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Notes & Tips

Using silica particles to isolate total RNA from plant tissues recalcitrant to extraction in guanidine thiocyanate

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Abstract

The most commonly used protocol of the RNA isolation, the guanidine thiocyanate method, was unsuitable for recalcitrant plant tissues containing a large amount of storage proteins and secondary metabolites. We demonstrated that RNA could bind to the silica particles, which have been used successfully in DNA isolation from various sources, under a high concentration of NaCl in the presence of ethanol and sodium acetate. Based on this observation, an efficient, inexpensive, and highly reproducible technique, the acid phenol-silica method, was developed to isolate high-quality RNAs from various plant tissues recalcitrant to extraction in guanidine thiocyanate.

Obtaining RNA of high quality and quantity is a crucial prerequisite for many molecular biology methods such as complementary DNA (cDNA)[1] library construction, reverse transcription–polymerase chain reaction (RT–PCR), differential display PCR, and rapid amplification of cDNA ends (RACE). RNA isolation from plant tissues, however, may be quite challenging. Some plant tissues, such as fruits, storage tubers, and seeds, contain a large amount of storage proteins, polysaccharides, and polyphenolics or other secondary metabolites that would coprecipitate with the RNA and constitute the major obstacle of RNA isolation [1,2]. The most commonly used protocol of the RNA isolation, based on guanidine thiocyanate or commercially available as TRIZOL reagent (Invitrogen), originally was developed for animal tissues [3], especially for those rich in RNase, but was unsuitable for recalcitrant plant tissues [2,4]. Several alternative methods have been developed to overcome this problem, including sodium dodecyl sulfate (SDS)/phenol [5], hexadecyltrimethylammonium bromide [2], and urea [6] methods. These improved methods, however, are labor-intensive and time-consuming.

We attempted to clone the cDNAs of storage proteins from mung bean (*Vigna radiata*) seeds but failed to isolate the intact RNA by using TRIZOL reagent and other common methods. The main problem was that the homogenate of mung bean seeds formed a very sticky glue-like gel in guanidine thiocyanate extraction buffer that could not be properly separated into two phases by centrifugation. Although a small amount of aqueous phase could be collected after extensive high-speed centrifugation, the pellet of RNA precipitated by isopropanol still was sticky and was difficult to completely dissolve in water even after incubation at 60 °C.

To address this problem, we developed a reproducible acid phenol-silica extraction protocol based on the acid phenol extraction method [3] and silica binding procedure [7]. Plant tissues (0.3–0.5 g) were frozen in liquid nitrogen and ground into a fine powder that was immediately mixed with 400 μl of extraction buffer (5 M NaCl and 100 mM Tris–HCl [pH 8.0]), 100 μl of 2-mercaptoethanol, and 1 ml of Tris-saturated phenol/chloroform (1:1, pH 8.0) in

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[1] *Abbreviations used:* cDNA, complementary DNA; RT–PCR, reverse transcription–polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; RT, room temperature; PVP, polyvinylpyrrolidone; DEPC, diethylpyrocarbonate; M-MLV, Moloney murine leukemia virus.

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a 2-ml polyethylene tube. After vortexing and incubating at room temperature (RT) for 1 min, the mixture was centrifuged at 12,000 rpm for 1 min. The aqueous phase was transferred to a new 2-ml tube. Then 25 μl of 10% polyvinylpyrrolidone (PVP, Sigma P-5288) solution, 25 μl of 5% *N*-lauroyl-sarcosine (Sigma L-5125), 300 μl of 3 M sodium acetate (pH 4.8), and 1 ml of water-saturated phenol/chloroform (1:1) were added and vortexed several times. After centrifugation for 5 min at 12,000 rpm, the aqueous phase was transfered to a 1.5-ml tube, followed by the addition of 200 μl ethanol and 100 μl silica suspension (1 g/ml) to absorb the RNA. The mixture was vortexed and incubated for 1 min at RT and then centrifuged at 12,000 rpm for 15 s to precipitate the silica particles. The pellet was washed with 70% ethanol two times to completely remove the detergent and salts from the silica particles. It is very important to resuspend the silica pellet by pipeting and vortexing during the ethanol wash steps. The pellet was collected by centrifugation at 12,000 rpm for 15 s and dried up in a vacuum to remove residual ethanol. The RNA was eluted by resuspending the silica pellet with 50 μl of water and centrifugation at 12,000 rpm for 5 min at RT. The supernatant was carefully transferred to a new tube without disturbing the silica particles. Incubating the silica pellet for 5 min at RT before centrifugation would increase the RNA yield. RNA elution efficiency is pH dependent, with optimal pH in the range of 7.0 to 8.5. Diethylpyrocarbonate (DEPC)-treated water to elute the RNA is not recommended here if the sample is prepared for RT–PCR because too much anti-RNase treatment might become a source of problems [8] when insufficient autoclave leaves a trace amount of DEPC that severely inhibits the enzymatic reaction. The whole procedure described here could be completed within 45 min.

