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ORIGINAL PAPER

Gene-specific markers for the wheat gene *Lr34/Yr18/Pm38* which confers resistance to multiple fungal pathogens

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Abstract The locus *Lr34/Yr18/Pm38* confers partial and durable resistance against the devastating fungal pathogens leaf rust, stripe rust, and powdery mildew. In previous studies, this broad-spectrum resistance was shown to be controlled by a single gene which encodes a putative ATP-binding cassette transporter. Alleles of resistant and susceptible cultivars differed by only three sequence polymorphisms and the same resistance haplotype was found in the three independent breeding lineages of *Lr34/Yr18/Pm38*. Hence, we used these conserved sequence polymorphisms as templates to develop diagnostic molecular markers that will assist selection for durable multi-pathogen resistance in breeding programs. Five allele-specific markers (*cssfr1–cssfr5*) were developed based on a 3 bp deletion in exon 11 of the *Lr34*-gene, and one marker (*cssfr6*) was derived from a single nucleotide polymorphism in exon 12. Validation of reference genotypes, well characterized for the presence or absence of the *Lr34/Yr18/Pm38* resistance locus, demonstrated perfect diagnostic values for the newly developed markers. By testing the new markers on a larger set of wheat cultivars, a third *Lr34* haplotype, not described so far, was discovered in some European winter wheat and spelt material. Some cultivars with uncertain *Lr34* status were re-assessed using the newly derived markers. Unambiguous identification of the *Lr34* gene aided by the new markers has revealed that some wheat cultivars incorrectly postulated as having *Lr34* may possess as yet uncharacterised loci for adult plant leaf and stripe rust resistance.

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Introduction

Selection and development of wheat cultivars with effective and durable rust resistance constitute a global breeding objective in wheat (*Triticum aestivum*). A majority of genes that confer race-specific resistance to rusts and other biotrophic fungi (*R* genes) remain effective for only a few years when deployed at larger scale. Due to this rapid adaptation of the pathogen, new varieties with different resistance genes are continuously needed to replace varieties which have become susceptible. In contrast, the *Lr34* gene which confers race non-specific, partial and slow rusting resistance to leaf (or brown) rust (caused by *Puccinia triticina*), has remained effective for many decades. The gene was first characterized in Canada by Dyck (1977, 1987), but *Lr34*-containing germplasm has been used in cross-bred cultivars since the early part of the twentieth century. Three

breeding lineages of *Lr34* in wheat germplasm have been identified: They consist of (1) Far-East germplasm, (2) spring wheat lines from North and South America that were traced back to *Lr34* cultivar sources developed in Italy and (3) some winter wheat material in Europe (Kolmer et al. 2008).

The *Lr34* gene, then called *Yr18*, also confers moderate resistance to stripe or yellow rust (*Puccinia striiformis*) (McIntosh 1992; Singh 1992a) and powdery mildew (*Blumeria graminis*), where it is called *Pm38* (Spielmeyer et al. 2005). The locus is further associated with tolerance to stem rust (*Puccinia graminis*) (Dyck 1992) and barley yellow dwarf virus, *Bdv1* (Singh 1993). Flag leaves of many wheat cultivars containing *Lr34* in certain environments develop a necrotic leaf tip. This morphological marker is referred to as leaf tip necrosis (*Ltn1*, Dyck 1991; Singh 1992b). The *Lr34* gene is globally used as a component of durable rust resistance in breeding programs. Additive effects of *Lr34* and 3–4 genetically unlinked slow rusting genes result in high levels of resistance comparable to immunity and form the basis of durable resistance to leaf and yellow rusts in the spring wheat germplasm developed by CIMMYT (Singh and Trethowan 2007) and other wheat cultivars worldwide.

Expression of the *Lr34* resistance is predominantly at the adult plant stage and is frequently masked by effective *R* genes in the field. Consequently, there is considerable interest in developing effective methods for *Lr34* detection. Phenotypic assays reliant on *Ltn1* (Singh 1992b) can often be confounded by the cultivar background, the multigenic effects on overall leaf tip necrosis (Ltn) expression and variable expression in different environments. Development of molecular markers for *Lr34* has long been a major objective for marker assisted selection in wheat. After considerable effort, two markers *SWM10* (Bossolini et al. 2006) and *csLV34* (Lagudah et al. 2006) closely linked to the *Lr34/Yr18/Pm38/Ltn1* locus have been shown to be specific diagnostic tools for this multi-pathogen resistance trait. However, rare recombination events between these markers and *Lr34* in certain wheat lineages as well as equivocal gene postulations revealed limitations in the diagnostic specificity of these markers (Kolmer et al. 2008; McCallum et al. 2008).

