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ORIGINAL PAPER

Fine scale genetic and physical mapping using interstitial deletion mutants of *Lr34/Yr18*: a disease resistance locus effective against multiple pathogens in wheat

W. Spielmeyer · R. P. Singh · H. McFadden · C. R. Wellings · J. Huerta-Espino · X. Kong · R. Appels · E. S. Lagudah

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Abstract The *Lr34/Yr18* locus has contributed to durable, non-race specific resistance against leaf rust (*Puccinia triticina*) and stripe rust (*P. striiformis* f. sp. *tritici*) in wheat (*Triticum aestivum*). *Lr34/Yr18* also cosegregates with resistance to powdery mildew (*Pm38*) and a leaf tip necrosis phenotype (*Ltn1*). Using a high resolution mapping family from a cross between near-isogenic lines in the "Thatcher" background we demonstrated that *Lr34/Yr18* also cosegregated with stem rust resistance in the field. *Lr34/Yr18* probably interacts with unlinked genes to provide enhanced stem rust resistance in "Thatcher". In view of the relatively low levels of DNA polymorphism reported in the *Lr34/Yr18* region, gamma irradiation of the single chromosome substitution line, Lalbahadur(Parula7D) that carries *Lr34/Yr18* was used to generate several mutant lines. Characterisation of the mutants revealed a range of highly informative genotypes, which included variable size deletions and an overlapping set of interstitial deletions. The mutants enabled a large number of wheat EST derived markers to be mapped and define a relatively small physical region on chromosome 7DS that carried *Lr34/Yr18*. Fine scale genetic mapping confirmed the physical mapping and identified a genetic interval of less than 0.5 cM, which contained *Lr34/Yr18*. Both rice and *Brachypodium* genome sequences provided useful information for fine mapping of ESTs in wheat. Gene order was more conserved between wheat and *Brachypodium* than with rice but these smaller grass genomes did not reveal sequence information that could be used to identify a candidate gene for rust resistance in wheat. We predict that *Lr34/Yr18* is located within a large insertion in wheat not found at syntenic positions in *Brachypodium* and rice.

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W. Spielmeyer and R. P. Singh contributed equally to the study through the "Thatcher" and "Lalbahadur" genetic stocks, respectively.

W. Spielmeyer · H. McFadden · E. S. Lagudah (✉)
CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia
e-mail: evans.lagudah@csiro.au

R. P. Singh
CIMMYT, Apdo. Postal 6-641, 06600 Mexico DF, Mexico

C. R. Wellings
Plant Breeding Institute, University of Sydney, Cobbitty, NSW 2570, Australia

J. Huerta-Espino
Campo Experimental Valle de México INIFAP, Apdo. Postal 10, 56230 Chapingo, Edo de México, Mexico

X. Kong
Chinese Academy of Agricultural Science, Beijing, China

R. Appels
Centre for Comparative Genomics, Murdoch University, Perth 6150, Australia

Introduction

The development of wheat (*Triticum aestivum*) cultivars with effective and durable rust resistance has been an important breeding objective worldwide. Although race-specific resistance provides highly effective protection, resistance which does not rely on a specific recognition event between host and the pathogen is often more stable. Non-race specific resistance is characterised by a non-hypersensitive and partial resistance response with varying levels of disease under field conditions (Rubiales and Niks 1995). Some of the most important, durable rust resistance

genes include *Sr2* for resistance to stem rust (*Puccinia graminis* f. sp. *tritici*) and *Lr34/Yr18* for leaf and stripe rust resistance (*Puccinia triticina* and *Puccinia striiformis* f. sp. *tritici*) (Dyck 1987; McFadden 1930). These genes used in combination with other resistance genes have been effective for many decades in wheat production areas worldwide.

The *Lr34/Yr18* locus is of particular interest to breeders, because it has contributed to durable resistance to two rust pathogens with both resistances always inherited together (Kolmer 1996; McIntosh 1992; Singh 1992). The locus was also associated with tolerance to barley yellow dwarf virus and resistance to powdery mildew (*Blumeria graminis* sp. *tritici*), which was recently designated *Pm38* (Singh 1993; Spielmeyer et al. 2005). Furthermore, leaf rust resistance conferred by unlinked race-specific genes was enhanced when combined with *Lr34/Yr18* (German and Kolmer 1992). Previous studies also linked the locus to improved stem rust resistance in the "Thatcher" background. Dyck (1987, 1991) reported that near-isogenic lines in "Thatcher" carrying *Lr34* showed higher level of stem rust resistance. Other studies examined the segregation of stem rust resistance in bi-parental crosses and postulated that *Lr34* was either tightly linked to a stem rust resistance gene or that the locus enhanced independent gene(s) present in the "Thatcher" background (Dyck 1992; Liu and Kolmer 1998; Nazareno and Roelfs 1981). In these studies, none of the known race-specific stem rust resistance genes in "Thatcher" (*Sr5, Sr9g, Sr12* and *Sr16*) could account for the level of resistance seen amongst the progeny in the field. More recently, the association of *Lr34* with enhanced stem rust resistance was confirmed in several crosses involving "Thatcher" derived backgrounds including 100 recombinant inbred lines derived from a cross between "Thatcher" and a near-isogenic line carrying *Lr34* (RL6058) (Gavin Vanegas et al. 2007). This study demonstrated that *Lr34* allowed the expression of additional adult stem rust resistance gene(s) in "Thatcher" and suggested that *Sr12* and *Sr16* may have contributed to resistance in adult plants.

