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Lr68: a new gene conferring slow rusting resistance to leaf rust in wheat

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Abstract The common wheat cultivar Parula possesses a high level of slow rusting, adult plant resistance (APR) to all three rust diseases of wheat. Previous mapping studies using an Avocet-*YrA*/Parula recombinant inbred line (RIL) population showed that APR to leaf rust (*Puccinia triticina*) in Parula is governed by at least three independent slow rusting resistance genes: *Lr34* on 7DS, *Lr46* on 1BL, and a previously unknown gene on 7BL. The use of field rust reaction and flanking markers identified two F_6 RILs, Arula1 and Arula2, from the above population that lacked *Lr34* and *Lr46* but carried the leaf rust resistance gene in 7BL, hereby designated *Lr68*. Arula1 and Arula2 were crossed with Apav, a highly susceptible line from the cross Avocet-*YrA*/Pavon 76, and 396 F_4-derived F_5 RILs were developed for mapping *Lr68*. The RILs were phenotyped for leaf rust resistance for over 2 years in Ciudad Obregon, Mexico, with a mixture of *P. triticina* races MBJ/SP and MCJ/SP. Close genetic linkages with several DNA markers on 7BL were established using 367 RILs; *Psy1-1* and *gwm146* flanked *Lr68* and were estimated at 0.5 and 0.6 cM, respectively. The relationship between *Lr68* and the race-specific seedling resistance gene *Lr14b*, located in the same region and present in Parula, Arula1 and Arula2, was investigated by evaluating the RILs with *Lr14b*-avirulent *P. triticina* race TCT/QB in the greenhouse. Although *Lr14b* and *Lr68* homozygous recombinants in repulsion were not identified in RILs, γ-irradiation-induced deletion stocks that lacked *Lr68* but possessed *Lr14b* showed that *Lr68* and *Lr14b* are different loci. Flanking DNA markers that are tightly linked to *Lr68* in a wide array of genotypes can be utilized for selection of APR to leaf rust.

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Introduction

Wheat, one of humankind's important staple foods, is grown on about 225 m ha worldwide. The three rust diseases of wheat caused by *Puccinia triticina*, *P. striiformis*, and *P. graminis* are the most important biotic constraints to wheat production. The most effective and environmentally sound method to control these diseases is through the deployment of resistant cultivars. Although a number of rust resistance genes have been identified in wheat (McIntosh et al. 2010), a major problem has been their short-lived effectiveness due to the fast emergence of virulent races of the pathogen that are capable of overcoming the resistance. However, at least four designated resistance genes, *Lr34/Yr18*, *Lr46/Yr29*, *Sr2/Yr30*, and *Lr67/Yr46*,

have conferred partial but durable resistance for a long period of time (Singh et al. 2000a; Hiebert et al. 2010; Herrera-Foessel et al. 2011; Singh et al. 2011). These genes confer a slow rusting type of resistance (Caldwell 1968) despite a compatible host reaction and are effective across all races of the pathogen. Slow rusting or retarded disease progress in the field results from a longer latent period, lower receptivity, smaller pustule size, and lower spore production when carefully quantified under controlled conditions and can be measured in the field by a smaller area under the disease progress curve or lower final disease severity compared to a susceptible check (Ohm and Shaner 1976; Wilcoxson 1981; Das et al. 1993). The additive gene action of this type of gene results in increased levels of resistance when combined, reaching what has been described as near immunity (Singh et al. 2000b; Lillemo et al. 2011). The pleiotropic action of these genes on other diseases such as powdery mildew caused by *Blumeria graminis* make them additionally valuable for breeding for broad-spectrum resistance (Lillemo et al. 2008; Mago et al. 2011).

Although only a few slow rusting genes have been cataloged, they seem to be common in bread wheat (*Triticum aestivum*); a dozen of these genes for leaf rust resistance were estimated to be present in CIMMYT bread wheat germplasm based on allelism tests (Singh et al. 2000b), and at least 23 QTLs (in chromosome arms 1BS, 1BL, 1DS, 2AL, 2BS, 2DS, 2DL, 3AL, 3BS, 3BL, 4AL, 4BS, 4BL, 4DL, 5AS, 5BL, 5DL, 6AL, 6BL, 7BS, 7BL, 7DL, 7DS) associated with durable or slow rusting resistance have been described in the literature (William et al. 1997; Nelson et al. 1997; Faris et al. 1999; Messmer et al. 2000; Suenaga et al. 2003; Schnurbusch et al. 2004, Navabi et al. 2005; Xu et al. 2005a; William et al. 2006; William et al. 2007; Rosewarne et al. 2008; Chu et al. 2009; Singh et al. 2009). Fine mapping of slow rusting APR genes remains a challenge as their often small individual effects pose difficulties in phenotyping when present alone. Cloning of the slow rusting gene *Lr34/Yr18/Pm38* (Krattinger et al. 2008) allowed a better understanding of the genetic nature of this gene and the development of gene-based DNA markers (Lagudah et al. 2009) to facilitate breeding for durable resistance in the presence of major genes or in environments that are not optimal for selection of this trait.

The spring bread wheat Parula (pedigree: FKN/3/2*Frontana//Kenya 350 AD.9C.2/Gabo 55/4/Bluebird/Chanate) displays high levels of durable APR to all three wheat rusts worldwide (Singh et al. 2011). Singh and Rajaram (1992) postulated the presence of race-specific resistance gene *Lr13* in Parula by testing its seedlings in the greenhouse with 12 Mexican *P. triticina* races. Field trials with *Lr13*-virluent races TCB/TD and TBD/TM indicated the presence of the slow rusting resistance gene *Lr34*. F_3 families from crosses with the susceptible parent Yecora 70 carrying *Lr13* and allelism tests on Frontana and RL6058 (a tester for *Lr34*) showed that Parula's APR was conferred by three additive genes, one of which was *Lr34* (Singh and Rajaram 1992).

