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Molecular markers linked to the aluminium tolerance gene *Alt1* in rye (*Secale cereale* L.)

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Abstract Rye has one of the most efficient group of genes for aluminium (Al) tolerance among cultivated species of Triticeae. This tolerance is controlled by at least two independent and dominant loci (*Alt1* and *Alt3*) located on chromosomes 6RS and 4R. We used two pooled DNA samples, one of Al-tolerant individuals and another of Al-sensitive plants from one F_2 that segregated for the *Alt1* locus. We also used two pooled DNA samples, one with genotypes *11* and another with genotypes *22* for the *Lap1* locus (leucin aminopeptidase) from another F_2 progeny that segregated for this locus, located on the 6RS chromosome arm. We identified several RAPD markers associated with the pooled Al-tolerant plants and also with one of the bulks for the *Lap1* locus. The RAPD fragments linked to *Alt1* and *Lap1* genes were transformed into SCAR markers to confirm their chromosomal location and linkage data. Two SCARs ($ScR01_{600}$ and $ScB15_{7900}$) were closely linked to the *Alt1* locus, $ScR01_{600}$ located 2.1 cM from *Alt1* and $ScB15_{790}$ located 5.5 cM from *Alt1*, on the 6RS chromosome arm. These SCAR markers can aid in the transfer of Al tolerance genes into Al-sensitive germplasms.

Key words Aluminium · Tolerance · RAPDs · SCARs · Rye

Introduction

The identification of molecular markers closely linked to agronomic genes is the first step in their isolation and is also a valuable tool in breeding programs. Aluminium (Al) stress is considered to be the primary cause of reduced crop production. However, the information about the genetic systems controlling plant response to Al stress is fragmentary and limited (Foy 1988; Aniol and Gustafson 1990).

A large number of genetic studies support the conclusion that Al tolerance in wheat is controlled by a single dominant gene (Kerridge and Kronstad 1968; Aniol and Gustafson 1984; Fisher and Scott 1987; Larkin 1987). Al tolerance, assessed on the basis of root elongation, segregated as a single dominant locus in wheat (Delhaize et al. 1993; Sommers and Gustafson 1995; Riede and Anderson 1996). Two RFLPs linked to the wheat Alt_{BH} gene, located on the 4DL chromosome arm, have been reported (Riede and Anderson 1996).

The genus *Secale* has the most efficient group of genes for Al tolerance among cultivated species of the Triticeae (Aniol and Kaczkowsky 1979). Many triticales have some degree of Al tolerance, but not as much as rye itself (Aniol and Gustafson 1984). Aluminium tolerance (*Alt*) genes were located on rye chromosomes 3R, 4R and 6RS (Aniol and Gustafson 1984) using wheat-rye addition lines. Isozyme loci have been linked to the *Alt1* gene in rye (Gallego and Benito 1997). However, only two DNA markers linked to genes related to aluminium tolerance in rye have been described to-date (Gallego et al. 1998).

The main objective of the present work was to obtain more molecular markers closely linked to the Al tolerance genes in rye (*Secale cereale* L.) using bulked segregant analysis (Michelmore et al. 1991).

Materials and methods

Plant material

To map the *Alt1* locus in rye, two F_2s (AR6-17⊗ and AR1-13⊗) were used. AR6-17⊗ was segregating for the *Alt1* locus located on the 6RS

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chromosome arm. AR1-13⊗ was segregating for the *Lap1* locus, also located on the 6RS chromosome arm. (Gallego and Benito 1997).

Hexaploid wheat, *Triticum aestivum* L., cv "Chinese Spring" (CS), rye, *Secale cereale* L., cv "Imperial" (I), amphiploid wheat-rye (CS-I), the seven disomic wheat-rye (CS-I) addition lines (1R, 2R, 3R, 4R, 5R, 6R and 7R) and the available ditelosomic wheat-rye (CS-I) addition lines (1RS, 1RL, 2RL, 3RS, 4RL, 5RL, 6RL, 7RS and 7RL), kindly supplied by Dr. A. J. Lukaszewski, were used to assign loci to chromosome arms.

Aluminum tolerance screening test

The Al tolerance test was carried out using the method of Aniol (1984) at an Al concentration of 150 μM (Gallego and Benito 1997).

