Genetic and Molecular Characterization of Leaf Rust Resistance in Two Durum Wheat Landraces

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ABSTRACT

Leaf rust, caused by *Puccinia triticina*, is a constraint to durum wheat (*Triticum turgidum* subsp. *durum*) production, and landraces are reported to be an important source of resistance. Two Portuguese landraces (Aus26582 and Aus26579) showed resistance against durum-specific *P. triticina* races and were crossed with a susceptible landrace (Bansi) to develop recombinant inbred line (RIL) populations. Monogenic segregation for leaf rust resistance was observed among both RIL populations. The underlying locus, temporarily named *LrAW2*, was mapped to the short arm of chromosome 6B in the Aus26582/Bansi population and five DArTseq markers cosegregated with *LrAW2*. Simple sequence repeat markers *sun683* and *sun684*, developed from the chromosome survey sequence (CSS) contig 6BS_2963854, identified through BlastN search of cosegregating DArTseq

markers in the International Wheat Genome Sequencing Consortium database, cosegregated with *LrAW2*. Comparison of the CSS contig 6BS_2963854-based sequences amplified from parental genotypes led to the development of marker *sunKASP_60*, which also showed close linkage with *LrAW2*. Markers *sun684* and *sunKASP_60* showed close association with *LrAW2* in both RIL populations. The amplification of *LrAW2*-specific products by linked markers in Aus26582, Aus26579, and Guayacan (*Lr61*) indicated that *LrAW2* may be *Lr61*. The alternate amplicon or haplotype produced with *LrAW2*-linked markers in Australian durum cultivars demonstrated their effectiveness in marker-assisted selection.

Additional keywords: breeding, genetic analysis, pyramiding, SNP.

Durum wheat (*Triticum turgidum* L. subsp. *durum*, $2n = 4x = 28$) is the commonly cultivated tetraploid wheat, especially in the Mediterranean region, which contributes 50% to the total global durum production (Henkhar et al. 2016). It is mostly used for making pasta, flat bread, and couscous in many regions of the world. The sustainability of durum wheat production is challenged by many abiotic and biotic stresses. Leaf rust, caused by *Puccinia triticina*, is among the important biotic stresses and in the event of severe epidemics up to 70% reduction in durum wheat production can occur (Herrera-Foessel et al. 2006; Singh et al. 2004).

Durum wheat showed a high level of leaf rust resistance against commonly prevailing *P. triticina* races (Ordoñez and Kolmer 2007a) until the detection of durum-specific races of *P. triticina* in Mexico (Singh et al. 2004). In 2001, appearance of the *P. triticina* race BBG/BN in northwestern Mexico carrying virulence to Altar C84, which carries *Lr72*, defeated resistance in CIMMYT durum wheat cultivars known to be resistant for over 25 years and caused economic losses of approximately US\$32 million (Herrera-Foessel et al. 2014; Singh et al. 2004). In 2003, the race BBG/BN was observed on durum wheat in southern California (Kolmer et al. 2007). At the same time, changes in virulence of *P. triticina* on durum wheat were also observed in Spain (Martinez et al. 2005) and France (Goyeau et al. 2006, 2012). Goyeau et al. (2012) stated that the detection of the Altar C84-virulent race in France in 1997 predated its emergence in Mexico. Phenotypic analysis and simple sequence repeat (SSR) genotyping of predominant durum-specific *P. triticina* races collected from North America, South America, Western Europe, and the Middle East indicated a high level of genetic similarity, whereas races collected from Ethiopia were genetically distinct and indicated a different origin (Kolmer 2005;

Kolmer and Acevedo 2016; Kolmer and Hughes 2014; Ordoñez and Kolmer 2007b).

Of more than 70 designated leaf rust resistance genes (McIntosh et al. 1995, 2013, 2014), very few are known to be present in durum wheat cultivars. Following the detection of durum-specific *P. triticina* races in Mexico, CIMMYT scientists initiated genetic analysis of leaf rust resistance among durum wheat germplasm, and this led to the postulation of complimentary gene pair *Lr27+Lr31* located in chromosomes 3BS and 4BS, respectively (Singh et al. 1993; Singh and McIntosh 1984), *Lr3a* in chromosome 6BL (Browder 1980), *Lr14a* in chromosome 7BL (Dyck and Samborski 1970), *Lr23* in chromosome 2BS (Watson and Luig 1961), *Lr61* in chromosome 6BS (Herrera-Foessel et al. 2008), and *Lr72* in chromosome 7BS (Herrera-Foessel et al. 2014). Loladze et al. (2014) showed the presence of some of these genes in the elite CIMMYT germplasm and discussed a complicated linkage or allelic relationships among genes located in chromosome 6BS. Other resistance genes found only in durum wheat cultivars include *LrCamyo* in chromosome 6BL (Herrera-Foessel et al. 2007) and *LrWo* in chromosome 5BS (Singh et al. 2010). *Lr3a*, *Lr14a*, *Lr23*, and *Lr27+Lr31* are also present in common wheat but are ineffective against predominant common wheat *P. triticina* races (McIntosh et al. 1995). However, *Lr23* is effective against the currently predominating *P. triticina* races in Australia (Chhetri et al. 2017).

