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Detection of DNA sequence polymorphisms among wheat varieties

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Summary. A DNA marker detection strategy that allows the rapid, efficient resolution of high levels of polymorphism among closely related lines of common wheat (*Triticum aestivum*) has been developed to circumvent the apparent lack of restriction fragment length polymorphism in many important self-pollinated crop species. The technique of randomly amplified polymorphic DNA (RAPD) was combined with a denaturing gradient gel electrophoresis system (DGGE) to explore DNA sequence polymorphisms among different genotypes of wheat. Of the 65 primer combinations used for the polymerase chain reaction (PCR) amplifications, over 38% of them produced readily detectable and reproducible DNA polymorphisms between a spring wheat line, SO852, and a winter wheat variety, 'Clark'. A high level of polymorphism was observed among a number of commercial varieties and breeding lines of wheat. This procedure was also used to detect polymorphisms in a recombinant inbred population to test the feasibility of its application in genome mapping. This DNA polymorphism detection system provides an opportunity for pedigree analysis and fingerprinting of developed wheat lines as well as construction of a high density genetic map of wheat. Without the need for ^{32}P and sophisticated DNA extraction procedures, this approach should make it feasible to utilize marker-based selection in a plant breeding program.

Key words: Common wheat – RAPD – DGGE – DNA polymorphism – DNA markers

Introduction

Perhaps the greatest barrier to the use of restriction fragment length polymorphism (RFLP) technology for DNA marker-aided selection in plant breeding is the low level of DNA polymorphism in a number of important crop species. This is a particularly difficult problem in the case of cultivated wheat (*Triticum aestivum*), a self-pollinated hexaploid crop species (Kam-Morgan et al. 1989). Polymorphic loci have been observed in wheat, including loci encoding alpha-amylase (Muthukrishnan et al. 1984), beta-amylase (Sharp et al. 1989), and glutenin subunits (Harberd et al. 1986). Ribosomal DNA and other miscellaneous probes were also reported to yield polymorphisms (May and Appels 1987; Gill et al. 1988). Most of these identified polymorphisms have been limited to loci for certain structural genes. Recently, a chromosome 3B-specific moderately repeated sequence was identified that demonstrates a high level of polymorphism among wheat lines (Harcourt and Gale 1991).

A higher level of polymorphism is detected among hexaploid wheat and related species in the tribe *Triticeae*. A set of wheat RFLP probes specific for the 14 chromosome arms of wheat have been reported to detect polymorphisms between wheat line 'Chinese Spring' and a number of other grasses such as *Hordeum vulgare*, *Secale cereale*, and *Aegilops umbellulata* (Sharp et al. 1989; Kam-Morgan et al. 1989). These probes are very useful for identifying alien introgressions in the exploitation of tertiary gene pools. However, because most wheat breeding programs are still seeking improved genotypes via sexual recombination within the species *Triticum aestivum*, it may be of greater importance to map the hexaploid wheat genome with DNA markers that show intervarietal polymorphisms. Markers of this type located on a high density map provide a more direct method for selecting desirable genes based on their linkage relationship. The integration of polymorphic DNA markers into a plant breeding program can greatly facilitate the manipulation of gene movement among lines and make

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possible the analysis of polygenic characters. Such markers would allow the breeder to identify and evaluate pedigrees of important lines as well as estimate degrees of relatedness among breeding materials (Tanksley et al. 1989).

