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ORIGINAL PAPER

Identification of novel genomic regions associated with resistance to *Pyrenophora tritici-repentis* races 1 and 5 in spring wheat landraces using association analysis

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Abstract Tan spot, caused by *Pyrenophora tritici-repentis*, is a major foliar disease of wheat worldwide. Host plant resistance is the best strategy to manage this disease. Traditionally, bi-parental mapping populations have been used to identify and map quantitative trait loci (QTL) affecting tan spot resistance in wheat. The association mapping (AM) could be an alternative approach to identify QTL based on linkage disequilibrium (LD) within a diverse germplasm set. In this study, we assessed resistance to *P. tritici-repentis* races 1 and 5 in 567 spring wheat landraces from the USDA-ARS National Small Grains Collection (NSGC). Using 832 diversity array technology (DArT) markers, QTL for resistance to *P. tritici-repentis* races 1 and 5 were identified. A linear model with principal components suggests that at least seven and three DArT markers were significantly associated with resistance to *P. tritici-repentis* races 1 and 5, respectively. The DArT markers associated with resistance to race 1 were detected on chromosomes 1D, 2A, 2B, 2D, 4A, 5B, and 7D and explained 1.3–3.1% of the phenotypic variance, while markers associated with resistance to race 5 were distributed on 2D, 6A and 7D, and explained 2.2–5.9% of the phenotypic variance. Some of the genomic regions identified in this study correspond to previously identified loci responsible for resistance to *P. tritici-repentis*, offering validation for our AM approach. Other regions identified were novel and could possess genes useful for resistance breeding. Some DArT markers associated with resistance to race 1 also were localized in the same regions of wheat chromosomes where QTL for resistance to yellow rust, leaf rust and powdery mildew, have been mapped previously. This study demonstrates that AM can be a useful approach to identify and map novel genomic regions involved in resistance to *P. tritici-repentis.*

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Introduction

Pyrenophora tritici-repentis (Died.) Drechs. [(anamorph: *Drechslera tritici-repentis* (Died.) Shoem.)], the causal organism of tan spot, is a major foliar pathogen of wheat (*Triticum aestivum* L.) worldwide (Hosford 1982). *P. tritici-repentis* infects leaves and heads of susceptible wheat cultivars inducing two distinct symptoms: tan necrosis and extensive chlorosis on leaves and grain shriveling and discoloration on heads (de Wolf et al. 1998). The necrosis and chlorosis symptoms are associated with the production of multiple host selective toxins (HSTs) such as Ptr ToxA, Ptr ToxB, and Ptr ToxC (Ballance et al. 1989; Ciuffetti et al. 1997; Ciuffetti and Tuori 1999; Effertz et al. 2002; Strelkov

et al. 1999; Tomas et al. 1990). The fungus can cause 5–15% yield loss, but in some cases more than a 50% reduction in yield can occur when conditions are favorable for disease (Singh and Hughes 2006). Severe epidemics have been reported in Australia, Brazil, the United States (Schilder 1989) and Europe (Cook and Yarham 1989; Gindrat et al. 1988). Eight pathogenic races of *P. tritici-repentis* have been identified based on the differential reactions across a set of wheat genotypes (Ali and Francl 2002; Lamari et al. 2003). Among these, races 1 and 5 are commonly found in several wheat-producing countries around the world (Adhikari et al. 2008; Ali and Francl 2002).

Host plant resistance is one of the most desirable strategies to manage tan spot of wheat. Previous reports have shown that the resistance trait can be inherited both qualitatively (Gamba and Lamari 1998; Lamari and Bernier 1989) and quantitatively (Elias et al. 1989; Friesen and Faris 2004). Several single recessive genes such as *tsn1*, *tsn2*, *tsn3*, *tsn4*, *tsn5*, *tsn6*, and *tsn-syn1*, and the dominant gene, *Tsn-syn2* are known to confer resistance to necrosis (Anderson et al. 1999; Singh et al. 2006, 2008; Tadesse et al. 2006a; Tadesse et al. 2006b), while the recessive genes *tsc1* and *tsc2* condition resistance to chlorosis (Abeysekara et al. 2010; Effertz et al. 2002; Friesen and Faris 2004).

Until now, quantitative trait loci (QTL) conferring resistance to *P. tritici-repentis* have been characterized and mapped using populations (e.g., recombinant inbred lines, double haploid lines) developed from crosses between resistant and susceptible wheat genotypes (Effertz et al. 2002; Friesen and Faris 2004). The association mapping (AM) approach can be used to detect QTL in population with complex-pedigrees such as germplasm collections or natural populations (Flint-Garcia et al. 2003). AM capitalizes on the recombinant events from many lineages and utilizes linkage disequilibrium (LD) to detect significant marker-trait associations (Nordborg et al. 2002; Buckler and Thornsberry 2002; Flint-Garcia et al. 2003; Yu and Buckler 2006). The AM approach was initially used to identify alleles at loci responsible for susceptibility to human diseases (Goldstein et al. 2003). Recently, AM has been used in various crops to detect molecular markers associated with a variety of complex traits. Agrama et al. (2007) found simple sequence repeat (SSR) markers significantly associated with various agronomic traits in rice, including kernel width, kernel length, kernel width/length ratio, and 1000-kernel weight. Emebiri et al. (2010) used diversity array technology (DArT) and reported markers associated with regions that harbor amy-1 genes responsible for late maturity α-amylase (LMA) activity in wheat. Beattie et al. (2010) detected several DArT markers associated with seven malting quality traits and QTL responsible for resistance to loose smut and net blotch in barley. Association studies have also been performed to detect major and minor QTL responsible for disease resistance in maize (Kump et al. 2011), potato (Malosetti et al. 2007), barley (Roy et al. 2010; Massman et al. 2011), and wheat (Crossa et al. 2007; Tommasini et al. 2007). AM utilizes populations from diverse origin and has been shown to detect QTL with adequate resolution (Emebiri et al. 2010; Neumann et al. 2011; Roy et al. 2010; Beattie et al. 2010). The main objective of this study was to use AM approach to identify the genomic regions in wheat that may contribute to tan spot resistance from a subset of spring landraces from the USDA-ARS National Small Grains Collection (NSGC).

Materials and methods

Spring wheat landraces

In this study, 567 spring growth habit hexaploid wheat accessions (*Triticum aestivum* L.) from the NSGC were chosen for association analysis. All of the selected accessions were classified as landraces and most ($n = 533$) were part of the wheat core subset, representative of diverse geographic origin. The accessions, originating from 55 countries, were obtained from single plant selections grown at the NSGC in Aberdeen, ID.

