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Extraction of RNA from Fresh, Frozen, and Lyophilized Tuber and Root Tissues

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A method for isolating trancriptionally competent RNA from fresh, frozen, and lyophilized plant storage tissues containing high levels of starch and phenolics is described. The protocol avoids the use of guanidium salts, which often lead to the formation of a viscous gel during extraction of high starch-containing tissues, and instead uses a borate-Tris buffer in combination with high concentrations of NaCl, Na_2SO_3, and sodium dodecyl sulfate in the extraction medium. RNA was extracted from fresh, frozen, and lyophilized tissues of potato tubers, storage roots of sweet potato, radish, and turnip, and rhizomes of ginger. The yield of RNA from potato tubers averaged 281 μg g fresh weight^{-1} and 1584 μg g dry weight^{-1} from frozen and lyophilized samples, respectively. A_{260}/A_{230} ratios of potato RNA extracts were 2.2 or greater, indicating minimal contamination by polyphenols and carbohydrates. Similarly, A_{260}/A_{280} ratios exceeded 1.9, demonstrating minimal contamination of the RNA by tuber protein. While A_{260}/A_{280} ratios of extracts from the other plant species were somewhat lower than those for potato (average = 1.56 and 1.80 for fresh and lyophilized samples, respectively), A_{260}/A_{230} ratios averaged more than 2.0, and the RNA extracted from fresh and lyophilized samples of all species was intact, as demonstrated by denaturing agarose-formaldehyde gel electrophoresis. The protocol yielded RNA suitable for downstream molecular applications involving reverse transcription-polymerase chain reaction from all five species. Transcriptionally competent RNA was also recovered from lyophilized potato tuber tissue stored for 6 years (ambient temperature) by a simple modification to the protocol involving extraction in cold acetone. Lyophilization can thus be used to preserve RNA in high starch- and phenolic-containing plant tissues for studies on gene expression.

KEYWORDS: ***Solanum tuberosum*** **L.; potato; radish; turnip; ginger; RNA extraction; lyophilized tissue; RT-PCR; starch; polyphenols**

INTRODUCTION

Isolation of RNA from plant storage organs such as roots and tubers is particularly challenging due to interference from high levels of polysaccharides (starch) and polyphenols. High concentrations of polymeric carbohydrates can greatly limit the recovery of nucleic acids and thus the quantification of RNA from plant tissues (*1*, *2*). Polysaccharides coprecipitate with RNA (*3*) and can directly inhibit reverse transcription-polymerase chain reaction (RT-PCR) (*4*). In addition, quinones, derived from the oxidation of phenolics (*5*), form high molecular weight complexes with nucleic acids and proteins (*6*) and thus inhibit RT-PCR (*7*, *8*). A simple and reproducible protocol for the isolation of RNA from plant tissues rich in polysaccharides and/or polyphenols would greatly facilitate further studies on gene expression in such species. Accordingly, a procedure for the extraction of trancriptionally competent RNA from fresh, frozen, and lyophilized plant tissues containing high levels of starch and phenolics was developed.

Recovery of intact RNA from lyophilized tissues for further downstream RNA-based methodologies offers several advantages as compared with fresh or frozen samples. Lyophilization potentially preserves the molecular integrity of samples for processing at a later date, eliminates the need for liquid nitrogen during tissue extraction, allows for efficient storage of samples (space requirements are less) without the need for ultra-low temperatures, and facilitates the transport and exchange of samples among collaborators. Despite these advantages, only three attempts at isolating RNA from lyophilized plant tissues have been reported (*8*−*10*), and only two of those were successful. The successful protocols used either guanidine hydrochloride (GHC) as a component of the extraction buffer (*8*) or involved elaborate steps such as CsCl centrifugation (*9*) to isolate the RNA. Since GHC or guanidine thiocyanate (GTC) in combination with phenol promote gelling of potato starch and poor recovery of RNA, both were avoided in our extraction medium. Our method is rapid and yields high quality RNA from fresh, frozen, and lyophilized tissues from potato tuber and a number of other starch-storing plant organs. Moreover, with a slight modification of the method, it was possible to isolate

