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Protocols

Detection of Single Sequence Repeat Polymorphisms in Denaturing Polyacrylamide Sequencing Gels by Silver Staining

S. CRESTE,[1] A. TULMANN NETO[2] and A. FIGUEIRA[2,*]
[1]*Departmento de Genética, Escola Superior de Agricultura "Luiz de Queiroz";*
[2]*Laboratório de Melhoramento de Plantas, Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Av. Centenário, 303- CP 96, Piracicaba, SP 13400-970, Brazil*

Abstract. Large-scale use of molecular markers in plant breeding is limited by the throughput capacity for genotyping. DNA polymorphisms can be detected in denaturing polyacrylamide gels indirectly by nucleotide labeling or directly by staining. Fluorescent-labeling or radiolabeling requires sophisticated infrastructure not always available in developing countries. We present an improved low-cost method for silver staining and compare it to 2 other methods for their ability to detect simple sequence repeat polymorphisms in denaturing polyacrylamide gels bound to glass plates. The 3 procedures differed in their requirement for an oxidation pretreatment, preexposure with formaldehyde during silver nitrate impregnation, inclusion of silver thiosulfate, and by their replacement of sodium carbonate for sodium hydroxide to establish alkaline conditions for silver ion reduction. All methods detected the same banding pattern and alleles. However, important differences in sensitivity, contrast, and background were observed. Two methods gave superior sensitivity, detecting down to 1 μL of loaded amplification products. Our improved method gave lower backgrounds and allowed reutilization of staining solutions. The use of thin (<1 mm) denaturing sequencing gels allows genotyping of 60-96 samples within 4 h. Use of smaller loading sample volumes and reutilization of staining solutions further reduced costs.

Key words: AFLP, banana, microsatellite, *Musa*, PAGE, silver staining, SSR

Abbreviations: SSR, simple sequence repeat; TBE, tris borate ethylenediaminetetracetic acid; TEMED, tetramethylethylenediamine.

Introduction

Molecular markers, such as simple sequence repeats (SSR) or sequence-tagged microsatellite sites (STMS), are now widely used in plant breeding (e.g., genome mapping, marker assisted selection) and in evolutionary and conservation studies. However, their routine application requires efficient methodology for mass genotyping (Mitchell et al., 1997). The most efficient method for visualizing single-

*Author for correspondence. e-mail: figueira@cena.usp.br; fax: +55-19-34294610; ph: +55-19-34294814.

strand DNA in polyacrylamide gels uses radioactive or fluorescent labeling of nucleotides. These procedures require special facilities and are expensive and time consuming, rendering them impracticable in most tropical countries where sophisticated infrastructures are lacking (Lagoda et al., 1998a).

Silver staining originally was described for ultrasensitive detection of polypeptides separated by polyacrylamide gel electrophoresis (Merril et al., 1981) and later adapted for nucleic acid detection (Sommerville and Wang, 1981; Herring et al., 1982; Blum et al., 1987). Beidler et al. (1982) devised a photochemical silver staining method for nucleic acid detection with increased sensitivity (5-7.5 pg DNA mm^{-2}). Since then, various small adaptations of these original silver staining methods have been proposed to increase sensitivity, to reduce steps of the procedure, to eliminate toxic staining components, and to better suit the DNA assay or method of fragment separation (Bassam et al., 1991; Santos et al., 1993; Sanguinetti et al., 1994). Bassam et al. (1991) enhanced the sensitivity (as low as 1 pg mm^{-2}) by including a gel preexposure with formaldehyde during silver nitrate impregnation and by lowering the concentration of silver nitrate. Backgrounds were reduced by inclusion of sodium thiosulfate and by eliminating an oxidation pretreatment with potassium dichromate and nitric acid. Sanguinetti et al. (1994) described a few protocol modifications, such as replacing sodium carbonate with sodium hydroxide to establish alkaline conditions for silver ion reduction to metallic silver by formaldehyde, with a reported sensitivity of 3 pg mm^{-2}. Detection of nucleic acids using silver stain has been demonstrated to be highly sensitive, with results similar to autoradiography and fluorescence labeling and detection (Comincini et al., 1995; Christensen et al., 1999).

