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ORIGINAL PAPER

Identification of Ug99 stem rust resistance loci in winter wheat germplasm using genome-wide association analysis

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Abstract The evolution of a new race of stem rust, generally referred to as Ug99, threatens global wheat production because it can overcome widely deployed resistance genes that had been effective for many years. To identify loci conferring resistance to Ug99 in wheat, a genome-wide association study was conducted using 232 winter wheat breeding lines from the International Winter Wheat Improvement Program. Breeding lines were genotyped with diversity array technology, simple sequence repeat and sequence-tagged site markers, and phenotyped at the adult plant stage for resistance to stem rust in the stem rust resistance screening nursery at Njoro, Kenya during 2009–2011. A mixed linear model was used for detecting marker-trait associations. Twelve loci associated with Ug99 resistance were identified including markers linked to known genes *Sr2* and *Lr34*. Other markers were located in the chromosome regions where no *Sr* genes have been previously reported, including one each on chromosomes 1A, 2B, 4A and 7B, two on chromosome 5B and four on chromosome 6B. The same data were used for investigating epistatic interactions between markers with or without main effects. The marker csSr2 linked to *Sr2* interacted with wPt4930 on 6BS and wPt729773 in an unknown location. Another marker, csLV34 linked to *Lr34*, also interacted with wPt4930 on 6BS and wPt4916 on 2BS. The frequent involvement of wPt4916 on 2BS and wPt4930 on 6BS in interactions with other significant loci on the same or different chromosomes suggested complex genetic control for adult plant resistance to Ug99 in winter wheat germplasm.

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Introduction

Stem rust, caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. and E. Henn., is one of the most destructive diseases of wheat worldwide. It caused yield losses from 10 to 50 % in many regions/countries including Europe, Asia, Australia and United States in the twentieth century (Zadoks 1963; Rees 1972; Joshi and Palmer 1973; Leonard 2001a, b). A number of stem rust resistance genes were successfully deployed in wheat cultivars worldwide since the 1950s that effectively controlled the disease. However, a new race of stem rust, Ug99 (Pretorius et al. 2000), was first identified in Uganda and has spread throughout much of Africa, the Middle East and West Asia. Ug99 and its variants differ from other strains of the stem rust pathogen due to their ability to overcome stem rust resistance (*Sr*) genes in wheat that have been effective for decades (Singh et al. 2006). Of nearly 50 stem rust resistance (*Sr*) genes identified in wheat, few are still effective against Ug99

(McIntosh et al. 1995; Singh et al. 2006, 2008). Among them, *Sr2* is one of the most widely used in wheat breeding programs worldwide and has provided durable adult plant rust resistance for more than 50 years (McIntosh et al. 1995). However, it only provides partial adult plant resistance (APR) and is associated with the pseudo black chaff trait (Hare and McIntosh 1979). Other effective *Sr* genes include *Sr13*, *Sr22*, *Sr25*, *Sr26*, *Sr32*, *Sr35*, *Sr39*, *Sr40*, *Sr44*, *Sr45*, *Sr46*, *Sr47* and a few unnamed genes (Singh et al. 2006; Jin et al. 2007).

Most of the resistance genes for stem rust of wheat are qualitative and race specific. They are effective for some isolates, but ineffective for others. The race-specific resistance genes are also known as "R" genes and follow the gene-for-gene model (Flor 1955). Host resistance requires the simultaneous presence of the resistance allele in the host and the corresponding avirulence allele in the pathogen. Most of the race-specific genes are expressed throughout all vegetative stages of wheat. Sources of resistance based on multiple genes, often termed quantitative resistance, which retards infection and also growth and reproduction of the pathogen in adult plants but not in seedlings, have been described as "adult plant resistance" (Gustafson and Shaner 1982). APR may be more durable than resistance based on single R genes and can be identified in cultivars with defeated or no race-specific resistance genes.

To improve the efficiency of wheat breeding for APR to stem rust, it is essential to understand the genetic basis of APR. APR to stem rust in wheat is much more complex than race-specific resistance because it is a quantitative trait. Selection for plants with APR is a time-consuming process involving extensive and precise quantitative measurements of the disease. Therefore, identification of novel resistance resources and development of molecular markers for APR is important for wheat breeding.

Association mapping (AM) is one of the several techniques to identify marker-trait associations using linkage disequilibrium (LD) and has been used in various plant species (see Zhu et al. 2008 for review). In common wheat (*Tritcum aestivum* L.), several studies using association mapping have been reported (Breseghello and Sorrells 2006; Roy et al. 2006; Jing et al. 2007; Tommasini et al. 2007; Crossa et al. 2007, Peng et al. 2009, Yu et al. 2011). Two approaches are commonly used in association mapping: one uses a whole-genome scan (Rafalski 2002; Kraakman et al. 2004, 2006) and another uses the candidate gene approach (Thornsberry et al. 2001; Szalma et al. 2005). The former, often called genome-wide association study (GWAS), identifies genomic regions throughout the genome associated with the trait of interest. High-density markers such as single nucleotide polymorphisms (SNPs) and diversity array technology (DArT) markers are often used for genotyping the whole genome because they can provide reasonable genome coverage. Success and resolution of genome scans is dependent on the genome coverage, extent of LD and effective population size. The candidate gene approach directly tests the effects of genetic variants of a gene that may affect a particular trait. However, the candidate gene approach is limited by existing knowledge about the biology of the trait of interest and the genes underlying the QTL interval.

