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ORIGINAL PAPER

Molecular genetic characterization of the *Lr34/Yr18* slow rusting resistance gene region in wheat

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Abstract Wheat expressed sequence tags (wESTs) were identified in a genomic interval predicted to span the *Lr34/Yr18* slow rusting region on chromosome 7DS and that corresponded to genes located in the syntenic region of rice chromosome 6 (between 2.02 and 2.38 Mb). A subset of the wESTs was also used to identify corresponding bacterial artificial chromosome (BAC) clones from the diploid D genome of wheat (*Aegilops tauschii*). Conservation and deviation of microcolinearity within blocks of genes were found in the D genome BACs relative to the orthologous sequences in rice. Extensive RFLP analysis using the wEST derived clones as probes on a panel of wheat genetic stocks with or without *Lr34/Yr18* revealed monomorphic patterns as the norm in this region of the wheat genome. A similar pattern was observed with single nucleotide polymorphism analysis on a subset of the wEST derived clones and subclones from corresponding D genome BACs. One exception was a wEST derived clone that produced a consistent RFLP pattern that distinguished the *Lr34/Yr18* genetic stocks and well-established cultivars known either to possess or lack *Lr34/Yr18*. Conversion of the RFLP to a codominant sequence tagged site (csLV34) revealed a bi-allelic locus, where a variant size of 79 bp insertion in an intron sequence was associated with lines or cultivars that lacked *Lr34/Yr18*. This association with *Lr34/Yr18* was validated in wheat cultivars from diverse backgrounds. Genetic linkage between *csLV34* and *Lr34/Yr18* was estimated at 0.4 cM

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Introduction

The leaf rust (*Puccinia triticina* f. sp. *tritici*) resistance gene, *Lr34* (formerly *LrT2*), was first described in a wheat line PI58548 by Dyck (1977) and was later shown to be present in a large number of wheat cultivars (Dyck and Samborski 1982; Singh 1993; Singh and Rajaram 1994). *Lr34* confers adult plant resistance (APR), although under appropriate conditions it may confer resistance in seedlings to certain rust races (Dyck and Samborski 1982). The efficacy of *Lr34*-mediated APR also varies between different cultivar backgrounds and growth conditions. More importantly *Lr34* has been recognized as a major component of durable rust resistance as it can act synergistically with other leaf rust resistance genes (German and Kolmer 1992). Several additional traits have been reported to be associated with *Lr34*, including the presence of leaf tip necrosis (Ltn) (Dyck 1991; Singh 1992a), re-designated *Ltn1* to differentiate it from other Ltn phenotypes (Rosewarne et al. 2006);

enhancement of stem rust resistance genes at independent loci in the cultivar Thatcher (Dyck 1987); cosegregation with the stripe rust (*Puccinia striiformis* f. sp. *tritici*) resistance gene *Yr18* (McIntosh 1992; Singh 1992b) and more recently adult plant powdery mildew resistance (Spielmeyer et al. 2005).

An important feature of *Lr34* is its apparent non race-specificity, a requirement for durable rust resistance. Non race-specific resistance to rusts often involves "slow rusting" (Caldwell 1968) genes that are associated with increased time between infection and sporulation (longer latent periods), production of fewer uredinia and smaller uredinia sizes all of which have been observed in *Lr34* containing wheat (Drijepondt and Pretorius 1989; Rubiales and Niks 1995; Kolmer 1996). The use of the slow rusting gene pair *Lr34/Yr18* in combination with other slow rusting genes in wheat cultivars has been suggested among some of the factors contributing to near immunity to leaf and stripe rust infection (Singh et al. 2000a).

Dyck (1987) located *Lr34* to wheat chromosome 7D. Subsequent studies using various inter-varietal crosses aimed at dissecting APR genes for leaf and stripe rust in wheat have invariably reported major QTL on chromosome 7DS (Nelson et al. 1997; Singh et al. 2000b; Bariana et al. 2001; Suenaga et al. 2003; Schnurbusch et al. 2004a, b) attributed to the slow rusting gene(s) *Lr34/Yr18*. In wheat it is not uncommon for cultivars to possess one or more unknown independent resistance genes for leaf and stripe rust and therefore quantitative rather than qualitative descriptions have been applied in most attempts at precision mapping of the *Lr34/Yr18* locus. However, segregating families produced from specifically developed genetic stocks such as Thatcher and its near-isogenic pair (RL6058) have enabled both quantitative as well as qualitative phenotypes to be classified (Spielmeyer et al. 2005). Such genetic stocks serve as a useful resource from which finer resolution of the *Lr34/Yr18* slow rusting resistance locus can be achieved as advances are made in identifying more useful molecular markers from this important agronomic region of wheat chromosome 7DS.

