Theor Appl Genet
DOI 10.1007/s00122-014-2285-z

ORIGINAL PAPER

Fine mapping of *LrSV2*, a race-specific adult plant leaf rust resistance gene on wheat chromosome 3BS

M. J. Diéguez · M. F. Pergolesi · S. M. Velasquez · L. Ingala · M. López · M. Darino · E. Paux · C. Feuillet · F. Sacco

Received: 11 July 2013 / Accepted: 4 February 2014

Abstract

Key message **Fine mapping permits the precise positioning of genes within chromosomes, prerequisite for positional cloning that will allow its rational use and the study of the underlying molecular action mechanism.**

Abstract Three leaf rust resistance genes were identified in the durable leaf rust resistant Argentinean wheat variety Sinvalocho MA: the seedling resistance gene *Lr3* on distal 6BL and two adult plant resistance genes, *LrSV1* and *LrSV2*, on chromosomes 2DS and 3BS, respectively. To develop a high-resolution genetic map for *LrSV2*, 10 markers were genotyped on 343 F2 individuals from a cross between Sinvalocho MA and Gama6. The closest co-dominant markers on both sides of the gene (3 microsatellites and 2 STMs) were analyzed on 965 additional F2s from the same cross. Microsatellite marker cfb5010 cosegregated with *LrSV2* whereas flanking markers were found at 1 cM distal and at 0.3 cM proximal to the gene. SSR markers designed from the sequences of cv Chinese Spring BAC clones spanning the *LrSV2* genetic interval were tested on the recombinants, allowing the identification of microsatellite swm13 at 0.15 cM distal to *LrSV2*. This delimited an interval of 0.45 cM around the gene flanked by the SSR markers swm13 and gwm533 at the subtelomeric end of chromosome 3BS.

Communicated by H.-C. Jing.

M. J. Diéguez and M. F. Pergolesi contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-014-2285-z) contains supplementary material, which is available to authorized users.

M. J. Diéguez (✉) · M. F. Pergolesi · L. Ingala · M. López · M. Darino · F. Sacco
Instituto de Genética "Ewald A. Favret" CICVyA-INTA CC25 (1712) Castelar, Buenos Aires, Argentina
e-mail: mdieguez@cnia.inta.gov.ar

S. M. Velasquez
Laboratorio de Fisiología y Biología Molecular IFIByNE (CONICET) FCEyN-UBA, Intendente Güiraldes 2160, C1428EGA Buenos Aires, Argentina

E. Paux · C. Feuillet
INRA UMR Genetics, Diversity and Ecophysiology of Cereals, Domaine de Crouelle, 5 Chemin de Bealieu, 63039 Clermont-Ferrand, Cedex 2, France

Introduction

Leaf rust, caused by the biotrophic fungus *Puccinia triticina*, is one of the most important diseases of wheat worldwide, causing in Argentina annual yield losses of about 5–10 % (Rodriguez Amieva et al. 1961; Macagno et al. 1993; Kolmer 1996).

The use of resistance genes eliminates or significantly reduces the utilization of pesticides to control this disease which in turn is both economical and environmentally advantageous (Pink 2002). However, the large amount of genetic variation for pathogenicity commonly observed in rust populations requires a constant effort to identify, evaluate and incorporate new resistant genes within breeding programs (Favret et al. 1983; Kolmer 1996; Germán et al. 2007).

Usually, new commercial varieties become susceptible over the years when widely grown due to the occurrence and/or selection of new virulent strains. In spite of this situation, some wheat varieties remained resistant for a long time, a kind of resistance operationally defined as "durable" by Johnson (1981). It could be explained by the

Published online: 20 February 2014

combination of genes expressed at the seedling and at the adult stages (Favret et al. 1983; Sawhney et al. 1989; Ingala et al. 2012). Adult plant resistance or APR genes are effective when the plant is completing its leaves development, in contrast to the more commonly studied seedling resistance genes that are expressed throughout the plant's life cycle (McIntosh et al. 1995). A common feature of varieties with durable resistance is the presence APR genes, suggesting that their presence plays an important role in this kind of resistance (Sawhney et al. 1989; Kolmer 1996; Messmer et al. 2000; Schnurbusch et al. 2004; Pretorius and Roelfs 1996).

Some Argentinian varieties such as Sinvalocho MA, Buck Manantial, El Gaucho FA, Buck Poncho, La Previsión 13 and Pergamino Gaboto are examples of durable resistance that were used in breeding programs locally and worldwide. In particular, Sinvalocho MA was used as 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 seedling resistance gene *Lr3* on distal 6BL and two dominant APR genes expressed at the flag leaf stage *LrSV1* and *LrSV2* were identified in this variety (Ingala et al. 2005). These three genes are race specific, exhibiting a typical hypersensitive response upon pathogen attack. Both APR genes are not effective at the seedling stage where Sinvalocho MA is susceptible but at the adult stage they confer a degree of resistance comparable to the highly resistant seedling *Lr* genes. This behavior is similar to the APR genes *Lr12* (Singh and Bowden 2011) and *Lr22a* (Hiebert et al. 2007) but contrasts with the apparent non race-specificity and partial resistance to leaf rust (*P. triticina*), stripe rust (*Puccinia striiformis*) and powdery mildew (*Blumeria graminis*) of *Lr34,* the only adult plant leaf rust resistance gene cloned to date (Krattinger et al. 2009). Non race-specificity resistance to rusts often involves "slow rusting" genes that are associated with increased time between infection and sporulation (longer latent periods) and production of fewer and smaller uredinia (Caldwell 1968). Indeed, Rubiales and Niks (1995) reported that *Lr34* is associated with reduced intercellular hyphal growth but not with a hypersensitive response or papilla formation. The other cloned adult plant resistance gene, *Yr36,* also confers partial resistance to a broad spectrum of stripe rust races and additionally has some effectiveness in seedlings at high temperatures (Fu et al. 2009).

