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Development of PCR markers for the wheat leaf rust resistance gene *Lr47*

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Abstract The leaf rust resistance gene *Lr47* confers resistance to a wide spectrum of leaf rust strains. This gene was recently transferred from chromosome 7 S of *Triticum speltoides* to chromosome 7 A of hexaploid wheat *Triticum aestivum*. To facilitate the transfer of *Lr47* to commercial varieties, the completely linked restriction fragment length polymorphism (RFLP) locus *Xabc465* was converted into a PCR-based marker. Barley clone ABC465 is orthologous to the type-I wheat sucrose synthase gene and primers were designed for the conserved regions between the two sequences. These conserved primers were used to amplify, clone and sequence different alleles from *T. speltoides* and *T. aestivum*. This sequence information was then used to identify the *T. speltoides* sequence, detect allele-specific mutations, and design specific primers. Cosegregation of the PCR product of these primers and the *T. speltoides* chromosome segment was confirmed in four backcross-populations. To complement this dominant marker, a cleavage amplified polymorphic sequence (CAPS) was developed for the 7 A allele of *Xabc465*. This CAPS marker is useful to select homozygous *Lr47* plants from F_2 or backcross-F_2 segregating populations, and in combination with the *T. speltoides*-specific primers is expected to facilitate the deployment of *Lr47* in new bread wheat varieties.

Key words Wheat · *Triticum speltoides* · Marker-assisted selection · Leaf rust · Resistance gene · PCR markers · Sucrose synthase

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Introduction

Introgression of resistance genes has been the method of choice for controlling rust diseases in wheat (McIntosh et al. 1995). The utilization of these genes has minimized the application of fungicides, contributing to reduce both environmental contamination and production costs. Therefore, it is not surprising that rust resistance genes were frequent targets in restriction fragment length polymorphism (RFLP) mapping studies in wheat (Schachermayr et al. 1994, 1995; Autrique et al. 1995; Dedryver et al. 1996; Nelson et al. 1997; Sun et al. 1997; Dubcovsky et al. 1998).

One recent example is the RFLP characterization of a chromosome segment carrying leaf rust resistance gene *Lr47*. This gene is located within an interstitial segment of *Triticum speltoides* (Taush) Gren. chromosome 7S#1 (Friebe et al. 1996) transferred to the short arm of chromosome 7 A of bread wheat translocation line T7AS-7S#1S-7AS·7AL (Dubcovsky et al. 1998). From telomere to centromere, the short arm of chromosome 7 A from this translocation line has a 40-cM segment of 7AS, a 20–30-cM interstitial segment of the short arm of chromosome 7S#1, and a 2–10-cM segment of chromosome 7AS. Any of the RFLP markers located within the 7S#1 chromosome segment can be used to monitor the introgression of *Lr47* because homoeologous chromosomes do not recombine in polyploid wheat in the presence of the wild-type *Ph1* locus (Riley and Chapman 1958). This is a valuable breeding objective because this gene confers resistance to a wide spectrum of leaf rust strains (Dubcovsky et al. 1998).

Although RFLP markers can be used to accelerate the deployment of *Lr47* in commercial bread wheat varieties, the use of these markers requires a large amount of DNA and the use of hybridization techniques. An attractive alternative for breeding programs is the conversion of RFLP markers to PCR-based allele-specific markers that require less DNA, are technically easier and faster than RFLP, and are also less expensive (Shattuck-Eidens et al. 1991; Paran and Michelmore 1993; Niewöhner et al. 1995).

