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Protocol

Protein Slot Blotting: An Easy, Rapid and Reliable Technique to Identify the Expression of a Protein in Transgenic Plants

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Abstract: A technique based on immunological recognition of a foreign protein in transgenic plants has been developed. It allows a quick and reliable screening of many plant samples, improves the accuracy of the results compared to ELISA and is easier to carry out and more sensitive than a western immunoblot. This technique has also been tested to recognize foreign proteins in rice and tobacco leaf extracts.

The goal of most studies with transgenic plants is to cause the accumulation of a foreign protein. Screening the regenerated plants for gene integration and expression is a process that involves different techniques, including PCR, ELISA, DNA- and RNA-blot hybridization analysis. Although western blots confirm that the introduced gene leads to new protein accumulation, they are somewhat laborious and time consuming. Furthermore, such analysis requires SDS-PAGE, which may modify antigenic epitope(s) resulting in a weaker, if not null, antibody reaction.

In order to establish a method for screening for gene expression in transgenic rice plants as an alternative to ELISA and western blots, we developed a simple slot-blot procedure that is rapid and non-denaturing. As our laboratory is involved in studies on tropical crops, we also

developed the technique in a way that it might be used even in laboratories that do not have access to sophisticated equipment.

Material and Solutions Required

Material

Small polytron (type PT 1200 from Kinematica, Switzerland), or other.
A slot- or dot-blot apparatus (we used one from Hoefer Scientific Instruments, USA).
Nitrocellulose membrane.
Agitator
Microfuge

Solutions

Grinding buffer: 100 mM Tris-HCl pH 6.8, 10 mM EDTA, 5 mM DTT
TBS: 20 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM KCl
TBST: TBS + 0.05% Tween 20
Substrate buffer: 100 mM Tris HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$
Nitro blue tetrazolium (NBT): 66 µL of stock solution prepared according to in Sambrook et al. (1989) per 10 mL of buffer
5-Bromo-4-chloro-3-indolyl phosphate (BCIP): 33 µL of stock solution per 10 mL buffer
Blocking solution: TBS + 5% (w/v) non fat dry milk or 1% (w/v) BSA[1]
Antibody against the protein of interest[2]
Antibody against the first antibody conjugated with alkaline phosphatase (anti-rabbit or anti-mouse)

Protocol

- In an Eppendorf tube grind quickly about 100 mg of fresh or frozen tissue, preferably from young leaves, in liquid nitrogen. Thaw powdered material in 500 µL of grinding buffer.
- Grind briefly (5 to 10 s) with a small polytron or similar grinder.[3]
- Centrifuge 10 minutes in a microfuge at 4°C. Decant the supernatant to a clean tube, keep on ice if used immediately, or freeze at -80°C for later use.
- Wet the nitrocellulose membrane in deionized water and assemble the blotter apparatus. Deposit 50 µL per sample of the supernatant in each well of the blotter applying vacuum an additional 15 min.
- Remove and wash the membrane briefly in TBS.

- Incubate at least one hour at room temperature with 10 mL of blocking solution with gentle agitation.
- Incubate the membrane 30 min. at room temperature under gentle agitation with an appropriate dilution of the primary antibody in 10 mL blocking solution.
- Wash 4 times for 5 min. in 30 mL of TBST.
- Dilute the secondary antibody in 10 ml of blocking solution,[4] and incubate the membrane 30 min. at room temperature with gentle agitation.
- Wash 4 times for 5 min. in 30 mL of TBST, and rinse briefly with TBS.
- Add 10 mL of the staining solution and leave in dark at room temperature or at 37°C.[5]
- Stop the reaction by washing with deionized water and record the results.

Notes

1. Better results, were obtained using dry milk rather than BSA.
2. Polyclonal rabbit antibodies were used in this study.
3. This step is not necessary if the primary grinding is thorough. The small polytron, however, is faster and the protein yield is better.
4. A dilution of 1/10,000 is generally sufficient.
5. Depending upon the protein concentration in the extract, the result will appear in from a few minutes to several hours. In our hands the background is negligible even after an overnight incubation with the staining solution.

Results and Discussion

The purpose of developing this technique was to reduce the time necessary to identify the accumulation of a foreign protein and have a clear qualitative response on multiple samples. Fig. 1 shows the type of results obtained when screening for the expression of the coat protein gene of the rice tungro bacilliform virus in transgenic rice plants. Positive results are easily distinguished from the green or yellow color caused by the pigments present in the plants extracts.

After the protein samples are prepared, the blots are easy and rapid: 48 samples can be blotted in 20 minutes; the total reaction is done in less than 4 hours.