Leucine aminopeptidase (LAP) electrophoresis

Electrophoresis was performed in horizontal 12% starch gels, using the buffers and staining methods described by Benito et al. (1991). The analysis were carried out on 12-day-old leaf extracts. The AR1-13⊗ plants were classified as homozygous for allele 1 (11), homozygous for allele 2 (22), and heterozygous (12).

Random amplified polymorphic DNA (RAPD) marker conditions

DNA for further analyses was extracted according to Dellaporta et al. (1983). RAPD reactions similar to those described by Williams et al. (1990) were performed with minor modifications. One-hundred-and-twenty 10-mer primers (Kits A, B, C, F, R and S from Operon Technologies) were used in this study. RAPD experiments were repeated at least three times before scoring the results (Gallego et al. 1998).

Bulked segregant analysis (BSA)

To identify RAPD fragments linked to the *Alt1* locus we used bulked segregant analysis (BSA) (Michelmore et al. 1991). Fragments in the *Alt1* region were identified by screening two different pairs of pools generated from two loci of this region: (1) locus *Alt1*, the two bulks consisted of 15 Al-tolerant individuals and 15 Al non-tolerant individuals (from AR6-17⊗ segregating for *Alt1*); and (2) locus *Lap1*, the two bulks consisted of 15 individuals each, with genotypes *11* and *22* for the *Lap1* locus, located on the 6RS chromosome arm. Primers showing differences within each pair of bulks were applied to all the individual plants of the respective progeny to determine their inheritance and linkage values.

Cloning and sequencing of RAPD products

The rye amplification products of interest were ligated and transformed using the pGEM-T vector system (Promega). Double-strand sequencing (DIG *Taq* DNA Sequencing kit, Boehringer Mannheim) was done by the non-radioactive PCR method. (Gallego et al. 1998).

Design and analysis of sequence characterized amplified regions (SCARs)

Two 21-mer oligonucleotides were designed from each cloned amplification product to be used as SCAR primers. Their sequences contained the original ten bases of the RAPD primer plus the next 11 internal bases, and are designated with the prefix Sc (Table 1).

Table 1 Sequences of the SCAR primer pairs generated from RAPD amplification products

Primer[a]	Sequence (5′ → 3′)[b]
$OPBO3_{655}$-F	CATCCCCCTGGAGAATAGGAC
$OPBO3_{655}$-R	CATCCCCCTGCAGTTCCTCG
$OPB15_{790}$-F	GGAGGGTGTTGTCGTGAAGCT
$OPB15_{790}$-R	GGAGGGTGTTCAGGACTAAAA
$OPB16_{1145}$-F	TTTGCCCGGACAACAGATGAG
$OPB16_{1145}$-R	TTTGCCCGGAGCTGTATAAAC
$OPS14_{705}$-F	AAAGGGGTCCAACATCACCTT
$OPS14_{705}$-R	AAAGGGGTCCTGACAATGTGG

[a] The first three letters and the two numbers refer to the kit and primer number (Operon Technologies) used to identify the progenitor RAPD marker. The subscript number refers to the size (bp) of the amplified RAPD product.
[b] The underlined sequence represents the sequence of the progenitor RAPD primer

Amplifications from genomic DNA were carried out in a 25-μl volume and contained 60 ng of template DNA, 100 μM of each dNTP, 15 pmol of each primer, $MgCl_2$ 2.5 mM and 1 u of *Amplitaq* DNA polymerase, and a Stoffel fragment (Perkin-Elmer Cetus) in 1 × reaction buffer (Tris-HCl 100 mM pH 8.3, KCl 100 mM). The amplifications were conducted in a PTC-100 thermal cycler (MJ Research) with the following programme: a preliminary step of 4 min at 94°, 35 cycles of 1 min at 94°, 1 min at 59–690°C (depending on the pair of primers), 6 min at 72° and a final step of 4 min at 72°. PCR reactions were stored at 4°C until their resolution by electrophoresis on 1.5% agarose gels stained with ethidium bromide (Sambrook et al. 1989). Experiments were repeated at least three times before scoring the results.

Some of the amplification fragments were digested with restriction endonucleases in order to detect variability in the progenies. A total of 17 restriction enzymes (Amersham) were used: *Hae*III, *Hha*I, *Rsa*I and *Taq*I (4-bp cutters) and *Bam*HI, *Bgl*I, *Eco*RI, *Hin*dIII, *Hin*fI, *Pst*I, *Sac*I, *Sma*I, *Xho*I, *Sal*I, *Msp*I, *Apa*I and *Kpn*I (6-bp cutters).