Table 1 Primer names, sequences and cycling conditions

Function	Name	Sequence	Touchdown	Additional cycles
Non-specific	**P7S3R** **P7S3L**	5'-AACTGGAAGCTGTACTCAGAG-3' 5'-GATGAACAATATGGGCAGG-3'	15 cycles[a] Annealing T: 60 → 46°C	30 cycles[a]. Annealing T: 46°C Extension: 72°C, 7'
T. speltoides-specific	**PS10R** **PS10L**	5'-GCTGATGACCCTGACCGGT-3' 5'-TCTTCATGCCCGGTCGGGT-3'	7 cycles Annealing T: 70 → 64°C	35 cycles. Annealing T: 63°C Extension: 72°C, 7'
CAPS	**PS10R** **PS10L2**	5'-GCTGATGACCCTGACCGGT-3' 5'-GGGCAGGCGTTTATTCCAG-3'	No touchdown	40 cycles. Annealing T: 55°C Extension: 72°C, 7'

[a] Each cycle includes a denaturation step at 94°C for 30", an annealing step at the indicated temperature for 30", and an extension step at 72°C for 30"

Development of PCR-based allele-specific markers in polyploid species is more complex than in diploid species because PCR-reactions can result in the amplification of multiple fragments of similar size from more than one genome. For this reason, few examples are available in hexaploid wheat (Mohler and Jahoor 1996; Schachermayr et al. 1997; Robert et al. 1999). One possible strategy to overcome this problem is to sequence different homoeoalleles and design specific primers for the targeted allele. The aim of the present work was to use this strategy to convert the RFLP marker ABC465 for the leaf rust resistance gene *Lr47* into a PCR marker.

Materials and methods

Plant material

Bread wheat line T7AS-7S#1S-7AS·7AL carrying the *T. speltoides* 7 S interstitial translocation in the short arm of chromosome 7 A (Dubcovsky et al. 1998) was the source of DNA for the *T. speltoides* sequences and the source of *Lr47* for the marker-assisted selection program. *T. speltoides* accessions DV562 and DV563 were kindly provided by Dr. J. Dvorak (University of California Davis, Calif., USA). A set of 166 backcross plants obtained from crosses between T7AS-7S#1S-7AS·7AL and varieties Yecora Rojo, RSI5, Express, and breeding line UC1041 were used to test the performance of *T. speltoides*-specific primers and to confirm the absence of recombination within the 7 S chromosome segment. Chinese spring nullisomic-tetrasomic lines N7AT7B, N7BT7 A and N7DT7B (Sears 1954) were used to assign amplified bands to homoeologous chromosomes. A diverse set of wheat varieties, including Anza, Attila, Avalon, Avocet, Brooks, Cavalier, Chinese Spring, Columbus, Cuyama, Express, Glupro, Hyack, Klasic, Len, Madsen, Marne, North Dakota 683, Opata, Pavon, RSI5, Sunfield, Tadinia, VPM1, Yolo and Yecora Rojo, was analyzed to validate the cleavage amplified polymorphic sequence (CAPS) marker for the 7 A allele.

RFLP procedures

Two methods of genomic DNA isolation were used to test *T. speltoides*-specific primers. The first one (Dvorak et al. 1988) was a large-scale DNA isolation procedure that resulted in high-quality DNA and was used to adjust the initial PCR conditions for amplification. The second one (Wining and Langridge 1991) was a small-scale and fast DNA isolation procedure that is more appropriate for marker-assisted selection programs. Procedures for Southern blotting and hybridization were previously described (Dubcovsky et al. 1994). Clone ABC465 was kindly provided by A. Kleinhofs (Washington State University).

Cloning and sequencing

Clone ABC465, selected to develop the PCR-based markers, was sequenced to complete the partial sequence previously produced by T. Blake (GrainGenes DNA L43955). A BLAST search of the complete sequence showed high levels of identity with the 3'-end of the wheat sucrose synthase type-1 cDNA (clone pST8, Maraña et al. 1988) and lower levels of identity with the wheat sucrose synthase type-2 cDNA (clone pST3, Maraña et al. 1988). The ABC465 clone hybridized with a 6.5-kb *Bam*HI restriction fragment from *T. speltoides* that was not present in any of the three genomes of bread wheat (Dubcovsky et al. 1998).

