Methylation-sensitive amplification polymorphism (MSAP) protocol to assess CpG and CpNpG methylation in the banana genome

Franc-Christophe BAURENS*, Sandrine CAUSSE, Thierry LEGAVRE

CIRAD-Bios, UMR DAP, TA A-96 / 03, Avenue Agropolis, 34398 Montpellier Cedex 5, France
franc-christophe.baurens@cirad.fr, sandrine.causse@cirad.fr, thierry.legavre@cirad.fr

Methylation-sensitive amplification polymorphism (MSAP) protocol to assess CpG and CpNpG methylation in the banana genome

Abstract — **Introduction**. The technique presented provides molecular markers for DNA methylation studies in the *Musa* sp. genome. This technique can be used for studying DNA methylation in natural populations, in mapping populations, and also in plant organs or during plant development. The principle, key advantages, starting plant material, time required and expected results are presented. **Materials and methods**. This part describes the required materials, and the protocols for producing MSAP autoradiography with *HpaII* / *MspI* (CCGG methylation) and for *EcoRII* / *PspGI* (CCWGG methylation). It mentions the main problems which can occur. **Results**. The protocol makes it possible to detect CCWGG and CCGG methylated sites.

France / *Musa* sp. / methods / methylation / DNA / marqueur génétique

Protocole MSAP pour évaluer les méthylations CpG et CpNpG dans le génome du bananier.

Résumé — **Introduction**. La technique présentée fournit des marqueurs moléculaires permettant des études de la méthylation de l'ADN du génome de l'espèce *Musa*. Cette technique peut être utilisée pour l'étude de la méthylation de l'ADN dans les populations naturelles de bananier, dans des populations de cartographie, dans les organes de la plante et au cours du développement. Le principe, les principaux avantages, le matériel végétal utilisé, le temps nécessaire et les résultats attendus sont présentés. **Matériel et méthodes**. Cette partie décrit le matériel de laboratoire nécessaire, et les protocoles pour produire des autoradiographies MSAP ave *HpaII* / *MspI* (méthylation de CCGG) et *EcoRII* / *PspGI* (méthylation de CCWGG). Elle mentionne les principaux problèmes pouvant se poser. **Résultats**. Le protocole permet de détecter les sites CCWGG et CCGG méthylés.

France / *Musa* sp. / méthode / méthylation / ADN / genetic markers

* *Correspondence and reprints*

Fruits, 2008, vol. 63, p. 117–123
© 2008 Cirad/EDP Sciences
All rights reserved
DOI: 10.1051/fruits:2007054
www.fruits-journal.org

1. Introduction

Application

This protocol aims at:

– identifying molecular markers linked to DNA methylation,

– identifying genomic regions with differential methylation patterns in relation to biological processes (*e.g.*, cell development, somaclonal variation).

Furthermore, this technique provides molecular markers for DNA methylation studies in plant natural populations or mapping populations.

Principle

The method is based on the principle of the amplified length fragment polymorphism (AFLP) technique [1]. DNA samples are cut with a rare cutter enzyme (usually *EcoRI*) and a frequent cutter (*MseI* in the original AFLP procedure). In the MSAP reaction [2],

Article published by EDP Sciences and available at http://www.fruits-journal.org or http://dx.doi.org/10.1051/fruits:2007054

two separated AFLP reactions are performed on each sample, using as a frequent cutter enzyme a pair of isochizomers with differential methylation sensitivity. The *HpaII* / *MspI* isoschizomer pair recognizes the same DNA sequence (5' CCGG 3'). Conversely to *MspI*, *HpaII* cannot cut the internally methylated $C^{m}CGG$ sequence. Comparison between DNA fragments issued from *EcoRI* / *HpaII* and *EcoRI/ MspI*, restrictions allows for identification of the methylation status of the CCGG site. The presence of bands in both patterns reflects an unmethylated site, presence in the *EcoRI* / *MspI* lane only, reflects an internally methylated site and, finally, the presence of bands in the *EcoRI* / *HpaII* lane only reflects a hemimethylated site [3]. As plant DNA methylation implies symmetrical CpG and CpNpG sites and also non-symmetrical CpX sites [4, 5], we propose here two different protocols for CpG and CpNpG methylation survey, based on the *HpaII* / *MspI* pair for CCGG sites and the *EcoRII* / *PspGI* pair for CCWGG sites.

