OPTIMIZED DNA EXTRACTION AND SOUTHERN HYBRIDIZATION IN DIFFERENT *SOLANACEAE* SPECIES

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We present here an optimized procedure for the isolation of polysaccharide-free DNA, as well as the identification of an interest gene, from different Solanaceae species, by Southern blot analysis. In this experiment an Arabidopsis ERL 1 cDNA-derived probe was used for the detection of ERL 1 gene in plant samples by Southern Blot hybridization. Use of this protocol resulted in sharp and intense hybridization signals where the presence and the copy number of an ERL 1 gene are determined.

Keywords: genomic DNA purification, DNA processing, alkaline blotting, Southern hybridization, *ERL-1* gene.

1. Introduction

Southern blotting has been one of the keystones of DNA analysis since its first description by E.M. Southern (1975) [1]. Immobilization of DNA by binding to nitrocellulose, either powdered or in sheet form, had been utilized in biochemistry and molecular biology for several years [2;3], but Southern analysis was the first to show how immobilization of size-fractionated DNA fragments could be carried out in a reliable and efficient manner. Other advances of Southern analysis include: more efficient methods of transfer of DNA from gel to membrane, downward capillarity transfer [4;5], vacuum blotting [6;7;8], bidirectional blotting and transfer in alkaline buffers [9], as well as the use of class II restriction endonucleases.

Southern blotting allows researchers to determine the molecular weight of a restriction fragment, to measure relative amounts in different samples, to locate a particular sequence of DNA within a complex mixture, as well as to study how genes are organized within genomes by mapping restriction sites in and around segments of genomic DNA for which specific probes are available. Genomic DNA is first digested with one or more restriction enzymes, and the resulting fragments are separated according to size by electrophoresis through a standard agarose gel. The DNA is then denatured *in situ* and transferred from the

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gel to a solid support - usually nylon or a nitrocellulose membrane [10]. The DNA attached to the membrane is then hybridized to a labelled DNA or oligonucleotide probe, and bands complementary to the probe are located by an appropriate detection system, for example, by autoradiography. By estimating the size and number of the bands generated after digestion of the genomic DNA with different restriction enzymes, separately or in combinations, it is possible to place the target DNA within a context of restriction sites. When setting up a Southern transfer, choices must be made between different types of membrane, transfer buffer and transfer method. The most popular membranes are made of nitrocellulose, uncharged nylon, or positively charged nylon [10]. Although these materials have different properties, the three types of membrane are virtually interchangeable for many applications. The main advantage of nylon membranes is that they are relatively robust and so can be reprobed ten or more times before falling apart, eliminating problems caused by leaching of nucleic acids from nitrocellulose membranes during incubation at elevated temperatures [10]. Nitrocellulose membranes are fragile and can rarely be reprobed more than three times; however, these are still extensively used, as they give lower backgrounds with some types of hybridization probe [10].

We present here an optimized procedure for the isolation of polysaccharide-free DNA, as well as the identification of an interest gene, from different *Solanaceae* species, followed by southern hybridization protocol to purify, fractionate, blot and hybridize plant genomic DNA. In this experiment, an arabidopsis *ERL 1* cDNA-derived probe was used for the detection of *ERL 1* gene in plant samples by *Southern Blot* hybridization. To understand what really occurs *in vivo*, it is necessary to use a protocol allowing full protection of the DNA integrity and recovery, moreover, an efficient transfer in the entire sizes range. Use of this protocol resulted in sharp and intense hybridization signals where the presence and the copy number of an *ERL 1* gene are determined.

2. Materials and Methods

Cactus DNA extraction buffer 50 mM Tris [Tris(hydroxymethyl)aminoethane] (pH 8)
5 mM EDTA (Ethylenediaminetetraacetic acid)
0.35 M sorbitol
→ Add 1 % β-mercaptoethanol directly prior to use

Cactus CTAB solution 50 mM Tris (pH 8.0)
25 mM EDTA
4 M NaCl (Sodium chloride)
1.8 % CTAB (hexadecyltrimethylammonium bromide)

Church hybridization buffer 0.5 M phosphate buffer (pH 7.2)

1 % BSA (Bovine serum albumin)
1 mM EDTA

Southern denaturation solution 1.5 M NaCl
0.5 M NaOH (Sodium hydroxide)

