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Full Length Research Paper

Comparative study of different protocols for isolation of RNA from polysaccharides and polyphenolics rich plant tissues

Subodh Gupta, A. K. Verma* and Laxmi Chand

Department of Biochemistry, College of Basic sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar – 263 145, India.

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The isolation of high quality ribonucleic acid (RNA) from plant has been a difficult problem due to presence of high amount of polysaccharides, polyplenolics and nucleases that mediate RNA degradation. After comparative analysis of four popular protocols for RNA isolation, a modified method was developed which gave maximum yield with purity. Modified method is not only suitable for the isolation of intact RNA from immature seeds of legumes, containing high amount of starch, polyphenolics and several nucleases but also works successfully with other plant organs like leaves, epicotyls and callus. The yield of total RNA extracted by this method varies from 71 to 417 μg/g tissues.

Key words: RNA isolation, polysaccharides and polyphenolics rich plant tissues, pegionpea and immature seeds.

INTRODUCTION

Purification of intact ribonucleic acid (RNAs) is fundamental for the study of gene expression and for isolation of new gene product via cDNAs. RT-PCR makes it now possible to detect and analyze the transcription products of virtually any gene. These studies require RNA from a definite part of different plant species.

A number of methods are available for isolation of RNA that involve the use of denaturants to inactivate ribonuclease (RNase) such as cetyltrimethyl ammonium bromide (CTAB) (Logemann, 1987) SDS, β marcaptoethanol, guanidinium salts (Cox 1968; Chirgwin et al., 1979; Chancznski and Sacchi, 1987). Triisopropyl naphthalene sulfonic acid (Monoz et al., 1997), Diethyl pyrocabonate (DEPC) (Fedoresak and Ehrerberg, 1966) and Phenol extraction or CsCl density gradient centrifugation (Sambrook et al., 1989). However, these methods are not much successful for isolation for total RNA from polysaccharide or starch and polyphenolics rich tissues, and gave very low yield due to degradation of RNA. RNA degradation starts just after the cell lysis which is due to binding of phenolic terpenoids to nucleic acids (Maliyaka, 1992). The isolation of high quality total of RNA. RNA degradation starts just after the cell lysis RNA from plant containing a high content of polyphenolics such as legume seed tissues was difficult and also gives poor yield of RNA which might be due to the conversion of insoluble polysaccharide into soluble polysaccharides, which contaminated the RNAs. Darvill and Albursheim (1984) also reported similar physicochemical properties and found that pallet did not dissolve completely in second step of several RNA isolation protocols. The author has isolated the total RNA by the method described from immature seeds of pigeonpea (Gupta et al., 2006, 2009).

In the present investigation we developed a method for the isolation of large amounts, high quality RNA from plant tissues; those were rich in polysaccharides or starch and polyphenolics.

MATERIALS AND METHODS

For providing RNase tree environment, all the glassware's were

*Corresponding author. E-mail:akv72@yahoomail.com.

Table 1. RNA preparation from Pigeon pea (*Cajanus cajan* L.) immature seeds (18 days after flowering) by modified and other methods (the values represent mean ±SD from three experiments and each experiment contains 3 replicates).

Method	Ratio $OD_{260/280}$	DNA	Yield μg RNA/g immature seeds	% Poly (A) + RNA from total RNA
CTAB	1.72 ±0.02	D	17.1 ±0.3	0.45
Guanidine + CTAB	1.27 ± 0.1	ND	101.1 ±0.46	0.72
Guanidine + CsCl	1.23 ±0.02	ND	6.1 ±0.4	ND
AGPC	ND	ND	ND	ND
Modified	1.98 ± 0.25	ND	198±0.42	1.01

ND = Not detectable D = Detectable. Yield was calculated assuming A_{1cm} at λ_{260mm} = 40 μg/ml

kept in oven at 180°C for 2 h and plastic wares were washed properly with tape water and boiled in IN KOH with 10 mM EDTA for 20 min, then rinsed with sterilized mill Q water or 0.1% (v/v) diethylpyrocarbonate (DEPC) treated water. All the chemicals were purchased from sigma.

Plant materials

The developing seeds, leaves and other tissues were collected from crop research centre, G.B. Pant University of Agriculture and Technology, Pantnagar (India). The materials were transported from field to laboratory in liquid nitrogen. It can be store in liquid nitrogen up to several weeks.

