

## Global DNA Methylation Analysis Using Methyl-Sensitive Amplification Polymorphism (MSAP)

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### Abstract

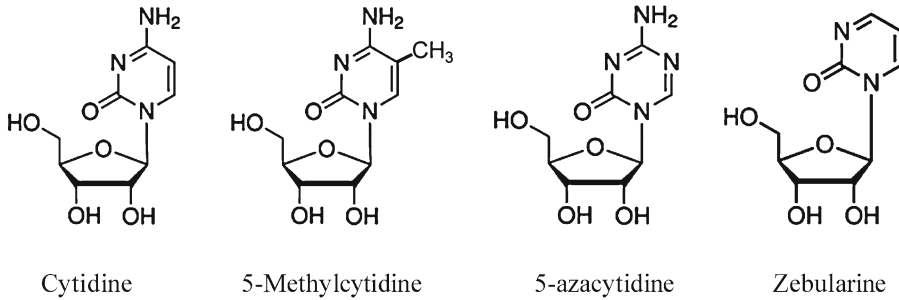
DNA methylation is a crucial epigenetic process which helps control gene transcription activity in eukaryotes. Information regarding the methylation status of a regulatory sequence of a particular gene provides important knowledge of this transcriptional control. DNA methylation can be detected using several methods, including sodium bisulfite sequencing and restriction digestion using methylation-sensitive endonucleases. Methyl-Sensitive Amplification Polymorphism (MSAP) is a technique used to study the global DNA methylation status of an organism and hence to distinguish between two individuals based on the DNA methylation status determined by the differential digestion pattern. Therefore, this technique is a useful method for DNA methylation mapping and positional cloning of differentially methylated genes. In this technique, genomic DNA is first digested with a methylation-sensitive restriction enzyme such as *HpaII*, and then the DNA fragments are ligated to adaptors in order to facilitate their amplification. Digestion using a methylation-insensitive isoschizomer of *HpaII*, *MspI* is used in a parallel digestion reaction as a loading control in the experiment. Subsequently, these fragments are selectively amplified by fluorescently labeled primers. PCR products from different individuals are compared, and once an interesting polymorphic locus is recognized, the desired DNA fragment can be isolated from a denaturing polyacrylamide gel, sequenced and identified based on DNA sequence similarity to other sequences available in the database. We will use analysis of *met1*, *ddm1*, and *atmbd9* mutants and wild-type plants treated with a cytidine analogue, 5-azaC, or zebularine to demonstrate how to assess the genetic modulation of DNA methylation in *Arabidopsis*. It should be noted that despite the fact that MSAP is a reliable technique used to fish for polymorphic methylated loci, its power is limited to the restriction recognition sites of the enzymes used in the genomic DNA digestion.

**Key words** DNA methylation, MSAP, Mutant lines, 5-azaC and zebularine

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## 1 Introduction

DNA methylation is an important epigenetic modification which usually takes place through the covalent attachment of a methyl group to the ring carbon 5 of the cytosine (C) in DNA without affecting the basic nucleotide sequence (Fig. 1). Methylated cytosines that are followed by guanines (G) are annotated as CpG, in which C



**Fig. 1** Chemical structure of cytidine, 5-methylcytidine, 5-azaC, and zebularine

binds to G by a phosphodiester bond (p) rather than the triple hydrogen bond between C and G in double-stranded DNA [1]. DNA methylation plays an important role in controlling gene expression in eukaryotes and it is typically associated with transcriptional gene repression [2, 3]. Determination of DNA methylation at particular locus provides important information on the gene expression pattern and gives detailed knowledge on the regulatory sequence for that gene. DNA methylation level in plants changes during different processes of plant growth and development and also when plants are exposed to biotic and abiotic stresses [4]. While some of these changes are transient, others are heritable through a process called transgenerational memory [5–7].

The DNA methylation pattern in *Arabidopsis* can be genetically manipulated via mutations in genes that maintain and/or are involved in establishing de novo DNA methylation. These include mutations in the *DNA methyltransferase MET1* gene, the chromatin remodeling factor gene *DDM1* (*Decrease in DNA Methylation 1*), and *methylcytosine-binding protein 9* (*AtMBD9*) which all lead to a significant alteration in genome-wide DNA methylation levels and consequently to the reactivation of transcriptionally silent genes and transposable elements [8, 9].

