Isolation of high quality RNA from pistachio (*Pistacia vera* L.) and other woody plants high in secondary metabolites

Maryam Moazzam Jazi¹ · Saideh Rajaei² · Seyed Mahdi Seyed¹

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Abstract The quality and quantity of RNA are critical for successful downstream transcriptome-based studies such as microarrays and RNA sequencing (RNA-Seq). RNA isolation from woody plants, such as *Pistacia vera*, with very high amounts of polyphenols and polysaccharides is an enormous challenge. Here, we describe a highly efficient protocol that overcomes the limitations posed by poor quality and low yield of isolated RNA from pistachio and various recalcitrant woody plants. The key factors that resulted in a yield of 150 µg of high quality RNA per 200 mg of plant tissue include the elimination of phenol from the extraction buffer, raising the concentration of β-mercaptoethanol, long time incubation at 65 °C, and nucleic acid precipitation with optimized volume of NaCl and isopropyl alcohol. Also, the A260/A280 and A260/A230 of extracted RNA were about 1.9–2.1 and 2.2–2.3, respectively, revealing the high purity. Since the isolated RNA passed highly stringent quality control standards for sensitive reactions, including RNA sequencing and real-time PCR, it can be considered as a reliable and cost-effective method for RNA extraction from woody plants.

Keywords *Pistacia vera* · Polyphenols · RNA extraction · RNA-sequencing · Real-time PCR · Tree

Introduction

RNA extraction from specific plant organs and tissues is an important upstream step for many molecular studies in plant biology. Currently, RNA-sequencing (RNA-seq) is being used as an efficient tool to investigate transcriptome of plants, especially non-model species (Healey et al. 2014). However, applying these technologies for recalcitrant plants are challenging as a result of high polyphenols and polysaccharides amounts that lead to poor quality and quantity of isolated RNA (Xiao et al. 2012). This is especially true for plants under abiotic and biotic stresses because of high accumulation of secondary metabolites (Winkel-Shirley 2002). The polysaccharides tend to co-precipitate with the RNA in the presence of alcohols, a major source of contamination in the final extract, and thus interfere with subsequent applications such as reverse transcription and cDNA library construction (Gambino et al. 2008). While several commercial kits are available for successful extraction of RNA from many tissues, they have proved to be less effective on tissues rich in polyphenols and polysaccharides (Kiefer et al. 2000). To reduce the effect of polyphenolic compounds and polysaccharides which are normally co-purified with RNA, compounds, like PVP (polyvinyl polypyrrolidone), cesium chloride, guanidine thiocyanate and CTAB (cetyltrimethylammonium bromide) have been used in different protocols (Wang and Rhee, 2001; Asif et al. 2006; Ouyang et al. 2014). However, most of these methods either require lots of plant tissue as a starting material or are not efficient for the isolation of good quality RNA to perform sensitive reactions, such as real-time PCR and RNA-seq.

✉ Seyed Mahdi Seyed
seyedi@nigeb.ac.ir

Maryam Moazzam Jazi
m_moazzam@nigeb.ac.ir

Saideh Rajaei
rajaee@nigeb.ac.ir

¹ Plant Biotechnology Department, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

² Environmental and Industrial Biotechnology Department, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

Pistachio (*Pistacia vera* L.), a dioecious nut crop, is indigenous to Asia and one of the most important commercial trees grown in Iran, Turkey, and USA (Karimi et al. 2009). To our knowledge, there is no protocol for total RNA extraction from pistachio tissues in the literature; therefore, an efficient method for high quality RNA extraction from pistachio tissues is necessary. Here, we present a versatile and cost-effective CTAB-based protocol for the isolation of high quality RNA from the leaves and roots of this tree. The isolated RNA was verified to be of high quality and suitable for real-time PCR analysis as well as RNA-seq library preparation and sequencing. The proposed protocol is the first report of RNA extraction from pistachio proven to be successful for RNA isolation from other woody species including pine, olive, barberry, rose, and weeping fig for using in very sensitive applications.

