African Journal of Biotechnology Vol. 10(78), pp. 17907-17915, 7 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2523
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Establishment of the total RNA extraction system for lily bulbs with abundant polysaccharides

Xueyan Li, Chunxia Wang, Hongmei Sun* and Tianlai Li

Shenyang Agricultural University, College of Horticulture, Key Laboratory of Protected Horticulture of Liaoning Province, Shenyang, Liaoning 110161, China.

Accepted 4 November, 2011

Isolating high quality RNA from lily bulbs is made difficult by the presence of plentiful polysaccharides. To solve this problem, an efficient and steady RNA extraction technique from different parts of scales in three hybrids lily bulbs has been developed through modifications of three common RNA extraction methods, cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS) and Trizol method. NaCl concentration of both CTAB and SDS protocols was increased in order to get rid of the polysaccharides in scales. The resulting RNA is of high-quality, as judged by agarose gel electrophoresis and UV-spectroscopic analysis. Furthermore, the middle scales obtain the highest RNA yield followed by the exterior and the interior scales. However, Trizol method was also improved to remove polysaccharides but failed. The results of reverse-transcription-polymerase chain reaction (RT-PCR) indicated that only the improved CTAB method is suitable for subsequent purposes, such as reverse transcription PCR and cDNA library construction.

Key words: *Lilium* bulbs, polysaccharides-rich tissues, RNA isolation, improved cetyltrimethylammonium bromide (CTAB) method.

INTRODUCTION

Total RNA extraction from plant tissues is a crucial prerequisite for plant molecular biology studies. Conventional RNA extraction procedures (guanidine thiocyanate (Chomczynski and Sacchi, 1987; Salzman et al., 1999), hot borate (Hamby et al., 1988; Moser et al., 2004), sodium dodecyl sulfate (SDS) (Zhou et al., 1999), cetyltrimethylammonium bromide (CTAB), (Pirttila et al., 2001; Zeng and Tang, 2002) and Trizol method (Curatti et al., 2008; Klotz and Haagenson, 2008) have been widely used for plant total RNA extraction, leaves and fruits, for example. However, there are many other special tissues, for example, seeds and abnormal organs, where problems have been encountered due to high concentrations of polysaccharides, phenols and other secondary metabolites. Therefore, how to remove or suppress the interference of those substances effectively is crucial to the acquirement of high-quality RNA. At present, RNA has already been extracted successfully from many polysaccharides-rich tissues such as peanut seeds (Yan et al., 2005), sweet potato roots (Scottjr et al., 1998), potato tubers (Stiekema et al., 1988; Garbarino et al., 1992) and *Fritillary thunbergii* bulbs (Hu et al., 2007).

Studying the development mechanism of lily bulbs, an important nutrient storage and reproductive organ, is the foundation to produce high-quality bulbs and cut flowers. Only on the premise of obtaining high quality total RNA, can molecular biology studies be carried out on lily bulbs. However, it may be quite challenging for the presence of considerable amounts of proteins and polysaccharides, which can coprecipitate with RNA (Sharma et al., 2002) as the result of many physical and chemical properties similar to RNA (Li and Wang, 1991; Logemann et al., 1987). Moreover, polysaccharides can inhibit the activity

*Corresponding author. E-mail: hmbh@sina.com. Tel: +86-24-88437885. Fax: +86-24-88487166.

Abbreviations: CTAB, Cetyltrimethylammonium bromide; **SDS,** sodium dodecyl sulfate; **DEPC,** diethylpyrocarbonate; **LiCl,** lithium chloride; **EDTA,** ethylenediaminetetraacetic acid; **NaAC,** sodium acetate; **RT-PCR,** reverse-transcription-polymerase chain reaction; **PVP,** polyvinylpyrrolidinone; **β-ME,** β-mercaptoethanol; **ddH_2O,** double-distilled water.

Table 1. SDS extraction buffer S1-S13, the absorbance ratios and the yields of RNA isolated by S1-S13.