The DNA methylation pattern in *Arabidopsis* genome can also be manipulated chemically. When chemical analogues of cytosine are incorporated into genomic DNA during replication, they inhibit catalytic activity of DNA methyltransferases by covalently binding to their active sites which leads to a general reduction in the DNA methylation level [10]. In plants, the most commonly used cytidine analogue is 5-azacytidine (5-azaC), in which the ring carbon 5 is replaced by nitrogen [10]. The chemical structure of cytidine, 5-methylcytidine, 5-azaC, and zebularine is illustrated in Fig. 1. 5-azaC induces hypomethylation and genome-wide transcriptional reactivation of silent genes and thus modifies plant growth and development [11–13]. Zebularine is also a cytidine analogue and inhibits DNA methylation in a similar way to 5-azaC. Compared to 5-azaC, zebularine is more stable and less toxic although the demethylation effect of zebularine is transient [14].

DNA methylation can be detected using sodium disulfide sequencing, with proteins with an affinity to the methyl group, with anti-methylcytosine antibodies, and using methylation-sensitive restriction endonucleases [15]. Global DNA methylation level can be detected using a southern blot hybridization technique. In this technique genomic DNA is digested with methylation-sensitive endonucleases such as *HpaII* and the methylation-insensitive isoschizomer *MspI*. Then the resulting DNA fragments are probed with an abundantly available gene in the genome such as the 120 bp 5S ribosomal RNA repeat. Global DNA methylation can also be studied using methyl-sensitive amplification polymorphism (MSAP). In this technique, genomic DNA from different samples is digested with the methylation-sensitive endonuclease *HpaII*, and adaptors are ligated to this DNA, followed by fragment amplification using PCR with specific primers (Fig. 2). Qualitative and quantitative differences in the amplification indicate variation in the global DNA methylation pattern, and the significant variation of methylation from site to site as well as from tissue to tissue can be studied. In this chapter, we describe the experimental protocol used to measure the global DNA methylation in methyltransferase mutants including *met1*, *ddm1*, and *atmbd9* mutants as well as in wild-type *Arabidopsis* plants after treatment with 5-azaC or zebularine using the MSAP technique.

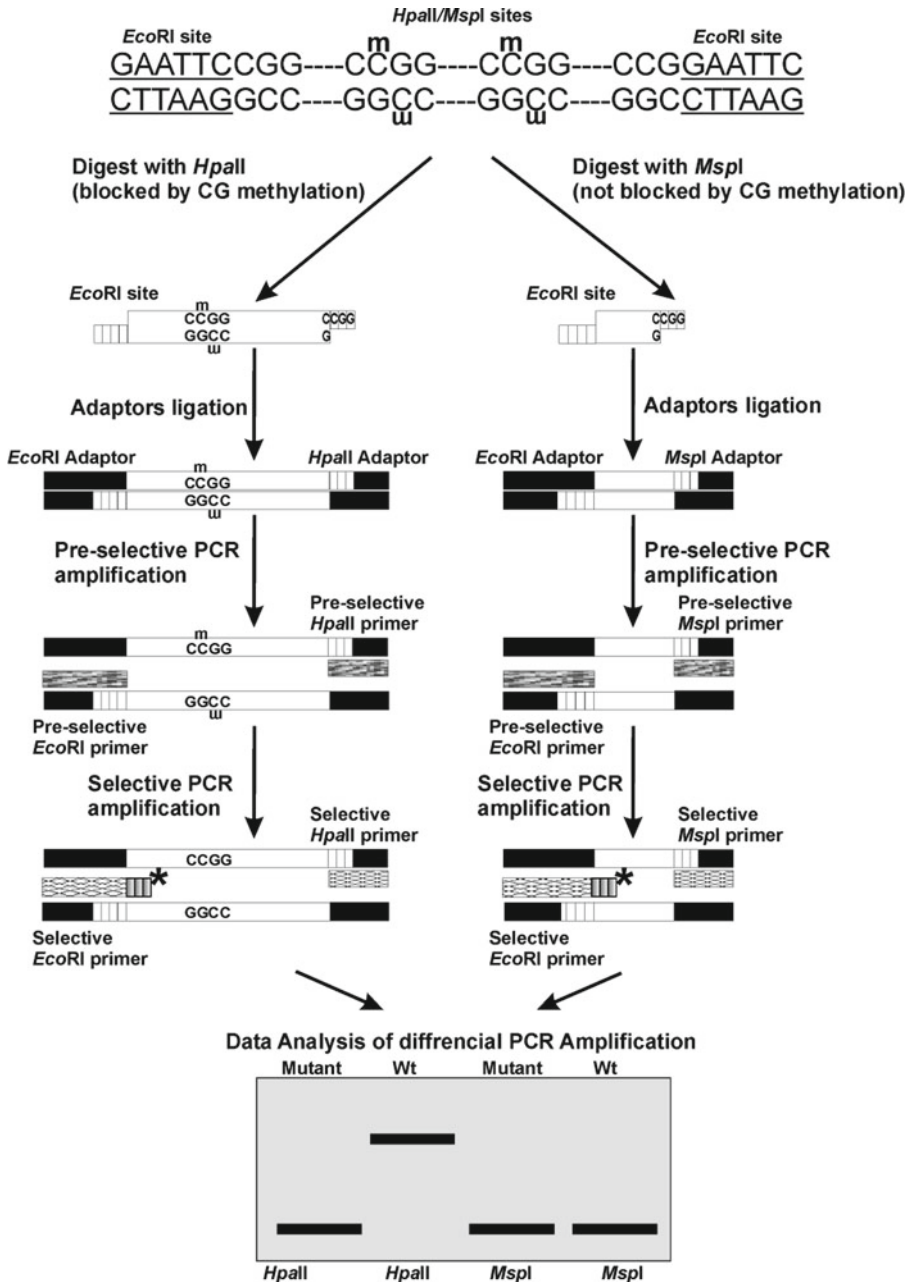
This MSAP technique can be used to identify differentially methylated genomic regions within and between populations of plants of different genetic backgrounds as well as in plants grown under different environmental conditions. In addition, this technique can be used for epigenetic mapping and positional cloning of target genes. MSAP is described here according to the previously published strategies and protocols designed for the amplified fragment polymorphism technique (AFLP) [16] and modified for the MSAP by Beaulieu et al. [17] and Madlung et al. [18]. Although MSAP is a reliable and easy to use technique, methods based on methylation-sensitive digestion limit the detection of methylation to the restriction sites of the endonuclease enzymes used.