One reason for the low frequency of RFLPs, aside from genome conservation, is that RFLPs result from base changes within restriction enzyme recognition sites or chromosomal structural rearrangements. DNA base mutations in the rest of the genome can not be detected using standard RFLP analysis methods. Three general strategies have been developed to detect point mutations outside of restriction enzyme recognition sites (Cotton 1989): (1) prepare DNA:DNA, DNA:RNA, or RNA:RNA heteroduplexes of wild-type and mutated sequences and cleave the heteroduplexes with an enzyme specific for single-base mismatches in the heteroduplexes (Gibbs and Caskey 1987); (2) use chemical methods to modify or modify and cleave unpaired bases in DNA heteroduplexes. The modified bases will then retard electrophoretic mobility or terminate polymerase chain reactions (PCR) (Ganguly and Prockop 1990; Gogos et al. 1990); (3) detect single-base mutations by differential melting properties that alter migration of DNA homoduplexes or heteroduplexes during electrophoresis under denaturing gel conditions (Fischer and Lerman 1983; Myers et al. 1987). This last strategy, with a number of modifications, has appeared to be most reliable and informative. Theoretically, up to 100% of the base changes can be resolved under proper conditions (Theophilus et al. 1989; Sheffield et al. 1989, 1990; Abrams et al. 1990). Riedel et al. (1990) used denaturing gradient gel electrophoresis (DGGE) to screen hybridization probes in maize, but in this cross-pollinated crop in which high levels of RFLPs exist, the resolved polymorphism on the denaturing gradient gel was only comparable to the incidence of the RFLP.

To explore DNA variation of the wheat genome and to simplify polymorphism detection, we have developed a strategy in which denaturing gradient gel electrophoresis (DGGE) is combined with a random amplified polymorphic DNA (RAPD) method (Williams et al. 1990; Welsh and McClelland 1990). This procedure was then evaluated using developed wheat lines.

Materials and methods

Wheat germplasm

The winter wheat var 'Clark' (Ohm et al. 1988), developed at Purdue University, and the spring wheat line, SO852, from the People's Republic of China, were used to screen polymerase chain reaction (PCR) primer combinations for percentage polymorphism. Twenty random lines derived by seven generations of single-seed descent from the cross 'Clark' × SO852 were analyzed for segregation of DNA polymorphism. Additionally, 11 other varieties and breeding lines, along with 'Clark' and SO852, were characterized for DNA polymorphisms (Table 1). All of the breeding lines, of different origins and varying degrees of relatedness according to their parentages, resulted from single plants in advanced generations (at least F_6) of inbreeding.

Template DNA preparation

High-molecular-weight genomic DNA was extracted from leaves of 2- to 4-week-old plants grown in the greenhouse or growth chamber. The DNA extraction procedure was a modification of that described by Keim et al. (1988). About 1 g of tissue was ground in liquid N_2 to a very fine powder and incubated with the lysis buffer (50 m*M* TRIS, pH 8.0, 1.0% CTAB, 50 m*M* EDTA, 1 m*M* 1,10 *o*-phenanthroline, 0.7 *M* NaCl, and 0.1% beta-mercaptoethanol) at 60 °C for 90 min. The slurry was chloroform extracted once and centrifuged at 6,000 rpm for 10 min. DNA was then precipitated in an equal volume of isopropanol, pelleted, and suspended in 200 µl TE (10 m*M* TRIS, pH 8, 1 m*M* EDTA). The genomic DNA was diluted in sterile water to a concentration of 0.05 µg/µl.

Primer combinations for PCR

Twenty arbitrarily designed primers of ten deoxyribonucleotides (Kit A) were obtained from Operon Technologies, Inc. All of the oligonucleotides were deprotected. They were suspended in TE buffer (pH 7.15) at 50 m*M* stock concentration. The primers were paired randomly for 65 combinations used to screen for polymorphic amplification.

Polymerase chain reaction

PCRs were conducted in a total volume of 100 µl with a Precision Scientific Genetic Thermal Cycler GTC-2. The reaction mixture contained 50 m*M* KCl, 10 m*M* TRIS-HCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100, 125 µ*M* each of dATP, dCTP, dGTP, and dTTP, 1 µ*M* each of 2 primers, 0.2–0.5 µg genomic template DNA, and 2.5 units of Taq DNA polymerase (Promega). The reactions were performed for 45 cycles of 1 min at 94 °C, 30 s at 36 °C, and 30 s at 72 °C. Amplifications were assayed by agarose gel electrophoresis at 35 V in 1 × TPE buffer on 1.2% agarose. The remaining sample was assayed by denaturing gradient gel electrophoresis.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis was carried out using vertical 16 × 16 cm, 0.75 mm thick, 12% polyacrylamide gels with a linear gradient of 10–50% denaturant [100% = 7 *M* urea, 40% formamide (v/v)]. The direction of the denaturant gradient was parallel to that of the electric field, with the highest concentration at the anode and the lowest at the cathode. Gels were run in a Bio-rad Protean II apparatus in 3.5 l TAE running buffer (40 m*M* TRIS acetate, pH 7.4, 20 m*M* sodium acetate, 1 m*M* EDTA) maintained at 60 °C with a thermal water circulator (Lauda). Electrophoresis was conducted at 150 V for 5 h. Gels were then stained with ethidium bromide and photographed.