Key advantages

DNA methylation survey at different sites of the genome (depending on the isoschizomers choosen for the study) provides an estimation of the methylation status of DNA; markers identified as differentially methylated in the biological process surveyed can be easily cloned; low quantities of DNA are required (*i.e.*, 500 ng per sample), DNA methylation assays can thus be performed with small samples of plant material (*i.e.*, small plant organs).

Starting material

DNA samples with sufficient purity for PCR amplification and enzymatic reactions (*i.e.*, restrictions, ligations) are required.

Time estimation

Starting from DNA samples, roughly 1 week is needed to obtain the first marker. Twelve samples in duplicate (48 lanes) can be compared on a 40 cm × 60 cm gel with one primer pair. Usually, 40 to 80 CCGG sites are revealed per primer pair.

2. Materials and methods

Materials

The protocol requires:

– PCR apparatus,
– agarose gel electrophoresis devices (minigel),
– a microcentrifuge,
– a high-voltage generator for acrylamide gel electrophoresis,
– acrylamide gel electrophoresis tools (usually 40 cm × 60 cm gel) and autoradiography facilities,
– 37 °C and 65 °C water bath or temperature block,
– pipetting material (from 1 µL to 1000 µL),
– sterile pipette tips,
– sterile 1.5-mL microcentrifuge tubes,
– 96-well PCR plaques or 0.5-mL PCR tubes,
– *EcoRI*, *HpaII* and *MspI, EcorII* and *PspGI* enzymes with specific buffers,
– 10 X Buffer Y+ for double digestion,
– T4 DNA ligase with buffer,
– polynucleotide kinase (PNK) with buffer,
– Taq DNA polymerase with PCR buffer,
– $MgCl_2$ 50 mM,
– dNTP mix (2.5 mM equimolar solution of dATP, dCTP, dGTP, dTTP),
– 1 M tris HCl (pH 8.0),
– 5 M NaCl,
– TBE buffer (0.5 X),
– mineral oil,
– γ^{33}P-ATP (2500 Ci·mmol^{-1}, 10 mCi·mL^{-1}),
– formamide dye-loading solution [0.5% (w/v) xylene cyanol, 0.5% (w/v) bromophenol blue, 12.5% saccharose, 10 mM NaOH in 95% formamide],
– acrylamide stock solution (5% acrylamide / bis-acrylamide 19 /1; 0.5 X TBE; 8 M urea),
– ammonium persulfate (10 mg·mL^{-1} solution in water),
– TEMED (N,N,N',N' tetramethylmethylene diamine),
– Oligonucleotide for linkers (stored as 100 µM solution in water):

- HLINK1: 5' GATCATGAGTCCTGCT 3',
- HLINK2: 5' CGAGCAGGACTCATGA 3',
- ELINK1: 5' CTCGTAGACTGCGTACC 3',
- ELINK2: 5' AATTGGTACGCAGTCTAC 3',
- EcoRIILINK1: 5'GATCATGAGTCCT-GCT 3',

- EcoRIILINK2: 5’CCWGGAGCAGGAC-TCATGAT.

– Pre-amplification primers:

- HPA + A: 5'ATCATGAGTCCTGCTCGGA3',
- ECO + A: 5'GACTGCGTACCAATTCA3',
- ECORII + A: 5’ATCATGAGTCCTGCTCCWGGA3'.

– *EcoRI* selective primers:

- ECO + AC: 5’ GACTGCGTACCAATTCAC3’,
- ECO + AG: 5’GACTGCGTACCAATTCAG3’.

– *HpaII* / *MspI* selective primers

- HPA2ATT: 5'ATCATGAGTCCTGCTCGGATT3',
- HPA2ATG: 5'ATCATGAGTCCTGCTCGGATG3',
- HPA2AAC: 5'ATCATGAGTCCTGCTCGGAAC3',
- HPA2AAG: 5'ATCATGAGTCCTGCTCGGAAG3'.

