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Denaturing gradient gel electrophoresis identifies genomic DNA polymorphism with high frequency in maize

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Summary. We have used denaturing gradient gel electrophoresis (DGGE) to identify genomic DNA polymorphism in maize (*Zea mays* L.). DGGE probes detect polymorphism in maize at a frequency comparable to the incidence of restriction fragment length polymorphism (RFLP). Probes identifying polymorphism were mapped to maize chromosome arms by utilizing DGGE and maize lines carrying B-A chromosomal translocations. The methods for library construction, probe screening, and genome analysis, described here for maize, can also be applied to the genomic analysis of other organisms.

Key words: Denaturing gradient gel electrophoresis – DNA polymorphism – Restriction fragment length polymorphism – *Zea mays* – B-A translocation

Introduction

DNA sequence variation can be detected by the screening of different genomes for restriction fragment length polymorphism (RFLP) using DNA fragments derived from one genotype to probe other genomic DNA of varying pedigree (Wyman and White 1980). RFLP markers have been used to construct genetic maps in human (White et al. 1985; Donis-Keller et al. 1987) and several other species, including maize (Helentjaris et al. 1986; Helentjaris 1987), tomato (Tanksley et al. 1987), lettuce (Landry et al. 1987), and *Arabidopsis* (Chang et al. 1988). These maps and markers are currently used as powerful analytical tools in experimental, diagnostic, forensic, and agricultural breeding applications (Beckmann and Soller 1986; Paterson et al 1988).

RFLPs result from base-pair change(s) within a restriction enzyme recognition site, deletions or insertions within a restriction fragment, or chromosomal rearrangements. For species in wich the occurrence of these genetic alterations is infrequent, such as self-pollinating species like soybean (Apuya et al. 1988), the construction of a genetic map of RFLP markers is difficult and may be of limited practical use.

Recently, an alternative to RFLP analysis has been described that utilizes denaturing gradient gel electrophoresis (DGGE) to identify DNA polymorphism (Lerman et al. 1986). Genomic DGGE analysis identifies DNA polymorphism on the basis of changes in the melting properties of genomic DNA fragments. Changes as small as a single base-pair difference between two DNA fragments of equal size can be detected by this technique (Lerman et al. 1984).

During DGGE, DNA fragments are electrophoresed through a polyacrylamide gel containing a chemical-denaturant gradient of increasing concentration that mimics a gradient of increasing temperature. At a particular denaturant concentration, an appropriately selected DNA fragment becomes partially melted and undergoes a dramatic decrease in electrophoretic mobility (Fischer and Lerman 1983). The region of a DNA fragment that melts first under partially denaturing conditions is called the early melting domain of the DNA fragment (Lerman et al. 1984). DNA fragments with different melting characteristics are separated in DG gels because denaturant concentration is associated with a particular physical location in the gel.

For the genomic analysis reported here, radioactively labeled, single-stranded DNA probes derived from the maize inbred B73 are hybridized in solution to restricted and denatured genomic DNA of different maize lines. The heteroduplex formed between the probe and

the genomic DNA fragment is analyzed by DGGE to identify polymorphism. Because base-pair mismatches significantly alter the melting behavior of a heteroduplex DNA molecule, DGGE detects virtually all base-pair mismatches in the early melting domain of the DNA fragment being assayed (Lerman et al. 1986). Myers et al. (1985) and Noll and Collins (1987) have used heteroduplex analysis with DGGE to detect DNA polymorphism in human genomic DNA for two specific loci.

In this paper we demonstrate that single-copy DNA probes, used in a heteroduplex assay with DGGE, identify polymorphism in maize inbred lines with high frequency. The frequency of allele identification utilizing DGGE is similar to the frequency reported for RFLP analysis in maize, an organism with a high RFLP incidence. We also show that DGGE probes can be mapped to chromosome arms by analyzing genomic DNA from a set of hypoploid maize lines that have been generated by the B-A translocation system (Roman 1947; Roman and Ullstrup 1951; Beckett 1978). Our methods of generating a library of DNA probes and performing genomic analysis with DGGE allow the rapid accumulation of a large collection of DNA probes for polymorphism analysis, and are applicable to the genetic analysis of other organisms.

Material and methods

Genetic stocks

Maize inbreds used in this work were H100, Pa91, Mo17, B73, A619, A632, A634, and L289. L289 lines carrying different B-A translocations (TB-1La, TB-1Sb, TB-3La, TB-3Sb, TB-4Lc, TB-4Sa, TB-5La, TB-6Lc, TB-6Sa, TB-7Lb, TB-9Lc, TB-9Sd, TB-10L19, and TB-10Sc) were crossed as male parents onto five inbred lines (A619, A632, A634, B73, and Mo17) to generate putative hypoploid progeny for each of 14 separate chromosome arms. F_1 progeny that were hypoploid for either chromosome arm *5S, 8L,* or *7S* were generated by pollinating four inbred lines (B73, Mo17, A632, or A619) by inbred A619, A632, B73 and Mo17 lines carrying TB-5Sc or TB-8Lb, or by an inbred A619 line carrying TB-7Sc, respectively. Each male was also crossed onto appropriate tester lines to verify that it carried the correct B-A translocation. Derivation of the B-A translocation lines and specific tester lines is described elsewhere (B.-Y. Lin and J. Beckett, in preparation). Progeny rows of all the crosses were subsequently planted. An example of the pollination strategy and of the chromosome constitution of progeny plants is diagrammed in Fig. 1. Hypoploid plants were identified by morphology and by analysis of pollen viability. Hypoploid plants produce pollen with only 50% viability; the abortive pollen carries the deletion of the chromosome arm involved in a translocation (B.-Y. Lin and J. Beckett, in preparation).

