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Genetic architecture of adult plant resistance to leaf rust in a wheat association mapping panel

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Leaf rust caused by *Puccina triticina* is one of the most destructive fungal diseases of wheat (*Triticum aestivum*). Adult plant resistance (APR) is an effective strategy to achieve long-term protection from the disease. In this study, findings are reported from a genome-wide association study (GWAS) using a panel of 96 wheat cultivars genotyped with 874 Diversity Arrays Technology (DArT) markers and tested for adult leaf rust response in six field trials. A total of 13 quantitative trait loci (QTL) conferring APR to leaf rust were identified on chromosome arms 1BL, 1DS, 2AS, 2BL, 2DS, 3BS, 3BL, 4AL, 6BS (two), 7DS, 5BL/7BS and 6AL/6BS. Of these, seven QTLs mapped close to known resistance genes and QTLs, while the remaining six are novel and can be used as additional sources of resistance. Accessions with a greater number of combined QTLs for APR showed lower levels of disease severity, demonstrating additive and significant pyramiding effects. All QTLs had stable main effects and they did not exhibit a significant interaction with the experiments. These findings could help to achieve adequate levels of durable resistance through marker-assisted selection and pyramiding resistance QTLs in local germplasm.

Keywords: association mapping, durable adult plant resistance, *Puccina triticina*, *Triticum aestivum*

Introduction

Leaf rust caused by *Puccina triticina* is one of the most destructive fungal diseases of wheat (*Triticum aestivum*). The pathogen is adapted to the different climates found in worldwide wheat growing areas and continually threatens global wheat production due to the appearance of new virulent races (Muhammad *et al.*, 2015). Effective control of the disease can be achieved by applying recommended fungicides. However, repeated application of the same groups of fungicides could cause slow erosion of disease control due to a gradual loss of sensitivity in the target pathogen and contribute to greater environmental pollution (Luo *et al.*, 2005). Therefore, planting genetically resistant varieties is the most profitable and environmentally friendly strategy to manage wheat rusts. Consequently, selection and development of cultivars with effective and durable leaf rust resistance are crucial and constitute a global breeding objective in wheat.

The constant development of resistant cultivars requires the availability of many sources of resistance to counter the continuing evolution of new virulence types within the pathogen population. The use of traditional breeding methods, such as linkage mapping, for identifying such resistance factors limits the extent of the germplasm that can be explored for new sources of resistance; such methods are also very expensive and time-consuming. Association mapping (AM) is an alternative approach that uses natural populations, eliminating the time and cost required to develop biparental populations and allowing a much larger and more representative gene pool to be surveyed simultaneously. Thus, plant varieties with different resistance genes can be identified and crossed to achieve transgressive segregation and produce superior progeny (Arraiano & Brown, 2017). In addition, as allelic variation and marker polymorphisms are observed at a higher frequency in AM panels compared to biparental populations, useful and novel alleles associated with the trait of interest may be identified when AM is used in conjunction with high-throughput marker technologies (Bajgain *et al.*, 2015). Moreover, AM offers higher resolution due to the exploitation of relatively higher numbers of meiotic events throughout the history of germplasm development. This mapping approach has been successfully used in various plant species to identify markers associated with different traits, as well as in uncovering the genetic basis of agronomically useful characters (Zhu *et al.*, 2008). Resistance to fungal diseases, and particularly to *P. triticina*, constitutes a valuable target trait for conducting genome-wide association studies (GWAS). Recent studies using this methodology have identified several quantitative trait loci (QTLs) for resistance to leaf rust (Crossa *et al.*, 2007; Kertho *et al.*,

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2015; Gao *et al.*, 2016; Jighly *et al.*, 2016; Li *et al.*, 2016). As the variation of complex traits usually shows QTL × environment interaction (QEI) (Mathews *et al.*, 2008), this should be considered to determine whether the genetic factors are reasonably stable across environments, determining their potential value in breeding programmes.

The objectives in the present study were to (i) characterize the reaction of an AM panel for adult plant resistance (APR) to leaf rust, (ii) identify stable QTLs for APR to leaf rust, and (iii) evaluate the effect of pyramiding QTLs for APR on disease severity level.

Materials and methods

Genetic resources

A core collection of 96 winter wheat accessions (mainly cultivars and advanced breeding lines) sampled from 20 countries across five continents was used as the AM panel (Table 1). The material included accessions from Europe (46%), America (31%), Asia (15%) and Oceania (8%). The genotypes included in the collection were carefully chosen at the Institute of Field and Vegetable Crops (Novi Sad, Serbia) from a larger collection of 710 genotypes from more than 50 countries of the wheat-producing regions of the world, based on their contrasting phenotypic expression for traits of agronomic importance. This led to the development of the core collection of 96 genotypes of highly contrasting phenotypic traits used in this study. The collection comprised an important number of 'founder genotypes' that have been widely used as parents in breeding programmes across the world. A detailed phenotypic and molecular characterization of the collection is reported in Kobiljski *et al.* (2002).

Experimental design and evaluation of phenotypic traits

The AM panel was evaluated under natural infection by *P. triticina* in six field trials performed at two locations: Faculty of Agriculture and Forestry Sciences (FALP), La Plata, Argentina and Julio Hirschhorn Experimental Station (JHES), Los Hornos, Argentina. At each location, three experiments (Exp1, Exp2 and Exp3) were planted on 21 June 2012, 14 June 2013 and 31 July 2013, respectively. The experimental design was a split-plot with two replications. The main plots were the experiments and the subplots were the 96 genotypes. The local cultivar Buck SY110, susceptible to prevailing races in the area, was included in all experiments as a susceptible control. Because many genotypes had winter growth habits and had vernalization requirements, seeds were germinated in Petri dishes and vernalized for 3 weeks at 4–8 °C in a growth chamber before planting in the field. Then, 10–15 seedlings of each genotype were planted in each row. Plots were 0.5 m long by 0.2 m (one row) wide. Between plots, one row of oat (*Avena sativa*) was sown to reduce interplot interference. The soil was a typical argiudol and the entire experiments were fertilized with 50 kg P_2O_5 ha^{-1} as calcium triple superphosphate at sowing and with 100 kg N ha^{-1} applied as granulated urea, one-half at sowing and the other-half at growth stage (GS) 3.3 (Zadoks *et al.*, 1974). In order to estimate APR, all the wheat accessions were scored for reaction to leaf rust by visually estimating the percentage of leaf area infected by pustules on the three upper leaves (flag leaf, flag leaf −1, flag leaf −2) of 7–10 plants in each plot. Additionally, averages of the three leaves for each plant were calculated. Data collection in each field experiment was initiated when the susceptible control cultivar showed clear infection by the disease in the top three leaves (at least 40%).

