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Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels

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A reliable, fast, cheap and sensitive silver staining method to detect nucleic acids in polyacrylamide gels was developed from two standard stain procedures. The main differences between the three methods concerned (i) the preexposure with formaldehyde during silver nitrate impregnation, (ii) the addition of sodium thiosulfate and sodium carbonate instead of sodium hydroxide during development; (iii) the removal of the stop reaction or the inclusion of absolute ethanol with acetic acid in the stop solution and (iv) the duration of the different reaction steps. All methods allowed the detection of similar polymorphisms for single sequence repeats with different cotton genotypes but important differences regarding the contrast, background and conservation duration of the gels were observed. Two methods gave superior sensitivity. The improved method was sensitive, fast (20 min), gave lower backgrounds, produced gels with long-term conservation ability, and allowed a reutilization of all the solutions used in the staining procedure from five to seven times, making it quite cheap.
Keywords. *Gossypium*, microsatellites, silver staining, cotton, polyacrylamide gels.

Développement d'une méthode fiable, rapide, économique et sensible de coloration au nitrate d'argent pour la détection des marqueurs SSR sur gels de polyacrylamide. Une méthode fiable, rapide, économique et sensible de coloration au nitrate d'argent pour la détection des acides nucléiques sur gels de polyacrylamide a été développée à partir de deux procédures standards. Les principales différences entre les trois méthodes comparées sont : (i) le prétraitement au formaldéhyde pendant l'imprégnation au nitrate d'argent, (ii) l'addition d'hydroxyde de sodium à la place du thiosulfate de sodium et du carbonate de sodium durant le développement, (iii) l'élimination de l'étape stop ou l'ajout d'éthanol absolu avec de l'acide acétique dans la solution stop et (iv) la durée des différentes réactions à chaque étape. Toutes les méthodes ont permis la détection du même polymorphisme pour une répétition de séquences simples avec différents génotypes de cotonnier mais d'importantes différences concernant le contraste, la couleur de fond du gel et la durée de conservation ont été observées. Deux méthodes ont donné une sensibilité supérieure. La méthode améliorée est sensible, rapide (20 min) et permet la réutilisation de toutes les solutions utilisées dans la procédure de coloration de cinq à sept fois, ce qui la rend très économique.
Mots-clés. *Gossypium*, microsatellites, coloration au nitrate d'argent, coton, gels de polyacrylamide.

1. INTRODUCTION

Radioactive or fluorescent detection approaches of DNA molecular markers are expensive, time-consuming and require special facilities that render them impracticable in most tropical countries where sophisticated infrastructure are lacking. On the opposite, silver staining is a relatively rapid, inexpensive alternative to these techniques. It was originally described for ultrasensitive detection of polypeptides separated by polyacrylamide gel electrophoresis (Merril *et al.*,1981) and later modified for nucleic acid detection with increased sensitivity due to various small adaptations of the original silver staining methods (Sommerville, Wang, 1981; Beidler *et al.*, 1982, Goldman, Merril, 1982; Blum *et al.*, 1987; Bassam *et al.*, 1991; Sanguinetti *et al.*, 1994). The last detection methods of nucleic acids using silver staining can be considered as sensitive as autoradiography and fluorescence labelling techniques (Christensen *et al.*, 1999).

Compared to the photochemical silver staining procedure devised by Merril *et al.* (1981) for polypeptide detection, 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 pre-treatment with potassium dichromate and nitric acid. This staining method is very sensitive and is often considered as a standard for non radioactive DNA detection. Its main constraints concern (i) its long duration; (ii) a relatively poor contrast of the DNA bands; (iii) the need to maintain the temperature at 10°C for the development and stop solutions; and (iv) the rather low rate of reuse of the staining solutions. The objective of this study was to develop a new method of silver staining that is as sensitive as the one of Bassam *et al.* (1991) but much simpler, faster and cheaper using interspecific hybrid of cotton as a model system. A quick staining procedure developed from the method of Sanguinetti *et al.* (1994) was modified in our laboratory in order to improve its level of sensitivity and the conservation ability of the gels after staining. The new staining procedures were compared to the standard method of Bassam *et al.* (1991) and the conditions for detection of SSRs were optimised using denaturing polyacrylamide gels bound to glass plates.

2. MATERIALS AND METHODS

Total genomic DNA was extracted from fresh young leaves of 12 greenhouse-grown cotton genotypes (*Gossypium hirsutum* L. cultivars, wild species and interspecific hybrid) using a modified procedure (Benbouza *et al.* submitted) adapted from Vroh Bi *et al.* (1996).

