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Chromosomal location of PCR fragments as a source of DNA markers linked to aluminium tolerance genes in rye

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Abstract To identify and locate rye DNA sequences homologous to three wheat c-DNAs (*wali1*, *wali2* and *wali5*) whose expression is induced by aluminium (Al) stress, we designed three pairs of specific primers. They were used in the amplification of genomic DNA from wheat-rye disomic addition lines. The *wali2* pair of primers amplified a 878-bp rye DNA fragment (*rali2*) located on chromosomes *4R* and *7R* that showed 79.37% homology with the corresponding wheat c-DNA. RAPD fragments were also used as genetic markers. We located 22 different RAPDs distributed on 11 different rye chromosome arms using wheat-rye disomic and ditelocentric addition lines. Thirteen of these markers were located on the chromosomes 3R, 4R and 6R, which also carry aluminium-tolerance genes. The $OPA08_{415}$ and $OPR01_{600}$ RAPD markers, located on the *6RL* and *6RS* chromosome arms, respectively, were converted to SCAR markers ($SCA08_{415}$ and $SCR01_{600}$) and linked to *Alt1* gene ($SCR01_{600}$-2.1 cM-*Alt1*-33.5 cM-$SCA08_{415}$). We propose that the chromosomal location of RAPDs and SCARs using wheat-rye addition lines is a source of DNA markers linked to aluminium-tolerance loci and offers a valuable strategy in marker-assisted selection for the introgression of tolerance genes in wheat.

Key words Chromosomal location · Aluminium tolerance · RAPDs · *Secale cereale* L.

Introduction

Aluminium (Al) is one of the most limiting factors of crop productivity in acid soils (Haug 1984). The phenotypic effect of Al toxicity is the inhibition of root elongation and subsequent inhibition of plant growth (Ryan et al. 1993). In spite of the large amount of research that has been conducted on Al toxicity, there is no consensus on the physiological mechanism of Al toxicity or tolerance in plants (Taylor 1991). Current hypotheses propose that the target of Al toxicity could be the root cap (Bennet et al. 1987), the plasma membrane (Kochian et al. 1991) or the direct binding of Al to the plant DNA (Matsumoto et al. 1977). Moreover, little is known about the plant molecular response to Al. Attempts were made to identify proteins in wheat roots that are induced by Al treatment using two-dimensional gel electrophoresis. Several protein "spots" induced by Al were identified, but none was further characterised (Delhaize et al. 1991; Picton et al. 1991; Rincon and Gonzáles 1991). Recently, seven different cDNAs, termed *wali1*-*wali7*, whose expression is induced by Al stress, were isolated from root tips of Al-treated wheat plants (Snowden and Gardner 1993; Richards et al. 1994).

Genetic variation in the response to Al has been found among both plant species and crop cultivars (Foy and Fleming 1978; Aniol and Gustafson 1984). A differential response of wheat to Al was reported, and several attempts were made to determine the inheritance of this character. Major genes controlling wheat tolerance to Al were located on chromosomes of the A and D genomes of hexaploid wheat (Aniol 1990), but the physiological processes controlled by these genes are still unknown. Most of the observed variability with respect to Al tolerance in wheat could be explained by two or three genes affecting the same character, with complete dominance in each locus. Studies on Al tolerance in the aneuploid series of the wheat cultivar 'Chinese Spring' (CS) revealed that genes controlling this character are located on the short arm of chromosome 5A and on the long arm of chromosome 2D and 4D (Aniol and Gustafson 1984; Aniol 1995). Genes for Al tolerance have been located also in chromosome 5D

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in a relatively tolerant wheat cultivar (Prestes et al. 1975).

Of the cultivated species of Titiceae the genus *Secale* has one of the most efficient group of genes for Al tolerance. Aluminium tolerance genes (*Alt*) were located in rye chromosomes 3R, 4R and 6R (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, DNA markers linked to genes related to aluminium tolerance in rye have not been described to date.

The purpose of this investigation presented here was to locate polymerase chain reaction (PCR) DNA fragments using wheat-rye addition lines in order to develop a source of DNA markers that could be linked to aluminium tolerance genes or to other important agronomic traits.

Materials and methods

Plant material

To study the genetic control of random amplified polymorphic DNA (RAPD) markers we used plants from one F_2 between the rye cultivar 'Ailés' (A) and the rye inbred line Riodeva (R) (AR⊗) and one backcross between the same lines (AR × R). To map the *Alt* loci and the linked molecular markers we used two F_2 progenies (AR6.17⊗ and AR1.13⊗) from crosses between Al-tolerant rye plants ('Ailés') and Al-non-tolerant rye plants ('Riodeva') which segregate separately for *Alt1* and *Alt3* loci, located on 6R and 4R chromosomes, respectively (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.