Most rust resistance genes that have been isolated from cereals including wheat are predicted to encode nucleotide binding site/leucine rich repeat proteins (NBS-LRR) (Collins et al. 1999; Feuillet et al. 2003; Huang et al. 2003; Cloutier et al. 2007) with the exception of *Rpg1*, a barely stem rust resistance gene that encoded a protein kinase (Brueggeman et al. 2002). These genes confer race-specific resistance relying on gene for gene interactions between host and the pathogen. There is no information on the molecular basis of durable, non-race specific rust resistance in wheat. The important role of *Lr34/Yr18* in providing stable resistance to multiple pathogens has focused attention on the detailed molecular mapping of this locus on chromosome 7DS (Spielmeyer et al. 2005; Schnurbusch et al. 2004) and the development of "breeder friendly" markers to predict the presence/absence of *Lr34/Yr18* in a wide range of genetic backgrounds (Bossolini et al. 2006; Lagudah et al. 2006). A recent study of the *Lr34/Yr18* region reported a major rearrangement in orthologous regions of rice and *Brachypodium* and outlined potential limitations of using smaller graminaceous genomes for fine mapping studies in wheat (Bossolini et al. 2007).

A major challenge with the *Lr34* region has been the difficulty of finding polymorphic markers (Bossolini et al. 2006; Lagudah et al. 2006). Interstitial deletion mutants can be used to generate additional markers without relying on polymorphism between two parental lines. In this study, we used gamma irradiation induced mutants to define a small physical interval for *Lr34/Yr18*, which was confirmed by high-resolution mapping using EST derived markers. We compared the DNA sequence of *Lr34/Yr18* region to orthologous regions in rice and *Brachypodium* to develop tightly linked markers that flanked the rust resistance locus in wheat. We investigated the possibility of deriving sequence information from rice and *Brachypodium* that would assist in the isolation of a candidate gene for *Lr34/Yr18* in wheat.

Materials and methods

Genetic material

The resistant line RL6058 is a backcross-derived line of Thatcher (Tc) (Thatcher*6/PI58548) and carries genes for resistance to leaf rust (*Lr34*) and stripe rust (*Yr18*) (Dyck 1977). In the present study RL6058 was crossed again to Thatcher to generate a large F2 population. Other genetic material included a backcross four derived line of Avocet carrying *Lr34/Yr18* (Avocet*5/Parula = Avocet + *Lr34*), which was crossed to Avocet to generate a large F2 population.

Development of mutants

Seeds from the single chromosome substitution line Lalbahadur (Parula 7D) with *Lr34*, *Yr18*, *Pm38* and *Ltn1* were obtained at CIMMYT, Mexico by bagging 4–6 spikes of various space sown plants before anthesis. To determine the purity, approximately 50 seeds of each plant were grown in the field under leaf rust pressure. Seeds of those plants that showed segregation for leaf rust resistance were discarded. Approximately 4,000 pure seeds were irradiated using a ^{60}Co source at a dosage of 20 krad. This gamma irradiation dosage was chosen for optimal mutagenic effects without excessive reduction on plant growth based on a previous protocol established by Mago et al. (2005). Mutagenised

M1 seeds were space sown in the field at Obregon, Mexico and single bagged heads from 2,000 plants harvested. M2 seeds from these single heads derived from each M1 plant were space sown in two 1-metre long paired row plots and evaluated for adult plant response to rust infection. M2 plots identified to carry both resistant and susceptible plants were then protected by applying the fungicide Tebuconazole. Leaf rust susceptible plants identified in M2 rows were selected along with their resistant sibs and were re-tested as M3 and M4 in two 1-m long row plots (about 20 plants per plot) to confirm their M2 genotypes. Hill plots of the same populations were also established under fungicide protection to obtain sufficient seeds and observations were made for the presence and absence of leaf tip necrosis (*Ltn1*) using the phenotypic differentiation between Lalbahadur and Lalbahadur(Parula 7D) as controls.

The methodology of field inoculation with leaf rust in Mexico was described in Lagudah et al. (2006). Rust response was monitored on adult plants of the gamma irradiated M2, M3 and M4 generations at CIMMYT, Mexico. At Cobbitty in Australia, replicated field sites of the M5 generation of the susceptible mutants and their corresponding resistant sibs were evaluated from the booting stage until grain maturity using the leaf and stripe rust pathotypes listed below. Observations for response to powdery mildew infection relied on natural field inoculum.

High resolution mapping families and rust screening

DNA was extracted from half seeds from the Tc x RL6058 and Avocet × Avocet*Lr34* F2 populations according to the protocol described in Mago et al. (2005). From each F2 population 1,150 half seeds were screened with SSR markers *gwm1220* and *csLV34* that were previously shown to flank the *Lr34/Yr18* locus (Spielmeyer et al. 2005; Lagudah et al. 2006). Twenty-one F2 lines from the Tc × RL6058 cross were identified with at least one recombination event between flanking markers. F3 families together with parental lines were grown in replicated field rows at two experimental sites at Cobbitty near Sydney (Australia) in 2005 to evaluate disease reaction to stripe and leaf rust. *Puccinia striiformis* f. sp. *tritici* pathotypes 134 E16A+ and 104 E137A− +Yr17 and *P. triticina* pathotypes 104-1,2,3, (6), (7), 9,11; 104-1,2,3, (6), (7), 11,13; 10-1,3,7,9,10,12; 76-3,5,9,10 +Lr37 were released at late stem elongation stage in 2005 at both sites. The subsequent infections on Thatcher were clearly higher (80 MS for stripe rust and 70 MS-80 MS for leaf rust) than on RL6058 (trace resistant with some small leaf rust pustules). F3 lines were rated according to the disease severity and response of the parental lines.

