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Isolation of high-quality RNA from gymnosperm and angiosperm trees

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*An improved protocol was developed for efficient and reliable extraction of high-quality total RNA and mRNA from various tissues of spruce (*Picea *spp.) and poplar (*Populus *spp.) trees, as well as other plant species. This method was specifically optimized for tissues with high content of polysaccharides, oleoresin terpenoids, and phenolic secondary metabolites, which often co-precipitate with RNA and inhibit subsequent reverse transcription. The improved protocol yielded up to 600 µg of total RNA per gram of tissue suitable for standard expressed sequence tags (ESTs), full-length cDNA library construction, and for microarray applications.*

INTRODUCTION

Development of large-scale expressed sequence tags (ESTs) and full-length cDNA resources as well as microarray RNA expression profiling with woody plants require efficient methods for isolation of high-quality RNA from a diverse array of tissues that vary widely in contents of polysaccharides and secondary metabolites. However, working with trees, cDNA library construction and RNA microarray analysis are often difficult due to RNA degradation and contamination of RNA with polysaccharides or secondary metabolites (e.g., polyphenolics and oleoresin terpenoids) that may inhibit reverse transcription. Ideally, methods for comparative tree genome studies should be efficient with a range of different species including angiosperms and coniferous gymnosperms. Existing protocols did not fulfill our demands of working with a range of tissues from both conifers and poplars. We developed an efficient protocol for RNA isolation based on two existing protocols reported by Chang et al. (1) and Wang et al. (2). Combination and modifications of these protocols allowed us to obtain high yields of intact RNA from various tissues of poplar and spruce using one standard protocol. The method reported here is technically straightforward, requires minimum prior training in handling of plant RNA, and uses little glassware, which makes it a method of choice for high-throughput tree genome projects. RNA quality was confirmed by different methods (e.g., UV absorbance scans, gel electrophoresis, and Northern blot analysis), with reverse transcription being the most important quality control for RNA used for successful construction of full-length cDNA libraries and microarray hybridizations. The protocol described here consistently produces high yields and quality of RNA from different tissues of different tree species, which makes it particularly suitable for comparative plant genome research.

MATERIALS AND METHODS

Plant Materials

Sitka spruce (*Picea sitchensis*) and hybrid spruce (*Picea glauca* × *Picea engelmannii*) trees were provided by CellFor (Vancouver, Canada). White spruce (*P. glauca*) trees were provided by the BC Ministry of Forests (Victoria, Canada). Poplar (*Populus trichocarpa* and *P. trichocarpa* × *Populus deltoides*) tissues were collected at the University of British Columbia.

Isolation of Total RNA and Purification of Poly(A) RNA

All solutions except Tris buffer were treated with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. Autoclaved