Under field conditions, recombinant inbred lines (RILs) carrying *LrSV1* or *LrSV2* reduce the pustule number to 60–65 %, respectively, and to 55 % when they are both present (Ingala et al. 2012). *LrSV1* was assigned to chromosome 2DS and is either an allele or closely linked to the *Lr22* gene. *LrSV2* was mapped on chromosome 3BS where no other adult plant leaf rust resistance gene was previously reported (Ingala et al. 2005). Using a population of 91 recombinant inbred lines (RILs) from the cross of Sinvalocho MA and Gama6, marker gwm533 was mapped completely linked to *LrSV2* gene and the closest marker was the AFLP P31/M37$_{150}$ at 0.6 cM proximal (Ingala et al. 2012). Interestingly, resistance genes for other wheat pathogens map to this region of 3BS: the *Sr2* gene of durable adult plant stem rust resistance caused by *Puccinia graminis f.* sp. *tritici* (Kota et al. 2006), *Qfhs.ndsu-3BS*, a major QTL for *Fusarium gramineum* resistance (Liu et al. 2006) and powdery mildew resistance, caused by *Blumeria graminis f. sp. tritici* (Mago et al. 2011). A BAC library of chromosome 3B has been constructed (Safar et al. 2004) and approximately 19,400 BAC-end sequences (BES) were generated, representing a cumulative length of nearly 11 Mb, 1.1 % of the chromosome length, which allowed the development of chromosome-specific markers (Paux et al. 2006). Moreover, a physical map of the wheat 3B chromosome became available (Paux et al. 2008; Rustenholz et al. 2011) and several megabases were sequenced by Choulet et al. (2010) in a pilot sequencing project aiming at gaining insight into the composition of Mb-sized contigs from chromosome 3B. Together with high-resolution mapping, this provides very solid ground to attempt the positional cloning the *LrSV2* gene as chromosome walking should be greatly facilitated or even not needed if the genetic to physical distance ratio is favorable in the target region. As a first step in progressing towards map-based cloning, a high resolution or "fine" genetic map should be constructed at the target locus (Feuillet et al. 2003; Yan et al. 2003; Huang et al. 2003; Cloutier et al. 2007; Krattinger et al. 2009, Fu et al. 2009). The aim of the present work was to develop a high-resolution map of the *LrSV2* region on chromosome 3BS.

Materials and methods

Plant material

For bulk segregant analysis, 14 resistant and 14 susceptible F9 recombinant inbred lines (RILs) from the cross between the resistant cv Sinvalocho MA and the susceptible cv Gama6 were used (Ingala et al. 2012).

For fine mapping, 6,815 F2 plants from the same cross were grown in the greenhouse and artificially inoculated at flag leaf stage with *P. triticina* race Ca2-G1R. This race gives an incompatible reaction in the presence of the *LrSV2* gene only at late developmental stages. 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. After inoculation, plants were kept overnight in moist chambers and thereafter grown in the greenhouse under a

14 h photoperiod at 18–24 °C. Three weeks later, disease symptoms were observed and plants were scored as resistant or susceptible according to the scale of Mains and Jackson (1926).

Molecular markers analysis

Genomic DNA was isolated according to Sacco et al. (1998) except that fresh material (grinded with liquid nitrogen) was used instead of lyophilized leaves. AFLPs were done as described previously (Diéguez et al. 2006).

The search of AFLP markers linked to *LrSV2* was carried out testing 405 primers combination by Bulk Segregant Analysis (BSA, Michelmore et al. 1991) using pools of six resistant and six susceptible RILs from the cross between the resistant cv Sinvalocho MA and the susceptible cv Gama6 (Ingala et al. 2012). Putative-associated bands were tested on eight additional resistant and eight susceptible individual RILs from the same population.

Eighty-five markers that were reported to map on subtelomeric 3BS region (listed in Tables S1 and S2) were tested for polymorphism between Sinvalocho MA and Gamma 6, they include SSR (Simple Sequence Repeats) and STS (Sequence-Tagged Sites) derived from genomic and EST (Expressed Sequence Tags) sequences, STM (Sequence-Tagged Microsatellites) and ISBP (Insertion Site-Based Polymorphisms).

PCR reactions were performed in a volume of 20 µl with 100 nM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.8 units Taq DNA polymerase (Invitrogen) and 50–100 ng of template DNA with the exception of cfb, cfp, gpw and swm markers where 1 µM of primers were used.

PCR primers and cycling conditions were as described for wms (Röder et al. 1998), barc (Song et al. 2005), wmc (Somers et al. 2004), gpw (Sourdille et al. 2004), cfb and cfp (Paux et al. 2006), sts (Liu and Anderson 2003), stm (Hayden et al. 2006), mag (Xue et al. 2008) and swm (Bossolini et al. 2006).

Amplification products were electrophoresed on denaturing 5 %-polyacrylamide gels in 0.5× TBE buffer. AFLP products were visualized by silver staining as described previously (Diéguez et al. 2006) and the rest of the markers were stained by the silver/NaOH method (Benbouza et al. 2006).

SCAR development

The $P40/M42_{325}$ AFLP band was reamplified with primers P40 and M42 (Vos et al. 1995) using as template a pipette tip-size plug from the silver-stained rehydrated gel. The PCR product was cloned in the pCR2.1-TOPO vector (Invitrogen) and sequenced with the M13 universal primers in an automated sequencer. Primers were designed with the software Primer3 (Rozen and Skaletsky 2000). PCR was performed using 100 ng of template genomic DNA in a volume of 20 µl with 100 nM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.8 units Taq DNA polymerase (Invitrogen). Cycling conditions were 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C and PCR products were electrophoresed on 2 % agarose gels with Ethidium Bromide in 0.5× TBE buffer.

Progeny tests

Progeny tests were carried out on 4–7 F3s from each recombinant F2 identified within the *LrSV2* containing interval between markers swm13 and gwm533. Plants were grown and artificially inoculated with *Puccinia triticina* race Ca2-G1R as described above. Markers swm13 and gwm533 were genotyped on each F3.