Southern depurination solution 0.2 N HCl (Hydrochloric acid)

Southern neutralisation solution 1 M Tris (pH 7.5)
1.5 M NaCl

SSC (saline sodium citrate)-20x, 1 litre 175.3 g NaCl
88.2 g SSC
water to 800 mL
→ Adjust pH to 7.0 with HCl
→ Add water to 1 litre
→ Autoclave
→ Use at desired final concentration

The transfer apparatus consist in filter paper, Whatman 3 mm (millimeter) filter paper, Nylon transfer membrane, 0.45 µm -Whatman Nytran® N and,
UV crosslinking chamber -Stratagene Stratalinker®

2.1. DNA extraction from plant tissue

For the extraction of 10-50 µg DNA (for each sample), *N. benthamiana, N. tabacum, Arabidopsis thaliana* and tomato leaves material was ground to a fine powder in liquid nitrogen using a mortar and pestle. 1 g of leaf powder was resuspended in 20 mL cactus DNA extraction buffer (for each sample), vortexed intensely, and centrifuged at 8000 x g (times gravity x relative centrifugal force) for 15 minutes (4 °C). The supernatant was discarded and the procedure repeated twice. These three initial washing steps remove large amounts of polysaccharides prior to cell lysis. After the third wash, the pellet was resuspended in 5 mL cactus DNA extraction buffer, followed by the addition of 3.5 mL cactus CTAB solution and 600 µL of a 15 % sarkosyl solution (*N*-lauroylsarcosine sodium salt). The mixture was vortexed and incubated in a water bath at 55 °C for 60-90 minutes. Subsequently, the lysate was extracted with an equal volume of chloroform and centrifuged at 8000 x g for 15 minutes (4 °C). 0.7 volumes isopropanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) were added to the supernatant to precipitate genomic DNA. After centrifugation at 8000 x g for 30 minutes (4 °C), the DNA pellet was washed once with 70 % ethanol and subsequently resuspended in 500 µL water containing 100 µg/mL RNase A. The DNA/RNase A solution was incubated at 37 °C for 30-60 minutes, followed by standard phenol/chloroform extraction and isopropanol precipitation. The RNase A-treated DNA pellet was finally resuspended in 100 µL of water and the DNA concentration was determined using the NanoDrop® spectrophotometer.

2.2. Southern analysis

With Southern blots the presence and/or copy number of genes and transgenes are determined. To do so, 15-20 µg of genomic DNA (for each sample) from *N. benthamiana, N. tabacum, Arabidopsis thaliana* and tomato, were digested with 50-80 u of appropriate restriction enzymes in 150 µL tubes containing the respective reaction buffer at one time concentration and 100 µM spermidine. The reactions were incubated at 37 °C for 5 hours. Additional 50-80 u of restriction enzymes were added after 2.5 h to compensate for loss of active enzyme during the incubation time. After incubation, samples were isopropanol-precipitated and resuspended in 40 µL of water. These prepared samples were loaded on 0.75 % agarose gels and run at 22 V over night. The following day, gels were set to run until the bromophenol blue band had migrated at least 10 cm to ensure for proper size separation. Finished gels were depurinated in 0.2 N HCl for 10 minutes, denatured in 1.5 M NaCl/0.5 M NaOH for 45 minutes and neutralised in 1 M Tris (pH 7.5)/1.5 M NaCl for 30+15 minutes (10 minute washes with water between all steps). After equilibrating the gel in 10 times (10x) SSC, the DNA is transferred to nylon membranes by classical capillary transfer as follows.

2.2.1. Capillary blotting procedure

Finished agarose southern gels were equilibrated in 10 times SSC and the size-fractionated DNA was blotted onto nylon membranes, essentially as described by Sambrook and Russell (2001) [11]. An agarose electrophoresis gel, containing the fractionated restriction fragments, is placed on a filter paper wick that forms a connection between the gel and a reservoir of high-salt buffer. The nylon membrane is placed on top of the gel and covered with a tower of paper towels that are held in place with a weight. Capillary action results in the buffer soaking through the filter paper wick, gel and membrane and into the paper towels. As the buffer passes through the gel the DNA fragments are carried with it into the membrane, where they become bound to the membrane. The only technical complication is the possibility that the buffer bypasses the gel by soaking directly from wick to paper towels, which is unlikely if the setup is assembled carefully. Effective transfer of fragments up to 15 kb in length takes approximately 18 h, roughly equivalent to ‘overnight’. After transfer, membranes were briefly rinsed in 2 times SSC and subsequently UV crosslinked using a Stratalinker® device (120 mJ/cm²).

