Theor Appl Genet (2011) 122:239–249
DOI 10.1007/s00122-010-1439-x

ORIGINAL PAPER

New slow-rusting leaf rust and stripe rust resistance genes *Lr67* and *Yr46* in wheat are pleiotropic or closely linked

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Received: 5 May 2010 / Accepted: 25 August 2010 / Published online: 17 September 2010

Abstract The common wheat genotype 'RL6077' was believed to carry the gene *Lr34/Yr18* that confers slow-rusting adult plant resistance (APR) to leaf rust and stripe rust but located to a different chromosome through inter-chromosomal reciprocal translocation. However, haplotyping using the cloned *Lr34/Yr18* diagnostic marker and the complete sequencing of the gene indicated *Lr34/Yr18* is absent in RL6077. We crossed RL6077 with the susceptible parent 'Avocet' and developed F_3, F_4 and F_6 populations from photoperiod-insensitive F_3 lines that were segregating for resistance to leaf rust and stripe rust. The populations were characterized for leaf rust resistance at two Mexican sites, Cd. Obregon during the 2008–2009 and 2009–2010 crop seasons, and El Batan during 2009, and for stripe rust resistance at Toluca, a third Mexican site, during 2009. The F_3 population was also evaluated for stripe rust resistance at Cobbitty, Australia, during 2009. Most lines had correlated responses to leaf rust and stripe rust, indicating that either the same gene, or closely linked genes, confers resistance to both diseases. Molecular mapping using microsatellites led to the identification of five markers (*Xgwm165*, *Xgwm192*, *Xcfd71*, *Xbarc98* and *Xcfd23*) on chromosome 4DL that are associated with this gene(s), with the closest markers being located at 0.4 cM. In a parallel study in Canada using a Thatcher × RL6077 F_3 population, the same leaf rust resistance gene was designated as *Lr67* and mapped to the same chromosomal region. The pleiotropic, or closely linked, gene derived from RL6077 that conferred stripe rust resistance in this study was designated as *Yr46*. The slow-rusting gene(s) *Lr67/Yr46* can be utilized in combination with other slow-rusting genes to develop high levels of durable APR to leaf rust and stripe rust in wheat.

Communicated by B. Keller.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1439-x) contains supplementary material, which is available to authorized users.

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Introduction

Leaf (or brown) rust and stripe (or yellow) rust, caused by *Puccinia triticina* and *P. striiformis*, respectively, are important constraints to common wheat (*Triticum aestivum*) production in most parts of the world. Growing resistant cultivars is the most effective and environmentally sound strategy for controlling rust diseases. Over 60 leaf rust and 40 stripe rust resistance genes are cataloged in wheat (McIntosh et al. 2008). Most of these resistance genes are race-specific and therefore succumb to new variants of the respective pathogen soon after their deployment. In contrast, slow-rusting resistance characterized by slow disease progress in the field despite a compatible host reaction (Caldwell 1968) is known to be durable. Although slow-rusting resistance genes have small-to-intermediate effects when present alone, high levels of resistance have been achieved by combining 4–5 such genes (Singh et al. 2000a). The slow-rusting gene *Lr34/Yr18*, located on chromosome arm 7DS, has provided durable resistance to leaf rust and stripe rust since the early twentieth century (Dyck 1977; 1987; Singh 1992a). *Lr34/Yr18* also confers resistance to powdery mildew (*Blumeria graminis*) (Spielmeyer et al. 2005), stem rust (*Puccinia graminis tritici*) (Dyck 1987), and barley yellow dwarf virus (Singh 1993). A morphological characteristic of *Lr34/Yr18* is its association with leaf tip necrosis (LTN) in adult plant stage, which can be used to identify it in certain environments (Singh 1992b). Recent cloning of *Lr34/Yr18* provided important information on its gene structure, which encodes a putative ATP-binding cassette (ABC) transporter and enabled the development of gene-based markers that facilitated the identification of *Lr34/Yr18* in different wheat backgrounds (Krattinger et al. 2009; Lagudah et al. 2009). Another gene that has a dual effect and confers slow-rusting resistance to leaf rust and stripe rust is *Lr46/Yr29*, which has also been shown to provide durable resistance in gene combinations (Singh et al. 2005).

'RL6077' (Tc*6/PI250413) is a near-isogenic line of 'Thatcher' that carries a slow-rusting leaf rust resistance gene transferred from accession PI250413 collected in Pakistan (Dyck and Samborski 1979). This gene was confirmed to be present in wheat accessions from Iraq, Afghanistan, and India, based on allelism tests indicating that it is not uncommon in germplasm from that region (Dyck and Samborski 1979; Shang et al. 1986). The leaf rust resistance gene in RL6077 also had an effect on stripe rust and was associated with LTN in the field (Dyck and Samborski 1979; Singh 1992a; Dyck et al. 1994). Similarities between this gene and *Lr34* led Dyck et al. (1994) to conclude that the resistance gene in RL6077 was likely to be *Lr34*, even though it is inherited independently. One of the reasons behind this conclusion was the presence of quadrivalents in pollen mother cells of F_1 hybrids of RL6077 and 'RL6058' (an *Lr34/Yr18* near-isogenic line) in cytological examinations indicating that *Lr34/Yr18* could have been translocated in a reciprocal manner to a different chromosome in RL6077 (Dyck et al. 1994). The presence of *Lr34/Yr18* in a Thatcher background is known to exhibit increased stem rust resistance to a number of isolates (Dyck 1987). A similar observation of the gene in RL6077 against stem rust was another contributing factor that led Dyck et al. (1994) to postulate the presence of *Lr34/Yr18* in RL6077. Until recently, this hypothesis was considered untenable; however, recent analysis of the diagnostic region in RL6077 from the ABC transporter encoded by the *Lr34/Yr18* gene showed the absence of *Lr34/Yr18* (Lagudah et al. 2009). Consequently, these results have prompted the mapping of the leaf rust and stripe rust resistance gene(s) in RL6077.

In a parallel study conducted with Canadian leaf rust isolates, Hiebert et al. (2010) mapped the leaf rust resistance in RL6077 to chromosome 4D from two populations (Thatcher × RL6077 and RL6058 × RL6077), leading to the designation of this gene as *Lr67*. In our study, we confirmed their finding using a different cross and a larger population, and investigated the genetic association of *Lr67* with stripe rust resistance, thereby providing evidence for a co-segregating gene designated as *Yr46* on chromosome arm 4DL. We also show through the comparison of the complete sequence of the ABC transporter gene at the *Lr34/Yr18* locus that RL6077 definitely does not carry the haplotype associated with *Lr34/Yr18*.

