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Compared Two Methods for Isolating RNA from Freezing and Nonfreezing Bread Wheat (*Triticum aestivum* L.) Plant Tissues

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Abstract: This report described an improved method for isolating intact purified RNA from freezing organs of bread wheat plants. High-quality RNA is important in studying gene expression. Common RNA extraction protocols have produced poor yields because freezing leaves contain polysaccharides and RNases. We used two methods for isolating RNA and comprised them. CTAB (cetyltrimethylammonium bromide) method protocol is based on a guanidine thiocyanate extraction combined with additional purification steps using butanol and the ionic detergent CTAB. Using this protocol, RNA yields ranged from 40-70 μg of total RNA 200 mg of fresh tissue. This method can be adapted to large-scale isolations, allowing the recovery of larger amounts of intact RNA (up to 250 μg g^{-1} of fresh tissue).

Key words: *Triticum aestivum*, bread wheat, freezing, RNA isolation

INTRODUCTION

A number of methods have been described for isolating RNA from plants. In most cases, procedures employ detergents such as SDS, phenol extraction and LiCl precipitation. These methods failed when used to isolate RNA from plants. RNA degraded, leading to low yield and poor-quality mRNA. Isolating high-quality RNA has proven difficult in a number of plant species, notably pine (Chang *et al*., 1993; Mackey and Chomczynski, 1996) and wounded tubers of potato (Solanaceae family) (Logemann *et al*., 1987; Chomczynski, 1992, 1993).

Poor yields and low-quality degraded RNA may result from inefficient cell lysis or high levels of nuclease activity. RNA degradation during extraction from bread wheat may be due in part to the increase in RNases associated with dehydrated tissue (Chang *et al*., 1993), because freezing of tissues cause dehydration. However, these problems can be alleviated using chaotropic agents, such as guanidinium isothiocyanate. A greater challenge in isolating high-quality RNA is the aqueous by products of secondary metabolism that accumulate in many higher plants, especially in mature tissue. These secondary metabolites, including phenolics and high molecular weight polysaccharides (Dang, and dunstan, 1996), can interfere with isolating and purifying biologically active nucleic acids if they copurify during extraction (Loomis, 1974; Chomczynski and Mackey, 1995). These problems may be worse in dehydrated and frozen plant tissues. Removing water in plant cells leads to an increase in solute concentration as the protoplast volume shrinks. Low yields of RNA may also be due to the high levels of polysaccharides that accumulate after dehydration (Hopkins, 1999) and freezing and bind to the RNA during the extraction process (Chang *et al*., 1993).

We comprised two methods for isolating high-quality RNA from bread wheat leaves and roots, based on the guanidinium thiocyanate-phenol extraction protocol (Chomczynski *et al*., 1987, 1993) and an improved purification protocol that employs the ionic detergent CTAB (Camacho-Villasana *et al*., 2002), originally developed for the extraction of nucleic acids from agarose gels (Dong and Dunstan 1996). RNA isolation protocol should be simple, fast, non-toxic and give good yields of high quality RNA. The procedure described in this manuscript appeared to satisfy many of these requirements. We are interested in studying in gene expression during cold stress and have found that the useful isolation procedure of Camacho-Villasana *et al*. (2002) can be successfully used to isolate RNA from leaves and roots. We also found that the method could be considerably simplified without compromising the quality or yield of the isolated RNA.

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MATERIALS AND METHODS

Plant material and cold stress treatment: Bread wheat (*Triticum aestivum* L.) was grown on Murashige and Skoog (MS) medium containing 1.5% sucrose. Seeds were sterilized and grown at 22°C for 3-5 days. When plants grown in plates were exposed to temperature of +2°C for 5-8 weeks (for hardening). Control plants were frozen immediately. Then plants were put at -4°C for low temperature and were frozen after 1, 4 and 16 h. Leaves and roots were harvested from 6-week-old plants. After the times indicated, detached organs were frozen in liquid nitrogen and kept at -70°C until needed.