Linkage analysis

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

A F2 population of 6,815 individuals from the cross Sinvalocho MA × Gama6 was grown in the greenhouse and inoculated at the flag leaf stage with the *P. triticina* race Ca2-G1R that gives an incompatible reaction in the presence of the *LrSV2* gene at this late developmental stage. In total, 5,507 resistant and 1,308 susceptible plants were identified. This segregation does not fit the expected 3:1 segregation for a single dominant gene ($p < 0.01$). However, since adult plant inoculations are operationally difficult and a number of plants may have escaped the infection, some susceptible plants may have been misclassified as resistant. This does not affect our linkage analysis with the F2 population since only the 1,308 susceptible individuals, in which there is certainty about the homozygosity of the susceptible *LrSV2* allele, were used as mapping population. In this type of mapping population, the recombinants are easily identified because marker alleles from the resistant parent are present in homozygous susceptible individuals. Therefore, in case of dominant markers like AFLP bands, only those in coupling phase with the resistance were useful in this fine mapping population. The search for linked AFLP markers was done using a Bulk Segregant Analysis approach. Bulks composed of six susceptible and six resistant RILs (recombinant inbred lines) from the cross between Sinvalocho MA and Gama6 (Ingala et al. 2012) were tested

with 405 AFLP primer combinations. Putative-associated bands, present only in the resistant pool, were tested on 16 (eight resistant and eight susceptible) additional RILs from the same population.

Five AFLP fragments were found in the resistant bulk and in the eight additional resistant individual RILs and not the susceptible bulk and the eight additional individual susceptible RILs. In order to identify the closest markers, a stepwise analysis was performed.

First, these 5 AFLPs, along with marker P31/M37$_{150}$ that was previously identified as the closest AFLP marker (Ingala et al. 2012), were tested on 89 of the 1,308 rust susceptible F2 plants of the mapping population. One of them, marker P40/M42$_{325}$ was not present in any of them suggesting complete linkage with *LrSV2* whereas markers P31/M37$_{150}$, P32/M36$_{240}$, P34/M44$_{330}$ and P46/M45$_{275}$ detected the same single recombinant. Marker P31/M42$_{100}$ was also present in this recombinant and in an additional one, positioning it further apart.

In a second step, from the analysis of those 89 F2 plants, 3 markers were selected for being tested on 254 additional susceptible plants from the mapping population: the completely linked AFLP P40/M42$_{325}$ and 2 of the markers that showed a single recombinant with *LrSV2* (P31/M37$_{150}$ and P32/M36$_{240}$). In addition, these 254 rust susceptible F2 plants and the 89 from the first step (343 in total) were genotyped with the previously identified linked SSR marker gwm533 (Ingala et al. 2012) and 6 polymorphic markers that were selected among 55 markers reported to map in the subtelomeric region of chromosome 3BS due to their codominance, allelic difference and quality of the amplified product (Table S1). In the resulting genetic map, the AFLP marker P40/M42$_{325}$ and the SSR cfb5010 cosegregated with the *LrSV2* gene. The closest flanking markers were cfb3417 at 1.2 cM distal and the group of markers stm560$_{tgag}$, stm559$_{tgag}$ and gwm533 cosegregating at 0.4 cM proximal to the *LrSV2* gene (Fig. 1a).

To increase the resolution of the genetic map, markers cfb3417, stm560$_{tgag}$, stm559$_{tgag}$, gwm533 and the cosegregating marker cfb5010 were genotyped on the rest of the rust susceptible F2 mapping population (totalizing 1,308 F2 individuals, 2,616 gametes). In this high-resolution map, cfb3417 mapped 1 cM distal, cfb5010 cosegregated and the group of markers formed by stm559$_{tgag}$, stm560$_{tgag}$ and gwm533 mapped 0.3 cM proximal to *LrSV2* (Fig. 1b).

With the aim of finding flanking markers encompassing a smaller interval suitable for future positional cloning, a sequence characterized amplified region (SCAR) marker was developed from the cosegregating AFLP P40/M42$_{325}$. The sequence of the AFLP band was homologous to position 561788-562080 of the 1.26 Mb contig0011b that was sequenced by Choulet et al. (2010) in a pilot sequencing project aiming at gaining insight into the composition of Mb-sized contigs from chromosome 3B. This region was annotated as a β-expansin 1a precursor, putatively expressed (Choulet et al. 2010). The primers designed on

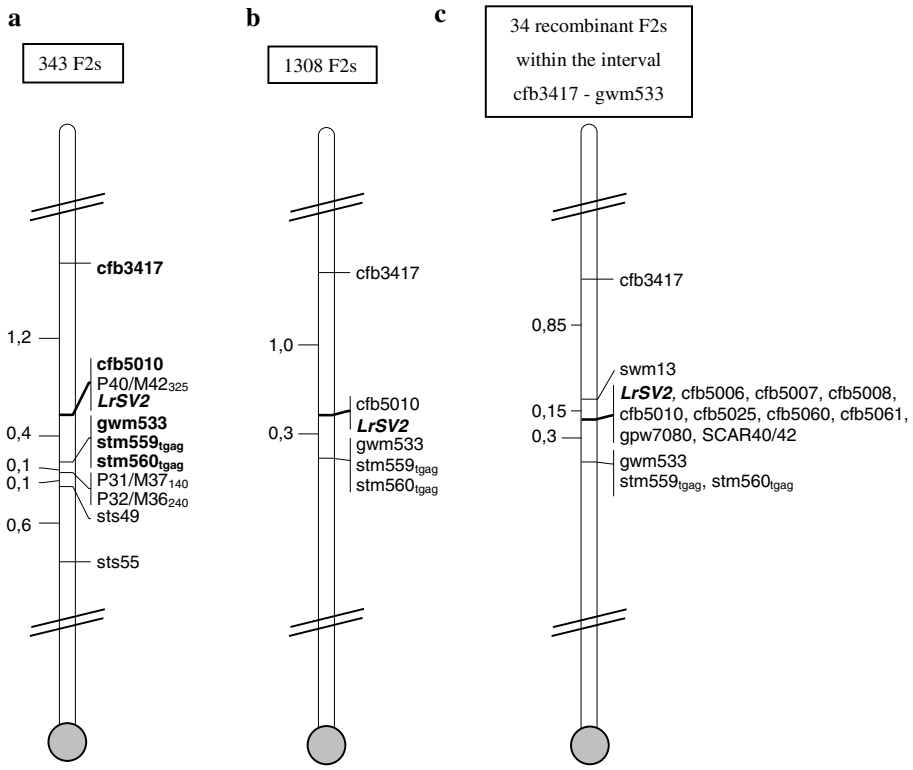

Fig. 1 Genetic maps of the *LrSV2* locus at the distal end of chromosome 3BS. **a** Genetic map of 343 F2s from the cross Sinvalocho MA x Gama6. Markers in *bold* were assayed in the rest of the population. **b** High-resolution genetic map of distal chromosome 3BS (1308 F2s from the cross Sinvalocho MA × Gama6). **c** Idem **b** with the addition of the BAC sequence-derived markers tested on the recombinants within the cfb3417 and gwm533 interval. The *grey circle* denotes the centromere. Genetic distances (cM) are shown on the left end side

this sequence amplified the expected 220 bp band only in the resistant Sinvalocho MA parental line (SCAR40/42, Fig. S1).