Extraction buffer	SDS (W/V, %)	Tris-HCl (mM, pH8.0)	EDTA (mM, pH8.0)	NaCl (M)	Absorbance ratio		Content (ng/µl)
					A260/A280	A260/A230	
S1	1.0	50	10	0.1	1.17±0.01^{cC}	0.25±0.01^{dD}	10.45±1.00^{fE}
S2	1.0	100	20	0.5	1.31±0.04^{cC}	0.28±0.02^{dCD}	21.28±0.90^{dCD}
S3	1.0	150	50	1.0	1.75±0.01^{bB}	1.42±0.16^{bB}	14.60±0.89^{efDE}
S4	1.5	50	20	1.0	1.87±0.08^{bAB}	1.26±0.03^{bBC}	16.18±0.60^{eDE}
S5	1.5	100	50	0.1	1.19±0.04^{cC}	0.25±0.01^{dD}	20.18±1.71^{deD}
S6	1.5	150	10	0.5	1.30±0.02^{cC}	0.29±0.01^{dCD}	17.90±1.27^{deD}
S7	2.0	50	50	0.5	1.78±0.19^{bB}	0.80±0.25^{cC}	14.98±0.35^{eDE}
S8	2.0	100	10	1.0	1.95±0.01^{abAB}	1.26±0.25^{bBC}	18.90±2.75^{cC}
S9	2.0	150	20	0.1	1.32±0.02^{cC}	0.55±0.25^{cdCD}	26.81±0.54^{cC}
S10	1.5	50	20	1.5	2.03±0.08^{abA}	1.57±0.06^{bAB}	32.87±3.51^{bB}
S11	1.5	50	20	2.0	2.11±0.01^{aA}	1.87±0.02^{abAB}	41.60±0.62^{aA}
S12	1.5	50	20	2.5	2.09±0.01^{aA}	1.93±0.13^{abAB}	34.28±0.78^{bB}
S13	1.5	50	20	3.0	2.10±0.01^{aA}	1.99±0.03^{aA}	31.30±1.04^{bB}

Results are means ± SE of three independent measurements, and the small letters as well as the capital letters denote the significances at $p \leq 0.05$ and $p \leq 0.01$, respectively; only those parts marked with different letters significantly differ.

of many enzymes (Manning, 1987). As a result, there is less research on RNA extraction from lily bulbs (Zheng, 2009; Li et al., 2008), while many studies have succeeded in extracting high quality RNA from other lily tissues such as leaves, stems and flowers (Yin et al., 2008; Tzeng et al., 2009; Wang et al., 2009; Wu et al., 2006; Hao et al., 2005; Nakatsuka et al., 2009; Kim et al., 2007). Therefore, the establishment of total RNA extraction system for lily bulbs is promptly needed for lily bulbs molecular biology investigations.

In this experiment, three RNA extraction protocols (CTAB procedure that is extensively used in abnormal organs, SDS method which is applied widely in polysaccharides-rich plant tissues and convenient Trizol reagent) were referred and modified. The aim of this study was to set up technology system for gaining high quality and biologically active RNA from lily bulbs, to set molecular biological basis for the gene cloning and functional analysis, cDNA library construction and lily genetically modification, furthermore, to provide the theory foundation for improving the bulb quality.

MATERIALS AND METHODS

Bulbs (10 to 12 cm circumference) in *Lilium davidii* var. *unicolor*, the species native to China, Asiatic hybrid lily bulbs cv. 'Elite' (12 to 14 cm circumference) and Oriental hybrid lily bulbs cv. 'Sorbonne' (14 to 16 cm circumference) were divided into the exterior, middle and interior scales, respectively according to Sun et al. (2004) (Sun et al., 2004) and the scales were frozen in liquid nitrogen immediately and then were preserved at -80°C until analysis.

Exogenous RNase removal

RNase-free labeled, unopened and disposable pipette tips and centrifuge tubes were directly autoclaved at 121°C for 40 min without treated by diethylpyrocarbonate (DEPC); glassware and solutions were sterilized at 121°C for 30 min; porcelain mortars, pestles and metal objects were wrapped in aluminum foil and baked at 180°C for more than 5 h.

Buffer modification

SDS extraction buffer S1 to S13: S1 to S9 was carried out by L_9 (3^4) orthogonal test that was applied according to different concentrations of SDS, Tris-HCl, ethylenediaminetetraacetic acid (EDTA) and NaCl in the extraction solutions. S10 to S13 was improved S4 buffer with higher NaCl concentration as the following four gradients 1.5, 2, 2.5 and 3 M. And 2% polyvinylpyrrolidinone (PVP) (W/V) was added into each extraction, respectively (Table 1).