Aluminium tolerance screening test

The Al tolerance test was done using the nutrient-culture pulse method (Aniol 1984).

RAPD analysis

DNA was extracted according to Dellaporta et al. (1983). RAPD reactions similar to those described by Williams et al. (1990) were performed with minor modifications. Eighty 10-mer primers (Kits A, B, F and R from Operon Technologies) were used in this study. The PCR volume was 25 µl and contained 60 ng template DNA, 100 µM of each dNTP, 5 pmoles primer and 0.4 U Biotaq DNA polymerase (Bioprobe Systems) in 1 × reaction buffer (20 m*M* TRIS-HCl, pH 8.55, 16 m*M* $(NH_4)_2SO_4$, 2.5 m*M* $MgCl_2$, 150 mg/ml BSA). Amplifications were done in a DNA Thermal Cycler 480 (Perkin Elmer) and a PTC-100 (MJ Research) using the following thermocycling program: a preliminary step of 4 min at 94°C, 45 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C, with a final step of 4 min at 72°C. Amplification 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 threefold before the score of the results.

The RAPD markers were designated, as follows: the first three letters and two numbers refer to the kit and primer number (Operon Technologies) used to amplify the RAPD marker. The subscript number refers to the size (bp) of the amplified RAPD product.

Southern analysis

Amplified products were transferred onto positively charged membranes (Boehringer Mannheim) under alkaline conditions (Sambrook et al. 1989). Some of the PCR fragments were selected as probes and subsequently extracted from the agarose gels. Labeling was carried out with digoxigenin (Dig-dUTP) by the random-primer method (Boehringer Mannheim).

Hybridizations were performed under standard conditions at 65°C in a solution containing 5 × SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine and 0.02% SDS. After hybridization, membranes were washed twice in 2 × SSC and 0.1% SDS for 15 min at 68°C and twice in 0.1 × SSC and 0.1% SDS for 15 min at 68°C and then subjected to detection with the DIG luminescent detection kit (Boehringer Mannheim).

Cloning and sequencing of RAPD products

PCR amplification products from rye were excised from the gel and ligated to the pGEM-T vector (Promega). Transformation of *E. coli* host DH5α resulted in recombinant clones containing the amplification fragment. The identity of the cloned product was verified by a digest with the restriction enzymes *Apa*I and *Sac*I (Amersham) and subsequent Southern analysis.

Double-strand sequencing (DIG Taq DNA Sequencing kit, Boehringer Mannheim) was done by the non-radioactive PCR method using the M13/pUC forward and reverse sequencing primers.

Sequence characterised amplified region (SCAR) design and analysis

Two pair of 21-mer oligonucleotides were designed for the cloned amplification products $OPA08_{415}$ and $OPR01_{600}$ to be used as SCAR primers. Their sequences contained the original 10 bases of the RAPD primers plus the subsequent 11 internal bases (Table 1).

Amplifications of genomic DNA were done in 25-µl volumes containing 60 ng template DNA, 100 µ*M* of each dNTP, 15 pmoles of each primer, 2.5 m*M* $MgCl_2$ and 1 U of Amplitaq DNA polymerase, Stoffel fragment (Perkin Elmer), in 1 × reaction buffer (100 m*M* TRIS-HCl, pH 8.3, 100 m*M* KCl). Amplifications were carried out in a PTC-100 thermal cycler (MJ Research) with the following thermocycling programme: a preliminary step of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 59°–69°C and 6 min at 72°C with a final step of 4 min at 72°C. PCR samples were stored at 4°C until their resolution by electrophoresis on 1.5% agarose gels stained with ethidium bromide (Sambrook et al. 1989).

Some of the amplification fragments were digested with restriction endonucleases to look for variability in the progenies. A total of 17 restriction enzymes (Amersham) were utilised: *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).