We also tested Thatcher, RL6077 (Thatcher + *Lr67/Yr46*), RL6058 (Thatcher + *Lr34/Yr18*) and 90RN2491 (Thatcher + *Lr34/Yr18* + *Lr67/Yr46*) against Ug99 stem rust in field tests in Kenya, since increased resistance of Thatcher and Thatcher derivatives in the presence of the *Lr34/Yr18* gene against the highly virulent Ug99 strain has been reported (Gavin Vanegas et al. 2008) but the effect of *Lr67/Yr46* in Thatcher against Ug99 was unknown.

Materials and methods

Development of mapping populations

An initial F_3 mapping population at CSIRO, Canberra, Australia, was generated by crossing RL6077 with a selection of the susceptible cultivar Avocet (Avocet S). This F_3 population was used for preliminary screening for leaf rust resistance at Cobbitty, Australia, during 2007; a subset (22 lines) of this population was also evaluated at Cd. Obregon, Mexico, during the 2007–2008 crop season. The F_3 population segregated for photoperiod sensitivity, and two different populations—an F_5 population with an F_4

segregation ratio (hereafter called F_4) developed at CIMMYT, and an F_4 population with an F_3 segregation ratio (hereafter called F_3) developed at CSIRO—were subsequently generated using selected photoperiod-insensitive F_3 lines. To develop the F_4 populations of 148 lines, two photoperiod-insensitive F_3 lines that were segregating for resistance were bulk harvested and F_4 plants space-planted for individual harvesting. To develop the F_3 population of 136 lines at CSIRO, leftover seeds of F_3 lines that were segregating according to previous phenotypic evaluations were space-planted and individually harvested. The F_4 population developed at CIMMYT was further advanced for two generations, and 148 recombinant inbred lines (RILs) with an F_6 genetic ratio were developed.

Field evaluations for characterizing leaf rust and stripe rust resistance

The Avocet × RL6077 F_3 and F_4 populations from CIMMYT and CSIRO, respectively, were evaluated for leaf rust response in Cd. Obregon, Mexico, during two crop seasons (2008–2009 and 2009–2010). The F_4 population was also evaluated for leaf rust at El Batan and for stripe rust in Toluca, Mexico, during the 2009 summer season, and the F_3 population was evaluated for stripe rust resistance at Cobbitty, Australia, during the same year. The RILs (F_6 Avocet × RL6077 population) were evaluated for leaf rust resistance in Cd. Obregon during the 2009–2010 crop season.

The 148 F_4 and the 136 F_3 lines of Avocet × RL6077, together with the parents, were grown in Cd. Obregon during the 2008–2009 crop season on different sowing dates (23 November 2008 and 21 January 2009, respectively) and during the 2009–2010 crop season on the same sowing date (18 November 2009). The 148 RILs were sown in Cd. Obregon during the 2009–2010 crop season on 5 December 2009. Plots of 1-m double rows with about 60 plants per line were grown for each population, and spreader rows of the susceptible variety 'Morocco' were planted around the experimental area. Morocco was also grown as hill plots on one side of each experimental plot in the middle of a 0.5-m pathway. To initiate the leaf rust epidemics, spreader rows and hills were inoculated using hand-sprayers containing urediniospores of two pathotypes of *P. triticina*; MCJ/SP and MBJ/SP, suspended in Soltrol Oil (Phillips 66 Co., Bartlesville, OK, USA). In 2008–2009, spreaders and hills around the F_4 population were inoculated on 21 and 23 January 2009, whereas spreaders and hills for the F_3 population were inoculated on 20 February of that same year. In the 2009–2010 crop cycle, the same inoculation dates (18 and 25 January 2010) were used for all populations. Disease severity on parents was scored according to the modified Cobb Scale where percentage of rusted tissue was visually estimated according to Peterson et al. (1948) and host response to infection was determined according to Roelfs et al. (1992), where for leaf rust, 'R' indicated resistant or miniature uredinia surrounded by necrotic tissue, 'MR' indicated moderately resistant or smaller to moderate-sized uredinia surrounded by necrotic or chlorotic tissue, 'MS' indicated moderately susceptible or moderate-sized uredinia without necrotic or chlorotic tissues, and 'S' indicated susceptible or large uredinia without necrotic or chlorotic tissue. Rust response assessments were performed when the susceptible parent Avocet reached 100% leaf rust severity. Lines in each population were scored as homozygous resistant (HR), homozygous susceptible (HS), and segregating (SEG), based on their phenotypic response to leaf rust. In 2009–2010 we evaluated each F_4 and F_6 line to obtain a score for mean leaf rust severity.

The F_4 Avocet × RL6077 population was sown in Toluca and El Batan on 25 and 23 May 2009, respectively. The same plot sizes were used as described earlier for the Cd. Obregon trials. In Toluca, the spreaders consisted of a mixture of six susceptible wheat lines derived from the cross 'Avocet × Attila' known to carry the *Yr27* stripe rust resistance gene. Mexican *P. striiformis* isolates Mex96.11 and Mex08.13 were used to inoculate spreaders and hills four times (on 16, 17, 24 and 30 June) to assure the development of the disease epidemic in Toluca. In El Batan, the spreader rows consisted of a mixture of Morocco and two other leaf rust susceptible wheat lines that were inoculated with *P. triticina* (a mix of pathotypes MCJ/SP and MBJ/SP) on 18 and 22 June 2009. The F_4 lines in El Batan were directly inoculated together with the spreaders. The percentage stripe rust severity (Peterson et al. 1948) and the host response to infection (Roelfs et al. 1992) were recorded on the parents, where 'R' indicated resistant with necrotic/chlorotic stripes without sporulation, 'MR' indicated moderately resistant with necrotic/chlorotic stripes with some sporulation, 'M' (or 'MRMS') was necrotic/chlorotic stripes with intermediate to abundant sporulation, 'MS' indicated moderately susceptible with chlorotic/occasionally necrotic stripes with abundant sporulation, and 'S' indicated stripes with or without chlorosis and with abundant sporulation. The F_4 lines were scored as HR, HS, and SEG for leaf rust at El Batan and for stripe rust at Toluca, when the susceptible parent Avocet was showing 100S response. The minimum and maximum plant severity response for stripe rust was also recorded for each of the 148 F_4 lines at Toluca.

