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High-resolution mapping and mutation analysis separate the rust resistance genes *Sr31*, *Lr26* and *Yr9* on the short arm of rye chromosome 1

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Abstract The stem, leaf and stripe rust resistance genes *Sr31*, *Lr26* and *Yr9*, located on the short arm of rye chromosome 1, have been widely used in wheat by means of wheat-rye translocation chromosomes. Previous studies have suggested that these resistance specificities are encoded by either closely-linked genes, or by a single gene capable of recognizing all three rust species. To investigate these issues, two 1BL·1RS wheat lines, one with and one without *Sr31*, *Lr26* and *Yr9*, were used as parents for a high-resolution F2 mapping family. Thirty-six recombinants were identified between two PCR markers 2.3 cM apart that flanked the resistance locus. In one recombinant, *Lr26* was separated from *Sr31* and *Yr9*. Mutation studies recovered mutants that separated all three rust resistance genes. Thus, together, the recombination and mutation studies suggest that *Sr31*, *Lr26* and *Yr9* are separate closely-linked genes. An additional 16 DNA markers were mapped in this region. Multiple RFLP markers, identified using part of the barley *Mla* powdery mildew resistance gene as probe, co-segregated with *Sr31* and *Yr9*. One deletion mutant that had lost *Sr31*, *Lr26* and *Yr9* retained all *Mla* markers, suggesting that the family of genes on 1RS identified by the *Mla* probe does not contain the *Sr31*, *Lr26* or *Yr9* genes. The genetic stocks and DNA markers generated from this study should facilitate the future cloning of *Sr31*, *Lr26* and *Yr9*.

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Introduction

The short arm of chromosome 1 (1RS) of rye (*Secale cereale*) carries important disease resistance genes that confer resistance to rust diseases (*Puccinia* spp.) and powdery mildew. Many successful wheat cultivars containing 1RS translocations from two different rye genotypes have been released, including the 'Veery' lines developed at CIMMYT (Rajaram et al. 1983). The Veery varieties were derived from crosses between a Mexican spring semi-dwarf and the winter wheat variety 'Kavkaz', which carries a 1BL·1RS translocation chromosome with the 1RS arm coming from 'Petkus' rye (Zeller 1973; Schlegel and Korzun 1997). This rye chromosome arm carries genes *Sr31, Lr26, Yr9* and *Pm8* conferring race-specific resistance to stem rust (caused by *Puccinia graminis* f. sp. *tritici*), leaf rust (*Puccinia triticina*), stripe rust (*Puccinia striiformis* f. sp. *tritici*) and powdery mildew (*Blumeria graminis* f. sp. *tritici*), respectively. The rust resistance genes *Sr31*, *Lr26*, and *Yr9* from 'Petkus' rye co-segregated among 214 testcross progeny and mapped to the end of chromosome 1RS, approximately 5 cM distal to the seed storage protein genes (*Sec-1*) (Singh et al. 1990; Lukaszewski 2000). In another study, the map location of *Lr26* was confirmed to be distal to the *Sec-1* locus of rye (Hsam et al. 2000). There is some evidence for the presence of *Yr9* in triticale in the absence of *Sr31* and *Lr26* (McIntosh et al. 1995; Adhikari 1996).

In our previous work we identified DNA markers linked to the *Sr31*, *Lr26* and *Yr9* resistance genes on 1RS (Mago et al. 2002). Because suitable rust strains and genetic stocks for mapping these resistance genes directly in rye were not available, we used a wheat translocation line containing 1RS and a set of *ph1*-induced recombinants between 1RS and homoeologous wheat 1S arms (Lukaszweski 2000) to position DNA markers relative to the rust resistance genes. The DNA markers included resistance gene analogs (RGAs) of the

nucleotide binding site–leucine rich repeat (NBS-LRR) class. We also developed a comparative map of a chromosomal region on 1S by combining previous Triticeae mapping data with results from rye and *Aegilops tauschii* (Mago et al. 2002).

Our objectives in this study were to lay the groundwork for map-based cloning of *Sr31* and to determine whether *Sr31*, *Lr26* and *Yr9* could be separated by recombination or mutation. We therefore established a wheat mapping family using parental wheat translocation lines carrying 1RS from 'Petkus' rye (*Sr31*, *Lr26*, *Yr9*) and 1RS mostly derived from 'King II' rye (no rust resistance). This mapping family was used initially to identify DNA markers flanking *Sr31*, *Lr26* and *Yr9*. Flanking DNA markers were then used to develop a high-resolution map of the *Sr31*, *Lr26* and *Yr9* region. A recombinant separating the leaf rust resistance gene *Lr26* from *Sr31* and *Yr9* was obtained. No recombinants were recovered between *Sr31* and *Yr9*. In addition we isolated mutants of the individual rust resistance genes thus separating the three rust resistance genes from each other.

Materials and methods

Plant material

A wheat F2 family of 143 individuals was derived from two parental lines each possessing a 1BL·1RS translocation. One parent, Federation *4/Kavkaz, carried a 'Petkus' 1RS with *Sr31*, *Lr26* and *Yr9*. The other parent contained 1RS that was partly of 'Petkus' rye origin (the proximal region) and partly of 'King II' rye origin (the distal region) that included the *Nor* and *Sec1* loci of 'King II' and the susceptible haplotype of *Sr31*, *Lr26* and *Yr9* (Fig. 1). The origin of the second parental line was described in Singh et al. (1990). The wheat line 'Federation', the recurrent parent of Federation*4/Kavkaz, was used as a control to establish that DNA markers originated from the 1RS arm backcrossed into 'Federation' from 'Kavkaz'. Chinese Spring (CS) ditelo (Dt) 1BL was used to differentiate 'King II' 1RS markers from wheat 1AS or 1DS.

To determine the *Sr31*, *Lr26* and *Yr9* genotypes of each of the 143 F2 plants, three lots of 25 F3 seedlings from each F2 plant were separately tested with cultures of the three rust pathogen species, namely *P. graminis* f. sp. *tritici* pathotype 98-1, 2, 3, 5, 6, *P. triticina* pathotype 104-2, 3, (6), (7), 11 and *P. striiformis* f. sp. *tritici* pathotype 110 E143 A+, respectively. The inoculation procedures were described in Mago et al. (2004).