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## 2 Materials

### 2.1 Treatment of *Arabidopsis* Seeds with 5-azaC and Zebularine

1. *Arabidopsis* seeds: The seeds of *Arabidopsis thaliana* ecotype Columbia (Col) wild-type and *met1*, *ddm1*, *atmbd9* mutants can be obtained from the Arabidopsis Stock Center (TAIR; [www.arabidopsis.org](http://www.arabidopsis.org)).
2. Sterilization solution (5 % sodium hypochlorite, 0.05 % Tween-20).
3. Ethanol 75 %.
4. 1 mm Whatman filter papers.



**Fig. 2** Schematic representation of the MSAP technique. DNA is digested first with methylation-sensitive (*HpaII*) and methylation-insensitive (*MspI*) endonucleases, then the resulting DNA fragments are ligated to specific adaptors. Subsequently, the ligated DNA fragments are used as templates in a preselective PCR reaction using specific primers. The resulting PCR products are used as DNA template in a selective PCR reaction using three selective nucleotides as fluorescently labeled primers (*asterisk*). The selective PCR products are loaded into an ABI Prism 310 Genetic Analyzer machine. *Bands* are scored for presence or absent

5. DNA demethylation chemicals 5-azaC and zebularine are available in Sigma. Preparing fresh 0.5 mM 5-azaC aqueous solution for each treatment. Never use stored 5-azaC solution (*see Note 1*). Prepare 40 mM zebularine stock solution in sterile distilled water and store at  $-20\text{ }^{\circ}\text{C}$  (*see Note 2*).
6. Preparing zebularine treatment medium: solid 0.5 $\times$  MS medium [19], 1 % sucrose, 1 % agar, 40  $\mu\text{M}$  zebularine in Petri dishes. Control medium is solid 0.5 $\times$  MS medium without zebularine.
7. Pots containing a mixture of universal substrate and vermiculite (3:1 v/v).

## 2.2 Genomic DNA Extraction and Purification

1. Liquid nitrogen.
2. Mortar and pestle.
3. DNA extraction buffer (150 mM Tris-HCl, pH 8.0, 15 mM EDTA (ethylenediaminetetraacetic acid), 1.0 M NaCl, 0.16 % (w/v) CTAB (cetyltrimethylammonium bromide), 20  $\mu\text{L}/\text{L}$  2-mercaptoethanol, and 0.1 % (w/v) PVP (polyvinylpyrrolidone)).
4. Phenol/chloroform/isoamyl alcohol (PCIM, 25:24:1, v/v/v), stored at  $4\text{ }^{\circ}\text{C}$ .
5. 100 % Isopropanol.
6. 75 % Ethanol.
7. Tris EDTA (TE) buffer (10 mM Tris-Cl, pH 7.5. 1 mM EDTA).
8. Sodium acetate 3 M (pH 5.2).
9. QIAGEN DNeasy Plant Maxi Kit (Catalogue number 68163).
10. NanoDrop spectrometer.
11. Agarose gel electrophoresis unit.

