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ORIGINAL PAPER

Recent emergence of the wheat *Lr34* multi-pathogen resistance: insights from haplotype analysis in wheat, rice, sorghum and *Aegilops tauschii*

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Abstract Spontaneous sequence changes and the selection of beneficial mutations are driving forces of gene diversification and key factors of evolution. In highly dynamic co-evolutionary processes such as plant-pathogen interactions, the plant's ability to rapidly adapt to newly emerging pathogens is paramount. The hexaploid wheat gene *Lr34*, which encodes an ATP-binding cassette (ABC) transporter, confers durable field resistance against four fungal diseases. Despite its extensive use in breeding and agriculture, no increase in virulence towards *Lr34* has been described over the last century. The wheat genepool contains two predominant *Lr34* alleles of which only one confers disease resistance. The two alleles, located on chromosome 7DS, differ by only two exon-polymorphisms. Putatively functional homoeologs and orthologs of *Lr34* are found on the B-genome of wheat and in rice and sorghum, but not in maize, barley and Brachypodium. In this study we present a detailed haplotype analysis of homoeologous and orthologous *Lr34* genes in genetically and geographically diverse selections of wheat, rice and sorghum accessions. We found that the resistant *Lr34* haplotype is unique to the wheat D-genome and is not found in the B-genome of wheat or in rice and sorghum. Furthermore, we only found the susceptible *Lr34* allele in a set of 252 *Ae. tauschii* genotypes, the progenitor of the wheat D-genome. These data provide compelling evidence that the *Lr34* multi-pathogen resistance is the result of recent gene diversification occurring after the formation of hexaploid wheat about 8,000 years ago.

Introduction

Plant diseases are a threat to global crop production. The three rusts of wheat, leaf rust (*Puccinia triticina*), stripe

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rust (*Puccinia striiformis* f. sp. *tritici*) and stem rust (*Puccinia graminis* f. sp. *tritici*) are among the most devastating diseases in wheat growing areas around the world. The release of cultivars with high levels of disease resistance is a major objective for breeders. The wheat genepool contains a large set of resistance genes; the wheat gene catalogue lists more than 50 resistance genes for each of the three rust species (McIntosh et al. 2008). However, many of these resistance genes confer resistance to only some races of a particular rust species. These race-specific resistance genes are frequently overcome in the field because of rapid evolution and adaptation of pathogen populations (Kolmer et al. 2003). In the year 2000 for example, a new leaf rust race with virulence against resistance gene *Lr24* evolved in Australia (Park et al. 2002). Previously, in Uganda, a devastating new stem rust race that overcame the widely used resistance gene *Sr31* was observed in 1998 and confirmed in 1999 (Pretorius et al. 2000). Wind dispersal of rust spores over long distances favours the rapid spread of new virulent races (Kolmer 2005). Pyramiding of race-specific resistance genes and the use of more durable, race non-specific resistance genes are strategies to avoid rapid adaptation of pathogens in the field (Brunner et al. 2010; Lagudah 2011; Mago et al. 2011; Sun et al. 2009).

The wheat gene *Lr34/Yr18/Sr57/Pm38* (subsequently referred to as *Lr34*) confers durable and race non-specific resistance against the four biotrophic fungal pathogens leaf rust, stripe rust, stem rust and powdery mildew (*Blumeria graminis* f.sp. *tritici*) (Krattinger et al. 2009). Because of its durability and broad-spectrum specificity *Lr34* has become one of the most important disease resistance genes in wheat breeding. The origin of *Lr34* in modern wheat breeding programmes has been traced back to two wheat cultivars, Mentana and Ardito, both of which were released in Italy in the early 1900s (Kolmer et al. 2008). In the 1940s a cross between Mentana and Fronteira, made in Brazil by Iwar Beckman, gave rise to Frontana (Camargo and Ferreira Filho 2001). This popular cultivar served as an important source of *Lr34* resistance in the breeding programmes at CIMMYT and in North America. Despite its extensive use in breeding and agriculture over the last century, no increase in virulence towards *Lr34* has been observed. Only a few genes with comparable durability and broad-spectrum specificity have been described in wheat, among them are *Lr46/Yr29/Pm39* and *Lr67/Yr49* (Lagudah 2011). Little is known about the molecular function and evolution of these durable resistance genes and only *Lr34* has been cloned thus far.