The high-resolution mapping family consisted of 36 F2 individuals that were recombinant for PCR markers flanking *Sr31*. These were identified among 1,580 F2 individuals from the above cross. F3 progeny of the 36 recombinants were phenotyped for stem rust (*Sr31*), leaf rust (*Lr26*) and stripe rust (*Yr9*) response.

DNA isolation

Genomic DNA was isolated from leaves as described previously in Mago et al. (2002, 2004). For high-resolution mapping a half seed method was used. DNA was extracted by placing individual half seeds (without the embryo) in wells of a 96 well plate. The half seeds were crushed with a stainless steel ball bearing using a Retsch MM300 mixer mill (Retsch, Haan, Germany). After a short spin at 100 rpm, 300 µl of pre-warmed (65°C) extraction buffer (0.1 M Tris–HCl pH8.0, 0.05 M EDTA pH8.0 and 25% SDS) was added. The plate was sealed and incubated at 65°C for 1 hr then cooled at 4°C for 30 min. To each well, 150 µl of 6 M ammonium acetate was added, the plate was resealed and shaken vigorously before leaving it at 4°C for 30 min. The plate was centrifuged at 3,000 rpm for 30 min and the supernatant was transferred to a fresh deep well plate containing 180 µl of iso-propanol in each well, mixed thoroughly and left at room temperature for 5 min to precipitate before centrifugation at 3,000 rpm for 30 min. The supernatant was carefully discarded and the DNA washed with 250 µl of 70% ethanol. The pellet was air dried and resuspended overnight in 150 µl 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 microtiter plate for storage at -20°C.

Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and PCR marker analysis

RFLP filters were made from 20 µg of DNA isolated from CS Dt1BL, the two parental lines, and from 'Federation', digested with 12 restriction enzymes (*Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Nco*I, *Nde*I, *Nsi*I, *Sac*I, *Xba*I and *Xho*I). Southern analysis was done under standard conditions using RFLP probes previously mapped in the region (Mago et al. 2002). DNA probes used for hybridization to blotted DNA digests were labeled with [^{32}P]-CTP using the megaprime DNA labeling system (Amersham Pharmacia). When an enzyme was identified that gave a polymorphism with a probe, DNA from all the lines comprising the mapping family was digested with the same enzyme, hybridized with the probe and the RFLP scored. RFLP probes were mapped using the restriction enzymes *Bam*HI (Mla-LRR, 540G23-T7, BF291707), *Dra*I (BE196644, MWG2245), *Eco*RI (MWG68, MWG 837, BF478880, BE444266, BE405749), *Eco*RV (BE196644, Mla-LRR, MWG 60) and *Xba*I (MWG 36, P2M11). Marker Iag95 was mapped as a co-dominant PCR marker using primers and conditions described in Mago et al. (2002).

Bulk segregant analysis was done on pooled DNA from ten homozygous resistant and ten homozygous susceptible F2 plants using the standard AFLP procedure with *Pst*I (5′ GATGGATCCAGTGCAG 3′) and

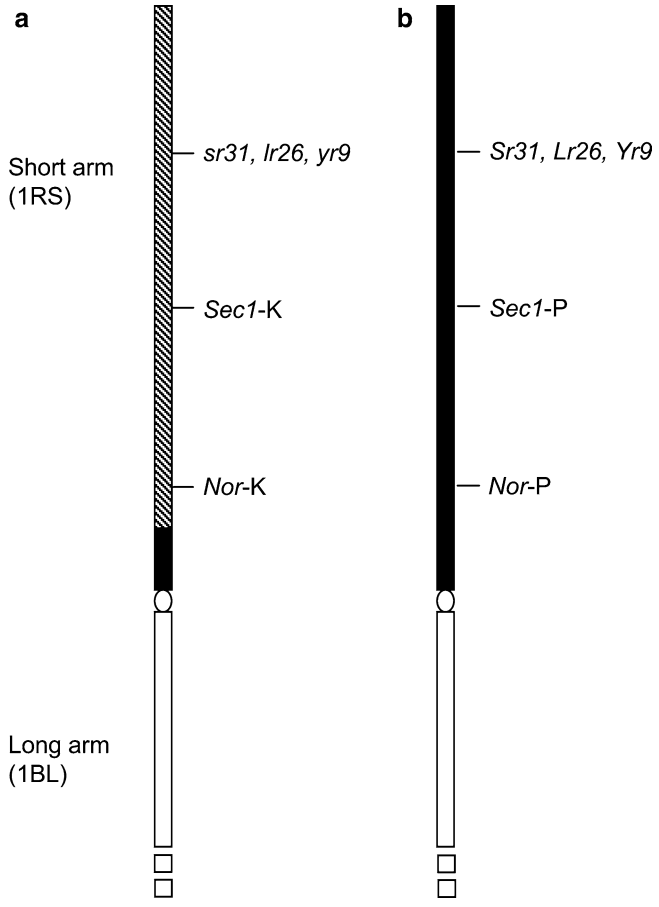

Fig. 1 Structure of the short arm of rye chromosome 1 in the two parents used for generating the F2 mapping family. **a** Susceptible parent 'King II' derivative line. The 'Petkus'– derived proximal region is shown in *solid black* and 'King II' distal region is *shaded*. **b** Resistant parent carrying the 1BL·1RS translocation from 'Petkus' rye, shown as *solid black* line. The inferred locations of the nucleolus organizer locus (*Nor*) and secalin seed storage gene locus (*Sec1*) are marked

*Mse*I (5′ GATGAGTCCTGAGTAA 3′) primer combinations with three additional nucleotides (Vos et al. 1995). CS Dt1BL and Federation were included to distinguish the 1RS bands from wheat bands of 1AS or 1DS origin. Cloning and sequencing of the polymorphic bands was done as described in Mago et al. (2002). One of the polymorphic bands amplified from Federation*4/Kavkaz, P-AGT/M-GTT-287 (P6M12), was cloned, sequenced and converted to a PCR-based marker (P6M12-*P*) that was amplified from the genomic DNA using the primers 5′ GTACTAGTATCCAGAGGTCA CAAG 3′ and 5′CAGACAAACAGAGTACGGGC 3′. Amplification conditions were initial denaturation at 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 40 s, and then one cycle of 72°C for 10 min in 20 µl reactions. The marker was dominant: two P6M12-*P* fragments of 360 and 260 bp were amplified from the resistant parent Federation*4/Kavkaz and the susceptible parent was null for this marker.