The *Lr34* gene has recently been isolated and predicted to encode a pleiotropic drug resistance (PDR)-like ATP-binding cassette (ABC) transporter (Krattinger et al. 2009). The same gene controlled resistance based on *Lr34*, *Yr18*, *Pm38* as well as Ltn. The nucleotide sequence of *Lr34* spans 11,805 bp and consists of 24 exons. Comparison of different wheat cultivars revealed only two distinct haplotypes, a susceptible *−Lr34* and a resistant *+Lr34* haplotype. The two haplotypes differed in only three nucleotide polymorphisms, two of which were located in exons. One single nucleotide polymorphism (SNP) was located in intron 4. The two exon polymorphisms comprise a 3 bp deletion in exon 11 and a second SNP in exon 12 of *Lr34*. The same *+Lr34* haplotype was found in the three independent breeding lineages of *Lr34*, indicating that a single progenitor was likely to account for the origin of *Lr34*. Here, we use sequence polymorphisms in two exons of the *Lr34* gene, located on chromosome 7D, to develop perfect markers that unambiguously detect the presence or absence of this multi-pathogen resistance trait in a wide range of wheat genotypes.

Materials and methods

Plant material

Genetic stocks comprising the Indian cultivar Lalbahadur and its near isogenic line Lalbahadur *+Lr34* developed at CIMMYT, the Canadian cultivar Thatcher and the near isogenic derivative Thatcher *+Lr34* (RL6058) together with a wide range of cultivars known to either possess or lack the *Lr34* gene were used in assessing the markers (Table 1). Genotypic variants at the *csLV34* and *Lr34* loci were detected in different introductions of the Italian cultivar Ardito, maintained at the Australian Winter Cereal Collection (Tamworth, Australia). Two introductions, AUS1815 and AUS1818, were chosen as the reference standard for Ardito as they showed variant alleles expected for the presence of *Lr34*. A different batch of Ardito, AUS1817 possessed alleles associated with non-*Lr34* genotypes. A deletion mutant stock of *Lr34*, designated mu12, obtained by gamma irradiation (Spielmeyer et al. 2008) was included as a control sample to validate the D genome specificity of PCR amplification products in the *Lr34* gene.

Genomic DNA analyses

Total genomic DNA from leaf samples was obtained using standard methods (Lagudah et al. 1991; Stein et al. 2001). For DNA extraction from single seeds, they were placed into wells of a 96-well plate and crushed with a stainless steel ball bearing using a Retsch MM300 mixer mill (Retsch, Germany). Five seeds from each genotype were pooled and crushed, and an aliquot of the wholemeal sample placed into the microwell plate. After a short spin at 1,000 rpm, 300 µl of pre-warmed (65°C) extraction buffer (0.1 M Tris–HCl pH 8.0, 0.05 M EDTA pH 8.0 and 25% SDS) was added to each well. Samples were incubated at 65°C for 1 h and cooled at 4°C for 30 min; 150 µl of 6 M ammonium acetate was added, shaken vigorously, and left at 4°C for 30 min. The plate was centrifuged at 3,000 rpm for 30 min and the supernatant was transferred

Table 1 *Lr34* gene-specific marker classification of a reference set of wheat genotypes

Genotype	*Lr34* status	Origin	*cssfr1* (+*Lr34*) (bp)	*cssfr2* (−*Lr34*) (bp)	*cssfr6*[a] (bp)
Far east lineage					
Chinese Spring	+	China	517	–	451 + 135
Chuanmai 18	+	China	517	–	
RL6058	+	China	517	–	451 + 135
Norin 10	?	Japan	517	–	
Akakomugi	?	Japan	–	523	
Fukuho	+	Japan	517	–	
N/S American lineage					
Frontana	+	Brazil	517	–	
Fronteira	–	Brazil	–	523	
Opata	+	CIMMYT	517	–	
Synthetic	–	CIMMYT	–	523	
Jupateco R	+	CIMMYT	517	–	451 + 135
Jupateco S	–	CIMMYT	–	523	589
Avocet	–	Australia	–	523	589
Avocet +*Lr34*	+	CIMMYT	517	–	451 + 135
Glenlea	+	Canada	517	–	451 + 135
Thatcher	–	Canada	–	523	589
Anza	+	USA	517	–	
Chris	+	USA	517	–	
Condor	+	Australia	517	–	
Penjamo 62	+	CIMMYT	517	–	
Inia 66	–	CIMMYT	–	523	
Lalbahadur +*Lr34*	+	CIMMYT	517	–	451 + 135
Lalbahadur	–	India	–	523	589
European lineage					
Mentana	+	Italy	517	–	
Ardito	+	Italy	517	–	
Forno	+	Switzerland	517	–	451 + 135
Arina	–	Switzerland	–	523	589
Pegaso	+	Italy	517	–	
Bezostaja	+	Russia	517	–	
Kavkaz	+	Russia	517	–	
Roazon	–	France	–	523	
Maris Huntsman	–	UK	–	523	
Renan	–	France	–	523	589

[a] Not all lines were analysed with marker *cssfr6*

to a fresh deep well plate containing 180 µl of isopropanol per well, mixed thoroughly, and left at room temperature for 5 min to precipitate before centrifugation at 3,000 rpm for 30 min. The pellet was washed with 250 µl of 70% ethanol and air dried before being re-suspended overnight in 150 µl of water at 4°C. The plate was centrifuged at 3,000 rpm for 30 min and 50 µl of the supernatant was transferred to a fresh microtitre plate for storage at −20°C. Between 2 and 4 µl of this DNA was used to perform PCR.

