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An effective method for extracting total RNA from *Dioscorea opposita* Thunb.

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ABSTRACT. *Dioscorea opposita* Thunb., included in the genus *Dioscorea* of the family Dioscoreaceae, is an important herb with great edible and medicinal value. In this study, the total RNA from leaves of Lichuan *Dioscorea opposita* Thunb. was isolated by an improved Trizol method. The results showed that the RNA extracted by the improved Trizol method had good integrity, and the RNA could be used for downstream molecular biology operations including reverse transcription-polymerase chain reaction.

Key words: *Dioscorea opposita* Thunb.; Total RNA extraction; Improved Trizol method

INTRODUCTION

Dioscorea opposita Thunb. (popular name shanyao in China), included in the genus *Dioscorea* of the family Dioscoreaceae, has been used as an important herb in traditional Chinese medicine for many years. Presently, many local cultivars are grown in China (Li et al., 2009), and according to planting region they were classified into Huai *D. opposita* Thunb., Lichuan *D. opposita* Thunb., Lixian *D. opposita* Thunb., Chenji *D. opposita* Thunb., and Xichen *D. opposita* Thunb. (Sun et al., 2010). Among these local cultivars, Lichuan *D. opposita* Thunb. is a particular local variety that is mainly distributed in Lichuan City, Hubei Province, China. Although it has been transplanted in Sichuan Province and other areas, the taste and many characteristics are worse than those of the local variety, which may be attributed to the unique terrain and good ecological environment in Lichuan City (Zhang, 1996).

As reported in the literature, there are many chemical components that are contained in *D. opposita* Thunb. including mannan, allantoin, dopamine, batatasine, phytic acid, abscisin II, amino acids, glucoprotein, choline, cholesterol, ergosterol, campesterol, saponins, starch, non-starch polysaccharides, and others (Wang et al., 2006, 2008; Yuan, 2008). Therefore, as an important medical resource, *D. opposita* Thunb. has great development and scientific research values. In molecular biology research, high-quality RNA extraction is a critical determinant of many molecular experiments, such as polymerase chain reaction (PCR), cDNA library construction, rapid amplification of cDNA ends (RACE), and Northern blot hybridization. However, RNA extraction methods of different plants are different because of differences of the plant itself, for example, structural and physiological differences (Chang et al., 1993; Li et al., 2008; Qin et al., 2009; Japelaghi et al., 2011; Yin et al., 2011). For this, an appropriate RNA extraction method is very necessary for a particular species.

In this study, a simple and effective method to isolate high-quality RNA from the leaves of *D. opposita* Thunb. was developed, and the extracted RNA could be used for downstream molecular biology experiments.

MATERIAL AND METHODS

Plant materials

The leaves of Lichuan *D. opposita* Thunb., which were collected from the experimental field of China West Normal University, China, were selected to extract total RNA.

Preparation of some important solutions and reagents

Trizol was obtained from Sangon Biotech (Shanghai, China). The PrimeScript® One Step RT-PCR Kit Ver. 2 (TaKaRa, Dalian, China) was used for reverse transcription-PCR (RT-PCR). Extraction buffer I consisted of 100 mM Tris-Cl, pH 8.0, 50 mM ethylenediaminetetraacetic acid (EDTA), and 500 mM NaCl, pH 8.0. Additional solutions include 2.5 M potassium acetate (KAc), polyvinylpyrrolidone 40 (PVP40), isopropanol, 70% ethanol, chloroform:isoamyl alcohol (24:1), and diethyl pyrocarbonate (DEPC)-treated water.

All of the solutions, reagents, and consumables that were used for RNA extraction needed to be treated with 0.1% DEPC.

Total RNA extraction

The total RNA of *D. opposita* Thunb. was extracted by the conventional Trizol and improved Trizol methods. The conventional Trizol extraction was performed according to the manufacturer instructions (Sangon Biotech, Shanghai Co., Ltd., China). The improved Trizol procedure was as follows.

1) Approximately 0.2 g leaves and small amounts of PVP40 powder (0.1 g per 1.0 g leaves) were ground into fine powder in a liquid nitrogen-chilled mortar.

2) The powder was then immediately transferred to a 2-mL centrifuge tube. Subsequently, 1 mL cold extraction buffer I was added into the tube. The contents were homogenized quickly by vortexing and allowed to stand at 4°C for about 10 min. Subsequently, the tube was centrifuged at 4°C and 8000 rpm for 10 min.

3) The supernatant was discarded, and the precipitate was suspended in 1 mL Trizol and supplemented with 200 µL β-mercaptoethanol (β-ME) and 200 µL 2.5 M KAc. After being mixed by vortexing, it was incubated at 4°C for 10 min and then centrifuged at 4°C and 10,000 rpm for 5 min.

4) The aqueous phase was transferred to a new 2.0-mL tube, and 300 µL 2.5 M KAc buffer and an equal volume of chloroform:isoamyl alcohol (24:1) were added. Then, the mixture was vortexed, incubated for 10 min at 4°C, and centrifuged at 10,000 rpm for 10 min.