William et al. (1997) identified that in addition to *Lr34*, QTLs on chromosome arms 1BS, 1DS and 7BL were associated with leaf rust resistance in Parula. Their analysis used Recombinant Inbred Lines (RILs) from a cross between Parula and the moderately susceptible Siete Cerros, with random amplified polymorphic DNAs (RAPD) and restriction fragment length polymorphism (RFLP) markers identified through bulked segregant analysis and subsequently mapped using nullitetrasomic and ditelosomic stocks of Chinese Spring. Leaf tip necrosis (LTN) was assumed to be contributed by *Lr34* on 7DS, and the two associated markers in 7BL explained most of the effect of leaf rust resistance in the population. Both Parula and Siete Cerros carry *Lr46*; therefore it did not segregate in this study.

An additional study to characterize the genetic loci associated with resistance to leaf and yellow rust in Parula was conducted using an F_6 RIL population derived from the cross with the susceptible parent Avocet-*YrA* (William et al. 2007; Lillemo et al. 2011). Bulked segregant analysis identified 181 polymorphic AFLPs, SSRs, and RFLP markers in the population. Simple and composite interval mapping revealed two QTLs associated with both leaf and yellow rust resistance on chromosome arms 7DS and 1BL, confirming the presence of known slow rusting genes *Lr34/Yr18* and *Lr46/Yr29* in Parula. Two additional QTLs were identified, one for leaf rust resistance on chromosome arm 7BL and another for yellow rust resistance on chromosome arm 3BS, identified as *Sr2/Yr30*.

The Thatcher near-isogenic line (RL6006) carrying *Lr14b*, located on chromosome arm 7BL, was reported to display APR in Australia (McIntosh et al. 1995) and India (Sawhney et al. 1992) with races virulent to *Lr14b*, indicating that either the *Lr14b* allele or a closely linked gene conferred adult plant resistance.

The objectives of our study were: (1) to characterize the slow rusting resistance gene, hereby designated as *Lr68*, located on chromosome arm 7BL of Parula, as a simple Mendelian trait and identify closely linked DNA markers; and (2) to establish the relationship between *Lr68* and race-specific resistance gene *Lr14b* located in the same region.

Materials and methods

Development of a mapping population segregating for single APR gene *Lr68*

Two F_6 RILs, Arula1 and Arula2 (CIMMYT GID 1847450 and 1847422) were selected from the Avocet-*YrA*/Parula

mapping population (William et al. 2007). These RILs had positive alleles for markers linked to the APR QTL on 7BL but lacked positive alleles for markers linked to *Lr46/Yr29* on 1BL (*Xgwm259*) and *Lr34/Yr18* on 7DS (*Xgwm295/Xgwm130*). These were subsequently verified with the gene-based marker for *Lr34/Yr18* (Lagudah et al. 2009). The two RILs also lacked strong leaf tip necrosis, and showed higher leaf rust responses compared to lines carrying either *Lr46* or *Lr34*, but lower severity responses than the susceptible parent. Arula1 and Arula2 were crossed with a highly susceptible F_6 RIL Apav (GID 1853706) derived from the Avocet-*YrA*/Pavon 76 mapping population. When used as the susceptible parent, Apav had shown higher susceptibility to leaf rust and yellow rust in field trials than Avocet-*YrA*. The 396 F_4-derived F_5 RILs, 198 from each cross, were generated by harvesting random spikes from each F_2 plant, thereafter growing hills (from 10 to 15 kernels) of each derived family, harvesting one spike in the F_3 and F_4 generations, and finally harvesting each F_5 generation hill plot as a bulk.

Phenotypic characterization of leaf rust resistance in the field

Field evaluations for leaf rust resistance of the parents and the 396 Arula × Apav F_4-derived F_5 RILs were conducted at CIMMYT's research station in Cd. Obregon, Mexico, during the 2007–2008 and 2008–2009 crop seasons. Approximately 80 seeds of each of the parents and RILs were grown as paired 1 m rows, 20 cm apart, on 75 cm wide raised beds. Spreaders of the susceptible cultivar Morocco were planted as hills in the middle of a 0.5 m pathway on one side of each plot and all around the experimental field. The spreaders were inoculated thrice by spraying urediniospores of the two Mexican *P. triticina* races MCJ/SP and MBJ/SP suspended in lightweight mineral oil Soltrol 170 (Chempoint.com) about 8 weeks after sowing. The avirulence/virulence formula of MCJ/SP is *Lr2a,2b,2c,3ka,9,16,19,21,24,25,28,29,30,32,33,36/1,3,3bg,10,11,12,13,14a,14b,15,17a,18,20,23,26,27+31*. The virulence formula of MBJ/SP is the same as that of MCJ/SP, except that it is only partially virulent to *Lr26*. Both races are virulent to seedling leaf rust resistance genes *Lr14a* and *Lr14b*, also located on chromosome 7BL (McIntosh et al. 1995), and to *Lr13*, which is present in all of the original parents that were used in developing the single gene population (Parula, Avocet-*YrA*, and Pavon 76) (Singh and Rajaram 1991, 1992).

Leaf rust severity on the parents was scored in the field using the modified Cobb Scale (Peterson et al. 1948) and host response to infection, as described in Roelfs et al. (1992). The 396 RILs were classified into three phenotypic categories (homozygous resistant, homozygous susceptible, and segregating) when the susceptible parent displayed 90–100% leaf rust severity. Timing of the phenotypic evaluation was important because the effect of this slow rusting resistance gene was relatively small. Repeated observations were made to assure that each RIL was correctly classified. Some RILs and the parents were re-evaluated at Cd. Obregon during the 2009–2010 and 2010–2011 seasons to further confirm their responses. Planting and inoculation procedures were the same, except that only one *P. triticina* race, MBJ/SP, was used.

