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ORIGINAL PAPER

Genetic analysis of leaf rust resistance genes and associated markers in the durable resistant wheat cultivar Sinvalocho MA

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Abstract In the cross of the durable leaf rust resistant wheat Sinvalocho MA and the susceptible line Gama6, four specific genes were identified: the seedling resistance gene *Lr3*, the adult plant resistance (APR) genes *LrSV1* and *LrSV2* coming from Sinvalocho MA, and the seedling resistance gene *LrG6* coming from Gama6. *Lr3* was previously mapped on 6BL in the same cross. *LrSV1* was mapped on chromosome 2DS where resistance genes *Lr22a* and *Lr22b* have been reported. Results from rust reaction have shown that *LrSV1* from Sinvalocho is not the same allele as *Lr22b* and an allelism test with *Lr22a* showed that they could be alleles or closely linked genes. *LrSV1* was mapped in an 8.5-cM interval delimited by markers gwm296 distal and gwm261 proximal. Adult gene *LrSV2* was mapped on chromosome 3BS, cosegregating with gwm533 in a 7.2-cM interval encompassed by markers gwm389 and gwm493, where other disease resistance genes are located, such as seedling gene *Lr27* for leaf rust, *Sr2* for stem rust, QTL *Qfhs.ndsu-3BS* for resistance to *Fusarium gramineum* and wheat powdery mildew resistance. The gene *LrG6* was mapped on chromosome 2BL, with the closest marker gwm382 at 0.6 cM. Lines carrying *LrSV1*, *LrSV2* and *LrG6* tested under field natural infection conditions, showed low disease infection type and severity, suggesting that this kind of resistance can be explained by additive effects of APR and seedling resistance genes. The identification of new sources of resistance from South American land races and old varieties, supported by modern DNA technology, contributes to sustainability of agriculture through plant breeding.

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Introduction

Leaf rust, caused by the biotrophic fungus *Puccinia triticina*, is one of the most important diseases of wheat worldwide. In Argentina, annual yield losses of 5–10% or more are caused, depending on the stage of crop development at which the initial rust infection occurs (Rodriguez Amieva et al. 1961; Macagno et al. 1993; Kolmer 1996). The large amount of genetic variation for pathogenicity commonly observed in rust populations as well as the short span of time in which these populations adapt to resistance genes in wheat cultivars, make the genetic control more difficult. Usually, most of the new wheat commercial varieties are resistant to rust populations. However, these varieties carrying different resistant gene combinations frequently become susceptible when widely grown over the years because of the occurrence and selection of new virulent strains.

Leaf rust resistance based on single genes has always been observed to be short-lived (McIntosh et al. 1995; Kolmer 1996). In the last decade, two argentine wheat varieties, Klein Don Enrique and ProINTA Gaucho, carrying *Lr26* and *Lr19* genes, respectively, became completely susceptible after a short period of time. However, there are some other wheat varieties that have remained resistant for a long time. This kind of resistance was operationally defined as "durable" by Johnson (1981). In contrast to the presence of single race-specific resistance genes alone, host genetic analysis carried out on durable varieties revealed

that some combinations of major seedling resistance genes, adult plant resistance (APR) genes and the accumulation of minor resistance genes confer resistance over long periods of time, in different environments and against diverse pathotypes of the fungus (Sawhney et al. 1989; Kolmer 1996; Messmer et al. 2000; Schnurbusch et al. 2004; Pretorius and Roelfs 1996). Specific combinations involving APR genes *Lr13* and *Lr34*, and some seedling resistance genes showed enhanced resistance, both in greenhouse and field tests (Sawhney et al. 1989; Kolmer 1992). A common feature of varieties with durable resistance is APR genes, suggesting that their presence plays an important role in this kind of resistance.

Some argentine wheat varieties such as La prevision 13, Pergamino Gaboto, El Gaucho FA, Sinvalocho MA, Buck Manantial and Buck Poncho, among others, show durable resistance (Favret et al. 1983). The cultivar Sinvalocho MA, obtained from the cross Klein Sin Rival × 38 MA, has been used as leaf rust resistance source in many breeding programs in Argentina and eastern Europe (Favret et al. 1983). Klein Sin Rival is derived from Americano 44d × Barleta 7D, and 38MA from Chino × Barleta. All these cutivars and land races were produced early in the twentieth century in Argentina, and some of them, such as Americano 44d and 38MA are known to carry APR genes (Pérez and Roelfs 1989; Roelfs 1988; Antonelli 1983). Dyck (1991) suggested that APR genes *Lr34* and *Lr12* could have been introduced into breeding programs through the cultivar 38MA derived from Chino, a Chinese introduction that may have been Chinese Spring, known to carry *Lr12*, *Lr31* and *Lr34*. Furthermore, Roelfs (1988) suggested that Americano 44D may be the source of genes *Lr34* and *Lr12* or *Lr13*.