2.2.2. Radioactive labelling of nucleic acid probes

For the detection of DNA sequences during Southern analyses, specific probes were labelled radioactively with ^{32}P. Depending on the experiment, radiolabelled probes were generated either by random-prime labelling of cDNA

fragments with Klenow Fragment, or by 5' end labelling of DNA oligonucleotides with T4 polynucleotide kinase.

2.2.2.1. Random-prime labelling of DNA probes

Random-primed DNA probes were used for Southern hybridisations using Invitrogen's RadPrime DNA Labelling system according to the manufacturer's recommendations. Purified restriction fragments or PCR (polymerase chain reaction) products between 200 and 500 nt (nucleotides) in length served as templates in RadPrime labelling reactions. 50-100 ng (nanograms)of template DNA were denatured at 95 °C for 2 minutes and quick-chilled on ice, followed by the addition of 20 µL 2.5 times RadPrime Buffer, 1 µL of 0.5 M dTTP (deoxythymidine triphosphate) and 0.5 M dGTP (deoxyguanosine triphosphate) each, 1 µL random primers (3 µg/µL), 2 µL [α-32P] ATP (adenosine triphosphate) and [α-32P] CTP (cytidine triphosphate) each (3000 Curie/mmol), 40 u (units) Klenow Fragment, and water to a final volume of 50 µL. The reaction mixture was incubated in a water bath at 37 °C for 1 hour and then purified using Amersham's MicroSpin™ S-200 spin columns according to the manufacturer's protocol. Purified random-primed DNA probes were denatured in a water bath at 95 °C for 5 minutes and quick-chilled on ice before adding them to the hybridization buffer.

2.2.2.2. *5' End* labelling of DNA oligonucleotides

End-labelled oligonucleotide probes (DNA) were created in 5' end labeling reactions using T4 polynucleotide kinase. To this end 8 pmol template DNA were mixed with 5 µL 10 times PNK(Polynucleotide kinase) reaction buffer, 6 µL [γ-32P] ATP (3000 Curie/mmol), 20 u T4 polynucleotide kinase, and water in a total reaction volume of 50 µL. After 1 hour incubation at 37 °C in a water bath, these labeled oligo-nucleotide probes were purified using commercial MicroSpin™ G-25 spin columns (Amersham), according to the manufacturer's specifications. Purified oligonucleotide probes were briefly denatured at 95 °C and quick-chilled on ice, prior to their addition to the hybridization buffer.

2.2.3. Hybridization, washing, and exposure of Southern membranes

Membranes were pre-hybridized in 10-15 mL pre-warmed Church hybridization buffer for 1 hour. Pre-hybridization and hybridization temperatures were dependent on the size of the DNA to be detected and/or the length of the probe used. Typically, DNA Southern were hybridized at 65 °C using random-primed DNA probes. Probes were denatured and added to the hybridization buffer, followed by rotating over-night incubation in a hybridization oven. The following day, membranes were washed with 10-15 mL of pre-warmed washing solutions in the following order: 1. rinse with 2 times SSC/0.1 % SDS; 2. wash 2

times 15 minutes with 2 times SSC/0.1 % SDS; 3. wash 2 times 10 minutes with 1x SSC/0.1 % SDS; 4. wash 2x 5 minutes with 0.5 x SSC/0.1 % SDS. All washing steps were performed at hybridization temperature. Washed membranes were rinsed in 2 times SSC, and subsequently sealed in plastic bags while still wet. Such prepared membranes were exposed to X-Ray films in appropriate exposure cassettes. Suited exposure times had to be determined empirically. Exposed X-Ray films were developed automatically, using a Curix 60 developer (Agfa). In special cases, X-Ray films were developed manually using the Curix 60's solutions for development and fixation, enabling a more precise timing of X-Ray film development.

3. Results and Discussions

Southern blot hybridization is a useful tool for analyzing genomic DNA, usually restriction fragments, in such a way that DNA becomes immobilized on the membrane and can be used as a substrate for hybridization analysis with radioactive labeled DNA or RNA probes that specifically target individual restriction fragments in the blotted DNA.