The Avocet × RL6077 F_3 population was evaluated for stripe rust resistance under field conditions using a mixture of *P. striiformis* strains 134E16A+J+*Yr27*+ and 134E16A+*Yr17*+ inoculated on spreader wheat rows at Cobbitty, Australia, during 2009. The F_3 lines were scored as HR, HS, and SEG response to stripe rust.

Field evaluation of RL6077 against stem rust in Kenya

Wheat genotypes Thatcher, RL6077, RL6058, and 90RN2491—a derivative from the RL6077 × RL6058 cross combining both *Lr34/Yr18* and *Lr67/Yr46* (Dyck et al. 1994)—were grown in field trials in Njoro, Kenya, in 2009. The prevalent Ug99 derivative race TTKST of *P. graminis tritici* with virulence for resistance gene *Sr24* was used to inoculate spreader rows in the field. Stem 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' indicated resistant or miniature uredinia surrounded by necrosis and chlorosis, 'MR' indicated moderately resistant or small uredinia surrounded with chlorosis or necrosis, 'MS' indicated moderately susceptible or moderate-sized uredinia without chlorosis or necrosis, and 'S' indicated susceptible or large uredinia without chlorosis and necrosis.

Comparison of *Lr67/Yr46* with other known slow-rusting resistance genes in field and greenhouse tests

Field evaluations and greenhouse tests were conducted on adult plants to investigate the expression of *Lr67/Yr46* and compare it with the expression of known slow-rusting resistance genes *Lr34/Yr18* and *Lr46/Yr29*. Plots of single gene lines carrying *Lr34/Yr18* (*Yr18*/3*Avocet) and *Lr46/Yr29* (Avocet-*YrA**3//Lalbmono1*4/Pavon) were grown in Cd. Obregon during the 2009–2010 crop season and in Toluca in 2009 adjacent to lines from the Avocet × RL6077 populations; leaf and stripe rust responses were recorded when Avocet displayed 100S severity response.

Greenhouse tests were conducted on RL6077 (*Lr67/Yr46*), RL6058 (*Lr34/Yr18*), Avocet-*YrA**3//LalbMono1*4/Pavon (*Lr46/Yr29*), Avocet, and two lines from the F_3 Avocet × RL6077 population that were HR for the *Lr67/Yr46* gene. Eight pots (4 plants/pot) of each genotype were used for the evaluation. Recently emerged, fully expanded flag leaves were inoculated with *P. triticina* pathotypes MCJ/SP and MBJ/SP, and *P. striiformis* pathotypes Mex96.11 and Mex08.13. Each race was evaluated separately. Inoculations were conducted by spraying urediniospores suspended in Soltrol oil using an atomizer. Inoculated plants were placed in a dew chamber overnight (*P. triticina*) or for two nights (*P. striiformis*) and then transferred to a greenhouse. A data logger (LogTag analyzer, ver. 1.9®) was installed in the greenhouse and programmed to measure the greenhouse temperature every 15 min during the time from when the plants were transferred to the greenhouse after dew exposure to the time when responses were recorded. Minimum, maximum, and average post inoculation temperatures for the leaf rust and stripe rust greenhouse tests were 10, 26, and 18°C, and 10, 25, and 17°C, respectively. Leaf rust infection type was recorded based on a 0–4 Scale (Roelfs et al. 1992) at 11 days post-inoculation, where infection types '0' = no visible symptoms, ';' = necrotic/chlorotic flecks, '1' = small uredinia surrounded by necrosis, '2' = small to medium uredinia surrounded by chlorosis or necrosis, 'X' = random distribution of variable-sized uredinia, '3' = medium-sized uredinia without chlorosis, '4' = large-sized uredinia without chlorosis, '+' and '−' were somewhat larger or smaller uredinia than normal for the infection type. Infection type '3' and '4' were considered susceptible while all other infection type was considered resistant. Responses for stripe rust were scored based on a 0–9 Scale (McNeal et al. 1971) at 20 days post-inoculation, where infection type '0' = no visible infection, '1' = necrotic/chlorotic flecks without sporulation, '2' = necrotic/chlorotic stripes without sporulation, '3' = necrotic/chlorotic stripes with trace sporulation, '4' = necrotic/chlorotic stripes with light sporulation, '5' = necrotic/chlorotic stripes with intermediate sporulation, '6' = chlorotic stripes with moderate sporulation, '7' = chlorotic stripes with abundant sporulation, '8' = stripes without chlorosis, moderate sporulation, '9' = stripes without chlorosis and abundant sporulation. Infection types '6' and '7' were considered moderately susceptible, whereas '8' and '9' were considered susceptible, and all other infection type were considered resistant.

Molecular mapping of *Lr67/Yr46*

Initial genome-wide scans to identify associated simple sequence repeat (SSR) markers were conducted at the Department of Primary Industries, Victorian AgriBiosciences Center, Australia, using a subsample of the original F_3 Avocet × RL6077 population. Leaf tissue from the parents, seven HR lines and seven HS lines was harvested and DNA extracted according to the method described by Lagudah et al. (1991b). A collection of SSR markers distributed on all wheat chromosomes was used for screening. Polymerase chain reaction (PCR) products were separated by capillary electrophoresis with an ABI3730xl instrument; allele sizes were determined using GeneScan software, version 3.7 (Applied Biosystems) (Hayden et al. 2008). A total of six SSR markers, *Xbarc98*, *Xbarc288*, *Xcfd23*, *Xcfd71*, *Xwmc48*, and *Xwmc89* from chromosome 4D differentiated the HR and susceptible genotypes and the parents. To establish genetic linkage and relative marker order with the *Lr67/Yr46* locus, markers were first screened on the 148 lines of the Avocet × RL6077- derived F_4-lines at CIMMYT's biotechnology laboratory, and a final linkage map was then generated using the 148 RILs. About 30 seeds per F_4 and F_6 line were grown in the greenhouse and leaf tissues harvested. DNA was extracted according to a CTAB

procedure, and PCR reactions were conducted based on standard methods (CIMMYT 2005) with annealing temperatures according to available information for each marker in graingenes database (http://wheat.pw.usda.gov/GG2/index.shtml). The PCR products were separated on 12% acrylamide gels (29:1), and silver staining was used to visualize the amplification products (CIMMYT 2005). The same markers were also screened in a subset of lines from the F_3 Avocet × RL6077 population at CSIRO using the DNA fragment analyzer ABI3730xl. Chromosome 4D short- and long-arm deletion lines, 4DS-01(fragment length FL0.53), 4DS-03 (FL0.67), 4DS-05 (FL0.63), 4DL-09 (FL0.31), 4DL-06 (FL0.38), 4DL-13 (FL0.56), 4DL-12 (FL0.71), and 4DL-14 (FL0.86) (kindly supplied by Dr. TR Endo, Japan), were used to determine the physical location of markers linked closely with *Lr67/Yr46*.

