

## RNA Isolation from Plant Tissues Recalcitrant to Extraction in Guanidine

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Guanidine is often used in RNA extraction buffers for obtaining high-quality RNA from a wide variety of plants (4,8). We attempted to isolate RNA from conifer developing secondary xylem using this chaotropic agent, but only very low yields were obtained. In addition, the RNA had very low  $A_{260}/A_{280}$  ratios (1.1–1.3), which did not improve with multiple LiCl precipitations. We also attempted to prepare cDNA libraries from gymnosperm developing secondary xylem for isolation of cDNA clones of enzymes involved in lignin biosynthesis for comparison to those of woody angiosperms. However, since the RNA was a poor substrate for reverse transcription, a well-represented cDNA library could not be prepared. Yields of RNA from sugarcane tissues were also low when extracted in the presence of guanidine. The  $A_{260}/A_{280}$  ratios generally ranged between 1.6 and 1.7 and did not significantly increase with LiCl precipitations. Others found that the yields of RNA were negligible from eastern white pine ovules, seedlings, needles or seeds with extraction in the presence of guanidine (1). This was attributed to the presence of unidentified secondary metabolites that interfered with resuspension of the RNA when extracted with guanidine. RNA was isolated from these tissues using a protocol (1) that includes extraction in buffer lacking guanidine followed by purification using a discontinuous CsCl gradient. Recently, another protocol was described for isolation of functional RNA from woody stems of gymnosperms that also used a discontinuous CsCl gradient, but the RNA yields were very low (7). In this paper, a short and rapid protocol (Table 1) is described to obtain total RNA from sugarcane, developing xylem tissue of conifers and tissues from other plants (tomato, barley, corn and pea) using a modified, simple extraction buffer based on a previously described buffer (3). The RNA obtained can be purified

Table 1. Protocol

### (A) RNA isolation

1. Freeze tissue in liquid nitrogen and grind to a fine powder using a mortar and pestle.
2. Weigh 10 g of powdered frozen tissue and place into a polypropylene beaker containing 20 mL homogenization buffer. Add 20 mL equilibrated phenol and 4 mL chloroform:isoamyl alcohol (24:1) to the beaker in an exhaust hood and homogenize the tissue using a polytron (Tekmar, Cincinnati, OH, USA) homogenizer at high speed for 1–2 min.
3. Add 1.4 mL 3 M sodium acetate (pH 5.2), homogenize another 30 s and transfer the homogenate to two polypropylene centrifuge tubes. Cool on ice for 15 min and centrifuge at 16 000 $\times$ g for 10 min at 4°C.
4. Carefully transfer the aqueous phase to new centrifuge tubes and add an equal volume of isopropanol.
5. Chill the solution at -70°C for 20 min and recover the precipitate by centrifugation at 10 000 $\times$ g for 10 min at 4°C.
6. Wash the pellet with 70% ethanol, centrifuge at 10 000 $\times$ g for 5 min and briefly dry the pellet.
7. Resuspend pellet in 900  $\mu$ L of diethylpyrocarbonate (DEPC)-treated dH<sub>2</sub>O at room temperature for 10 min.
8. Remove insoluble material by centrifugation at 10 000 $\times$ g for 5 min at 4°C and transfer supernatant to a microcentrifuge tube. Adjust volume to 900  $\mu$ L with DEPC-treated dH<sub>2</sub>O if needed.
9. Precipitate the RNA by addition of 300  $\mu$ L 8 M LiCl (2 M final concentration) and incubate on ice for 3 h. (If RNA concentration is low, precipitate overnight in 2.5 M LiCl at 4°C.)
10. Recover RNA by centrifugation at 14 000 $\times$ g for 10 min at 4°C.
11. Wash RNA pellet with 70% ethanol, centrifuge at 10 000 $\times$ g for 5 min and briefly dry the pellet.
12. Resuspend the RNA in 500  $\mu$ L of DEPC-treated dH<sub>2</sub>O for 10 min at room temperature and centrifuge at 10 000 $\times$ g for 5 min to remove any residual insoluble material if present.

### (B) Reagents

#### Homogenization buffer

- 100 mM Tris-HCl (pH 9.0)
- 200 mM NaCl
- 15 mM EDTA
- 0.5% Sarkosyl

Prior to use, add 2-mercaptoethanol to 100 mM.

All reagents were prepared in DEPC-treated water. Polypropylene centrifuge tubes and beakers were soaked in AbSolve™ RNase Decontaminant (Du Pont NEN, Boston, MA, USA) and rinsed with dH<sub>2</sub>O.

without CsCl gradient purification with comparable or better yields.