Genetic mapping

Linkage analysis was performed on F_2 segregation data using the MAPMAKER 3.0 computer program (Lander el al. 1987). Two loci were considered linked if the LOD score was greater than 4, and if the two-point distance was less than 45 cM. Genetic distances were calculated using the Kosambi function (Kosambi 1944).

Results

Identification of RAPD markers linked to the *Alt1* locus

To find RAPDs around the *Alt1* region, bulks from two loci of this region (*Alt1* and *Lap1*) were constructed using two different F_2s (see Materials and methods). Eight out of the one-hundred-and-twenty 10-mer primers used yielded no amplification fragments and the remaining primers amplified 452 fragments (1–10 bands per primer).

BSA with the *Alt1* locus (AR6-17⊗)

Two fragments ($OpB03_{655}$ and $OpR01_{600}$) were present in the block of tolerant plants and absent in the block of non-tolerant plants. When tested on the individual F_2 plants, the $OpB03_{655}$ fragment segregated 3:1 and was used in the analysis of linkage (Fig. 1). The $OpR01_{600}$ fragment is the same one that we located previously, using the CS-I addition lines transformed in a SCAR marker, and mapped at 2.1 cM from the *Alt1* locus (Gallego et al. 1998).

BSA with the *Lap1* locus (AR1-13⊗)

Alt1 and *Lap1* loci are both located on the 6RS chromosome arm. This is the reason why we decided to construct two bulks based on the genotype for the *Lap1* locus to obtain RAPD markers in the *Alt1* region (the *Alt1* locus did not segregate in this cross). The screening of the 120 10-mer primers on the AR1-13⊗ progeny revealed that the $OpB15_{790}$ and $OpB16_{1145}$ bands were associated to the 11 (homozygous for allele 1) and 22 (homozygous for allele 2) *Lap1* bulks, respectively. Therefore, these two fragments were selected to subsequently analyze their possible linkage to *Alt1*.

Amplification with OPB15 and OPB16 primers in the AR6-17⊗

The amplification with OPB15 and OPB16 on individual plants of the AR6-17⊗ progeny (segregating for *Alt1*) gave different results for the two fragments of interest ($OpB15_{790}$ and $OpB16_{1145}$). We could not detect amplification of the $OpB16_{1145}$ fragment. However, $OpB15_{790}$ was amplified in this progeny showing a 3:1

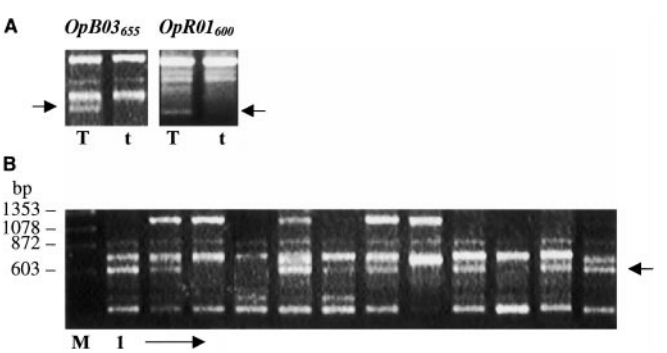

Fig. 1 **A** Two different RAPD fragments showing differences between a bulk of Al-tolerant plants and another of Al non-tolerant plants. **B** Individual segregation of the $OpB03_{655}$ fragment amplified on 12 individuals from the AR6-17⊗ progeny, segregating for the *Alt1* locus. *M* molecular-weight marker (*Hae*III digests of ϕX174 phage). *T* bulk of Al-tolerant plants. *t* bulk of Al non-tolerant plants. *Arrows* show the segregating fragments

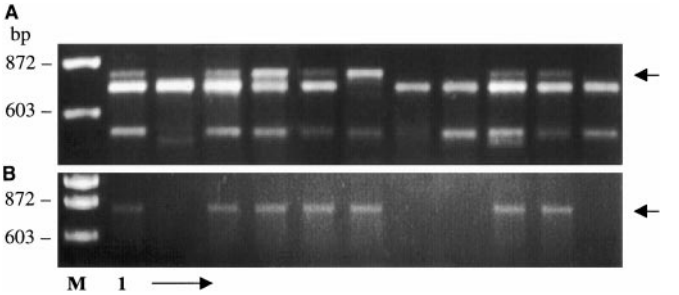

Fig. 2 Segregation of the 790-bp fragment amplified with **A** the OPB15 10-mer RAPD primer and **B** with the OPB15 21-mer SCAR-specific pair of primers on 12 individuals from the AR6-17⊗ progeny segregating for the *Alt1* locus. *M* molecular-weight marker (*Hae*III digests of ϕX174 phage). *Arrows* show the segregating fragment

segregation ratio and no recombinants were found between this locus and *Alt1* (Fig. 2a).