Analysis of phenotypic data

The leaf rust phenotypic data collected across six experiments were subjected to analysis of variance (ANOVA). Data analyses were conducted with GenStat 12th edn. (Payne *et al.*, 2009) by combined analysis of ANOVA with split-plot design, considering the experiments and the genotypes as fixed effect and blocks as random effects. To compare the response of the genotypes to leaf rust across environments, the least significant differences (LSDs) of means of genotype severity values were calculated. In addition, to evaluate the consistency in behaviour of genotypes throughout the experiments, Pearson correlation coefficients were calculated. Furthermore, the phenotypic and genotypic variance components were also estimated from the ANOVA and the information was used to calculate broad-sense heritability (h^2) as the ratio of genotypic to phenotypic variance: $h^2 = \sigma^2 g / (\sigma^2 g + \sigma^2 ge/E + \sigma^2/ER)$, where $\sigma^2 g$ denotes the genotypic variance, $\sigma^2 ge$ the variance of genotype × environment interaction and σ^2 the variance of error. E and R are the number of environments and replications, respectively.

Molecular markers and map construction

The AM panel was genotyped with high-density Diversity Arrays Technology (DArT) markers. The DArT technology was provided by Triticarte Pty Ltd (Canberra, Australia; http://www.triticarte.com.au), a whole-genome profiling service laboratory. A total of 874 polymorphic DArT loci was used named using the prefix wPt followed by a unique numerical identifier. Those with <5% allele frequency were excluded from all analysis, resulting in the removal of 39 markers. An integrated map of the DArT markers developed by Crossa *et al.* (2007) was used to assign 525 trait-associated markers to chromosome arms. Additionally, the chromosomal locations of at least 177 of the 310 unmapped markers were provided by Triticarte.

Population structure and linkage disequilibrium

The genetic structure of the collection was investigated using a subset of 219 DArT markers distributed evenly across the genome. An admixture model with correlated allele frequency was applied using the program STRUCTURE (Pritchard *et al.*, 2000). A burn-in of 10 000 iterations followed by 10 000 Markov chain Monte Carlo (MCMC) replicates was conducted to test K values in the range of 1–12. Number of subgroups (ΔK) in the panel was estimated following the approach of Evanno *et al.* (2005), in which the number of ΔK is maximized and then the likelihood distribution of K was examined. In addition, estimation of kinship relations was investigated using TASSEL v. 2.01 (Bradbury *et al.*, 2007), which generates a pairwise relationship matrix (kinship matrix). Finally, an ANOVA for leaf rust response using population subgroups was performed to determine the significance of the confounding effect of population structure on the phenotypic trait. On the other hand, the pairwise linkage disequilibrium (LD) among the pairs of markers was calculated using observed versus expected allele frequencies of the markers using TASSEL v. 2.01. Allele frequency correlations (r^2) were

Table 1 Name, growth type and country of origin of the 96 wheat genotypes in the germplasm set analysed, along with their subgroup Q as defined by STRUCTURE analysis (assigned to a subgroup if $P > 0.5$)

Genotype	Origin	Growth type	Q	Genotype	Origin	Growth type	Q
Magnif 41	AR	Winter	1	NS 559	RS	Winter	1
Cook	AU	Spring	1	NS 602	RS	Winter	1
Kite	AU	Spring	1	NS 63-24	RS	Winter	1
Min. Dwarf	AU	Spring	1	NS 66/92	RS	Winter	1
Timson	AU	Winter	1	NS 79/90	RS	Winter	1
Triple Dirk B	AU	Spring	1	Renesansa	RS	Winter	1
Triple Dirk S	AU	Spring	1	Sava	RS	Winter	1
Rusalka	BG	Winter	1	Slavija	RS	Winter	1
Lambriego Inia	CL	Winter	1	Donska Polupat.	RU	Winter	1
Al-Kan-Tzao	CN	Spring	1	Mironovska 808	UA	Winter	1
Ching-Chang 6	CN	Spring	1	Benni Multifloret	US	Winter	1
Peking 11	CN	Facultative	1	Florida	US	Winter	1
Tibet Dwarf	CN	Spring	1	Hays 2	US	Winter	1
Tom Thumb	CN	Winter	1	Helios	US	Winter	1
Capelle Desprez	FR	Winter	1	Holly E	US	Winter	1
Durin	FR	Winter	1	Hope	US	Spring	1
Avalon	GB	Winter	1	INTRO 615	US	Winter	1
Brigant	GB	Winter	1	Lr 10	US	Spring	1
Highbury	GB	Spring	1	Norin 10/Brev.14	US	Winter	1
TJB 990-15	GB	Winter	1	Phoemix	US	Winter	1
Ana	HR	Winter	1	Purd./Loras	US	Winter	1
ZG 1011	HR	Winter	1	Purd. 38120	US	Winter	1
ZG 987/3	HR	Winter	1	Purd. 5392	US	Winter	1
ZG K 3/82	HR	Winter	1	Red Coat	US	Winter	1
ZG K 238/82	HR	Winter	1	Semilla Eligulata	US	Winter	1
ZG K T 159/82	HR	Winter	1	UC 65680	US	Spring	1
Bankut 1205	HU	Winter	1	Vel	US	Winter	1
L-1	HU	Winter	1	WWMCB 2	US	Spring	1
Szegedi 768	HU	Winter	1	Gala	AR	Winter	2
Hira	IN	Spring	1	Triple Dirk B (bulk)	AU	Spring	2
Sonolika	IN	Spring	1	BCD 1302/83	MD	Winter	2
Suwon 92	IN	Winter	1	Cajeme 71	MX	Spring	2
UPI 301	IN	Spring	1	L 1/91	RS	Winter	2
Acciaio	IT	Facultative	1	L 1A/91	RS	Winter	2
Ai-bian	JP	Spring	1	Nizija	RS	Winter	2
Norin 10	JP	Winter	1	Nov. Crvena	RS	Winter	2
Saitama 27	JP	Spring	1	Nova Banatka	RS	Winter	2
Triticum compactum	LV	Winter	1	NS 33/90	RS	Winter	2
Inia 66	MX	Spring	1	NS 46/90	RS	Winter	2
Mex. 120	MX	Spring	1	NS 55-25	RS	Winter	2
Mex. 17 bb	MX	Winter	1	NS 74/95	RS	Facultative	2
Mex. 3	MX	Spring	1	PKB Krupna	RS	Winter	2
S. Cerros	MX	Spring	1	Pobeda	RS	Winter	2
Vireo S	MX	Winter	1	Sofija	RS	Winter	2
F 4 4687	RO	Winter	1	Bezostaja 1	RU	Winter	2
Ivanka	RS	Winter	1	Centurk	US	Winter	2
Mina	RS	Winter	1	Lr 12	US	Spring	2
NS 22/92	RS	Winter	1	*Triticum sphaerococcum*	US	Winter	2

