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Title: An improved method for high quality RNA isolation from needles of adult maritime pine trees

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Abstract

Conventional RNA isolation methods optimised for pine seedlings have been shown to produce poor quality RNA when applied to needles of adult pine trees. We describe here a modified procedure to isolate high pure RNA from needles of thirty-year-old maritime pines, exhibiting high levels of phenolics, polysaccharides and RNases. Major changes were the inclusion of proteinase K in the extraction medium followed by incubation at 42°C. Integrity and purity were evaluated by denaturing gel electrophoresis and by spectrophotometry (A_{260}/A_{230} and A_{260}/A_{280}). Total RNA could be successfully used for poly$(A)^+$-RNA isolation and cDNA library construction.

Key words: cDNA library construction, maritime pine needles, RNA isolation

Abbreviations: CTAB, hexadecyltrimethylammonium bromide; DEPC, diethyl pyrocarbonate; FW, fresh weight

Introduction

Isolation of intact and high-quality RNA from conifers, suitable for gene isolation by RT-PCR, cDNA library construction, and gene expression studies is difficult due to the high levels of phenolics, polysaccharides and endogenous RNases. Phenolics are readily oxidized to form covalently linked quinones and easily bind nucleic acids (Loomis, 1974). In addition to phenolics and other secondary metabolites, polysaccharide contamination hinders resuspension of precipitated RNA, interferes with absorbance-based quantification, and may inhibit enzymatic manipulations, poly(A)$^+$-RNA isolation and electrophoretic migration (Wilkins and Smart, 1996). Also, endogenous ribonucleases may drastically reduce the integrity of RNA, particularly when their amount is increased, such as during senescence, wounding and pathogen attack (Logemann et al., 1987; López-Gómes and Gómes-Lim, 1992; Green, 1994). In order to overcome these problems, specific protocols for the isolation of RNA from pine embryos and seedlings have been developed (Chang et al., 1993; Xu et al., 1997; Claros and Cánovas, 1998; Avila and Cánovas, 2000), but few methods for RNA isolation from adult pine needles are available. Here we describe a protocol that can produce high purity and high integrity RNA from needles of thirty-year-old maritime pine trees, suitable for reverse transcription and cDNA library construction.

Materials and Methods

Plant material

Maritime pine (*Pinus pinaster* Ait.*)* needles were harvested from native thirty-year-old trees, and from two-month-old seedlings, growing at 24°C under 16 h day. After harvesting, needles were immediately frozen in liquid nitrogen and stored at -80ºC until used.

Solutions and reagents

All the solutions were prepared with DEPC-treated and autoclaved MilliQ-water (Millipore).

- Extraction buffer: 100 mM Tris-HCl, pH 8.0; 2% (w/v) CTAB; 30 mM EDTA; 2 M NaCl; 0.05% (w/v) spermidine. Add 2% (w/v) PVPP (polyvinylpyrrolidinone; Sigma P-6755) and 3% (v/v) 2-mercaptoethanol prior to use.
- Chloroform:isoamyl alcohol (24:1, v/v)
- 10 M LiCl
- 2 M LiCl
- 10 mg/mL proteinase K
- DEPC-treated MilliQ-water

All non-disposable plastic material was treated with DEPC and autoclaved. Glass material, mortar and pestle were treated for 4 hours, at 180°C.

Protocol

RNA extraction

1. Grind the frozen material to a fine powder with a mortar and pestle under liquid nitrogen. Do no let the tissue thaw.
2. In a 50 mL Falcon tube, complete 15 mL of the extraction buffer by adding 2% (w/v) PVPP and 2% (v/v) 2-mercaptoethanol. Add proteinase K (10 mg/mL) to a final concentration of 2 mg/mL[1].
3. Incubate the extraction buffer for 10 min at 42ºC.
4. Quickly add 1.3 g of ground frozen tissue to the extraction buffer[2,3] and mix vigorously by vortexing.
5. Incubate for 90 min at 42ºC.
6. Extract by adding 1 vol of chloroform:isoamyl alcohol (24:1, v/v). Vortex and centrifuge at 15,000 *g* for 15 min at 4ºC to separate phases[4].
7. Transfer the top aqueous phase to another centrifuge tube, repeat the extraction with chloroform:isoamyl alcohol and centrifuge in same conditions.
8. Recover the top phase and add 1/4 vol of 10 M LiCl. Store at 4ºC, allowing overnight precipitation.
9. Centrifuge at 15,000 *g* for 25 min at 4ºC. Discard the supernatant.
10. Wash the pellet in 2 mL of 2 M LiCl and centrifuge at 15,000 *g* for 25 min at 4ºC.
11. Discard the supernatant and dissolve the pellet in 100-200 μL of DEPC-treated water[5].

