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Physical and genetic mapping of amplified fragment length polymorphisms and the leaf rust resistance *Lr3* gene on chromosome 6BL of wheat

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Abstract The Argentinian wheat cultivar Sinvalocho MA carries the *Lr3* gene for leaf rust resistance on distal chromosome 6BL. In this cultivar, 33 spontaneous susceptible lines were isolated and cytogenetically characterized by C-banding. The analysis revealed deletions on chromosome 6BL in most lines. One line was nulli-6B, two lines were ditelo 6BS, two, three, and ten lines had long terminal deletions of 40, 30, and 20%, respectively, three lines showed very small terminal deletions, and one line had an intercalary deletion of 11%. Physical mapping of 55 amplified fragment length polymorphism (AFLP) markers detected differences between deletions and led to the division of 6BL into seven bins delimited by deletion breakpoints. The most distal bin, with a length smaller than 5% of 6BL, contained 22 AFLP markers and the *Lr3* gene. Polymorphism for nine AFLPs between Sinvalocho MA and the rust leaf susceptible cultivar Gamma 6 was used to construct a linkage map of *Lr3*. This gene is at a genetic distance of 0.9 cM from a group of seven closely linked AFLPs. The location of the gene in a high recombinogenic region indicated a physical distance of approximately 1 Mb to the markers.

Keywords Wheat · *Triticum aestivum* · Leaf rust resistance · Deletion lines · Amplified fragment length polymorphisms · *Lr3* gene

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Introduction

Common wheat, *Triticum aestivum* L., is an allohexaploid species, whose large genome (about 17,300 Mb) contains three related genomes, called A, B, and D, which were derived from different diploid progenitors. The ploidy level of common wheat provides genetic duplications that enable extensive cytogenetical manipulation (Sears 1954). Different aneuploid stocks, such as nulli-tetrasomic and ditelosomic lines or wheat-alien addition lines, were developed in cultivar Chinese Spring of hexaploid wheat. These lines continue to be instrumental in the cytogenetic mapping of many characters and DNA markers such as RFLPs, SSRs, amplified fragment length polymorphisms (AFLPs), and ESTs (Röder et al. 1995; Huang et al. 2000; Qi et al. 2003). The high degree of tolerance to aneuploidy of hexaploid wheat confers this species the possibility of obtaining chromosome deletions in homozygous condition. Endo (1988, 1990) reported a genetic system for the systematic production of deletions in individual chromosome arms of wheat. The monosomic addition of a single chromosome from *Aegilops cylindrical*, or *Aegilops triuncialis*, or a chromosome segment from *Aegilops speltoides*, to Chinese Spring causes chromosome breaks in gametes lacking the alien chromosome or chromosome segment. Endo and Gill (1996) used this system to obtain 436 heterozygous deletions, which were identified by C-banding. Most of these deletions covering all 21 wheat chromosomes (80%) were established as homozygous lines. Deletion stocks have been extensively used for molecular mapping in Chinese Spring, providing information on the physical positions of genes controlling phenotypic traits, and different aspects of chromosome structure and function (Werner et al. 1992; Gill et al. 1993a, b, 1996; Faris et al. 2000; Qi et al. 2004).

However, deletion stocks of cultivar Chinese Spring require additional manipulations to be incorporated in the study of useful traits that appear in other cultivars of bread wheat. Deletions spontaneously arising in such

cultivars can be used for similar purposes. Deletions with sufficient size to be detected microscopically can be characterized by C-banding, but identification of small differences between deletions, or submicroscopic-sized deletions, requires the use of more sensitive techniques such as the constructions of genetic maps with molecular markers. AFLPs (Vos et al. 1995) are a powerful DNA marker technique, originally conceived to construct high-density DNA marker maps, with applications in genome research and positional cloning of genes. This technique has the advantage that previous knowledge of the DNA sequence is not required and is particularly effective for the simultaneous screening of very large numbers of loci. It has been widely used in different applications, in species such as barley (Becker et al. 1995), rice (Hashimoto et al. 2004), maize (Vuylsteke et al. 1999) and wheat (Parker et al. 1998; Shan et al. 1999). On the other hand, the integration of deletion physical maps and genetic linkage maps is helpful in aligning genetic and physical distances and can assist for estimating the frequency and distribution of recombination along chromosomes. Map-based cloning strategies enhance the utility of genetic linkage maps when a comparison of physical and genetic distances is possible in a given chromosome region (Harper and Cande 2000).

