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A simple PCR-based method for scoring the *ph1b* deletion in wheat

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Abstract The amplified fragment length polymorphism (AFLP) technique was used to isolate DNA sequences present in the euploid wheat Chinese Spring but not in the Chinese Spring *ph1b* mutant (which has a deletion of the *Ph1* gene, a suppressor of homoeologous chromosome pairing). The polymorphic DNA fragments identified by AFLP were then cloned, sequenced, and used to design two primer pairs. These primers were used in a PCR-based assay to specifically amplify products from the Chinese Spring euploid but not from the *ph1b* mutant. This PCR assay can be carried out from extracted genomic DNA or directly from alkaline-treated wheat leaves, and the reaction products can be scored on a plus-minus basis, making the screening amenable to automation. The reliability of the assay was tested using a F_1-derived doubled-haploid population of 55 lines which segregate for the *ph1b* deletion. This PCR-screening technique is less time and labour consuming, and more accurate and reliable, than cytologically based conventional methods.

Key words AFLP · Chinese Spring · Chromosome pairing · PCR assay · *ph1b* deletion

Introduction

Bread wheat (*Triticum aestivum* L. em Thell. 2n = 6x = 42) is an allohexaploid with three related (homoeologous) genomes (*A*, *B* and *D*) (Sears 1952), each derived from a different ancestor. There are seven pairs of homologous chromosomes per ancestral genome. Chromosome pairing is strictly controlled during premeiotic interphase, and only homologous chromosomes pair and recombine, thus maintaining the integrity of the three genomes. Several genes controlling chromosome pairing (*Ph* genes) have been identified, but the strongest effect has been observed for the *Ph1* locus, located on the long arm of wheat chromosome *5B*, which suppresses pairing and recombination between homoeologous chromosomes (Sears and Okamoto 1958; Riley and Chapman 1958). In the absence of *Ph1*, homoeologous recombination can occur between wheat chromosomes and those from related species and genera (Riley et al. 1968; Koebner and Shepherd 1985), which is useful for the introduction of new genetic traits into wheat (reviewed Ji and Langridge 1990).

Lines carrying deletions of the *Ph1* locus have been identified for hexaploid (*ph1b*) (Sears 1977) and tetraploid (*ph1c*) (Giorgi 1978) wheat. Using these lines, markers located within the region defined by the *ph1b* deletion can be identified using restriction fragment length polymorphism (RFLP). The *ph1b* deletion is estimated to span around 70 Mb in wheat (Dunford et al. 1995) but it is difficult to identify lines containing novel deletions which either exclude or include parts of the *ph1b* deletion region that would facilitate chromosome walking to the *Ph1* locus.

Several methods have been developed to replace the time- and labour-consuming cytological analysis routinely used to score plants for chromosome pairing. Primers derived from the sequences of RFLP markers located within the region defined by the *ph1b* deletion have been used in two PCR-based assays to identify plants lacking the PCR product and hence the *Ph1* locus (Gill and Gill 1996; Segal et al. 1997). These methods could be further improved if the PCR amplifications could be carried out directly from leaf material.

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AFLP is a recently developed powerful fingerprinting technique for genomic DNA of any origin or complexity (Zabeau and Vos 1993; Vos et al. 1995). Results of AFLP fingerprinting are reliable and reproducible. In the present study, AFLP was used to detect polymorphisms between the total genomic DNA of the wheat cultivar Chinese Spring and the Chinese Spring *ph1b* deletion mutant and other relevant lines. Primers that selectively amplify such polymorphic sequences were designed and used in a PCR assay to specifically determine the presence or absence of the *ph1b* deletion.

PCR can be carried out using genomic DNA, but the direct use of plant tissues as a source of DNA in PCR reactions is the ideal target for practical purposes. Indeed, intact fresh or frozen barley leaves can be used as templates in PCR reactions after a brief alkaline treatment (Clancy et al. 1996). Thus, employing a method adapted from Klimyuk et al. (1993), we used DNA from intact leaf material in the PCR assay, which provided a rapid and inexpensive method for screening large numbers of individual plants. In addition, since it is based on a plus-minus screen, the technique could be easily automated.

Materials and methods

Plant material

For AFLP analysis, genomic DNA was extracted from hexaploid wheat cv Chinese Spring, the Chinese Spring *ph1b* deletion mutant (Sears 1977), and homoeologous group-five nullisomic-tetrasomic lines N5AT5B, N5BT5D and N5DT5A (Sears 1966). Leaves were harvested from 6-week-old Chinese Spring euploid and Chinese Spring *ph1b* mutant plants for testing the PCR-based scoring method.

