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ORIGINAL PAPER

Fine-mapping of the leaf rust *Lr34* locus in *Triticum aestivum* (L.) and characterization of large germplasm collections support the ABC transporter as essential for gene function

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Abstract Leaf rust resistance gene *Lr34* is likely the most important leaf rust gene characterized to date. It has been characterized as an adult plant resistance gene and is known to enhance the resistance of other leaf rust resistance genes and to condition resistance to a number of other diseases. Located on chromosome 7D, this gene was identified to be one of six co-located genes of which, an ABC transporter was shown to be the only valid candidate. Ten new molecular markers were developed spanning the *Lr34* locus, including six novel microsatellite markers (cam), one insertion site-based polymorphism marker (caISBP), two single nucleotide polymorphisms (caSNP), and one gene-specific marker (caIND). Using these new markers and others that were previously published, a comparative fine map of the locus was constructed from five segregating populations representing 1,742 lines. Identification of a susceptible line with a recombination in the 4.9 kb interval between caSNP4 located in the ABC transporter gene and cam8 located just upstream of this gene provided further evidence to support the identity of the ABC transporter as *Lr34* by ruling out four of the adjacent genes. Originally, three mutations forming two haplotypes had been described for the ABC transporter gene. A third combination of the three mutations and an additional rare mutation in exon 22 were subsequently described. We identified an additional novel mutation in exon 10 that would cause a frameshift and is likely non-functional. This mutation was only found in *Lr34*− lines and constituted a novel molecular haplotype. Characterization of two germplasm collections of 700 *Triticum aestivum* lines permitted us to gain an understanding of the frequency of the ABC haplotypes characterized to date and their distribution in germplasm from and around the world. In addition to the four haplotypes previously described, a fifth haplotype was found in two of the 700 lines from the germplasm collections. These lines displayed the deletion in indel 11 characteristic of *Lr34*+ lines, but are likely susceptible to leaf rust. Mapping and haplotyping data suggest that of all the markers described herein, marker caIND11 is the best diagnostic marker for marker-assisted selection of *Lr34* because it is co-dominant, robust and with the exception of 2/700 lines, it is highly diagnostic. Other markers are also described to provide alternatives for laboratories with different technologies.

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Introduction

Leaf rust caused by the fungal pathogen *Puccinia triticina* is an important disease of wheat, a staple food for 50% of the world population. Leaf rust is the most frequent of the rust diseases (Kolmer 2005), causing significant yield loss reaching 5–15% on susceptible wheat varieties (Samborski 1985). Genetic resistance has been the strategy of choice to manage leaf rust (McCallum et al. 2007). To date, leaf rust resistance genes named *Lr1* to *Lr60* have been

characterized in bread wheat, durum wheat, and several diploid wheat species. Many of these genes are race-specific resistance genes and they have been overcome by the development of new races of leaf rust pathogen (Samborski 1985; McCallum et al. 2007). However, *Lr34,* an adult plant resistance gene (Dyck et al. 1966) confers similar levels of resistance to all races of leaf rust (Spielmeyer et al. 2005). *Lr34* has provided effective field resistance to leaf rust since the 1970s in Canada (McCallum et al. 2007). Besides providing resistance to leaf rust, *Lr34* enhances the level of resistance conditioned by other leaf rust resistance genes (German and Kolmer 1992), and is associated with leaf tip necrosis (Ltn) (Singh 1992), later renamed *Ltn1* to distinguish it from other Ltn phenotypes (Rosewarne et al. 2006). *Lr34* enhances resistance to stem rust (*Puccinia graminis*) (Dyck 1992) and barley yellow dwarf virus (Singh 1993). It co-segregates with *Yr18* conferring resistance to stripe rust (*Puccinia striiformis*) and was shown to have a positive interaction with *Pm38* conferring resistance to powdery mildew (*Blumeria graminis*) (Spielmeyer et al. 2005). *Lr34* is durable and its pleiotropic gene action renders it the most important wheat resistance gene characterized to date.

Using QTL analysis, *Lr34* was first mapped to the short arm of chromosome 7D (Dyck 1987). In the last few years, substantial efforts have been dedicated to map-based cloning of *Lr34* which was facilitated by the development of closely linked polymorphic markers combined with the development of interstitial deletion mutant lines (Spielmeyer et al. 2008; Krattinger et al. 2009). Spielmeyer et al. (2005) first positioned *Lr34* between two microsatellite markers, namely gwm1220 and gwm295. Chromosome walking efforts led to the development of the closely linked microsatellite marker swm10 (Bossolini et al. 2006). An RFLP marker converted to a PCR-based marker (csLV34) was developed almost at the same time (Lagudah et al. 2006). This marker has been used extensively to postulate the presence or absence of *Lr34* because of its ease of use, the simple allele patterns produced and the relatively close linkage to *Lr34*. Marker csLV34 was used to characterize *Lr34* in Australian wheat cultivars (Singh et al. 2007) and in a world wide collection (Kolmer et al. 2008), but it was not diagnostic in some genetic backgrounds, including broadly used Canadian wheat germplasm (McCallum et al. 2008; Lagudah et al. 2009). Using interstitial deletion mutants, Spielmeyer et al. (2008) were able to position microsatellite marker csLVMS1 only 0.13 cM from *Lr34* and assessed the position of gwm1220 to be flanking with an estimated distance of 0.4 cM. More recently, Krattinger et al. (2008) reported the development of markers csLVE17 and csLVA1/SWSNP3 positioning *Lr34* in a 0.15 cM interval that contained six predicted genes, namely a hexose carrier (HC), an ABC transporter (ABC), two cytochrome P450s (CYP-1, CYP-2), and two lectin receptor kinases (LRK-1, LRK-2), as well as two pseudogenes.

