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Identification of molecular markers linked to *Pm13*, an *Aegilops longissima* gene conferring resistance to powdery mildew in wheat

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Abstract RFLP, RAPD, STS and DDRT-PCR techniques were applied to find molecular markers linked to *Pm13*, an *Aegilops longissima* gene conferring resistance to powdery mildew in wheat. The experimental strategy was based on the differential comparison of DNAs from common wheat and from common wheat/*Ae. longissima* recombinant lines carrying short segments of the $3S^lS$ chromosome arm containing the *Pm13* gene. Sixteen RFLP clones that detect loci previously located in the short arms of group-3 wheat chromosomes were screened for their ability to hybridise to *Ae. longissima* restriction fragments derived from the $3S^lS$ segments introgressed into the recombinant lines. Eight RFLP clones and one STS marker detected $3S^lS$-specific fragments whose location relative to the wheat-alien chromatin breakage point of the recombinant lines was determined. Four amplification products were identified through the screening of about 200 RAPD primers. Their polymorphism was associated with the introgression of the alien DNA. One of the differential fragments was derived from the $3S^lS$ DNA segment, while the remaining three corresponded to the replaced 3DS DNA. Further analyses carried out using 40 combinations of DDRT-PCR primers detected an additional reproducible polymorphism associated with the presence of $3S^lS$ DNA. In view of their possible utilisation in *Pm13* marker-assisted selection, differentially amplified RAPD and DDRT-PCR fragments were cloned, transformed into RFLP markers and converted into STS markers.

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Introduction

Powdery mildew, caused by *Erisiphe graminis* D.C. ex Merat f.sp. *tritici* Em. Marchal, is one of the most serious diseases of cultivated wheats (*Triticum turgidum* L. var. *durum* and *Triticum aestivum* L.). Several genes conferring powdery mildew resistance are known within cultivated wheat germplasm, but most of them have already been overcome by new virulent *E. graminis* strains. It is therefore necessary to extend the search for new sources of genetic resistance to powdery mildew to a wider gene pool, including the wild relatives of cultivated wheats. Moreover, the availability of several different resistance genes permits their pyramiding into the same cultivar, thus producing stronger and more durable resistance. For efficient transfer of resistance genes, linked molecular markers can be of great help, particularly when dealing with multiple resistance genes. Furthermore, the construction of a highly saturated map around target genes represents an essential requirement for map-based cloning (Tanksley et al. 1995).

Near-isogenic lines (NILs) have been used to select molecular markers linked to resistance genes (Young et al. 1988). A similar strategy, based on the use of recombinant lines carrying short chromosome segments introgressed from related species, has been applied for selective identification, by differential analysis, of molecular markers detecting polymorphisms between alien and substituted DNA (Schachermayr et al. 1994, 1995; Autrique et al. 1995; Procunier et al. 1995; Qi et al. 1996; Talbert et al. 1996).

RAPD marker technology (Williams et al. 1990) is widely used to find markers linked to target genes. The use of the RAPD analysis in wheat appears to be

particularly suited for the identification of introgressed alien chromatin (Devos and Gale 1992). In fact, RAPDs have been successfully employed in wheat to identify markers linked to the powdery mildew resistance gene *Pm18* (Hartl et al. 1995), to the *Pm21* gene introgressed from *Dasypyrum villosum* (Qi et al. 1996), to *Lr* genes (conferring resistance to leaf rust) introgressed from *Ae. umbellulata* (Schachermayr et al. 1994) and from *Agropyron elongatum* (Procunier et al. 1995; Schachermayr et al. 1995), and to the *Ccn-D1* nematode resistance gene introgressed from *T. tauschii* (Eastwood et al. 1994).

The Differential Display Reverse Trascriptase-PCR (DDRT-PCR) technique enables the amplification of differentially expressed mRNA deriving from identical genotypes exposed to different treatments (Liang and Pardee 1992). Hannappel et al. (1995) applied DDRT-PCR on tomato NILs to isolate anonymous cDNAs from a specific chromosome region associated with the *Tm-2a* gene for resistance to Tomato Mosaic Virus.