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Table 1. Forward (F) and Reverse (R) Primer Sequences for β-Tubulin (TUBST1) of Potato, Acetyl-CoA Carboxylase (BnACCg8) of Turnip, Phenylalanine Ammonia Lyase (PAL) of Radish and Ginger, and Actin of Sweet Potato[a]

species	gene	primer sequence (5 → 3)	product	accession	ref
radish	PAL	F: GCGATCATG GAGCACATCCT R: GCGGTTAAA TTCGACGGAAG	380 bp	AB087212	direct submission
potato	TUBST1	F: AAATGTGCAGAACAAGAACTCATCC R: CATAACAAGTTCACTTTGGCAG	380 bp	Z33382	*29*
sweet potato	actin	F: GATGCTCGTGGCATACAAGTATTC R: CAAATCATACATCCAACATGACAA	115 bp	AY905538	direct submission
ginger	PAL	F: ATCGAGGCT GCTGCCATTATG R: AACTGGAG CTCGGAGCAATAGG	470 bp	DQ418481	direct submission
turnip	BnACCg8	F: TTCAACAATAACATGCTGGTAATA R: GTGACGCATACGTTCTATAACATC	115 bp	X77576	*30*

[a] The results of RT-PCR are presented in **Figure 3**.

transcriptionally competent RNA from lyophilized potato tuber tissue stored at ambient temperature for 6 years.

MATERIALS AND METHODS

Plant Materials. RNA was extracted from fresh, frozen, and lyophilized tissues of potato tuber (*Solanum tuberosum* L.), sweet potato root (*Ipomea batata* L.), turnip root (*Brassica rapa* L.), radish root (*Raphanus sativus* L.), and ginger rhizome (*Zingiber officinale* L.). Slices of root or tuber tissue (approximately 1.5 mm thick) were cut with a sterile razor blade that had been pre-rinsed with RNAaseZap (Ambion, Austin, TX). The freshly cut tissue slices were weighed and either transferred directly to a mortar (pre-rinsed with RNAaseZap) and ground (mortar and pestle) to a fine powder in liquid N_2 or frozen in liquid N_2 and lyophilized prior to extraction as described next. A modified extraction procedure was used to recover intact RNA from lyophilized slices of potato tuber tissue that had been stored for 6 years at ambient temperature.

RNA Extraction. Mortar and pestles were washed with 70% (v/v) EtOH and rinsed with RNAaseZap in preparation for extractions. Two grams of fresh tissue was ground (mortar and pestle) to a fine powder in liquid N_2 and transferred to a 15 mL Falcon tube. All solutions and reagents were prepared in diethylpyrocarbonate (DEPC) treated water. RNA was extracted by the sequential addition of 1.0 mL of 5 M NaCl, 0.5 mL of 10% (w/v) SDS, 1.65 mL of 1.95% (w/v) Na_2SO_3, 1.75 mL of borate-Tris buffer (0.2 M, pH 8.0 containing 10 mM EDTA), and 0.1 mL of β-mercaptoethanol. The extract was vortexed and incubated at 65 °C for 5 min. The extract was then centrifuged (1800*g*, 5 min, 23 °C), and an equal volume of Tris-saturated phenol (pH 7.9) was mixed with the supernatant. The mixture was centrifuged to achieve phase separation. Occasionally, phase separation did not occur. In such cases, additional borate-Tris buffer was added in 1.0 mL increments until centrifugation resulted in satisfactory phase separation. The upper phase was extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged to facilitate phase separation. One milliliter of the upper phase was transferred to a 2 mL microfuge tube containing 0.9 mL of isopropyl alcohol. The extract was incubated at 4 °C for 1 h, and the RNA was pelleted at 20 000*g* (15 min, 4 °C). The RNA pellet was washed free of salts with 70% EtOH (4−5 times), the residual EtOH was evaporated at 65 °C on a block heater, and the pellet was solubilized in DEPC treated water. The RNA was diluted 100-fold and quantified at A_{260}, and the extent of protein and carbohydrate/phenolic contaminations was assessed by A_{260}/A_{280} and A_{260}/A_{230} ratios, respectively. The integrity of the RNA was determined by agarose-formaldehyde gel electrophoresis.