Krattinger et al. (2009) identified the ABC transporter gene as the sole candidate for *Lr34*. Although they did not demonstrate the gene function by complementation, analysis of mutants exclusively pointed to the ABC transporter gene as *Lr34*. The 11,805 bp nucleotide genomic sequence of this ABC transporter comprised 24 exons and was predicted to encode a protein of 1,401 amino acids. Krattinger et al. (2009) discovered three mutations within the ABC transporter sequence. Two single nucleotide polymorphisms (SNPs) in intron 4 and exon 12 and one 3 bp indel in exon 11 were observed between the resistant cultivar Chinese Spring (*Lr34+*) and susceptible cultivar Renan (*Lr34−*). Based on their assessment of 27 wheat accessions of diverse origins, the three polymorphisms were reported to constitute only two haplotypes, namely A/Del/C and T/TTC/T for *Lr34+* and *Lr34−* lines, respectively. Based on this observation, they hypothesized that *Lr34* had a single origin. When a more diverse germplasm collection was assayed, Lagudah et al. (2009) identified a third *Lr34* haplotype, i.e., A/TTC/T for which no lines were found to carry the reciprocal allele. Hence, they concluded that mutation rather than recombination gave rise to this haplotype. Lagudah et al. (2009) also identified a fourth mutation in the ABC transporter gene, i.e., a G/T SNP in exon 22 in cultivar 'Jagger'. This mutation is predicted to result in a premature stop codon, be non-functional, and likely explain why Jagger has the *Lr34+* haplotype A/Del/C, but does not exhibit *Lr34* field resistance. The frequency of this mutation is still unknown.

Here, we report on the development of several novel markers spanning the *Lr34* locus, including dominant and co-dominant markers characterizing the various alleles of the ABC transporter. These markers were assessed on two large germplasm collections consisting of 700 *Triticum aestivum* lines, and two novel haplotypes are reported. The novel markers were used to screen more than 1,700 recombinants from five populations that were extensively characterized for leaf rust resistance, thereby permitting fine-mapping of *Lr34* and providing further evidence to support the identity of the ABC transporter gene as *Lr34*. A novel mutation in exon 10 of the ABC transporter is also described.

Materials and methods

Plant materials

Two different collections of germplasm were used as sources for our genetic materials. A world collection (WC),

representing germplasm from various geographical areas of the world, comprised 337 hexaploid wheat (*T. aestivum*) accessions. The second collection (AM) included mostly North American germplasm (cultivars and breeding lines) and some historical germplasm totaling 363 accessions. To assess the 7D specificity of markers, Chinese Spring (CS) and its 21 nulli-tetrasomic lines (CSNT) were used. A subset of ten parental lines was used simultaneously to assess potential marker polymorphism. In addition, five segregating populations derived from three crosses and totaling 1,742 segregating lines were used to fine map the *Lr34* locus (Table 1). All populations were F_1 derived (Radovanovic and Cloutier 2003; McCartney et al. 2005, 2006; Huang et al. 2006). Populations were grown in inoculated field leaf rust nurseries at Glenlea, Manitoba, and Portage La Prairie, Manitoba, during the years 2005–2008. A mixture of *P. triticina* virulence phenotypes, representative of those found in Western Canada was used to inoculate rows of susceptible wheat lines, which were interspersed among the test lines and served as an inoculum source. Flag leaves on each progeny line were scored at maturity for leaf rust severity using a modified Cobb scale (Peterson et al. 1948). Each population was phenotyped from two to four times in randomized replicated trials over several years and locations to ensure accurate and reliable rust phenotyping.

Genomic DNA extraction

Plants were grown in a greenhouse up to the 3–4 leaf stage. A total of 100 mg of leaf tissue was collected from each line, immediately frozen in liquid nitrogen and then lyophilized in a freeze dryer for approximately 48 h. The freeze-dried tissues were ground to a fine powder using a tissue lyser (Qiagen, Newtown City, USA). DNA extraction was performed using the DNeasy® 96 plant kit as per manufacturer's instruction (Qiagen). The genomic DNA samples were quantified by fluorometry and diluted to 15 ng/μl.

Molecular marker development

The hexaploid wheat cultivar 'Glenlea' BAC library (Nilmalgoda et al. 2003) was screened with microsatellite marker swm10 (Bossolini et al. 2006) and five BAC clones were identified, fingerprinted and BAC end sequenced. Chromosome walking steps were repeated with BAC end markers and a contig of several overlapping clones was obtained from which four BAC clones spanning 275,208 nucleotides were sequenced (Supplementary data Fig. 1S). Putative microsatellites were predicted as previously described (Cloutier et al. 2009) using the default settings of the MISA software (Thiel et al. 2003).

Genomic sequences from the *Lr34* locus of Chinese Spring (FJ436983), Glenlea (FJ436984), Renan (FJ436985), and *Aegilops tauschii* (FJ436986) (Wicker et al. 2009, Krattinger et al. 2009) were used to develop additional microsatellite, single nucleotide polymorphism (SNP), insertion site-based polymorphism (ISBP) and gene-specific markers. For microsatellite development, the ~250 kb sequences were individually analyzed as described above. The genomic sequence information was further mined for ISBP by DNA sequence alignments. Dominant markers were developed to assess the SNP located in intron 4 (caSNP4) and exon 12 (caSNP12) of the ABC transporter sequence. A co-dominant marker was developed to flank the three nucleotide indel of exon 11 (caIND11).

Marker specificity and polymorphism assessment

The designed primers were assessed on CSNT lines and a small collection of ten parental wheat lines to verify 7D specificity and potential polymorphism. Markers polymorphic between parents were mapped onto 1–5 segregating populations (Table 1). Parents 87E03-S2B1 and RL4452 are known as white and short Glenlea,

Table 1 Description of the five populations used to fine map the *Lr34* locus

Cross	Name	Type	No. of lines	Lr34−	Lr34+
AC Karma × 87E03-S2B1[a]	98C18-DH	DH	372	AC Karma	87E03-S2B1[b]
AC Karma × 87E03-S2B1[a]	98C18-SSD	RIL	372	AC Karma	87E03-S2B1[b]
AC Domain × RL4452[c]	93E54[d]	DH	271	AC Domain	RL4452[d]
Thatcher × RL6058-886C	03B10A	DH	217	Thatcher-885J	RL6058-886C
Thatcher × RL6058-886A	03B10B	DH	510	Thatcher-885B	RL6058-886A

[a] Radovanovic and Cloutier (2003), Huang et al. (2006)

