Article

Mapping of Adult Plant Leaf Rust Resistance in Aus27506 and Validation of Underlying Loci by In-Planta Fungal Biomass Accumulation

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Abstract: Among the rust diseases, leaf rust of wheat caused by *Puccinia triticina*, is the most prevalent worldwide and causes significant yield losses. This study aimed to determine the genomic location of loci that control adult plant resistance (APR) to leaf rust in the pre-Green Revolution landrace accession, Aus27506, from the “Watkins Collection”. An Aus27506/Aus27229-derived F₇ recombinant inbred line (RIL) population was screened under field conditions across three cropping seasons and genotyped with the iSelect 90K Infinium SNP bead chip array. One quantitative trait loci (QTL) on each of the chromosomes 1BL, 2B and 2DL explained most of the leaf rust response variation in the RIL population, and these were named *QLr.sun-1BL*, *QLr.sun-2B* and *QLr.sun-2DL*, respectively. *QLr.sun-1BL* and *QLr.sun-2DL* were contributed by Aus27506. *QLr.sun-1BL* is likely *Lr46*, while *QLr.sun-2DL* appeared to be a new APR locus. The alternate parent, Aus27229, carried the putatively new APR locus *QLr.sun-2B*. The comparison of average severities among RILs carrying these QTL in different combinations indicated that *QLr.sun-2B* does not interact with either of the other two QTL; however, the combination of *QLr.sun-1BL* and *QLr.sun-2DL* reduced disease severity significantly. In planta fungal quantification assays validated these results. The RILs carrying *QLr.sun-1BL* and *QLr.sun-2DL* did not differ significantly from the parent Aus27506 in terms of resistance. Aus27506 can be used as a source of adult plant leaf rust resistance in breeding programs.

Keywords: wheat; leaf rust; QTL mapping

1. Introduction

Leaf rust, caused by *Puccinia triticina* (Eriks and E. Henn), is an important disease of wheat. This rust pathogen is common across a range of geographical environments [1] and was introduced into Australia by early migrant settlers [2]. Several subsequent introductions, combined with the evolution of *P. triticina* (Pt) pathotypes in Australia, have defeated many of the available leaf rust resistance genes. For example, after 25 years of deployment, *Lr24* was overcome in South Australia in 2000 due to the evolution of virulence in Pt pathotype 104-1,2,3,(6),(7),11. One of the more recent exotic

leaf rust incursions, Pt pathotype 104-1,3,4,6,7,8,9,10,12+Lr37 (which has combined virulence for the leaf rust resistance genes *Lr1*, *Lr3a*, *Lr13*, *Lr14a*, *Lr15*, *Lr17a*, *Lr17b*, *Lr20*, *Lr26*, *Lr28*, *Lr27+Lr31* and *Lr37*) currently predominates in Australia [3]; (http://sydney.edu.au/agriculture/documents/pbi/cereal_rust_report_2014.pdf).

The transfer of genetically diverse resistance genes in wheat cultivars is the most cost-effective way to control rust diseases [4]. Leaf rust resistance genes can be divided into two classes based on the plant growth stage at which resistance is expressed [5]. Most leaf rust resistance genes condition all stage resistance (ASR) that is effective against avirulent pathotypes throughout the plant growth [6]. ASR genes often confer high levels of resistance, but they can be rapidly defeated by pathogen evolution. In contrast, adult plant resistance (APR) genes, only provide resistance in post seedling stages. APR is typically partially effective and not associated with hypersensitive host cell death [4]. However, combinations of two or more APR genes can provide moderate levels of resistance and this type of resistance is assumed to be durable [7,8]. However, a few atypical APR genes also exist; for example, *Lr22b* is race-specific and expresses high levels of hypersensitive resistance at the adult plant stages suggesting that mechanistically it is more similar to ASR genes.