Materials and methods

Plant materials

Leaves of 20-year-old field-grown pistachio (*P. vera*) trees were collected in April and September, which can be considered as adult leaves; for young ones, 40-day-old greenhouse-grown plants (*P. vera* L. C Ghazvini and *P. vera* L. C Sarakhs) with 16-h-light at 25 °C to 28 °C, and 8-h-dark at 20 °C were used after applying salt treatment by adding 250 mM NaCl to the Hoagland's nutrient solution; leaves as well as roots collected after four days of salt stress. Moreover, leaves of pine (*Pinus* spp), olive (*Olea europaea*), barberry (*Berberis thunbergii*), rose (*Rosa hybrida*) and weeping fig (*Ficus benjamina*) were collected from adult field-grown trees. All samples were immediately frozen in liquid nitrogen and stored at −70 °C until use.

Solutions and reagents

The CTAB extraction buffer was modified from Chang et al. (1993) and composed of filter-sterile 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, 2 % CTAB (w/v), 2 % PVP (w/v), double-distilled water (ddH₂O), and 4 % β-mercaptoethanol. Other reagents comprised of chloroform: isoamyl alcohol (24:1; v/v), 5 M NaCl, isopropanol, 0.1 % (v/v) DEPC-treated water, 12 M LiCl, and 70 % ethanol.

RNA extraction protocol

About 200 mg of plant tissue was ground into a fine powder using liquid nitrogen and transferred to a 15 ml polypropylene tube containing 4 ml of extraction buffer with 4 % β-mercaptoethanol, which was added just prior to use. The mixture was shaken vigorously to homogenize the tissue by vortexing for 40 s and incubated at 65 °C for 35 min, inverting

the tube every 7 min during incubation. Then, an equal volume (4 ml) of chloroform:isoamyl alcohol (24:1, v/v) was added and mixed by inverting the tube. The mixture was incubated at room temperature for 15 min with gentle shaking, followed by centrifugation at 5500 g for 20 min at 4 °C. The supernatant was recovered and a second extraction with chloroform:isoamyl alcohol (24:1, v/v) was performed. The final supernatant was transferred to a new tube and an equal volume of NaCl 5 M, and chilled isopropanol were added to it, mixed by inverting the tube and the final mixture was kept at −20 °C for at least 2 h. The samples were then centrifuged at 20,000 g for 35 min at 4 °C, then the supernatant was removed and pellet was suspended in 500 μl of DEPC-treated water and incubated overnight at 4 °C or 30 min at −80 °C after adding 12 M LiCl (113 μL). The RNA pellet was collected by centrifugation at 12,000 g for 15 min at 4 °C. The supernatant was removed and the pellet was washed using 5 M NaCl followed by centrifugation. Finally, the pellet was washed with chilled 70 % ethanol, which its volume depends on the pellet amount, air dried at room temperature and re-suspended in DEPC-water. To eliminate possible genomic DNA contamination, the RNA sample was treated with RNase-free DNase I (Fermentas); 1 μg of total RNA was used in the reaction containing 1 μl reaction buffer with MgCl₂ and 1 μl DNase I (1u/μl). Reaction was incubated at 37 °C for 30 min and terminated by adding 1 μl 25 mM EDTA and incubating at 65 °C for 10 min. All treated RNA samples were kept at −70 °C until use.

Estimation of RNA purity, yield and integrity

The purity and quantity of the collected RNA was evaluated by determining the spectrophotometric absorbance of the samples at 230, 260 and 280 nm. RNA integrity was estimated from different distinct rRNA bands on 1.2 % formaldehyde–agarose gel electrophoresis after staining with ethidium bromide and visualization under UV light (Rodrigues et al. 2007).