An effective gene for resistance to wheat powdery mildew, named *Pm13*, was transferred to bread wheat cv Chinese Spring (CS) from the short arm of chromosome $3S^l$ of *Ae. longissima* by using the *ph1b* mutation as the pairing-promoting system (Ceoloni et al. 1988). Both monosomic analysis and C-banding indicated the introgressed chromosome segment to be located in the short arm of either the 3B or 3D wheat chomosome in different recombinant lines (Ceoloni et al. 1992).

Recombinant lines carrying the *Pm13* gene were later characterised by RFLP markers and telocentric mapping analysis (Donini et al. 1995). It was thus clearly shown that, although in all cases the alien-wheat breakage point (BP) had a distal location, the amount of *Ae. longissima* chromatin introgressed in each line varied.

The work reported in this paper was carried out to develop a more detailed map of the region around *Pm13* than previously attained (Donini et al. 1995). Some RFLP clones and one STS marker, previously located in the short arms of group-3 chromosomes of wheat, were used for the analysis of recombinant lines deriving from the introgression of $3S^lS$ chromosome segments of *Ae. longissima* into CS. About 200 RAPD primers and about 40 DDRT-PCR primer combinations were screened in order to enrich the introgressed chromosome segments with more molecular markers. Some of these markers were converted into STSs (Olson et al. 1989; Paran and Michelmore 1993) for improving the efficiency of their use.

Materials and methods

Plant material

The following genotypes were used for molecular-marker identification and localisation: *T. aestivum* cv Chinese Spring (CS); CS group-3 compensating nulli-tetrasomic lines (Sears 1966) and 3L ditelosomic lines (Sears and Sears 1978); *Ae. longissima* accession TL01 and TL01-derived $3S^l$, $3S^lS$ and $3S^lL$ addition lines into CS: TLDA$3S^l$, TLDTA$3S^lS$ and TLDTA$3S^lL$ respectively (Feldman 1975; Ceoloni 1983); a complete set of addition lines deriving from *Ae. longissima* accession No. 4 (Friebe et al. 1993). The $3S^lS$-3BS (R6A, R5A, R1A, R4A and R1B) and $3S^lS$-3DS (R2A, R1D and R2B) wheat-*Ae. longissima* recombinant lines (Ceoloni et al. 1988) were used to locate the molecular markers along the $3S^lS$ chromosome arm. The recombinant lines carrying the shortest chromosome segments (R4A, R1B and R2B, see Donini et al. 1995) were used for RAPD and DDRT-PCR analysis.

Powdery mildew infection and tissue sampling

An Italian wheat powdery mildew isolate, virulent towards CS but not towards the *Pm13* recombinant wheat lines, was provided by M. Pasquini (Cereal Research Institute, Rome). Seedlings at the 1st leaf stage were infected by spreading a conidial water suspension on the upper leaf surface. After infection, seedlings were placed in a growth chamber at 16–18°C, with a 6-h dark and an 18-h light photoperiod. The first leaf of three infected seedlings/genotype was sampled after 12 and 20 h, respectively. Non-infected seedlings, grown under the same conditions as above, were sampled coincident with the first sampling of infected material. After sampling, leaves were frozen in liquid nitrogen and stored at −75°C until RNA extraction. Infection efficiency was controlled on two other seedlings about 10 days after inoculation.

DNA and RNA extraction

Genomic DNA was extracted either from 5 g (D'Ovidio et al. 1992) or from 0.5 g (Dellaporta et al. 1983) of fresh leaves. Total RNA extraction was performed with the RNeasy kit (Qiagen) according to the protocol provided by the manufacturer.

RFLP analysis

The following 16 probes were used for RFLP analysis: the genomic clones UTV103 from *Ae. squarrosa*, UTV135 from *Ae. longissima* and UTV1015, UTV1022 and UTV1538 from *T. urartu*, isolated at the University of Tuscia, Viterbo (Italy), and previously located on the short arms of CS group-3 chromosomes by nulli-tetrasomic analysis (D'Ovidio et al. 1994); the cDNA barley clones BCD907, BCD1428, BCD1532, BCD1823 and the cDNA oat clones CDO395, CDO460, CDO549, CDO638, CDO1164, CDO1345, CDO1435 previously shown to map on the short arms of group-3 wheat chromosomes (Nelson et al. 1995c; Sorrells and Anderson 1997), and kindly provided by Mark E. Sorrells (Departement of Plant Breeding and Biometry, Cornell University, Ithaca, USA).