Experimental designs

For each race of *P. tritici-repentis*, two separate experiments were conducted in growth chambers using procedures as described previously (Chu et al. 2008). Three seeds of each accession were planted in Fison sunshine mix #1 (Fison Horticulture, Vancouver, BC) contained in a single 3.8 cm wide × 20 cm long plastic cone (Stuewe and Sons, Inc., Corvallis, OR, USA). In total, nine seeds of each wheat accession were planted in three cones, and cones were placed in trays (Stuewe and Sons, Inc., Corvallis, OR, USA). Each cone was considered as experimental unit and each single plant in a cone was regarded as sample. Wheat accessions were arranged in a randomized complete block design with three replications. In each experiment, replications were random effect and spring wheat accessions were fixed effect. All experiments were conducted in growth chambers at North Dakota State University (NDSU), Fargo, ND from 2009 to 2010.

Inoculum and inoculation

To evaluate wheat accessions for resistance to *P. tritici-repentis*, seedlings of wheat accessions were inoculated as described by Lamari and Bernier (1989) using race 1 (isolate Pti2) (Adhikari et al. 2008; Ali and Francl 2002) and race 5 (isolate DW7) (Martinez et al. 2001; Friesen and

Faris 2004). The isolate Pti2 was originally collected from a wheat field in South Dakota and maintained at the Department of Plant Pathology, NDSU. Similarly, isolate DW7 was collected from durum wheat field in North Dakota (1998) and used for the production and characterization of Ptr ToxB (Martinez et al. 2001).

To prepare inoculum, a single mycelial plug (0.5-cm in diameter) was placed on V8 potato dextrose agar in 10-cm petri plates. Agar medium was prepared using 150 ml of V8 juice (Campbell Soup Company, Camden, NJ, USA), 10 g of Difco PDA (Becton, Dickinson and Company, Sparks, MD), 10 g of Difco agar, 3 g of calcium carbonate, and 850 ml of sterile distilled water. After transferring mycelia plug in petri plates, plates were wrapped with aluminum foil and incubated at 21°C for 5 days. The mycelial growth was flattened using the bottom of a surface disinfested test tube and excess water was removed from plates. The resulting cultures were incubated under continuous light for 24 h at 21°C followed by 24 h in the dark at 16°C. Approximately, 25 ml sterile distilled water was added to each plate and the conidia were dislodged by scraping the agar surface with a platinum loop/spatula. The resulting conidial suspensions were filtered through cheese-cloth and the inoculum concentration of each race was adjusted to 3,000 conidia/ml using hemacytometer. The surfactant polyoxyethylene-20-sorbitan monolaurate was added (100 µl/l) to each conidial suspension to break surface tension and 2-week-old seedlings were spray-inoculated until run-off. Following inoculation, seedlings were incubated in a mist chamber for 24 h at 100% relative humidity at 24°C. Seedlings were then moved to a growth chamber programmed for a 23/19°C diurnal cycle (day/night) with a 16-h photoperiod.

Phenotypic data analysis

Disease was assessed on second leaves 7-day post inoculation. A 1 to 5 disease rating scale (Lamari and Bernier 1989) was used, where scores ≤ 2 were considered resistant and those >2 were considered susceptible. Non-parametric analysis (Shah and Madden 2004) was performed for *P. tritici-repentis* races 1 and 5 separately using Software Proc Mixed of SAS (SAS Institute Inc. 2010) to test if the results of both the experiments were similar in order to combine the data for further analysis. Similarly, SAS program (SAS Institute Inc. 2010) also was used to calculate Bartlett's χ^2 to test the homogeneity of variances among two experiments for each race as described previously (Chu et al. 2008, 2010).

DNA extraction and genotyping

A single plant of each wheat accession was grown in the greenhouse at USDA-ARS, Small Grains and Potato Germplasm Research Unit, Aberdeen, ID. Primary leaves were harvested once the secondary leaves were completely emerged. DNA was extracted using a cetyl-trimethyl ammonium bromide (CTAB) protocol. In brief, seedling leaf tissue was placed in 2.0-ml microcentrifuge tubes, frozen at −80°C until ready for DNA extraction. Tubes containing the leaf tissue were placed in liquid nitrogen and tissue was macerated into fine powder using polybutylene (PBTP) pestles (VWR cat. no. 47747-358). The ground tissue was incubated with 1-ml extraction buffer (0.35 M sorbitol, 0.3 M TrisHCl pH 8.0, 5 mM EDTA pH 8.0, 2 M NaCl, 2% CTAB, 5% (w/v) *N*-Lauroylsarcosine, 2% (w/v) Polyvinylpyrrolidone (PVP40, K29-32), and 0.5% (w/v) sodium metabisulfite) at 65° for 1 h. DNA was extracted with 24:1 chloroform:isoamyl alcohol, precipitated with isopropanol, washed with 70% EtOH, and resuspended in 10 mM Tris buffer. DNA quantity and quality were checked using a spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE) and test gels were run for 1 h at 90 v. The samples were diluted to a concentration of 80–100 ng/µl and 50 µl aliquots were sent for DArT genotyping to Triticarte Pty. Ltd. (Canberra, Australia; http://www.triticarte.com.au). In total, 2,500 DArT loci were interrogated in the accessions.

DArT markers from a *Pst*I/*Bst*NI representation were used to genotype 567 wheat accessions as described previously (Akbari et al. 2006). To develop consensus maps, a set of whole genome-derived DArT markers were genotyped on over 80 wheat populations (recombinant-inbred lines, double haploid, F_2s) (Akbari et al. 2006; Andrzej Kilian, personal communication).

DArT marker analysis

The TASSEL program, version 2.1 (http://www.maizegenetics.net) was used to calculate LD statistic (r^2) between DArT markers. LD values were calculated for each chromosome and then combined across all three wheat genomes (Emebiri et al. 2010). A critical value of r^2 was derived using the 95 percentile of unlinked LD estimates (Breseghello and Sorrells 2006). The intergenic r^2 values were plotted against genetic distance and a curve was fitted to the plot in SAS 9.2. We plotted the intra-chromosomal r^2 values against the genetic distance, and later a distance at which the critical r^2 was intercepted was determined by plotting a LOESS curve to the plot. This would help to determine the exact location at which point the LD occurred (Neumann et al. 2011). The LD decay estimate was the point at which the critical r^2 value intercepts the spline (Neumann et al. 2011).

Association mapping

Missing data were imputed using the FastPHASE version 2.0 (Scheet and Stephens 2006) with default settings.

