Optimization of total RNA isolation method from the aromatic medicinal plant *Artemisia annua* L.

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Abstract

This paper is the first report on the development of a protocol that allows rapid and simplified extraction of total RNA from *Artemisia annua* L., an aromatic medicinal plant. This innovative protocol ensures a consistently high quantity and good quality of total RNA without any contamination of polyphenols, polysaccharides and proteins. The total RNA obtained is also free of fungal RNA even when extracted from fungal infested plants. The extraction buffer used in the proposed modified protocol was made up of non-hazardous chemicals. High concentrations of polyphenols of *A. annua* L. could be successfully eliminated and the prepared total RNA could be used for downstream reactions.

Introduction

Artemisia, one of the largest genera in the Asteraceae family, consists of more than 400 species. Some of the species of this genus have been well studied for their medicinal value.[1] *Artemisia annua* L. (annual wormwood) is an aromatic herb native to Asia and Eastern European countries, and naturalized in Argentina, India, Vietnam and Brazil.[2] *A. annua* L. produces artemisinin, a sesquiterpene lactone that serves as a potential antimalarial drug. Bioactivity studies of artemisinin have indicated extraordinary endoperoxide bridges in the chemical structures which largely contributed to the schizonticide action against *Plasmodium falciparum*.[3,4] *P. falciparum* has started to develop resistance against chloroquinine and quinine, the commonly administered drugs. This multidrug resistant strain began to spread in the 1970s, especially in Southeast Asian countries such as Myanmar, Vietnam, Thailand and Malaysia.[5] The current production of artemisinin does not meet the rising world demand for the bioactive compound and its derivatives. Furthermore, synthetic artemisinin is expensive and has not been commercialized in the drug market.[4,5]

During the past decades, *in vitro* culture technique has been reported to be successful for propagation of *A. annua* L.[6,7] However, the production of artemisinin is still very limited,[8,9] and one of the important setbacks is that the expression and accumulation of artemisinin in the plant are easily affected by environmental, nutritional and physiological factors. Hence, researchers around the world are currently working on enhancing the production of artemisinin with most of the work focusing not only on breeding but also on manipulation of the artemisinin biosynthetic genes. A detailed knowledge of the artemisinin biosynthetic pathway, as well as its regulation and expression in the plant, can certainly help to provide novel approaches for the manipulation of the expression of key genes *in vitro*. A combination of *in vitro* culture and manipulation of gene expression would be an interesting technique to enhance artemisinin production which will be of great benefit in tropical countries like Malaysia where the climate is not suitable for cultivation of *A. annua* L.

A. annua L. is known for its rich mono- and sesquiterpene lactones.[10,11] High quantity and good quality of total RNA is needed to construct a high quality cDNA library of tissue culture-derived plant materials of *A. annua* L. There are many available protocols for total RNA extraction from plant leaves.[12-16] However; it is difficult to extract total RNA from plants rich in secondary metabolites due to the interference of polyphenols and polysaccharides. These secondary metabolites tend to co-precipitate with RNA during extraction and inhibit downstream enzyme modifications of the RNA.[17] A method for total RNA extraction from plants rich in secondary metabolites has been established but it involves tedious preparation of the buffer. Our preliminary studies indicated that extraction of total RNA of *A. annua* L. using commercial kits and the available protocol established for plants with high secondary metabolites were not satisfactory.[18] Hence, this paper reports on the development of a modified protocol for an effective and rapid extraction of high quantities of good quality total RNA from *A. annua* L.

Materials and Methods

Plant material

Seeds of *A. annua* L. were collected from three different clones (TC1, TC2 and Highland) from Vietnam and cultured in half-strength MS medium for germination.[19] The *in vitro* seedlings were maintained in a culture room at 25±2°C with light intensity of 32.5 µmol m^{-2} s^{-1}. Some of the seeds were also germinated in polybags and placed in a greenhouse. The leaves of 10-week old *in vitro* plantlets and seed-derived greenhouse seedlings were used for total RNA isolation.

Total RNA isolation

Total RNA isolation was first carried out following two well established protocols: IQeasy™ Plus Plant RNA Extraction Mini Kit (iNtRON Technologies) and the standard protocol for plants with high content of secondary metabolites using cetyltrimethyl-ammonium bromide (CTAB) and glacial acetic acid.[18] The efficiency of these two protocols was compared for *A. annua* L.

Total RNA isolation was also carried out using our modified CTAB protocol. The total RNA from 0.5 g of *A. annua* L. cultured leaves was isolated using 3 mL of sterile extraction buffer comprised of 3.0% CTAB (w/v), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 3.0 M NaCl, 2.5% polyvinylpyrrolidone (PVP) K-40 (w/v). The lysis buffer was pre-heated in a water bath (Grant Instruments, Cambridge,

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Key words: *Artemisia annua* L., tissue cultured leaves, total RNA extraction, PCR.