To obtain additional markers at the *LrSV2* locus, we took advantage of the construction of the physical map of the 3B chromosome of cv Chinese Spring (Paux et al. 2008; Rustenholz et al. 2011). In this map, marker cfb3417 is located in a contig of 820 kb whereas marker gwm533 is found in another contig of 1,442 kb within the 3BS8-0.78-0.87 deletion bin. Additional anchoring information from the physical map indicated that the contigs containing the 2 markers are separated by two additional contigs of 980 and 1,552 kb (Paux et al. 2008; Rustenholz et al. 2011) suggesting that, if no big insertion/deletions occurred in cv Sinvalocho, the defined *LrSV2*-containing interval could span around 4.8 Mb. Thirty additional SSR markers designed from the sequence of cv Chinese Spring BAC clones spanning this physical interval (Choulet et al. 2010) were tested for polymorphism between the parental lines (Table S2). Eight markers were selected along with the SCAR40/42 for testing the 34 F2s recombinants identified within the target interval (27 recombinants between cfb3417 and *LrSV2* and 7 between *LrSV2* and the cluster including gwm533). Seven of the new markers cosegregated with the *LrSV2* gene, cfb5010 and SCAR40/42 whereas the microsatellite swm13 mapped distal at 0.15 cM (Fig. 1c). Primer sequences for the amplification of the markers used to develop the map are shown in Table 1.

Progeny tests were carried out for the four recombinant F2s identified between swm13 and *LrSV2* and the seven between *LrSV2* and the cluster including gwm533. All F3 progeny plants were rust susceptible and either marker swm13 or gwm533 showed segregation within each F3 progeny. Therefore, all these F2s were homozygous susceptible for the *LrSV2* gene and heterozygous for only one of the flanking markers and were true recombinants (Fig. 2).

Thus, our results show that the *LrSV2* gene is located in a genetic interval of 0.45 cM defined by the flanking microsatellites swm13 and gwm533.

Discussion

In this work, we mapped the *LrSV2* gene for adult plant wheat leaf rust resistance in a genetic interval of 0.45 cM, flanked by microsatellite markers swm13 (distal) and gwm533 (proximal). The marker order in Sinvalocho MA × Gama6 cross analyzed was similar to published maps constructed from other crosses (Liu and Anderson 2003) and to the Chinese Spring physical map (Paux et al. 2008).

In the variety Chinese Spring, markers swm13 and gwm533 were physically mapped on subtelomeric chromosome 3BS deletion bin 3BS8-0.78-0.87 in two adjacent contigs, Ctg0011 and Ctg0344, respectively (Paux et al. 2008). Ctg0011 was sequenced by Choulet et al. (2010) in a pilot sequencing project aiming at gaining insight into the composition of Mb-sized contigs from chromosome 3B. Marker swm13 was found at the distal end of this 1266078 bp sequence (Accession |FN645450.1|) and therefore, the physical distance to gwm533 is larger than 1.2 Mb in Chinese Spring (Choulet et al. 2010). A reference sequence of Chinese Spring chromosome 3B has been produced recently (Choulet et al. in prep) and the interval swm13–gwm533 spans roughly 2.5 Mb. If no big insertion/

Table 1 Primer sequences for the amplification of the markers used to develop the map of Fig. 1

Marker	Forward primer	Reverse primer	Reference
cfb3417	TTGTTAAGGCGAGACATTGG	GATACACAGAGGGCGGAG	P. Sourdille, personal communication
cfb5006	ATCGGCGAGAAGTCACGG	GAGTGGAGGTGGGTGGAG	
cfb5007	CATCCGATCCAATTTGTGGAG	CCGGTGGAATAGTTGTCTTG	
cfb5008	GTCTTGGGTCAATCCGTTTG	TGTGTGTGTGTGGGGG	
cfb5010	ACAACGACGGGAGAGGGG	AAGGAGGCGGCGGAAGAG	
cfb5025	GGTATGCAGGTCCTGGAATG	GCCTTTGAAATTAAGTCAACCC	
cfb5060	GGACCCTTAGCTTTGATGATG	GTGCCAACAAAACATGCAG	
cfb5061	ACTAGACCCTTGCTAAAGCC	ACTCACTGCCAACTGCTC	
gpw7080	ATGCCAACCAGACATCACAG	CAAAACCTACAGCTCCCTCG	
gwm533	AAGGCGAATCAAACGGAATA	GTTGCTTTAGGGGAAAAGCC	Röder et al. (1998)
SCAR40/42	CCACACCACTCACCAGTGTC	CCTACCACTTCGACCTCAGC	This work
$stm559_{tgag}$	AAGGCGAATCAAACGGAATA	TGTGTGTGTGTGTGAGAGAGAG	Hayden et al. (2004)
$stm560_{tgag}$	GGAGGGAAACTATCAAAATATGCTGGT	TGTGTGTGTGTGTGAGAGAGAG	Hayden et al. (2006)
sts49	ATTGCGAAGAACACCCTCAC	ACGTCTTCAGCACCGAGTTC	Ling et al. (2003)
sts55	CGGCAACAACAACAACCA	ATGAGGCTCCCAACTCCAC	
swm13	TCGAAGAGCTAGCTATGGAC	CCAGGACGAATTCGTACAAC	B. Keller, personal communication

