Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch

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Isolation of high-quality RNA from plant seeds is very critical for seed-specific gene analysis. However, seed endosperm contains very high levels of starch, which cause the solidification of samples in the guanidine isothiocyanate (GITC)-based RNA extraction buffers, such as GITC-phenol-chloroform buffer (1), TRizol® reagent (Invitrogen, Carlsbad, CA, USA) and the RNeasy® kit (Qiagen, Valencia, CA, USA). These current RNA small- and large-scale extraction protocols typically either fail to yield RNA or result in reduced yields with poor quality. Tissue with a high level of starch can also hinder resuspension of precipitated RNA or contaminate the RNA pellet by co-precipitation (2). Although several protocols were developed to remove polysaccharide and phenolics contamination from plant RNA (3–5), most of the existing protocols are time-consuming and labor-intensive. Additionally, there are no satisfactory protocols for isolating RNA from starch-rich seeds, such as wheat, rice, and maize, using <1 g of seed material. In this report, we developed a rapid method for extracting high-quality RNA from wheat, rice, and maize seed endosperms. It can be used to isolate seed-derived RNA in both small- and large-scale extraction protocols, and it directly overcomes the problems of solidification of samples in extraction buffer, starch contamination, and starch co-precipitation.

Wheat (Triticum aestivum L.) seeds “Bobwhite” were collected around 20 days post-anthesis. Rice (Oryza sativa L.) and maize (Zea mays L.) seeds were collected 23 days post-anthesis. For the new RNA extraction procedure, 50–100 mg seeds were ground to a fine powder in liquid nitrogen with prechilled mortar and pestle. The flour sample was then transferred into a prechilled 1.5-mL RNase-free microcentrifuge tube. A 400-µL extraction buffer I [100 mM Tris, pH 8.0, 150 mM LiCl, 50 mM EDTA, 1.5% sodium dodecyl sulfate (SDS), 1.5% 2-mercaptoethanol] aliquot was immediately added to the seed powder. After mixing the content with vigorous vortex mixing, 250 µL phenol-chloroform mixture (1:1, pH 4.7) were added, and the samples were mixed well by inversion. Samples were then centrifuged immediately at 13,000x g for 15 min at 4°C. The upper aqueous phase (around 250 µL) was carefully transferred to a new 1.5-mL RNase-free microcentrifuge tube containing 250 µL extraction buffer II [70% guanidinium sulfate (w/v), 0.75 M sodium citrate, 10% lauryl sarcosine, 2 M sodium acetate, pH 4.0]. Samples were mixed by gentle inversion and incubated at room temperature for 10 min. After the incubation, 200 µL chloroform-isooamyl alcohol (24:1) were added, and the samples were then centrifuged at 13,000x g for 15 min at 4°C. To the recovered supernatant (around 450 µL), 300 µL isopropanol, and 250 µL 1.2 M sodium chloride were added. The samples were then mixed by inversion and put on ice for 15 min. The sample was centrifuged at 13,000x g for 15 min at 4°C, then the supernatants were discarded, and the RNA pellets were washed carefully with 400µL 70% ethanol. The RNA pellets were then dried for 15–20 min at room temperature in a laminar flow hood and were resuspended in the appropriate volume of RNase free water (e.g., 50 µL) and stored at -70°C. This protocol can also be used for seed tissue above 1 g by adjusting the volumes of buffers accordingly. All the solutions were prepared from 0.1% diethylpyrocarboxate (DEPC)-treated water and powder stocks. Glassware used in this protocol was cleaned with detergent, filled with 0.1% DEPC, incubated at 37°C overnight, and then autoclaved for 30 min.

Standard RNA extraction methods using GITC-phenol-chloroform (1), RNeasy kit, or TRizol® reagent failed to produce satisfactory RNA when attempting to extract RNA from starch-rich seed species such as wheat, rice, and maize (data not shown). A large starch percentage causes sample solidification when RNA extraction is attempted with buffers from the above mentioned protocols. In addition, starch tended to co-precipitate with the RNA pellet, making it very difficult to redissolve the RNA in water. Although a seed flour sample could be easily disrupted in phenol-SDS RNA extraction buffer when a standard phenol-SDS protocol was used (2), this protocol failed to produce high-quality RNA due to the serious degradation of the RNA as indicated by the degradation of 28S and 18S ribosomal RNA (rRNA) as well as a smear of smaller sized RNAs (Figure 1). The quality of RNA produced by the phenol-SDS method was not sufficient for reverse transcription PCR (RT-PCR) analysis (data not shown).