At least 12 F3 plants from each of the 21 recombinant F2 lines from the Tc x RL6058 cross were screened with markers *gwm1220* and *csLV34* to identify one individual F3 plant which was homozygous at both marker loci. F4 plants from these 21 homozygous F3s from Tc × RL6058 together with 19 F3 families from recombinant F2 plants from the Avocet × Avocet*Lr34* cross were grown in replicated field rows in 2006 at one site in Cobbitty to evaluate the response to leaf and stripe rust using the same pathotypes as in 2005. In addition, *Puccinia graminis* f. sp. *tritici* pathotypes 98-1,2,3,5,6; 34-2,12,13; 34-1,2,7 +Sr38; 343-1,2,3,4,5,6 were released at booting stage in 2006. "Thatcher" was scored 50 MS-60 MS with most of the peduncular internode covered with susceptible-type stem rust pustules and "RL6058" as trace resistant with only a few small stem rust pustules. Differences in disease reactions to stripe, leaf and stem rust were qualitative between parental lines and progeny, resulting in bi-modal distributions. There was no segregation for stem rust resistance in the Avocet-derived population, because of the presence of *Sr26* in "Avocet", which is effective against all current Australian pathotypes.

Molecular marker analysis

Genomic DNA was extracted from leaves and used for RFLP analysis according to Lagudah et al. (1991a, b). Wheat EST derived markers, SSR and STS markers from the *Lr34* region (Table 1) were used as probes in RFLP analysis and in PCR to compare DNA profiles between mutants and their parental genotypes. For genetic mapping DNA probes (approximately 300–500 bp) were hybridised to genomic DNA of parental lines that was previously digested with up to 24 restriction enzymes and fixed on nylon membranes according to standard DNA hybridisation protocols. RFLPs were identified between parental lines for the following ESTs and restriction enzymes: BQ788742 (*Eco*RV), CA500527 (*Apa*LI), BF473324 (*Acc*I) and BJ280740 (*Nco*I). These RFLPs were mapped using the 21 homozygous recombinant F3 lines from the Tc × RL6058 cross. Recombination frequency was converted into genetic distance estimates without the use of a mapping function. Primer sequences and PCR protocols for *gwm295* and *gwm1220* were previously published or kindly provided by Dr. M. Ganal, TraitGenetics Germany (Röder et al. 1998). Standard PCR conditions using 58°C annealing were used to amplify products, which were resolved on 1.5% agarose electrophoresis gels.

BAC library screening and sequencing

BAC clones were isolated from two sources of the diploid D genome progenitor, *Aegilops tauschii*, AUS 18913 (Moullet et al. 1999) and AL8/78 (http://wheat.pw.usda.gov/PhysicalMapping/) using a subset of the wheat EST derived markers, BJ280740, CA500527, BF473324 and AL810355 from the *Lr34* region. Additional BAC clones

Table 1 Mutants showing variable size deletions detected by wheat ESTs and STS markers and arranged against the corresponding order of the rice orthologs

Wheat EST & STS	Mutants						Rice gene	Location (Mb)	Gene description
	#7	#9	#12	#15	#16	#20			
1-gwm4197	+	+	+	+	–	+			
2-BE493812	–	+	+	–	–	+	Os06g04660	2.031	Oxidoreductase Fe(II) oxygenase
3-BE495774	–	–	+	–	–	+	Os06g04800	2.09	Beta 1 proteasome subunit
4-BF483741	–	–	+	–	–	–	Os06g04820	2.1	Leucine carboxyl methyl transferase
5-BE499296	–	–	+	–	–	–	Os06g04850	2.13	Homeobox protein
6-gwm1220	–	–	–	–	–	–			(On same BAC clones as BJ280740)
7-BJ280740	–	–	–	–	–	–	Os06g04880	2.144	Kinase-U box
8-KUDS-STS	–	–	–	–	–	–	Os06g04880	2.144	Kinase-U box
9-TC239710	–	–	–	–	–	–	Os06g04890	2.15	Hypothetical protein
10-BJ218124	+	+	+	+	+	+	Os06g04910	2.156	Similar to AT5g20070
11-CD452485	+	+	+	+	+	+	Os06g04920	2.162	Zn finger in Ran binding protein
12-DQ013359	–	+	+	+	+	+	Os06g05050	2.23	Wall associated protein kinase
13-CA595218	–	+	+	+	+	+	Os06g05080	2.242	Cytochrome c oxidase subunit
14-BE400881	+	+	+	+	+	+	Os06g05090	2.248	Arginine methyl transferase
15-BE490148	+	+	+	+	+	+	Os06g05110	2.257	Superoxide dismutase
16-CA500527	–	+	–	+	+	+	Os06g05130	2.274	acyl-ATP thioesterase
17-BQ788742	–	+	–	+	+	+	Os06g05160	2.292	Probable sulfate transporter 3.4
18-BE515883	–	+	–	+	+	+	Os06g05190	2.33	DNA repair protein XRCC1
19-CK208877	–	–	–	+	–	–	Os06g05240	2.36	Zinc carboxypeptidase
20-BF473324	–	–	–	+	–	–	Os06g05250	2.367	GTP-binding protein
21-AL810355	–	–	–	+	–	–	Os06g05272	2.371	Pectate lyase
22-BE22-STS	–	–	–	–	–	–			(On same BAC clone as AL810355)
23-Gwm295	+	+	+	+	+	+			

were obtained from genomic libraries of "Chinese Spring" by screening with the STS marker KUDS (Lagudah et al. 2006) and from the cultivar Glenlea using the wheat EST AL810355. The relative position of different wheat EST derived markers on BAC clones were determined from their relative order on an overlapping set of BACs that hybridized to one or more ESTs which constituted a mini contig (Lagudah et al. 2006). A fragment that hybridised with AL810355 (a pectate-lyase like gene) was subcloned from Bac22, which was isolated from the *Ae. tauschii* (AUS18913) library (Moullet et al. 1999). Sequence from the subclone was used to develop a new SSR marker *csLVMS1*. Primer sequences for *csLVMS1* are as follows: csLVMS1F-5′CTCCCTCCCGTGAGTATATTC3′ and csLVMS1R- 5′ATCAAAATCCCATTGCCTGAC3′. The marker amplified a 212 bp product from "Avocet" and "Thatcher" and a 210 bp product from "Avocet*Lr34*" and "RL6058". These PCR products were separated using a DNA fragment analyzer.