Minor allele frequencies (MAF) refer to the frequency at which the less common allele of the DArT markers occurs in a given population and were detected using PowerMarker 3.25 (Liu and Muse 2004). Markers with MAF <0.05 were removed from our data set in subsequent analysis.

Among the 832 polymorphic DArT markers detected, we used 309 DArT markers that have an LD <0.5 with all other random markers as described previously (Weber et al. 2007). These 309 DArT markers were used for estimating PCA. The threshold of LD <0.5 was selected as defined by Hill and Robertson (1968), and this has been previously implemented by Weber et al. (2007; 2008). PROC PRINCOMP in SAS 9.2® was used to estimate principal components (PC) that explain 25% variability (P matrix) and used in the regression model. The underlying regression statistics was:

$$y = X\alpha + P\beta + Iv + \varepsilon,$$

where y is a vector of phenotypic values, α is the fixed effect for the candidate marker, β is a vector of fixed effects regarding population structure, X is the vector of genotypes at the candidate marker. P is a matrix of the significant principal component, v is a vector of the random effects pertaining to co-ancestry, I is an identity matrix, and ε is a vector of residuals. The variances of the random effects are assumed to be $\mathrm{Var}(v) = 2KV_g$ and $\mathrm{Var}(e) = IV_R$, where K is the kinship matrix, V_g the genetic variance, and V_R the residual variance (Yu and Buckler 2006).

A permutation test using 10,000 permutations was conducted to correct the p value for multiple comparisons (Roy et al. 2010). The experiment-wise p value provides a test of significance (adjusted p value) that corresponds to the experiment-wise error which was used to make decision about the significance of marker effects. For the significant markers, we calculated the phenotypic variation (R^2) using a simple regression equation.

In addition to the regression-based association analysis, tests based on more stringent criteria were performed using JMP Genomics 5.0 (SAS Institute Inc. 2010). Initially, MAF and the proportion of missing genotypes were calculated on the 832 marker variables. DArT loci with MAF >0.05 and missing data >0.10 were removed and principal component analysis (PCA) was used to compute the Q matrix (fixed), and a pair-wise allele-sharing similarity was used to compute the Kinship (K) matrix (random). Ward's method involves an agglomerative clustering algorithm, which starts out with n clusters of size 1 and continues until all the observations are included into one cluster (Ward 1963). This method is also an alternative approach for cluster analysis and thus was used to determine the number of clusters for 567 spring wheat landraces. Data from these analyses were used to account for structure in a mixed linear model to associate numeric DArT genotypes with ordinal phenotypes using residual denominator degrees of freedom. Both genotype (categorical, ANOVA) and trend (quantitative, regression) association tests were performed using a PROC GLIMMIX procedure in SAS. Since the disease phenotypes were recorded using an ordinal scale, and the distribution was not normal, the residual pseudo-likelihood estimation (RSPL) method was applied. The negative Log_{10} (NegLog_{10}) conversion was used on all p values and the false discovery rate (FDR) multiple testing corrections were applied with no p value adjustment (Benjamini and Yekutieli 2001).

Results

Resistance to *P. tritici-repentis* races 1 and 5

All experiments had uniform infection of *P. tritici-repentis*, thus allowing ordinal classification of disease reactions as described previously (Lamari and Bernier 1989). Non-parametric analysis (Shah and Madden 2004) of phenotypic data showed that the results of both the experiments were consistent and interactions between the two experiments were not significant ($p \leq 0.05$). Homogeneity tests also indicated the data from the two experiments for each race were homogeneous. Bartlett's χ^2 were 2.48 and 3.16, and the associated p values with 1 degree of freedom were 0.13 and 0.11 for reaction to *P. tritici-repentis* races 1 and 5, respectively. Therefore, data from homogenous experiments were pooled and combined for AM analysis. Of the 567 spring wheat landraces assessed, nearly 12.9% (73 wheat accessions) were resistant to race 1 and 34% (193 wheat accessions) were resistant to race 5 (Fig. 1; Supplementary Tables 1, 2). Sources of resistance to *P. tritici-repentis* races 1 and 5 were distributed among accession of diverse origin (Supplementary Tables 3, 4).

DArT marker analysis

Nearly 33% of the markers (832 out of 2,500) were polymorphic of which 46 had MAF <0.05 and 30 had >0.10 missing genotypes across the population. Among 832 polymorphic markers, 30.6, 31.6, and 19.2% were distributed on wheat chromosome A, B, and D, respectively. Nearly 18.5% (154 DArT markers) remained unlinked due to unknown map position (*data not shown*). The highest numbers of polymorphic markers were distributed on chromosome 6A (77 DArT markers) (Fig. 2). In contrast, none of the polymorphic DArT markers were distributed on chromosome 4D (Fig. 2). The average frequency of DArT polymorphisms per chromosome was 38.3.

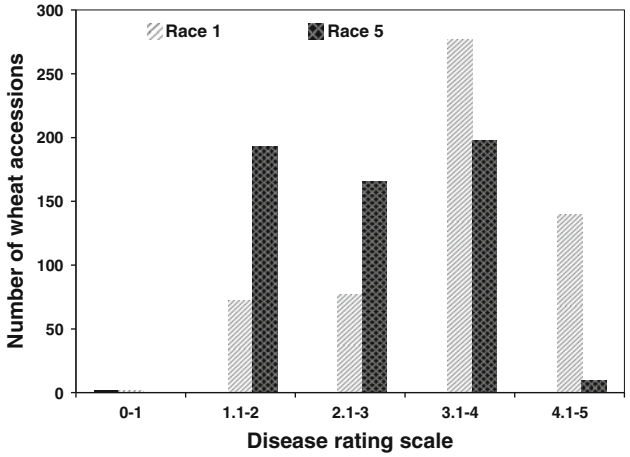

Fig. 1 Disease reactions of spring wheat landraces ($n = 567$) to *Pyrenophora tritici-repentis* races 1 and 5. Materials were evaluated in the growth chambers at North Dakota State University, Fargo, ND. Following inoculation, seedlings were incubated in a mist chamber for 24 h at 100% relative humidity at 24°C. Seedlings were then moved to a growth chamber programmed for a 23/19°C diurnal cycle (day/night) with a 16-h photoperiod. The wheat accessions were classified as resistant (R) if the average disease scores ≤2 and susceptible if the average disease scores >2 seven days post inoculation (Lamari and Bernier, 1989)