Over the last two decades, rust resistance breeding has reduced the deployment of ASR in favor of APRs and/or combinations of both types through marker-assisted selection [6]. APR expression under field conditions can be detected by different methods, such as the area under disease progress [9], size and number of uredinia produced during disease development and latent period [10,11]. These measurements are laborious and require specialized skills. To overcome the difficulties of these time-consuming disease assessment methods, Ayliffe et al. [12] introduced a simple and quick method to quantify the fungal biomass in plant tissues by the detection of chitin. Using this assay, the additive effects of different gene combinations of ASR and APR (*Sr33+Sr2*, *Sr2+Sr45*) were compared with individual gene effects (*Sr33*, *Sr45* and *Sr2*). Similarly, the comparative effectiveness and additivity of the APR loci *Lr34/Yr18*, *Lr46/Yr29* and *Lr67/Yr46* was also examined [12].

Eighty quantitative trait loci (QTL) for leaf rust resistance have been mapped [13], and many have been detected in multiple studies. Both wheat landraces and close relative species of wheat have been used to discover new sources of disease resistance [14,15]. In this study, a pre-Green Revolution wheat landrace collected from France (Aus27506), that showed a susceptible response to leaf rust at the seedling stage, but exhibited adult plant resistance, was used for mapping putatively new source(s) of resistance.

2. Materials and Methods

2.1. Development of Mapping Population

The leaf rust resistant landrace Aus27506 was selected from the “Watkins Collection” [14] and crossed with the moderately susceptible landrace Aus27229. A recombinant inbred line (RIL) population consisting of 106 RILs ($F_{6:7}$) was developed.

2.2. Greenhouse Tests

Aus27506 and Aus27229 were tested at the two-leaf stage with the Pt pathotypes 104-1,3,4,6,7,8,9,10,12+Lr37, 104-2,3,6,(7),12, 76-3,5,7,9,10,12,13+Lr37 and 10-1,3,9,10,11,12 and at the 4th leaf stage with the Pt pathotype 104-1,3,4,6,7,8,9,10,12+Lr37 (https://www.sydney.edu.au/content/dam/corporate/documents/sydney-institute-of-agriculture/research/plant-breeding-and-production/cereal_rust_report_2016_14_3.pdf). The leaf rust inoculation, incubation and disease assessments were conducted according to McIntosh et al. [16].

2.3. Field Evaluation

Eight to ten seeds of each RIL and both parents were sown as hill-plots at the experimental sites Lansdowne (LDN), Karalee (KAR) and Horse research unit (HRU) of the University of Sydney Plant

Breeding Institute (PBI), Cobbitty, during the 2014, 2015 and 2016 cropping seasons in two replications. The leaf rust spreader rows (a mixture of the susceptible genotypes MacKellar, Sonora, QAL2000, Morocco, Yitpi, Westonia and Stylet) were sown after every fifth row. In addition, each block of 35 × 2 experimental hill-plots was surrounded by a 30 cm susceptible spreader row. Leaf rust epidemic was created by spraying mixtures of urediniospores (Pt isolates 104-1,3,4,6,7,8,9,10,12+Lr37, 104-2,3,6,(7),12, 76-3,5,7,9,10,12,13+Lr37 and 10-1,3,9,10,11,12) suspended in light mineral oil on the spreaders using an ultra-low-volume applicator (Microfit™, Micron Sprayer Ltd. Herefordshire, U.K.). The experimental area was irrigated using a sprinkler irrigation system when required to enhance crop growth and to create congenial conditions for rust development.

Adult plant leaf rust responses were scored from flag leaf initiation to grain filling at weekly intervals based on a 1–9 scale [5]. The 1–9 scale was converted to a disease severity score [17] to compare responses of RILs carrying different combinations of the QTL for leaf rust resistance.

2.4. Molecular Mapping

2.4.1. DNA Isolation and Quantification

Genomic DNA was extracted from the RILs and parents using a modified CTAB method [18] and quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA).