The presence of more than one sucrose synthase related-sequence per genome was suggested by the complex RFLP pattern obtained when hexaploid wheat DNA was hybridized with ABC465 (Dubcovsky et al. 1998). Because the presence of these related sequences could complicate the detection and cloning of the amplification product from the *T. speltoides* sucrose synthase gene, the following strategy was used to enrich the DNA sample with the targeted sequence. Fifteen micrograms of DNA from T7AS-7S#1S-7AS·7AL were digested with the restriction enzyme *Bam*HI and separated by electrophoresis in a 1% low melting point (LMP) agarose gel. The gel was stained with ethidium bromide and a 1-cm section was excised from the gel in the 6.5-kb region were the *T. speltoides* RFLP was previously detected. Digested DNA was extracted from the LMP agarose using the Wizard PCR Preps DNA Purification kit (Promega) and used as a template for PCR-amplification.

Primers P7S3R and P7S3L (Table 1) were designed based on the conserved sequences between the barley clone ABC465 and the wheat clone pST8 (Maraña et al. 1988). Primer pair design and compatibility were tested using the program OLIGO version 4.0. PCR products were purified using the Wizard PCR Preps DNA Purification kit (Promega) and cloned into pGEM-T Easy Vector System I (Promega) according to the manufacturer's protocols. Recombinant clones were purified and sequenced using an ABI377 automatic sequencer. Sequences were aligned using computing program ClustalW 1.7 (Fig. 1) and identity values for best-fit alignments were calculated using the SeqWeb version 1.1 of the GCG Wisconsin Package (Table 2). Intron/exon junctions were edited manually.

PCR procedures

All reactions were performed in a Perkin Elmer GeneAmp PCR system 9700 using 120 ng of genomic DNA and a final volume of 25 µl. The final concentration of the different products used in the PCR reaction were: 1 × *Taq* polymerase buffer (Promega), 1.5 U of *Taq* polymerase (Promega), 3.0 mM of $MgCl_2$, 0.2 µM of each primer, and 200 µM of each dNTP. Primer names, sequences and PCR cycling conditions are summarized in Table 1. Following amplification with the CAPS primers PS10R – PS10L2, PCR products were digested with the restriction enzyme *Sac*I. PCR and digestion products were separated by electrophoresis in 2% agarose gels and visualized using ethidium bromide and UV light.

Table 2 Identity values between best-fit alignments of pairs of sequences. Sequence pST8 is a cDNA from wheat sucrose synthase type 1, pST3 is a cDNA from wheat sucrose synthase type 2 (Maraña et al. 1988), and ABC465 is a cDNA from barley. Sequence pS10 is a genomic clone from *T. speltoides* and pS7, pS11, pCAPSa, and pI2 are genomic clones from *T. aestivum*.

Sequence	pST8	pS10	pS7	pS11	pCAPSa	pI2	ABC465
pS10	96.4%						
pS7	99.2%	96.0%					
pS11	95.2%	93.1%	93.7%				
pCAPSa	96.9%	94.2%	94.9%	98.6%			
pI2	86.0%	90.3%	88.4%	88.0%	89.0%		
ABC465	92.9%	92.6%	92.6%	89.3%	90.5%	84.6%	
pST3	79.4%	81.7%	79.2%	80.9%	80.9%	81.0%	78.7%

Results and discussion

Recombination within the *T. speltoides* segment

No recombination was expected between the *T. speltoides* 7 S chromosome segment and wheat chromosome 7 A in the presence of the wild-type *Ph1* locus. To confirm this hypothesis, 110 individuals from four different *T. speltoides* backcross populations were screened with RFLP clones ABC465 and WG834. These two loci are 27 cM apart in *Triticum monococcum* (Dubcovsky et al. 1996) and are the most external markers on the *T. speltoides* interstitial chromosome segment (Dubcovsky et al. 1998). No recombination was detected here between these two RFLP markers. This result confirmed that recombination is suppressed within the translocated chromosome segment and that, therefore, only one molecular marker is necessary to monitor the introgression of the *Lr47* gene carried by the *T. speltoides* chromosome segment.

