgroup 5 (S5) late 1980s CIMMYT (35)		
	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
DZo-2009	40.6	9.0	70.0	41.6	0.2	70.0	32.3	0.2	60.0	44.9	6.0	80.0	45.4	4.0	70.0
DZm-2009	27.4	6.0	54.0	34.5	2.0	80.0	22.7	0.0	63.0	42.7	3.0	80.0	44.0	3.0	80.0
DZo-2010	45.7	27.0	70.0	44.5	3.0	80.0	38.3	0.0	80.0	49.0	9.0	90.0	66.2	12.0	80.0
DZm-2010	43.5	9.4	60.5	32.2	7.9	60.5	27.4	7.9	60.5	33.7	11.0	52.7	36.2	7.9	68.2
Mean	39.6	14.6	58.0	38.6	3.5	67.5	29.4	3.8	58.7	42.7	7.5	66.2	47.5	9.5	72.0

Least significant difference (LSD) among subgroups = 4.99 ($P = 0.05$)

[a] Number of accessions belonging to each subgroup

well-suited for AM purposes. Considering the mean subgroup values across seasons and based on the least significant difference among subgroups, S4 and S5, which mainly included CIMMYT elite germplasm, showed significantly higher stem rust susceptibility than S1, S2 and S3. The complete dataset of phenotypic response and population structure membership coefficients for each of the 183 accessions included in the association panel is reported as supplemental Table 2.

Association mapping for stem rust response

In view of the strong genotype by season interaction, marker-phenotype association tests were conducted separately for each season as well as for the responses averaged over the four seasons.

The association mapping (AM) analysis was conducted by performing single-marker *F* tests using both the General Linear Model with Q covariate matrix (population structure correction: Q GLM) and the mixed linear model with Q + K matrices (population structure and familial relatedness correction: Q + K MLM). The genome-wide scan revealed chromosome regions harbouring putative QTLs for stem rust response on all chromosomes except for 3B. Overall, 45 chromosome regions harboured markers that were significant ($P \leq 0.05$) in at least two seasons under the Q GLM model as well as across the averaged data of the four seasons; 36 of these 45 chromosome regions showed significant effects also using the Q + K MLM model.

Introducing the experiment-wise correction, eight chromosome regions showed significant ($P \leq 0.05$) effects in the Q GLM model while in the Q + K MLM model the significance was limited to one region on chromosome 6A which showed the strongest association with stem rust response. Based on these findings, we decided to present detailed results of the 36 chromosome regions which were detected in the marker-wise analysis and considered as putative QTLs.

Figure 1 summarizes the results of the Q + K MLM genome scan for the disease response averaged across the four seasons. In several cases, the presence of a QTL was evidenced by multiple SSR and DArT markers significantly associated with the phenotype, located within chromosome regions of 10 cM or less (linked markers) and with the same directional effect, as estimated from the durum consensus map, and, in most cases, with LD r^2 values higher than 0.6. For each of the QTLs that were identified as linkage blocks of adjacent markers, all the markers significantly associated with the phenotype were checked for consistency of their effects and the marker with the most significant association to the trait was considered as the QTL-tagging marker.

For 12 of the 36 chromosome regions considered as putatively harbouring QTLs, the significance of the effects on stem rust response was confirmed across three or four seasons (QTL features reported in Table 4; see also Fig. 1), while the other 24 regions showed significant, consistent effects in two seasons (Table 5; Fig. 1). The QTLs with consistent effects across three or four seasons (Table 4) were also those with the highest overall R^2 values based on the combined analysis over seasons (in most cases comprised between 4.0 and 7.0 %) as well as for single seasons (values ranging from 1.0 to 11.3 %). In particular the regions on chromosomes 1BS (QTL-tagging marker *barc8*), 2AS (*gwm1045*), 3AS (*wPt-7972*), 6AL (*gwm427* and *CD926040*) and 7AS (*wPt-2799*) showed the highest R^2 values and all of these QTLs were tagged by a series of adjacent markers that supported the primary QTL effect. Regions on chromosomes 2BL, 3AL and 5AL had consistently high R^2 values, but were identified by single markers.

The QTL tagged by *barc8* on chromosome 1BS at 32.0 cM showed strong LD (r^2 range of 0.60–0.67) along with a 9.0 cM interval that included nine DArT markers (following the mapping order of the consensus map: wPt-