The techniques used to prepare DNA for Southern blotting depend on the type of DNA that is being studied. For genomic DNA, the objective is to obtain molecules that have not become extensively fragmented by random shearing during the extraction process, so that specific restriction fragments of 20 kb (kilobase) and more can be obtained. After cell disruption, genomic DNA extraction procedures continue with steps aimed at removing the major biochemicals other than DNA present in the initial extract. One of these steps is deproteinization, which is carried out by phenol extraction or a 1: 1 mixture of phenol and chloroform, in such a way that after centrifugation, the precipitated proteins migrate to the interface between the organic and aqueous phases, whereas the nucleic acids remain in the aqueous phase [12].

Preparations of plant DNA are often contaminated by polysaccharides, namely pectins, which co-precipitate with DNA after alcohol addition, and finally lead to highly viscous solutions. In this experiment, essentially polysaccharide-free genomic DNA from *N. benthamiana, N. tabacum, Arabidopsis thaliana* and tomato leaves, were extracted using a modified CTAB (cetyl-trimethyl-ammonium bromide) extraction method [12]. This compound specifically binds nucleic acids, improving their recovery during phenol extraction. Most extraction procedures also include digestion of RNA with ribonuclease (RNase A), and a final treatment with ethanol, which precipitates the remaining nucleic acid polymers, enabling ribonucleotides and other low-molecular weight contaminants to be removed (see Fig.1).

With Southern blots the presence and/or copy number of genes and transgenes are determined. To do so, genomic DNA from *N. benthamiana, N. tabacum, Arabidopsis thaliana* and tomato were digested with 50-80 u of appropriate restriction enzymes, and incubated at 37 °C for 5 hours. Additional 50-80 u of restriction enzymes were added after 2.5 h, to compensate for loss of active enzyme during the incubation time. These prepared samples were loaded on 0.75 % agarose gels and run at 22 V over night. The following day, gels were let to run until the bromophenol blue band had migrated at least 10 cm to ensure for proper size separation (see Fig. 1).

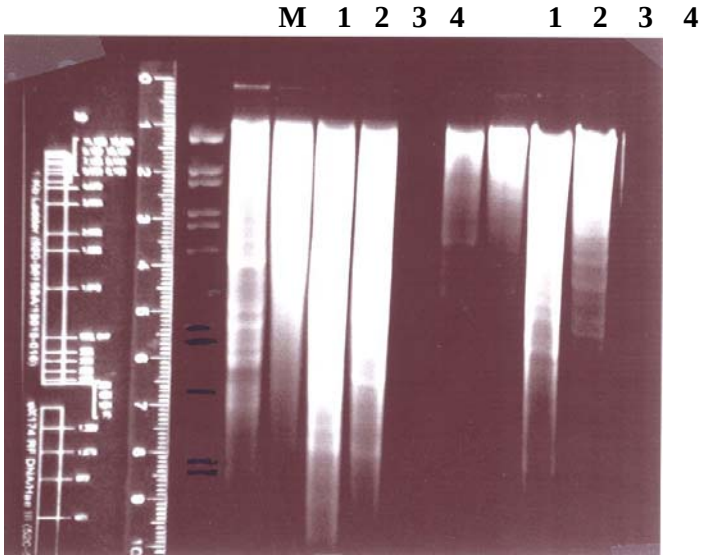

Fig. 1. Digesting genomic DNA with EcoRI and BamHI restriction endonucleases

Genomic DNA samples from different *Solanaceae* species were digested with EcoRI and BamHI restriction enzymes, ran in an agarose gel with DNA size marker on first line, stained with *ethidium bromide*, and photographed with a ruler laid alongside the gel, so that band positions can later be identified on the membrane. Where M is the marker, line 1 *Arabidopsis*, line 2 *N.benthamiana*, line 3 *N. tabacum* and line 4 tomato samples.

The transfer of electrophoretically separated DNA from gels to two-dimensional solid supports *via* upward capillary is a key step in *Southern hybridization*. First, the agarose gel is pretreated by soaking in a series of solutions that depurinate, denature, and neutralize the DNA and gel matrix, and the second stage is the transfer itself, which occurs by upward capillary action.