Primer design

Since the nucleotide sequences of *actin* and *β-tubulin* genes have not still been reported in pistachio, sequence homology among the genes of interest from other plants, available in the GenBank of the National Center for Biotechnology Information (NCBI) followed by identifying the conserved regions were used to design the primers by Oligo7 software. PCR was performed using PCR Master Mix kit (Fermentas), as the initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C–58 °C for 30 s and extension at 72 °C for 50 s with a final extension at 72 °C for 10 min. Amplicons were purified using Gel Extraction Kit (Roch, Germany) and ligated into the TA cloning vector according to the manufacturer's

instructions (InsTAclone PCR Cloning Kit, Fermentas). The constructed plasmids were introduced into *Escherichia coli* DH5 α competent cells and spread on X-gal/IPTG plate. White colonies were picked after 15 h incubation at 37 °C and the corresponding DNA was amplified through colony PCR to confirm positive colonies. Finally, the colonies of interest were subjected to plasmid extraction using the Plasmid Extraction Kit (Roch, Germany) and then sequenced. After confirming the resulting sequences with available ones in the GenBank (NCBI) using the BLASTN algorithm, they were submitted to NCBI (Table 1).

Quantitative real-time PCR

For real-time PCR analysis, single-stranded cDNA was synthesized from 2 μ g total RNA according to the manufacturer's instructions (RevertAid First Strand cDNA Synthesis Kit, Fermentas). Real-time PCR was performed using the SYBR Green Real-time PCR master mix (Ampliqon, Denmark) to amplify the *actin* and *β -tubulin* genes. The reaction mixture contained 1 μ L of cDNA sample, 0.5 μ L each of the forward and reverse primers (10 μ M) and 10 μ L real-time master mixes in a final volume of 20 μ L. The thermal cycling conditions were as follows: initial denaturation and polymerase activation step at 94 °C for 15 min, followed by 40 cycles 94 °C for 30 s, 63 °C for 30 s and 72 °C for 20 s. A melting-curve analysis was included (65 °C to 95 °C with fluorescence measured every 0.5 °C) at the end of each reaction to confirm further the specificity of primer pairs. Additionally, each PCR reaction included a reverse transcription negative control and a negative control with no template to check for potential genomic DNA and reagent contamination, respectively. The amplified products were monitored using melt curve analysis and visualized on 2 % agarose gel after staining with ethidium bromide. All samples were amplified in triplicate and the qPCR efficiency for each primer pair was calculated based on the slope of the standard curve using the following equation: ($E = 10^{-1/\text{slope} - 1}$) (Radonic et al. 2004).

RNA sequencing

High quality RNA extracted from the roots of *P. vera* L. C Ghazvini and *P. vera* L. C Sarakhs following salt treatment and control were used for RNA-seq library preparation and sequencing. To this end, mRNA samples were purified from

1 μ g extracted total RNAs using the Oligotex mRNA Mini Kit (Qiagen), followed by library preparation and sequencing on Illumina Hiseq 2000 as a 2 $\times$ 100 paired-end run according to the manufacturer's protocols.

Results and discussion

High quality RNA isolation is a critical step for studying genes expression, regulation, and function. After unsuccessful attempts with existing RNA isolation protocols for recalcitrant plants, as shown in Table 2, the present study aimed at developing a method that works reliably for RNA isolation from pistachio plants to conduct subsequent applications in comparative functional genomics (e.g., real-time PCR and RNA-seq analysis). Here, we report an improved CTAB-based method that does not require either commercial kits or several clean up steps to obtain the satisfactory results.

In the proposed method, chloroform and isoamyl alcohol (24:1, v/v) was used for the denaturation of contaminating proteins. Phenol, a very hazardous chemical, is usually used for removing proteins, but has detrimental influence on subsequent applications, such as reverse transcription (Liao et al. 2004). Phenol-based methods may produce unusable brown RNA pellet, also observed in our study, which can be as a result of oxidation. Additionally, it has been reported that phenol-based extraction could reduce poly (A) ⁺ RNA transcript levels (Chang et al. 1993); it was therefore important to eliminate the phenol. We found out that if nucleic acids were precipitated with sodium acetate and chilled absolute ethanol instead of NaCl/isopropanol, a large water-insoluble precipitate pellet is formed, and RNA would be lost; Tong et al. in 2012 reported a similar finding (Tong et al. 2012). However, in the presence of an equal volume of 5 M NaCl and chilled isopropanol, the polysaccharides stayed in solution, whereas the nucleic acids formed a jelly-like precipitate which was then precipitated by LiCl, resulting in high quality total RNA. Although real time PCR reaction with reverse transcription negative control template indicated that there is no significant DNA contamination in RNA samples, small DNA contamination is inevitable, so the isolated RNA was further treated with RNase-free DNase I in order to get rid of any co-extracted DNA during LiCl precipitation. Unlike some available methods (Chang et al. 1993; Gasic et al. 2004), we observed that the application of LiCl precipitation directly after a

