Electronic Journal of Biotechnology 23 (2016) 21–27

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Isolation of high-quality RNA from *Platycladus orientalis* and other Cupressaceae plants

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ARTICLE INFO

Article history:
Received 24 February 2016
Accepted 1 August 2016
Available online 18 August 2016

Keywords:
Cupressaceae plants
Polysaccharides
Polyphenols
Total RNA isolation
Two-step removal of polyphenols and polysaccharides

ABSTRACT

Platycladus orientalis has a lifespan of several thousand years in China, making it a good plant in which to study aging at the molecular level, but this requires sufficient quantities of high-quality *P. orientalis* RNA. However, no appropriate methods have been reported for total RNA isolation from *P. orientalis* leaves. The TRIzol method did not extract RNA, while cetyltrimethylammonium bromide, sodium dodecyl sulfate-phenol, and plant RNAout kit (Tianz, Inc., China) protocols resulted in low yields of poor quality RNA. Isolating total RNA using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) resulted in a high-quality product but a low yield. However, the two-step removal of polyphenols and polysaccharides in the improved plant RNAout kit protocol resulted in the isolation of RNA with a 28S:18S rRNA ratio of band intensities that was ~2:1, the A260/A280 absorbance ratio was 2.03, and the total RNA yield from *P. orientalis* leaves was high. This protocol was tested on different *P. orientalis* tissues of different ages and on leaves of five other Cupressaceae plants. The total RNAs were successfully used in complementary DNA synthesis for transcriptome sequencing and would be suitable to use in additional experiments. The results of this study will benefit future studies in Cupressaceae plants.

1. Introduction

Platycladus orientalis is used extensively as a medicinal ingredient and as an ornamental landscape plant that can tolerate a wide range of environmental extremes [1]. *P. orientalis* is rich in many longevity- and resistance-related genes [2]. Recently, many researchers have analyzed the resistance mechanisms using transcriptome and digital expression profiles [3,4,5,6]. Obtaining high-quality RNA is a prerequisite to success in subsequent steps of gene expression analyses, such as quantitative reverse transcription polymerase chain reactions (qRT-PCR), the synthesis of complementary DNA (cDNA) libraries, differential displays, and microarrays. However, no improved protocols have been reported for total RNA isolation from the leaves of *P. orientalis*.

Traditional methods using TRIzol reagent, cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS)-phenol could not produce high-quality RNA from gymnosperms that contain abundantpolyphenols, polysaccharides, pectin, and other secondary metabolites [7]. When the CTAB method is used to extract RNA, polysaccharides are released and coprecipitated with RNA at 65°C because the physical and chemical properties of polysaccharides are similar to those of RNA, resulting in the RNA and polysaccharides being removed together [8]. At present, many commercial kits result in the extraction of sufficient amounts of high quality RNA to perform subsequent experiments. The common and commercial method, Talent Total Quick RNA kit (Talent S.R.L., Italy), were used together [8] to extract RNA of high quality and integrity from *Cupressus arizonica* pollen.

It is difficult to effectively remove polysaccharides, polyphenols, gum, and other substances while minimizing the loss of the RNA of *P. orientalis* and other Cupressaceae plants. It is even more challenging to consistently obtain pure RNA for RNA-seq, the rapid amplification of cDNA ends, northern blotting, cDNA synthesis, and other experiments [11,12]. Our modified protocol can be applied to the isolation of high levels of high quality total RNA form *P. orientalis* and other Cupressaceae plants.

2. Material and methods

2.1. Sample preparation

Leaves of 100-, 1000-, 2000-, and 3000-year-old *P. orientalis* leaves were collected from the Mausoleum of the Yellow Emperor, Shaanxi, China. Different tissues, including seeds, roots, stems, and fruits, were collected from five-year-old seedlings of *Sabina chinensis*,

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2016.08.003

Table 1
Descriptions of reference genes and *NAC* from *P. orientalis* used in qRT-PCR.

Gene abbreviation	Gene name	*Arabidopsis* homologlocus	Primer sequence (5′–3′)	Size (bp)	PCR efficiency
αTUB	Alpha-tubulin	AT5G19770	CCACATCTCTTAGGTTTGATGGAG GGGTCACACTTGGCCATCAT	205	103.6%
UBC	Ubiquitin-conjugating enzyme E2	AT3G57870	TCTTGCTGAAGAGCGGAAGG ATGCACTGCCAGACCATCAA	108	99.2%
NAC	NAC domain protein	AT5G39610	AGAGGAGAAGGAAGCGAAGG TGGCGTATGATGAGTCCAAA	169	104.3%

Chamaecyparis lawsoniana, *Thuja occidentalis*, *Procumbent juniper*, and *Thuja standishii*, which were cultivated in soil-filled pots in the greenhouse at the Chinese Academy of Forestry, Beijing, frozen immediately in liquid nitrogen and preserved for long-term storage at -80°C.