A map-based cloning approach identified the *Lr34* gene as a single full-size ATP-binding cassette (ABC) transporter gene of the ABCG subfamily. The nucleotide sequence of *Lr34* contains 24 exons, spans 11,805 bp and translates into a predicted protein of 1,401 amino acids. Mutations in this gene resulted in enhanced susceptibility to leaf rust, stripe rust and powdery mildew (Krattinger et al. 2009). Transgenic studies showed that this ABC transporter was sufficient and required for broad-spectrum disease resistance (Risk et al. 2012). Full-size ABC transporters share a conserved structure consisting of two cytosolic nucleotide binding domains (NBDs) and two transmembrane domains (TMDs). The TMDs shuttle a specific substrate across a biological membrane. Each TMD is predicted to consist of six membrane-spanning helices (Krattinger et al. 2011). The current model suggests that this protein class transports a set of structurally and functionally diverse molecules (Rea 2007).

Lr34 exists as a single-copy gene on chromosome 7DS. In the genepool of domesticated hexaploid wheat two highly similar alleles are predominantly found, *Lr34res-D* and *Lr34sus-D*. *Lr34res-D* is the only *Lr34* allele known to confer disease resistance. It has been found in all wheat varieties with *Lr34*-based disease resistance. Interestingly, both alleles are expressed and may encode for functional ABCG transporters. Their nucleotide sequences differ by only two exon-polymorphisms (Krattinger et al. 2011). A deletion of the three base pairs 'TTC' in exon 11 of *Lr34res-D* results in the deletion of a phenylalanine residue at position 546 of the LR34res-D protein. A C/T single nucleotide polymorphism (SNP) in exon 12 results in a tyrosine to histidine conversion at position 634 of LR34res-D. Both of these mutations are predicted to be within the first TMD; amino acid 546 within transmembrane helix 2 and residue 634 at the cytosolic end of transmembrane helix 4. The tyrosine residue at position 634 of LR34sus-D is highly conserved among different ABCG proteins in various plant species (Krattinger et al. 2011). Several additional *Lr34* alleles have been identified at lower frequencies in the wheat genepool (Dakouri et al. 2010; Lagudah et al. 2009; McCallum et al. 2012). However, none of these were associated with disease resistance and they either represent mutated versions of *Lr34res-D* or variants of *Lr34sus-D*. The susceptible American cultivar Jagger for example, carries the *Lr34res-D* allele with an additional point mutation that results in a premature stop codon (Lagudah et al. 2009).

Beside the *Lr34* gene on chromosome 7DS, the genome of hexaploid wheat contains two homoeologous *Lr34* genes on chromosomes 7AS and 4A (contains a translocated part of chromosome 7BS), respectively. While the gene on chromosome 4A is expressed and putatively functional, the copy on chromosome 7A is interrupted by insertion of several repetitive elements. We named the putatively functional homoeologous gene on chromosome 4A as

Lr34-B. The LR34-B protein is 97 % identical to LR34-D. For the two critical codons that distinguish *Lr34res-D* from *Lr34sus-D*, *Lr34-B* carried the same nucleotides as *Lr34sus-D* in a set of 16 wheat cultivars (Krattinger et al. 2011).

Lr34 orthologs are found in the sequenced genomes of rice (*Oryza sativa*) cultivar Nipponbare and *Sorghum bicolor* genotype BTx623. The genome sequences of *Brachypodium distachyon* line Bd21 and maize accession B73 lack *Lr34* orthologs. Furthermore, Southern blot and EST sequence analysis suggested absence of *Lr34* orthologs in barley and rye (Krattinger et al. 2011). The orthologous rice protein found in cultivar Nipponbare, OsABCG50, is 86 % identical to LR34. The genome sequence of sorghum genotype BTx623 contains two adjacent *Lr34* orthologs that most likely arose through gene duplication. The predicted protein sequences of Sb01g016775 and Sb01g016770 are 75 and 71 % identical to LR34, respectively. Sb01g016770 is most likely a pseudogene because intron 4 spans more than 20 kb and contains signatures of CACTA transposons. We found expression of Sb01g016775, but not Sb01g016770 in BTx623 plants at the 4–5 leaf stage. At the critical position in exon 11, Sb01g016775 like *Lr34sus-D* carries the codon 'TTC' which translates into the nonpolar amino acid phenylalanine. OsABCG50 and Sb01g016770 have a 'TTA' translating into the nonpolar amino acid leucine (Fig. 1). The presence of a nonpolar residue at this position is in accordance with its predicted location within a transmembrane helix. LR34res-D is the only protein that carries a deletion at this position. This deletion is likely to alter the residues exposed to the substrate during transportation through the membrane. The tyrosine residue at the second critical position in exon 12 is conserved among LR34sus-D, LR34-B, OsABCG50 and the two sorghum orthologs. The only exception is the histidine found in LR34res-D suggesting that this change may alter substrate specificity. This sequence comparison demonstrates that LR34res-D has a unique sequence composition which we refer to as 'resistant haplotype'. There is currently no evidence that *Lr34sus-D*, *Lr34-B*, OsABCG50, Sb01g016770 or Sb01g016775 confer disease resistance. We therefore refer to their sequence compositions at the two critical codons as 'susceptible haplotype'.