Table 1 Primer sequence of *ACT* and *β -TUB* for quantitative real-time PCR in *P. vera*

GenBank accession number	Gene abbreviation	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Tm (°C)
JZ896671	<i>ACT</i>	GTATCCACGAGACCACCTACA	GGAGCAACGACCTTGATCTTC	60
JZ896672	<i>β-TUB</i>	TGGGACCCACGTGAAGTCAG	GAGTGGTGTAACTTGCTGCTTG	60

Table 2 Average quality and quantity of total RNA extracted from different woody plants determined using the spectrophotometer in comparison to other available methods. RNA was isolated in triplicate for each sample

Species	Organ	A260/A280 ^a	A260/A230 ^a	Yield (μg/mg) ^a	Method
<i>Pinus</i> spp	Leaf	2.11 ± 0.03	2.27 ± 0.05	0.74 ± 0.047	Present
<i>Olea europaea</i>	Leaf	1.93 ± 0.012	2.260 ± 0.026	1.07 ± 0.06	Present
<i>Berberis thunbergii</i>	Leaf	2.103 ± 0.026	2.290 ± 0.032	0.7 ± 0.03	Present
<i>Rosa</i> spp	Leaf	2.140 ± 0.017	2.307 ± 0.038	0.63 ± 0.044	Present
<i>Ficus benjamina</i>	Leaf	1.9 ± 0.033	2.290 ± 0.021	0.75 ± 0.05	Present
<i>P. vera</i> (adult)	Leaf	2.107 ± 0.041	2.267 ± 0.032	0.94 ± 0.055	Present
<i>P. vera</i> L. C Ghazvini	Leaf	2.117 ± 0.023	2.27 ± 0.041	1.09 ± 0.05	Present
<i>P. vera</i> L. C Sarakhs	Leaf	1.89 ± 0.020	2.29 ± 0.024	0.84 ± 0.044	Present
<i>P. vera</i> L. C Ghazvini	Root	2.103 ± 0.018	2.26 ± 0.027	0.74 ± 0.03	Present
<i>P. vera</i> L. C Sarakhs	Root	2.13 ± 0.01	2.24 ± 0.020	0.67 ± 0.037	Present
<i>P. vera</i> (adult)	Leaf	0.8 ± 0.023	1.3 ± 0.031	0.06 ± 0.022	Provost et al. 2007
<i>P. vera</i> L. C Ghazvini	Leaf	1.12 ± 0.012	1.3 ± 0.082	0.44 ± 0.036	Ghawana et al. 2011
<i>P. vera</i> L. C Sarakhs	Leaf	0.95 ± 0.067	1 ± 0.071	0.13 ± 0.031	Meisel et al. 2005
<i>P. vera</i> L. C Ghazvini	Root	0.82 ± 0.043	1.28 ± 0.074	0.06 ± 0.037	Ghawana et al. 2011
<i>P. vera</i> L. C Sarakhs	Root	1.05 ± 0.016	1.18 ± 0.042	0.15 ± 0.074	Gasic et al. 2004

^a Results are expressed as the average of three samples (± standard error)

second chloroform extraction led to poor quality RNA with low yield from pistachio and other woody plants. Thus, nucleic acids were precipitated by adding NaCl/isopropanol to the supernatant and then the RNA pellet was precipitated by LiCl solution. Real-time PCR with a reverse transcription negative control sample as template showed that there was no significant DNA contamination in the extracted RNA, revealing the high purity of extracted RNA. However, small DNA contamination was inevitable, so the isolated RNA was further

treated with RNase-free DNase I in order to get rid of any co-extracted DNA during LiCl precipitation.