[b] 87E03-S2B1 = Glenlea*7/C7932 = White Glenlea

[c] RL4452 = Glenlea*6/Kitt = Short Glenlea

[d] McCartney et al. 2005, 2006

Table 2 List of primers used as markers and for sequencing the *Lr34* locus

Marker name	Primer sequence[a]	PCR conditions	Resolution method	Observed allele sizes[b]
cam1	F: TGGCATGAGAAGAAAGCGTA	Initial denaturation: 93°C, 2 min	ABI3130x1	338–361 (11 alleles)
	R: CCGCTACCGCAGTAATGTCT	Denaturation: 94°C, 1 min		
cam2	F: GACAACAAACAGGACCTGGCACT	Annealing: 58°C, 50 s	ABI3130x1	179/N
	R: CACCCAAAGAGAAGGAACCA	Extention: 72°C, 1 min		
cam8	F: GCTGGATCTCAACCTCCTGA	Cycles: 30	ABI3130x1	207/N
	R: CAGGTCACAGATGTGGATGG	Final extension: 72°C, 10 min		
cam11	F: CCAGGGTGCATCCCAAGTA	End: 15°C constant	ABI3130x1	298–303/N (7 allele sizes)
	R: ACCGAAAGTGTTTTGGAGTG			
cam16	F: AACAATGAATACCCTAGCAGAGC		ABI3130x1	308–314 (7 allele sizes)
	R: CGAACGCGTGGTTGTCAT			
cam23	F: CGGCCCTGAAAATCGTACT		ABI3130x1	373/383/385/389
	R: CATGTATGGTGCGTGCTTGT			
caIND11	F: GTCTCCCAATCTGCATGCTC		ABI3130x1	394/397
	R: TACCTCCCAAAAGCCAGTTG			
caSNP4	F: GCGTTTCTGTCACCAGAAGT	Initial denaturation: 94°C, 5 min	1.5% agarose gel	390/N
	R: AATAAACTCGCGCCTCTTGA	Denaturation: 94°C, 1 min		
caSNP12	F: TCCCCAGTTTAACCATCCTG	Annealing: 65°C, 30 s	1.5% agarose gel	234/N
	R: CATTCAGTCACCTCGCAGC	Extention: 72°C, 1 min		
		Cycles: 35		
		Final extension: 72°C, 10 min		
		End: 15°C constant		
caISBP1	F: CATATCGAGCTTGCCAAACG	As SNP4 and 12 but annealing at 60°C	1.5% agarose gel	391/509
	F: TCAGCCACACAATGTTCCAT			
	R: CGTGAGCACAGAGAAAACCA			
SEQ-SNP4	F: TAGCCAAAGAGCCAAACTTA[c,d]	As ISBP1	ABI3130x1—seq	604
	R: TGATCGCCTAGACGCCTACT[c,d]			
SEQ-IND10/ IND11/SNP12	F: CATATGCCCAGAAAGAAAAG[c,d]		ABI3130x1—seq	1,159
	R: AAATCCCCAGTTTAACCATCCT[c,d]			
	F: CATATGCAATACCAGCTTCA[d]			
	R: GCTGCATCAAGGAGTAAATC[d]			

[a] Forward primer of markers resolved on the ABI3130×1 were tailed at the 5′ end with sequence CACGACGTTGTAAAACGAC

[b] Size as estimated by Genographer and including the tail sequence

[c] Primers used for amplification of a portion of the 7D ABC transporter gene identified as Lr34

[d] Primers used to sequence the PCR amplicons flanking SNP4 or IND10/IND11/SNP12

respectively. They were derived by backcrossing using Glenlea as the recurrent parent and both carry the Glenlea *Lr34* locus.

All markers, primer sequences, PCR conditions, resolution methods and observed allele sizes are reported in Table 2. For microsatellite markers resolved on the ABI3130xl (Applied Biosystems, Foster City, CA), three separate reactions were resolved simultaneously. They were amplified independently with one of three M13-labeled fluorochromes (FAM, HEX, or NED) as described in Schuelke (2000). Aliquots of 2 μL from each reaction were pooled and diluted with 24 μL of water. A 2 μL aliquot of the combined diluted products was subsequently transferred to an ABI compatible plate. A total of 4 μL of Hi-Di formamide/GenScan Rox500 marker in a 3.9:0.1 ratio was added prior to denaturation and resolution on an ABI3130xl (Applied Biosystems). The ABI files were subsequently converted into a gel-like image using a modified version of Genographer (Benham et al. 1999, modified by Travis Banks and available at http://sourceforge.net/projects/genographer/) and sized using the peak size information.

Fine-mapping of *Lr34* locus markers

Novel microsatellite markers (cam1, cam2, cam8, cam11, cam16, cam23), ISBP marker (caISBP1), SNP markers (caSNP4, caSNP12), and co-dominant gene-specific marker (caIND11) as well as other previously described markers located near *Lr34* (gwm1220, Spielmeyer et al. 2005; swm10, Bossolini et al. 2006; csLV34, Lagudah et al. 2006; csLVMS1, Spielmeyer et al. 2008) were assessed on 1–5 of the segregating populations characterized for *Lr34* (Tables 1, 2). The genetic maps were constructed with MAPMAKER/Exp version 3.0b (Lander et al. 1987). The Kosambi mapping function (Kosambi 1944) was used to convert recombination fractions into centimorgans (cM) as map distances.

Germplasm characterization of the *Lr34* locus

ABC transporter-specific markers caSNP4, caSNP12 and caIND11 were assessed on 700 wheat lines using genomic DNA from the two germplasm collections (WC and AM) following PCR and resolution methods outlined in Table 2.

Sequencing of the ABC transporter

Gene-specific primers (Table 2) were designed to flank the three mutations (SNP4, SNP12 and IND11) previously identified in the ABC transporter gene (Krattinger et al. 2009). One primer pair amplified a 604 bp fragment flanking the SNP in intron 4 and a second primer pair amplified an 1,159 bp fragment flanking both SNP12 and IND11 mutations (Table 2). Single band amplification and chromosome 7D specificity of the primers were determined using the CSNT lines as described above. Genomic DNA from a subset of 172 lines from the two germplasm collections were PCR amplified in triplicate (3 × 10 μL) for each of the two primer pairs. The PCR amplicons from the triplicate reactions were combined and purified using MultiScreen-384 plates (Millipore, Billerica, MA) and re-suspended in 30 μL of water. A 2 μL aliquot was used to sequence the purified amplicon using BigDye V3.0 in 6 μL reactions as previously described (Huang and Cloutier 2008). Primers used for amplification were also used for sequencing. For the 1,159 bp fragment flanking SNP12 and IND11, two additional internal primers (Table 2) were used thereby generating four overlapping sequences ensuring that at least one sequence from both strands was generated for each of the mutation points. Sequencing reactions were resolved on an ABI3130xl. The sequences were processed using PHRED (Ewing et al. 1998) and assembled using CAP3 (Huang and Madan 1999) as implemented in SOOMOS (T. Banks, unpublished). The assembly was visualized in Jalview (Waterhouse et al. 2009) and SNPs and indels were recorded.