Sequence comparisons

Many recombinant clones were obtained from the PCR-amplification products of the size-selected *Bam*HI-digested T7AS-7S#1S-7AS·7AL DNA using conserved primers P7S3R and P7S3L. Two PCR fragments of approximately 490- and 410-bp were amplified with these primers and were cloned into pGEM-T Easy. PCR-amplified inserts were gel-sized, and four recombinant genomic clones with inserts of about 490 bp (pS3, pS7, pS10, pS11) and one genomic clone with an insert of about 410 bp (pI2) were sequenced. As expected from the source of the sequence information used to design these primers, the sequences of these five clones were more similar to type-1 than to type-2 sucrose synthase genes, particularly in the 3' non-coding region (average identity 81%, Table 2).

Comparisons among the five genomic clones showed that pI2 (EMBL accession number AJ249624) had the most divergent sequence (average identity approximately 88%). A large deletion in the 3' non-coding region and various deletions in the intron region determined the smaller size of clone pI2 (Fig. 1). This 410-bp amplification fragment was assigned to chromosome 7B by nullisomic-tetrasomic analysis and therefore was discarded as candidate for a *T. speltoides* clone. The other four genomic clones with inserts of 490 bp were more similar among each other and to wheat clone pST8 than to barley clone ABC465 (Table 2). Seven deletions/insertions and 20 point mutations, concentrated in the 3' non-coding region, differentiated the barley sequence from the *Triticum* sequences, excluding pI2 (Fig. 1). Comparison between the cDNA sequences from ABC465 and pST8 with the genomic sequences allowed the identification of a 99 bp intron also present in the pS10 and pS11 clones (Fig. 1).

Clones pS3 and pS10 (EMBL accession number AJ238219) were identical and, therefore, clone pS3 was eliminated from further analyses. Clone pS7 (EMBL accession number AJ238218) differed only in four base pairs (99.2% identity, Table 2) from *Triticum aestivum* cDNA clone pST8 (Maraña et al. 1988) suggesting that pS7 was not the *T. speltoides* sequence. This level of sequence divergence has been observed between allelic variants of the same genome among different wheat varieties (Bryan et al. 1999)

Clone pS11 (EMBL accession number AJ238219) had a point mutation at position 264 that disrupted a *Sac*I restriction site (Fig. 1). This mutation was absent in pS10 and was used to determine which of these clones was from *T. speltoides*. DNAs from two different accessions of *T. speltoides* (DV562 and DV563) were amplified by PCR with the same set of primers and the amplification products were digested with the restriction enzyme *Sac*I. The *Sac*I restriction site was present in both *T. speltoides* accessions suggesting that pS10 was the best candidate for a *T. speltoides* clone.

Development of pS10 allele specific PCR primers

The region between positions 288 and 295 in the 3' non-coding region showed six bp differences between pS10 and the other sequences. These mutations were selected to match the 3' end of selective primer PS10L. To reinforce the selectivity of this primer, the 3' end of the complementary primer PS10R was designed to match a single point mutation (position 42) that differentiated pS7 and pST8 from the other clones (Fig. 1).

