

# Short Technical Reports

## Isolation and Purification of Functional Total RNA from Woody Branches and Needles of Sitka and White Spruce

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### ABSTRACT

The isolation of intact, functional RNA from conifer spp. is not easy, especially from those tissues that are heavily lignified and characterized by a low number of living cells. An efficient procedure for isolating RNA from combined wood and bark tissues of conifers was developed based on a protocol optimized for the extraction of RNA from pollen and one for the isolation of RNA from woody stems. This protocol does not involve the use of phenol, and no ultracentrifugation was required. In addition, the protocol overcame the problems of RNA degradation and low yield due to oxidation by polyphenolics and co-precipitation with polysaccharides, both of which are abundant components in conifer bark tissues. The isolated RNA was of high quality and undegraded as gauged by spectrophotometric readings and electrophoresis in denaturing agarose gels. Quality was further assessed through the subsequent use of the RNA in reverse transcription and RT-PCR, indicating that it could be used for a number of downstream purposes including Northern blot hybridization and cDNA library construction. Using this modified protocol, 80–150  $\mu\text{g}$  of RNA was routinely obtained from 1 g of fresh material. This protocol was also used for the isolation of RNA from needles of spruce spp., from which 750–950  $\mu\text{g}$  RNA per gram of starting material could routinely be obtained.

### INTRODUCTION

One of the challenges associated with conducting research of a molecular nature in conifer spp. is the isolation of intact nucleic acids, especially RNA. This is particularly troublesome for bark and woody tissues, due not only to the low number of living cells in these

tissues but also to their lignified nature, which makes disruption of the cells difficult. In addition, there are other problems associated with the fact that conifer cells contain high concentrations of polyphenolics and polysaccharides (1,9) that may co-precipitate with DNA and RNA upon isolation (5,6). These tissues also contain other unidentified compounds that affect the isolation of functional RNA for downstream applications, such as RT-PCR or cDNA library construction (1).

In this report, we describe a successful and reliable procedure for the isolation of RNA from combined bark and wood tissues of Sitka (*Picea sitchensis* Bong. Carr.) and white spruce (*P. glauca* Moench Voss.). The protocol, which does not use phenol or require ultracentrifugation, is primarily a modification of one that was optimized for the isolation of RNA from cotton leaves and pollen (4). The modifications directly overcome the problems associated with polyphenol and polysaccharide contamination and were derived from a protocol optimized for the isolation of RNA from woody conifer stems (8). The quality of the isolated RNA was consistently high as judged by spectrophotometric readings and its separation in denaturing agarose gels. The yields were reasonable with respect to their use for RT-PCR, the preparation of poly (A)+ RNA and Northern blot hybridization. We have used this protocol routinely for the isolation of functional RNA from both combined wood and bark tissues, as well as mature needles.

### MATERIALS AND METHODS

#### Plant Materials

One-year-old lateral branches were collected from six-year-old Sitka spruce grown at the University of British Columbia research forest, Maple Ridge, BC and from sixteen-year-old white spruce grown at Clearwater, BC, Canada.

#### Preparation of Buffers and Chemical Solutions

All solutions were treated with 0.1% (vol/vol) diethylpyrocarbonate (DEPC)

with the exception of Tris buffers that were made with DEPC-treated water. All glassware and plasticware were soaked in 0.1% (vol/vol) solutions of DEPC for 2 h, then autoclaved before use. The homogenization buffer contained: 200 mmol/L Tris-HCl, pH 8.5, 1.5% (wt/vol) lithium dodecylsulfate, 300 mmol/L LiCl, 10 mmol/L Na<sub>2</sub>EDTA, 1% (wt/vol) sodium deoxycholate and 1% (vol/vol) NP-40. Following autoclaving, the following chemicals were added to the homogenization buffer immediately before use: 1 mmol/L aurointricarboxylic acid, 10 mmol/L dithiothreitol, 5 mmol/L thiourea and 2% (wt/vol) polyvinylpyrrolidone (PVPP).