Southern blotting, digoxigenin-labelling of probes, hybridisation and immunological detection were performed as described by D'Ovidio et al. (1994).

RAPD analysis

A preliminary screening for genetic polymorphism between CS and recombinant lines R4A, R1B and R2B was carried out using 193 random 10-mers (series B, R–Z; Operon Technologies). When polymorphic amplification patterns were detected, the results were further confirmed by amplifying the DNA from the TL01, TLDTA$3S^lS$, DT3BS, CS, R4A, R1B and R2B genotypes. The amplifications of the last four genotypes were repeated in two independent reactions.

Amplification reactions were performed in a 20-µl vol containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.25 µM of random primer (Operon), 1 ng/µl of genomic DNA and 25 mU/µl of *Taq* polymerase (Stoffel Fragment, Perkin Elmer). Amplification reactions were performed in a Programmable Thermal Controller (MJ-Research) programmed at 94°C for 2 min, followed by 45 cycles of 1 min at 94°C, 2 min at 37°C and 2 min at 72°C. The final extension of the amplified fragments was achieved in a 5-min step at 72°C. Amplification products were separated on 1.6% agarose gels and detected by ethidium-bromide staining.

DDRT-PCR analysis

Primers $(dT)_{12}VN$ (V = A + C + G, N = A or C or G) and arbitrary 10-mer sets (Bauer et al. 1993) (Operon Technologies) were used to perform reverse transcription and cDNA amplification.

Reverse transcriptions were carried out at 42°C for 15 min in either a 20- or a 100-µl vol containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 2.5 µM of $(dT)_{12}VN$ primer, 1 U/µl of RNase inhibitor, 50 ng/µl of total RNA and 2.5 U/µl of MuLV Reverse Transcriptase (Perkin Elmer). Reactions were stopped by heat inactivation at 99°C for 5 min. A 5-µl vol of reverse-transcription reaction products was amplified in 25-µl of the same buffer solution (the $MgCl_2$ concentration was adjusted to 2.5 mM), to which 1 µl of 50 µM $(dT)_{12}VN$, 1 µl of 10-mer primer 20 mM and 0.625 µl of 50 mU/µl of Ampli*Taq* polymerase (Perkin Elmer) were added. Amplification reactions were performed by 40 cycles of 30 s at 94°C, 1 min at 42°C and 1 min at 72°C. The extension of the amplified fragments was achieved with 5 min at 72°C. Amplification products were separated on 6% non-denaturing polyacrylamide gels and detected by silver staining. Only amplification products larger than 300 bp were considered.

Cloning and sequencing of RAPD and DDRT-PCR polymorphic products

The polymorphic amplification products identified by RAPD and DDRT-PCR analyses were recovered and re-amplified by PCR using the same experimental conditions. The amplified sequences were cloned into a pUC18 vector (Sure Clone, Pharmacia) by blunt-end ligation and inserted into *Escherichia coli* strain DH5α. The correspondence of the cloned fragments to the original polymorphic amplification products was verified by Southern hybridization with the RAPD or DDRT-PCR original products.

RAPD and DDRT-PCR products were sequenced using the AmpliCycle kit (Perkin Elmer).

Design and analysis of STS primers

Based on the sequence information of the cloned RAPD and DDRT-PCR differential fragments, the following pairs of STS primers were synthesised:

UTV13F: CCCCCTGAAATCAAATGTGT and UTV13R: CCCCC TGAAAAACAAGAAT, from RAPD fragment $OPV13_{800}$;
UTV14F: CGCCAGCCAATTATCTCCATGA and UTV14R: AGCCATGCGCGGTGTCATGTGAA, from RAPD fragment $OPX12_{570}$;
UTV15F: GGGCATTGTCACCTGGGTTACTG and UTV15R: GTGTATCATGTTCCTCCACATGGC, from DDRT-PCR fragment DD02-A_{600};
UTV19F: TCAACGGGACGTAGATTTTACCCA and UTV19R: TCAACGGGACACGTTAGTTGTTGT, from RAPD fragment $OPZ12_{550}$.

Moreover, a pair of STS primers corresponding to the clone pTtksuG53 (University of Kansas) was obtained from the GrainGenes database:

KSUG53L: GCTGGCAGAGAGAGATTGAG and KSUG53R: CCAAATGACACAAACAACAT.