DNA from Federation*4/Kavkaz and mutants in class 6, 7 and 8 was used to develop AFLP markers using the same procedure as described above. A mutant from class 1 with a deletion of the 1RS chromosome arm was included to identify the 1RS specific bands from any wheat AFLPs.

Four microliter of the DNA was used to perform a multiplex PCR using the primers for Iag95 and P6M12-*P*. Amplification conditions were initial denaturation at 95°C for 3 min followed by 30 cycles of 94°C for 30 s, 57°C for 1 min and 72°C for 1 min and then 1 cycle of 72°C for 10 min in 20 µl reaction. Recombinants were confirmed by repeating the PCR using the individual marker amplification. Recombinant individuals were recovered by germinating the retained half seed with the embryo and confirmed as recombinants by repeating the PCR on genomic DNA from the same plant.

Wheat and rice sequence comparisons and cloning of wheat ESTs

Synteny between wheat 1AS chromosome and rice was exploited in order to identify potential new markers in the region. Wheat ESTs previously mapped to deletion bin 1AS-3, which showed a nucleotide sequence identity with rice chromosome 5S BAC/PAC clones (Guyot et al. 2004; Spielmeyer and Richards 2004), were downloaded from http://wheat.pw.usda.gov/NSF/. Primers designed on the basis of published EST sequences were used to amplify CS genomic sequences that were gel purified (Qiagen, Germany) and cloned into pGemT-Easy Vector system (Promega). Insert identity was confirmed by DNA sequencing and cloned fragments were amplified by PCR to produce probes for DNA gel-blot analysis.

Linkage analysis

Segregations of the markers and rust resistance loci (*Sr31*, *Lr26* and *Yr9*) were tested using chi-squared test for the expected 1:2:1 or 3:1 ratios. Linkage analysis and map construction for markers and resistance loci were performed with the MAPMAKER Version 2.0 (Lander and Green 1987). An LOD score of 3.0 was used to develop the linkage map and the Kosambi mapping function was used to convert recombination frequencies into centimorgans (cM).

Mutagenesis and mutant screening

Two sets of mutation experiments were conducted. In one experiment seed from wheat varieties 'Pakistan 81', 'Sarhad 82' and 'Kohinoor 83' that carry the 'Petkus' 1RS translocation were treated with ethyl methyl sulfonate (EMS). In the second mutagenesis experiment Federation*4/Kavkaz was mutagenized with EMS,

γ-irradiation or sodium azide. EMS and γ-irradiation treatments were according to the methods described in Mago et al. (2004). For treatment with sodium azide seeds were soaked overnight in water then drained and treated for 2 h in 7 mM or 10 mM sodium azide in a pH 3.0 buffer with gentle shaking. The seeds were washed thoroughly under tap water overnight and dried on filter paper before sowing in soil. In the first experiment, M2 families from single ears of independent M1 plants were screened for either *Lr26* or *Yr9* mutants. In the second experiment, M2 families were screened for either *Sr31* or *Lr26* mutants. M3 families of each mutant were phenotyped with all three rust species (those detecting *Sr31*, *Lr26* and *Yr9*).

Results

Segregation of resistance genes

For the molecular mapping of the rust resistance genes *Sr31*, *Lr26* and *Yr9*, a total of 143 F3 families from the cross between the rust susceptible 'King II' derivative line and the rust resistant Federation*4/Kavkaz were tested for inheritance of the three rust resistances. Consistent with earlier analyses (Singh et al. 1990; Lukaszewski 2000) no recombination was observed between the three resistances. The observed segregation of 47 homozygous resistant, 65 heterozygous and 31 homozygous susceptible families is consistent with a 1:2:1 monogenic segregation ratio ($\chi^2 = 4.81$, $P > 0.05$). Because the *Sr31*, *Lr26* and *Yr9* specificities co-segregated with each other, this study provided no evidence as to whether these specificities are determined by the same gene, or by separate, closely linked genes.

Genetic mapping

Twenty-five RFLP probes previously mapped on wheat chromosome 1S, barley 1HS (Wei et al. 1999) and rye 1RS (Mago et al. 2002), in combination with 12 restriction enzymes, were used to identify RFLPs. Only eight of the 25 probes detected a polymorphism between the parents. Seven of these RFLP markers, one AFLP marker (P6M12-*P*, see below), one RFLP-derived PCR marker (Iag95) and the rust resistance genes were mapped using the 143 F2 plants and their F3 progenies (Fig. 2). RFLP marker MWG60 mapped 4.5 cM distal and Iag95 1.7 cM distal to the rust resistance locus. Iag95 behaved as a co-dominant PCR marker. We previously showed that probes derived from the powdery mildew locus *Mla* in barley identified markers in the rust resistance region on 1RS (Mago et al. 2002, 2004). The Mla-LRR probe detected multiple restriction fragments and comparisons of 'Federation' and Federation*4/Kavkaz and CS Dt1BL and the King II derivative identified RFLPs derived from rye 1RS (Fig. 3). Some of these rye fragments were also