Results

Lr34 locus molecular marker development and physical location

A total of 21 and 24 putative microsatellite markers were identified by MISA from the ~275 kb and ~250 kb sequences contiguous to the swm10 marker and the ABC transporter gene, respectively. A total of 31 primer pairs were designed from these 45 putative microsatellites and tested on the CSNT lines for 7D specificity; 6 of the 31 primer pairs were 7D specific and polymorphic. Microsatellite markers cam1 and cam2 were developed from the swm10 contiguous sequence (Supplementary data Fig. 1S) and cam8, cam11, cam16, and cam23 were derived from the Chinese Spring, Renan, Glenlea, and *A. tauschii* sequences containing the ABC transporter gene. Primer sequences, amplification conditions, resolution methods, and observed allele sizes for all novel microsatellite and other markers used in this study are listed in Table 2. Figure 1a illustrates the 7D specificity and polymorphism of one of the polymorphic microsatellites, namely cam23. Primers were designed for 11 potential ISBPs and were similarly tested. Marker caISBP1 was developed as a co-dominant marker using a 3 primer combination PCR (Fig. 1b).

Microsatellite markers cam8, cam11, cam16, cam23, and marker caISBP1 further saturate the *Lr34* locus as previously defined to be located between markers csLVA1/SWSNP3 and csLVE17 (Fig. 2). Marker cam8 is located just upstream of the ABC transporter gene. In fact, marker cam8, cam11, and caISBP1 are all positioned between the ABC transporter and the first cytochrome P450 (CYP-1). Marker cam16 is located between CYP-1 and LRK-1 while cam23 was distal to CYP-2 (Fig. 2). These five novel markers will be useful in dissecting the role of the six genes found at the *Lr34* locus thus defined by the 0.15 cM csLVA1/SWSNP3-csLVE17 interval (Krattinger et al. 2009).

ABC transporter gene-specific SNP and indel-based markers

Two dominant markers specific for *Lr34* were developed to target the SNPs located in intron 4 (caSNP4) and in exon 12 (caSNP12) of the ABC transporter gene using primers with a selective 3′ end nucleotide and stringent PCR conditions (Table 2). In addition, an *Lr34*-specific co-dominant marker was developed to flank the three nucleotide indel of exon 11 (Fig. 1c). The 394 bp fragment represented lines with a TTC deletion (coding for a phenylalanine residue) in exon 11, characteristic of *Lr34*+ lines. The most common alternate allele (*Lr34*− associated) was

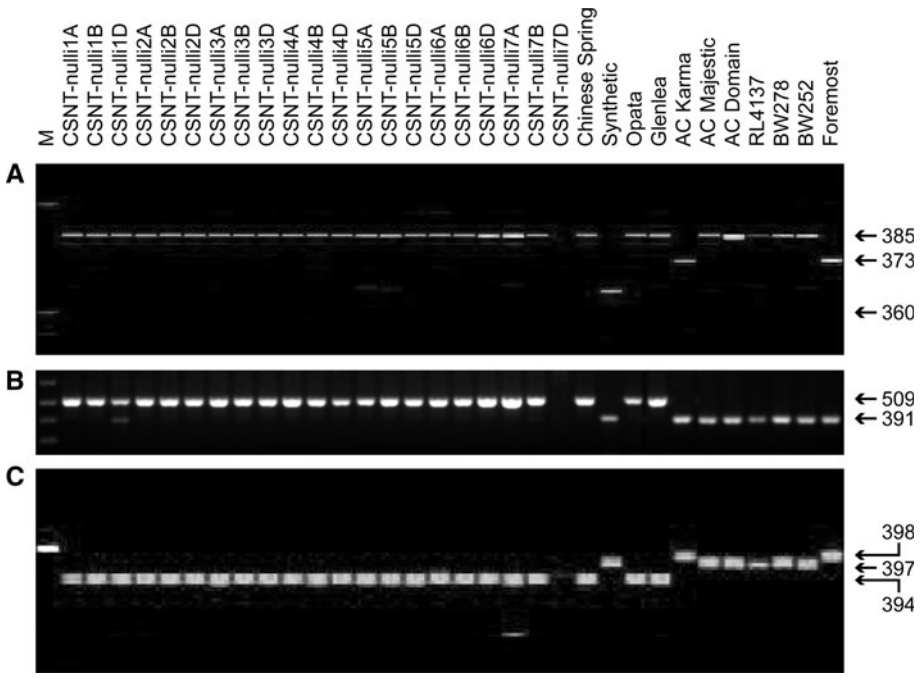

Fig. 1 PCR amplification showing 7D specificity and polymorphism of a subset of the markers developed herein: **a** cam23, **b** caISBP1, and **c** caIND11. Chinese Spring nulli-tetrasomic lines and a subset of 10 parental lines were used and are *labeled* at the *top*. *M* is for marker GenScan Rox-500 (Applied Biosystems) or 1 kb plus DNA ladder (Invitrogen). Fragment sizes are indicated on the *right*

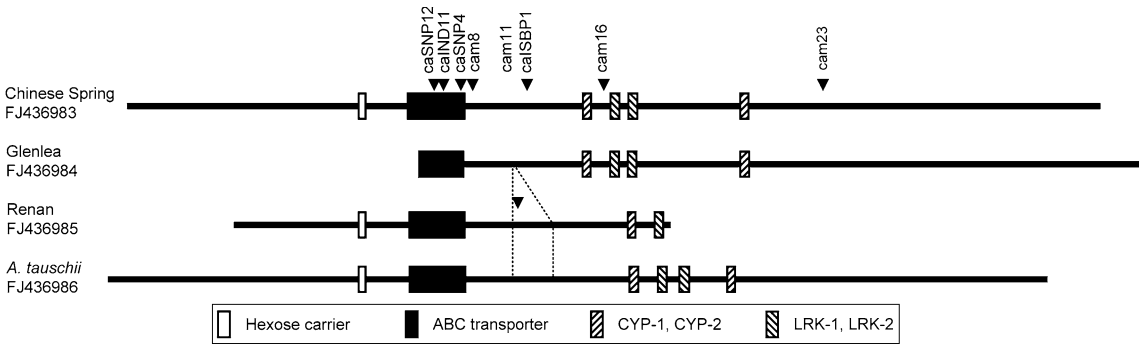

Fig. 2 Physical map of the *Lr34* locus BAC clones previously sequenced showing the positions of the novel markers in reference to the hexose carrier, the ABC transporter, the two cytochrome P450s, and the two lectin receptor kinases. *Filled triangles* indicate marker position

397 bp in size. However, some lines, e.g., AC Karma and Foremost (Fig. 1c, lane 26 and 32) produced a 398 bp amplicon, indicating the presence of an additional nucleotide in these amplicons (see below). Physical location of these three markers within the ABC transporter sequence is illustrated (Fig. 2).