In the present study, our objective was to determine the genetic control of APR to Ug99 stem rust in an international winter wheat germplasm. Genome-wide markers as well as markers linked to known *Sr* genes were used to identify loci associated with stem rust resistance and evaluate their epistatic interactions.

Materials and methods

Genetic resources

The wheat accessions for this study were selected by International Winter Wheat Improvement Program (IWWIP) representing winter wheat breeding programs in Turkey, Eastern Europe, West Asia and USA. A total of 232 wheat lines of diverse origins, including 100 lines from the first Winter Wheat Stem Rust Resistance Nursery (1st WWSRRN) and 132 lines from the third Winter Wheat Stem Rust Resistance Nursery (3rd WWSRRN). Their names, types, origins and pedigrees are presented in supplemental Tables S1 and S2.

Phenotyping and data analysis

Wheat lines were evaluated for stem rust response in Kenya as previously described (Yu et al. 2011) subject to the following modifications. Winter wheat germplasm was first vernalized for 6 weeks at a temperature of 2–4 °C. Then, the pots were transferred into the field, transplanted and provided with optimal nutrient and water management. A mixture of stem rust susceptible entries was planted after every two rows of winter wheat hill plots to increase disease pressure. Due to the winter habit, the germplasm flowered about 2 weeks later than the spring wheat germplasm planted in the same field and for this reason was subjected to heavy stem rust pressure.

Disease severity and host response data were combined in a single value called the coefficient of infection (C.I.) that was calculated by multiplying the disease severity and a constant value for host response. These values of host response were 0.0, 0.2, 0.4, 0.8 and 1.0 for immune, resistance (R), moderate resistance (MR), moderate susceptible (MS) and susceptible (S), respectively. Where

cultivars carried seedling resistance genes that were effective in one or more field rust nurseries, the C.I. value provided an indication of the level of protection afforded by the resistance gene and other minor additive resistance genes the cultivar may contain. Where cultivars lacked seedling resistance genes that were effective to the pathotype TTKST, the C.I. values provided an estimation of the level of APR present. A summary of the level of APR present in the wheat materials is provided in Tables S1 and S2 by grouping the materials in different resistance categories based on the relative effectiveness of APR compared to susceptible checks.

Phenotypic data were first analyzed using ANOVA with the model of year, location and genotype. A highly significant ($P < 0.001$) difference in rust scores was observed among the panel of accessions. Least square means for individuals were calculated by SAS PROC using a mixed model (SAS Institute, NC).

DArT genotyping and data analysis

DNA was extracted from young leaves of seedlings using the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle 1987) and sent to Diversity Arrays Technology Pty Ltd, Australia (http://www.diversityarrays.com) for a whole-genome profiling using DArT markers. Two DArT arrays were used for genotyping the 1st WWSRRN and 3rd WWSRRN, and 1,423 and 1,510 polymorphic DArT markers were scored, respectively. To increase the population size, genotyping data for common markers between the 1st WWSRRN and 3rd WWSRRN were selected and formed into a combined set for further analysis. The Wheat Interpolated Maps v4 (Diversity Arrays Technology Pty Ltd, personal communication) were used as a reference to locate the positions of DArT markers. This consensus map was generated from multiple populations (http://www.triticarte.com.au/). It contains 4,721 marker loci including 4,606 DArT and 115 simple sequence repeat (SSR) markers. Among them, 1,598, 2,229 and 894 markers were distributed in A, B and D genomes, respectively. For the 1,267 common polymorphic DArT markers analyzed in the present study, 988 were mapped and of those, 357, 403 and 228 were located in A, B and D genomes, respectively. For markers without map positions, r^2 values between markers were used as surrogates for estimating map location. If $r^2 = 1$ was detected between two markers, we removed one from the data set because they were likely to be completely linked. Markers with a minor allele frequency of <5 % were also removed from the data before analysis. Eight SSR or sequence-tagged site (STS) markers were used for genotyping markers linked to known stem rust resistance genes, according to our previously reported procedure (Yu et al. 2010). Specific alleles for the target loci were scored "1" as presence, "0" as absence and "–" for missing and added to the DArT set for association analysis. The missing data was imputed using the data imputation function in TASSEL (http://www.maizegenetics.net/tassel/).

Principal component analysis (PCA)

A PCA was performed using SAS PROC PRINCOMP (SAS Institute, NC) on the marker data for lines within each nursery as well as across both nurseries, as these nurseries contained different sources of stem rust resistance. A covariance matrix was obtained and used for association analysis. To display results, principal component 1 scores were plotted against principal component 2 scores for each of the lines.

