RNA Isolation and Expression from Different Dormant and After-Ripened Wheat 
(Triticum aestivum) Seed Tissues Rich in Polysaccharides and Proteins

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Abstract: RNA extraction with high quality and quantity is a prerequisite to study analytical methods in plant 
genetic, molecular biology and related physiological investigations. However, extracting RNA from seed tissues 
can be difficult and often require the modifications of existing protocols or the development of new procedures. 
Seeds and fruits containing large amount of polysaccharides, polyphenol compounds and proteins are the most 
difficult plant tissues for nucleic acid isolation. In this study, we described a low-cost extraction protocol that has efficiency of integrity and quality of RNA from various wheat seed tissues (embryo, 
endosperm with aleurone layers and whole seed). High quality RNA with no DNA contaminations was obtained 
through the protocol described here. The results of the same materials revealed that after-ripened seeds have 
sharper band of RNA than dormant seeds. Similarly, spectrophotometric results exhibited that at the same 
material weight the RNA contents were more in after-ripened embryos and endosperm than in dormant state. 
The protocol resulted in high-quality RNA, as supported by performing RT-PCR.

Key words: After-ripened, dormant, polysaccharides, RNA isolation, wheat seed

INTRODUCTION

RNA extraction with high quality and quantity is a prerequisite to study analytical methods in plant 
genetic, molecular biology and related physiological investigations (Gen et al., 2002). These analytical 
methods like transcriptom analysis by RT-PCR provide simple tools for studying gene expression at the 
molecular level, segregation of mutation and monitoring the transcription of different type of gene alleles in 
various plant tissues (Gen et al., 2002, Berendzen et al., 2005).

Also quality and quantity of RNA is essential for ensuring that the sample represents all expressed gene in 
a cDNA library. However, extracting RNA from plant tissues can be difficult and often require the modification of existing protocols or the development of new procedures (Gasie et al., 2004). Between them, seeds and 
fruits because of containing large amount of polysaccharides, polyphenol compounds and proteins are one of the most difficult plant tissues for nucleic acid isolation (Gen et al., 2002; Singh et al., 2003).

Common protocols for RNA extraction from seed tissues are tedious and usually result in poor yields. We 
have systematically tested some protocols for RNA isolation from wheat seeds for the efficiency of integrity 
and quality of RNA.

In this study, we evaluated a low-cost extraction protocol that has efficiency of integrity and quality of RNA from various wheat seed tissues (embryo, endosperm with aleurone layers and whole seed).

MATERIALS AND METHODS

Plant materials: Embryo, endosperm with aleurone layer and whole seed of dormant and after-ripened wheat 
(Triticum aestivum) cv. RL4137 (a Canadian hard red spring wheat) were used in this research. Wheat seeds 
were harvested at Zadoks’ Growth Stage 92 (Zadoks et al., 1974) grown in the experimental farm at the University of 
Tehran in 2004. RL4137 expresses high levels of seed dormancy at this stage (Tavakkol Afshari and Huel, 2002). 
This experiment was conducted in 2004-2005 in the Plant Molecular Breeding Laboratory, Department of Agronomy 
and Plant Breeding, University of Tehran. For after-ripening treatments, seeds were kept in germinator at 23°C 
under dark and dry conditions for six weeks. Following 
tissues were collected at different experimental treatments; namely: Dormant and after-ripened embryo, dormant 
and after-ripened endosperm with aleurone layers and dormant and after-ripened of intact seed. The above-
mentioned tissues were collected and frozen in liquid nitrogen and kept at -80°C until it is used.

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RNA extraction protocol according to Naito et al. (1994):
Add 300 µL of EB and 300 µL of PCI to an eppen tube on ice. Cool the mortar and pestle with liquid N2, add 10 seed pods (or 0.05 g of 8DAF silicles) per sample and homogenise very well, adding N2 as necessary to keep sample frozen. Once fully ground, use small spoon to transfer sample to the eppen tube with the buffer. Vortex for 3 min and divide the sample into 2 eppen tubes. Centrifuge for 1 min and collect sup into new eppen. Add an equal volume of PCI and vortex. Centrifuge for 5 min and collect sup into new eppen. Centrifuge again for 5 min and collect sup into new eppen. Add 0.1 vol 3 M NaOAc and 3 vol 100% EtOH, invert 4 times to mix. Place at -80°C (dry ice) for 10 min. Centrifuge for 5 min, discard sup and vacuum dry pellet. Dissolve pellet with 100 µL ddH₂O (may take time). Centrifuge for 10 min and collect sup into new eppen. Add equal volume of 4 M LiCl, invert 4 times to mix. Place on ice on 4°C cold room overnight or -80°C (dry ice) for 1 h.

