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Protocols

A Simple and Efficient Protocol for Isolation of Functional RNA from Plant Tissues Rich in Secondary Metabolites

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Abstract. A protocol is described for rapid RNA isolation from various plant species and tissues rich in polyphenolics and polysaccharides. The method is based on the Nucleon PhytoPure™ system without the use of phenol. The procedure can be completed within 1 h and many samples can be processed at the same time. The yield ranged from 240 μg up to 3 mg per gram of tissue with an average purity measured as $A_{260/280}$ of 1.85. The RNA was of sufficient quality for use in RT-PCR reactions. Quantitation of single-stranded cDNA was carried out with the RiboGreen™ reagent and of PCR products with the PicoGreen™ reagent.

Key words: RNA extraction, RT-PCR

Abbreviation: CTAB; cetyltrimethylammonium bromide.

Introduction

Molecular tools are increasingly important in modern plant research. RNA isolation from usual herbaceous model plants like arabidopsis, tobacco, tomato, potato or maize is usually achieved by classical phenol/lithium chloride and guanidinium based methods (Sambrook et al., 1989; De Vries et al., 1993; Soni and Murray, 1994). However, there are many other plant species and special tissues, e.g. trees, ornamental plants, flowers or buds, where problems have been encountered due to high concentrations of polysaccharides, polyphenolics or latex. Various protocols for RNA isolation from plant species rich in polyphenolics or polysaccharides exist (Schneiderbauer et al., 1991; John, 1992; Chang et al., 1993; Ainsworth, 1994; Grosskopf et al., 1994; Lewinsohn et al., 1994; Schultz et al., 1994; Wan and Wilkins, 1994; Dong and Dunstan, 1996; Claros and Cánovas, 1998; Geuna et al., 1998). However, these methods have been developed for specific plant tissues, and are generally time consuming. Moreover, modern PCR techniques like RT-PCR, used for transcript quantitations, often cannot be successfully applied to such RNA preparations. Several commercially available RNA isolation kits frequently used in plant science (Lewis, 1997) gave only unsatisfactory results with all plant tissues listed in Table 1. The new, short and generally applicable protocol

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Table 1. RNA isolated from various plant species and different plant tissues.

Plant species/tissue	Addition to standard protocol	$A_{260/280}$
Crops and model plants		
Arabidopsis thaliana, leaf		2.0
Hordeum vulgare, leaf		2.0
Lycopersicon esculentum, leaf, fruit		2.0
Solanum tuberosum, leaf		2.0
Phaseolus vulgaris, leaf, root cotelydon	100 µl proteinase K (10 mg/ml)	2.0, 1.8, 1.6
Glycine max, cotelydon	100 µl proteinase K (10 mg/ml)	1.4
Nicotiana tabacum, leaf		2.0
Ornamentals (leaves)		
Tradescantia pallida		2.0
Coleus blumei		1.9
Euphorbia milii		2.0
Nerium oleander		2.0
Ficus benjamina	XAD2 (tip of a spatula)	1.2
Stapelia sp.		1.4
Ornamentals, weeds (flowers)		
Saintpaulia ionantha		1.4
Ranunculus repens		1.7
Syringa hybrids		1.7
Rosa hybrid		1.8
Pelargonium hybrids		1.6–1.8
Cyclamen persicum		1.8
Gerbera hybrids		1.8
Petunia hybrida		1.9
Veronica chamaedrys		1.9
Trees (leaves and other tissues)		
Tilia cordata, leaf, flower	200 µl KCl (1 M), 100 µl benzylchloride	1.1
Corylus avellana		1.9
Sorbus aucuparia		1.8
Malus floribunda		1.5–1.8
Quercus robur		1.9
Betula pendula	100 µl $CaCl_2$ (20%, w/v)	1.9
Acer platanoides	100 µl $CaCl_2$ (20%, w/v)	1.7
Fagus sylvatica		1.7
Picea abies, needle		1.7
Pinus sylvestris, needle, phloem, cone		1.5–2.0, 1.5, 1.8
Fungi (mycelium)		
Phanerochaete chryosporium		1.6
Lophodermium piceae		1.4
Leptographium wingfieldii		1.3–1.8

given here combines a cetyltrimethylammonium bromide (CTAB)/polyvinylpyrrolidone (PVP) method with the Nucleon PhytoPure system (Ernst et al., 1999). The method is appropriate for the simultaneous processing of many samples in Eppendorf tubes. The RNA was suitable for RT-PCR and northern blot analysis.

Materials and Methods

Plant materials

All plant species and plant tissues used are given in Table 1.

