Characterization and mapping of leaf rust resistance in four durum wheat cultivars

Dhouha Kthiri[1], Alexander Loladze[2], P. R. MacLachlan[1], Amidou N'Diaye[1], Sean Walkowiak[1], Kirby Nilsen[1], Susanne Dreisigacker[2], Karim Ammar[2], Curtis J. Pozniak[1]*

1 Department of Plant Sciences, Crop Development Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, **2** International Maize and Wheat Improvement Center (CIMMYT), Mexico, D.F., Mexico

* curtis.pozniak@usask.ca

Abstract

Widening the genetic basis of leaf rust resistance is a primary objective of the global durum wheat breeding effort at the International Wheat and Maize Improvement Center (CIMMYT). Breeding programs in North America are following suit, especially after the emergence of new races of *Puccinia triticina* such as BBG/BP and BBBQD in Mexico and the United States, respectively. This study was conducted to characterize and map previously undescribed genes for leaf rust resistance in durum wheat and to develop reliable molecular markers for marker-assisted breeding. Four recombinant inbred line (RIL) mapping populations derived from the resistance sources Amria, Byblos, Geromtel_3 and Tunsyr_2, which were crossed to the susceptible line ATRED #2, were evaluated for their reaction to the Mexican race BBG/BP of *P. triticina*. Genetic analyses of host reactions indicated that leaf rust resistance in these genotypes was based on major seedling resistance genes. Allelism tests among resistant parents supported that Amria and Byblos carried allelic or closely linked genes. The resistance in Geromtel_3 and Tunsyr_2 also appeared to be allelic. Bulked segregant analysis using the Infinium iSelect 90K single nucleotide polymorphism (SNP) array identified two genomic regions for leaf rust resistance; one on chromosome 6BS for Geromtel_3 and Tunsyr_2 and the other on chromosome 7BL for Amria and Byblos. Polymorphic SNPs identified within these regions were converted to kompetitive allele-specific PCR (KASP) assays and used to genotype the RIL populations. KASP markers *usw215* and *usw218* were the closest to the resistance genes in Geromtel_3 and Tunsyr_2, while *usw260* was closely linked to the resistance genes in Amria and Byblos. DNA sequences associated with these SNP markers were anchored to the wild emmer wheat (WEW) reference sequence, which identified several candidate resistance genes. The molecular markers reported herein will be useful to effectively pyramid these resistance genes with other previously marked genes into adapted, elite durum wheat genotypes.

Introduction

Durum wheat (*Triticum turgidum* L. ssp. *durum*) is a widely grown crop used in the preparation of diverse food products including bread, couscous, bulgur and pasta. As the main global provider of improved wheat germplasm, the International Wheat and Maize Improvement Center (CIMMYT) sustains a breeding effort addressing all issues important to the viability of durum wheat crops worldwide. Canada is among the world's largest producers and is the largest exporter of durum wheat. Canada also maintains an extensive genetic improvement effort of durum wheat, aiming at enhancing the competitiveness of this crop for Canadian farmers. Leaf rust, caused by the fungal pathogen *Puccinia triticina* Eriks, is a major biotic constraint threatening the productivity of durum wheat worldwide, thereby representing an important breeding objective for both programs. This foliar disease is capable of causing considerable grain yield losses and quality downgrades [1,2]. Improvement of resistance is the most cost-effective and environmentally viable strategy for controlling leaf rust, and deployment of cultivars with durable resistance is a major target of wheat breeding programs globally [3–5].

Over 76 genes conferring resistance to leaf rust (*Lr* genes) have so far been identified and localized to specific wheat chromosomes. Most of these genes originated from hexaploid bread wheat (*T. aestivum* L.) or wild grass species related to wheat, while a limited number have been found and characterized in tetraploid durum wheat [6]. In both durum and bread wheat, monogenically inherited *Lr* genes have usually been defeated by new, rapidly evolving races of *P. triticina* with different virulence patterns [2,7]. Durum wheat has historically been more resistant to leaf rust than bread wheat [8,9], and most of the predominant *P. triticina* isolates found on common wheat are avirulent on a large number of durum wheat genotypes [10–12]. However, with the appearance of more durum-specific races of the pathogen, and the breakdown of resistance in several countries during the last decade [2,13,14], leaf rust has become a primary challenge for durum breeders globally. The detection and spread of the new *P. triticina* race BBG/BN with virulence to *Lr72*, a widely-deployed gene in the CIMMYT durum germplasm, has led to severe epidemics in northwestern Mexico, from 2001 to 2003 [8,15]. Since then, genetic studies conducted at CIMMYT have led to the identification of effective resistance genes in modern durum wheat germplasm, including the linked genes *Lr3* and *Lr_{Camayo}*, both mapped to chromosome 6BL [16], the complementary gene pair *Lr27+31*, located on chromosome arms 3BS and 4BS, respectively [17], *Lr14a* on chromosome 7BL [18] and the newly designated *Lr61* on chromosome 6BS [19]. As the race BBG/BN continued to evolve, a new variant identified as BBG/BP acquired virulence to the complementary resistance genes *Lr27+Lr31* in 2008 [2]. Race BBBQD, with a similar virulence pattern to the Mexican races of *P. triticina*, was detected in durum fields in California, USA, during 2009 [20]. In 2013, this highly virulent race was reported in Kansas, USA, increasing the risk of its spread northward to the major durum-producing areas of North Dakota, USA and Saskatchewan, Canada [21,22].

Diversification and widening of the genetic basis for leaf rust resistance in durum wheat, and breeding for durable resistance, are both critical for the sustainability of its production. The recent revolution in next generation sequencing technologies [23–25] and the development of low-cost and high-throughput SNP genotyping systems [26–29] have promoted the rapid development of reliable markers for marker-assisted breeding in wheat, while providing efficient tools for mapping resistance genes.

Selective genotyping [30] and pooled DNA analysis or bulked segregant analysis (BSA) [31,32] are two cost-saving, yet effective approaches to rapidly identify candidate regions for genes of interest, by genotyping selected individuals or pooled DNA samples from the high and low tails of the phenotypic distribution of a population. Linkage between the phenotype

and the markers is then inferred by analyzing allele frequencies between the groups of individuals or bulks with contrasting phenotypes. Both approaches have been used to map rust resistance genes in wheat [19,33,34]. The objectives of the present study were to (1) characterize the genetic basis of leaf rust resistance in the four durum genotypes Amria, Byblos, Geromtel_3 and Tunsyr_2, which express resistance to all currently known races of *P. triticina* in Mexico, and (2) to develop tightly linked molecular markers that would be useful for marker-assisted breeding and gene pyramiding.

Material and methods

Plant materials

Four RIL populations were developed by crossing the highly susceptible CIMMYT line ATRED #2 (pedigree: Atil*2/LocalRed) to four resistance sources, namely, Geromtel_3 and Tunsyr_2 from the International Center for Agricultural Research in the Dry Areas (ICARDA) (pedigrees: Gersabil_1/4/D68.1.93A.1A//Ruff/Flamingo/3/Omtel_5 and D68.1.93A.1A//Ruff/Flamingo/3/Omtel_5/4/Lahn, respectively), Amria, from Morocco (pedigree: HadjMouline/Saada//Karim), and Byblos, a French durum cultivar of unknown pedigree. These sources of resistance were selected based on information generated by CIMMYT's durum wheat breeding team, for their seedling and adult plant resistance at several locations and over multiple cropping cycles, in Mexico and worldwide [35]. Crosses and generation advancement were made at CIMMYT's experimental stations in Mexico, as described in detail in Loladze et al. [35].

Field experiments and phenotyping

Three generations of the RILs and the parental genotypes were evaluated for the disease response in the field, in two different environments in Mexico. During the summer of 2011, the F_2-derived F_3 ($F_{2:3}$) families from each cross were space planted in double 1.2-meter-long rows in the CIMMYT field leaf rust nurseries at the El Batán experimental station, which allowed us to observe approximately 20 to 30 individual plants per family. The El Batán experimental station is located at CIMMYT headquarters near Mexico City (latitude 19.53, longitude -98.84, altitude 2250 m asl), where wheat is sown in mid-May and harvested in mid-October. In 2013, the $F_{2:6}$ RILs were grown at the CENEB station in Ciudad Obregon, situated in the State of Sonora (latitude 27.33, longitude -109.93, altitude 35 m asl) in Northwestern Mexico, with a wheat crop season from mid-November to late April. Finally, the F_8 RILs were phenotyped during the summer of 2014, at the field leaf rust nurseries in El Batán station. Field plots for the 2013 and 2014 trials were grown in 1.2-meter-long rows, with two replicates (paired rows) for each line and approximately 30 plants per replicate. Parental genotypes and susceptible and resistant checks were included in all field evaluations. A mixture of the susceptible cultivars Banamichi C2004 and Jupare C2001 (resistant to most Mexican races, except BBG/BP) was used as rust spreader rows. At the tillering stage, all plant materials were inoculated with race BBG/BP urediniospores suspended in light mineral oil (Soltrol 170), at a concentration of 5 to 10 mg of urediniospores per 5 ml of oil. The race BBG/BP of *P. triticina* was the predominant durum-specific race in Mexico, with the following avirulence/virulence formula: *Lr1*, *2a*, *2b*, *2c*, *3*, *3ka*, *3bg*, *9*, *13*, *14a*, *15*, *16*, *17*, *18*, *19*, *21*, *22a*, *24*, *25*, *26*, *28*, *29*, *30*, *32*, *35*, *37/Lr10*, *11*, *12*, *14b*, *20*, *23*, *27 + 31*, *33*, *72* [2,35].

The scoring of leaf rust reactions was performed at least twice during each growing season. The percentage of infected leaf area (disease severity) was estimated according to the modified Cobb scale [36]. Host reaction was also recorded using four categories: resistant (R) with miniature uredinia; moderately resistant (MR) as indicated by presence of small uredinia, moderately susceptible (MS) expressed as moderate sized uredinia and full susceptibility (S), with

presence of many large uredinia [37]. In the F_3 generation, depending on the host reactions of plants within a family, families were categorized as homozygous resistant (all plants resistant), homozygous susceptible (all plants susceptible) and segregating (plants originating from a heterozygous plant). The F_6 and F_8 RILs were scored as resistant (R) or susceptible (S), based on their host reaction. The chi-square (χ^2) test was applied to determine the goodness of fit of the observed phenotypic distributions of the host reaction in the segregating populations to the expected genetic ratio for a monogenic inherited resistance.