A BAC library of *Brachypodium sylvaticum* was kindly provided by Dr. G. Moore, John Innes Centre, UK. High-density filters representing approximately 5× genome coverage were screened with wheat EST probes CA500527 and BJ280740. BAC DNA was isolated, digested with *Hind*III restriction enzyme and fixed to nylon membranes for DNA hybridisation. Wheat ESTs BE499296, BJ280740, BE490148, CA500527, BQ788742, BE515883, CK208877, BF473324, AL810355 (Lagudah et al. 2006) that corresponded to rice genes located between Os06g04850 and Os06g05260 hybridised to BAC A4 confirming that this clone represented orthologous *Brachypodium* genome sequence.

The sequencing of BAC clone A4 was carried out as described previously (Kong et al. 2004). The shot-gun library was sequenced to 5.2× coverage of the BAC clone and then assembled into contigs. Direct sequencing from the BAC clone using primers from the contig ends enabled gap closures. The Rice GAAS program (http://rice-gaas.dna.affrc.go.jp/) was used for gene prediction of the *Brachypodium* sequence.

Results

Rust screening

Previously, leaf and stripe rust resistance genes *Lr34* and *Yr18* were mapped to a genetic interval of approximately

3 cM on chromosome 7DS using 110 F7 recombinant inbred lines from a cross between "Thatcher" (susceptible) and a near-isogenic line carrying *Lr34/Yr18* (RL6058) (Spielmeyer et al. 2005). In this study, the flanking PCR-based markers *gwm1220* and *csLV34* (Lagudah et al. 2006) were used to identify 21 recombinant F2s. The 21 F3 rows were grown in replicated field trials and scored for stripe and leaf rust resistance in 2005. The qualitative differences in disease reaction to stripe and leaf rust allowed each F3 row to be classified as homozygous resistant (HR), homozygous susceptible (HS) or segregating. Resistance to stripe rust cosegregated with resistance to leaf rust. With the same flanking markers (*gwm1220* and *csLV34*) another high resolution mapping family was developed from the Avocet × Avocet*Lr34* cross. Phenotypic analysis of F3 rows demonstrated that resistance to leaf rust and stripe rust also co-segregated in this population. Leaf and stripe rust resistance therefore failed to recombine in populations derived from 4,600 gametes (2,300 F2s) consistent with earlier studies reporting tight linkage between *Lr34* and *Yr18* (Singh 1992; McIntosh 1992; Spielmeyer et al. 2005).

At least twelve F3 plants were screened from each of the 21 F2 lines (Thatcher × RL6058) with markers *gwm1220* and *csLV34* to identify at least one individual F3 plant that was homozygous at both marker loci. F4 families from these homozygous F3 plants were grown in replicated field rows in 2006 in Cobbitty to confirm response to leaf and stripe rust as described above. Rust scores in 2006 were identical to the data obtained in 2005. In 2006, homozygous recombinant lines were also inoculated in the field with stem rust. While stripe and leaf rust infections were scored early (between head emergence and anthesis), stem rust symptoms became visible during late grain filling. Parental lines showed clear differences in their level of stem rust resistance with "Thatcher" susceptible (50 MS) and "RL6058" resistant (TR). Homozygous lines, which lacked *Lr34/Yr18* were all susceptible to stem rust, whereas lines with *Lr34/Yr18* were all resistant. In summary, stripe rust resistance conditioned by *Yr18* and leaf resistance conferred by *Lr34* cosegregated with stem rust resistance in homozygous recombinant lines derived from a population of 1150 F2s. The Avocet × Avocet*Lr34* population was not evaluated for stem rust response because "Avocet" carried *Sr26*, which is effective against all current Australian stem rust isolates.

Isolation of mutants

Gamma irradiation-induced mutants of the wheat line Lalbahadur(Parula7D) were assessed in the field for response to leaf rust infection at Obregon, Mexico. Among 2000 M2 single spike rows, 21 rows with leaf rust susceptible plants were identified. All susceptible plants failed to show leaf tip necrosis, in contrast to the *Ltn1* phenotype found in the parental line and the remaining resistant M2 lines. Single plant selections of susceptible individuals and their resistant sibs from the 21 M2 rows were grown and advanced to the M4 generation. Homozygous susceptible and resistant sib lines were used to further characterize these mutants.

Simple sequence repeat (SSR) markers gwm1220 and gwm295 that flank the *Lr34/Yr18* locus were initially used to assess the extent of potential deletions induced in the gamma irradiated mutants. Thirteen out of the 21 mutants had lost both SSR markers while the resistant sibs retained these markers. No further analysis was performed on this group of mutants as they were predicted to carry large deletions and would not provide additional information to narrow down the *Lr34/Yr18* interval. Of the eight remaining mutants, six (#7, #9, #12, #15, #16, #20) were shown to be deleted for the SSR gwm1220 but retained gwm295; two mutants (#19 and #21) retained both SSR markers.