Methodology to identify rye DNA fragments homologous to *wali* cDNAs

In order to locate those rye DNA fragments homologous to wheat cDNAs whose expression is induced by Al stress (*wali1* to *wali7*) (Snowden and Gardner 1993; Richards et al. 1994), three pairs of specific primers were designed from *wali1*, *wali2* and *wali5* cDNA

Table 1 Sequences of the primer pairs utilised in this study

Primer	Sequence (5′ → 3′)	T_1[a]	T_2[a]
$OPA08_{415}$-F	GTGACGTAGGGTGCGTATGCA[b]	69°C	69°C
$OPA08_{415}$-R	GTGACGTAGGCAGGCTGTAAG[b]		
$OPR01_{600}$-F	TGCGGGTCCTGTCACCCGGTA[b]	59°C	63°C
$OPR01_{600}$-R	TGCGGGTCCTCCATAGTGAGT[b]		
wali1-F	CAGGTCGCCGCCGTGGTCGT	60°C	–
wali1-R	ATTCATCACTCCATCCATAG		
wali2-F	TCCGACAAGAAGCGTAGCAG	60°C	60°C
wali2-R	CTCACTACGAACGACTACTT		
wali5-F	GAGCATTCTACCACCGCACC	60°C	–
wali5-R	CTGAACAACAAGCAACACCA		

[a] T_1 and T_2 are the annealing temperatures utilised for the amplification of the fragments in the location and mapping experiments, respectively
[b] The underlined sequence represents the sequence of the progenitor RAPD primer

sequences (Table 1). Genomic DNA of wheat (CS), rye (I), the seven wheat-rye addition lines (1R–7R) and the amphiploid wheat-rye (CS-I) were amplified using the three different specific pairs of primers at an annealing temperature of 60°C. Southern experiments were subsequently carried out to identify those amplified rye fragments that were homologous to the wheat *wali* genes, using the original wheat clones as probes (*wali1*, *wali2* and *wali5* clones kindly supplied by Dr. R. C. Gardner and Dr. K. Richards). Those PCR fragments showing hybridisation signals were sequenced to determine their degree of homology with the original clones.

Genetic mapping

Linkage analysis was performed on F_2 segregation data using the MAPMAKER 3.0 computer programme (Lander el al. 1987). Genetic distances were calculated using the Kosambi function (Kosambi 1944).

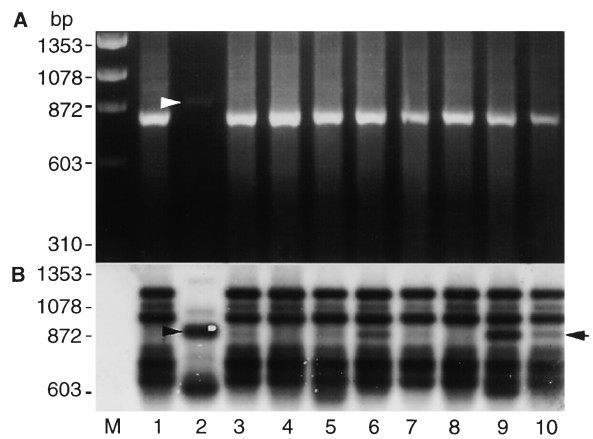

Fig. 1A, B Chromosomal location of a rye fragment (*rali2*) with homology to *wali2* cDNA. **A** Amplification of rye genomic DNA using *wali2* primers, **B** hybridisation of the *wali2* c-DNA to a Southern blot of the agarose gel shown in panel **A**. *M* Molecular-weight marker (*Hae*III digests of 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. *Arrows* show the located rye fragment (*rali2*)

Results

Chromosomal location of DNA fragments homologous to *wali* cDNAs

Several fragments were amplified from 'Imperial' rye using the pair of primers designed from the sequence of *wali1*. While two of these fragments (that we named *rali1.1* and *rali1.2*) showed hybridisation signals with *wali1*, their sequences revealed a very low homology to *wali1*. Moreover, *rali1.1* and *rali1.2* were products of the amplification with only the wali1-F primer, whose sequence was found in both extremes of these fragments. These PCR fragments did not appear in any of the addition lines.

The amplification with the *wali2* primers in 'Imperial' rye produced a fragment of 878 bp (*rali2*) located on chromosomes 4R and 7R by hybridisation with the wheat probe (Fig. 1). The *rali2* sequence showed a 79.37% homology with *wali2* (Fig. 2). The *rali2* fragments did not show variability in the F_2 progeny AR1.13⊗ segregating 3 : 1 (tolerant : non-tolerant) for the Al tolerance gene *Alt3* located on chromosome 4R. The digestion of the *rali2* fragments with 17 different restriction endonucleases did not reveal variability in this progeny.

The *wali5* specific primers amplified several rye fragments but none of these DNA segments hybridised with the *wali5* probe.