Sinvalocho MA has been used as a differential local variety in leaf rust studies for more than 50 years in Argentina (Sacco et al. 1995). The studies on seedling leaf rust resistance indicated the presence of an allele or a closely linked gene of the *Lr3* locus on chromosome 6B (Haggag and Dyck 1973; Favret et al. 1983). Subsequent studies confirmed this location on the long arm of chromosome 6B (Suárez and Favret 1984; Sacco et al. 1992). A more detailed study using a set of 33 Sinvalocho MA, spontaneous deletion lines of the long arm of chromosome 6B, cytogenetically and molecularly characterized by C-banding and AFLPs, allowed to locate *Lr3* gene in a distal bin, with a length smaller than 5% of 6BL (Diéguez et al. 2006). No additional genes for seedling reaction to leaf rust were reported in this cultivar. However, Favret and Cenoz (1963) suggested the presence of inhibitor genes for leaf rust reaction in Sinvalocho MA by inducing mutants on this cultivar and carrying out their subsequent genetic analysis. Concerning APR, Antonelli (1983) reported some rust races where the reaction changed from susceptibility at seedling stage to resistance at adult stage, suggesting the presence of APR genes. However, this author did not carried out genetic studies to identify genes in segregating populations.

More than 60 genes for leaf rust reaction have been characterized in wheat, both from hexaploid wheat and from related species that have been introgressed through genetic manipulation (McIntosh et al. 1995). Most of these resistance genes were incorporated by backcrossing into the wheat variety Thatcher, so that there are single-gene differential lines in a common isogenic background that were used extensively throughout the world to characterize the pathogenic variability of *P. triticina* (Kolmer 1999; Kolmer and Liu 2000; Kolmer et al. 2005).

The progress made in DNA marker technology has been remarkable in the last few years. The use of molecular markers linked to genes of interest facilitates assisted selection tasks when they are introgressed into commercial varieties (Harper and Cande 2000). Also, this information may be useful for positional cloning of genes (Yan et al. 2003; Feuillet et al. 2003). Seedling resistance genes *Lr1*, *Lr10*, and *Lr21* and the adult plant leaf rust resistance gene *Lr34* have been cloned in the last decade (Huang et al. 2003; Feuillet et al. 2003; Cloutier et al. 2007; Krattinger et al. 2009). Given the importance of durable varieties and their potential use as a source of resistance, the studies about the genetic basis, the identification of the genes involved, their mode of inheritance and chromosomal location are very useful. In addition, the identification of markers associated to resistance genes would allow their introgression into defeated commercial varieties as well as the construction of low resolution maps as a starting point for fine mapping and subsequent map-based cloning.

The objectives of the present work were to identify leaf rust resistance genes in the wheat cultivar Sinvalocho MA both at seedling and adult stage, to study their mode of inheritance, to search for associated DNA markers, and the testing of gene combinations under natural field infections over a period of years.

Materials and methods

Plant material

An F_3 population of 93 families and an F_8 population of 91 RILs (recombinant inbred lines) obtained by single seed descendent (SSD) were derived from a cross between Sinvalocho MA × Gama6. Gama6 was usually used as susceptible parent in different experiments, however, in recent years it has shown resistance to some leaf rust isolates (Sacco unpublished).

Table 1 Sinvalocho MA, Gama6 and differential lines *Lr22a*, *Lr22b* and *Lr27 + Lr31* seedling and adult plant reactions to 13 races of *Puccnia triticina*

Race	Nomenclature*	Sinvalocho MA		Gama6		*Lr22a*		*Lr22b*		*Lr27 + Lr31*	
		Seedling	Adult	Seedling	Adult	Seedling	Adult	Seedling	Adult	Seedling	Adult
66	DBBS	0; 1	0; 1	4	4						
Ca02-12	DBBR	0; 1	0; 1	34	34						
Ca03-PIM	MBBS	34		0; 1							
Ca04-KDE	MCTS	34		0; 1							
Ca04-PG	MCPS	34		0; 1							
Ma04-Bg11	MCNS	34		0; 1							
Ma04-KDE	MCPQ	34		0; 1							
99-28	MCTD	34	0; 1	34	34	34	1+2−	34	34		
Ca00-5	CCTP	34	0; 1	34	34						
Ca02-G1R	MBGJ	34	0; 1	34	34					0; 1	0; 1
Ca02-G6	BLGB	34	0; 1	34	34						
Ca04-19	MCLS	34	0; 1	34	34						
F05	FBBS	34	0; 1	4	4						

Races in boldface detected adult plant resistance

* According to North American System (Long and Kolmer 1989)

Rust infections

Plants were infected by artificial inoculation, both at seedling (first to second leaf) and adult stage (flag leaf at ear emergence), using a collection of 13 different races derived from single spore isolates of *P. triticina* (Table 1). Infections with each race were performed on different sets of plants.