These eight mutants and their resistant sibs (M5 generation) were further characterised for adult plant response to stripe and leaf rust at Cobbitty, Australia. All mutants were completely susceptible to stripe rust and leaf rust while their resistant sibs and the parental genotype "Lalbahadur(Parula7D)" were resistant. Observations on stem rust and powdery mildew infection were also noted. Natural field infections of powdery mildew during late booting and anthesis stage covered approximately 70–80% of the lower leaves and leaf sheaths of all the rust susceptible mutants. In contrast, the resistant sibs and the wild type parental genotype only showed moderate levels of infection of sheaths and leaves (approximately 20–30%). Thus, leaf rust susceptible mutants that were first identified under field conditions in Mexico were confirmed under Australian field conditions to have lost resistance to leaf rust but also resistance to stripe rust and powdery mildew. In the "Lalbahadur" background stem rust response revealed no significant differences between the susceptible mutants, resistant sibs and the wild type parent as they were all susceptible to the prevailing stem rust field isolates. Other genetic stocks sown alongside the mutants included "Chinese Spring" which carries *Lr34/Yr18* but which was as susceptible to stem rust as the mutants and "Lalbahadur(Parula7D)" suggesting that the presence of *Lr34* in the "Lalbahadur" and "Chinese Spring" backgrounds failed to contribute to effective stem rust resistance.

Defining a small physical interval for *Lr34/Yr18*

To assess the extent of induced deletions in the eight mutants, wheat EST and BAC derived markers from the genomic interval spanning the *Lr34/Yr18* locus were evaluated (Lagudah et al. 2006). Variable size deletions were detected based on the number of markers lost in the mutants

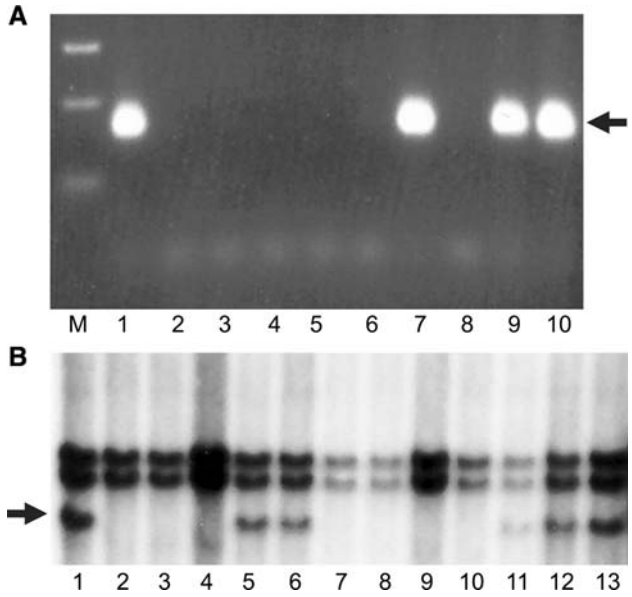

Fig. 1 Analysis of gamma irradiated mutants from Lalbahadur(Parula7D) based on PCR detection with the microsatellite, gwm 1220, (**a**) and a genomic blot probed with the wheat EST BF483741 (**b**). **a** Absence of PCR products corresponds to deleted regions. *Lane M* 100 bp size ladder, *lane1* resistant sib of mutant#7, *2* mutant #7, *3* mutant#9, *4* mutant#12, *5* mutant#15, *6* mutant#16, *7* mutant#19, *8* mutant#20, *9* mutant#21, *10* Lalbahadur(Parula7D) parental line. **b** Deleted fragments are shown by *arrows*. *Lane 1* resistant sib from mutant# 7, *2*, *3* susceptible mutant #7, *4* mutant#9, *5*, *6* mutant #12, *7*, *8* mutant #15, *9*, *10* mutant #16, *11*, *12* mutant#20, *13* mutant#19

(Figs. 1, 2; Table 1). Two mutants (#19 and #21) retained all the markers listed in Table 1. The relative size of deleted segments was estimated in the remaining six mutants between markers *gwm1220* and *gwm295*: #7 > #12 > #9 = #16 = #20 > #15. The gene order between rice and predicted orthologs in wheat was not conserved. All six mutants lost wheat orthologs of two rice genes near the distal end of the chromosomal region, namely wheat ESTs *BJ280740* (including KUDS, a D genome specific PCR marker derived from BJ280740) and *TC239710* STS (BAC derived sequence specific for the wheat EST TC239710 D genome member). Up to six wheat orthologs were also deleted in the mutants near the proximal end, while wheat ESTs that corresponded to rice genes from the middle region between 2.15 and 2.37 Mb were present in these mutants (Table 1). The patterns of deleted markers suggest that the chromosomal region between ESTs *BJ218124* and *AL810355* was inverted in wheat with respect to rice (Table 1). These results confirm previous reports of a large inversion within this region (Bossolini et al. 2006, 2007). Additional rearrangements may have occurred between wheat and rice as revealed in mutant #7 which also lost wheat ESTs *DQ013359* and *CA595218*. In summary, the smallest deleted region predicted to carry *Lr34/Yr18* was defined on the distal end by a deletion break point in mutant #12 between EST *BE499296* and *gwm1220* and on the proximal side by a breakpoint in mutant #15 between the EST *AL810355* (pectate lyase-like gene) and *BE22STS*, a BAC marker from the distal end of the same BAC clone carrying *AL810355*. Because mutant #15 retained the pectate lyase-like gene, this gene was not a candidate gene for *Lr34/Yr18*.

Defining a small genetic interval for Lr34/Yr18

To complement the analysis of deletion mutants, we mapped wheat ESTs *BJ280740*, *CA500527*, *BQ788742* and *BF473324* as RFLP markers in the Tc × RL6058 high