Genomic DNA isolation

Tissue from different inbred and hypoploid lines was harvested for DNA isolation from unpollinated, immature ear shoots of 3-month-old plants, quick-frozen in liquid nitrogen, and stored at −80 °C until use. Genomic DNA was prepared by a standard procedure (Sheldon 1982) modified as follows: 100–1,000 g aliquots of tissue were ground briefly under liquid nitrogen and

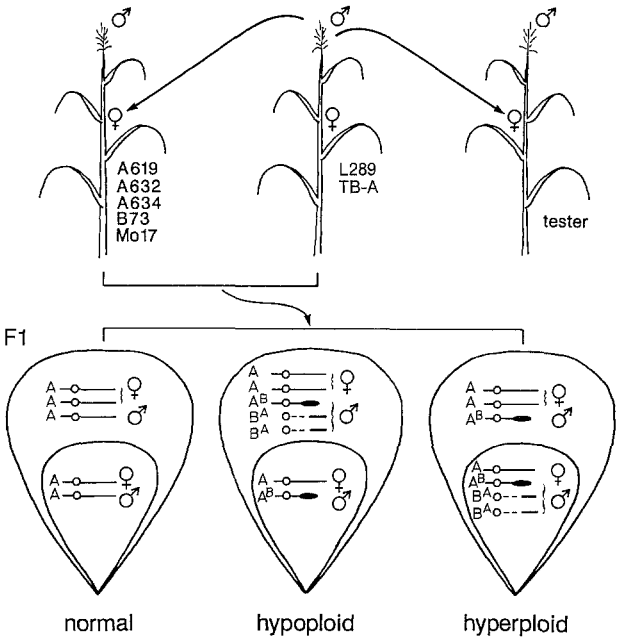

Fig. 1. Pollination scheme for the generation of hypoploid progeny plants. A plant carrying an appropriate B–A translocation in the background of L289 (L289 TB-A) is used as the pollen parent, and crossed onto all five inbred lines, as well as the appropriate tester strain. The B–A translocation line is maintained by using the line as the female parent in a cross with inbred L289 (Beckett 1987). Since the B–A translocation chromosome is maintained in a heterozygous state and segregates during its propagation, it is essential to test each B–A translocation pollen donor

then at room temperature in the presence of 2 vol. lysis buffer [1% sodium dodecylsulfate (SDS), 0.1 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.0, 0.15 *M* NaCl] and 1 vol. phenol (containing 0.1% 8-hydroxyquinoline and saturated with 10 m*M* TRIS, pH 8.0, 1 m*M* EDTA, 10 m*M* NaCl). The extract was incubated at 60 °C for 10 min, followed by centrifugation at 10,000 × g for 10 min. The aqueous phase was extracted twice with water-saturated phenol solution (49.5% phenol, 49.5% chloroform, 1% isoamyl alcohol), brought to 0.3 *M* sodium acetate, and ethanol precipitated. DNA was gently spooled out with a bent glass rod, redissolved in water at the ratio of 1 ml per 4 g tissue, and treated with 50 µg RNase A (boiled). The genomic DNA was further purified by cesium chloride centrifugation, dissolved in TE (10 m*M* TRIS, pH 8.0, 1 m*M* EDTA) at 1 mg/ml final concentration, and stored over chloroform at 4 °C until use. Typical yields were approximately 25–75 µg per gram tissue.

Generation of maize DGGE probes

Molecular cloning procedures were as described by Maniatis et al. (1982) unless otherwise noted. Genomic DNA of maize inbred B73 was digested with BamHI and EcoRI and size-fractionated by gel electrophoresis in 0.8% low-melting agarose. DNA of approximately 400–1,500 bp in length was purified on Elutip-d columns (Schleicher and Schuell) according to the manufacturer's instructions, and ligated to BamHI- and EcoRI-digested phage M13mp19 RF DNA. The ligation mixture was used to transform *E. coli* strain XL1-Blue (Stratagene). To select maize DNA clones present in the genome in low-copy number (Landry and Michelmore 1985), each clone was tested for its

ability to hybridize to total B73 maize DNA. M13 clones containing maize DNA were transferred by toothpick onto duplicate plates overlaid with a lawn of XL1-Blue, grown overnight, and transferred to Biodyne nylon filters (Pall Ultrafine Filtration, Inc.) according to the manufacturer's instructions. Filters were probed with B73 total genomic DNA that had been cleaved with EcoRI and BamHI and labeled by nick translation. After autoradiography, clones that did *not* show hybridization to B73 above background were selected as candidate "low-copy" clones and were further characterized.