Next day: Centrifuge for 15 min, discard sup. Small pellet should be visible, be careful as it moves easily. Add 1 mL of 2 M LiCl and carefully invert once to mix. Centrifuge for 2 min and discard sup. Add 1 mL of 70% EtOH and invert once to wash. Centrifuge for 2 min, discard sup and vacuum dry. Dissolve pellet in 5-10 µL ddH₂O. Combine the 2 solutions/sample into one eppen. Centrifuge for 5 min and collect sup into new tube. Store at -30°C or lower.

Solutions and reagents: Diethyl Pyrocarbonate (DEPC) treated water was used for all solutions except Extraction Buffer (EB). Extraction Buffer includes; 1M Tris-HCl (MERCK) pH 9.0, 1% SDS. Other used solutions: DEPC water-saturated Phenol-Chloroform-Isoamylalcohol (PCI) 25:24:1 V/V, it’s better to mix them just before the extraction, (in Naito et al. protocol phenol was satu rated with TE). 3M NaOAc (MERCK) pH 5.6, 4M LiCl (MERCK), 2M LiCl, 100 and 70% EtOH and DEPC-treated water. All the solutions except phenol and chloroform Isoamylalcohol were autoclaved.

Experimental protocol for total RNA extraction: This protocol is an improvement and modification of the soybean seed RNA extraction method described by Naito et al. (1994). Our experimental protocol was conducted in four steps in the following pattern:

Step 1: Homogenization
• The EB was pre-warmed and 1.5 mL of EB put in 10 mL falcon.
• Three hundred milligram embryo was added in a chilled mortar and grinded very well with liquid nitrogen.

• Sample was transferred to 10 mL falcon containing EB.
• Sample was vortexed immediately then equal volume of (1.5 mL) PCI was added to falcon contain sample and EB (in Naito et al. protocol both PCI and EB add in a same time in a tube and EB was not warmed). It was vortexed again for 1 min and the sample divided in two 2 mL tubes.

Step 2: Phase separation
• It was centrifuged at 14000 x g for 3 min (use 14000 g instead of 10000 g in Naito et al. protocol) at 4°C. Then supernatant collected in to fresh chilled tubes on ice.
• An equal volume of PCI was added and vortexed.
• It was centrifuged at 14000 x g for 7 min at 4°C. Supernatant was collected in to fresh tube on ice (carefully remove it because the interphase is removed and mix to upper phase easily).
• Re-extracted with an equal volume of PCI next vortexed and centrifuged again, carefully pipette aqueous phase in to fresh tubes and do not collect the white phase at the bottom. (This step repeats twice for improving removal of embryo residual but it is once in Naito et al. protocol).
• The aqueous phase was centrifuged at 16000 x g for 7 min at 4°C (either time or centrifuge g is increased in order to remove whole phenol).
• Supernatant was collected in to fresh chilled tube, then 0.1 VOL 3 M NaOAc and 3 VOL 100% ethanol were added and mixed by inversion, stored at -80°C for 20 min.

Step 3: RNA precipitation
• Sample was centrifuged at 16000 x g for 8 min at 4°C. Then supernatant and white pellet on the side of tube discarded (do carefully because RNA pellet is at bottom and may move). The tube was kept reverse on an autoclaved paper at the room temperature (In this step use 16000 g instead of 10000 g. Also the time was increased, you can not see the RNA pellet easily. The one you see should be discarded).
• Pellet was dissolved with 100 µL DEPC-treated water, equal volume of 4M LiCl added and inverted 4 times to mix.
• The sample was put in -80°C for 2 h.
• Sample was centrifuged at 16000 x g for 15 min at 4°C.
• Supernatant discarded with pipetting and 1 mL 2M LiCl was added. Inverted once to mix.
• Again it was centrifuged at 16000 x g for 2 min at 4°C.
Step 4: RNA recovery

- RNA was washed by 1 mL 70% Ethanol. Centrifuged, supernatant discarded and put in room temperature until dry (put in room temperature instead of vacuum dry).
- Pellet dissolved in 10 μL DEPC-treated water and combined the two samples in to one tube.
- Stored at -80°C.

RNA analysis: RNA quantification was performed spectrophotometrically (JENWAY-6405UV/vis Spectrophotometer) at wave lengths of 230, 260 and 280. To confirm the RNA quality, the RNA was electrophoresed on 1.1% agarose gel containing formaldehyde.

RT-PCR analysis: First strand cDNA was synthesized from 0.2 μg of dormant and after-ripened RNA with oligo dT used as a primer following the RT manufacture's instruction of Fermentaz Company. A touch down PCR (MgCl$_2$, 50 mM, dNTPs 10 mM, forward primer 20 μM, reverse primer 20 μM, PCR buffer 10 X, Taq DNA polymerase, all material prepared from Fermentaz company and followed the instruction) was performed from 1 μL cDNA by primers which were constructed based on the conserved sequence for wheat MAP Kinases (Sambrook and Russell, 2001). PCR amplification was performed to demonstrate that the RNA could also be used for other analysis (Data about MAP kinases research not shown). For electrophoresis PCR products were loaded on 6% polyacrylamide denaturing gels and stained by silver nitrate method.