Infections were carried out spraying a suspension of 20 mg of *P. triticina* urediospores in 50 ml of water with one drop of Tween 20. Incubation was performed in moist chambers (100% humidity) for 16 h. Afterwards, plants were kept in the greenhouse at temperatures that ranged between 15 and 25°C. Reactions were scored after 12–14 days for seedlings and 14–21 days for adult plants, according to Mains and Jackson's scale (Mains and Jackson 1926).

F_3 families were tested using an average of 15 plants each, which allowed classification of F_2 plants in homozygous resistant, homozygous susceptible or heterozygous. RIL families were tested infecting five plants per line, and classified as homozygous resistant or susceptible. Segregations were tested by Chi Square analysis.

DNA isolation

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.

Molecular markers

In order to identify DNA markers linked to resistance genes, bulk segregant analysis (BSA) was performed (Michelmore et al. 1991). The method is based on the search of DNA markers in specific genome regions by comparing two groups of pooled DNA of individuals from a single cross, where individuals that make up each bulk or mixture are identical for the character or gene of interest. For each resistance gene detected in this study, 12 homozygous resistant families and 12 homozygous susceptible were evaluated.

Genetic markers associated with resistance genes were allocated at the chromosomal level using different strategies. In some cases, comparisons among hexaploid wheat, tetraploid wheat (*Triticum durum*) and diploid wheat (*Triticum monoccoccum*) allowed to identify the genome where markers were located, and then using nulli-tetrasomic lines, as well as ditelosomic lines developed in the cultivar Chinese Spring, markers were assigned to chromosome arm level. In others, directly nulli-tetrasomic and ditelosomic lines were used. Also a genetic linkage map from the cross Sinvalocho MA × Gama6 was used (L Ingala unpublished), when a marker associated to a resistance gene had been mapped in a specific linkage group of this cross.

Once the chromosomal arm position of the resistance genes was confirmed, SSR markers which had been mapped in other populations were used to develop genetic maps.

SSRs

The PCR reactions were performed in a volume of 20 µl. The reaction mixture contained 20 mM Tris–HCl pH 8, 0.5 mM KCl, 0.1 µM of each primer, 0.05 mM dNTPs, 1.5 mM MgCl2, 0.8unit *Taq* Polymerase (Invitrogen) and 20 ng of template DNA (Röder et al. 1998). DNA amplification was performed fixing the annealing temperature (50, 55 or 60°C) or dropping it 1 degree per cycle, depending on micro-satellite: 94º/3 min, (94º/1 min, 50–55–60ºC/1 min, 72°/2 min) 45 times, 72°/10 min.

AFLPs

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 37ºC. The preamplification was done in a final volume of 25 µl with 1.32 mM $MgCl_2$, 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 94ºC, 30 s at 60ºC and 1 min at 72ºC. The amplification products were diluted 1:3 in low EDTA-TE buffer (10 mM Tris–HCl pH 8, 0.1 mM EDTA) and 2 µl were used for specific amplification in 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 94ºC, 30 s annealing (see below) and 1 min at 72ºC. The annealing temperature of 65ºC in the first cycle was subsequently reduced by 0.7ºC per cycle for the next 12 cycles, and maintained at 56ºC for 25 more cycles. The sequence of the primers and adaptors was as described by Vos et al. (1995).

PCR products visualization

One volume of loading buffer (10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol and 95% deionized formamide) was added to both SSR and AFLP reaction products and after 5 min incubation at 95ºC, 8 µl were loaded in 6% denaturing polyacrylamide gels and electrophoresed in 0.5X TBE buffer 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 and developed in chilled 30 g/l $NaCO_3$, 0.06% formaldehyde and 0.0002% sodium thiosulfate (Bassam et al. 1991).

Field testing

In order to validate the effects of individual genes and gene combinations, the RIL population was exposed to natural pathogen populations, without infector rows. The inoculum was constituted only by the population of spores present in the environment, without the addition of previously isolated races or mixtures of races. Nine field trials were conducted in three different locations in Argentina where wheat leaf rust is endemic and occurs yearly: Reconquista (29°S-60°W) and Maciel (32°S-60°W), both in the province of Santa Fe and Castelar (34°S-58°W) in the province of Buenos Aires. These three locations are representative of different wheat agro ecosystems in the central plain of Argentina, the Pampas Region. This flat area is usually considered as an epidemiological unit for leaf rust disease, together with Uruguay and South Brazil (German and Kolmer 1994).