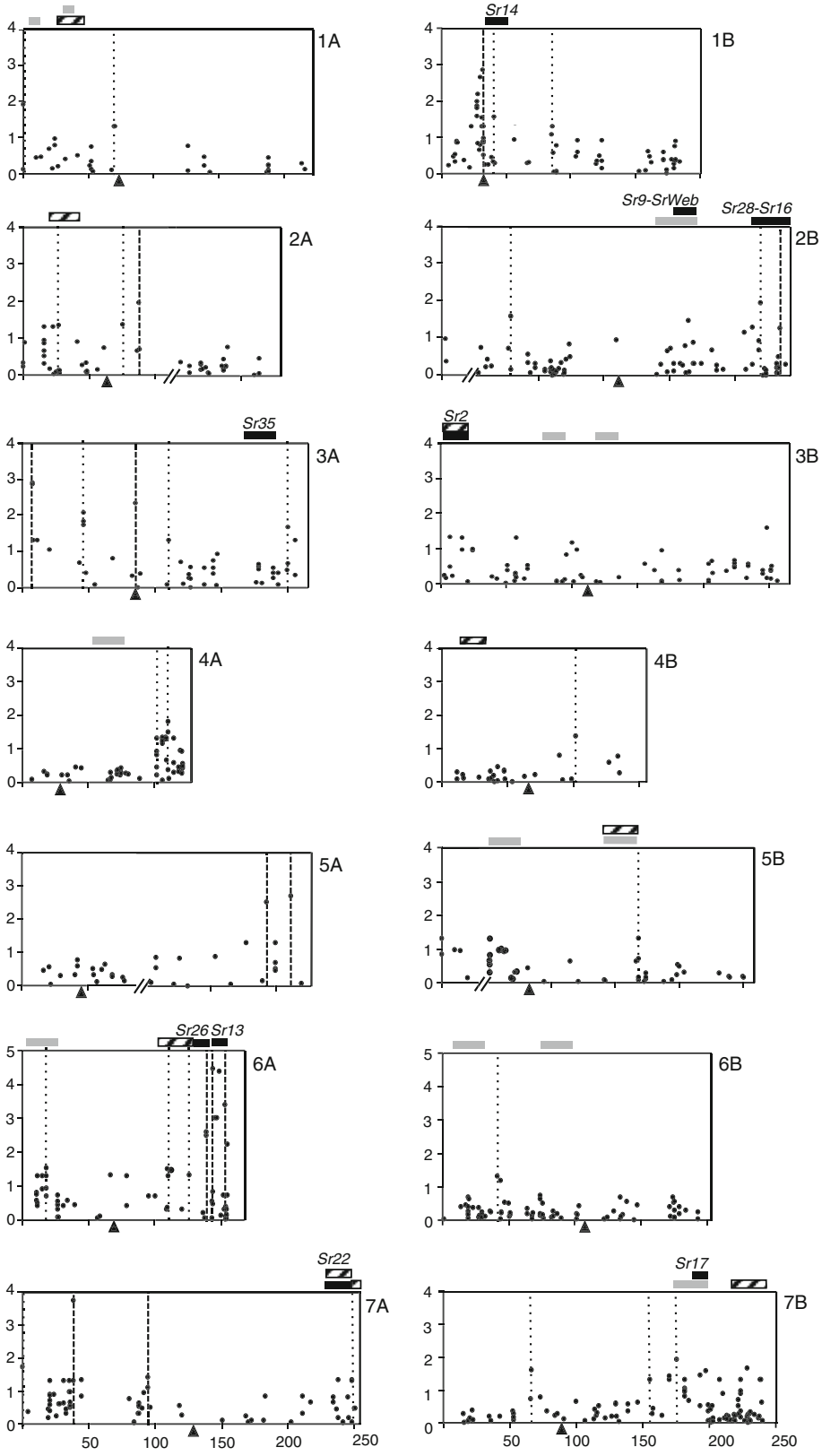

Fig. 1 Association mapping probabilities, reported as −log (*p*), of the mapped markers tested for association to stem rust response of 183 elite accessions of durum wheat. Results are shown for the stem rust response averaged over four evaluation seasons, reported on a chromosome-by-chromosome basis. The −log 0.05 significance threshold value is equal to 1.35. Centromeres have been indicated as *solid filled triangles*. *Vertical, dashed lines* indicate the 12 markers with significant effects ($P < 0.05$) in three or four seasons; vertical, dotted lines indicate the 24 markers tagging QTL regions with significant effects ($P < 0.05$) in two seasons only. Chromosome intervals corresponding to the locations of stem rust (*Sr*) resistance loci reported by previous studies in hexaploid and tetraploid wheat have been reported as *black bars* above the graph of each chromosome. Chromosome linkage blocks associated with stem rust response in hexaploid wheat (Crossa et al. 2007; Yu et al. 2011) and in tetraploid wheat (Haile et al. 2012) have been reported as *grey* and *crossed-bars*, respectively

2999, wPt-4605, wPt-3582, tPt-8831, wPt-9864, wPt-4133, wPt-1876, wPt-5899 and wPt-4729) and one SSR marker (*gwm1100*). In the distal region of chromosome 6AL, highly significant effects were detected at three adjacent chromosome regions/linkage that overall spanned 15.8 cM on the durum consensus linkage map, but showed low LD

Table 4 Quantitative trait loci (QTLs) for stem rust response identified through association mapping in a panel of 183 elite durum wheat accessions evaluated in Ethiopia, with significant effects observed over three to four evaluation seasons

Chrom.	Most associated marker	Position (cM)[a]	Seasons with significant marker-trait associations	R^2 range (%)[b]	R^2 (%)[c]	Associated markers in the QTL region	Interval width (cM)[a]
1BS	*barc8*	32.0	DZm-2009, DZo-2010, DZm-2010	3.2–5.6	4.6	*gwm1100*, wPt-2999, wPt-4605, tPt-8831, wPt-9864, wPt -3582, wPt-4133, wPt-1876, wPt-5899, wPt-4729	9.0
2AS	*gwm1045*	87.7	DZm-2009, DZo-2010, DZm-2010	3.3–5.8	3.9	*gwm425*, *cfa2263*	12.5
2BL	*wmc356*	220.0	DZo-2009, DZm-2009, DZo-2010	3.2–6.6	4.1	–	0.0
3AS	wPt-7992	8.0	DZo-2009, DZm-2009, DZo-2010, DZm-2010	1.7–4.7	3.3	wPt-6854, *barc12*, wPt-1111	3.5
3AL	*wmc388*	85.6	DZo-2009, DZo-2010, DZm-2010	1.9 -4.1	4.0	–	0.0
5AL	*gwm126*	93.3	DZo-2009, DZo-2010, DZm-2010	1.8 -4.8	4.1	–	0.0
5AL	*gwm291*	111.7	DZo-2009, DZm-2009, DZo-2010,	2.7 -5.7	4.4	–	0.0
6AL	*gwm427*	139.5	DZo-2009, DZm-2009, DZm-2010	1.7 -6.8	3.5	*wmc580*	0.1
6AL	*CD926040*	144.0	DZo-2009, DZm-2009, DZo-2010, DZm-2010	3.5–11.3	7.1	wPt-9474, wPt-4229, wPt-5654, wPt-3247, wPt-4663	9.3
6AL	*barc104*	155.3	DZo-2009, DZm-2009, DZm-2010	6.1–9.7	4.5	–	0.0
7AS	wPt-2799	38.2	DZo-2009, DZm-2009, DZo-2010, DZm-2010	1.7–4.9	5.2	*barc70*, *gwm1187*, *wmc479*	6.3
7AS	wPt-7785	94.8	DZo-2009, DZo-2010, DZm-2010	1.1–2.3	1.5	–	0.0

For each QTL, the chromosome position, the associated markers and the QTL features are reported

[a] Position of the QTL most associated marker as from the durum consensus map used as reference

[b] Range of R^2 value across the three to four evaluation seasons with significant marker-trait association

[c] R^2 value for the marker most associated with the QTL (averaged over the four evaluation seasons)

with each other. Each of these three chromosome regions were identified, respectively, by: (i) the marker pair *gwm427-wmc580* (at chromosome position 139.5 cM, r^2 LD value between the two markers = 0.98), (ii) the EST-derived marker *CD926040* (chromosome position 144.0 cM), associated with wPt-9474, wPt-4229, wPt-5654, wPt-3247 and wPt-4663 (spanning a 9.3 cM interval with moderate LD among markers and r^2 values ranging from 0.12 to 0.58) and (iii) *barc104* (chromosome position 155.3 cM). The marker pair *gwm427-wmc580* showed low LD values with all the other markers in the region (r^2 values from 0.01 to 0.20) while LD was detected between the linkage block of markers associated with *CD926040* and *barc104* (r^2 from 0.26 to 0.55).

As compared to the QTLs identified across three or four seasons, those (24 in total) with significant effects in only two seasons (Table 5) showed in general lower effects and R^2 values both on a mean-(values from 1.0 to 3.8 %) and single-season basis. Nonetheless, some of these QTLs (e.g. those on chrs. 1AS, 1BL, 2B, 3AL, 6A and 7B) showed relatively high R^2 values in specific seasons (from 3.6 to 8.0 %).

The least square phenotypic means (based on CIs) of non-rare alleles at the QTL-tagging markers with significant effects in three to four seasons are reported in Table 6.