Sinvalocho MA is an Argentinian wheat cultivar used as a donor of leaf rust resistance in many crosses and as a differential local variety in leaf rust studies for more than 50 years in Argentina (Sacco et al. 1995). The *Lr3* gene, which confers recessive resistance to Argentine race 66 of *Puccinia triticina*, was located at the distal end of the long arm of chromosome 6B (Sacco et al. 1992). Some spontaneous naturally occurring deletions of chromosome arm 6BL, containing the *Lr3* gene for leaf rust resistance, were isolated in this cultivar (Sacco et al. 1995, 1998). Rust susceptibility derived from mutation or loss of the *Lr3* gene has facilitated the production of 33 susceptible lines. These lines were assumed to be the result of deletions of the 6BL arm that included the *Lr3* gene. In order to characterize this deletion stock and to search for molecular markers linked to the *Lr3* gene, these 33 susceptible Sinvalocho MA lines are analyzed at the karyotypic level by C-banding and at the molecular level by AFLPs analysis. In addition, nine AFLP markers, together with the *Lr3*, were genetically mapped using a recombinant inbred lines (RILs) population.

Materials and methods

Plant material

Lines of Sinvalocho MA susceptible to *P. triticina* race 66 were derived from three resistant plants, termed T_A, T_B, and T_C. Thirteen spontaneous susceptible plants found among the progeny of T_A, T_B, and T_C were selfed for two generations and tested with race 66 (Sacco et al. 1995) to obtain homozygous susceptible plants. A total of 33 different susceptible lines were subsequently isolated and named A, B, or C according to their T_A, T_B, or T_C progenitor (Fig. 1). The RIL population used in the construction of the genetic map consisted of 60 F7 lines derived from the cross between Gamma 6 (susceptible) and Sinvalocho MA (resistant). C-banding and AFLP analyses were carried out on sib seedlings.

C-banding analysis

The structure of chromosome 6B in each rust susceptible line was studied by C-banding according to the procedure of Sacco et al. (1998). Five to ten 6B chromosomes were measured and the ratio of short arm (without the satellite) to long arm (6BS:6BL) was calculated. For each line, a fraction length (FL) was estimated as the ratio of (6BS:6BL) of the normal chromosome to (6BS:6BL) of the susceptible line chromosome (Endo and Gill 1996).

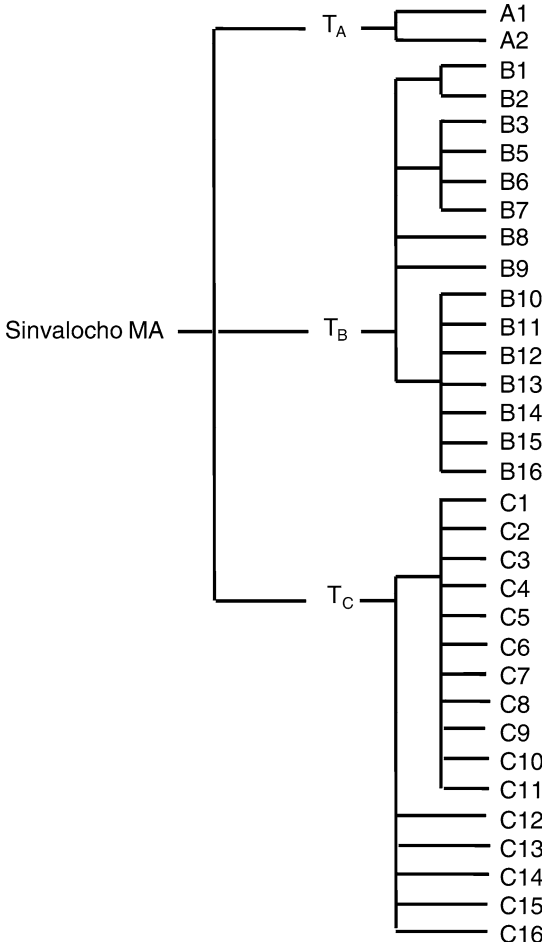

Fig. 1 Genealogy tree of the leaf rust susceptible lines derived from Sinvalocho MA

