radiation, while expression of the housekeeping gene *GAPD* was not appreciably affected. This approach has given useful and consistent results in our laboratory in a wide variety of stress-gene expression experiments. In addition, it has been effective in comparing the expression of genes between different cell lines (Amundson et al., manuscript submitted) in which the expression of housekeeping genes often varied. In these studies, there was a good correlation between relative expression of specific transcripts and their expression at the protein level.

In an era when high-throughput expression screening techniques such as microarray analysis are becoming increasingly widespread, there is a pressing need for rapid and accurate methods of confirming the results of such studies. Our single-probe hybridization with normalization to polyU is one such technique. With the current RNA Think program, up to 50 RNA samples can be analyzed in one run and ten to twenty different probes can be used in one day, allowing the analysis of hundreds of samples. Unlike techniques such as RNase protection and real-time PCR, no specialized probes need to be developed before a new gene can be analyzed with this technique. Finally, this single-probe hybridization technique is more sensitive and more quantitative than hybridizations using complex probes, such as those used in microarray analysis, thus providing an important complement to such high-throughput techniques.

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Reliable and Reproducible Method to Extract High-Quality RNA from Plant Tissues Rich in Secondary Metabolites

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The extraction of high-quality RNA for Northern blot and enzymatic reactions is an important prerequisite for studies of molecular responses to stress and developmental processes in trees. In the same way, ideally, the efforts to achieve high-quality RNA should not substantially restrict its yield. In most plants, this is not a major problem and reliable methods have been developed (1,17,18). Also, commercial kits are available for a range of applications. However, many plants, such as most gymnosperms and many woody angiosperm species, are rich in polyphenols, terpenes, carbohydrates and other secondary metabolites that severely restrict the use of common methods developed for RNA extraction. The available RNA extraction kits are also of limited use.

The need for a method to extract RNA from these particular species is evident and is mirrored in several papers published on tree molecular biology (2-6,10-16,19). However, none of these methods has performed satisfactorily in our hands, and we developed a novel method.

The materials used for our RNA extraction method were roots, needles and bark of Norway spruce (*Picea abies*). These tissues contain high amounts of different secondary metabolites. The roots are high in polyphenols and other phenolic compounds; the needles are high in carbohydrates, and the bark is high in both polyphenols and terpenes. Here, we present a method for RNA extraction that has worked reliably in our laboratory and results in high-quality RNA that is useful for both Northern blot hybridization and enzymatic reactions such as reverse transcription and RT-PCR. No ultracentrifugation is required, and the yield is sufficient for subsequent analysis. The Protocol presents the method that is modified from previous publications, primarily those

Benchmarks

by Bugos et al. (5) and Dong and Dunstan (6).

The extraction buffer has been described previously (6). It does not include guanidine since it has been shown that this chaotropic agent interferes both with the yield and quality of RNA from secondary xylem of white spruce and from internodes of sugarcane (5). We have also experienced similar results with roots of Norway spruce. Note that the buffer has a relatively high pH that reduces the binding capacity of polyphenols to RNA and other molecules. The polyvinylpyrrolidone (PVP) in the buffer interacts with the polyphenols, and a 1% concentration has been reported as optimal for RNA extraction (6). We and others (2,6,8,10) have observed that a homogenization buffer containing PVP improves both the yield and quality of the RNA extracted from polyphenol-rich tissues. Aurintricarboxylic acid (ATA) was included in the buffer as a general RNase inhibitor, and it is also an effective inhibitor of other nucleases (7). ATA has been shown to be an efficient RNase inhibitor down to concentrations as low as 1 mM (7). It has been included successfully in the extraction buffer of a few previously reported RNA and DNA extraction methods (3,6,10,12).

After a thorough homogenization, the samples are heated at 65°C for 10 min to improve cell lysis. Because ATA is included, RNA degradation is not a problem during this incubation, and the time can be extended without risk of RNA degradation. For bark, we have extended the time to 40–60 min to improve cell lysis and yield without detectable degradation of RNA. Depending on growth conditions, needles and sometimes other tissues may contain high amounts of insoluble carbohydrates as well. These carbohydrates can severely reduce the yield of RNA.