Clones were subsequently screened for the absence of BamHI and EcoRI sites internal to the inserted DNA. Clones with unique BamHI and EcoRI sites then were screened by DGGE: maize DNA fragments showing a significant decrease in electrophoretic mobility during DGGE were used for polymorphism screening.

Manual isolation of single-stranded or M13 RF DNA was performed as described (Maniatis et al. 1982). Robotic isolation of M13 RF DNA was as described by DeBonville and Riedel (1986).

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) was performed as described previously (Myers et al. 1987). Briefly, 14% polyacrylamide gels (25 ml) with a linear gradient of 0%–80% denaturant [100% = 7*M* urea, 40% formamide (v/v)] were poured between glass plates separated by teflon spacers (0.75 mm thick), with sample well width of 8 mm. The gel was poured so that a 1-cm region of 0% denaturant was below the sample wells. Gels were run in an apparatus similar to that described by Myers et al. (1987) and immersed in an aquarium (the cathode) of running buffer (40 m*M* Tris-acetate, pH 7.4, 20 m*M* sodium acetate, 1 m*M* EDTA) kept at 60 °C with a circulating heater. Gel electrophoresis was for 16 h at 150 V, after which gels were stained with ethidium bromide and photographed, or dried for autoradiography. Exposure of Kodak XAR5 film was done at −80 °C for 12–168 h with or without an intensifying screen and flashing the film.

DNA probe biochemistry

Restriction enzymes were obtained from New England Biolabs, streptavidin agarose and Klenow polymerase from Bethesda Research Laboratories, and α-[^{32}P] dATP from New England Nuclear. A phage M13 sequencing oligonucleotide primer (ACGTTGTAAAACGACGGCC), synthesized using an Applied Biosystems DNA synthesizer and biotinylated at the 5′ end, was generously provided by the DNA synthesis laboratory of Dr. G. Brown at Genetics Institute. Single-stranded radiolabeled probes with a specific activity of 2.4×10^8 cpm/μg (Cerenkov) were synthesized using M13 primer extension by a modification of standard procedures (Noll and Collins 1987). Specifically, 1 μg M13 template DNA containing the appropriate insert was annealed with 5 p-mol biotinylated M13 universal sequencing primer and extended using the large fragment of DNA polymerase I in a reaction mixture consisting of 10 m*M* TRIS-HCl, pH 8.0, 10 m*M* $MgCl_2$, 50 m*M* NaCl, 8.3 m*M* DTT, 13.3 μ*M* dCTP, dGTP and dTTP, 6.7 μ*M* dATP, and 40 μCi of [α-^{32}P]-dATP (3,000 Ci/mmol, 1 Ci = 37 GBq) at 37 °C for 60 min in a 16-μl total volume. The radiolabeled probe was cleaved at a position immediately 3′ of the insert using BamHI in the presence of 150 m*M* NaCl at 37 °C for 60 min. The reaction mixture was adjusted to 110 μl and 0.1 *N* NaOH, incubated at 65 °C for 10 min to denature the probe, and diluted to 1 ml with buffer A (0.1 *M* TRIS-HCl, pH 8.0, 5 × Denhardt's, 0.2 *M* NaCl). The probe was bound to a streptavidin agarose column (BRL, 0.2-ml bed volume), washed extensively in succesion with 10-column vol. each of buffer B (10 m*M* TRIS-HCl, pH 8.0, 5 × Denhardt's, 0.2 *M* NaCl), buffer C (100 m*M* TRIS base, 5 × Denhardt's, 0.2 *M* NaCl, pH 10.5), buffer B, and biotin buffer (1 m*M* biotin, 10 m*M* TRIS-HCl, pH 8.0, 0.2 *M* NaCl) to remove free nucleotides, nonspecific radiolabeled fragments, and M13 template. Bound probe was eluted by resuspending the column matrix in biotin buffer, incubating in a boiling water bath for 10 min, and collecting the void volume of the column at room temperature.

Genomic DNA polymorphism analysis by DGGE

Genomic DNAs from different inbred lines were digested to completion with EcoRI and BamHI in the presence of 150 m*M* NaCl, 0.1 mg/ml gelatin, 10 m*M* $MgCl_2$, and 6 m*M* 2-mercaptoethanol at 37 °C overnight. Digests were purified by extraction with phenol and chloroform, ehtanol precipitation, and were dissolved in water at a final concentration of 1 mg/ml.

Hybridizations were carried out in 1.5-ml eppendorf tubes or sterile, flat-bottomed microtiter plates sealed with parafilm. Genomic DNA aliquots (5–10 μg) were denatured in 0.1 *N* NaOH in a total volume of 11 μl at 37 °C for 30 min , and neutralized with 1.1 μl 0.3 *M* citric acid. Hybridizations were performed in 20 μl total volume using a 1.5–3 molar excess (generally 800–1,000 cpm (Cerenkov)/1,000 base fragment) of single-stranded, radiolabeled probe in the presence of 100 m*M* TRIS-HCl, pH 8.0, and 350 m*M* NaCl at 65 °C for 16 h. Background reduction was essentially as described (Noll and Collins 1987) using 10 ng of the appropriate M13 template to capture excess radiolabeled probe. Loading buffer was added to a final concentration of 10 m*M* TRIS-HCl, pH 8.0, 1 m*M* EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, 1.5% Ficoll Type 400, and the samples were analyzed by denaturing gradient gel electrophoresis.