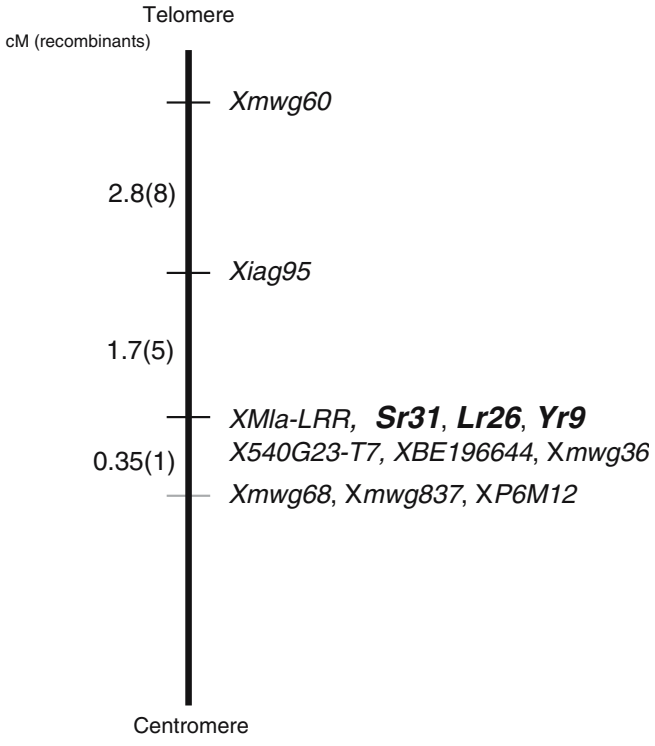

Fig. 2 Genetic map of the genomic region carrying rust resistance genes *Sr31*, *Lr26* and *Yr9* on the short arm of rye chromosome 1 based on 143 F2 individuals. Genetic distances (cM) as well as number of recombinants (*shown in brackets*) observed for specific intervals are shown on the *left hand side* and genetic markers on the *right*

polymorphic between the rust resistant and susceptible parents of the mapping family. These RFLPs co-segregated with rust resistance. RFLP marker MWG36, which maps distally to the *Mla* locus in barley (Wei et al. 1999) and a marker detected with barley EST BE196644 (Wei et al. 2002), also co-segregated with Mla-LRR markers and rust resistance. A barley BAC end marker 540G23-T7 and two RFLP probes, MWG68 and MWG837, which were previously mapped proximally to *Mla* in barley (Wei et al. 1999), were placed 0.35 cM proximal to the rust resistance locus. The map order of all these markers on 1RS was in agreement with previously published maps in other members of the Triticeae (Peng et al. 2004). Markers MWG36, MWG68 and MWG837 were dominant and efforts to convert MWG68 and MWG837 to PCR-based markers were not successful.

One AFLP marker (P6M12), which mapped proximal to the rust resistance locus and co-segregated with MWG68 and MWG837 (Fig. 2), was converted to a PCR-based marker (P6M12-*P*). None of the markers proximal to MWG68 or MWG837 in previously published wheat and barley maps showed polymorphism between the parents. For high-resolution mapping PCR markers Iag95 and P6M12-*P*, which flank the rust resistance gene(s), were used to identify individual F2 recombinants in the *Sr31*, *Lr26* and *Yr9* region.

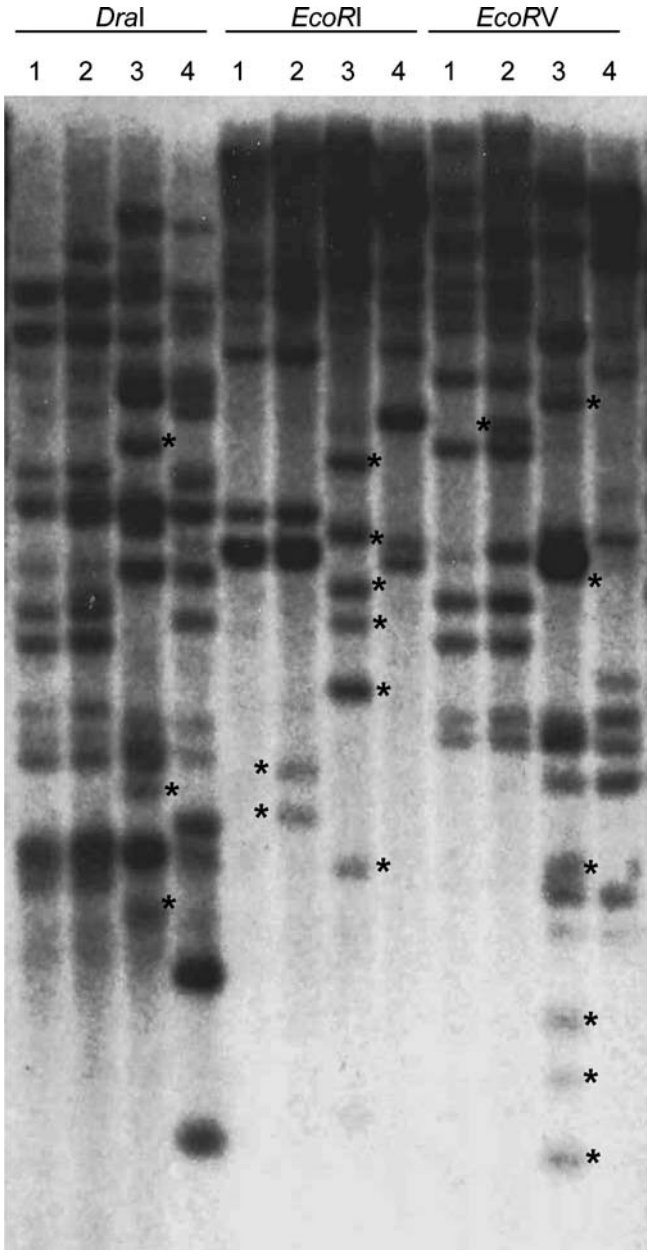

Fig. 3 DNA gel blot hybridized with barley probe Mla-LRR. *Lanes 1*, CS Dt1BL, *2*, 'King II' derivative line, *3*, Federation*4/Kavkaz, *4*, 'Federation'. Twenty micrograms of genomic DNA per lane from each line was digested with the indicated restriction enzymes. The polymorphic rye specific bands in both the parents are indicated by an *asterisk* (*)

Selection of recombinants for high-resolution mapping

DNAs from 1,580 F2 seeds were screened for recombination between the flanking PCR markers Iag95 and P6M12-*P*. Because Iag95 is a co-dominant marker and P6M12-*P* is dominant marker, only 50% of F2 individuals carrying a single recombinant chromosome and 50% of those carrying two recombinant chromosomes could be identified. Thirty-six F2 recombinants were recovered. Figure 4 shows the PCR amplification of Iag95 and P6M12-*P* in the parents and three recombinants and a schematic diagram representing the genotypes of the recombinants that could be recovered. The F3 progeny of the recombinants were phenotyped for rust resistance. One recombinant separated *Lr26* from *Sr31* and *Yr9*. This recombinant was of type 1 (Fig. 4) and was homozygous for Iag95-K, homozygous susceptible to leaf rust and heterozygous for *Sr31*, *Yr9* and the RFLP probes Mla-LRR and BE196644.