Despite major efforts at identifying molecular markers closely linked to *Lr34/Yr18*, to date there are no useable markers that can be considered to have a wide application across diverse wheat backgrounds. In a number of studies, molecular markers derived from cereal ESTs mapped to chromosome 7DS spanning the *Lr34/Yr18* QTL and/or homoeologous group 7S have been shown to have corresponding orthologs in a region of rice chromosome 6S (Nelson et al. 1997; Singh et al. 2000b; Kudrna et al. 2000; Schnurbusch et al. 2004b; Rosewarne et al. 2006). In this study we have utilized the knowledge accrued from colinearity of rice chromosome 6S and the *Lr34/Yr18* region of wheat chromosome 7DS to identify orthologous wheat expressed sequence tags (wESTs) (see Table 1) as well as diploid D genome bacterial artificial chromosomes (BACs) in an attempt to further characterize and develop potentially useful molecular markers for the *Lr34/Yr18* gene region. This is coupled with the use of genetic stocks specially developed to enhance qualitative phenotypic evaluation of the *Lr34/Yr18* slow rusting resistance locus and to enable marker validation.

Materials and methods

Plant material

Genetic stocks developed from 7D monosomics of the bread wheat cultivar Lalbahadur were used to develop single chromosome substitutions that retained chromosome 7D derived from the cultivar Parula, a source of *Lr34/Yr18*. The resulting line Lalbahadur (Parula7D) was crossed to Lalbahadur and a family of 150 (inclusive of the parents) F3 lines was phenotyped for the adult plant leaf rust resistance, *Lr34* and leaf tip necrosis, *Ltn1*. For simplicity Lalbahadur (Parula7D), hereafter will be referred to as Lalb + *Lr34*. A moderately high-resolution mapping family was generated from 768 F2 seeds (Lalbahadur × Lalb + *Lr34*) with 131 recombinant plants selected using a pair of microsatellite markers gwm4187 and gwm295 that flank the *Lr34* locus. The microsatellite markers gwm1220 and gwm295 were previously shown to flank *Lr34* on the distal and proximal end, respectively (Spielmeyer et al. 2005); gwm1220 was not polymorphic between Lalbahadur and Lalb + Lr34, and the polymorphic marker gwm4187 was used instead. Primer sequences of the gwm markers were kindly supplied by Dr. M. Ganal, TraitGenetics, Gatersleben, Germany. Other genetic stocks previously phenotyped for adult plant leaf and stripe rust resistance trait included the near isogenic lines (NIL), Avocet, Avocet + *Lr34/Yr18*; Thatcher, RL6058 (= Thatcher + *Lr34/Yr18*); RL6055, line 896, line 897; Jupateco73R and Jupateco73S (see Table 2). Other cultivars well characterized and known either to possess or lack *Lr34/Yr18* used in the study include Frontana, Anza, WW15, Otane, Forno, Bezostaja1, Opata, "Synthetic", Fukuho, Oligoculm, Condor, Janz, Cook (Table 2). Recombinant inbred lines (RILs) derived from Jupateco73R × Jupateco73S that segregated for the presence or absence of *Yr18* and *Ltn1* were also used in the study. Genetic linkage of a subset

Table 1 Rice genes from a region (2.02–2.4) of chromosome 6S and their potential orthologous wheat ESTs

Rice gene	Rice position (Mb)	Wheat EST	Gene description
Os06g04640	2.026	CD925913	DNA binding bromodomain
Os06g04660	2.031	BE493812	Oxidoreductase 2OG-Fe(II) oxygenase
Os06g04800	2.09	BE495774	Beta 1 proteasome subunit
Os06g04810	2.095	Not available	Leucine rich repeat
Os06g04820	2.1	BE499296	Leucine carboxyl methyl transferase
Os06g04830	2.106	Not available	Leucine rich repeat
Os06g04840	2.111	Not available	Leucine rich repeat
Os06g04850	2.13	BE499296	Homeobox protein
Os06g04880	2.144	BJ280740	Kinase-U box
Os06g04900	2.155	CV779862	Sugar transporter
Os06g04910	2.156	BJ218124	Similar to AT5g20070
Os06g04920	2.162	CD452485	Zn finger in Ran binding protein
Os06g04940	2.209	CD903842	Early nodulin93 protein
Os06g04950	2.18	BE414640	Nodulin protein
Os06g04980	2.192	CV774732	F-box domain
Os06g05010	2.209	CD903842	Early nodulin93 protein
Os06g05020	2.212	CD904552	Early nodulin93 protein
Os06g05050	2.23	DQ013359	Wall associated protein kinase
Os06g05080	2.242	CA595218	Cytochrome *c* oxidase subunit
Os06g05090	2.248	BE400881	Arginine methyl transferase
Os06g05110	2.257	BE490148	Superoxide dismutase
Os06g05130	2.274	CA500527	Acyl-ACP thioesterase
Os06g05140	2.282	BAC subclone	Pentatricopeptide repeat
Os06g05150	2.285	BE606973	Hypothetical protein
Os06g05160	2.292	BQ788742	Probable sulfate transporter 3.4
Os06g05180	2.32	BE406581	Coatomer complex subunit
Os06g05190	2.33	BE515883	DNA repair protein XRCC1
Os06g05240	2.36	CK208877	Zinc carboxypeptidase
Os06g05250	2.367	BF473324	GTP-binding protein
Os06g05250	2.371	AL810355	Pectate lyase
Os06g05310	2.388	CD881658	Transferase family

of polymorphic wESTs was determined from a sample of 107 RILs derived from the cross Opata × Synthetic (Nelson et al. 1997).

