Theor Appl Genet (1992) 84: 567–572

THEORETICAL AND APPLIED GENETICS

The use of random amplified polymorphic DNA markers in wheat

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Received November 11, 1991; Accepted December 19, 1991
Communicated by G. Wenzel

Summary. An evaluation was made of the use of random amplified polymorphic DNA (RAPD) as a genetic marker system in wheat. Reproducible amplification products were obtained from varietal, homozygous single chromosome recombinant line and wheat/alien addition line genomic DNA with selected primers and rigorously optimized reaction conditions. Factors influencing the RAPD patterns are DNA concentration, Mg^{2+} concentration, polymerase concentration and denaturing temperature. In wheat, the non-homoeologous, non-dose responsive and dominant behaviour of RAPD products devalues their use as genetic markers for the construction of linkage maps, and the high probability that the amplified fragments derive from repetitive DNA limits their use as a source of conventional RFLP probes. However, RAPD markers will most certainly find many applications in the analysis of genotypes where single chromosomes or chromosome segments are to be manipulated.

Key words: RAPD – PCR – Markers – Wheat – *Triticum aestivum*

Introduction

Random amplified polymorphic DNA (RAPD) has been proposed as a new species of genetic marker, one that overcomes many of the technical limitations of RFLP analysis (Williams et al. 1990; Welsh and McClelland 1990; Rafalski et al. 1991). RAPD would find applications in the construction of linkage maps (Williams et al. 1990), in the identification of strains and varieties by genomic fingerprinting (Welsh and McClelland 1990; Goodwin and Annis 1991; Weining and Langridge 1991) and, following cloning of amplified fragments, as a further source of conventional RFLP probes. In this paper, we evaluate the use of RAPD as a source of genetic markers in wheat.

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Materials and methods

Polymerase chain reaction (PCR) materials

The 10-mers used as random primers in the PCR were purchased from Operon Technologies, Alameda Calif. Taq DNA polymerase, together with 10 × concentrated PCR buffer, was supplied by Boehringer. Other brands of Taq DNA polymerase were tested (BRL, Perkin-Elmer Cetus) and performed equally well. Amplifications were carried out in a Perkin-Elmer Cetus DNA Thermal Cycler and the Techne PHC-3. Agarose (Koch Light) was supplied by New Brunswick Scientific.

Sources of genomic DNA

RAPD analyses were carried out using genomic DNA, extracted as described by Sharp et al. (1988), from 12 bread wheat (*Triticum aestivum* L.) varieties – 'Chinese Spring' (CS), 'Synthetic' (IPSR1190903, McFadden and Sears 1946; Sears 1976), 'Hope', 'Bezostaya I', 'Cappelle-Desprez', 'Mara', 'Sportsman', 'Highbury', 'Timgalen', 'RL4137', 'Hobbit'S" and 'VPM1' (Maia 1967) – all obtained from the IPSR wheat collection maintained at The Centre for Plant Science Research, Norwich. Twenty-one nullisomic-tetrasomic (NT) and the appropriate ditelosomic (DT) lines of the cv 'Chinese Spring' (Sears 1954) were used to assign RAPD bands to chromosome arms. Segregation of a RAPD polymorphism was tested using 22 single chromosome recombinant lines derived from 'Hobbit'S" × 'Hobbit'S" (VPM1 7D) (Worland et al. 1988). The applicability of RAPD for the analysis of wheat/alien addition and substitution lines was tested using the CS/*Hordeum vulgare* cv 'Betzes' single chromosome addition lines (Islam et al. 1981).

Amplification conditions

The amplification conditions were rigorously tested in optimization experiments described below in the results section. A

standard procedure was arrived at, based on the protocol of Williams et al. (1990). The PCR volume was 50 µl and contained 200 n*M* primer, 100 µ*M* each of dATP, dCTP, dGTP and TTP, 20 ng template DNA and 0.8 U of Taq DNA polymerase in 1 × PCR buffer (10 m*M* TRIS-HCl pH 8.3, 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 100 µg/ml gelatine) containing the detergents NP40 (0.05%) and Tween 20 (0.05%). During the manipulations the tubes were kept on ice. The reaction mixtures were overlaid with mineral oil and subjected to PCR. Standard amplifications were performed in a Perkin-Elmer Cetus DNA Thermal Cycler programmed for 60 cycles of 1 min at 94 °C, 1 min at 36 °C and 2 min at 72 °C, using the fastest possible transition between each temperature. After the last cycle, the samples were kept at 72 °C for an additional 5 min and then cooled to 4 °C. Samples of 12 µl were analyzed by electrophoresis on a 1.5% agarose gel, and amplified products were detected by staining with ethidium bromide.