Allelism tests

Allelism tests were conducted as described in Loladze et al. [35], by screening F_2 populations from crosses between the resistant parental lines for the presence of susceptible recombinants. A minimum of 181 and up to 304 F_2 plants per cross were evaluated in these tests. Also, allelism to the known resistance gene *Lr61* was studied in F_2 populations from crosses between the *Lr61*-carrying line Sooty_9/Rascon_37//Guayacan INIA and each of the four parental lines Amria, Byblos, Geromtel_3 and Tunsyr_2. In addition, in April 2013, 200 F_2 plants generated from the cross Amria/Byblos were evaluated for their reaction to BBG/BP, at seedling stage in the greenhouse at CIMMYT. The infection types (ITs) of the resulting F_2 seedling progenies were assessed using the 0 to 4 scale described by McIntosh et al. [38], where "0" = no visible leaf rust symptoms; ";" = hypersensitive flecks without any uredinia; "1" = small uredinia surrounded by necrosis; "2" = small to medium uredinia surrounded by chlorosis or necrosis; "3" = medium-sized uredinia with or without chlorosis; "4" = large uredinia without chlorosis or necrosis; "X" = random distribution of variable-sized uredinia, and "+" and "-" were used when uredinia were somewhat larger or smaller than the average for the IT class. ITs of 3, 3+ and 4 were considered to be susceptible host reactions, whereas all of the other ITs were considered resistant. In case of absence of susceptible recombinants in the F_2 progenies, it was assumed that the two resistant parents carried allelic or closely linked leaf rust resistance genes.

Bulked segregant analysis using the iSelect 90K SNP array

A bulked segregant analysis (BSA) approach was carried out on 15 resistant and 15 susceptible F_3 families from each population, using the Illumina iSelect 90K Infinium SNP genotyping array [28]. Genomic DNA was extracted from the parental lines and the selected families using a modified CTAB method [39]. The quality of DNA was assessed on 2% agarose gel. DNA quantification was performed using PicoGreen fluorescence detection, and all DNA samples were diluted to 50 ng/μl. One resistant and one susceptible bulk DNA samples were created for each population, by pooling equal quantities of genomic DNA from the previously selected families. The parental lines, the selected families, and the bulks from each population were genotyped with the wheat 90K Infinium iSelect assay, using BeadStation and iScan, according to the manufacturer's protocol from Illumina. SNP clustering and data analysis were performed using GenomeStudio software (Illumina, San Diego, CA, USA). Polymorphic SNPs that distinguished the parental lines and co-segregated with the leaf rust reaction of selected families and their resulting bulks were identified in each population.

Genotyping polymorphic SNPs for F_8 RILs using KASP and Fluidigm assays

Genomic DNA was extracted from the four F_8 RIL populations as well as the parental lines, according to CIMMYT's automated DNA extraction protocol, using a BIOMEK FX^p liquid handling station and the Sbeadex mini plant kit from LGC Genomics (LGC, Teddington,

Middlesex, UK)[39]. After DNA quantification and quality assessment, all samples were diluted to approximately 50 ng/μl. Based on the results from the BSA done in the F_3 generation, and the source sequence from which the 90K iSelect probes were originally developed, polymorphic SNPs identified within the candidate regions for leaf rust resistance were converted into KASP markers. For each SNP, two allele-specific forward primers and one common reverse primer were designed using the Primer3 software [40]. Seven additional publicly available KASP primers that were designed at the University of Bristol (http://www.cerealsdb. uk.net) were also used for genotyping. Primer sets of all 66 KASP markers used in the mapping of leaf rust resistance in Amria, Byblos, Geromtel_3 and Tunsyr_2 are listed in S1 Table.

KASP genotyping was performed for the Amria/ATRED #2 and Byblos/ATRED #2 RIL populations, according to the guidelines in the KBIOscience KASP SNP genotyping manual (http://www.kbioscience.co.uk/). Reactions were performed in 384 well plates, with a final reaction volume of 8 μl, which contained 2.5 μl of KASP 2X reaction mix, 50 ng of template DNA, 0.165 μM Hex forward primer, 0.165 μM FAM forward primer and 0.412 μM universal reverse primer. The following cycling conditions were used: 15 min at 94°C followed by 10 touchdown cycles of 20 s at 94°C and 60 s at 61°C (dropping 0.8°C per cycle); after the final annealing temperature of 57°C was achieved, there were 26 cycles of 20 s at 94°C and 60 s at 57°C, with a final fluorescence plate reading taken at 10°C. Thermocycling and fluorescence readings were performed on a Bio-Rad C1000 thermocycler and the data were analyzed using Bio-Rad CFX Manager software (Bio-Rad Laboratories Ltd, Hercules, CA, USA).

Because of the large number of markers to be genotyped for the populations derived from the crosses Geromtel_3/ATRED #2 and Tunsyr_2/ATRED #2 (55 and 35 markers, respectively), a high-throughput SNP genotyping platform was selected. The Fluidigm 192.24 Dynamic Array IFC (Integrated Fluidic Circuit) (Fluidigm Corp., South San Francisco, CA, USA) provides a solution for targeted high sample throughput SNP genotyping. It is designed to genotype 192 samples against 24 assays in a single run. Genotyping was carried out on the parental lines Geromtel_3, Tunsyr_2, ATRED #2 and the F_8 RILs from both populations, following the procedures detailed in the manufacturer's SNP genotyping analysis user guide (https://www.fluidigm.com). Specific Target Amplification (STA) primers were designed for each SNP, and STA was performed for all genomic DNA samples. A 1:100 dilution of the STA products was then used for sample mix preparation. The assay mix and sample mix were then loaded onto a 192.24 dynamic array chip, mixed and thermal-cycled using an IFC Controller HX and FC1 thermal cycler (Fluidigm Corp., South San Francisco, CA, USA), according to the manufacture's protocols. End-point fluorescent images of the chip were acquired on an EP-1 imager, and the data was analyzed with the Fluidigm SNP Genotyping Analysis software (Fluidigm Corp., South San Francisco, CA, USA).

Linkage mapping

SNP markers that showed a good quality of allele calling, based on the clusters of the scatter plots, were used for linkage analysis. Linkage maps of chromosome arms that carried the *Lr* genes were constructed using MapDisto 1.7.7 software [41], at a minimum logarithm of odds (LOD) score of 3 and maximum recombination fraction of 0.3. Co-segregating markers were identified in each population, and the marker with the lowest percentage of missing data was chosen to represent each cluster. Double recombinants were corrected using the functions 'Show double recombinants,' 'Show error candidates' and 'Replace error candidates by flanking genotype' as implemented in the MapDisto software [42]. The Kosambi function was used to convert the recombination fractions to centimorgans (cM) [42]. The final linkage maps were prepared using MapChart software [43].

Genotyping simple sequence repeat (SSR) markers

Two SSR markers, *Xgwm344-7B* and *Xgwm146-7B*, which were previously shown to be linked to *Lr14a* [18] were used to screen the two resistant parents Amria and Byblos, as well as the susceptible parent ATRED #2. Subsets of resistant and susceptible RILs from both populations were also included. The French cultivar Sachem, previously reported to carry *Lr14a* [44], was included as a positive check. The same lines were also tested using four combinations of nucleotide-binding site leucine-rich repeat (NBS-LRR)-specific primers previously determined to be linked to *Lr14a* in a segregating population (*4406F*: CACGACGTTGTAAAACGACTTTCATT TTGTTCTCTCAGCCATA; *4407F*: CACGACGTTGTAAAACGACTTCATTTTGTTCTCTCAGCCA TAC; *4840R*: GATGGATGATTTGGGTTTTTCTAC and *4852R*: TTACATGTGGATGATGGATGAT TT) (C. Pozniak, *unpublished data*). The SSR marker *Xwmc487*, previously reported to be linked to *Lr61* on chromosome arm 6BS [19], was also used to genotype Geromtel_3 and Tunsyr_2. The durum wheat cultivar Guayacan INIA was used as a positive control that carries *Lr61*. The primer sequences of these SSR markers were obtained from the GrainGenes database (http://wheat.pw.usda.gov/GG3). PCR reactions were performed in 96 well plates with total reaction volumes of 25 μl, according to the protocols described by Herrera-Foessel et al. [19] and Pozniak et al. [45]. Dye-labeled M13 primer was added to the PCR mix for *Xgwm344-7B* and *Xgwm146-7B*; this allowed polymorphisms to be resolved using capillary electrophoresis on an ABI3130 genetic analyzer (Applied Biosystems®, Foster City, CA, USA). Polymorphisms for the NBS-LRR-specific primers and the SSR marker *Xwmc487* were scored on 2% agarose gels.

Physical mapping of the polymorphic SNPs

The probe source sequences of all the SNP markers used in the final mapping of the *Lr* genes from all sources of resistance were physically mapped against the reference sequence of WEW accession 'Zavitan' [46] using GMAP [47]. The corresponding physical intervals for SNPs associated with leaf rust resistance in each population were identified under stringent parameters of coverage > 90% and identity > 95%. Genes falling within these physical intervals were identified using the available annotations for the WEW genome [46].

Results

Genetic characterization of the leaf rust resistance

The observed phenotypic distributions of host reactions at the F_3, F_6 and F_8 generations supported segregation of a single dominant gene for leaf rust resistance in Amria, Geromtel_3 and Tunsyr_2 (Table 1). However, only the F_3 and F_6 RILs from the cross Byblos/ATRED#2 fit the ratios expected for segregation of a single gene (Table 1). The F_8 RILs from the latter cross did, however, fit a ratio of 9:7, expected for resistance controlled by two complementary genes ($p = 0.665$).

The frequency distributions of the disease severity (DS) scores for the four F_6 populations are represented in Fig 1. The four resistant parents showed the lowest DS scores (0–5%), and the highest scores (90–100%) were observed for the susceptible parent ATRED #2. Histograms of the DS recorded for Byblos/ATRED #2 (Fig 1A) and Tunsyr_2/ATRED #2 (Fig 1B) F_6 populations revealed bimodal distributions, which is typical of traits under control of a major genetic factor. However, the DS data from Amria/ATRED #2 (Fig 1C) and Geromtel_3/ ATRED #2 (Fig 1D) showed a skewed distribution towards increased resistance.

