HIGH RESOLUTION METAPHOR AGAROSE GEL ELECTROPHORESIS FOR GENOTYPING WITH MICROSATELLITE MARKERS

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Microsatellites are the target sequences of choice for a wide range of applications in genomics for genotyping, genome mapping, QTL analysis and assessment of genetic diversity. We have effectively employed MetaPhor agarose gel electrophoresis (MAGE) for genotyping with microsatellite markers. Separation of SSRs on MetaPhor agarose gels appeared to be easier and economical. By employing MAGE, we were successful to clearly separate SSR alleles with a size difference of five bp. MAGE is a reliable and appropriate approach for identification of small length polymorphisms while screening large number of samples, therefore it is a valuable tool for the researchers involved in DNA fingerprinting work with microsatellite markers.

Keywords: Simple sequence repeats (SSRs); polymerase chain reaction (PCR); polyacrylamide gel electrophoresis (PAGE); MetaPhor agarose gel electrophoresis (MAGE).

INTRODUCTION

DNA markers like restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) are important tools in genomics. These DNA fingerprinting approaches facilitate the assessment of genetic diversity (Rahman *et al.*, 2002a & 2007; Asif *et al.*, 2006), monitoring of introgression, genetic mapping, QTL analysis (Paterson *et al.*, 2003) and marker assisted selection (MAS), which can also facilitate the development of new crop varieties. Ideal DNA markers are abundant, non-specific to stage and tissue, and unaffected by the environment. All these properties make molecular markers important for crop improvement. Therefore, molecular markers for agronomically important traits are available for nearly all crops (Jauhar *et al.*, 2006).

In recent years, microsatellites have become the target sequences of choice for a wide range of applications in genomics for genotyping, genome mapping, QTL identification, estimation of genetic diversity and relatedness (Lubberstedt *et al.*, 2000). Satellite DNA or repetitive sequences were first observed in the form of dinucleotide repeats of poly CA and poly GT (Hamada *et al.*, 1984) when eukaryotic genomic DNA was subjected to isopycnic cesium chloride density gradient centrifugation and distinctive bands of DNA of lesser or greater density than the bulk of the genomic DNA were frequently observed (Beridze, 1986). These repetitive sequences were named as Simple Sequence Repeats (SSRs) (Tautz and Renz, 1984), later on named as Microsatellites (Litt and Luty, 1989) or Simple/ Short Tandem Repeats (STRs) (Edwards *et al.*, 1991). Microsatellites are direct tandem repeated sequences of DNA with a repeat size ranging from one to six bases. Microsatellites are abundant and ubiquitously distributed in the genomes of plants and animals (Burow and Blake, 1998). The most abundant type of microsatellites in plants is $(AT)_n$ dinucleotides (Morgante and Olivieri, 1993). SSRs are present in both non-coding and coding regions of the genome. Expressed sequence tags derived SSRs (EST-SSRs) from transcribed regions of the DNA are generally more conserved across species than those from the untranscribed regions (Qureshi *et al.*, 2004).

Microsatellites are codominant markers and extremely useful because of highly variable nature among taxa (Fisher *et al.*, 1998) and this polymorphism is mainly based on differences in number and length of the repeat motifs. The length polymorphism of a microsatellite marker is commonly detected through polymerase chain reaction (PCR) amplification using pairs of specific SSR primers flanking tandem arrays of microsatellite repeats and followed by electrophoresis (Rahman *et al.*, 2002b). For SSR analysis there are three electrophoresis methods currently employed to determine the length polymorphisms. The polyacrylamide gel electrophoresis (PAGE) is the most common and excellent method (Cregan and Quigley, 1997). The amplification products in polyacrylamide gels are typically visualized with radioactive labeling, fluorescent dye labeling, and silver staining. However, these visualization techniques require either expensive or hazardous radioactive chemicals and are time consuming. Capillary electrophoresis has also been used to determine length polymorphisms of microsatellite markers (Marino *et al.*, 1995), but this method requires highly sophisticated instruments and fluorescently tagged primers, which are much expensive.