The RNA extraction protocol for lyophilized potato tuber tissue depended on the duration of storage. Lyophilized tissue slices stored for 6 years (ambient temperature) were first ground to a fine powder with mortar and pestle at room temperature. Two hundred milligrams of lyophilized powder was mixed with 2 mL of chilled (liquid N_2) acetone. The extract was centrifuged at 20000*g* (10 min, 4 °C), and the pellet was transferred to a 15 mL Falcon tube by sequential addition of sodium salts and borate-Tris buffer, as described previously for fresh and frozen tissues. The acetone extraction was not necessary for lyophilized potato tuber slices stored less than 1.5 years or samples of the other plant species that had been stored for less than a month.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Suitability of the RNA for RT-PCR was assessed for all species. Following DNase treatment (DNA-free kit, Ambion, Inc., Austin, TX), first-strand cDNA synthesis was accomplished with 1 μg of total RNA and oligo$(dT)_{20}$ primer using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendations. Separate reactions containing no-RT and no-template were included as controls. PCR was carried out with GoTaq DNA polymerase (Promega Corporation, Madison, WI) using gene-specific primers. Forward and reverse primer sequences for the genes of interest are listed in **Table 1**. For PCR, samples were initially denaturated at 94 °C for 2 min, followed by annealing for 30 cycles of 30 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C. Final extension occurred at 72 °C for 5 min. Occasionally, the isolated RNA was passed through a spin column (Qiagen, Valencia, CA) to facilitate RT-PCR.

RESULTS AND DISCUSSION

Methods of RNA isolation are devised around the basic principle of preventing degradation by extracting RNA from cells/tissues in an environment inhibitory to RNases. This is achieved by disrupting cells in the presence of chaotropic agents such as guanidine hydrochloride (GHC) or guanidine thiocyanate (GTC) in combination with SDS or phenol or both (*11*−*14*). Many methods for extraction of RNA from plant tissues use GHC in the homogenization buffer (*12*, *15*−*17*). Our attempts to extract RNA from potato tuber tissue using GHC- or GTC-based protocols or commercial kits (TRIzol, Life Technologies, Carlsbad, CA; Qiagen RNAeasy, Qiagen Inc., Valencia, CA) often led to the swelling of starch, which yielded a viscous gel that greatly limited the recovery of supernatant for further processing of RNA.

High amylopectin content (*18*, *19*) and phosphorylation of starch (*20*, *21*) promote gelling. In contrast to starch from cereals, potato starch is phosphorylated (*21*−*23*), and the negatively charged phosphate groups cause repulsion of adjacent starch chains, which facilitates hydration and the gelling process (*20*). Such gelling characteristics can be demonstrated in maize by phosphorylation of the starch in vitro (*20*). The food industry uses sodium caseinate to overcome the gelling property of potato starch, and sodium chloride can mimic the effect of sodium caseinate, suggesting a role for sodium ions in limiting the gelling process (*24*). In working with potato tuber, we found that the combination of sodium chloride, sodium sulfite, and SDS in the initial extraction medium eliminated gel formation, thus facilitating maximum recovery of RNA.

Potato tubers are also rich in phenolics (*25*, *26*), which can affect the quality of isolated RNA (*7*). Quinones, derived from the oxidation of phenolics (*5*), form high molecular weight

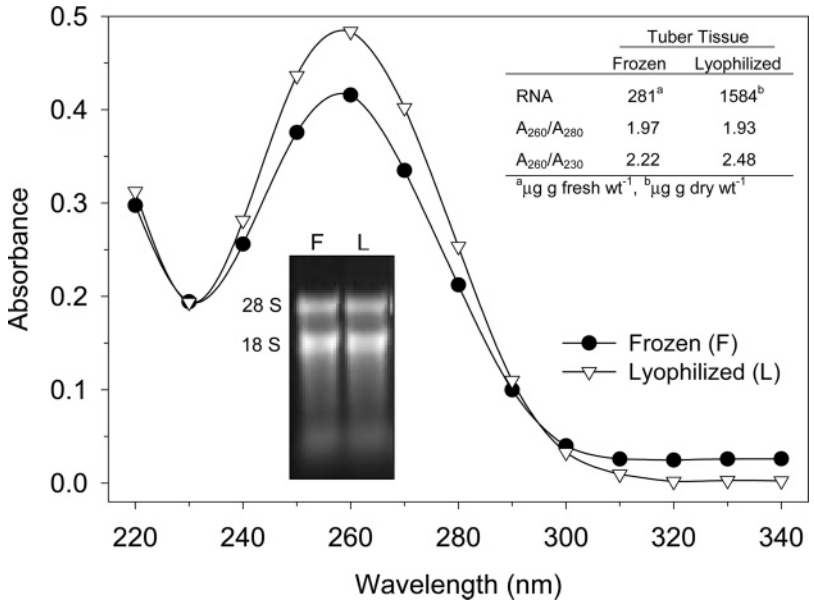

Figure 1. Absorbance spectra of RNA from frozen (stored at –80 °C, 6 months) and lyophilized potato tuber tissue. The yields of RNA and the A_{260}/A_{280} and A_{260}/A_{230} ratios are shown in the table inset. Integrity of the RNA was examined on a 1.2% agarose-formaldehyde gel (inset, 5 μg of RNA per lane). Extractions were repeated at least 5 times with similar results.