Table 1. Classification of field reactions to the race BBG/BP of *P. triticina* of F$_3$, F$_6$ and F$_8$ progenies from four crosses involving four sources of resistance crossed to the susceptible genotype ATRED #2.

| Cross	F$_{2:3}$ Families			F$_{2:6}$ Families			F$_8$ RILs		
	Hr:Seg:Hs	Ratio	P	Hr:Seg:Hs	Ratio (%)	P	R:S	Ratio	P
Amria/ATRED #2	48:123:48	1:2:1	0.189	121:3:92	48.5:3:48.5	0.065	113:100	1:1	0.411
Byblos/ATRED #2	51:120:60	1:2:1	0.591	105:5:114	48.5:3:48.5	0.618	128:93	1:1	0.046*
Geromtel_3/ATRED #2	46:98:35	1:2:1	0.227	107:8:76	48.5:3:48.5	0.052	102:87	1:1	0.309
Tunsyr_2/ATRED #2	43:108:52	1:2:1	0.443	97:12:97	48.5:3:48.5	0.084	103:101	1:1	0.944

F$_{2:3}$ and F$_{2:6}$ families were classified as homozygous resistant (Hr); segregating (Seg); and homozygous susceptible (Hs), based on the host reactions of the plants within each family. The F$_8$ RILs host reactions were scored as resistant (R) or susceptible (S). The level of significance for segregation ratios determined by χ^2 tests are indicated by P, p-value. The null hypothesis for the χ^2 test was rejected at p-value < 0.05.

*p-value < 0.05 indicating that the observed segregation ratio is significantly different from the expected segregation ratio at a 95% level of confidence.

https://doi.org/10.1371/journal.pone.0197317.t001

Allelism tests

Allelism tests were performed to determine if the sources of resistance used in this study carried genes that were either allelic to each other or tightly linked. The absence of susceptible F$_2$ plants from the cross involving Amria and Byblos suggests that these two genotypes may be
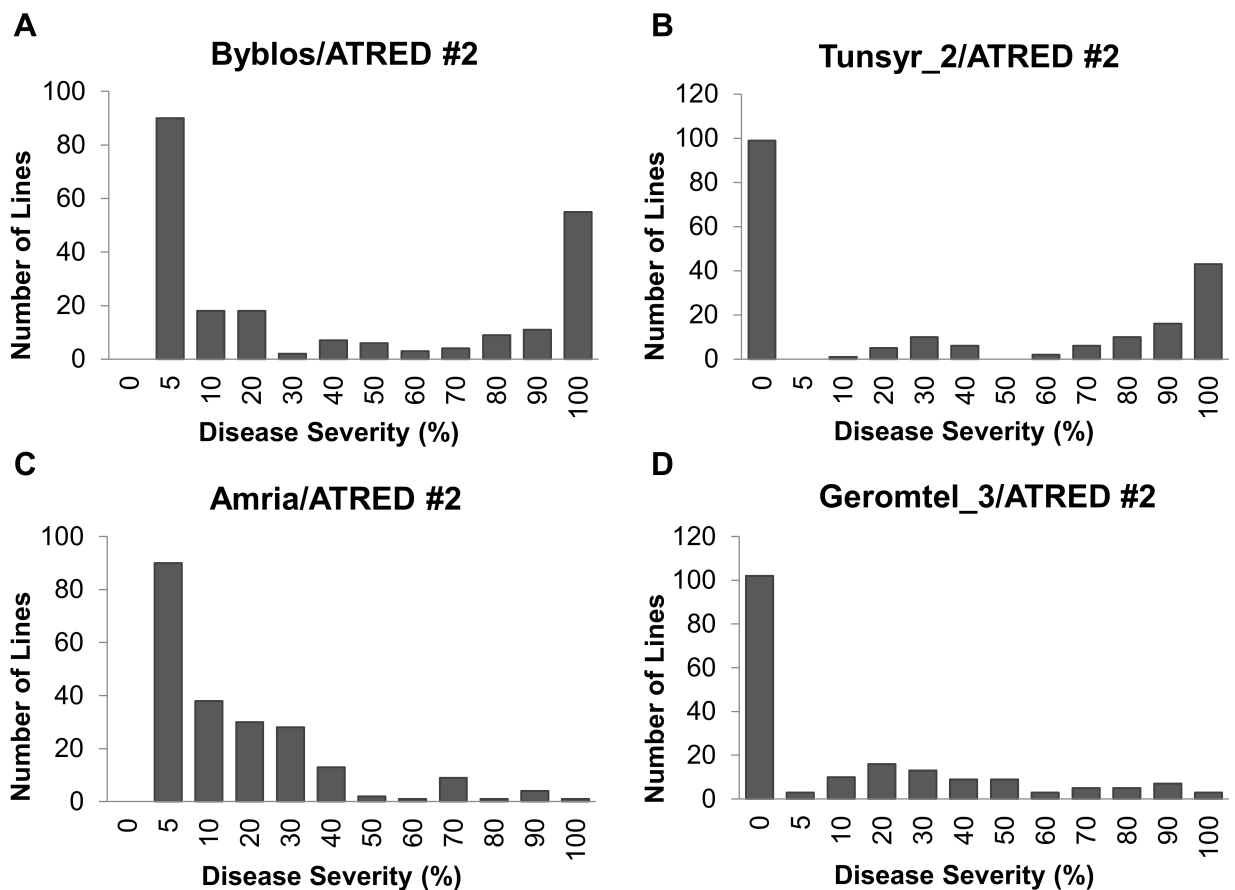
Fig 1. Frequency distributions of the disease severity (DS) scores in the F$_6$ generation of four RIL populations. (A) Frequency distribution of DS for the Byblos/ATRED #2 population. (B) Frequency distribution of DS for the Tunsyr_2/ATRED #2 population. (C) Frequency distribution of DS for the Amria/ATRED #2 population. (D) Frequency distribution of DS for the Geromtel_3/ATRED #2 population.

https://doi.org/10.1371/journal.pone.0197317.g001

Table 2. Number of resistant and susceptible F$_2$ plants from crosses between different sources of resistance to leaf rust used for allelism testing.

| Cross	Total F$_2$ plants	Resistant	Susceptible
Amria/Byblos	200	200	0
Amria/Geromtel_3	250	220	30
Amria/Tunsyr_2	304	250	54
Geromtel_3/Tunsyr_2	275	275	0
Amria/Sooty_9/Rascon_37//Guayacan INIA	310	223	87
Byblos/Sooty_9/Rascon_37//Guayacan INIA	280	173	107
Geromtel_3/Sooty_9/Rascon_37//Guayacan INIA	301	301	0
Tunsyr_2/Sooty_9/Rascon_37//Guayacan INIA	276	276	0

carrying allelic or closely linked genes for leaf rust resistance (Table 2). The presence of susceptible plants in all the other crosses involving Amria indicates that the resistance in this genotype is different from those in Geromtel_3 and Tunsyr_2, and is neither allelic nor it is linked to *Lr61*, carried by the line Sooty_9/Rascon_37//Guayacan INIA. No susceptible plants were identified in all the crosses involving Geromtel_3, Tunsyr_2 and the *Lr61*-carrying Sooty_9/Rascon_37//Guayacan INIA, which suggested that the leaf rust resistance genes present in these three genotypes are either allelic or closely linked to each other. Altogether, these results indicate that Geromtel_3 and Tunsyr_2 carry genes that are the same, allelic, or tightly linked to *Lr61* and that Amria and Byblos share a separate resistance locus independent of *Lr61*.

Linkage mapping

Leaf rust resistance in Amria and Byblos. BSA identified 28 and 24 SNPs that were genetically linked to the leaf rust resistance from Amria and Byblos, respectively; 22 of them were common between the two populations, supporting the allelism test results that indicated that Amria and Byblos may carry the same gene, alleles, or closely linked genes for leaf rust resistance. Based on the high-density consensus map of tetraploid wheat [48], a candidate region for leaf rust resistance in both genotypes was identified on the long arm of chromosome 7B. A list of all the SNP markers linked to the resistance in Amria and Byblos and their position on the consensus map is presented in S2 Table. Corresponding sequences from the Infinium assay were used to develop allele-specific KASP primers for 19 SNPs on chromosome 7BL, which were later tested on the parental lines. Only the markers that produced clear clusters for accurate genotype assignment were assayed on the entire RIL populations (S1 Table). Among the 19 KASP markers developed, only 14 and 10 were considered reliable for mapping in Amria/ATRED #2 and Byblos/ATRED #2 populations, respectively (Fig 2). All 14 markers that were closely linked to the leaf rust resistance gene in Amria (referred to as *Lr_Amria*) mapped within a 6.7 cM interval from the gene (Fig 2A). The KASP marker *usw260* was the closest to *Lr_Amria*, mapping at 4.8 cM proximal to the gene. In the Byblos/ATRED #2 population, 10 KASP markers mapped within 2.7 cM of the resistance gene in Byblos (Fig 2B). *Lr_Byblos* was located at 1.3 cM from the co-segregating markers *usw259*, *usw262*, *usw255*, *usw260*, *usw263* and *BS00004171*. All of these markers from both populations, mapped within an interval of 3 cM, spanning positions 208.7–211.5 cM on the tetraploid wheat consensus map (Fig 2C), providing compelling map-based evidence supporting the allelism or tight linkage between the resistance genes from both sources.