To reduce the amount of insoluble carbohydrates, a precipitation step with 0.7 mL potassium acetate at a low pH of 4.8 is included before the extraction with phenol and chloroform:isoamyl alcohol. The extraction, with both phenol and chloroform:isoamyl alcohol at 24:1, is important for the removal of phenols, polyphenols, terpenes and other hydrophobic compounds. This step has to be repeated until no cloudy interface can be detected. We have usually

Table 1. Yield and Absorbance Measurements from Different Tissues of Norway Spruce

Tissue	Plant age	Yield μg RNA/g tissue	A_{260}/A_{280}	A_{260}/A_{230}	n
Healthy roots	2 months	299	1.89	2.46	5
Infected roots ^a	2 months	212	1.86	2.18	4
Drought stressed roots	2 months	n.a. ^b	1.79	2.53	8
Healthy needles	2 months	189	1.84	2.52	5
Infected needles ^a	2 months	267	1.91	2.09	4
Bark	35 years	102	1.75	2.62	9

^a The plants were infected with a pathogenic isolate of *Rhizoctonia* sp.
^b n.a., not applicable. Since these roots can be very dry, the apparent yields are not comparable with other samples.

repeated this extraction 5–7 times. This method also removed much of the PVP, any remaining PVP or traces of polyphenols are removed with insoluble polyvinylpyrrolidone (PVPP). The combination of PVP and PVPP has efficiently been used for RNA extraction (10,12) and for the purification of active

enzymes from polyphenol-rich tissues of different gymnosperm plants (9). Nucleic acids are precipitated with isopropanol, and the RNA fraction is then precipitated with LiCl following resuspension (see Protocol). Some ATA may remain in the samples and can interfere with further enzymatic manipulations.

Figure 1. Agarose gel electrophoresis and Northern blot analysis of RNA from roots of Norway spruce. (A) Electrophoresis was performed in a non-denaturing TAE-agarose gel. Each lane represents 2 μg RNA. The bands of 28S and 18S rRNA are seen. (B) Northern blot analysis; each lane represents 20 μg RNA that was separated on a denaturing formaldehyde-agarose gel and transferred to a nylon membrane by capillary transfer. The RNA was fixed by UV cross-linking to the membrane using an Ultraviolet Crosslinker (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization was performed at 40°C in 50% formamide as described (1) with a ^{32}P -labeled Chalcone synthase cDNA-probe from Norway spruce. Stringent washes of the membrane were performed twice at 65°C for 15 min with 0.1 $\times$ SSC and 0.1% SDS, and the membrane was then exposed to a Kodak® BioMax® MS film overnight at room temperature. The size of the hybridizing RNA is 1.5 kb. Four samples of total RNA, independently isolated from healthy roots of Norway spruce, were analyzed in both (A) and (B).

Protocol (0.5–3 g of Tissue)