– *EcoRII / PspGI* selective primers

- E2ATC: 5’ATCATGAGTCCTGCTCCWGGATC3',
- E2AGC: 5’ATCATGAGTCCTGCTCCWGGAGC3',
- E2AGG: 5’ATCATGAGTCCTGCTCCWGGAGG3',
- E2AGT: 5’ATCATGAGTCCTGCTCCWGGAGT3'.

Note: A: adenine; C: cytosine; G: guanine; T: thymine; W: adenine or thymine.

Protocol for *HpaII* / *MspI* (CCGG methylation)

Caution: Preliminary experiments should be performed before starting this protocol to ensure that all restriction enzymes are not inhibited by DNA extracts.

• Step 1
Restriction no. 1:
– in a 1.5-mL Ependorf tube, add the following components: (10 µL of DNA 50 ng·µL^{-1}) + (10 µL of Buffer Y+) + [2 µL of *EcoRI* enzyme (10 U·µL^{-1})] + (33 µL of distilled water). Total volume: 50 µL;
– incubate the mix for 2 h at 37 °C;
– inactivate enzymes by heating at 65 °C for 15 min.

• Step 2
Divide each sample into two separate series (one for *HpaII*, one for *MspI*).

• Step 3
Restriction no. 2:
– in each tube add: (25.0 µL of mix from step 1) + [1.5 µL of enzyme (10 U·µL^{-1}) (*HpaII* or *MspI*)] + (23.5 µL of distilled water). Total volume: 50.0 µL;
– incubate overnight at 37 °C;
– inactivate enzymes by heating at 65 °C for 15 min;
– briefly centrifuge tubes and let them cool to room temperature.
Note: The protocol for *HpaII / MspI* has been optimized for MBI Fermentas (Vilnius, Lithuania) enzymes and buffers. If other suppliers of enzymes are used, check buffer compatibility between step 1 and step 3. If buffers are not compatible, a purification step by anion exchange column or a phenol / chloroform purification procedure should be added.

• Step 4
Linker preparation and ligation:
– prepare 10 µM linker solution (for the *EcoRI* linker use ELINK1 and ELINK2, for the *HpaII / MspI* linker use HLINK1 and HLINK2) by mixing: (100 µL of LINK1 100 µM) + (100 µL of LINK2 100 µM) + [10 µL of tris 1M (pH 8.0)] + (50 µL of NaCl 5M) + (740 µL of distilled water qsp 1 mL). Total volume: 1000 µL;
– heat to 100 °C and let the solution cool to room temperature;
– add to the restricted DNA mix from step 3 the following components: (10 µL *EcoRI* linker 10 µM) + (10 µL *HpaII* linker 10 µM) + [2 µL T4 DNA ligase (10 U·L^{-1})] + (10 µL ligase buffer 10 X) + (18 µL distilled water). Total volume: 100 µL;
– incubate for 2 h at room temperature (or overnight at 4 °C);
– dilute an aliquot (usually 10 µL or 20 µL) to 1/10th;
– keep the remaining restriction ligation mix at –20 °C for subsequent use.

