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Simple and Efficient RNA Extraction Method for Gene Expression Studies in *Triticum aestivum*

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ABSTRACT: *The extraction of high quality total RNA from plant tissue containing high level of polysaccharide and polyphenols is a critical step in gene expression studies. Although several protocols and commercial productsare available for RNA isolation, most have been failed to yield high quality RNA from* Triticum aestivum *tissue. An attempt is made to establish an efficient RNA extraction method, which yielded high-quality RNA from different tissues of the* Triticum aestivum. *The average A260/A280 ratio and yield of the total RNA isolated were around 1.75 and 161.4ìg/g, respectively. It also gives good electrophoretic profile on denaturing formaldehyde agarose gel. The quality of extracted RNA was further assessed by cDNA synthesis and RT-PCR for gene expression study.*

Key words: *RNA extraction, Polyvinylpyrrolidone (PVPP), Gene expression,* Triticum aestivum, *polysaccharides, cDNA, RT-PCR*

INTRODUCTION

To study the gene expression or transcriptome analysis in plant; the isolation of high-quality RNA depends greatly on the treatment and handling of the tissue prior to RNA extraction. Cellular ribonucleases act quickly and efficiently to degrade RNA upon cell lysis; therefore, tissue frozen in liquid nitrogen or storage at -70°C is a prerequisite. Commercial reagents such as RiboReserve™ (AMRESCO) may increase storage stability without compromising the quality or quantity of the extracted RNA (Accerbi *et al*., 2010).

Plant material contains high levels of lipids, complex polysaccharides and phenols and many other secondary metabolites that interfere with RNA extraction, by degrading or co-precipitating with the extracted RNA (Asif *et al*., 2000). Furthermore, samples from different plants and tissues show differences in their composition (Hibino *et al*., 2001; Wu *et al*., 2008). The difficulties of RNA extraction have been reported by numerous publications (Fu *et al*., 2004; Miyama *et al*., 2006; Zeng *et al*., 2006; Yang *et al*., 2008). These RNA isolation protocols using acidic guanidiniumthiocynate, CTAB and SDS/Phenol, suggests that it requires different method for successful RNA isolation.

Surfactants agents (CTAB and SDS) are widely used in RNA isolation to inhibit RNase activity. However, the efficiency of such methods varies depending on the composition of the treated tissues (Chan *et al*., 2004; Rodrigues *et al*., 2007). The potassium acetate is used to precipitate polysaccharides (Song *et al*., 2011). Organic solvents like phenol and chloroform are used to dissociate RNA from proteins, separating them into two different phases. Lithium chloride is used as a strong dehydrating agent to precipitates RNA especially not DNA (Chen *et al*., 1997; Sambrook and Russell, 2001).

There are several reports on RNA extraction from wheat tissue (Jianweigao *et al*., 2001). The standard procedures that use these reagents, rarely result in RNA recovery with enough integrity or purity, when performed on tissues with high polysaccharide and polyphenol content (Rubio-Pina and Vazquez-Flota, 2008). These procedures are frequently modified to suit specific tissues and conditions.

In this study, RNA extraction method was designed to extract high quality RNA. Triton x-100

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was used to soluble the membrane protein, Polyvinylpyrrolidone (PVPP) was used to remove polyphenolics compound, Potassium acetate (KAc) was used to precipitate polysaccharides and Lithium chloride (LiCl) was used to precipitates the RNA from wheat tissue. It was found that extracted RNA was suitable for downstream applications.

Wheat (*Triticum aestivum*) is a good model plant to study transcritome analysis since draft sequences are now available in Gene Bank. Thus, the aim of the study described here was to standardize the RNA extraction protocol for cDNA synthesis and other RT-PCR based downstream studies.