2.2. Experimental Methods

2.2.1. CTAB method

Frozen *P. orientalis* leaves (0.1 g) were finely ground and quickly transferred to 1 mL CTAB RNA extraction buffer (2% CTAB; 100 mM Tris–HCl, pH 8.0; 2.0 M NaCl; 20 mM EDTA, pH 8.0) pre-warmed to 65°C for 5 min. β-Mercaptoethanol (30 μL) was added, and samples were placed at 65°C for 30 min with gentle inversion every 5 min and then cooled to room temperature. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed vigorously in a vortex for 30 s. The contents were centrifuged at 14,000 × g for 10 min at 4°C. The aqueous supernatant was transferred to a new tube, and the chloroform:isoamyl alcohol (24:1) extraction was repeated. To the upper phase,10 M LiCl (final concentration, 3 M) was added, mixed well, and incubated at -80°C for 1 h or overnight at -20°C. RNA was pelleted at 12,000 × g and 4°C for 20 min, The pellets were washed two times with 1 mL 70% ethanol and air-dried at room temperature for 10 min. The total RNA pellet was resuspended in 50 μL RNase-free water and stored at -80°C until use.

2.2.2. SDS-phenol protocol

P. orientalis leaves (0.1 g) were ground into a powder and 600 μL SDS extraction buffer [2% SDS; 25 mM ethylenediaminetetraacetic acid (EDTA); 100 mM Tris–HCl, pH 8.0; 2% Polyvinylpyrrolidinone (PVP)] was added. Then, 300 μL chloroform and 300 μL water-saturated phenol were added, and the sample was mixed and incubated for 20 min on ice. The sample was vortexed and centrifuged at 12,000 × g for 15 min at 4°C to separate phases. The supernatant was transferred to a new centrifuge tube. The extraction with 300 μL chloroform and 300 μL water-saturated phenol was repeated with an incubation and centrifugation under the same conditions as described above. The top phase was recovered and an equal volume of isopropyl alcohol was added to precipitate the RNA. RNA was pelleted at 14,000 × g for 20 min at 4°C. The RNA was ethanol precipitated, washed, resuspended, and stored as described in the CTAB protocol.

2.2.3. TRIzol protocol

Total RNA was extracted according to the of TRIzol manufacturer's protocol (Life Technologies Corp., Carlsbad, USA) and resuspended in a total volume of 50 μL RNase-free water.

2.2.4. Plant RNAout kit

The plant RNAout kit was used according to the manufacturer's instructions (Tianz, Inc., China), and RNA was resuspended in a total volume of 50 μL RNase-free water.

2.2.5. Improved plant RNAout kit protocol

Approximately 0.4 g of *P. orientalis* leaves were ground to a fine powder in liquid nitrogen. PVP (0.02 g) was added, the sample was transferred to four 2-mL centrifuge tubes, and 1 mL extraction buffer and 50 μL β-mercaptoethanol were added. Samples were vortexed, and 200 μL high-salt solution (0.8 M sodium citrate; 5 M KAc, pH 4.8) was added. Then, 300 μL water-saturated phenol and 300 μL chloroform were added, and the mixture was shaken for 30 s and then incubated on ice for 20 min. The sample was vortexed and centrifuged at 14,000 × g for 15 min at room temperature. After centrifugation, ~700 μL supernatant was transferred into a new tube without disturbing the pellet. Then, the remaining steps followed the manufacturer's instructions. Four 50-μL RNA samples were obtained using the kit's protocol. DNA removal reagents (Tianz, China), 15 μL 10× DNase I buffer and 30 μL DNase I, were added to each tube, and the mixture was incubated at 20–25°C for 20 min. The four samples were transferred to a 2-mL centrifuge tube, and equal volumes of the polysaccharide scavenger agents were added (Tianz, China). Samples were vortexed, and an equal volume of chloroform was added. Samples were centrifuged at 14,000 × g for 10 min, and the supernatant layer was retained. The RNA was ethanol precipitated, washed, resuspended, and stored as described in the CTAB protocol.

2.2.6. Spectrum™ Plant Total RNA Kit

Total RNA was extracted according to the protocol of the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO, USA) and resuspended in a total volume of 50 μL RNase-free water.