Analysis of *Lr34* ABC transporter and homoeologs in RL6077

The total gene sequence was determined to ensure no additional variants were present at the *Lr34/Yr18* locus in RL6077 that could confer *Lr67/Yr46* resistance. D-genome-specific primers that amplify the *Lr34/Yr18* ABC transporter sequence on wheat chromosome 7D (Krattinger et al. 2009) were used to generate PCR products from RL6077. Full coverage of the corresponding *Lr34/Yr18* allele in RL6077 was obtained and compared against the reference *Lr34/Yr18* haplotype. Restriction fragment length polymorphism (RFLP) analysis of the homoeologs of the *Lr34/Yr18* ABC transporter were investigated using the *Lr34/Yr18* cDNA fragment as a probe on membrane filters containing DNA of RL6077, Thatcher and the complete set of nullitetrasomic stocks of Chinese Spring. Genomic DNA of Thatcher and RL6077 were restricted with 14 restriction endonucleases (*Acc*I, *Bam*HI, *Bg*III, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*II, *Nde*I, *Nco*I, *Nsi*I, *Pst*I, *Xba*I, *Xho*I). DNA transfer and hybridization conditions were as described in Lagudah et al. (1991a).

Linkage and statistical analysis

MAPMAKER/EXP version 3.0 (Lander et al. 1987) at minimum log of odds (LOD) of 3.0 was used for the linkage analysis. The Kosambi mapping function was used to calculate the genetic distances. MapChart (Voorrips 2002) was used for drawing the genetic linkage map of the associated markers and *Lr67/Yr46*. The χ^2 test was used to test the goodness of fit of observed segregation with expected ratios for the genotypic classes for all markers and rust response phenotypes.

Results

Characterization of leaf rust and stripe rust resistance in field and greenhouse

Displayed in Table 1 are the field responses of RL6077 (*Lr67/Yr46*); the two single gene lines carrying *Lr34/Yr18* and *Lr46/Yr29*; the average leaf rust and stripe rust responses of HR lines of the Avocet × RL6077 populations; and the susceptible line Avocet. Since RL6077 was very late maturing, especially in the Cd. Obregon field site due to its day-length sensitivity, responses could only be recorded on the lower leaves. The average response of the HR lines therefore allows a better comparison between *Lr67/Yr46* and the two known slow-rusting genes, *Lr34/Yr18* and *Lr46/Yr29*, since the populations were developed from photoperiod-insensitive lines. *Lr34/Yr18* displayed a slightly lower leaf rust severity compared with the average response of the homozygous *Lr67/Yr46* lines, whereas their stripe rust responses were similar in the field. *Lr46/Yr29* showed higher leaf rust and stripe rust responses than *Lr67/Yr46* and *Lr34/Yr18*. The *Lr67/Yr46* lines showed strong LTN associated with resistance comparable to the LTN manifested by the *Lr34/Yr18*-carrying line, whereas the line carrying *Lr46/Yr29* showed weaker LTN, as is usually observed in Mexican environments (Fig. S1).

In the greenhouse adult plant test with *P. triticina* races, the Thatcher derivative with *Lr34/Yr18* (RL6058) initially displayed somewhat lower responses than lines carrying *Lr46/Yr29* and *Lr67/Yr46* (Table 2). However, this difference was less evident in a later scoring (results not shown). RL6058 displayed similar, or slightly lower, stripe rust responses compared with RL6077, whereas *Lr46/Yr29* showed higher responses with the more aggressive race Mex08.13. *Lr34/Yr18* is known to express better in lower temperatures (Dyck and Samborski 1982), whereas the temperature effect on *Lr67/Yr46* and *Lr46/Yr29* is unknown. The average temperature for both leaf rust and stripe rust greenhouse test was 18 and 17°C, respectively, and use of different (lower and higher) temperature regimes in the future would provide additional information on the comparative expression of these slow-rusting resistance genes. In our study, the stripe rust response of each slow-rusting gene seems to have been influenced by the cultivar background, since the two HR lines from the Avocet × RL6077 population showed higher responses than RL6077, and Thatcher displayed lower responses than Avocet to isolate Mex08.13 of *P. striiformis* (Fig. S2).

Table 1 Leaf rust and stripe rust responses of RL6077 (RL6077-*Lr67/Yr46*), HR lines carrying *Lr67/Yr46* from the Avocet × RL6077 F_4 and F_6 populations (average response) (*Lr67/Yr46*), *Yr18*/3*Avocet (*Lr34/Yr18*), Avocet-*YrA**3//LalbMono1*4/Pvn (*Lr46/Yr29*), and Avocet when evaluated in Mexican sites under artificially created epidemics with two *Puccinia triticina* races at Cd Obregon during 2009–2010 crop cycle and with two *P. striiformis* races at Toluca in 2009

Genotype	Pathogen (race) and field response[a]	
	P. triticina (MCJ/SP + MBJ/SP)	*P. striiformis* (Mex96.1 + Mex08.13)
RL6077-Lr67/Yr46	1 MS[b]	1 M[b]
Lr67/Yr46	5 MS	30 M
Lr34/Yr18	1 MS	30 M
Lr46/Yr29	15 MS	40 MSS
Avocet	100 S	100 S

[a] Field responses follow the Modified Cobb Scale (Peterson et al. 1948) and host response to infection as described in Roelfs et al. (1992)