PCR amplification with each primer pair was performed in a 50–100 µl vol containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 µM of each dNTP, 1 ng/µl of specific primers, 1 ng/µl of genomic DNA and 25 mU/µl of *Taq* polymerase (Stoffel Fragment, Perkin Elmer). Monomorphic amplified products were digested with either 4- or 6-cutter restriction enzymes and separated on a 1.5% Super Fine Resolution agarose gel (Amresco).

Results and discussion

RFLP analysis

To identify restriction fragments derived from the $3S^l$ short arm, 16 RFLP probes were hybridised on Southern blots containing DNA of CS and TLDTA3S^{l}S digested with different restriction enzymes (*Eco*RI, *Hin*dIII, *Bam*HI, *Eco*RV and *Pst*I). RFLP clones UTV103, UTV135, UTV1022, UTV1538, BCD907, BCD1532, CDO395, CDO460, CDO549 and CDO1435 hybridised to additional restriction fragments of the TLDTA3S^{l}S-line DNA. The fragments were thus unequivocally located in the 3S^{l}S arm. The probes were then hybridised on Southern blots containing DNA of all the *Pm13* recombinant lines digested with the most appropriate restriction enzyme. The distribution of the tested RFLP markers on chromosome arm 3S^{l}S was inferred by comparing the hybridization patterns of the wheat-*Ae. longissima Pm13* recombinant lines (Fig. 1) and agreed with their locations on a recently published group-3 consensus map derived from wheat and related Triticeae species (Nelson et al. 1995 c). The presence of additional hybridisation fragments in a given recombinant line was indicative of a distal location of the corresponding restriction fragments with respect to the CS-*Ae. longissima* breakage point (BP) of that line; whereas their absence demonstrated their location in a proximal position.

The results obtained add further support to the well-established evidence of the largely conserved synteny between homoeologous Triticeae chromosomes (Nelson et al. 1995 a, b, c; Van Deynze et al. 1995; Marino et al. 1996). The DNA markers employed allowed discrimination between recombinant lines containing *Pm13*, which could not be distinguished with previously available RFLP markers (Donini et al. 1995). This applies to probes CDO395 and UTV1538, which discriminated between 3D recombinant lines R2A and R1D, and probes CDO460 and UTV135, which distinguished between the BP of the 3B recombinant lines R4A and R1B. The *Ae. longissima*-specific loci *Xcdo460* and *Xutv135* in particular, whose characteristic fragments are shown by all recombinant lines except R1B,

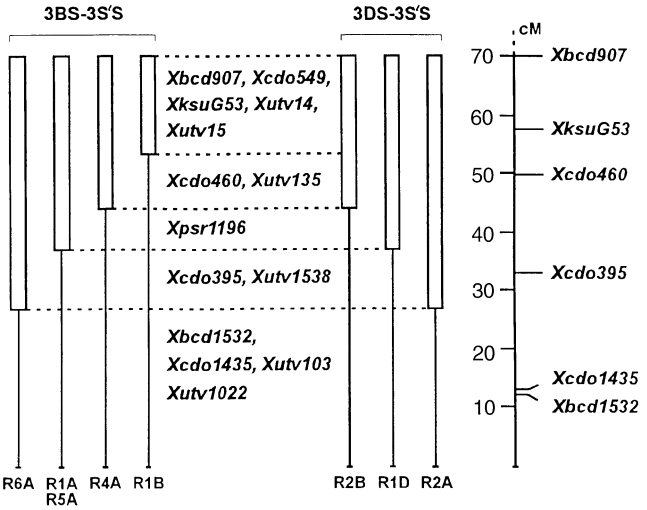

Fig. 1 Distribution of DNA markers relative to the *Pm13* recombinant-line breakpoints. Marker and breakpoint relative positions are inferred from RFLP and STS analysis. On the right side: genetic distances of markers derived from a group-3 consensus map (Nelson et al. 1995c)

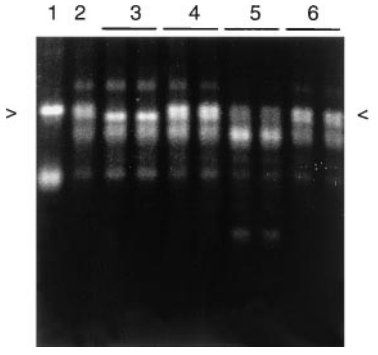

Fig. 2 Amplification pattern of the RAPD primer OPX12: *1* TL01; *2* TLDTA3S^{l}S; *3* CS; *4* R4A; *5* R1B; *6* R2B. Arrowheads show the polymorphic fragment

can thus be located in the *Ae. longissima* chromosome region adjacent to the shortest of the distal 3S^{l}S segments introgressed in the various *Pm13* recombinant lines. On the basis of the published data on the consensus map of wheat and other Triticeae species, this region should span about 20 cM.