Tests were carried out during 4 years in the experimental field of the Institute of Genetics at Castelar, 3 years in Maciel and 2 years in Reconquista. An analysis of variance (ANOVA) was carried out to test the effect of gene combinations. Every RIL containing the same gene combination was considered as an experimental unit for different years and places, and the total number of RILs containing the same gene combination (an average of 11 RILs for each gene combination) as repetitions of the same treatment. Ten to 12 seeds for each RIL were sown and grown in 1-m rows. Infection data score was based on the scale of Mains and Jackson (1926) and infection severity was estimated counting the number of pustules per square centimeter on five independent flag leaves observations in each row.

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 and the maps were drawn with the MapChart software (Voorrips 2002).

Results

Identification of leaf rust resistant genes

In order to test polymorphism for *P. triticina* seedling reaction, both Sinvalocho MA and Gama6 were inoculated at seedling stage with 13-rust isolates. Rust races to which both parents were susceptible at seedling stage were further used to inoculate adult plants to test APR, as indicated in Table 1.

Races 66, Ca02-12, Ca03-PIM, Ca04-KDE, Ca04-PG, Ma04-Bg11 and Ma04-KDE detected seedling resistance, while races 99-28, Ca00-5, Ca02-G1R, Ca02-G6, Ca04-19

Table 2 Segregation for reaction to races of *P. triticina* in F_3 and F_8 RILs populations from the cross Sinvalocho MA × Gama6

Mapping population	Race	Gene detected	Rust reaction			Expected ratio	*P*
			R	Seg	S		
F_3 (seedling)	66, Ca02-12	Lr3	24	51	18	1:2:1	0.3–0.5
F_3 (adult)	99-28, Ca00-5	*LrSV1*	23	49	21	1:2:1	0.8–0.9
F_3 (adult)	F05	*LrSV2*	25	45	23	1:2:1	>0.9
F_8 RILs (seedling)	66, Ca02-12	*Lr3*	48		34	1:1	0.1–0.2
F_8 RILs (seedling)	Ca03-PIM, Ca04-KDE, Ca04-PG, Ma04-Bg11, Ma04-KDE	*LrG6*	46		39	1:1	0.3–0.5
F_8 RILs (adult)	99-28, Ca00-5	*LrSV1*	49		36	1:1	0.2–0.3
F_8 RILs (adult)	F05, Ca02-G1R, Ca02-G6, Ca04-19	*LrSV2*	41		45	1:1	0.8–0.9

R resistant, *Seg* segregant, *S* susceptible

Table 3 Chi-squares for association of seedling and adult resistance genes detected in the cross Sinvalocho MA × Gama6

Mapping population	Genes	Segregation									Expected ratio	*P**
		R	R	R	Seg	Seg	Seg	S	S	S		
		R	Seg	S	R	Seg	S	R	Seg	S		
F_3 (adult–adult)	*LrSV1* versus *LrSV2*	6	9	8	14	24	11	5	12	4	1:2:1:2:4:2:1:2:1	0.5–0.6
F_3 (adult-seedling)	*LrSV1* versus *Lr3*	7	11	5	11	31	7	6	9	6	1:2:1:2:4:2:1:2:1	0.3–0.5
F_3 (adult-seedling)	*LrSV2* versus *Lr3*	10	11	4	9	28	8	4	8	6	1:2:1:2:4:2:1:2:1	0.3–0.5
F_8 RIL (seedling–seedling)	*LrG6* versus *Lr3*	26		20				22		14	1:1:1:1	0.5–0.7
F_8 RIL (seedling-adult)	*LrG6* versus *LrSV1*	24		22				25		14	1:1:1:1	0.2–0.3
F_8 RIL (seedling-adult)	*LrG6* versus *LrSV2*	22		25				19		20	1:1:1:1	0.8–0.9

R resistant, *Seg* segregant, *S* susceptible

* Independent Chi square

and F05 detected APR, all fitting a one-gene ratio (Tables 1, 2).

The rust reaction cosegregated when tested in the seedling stage with races 66 and Ca02-12 in the F_3 families and the F_8 RILs, indicating that both identified the same gene (Table 2). Race 66 was previously used to detect the seedling resistance *Lr3* gene on distal 6BL of Sinvalocho MA (Sacco et al. 1998; Diéguez et al. 2006).

Races Ca03-PIM, Ca04-KDE, Ca04-PG, Ma04-Bg11 and Ma04-KDE, identified the same seedling resistance gene coming from Gama6 line, temporarily designated *LrG6* (Table 2). This gene was inherited independently from *Lr3* (Table 3).