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USA) at 65°C and 2% β-mercaptoethanol (v/v) was added into the buffer before pre-heating. The leaves of tissue-cultured plants were ground in liquid nitrogen using a sterile mortar and pestle. The fine powdered leaves were added to the pre-heated buffer using a sterile spatula. Vigorous vortex (Chilterin) was applied for 2 min before the mixture was incubated at 65°C for 30 min. The vortex and incubating steps were repeated 4-6 times. An equal amount of chloroform: isoamyl alcohol (24:1) was added to the mixture and vortexed vigorously for 2 min until a homogenized mixture was obtained. The homogenized mixture was then centrifuged (Kubota 6500, Tokyo) at 10,000 g for 20 min at 4°C. After centrifugation, two phases were visible. The aqueous supernatant was transferred into another centrifuge tube and an equal volume of chloroform: iso-amyl alcohol (24:1) was added and vortexed for 2 min. The mixture was again centrifuged at 13,000 g for 20 min at 4°C. This separation step was repeated until there was no flocculent-like layer between the aqueous and organic phase. After the final centrifugation, the yellowish aqueous phase was transferred into a sterile centrifuge tube and 3M NaOAc was added in the ratio of 3:10. The tube was inverted a few times to ensure the solutions were well mixed. The tube was then stored at 4°C overnight or for 12-15 h; then centrifuged at 10,000 g for 30 min at 4°C. The supernatant was discarded and the white pellet was left to air dry for 1 min. The white pellet was washed with 250 µL 70% absolute ethanol and rapidly centrifuged at 13,000 g for 20 min at 4°C. The washing step was repeated two times before the pellet was left to air dry for 15 min. The dry and ethanol-free pellet was dissolved in 50 µL DEPC-treated RNase-free water. The same procedure was repeated for greenhouse-grown *A. annua* L. leaf materials. The leaves were harvested and immediately wiped thoroughly on both surfaces with 70% ethanol and used for extraction.

Measurement of total RNA quality

The purity and quantity of total RNA were quantified with a UV spectrophotometer (Hitachi U1900) at 230 m, 260 nm and 280 nm wavelengths, and 2 µL of total RNA was electrophoresed on 1% heat-denaturing agarose gel.[20]

mRNA isolation and first strand cDNA synthesis

Poly(A)$^+$mRNA was isolated from total RNA from each clone by using the MagneSphere® Magentic Separation Products (Promega). The isolated poly(A)$^+$mRNA was reverse transcribed into cDNA using oligo$_{18}$(dT) primer and M-MuLV RT enzyme (Promega).[20]

Detection of DNA contamination by polymerase chain reaction amplification

Polymerase chain reaction amplification was performed using the universal primers ITS1F and ITS4R.[21] The amplification of cDNA preparation contained 20 ng of cDNA, 1 X buffer, 2.5 mmol/L $MgCl_2$ solution, 10 mmol/L dNTPs in equimolar ratio, 10 umol/L primers, and 2.5 units of Flexi GoTaq (Promega). The amplifications were carried out using BioRad Thermal Cycler.[21] The amplified products were electrophoresed using 1% agarose gel and run at 100V for 50 min. The gel was then stained with 1% ethidium bromide and destained in sterile distilled water. The gel was viewed and documented using Gel Doc XR System (Bio-Rad).

Results

Extraction of total RNA using the commercial kit gave a low yield (13.34-16.42 µg g^1 FW) and the preparation was contaminated with polyphenols and polysaccharides, as indicated by the $A_{260/230}$ reading which ranged between 0.76 and 0.81. It also showed it was contaminated with protein, as indicated by the $A_{260/280}$ which was not within the normal range of 1.8-2.0. Using the standard protocol for plants with high secondary metabolites,[18] the yield of total RNA acquired for 0.5 g of fresh leaves was very much lower than that obtained using the commercial kit protocol (1.3-9.7 µg g^{-1} FW) for the three clones of *A. annua* L. Polyphenol contaminations were still found in the RNA preparations using this standard protocol of CTAB and glacial acetic acid, although RNA purity reading $A_{260/280}$ indicated the preparations were free of other protein contamination (Table 1).