2.4.2. iSelect 90K Infinium Bead Chip Array Genotyping

The RIL population was genotyped at AgriBio, La Trobe University, Melbourne, using the iSelect 90K Infinium SNP bead chip array described by Wang et al. [19]. The RILs that did not produce high quality genotypic data ($\geq 10\%$ missing data) were excluded from downstream analyses. Monomorphic markers were also excluded. The remaining marker data were evaluated using Chi-squared analysis, and markers that deviated from a 1:1 segregation ratio were also discarded. Markers with 5% or fewer heterozygous calls were retained to avoid the false purging of heterozygous loci [20].

2.4.3. Linkage Map Construction and QTL Analysis

MapManager version QTXb20 [21] was used for the genetic linkage map construction. The Kosambi mapping function [22] was used to convert recombination fractions into centiMorgans (cM). The redundant markers were excluded using the command “hide redundant loci”, option and the phenotypic data were imported into MapManager. QTL cartographer [23] was used for composite interval mapping (CIM) based on 1000 permutations.

2.4.4. Genotyping with Markers Linked with Known APR Genes

To screen the parental lines for the presence of the previously characterised leaf rust APR genes, the following markers were tested; *csLV34* for the *Lr34* gene [24], *SNPLr46G22* for the *Lr46* gene (Lagudah unpublished) and *csGS*, *cs7BLNLRR* and *Psy1-1* for the *Lr68* gene [25]. These markers were amplified using standard PCR conditions, except for the KASP marker *SNPLr46G22*, for which the KASP assay described in Chhetri et al. was used [17]. The markers revealing polymorphism between the parents were tested on the entire RIL population and incorporated into the genetic map.

2.5. Statistical Analysis

Chi-squared analysis was used to test the goodness of fit of the observed segregation to the expected genetic ratios. Wright's formula [26] was used to estimate the number of adult plant leaf rust resistance loci segregating in the Aus27506/Aus27229 RIL population. Pearson's correlation coefficients were calculated among the different leaf rust response data sets using the function PROC CORR in SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). The least significant difference (LSD) was calculated to compare the mean disease severity of the RILs carrying different combinations of QTL.

2.6. Fungal Quantification Using Chitin Assay

2.6.1. Sample Collection

Three leaf rust infected flag leaves, each from Aus27506, Aus27229 and RILs carrying different QTL combinations, were harvested from the field. An un-inoculated Aus27506 from the greenhouse was used as a negative control. The samples were weighed, cut into three-centimeter pieces and placed into 50 mL falcon screw cap tubes filled with 1M KOH containing 0.1% Silwet. The samples from each genotype were replicated four times.

2.6.2. Wheat Germ Agglutinin Chitin Assay (WAC)

The fungal chitin was quantified in these samples using the method described by Ayliffe et al. [12]. Briefly, each sample was autoclaved at 121 °C and 15 psi for 20 min with loosened caps. The KOH solution was decanted, and the samples were washed twice with 50 mM Tris-HCl pH 7.0 (Tris buffer) and left to neutralize in this buffer for at least 20 min. The tissue was then resuspended in Tris buffer at 200 mg/mL fresh weight and homogenized with a probe sonicator. The homogenates (200 µL) were aliquoted into 0.5 mL PCR tubes with four replicates per sample, and 10 µL of a 1 mg/mL solution of wheat germ agglutinin-fluorescein isothiocyanate (WGA-FITC) was added to each tube. After 15 min, the staining tubes were centrifuged at 600 rpm and the supernatant was removed from each sample. The samples were washed three times with 200 µL of Tris buffer and finally resuspended in 100 µL of Tris buffer. The fluorescence was quantified in a fluorometer using 485 nm absorption and 535 nm emission wavelengths for 1.0 s.

3. Results

3.1. Greenhouse Tests

Aus27506 and Aus27229 were susceptible [infection type (IT) 3+] at the two and three leaf stages against Pt pathotypes 104-1,3,4,6,7,8,9,10,12+Lr37, 104-2,3,6,(7),12, 76-3,5,7,9,10,12,13+Lr37 and 10-1,3,9,10,11,12 (https://www.sydney.edu.au/content/dam/corporate/documents/sydney-institute-of-agriculture/research/plant-breeding-and-production/cereal_rust_report_2016_14_3.pdf). The testing at the fourth leaf stage of these parental lines also showed susceptible IT 3+ against the Pt pathotype 104-1,3,4,6,7,8,9,10,12+Lr37. These results indicated the absence of seedling and intermediate stage leaf rust resistance in both parents.

