

Detection of Transposable Element Insertion Site Polymorphisms by Sequence-Specific Amplification Polymorphism (SSAP)

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[Abstract] Transposable elements represent a major part of any eukaryotic genomes. Notably in plants they can account for more than 80% of the whole genomic sequence (such as in maize). Due to their mobility across the genome, they can act as mutagens but can also be considered as an important source of genetic diversity. It has been shown that they may be activated following various stresses, and it has been assumed that they may contribute to genome evolution and adaptation. Molecular methods have thus been proposed to allow identification of new transposition events, or more generally to tag transposable element insertion site polymorphisms. Sequence-Specific Amplification Polymorphism (SSAP) is a high throughput method derived from AFLP, which has been first tested on the barley genome (Waugh *et al.*, 1997). Its efficiency in tagging TEs in comparison to AFLP is based on the use of specific primers anchored in the TE sequences of interest, requiring the TEs under survey to be previously characterized. SSAP can thus be used to identify any genomic reorganization in the vicinity of TE insertion sites, and still represents an efficient approach to analyse evolutionary dynamics of TEs.

Materials and Reagents

A. Restriction of genomic DNA using endonucleases

1. Genomic DNAs from the samples to be analysed at concentrations of 100 ng/μl
2. Endonuclease enzyme insensitive to DNA methylation, generating cohesive ends

Note: TE sequence ends have to be devoid of any corresponding restriction sites.

B. Adapter ligation

3. Two oligonucleotides which form a double-stranded adapter, with an overhang complementary to the overhang left by the restriction enzyme used. In the case of *EcoRI* (Thermo Fisher Scientific, Fermentas) the 5' overhang is AATT and the oligonucleotide sequences are 5'-CTCGTAGACTGCGTACC-3' and 5'-AATTGGTACGCAGTCTAC-3'. Annealing of the two oligonucleotides will constitute the adapter (purple rectangles in Figure 1).

4. T4 DNA ligase (1-3 U/μl) with the corresponding ligase buffer (Promega corporation, catalog number: M1801)
 5. Sterile ultrapure water
- C. Pre-amplification
6. PCR reagents
 - a. Homemade *Taq* DNA polymerase is of sufficient quality for the PCR reactions but *Taq* Promega has also been used (Promega corporation, catalog number: M8301) with the corresponding *Taq* polymerase buffer.
 - b. 25 mM MgCl₂
 - c. dNTPs (10 μM each)
 7. Pre-amplification primers (10 μM each)
 - a. One primer is anchored in the adapter: 5'-GACTGCGTACCAATTC-3' (primer P1 in Figure 1).
 - b. The other primer is anchored in the TE of interest, close to its end to limit the size of amplicon (primer P2 in Figure 1).
- D. Selective amplification
8. PCR reagents (see pre-amplification step)
 9. Selective amplification primers (10 μM each)
 - a. One primer is anchored in the adapter, with the addition of 3 nucleotides at the 3' end (primer P3 in Figure 1) to limit the number of bands to be visualized and analyzed by gel electrophoresis. Several combinations of A, T, G and C should be used to target different genomic localizations of TE insertion sites.
 - b. The other primer is defined to perform a nested PCR in comparison with the pre-amplification step (primer P4 in Figure 1); this primer is labelled with a 5'-IRDye. DNA labelled with IRDye (infrared dye) has to be stored in the dark at -20 °C. To minimize exposure to light, wrap the tube in aluminium foil.
- E. Acrylamide gel for SSAP profile analysis
10. Kimwipes
 11. Urea (Merck KGaA, catalog number: 1.08488.1000)
 12. Long Ranger Acrylamide (50%) (Lonza, catalog number: 50611)
 13. APS (Bio-Rad Laboratories, catalog number: 161-0700)
 14. Temed (Bio-Rad Laboratories, catalog number: 161-0801)
 15. Absolute ethanol
 16. Formamide (Sigma-Aldrich, catalog number: F9037)