Molecular markers based on the *Lr34* sequence polymorphism in exon 11

PCR amplification for markers *cssfr1* to *cssfr5* was performed using various primer combinations and 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 optimized annealing temperatures. Agarose gels (1%) were used to resolve the PCR products.

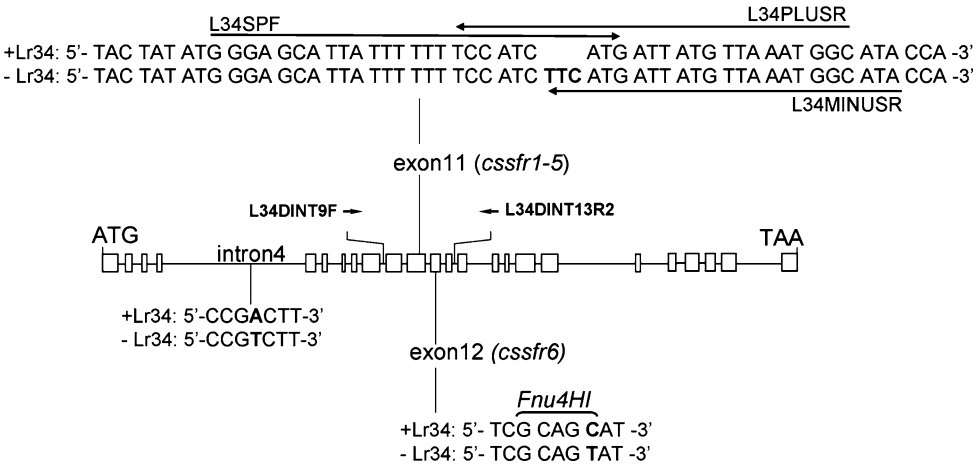

Fig. 1 Schematic representation of *Lr34* gene structure showing introns (*horizontal lines*) and exons (*boxes*) encoding the *Lr34* ABC transporter. Haplotype sequence differences are shown in *bold*, the locations of various primers are indicated by *arrows* and the restriction endonuclease site, *Fnu*4HI, created in exon 12 is indicated

The region spanning intron 9 to intron 13 of the *Lr34* gene was targeted for primers specific to the D genome which were then used in combination with primers anchored to sequence changes in exon 11 that differentiated *+/−Lr34* genotypes (Fig. 1). Oligonucleotides that were selected are as follows:

L34DINT9F	5′TTGATGAAACCAGTTTTTTTTCTA3′
L34PLUSR	5′GCCATTTAACATAATCATGATGGA3′
L34MINUSR	5′TATGCCATTTAACATAATCATGAA3′
L34SPF	5′GGGAGCATTATTTTTTTCCATCATG3′
L34DINT13R2	5′ACTTTCCTGAAAATAATACAAGCA3′.

The primer combinations L34DINT9F/L34PLUSR (designated as marker *cssfr1*) and L34DINT9F/L34MINUSR (*cssfr2*) were used in allele-specific amplification of the presence and absence of the *Lr34* gene, respectively. From primer concentration stocks of 10 µM we used 0.5 µl of each primer per 20 µl total volume of PCR reaction using an annealing temperature of 58°C. A multiplex PCR reaction was developed where each pair of the allele-specific primers were used in conjunction with the csLV34F/R primers (Lagudah et al. 2006) to ensure amplification had occurred in every reaction. Primer ratios in the multiplex reaction were 0.5 µl:0.25 µl for the allele specific and csLV34 primers, respectively (*cssfr3* and *cssfr4*). Another multiplexed system based on contrasting *+/−Lr34* amplification products was optimised for the primer pairs L34SPF/L34DINT13R2 and L34DINT9F/L34MINUSR (*cssfr5*). Primer ratios of 0.5 µl:0.5 µl or 0.5 µl:0.4 µl per 20 µl PCR of L34SPF/L34DINT13R2 and L34DINT9F/L34MINUSR, respectively, at an annealing temperature of 58°C were used.

Molecular markers based on the *Lr34* sequence polymorphism in exon 12 and on the mutation in exon 22 in cv. Jagger

A fragment spanning the SNP in exon 12 was amplified using primers cssfr6_f: 5′CTGAGGCACTCTTTCCTGTACAAAG3′ and cssfr6_r: 5′GCATTCAATGAGCAATGGTTATC3′. The 20 µl PCR reaction contained 0.125 mM dNTPs each, 0.5 µM of each primer, PCR buffer (10× stock concentrations were 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris pH 9.0), and 5 units Taq polymerase. The PCR was performed at an annealing temperature of 67.5°C and 35 cycles. PCR products were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) and eluted in 50 µl elution buffer. Forty microlitres purified PCR product were subsequently digested with 5 units of the restriction enzyme *Fnu*4HI (5′GCNGC3′, New England $BioLabs_{Inc.}$) over night. Digested fragments were separated on a 2% agarose gel.