Amplification of genomic DNA was done according to the protocol of Liu *et al.* (2000). PCR reactions were performed in 10 μl volume containing 10 ng of cotton DNA template, 10 x PCR Buffer (100 mM Tris-HCL, pH 9 and 500 mM KCl, 15 mM $MgCl_2$) (Amersham Pharmacia Biotech, Inc), 2-3.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μM of each single primer (Invitrogen), 0.4 U of AmpliTaq (Amersham Pharmacia Biotech, Inc).

The amplification profile consisted of an initial period of DNA denaturation and AmpliTaq activation at 95°C for 12 min, followed by 40 cycles of 93°C (step 1) for 15 s, 55°C (step 2) for 30 s, and 72°C (step 3) for 1 min. The ramp time was 42 s to step 1, 36 s to step 2, and 40 s to step 3. After 40 cycles, the extension temperature of 72°C was held for 6 min.

The polyacrylamide electrophoresis gels were prepared as follows. Wash a 33.2 x 38.2 cm glass plate with 5 ml of absolute ethanol using paper towel. Let dry for 2 min and repeat the operation again. Treat the glass with 15 μl of γ-methacryloxy-propyl-trimethoxysilane (Bind Silane, M-6514, Sigma) in 4 ml of absolute ethanol and 1.25 ml of 10 % acidic acid. Let dry 5 min, and then polish using Kimwipes paper. Then, remove the excess using Kinwipe paper moistened with absolute ethanol.

Treat a larger glass plate (33.2 x 41.6 cm) 5 ml of absolute ethanol using Kimwipes paper. Let dry for 2 min, repeat the operation again and polish with Kimwipes paper.

Prepare a 40 % acrylamide solution with 38 g acrylamide and 2 g N, N'-methylene bisacrylamide in 100 ml distilled water.

Prepare a urea: acrylamide solution with 75 ml of 40 % acrylamide solution, 50 ml 10x TBE (1M Trizma, 89 mM boric acid, 20 mM Na_2 EDTA) and 210 g urea. Add distilled water to a final volume of 500 ml, filter and stock at 4°C in a brown bottle.

Prepare gels (6 % polyacrylamide; 8M urea) by mixing 60 ml of the urea: acrylamide solution in 10x TBE with 250 μl of ammonium persulfate and 50 μl of TEMED (Sigma). Apply the gel solution to the assembled gel plates (1.5 mm thick) using S2 Sequencing gel electrophoresis apparatus (Invitrogen). Allow the gel to polymerize during 30 min.

The amplification products were denatured for 2 min at 92°C in the thermocycler and placed on ice before being applied to the gel in 5 μl containing an equal volume of stop solution (10 mM NaOH, 95% Formamide, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol). The running conditions were 1680 V, 40 mA, 80 W for 75 min, after a pre-run of the gels for 1 h. The electrophoresis running buffer (TBE 1x) contained 10 mM Trizma, 8.9 mM boric acid, 2 mM Na_2 EDTA.

Gels were silver stained using three methods according to the steps described in **table 1**. The protocol of Bassam *et al.* (1991) (Method 1) was followed exactly as recommended. The method derived from the procedure of Sanguinetti *et al.* (1994) (Nachit, Elouafi, personnal communication) (Method 2) reduces drastically the time devoted to the different steps, by adding a very short rinse step (1 s) in distilled water after the impregnation of the gel with silver nitrate, by using NaOH as reagent to increase the pH of the development solution which also includes a higher content of formaldehyde and by suppressing the stop reaction. The improved staining method we developed (Method 3) is a combination of the different steps proposed by Bassam *et al.* (1991) and Sanguinetti *et al.* (1994). It consisted in the following steps: after electrophoresis, gels were washed in 2000 ml cold (10-12°C) fixing solution (10% absolute ethanol, 0.5%

Table 1. Steps of the three compared procedures for silver staining — *Les étapes des différentes méthodes de coloration au nitrate d'argent utilisées.*

Step	Method 1 Bassam *et al.* (1991)	Method 2 Modification of Sanguinetti *et al.* (1994)	Method 3 Improved procedure
1 - Fixation	10% acetic acid 20 min	10% ethanol 0.5% acetic acid 3 min	10% absolute ethanol 0.5% acetic acid 5 min
2 - Rinse	H2O 3 times	-	-
3 - Impregnation	Per liter: 1 g $AgNO_3$, 1.5 ml 37% HCOH 30 min	Per liter: 2 g $AgNO_3$ 5 min	Per liter: 1,5 g $AgNO_3$, 1.5 ml 37% HCOH (22-24°C) 6-7 min
4 - Rinse	H_2O, 20 s, optional	Distilled H_2O, 1 s	Distilled H_2O, 10^{-1} s
5 - Development	Per liter: 30 g Na_2CO_3, 1.5 ml 37% HCOH; 2 mg Na_2SO_3.5 H_2O (10°C) 2-5 min	Per liter: 15 g NaOH, 2 ml 37% HCOH 5 min	Per liter: 15 g NaOH, 2 ml 37% HCOH (22-24°C) 3-5 min
6 - Stop	10% acetic acid (10°C) 5 min	-	10% absolute ethanol 0.5% acetic acid 2 min