Comparison of APR conferred by *Lr68* and other slow rusting genes

Trials were also established at Cd. Obregon during the 2008–2009, 2009–2010, and 2010–2011 crop seasons to compare the effects of *Lr68* with those of other designated slow rusting leaf rust resistance genes present in various backgrounds (Table 1). Planting and inoculation procedures were the same as described above for phenotyping of the mapping population. Leaf rust severity and host response to infection were recorded in the same manner as that described for the parents of the population. The leaf rust responses of Avocet-*YrA*, Apav and the two Arula parents recorded during the 1996–1997 and 1997–1998 seasons are also included in Table 1.

Characterization of *Lr68* and *Lr14b* in the greenhouse

The parents and the differential set carrying known seedling resistance genes mostly in a Thatcher background were characterized in the greenhouse at the seedling stage with races MCJ/SP and MBJ/SP, also used in field trials. Comparisons were made between the infection types of the parents and those of Thatcher (*Lr22b*), *Lr1* (RL6003), *Lr14a* (RL6013), *Lr14b* (RL6006), and Manitou (*Lr13*).

The 396 Apav × Arula RILs, the parents, the leaf rust differential set, and several additional varieties reported to carry *Lr14b* or a QTL on chromosome 7BL (Table 2) were evaluated at the seedling stage with the *Lr14b*-avirulent *P. triticina* race TCT/QB. Seed of the *Lr14ab* (RL6039) tester and a second *Lr14b* seed source was provided by RA McIntosh, the University of Sydney. The avirulence/virulence formula of race TCT/QB is: *Lr9,10,14b,15,16,18,19,21,23,24,27+31,36/1,2a,2b,2c,3a,3bg,3ka,11,13,14a,17a,17b,26,30,32,33*.

About ten kernels per line were sown in plastic trays as hills and inoculated 10 days after planting at the 2-leaf stage, as described in Singh (1991), using an atomizer with urediniospores suspended in Soltrol 170. Plants were placed in a dew chamber overnight and then transferred to a greenhouse with minimum/maximum and average temperatures of 15.3/28.4 and 21.8°C, respectively. Infection

Table 1 Seedling (2-leaf stage) greenhouse infection type to *Puccinia triticina* races MCJ/SP and MBJ/SP and adult plant leaf rust response recorded at Cd. Obregon, Mexico, for the two slow rusting resistant parents carrying *Lr68*, Arula1 and Arula2, the susceptible parent, Apav, and testers for different genes and checks

Entry	*P. triticina* race and seedling infection type response[a,b]		Year, field leaf rust severity, and host response to infection[c]					
	MCJ/SP	MBJ/SP	1996–1997	1997–1998	2007–2008	2008–2009	2009–2010	2010–2011
Avocet-*YrA*	–	–	90	100	90S	80S	100S	90S
Apav	3+	3+	100	100	100S	100S	100S	100S
Lr68 (Arula1)	3+	3+	30	60	15MSS	50MSS	50MSS	10MS
Lr68 (Arula2)	3+	3+	40	50	20MSS	40MSS	–	–
Lr34 (YR18/3*Avocet-*YrA*)	–	–	–	–	–	10MSS	1MS	5MS
Lr46 (Avocet-*YrA**3//Lalbmono1*4/ Pavon)	–	–	–	–	–	30MSS	15MS	20MS
*Lr67 (RL6077/*Avocet-*YrA)*	–	–	–	–	–	–	1MSS	10MS
Lalbahadur	–	–	–	–	–	100S	100S	100S
Lr34 (Lalb/Prl7D)	–	–	–	–	–	10MSS	5MSS	5MS
Lr46 (Lalbmono1*4/Pavon)	–	–	–	–	–	30MSS	20MSS	20MS
Parula (*Lr34+Lr46+Lr68*)	–	–	1	5	–	1MS	1MS	1MS
Pavon F76 (*Lr46+*)	–	–	–	–	–	10MSS	10MSS	15MS
Thatcher (*Lr22b)*	4	4	–	–	–	–	–	–
Lr1 (RL6003)	3+	4	–	–	–	–	–	–
Manitou (*Lr13*)	4	4	–	–	–	–	–	–
Lr14a (RL6013)	4	4	–	–	–	–	–	–
Lr14b (RL6006)	4	4	–	–	–	–	–	–

[a] Infection types are based on a '0–4' scale (Roelfs et al. 1992), where '0' = no uredinia or other macroscopic signs of infection; ';' = no uredinia, but hypersensitive necrotic or chlorotic flecks of varying sizes present; '1' = small uredinia surrounded by necrosis; '2' = small to medium uredinia surrounded by green islands; 'X' = random distribution of variable-sized uredinia on a single leaf with a pure culture; '3' and '4' = medium and large uredinia, respectively, without chlorosis or necrosis; '+' and '−' = uredinia somewhat larger and smaller, respectively, than normal for infection type

[b] Post-inoculation temperature: min = 15.3°C, max = 28.4°C, average = 21.8°C

[c] Artificial epidemics in field evaluations were initiated with *Puccinia triticina* race MCJ/SP and MBJ/SP in all years except the last two, when only MBJ/SP was used. Leaf rust severity was scored on adult plants using the modified Cobb Scale (Peterson et al. 1948), and host response to infection was evaluated as described in Roelfs et al. (1992), where 'R' = resistant or miniature uredinia surrounded by necrosis and chlorosis, 'MR' = moderately resistant or small uredinia surrounded with chlorosis or necrosis, 'MS' = moderately susceptible or moderate-sized uredinia without chlorosis or necrosis, and 'S' = susceptible or large uredinia without chlorosis and necrosis

types were recorded 10 days after inoculation using the 0–4 scale as described in Roelfs et al. (1992).