Infections carried out at adult plant stage, both in F_3 families and F_8 RIL populations, with races 99-28 and Ca00-5 allowed detecting the segregation of the same APR gene (Table 2). This gene was temporarily designated *LrSV1* and was inherited independently from *Lr3* and *LrG6* (Table 3).

Adult plant infections performed using race F05 on F_3 and races F05, Ca02-G1R, Ca02-G6 and Ca04-19 on F_8 RILs populations derived from the same cross detected segregation of the same gene for APR (Table 2). This gene was designated *LrSV2*, and was inherited independently from *Lr3*, *LrG6* and *LrSV1* (Table 3).

Associated markers and chromosomal location

AFLP analysis was performed using 44 primer combinations. It was found that every primer combination detected at least one polymorphic band between Sinvalocho MA and Gama6. Therefore, all primer combinations were tested by BSA on resistant and susceptible families carrying *LrG6*, *LrSV1* or *LrSV2* genes. This strategy allowed to detect two associated AFLP bands to each gene: $P31/M33_{175}$ and $P40/M38_{140}$ to *LrG6*, $P31/M42_{125}$ and $P40/M35_{125}$ to *LrSV1* and $P31/M37_{150}$ and $P31/M39_{175}$ to *LrSV2*. The AFLP bands cosegregating with *LrG6* also cosegregated with SSR gwm526 which maps on chromosome 2BL (Röder et al. 1998) in a linkage map of this cross (L Ingala unpublished). Testing hexaploid, tetraploid and diploid wheat, the bands associated to *LrSV1* were located on the D genome and those associated to *LrSV2* on the B genome. Using ditelosomic/nulli-tetrasomic stocks, *LrSV1* was allocated on chromosome 2DS and *LrSV2* on chromosome 3BS. Several

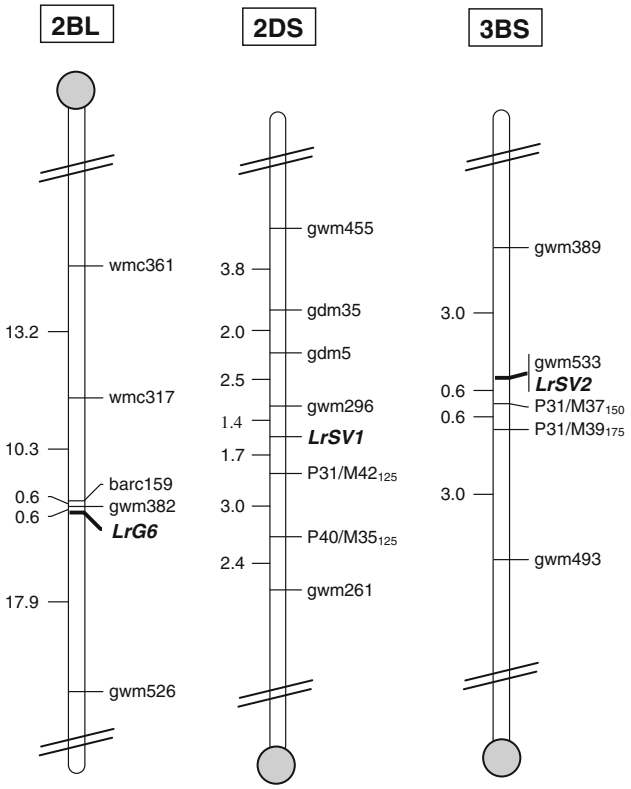

Fig. 1 Genetic maps of chromosome arms 2BL, 2DS and 3BS of the cross Sinvalocho MA × Gama6 (91RILs). Distance (cM) is on the *left*. The *gray circle* denotes the centromere

public SSRs previously reported on chromosomes 2BL, 2DS and 3BS were tested by BSA. It was found that markers barc159, gwm382, gwm526, wmc317 and wmc361 were associated to *LrG6,* gwm261, gwm296, gwm455, gdm5 and gdm35 to *LrSV1*, and gwm389, gwm493 and gwm533 to *LrSV2*. These markers were subsequently mapped on the F_8 RILs population, along with AFLPs markers (Fig. 1). The closest marker to *LrG6* was gwm382 at 0.6 cM, to *LrSV1* was gwm296 at 1.4 cM and gwm533 completely linked to *LrSV2*.