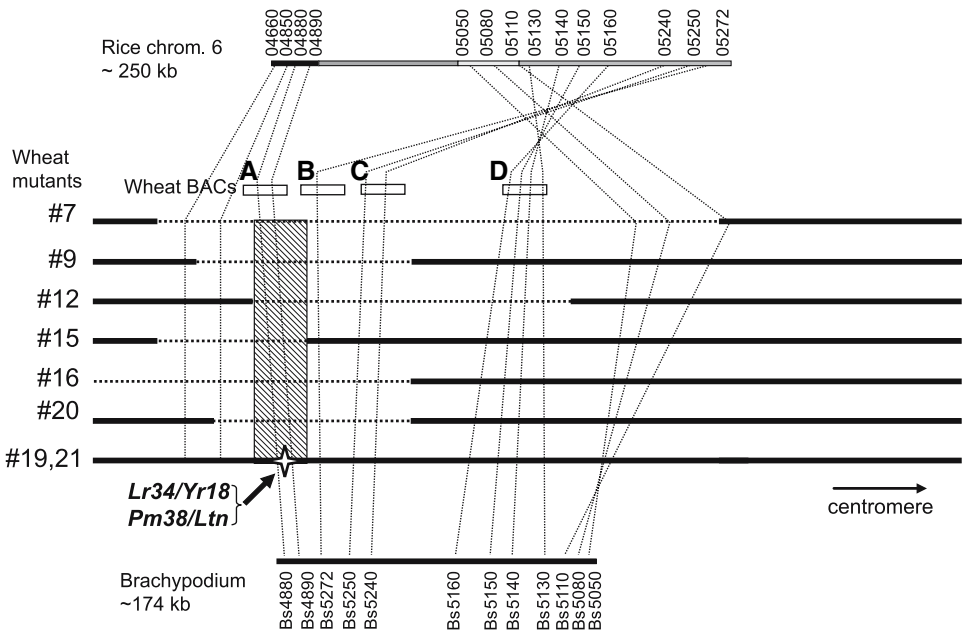

Fig. 2 Schematic representation of the wheat mutants showing deleted regions in *horizontal dashed lines* compared with the corresponding regions in rice and *Brachypodium sylvaticum*. The *dashed lines* link up orthologous genes from *Brachypodium* (Bs4880 to Bs5050) and rice (Os06-04880 to 05272). The D genome BAC clones carry orthologous genes including *A* from "Chinese Spring": *BJ280740* and *TC239710*; *B* from *Ae tauschii*: *BAC22* and *AL810355*; *C* from *Ae tauschii*: *BF473324* and *D* from *Ae tauschii*: *CA500527* and *BQ788742*. The *shaded area* corresponds to smallest deleted region predicted to carry *Lr34/Yr18*

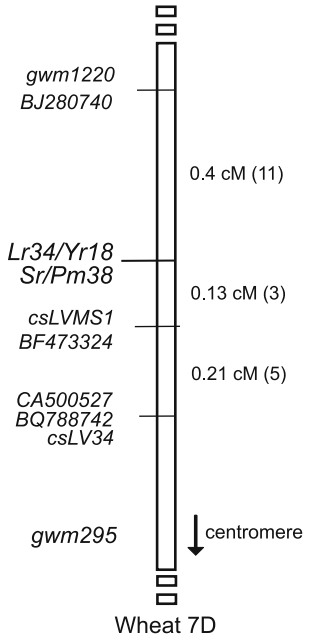

Fig. 3 High-resolution genetic map of the *Lr34/Yr18* region using a population of 1150 F2 lines derived from a cross between "Thatcher" (susceptible) and "RL6058" (*Lr34/Yr18*). The genetic distance is shown as centi-Morgans cM with number of recombinants included in *brackets*

resolution mapping family (Fig. 3). Wheat EST *BF473324* was positioned between *BJ280740* and *CA500527* and not on the proximal side as the corresponding genes in rice (Fig. 2). Genetic mapping therefore supported the findings from the mutant analysis confirming that gene order was disrupted between rice and wheat. *BJ280740* was separated on the distal side by 11 recombinants (0.4 cM) from *Lr34/Yr18* locus and *BF473324* by four recombinants (0.13 cM) on the proximal side. Wheat EST AL810355 (pectate lyase-like gene) was not mapped because the probe failed to detect an RFLP between parental lines; the SSR marker *csLVMS1* was mapped instead which was located within 3 kb on the proximal side of the putative orthologous pectate lyase-like gene isolated from *Aegilops tauschii* BAC clone (Fig. 4). SSR marker *csLVMS1* cosegregated with *BF473324* and was therefore genetically separated from *Lr34/Yr18*. In summary, a genetic interval of approximately 0.5 cM was defined on chromosome 7D that contained *Lr34/Yr18*. The genetic analysis also excluded the protein kinase-like gene (*BJ280740*) as a candidate gene for rust resistance.

Comparative analysis of the Lr34/Yr18 region with rice and Brachypodium

To gain a better understanding of the evolution of the *Lr34/Yr18* region, a *Brachypodium sylvaticum* BAC library was screened with wheat ESTs BJ280740 and CA500527. Nine BAC clones were identified and the largest one BAC A4 (28 A13) with an insert size of 174 kb was sequenced. A4 contained closely related sequences to wheat ESTs from the *Lr34/Yr18* region (Table 2) and corresponded to the same clone (common BAC library and coordinate A13 from plate 28), which was previously sequenced by Bossolini et al. 2007 (Genbank accession #EF059989). The gene order in the *Brachypodium* BAC closely matched the order of wheat genes as predicted from the overlapping set of deletion mutants (Fig. 2). A small change in gene order, however, was noted. Cytochrome c oxidase subunit and the protein kinase orthologs were deleted in mutant #7 but the arginine methyl transferase and superoxide dismutase orthologs