Molecular mapping and linkage analysis

Leaf tissues of the parents, RILs, checks and other wheat varieties were harvested for DNA extraction either from the field plots 8 weeks after sowing or from seedlings grown in the greenhouse. Leaves were lyophilized and ground, and a cetyltrimethylammonium bromide (CTAB) method was used for DNA extraction (CIMMYT 2005). SSR markers on chromosome arm 7BL (Somers et al. 2004) and those in the *Lr14a* region (Herrera-Foessel et al. 2008) were tested on the parents. Entries of the population were genotyped with markers found to be polymorphic. The microsatellite assay procedure was performed according to established protocols (CIMMYT 2005), adjusting for $MgCl_2$ and primer concentration. The polymerase chain reaction (PCR) program was adjusted based on the optimum annealing temperature for each marker. The PCR product was loaded on 12% acrylamide (29:1) gels for a better definition of the bands. Visualization of the bands was achieved using silver staining for acrylamide gels (CIMMYT 2005). CAPS marker *Psy1-1* (Pozniak et al. 2007) and STS marker *PsyB1* (Zhang and Dubcovsky 2008), both associated with yellow pigment in the endosperm, were also applied to genotype the parents and RILs for saturating the 7BL region. Marker *PsyB1* gave multiple banding alleles in the population that were different from the parents and was therefore not further used in any

Table 2 Seedling (2-leaf stage) infection type of the *Lr68*-carrying parents (Arula1 and Arula2) and the susceptible parent (Apav) used for the development of the single gene-based mapping population and comparison with testers and other lines when inoculated with the *Lr14b*-avirulent *P. triticina* race TCT/QB

Line	Infection type[a,b]
Thatcher	3+
Siete Cerros	3+
Lr14b (RL6006)	X
Lr14ab (RL6039)	X
Lr68 (Arula1)	X
Lr68 (Arula2)	X
Maria Escobar (*Lr14b*)	X
CI13227	X
Weebill 1 (*Lr14b*)	X
Lr10 (RL6004)	;
Apav	;
Avocet-*YrA*	;
Pavon 76	;
Parula 81	;1−
Pastor	;
Brambling	;
Attila	;1−
Frontana	X−
Saar	;
Alpowa	;12
Colosseo (DW)	;

[a] Infection type according to the 0–4 scale described by Roelfs et al. (1992) where '0' = no uredinia or other macroscopic signs of infection; ';' = no uredinia, but hypersensitive necrotic or chlorotic flecks of varying sizes present; '1' = small uredinia surrounded by necrosis; '2' = small to medium uredinia surrounded by green islands; 'X' = random distribution of variable-sized uredinia on a single leaf with a pure culture; '3' and '4' = medium and large uredinia, respectively, without chlorosis or necrosis; '+' and '−' = uredinia somewhat larger and smaller, respectively, than normal for infection type. More than one designation represents a range of infection types

[b] Post-inoculation temperature: min = 15.3°C, max = 28.4°C, average = 21.8°C

analysis. Two additional markers, *csGS* and *cs7BLNLRR*, developed by comparative genomics of the synteny between *Brachypodium* and the 7L region of wheat, were also used. The primer sequence of STS marker *csGS* is F1 5′AAGATTGTTCACAGATCCATGTCA 3′ and R1 5′ GAGTATTCCGGCTCAAAAAGG 3′, and the annealing temperature is 60°C (expected size about 385 bp). The primer sequence of the CAPS marker *cs7BLNLRR* is F 5′GAAGGAGTGCTTCCTCCACTG 3′ and R1 5′ CTTGG TTCTCCTGTTCTTCCC 3′, with an annealing temperature of 60°C. The PCR product of the CAPS marker was digested by adding a premix of 0.5 µl *HaeIII* restriction enzyme, 2 µl of 10 × restriction buffer, and 8 µl of water, and then incubated at 37°C for 60 min on the PCR thermocycler. Polymorphic fragments of 738, 478 and 270 bp were obtained (Fig. 1).

A total of 367 RILs (RILs with missing values for genes or markers were excluded) were used to establish a linkage map of *Lr68* and associated markers on chromosome arm 7BL. ICImapping 3.1 (http://www.isbreeding.net) was used to generate the map at minimum 3.0 log of odds (LOD). Distances were calculated using the Kosambi mapping function, and marker ordering was validated by three different ordering algorithms [Seriation, Record and Multi Fragment algorithm (MF)]. The rippling command was used for fine-tuning the order of the markers in the linkage maps. The linkage map was drawn using MapChart (Voorrips 2002).

The Apav × Arula population also segregated for race-specific resistance gene *Lr10* in tests with *P. triticina* race TCT/QB, and the presence of *Lr14b* could not be determined in lines carrying *Lr10*. To investigate the association of *Lr68* with *Lr14b*, RILs carrying *Lr10* were excluded from further analysis. The presence of *Lr10* in RILs based on low infection type with *P. triticina* race TCT/QB was corroborated with the *Lr10*-linked marker, *Lrk10-6* (Schachermayr et al. 1997).

Chi-squared statistical tests were used to determine goodness-of-fit of the observed ratios of phenotypic and marker responses with expected ratios for a single gene model. A total of 370 and 188 RILs of the Apav × Arula population were used for the Chi-squared tests for *Lr68* and *Lr14b*, respectively. Lines with inconclusive phenotypic responses were excluded from the analysis.