## 2.3 Methyl-Sensitive Amplification Polymorphism (MSAP)

1. Restriction enzymes and their buffers (*EcoRI*, *HpaII* and *MspI*).
2. T4 DNA ligase and ligase buffer.
3. Adapters: *EcoRI* adapter: (5'-CTCGTAGACTGCGTACC-3') and (5'-AATTGGTACGCAGTCTAC-3'). *HpaII-MspI* adapter: (5'-GATCATGAGTCCTGCT-3' and 5'-CGAGCAGGACTCATGA-3'). Primers should be HPLC purified and synthesized at 0.2  $\mu\text{M}$  scale.
4. Oligonucleotide primers: preselective *EcoRI* oligonucleotide primer (5'-GACTGCGTACCAATTC-3'), preselective oligonucleotide primer *HpaII-MspI* (5'-ATCATGAGTCCTGCTCGG-3'), selective *EcoRI* oligonucleotide primer (5'-GACTGCGTACCAATTC-AAC, ACC, ACA or AAG-3') (Applied Biosystems) (*see Note 3*), and *HpaII-MspI* selective

oligonucleotide primers (5'-ATCATGAGTCCTGCTCGGTCAA-3' and 5'-ATCATGAGTCCTGCTCGGTCCA-3'). Primers should be HPLC purified and synthesized at 0.2  $\mu$ M scale.

5. *Taq* DNA polymerase, buffer, and dNTPs.
6. A thermocycler such as Perkin-Elmer GenAmp PCR System 9700.
7. Mini agarose gel electrophoresis unit.
8. 1 $\times$  Tris Borate EDTA (TBE) buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA).
9. 100 Kb DNA GeneRuler ladder (Fermentus, catalogue number SM0241).
10. 6 $\times$  DNA loading dye (Fermentus, catalogue number R0611).
11. GeneScan-500 [ROX] internal size standard (Applied Biosystems, catalogue number 401734).
12. Deionized formamide (Applied Biosystems, Catalogue number 400596).
13. ABI Prism 310 Genetic Analyzer (Applied Biosystems).
14. ABI Prism GeneScan 3.1 software.

#### **2.4 Identification of the Polymorphic DNA**

1. Selective *Eco*RI oligonucleotide primers end labeled with radioisotope (ATP [ $^{32}$ P]) end-labeling grade from ICN Radiochemicals, Solon, OH, USA.
2. 40 % Acrylamide solution (37:5:1 acrylamide-bis-acrylamide solution) can be obtained from Bio-Rad Life Science.
3. 1 M Tris-HCL buffer (pH 8.0) can be obtained from Sigma Aldrich.
4. 10 % ammonium persulfate (10 mg/mL) can be obtained from Bio-Rad Life Science.
5. TEMED (*N,N,N',N'*-tetramethylethylenediamine) can be obtained from Bio-Rad Life Science.
6. TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA).
7. 10 % Acetic acid.
8. Formamide loading dye: formamide dye (98 % formamide, 10 mM EDTA pH 8.0) and bromophenol blue and xylene cyanol as tracking dyes.
9. Power supply.
10. Fuji BAS-2000 phosphoimage analysis system (Fuji Photo Film Company Ltd, Japan).
11. QIAEX II Gel Extraction Kit (QIAGEN, Catalogue number 20021).

12. QIAquick PCR Purification Kit (QIAGEN, Catalogue number 28104).
13. Sequi-Gen 38 cm × 50 cm gel apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA).

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### 3 Methods

As a general precaution, in order to obtain a constant temperature and time accuracy during the experiments, a thermocycler machine should be used in the incubation steps during the digestion and ligation.

#### **3.1 Surface Sterilization of Arabidopsis Seeds**

1. Suspend 100 mg seeds of Col or a mutant line in 1 mL 75 % ethanol in an Eppendorf tube for 5 min.
2. Remove the ethanol solution and wash the seeds two times with sterile distilled water.
3. Suspend the seeds in the sterilization solution for 5 min in an Eppendorf tube with frequent mixing.
4. Remove sterilization solution from the tube, and wash the seeds with sterile distilled water six times.
5. Stratify the surface-sterilized seeds by storing them in the dark at 4 °C for 2 days. The seeds are ready for demethylation treatment by 5-azaC and zebularine.