A similar approach was used for marker *cssfr7*. A fragment of 247 bp was amplified spanning the mutation discovered in cultivar Jagger (Primers: cssfr7_f: 5′GCGTATTGTAATGTATCGTGAGAG3′ and cssfr7_r 5′CATAGGAATTTGTGTGCTGTCC3′, annealing temperature 65°C, 35 cycles). Forty microlitres of purified PCR product were digested with 10 units *Nla*III (5′CATG3′, New England $BioLabs_{Inc.}$) for three hours and fragments were separated on a 2% agarose gel.

Results

Haplotypes of *+/−Lr34* provide the molecular basis for diagnostic markers

The *Lr34* gene has recently been isolated and its complete nucleotide sequence is known (Krattinger et al. 2009). Comparative analysis of resistant and susceptible alleles of diverse wheat genotypes identified two haplotypes (Fig. 1). Three sequence polymorphisms were present in intron 4, exon 11, and exon 12 of the putative ABC transporter encoding gene. Nucleotide polymorphisms that distinguished the haplotypes were an A/T SNP in intron 4, an indel of TTC in exon 11 and a C/T SNP in exon 12

corresponding to the presence or absence of the resistant and susceptible alleles, respectively. PCR-based markers were developed around the sequence changes in exons 11 and 12 (Fig. 1).

Molecular markers *cssfr1* to *cssfr5* are based on indel polymorphism in exon 11

Allele-specific markers for genotypes with and without the resistant *Lr34* allele were developed based on the indel polymorphism in exon 11. This resulted in the design of primers L34PLUSR and L34MINUSR, respectively, in combination with L34DINT9F (Fig. 1). Initial tests with the primer pair L34DINT9F/L34PLUSR (*cssfr1*) carried out on two pairs of near isogenic *Lr34* lines (Lalbahadur and Lalb + *Lr34* as well as Thatcher and Tc + *Lr34*) resulted in the amplification of a 517 bp product specific for the *Lr34* isolines (Fig. 2a). Conversely the recurrent parents Lalbahadur and Thatcher lacking *Lr34* amplified a 523 bp product using the primer pair L34DINT9F/L34MINUSR (*cssfr2*, Fig. 2b). These markers did not amplify from a susceptible mutant (mutant 12) with an interstitial deletion of chromosome 7DS that includes the *Lr34* gene confirming the D genome specificity of the primers (Fig. 2).

Markers *cssfr1* and *cssfr2* are dominant markers. While they can be readily assayed by the presence or absence of amplification products when dealing with homozygous lines, progeny tests will be required with lines heterozygous at the *Lr34* locus. Furthermore, a failed PCR reaction will be misinterpreted as the absence of a specific allele typical of dominant markers. To overcome these limitations, we combined the robust codominant sequence tagged site marker, *csLV34*, tightly linked to *Lr34* (Lagudah et al. 2006) in a multiplex reaction with the aforementioned allele-specific markers for the presence or absence of *Lr34*. The codominant marker system for *csLV34* eliminates the problem of failed PCR and therefore ensures a valid diagnosis of the *+Lr34* and *−Lr34* alleles (*cssfr3* and *cssfr4*, Fig. 2c, d).

A third multiplex reaction was developed based on primer combinations L34SPF/L34DINT13R2 and L34DINT9F/L34MINUSR. This reaction amplified two bands of contrasting size which can easily be resolved in a 1% agarose gel: a 751 bp fragment specific for the *+Lr34* allele and a 523 bp fragment specific for the *−Lr34* allele. Hence, this primer combination provides a co-dominant marker (*cssfr5*, Fig. 2e) that allows rapid, economical, and reliable tracking of both the *+Lr34* and the *−Lr34* allele in the same PCR reaction.

Molecular marker *cssfr6* based on single nucleotide polymorphism in exon 12

The C/T SNP found in exon 12 provided an additional target to develop a codominant, *Lr34* gene-specific marker. The endonuclease *Fnu*4HI (5′GCNGC3′) cleaves the resistant allele, but not the susceptible one (Fig. 1). This restriction polymorphism was used for the development of a cleaved amplified polymorphic sequence (CAPS)-based marker, named *cssfr6*. The primer pair cssfr6_f/cssfr6_r