All the RFLP markers that mapped to the Iag95-P6M12-*P* region (Fig. 2) were mapped in the high-resolution mapping family (Fig. 5). Iag95 mapped 1.99 cM distal to *Lr26*. Of the 36 recombination events identified between Iag95 and P6M12, 30 were located between Iag95 and *Lr26*. RFLP marker MWG36, which previously co-segregated with the rust loci, was separated from *Lr26* by 13 recombination events (within ~1 cM; Fig. 5). Two recombination events were identified between the barley BAC end marker 540G23-T7, which maps proximal to the *Mla* locus in barley (Wei et al. 1999; Mago et al. 2004) and the *Sr31*/*Yr9* locus (0.13 cM). The polymorphisms detected with the barley Mla-LRR probe using restriction enzymes *Bam*HI, *Eco*RI and *Eco*RV, co-segregated with *Sr31* and *Yr9*, but recombined with *Lr26*. Barley EST marker BE196644 also co-segregated with *Sr31* and *Yr9*. The barley BAC end probes derived from 'Morex' in the region distal to the *Mla* locus (Wei et al. 1999) were either monomorphic or did not hybridize to the 1RS region of the parents. In barley, this region contains several retro transposon-like elements and may have significantly diverged from the orthologous rye region (Wei et al. 2002).

Identification of AFLP markers associated with the resistance genes

To increase the number of markers in the region of the rust resistance genes, we screened AFLPs across bulk DNA from homozygous resistant and homozygous susceptible F2 plants as described previously. A total of 144 *Pst*I–*Mse*I primer combinations identified 15 AFLPs specific to the rye chromosome arm 1RS. Eight of these originated from the susceptible parent and seven from the resistant parent. Only four of the 15 markers mapped to the Iag95-P6M12-*P* interval and one of these P-ATG/M-GTG (P8M11) co-segregated with Iag95 (Fig. 5). Three AFLP markers P-ACC/M-GTG, P-AGT/M-GAC and P-ATC/M-GAC (P2M11, P6M22 and P7M2) co-segregated with RFLP probes MWG68 and MWG837. No AFLP marker mapped between the rust locus and the distal RFLP marker MWG36.

Wheat – rice synteny in the Sr31, Lr26 and Yr9 region and generation of additional markers

To find extra markers in the region we explored the synteny of wheat 1S to rice. Guyot et al. (2004) and