Sequencing the ABC transporter polymorphisms

To confirm the accuracy of the caSNP4-dominant marker, a subset of 172 lines from the two germplasm collections were amplified with the 7D specific SEQ-SNP4 primer pair and the 604 bp fragments were sequenced. Sequencing confirmed the A/T SNP4 results obtained with the dominant caSNP4 marker. Similarly, the same subset of lines was amplified with primer pair SEQ-IND10/IND11/SNP12 and the 1,159 bp amplicons were sequenced in both orientations with a total of four primers (Table 2). Sequencing confirmed the C/T SNP12 results obtained with the dominant caSNP12 marker and the three nucleotide TTC indel of exon 11 obtained with the co-dominant marker caIND11. A new mutation in indel 10 was also revealed. Lines that amplified the 398 bp amplicon with the caIND11 primer pair had an additional A in exon 10. This frameshift mutation would cause amino acid changes in the last 26 residues of this exon. This A/− mutation was termed IND10. Cultivar Invader was one of the 71 accessions with the A indel in exon 10. This haplotype could be traced back in its pedigree to accessions Sinton, CT-262, and Lee (Supplementary data Fig. 2S) confirming that it is stably inherited.

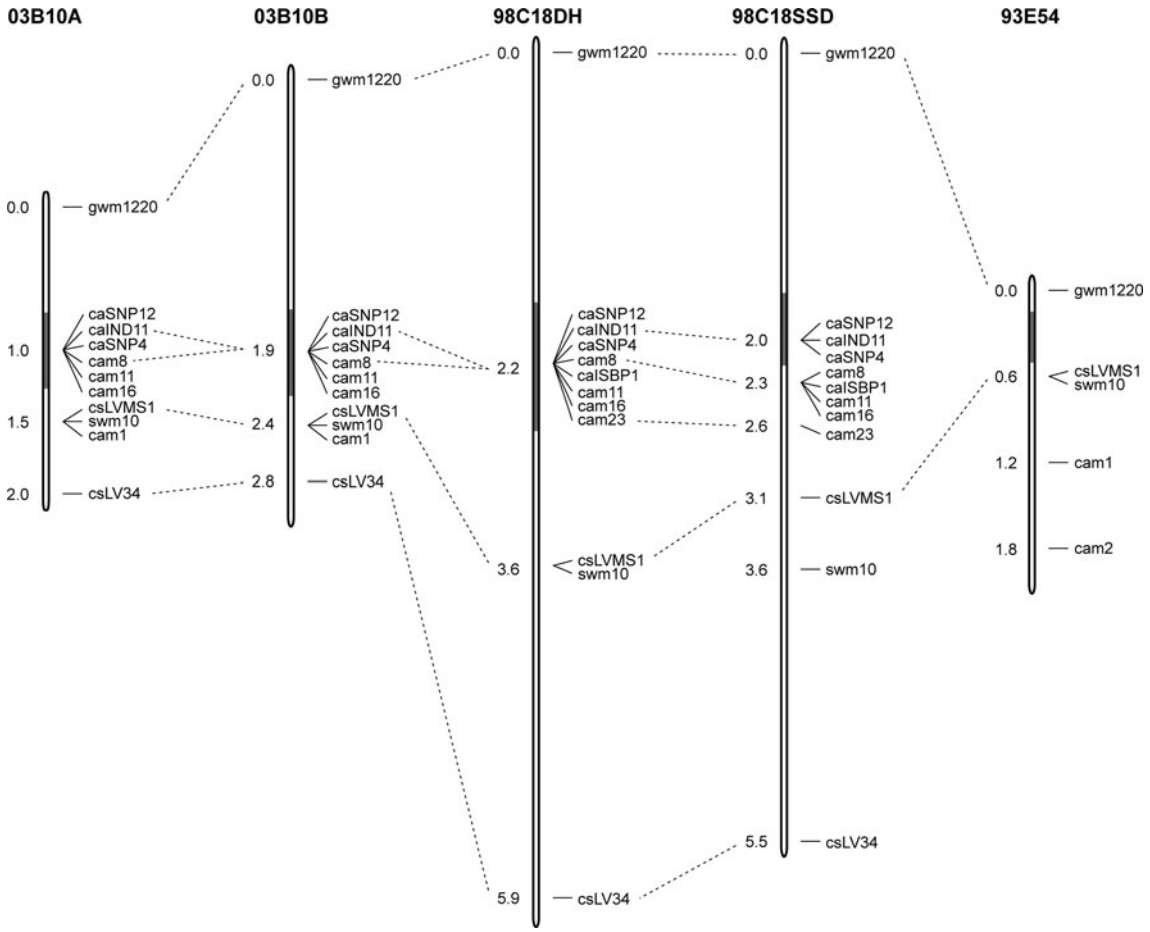

Fig. 3 Comparative fine-mapping of the *Lr34* locus using five segregating populations shows co-segregation of *Lr34* with all three ABC transporter markers (caSNP4, caIND11, andcaSNP12). A 0.3-cM interval between the ABC transporter markers and cam8 in population 98C18SSD is illustrated. *Filled sections* illustrate the location of *Lr34* in each population

Fine-mapping of the *Lr34* locus

All novel markers developed herein (cam1, cam2, cam8, cam11, cam16, cam23, caISBP1, caSNP4, caSNP12, and caIND11) and markers previously described (gwm1220, csLVMS1, swm10, and csLV34) were mapped in one to five segregating populations phenotypically characterized for *Lr34* (Table 1; Fig. 3). The gwm1220/csLV34 interval spanned 1.8–6 cM and included 5–12 of the 14 markers depending on the population; 23 of 1,742 segregating lines had a recombination between marker gwm1220 and csLVMS1 (Fig. 4). Line 98C18SSD-K16 displayed an *Lr34*+ phenotype and the ABC transporter *Lr34*+ genotype, namely caSNP4 A, caIND11 394 (Del), and caSNP12 C, but had the *Lr34*− genotype for cam8 (null), caISBP1 (391), cam11 (299), cam16 (313), and cam23 (385). The recombination in this line physically mapped to the 4.9 kb interval between cam8 and caSNP4, thereby providing evidence to eliminate the two cytochrome P450s and the two kinases as being essential for the functioning of *Lr34*.