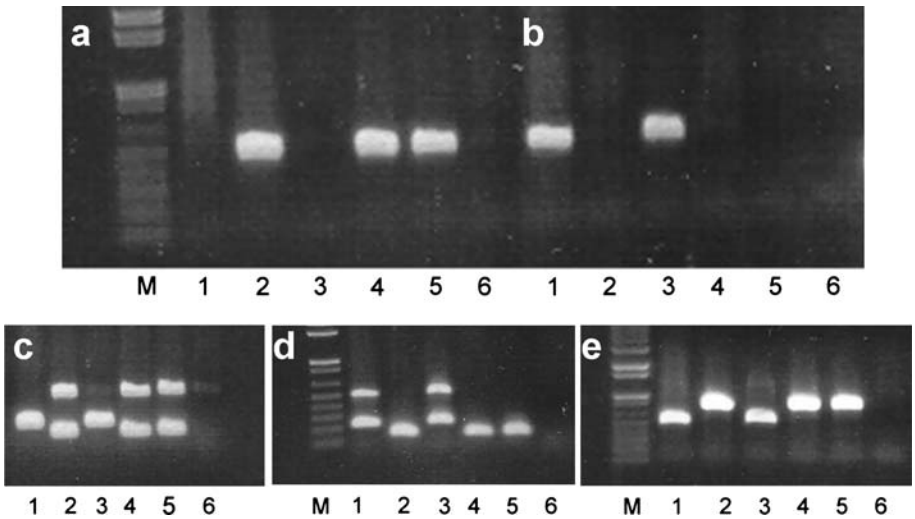

Fig. 2 PCR amplification products generated using a variety of markers for assaying allelic variants of the *Lr34* gene. **a**, **b** Allele-specific products from six genotypes based on markers *cssfr1* and *cssfr2* detecting the presence (*+Lr34*) and absence (*−Lr34*) of the *Lr34* gene, respectively. **c**, **d** To exclude failure of PCR of these dominant markers, *cssfr1* and *cssfr2* can be multiplexed with the codominant marker *csLV34* (*cssfr3* in **c** and *cssfr4* in **d**). **e** A third multiplex PCR amplified two bands, one for the *+Lr34* allele and one for the *−Lr34* allele (*cssfr5*, **e**). The tested lines are *1* Lalbahadur(Lalb), *2*Lalb + *Lr34*, *3* Thatcher(Tc), *4* Tc +*Lr34*, *5* Chinese Spring, *6 Lr34* deletion mutant mu12. *M* 100 bp size ladder

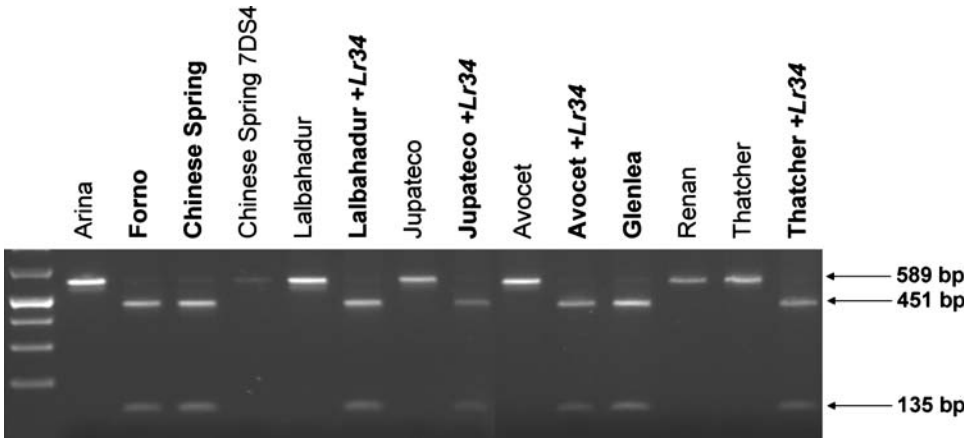

Fig. 3 Marker *cssfr6* tested on a set of wheat lines. Lines known to carry the *+Lr34* allele are marked in *bold*. Chinese Spring 7DS4 is a deletion line derived from Chinese Spring (Endo and Gill 1996) that lacks *Lr34*. The ladder corresponds to the GeneRulerTm 1 kb DNA Ladder Plus (Fermentas)

amplified a 652 bp fragment from the *−Lr34* allele and a 649 bp fragment from the *+Lr34* allele covering the SNP in exon 12. Subsequent digestion of the amplified fragments with *Fnu*4HI resulted in cleavage of the 649 bp amplicon into three fragments of 63, 135, and 451 bp for the *+Lr34* haplotype (Fig. 3). The 652 bp amplicon from the susceptible haplotype was digested into only two fragments of 63 and 589 bp in size.

Validation of markers using reference genotypes

A set of wheat cultivars that have been well characterised for the presence or absence of *Lr34* was used to validate the newly developed gene-specific markers. The cultivars included genotypes representing the three different breeding lineages from which *Lr34* cultivars have been derived (Table 1). Regardless of the breeding lineage, all *Lr34* carrying genotypes possessed the same allele-specific product generated by *cssfr1*. All non-*Lr34* containing genotypes were positive for *cssfr2*. Similarly, *cssfr6* reliably distinguished between resistant and susceptible cultivars. Thus, the newly developed gene-specific markers for *Lr34* confirmed the nucleotide sequence variants, as detected by direct sequencing of the *Lr34* alleles (Krattinger et al. 2009).

Detection of a third *Lr34* haplotype

Earlier findings reported the presence of only two distinct *Lr34* alleles, one conferring resistance in *+Lr34* cultivars, and a second allele found in *−Lr34* varieties (Krattinger et al. 2009). However, when testing the diagnostic potential of the SNP located in intron 4, we found a new, third allele. The winter wheat cultivars Zinal, Allalin and Galaxie, as well as the spelt (*Triticum spelta*) varieties Ostro and Rouquin showed the *+Lr34* haplotype in intron 4, but had the *−Lr34* haplotype for the two markers in exons 11 and 12. Hence, these lines form a third haplotype (Table 2). Interestingly, the reciprocal allele (T, for SNP in intron 4 and *+Lr34* for both exon markers) was never observed. This finding suggests that this haplotype arose through mutation rather than recombination and probably represents the progenitor of the functional *+Lr34* haplotype. Two independent mutations in exons 11 and 12 in a landrace carrying the third haplotype would have resulted in the functional *+Lr34* allele.