Preparation of homogenate and extraction of RNA

Plant materials (one gram), mainly new growing but not mature because they became hard and contain less quantities of RNA, was harvested and prepare homogenate by grinded in liquid nitrogen inpre-cooled mortar and pestle till fine powder was obtained. Homogenate was collected in 50 ml centrifuge tube and immediately dissolve in buffer I containing 5 ml 4 M Guanidine Thiocynate (pH 7.0) mM Tris- HCl (pH 7.5), 0.1% (w/v) insoluble polyvinyl pyrrollidone (PVP), 100 mM β marcaptoethanol, the tube was kept for 30 min on ice. The homogenate may be stored for 1 to 2 days at 4°C. The homogenate was centrifuged at 10,000 g for 30 min at 4°C**.** Supernatant was collected in a fresh 50 ml centrifuge tube and added 225 μl 2 M sodium acetate (pH 4.1) and then 2 volume of absolute ethanol. The contents were mixed by inverting the tube and placed in –20°C for at least 1 h for precipitation of nucleic acids. The tube was centrifuged at 10,000 g for 20 min at 4°C. The pellet was dissolved in 10 ml of buffer II containing 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 100 mM NaCl, and 0.2 (w/v) SDS. The mixture was homogenized in slow speed homogenizer and then added 10 ml phenol (equilibrated with 0.1M Tris HCl (pH 8.0): chloroform: isoamyl alcohol (25:24:1). The mixture was vortexed for one min and centrifuged at 1000 g for 5 min at 20°C. Aqueous layer was collected in 50 ml fresh tube and added 10 ml chloroform: isoamyl alcohol (24:1) mixture. This mixture was again mixed and vortexed followed by centrifugation at 10,000 g for 5 min and collect aqueous phase in fresh tube (50 ml) and adjusted pH 5.0 with 0.1 M acetic acid (check the pH with pH indicator). In this mixture 10 μl 1M MgCl2 and 2.5 ml 8M LiCl were added for selective RNA precipitation. The tube was kept at 4°C for overnight and then centrifuged at 14,000 g for 20 min. The supernatant was discarded tubes to store at –20°C for one weak and at –70°C for one month.

This method was also used for small scale (100 to 200 mg tissues) RNA isolation. The plant materials were kept in 1.5 ml eppendorf tube and grinded in liquid nitrogen with the help of blunt end needle to make fine powder. A volume of 500 μl buffer I was added. The tube was centrifuged in microfuge at 12,000 rpm for 20 min. The supernatant was collected and added 25 μl 2 M sodium acetate and 2 volumes ethanol for RNA precipitation at 20°C for 1 h. The pellet was collected by centrifugation at 12,000 rpm and dissolved in 700 μl Buffer II by vortexing. The mixture was centrifuged at 12,000 rpm for 10 min at room temperature. The aqueous phase was removed and one drop of 0.1 M acetic acid was added followed by addition of 1.0 μl 1 M $MgCl_2$ and 20 μl of 8 M LiCl. The eppendrof tube was then kept at 4°C for overnight. The pellet was recovered by centrifugation at 14,000 rpm for 20 min and dissolved in 50 μl of RNase free water.

The RNA concentration and purity were checked by taking absorbance at λmax 260 nm and $OD_{260\ 280}$ ratio, respectively. Integrity of the product was checked on 1.5% (w/v) agarose gel treated with 3% (v/v) formaldehyde. The gel was run in MOPS buffer (Lehraeh et al., 1977) and then stained in ethidium bromide (1 μg/ml) solution for 10 min and distained in sterilized distilled water for 5 min. This gel was visualized in UV light and then photographed.

RESULTS AND DISCUSSION

Several methods were used like CTAB, acid- guanidinium thiocynate–phenol–chlorform extract (AGPC), Guanidium –CsCl cushion centrifugation and also the combination of these methods to isolate the RNA from pigeonpea of the late stage of developing seeds. The yield from these methods and modified method are given in Table 1. It was observed that well established methods did not give high yield and good quality RNA. The modified method when used to isolate RNA from immature seeds, that were having high amount of polysaccharides or starch and polyphenolic compounds gave good quality (ratio OD 260 280 1.9 ± 0.2) and high yield (198 ± 0.4 μg RNA/g immature seeds). When poly RNA was fractionated from the isolated total RNA by Oligo dT column, the modified method gave highest yield (1% of total RNA) in comparison to other methods. While Poly (A) RNA was not detected in guanidine with CsCl and AGPC methods.

The method AGPC was found very poor for total RNA isolation from 18 days old immature seeds of pigeonpea.