AFLP analysis

Genomic DNA was isolated according to Sacco et al. (1998) except that fresh material (ground in a mortar with liquid nitrogen) was used instead of lyophilized leaves. Seven hundred and fifty nanograms of DNA was simultaneously digested and ligated to adaptors in a total volume of 20 µl with 3 units (U) of *Pst*I, 1.6 U of *Mse*I, 1.2 U of T4 DNA ligase, 0.2 mM ATP, 75 nM *Pst*I adaptor and 750 nM *Mse*I adaptor in 10 mM Tris–HAc pH 7.5, 10 mM MgAc, 50 mM potassium acetate, 5 mM DTT, 50 ng/µl BSA for 6 h at 37°C. The pre-amplification was done in a final volume of 25 µl with 1.32 mM MgCl2, 0.2 mM dNTPs, 75 ng each of primers P01 and M01 and 1 U of Sequencing grade Taq DNA Polymerase (Promega, Madrid, Spain) using 3 µl of the ligation mix as template. Thermal cycling consisted of 30 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C. The amplification products were diluted 1:3 in low EDTA-TE buffer (10 mM Tris–HCl pH 8, 0.1 mM EDTA) and 1 µl was used for specific amplification in a 22 µl reaction as follows: 1.6 mM $MgCl_2$, 0.2 mM dNTPs, 30 ng of each primer and 1 U of Taq DNA Polymerase (Promega or Gibco, Madrid, Spain). The cycling profile was 30 s at 94°C, 30 s annealing (see below) and 1 min at 72°C. The annealing temperature of 65°C in the first cycle, was subsequently reduced by 0.7°C per cycle for the next 12 cycles, and maintained at 56°C for 25 more cycles. The sequence of the primers and adaptors was as described by Vos et al. (1995). After addition of one volume of loading buffer (10 mM NaOH, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol and 95% deionized formamide) and 5 min incubation at 95°C, 8 µl were loaded in 6% denaturing polyacrylamide gels and electrophoresed in TBE buffer (0.5 time in the upper chamber and one time in the lower) during 3 h at 60 W. The separated fragments were silver stained as follows: 30 min incubation in 10% HAc, three washes in double distilled water, 30 min incubation in 1 g/l $AgNO_3$ plus 0.06% formaldehyde, 15 s wash in chilled double distilled water, developed in chilled 30 g/l $NaCO_3$, 0.06% formaldehyde and 0.0002% sodium thiosulfate (Bassam et al. 1991).

Genetic map

The linkage groups and the genetic distances were calculated using MAPMAKER Version 3.0 (Lander et al. 1987) at min LOD 3.0.

Results

C-banding analysis of the structure of chromosome 6B was carried out in control rust resistant plants derived from progenitors T_A, T_B, and T_C, and in the 33 susceptible lines. All plants studied were homozygous for the constitution of chromosome 6B. The C-banding pattern of this chromosome in the control and susceptible lines is shown in Fig. 2. Line C13, with $2n = 40$, was nullisomic for chromosome 6B and lines A1 and B8 were ditelocentric 6BS (the complete 6BL arm was lost). Most of the remaining rust susceptible lines bore deletions in chromosome arm 6BL, with the exception of lines C1, C2, C3, C4, C6, C7, C8, C9, C10, C11, and C15 that