#### **3.2 Treating Arabidopsis Seeds with 5-azaC**

1. Wet 1 mm Whatman filter papers with 0.5 mM 5-azaC aqueous solution (2 mL/paper) or with sterilized distilled water as the control.
2. Place the wetted filter papers in Petri dishes.
3. Sow the surface-sterilized Col seeds on the filter papers, and wrap up the Petri dishes with parafilm to keep humidity.
4. Allow the seeds to germinate by placing the Petri dishes in the dark at 4 °C for 6 days.
5. Plant the seedlings in pots filled with universal substrate and vermiculite under the following growth conditions: 24 °C (day)/20 °C (night), 16 h light/8 h dark, 200 μE light intensity, and 60 % humidity.
6. Record plant growth and development phenotype with and without treatment.

#### **3.3 Treating Arabidopsis Seeds with Zebularine**

1. Sow surface-sterilized Col seeds on zebularine treatment medium and control medium, respectively.
2. Incubate the seeds under the following environmental condition: 24 °C (day)/20 °C (night), 16 h light/8 h dark, 200 μE light intensity.

3. At 14 days after seed germination, transfer the seedlings growing to a freshly prepared media containers (*see Note 4*).
4. Record plant growth and development phenotype with and without treatment.

### **3.4 Genomic DNA Isolation**

1. Collect rosette leaves from ten plants of each treatment, mutant line, and wild-type *Arabidopsis* plants before flowering, freeze in liquid nitrogen, mill to powder, and store at  $-80\text{ }^{\circ}\text{C}$  for DNA extraction and genome-wide analysis of DNA methylation.
2. Add 3 mL of DNA extraction buffer to a 50-mL polypropylene tube for each 1 g of fine grounded tissue.
3. Incubate at  $65\text{ }^{\circ}\text{C}$  in a water bath for 45 min with frequent shaking then allow the extract to cool down to room temperature.
4. Extract the homogenate with phenol/chloroform/isoamyl alcohol (25:24:1).
5. Centrifuge at  $10,000\times g$  for 10 min at room temperature and transfer the aqueous layer to a new tube.
6. Extract again with chloroform/isoamyl alcohol (24:1).
7. Centrifuge at  $10,000\times g$  for 10 min at room temperature.
8. Transfer the aqueous layer to a new tube and precipitate the nucleic acids in the aqueous phase by adding 10 % volume of sodium acetate 3 M (pH 5.2) and 60 % volume of cold isopropanol and incubated 2 h at  $-80\text{ }^{\circ}\text{C}$ .
9. Centrifuge at  $10,000\times g$  for 30 min at  $4\text{ }^{\circ}\text{C}$  and wash the nucleic acids pellet with 1 mL of cold 75 % ethanol.
10. Dissolve the pellet in 300  $\mu\text{L}$  of TE buffer.
11. Purify the extracted DNA from contaminants and enzyme inhibitors using the QIAGEN DNeasy Plant Maxi Kit following the manufacturer's instructions.
12. Determine the quantity and the quality of the DNA using the NanoDrop spectrometer and run 10  $\mu\text{L}$  in a 1 % agarose gel (*see Note 5*).

### **3.5 Methyl-Sensitive Amplified Polymorphism (MSAP)**

#### *3.5.1 DNA Digestion, Adaptor Ligation, Preselective, and Selective PCR Amplification*

1. Digest genomic DNA (100 ng) of ten individual *Arabidopsis* plants per treatment using 4 U each of *EcoRI* and either methylation-sensitive *HpaII* or methylation-insensitive *MspI* in a final volume of 10  $\mu\text{L}$  using the thermocycler as an incubator for the reaction.
2. When the incubation time is finished, deactivate the digestion enzymes by heating the reaction at  $80\text{ }^{\circ}\text{C}$  for 10 min.
3. Anneal the complementary oligonucleotides (*EcoRI* adapter primers) and (*HpaII-MspI* adapter primers) in two different



