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Protocols

An Improved and Rapid Protocol for the Isolation of poly(A)$^+$-RNA from Small Samples of Scots Pine Seedlings

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Abstract. A simple rapid method for the isolation of poly(A)$^+$-RNA from pine seedlings (*Pinus sylvestris*) was developed. The entire procedure can be carried out in Eppendorf tubes, thus greatly minimizing the time, starting material (as little as 3-5 μg of total RNA) and the amount of chemicals required. The poly(A)$^+$-RNA recovered is a suitable substrate for RT-PCR.

Key words: mRNA extraction, pine seedlings, RT-PCR application

Introduction

Expression of specific genes at the level of mRNA can be studied using techniques such as northern blot, slot/dot blot, RNase protection assay, *in situ* hybridization and RT-PCR. Among these, RT-PCR offers a high level of specificity and sensitivity and thus it is frequently the method of choice for studies of gene expression. Numerous protocols for extraction and quantification of RNA are available (O'Driscoll et al., 1993; Krumlauf, 1994; Gupta, 1999), and include many plant species (Chirgwin et al., 1979), and procedures for samples with a high content of polyphenols and polysaccharides (Chomczynski and Mackey, 1995; Staub et al., 1995). Specific protocols have been developed for conifers (Chang et al., 1993; Bugos et al., 1995; Claros and Cánovas, 1998) and RNA extracted by these methods is suitable for most purposes. We are studying the expression of a gene whose transcript has low abundance in pine seedlings, but can be quantitated by RT-PCR. The protocol developed in our lab for RNA extraction from pine seedlings (Claros and Cánovas, 1998) was suitable for most samples but proved unreliable for estimation of transcript levels in roots. Here we describe a method for microextraction of poly(A)$^+$-RNA from as little as 3-5 μg total RNA. The method can be performed in Eppendorf tubes and the resulting poly(A)$^+$-RNA is suitable for RT-PCR analyses. In our hands it works independently of the tissue origin for all tested samples.

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Materials and Methods

Plant material

Scots pine seeds (*Pinus sylvestris*) used in all experiments were from Servicio de Material Genético, ICONA (Instituto de Conservación de la Naturaleza), Guadalajara (Spain). Seed germination and growth of seedlings was as described elsewhere (Cánovas et al., 1991).

Total RNA extraction

Total RNA from various tissues and developmental stages of the pine seedling was extracted (using the protocol of Cantón et al., 1993, or Claros and Cánovas 1998), and dissolved in sterile distilled water. The A_{260}/A_{280} ratio was 1.8 - 2.0 for most of the samples, except root RNA samples which ranged 1.5 - 1.7.

Poly(A)$^+$-RNA preparation

The poly(A)$^+$-RNA fraction was isolated using oligo-$(dT)_{15}$ cellulose (Boehringer) contained in a hand-made spin column assembly described in the following protocol.

Oligo-(dT) cellulose was prepared by adding to the dry resin a 4-5 volume equivalent of BB (binding buffer) containing 500 mM LiCl, 10 mM Tris pH 7.5, 1 mM EDTA and 0.5% SDS. The affinity adsorbent was allowed to settle for 1 h at room temperature. The supernatant was then discarded and 6 volumes of the equivalent wet resin volume of BB buffer were added. Oligo-(dT) cellulose prepared in this way was used routinely for poly(A)$^+$-RNA preparation.

Samples containing total RNA in water were heated at 65ºC for 5 min and then cooled on ice. These were mixed in Eppendorf tubes with an equal volume of 2x BB and with 50 µl of the suspension containing the wet resin, prepared as described above. Mixes were allowed to bind to the resin using a rotary mixing apparatus during 15 min at room temperature. The samples were then transferred to a 0.5 mL Eppendorf tube with a hole at the bottom where we have placed a small piece of glass wool, and the entire assembly was fitted into a 1.5 mL Eppendorf tube without lid. Samples prepared in this manner were centrifuged at 3500 rpm in a microcentrifuge for 30 s as described in Figure 1. The 1.5 mL tubes were discarded and replaced by fresh ones and the resin was washed with 75 µl of BB buffer and centrifuged as before, this step was repeated three times.