Development and phenotyping of deletion mutants for *Lr68* and *Lr14b*

Mutagenesis of Arula1 (GID 1847450) was conducted by γ-irradiation using a ^{60}Co source at CSIRO Plant Industry,

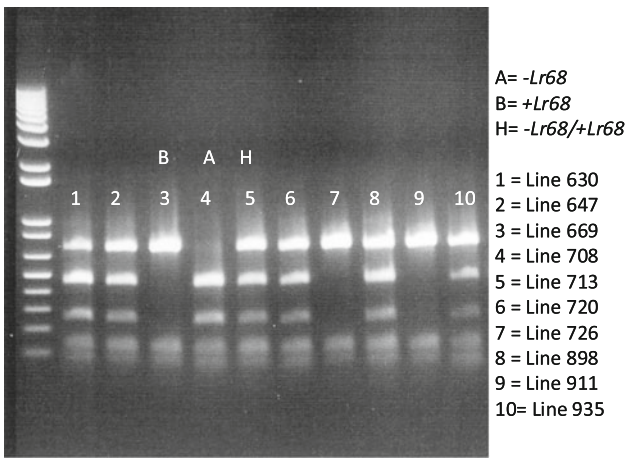

Fig. 1 Co-dominant CAPS marker *cs7BLNLRR* associated with *Lr68* when evaluated on 10 RILs from the Apav × Arula population

Canberra, Australia. The dosage selected based on LD_{50} was 25 krads. The M_1 plants were grown during 2009 at El Batan, Mexico, and 2,000 were harvested. Twenty seeds of each of the 2,000 M_2 lines and susceptible check Apav were sown 10 cm apart in paired row plots 1 m in length with a 0.5 m pathway in the field in Ciudad Obregon, Mexico, during the 2009–2010 crop season. Planting of spreader rows and inoculation procedures were the same as described above for phenotyping of the mapping population. M_2 families containing one or more susceptible plants were identified. Ten plants, plants identified to be susceptible and several resistant plants from each of the families, were harvested to obtain M_3 progenies. The M_3 lines and susceptible check Apav were grown in the field in El Batan and Cd. Obregon during the 2010 and 2010–2011 seasons, respectively, under leaf rust epidemics was artificially initiated with race MBJ/SP. Each line was classified as homozygous susceptible, segregating or homozygous resistant. These M_3 lines (about 12 kernels per line) and the leaf rust differential set were evaluated twice in the greenhouse at the 2-leaf stage with the *Lr14b*-avirulent race TCT/QB to detect the presence or absence of *Lr14b*. Infection type responses were recorded 10 days post inoculation based on the 0–4 Scale. Leaf tissue of each M_3 line (about 20 plants) was also harvested for DNA extraction as previously described. The M_3 lines were confirmed to be susceptible; their resistant sibs and the *Lr68* parent were screened with markers linked to *Lr68* on chromosome arm 7BL and also with random markers from other chromosomes to investigate the size of the deletion and exclude any possibility of outcrossing or contamination.

Haplotyping of lines carrying *Lr68* or other genes reported in 7BL

The four most closely linked markers to *Lr68* in 7BL, *csGS*, *cs7BLNLRR*, *Psy1-1*, *gwm146*, and two additional markers, *gwm344* and *wmc526,* monomorphic in the parents for the *Lr68* population, but previously reported to be associated with *Lr14a* (Herrera-Foessel et al. 2008; Maccaferri et al. 2008), were used to genotype wheat lines with *Lr68* and wheat lines with *Lr14a* or *Lr14b*. Haplotype data from these markers were used to investigate whether lines carrying *Lr68* can be differentiated from those with either *Lr14a* or *Lr14b*. Lines previously reported to carry a QTL for slow rusting in the same region (Table 3) were also included, together with susceptible checks.

Results

Characterization of *Lr68* in the greenhouse and field

The two *Lr68*-carrying parents, Arula1 and Arula2, and the susceptible Apav parent displayed compatible or high infection types (infection type '3+') in seedling stage when tested with *P. triticina* races MCJ/SP and MBJ/SP used in field evaluations (Table 1). In the same greenhouse test, Thatcher (*Lr22b*), *Lr1* (RL6003), *Lr14a* (RL6013), *Lr14b* (RL6006) and Manitou (*Lr13*) displayed infection type '4' or '3+'.

In the field, parents Arula1, Arula2, and Apav displayed moderately susceptible to susceptible host responses to leaf rust infection with *P. triticina* races MCJ/SP and MBJ/SP (Table 1). The *Lr68*-carrying Arula1 and Arula2 exhibited slow rusting resistance in the field and showed final leaf rust severities ranging from 10 to 60 MSS, depending on the crop season, when susceptible parent Apav or Avocet-*YrA* reached 100S (Table 1). The effect of *Lr68* was smaller than those of *Lr34*, *Lr46* and *Lr67*, except for 2010–2011, when the effect of *Lr68* was larger than that of *Lr46* and comparable to the effect of *Lr67* (Table 1; Figs. 1S, 2S). The combined effect of the three slow rusting genes *Lr34*, *Lr46* and *Lr68* in the Parula background resulted in near immunity in all years of evaluation (Table 1, Fig. 2S). Post-flowering LTN was observed on *Lr68* parents and RILs but was much smaller than the LTN expressed by *Lr34*, *Lr67* or *Lr46* (Fig. 1S).

Segregation for *Lr68* in 370 Apav × Arula F_4-derived F_5 RILs conformed to the expected ratio for segregation of a single resistance gene (Table 4). Several RILs had to be re-scored for multiple seasons to establish the phenotypic classes, given that the effect of *Lr68* was small and it was sometimes challenging to distinguish between homozygous resistant and segregating, or between homozygous susceptible and segregating categories.