MetaPhor agarose gel electrophoresis (MAGE) is another approach to separate alleles of microsatellite markers (Abdurakhmonov *et al.*, 2007). MetaPhor agarose (FMC or Cambrex Corporation, USA) is an intermediate melting temperature agarose (75°C) that provides twice the resolution capabilities of the finest sieving agarose products. Using submarine gel electrophoresis, MetaPhor agarose gives high resolution separation of 20 to 800 bp DNA fragments that differ in size by 2%, which approximates the resolution of polyacrylamide gels. MetaPhor agarose gels (2% to 4%) made in either TAE or TBE and stained with ethidium bromide are ideal for resolving SSRs (Anonymous, 2004). The present research work was conducted to optimize and establish a more rapid and efficient method for genotyping with microsatellite markers in cotton, which is our main fiber and cash crop. Efficiency of MetaPhor agarose gel electrophoresis was evaluated successfully for SSR analysis, which would be a valuable tool for the researchers especially in a country like Pakistan.

MATERIALS AND METHODS

Plant material and DNA isolation

Two cotton genotypes (FH-883 and FH-631S) contrasting for fiber traits and their $F_{2:3}$ population were used for the present study. DNA was extracted from the two selected cotton parents, their F_{1s} and $F_{2:3}$ population by CTAB method (Murray and Thompson, 1980). Young leaves were ground to a very fine powder in liquid nitrogen and transferred to a 50 ml Falcon tube. The 15 ml of hot 2x CTAB was added and incubated for 30-45 minutes at 65°C with occasional swirling. Equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed, then spun at 4000 rpm for 10 min. The supernatant was collected into a new 50 ml tube. Nucleic acid was precipitated with 0.6 volume of chilled isopropanol. Nucleic acid was pelleted at 4000 rpm for 5 minutes and supernatant was discarded. Pellet was washed with 70% ethanol and air dried before resuspending in 0.5 ml 0.1x TE buffer. The suspension was transferred to 1.5 ml eppendorf tube, added 7 µl of RNase and incubated at 37°C for 1 hour. Equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed, centrifuged for 10 min at 13000 rpm and then supernatant was transferred to a new eppendorf. $1/10^{th}$ 3M NaCl was added, mixed gently and chilled ethanol (2 volumes) was added. After a spin at 13000 rpm for 10 minutes, discarded the supernatant, washed the pellet with 70% ethanol, air dried and resuspended it in 0.1x TE buffer. DNA concentration was measured with DyNAQuant 200 Fluorometer and also comparing with Quantification Standards, Phage λ DNA (GibcoBRL).

PCR for amplification of microsatellite markers

SSR primers (including EST-SSRs) were surveyed to screen two cotton parents (FH-883 and FH-631S) and subsequently screening their F_1 and $F_{2:3}$ population with polymorphic SSRs. These primers belonged to the series of BNL, CM, JESPR and MGHES (EST based SSR primers). The sequences of these primers were obtained from publically available cotton microsatellite data (CMD) (Blenda *et al.*, 2006) and synthesized from GeneLink, USA. Using eppendorf mastercycler gradient, PCR amplification was performed in 20 µl reaction volumes containing 30 ng/µl cotton DNA, 10x PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs (each of dATP, dGTP, dCTP and dTTP), 30 ng/µl primer and 1.0 unit of *Taq* DNA polymerase. *Taq* DNA polymerase, 10x PCR buffer, $MgCl_2$ and dNTPs were from Fermentas. PCR profile was 94^{o}C for 5 min, then 35 cycles of 94°C for 30 sec, 55- 65°C (variable) for 30 sec, 72°C for 1 min and finally 72°C for 4 min.