CTAB extraction buffer included 2% CTAB (W/V), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0) and 2% PVP. For treatment C1 to C3, the NaCl concentrations were set as 1.4, 2 and 3 M. Above all the processing, 2% β-mercaptoethanol(β-ME) (W/V) was added into just before use.

RNA extraction protocols

Modified CTAB procedure and improved SDS method

(1) Scales at -80°C were ground to fine powders in porcelain mortars which were pre-cooled in liquid nitrogen. The powders were transferred to RNase-free tubes filled with 1 ml pre-warmed extraction buffer and 20 µl β-ME, vortexed to mix completely. The samples were incubated at 65°C for 20 min and vortexed every 5 min; (2) following mixing, an equal volume of chloroform-isoamyl alcohol (24:1) was added, mixed well, and centrifuged for 10 min at 15 000 g at 4°C; (3) after centrifugation, the aqueous phase was again extracted with an equal volume of chloroform-isoamyl alcohol, centrifuged as previously described. Repeat this step one or two times; (4) the supernatant was then mixed with 1/3 volume of 8 M lithium chloride (LiCl). The RNA was precipitated overnight at -20°C and harvested by centrifugation for 10 min at 15 000 *g* at 4°C; (5) the supernatant was discarded and the pellet was dissolved with 400 µl double-distilled water (ddH_2O). The solution was extracted once with an equal volume of chloroform-isoamyl alcohol; (6) 1/10 volume of 3 M sodium acetate (NaAC) (pH 5.2) and 3 volumes of 100% ethanol was added to the supernatant, precipitated at -20°C for 30 min and cen-trifuged as previously described; (7) the supernatant was removed without disturbing the RNA pellet. The

RNA pellet was washed with 75% ethanol. After centrifugation for 10 min the ethanol was care-fully removed by pipetting and the RNA dried at room temperature and subsequently resuspended in 50 µl ddH_2O. RNA was ready for quantification and quality.

Improved Trizol method

(1) Using chilled porcelain mortars and pestles, scales were ground in the presence of liquid nitrogen. The frozen powder was transferred quickly to the 1.5 ml tube with 1 ml extraction buffer, 20 µL β-ME and 0.1 g PVP, mixed completely by vortexing the tube, and centrifuged for 5 min at 15 000 g at 4°C after let stand for 10 min; (2) the supernatant was transferred to a new tube with 1/10 volume of 5 M KAC (pH 4.8), 1/10 volume of 100% ethanol and an equal volume of chloroform, vortexed vigorously, and centrifuged for 10 min at 15 000 g at 4°C after let stand for 10 min on ice. (3) The aqueous phase was mixed with an equal volume of isopropanol, left for 10 min, and centrifuged as previously described; (4) the supernatant was discarded and the RNA pellet was given a wash with 75% ethanol, followed by centrifugation for 10 min. The pellet was then dried at room temperature and subsequently resuspended in 50 µl ddH_2O. RNA was ready for quantification and quality.

DNase treatment

Extracted crude RNA was treated with RNase-free DNase I (Tiangen) according to the manufacturer's instructions to remove contaminant genomic DNA. RNA was then extracted further with chloroform-isoamyl alcohol 24:1 step as described earlier and precipitated with 1/10 volume of 3 M NaAC (pH 5.2) and 3 volumes of 100% ethanol. The RNA pellet was washed with 75% ethanol. After centrifugation for 10 min the ethanol was carefully removed. The RNA was dried at room temperature and subsequently resuspended in ddH_2O for RT-PCR.

Assessment of RNA quality and quality

The RNA purity was estimated by the absorbency ratios A260/A230 and A260/A280, a measure of contamination by the polysaccharides and proteins, respectively. The RNA integrity was judged by the clarity of ribosomal RNA bands visualized on agarose gel electrophoresis.

RT-PCR analysis

The first strand cDNA was synthesized by M-MLV reverse transcriptase (Promega) according to the manufacturer's specifications. RT-PCR amplifications were performed using Actin specific primers designed according to the nucleotide sequence of *Lilium* Actin (# AB438963.1).

The sequence of the Actin forward primer is 5´-gTgCTTTCCCTCTACgCCAg-3´, the reverse primer is 5´-CgATggTTATCACCTGCCCg -3´. The PCR protocol was as follows: 25 µl reaction volume with 3 µl 10× PCR buffer (contains Mg^{2+}), 0.8 µl dNTP, 0.4 µl Taq polymerase, 0.8 µl forward primer, 0.8 µl reverse primer, 1 µl cDNA, 18.2 µl ddH2O. PCR conditions were: 94°C for 5 min, 30 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 30 s and 72°C for 10 min.