The distal region of chromosome 7B is known to carry the major leaf rust resistance gene *Lr14a*. Several markers reported to be linked to *Lr14a* were used to genotype the parental lines Amria, Byblos, ATRED #2, as well as resistant (R) and susceptible (S) RILs from the two
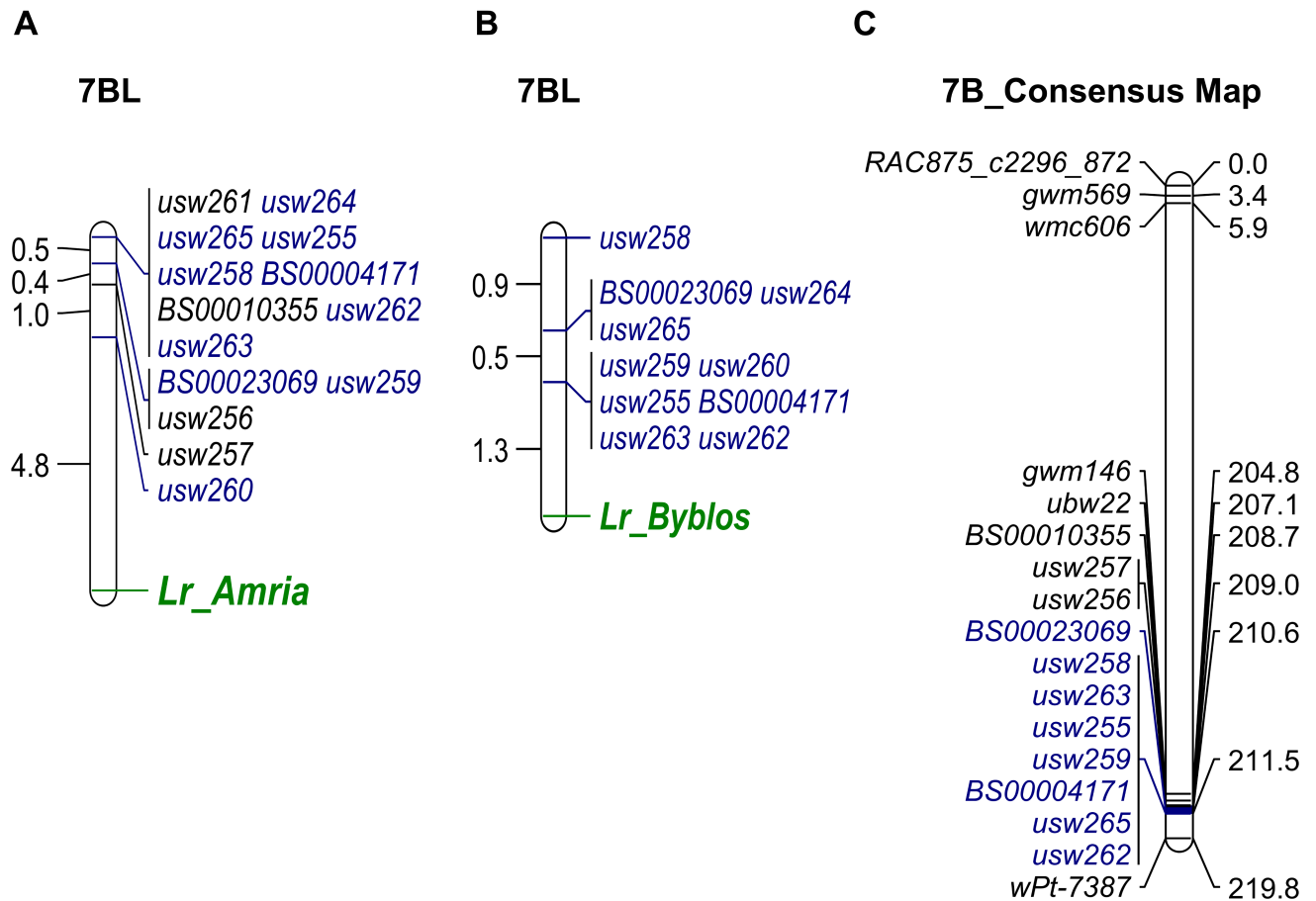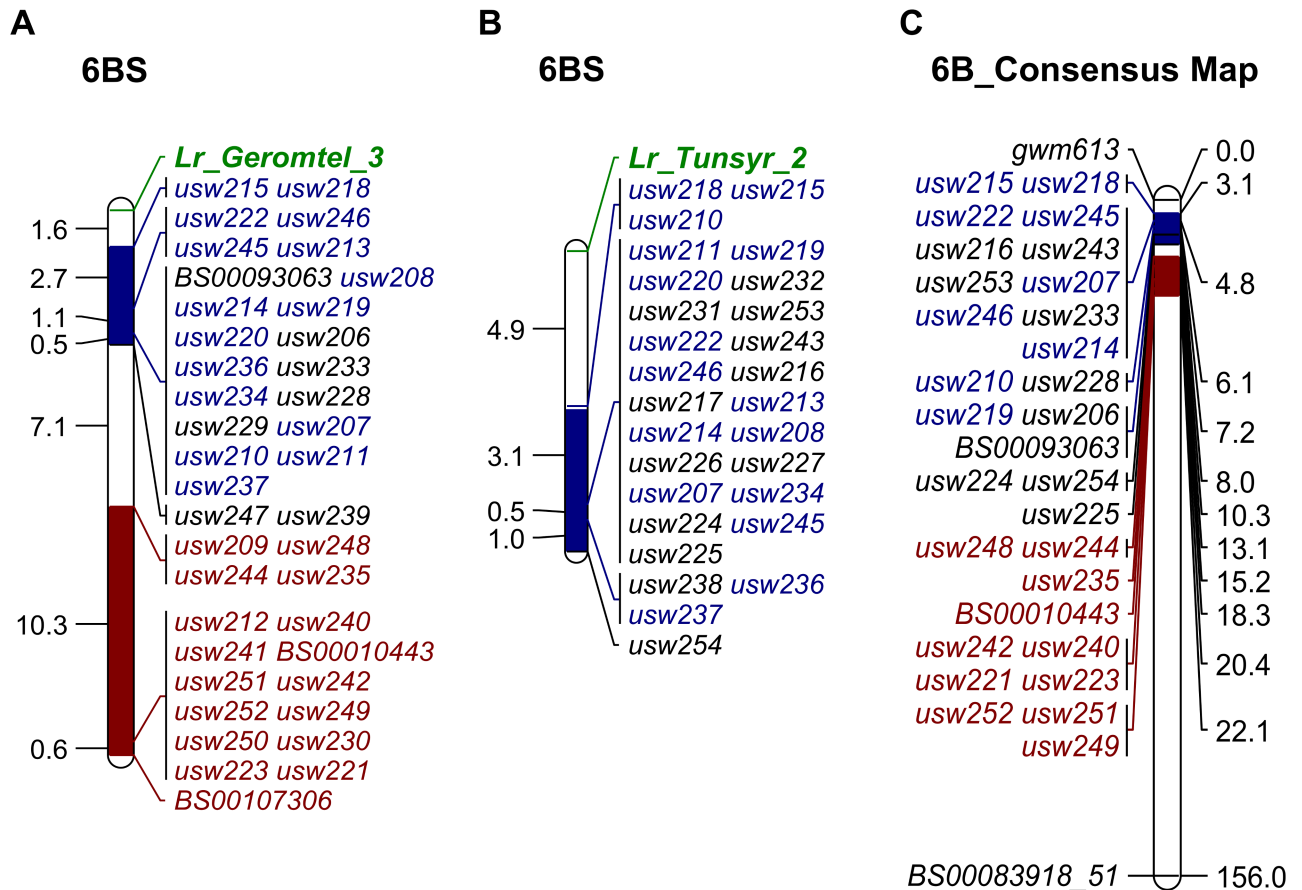
Fig 2. Linkage groups of KASP markers associated with the leaf rust resistance genes *Lr_Amria* and *Lr_Byblos* and their positions on the consensus map. (A) Markers associated with resistance in Amria. (B) Markers associated with resistance in Byblos. (C) High-density tetraploid consensus map for chromosome 7B [48]. Markers highlighted in blue are linked to the resistance in both Amria and Byblos. Genetic distances are displayed in cM.

mapping populations. The *Lr14a*-carrier "Sachem" was used as a positive control [44]. All lines, including the parents, showed polymorphism for both markers *Xgwm344* (S1 Fig) and *Xgwm146* (S2 Fig), when compared to Sachem. Furthermore, capillary electrophoresis analysis revealed different amplicon sizes for the parental lines Amria and Byblos compared to Sachem, for both markers (S1 and S2 Figs). For the *Xgwm344* marker, a single 152 base pairs (bp) DNA fragment was amplified for each of the parental lines Amria, Byblos and ATRED #2, clearly different from the 122 bp DNA fragment amplified for Sachem (S1 Fig). For the *Xgwm146* marker, two different amplicons were amplified for Sachem (174 bp and 189 bp) whereas both Amria and ATRED #2 were characterized by two amplicons of 172 bp and 206 bp, and Byblos had a single 179 bp fragment amplified (S2 Fig). Different combinations of NBS-LRR primers were used to screen the parental lines and the *Lr14a*-carrying Sachem, which were able to show either presence or absence of the NBS-LRR associated with *Lr14a* (S3 Fig). Only a single fragment was amplified from Sachem, indicating the presence of *Lr14a* in this cultivar; however, no PCR products were observed for any of the other lines tested. Altogether, our current data suggests that the major *Lr* gene present in Amria and Byblos is likely different from *Lr14a*.

Leaf rust resistance in Geromtel_3 and Tunsyr_2. BSA revealed that 115 SNPs were linked to the leaf rust resistance in Geromtel_3 and 67 SNPs were associated with the

Fig 3. Linkage groups of KASP markers associated with the leaf rust resistance genes *Lr_Geromtel_3* and *Lr_Tunsyr_2* and their positions on the consensus map. (A) Markers associated with resistance in Geromtel_3. (B) Markers associated with resistance in Tunsyr_2. (C) High-density tetraploid consensus map for chromosome 6B [48]. Markers highlighted in blue are linked to the resistance in both Geromtel_3 and Tunsyr_2. Markers highlighted in red are linked only to the resistance in Geromtel_3. Genetic distances are displayed in cM.

https://doi.org/10.1371/journal.pone.0197317.g003

resistance in Tunsyr_2, including 52 common SNPs between the two sources of resistance. Based on the tetraploid wheat consensus map [48], a candidate region for the leaf rust resistance carried by both sources was identified on the short arm of chromosome 6B. A summary of all the SNP markers associated with the leaf rust resistance in Geromtel_3 and Tunsyr_2, and their positions on the consensus map are presented in S3 Table. Allele-specific KASP primers were developed for 56 SNPs on chromosome 6BS, which were used to genotype the parental lines as well as selected resistant and susceptible lines from each population. A total of 40 KASP markers that produced clear clusters were assayed on the Geromtel_3/ATRED #2 F_8 RILs, whereas, only 28 markers were retained to genotype the Tunsyr_2/ATRED #2 RIL population. Primer sets of all KASP markers linked to leaf rust resistance in Geromtel_3 and Tunsyr_2 are listed in S1 Table.

The genetic map for the leaf rust resistance gene in Geromtel_3 (*Lr_Geromtel_3*) spanned an interval of approximately 23.9 cM (Fig 3A), with markers *usw215* and *usw218* mapping 1.6 cM from the gene and *usw222*, *usw245*, *usw213* and *usw246* mapping 4.3 cM from it. Fifteen other SNP markers mapped at approximately 5.4 cM from *Lr_Geromtel_3*. Another set of 17 markers (highlighted in red in Fig 3A) were linked exclusively to *Lr_Geromtel_3*, mapping between 13 to 23.9 cM distal to the gene, but were monomorphic in the Tunsyr_2 progenies.

A total of 28 markers were used to construct the genetic map for the leaf rust resistance locus in Tunsyr_2 (*Lr_Tunsyr_2*), which spanned a 9.5 cM interval (Fig 3B). Markers *usw210*, *usw215* and *usw218* mapped at 4.9 cM distal to *Lr_Tunsyr_2*.