Field evaluation of rust phenotypes

The F3 lines from Lalb × Lalb + *Lr34* were phenotyped during the 2002–2003 crop season at CIMMYT's research station at Ciudad Obregon, Sonora State, Mexico for the APR to leaf rust based on *Lr34* and for the presence/absence of Ltn. Between 60 and 80 seeds of each F3 lines were grown as two rows 20 cm apart and 1 m long on top of 80 cm wide raised beds. Spreader rows of susceptible cultivar Morocco were sown as hill plots in the middle of 0.5 m wide pathway on one side of the plot. Rust infections were initiated by spraying the spreader rows with a suspension of urediniospores of *P. triticina* race MCJ/SP in a lightweight mineral oil, Soltrol 170 about 6 weeks after planting. The F3 lines were scored as homozygous resistant, segregating and homozygous susceptible. Ninety RILs from the cross Jupateco73R × Jupateco73S were evaluated in the field for reaction to leaf and yellow (stripe) rusts at CIMMYT research stations at Ciudad Obregon in 1998–1999 and Toluca research station in Mexico State in 1999 crop seasons, respectively, in a similar fashion as described above. Sixty RILs were maintained at CSIRO, Canberra, Australia for DNA marker analysis. Subsamples from CSIRO were then returned for phenotypic re-evaluation in Obregon, Mexico in 2005–2006. The moderately high-resolution mapping family of Lalbahadur × Lalb + *Lr34* was also evaluated in Obregon, Mexico during 2005–2006. The rust cultures and disease ratings were as previously described (Nelson et al. 1997; Singh et al. 2000a, b). Leaf rust evaluations in Australia (Plant Breeding Institute, Cobbitty) were conducted on the moderately high-resolution mapping family, with each line sown on 1 m row plots. A mixture of susceptible cultivars Krickauff, Camm and Morocco was planted as infector rows to facilitate development of leaf rust epidemic. Urediniospores of *P. triticina* pathotypes 104-1,2,3,(6),(7),11,13; 104-1,2,3,(6),(7),11 + Lr37 and 76-1,3,5,10,12 were used to inoculate infector rows in the field. All these pathotypes produce similar responses on

Table 2 Association of *Lr34/Yr18* with the molecular marker csLV34 in genetic stocks/cultivars of wheat

Cultivar/accession	Pedigree[a]	*Lr* gene(s)[b]	csLV34 allele	Source/origin
Thatcher (Tc)		−ve *Lr34*	a	USA
RL6058	Tc*6/PI5848	*Lr34*	b	Canada
RL6050	Tc*6/Terenzio	*Lr34, LrT3*	b	Canada
Line 896	Tc*6/Terenzio	*LrT3*	a	Canada
Line 897	Tc*6/Terenzio	*Lr34*	b	Canada
RL6059	Tc*6/PI58548	*Lr34, Lr33*	b	Canada
RL6057	Tc*6/PI58548	*Lr33*	a	Canada
RL6069	Tc*6/Lageadinho	*Lr34, LrT3*	b	Canada
Glenlea		*Lr34*	b	Canada
Frontana		*Lr34*	b	Brazil
Jupateco R		*Lr34*	b	CIMMYT
Jupateco S		−ve *Lr34*	a	CIMMYT
Opata		*Lr34*	b	CIMMYT
"Synthetic"		−ve *Lr34*	a	CIMMYT
Fukuho-komugi		*Lr34*	b	Japan
Oligoculm		−ve *Lr34*	a	Israel
Condor		*Lr34*	b	Australia
Cook		*Lr34*	b	Australia
Olympic		−ve *Lr34*	a	Australia
Anza		*Lr34*	b	USA
Forno		*Lr34*	b	Switzerland
Bezostaya		*Lr34*	b	Russia
Otane		*Lr34*	b	New Zealand
Chinese Spring		*Lr34*	b	China

[a] Pedigree information is only provided for the near isogenic stocks

[b] The presence of *Lr34* also indicates the presence of the associated *Yr18* stripe rust resistance gene

cultivars carrying *Lr34*. Urediniospores dispersed in light mineral oil were sprayed on infector rows using an ultra-low volume applicator

Isolation and characterisation of wESTs

Wheat ESTs identified through bioinformatic analysis and predicted to be orthologous to rice genes in a region of rice chromosome 6 (see Table 1) known to be colinear with wheat 7DS genomic region carrying *Lr34/Yr18* were cloned. Primers were designed from conserved regions of exons from the rice genes and wheat ESTs (all located in GenBank) and were used in PCR amplification of genomic DNA templates from Chinese Spring and/or *Aegilops tauschii* accession AUS18913. PCR amplification conditions, cloning into a pGEMT vector and sequencing of cloned products were performed as described by Seah et al. (1998).

Isolation of D genome BACs

Selected wheat ESTs (BE495774, CA500527 and BF473324) distributed across the targeted 7DS region were used to screen a BAC library derived from the diploid D genome progenitor, *A. tauschii* (Moullet et al. 1999). These BAC clones were used as an additional template to generate markers for analysis of the *Lr34/Yr18* region. Subclones of the BACs were generated from short-gun *Hin*dIII restricted DNA libraries and random clones were picked and arrayed in 384 microwell plates and subsequent fixation of colonies on membrane filters. Potential low copy subclones were developed from the BAC clones after the subclones were hybridized using total genomic DNA from Chinese Spring as a probe. Subclones for which hardly any visible or no hybridization signals were detected upon overnight autoradiography were inferred to be potentially low copy fragments and likely to be devoid of most of the frequent repeated sequences in the wheat genome.