AR, Argentina; AU, Australia; BG, Bulgaria; CL, Chile; CN, China; FR, France; GB, United Kingdom; HR, Croatia; HU, Hungary; IN, India; IT, Italy; JP, Japan; LV, Latvia; MD, Moldova; MX, Mexico; RO, Romania; RS, Serbia; RU, Russia; UA, Ukraine; US, United States of America.

calculated according to Weir (1996) with the LD function using 1000 permutations. From the interchromosomal pairs, a critical r^2 value, beyond which LD is due to true physical linkage, was estimated by taking the 95th percentile of the square root-transformed r^2 data (Breseghello & Sorrells, 2006). Results concerning population structure and LD of this AM panel have been described previously by Neumann *et al.* (2011).

Marker-trait association analysis

For association analysis, APR to leaf rust was represented by the mean values of severity of the two replications in each experiment and the analyses were performed separately for every experiment. The program TASSEL v. 2.01 was used to calculate associations between the markers and the phenotypic trait,

employing the general linear model (GLM) based on Q matrix (contribution of a genotype to each of the ancestor groups) derived from STRUCTURE. The Q matrix was introduced in the model as covariate to control the structure and avoid false positives (GLM (Q) model). With TASSEL v. 2.1 the mixed linear model (MLM) suggested by Yu *et al.* (2006) was additionally implemented using both Q matrix and the kinship matrix (MLM (Q + K)). The efficient mixed model analysis (Kang *et al.*, 2008) was chosen to reduce computing time and the MLM parameters were left at the default settings from TASSEL. In all cases, marker-trait associations (MATs) significant ($P < 0.05$) in at least four of the six experiments and highly significant ($P < 0.01$) in at least two experiments were considered to identify leaf rust resistance loci. Those markers significantly associated with resistance that overlapped or showed r^2 values higher than 0.263 (critical r^2) and consistent direction of their effects, were assigned to the same QTL. In addition, an ANOVA of a multiple regression, using the values of severity of leaf rust as a dependent variable and experiments and QTLs as independent for each QTL separately, was used to test if the QTLs were reasonably stable across environments. Finally, the significant QTLs identified in this study were inspected for correspondence with chromosomal regions associated to known *Lr* resistance genes and QTLs based on the genetic linkage map reported by Crossa *et al.* (2007).

Furthermore, in order to assess the pyramiding effect of significant QTLs for APR, the average severity of all environments was regressed against number of accumulated resistance alleles in each genotype of the AM panel. In addition, the genotypes were grouped according to the number of resistant alleles of QTLs for APR and differences between the means of the groups were compared using the Duncan multiple range test ($P < 0.05$).

Results

Analysis of leaf rust response

The response of the AM panel showed a wide and continuous distribution of leaf rust severity across field trials (Fig. 1). The mean values in the 96 genotypes ranged from 0 to 74.1%, with higher infection levels in Exp3 at both locations, due to the more favourable weather conditions for rust development (Fig. 2). The ANOVA revealed significant differences among genotypes to leaf rust infection at both test locations. The effect of experiments, genotypes, and their interactions were highly significant ($P < 0.001$). In all experiments, the frequency distribution was skewed to the left (genotypes with low disease severity score), with a considerable proportion of genotypes (ranging from 24 to 61 in JHES-Exp3 and JHES-Exp1, respectively) showing a resistant response.

Significant Pearson correlation coefficients ($P < 0.001$) for leaf rust severity level were observed among environments, which ranged from 0.61 to 0.95 indicating a