17. 10x TBE buffer (Tris/Borate/EDTA) (see Recipes)
18. 5.5% acrylamide gel (see Recipes)
19. Loading buffer (see Recipes)

Equipment

1. 1.5 ml Eppendorf tubes
2. Centrifuge
3. Parafilm
4. Incubator or oven
5. Water bath
6. 96-well PCR plates
7. 0.45 µm filters
8. LI-COR DNA analyzer (LI-COR) and corresponding equipment (plates, combs, spacers...)
9. Thermal cycler
10. Horizontal shaker

Procedure

Note: Adapted from Waugh et al. (1997) and Pouilly et al. (2008); as used in Sarilar et al. (2013).

A. Targeting TE insertion sites

1. Restriction of genomic DNAs
 - a. Make a restriction reaction mixture that will be distributed equally into individual Eppendorf tubes. The reaction mixture combines (in order) sterile ultrapure water (with a final volume of 36 µl per reaction), the 1x enzyme buffer and the selected endonuclease (6 U per reaction), taking into account that 5 µl of genomic DNA will be added into the mix.
 - b. Distribute 31 µl of the reaction mixture into each Eppendorf tube.
 - c. Add 5 µl of each DNA solution to be analysed (which correspond to 500 ng of genomic DNA) to the corresponding tube.
 - d. Mix gently the solution in each tube by pipeting, and centrifuge shortly.
 - e. Incubate at least 3 h at 37 °C.
 - f. Stop the enzyme activity by incubating the tubes 20 min at 65 °C.
 - g. Put the tubes on ice, centrifuge briefly and replace them on ice. Proceed immediately to the ligation step.
2. Prepare the adapters and ligate them to the restricted DNAs

- a. In a 0.5 ml Eppendorf tube, mix the two oligonucleotides (100 μ M each) in equal proportions to make the adapter solution (final concentration of 50 μ M).
 - b. Incubate the mix at 95 °C for 5 min (in a thermocycler) and let cool it down on the bench at room temperature.
 - c. Make a ligation reaction mixture, by combining (in order) sterile ultrapure water (with a final volume of 50 μ l per reaction), 1x ligase buffer, adapter solution (50 pmol) and T4 DNA ligase (1 U per reaction).
 - d. Distribute 14 μ l of the reaction mixture into the Eppendorf tubes containing each 36 μ l of restricted DNAs.
 - e. Mix gently using a pipette and then centrifuge shortly.
 - f. Incubate at 20-25 °C overnight (on the bench) to allow ligation.
 - g. Dilute the ligation products to 1/10 in 96-well PCR plates: 90 μ l of sterile ultrapure water + 10 μ l of the restriction-ligation products.
- Note: Store non-diluted restriction-ligation products at -20 °C, but keep 1/10 diluted restriction-ligation products at 4 °C to avoid DNA degradation because of freezing - thawing cycles.*
3. Pre-amplification
 - a. Use the first primer pair P1/P2 (Figure 1b) for the pre-amplification.
 - b. Make a pre-amplification PCR reaction mixture by combining (in order) sterile ultrapure water (with a final volume of 20 μ l per reaction), 1x *Taq* polymerase buffer, MgCl₂ (2.5 mM), dNTP solution (200 μ M), primer P1 anchored in the adapter (8 pmol), primer P2 anchored in the TE under study (8 pmol) and *Taq* DNA polymerase (3-4 U).
 - c. Distribute 17 μ l of the pre-amplification reaction mixture into the wells of a 96-well PCR plate.
 - d. Add 3 μ l of the 1/10 diluted restriction-ligation products. Mix gently for each reaction by pipeting 2-3 times.
 - e. Centrifuge.
 - f. PCR program
 - i. 1 cycle at 72 °C for 2 min
 - ii. 1 cycle at 94 °C for 3 min
 - iii. 25 cycles of (94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min)
 - iv. 1 cycle at 72 °C for 5 min
 - v. Keep at 10 °C
 - g. Verification of the pre-amplification products can be performed by agarose gel electrophoresis: Load 4 μ l of the reaction product (with loading buffer) and run the electrophoresis for ~20 min at 100 V.