In this study we examined *Lr34* homoeologs and orthologs in a set of wheat, rice and sorghum genotypes. We only found the susceptible haplotype for *Lr34-B*, OsABCG50 and the two sorghum orthologs. Furthermore, we only found the *Lr34sus-D* allele in a broad range of 252 accessions of *Aegilops tauschii*, the donor of the wheat D-genome. These data indicate that *Lr34res-D*, which confers broad-spectrum disease resistance in hexaploid wheat, is unique to the wheat D-genome and originated after the formation of hexaploid wheat.

Materials and methods

Genotype collection

Hexaploid wheat

A selection of 77 *Triticum aestivum* landraces from the collections maintained at the Australian Winter Cereals Collection (AWCC), Tamworth, Australia was analysed. The set contained landraces from China (66), Afghanistan (3), Nepal (3), Iran (2), Georgia (1), India (1) and Pakistan (1). Table S1 contains a detailed list of all the accessions used in this study.

Sorghum

We analysed a set of 97 sorghum accessions, 87 of them represent cultivated grain sorghums, sweet sorghums and forage sorghums from Africa, Asia, Australia, Central America and North America. All cultivated sorghums belong to the species *Sorghum bicolor* Moench. The set further included 10 wild sorghum accessions belonging to different species; *S. arundinaceum* (progenitor of cultivated *S. bicolor*), *S. drummondii* (natural cross between *S. bicolor* and *S. arundinaceum*) and *S. propinquum* are weedy species that naturally intercross with *S. bicolor*. The wild species *S. brachypodum*, *S. bulbosum*, S. *interjectum*, *S. stipoideum* and *S. intrans* are endemic to northern Australia and do not naturally hybridize with *S. bicolor* (Dillon et al. 2004).

Rice

The rice set contained 32 accessions. Based on their diversity and geographical origin, 20 of them had previously been selected by the OryzaSNP project to study genomewide SNP variation (McNally et al. 2009). They represent a set of genetically diverse varieties and landraces from the variety groups *indica* (nonsticky, lowland rices, tropical Asia), *japonica* (sticky, temperate East Asia, upland areas of Southeast Asia, and high elevations in South Asia), *aromatic* (e.g. basmati), *aus* (drought-tolerant, early maturing rice grown in Bangladesh) and deep-water (Garris et al. 2005).

Aegilops tauschii

A genetically and geographically diverse set of 252 *Aegilops tauschii* accessions was selected for this haplotype study. The accessions derived from the collections maintained at the Australian Winter Cereals Collection in Tamworth, Australia, the Commonwealth Plant Introduction collection (CPI) and UC Davis. To maximize potential