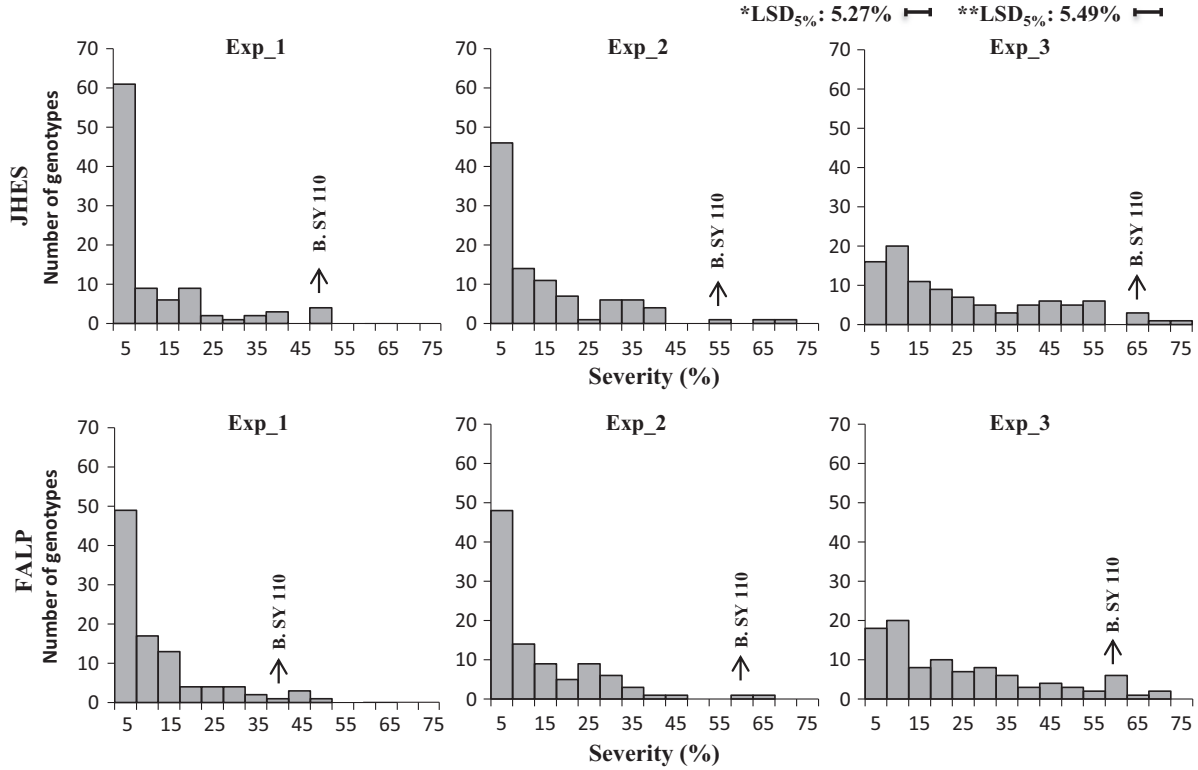
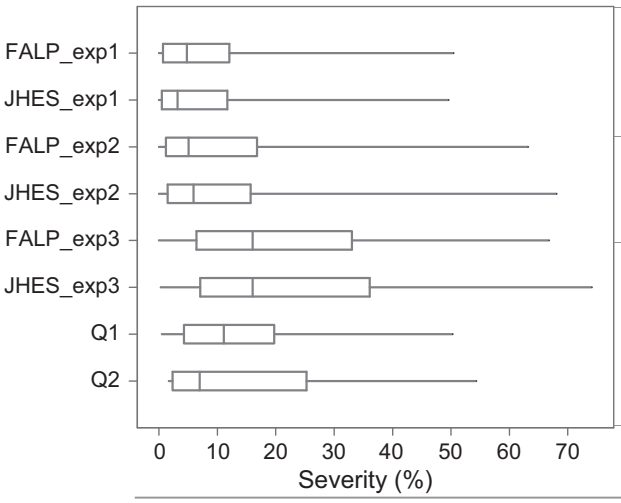


Figure 1 Frequency distribution of scores for leaf rust severity (average percentage of leaf area covered by pustules on the three upper leaves) of 96 winter wheat accessions used as an association mapping panel in six experiments carried out at the Faculty of Agricultural and Forestry Sciences (FALP) and Julio Hirschhorn Experimental Station (JHES) in 2012 and 2013. Arrows indicate susceptible control Buck SY110 (B.SY110); *least significant difference (LSD) within experiments and ** LSD between experiments.



		Environment					
	Statistic	FALP_Exp1	JHES_Exp1	FALP_Exp2	JHES_Exp2	FALP_Exp3	JHES_Exp3
	Minimum	0	0	0	0	0	0.3
	Mean	9.1	8.6	10.5	11.9	21.6	22.4
	Maximum	50.4	49.6	63.2	68.1	66.8	74.1
Correlation**	FALP_Exp1	-	0.86	0.61	0.63	0.61	0.64
	JHES_Exp1		-	0.71	0.68	0.68	0.68
	FALP_Exp2			-	0.95	0.67	0.68
	JHES_Exp2				-	0.66	0.68
	FALP_Exp3					-	0.93
	JHES_Exp3						-
	h^2		90.15				

Figure 2 Boxplot for severity of leaf rust disease (average percentage of leaf area covered by pustules on the three upper leaves) evaluated at adult plant stage in six field environments at two locations: Faculty of Agricultural and Forestry Sciences (FALP) and Julio Hirschhorn Experimental Station (JHES) in 2012 and 2013, and for the two Q-groups (Q1 and Q2) revealed from STRUCTURE analysis. The adjacent table indicates the minimum, maximum and mean disease severity values measured in each environment, the correlation between them and the broad-sense heritability (h^2). **Correlations were significant at $P < 0.01$ for all experiments.

stable response of wheat genotypes to leaf rust disease across experiments. The broad-sense heritability was consistent with these results, showing a value of 0.91 for the experiments average. Additionally, of the two population subgroups defined by STRUCTURE (Neumann *et al.*, 2011), Q1 presented a slightly higher phenotypic variation; however, there were no significant phenotypic differences for leaf rust resistance within the two groups of the Q matrix in all experiments (Fig. 2).

Analysis of DArT markers

The genome-wide distribution of markers was 190 on genome A, 310 on genome B, and 47 on genome D, covering a genetic distance of 949.2, 1060.1 and 283.5 cM, respectively. This resulted in an average marker distance of 4.9, 3.5 and 6.0 cM for the A, B and D genomes, respectively. All chromosomes except 6D were tagged by markers. Chromosome 6B had the highest number of markers (60), while chromosome 5D had the least number of markers with only two. Most markers mapped to only one position in the genome but 24 markers mapped to more than one and were considered as multilocus.

Whole-genome association mapping for leaf rust response

The results of the analysis of the GWAS using GLM (Q) and MLM (Q + K) models are shown in Table 2. In general, both models revealed similar significant markers, but GLM (Q) usually had higher *P*-values compared to the MLM (Q + K) model. For the entire dataset, a total of 14 significant marker-trait associations assigned to 13 QTLs were identified to be significantly associated with leaf rust resistance on chromosome arms 1BL, 1DS, 2AS, 2BL, 2DS, 3BS, 3BL, 4AL, 5BL/7BS, 6AL/6BS, 6BS (two) and 7DS. The proportion of phenotypic variance explained by the individual QTLs across all environments ranged from 4.6% to 14.6%. The most important R^2 effect was observed for QLr.wpt-3BL on 3BL (Table 2).