While markers *usw215* and *usw218* were the closest to both *Lr_Geromtel_3* and *Lr_Tunsyr_2* (Fig 3A and 3B), only 16 markers were linked to the resistance in both populations (markers highlighted in blue in Fig 3). Twenty-four markers were exclusively associated to the resistance in Geromtel_3 and 12 markers were only linked to the resistance in Tunsyr_2.

The leaf rust resistance gene *Lr61* was identified in the Chilean durum wheat cultivar Guayacan INIA, and was mapped to the short arm of chromosome 6B, at approximately 28 cM from the SSR marker *Xwmc487* [19]. PCR amplicons for the marker *Xwmc487* were generated for the parental lines Geromtel_3, Tunsyr_2 and ATRED #2, as well as the cultivar Guayacan INIA (S4 Fig). The agarose gel revealed polymorphism between Guayacan INIA and the parental lines Geromtel_3 and ATRED #2, suggesting that *Lr61* may not be present in these genotypes. In this case, the results from the allelism tests would more likely indicate close linkage between the two genes (*Lr_Geromtel_3* and *Lr61*) rather than allelic relationship. Alternatively, the large distance between *Xwmc487* and *Lr61* could make this marker non-diagnostic for the presence of *Lr61* in genetic backgrounds that are different from the one that was used for its mapping. On the other hand, the PCR product amplified for Tunsyr_2 was similar to that amplified in Guayacan INIA, hampering our ability to determine whether the resistance from these two genotypes was due to the same gene or to different but closely linked genes.

Physical mapping

DNA sequences associated with 10 SNP markers linked to the resistance in Amria and Byblos were positioned on the "Zavitan" reference sequence of tetraploid wheat (Table 3). Except for the marker *Ku_c6566_3086*, all SNP markers spanned a physical interval of about 6.7 megabase pairs (Mb) (746,587,151–753,310,876 bp). Five markers, namely *Tdurum_contig62213_423*, *tplb0045c05_154*, *BS00023069_51*, *BS00064933_51* and *Kukri_c20875_997*, were linked to the resistance in both Amria and Byblos and mapped within a 42,778 bp interval (747,105,190–747,147,968 bp). Likewise, sequences of 27 SNPs associated with the resistance in Geromtel_3 and/or Tunsyr_2 were physically mapped on the WEW genome (Table 4). All of the SNP markers linked to the resistance in Tunsyr_2 mapped within a 4Mb interval (5,812,642–

Table 3. Map positions of the SNP markers linked to leaf rust resistance in Amria and Byblos and their corresponding physical intervals in the WEW sequence of chromosome 7B.

SNP marker	KASP marker	Linkage to resistance[a]	Position Chr. 7B[b]	Position in WEW
Ku_c6566_3086	*usw260*	A and B	N/A	653,676,250
Tdurum_contig30909_76	*usw258*	A and B	211.5	746,587,151
Tdurum_contig62213_423	*usw264*	A and B	N/A	747,105,190
tplb0045c05_154	*usw265*	A and B	211.5	747,108,023
BS00023069_51	*BS00023069*	A and B	210.6	747,110,507
BS00064933_51	*usw255*	A and B	211.5	747,145,702
Kukri_c20875_997	*usw262*	A and B	211.5	747,147,870
RAC875_c525_1372	*usw257*	A	209.0	751,585,860
BS00010355_51	*BS00010355*	A	208.7	751,588,580
Kukri_c17115_372	*usw261*	A	N/A	753,310,785

[a] A, Amria; B, Byblos.

[b] Chr, chromosome; N/A, SNP map position not available from the consensus map.

https://doi.org/10.1371/journal.pone.0197317.t003

Table 4. Map positions of the SNP markers linked to leaf rust resistance in Geromtel_3 and Tunsyr_2 and their corresponding physical intervals in the WEW sequence of chromosome 6B.

SNP marker	KASP marker	Linkage to resistance[a]	Position Chr. 6B[b]	Position in WEW
Tdurum_contig43538_1687	*usw246*	G and T	4.8	5,812,642
Tdurum_contig43538_1582	*usw245*	G and T	4.8	5,812,747
Excalibur_c96134_182	*usw222*	G and T	4.8	5,812,813
CAP7_rep_c6852_87	*usw216; usw217*	T	4.8	5,814,978
BobWhite_c39821_195	*usw213*	G and T	N/A	5,821,656
BS00093063_51	*BS00093063*	G	7.2	8,254,653
RAC875_c31381_820	*usw237*	G and T	7.2	8,438,520
RAC875_c31381_883	*usw238*	T	N/A	8,438,666
Excalibur_c31801_48	*usw219; usw220*	G and T	7.2	9,232,277
IACX9205	*usw224*	T	8	9,506,064
Tdurum_contig52819_287	*usw247*	G	N/A	9,546,765
RAC875_c33407_350	*usw239*	G	N/A	9,546,765
RAC875_c1305_120	*usw254*	T	8	9,797,072
Wsnp_CD453605B_Ta_2_1	*usw248*	G	13.1	12,461,455
Tdurum_contig42655_1727	*usw244*	G	13.1	12,470,253
RAC875_c18689_1950	*usw235*	G	15.2	15,305,559
BS00010443_51	*BS00010443*	G	18.3	18,454,200
BobWhite_c34318_375	*usw212*	G	N/A	19,978,536
Kukri_c24795_267	*usw230*	G	N/A	19,978,605
RAC875_c38592_187	*usw240; usw241*	G	20.4	19,983,498
Excalibur_rep_c114123_366	*usw223*	G	20.4	19,983,705
Excalibur_c64989_556	*usw221*	G	20.4	19,983,996
RAC875_rep_c105906_124	*usw242*	G	20.4	19,984,203
Wsnp_Ex_c702_1383612	*usw252*	G	22.1	21,730,858
wsnp_Ex_c702_1382859	*usw251*	G	22.1	21,733,535
Wsnp_Ex_c4728_8444212	*usw249; usw250*	G	22.1	21,736,168
BS00107306_51	*BS00107306*	G	N/A	23,093,453

[a] G, Geromtel_3; T, Tunsyr_2.

[b]Chr, chromosome; N/A, SNP map position not available from the consensus map.

https://doi.org/10.1371/journal.pone.0197317.t004

9,797,172 bp). However, SNP markers linked to the resistance in Geromtel_3 spanned an interval of about 17.3 Mb (5,812,642–23,093,553 bp).

Several transcripts coding for putative NBS-LRR proteins, resistance gene analogues (RGA2), RPM1 and RPP13-like disease resistance proteins, as well as proteases and ABC transporter were identified within the 7BL interval for the leaf rust resistance in Amria and Byblos (S4 Table). Several NBS-LRR-encoding sequences have also been identified in the interval for Geromtel_3 and Tunsyr_2 on chromosome 6BS (S5 Table). Other candidate genes for the leaf rust resistance in Geromtel_3 and Tunsyr_2 include a HR-like lesion-inducing protein as well as a zinc finger peptidase and sugar transporters. The marker *usw224* that was linked to the resistance in Tunsyr_2 was mapped within an RPP13-like disease resistance gene (S5 Table).

Discussion

To sustain the economic viability of durum wheat production globally, it is necessary to protect crops from the potentially destructive impact of rusts, including leaf rust. This is most effectively done by identifying and deploying new sources of resistance that are able to durably

mitigate the threat of a dynamic and rapidly evolving pathogen population. In an effort to identify new sources of resistance to leaf rust, CIMMYT selected to characterize four resistant genotypes, namely Amria, Byblos, Geromtel_3 and Tunsyr_2, for their all stage resistance to all Mexican and Mediterranean pathotypes of *P. triticina* [35]. Studies of the inheritance of the leaf rust resistance from these four sources suggested that they all carry major resistance genes. Allelism testing results suggested that Amria and Byblos may share the same or closely linked resistance genes. Likewise, the resistance genes in Geromtel_3 and Tunsyr_2 were shown to be either allelic or tightly linked to each other and to the previously designated gene *Lr61* [19]. Furthermore, pedigree analysis showed that the two ICARDA lines Geromtel_3 and Tunsyr_2 share a common parent, namely the Tunisian breeding line D68.1.93A.1A (M.S. Gharbi, *personal communication*), which may be the original source of their leaf rust resistance [35]. The hypotheses of allelic or tightly linked genes were confirmed by the results of the BSA approach that identified two genomic regions associated with leaf rust resistance in these genotypes; one on chromosome 7BL for the resistance in Amria and Byblos, and the other on chromosome 6BS for the resistance in Geromtel_3 and Tunsyr_2.

Several studies, including association mapping and QTL analyses, have reported the importance of the distal region of chromosome arm 7BL in wheat resistance to leaf rust [44,49,50]. Indeed, three leaf rust resistance genes (i.e. *Lr14a*, *Lr14b* and *Lr68*) have been located on chromosome 7BL [51]. *Lr68* is an adult-plant resistance gene, with a partial resistance effect that confers a slow-rusting phenotype, identified in the common wheat cultivar Parula and is flanked by markers *Psy-1-1* and *Xgwm146* [51]. In contrast, the resistance in Amria and Byblos is expressed at the seedling stage [35] and shows a more complete resistance than *Lr68*, making *Lr68* an impossible candidate for the resistance in these two sources. The race-specific resistance gene *Lr14b* is very closely linked to *Lr14a* [52]. However, virulence against *Lr14b* is very common among *P. triticina* races that infect durum wheat, including the Mexican race BBG/BP that was used in the present study [2,51]. Since Amria and Byblos were resistant to this race, it can be concluded that their *Lr* genes are distinct from *Lr14b*. The final candidate gene located on chromosome 7BL, *Lr14a*, is linked to SSR markers *Xgwm344-7B* and *Xgwm146-7B* [18]. According to studies conducted by CIMMYT on a wide range of germplasm groups worldwide, *Lr14a* is present in the great majority of the resistant durum genotypes, and could represent the most common source of leaf rust resistance currently exploited by durum wheat breeders, globally. The over-reliance on *Lr14a* is dangerous since this gene was overcome in several areas around the Mediterranean Basin, including France [13], Tunisia [53] and Spain [14]. The ITs of Amria and Byblos were contrastingly different from those of *Lr14a*-carrying durum wheat genotypes indicating that the former cultivars were not carrying the gene (K. Ammar, *unpublished data*). In addition, Goyeau et al. [54] investigated the structure and evolution of the French durum *P. triticina* population using a durum wheat differential set, including Byblos. The French commercial cultivar Byblos was the only genotype displaying low infection types to all French pathotypes, including a pathotype which was virulent for both *Lr14a* and *Lr14b* alleles. Furthermore, the ITs of Byblos and those of Thatcher isolines, which carry known resistance genes, were different, leading to the conclusion that Byblos carried unknown gene(s) for resistance. Finally, neither Amria nor Byblos were positive for the molecular markers known to be linked to *Lr14a*, including *Xgwm344* (S1 Fig), *Xgwm146* (S2 Fig) and the NBS-LRR-specific primers *4406F/4840R*, *4406F/4852R*, *4407F/4840R* and *4407F/4852R* (S3 Fig). Altogether, these results suggest that the major *Lr* gene present in Amria and Byblos is different from *Lr14a* and is likely to be previously uncharacterized leaf rust resistance gene, making these two cultivars good candidates for exploring alternative sources of resistance for durum rust breeding.