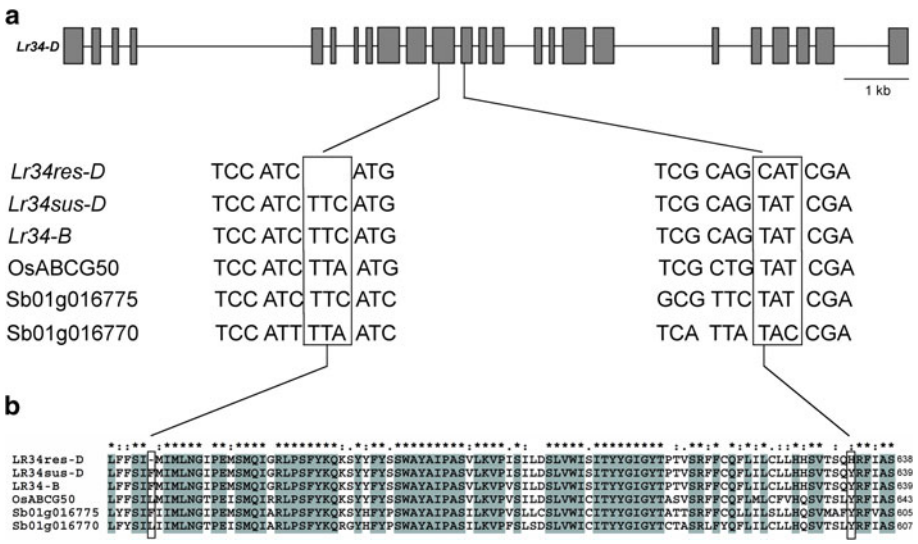

Fig. 1 Resistant and susceptible *Lr34* haplotypes **a** Gene structure of *Lr34*. *Grey boxes* indicate exons; introns are shown as adjoining lines. The two critical codons in exons 11 and 12 that differ between *Lr34* alleles of resistant and susceptible wheat cultivars are framed. **b** Alignment showing residues 541–639 of the wheat LR34 protein and the corresponding regions from the homoeologous LR34-B protein as well as the orthologous proteins from rice and sorghum. The two critical residues that differ between LR34res-D and LR34sus-D are framed. Residues marked in *grey* are conserved in all the six proteins. *Asterisk* represents identical amino acids, *colon* represents highly conserved amino acids and *dot* represents similar amino acids. Accession numbers: *Lr34res-D* (FJ436983), *Lr34sus-D* (FJ436985), *Lr34-B* (HM775493). Sequences of OsABCG50, Sb01g016775 and Sb01g016770 have been taken from the sequenced genomes of rice cultivar Nipponbare (International Rice Genome Sequencing Project 2005) and sorghum genotype BTx623 (Paterson et al. 2009), respectively

genetic differences, genotypes from both ssp. *tauschii* (morphological varieties *typica* and *meyeri*) and ssp. *strangulata* as well as intermediate forms were selected (Lagudah et al. 1989). Subspecies *tauschii* occurs from eastern Turkey to China and Pakistan, whereas ssp. *strangulata* is found in southeastern Caspian Iran and Transcaucasia.

Sequence analysis

Specific primers that spanned exon/intron 9 to exons 12–13 were designed for each gene (Table 1). PCR reactions were performed using GoTaq® Flexi DNA Polymerase (Promega) or HotStar Taq DNA polymerase (Qiagen) including 0.2 mM dNTPs each, 0.5 µM of each primer, 200 ng DNA and 1U polymerase in a 20 µl reaction. After amplification excess nucleotides and primers were eliminated by incubating 1 µl of the PCR reaction with 0.25 µl Shrimp Alkaline Phosphatase (Fermentas, 1 U/µl) and 0.15 µl Exonuclease (New England Biolabs$_{Inc}$, 20 U/µl) at 37 °C for 45 min followed by an inactivation step at 80 °C for 15 min. Purified PCR products were sequenced using BigDye® Terminator v3.1 (Applied Biosystems). Sequences were aligned using ClustalX2 with a gap opening penalty of 10 and a gap extension penalty of 0.2 (Larkin et al. 2007). Sequences were analysed for the two critical codons that distinguish *Lr34res-D* from *Lr34sus-D* as well as for overall sequence variation.

Expression analysis of sorghum Lr34 *orthologs*

RNA of leaves of *Sorghum bicolor* genotype BTx623 and *Sorghum propinquum* accession AusTRCF 302546 was extracted using the RNeasy® Plant Mini Kit (Qiagen). SuperScript® III Reverse Transcriptase (Invitrogen) was used to produce cDNA. *Lr34* orthologs were amplified with gene-specific primers for Sb01g016770 (Pf: CAA CAA TTG GCC AAC ATC AAT G, Pr: GAG ATA CCC AAA ATC CCC AG) and Sb01g016775 (Pf: CAG CAA ATG GCA AAC ATC AAT G, Pr: GAG ATG TCC AAA ATC CCC AC) using HotStar Taq DNA polymerase (Qiagen) at an annealing temperature of 60 °C and a PCR set-up as described above.