MATERIALS AND METHODS

Plant Materials and Growth Condition

Wheat (*Triticum aestivum*) Lokwan variety seed was primarily washed with running tape water then with distilled water. Following seeds were soaked in distilled water for 3 hr and then kept for germination in Petri dish (9 cm) containing distilled water under dark condition at 25±2°C. On the following day uniformly germinated seeds were selected and transferred to Petri dishes containing two sheets of filter paper moistened with 5 mL of distilled water. Each Petri dish contained around 20 germinated seeds. The germinated seeds were allowed to grow at 25±2°C in day light period for 10-11 hr and darkness for 13-14 hr. To avoid the loss by evaporation glass plates was kept on the tray containing Petri dish and uptake by the seeds, a further 3 mL of distilled water was added to the Petri dish on day three. From the third day (about 72 hr) of germination equal size of leaves, coleoptiles, seeds and roots were harvested for total RNA isolation.

Total RNA Extraction Method

Glassware used in this protocol was thoroughly washed with detergent, filled with 0.1% DEPC water, incubated at room temperature overnight, and then autoclaved for 30 min. Solutions was also prepared by 0.1% DEPC treated water. For the RNA extraction procedure 200 mg of wheat tissues (leaf, coleoptile, seed and root) was taken and crushed to powder with 100 mg of PVPP in pre-chilled mortar and pestle using liquid nitrogen. Then 1.6 mL pre-heated Triton-X-100 extraction buffer [0.6 M Tris-Base, 5M NaCl, 0.5 M EDTA, 10% SDS, 40ìL of Triton-x, 25 mg of DTT, D/W] was added to the crushed material. The content was transferred into 2.5 mL RNase-free micro centrifuge tube. After mixing the content with vigorous vortex mixing tubes were incubated in boiling water bath at 65°C for 20 min. Then 300 µL 5 M KAc was added and kept at 4°C for 20 min. After mixing by gentle inversion samples were centrifuged immediately at 15,870 x g for 15 min at 4°C. The aqueous phase was carefully transferred to a new 2.5 mL tube and extracted primarily two times with Phenol:Chloroform (25:24 pH-4.5) and then single time with Chloroform:Isoamyl alcohol (24:1) at 15,870 x g for 15 min at 4°C. The upper aqueous phase was carefully transferred to a new 2.5 mL tube and fourth volume of 10 M LiCl was added for RNA precipitation. The tubes were then mixed by inversion and kept at 4°C for 1.5 hr. The samples were centrifuged at 15, 870 x g for 15 min at 4°C and then supernatants were discarded. RNA pellet was washed with 70% chilled ethanol and air dried in laminar air flow hood for 15 min. Total RNA pellet was suspended in appropriate volume of RiboReserve™ (RNA storage solution AMRESCO made) and stored at -20°C. All the solutions were prepared in 0.1% DEPC treated water. Glassware used in this protocol was thoroughly washed with detergent, filled with 0.1% DEPC water, incubated at room temperature overnight, and then autoclaved for 30 min.

RNA Quality Analysis and Visualization

Total RNA samples were quantified by absorbance at 230, 260 and 280 nm using a spectrophotometer (BioTek Instruments Incorporation, Winooski, VT.). The RNA concentration was calculated in µg/g. The ratio A260/A280 was used to assess the purity of the isolated RNA. For the RNA visualization, RNA samples (100 ng) were first denatured by addition of 4.2 µL of formamide and 2 µL of gel loading dye (Genei, Bangalore) and boiled at 65°C in boiling water bath and then chilled at -20°C for 10 min. To analyze the RNA banding pattern, gel electrophoresis was carried out on 1.2% agarose gel stained with ethidium bromide. Low Range Ruler from Bangalore Genei, India, was used as molecular size standard. Total 10 µL of mixture was run in 1x TAE buffer for 50 min at 65 V. The RNA bands were visualized on a UV transilluminator.

cDNA synthesis: cDNA was synthesized by MultiScribe™ Reverse Transcriptase (50 U μL^{-1}) in a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. For the cDNA synthesis 2.5 µg/µL total RNA was taken as template. The reaction mixtures (20 µL) were incubated in a Veriti (96 Well Fast Thermal Cycler), Applied Biosystems, USA. The thermal cycling condition of reverse transcription reaction used as

follow:- step-1: 25°C for 10 min; step-2: 37°C for 120 min; step-3: 85°C and step-4: 4°C for infinite.