#### Isolation of Total RNA

Strips (2–3 cm long) of bark and wood tissues were peeled from lateral branches with a razor blade. Approximately 5 g bark/wood tissue were used per sample. For mature needles, 3 g of leaf material were used. Tissue was frozen in liquid nitrogen, transferred quickly into a kitchen coffee grinder containing a small amount of sea sand (DEPC-treated) and ground to a fine powder. The resulting powder was transferred to a chilled mortar and extracted with 5 volumes homogenization buffer on ice. The homogenate was transferred to a plastic weighing tray, allowed to freeze slowly at -80°C for at least 2 h and then placed in a 37°C water bath until just thawed. The homogenate was then transferred immediately to centrifuge tubes and centrifuged at 5000 $\times$  g for 20 min at 4°C.

The supernatant was filtered through one layer of Kimwipe<sup>®</sup> tissues, and the pellet was discarded. The supernatant was mixed with 1/30 volume 3.3 mol/L sodium acetate, and ethanol was added to a final concentration of 10% vol/vol. The mixture was incubated on ice for 10 min, followed by centrifugation at 5000 $\times$  g for 20 min at 4°C.

The supernatant was collected, to which was added 1/9 volume 3.3 mol/L sodium acetate and isopropanol, to a final concentration of 33% vol/vol. The resulting mixture was placed at -20°C for 2 h. The mixture was then centrifuged at 5000 $\times$  g for 30 min at 4°C, and the resulting pellet was resuspend-

# Short Technical Reports

ed in 3 mL TE buffer (10 mmol/L Tris-HCl, pH 7.5, and 1 mmol/L EDTA) and incubated on ice for 30 min followed by centrifugation as before. The supernatant was retained, mixed with 1/4 volume 10 mol/L LiCl and incubated on ice overnight. The following day the sample was centrifuged at 10 000× *g* for 30 min at 4°C. The pellet was resuspended in 1.5 mL TE buffer, following which 1.5 volume 5 mol/L potassium acetate (pH not adjusted) were added and the mixture incubated for 3 h on ice. The mixture was centrifuged at 10 000× *g* for 30 min at 4°C, and the pellet obtained was resuspended in 1 mL TE buffer, incubated on ice for 1 h, then it was centrifuged as before to remove any undissolved material.

The clear supernatant was transferred to a clean centrifuge tube (if the supernatant was not clear, a second centrifugation was performed) and mixed with 1/9 vol 3.3 mol/L sodium acetate and 2 volumes ethanol before being placed at -20°C for a minimum of 2 h. The RNA pellet was then obtained following centrifugation at 10 000× *g* for 30 min at 4°C and was washed with 500 µL absolute ethanol followed by centrifugation at 10 000× *g* for 10 min. The pellet was air-dried and resuspended in DEPC-treated water.

The smallest amount of tissue that could be ground efficiently by the coffee mill was approximately 4 g. The optimum ratio of bark/wood tissue to extraction buffer was 1:5.

## Purification of poly(A)+ RNA

Poly (A)+ RNA was purified from total RNA using a Dynabead kit® (DynaL, Lake Success, NY, USA) following the manufacturer's instructions.

## Electrophoresis of RNA

Ten micrograms of RNA were electrophoresed in a denaturing formaldehyde 1% agarose gel according to the protocol described in Lehrach et al. (7).

## Synthesis and Labeling of cDNA

Reverse transcription reactions and labeling of cDNA were carried out with 5 µg total or 0.6 µg poly (A)+ RNA. RNA was combined with 4.5 µmol/L

oligo(dT)<sub>16</sub> primer, 10 mmol/L DTT, 500 µmol/L dNTPs, 0.37 MBq <sup>32</sup>P-dATP (1.1 × 10<sup>14</sup> Bq/mmol; Amersham Canada Ltd., Oakville, ON, Canada), and 10 U M-MLV reverse transcriptase supplied with its own buffer (Life Technologies, Burlington, ON, Canada). Reverse transcription in a total volume of 6 µL was conducted at 37°C for 1 h, following which the reaction was stopped by the addition of 19 µL stop buffer containing 10 mmol/L EDTA and 10 mmol/L Tris-HCl, pH 8.0. Unincorporated label was removed on a Microspin™ S-300 gel filtration column (Amersham Pharmacia Biotech, Baie d'Urfe, QC, Canada). Labeled cDNA was separated in a 1% agarose neutral gel, after which the gel was dried and exposed to Kodak X-Omat™ blue XB-1 film (NEN Life Science products, Boston, MA, USA) for 6 h.

