Journal of Medicinal Plants Research Vol. 5(13), pp. 2834-2838, 4 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Comparison of three different methods for total RNA extraction from *Fritillaria unibracteata*: A rare Chinese medicinal plant

Pei Hou[1], Ziyin Xie[2], Le Zhang[2], Zhen Song[3], Jiao Mi[3], Yang He[3]* and Yufeng Li[2]

[1]College of Life Sciences, Sichuan University, Chengdu 610064, China.
[2]College of Bioengineering, Xihua University, Chengdu 610039, China.
[3]College of Medical Technology, Chengdu University of Traditional Chinese Medicine, Chengdu 610075, China.

Accepted 3 May, 2011

Efficient RNA isolation is a prerequisite to elucidate the molecular mechanisms of peimine metabolism in the medicinal plant *Fritillaria unibracteata*, a rare and famous region drug in Sichuan, China. Three different methods were tested: modified SDS acid phenol, modified CTAB and SDS-LiCl. Compared with the modified CTAB and SDS-LiCl methods, the method of modified SDS acid phenol would obtain initial, abundant and high-quality intact RNA from *F. unibracteata*. Meanwhile, the RNA-containing bands are clear and light, and the lightness of 28S rRNA is two times than 18S rRNA. The ratio of A_{260}/A_{280} is approximately 1.85, and the yield of total RNA is 73 µg per g starting tissue. The specific band of *3-hydroxy-3-methylglutaryl-CoA reductase* (*HMGR*) gene by the reverse transcription-polymerase chain reaction (RT-PCR) showed that RNA extraction using the modified SDS acid phenol method can provide fundamental materials for further study of genetic modification in this precious Chinese medicinal plant *F. unibracteata*.

Key words: *Fritillaria unibracteata*, RNA extraction, modified SDS acid phenol.

INTRODUCTION

Fritillaria unibracteata (Chen and Hsia, 1977) (Anzibeimu in Chinese) is a well-known and precious Traditional Chinese Medicine. Its original herb belongs to the family of Liliaceae. In Chinese herbal practices, in which the bulb can be generally eaten, moreover, it is highly effective for the treatment of relieving cough, removing phlegm, reducing fever and moistening lungs. It is especially effective in the treatment of the elderly and children, particularly in cases of difficult recovery after a lengthy treatment (Gao et al., 1999). With the development of genomics and functional genomics, it has been widely studied for its roles in world medicine and identifying key genes from this valuable officinal plant. As a rare Chinese medicinal plant, *F. unibracteata* has been incipiently identified by the 5S-rRNA sequence (Li et al., 2003), which is a potential combination of spacer regions for barcoding medicinal plants. However, the vegetative organs of *F. unibracteata* species including bulbs contain considerable amounts of phenolic compounds, secondary metabolites and polysaccharides, in addition, RNA molecules are subject to enzymatic degradation by RNases (Schneiderbauer et al., 1991; Graham, 1993), which make the isolation of RNA difficult and reduce the quality and quantity of RNA. Thus lots of RNA isolation methods either result in very low yields of RNA or form complexes with these contaminants resulting in low quality poly$(A)^+$ RNA unsuitable for first strand cDNA synthesis and RT-PCR (Koonjul et al., 1999). Such undesirable outcomes prompted many researchers to develop new improved protocols for RNA extraction from several recalcitrant plant tissues (Gasic et al., 2004; Iandolino et al., 2004).

The availability of high-quality RNA is the primary

*Corresponding authors. E-mail: hawkheyang@yahoo.cn. Fax: +86-28-87725225 or +86-28-86125763.