Results

Frequency of polymorphic amplification

We used genomic DNA prepared from a Chinese spring wheat line, SO852, and a soft red winter wheat var, 'Clark', to screen primer combinations for readily detectable and reproducible polymorphic PCR amplifications.

Although single random primers can be used effectively for this purpose (Welsh and McClelland 1990; Williams et al. 1990), random pairing of primers should give rise to n/2 times as many combinations, reducing the cost for designing primers. To test reproducibility of results, each PCR reaction was repeated 2–4 times.

Contrasting results were obtained using agarose gel electrophoresis versus denaturing gradient gel electrophoresis. Agarose gel electrophoresis revealed little or no reproducible polymorphic amplification (Fig. 1). The PCR conditions employed in our study allowed amplification of one to three major bands on an agarose gel plus a few minor bands in many cases. These minor bands, although occasionally polymorphic in migration, were not always reproducible and, therefore, not reliable as markers.

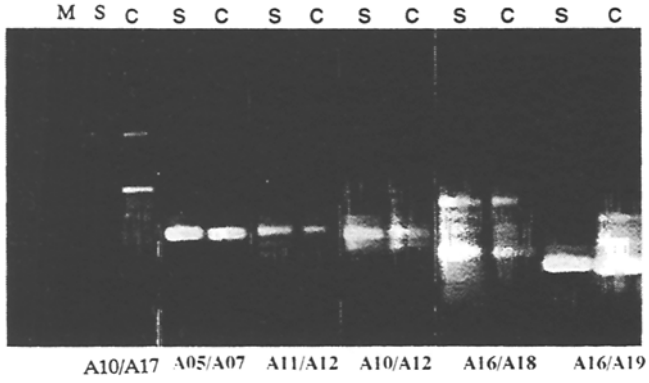

Fig. 1. Agarose gel electrophoresis of randomly primed DNA amplifications. *M* represents molecular weight markers of lambda phage digested with *Pst*I, *S* a spring wheat line SO852, and *C* the winter wheat var 'Clark'. Primer pairs used for each pair of the wheat lines are designated *below* each *lane*. The sequences (5'-3') of the primers are: *A05* AGGGGTCTTG, *A07* GAAACGGGTG, *A10* GTGATCGCAG, *A11* CAATCGCCGT, *A12* TCGGCGATAG, *A16* AGCCAGCGAA, *A17* GACCGCTTGT, *A18* AGGTGACCGT, *A19* CAAACGTCGG

By contrast, a relatively high level of readily detectable and reproducible polymorphisms was observed when PCR samples were subjected to denaturing gradient gel electrophoresis. Figure 2 (panels A and B) shows the results of two replications of PCR reactions. Rather than molecular weight as the primary determinant of DNA migration pattern, the thermal stability of DNA fragments greatly influences DNA fragment migration. Of the 65 pairs of primers screened, over 38% of them led to reproducible polymorphic amplifications. Typically, more bands were resolved on a denaturing gradient gel than on an agarose gel for the same sample, presumably because DNA fragments of approximately the same size but different sequence were separated on the denaturing gradient gel. We observed three types of polymorphisms using DGGE: shifts in the migration of fragments amplified in both lines, missing bands from 1 or the other line (null phenotypes), and bands present in both lines but quantitatively different (Fig. 2).