Recently, the leaf rust resistance gene *LrBi16* has been mapped on chromosome 7BL of the Chinese bread wheat cultivar Bimai 16, and was reported to be allelic to *Lr14a* [55]. Xing et al. [56] also identified *LrFun* on the long arm of chromosome 7B of the Romanian bread wheat line Fundulea 900 and mapped it at 4.4 cM from the SSR marker *Xgwm344-7B*. Additional studies will be required to determine the relationship between these resistance genes and the genes from Amria and Byblos.

Linkage analysis positioned the leaf rust resistance locus in Amria and Byblos at the distal end of chromosome 7BL. The KASP marker *usw260* was the most tightly linked marker to the resistance in both genotypes; however, the distance to the *Lr* gene varied from 1.3 cM in Byblos to 4.8 cM in Amria. The variation in the length of the *Lr_Amria-usw260* and *Lr_Byblos-usw260* intervals between mapping populations suggests that recombination rates in this region of chromosome 7BL may vary between crosses or that insertion/deletion occurred in Amria or Byblos in the interval harboring the gene and the *usw260* SNP marker. Since this variation in marker-gene genetic distances was also observed for other SNP markers such as *usw255*, *usw258*, *usw262*, *usw263*, *usw264*, *usw265*, *BS00023069* and *BS0004171*, *usw260* remains the best marker to use in breeding, given its tight linkage to the resistance in both sources.

Three leaf rust resistance genes have been reported to map to chromosome arm 6BS, namely, *Lr36*, *Lr53* and *Lr61*. Both *Lr36* and *Lr53* originate from wild grasses relatives. *Lr36* was derived from *T. speltoides* and backcrossed into hexaploid wheat [57]. Marais et al. [58] reported the introgression of *Lr53* from *T. dicoccoides* to the short arm of chromosome 6B in common wheat. However, no reports are available to indicate that either *Lr36* or *Lr53* have been transferred to durum wheat. Furthermore, pedigree information of Geromtel_3 and Tunsyr_2 do not indicate any relationship to any of the wild relatives carrying these genes [35], though, *Lr53* cannot be fully ruled out as a candidate, since *T. dicoccoides* (genome AABB) is the wild progenitor of durum wheat. Herrera-Foessel et al. [19] identified *Lr61* on chromosome arm 6BS in the durum wheat cultivar Guayacan INIA to be linked to the SSR marker *Xwmc487*, but at a rather large distance (28.5 cM). *Lr61* is a partially dominant gene [19], but phenotypic analyses of the F_1 plants from the crosses Geromtel_3/ATRED #2 and Tunsyr_2/ATRED #2 suggested that these cultivars carry completely dominant genes for leaf rust resistance [35]. Genotyping with *Xwmc487* revealed polymorphism between the fragments amplified for Geromtel_3 compared to Guayacan INIA, but not in the case of Tunsyr_2, when compared to the same check (S4 Fig), which suggests that the resistance in Tunsyr_2 may be allelic to *Lr61*, whereas Geromtel_3 carries a potentially different but closely linked gene. However, these marker results cannot be considered conclusive given the large distance between *Xwmc487* and *Lr61*, and the absence of high-density maps for the original *Lr61* mapping population.

The distributions of markers linked to the leaf rust resistance in Geromtel_3 on both the consensus linkage map (Fig 3C) and the WEW pseudomolecules (S3 Table) suggest that Geromtel_3 may be carrying two different but tightly linked major *Lr* genes. One of these genes is likely allelic to *Lr61* and to *Lr_Tunsyr_2*, mapping to the distal end of chromosome 6BS, and the second is only present in Geromtel_3 and located centrally at about 17 Mb from the first *Lr* gene.

Physical mapping of the SNP markers linked to the resistance in Amria, Byblos, Geromtel_3 and Tunsyr_2 to the WEW reference sequence enabled the identification of candidate genes for leaf rust resistance, including NBS-LRR disease resistance proteins, RPP13-like and RPM1 disease resistance proteins, as well as several receptor kinases (S4 and S5 Tables). NBS-LRR proteins are the most abundant class of disease resistance genes in plants. This protein family includes two major subfamilies, based on the features of their N-terminal

structures: the Toll-interleukin (TIR-NBS-LRR) subfamily, and the coiled-coil (CC-NBS-LRR) subfamily [59–61]. Leaf rust resistance genes *Lr21* [62], *Lr10* [63], and *Lr1* [64], stem rust resistance genes *Sr33* [65] and *Sr35* [66], and the powdery mildew resistance gene *Pm3b* [67], are six resistance genes that have been cloned in wheat. All six proteins contain CC, NBS and LRR motifs. TIR-NBS-LRR genes represent the majority of the *R* genes in *Arabidopsis*; however, disease resistance proteins with a TIR N-terminal domain have not yet been reported in cereals [59]. These abundant NBS-LRR disease resistance proteins act as immune receptors and are involved in the detection of diverse pathogens through direct or indirect perception of pathogen *Avr* proteins [61,68,69]. Pathogens are able to evade recognition and overcome plant resistance through *Avr* gene mutation. Hence, both pathogen and host plant undergo parallel molecular diversification to secure their survival, leading to the concept of evolutionary race between pathogen virulence and plant defense [60,69–71]. This plant-pathogen coevolution could explain the rapid breakdown of leaf rust resistance conferred by most seedling, race-specific *Lr* genes in wheat, especially when the same genes are deployed in many cultivars grown over large areas, allowing for the rapid adaptation and spread of new virulent races [71]. Several NBS-LRR proteins were identified within the physical intervals associated with the leaf rust resistance in Amria, Byblos, Geromtel_3 and Tunsyr_2 (S4 and S5 Tables). Resistance gene clusters have been reported in several plant genome studies and are the result of either segmental duplications that involve many genes, or ectopic duplications that move single genes or small gene clusters to unlinked loci [59–61]. Plant *R* genes are subject to several selective forces to cope with the rapidly evolving pathogen *Avr* genes. Alteration of *R* gene clusters, through diversification and gene conversion, results in increased variation and promotes the generation of novel resistance specificity [64]. Further investigations will be required to confirm the specific identity of the genes associated with leaf rust resistance in Amria, Byblos, Geromtel_3, and Tunsyr_2.

Conclusions

Results from this study indicated that the durum wheat cultivars Amria and Byblos carry allelic or closely linked *Lr* genes on the long arm of chromosome 7B. Based on molecular marker analysis and previous genetic studies, it was concluded that none of these cultivars carried *Lr14a*, a widely-deployed resistance gene in durum wheat that is located on chromosome 7BL. Similarly, the leaf rust resistance in Geromtel_3 and Tunsyr_2 was mapped to chromosome 6BS. Allelism tests revealed that the genes in these lines are either allelic or closely linked to each other and to *Lr61*. Linkage map analysis in the genomic region responsible for this resistance suggested that Geromtel_3 may carry an additional gene, different from the one carried by Tunsyr_2.

Physical mapping identified several candidate genes for the leaf rust resistance in these lines that were mainly NBS-LRR proteins, which commonly act as *R* genes in plants. The results from the present study highlight the importance of chromosome arms 6BS and 7BL as regions rich in leaf rust resistance genes, which can be valuable in breeding programs for pyramiding multiple genes, to achieve more durable resistance. KASP markers tightly linked to these *Lr* genes have been produced and tested, and are ready to be used in applied breeding programs with high reliability and throughput.

Supporting information

S1 Table. KASP primer sets for the SNP markers associated with leaf rust resistance in Amria, Byblos, Geromtel_3 and Tunsyr_2. Markers starting with "*usw*" were designed by the durum wheat molecular lab at the University of Saskatchewan. Markers starting with "*BS*"

were designed at the University of Bristol (http://www.cerealsdb.uk.net).
(XLSX)

S2 Table. List of the SNP markers linked to leaf rust resistance in Amria and Byblos and their positions on the tetraploid wheat consensus map and in the wild emmer wheat (WEW) reference sequence. N/A, mapping information not available.
(XLSX)

S3 Table. List of the SNP markers linked to leaf rust resistance in Geromtel_3 and Tunsyr_2 and their positions on the tetraploid wheat consensus map and in the wild emmer wheat (WEW) reference sequence. N/A, mapping information not available.
(XLSX)

S4 Table. List of annotated genes within the *Lr_Amria* and *Lr_Byblos* intervals.
(XLSX)

S5 Table. List of annotated genes within the *Lr_Geromtel_3* and *Lr_Tunsyr_2* intervals.
(XLSX)

S1 Fig. PCR amplicons for the SSR marker *Xgwm344* linked to *Lr14a*. 1Kb+ DNA ladder; lanes 1–2, Amria; 3–4 resistant RILs from Amria/ATRED #2; 5–6 susceptible RILs from Amria/ATRED #2; 7–8 Byblos; 9–10 resistant RILs from Byblos/ATRED #2; 11–12 susceptible RILs from Byblos/ATRED #2; 13–14 Sachem (*Lr14a*+); and 15–16 ATRED #2.
(PDF)

S2 Fig. PCR amplicons for the SSR marker *Xgwm146* linked to *Lr14a*. 1Kb+ DNA ladder; lanes 1–4 Amria; 5–8 Byblos; 9–10 Sachem (*Lr14a*+); 11–14 resistant RILs from Amria/ATRED #2; 15–18 resistant RILs from Byblos/ATRED #2; 19–22 susceptible RILs from Amria/ATRED #2; 23–26 susceptible RILs from Byblos/ATRED #2; 27–30 ATRED #2; and 31–32 Sachem (*Lr14a*+).
(PDF)

S3 Fig. Banding pattern of the PCR products for the NBS-LRR-specific primers associated with *Lr14a*. (A) *4406F/4840R*. (B) *4406F/4852R*. (C) *4407F/4840R*. (D) *4407F/4852R*. 1Kb+, DNA ladder; Am, Amria; By, Byblos; AT, ATRED #2; Sa, Sachem *(Lr14a*+).
(PDF)

S4 Fig. PCR amplicons for the SSR marker *Xwmc487* linked to *Lr61*. 1Kb+, DNA ladder; Gr_3, Geromtel_3; Tn_2, Tunsyr_2; G. INIA, Guayacan INIA (*Lr61*+); ATRED, ATRED #2.
(PDF)

Acknowledgments

Sonora (PIEAES)" / Fundacíon Produce Sonora, México, and (2) the "Durable Rust Resistance Wheat (DRRW)" Project as part of the Borlaug Global Rust Initiative (BGRI).