In general, these silver staining methods were optimized for native or denaturing gels unbound to any backing surface, such as glass plates or plastic. The use of polyester backing film affected the quality of silver staining, requiring longer development time and increased background staining (Bassam et al., 1991). For an increased throughput analysis, large numbers of samples can be analyzed at once in larger and thinner (<1 mm) sequencing gels, but manipulations during silver staining require gel binding to the glass surface.

The objective of this study was to evaluate a new low-cost method of silver staining adapted from Beidler et al. (1982) using banana (*Musa* spp.) as a model system. We compared sensitivity of this method to the 2 other commonly used procedures (Bassam et al., 1991; Sanguinetti et al., 1994) and optimized the conditions for detection of SSRs using denaturing polyacrylamide gels bound to glass plates.

Materials and Methods

Total genomic DNA was extracted from the leaves of 34 field-grown *Musa* triploid and tetraploid cultivars and 1 diploid genotype using a modified procedure adapted from Doyle and Doyle (1990). The DNA was then quantified by fluorometry (DyNA Quant 2000 Fluorometer, Amersham Pharmacia Biotech, Buckinghamshire, UK). Amplification reactions were performed as described by Lagoda et al. (1998b) with minor modifications. The DNA amplification reaction volume was 25 µL, containing 50 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Triton-X, 1.5 mM $MgCl_2$, 100 µM each of the

4 dNTPs, 0.2 μM of each primer AGMI24 and AGMI25 (Lagoda et al., 1998b), and 1.5 units of *Taq* polymerase (Life Biotech do Brasil, São Paulo, SP, Brazil). The SSR primers were synthesized by Life Biotech do Brasil and were based on sequences obtained from Lagoda et al. (1998b). Amplifications were conducted in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), programmed with an initial denaturing step at 94°C for 3 min, followed by 9 touchdown cycles of 40 s at 94°C; 40 s at 60°C; and 60 s at 72°C, decreasing 1°C per cycle down to 51°C; ending with 25 cycles of 40 s at 94°C; 40 s at 50°C; and 60 s at 72°C.

Gel electrophoresis

- Treat the smaller glass plate (33.3 x 39.4 cm) with 4 μL of γ-methacryloxypropyl-trimethoxysilane (PlusOne Bind Silane, cat. # 17-1330-01; Amersham Pharmacia Biotech), in 1 mL of acidic ethanol (0.5% glacial acetic acid in 95% ethanol) to covalently attach the gel onto the glass plate. Let dry for 5 min and remove the excess using a paper tissue moistened with 95% ethanol;
- Treat the larger glass plate (33.3 x 41.9 cm) with 1 mL of a 2% solution of dimethyldichlorosilane in octamethyl cyclo-octasilane (PlusOne Repel-Silane ES, cat # 17-1332-01; Amersham Pharmacia Biotech) to assure gel release. Let dry for 5 min and remove excess with a tissue moistened in distilled water.
- Prepare a 30% acrylamide solution with 29 g acrylamide and 1 g N,N'-methylene bisacrylamide in 100 mL ultrapure water.
- Prepare a urea:acrylamide solution with 100 mL of 30% acrylamide solution, plus 100 mL 5 X TBE (445 mM Tris-base; 445 mM boric acid; 10 mM EDTA), and 210 g urea. Add ultrapure water to a final volume of 500 mL, filter in paper, and stock at 4°C in a brown bottle.
- Prepare gels (6% polyacrylamide; 7 M urea) by mixing 50 mL of the urea:acrylamide solution in TBE with 200 μL of freshly prepared 10% ammonium persulfate and 84 μL of TEMED. Apply the gel solution to the assembled gel plates (0.4 mm thickness) using a SQ3 sequencing apparatus (Amersham Pharmacia Biotech). Allow 60 min for the gel to polymerize.
- Run the sequencing gel at 60 W (42 mA; 1500 V) for 60 min or until the gel temperature reaches 55°C in 1 X TBE (89 mM Tris; 2 mM EDTA; 89 mM boric acid);
- Add 12.5 μL of denaturing buffer (10 mM NaOH; 0.05 % xylenecyanol [w/v]; 0.05% bromophenol blue [w/v]; 20 mM EDTA in formamide) to the 25-μL amplification reaction. When using different PCR reaction volumes, keep the same ratio of denaturing buffer.
- Denature the samples for 3 min at 94°C in the thermocycler. Immediately place the samples on ice.
- Apply samples (8 μL) to the gel as quickly as possible. Run the gel at 60 W for 80-100 min at 50-55°C.