The SSR marker *gwm427* (chromosome 6AL) showed two common alleles (212 and 188 bp), with the 188 bp allele being associated with significantly ($P \leq 0.05$) lower CI values. The EST-derived marker *CD926040* (chromosome 6AL) carried three common alleles with phenotypic effects that were estimated to be beneficial for one allele (855 bp) over all seasons and detrimental (i.e. associated with increased susceptibility) for the other two alleles (851 and 845 bp). At *barc104* (chromosome 6AL) the 202 and 206 bp alleles were both considered as beneficial as compared to the 172 bp allele (detrimental).

The genotypes of the 183 accessions of the AM durum panel for all the QTL-tagging markers identified in this

Table 5 Quantitative trait loci (QTLs) for stem rust response identified through association mapping in a panel of 183 elite durum wheat accessions evaluated during four seasons in Ethiopia, with significant effects observed in two out of four evaluation seasons

Chrom.	Most associated marker	Position (cM)[a]	Seasons with significant marker-trait associations	R^2 range (%)[b]	R^2 (%)[c]	Associated markers in the QTL region	Interval width (cM)[a]
1AS	*gpw2246*	0.0	DZo-2009, DZm-2009	2.3–4.7	3.1	–	0.0
1AS	wPt-5411	69.6	DZm-2009, DZo-2010	1.4–1.6	1.3	*gwm164*	1.0
1BL	*cfd65*	40.8	DZm-2009, DZm-2010	3.5–3.8	2.4	wPt-8168, *gwm947*	11.0
1BL	wPt-0202	85.7	DZo-2009, DZm-2009	1.4–2.0	1.0	wPt-0506, wPt-3227	0.6
2AS	wPt-7049	26.9	DZo-2010, DZm-2010	1.7–3.2	1.6	*barc212*	4.0
2BS	wPt-8404	75.7	DZm-2009, DZm-2010	2.2–6.1	1.6	*wmc257*, *wmc243*, *wmc25*	2.0
2BL	*wmc361*	29.0	DZm-2009, DZo-2010	2.5–4.3	2.0	–	0.0
2BL	*gwm1300*	169.1	DZo-2009, DZm-2010	1.6–8.0	1.7	wPt-5242	1.0
3AL	wPt-1923	46.4	DZm-2009, DZm-2010	2.2–4.5	2.2	wPt-3348, wPt-1652	0.0
3AL	*wmc428*	110.5	DZo-2009, DZm-2009	4.4–6.8	3.8	–	0.0
3AL	wPt-8203	200.3	DZo-2009, DZm-2009	1.9–2.5	1.8	*barc1177*	5.9
4AL	wPt-9196	102.4	DZo-2010, DZm-2010	1.0–1.5	1.0	wPt-2985, wPt-8886 wPt-8271 wPt-8167 wPt-3108 wPt-3796 wPt-6502 wPt-7821	6.9
4AL	wPt-0798	111.0	DZo-2009, DZm-2010	2.8–2.9	1.9	wPt-5055	0.0
4BL	wPt-8543	101.9	DZo-2009, DZo-2010	1.2–2.9	1.4	–	0.0
5BL	wPt-9300	118.9	DZm-2009, DZo-2010	1.2–1.9	1.1	wPt-2453, wPt-1733	0.0
6AS	wPt-7330	18.6	DZm-2009, DZo-2010	1.2–3.6	1.6	wPt-1742, wPt-5395, wPt-5633, tPt-6710, wPt-1377, wPt-9075, wPt-6520, wPt-7754, wPt-4016, wPt-4017, wPt-3468	7.5
6AL	tpt-4209	109.6	DZm-2009, DZo-2010	2.3–2.6	1.5	*gwm1150*	8.4
6AL	*gwm169*	126.6	DZo-2009, DZm-2010	2.0–3.0	1.5		0.0
6BS	wPt-1437	41.9	DZo-2009, DZo-2010	2.2–2.3	1.3	wPt-2095, wPt-7935	2.4
7AS	wPt-5489	0.0	DZo-2009, DZo-2010	1.5–1.8	2.0		0.0
7AL	wPt-0745	248.4	DZo-2009, DZm-2010	1.7–2.2	1.3	wPt-7763	0.0
7BS	*gwm573*	66.6	DZo-2009, DZm-2009	2.9–5.7	3.4	*gwm1184*, *wmc182*	6.2
7BL	*wmc517*	155.6	DZm-2009, DZm-2010	3.5–3.6	2.3	–	0.0
7BL	wPt-8615	175.9	DZo-2010, DZm-2010	2.3–2.7	2.1	wPt-5343, wPt-1715, wPt-4298, wPt-4869, wPt-7362, wPt-4010, wPt-7191, wPt-7351, Pt-8417, wPt-4045, *gwm611*	21.0

For each QTL, the chromosome position, the associated markers and QTL features are reported

[a] Position of the QTL most associated marker as from the durum consensus map used as reference

[b] Range of R^2 value across the evaluation seasons with significant marker-trait association

[c] R^2 value for the marker most associated with the QTL (average over the four evaluation seasons)

study are reported in Supplemental Table 3 and 4, with the durum accessions that were sorted based on their population structure (supplemental Table 3) and mean stem rust response (supplemental Table 4) over the four evaluation seasons. For the same QTL-tagging markers, the least square means of the corresponding alleles are reported in supplemental Table 5.

Table 7 reports the frequencies in the five main germplasm subgroups of the non-rare alleles at the QTL-tagging markers that were significant in three to four seasons. Inspection of allele frequencies as reported in Table 7 indicates that allele fixation within subgroups was rare and further suggests that, in most cases, the frequency of the resistant alleles and of the other common alleles can be considered as balanced (>0.20), hence informative. In general, common alleles were present with balanced frequencies—the best condition to maximise the reliability of the association assay—in two or three subgroups; while *barc104* (chromosome 6AL), wPt-2799 (chromosome 7AS) and wPt-7785 (chromosome 7AS) showed balanced allele frequencies across four or five subgroups. For each QTL-tagging marker, the frequency of the beneficial allele/

Table 6 Allele frequencies and phenotypic coefficients of infection (CI) least square means for the markers most associated with the QTLs consistently observed over three to four evaluation seasons