2.2.7. Yield and quality of total RNA

The integrity and purity of the total RNA were evaluated using 2% denaturing gel electrophoresis and NanoDrop DU8000 spectrophotometry (A_{260}/A_{280} and A_{260}/A_{230}). Volumes of 5 μL RNA were loaded in each lane for gelelectrophoresis. In addition, the RNA from 100-, 1000-, 2000-, and 3000-year-old *P. orientalis* leaves were also analyzed on an Agilent 2100 Bioanalyzer.

2.2.8. Transcriptome sequencing

The improved plant RNAout kit protocol was used to isolate RNA from 20-, 1000-, and 2000-year-old *P. orientalis* leaves for RNA sequencing and raw data processing by BGI Tech. The mRNA-seq library was constructed following the manufacturer's instructions from a mRNA-seq Sample Preparation Kit (Cat# RS-930-1001,

Table 2
Yield and quality of total RNA isolated from *P. orientalis* leaves using five RNA extraction methods.

Sample	CTAB	SDS	TRIzol	Plant RNAout kit	Improved plant RNAout kit	Spectrum™ Plant Total RNA Kit
RNA yield (μg/g FW)	10.6	3.7	–	57.1	86.6	34
Absorbance ratio (A_{260}/A_{280})	1.05	0.92	–	1.58	2.03	2.20
Absorbance ratio (A_{260}/A_{230})	0.79	0.33	–	0.87	1.89	2.12

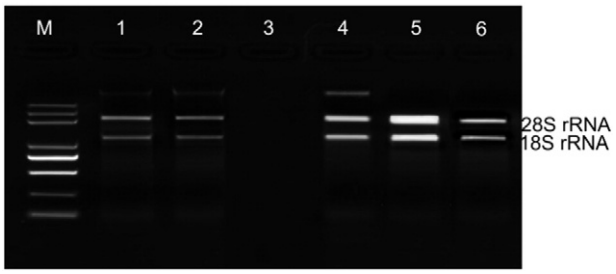

Fig. 1. Agarose gel electrophoresis showing total RNA extracted from *P. orientalis* leaves using five RNA isolation methods: CTAB method (lane 1), SDS method (lane 2), TRIzol method (lane 3), plant RNAout kit protocol (lane 4), and Improved plant RNAout kit protocol (lane5).

Illumina Inc., San Diego, CA, USA). Briefly, mRNA was purified from 20 μg of total RNA using oligo (dT) magnetic beads. Following purification, the mRNA was fragmented into small pieces using divalent cations under an elevated temperature, and the cleaved RNA fragments were used for first strand cDNA synthesis using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA polymerase I and RNaseH. Sequencing adapters were ligated to short fragments after purifying with a QiaQuick PCR extraction kit and were used to distinguish different sequencing samples. Fragments with lengths ranging from 200 to 500 bp were then separated by agarose gel electrophoresis and selected for PCR amplification as sequencing templates. The final cDNA library was sequenced using the Illumina GAIIx system according to the manufacturer's protocols, with a paired-ends (PE) read length of 90 bp. The raw data are available at the NCBI Sequence Read Archive under the accession number SRP074805.

2.2.9. De novo assembly and gene annotation

The raw reads were cleaned by removing adaptor sequences, empty reads and low quality sequences, which included reads containing an N percentage (*i.e.*, the percentage of nucleotides in a read that could not be sequenced) over 10% and reads containing more than 50% nucleotides with a Q-value ≤5. Transcriptome *de novo* assembly was performed separately with the short reads assembling programs SOAPdenovo and Trinity. The longest transcript was selected as the unigene in this study.

2.2.10. cDNA synthesis and qRT-PCR

First-strand cDNA was synthesized from 600 ng total RNA in a volume of 20 μL using the PrimeScript® RT reagent kit (Takara, Japan) according to the manufacturer's protocol. All of the candidate gene sequences were obtained from the *P. orientalis* transcriptome. Gene primers were designed by Primer3.0 software (http://frodo.wi.mit.edu/primer3/) (Table 1), and all of the primer pairs were initially tested using standard RT-PCR with Premix Ex Taq (TaKaRa, Japan). A single amplification product of the expected size for each gene was verified by electrophoresis on a 2% agarose gel and staining with ethidium bromide. qRT-PCR was carried out in 96-well blocks on an Applied Biosystems 7500 Real-Time PCR system using SYBR® Premix

Table 3
Total RNA extracted from leaves of different ages, as well as seeds, roots, stems, and fruits of *P. orientalis* and the leaves of other Cupressaceae species, *S. chinensis*, *C. lawsoniana*, *T. occidentalis*, *P. juniper*, and *T. standishii* using a Spectrum™ Plant Total RNA Kit.