acetic acid) for 5 min. Washed gels were soaked for 6-7 min at room temperature (22-24°C) in a 2000 ml solution of 0.15% $AgNO_3$, 2 ml 37% HCOH. Gels were rinsed quickly (10-15 s) once with 2000 ml distilled H_2O. They were then developed by soaking them at room temperature (22-24°C) in a 2000 ml developing solution (1.5% NaOH, 3 ml 37% HCOH) until the bands appear with a sufficient intensity (3-5 min). When the desired intensity was achieved development was stopped by impregnating the gel in a 2000 ml stop solution (10% absolute ethanol, 0.5% acetic acid) for 2 min. All steps were done in plastic containers. The gel plates were agitated in a shaker throughout the staining process. All solutions were prepared using ultra pure distilled water (12 M .cm). The fix stop, developer and silver nitrate solutions were prepared in advance but formaldehyde was added just before use. Impregnation with silver nitrate was performed under room lighting. We added systematically 1 ml of formaldehyde during gels development after the first use of silver nitrate solution. Gels were dried at room temperature and DNA bands were viewed directly with aid of a white light box, then photographed or scanned.

We used absolute ethanol and sodium hydroxide from Merck Eurolab. Acetic acid was from Sigma. Formaldehyde and silver nitrate were ACS reagent from Sigma and Aldrich respectively.

3. RESULTS AND DISCUSSION

DNA from twelve cotton genotypes (diploid species, tetraploid cultivars and interspecific tetraploid hybrids) was amplified by PCR using SSR primer pairs BNL1673 and BNL3792 on polyacrylamide gel. These primer pairs were developed at Brookhaven National Laboratory. Clone sequences used for primer definition are available at http://ukcrop.net/perl/ace/search/cottonDB. Samples of the same DNA amplication reactions were applied to three identical 6% polyacrylamide gels bound to glass plates. The three silver staining methods were used for detection of SSR polymorphism. The methods of Bassam *et al.* (1991) and the improved procedure disclosed identical banding patterns with identical alleles (**Figure 1**) while some bands were not visible for the method inspired from Sanguinetti *et al.* (1994). The methods also differed regarding the contrast, the conservation period of the gels and the number of possible reuse of the different solutions involved in the staining procedure.

Method 1 was long (63 min) and exhibited a relatively poor image contrast when applied in our laboratory, yielding grey-black bands on a light grey background. It was however as sensitive as the improved method we developed allowing the detection of the smallest fragments of the standard molecular weight

a b

Figure 1. (a) Revelation of the amplification products of SSR primer pair BNL3792 SSR on polyacrylamide gel using method 2; (b) Revelation of the amplification products of SSR primer pair BNL3792 on polyacrylamide gel using the optimised silver staining method — (a) *Révélation des produits d'amplification du couple d'amorces SSR BNL3792 sur gel polyacrylamide par la méthode 2. (b) Révélation des produits d'amplification du couple d'amorces SSR BNL3792 sur gel polyacrylamide par la méthode de coloration au nitrate d'argent améliorée.*

Lane 1: G. *hirsutum* var. STAM F; Lane 2: G. *hirsutum* var. NC8; Lane 3: G. *sturtianum*; Lane 4: G. *raimondii*; Lane 5: G. *hirsutum* var. TM1; Lane 6-7: trispecific hybrid G. *hirsutum* x G. *raimondii* x G. *sturtianum* (HRS); Lane 8-9: BC1; Lane 10-11: BC2; Lane 12: BC2S1. MPM: Standard molecular weight (Phi X 174 DNA/Hinf I) — *Marqueur du poids moléculaire (Phi X 174 DNA/Hinf I).*

(Phi X 174 DNA/Hinf I) (48 bp). The fixing solution could be reused three times and the stopping solutions five times while the silver nitrate solution could be reused only three times without loss of sensitivity. Method 2 was very fast (12 min) without need of controlling the temperature during the development step but it gave the lowest sensitivity producing a strong yellow background with discrete bands that were not visible for the smallest DNA fragments. The different solutions used in the staining process could be reused from three (coloration and development) to five times (fixation). The major problem with this method was the very rapid degradation (appearance of cracks) of the gels bound on glass plates a few hours after the end of the developing step.