Identification and characterization of genetically diverse sources of leaf rust resistance are essential to control this disease. Durum wheat landraces were cultivated until the mid-20th century (Soriano et al. 2016). The cultivation of landraces was replaced by improved semidwarf cultivars (Lopes et al. 2015), thus leaving behind some unused genetic diversity. Recent studies clearly demonstrated the potential of landraces to contribute diverse alleles for economic traits (Bansal et al. 2013; Bariana et al. 2013; Soriano et al. 2016). Two durum wheat landraces (Aus26582 and Aus26579) collected from Portugal (Miller et al. 2000) exhibited resistance against durum-specific *P. triticina* races (U. Bansal and H. Bariana, unpublished). This investigation was planned to conduct genetic

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analysis of leaf rust resistance carried by Aus26582 and Aus26579, to determine genomic locations of underlying resistance genes, and to identify molecular markers closely linked with resistance genes.

MATERIALS AND METHODS

Plant materials. Aus26582 and Aus26579 collected from Portugal were kindly supplied by the Australian Winter Cereal Collection, Tamworth, NSW, Australia (now the Australian Grains Gene Bank, Horsham, VIC, Australia). These landraces were crossed with the susceptible landrace Banshi from India to generate recombinant inbred line (RIL) $F_{5:6}$ populations comprising 155 and 79 lines, respectively. Eleven Australian durum cultivars were used to test the effectiveness of linked markers for marker-assisted selection (MAS).

Pathogen materials. Durum-specific *P. triticina* races CA 1.2 and ETH 12.5-2 from California and Ethiopia, respectively, were used for testing both RIL populations (Aus26582/Banshi and Aus26579/Banshi) at the Cereal Disease Laboratory (CDL), St. Paul, MN. Aus26582, Aus26579, and Banshi were also tested against six Australian *P. triticina* races: 104-2,3,6,(7) (Plant Breeding Institute culture number 231), 10-1,2,3,4 (348), 68-1,2,3,4 (469), 104-1,(2),3,(6),(7),11,13 (547), 76-1,3,5,7,9,10,12+*Lr37* (621), and 104-1,3,4,6,7,8,9,10,12+*Lr37* (634) from the cereal rust race collection of the University of Sydney Plant Breeding Institute, Cobbitty. The virulence or avirulence details of *P. triticina* races are given in Table 1.

Greenhouse screening. *Evaluation against durum-specific P. triticina races.* Seedling leaf rust tests were carried out on Aus26582/Banshi and Aus26579/Banshi RIL populations along with the parents Aus26582, Aus26579, and Banshi at the CDL. The RIL populations were planted in 3.5-cm² plastic pots, with 5 to 6 seeds/RIL and four RILs per pot planted in the quarterly corners that were filled with vermiculite and placed in plastic trays as six pots per tray. When the primary leaves were fully expanded, seedlings of the RILs, parents, and common wheat ‘Thatcher’ were inoculated with races CA1.2 and ETH 12.5-2. For seedling inoculations, approximately 1 mg of urediniospores was mixed with 0.2 ml of Soltrol 170 oil (Phillips Petroleum, Borger, OK) and then spray inoculated onto the plants. After inoculation, seedlings were allowed to dry for 1 h and then placed in a mist chamber overnight at 18°C and 100% relative humidity. After incubation, the seedlings were placed on a greenhouse bench with supplemental metal halide lighting at a temperature of 18 to 23°C. Seedlings were fertilized with a 20-20-20 NPK solution immediately after inoculation and at 14 days after planting. The infection types (IT) on the primary leaves of individual plants were recorded 10 days after inoculation using a 0-to-4 scale, as detailed by Long and Kolmer (1989) and McIntosh et al. (1995). The most commonly expressed ITs in this study were IT₀ = pinhead necrotic spots, IT₁ = presence of pinhead necrotic spots (·) and some with small reddish dots (1) on the same leaf, and IT₃₊ = pustules showing sporulation (red powdery mass). The signs plus (+) and minus (–) were used to reflect ITs higher and lower than those expected for that class.