Sample		RNA yield (μg/g FW)	Absorbance ratio (A_{260}/A_{280})	Absorbance ratio (A_{260}/A_{230})
P. orientalis	100 yr	26.3	2.19	2.11
	1000 yr	22.1	2.22	2.12
	2000 yr	30.4	2.15	1.91
	3000 yr	25.5	2.20	2.12
	Seed	56.3	2.05	2.03
	Root	19.9	2.16	2.13
	Stem	54.3	2.14	1.96
	Frite	38.9	2.08	1.98
S. chinensis	Leaf	19.6	2.17	1.84
C. lawspnian		38.1	2.19	2.01
T. occidentalis		29.1	2.22	1.94
P. juniper		53.9	2.16	2.17
T. standishii		26.4	1.98	1.86

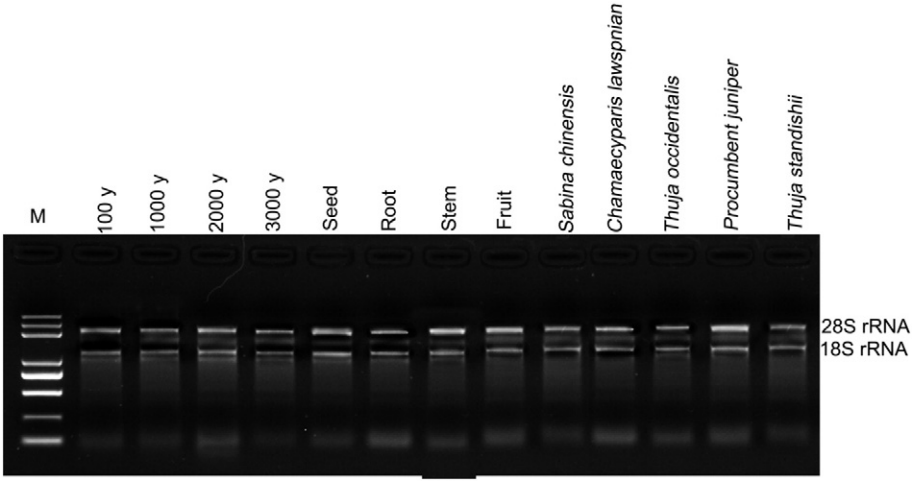

Fig. 2. Total RNA extracted from leaves of different ages, seeds, roots, stems, and fruits of *P. orientalis* and the leaves of other Cupressaceae species: *S. chinensis*, *C. lawsoniana*, *T. occidentalis*, *P. juniper*, and *T. standishii* by Spectrum™ Plant Total RNA Kit.