Re-assessment of cultivars postulated to carry *Lr34*

In the pioneering studies of Dyck and colleagues in Canada, *Lr34* was identified in a wide range of wheat genotypes, and several near isogenic lines were developed in the cultivar Thatcher (Tc). One of the isolines, RL6077 (Tc*6/PI250413), with adult plant resistance to leaf and stripe rust and Ltn has been referred to as an *Lr34*-like gene or another source of *Lr34* (Dyck and Samborski 1979; Dyck 1987; Singh 1992a). In a genetic study from an intercross between a reference *Lr34* isoline, RL6058, and RL6077, the genes from the two lines were independent of each other. The *Lr34*-like gene from RL6077 was inferred to have translocated onto another chromosome based on the presence of quadrivalents in the RL6077/RL6058 hybrid (Dyck et al. 1994). Previous tests with the tightly linked *Lr34* marker, *csLV34*, showed that RL6077 carried the allele not associated with *Lr34*. Since a rare recombination event could not be ruled out, no conclusive evidence on the *Lr34* status of RL6077 could be established. With the availability of the gene-specific markers for *Lr34* in the current study, we demonstrated that RL6077 has the *−Lr34* allele (Table 3). Thus the adult plant *Lr/Yr* gene in RL6077 is different from *Lr34* indicating that only a single *Lr34* gene has so far been verified in bread wheats. Our findings do not lend support to the hypothesis that two *Lr34* genes are located on different chromosomes as previously suggested (Dyck et al. 1994).

Another cultivar, Cappelle Desprez, was postulated to have *Lr34* based on the observed genetic association of leaf

Table 2 Detection of a third *Lr34* allele in wheat breeding lines

Genotype	Origin	+/− *Lr34*[a]	A/T SNP intron 4	*cssfr1*/*cssfr2*	*cssfr6*
+*Lr34* haplotype representing the three independent sources of *Lr34*					
Chinese Spring	China	+	A	+*Lr34*	+*Lr34*
Avocet +*Lr34*	CIMMYT	+	A	+*Lr34*	+*Lr34*
Forno	Switzerland	+	A	+*Lr34*	+*Lr34*
−*Lr34* haplotype found in susceptible wheat cultivars					
Renan	France	−	T	−*Lr34*	−*Lr34*
Arina	Switzerland	−	T	−*Lr34*	−*Lr34*
Lines showing a third, new haplotype					
Zinal	Switzerland	?	A	−*Lr34*	−*Lr34*
Allalin	Switzerland	?	A	−*Lr34*	−*Lr34*
Galaxie	France	?	A	−*Lr34*	−*Lr34*
Ostro	Switzerland	?	A	−*Lr34*	−*Lr34*
Rouquin	Belgium	?	A	−*Lr34*	−*Lr34*

[a] Presence (+) and absence (−) of *Lr34*-based disease resistance in these lines has been well characterized during the map-based isolation of *Lr34*

Table 3 *Lr34* allele status of genotypes with previously uncertain *Lr34* gene postulations

Wheat genotype	*cssfr1* (presence of *Lr34*)	*cssfr2* (absence of *Lr34*)	*csLV34* allele[a]
RL6077	−	+	a
Capelle Desprez	−	+	a
AC Domain	−	+	b
Jagger[b]	+	−	b
H45	+	−	b
Salamanca75	−	+	a
Rayon89	−	+	a
Newton	−	+	b
Titlis	−	+	a
Westphal12A	−	+	a

[a] "a" allele: indicates absence of *Lr34*, "b" allele indicates presence of *Lr34*

[b] Jagger carries an additional point mutation, resulting in a premature stop codon and a non-functional protein

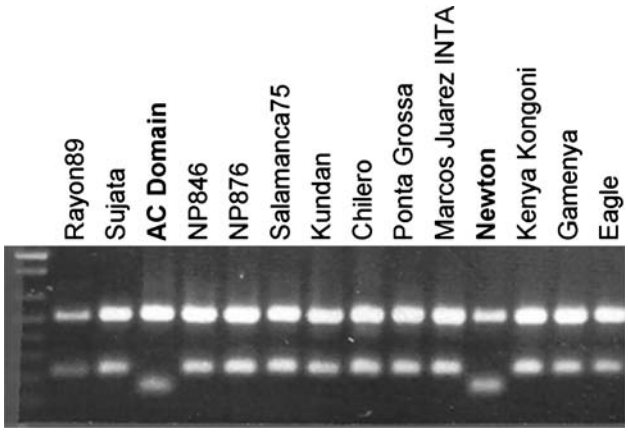

Fig. 4 Multiplex PCR simultaneously targeting the *Lr34* and tightly linked *csLV34* loci using marker *cssfr4*. Top fragment is the non-*Lr34* allele (523 bp) and the two bottom bands are the *csLV34* alleles. Rare recombinants are present in cultivars AC Domain (*lane 3*) and Newton (*lane 11*)

and stripe rust resistance (McIntosh 1992). Tests with the *Lr34* gene-specific marker indicate that it lacks *Lr34* and we conclude that Cappelle Desprez carries a different *Lr/Yr* adult plant resistance gene.