Genomic analysis at the waxy locus

Procedures used for DNA blots were as described in Maniatis et al. (1982). A 660-bp *waxy* BamHI fragment (Shure et al. 1983) was radioactively labeled by the random primer method (Feinberg and Vogelstein 1983) and used to probe Southern blots of maize inbred DNA. For DGGE analysis, a labeled 660-bp *waxy* BamHI probe was prepared from an M13 clone as described above, except that XbaI was used in place of BamHI to cleave the primer extended probe (in this case, cleavage by BamHI would have separated the newly synthesized probe from the biotinylated primer sequence).

Results

Comparison of DGGE and RFLP analysis at the waxy locus

As an initial experiment to test the usefulness of DGGE for genomic DNA analysis in maize, we compared the detection of DNA polymorphism by RFLP and DGGE techniques at a well-characterized genetic locus. For the probe in this experiment, we used a 660-bp BamHI fragment of the *waxy* gene derived from the F_2 generation of the W23 × K55 hybrid strain (Shure et al. 1983). The *waxy* gene is located on the short arm of chromosome 9. When this DNA fragment is subjected to DGGE, it exhibits a significant reduction in electrophoretic mobility (data not shown); it is thus a potentially useful probe for DGGE analysis (see below).

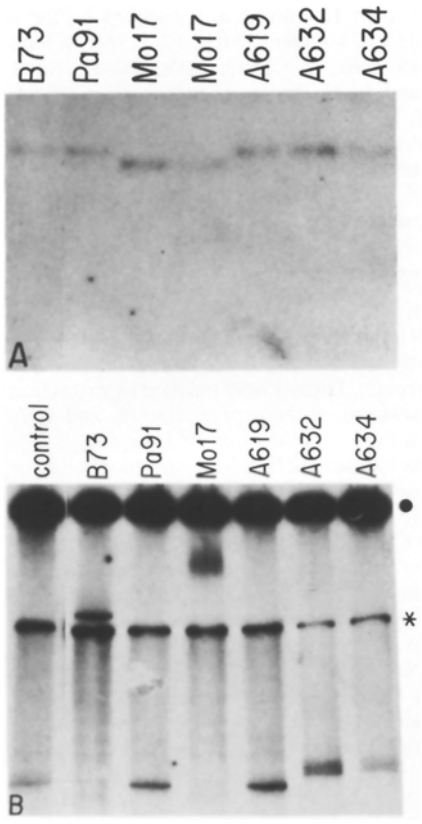

Fig. 2 A and B. Comparison of Southern blot and DGGE analysis with the same maize strains and probe. **A** Southern blot analysis of BamHI- and EcoRI-digested genomic DNA from the six maize strains indicated with the 660-bp *waxy* BamHI fragment (radiolabelled by random primer method) as a probe. The genomic DNA in the right-most Mo17 lane was digested with BamHI only. The hybridization band in the B73 lane corresponds to a 660-bp fragment. Direction of electrophoresis is from *top* to *bottom*. **B** BamHI- and EcoRI-digested genomic DNA from the same six maize strains analyzed by DGGE with the 660-bp *waxy* BamHI fragment (cloned in M13 and radiolabelled by primer extension) as the hybridization probe. The lane marked "control" contains the M13 clone with the inserted 660-bp *waxy* fragment, digested appropriately and hybridized with the *waxy* probe. "•" marks the location of excess probe "captured" by incubation with single-stranded template DNA prior to loading the gel; "*" marks the location of excess single-stranded probe not captured incubation with template DNA

The 660-bp *waxy* probe identifies two alleles in a Southern blot of six inbred genomic DNAs digested with EcoRI and BamHI (Fig. 2A). Digestion of Mo17 DNA with BamHI alone yields the same RFLP. Although EcoRI digestion did not affect the sizes of the DNA fragments homologous to the probe in this experiment, we included EcoRI in the digestion of these genomic DNAs to simulate the procedures we planned to use in genomic DGGE analysis.

In contrast to the Southern analysis, genomic analysis by DGGE using the 660-bp *waxy* probe identified four alleles for the same six inbreds (Fig. 2B). DGGE analysis not only detected the polymorphism in inbred Mo17, but also identified polymorphisms that were not resolved by conventional Southern blot analysis of identically restricted genomic DNAs.

Identification of random DNA probes suitable for genomic DGGE analysis in maize

In order to assess whether DGGE analysis would identify DNA polymorphism at reasonable frequency, we constructed a genomic DNA library from maize inbred B73 with fragments ranging in length from 400 to 1,500 bp. Each fragment, flanked by a BamHI site at one end and an EcoRI site at the other, was cloned in bacteriophage M13mp19. The cloning procedures, screening and identification of clones containing low-copy genomic DNA fragments, and subsequent characterization of the cloned DNA fragments are described in "Materials and methods".