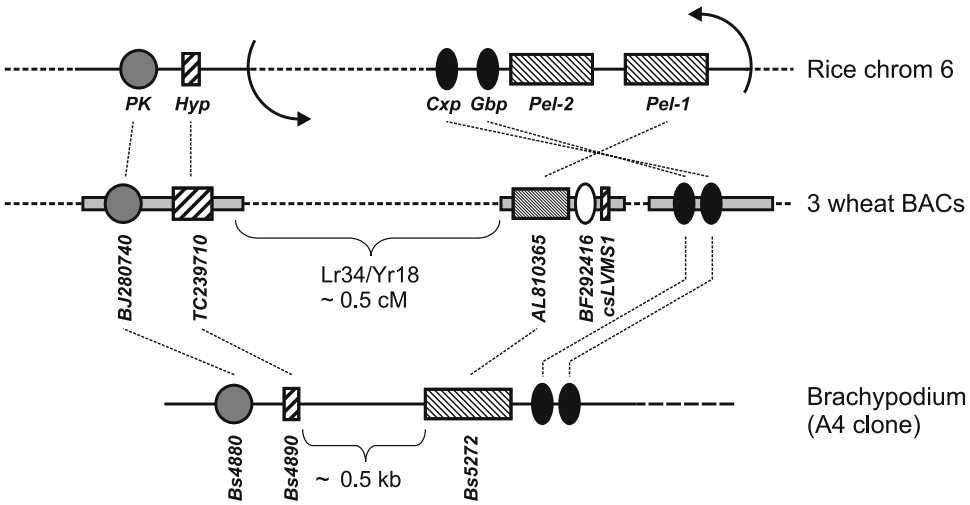

Fig. 4 Orthologous region of *Lr34/Yr18* in rice and *Brachypodium sylvaticum* including *PK*, protein kinase like gene, *Hyp*, hypothetical protein, *Pel*, pectate lyase-like gene, *Gbp*, GTP binding protein-like gene and *Cxp*, carboxypeptidase-like gene. The hypothetical protein in wheat is at least two times larger in size compared to the orthologs in rice and brachypodium. The *curved arrows* refer to the region in rice that is inverted relative to the corresponding gene regions in wheat. The corresponding genes in wheat are located on three separate BAC clones. The wheat BAC clone with pectate lyase-like gene also contained sequences that were closely related to EST BF229416 and the SSR marker *csLVMS1*

Table 2 Predicted gene order in *Brachypodium* BAC A4 compared with wheat ESTs tested on mutants and corresponding rice orthologs

B sylvaticum BACA4	Gene description	Wheat EST*	Rice ortholog
Predicted genes			
Bs4880	Kinase-U box	BJ280740	Os06g04880
Bs4890	Hypothetical protein	TC239710	Os06g04890
Bs5272	Pectate lyase	AL810355	Os06g05272
Bs5250	GTP-binding protein	BF473324	Os06g05250
Bs5240	Zinc carboxypeptidase	CK208877	Os06g05240
Bs5190	DNA repair protein XRCC1	BE515883	Os06g05190
Bs5180	Coatomer complex subunit	BE406581	Os06g05180
Bs5160	Probable sulfate transporter 3.4	BQ788742	Os06g05160
Bs5150	Hypothetical protein	BE606973	Os06g05150
Bs5140	Pentatricopeptide repeat	Bac subclone	Os06g05140
Bs5130	acyl-ATP thioesterase	CA500527	Os06g05130
Bs5120	Hypothetical protein		Os06g05120
Bs5110	Superoxide dismutase	BE490148	Os06g05110
Bs5090	Arginine methyl transferase	BE400881	Os06g05090
Bs5080	Cytochrome c oxidase subunit	CA595218	Os06g05080
Bs5050	Wall associated protein kinase	DQ013359	Os06g05050

were retained. The order of corresponding genes in *Brachypodium* represented by *Bs5050* (protein kinase-like), *Bs5080* (cytochrome c oxidase subunit-like), *Bs5090* (arginine methyl transferase-like) and *Bs5110* (superoxide dismutase-like) was not consistent with the order of the wheat orthologs as predicted in mutant #7 (Tables 1, 2; Fig. 2).

Delineating the Lr34/Yr18 locus

The mutant analysis identified a physical interval flanked by *BE499296* and *AL810355* (pectate lyase-like gene) that was predicted to contain the *Lr34/Yr18* locus. Because the deletion breakpoint in mutant #15 was located between the pectate lyase-like gene and one BAC end (BE22STS), we sub-cloned a 7 kb fragment that contained the pectate lyase-like sequence to check for any additional genes. An additional sequence with very high similarity (95%) to *Ae speltoides* cDNA (BF292416) and a new simple sequence repeat (SSR), *csLVMS1* were present in the BAC subclone (Fig. 4). The BF292416 related sequence was located on the proximal side of the pectate lyase-like gene and was therefore not a candidate gene for *Lr34/Yr18*. The *csLVMS1* marker, with an SSR-like motif of $(AT)_6TT(AT)_6$, detected allelic variants in the parental lines with and without *Lr34/Yr18* (Fig. 4).

Fine scale genetic mapping narrowed the interval to a region flanked by *BJ280740* (protein kinase like) and *csLVMS1*. The corresponding genes *Bs4880* (protein kinase like) and *Bs5260* (pectate lyase like) in *Brachypodium* were separated by approximately 5 kb of DNA. The only annotated gene *Bs4890* (*57h21.17* in Genbank accession EF059989) within this small region in *Brachypodium* had no match in the protein database. A relatively short sequence in *Brachypodium* appeared to span a region of approximately 0.5 cM in wheat. A wheat EST *TC239710* closely related to *Bs4890* was located on the same wheat BAC clone as *BJ280740* (within 150 kb) (Fig. 2). Given the relative large genetic distance of 11 recombinants between *BJ280740* and *Lr34/Yr18*, it is unlikely that *TC239710* was located at the resistance gene locus and part of a candidate gene. We therefore predict that rust resistance gene(s) are located between *TC239710* and the pectate lyase-like gene in wheat (Fig. 4). The same interval was associated with a break in collinearity between rice and *Brachypodium*. A TC239710-like sequence (*Os06g04890*) was located close to the protein kinase-like gene (*Os06g04880*) in rice but these sequences were separated from the pectate lyase-like gene (*Os06g05260*) by approximately 210 kb suggesting that collinearity was disrupted on the proximal side of *Os06g04890* which corresponded to the inversion breakpoint in rice. In summary, we were unable to find information in the orthologous genome sequence of rice and *Brachypodium* that could be used to isolate a candidate gene for *Lr34/Yr18*. We predicted that the rust resistance gene locus was located on a large insertion in wheat not found at syntenic positions in *Brachypodium* and rice.