requirement for molecular cloning and gene expression studies (Liu et al., 2005; Wang et al., 2008). There are a large number of publications reported various successful RNA isolation protocols for different species, and for the same species grown under different environmental conditions (Salzman et al., 1999; Li et al., 2006; Chun et al., 2008; Thanh et al., 2009; Takahashi et al., 2010), among which the method of total RNA isolation by a single-step extraction with an acid guanidine thiocyanate-phenol-chloroform mixture is the most classical (Chomczynski et al., 1987). And other RNA extraction protocols from plants which rich in phenolics and polysaccharides mainly utilized the steps of sodium dodecyl sulfate (SDS), solublepolyvinylpyrrolidone (PVP) and ethanol precipitation (Dong and Dunstan, 1996). One representative protocol used acetone treatment of freeze-dried and powdered plant materials (Schneiderbauer et al., 1991), and more groups modified the cetyltrimethylammonium bromide (CTAB) method (Apt et al., 1995; Gareth et al., 2006; Wang et al., 2008), but the acidguanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) has been a classical reference for all scientific institutions. Moreover, several commercial reagents and kits are available for isolating RNA from plants (e.g., Trizol, Gibco-BRL Life Technologies; RNeasy plant kit, QIAGEN), but they are not always effective for all plant tissues, particularly for medicinal plant tissues. Therefore, we decided to test different methods to eliminate complex contaminants in RNA preparations and obtain the high quality RNA from *F. unibracteata*.

In this study, we explored three different methods of RNA extraction from *F. unibracteata*. Compared with the methods of modified CTAB and SDS-LiCl, the modified SDS acid phenol method can obtain initial, abundant and high-quality intact RNA from *F. unibracteata*. RT-PCR analysis revealed RNA producing by the method of modified SDS acid phenol is stable and effective. This study provided fundamental information for the future study of genetic engineering in this precious Chinese medicinal plant *F. unibracteata*.

MATERIALS AND METHODS

Plant materials and equipments preparation

Bulbs of *F. unibracteata* (Chen and Hsia, 1977) were collected from the Songpan District of Sichuan, China, and snap-frozen in liquid nitrogen. The frozen bulbs were transported in liquid nitrogen, and stored at -80ºC upon reaching the laboratory. Mortars, pestles, and glassware were baked at 180ºC for 2 h, and non-disposable plastic ware was autoclaved before use.

Protocol

Method 1: modified CTAB method

1. Grind 500 mg of bulbs with liquid nitrogen using an RNase-free mortar and pestle. Transfer to clean 65ºC tube with 1.5 ml CTAB extraction (2% CTAB (w/v), 2% PVP (w/v), 25 mmol·L^{-1} EDTA, 100 mmol·L^{-1} Tris-C1, 2.0 mmol·L^{-1} NaCl, 0.125 mol·L^{-1} boric acid (pH=8.0), and add β-mercaptoethanol to 2% final concentration).
2. Vortexing for 30 s, heating 5 min in a 65ºC water bath. Add 1.5 ml chloroform/ isopr- opanol (24:1, v/v) to extract two times.
3. Add 8 mol·L^{-1} LiCl to supernatants and make the final concentration to 2 mol·L^{-1}. Precipitate at 4ºC over night, then centrifuge samples at 10,000 g at 4ºC for 1 h.
4. Dissolve pellet with 500 µl solution (1.0 mol/L NaC1, 0.5% SDS (w/v), 10 mmol·L^{-1} Tris-HCl, 1 mmol·L^{-1} EDTA, pH=8.0).
5. After extraction with chloroform, supernatants were transferred to a fresh tube, add 2 times volume ethanol. Incubate 30 min at -70ºC, centrifuge samples at 10,000 g at 4ºC for 20 min.
6. Eliminate supernatants and add 1 ml 70% ethanol (v/v). After centrifuge, dissolve pellet with 50 µl DEPC-treated H_2O and store at -70℃.