Genomic DNA analysis

Total genomic DNA was extracted from leaves using the method described by Lagudah et al. (1991b) and RFLP analysis performed according to Lagudah et al. (1991a); however, longer gels (20 cm × 14 cm) were used. Genomic DNA from parental lines Lalbahadur and Lalb + *Lr34* as well as Thatcher and RL6058 (Thatcher + *Lr34*) were restricted with 12 restriction endonucleases (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Nde*I, *Nco*I, *Nsi*I, *Pst*I, *Xba*I, *Xho*I) and hybridized with radiolabeled inserts from the cloned ESTs. RFLPs located on chromosome 7D were confirmed using Chinese Spring and nullisomics for lines deficient for chromosome 7D.

In addition to RFLP analysis, several wEST derived PCR amplification products from the parental pairs Avocet/Avocet + *Lr34*, Lalb/Lalb + *Lr34*, Thatcher/Thatcher + *Lr34* and Opata/Synthetic were analysed for single nucleotide polymorphism (SNP). Sequencing

of cloned products and direct sequencing of PCR products was conducted using Bigdye® Terminator v3.1 Cycle Sequencing in accordance with ABI protocol (Applied Biosystems, Melbourne, Australia).

Conversion of RFLP to a PCR-based marker

An *Eco*RV fragment (~3.8 kb) that hybridized to wEST BQ788742 was subcloned from the diploid D genome BAC and the entire fragment sequenced. The end sequences were then used to generate primers to sequence the immediate flanking regions. The following primer pairs (see Fig. 1b): INT4F2-5′AATAGCAC AGTGTTTAGTTCC3′ and 788742R-5′CGTGGTGA CGTAGCAGGAGGC3′, SULTEx10F-5′ACCTGG TGCTGGCGAACCCGG3′ and L34OREV-5′CTCC AAAGTGGCACACACATATGC3′, 788742F-5′TCC AAGGCTTTTCTGGGTGTC3′, and L34R2-5′AGG CAGGCTGCAATGGGTCAC3′ were designed from the BAC subclone sequences (Fig. 2b) and were used to PCR amplify the equivalent sequence from the allohexaploid D genome of Lalbahadur and Lalb + *Lr34*. Comparative sequence analysis led to the identification of a polymorphic region from which a sequence tagged site (STS), csLV34, was developed. The primer pair used for csLV34 amplification comprised of csLV34F 5′GTTGGTTAAGACTGGTGATGG3′ and csLV34R 5′TGCTTGCTATTGCTGAATAGT3′. PCR amplification using csLV34F + R primers was performed using HotStar® *Taq* polymerase (Qiagen Pty Ltd, Vic., Australia) as per the manufacturer's recommendations. PCR conditions were as described by Seah et al. (1998) with annealing temperatures between 55 and 60°C being effective. Genetic linkage between csLV34 and other wESTs was determined from 107 RILs derived from the Opata × Synthetic family using Mapmaker-v2 (Lander et al. 1987). Instead of RFLP analysis, mapping of the locus associated with the wEST BJ280740 was conducted using a PCR-based polymorphism with the primer pair KUDSF2-5′ACGTTTCAG CATCAACCTGAA3′ and KUDSR1-5′GAACTTG CAATCAAGTAGGAG3′ at an annealing temperature of 58°C and run in 2.5% agarose gels.

Results

Isolation and characterisation of wheat ESTs from the Lr34/Yr18 region

From previous studies using cDNA clones from homoeologous group 7 chromosomes of wheat and barley, the adult plant disease resistance *Lr34/Yr18* was