Pistachio tissues, like other recalcitrant species, contain high levels of polyphenols and polysaccharides, which are known to interfere with the isolation of RNA. These compounds can co-precipitate and contaminate RNA during extraction, affecting its quality, quantity and subsequent applications (Dash 2013; Singh et al. 2003). Given that pectic substances in the cell wall of trees and the high content of

Fig. 1 Agarose gel electrophoresis of extracted RNA and real-time PCR products. **a** and **b** 2 μg of isolated total RNA by the proposed method and other methods, respectively, resolved on 1.2 % formaldehyde denaturing agarose gel and stained with ethidium bromide. **c** and **d** real-time PCR products resulted from amplification of *actin* (178 bp) and *β-tubulin* (138 bp) amplicons from the proposed method-extracted RNA samples resolved on 2 % agarose gel, respectively. In (a), (c), and (d), lane 1) adult leaf of *Pinus* spp., lane 2) adult leaf of *O. europaea*, lane 3) adult leaf of *B. thunbergii*, lane 4) adult leaf of *R. hybrida*, lane 5) adult leaf of *F. benjamina*, lane 6) adult leaf of *P. vera*, lane 7) young leaf of *P. vera* L. C

Ghazvini, lane 8) young leaf of *P. vera* L. C Sarakhs, lane 9) root of *P. vera* L. C Ghazvini, lane 10) root of *P. vera* L. C Sarakhs. Lane M represents 100 bp DNA marker (Fermentas). In (b), lane 1) root of *P. vera* L. C Sarakhs (Gasic et al.'s method 2004), 2) root of *P. vera* L. C Ghazvini (Ghawana et al.'s method 2011), 3) young leaf of *P. vera* L. C Ghazvini (Ghawana et al.'s method 2011), 4) adult leaf of *P. vera* (Provost et al.'s method 2007), 5) young leaf of *P. vera* L. C Sarakhs (Meisel et al.'s method 2005), 6) young leaf of *P. vera* L. C Ghazvini (Gasic et al.'s method 2004)

Table 3 Quantitative real-time PCR (qRT-PCR) analysis of total RNA extracted from different woody species. QRT-PCR was performed in triplicate for each sample

Species	Organ	<i>Actin</i> Ct ^a	<i>β-tubulin</i> Ct ^a
<i>Pinus</i> spp	Leaf	14.225 ± 0.141	17.06 ± 0.0346
<i>Olea europaea</i>	Leaf	17.875 ± 0.031	15.58 ± 0.144
<i>Berberis thungergii</i>	Leaf	19.835 ± 0.894	18.85 ± 0.178
<i>Rosa</i> spp	Leaf	17.875 ± 0.106	15.73 ± 0.0519
<i>Ficus benjamina</i>	Leaf	17.955 ± 0.089	15.785 ± 0.0144
<i>P. vera</i> (adult)	Leaf	19.02 ± 0.161	16.59 ± 0.005
<i>P. vera</i> L. C Ghazvini	Leaf	16.94 ± 0.023	15.24 ± 0.202
<i>P. vera</i> L. C Sarakhs	Leaf	18.1 ± 0.184	16.105 ± 0.124
<i>P. vera</i> L. C Ghazvini	Root	21.18 ± 0.092	19.71 ± 0.080
<i>P. vera</i> L. C Sarakhs	Root	20.73 ± 0.051	19.645 ± 0.349

^a The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. The results were expressed as the average of three samples (± standard error)

polyphenols complicate nucleic acid extraction, high concentrations of PVP and β-mercaptoethanol were used in order to avoid polyphenolic oxidation and formation of high-molecular-weight complexes with nucleic acids and proteins (Xu et al. 2009). A high ionic strength buffer resulting from 2 M NaCl, along with an extended incubation time at 65 °C would enhance the solubility of polysaccharides and help to reduce their co-precipitation with RNA.