A F_1-derived doubled-haploid population of 55 lines, developed from a cross between the Chinese Spring *ph1b*/*ph1b* mutant line and a Chinese Spring (Cappelle-Desprez *5BL*/*5BS*) single-chromosome substitution line (Snape et al. 1995), was used to test the reliability of the PCR-based scoring method. The doubled-haploid plants were previously classified cytologically for the presence or absence of the *Ph1* gene(s), using crosses with *Aegilops variabilis*. In the presence of the *Ph1* locus, the chromosomes were low-pairing (mainly univalents), whereas in the absence of the gene(s) the chromosomes were high-pairing (bivalents or multivalents were found). Confirmation that a marker is located within the *ph1b* deletion region is obtained if it co-segregates with the high-chromosome-pairing phenotype.

DNA extraction

DNA was extracted according to Sharp et al. (1988) with the following modifications. Leaf material frozen at −70°C was ground with liquid N_2, and the DNA extracted with occasional gentle inversion at 65°C. A second phenol/chloroform extraction was carried out after RNase treatment and the DNA isopropanol-precipitated and dissolved in TE.

AFLP fingerprinting

AFLP fingerprinting was carried out according to Vos et al. (1995) using *Pst*I and *Mse*I adaptors:

*Pst*I adaptor 5′-CTCGTAGACTGCGTACATGCA-3′
3′-CATCTGACGCATGT-5′

*Mse*I adaptor 5′-GACGATGAGTCCTGAG-3′
3′-TACTCAGGACTCAT-5′.

A two-step amplification was used. The first step was a non-selective pre-amplification using *Pst*I and *Mse*I primers:

*Pst*I non-selective primer 5′-GACTGCGTACATGCAG-3′

*Mse*I non-selective primer 5′-GATGAGTCCTGAGTAA-3′.

The products were diluted 50-fold, and a second amplification was performed using *Pst*I and *Mse*I primers with three selective bases. *Taq* DNA polymerase and its buffer were supplied by Boehringer Mannheim Ltd. All the AFLP reactions were performed in a PTC-100 thermocycler (MJ Research, Inc., USA). Radiolabelled products of selective amplification were loaded onto 6% polyacrylamide denaturing sequencing gels. Gels were transferred to chromatography paper (Whatman 3 mm), dried, and exposed to X-ray films (Kodak Biomax-MR) for about 50 h.

Cloning and sequencing of the polymorphic DNA fragments

The X-ray films were aligned with the original dried gels and the gel slices containing the AFLP products of interest were excised. DNA was eluted from the gel slices by soaking in 100-µl of dH_2O overnight. The elutions were diluted 10-fold and a re-amplification step was carried out to generate sufficient DNA for cloning. With 1-µl of the diluted elution as the template, a PCR amplification was performed as described above in 20-µl using the same cycle profile and conditions as for the selective AFLP amplification, with the exception that 75 ng of both unlabelled AFLP primers were used. The re-amplified products were electrophoresed on a 2% agarose gel, the bands excised, and the DNAs purified using the Qiaquick PCR purification system (Qiagen). Ligation into the pMOSBlue T-vector (Amersham), transformation and screening of recombinants were carried out according to the instructions of the manufacturer. Sequencing of the clones was carried out with a Cycle Sequencing Kit (Pharmacia) following the manufacturers instructions. All oligonucleotide primers were synthesized using the Expedite Nucleic Acid Synthesis System (Millipore).

PCR-based scoring of the *ph1b* deletion region

The method for PCR amplification from intact leaf material was adapted from Klimyuk et al. (1993). Single leaves from each wheat plant were harvested into sterile 1.5-ml Eppendorf tubes then stored at −70°C. Immediately prior to use, the leaves were cut into 3–5 mm^2 pieces (5–6 pieces were sufficient), placed into 40-µl of 0.25 M NaOH and boiled for 30 s. 40-µl of 0.25 M HCl, 20-µl of 0.5 M Tris-Cl and 0.25% (v/v) Nonidet P-40 (Sigma) was then added and the tubes were boiled for a further 2 min. A single 3–5 mm^2 piece of the leaf material was transferred into a sterile tube for the PCR reaction; the remaining alkaline-treated samples were stored at 4°C, and these could be used for subsequent PCR analysis by incubating at 100°C for 2 min immediately prior to use. PCR reactions were performed in 50-µl using the same cycle-profile and conditions as for the selective AFLP amplification, except that 75 ng each of the primers specific to the *ph1b* deletion region (see Results and discussion) were employed. In addition, 50 ng each of the primers LJC2F (5′ - ACT GGA TAC AAG CAG GTC C) and LJC2R (5′ - ACC CGT GGT GGA AAT AAG), derived from an RFLP marker *Xpsr137* located on wheat chromosome 2*A* (Smith et al. 1997), were included in the leaf-based PCR reaction as a positive control. To visualize the DNA, 15-µl of the 50-µl amplified reaction mix was electrophoresed on a 2% agarose gel.