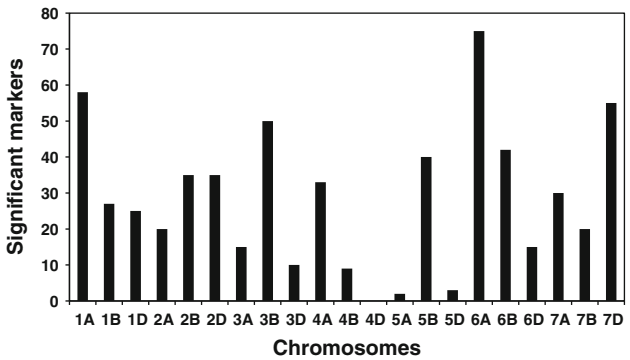

Fig. 2 Distribution of significant diversity array technology (DArT) markers throughout the wheat chromosomes

Linkage disequilibrium

The 683 polymorphic markers with known chromosomes and positions were used to calculate LD (Fig. 3). In the entire collection, 6506 (~44%) of the 14938 intra-chromosomal marker pairs showed a significant level of LD ($p < 0.01$) (Table 1). The average r^2 for all pairs was 0.058. To examine the LD value in our spring wheat accessions, a critical value for significance was fixed at $r^2 = 0.049$. This was considered the upper limit and all the LD values beyond this critical r^2 value (0.049) were considered to be result of genetic linkage (Breseghello and Sorrells 2006). We followed Maccaferri et al.'s (2005) marker classification system; where class 1 = distance <10 cM; class 2 = distance 10–20 cM; class 3 = distance 20–50 cM; and class 4 = distance >50 cM (Table 1). In the entire population, 2,546 of the pairs were in LD ($p < 0.01$) because of physical linkage, as their r^2 values were higher than the critical value of 0.049. The LD analysis showed 786 (94.5%) of the DArT loci were significant and most of these associated markers were located within 10 cM.

Relationship matrix

Hierarchal clustering using Ward's method revealed at least 11 distinct sub-clusters in the population (Fig. 4; Supplementary Table 5). Cluster 11 (baby blue group) had the greatest number of accessions ($n = 112$) followed by cluster 1 (red group; $n = 89$) and cluster 10 (army green group; $n = 76$) each, respectively. Cluster 2 (green group; $n = 26$) and cluster 7 (gold group; $n = 28$) had the fewest accessions while accessions in the remaining clusters ranged from 31 to 55 (Supplementary Table 5). The results based on pair-wise allele sharing, as displayed in the heat map (Fig. 4), showed a large portion of accessions in clusters 9, 10, and 11 shared similar alleles. Accessions in these clusters also shared about 60% of alleles with those in clusters 5, 6 and 7. Three accessions in cluster 2 shared many alleles with some accessions in cluster 4. A sub-cluster in cluster 3 shared many alleles with all accessions in cluster 9 and 2 sub-clusters of cluster 10 (Fig. 4).

PCA of the populations showed that the first PC accounted for 39.0% of the variation, while the second and third PCs accounted for 10.9 and 7.8%, respectively (Supplementary Fig. 1). Overall, the three PCs accounted for almost 60% of the genetic variation in the populations. Structure as defined by three PCs was similar as that defined by the allele sharing similarity matrix (Fig. 4) and the three main groups corresponded roughly to geographic origin with most of the Eastern Africa, Eastern Asia, South America, and Southern Europe accessions grouping in one cluster; most of the Western Europe accessions in a second cluster, and most of the South-central Asia accessions in a third cluster.

Association mapping of QTL for resistance to *P. tritici-repentis* race 1

From the AM analysis, seven putative genomic regions conferring resistance to race 1 were identified (Table 2). One distinct candidate genomic region on chromosome 1D spanning a gap of 62.1–67.0 cM was found significantly associated with five co-segregating DArT markers (Table 2). These five co-segregating DArT markers were able to explain phenotypic variations ranging from 1.8 to 2.3%. Two best putative genomic regions were identified

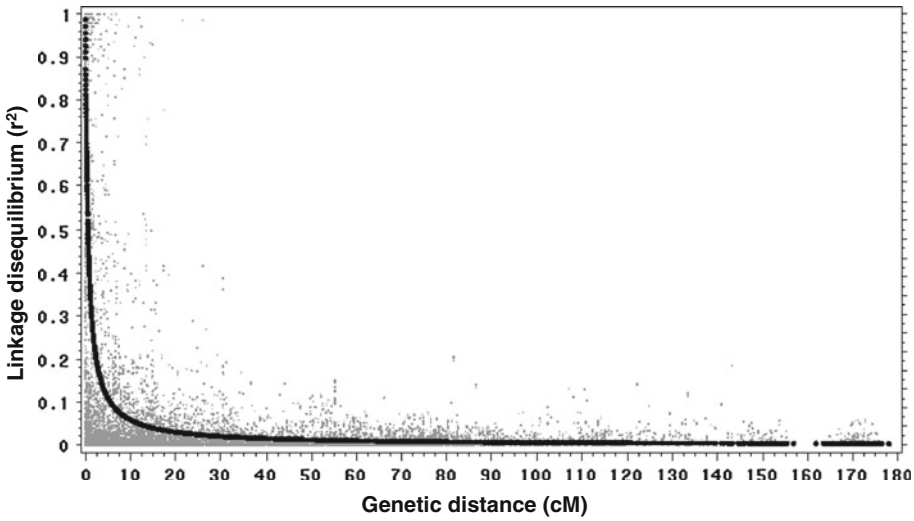

Fig. 3 Linkage disequilibrium (r^2) as a function of map distance of 683 diversity array technology (DArT) markers in a set of 567 spring wheat landraces

Table 1 Summary of linkage disequilibrium (LD) in the intra-chromosomal pairs in the entire spring wheat accessions

Class[a]	Pairs total	Mean r^2 of all pairs	No. of significant pairs	% significant pairs	No. of physically linked pairs	% of physically linked pairs	Mean r^2 for physically linked	No. of pairs in complete LD
Class 1	3,925	0.173	2,549	64.94	1,670	65.51	0.414	100
Class 2	2,071	0.034	979	47.27	342	34.93	0.148	62
Class 3	3,156	0.016	1,120	35.48	256	22.85	0.093	126
Class 4	5,786	0.012	1,858	32.11	278	14.96	0.077	220
Total	14,938	0.058	6,506	44.95	2,546	34.56	0.183	508

[a] Class 1 = genetic distance <10 cM; class 2 = distance >10; <20 cM; class 3 = distance >20; <50 cM, and class 4 = >50 cM (Maccaferri et al. 2005). Mean allele frequency correlations (r^2) for all pairs, number and percentage of significant pairs in LD ($P < 0.01$), number and percentage of physically linked pairs ($r^2 >$ critical r^2, $P < 0.01$), and number of pairs in complete LD ($r^2 = 1$) were estimated according to Neuman et al. (2010)

on chromosome 2B significantly associated with DArT marker wPt-0950 and two co-segregating DArT markers (wPt-4125 and wPt-7757). The markers on 2B explained phenotypic variations ranging from 1.6 to 3.1%. At least one putative genomic region for race 1 resistance was identified on each of the chromosome 2A, 4A and 7D. DArT markers on chromosomes 2A, 4A and 7D were able to explain phenotypic variations ranging from 1.3 to 2.3% (Table 2).

