Representational difference analysis (RDA)

Restriction endonuclease digestion of genomic DNA

For RDA, the technique outlined by Lisitsyn et al. (1993) with some modifications is followed. Figure 1 outlines the single steps involved in the process.

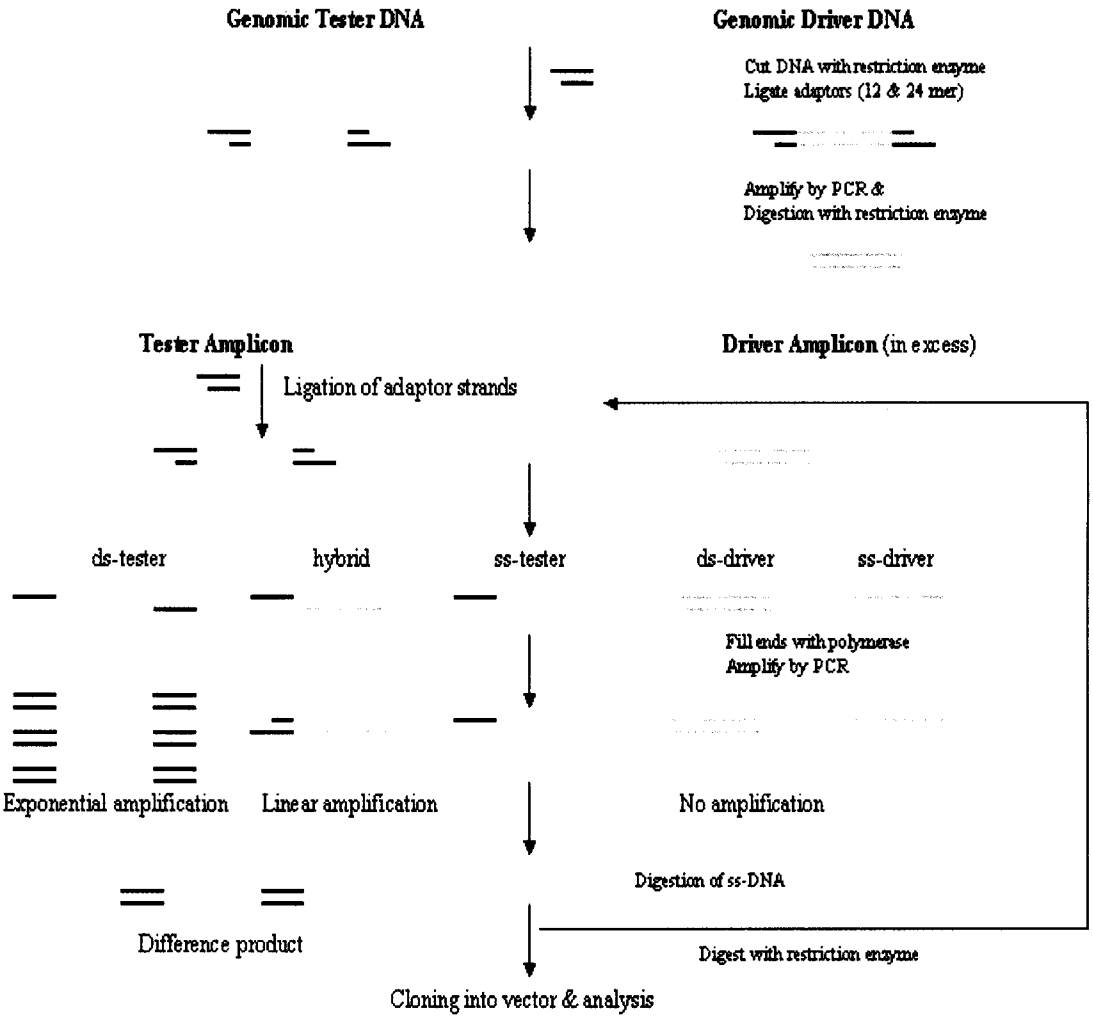

Figure 1: Steps involved in the DNA subtraction procedure of Representational Difference Analysis.

To produce the representations for RDA, total cellular DNA is isolated from 1g of tobacco leaves (Gawel and Jarret, 1991) and digested with the restriction enzymes *Hind*III (methylation-insensitive) and *Hpa*II (methylation-sensitive). For production of amplicons (representations), two micrograms of DNA derived from one non-modified tobacco plant used as driver DNA and two micrograms of DNA derived from two genetically modified tobacco plants as tester DNA after mixing their individual DNAs (1:1), is digested in the first and third experiments (experiment 1 and 3) with 80 units of either the enzyme *Hpa*II (methylation-sensitive) or the enzyme *Hind*III. In the second and forth experiment (experiment 2 and 4) under identical conditions, two micrograms of DNA derived from one non-modified tobacco plant used as tester DNA and two micrograms of DNA derived from two transgenic tobacco plants as driver DNA after mixing their individual DNAs (1:1) is digested in 50 µl digestion mixture at 37°C for 1 h with 80 units of an appropriate restriction enzyme (Roche, Switzerland). Before further processing, DNA is first analyzed on a 1% agarose gel in TAE buffer, after digestion.