Germplasm characterization

We characterized 700 accessions of hexaploid wheat from the WC and AM collections for the ABC-specific markers caSNP4, caSNP12 and caIND11. A subset of 172 lines including all the lines with a putative novel haplotype and all the lines that produced a 398 bp IND11 amplicon were sequenced over SNP4 and IND10/IND11/SNP12. Incomplete data, i.e., missing marker data for one or more markers was obtained for 47 of the 700 lines. The remaining 653 lines were characterized for all four polymorphisms. They partitioned into five different haplotypes (Table 3). Haplotypes 1 and 2 represent the two haplotypes originally described by Krattinger et al. (2009). Haplotype 1 is the only haplotype resulting in a *Lr34*+ phenotype and was found in 29.5 and 21.9% of the AM and WC collections, respectively. Haplotype 2 (T/N/TTC/T) is associated with the *Lr34*− phenotype and is the most common haplotype representing 55.4% of all accessions from the two collections combined. Haplotype 3, recently described by Lagudah et al. (2009) was present in

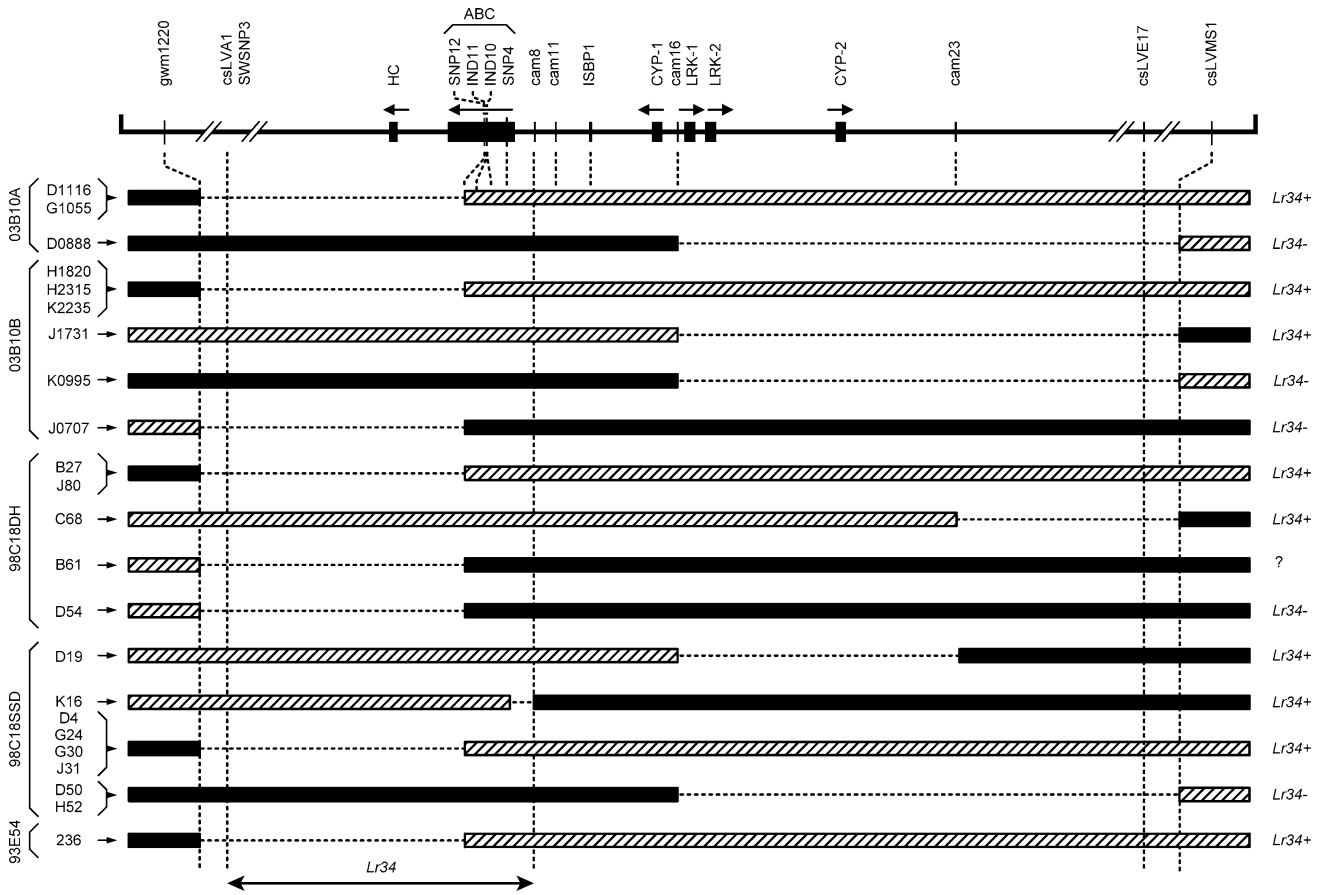

Fig. 4 Schematic representation of the 23 gwm1220-csLVMS1 recombinant lines from the five segregating populations (Table 1) showing the narrower *Lr34* locus defined to be between csLVA1/SWSNP3 and cam8. *Solid bars* represent *Lr34*-alleles and hatched bars *Lr34+*. *Arrows* indicated gene orientation

Table 3 Haplotypes of the ABC transporter for SNP4, IND10, IND11, and SNP12 determined in two hexaploid wheat collections