Results and discussion

AFLP analysis

AFLP analysis was carried out on seven genomic DNA samples: two each from the Chinese Spring and *ph1b* mutant lines and one each from the N5DT5A, N5BT5D and N5AT5B lines. Two *Pst*I primers (P64 and P65) were used with 64 different *Mse*I primers (M31 to M94). Three selective base primers were used to reduce the complexity of the banding pattern on the AFLP gel, given the large genome size of wheat. These conditions generated about 70–100 reaction products. The occurrence of amplified fragments from the Chinese Spring, N5AT5B and N5DT5A DNA, but not from *ph1b* or N5BT5D DNA, indicated that the product was derived from the region defined by the *ph1b* deletion. Six reaction products ranging in length from 50 bp to around 220 bp (termed PC1 to PC6) were identified as being absent in the N5BT5D and *ph1b* lanes when P65 was used. Two of the products identified on the AFLP fingerprint of the seven wheat lines (PC2 and PC3) are shown in Fig. 1(A) and (B). No such products were identified when P64 was used.

Cloning and sequencing

Twenty clones were obtained from the six Chinese Spring reaction products, and all 20 were sequenced (data not shown). All the sequences had a *Pst*I primer sequence at one end and an *Mse*I primer sequence at the other, which demonstrated the preferential amplification of fragments with a different adaptor at each end (Vos et al. 1995) and showed the reliability of the amplification. Four of the AFLP sequences were too short for suitable sequence-specific primers to be designed, and eight sequences were eliminated because they were identical to others already determined. The eight remaining sequences were checked against sequences in the EMBL database, but no significant DNA sequence homology was found.

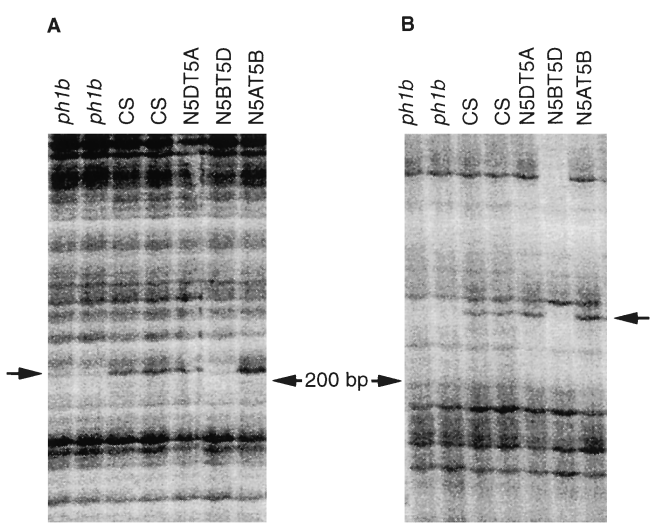

Fig. 1A, B Partial AFLP profiles of wheat genomic DNA. *Lanes 1 and 2 ph1b*; *lanes 3 and 4* Chinese Spring; *lanes 5–7* homoeologous group-5 nullisomic-tetrasomics. Panel **A** AFLP fingerprint using the *Pst*I primer P65 and the *Mse*I primer M47. *Arrow* indicates band PC2-1 (206 bp), absent in the *ph1b* and N5BT5D lanes. Panel **B** AFLP fingerprint using the *Pst*I primer P65 and the *Mse*I primer M56. *Arrow* indicates band PC3-9 (232 bp), also absent in the *ph1b* and N5BT5D lanes