Reagents

Extraction buffer (pre-warmed at 65°C): 100 mM Tris-HCl (pH 8.0); 25 mM EDTA; 2 M NaCl; 2% CTAB (w/v); 2% PVP (w/v); 0.5% spermidine (w/v); 2% ß-mercaptoethanol (v/v)

Chloroform/isoamylalcohol (24:1; v/v)

Nucleon™ PhytoPure™ DNA extraction resin (Amersham Pharmacia Biotech, Freiburg, Germany)

Isopropanol

70% ethanol

TE: 10 mM Tris-HCl; 1 mM EDTA; pH 8.0

DNase buffer: 400 mM Tris-HCl; 60 mM $MgCl_2$; pH 7.5.

Protocol

Between 30-50 mg of liquid N_2 frozen fresh material, or lyophilized tissue was used for microextraction in 500 µl buffer. It is important to note, that the temperature of the extraction buffer must be 65°C. The procedure is as follows:

- Grind the lyophilized or fresh material with a mortar and pestle under liquid nitrogen.
- Put an exact amount of powdered tissue into a 2 mL Eppendorf tube, add 500 µL pre-warmed extraction buffer at 65°C.
- Add 250 µL chloroform/isoamylalcohol and 100 µL Nucleon PhytoPure DNA extraction resin and vortex (1,800 rpm) at room temperature for 10 min. Centrifuge for 5 min at 4°C and 13,000 rpm.
- Transfer the supernatant to a 2.0 mL Eppendorf tube, add 125 µL chloroform/isoamylalcohol, vortex at room temperature and centrifuge for 2 min at 4°C and 13,000 rpm. (This step increases the purity of the RNA sample considerably).
- Transfer the supernatant to a 2.0 mL Eppendorf tube and add 2 vol of cold isopropanol (4°C). Incubate for 5 min on ice and centrifuge for 5 min at 4°C and 13,000 rpm.
- Dissolve the pellet in 13 µL H_2O, add 2 µL DNase buffer and 5 µL (5 U) DNase I (Amersham Parmacia Biotech) and incubate for 15 min at 37 °C.
- Add 2 vol of cold isopropanol (4°C), mix and centrifuge for 2 min at 4°C and 13,000 rpm. Wash the pellet with 1 mL 70% ethanol (v/v) (-20 °C) and centrifuge again for 5 min at 4°C and 13,000 rpm.
- Aspirate the supernatant, air-dry the pellet for 20 min and dissolve it in 50 µL H_2O or TE buffer.

Quantitative RT-PCR

- To 1 µL oligo dT-primer (Life Technologies, Karlsruhe, Germany) add 5 µg of total RNA, bring to a final volume of 12 µL and incubate for 10 min at 70°C and then for 1 min on ice.
- Add 4 µL of 5 x first strand buffer, 1 µL dNTP mix (2.5 mM each) (AGS/Hybaid, Heidelberg, Germany), 2 µL DTT, 0.5 µL RNase inhibitor (Life Technologies) and 1 µL of SUPERSCRIPT II reverse transcriptase (Life Technologies). Incubate for 1 h at 42°C, then for 15 min at 70°C and finally transfer to ice.
- Add 1 µL RNase H (Amersham Pharmacia Biotech) and 5 µL RNase A (10 mg/mL) (Amersham Pharmacia Biotech) and incubate for 20 min at 37°C. Stop the reaction by transfer to ice or –20°C. (Addition of RNase A is important for degradation of total RNA).
- Quantitate the single stranded cDNA at a dilution of 1:100, using the RiboGreen™ RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). For standard curves and sample analysis, use 100 µL of the probe and 100 µL of 1:2000 diluted RiboGreen reagent according to the product information sheet.
- For PCR, use 10 ng of single stranded cDNA and follow the described methods. Adapt the temperature conditions for each primer. We used 30 cycles with a final extension step of 10 min at 72°C.
- Quantitate PCR products at a dilution between 1:100 and 1:500, using the PicoGreen™ dsDNA quantitation kit (Molecular Probes). For standard curves and sample we used 100 µL of probe and 100 µL of PicoGreen reagent (1:200 diluted) as recommended by the manufacturer. In addition, PCR products can be resolved in 1% agarose gels and visualized by ethidium bromide staining.