However, high quality and good yield of total RNA could be successfully obtained from tissue-cultured *A. annua* L. using our modified CTAB protocol. The extracted total RNA ranged from 33 to 54 µg g^{-1} FW for the three clones. This indicates that as little as 0.5 g fresh leaf material is sufficient to yield enough total RNA for downstream applications such as reverse transcription to first strand and PCR. As for the three clones using the proposed modified CTAB method, the $A_{260/230}$ were between 2.11 and 2.56, higher than 2.0, indicating absence of polysaccharide or polyphenol contamination. For $A_{260/280}$ readings, the values were between 1.89 and 1.96, indicating the total RNA preparation using the method described above was free of protein contamination (Table 1). Electrophoresis of total RNA on heat denaturing gel showed two sharp bands, evidence of the integrity of the total RNA extracted (Figure 1).

The standard protocol for total RNA extraction and isolation applied to the greenhouse clones of *A. annua* L. gave reasonably high yields but the RNA was still contaminated with polyphenol, polysaccharides and proteins. Our modified CTAB protocol again proved that good yields of total RNA (37.92-43.38 µg g^{-1} FW) without any contaminants could be obtained

Table 1. Yield of RNA and absorbance ratios from three different clones for three different methods of total RNA extraction.

	Extraction method								
	IQeasyTM Plus Plant RNA Extraction Mini Kit			CTAB and glacial acetic acid			Modified CTAB		
In vitro clone	Absorbance ratios		RNA yield	Absorbance ratios		RNA yield	Absorbance ratios		RNA yield
	OD 260/230	OD 260/280	(µg g^{-1} FW)	OD 260/230	OD 260/280	(µg g^{-1} FW)	OD 260/230	OD 260/280	(µg g^{-1} FW)
TC1	0.76	1.19	13.34	1.53	1.84	9.7	2.28	2.03	37.92
TC2	0.81	1.15	16.42	1.01	1.86	1.3	2.13	1.85	43.38
Highland	0.77	1.21	14.84	1.28	1.92	3.6	2.50	1.90	39.72

CTAB, cetyltrimethyl-ammonium bromide; OD, optical density.

from the greenhouse-grown *A. annua* L. leaves (Table 2). Heat-denatured gel electrophoresis of total RNA extracted from greenhouse-grown *A. annua* L. clones using the modified protocol showed two distinct bands, indicating high integrity of RNA (Figure 2).

Discussion

A. annua L. is reported to be rich in secondary metabolites which can hamper total RNA preparation and inhibit further downstream applications if not eliminated during total RNA extraction. Available commercial kits like IQeasy™ Plus Plant RNA Extraction Mini Kit (iNtRON Technologies) or other protocols based on guanidium isothiocyanate,[12,15] or cesium chloride,[13] are very often used for total RNA preparations from plant material. However, the stated methods have been shown to produce low quality of total RNA contaminated with polyphenols and inhibitors that hamper the amplification process during PCR.[18] The yellow pellets produced in the present investigation using the commercial kit indicated presence of impurities in the total RNA preparation. Similarly, the conventional method proposed for medicinal plants,[18] which promised elimination of secondary metabolites and polyphenols in RNA preparation using insoluble polyvinyl polypyrrolidone (PVPP), gave low yields and poor quality of RNA from *A. annua* L. The yellow pellets produced after washing at the end of the protocol indicated the presence of polyphenols. Hence this showed that the use of PVPP did not successfully eliminate polyphenols from the total RNA preparations of *A. annua* L. When not fully separated from the total RNA, polyphenols will inhibit the sensitive downstream reactions like PCR. The total RNA of *A. annua* L. isolated using our proposed modified protocol was free of contamination and they could be used effectively in the PCR. In our modified protocol, a high amount of PVP and β-mercaptoethanol added to the lysis buffer successfully eliminated polyphenols and polysaccharide that were present in *A. annua* L. leaves.[22-24] Using our proposed modified protocol, an almost colorless pellet was produced after overnight precipitation with sodium acetate which was found to ensure better elimination of co-precipitated polysaccharides, which are normally used in isolation of total RNA, compared to lithium chloride. The clear pellets produced with our protocol further indicated that the preparations were free of polysaccharides. Chloride ions in lithium chloride have been found to suppress RNA-dependent DNA polymerases in downstream reactions.[25]