Chromosomal location of RAPD fragments

We carried out RAPD amplifications on genomic DNA of wheat (CS), rye (I), the seven wheat-rye addition lines (1R–7R) and the amphiploid wheat-rye (CS-I) with 60 different 10-mer oligonucleotides (Kits A,

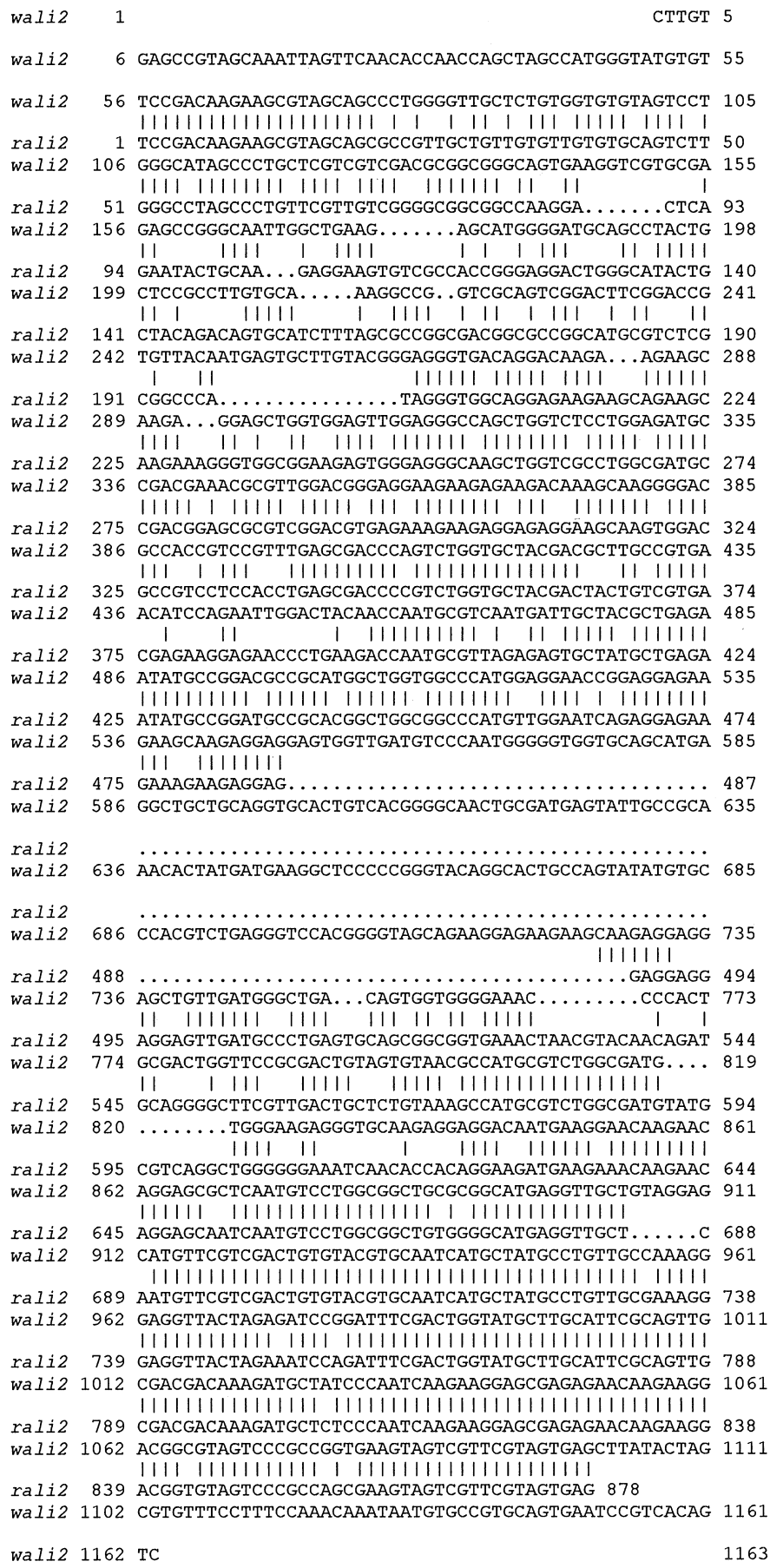

Fig. 2 Alignment of the nucleotide sequences of the *rali2* fragment and the wheat *wali2* c-DNA. The conserved nucleotides are marked with *vertical bars.* Gaps (represented by *dots*) were introduced into the sequences to maximise the alignment. The *wali2* sequence was obtained from the GenBank data base, L11880 (Snowden and Gardner 1993)

B and F from Operon Technologies). Out of 137 amplified fragments in rye (1–7 bands per primer), 46 (33%) presented the same electrophoretic mobility of some of the wheat fragments and 21 (15%) could be located, being distributed all along the seven rye chromosomes. These fragments were assigned to chromosome arms by amplification on the corresponding ditelocentric addition lines. The chromosome arm locations and the sizes (bp) of the different fragments are shown in Table 2. The 70 (51%) remaining fragments did not present the same electrophoretic mobility of any wheat DNA segment, but they did not appear in any of the seven addition lines.