Hybridization conditions

Methods for Southern transfer, probe labelling and filter hybridization were as described by Devos et al. (1992).

Results and discussion

Aneuploid analysis

Figure 1 A shows the amplification result using a single primer and 21 NT lines as template DNA. The banding patterns obtained in wheat with 10-mers as primers usually consisted of one to five products, which, however, could not all be assigned to individual chromosomes using standard nullisomic-tetrasomic analysis. Of the three bands, two major and one minor, obtained with primer B-08, only the smallest could be unequivocally located (Fig. 1 A). A 3BS location was confirmed by ditelosomic analysis (Fig. 1 B). No increase in signal strength was observed in the N3D-T3B sample, which carries four copies of chromosome 3B. The absence of the high-molecular-weight minor band in the nullisomic 5A line (Fig. 1 A) could not be confirmed in subsequent analysis of the appropriate ditelosomic lines. Similarly, the reduced signal strength of the smallest and middle RAPD product in the N5AT5B and N1AT1D line, respectively, was not reproducible.

Three out of 10 primers tested gave patterns where more than one band could be assigned to a chromosome by NT analysis. In each case, non-homoeologous locations were observed. For example, primer A-17 generated one major band, located on chromosome 7D, and three minor bands, of which two could be allocated to chromosome 2A and the other to 5D (results not shown). This is in contrast to results obtained by RFLP analysis where most probes hybridize to sites on homoeologous chromosomes.

The efficacy of RAPD for the analysis of alien chromosome addition lines was demonstrated using the CS/*H. vulgare* single chromosome addition lines. Figure 1 C shows the result obtained with primer B-08, which shows a single major band in the barley cv 'Betzes' and three bands in CS, while the CS/4H addition line clearly displays a barley band in addition to the wheat bands. The allocation of a B-08 RAPD band to 3BS in wheat (Fig. 1 A, B) and to barley chromosome 4 (Fig. 1 C), which has no known homoeology with wheat chromosome 3B, provides further evidence that each amplification product has to be considered as an independent marker.

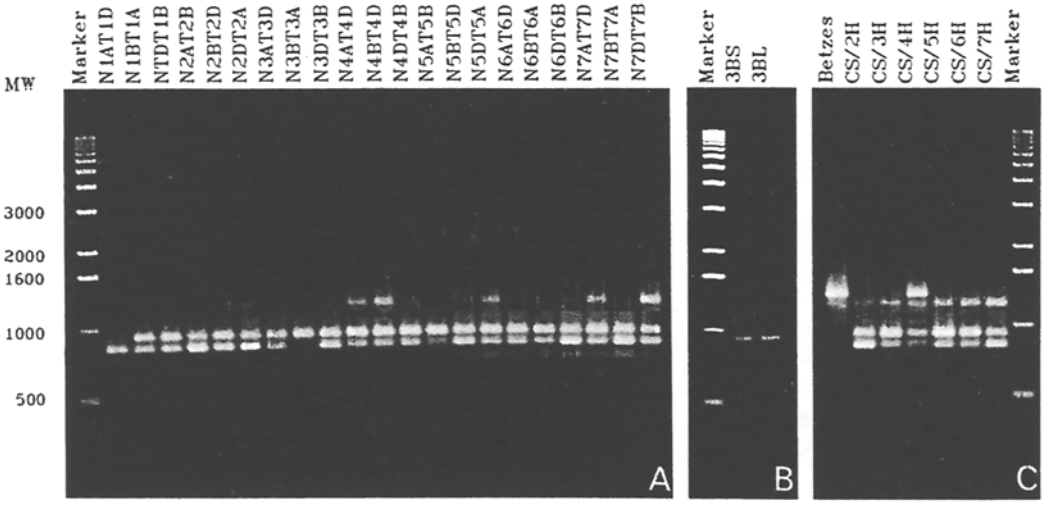

Fig. 1 A–C. RAPD patterns obtained with primer B-08 (5'GTCCACACGG3') with wheat aneuploid and wheat-alien genetic stocks. **A** Twenty-one wheat nullisomic-tetrasomic lines; **B** chromosome 3B ditelosomic stocks; **C** CS/*Hordeum vulgare* cv 'Betzes' single chromosome addition lines. In each case a 1-kb ladder is included as a molecular weight marker