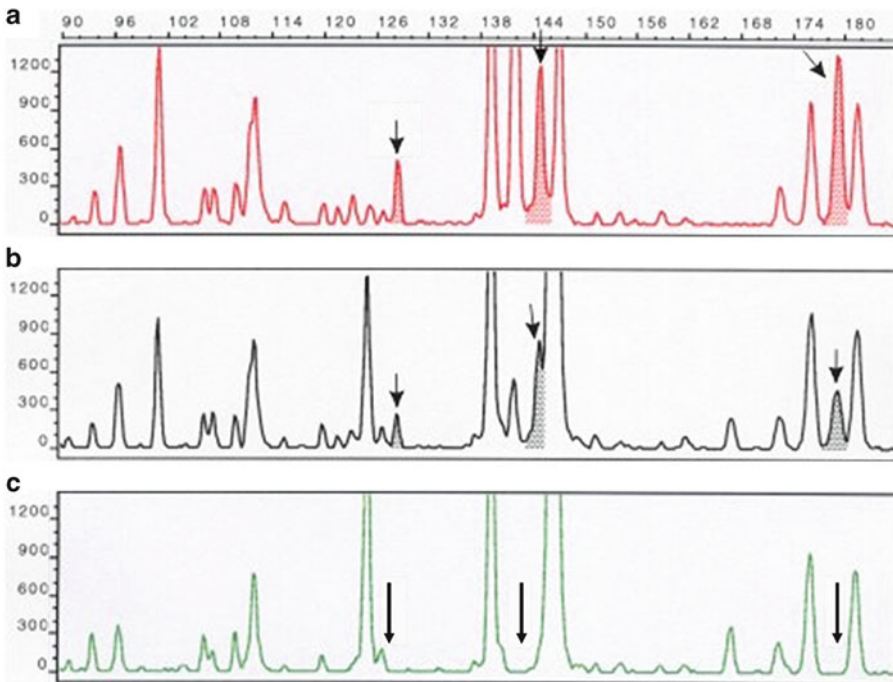
tubes by adding 20  $\mu\text{L}$  of 30 pmol from each complementary primer in a 100  $\mu\text{L}$  PCR tubes, heat up to 72  $^{\circ}\text{C}$  for 10 min, and then allow the reaction to cool down to room temperature (*see Note 6*).

4. Ligate the digested genomic DNA fragments (10  $\mu\text{L}$ ) to the two adapters by adding ligation mixture (2  $\mu\text{L}$  of 1.5 pmol of *EcoRI* adapter, 2  $\mu\text{L}$  of 15 pmol of *HpaII-MspI* adapter, 4 U of T4 DNA ligase, 1 $\times$  ligase buffer) in a total volume of 25  $\mu\text{L}$  and incubate overnight at 18  $^{\circ}\text{C}$ .
5. Subsequently, dilute the ligation reaction four times using  $\text{H}_2\text{O}$  Milli-Q.
6. Use 3  $\mu\text{L}$  of the diluted ligation reaction, 10 pmol of preselective *EcoRI* and *HpaII-MspI* primers, 0.2 mM of dNTPs, and 0.5 U of Taq DNA polymerase. Set the thermocycler using the following conditions: 94  $^{\circ}\text{C}$ , 30 s; 56  $^{\circ}\text{C}$ , 1 min; 72  $^{\circ}\text{C}$ , 1 min for 20 cycles of amplification.
7. Check the size of the amplified fragments by running 10  $\mu\text{L}$  of the PCR products using agarose gel electrophoresis 1.5 % in 1 $\times$  TAE buffer at 4 V/cm for 3–4 h (*see Note 7*).
8. Stain with ethidium bromide (*see Note 8*).
9. View the gel on a UV transilluminator (*see Note 9*).
10. Dilute 10  $\mu\text{L}$  of the PCR products ten times with  $\text{H}_2\text{O}$  Milli-Q and use the dilution as a template for the selective amplification.
11. Use 3  $\mu\text{L}$  of the diluted PCR products, 0.5 pmol of one *EcoRI* selective labeled primers and 10 pmol of one *HpaII/MspI* selective primers, 0.04 mM dNTP, and 0.5 U Taq polymerase in a 11  $\mu\text{L}$  PCR reaction using a touchdown program of a thermocycler using the following: 94  $^{\circ}\text{C}$  for 2 min and 20 cycles of 94  $^{\circ}\text{C}$  for 20 s, 66  $^{\circ}\text{C}$  for 30 s, 72  $^{\circ}\text{C}$  for 2 min. The annealing temperature of the first ten cycles follows the shutdown program in which each cycle falls by 1  $^{\circ}\text{C}$ . At the end of these cycles, maintain the reaction at 60  $^{\circ}\text{C}$  for 30 min to get better extension.