Relative Quantification by RT PCR

To assess the quality of extracted RNA, Relative quantification assay was performed using cDNA as template and primers pair of Cellulose synthase gene (*HvCesA*1F: 52 TGT GGC ATC AAC TGC TAG GAA A 32 and R: 52 CGT ACA AAG TGC CTC ATA GGA AA 32) as a target gene. Primer pair of *HvCesA*1 was selected from Rachel *et al.(* 2004).

PCR Amplification of *Triticum aestivum* cDNA

To check quality of synthesized cDNA, PCR amplification was carried out using cDNA as template and primers pair of *HvCesA*1 and Anionic peroxidase gene (*Ana* 3F: 52 AGG TCA CCA TCA TCT GGT CA 32 and R: 52 TTC TTC CGC CGG ACC AAC TC 32) as a target gene. Primer pair of anionic peroxidasegene *Ana* 3 was designed in our lab from *Zea mays* full-length cDNA clone sequences obtained from NCBI. For PCR amplification 25 µL of reaction mixture was used. PCR was carried out in a Veriti (96 Well Fast Thermal Cycler, Applied Biosystems, USA).

RESULTS

RNA Extraction, Purity and Yield Analysis

It gives good electrophoretic profile on denaturing formaldehyde agarose gel (Figure. 1). Leaf RNA purity average ratio A260/A280 was 1.85 and average A260/A230 ratio was 2.02 with RNA yield average of 331.07µg/g. While samples from coleoptile shown RNA purity average ratio A260/A280 was 1.82 and average A260/A230 ratio was 1.81 with yield average of 166.4µg/g. RNA extracted from seed samples that shown average purity ratio A260/A280 of 1.72 and averageA260/A230 ratio of 1.53 with RNA yield average of all seed samples was 91.35µg/g. In case of root samples, RNA purity ratio A260/A280 was average 1.63 and A260/A230 ratio was average 1.51 with RNA yield average of all root samples was 56.77µg/g (Table 1). In general, average RNA purity ratio A260/A280 of all parts of wheat seedlings calculated together was 1.75 and A260/A230 ratio was average 1.72 with RNA yield average of 161.4µg/g (Table 1). The result shown that highest RNA yield extracted from leave sample while lowest RNA yield extracted from root sample.

Relative Quantification by RT PCR

Quantification of target gene at successive cycle was confirmed by Run Vs Cycle number amplification plot

Table 1
Average Purity and Yield of RNA from Triticum Aestivum Leaf, Coleoptiles, Seed and Root

Tissue	*A260/A280*	*A260/A230*	*RNA µg/g*
Leaf	1.85	2.02	331.07
Coleoptile	1.82	1.81	166.4
Seed	1.72	1.53	91.35
Root	1.63	1.51	56.77
Average	1.75	1.72	161.4

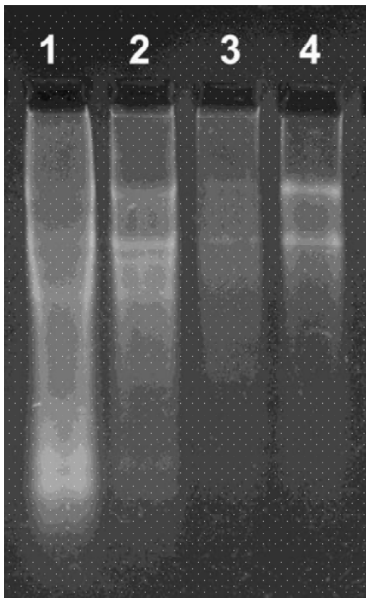

Figure 1: Agarose gel electrophoresis of RNA extracted from *Triticum aestivum* (1) Leaf, (2) Coleoptile, (3) Seed and (4) Root respectively

(Figure 2). Furthermore dissociation curve was developed after completion of RT-PCR reaction. Dissociation curve shows single, tight peak indicates specific amplification for each well on the real-time PCR plate (Figure 3).