The *LrSV1* gene was mapped on chromosome 2DS where the resistance genes *Lr22a* and *Lr22b*, either closely linked or allelic, have been reported (Rowland and Kerber 1974; Dyck 1979). Therefore, the differential lines carrying either *Lr22a* or *Lr22b* were inoculated at the flag leaf stage with *P. triticina* race 99-28 which identifies *LrSV1*. These lines turned to be resistant and susceptible, respectively, showing that *LrSV1* from Sinvalocho MA is either a different locus or a different allele of *Lr22b*. To study the relationship between the gene *LrSV1* and the *Lr22* locus, an allelism test was performed, using race 99-28 which is avirulent to gene *Lr22a* (no avirulent races to gene *Lr22b* were available in our *P. triticina* collection). Two lines from the RIL population carrying *LrSV1* were crossed to *Lr22a*, and an adult plant F2 population of 373 individuals was infected with race 99-28. All plants tested were resistant (0; 11+ infection type), except a small sterile plant that showed a 2++3 infection. This moderately susceptible plant probably had some chromosome abnormality which rendered sterile flowers, and subsequently absence of progeny. The absence of susceptible recombinant plants among F2 progenies indicated that *LrSV1* and *Lr22a* are alleles or closely linked genes, with a probable recombination value (p) of less than 11%.

Field treatments

The average number of pustules per square centimeter in flag leaves of parental lines and RILs with each combination of genes are indicated in Fig. 2. *Lr3* gene was excluded from the analysis, since its presence had no effect on any gene combination.

The analysis of variance to determine the significance of the effect of genes on the number of pustules/cm^2 was highly significant for the F test ($p < 0.01$).

Five orthogonal comparisons, planned a priori, were made and three were significant (Fig. 2b). Treatments with three genes (average of 17 pustules/cm^2) were significantly different from the group with all the possible combinations of two genes (average of 33 pustules/cm^2). This later group was significantly different from the group with single gene combinations (average of 39 pustules/cm^2) and the group without genes (average of 63 pustules/cm^2) was significantly different from the group with three genes.

No significant differences were observed among single genes, as well as among different combinations of two genes.

The original variety Sinvalocho MA was also evaluated in these 9 field trials and showed an average of 31 pustules/cm^2 which did not differ significantly from the value observed in RILs carrying both *LrSV1* and *LrSV2* (average of 34 pustules/cm^2).The line Gama6 showed an average of 38 pustules/cm^2 which did not differ from the value observed in lines carrying single genes.

Lines that stacked *LrSV1*, *LrSV2* and *LrG6* genes were those that showed the lowest average number of pustules/cm^2, about 73% less than the average observed in the control that had none of these genes. The pustule number was reduced about 48% by the presence of any combination of two of the three genes, and 38% by each single gene alone.

Discussion

In the cross between the durable resistant variety Sinvalocho MA × Gama6, four specific genes for leaf rust resistance were identified by Mendelian genetic analysis, three

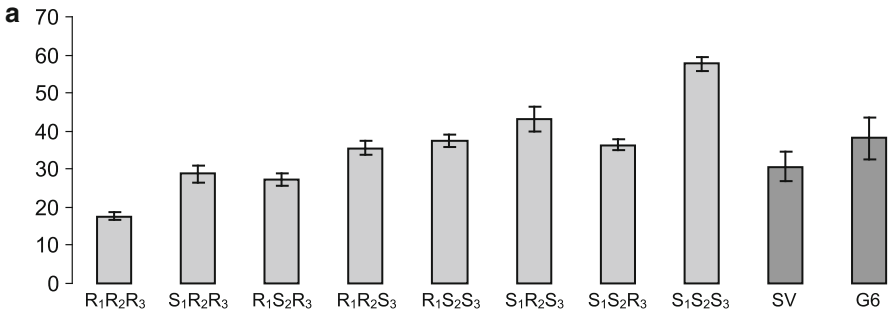

Fig. 2 **a** Average pustule number per square centimeter in the flag leaf and standard error of parental lines Sinvalocho MA (SV) and Gama6 (G6) and RILs carrying the indicated gene composition. *R* stands for presence of a gene and *S* for absence. R_1, *LrSV1*; R_2, *LrSV2* and R_3, *LrG6*. **b** Orthogonal comparisons among five treatments. *Asterisk*s indicate highly significant

b

Treatment comparison		F value	Probability
Comparison 3 genes *versus* any combination of 2 genes	(*LrSV1,LrSV2,LrG6*) *versus* (*LrSV1,LrSV2+LrSV1,LrG6+LrSV2,LrG6*)	48.82**	< 0.01
Comparison between all possible combinations of 2 genes	(*LrSV1+LrSV2*) *versus* (*LrSV1+LrG6*) *versus* (*LrSV2+LrG6*)	0.4	
Comparison between no gene and 3 genes	(none) *versus* (*LrSV1+LrSV2+LrG6*)	98.44**	<0.01
Comparison between any combination of 2 genes and single genes	(*LrSV1,LrSV2+LrSV1,LrG6+LrSV2,LrG6*) *versus* (*LrSV1+LrSV2+LrG6*)	10.02**	<0.01
Comparison between single genes	(*LrSV1*) *versus* (*LrSV2*) *versus* (*LrG6*)	0.48	

coming from Sinvalocho MA (*Lr3*, *LrSV1* and *LrSV2*) and one from Gama6 (*LrG6*).