Linkage disequilibrium

Linkage disequilibrium between markers was assessed by calculation of r^2 between markers using TASSEL. Linkage disequilibrium statistics were calculated per chromosome and subsequently aggregated over all chromosomes. The LD decay with genetic distance was evaluated by an exponential probability density function (pdf) using PROC NLIN in SAS software. The analysis found that the exponential pdf with lambda equal to 0.38 explained the most variation; therefore, this function was used to calculate predicted r^2 values.

Association analysis

The trimmed marker data sets were used to generate a marker similarity matrix containing all lines (Kinship or K matrix) using TASSEL. TASSEL calculates kinship as the proportion of alleles shared between each pair of lines. Once this matrix is calculated, the numbers are rescaled so that the numbers fall between 0 and 2 (Peter Bradbury, Personal communication). Substructure within the germplasm accessions was also investigated using PCA and the covariance matrix (Q matrix) was used to correct the effect of population substructure. Both Q and K matrices were used in the mixed linear model (MLM) to correct for both population and family structure. A false discovery rate (FDR) of 0.05 was used as a threshold for significant association (Benjamini and Hochberg 1995).

Epistatic interaction

The same data sets including genotyping, phenotyping and Q matrices were used to analyze epistatic interactions between markers with significant main effects and all other markers regardless of whether they were significant or not.

A linear regression model was used to calculate P values for pairwise marker interactions, and a FDR of 0.05 was used as a threshold for significant interaction. The epistatic effect was analyzed according to Xu and Jia (2007) and modeled as follows:

$$y = 1\mu + Z_l\gamma_1 + (Z_1 \times Z_{l'})\gamma_{ll'} + e$$

where y is $n \times 1$ vector for phenotypic observation, μ is the population mean, Z_l is a vector $(Z_{11}\ldots Z_{nl})^T$ for the genotype indicators of locus l, γ_1 is the main effect for locus l and $\gamma_{ll'}$ is the epistatic effect between loci l and l' (l' is another marker), and e is the error. To partition the full genetic effect, a reduced model was used as follows:

$$y' = 1\mu + Z_l\gamma_1 + e$$

The interaction effect was obtained by the difference between the genetic variations explained by the two models (full vs. reduced models).

Results

Population structure

Principal component analysis was used for analyzing the genetic relationships in the diverse germplasm represented in the 1st WWSRRN and 3rd WWSRRN. Five groups, including two major and three minor groups, were identified by PCA (Fig. 1). The first group consisted of 106 genotypes representing IWWIP germplasm from the Turkey and Eastern European gene pools and was characterized by the frequent occurrence of Bezostaya 1 and its descendants in the pedigrees. Bezostaya 1 was also a member of this cluster. The second cluster comprising 73 genotypes included US germplasm or germplasm with US varieties in the pedigree. Many varieties and breeding lines in this cluster possess the 1A.1R translocation. Cluster 3 with 15 genotypes originating from Canada, Bulgaria and Turkey was difficult to characterize because of a lack of pedigree information. Eleven lines from Romania, Russia and Turkey were clustered into group 4 but do not have an obvious common parentage. Cluster 5 comprised ten sister doubled haploid lines from the International Center for Agricultural Research in the Dry Areas (ICARDA) and several breeding lines from Iran with similar pedigrees. The diversity within the population represents the modern winter wheat germplasm with the exception of Western Europe and China. The result obtained by PCA indicated that subpopulations exist in the association panel and this covariance matrix was used in the association analysis to correct for population structure.

LD decay

For analyzing LD decay, genetic distances for 401 DArT markers were obtained from a consensus linkage map constructed by Crossa et al. (2007). Although they only represented half of the markers used in the present study, they covered an estimated 2,149 cM or 83 % of the wheat genome (Somers et al. 2004). The predicted r^2 value declined to 0.1 within 3.5 cM (Fig. 2).