Data analysis

L_9 (3^4) orthogonal test were analyzed to test the effects of ingredients(EDTA, SDS, Tris-HCl and NaCl) in extraction buffer S1 to S9, the A260/A280 ratio and the A260/A230 ratio of RNA isolated by S1 to S9 was calculated.

For all comparisons between extraction buffers, statistical analysis was performed by applying one-way ANOVA and multiple comparisons of means of each sowing date using the least significant difference (L.S.D.) test at $p \leq 0.05$ and $p \leq 0.01$ to distinguish significantly different means. All statistical analyses were performed with the SPSS 17.0 program.

RESULTS

Selection and optimization of RNA extraction from the bulbs in *L. davidii* var. *unicolor*

SDS extraction buffer S1 to S9

Total RNA examined by electrophoresis on 1.2% agarose gels showed no degradation and little DNA contaminants (Figure 1a). From Figure 1a and Table 1, we can see RNA isolated by S4 had the clearest 28S and 18S rRNA bands, less protein and polysaccharides contaminants and the higher yield. Therefore, S4 was the best treatment. According to Table 2, NaCl concentration had extremely significant effect on the value of both A260/A280 and A260/A230, which indicated that the high NaCl concentration of the extractions was useful to remove polysaccharides effectively.

SDS extraction buffer S10 to S13

In all tested samples, the quality of RNA obtained by SDS extractions S10 to S13 with higher NaCl concentration was better than S4. Distinct 28S and 18S rRNA bands were found in agarose gel electrophoresis and no degradation was detected (Figure 1b). Moreover, the ratios of A260/A230 and A260/A280 showed weak contamination by polysaccharides or polyphenols and little or no protein contamination (Table 1). Taking into account all the earlier mentioned analysis, the best treatment was S11 (2 M NaCl concentration).

CTAB extraction buffer C1 to C3

With all RNA samples tested, there is a 28S rRNA band almost twice as bright as 18S rRNA band which indicated that little or no RNA degradation occurred (Figure 1c). The A260/A230 ratio was higher than 2.13, which showed the RNA was of high purity and free of polyphenol and polysaccharide contamination. The A260/A280 ratios ranged from 1.92 to 2.16, indicating a lack of protein contamination (Table 3). Among the three treatments, C1 (1.4 M NaCl concentration) is better than the others.

