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Protocols

An Improved Method for Isolating RNA From Dehydrated and Nondehydrated Chili Pepper (*Capsicum annuum* L.) Plant Tissues

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Abstract. High-quality RNA is important in studying gene expression. This report describes an improved method for isolating intact purified RNA from dehydrated organs of chili pepper plants. Common RNA extraction protocols have produced poor yields because dehydrated leaves accumulate polysaccharides and RNases. Our protocol is based on a guanidine thiocyanate extraction combined with additional purification steps using butanol and the ionic detergent CTAB (cetyltrimethylammonium bromide). Using this protocol, RNA yields ranged from 40-70 μg of total RNA per 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 per gram of fresh tissue).

Key words: *Capsicum annuum,* chili pepper, dehydrated tissue, polysaccharides, RNA isolation

Abbreviations: CTAB, cetyltrimethylammonium bromide; DEPC, diethyl pyrocarbonate; ELIP, early light induced protein.

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 dehydrated chili pepper (*Capsicum annuum* L.) leaves. RNA degraded, leading to low yields and poor-quality mRNA. Isolating high-quality RNA has proven difficult in a number of plant species, notably pine (Chang et al., 1993; Stokes et al., 1990) and wounded tubers of potato (Solanaceae family) (Logemann et al., 1987). Isolating high-quality genomic DNA from *Capsicum* is also reportedly difficult, particularly in infected tissues (Prince et al., 1997).

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Poor yields and low-quality degraded RNA may result from inefficient cell lysis or high levels of nuclease activity. RNA degradation during extraction from chili pepper may be due in part to the increase in RNases associated with dehydrated tissue (Chang et al., 1993). However, these problems can be alleviated using chaotropic agents, such as guanidinum isothiocyanate. A greater challenge in isolating high-quality RNA is the aqueous byproducts of secondary metabolism that accumulate in many higher plants, especially in mature tissues. These secondary metabolites, including phenolics and high molecular weight polysaccharides, can interfere with isolating and purifying biologically active nucleic acids if they copurify during extraction (Loomis, 1974; Stokes et al., 1990). These problems may be worse in dehydrated 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 bind to the RNA during the extraction process (Chang et al., 1993).

We developed a method for isolating high-quality RNA from dehydrated chili pepper leaves, stems, and roots, based on the guanidinium thiocyanate–phenol extraction protocol (Chomczynski and Sacchi, 1986). It is combined with a purification protocol that employs the ionic detergent CTAB (cetyltrimethyl-ammonium bromide), originally developed for the extraction of nucleic acids from agarose gels (Langridge et al., 1979).

Materials and Methods

Plant material and water-deficit treatment

In a greenhouse in Riverside, Calif, plants of *C. annuum* (L.) Serrano var. Tampiqueño 74 were grown at 26°C without supplemental lighting. Leaves, stems, and roots were harvested from 6-week-old plants. Control organs were frozen immediately. To achieve a water-deficit stress, detached leaves, stems, or roots were weighed and incubated at room temperature to allow a loss of 15% fresh weight. This condition was the time 0 treatment. The detached plant organs were transferred to plastic bags, sealed, and maintained at room temperature for 6 and 12 h. Nondehydrated detached leaves, stems, or roots were placed in water for the times indicated above and used as additional controls. For the salinity treatment, detached leaves were placed in 0.25 M NaCl until the leaves in the dehydration treatment at time 0 were ready (~1 h). After the times indicated, detached organs were frozen in liquid nitrogen and kept at -70°C until needed.