Chromosome	Marker	Allele[a,b]	Allele frequency	CI least square means				
				DZo-2009	DZm-2009	DZo-2010	DZm-2010	Mean over four seasons
1BS	*barc8*	257	0.23	52.7	**70.0**	**87.2**	**48.3**	**63.6b**
		255*	0.77	46.9	**46.4**	**67.9**	**36.3**	**49.4a**
2AS	*gwm1045*	Null	0.12	52.3	**60.9**	**84.8**	**51.9**	**62.1b**
		180*	0.76	50.5	**47.5**	**74.4**	**39.1**	**52.9a**
		172	0.12	56.5	**64.1**	**95.2**	**48.1**	**65.9c**
2BL	*wmc356*	180*	0.12	**22.4**	**20.0**	**61.1**	23.3	**32.4a**
		178	0.69	**40.2**	**34.2**	**74.3**	33.7	**45.5b**
		176	0.19	**47.6**	**37.9**	**89.9**	27.5	**49.9c**
3AS	wPt-7992	1	0.21	**59.9**	**64.1**	**80.9**	**47.8**	**62.3b**
		0*	0.79	**50.2**	**49.9**	**73.3**	**40.4**	**53.3a**
3AL	*wmc388*	250	0.29	**61.4**	53.3	**75.7**	**43.4**	**57.4b**
		258	0.38	**60.2**	55.9	**81.6**	**44.6**	**60.8c**
		275*	0.33	**47.9**	45.9	**70.7**	**35.5**	**49.6a**
5AL	*gwm126*	Nu11	0.46	**51.0**	48.8	**74.8**	**41.3**	**53.7b**
		214*	0.42	**41.8**	41.0	**67.2**	**32.9**	**44.8a**
		208	0.12	**49.8**	44.9	**77.2**	**42.3**	**52.5b**
5AL	*gwm291*	166	0.45	**49.9**	**48.3**	**71.8**	39.1	**51.9b**
		160	0.40	**54.6**	**59.2**	**81.9**	43.2	**59.6c**
		139*	0.15	**42.9**	**44.9**	**60.7**	39.7	**47.3a**
6AL	*gwm427*	212	0.72	**55.1**	**53.6**	**76.4**	45.1	**57.5b**
		188*	0.28	**45.8**	**43.6**	**69.9**	33.4	**48.5a**
6AL	*CD926040*	855*	0.32	**50.1**	**49.8**	**73.1**	**39.8**	**53.4a**
		851	0.40	**68.9**	**61.3**	**83.8**	**55.7**	**66.9b**
		845	0.28	**61.8**	**67.8**	**89.6**	**48.6**	**67.3b**
6AL	*barc104*	206*	0.21	**50.6**	**68.6**	76.6	**32.9**	**56.2b**
		202*	0.30	**50.0**	**48.9**	73.7	**37.9**	**52.4a**
		172	0.49	**62.9**	**68.4**	81.9	**49.1**	**63.8c**
7AS	wPt-2799	1*	0.42	**48.2**	**44.7**	**70.5**	**36.6**	**49.9a**
		0	0.58	**55.6**	**59.3**	**78.2**	**45.3**	**59.6b**
7AS	wPt-7785	1	0.78	**48.6**	46.0	**71.8**	**38.7**	**50.9b**
		0*	0.12	**40.9**	41.9	**64.9**	**31.3**	**45.1a**

Data are reported for the common allelic variants only (frequency $\geq$0.10). Least square means reported with bold font refer to the marker-environment pairs showing significant associations. For each locus, the least significant difference between the allele means over four seasons was calculated: means followed by different letters are significantly different ($P \leq 0.05$)

[a] Molecular weight (bp) of the alleles at SSR markers; presence (1) or absence (0) of the band at DArT markers (wPt-)

[b] "*" indicates the most resistant allele

s was highly variable across the five germplasm subgroups. As an example, in five cases beneficial alleles were observed at relatively high frequencies (>0.50) in more than one subgroup, i.e. in all the five subgroups (wPt-7992 on chromosome 3AS), in four subgroups (*barc8* and *gwm1045* on chromosomes 1BS and 2AS, respectively), in three subgroups (*barc104* on chromosome 6AL) and in two subgroups (*wmc388* on chromosome 3AL).

Overall, subgroup 1 (ICARDA accessions bred for dryland conditions) had higher frequencies of the resistance alleles at the QTLs on chromosome 5A than the other subgroups. Subgroup 5 (CIMMYT accessions released in the late 1980s–early 1990s), though characterized by relatively high mean phenotypic responses, had higher frequencies of resistance allele at QTLs on chromosome 6A than the other subgroups.

For each locus consistently associated with stem rust resistance over seasons, in addition to reporting the allelic effects estimated as phenotypic least squared means over the whole association panel and the consistency of their

significant differences (Table 6) were further inspected within subgroups. Markers associated with the main QTLs for stem rust resistance on chromosomes 1B (*barc8*), 6A (*CD926040* and *barc104*) and 7A (wPt-2799) were considered for the comparison of the allelic phenotypic values in the entire panel and its subpopulations as these markers accounted for the largest proportion of phenotypic variation. Accessions carrying the 255-bp allele at *barc8*, the 855-bp allele at *CD926040,* the 202- or 206-bp allele at *barc104* as well as the presence of the band at wPt-2799 had significantly ($P \leq 0.05$) lower stem rust infection than the other accessions across three or more of the five subgroups that composed the panel.

The relevance of the QTL-tagging markers significant over three or four seasons in predicting the accessions' stem rust response was further investigated by regressing CI values on the cumulated number of beneficial alleles of the accessions. The scatter plot thus obtained is reported in Fig. 2. Although the significance of the linear regression was high ($P \leq 0.001$), the R^2 value of the regression was

Table 7 Allele frequency within each of the five germplasm subgroups (S1–S5) for the markers most associated with the QTLs consistently observed over three to four evaluation seasons

Chromosome	Marker	Allele[a,b]	Frequency within subgroups				
			Subgroup 1 (S1) ICARDA drylands (11)[a]	Subgroup 2 (S2) ICARDA temperate (55)	Subgroup 3 (S3) Italian and early 1970 CIMMYT (26)	Subgroup 4 (S4) late 1970 CIMMYT (56)	Subgroup 5 (S5) late 1980 CIMMYT (35)
1BS	*barc8*	257	0.00	**0.20**	**0.73**	0.12	0.15
		255*	1.00	**0.80**	**0.27**	0.89	0.85
2AS	*gwm1045*	null	**0.20**	**0.18**	**0.13**	0.10	0.07
		180*	**0.80**	**0.79**	**0.25**	0.88	0.93
		172	**0.00**	**0.03**	**0.69**	0.02	0.00
2BL	*wmc356*	180*	0.00	**0.30**	0.00	0.05	0.00
		178	1.00	**0.62**	0.17	0.84	1.00
		176	0.00	**0.08**	0.83	0.11	0.00
3AS	wPt-7992	1	0.09	**0.33**	**0.29**	0.17	0.06
		0*	0.91	**0.67**	**0.71**	0.83	0.94
3AL	*wmc388*	250	0.00	**0.37**	0.65	**0.28**	**0.06**
		258	0.00	**0.43**	0.25	**0.52**	**0.25**
		275*	1.00	**0.20**	0.10	**0.20**	**0.69**
5AL	*gwm126*	Nu11	0.18	**0.43**	**0.20**	**0.43**	0.85
		214*	0.82	**0.41**	**0.48**	**0.48**	0.15
		208	0.10	**0.16**	**0.32**	**0.09**	0.00
5AL	*gwm291*	166	0.10	0.47	**0.13**	0.42	0.77
		160	0.00	0.39	**0.63**	0.54	0.20
		139*	0.90	0.14	**0.24**	0.04	0.02
6AL	*gwm427*	212	0.00	**0.63**	0.87	**0.80**	**0.58**
		188*	1.00	**0.37**	0.13	**0.20**	**0.42**
6AL	*CD926040*	855*	0.18	**0.24**	0.08	**0.22**	**0.79**
		851	0.72	**0.44**	0.65	**0.36**	**0.12**
		845	0.10	**0.32**	0.27	**0.42**	**0.09**
6AL	*barc104*	206*	**0.33**	**0.28**	**0.24**	**0.20**	0.06
		202*	**0.33**	**0.24**	**0.05**	**0.12**	0.84
		172	**0.33**	**0.48**	**0.71**	**0.67**	0.10
7AS	wPt-2799	1*	**0.30**	**0.37**	**0.45**	**0.25**	**0.73**
		0	**0.70**	**0.63**	**0.55**	**0.75**	**0.27**
7AS	wPt-7785	1	**0.50**	**0.78**	**0.73**	**0.80**	1.00
		0*	**0.50**	**0.22**	**0.27**	**0.20**	0.00