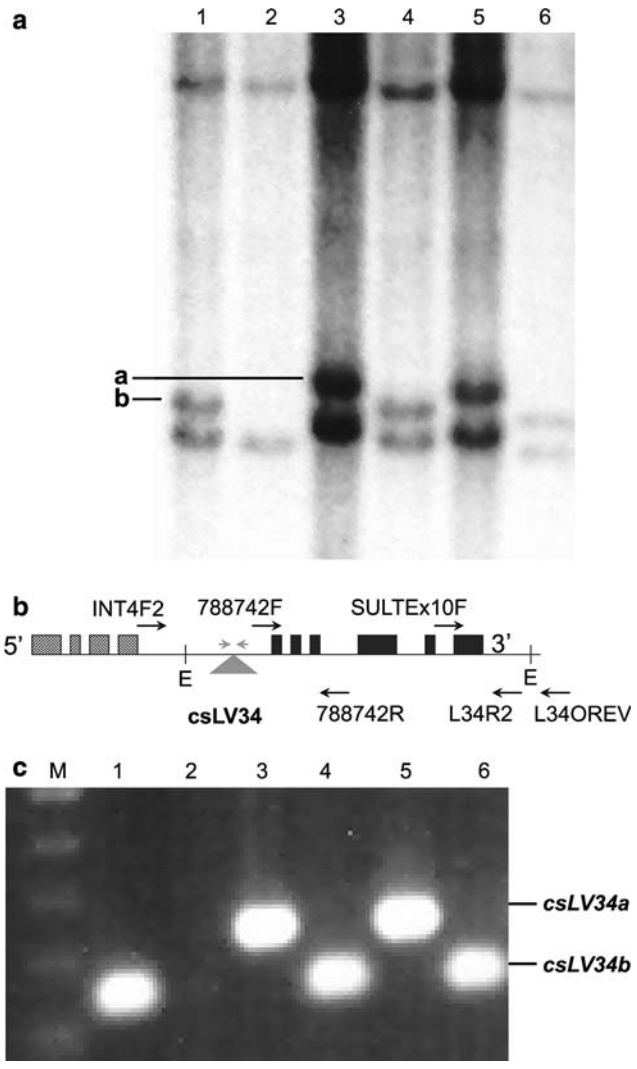

Fig. 1 **a** RFLP patterns of *Eco*RV restricted genomic DNA from different wheat lines probed with the wheat expressed sequence tag (wEST) BQ788742. (1) Chinese Spring, (2) nullisomic chromosome 7D, (3) Lalbahadur, (4) Lalbahdur + *Lr34/Yr18*, (5) Avocet, (6) Avocet + *Lr34/Yr18*. The *arrowed fragments* "a" (~3.8 kb) and "b" (~3.7 kb) refer to fragments associated with the absence and presence of *Lr34/Yr18*, respectively. Chromosomal location of the D genome fragment in Chinese Spring is confirmed through the 3.7 kb fragment loss in a nullisomic 7D line (*lane 2*). **b** Schematic representation of the gene, Os06g05160, an ortholog of the wEST BQ788742 that encodes a putative sulfate transporter class 3.4. The *boxes* shown represents exons and those in *black* are present in the *Eco*RV (*E*) subclone derived from the *A. tauschii* BAC. The *grey-hatched boxes* are the remainder of the exons found in the rice gene. The *horizontal arrows* depict various primers used to amplify equivalent regions from Lalbahadur and Lalb + *Lr34/Yr18*. The *broad arrow* in intron4 shows the location of the sequence tagged site csLV34 flanked by the *grey arrows* representing the forward (csLV34F) and reverse primers (csLV34R). **c** PCR amplification of the same set of genomic DNA in the same order as in (**a**) using the csLV34 primers. M-100 bp ladder molecular size markers; the csLV34a amplification product of 229 bp is associated with lines that lack *Lr34/Yr18* and the csLV34b product of 150 bp is associated with wheat lines or cultivars that possess *Lr34/Yr18*

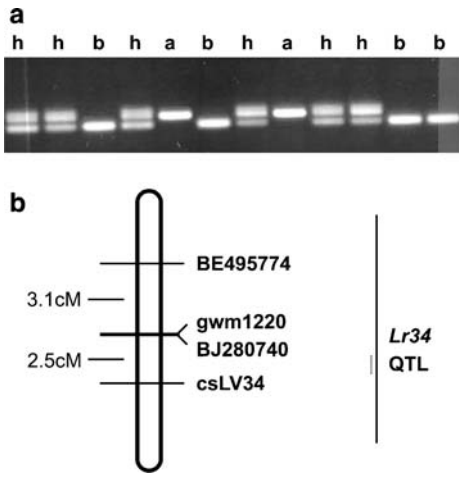

Fig. 2 **a** csLV34 PCR amplification products obtained from the F3 lines segregating for *Lr34/Yr18* from the Lalbahadur × Lalb + *Lr34* family. The codominant amplification products obtained as either "a" or "b" or "h" correspond with homozygous susceptible, resistant and heterozygous, respectively. **b** The linkage order and map distances in centi Morgans (cM) of the *csLV34* locus relative to the wESTs for beta 1 proteasome (BE495774) and the protein kinase with a U box domain gene (BJ280740) mapped in the Opata × "Synthetic" recombinant inbred lines (RILs). The vertical line shows the position of the *Lr34* QTL in Opata × "Synthetic" RILs. The *dotted vertical line* shows the most likely position of *Lr34* as against the broader QTL encompassing all the markers

positioned as a QTL on chromosome 7DS (Nelson et al. 1997; Kudrna et al. 2000; Schnurbusch et al. 2004b; Rosewarne et al. 2006). All of the cDNA clones or derived wheat ESTs from the *Lr34/Yr18* region identified orthologous sequences within a syntenic region located on rice chromosome 6. In an attempt to find additional markers from the *Lr34/Yr18* region, bioinformatic analyses based on Blast searches of genes located between 2.026 and 2.388 Mb of the rice chromosome 6 (Table 1) were used to identify additional corresponding wheat ESTs. Over 30 wheat ESTs were cloned and sequenced to confirm their identity. Wheat ESTs with >70% sequence identities over a minimum of 100 bp length relative to orthologous rice genes were used as probes in RFLP analysis. In a few cases, no wEST or other Triticeae homologs of the corresponding rice genes were identified such as the loci Os06g04810, Os06g04830 and Os06g04840 (Table 1) that encode leucine rich repeat (LRR) sequences. For these genes, rice genomic DNA was used as a PCR template to amplify and clone exon regions for use as probes. DNA hybridization in wheat using the rice LRR probes was weak and produced poor signals even under low stringency of hybridization.