Previously located markers were used as specific probes in hybridisations with the amplified products (Southern blots) and with genomic DNA (Dot blots) in order to confirm the above chromosomal location results.

Figure 3A shows amplification results with the OPA08 10-mer primer. The direct observation of the amplification products revealed 1 rye RAPD fragment located on chromosome 6R. The identities of fragments of the same size (415 bp) amplified in rye (I), the 6R addition line and the amphiploid (CS-I) were verified by hybridisations with the rye fragment used as a probe in a Southern blot with the amplification products (Fig. 3B). Furthermore, dot blot assays with genomic DNA from rye (I), wheat (CS), the seven wheat-rye addition lines and the amphiploid were performed to verify the results obtained above. These experiments were done with some rye RAPD bands obtained with the primers OPA01, OPA02, OPA05, OPA10, OPA13 and OPB04. The probes hybridised exclusively to the DNA from rye, the amphiploid and the corresponding wheat-rye addition line. Thereby, we were able to confirm the chromosomal location results in all the situations analysed by means of the hybridisation results.

Using the dot blot technique we tried to locate 10 fragments that could not be assigned to any rye chromosome. These localisations were not possible because the hybridisation signal appeared in all dots.

Table 2 RAPD rye fragments located in this work with their chromosomal location

RAPD fragment	Chromosomal arm	RAPD fragment	Chromosomal arm
$OPA17_{466}$	1RL	$OPF14_{370}$	4RL
$OPF01_{263}$	2RS	$OPA01_{570}$	5RS
$OPF08_{311}$	2RL	$OPA20_{564}$	5RS
$OPF19_{221}$	2RL	$OPR01_{600}$	6RS
$OPB17_{315}$	3RS	$OPA02_{304}$	6RL
$OPB18_{330}$	3RS	$OPA08_{415}$	6RL
$OPF01_{570}$	3RS	$OPA13_{230}$	6RL
$OPB04_{325}$	3RL	$OPB15_{365}$	6RL
$OPB07_{276}$	3RL	$OPA10_{370}$	7RL
$OPB16_{175}$	3RL	$OPF01_{476}$	7RL
$OPA05_{385}$	4RS	$OPF05_{257}$	3RS and 6RL

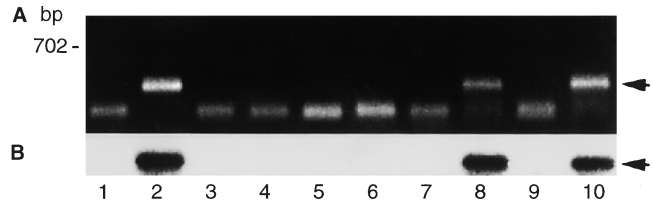

Fig. 3A, B Chromosomal location of the $OPA08_{415}$ RAPD rye fragment. **A** Amplification of genomic DNA using the OPA08 primer, **B** hybridisation of the 415-bp 'Imperial' rye fragment to a Southern blot of the agarose gel shown in panel **A.** *1 Triticum aestivum* 'Chinese Spring', *2 Secale cereale* 'Imperial', *3–9* disomic addition lines 'Chinese Spring'-'Imperial' 1R, 2R, 3R, 4R, 5R, 6R y 7R, respectively, *10* amphiploid CS-I. *Arrows* show the located rye fragment ($OPA08_{415}$)

The *Alt1* gene was located on the 6RS chromosome arm in rye (Aniol and Gustafson 1984) using wheat-rye addition lines. To find RAPD markers specifically located on this chromosome arm we compared the amplification patterns of wheat (CS), the amphiploid wheat-rye (CS-I) and the 6R and 6RL wheat-rye addition lines (the 6RS addition line was not available) with 20 additional 10-mer oligonucleotides (Kit R from Operon Technologies). Rye RAPD markers present in the amphiploid (CS-I) and 6R addition line and absent in the wheat (CS) and 6RL addition line were selected. With this strategy we isolated the $OPR01_{600}$ fragment located on 6RS (Table 2).