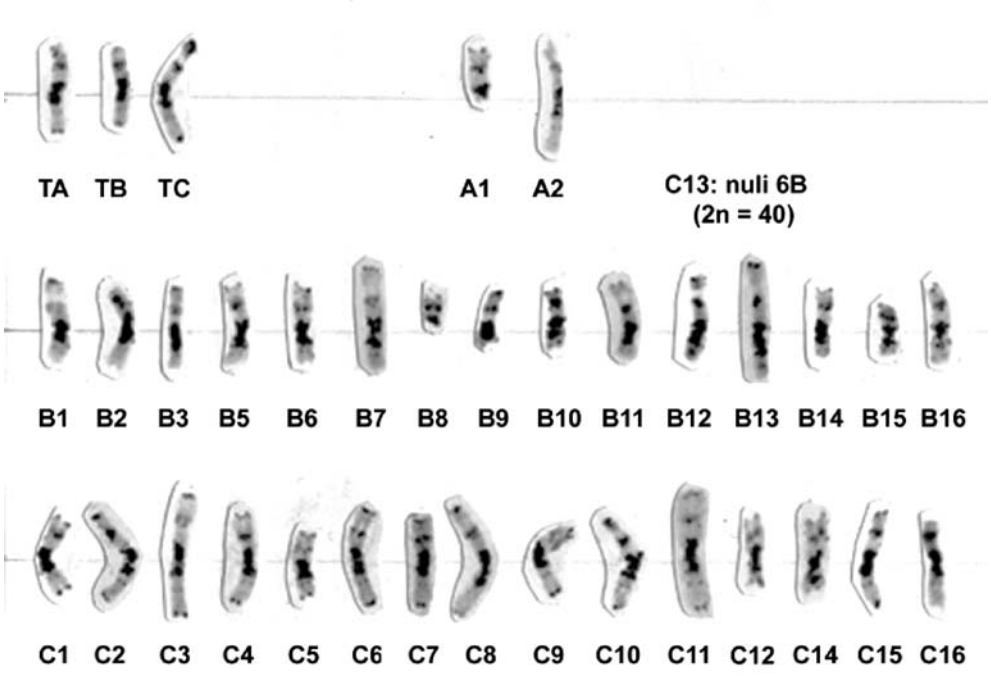

Fig. 2 C-banding of chromosome 6B in the three rust resistant control Sinvalocho MA lines (TA, TB, and TC) and in 32 (An, Bn, and Cn,) rust susceptible lines. (C13, $2n = 40$, was nulli 6B)

Table 1 6BS:6BL ratio (6BS-satellite:6BL) and fraction length (*FL*) of the 6BL arm in the rust resistant control and susceptible lines

Line	6BS:6BL	FL	Line	6BS:6BL	FL
Control	0.74	1.00	B5	0.85	0.87
A2	0.74	1.00	B16	0.89	0.83
B2	0.73	1.01	B1	0.90	0.82
C1	0.74	1.00	B12	0.91	0.81
C2	0.75	0.99	B6	0.94	0.79
C3	0.72	1.03	B11	0.95	0.78
C4	0.74	1.00	B14	0.98	0.76
C6	0.74	1.00	B7	0.98	0.76
C7	0.74	1.00	B13	0.99	0.75
C8	0.76	0.97	B10	1.03	0.72
C9	0.74	1.00	B15	1.05	0.70
C10	0.72	1.03	C12	1.10	0.67
C11	0.73	1.01	B9	1.27	0.58
C15	0.73	1.01	C14	1.27	0.58
C16	0.76	0.97	A1	0.00	0.00
C5	0.83	0.89	B8	0.00	0.00
B3	0.85	0.87			

showed no apparent modification of this arm. Among the lines with deletions, C5 was the only one lacking an intercalary segment subdistally located. The absence of the telomeric C-heterochromatin band of the standard 6BL arm denotes the existence of terminal deletions in the remaining lines.

The values of the 6BS:6BL ratio and FL in the control and susceptible lines are listed in Table 1. Lines B9 and C14 had an FL of 0.58, which means that approximately 40% of the 6BL arm was deleted. Lines B10, B15, and C12, with an FL of 0.70, have lost approximately 30% of 6BL while in lines B1, B3, B5, B6, B7, B11, B12, B13, B14, and B16, with FL values between 0.75 and 0.87, the deletion size is close to 20% of the arm. The internal segment deleted in line C5 represents approximately 11% of 6BL. Lines A2, B2, and C16 had a 6BS:6BL ratio similar to the standard chromosome suggesting no substantial chromatin loss. Because these three lines showed the terminal 6BL banding weaker than the standard chromosome, they could carry a very short terminal deletion. From the C-banding analysis, five different subregions were established for the 6BL arm (Fig. 2).