To maximize the probability of finding RFLPs linked to *Lr34/Yr18*, three different genetic backgrounds from pairs of genetic stocks (Avocet and Avocet + *Lr34*, Lalbahadur and Lalb + *Lr34*, Thatcher and Thatcher + *Lr34*) were analysed using the wheat ESTs listed in Table 1 as probes. With the exception of wEST, BQ788742, all the ESTs failed to distinguish between lines that differed for the presence/absence of *Lr34*.

A wheat EST derived clone reveals polymorphism in Lr34/Yr18 wheat lines

The *Lr34/Yr18* region of wheat chromosome 7D lacked polymorphism with many wESTs when used as probes in RFLP analyses. This was despite electrophoresis conditions set-up to maximize resolution through longer runs of at least 16 h and relatively long gels of 20 cm. However, one clone derived from the wEST, BQ788742 (rice ortholog Os06g05160, encoding a putative sulfate transporter class 3.4) detected the same RFLP in all three pairs of parental lines differing for the presence or absence of *Lr34/Yr18* (Fig. 1a). Consistent RFLP patterns in the parental lines were observed with the restriction endonucleases, *Dra*I, *Eco*RV, *Nco*I and *Sac*I. RFLP analysis using *Eco*RV and *Nco*I was extended to other wheat cultivars from diverse backgrounds known to either possess or lack *Lr34/Yr18* (Table 2). The results were identical to the observations of the initial three pairs of parental cultivars; all *Lr34/Yr18* containing cultivars possessed an approximately 3.7 kb *Eco*RV restriction fragment while those that lacked this pair of slow rusting genes contained an ~3.8 kb fragment (Fig. 1a).

Because of the consistency in the ±*Lr34/Yr18* associated restriction fragments across different backgrounds, conversion of the RFLP into a STS based on PCR analysis was highly desirable for rapid genetic and breeding applications. A single hybridizing fragment, identical in size to the RFLP found in cultivars lacking *Lr34/Yr18*, was present in the diploid D genome progenitor, *A. tauschii* accession AUS18913, used in constructing the BAC library. Among the BAC clones that carry wEST CA500527, one clone contained the D genome homolog of BQ788742. The ~3.8 kb *Eco*RV restriction fragment present in *A. tauschii* and also associated with non *Lr34/Yr18* wheat cultivars was identified in the BAC, subcloned and sequenced. Sequence analysis confirmed the *A. tauschii* D genome fragment corresponded to the rice ortholog Os06g05160 and was a partial clone encoding a putative sulfate transporter class 3.4 that contained six exons (Fig. 1b), five introns and the 3′UTR (Fig. 1b). Newly designed primer pairs, with one primer specific to the introns or the 3′UTR from the diploid D

genome and the other from the exons (Fig. 1b), were used to amplify the equivalent D genome ±*Lr34* associated sequences from the hexaploid wheat cultivars Lalbahadur (that lacks *Lr34*) and Lalb + *Lr34*. Comparative analysis of these D genome members revealed complete sequence identity except for a 79 bp insertion found in one of the introns in *A. tauschii* and Lalbahadur but absent in Lalb + *Lr34*. Primer sequences designed to flank the insertion site and designated csLV34F and csLV34R were used to generate an STS that amplifies 150 and 229 bp PCR products in Lalb + *Lr34* and Lalbahadur (and *A. tauschii* AUS18913), respectively (Fig. 1c). The csLV34 STS provided a simpler analysis and correlated with the results obtained on the diagnostic RFLP between the wider set of wheat cultivars with and without *Lr34/Yr18* (Fig. 1c, Table 2). Two allelic variants based on size polymorphisms for the csLV34-STS have so far been detected; the larger "a" allele is associated with the non-*Lr34* carrying cultivars and smaller "b" allele with *Lr34* containing cultivars.

With the availability of a simple and robust PCR assay that was proving to be "diagnostic" for *Lr34/Yr18* from diverse wheat cultivars sourced from different parts of the world (Table 2), we extended the analysis to a range of *Lr34* NILs that were first developed in wheat (Dyck 1979). Three different sources of *Lr34* plus other *Lr* genes derived from the wheat genotypes PI58458, Terenzio and Lageadinho were backcrossed five times into cultivar Thatcher. Without exception all NILs that inherited *Lr34* also carried the *csLV34b* allele and those without *Lr34* but carrying other *Lr* genes possessed the *csLV34a* allele (Table 2).