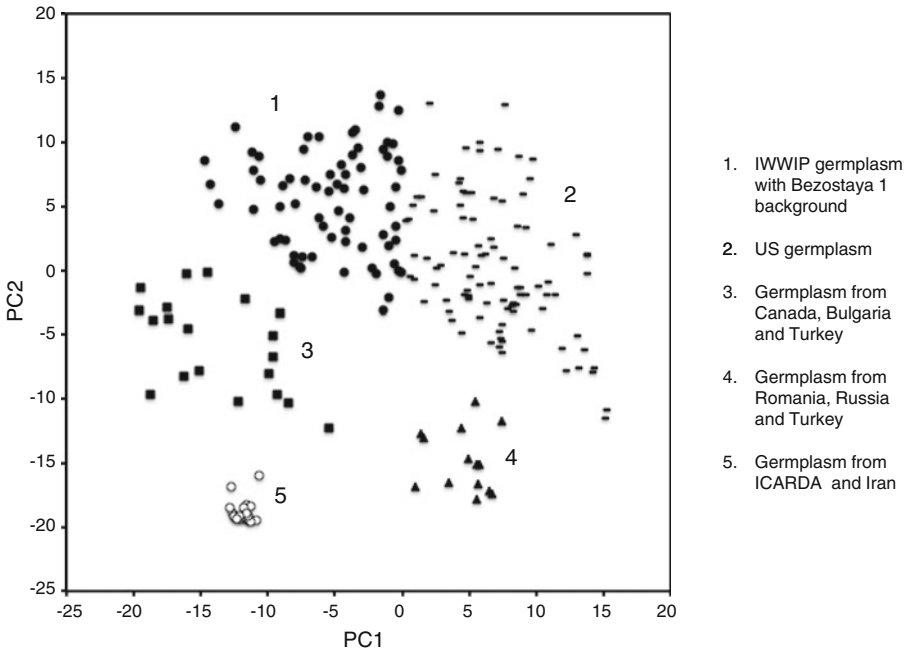

Fig. 1 The first and second axes from the principal component analysis of the winter wheat germplasm using DArT and SSR genotyping data. Each data point represents a genotype. Five clusters are distinguishable. Representative genetic background or origins for individuals in each subgroup were indicated in the legend (*right panel*). *IWWIP* International Winter Wheat Improvement Program, *ICARDA* International Center for Agricultural Research in the Dry Areas

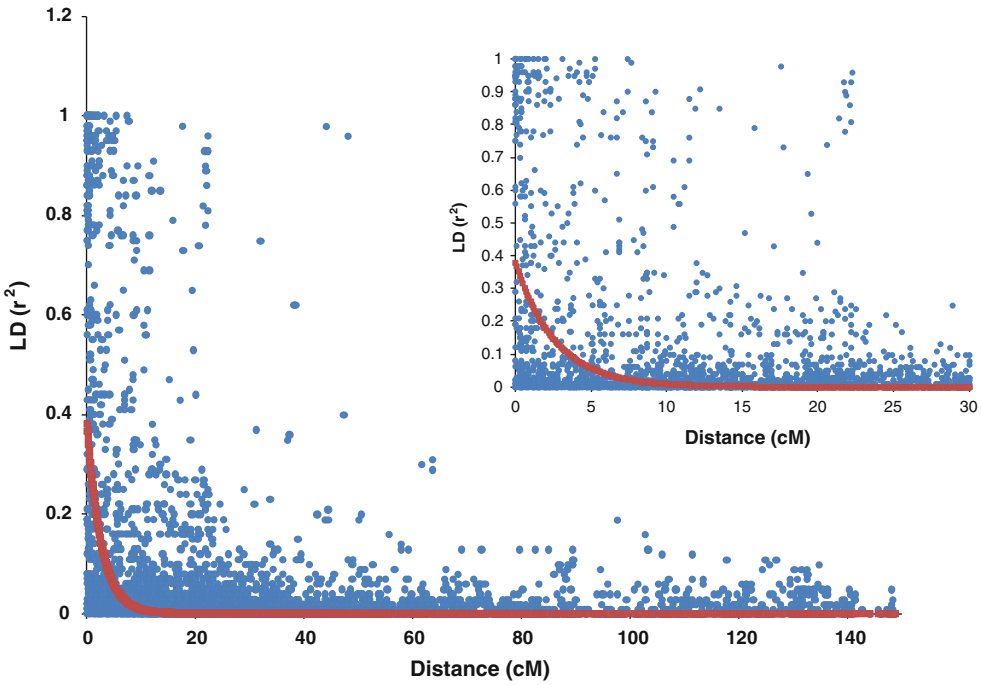

Fig. 2 Scatterplot of estimates of r^2 for pairs of DArT markers across chromosomes and genomes, showing LD decay, as measured by r^2 against genetic distance (cM). *Inset panel* shows a more detailed view of LD decline for markers located within the first 30 cM. The *decay curves* were plotted with predicted LD values according to Andreescu et al. (2007)

Analysis of marker-trait associations

Using a MLM, 12 significant markers including 2 STS and 9 DArT markers were associated with stem rust resistance, with a total of $r^2 = 0.73$ (Table 1). Two STS markers, csSr2 linked to *Sr2* on chromosome arm 3BS and csLV34 linked to *Lr34* on 7DS (Fig. 3), respectively, were significantly associated with stem rust resistance ($P < 0.0003$ and 0.00002, respectively) with an $r^2 = 0.18$ (Fig. 3). Among the nine DArT markers associated with stem rust resistance, wPt730213 was located in the distal region of 1AS (Fig. 3). DArT marker wPt4916 was located in the distal region of 2BS. On chromosome 4AL, DArT marker wPt3349 was located about 7 cM proximal to another resistance locus, wPt5749, previously identified (Yu et al. 2011). Two markers wPt1302 and wPt3873 were identified on 5BS. The latter was located 1 and 4 cM proximal to two markers: wPt1149 and wPt5346 associated with stem rust resistance reported by Crossa et al. (2007) and Yu et al. (2011), respectively. Four DArT markers associated with stem rust resistance were identified on chromosome 6B. Among them, wPt4930 and wPt1241 were located on 6BS within 14 cM and probably identify the same resistance gene (Fig. 3). Two previously reported resistance loci,