MetaPhor agarose gel electrophoresis for microsatellite analysis

For the gel preparation, 2% standard agarose and 2% Metaphor agarose was slowly added to the chilled 1x TBE buffer with continuous swirling. It was soaked in that cold buffer for about 10-15 minutes before heating in the microwave (to prevent it from foaming and not to over boil the gel). Just prior to pouring the gel, ethidium bromide (0.3 µg/ml) was mixed to the dissolved agarose. Once the molten gel was solidified after being poured into a cast, it was kept at 4°C for 20 minutes before use to obtain a better resolution and for easier handling of the gel (Anonymous, 2004). After adding 3 µl 6x loading dye to the 20 µl SSR PCR product, 10 µl of it was loaded on the Metaphor agarose gel submerged in chilled 1x TBE buffer and electrophoresed for about 3-4 hours at 6 V/cm using horizontal electrophoresis system HU-13 (Scie-Plas, UK). After electrophoresis, the finely resolved SSR products were visualized under UV transilluminator and photographed using the Stratagene Eagle Eye still video system or Kodak (1D 3.5) imaging system. After gel electrophoresis and imaging good quality gel photographs were used to score the all visible and unambiguously scorable fragments amplified by SSR primers. The primers that produced polymorphic fragments between the two cotton parents were used to survey their $F_{2:3}$ lines, and finally that data was analysed for further genome mapping studies.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of two alleles of the microsatellite marker MGHES-17 after electrophoresis at 80 V for about two hours. The two alleles of MGHES-17 differed by 20 bp (200 and 220 bp). Longer electrophoresis period was required for better separation of the alleles with less difference. Fig. 2 shows the difference of one versus two and four hour electrophoresis at 80 V for the separation of 95 and 100 bp alleles of marker MGHES-06. In this case, one hour of electrophoresis did not separate the two alleles (Fig. 2a) while two hours of electrophoresis barely separated the two alleles (Fig. 2b), however, four hours of electrophoresis clearly separated the two alleles (Fig. 2c). By employing MAGE, we were successful to clearly separate alleles of microsatellite markers with a size difference of five bp, because the product amplified by the primers used were of minimum five bp differences, however it can also resolve PCR products that differ in size by 2% (Anonymous, 2004).

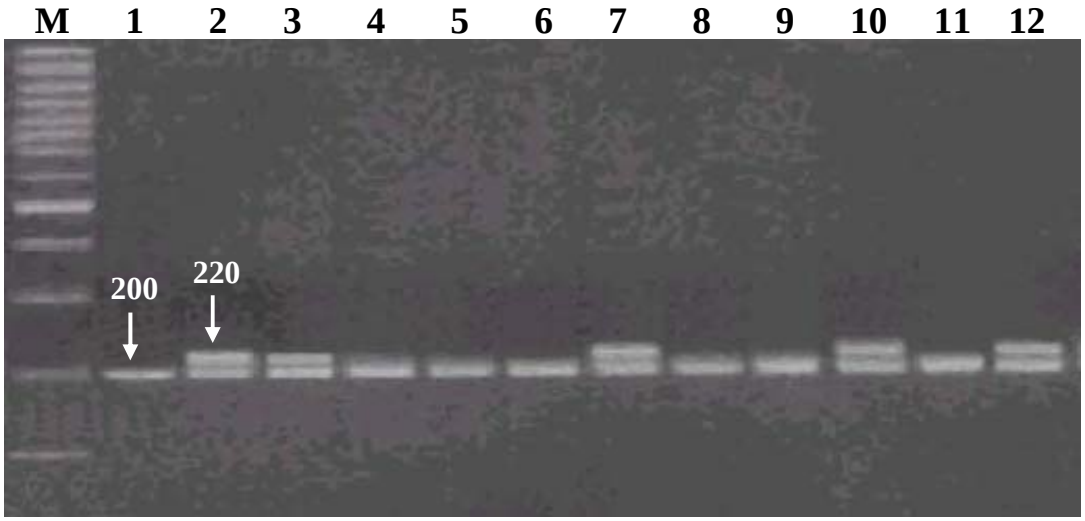

Fig. 1. Metaphor agarose gel image of amplification profile with SSR primer MGHES-17. M: DNA marker, 1: FH-883, 2: FH-631S, 3 to 12: ten lines of (FH-631S x FH-883) $F_{2:3}$ population.