Ligation of DNA adaptors

A pair of single-stranded oligonucleotide adaptors of different length is used to alter the ends of DNA fragments to enable DNA amplification. The longer adaptor is also used as primer for DNA amplification after adaptor ligation. For adaptor ligation, digested tester and driver DNA (between 0.5 and 1 µg) is mixed in a total volume of 30 µl with 7.5 µl of a 12-mer and 24-mer adaptor (adaptor pair set 1, Table A.1) from a 62 pmol/µl adaptor stock solution and a ligase buffer consisting of 66 mM Tris-HCl (pH 7.6); 6.6 mM $MgCl_2$; 10 mM DDT and 66 µM ATP. To anneal the adaptors, the ligation mixture is incubated at 55°C for 5 min in a heating block after which, the block is immediately placed into a cold room for approximately 1h until the temperature drops in the ligation mixture to 10-15°C. The reaction tubes are incubated on ice for 3 min after which 4 µl (1U/µl) of T4 DNA ligase (Amersham Life Science, UK) is added to the mixture and then incubated overnight at 16°C for ligation.

Amplification of tester and driver DNA

For preparation of tester and driver amplicons by PCR, ligated DNA is diluted with 500 µl TE buffer containing 10 mM Tris-HCl (pH 8) and 0.1 mM EDTA. For DNA amplification, a PCR tube containing a PCR amplification mixture (100 µl), which contains 40 ng of ligated DNA; 372 pmol of the 24-mer adaptor (adaptor pair set 1, Table A.1); 10 mM dNTP's (4 µl); 25 mM $MgCl_2$ (6 µl) and PCR buffer consisting of 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$ and 0.001% w/v gelatine, is placed into a pre-warmed (72°C) thermocycler (GeneAmp PCR System, Perkin Elmer, USA). To fill-in the 3'-recessed ends of the ligated fragments, 6 units of Taq DNA polymerase (Amersham Life Science, UK) is added to the PCR amplification mixture. DNA amplification by PCR is carried out for 25 cycles (11 sec at 95°C; 2:07 min at 72°C) with the last cycle followed by a DNA extension period for 10 min at 72°C. Approximate total amount of DNA of amplified tester and driver amplicon is determined on a 1.5% agarose gel in TAE buffer with sheared herring sperm DNA as a standard. Amplified DNA is then phenol/chloroform purified and after ethanol precipitation (Sambrook et al., 1989) amplicon DNA is dissolved in TE buffer to obtain a DNA concentration of about 0.5µg/µl.

To cleave adaptors from amplified DNA, driver DNA and tester DNA (40 µg) are digested for 1 h at 37°C with the initially selected restriction enzyme (20 units enzyme/µg DNA). Yeast tRNA (10 µg) is added to digested DNA, which is phenol/chloroform purified, ethanol precipitated and finally dissolved in 70 µl of sdH_2O.

The tester amplicon DNA (1µg) from which adaptors were cleaved is then ligated to a second adaptor pair (adaptor pair set 2; Table 1) following the procedure outlined under "Ligation of DNA adaptors". Ligated tester amplicon DNA is then amplified following the procedure outlined under "Amplification of DNA by PCR and adaptor removal" but with the addition of 10 extra DNA amplification cycles (35 cycles).

Kinetic enrichment of DNA

Ligated tester DNA is diluted to 50 ng/µl in a total volume of 70 µl with TE buffer (10 mM Tris-HCl, pH8; 0.1 mM EDTA). For hybridization, diluted tester DNA (4 µl) is mixed with driver amplicon DNA (8 µl) and then 10 M ammonium acetate (3 µl) solution and 96% ethanol (38 µl) are added to the two DNAs and mixed with DNAs by sucking and blowing using an Eppendorf pipette. The mixture is chilled at -70°C for 10 min followed by an incubation period of 2 min at 37°C. DNA is precipitated by centrifugation for 10 min at 13000 x g and the DNA containing pellet is washed twice with 70% ethanol and dried. The DNA pellet is resuspended in 4 µl EE buffer containing 30 mM EPPS (N-(2-hydroxyethyl piperizine)-N-(3-propene sulfonic acid) (pH 8) and 3 mM EDTA. The DNA is overlaid with 35 µl of sterile mineral oil and the sample is incubated at 98°C for 4 min to denature the DNA. A 5 M sodium chloride solution (1 µl) is directly injected into the DNA drop and the mixture was incubated at 67°C overnight.