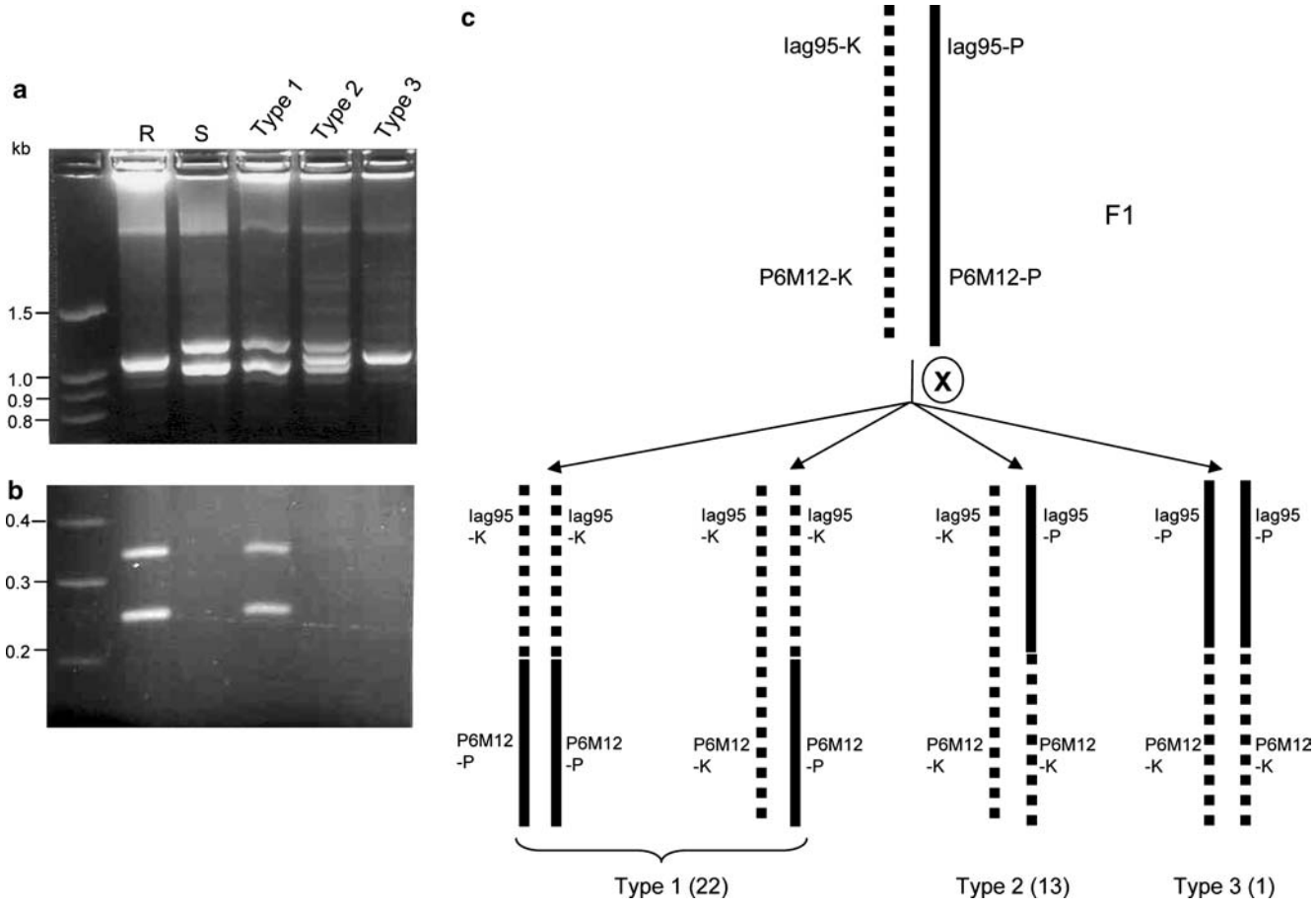

Fig. 4 PCR amplification of the flanking co-dominant marker Iag95 (**a**) and dominant marker P6M12-*P* (**b**) used for high resolution mapping of genomic region carrying rust resistance genes on the rye chromosome arm 1RS. *Lanes* R (resistant parent), Federation*4/Kavkaz; S (susceptible parent), 'King II' substitution line derivative; Type 1–3, three types of recombinants. (**c**) Crossing scheme used to generate the recombinants and the different types (1–3) that were recovered using PCR markers Iag95 and P6M12-*P*. Numbers of recombinants recovered of each type are indicated in *brackets*. The 'Petkus' alleles are designated Iag95-P and P6M12-P and the 'King II' derived alleles are Iag95-K and P6M12-K

Spielmeyer and Richard (2004) demonstrated that genes on BAC/PAC contigs from rice chromosome 5S were related to genes in wheat chromosome 1S deletion bin 1AS3-0.86-01.00. Wheat RFLP marker MWG2245, which co-segregated with Iag95 (Fig. 5), provides a link between these wheat genes, the *Sr31*, *Lr26*, *Yr9* region and the rice genome. We designed primers to the 21 wheat ESTs that were mapped proximally to MWG2245 by Guyot et al. (2004) and Spielmeyer and Richard (2004). PCR products amplified from wheat were used as probes and the resultant RFLPs were mapped. Four RFLPs (BF291707, BE405749, BE444266 and BF478880) mapped to the Iag95-P6M12-*P* region (Fig. 5) but none were closer to the resistance genes than previously mapped DNA markers. Comparison of the map location of these markers on 1RS and the rice 5S BAC/PAC contig is shown in Fig. 5. The order of the distal markers BF291707 and BE405749 is reversed and the proximal markers BF478880 and BE444266, which co-segregate in our high-resolution mapping family, are 600 kb apart in rice. The markers BE405749 and BF478880 that are 2.1 cM apart in rye are separated by 200 kb in rice. If there is synteny between rye and rice in this region, then rice orthologs of rust resistance genes could be expected to occur in this interval. No clear candidates for *Sr31*, *Lr26 and Yr9* were identified in this rice interval, similar to a previous study looking for orthologs of the barley stem rust resistance gene *Rpg1* in rice (Kilian et al. 1997).

Isolation and analysis of rust susceptible mutants

Two independent mutation experiments were conducted on wheat varieties carrying 'Petkus' 1RS. In the first experiment, using EMS, 70 mutants were recovered from 6,737 M2 families. Thirty-six mutants were for *Yr9* alone, 32 were for *Lr26* alone and two were mutant for both *Lr26* and *Yr9*. *Sr31* mutants were not selected in this experiment. In the second experiment, a total of 43 mutants were recovered from 4,478 M2 families. These include 35 from radiation treatment (from 2,540 M2 families) and two from EMS treatment (980 M2 families). Treatment with sodium azide yielded six mutants