Identification of linked markers and development of the linkage map

Genetic linkage was established between *Lr68* and nine markers (*gwm577, wmc273, barc182, wmc232, cfa2257b, csGS, cs7BLNLRR, Psy1-1* and *gwm146*) positioned on chromosome arm 7BL (Fig. 2). Two markers, *Psy1-1* at 0.5 cM, and *gwm146* at 0.6 cM, were identified on each side of the *Lr68* locus. Two other markers, *gwm344* and *wmc526*, previously reported to tag *Lr14a* (Herrera-Foessel et al. 2008; Maccaferri et al. 2008) and distally located from *Lr68* flanking markers, were monomorphic on the parents and displayed the banding allele that was not associated with the seedling gene *Lr14a* (Table 3).

Haplotyping of lines carrying *Lr68* or other genes reported in 7BL

Five haplotype groups (Table 3) could be distinguished using the three *Lr68*-associated markers, *csGS, cs7BLNLRR* and *Psy1-1,* and the *Lr14a*-associated markers *gwm344* and

Table 3 Marker haplotypes in various wheat lines using *Lr68* and *Lr14a* linked markers from chromosome arm 7BL

Line	Marker, group and marker allele					
	csGS	*cs7BLNLRR*	*Psy1-1*	*gwm146*	*wmc526*	*gwm344*
	Lr68+Lr14b					
Lr14b (RL6006)	B	B	B	C	A	A
Lr68 (Arula1)	B	B	B	E	A	A
Parula	B	B	B	E	A	A
Frontana	B	B	B	B	A	A
Amadina	B	B	B	B	A	A
Weebill 1	B	B	B	B	A	A
Maria Escobar	B	B	B	C	.[a]	A
	Lr14ab					
Lr14ab (RL6039)	B	B	B	C	.	B
	Lr14a					
Lr14a (RL6013)	A	A	A	B	B	B
Alpowa	A	A	A	B	B	B
Attila	A	A	A	B	B	B
Brambling	A	A	A	B	B	B
Saar	A	A	A	B	B	B
Opata	A	A	A	B	B	B
Pastor	A	A	A	B	B	B
Colosseo (DW)	A	A	A	B	B	B
Llareta (DW)	A	A	A	B	B	B
	Susceptible					
Thatcher	A	A	A	A	A	A
Avocet-*YrA*	A	A	A	D	A	A
Apav	A	A	A	D	A	A
Synthetic	A	.	A	D	A	A
Atil C2000 (DW)	A	A	A	E	A	A
	Other					
Fukuho-komugi	A	A	A	D	B	A
Oligoculm	A	A	A	E	B	A

[a] '.'= missing data

wmc526. Marker *gwm146* was multi-allelic and did not contribute to distinguishing the haplotypes (Table 3). The *Lr68*-carrying lines and the genetic stocks/cultivars previously reported to carry *Lr14b* could not be distinguished from each other based on the marker genotype but could be distinguished from *Lr14a*-carrying lines and susceptible checks. The marker response of Oligoculm and Fukuho-komugi was somewhat different compared to the other groups. A QTL in 7BL had previously been reported to reduce stripe rust in Oligoculm × Fukuho-komugi population (Suenaga et al. 2003) and the two parents were therefore included in the haplotyping study.

Identification of race-specific resistance gene *Lr14b* in greenhouse seedling tests

The Apav × Arula RILs segregated for two resistance genes, *Lr14b* and *Lr10,* with race TCT/QB. *Lr10* originated from the susceptible parent Apav and was identified by the very low infection type ';' (Fig. 3S). *Lr14b* originated from Arula1 and Arula2 and conferred the mesothetic reaction 'X'. The *Lr10*-linked marker, *Lrk10-6*, confirmed the presence of *Lr10*. Lines that were homozygous resistant for *Lr10* were excluded from further analysis, since the presence of *Lr14b* could not be determined in these lines. The remaining 188 lines that were either segregating, susceptible or homozygous resistant for *Lr14b* were used to investigate the relationship between *Lr68* and *Lr14b*. The segregation of *Lr14b* in these lines, without exception, coincided with the response of *Lr68* identified using *Lr14b* (and *Lr10*) virulent races in the field, indicating that there was no recombination between these loci in the 188 lines tested. The resistance reaction conformed to the segregation ratio expected for a single gene (Table 4). Additional lines reported to carry *Lr14a*, *Lr14b,* or a QTL for slow rusting resistance on chromosome arm 7BL, were evaluated in the same experiment with race TCT/QB (Table 2). Maria Escobar, Weebill 1 and CI13327 had the same

Table 4 Goodness of fit tests for single gene model for *Lr68* and *Lr14b* phenotypic data for F_4-derived F_5 families of Arula × Apav population

	Observed frequency for each category[a]			Expected frequency for each category[ab]			*P* value for χ^2 test
	HR	*Seg*	*HS*	*HR*	*Seg*	*HS*	
Lr68[c]							
Apav × Arula1	69	31	86	81	23	81	0.09
Apav × Arula2	78	24	82	81	23	81	0.93
Overall	147	55	168	162	46	162	0.20
Lr14b[d]							
Apav × Arula1	44	17	51	49	14	49	0.54
Apav × Arula2	38	12	35	37	11	37	0.85
Overall	79	28	81	82	24	82	0.60

[a] *HR* Homozygous resistant, *Seg* Segregating, *HS* Homozygous susceptible

[b] The expected frequency of one gene in F_4-derived F_5 is 0.4375 (*HR*):0.125 (*Seg*):0.4375 (*HS*)

[c] Based on phenotypic evaluations in the field with *P. triticina* race MCJ/SP and MBJ/SP during the 2007–2008 and 2008–2009 crop cycles in Ciudad Obregon, Mexico, and after re-evaluation of some lines in 2009–2010 and 2010–2011

[d] Based on evaluations conducted in seedlings (2-leaf stage) in the greenhouse with *P. triticina* race TCT/QB. Lines carrying *Lr10* were subtracted from the analysis since the response to *Lr14b* could not be detected in these lines