In the AFLPs analysis, approximately 10.000 loci, 178 primer pair combinations with an average of 60 amplified bands per primer pair, were screened. When a band was absent in the B8 ditelo 6BS line, the primer combination that generated such a band was tested on the rest of the lines to assign markers on 6BL subregions. Forty-four of these primer combinations yielded 55 bands located on 6BL. The physical position of such markers on chromosome arm 6BL subregions was

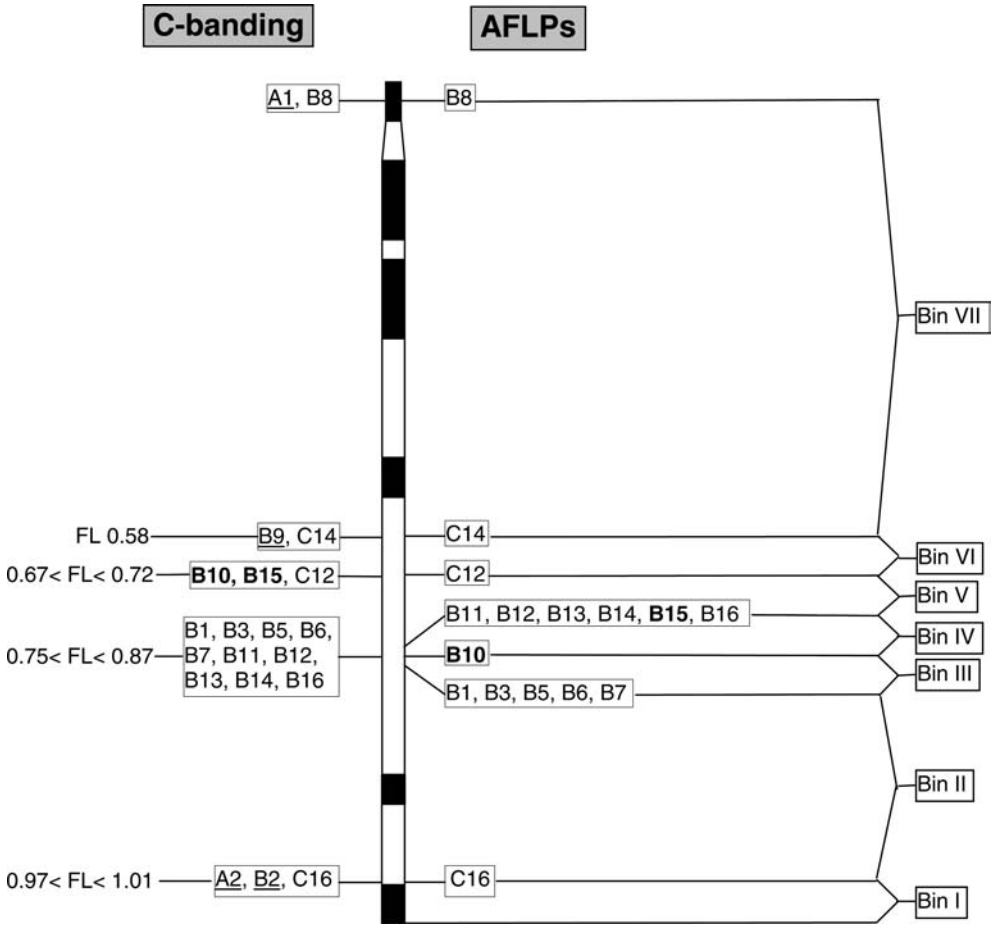

Fig. 3 The position of the breakpoints in different deletions (An, Bn, and Cn) of chromosome arm 6BL of hexaploid wheat cv. ''Sinvalocho'' deduced from the C-banding pattern (*left*) and the AFLP markers mapping (*right*). *Underlined* deletions were only cytologically analyzed. Lines with differences between the molecular and the cytological analyses are shown in *bold*