Table 1 Representative markers significantly associated with stem rust resistance in the IWWIP germplasm

Marker	Chromosome	cM	*P* value	r^2
wPt730213	1A	0.20	3.01E−04	0.06
wPt4916	2B	1.80	5.52E−04	0.07
csSr2	3B	0.00	2.90E−04	0.08
wPt3349	4A	84.78	4.71E−04	0.05
wPt1302	5B	1.23	4.33E−04	0.05
wPt3873	5B	27.32	6.80E−04	0.05
wPt4930	6B	9.83	5.92E−04	0.05
wPt1241	6B	24.10	8.23E−04	0.06
wPt4648	6B	51.52	4.34E−04	0.05
wPt6116	6B	107.22	5.33E−04	0.05
wPt0318	7B	78.09	1.61E−04	0.06
csLV34	7D	50.51	2.01E−05	0.10

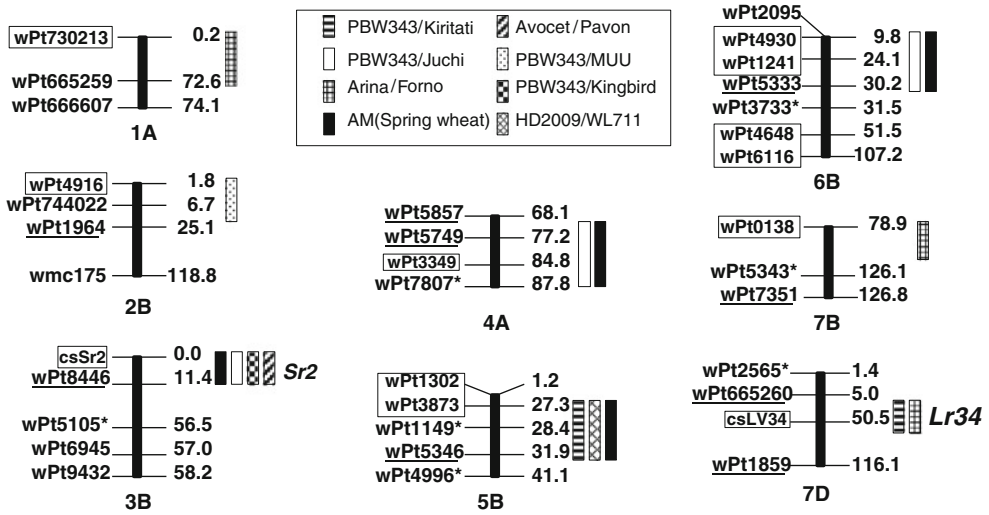

Fig. 3 Chromosome positions of significant markers associated with stem rust resistance in the present (labeled by *rectangle*) and previous studies [Yu et al. 2011 (*underlined*); Crossa et al. 2007 (labeled by *asterisk*)]. The location of DArT markers was based on the Wheat Interpolated Maps v4 (Triticarte Pty Ltd, Australia, personal communication). The approximate location of reported *Sr* genes was indicated to the *right* of each chromosome. Resistance QTL were indicated by *bars* on the right side of chromosome regions with different populations distinguished by different patterns or shading

wPt5333 (Yu et al. 2011) and wPt3733 (Crossa et al. 2007) were also identified in a similar region. On 6BL, marker wPt4648 was 27 cM from wPt1241 and wPt6116 was more than 50 cM from wPt4648 and therefore are likely different QTLs. No *Sr* gene has been reported in the regions near wPt4648 and wPt6116. Marker wPt0138 on 7BL was associated with stem rust resistance and its location was about 40 cM distal to previously reported markers wPt5343 (Crossa et al. 2007) and wPt7351 (Yu et al. 2011).

Two SSR markers, gwm533 (Spielmeyer et al. 2003) and csSr2 (Mago et al. 2010) were used for haplotyping *Sr2* in the association panel. Marker gwm533 identified 44 and 68 positives in the 1st WWSRRN and 3rd WWSRRN, respectively, while 34 and 37 positives were detected by marker csSr2 (Tables S1 and S2). However, only marker csSr2 was significantly associated with the rust resistance in the AM (Table 1; Fig. 3).

For identifying the *Lr34* locus, marker csLV34 (Lagudah et al. 2006) was used for genotyping the germplasm in both nurseries. Twenty-nine and 53 positives were identified in the 1st WWSRRN and the 3rd WWSRRN, respectively (Tables S1 and S2).