Table 2. Yield and Quality of RNA Isolated from Fresh and Lyophilized Radish, Sweet Potato, and Turnip Roots and Ginger Rhizome[a]

	radish	sweet potato	ginger	turnip
	Fresh Tissue			
RNA (μg g fresh wt^{-1})	146	237	42.1	105
A_{260}/A_{280}	1.59	1.53	1.48	1.63
A_{260}/A_{230}	2.57	2.42	2.30	2.61
	Lyophilized Tissue			
RNA (μg g dry wt^{-1})	1423	926	407	688
A_{260}/A_{280}	1.67	1.58	1.86	2.10
A_{260}/A_{230}	2.44	2.20	1.50	1.95

[a] A_{260}/A_{280} and A_{260}/A_{230} ratios indicate relative contamination of the RNA by protein and carbohydrate/phenolics, respectively, and are thus coarse indicators of purity of the RNA extracts.

complexes with nucleic acids and proteins (*6*) and thus inhibit RT-PCR (*7*, *8*). Sodium sulfite inhibits polyphenol oxidase activity (*27*) and eliminates the need for proteinase K during tissue extraction (*7*). Sodium sulfite was therefore included as a component of our extraction medium.

The absorbance spectra, yield, quality, and integrity of the RNA isolated from frozen and lyophilized potato tuber tissue are compared in **Figure 1**. The reproducibility of our RNA isolation protocol was confirmed with five independent RNA isolations, and the average is presented. On average, 281 and 1584 μg of RNA was recovered per gram of frozen and lyophilized tuber tissue, respectively (**Figure 1**, inset table). The lyophilized tissue came from tubers averaging 18% dry matter. Therefore, the yield of RNA from lyophilized samples was comparable with that from frozen samples. The A_{260}/A_{230} ratios of the isolated RNA were 2.2 or greater, indicating minimal contamination by polyphenols and carbohydrates (*1*, *2*, *17*). The A_{260}/A_{280} ratios exceeded 1.9, indicating minimal interference by protein (*1*, *14*). Electrophoresis on agarose-formaldehyde gels resolved 28S and 18S rRNA bands (**Figure 1**, inset), revealing intact RNA.

The suitability of this protocol for isolating RNA from fresh and lyophilized tissues from other starch-bearing plant organs was also examined. Yield and quality parameters of the RNA

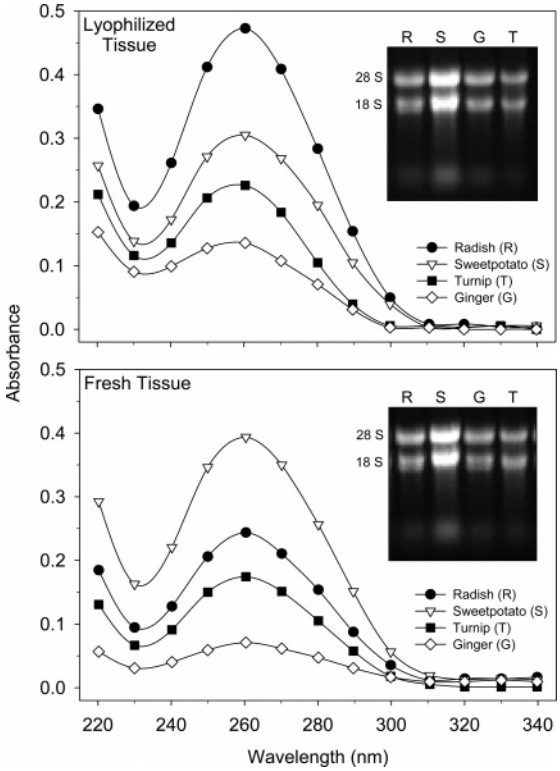

Figure 2. Absorbance spectra of RNA from fresh and lyophilized storage roots of radish, sweet potato, and turnip, and rhizomes of ginger. Integrity of the RNA was examined on a 1.2% agarose-formaldehyde gel (inset, 4–6 μg of RNA per lane). The RNA yields and A_{260}/A_{280} and A_{260}/A_{230} ratios are presented in **Table 2**. Extractions were repeated 3 times with similar results.