After determining that a cloned DNA fragment represented low-copy DNA in the maize genome and contained the appropriate restriction sites at its end, we examined its behavior in DGGE. We established two criteria for fragments to be useful as probes for genomic DGGE analysis: the mobility of a cloned DNA fragment should significantly decrease during electrophoresis, and the band corresponding to the partially melted DNA fragment on the denaturing gradient (DG) gel should remain as a sharp, rather than diffuse, band. Figure 3 shows the electrophoretic behavior of four cloned DNA fragments in our collection. After digestion of M13 clones with BamHI and EcoRI, aliquots were loaded onto the gel so that the cloned DNA fragments would undergo electrophoresis for 10, 12, 14, and 16 h. For each sample, a substantial decrease in the relative mobility of the cloned DNA fragment was observed during electrophoresis. We originally selected DNA fragments that demonstrated virtually total loss of electrophoretic mobility after 14 h of electrophoresis (e.g., clone C); we later selected, in addition, DNA fragments that demonstrated a significant, but not complete, reduction in electrophoretic mobility after 14 h of electrophoresis (clones A, B, and D). Both types of DNA fragments are useful for detecting genomic DNA sequence heterogeneity: they generate sharp genomic DNA hybridization bands and provide clear resolution of different alleles (see below). A screen of 3,100 low-copy cloned maize DNA fragments yielded 770 clones that satisfied these standards for potential usefulness as DGGE probes.

DGGE probes identify polymorphic alleles in maize with high frequency

Genomic DNAs from seven maize inbred lines were processed as described in "Materials and methods", hybridized to radioactively labeled single-stranded DNA

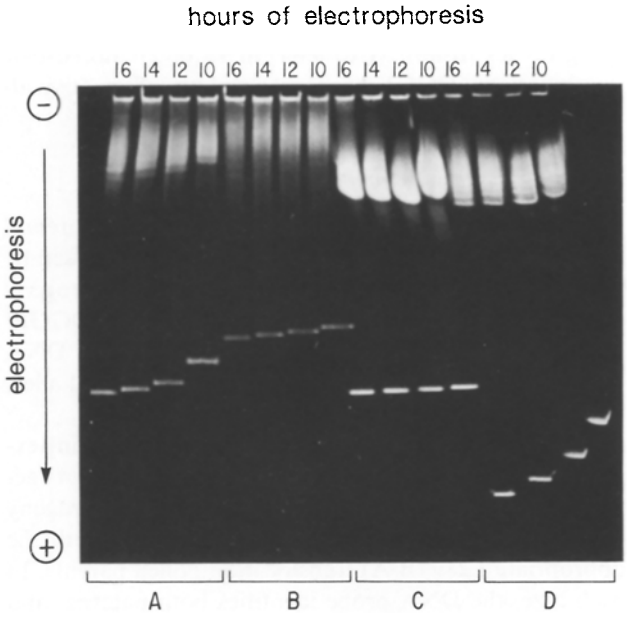

Fig. 3. Mobility of cloned DNA fragments in DGGE. After digestion with BamHI and EcoRI, M13 clones *A* through *D* were subjected to DGGE, as described in "Materials and methods", for the times indicated above each gel lane

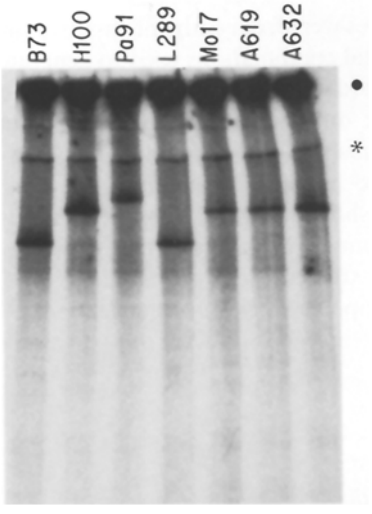

Fig. 4. Autoradiogram of DNA polymorphism screen using DGGE. Genomic DNA of inbred maize lines was processed, hybridized with probe A60 and electrophoresed as described in "Materials and methods." The relative mobility of the heteroduplex formed between the probe and the genomic DNA is affected by the number and position of mismatched base pairs within the heteroduplex. •: captured single-stranded probe. *: free single-stranded probe

probes, and analyzed by DGGE. The resulting DG gels were subjected to autoradiography. The probes functioned as single-copy probes, because they generated signal intensities that corresponded to single-copy genes in reconstruction experiments (data not shown), and because they consistently identified single bands in the seven inbred genomic DNAs used in this study. Figure 4 shows a typical result for one DNA probe. Genomic DNA of inbred maize lines was processed, hybridized with probe A60, and electrophoresed as described in "Materials and methods". The relative mobility of the heteroduplex formed between the probe and the genomic DNA is altered as a result of the number and position of mismatches within the probe-genomic DNA heteroduplex. In this screen, probe A60 identifies three distinguishable alleles: one carried by H100, Mo17, A619, and A632; another carried by B73 and L289; and a third carried by Pa91.