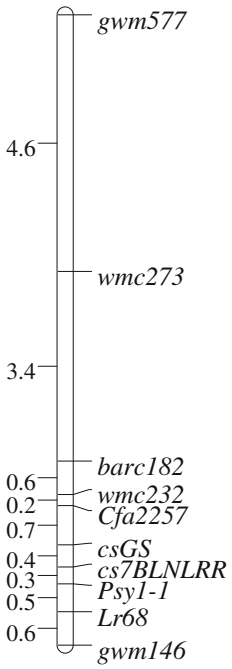

Fig. 2 Genetic linkage map of the slow rusting resistance gene *Lr68* and relative distance to markers located on chromosome arm 7BL based on 367 F_4-derived F_5 lines from the Apav × Arula population. Distances are shown in cM

infection types as *Lr14b*. Other lines included in the test had lower infection types, which could indicate the presence of *Lr10* or other genes that may have masked the effect of *Lr14b*. Therefore, the presence of *Lr14b* in these lines could not be determined solely based on the infection type.

Identification of mutants for *Lr68* and *Lr14b*

Mutants from eight independent events were identified among M_3 progenies derived from 2,000 M_1 plants based on susceptible and resistant responses of the sibs in the field with race MBJ/SP (Table 5). These mutants, originating from different M_1 plants, are presumed to carry deletion fragments of different sizes in the *Lr68* region. The deletion events were confirmed by mutants that had lost flanking markers expected of larger deletions and mutants that retained markers *csGS, cs7BLNLRR, Psy1-1* and *gwm146* (Table 5). The latter are presumed to be either small interstitial deletions or putative point mutations in the *Lr68* gene. Based on seedling infection types of the mutants with *P. triticina* race TCT/QB and adult plant resistance in the field with race MBJ/SP, three mutants carried *Lr14b* but lacked *Lr68*, indicating that *Lr68* and *Lr14b* are different loci (Table 5).

Discussion

Our studies have resulted in the mapping and identification of a new slow rusting APR gene, *Lr68*, on chromosome arm 7BL of Parula. The RIL mapping population where *Lr68* segregated as a simple Mendelian trait allowed us to develop a more precise linkage map for the genomic region harboring *Lr68* and linked DNA markers (Fig. 2). *Lr68* was mapped to a specific gene-rich area on chromosome 7BL between the locus associated with yellow endosperm color (*Psy1-1*) and marker *gwm146*. Furthermore, we were able to confirm that the *Lr68* locus is different from previously reported loci carrying the race-specific resistance genes *Lr14a* and *Lr14b*. The position of *Lr68* did not correspond to the previously mapped *Lr14a* locus that maps 7.5 cM distal to *gwm146* (Herrera-Foessel et al. 2008). Although homozygous repulsion recombinants between *Lr68* and *Lr14b* could not be found in the

Table 5 Gamma-irradiation-induced deletion stocks identified that lack *Lr68*, or both *Lr68* and *Lr14b*, CIMMYT identification number (GID), and their response to associated markers

GID	Origin	Flanking markers				Response field (MBJ/SP)	Greenhouse 2-leaf (TCT/QB)
	Different M_1	*CsGs*	*7BLNRR*	*Psy1-1*	*gwm146*	Presence of *Lr68*	Presence of *Lr14b*
6346367	A	I[a]	I	I	I	S (no *Lr68*)	R (*Lr14b*)
6346544	B	D[b]	D	D	I	S (no *Lr68*)	S (no *Lr14b*)
6346773	C	D	D	D	I	S (no *Lr68*)	S (no *Lr14b*)
6346891	E	D	D	D	I	S (no *Lr68*)	SEG (*Lr14b/no Lr14b*)
6347059	F	I	I	I	I	S (no Lr68)	S (no *Lr14b*)
6347060	F	I	I	I	I	S (no *Lr68*)	S (no *Lr14b*)
6347120	G	I	I	I	I	S (no *Lr68*)	R (*Lr14b*)
6347140	H	I	I	I	I	S (no *Lr68*)	S (no *Lr14b*)
6347141	H	I	I	I	I	S (no *Lr68*)	S (no *Lr14b*)
6347455	I	I	I	I	H[c]	S (no *Lr68*)	R (*Lr14b*)

[a] Marker intact

[b] Marker deleted

[c] Marker allele heterozygous

Apav × Arula RIL population, we identified three deletion mutants, originating from different M_1, that lacked *Lr68* but possessed *Lr14b*.

Lr14b, a race-specific resistance gene, conferred a mesothetic reaction on seedlings to race TCT/QB. This resistance gene is ineffective against most races worldwide and was at first thought to be an allele of *Lr14a*. However, recombinants that carry both genes were identified by Dyck and Samborski (1970) and showed that *Lr14a* and *Lr14b* were located at different loci. *Lr14b* was originally transferred to Thatcher from the South American cultivar Maria Escobar. It is possible that Maria Escobar also carries the closely linked gene *Lr68*, which as a result was transferred along with *Lr14b* during back-crossing with Thatcher. This would explain why a Thatcher near-isoline for *Lr14b* was reported to display APR in field trials with races virulent to *Lr14b* (Sawhney et al. 1992; McIntosh et al. 1995). The Thatcher near-isoline that carries both *Lr14a* and *Lr14b* was also reported to display APR (Sawhney et al. 1992). Zhang et al. (2011) recently mapped another race-specific leaf rust resistance gene, likely to be different from *Lr14a* and *Lr14b,* in the same region in the Chinese wheat cultivar Bimai 16. The relationship between *Lr68* and this undesignated gene will require further investigation. QTLs for slow rusting APR in this 7BL region have been reported in various studies involving different populations; however, the relationship between *Lr68* and these QTLs will also require further study.