Author Contributions

Conceptualization: Karim Ammar, Curtis J. Pozniak.

Formal analysis: Dhouha Kthiri, Alexander Loladze, P. R. MacLachlan, Amidou N'Diaye, Kirby Nilsen, Curtis J. Pozniak.

Funding acquisition: Curtis J. Pozniak.

Investigation: Dhouha Kthiri.

Methodology: Dhouha Kthiri, Susanne Dreisigacker, Curtis J. Pozniak.

Project administration: Karim Ammar.

Resources: Alexander Loladze, P. R. MacLachlan, Amidou N'Diaye, Susanne Dreisigacker, Karim Ammar, Curtis J. Pozniak.

Supervision: Karim Ammar, Curtis J. Pozniak.

Validation: Dhouha Kthiri.

Visualization: Dhouha Kthiri.

Writing – original draft: Dhouha Kthiri.

Writing – review & editing: Dhouha Kthiri, Alexander Loladze, Sean Walkowiak, Kirby Nilsen, Karim Ammar, Curtis J. Pozniak.

References

1. Kolmer JA. Tracking wheat rust on a continental scale. Current Opinion in Plant Biology. 2005; 8: 441–449. http://dx.doi.org/10.1016/j.pbi.2005.05.001. PMID: 15922652

2. Huerta-Espino J, Singh RP, German S, McCallum BD, Park RF, Chen WQ, et al. Global status of wheat leaf rust caused by *Puccinia triticina*. Euphytica. 2011; 179: 143–160. https://doi.org/10.1007/s10681-011-0361-x

3. Ellis JG, Mago R, Kota R, Dodds PN, McFadden H, Lawrence G, et al. Wheat rust resistance research at CSIRO. Aust J Agric Res. 2007; 58: 507–511.

4. Rosewarne GM, Singh RP, Huerta-Espino J, Rebetzke GJ. Quantitative trait loci for slow-rusting resistance in wheat to leaf rust and stripe rust identified with multi-environment analysis. Theor Appl Genet. 2008; 116: 1027–1034. https://doi.org/10.1007/s00122-008-0736-0 PMID: 18335201

5. Vida G, Gal M, Uhrin A, Veisz O, Syed NH, Flavell AJ, et al. Molecular markers for the identification of resistance genes and marker-assisted selection in breeding wheat for leaf rust resistance. Euphytica. 2009; 170: 67–76. https://doi.org/10.1007/s10681-009-9945-0

6. McIntosh RA, Dubcovsky J, Rogers WJ, Morris C, Appels R, Xia XC. Catalogue of gene symbols for wheat: 2015–2016 supplement. 2015. https://shigen.nig.ac.jp/wheat/komugi/genes/macgene/supplement2015.pdf.

7. Schnurbusch T, Paillard S, Schori A, Messmer M, Schachermayr G, Winzeler M, et al. Dissection of quantitative and durable leaf rust resistance in Swiss winter wheat reveals a major resistance QTL in the *Lr34* chromosomal region. Theor Appl Genet. 2004; 108: 477–484. https://doi.org/10.1007/s00122-003-1444-4 PMID: 14523520

8. Singh RP, Huerta-Espino J, Pfeiffer W, Figueroa-Lopez P. Occurrence and impact of a new leaf rust race on durum wheat in Northwestern Mexico from 2001 to 2003. Plant Dis. 2004; 88: 703–708. https://doi.org/10.1094/PDIS.2004.88.7.703

9. Herrera-Foessel S, Singh RP, Huerta-Espino J, Crossa J, Yuen J, Djurle A. Effect of leaf rust on grain yield and yield traits of durum wheats with race-specific and slow-rusting resistance to leaf rust. Plant Dis. 2006; 90: 1065–1072. https://doi.org/10.1094/PD-90-1065

10. Singh RP. Pathogenicity variations of *Puccinia recondita* f. sp. *tritici* and *P. graminis* f. sp. *tritici* in wheat-growing areas of Mexico during 1988 and 1989. Plant Dis. 1991; 75, 8: 790–794. https://doi.org/10.1094/PD-75-0790

11. Ordonez ME, Kolmer JA. Virulence phenotypes of a worldwide collection of *Puccinia triticina* from durum wheat. Phytopathology. 2007; 97: 344–351. https://doi.org/10.1094/PHYTO-97-3-0344 PMID: 18943655

12. Mantovani P, Maccaferri M, Tuberosa R, Kolmer J. Virulence phenotypes and molecular genotypes in collections of *Puccinia triticina* from Italy. Plant Dis. 2010; 94: 420–424. https://doi.org/10.1094/PDIS-94-4-0420

13. Goyeau H, Ammar K, Berder J. Virulence in *Puccinia triticina* for durum wheat cultivar *Creso* and other durum wheat cultivars carrying resistance gene *Lr14a* in France. Plant Dis. 2010; 94: 1068–1068. https://doi.org/10.1094/PDIS-94-8-1068A

14. Soleiman N., Solis I., Soliman M., Sillero J., Villegas D., Alvaro F., Royo C., et al. Short communication: Emergence of a new race of leaf rust with combined virulence to *Lr14a* and *Lr72* genes on durum wheat. Spanish J Agric Res. 2016; 14. https://doi.org/10.5424/sjar/2016143-9184

15. Herrera-Foessel S, Huerta-Espino J, Calvo-Salazar V, Lan CX, Singh RP. *Lr72* confers resistance to leaf rust in durum wheat cultivar Atil C2000. Plant Dis. 2014; 98: 631–635. https://doi.org/10.1094/PDIS-07-13-0741-RE

16. Herrera-Foessel SA, Singh RP, Huerta-Espino J, William M, Rosewarne G, Djurle A, et al. Identification and mapping of *Lr3* and a linked leaf rust resistance gene in durum wheat. Crop Sci. 2007; 47: 1459–1466. https://doi.org/10.2135/cropsci2006.10.0663

17. Singh RP, Bechere E, Abdalla O. Genetic analysis of resistance to leaf rust in nine durum wheats. Plant Dis. 1993; 77: 460–463.

18. Herrera-Foessel S, Singh RP, Huerta-Espino J, William HM, Garcia V, Djurle A, et al. Identification and molecular characterization of leaf rust resistance gene *Lr14a* in durum wheat. Plant Dis. 2008; 92: 469–473. https://doi.org/10.1094/PDIS-92-3-0469

19. Herrera-Foessel S, Singh RP, Huerta-Espino J, William HM, Djurle A, Yuen J. Molecular mapping of a leaf rust resistance gene on the short arm of chromosome 6B of durum wheat. Plant Dis. 2008; 92: 1650–1654. https://doi.org/10.1094/PDIS-92-12-1650

20. Aoun M, Breiland M, Turner MK, Loladze A, Chao S, Xu SS, et al. Genome-wide association mapping of leaf rust response in a durum wheat worldwide germplasm collection. The Plant Genome. 2016; 9. https://doi.org/10.3835/plantgenome2016.01.0008 PMID: 27902791

21. Kolmer JA. First Report of a wheat leaf rust (*Puccinia triticina*) phenotype with high virulence to durum wheat in the Great Plains region of the United States. Plant Dis. 2015; 99: 156–156. https://doi.org/10.1094/PDIS-06-14-0667-PDN

22. Kolmer JA, Hughes ME. Physiologic specialization of *Puccinia triticina* on wheat in the United States in 2013. Plant Dis. 2015; 99: 1261–1267. https://doi.org/10.1094/PDIS-12-14-1277-SR

23. Zhou X, Ren L, Meng Q, Li Y, Yu Y, Yu J. The next-generation sequencing technology and application. Protein Cell. 2010; 1: 520–536. https://doi.org/10.1007/s13238-010-0065-3 PMID: 21204006

24. Deschamps S, Campbell MA. Utilization of next-generation sequencing platforms in plant genomics and genetic variant discovery. Mol Breed. 2010; 25: 553–570. https://doi.org/10.1007/s11032-009-9357-9

25. Edwards D, Batley J, Snowdon RJ. Accessing complex crop genomes with next-generation sequencing. Theor Appl Genet. 2013; 126: 1–11. https://doi.org/10.1007/s00122-012-1964-x PMID: 22948437

26. Akhunov E, Nicolet C, Dvorak J. Single nucleotide polymorphism genotyping in polyploid wheat with the Illumina GoldenGate assay. Theor Appl Genet. 2009; 119: 507–517. https://doi.org/10.1007/s00122-009-1059-5 PMID: 19449174

27. Cavanagh CR, Chao S, Wang S, Huang BE, Stephen S, Kiani S, et al. Genome-wide comparative diversity uncovers multiple targets of selection for improvement in hexaploid wheat landraces and cultivars. Proc Natl Acad Sci USA. 2013; 110: 8057–8062. https://doi.org/10.1073/pnas.1217133110 PMID: 23630259

28. Wang S, Wong D, Forrest K, Allen A, Chao S, Huang BE, et al. Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. Plant Biotechnol J. 2014; 12: 787–796. https://doi.org/10.1111/pbi.12183 PMID: 24646323

29. Allen AM, Winfield MO, Burridge AJ, Downie RC, Benbow HR, Barker GLA, et al. Characterization of a wheat breeders' array suitable for high-throughput SNP genotyping of global accessions of hexaploid bread wheat (*Triticum aestivum*). Plant Biotechnol J. 2017; 15: 390–401. https://doi.org/10.1111/pbi.12635 PMID: 27627182

30. Darvasi A, Soller M. Selective genotyping for determination of linkage between a marker locus and a quantitative trait locus. Theor Appl Genet. 1992; 85: 353–359. https://doi.org/10.1007/BF00222881 PMID: 24197326

31. Michelmore RW, Paran I, Kesseli RV. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci U S A. 1991; 88: 9828–9832. PMID: 1682921