The silica suspension (1 g/ml) used in our protocol was prepared as reported previously [7,9,10]. Briefly, 6 g silica (Sigma S-5631) was resuspended with 50 ml of water, vortexed, and allowed to settle for 24 h. The supernatant containing very fine particles was aspirated and discarded. This process guarantees that the silica particles used in the purification step have the proper size (1–5 μm) that could be recovered/removed completely by centrifugation. The pellet was resuspended with 6 ml of 0.1 M HCl and then aliquoted and stored at 4 °C.

Using this protocol, we successfully isolated high-quality RNAs from developing seeds (Fig. 1A, lanes 1 and 2), germinating seeds (lane 3), dry seeds (lanes 4–12), leaves (lane 13), and shoots (lane 14) of various plant species. RNA samples showed intact 25S and 18S bands on agarose gels (Fig. 1A), suggesting that little or no RNA degradation had occurred during the extraction. Spectrophotometer analysis revealed that the A_{260}/A_{280} ratios of most RNA samples ranged from 1.9 to 2.0 (Supplementary Table 1), indicating that the RNA isolated by this approach was largely free of contaminating proteins, DNA, polyphenolics, and polysaccharides. A comparison of yields based on the spectrophotometric determination

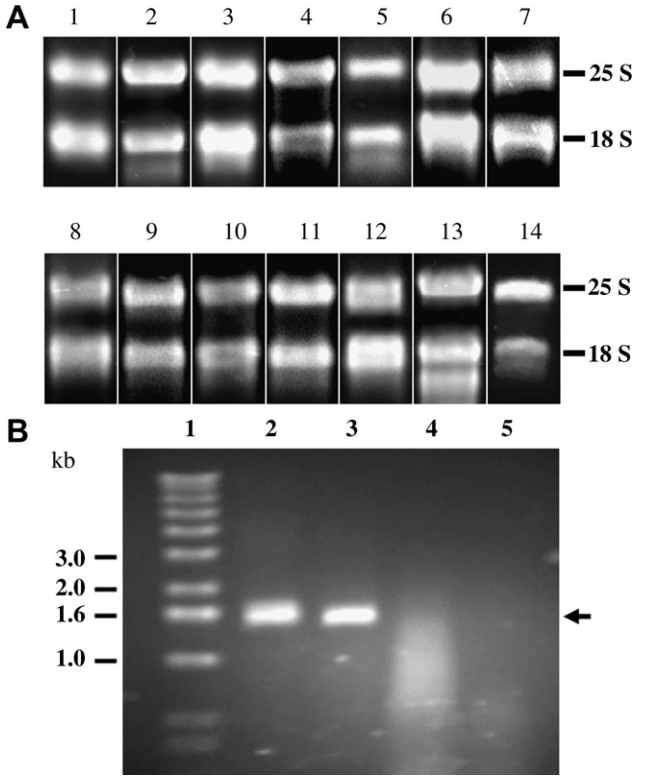

Fig. 1. (A) Agarose gel electrophoresis of RNA samples isolated by the acid phenol-silica extraction method. Lanes 1 and 2: developing seeds of *Vigna radiata* and *Bauhinia blakeana;* lane 3: germinating (36 h) seeds of *V. radiata;* lanes 4 to 12: dry seeds of *V. radiata, Sesbania rostrata, Albizzia julibrissin, Ricinus communis, Jatropha curcas, Solanum americanum, Malus pumila, Oryza sativa,* and *Daucus carota;* lane 13: leaves of *J. curcas;* lane 14: shoots of *Potentilla griffithii.* (B) RT–PCR of mung bean 8S globulin gene from RNA isolated by the acid phenol-silica extraction method (lanes 2 and 3) and by use of TRIZOL reagent (lanes 4 and 5). Lane 1: molecular weight marker; lanes 2 and 4: amplification with 8S globulin α-subunit gene-specific primers; lanes 3 and 5: amplification with 8S globulin β-subunit gene-specific primers. Arrow indicates the RT–PCR products.