Out of the 13 genomic regions associated with leaf rust resistance identified in this study, the QTLs QLr.wpt-2AS and QLr.wpt-2BL on 2AS and 2BL were detected in all experiments. In addition, QLr.wpt-2DS, QLr.wpt-3BL and QLr.wpt-5BL/7BS on 2DS, 3BL and 5BL/7BS, respectively, showed significant association with leaf rust severity for five out of the six experiments. The remaining eight QTLs were significant in four of the six experiments (Fig. S1). Furthermore, in the ANOVA conducted to test QEI, none of the QTLs revealed a significant interaction with the environment. The mean squares of QEI were much smaller than those corresponding to the QTL effects (Table 3). Thus, the QTLs identified were reasonably stable and with consistent behaviour across experiments.

Relationship between number of favourable alleles and resistance to leaf rust

Considering the 14 DArT markers significantly associated with the 13 QTLs identified in this study, the number of favourable alleles present in a specific genotype ranged from 1 to 12. Genotypes with a greater number of combined QTLs for APR showed lower levels of disease severity, demonstrating a significant additive effect. As the number of combined QTLs increased, disease severity was reduced, with a regression coefficient of

Table 2 Quantitative trait loci (QTLs) and significant markers associated with adult plant resistance to leaf rust in 96 winter wheat accessions

QTL	Marker	Chr[a]	Pos[b]	Experiment	*P* (Q)[c]	*P* (Q + K)[d]	R^2	Similar region to	References
QLr.wpt-1BL	wPt9809	1BL	71.1	FALP-Exp1	0.0083	0.0096	0.087	QLr.stars-1BL3	Li *et al.* (2016)
				JHES-Exp2	0.0077	0.0345	0.092		
				FALP-Exp2	0.0259	0.0500	0.064		
				JHES-Exp3	0.0485	0.0485	0.046		
QLr.wpt-1DS	wPt0413	1DS	49.2	JHES-Exp1	0.0017	0.0016	0.105	Novel	–
				FALP-Exp1	0.0138	0.0117	0.078		
				JHES-Exp2	0.0086	0.0224	0.094		
				FALP-Exp2	0.0006	0.0013	0.135		
QLr.wpt-2AS	wPt3114	2AS	68.3	JHES-Exp1	0.0366	0.0366	0.048	QLr.sfr-2AL	Schnurbusch *et al.* (2004)
				FALP-Exp1	0.0119	0.0129	0.081		
				JHES-Exp2	0.0025	0.0100	0.115		
				FALP-Exp2	0.0037	0.0078	0.103		
				JHES-Exp3	0.0135	0.0151	0.075		
				FALP-Exp3	0.0134	0.0147	0.078		
QLr.wpt-2BL	wPt8460	2BL	92.8	JHES-Exp1	0.0076	0.0077	0.078	QLr.osu-2B	Xu *et al.* (2005)
				FALP-Exp1	0.0145	0.0160	0.079		
				JHES-Exp2	0.0026	0.0025	0.117		
				FALP-Exp2	0.0024	0.0025	0.112		
				JHES-Exp3	0.0036	0.0026	0.101		
				FALP-Exp3	0.0104	0.0112	0.085		
QLr.wpt-2DS	wPt0330	2DS	8.0	JHES-Exp1	0.0056	0.0058	0.084	*Lr22*; *LrSV1*; QLr.cimmyt-2DS, QLr.sfr-2DS and QLr.hbau-2DS	Dyck (1979); Ingala *et al.* (2012); Rosewarne *et al.* (2012); Schnurbusch *et al.* (2004); Zhang *et al.* (2009)
				FALP-Exp1	0.0015	0.0017	0.116		
				JHES-Exp2	0.0373	0.0302	0.064		
				JHES-Exp3	0.0052	0.0052	0.091		
				FALP-Exp3	0.0220	0.0173	0.066		
QLr.wpt-3BS	wPt7212	3BS	23.2	JHES-Exp1	0.0202	0.0202	0.058	*Lr27/Sr*; *LrSV2*	Singh & McIntosh (1984); Ingala *et al.* (2012)
				FALP-Exp1	0.0014	0.0016	0.118		
				JHES-Exp3	0.0046	0.0080	0.092		
				FALP-Exp3	0.0078	0.0080	0.086		
QLr.wpt-3BL	wPt7502	3BL	57.4	JHES-Exp1	0.0007	0.0007	0.118	Novel	–
				FALP-Exp1	0.0004	0.0004	0.141		
				JHES-Exp2	0.0004	0.0011	0.146		
				FALP-Exp2	0.0011	0.0015	0.125		
				JHES-Exp3	0.0344	0.0500	0.058		
QLr.wpt-4AL	wPt7280	4AL	142.9	JHES-Exp1	0.0217	0.0217	0.057	*Lr28/Sr7*	McIntosh *et al.* (1982)
				FALP-Exp1	0.004	0.0045	0.097		
				JHES-Exp2	0.0048	0.0064	0.100		
				FALP-Exp2	0.0331	0.0251	0.060		
QLr.wpt-5BL/7BS	wPt9467	5BL/7BS	77/50	JHES-Exp1	0.0097	0.0069	0.068	Novel	–
				FALP-Exp1	0.0063	0.0039	0.088		
				JHES-Exp2	0.0369	0.0088	0.062		
				JHES-Exp3	0.0163	0.0047	0.067		
				FALP-Exp3	0.0218	0.0096	0.064		
QLr.wpt-6AL/6BS	wPt8833	6AL/6BS	56.5/5.2	JHES-Exp1	0.0075	0.0075	0.078	Novel	–
				FALP-Exp2	0.0081	0.0081	0.086		
				JHES-Exp3	0.0392	0.0392	0.054		
				FALP-Exp3	0.0449	0.0449	0.054		
QLr.wpt-6BS.1	wPt6282	6BS	41.4	JHES-Exp1	0.0116	0.0116	0.073	Novel	–
				FALP-Exp1	0.0131	0.0131	0.081		
				JHES-Exp2	0.0031	0.0094	0.114		
				FALP-Exp2	0.0025	0.0076	0.114		
	wPt3116	6BS	41.4	JHES-Exp1	0.0052	0.0052	0.083		
				FALP-Exp1	0.0135	0.0135	0.077		
				JHES-Exp2	0.0124	0.0311	0.086		
				FALP-Exp2	0.0054	0.0100	0.095		
QLr.wpt-6BS.2	wPt2175	6BS	64.9	JHES-Exp1	0.0299	0.0303	0.052	QLr.caas-6BS.1	Ren *et al.* (2012)
				JHES-Exp2	0.0195	0.0218	0.073		
				FALP-Exp2	0.0055	0.0094	0.091		
				JHES-Exp3	0.0398	0.0350	0.051		