For other known *Sr* genes, specific SSR markers were used for genotyping. Lines positive for the presence of *Sr24, Sr36* and *1A.1R* (Tables S1 and S2) were identified. However, none of these loci were significantly associated with Ug99 APR resistance in this study (Table 1; Fig. 3). The absence of *Sr25* and *Sr26* in this germplasm (based on pedigree) was confirmed by genotyping using marker BF145935 and the multiplexed markers Sr26#42 and BE518379, respectively (Liu et al. 2010). Marker scm9 was used for genotyping the *IA.1R* translocation (Saal and Wricke 1999) and 40 positive lines for 1A.1R were found in the association panel (Table S1 and S2). However, scm9 was not significantly associated with stem rust resistance.

For further validation of the new *Sr* loci identified in this study, we compared their locations with DArT markers previously reported by Crossa et al. (2007) (Fig. 3, labeled by asterisk). All significant QTL in this study except those on 6BL co-located with those previously reported. Moreover, we compared them with the stem rust resistance QTL identified in biparental winter populations (Bansal et al. 2008, Sridhar Bhavani et al. Personal communication), and all significant marker loci identified on 1AS, 2BS, 3BS, 4AL, 5BS, 6BS and 7BL in this study were co-located with QTL in biparental populations (Fig. 3, right side bars).

Epistatic interactions

Epistatic interaction analysis was carried out between markers with significant main effects and all other markers (Table 2). All marker loci significantly associated with rust resistance, also significantly interacted with one or more markers (Fig. 4). Nine and eight markers interacted with wPt4930 and wPt4916, respectively. Among them, seven interacted with both wPt4930 and wPt4916 ($P < 0.0001$) (Fig. 4). Markers wPt4930 and wPt4916 were significantly associated with stem rust resistance and interacted each other with high values of main (0.23 and 0.17) and interaction (0.08 and 0.06) effects, respectively (Table 2; Fig. 4). Marker csSr2 linked to *Sr2* on 3BS interacted with DArT markers wPt4930 and wPt2095 (same locus) on 6BS and wPt729773 with unknown location (Fig. 4; Table 2), although the latter two markers

Table 2 Epistatic interactions for markers with significant main effects in the IWWIP germplasm

Resistance marker	Interacting markers	Average *P* value	Main effect	Interaction effect
csLV34	wPt4916, wPt4930	4.02E−06	0.11	0.03
csSr2	wPt2095, wPt4930, wPt729773	1.96E−05	0.16	0.06
wPt0318	wPt2095, wPt4930, wPt4916	1.54E−05	0.08	0.02
wPt1241	wPt4916, wPt4930	2.34E−06	0.09	0.03
wPt3349	wPt4916, wPt4930	1.14E−05	0.13	0.05
wPt3873	wPt4930	1.05E−05	0.14	0.05
wPt4648	wPt2095, wPt4916	1.24E−05	0.09	0.03
wPt4916	wPt1241, wPt4930, wPt7024	1.01E−05	0.17	0.06
wPt4930	wPt4916	2.38E−06	0.23	0.08
wPt6116	wPt2095, wPt4916, wPt4930	1.54E−05	0.15	0.08
wPt1302	wPt1973, wPt4916, wPt4930, wPt7024, wPt730213	3.03E−05	0.13	0.01
wPt730213	wPt4930, wPt4916, wPt1302	5.56E−06	0.13	0.03