Least square means reported with bold font refer to the marker-environment pairs showing significant associations

[a] Molecular weight (bp) of the alleles at SSR markers; presence (1) or absence (0) of the band at DArT markers (wPt-)

[b] "*" indicates the most resistant allele

very low (5.6 %). As expected, the regression coefficient was negative ($b = -1.75$). The increase in resistance associated with the cumulative effects of the beneficial alleles is also revealed by the comparison between the response values predicted for zero beneficial alleles (CI = 48.3) and the maximum number (9) of cumulated beneficial alleles (CI = 32.5). The significance of the regression was also tested for the pool of QTL-tagging markers when considering only the accessions with the susceptible allele at *CD926040*, the marker most associated with the *Sr13* region; also in this case the regression on the number of beneficial alleles was highly significant ($P \leq 0.001$), with the b coefficient and the R^2 value equal to −3.52 and 16.1 %, respectively.

Discussion

A better understanding of the genetic basis underlying the durum wheat response to Ug99 and durum-specific Ethiopian races of stem rust will help enhancing disease resistance of this crop globally, while shedding light on the evolution of durum wheat-stem rust relationships in East Africa. To this end, association mapping (AM) is a useful approach as indicated by the growing interest in its application to identify disease-resistance genes/QTLs in a wide range of crops (Ersoz et al. 2009; Hall et al. 2010; Maccaferri et al. 2010).

The AM durum panel evaluated in the present study encompasses a large portion of the genetic variation present in the elite germplasm pools commonly used by durum breeders. Only very few landraces/pre-Green Revolution genotypes were kept because of their "founders" role and significant contribution to the development of some of the modern germplasm groups. The predominance of elite germplasm in this panel was justified for several reasons. First, the presence in the elite germplasm of LD which extends over rather long distances, as shown in Maccaferri et al. (2005, 2006, 2011a, b) enabled us to conduct a genome-wide scan with an average marker density matching the genotyping capacity allowed by the marker systems currently available for durum wheat, mainly SSR and DArT markers (Maccaferri et al. 2003, 2008). Second, very little information about useful loci for quantitative stem rust field resistance is available in durum wheat and, thus, the modern germplasm pool was considered as the primary target for such investigation. Finally, the high homogeneity in phenology of the elite materials herein considered (Maccaferri et al. 2006) as compared to the higher heterogeneity in phenology observed in other AM collections, particularly those including landraces (Wang et al. 2012), allowed for a more meaningful assessments of the disease responses.

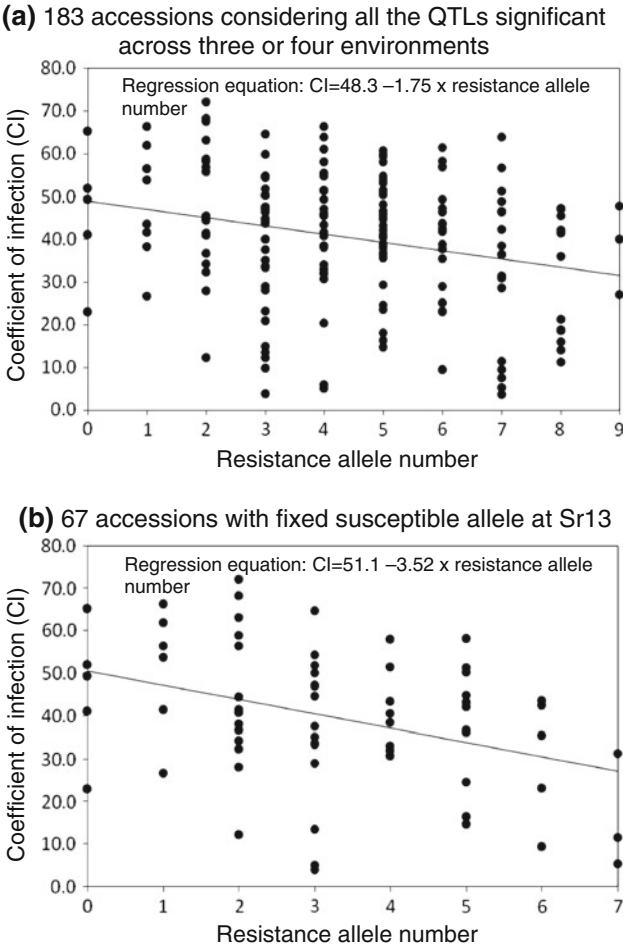

Fig. 2 Scatterplot of the coefficient of infection values of the elite accessions of durum wheat on the cumulated number of beneficial alleles at the QTL-tagging markers significant ($P < 0.05$) in three to four seasons. Results are shown for the stem rust response averaged over four evaluation seasons of the 183 accessions (**a**) and of the 67 accessions (**b**) with the susceptible allele at *CD926040*, the marker most tightly associated with the *Sr13* region

Response of the elite durum wheat germplasm to stem rust under field conditions

Highly significant genotype × season interactions were detected within the AM panel used in this study. These interactions were not only due to magnitude effects, since the stem rust response of some accessions varied from resistant in one season to clearly susceptible in another season. This finding was confirmed by the values of correlation coefficients between accession responses in different seasons that even if highly significant were quite low ($r < 0.58$). These interactions could be explained in part by the different growing conditions prevailing in different seasons, which are known to affect disease incidence and intensity. Such inter-season effect on disease intensity is clearly seen in the increase in average intensity in the

warmer off-seasons compared to the more temperate conditions during the main-seasons. Most importantly perhaps, genotype × season interactions may have been due to the use of a mixture of races with different virulence spectra rather than a single-race. The different races, especially the least characterized durum-specific ones, may have impacted differently on final reaction in different seasons, due to different starting relative quantities of inoculum, fitness or interactions with season-specific environmental and/or inoculation conditions. However, the use of such a mixture rather than single race inoculum, while predictably complicating the interpretation of the results, was essential for this study to address comprehensively stem rust threats that are relevant to durum wheat breeding under field conditions. The use of Ug99 or its more recent variants alone, all avirulent on *Sr13*, would have had limited relevance to global durum wheat breeding as resistance to them is present in most germplasm groups worldwide. On the other hand, the exclusive use of the Ethiopian races, as single isolates or mixtures, because of their unclear virulence spectrum, would have likely provided incomplete information as to the global usefulness of sources of resistance or genomic regions involved in controlling such resistance. Also, the presence of Ug99 in the mixture was important, since this is the only race that so far has migrated out of Africa into Asia and could, therefore, become the first threat to the South Asian durum-growing areas. Whatever the reason for the highly significant genotype × season interaction, its effects were mitigated and robustness of our conclusions was supported by the analysis of single-season data in addition to the results averaged over seasons. Genotypes were considered resistant or susceptible only when they performed as such consistently across seasons, and phenotype-marker associations, as discussed below, were considered relevant only when they were significant in at least three of the four seasons.