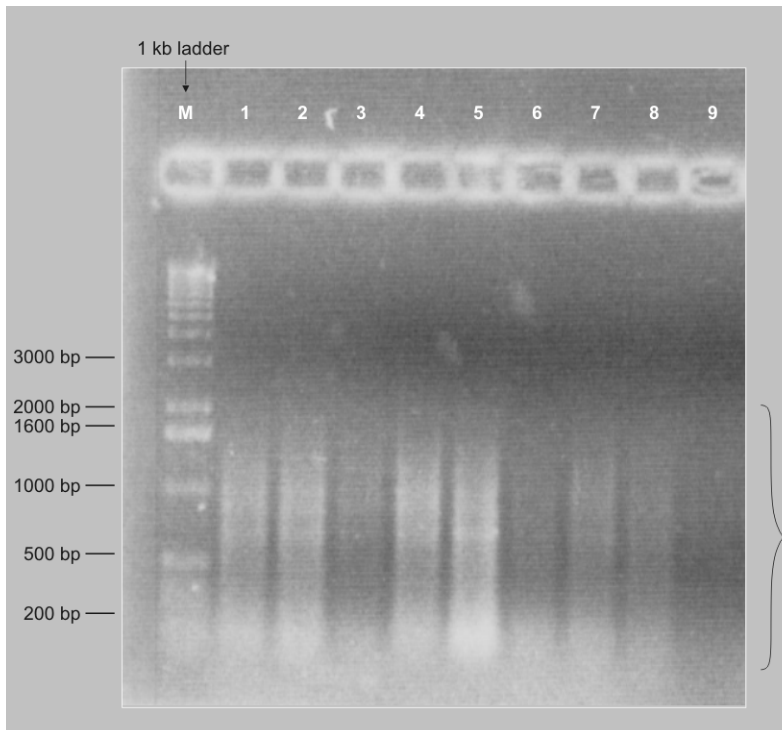

Figure 1.
Pre-amplification visualization on agarose gel electrophoresis (see step 6 of the protocol and troubleshooting section). Lanes 1 to 9: banana DNA samples of Grande Naine cv. Note the size of the smear (bracket on the right) and the heterogeneity between samples. Lanes 3 and 6: weak amplification. Lane 9: no amplification. Heterogeneity at this step will produce the type B pattern of figure 2.

• Step 5
Pre-amplification (*Note:* depending on the number of samples, 0.5-mL PCR tubes or 96-well microplaques can be used):
– for each sample: (5 µL of diluted DNA from step 4) + (5 µL of Hpa + A primer 2 µM) + (5 µL of Eco + A primer 2 µM) + (5 µL of 10 X PCR buffer) + (2 µL of $MgCl_2$ 50 mM) + (3 µL of dNTP 10 mM) + [1 µL of Taq DNA polymerase (1 U·µL^{-1})] + (4 µL of distilled water). Total volume: 50 µL;
– add a drop of mineral oil and run the PCR program 'PREAMP': 94 °C, 5 min; 20 times [94 °C, 30 s; 56 °C, 1 min; 72 °C, 1 min]; 72 °C, 5 min.

• Step 6
Pre-amplification control: check 5 µL of PCR reaction on agarose minigel.

Caution: the ligation step is the most critical step of this method; pre-amplification control on agarose gel electrophoresis is the first and the most important control of the method. Pre-amplified PCR products should appear as a smear with equal intensities between samples (see also troubleshooting, *figures 1, 2*).

• Step 7
Dilution of pre-amplification products: dilute the pre-amplification solution to 1/20th with distilled water.

• Step 8
Primer labeling:
– for 30 reactions, prepare: (0.5 µL of 100 µM selective primer) + (2.0 µL of 10 X buffer) + (2.0 µL of γ^{33}P-ATP) + [1.0 µL of PNK (10 U·µL^{-1})] + (14.5 µL of distilled water). Total volume: 20 µL;
– incubate at 37 °C for 1 h;
– inactivate enzyme at 65 °C for 10 min and store at 4 °C.
Note: if selective PCR amplification is performed immediately after primer labeling, the PNK inactivation step is not necessary.

• Step 9
Selective radiolabeled amplification:
– For one reaction, prepare: [5.0 µL of diluted pre-amplified DNA (step 7)] + [2.0 µL of *EcoRI* selective primer (2 µM)] + [0.6 µL of labeled selective primer (step 8)] + (0.4 µL of $MgCl_2$ 50 mM) + (1.0 µL of dNTP 10 mM) + (2.0 µL of PCR buffer 10 X) + [1.0 µL of Taq DNA polymerase (1 U·µL^{-1})] + (8.0 µL of distilled water). Total volume: 20.0 µL;
– add a drop of mineral oil to each well and run the PCR program 'AFLP': 94°, 5 min; 12 times [94 °C, 30 s, 65 °C, 1 min (– 0.7 °C per cycle); 72°, 1 min]; 23 times [94 °C, 30 s; 56 °C, 1 min; 72 °C, 1 min]; 72 °C, 5 min.