Based on Q + K mixed model association analysis without multiple testing correction, two DArT markers were associated with resistance to race 1 on chromosome 1D and 2B. Of these markers, only wPt-1505 was not significantly associated with resistance when using the regression model. Once multiple test correction was applied, a single DArT marker (wPt-5688) was significantly associated with resistance to *P. tritici-repentis* (Fig. 5a; Table 3). Interestingly, resistance association with this marker was not revealed when using PCA to account for population structure.

Association mapping of QTL for resistance to *P. tritici-repentis* race 5

Three putative genomic regions conferring resistance to race 5 were detected. One distinct candidate genomic region on chromosome 6A spanning a gap of 30.3–36.7 cM was found significantly associated with seven co-segregating markers (Table 2). These co-segregating DArT markers explained phenotypic variations ranging from 2.2 to 5.6% as assessed by R^2 value, respectively. Two co-segregating DArT markers were significantly ($p < 0.01$) associated with one putative genomic region at chromosome 7D, and explained 4.3 and 4.9% of the phenotypic variation as assessed by R^2 value, respectively. One DArT marker, wPt-664805 on chromosome 2D was found significantly ($p < 0.05$) associated and explained 3.9% of the phenotypic variations as assessed by R^2 value.

Based on Q + K mixed model association analysis without multiple testing correction, the aforementioned markers on chromosomes 2D, 6A, and 7D were significantly

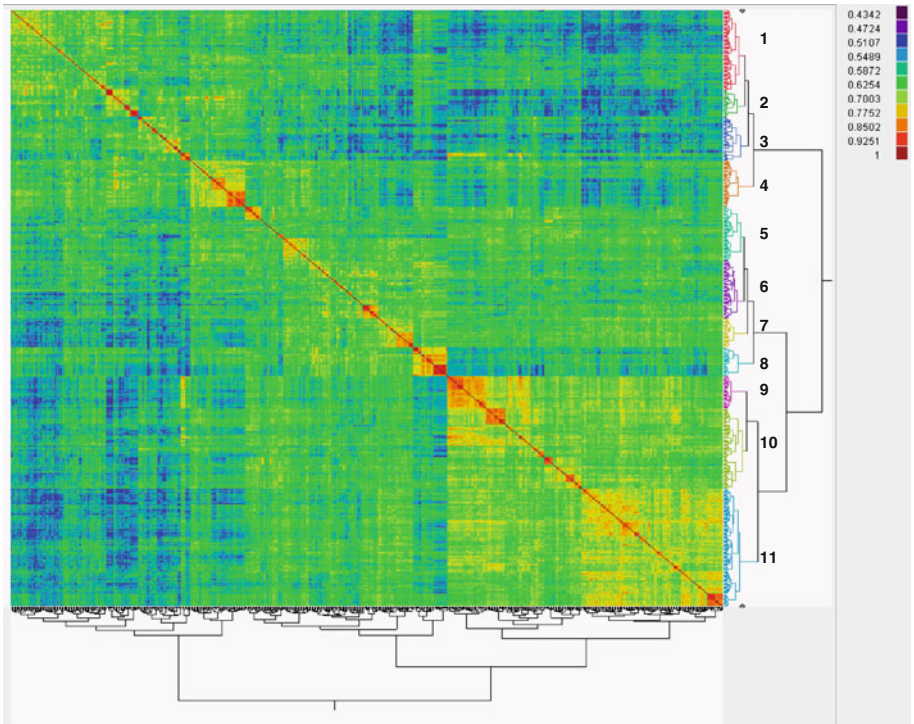

Fig. 4 Hierarchal cluster analysis of 532 wheat accessions using Ward's method with heat plot based on a pair-wise allele-sharing similarity matrix. Individual clusters (*colored*) were arbitrarily determined by genetic distance. Clusters are arranged from 1 (*top right/left bottom*) to 11 (*bottom right* and *right bottom*) in an ascending order. Accessions within each cluster are denoted in Supplementary Table 5. Heat plots indicate pair-wise marker-to-marker correlation between two accessions. *Red blocks* indicate accession with numerous shared alleles, while *blue block* indicate few shared alleles

associated with resistance to race 5 (Fig. 5b, c; Table 3). In addition, the DArT loci wPt-664400 on chromosome 7D was also significantly associated with resistance. Once multiple test correction was applied, only association with the markers on chromosomes 2D and 7D were significant.

Discussion

Host resistance is the most economically and environmentally sustainable method of controlling tan spot disease in wheat (de Wolf et al. 1998). Until now, six genes and few minor QTL responsible for tan spot resistance have been identified in cultivated wheat and advanced breeding lines (Anderson et al. 1999; Chu et al. 2008, 2010; Faris et al. 1996; Faris and Friesen 2005; Gamba and Lamari 1998; Li et al. 2011; Tadesse et al. 2006a, 2006b; Singh et al. 2008, 2009, 2010). Since new *P. tritici-repentis* races are emerging through natural selection (Ali et al. 2010), development of new resistance sources is imperative. In this study, we applied the AM approach to identify marker-trait associations in a subset landraces representing the broad range of geographic origin in the NSGC. Although the wheat genome has not been sequenced, DArT markers could be an ideal marker system to identify QTL using the AM (Raman et al. 2010). The present study is the first DArT marker-based the AM approach used to detect genomic regions in spring wheat landraces for resistance to prevalent races 1 and 5 of *P. tritici-repentis*.

To determine the extent of LD in this study, we used 683 polymorphic markers which have known chromosomal location in wheat. Data analysis revealed rapid decay of LD within about 10 cM. LD decay from 10 to 40 cM has been observed in several previous studies (Chao et al. 2007; Crossa et al. 2007; Emebiri et al. 2010) using different sample sizes and different marker systems. Nearly 30.6% of DArT markers were significant and located on chromosome A with marker coverage of 1/10 cM. However, chromosomes B and D had 31.6 and 19.2% significant markers, respectively. It appeared that the marker system used in this study did not provide full coverage for discovering all causal variants in such a diverse spring wheat landraces analyzed. For complete genome coverage, LD values between all neighboring markers should be high ideally ranging from 0.8 to 1. Although low density DArT markers were used, we were able to discover several significant marker trait associations, indicating the utility of AM approach for investigating the genetic basis of resistance to *P. tritici repentis*.