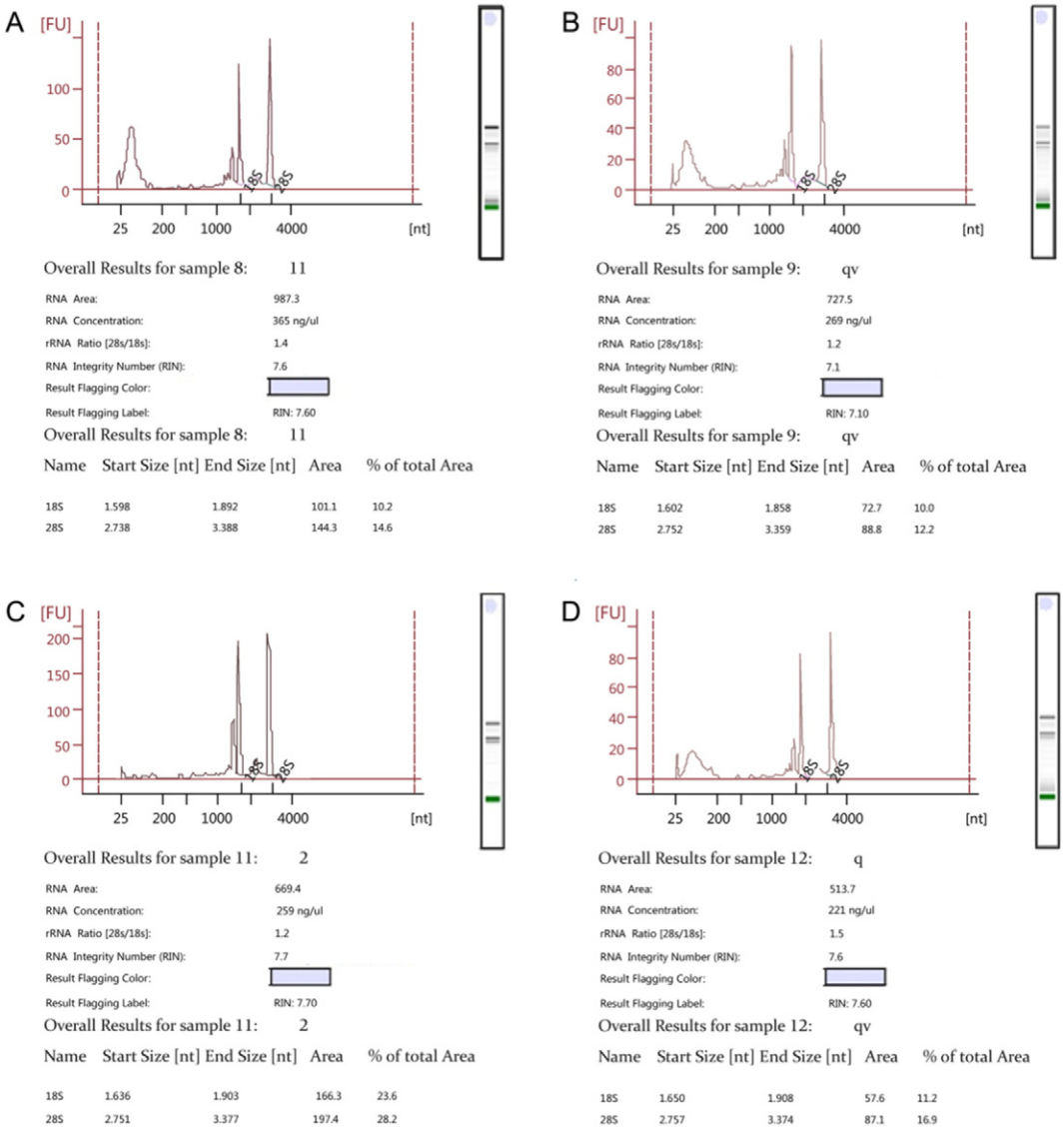

Fig. 3. Agilent 2100 Bioanalyzer analysis of total RNA extracted from 100- (a), 1000- (b), 2000- (c), and 3000-year-old (d) *P. orientalis* leaves using the improved plant RNAout kit protocol.

Ex Taq™ (Takara, Japan) in a 20 μL reaction volume according the protocol.

3. Results

3.1. Yield and quality of total RNA isolated from P. orientalis leaves using five RNA extraction methods

We examined five different RNA extraction methods to isolate RNA from *P. orientalis* leaves. Total RNA could not be isolated by the TRIzol method, as evidenced by the lack of 28S and 18S bands. The yields of the CTAB and SDS-phenol methods were 10.6 and 3.7 μg/g, respectively, which were low, and the A_{260}/A_{280} ratios were 1.05 and 0.92, respectively, and the A_{260}/A_{230} ratios were 0.79 and 0.33, respectively, indicating poor RNA quality (Table 2 and Fig. 1). RNA was extracted using the plant protocol of the RNAout kit. The A_{260}/A_{280} and A_{260}/A_{230} ratios were 1.29 and 0.48, respectively. These ratios indicated that the RNA samples contained protein and polysaccharide contamination. These methods did not allow us to obtain sufficient amounts of high-quality RNA for subsequent experiments. High-quality RNA was obtained using the Spectrum™ Plant Total RNA Kit, but the yield, at 34 μg/g, was low. However, the improved plant RNAout kit isolated RNA of a high purity, lacking contaminating polyphenols and polysaccharides, as demonstrated by the absorbance ratio values, in which the A_{260}/A_{280} ratio was 2.03, the A_{260}/A_{230} ratio was 1.89. Additionally, the RNA yield was high at 86.6 μg/g. The

Table 4
Total RNA extracted from *P. orientalis* leaves of different ages using the improved plant RNAout kit.