Nevertheless, clear trends in the distribution of genetic resistance present in this AM panel were observed and reliable conclusions could be drawn. First the very low frequency (5 % of all accessions) of high-level resistance, expressed as reactions that are consistently close-to-immune or always below 10 % DSS, supported the conclusions from previous studies that elite durum wheat germplasm is relatively poor in genes with major effects providing complete field resistance to stem rust (Singh et al. 1992; Bonman et al. 2007). This also agree with results from evaluations conducted in Ethiopia at the onset of the Borlaug Global Rust Initiative in 2007–2008 which showed only 3 % of resistant lines within the CIMMYT elite germplasm tested in that year (Ammar and Badebo, unpublished). This trend seems to extend to wider germplasm groups as shown by Olivera et al. (2010), who reported 5.2 % of field resistance in a worldwide collection of 996 durum wheat accessions and other tetraploid relatives under conditions and with races similar to those used in the present study.

Another interesting reaction group includes genotypes showing DSS between 10 and 20 %, most with R-MR to MS type pustules, with a reaction type very similar to that of local Ethiopian cultivars such as Boohai or Ude, considered adequately resistant to be competitive in most areas of Ethiopia. In the present study, 9 % of the genotypes were consistently classified in this group and, therefore, could be considered as valuable resistance sources for breeding programs, possibly providing usable resistance genes.

In contrast to the low frequency of accessions with high levels of resistance, a sizeable portion (at least 28 %) showed a DSS consistently between 30 and 40 %. Such intermediate, albeit susceptible, values could indicate, if accompanied by seedling susceptibility to the races investigated in this study, relatively high frequencies of minor genes conferring quantitative and partial field resistance to both Ug99 and the Ethiopian durum races of stem rust. The accumulation of such genes in a single genotype might result in durable race non-specific resistance at levels comparable to that conferred by major gene-based resistance (Skovmand et al. 1978; Lagudah 2011; Singh et al. 2011). Along with this hypothesis, genotypes useful as sources of minor gene-based resistance to leaf rust have already been identified in durum wheat (Herrera-Foessel et al. 2007) and the improvement of resistance through the cumulation of such genes has been demonstrated (Herrera-Foessel et al. 2009).

Alternatively, the low rust response observed in some accessions included in the present study may be due to the presence and, possibly, cumulation of race-specific seedling genes, which exhibit moderate resistance to moderate susceptibility at adult plant growth stage.

Genetic basis of the resistance to stem rust in durum wheat and relevance to breeding

Based on the observation that complete immunity to the Ethiopian races was seldom observed in the field under heavy infection conditions, it has been suggested that resistance in durum wheat elite germplasm was likely to be based on additivity, i.e. resulting from the cumulative effect of additive beneficial alleles from multiple loci (major and minor) of variable effect (Osman Abdallah, personal communication; Ayele Badebo, personal communication). This hypothesis is clearly supported in the present study by the fact that improved resistance response was always associated with several genomic regions (36 in total), each contributing a small fraction of the variability associated with field reaction while none of them was

individually capable of providing a high level of resistance. When estimated in single seasons, each QTL identified in this study explained not more than 13 % of the phenotypic variation for stem rust resistance. Even though QTL effects estimated via AM are usually lower than those estimated through biparental mapping, due to the higher complexity of the genetic control in association panels as compared to biparental populations (Brachi et al. 2011), the fact that even the most resistant, close-to-immune genotypes did not owe their resistance to a single major QTL, indicates the marginal role of classical major genes in determining field resistance, as often seen in bread wheat.

However, it is also known that most of the seedling major genes described for stem rust resistance in wheat, including *Sr13* of tetraploid wheat, when evaluated at the adult plant stage confer medium-resistance to medium-susceptibility rather than complete resistance/immunity.

Recently, the hypothesis regarding the presence of a relatively complex control (oligogenic or polygenic) has been strengthened by the results obtained from the genetic mapping of the factors responsible for the resistant response of the ICARDA elite cultivar Sebatel (Haile et al. 2012), also included in this study. The genetic basis of Sebatel resistance turned out to be oligogenic, with nine QTLs (including major and minor ones) identified in the RIL population and R^2 values ranging from 5.0 to 34.0 %.

Another aspect that can contribute to explain the different results found between durum and bread wheat is that the elite breeding germplasm of durum wheat has not been improved in the past decades by means of an extensive use of wide-crosses to introgress alleles with strong phenotypic effects (Maccaferri et al. 2005), as has been the case with hexaploid wheat.

In the absence of single-race analysis at seedling and adult stages with a wide collection of races, conclusive evidence cannot be drawn as to the nature of the resistance observed in the present study. Nevertheless, this report on the oligogenic nature and likely minor gene basis of stem rust resistance in durum wheat has important implications for breeding activities. It suggests that deploying the sources identified in this study in a resistance breeding program would result in an increase of resistance that would likely be more durable as compared to a monogenic, major gene resistance. However, unlike the large-effect QTLs that are easily identified and maintained in breeding populations through phenotypic selection and can be easily managed via marker-assisted selection (MAS), the simultaneous handling of small-effect QTLs is much more complex. In fact, an effective phenotypic selection for small-effect loci requires well-planned populations and intense, uniform epidemics at every cycle of visual selection to readily detect and accurately score transgressive segregants. Under these conditions, the availability of useful markers reliably tagging the minor QTLs and the ready access to MAS facility becomes critical. In the near future, the availability of high-density single nucleotide polymorphism (SNP) platforms including thousands of highly multiplexed assays will allow for a nearly complete genome coverage and the possibility to switch from single-marker to haplotype-based analyses, thus enabling a full exploitation of the potential of AM (Akhunov et al. 2009; Trebbi et al. 2011; You et al. 2011; Kaur et al. 2012; van Poecke et al. 2012). The use of the same SNP assays in applied breeding programs will also facilitate the simultaneous selection of multiple beneficial alleles for partial resistance. Thus, MAS strategies that can effectively deal with a relatively high number of markers and haplotypes are required to accumulate and maintain the beneficial alleles at these small-effect QTLs in order to achieve an acceptable and durable level of resistance for stem rust within durum breeding populations (Kuchel et al. 2007). With this aim, recent advances in the implementation of genomic selection in crop species, in particular to improve stem rust resistance in hexaploid wheat (Rutkoski et al. 2010), indicate that this could be the most efficient approach to exploit the potential of high-density molecular marker screening tools.