Evaluation against Australian P. triticina races. Six sets of eight seeds each of Aus26582, Aus26579, and Banshi were sown in 9-cm pots filled with a potting mix containing a mixture of pine bark and river sand at a ratio of 2:1. Water-soluble fertilizer Aquasol (20 g) dissolved in 10 liters of water was applied to all pots before sowing. The pots were placed in a rust-free microclimate room maintained at 20°C. At 1 week after sowing, nitrogenous fertilizer urea was applied to all pots at the same rate as the Aquasol.

Inoculations were carried out at the two-leaf stage. Urediniospores of each pathotype (Table 1) were suspended in light mineral oil Isopar-L (10 ml) and atomized using an aerosol pressure pack on each set separately, according to Bariana and McIntosh (1993). Inoculated seedlings were placed in a dark humid chamber fitted with an ultrasonic humidifier for 24 h. After 24 h, inoculated seedlings were transferred to microclimate rooms maintained at 25 ± 2°C. Leaf rust response was recorded after 12 to 14 days of inoculation using the 0-to-4 scale described in McIntosh et al. (1995). Both RIL populations were also tested against *P. triticina* race 104-1,(2),3,(6),(7),11,13 (547) using the above-mentioned procedure.

DNA extraction. Genomic DNA was extracted from 10-day old seedlings of both RIL populations along with the parents, following the procedure described by Bansal et al. (2014b). The quality of DNA was tested using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies) and final dilutions of 30 ng/μl were prepared.

Genomic location of resistance genes. A DArTseq map of the Aus26582/Banshi RIL population containing 5,802 markers was available from another study pertaining to quantitative trait locus mapping of stripe rust resistance (U. Bansal and H. Bariana, unpublished). Phenotypic data were converted to genotypes and incorporated into the Aus26582/Banshi DArTseq map using MapManager, version QTXb20 (Manly et al. 2001). Previously reported SSR markers located in the short arm of chromosome 6B were also used (the GrainGenes 2.0 database; <https://wheat.pw.usda.gov>). Both RIL populations were tested with polymorphic markers.

Development of new SSR markers. Sequences of DArTseq markers that showed close linkage with the resistance gene were blasted against the flow-sorted chromosome survey sequence (CSS) contigs of Chinese Spring using BlastN query (https://urgi.versailles.inra.fr/blast/?dbgroup=wheat_all&program=blastn). The CSS contigs having 100% similarity (1e⁻²⁸ to 5e⁻⁰⁸) were selected and used to design SSR markers using the BatchPrimer3 software (<http://batchprimer3.bioinformatics.ucdavis.edu/cgi-bin/batchprimer3/batchprimer3.cgi>). Each forward primer had an M13 sequence tail added to it at the 5' end. These primers were named as “sun” (Sydney University) and sequences are given in Table 2.

Development of kompetitive allele-specific polymerase chain reaction markers. The CSS contig 6BS_2963854 (from which closely linked SSR markers were identified) was used to develop sequence-tagged site (STS) markers in order to identify single-nucleotide polymorphisms (SNPs) between the parents Aus26582 and Banshi. Primers were designed using software Primer3 (v. 0.4.0) (Koressaar and Remm 2007; Untergasser et al. 2012). Majority