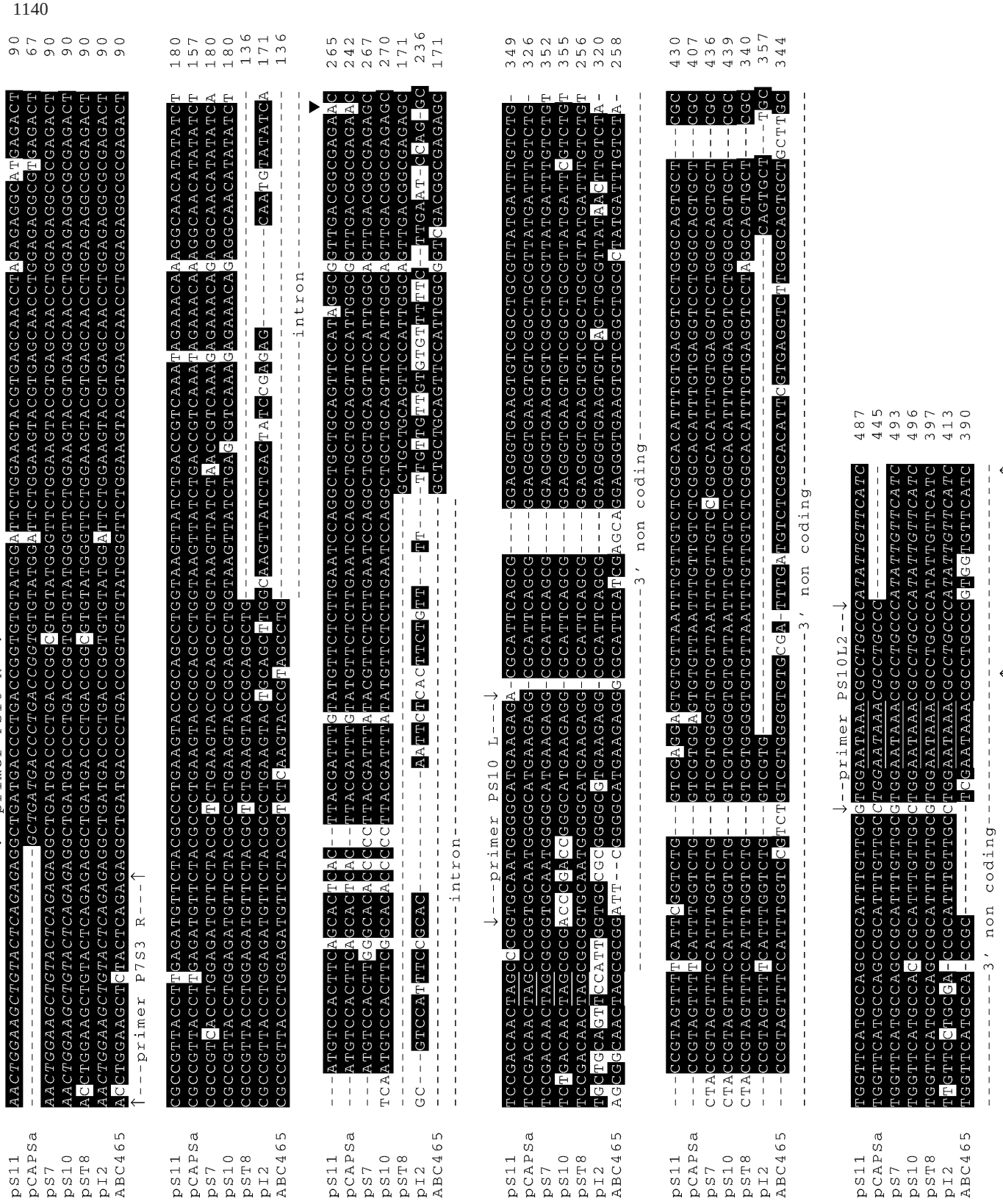

Fig. 1 Alignment of nucleotide sequences from wheat clones pS11, pCAPSa, pS7, pS10, pST8, pI2 and barley clone ABC465. Identities are indicated with *black boxes*. Gaps were introduced to maximize nucleotide alignment and are indicated with *dashes*. Locations of PCR primers P7S3R, PS10R, PS10L, PS10L2, and P7S3L are indicated with *arrows* and sequences from the primers are *italicized*. An *arrowhead* indicates the point mutation disrupting the *Sac*I restriction site used to develop the CAPS marker. Stop codons (TAG) and polyadenylation signals (AATAAA) are *underlined.* The intron and the 3' non-coding regions are indicated by *dashes* below the sequences