• Step 10
Acrylamide gel electrophoresis:
– prepare acrylamide gel by mixing 60 mL of acrylamide stock solution, 270 µL of ammonium persulfate (10 mg·mL^{-1}) and 90 µL of TEMED,
– pour the gel between the glass plaques, and let it polymerize for at least 3 h,
– prepare gel for electrophoresis in 0.5 X TBE buffer,
– run gel for 30 min at 55 W,
– rinse the wells with buffer to remove urea and unpolymerized acrylamide,
– add 20 µL of formamide dye to each of the PCR reactions from step 9,
– mix carefully by repeated pipetting,
– heat the reaction mix to 95 °C for 5 min and keep at 65 °C during the next step,
– load 5 µL onto the gel,

– run the gel at 55 W constant power for 2 h,
– autoradiograph dried gel for 3–4 d.

Protocol for *EcoRII* / *PspGI* (CCWGG methylation)

Caution: The classical isoschizomer pair for CCWGG methylation study is *EcoRII / MvaI*. We present here a protocol with *EcoRII / PspGI*. In fact, *MvaI* and *PspGI* have the same methylation sensitivity at the CCWGG site but differ in their cleavage location within the site. Only *PspGI* is compatible with *EcoRII* for this protocol.

• Step 11
Restriction no. 1:
– prepare two series of 1.5-mL Ependorf tubes for *EcoRII* and *PspGI*, labeled with the sample number;
– in *EcoRII* samples, add the following components: (5 µL of DNA 50 ng·µL^{-1}) + (5 µL of buffer 10 X) + [2 µL *of EcoRII* enzyme (10 U·µL^{-1})] + (38 µL of distilled water). Total volume: 50 µL;
– incubate for 2 h at 37 °C;
– in *PspGI* samples, add the following components: (5 µL of DNA 50 ng·µL^{-1}) + (5 µL of buffer 10 X) + [1 µL *of PspGI* enzyme (10 U·µL^{-1})] + (39 µL of distilled water). Total volume: 50 µL;
– incubate for 2 h at 75 °C.

• Step 12
Sample purification:
– purify all samples by phenol / chloroform extraction followed by ethanol precipitation,
– resuspend DNA in 10 µL of distilled water.

• Step 13
Restriction no. 2:
– In each tube mix (10 µL of purified DNA from step 12) + (5 µL of buffer 10 X) + [2 µL of *EcoRI* enzyme (10 U·µL^{-1})] + (38 µL of distilled water). Total volume: 50 µL;
– incubate for 2 h at 37 °C;
– inactivate enzyme by heating at 65 °C for 15 min;
– briefly centrifuge tubes and let them cool to room temperature.