1. Frozen (-80°C) or fresh tissue is thoroughly homogenized to a fine powder in liquid nitrogen using a mortar and pestle.
2. The tissue powder is transferred to a 50-mL centrifuge tube containing 15 mL extraction buffer. Mix well. The sample can stand on ice up to 60 min to allow several samples to be homogenized and later extracted together.
3. Incubate the samples at 65°C in a water bath for 10 min with occasional gentle mixing and centrifuge at 14 000× *g* for 15 min at room temperature.
4. Transfer the supernatant to a new tube and add 0.7 mL 3 M KAc, pH 4.8. Mix well and let sit on ice for 30 min. Centrifuge the samples at 10 000× *g* for 10 min at 4°C.
5. Transfer the supernatant to a new tube and add 2 mL phenol equilibrated with 0.1 M TE, pH 8.0, and 0.4 mL chloroform:isoamyl alcohol (24:1). Mix well and centrifuge at 16 000× *g* for 10 min at 4°C.
6. Repeat Step 5 until no whitish interface can be seen.
7. Extract once with 2 mL chloroform:isoamyl alcohol (24:1) and centrifuge as above.
8. Add 100 mg PVPP/g original tissue to the water phase and incubate for 20 min at 75°C–80°C in a water bath with occasional mixing.
9. Cool the samples to room temperature and add 1 volume each of chloroform and 5 mL water. Mix to an emulsion and centrifuge at 10 000× *g* for 10 min at 4°C.
10. Transfer the water phase to a new tube. If there is still PVPP present in the water phase, repeat the chloroform extraction once more.
11. Add 1 volume isopropanol and let precipitate at -20°C overnight.
12. Centrifuge at 10 000× *g* and 4°C for 10 min. Wash the pellet once with 2 M LiCl. Centrifuge at 10 000× *g* and 4°C for 10 min.
13. Wash the pellet once with 70% ethanol, centrifuge as above and dry the pellet well.
14. Resuspend the pellet in 900 µL water at room temperature and pellet insoluble material with a centrifugation at 10 000× *g* and 4°C for 5 min.
15. Transfer the supernatant to a microcentrifuge tube and adjust the volume to 900 µL with water, if necessary.
16. Precipitate the RNA by adding 300 µL 8 M LiCl (final concentration 2 M) and incubate on ice for 3 h. If the RNA concentration is low, it may improve the yield if the samples are incubated overnight at 4°C with a final LiCl concentration of 2.5 M.
17. Centrifuge at 14 000× *g* and 4°C for 10 min. Wash the pellet first with 2 M LiCl and centrifuge at 10 000× *g* and 4°C and then with 70% ethanol. Centrifuge as above and dry the pellet well.
18. Resuspend the pellet in 50–100 µL water at room temperature. Centrifuge at 10 000× *g* and 4°C for 7 min and remove insoluble material if present.

Reagents:	Before use, add ATA to 0.5 mM and
Extraction buffer	β-mercaptoethanol to 14.3 mM.
50 mM Tris-HCl (pH 8.0)	ATA is prepared in a stock solution of
300 mM NaCl	100 mM and adjusted to pH 8.0 with
5 mM EDTA	NaOH. All reagents were prepared in
2% (w/v) SDS	DEPC-treated water.
0.5% (w/v) PVP (MW 360 000)	

Benchmarks

Figure 2. RT-PCR of transcript encoding the putative peroxidase SPI2. RNA isolated according to the protocol was reverse transcribed with MoMLV reverse transcriptase (Promega, Madison, WI, USA) and amplified with Red Hot *Taq* DNA polymerase (Advanced Biotechnologies, Columbia, MD, USA) using two specific primers. The PCR conditions were 35 cycles with 94°C for 30 s, 58°C for 30 s and 72°C for 2 min. St. = DNA Standard (1-kb Plus DNA Ladder; Life Technologies, Rockville, MD, USA).

To avoid this potential problem, mRNA can be purified with polyT magnetic beads (e.g., Dynabeads; Dynal, Lake Success, NY, USA). When the RNA is bound to these beads, any remaining ATA is removed by washes with the appropriate buffer (i.e., 150 mM LiCl, 10 mM Tris, 1 mM EDTA, pH 8.0), and then, if necessary, released from the beads with DEPC-treated water.

In the past, we have used this method, and it has always given reproducible results with regard to quality and yield. This is not a quick procedure, but all attempts to save time have resulted in compromised quality and yield of the RNA. The yield varies with the tissue type but varies little between samples of the same tissue and treatment. The mean values for both yield and quality measurements are presented in Table 1. Evidence of RNA integrity is provided in Figure 1. Figure 2 shows a successfully amplified fragment of the putative peroxidase gene, spruce pathogen induced 2 (*spi2*). Our method has been used successfully by other laboratories that are working on tissues with similar features.

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