(continued)

Table 2 (continued)

QTL	Marker	Chr[a]	Pos[b]	Experiment	*P* (Q)[c]	*P* (Q + K)[d]	R^2	Similar region to	References
QLr.wpt-7DS	wPt2565	7DS	1.3	JHES-Exp1	0.0119	0.0119	0.066	Novel	–
				FALP-Exp1	0.0098	0.0098	0.080		
				JHES-Exp3	0.0329	0.0469	0.055		
				FALP-Exp3	0.0035	0.0045	0.098		

[a]Chr: chromosome.
[b]Pos: the marker position on the linkage map.
[c]*P* (Q): *P*-values using Q matrix.
[d]*P* (Q + K): *P*-values using both Q matrix and kinship matrix.

−3.44 ($P < 0.0001$) and R^2 values of 0.33 (Fig. 3a). Thus, statistically significant reduction in the severity levels generated by accumulation of resistant alleles was observed, reaching values below 15% when seven or more genes were present in the same genotype (Fig. 3b).

The frequency of resistant alleles for each QTL within the AM panel ranged from 26% (QLr.wpt-6BS.1) to 89% (QLr.wpt-3BL and QLr.wpt-4AL). Considering the population structure, the greatest difference in the frequency of favourable alleles was observed for QLr.wpt-5BL/7BS, which was present in 2 (10%) and 38 (50%) genotypes of subpopulation Q1 and Q2, respectively. In addition, most of the QTLs identified showed relatively stable frequency of resistant alleles across the different origins. One exception is QLr.wpt-2AS, which was only observed in one genotype from Oceania, while it was present in 10, 13 and 20 genotypes from Asia, America and Europe, respectively (Table 4).

Discussion

The present study reports the results of a GWAS using a worldwide AM panel of 96 genotypes. Although the panel size is not large, it includes genotypes that have been selected from a larger population based on their contrasting phenotypic expression in order to accumulate as much variation as possible. Thus, considerable phenotypic variation in leaf rust severity among genotypes was observed. These results are in agreement with Neumann *et al.* (2011) and Gerard *et al.* (2017), who reported wide phenotypic variation in this AM panel for several agronomic traits, including resistance to leaf rust and septoria leaf blotch (*Zymoseptoria tritici*). In addition, genetic diversity studies using simple sequence repeat (SSR) markers have concluded that large genetic variability has been successfully achieved in the selected material (Kobiljski *et al.*, 2002).

The two groups of structure identified in the population were consistent with the origin and pedigree of the materials. However, the genotypes of both groups did not show significant differences in leaf rust resistance. In the entire collection only 14.9% of the marker pairs showed a significant level of LD ($P < 0.01$). These findings are in line with those reported by Arraiano & Brown (2017), but are slightly lower than the level described by Crossa *et al.* (2007), who found 26% of marker pairs in significant LD. In addition, the overall LD decayed fast (local regression curve does not intercept the critical r^2), similar to that reported by Breseghello & Sorrells (2006).

Genome-wide association studies have been reported as being a useful approach to identify linked markers with genes controlling important agronomic traits (Zhu *et al.*, 2008). In recent GWAS, using DArT and SNP (single nucleotide polymorphism) markers, several genetic regions associated with resistance to leaf rust in wheat have been identified (Crossa *et al.*, 2007; Kertho *et al.*, 2015; Gao *et al.*, 2016; Jighly *et al.*, 2016; Li *et al.*, 2016). In the present study, 13 QTLs associated with resistance to leaf rust were found linked to 14 DArT markers on the chromosomes 1BL, 1DS, 2AS, 2BL, 2DS, 3BS, 3BL, 4AL, 6BS (two), 7DS, 5BL/7BS and 6AL/6BS. Of the total, the QTLs QLr.wpt-1BL, QLr.wpt-2AS, QLr.wpt-2BL, QLr.wpt-2DS, QLr.wpt-3BS, QLr.wpt-4AL and QLr.wpt-6BS.2 were mapped on similar genomic regions harbouring known genes or QTLs. In contrast, QLr.wpt-1DS, QLr.wpt-3BL, QLr.wpt-6BS.1, QLr.wpt-7DS, QLr.wpt-5BL/7BS and QLr.wpt-6AL/6BS were found in regions where, to the authors' knowledge, no previous evidence of *Lr* resistance genes or QTLs has been reported.

In order to compare the map positions of the QTLs identified with previously reported genetic factors, an integrated genetic linkage map reported by Crossa *et al.* (2007) was used. The QLr.wpt-1BL identified by wPt9809 (71.1 cM) on 1BL resides in a similar genomic region to where QLr.stars-1BL3 was located by Li *et al.* (2016). In that study, QLr.stars-1BL3 was linked to the SNP marker IWA579 in a winter wheat worldwide collection, but the study was carried out at the seedling stage. The QTL QLr.wpt-2AS linked to wPt3114 (68.3 cM) was mapped close to the centromere on 2AS. Schnurbusch *et al.* (2004) reported the location of the leaf rust resistance QTL QLr.sfr-2AL in a similar chromosomal region, which was closely linked to cfa2263c in the winter wheat cultivar Forno. The marker cfa2263c was found at an approximate distance of 3.3 cM from wPt3114, but on the other side of the centromere. The cultivar Forno is descended from the French cultivar Cappelle Desprez, which is present in the AM panel used in the present study, and has been widely used in Europe because it confers partial leaf rust resistance (McIntosh,

Table 3 Mean squares for the analysis of variance of DArT markers associated with the 13 quantitative trait loci (QTLs) for resistance to leaf rust in 96 winter wheat accessions