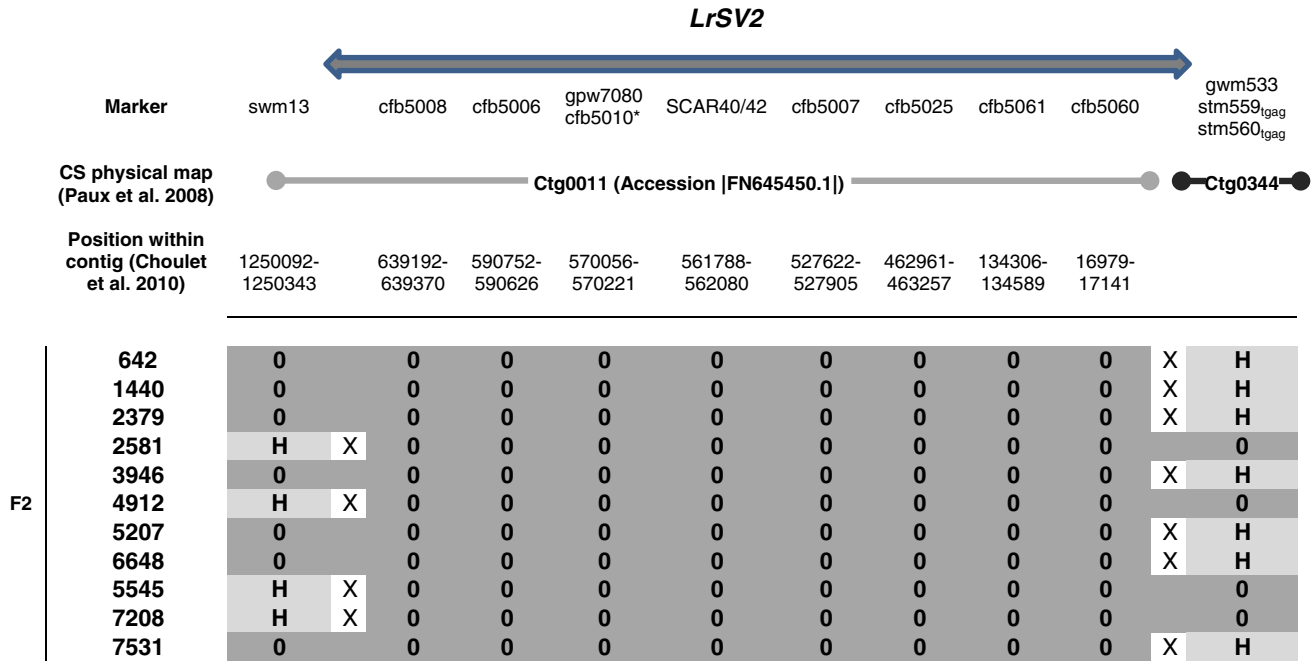

Fig. 2 Graphical genotype matrix of the 11 critical recombinant F2s found between swm13 and gwm533. The physical position of markers on the Chinese Spring (CS) Ctg0011 is shown. The crossing over is denoted by "×". *0* susceptible Gama6 genotype, *H* heterozygous. * approximate position

deletions occurred in cv Sinvalocho, the genetic/physical distance ratio within the defined *LrSV2*-containing interval could be around 0.18 cM/Mb. This interval can be divided into three regions: swm13–cfb5008 with a ratio of 0.25 cM/Mb; cfb5008–cfb5060, 622 kb where no recombination was observed and cfb5060–gwm533 with 0.24 cM/Mb. *LrSv2* cosegregated with the markers physically mapped to this central region where no recombinants were detected.

An important feature of wheat genome organization is that gene density and recombination rates increase with the distance to the centromere, with mean recombination rates ranging between 0.06 and 0.87 cM/Mb for the proximal and distal intervals, respectively (Gill et al. 1993; Lukaszewski and Curtis 1993; Akhunov et al. 2003). A detailed study of recombination along chromosome 3B in the crosses Chinese Spring × Renan and Chinese Spring × Courtot (Saintenac et al. 2009) showed that the 3BS8-0.78-0.87 deletion bin has the highest crossover frequency within this chromosome, with an average of 0.85 cM/Mb. The distribution of cross over events may not be homogeneous throughout the deletion bin and indeed, recombination hotspots have been reported in this region (Saintenac et al. 2011). A possible cause of reduction in recombination could be the presence of large insertions/deletions between the parental lines since the wheat genome has a high buffering capacity to tolerate rearrangements due to its polyploidy. In fact, by sequencing 357 kb of this contig, Breen et al. (2010) reported that cultivar Hope lacks a 51,666 bp mitochondrial DNA genome insert yet found in Chinese Spring. In addition, it should be noted that in our work recombination frequency may be underestimated because some putative recombinants with no F3 offspring, in which the rust and marker segregation could not be confirmed by progeny tests, were eliminated from the mapping population. This introduced a bias towards less number of recombinants, subsequently rendering shorter genetic distances.

When comparing the *LrSV2* locus genetic maps constructed with 343 and 1,308 F2 individuals, we observed very similar genetic distances, suggesting that low or medium resolution maps are sufficient to define a confident genetic interval spanning the target gene and identify flanking markers in a region with high recombination rates such as bin 3BS8-0.78-0.87. Once a confident interval is established, the flanking markers can be used to genotype a larger population to identify new recombinants within the region. The genotyping of additional markers within the interval would resolve all recombination breakpoints until the gene is restricted to a small region that allow the establishment of a physical contig of BAC clones containing the flanking markers. The number of recombinants that will need to be genotyped is correlated to the genetic distance between flanking markers. Therefore, the determination of an accurate small genetic interval is invaluable for the acceleration of a positional cloning project. This strategy was successfully

performed for map-based cloning of leaf rust genes *Lr1*, *Lr10*, *Lr21* and *Lr34* in which intervals of 0.8, 0.13, 1.7 and 0.15 cM, respectively, were defined (Cloutier et al. 2007; Feuillet et al. 2003; Huang et al. 2003; Krattinger et al. 2009). These authors used markers developed from wheat ancestors or related species (Stein et al. 2000; Ling et al. 2003; Huang et al. 2003; Krattinger et al. 2009). In the present work, we were able to develop a fine map taking advantage of the sequence available from BAC-end sequences (Paux et al. 2006, 2008) and from the complete sequence of contigs of the physical map of chromosome 3B of the reference cultivar Chinese Spring (Choulet et al. 2010).