The two seedling resistance genes (*Lr3* and *LrG6*), and the two dominant genes expressed at flag leaf stage, *LrSV1* and *LrSV2*, are race-specific and exhibit a typical hypersensitive response according to the race that identify each gene.

The gene *LrG6* from Gama6 mapped on chromosome 2BL. No seedling leaf rust resistance gene has been reported on this arm. However, genes for stem rust, stripe rust and APR to leaf rust have been mapped on 2BL in recent years (Sui et al. 2009; Kolmer et al. 2011; Singh et al. 2011), yet none of the associated molecular markers used in these studies could be mapped on Sinvalocho MA × Gama6, preventing any chromosome location comparison. Additional studies should be carried out to precisely locate this gene on 2BL and to elucidate its relationship to other resistance genes.

The *LrSV1* gene was mapped on chromosome 2DS. On this chromosome arm, resistance genes *Lr22a* and *Lr22b*, either closely linked or allelic, have been reported (Rowland and Kerber 1974; Dyck 1979). Lines carrying *Lr22a* and *Lr22b* turned to be resistant and susceptible, respectively, when inoculated at the flag leaf stage with *P. triticina* race 99-28 which identifies *LrSV1*. These results showed that *LrSV1* from Sinvalocho is either a different locus or a different allele of *Lr22b*, first described in the old American variety Marquis (Bartos et al. 1969). On the other hand, the gene *Lr22a* was introgressed from *Triticum tauschii* into hexaploid wheat in the 70s (Dyck and Kerber 1970) and therefore it seems unlikely that it is the same allele as *LrSV1* since Sinvalocho MA is an Argentinean traditional variety developed in the 30s and according to the available pedigree is not related to neither of those mentioned above. The absence of susceptible plants among 373 F2 progenies infected at adult stage with race 99-28 supports the hypothesis that *LrSV1* and *Lr22a* are either alleles or closely linked genes, with a probable recombination value (*p*) less than 11%. Hiebert et al. (2007) reported the location of gene *Lr22a* on chromosome 2DS, with the closest marker gwm296 distal at 2.9 cM. In our cross, this marker was mapped at 1.4 cM distal from *LrSV1*. The order and the distances of *Lr22a* and the linked markers gwm261, gwm296 and gwm455 in the maps published by these authors are completely consistent with the map of Sinvalocho MA × Gama6 reported here. Furthermore, the present map encompasses *LrSV1* in an interval delimited by markers gwm296 distal and gwm261 proximal, with the closest proximal marker, AFLP $P31/M42_{125}$, at 1.7 cM. In a study of durable resistance in the Swiss winter wheat "Forno", a QTL analysis revealed a significant QTL close to gwm296 marker (Schnurbusch et al. 2004). These authors speculated that this QTL on 2DS could be *Lr22*.

Adult gene *LrSV2* was mapped on chromosome 3BS completely linked to marker gwm533. Other resistance genes are located in this region, such as leaf rust seedling resistance *Lr27*, *Sr2* gene for resistance to stem rust *P. graminis f. sp. tritici* (Kota et al. 2006), *Qfhs.ndsu-3BS*, a main QTL for resistance to *Fusarium gramineum* Schwabe (Liu et al. 2005) and powdery mildew resistance, caused by *Blumeria graminis* f. sp. *tritici* (Mago et al. 2011). Gene *Sr2* was allocated in the same interval encompassed by markers gwm389 and gwm493 used in the present study (Spielmeyer et al. 2003). In addition, an analysis of recombinants and mutants suggests that *Lr27* and *Pm* resistance cosegregated with *Sr2*, indicating that a single gene on chromosome arm 3BS may be responsible for resistance to these three fungal pathogens (Mago et al. 2011). The race Ca02-G1R that detected *LrSV2* at adult stage, tested at seedling behaved as virulent on *LrSV2* and avirulent on *Lr27* + *Lr31*. The same results were obtained in Australia in seedling inoculations (Spielmeyer, personal communication). The fact that *Lr27* and *LrSV2* genes are detected at different developmental leaf stages (*Lr27* at seedling and *LrSV2* at adult plant stage, approximately at flag leaf −2) does not rule out that they are allelic or even that they are the same allele with different expression due to background effects. Fine mapping and additional genetic studies could address this question.

Chromosome 3B is the largest wheat chromosome and could be sorted by flow cytometry as a well-discriminated peak that allowed the development of the first chromosome-specific BAC library (Safar et al. 2004). The subsequent construction of a physical map of this chromosome (Paux et al. 2008) and the annotation of several megabases of continuous sequence (Choulet et al. 2010) made available an enormous amount of markers to accelerate the possibility of the fine mapping of *LrSV2* in order to attempt its positional cloning.