3.2. Genotyping with Markers Linked with Known Genes

The genotyping of the parental lines with the marker *csLV34* indicated the absence of *Lr34*. The marker *SNPLr46G22* produced the same allele in both parents, suggesting the presence of *Lr46*. The markers *csGS*, *cs7BLNLRR* and *Psy1-1* were monomorphic between parents and produced an *Lr68*-specific amplicon, indicating the presence of *Lr68* in both parents.

3.3. Field Tests

On a 1–9 scale, adult plant leaf rust response of Aus27506 varied from 3 (resistant to moderately resistant) to 4 (moderately resistant leaf rust response), whereas Aus27229 showed moderate susceptibility (score 6) across all the data sets over three years. The adult plant leaf rust responses among the RILs varied from 2 to 6 (Figure 1). The analysis of the leaf rust response variation among the Aus27506/Aus27229 RIL population using Wright's formula estimated the involvement of two to three resistance loci (2.42, 2.38 and 2.42 in the 2014-LDN, 2015-KAR and 2016-HRU experiments, respectively). The leaf rust responses across seasons were significantly correlated, and the Pearson correlation coefficients between data sets varied from 0.5 to 0.6 at $P < 0.001$.

Figure 1. Leaf rust response variation among the Aus27506/Aus27229 recombinant inbred lines (RILs) when tested under field conditions. LDN: Lansdowne; KAR: Karalee; HRU: Horse research unit.

3.4. Linkage Map Construction

The Aus27506/Aus27229 RIL population was genotyped using a 90K wheat Infinium SNP bead chip array. The fifteen RILs with poor genotype calling were discarded, and a linkage map consisting of 2334 SNP showing 1:1 segregation was generated from 91 RILs. These markers formed 36 discrete linkage groups representing the 21 wheat chromosomes. The Aus27506/Aus27229 linkage map covered 6327.5 cM with an average marker density of 2.71 cM. The total map length of the “A” genome was 2485.6 cM with 951 markers, the “B” genome was 2915.7 cM with 1130 markers, and the “D” genome was 926.2 cM with 253 markers. The average marker densities of the “A”, “B” and “D” genomes were 2.61 cM, 2.58 cM and 3.66 cM, respectively. The number of polymorphic markers for the “B” genome was the highest and for the D genome, the lowest.

3.5. QTL Analysis

Composite interval mapping (CIM) performed using the QTL Cartographer for the Aus27506/Aus27229 RIL population identified three QTL for leaf rust resistance on chromosomes 1BL, 2B and 2DL. These QTL were named *Q_{Lr.sun-1BL}*, *Q_{Lr.sun-2B}* and *Q_{Lr.sun-2DL}*.

Q_{Lr.sun-1BL}, contributed by Aus27506, explained 22%, 18% and 11% of the phenotypic variation, with logarithm of the odds (LOD) scores of 6.9, 4.76 and 2.7 in the 2014-LDN, 2015-KAR and 2016-HRU data sets, respectively. The QTL peaked at marker *IWA8332*, located on the long arm of the chromosome 1B, and it was flanked by the markers *IWB74914* (IWGSC_RefSeq_v1.0 667,717,100 bp) and *IWB72835* (679,898,801 bp) (Table 1; Figure 2a).

The leaf rust QTL on chromosome 2B (*Q_{Lr.sun-2B}*) explained 6–12% of the phenotypic variation and was derived from Aus27229. This QTL was statistically significant in the 2014-LDN and 2015-KAR data sets, with LOD scores of 3.53 and 2.98, respectively. *Q_{Lr.sun-2B}* peaked at *IWB63020*, with the QTL interval defined by markers *IWB68511* (313,499,584 bp) and *IWB16756* (532,502,609 bp) (Table 1; Figure 2b).