TABLE 1. Virulence/avirulence formulae of *Puccinia triticina* races used

Leaf rust pathotype	Culture number	Virulence/avirulence formula ^a
104-2,3,6,(7)	231	<i>Lr14a</i> , (<i>Lr17a</i>), <i>Lr17b</i> , <i>Lr23</i> , <i>Lr27+Lr31/Lr3ka</i> , <i>Lr13</i> , <i>Lr15</i> , <i>Lr16</i> , <i>Lr20</i> , <i>Lr24</i> , <i>Lr26</i> , <i>Lr28</i> , <i>Lr37</i> , <i>Lr61</i> , <i>Lr72</i>
10-1,2,3,4	348	<i>Lr14a</i> , <i>Lr15</i> , <i>Lr20</i> , <i>Lr23/Lr3ka</i> , <i>Lr13</i> , <i>Lr16</i> , <i>Lr17a</i> , <i>Lr17b</i> , <i>Lr24</i> , <i>Lr27+Lr31</i> , <i>Lr26</i> , <i>Lr28</i> , <i>Lr37</i> , <i>Lr61</i> , <i>Lr72</i>
68-1,2,3,4	469	<i>Lr14a</i> , <i>Lr15</i> , <i>Lr20</i> , <i>Lr23/Lr3ka</i> , <i>Lr13</i> , <i>Lr16</i> , <i>Lr17a</i> , <i>Lr17b</i> , <i>Lr24</i> , <i>Lr26</i> , <i>Lr27+Lr31</i> , <i>Lr28</i> , <i>Lr37</i> , <i>Lr61</i> , <i>Lr72</i>
104-1,(2),3,(6),(7),(9),11,13 + <i>Lr24</i>	547	<i>Lr14a</i> , <i>Lr17a</i> , <i>Lr20</i> , <i>Lr23</i> , <i>Lr24</i> , <i>Lr26</i> , <i>Lr27+Lr31/Lr3ka</i> , <i>Lr13</i> , <i>Lr15</i> , <i>Lr17b</i> , <i>Lr28</i> , <i>Lr37</i> , <i>Lr61</i> , <i>Lr72</i>
76-1,3,5,7,9,10,12+ <i>Lr37</i>	621	<i>Lr13</i> , <i>Lr14a</i> , <i>Lr16</i> , <i>Lr17a</i> , <i>Lr17b</i> , <i>Lr20</i> , <i>Lr26</i> , <i>Lr37/Lr15</i> , <i>Lr23</i> , <i>Lr24</i> , <i>Lr28</i> , <i>Lr27+Lr31</i> , <i>Lr61</i> , <i>Lr72</i>
104-1,3,4,6,7,8,9,10,12+ <i>Lr37</i>	634	<i>Lr12</i> , <i>Lr13</i> , <i>Lr14a</i> , <i>Lr17a</i> , <i>Lr17b</i> , <i>Lr20</i> , <i>Lr26</i> , <i>Lr27+Lr31</i> , <i>Lr28</i> , <i>Lr37/Lr1</i> , <i>Lr3a</i> , <i>Lr16</i> , <i>Lr24</i> , <i>Lr23</i> , <i>Lr61</i> , <i>Lr72</i>
CA 1.2		<i>Lr1</i> , <i>Lr2a</i> , <i>Lr2c</i> , <i>Lr3a</i> , <i>Lr3ka</i> , <i>Lr3bg</i> , <i>Lr9</i> , <i>Lr11</i> , <i>Lr14a</i> , <i>Lr16</i> , <i>Lr17a</i> , <i>Lr18</i> , <i>Lr21</i> , <i>Lr24</i> , <i>Lr26</i> , <i>Lr28</i> , <i>Lr30</i> , <i>Lr39</i> , <i>Lr42</i> , <i>Lr72/LrB</i> , <i>Lr10</i> , <i>Lr14b</i> , <i>Lr20</i> , <i>Lr23</i>
ETH 12.5-2		<i>Lr72</i> -virulent and avirulent on Thatcher

^a Taken from the University of Sydney Cereal Rust Collection database and Cereal Disease Laboratory database, United States.

parameters used for designing primers were with default settings but minimum melting temperature was set to 57°C and maximum to 63°C. The fragment length was set to 1,000 to 1,300 bp.

STS markers were used to amplify products from Aus26582 and Bansi and the amplicons were sequenced at the Australian Genomic Research Facility, Sydney (www.agrf.org.au) using the Sanger sequencing method. Sequences were aligned using the Sequencher software (www.genecodes.com). The SNP in the parental sequences were used to design kompetitive allele-specific polymerase chain reaction (KASP) markers using BatchPrimer3 v1.0 software (http://batchprimer3.bioinformatics.ucdavis.edu/cgi-bin/batchprimer3/batchprimer3.cgi). Each KASP marker was named as “*sunKASP*” followed by the consecutive number. KASP genotyping was performed according to Nsabiye et al. (2016).

SSR and STS genotyping. Polymerase chain reaction (PCR) volumes of 10 µl were used for SSR genotyping. The reaction mixture contained 30 ng of genomic DNA, 1× Immolase buffer containing 1.5 mM MgCl₂, 200 mM dNTP, 100 nM M13 tailed forward primer, 200 nM reverse primer, 100 nM M13 dye with labeled fluorescence (IR-700 or IR-800), and 0.04 U of Immolase DNA polymerase (Bioline). PCR was performed using a touchdown profile comprising a initial denaturing step of 95°C for 10 min for the activation of DNA polymerase (Hot Start); followed by 92°C for 30 s, 62°C (with 1°C drop down for 7 cycles) for 30 s, and 72°C for 30 s; 35 cycles of 30 s at 92°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step at 72°C for 10 min. PCR products were separated on 2% agarose gels. For smaller base pair differences, the products were separated on 6.5% polyacrylamide gels on a LICOR 4300 DNA analyzer (Bansal et al. 2014a).

For STS markers, 20 µl of reaction volumes were used. The reaction mixture contained 30 ng genomic DNA, 1× Immolase buffer containing 1.5 mM MgCl₂, 200 mM dNTP, 100 nM forward primer, 200 nM reverse primer, and 0.04 U of Immolase DNA polymerase (Bioline). PCR was carried out using a touchdown profile comprising initial denaturation of 95°C for 10 min; followed by 5 cycles of denaturation at 92°C for 30 s, annealing at 65°C (with 1°C drop down for five cycles) for 40 s, and extension at 72°C for 50 s; 35 cycles of 30 s at 92°C, 40 s at 60°C, and 50 s at 72°C; and a final extension step at 72°C for 5 min. PCR products were eluted from 1% agarose gels.