Flor (1971) pointed out that cultivars with two or more genes for rust resistance should be less apt to succumb to new races than cultivars possessing a single resistance gene. In this context, the genetics of host–parasite interaction is a subject of great practical importance. When several genes are involved in host–parasite interactions, usually low infection type is epistatic to high infection type. The dissection of resistance into Mendelian factors using single rust isolates enables the identification of important genes in a specific cross, and also to study the association between these genes and leaf rust resistance under field conditions. The fact that lines carrying genes *LrSV1*, *LrSV2* and *LrG6* showed the lower number of pustules per square centimeter, corroborates the hypothesis that the accumulation of genes is determinant for a higher resistance response in the field, with both seedling resistance and APR genes being important to achieve higher protection towards the pathogen. In the present study, it was found that mainly additive effects of major resistance genes explained not only a significant reduction of pustule number but also the infection type towards resistance. In order to have a more objective statistical analysis, it was preferred to score number of pustules per square centimeter instead of using the modified Cobb scale (Peterson et al. 1948) in the field tests. It is worth to mention that scores over 50–60 pustules per square centimeter clearly corresponded to moderately susceptible to susceptible infection types. On the other hand, scores under 50 pustules/cm^2 gradually become more resistant (from 22+ to 0; 1 infection types in RILs that stacked the 3 resistance genes). The durable resistance observed in Sinvalocho MA can be explained by the presence of at least two APR genes. This resistance was additionally enhanced by the presence of the seedling resistance gene *LrG6*.

Results observed in this study may suggest that resistance due to race-specific APR genes is enhanced by the presence of additional genes, inducing a kind of unspecific gene dosage effect, which render the variety durable against any pathogen population. The combination of adult plant and seedling resistance genes conferring durable resistance to leaf rust has been reported in different wheat cultivars. Pretorius and Roelfs (1996) reported the presence of *Lr10*, *Lr13* and *Lr34* in the highly resistant wheat cultivar Era. However, they pointed out that the combination of these genes is not completely responsible for the adult plant resistance observed in this cultivar, and that additional genes for resistance could be involved. Samborski and Dyck (1982) also reported enhancement of resistance to *P. triticina* involving genes *Lr13* and *Lr16*, an adult and seedling resistance gene, respectively, in the wheat cultivar Columbus. These authors suggested a residual or ghost effects, a term used by Riley (1973) to describe less than complete compatibility associated with genes for virulence. The same phenomenon was reported for the *Triticum aestivum: Erysiphe graminis* system by Martin and Ellingboe (1976) and Nass et al. (1981), who reported that combination of some "defeated" powdery mildew resistance genes had measurable ability to restrict disease increase and severity. Kolmer (1992) studied paired combinations of *Lr13* with additional leaf rust resistance genes and found that in field tests, only those genes that conferred some degree of effective resistance relative to Thatcher when present singly, combined with *Lr13,* condition higher levels of resistance than either *Lr13* or the additional gene. Sawhney et al. (1989) reported that the presence of *Lr10* and *Lr23* genes in Chinese Spring background containing *Lr12* and *Lr34* enhanced the level of resistance in field tests. The gene *Lr10* also has been identified in the Argentine durable wheat cultivar Buck Poncho, along with *Lr11* and an unidentified adult resistance gene, as the main determinants of resistance (Sacco et al. unpublished).

An alternative hypothesis concerning durable resistance to wheat leaf rust refers to combinations of several specific disease reaction genes (seedling and/or adult resistance genes) for which the pathogen population is not able to accumulate in a single genotype all the corresponding alleles for virulence due to a biological restriction, probably associated with their survival and/or fitness (Saione et al. 1993).

The identification of new sources of resistance genes is of considerable importance for plant breeding. The trend in modern agriculture is the release of homogeneous selected high yielding varieties, usually genetically related, in vast areas, which provokes not only disease epidemics but also a genetic erosion of useful genes. Varieties from South America, particularly from land races and old varieties brought by immigrants at the beginning of the 20th century in Argentina, Uruguay and Brazil are an invaluable source of useful germplasm for leaf rust resistance. Several genes have been isolated from these materials (Favret et al. 1983; McIntosh et al. 1995; Kohli 1986) and others remain to be identified.

Modern DNA technology provides a powerful tool to accurately identify useful genes from old varieties and to introgress them in modern ones without dragging undesirable traits. In the near future, not only more genes for rust resistance are expected to be identified, tagged and cloned but also that molecular markers would massively be used by plant breeders to make an outstanding contribution to sustainable agriculture.

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