### 3.5.2 Separating the PCR Products of Selective Amplifications by Capillary Electrophoresis on an ABI Prism 310 Genetic Analyzer

The ABI Prism 310 Genetic Analyzer is able to detect the fluorescence as the *EcoRI* site-specific primers are labeled with yellow (NED), blue (FAM), or green (JOE) fluorescent dyes. Each selective primer can be labeled with one of the three fluorescent colors to allow loading together three different reactions. An internal size marker, GeneScan Rox-500 (35–500 bp) labeled with a red (ROX) dye, should be added in order to determine the size of the separated fragments.

1. Prepare a loading buffer for each sample by mixing 24.0  $\mu\text{L}$  of deionized formamide and 1.0  $\mu\text{L}$  of GeneScan-500 [ROX] size standard.



**Fig. 3** A sample chromatogram of results obtained from the ABI Prism 310 Genetic Analyzer machine. While the horizontal scale represents the molecular weight of the fragments, the vertical scale represents the quantity of the amplicon. Each peak represents an amplicon (a fragment of DNA produced during the selective PCR amplification) (a–c). Differentially amplified and polymorphic peaks are indicated by *arrow*. Smaller peaks indicate the presence of a heteromorphic allele in terms of DNA methylation status (b). Absence of peaks indicated by *arrows* may represent genetic modulation of DNA methylation in *Arabidopsis* (c)

2. Add 25  $\mu\text{L}$  of the loading buffer mix to a genetic analyzer sample tube. One tube was used for each sample.
3. Add 2  $\mu\text{L}$  of the selective amplified PCR products to the tube.
4. Heat the tubes to 95  $^{\circ}\text{C}$  for 3 min using a thermocycler machine.
5. Then, snap chill the tubes on ice (*see Note 10*).
6. Using the ABI Prism 310 Genetic Analyzer machine, inject each sample for 12 s, at 15 kV, and use 15 kV as running voltage for 26 min (*see Note 11*).

### 3.5.3 Data Analysis

Genomic DNA of ten individual plants (ten replicates) is usually treated and screened for each *Arabidopsis* genetic line and treatment. The DNA methylation deviation pattern from the wild-type can be assured using these replicates which are represented as presence or absent of particular polymorphic DNA fragment (amplicon) in every treatment using the same primer pair in the selective PCR amplification. Quantitative amplification can indicate the presence of a heteromorphic allele in terms of DNA methylation (Fig. 3).

Selectively amplified DNA fragment data can be collected by the ABI Prism 310 and analyzed using the ABI Prism GeneScan 3.1 software which will size and quantify the detected fragments. The same software can be used to compare the graphical representations of amplified fragments from all individual plants. A peak size between 60 and 500 bp should be selected to study the polymorphic DNA fragments (peaks) between the two genetic lines (Fig. 3). MSAP products can be scored as present (1) or absent (0) on the chromatogram to create a binary matrix. The proportion of polymorphic peaks can be estimated as the ratio of the number of polymorphic peaks to the total number of bands. This data can be treated and arranged depending on the purpose of the study. Partial methylation, due to differences in methylation status between copies of the same locus, results in changes in product intensity between genotypes and 5-azaC- and zebularine-treated plants.

Once an interesting peak is identified based on the polymorphic pattern in the chromatogram, the DNA fragment corresponding to that peak can be amplified using the same primer pair, isolated and sequenced by running the selective PCR products in a vertical denaturing 5 % polyacrylamide gel.

### **3.6 Identification of the Polymorphic DNA Fragment**

1. Perform the selective PCR as mentioned above using the preselective PCR products as a DNA template and the suitable selective [<sup>32</sup>P-ATP] end-labeled EcoRI primer. Run the PCR using the thermocycler and the same conditions as mentioned above (*see Note 12*).
2. Prepare denaturing 5 % acrylamide gel by mixing 12.5 mL of 40 % of acrylamide-bis solution, 7.5 M urea in 50 mM TBE, 500 μL 10 % ammonium persulfate, and 100 μL TEMED (*see Note 13*).
3. Cast the solution in a Sequi-Gen 38 cm × 50 cm gel apparatus and allow the gel to solidify for 4 h.
4. Denature PCR samples by mixing 20 μL of formamide loading dye with equal amount of PCR sample, heat at 90 °C for 3 min, and then quickly chill on ice for at least 2 min.
5. Wash the gel wells from unpolymerized polyacrylamide and urea then load an equal amount of every sample in the well.
6. Run the gel electrophoresis using TBE buffer at constant power, 110 W, for 2 h.
7. Fix the DNA in the gel for 30 min in 10 % acetic acid, dry it on the glass plates, and expose it to Fuji phosphoimage screens for 16 h. Fingerprint patterns can be visualized using a Fuji BAS-2000 phosphoimage analysis system.
8. Isolate the polymorphic DNA by cutting the band from the gel.
9. Rehydrate the band by boiling in 100 μL H<sub>2</sub>O Milli-Q for 5 min.