QTLs identified through association mapping and relationship with previously described QTLs and Sr loci

The joint Q GLM and Q + K MLM association analyses highlighted several chromosome regions putatively harbouring QTLs with main effects of varying magnitudes on field stem rust response. As expected, multiple-test correction drastically reduced the number of significant regions, a condition not well-suited for an exploratory analysis like the present one. In addition, our goal was to keep a reasonable power to identify loci conferring partial resistance with alleles characterized by relatively small effects. Therefore, the most significant chromosome regions based on the less stringent marker-wise significance test have also been considered, provided that the associations were significant on the season average data and in at least two of the four seasons.

Several QTLs identified in this study co-located with previously reported major *Sr* loci as well as with a number of QTLs recently identified through AM in hexaploid wheat (Yu et al. 2011) and in tetraploid wheat (Haile et al. 2012). Others, namely those discovered on chromosomes 1A, 1B, 3A, 4A, 4B, 5A, 5B, 7A and 7B were not reported elsewhere. These results highlight the effectiveness of AM to dissect and target the genetic basis of moderately complex traits while showing its potential to unveil the presence of previously unknown QTLs, provided that an

appropriately balanced and phenologically suitable set of accessions are evaluated.

On chromosome 1A, significant effects were identified in the distal end and in the short arm near the centromere. In both cases, significant effects were also reported in hexaploid and tetraploid wheat, respectively, within 10 cM distance from the significant markers, with associated R^2 values of ca. 5.0 %. Highly significant effects were detected near to the centromere of chromosome 1B, between 30 and 40 cM from the top of the chromosome. In the cultivated hexaploid wheat germplasm, these markers could either tag *Sr14* or *Sr31*. In several hexaploid wheat cultivars, chromosome 1BS is known to harbour the 1B·1R translocated gene *Sr31* (Zeller 1973), which is present in the hexaploid wheat germplasm only, while the centromeric region of chromosome 1B is known to harbour *Sr14* (McIntosh 1980), which originated from the tetraploid wheat (Khapli emmer; Heermann and Stoa 1956) and has been shown to be effective against Ug99 races (Singh et al. 2006). *Sr14* should be located on chromosome 1BL very close to the centromere (McIntosh 1980), a location compatible with the mapping position of the significant markers represented by the QTL-tagging markers *barc8* (in close linkage with *gwm1100* and nine DArT markers) and *cfd65* (in close linkage with the significant wPt-8168 and *gwm947*). Due to the absence of the 1B·1R translocation in the present durum panel, the effect herein detected is likely due to *Sr14*. A recent AM study in a panel of spring hexaploid wheat (Yu et al. 2011) showed the presence of a QTL associated with stem rust response precisely in the same region of chromosome 1BS, near the centromere (wPt 1560 at 8.6 cM and wPt5678 at 33.7 cM). Notably, the DArT markers identified by Yu et al. (2011) as associated with stem rust resistance in spring hexaploid wheat were reported to tag resistance gene loci located on chromosome 1BS instead of 1RS as it would be the case in presence of a functional *Sr31* allele. Moreover, three DArT markers significantly associated with stem rust response were reported in the same region by Crossa et al. (2007). The presence of the *Sr14* resistance allele in durum wheat germplasm can be traced back to *Triticum dicoccum* Schrank accessions such as Khapli emmer, which is known to carry *Sr14* and is also considered as one of the few founders of modern durum wheat germplasm (Autrique et al. 1996). *Sr14* has been considered as one of the causes of stem rust resistance in some synthetic wheat-derived lines (Njau et al. 2010).

Additional overlap with a minor QTL for stem rust resistance in durum wheat described by Haile et al. (2012) occurred on chromosome arm 2AS.

On chromosome arm 2BL, *gwm1300* and *wmc356* (50.9 cM apart) were significantly associated with stem rust resistance for two and three seasons, respectively. These markers mapped in regions corresponding to the putative locations of *Sr9/SrWeb* and *Sr28/Sr16*, respectively. At the *Sr9* region, two alleles are known: *Sr9e* which was reported to be ineffective against Ug99 at the seedling stage while showing MR to MS infection types in the field nurseries (Jin et al. 2007) and *Sr9g*, which provides field resistance to Ug99 and to the Ethiopian races. *Sr9e* is present in many durum wheat genotypes, including the CIMMYT landmark Yavaros C79 and its sister line Karim 80, which in the present study were classified as moderately resistant to moderately susceptible. *Sr9g* is one of the resistance alleles reported to be present in the durum cultivar Iumillo (McIntosh et al. 1995).

Several regions with significant associations to field reaction to stem rust were detected on chromosome 3A where *Sr27* and *Sr35*, both effective against Ug99, have been reported (McIntosh et al. 1995; Singh et al. 2006). However, as *Sr27* originated from a wheat-rye translocation engineered exclusively in bread wheat and *Sr35* from *Triticum monococcum*, transferred to some tetraploids of Canadian origin, none of which was present in this study or in the pedigree of the accessions of the AM panel, the chromosome 3A related associations detected herein are likely to involve novel loci or alleles.

The distal region of chromosome 3BS is known to harbour *Sr2*, a gene that confers effective partial resistance to Ug99 at the adult plant stage (Mago et al. 2011b). In hexaploid wheat, the beneficial *Sr2* allele is actively selected by MAS (Mago et al. 2011a). Although the effective alleles originates from the tetraploid wheat germplasm (Yaroslav emmer), *Sr2* was not detected as a locus relevant for stem rust response in the durum elite germplasm considered in this study. Nevertheless, *Sr2* has been reported as the major component of resistance in the durum RIL population developed from the Sebatel × Kristal (R^2 = 34.0 %, Haile et al. 2012). The SSR markers used to characterize the *Sr2*-associated haplotype (*gwm533* and *barc133*) showed that this haplotype was rare in the sample of elite durum wheat accessions herein considered and the corresponding marker alleles were, therefore, not considered for the association test. This observation can be considered as an instructive example regarding one of the limitations of association mapping versus the use of specifically developed mapping populations.

On chromosome 6A, AM highlighted six QTLs with significant effects on field stem rust reaction. One of these regions (approximately 8 cM wide) tagged by wPt-7330, in the distal portion of chromosome arm 6AS colocates with the region known to harbour *Sr8*, a gene known to be ineffective against Ug99 (Singh et al. 2006). Interestingly, this region completely overlapped with a QTL for stem rust resistance recently reported in an AM study in hexaploid

wheat (Yu et al. 2011). A wide region on chromosome arm 6AL, about 40 cM wide, plays a major role in controlling stem rust response in the durum wheat germplasm tested herein. This region in our association mapping panel includes two distinct sub-regions harbouring effective but most probably distinct genes, including *Sr13*.