Results

For the haplotype analysis we designed gene-specific primers that amplified exons 9–13 of *Lr34-D,* the wheat homoeolog *Lr34-B* and *Lr34* orthologs from rice and sorghum (Table 1). PCR products were sequenced and analysed for the two critical codons in exons 11 and 12 that

Table 1 Primer sequences to amplify *Lr34*-like genes in wheat, rice and sorghum

Gene	Accession number/gene identifier	Forward primer	Binding site	Reverse primer	Binding site	T_m (°C)	Product size (bp)	DNA polymerase
Lr34-D	FJ436983/ FJ436985	AGC AAC TGA GAT GAT TTG CAC	Intron 9	ATT TGT TCA GAT TTG CTA GGC	Intron 12	62.5	1,038	Go Taq
Lr34-B	HM775493	GTG GGA CCC GCA AAA GCA TAC	Exon 9	GCC ATT CTG GCA TGG AGG CTG	Intron 12/ Exon 13	67.5	1,401	Go Taq
OsABCG50	LOC Osl2g32820	GTT GTT TCC AGC AAC TGA CG	Exon 9	GAG TGA AGC CTC CAA ACA TG	Exon 12	60	1,228	HotStar
Sb01g016775		CAG CAA ATG GCA AAC ATC AAT G	Exon 9	GAG ATG TCC AAA ATC CCC AC	Exon 13	60	1,371	Go Taq
Sb01g016770		CAA CAA TTG GCC AAC ATC AAT G	Exon 9	GAG ATA CCC AAA ATC CCC AG	Exon 13	60	1,367	Go Taq

distinguish *Lr34res-D* found in resistant wheat cultivars from *Lr34sus-D* present in susceptible wheat varieties.

Hexaploid wheat

In addition to the *Lr34* gene on chromosome 7DS, the hexaploid wheat genome contains an expressed and putatively functional *Lr34* homoeolog on chromosome 4A. The putative protein, named LR34-B, is 97 % identical to LR34 and showed the susceptible haplotype in an initial set of 16 different wheat cultivars (Krattinger et al. 2011). Here we obtained sequence information from 77 hexaploid landraces, predominantly from China, of which 37 showed the resistant haplotype for *Lr34-D*. Using the closely linked diagnostic marker *csLV34* (Lagudah et al. 2006), two studies reported a very high frequency of the resistant *Lr34* haplotype in Chinese hexaploid wheat landraces (Kolmer et al. 2008; Yang et al. 2008). Surprisingly, the resistant *Lr34* haplotype was not found in many landraces from other parts of the world (Kolmer et al. 2008). We therefore reasoned that landraces from China were a good starting point to initiate diversity analysis of the *Lr34-B* homoeologs. All of the 77 landraces possessed the susceptible haplotype for *Lr34-B*. They carried a 'TTC' at the critical codon in exon 11 of *Lr34-B* and a 'TAT' at the second critical site in exon 12 (Fig. 1; Table 2; Table S1).

Sorghum

The genome sequence of sorghum genotype BTx623 contains two neighbouring *Lr34* orthologs, Sb01g016770 and Sb01g016775, respectively. We used a set of 97 sorghum genotypes to study diversity at the two critical codons.

Sb01g016770

We obtained sequences for Sb01g016770 from 95 genotypes, 94 of the 95 accessions showed the susceptible *Lr34* haplotype (Table 2; Table S1). An interesting variant was found in the wild *S. propinquum* accession AusTRCF 302546 from Asia. A point mutation in exon 12 altered the critical codon 'TAC' to 'TGC'. This polymorphism would result in a tyrosine to cysteine conversion in the respective protein. Using RT-PCR, we found expression of Sb01g016770 in the wild *S. propinquum* (Fig. 2). Sb01g016770 was not expressed in the sequenced *S. bicolor* genotype BTx623. This was attributed to transposon insertions in intron 4 which resulted in an increase in length of the respective intron by more than 20 kb. Although being a pseudogene in genotype BTx623, Sb01g016770 may still be functional in other sorghum accessions. *S. propinquum* is considered to be a perennial rhizomatous form of *S. bicolor* (Dillon et al. 2004). To our knowledge there is no information on disease resistance of *S. propinquum* accession AusTRCF 302546.