The new protocol reported here resulted in the rapid isolation of high-quality RNA from starchy seed samples of 50–100 mg. The quality of the RNA prepared by this method was demonstrated by intact sharp 28S and 18S rRNA bands and the lack of RNA degradation on agarose gels (Figure 1). We have successfully used this RNA for both RT-PCR and Northern blot analyses (data not shown). In this protocol, the following steps and rationale were used: (i) Extraction buffer I, which contains SDS, effectively dissolved seed sample containing high concentrations of starch. Thus, the problem of sample solidification due to excess starch in the seed was resolved. Solidification could not be avoided when using other TRizol®- or GITC-containing buffers. The addition
of 2-mercaptoethanol aided in RNase and oxidation protection. (ii) Acid phenol-chloroform (1:1, pH 4.7) was used to promote cell lysis and for DNA and protein separation. After phenol-chloroform extraction, the bulk of the starch was removed. (iii) A GITC-based extraction buffer II ensured RNase inhibition. Extraction buffer II, which was added immediately after the phenol-chloroform extraction, prevented the degradation of RNA. Guanidinium sulfate, a strong protein denaturant, may be replaced by other guanidine salts such as in TRIZol. (iv) High concentration of salt (1.2 M sodium chloride) and low isopropanol concentration (30%) were added into the RNA precipitation step to provide maximum starch solubility. This modification effectively maintained carbohydrates in a soluble form, while helping to precipitate the RNA in high purity. With little carbohydrate impurities, the RNA pellet was easily redissolved in water.

Wheat seed RNA prepared by our protocol was of high purity with low polysaccharide and protein contamination, which was indicated by the A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios (6), which ranged from 1.96 to 2.00 and 1.80 to 1.90, respectively. In addition, high purity RNA was also prepared by this protocol from rice and maize seeds. A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios of rice seed RNA prepared by our protocol ranged from 1.93 to 1.97 and 1.99 to 2.03, respectively, and A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratios of maize seed RNA prepared by our protocol ranged from 1.95 to 2.01 and 1.86 to 1.92, respectively (Table 1). The average RNA yields from 100-mg seed samples of wheat, rice, and maize were 48.5, 50.3, and 51.5 µg respectively (Table 1), which were approximately double the corresponding RNA yields from the phenol-SDS protocol. The RNA purity from a 100-mg seed sample was acceptable for RT-PCR, cDNA library construction, or Northern blot analysis. On the other hand, the average A₂₆₀/A₂₈₀ of wheat, maize, and rice seed RNA prepared by the standard phenol-SDS protocol ranged from 1.54 to 1.69, which indicated that the samples contained protein contamination. Furthermore, additional impurities in these samples were noted as the average ratio at A₂₆₀/A₂₃₀ of wheat, rice, and maize seed RNA prepared by the standard phenol-SDS protocol ranged from 0.34 to 0.90, far less than the accepted A₂₆₀/A₂₈₀ value of 1.80. These results indicated that RNA prepared by phenol-SDS was low in purity and that the purity was highly variable as implied by the high standard errors of A₂₆₀/A₂₈₀ or A₂₆₀/A₂₃₀ (Table 1).

In conclusion, we report a new, efficient, and reliable RNA extraction method for wheat seed endosperm. This method can also be used for RNA extraction from other plant seeds with high starch content, such as rice and maize. By this method, the isolated RNA from 50–100 mg seed tissue was of high quality and quantity, and it could be used for RT-PCR analysis and Northern blot analysis. Therefore, this method would be especially useful for rapid production of RNA for gene expression analysis of plant tissue with high starch content.

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**COMPETING INTERESTS STATEMENT**

The authors declare no competing interests.
REFERENCES


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