Polymorphisms among varieties of different relatedness

A number of commercial wheat varieties, breeding lines, and accessions of different origins (Table 1) were tested for polymorphism using different primer pairs. Figure 3 shows the results of PCR using primers A11/A16 to amplify sequences from the 13 wheat lines and subjecting samples to DGGE. When agarose gel electrophoresis was carried out, no reproducible polymorphism was detected (data not shown). However, multiple polymorphic bands were detected among the 13 lines using these particular primers in combination with DGGE. Using 4 pairs of primers, the overall level of polymorphism among these varieties, measured as the proportion of

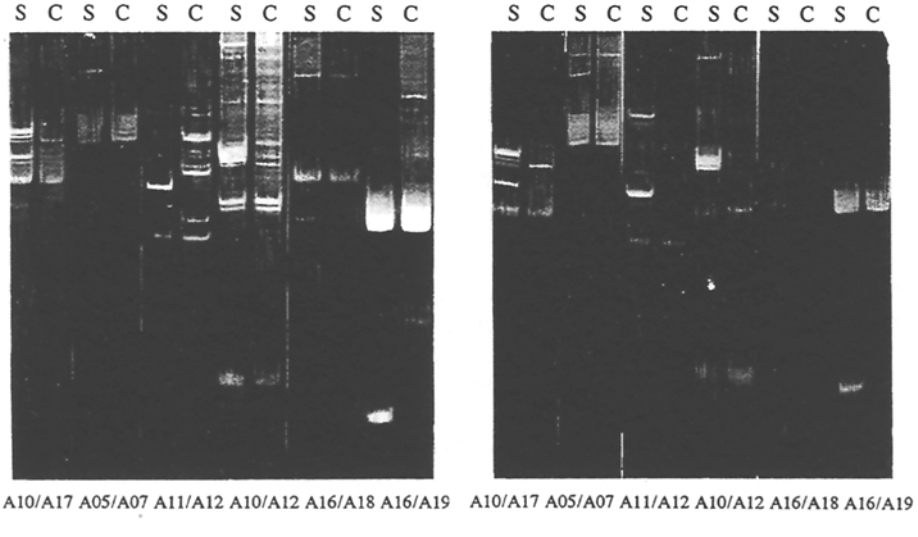

Fig. 2. Denaturing gradient gel electrophoresis of randomly amplified PCR products. The DNA samples in *panel A* are the same as that in Fig. 1. *Panel B* shows the DGGE results of a repeating set of PCR reactions. DNA samples in both panels are presented in the same order as that in Fig. 1. *S* represents the spring wheat line SO852 and *C* the winter wheat var 'Clark'. Primers used are designated *below* the *lanes*

Table 1. Varieties and breeding lines used in fingerprinting analysis

Wheat line	Pedigree	Origin
A. Tyler	Blueboy//Thorne*5/ 199-4/3/Blueboy	Virginia
B. Cardinal	Logan*2/3/Va 63-52-12/ Logan//Blueboy	Ohio
C. 85138D1-3-2	Newton*7/Ella	Purdue
D. 831800A1-7-2-5-2-12	Roazon/Caldwell	Purdue
E. Szeged 215	Jubiljnaja 50/Szava// Ramaja h7/Partizanka	Hungary
F. 871329B11-X-14-2	Acc1719(VPM/Moisson)/ Clark//Dynasty	Purdue
G. 811670A9-10-6-7-22-1	Caldwell//Beau/Kavkaz	Purdue
H. 79410D1-3-3-5-2-84-3	Auburn//Caldwell/ Sullivan	Purdue
I. 85809A5-2-6-2	Clark/79424H1-20/4/ Auburn//Acc1690/ Caldwell/3/Caldwell	Purdue
J. 8138I1-16-X-2-2-1-1-3-3-2	Auburn//751814A6-1/ 72468C21-186-1	Purdue
K. Cotipora	Veranopolis*2/Egypt101	Brazil
L. SO852	–	China
M. Clark	72468C21-17-1// 68283A1-11/71516C1-5-9-1	Purdue

differences detected in all possible pairwise comparisons between varieties, was about 90%. The high level of polymorphism exists even among closely related lines. For example, the winter wheat var 'Auburn' was a common parent in the final crosses leading to the elite breeding lines 79410D1-3-3-5-2-84-3 and 8138I1-16-X-2-2-1-1-3-3-2. Of 4 pairs of primers used for PCR amplification, 3 generated distinct polymorphic amplifications between the 2 lines. We are currently evaluating this system for estimation of relatedness among wheat and oat varieties. What is perhaps a more important implication of these observations to wheat breeders is the possibility that such polymorphism may be mapped and utilized for marker-assisted selection in crosses of relatively closely related materials.