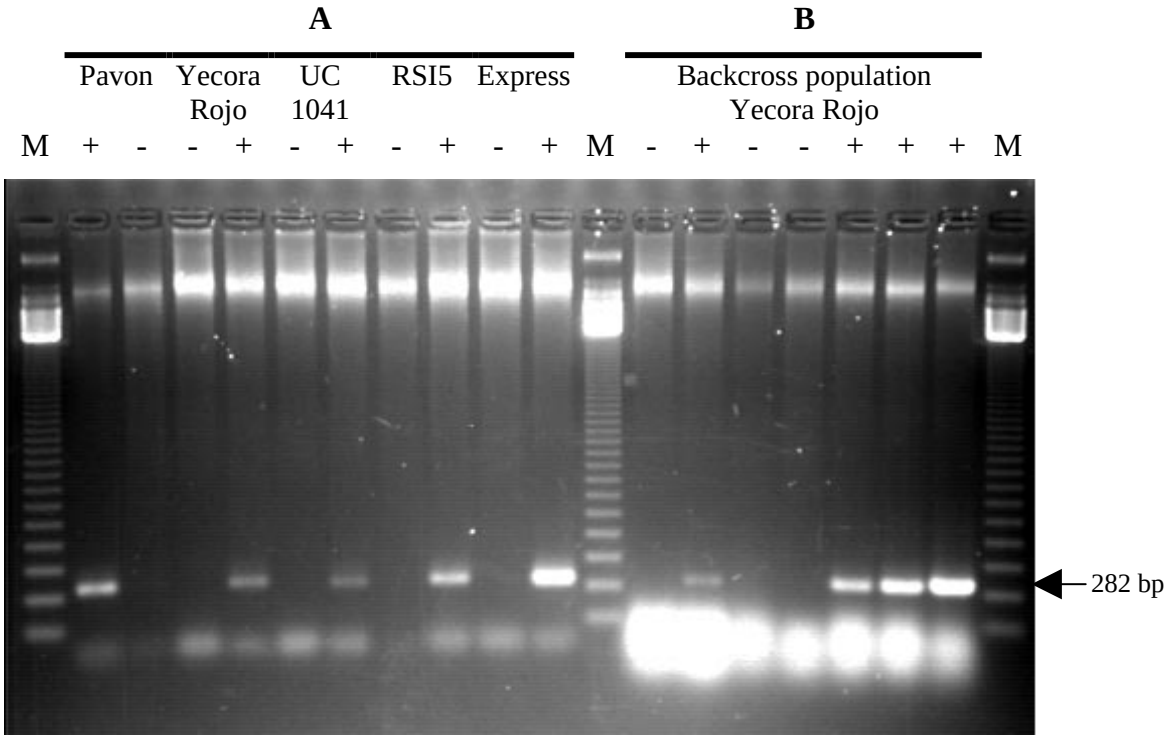

Fig. 2 PCR amplification using *T. speltoides*-specific primers PS10R and PS10L. *A* Genomic DNAs from backcross plants in five different genetic backgrounds obtained by a high-quality DNA isolation protocol (Dvorak et al. 1988). *B* Genomic DNAs from Yecora Rojo backcross plants using a fast DNA-isolation protocol (Wining and Langridge 1991). Symbols "+" and "–" indicate backcross plants with and without the *T. speltoides* segment respectively, determined by RFLP data using ABC465 and WG834 as probes. An *arrow* indicates the *T. speltoides* specific 282-bp amplification product. *"M"* indicates the molecular markers (123-bp ladder, Gibco BRL).

PCR-amplification of DNA from Pavon and T7AS-7S#1S-7AS·7AL using primers PS10R–PS10L showed a 282 bp fragment in T7AS-7S#1S-7AS·7AL that was absent in Pavon (Fig. 2). The specificity of this pair of primers for the *T. speltoides* sequence was tested using DNA isolated by a fast small-scale procedure (Wining and Langridge 1991) from 166 backcross plants in five different genetic backgrounds (Fig. 2). The 282 bp PCR fragments were present only in the samples that showed the characteristic 6.5 kb *T. speltoides Bam*HI restriction fragment after hybridization with ABC465. These data confirmed that the pS10 sequence belonged to the *T. speltoides* 7 S allele and that these primers were efficient in differentiating the *T. speltoides* allele in different bread wheat genetic backgrounds and using different DNA extraction procedures.

Cleavage amplified polymorphic sequence

Though *T. speltoides*-specific primers PS10R – PS10L were useful to select heterozygous plants carrying *Lr47*, they were not appropriate to differentiate heterozygous from homozygous *Lr47* plants. This differentiation is critical to select *Lr47* homozygous plants in F_2 segregating populations or after self-pollination of the heterozygous BC plants from the last cycle of a backcrossing program.