Next, the resin was washed with 50 µl of WB (washing buffer), containing 150 mM LiCl, 10 mM Tris and 1 mM EDTA, and centrifuged as before. This step was repeated two times. Then the resin was completely dried by centrifugation at 5000 rpm in a microcentrifuge for two min and the poly(A)$^+$-RNA samples were eluted from the resin with 20 µl of EB (elution buffer) containing 10 mM Tris pH 7.5 and 1 mM EDTA. The final yield was about 50-100 ng in 20 µl. Fresh 1.5 mL tubes were used for each centrifugation step. All buffers and material used in this procedure were sterile.

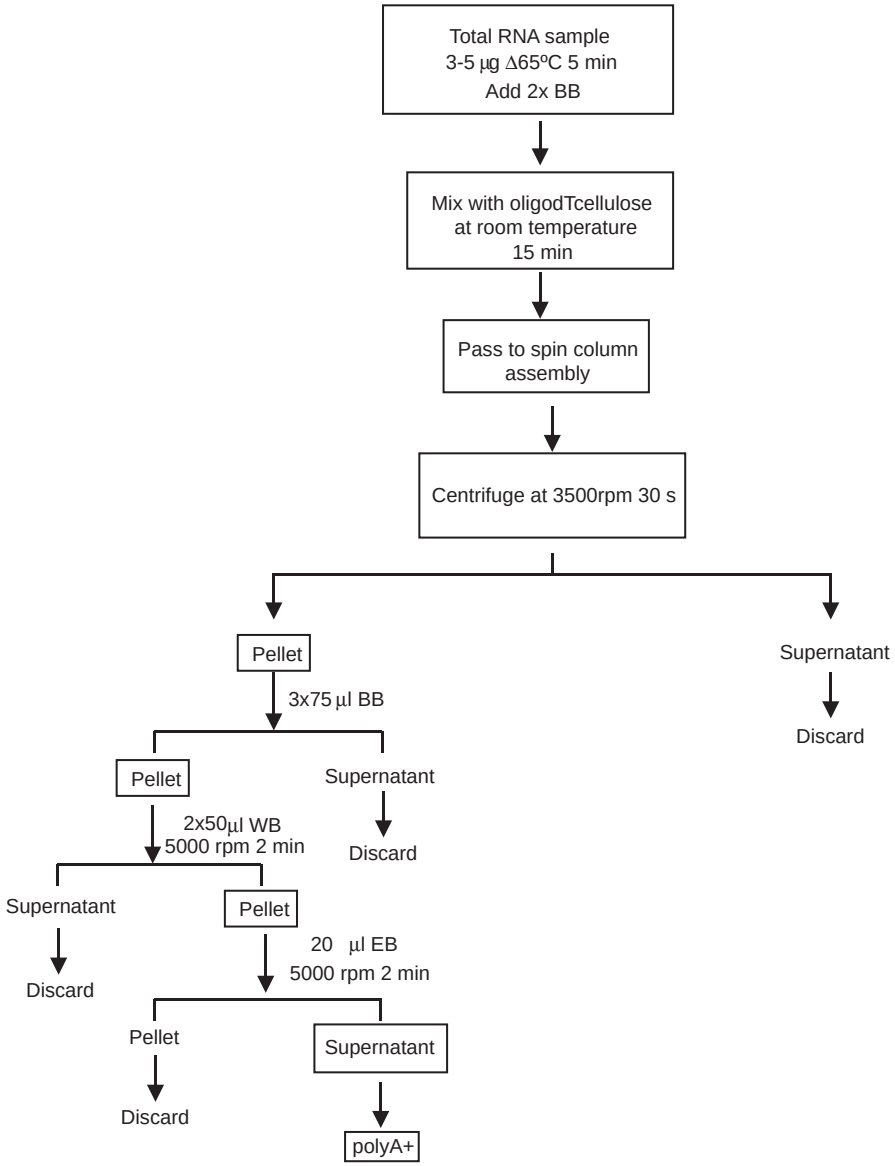

Figure 1. Protocol for microscale preparation of poly(A)$^+$-RNA from Scots pine seedlings.

RT-PCR sample analysis

We have used the RT-PCR kit, ProStar HF single-tube RT-PCR system from Stratagene to analyze the transcript levels in RNA samples prepared as described above from pine seedlings. We tested the transcript from the *upk* gene, recently cloned in our laboratory from Scots pine (accession number AJ250467). The *upk* gene was undetectable using traditional northern blot assay. We designed two

specific primers derived from the cDNA as follows: 5′-GCGGTAGGGTTCGG-TGA-3′ and 5′- GACCCGACCATTCCCGA-3′. As little as 1 ng of poly(A)$^+$-RNA prepared as above was used for each RT-PCR reaction. RT-PCR was performed using the manufacturer's specifications and with the following cycle conditions: 95ºC 1 min, followed by 95ºC 30 s, 54ºC 30 s and 68ºC 2 min for 40 cycles and then an extension time of 10 min at 68ºC.