Table 1. Alleles identified by DGGE probes in seven inbred maize strains

Single-copy probes tested		45
Probes identifying:	1 allele	9
	2 alleles	17
	3 alleles	11
	4 alleles	6
	5 alleles	2
Probes identifying polymorphism		36

Table 2. Frequency with which DGGE probes reveal DNA sequence heterogeneity among each pair of maize strains tested

	B73	H100	Pa91	Mo17	A619	A632	L289
B73	–	0.44	0.58	0.75	0.84	0.56	0.60
H100		–	0.64	0.69	0.74	0.31	0.71
Pa91			–	0.67	0.68	0.58	0.43
Mo17				–	0.61	0.61	0.66
A619					–	0.74	0.65
A632						–	0.57
L289							–

36 probes were tested for each pairwise combination of strains, except for combinations with L289 (35 probes), and A619 (31 probes, including pairings with L289)

Table 1 groups 45 single-copy DGGE probes on the basis of the number of alleles they identify in seven inbred maize lines. These 45 probes were randomly selected from the 770 clones that satisfied our criteria for usefulness as DGGE probes. Thirty-six (80%) of these DGGE probes identify at least two alleles among the seven strains tested.

Table 2 lists the frequency with which the 36 informative probes identify polymorphisms in pairwise comparisons of all the inbred lines tested. This frequency is relevant to the practical usefulness of DGGE analysis in tracking parental alleles during a breeding program. The frequency of polymorphism occurrence for each inbred pairing is calculated as the ratio of the number of probes that identify differing alleles to the total number of probes tested for that combination of inbreds. The frequency with which DGGE probes identify an informative pair of

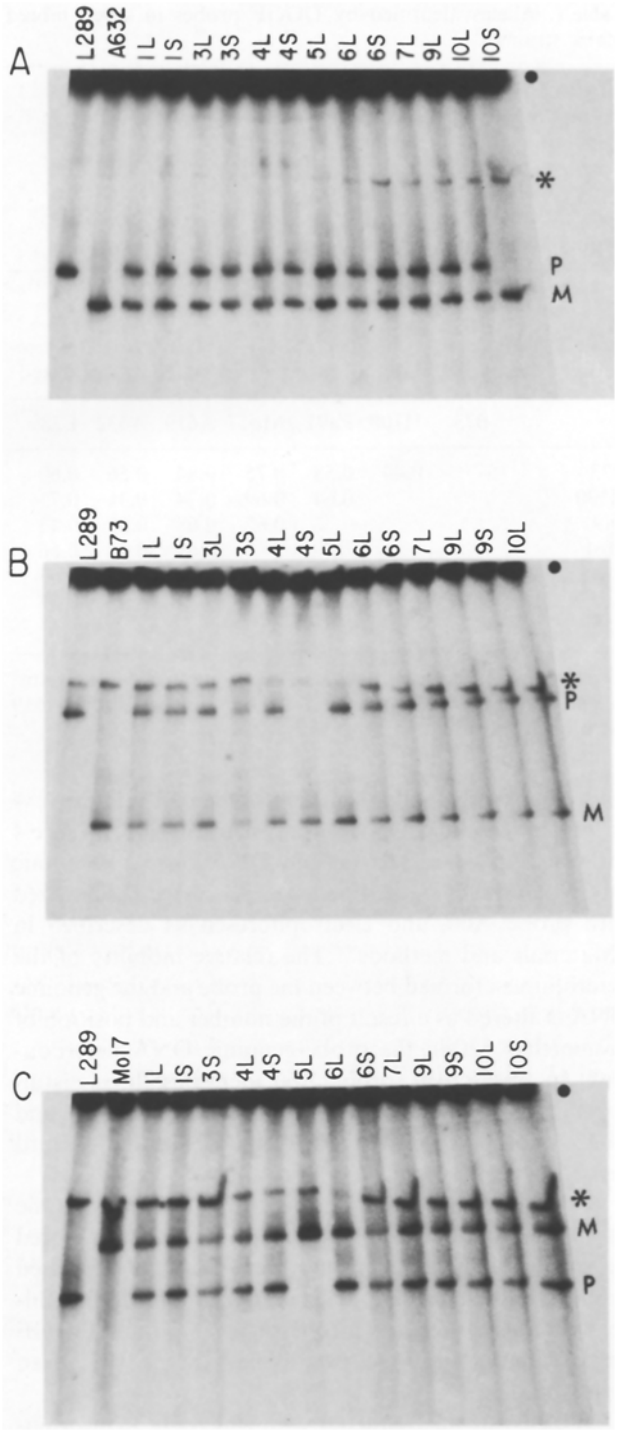

Fig. 5A–C. Autoradiograms of chromosome arm mapping screen using collections of hypoploid genomic DNAs. Inbred and hypoploid genomic DNAs were processed, hybridized with DNA probes, electrophoresed, and gel autoradiography was performed as described in "Materials and methods". Genomic DNA was isolated from hypoploid progeny generated by the following sets of crosses: (**A**) A632 × L289 (B–A); (**B**) B73 × L289 (B–A); (**C**) Mo17 × L289 (B–A). The DNA probes used these experiments were (**A**) A15; (**B**) A95; (**C**) A52. ●: captured single-stranded probe. *: free single-stranded probe. P: paternal allele. M: maternal allele

alleles for any combination of two inbreds in this sampling ranges from 0.31 to 0.84. In 18 of 21 inbred-pair combinations, DGGE probes yielded an informative allele pair more than 50% of the time.

Chromosome arm mapping of DGGE probes

The result shown in Fig. 4 indicates that the parental combinations of either Mo17, A619, or A632 crossed by the collection of L289 (B-A) lines will yield F_1 progeny with alleles that are easily distinguishable by DGGE. Chromosome arm assignments can be made for DNA probes on the basis of the absence of the paternal allele in a specific hypoploid genomic DNA.