Method 2: modified SDS acid phenol method

1. Grind 500 mg of bulbs with liquid nitrogen using an RNase-free mortar and pestle. Transfer to 1.5 ml clean tube and add 600 ml RNA extraction (100 mmol·L^{-1} Tris-HCl, 400 mmol·L^{-1} NaCl, 1.0% SDS (w/v), pH=7.5, and add β-mercaptoethanol to 2% final concentration). After homogenizing, incubate 3 min.
2. Add 200 µl acid phenol (pH=4.0) and 200 µl chloroform, then centrifuge samples at 10,000 g at 4℃ for 10 min.
3. Transfer supernatants to fresh tubes and add 200 µl 5 mol·L^{-1} NaCl, 200 µl acid phenol (pH=4.0) and 200 µl chloroform, centrifuge samples at 10,000g at 4℃ for 10 min.
4. Transfer supernatants to fresh tubes and add the same volume isopropyl alcohol, incubate at room temperature for 10 min. Centrifuge samples at 10,000 g at 4℃ for 10 min.
5. Wash the pellet with 1 ml 70% ethanol (v/v), centrifuge samples at 10,000 g at 4℃ for 10 min.
6. Dissolve pellet with 50 µl DEPC-treated H_2O and store at -70℃.

Method 3: SDS-LiCl method

1. Grind 500 mg of bulbs with liquid nitrogen using an RNase-free mortar and pestle. Transfer to 1.5 ml clean tube and add pre-cold 500 µl RNA extraction (100 mM Tris-HCl, pH=9.0, and add β-mercaptoethanol to 2% final concentration). After homogenizing, incubate 15 min.
2. Add 25 µl 10% SDS (w/v), centrifuge samples at 10,000 g at 4℃ for 15 min. Then, add 1/3 volume 8 mol·L^{-1} LiCl, incubate on ice for 2 h after homogenizing. Centrifuge samples at 10,000 g at 4℃ for 20 min.
3. Wash the pellet with 1.5 ml of 70% ethanol (v/v), centrifuge samples at 10,000 g at 4℃ for 1 min. Dissolve pellet with 500 µl of DEPC-treated H_2O, add the same volume saturation phenol to extract.
5. Extract supernatants using the same volume phenol/chloroform/ isopropanol (25:24:1, v/v/v), then extract with the same volume chloroform/isopropanol (24:1, v/v).
6. Add 1/10 volume 3 mol·L^{-1} NaAC (pH=6.0) and 1.5 times ethanol, incubate 15 min. Centrifuge samples at 10,000 g at 4℃ for 20 min.
7. Dissolve pellet with 50 µl of DEPC-treated H_2O and store at -70℃.

Total RNA analysis

The quality and quantity of total RNA from three different methods were determined by running 1.2% of formaldehyde agarose gel electrophoresis and visualizing under UV light. 2 µl RNA was diluted to 100 µl, and the RNA concentration and purity were determined by analyzingA_{260}/A_{280} and A_{260}/A_{230} absorbance ratios.

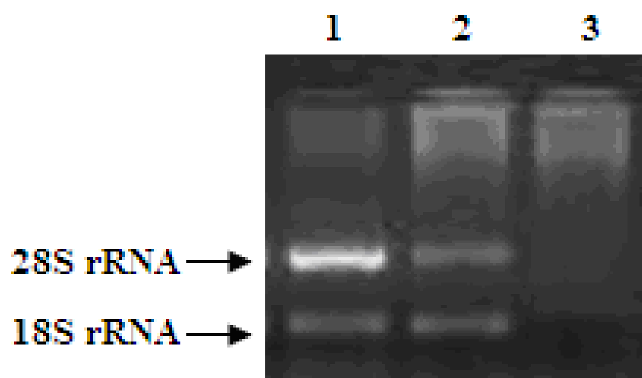

Figure 1. Electrophoretic analysis of *F. unibracteata* RNAs isolated through the three different extraction methods. Lane 1-3, RNAs using modified SDS acid phenol method (Lane 1), SDS-LiCl method (Lane 2) and modified CTAB method (Lane 3).