Homozygous *Lr47* plants can be identified by the absence of the 7 A allele. Therefore, a cleavage amplified polymorphic sequence (CAPS) marker was developed for the 7 A allele of the sucrose synthase locus. Amplification of wheat genomic DNA with CAPS primers PS10R and PS10L2 resulted in two fragments of 450 (pCAPSa)- and 380 (pCAPSb)-bp that were cloned and sequenced. Primer PS10L2 (Table 1) was preferred over primer P7S3L because the latter produced lower yields of the amplification product from the 7 A allele (data not shown). Nullisomic-tetrasomic analysis in Chinese Spring showed that the 450-bp product was amplified from chromosome 7 A (Fig. 3A, lane 1) while the 380-bp product was amplified from chromosome 7B (Fig. 3A, lane 2). Sequence analysis showed that pCAPSa (EMBL accession number AJ249623) was more closely related to *Triticum* clones pS7, pST8, pS10 and pS11 than to the barley clone ABC465 (Table 2). Clone pCAPSb showed an identical sequence to clone pI2 and, therefore, was not included in Fig. 1. Nullisomic-tetrasomic analysis (Fig. 3, lines 1, 2, and 3) suggested that the 7D allele was not efficiently amplified using the CAPS primers.

Unfortunately, CAPS primers PS10R and PS10L2 amplified a fragment from *T. speltoides* chromosome 7 S of identical mobility to the 7 A allele (Fig. 3A, lane 4). Sequences of both alleles (pCAPSa and pS10, Fig. 1) were compared and a *Sac*I restriction site was found at position 264 in *T. speltoides* that was absent in the 7 A allele and in the 380-bp 7B fragment. Digestion of the