[b] Responses of flag-1 and flag-2 leaves

Table 2 Greenhouse infection type responses for leaf rust and stripe rust recorded on flag leaves of RL6077 (RL6077-*Lr67/Yr46*), two HR F_3 lines of the Avocet × RL6077 population (*Lr67/Yr46*), RL6058 (*Lr34/Yr18*), Avocet-*YrA**3//LalbMono1*4/Pvn (*Lr46/Yr29*), Avocet and Thatcher after inoculation with two *Puccinia triticina* (MCJ/SP and MBJ/SP) and two *P. striiformis* (Mex96.11 and Mex08.13) races

Genotype	Pathogen and infection type responses			
	P. triticina[a]		*P. striiformis*[b]	
	MCJ/SP	MBJ/SP	Mex96.11	Mex08.13
RL6077-Lr67/Yr46	3+	3+	67	7
Lr67/Yr46 (1)	3+	3+	8	78
Lr67/Yr46 (2)	3+	3+	89	78
Lr34/Yr18	3	33+	6	67
Lr46/Yr29	3+	3+	67	9
Avocet	3+	3+	89	9
Thatcher	3+	3+	8	78

[a] The infection type responses for leaf rust followed the 0–4 Scale as described in Roelfs et al. (1992) where infection types '3' and '4' are considered high or susceptible, and '+' or '−' are somewhat larger or smaller uredinia, respectively, than normal for the infection type; more than one designation represents a range of infection types. Plants were maintained at minimum, maximum, and average temperatures of 10, 26, and 18°C, respectively, and responses were recorded 11 days post-inoculation

[b] The infection type responses followed the 0–9 Scale as described by McNeal et al. (1971), where infection type '6 'and '7' are considered moderately susceptible, whereas '8' and '9' are considered susceptible; more than one designation represents a range of infection types. Plants were maintained at minimum, maximum and average temperatures of 10, 25, and 17°C, respectively, and responses were recorded 20 days post-inoculation

Distribution of phenotypic categories in Avocet × RL6077 populations

In all leaf rust and stripe rust evaluations in Mexico and Australia, the lines in each population were classified based on their response to the presence and absence of *Lr67/Yr46* and grouped into three categories (HR, HS and SEG). The observed ratio for the three phenotypic categories showed a good fit with the ratio expected for segregation at a single locus (Table 3). Evaluations conducted at different sites and during different years helped to determine a final conclusion for each line and to define the category they belonged to for a better marker-trait association.

A majority of lines showed the same response category for leaf rust and stripe rust, indicating that the same gene or closely linked genes conferred resistance to both diseases. The distribution of stripe rust responses for the HR (*+Lr67*) and the HS *(−Lr67*) leaf rust categories of the Avocet × RL6077 F_4-lines is displayed in Fig. 1. The stripe rust responses were clearly lower for the *+Lr67* category compared with the *−Lr67* category. The phenotypic correlation between the mean severity to leaf rust (data from the 2009–2010 crop cycle in Obregon) and mean severity to stripe rust (Toluca, 2009 crop cycle) for the Avocet × RL6077 F_4 lines was high ($r = 0.78$, $P < 0.01$).

Segregation for additional minor gene(s) was observed for both leaf rust and stripe rust as evidenced by the continuous distribution of lines from the Avocet × RL6077 F_4 population for stripe rust severity (Fig. 2), and by the continuous distribution of the Avocet × RL6077 F_6 lines in the *-Lr67* category for their leaf rust severity (Fig. 3).

Molecular mapping of *Lr67/Yr46*

Six SSR markers, *Xbarc98*, *Xbarc288*, *Xcfd23*, *Xcfd71*, *Xwmc48*, and *Xwmc89*, from chromosome 4D were identified to be potentially associated with *Lr67/Yr46* in the genome-wide scan conducted using the DNA of the parents and the subsample of HR and HS lines from the original F_3 population. An initial linkage map was generated using the Avocet × RL6077 F_4 population with the consensus leaf rust and stripe rust responses excluding lines whose phenotypic classification remained unclear in the genetic linkage analysis. Additional markers, including *Xgwm165* and *Xgwm192* in this region of chromosome 4D, were screened on the Avocet × RL6077 F_4 population to investigate the trait-marker association. *Xwmc48* and *Xwmc89* were not linked with *Lr67/Yr46*, and *Xbarc288* was monomorphic in the population but not among the parents (probably due to allele fixation in the two F_3 families that were used to generate the advanced populations). Markers *Xgwm165*, *Xbarc98*, *Xcfd23* and *Xgwm192* were scored as dominant markers in the F_4 population. The most closely linked markers were *Xgwm165* and *Xgwm192*

Table 3 Number of HR, SEG and HS lines in the Avocet × RL6077 F_3, F_4 and F_6 populations and *P* value from the χ^2 tests when tested against segregation for a single gene (*Lr67/Yr46*)

Population	Disease	Number of observed lines			Number of expected lines[a]			
		HR	SEG	HS	HR	SEG	HS	*P* value
F_3	Leaf rust[b]	34	66	36	34	68	34	0.92
F_3	Stripe rust[c]	27	75	34	34	68	34	0.34
F_4	Leaf rust[d]	56	42	50	56	37	56	0.54
F_4	Stripe rust[e]	57	47	44	56	37	56	0.08
F_6	Leaf rust[f]	67	8	72	69	9	69	0.84

[a] Genetic ratio expected for segregation of a single gene in F_3 (1:2:1), F_4 (3:2:3), F_6 (15:2:15)

[b] Based on evaluations conducted in Cd. Obregon, Mexico, in the 2008–2009 and 2009–2010 crop seasons

[c] Based on evaluation conducted in Cobbitty, Australia, in 2009

[d] Based on evaluations conducted in Cd. Obregon, Mexico, in the 2008–2009 and 2009–2010 crop seasons and in El Batan, Mexico, in 2009

[e] Based on evaluation conducted in Toluca, Mexico, in 2009

[f] Based on evaluation conducted in Cd. Obregon, Mexico, in the 2009–2010 crop season