Using an antibody with a IgG concentration of 1mg/mL (stock), less than 250 pg of virus was detected and a positive signal is given when as little as 25 µg of total protein from a transgenic rice plant (Fig. 1). The percentage of the product of the transgene has not been determined. In order to detect the same protein with ELISA, at least 100 µg of total protein from rice leaves was required and led to some misinterpretations.

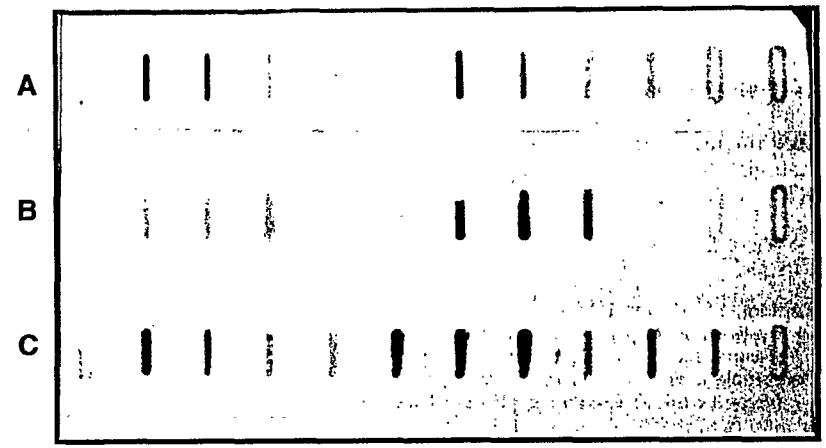

Fig. 1. Slot-blotting of various dilutions of virus particles, crude extracts from transformed rice plants with the RTBV CP, and offspring from a transgenic rice line. A: serial dilution of RTBV particles, 2.5 µg to 2.5 ng. A2-5 in grinding buffer, A7-10: in negative control crude extract. B2-4: 70-140-210 µg of total protein from negative control. B5-7: 15-30-45 µg of total protein from a transformed rice plant with the RTBV CP coding sequence. C: 50 µL/slot of crude extract from different offspring of a transgenic rice line (approximately 60 µg of total protein).

As the technique was being developed, a negative response was observed after concentrating the proteins by precipitation with cold acetone. In this case the acetone precipitation apparently denatured the protein so that it was no longer recognized by the antibody, or the protein in question was not redissolved.

This technique has also been applied to identify rice plants infected with rice yellow mottle virus, and the presence of RYMV particles in leaves crude extracts of tobacco leaves.

Some parameters of this procedure may vary from one protein to another as well as from one antibody to another. Modifying the concentration of the blocking agent used, the blocking time, the incubation times (with the first and/or second antibody) the concentration of the first antibody, etc. might be necessary for each particular situation.

If there is a very low level of protein accumulation, techniques using a radioactive label, a more complicated extraction procedure in the preparation of the samples before blotting, or longer exposure to antibodies may be applied (Lapidot et al.,1993).

The technique described here is simple, rapid and reliable. It is not proposed as a substitute for ELISA in general, but in some cases it is more convenient than other methods.

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Reports of Meetings

Recent Advances in the Molecular Genetics of Tree Species

Sixth International Meeting: Molecular Genetics Working Party of the International Union of Forestry Research Organizations

On 20–23 May 1994 a group of 70 scientists gathered at Prouts Neck, Maine, USA, to discuss progress toward understanding and manipulating the molecular genetics of a diverse group of ecologically and economically important species, forest trees. Like the organisms themselves, the presentations were diverse, spanning genes, genomes, and populations of both angiosperms and gymnosperms. The meeting was organized by Michael Greenwood and Keith Hutchison of the University of Maine (USA).

Compared to previous meetings of this group, significant progress has been made in several areas. Research teams continue to isolate and characterize new genes, but transgenic plants are increasingly being used to study gene function *in vivo*. Genome mapping has also matured. In earlier meetings presentations described the construction of genetic linkage maps, but at this meeting maps were being used as tools to identify and dissect quantitative trait loci (QTL).

The biological distinctiveness of forest trees provides both challenges and opportunities for forest biologists. Transformation systems remain recalcitrant, especially for conifers, but progress is apparent even here. On the other hand, advantages of the haploid genetics offered by conifer gametophytes continues to be exploited. Recent advances in model organisms such as *Arabidopsis* continue to influence studies in forest

Abbreviations: 4-CL, 4-coumarate-CoA ligase; BSP, bark storage protein; CAD, cinnamyl alcohol dehydrogenase; CHS, chalcone synthase; OMT, O-methyltransferase; PAL, phenylalanine-ammonia lyase; QTL, quantitative trait loci; RLGS, restriction landmark genomic scanning; SSR, simple sequence repeat.