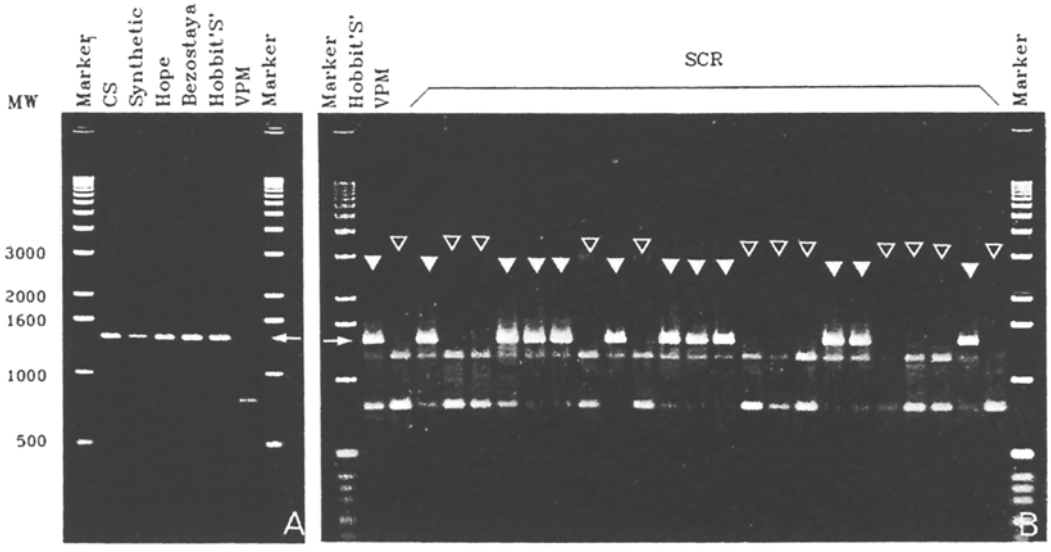

Fig. 2A, B. RAPD polymorphism in wheat obtained with primer A-17 (5'GACCGCTTGT3'). **A** Six varieties; **B** segregation of a RAPD band in a population of 22 single chromosome recombinant lines from the cross 'Hobbit'S'' × 'Hobbit'S'' (VPM1 7D)

RAPD as genetic markers

A major problem associated with the construction of RFLP-based genetic maps in wheat is the particularly low level of polymorphism. Thus, 12 diverse varieties were screened for RAPD profiles with 6 primers, e.g. Fig. 2A, to assess the relative degree of polymorphism. Little variation was observed, and the results with these primers suggest a level of polymorphism for RAPD comparable with RFLP.

Figure 2A shows a polymorphism between VPM1 and 5 other wheat varieties, where VPM1 lacks a RAPD product generated with primer A-17 that is present in CS and located on chromosome 7D by nullisomic-tetrasomic analysis. This polymorphism is likely to be connected with the well-established presence of *Aegilops ventricosa* chromatin in chromosome 7D of VPM1. Its inheritance was followed in a population of 22 homozygous single chromosome recombinant lines from a 'Hobbit'S'' × 'Hobbit'S'' (VPM1 7D) cross, and the segregation did not deviate from the expected 1:1 ratio. However, as RAPD markers are dominant, they are less informative than codominant RFLP markers, and the construction of linkage maps based on the segregation of dominant markers in F_2 populations will require some adaptation of the currently used linkage programs.

In order to investigate the nature of the amplified sequences, the PCR products of 3 primers were transferred to a charged nylon membrane and hybridized with radiolabelled total genomic wheat DNA. Overnight exposure revealed strong signals for most of the products (results not shown), indicating their highly repetitive character. Furthermore, when putative low-copy sequences were isolated and used as hybridization probes in Southern blots of 21 NT lines of wheat cv 'Chinese Spring' where each chromosome was nullisomic in turn, one or more bands were present in every line after short exposure times. Even the barley RAPD product generated by primer B-08 and shown to be located on barley chromosome 4 hybridized uniformly with the wheat NT lines (results not shown). These observations strongly suggest that the majority of amplification products using random 10-mers arise from repetitive DNA sequences, which is not altogether unexpected, since the ratio of repetitive to unique sequences is exceptionally high in the large wheat genome. This almost certainly excludes the general use of the amplified sequences as RFLP probes in wheat.

