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Southern hybridisation of tobacco TA29 and sesame GN13 promoter fragments in transgenic plants

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We use this protocol and it's working

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Disclaimer

Abstract

The protocol describes the Southern blot hybridisation steps of two genomic upstream elements (hereafter, promoters): TA29 of tobacco (*Nicotiana tabacum* L.) and GN13 of sesame (*Sesamum indicum* L.). The TA29 is a well-known anther-specific promoter of the tobacco *TA29* gene. The GN13 is the anther-specific promoter of the sesame *β-1,3-glucanase* gene. In this protocol, the genomic DNA(s) were isolated from the transgenic plants, *Eco*RI digested, purified, and electrophoresed in an agarose gel. The DNA(s) of the gel were mobilised into a positively charged nylon membrane using the capillary transfer method and hybridised with radiolabelled probes prepared from the respective promoter fragments. Finally, the blots were visualised by phosphor-imaging. This protocol is useful to confirm the transgene integration in the transgenic plants, thereby confirming the transformation process. The transgene copy number could also be quantified using this protocol.

Materials

[α-32P]-dCTP = BRIT (India)
Bovine Serum Albumin (BSA) = SRL (India)
DecaLabel DNA Labeling Kit = Thermo Fisher Scientific (USA)
DNA gel loading dye = Fermentas (USA)
DNeasy plant maxi kit =
QIAGEN (Germany)
*Eco*RI = Farmentas (USA)
Ethanol = Merck (Germany)
Ethidium bromide = Fermentas (USA)
Gel doc, model Gel Doc™ XR+ System = Bio-Rad (USA)
Gel rocker, model Rocker 25 = Labnet International (USA)
HCL = SRL (India)
*Hind*III = Farmentas (USA)
Hybridisation oven cum shaker = Amersham Biochem (UK)
NaCl = SRL (India)
NaOH = SRL (India)
$NaPO_4$ = SRL (India)
*Nco*I = Farmentas (USA)
Nuclease-free water = Sigma-Aldrich (USA)
Nylon membrane, model N+-nylon membrane = GE Healthcare Life Sciences (UK)
QIAquick Gel Extraction Kit = QIAGEN (Germany)
QIAquick PCR Purification Kit = QIAGEN (USA)
RNase A = GeNei (India)
Sodium dodecyl sulfate (SDS) = SRL (India)
Sodium acetate = SRL (India)
Speed vacuum drier = OPERON (South Korea)
Tris buffer = Sigma-Aldrich (USA)
Tris-equilibrated phenol-chloroform solution = SRL (India)
Trisodium citrate = SRL (India)
Typhoon TRIO+ Variable Mode Imager = GE Healthcare Life Sciences (UK)

Troubleshooting

GENOMIC DNA ISOLATION AND RESTRICTION DIGESTION

1 Total genomic DNA from young leaves of transformed plants was isolated using the DNeasy plant maxi kit (QIAGEN, Germany) according to the kit-specified protocol. For Southern blot analysis (Sambrook and Russell 1989), approximately 15 µg of genomic DNA was digested with EcoRI (Farmentas, USA). The digestion process was carried out in the presence of RNase A (GeNei, India) to minimise the RNA contamination. The digestion protocol was as follows:

	A	B
	Components	**Volume**
	Double Distilled H2O	58 µl
	10x Digestion buffer	20 µl
	RNase A (10 mg/ml)	2 µl
	EcoRI (10 Units/µl)	20 µl
	Genomic DNA (~150 ng/µl)	100 µl
	Total	***200 µl***

DIGESTED DNA PURIFICATION AND AGAROSE GEL ELECTROPHORESIS

2 The digested DNA was purified by the phenol-chloroform method. An equal volume of Tris-equilibrated phenol-chloroform solution (phenol:chloroform 1:1, SRL, India) was mixed with the digested DNA and centrifuged at 10,000 rpm for 20 minutes to separate the organic and aqueous phases. With the collected aqueous phase, the phenol-chloroform centrifugation process was repeated. To the finally collected volume of aqueous phase, the $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2) and three volumes of chilled ethanol (Merck, Germany) were added and incubated at -20°C for 16 hrs. to precipitate the DNA. After the incubation period, it was centrifuged at 10,000 rpm for 20 minutes at 4°C. After that, the supernatant was discarded and the DNA pellet was washed with 70% ethanol twice by centrifugation at 10,000 rpm for 20 minutes each at room temperature. Finally, the pellet was dried in a speed vacuum drier (OPERON, South Korea) for 2 minutes and was re-suspended in 20 µl of nuclease-free water (Sigma-Aldrich, USA). Subsequently, it was mixed with ten µl of DNA gel loading dye (Fermentas, USA) containing bromophenol blue and electrophoresed in a 0.8% agarose gel (without ethidium bromide) at 30 V at 4°C for 16 hrs.