Segregation of polymorphic loci in a single-seed descent population

To test the feasibility of using this rapid screen to facilitate genetic mapping, we have applied this procedure to detect DNA sequence polymorphisms in a recombinant inbred population segregating for a number of important phenotypic traits. The recombinant inbred population was derived from the cross 'Clark' × SO852 by means of single-seed descent (SSD). After seven generations of inbreeding, it is highly inbred; therefore, for any single locus there are only 2 genotypes expected. The population contains 80 genotypes. Twenty genotypes were randomly sampled for PCR amplification. Figure 4 shows the results of amplification using primers A11/A16 with DNAs from 10 genotypes on a denaturing gradient gel. Although no reproducible polymorphisms were observed using agarose gel electrophoresis (data not shown), two patterns of amplification at a specific locus were detected using DGGE. The two patterns correspond to the parental patterns. Our results suggested Mendelian segregation of the DNA sequence polymorphism in the recombinant inbred population.

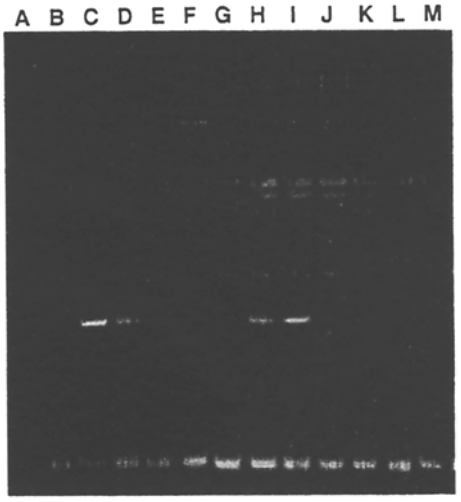

Fig. 3. Denaturing gradient gel electrophoresis of randomly amplified DNAs from 13 different wheat lines. The primer combination is A11/A16 (primer sequences are shown in Fig. 1). Wheat lines used are listed in Table 1

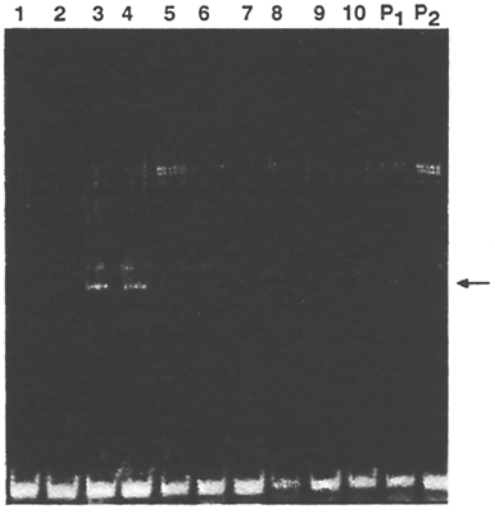

Fig. 4. Denaturing gradient gel electrophoresis of randomly amplified DNAs from a recombinant inbred population. The primer combination is A11/A16 (primer sequences are shown in Fig. 1). P_1 and P_2 designate the parents SO852 and 'Clark', respectively, and *lanes 1–10* are located with DNAs from ten F_7 progeny in the recombinant inbred population. The marker shows the segregation at a locus

Discussion

Levels of DNA polymorphisms

By applying the randomly primed PCR products to denaturing gradient gels, we have been able to detect high levels of reproducible polymorphisms measured both as the percentage of primer pairs resulting in polymorphic amplification (38%) and as the proportion of polymorphisms in all possible pairwise comparisons of genotypes (90%) for a given pair of primers. Three types of polymorphisms appeared to be present in our study: DNA fragment shifts, absence of a fragment (null phenotype), and differences in fragment stoichiometry. We have not yet determined the causes of these different phenotypes, but their consistent repeatability suggests that they are not an artifact of the PCR technique and may be useful as markers. One of the advantages of the RAPD method is that arbitrarily designed random primers for PCR provide a broadened scope of potential template DNA. In theory, the entire plant genome is targeted for primer annealing, facilitating development of a high density map.