RAPD analysis

Comparative RAPD analysis was performed between CS and the R4A, R1B and R2B recombinant lines, carrying the shortest introgressed segments from TL01 DNA. A total of 148 RAPD primers were screened and they produced 1080 distinguishable amplification products. In order to assess the polymorphism between CS and TL01, 45 additional RAPD primers were analysed on genomic DNA from TL01, TLDTA3S^{l}S, CS, R4A, R1B and R2B. These RAPD reactions amplified 359 products in CS (average of 8.0 bands/primer) and 295 in TL01 (average of 6.6 bands/primer); about 52% (152/295) of the TL01 amplification products were polymorphic when compared with the CS pattern. The ratio of amplified products observed between CS and TL01 was about 1.2, much lower than the ratio of their DNA contents, which is about 3. Three causes could explain this inconsistency: (1) reduced competition among the amplification products, which increased the number of distinguishable bands in TL01; (2) co-migration of amplification products which are monomorphic between the three CS genomes; and (3) abundance of repetitive DNA in CS.

Four primers yielded reproducible polymorphic amplification patterns, which could be associated with regions of either wheat (OPU20, OPV13 and OPZ12) or alien (OPX12) DNA involved in introgression. In particular, OPX12 amplified a 570 bp product in TL01 and TLDTA3S^{l}S as well as in all the recombinant lines, but not in CS (Fig. 2). Primers OPU20, OPV13 and OPZ12 produced amplification fragments of 300, 800 and 550 bp, respectively (data not shown) that were present in CS but absent in recombinant line R2B. Since these fragments were also lacking in the 3DL ditelosomic-line amplification pattern, they could be located in the CS 3DS arm, within the region distal to the breakage point of all the recombinant lines.

DDRT-PCR analysis

Seedlings of CS and of the R4A recombinant line were infected with the powdery mildew strains 0–10. When infection efficiency was checked, 10 days after inoculation, CS seedlings showed more than 100 infection sites on the infected leaf. DDRT-PCR analysis was performed using 43 primer combinations (poly-T anchor primers and random 10-mers) on cDNA produced by reverse transcription of total RNA extracted from the first leaf of CS and R4A plantlets, both non-infected (0 h), as well as after 12 and 20 h following infection. An average of 22 amplification products larger than 300 bp for each primer combination was obtained. The comparison of the DDRT-PCR pattern of the R4A recombinant line with that of Chinese Spring detected a total of 23 differential fragments. Seventeen of them (14 in R4A and three in CS) were found only at a single induction time, four fragments (three in R4A and one in CS) at two induction times, whereas only two R4A-specific amplification products, of 600 and 700 bp, were observed at all three induction times using DD02-$(dT)_{12}$VA (Fig. 3) and DD07-$(dT)_{12}$VA primer combinations, respectively. One of these differential DDRT-PCR fragments (DD02-A_{600}) was related to the introgression of the *Ae. longissima* chromosome segment and was converted into the RFLP marker

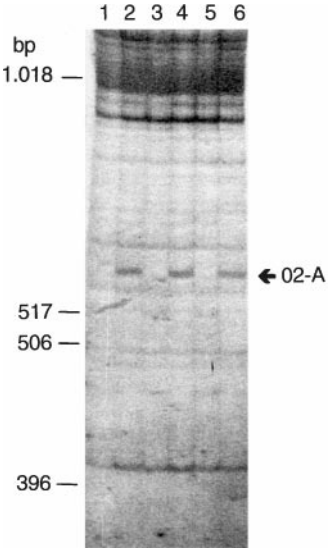

Fig. 3 DDRT-PCR pattern obtained with the primer combination DD02-$(dT)_{12}$VA. *Arrow* shows the polymorphic fragment. *Lines 1, 3, 5* CS; *2, 4, 6* R4A. *Lines 1 and 2* not infected; *3 and 4* 12 h after infection; *5 and 6* 20 h after infection

Xutv15, whilst the other fragment (DD07-A_{700}), not detecting any RFLP between CS and TL01, was discarded.