A linear model that incorporates population stratification control by PCA (Price et al. 2006) was initially used in this study. Based on the model, putative genomic regions affecting resistance to race 1 on chromosomes 1D, 2A, 2B, 2D, 4A and 7D and three putative genomic regions conferring resistance to race 5 on chromosomes 2D, 6A, and 7D were detected. Intriguingly, no markers were identified on chromosome 4D. Sadeque and Turner (2010) also did not find any markers on chromosome 4D from DArT

Table 2 Quantitative trait loci (QTL) for resistance to *Pyrenophora tritici-repentis* races 1 and 5 identified through association mapping in a group of spring landraces of diverse origin from the USDA-ARS National Small Grains Collection (NSGC)

QTL[a]	Position (cM)[b]	*p* value[c]	R^2 (%)[d]	Type	Reference
Rts1 1D-wPt-0413	62.09	0.001285299**	2.34	Novel	
Rts1 1D-wPt-9380	66.14	0.000199683**	1.79		
Rts1 1D-wPt-666832	66.97	4.62729E−05***	2.1		
Rts1 1D-wPt-664609	66.97	0.001285299**	2.31		
Rts1 1D-wPt-671545	66.97	0.001285299**	2.31		
Rts1 2A-wPt-6662	87.22	0.001312906**	1.25	QTL	Chu et al. (2008)
Rts1 2B-wPt-0950	86.57	0.001695239**	1.58	QTL	Li et al. (2011)
Rts1 2B-wPt-4125	63.23	0.003518832*	3.06	Novel	
Rts1 2B-wPt-7757	63.23	0.003518832*	3.06		
Rts1 2D-wPt-730744	73.00	0.002658452*	2.02	Novel	
Rts1 4A-wPt-2291	92.05	0.002324411*	2.3	QTL	Chu et al. (2008)
Rts1 7D-wPt-730876	170.73	0.001186916**	2.26	Novel	
Rts5 2D-wPt-664805	88.60	0.000863958*	3.9	Novel	
Rts5 6A-wPt-729806	36.71	5.85577E−05**	5.85	Novel	
Rts5 6A-wPt-730456	30.30	8.38719E−05**	5.69		
Rts5 6A-wPt-730711	30.30	0.000120278*	5.56		
Rts5 6A-wPt-730109	30.30	0.000304242*	5.21		
Rts5 6A-wPt-671708	30.30	0.000677904*	2.34		
Rts5 6A-wPt-731413	30.30	0.000677904*	2.34		
Rts5 6A-wPt-730368	30.30	0.000891778*	2.19		
Rts5 7D-wPt-1859	116.90	0.000111899*	4.86	Novel	
Rts5 7D-wPt-665687	116.90	0.000749254*	4.32		

[a] DArT marker system was for detecting QTL for resistance to *Pyrenophora tritici-repentis* races 1 and 5 in the collection. In a column, the QTL identified in the present study have been named as follows: first: Rts1 or Rts5 indicates resistance to *P. tritici-repentis* race 1 or race 5; second: the chromosome number where the DArT marker is mapped based on WheatDArTmaps version 1.2; third: DArT marker which showed significant association with QTL for resistance *to P. tritici-repentis* races 1 and 5 after correction for multiple comparisons ($P < 0.05$)

[b] Genetic distance centi Morgan (cM)

[c] The *p* value adjusted after multiple tests (experiment-wise *P* value) where ***, ** and * indicates significant at <0.001, <0.01 and <0.05, respectively. The significance was assessed using 10,000 permutations (Roy et al. 2010)

[d] Phenotypic variation explained by the markers, not including the other items in the model

analysis. One possible reason for not finding any marker on chromosome 4D in our DArT marker analysis may be due to the low density of DArT markers present on the array from the wheat D genome.

Three of the genomic regions conferring resistance to race 1 detected in this study were in the same genetic regions as previously reported genes for tan spot resistance. For instance, the genomic region containing Rts1 2A-wPt-6662 corresponds to the region containing SSR markers reported by Chu et al. (2008). The genomic region containing Rst1 2B-wPt-0950 corresponds to a similar region identified by Li et al. (2011) while the genomic region containing Rts1 4A-wPt-2291 appears to be the same region identified previously by Faris et al. (1997, 1999); Friesen and Faris (2004), Faris and Friesen (2005); Effertz et al. (2001) and Chu et al. (2008). Li et al. (1999) found that the marker 'Xksu916 (Oxo)' that detect QTL on 4A represents a gene encoding Oxalate oxidase, and Friesen and Faris (2004) further suggested that Oxalate oxidase gene on 4A chromosome might play a significant role for resistance to other multiple pathogens. These genomic regions conferring resistance to race 1 have shown resistance to yellow rust, leaf rust, and powdery mildew, and genes responsible for grain yield (Crossa et al. 2007). The DArT marker, wPt-9380 located on chromosome 1D was significantly associated with resistance to race 1, and the same DArT marker was previously reported to be associated with QTL responsible for grain yield (Arbelbide and Bernardo, 2006) and other QTL responsible for yellow rust resistance (Crossa et al. 2007). Other DArT markers, such as wPt-4125 and wPt-7757 mapped to chromosome 2B, were significantly associated with race 1 resistance in this study, and the same DArT markers were previously reported to be associated with QTL responsible for grain yield (Arbelbide and Bernardo 2006) and yellow rust resistance (Crossa et al. 2007). Crossa et al. (2007) also