0.1% DEPC-treated water was used for preparation of Tris buffers. Glassware was baked for at least 4 h at 180°C. Extraction buffer [200 mM Tris-HCl, pH 8.5, 1.5% lithium dodecylsulfate, 300 mM LiCl, 10 mM disodium salt EDTA, 1% (w/v) sodium deoxycholate, 1% (w/v) Tergitol Nonidet® P-40 (NP40)] was autoclaved and, just before use, 5 mM thiourea, 1 mM aurintricarboxylic acid, 10 mM dithiothreitol, and 2% (w/v) polyvinylpolypyrrolidone (PVPP) were added. Plant tissue (5 g) was ground to a very fine powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a 50-mL polypropylene Falcon® tube. Twenty milliliters extraction buffer per gram tissue was added and vigorously shaken. The suspension was then frozen at -80°C for 1 h (alternatively, extracts may be snap-frozen in liquid nitrogen). After thawing, the extracts were centrifuged at 3000× *g* for 20 min at 4°C. If tissue particles did not precipitate, the supernatant was filtered through one layer of Kimwipe® tissue paper, using a funnel, into a fresh 50-mL polypropylene tube and kept on ice. One-thirtieth volume of 3.3 M sodium acetate (pH 6.1) and 0.1 volume 100% ethanol were added, and the mixture was chilled on ice for 10 min to precipitate polysaccharides. Polysaccharides were pelleted by centrifugation at 3000× *g* for 30 min at 4°C. Polysaccharide precipitation was very effective for poplar tissues, but was omitted for spruce tissues due to their relatively small polysaccharide content. In order to precipitate nucleic acids, one-nineth volume of 3.3 M sodium acetate and 0.6 volume of ice-cold isopropanol were added to the supernatant, and the solution was left at -20°C for 2 h or at -80°C for 30 min. Nucleic acid pellets were collected by centrifugation for 45 min at 3000× *g* at 4°C. The supernatant was removed, and the pellet was resuspended in 8 mL of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 8 mL 5 M NaCl and kept on ice for 30 min with periodic vortex mixing. Samples were mixed with 4 mL of 10% cetyltrimethylammonium bromide (CTAB) at room temperature, vortex mixed, and incubated for 5 min at 65°C to remove residual polysaccharides. Mixtures were extracted twice with an equal volume of chloroform/isoamylalcohol (24:1, v/v). One-fourth volume of 10 M LiCl was added to the supernatant, mixed, and kept at 4°C overnight. At this stage, samples from tissues rich in polysaccharides (e.g., poplar leaves and bark or spruce xylem) should not be cooled down below 4°C to avoid residual polysaccharides co-precipitating with RNA. Overnight precipitation substantially increased the RNA yield. For tissues with small amounts of polysaccharides (e.g., spruce needles), LiCl precipitation can be performed for 2 h at -20°C. RNA was pelleted by centrifugation at 3000× *g* for 30 min at 4°C. The supernatant was poured off, and the residual liquid was carefully removed with a pipet. The RNA pellet was dissolved in 2 mL TE buffer on ice, which took up to 1 h. Samples were transferred to 2-mL microcentrifuge tubes, and 0.9 volume of chilled isopropanol and 0.1 volume of 3.3 M sodium acetate were added, followed by precipitation at -20°C for 1 h or at -80°C for 30 min. RNA pellets were collected by centrifugation in a microcentrifuge at 14,000× *g* at 4°C for 30 min, washed with 1 mL 70% ethanol, and collected by centrifugation at 14,000× *g* at 4°C for 10 min. Pellets were dried for 10 min at room temperature, and RNA was resuspended in 0.5–1 mL autoclaved DEPC-treated water on ice. For the first quality check, RNA absorption was scanned with a spectrophotometer between 200 to 300 nm, and 2 µg total RNA was separated by gel electrophoresis using a nondenaturing 1% agarose gel. Poly(A) RNA was purified from total RNA using the Poly(A) Pure™ kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol.

Evaluation of RNA Quality by Reverse Transcription

cDNA synthesis was performed using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Invitrogen Canada, Burlington, ON, Canada) according to the manufacturer's protocol with minor modifications as follows. Five micrograms total RNA or 200 ng mRNA were used in each reaction. [^{32}P]dGTP (Amersham Biosciences, Piscataway, NJ, USA) was used as tracer to monitor cDNA synthesis. After cDNA synthesis, reactions were purified from unincorporated nucleotides using a MicroSpin™ S300 column (Amersham Biosciences), and cDNA was separated on a denaturing 1% alkaline agarose gel. The gel was dried, exposed on a Phosphorimager Screen (Amersham Biosciences), and scanned on a Storm™ 860 phosphorimage scanner (Amersham Biosciences).

RESULTS AND DISCUSSION

The main objective of this study was to develop one method that works reliably for RNA isolation from different woody plants, including angiosperms and gymnosperms, for subsequent application in tree genomics (e.g., reverse transcription of RNA for EST and full-length cDNA library construction and for microarray RNA expression profiling). The protocol described here was specifically optimized for use in comparative tree genome research, which requires large numbers of isolations of consistently high-quality RNA from diverse species and tissues.

Several existing methods deal with different problems of RNA isolation from conifers. A widely used method described by Chang et al. (1), as well as a number of recent protocols (3,4), use preheated CTAB and extraction buffers with high concentrations of NaCl to remove high levels of polysaccharides and proteins during RNA isolation. The method described in Reference 1 was developed for RNA isolation from conifers, and in our hands, it yielded RNA of good quality when applied to conifer tissues. However, other reports (5,6) and our test of this method with poplar tissues revealed some limitations of the method. Lewinsohn et al. (5) could not obtain intact RNA from conifers using this protocol, and Wu et al. (6) describe similar problems when isolating RNA from cotton. When we extracted RNA from poplar leaves using the protocol described in Reference 1, the RNA quality was inconsistent. In several instances, use of the method reported in Reference 1 produced largely degraded RNA, probably due to the presence of active RNases during incubation at 65°C right after tissue homogenization.