Sb01g016775

We got amplification products for 88 genotypes using the gene-specific primers for the second *Lr34* ortholog Sb01g016775. The primers did not amplify in the endemic Australian wild sorghum species. This indicates absence of this particular gene or divergence in the primer binding region in this particular germplasm. The two critical codons 'TTC' in exon 11 and 'TAT' in exon 12 were conserved among all of the 88 genotypes, indicating the presence of the susceptible haplotype.

In summary, with exception of one genotype, all the sorghum accessions showed the susceptible haplotype for the two *Lr34* orthologs Sb01g016770 and Sb01g016775. A wild *Sorghum propinquum* accession showed a new and putatively interesting variant for the critical residue in exon 12 of Sb01g016770.

Rice

The orthologous rice protein, OsABCG50, is 86 % identical to LR34-D. We obtained 838 bp of nucleotide

Table 2 Number of wheat, sorghum, rice and *Ae. tauschii* accessions that showed the resistant, susceptible or a novel haplotype for *Lr34-D*, the wheat homoeolog *Lr34-B* and the orthologs OsABCG50, Sb01g016770 and Sb01g016775, respectively

Species	Gene name	Country of origin of accessions	Nr. of accessions with resistant *Lr34* haplotype	Nr. of accessions with susceptible *Lr34* haplotype	Nr. of accessions with novel *Lr34* haplotype
Hexaploid wheat	*Lr34-D*	China	33	33	0
		Afghanistan	0	3	0
		Nepal	1	2	0
		Iran	2	0	0
		Georgia	0	1	0
		India	0	1	0
		Pakistan	1	0	0
		Total	**37**	**40**	**0**
	Lr34-B	China	0	66	0
		Afghanistan	0	3	0
		Nepal	0	3	0
		Iran	0	2	0
		Georgia	0	1	0
		India	0	1	0
		Pakistan	0	1	0
		Total	**0**	**77**	**0**
Sorghum	Sb01g016770	USA	0	26	0
		Africa[a]	0	18	0
		Australia	0	14	0
		China	0	9	0
		India	0	3	0
		Central America[a]	0	2	0
		Former USSR	0	1	0
		Asia	0	0	1
		Africa/USA[b]	0	18	0
		India/USA[b]	0	3	0
		Total	**0**	**94**	**1**
	Sb01g016775	USA	0	27	0
		Africa[a]	0	18	0
		Australia	0	8	0
		China	0	8	0
		India	0	2	0
		Central America[a]	0	2	0
		Former USSR	0	1	0
		Asia	0	1	0
		Africa/USA[b]	0	18	0
		India/USA[b]	0	3	0
		Total	**0**	**88**	**0**
Rice	OsABCG50	Philippines	0	8	0
		India	0	6	0
		China	0	4	0
		Bangladesh	0	2	0
		Colombia	0	2	0
		Korea	0	2	0
		West Africa	0	2	0
		USA	0	2	0
		Brazil	0	1	0
		Iran	0	1	0
		Japan	0	1	0
		Taiwan	0	1	0
		Total	**0**	**32**	**0**

Table 2 continued

Species	Gene name	Country of origin of accessions	Nr. of accessions with resistant *Lr34* haplotype	Nr. of accessions with susceptible *Lr34* haplotype	Nr. of accessions with novel *Lr34* haplotype
Aegilops tauschii	*Lr34-D*	Afghanistan	0	87	0
		Iran	0	40	0
		Azerbaijan, Georgia, Dagestan	0	33	0
		Turkey	0	16	0
		Armenia	0	9	0
		China	0	5	0
		Turkmenistan, Uzbekistan	0	4	0
		Pakistan	0	2	0
		Former USSR	0	23	0
		Unknown	0	33	0
		Total	**0**	**252**	**0**