Genetic linkage analysis of csLV34 relative to Lr34/Yr18

Linkage of the *csLV34* STS locus to the *Lr34/Yr18* locus was analysed in families for which the APR trait could be scored as a single Mendelian trait rather than a quantitative trait. In a previous study of 115 RILs from a Thatcher × RL6058 (= Thatcher + *Lr34*) cross, *Lr34/Yr18* unambiguously segregated as a single locus (Spielmeyer et al. 2005). In the current study, the Thatcher × RL6058 family was genotyped for the *csLV34* locus and it was found to cosegregate with the *Lr34/Yr18* locus. We extended the analysis to a different family of 60 RILs obtained from a cross between Jupateco73R and Jupateco73S in which *Lr34/Yr18* also segregated as a single genetic locus and, again, no recombinant individuals were identified. Therefore we examined *csLV34*STS–*Lr34/Yr18* in a third family of 148 F3 lines derived from the progeny of the single chromosome substitution, Lalbahadur × Lalb + *Lr34*. In this family *Lr34* segregated as a single Mendelian trait (32 homozygous resistant:78 segregating:38 homozygous susceptible; $\chi^2 = 0.91$, $P < 0.01$). All the homozygous resistant lines carried only the *csLV34b* allele and all but one of the homozygous susceptible lines possessed only the *csLV34a* allele (Fig. 2a). The one exception carried both alleles. Among the segregating lines, 75 out of the total of 78 were heterozygous at the *csLV34* locus (Fig. 2a); of the three exceptions, two lines contained the *csLV34b* allele while the third line possessed a *csLV34a* allele. Because the DNA used in the analysis was subsampled from F3 plants rather than the original F2 plant and pooled, it raises the question of the true genotypic representation found in the samples for a given F3 line. Further tests on the four apparent recombinants between csLV34 and *Lr34* are required to confirm the authenticity of the recombination. A reliable estimate obtained so far on recombination between *Lr34/Yr18* and csLV34 is from the moderately high-resolution mapping family, Lalbahadur × Lalb + *Lr34* (1536 gametes), is 0.4 cM. Five recombinant lines were identified between csLV34 and *Lr34* from field evaluations of leaf rust resistance conducted at two sites in Australia and Mexico.

Since none of the other derived wEST clones, apart from BQ788742—the entry point for developing csLV34, detected polymorphism between pairs of parental lines in crosses where *Lr34/Yr18* segregated as a single locus, the genetic position in wheat of *csLV34* relative to other wESTs was determined in a different family. Rosewarne et al. (2006) previously mapped the wEST BE495774, which encodes part of the exons of β1 proteasome subunit, within the *Lr34* QTL region in the Opata × "Synthetic" recombinant inbred family. In the current study one other wEST, BJ280740 (Table 1), which represents part of a gene encoding a protein kinase with a domain implicated in ubiquitination (U box) could differentiate the D genomes of the parental pair Opata and "Synthetic". This differentiation was due to an insertion of 11 bp sequence in intron4 (obtained by PCR using the primers KUDSF2 and KUDSR1) of the predicted gene found in the D genome of *T. aestivum* cv Opata and other common wheat cultivars. By contrast the deletion variant was found in the D^t genome of *A. tauschii* present in "Synthetic" (Nelson et al. 1997) and other *A. tauschii* accessions (data not shown). Thus *csLV34* could be mapped relative to the β1 proteasome (BE495774) and the protein kinase-U box (BJ280740) genes and followed the order: BE495774-3.0 cM-BJ280740-2.5 cM-*csLV34* (Fig. 2b). The micosatellite marker gwm1220 was

polymorphic in this family and was found to cosegregate with BJ280740 (Fig. 2b).

Isolation of diploid D genome BACs from the Lr34/Yr18 region and micro-colinear gene arrangements relative to rice

In view of the very low success rate in finding RFLPs based on wESTs, further attempts were made to identify low copy sequences that could be used as probes. Three wheat ESTs, BE495774, CA500527 and BF473324 (Table 1) were used to identify BAC clones from the diploid (*A. tauschii*) D genome library. DNA hybridization analysis of nulli-tetrasomic genetic stocks using CA500527 and BF473324 as probes showed these ESTs to be present on only homoeologous group 7, and thus the corresponding BAC clones were also derived from chromosome 7D. BE495774; however, identified sequence homologies located on group 1 and 7 chromosomes; BAC clones corresponding to chromosomes 1D and 7D were distinguished by RFLP analysis as previously reported by Rosewarne et al. (2006). BE495774 has been shown to be linked to the *Lr34* QTL in the wheat population of Opata × "Synthetic" (Rosewarne et al. 2006). RFLP analysis using low copy subclones from wEST BE495774 derived BAC could not distinguish between the *Lr34/Yr18* parental pairs, Avocet and Avocet + *Lr34*, Lalbahadur and Lalb + *Lr34*, or Thatcher and Thatcher + *Lr34*. Considerable effort was made to find other forms of polymorphism beside RFLP, by looking for SNPs. Sequence analysis of the intergenic regions surrounding the β1 proteasome gene (an ortholog of Os06g04800, Table 1) isolated from the 7D BAC as well as three of the seven introns present in the gene (sequence deposited as GenBank no. DQ023304) failed to reveal any consistent SNP that distinguished the three pairs of wheat parental lines with or without *Lr34*.

In the course of sequencing the upstream region of the β1 proteasome gene, a sequence encoding a leucine carboxyl methyl transferase (rice ortholog Os06g04820) was identified (GenBank no. DQ023304) instead of the ortholog of Os06g04810 that encodes an LRR protein (see Fig. 3). The absence of the LRR sequence next to the β1 proteasome gene on the 7D BAC clone is consistent with our earlier observation that no wEST orthologs of this gene could be identified. Moreover, the rice LRR as a probe failed to hybridise to wheat. Disruption of micro-colinearity in the β1 proteasome gene region between rice chromosome 6 and wheat chromosome 7D is thus evident through the deletion of this class of rice LRR sequences in wheat.