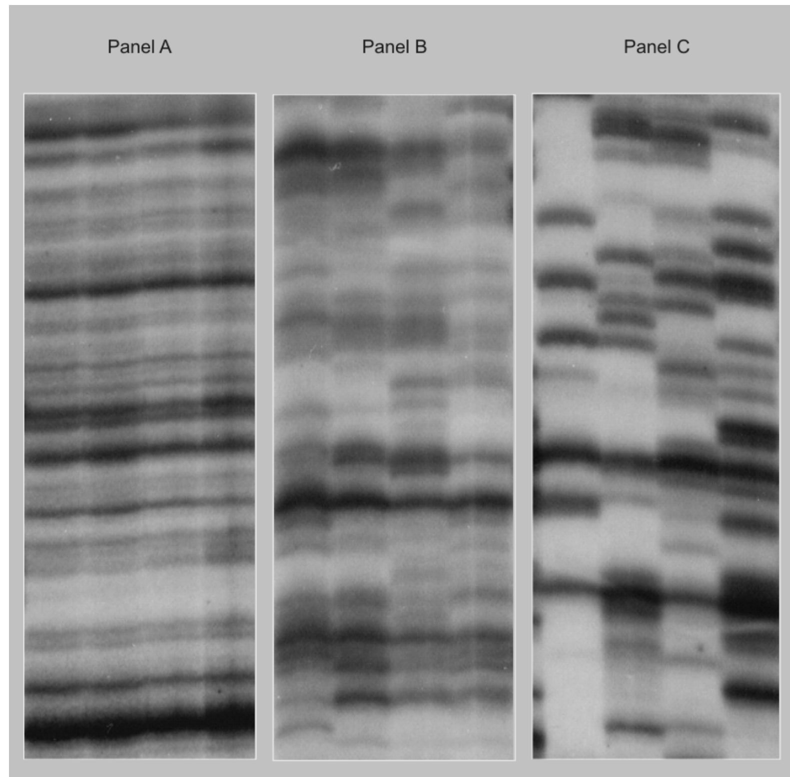

Figure 2.
MSAP pattern obtained with optimal, sub-optimal and stochastic ligation.
Each panel corresponds to MSAP amplification of four identical banana samples: panel A: example of optimal conditions (restriction with *EcoRII*, Eco-AG / E2-AGG primer pair); panel B: example of sub-optimal conditions (restriction with *PspGI*, Eco-AG / E2-AGG primer pair), note the differences in intensities between the lanes and the lack of several bands; panel C: another critical example of sub-optimal conditions (restriction with *PspGI*, Eco-AG / E2-AGG primer pair), note there is no reproducibility of band levels between the lanes.

• Step 14
Linker preparation and ligation:
For the *EcoRI* linker, use ELINK1 and ELINK2; for the *EcoRII* / *PspGI* linker, use EcoRIILINK1 and EcoRIILINK2.
– prepare 10 µM working linker solution by mixing: (100 µL of LINK1 100 µM) + (100 µL of LINK2 100 µM) + [10 µL of tris 1M (pH 8.0)] + (50 µL of NaCl 5M) + (740 µL of distilled water qsp 1 mL). Total volume: 1000 µL;
– heat to 100 °C and let the solution cool to room temperature;
– add to restricted DNA mix from step 13: (10 µL *of EcoRI* linker 10 µM) + (10 µL of *EcoRII* linker 10 µM) + [2 µL of T4 DNA ligase (10 U·µL^{-1})] + (10 µL of ligase buffer 10 X) + (18 µL of distilled water). Total volume: 100 µL;
– incubate for 2 h at room temperature (or overnight at 4 °C);
– dilute an aliquot (usually 10 µL or 20 µL) to 1/10th;
– keep the remaining restriction ligation mix at –20 °C for subsequent use.