Chomczynski method:

- Grind 100 mg of tissue in liquid nitrogen and then add 1 mL of solution D and 0.1 mL 2M sodium acetate (pH 4) and 1 mL phenol saturated with TE (10 mM Tris-HCl, 1 mM EDTA [pH 8]) and 0.2 mL chloroform
- Vortex the sample vigorously and place on the ice for 15 min
- Spin at maximum speed in a microfuge for 20 min at 4°C
- Transfer the aqueous phase to a new microfuge tube.
- Add 1 mL of isopropanol and mix by inversion. Place the sample at -20°C for at least 1 h
- Spin at 10000 g at 4°C in a microfuge for 20 min. Discard the supernatant
- Dissolve the pellet in 0.3 mL of solution D and add volume of isopropanol. Let the sample stand at -20°C for 1 h
- Spin in a microfuge at 10000 g at -4°C for 10 min and then dry the pellet
- Dissolve the RNA pellet in DEPC-treated water

Solution D: 4M guanidine thiocyanate, 25 mM sodium citrated (PH 7), 0.5% sarcosyl prepare the solution with DEPC-treated H_2O.

This solution after filtration store at room temperature. Add in use 0.1 mL 2-mercaptoethanol for it.

RNA extraction protocol by CTAB:

- Place 500 µL of extraction buffer and 500 µL of phenol saturated with TE in a microfuge tube. Add 3.5 µL of 2-mercaptoethanol and 50 µL of 3 M sodium acetate
- Grind 200 mg of tissue in liquid nitrogen. Transfer the homogenized tissue-liquid nitrogen slurry into the microfuge tube prepared in as mentioned above
- Vortex the sample vigorously and incubate at room temperature for 5 min
- Add 200 µL of chloroform-isoamylalcohol (24:1). Vortex for 1 min. Let the sample stand at room temperature for 12 min
- Spin at maximum speed in a microfuge for 15 min. Transfer the aqueous phase to a new microfuge tube
- Add 500 µL of isopropanol and mix by inversion. Let the sample stand at room temperature for 7 min
- Spin at maximum speed at room temperature in a microfuge for 10 min. Discard the supernatant. Wash the pellet with 1 mL of 75% ethanol
- Spin in a microfuge at maximum speed at 4°C for 5 min. Discard the supernatant. Dry the RNA pellet at room temperature for 10 min
- Dissolve the RNA pellet in 200 µL of TESAR
- Add 200 µL of aq/CTAB and 200 µL of bu/CTAB. Vortex for 2 min. Note: 2 min of vortexing is necessary to ensure good recovery
- Spin in a microfuge at maximum speed at room temperature for 5 min to resolve the phases
- Remove the upper butanol phase and transfer to new microfuge tube
- Re-extract the lower layer with 200 µL of bu/CTAB
- Remove the upper layer and combine with the bu/CTAB collected in as mentioned earlier. The RNA is a CTAB salt and is soluble in butanol
- Add 150 µL of 0.2 M NaCl to the combined butanol phases. Vortex for 30 sec and spin for 5 min
- Transfer upper butanol layer to a new microfuge tube. Retain the lower aqueous phase. The RNA is now a sodium salt and is soluble in the aqueous phase
- Re-extract the butanol layer with 150 µL of 0.2 m NaCl
- Collect the lower layer and combine with the aqueous phase retained in as mentioned earlier. Add 300 µL of chloroform to the combined aqueous phases. Vortex for 30 sec
- Spin at maximum speed at 4°C for 5 min. Transfer the upper aqueous phase to a new tube
- Add 1/10 vol of sodium acetate and 2.5 vol of ethanol to the aqueous phase to precipitate the RNA. Incubate the sample at -20°C for at least 1 h
- Spin at maximum speed at 4°C for 10 min
- Dry the pellet and resuspend in DEPC-treated H_2O

Solutions required

Extraction buffer: 4 M guanidinium thiocyanate (guanidine thiocyanate), 25 mM sodium citrate (pH 7) and 0.5% sarcosyl. Prepare the solution with DEPC-treated H_2O. The pH was adjusted to 8 using pH indicator strips instead of a pH electrode. Sterilize the solution by filtration and store at room temperature in a sterile dark bottle.