Aus27506 contributed *Q_{Lr.sun-2DL}* and it peaked at *IWB64805*. It explained 6–19% of the phenotypic variation, with LOD values ranging from 2.3 to 4.89 across years/sites (Table 1; Figure 2c). *Q_{Lr.sun-2DL}* spanned from *IWB25696* (518,808,347 bp) to *IWB23831* (518,808,247 bp).

Table 1. Leaf rust quantitative trait loci (QTL) detected in the Aus27506/Aus27229 RIL population.

QTL	Season/Site	Peak Marker	Flanking Markers	LOD	R ²	Parent
<i>Q_{Lr.sun-1BL}</i>	2014-LDN	IWA8332	IWB74914-IWB72835	6.90	22	Aus27506
	2015-KAR	IWA8332	IWB74914-IWB72835	4.76	18	Aus27506
	2016-HRU	IWA8332	IWB74914-IWB72835	2.70 ^{ns}	11	Aus27506
<i>Q_{Lr.sun-2B}</i>	2014-LDN	IWB63020	IWB68511-IWB16756	3.53	12	Aus27229
	2015-KAR	IWB63020	IWB68511-IWB16756	2.98	09	Aus27229
	2016-HRU	IWB63020	IWB68511-IWB16756	1.48 ^{ns}	06	Aus27229
<i>Q_{Lr.sun-2DL}</i>	2014-LDN	IWB64805	IWB25696-IWB23831	4.89	19	Aus27506
	2015-KAR	IWB64805	IWB25696-IWB23831	2.30 ^{ns}	06	Aus27506
	2016-HRU	IWB64805	IWB25696-IWB23831	3.40	12	Aus27506

RIL: recombinant inbred line; LDN: Lansdowne; KAR: Karalee; HRU: Horse research unit; ns: non-significance.

Figure 2. Leaf rust resistance QTL detected on chromosomes (a) 1BL, (b) 2B and (c) 2DL among Aus27506/Aus27229 RIL population.

4. Interaction among QTL

4.1. Average Disease Severity among RILs with Different Combinations of QTL

To study the interaction among the different QTL identified in this study, the RILs were categorized based on their QTL peak marker alleles and average disease severity scores. When *Q_{Lr.sun-2B}*, *Q_{Lr.sun-1BL}* and *Q_{Lr.sun-2DL}* were present individually, the average leaf rust severities over the three field seasons were 32.0%, 19.1% and 18.3%, respectively (Table 2). The phenotypic effects of *Q_{Lr.sun-2DL}* and *Q_{Lr.sun-1BL}* were statistically similar, whereas *Q_{Lr.sun-2B}* showed significantly more disease severity.

Table 2. Mean leaf rust severities of Aus27506/Aus27229 RILs carrying different QTL combinations.

QTL	Mean Leaf Severities			
	2014-LDN	2015-KAR	2016-HRU	Average
<i>Q_{Lr.sun-1BL}</i>	18.2 ^b	20.00 ^b	19.20 ^b	19.13 ^b
<i>Q_{Lr.sun-2B}</i>	31.00 ^c	28.00 ^c	37.00 ^c	32.00 ^c
<i>Q_{Lr.sun-2DL}</i>	17.00 ^b	19.00 ^b	19.00 ^b	18.33 ^b
<i>Q_{Lr.sun-1BL}+Q_{Lr.sun-2B}</i>	17.50 ^b	18.46 ^b	19.00 ^b	18.32 ^b
<i>Q_{Lr.sun-2B}+Q_{Lr.sun-2DL}</i>	17.00 ^b	19.44 ^b	19.28 ^b	18.57 ^b
<i>Q_{Lr.sun-1BL}+Q_{Lr.sun-2DL}</i>	12.25 ^a	14.28 ^a	14.44 ^a	13.74 ^a
<i>Q_{Lr.sun-1BL}+Q_{Lr.sun-2B}+Q_{Lr.sun-2DL}</i>	11.42 ^a	12.85 ^a	13.21 ^a	12.49 ^a
RILs not carrying any QTL (Nil)	42.00 ^d	41.00 ^d	40.83 ^d	40.87 ^d
LSD	4.05	3.00	2.98	3.34

Means of disease severity followed by different letters (a, b, c and d) are significantly different based on LSD test at $P = 0.05$. Same letter shows non-significance (ns). LSD: least significant difference.