Statistical analysis and linkage map construction. To determine the goodness of fit of the observed segregation to the expected genetic ratios, χ^2 tests were performed on the phenotypic segregation data of RIL populations. A genetic linkage map was constructed using MapManager, version QTXb20 (Manly et al. 2001) and the final linkage maps were drawn using Map Chart, version 2.2 (Voorrips 2002).

RESULTS

Parental assessments and inheritance studies. Aus26582 and Aus26579 produced IT ; (= near immune) against durum-specific races CA 1.2 and ETH 12.5-2 when tested at the CDL, Minnesota, and the susceptible control Bansi produced IT 3+ against both races. RIL populations were tested at the seedling stage against races CA 1.2 and ETH 12.5-2. The χ^2 analysis of seedling leaf rust response variation among both RIL populations against races CA 1.2 and ETH 12.5-2 conformed to segregation at a single locus (Table 3). Homozygous resistant lines among both RIL populations produced IT ; to ;1 similar to the resistant parents, and homozygous susceptible RILs displayed IT 3+, identical to the susceptible parent Bansi.

Aus26582 and Aus26579 displayed IT ; to ;1+ against six common wheat *P. triticina* races in Australia (Fig. 1). The Aus26579/Bansi RIL population showed results identical to those observed with races CA 1.2 and ETH 12.5-2 when tested against Australian *P. triticina* race 104-1,(2),3,(6),(7),11,13. In contrast, digenic (118 [IT ;1 to ;22+]:37 [IT 3+]; $\chi^2_{(3;1)} = 0.11$, nonsignificant at $P = 0.05$ and 1 degree of freedom [df]) inheritance of leaf rust resistance was noted among the Aus26582/Bansi RIL population against *P. triticina* race 104-1,(2),3,(6),(7),11,13. Sixty-nine RILs that were scored homozygous susceptible (Table 3) against the durum-specific races showed monogenic inheritance when tested against 104-1,(2),3,(6),(7),11,13 (32 [IT 12- to ;22+]:37 [IT 3+]; $\chi^2_{(1;1)} = 0.36$, nonsignificant at $P = 0.05$ and 1 df). The second locus in Aus26582 was ineffective against durum-specific races. This investigation covers results on characterization of the locus conferring resistance to durum-specific races segregating in both RIL populations. The locus conferring leaf rust resistance in Aus26582 against

TABLE 3. Frequency distribution of Aus26582/Bansi and Aus26579/Bansi recombinant inbred lines (RILs) when tested against durum-specific *Puccinia triticina* races CA 1.2 and ETH 12.5-2

Population	Phenotype	Number of RIL		$\chi^2_{(1;1)}^a$
		Observed	Expected	
Aus26582/Bansi	Resistant	86	77.5	0.93
	Susceptible	69	77.5	0.93
	Total	155	155.0	1.86
Aus26579/Bansi	Resistant	38	39.5	0.06
	Susceptible	41	39.5	0.06
	Total	79	79.0	0.12

^a Table value of χ^2 at $P = 0.05$ and 1 degree of freedom = 3.84.

TABLE 2. Polymerase chain reaction-based markers developed in this study from the DArTseq marker sequences

DArTseq marker	Loc (cM) ^a	CSS ^b contig	E-value	Repeat motif	Marker name	Primer (5'–3')	Amplicon (bp)		
							Aus26582	Aus26579	Bansi
1089713	5.78	6BS_3028198	1e-28	(TC) ¹³	<i>sun677-F^c</i>	tcgattaatgaaacaggaac	151	151	Null
1725149	6.45	6BS_2935897	1e-22	(TGAGAC) ³	<i>sun677-R</i>	aggagagagaaacttgctta	146	146	146
					<i>sun678-F</i>	gtgttggttaaagcaagactga			
					<i>sun678-R</i>	ttgatcatttgaagatgtgac			
3024107	6.86	6BS_2938796	2e-13	(ATGC) ³	<i>sun679-F</i>	tgctagatcggtttgattcta	Null	Null	162
3939152 ^d	6.86	6BS_2996377	2e-06	(TGA) ⁴	<i>sun679-R</i>	agtgaagcaagtttaacacacg	142	142	142
					<i>sun680-F</i>	tgattattccactatgaccag			
					<i>sun680-R</i>	tatttagaaacggaggagtc			
4261696 ^d	6.86	6BS_2963854	5e-08	(TTCTT) ³	<i>sun681-F</i>	ctgtcctgtttgtttgttc	180	180	180
					<i>sun681-R</i>	agaaacgtgaacaagtgtgaa	141	141	Null
					<i>sun682-F</i>	ctctctttctgttactcgtc			
4261696 ^d	6.86	6BS_2963854	5e-08	(CCACA) ⁴	<i>sun682-R</i>	gggttgaggagtaagaatgttt	160	Null	Null
					<i>sun683-F</i>	gacgttcaaaaagtgaagaag			
					<i>sun683-R</i>	aacctctagacacaatgcaa			
					<i>sun684-F</i>	cgttcgtttctatcgtttta	124	124	128
				(ATGG) ³	<i>sun684-R</i>	ggttatccatgtgattcaaga			