32. Darvasi A, Soller M. Selective DNA pooling for determination of linkage between a molecular marker and a quantitative trait locus. Genetics. 1994; 138: 1365–1373. PMID: 7896115

33. Basnet BR, Singh S, Lopez-Vera E, Huerta-Espino J, Bhavani S, Jin Y, et al. Molecular mapping and validation of *SrND643*: A new wheat gene for resistance to the stem rust pathogen Ug99 race group. Phytopathology. 2015; 105: 470–476. https://doi.org/10.1094/PHYTO-01-14-0016-R PMID: 25870921

34. Qureshi N, Bariana H, Forrest K, Hayden M, Keller B, Wicker T, et al. Fine mapping of the chromosome 5B region carrying closely linked rust resistance genes *Yr47* and *Lr52* in wheat. Theor Appl Genet. 2017; 130: 495–504. https://doi.org/10.1007/s00122-016-2829-5 PMID: 27866228

35. Loladze A, Kthiri D, Pozniak C, Ammar K. Genetic analysis of leaf rust resistance in six durum wheat genotypes. Phytopathology. 2014; 104: 1322–1328. https://doi.org/10.1094/PHYTO-03-14-0065-R PMID: 24906010

36. Peterson RF, Campbell AB, Hannah AE. A diagrammatic scale for estimating rust intensity on leaves and stems of cereals. Can J Res. 1948; 26c: 496–500. https://doi.org/10.1139/cjr48c-033

37. Roelfs AP, Singh RP, Saari EE. Rust diseases of wheat: concepts and methods of disease management. Mexico DF, Mexico: CIMMYT; 1992.

38. McIntosh RA, Wellings CR, Park RF. Wheat Rusts: An Atlas of Resistance Genes. Australia: CSIRO PUBLISHING; 1995.

39. Dreisigacker S, Sehgal D, Reyes-Jaimez AE, Luna-Garrido B, Muñoz-Zavala S, Núñez-Ríos C, et al. CIMMYT wheat molecular genetics: laboratory protocols and applications to wheat breeding. Mexico, D.F.: CIMMYT; 2016.

40. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 2000; 132: 365–386. PMID: 10547847

41. Lorieux M. MapDisto: fast and efficient computation of genetic linkage maps. Mol Breed. 2012; 30: 1231–1235. https://doi.org/10.1007/s11032-012-9706-y

42. Kosambi DD. The estimation of map distances from recombination values. Ann Eugenics. 1943; 12: 172–175. https://doi.org/10.1111/j.1469-1809.1943.tb02321.x

43. Voorrips RE. MapChart: Software for the graphical presentation of linkage maps and QTLs. J Hered. 2002; 93: 77–78. PMID: 12011185

44. Singh A, Pandey MP, Singh AK, Knox RE, Ammar K, Clarke JM, et al. Identification and mapping of leaf, stem and stripe rust resistance quantitative trait loci and their interactions in durum wheat. Mol Breed. 2013; 31: 405–418. https://doi.org/10.1007/s11032-012-9798-4 PMID: 23396999

45. Pozniak CJ, Knox RE, Clarke FR, Clarke JM. Identification of QTL and association of a phytoene synthase gene with endosperm colour in durum wheat. Theor Appl Genet. 2007; 114: 525–537. https://doi.org/10.1007/s00122-006-0453-5 PMID: 17131106

46. Avni R, Nave M, Barad O, Baruch K, Twardziok SO, Gundlach H, et al. Wild emmer genome architecture and diversity elucidate wheat evolution and domestication. Science. 2017; 357: 93–97. https://doi.org/10.1126/science.aan0032 PMID: 28684525

47. Wu TD, Watanabe CK. GMAP: a genomic mapping and alignment program for mRNA and EST sequences. Bioinformatics. 2005; 21: 1859–1875. https://doi.org/10.1093/bioinformatics/bti310 PMID: 15728110

48. Maccaferri M, Ricci A, Salvi S, Milner SG, Noli E, Martelli PL, et al. A high-density, SNP-based consensus map of tetraploid wheat as a bridge to integrate durum and bread wheat genomics and breeding. Plant Biotechnol J. 2015; 13: 648–663. https://doi.org/10.1111/pbi.12288 PMID: 25424506

49. Maccaferri M, Sanguineti MC, Mantovani P, Demontis A, Massi A, Ammar K, et al. Association mapping of leaf rust response in durum wheat. Mol Breed. 2010; 26: 189–228. https://doi.org/10.1007/s11032-009-9353-0

50. Terracciano I, Maccaferri M, Bassi F, Mantovani P, Sanguineti MC, Salvi S, et al. Development of COS-SNP and HRM markers for high-throughput and reliable haplotype-based detection of *Lr14a* in durum wheat (*Triticum durum* Desf.). Theor Appl Genet. 2013; 126: 1077–1101. https://doi.org/10.1007/s00122-012-2038-9 PMID: 23292293

51. Herrera-Foessel S, Singh RP, Huerta-Espino J, Rosewarne GM, Periyannan SK, Viccars L, et al. *Lr68*: a new gene conferring slow rusting resistance to leaf rust in wheat. Theor Appl Genet. 2012; 124: 1475–1486. https://doi.org/10.1007/s00122-012-1802-1 PMID: 22297565

52. Dyck PL, Samborski DJ. The genetics of two alleles for leaf rust resistance at the *Lr14* locus in wheat. Can J Genet Cytol. 1970; 12: 689–694. https://doi.org/10.1139/g70-091

53. Gharbi MS, Ammar K, Berrias S, Karboul K, Manel A. Mitigating the threat of leaf rust to durum yield stability in new *Septoria tritici* blotch resistant germplasm in Tunisia. Borlaug Global Rust Initiative Tech Workshop. 2013.

54. Goyeau H, Berder J, Czerepak C, Gautier A, Lanen C, Lannou C. Low diversity and fast evolution in the population of *Puccinia triticina* causing durum wheat leaf rust in France from 1999 to 2009, as revealed by an adapted differential set. Plant Pathol. 2012; 61: 761–772. https://doi.org/10.1111/j.1365-3059.2011.02554.x

55. Zhang P, Zhou H, Lan C, Li Z, Liu D. An AFLP marker linked to the leaf rust resistance gene *LrBi16* and test of allelism with *Lr14a* on chromosome arm 7BL. Crop J. 2015; 3: 152–156. http://dx.doi.org/10.1016/j.cj.2014.11.004.

56. Xing L, Wang C, Xia X, He Z, Chen W, Liu T, et al. Molecular mapping of leaf rust resistance gene *LrFun* in Romanian wheat line Fundulea 900. Mol Breed. 2014; 33: 931–937. https://doi.org/10.1007/s11032-013-0007-x

57. Dvorak J, Knott DR. Location of a *Triticum speltoides* chromosome segment conferring resistance to leaf rust in *Triticum aestivum*. Genome. 1990; 33: 892–897. https://doi.org/10.1139/g90-134

58. Marais GF, Pretorius ZA, Wellings CR, McCallum B, Marais AS. Leaf rust and stripe rust resistance genes transferred to common wheat from *Triticum dicoccoides*. Euphytica. 2005; 143: 115–123. https://doi.org/10.1007/s10681-005-2911-6

59. Dangl JL, Jones JDG. Plant pathogens and integrated defence responses to infection. Nature. 2001; 411: 826–833. https://doi.org/10.1038/35081161 PMID: 11459065

60. McHale L, Tan X, Koehl P, Michelmore RW. Plant NBS-LRR proteins: adaptable guards. Genome Biol. 2006; 7: 212. https://doi.org/10.1186/gb-2006-7-4-212 PMID: 16677430

61. McDowell JM, Simon SA. Molecular diversity at the plant–pathogen interface. Dev Comp Immunol. 2008; 32: 736–744. http://dx.doi.org/10.1016/j.dci.2007.11.005. PMID: 18191204

62. Huang L, Brooks SA, Li W, Fellers JP, Trick HN, Gill BS. Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploid genome of bread wheat. Genetics. 2003; 164: 655. PMID: 12807786

63. Feuillet C, Travella S, Stein N, Albar L, Nublat A, Keller B. Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome. Proc Natl Acad Sci U S A. 2003; 100: 15253–15258. https://doi.org/10.1073/pnas.2435133100 PMID: 14645721

64. Cloutier S, McCallum BD, Loutre C, Banks TW, Wicker T, Feuillet C, et al. Leaf rust resistance gene *Lr1*, isolated from bread wheat (*Triticum aestivum* L.) is a member of the large *psr567* gene family. Plant Mol Biol. 2007; 65: 93–106. https://doi.org/10.1007/s11103-007-9201-8 PMID: 17611798

65. Periyannan S, Moore J, Ayliffe M, Bansal U, Wang X, Huang L, et al. The gene *Sr33*, an ortholog of barley *Mla* genes, encodes resistance to wheat stem rust race Ug99. Science. 2013; 341: 786–788. https://doi.org/10.1126/science.1239028 PMID: 23811228

66. Saintenac C, Zhang W, Salcedo A, Rouse MN, Trick HN, Akhunov E, et al. Identification of wheat gene *Sr35* that confers resistance to Ug99 stem rust race group. Science. 2013; 341: 783–786. https://doi.org/10.1126/science.1239022 PMID: 23811222

67. Yahiaoui N, Srichumpa P, Dudler R, Keller B. Genome analysis at different ploidy levels allows cloning of the powdery mildew resistance gene *Pm3b* from hexaploid wheat. Plant J. 2004; 37: 528–538. https://doi.org/10.1046/j.1365-313X.2003.01977.x PMID: 14756761

68. Bent AF, Mackey D. Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. Annu Rev Phytopathol. 2007; 45: 399–436. https://doi.org/10.1146/annurev.phyto.45.062806.094427 PMID: 17506648

69. Ellis JG, Dodds PN, Lawrence GJ. Flax rust resistance gene specificity is based on direct resistance-avirulence protein interactions. Annu Rev Phytopathol. 2007; 45: 289–306. https://doi.org/10.1146/annurev.phyto.45.062806.094331 PMID: 17430087

70. Gohre V, Robatzek S. Breaking the Barriers: Microbial effector molecules subvert plant immunity. Annu Rev Phytopathol. 2008; 46: 189–215. https://doi.org/10.1146/annurev.phyto.46.120407.110050 PMID: 18422429

71. Krattinger SG, Keller B. Molecular genetics and evolution of disease resistance in cereals. New Phytol. 2016; 212: 320–332. https://doi.org/10.1111/nph.14097 PMID: 27427289