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 7.7])

RNA extraction protocol

1. 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.
2. Grind 200 mg of tissue in liquid nitrogen. Transfer the homogenized tissue-liquid nitrogen slurry into the microfuge tube prepared in Step 1.
3. Vortex the sample vigorously, and incubate at room temperature for 5 min.
4. Add 200 µL of chloroform-isoamylalcohol (24:1). Vortex for 1 min. Let the sample stand at room temperature for 12 min.
5. Spin at maximum speed in a microfuge for 15 min. Transfer the aqueous phase to a new microfuge tube.
6. Add 500 µL of isopropanol and mix by inversion. Let the sample stand at room temperature for 7 min.
7. 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.
8. 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.
9. Dissolve the RNA pellet in 200 µL of TESAR.
10. 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.
11. Spin in a microfuge at maximum speed at room temperature for 5 min to resolve the phases.
12. Remove the upper butanol phase and transfer to a new microfuge tube.
13. Re-extract the lower layer with 200 µL of bu/CTAB.
14. Remove the upper layer and combine with the bu/CTAB collected in Step 12. The RNA is a CTAB salt and is soluble in butanol.

15. Add 150 µL of 0.2 M NaCl to the combined butanol phases. Vortex for 30 s and spin for 5 min.
16. 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.
17. Re-extract the butanol layer with 150 µL of 0.2 M NaCl.
18. Collect the lower layer and combine with the aqueous phase retained in Step 16. Add 300 µL of chloroform to the combined aqueous phases. Vortex for 30 s.
19. Spin at maximum speed at 4°C for 5 min. Transfer the upper aqueous phase to a new tube.
20. 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.
21. Spin at maximum speed at 4°C for 10 min.
22. Dry the pellet and resuspend in DEPC-treated H_2O.

RNA blot analysis

To confirm the integrity of RNA samples, the RNA was resolved using gel electrophoresis and/or blotted and hybridized. RNA quantification was performed spectrophotometrically at wavelengths of 260 and 280 nm. Of the total RNA isolated from chili pepper leaves, stems, or roots stressed by dehydration, 15 µg was electrophoresed on a 1.2% denaturing agarose gel. RNAs were visualized by staining with ethidium bromide to confirm equivalent RNA loading per lane. RNAs were blotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech) and autocrosslinked with UV light for 3 min. Membranes were hybridized in hybridization buffer (50% formamide, 5 X SSPE, 2 X Denhardt's solution, 0.075 mg/mL herring sperm DNA) at 55°C with a ^{32}P-dCTP-labeled early light induced protein (ELIP)–like cDNA fragment from chili. The ELIP fragment, ODE1 (accession number AFI69203), was isolated from differential display experiments (unpublished results). The membranes were washed with 2 solutions. Solution 1 was 1 X SSPE and 0.1% SDS. Solution 2 was 2 X SSPE and 0.1% SDS. After one wash in solution 1 at 55°C for 5 min, the membranes were washed twice with solution 1 (room temperature, 10 min each) and twice with solution 2 (room temperature, 5 min each). Washed membranes were exposed to BIOMAX X-ray films using an intensifying screen for 48 h.

Results and Discussion

Several established methods were initially used to isolate RNA from dehydrated chili pepper tissues. These included standard and modified LiCl precipitation, nonphenolic extractions, and precipitations with various salts (Schuler et al., 1990; Chang et al., 1993; Bugos et al., 1993). All failed to render good quality and high yields of RNA (Figure 1). Yields 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 extractions were largely insoluble.