Most of the *A. annua* L. plants that were grown in the greenhouse were found to be infested with fungi. In order to confirm the presence of fungal RNA in the total RNA preparation, PCR was run with fungal-specific internal transcribed spacer (ITS) primers, ITS1F and ITS4R to ensure the purity of the isolated RNA.[21] The results obtained from PCR of first strand cDNA derived from the total RNA of the greenhouse-grown *A. annua* L. clones using the standard protocol indicated the presence of fungal RNA. The PCR profile was run on the total RNA isolated using our modified protocol and did not show any cDNA amplification, which indicates absence of fungal RNA contamination (Figure 3). The absence of fungal RNA in the total RNA preparation using modified protocol might be due to the repeated heating of the leaf samples at 65°C. Fungal RNA has been reported to be sensitive to high temperature and the continuous exposure of leaf samples to high temperature in the modified protocol might be the cause of fungal RNA denaturation. It was reported that fungal RNA were made up of mostly 23S and 16S, and when exposed to high temperature, nicking occurred and they were denatured to smaller subunits.[26-28] These small subunits (which were mainly proteins) were then eliminated from the total RNA preparations during the chloroform/isoamyl alcohol separation stage. Hence, the total RNA isolated using the modified protocol did not show cDNA amplification in the PCR. A high concentration with reduced volume of extraction buffer was used to extract total RNA in order to reduce the amount of con-

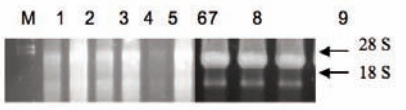

Figure 1. Heat-denatured gel electrophoresis of total RNA extracted from *in vitro A. annua* L. clones using three different methods. M, RiboRuler™ RNA Ladders (Fermentas); 1-3, TC1, TC2 and Highland RNA using IQeasy™ Plus Plant RNA Extraction Mini Kit; 4-6, TC1, TC2 and Highland RNA using cetyltrimethyl-ammonium bromide (CTAB) plus glacial acetic acid; 7-9, TC1, TC2 and Highland RNA using modified CTAB.

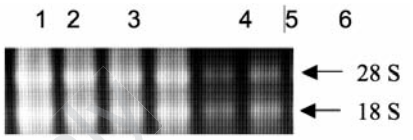

Figure 2. Heat-denatured gel electrophoresis of total RNA extracted from greenhouse-grown *A. annua* L. clones using two different methods. 1-3, TC1, TC2 and Highland RNA using modified cetyltrimethyl-ammonium bromide (CTAB); 4-6, TC1, TC2 and Highland RNA using CTAB and glacial acetic acid.

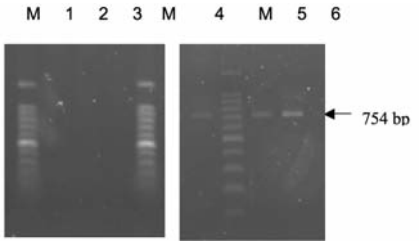

Figure 3. Polymerase chain reaction profiles of first strand cDNA amplified from greenhouse-grown *A. annua* L. RNA isolated through modified cetyltrimethyl-ammonium bromide (CTAB) protocol (1-3) and CTAB plus glacial acetic acid method (4-6) using universal primers ITS1F and ITS4R. 1 and 4, TC1; 2 and 5, TC2; 3 and 6, Highland; M, 100bp DNA Marker (Fermentas).

Table 2. Yield of RNA and absorbance ratios from three different greenhouse-grown clones for two different methods of total RNA extraction.

	Extraction method					
	CTAB and glacial acetic acid			Modified CTAB		
Greenhouse clone	Absorbance ratios		RNA yield (µg g^{-1} FW)	Absorbance ratios		RNA yield (µg g^{-1} FW)
	OD 260/230	OD 260/280		OD 260/230	OD 260/280	
TC1	0.45	3.74	10.92	2.15	1.86	54.00
TC2	1.47	2.88	40.98	2.21	1.96	40.81
Highland	1.36	1.40	67.32	2.08	1.81	33.00

CTAB, cetyltrimethyl-ammonium bromide; OD, optical density.

taminating polysaccharides.[13,24,25] Use of DPEC-treated double distilled water in the modified protocol eliminated ribonucleases which would degrade RNA during total RNA preparation. Spraying RNAse Quiet (Fermentas) before and during the total RNA extraction also promotes the elimination of ribonucleases. Furthermore, fewer centrifugation steps were used in the modified protocol compared to CTAB plus glacial acetic acid, and this reduced the amount of total RNA lost during total RNA extractions. Washing with absolute ethanol was repeated twice to ensure complete removal of CTAB from total RNA. The simple CTAB protocol that used non-hazardous chemicals further simplifies the modified protocol and is helpful in obtaining high quantity and good quality total RNA from *in vitro* and greenhouse-grown leaves of *A. annua* L.

The high yield and quality of the extracted total RNA proved that the modified CTAB protocol could be effectively used for extraction from delicate leaf samples such as tissue-cultured *A. annua* L.

Conclusions

Our modified CTAB protocol ensured isolation of high quantity and good quality total RNA from *A. annua* L., a medicinal plant containing large amounts of secondary metabolites.

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