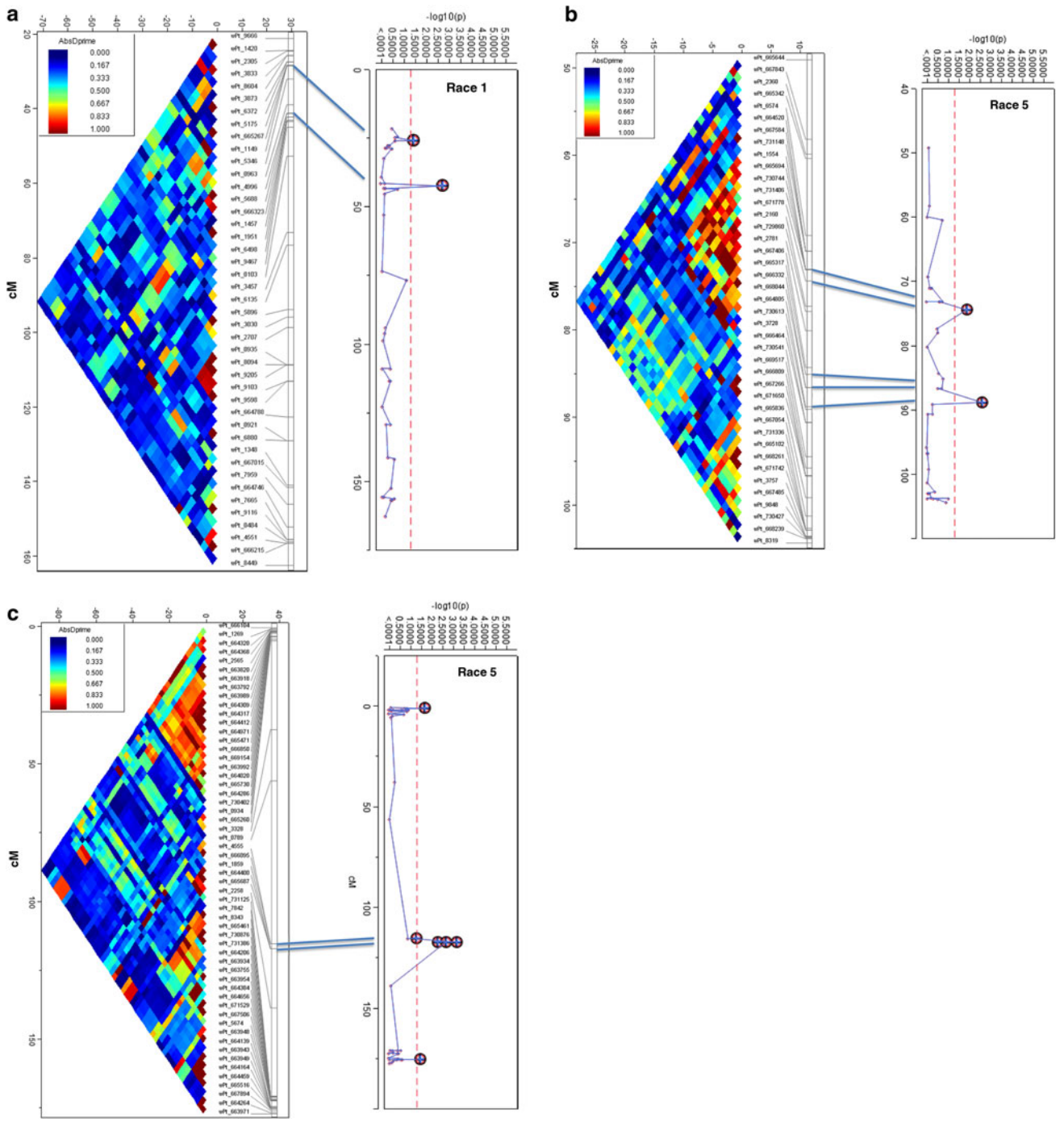

Fig. 5 **a** Association mapping results using a Q + K mixed model with false discovery rate (FDR) and multiple testing correction with *Pyrenophora tritici-repentis* race 1. Diversity array technology (DArT) markers (wPt) on chromosome 5B are shown to the left of linkage group while LD heat map shown to the far left (Abs *Dprime*). Negative $\log_{10}(p)$ graphs are shown to the right of each linkage group with significance threshold indicated by red line. Blue lines indicate DArT markers associated with resistance to race 1 and significant markers are in Table 2. Genetic distances are in centi Morgan (cM) **b** Association mapping results using a Q + K mixed model with false discovery rate (FDR) and multiple testing correction with *Pyrenophora tritici-repentis* race 5. Diversity array technology (DArT) marker (wPt) on chromosome 2D are shown to the left of linkage group while LD heat map shown to the far left (Abs *Dprime*). Negative $\log_{10}(p)$ graphs are shown to the right of each linkage group with significance threshold indicated by *red line*. *Blue lines* indicate DArT markers associated with resistance to race 5 and significant markers are in Table 2. Genetic distances are in centi Morgan (cM) **c** Association mapping results using a Q + K mixed model with false discovery rate (FDR) and multiple testing correction with *Pyrenophora tritici-repentis* race 5. Diversity array technology (DArT) marker (wPt) on chromosome 7D are shown to the left of linkage group while LD heat map shown to the far left (Abs *Dprime*). Negative $\log_{10}(p)$ graphs are shown to the right of each linkage group with significance threshold indicated by red line. *Blue lines* indicate DArT markers associated with resistance to race 5 and significant markers are in Table 2. Genetic distances are in centi Morgan (cM)

Table 3 Properties and statistics of diversity array technology (DArT) markers significantly associated with QTL for resistance to *Pyrenophora tritici-repentis* races 1 and 5 using both categorical and quantitative Q + K mixed linear model

Marker	cM[a]	CH[b]	MAF[c]	Race	Categorical/quantitative		
					F	*F* (*p*)	−Log10 (*p*) FDR
wPt_9380	66.1	1D	0.29	1	9.20986	2.5985	0.496089*[,d]
wPt_666832	67.0	1D	0.28	1	9.23631	2.6037	0.496089*
wPt_0950	86.6	2B	0.21	1	7.52656	2.2023	0.404372*
wPt_1505	106.6	2B	0.29	1	6.30826	1.9095	0.307061*
wPt_4996	41.1	5B	0.44	1	30.1685	7.8930	2.760218
wPt_664805	88.7	2D	0.37	5	13.4824	3.5773	1.305614
wPt_1859	116.9	7D	0.44	5	16.3219	4.212	1.413996
wPt_664400	116.9	7D	0.44	5	13.163	3.5015	1.305614
wPt_665687	116.9	7D	0.44	5	14.3814	3.7798	1.305614

[a] Genetic distance centi Morgan (cM)

[b] Chromosome number where the DArT marker is mapped

[c] Marker allele frequency

[d] Indicate markers not quite meeting the significant threshold with FDR correction

* Significantly associated with resistance ($P < 0.05$)

found a few genomic regions with QTL for resistance to multiple diseases such as powdery mildew, yellow rust, and leaf rust and suggested that loci responsible for resistance to powdery mildew might have been selected indirectly when selection was made for leaf rust or yellow rust resistance.