SCAR analyses in the AR6-17⊗

In order to obtain a reliable assay for the polymorphism detected by $OpB03_{655}$ and $OpB15_{790}$, and to find polymorphism for the $OpB16_{1145}$ fragment, four pairs of 21-mer primers were obtained using the sequences of the fragments of interest. To optimise the reaction conditions for each pair of specific primers we employed genomic DNA from the parents and offspring of the F_2 under study (AR6-17⊗) as a template in amplifications with different annealing temperatures and primer concentrations. Under these conditions only the DNA products of interest were amplified.

The observed variability for the $OpB03_{655}$ and $OpB15_{790}$ RAPD fragments was also present in the respective SCAR fragments, and the individual SCAR data (presence or absence) were consistent with the RAPD scores (Fig. 2b). Moreover, we tried to locate the $ScB03_{655}$, $ScB15_{790}$ and $ScB16_{1145}$ loci by amplification in wheat-rye addition lines. The $ScB03_{655}$ fragment could not be assigned to a specific rye chromosome because we could not find an annealing temperature that amplified the "Imperial" DNA. The $ScB15_{790}$ locus was located on the 6*RS* chromosome arm (Fig. 3).

The $ScB16_{1145}$ fragment was present in all F_2 individuals of AR6-17⊗. Subsequently, we carried out separate digestions of the amplified product on F_2 plants with the 17 restriction enzymes listed in Materials and methods. However, no polymorphisms were found. The chromosomal location on the 6RS chromosomal arm was confirmed by amplification in the wheat-rye addition lines at 61°C (Fig. 3).

A genetic map of rye chromosome 6R integrating the *Alt1* locus, the SCAR markers presented above and several isozyme loci (*Aco1*, *Ndh2*, *Est6* and *Est8*) previously mapped (Gallego and Benito 1997), was generated in the AR6-17⊗ population (Fig. 4).

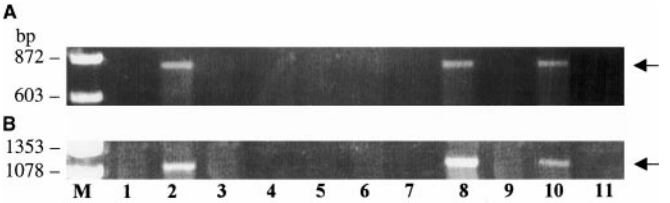

Fig. 3 Chromosomal location of **A** $ScB15_{790}$ and **B** $ScB16_{1145}$ rye fragments. Amplifications were performed with the $OPB15_{790}$ and $OPB16_{1145}$ pair of primers at annealing temperatures of 63°C and 61°C, respectively. *M* molecular-weight marker (*Hae*III digests of ϕX174 phage). *1 Triticum aestivum* "Chinese Spring". *2 Secale cereale* "Imperial". *3–9* disomic addition lines "Chinese Spring"–"Imperial" 1R, 2R, 3R, 4R, 5R, 6R and 7R, respectively. *10* amphiploid CS-I. *11* ditelocentric addition line 6RL. *Arrows* show the located SCAR rye fragments

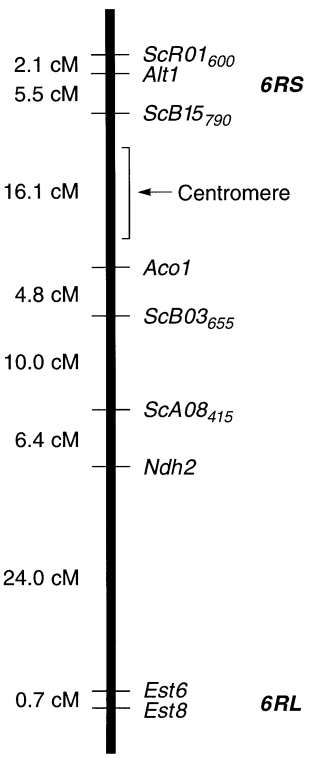

Fig. 4 Linkage map of chromosome 6R showing the *Alt1* locus, the SCAR markers developed in this work and other markers previously mapped (Gallego and Benito 1997). Genetic distances were calculated using the Kosambi function. Map distances are in Morgans (cM)

The two-point linkage studies and the genetic distances obtained showed that $ScB15_{790}$ and $ScR01_{600}$ were the two loci closest to *Alt1* (Table 2).