Cloning of *Lr34* and *Lr1* genes allowed the estimation of genetic/physical distance ratios of 0.4 and 19 cM/Mb, respectively (Cloutier et al. 2007; Krattinger et al. 2009). This difference in recombination frequency between both regions could be attributed to the uneven distribution of recombination hot spots which mainly occur nearby or within genes (Saintenac et al. 2011) and prevents extrapolations beyond the reasonable genetic intervals that should be defined prior to a positional cloning attempt in wheat.

Given the high number of segregating individuals needed for fine mapping projects, the development of RILs populations would be too laborious and is more convenient to use F2 individuals from the cross between the line carrying the gene of interest and a line with a contrasting phenotype. Dealing with dominant genes, like the resistance gene *LrSV2* studied here, allows having genotype certainty only in the susceptible F2s. If they are used as mapping population, the need of progeny testing for rust might be reduced to only those few critical recombinants. In addition, in this type of mapping population, recombination with the markers can be easily identified because marker alleles from the resistant parent are present in homozygous susceptible individuals, minimizing scoring errors, which is not a minor issue when dealing with high number of samples. Most of the STSs, STMs, and SSRs markers are co-dominant, allowing the detection of heterozygous individuals. However, in the case of dominant markers like AFLP bands and some ISBPs and SSRs, only those in coupling phase with the resistant allele can be used.

Taking into account that *LrSV2* is a single dominant gene, a 3:1 segregation was expected for resistance:susceptibility in an F2 population. In the observed segregation, however, a deficit of susceptible individuals was noticed. This could be due to the difficulties inherent to adult plant rust inoculations where the inoculums might not reach every leaf. Thus, several individuals might have escaped infection and were misclassified as resistant. In addition, a very conservative criterion was applied and plants not showing clear infection symptoms were eliminated from the analysis.

Within the sequenced 1.25 Mb proximal to swm13 in the variety Chinese Spring, 21 genes and 15 pseudogenes were annotated by Choulet et al. (2010). Among them, perhaps the most promising candidates for *LrSV2* could be two pseudogenes annotated as RGAs (resistant gene analogs) similar to a LZ-NBS-LRR protein and to a disease resistance RPM1-like protein, three with similarity to tyrosine kinases that could be involved in signal transduction pathways and one homologous to a receptor kinase. In addition, two putatively expressed genes and one pseudogene were identified as β-expansin 1a precursors that, as cell wall components, have been associated with disease resistance in rice (Fu et al. 2011). However, it should be noted that this is not the complete *LrSV2* interval and that Chinese Spring does not carry *LrSV2* and therefore it will be essential to obtain the sequence of the resistant cultivar Sinvalocho MA. The map-based cloning strategy should be performed together with a detailed haplotype characterization of the target sequence regions since rearrangement may have occurred at homologous loci between different varieties.

Interestingly, the *Sr2* gene for stem rust resistance was also mapped on Ctg0011 (Mago et al. 2011) in a physical interval included within the one defined here for the *LrSV2* gene. Therefore, the APR gene *LrSV2* could belong to the same multiple resistance *locus* as *Sr2* or, alternatively, given that resistance genes are often found in clusters (Michelmore and Meyers 1998; Helguera et al. 2003) they could belong to the same gene cluster.

If a smaller physical interval is defined, the sequencing effort and the number of candidate ORFs could be reduced. Both swm13 and gwm533 are co-dominant markers and suitable to be used as flanking markers to find additional recombinants. It should be highlighted that eight SSRs and the SCAR40/42 cosegregate with *LrSV2* and that new markers could be developed from the sequence information available for this region in the reference cultivar Chinese Spring (Choulet et al. 2010). The analysis of these nine cosegregating markers and additional ones in the recombinants should allow the definition of a smaller interval suitable for the postulation of a reasonable number of candidate ORFs for *LrSV2*.

Acknowledgments M. López thanks for doctoral fellowship from the Argentinean Scientific and Technological Research Council (CONICET) and M.F. Pergolesi from the Argentinean Agency for Science and Technology Promotion (ANPCyT). The authors wish to thank Grants PICT 2005-38150 and PAE 2007-37108-PID 121 from the Argentinean Agency for Science and Technology Promotion (ANPCyT) and Grants No 522305 and 522312 from the Argentinean Institute of Agricultural Technology (INTA).They also wish to thank Mr. C. Romero for excellent assistance with the greenhouse work.

Conflict of interest The authors declare that they have no conflict of interest.

References

Akhunov D, Goodyear AW, Geng S, Qi L, Echalier B et al (2003) The organization and rate of evolution of wheat genomes are correlated with recombination rates along chromosome arms. Genome Res 13:753–763

Benbouza H, Jacquemin J-M, Baudoin J-P, Mergeai G (2006) Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechnol Agron Soc Environ 10(2):77–81

Bossolini E, Krattinger S, Keller B (2006) Development of simple sequence repeat markers specific for the *Lr34* resistance region of wheat using sequence information from rice and *Aegilops tauschii*. Theor Appl Genet 113(6):1049–1062

Breen J, Li D, Dunn DS, Békés F, Kong X, Zhang J, Jia J, Wicker T, Mago R, Ma W, Bellgard M, Appels R (2010) Wheat beta-expansin (EXPB11) genes: identification of the expressed gene on chromosome 3BS carrying a pollen allergen domain. BMC Plant Biol 10:99–109

Caldwell RM (1968) In: Finley K.W, Shepherd K.W(eds) Breeding for general and/or specific plant disease resistance. Proc. 3rd Int. Wheat Genetics Symp Aust Acad Sci, Canberra: Australia, pp 263–272