The first, proximal sub-region, tagged by tPt-4209, *gwm1150* and *gwm169* and associated with stem rust resistance in this study, colocates with *Sr26*, a gene effective against Ug99 (Singh et al. 2006) and the Ethiopian races (Ayele and Ammar, unpublished results). However, the presence of the known *Sr26* allele in the AM panel or in the durum wheat germplasm at large is unlikely, since the *Sr26*-resistant allele has been introgressed from the wild relative *Thinopyrum ponticum* exclusively into bread wheat. A gene/allelic variant other than *Sr26* should be located in this sub-region. This QTL region has been independently confirmed in the Sebatel × Kristal durum population and reported as *QSr.IPK-6A* (Haile et al. 2012), a QTL with R^2 value equal to 9.3 % and tagged by the SSR markers *gwm494-gwm1150*.

The second, distal sub-region of chromosome 6AL includes three further sub-regions (tagged by *gwm427*, CD926040 and *barc104*) strongly associated with stem rust response. These sub-regions colocate with *Sr13*, which has been mapped in tetraploid wheat to chromosome 6AL within a 1.2–2.8 cM interval, flanked by the EST-derived markers *CD926040* and *BE471213* (Simons et al. 2011). In our study, *CD926040* showed the maximum R^2 value and was consistently significant across all four seasons. *Sr13* is effective against the TTKS complex of *Puccinia graminis* ssp. *tritici*, namely TTKSK (Ug99), TTKST and TTTSK. However, virulence for *Sr13* within Ethiopian stem rust populations has been suspected for some time, and recently confirmed by the characterization of the TRTTF and JRCQC isolates collected from the Ethiopian site in Debre Zeit (Olivera et al. 2010, 2012). Therefore, while very effective against the TTKSK or Ug99 lineage—the only ones so far to have migrated out of Africa, its presence alone is not sufficient for adequate protection in Ethiopia. This is clearly seen when comparing the field reaction and the long-range haplotype in the extended *Sr13* chromosome region of two US desert-durum cultivars, namely Kronos and Kofa, which were considered in the present study. While both cultivars exhibited the haplotype of Khapli Emmer, known to carry *Sr13* (Knott 1962), Kronos had one of the most consistently resistant reactions over seasons while Kofa was regularly susceptible. Taking into account all of the above information, the presence of the resistant allele(s) in the *Sr13* region is valuable for breeding activities and should be pursued for pyramiding multiple useful alleles.

Specifically for the *Sr13* locus, Simons et al. (2011) found different linked marker alleles among the *Sr13* donors, suggesting that breeding programs used different sources of *Sr13* or that independent recombination events occurred between loci. In our study, the durum wheat accessions Khapli, Kofa and Kronos were the donors of resistant *Sr13* alleles (Simons et al. 2011). The LD decay among the three main linkage blocks (tagged by *gwm427-wmc580*, *CD926040* and *barc104*) near *Sr13* and the variation in band sizes of the marker alleles indicate that the current markers are not fully diagnostic in a wide range of backgrounds and, therefore, cannot be used to predict with high confidence the presence of *Sr13* in unknown sets of germplasm. This notwithstanding, these markers can be used to follow the *Sr13* resistant alleles in segregating populations involving parental lines (e.g. Khapli, Kofa and Kronos) related to any of the known *Sr13* sources. The future availability of high-density, SNP platforms (Trebbi et al. 2011; van Poecke et al. 2012) will likely provide much better haplotype resolution.

The significant effects identified in chromosome arms 6BS, 7AS and 7BS are specific to the durum germplasm considered here. They have not been reported as QTL locations in bread wheat nor in the Sebatel × Kristal population.

On the distal portion of chromosome 7AL, DArT markers with significant effects on stem rust resistance in our study overlapped with the locations of *Sr22* and *QSr.ipk-7AL* (Haile et al. 2012), providing independent evidence for the relevance of this chromosome region for stem rust response. Finally, AM detected QTLs at the distal end of chromosome arm 7BL (QTL-tagging marker wPt-8615), with several DArT markers associated with stem rust resistance. This region colocates with that known to harbour *Sr17*, a gene linked to *Lr14a* and *Pm5* in bread wheat (Crossa et al. 2007). It also is consistent with a region reported to include a stem rust QTL in the Arina × Forno RIL population (Bansal et al. 2008). *Sr17*-related resistance to stem rust has been reported in tetraploid wheat or synthetic bread wheat (Bansal et al. 2008). Consistent with this, in the present study, there was no relationship, either in coupling or repulsion, between stem rust resistance and the presence of *Lr14a* (known from previous studies on the same panel). This may indicate that the two genes are far enough apart that no linkage was detected.

Based on the results herein presented, it is clear that quantitative, additive variation is present in the elite germplasm at chromosome regions known to carry well-characterized resistance genes (*Sr14*, *Sr28-Sr16*, *Sr8* and particularly *Sr13*) whose alleles are tagged by known molecular markers and are known to be frequently defeated by specific races or non-effective at seedling stage. Although further work would be required to confirm the presence of known alleles of these genes, our results may reflect appreciable residual quantitative and additive effects of seedling resistance genes at the adult, open field stage.

The low rust response phenotypes observed in the present study seem to be due to the presence of combinations of resistance genes including previously designated genes and novel genes/QTLs.

Implications for marker-assisted breeding

Association mapping in elite germplasm has the potential to accelerate the translation of basic genetic information towards applications in crop improvement and cultivar release. Our study shows that AM effectively complement bi-parental mapping studies by providing independent validation of previously detected QTLs and discovering new QTLs. In addition, our study highlighted the presence of valuable genetic variation that could be exploited to sustainably enhance stem rust resistance in durum wheat. This study clearly documented the oligogenic or minor gene-based nature of resistance to Ug99 and the Ethiopian races of stem rust in durum wheat. Several chromosome regions harbouring putative QTLs involved in the stem rust response in the field under high infection rate were consistently detected across seasons; the allelic variation at these QTLs can be exploited for further validation studies and utilization in MAS programs. The AM results reported herein confirm the important role of the *Sr13* region, but also its limitation in individually addressing the presence of the Ethiopian races. Our analysis also highlighted the role of chromosome regions putatively harbouring *Sr14*, *Sr9* and *Sr17,* to be further dissected as providing alleles with beneficial effects on final resistance, but again not sufficiently strong individually. In addition, the AM analysis strengthens the role of five QTLs recently described in the Sebatel × Kristal durum mapping population and located in chromosome regions where no designated resistance genes were mapped. Novel regions, previously not reported to be associated with stem rust resistance either in durum or bread wheat, have been detected. These regions contribute minor genes that can be cumulated through either traditional cycles of recurrent selection or MAS using markers in classical cross and backcross schemes or by means of a more comprehensive genomic selection approach towards the release of durum wheat cultivars with a more durable resistance to stem rust.

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