Inheritance of RAPD markers in rye

The amplification patterns of 4 different primers (OPA02, OPA04, OPA05 and OPA08) were analysed in the backcross AR × R, and the profiles generated with OPB03, OPB15 and OPR01 primers were analysed in the F_2 AR⊗ progeny. These primers were selected because they produced segregating fragments. The number of fragments amplified with each primer ranged from 1 to 5, but only 1 DNA segment segregated with each primer. The observed segregation ratios are shown in Table 3.

Conversion of a RAPD to a SCAR

Plants from the F_2 AR6.17⊗ that segregated 3:1 (tolerant : non-tolerant) for the *Alt1* gene located on chromosome arm 6RS were obtained (Gallego and Benito 1997). The primers that yielded RAPD fragments located on 6RL ($OPA02_{304}$, $OPA08_{415}$, $OPA13_{230}$, $OPB15_{365}$ and $OPF05_{257}$) and 6RS ($OPR01_{600}$) chromosome arms were selected and used to amplify in this progeny, in order to find markers linked to the *Alt1* locus. The results of the RAPDs were uniform in all individuals, with the exception of the $OPR01_{600}$ fragment. The $OPA02_{304}$, $OPA13_{230}$ and $OPB15_{365}$ loci were

Table 3 Segregation of rye RAPD markers analysed in one backcross (BC) and one F_2

Cross		RAPD marker	Segregation		χ^2 (1:1)	χ^2 (3:1)
			Presence (+)	Absence (−)		
AR × R	BC	$OPA02_{304}$	44	46	0.02	–
		$OPA04_{325}$	42	48	0.2	–
		$OPA05_{280}$	48	42	0.2	–
		$OPA08_{330}$	69	30	4.35*	1.48
AR⊗	F_2	$OPB03_{655}$	71	26	–	0.17
		$OPB15_{790}$	70	27	–	0.41
		$OPR01_{600}$	73	24	–	0.003

*$P < 0.05$

absent whilst $OPA08_{415}$ and $OPF05_{257}$ were present in all plants of the F_2 offspring. The $OPR01_{600}$ marker segregated in this progeny (Fig. 4A) and behaved as if it were linked to the *Alt1* locus at 2.1 cM.

The $OPA08_{415}$ and $OPR01_{600}$ products were sequenced in order to design specific SCAR primers for these fragments (Table 1). With this approach the unique and robust amplification of the fragments of interest ($SCA08_{415}$ and $SCR01_{600}$) could be obtained. To optimise the reaction conditions for each pair of specific primers we used genomic DNA from the parents and offspring of the F_2 under study (AR6.17⊗) as template in amplifications with different annealing temperatures and primer concentrations. We determined that 15 pmoles the primer pairs $OPA08_{415}$ and $OPR01_{600}$ in amplifications carried out at annealing temperatures of 69°C and 63°C, respectively, provided the optimum conditions. Under these conditions only the DNA products of interest were amplified.

Amplification with the $OPA08_{415}$ pair of primers at 69°C yielded only the DNA product of interest in all plants of the progeny, whereas amplification at 70°C resulted in the absence of the $SCA08_{415}$ fragment. Subsequently, separate digestions of the $SCA08_{415}$ product on F_2 plants were done using the 17 restriction enzymes listed in the Materials and methods. The $SCA08_{415}$ fragment appeared as a codominant marker with a 1:2:1 segregation ratio in *Rsa*I digests (Fig. 5). The linkage analysis showed that this marker behaved as being linked to the *Alt1* locus at a distance of 33.5 cM. The observed variability for the $OPR01_{600}$ RAPD fragment was also present in the respective SCAR fragment, and individual SCAR data (presence or absence) were totally coincident with the RAPD scores (Fig. 4B). $SCR01_{600}$ was linked to the *Alt1* locus at 2.1 cM (Table 4).

Thus we have confirmed the location of $SCA08_{415}$ and $SCR01_{600}$ on the 6RL and 6RS chromosome arms by SCAR amplification on genomic DNA of wheat (CS), rye (I), the seven wheat-rye addition lines (1R–7R), the amphiploid wheat-rye (CS-I) and the 6RL ditelosomic wheat-rye (CS-I) addition lines (Fig. 6).