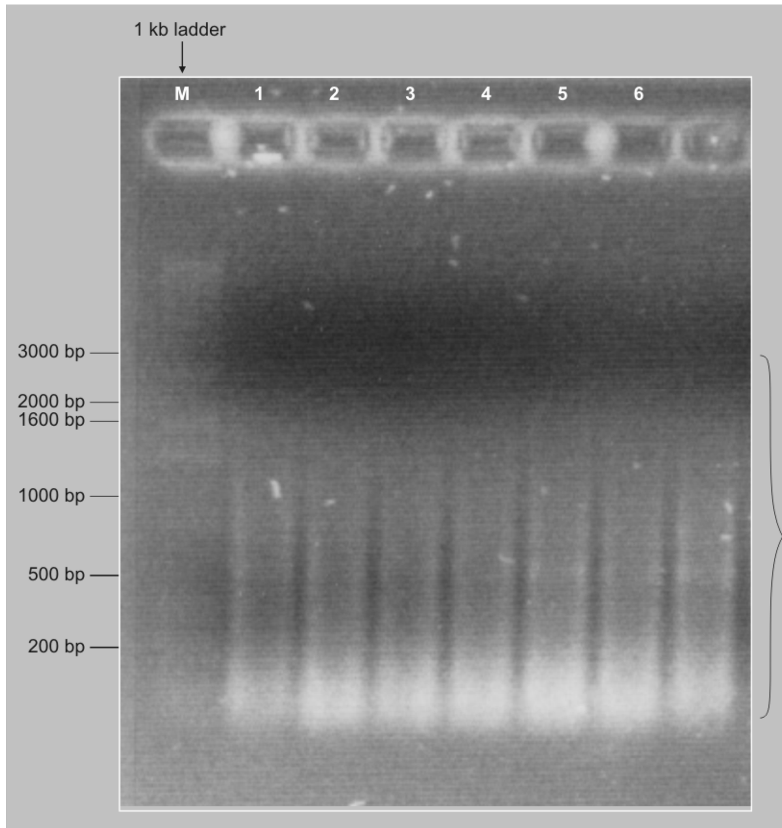

Figure 3.
Pre-amplification visualization on agarose gel electrophoresis. Lanes 1 to 6: banana DNA samples of commercial selections of Grande Naine cv. Note the size of the smear (bracket on the right) and the homogeneity between samples.

• Step 15
Pre-amplification:
– For each sample, mix (5 µL of diluted DNA from step 1) + (5 µL of *EcoRII* + A primer 2 µM) + (5 µL of Eco + A primer 2 µM) + (5 µL of 10 X PCR buffer) + (2 µL of $MgCl_2$ 50 mM) + (3 µL of dNTP 10 mM) + [1 µL of Taq DNA polymerase (10 U·μL^{-1})] + (24 µL of distilled water). Total volume: 50 µL;
– add a drop of mineral oil and run the PCR program 'PREAMP': 94 °C, 5 min; 20 times [94 °C, 30 s; 56 °C, 1 min; 72 °C, 1 min]; 72 °C, 5 min.

• Step 16
To check the pre-amplification, check 5 µL of PCR reaction on agarose minigel.

• Step 17
Follow the same protocol as for *HpaII / MspI* starting from step 7.

Troubleshooting

Four main problems can occur:

(a) There is no pre-amplification or 'bad pattern' (*figure 1*), which can result from no enzymatic reactions.

Solutions:
– check carefully all components of the reactions, especially for the ligation step,
– use fresh aliquots of T4DNA ligase buffer,
– test the functionality of the all restriction enzymes on DNA extracts,
– add a positive control to PCR reaction.

(b) The signal on autoradiography is weak, which can be due to a low quantity of DNA or inhibition of reaction efficiency by unwanted components from DNA extraction.

Solutions:
– check the quantity and quality of DNA before starting experiments,
– increase the time of exposure,
– decrease the dilution factor of pre-amplification PCR products,
– use α^{33}P.dCTP in PCR reaction (incorporation) instead of labeled primers with PNK (end labeling).

(c) There is an abnormal pattern of amplification (*figure 2*): it is due to a poor ligation.

Solution: restart the experiment at step 4.

(d) There is an incorrect migration due to a distortion of the electric field during migration.

Solutions:
– carefully clean the wells before loading samples onto the gels,
– increase the time of polymerization of the gel,
– try 1 X TBE as running buffer.

3. Typical results obtained

The protocol makes it possible to obtain:
– control of the pre-amplification step using agarose gel electrophoresis (*figure 3*),

– autoradiography of *HpaII / MspI* and *EcoRII / PspGI,* MSAP analysis: detection of CCWGG and CCGG methylated sites (*figure 4*).

References

[1] Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J., Kuiper M., Zabeau M., AFLP: a new technique for DNA fingerprinting, Nucleic Acids Res. 23 (21) (1995) 4407–4414.

[2] Reyna-López G.E., Simpson J., Ruiz-Herrera J., Differences in DNA methylation pattern are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms, Mol. Gen. Genet. 253 (1997) 703–710.

[3] Xiong L.Z., Xu C.G., Saghai-Maroof M.A., Zhang Q., Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique, Mol. Gen. Genet. 261 (1999) 439–446.

[4] Schmitt F., Oakeley E.J., Jost J.P., Antibiotics induce genome-wide hypermethylation in cultures of *Nicotiana tabacum* plants, J. Biol. Chem. 272 (3) (1997) 1534–1540.