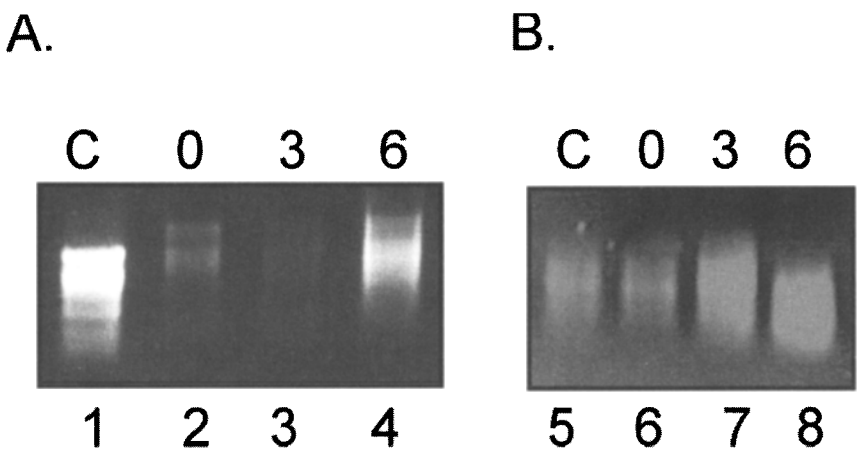

Figure 1. Agarose gel electrophoresis of total RNA from chili pepper using 2 extraction procedures. The methods described by (A) Bugos et al. (1995) and (B) Schuler et al. (1993) were used to isolate RNAs from chili pepper plant leaves: untreated control (lanes 1 and 5) and leaves dehydrated for 0 (lanes 2 and 6), 3 (lanes 3 and 7), and 6 (lanes 4 and 8) h. The RNAs were resolved on agarose gels.

Polysaccharide contamination and an increased amount of RNases may have limited RNA isolation from dehydrated chili pepper 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).

Our protocol allowed recovery of intact, high-quality RNA from leaves (Figure 2A), stems, and roots (Figure 2B). Distinct rRNA bands were apparent in the nontreated and dehydrated 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 per gram of fresh weight, depending on the tissue. In stems, the yield was approximately 200 μg per gram of fresh weight from unstressed and stressed tissues, whereas in leaves and roots, the yield was higher and included tissues from severely water-stressed plants. Yields for unstressed and stressed leaves (0 and 3 h) were 250 μg. In all cases, the RNA obtained was of high quality and integrity.

The quality of the RNA was also judged by hybridization to a specific RNA. Total RNA was used in RNA blot analyses. A cDNA fragment from chili pepper exhibiting 75% identity with genes encoding ELIP from *Craterostigma plantagineum* (accession number Q01931; DSP-22) and *Arabidopsis thaliana* (accession number 15228866; At3g22840) was used as a probe. RNA blots showed that ELIP-like RNAs accumulated to higher levels in nondehydrated than in dehydrated leaves (Figure 3A). Compared with the 0 h treatment, the RNA level increased in leaves that were maintained in a dehydrated state for 12 h. In contrast, no differences in RNA levels were detected in stressed and unstressed stems (Figure 3B). ELIP-like RNAs were not detected in stressed or unstressed roots