Loblolly pine (*Pinus taeda* L.) and Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) seeds were obtained from Weyerhaeuser Corporation (Washington, NC, USA) and germinated in peat:vermiculite (3:1). The seedlings were transferred to soil and maintained in a greenhouse for 3 years at 22°C with a day length of 16 h. Tissue was

also obtained from the trunk of a 15-year-old white spruce (*Picea glauca* [Moench] Voss) on July 25, 1991, approximately 10 miles from the MTU campus. The bark (with phloem and cambial tissues attached) was peeled from these species, exposing the developing secondary xylem on the stem or trunk. Approximately 0.2–0.5 mm of this xylem layer was scraped from the woody stem and immediately frozen in

Table 2. Yield and Absorbance Ratios for RNA Isolated from Sugarcane and Conifers

Tissue	$A_{260}/A_{280}$	Yield μg RNA/g FW
<b>Protocol</b>		
Loblolly pine developing secondary xylem	1.74	119
Douglas fir developing secondary xylem	2.01	183
White spruce developing secondary xylem	2.06	211
Sugarcane internodes 1–5	1.99	235
Sugarcane mature leaf	1.87	245
<b>Modified Protocol</b>		
Loblolly pine developing secondary xylem	1.90	200

liquid nitrogen. Sugarcane (*Saccharum* spp. hybrid cv H65-7052) tissue was obtained from approximately 9-month-old field plants growing near the experimental station of the Hawaiian Sugar Planters' Association in Aiea, HI.

RNA yields ranged from 119–211 μg/g fresh weight (FW) from xylem tissue of conifers, with consistently lower yields from loblolly pine xylem (Table 2). The  $A_{260}/A_{280}$  ratios for RNA isolated from xylem tissue of Douglas fir and white spruce were near 2.0, indicating a lack of contamination from polysaccharides and phenolics. Howev-

er, the ratio was somewhat low for loblolly pine RNA, which consistently had a ratio around 1.7, and the ratio did not improve with multiple LiCl precipitations. It was recently reported that LiCl precipitation of RNA isolated from woody stems of gymnosperm species causes severe browning of extracts (7). However, this problem was not observed when isolating RNA from the developing secondary xylem of conifers using our protocol, suggesting that the contaminating phenolics are removed in this procedure before the LiCl precipitation. Denaturing agarose

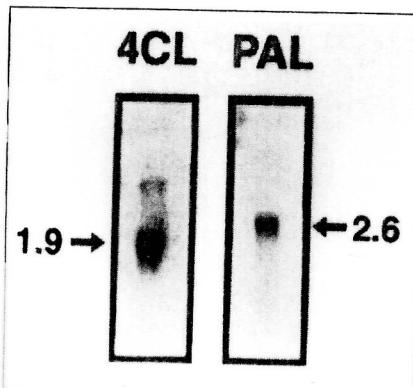


Figure 1. Northern analysis of total RNA from loblolly pine. Total RNA (20 μg/lane) isolated from the developing secondary xylem of loblolly pine was electrophoresed in a formaldehyde-agarose gel, transferred to nylon by capillary transfer and hybridized with a random-prime  $^{32}\text{P}$ -labeled probe prepared from a parsley 4-coumarate:CoA ligase cDNA (4CL) and from a hybrid poplar phenylalanyl-ammonia-lyase cDNA (PAL) overnight at 37°C in standard hybridization solution containing 50% formamide (9). The blots were washed twice at 55°C for 20 min with 4x standard saline citrate (SSC), 1% sodium dodecyl sulfate (SDS) and once at 55°C for 10 min with 2x SSC, 1% SDS followed by exposure to film for 48 h at -70°C using intensifying screens. Hybridizing bands are indicated in kilobases.

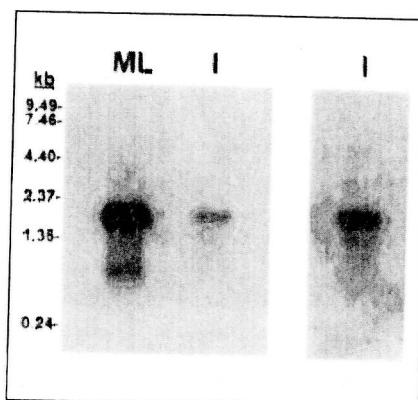
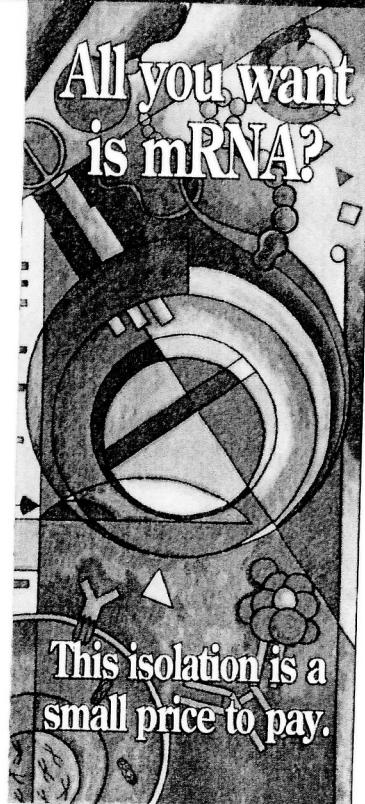