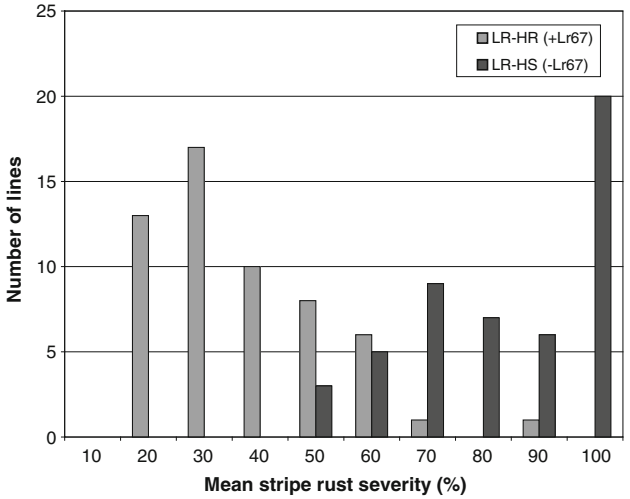

Fig. 1 Mean stripe rust severity of leaf rust homozygous resistant (LR-HR) and leaf rust homozygous susceptible (LR-HS) F_4 lines from the Avocet × RL6077 population

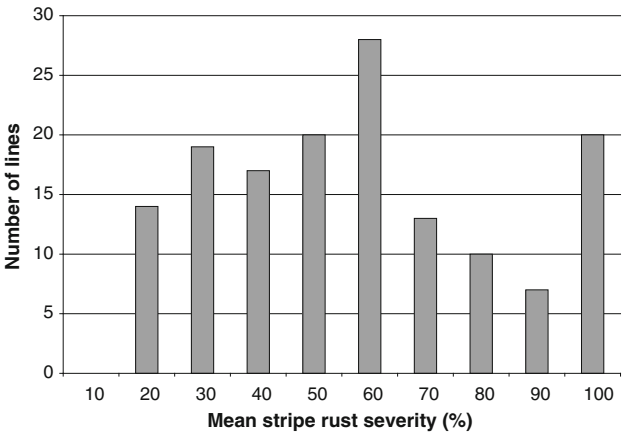

Fig. 2 Distribution of mean stripe rust severity among 148 F_4 lines from the Avocet × RL6077 population when tested at Toluca, Mexico, in 2009

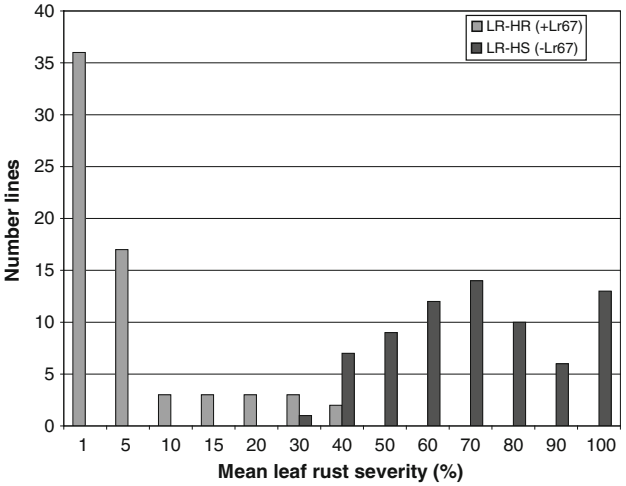

Fig. 3 Distribution of mean leaf rust severity among the homozygous resistant (LR-HR) and homozygous susceptible (LR-HS) F_6 lines from the Avocet × RL6077 population

based on the initial analysis conducted using the F_4 population. The markers that were initially identified to be linked with *Lr67/Yr46*, *Xcfd71*, *Xcfd23, and Xbarc98*, were also screened on a subset of the Avocet × RL6077 F_3 population, and similar linkage distances were obtained (results not shown). Co-dominant amplification products were detected using the ABI3730xl DNA fragment analyzer; markers *Xcfd71*, *Xcfd23 and Xbarc98* produced amplification of 231, 229, and 192 bp products, respectively, that were associated with *Lr67/Yr46*. The corresponding alternate alleles linked with susceptibility and inherited from Avocet were 242, 238, and 189 bp, respectively. The final genetic linkage map was generated using the Avocet × RL6077 F_6 RIL population (Fig. 4), and the closest markers, *Xgwm165* and *Xgwm192*, were

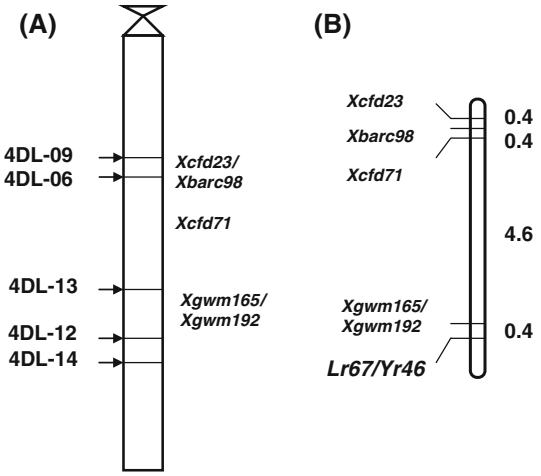

Fig. 4 **a** Physical map of *Lr67/Yr46*-associated SSR markers based on 'Chinese Spring' chromosome arm 4DL deletion lines, and **b** Genetic linkage map of *Lr67/Yr46* and associated SSR markers of chromosome arm 4DL in Avocet × RL6077 F_6 population. *Numbers* to the *right* are genetic distances in centiMorgans

located at 0.4 cM proximal to *Lr67/Yr46*. The PCR amplification products of these two markers using two resistant and four susceptible F_6 lines from the Avocet × RL6077 population are shown in Fig. 5.

Physical mapping of *Lr67/Yr46* associated markers using 4D deletion lines

The five markers identified to be associated with *Lr67/Yr46* were tested on a set of lines lacking different segments of the 4D short and long arms to establish the physical position of *Lr67/Yr46*. Amplification products were obtained in all of the lines with deletions in the short arm, whereas one or more lines with deletions in the long arm showed absence of amplification product, confirming that *Lr67/Yr46* is present in the long arm (Table 4). The relative marker order of the genetic linkage map was confirmed in the chromosomal deletion bins that span break points between long-arm fraction lengths of 0.3–0.56. The gene *Lr67/Yr46* was inferred to be located in a deletion bin distal to the 0.56 chromosomal fraction length break point, based on the location of the most closely linked markers, *Xgmw165* and *Xgwm192*.