The Canadian cultivar, AC Domain was postulated to contain *Lr34* (Kolmer 1996) and also carries the *Lr34*-linked allele at the *csLV34* locus (Kolmer et al. 2008). However, a rare recombination between *Lr34* and the tightly linked *csLV34* locus has been reported in certain Canadian parental breeding lines (McCallum et al. 2008) which are contained in the pedigree of AC Domain. Application of the *Lr34* gene-specific markers confirmed the recombination event in AC Domain, and consequently the absence of *Lr34* in the cultivar (Fig. 4). Similarly, another North American cultivar, Newton, turned out to be a recombinant as observed in AC Domain and *Lr34* is absent, supporting the observations made by Robert Bowden (USDA, personal communication) on the absence of *Lr34* in Newton (Fig. 4).

Two wheat cultivars, Jagger and H45, classified as susceptible to leaf rust and stripe rust, respectively, on adult plants were shown to have *Lr34*-associated alleles of the *csLV34* marker (Kolmer et al. 2008). Additional tests with the gene-specific markers confirmed that both cultivars indeed carry the *Lr34* gene (Table 3). However, field observations have shown full susceptibility of cv. Jagger. Sequencing of the *Lr34* gene in Jagger identified a G/T point mutation that resulted in a premature stop codon. Consequently, the predicted protein of cultivar Jagger lacks 185 amino acids of the C-terminus and this allele is most likely not functional. This point mutation probably occurred in a resistant cultivar that carried the +*Lr34* allele. An additional CAPS marker (*cssfr7*) was developed, allowing

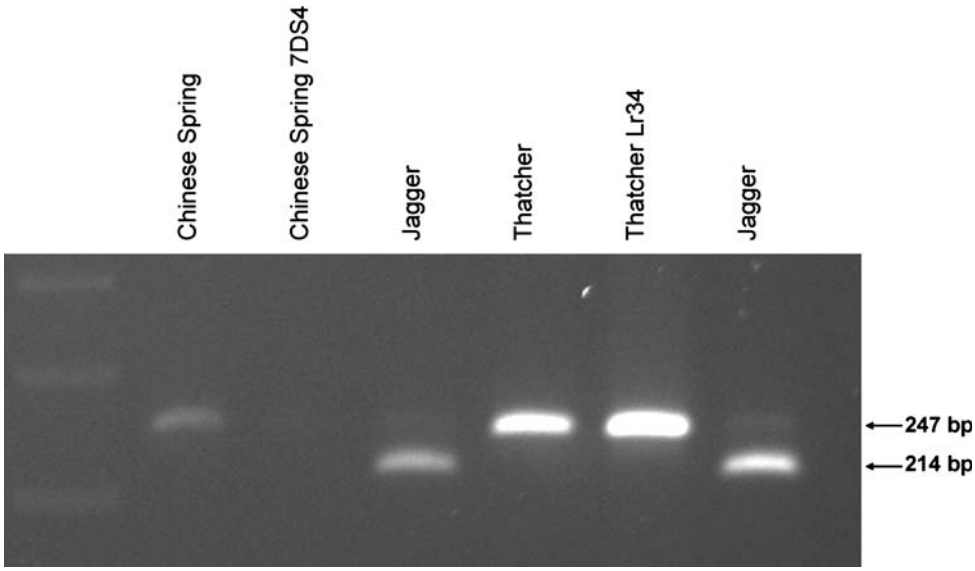

Fig. 5 Marker *cssfr7* that allows detection of a mutated and probably non-functional *Lr34* allele as it was found in cultivar Jagger. Chinese Spring 7DS4 is a deletion line derived from Chinese Spring that lacks *Lr34*. The bands of the ladder (GeneRulerTm 1 kb DNA Ladder Plus, Fermentas) correspond to 400, 300, and 200 bp (*top* to *bottom*)

diagnostic detection of the mutated Jagger allele. The restriction enzyme *Nla*III cleaves the mutated allele found in Jagger, but not +/−*Lr34* alleles that do not carry the premature stop codon (Fig. 5). Complete sequencing of the *Lr34* gene in the cultivar H45 will also be necessary to determine if there is a mutation. It is also possible that a second site mutation in a gene required for *Lr34* resistance may have occurred in H45 if no change in the encoding *Lr34* sequence is detected.

Leaf tip necrosis, a morphological marker which under certain environmental conditions can be used to predict the presence of *Lr34* was previously assessed in 127 wheat genotypes at CIMMYT, Mexico (Kolmer et al. 2008). From these genotypes, 52 lines lacked the *Ltn* phenotype and carried the non-*Lr34* associated csLV34a allele. Of the 75 genotypes that expressed Ltn, 62 possessed the *Lr34* associated csLV34b allele, while the remaining 13 genotypes had the csLV34a allele. Use of the *Lr34* gene-specific markers confirmed that all 13 genotypes carried the −*Lr34* allele (Table 4; Fig. 4). Clearly, these observations demonstrate the existence of −*Lr34* containing wheat lines with *Ltn* phenotypes. Reports of a single locus with Ltn and dual leaf and stripe rust resistance have been made in wheat carrying the adult plant resistance gene(s) *Lr46/Yr29* (Rosewarne et al. 2006). Furthermore, additional adult plant rust resistance genes other than *Lr34* or *Lr46/Yr29* must be present in the 13 genotypes, as only a subset of them have been predicted to carry *Lr46/Yr29* (Kolmer et al. 2008).