## RT-PCR

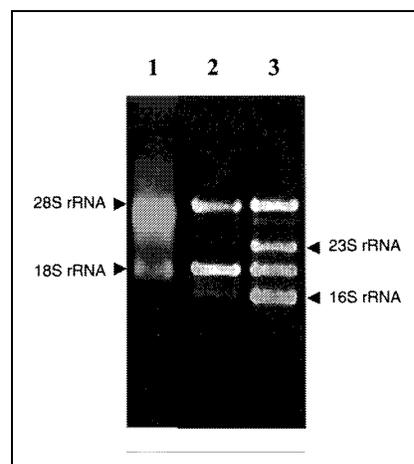
Single-stranded cDNA was synthesized from 100–300 µg total RNA using M-MLV reverse transcriptase supplied with its own buffer. The RNA template was then removed by alkaline hydrolysis. The cDNA volume was adjusted to 80 µL and 180 µL salt solution (50 mmol/L NaCl, 25 mmol/L EDTA), and 63 µL 2 mol/L NaOH were added followed by incubation at 50°C for 30 min. After incubation, 63 µL each of 1 mol/L HCl and 1 mol/L Tris-HCl, pH 8.0, were added, followed by the addition of an equal volume 4 mol/L NH<sub>4</sub>OAc and 1.6 mL ethanol and incubation at -20°C overnight. The cDNA was recovered by centrifuging at 12 000× *g* for 20 min. The pellet was washed with salted ethanol (50 µL 2 mol/L NH<sub>4</sub>OAc plus 100 µL 70% ethanol) followed by a wash with 70% ethanol and finally with 100% ethanol.

The cDNA pellet was air-dried and resuspended in 100 µL distilled water. The cDNA was used for PCR amplification as follows: a typical PCR (50 µL) contained 150 ng cDNA template, 0.1 mmol/L dNTP, 0.5 µmol/L of the degenerate forward primer Mult-F2 (GCI C/TTI GAC/T TAC/T GTI TAC/T), 0.5 µmol/L of either of the degenerate reverse primers Mult-R4 (ACA/G TTA/G TAI CCA/G TGI A) or Mult R6 (CCI G/CA/TT/C TCT/C TTC

CAC CA), 3 mmol/L MgCl<sub>2</sub>, and 2.5 U *Taq* DNA polymerase (Life Technologies) supplied with its own buffer. The PCR mixture was initially denatured at 94°C for 4 min and then subjected to 45 cycles at the following conditions: 94°C for 1 min, 45°C for 1 min, 72°C for 2 min with a final extension at 72°C for 10 min.

## RESULTS AND DISCUSSION

Conifer bark and woody tissues are highly lignified and characterized by a low number of living cells, which together make the isolation of high quality RNA a challenge. Several protocols exist for isolating RNA from conifers, including those optimized for needles (3,10) and those used for seedlings (1) or roots (3). The protocol outlined by Chang et al. (3) was attempted for combined wood/bark of spruce but resulted in an unacceptably low yield (<10 µg/g FW). In addition, one other method optimized for the isolation of functional RNA from woody gymnosperm stems was used (8). However, in our hands, it too resulted in a low yield of RNA. The isolation of RNA from woody tissues was also attempted using several commercially available kits (Tri Reagent®, Molecular Research Center, Cincinnati, OH, USA; FastRNA™, Bio101, Vista, CA, USA; RNeasy®, Qiagen, Mississauga, ON, Canada), all of which were



**Figure 1. Denaturing agarose gel electrophoresis of total RNA.** RNA was isolated by the unmodified protocol of Hughes and Galau (3) from bark/wood (lane 1), and by the modified protocol from bark/wood (lane 2) and mature needles (lane 3).