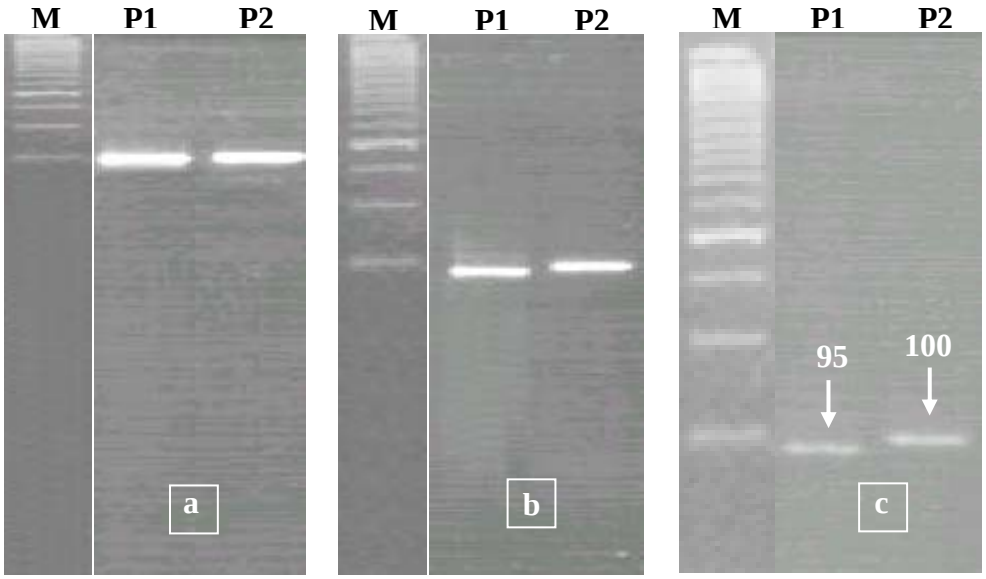

Fig. 2. Metaphor agarose gel image of amplification profile with SSR primer MGHES-06, after electrophoresis of a) one hour, b) two hours, c) four hours. M: DNA marker, P1: FH-883, P2: FH-631S.

Polyacrylamide gel electrophoresis, MetaPhor agarose gel electrophoresis and automated capillary electrophoresis are used for microsatellite analysis and all these methods produce comparable and reproducible results (Ochsenreither *et al.*, 2006). However, PAGE is a tedious and time-consuming process, while capillary electrophoresis can be performed more quickly and good for high-throughput analysis but it is more expensive and requires more sophistication and expertise. In the present research work we have effectively employed MetaPhor agarose gel electrophoresis for genotyping with microsatellite markers in cotton. Other researchers have also used Metaphor agarose for SSR analysis in cotton (Mei *et al.*, 2004; Bolek *et al.*, 2005; Abdurakhmonov *et al.*, 2007). Electrophoresis of SSRs on MetaPhor agarose gels appeared to be easier and economical. For screening large populations with SSRs MAGE is a reliable and appropriate approach for identification of small length polymorphisms.

Ethidium bromide is commonly used to stain nucleic acid (Sambrook and Russel, 2001) in Metaphor gels for visualization of SSR products. Gels can be stained either before or after electrophoresis. To stain prior to electrophoresis, ethidium bromide was added to the dissolved Metaphor agarose just before pouring the gel. The ethidium bromide stain runs in the opposite direction of the DNA therefore the upper and lower portions of the gels appeared differentially stained especially when gels were electrophoresed at high voltages for a short period of time. Therefore for even staining, ethidium bromide could be included in the electrophoresis buffer.

SSRs are being extensively used for genome mapping studies (Abdurakhmonov *et al.*, 2007; Guo *et al.*, 2007). To achieve better resolution, it is better to use fresh Metaphor gels for surveying new SSRs during screening of parents in genetic mapping. However, for subsequent screening of their population with same SSRs, we reused the Metaphor agarose gels for five to six times and hence it became more economical. For further enhancement in throughput of MAGE, two or three amplification products can be loaded simultaneously in the same well if sizes of the alleles of these SSRs are known not to overlap. Alternatively, if sizes of the alleles of the microsatellite markers are same, one sample set can be electrophoresed for some time, and then additional samples can be loaded into the same wells followed by further electrophoresis. Our results clearly demonstrate that the MAGE system has a high resolution to distinguish SSR alleles and is ideal for the researchers currently involved in genotyping with microsatellite markers.

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