Table 4 *Lr34* allele status of genotypes with Ltn but carrying the non-*Lr34* associated *csLV34* allele

Wheat genotype	*cssfr1* (presence of *Lr34*)	*cssfr2* (absence of *Lr34*)
Eagle	–	+
M10 (Sujata)	–	+
NP876	–	+
Rayon	–	+
Salamanca	–	+
Tarachi	–	+
Chilero	–	+
Gamenya	–	+
Marcos Juarez INTA	–	+
Ponta Grossa	–	+
Kenya Kongoni	–	+
Kundan	–	+
NP846	–	+

Ltn phenotypes obtained from Kolmer et al. (2008)

Discussion

In this study, we describe the development of highly diagnostic molecular markers based on the nucleotide sequence of the durable disease resistance gene *Lr34* active against several pathogen species. The sequences of the *Lr34* genes from resistant (+*Lr34*) and susceptible alleles (−*Lr34*) had been found to be mostly identical, with only three polymorphic nucleotide positions in the total gene length of 11,805 bp (Krattinger et al. 2009). This left very few opportunities for the development of diagnostic markers.

Markers *cssfr1* to *cssfr5* were designed on a 3 bp indel polymorphism in exon 11 of the *Lr34* gene. These markers can be resolved on standard agarose gels and therefore they provide simple and economical tools to reliably track *Lr34* and should be valuable particularly for breeders who do not use high-throughput SNP marker technology. This is especially true for *cssfr5*, a co-dominant marker that amplified both, the +*Lr34* and the −*Lr34* allele in a single multiplex reaction. On the other hand, the SNP in exon 12 that served

as template for the CAPS marker *cssfr6* may further be used to develop-specific markers for various SNP genotyping technologies which allow rapid and high-throughput screening.

All the markers presented in this study are suitable for marker assisted selection and various end users may choose which marker system fits well with their protocols for marker assisted selection. However, *cssfr5* and *cssfr6* are probably the best primer combinations for marker assisted selection: *cssfr5* allows detection by simple agarose gel electrophoresis while *cssfr6* is well suited for high-throughput analysis.

Validation of the newly developed markers on a set of reference genotypes representing the three independent breeding lineages of *Lr34* demonstrated perfect diagnostic values. Of the cultivars analysed, Mentana and Ardito are among the oldest +*Lr34* cross-bred genotypes and therefore provide an indication of how long the *Lr34* gene has been deployed in commercial agriculture. Both cultivars were released by Nazareno Strampelli at the beginning of last century (Borghi 2001), demonstrating how durable the *Lr34* gene has been. Interestingly, Mentana and Ardito are sib-lines linking the North/South American and European winter wheat lineages carrying *Lr34*. The founder cultivars for *Lr34* germplasm in North/South American wheats (Frontana) and European winter wheats (Bezostaya) have their pedigrees traced back to Mentana and Ardito, respectively (Kolmer et al. 2008).

The morphological trait Ltn and the tightly linked molecular markers *SWM10* and *csLV34* have so far been used to assist selection for *Lr34* (Singh 1992b; Bossolini et al. 2006; Lagudah et al. 2006). Selection based on the Ltn can sometimes be misleading, because overall Ltn is a multigenic effect and its expression varies among different environments. In this study, we demonstrate the existence of −*Lr34* containing wheat lines with *Ltn* phenotype. The genetic distances between *Lr34* and the markers *SWM10* and *csLV34* are 0.06 and 0.31 cM, respectively. Although these markers map very close to *Lr34*, recombination between these markers and the *Lr34* disease resistance gene may occur, resulting in a wrong determination of the *Lr34* status. Such rare recombination events could be identified in the Canadian cultivar AC Domain and the North American cultivar Newton. Hence, the newly derived markers based on the *Lr34* gene sequence provide more precise tools to determine the *Lr34* status of wheat cultivars. However, even these markers may fail to predict presence of *Lr34*-based resistance in rare cases where an additional mutation in a +*Lr34* cultivar results in a non-functional allele. We have identified such a mutation in the cultivar Jagger, where a single point mutation resulted in a truncated protein. Thus, molecular markers will greatly facilitate selection of complex traits such as durable disease resistance, but careful phenotypic evaluation will still be necessary to ultimately confirm the functionality of the genes that were selected by marker assisted breeding.

In summary, we have developed a new set of gene-based markers that provides unambiguous identification of *Lr34* carrying wheat genotypes. The unequivocal determination of *Lr34* in the primary gene pool of wheat will help to focus on other uncharacterised quantitative trait loci for slow rusting adult plant resistance. This will allow a more efficient combination of *Lr34* with other quantitative trait loci contributing to durable resistance.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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