**Table 1. Yield and Absorbance Ratio of Total RNA Isolated from Bark/Wood and Needle Tissues of Sitka Spruce and White Spruce**

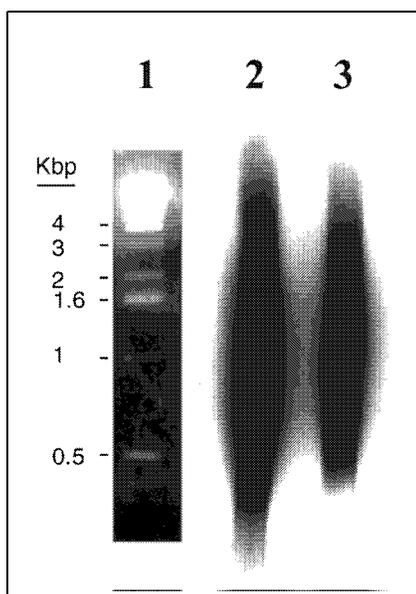
Method	Species	Tissues	Absorbance Ratio (A <sub>260</sub> /A <sub>280</sub> )	Yield (µg RNA/g tissue)
Original protocol <sup>a</sup>	Sitka spruce	Bark/wood	1.81	33
Modified protocol <sup>b</sup>	Sitka spruce	Bark/wood	1.85	83–150
Modified protocol <sup>b</sup>	Sitka spruce	Needles	1.92	750–950
Modified protocol <sup>b</sup>	White spruce	Bark/wood	1.83	95–128

<sup>a</sup>Protocol described by Hughes and Galau (3).  
<sup>b</sup>Protocol described in this report.

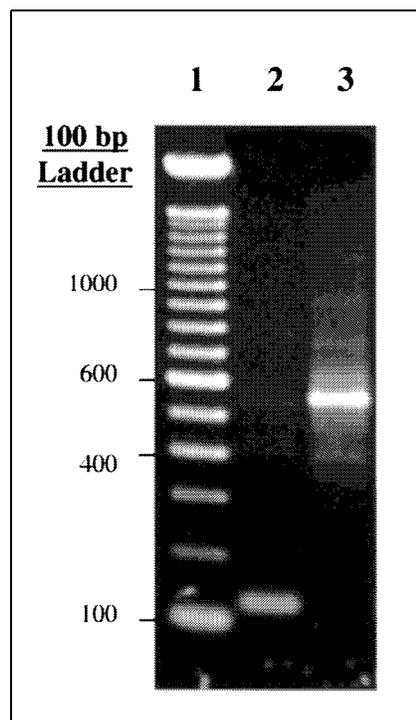
unsuccessful in our hands. Some success was finally obtained when we used a procedure described by Hughes and Galau (4) and optimized for the isolation of RNA from cotton pollen and leaf tissue (Table 1).

To isolate RNA from bark/wood tissues, an essential first step is to grind the sample as efficiently as possible while minimizing RNA degradation. This was achieved by freezing bark and wood shavings in liquid nitrogen and then grinding them in a kitchen coffee mill together with a small amount of sea sand. It was thus possible to obtain a fine powder in less than one minute. Although the protocol described by

Hughes and Galau (4) yielded RNA, the amount obtained was still low (Table 1), degradation was consistently observed and contaminating material of high molecular weight was present (Figure 1, lane 1). Furthermore, during the isolation procedure, browning of the extract was frequently observed. This phenomenon has been reported by others and is usually accompanied by RNA damage caused by the high concentration of polyphenolics present in conifer



**Figure 2.** <sup>32</sup>P-labeled cDNA synthesized from total RNA (lane 2) and poly (A<sup>+</sup>) RNA (lane 3) templates.



**Figure 3.** RT-PCR products obtained using the degenerate terpene synthase primer combinations: Mult-F2/Mult-R4 (lane 2) and Mult-F2/Mult-R6 (lane 3).

# Short Technical Reports

cells (3,9). The low RNA yield could be the result of co-precipitation with contaminating polysaccharides (8).

To overcome these two problems, the protocol of Hughes and Galau was modified according to Lewinsohn et al. (8), who introduced insoluble PVPP to the extraction buffer to absorb the phenolics and removed contaminating polysaccharides by precipitation with 10% ethanol. However, unlike the protocol described by Lewinsohn et al. (8), it was not necessary to incorporate a subsequent CsCl ultracentrifugation step; RNA was recovered by precipitation with LiCl. When this modification was incorporated, a green supernatant/pellet was observed at all steps of the modified protocol, indicating that oxidation was minimal throughout. In addition, the yield of total RNA obtained from spruce bark/wood tissues increased approximately three to fivefold (Table 1).