12. Determine RNA concentration and purity at this stage. If RNA is contaminated by polysaccharides, incubate RNA sample at 65ºC for 10 min and immediately transfer to 4ºC for 10 min. Centrifuge at 18,000 *g* for 15 min at 4ºC and gently recover RNA solution that should be immediately stored at –80ºC.

Notes

1. Proteinase K incubation is mandatory for decreasing RNase activity during the extraction protocol.
2. The ratio of tissue/buffer used was optimised. The best results for adult pine needles were obtained for a relation of 1.3 g FW (ground tissue) to 15 mL (extraction buffer).
3. The protocol can be scaled-up by increasing proportionally the quantities used.
4. The centrifugation conditions given throughout the protocol should not be decreased. Use fixed-angle rotors.
5. There is no need for further RNA precipitation using alcohols.

Purification of poly(A)$^+$-RNA

Poly(A)$^+$-RNA was purified from total RNA using *Dynabeads® Oligo(dT)$_{25}$ Biomagnetic Separation System* (Dynal), according to manufacturer's instructions.

Construction of a cDNA library

Construction of a maritime pine needles cDNA library was performed according to the Instructions Manuals provided with *ZAP Express™ Synthesis Kit* (Stratagene) and *ZAP Express™ Gigapack® III Gold Cloning Kit* (Stratagene).

Results and Discussion

Standard methods for RNA isolation, including hot borate method (Wilkins and Smart, 1996) and CTAB/NaCl method (Chang et al., 1993), have been applied for the isolation of RNA from needles harvested from thirty-year-old *Pinus pinaster*. Both protocols failed to yield high quality RNA for downstream enzymatic procedures, such as RT-PCR or cDNA synthesis, mainly due to contamination by polysaccharides and degradation of RNA. A new method for isolation of RNA from adult maritime pine needles was devised using high concentration of proteinase K combined with prolonged 42°C incubation for enzymatic digestion of ribonucleases. The inclusion of proteinase K is critical for obtaining intact RNA (Figure 1). When proteinase K was not added to the extraction buffer, the integrity of isolated RNA was extremely low. Even in the presence of proteinase K, the integrity of RNA highly depends on the ratio of the amount of plant material (FW) to the volume of extraction buffer, being significantly reduced as the amount of starting material increases (Figure 1; lanes 2-5). In addition to high

integrity, RNA prepared by this method also revealed high purity, as judged by A_{260}/A_{230} and A_{260}/A_{280} ratios that indicate low polysaccharide and reduced protein contamination, respectively (Table 1). To verify the applicability of this method, two-month-old seedlings were used and high quality RNA was also obtained (Figure 1; lane 6). The RNA yield of 143 µg/g FW obtained using this protocol for adult *P. pinaster* needles is lower than that obtained for seedlings (Table1), but is higher than that referred by Chang et al. (1993) for five-year-old loblolly pine needles.

In order to evaluate the suitability of isolated RNA in downstream enzymatic procedures, RNA prepared from adult pine needles was used for cDNA library preparation. Poly$(A)^+$-RNA was isolated from RNA by biomagnetic separation using beads coupled to oligo dT, with a yield of 34 µg mRNA/mg total RNA. After synthesis of first- and second-cDNA strands, the bulk of radioactive cDNA fragments lied between 1 kb and 3 kb, which is an indication of effective cDNA synthesis with high-quality mRNA used as starting material. After size fractionation on a Sepharose® CL-2B gel filtration column (Figure 2), the longer cDNA fragments were used for cloning in ZAP Express™ vector (Stratagene). An efficiency of 3.5×10^5 pfu/µg of packaged cDNA library was obtained, from which about 96% were recombinant clones as determined by colour selection using IPTG and X-gal. As a result, after cDNA library preparation, a total number of 1.6×10^6 recombinant clones were obtained, which can be considered as a good representational primary library size. As the high quality of a cDNA library depends not only on the number of clones but also on the size of