Table 2 AFLP markers physically mapped in the seven 6BL bins of Fig. 3

Bin	Markers	Total number of markers
Telomere I	**P31/M32$_{125}$**, **P34/M32$_{190}$**, P34/M39$_{150}$, P35/M31$_{190}$, P35/M33$_{110}$, **P35/M38b$_{180}$**, P35/M38d$_{50}$, P35/M39$_{150}$, P35/M40a$_{150}$, P35/M40b$_{60}$, P35/M45b$_{275}$, P36/M33$_{165}$, P36/M37$_{195}$, **P36/M39$_{175}$**, P38/M34$_{240}$, P39/M34a$_{185}$, **P39/M34b$_{135}$**, P41/M35$_{120}$, P41/M38$_{80}$, P42/M33$_{245}$, **P43/M40$_{200}$**, P46/M31$_{140}$	**22**
II	P34/M31$_{120}$, P34/M36$_{180}$, P35/M36$_{90}$, P35/M38c$_{60}$, **P36/M34$_{245}$**, P36/M35b$_{90}$, P37/M39$_{100}$, P37/M41$_{200}$, P38/M36$_{80}$, P43/M32$_{175}$,P43/M37a$_{325}$	11
III	P33/M39$_{235}$, P33/M41a$_{275}$, P34/M33a$_{210}$, P34/M34$_{120}$, P36/M35a$_{185}$,P41/M43b$_{160}$, P42/M41$_{200}$, P43/M37b$_{210}$, P46/M44$_{210}$	9
IV	P38/M42$_{100}$	1
V	P37/M42$_{230}$, P46/M39$_{350}$	2
VI	P33/M41b$_{255}$, P34/M41$_{140}$	2
VII	P34/M33b$_{160}$, P34/M37$_{70}$, P34/M42$_{100}$,P35/M38a$_{260}$,	8
centromere	P35/M45a$_{325}$,P37/M35$_{95}$, **P41/M43a$_{275}$**, P46/M42$_{275}$	

Markers lettered in bold were polymorphic in Sinvalocho MA and Gamma 6, the parents of the mapping population

inferred from their presence or absence in the rest of the deletion stocks. The AFLP patterns of Sinvalocho MA controls T_A, T_B, and T_C differed neither from that of lines C1, C2, C3, C4, C6, C7, C8, C9, C10, C11, and C15 with the standard C-banding karyotype nor from that of line C5 with an intercalary deletion. Deletion of lines B10, B15, and C12, with similar FL values, was found to differ in size after the presence/absence of AFLP test. The size of the deleted segment increased in the order B10, B15, and C12. Likewise, deletions B1, B3, B5, B6, B7, B11, B12, B13, B14, and B16, with FLs ranging between 0.75 and 0.87, were separated in two different groups. Thus, a total of seven chromosome bins flanked by the breakpoint of the different deletions were obtained. The deletion map is represented in Fig. 3. AFLPs were designated according to the primers used (*Pst*I primer/*Mse*I primer) and the base pairs number estimated from the mobility of molecular size markers run in the same gel. Letters a–c or d indicate different bands obtained with one primer pair. The distribution of the 55 amplified bands on chromosome 6BL is shown in Table 2. A total of 22 AFLP markers in addition to *Lr3* were found to be located on the most distal region flanked by the telomere and the deletion C16 breakpoint. Six of these AFLPs, P31/M32$_{125}$, P34/M32$_{190}$, P35/M38b$_{180}$, P36/M39$_{175}$, P39/M34b$_{135}$, and P43/M40$_{135}$, and markers P36/M34$_{245}$ and P41/M43a$_{275}$, located on bins II and VII, respectively, showed no band in the rust leaf susceptible cv. Gamma 6. In addition, after testing 53 primer combinations, an AFLP band, P46/M42$_{310}$, present in Gamma 6 and absent in Sinvalocho MA, was found to be associated with the *Lr3* gene by using bulk segregant analysis. The nine polymorphic AFLPs and the *Lr3* gene were assayed in the mapping population. All markers fitted 1:1 segregations at *P* between 0.1 and 0.5 (Table 3), suggesting the high degree of homozygosity achieved. The use of RILs as mapping population implicates that the actual number of meiosis being analyzed was approximately 120 (2*n*). The linkage groups, best order and distances were estimated by using MAPMAKER Version 3.0 at min LOD 3.0. Segregations and *P* values for chi-square analysis and linkage groups are shown in Table 3. Seven distal AFLP bands, *Lr3* and the P46/M42$_{310}$ of Gamma 6, which could not be physically positioned, were in one group, and the proximal P41/M43a$_{275}$ marker segregated independently. The most likely order and genetic distance are depicted in Fig. 4. The *Lr3* gene is flanked by the P36/M39$_{175}$ AFLP marker at 4.6 cM and by seven markers, P31/M32$_{125}$, P34/M32$_{190}$, P35/M38b$_{180}$, P39/M34b$_{135}$, P43/M40$_{135}$, P36/M34$_{245}$, and P46/M42$_{310}$ at 0.9 cM. Because P36/M34$_{245}$ is in bin II, these seven markers are proximal and P36/M39$_{175}$ distal relative to the *Lr3* locus.