Influence of different parameters on the random amplification reaction

The establishment of standard conditions for RAPD analysis in wheat, a hexaploid species with a haploid DNA content of 17.3 pg, proved to be difficult due to the sensitivity of the system to experimental variables. Consequently, amplification of random genomic sequences in a reproducible way, as above, is only possible with rigorously optimized reaction conditions.

Optimization of the DNA concentration. To determine the optimal template concentration, DNA amounts ranging from 0.1 ng to 100 ng were added to the standard reaction mixture. All reactions were replicated 4 times to examine the possible influence of DNA concentration on the fidelity of the amplification. The results (Fig. 3) clear-

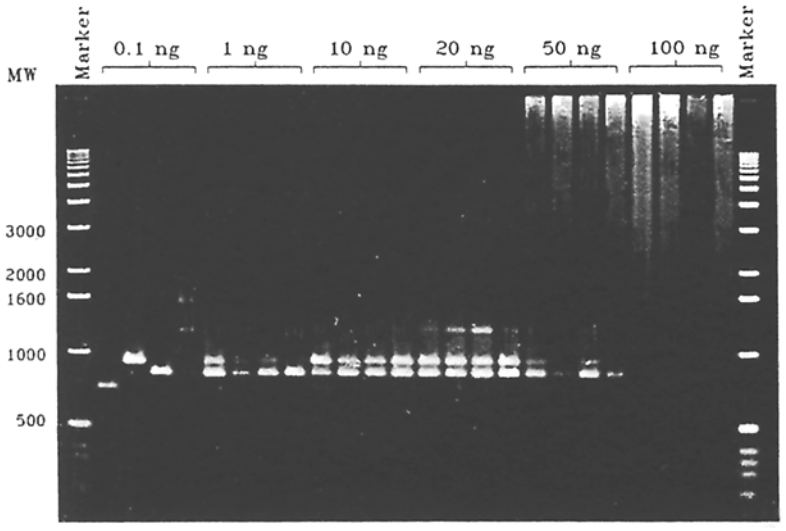

Fig. 3. The effect of genomic DNA reproducible concentration on the generation of reproducable amplification products, using primer B-08 with 'Chinese Spring' genomic DNA

ly show that only the concentration range 10–20 ng yielded reproducible patterns with a primer concentration of 200 nM: too little template DNA gave variable banding patterns, while too much DNA resulted in non-specific amplification. Variability at low template concentrations may be the result of a reduced probability in initiating amplification reactions and reflects the inefficiency of the priming events (Welsh and McClelland 1990). The conversion of PCR products to random-length, higher-molecular-weight fragments, visible as smears on ethidium bromide-stained agarose gels, has also been reported by Bell and DeMarini (1991). Their results suggest that when most of the oligonucleotide primers have been converted into PCR product further priming events will mainly involve the annealing of the 3′-OH ends of these products to genomic template or to themselves. Extension and random termination of these molecules is the likely cause for the observed smear. This implies that the generation of non-specific amplification products can be prevented by manipulating either the ratio of primer/template concentration and/or the number of PCR cycles.

Optimization of the Mg^{2+} concentration. To examine the influence of Mg^{2+} on the efficiency and fidelity of the RAPD amplification, $MgCl_2$ was added to the reaction buffer, which already contains a concentration of 1.5 m*M* $MgCl_2$, to give final concentrations of 1.5 m*M*, 2.0 m*M*, 2.5 m*M* and 3.0 m*M*. The result of the experiment, carried out in triplicate, is shown in Fig. 4. Specific and reproducible results were obtained only in the presence of 1.5 m*M* Mg^{2+}. However, when the amount of template DNA in the reaction was lowered from 20 ng to 5 ng, an increase in Mg^{2+} concentration resulted in the generation of novel bands and/or the disappearance of bands (not shown). This clearly demonstrates a relationship between Mg^{2+} and DNA concentrations and suggests that the stringency of the annealing process is decreased at higher Mg^{2+} concentrations.

Influence of the Taq DNA polymerase concentration. In the determination of the optimal enzyme concentration, both the cost of Taq DNA polymerase and the generation of clear and stable amplification products have to be considered. An increasing number of bands were obtained with increasing enzyme concentration, finally merging into non-specific DNA amplification at enzyme concentrations of about 2.5 U/50 µl reaction volume (results not shown). In our hands, 0.8 U Taq polymerase/50 µl reaction volume consistently gave simple and reliable banding patterns that lacked the minor bands generated under conditions with higher enzyme concentrations.

