Detection of DNA Methylation Changes Surrounding Transposable Elements

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[Abstract] Transposable elements (TEs) are a major component of all genomes, thus the epigenetic mechanisms controlling their activity is an important field of study. Cytosine methylation is one of the factors regulating the transcription and transposition of TEs, alongside Histone modifications and small RNAs. Adapter PCR-based methods [such as Amplified Fragment Length Polymorphism (AFLP)] have been successfully used as high-throughput methods to genotype un-sequenced genomes. Here we use methylation-sensitive restriction enzymes, in combination with PCR on adaptor-ligated restriction fragments, to evaluate epigenetic changes in TEs between genomic DNA samples.

Materials and Reagents

1. Two oligonucleotides which form the double-stranded adapter, with an overhang complementary to the overhang of the restriction enzyme used. In the case of HpaII or MspI, the overhang is a 5' CG, and the adapter sequences are 5'-GATCATGAGTCCTGCT-3' and 5'-CGAGCAGGACTCATGA-3'. The two nucleotides at the 5' end of the latter oligonucleotide will constitute the 5' CG overhang, after hybridization of the two sequences (black rectangles in Figure 1, Shaked *et al.*, 2001). These oligonucleotides should be designed such that they do not resemble know sequences in the examined species.
2. Pre-selective primers, one complementary to the adapter with the addition of a G nucleotide at the 3' end (5'-ATCATGAGTCCTGCTCGG-3'; primer P2 in Figure 1), and the other complementary to the TE of interest (primer P1 in Figure 1). The TE-specific primer should be designed as a reverse-complement of the 5' end of the TE with a Tm=60 °C, between 30-50 bp into the TE (to allow for sequence validation in downstream assays). Restriction enzyme recognition sites (CCGG) between the primer and the 5' end of the TE should be avoided.
3. Selective primers, one identical to the above TE-specific primer with the addition of a fluorescent tag (*e.g.* 6-FAM) or radioactive tag (end label with ^{32}P), and one similar to the pre-selective primer complementary to the adapter with the addition of random

nucleotides at the 3' end (*e.g.* 5'-CATGAGTCCTGCTCGGTCAG-3', includes an extra TCAG at the 3' end).

4. NaCl
5. T4 DNA ligase and buffer (New England Biolabs, catalog number: M0202)
6. Restriction enzymes *Hpa*II and *Msp*I (New England Biolabs, catalog number: R0171 and R0106)
7. Taq DNA polymerase and Taq DNA polymerase buffer (EURx, catalog number: E2500)
8. $MgCl_2$
9. dNTP mix
10. Polynucleotide Kinase (PNK) enzyme and PNK buffer (New England Biolabs, catalog number: M0201)
11. Gamma-phosphate (^{32}P)-labeled ATP (or fluorescently-labeled primers)
12. GS-500 ROX-labeled size standard (for fluorescently-labeled products only) (Applied Biosystems)
13. Hi-Di Formamide (for fluorescently-labeled products only) (Applied Biosystems, catalog number: 4311320)