Discussion

The resistance of root apical meristem to aluminium shock corresponds well with Al tolerance and might be an important part of the Al tolerance mechanism but is not aluminium-tolerant per se. *Alt* genes have been located on chromosome arm 6RS (*Alt1*) and chromosome 4R (*Alt3*) and 3R (*Alt2*) using wheat-rye addition lines and root re-growth tests (Aniol and Gustafson 1984). Rye is the most Al-tolerant triticeae species; however, to-date, only two isozyme loci (*Aco1* and *Ndh2*) and two DNA markers have been shown to be linked to the *Alt1* gene (Gallego and Benito 1997; Gallego et al. 1998).

BSA is an efficient procedure to detect markers linked to target loci. We have found SCAR markers linked to the *Alt1* locus by analyzing 120 10-mers. On the basis of Al tolerance, the homozygous dominant and the heterozygous genotypes were indistinguishable and were bulked together in the F_2. Therefore, only RAPD bands associated in cis (coupling phase) with the dominant Al tolerance allele could be detected. Analysis of F_3 families would have been needed to exclude heterozygous plants. However, we think that it is more efficient to increase the number of RAPD primers than to analyse approximately 1000 F_3 individuals per F_2 family. The number of markers linked to the target loci is similar to previously reported data: Michelmore et al. (1991; 100 primers and three fragments linked to Dm5/8), Schachermayr et al. (1994; 495 primers and three fragments linked to Lr9) and Woeste et al. (1996; 400 primers and two markers linked to hypersensitivity to CLRV). To find markers associated in trans (repulsion phase) with the *Alt1* locus we constructed bulks for the *Lap1* locus (with co-dominant inheritance) that is situated on the same chromosome arm as *Alt1* but was not segregating in the same cross. In this way it was possible to demonstrate that the $OpB15_{790}$ fragment is linked to the *Lap1* locus. The OpB15 oligo was later used to obtain the $OpB15_{790}$ marker in the AR6-17⊗ population, this fragment being linked to the *Alt1* locus (repulsion phase). However, the distance between *Lap1* and *Alt1* has not been estimated because both loci are not segregating simultaneously in the same cross. Therefore, the study of bulks for loci located on the same chromosome arm as the *Alt1* locus gave good results.

The $ScR01_{600}$ and $ScB15_{790}$ markers were located on the 6RS chromosome arm. They closely flank the *Alt1* gene at 2.1 and 5.5 cM, respectively, and can therefore be used in marker-assisted selection programs. We have not found recombinants between $ScB15_{790}$ and the *Alt1* gene (distance = 0.0 cM, Table 2); these loci are in repulsion and in this phase the error of the distance is higher than in the coupling phase. However, the MAPMAKER multipoint analysis (the LOD score was greater than 4) indicates that the best estimation of the distance between them is 5.5 cM. Suppressor and enhancer genes of Al tolerance have been located on the 6BS chromosome arm in wheat (Aniol 1990; Gustafson and Ross 1990) but there is no additional data about the existence of Al tolerance genes located on the short arms of homoelogous group 6 in wheat.

Taking into account that the *Alt1*, *Alt2* and *Alt3* loci are located on the 6RS, 3R and 4R chromosomes, respectively (Aniol and Gustafson 1984), another

Table 2 Two-point linkage analyses between the *Alt1* gene and the molecular markers segregating in AR617⊗. T = Al tolerant plants, t = Al non-tolerant plants. The *Aco1* locus presents two active and co-dominant alleles (*1* and *2*); (*11*) and (*22*) are homozygous plants and (*12*) are heterozygous plants. The remaining isozyme loci present one active allele and one null allele, therefore it is not possible to distinguish plants homozygous for the active allele from heterozygous ones, + = presence, − = absence, r = repulsion, c = coupling