Sample	RNA yield (μg/g FW)	Absorbance ratio (A_{260}/A_{280})	Absorbance ratio (A_{260}/A_{230})	RNA integrity number	28S:18S
100 yr (a)	45.6	2.17	2.09	7.6	1.4
1000 yr (b)	33.6	2.09	2.05	7.1	1.2
2000 yr (c)	32.4	2.02	1.98	7.7	1.2
3000 yr (d)	27.6	2.06	1.93	7.6	1.5

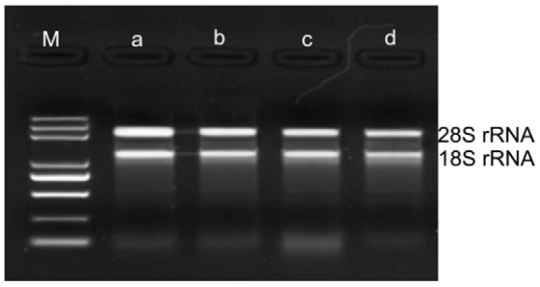

Fig. 4. Agarose gel electrophoresis showing total RNA extracted from 100- (line a), 1000- (line b), 2000- (line c), and 3000-year-old (line d) *P. orientalis* leaves using the improved plant RNAout kit protocol.

intensities of the 28S rRNA bands were approximately twice that of the 18S rRNA bands using all of the methods, except for the TRIzol method. Thus, the improved protocol of the plant RNAout kit yielded high levels of high quality RNA.

3.2. Total RNA extraction from P. orientalis and other Cupressaceae plants using the Spectrum™ Plant Total RNA Kit

The yields of total RNA were as follows: 22.1–30.4 μg/g for leaves of different ages, 19.9–56.3 μg/g for different tissues, and 19.6–53.9 μg/g for leaves of other Cupressaceae species using a Spectrum™ Plant Total RNA Kit (Table 3). The extraction protocol described here was efficient in yielding high quantities of high quality total RNA from leaves of different ages, from the seeds, roots, stems, and fruits of *P. orientalis*, and from the leaves of other Cupressaceae species, *S. chinensis*, *C. lawsoniana*, *T. occidentalis*, *P. juniper*, and *T. standishii*. For all of the samples, the $A_{260}/_{230}$ ratio was higher than 1.79. This indicated that the RNA was of a high purity, without polyphenol and polysaccharide contamination. The $A_{260}/_{280}$ ratios ranged from 1.93–2.14, indicating a lack of protein contamination. The RNA integrity was assessed by the sharpness of ribosomal RNA bands visualized on a denaturing 1.2% agarose gel. For all of the RNA samples tested, distinct 28S and 18S rRNA bands without degradation were observed (Fig. 2).

3.3. Total RNA extraction from leaves of P. orientalis plants of different ages using the improved plant RNAout kit

The improved protocol of the plant RNAout kit was used to extract RNA from 100-, 1000-, 2000-, and 3000-year-old *P. orientalis* seedlings. RNA integrity was analyzed by the Agilent 2100 Bioanalyzer (Fig. 3) and subsequently with the RNA integrity number algorithm. The RNA integrity number quality indicators were greater than 7.0, the RNA concentrations were greater than 27.6 μg/g, and the RNA yield was more than 10 μg in all of the detected samples (Table 4). The A_{260}/A_{280} and A_{260}/A_{230} ratios were greater than 1.93 according to

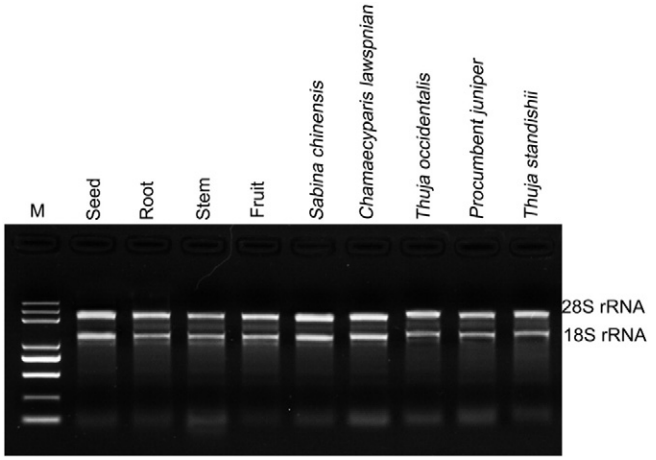

Fig. 5. Agarose gel electrophoresis showing total RNA extracted from seeds, roots, stems, and fruits of *P. orientalis* and the leaves of other Cupressaceae species: *S. chinensis, C. lawsoniana, T. occidentalis, P. juniper,* and *T. standishii.*

an ultraviolet spectrophotometer. RNA integrities were assessed by 2% agarose gel electrophoresis, which showed clear bands without DNA contamination and other impurities. The 28S:18S rRNA band intensities ratios were ~2:1 (Fig. 4). These results indicated that the RNAs extracted by the modified protocol had high concentrations and were of high quality.