GEL TREATMENT

3 After the completion of electrophoresis, the gel was stained with ethidium bromide (Fermentas, USA) and photographed in a gel doc (Gel Doc™ XR+ System, Bio-Rad, USA) for further representation purposes. After that, the gel was de-purinated by 0.25 N HCl for twenty minutes or until the colour of the gel loading dye changed from blue to yellow. The de-purinated gel was washed in the denaturation solution (0.5 N NaOH, 1.5 N NaCl, pH 12.5) for twenty minutes or until the colour of the dye turned back to blue. Finally, the gel was washed with neutralisation solution (1 M Tris buffer, 1.5 M NaCl) for twenty minutes. Neutralisation of the gel was done twice. In each passage of the solution transfer, the gel was washed with sterile double distilled water twice, for five minutes each. The total washing process was done in a gel rocker (Rocker 25, Labnet International, USA).

CAPILLARY TRANSFER OF DNA

4

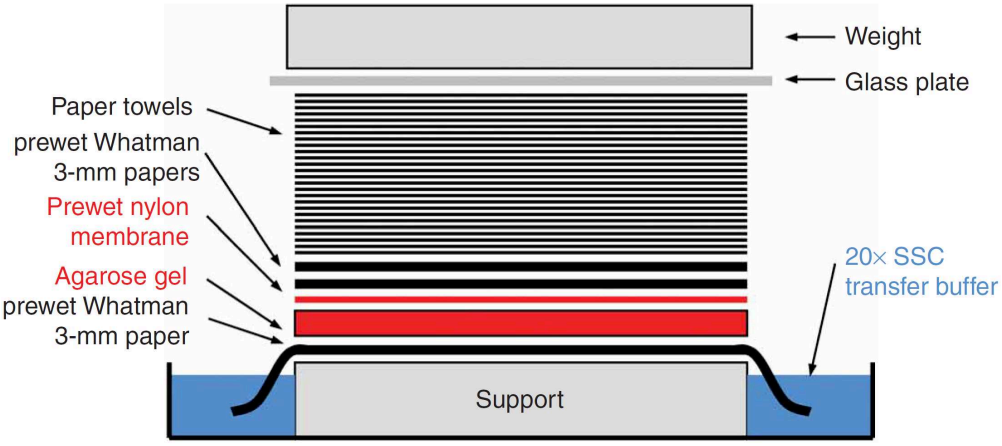

Fig. 1. Southern hybridisation apparatus for capillary transfer method

The washed membrane was subsequently transferred to the N+-nylon membrane (GE Healthcare Life Sciences, UK) by capillary transfer method in the presence of 20x SSC buffer (3 M NaCl, 300 mM trisodium citrate, pH 7.0) (Fig. 1). After the transfer, the membrane was cross-linked at 1600 µJ/cm^2 UV radiation.

PROBE LABELLING

5 The 871-bp tobacco TA29 and the 793-bp sesame GN13 upstream elements were digested out from the recombinant TA29::GUS and GN13::GUS cassettes, respectively, with *Hind*III and *Nco*I restriction enzymes. The digested fragments were gel purified (QIAquick Gel Extraction Kit, QIAGEN, Germany) and used as a probe after labelling with the [α-32P]-dCTP (BRIT, India) using the DecaLabel DNA Labeling Kit (Thermo Fisher Scientific, USA) according to the kit-specified protocol. The radio-labelled probe was purified using the QIAquick PCR Purification Kit (QIAGEN, USA) according to the kit-specified protocol.

HYBRIDISATION AND BLOT IMAGING

6 The membrane was pre-hybridised with pre-hybridisation buffer (0.5 M $NaPO_4$, pH 7.2, 7% SDS, 1% BSA) at 65°C in a hybridisation oven/shaker (Amersham Biochem, UK). The radiolabelled probe was heated at 95°C, snap-chilled on ice and added to the pre-hybridisation buffer for hybridisation. The hybridisation was carried out at 65°C for 16 hrs. After that, the hybridisation solution was discarded and the membrane was washed at 65°C twice in buffer 1 (2x SSC + 0.1% SDS) for thirty minutes per wash. Two subsequent washes were carried out in buffer 2 (1x SSC + 0.1% SDS) for thirty min per wash, and finally, it was placed in buffer 2 at room temperature. The membrane was exposed for 16 hrs. to phosphor imaging plates and detected in Typhoon TRIO+ Variable Mode Imager (GE Healthcare Life Sciences, UK).

Protocol references

Sambrook J, Rusell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, USA