Touch down PCR program

Denaturation 94°C 4 min

[Denaturation 94°C 30 sec] 14 cycle. Each cycle the

Annealing 57°C 30 sec

Extension 72°C 1 min

[Denaturation 94°C 30 sec] Annealing temperature reduced 0.7°C

Really extension 72°C 10 min

RESULTS AND DISCUSSION

RL4137 cultivar exhibited a gradual loss of dormanacy during after-ripening treatment (Table 1). A six weeks

<table>
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<td>67</td>
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Fig. 1: The results of Naito RNA extraction protocol on wheat seeds, electrophoresed on 1.1% agarose gel containing formaldehyde. Contamination of DNA and other components like proteins were observed of after-ripening period resulted in seed dormancy loss as demonstrated by higher germination (80%).

The Naito et al. (1994) unmodified protocol was used at first for RNA extraction from wheat seeds, but the result was poor in quality because the extracted RNA had contamination of DNA, protein and polysaccharides. It is likely that because of TE saturated phenol and basic pH of extraction buffer DNA also separated by extraction buffer. Based on modified protocol, it is recommended to use DEPC-treated water saturated phenol and lower pH to isolate and purify RNA more efficiently (Fig. 1).

Also method of Chomezynski and Sacchi (1987) was examined in this research, but the results were poor for isolating good quality of RNA from wheat seed (at the end of protocol we obtained an insoluble pellet). Singh et al. (2003) reported several methods for RNA isolating from plant tissue rich in starch (Chomezynski and Sacchi, 1987; Logemann et al., 1987; Gehrig et al., 2000; Salzman et al., 1999; Hosein, 2001), but these methods were not good enough for isolating good quality of RNA from wheat seed.
Fig. 2: RNA extraction from wheat seed with modified protocol, electrophoresed on 1.1% agarose gel containing formaldehyde. There was no evidence for contamination of DNA and other components.

Fig. 3: RT-PCR results on denaturing polyacrylamide gel electrophoresis, Lane 1: Embryo, Lane 2 and 4: Control samples that their cDNA synthesis were performed without RT, respectively. Lane 3: Endosperm and S. Size marker.

High quality RNA with no DNA contaminations was obtained through our modified protocol. This protocol kept the integrity of the RNA while disrupting cells and dissolving cell components. For all RNA samples, distinct 28s and 18s rRNA bands without degradation were observed (Fig. 2). RNA quantification was performed spectrophotometrically at wave length of 260 and 280. RNA samples were diluted in water instead of TE prior to spectrophotometric analysis. The 260/280 OD ratio ranged from 1.9-2.01 indicating a lack of protein contamination (Table 2). Also 260/230 OD ratio ranged from 2.12-2.35 suggesting less polysaccharides contamination (Table 2).

Moreover, the protocol resulted in high-quality RNA, as evidenced by performing RT-PCR (Fig. 3). After synthesizing ssDNA, MAP kinases fragments were amplified from embryo and endosperm with aleurone layers of wheat with the expected size according to degenerate primers of MAP kinases between 150-300 bp. These MAP kinases fragments had sharper band in after-ripened embryo than endosperm (data not shown). In addition the control samples showed no bands, indicating no DNA contamination. However endosperm tissue produced high quality but low yielded total RNA especially when seeds were dormant (Fig. 4). It was necessary to extract RNA from 600-900 mg of endosperm. Moreover, high volume of extraction buffer was needed.

In dormant state, RNA electrophoresis for the same weight materials of embryo, whole seed and endosperm with aleurone layers showed sharper RNA bands for embryo, endosperm and whole seed, respectively (Fig. 5). In addition embryo had higher RNA content than endosperm at the same material weight in dormant state (Fig. 5).

The results for the same embryo material revealed that after-ripened seed had sharper band of RNA than dormant seeds (Fig. 6). Spectrophotometric results approved that RNA content were more in after-ripened seed than in dormant. Gasic et al. (2004) reported similar results about dormant and active lateral buds showing active lateral buds have more RNA content than dormant ones.

The average extraction time of this protocol was approximately 5-8 h. One of the major disadvantages of
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ABBREVIATIONS

RT-PCR : Reverse Transcriptase Polymerase Chain Reaction
DEPC : Diethyl Pyrocarbonate
PCI : Phenol Chloroform Isoamylalcohol
EB : Extraction Buffer
MAP
Kinases : Mitogen Activated Protein Kinases
sscDNA : Single Strand Complementary DNA
tRNA : Ribosomal RNA and OD, optical density

REFERENCES