Typical results obtained from hypoploid mapping experiments are shown in Fig. 5. This figure represents genomic analysis by DGGE of the hypoploid F_1 progeny generated by crosses of A632, B73, or Mo17 with the appropriate L289 (B-A) translocation pollen parents. In each case, the DNA probe identifies both paternal and maternal alleles in all hypoploid progeny except one. In that particular progeny, the probe hybridizes only with the maternal allele, with no detectable signal for the paternal allele. On that basis, the DGGE probe is assigned to the chromosome arm for which the paternal allele is missing.

Many DGGE probes were frequently informative for several different parental combinations used to generate the hypoploid progeny (data not shown). We used these probes as controls for our mapping experiments, and have found that chromosome arm assignments are consistent regardless of the parental combination used for the mapping (data not shown). Table 3 lists the F_1 hypoploid progeny and genomic DNA samples that were generated for the mapping studies. Table 4 lists examples of probes that we have mapped using several collections of hypoploid progeny.

Discussion

Experimental considerations for genomic analysis by DGGE

We expected several factors to influence the ability of genomic probes to identify genetic polymorphism at high frequency using DGGE: these factors included the choice of inbred DNA from which the library was constructed, the size of the DNA fragments used as probes, the melting characteristics of each cloned fragment, and the gel conditions used for DGGE.

We isolated DGGE probes from a genomic library of maize inbred B73. This inbred line has been widely used as a parent for many commercially successful hybrid maize varieties, and is frequently used as germ plasm in developing improved inbred lines for commercial corn

Table 3. F_1 hypoploid progeny and genomic DNA used in this study

Chromosome	Parental lines					
	A632/L289	B73/L289	Mo17/L289	A619/L289 A632/A619	Mo17/A619 B73/A619 Mo17/A632	B73/A632 Mo17/B73
1L	ab	ab	ab	a		
1S	ab	ab	ab	a		
3L	ab	ab		a		
3S	ab	ab	ab	a		
4L	ab	ab	ab			
4S	ab	ab	ab	a		
5L	ab	ab	ab			
5S					c	c
6L	ab	ab	ab			
6S	ab	ab	ab	a		
7L	ab	ab	ab			
7S					c	
8L					c	c
9L	ab	ab	ab	a		
9S		ab	ab	a		
10L	ab	ab	ab	a		
10S	ab		ab	a		

a: tissue collected from both hypoploid and normal F_1 progeny
b: genomic DNA isolated from hypoploid F_1
c: hypoploid progeny generated

Table 4. Confirmation of chromosome arm assignments using different hypoploid progeny series

Probe	Chromosome arm	Parent lines of hypoploid progeny
A30	*3L*	A632/L289 Mo17/L289
A52	*5L*	Mo17/L289 A632/L289
A95	*4S*	B73/L289 Mo17/L289
F5	*4S*	B73/L289 A632/L289
I96	*1L*	B73/L289 Mo17/L289
N19	*6L*	B73/L289 A632/L289
040	*4S*	B73/L289 A632/L289

production. The extensive use of B73 and its success as a parental line for hybrid seed production (Darrah and Zuber 1986) led us to expect that the B73 genome would not contain substantial genomic deletions that would create gaps in the map coverage provided by this collection of probes.

We selected the length range of the DNA probes for the following reasons: the lower size limit (400 bp) was determined by our observation that maize DNA fragments of this size or smaller infrequently yielded sharp bands after DGGE (data not shown); the upper limit (1,500 bp) was chosen because the average interspersion of repetitive DNA in maize is approximately 2,000 bp (Hake and Walbot 1980). It should be noted that there are no probe size restrictions imposed by DGGE; the acrylamide concentration and voltage gradient can be adjusted as desired. We chose to use only single-copy probes to simplify analysis and to allow us subsequently to combine these probes with a two-dimensional gel system (K. Kuranda, P. Bledsoe, G.E. Riedel, in preparation). Fragments with different restriction site ends were chosen for the ease this feature provided in single-strand probe synthesis from M13 clones.

The melting behavior of the DNA fragment is the major parameter that determines whether its electrophoretic mobility will decrease significantly during DGGE. Other parameters include the voltage gradient, the magnitude and shape of the gradient of chemical denaturants, the time of electrophoresis, and the acrylamide concentration in the gel. We determined empirically that the gel electrophoresis conditions described in "Materials and methods" generally were useful for screening cloned DNA fragments and detecting DNA polymorphisms at reasonable frequencies in maize. It was surprising to us that we could utilize such a steep (0%–80%) denaturing gradient for DGGE analysis in maize and still detect polymorphism at high frequency. Different DGGE conditions may be appropriate for the genomic analysis of other species and may increase the number of alleles identified by a particular probe. For

example, decreasing the steepness of the denaturing gradient in the gel allows one to increase substantially the resolving power of DGGE (Lerman 1986).