The results revealed that high quality total RNA was obtained from pistachio and other woody species using this method (Fig. 1a and Table 2). We assessed the integrity of the isolated RNA by visualization of ribosomal RNA bands on 1.2 % formaldehyde–agarose gel (Fig. 1a). The gel revealed distinct 25S, 23S, 18S, 16S, and 5S rRNA bands for the leaf samples, and 25S and 23S rRNA bands for the root samples (Fig. 1a). Moreover, the quantity and quality of extracted RNA were estimated using the spectrophotometer (Table 2). As shown in Table 2, the A260/A280 and A260/A230 ratios for all the samples evaluated were about 2.1 and 2.3, respectively, indicating that the extracted RNA was of high purity. The total RNA was isolated in triplicate for each sample and yields ranged from 0.67 to 1.09 μg per mg of fresh weight, which was considerably high in terms of the quantity and quality of the RNA relative to other reports as presented in Fig. 1b and Table 2 (Gasic et al. 2004; Meisel et al. 2005; Provost et al. 2007; Ghawana et al. 2011).

The suitability of the extracted RNA for downstream applications was determined by running real-time PCR and RNA-seq. These applications were chosen because of their high sensitivity to impurities and integrity of RNA (Tajner et al. 2013). Figures 1c, d show the resolved PCR products on 2 % agarose gel following amplification of *actin* and *β-tubulin* genes, respectively. According to our real-time PCR results, while the desired detectable fluorescence signal of *actin* and *β-tubulin* genes appeared after 14.2–21.8 and 15.24–19.71 cycles in different positive samples, respectively

Fig. 2 Read quality distributions of pistachio's root samples from Illumina HiSeq 2000 sequencing. **a** *P. vera* L. C Ghazvini, control. **b** *P. vera* L. C Ghazvini, salt stress. **c** *P. vera* L. C Sarakhs, control. **d** *P. vera* L. C Sarakhs, salt stress. The PHRED quality score and percentage of sequences with a particular score, normalized to the total number of sequences were illustrated as the x and y axes, respectively

(Table 3), the fluorescence signal emerged after 38.3 cycles in reverse transcription negative control and after 39 cycles in the no template control samples, implying that there was very little DNA contamination present in the isolated RNA samples. The linear correlation coefficient (R^2) of the standard curve during the amplification of 10-fold serial dilution of pooled cDNA samples from different RNA samples using *actin* and β -*tubulin* primers was 0.9876 and 0.9934, respectively. PCR efficiency of 100 % is determined with a slope value of -3.322 , while higher or lower values denote a PCR efficiency higher or lower than 100 %, yet slope values up to -3.0 or down to -4.1 are tolerated (Cury and Koo 2007; Overbergh et al. 2003). The amplification slope value for cDNA samples of interest was determined to be -3.43 and -3.614 for *actin* and β -*tubulin* primers, respectively, which is within the range of acceptable slope, further confirming the quality of extracted RNA.

Providing high quality RNA is a critical requirement of any RNA-seq experiment as the isolated RNA should pass stringent quality control standards for sequencing on the available platforms (Healey et al. 2014). The quality of sequencing data were assessed by CLC Bio Genomics WorkBench software, version 6.5 (CLC Bio, Denmark) and illustrated in Fig. 2. The quality of raw reads is calculated based on PHRED score, which is logarithmically related to the base-calling error probability (Ewing and Green 1998). The generation of about 50 million 100-bp paired-reads per library with the mean quality score of 38 implies the almost a 99.99 % base call accuracy, reflecting the high quality of the isolated RNA for library preparation and high-throughput sequencing. The results are particularly important as two sequencing datasets (Fig. 2b, d) were derived from the isolated RNA from the salt-stressed plants, which accumulated higher amounts of phenolics and polysaccharides as compared to the unstressed plants. In conclusion, our results demonstrated that the extracted RNA using the proposed protocol was transcriptionally quite acceptable and well-suited for sensitive downstream studies including real-time PCR and RNA-seq analysis.

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Conflict of interest The authors declare that they have no conflict of interest.

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