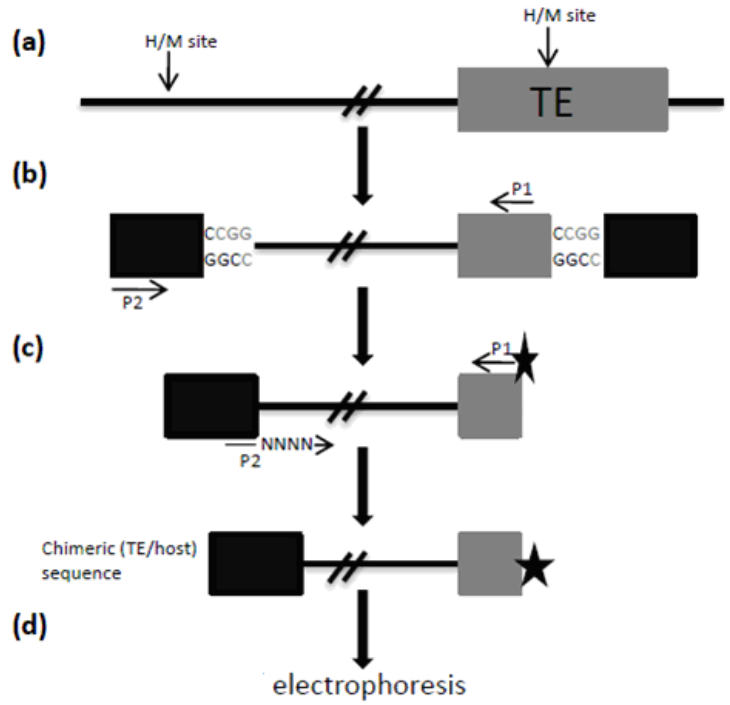

Figure 1. An overview of the TMD method, adapted from (Yaakov and Kashkush, 2011). The method steps include: (a) Restriction of genomic DNA with *Hpa*II (H) or *Msp*I (M); (b) The first round of PCR amplification, using a primer from within the TE (P1) and a primer from the adapter (P2); (c) The second round of PCR amplification, using primer P1 from

within the TE, labeled with a radioactive or fluorescent tag, and primer P2 with the addition of random nucleotides at the 3' end; and (d) Electrophoresis of the resulting PCR amplicons on a polyacrylamide gel (in the case of a radioactive tag) or in a capillary fluorescence detection machine (in the case of a fluorescent tag).

Equipment

1. Thermal cycler
2. Agarose gel electrophoresis machine
3. 43 cm PAGE machine (Thermo Scientific Owl Aluminum-Backed Sequencer S3S, for radiolabeled products only)
4. Capillary electrophoresis machine (such as the Applied Biosystems 3730xl DNA analyzer; for fluorescently-labeled products only)

Procedure

A. Adapter pair preparation
   1. Mix the two adapter oligonucleotides to a final concentration of 250 ng/µl.
   2. Incubate them at 95 °C for 5 min and then at room temperature for 10 min.

B. Restriction/Ligation
   1. Add to a 0.2 ml tube: 1 µl of 10x ligase buffer, 1 µl of 0.5 M NaCl, 1 µl of the adapter pair 120 units of T4 ligase, 2 units of *HpaII* or *MspI*, 300-500 ng of genomic DNA and ddH_2O to a final volume of 10 µl.
   2. Mix well and incubate at 37 °C for 2-3 h.
   3. Dilute reaction 1: 10 by adding 90 µl of ddH_2O.
   4. This reaction can be stored at -20 °C.

C. Pre-selective amplification
   1. Add to a 0.2 ml tube: 2 µl of 10x Taq DNA polymerase buffer, 2 µl of 25 mM $MgCl_2$, 0.8 µl of dNTP mix, 1 unit of Taq DNA polymerase, 1 µl of 50 ng/µl adapter-specific pre-selective primer, 1 µl of 50 ng/µl transposon-specific primer, 4 µl of Restriction/Ligation reaction products (cut with *Hpa*II or *Msp*I) and ddH_2O to a final volume of 20 µl.
   2. Use the thermal cycler to PCR with the following program:
      a. 94 °C for 3 min
      b. 94 °C for 30 sec
      c. 60 °C for 30 sec

d. 72 °C for 1 min

Return to step b 29 times

3. Run 10 µl of the resulting products on a 1.5% agarose gel to validate amplification.
4. Dilute the remaining 10 µl with 190 µl of ddH_2O.
5. This reaction can be stored at -20 °C.

D. Radiolabeling

1. For 20 reactions, add to a 0.2 ml tube: 6 µl of ddH_2O, 6 µl of transposon-specific primer, 2 µl of 10x PNK buffer, 1 µl of PNK and 5 µl of radiolabeled ATP.
2. Mix well and incubate at 37 °C for 1 h and then at 70 °C for 10 min.