		Source of variation			
Name of QTL	Marker	Experiments	QTL	QEI[a]	Error
d.f.		5	1	5	552
QLr.wpt-1BL	wPt9809	2968.9***	4279.3***	112.2 ns	174.7
QLr.wpt-1DS	wPt0413	3755.5***	8194.2***	178.9 ns	179.7
QLr.wpt-2AS	wPt3114	3749.1***	3760.1***	182.5 ns	189.2
QLr.wpt-2BL	wPt8460	3746.3***	6027.7***	163.8 ns	185.8
QLr.wpt-2DS	wPt0330	2466.9***	5466.5***	94.9 ns	186.3
QLr.wpt-3BS	wPt7212	3911.9***	4798.6***	167.5 ns	187.1
QLr.wpt-3BL	wPt7502	1642.7***	6903.4***	124.5 ns	184.0
QLr.wpt-4AL	wPt7280	1331.4***	2934.6***	142.2 ns	189.1
QLr.wpt-5BL/7BS	wPt9467	3501.8***	1995.1***	111.2 ns	191.7
QLr.wpt-6AL/6BS	wPt8833	3209.7***	4820.9***	90.3 ns	187.5
QLr.wpt-6BS.1	wPt6282	2992.7***	5525.8***	143.6 ns	186.9
	wPt3116	2671.3***	4646.7***	86.3 ns	186.1
QLr.wpt-6BS.2	wPt2175	3663.7***	5677.1***	165.6 ns	176.6
QLr.wpt-7DS	wPt2565	3603.5***	5582.2***	235.9 ns	181.9

***Significant at $P < 0.001$; ns: not significant.
[a]QEI: QTL × environment interaction.

1992). Meanwhile, the QLr.wpt-2BL linked to wPt8460 (92.8 cM) was found in the proximity of QLr.osu-2B, previously identified by Xu *et al.* (2005) on 2BL. QLr.osu-2B was flanked by markers Xagc.tgc135 and Xcatg.atgc60 in the wheat cultivar CI 13227 and reported to provide a high level of slow leaf-rusting resistance (Shaner *et al.*, 1997).

The APR QTL for leaf rust QLr.wpt-2DS, linked to wPt0330 (8 cM), was located in a similar position to *Lr22* (Dyck, 1979). In addition, in this region, Ingala *et al.* (2012) recently identified the APR gene *LrSV1* linked to marker gwm296 in the Argentinean cultivar Sinvalocho MA. In the present study, the marker wPt0330 is 1 cM from gwm296 and, interestingly, the AM panel includes the Argentinean cultivar Magnif 41, which is descended from Sinvalocho MA. The cultivar Sinvalocho MA has been successfully used as a source of leaf rust resistance in Argentina and has remained durable over time. Additionally, one of its parents, 38 MA, has been extensively reported as carrying APR genes (Antonelli, 1983). Three additional QTLs, QLr.sfr-2DS, QLr.hbau-2DS and QLr.cimmyt-2DS derived from the cultivars Forno, Saar and Avocet, respectively, were previously mapped to a similar position (Schnurbusch *et al.*, 2004; Zhang *et al.*, 2009; Rosewarne *et al.*, 2012). In particular, QLr.hbau-2DS has shown a high level of APR to leaf rust, stripe rust and powdery mildew in Europe, Asia and South America.

The QTL QLr.wpt-3BS, tagged by wPt7212 (23.2 cM) on 3BS, mapped close to the gene *Lr27* (<10 cM); this gene confers resistance to leaf rust at the seedling stage, but only when the complementary gene *Lr31* on 4B is also present (Singh & Bowden, 2010). *Lr27* is linked to *Sr2*, which confers durable race-nonspecific APR to stem rust (*Puccinia graminis*) and resistance to powdery mildew. Mago *et al.* (2011) have suggested that a single gene may be responsible for the resistance to these three fungal pathogens. On this chromosomal region, Ingala *et al.* (2012) also identified an *Lr* gene, designated *LrSV2*, expressed only at the adult stage. Likewise, QLr.wpt-4AL, identified by wPt7280 (142.9 cM), was mapped on the long arm of chromosome 4A, which colocated with the region known to host the gene *Lr28*, mapped by McIntosh *et al.* (1982). *Lr28* is linked to *Sr7* and so this region is also involved in multiple-rust resistance. In the spelt winter line Oberkulmer, Messmer

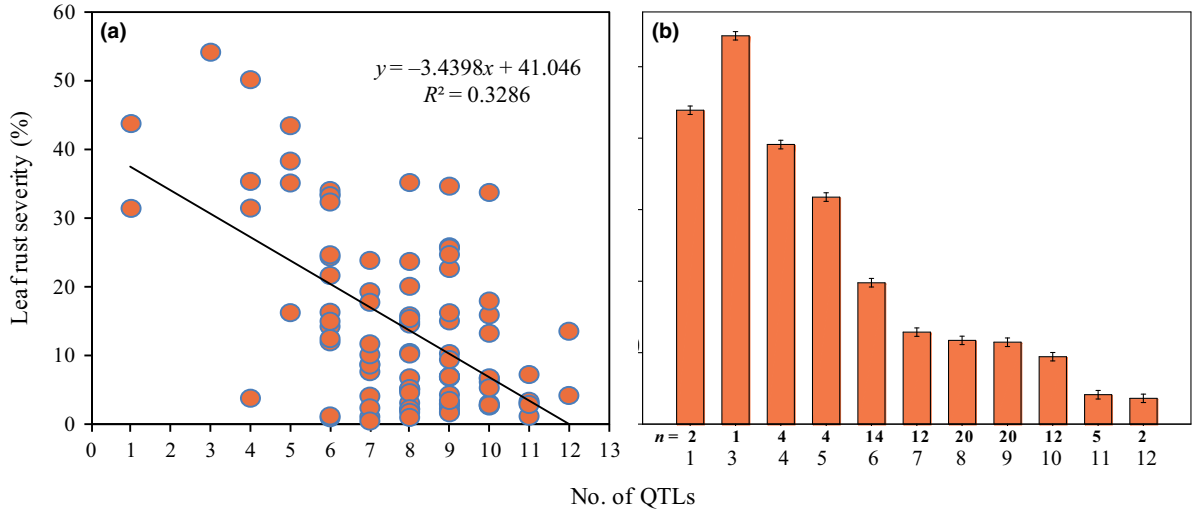


Figure 3 (a) Regression between disease severity levels (average percentage of leaf area covered by pustules on the three upper leaves) and the number of combined quantitative trait loci (QTLs), (b) effect of the accumulation of QTLs on leaf rust severity among different classes. The error bars denote 1 SE, $P < 0.05$ and *n* indicates the number of genotypes within each class.