PCR-based scoring of the *ph1b* deletion region

Sequence-specific primers were designed and synthesized for the eight remaining DNA products. The primer sequences included the restriction site, the selective bases, and an additional short sequence 3′ from the selective nucleotides. The Tms of these primers are 58°C, which is suitable for the PCR reaction conditions. Using the diluted pre-amplified products as template, PCR reactions were carried out with these sequence-specific primers. The primers for the reaction products PC2-1 (206 bp) and PC3-9 (232 bp) (primers and reaction products shown in Fig. 2) generated the same pattern as that on the AFLP fingerprint with products missing in the N5BT5D and *ph1b* lanes (Fig. 3). [The AFLP primers used to generate the reaction product PC2-1 were P65 (*Pst*I non-specific primer + selective nucleotide sequence GAG) and M47 (*Mse*I non-specific primer + selective nucleotide sequence CAA), and those used to generate PC3-9 were P65 (+GAG) and M56 (+CGC)]. Furthermore, when used in the PCR-based assay, PC3-9 specific primers generated a product from Chinese Spring DNA but not from *ph1b* DNA when either extracted genomic DNA or alkaline-treated leaf extracts were used as templates (Fig. 4). Two additional primers, LJC2F and LJC2R, were used that amplify a specific 137-bp sequence from wheat chromosome 2*A* (Fig. 4). Inclusion of these positive control primers distinguishes between failed PCR reactions (absence of both 137-bp and 210-bp PCR products), wheat plants with the *ph1b* deletion (presence of 137-bp and absence of 210-bp PCR products), and wheat plants without the *ph1b* deletion (presence of both 137-bp and 210-bp PCR products).

The PC3-9 and PC2-1 specific primers were used to check the segregation of the amplified PCR products with the chromosome-pairing phenotype of the doubled-haploid plants. The products of the PC3-9 primers co-segregated with the high-pairing phenotype and hence with the *ph1b* deletion (Fig. 5). PC2-1 primers created an ambiguous pattern, with faint bands appearing in the lines that had a high-chromosome-pairing phenotype. The PC3-9 primers can therefore be used to detect plants homozygous for the *ph1b* deletion using a small piece of wheat leaf in a PCR reaction. The same

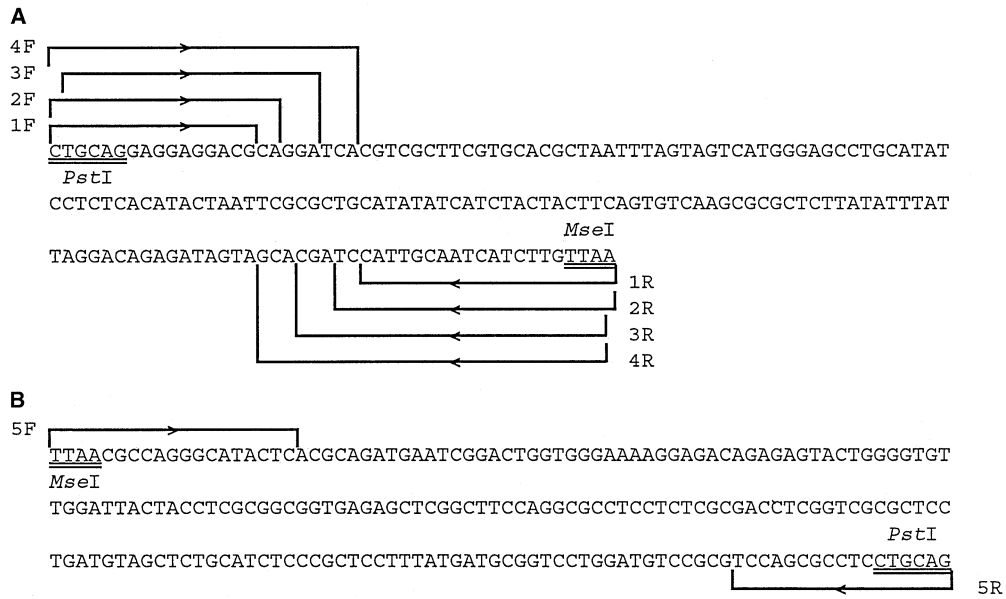

Fig. 2A, B Genomic sequences of two polymorphic amplified fragments. *Pst*I and *Mse*I restriction enzyme sites are underlined; *1F–5F*, sequence-specific forward primers; *1R–5R* sequence-specific reverse primers. **A** PC2-1 (206 bp); **B** PC3-9 (232 bp)