E. Selective amplification

1. Add to a 0.2 ml tube: 2 µl of 10x Taq DNA polymerase buffer, 2 µl of 25 mM $MgCl_2$, 0.8 µl of dNTP mix, 1 unit of Taq DNA polymerase, 1 µl of 50 ng $µl^{-1}$ adapter-specific selective primer, 1 µl of radiolabeled (or fluorescently labeled) transposon-specific primer, 3 µl of pre-selective amplification PCR products and ddH_2O to a final volume of 20 µl.
2. Use the thermal cycler to PCR with the following program:
   a. 94 °C for 2 min.
   b. 63 °C for 30 sec (decrease temperature by 1 °C every cycle until 56 °C).
   c. 72 °C for 1 min.

   Return to step b 32 times.
3. Run the resulting products on a denaturing 5% polyacrylamide gel (for radio-labeled products, see Figure 2 for an example); or add 0.5 µl of GS-500 ROX-labeled size standard, 1-2.5 µl of PCR product (add less PCR product if fluorescence intensity is too high) and complete to 13 µl with formamide (for fluorescently-labeled products only).

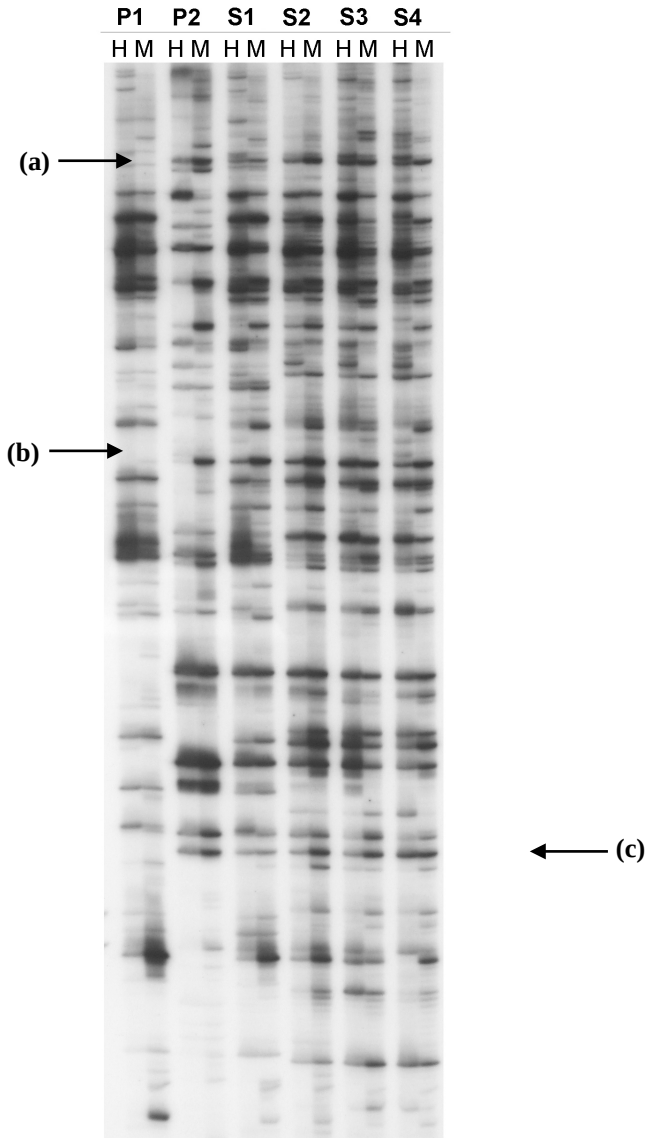

Figure 2. An example (Yaakov and Kashkush, 2011) of an autoradiogram showing TMD products for 6 DNA samples representing two parental plants (P1 and P2) and their allopolyploid offspring (S1-S4). The TE analyzed is a *Stowaway*-like miniature inverted repeat transposable element (MITE), called *Thalos*. Arrow (a) shows a change in methylation between S1 and S2, as only the *Msp*I band is present in P1 and S1-S2, but both *Hpa*II and *Msp*I bands are present in S3-S4. The disappearence (b) or appearance (c) of bands can also be seen, which may arise as a result of complete methylation of the restriction site, or a mutation in the restriction or primer binding sites. The total percent methylation of all sites for a given sample analyzed can be calculated by dividing the number of polymorphic sites (those resenting bands for only one restriction enzyme) by the total number of sites (presenting bands for one and both restriction enzymes).

Acknowledgments

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