Influence of the annealing and denaturing temperature. Although annealing was carried out at 36 °C, the effect of a rise of the annealing temperature is minimal up to 40 °C, while all priming events are prevented at 42 °C (results not shown). Consequently, small temperature differences across the block (2 °C in our Perkin Elmer Cetus DNA Thermal Cycler) have negligible effects on the RAPD banding patterns, provided that the priming threshold is not exceeded. A much larger effect was observed when the standard denaturing temperature of 94 °C was lowered to 90 °C. Figure 5 shows the same experiment as that shown in Fig. 1 A, but at a denaturing temperature of 90 °C instead of 94 °C. A comparison of the patterns reveals that major bands produced under a denaturing temperature of 94 °C disappeared and that additional minor bands were formed when denaturing was carried out at 90 °C. A test of the block of our DNA Thermal Cycler revealed a 1 °C difference across the

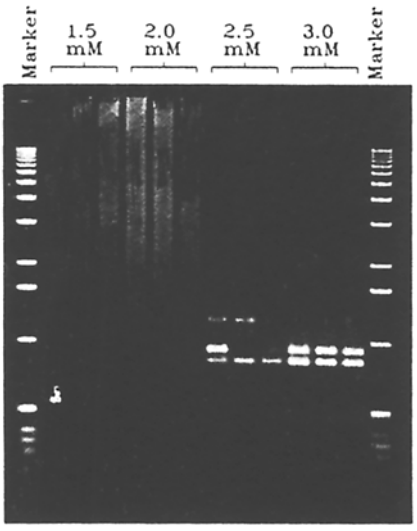

Fig. 4. The effect of Mg^{2+} concentration, using primer B-08 with 'Chinese Spring' genomic DNA

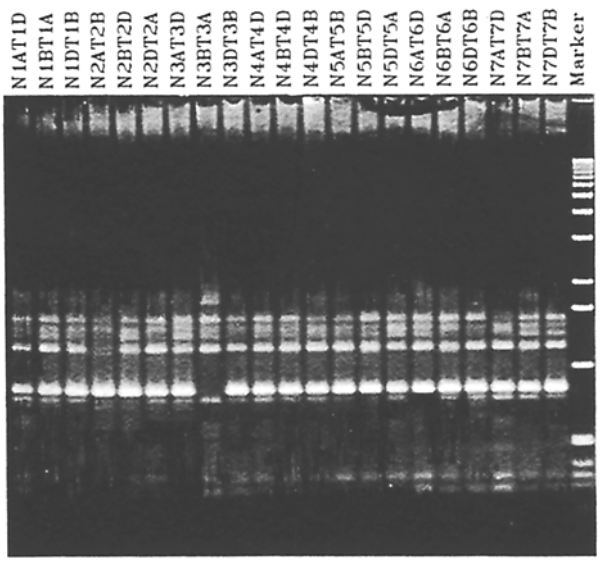

Fig. 5. The effect of denaturing temperature on RAPD profiles. This figure should be compared with Fig. 1 A in which all parameters are identical except that a denaturation temperature of 90 °C was used instead of 94 °C

block at a nominal 94 °C, and thus the minor bands, present in some amplification reactions, might be the result of this unequal heat distribution. Analysis of a similar experiment carried out in a PHC-3 thermal cycler (Techne) revealed no detectable amplification products when a denaturing temperature of 94 °C was employed, and RAPD patterns comparable with these obtained at a denaturing temperature of 94 °C in the Perkin-Elmer Cetus DNA Thermal Cycler were obtained only at a denaturing temperature of 90 °C. These results clearly indicate the different performances of different types of DNA thermal cyclers and the need to test each machine for optimal cycling conditions.

Primer performances. Although our standard amplification conditions were adequate for the generation of RAPD patterns with all 30 primers tested, the optimal template concentrations were not identical for all primers. This can be observed in a comparison of Fig. 3 and Fig. 6; a DNA concentration of 50 ng yielded both specific and non-specific amplification products with primer B-08 (Fig. 3), while only a high-molecular-weight smear was generated with primer A-16 (Fig. 6). Both primer/template ratios, however, were within the range 200 n*M* to 10–20 ng. Furthermore, not all primers performed equally well. Some, presumably due to the lack of suitable priming sites in the wheat genomic DNA, gave poorly amplified banding patterns, while others created "polymorphic" bands, even within a set of identical replicated DNA samples (Fig. 6). Thus, oligonucleotides should be rigorously tested for priming ability and reproducibility before they can be employed as genetic markers.