their inserts, the cloning efficiency of long cDNAs was evaluated by determining the insert size from 33 randomly chosen. The analysed recombinant clones had inserts with an average size of about 2 kb and a modal size between 1 kb and 1.5 kb (Figure 3). This indicates that the cDNA library prepared from the RNA isolated using the described protocol fulfils two key characteristics of a high quality cDNA library: (i) is large enough to contain representatives of all genes being expressed and (ii) has a reduced number of clones containing small cDNA inserts (less that 10% of random clones contained cDNA inserts < 1 kb), increasing the probability of finding full-length cDNAs.

The method described is an efficient, simple and reproducible procedure for the isolation of RNA from needles of adult maritime pine trees, which could serve as a robust template for reverse transcription as indicated by the construction of a good-quality cDNA library. Using high concentration of proteinase K combined with prolonged 42°C incubation allows enzymatic digestion of ribonucleases, avoiding the need for phenol extraction to denature and partition proteins and thereby inhibiting RNases. As phenol extraction can damage poly(A)$^+$-RNA, the devised method becomes more suitable when isolated RNA is to be used for RT-PCR or for synthesis of cDNA libraries.

Table 1. Purity and yield of two isolation methods used for RNA isolation from *Pinus pinaster* needles.

Method	Plant material	A_{260}/A_{230}	A_{260}/A_{280}	µgRNA/g FW
Chang et al. (1993)	Neddles from adult trees	1.73±0.06	1.94±0.05	113±12
In this work	Neddles from adult trees	2.12±0.09	2.07±0.14	143±20
In this work	Neddles from seedlings	2.14±0.03	1.92±0.10	340±17

Legends to figures

Figure 1 – Electrophoretic analysis of *Pinus pinaster* RNA isolated using different extraction procedures. Total RNA preparations (5 µg) were analysed on 0.8% formaldehyde-denaturing agarose gel stained with ethidium bromide. RNA from needles of thirty-year-old pine trees was isolated using the described protocol without the addition of proteinase K (1), or in the presence of proteinase K at ratio of FW to extraction buffer of 1.3 g/15 ml (2); 2.0 g/15 ml (3); 2.6 g/15 ml (4) and 3.3 g/15 ml (5). RNA from seedlings was isolated in the presence of proteinase K at ratio of FW to extraction buffer of 1.3 g/15 ml (6).

Figure 2 – Electrophoretic analysis of *Pinus pinaster* size-fractionated cDNA fragments after Sepharose CL-2B gel filtration. Aliquots (8µl) of radioactive fractions were separated on a 5% nondenaturing polyacrylamide gel and subjected to autoradiography. Molecular markers: 1kb DNA ladder.

Figure 3 – Insert size frequency of *Pinus pinaster* cDNA fragments cloned in ZAP Express™ vector (Stratagene). Thirty three cDNA clones were randomly chosen and the corresponding inserts were analysed upon *in vivo* excision, digestion with *Eco*R I/*Xho* I and agarose electrophoresis.

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characterization of differentially expressed genes from loblolly pine embryos. Plant Mol Biol Reptr 15: 377-391.