With a positively charged nylon membrane, the transferred DNA becomes covalently linked to the membrane. The advantage of this combination is that no post-transfer immobilization step is required, as the positively charged membrane binds DNA irreversibly under alkaline transfer conditions. Capillary transfer is still the most popular method of Southern blotting, as previously described on methods. And finally, in the last stage the DNA is immobilized on the membrane by UV irradiation. When immobilizing DNA on a nylon membrane by UV crosslinking, the intensity of irradiation is critical; too little results in submaximal immobilization, and too much causes DNA degradation. Here, we subsequently crosslinked the DNA using a Stratalinker® device, at the intensity of 120 mJ/cm². After immobilization, the DNA can be subjected to hybridization analysis, enabling bands with sequence similarity to a labeled probe to be identified. Experimentally, the analysis is usually carried out with a probe, which has been labeled, and target DNA that has been immobilized on a membrane support.

In this experiment, an arabidopsis *ERL 1* cDNA-derived probe was used for the detection of a duplicated *ERL 1* gene in plant samples by Southern hybridization. Genomic DNA was cleaved with *EcoR I* and *BamH I* restriction enzymes for comparison. In both cases, single major bands were detected in *Arabidopsis*, *N. benthamiana*, *N. tabacum* and tomato, while 2 distinct bands were detected in both *N. benthamiana* samples (see Fig. 2).

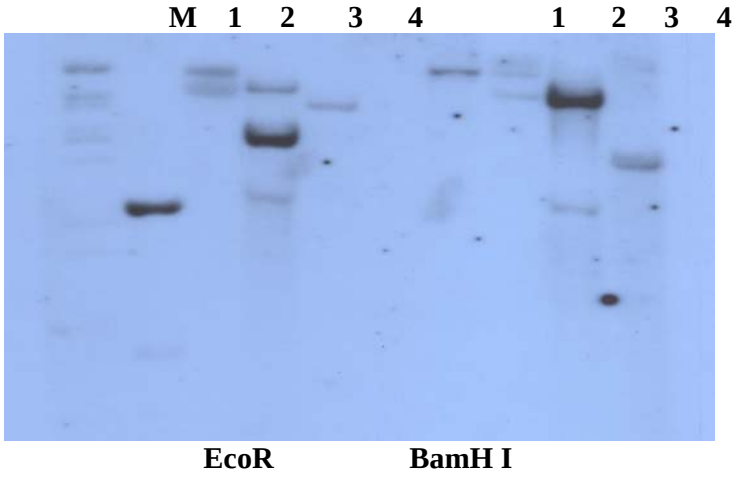

Fig. 2. Southern analysis of *ERL 1* in different *Solanaceae* species.

An arabidopsis *ERL 1* cDNA-derived probe was used for the detection of *ERL 1* genes by Southern hybridization. Genomic DNA was cleaved with EcoR I and BamH I for comparison. In both cases single major bands were detected in *Arabidopsis* , tobacco, and tomato, while 2 distinct bands were detected in both *N.*

benthamiana samples. Where M is the marker, line 1 *Arabidopsis*, line 2 *N.benthamiana*, line 3 *N.tabacum* and line 4 tomato samples.

Hybridization analysis is sensitive and permits detection of single-copy genes in complex genomes. Its advantages are simplicity, reliability, and the lack of special equipment requirements.

4. Conclusions

In this experiment, essentially polysaccharide-free genomic DNA from *N. benthamiana, N. tabacum, Arabidopsis thaliana* and tomato leaves were extracted using a modified CTAB (cetyl-trimethyl-ammonium bromide) extraction method [12]. This compound specifically binds nucleic acids, improving their recovery during phenol extraction. After immobilization on the membrane by UV irradiation, the DNA can be subjected to southern hybridization analysis, enabling bands with sequence similarity to a labeled probe to be identified.

The principle of *Southern hybridization* analysis is that single-stranded DNA molecules of defined sequence (the "probe") can base-pair to a second DNA molecule that contains a complementary sequence (the "target"), with the stability of the hybrid depending on the extent of base pairing that occurs [1]. Experimentally, the analysis is usually carried out with a probe, which has been labeled, and target DNA that has been immobilized on a membrane support. Therefore, in this experiment, an arabidopsis *ERL 1* cDNA-derived probe was used for the detection of *ERL 1* gene in plant samples by Southern hybridization analysis, as previously described.

R E F E R E N C E S:

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