cDNA synthesis and RT-PCR

For reverse transcription (RT)-PCR, first-strand cDNA was synthesized using the PrimeScript^{TM} First-Strand cDNA Synthesis kit (TaKaRa Biotechnology (Dalian) Co., Ltd.) according to the manufacturer's instructions. The following degenerate oligonucleotide primers were used with 1 g of isolated RNA samples to amplify *3-hydroxy-3-methylglutaryl-CoA reductase* (*HMGR*) gene. Forward primer: 5'-THYTNGGNCARTGYTGYGARATGCC-3' and reverse primer: 5'-CDATC CARTTNACNGCNGGYTT-3'. The PCR amplification was carried out in a GeneAmp® PCR System 9700 (PE Applied Biosystems Inc.) with a 25 µl reaction mixture containing 1 µl 10 µmol each primer, 1 µg template DNA and 2 x Master Mix (0.1U Taq Plus Polymerase, 500 µmol dNTP each, 20 mmol Tris-HCl, 100 mmol KCl, 3 mmol $MgCl_2$) 12.5 µL. PCR amplification was carried out under the following conditions: initial denaturation at 94°C (3 min); followed by 30 cycles of 94°C denaturation (30 s), 55°C annealing (30 s), and 72°C extension (1 min); a final extension at 72°C for 10 min. Successful PCR amplification produced a single DNA band of approximately 300 bp in length on a 1% agarose gel and was visualized by ethidium bromide staining under UV light.

RESULTS AND DISCUSSION

RNA isolation methodology

Several standard methods for RNA isolation including AGTPC-LiCl method (Chomczynsci and Sacchi, 1987), and Qiagen RNeasy were applied to RNA isolation in *F. unibracteata*, but failed to yield available RNA in this medicinal plant. The main reasons were the presence of phenolic compounds, polysaccharides and complex secondary products (Schultz et al., 1994). Upon this status, we tested three different methods (modified SDS acid phenol, modified CTAB and SDS-LiCl) to evaluate which method is the best and effective RNA extraction methods. The results (Figure 1) revealed that the method of modified CTAB can not produce clear and light RNA band. Using the methods of modified SDS acid phenol and SDS-LiCl, clear and light RNA bands were shown, and DNA contamination was not existed. Furthermore, the clear and light RNA band producing by modified SDS acid phenol method is better than that of SDS-LiCl method.

Low pH extraction may inhibit phenolic compounds to ionize and oxidize (Guillemaut and Marechal-Drouard, 1992; Ainsworth, 1994). This reason may result in abundant and high-quality intact RNA using modified SDS acid phenol method. The solution of NaAC was proved usefully to precipitate polysaccharides (Bahloul and Burkard, 1993), in the method of SDS-LiCl, we used 3 mol/L NaAC to produce high quality and abundant RNA.

We explored many crucial steps in the extraction process which possibly influenced the efficiency of RNA extraction. For instance, in order to fully inhibit the activity of RNase, all eppendorf tubes and tips used were RNase-free. After extraction, aliquots of extracted RNA were stored at -80°C to avoid RNA degradation. Overall, modified SDS acid phenol method and SDS-LiCl method can produce initial, high quality and abundant total RNA. Compared with the modified CTAB method (no available RNA band was shown). So, further experiments would be carried out to judge the stable and effective of RNA.

Assessment of the quality and quantity of total RNA

The success of an RNA isolation protocol may be judged by the quantity, purity, and integrity of RNA recovered (Wang et al., 2011). Estimation of RNA purity using absorbance ratio at 260 and 280 nm (A260/A280), A260/A280 ratio of 1.8～2.0 corresponding to a better quality of RNA (Sambrook et al., 1989). The RNA isolated by the modified SDS acid phenol method also exhibited good purity ($A_{260}/_{A280}$ ratio was 1.85), and the $A_{260}/_{A280}$ ratio of SDS-LiCl method was 1.53, lower than that of modified SDS acid phenol method. In both two method,

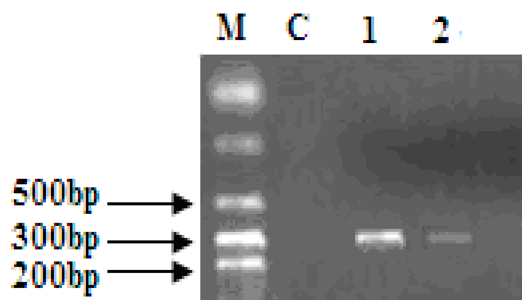

Figure 2. Agarose gel electrophoresis analysis of 300-bp fragments of *FuHGMR* by RT-PCR. Lane M, DNA Marker; Lane C, negative control; Lane 1-2, RT-PCR amplicons from total RNA using the modified SDS acid phenol method (Lane 1) and the SDS-LiCl method (Lane 2), respectively.