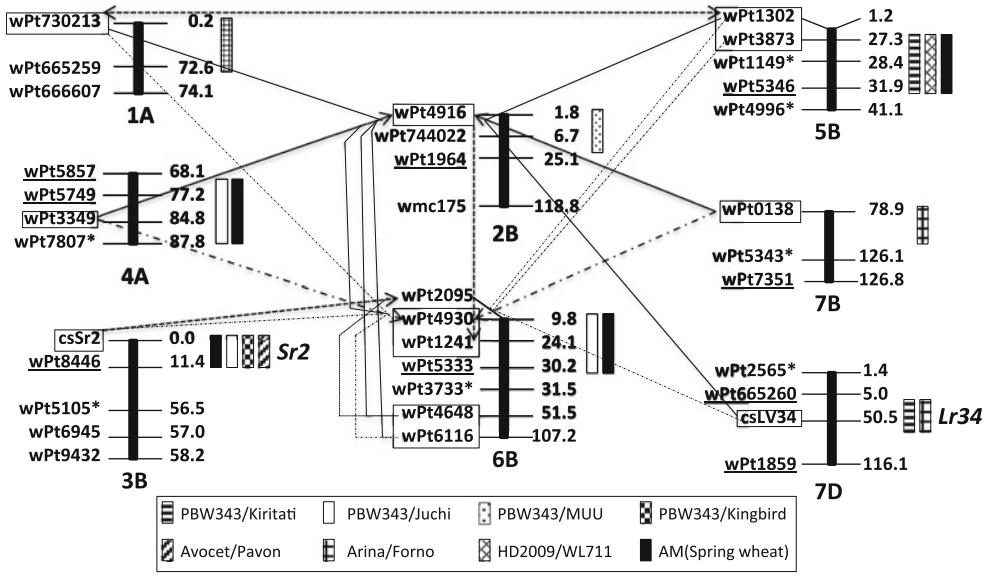

Fig. 4 The network of gene–gene interactions between the marker loci for stem rust resistance and all other markers. The chromosome positions of interacting markers and related markers were indicated on genetic maps. Significant markers associated with stem rust resistance were labeled by *rectangle* (this study), *asterisk* (Crossa et al. 2007) and *underlined* (Yu et al. 2011). The regions of stem rust resistance loci identified in other populations were indicated by *bars* on the *right side*. *Arrows* illustrate the directions of pairwise interactions between markers and different line patterns represent different interactions

(individually) were not significantly associated with the rust resistance in this study (Table 1; Fig. 3). Marker csSr2 had a higher value for both main and interaction effects indicating its significant contribution to the resistance (Table 2). Marker csLV34 linked to *Lr34* on chromosome 7BS interacted with wPt4916 on 2BS and wPt4930 on 6BS (Table 2; Fig. 4). The main effect of marker wPt1241 was not significant, however, it interacted with wPt4930 and wPt4916 (Table 2). Significant marker wPt1302 interacted with five markers at different chromosome locations including markers with main effects, wPt4916, wPt4930 and wPt730213, and those without, wPt1973 and wPt7024 (Table 2; Fig. 4). Other significant markers interacted with one to three markers on the same or different chromosomes and had main effect values of 0.08–0.15 and interaction effects of 0.01–0.08 (Table 2).

Discussion

Comparison of stem rust resistance loci in the spring and winter germplasm

Crossa et al. (2007) identified a number of significant DArT markers associated with stem rust resistance by

association mapping in the Elite Spring Wheat Yield Trial population from CIMMYT. We reported previously 15 loci on 9 chromosomes associated with Ug99 stem rust resistance in the CIMMYT spring stem rust screening nursery germplasm (Yu et al. 2011). In the present study, we identified 12 loci on 7 chromosomes in the IWWIP winter germplasm. Significant ($P < 0.01$) marker loci were compared for their chromosome locations in the same consensus map between spring and winter germplasm identified in the previous (2011) and the present studies. Markers spaced across the genome within 15 cM intervals were considered to be the same QTL. Using these criteria, four loci associated with stem rust resistance were found in the same chromosome locations in both spring and winter growth habits, including the csSr2 locus (present study and Yu et al. 2011) on 3BS; wPt3349 (present study) and wPt7807 (Crossa et al. 2007) on 4A; wPt3873 (present study), wPt1149 (Crossa et al. 2007) and wPt5346 (Yu et al. 2011) on 5BS; and wPt1241 (present study), wPt5333 (Yu et al. 2011) and wPt3733 (Crossa et al. 2007) on 6BS. The rest were in different locations in the spring and winter nurseries. Of those identified in the spring wheat panel, loci associated with stem rust resistance on 1B, 2BL and 6A were not found in the winter wheat. The significant locus, wPt730213 on 1AS identified in the winter wheat, was not associated with stem rust resistance in the spring wheat. However, a resistance locus was reported in a similar region on 1AS in another winter wheat population, Arina/Forno (Bansal et al. 2008). Significant markers associated with stem rust resistance were identified on 7BL in both spring and winter germplasm; however, wPt0138 identified in the winter wheat was 48 cM away from wPt7351 identified in the spring wheat ($P < 0.01$) and are likely different QTL (Fig. 3, 7B). A QTL for stem rust resistance was reported on 7BL in the Arina/Forno population (Bansal et al. 2008) and its location was closer to wPt0138. The *Lr34* locus was highly associated with rust resistance in the winter germplasm. *Lr34* was also reported to be associated with APR to stem rust in the Arina/Forno population (Bansal et al. 2008). Although the main effect of *Lr34* was not significant in the spring germplasm, it had significant interactions with other loci (Yu et al. 2011). Different loci associated with stem rust resistance were identified in the spring and winter germplasm because they represent different gene pools.

Significant markers linked to previously identified APR loci

Among the markers significantly associated with stem rust resistance in this study, *Sr2* is known to be partially effective against race TTKSK (Ug99). *Sr2* is one of the most widely used stem rust resistance genes (McIntosh et al. 1995) and has provided durable adult plant rust resistance for more than 50 years. As described in "Materials and methods", the disease severity and host response data were combined into a single coefficient of infection value that represented quantitative resistance to stem rust to estimate the level of APR present. The use of coefficient of infection allowed the identification of QTLs for APR to Ug99 as well as the presence of major genes. Indeed, the *Sr2*-linked marker, csSr2 (Mago et al. 2010) was strongly associated with Ug99 resistance at the adult plant stage in the present study (Table 1). It was reported earlier that *Sr2* contributes to APR through the interaction between *Sr2* and other unknown genes to form an *Sr2* complex (Singh et al. 2009). The associations of the *Sr2* locus with stem rust resistance and its interactions with other loci in the present study as well as in the study by Yu et al. (2011) also support this hypothesis.