Note: A smear should be obtained (an example of the gel is shown in Figure 1b).

- h. Dilute the pre-amplification product in 96-well PCR plates according to the plate layout to carry out the separation of fragments using the LI-COR DNA analyzer.

Note: The factor of dilution is usually 1/10 (36 μ l of sterile ultrapure water + 4 μ l of pre-amplification product) but it has to be adapted (1/2, 1/5, 1/10, or no dilution) according to the intensity of the smear observed after electrophoresis, in order to get homogeneous selective amplification products.

4. Selective amplification

- a. Use the second primer pair P3/P4 (Figure 1c) for the selective amplification to perform a nested PCR (to increase the specificity of the PCR reaction).
- b. Make a selective PCR reaction mixture by combining (in order) sterile ultrapure water (with a final volume of 20 μ l per reaction), 1x Taq polymerase buffer, MgCl₂ (2.5 mM), dNTP solution (200 μ M), selective primer P3 anchored in the adapter with 3 additional selective nucleotides (4 pmol), selective primer P4 anchored in the TE under study and labelled with IRDye (4 pmol) and Taq DNA polymerase (1.5-2 U).

Note: The primer labelled with the IRDye is sensitive to the light; the PCR plate has to be covered with aluminium foil to avoid degradation.

- c. Distribute 15 μ l of the selective PCR reaction mixture into the wells of a 96-well PCR plate.
- d. Add 5 μ l of the diluted pre-amplification products. Mix gently for each reaction by pipeting 2-3 times.
- e. Centrifuge.
- f. PCR program: a typical touchdown program is performed (Vos *et al.*, 1995) to increase the specificity of the PCR amplification.
 - i. 1 cycle at 94 °C for 4 min
 - ii. 13 cycles of [94 °C for 30 sec, 65 °C (then -0.7 °C per cycle) for 30 sec and 72 °C for 1 min]
 - iii. 25 cycles of (94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 1 min)
 - iv. 1 cycle at 72 °C for 5 min
 - v. Keep at 10 °C

Note: Maintain the 96-well PCR plates out of the light by covering them with aluminium foil and once the program is finished keep them at -20 °C.

B. Electrophoresis run on LI-COR DNA analyzer to separate amplified products

1. Prepare the DNA analyzer according to manufacturer's instructions.
2. Use 0.25 mm spacers between glass plates and prepare 40 ml of 5.5% acrylamide gel.
3. Do pre-run with autofocus and following parameters: 45 °C; 2,000 W; 25 mA.