The quality of the RNA obtained from bark/wood tissue of spruce was

good as assessed by the ratio of its absorbance at 260 and 280 nm, which was approximately 1.85. Further assessment of RNA quality was obtained by visualizing the major ribosomal RNAs following their separation on denaturing agarose gels (Figure 1, lane 2). No significant degradation of the RNA was observed on these gels. To determine whether the RNA was suitable for such downstream purposes as cDNA library construction and RT-PCR, it was used as a template for a reverse transcription reaction. For this purpose, both total and poly (A)<sup>+</sup> RNA were used, the reverse transcription reaction was performed in the presence of [ $\alpha$ -<sup>32</sup>P]-dATP and the products were separated in an agarose gel (Figure 2). The size of the labeled cDNA ranged from 0.5–4 kbp when either total or poly (A)<sup>+</sup> RNA was used as a template. This indicates that the quality of the RNA isolated by the protocol described here was acceptable for a range of downstream purposes including cDNA library construction and RT-PCR. This RNA has been used to clone DNA fragments corresponding to wound-induced terpene synthase genes that are expressed in the bark and/or wood tissues of spruce. Total RNA was isolated from the stems of Sitka spruce seven days after drill-wounding (achieved using a 0.95 mm drill bit). RT-PCR was carried out with degenerate primers designed to anneal to conserved regions of terpene synthase genes (2). Two cDNA products of the expected size were obtained (Figure 3).

This protocol was also used to isolate RNA from mature needles of Sitka spruce. Total RNA obtained from needles was also of good quality as assessed by the  $A_{260}/A_{280}$  ratio and its appearance in denaturing agarose gels (Table 1 and Figure 1, lane 3). The yield of RNA from mature needles was significantly higher than that for bark/wood tissues (Table 1).

## REFERENCES

1. Baker, S.M., E.E. White and S. Misra. 1992. Isolation of inhibitor-free poly (A)<sup>+</sup> RNA from *Pinus monticola* and *Pinus lambertiana* seedlings. *Can. J. For. Res.* 22:622-624.
2. Bohlmann, J., V. Ramachandiran, S. Katoh and R. Croteau. 1999. cDNA cloning, characterization and functional expression of four new monoterpene synthase members of the Tpsd gene family from grand fir (*Abies grandis*). *Arch. Biochem. Biophys.* 368:232-243.
3. Chang, S.J., J. Puryear and J. Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11:113-116.
4. Hughes, D.W. and G. Galau. 1988. Preparation of RNA from cotton leaves and pollen. *Plant Mol. Biol. Rep.* 6:253-257.
5. Hutchinson, K.W., P.B. Singer and M.S. Greenwood. 1988. Molecular genetic analysis of development and maturation in larch. In *Proceedings of the 2<sup>nd</sup> IUFRO Working Party on Molecular Genetics*, 15-18 June 1987, Petawawa, Ont. Canada. Edited by W.M. Cheliak and A.C. Yapa. *Can. For. Serv. Petawawa Natl. For. Inst. Inf. Rep. PI-X-80*. p. 26-33.
6. Jansson, S. and P. Gustafsson. 1988. Cloning of a cDNA encoding the light-harvesting chlorophyll A/B-binding protein (CAB) from Scots pine (*Pinus sylvestris* L.). In *Proceedings of the 2<sup>nd</sup> IUFRO Working Party on Molecular Genetics*, 15-18 June 1987, Petawawa, Ont. Canada. Edited by W.M. Cheliak and A.C. Yapa. *Can. For. Serv. Petawawa Natl. For. Inst. Inf. Rep. PI-X-80*. p. 19-25.
7. Lehrach, H., D. Diamond, J.M. Wozney and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry* 16:4743-4751.
8. Lewinsohn, E., C.L. Steele and R. Croteau. 1994. Simple isolation of functional RNA from woody stems of gymnosperms. *Plant Mol. Biol. Rep.* 12:20-25.
9. Schneiderbauer, A., H. Sandermann Jr. and D. Ernst. 1991. Isolation of functional RNA from plant tissues rich in phenolic compounds. *Anal. Biochem.* 197:91-95.
10. Schultz, D.J., R. Craig, D.L. Cox-Foster, R.O. Mumma and J.I. Medford. 1994. RNA isolation from recalcitrant plant tissue. *Plant Mol. Biol. Rep.* 12:310-316.

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