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

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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 méthylationns 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 avec 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

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 (MspI in the original AFLP procedure). In the MSAP reaction [2],
two separated AFLP reactions are performed on each sample, using as a frequent cutter enzyme a pair of isochizomers with differential methylation sensitivity. The \textit{HpaII} / \textit{MspI} isochizomer pair recognizes the same DNA sequence (5’ CCGG 3’). Conversely to \textit{MspI}, \textit{HpaII} cannot cut the internally methylated C\textsuperscript{m}CGG sequence. Comparison between DNA fragments issued from \textit{EcoRI} / \textit{HpaII} and \textit{EcoRI} / \textit{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 \textit{EcoRI} / \textit{MspI} lane only, reflects an internally methylated site and, finally, the presence of bands in the \textit{EcoRI} / \textit{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 \textit{HpaII} / \textit{MspI} pair for CCGG sites and the \textit{EcoRII} / \textit{PspGI} pair for CCWGG sites.

**Key advantages**

DNA methylation survey at different sites of the genome (depending on the isochizomers chosen 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 (\textit{i.e.}, 500 ng per sample), DNA methylation assays can thus be performed with small samples of plant material (\textit{i.e.}, small plant organs).

**Starting material**

DNA samples with sufficient purity for PCR amplification and enzymatic reactions (\textit{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 \times 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 (mini-gel),
- a microcentrifuge,
- a high-voltage generator for acrylamide gel electrophoresis,
- acrylamide gel electrophoresis tools (usually 40 cm \times 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,
- \textit{EcoRI}, \textit{HpaII} and \textit{MspI}, \textit{EcorII} and \textit{PspGI} enzymes with specific buffers,
- 10 X Buffer Y+ for double digestion,
- T\textsubscript{4} DNA ligase with buffer,
- polynucleotide kinase (PNK) with buffer,
- Taq DNA polymerase with PCR buffer,
- MgCl\textsubscript{2} 50 mM,
- dNTP mix (2.5 mM equimolar solution of dATP, dCTP, dGTP, dTMP),
- 1 M tris HCl (pH 8.0),
- 5 M NaCl,
- TBE buffer (0.5 X),
- mineral oil,
- \textsuperscript{33}P-ATP (2500 Ci·mmol\textsuperscript{-1}, 10 mCi·mL\textsuperscript{-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\textsuperscript{-1} solution in water),
- TEMED (N,N,N’,N’ tetramethylmethylenediamine),
- 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’GATCATGAGTCCTGCT 3’,
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- EcoRIILINK2: 5'CCWGGAGCAGGACTCATGAT.
- Pre-amplification primers:
  - HPA + A:
    5'5'ATCATGAGTCTCTGCTCGGA3',
  - ECO + A:
    5'5'GACTGCGTACCAATTCA3',
  - ECORI + A:
    5'5'ATCATGAGTCTCTGCTCCWGGA3'.
- EcoRI selective primers:
  - ECO + AC:
    5'GACTGCGTACCAATTCA3',
  - ECO + AG:
    5'GACTGCGTACCAATTCA3'.
- HpaII / MspI selective primers
  - HPA2ATT:
    5'5'ATCATGAGTCTCTGCTCCWGATT3',
  - HPA2ATG:
    5'5'ATCATGAGTCTCTGCTCCWGATG3',
  - HPA2AAC:
    5'5'ATCATGAGTCTCTGCTCCWGGAAC3',
  - HPA2AAG:
    5'5'ATCATGAGTCTCTGCTCCWGGAAG3'.
- EcoRII / PspGI selective primers
  - E2ATC:
    5'5'ATCATGAGTCTCTGCTCCWGATC3',
  - E2AGC:
    5'5'ATCATGAGTCTCTGCTCCWGAGC3',
  - E2AGG:
    5'5'ATCATGAGTCTCTGCTCCWGAGG3',
  - E2ATG:
    5'5'ATCATGAGTCTCTGCTCCWGAGT3'.
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 of EcoRI linker 10 µM) + (10 µL HpaII linker 10 µM) + [2 µL T4 DNA ligase (10 U·µL–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.
Step 5
Pre-amplification (Note: depending on the number of samples, 0.5-mL PCR tubes or 96-well microplagues 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 MgCl₂ 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 γ³₂P-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 MgCl₂ 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°C, 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,
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 EcoRII enzyme (10 U·µL−1)] + (38 µL of distilled water). Total volume: 50 µL;
– incubate for 2 h at 37 °C;
– briefly centrifuge tubes and let them cool to room temperature.

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 1 M (pH 8.0)) + (50 µL of NaCl 5 M) + (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.
Step 15
Pre-amplification:
– For each sample, mix (5 µL of diluted DNA from step 1) + (5 µL of EcoRII primer 2 µM) + (5 µL of Eco + A primer 2 µM) + (5 µL of 10X PCR buffer) + (2 µL of MgCl2 50 mM) + (2 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 α33P.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),

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

References


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.