PCR Amplification of *HvCesA*1 and *Ana* 3 gene

The PCR product of target genes were loaded on agarose gel electrophoresis in following manner: leaf, coleoptile, seed and root. The amplified product showed clear band pattern (Figure. 4 and 5 for *HvCesA*1 and *Ana* 3 genes, respectively).

DISCUSSION

The quality and quantity of RNA is an important parameter for molecular biology research. Plant material contains high levels of lipids, complex polysaccharides and phenols and many other secondary metabolites that interfere with RNA extraction, by degrading or co-precipitating with the extracted RNA (Asif *et al.*, 2000). Phenolic compounds

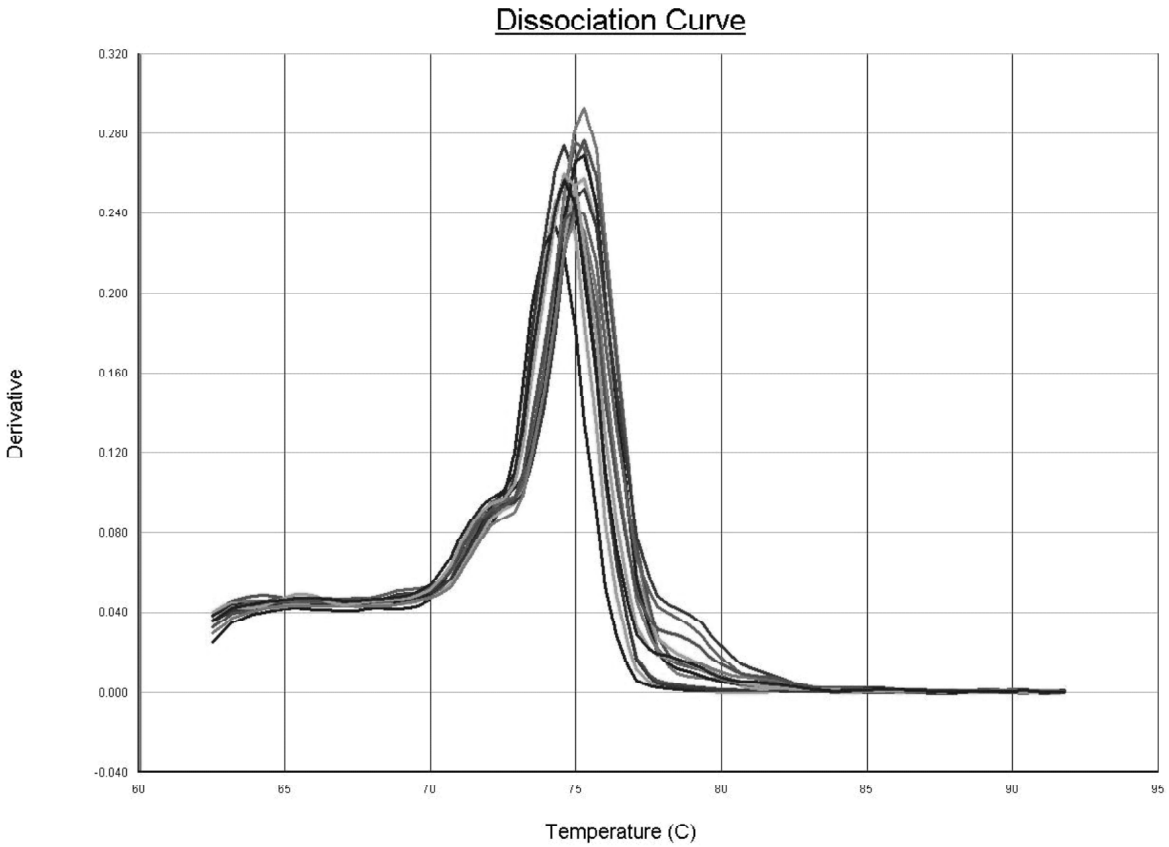

Figure 2: Amplification plot obtained during RT-PCR with *HvCesA*1primer

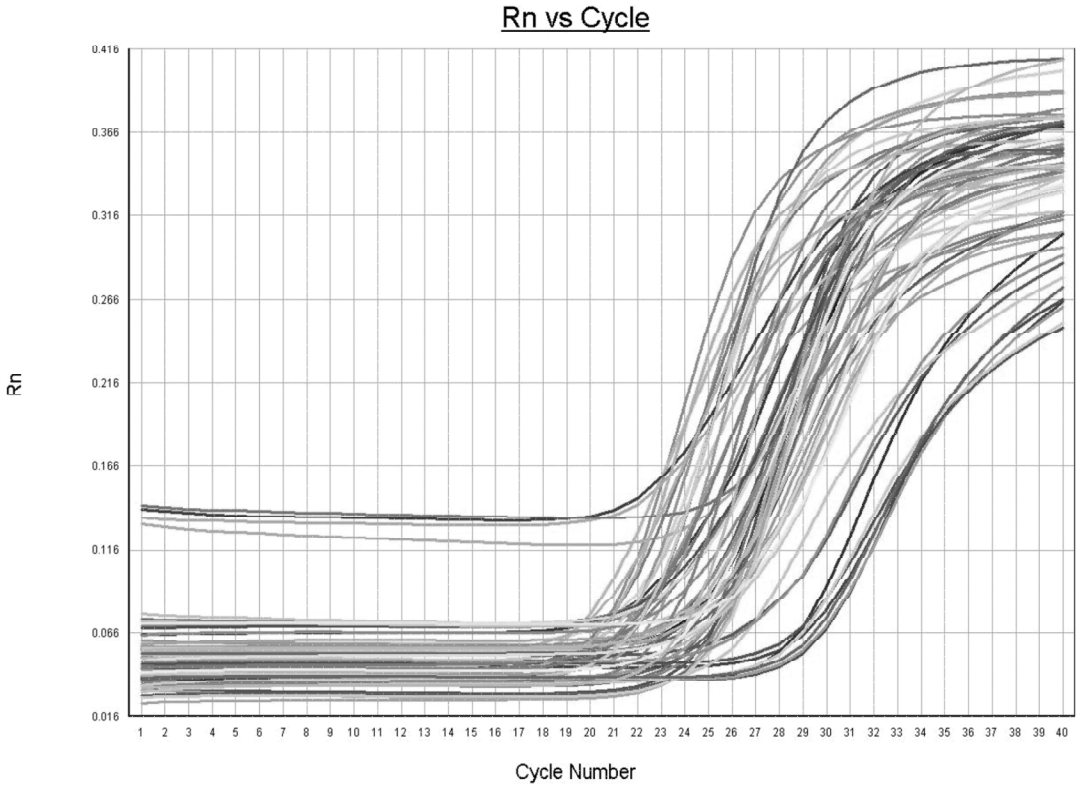

Figure 3: Dissociation curve developed after completion of RT-PCR reaction with *HvCesA*1 primer

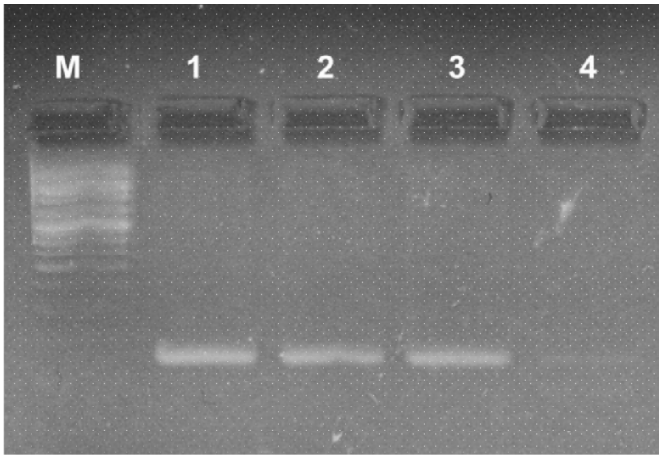

Figure 4: The amplification pattern of *HvCesA*1 primers on *Triticum aestivum* (1) Leaf, (2) Coleoptile, (3) Seed, (4) Root respectively. (M) Was Law range DNA Ladder