Table 4 Comparison of marker genotypes on Chinese Spring chromosome 4D deletion lines

Deletions lines		Response of *Lr67/Yr46* associated markers				
Line	Ratio intact	*Xbarc98*	*Xcfd23*	*Xcfd71*	*Xgwm165*	*Xgwm192*
4DS-01	0.53	+	+	+	+	+
4DS-03	0.67	+	+	+	+	+
4DS-05	0.63	+	+	+	+	+
4DL-09	0.31	−	−	−	−	−
4DL-06	0.38	+	+	−	−	−
4DL-13	0.56	+	+	+	−	−
4DL-12	0.71	+	+	+	+	+
4DL-14	0.86	+	+	+	+	+

Response to Ug99 stem rust infection

Thatcher, RL6077, RL6058, and 90RN2491 were tested in Kenya. Stem rust scores showed Thatcher with a moderate severity of 25MS, while increased resistance scores of 1MR were observed in RL6058 and 90RN2491, and RL6077 showed a score of 10R-MR. These results indicated that *Lr67/Yr46* in a Thatcher background also produces elevated stem rust resistance, as has been found for *Lr34/Yr18* against Ug99. However, the additive effect of *Lr67/Yr46* + *Lr34/Yr18,* gene combinations present in 90RN2491, could not be determined because RL6058 with *Lr34/Yr18* was already displaying very low disease severity.

ABC transporter sequence and RFLP analysis of RL6077

The complete gene sequence of *Lr34/Yr18* allele in RL6077 did not reveal any additional variants, except for the characteristic haplotype of the susceptible allele found in intron 4 and the previously determined variants in exons

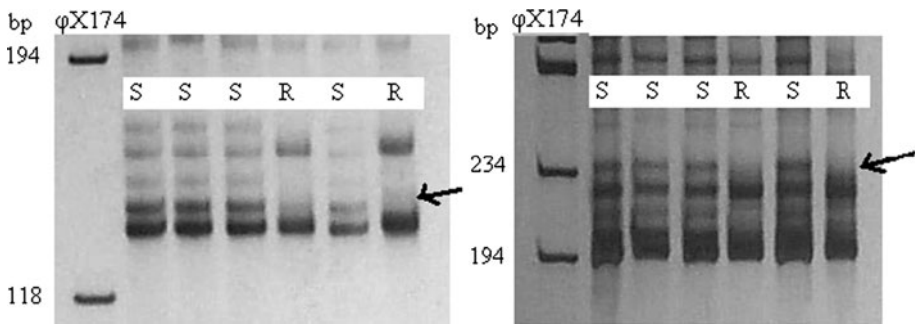

Fig. 5 Polymerase chain reaction (*PCR*) amplification products resolved in 12% acrylamide gels when using *Xgwm192* (*left*) and *Xgwm165* (*right*) for four resistant and two susceptible F_6 lines from the Avocet × RL6077 population, and size of bands produced by the size marker (*φ*X174/*Hae*III)

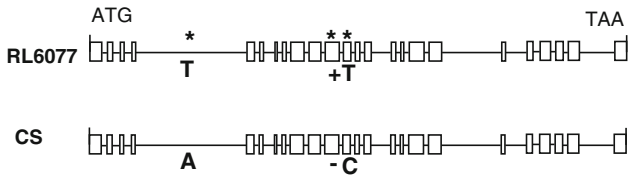

Fig. 6 Diagrammatic representation of the complete sequence of the *Lr34/Yr18* allele from RL6077 compared with the Chinese Spring (*CS*) haplotype that carries the resistance allele (from Krattinger et al. 2009). The *boxes* denote exons. The *asterisks* denote positions of SNPs or indels at intron 4 (T/A), exon 11(±indel TTC) and exon 12 (T/C)

11 and 12 (Fig. 6), nor did it reveal any features of the resistant haplotype (Krattinger et al. 2009). The RL6077 sequence is identical to the susceptible haplotypes found in the wheat cultivar Renan (Genbank FJ436985, nucleotide positions 48885–36898) and the diploid D genome, *Aegilops tauschii* accession AL8/78 (FJ436986, 73713–61747). Thus, *Lr67/Yr46* cannot be attributed to the presence of a closely related variant of *Lr34/Yr18*. RFLP analysis based on 14 restriction enzyme-probe combinations did not reveal any differences between RL6077 and Thatcher (Fig. S3). Thus, at this level of resolution, *Lr34* homoeologs did not distinguish the recurrent parent Thatcher from the addition of the *Lr67/Yr46* gene.

Discussion

From a comparatively larger set of wheat lines, albeit a different wheat population, the present study confirms Canadian reports of adult plant leaf rust resistance at the *Lr67* locus located on chromosome 4D (Hiebert et al. 2010). The use of photoperiod-insensitive genotypes selected from the Avocet × RL6077 population enabled field evaluations in diverse field locations without the limitations of variable day length during the growing season. We also provide conclusive evidence of the same gene, or a closely linked gene hereby designated as *Yr46*, being effective against stripe rust. Hiebert et al. (2010) also reported segregation of stripe rust resistance in F_4 lines of Thatcher × RL6077 population and in an F_3 population developed from one of the HR F_4 lines, 'H1777', from the Thatcher × RL6077 population. However, the correlated responses of *Lr67* and stripe rust resistance based on phenotypic response was only based on seven F_4 lines. Stripe rust resistance based on Australian observations was found to be associated with marker *Xcfd71* (*Lr67* map location) in the H1777 population, but a comparison with Canadian data on leaf rust response was not made in their study.

A minor QTL for leaf rust adult plant resistance inherited from the wheat line, ND495, was recently reported by Chu et al. (2009) in the same chromosomal region in 4DL. The relationship of this QTL to *Lr67* is unknown and further studies to determine whether adult plant stripe rust response cosegregates with the QTL will be required to ascertain whether *Lr67/Yr46* is also present in ND495. Two seedling stripe rust resistance genes, *Yr22* (Chen et al. 1995) and *Yr28* (Singh et al. 2000b), have been reported in this chromosome. *Yr28* is associated with marker *mwg634* located at the distal end of the short arm of chromosome 4D. Suenaga et al. (2003) identified a QTL for stripe rust resistance in the same chromosome arm at a more distally located position compared with *Lr67/Yr46* in bread wheat 'Oligoculm', but the associated effect of leaf rust resistance was not detected.