5) The supernatant was carefully transferred to another new tube, and the chloroform:isoamyl alcohol (24:1) treatment was repeated as above.

6) The upper aqueous phase was taken out, and two-thirds volume of ice-cold isopropanol was added to the tube. The tube was maintained at -20°C for more than 30 min, and centrifuged at 12,000 rpm for 10 min at 4°C.

7) The supernatant was discarded, and the pellet was washed once or twice with 75% ethanol and then dried under the natural environment.

8) Finally, the obtained RNA precipitate was dissolved in 30-50 µL sterilized DEPC-treated water.

9) RNA samples were stored at -70°C or the pellet with isopropanol was stored at -20°C.

Quality and yield of the obtained total RNA

The purity and yield of the total RNA were determined by monitoring the A_{260}/A_{280} absorbance ratio using the NanoDrop 2000. The sample integrity was tested by 1.0% agarose gel electrophoresis and stained with ethidium bromide (EB).

RT-PCR analysis

The specific PCR primers were designed by Primer Premier 5.0 according to the reported mRNA sequence of the *Actin* gene, a housekeeping gene. The specific primers of the cDNA sequence are as follows: Actin-F: 5'-GTGACAATGGAACAGGAATG-3'; Actin-R: 5'-CACTTCCGGTGGACAATG-3'.

Total RNA was synthesized into cDNA using the PrimeScript® One Step RT-PCR Kit Ver. 2 according to manufacturer instructions. The 25-µL reaction system included 1 µL 1-step Enzyme Mix (containing PrimeScript RTase, Ex Taq HS, and RNase inhibior), 12.5 µL 2X

1-step buffer (including reaction buffer, dNTP mixture, and enhance solution), 0.5 µL forward primer, 0.5 µL reverse primer, 2 µL template RNA, and 8.5 µL RNase-free H_2O. The RT reaction was carried out at 50°C for 30 min. The amplification reaction was as follows: 94°C for 2 min; 30 cycles of 94°C for 30 s, 46°C for 30 s, and 72°C for 1 min; and a final elongation step at 72°C for 7 min. The PCR products were electrophoresed on a 1.0% agarose gel containing 0.5 µg/mL EB in Tris/borate/EDTA (TBE) buffer and photographed on ultraviolet light.

RESULTS

Purity and integrity of total RNA

To determine the integrity of RNA, 5 µL of each sample was electrophoresed on a 1.0% agarose gel in TBE buffer. The RNA samples that were prepared by the improved Trizol method demonstrated intact, sharp 28S and 18S ribosomal RNA (rRNA) bands and a lack of RNA degradation on agarose gels, indicating a high quality of total RNA (Figure 1). In contrast, no legible band was observed by the conventional Trizol method (the first lane in Figure 1).

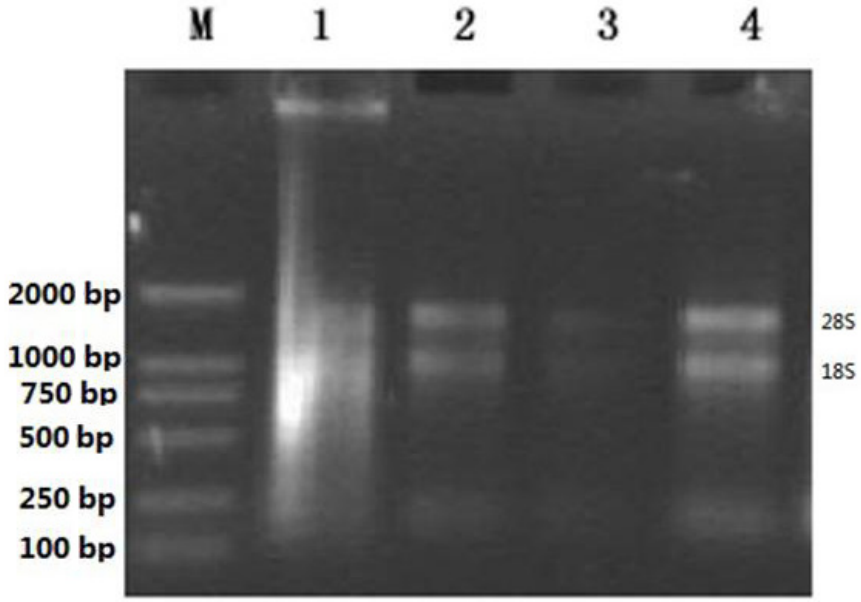

Figure 1. Agarose gel electrophoresis of total RNA products from *Dioscorea opposita* Thunb. *Lane M* = DNA marker; *lane 1* = Trizol method; *lanes 2-4* = improved Trizol method.

