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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 and ribonucleic 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 procedure (Gasic *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 systemically 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 Hucl, 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 mL 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 siliques) 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 ddH_2O (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–Isoamylalchol (PCI) 25:24:1 V/V, it's better to mix them just before the extraction, (in Naito *et al.* protocol phenol was saturated 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 Isoamylalchol 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.

- Supernatant discarded and dried by cut autoclaved paper (a gel like pellet is visible).

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 and 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 Fermentas 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
Denaturing 94°C 4 min

Denaturing 94°C 30 sec Annealing 67°C 30 sec Extension 72°C 1 min	14 cycle. Each cycle the annealing temperature reduces 0.7°C
Denaturing 94°C 30 sec Annealing 56°C 30 sec Final extension 72°C 10 min	30 cycle repeat

RESULTS AND DISCUSSION

RL4137 cultivar exhibited a gradual loss of dormancy during after-ripening treatment (Table 1). A six weeks of after-ripening period resulted in seed dormancy loss as demonstrated by higher germination (80%).

Table 1: Germination percentage of RL4137 cultivar after six weeks of after-ripening

	After-ripening (weeks)						
	0	1	2	3	4	5	6
Germination (%)	0	4	7.5	23	49	67	80

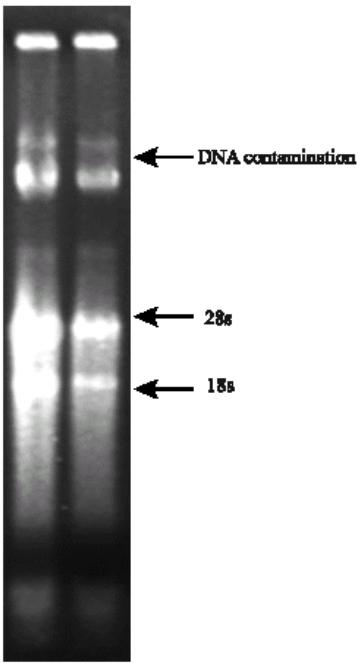

Fig. 1: The results of Naito RNA extraction protocol on wheat seeds, electrophoresed on 1.1% agarose gel containing formaldehyde. Contamination of DNA and other components like proteins were observed

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 Chomczynski 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 (Chomczynski 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.

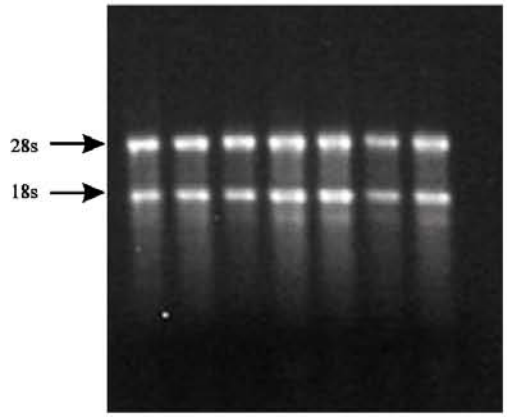

Fig. 2: RNA extraction from wheat seed with modified protocol, electrophoresed on 1.1% agarose gel containing formaldehyde. There was no evidence for contamination of DNA and other components

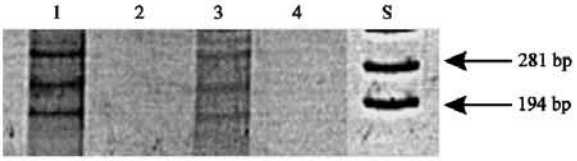

Fig. 3: RT-PCR results on denaturing polyacrylamide gel electrophoresis, Lane 1: Embryo, Lane 2 and 4: Control samples that their cDNA synthesis were performed without RT, respectively. Lane 3: Endosperm and S. Size marker

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 sscDNA, 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,

Table 2: RNA yield and quantity using spectrophotometric method

Sample		Absorbency ratio OD 260/OD230	Absorbency ratio OD260/OD280	Yield ($\mu g\ g^{-1}$ FW)
Embryo	D	2.35	1.98	650
	AR	2.20	1.92	875
Endosperm with	D	2.33	2.00	520
aleurone layers	AR	2.16	1.96	600
Whole seed	D	2.25	2.01	190
	AR	2.15	1.98	230

D: Dormant, AR: After-ripened, 300 mg material was used for each sample

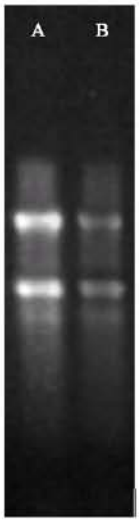

Fig. 4: Total RNA extracted from 700 mg endosperm tissues, electrophoresed on 1.1% agarose gel containing formaldehyde. A: After-ripened tissue B: Dormant tissue

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

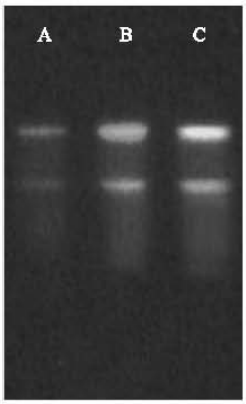

Fig. 5: Total RNA from equal materials; A: Whole seed, B: Endosperm, C: Embryo, all materials were in dormant state. (RNA electrophoresed on 1.1% agarose gel containing formaldehyde). In equal materials, more µg RNA were achieved initially from embryo, then endosperm and finally whole seed

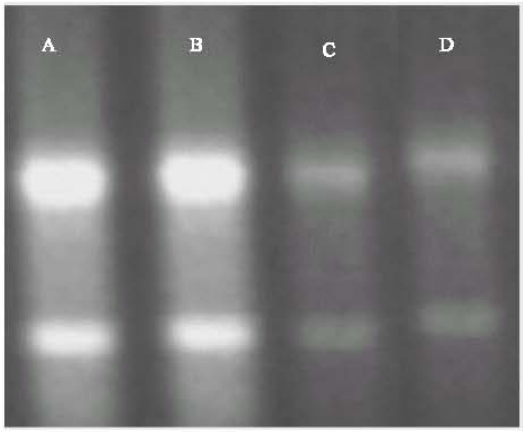

Fig. 6: Compassion between total RNA extracted from dormant and after-ripened tissues. RNA electrophoresed on 1.1% agarose gel containing formaldehyde. A: After-ripened embryo (300 mg material), B: After-ripened endosperm with aleurone layers (900 mg material), C: Dormant embryo (300 mg material), D: Dormant endosperm with aleurone layers (900 mg material)

this isolation method is that toxic chemicals such as phenol were used and therefore the lab safety should be considered. The results of this method could be useful for seeds and tissues containing high level of protein and polysaccharides.

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ABBREVIATIONS

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

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