Table 4 Resistance allele frequency of DArT markers linked to quantitative trait loci (QTLs) for resistance to leaf rust in association mapping panel of wheat cultivars, two subpopulations (Q1 and Q2) and the four origins

	Frequency						
Resistance allele	Overall	Subpop. Q1	Subpop. Q2	Europe	America	Asia	Oceania
QLr.wpt-1BL	0.64	0.63	0.65	0.73	0.48	0.60	0.75
QLr.wpt-1DS	0.66	0.62	0.80	0.64	0.66	0.67	0.75
QLr.wpt-2AS	0.46	0.50	0.30	0.45	0.45	0.67	0.13
QLr.wpt-2BL	0.43	0.38	0.60	0.55	0.34	0.27	0.38
QLr.wpt-2DS	0.80	0.76	0.95	0.80	0.86	0.60	1.00
QLr.wpt-3BS	0.52	0.46	0.75	0.66	0.34	0.53	0.38
QLr.wpt-3BL	0.89	0.88	0.90	0.93	0.83	0.87	0.88
QLr.wpt-4AL	0.89	0.86	1.00	0.95	0.90	0.73	0.75
QLr.wpt-5BL/7BS	0.42	0.50	0.10	0.41	0.38	0.40	0.63
QLr.wpt-6AL/6BS	0.74	0.74	0.75	0.77	0.62	0.80	0.88
QLr.wpt-6BS.1	0.33	0.34	0.30	0.39	0.28	0.33	0.25
QLr.wpt-6BS.1	0.26	0.25	0.30	0.36	0.24	0.20	0.25
QLr.wpt-6BS.2	0.66	0.70	0.50	0.59	0.79	0.60	0.63
QLr.wpt-7DS	0.77	0.75	0.85	0.84	0.72	0.67	0.75

et al. (2000) reported the QTL QLr.sfrs-7B.1, which appears to be close to the multilocus marker wPt9467 associated with QLr.wpt-5BL/7BS on 7BS. However, the panel used in the present study did not contain spelt wheat, and it is probable that QLr.wpt-5BL/7BS is a new locus for APR to leaf rust in bread wheat. Finally, the QLr.wpt-6BS.2 linked to wPt2175 (64.9 cM) was found in a region close to the centromere on 6BS. In the same region, QLr.caas-6BS.1, derived from the wheat cultivar Bainong 64, was detected by Ren *et al.* (2012). Further genetics studies and allelism tests are required to confirm whether the positions of the genomic regions found here correspond to the known *Lr* genes and QTLs. This confirmation would enable the use of the DArT markers found in this study for marker-assisted selection (MAS).

QTL mapping has been widely used in plant breeding; however, approaches taking into account the QEI are limited (Crossa, 2012). The differentiation of QTLs into stable or environment-dependent is important for achieving maximum effectiveness in different environments and making them suitable for MAS. The QTLs for resistance to leaf rust identified in the present study did not show significant interactions with the experiments, indicating consistent behaviour across environments. These results contrast with previous reports in which QEI for resistance to leaf rust has been reported (Gutiérrez *et al.*, 2015). The QTLs identified in the present study were most probably stable across environments because only those that were significant in at least four of the six experiments were considered. Such QTLs, free from environmental interactions, are probably suitable for wide application in plant breeding in the target environments.

In the AM panel used here, the resistance to leaf rust was determined by several QTLs with small to moderate effect. In addition, genotypes in the panel showed a significant correlation between the number of QTLs and the final leaf rust severity. This finding indicates the presence of additive effects, and therefore pyramiding QTLs in a single genetic background would contribute to reducing leaf rust. These results are in agreement with Lagudah (2011), who mentioned the enhanced reduction of rust severity when multiple partial APR genes are combined. On the other hand, in some of the QTLs identified, the frequency of resistant alleles was lower in some subpopulations or origins than in others, suggesting that overall resistance could be improved by increasing crosses among subgroups.

Finally, although the AM panel was naturally infected, several studies reported the same races of *P. triticina* in the eastern epidemiological zone (Argentina, Brazil, Paraguay and Uruguay) from South America, probably due to the lack of geographic barriers and similarity of the cultivars used in the area (Germán *et al.*, 2007; Ordoñez *et al.*, 2010). In recent years, the most common virulence phenotypes found in Brazil were also commonly found in Argentina and Uruguay, confirming that the eastern Atlantic region is a single epidemiological zone for wheat leaf rust (Ordoñez *et al.*, 2010). These studies have shown a complex composition of races in the region, with predominance of the known leaf rust races MFP, MDP and TDT 10-20 (Campos, 2014). Seedling resistance genes *Lr27* and *Lr28* and APR gene *Lr22*, whose positions are similar to the QTLs QLr.wpt-3BS, QLr.wpt-4AL and QLr.wpt-2DS, respectively, have been reported as effective resistance genes against the mentioned leaf rust races (Ordoñez *et al.*, 2010; Campos, 2014). However, their frequency in the most widespread local cultivars is low, so their introduction in new cultivars could be an additional alternative to the QTLs of additive effects identified in this study to increase the diversity of effective leaf rust resistance into local high-yielding cultivars.

The QTLs for APR found in this study showed additive effects, relatively small R^2 and consistent behaviour across experiments. These minor QTLs could be pyramided using MAS for developing new combinations achieving high durable resistance in the target environments. In addition, accessions in the panel with several

and different QTLs for resistance to leaf rust could serve as useful parental breeding lines to achieve transgressive segregation and thus allow more efficient selection of progeny with better resistance than either parent.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Manhattan plots showing *P* values across 21 wheat chromosomes for DArT markers associated with field adult plant resistance to leaf rust. (a, c, e) experiments 1, 2 and 3 at Julio Hirschhorn Experimental Station (JHES); (b, d, f) experiments 1, 2 and 3 at Faculty of Agriculture and Forestry Sciences (FALP). The horizontal blue line indicates significant threshold at $P = 0.05$ and 0.01. DArT markers significant in at least four of the six environments $P < 0.05$ or $P < 0.01$ are shown in red.