Loci		Distribution of progeny (phenotype)								$\chi^2_{contingency}$	cM
		T, 11	*s, 11*	*T, 12*	*s, 12*	*T, 22*	*s, 22*				
Alt1, $ScA08_{415}$		22	2	42	9	9	13			18.75***	26.9
Alt1, *Aco1*		8	16	43	6	22	2			31.17***	20.7
		T, +		*T*, −	*s*, +		*s*, −				
Alt1, *Est6*	r	58		15	21		3			0.75	–
Alt1, *Est8*	c	53		20	12		12			3.18	–
Alt1, *Ndh2*	c	58		15	11		13			9.94**	35.5
Alt1, $ScB03_{655}$	c	64		9	7		17			31.51***	18.7
Alt1, $ScB15_{790}$	r	46		27	24		0			12.28***	0
Alt1, $ScR01_{600}$	c	72		1	1		23			86.64***	2.1
	11,11	*11,12*	*11,22*	*12,11*	*12,12*	*12,22*	*22,11*	*22,12*	*22,22*		
$ScA08_{415}$, *Aco1*	0	7	17	5	39	7	19	3	0	53.71**	13.0
		11, +	11, −		12, +	12, −	22, +		22, −		
$ScA08_{415}$, *Est6*		14	10		44	7	21		1	12.11**	33.8
$ScA08_{415}$, *Est8*		22	2		38	13	5		17	27.13***	23.8
$ScA08_{415}$, *Ndh2*		24	0		45	6	0		22	71.13***	6.1
$ScA08_{415}$, $ScB03_{655}$		24	0		45	6	2		20	29.71***	8.4
$ScA08_{415}$, $ScB15_{790}$		9	15		40	11	21		1	36.57***	26.7
$ScA08_{415}$, $ScR01_{600}$		21	3		41	10	9		13	15.56**	26.9
Aco1, *Est6*		24	0		39	10	16		8	9.33**	32.9
Aco1, *Est8*		8	16		35	14	22		2	19.92***	28.2
Aco1, *Ndh2*		5	19		40	9	24		0	43.29***	14.8
Aco1, $ScB03_{655}$		1	23		46	3	24		0	80.19***	4.1
Aco1, $ScB15_{790}$		24	0		40	9	6		18	38.41***	16.2
Aco1, $ScR01_{600}$		8	16		43	6	22		2	31.16***	20.7
		+ , +			+ , −	− , +			− , −		
Est6, *Est8*	r	47			32	18			0	10.86***	0
Est6, *Ndh2*	r	52			27	17			1	5.83*	23.3
Est6, $ScB03_{655}$	r	54			25	17			1	5.08*	25.2
Est6, $ScB15_{790}$	c	61			18	9			9	5.33*	–
Est6, $ScR01_{600}$	r	58			21	15			3	–0.77	
Est8, *Ndh2*	c	57			8	12			20	26.29***	22.2
Est8, $ScB03_{655}$	c	56			9	15			17	16.89***	28.8
Est8, $ScB15_{790}$	r	43			22	27			5	3.41	–
Est8, $ScR01_{600}$	c	53			12	20			12	3.19	–
Ndh2, $ScB03_{655}$	c	63			6	8			20	39.97***	15.4
Ndh2, $ScB15_{790}$	r	44			25	26			2	8.37**	26.8
Ndh2, $ScR01_{600}$	c	58			11	15			13	9.96**	35.5
$ScB03_{655}$, $ScB15_{790}$	r	45			26	25			1	10.17**	18.7
$ScB03_{655}$, $ScR01_{600}$	c	64			7	9			17	31.53***	18.7
$ScB15_{790}$, $ScR01_{600}$	r	46			24	27			0	12.29***	0

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

strategy to obtain RAPD markers linked to these genes is to compare the DNA fragments amplified in wheat (CS), the amphiploid (CS-I), and the corresponding ditelocentric wheat-rye addition lines. Rye RAPD markers present in the amphiploid (CS-I), and in the corresponding disomic or ditelocentric addition line, but absent in wheat (CS), can be selected. Using this system we identified 15 RAPD markers on these chromosomes and linked two of them to the *Alt1* locus. One of these fragments is $OpR01_{600}$, the same one that we have obtained using BSA analyses in this work, and another is the $OpA08_{415}$ fragment indicated on the map of the 6R chromosome (Fig. 4) (Gallego et al. 1998).

The availability of genetic markers linked to the *Alt* rye genes is of interest in breeding programs, for the selection of plants with a high number of Al tolerance alleles. For example, we have obtained F_2 progenies segregating simultaneously for the *Alt1* and *Alt3* loci (segregation 15:1) and are currently selecting homozygous plants with four Al-tolerant alleles.

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