Results and Discussion

After isopropanol precipitation, a DNase treatment was performed in order to obtain DNA-free RNA. For some preparations, proteinase K, a polymeric adsorbent (Amberlite® XAD-2), benzylchloride or $CaCl_2$ was included in the extraction buffer (Table 1). The entire procedure, which is completed within 1 h resulted in RNA preparations suitable for RT-PCR. We isolated RNA from more than 30 herbaceous species and trees, using different tissues such as leaves, cotelydons, roots, needles, phloem, cones, fruits or flowers (Table 1), as well as fungi. Agarose-formaldehyde gel electrophoresis showed distinct ribosomal bands, indicating that no RNA degradation occurred (Figure 1). Between 30-50 mg tissue was sufficient to isolate enough RNA suited for RT-PCR, which was demonstrated using specific primers for actin or glycaldehyde-3-phosphate dehydrogenase (Figure 2). RNA yields depend on the species and tissue, and gave 24-300 µg per 100 mg FW with an average $A_{260/280}$ of 1.85 (Table 1). In single cases, e.g. lime, the absorption ratio $A_{260/280}$ was lower. However, this RNA preparation also was suitable for RT-PCR (Figure 2).

RNA quantitation by fluorescence-based RiboGreen reagent has been shown to be linear over the range of several orders of magnitude (Jones et al., 1998). In this protocol, we used RiboGreenRNA reagent for single-stranded cDNA

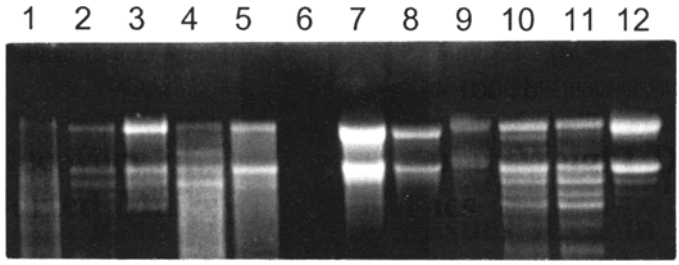

Figure 1. Ethidium bromide stained 1.5% agarose formaldehyde gel of total RNA (4 µg) from various plant species and tissues. *Tilia cordata*, leaf (1); *Quercus robur*, leaf (2); *Fagus sylvatica*, leaf (3); *Pinus sylvestris*, needle (4), phloem (5); *Leptographium wingfieldii*, mycel (6); *Syringa hybrid*, flower (7); *Ranunculus repens*, flower (8); *Lycopersicon esculentum*, fruit (9); *Nicotiana tabacum*, leaf (10); *Solanum tuberosum*, leaf (11); *Solanum tuberosum*, leaf, TRIzol™ -reagent (12).

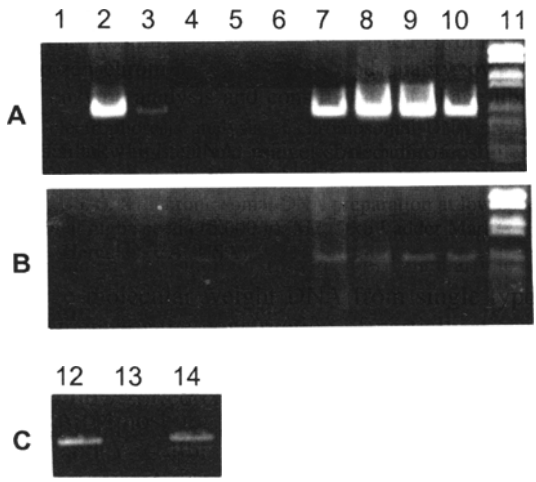

Figure 2. Agarose gel electrophoresis of RT-PCR products. A. Glycaldehyde-3-phosphate dehydrogenase; B. Actin: *Tilia cordata*, leaf (1); *Quercus robur*, leaf (2); *Fagus sylvatica*, leaf (3); *Pinus sylvestris*, needle (4), phloem (5); *Leptographium wingfieldii*, mycel (6); *Nicotiana tabacum*, leaf (7); *Solanum tuberosum*, leaf (8); *Syringa hybrid*, flower (9); *Ranunculus repens*, flower (10); DNA marker (11). C. Stilbene synthase: ozone-exposed *Pinus sylvestris*, needle (12), phloem (13); actin: *Leptographium wingfieldii* (14).

quantitation. Starting with an exact amount of single stranded cDNA, quantitative RT-PCR was carried out. After conventional first strand cDNA synthesis, residual RNA was degraded by RNase A and RNase H. In general, 5 µg of total RNA was used for first strand cDNA synthesis. This produced 80-260 ng of single stranded cDNA, depending on the material used. For quantitation of cDNA it is important to note that the relative effects of oligo(dT) on the RiboGreen assay (Jones et al., 1998) have to be considered. For quantitative RT-PCR we routinely used 10 ng of single stranded cDNA, running 30 cycles. This cycle number was reliable for measuring changes in gene expression (Halford et al., 1999). Quantitation of PCR products was carried out with different diluted end product samples, using the

PicoGreen dsDNA quantitation, which has been shown to be an effective fluorescence quantitation method for DNA (Ahn et al., 1996).

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