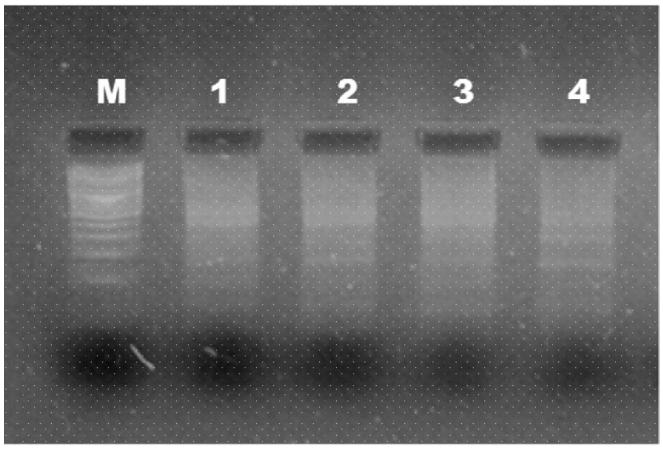

Figure 5: The amplification pattern of *Ana* 3 primer on *Triticum aestivum* (1) Leaf, (2) Coleoptile, (3) Seed, (4) Root respectively. (M) M was Law range DNA Ladder

are readily oxidized to form covalently linked quinones, which binds to RNA. This RNA is unusable for downstream procedures such as reverse transcription and cDNA library construction.

Numerous reagents and kits are commercially available for RNA isolation from various plant tissues (e.g. TRIZOL® and TRIZOL LS®-Invitrogen Life Technologies, USA; TRI Reagent®-Molecular research Center, USA; RNeasy plant kit; and QIAGEN), but none of these have high success rate for all type of plant tissues and doesn't fulfill all desirable criteria like purity and concentration of RNA. Most RNA isolation methods compromise with quantity of RNA to achieve high quality of RNA. In routine for the isolation of total RNA from plant, different solutions are uses like cetyltrimethylamonium bromide, dodecyl sulphate salts, phenol/chloroform, guanidine hydrochloride, benzyl chloride and guanidine isothiocyanate (Suzuki *et al*., 2001; Kolosova *et al*., 2004).

Several methods utilize EDTA, LSS, and CTAB to successfully inhibit RNase activities. Sodium lauryl sarcosinate (sarcosyl) uses for RNA solubility, which ensure maximum RNA solubility in the aqueous phase and the removes most polysaccharides and other insoluble material. In addition, secondary metabolites and other interfering compounds effectively remove using sodium borate and PVPP under a deoxidized condition.

The 200 mg plant material used to extract RNA and it was found that extracted RNA was of high quality and suitable for downstream applications (e.g. cDNA synthesis, Gene expression analysis). The pattern of RNA yield in terms of µg/g obtained from four parts of wheat seedlings were leaf > coleoptile>seed > root. In general leaf gives highest RNA yield whereas seed gives lowest RNA yield. The purity ratio A260/A280 was ranged from 1.6 to 1.8 in all four kind of plant tissues.

Thus, established method was proven to be success, in that PVPP treatment efficiently removes secondary metabolites, Triton x-100 solubilize the membrane proteins, KAc efficiently precipitates polysaccharides and LiCl can successfully precipitated high quality RNA from wheat tissue. This method not requires toxic chemicals or costly reagents.

Furthermore it doesn't require proteinase K treatment to remove protein impurity from extract. It reduces the cost, time of procedure and product loss. So, this protocol is very simple, short, cost effective and exceedingly success for extracting high quality RNA. In this experiment, distilled water control and hormonal treated tissues like leaf, coleoptile, seed and root showed recovery of good quality (Average A260/A280: 1.75) and quantity (Average: 161.4 µg/g) of RNA and has successfully converted into cDNA for downstream application.

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