Silver staining

Gels were silver stained using 3 methods according to the steps described on Table 1. The protocol described by Bassam et al. (1991) was followed exactly as described. The method described by Sanguinetti et al. (1994) was slightly modified,

Table 1. Summary of steps for the 3 silver stain methods.

Step	Improved Procedure (adapted from Beidler et al., 1982)	Bassam et al., 1991	Sanguinetti et al., 1994
1. Fix	10% ethanol, 1% acetic acid,10 min	10 % acetic acid, 20 min	10% ethanol, 0.5% acetic acid, 20 min
2. Wash	H_2O, 1min	-	-
3. Pretreat	1.5% nitric acid, 3 min	-	-
4. Rinse	H_2O, 1min	H_2O, 2 min, 3 times	-
5. Impregnate	0.2% $AgNO_3$, 20 min	0.1% $AgNO_3$, 1.5 mL 37% formaldehyde $liter^{-1}$, 30 min	0.2% $AgNO_3$, 30 min
6. Rinse	H_2O, 30 s, 2 times	H_2O, 20 s (optional)	-
7. Develop	30 g L^{-1} Na_2CO_3, 0.54 mL 37% formaldehyde $liter^{-1}$, 4-7 min	3% Na_2CO_3, 1.5 mL 37% formaldehyde $liter^{-1}$, 2 mg $Na_2S_2O_3.5H_2O$ $liter^{-1}$, 2-5 min	3% NaOH, 5 mL 37% formaldehyde $liter^{-1}$, 5-10 min
8. Stop	5 % acetic acid, 5 min	10 % acetic acid, 5 min	10% ethanol, 0.5% acetic acid

with altered time for improved detection. An improved method adapted from Beidler et al. (1982) was optimized. All chemicals used for staining were analytical grade, either from Mallinckrodt Baker S.A. (Xalostoc, Mexico) (ethanol, nitric acid, sodium carbonate) or from Merck (nitric acid, silver nitrate, sodium hydroxide, formaldehyde). All solutions were prepared using ultrapure distilled water. The gel plates were agitated gently in a shaker throughout the staining processes.

- Disassemble gel apparatus carefully, separating the glass plates.
- Place glass plate with bound gel onto plastic tray.
- Apply 1000 mL fixing solution (10% ethanol, 1% acetic acid) and shake gently for 10 min.
- Wash gel with distilled H_2O for 1 min.
- Pretreat gel (oxidize) with 1000 mL of 1.5% nitric acid for 3 min, shaking gently.
- Rinse gel with 1000 mL distilled H_2O for 1 min.
- Impregnate gel with 1000 mL of 0.2% $AgNO_3$ solution for 20 min, shaking gently.
- Rinse gel with 1000 mL distilled H_2O for 30 s, twice.
- Develop gel by applying, initially 250 mL of cold (ca. 12°C) developing solution (30 g L^{-1} Na_2CO_3; 0.54 mL 37% formaldehyde $liter^{-1}$) and gently shake until the solution becomes dark. Replace the solution with 750 mL of fresh cold solution, for 4-7 min, until the bands appear with desirable intensity (ca. 5 min). Remove developing solution.
- Stop developing reaction by adding 1000 mL of 5% acetic acid for 5 min.
- Wash gel in distilled water.
- Air dry and photograph or scan.

Results and Discussion

DNA from 35 banana genotypes (diploid, triploid, and tetraploid cultivars) was amplified by PCR using primers AGMI 24/25 (Lagoda et al., 1998b). Samples

from the same DNA amplification reactions were applied to 3 identical 6% denaturing polyacrylamide gels. All 3 silver staining methods were used for detection of SSR polymorphism. All methods disclosed identical banding patterns with identical alleles (Figure 1). However, the methods differed in sensitivity and contrast. Our improved method based on Beidler et al. (1982) had a similar sensitivity and contrast to the method proposed by Bassam et al. (1991), utilized by Lagoda et al. (1998a), but had less background probably because of the smaller formaldehyde concentration. The method developed by Sanguinetti et al. (1994) gave the lowest sensitivity in our hands and produced a strong yellow background but with visible and discrete bands. A major problem with this method was interference of the developing solution containing NaOH, which releases the gel from the glass plates.