SDS to make cells soluble and inhibit RNase activity (Farrell, 1993), but the acid phenol maybe decrease the contamination of protein, polysaccharides, polyphenol, secondry products and DNA compare to LiCl. After UV spectrophotometric and electrophoretic analysis, the average yields of the RNA extracted by the SDS-LiCl method was 48 µg/g as the same as the modified SDS acid phenol method. The average yields of the RNA extracted by the two methods attributed to young and fresh bulb of *F. unibracteata*, with the tissues contain low levels of phenolic and polysaccharides compounds. At the same time, sample preparation may have released many secondary metabolites that caused RNA to be lost when those metabolites are eliminated during extraction or precipitation (Yao et al., 2009).

The integrity of the RNA was evaluated by gel electrophoresis. Total RNA isolated by modified SDS acid phenol method and SDS-LiCl method showed two bright bands (28S rRNA and 18S rRNA). But the gel electrophoresis result of SDS-LiCl method showed that the band of RNA was not enough clear, which demonstrating that the purity of RNA is not good. Moreover, RNA from modified SDS acid phenol method, the lightness of the 28S band was approximately twice than that of the 18S band, which demonstrating that the RNA was free of RNase and intact. In addition, there were no bands visible around the loading wells, which indicating that the RNA was free of genomic DNA contamination. In contrast, the RNA from the modified SDS acid phenol method was better than that of the SDS-LiCl method.

RT-PCR analysis

RNA samples were further characterized with RT-PCR using gene-specific primers. Mevalonate synthesis through the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is a key step in isoprenoid biosynthesis. HMG-CoA reductase is inactivated by phosphorylation by SNF1-related protein kinase 1 (SnRK1) (Hey et al., 2006). A 300-bp fragment of the *HMGR* gene was reverse-transcripted and amplified from total RNA isolated using the modified SDS acid phenol method and SDS-LiCl method. Using the SDS-LiCl method, RT-PCR amplification resulted in a faint band, but through the SDS acid phenol method, a bright band resulted from RT-PCR (Figure 2). Regarding the comparative experiments described in this paper, we successfully amplified the HMGR gene, and our results indicated that the SDS acid phenol method is found to be effective in extracting total RNA from *F. unibracteata*.

ACKNOWLEDGEMENTS

This work was supported by the grant from the Project of Sichuan Provincial Education Department (no.09ZB017).

REFERENCES

Ainsworth C (1994). Isolation of RNA from floral tissue of *Rumex acetosa* (sorrel). Plant Mol. Biol. Rep., 12: 198-203.

Apt KE, Clendennen SK, Powers DA, Grossman AR (1995). The gene family encoding the fucoxanthin chlorophyll proteins from the brown alga *Macrocystis pyrifera*. Mol. Gen. Genet., 246: 455-464.

Bahloul M, Burkard G (1993). An improved method for the isolation of total RNA from spruce tissues. Plant Mol. Biol. Rep., 11: 212-215.

Chen SC, Hsia KC (1977). A taxonomic study of the Chinese drug Bei-mu (*Fritillaria*). Acta Phytotaxon. Sin., 15: 31-46.

Chun J, Zheng YF, Wang SH (2008). A RNA isolation method suitable for a wider ange of materials. Prog. Biochem. Biophys., 35(5): 591-597.

Chomczynski P, Sacchi N (1987). Single-step method of RNA isolation by acid guani-diniumthiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156-159.

Dong JZ, Dunstan DI (1996). A reliable method for extraction of RNA from various conifer tissues. Plant Cell Rep., 15: 516-521.

Farrell RE Jr (1993). RNA methodologies: A laboratory guide for isolation and characterization. Academic Press, London.