Discussion

The *Lr34/Yr18* resistance gene has been deployed worldwide because it contributes to durable resistance against a broad range of leaf and stripe rust races. Stem rust resistance cosegregated with *Lr34/Yr18* in a high resolution

mapping population derived from a cross between "Thatcher" and a near-isogenic line RL6058, demonstrating that a single genetic locus conferred adult plant resistance to leaf, stripe and stem rust in the field. These results confirm earlier reports of a strong association of *Lr34* with adult stem rust resistance in approximately 100 recombinant inbred lines from the same cross between "Thatcher" and "RL6058" (Gavin Vanegas et al. 2007). There are several possible explanations for the enhanced stem rust resistance in "Thatcher" with *Lr34*: It is unlikely that the *Lr34/Yr18* locus by itself confers stem rust resistance given many wheat lines with *Lr34/Yr18* are susceptible to stem rust including "Chinese Spring" and "Lalbahadur(Parula7D)". It is also unlikely that allelic variation at the locus is responsible because when *Lr34/Yr18* from "Chinese Spring" was backcrossed into "Thatcher", the backcross line (Tc*5/Chinese Spring) was also showing enhanced resistance to stem rust (Dyck 1991). The likelihood of a tightly linked gene conferring stem rust resistance is very small given the large number of meiotic events screened in this study. The most likely explanation for the observed effect is that *Lr34/Yr18* interacts with unlinked gene(s) in the "Thatcher" background that result in enhanced stem rust resistance of adult plants. Recent evaluation of European wheat cultivars attributed improved stem rust resistance in "Forno", a European winter wheat unrelated to "Thatcher", in part to the presence of *Lr34/Yr18* suggesting that a positive effect of *Lr34/Yr18* on stem rust resistance may not be limited to the "Thatcher" background (Patham and Park 2007). Studies are underway to determine the genetic basis of the enhanced stem rust resistance gene(s) in "Thatcher" and to develop a molecular marker assay that will assist in the selection for these favourable gene combinations.

Both the rice genome and *Brachypodium* BAC sequence provided useful information for fine mapping of the *Lr34/Yr18* region in wheat. For most of the annotated rice genes, closely related wheat ESTs were identified that mapped to the predicted deletion bin on chromosome 7DS. However, the low level of polymorphism generally found in the 7DS region limited the number of wheat ESTs, which could be mapped genetically. We therefore, isolated gamma irradiation induced mutants that harboured deletions of various sizes. These interstitial deletion mutants were useful to map a large number of additional markers that were previously not polymorphic between parental lines. This enabled us to narrow down the physical interval carrying the *Lr34/Yr18* locus which led to the identification of a SSR marker *csLVMS1* with an atypical repeat structure, characterized by stretches of six CT dinucleotides on either side of two thymine bases, and was located within 0.13 cM of the resistance locus. In close proximity to *csLVMS1* an additional sequence with high homology to BF292416 was identified which was not present in the corresponding regions in rice or *Brachypodium*. The pattern of lost wheat markers in the overlapping interstitial mutants did not match the gene order of orthologous rice genes. Rearrangements, mainly due to an inversion in the gene order between rice and wheat, could account for these differences. The gene order in *Brachypodium* closely matched the overall order of wheat genes suggested by the wheat mutants. Our data was consistent with previous findings indicating that wheat shared greater gene order with *Brachypodium* than with rice (Foote et al. 2004) and was also in agreement with a recent study of the *Lr34/Yr18* region describing an inversion of approximately 220 kb in rice relative to *Brachypodium* (Bossolini et al. (2007).

Two of the mutants (#19 and #21) showed no loss of markers in the *Lr34/Yr18* region suggesting that gamma irradiation is capable of generating relatively small changes in the genome. It still needs to be confirmed that mutations within these lines occurred at the resistance locus and are not due to independent, second site events. The small deletion mutants generated in this study together with chemically induced mutants (Lagudah ES and Spielmeyer W, unpublished) will be required to confirm the identity of resistance gene(s).

A contiguous genomic sequence in *Brachypodium* contained putative orthologs of wheat genes that were flanking the *Lr34/Yr18* locus. A physical distance of approximately 5 kb in *Brachypodium* (between *Bs04880* and *Bs05260*) corresponded to more than 0.5 cM of genetic distance in wheat. Although the relationship of genetic to physical distance in this region has not yet been resolved, we predict that the *Lr34/Yr18* locus is associated with a large insertion in wheat with respect to rice and *Brachypodium*. The same interval was associated with an interruption of gene order leading to the inversion between rice and *Brachypodium*. The results suggest that the ancestral chromosomal region of *Lr34/Yr18* may have undergone at least two independent changes that altered gene order and possibly gene content between rice, *Brachypodium* and wheat. *Brachypodium* genomic sequences did not provide additional markers or genes, which were not already identified from the rice sequence.

The possibility of a single gene being responsible for durable resistance to leaf rust, stripe rust and powdery mildew should facilitate breeding of resistant cultivars. Additional stem rust resistance in "Thatcher" may be due to the interaction of *Lr34/Yr18* with other genes in the background. Future work will focus on the identification and development of markers for these "interacting" gene(s) to assist in the future selection of useful gene combinations. We reported previously a tightly linked marker *csLV34*, which can predict the presence of the disease resistance locus in a wide range of wheat backgrounds (Lagudah et al.

2006). We expect to answer more fundamental questions about the molecular basis of durable rust resistance in wheat by ultimately isolating the *Lr34/Yr18* gene(s).

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