[a] For detailed list of countries see Table S1

[b] Landraces from Africa/India converted to temperate adaptation in USA

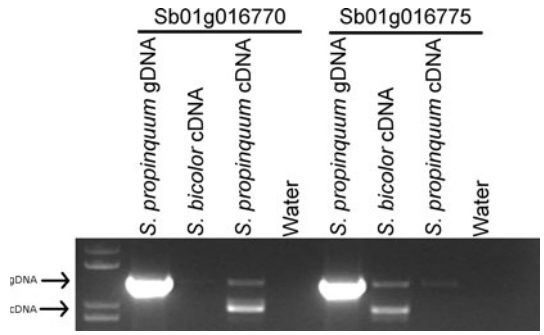

Fig. 2 Leaf expression of the *Lr34* orthologs Sb01g016770 and Sb01g016775 in leaves of *Sorghum bicolor* genotype BTx623 and *Sorghum propinquum* accession AusTRCF 302546. The PCR products have an expected size of 1,367 bp on genomic DNA and 1,000 bp on cDNA. The upper band in the cDNA samples may be due to residual genomic DNA or unspliced transcript. The band sizes of the 1 Kb plus DNA Ladder (Invitrogen) correspond to 850, 1,000, 1,650 and 2,000 bp from *bottom* to *top*. Amplified fragments from cDNA were verified by sequencing

sequence spanning exons 10–12 of the OsABCG50 gene from all 32 genotypes. All the accessions had exactly the same sequence as the reference sequence of Nipponbare, including the two critical codons. We therefore only found the susceptible haplotype among the 32 tested rice genotypes.

Aegilops tauschii

A diverse set of *Aegilops tauschii* accessions with different geographical origins was analysed using *Lr34-D* specific primers. The set included accessions from both ssp. *tauschii* and ssp. *strangulata.* Their geographical origin ranged from Turkey and Transcaucasia to the Caspian Sea region, Pakistan and China. All the 252 accessions carried the *Lr34sus-D* allele, which is found in susceptible wheat cultivars.

In total we obtained 720 bp of nucleotide sequence spanning exons 10–12 from all the 252 accessions. While there was relatively little sequence diversity in *Lr34* among cultivated wheat varieties, we found seven sequence variants among the *Ae. tauschii* accessions within the 720 bp of sequence (Table S1). The different subtypes showed SNPs or small deletions compared to the reference *Lr34sus-D* sequence of hexaploid wheat. Some SNPs affected the coding sequence and would result in amino acid substitutions. The most frequent subtype was found in 116 (46 %) genotypes representing accessions from both subspecies spanning the whole geographical range. This particular subtype carried one SNP in exon 11 that would result in a Tyrosine to Cysteine substitution in a cytosolic loop of TMD1. Other subtypes were less frequent and confined to accessions from a particular region. 103 genotypes (41 %) showed the same sequence as the wheat *Lr34sus-D* reference sequence within the 720 bp. These data indicate that although *Lr34* sequence diversity in cultivated wheat is limited, there is greater structural and probably functional variation among wild *Ae. tauschii* accession.

Discussion

Lr34 *haplotypes in wheat, rice and sorghum*

Map-based cloning of *Lr34* identified a single ABCG transporter gene in wheat that conferred resistance against leaf rust, stripe rust, stem rust and powdery mildew (Krattinger et al. 2009; Risk et al. 2012). Interestingly, both wheat cultivars with and without *Lr34*-based disease

resistance carried an expressed and putatively functional *Lr34* allele. The two alleles *Lr34res-D* and *Lr34sus-D* differed by only two exon-polymorphisms (Fig. 1). This diversity study strongly suggests that the resistant *Lr34* haplotype is evolutionary younger than the susceptible haplotype and is unique to the wheat D-genome. The absence of *Lr34res-D* in a set of 252 *Ae. tauschii* accessions indicates that this particular allele originated after the hybridization of tetraploid wheat with *Ae. tauschii*, the event that gave rise to modern hexaploid bread wheat about 8,000 years ago (Feldman 2001; Salamini et al. 2002). Most likely, two gain-of-function mutations in *Lr34sus-D* in an ancestral wheat landrace resulted in the novel *Lr34res-D* allele that conferred enhanced tolerance against fungal diseases.