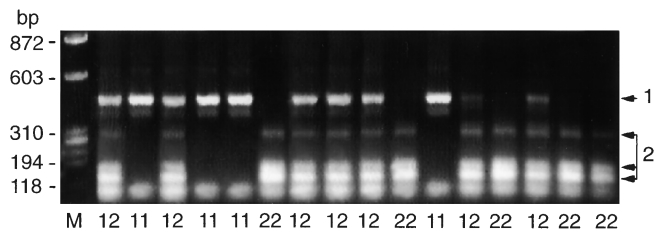

Fig. 5 Segregation of $SCA08_{415}$ locus. Amplification was performed with the specific $OPA08_{415}$-F and $OPA08_{415}$-R primers (69°C) on 16 individuals from the F_2 progeny segregating for the *Alt1* locus. The amplification products were digested with *Rsa*I to reveal length polymorphisms. The different homozygotes and heterozygotes are indicated at the *bottom* of Fig. *1* Undigested PCR product, *2* fragments that appear after digestion with *Rsa*I. *M* Molecular-weight marker (*Hae*III digests of ϕX174 phage)

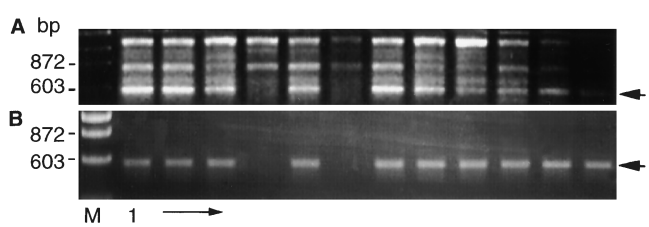

Fig. 4A, B Segregation of the 600-bp fragment amplified with **A** the OPAR01 10-mer (RAPD) and **B** the OPAR01 21-mer specific pair of primers (SCAR) at an annealing temperature of 63°C on 12 individuals from the F_2 progeny segregating for the *Alt1* locus. *M*: Molecular-weight marker (*Hae*III digests of ϕX174 phage)

Discussion

The analysis of products amplified with *wali* primers in rye identified a fragment (*rali2*) with high similarity to *wali2*. However, the chromosomal location results indicated the existence of two *rali2* fragments of the same size and homologous to *wali2*, one located on chromosome 4R and the other on 7R. Since *rali2* was present in two different loci, it was difficult to find plants variable at both loci simultaneously. This is probably the reason why polymorphism could not be found for this

Table 4 Multipoint linkage analyses between *Alt1*, $SCA08_{415}$ and $SCR01_{600}$ loci

Loci	Distribution of progeny (phenotype)[a]						χ^2	Distance (cM)
Alt1, $SCA08_{415}$	T,11	t,11	T,12	t,12	T,22	t,22		
	22	2	42	9	9	13	18.75*	33.5
Alt1, $SCR01_{600}$	T, +		T, −	t, +		t, −		
	72		1	1		23	86.64*	2.1
$SCA08_{415}$, $SCR01_{600}$	11, +	11, −	12, +	12, −	22, +	22, −		
	21	3	41	10	9	13	15.56*	34.5

[a] T, Al-tolerant plants; t, Al-non-tolerant plants. The $SCA08_{415}$ locus presented two codominant alleles (1 and 2); 11 and 22 are homozygous plants and 12 are heterozygous plants. The $SCR01_{600}$ locus showed a dominant allele (+ , presence) and a null allele (− , absence)
* $P < 0.001$

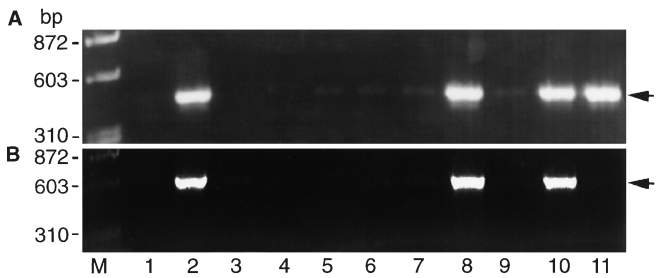

Fig. 6A, B Chromosomal location of **A** $SCA08_{415}$ and **B** $SCR01_{600}$ rye fragments. Amplifications were performed with the $OPA08_{415}$ and $OPR01_{600}$ pair of primers at an annealing temperature of 69°C and 59°C, respectively. *M* Molecular-weight marker (*Hae*III digests of ϕX174 phage). *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

fragment. It should be noted that *wali2* is the only mRNA that shows a differential expression pattern between Al-tolerant and Al-sensitive wheat genotypes (Snowden and Gardner 1993). The absence of variability for *rali2* has prevented linkage analysis between this fragment and the *Alt3* locus segregating in the F_2 offspring. The relationship found between the *Alt3* locus and the *rali2* fragment is that they were located on 4R chromosome. The Al-tolerance genes and the mRNAs whose expression is induced by Al stress could be different genes and not necessarily related. Nevertheless, the existence of a relationship between the *rali2* and the *Alt3* locus could be also possible.