Discussion

The hexaploid condition confers to common wheat a high tolerance to chromosome alterations since homoeologous chromosomes can compensate for the loss of chromosome segments or complete chromosomes. This feature made possible the systematic production of deletion stocks in cv. Chinese Spring of hexaploid wheat exploiting the gametocidad effects of *A. cylindrica*,

Table 3 Physical location and linkage groups of the *Lr3* gene and nine AFLP markers

Marker	Physical location	*P* χ^2 1:1	Linkage group
Lr3	Bin I	0.3 < 0.5	1
P43/M40$_{200}$		0.2 < 0.3	1
P31/M32$_{125}$		0.2 < 0.3	1
P34/M32$_{190}$		0.2 < 0.3	1
P35/M38b$_{180}$		0.2 < 0.3	1
P36/M39$_{175}$		0.3 < 0.5	1
P39/M34b$_{135}$		0.2 < 0.3	1
P36/M34$_{245}$	Bin II	0.2 < 0.3	1
P41/M43a$_{275}$	Bin VII	0.1 < 0.2	2
P46/M42$_{310}$	ND[a]	0.2 < 0.3	1

[a]The P46/M42$_{310}$ marker could not be physically allocated owing to the lack of deletion stocks in Gamma 6

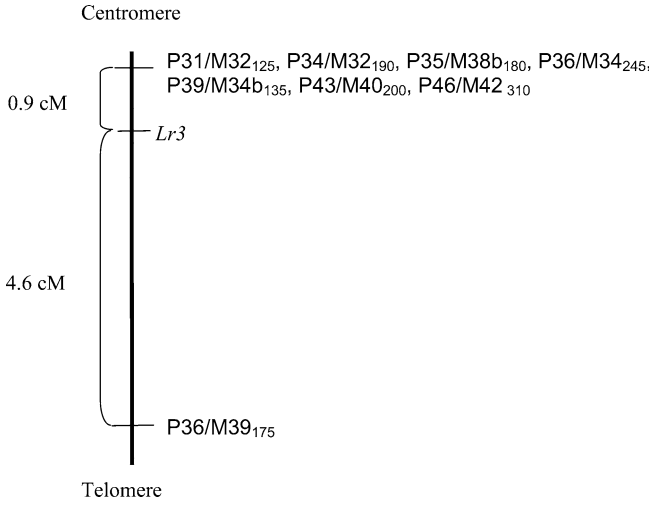

Fig. 4 Genetic map of the *Lr3* gene and eight AFLPs. Log likelihood = −27.74

A. triuncialis, or *A. speltoides* chromosomes added to wheat (Endo and Gill 1996) as well as the isolation of homozygous lines with the spontaneous deletions reported here. These spontaneous deletions were easily detected because of their association with leaf rust susceptibility. However, this limited them to chromosome arm 6BL that carries the *Lr3* gene. The underlying mechanism is still unknown.