of the RNA samples (Supplementary Table 1) showed that the RNA yields of the acid phenol-silica method were significantly lower than those of the guanidine thiocyanate method. The A_{260}/A_{280} ratios of the RNA samples isolated by the guanidine thiocyanate method, however, were very low (Supplementary Table 1), indicating that the heavy contamination of proteins, DNA, polyphenolics, and polysaccharides contributed to the high calculated yields. To examine the quality of the RNA isolated by the acid phenol-silica method, RT–PCR of the mung bean 8S globulin gene was performed using Moloney murine leukemia virus (M-MLV) RNase H^- (Promega) and LA Taq DNA polymerase (TaKaRa). To synthesize the cDNAs, 1 μg total RNA was annealed with the adapter primer ZF389 (5′-GTAATACGACTCACTATAGGGCACGCGTGGT TTTTTTTTTTTTTTTTTTT-3′). Two pairs of the gene-specific primers ZF373 (5′-TTACAACTCCGCCAATAT-3′) and ZF373 (5′-TCAGGTTGGGCTCATTTA-3′) for the mung bean 8S globulin α-subunit (Vr8SGa) and ZF377 (5′-GGCACGAGTATGGTGAGA-3′) and ZF378

(5′-GTCGGACACGTTAATTCG- 3′) for the β-subunit (Vr8SGb), based on the sequences of GenBank DQ538333 and DQ538335, were designed to obtain the full-length cDNAs of *Vr8SGa* and *Vr8SGb*. Expected sizes (~1.5 kb) of two specific RT–PCR products were amplified with total RNA samples isolated by our acid phenol-silica extraction method (Fig. 1B, lanes 2 and 3), whereas no RT–PCR product was amplified with total RNA samples isolated by using TRIZOL reagent (lanes 4 and 5). Sequencing analysis revealed that the two RT–PCR products were the cDNAs of *Vr8SGa* and *Vr8SGb*, respectively, which were submitted to GenBank under accession numbers EF990627 (*Vr8SGa*) and EF990626 (*Vr8SGb*). The failure of the RT–PCR with total RNA isolated by using TRIZOL reagent (Fig. 1B, lanes 4 and 5) could be attributed to the impurities in the RNA samples. We noted that the color of the RNA sample isolated by using TRIZOL reagent from *Jatropha curcas* seeds was dark brown, whereas the RNA sample isolated from the same tissues by the acid phenol-silica extraction method was clear.

Because we observed that maintaining an appropriate pH (~8.0) of the extraction solution is a crucial factor in the success of RNA isolation from plant tissues, especially from the fresh plant tissues that are rich in organic acids, 100 mM Tris–HCl (pH 8.0) was included to increase the buffer capacity of the extraction solution. To monitor the pH change of the homogenate during extraction, pH indicator dye (phenol red or litmus) could be added to the extraction buffer at a 0.0001% (w/v) concentration. Alternatively, a pH test strip could be used to check the pH of the homogenate in case the pigments interfere with the pH indicator dye. A major obstacle of RNA isolation from plant tissues is the presence of polyphenolics and polysaccharides that coprecipitate with the RNA and inhibit the enzymatic reactions in subsequent steps [11]. In our protocol, the PVP and *N*-lauroyl-sarcosine were employed to remove the contaminants.

Silica particles (glass beads) and glass milk were widely used in DNA isolation from various sources [7,10,12,13]. In this study, we demonstrated that RNA could also bind to the silica particles under a high concentration (> 2 M) of NaCl in the presence of ethanol and sodium acetate. Therefore, silica particles provide a very efficient, inexpensive, and rapid means for RNA isolation from various plant species, as shown in Fig. 1. Although a variety of commercial silica-based columns, including the columns for DNA recovering from agarose gel, PCR cleanup columns, plasmid purification columns, and genomic DNA extraction columns, could also bind RNA, most of them showed poor discrimination between RNA and contaminants derived from plant tissues (e.g., polysaccharides, polyphenolics) and, hence, are not reliable for RNA isolation from plants (data not shown).

In conclusion, the method we have proposed in this report is an efficient, inexpensive, and highly reproducible technique to isolate total RNA from plant tissues recalcitrant to extraction in guanidine thiocyanate. By using this acid phenol-silica extraction protocol, a large amount of high-quality RNA, which is required for cDNA library construction, RT–PCR, RACE, and Northern blot analysis, could be obtained from various plant species and tissues.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2007.11.030.

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