Cloning of RAPD and DDRT-PCR amplification products

Three RAPD ($OPV13_{800}$, $OPX12_{570}$, $OPZ12_{550}$) and two DDRT-PCR (DD02-A_{600}, DD07-A_{700}) polymorphic amplification products were recovered from gels and cloned. In order to verify the correspondence between the cloned fragments and the amplified products, the clones were employed as probes on Southern blots containing the amplification products from the original RAPD and DDRT-PCR reactions using R4A and CS DNA. The clones deriving from the RAPD and DDRT-PCR amplification products were probed on CS and TLDTA3S^{l}S ditelosomic addition-line DNAs digested with different restriction enzymes. The clones from $OPV13_{800}$ (pUTV13) and $OPZ12_{550}$ showed non-specific hybridisation patterns. The clone from the DDRT-PCR product DD07-A_{700} gave non-specific hybridisation and a strong monomorphic fragment. The clone pUTV14 from the RAPD fragment $OPX12_{570}$ detected either two or three fragments, according to the enzyme used, on TLDTA3S^{l}S and a weak and non-specific hybridisation on CS (Fig. 4 a). The pUTV15 clone from the DDRT-PCR fragment DD02-A_{600} hybridised to additional restriction fragments on TLDTA3S^{l}S (Fig. 4 b). The hybridisation patterns of pUTV14 and pUTV15 with all of the recombinant lines were identical to the TLDTA3S^{l}S pattern (data not shown), indicating that they hybridised to fragments located within the chromosome region distal to all the recombination breakage points (Fig. 1). Both clones pUTV14 and pUTV15 detected additional restriction fragments on TL01 DNA (data not shown), likely to be located on chromosomes other than 3S^{l}S.

STS analysis

Terminal regions of the clones deriving from the RAPD amplification products $OPV13_{800}$ (pUTV13), $OPX12_{570}$ (pUTV14), and $OPZ12_{550}$ (pUTV19), as well as from the DDRT-PCR fragment DD02-A_{600} (pUTV15), were sequenced and specific primers of 19–24 nucleotides in length were synthesised. Primers deriving from the RAPD fragment $OPZ12_{550}$ and from the DDRT-PCR fragment DD02-A_{600} did not detect any polymorphism, even after digestion with several 6- or 4-cutter restriction enzymes (data not shown). Primers from the RAPD fragment $OPV13_{800}$ (UTV13F/R) amplified two products in CS and none in TL01 DNAs (Fig. 5 a). The largest fragment (from Chinese Spring) is missing in the R2B recombinant line (Fig. 5 a) as well as in the N3DT3A and N3DT3B nulli-tetrasomic lines (data not shown). This product, therefore, derives from the RAPD fragment $OPV13_{800}$ from the wheat 3DS region distal to all the recombination breakage points on this arm. Primers from the $OPX12_{570}$ RAPD fragment (UTV14F/R) amplified a product of the expected size in TL01, but not in CS (Fig. 5 b). Since the amplification product was also present in TLDTA3S^{l}S and in all the recombinant lines, it was assumed that it corresponded to the $OPX12_{570}$ fragment. The amplification pattern of the STS marker derived from the pTtksuG53 RFLP probe did not show any polymorphism between CS and TL01. However, cleavage of the amplification products with the *Rsa*I restriction enzyme produced two restriction fragments of 850 and 750 bp specific for the 3S^{l}S arm (data not shown) which were distal to all the recombination sites. Another restriction fragment of 600 bp was present in CS, but not in TL01 and in recombinant line R2B. The location of this marker on chromosome 3D was further confirmed by nulli-tetrasomic analysis (data not shown).