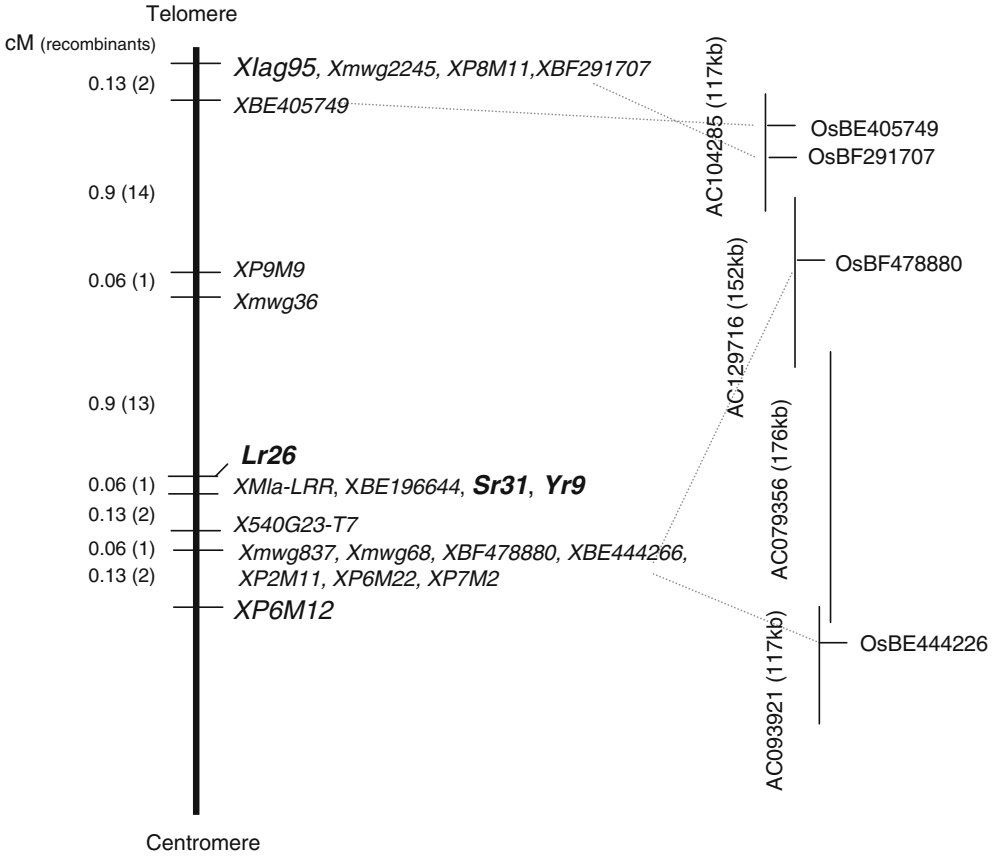

Fig. 5 High-resolution genetic map of the distal portion of the short arm of rye chromosome 1S harboring resistance genes *Sr31*, *Lr26* and *Yr9* and comparison to the BAC/PAC contig of distal end of short arm of rice chromosome 5. Genetic distances (cM) as well as number of recombinants (*shown in brackets*) observed for specific intervals on the high-resolution map are shown on the *left* and genetic markers are shown on the *right*

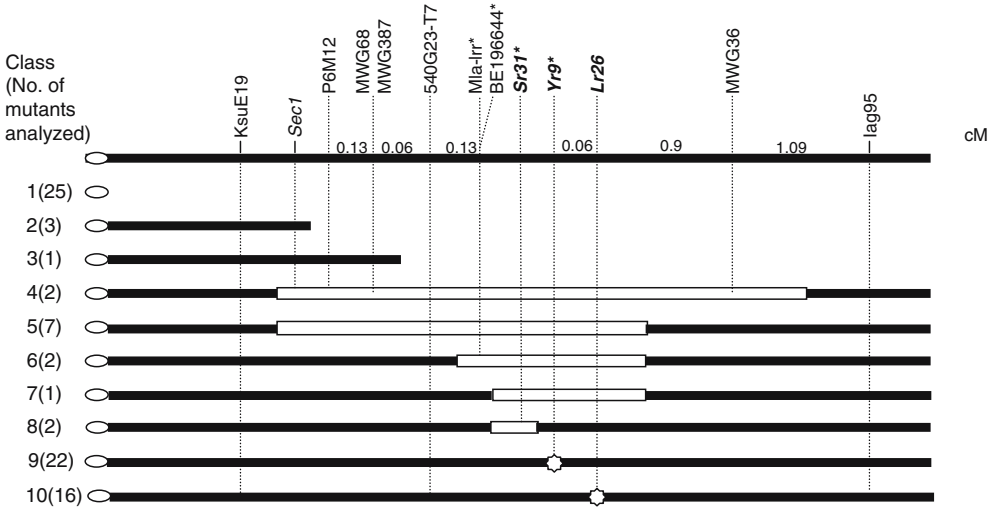

Fig. 6 Diagrammatic representation of the various classes of rust susceptible mutants. Numbers of mutants analyzed in each class are in *brackets*. Class 1 mutants may include aneuploids and complete 1RS deletions. Mutants in class 4–8 were interstitial deletions and are represented as *empty box*. *Asterisk* the order of *Sr31* and *Yr9* with respect to Mla-LRR could not be determined

(958 M2 families).The rust resistance genotypes of the mutants were confirmed by analyzing their M3 progenies using all three rust species. Forty-one of the 43 mutants were mutant for *Sr31*, *Lr26* and *Yr9*. Two irradiation mutants were mutant for *Sr31* only. No *Lr26* or *Lr26*, *Yr9* double mutants were recovered in experiment 2. All mutants that lost more than one resistance were deletions. These experiments indicate that these resistances can be mutated independently and are probably controlled by separate genes.

DNA markers from 1RS (Mago et al. 2002 or present study) were used to examine the majority of the mutants. Ten classes of mutants were distinguished and these are depicted in Fig. 6 together with the number of mutants analyzed in each class. Mutants in classes 1–3 are likely terminal deletions of varying sizes with some of those in class 1 possibly being whole arm or entire chromosome deletions. These included the two *Lr26*, *Yr9* double mutants from experiment 1, which had not been characterized for their *Sr31* phenotype. Mutants in classes

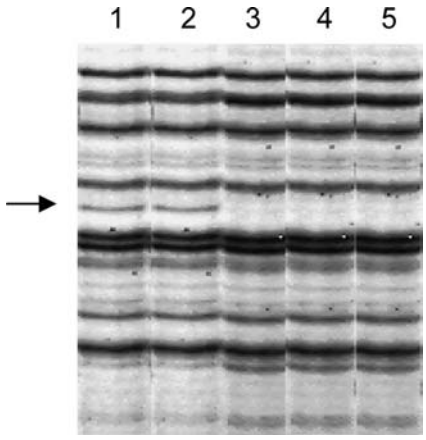

Fig. 7 An example of AFLP analysis of mutants used to develop markers in the *Sr31*, *Lr26* and *Yr9* region. *Lanes 1*, Susceptible parent ('King II' derivative), *2*, Federation*4/Kavkaz, *3*, Mutant class1, *4*, Mutant Class 6, *5*, Mutant Class *8*. *Arrow* shows the AFLP P-ACC/M-GAG-384 in the parents (*lanes 1 and 2*) and is absent in interstitial deletion mutants in classes 1, 6 and 8

4–6 are interstitial deletions that lost all resistance genes and at least two DNA markers. Although the mutant in class 7 had lost no DNA marker it is assumed to be an interstitial deletion because it lacks all three rust resistances. The two mutants in class 8, which lack only *Sr31*, could be point mutations or involve small interstitial deletions not identified by any of the markers used in the study. Other evidence (see below) indicates that mutants in classes 7 and 8 do involve interstitial deletions. Hybridization of the Mla-LRR probe with the genomic DNA of mutants in classes 7 and 8 digested with multiple restriction enzymes (6 enzymes for class 7 and 16 enzymes for class 8; data not shown) did not detect loss of any members of the complex *Mla* cluster on 1RS. The barley EST probe BE196644 was also present in mutants in classes 7 and 8 but was deleted in all other classes. Mutants in classes 9 and 10, all derived from EMS treatment, may be point mutations as they lack none of the molecular markers and retain *Sr31*.