Additionally, the RNA quality was measured by means of spectrophotometric ratios that reveal differences in absorption spectra maxima of pure nucleic acids (Amax = 260 nm), proteins (Amax = 280 nm), and polysaccharides (Amax = 230 nm). The A_{260}/A_{280} ratio for all RNA samples that were prepared by the improved Trizol method ranged from 1.9 to 2.2 (Table 1), indicating low protein contamination. The A_{260}/A_{230} ratio was higher than 1.6, demonstrating that the RNA samples were of high purity and without polyphenol and polysaccharide con-

tamination. In addition, we also found that the RNA quality could be further improved when the RNA samples were precipitated with isopropanol again after the samples were washed with 75% ethanol and dissolved.

Table 1. Purity and yield of the total RNA extracted from the leaves of *Dioscorea opposita* Thunb.

Extraction methods	A_{260}	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/μL)
Conventional Trizol method	4.893	1.43	1.0	195.7
Improved Trizol method	9.685	1.93	1.94	387.4
	4.746	2.16	1.63	189.8
	9.275	2.13	1.74	371.0

RT-PCR analysis

To further evaluate the integrity of the RNA that was extracted by the improved Trizol method, the RNA was used to amplify the housekeeping gene *Actin* by RT-PCR. The results showed that an expected fragment of approximately 1.1 kb was amplified (Figure 2), suggesting that the RNA products that were obtained by the improved method could be used for many additional studies at the molecular level.

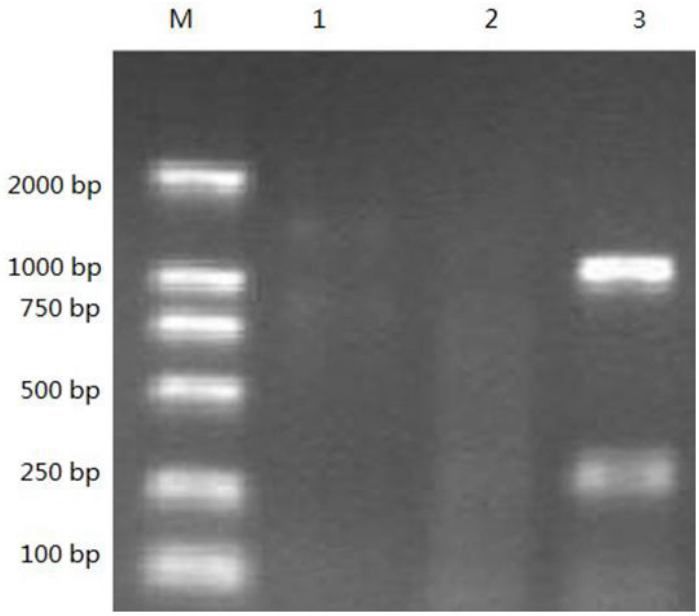

Figure 2. Agarose gel electrophoresis of RT-PCR products of the *Actin* gene cDNA from *Dioscorea opposita* Thunb. *Lane M* = DNA marker; *lane 1* = 2 μL RNA template; *lane 2* = negative control; *lane 3* = RT-PCR product.

DISCUSSION

The isolation of high-quality RNA from plant tissues that are rich in polysaccharides and polyphenols is quite challenging. Although many procedures have been published for

the extraction of RNA from plant tissues so far, the vast majority of them are not completely satisfying because some plant tissues, such as fruits, storage tubers, and seeds, contain a large amount of storage proteins, polysaccharides, polyphenols, or other secondary metabolites (Li and Wang, 1999; Liu et al., 2008; Ma and Yang, 2011). The presence of these substances can affect the quality and quantity of the isolated RNA (Salzman et al., 1999). Polyphenolic compounds (particularly tannins) are readily oxidized to form covalently linked quinones and can irreversibly bind proteins and RNA to form high molecular weight complexes (Salzman et al., 1999; Li et al., 2008). In contrast, polysaccharides tend to coprecipitate with the RNA and constitute the major obstacle of RNA isolation in low ionic strength buffers (Yuan et al., 2005). In addition, these contaminating substances severely interfere with RNA-dependent RT (Koonjul et al., 1999). Therefore, these compounds must be eliminated during RNA isolation.

It was demonstrated that *D. opposita* Thunb. contains abundant mucus, a mannosan-protein complex, and certain secondary metabolites (Zhang, 1996; Yuan, 2008). Therefore, in this study, PVP40 was added additionally at the stage of pestling leaves, which can effectively prevent polyphenols from being oxidized into reddish-brown substances (Liu et al., 2008; Japelaghi et al., 2011). Additionally, extraction buffer I was first used to partly eliminate polysaccharide contamination. The following step included adding high concentration KAc (2.5 M) to remove effectively polysaccharides (Liu et al., 2008; Japelaghi et al., 2011).

In conclusion, the method described here is an efficient and reliable method to isolate total RNA from *D. opposita* Thunb. Using this improved Trizol extraction protocol, a large amount of high-quality RNA, which is required for cDNA library construction, RT-PCR, RACE, and Northern blot analysis, could be obtained from *D. opposita* Thunb.

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