Figures

Figure 1

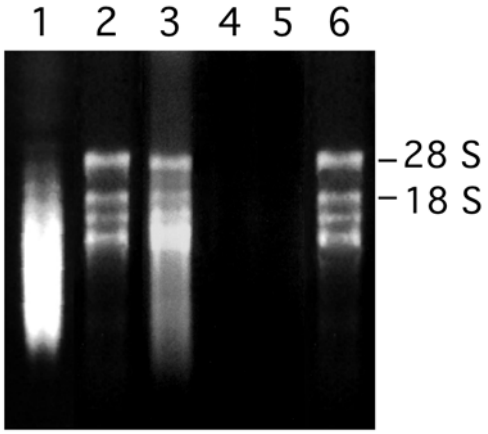


Figure 2

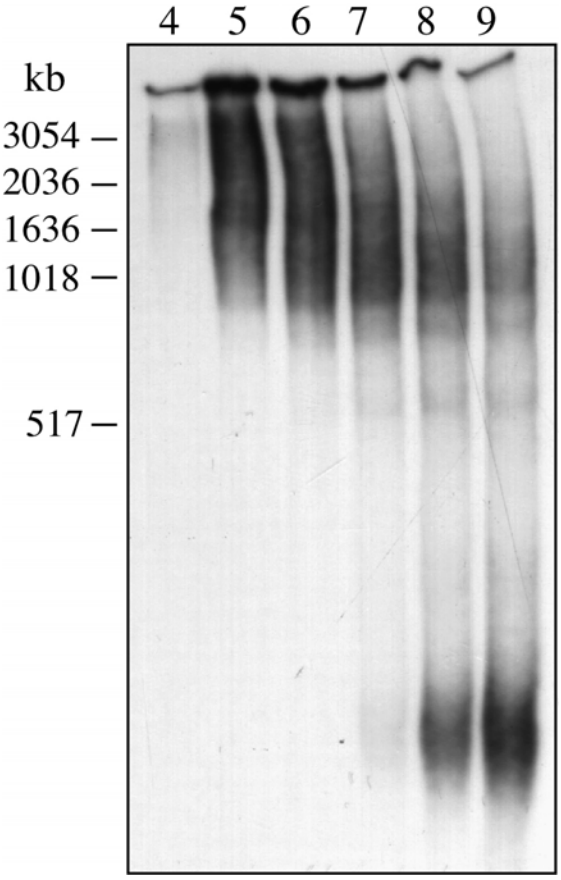
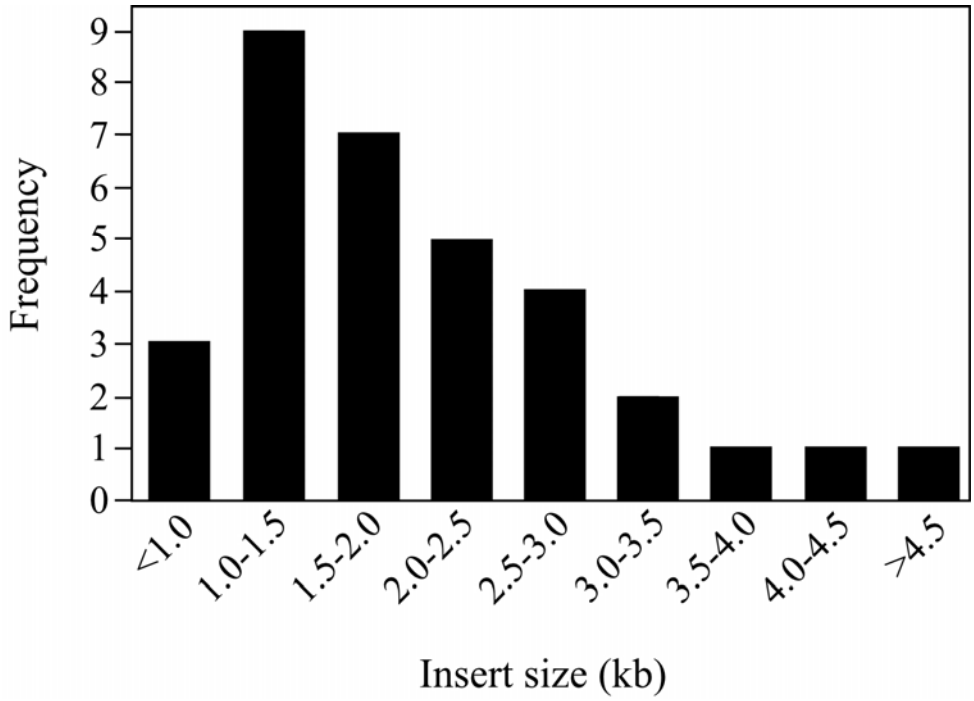


Figure 3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Insert size (kb)