The purpose of the experiments on chromosomal location of RAPDs was to obtain a pool of RAPD markers distributed all along the rye chromosome arms and, subsequently, to convert these RAPDs in SCARs by designing pairs of specific primers for each DNA fragment located. RAPD markers have been studied in nullisomic-tetrasomic lines and in wheat-barley addition lines (Devos and Gale 1992). These authors argue several problems that devalue the use of RAPDs as genetic markers for the construction of linkage maps in wheat: non-homoelogous, non-dose responsive and dominant behaviour. We have overcame the problems inherent to these markers by obtaining a pool of RAPD markers which could be transferred into SCAR markers that could be linked to interesting agronomic traits. In this preliminary study, we have located 22 RAPD markers using this method and have confirmed six chromosomal location results by means of hybridisation in dot blot assays with genomic DNA. PCRs with specific primers have been used in wheat-barley addition lines to locate the α-amylase gene family in barley (Weining and Langridge 1991) and to amplify repeated DNA sequences in rye (Rogowsky et al. 1992). These PCR products can be mapped as standard genetic markers.

It should be noted that 15 out of the 22 located fragments were on chromosomes 3R ($OPB04_{325}$, $OPB07_{276}$, $OPB16_{175}$, $OPB17_{315}$, $OPB18_{330}$, $OPF01_{570}$ and $OPF05_{257}$), 4R ($OPA05_{385}$ and $OPF14_{370}$) and 6R ($OPA02_{304}$, $OPA08_{415}$, $OPA13_{230}$, $OPB15_{365}$, $OPF05_{257}$ and $OPR01_{600}$). Aniol and Gustafson (1984) described the existence of *Alt* loci on these chromosomes. Therefore, these markers are being studied in F_2s with respect to their linkage to *Alt* genes. Additionally, the remaining RAPD markers can be useful in further studies.

There is a high percentage of rye RAPD fragments (51%) which were not found in any of the wheat-rye addition lines. This fact could be attributed to the existence of variability in 'Imperial' rye or to primer competition for the homologous rye and wheat target sequences. The dot blot technique was not useful in locating these fragments because they probably contain repetitive interspersed sequences (Williams et al. 1990) that cross-hybridise with the DNA of wheat and rye chromosomes in all the addition lines, thereby preventing their location on specific chromosomes.

The analysis of the F_2 AR⊗ and the backcross AR × R indicated that RAPDs behave in a dominant Mendelian mode. Similar results have been previously described in rye (Phillip et al. 1994; Loarce et al. 1996). However, we found a 3:1 segregation in

the backcross that could be explained if 2 independent fragments with the same size were segregating simultaneously.

We have obtained two SCAR fragments ($SCR01_{600}$ and $SCA08_{415}$) linked to the *Alt1* locus on the 6R chromosome. The $SCR01_{600}$ fragment has been narrowly linked to *Alt1* at a distance of 2.1 cM, and it could be used as a starting point for the analysis and grouping of progenies segregating for this specific Al-tolerance locus. Therefore, another strategy to obtain RAPD markers linked to a previously located gene is to compare the DNA fragments amplified in wheat (CS), amphiploid (CS-I) and the corresponding ditelocentric wheat-rye addition lines. Two RFLPs linked to the wheat Alt_{BH} gene located on the 4DL chromosome arm (Riede and Anderson 1996) and two microsatellite markers linked to the *als1-1* and *als4* genes (Al-sensitive mutants) located on the chromosome 5 of *Arabidopsis thaliana* (Larsen et al. 1996) have been reported. $SCR01_{600}$ and $SCA08_{415}$ are the first molecular markers linked to a rye *Alt* gene. Therefore, the results obtained in this work indicate that this methodology can produce positive linkage relationships with genes of agronomic interest.

The development of SCARs offers additional advantages over RAPDs. The SCAR primers amplify only the specific RAPD fragment of interest, allowing their easy transformation into codominant markers and their transference to other progenies and populations different from those where they were obtained. Currently, the transformation into SCAR of the remaining RAPD fragments that we located on 3R, 4R and 6R chromosomes is being done in order to increase the number of markers linked to the *Alt1* locus and to obtain makers linked to other *Alt* loci. Moreover, these SCAR markers could be used to detect rye segments in a wheat background and to characterise their composition (Iqbal and Rayburn 1995).

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