^a Map location in centimorgans (cM).

^b CSS = chromosome survey sequence.

^c Forward primer labeled with M13: CACGACGTTGTAAAACGAC.

^d Position not available in DArTseq map but cosegregated with 6BS_2938796.

durum-specific pathotypes was temporarily named *LrAW2* and the DArTseq map of Aus26582/Bansi RIL population was used for genomic location of this resistance locus.

Genomic location of *LrAW2*. The DArTseq linkage map of 92 Aus26582/Bansi RIL (U. Bansal and H. Bariana, unpublished results) covering 14 linkage groups assigned to all 14 chromosomes with 5,802 markers was used to determine genomic location of location of *LrAW2*. Because the Aus26582/Bansi RIL population exhibited monogenic segregation for *LrAW2* (Table 3), the phenotypic segregation data were converted into genotypes: *LrAW2* = A and *lrAW2* = B and *LrAW2* was incorporated into the DArTseq linkage map using the MapManager program (version QTXb20) through the link report function. *LrAW2* was mapped in the 3.1-centimorgan (cM) marker interval flanked by markers 1270844 to 7914776 in the short arm of

chromosome 6B (Fig. 2A) and five DArTseq markers (1089713, 1725149, 3024107, 3939152, and 4261696) cosegregated with *LrAW2* ($R^2 = 15\%$). The sequences of DArTseq markers 1089713, 1725149, 3024107, 3939152, and 4261696 showed homology with the CSS contigs 6BS_3028198, 6BS_2935897, 6BS_2938796, 6BS_2996377, and 6BS_2963854, respectively, when BlastN searched against the flow-sorted chromosome 6B sequence of the reference wheat ‘Chinese Spring’ (Table 2). Eight SSR markers designed from the CSS contigs listed in Table 2 were tested for polymorphism on parental genotypes. Five SSR markers (*sun677*, *sun679*, *sun682*, *sun683*, and *sun684*) showed polymorphisms between Aus26582 and Bansi. Marker *sun679* did not amplify any product in Aus26582, whereas Bansi was null for markers *sun677*, *sun682*, and *sun683*. Three markers (*sun677*, *sun682*, and *sun684*) were polymorphic between Aus26579 and Bansi.

Fig. 1. Infection types produced by Aus26582 (lane 1), Aus26579 (lane 2), and Bansi (lane 3) against six common wheat *Puccinia triticina* pathotypes.

Fig. 2. Genetic linkage maps of chromosome arm 6BS: A, DArTseq map of AUS26582/Bansi and polymerase chain reaction-based simple sequence repeat and single-nucleotide polymorphism marker maps of B, Aus26582/Bansi and C, Aus26579/Bansi RIL populations showing the location of *LrAW2*.

Markers *sun683* and *sun684* (designed from the CSS contig 6BS-2963854) cosegregated with *LrAW2* in the Aus26582/Bansi population (Fig. 2B), whereas only *sun684* cosegregated with *LrAW2* in the Aus26579/Bansi population (Fig. 2C). Marker *sun682* mapped 0.7 and 0.6 cM distal to *LrAW2* in Aus26582/Bansi and Aus26579/Bansi RIL populations, respectively (Fig. 2B and C). These results indicated presence of the same gene for leaf rust resistance in Aus26582 and Aus26579.