3.4. Total RNA extraction from P. orientalis and other Cupressaceae plants by the improved plant RNAout kit

High-quality RNAs were isolated successfully from seeds, roots, stems, and fruits of *P. orientalis* and from the leaves of other Cupressaceae species, *S. chinensis*, *C. lawsoniana*, *T. occidentalis*, *P. juniper*, and *T. standishii*. The purities of the total RNAs were determined using a NanoDrop DU8000 spectrophotometer. The yields of the total RNAs from the different *P. orientalis* tissues were greater than 41.8 μg/g, which were considered to be high enough for subsequent experiments. The ratios of A_{260}/A_{280} were greater than 1.92, and the ratios of A_{260}/A_{230} were greater than 1.79 (Table 5), indicating that there were no polysaccharide, polyphenol, or protein contamination in the extracted RNA samples. Moreover, the 28S and 18S rRNA bands were clearly shown, without degradation, by 2% agarose gel electrophoresis (Fig. 5), which suggested that the RNAs were pure and could be used for further analyses.

3.5. Transcriptome analysis

To obtain an overview of the *P. orientalis* gene expression profile at different ages, high-quality total RNA samples of polysaccharide-rich tissues, such as those from 20-, 1000-, and 2000-year-old individuals

Table 5
Total RNA extracted from seeds, roots, stems, and fruits of *P. orientalis* and the leaves of other Cupressaceae species, *S. chinensis*, *C. lawsoniana*, *T. occidentalis*, *P. juniper*, and *T. standishii.*

Sample		RNA yield (μg/g FW)	Absorbance ratio (A_{260}/A_{280})	Absorbance ratio (A_{260}/A_{230})
P. orientalis	Seed	82.1	2.14	2.23
	Root	49.8	1.92	1.79
	Stem	41.8	1.96	1.82
	Frite	47.4	2.02	1.87
S. chinensis	Leaf	76.6	1.96	1.90
Chamaecyparis lawspnian		66.1	2.07	2.02
T. occidentalis		43.4	1.93	1.83
P. juniper		44.5	2.01	1.92
T. standishii		56.5	1.98	1.86

Table 6
Transcriptome data from *P. orientalis* leaves.

Total number of reads	13,600,516
Total base pairs (bp)	1,224,046,440
Average read length	90 bp
Total number of contigs	363,885
Mean length of contigs	142
N50	105
Total number of scaffolds	68,219
Mean length of scaffolds	389
N50	569
Total number of unigenes	51,664
Mean length of unigenes	475
N50	631

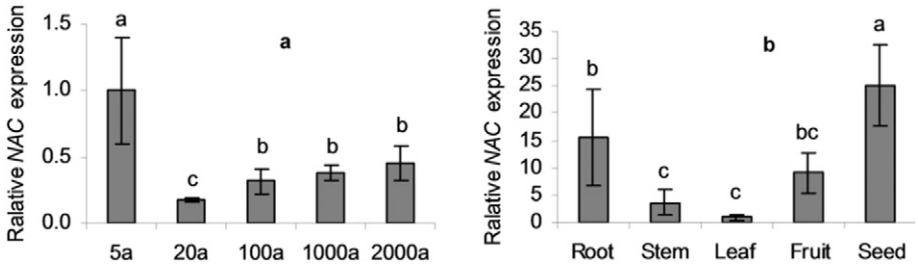

Fig. 6. The expression profiles of *NAC* in leaves of different ages (a) and different tissues (b) of *P. orientalis* (as determined by qRT-PCR with *ubiquitin C* and *α-tubulin* as reference genes).

of *P. orientalis*, were successfully sequenced on the Illumina platform. After cleaning and quality checks, 1.2 billion reads were assembled into 363,885 contigs with an N50 length, the median contig size of the genomic assembly, of 105 bp and a mean length of 142 bp (Table 6). The 68,219 scaffolds generated 51,664 unigenes with an N50 length of 631 bp and mean length of 475 bp. Our modified protocol yielded RNA that could be used as a template for the transcriptome sequencing of *P. orientalis*.