[5] Zhou Y., Magill C.W., Magill J.M., Newton R.J., An apparent case of nonsymetrical and sustained strand-specific hemimethylation in the Dc8 gene of carrot, Genome 41 (1998) 23–33.

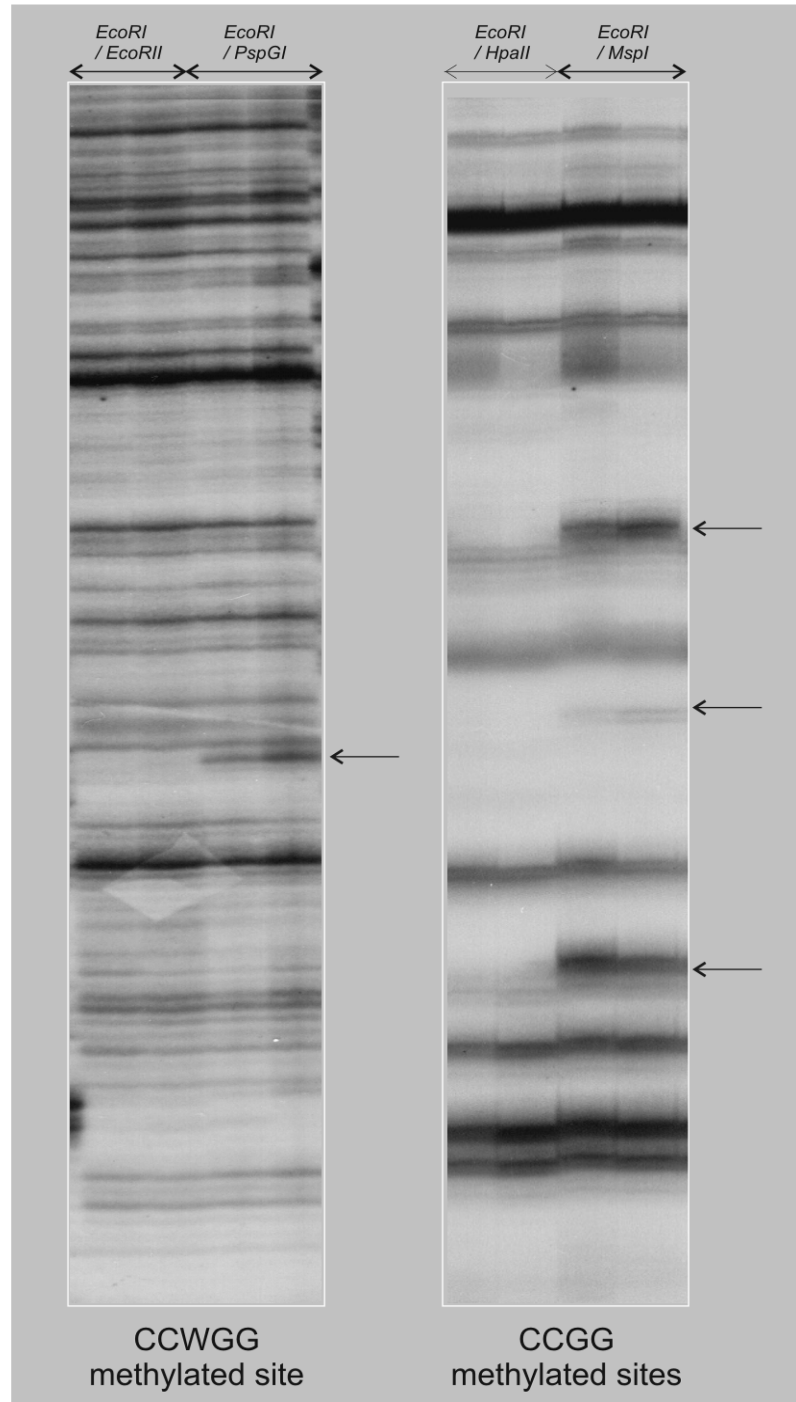

Figure 4.
Typical results of MSAP autoradiography detecting CCWGG and CCGG methylated sites (arrows). Each sample is in duplicate and corresponds to leaf DNA of Grande Naine cv.