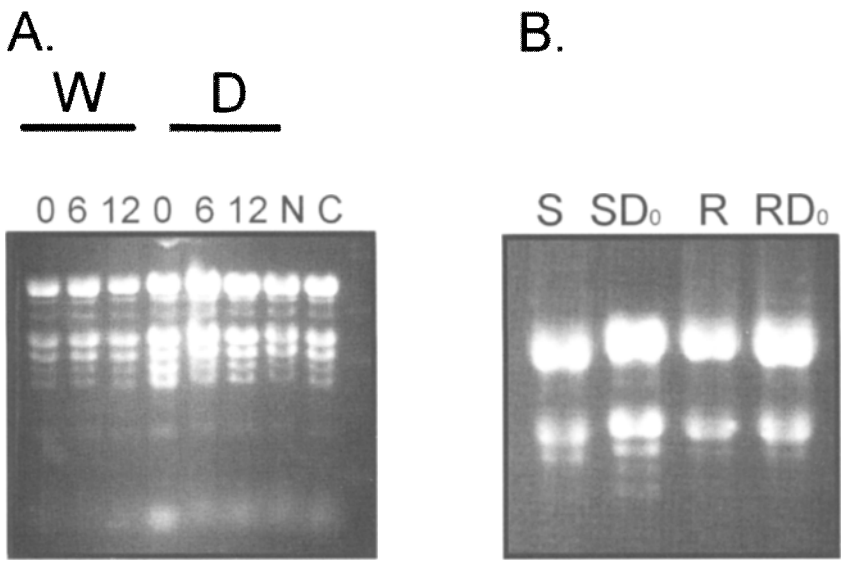

Figure 2. Agarose gel electrophoresis of total RNA extracted from chili pepper organs using guanidine thiocyanate and purified using a CTAB/butanol extraction. (A) RNA extracted from detached leaves. RNA from leaves placed in water (W) for 0, 6, and 12 h. RNA from detached leaves stressed by dehydration (D) for 0, 6, and 12 h. RNA from leaves subjected to NaCl treatment (N). Control leaves without treatment (C). (B) RNA from stems and roots. RNA from stems with no treatment (S). RNA from stems stressed by dehydration at time 0 (SD_0). RNA from roots with no treatment (R). RNA from roots stressed by dehydration at time 0 (RD_0).

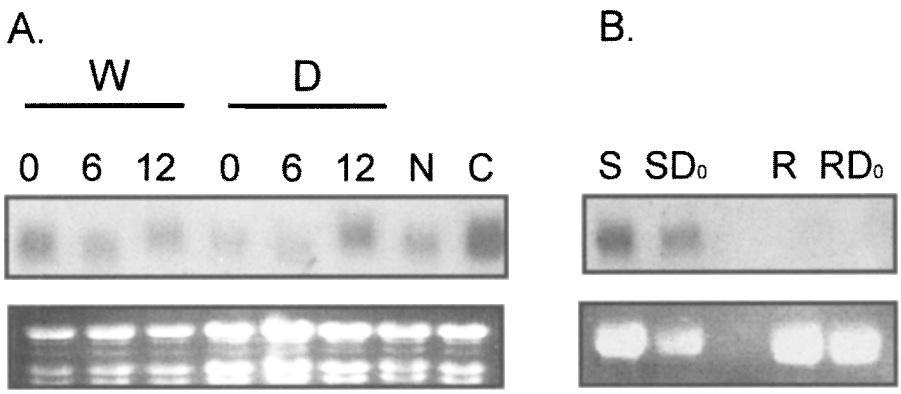

Figure 3. RNA blot analysis of total RNA hybridized with ^{32}P-labeled–ELIP cDNA. In each lane, 15 µg total RNA of chili pepper was loaded, blotted, and hybridized. Total RNA shown in the lower panels was stained with ethidium bromide. **(A)** Total RNA was extracted from detached leaves placed in water (W) for 0, 6, and 12 h or stressed by dehydration (D) for 0.6 and 12 h. RNA from leaves subjected to NaCl treatment (N). Control leaves with no treatment (C). (B) RNA from stems and roots. RNA from stems with no treatment (S). RNA from stems stressed by dehydration at time 0 (SD_0). RNA from roots with no treatment (R). RNA from roots stressed by dehydration at time 0 (RD_0).

(Figure 3B) because the transcript of this ELIP accumulates in cells that contain chloroplasts.

This two-part RNA isolation procedure was successful in isolating RNA from a species that is recalcitrant to RNA isolation. The efficiency of this method is evident in that it (1) is a rapid, single-step protocol; (2) promotes efficient lysis of plant cells; and (3) inhibits ribonuclease activity due to the presence of 4 M guanidinium thiocyanate (Cox, 1968; Chomczynski et al., 1987). RNA yields increased to 250 μg of RNA per gram of fresh tissue by combining the guanidinium extraction and the CTAB/butanol purification protocols. When CTAB is added to the RNA sample, the RNA partitions into the butanol as a quaternary ammonium salt (CTAB salt) leaving neutral contaminants in the aqueous phase (Langridge et al., 1980). 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 into the aqueous phase so CTAB, polysaccharides, and other contaminants can be removed by chloroform extraction (Chang et al., 1993).

This 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. The average execution time for this protocol was approximately 3-4 h, handling 10 samples at the same time.

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