Analysis of 3,100 low-copy cloned maize DNA fragments by DGGE identified 770 that partially denatured and thus significantly decreased in mobility during electrophoresis through a DG gel. Nearly all the remaining 2,330 cloned DNA fragments denatured completely when they migrated to a sufficiently high concentration of denaturant. We found that these cloned DNA fragments were not useful for detecting sequence heterogeneity in our heteroduplex analysis protocol (data not shown), primarily due to the diffuse nature of the band on the DG gel. However, a modification of DGGE analysis has been recently developed by Sheffield et al. (1989), which has the potential of rendering all DNA fragments of appropriate length suitable for DGGE analysis. Use of this modification may significantly increase the number of probes available for DGGE genomic analysis, as well as reduce the initial effort required to generate the probes.

Classes of DNA polymorphism identified by DGGE analysis

Much of the polymorphism we detected in the DGGE assay resulted in large displacements of hybridization bands (Figs. 2B and 3). These large displacements presumably reflect substantial sequence difference between the probe and the genomic DNA used in the assay. Several DGGE probes yielded a "null allele", i.e., no hybridization band for one or more genomic DNA samples (data not shown). This phenomenon was reproducible and was observed for several different probe and genomic DNA combinations. Null alleles may result from an absence of the sequence homologous to the probe in a particular genome or from changes in the genomic DNA, such as large insertions or deletions, that cause the heteroduplex of probe and genomic DNA to be so unstable that no hybridization band can be detected. Null alleles may also result from the loss of the BamHI or EcoRI sites in the genomic DNA being probed, so that the probe/genomic DNA heteroduplex is too large to enter the DG gel.

Usefulness of DGGE probe for genomic analysis

Of the 45 single-copy DGGE probes used in this study, 80% identified two or more alleles when screened against seven maize inbreds (Table 1). This frequency is roughly equivalent to the frequency reported for RFLP identification in maize, which is approximately 90%, depending upon the choice of restriction enzyme (T. Helentjaris, personal communication).

The usefulness of the DGGE protocol in identifying polymorphism at maize loci can also be compared with the corresponding usefulness of a collection of RFLP probes. For this comparison, we used polymorphism frequency data generated by the random collection of 45 probes in this study. We also used RFLP frequency data generated by T. Helentjaris and V. Turner (personal communication) for 56 single-copy probes that are uniformly distributed on all ten chromosomes of the maize genome (Helentjaris 1987). Walton and Helentjaris (1986) have previously determined, in a separate screen, the single enzyme treatment of genomic DNA (either EcoRI, HindIII, or SstI) which identifies the most alleles for each probe. The comparison used the allele information from the enzyme treatment that had been previously determined to be most informative. Allele information from five inbred lines (B73, Mo17, Pa91, A619, and A632) was used for the comparison.

The 56 RFLP probes identify 158 alleles in the five inbred lines (2.82 alleles/probe); the 45 DGGE probes identify 105 alleles (data not shown) in the same five inbred lines (2.33 alleles/probe). More informative sets of probes can be generated for either RFLP or DGGE protocols by utilizing additional genomic digests for RFLP probes or by screening additional probes and discarding less informative ones. It is interesting to note that while these DGGE probes appear to yield nearly the same amount of allele information in maize as the collection of mapped RFLP probes, they do so by measuring parameters that are different from those used for RFLP analysis.

The DGGE probes described in this study frequently discriminated between any pairwise combination of the seven inbreds we screened. The probability that any informative DGGE probe would discriminate between any two inbreds ranged from 31% to 84%.

Genetic behavior of DGGE markers

This work demonstrates that DGGE probes can be mapped to chromosome arms in a fashion analogous to other maize genetic markers. The collection of hypoploid maize lines we have generated can be used to assign DNA probes rapidly to chromosome arms. Burr et al. (1988) first demonstrated the usefulness of hypoploid analysis by confirming the map locations of RFLP probes that had been previously mapped through the use of recombinant inbred lines; we extend that work in this report now to include DGGE probes.

Potential usefulness of DGGE genomic analysis in plant breeding programs

The protocols we have utilized for routine DGGE genomic analysis were selected for their compatibility with large-scale plant breeding applications, such as the rapid screening of unknown genotypes or the screening of offspring of genetic breeding programs. For example, the DGGE protocols in this study have been designed so that

virtually all the steps of the protocols, including the generation of probes, can be performed in microtiter plate wells. These adaptations will contribute to the eventual automation of DGGE analyses.

We have also developed devices to facilitate DGGE analyses. For example, we have modified a two-dimensional (2-D) gel system described by Fischer and Lerman (1979), in which the first dimension separates DNA fragments by size and the second dimension separates by DGGE. The modified gel system (K. Kuranda, P. Bledsoe, G.E. Riedel, in preparation) runs both dimensions in the same gel without user manipulation and may allow the analysis of several hundred probes on a single 2-D gel. Combining several hundred probes in one liquid hybridization mixture is feasible, because only a small quantity of each probe [800 cpm (Cerenkov)] is required to generate a full-strength signal for a single-copy gene. Thus, eventually it may be possible to assay an individual plant with a set of several hundred mapped DGGE probes, using only 2–10 μg of genomic DNA and one gel.

The informativeness of either DGGE probes or RFLP probes is high in maize, but is derived from the analysis of different properties of genomic DNA. Accordingly, the DGGE method may provide a useful alternative for genetic analysis in species where RFLP incidence is limited.

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