The optimised method was fast (20 min) and at least as sensitive as the method of Bassam *et al.* (1991) with a better contrast. All the steps can be carried out at room temperature. The polyacrylamide gels stained using this procedure could be stored up to 12 months and all the solutions used in the different staining steps could be reused at least five times, with a maximum of seven times for the silver nitrate solution, without loss of sensitivity. Preparation and handling of the solutions were the same for all methods. The silver nitrate and developing solutions were stored at room temperature while the fixing solution was stored at 4°C.

The presence of absolute ethanol at a rate of 10% in the fixing solution permits elimination of molecules that interfere with silver staining such as electrophoresis buffer, urea, denaturants and detergents. It also allowed a drastic reduction of the concentration of acetic acid (from 10% to 0.5%) necessary to render the macromolecules insoluble in the gel and prevent them from diffusing out of the matrix during the subsequent staining steps.

A concentration of $1.5 g \cdot l^{-1}$ $AgNO_3$ gives optimal sensitivity in relatively short time of impregnation (6-7 min). The presence of formaldehyde in the silver impregnation solution improves both sensitivity and intensity of the detected bands. Washing the gels briefly following this step removes the excess of silver from their surface.

Replacing sodium carbonate by sodium hydroxide permits to establish alkaline conditions (pH 13) for silver ion reduction to metallic silver by formaldehyde. A high formaldehyde concentration (3 ml 37% HCOH per liter) in the development solution combined with a development at room temperature (22-24°C) apparently increased band intensity (**Figure 2**) and therefore also the sensitivity of the method and it decreased development time. The reduction of silver by formaldehyde is temperature-dependent. It is enhanced by increasing temperature; conversely, decreasing the temperature of the development solution below the 18-25°C range (Blum *et al.*, 1987) increases developing time. Bands appeared dark brown-black on an uniformly pale yellow background; over development resulted in an orange brown background with low contrast bands. For some amplified primers, minor shadow bands arising from PCR slippage were commonly visible generally two bases but sometimes three or more, below the main band. As for the fixing step, the addition of absolute ethanol permits to decrease the concentration of acetic acid to 0.5% in the solution that stops the reduction of Ag^+ ions to metallic silver.

We have used the improved method presented here routinely in our laboratory for analyses of SSRs in various tropical crops (**Figure 3**) (*Phaseolus polyanthus* Greenm, *P. coccineus* L., *P. vulgaris* L., *Oryza sativa* L.) (Silué *et al.*, 2004). It gives satisfying results and is simple and cheap without the risks associated to radioisotope use. Its short duration permits the staining of a large number of gels per day.

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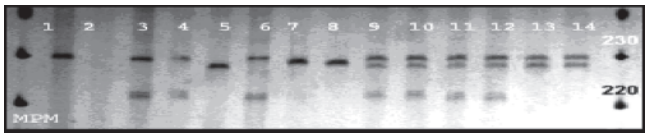

Figure 2. Revelation of the amplification products of SSR primer pair BNL1673 on polyacrylamide gel using the optimised silver staining method — *Révélation des produits d'amplification du couple d'amorce SSR BNL1673 sur gel polyacrylamide par la méthode de coloration au nitrate d'argent améliorée*.

Lane 1: G. *raimondii*; Lane 2: G. *hirsutum* (var. STAM F); Lane 3: G. *hirsutum* (var. NC8); Lane 4: G. *hirsutum* (var.C2); Lane 5: G. *sturtianum*; Lane 6: G. *hirsutum* (var.TM1); Lane 7-8: HRS (G. *hirsutum* x G. *raimondii* x G. *sturtianum*); Lane 9-10: BC1; Lane 11-12: BC2; Lane 11-12: BC2S1; MPM: Standard molecular weight (Phi X 174 DNA/Hinf I) — *Marqueur du poids moléculaire (Phi X 174 DNA/Hinf I).*

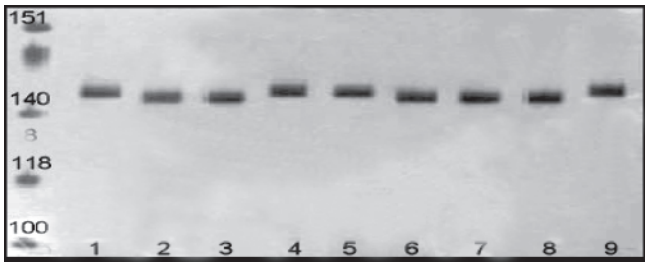

Figure 3. Revelation of the amplification products of SSR primer pair AG1 applied to different *Phaseolus* genotypes on polyacrylamide gel using the optimised silver staining method — *Révélation de l'amplification du couple d'amorces SSR AG1 sur différents genotypes de* Phaseolus *sur gel polyacrylamide par la méthode de coloration au nitrate d'argent améliorée*.

Lane 1-9 : *P. vulgaris*

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