Our genotype collection captured a broad genetic diversity by including various subspecies and morphological varieties spanning a wide geographical range. We assumed that the resistant *Lr34* haplotype conferred a selective advantage over the susceptible haplotype by providing enhanced disease resistance. Hence, we would have expected selection in favour of the resistant haplotype if two similar mutations had occurred in any of the homoeologous or orthologous *Lr34* genes. The absence of the resistant haplotype in rice and sorghum suggests that the two critical mutations only occurred in hexaploid wheat. Alternatively, the resistant *Lr34* haplotype may cause deleterious effects in diploid species and may only be tolerated in a buffered polyploid background. At the molecular level the two critical amino acid changes in LR34res-D may affect conformational structure, binding specificity, or protein stability. In *Arabidopsis thaliana*, a single gain-of-function mutation in the highly conserved NBD2 of the ABCG transporter ABCG37 resulted in increased protein stability and enhanced tolerance to the auxin herbicide 2,4-dichlorophenoxyacetic acid (Ito and Gray 2006). In contrast, a single amino acid substitution in transmembrane helix 3 of the human half-size transporter ABCG2 was sufficient to alter its substrate specificity (Honjo et al. 2001).

The susceptible *Lr34* haplotype is evolutionary older than *Lr34res-D* which raises the question about its function in hexaploid wheat, rice and sorghum. Absence of *Lr34* orthologs in maize, Brachypodium, barley and rye suggest that this particular ABCG transporter is not crucial for the survival of these grasses or that genetic redundancy compensates for the loss of this gene. However, it is possible that *Lr34sus-D*, *Lr34-B*, OsABCG50 and the two sorghum orthologs have conserved functions. For example, two recent studies identified three orthologous ABCG transporters from barley, rice and Arabidopsis that are all involved in the formation of cutin layers (Bessire et al. 2011; Chen et al. 2011).

Evolution of Lr34 *resistance in hexaploid wheat*

Because of its durability, broad-spectrum resistance and race non-specificity, *Lr34* has become one of the most important genes in wheat disease resistance breeding. The absence of the resistant *Lr34* haplotype in *Lr34-B*, the rice and sorghum orthologs and *Ae. tauschii* provides strong evidence that *Lr34res-D* is evolutionary very young and emerged after the formation of hexaploid wheat approximately 8,000 years ago (Feldman 2001). Two subsequent gain-of-function mutations in *Lr34sus-D* gave rise to the resistant *Lr34res-D* allele. Spontaneous emergence of disease resistance has been described for *mlo* genes in barley, Arabidopsis, tomato and pea (Bai et al. 2008; Consonni et al. 2006; Humphry et al. 2011; Piffanelli et al. 2004). Several induced and natural loss-of-function mutations in *Mlo* genes in these species conferred recessive, durable and broad-spectrum resistance against powdery mildews. *Lr34res-D* on the other hand is the result of two gain-of-function mutations. We therefore consider it likely that the two specific mutations in *Lr34sus-D* that gave rise to *Lr34res-D* occurred only once during the evolution of wheat. Selection by early farmers may have played an important role in fixing the new *Lr34res-D* allele. Kolmer et al. (2008) reported the absence of *Lr34res-D* in landraces from most countries with the exception of China where the resistant *Lr34res-D* allele was found in more than 30 % of landraces. We therefore speculate that the *Lr34res-D* allele emerged in a Chinese landrace. The origin of *Lr34* in modern breeding has been traced back to two cultivars, Mentana and Ardito, released in Italy by Nazareno Strampelli in the early 1900s (Borghi 2001; Kolmer et al. 2008). It is possible that *Lr34res-D* containing landraces from China were introduced to Italy where they were subsequently incorporated into breeding programmes. Dakouri et al. (2010) described two cultivars, Odesskaja 13 and Koktunkulskaja 332, which carried an intermediate *Lr34* haplotype; the resistant haplotype for the deletion of the phenylalanine in exon 11 but the susceptible haplotype for the second critical site in exon 12. These two cultivars, which originated from the Ukraine and Kazakhstan, may point to a different origin of the *Lr34res-D* allele. Alternatively, the intermediate haplotype in these two cultivars arose through recombination. The two cultivars were classified susceptible to leaf rust (Dakouri et al. 2010), which suggest that both mutations are required for disease resistance.

Today, little is known about the molecular function and evolution of durable, race non-specific disease resistance. A few wheat genes such as *Lr46/Yr29/Pm39* and *Lr67/Yr49* confer a very similar phenotype to *Lr34*. The recent emergence of the *Lr34* multi-pathogen resistance raises intriguing questions about the evolution of these genes.

The future cloning of *Lr46/Yr29/Pm39* and *Lr67/Yr49* will shed more light on the function and evolution of durable disease resistance in wheat.

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