The mineral oil is then removed and tRNA (10 µg) must be added to hybridized DNA and the DNA sample is then diluted by adding 100 µl TE buffer to the mixture. To fill the adaptor ends, diluted hybridized DNA (20 µl) is added to 180 µl standard PCR reaction mixture as outlined under "Polymerase chain reaction (PCR)". The solution is divided into 2 separate PCR tubes and 1µl of Taq DNA polymerase is added to each tube. The solution is incubated at 72°C for 5 min after which 5 µl of 24-mer primer (adaptor pair set 2; Table A.1) is added to the solution. Ten cycles of PCR (1min at 95°C and 3min at 70°C) are performed using an extension at 70°C for 10 min after the last cycle. To evaluate the effectiveness of hybridization step, 20 µl of the hybridization mixture is amplified for an additional 20 cycles of amplification and any amplification products are visualized on a 2% agarose gel in TAE buffer. If amplification products are visible, 20 µl of the hybridization mixture is digested with 20 units of mung bean nuclease at 30°C for 30 min. The reaction is stopped by the addition of TE buffer (160 µl). The digested product is amplified in a standard PCR reaction mixture containing 6

µl of the 24-mer primer (adaptor pair set 2; Table 1). Amplified DNA subtraction products are purified with phenol/chloroform and precipitated with ethanol and finally dissolved in 100 µl of sdH_2O.

For a second round DNA subtraction/kinetic enrichment the DNA subtraction products (5 µg) are digested with 100 units of an appropriate restriction enzyme in a total volume of 100 µl. The DNA is phenol/chloroform purified after addition of tRNA (10 µg), ethanol precipitated and resuspended in sdH_2O to obtain a concentration of 20 µg DNA/ml. DNA (100 ng) is ligated to a third set of adaptors (adaptor pair set 3; Table A.1) in a total volume of 30 µl as described above. To ligated DNA 50µl of sdH_2O containing tRNA (20 µg/ml) is added so that the mixture (80 µl) conatins about 100 ng of DNA. DNA hybridization and kinetic enrichment is carried out with 50 ng ligated DNA (40 µl) and an appropriate amount of driver amplicon DNA as described above. For a third round DNA subtraction/kinetic enrichment the procedure is repeated but using a fourth adaptor pair set or reusing the first adaptor pair set.

Cloning of DNA subtraction products

RDA subtraction products were are with appropriate restriction enzymes to remove ligated adaptors, separated on a 1.5% agarose gel in TAE buffer and visualized by ethidium bromide staining. DNA fragments are then eluted from the agarose gel and purified using a Sephaglas BandPrep Kit following the protocol given by the supplier (Pharmacia Biotech, USA). Purified DNA fragments are cloned into the *Eco*RV vector pMOSBlue according to protocol of the supplier (Amersham Life Science, UK) with a ligation buffer (20 µl) consisting of 66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM DDT, 66 µM ATP, and 2 U T4 DNA ligase. Ligation is done at 16°C overnight and MOSBlue competent cells (Amersham Life Science, UK) are transformed with ligated plasmid DNA by heat shock treatment of cells for 40 seconds at 42° in a standard procedure as outlined by Sambrook et al. (1989). Transformed

cells are then plated onto LB (Luria Bertani) agar plates containing 10 g/l NaCl, 10 g/l tryptone and 5g/l yeast extract. Plates are supplemented with 100 µg/ml ampicillin, 50 µl 10% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 10 µl 100 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to allow blue/white selection of colonies. White colonies containing the cloned DNA fragments are randomly picked and after plasmid purification from these colonies according to the method outlined by Sambrook et al. (1989), cloned DNA fragments are analyzed after restriction enzyme digest with *Bam*HI and *Hind*III by gel electrophoresis on a 1% agarose gel in TAE buffer.

Table 1: Representational difference analysis adaptors.

Adaptor Pair Set	**Name**	**Sequence**
1	R Hind 24	5' AGC ACT CTC CAG CCT CTC ACC GCA 3'
	R Hind 12	5' AGC TTG CGG TGA 3'
2	J Hind 24	5' ACC GAC GTC GAC TAT CCA TGA ACA 3'
	J Hind 12	5' AGC TTG TTC ATG 3'
3	N Hind 24	5' AGG CAG CTG TGG TAT CGA GGG AGA 3'
	N Hind 12	5' AGC TTC TCC CTC 3'
1	R Hpa 24	5' AGC ACT CTC CAG CCT CTC ACC GAC 3'
	R Hpa 11	5' CGG TCG GTG AG 3'
2	J Hpa 24	5' ACC GAC GTC GAC TAT CCA TGA AAC 3'
	J Hpa 11	5' CGG TTT CAT GG 3'
3	N Hpa 24	5' AGG CAA CTG TGC TAT CCG AGG GAC 3'
	N Hpa 11	5' CGG TCC CTC GG 3'
4	S Hpa 24	5' ACT TCT ACG GCT GAA TTC CGA CAC 3'
	S Hpa 12	5' CGG TGT CGG AAT 3'

Gawel N.J. and Jarret R.L. 1991. A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea*. Plant Molecular Biology Reporter 9: 262-266.

Lisitsyn N.A., Lisitsyn N.M. and Wigler M. 1993. Cloning the differences between two complex genomes. Science 259: 946-951.

Sambrook J., Fritsch E.F., Maniatis T. 1989. Molecular cloning: A laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.