The combination of *Q_{Lr.sun-2B}* with either *Q_{Lr.sun-1BL}* or *Q_{Lr.sun-2DL}* did not significantly reduce leaf rust severity compared to the RILs carrying either of the later QTL singly. In contrast, rust severity in the RILs possessing both *Q_{Lr.sun-1BL}* and *Q_{Lr.sun-2DL}* was significantly lower than those RILs that carried them singly (Table 2). The three-QTL combination, *Q_{Lr.sun-1BL}+Q_{Lr.sun-2B}+Q_{Lr.sun-2DL}*, produced an average disease severity of 12.49%, which was similar to that with the two-QTL combination (*Q_{Lr.sun-1BL}+Q_{Lr.sun-2DL}*).

4.2. Quantification of Fungal Biomass by Chitin Assay

To assess the fungal biomass, infected flag leaves of the parents and representative RILs carrying different QTL combinations were collected and used for the chitin assay. Aus27506 and Aus27229 differed significantly for fungal biomass (Figure 3). The fungal growth of parent Aus27506, which carried *Q_{Lr.sun-2DL}* and *Q_{Lr.sun-1BL}*, was 61% lower than that of Aus27229, which carried *Q_{Lr.sun-2B}*. The RILs carrying all three QTL did not differ significantly for fungal biomass accumulation compared with Aus27506. RILs with *Q_{Lr.sun-2DL} + Q_{Lr.sun-1BL}* had more fungal colonization compared with Aus27506 and the RILs with all three QTL significantly reduced fungal growth compared to the RILs with the other two dual combinations (*Q_{Lr.sun-1BL}+Q_{Lr.sun-2B}* and *Q_{Lr.sun-2B}+Q_{Lr.sun-2DL}*) (Figure 3). The dual combinations involving *Q_{Lr.sun-2B}*, contributed by Aus27229 and either *Q_{Lr.sun-1BL}* or *Q_{Lr.sun-2DL}*, showed similar levels of fungal growth, which were slightly less than that of Aus27229 (*Q_{Lr.sun-2B}*).

Figure 3. Quantification of rust growth in Aus27506, Aus27229 and RILs carrying different QTL combinations.

5. Discussion

The composite interval mapping of adult plant leaf rust response variation among an Aus27506/Aus27229 RIL population identified three QTL on chromosomes 1BL, 2B and 2DL, respectively. Chromosome 1B carries the formally designated leaf rust resistance genes *Lr26/Yr9/Sr31* [27], *Lr33* [28], *Lr44* [29], *Lr46* [30], *Lr51* [31], *Lr55* [32], *Lr71* [33] and *Lr75* [34]. Of these genes, only *Lr33* and *Lr46* are located on the long arm. *Lr33* is located 3 cM distal to the centromere [28], and *Lr46* is located in the most distal deletion bin (1BL-0.84-0.89) of the long arm [35]. *QLr.sun-1BL*, detected in this study, was located in the distal region of chromosome 1BL (137 cM of a total map length of 174 cM). Screening with the *Lr46*-linked marker *SNPLr46G22* did not differentiate the parents, presumably due to the non-diagnostic nature of this marker and, consequently, false positive amplification in Aus27229. *QLr.sun-1BL* explained 11–22% of the phenotypic variation, which is similar to that reported for *Lr46* in other studies [13]. Furthermore, *Lr46* appears to be better expressed in cooler climates [36]. The field trial sites in this study had an average temperature of 26.2 to 28.4 °C during the months of October and November in 2014, 2015 and 2016 when the phenotypic scoring was performed, with occasional extremes of 35–38 °C (<http://www.bom.gov.au/climate/current/annual/nsw/summary.shtml#recordsTmaxAvgHigh>).