Methods described by Lewinsohn

Table 1. Yields of Total RNA Isolated from Different Tissues of Spruce and Poplar Trees Using Different RNA Isolation Methods

Species	Tissue	RNA Yield[a] (µg/g tissue)	RNA Yield[b] (µg/g tissue)	Absorbance Ratio (A_{260}/A_{280})	Absorbance Ratio (A_{260}/A_{230})
Spruce	Needles	50–100	200–450	2.2–2.3	2.3–2.8
	Bark	100–400	150–450	2.2–2.4	2.5–3.6
	Xylem	50–200[c]	200–350	2.4	2.5–2.6
Poplar	Leaves	100–600[c]	200–600	2.3	2.4–2.5
	Bark	30–80[c]	50–100	2.2	2.2–2.3
	Xylem	100–200	200–300	2.3	2.3–2.4

Absorbance at 230, 260, and 280 nm was determined for RNA samples isolated using the method described in this paper.
[a]RNA yield obtained following the protocol by Wang et al. (2).
[b]RNA yield obtained following the protocol described in this paper.
[c]RNA samples were often heavily contaminated with gelatinous polysaccharides and were unsuitable for quality cDNA synthesis.

et al. (5) and Wang et al. (2) efficiently remove excessive phenolics with PVPP in the extraction buffer. However, in our experiments, the protocol described in Reference 2 yielded relatively low amounts of RNA from spruce needles and spruce xylem (Table 1), and RNA from spruce xylem and all poplar tissues tested sometimes contained large amounts of gelatinous polysaccharides. Polysaccharides co-precipitate with RNA (as mentioned in Reference 5) and do not separate from RNA in later steps of mRNA isolation. The resulting mRNA was not suitable on a reliable basis for reverse transcription reactions and subsequent cDNA library construction. Another RNA isolation method, described by Mattheus et al. (7), requires time-consuming cesium chloride ultracentrifugation and was therefore not taken into consideration for application for large sample numbers typical of genomics research. In conclusion, none of the existing methods tested in our initial trials fulfilled the requirements for a protocol that can be widely used in tree genomics research on a large sample number with both gymnosperms and angiosperms.

By combining key features of the methods described by Chang et al. (1) and Wang et al. (2), we developed an improved RNA isolation protocol that was suitable for RNA isolation from conifer and poplar tissues rich in oleoresins, phenolics, or polysaccharides. Using this method, we consistently obtained high yields of intact RNA (Table 1). We adopted the extraction buffer described by Wang et al. (2) because it inactivates polyphenolics, includes RNase inhibitors, and does not require incubation of tissue extracts at elevated temperatures prior to removal of RNases. After removal of RNases, we introduced a polysaccharide purification step using the CTAB method described in Reference 1. Incubation at elevated temperatures was kept to a minimum.

We evaluated the new protocol for quantity and quality of RNA yields from

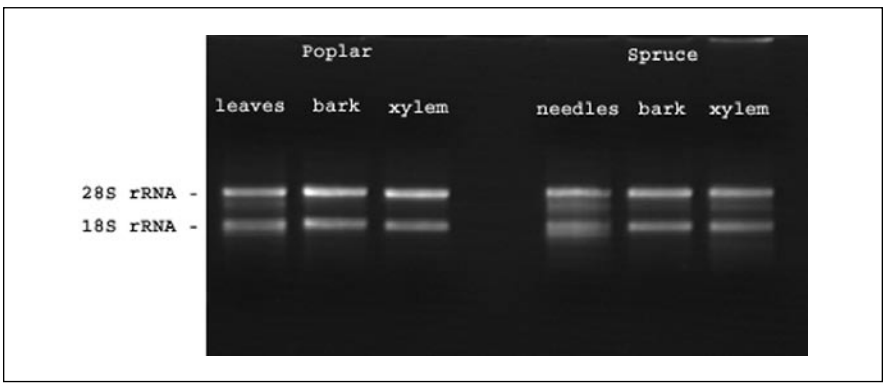

Figure 1. Agarose gel electrophoresis of total RNA. RNA was isolated with the protocol described here from poplar leaves, poplar bark, poplar xylem, spruce needles, spruce bark, and spruce xylem.