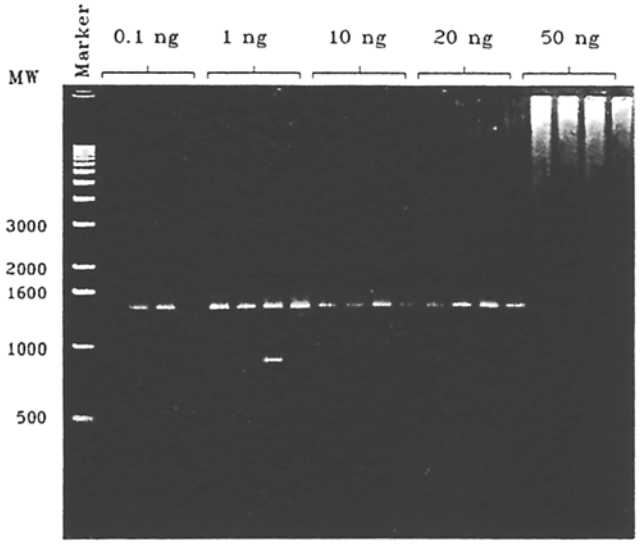

Fig. 6. Primer performance. This figure should be compared with Fig. 3 in which the parameters are identical except that primer A-16 (5'AGCCAGCGAA3') replaces B-08

Conclusions

The sensitivity of the random amplification system indicates that the use of RAPD to produce genetic markers for the construction of linkage maps in wheat is probably not worthwhile at present, in our hands at least. Firstly, the transfer of any RAPD variant from one laboratory to another is likely to result in ambiguities, since amplification patterns on different DNA thermal cyclers, even set

at nominally identical conditions, can be quite different with identical primers and template DNA. Secondly, since RAPD products are the result of arbitrarily priming events, a given primer can amplify a number of non-homologous sequences that may vary between varieties. However, since the chromosomal locations of the bands can not be predicted (this is in contrast to the majority of conventional biochemical and molecular markers in wheat), there will always be a question whether RAPD bands, even those of similar molecular weight, derive from the same locus in different varieties. Cross-hybridization of the product of interest with bands of similar or distinct molecular weight in the varietal RAPD profiles will provide strong evidence for allelism, but since both homologous and homoeologous sequences will generate positive signals in hexaploid wheat, segregation analysis is needed to confirm the identity of the RAPD bands. Cloning the band and Southern hybridization, however, will often not be possible in wheat because the majority of the amplified products originate from repeated DNA sequences. Thirdly, the non-dose-responsive behaviour, as seen in NT analysis, and the non-codominant phenotype further devalue the use of RAPD as a generally applicable marker system in wheat for many applications. Although mapping can be carried out efficiently using doubled haploid or other homozygous recombinant mapping populations, their eventual use in genetic and breeding situations will often be limited by their inability to detect heterozygotes. Finally, our results suggest that the level of polymorphism obtained is likely to be as low as with conventional RFLP analysis. This is countered by the fact that analysis with many primers is easier than using many RFLP probes. However, DNA sequences used as probes have the advantage that a wide range of probe/enzyme combinations can be assayed to reveal RFLP.

There are, however, many applications where RAPD will find great utility in wheat genetics. In general, these are in experiments that are carried out in a single laboratory on a single genotype or population. The production of wheat/alien chromosome addition and substitution lines will benefit from RAPD analysis, as will the introgression, by translocation or recombination, of alien chromosome segments into the wheat genome. The tagging of genes controlling quantitative or other agronomic characters in individual populations of single chromosome recombinant lines, doubled haploid or recombinant inbred populations is likely to be facilitated by use of RAPD, providing the sensitivity of the system to experimental conditions is kept in mind.

Acknowledgement. This publication results from work undertaken in the Agricultural Genetics Company RFLP Club programme supported by Cambridge Plant Breeders Ltd., ICI plc, Ciba-Geigy plc, Plant Breeding International Cambridge Ltd. and Nickerson International Seed Company Ltd.

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