AFLP analysis using 144 *Pst*I–*Mse*I primer combinations of mutants in classes 6, 7 and 8 and their resistant and susceptible parents, identified five new markers P-ACC/M-GAG-384, P-ACG/M-GCG-339, P-ACG/M-GCG-411, P-ACA/M-GCG-553 and P-ATG/M-GTC-456 that were absent in one or more of the five independent mutant lines. The sequences of these AFLP markers are available in the GenBank (Accession Nos. DQ167393–DQ167397). Four of the AFLPs P-ACC/M-GAG-384, P-ACG/M-GCG-339, P-ACG/M-GCG-411 and P-ATG/M-GTC-456 were absent in mutant classes 6, 7 and 8 while P-ACA/M-GCG-553 was absent in classes 6 and 7 but present in class 8. Loss of four markers in class 8 mutants suggests that they also contain deletions. Mutant classes 9 and 10 have not been analyzed. Importantly the AFLPs were present in rust resistant sibs of each mutant class. Figure 7 shows the amplification of P-ACC/M-GAG-384. The marker was amplified in both parents but not in any of the mutants of class 1, 6 and 8. None of the AFLPs that distinguish the mutants from the resistant parent could be mapped in our mapping family because they failed to identify polymorphisms between the resistant and susceptible parents either as AFLP, RFLP or PCR markers. However loss of these AFLPs in class 6 mutants, together with the mapped molecular markers detected by the Mla-LRR and BE196644 probes (Fig. 6), indicates that they map to the rust resistance gene region. Additionally, loss of these markers in class 7 and 8 mutants, which have not lost any of the mapped molecular markers (Fig. 6), indicates that these mutants also result from deletion linked to, and most likely including, the rust resistance genes.

Discussion

Wild relatives of common wheat, *Triticum aestivum*, and related cultivated species like rye, provide a source of genes for wheat improvement (Friebe et al. 1996) and many disease and pest resistance genes have been introgressed into wheat from alien sources by translocation. The short arm of rye chromosome 1 (1RS) from 'Petkus' rye has been the most widely used alien translocation in wheat because of the agronomic and disease resistance advantages it has provided over a wide geographical area. A major difficulty for map-based cloning from alien sources is that normally the alien chromatin does not recombine with its wheat homoeologues. Consequently, it is difficult to develop genetic stocks to facilitate the cloning of such genes. Recombination between alien translocations and a wheat homoeologous chromosome can be achieved to some extent by inducing recombination in a *ph-1* mutant background. Lukaszewski (2000) induced recombination between wheat and rye in a *ph-1* mutant background which allowed limited mapping of DNA markers and the rust resistance genes on 1RS (Mago et al. 2002). In another approach to map resistance genes on the wheat rye translocation chromosome 1BL·1RS, Singh et al. (1990) testcrossed a heterozygote with 1BL·1RS derived from 'Petkus' rye (*Sr31, Lr26, Yr9*) and 1R from King II rye (*sr31, lr26, yr9*) with CS Dt1BL. Recombination between the two 1RS arms permitted a map to be generated in which the rust resistance genes co-segregated. With the aim to develop tools to clone *Sr31* we used two 1BL·1RS lines, one with and one without *Sr31, Lr26* and *Yr9*, as parents to establish an F2 mapping family. Initial mapping in a low resolution family based on 143 F2s showed that chromosomes from the two rye segments ('Petkus' and 'King II') recombined and also confirmed the co-segregation of *Sr31*, *Lr26* and *Yr9*.

High-resolution mapping, which gives precise placement of the target gene amongst closely linked markers, is essential for map-based cloning projects in cereals (Blair et al. 2003; Yan et al. 2003; Bulgarelli et al. 2004; Yahiaoui et al. 2004). We identified 36 F2 individuals

recombinant for the two markers 2.3 cM apart that flanked the rust resistance genes. One of these recombinants separated *Lr26* from *Sr31* and *Yr9* (0.06 cM) providing a partial answer to the question of whether one or more genes located on the chromosome arm 1RS confer resistance against the three rust species. As no recombinants between *Sr31* and *Yr9* were obtained, we used mutagenesis to further investigate whether *Sr31* and *Yr9* are different genes. The recovery of several mutants for *Sr31*, *Lr26* or *Yr9* alone indicates that they are different closely linked distinct loci (Fig. 6).

Previously we mapped the stem rust resistance gene *SrR* on 1RS from 'Imperial' rye using deletion mutation analysis (Mago et al. 2004). Unlike *Sr31*, *SrR* is not linked to any leaf rust or stripe rust resistance gene. It is presently unknown whether *Sr31* and *SrR* express the same or different resistance specificities which could indicate whether they might be the same or different genes. An analysis of *SrR* mutants revealed that it maps in the same region as *Sr31* and that homologues of the barley powdery mildew resistance gene *Mla* were candidates for *SrR* (Mago et al. 2004). An Mla-LRR probe identified about 20 fragments on 1RS and some interstitial deletion mutants showed loss of only three of these bands (Mago et al. 2004). In the present study, we were interested in the relationship between the rye *Mla* homologues and *Sr31*, *Lr26* and *Yr9*. High-resolution mapping of the region also showed that all *Mla* RFLPs on 1RS co-segregated with *Sr31*and *Yr9* resistance genes and recombined with *Lr26*. However, the retention of all *Mla* restriction fragments in the single deletion mutant in class 7, that has lost all three rust resistance genes, and the two mutants in class 8, that have lost only *Sr31*, suggest that a rye homologue of barley *Mla* is unlikely to be a candidate for any of the three resistance genes of 'Petkus' origin. Two other RGA classes (RGH2 and RGH3; Wei et al. 1999) at the barley *Mla* locus failed to identify any rye homologues in our study. Nevertheless, the mapping information and mutant stocks provided here will be important resources for the future cloning of these genes by either map-based or candidate–gene cloning approaches.

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