Identical sample serial dilutions of the amplified reactions were loaded to test the method sensitivity (Figure 2). The test samples contained a total DNA concentration of 24 ng in 8 μL estimated by fluorometry, and decreasing volumes (4 μL, 2 μL, and 1 μL) were applied. The improved method was sensitive with less background, detecting discrete and sharp bands even at the lowest volume. The method proposed by Sanguinetti et al. (1994) could only detect samples of 4 μL.

Preparation and handling of the solutions were the same for all methods. The fixing solution was reused at least 3 times, while the oxidation (nitric acid) and stopping (acetic acid) solutions could be reused 5 times. The silver nitrate solution could be reused twice without loss of sensitivity. The silver nitrate solution was stored in the dark in amber bottles at room temperature.

Previously, SSR analyses were conducted using medium-size (20 x 20 cm) nondenaturing gels (1.5-mm thickness). Because the gel size and thickness supported staining manipulations, both surfaces were free from glass and were silver stained according to the method described by Sanguinetti et al. (1994). However, when larger sequencing gels (33.3 x 39.4 cm) were used, this staining method was not appropriate because of the size and thickness (0.4 mm). Lagoda et al. (1998a) reported the use of SSR polymorphisms for banana based on small (10 x 12 cm) denaturing gels bound to a polyester film and silver stained using the method of Bassam et al. (1991). The same staining method gave good results when tested for large sequencing gels bound to a glass surface. However, the developing reaction was too fast for a suitable control of contrast, even using the solutions at lower temperatures. The improved staining method for large sequencing gels bound to glass based on Beidler et al. (1982) presented various advantages. A large number of samples could be analyzed in a single run, allowing a direct comparison of alleles. Because the sequencing gels are thinner but larger, a comparable volume of polyacrylamide and other reagents are used for a larger number of samples (up to 96, compared to 20). Most of the staining reagents were reused without loss of quality. A higher sensitivity of fragment detection allowed small volumes of samples to be loaded, consequently reducing the amplification reaction volumes required (down to 13 μL).

We have used the improved method based on Beidler et al. (1982) routinely in our laboratory for analyses of AFLPs and SSRs in various tropical crops (*Theobroma grandiflorum* [cupuassu], *Theobroma cacao* [cocoa], sugarcane,