Gao SL, Zhu DN, Cai ZH, Jiang Y, Xu DR (1999). Organ culture of a precious Chinese medicinal plant – *Fritillaria unibracteata*. Plant Cell Tissue Organ Cul., 59: 197-201.

Gareth P, Asuncion LL, Marta V, Ester S (2006). Simple and rapid RNA extraction from freeze-dried tissue of brown algae and seagrasses. Eur. J. Phycol., 41: 97-104.

Gasic K, Hernandez A, Korban SS (2004). RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol. Biol. Rep., 22: 437.

Graham GC (1993). A method for extraction of total RNA from *Pinus radiata* and other conifers. Plant Mol. Biol. Rep., 11: 32-37.

Guillemaut P, Marechal DL (1992). Isolation of plant DNA: A fast inexpensive and reliable method. Plant Mol. Biol. Rep., 10: 69-74.

Hey SJ, Powers SJ, Beale MH, Hawkins ND, Ward JL, Halford NG (2006). Enhanced seed phytosterol accumulation through expression of a modified HMG-CoA reductase. Plant Biotechnol. J., 4: 219-229.

Iandolino AB, DaSilva FG, Lim H, Choi H, Williams LE, Cook DR (2004). High quality RNA, cDNA, and derived EST libraries from Grapevine (*Vitis vinifera* L.). Plant Mol. Biol. Rep., 22: 269-278.

Koonjul PK, Brandt WF, Farrant JM, Lindsey GG (1999). Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA. Nucl. Acids Res., 27: 915-916.

Li JH, Tang CH, Song CY, Chen MJ, Feng ZY, Pan YJ (2006). A simple, rapid and effective method for total RNA extraction from *Lentinula edodes*. Biotechnol. Lett., 28: 1193-1197.

Li YF, Li YX, Lin J, Xu Y, Yan F, Tang L, Chen F (2003). Identification of bulb from *Fritillaria cirrsoha* by PCR with specific primer. Planta Med., 69: 186-188.

Liu W, Wang B, Duan C, Li B (2005). A method for isolating functional RNA callus of *Dendrobium candidum* contented rich polysaccharides. Colloid. Surface B, 4: 259-262.

Salzman RA, Fujita T, Zhu SK, Hasegawa PM, Bressan RA (1999). An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol. Biol. Rep., 17: 11-17.

Sambrook J, Fritsch E, Maniatis T (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor, New York.

Schneiderbauer A, Sandermann JH, Ernst D (1991). Isolation of function RNA from plant tissues rich in phenolic compounds. Anal. Biochem., 197: 91-95.

Schultz DJ, Craig R, Cox FDL, Mumma RO, Medford JI (1994). RNA isolation from recalcitrant plant tissue. Plant Mol. Biol. Rep., 12(4): 310-316.

Takahashi H, Kamakura H, Shiono K, Abiko T, Tsutsumi N, Nishizawa NK, Nakazono M (2010). A method for obtaining high quality RNA from paraffin sections of plant tissues by laser microdissection. J. Plant Res., 123: 807-813.

Thanh T, Omar H, Abdullah MP, Chi VTQ, Noroozi M, Ky H, Napis S (2009). Rapid and effective method of RNA isolation from green microalga *Ankistrodesmus convolutes*. Mol. Biotechnol., 43: 148-153.

Wang XC, Tain WM, Li YX (2008). Development of an efficient protocol of RNA isolation from recalcitrant tree tissues. Mol. Biotechnol., 38: 57-64.

Wang XH, Xiao HL, Chen GX, Zhao X, Huang CH, Chen CY, Wang F (2011). Isolation of high-Quality RNA from *Reaumuria soongorica*, a desert plant rich in secondary metabolites. Mol. Biotechnol., 48: 165-172.

Yao JT, Fu WD, Wang XL, Duan DL (2009). Improved RNA isolation from *Laminaria japonica* Aresch (Laminariaceae, Phaeophyta). J. Appl. Phycol., 21: 233-238.