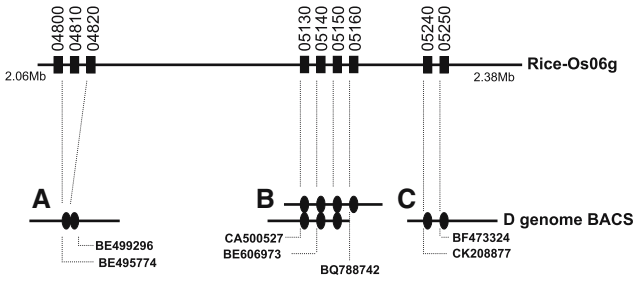

Fig. 3 Regions of micro-colinearity between rice genes and potential orthologous sequences derived from wheat ESTs found in corresponding diploid D genome BAC. In region A disruption between the rice genes, Os06g04800, -04810 and -04820 and their corresponding orthologs in the diploid D genome BAC is shown. The wheat D genome equivalent for -04810 is deleted and the remaining two collinear genes are in the same orientation as in rice. Conservation of micro-colinearity between rice genes, Os06g05130, -05140, -05150, and -05160 and their orthologs from an overlapping set of diploid D genome BACs is shown in region B (the D genome ortholog of -05150 was identified in a BAC subclone). Similarly in region C, conservation of micro-colinearity between the rice genes, Os06g05240, -05250 and their potential orthologs in the diploid D genome BAC. The overall colineraity between rice and the Lr34 region of wheat with major rearrangements has been described in a recent publication (Bossolini et al. 2006)

Although RFLP and SNP analysis of subclones from the wEST BE495774 derived BAC clone showed very high sequence conservation between the three pairs of parental lines (±*Lr34*), the analysis also revealed disruption of micro-colinearity with rice. We therefore focused on micro-colinearity comparisons in the remainder of the wEST derived D genome BAC clones from the *Lr34/Yr18* region. An overlapping set of BAC clones containing the wEST CA500527 that encodes an acyl thioesterase (rice ortholog Os06g05130) was found to contain sequences that were colinear with three adjacent rice genes (Fig. 3). Similarly the wEST BF473324 (encoding part of a GTP-binding protein) derived D genome BAC clones also contained the adjacent gene for a putative zinc carboxypeptidase, as is the case in rice (ortholog Os06g05240) (see Fig. 3).

Discussion

An extensive search for genetic differentiation based on RFLPs from wESTs located in a region of wheat chromosome 7DS revealed a paucity of polymorphism between three different pairs of wheat genotypes that differ for the presence or absence of the slow rusting genes *Lr34/Yr18*. Albeit on a limited scale, SNP analysis confirmed sequence conservation in this region of the genome. Additional polymorphism may be identified from a larger scale SNP analysis, expanding the range of restriction endonucleases to include 4 bp cutters for

RFLP identification, or other higher resolution marker systems such as single stranded conformational polymorphism (SSCP). It is not unexpected to find limited variation in the D genome as this observation reflects the overall low level of polymorphism of the D genome relative to the A and B genomes that has been reported in wheat (Cadalen et al. 1997; Ogbonnaya et al. 2005). The high level of gene sequence conservation inferred from the RFLP analyses of the *Lr34/Yr18* region has made finding diagnostic markers for this important disease resistance gene difficult.

The sole wEST derived clone, BQ788742, which produced an RFLP for the *Lr34/Yr18* genotypes enabled the development of a sequenced tagged site marker, csLV34, located within an intron of a predicted sulfate transporter-encoding gene. Amplification products from the *csLV34* locus have proven to be "diagnostic" for *Lr34/Yr18* across a range of wheat cultivars and NILs. These genotypes are from well-established material where previous genetic analysis has shown that the adult plant rust resistance trait is located in the 7DS region expected of *Lr34/Yr18*. This delineation is important in light of recent findings by Rosewarne et al. (2006) about the occurrence of other loci independent of *Lr34* that also exhibit cosegregation of adult plant stripe and leaf rust resistance as well as Ltn. csLV34 therefore is a valuable molecular marker for use in selection and breeding for one of the key genes utilized as a component of durable rust resistance in wheat. While we show that the *csLV34* locus is tightly linked to *Lr34/Yr18*, and the marker validated to be "diagnostic" in this set of germplasm, csLV34 is not a "perfect" marker for *Lr34/Yr18* based on high-resolution mapping. This distinction between "diagnostic" and "perfect" is important because of the occasional rare recombinants that may be encountered when selecting for *Lr34/Yr18* in breeding using csLV34.

In this study we have shown that some blocks of micro-colinearity of genes are conserved between orthologous loci from rice and wheat based on the D genome BACs. However, in view of mosaic patterns of rearrangements at a micro-colinear level that have been encountered in comparative cereal genomics (Keller and Feuillet 2000; Bennetzen and Ramakrishna 2002; Song et al. 2002) the overall precise order of the targeted wESTs in the *Lr34/Yr18* region will require large scale sequencing or generation of an overlapping series of deletion mutants. We are currently pursuing both approaches towards the positional cloning of *Lr34/Yr18*. In a recently published study, Bossolini et al. (2006) have reported on a large inversion in the collinear regions between rice and the *Lr34* region of wheat

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