- Phenol saturated with TE (10 mM Tris-HCl, 1 mM EDTA [pH 8])
- 3 M sodium acetate
- Chloroform-isoamylalcohol (24:1)
- Isopropanol
- 75% ethanol
- TESAR (10 mM Tris-HCl [pH 7.6], 1 mM EDTA. 1% sarcosyl): Prepare with DEPC-treated water
- Bu/CTAB and aq/CTAB: Agitate 75 mL of 1-butanol and 75 mL of ddH_2O in a separatory funnel. Allow the 2 phases to separate (butanol, upper phase) for about 4 h. Add 1.84 g of CTAB to 50 mL of water-saturated butanol. Add 50 mL of butanol-saturated water and shake in a separatory funnel. Allow the 2 phases to separate overnight. (Bu/CTAB was the upper phase and aq/CTAB was the lower phase). Store separately
- 0.2 M NaCl
- Chloroform
- Absolute ethanol
- DEPC-treated water: Add 1 mL of DEPC to 1000 mL of deionized water and shake vigorously. Incubate for 1 h at 37°C, autoclave to inactivate the DEPC and store at room temperature
- 20 X SSPE (3 M NaCl, 0.2 M sodium phosphate monobasic NaH_2PO_4, 0.02 M EDTA [pH 7.71])

RNA analysis: To confirm the integrity of RNA samples, the RNA was resolved using gel electrophoresis. RNA quantification was performed spectrophotometrically at wavelengths of 260 and 280 nm. Of the total RNA isolated from bread wheat leaves and roots treatment by cold stress, 15 µg was electrophoresed on a 1.5% denaturing agarose gel. RNAs were visualized by staining with ethidium bromide to confirm equivalent RNA loading per lane.

Volume of RNA sample = 100 µL
Dilution = 10 µL of RNA sample + 490 µL distilled water ($\frac{1}{50}$ dilution)
e.g., $A_{260} = 0.23$
Concentration of RNA sample = $40 \times A_{260} \times$ dilution factor
Concentration of RNA sample = $40 \times 0.23 \times 50$
Concentration of RNA sample = 40×11.5 µg mL^{-1}
Total yield = Concentration × Volume of sample in mm
Total yield = 460 µg $mL^{-1} \times 0.1$ mL = 46 µg

RESULTS AND DISCUSSION

Several established methods were initially used to isolate RNA from plants tissues. These included standard and modified LiCl precipitation, nonphenolic extractions and precipitations with various salts (Maes and Messens, 1992; Chang *et al.*, 1993; Bugos *et al.*, 1993). All failed to render good quality and high yields of RNA.

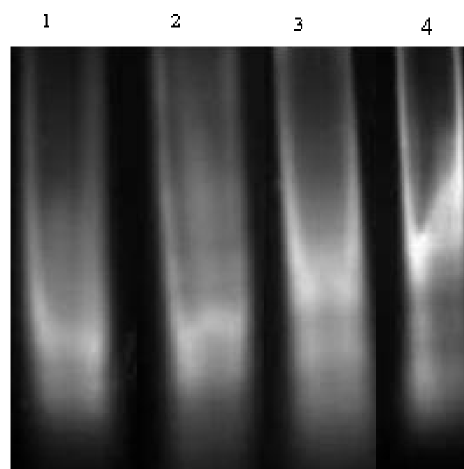

Fig. 1: Agarose gel electrophoresis of total RNA from bread wheat using Chomczynski's protocol. The method described in text was used to isolate RNAs from bread wheat plant leaves: untreated control (Lane 1) and cold stress leaves for 1 h (Lane 2), 4 h (Lane 3) and 16 h (Lane 4). The RNAs were resolved on agarose gels

Polyscchorides contamination and an increased amount of RNases may have limited RNA isolation from cold stress bread wheat plants, particularly from leaves (Chang *et al.*, 1993). Yield and purity problems may also be due to the oxidation of phenolic compounds, which can bind irreversibly to nucleic acids and coprecipitate with RNA (Loomis, 1974; Chang *et al.*, 1993). In Chomczynski method yields of RNA were consistently less than 60 µg total RNA per gram of fresh tissue. The RNA appeared to be partially degraded and the rRNA bands were not distinct. The pellets of these extraction were largely insoluble (Fig. 1).