Southern blot and filter hybridization

Gel transfer was essentially as described by Cantón et al. (1993). The probes were labeled with the high prime kit from Boehringer and hybridizations were performed in a solution containing 6x SSC, 5x Denhardt's solution (1x Denhardt's solution is 0.02% (w/v) ficoll, 0.02% (w/v) polyvinylpyrrolidone, 0.02% (w/v) bovine serum albumin), 0.1% SDS and 100 mg/mL of denatured salmon sperm DNA at 65ºC. Filters were washed twice in 2x SSC, 0.1% SDS for 30 min at 65ºC followed by 0.2x SSC, 0.1% SDS for 15 min at 72ºC.

Results and Discussion

Removal of the minor interfering contaminants from total RNA samples without excessive loss of material is difficult. In this protocol, the problem is overcome by preparation of poly(A)$^+$-RNA in a spin column assembly containing oligo-(dT) cellulose and by using Eppendorf tubes. This minimizes the amount of starting material and reagents and increases the number of samples that can be processed simultaneously. Up to 24 samples can be prepared in a standard microcentrifuge, and the process can be completed within two hours.

Since we could not detect mRNA from the *upk* gene by northern blot analysis we used RT-PCR to quantify the level of message. We performed the above procedure using 100 ng of total RNA and analyzed the results in an agarose gel (Figure 2A). Only embryo RNA was a suitable template for amplifying the *upk* product. The other samples that included RNA from roots at different developmental stages were a poor template for the RT-PCR reaction. To increase the sensitivity of our method, the agarose gels containing the samples were blotted and hybridized with a specific DNA-labeled probe (Figure 2B). Since radioactive detection of nucleic acids is a more sensitive procedure than staining with ethidium bromide, this allowed us to verify the amplification of the *upk* transcript in the germinating seed samples. No amplification of *upk* product was observed in the other tracks on the gel.

However when we used poly(A)$^+$-RNA, prepared from RNA used in the experiment shown in Figure 2, all samples were suitable for amplifying the *upk* fragment. All root samples independent of growing stage were suitable templates for the amplification reaction, even when ethidium bromide was used for detection (Figure 3A). This indicates that the amplification reaction can be used to analyze specific PCR products that can be identified and quantitated by Southern blotting (Figure 3B).

We have successfully used poly(A)$^+$-RNA samples prepared from tissues representing five developmental stages of pine seedlings: embryos, germinating seeds, and plants with cotyledons of 0.5 cm, 1.0 cm and 1.5 cm, respectively. The

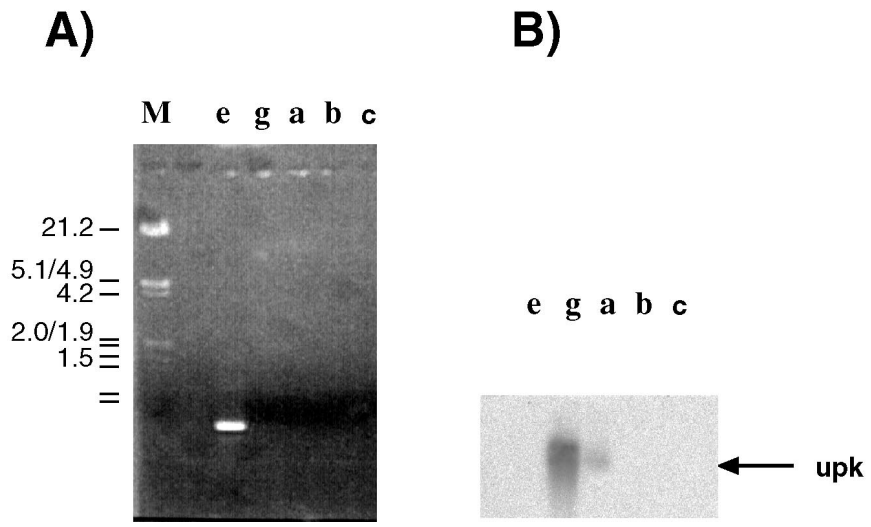

Figure 2. A) Agarose electrophoresis of the RT-PCR *upk* amplification reaction using *P. sylvestris* total RNA. (e) Embryos, (g) Germinating Seed, (*a*) Plants with cotyledon of 0.5 cm, (*b*) Plants with cotyledon of 1.0 cm and (*c*) Plants with cotyledon of 1.5 cm. M is DNA molecular weight marker, an *Eco*R I/*Hin*d III digest of bacteriophage λ DNA. B) Result obtained when the gel shown in A) was blotted and hybridized to the specific probe for *upk*.