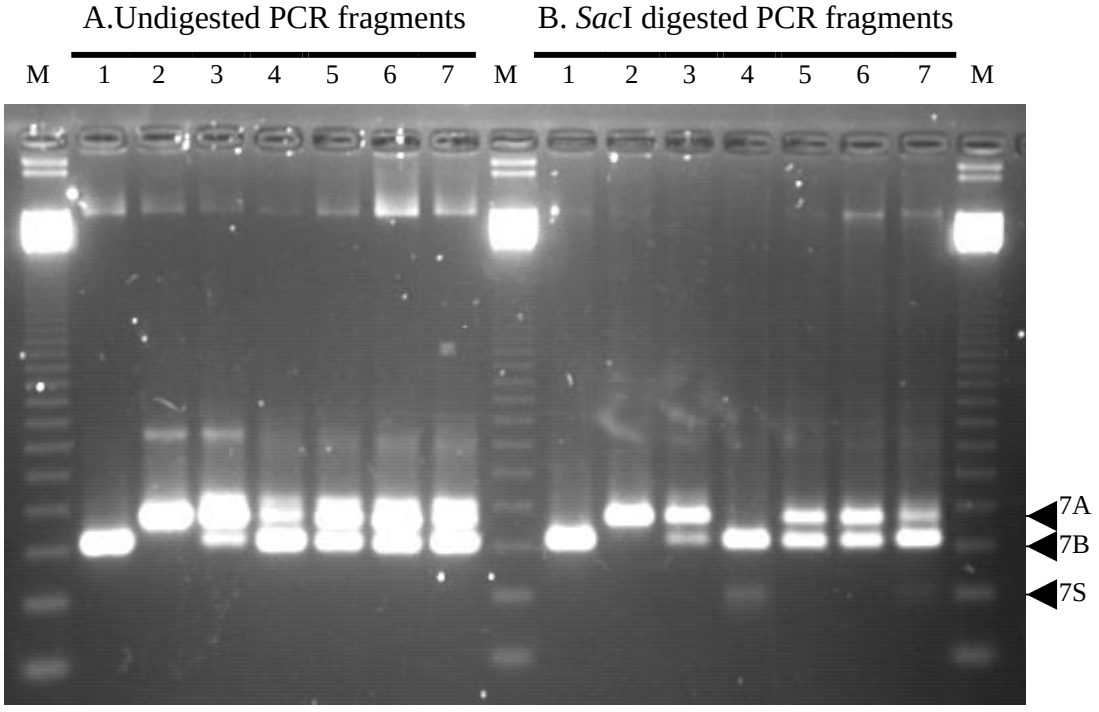

Fig. 3 A PCR fragments amplified with primers PS10R and PS10L2. **B** Same amplification products digested with the restriction enzyme *Sac*I. **A***1* and **B***1* Chinese spring nullisomic-tetrasomic lines N7AT7B; **A***2* and **B***2* nullisomic-tetrasomic N7BT7 A; **A***3* and **B***3* nullisomic-tetrasomic N7DT7B; **A***4* and **B***4* homozygous *Lr47* plant (T7AS-7S#1S-7AS·7AL); **A***5* and **B***5* homozygous 7 A plant (Pavon); **A***6* and **B***6* homozygous 7 A plant (BC_5 in Yecora Rojo); **A***7* and **B***7* heterozygous plant (BC_5 in Yecora Rojo). *"M"* indicates the molecular markers (123-bp ladder, Gibco BRL)

amplification products from homozygous *Lr47* plants with the restriction enzyme *Sac*I showed no 450-bp fragment (Fig. 3B, lane 4) and two fragments of approximately 260- and 190-bp. These two fragments were frequently observed as a single band as in Fig. 3B, lane 4. *Sac*I digestion of the amplification products from heterozygous plants (Fig. 3B, lane 7), showed a low-intensity 450-bp fragment and very faint 260- and 190-bp fragments. Finally, *Sac*I digestion of the amplification products of plants homozygous for normal chromosome 7 A showed an intense 450-bp fragment and no 260- or 190-bp fragments (Fig. 3B, lanes 5 and 6).

These CAPS primers are not very efficient in amplifing the *T. speltoides* allele (Fig. 3A and B, lane 4) and, therefore, are not very useful for positive selection of *Lr47*. The best strategy is first to select plants carrying one or two doses of *Lr47* using the *T. speltoides*-specific primers PS10L –PS10R, and then select the homozygous *Lr47* plants using the CAPS markers. After digestion of the amplification products with *Sac*I, the absence of the 450-bp fragment indicates the absence of the 7 A allele, and therefore the presence of two chromosomes carrying the *T. speltoides Lr47* resistance gene. Although partial *Sac*I digestion of the 450-bp amplification product may determine the elimination of some homozygous plants (false negatives), this strategy minimizes the possibility of selecting false homozygous *Lr47* plants (false positives). An additional advantage of this CAPS marker is that the 380-pb 7B allele can be used as an internal control of amplification efficiency.

Twenty five wheat varieties from different classes, including hard red spring, soft red spring, hard white spring, hard red winter, soft white winter and club (see Materials and methods), were evaluated with this CAPS marker to determine the frequency of the 7 A allele mutation eliminating the *Sac*I restriction site. After digestion with *Sac*I, all varieties showed the expected 450-bp fragment with the exception of translocation line T7AS-7S#1S-7AS·7AL used as a control.

Conclusions

The combined use of the *T. speltoides*-specific PCR primers and the A genome-specific CAPS marker will facilitate the deployment of *Lr47* in commercial bread wheat varieties. Translocation line T7AS-7S#1S-7AS·7AL, released as germplasm under PI603918 (Lukaszewski et al. 1999), is a valuable source for the resistance gene *Lr47*. This line had three backcrosses with Pavon, a hard white spring wheat with good breadmaking quality and additional rust resistance genes. Resistance gene *Lr10* is present in Pavon and is still useful in combination with other resistance genes (McIntosh et al. 1995). This gene can be also targeted by marker-assisted selection because PCR markers are also available (Schachermayr et al. 1997). Additionally the variety Pavon carries a seedling leaf rust resistance gene *Lr1* on the long arm of chromosome 5D (McIntosh et al. 1995) and an adult slow rusting gene on chromosome 1B (Singh et al. 1998). Howev-

er, molecular markers are not yet available for these additional genes.

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