10. Clean up the DNA fragment from the gel impurities using the QIAEX II Gel Extraction Kit.
11. Use the purified fragment as a template for a PCR reaction containing 5.0  $\mu\text{L}$  of the eluted DNA, 10.0 pmol of selective EcoRI primer, 10.0 pmol HpaII/MspI, PCR buffer containing  $\text{MgCl}_2$ , 2.5 mM dNTP, and 1.0 U Taq polymerase. The PCR cycle should be used as mentioned above for the selective PCR reaction (*see* **Note 14**).
12. Purify the PCR reaction using QIAquick PCR Purification Kit following the manufacturer's instructions.
13. Sequence the PCR products by using the selective EcoRI primer and the routine sequencing reaction and conditions.

### 3.6.1 Data Analysis

In order to identify the differentially methylated DNA fragments, information obtained from the sequencing reaction can be used in a BLAST search against the National Center of Biotechnology databases searching for sequence similarity. The BLAST website is available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. A gene can be identified based on the similarity between the sequence in the database and the obtained one.

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## 4 Notes

1. 5-azaC is white crystalline powder and soluble (50 mM) in water. However, 5-azaC is unstable in aqueous solution and sensitive to light and oxidation. Therefore, storing 5-azaC is not recommended. Treatment of *Arabidopsis* seeds should use freshly prepared 5-azaC solution kept in the dark and at low temperature.
2. Zebularine is an off-white solid and soluble (100 mM) in water. A zebularine aqueous solution is stable for up to 3 months at  $-20\text{ }^\circ\text{C}$ .
3. The EcoRI site-specific primers can be labeled with yellow (NED), blue (FAM), or green (JOE) fluorescent dyes to allow one to load three different reactions simultaneously.
4. The demethylation effect of zebularine is transient. Growing *Arabidopsis* seedlings on zebularine treatment medium and transferring them to control medium can be used to find zebularine transiently reduced *Arabidopsis* genomic DNA methylation.
5. The DNA concentration can be measured using a NanoDrop spectrophotometer adjusted to a wavelength of 260 nm. The purity of the DNA is determined by measuring the absorbance ratio 260/280 nm. A good quality DNA should have a ratio between 1.8 and 2.0. Good quality DNA appears in the agarose

gel stained with ethidium bromide as a high molecular weight sharp single DNA band. Bad quality DNA appears as several DNA bands or a smear in the same gel. Smears in the gel indicate the presence of low molecular weight DNA which is due to degradation during DNA extraction. This is not suitable for MSAP analysis.

6. DNA digestions and adapter ligations should be carried out separately to avoid the formation of a long continuous DNA molecule that contains multiple copies of the same DNA sequences linked together in series (concatemers).
7. The ligation step is a very critical step of this protocol. Pre-amplified PCR products should appear as a smear with equal intensities between samples using agarose gel electrophoresis and ethidium bromide staining.
8. Ethidium bromide is a mutagen chemical and is moderately toxic. Apply extra cautions when you use it. Wear gloves, a lab coat, and safety glasses when using this dye.
9. Good amplification products for MSAP should appear as a smear of molecular weight between 100 and 1,500 bp in a 1.5 % agarose gel.
10. The genetic analyzer sample tubes can be placed in the 48- or 96-well sample tray.
11. To verify the reproducibility of each fragment, each MSAP procedure should be repeated at least twice.
12. PCR labeling of the DNA fragment, excision of the DNA fragment from the chromatogram, and purification of the radio-labeled PCR should be carried out behind 3/8 or 1/2 inch-thickness glass or transparent acrylic plates.
13. Unpolymerized acrylamide and TEMED should be handled carefully because they are widely considered as neurotoxic and reproductive toxic materials, respectively.
14. Often, the eluted amount of DNA is not enough to be used in the sequencing reactions, therefore PCR is used to amplify and increase the original amount of eluted DNA.

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