Five overlapping STS markers (*sun665*, *sun666*, *sun667*, *sun668*, and *sun669*) were designed from the CSS contig 6BS_2963854 (Table 4; Fig. 3). Three of the five markers (*sun667*, *sun668*, and *sun669*) produced >1-kb amplicons, whereas two markers (*sun665* and *sun666*) produced multiple bands in parental genotypes Aus26582 and Bansi. The amplicons produced by three markers (*sun667*, *sun668*, and *sun669*) in Aus26582 and Bansi were sequenced, aligned, and compared for SNP. No polymorphism was observed between the sequences of parental amplicons of marker *sun668*, whereas one and four SNP were observed in amplicons produced by *sun667* and *sun669*,

TABLE 4. List of sequence-tagged site markers designed from the chromosome survey sequence contig 6BS_2963854

Marker name	Primer sequence (5'–3')	Product size (bp)
<i>sun665-F</i>	tggcaagcaagttgtcactc	1,400
<i>sun665-R</i>	gccatactgatccaaaggt	
<i>sun666-F</i>	ttccatactatccagcgtcgt	1,212
<i>sun666-R</i>	gaccctgccgttactcacc	
<i>sun667-F</i>	atcgagcagagattgtgtac	1,283
<i>sun667-R</i>	ggaacgtcttcctgacgtgt	
<i>sun668-F</i>	tagaagggtgggtgctaga	1,267
<i>sun668-R</i>	gctctgacgatgcgcaata	
<i>sun669-F</i>	tattgcgacatcgtcagagc	1,288
<i>sun669-R</i>	gacacgtcgatccgggtactt	

respectively. Five allele-specific primers *sunKASP_57* (*sun667*), *sunKASP_58*, *sunKASP_59*, *sunKASP_60*, and *sunKASP_61* (*sun669*) were developed (Table 5; Fig. 3). These markers were tested on the parents to check allele clustering. Two markers, *sunKASP_59* and *sunKASP_60*, produced well-differentiated clusters and were tested on both RIL populations and incorporated into the map.

SSR markers previously reported on the short arm of chromosome 6B were also used. Markers *wmc487*, *gwm133*, *gwm193*, and *cfld13* showed polymorphism among three parental genotypes but did not show association with *LrAW2* in both RIL populations. The linkage maps of the *LrAW2* region included seven markers (five SSR and two KASP) in the Aus26582/Bansi and five markers (three SSR and two KASP) in the Aus26579/Bansi population (Fig. 2). Marker *sunKASP_60* was mapped 0.2 and 0.6 cM proximal to *LrAW2*, whereas *sunKASP_59* was mapped 1.5 and 2.9 cM proximal to *LrAW2* in Aus26582/Bansi and Aus26579/Bansi RIL populations, respectively.

Validation of *LrAW2*-linked markers. Markers *sun683*, *sun684*, and *sunKASP_60* were genotyped on 11 Australian durum cultivars (Table 6). All of these cultivars were susceptible to the race CA 1.2 and produced amplicons or haplotypes different from those associated with *LrAW2* for three markers. These results indicated the effectiveness of markers *sun683*, *sun684*, and *sunKASP_60* for MAS of *LrAW2* in durum breeding programs.

DISCUSSION

Although Australia is geographically separated from all other wheat-growing continents, incursions of exotic rust races have occurred (Kolmer 2005; Park et al. 2002; O'Brien et al. 1980; Wellings et al. 2003). The two separate detections of *P. striiformis* f. sp. *tritici* (causal organism of wheat stripe rust) in 1979 in eastern Australia (O'Brien et al. 1980) and in 2002

Fig. 3. Graphical presentation of simple sequence repeat, sequence-tagged site, and kompetitive allele-specific polymerase chain reaction (KASP) markers developed from the chromosome survey sequence (CSS) contig 6BS_2963854.

TABLE 5. Development of kompetitive allele-specific polymerase chain reaction (KASP) assays from the single-nucleotide polymorphisms (SNP) detected between the parental sequences

STS marker ^a	Marker name	Allele 1 primer ^b	Allele 2 primer ^c	Common primer	SNP
<i>sun667</i>	<i>sunKASP_57</i>	ctactaaagactccacaaccacaa	ctactaaagactccacaaccacac	gccgtagcagtcattgaag	[A/C]
<i>sun669</i>	<i>sunKASP_58</i>	ccctctacaacaccataattgg	gccctctacaacaccataattga	tttatcagggtgccatggt	[G/A]
<i>sun669</i>	<i>sunKASP_59</i>	gtccaccaattctacatctga	gtccaccaattctacatctgc	atgaggctgggtaggagag	[A/C]
<i>sun669</i>	<i>sunKASP_60</i>	cactgaagcttccggat	cactgaagcttccggac	ggatgatcgtcggtagg	[T/C]
<i>sun669</i>	<i>sunKASP_61</i>	ctatgcgcgtcctgtgg	gctatgcgcgtcctgtgt	cattggcaccactataatca	[T/G]

^a STS = sequence-tagged site.

^b A1 primer labeled with FAM: GAAGGTGACCAAGTTCATGCT.

^c A2 primer labeled with HEX: GAAGGTCGGAGTCAACGGATT.