Choulet F, Wicker T, Rustenholz C, Paux E, Salse J, Leroy P, Schlub S, Le Paslier M-C, Magdelenat G, Gonthier C, Couloux A, Budak H, Breen J, Pumphrey M, Liu S, Kong X, Jia J, Gut M, Brunel D, Anderson JA, Gill BS, Appels R, Keller B, Feuillet C (2010) Megabase level sequencing reveals contrasted organization and evolution patterns of the wheat gene and transposable element spaces. Plant Cell 22:1686–1701

Cloutier S, McCallum BD, Loutre C, Banks TW, Wicker T, Feulliet C, Keller B, Jordan MC (2007) Leaf rust resistance gene *Lr1*, isolated from bread wheat (*Triticum aestivum* L.) is a member of the large psr567 gene family. Plant Mol Biol 65(1–2):93–106

Diéguez MJ, Altieri E, Ingala LR, Perera E, Sacco F, Naranjo T (2006) Physical and genetic mapping of AFLPs and the leaf rust resistance *Lr3* gene on chromosome 6BL of wheat. Theor Appl Genet 112:251–257

Favret EA, Saione HA, Franzone PM (1983) New approaches in breeding for disease resistance. Cereal breeding and production Symp. Argentina Special Report 718. Oregon State University

Feuillet C, Travella S, Stein N, Albar L, Nublat A, Keller B (2003) Molecular cloning of a new receptor like–kinase gene encoded at the *Lr10* disease resistance locus of wheat. Plant J 11:45–52

Fu D, Uauy C, Distelfeld A, Blechl A, Epstein L, Chen X, Sela H, Fahima T, Dubcovsky J (2009) A kinase-start gene confers temperature-dependent resistance to wheat stripe rust. Science 323:1357–1360

Fu J, Liu H, Li Y, Yu H, Li X, Xiao J, Wang S (2011) Manipulating broad-spectrum disease resistance by suppressing pathogen-induced auxin accumulation in rice. Plant Phys 155:589–602

Germán S, Barcellos A, Chaves M, Kohli M, Campos P, de Viedma L (2007) The situation of common wheat rusts in the Southern Cone of America and perspectives for control. Aust J Agric Res 58(6):620–630

Gill KS, Gill BS, Endo TR (1993) A chromosome region-specific mapping strategy reveals gene rich telomeric ends in wheat. Chromosoma 102:374–381

Hayden MJ, Kuchel H, Chalmers KJ (2004) Sequence tagged microsatellites for the Xgwm533 locus provide new diagnostic markers to select for the presence of stem rust resistance gene *Sr2* in bread wheat (*Triticum aestivum* L.). Theor Appl Genet 109:1641–1647. doi:10.1007/s00122-004-1787-5

Hayden MJ, Stephenson P, Logojan AM, Khatkar D, Rogers C, Elsden J, Koebner MD, Snape JW, Sharpe PJ (2006) Development and genetic mapping of sequence-tagged microsatellite (STMs) in bread wheat (*Triticum aestivum* L.). Theor Appl Genet 113:1271–1281

Helguera M, Khan IA, Kolmer J, Lijavetzky D, Zhong-qi L, Dubcovsky J (2003) PCR assays for the *Lr37-Yr17-Sr38* cluster of rust resistance genes and their use to develop isogenic hard red spring wheat lines. Crop Sci 43:1839–1847

Hiebert CW, Thomas JB, Somer DJ, McCallum BD, Fox SL (2007) Microsatellite mapping of adult-plant leaf rust resistance gene *Lr22a* in wheat. Theor Appl Genet 115:877–884

Huang L, Brooks SA, Li W, Fellers JP, Gill BS, Trick HN, Gill BS (2003) Map-based cloning of leaf rust resistance gene Lr21 from the large and poliploid genome of bread wheat. Genetics 164:655–664

Ingala L, Saione H, Helguera M, Nisi M, Sacco F (2005) Inheritance of adult plant resistance genes, and associated markers, from a durable resistant cultivar to leaf rust. In: Buck HT, Nisi JE, Salomon N(eds) Proceedings of the seventh international wheat conference. Mar del Plata, Argentina. Wheat production in stressed environments. Develpments in plant breeding. vol 12. Springer, Dordrecht,2007,pp 59–64

Ingala L, López M, Darino M, Pergolesi MF, Diéguez MJ, Sacco F (2012) Genetic analysis of leaf rust resistance genes and associated markers in the durable resistant wheat cultivar Sinvalocho MA. Theor Appl Genet 124(7):1305–1314. doi:10.1007/s00122-012-1788-8

Johnson R (1981) Durable resistance: definition of, genetic control, and attainment in plant breeding. Phytopathology 71:567–568

Kolmer JA (1996) Genetics of resistance to wheat leaf rust. Annu Rev Phytopathol 34:435–455

Kota R, Spielmeyer W, McIntosh RA, Lagudah ES (2006) Fine genetic mapping fails to dissociate durable stem rust resistance gene Sr2 from pseudo-black chaff in common wheat (*Triticum aestivum* L.). Theor Appl Genet 112:492–499

Krattinger S, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter L, Keller B (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323:1360–1363

Lander E, Green P, Abrahamson J, Barlow A, Daley M, Lincoln S, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Ling H-Q, Zhu Y, Keller B (2003) High-resolution mapping of the leaf rust disease resistance gene Lr1 in wheat and characterization of BAC clones from the Lr1 locus. Theor Appl Genet 106:875–882

Liu S, Anderson JA (2003) Targeted molecular mapping of a major wheat QTL for *Fusarium* head blight resistance using wheat ESTs and synteny with rice. Genome 46:817–823

Liu S, Zhang X, Pumphrey MO, Snack RW, Gill BS, Anderson JA (2006) Complex microcolinearity among wheat, rice and barley revealed by fine mapping of the genomic region harbouring a major QTL for resistance to fusarium head blight in wheat. Funct Integr Genomics 6:83–89

Lukaszewski AJ, Curtis CA (1993) Physical distribution of recombination in B-genome chromosomes of tetraploid wheat. Theor Appl Genet 86:121–127

Macagno LF, Pizarro JB, Cordone GE (1993) Dirección Nacional Asistente de Planificación. Publicación miscelánea N° 4. INTA 15–28