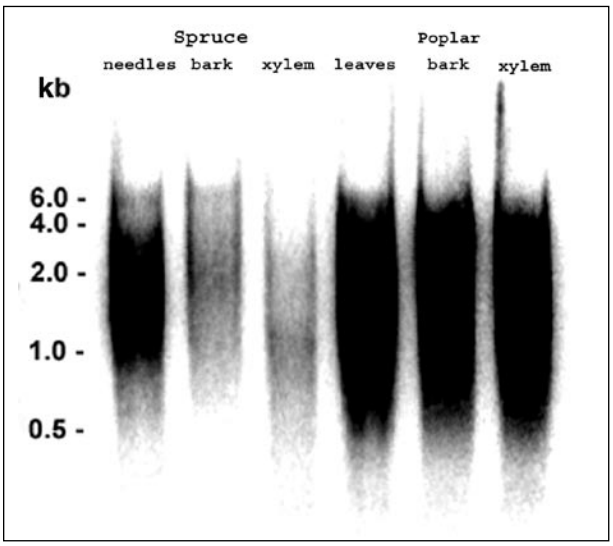

Figure 2. Autoradiogram of labeled cDNA obtained from total RNA by reverse transcription using an oligo(dT) primer. RNA was isolated from spruce needles, spruce bark, spruce xylem, poplar leaves, poplar bark, and poplar xylem.

a suite of different tissues from spruce (needles, bark, and xylem) and poplar (leaves, bark, and xylem) (Table1 and Figure 1). RNA from all samples showed highly intact ribosomal RNA bands detected after agarose gel electrophoresis (Figure 1). For all tissues tested, yields were as high or higher when compared with the method reported in Reference 2 (Table 1). RNA purity was evaluated by the ratios of absorbance at 260:230 nm and 260:280 nm (Table 1). We commonly obtained ratios above 2, indicating that RNA samples were free of polysaccharides, polyphenols, or proteins. RNA integrity and purity was further confirmed by cDNA synthesis from total RNA (Figure 2) and mRNA (Figure 3). Effective cDNA synthesis is a suitable indicator for high-quality RNA, because reverse transcription is sensitive to impurities and therefore is a relevant test of RNA quality for applications in genomics research. Reverse transcription reactions were performed with total RNA or mRNA using an oligo(dT) primer and radiolabeled dNTPs. The quality of first strand cDNA was analyzed by denaturing gel electrophoresis. The typical size of cDNA products ranged from 0.5 to 5 kb (Figure 2). When used for full-length library construction (8), mRNA isolated with our protocol consistently produced cDNA ranging in size from 0.5 to 6 kb and higher (Figure 3), which is indicative of a very high quality RNA substrate. Several cDNA libraries from different spruce and poplar tissues were successfully constructed using RNA isolated with this protocol. RNA was also used in gene expression analysis by Northern blot analysis (data not shown). Hybridization signals were distinct without smear from degraded RNA. The method described here was also successfully used for high-quality RNA isolation from other plant tissues, such as grapevine berries and cycas roots (data not shown). In summary, we describe an efficient and robust procedure for the isolation of high-quality RNA from different tissues of gymnosperm and angiosperm trees and other plants, which makes it suitable as a reliable procedure in plant genome research. The protocol is particularly useful for RNA isolation from tissues rich in secondary metabolites and polysaccharides.

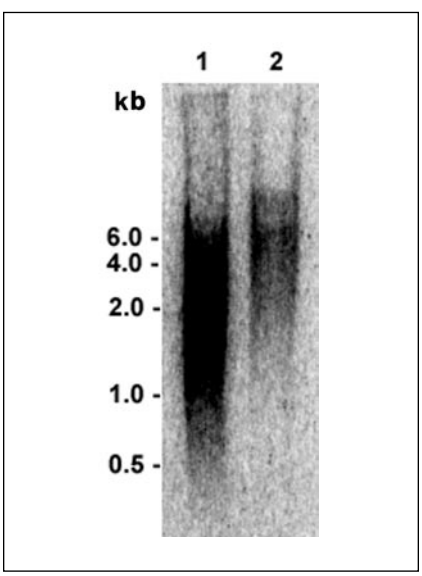

Figure 3. Autoradiogram of cDNA synthesis from poplar leaf mRNA. Synthesis was performed using a full-length cDNA library construction method described in Carninci et al. (8). Lane 1, first strand synthesis; lane 2, second strand synthesis.

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