Figure 2. Northern analysis of poly(A)<sup>+</sup> RNA from sugarcane. Poly(A)<sup>+</sup> RNA (1 μg/lane) from mature leaves (ML) and a pooled sample of internodes 1 through 5 (I) were electrophoresed in a formaldehyde-agarose gel and transferred to nylon by capillary transfer. Hybridization was performed at 65°C in 50% formamide as described (5), except for the omission of dextran sulfate, with a  $^{32}\text{P}$ -labeled antisense RNA probe prepared from a *Bam*H- $\lambda$ hol fragment of the coding region of a glucose transporter cDNA (SGT2) of sugarcane (2). The membrane was washed twice at 65°C for 20 min with 0.1x SSC, 0.1% SDS and exposed to preflashed film for 4 h (left panel) and 16 h (right panel) at -70°C using intensifying screens. RNA molecular weight markers (Life Technologies, Gaithersburg, MD, USA) are indicated in kilobases.



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# Benchmarks

gel electrophoresis of the total RNA demonstrated discrete rRNA bands with no apparent degradation (data not shown).

Since the lower  $A_{260}/A_{280}$  ratios for loblolly pine total RNA suggested that contaminants were still present, a modified protocol was developed in order to obtain higher quality RNA. Modifications to the described protocol include the addition of 0.2 g/g FW polyvinyl-pyrollidone (PVP) (mol wt 24 000) to the homogenization mixture (step 2, Table 1), an additional phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol extraction before precipitation with isopropanol (step 4, Table 1) and a wash of the precipitated pellet with 2 M LiCl prior to the wash with 70% ethanol (steps 6 and 11, Table 1). Total RNA isolated from loblolly pine xylem with this modified protocol had higher yields and an  $A_{260}/A_{280}$  ratio of 1.90 (Table 2). It was

also observed that RNA isolated from actively growing, developing xylem tissue always resulted in higher  $A_{260}/A_{280}$  ratios. Poly(A)<sup>+</sup> RNA was isolated from this total RNA and a cDNA library was prepared resulting in a titer of  $2.75 \times 10^7$  pfu/ $\mu\text{g}$  input poly(A)<sup>+</sup> RNA. To determine the quality of the mRNA, Northern blots were done using loblolly pine total RNA. One blot was hybridized with a heterologous probe prepared from a 4-coumarate:CoA ligase cDNA (6), while the other was hybridized with a heterologous probe prepared from a phenylalanine ammonia-lyase cDNA (11) (Figure 1). The Northerns demonstrated discrete hybridizing bands of the approximate sizes for the corresponding mRNAs, with virtually no tailing of the bands.

Isolation of RNA from sugarcane was also improved with this protocol. Yields of RNA from mature leaf and young internodes ranged from 235–245

$\mu\text{g/g FW}$ , and the  $A_{260}/A_{280}$  ratios indicated that the RNA had minimal contamination (Table 2). cDNA libraries were prepared from the corresponding poly(A)<sup>+</sup> RNA, and the titers ranged from  $1.6 \times 10^7$  to  $1.3 \times 10^8$  pfu/ $\mu\text{g}$  input poly(A)<sup>+</sup> RNA, with cDNA ranging in size of 500–4000 bp. The sugarcane mature leaf cDNA library was screened with a glucose transporter cDNA from *Arabidopsis* (10), and a cDNA containing the entire coding region of a glucose transporter from sugarcane was isolated (2). Poly(A)<sup>+</sup> RNA was isolated from sugarcane total RNA using the PolyATtract® mRNA purification system (Promega, Madison, WI, USA) and hybridized with a  $^{32}\text{P}$ -labeled antisense RNA probe of the glucose transporter cDNA of sugarcane. The Northern blot produced discrete hybridizing bands with virtually no degradation, indicating the poly(A)<sup>+</sup> RNA was intact (Figure 2).



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- 143 to RCB. The parsley 4-coumarate:CoA ligase and hybrid poplar phenylalanine ammonia-lyase cDNAs were kindly provided by Carl Douglas at the University of British Columbia, Vancouver, BC, Canada. Address correspondence to Robert C. Bugos, Dept. of Plant Molecular Physiology, University of Hawaii, 3190 Maile Way, Honolulu, HI 96822, USA.

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