extracted from radish, sweet potato, ginger, and turnip are presented in **Table 2**. Absorbance spectra and integrity of the RNA as determined by gel electrophoresis are presented in **Figure 2** and insets. The yield of RNA varied considerably among the different plant species (**Table 2**); however, the isolation protocol effectively minimized carbohydrate contamination, as evidenced by consistently high (>2, except for ginger)

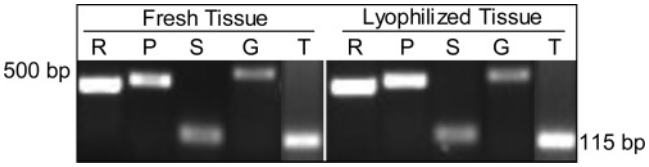

Figure 3. RT-PCR analysis of mRNA from fresh and lyophilized storage roots of radish (R), sweet potato (S), and turnip (T), rhizomes of ginger (G), and potato tuber (P). PCR products were run on 1.2% agarose-ethidium bromide gels. A list of the genes probed and their primer sequences are given in Table 1. The lyophilized potato tuber tissue had been stored for 1.5 years prior to extraction. Results are representative of 3 replications.

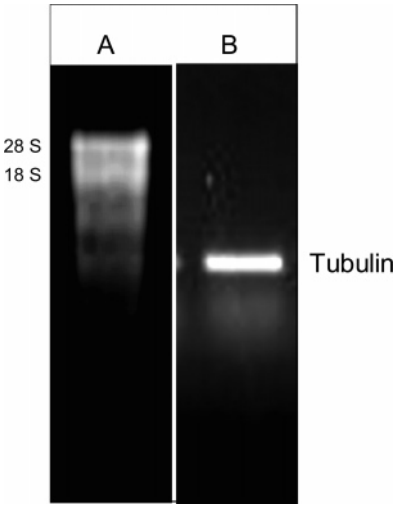

Figure 4. (**A**) Total RNA isolated from 6-year-old lyophilized potato tuber tissue resolved on a 1.2% agarose-formaldehyde gel (10 μg of RNA per lane). (**B**) RT-PCR analysis of β-tubulin. Extractions were repeated at least 3 times with similar results.

A_{260}/A_{230} ratios. Although the A_{260}/A_{280} ratios were lower than those for the potato tuber tissue, RT-PCR demonstrated that the RNA was transcriptionally competent and thus well-suited for downstream applications involving analysis of gene expression (**Figure 3**). For example, using the protocol, we effectively resolved the wound-induced differential expression of *S. tuberosum* respiratory burst NADPH oxidase homologue A (*Strboh A*), along with four other homologues (data not shown), providing an example of its utility for amplifying low and high abundance genes from potato tuber. In all studies, no PCR products were obtained from the no-RT and no-template controls.

The method described previously was not effective for isolating RNA from lyophilized tuber tissue stored for 6 years at ambient temperature. However, a slight modification of the method, involving initial extraction of the tissue with cold acetone, resulted in excellent recovery of trancriptionally competent RNA from these tissue samples (**Figure 4**). Similar acetone extractions have been shown to eliminate interference by phenolic compounds during the isolation of RNA from pine needles, spruce needles (*9*), and apple fruit (*28*). On average, the yield of RNA from 6-year-old samples was 884 μg per g dry wt, and the A_{260}/A_{280} and A_{260}/A_{230} ratios were 1.73 and 2.27, respectively, indicating minimal protein and carbohydrate/phenolic contamination.

In summary, the high salt extraction procedure reported herein is suitable for isolating RNA from fresh, frozen, and lyophilized tissues from a range of plant storage organs containing high concentrations of starch and phenolic compounds. The protocol is highly reproducible and yields intact and thus transcriptionally competent mRNA suitable for downstream analysis of gene expression. As such, this protocol has become the standard in our lab for routine RNA extraction from potato tubers.

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