Among the 33 susceptible lines, 11 lines (C1, C2, C3, C4, C6, C7, C8, C9, C10, C11, and C15) showed the standard chromosome structure and the 55 AFLP bands detected on chromosome 6BL. This suggests that the loss of leaf rust resistance was the result of a point mutation or a small intercalary deletion involving the *Lr3* gene but not the flanking markers. Lines C1–11, all of them derived from the same initial susceptible plant (Fig. 1), might carry the same mutation, but C15 with an independent origin probably represents a different mutation event. C5 with an internal deletion showed the presence of all AFLP bands. Because of its origin, this line might carry the same mutation as lines C1–4 and C6–11, the reduction of the 6BL arm length being the result of a further rearrangement. Likewise, lines B1 and B2, although derived from the same susceptible plant have different chromosome 6BL structures and represent different mutational events. Lines B3, B5, B6, and B7, with a common origin and similar C-banding and AFLP patterns, probably carry the same deletion inherited from their susceptible progenitor. Lines B11–16 could also be identical. Independent origins in the remaining lines agree with their variable chromosome 6B structures.

Apparent differences of the deleted segments in C-banded chromosomes of susceptible lines permitted five subregions of the 6BL arm to be delimited. Combined cytological and AFLP analyses provided a more precise characterization of deleted chromosomes with FL values ranging between 0.72 and 0.87, allowing subdivision of the subdistal region in three segments (Fig. 3). The lack of C-band markers in this subdistal region and a variable degree of chromosome condensation minimized the possibility of cytological identification of small differences between deletions. This might explain the shift of the B10 line breakpoint to a more distal position relative to lines B11–16 after AFLP mapping. Nevertheless, the presence of an additional internal deletion on B10 cannot be excluded.

Physical mapping of AFLPs showed an uneven distribution of markers along 6BL, with the majority at the distal end. This fact could be related to the use of the *Pst*I restriction enzyme, which is sensitive to cystein methylation (Nelson and McClelland 1988). Methylcystein-rich regions are known to exist more frequently around wheat centromeres causing a low frequency of cutting, and as a consequence, the generation of fewer markers in these regions. Polymorphic loci were also non-randomly distributed and most of them were distally located. This is in agreement with a report of Dvorak et al. (1998) suggesting that distal loci in *Triticeae* species are more polymorphic than proximal ones. The nine polymorphic markers formed two linkage groups with one proximal and eight distal loci. *Lr3* was included in the distal group with seven proximal markers ($P31/M32_{125}$, $P34/M32_{190}$, $P35/M38b_{180}$, $P39/M34b_{135}$, $P43/M40_{135}$, $P36/M34_{245}$, and $P46/M42_{310}$) at 0.9 cM and a distal $P36/M39_{175}$ marker at 4.6 cM. Given the economic importance of the *P. triticina*-caused rust disease, these markers will be useful to develop single characterized amplified regions (SCARs, Shan et al. 1999) for marker-assisted selection of rust resistance.

An important feature of wheat genome organization is that gene density and recombination rates are not uniform along the chromosomes and increase with the distance from the centromere (Gill et al. 1993a; Lukaszewski and Curtis 1993; Akhunov et al. 2003). Mean recombination rates range between 0.06 and 0.87 cM/Mb for the proximal and distal intervals, respectively (Akhunov et al. 2003). Taking into account that *Lr3* is distally located and assuming no variation of the recombination rate at this region of 6BL, we can estimate a length of approximately 6.3 Mb for the segment delimited by the AFLP markers and 1 Mb for the distance between *Lr3* and the nearest group of seven AFLP markers. A higher density map would facilitate *Lr3* isolation. For this purpose mapping strategies using bands similar to $P46/M42_{310}$ of Gamma 6, in coupling with leaf rust susceptibility, could facilitate the detection of recombinants in large F2 populations, circumventing the problem derived from dominance in this type of marker amplified by PCR. Screening for recombinants in such F2 can be carried out in two steps. First, rust resistant (recessive) homozygous seedlings can be selected, and second, any plant of this subpopulation with the AFLP band inherited from the susceptible parent will be recombinant.

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