Haplotype	SNP4	IND10	IND11	SNP12	No. of lines in the AM collection	No. of lines in the WC collection	Total	Phenotype association
1	A	N	N	C	104	66	170	*Lr34+*
2	T	N	TTC	T	206	156	362	*Lr34−*
3	A	N	TTC	T	11	37	48	*Lr34−*
4	T	A	TTC	T	31	40	71	*Lr34−*
5	T	N	N	T	0	2	2	*Lr34−*
Missing data	–	–	–	–	11	36	47	
Total					363	337	700	

7.4% of all accessions. Haplotypes 4 and 5 were novel. Haplotype 4 possessed the additional A in exon 10 and was found only in *Lr34−* lines with the T/TTC/T haplotype for SNP4/IND11/SNP12. Haplotype 5 was rare as it was found in only two lines. Varieties 'Odess kaja 13' and 'Koktunkulskaja 332' were obtained from the Plant Gene Resource Centre of Canada and had been donated by the Russian Federation. They originated from Ukraine and Kazakhstan respectively, and were considered susceptible to leaf rust (http://pgrc3.agr.gc.ca/search_grinca-recherche_rirgc_e.html).

Discussion

The *Lr34* gene has been an important source of resistance to leaf rust in wheat breeding programs due to its durability, its enhancement of other resistance genes, and the

ability to confer resistance to some other major pathogens of wheat (i.e., yellow and stem rusts and powdery mildew). Developing diagnostic markers to determine the presence or absence of *Lr34* in wheat germplasm is an important objective for wheat breeders and geneticists worldwide because phenotypic characterization of this adult plant resistance gene can be challenging due to environmental conditions or genetic background. A good marker is defined as robust, user-friendly, co-dominant and closely linked. Ideally, it would be derived from the gene itself and be based on the completely linked polymorphism(s). In some instances, genetic differences between resistant and susceptible alleles are small, rendering this task challenging (Krattinger et al. 2009). Here, we reported on the development of several new markers at the *Lr34* locus, provided recombination-based evidence for the identity of the ABC transporter as *Lr34*, identified new ABC transporter haplotypes and described a new mutation in this gene. We also described the development of marker caIND11, a co-dominant, robust, user-friendly marker that promises to be highly useful in breeding programs and for germplasm characterization. Alternative markers suitable for different technology platforms are also described.

Fine-mapping of the *Lr34* locus

Cloning of *Lr34* has been a major goal of wheat geneticists, breeders and pathologists alike. Sequence analysis of the *Lr34* locus resulted in the identification of a gene-rich region comprising six genes: HC, ABC transporter, CYP-1, CYP-2, LRK-1, and LRK-2 (Krattinger et al. 2009), of which the ABC transporter was shown to be the only possible candidate responsible for *Lr34* function. Postulation of the *Lr34* gene was based on the identification of a number of mutant lines, which had lost *Lr34* resistance and had mutations in the ABC transporter gene, but not in any of the five adjacent genes of the csLVA1/SWSNP3-csLVE17 interval. Here, we developed seven novel markers (cam1, cam2, cam8, cam11, cam16, cam23, and ISBP1), of which the last five are interstitial to the XcsLVA1/SWSNP3-XcsLVE17 interval (Fig. 2). In addition, three markers (caSNP4, caIND11, and caSNP12) were developed to characterize the three previously identified ABC transporter polymorphisms. These markers were mapped on 1,742 recombinant lines from five populations thereby generating a fine map of the locus showing the co-segregation of the new markers. A recombination event in line 98C18-SSD-K16 was identified in the 4.9 kb interval between caSNP4, a marker developed in intron 4 of the ABC transporter gene and cam8, a microsatellite marker located just upstream of the ABC transporter (Fig. 4). This recombination, located between CYP-1 and ABC, ruled out the possibility that CYP-1, CYP-2, LRK-1, and LRK-2 were needed for *Lr34* function and thereby provided recombination-based evidence to further support the *Lr34* functional identity of the ABC transporter. The hexose carrier of Renan, an *Lr34*- line, has a 6 bp deletion when compared with that of Chinese Spring (*Lr34*+). This polymorphism can be detected by marker SWDEL1 (Krattinger et al. 2009). Parental lines of the five populations used herein all had the HC of Chinese Spring and were monomorphic for SWDEL1 (data not shown). Therefore, no additional polymorphic markers for the HC-ABC transporter interval were identified between the parents of our five segregating populations and recombination-based evidence to rule out the hexose carrier could not be provided using these populations.

Marker development

Genomic DNA sequences can be mined for different types of markers. Microsatellites are ubiquitous in plant genomes and are found in coding and non-coding regions (Moxon and Wills 1999; Morgante et al. 2002). Putative microsatellites can be readily identified using software, such as MISA (Thiel et al. 2003) and SSRIT (Temnykh et al. 2001). Although only a fraction of putative microsatellites are successfully developed into polymorphic markers, their sheer number makes them highly suitable for fine-mapping. Indeed, we successfully developed four new polymorphic microsatellite markers (cam8, cam11, cam16, and cam23) in a short region of only 75 kb (Fig. 2) of the *Lr34* locus of wheat located on the D genome, a genome known for its low polymorphism level (Banks et al. 2009; Chao et al. 2009).

Repetitive elements represent an estimated 90% of the wheat genome, of which transposable elements (TE) are the most important component (Li et al. 2004). The insertion points of these elements constitute unique signatures that can be capitalized upon for the development of markers referred to as insertion site-based polymorphisms. Although the majority of the ISBP markers are dominant, i.e., characterizing the presence or absence of a TE border, caISBP1 was designed to be co-dominant using a second forward primer unique to the interstitial region between the *copia* and the CACTA elements in Renan. This robust and easy to use PCR marker is located only 13 kb upstream of the ABC transporter. Easily resolved on an agarose gel due to the large allele size difference, this co-dominant marker is well suited for laboratories that do not possess acrylamide or polymer-based instruments.

Previous studies reported on several molecular markers closely linked to the *Lr34* locus including gwm1220, csLV34, csLVMS1, swm10, SWSNP3, csLVA1, and csLVE17 (Spielmeyer et al. 2005; Bossolini et al. 2006; Lagudah et al. 2006; Spielmeyer et al. 2008; Krattinger

et al. 2009). These markers were used to map *Lr34* to its most restricted interval bordered by markers csLVA1/SWSNP3 and csLVE17 (Krattinger et al. 2009). Additional markers were developed from gene sequences. SWDEL3 was developed from the sequence of one of the LRK genes, SWDEL2, and csLVD2 originated from the ABC transporter and SWDEL1 from the hexose carrier. These last four markers all co-segregated with *Lr34* and did not enhance the fine-mapping of the gene (Krattinger et al. 2009). The microsatellite and ISBP markers described herein provide alternate markers that can be readily used in germplasm characterization. When combined with segregating populations, they rendered it possible to further define the *Lr34* interval (Fig. 4).