The five markers, *Xgwm165*, *Xgwm192*, *Xcfd71*, *Xbarc98,* and *Xcfd23*, identified to be associated with *Lr67/Yr46* in this study, positioned this gene to the long arm of chromosome 4D. Based on the physical mapping of the two closely linked markers *Xgwm165* and *Xgwm192* which map proximal to *Lr67/Yr46* the resistance locus/loci is predicted to be located in a deletion bin distal to the break point of 0.57. In the Hiebert et al. (2010) study no recombinants were found in an introgressed region spanning *Lr67* and markers *Xcfd71*, *Xbarc98*, *Xcfd23*, *Xwmc457* probably due to the small population size evaluated. Furthermore, they suggested from their data that *Lr67* is on the distal side of marker *Xcfd71* located in the 0.38–0.41 deletion bin. A precise definition of the break points will provide entry points into syntenic regions for comparative genomic analysis to enable a more targeted approach to enrich the region with more markers in refining the position of *Lr67/Yr46*.

Given the striking similarities between *Lr67/Yr46* and *Lr34/Yr18* for leaf, stripe, and stem rust responses, we undertook further characterization of the ABC transporter sequence at the *Lr34/Yr18* locus and its homoeologs in RL6077 to ascertain whether variant forms of the *Lr34/Yr18* locus represent *Lr67/Yr46*. Because the specific nature of the chromosome translocation present in RL6077 after several backcrosses remains unknown (Dyck et al. 1994), the presence of a duplicated *Lr34/Yr18* variant or homoeolog on the translocated chromosome cannot be ruled out. We previously reported only one section of the coding region containing the sequence variants of the *Lr34/Yr18* ABC transporter that differentiate resistance and susceptible alleles, with the latter alleles present in RL6077 (Lagudah et al. 2009). With the complete sequence of the gene from RL6077, we now have strong evidence that neither a closely related variant nor a homoeolog of the *Lr34/Yr18* is associated with *Lr67/Yr46*.

The robustness of the closely associated markers *Xgwm192* and *Xgwm165* for postulating *Lr67/Yr46* in a wide range of germplasm, as well as the utility of these

markers in a breeding program, needs to be investigated. Since the presence of *Lr34/Yr18* has often been deduced by the phenotypic response of adult plants to leaf rust and stripe rust in association with LTN in the field, it is possible that lines have erroneously been attributed to *Lr34/Yr18* instead of *Lr67/Yr46*. However, Kolmer et al. (2008) showed that very few exceptions were found among lines that had these characteristics and lacked the *Lr34/Yr18* positive marker allele. Subsequent analysis showed that these lines carried the susceptible *Lr34/Yr18* allele based on the diagnostic gene sequence marker (Lagudah et al. 2009); hence, the need to ascertain the presence of *Lr67/Yr46* in such genotypes.

Overall, the presence of *Lr67/Yr46* gave lower rust severity in association with LTN compared with the susceptible parent in the Avocet × RL6077 population. This allowed us to group lines in each population as HR, HS, and SEG based on their phenotypic response to leaf rust and stripe rust and appearance of LTN. An independent mean severity scoring (%) was in addition made for each line of the population. While the three groups HR, HS, and SEG allowed us to discriminate *Lr67/Yr46* in the populations, the mean severity response revealed segregation of additional minor genes in populations, as can be observed in Figs. 1, 2, 3. There were few cases where lines scored as S, showed lower mean severity response than some lines belonging to the R group as can be observed in Fig. 3. Phenotypes of these S lines exhibited severities that were relatively low and lacked LTN compared with the susceptible parent and other S lines in the population. We inferred that these S lines with lower responses most likely carried minor resistance gene(s) in the absence of *Lr67/Yr46* (and lack of LTN). Selection of homozygous lines lacking *Lr67/Yr46* from the Avocet × RL6077 F_6 population that showed relatively lower leaf rust and stripe rust responses can be used to develop new mapping populations for characterization of additional minor gene(s) derived from RL6077.

The expression of *Lr67/Yr46* is known to be variable as also has been observed by other authors (Dyck and Samborski 1979) and in some backgrounds relatively high severity levels were observed. A majority of the lines had correlated responses to leaf rust and stripe rust, but there were some lines that fell into different category for leaf rust and stripe rust responses (Table 3). These lines may possibly have been misclassified, from the early generation populations, and hence the use of the F_6 population where most of the lines are approximating homozygosity were used for the genetic mapping exercise. Biologically, this is not entirely surprising given the quantitative nature of adult plant slow-rusting phenotypes. This is more so with the stripe rust as the expression of *Yr46* was moderate, i.e., severity levels were intermediate.

Additive gene effects have been postulated among gene interactions, whereby up to four or five slow-rusting genes can confer near immunity to rust infection (Singh et al. 2000a). What remains unclear is which specific gene combinations produce optimal additive gene effects when working with fewer genes. In the present study, the level of gene additivity for *Lr67/Yr46* + *Lr34/Yr18* for enhanced stem rust resistance against Ug99 could not be observed when compared with the effect of *Lr34/Yr18* alone because RL6058, which carries *Lr34/Yr18*, was itself almost free from stem rust. Dyck et al. (1994), investigating the genotype 90RN2491, also failed to observe additive gene effects against leaf rust due to the combined effects of *Lr34/Yr18* and what we now know as *Lr67/Yr46*. Lillemo et al. (2008) examined gene interactions between *Lr34/Yr18* and another well-defined dual adult plant rust resistance gene *Lr46/Yr29* and found very little evidence for strong additive interaction. However, in each case this could have been due to low disease severity caused by the presence of *Lr34/Yr18*. Apart from *Lr34/Yr18*, *Lr46/Yr29,* and *Lr67/Yr46*, there is clearly a need to define other slow-rusting resistance loci to enable further characterization of which gene combinations give rise to a stronger synergistic effect against rust infection. *Lr34/Yr18* is known to enhance resistance levels when combined with other minor genes, and exploring this phenomenon with *Lr67/Yr46* may lead to new gene combinations that allow making more effective use of slow-rusting resistance genes in wheat breeding.

Acknowledgments We are grateful to Violeta Calvo Salazar, CIMMYT, and Libby Viccars, Sutha Chandramohan, and Lynette Rampling, CSIRO, for their skilled technical support on the molecular studies; to the technical field and greenhouse staff at CIMMYT; to Hanif Miah for the field rust studies at Cobbitty, Australia; to Dr. TR Endo, Japan, for providing the deletion lines used in this study, and to the Grains Research and Development Corporation (GRDC) of Australia for funding the work (Grants CSP00099 and CIM00013). We also thank Alma McNab for the technical editing.

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