The ratio of $OD_{260/280}$ for total RNA was 1.98 ± 0.2 in modified method which indicated the low amounts of

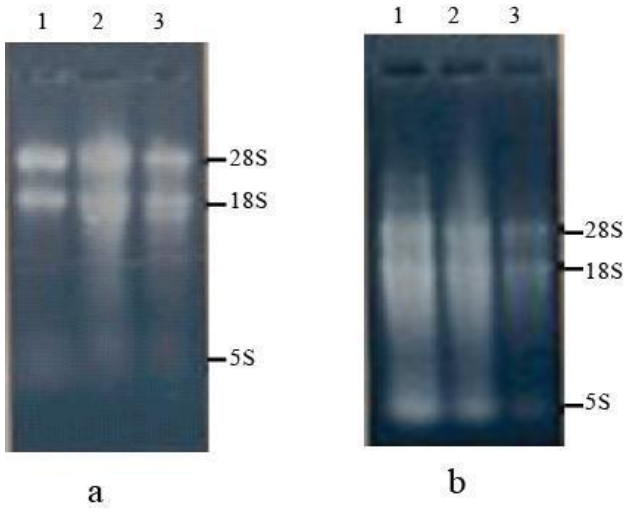

Figure 1. a) Denaturing agrose (1.5% w/v) gel electrophoresis of total RNA isolated from pigeonpea leaves, Lane 1, 2 and 3: replicates of one experiment. b) Native agrose (1.5% w/v) gel electrophoresis of total RNA isolated from 20 days old immature seeds of pigeonpea, Lane 1, 2 and 3: replicates of one experiment.

Table 2. RNA yields from different tissues using modified methods (The values represent mean ± SD from three experiments and each experiment contains 3 replicates).

Sources	RNA yield (μg/g tissues)	% Poly A mRNA from total RNA
Pigeonpea leaves	402 ± 1.6	1.76
Pigeonpea Epicotyls	212 ± 1.2	1.46
Pigeonpea Immature seeds 12 days old	172 ± 0.9	1.12
Pigeonpea immature seeds 18 days old	197 ± 1.1	1.01
Pigeonpea immature seeds 20 days old	198± 0.31	1.00
Pigeonpea immature seeds 24 days old	71± 0.6	0.78
Mustard callus	123 ± 1.1	0.72
Spinach leaves	417 ± 1.3	1.76

contamination of proteins and polysaccharides.

Native and denaturating formaldehyde agarose gel electrophoresis of total RNA showed three bands representing the 28S, 18S and 5S respectively (Figures 1 a and b). While in leaves gave extra bands (16S) of chloroplastic and mitochodrial RNA. This could be the reason for high yield of total RNA that was found in pigeonpea and spinach leaves (Table 2).

Modified method as well as, miniprep method for the total RNA isolation from many different tissues viz., pigenonpea leaves, epicotyls, immature seeds (12, 18, 20 and 24 days old), mustard callus and spinach leaves have also been standardized (Table 2). The yield of RNA from pigeonpea leaves (402 ± 1.6 μg tissues) and spinach leaves (417 ± 1.3 μg/g tissues) were approximately double then the pigeonpea epicotyls (212 ± 1.2 μg/g tissues). Mustard callus showed fewer yields (123 ± 1.1 μg/g tissues) of total RNA because callus contains high amount of water in fresh weight. The total RNA yield and percent Poly (A) RNA were increases from 12 days old to 20 days old immature seeds of pigeonpea. However it was decrease after 24 days old immature pigeonpea seeds (Gupta et al., 2006; Gupta et al., 2009).

The whole RNA isolation procedure relies on buffer 1 containing guanidinium thiocyanate (a strong protein denaturant), β-marcaptoethanol (a reluctant protein denaturant) together with polyphenolics adsorbent polyvinyl pyrrollidone (PVP), followed by an ethanol precipitation. After this step, the pellet was found to be very difficult to redissolve in further step, especially due to mixture rich in polysaccharides, and polypenolics which

are co-precipitated with RNA (Logemann et al., 1987; Sambrook et al., 1989). Other methods (Bahloul and Burkard, 1993; Ainsworth 1994 and Schultz 1994; Lopez-Gomez and Gomez-lim, 1992) also described the preventing oxidation and phenolic compounds but gave poor quality of RNA preparation. This problem was solved in modified method by re-dissolving the pellet in buffer II followed by phenol or chloroform extraction leads to an efficient inactivation of nucleases and provide the good quality of RNA.

This modified method can be used for isolation of high quality of intact RNA from different parts of plants without contaminating DNA and proteins, and it is especially well suited for isolation of RNA from tissues that contain high polysaccharide or starch content. Furthermore, this method can be exploited for Northern and dot blot analysis, cDNA synthesis for seed storage genes as well as for *in vitro* translation experiments.

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