CTAB protocol allowed recovery of intact, high-quality RNA from leaves (Fig. 2a) and roots (Fig. 2b). Distince rRNA bands were apparent in the samples; therefore, the quality of the RNA was independent of the treatment. In addition, the RNA was pure, as judged by an A_{260}/A_{280} ratio of approximately 2. The yield ranged from 200-250 µg g^{-1} of fresh weight, depending on the tissue.

In the leaves, the yield was higher than roots. Yields for unstressed and stressed leaves (1 and 16 h) were 250 µg. In all cases, the RNA obtained was of high quality and integrity. This two-part RNA isolation procedure was successful in isolating RNA is evident in that it (1) is rapid, single-step protocol (2) promotes efficient lysis of plant cells and (3) inhibits ribonuclease activity due to the presence of 4 M guaidinium thiocyanate (Cox, 1968; Chomczynski and Sacchi, 1987; Wilkins and Smart, 1996; Camacho-Villasana *et al.*, 2002). RNA yields

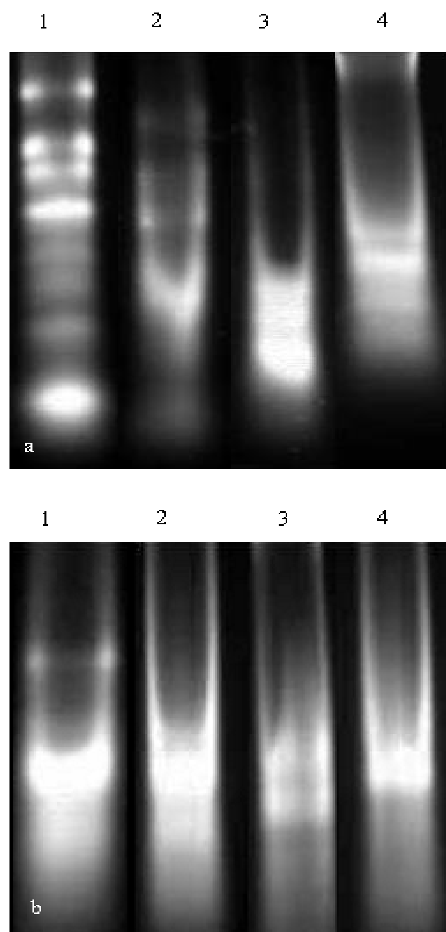

Fig. 2: Agarose gel electrophoresis of total RNA extracted from bread wheat tissues using guanidine thiocyanate and purified using a CTAB/butanol extraction. (a) RNA extracted from detached leave. Lane 1 (control) and cold stress leaves for 1 h (Lane 2) , 4 h (Lane 3) and 16 h (Lane 4) and (b) RNA from roots. Lane 1 (control) and Lane 2-4 cold stress at 1, 4 and 16 h, respectively

in creased to 250 μg of RNA per gram of fresh tissue by combining the guanidinium extraction and the CTAB/butanol purification protocols (Camacho-Villasana *et al.*, 2002). When CTAB is added to the RNA sample, the RNA partitions in to the butanol as a quaternary ammonium salts (CTAB salt) leaving neutral contaminants in the aqueous phase (Langridge *et al.*, 1980; Mackey and Chomczynski, 1996).

Thus, this purification step can remove proteins and insoluble material from nucleic acid samples. The use of NaCl in the purification steps dissolves the CTAB-RNA complex, allowing the RNA to be partitioned back in to the aqueous phase so CTAB, polysaccharides and other contaminants can be removed by chloroform extraction (Chang *et al.*, 1993). CTAB method may be useful for other plant species containing high levels of polysaccharides and RNases as a result of previous treatments such as dehydration or water deficit (Camacho-Villasana, 2002). The average execution time for this protocol was approximately 3-4 h, handling 10 samples at the same time.

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