The results of this study also are in agreement with the previous findings and demonstration that the wheat—*P. tritici-repentis* pathosystem is more complex than previously thought. Abeysekara et al. (2010) found some of the Ptr ToxB-insensitive wheat lines to be susceptible to *P. tritici-repentis* and suggested that there are other factors affecting disease besides the *Tsc2*-Ptr ToxB interaction. Friesen and Faris (2004) identified three QTL for resistance to *P. tritici-repentis* race 5 on chromosomes 2AS, 4AL, and 2BL. Additionally, few QTL for resistance to *P. tritici-repentis* races 1, 2, and 5 were identified on 2AS and 5BL (Chu et al. 2008). In another study, Faris and Friesen (2005) identified QTL responsible for resistance to *P. tritici-repentis* races 1, 2, 3, and 5 on 1BS and 3BL chromosomes. Race 5 produces toxins Ptr ToxB and Ptr ToxC and sensitivity to these genes has been reported to be governed by *Tsc1* and *Tsc2* genes located on chromosomes 1AS and 2BS, respectively (Effertz et al. 2002; Friesen and Faris, 2004). None of the previously identified loci for *Tsc1* and *Tsc2,* responsible for resistances to race 5, were detected in this study. The frequency of causal allele in population and sample size are the major factors that determine the probability of finding association in an AM and this may be one of the reasons for not finding associations with all previously mapped QTL. The three putative genomic regions on chromosomes 2D, 6A and 7D identified in this study for resistance to race 5 do not correspond to any previously identified QTL for resistance to *P. tritici-repentis*, and thus are novel. Chu et al. (2010) also identified QTL on chromosome 3AS, 3BL, 5AL and 7BL that did not correspond to any of the previously identified QTL for resistance to *P. tritici-repentis*. This result further confirms the previous study that Ptr ToxB and Ptr ToxC are not the only significant factors in causing the disease susceptibility, race 5 isolate 'DW7' but also can have additional virulence factors in addition to toxins Ptr ToxB and Ptr ToxC (Chu et al. 2008). We found a total of three novel QTL regions for race 5 that could harbor the toxin insensitivity genes which have not yet been discovered. It is also possible that these QTL are responsible for sensitivity to a novel toxin, designated as Ptr ToxD (Manning et al. 2002; Meinhardt et al. 2003). Since these materials are landraces and have not been fully exploited in wheat breeding programs, further study of the identified associations should reveal resistance genes not currently deployed in modern cultivars.

We hypothesized that not all the loci identified in this study are responsible for resistance to *P. tritici-repentis* races 1 and 5 because their detection was due to a type I error. Based on this possibility, we used more stringent model including FDR multiple testing correction and Q + K to account for structure. This analysis failed to identify the genomic regions defined as Rts1 2A-wPt-6662 on chromosome 2A and Rst1 2B-wPt-0950 on chromosomes 2B, which are known to be associated with resistance to *P. tritici-repentis* race 1 (Chu et al. 2008; Li et al. 2011). Therefore, using more stringent criteria could result in failure to identify agronomically useful loci. In a similar

way, Raman et al. (2010) failed to identify the true Al^{3+} resistance locus on chromosome 4B using more stringent analysis (MLM) versus a less stringent GLM approach. Conversely, in the present study, the more stringent model did detect an association with resistance to race 1 on chromosome 5B, which has been shown to confer resistance in other studies (Anderson et al. 1999; Faris et al. 1996), that was not detected using the simple regression model. These findings suggest that both types of analysis are useful in avoiding type I and II errors and should be considered when doing these analyses.

Genomic regions associated with multiple disease resistance could be important breeding tools and could facilitate introgression of multiple disease resistance, if the resistance is present in coupling rather than in repulsion (Neu and Keller 2002). There is some precedence for this circumstance since pleiotropy or linkage between adult plant resistance genes for different pathogens has been previously reported (Crossa et al. 2007). Most of the loci on chromosomes 1D, 2D, 6A and 7D identified in this study are novel because the genomic regions of where we have identified these QTL have no reports of mapped resistance to tan spot races 1 and 5. These results suggest that the DArT markers associated with resistance to *P. tritici-repentis* races 1 and 5 should be useful for genetic studies in the future.

The DArT markers associated with resistance to *P. tritici-repentis* explained small phenotypic variation effects (R^2) ranging from 1.3 to 3.1% for race 1 and 2.2–5.9% for race 5 (Table 2). Resistance to *P. tritici-repentis* is quantitative and governed by several minor genes (Chu et al. 2008; Friesen and Faris 2004; Singh et al. 2010). Small phenotypic variation effects also were observed in the AM of spot blotch disease resistance in wild barley (Roy et al. 2010), and late maturity α-amylase (LMA) activity in wheat (Emebiri et al. 2010). We found the 'total of 22 significant DArT markers associated with 10 putative genomic regions responsible for resistance to *P. tritici-repentis* races 1 and 5. It is possible that any accession could carry a resistance allele at more than one QTL and thus affect the estimate of the QTL effect (Roy et al. 2010). However, this needs to be confirmed in future studies.

AM approach identified novel genomic regions involved in tan spot disease resistance in wheat. One standard method for validating these QTL would be to develop near-isogenic lines in different genetic background, and test them in genetically diverse populations and breeding lines across multiple environments. Such near-isogenic lines could also be used to examine effectiveness of QTL against multiple races of *P. tritici-repentis* and to accurately measure allelic effects of the QTL (Roy et al. 2010). Alternatively, future work should seek to validate these QTL for resistance to *P. tritici-repentis* races 1 and 5 by developing bi-parental populations from crosses between the resistant accessions identified in this study and the susceptible agronomically accepted wheat cultivar. The frequency of all of the favorable QTL alleles in the population could be increased through cycles of marker-assisted selection (MAS) for multiple QTL and then intermate the selected individuals in the population using a recurrent selection scheme (Bernardo 2008).

In summary, results of this study suggest that the AM study identified marker-trait associations in a set of spring wheat landraces of diverse origin. Seven and three genomic regions were significantly associated with resistance to *P. tritici-repentis* races 1 and 5, respectively. DArT markers associated with resistance to race 1 were detected on chromosomes 1D, 2A, 2B, 2D, 4A, 5B, and 7D while markers associated with resistance to race 5 were distributed on 2D, 6A and 7D. Some of these genomic regions associated with resistance to *P. tritici-repentis* are novel and co-localized in the same chromosomal regions where genes or QTL for other important traits have been previously reported. After validating the DArT markers, they can be utilized to pyramid multiple QTL in order to develop durable resistance to *P. tritici-repentis*. To complete whole genome coverage and develop complete genetic maps, further research is in progress to use high density DArT and single nucleotide polymorphism (SNP) markers on a subset of selected wheat accessions. Such follow-up study would help develop precise genetic maps and identify markers that may provide largest phenotypic variation effects in this population.

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