Taking into consideration the genetic map position of *QLr.sun-1BL* and percent phenotypic variation that it explained, we concluded that this QTL likely represents *Lr46*. Many other studies have also related QTL in chromosome 1BL to *Lr46* [37,38].

The position of the QTL interval for *QLr.sun-2B* between 313,499,584 and 532,502,609 bp in the IWGSCv1.0 reference genome sequence indicated that it is located in the centromeric region. *Lr48*, an APR gene producing a low IT (23N to X) at the fourth leaf stage, co-segregated with the markers positioned at 59 cM [39] in the published 90K SNP consensus map of Wang et al. [19]. However, the susceptibility of the parents in multi-pathotype greenhouse tests at different growth stages indicate that *QLr.sun-2B* does not correspond to *Lr48*.

Several QTL are reported on chromosome 2B, including *QLr.ifa-2BS* [40], *QLr.ksu-2BS* [41], *QLr.sfrs-2BL* [42], *QLr.lp.ous-2B* [38], *QLr.cimmyt-2BS* [37], *QLr.lp.osu-2B* [43] and *QLr.osu-2B* [44]. *QLr.ifa-2BS* is an allele of *Lr13* from cultivar Forno and *QLr.lp.osu-2B*, mapped closer to the centromere between the amplified fragment length polymorphism (AFLP) markers XCAGCGAT70 and XCATGATGC60. To demonstrate whether *QLr.sun-2B* represents a new APR locus, it will be

important to enrich the target region with markers previously known to map onto chromosome 2B and are associated with known leaf rust resistance loci.

The markers delineating the *QLr.sun-2DL* interval were positioned between 518,808,347 and 518,808,247 bp in the IWGSC_RefSeq_v1.0 genome assembly. Taking into consideration the position of the linked markers, this QTL was located in the long arm of chromosome 2D. *QLr.sfr-2DL* [45], present on the long arm of chromosome 2D, is reported to be closer to the centromere. It peaks at the simple sequence repeat (SSR) marker *gwm539* and explains 11.4–12% of the phenotypic variation. The marker *gwm539* was mapped at the 116 cM position of the total 136 cM length of chromosome 2D [46]. Hence, *QLr.sun-2DL* appears to be a new locus.

Of the three QTL detected in this study, *QLr.sun-2DL* and *QLr.sun-1BL* contributed more towards disease reduction compared to *QLr.sun-2B*. The interaction studies indicated that *QLr.sun-2B* does not interact with *QLr.sun-2DL* or *QLr.sun-1BL* to reduce disease severity. Many studies have reported disease severity to be lowered by combinations of APR genes; for example, *Lr34* and *Lr46*, *Lr34* and *Lr68*, and *Lr75* and *QLr.sfr-7BL* showed enhanced resistance through additive gene action in different trials [34]. In-plant fungal quantification using the chitin assay is another measure to show the additive effect of resistance loci [12]. The RILs carrying two QTL combinations involving *QLr.sun-2B* showed more fungal biomass compared to the combination of *QLr.sun-1BL* and *QLr.sun-2D*. These results confirm conclusions drawn from the field disease severity score comparison of the RILs possessing different combinations of QTL.

The failure to observe segregation for a leaf rust response score beyond 6 suggests that Aus27506 and Aus27229 may have an APR locus in common. The monomorphism of the *Lr68*-linked marker is indicative of the presence of this gene in both parents. The development of single locus populations for *QLr.sun-2B* and *QLr.sun-2DL* is underway to enable the detailed mapping of the regions carrying these loci and to identify closely linked markers for their marker-assisted pyramiding in wheat breeding programs.

Author Contributions: U.B. and H.B. identified material and made initial cross. M.C. developed R.I.L. population. P.K. and H.B. conducted greenhouse and field screening. M.H. and M.A. conducted S.N.P. genotyping in-plant fungal biomass accumulation assays, respectively. P.K. drafted the manuscript. U.B., H.B., M.H. and M.A. edited drafts. All authors have read and agreed to the published version of the manuscript.

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