Fig. 4. Amplicons produced by markers *sun684* (left) and *sun683* (right) in genotypes: lane 1, Guayacan carrying *Lr61*; lane 2, Aus26582; lane 3, Aus26579; and lane 4, the susceptible control Bansi; lane L = 1-kb DNA ladder (right).

TABLE 6. Validation of markers closely linked with *LrAW2* among Australian durum wheat cultivars

Australian wheat	IT (CA 1.2) ^a	<i>sun684</i> (bp)	<i>sun683</i> (bp)	<i>sunKASP_60</i>
Aus26582, Aus26579	;	124	160	T:T
Bansi	3+	128	Null	C:C
Bellaroi	3+	128	Null	C:C
Kalka	3+	128	Null	C:C
Arrivato	3+	128	Null	C:C
Caparoi	3+	128	Null	C:C
DBA-Aurora	Not tested	128	Null	C:C
Gundaroi	3+	128	Null	C:C
Jandaroi	3+	128	Null	C:C
Tjilkuri	3+	128	Null	C:C
Tamaroi	3+	128	Null	C:C
WID802	3+	128	Null	C:C
Wollaroi	3+	128	Null	C:C

^a Infection type.

in Western Australia, arguably from two different geographic regions (Wellings et al. 2003), clearly show the international spread of rust pathogens and, hence, the introduction of durum-specific *P. tritricina* races is perhaps only a matter of time. Current Australian durum wheat cultivars are resistant to all prevalent Australian *P. tritricina* races and are susceptible to durum-specific races of Mexico and Ethiopia (H. Bariana and U. Bansal, unpublished).

Transfer of genetic variation from landraces and wild relatives is crucial to increase genetic diversity among modern wheat germplasm (Bariana et al. 2013; Randhawa et al. 2014; Soriano et al. 2016). Aus26582 and Aus26579 selected in this study appeared to carry the same gene (*LrAW2*) for resistance to *P. tritricina* races CA 1.2 and ETH 12.5-2 based on molecular mapping results and their effectiveness against durum-specific *P. tritricina* races. The closely linked markers *sun683* (dominant), *sun684* (codominant), and *sunKASP_60*, developed in this study, will facilitate MAS of *LrAW2* to prepare against the introduction of durum-specific races in Australia and other nations.

Leaf rust resistance gene *Lr61* has also been mapped in the chromosome 6BS of a Chilean wheat 'Guayacan INIA' (Guayacan) by Herrera-Foessel et al. (2008) using amplified fragment length polymorphism (AFLP) markers. Guayacan produces low IT ;1 to ;12 against Mexican *P. tritricina* races BBG/BN, NCJ/BN, and MCJ/SP and X⁺ against the race BBB/BN. The use of AFLP markers for mapping *Lr61* and DArTseq-derived markers in the current study did not allow a direct comparison of map locations in these investigations. Markers *sun683*, *sun684*, and *sunKASP_60* were tested on Guayacan (DNA kindly provided by Dr. Mandeep Randhawa, CIMMYT INT, Mexico). Guayacan produced amplicons or haplotypes similar to Aus26582 and Aus26579 (Fig. 4), suggesting that *LrAW2* and *Lr61* may represent the same locus; however, the possibility of either close linkage or the allelic relationship

between these genes cannot be ignored. *Lr61* was effective against prevalent durum-specific Mexican *P. tritricina* races, except a variant of an old race, BBB/BN (Herrera-Foessel et al. 2014), and it is still effective against European and Ethiopian races and the durum-specific *P. tritricina* races from the United States. Tests on Aus26582 and Aus26579 against six Australian common wheat *P. tritricina* races also suggested its effectiveness in Australia. No durum-specific *P. tritricina* race has yet been detected in Australia; however, previous incursions of foreign races of all three rust pathogens in Australia (O'Brien et al. 1980; Park 2007; Park et al. 2002; Wellings et al. 2003) stress the need to perform anticipatory breeding for resistance against durum-specific races.

LrAW2 is not present in any of the current Australian durum wheat cultivars. All Australian durum cultivars carried *sun683*, *sun684*, and *sunKASP_60* alleles alternate to those linked with *LrAW2*, indicating their effectiveness for MAS. Leaf rust resistance genes *Lr23* and *LrWo*, present either singly or in combination in some Australian durum wheat cultivars, are effective against predominant common wheat *P. tritricina* races in Australia. The adult plant resistance gene *Lr46* is also present in some Australian durum wheat cultivars (U. Bansal and H. Bariana, unpublished). Markers closely linked with *LrAW2* will be useful in its pyramiding with other marker-tagged resistance genes for preemptive breeding against the durum-specific races in Australia and elsewhere. Overall, markers developed in this study will be useful for both low-throughput (SSR) prebreeding and high-throughput (SNP) commercial breeding programs.

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