Use of RAPD/DGGE DNA polymorphisms in genotype fingerprinting

We have applied this DNA polymorphism detection procedure to a number of wheat commercial varieties, breeding lines, and accessions of known pedigree. Characteristic amplification patterns were demonstrated for these varieties. These characteristic patterns allow one to distinguish between related genotypes, suggesting their usefulness in pedigree analysis or genotype fingerprinting. Although the origin of the amplification products is still being investigated, the complexity of the banding pattern resulting from DGGE suggests that primer annealing occurs at multiple sites within this hexaploid genome, allowing one to scan a number of sites for polymorphisms per reaction. The relative ease of the assay, in comparison to some conventional protein marker assays, may make this system amenable for use by plant breeding programs and the seed industry.

Application of the DNA polymorphism in genetic mapping

The Mendelian segregation pattern observed in the recombinant inbred population indicates that the detected polymorphisms may be used in the construction of a high density genetic map. More importantly, the level of polymorphism detected among wheat varieties should provide wheat breeders with DNA markers that will facilitate field selection. The DNA polymorphisms detected using this procedure, in comparison to conventional RFLPs, are not only detected at a higher level among wheat varieties, including closely related genotypes, but also visualized without the use of [^{32}P]radioisotope. Furthermore, the polymorphism screening can be carried out fairly rapidly on a large scale. A polymerase chain reaction took about 4 h, and the electrophoresis 5 h in our study. Prepared gels could be stored in the cold room (4 °C) for at least 2 days prior to electrophoresis, and electrophoretic apparatus with multiple gel assembly allows one to run over 100 samples in a single experiment.

Limitations of PCR

While providing a powerful tool in biological research, PCR has its limitations. For instance, PCR can only efficiently amplify within a certain size range of DNA, and TaqDNA polymerase will introduce errors. The accumulated mutation rate after 20–30 cycles was reported to be as high as 0.3–0.8% (Keohavong and Thilly 1989; Belyavsky et al. 1989). The latter drawback may be of consequence when using the DGGE system because the gel system may be sensitive enough to detect such errors as polymorphisms. With the RAPD method of fragment amplification, an often occurring artifact on agarose gels is the resolution of some minor bands that are not repeatable. These unstable bands have been suggested to result from the formation of artificial heteroduplexes between multiple amplified fragments (Wenger and Nielson 1991) or from non-specific amplification, that is, amplification when primer/template homology is not perfect. These artifacts were minimized by optimizing PCR components such as the concentrations of each reaction component and the duration and temperature for template denaturation, primer annealing, and extension. The gradient gel, run at 60 °C, may also, to some extent, control the consistency of PCR products by denaturing artificial heteroduplexes. We verified the polymorphic amplifications by denaturing and reannealing PCR products prior to gradient gel electrophoresis and comparing these samples with the nascent ones. Electrophoretic patterns from the reannealed samples did not differ significantly from the original samples (data not shown).

In some lines derived from the recombinant inbred population, we observed reproducible polymorphic bands that were present in neither parent nor in any of the other progeny lines (data not shown). Because this type of anomaly was observed in more than 1 line, using different primers, and the lines demonstrating such an unusual polymorphism did so with only 1 primer combination, we concluded that this was not due to germ plasm contamination. Further investigation is needed to determine the cause of these aberrant polymorphic patterns. However, these extra bands are distinct from the identified polymorphic bands and, therefore, do not interfere with interpretation of the segregation pattern.

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Note added in proof

It should be noted that acrylamide gel electrophoresis has been used to enhance resolution of RAPD products previously (Caetano-Anollés, G., Bassam, B and Gresshoff, P. 1991, Biotechnology 9:553–557). However, we find that the addition of denaturant produces a significant difference in banding pattern and polymorphism level.