Lr34 was initially identified as a leaf rust resistance gene in wheat (Dyck 1987). Previous reports suggested that *Lr34* also provided APR to stripe rust (Singh 1992) and stem rust (Kerber and Aung 1999). In this study, the *Lr34*-linked marker csLV34 was significantly associated with Ug99 resistance and interacted with resistance loci on 2B and 6B (Tables 1, 2). These interactions are discussed in detail below.

Significant markers for novel stem rust resistance

The map positions of the remaining significant DArT markers except three in the same location on 6BL were in chromosome regions similar to those previously reported. They included a single QTL on 1AS, 2BS, 3BL, 4AL, and two on 5B. A locus associated with stem rust resistance in a similar location to marker wPt730213 on 1AS was reported in the biparental population Arina/Forno (Bansal et al. 2008). They suggested that it may be a new Ug99 resistance gene and this study provided supporting evidence. A significant marker wPt4916 on 2BS was co-localized with a locus associated with stem rust resistance identified in the PBW343 × MUU population (Sridhar Bhavani et al. Personal communication). No *Sr* gene has been reported in the location of significant marker wPt3349 on 4AL, although the association of the DArT marker wPt7807 located on 4AL in a similar location was reported by Crossa et al. (2007) in the CIMMYT Elite Spring Wheat Yield Trial (ESWYT) association panel. All significant markers identified on 5BS and 6B did not coincide with any reported major *Sr* gene; however, QTLs associated with stem rust resistance have been mapped in similar locations on both 5BS and 6BS in the PBW343/Kiritati and PBW343/Juchi populations, respectively (Sridhar Bhavani et al. Personal communication). The significant locus wPt0138 on 7B co-located with the reported APR locus in the Arina/Forno population (Bansal et al. 2008). With further

characterization and successful validation, diagnostic markers linked to these resistance genes should be useful for breeding wheat varieties with resistance to Ug99 and related stem rust races. Further, the germplasm in this study and that of Yu et al. (2011) provide information on genotypes containing multiple QTL that are useful in breeding programs.

Epistatic analysis reveals complex interactions contributing to stem rust APR

Based on the analysis for epistasis, numerous significant interactions were identified that contributed to stem rust resistance in this population (Table 2). Hot spots were found at the marker loci wPt4916 and wPt4930 (Fig. 4). Marker wPt4916 was located on 2BS in the region where several stem rust resistance genes have been reported (McIntosh et al. 1995), and among them, *Sr39* and *Sr40* are effective against Ug99 (Jin et al. 2008). However, based on the pedigree information, none of the genotypes studied contained *Sr39* or *Sr40* (Table S1 and S2). Marker wPt4930 was located on 6BS in the region where no *Sr* gene was reported. The frequent involvement of wPt4916 and wPt4930 in interactions with other significant loci on the same or different chromosomes suggested that they were critical to Ug99 APR in this germplasm. The interactions of *Sr2*-linked marker csSr2 and *Lr34*-linked marker csLV34 with other loci on the same and different chromosomes were established in spring wheat (Yu et al. 2010), and the present study indicated similar interaction trends in winter wheat germplasm. The frequent involvement of *Sr2* and *Lr34* in interactions with other loci on the same and different chromosomes in our studies substantiates earlier reports of interactions of *Sr2* (Singh et al. 2009) and *Lr34* (Kolmer et al. 2011) with other genes enhancing stem rust APR in wheat.

This study identified the presence of 12 loci associated with stem rust resistance on 7 chromosomes in the winter wheat breeding germplasm of which 4 loci namely, csSr2 locus on 3BS, wPt3349 on 4A, wPt3873 on 5BS and wPt1241 on 6BS were common in both spring and winter wheat. The *Lr34* locus was also observed to be highly associated with the rust resistance in the winter germplasm. As winter wheat represents different genetic backgrounds and gene pools, eight different loci associated with stem rust resistance were identified in the winter wheat germplasm that confer resistance to stem rust. Their chromosome positions were based on the consensus map generated from multiple mapping populations, and several SSR markers linked to the reported *Sr* genes were also in the same map. Further investigation using SNP markers for GWAS may provide more information for validating the loci associated with stem rust resistance identified by the previous (Yu et al. 2011) and current approaches. Additionally, higher density SNP markers may fill the gaps in the chromosome maps (especially in the D genome) and increase the chance for identifying new loci that were not identified using DArT and SSR markers. The development of near isogenic lines for specific QTL associated with stem rust resistance would be useful for fine mapping to narrow the QTL regions. Moreover, the near isogenic lines can also be used for analyzing epistatic interactions that contribute to stem rust APR. Further investigation may provide insight for understanding the complex gene interactions observed in this and previous studies, as well as mechanisms that contribute to the stem rust resistance gene network.

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