The origin of *Lr68* is likely to be the Brazilian wheat cultivar Frontana, which appears in the pedigree of Parula and various other CIMMYT wheats. Frontana is known for its APR to leaf rust based on the interaction of slow rusting APR gene *Lr34* and 2–3 additional unidentified slow rusting genes (Singh and Rajaram 1992). Frontana showed similar marker haplotypes when genotyped with markers closely linked to *Lr68* and *Lr14b*. An allelism test between Frontana and Parula, conducted by Singh and Rajaram (1992), indicated that these cultivars carried *Lr34* and other gene(s) in common. It is likely that other CIMMYT lines, such as Weebill 1, also carry *Lr68*, as Zhang et al. (2008a) reported the association of *Lr14b* with one of the APR genes present in Weebill 1. Weebill 1 and Amadina, a parent of Weebill 1, had the same marker haplotypes as *Lr68* and *Lr14b* in our study. *Lr14b* had a much lower infection type, 'fleck', with the two *Lr14b* avirulent races in the Zhang et al. (2008a) study, which was conducted at cooler temperatures, indicating the temperature sensitivity of this gene (McIntosh et al. 1995). A QTL for leaf rust resistance was identified in the same genomic region in the Avocet-*YrA* × Amadina population by Nyori (2010). Xu et al. (2005a, b) identified a QTL for slow rusting leaf rust resistance on chromosome arm 7BL in winter wheat CI13327 that was also associated with a longer latent period in the greenhouse. CI13327 showed a similar infection type as *Lr14b* in our greenhouse tests with *P. triticina* race TCT/QB and, therefore, likely carries closely linked gene *Lr68*.

Other lines reported to carry QTLs for slow rusting resistance to leaf rust on 7BL that were included in our haplotyping study were Attila (Rosewarne et al. 2008) and Opata (Faris et al. 1999). These lines displayed lower infection types than expected for *Lr14b* with the *Lr14b*-avirulent race TCT/QB, indicating that they possessed other seedling resistance genes. Hence the presence of

Lr14b in Attila and Opata could not be determined. Their marker haplotypes were similar to those of the *Lr14a* testers, of Brambling, another *Lr14a*-carrying bread wheat (Zhang et al. 2008b), and of durum wheats Llareta INIA (Herrera-Foessel et al. 2008) and Colosseo (Maccaferri et al. 2008). Results were similar for Alpowa and Saar, which are reported to possess a QTL for non-race-specific resistance to stripe rust or powdery mildew in similar genomic regions (Lin and Chen 2007; Lillemo et al. 2008). Suenaga et al. (2003) also reported a QTL for stripe rust resistance on 7BL in the Oligoculm × Fukuho-komugi population but the marker genotypes were somewhat different from any of the haplotype groups in our study. In addition to these studies, Messmer et al. (2000) and Schnurbusch et al. (2004) have also identified, in the same region of winter wheat Forno, a QTL for APR to leaf rust associated with LTN, which could indicate the presence of *Lr68*.

Precise information on the environmental effects and the interaction of slow rusting resistance genes will allow breeders to utilize them in optimal combinations to provide protection across diverse environments. In our study, we found that in Ciudad Obregon, Mexico, the effect of *Lr68* on leaf rust resistance was smaller than the effect of *Lr34*, *Lr67*, or *Lr46* (Fig. 1S). However, *Lr68* showed stronger effects than *Lr46* in the 2010–2011 season at Ciudad Obregon (Fig. 2S). This season was unusually cool compared to other years, indicating that *Lr68* may express better at lower temperatures. However, high and stable levels of resistance were obtained in Mexico when *Lr68* was combined with *Lr34* and *Lr46* in Parula. Zhang et al. (2008a) suggested that the APR gene in 7BL present in Weebill 1 showed stronger effects at higher temperatures, which is contradictory to our observations with *Lr68*.

Lillemo et al. (2011) determined the effect of *Lr68* (previously designated *LrP*), *Lr34* and *Lr46* on leaf rust in the Avocet-*YrA* × Parula F_6 RIL population using linked markers in nine field environments in Mexico, Brazil, Argentina, Uruguay and Chile. Compared to *Lr34*, the effect of *Lr68* was stronger at sites in Uruguay and Argentina. The additive effects of *Lr68* in combination with slow rusting genes *Lr34* and *Lr46* were confirmed at each site, indicating the value of *Lr68* for breeding durable and stable APR to leaf rust.

For marker-assisted selection of *Lr68*, we recommend the co-dominant marker *cs7BLNLRR* positioned at 0.8 cM or the dominant marker *csGS* at 1.2 cM from the gene (Fig. 2). The *csGS* marker was used in diagnosing *Lr68* in the crossing block of CIMMYT's bread wheat breeding program. *Lr68* was found to be more common than *Lr34* and the pedigrees of positive lines could often be traced back to Parula, Weebill 1 and Rayon F89 (results not shown). *Psy1-1*, which was found to be more closely linked to the gene, is not recommended for marker-assisted selection due to the difficulty in achieving consistent amplification. As SSR marker *gwm146* is multi-allelic, finding the correct *Lr68*-associated band can be problematic.

The γ-irradiation-induced deletion stocks identified in this study that lack *Lr68*, or both *Lr68* and *Lr14b* (Table 5), and the recombinants identified between the flanking markers in the Apav × Arula RIL population will be useful for fine mapping and cloning *Lr68* to develop a diagnostic gene-based marker. *Lr68* was also associated with slight LTN (Fig. 1S), smaller than that associated with other known slow rusting genes, which indicates that *Lr68* and *Lr34* may share a common defense mechanism. The multiple or broad-spectrum disease resistance conferred by slow rusting genes *Lr34* and *Lr46* implies an added value for breeding. The effect of *Lr68* on other diseases needs further investigation and deletion mutants can be highly valuable in such studies.

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