Mago R, Tabe L, McIntosh RA, Pretorius Z, Kota R, Paux E, Wicker T, Breen J, Lagudah ES, Ellis JG, Spielmeyer W (2011) A multiple resistance locus on chromosome arm 3BS in wheat confers resistance to stem rust (*Sr2*), leaf rust (*Lr27*) and powdery mildew. Theor Appl Genet 123(4):615–623

Mains EB, Jackson HS (1926) Physiologic specialization in the leaf rust of wheat; *Puccinia triticina* Erikss. Phytopathology 16:89–120

McIntosh RA, Welllings CR, Park RF (1995) Wheat rusts: an atlas of resistance genes. Kluwer, Dordrecht

Messmer MM, Seyfarth R, Keller M, Schachermayr G, Winzeler M, Zanetti S, Feuillet C, Keller B (2000) Genetic analysis of durable leaf rust resistance in winter wheat. Theor Appl Genet 100:419–431

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130. doi:10.1101/gr.8.11.1113

Michelmore RW, Paran I, Kesseli V (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Paux E, Roger D, Badaeva E, Gay G, Bernard M, Sourdille P, Feuillet C (2006) Characterizing the composition and evolution of homoeologous genomes in hexaploid wheat through BAC-end sequencing on chromosome 3B. Plant J 48:463–474

Paux E, Sourdille P, Salse J, Saintenac C, Choulet F, Leroy P, Korol A, Michalak M, Kianian S, Spielmeyer W, Lagudah E, Somers D, Kilian A, Alaux M, Vautrin S, Berges H, Eversole K, Appels R, Safar J, Simkova H, Dolezel J, Bernard M, Feuillet C (2008) A physical map of the 1 gigabase bread wheat chromosome 3B. Science 322:101–104

Pink DAC (2002) Strategies using genes for non-durable resistance. Euphytica 1:227–236

Pretorius ZA, Roelfs AP (1996) The role of *Lr10*, *Lr13*, and *Lr34* in the expression of adult-plant resistance to leaf rust in the wheat cultivar Era. Plant Dis 80(2):199–202

Röder M, Korzun V, Wendehake K, Plaschke J, Tixier M-H, Leroy P, Ganal MW (1998) A microsatellite map of wheat. genetics 149:2007–2023

Rodriguez Amieva, PJ, Tessi JL, Frecha JH, Vallega J (1961) Estimación de los daños producidos en la Argentina por las royas del tallo y de la hoja del trigo durante el período 1949–1958. Robigo 12:18–22

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Rubiales D, Niks RE (1995) Characterization of *Lr34*, a major gene conferring nonhypersensitive resistance to wheat leaf rust. Plant Dis 79:1208–1212

Rustenholz C, Choulet F, Laugier C, Safar J, Simkova H, Dolezel J, Magni F, Scalabrin S, Cattonaro F, Vautrin S, Bellec A, Berges H, Feuillet C, Paux E (2011) A 3000-loci transcription map of chromosome 3b unravels the structural and functional features of gene islands in hexaploid wheat. Plant Physiol 157:1596–1608

Sacco F, Favret EA, Suarez EY, Solari RM, Saione HA (1995) Spontaneous genetic variation for leaf rust reaction in Sinvalocho MA wheat. J Phytopathol (Berl.) 143:251–255

Sacco F, Suárez EY, Naranjo T (1998) Mapping of the leaf rust resistance gene *Lr3* on chromosome 6B of Sinvalocho MA wheat. Genome 41:686–690

Safar J, Bartos J, Janda J et al (2004) Dissecting large and complex genomes: flow sorting and BAC cloning of individual chromosomes from bread wheat. Plant J. 39:960–968

Saintenac C, Falque M, Martin O, Paux E, Feuillet C, Sourdille P (2009) Detailed recombination studies along chromosome 3b provide new insights on crossover distribution in wheat (*Triticum aestivum* L.). Genetics 181:393–403

Saintenac C, Faure S, Remay A, Choulet F, Ravel C, Paux E, Balfourier F, Feuillet C, Sourdille P (2011) Variation in crossover rates across a 3-Mb contig of bread wheat (*Triticum aestivum*) reveals the presence of a meiotic recombination hotspot. Chromosoma 120:185–198. doi:10.1007/s00412-010-0302-9

Sawhney RN, Nayar SK, Sharma JB, Bedi R (1989) Mechanism of durable resistance: a new approach. Theor Appl Genet 78:229–232

Schnurbusch T, Paillard S, Schori A, Messmer M, Schachermayr G, Winzeler M, Keller B (2004) Dissection of quantitative and durable leaf rust resistance in swiss winter wheat reveals a major resistance QTL in the *Lr34* chromosomal region. Theor Appl Genet 108:477–484

Singh S, Bowden R (2011) Molecular Mapping of adult-plant race-specific leaf rust resistance gene *Lr12* in bread wheat. Mol Breed 28:137–142

Somers DJ, Isaac P, Edwards K (2004) A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). Theor Appl Genet 109:1105–1114

Song QJ, Shi JR, Singh S, Fickus W, Costa JM, Lewis J, Gill BS, Ward R, Cregan PB (2005) Development and mapping of microsatellite (SSR) markers in wheat. Theor Appl Genet 110:550–560

Sourdille P, Singh S, Cadalen T, Brown-Guedira G, Gay G, Qi L, Gill BS, Dufour P, Murigneux A, Bernard M (2004) Microsatellite-based deletion bin system for the establishment of genetic-physical map relationships in wheat (*Triticum aestivum* L.). Funct Integr Genomics 4(1):12–25

Stein N, Feuillet C, Wicker T, Schlagenhauf E, Keller B (2000) Subgenome chromosome walking in wheat: a 450- kb physical contig in *Triticum monococcum* L. spans the *Lr10* resistance locus in hexaploid wheat (*Triticum aestivum* L.). Proc Natl Acad Sci 97:13436–13441

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Hered 93(1):77–78

Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Friters A, Pot J, Paleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4441

Xue S, Zhang Z, Lin F (2008) A high-density intervarietal map of the wheat genome enriched with markers derived from expressed sequence tags. Theor Appl Genet 117:181–189

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J (2003) Positional cloning of the wheat vernalization gene *VRN1*. Proc Natl Acad Sci 100(10):6263–6268