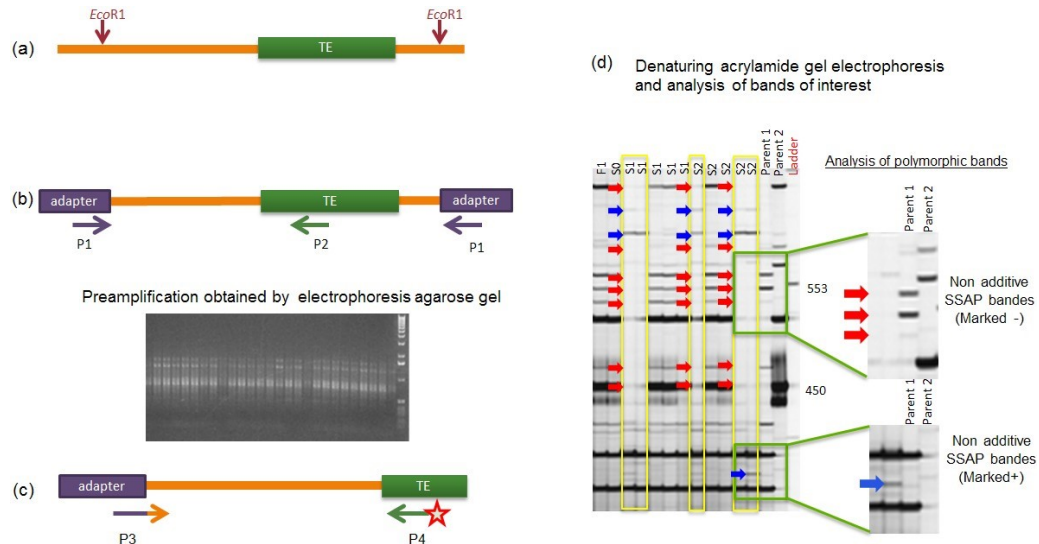


Figure 1. Schematic description of the different steps of the SSAP procedure to detect TE insertion site polymorphisms. (a). The genomic DNAs to be analysed are restricted using an endonuclease (here *EcoRI*, as used in Sarilar *et al.*, 2013) and then ligated to corresponding adapters. (b). A pre-amplification is performed on the restriction-ligation products using a specific PCR primer pair (to be designed) with one primer (P1) complementary to the adapter and the other primer (P2) anchored in the TE of interest. As illustrated by the picture, the pre-amplification should lead to a PCR product visualized as a smear after agarose gel electrophoresis. (c). The resulting PCR products are then subjected to a selective PCR amplification using another specific PCR primer pair (to be designed) with one primer (P3) complementary to the adapter with 3 additional selective nucleotides and the other primer (P4) anchored in the TE and labeled with an IRDye (at the 5' end). (d). The resulting amplicons are finally resolved in a denaturing acrylamide gel; polymorphic bands can be visualized and scored as non-additive SSAP bands (being additional + or missing -) when comparing the progeny profiling with the parental ones (as illustrated here for the analysis of resynthesized allotetraploid lines in comparison with their diploid progenitors, Sarilar *et al.*, 2013). Identification of the nature of the polymorphism responsible for non-additivity (e.g. transposition, genomic rearrangement, polymorphism at the endonuclease cutting site) requires further cloning and sequencing of the corresponding SSAP amplicon.

4. Load the gel
 - a. Mix the PCR products of selective amplification (2 μ l) with equal amount of loading buffer.
 - b. Heat the samples at 95 °C for 5 min for DNA denaturation and snap cool on ice before loading. Keep also on ice while loading the samples on the acrylamide gel.

- c. Load 0.5 μ l of each sample in each well. Between each deposit, rinse the multi-syringe in water. Load on the same acrylamide gel the samples which have to be compared, and load the controls on each gel if necessary.
5. Analyze the SSAP multiband fingerprints by scoring polymorphic bands which may correspond to TE insertion site polymorphisms (Figure 1d).

Note: Wear gloves when working with acrylamide because of its carcinogenic and toxic properties.

Recipes

1. 10 \times TBE buffer (1 L)
 - Tris Base 108 g
 - Boric acid 55 g
 - 40 ml of EDTA (0.5 M, pH 8)
 - ddH₂O qsp 1 L
 - Filter sterilize (0.45 μ M)
2. 5.5% acrylamide gel (40 ml)
 - Add 15.6 g urea to 20 ml ddH₂O; agitate while heating until obtain a translucent solution
 - Add 4.8 ml of 10x TBE buffer
 - Filter 0.45 μ m to remove any urea crystals
 - Add 4.4 ml Long ranger (50%)
 - Complete with ddH₂O up to 40 ml
 - When ready to inject gel solution, add for polymerization
 - a. 264 μ l of 10% APS
 - b. 26.4 μ l Temed
 - Mix completely to homogenate and draw the gel solution into a 50 ml syringe (with no needle)
3. Loading buffer
 - Mix 40 mg of bromophenol blue with 2 ml of EDTA (0.5 M, pH 8) and 0.5 ml of ddH₂O
 - Complete with 47.5 ml of formamide
 - Note: Loading buffer is carcinogenic because of formamide, it has to be handled with gloves and under the flow hood.*

Acknowledgments

This protocol is adapted from Waugh *et al.* (1997) and Sarilar *et al.* (2013).

References

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