3.6. Choosing suitable reference genes by qRT-PCR

The extracted RNA could be used for further analyses as demonstrated by cDNA library construction and qRT-PCR. We identified 10 reference genes that were suitable for the normalization of qRT-PCR data obtained from *P. orientalis* samples from 100-, 1000-, 2000-, and 3000-year-old tissues of seeds, roots, stems, and fruits. The reference genes were expressed in all of the tested samples with minimal variation; we chose *α-tubulin* and *ubiquitin C* as the most suitable reference genes in *P. orientalis* [3] because the *NAC* expression level showed that they were reliable housekeeping genes in plants subjected to various treatments as analyzed by qRT-PCR (Fig. 6 and Fig. 7). The successful qRT-PCR of fragments using amplified library samples further confirmed that the improved plant RNAout kit protocol yielded high-quality RNA from *P. orientalis*.

4. Discussion

Cupressaceae plants are rich in polysaccharides and polyphenols, which affect the ability to isolate high quality RNA. TRIzol is a quick method of RNA extraction, but it could not be used to extract RNA from Cupressaceae plants. This was indicated by the dark brown color of plant tissues treated with TRIzol reagent, indicating the oxidation of phenolic compounds in the plant tissues [13]. Generally, the CTAB and SDS-phenol methods used for RNA extractions from plants are modified for plant tissues that are rich in polysaccharides and polyphenols [14,15]. This might be attributed to the coprecipitation of polysaccharides and the oxidation of phenolic compounds that interact irreversibly with nucleic acids [8]. LiCl is used to precipitate RNA at certain concentrations, while potassium acetate is more effective in removing polysaccharides from the solution [7]. High LiCl concentrations may result in increased impurities (polysaccharides and polyphenols) in RNA extractions from plant tissues, limiting the concentration and quality of the RNA [9]. Additionally, the brown color of the supernatant that develops upon oxidation of the homogenate interferes with RNA extraction procedures [10]. However, commercial kits are quick methods of RNA extraction [8]. The plant RNAout kit (Tianz, China) for total RNA isolation from the root of *Paeonia suffruticosa* yielded high-quality total RNA that was free of contaminants. In this work, the plant RNAout kit was modified to overcome the problems associated with polyphenol, polysaccharide, and metabolite contamination. The Spectrum™ Plant Total RNA Kit works well with *Vitis vinifera, Chaenomeles sinensis*, and other difficult plants, and producing good quality RNA [16,17]. Leaves of different ages from other Cupressaceae species produced low yields of high-quality total RNA using the Spectrum™ Plant Total RNA Kit. After the enrichment process, the RNA concentrations were higher than the initial concentrations.

Based on these results, the plant RNAout kit method was improved by adding an appropriate amount of PVP to avoid the release of phenolic substances when crushing plant materials [18] and by adding up to 5% β-mercaptoethanol to inhibit RNase activity and prevent sample oxidation [19]. A high-salt method was used to remove the polysaccharides of spruce and pine tree tissues [20,21]. A low-concentration ethanol precipitation method was used to extract RNA from grape berry tissues and the woody stems of gymnosperms [22,23], while RNA extraction from mango peel and marine macro-algae involved an acetate precipitation method to remove polysaccharides [24,25]. This modified method added a high salt concentration (0.8 M sodium citrate and 5 M KAc, pH 4.8) to the extraction buffer and incubating samples on ice prevented the precipitation of the polysaccharides along with the RNA. Additionally, a polysaccharide scavenger agent was used to remove any polysaccharide residue. The yield and quality of the total RNA obtained from *P. orientalis* using the improved plant RNAout kit method were consistently high, and the RNA could be used for further analyses as demonstrated by transcriptome sequencing, cDNA library construction, and RT-PCR. Thus, the described improved method using the plant RNA out kit is an efficient and reproducible procedure for the isolation of RNA from *P. orientalis* and other Cupressaceae plants.

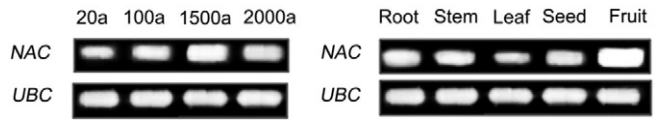

Fig. 7. The *NAC* expression profile in different tissue of different ages from *P. orientalis* (as determined by RT-PCR with *ubiquitin C* as the reference gene).

Conflict of interest

There is no conflict of interest.

Financial support

This work was supported by the National Natural Science Foundation of China [grant number 31300555]; and the National Non-Profit Research Institutions of Chinese Academy of Forestry [grant numbers RIF2013-12, RIF2014-11, CAFYBB2014QB004].

Acknowledgments

The authors thank the Mausoleum of the Yellow Emperor (Shaanxi, China) for providing the *P. orientalis* samples.

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