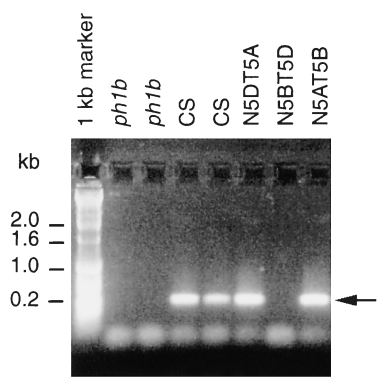

Fig. 3 PCR amplification using the AFLP pre-amplification products as templates and primers specific to PC3-9 [Fig. 2(B)]. *Lane 1* 1-kb DNA marker; *lanes 2 and 3 ph1b*; *lanes 4 and 5* Chinese Spring; *lanes 6–8* homoeologous group-5 nullisomic-tetrasomics. *Arrow* indicates the amplification product missing in the N5BT5D and *ph1b* lanes

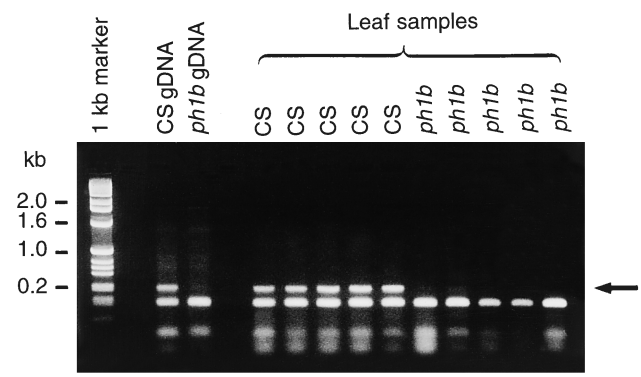

Fig. 4 PCR amplification products using primers specific to PC3-9 [Fig. (2B)]: *Lane 1* 1-kb DNA marker (Gibco BRL); *lane 2* blank; *lane 3* 10 ng Chinese Spring gDNA; *lane 4* 10 ng *ph1b* gDNA; *lane 5* blank, *lanes 6–10* DNA from intact Chinese Spring leaf samples; *lanes 11 to 15* DNA from intact *ph1b* leaf samples. *Arrow* indicates specific amplification in Chinese Spring and absence in *ph1b*. Lower band is a positive control

result was obtained when anthers were used instead of leaf material (N. Iqbal, personal communication). In our hands, the primers of Gill and Gill (1996) and Segal et al. (1997) detected the presence of the *ph1b* deletion when extracted genomic DNA was used in the PCR reaction, but could not detect the presence of the *ph1b* deletion when intact leaf material was employed (data not shown).

In conclusion, we have used AFLP to identify DNA sequences deleted in the *ph1b* mutant of wheat and have developed a simple PCR-based assay to detect plants homozygous for the *ph1b* deletion. The consistent and accurate amplification of specific bands using intact wheat leaves has also been demonstrated. The assay is amenable to automation using systems such as the ABI PRISM 7700 sequence detection system (Perkin Elmer) which allows fluorescent primers to be used during PCR such that, if the target of interest is present, the fluorescent emission can be detected.

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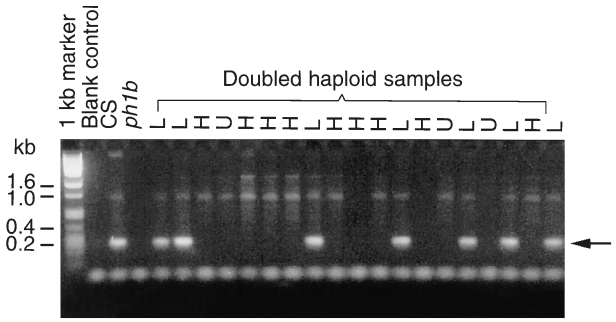

Fig. 5 PCR amplification of wheat doubled-haploid lines segregating for high (*H*) or low (*L*) chromosome pairing, using primers specific to PC3-9 [Fig. (2B)]. *Lane 1* λ*Pst*I DNA marker; *lane 2* blank control, no template DNA; *lane 3* positive control, Chinese Spring euploid gDNA; *lane 4* negative control, *ph1b* gDNA; *lanes 5–24* gDNA of doubled-haploid progeny derived from a cross between the Chinese Spring *ph1b*/*ph1b* mutant line and a Chinese Spring (Cappelle-Desprez *5BL*/*5BS*) substitution line. The specific amplification product for a sequence included in the *ph1b* deletion region is missing in those lines showing a high-pairing phenotype (*arrow*). For three of the lines the chromosome pairing status was undetermined (*U*)

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