ABC transporter-specific markers

The ABC transporter gene proposed as the *Lr34* gene is predicted to have 1,401 amino acids in 24 exons spanning 11,805 bp (Krattinger et al. 2009). Sequencing of the gene from a few genotypes revealed three polymorphisms, namely an A/T SNP in intron 4, a 3 bp TTC indel in exon 11, and a C/T SNP in exon 12. Like microsatellites, SNPs are widely distributed and provide a rich source of markers (Phillips 2007). To evaluate the polymorphism at these three positions, we developed two co-dominant SNP markers for SNP4 and SNP12, respectively, and a co-dominant marker for the 3 bp indel in exon 11 (caIND11). This last marker generated fragments of 394 bp in *Lr34*+ lines and either 397 or 398 bp in *Lr34*− lines (Fig. 1c). Lagudah et al. (2009) reported markers cssfr1 to cssfr5 based on the indel polymorphism in exon 11 and cssfr6 marker based on the SNP in exon 12. They did not report on markers for the SNP in intron 4. The markers reported herein are therefore complementary or additional to these recently reported markers. Sequencing of two 7D-specific amplicons spanning SNP4 and IND11/SNP12 confirmed the robustness of the SNP markers and revealed the presence of an A/− indel in exon 10 of lines that generated the 398 bp fragment with the caIND11 marker. The A indel in exon 10 would cause a frameshift and lines with the additional nucleotide may not produce a functional ABC transporter. Similar findings were recently reported for a G/T mutation in exon 22 of cultivar Jagger (Lagudah et al. 2009). This latter mutation was predicted to result in an early stop codon and be non-functional. The A indel in exon 10 is a widespread mutation and was found in 71 of the 700 accessions of the two collections (see below) while Jagger's exon 22 SNP mutation is rare. We tested the reported marker on both AM and WC collections and none of the 700 lines displayed this mutation (data not shown).

Lr34 phenotype may result from non-functional transcripts through various mechanisms such as frameshift mutation (e.g., indel in exon 10) and early stop codon mutation (e.g., SNP in exon 22). Alternate splicing, a well-documented mechanism in mammalian ABC transporter genes (Stojic et al. 2007; Piehler et al. 2008) may also be involved in *Lr34* function in plants.

Germplasm characterization

Krattinger et al. (2009) hypothesized that *Lr34* had a single origin because they found only two haplotypes for the three mutations identified in the ABC transporter in 27 wheat lines of various origins. *Lr34*+ lines had the A/Del/C haplotype for caSNP4, caIND11, caSNP12, and *Lr34*− lines displayed the T/TTC/T haplotype for the same mutation positions. Indeed, in the 700 accessions surveyed, these two haplotypes were the most common, but they were not the only haplotypes. Recently, Lagudah et al. (2009) reported on a third haplotype (A/TTC/T) discovered in three winter and two spelt wheat accessions. In our survey of 700 accessions, we found 48 lines with this haplotype (Table 3). It was found in wheat accessions originating from all areas of the world. In agreement with Lagudah et al. (2009), we also did not observe the reciprocal allele of haplotype 3 supporting the hypothesis that it originated from mutation rather than recombination.

The discovery of a new mutation in exon 10 defined a new haplotype (haplotype 4, Table 3). This haplotype was only found in *Lr34*− lines (T/A/TTC/T). The reciprocal of this haplotype was also not observed, suggesting that it similarly arose through mutation (Lagudah et al. 2009) and that this mutation occurred in a T/TTC/T genotype, therefore, after the formation of the original haplotype. This haplotype was surprisingly broadly distributed and represented more than 10% of the 700 lines tested. As with haplotype 3, its distribution was ubiquitous among the WC and AM collections. Marker caIND11 is diagnostic for both indels in exon 10 (A/−) and exon 11 (TTC/−) because the two mutations will result in different size fragments (Fig. 1c).

A rare fifth haplotype (T/N/N/T) was found in 2 of the 700 lines tested, i.e., Odess Kaja 13 and Koktunkulskaja 332. We believe these lines to be *Lr34*−. The Plant Gene Resource Centre (PGRC) reported them as susceptible to rust and that is in agreement with our own field observations where rust severity for these two lines, evaluated in a total of five environments over 3 years, ranged from 10 to 80% (data not shown). These lines lack the TTC indel in exon 11 typically associated with the *Lr34*+ phenotype. The phenylalanine residue encoded by the TTC indel may therefore not play a role in the *Lr34*− phenotype. On the other hand, we cannot rule out that these lines may possess other silencing or altering mutations, such as the rare mutation in cultivar Jagger (Lagudah et al. 2009).

Complete sequencing of their ABC transporter may provide such information. Other mutations in this ABC transporter will likely be discovered as more in-depth information on this gene from large germplasm collections is gathered and becomes available. Preliminary field assessment on the two collections indicated rust severity up to 70% on some of the lines with the A/N/N/C haplotype predicted to be associated with the *Lr34*+ phenotype. This brings the question whether such lines have a functional *Lr34* gene and if not, what are the additional causes for the non-functionality of *Lr34*. Additional phenotyping in different environments and more in-depth characterization of the ABC transporter sequence and gene expression will be necessary to confirm association.

Conclusion

We described the development of seven new markers located at the *Lr34* locus in addition to three markers specifically targeting the ABC transporter gene previously identified as *Lr34*. Physical and genetic maps of the locus confirm the close proximity of these markers to *Lr34*. They provide multiple alternatives for marker-assisted selection. Marker caIND11 is likely the most informative, while caISBP1 is the most user-friendly. We were able to map *Lr34* distal to four of the six genes previously identified to the interval thereby ruling out CYP-1, CYP-2, LRK-1, and LRK-2 as having a role in *Lr34* function. We described a new mutation in exon 10 that would likely be non-functional and that constituted a new haplotype. The characterization of a large germplasm collection identified a fourth and fifth haplotype of the ABC transporter.

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