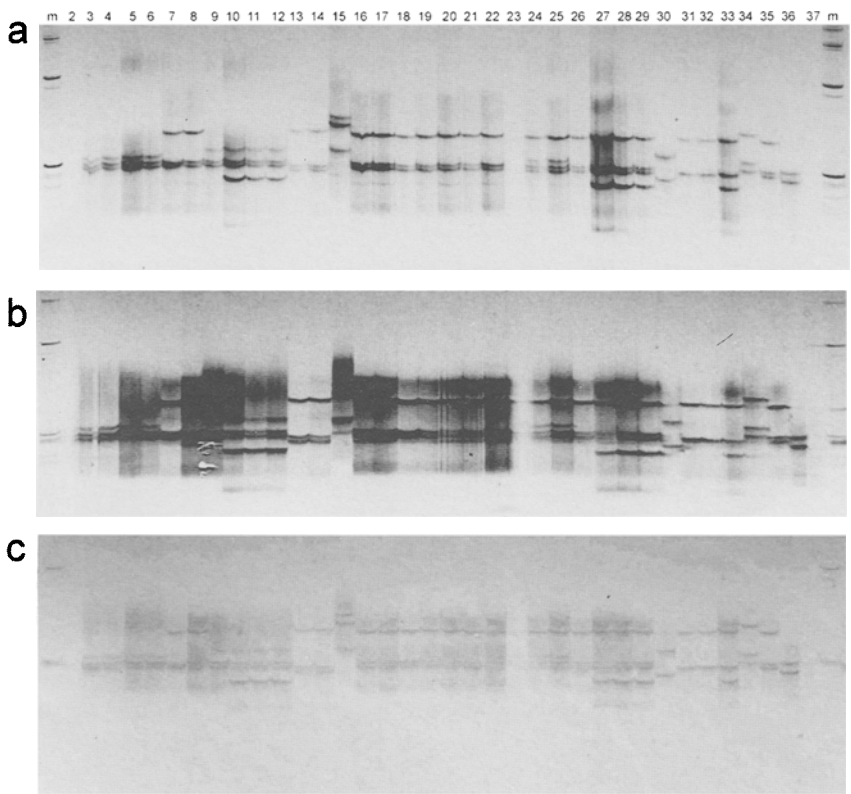

Figure 1. Sensitivity of 3 silver staining methods of *Musa* simple sequence repeat polymorphism amplified using primer AGMI 24/25 and separated in 6% denaturing polyacrylamide gels. (a) Our improved method based on Beidler et al. (1982), (b) method of Bassam et al. (1991), and (c) method of Sanguinetti et al. (1994). Lane m, 123 ladder; lane 2, 'Williams'; lane 3, 'Grand Naine'; lane 4, 'Nanica'; lane 5, 'Nanicão'; lane 6, 'PBN'; lane 7, 'Ouro da Mata'; lane 8, 'Branca'; lane 9, 'Gros Michel'; lane 10, 'Bucaneer'; lane 11, 'Ambrosia'; lane 12, 'Calypson'; lane 13, 'Mysore'; lane 14, 'Thap Maeo'; lane 15, 'Prata Jau'; lane 16, 'Prata'; lane 17, 'Prata Cruz das Almas'; lane 18, 'Prata Santa Maria'; lane 19, 'Prata Ponta-Aparada'; lane 20, 'Enxerto'; lane 21, 'Prata Anã'; lane 22, 'Pioneira'; lane 23, 'FHIA 01'; lane 24, 'FHIA 18'; lane 25, 'SH3640'; lane 26, 'Pacovan'; lane 27, 'PV42-142'; lane 28, 'PV42-85'; lane 29, 'PV42-68'; lane 30, 'Caipira'; lane 31, 'Maçã'; lane 32, 'Yangambi km 2'; lane 33, 'YB42-21'; lane 34, 'Terra'; lane 35, 'Prata Porte Baixo'; lane 36, 'Lidi'; lane 37, negative control (no DNA); m, 123 ladder. Samples 2-6, 9, and 30 are genomic constitution AAA (3*x*); samples 10-12 are AAAA (4*x*); samples 8, 13-21, 26, 31, 32, 34, and 35 genome AAB (3*x*); 7, 22-25, 27-29, and 33 genome AAAB (hybrids 4*x*); 36 AA diploid (2*x*). No amplification occurred for samples on lanes 2 and 27.

common bean, apple, and citrus). It gives satisfactory results and is simple and affordable without the risks associated with radioisotope use. However, silver nitrate solutions must be discarded properly to avoid environmental contamination. The amplification reaction, separation by electrophoresis and staining can be easily conducted in a single day.

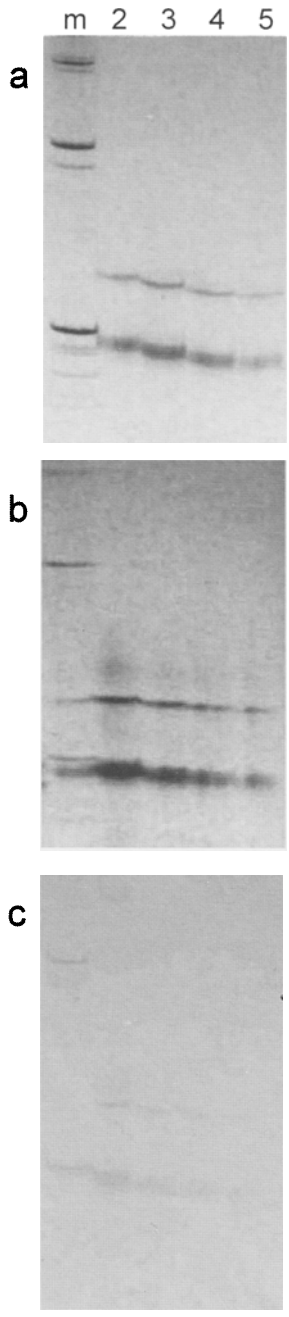

Figure 2. Sensitivity of detection of simple sequence repeat amplification from banana cultivar 'Ouro da Mata' (genome AAAB) using primers AGMI 24/25 with 3 silver staining methods in denaturing 6% polyacrylamide gels. Decreasing volumes of amplified products were loaded per lane. Lane m, 123 ladder; lane 2, 8 μL; lane 3, 4 μL; lane 4, 2 μL; lane 5, 1 μL. (a) Improved method based on Beidler et al. (1982); (b) method of Bassam et al. (1991); (c) method of Sanguinetti et al. (1994).

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