UTV14F/R amplifies a specific product from the introgressed segment, that could be very useful for marker-assisted selection in breeding programs aimed at the transfer of the *Pm13* gene into cultivated varieties of bread and durum wheat. Because of its homoeologous nature, the introgressed portion does not recombine with the corresponding regions of wheat 3B and 3D. This makes *Xutv14*, as well as other markers located within the alien region, completely linked to the *Pm13* gene. In particular, the characteristics of an STS marker such as *Xutv14* allow the easy identification of *Pm13* genotypes through a simple and reliable PCR reaction. The PCR reaction can be carried out on small amounts of plant tissue, thus allowing an early selection of the *Pm13* gene in segregating populations. The STS marker *Xutv13* could be exploited in backcross

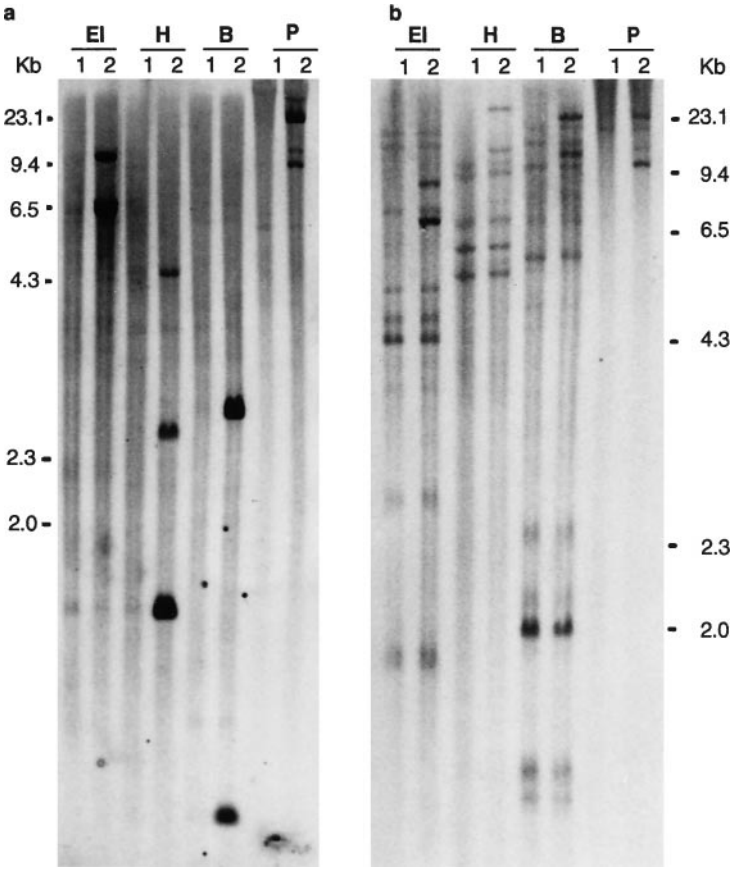

Fig. 4 RFLP patterns of (**a**) $OPX12_{570}$ (*Xutv14*)- and (**b**) $DD02\text{-}A_{600}$ (*Xutv15*)-derived clones. *1* CS and *2* TLDTA3S^{l}S. *EI* = *Eco*RI, *H* = *Hin*dIII, *B* = *Bam*HI, *P* = *Pst*I

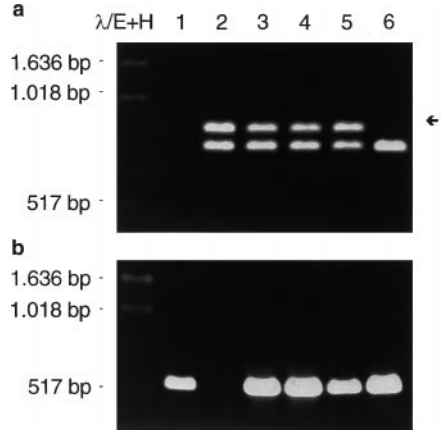

Fig. 5 Amplification patterns of (**a**) UTV13F/R and (**b**) UTV14F/R primers. *1* TL01; *2* CS; *3* TLDTA3S^{l}S; *4* R4A; *5* R1B; *6* R2B. *Arrow* shows the polymorphic fragment

programs for the early identification of bread wheat plants which are homozygous for the *Pm13* gene located on the 3DS chromosome.

The reported markers, namely *Xbcd907*, *Xcdo460*, *Xcdo549*, *XksuG53*, *Xutv14*, *Xutv15* and *Xutv135*, which map within an about 20-cM chromosome region introgressed from *Ae. longissima* and comprising the *Pm13* gene, could represent useful tools for map-based cloning (Tanksley et al. 1995) of the *Pm13* gene sequence.

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