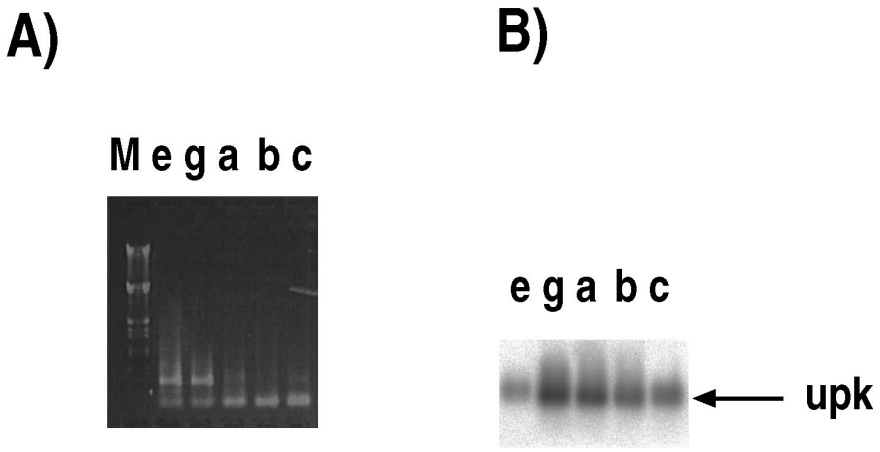

Figure 3. A) Agarose electrophoresis of RT-PCR products from *P. sylvestris* poly(A)$^+$-RNA. The order of samples as in Figure 2A. B) The gel shown in A) was blotted and hybridized to the *upk*-specific probe.

results were reproducible in independently duplicated poly(A)$^+$-RNA preparations. The poly(A)$^+$-RNA micropreparation was effective when the RNA samples contained interfering material that otherwise rendered them poor templates for the RT-PCR reaction. In addition, little starting material was necessary for RT-PCR

reactions, however, we think our procedure could be scaled up to prepare larger quantities of poly(A)$^+$-RNA for other uses.

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References

Bugos RC, Chiang VL, Zhang X-H, Campbell ER, Podila GK and Campbell WH (1995) RNA isolation from plant tissues recalcitrant to extraction in guanidine. Biotechniques 19: 734-737.

Cánovas FM, Cantón FR, Gallardo F, García-Gutiérrez A and de Vicente A (1991) Accumulation of glutamine synthetase during early development of maritime pine (*Pinus pinaster*) seedlings. Planta 185: 372-378.

Cantón FR, García-Gutierrez A, Gallardo F, de Vicente A and Cánovas FM (1993) Molecular characterization of a cDNA clone encoding glutamine synthetase from a gymnosperm, Pinus sylvestris. Plant Mol Biol 22: 819-828.

Chang S, Puryear J and Cairney J (1993) Simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Reptr 11: 113-116.

Chirgwin JM, Przybyla AE, Macdonald RJ and Ratter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in Ribonuclease Biochemistry 18: 5294-5299.

Chomczynski P and Mackey K (1995) Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques 19: 942-945.

Claros MG and Cánovas FM (1998) Rapid High Quality RNA Preparation from pine seedlings. Plant Mol Biol Reptr 16: 9-18.

Gupta A (1999) RT-PCR: characterization of long multi-gene operons and multiple transcript gene clusters in bacteria. Biotechniques 27: 966-70, 972.

Krumlauf R (1994) Analysis of gene expression by northern blot. Mol Biotechnol 2: 227-42.

O'Driscoll L, Daly C, Saleh M and Clynes M (1993) The use of reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate specific gene expression in multidrug-resistant cells. Cytotechnology 12: 289-314.

Staub U, Polivka H and Gross HJ (1995) Two rapid microscale procedures for isolation of total RNA from leaves rich in polyphenols and polysaccharides: application for sensitive detection of grapevine viroids. J Virol Methods 52: 209-218.