RNA isolation from different parts of scales by S11 and C1 to C3

The quality of RNA extracted from different scales of *L.*

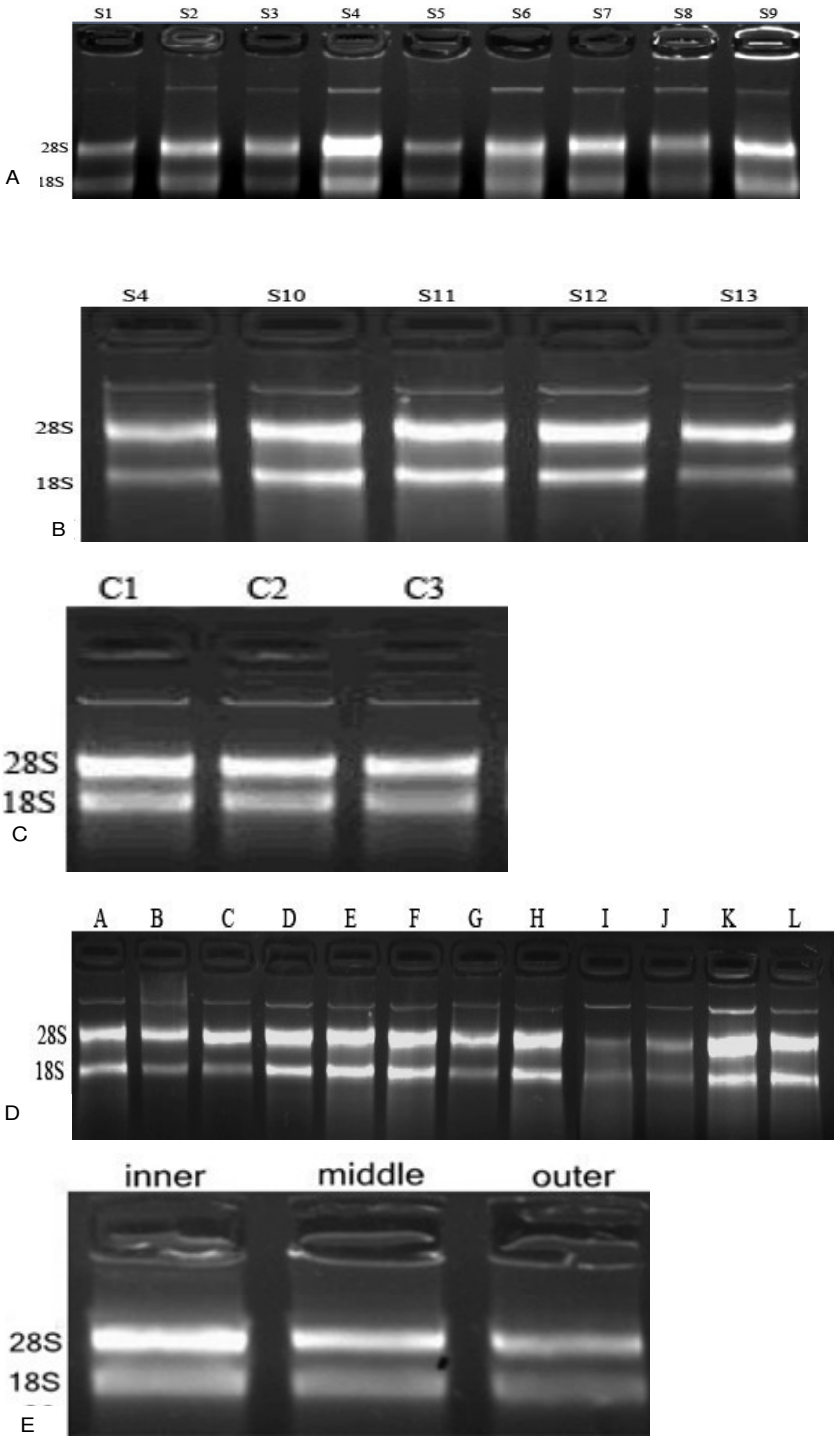

Figure 1. Agarose gel electrophoresis of RNA extracted from *L. davidii* var. *unicolor* (A) RNA isolated by SDS extraction buffer S1 to S9; (B) RNA isolated by SDS extraction buffer S4 and S10 to S13; (C) RNA isolated by CTAB extraction buffer C1 to C3; (D) A-RNA isolated from the inner scales by S11; B-RNA isolated from the inner scales by C3; C-RNA isolated from the inner scales by C2; D-RNA isolated from the inner scales by C1; E-RNA isolated from the middle scales by S11; F-RNA isolated from the middle scales by C3; G-RNA isolated from the middle scales by C2; H-RNA isolated from the middle scales by C1; I-RNA isolated from the outer scales by S11; J-RNA isolated from the outer scales by C3; K-RNA isolated from the outer scales by C2; L-RNA isolated from the outer scales by C1; (E) RNA isolated by improved Trizol method from different parts of scales).

Table 2. Analysis of variance of A260/A280 and A260/A230 of RNA extracted by SDS extraction buffer S1-S9.

Variation Sources	A260/A280				A260/A230				$F_{0.05}$	$F_{0.01}$
	SS	DF	MS	F	SS	DF	MS	F		
Block	102.651	1	102.651	1.156	16.646	1	16.646	221.974	3.35	5.49
SDS	0.392	2	0.916	2.204	0.239	2	0.120	1.596		
Tris-HCl	0.261	2	0.130	1.467	0.173	2	0.087	1.155		
EDTA	0.851	2	0.426	4.790*	0.371	2	0.185	2.473		
NaCl	8.754	2	4.373	49.222**	7.395	2	3.697	49.304**		
Error	2.399	27	0.089		2.025	27	0.075			
Total Error	115.298	36			26.850	36				

**Means significantly different (P≤ 0.01), *means significantly different (P≤0.05). SS, Sum of square; DF, degree of freedom; MS, mean of square.

Table 3. The absorbance ratios and yields of RNA isolated by CTAB extraction buffer C1-C3.

Extraction buffer	NaCl Concentration (M)	Absorbance Ratio		Contents (ng/µl)
		A260/A280	A260/A230	
C1	1.4	2.09 ± 0.01^{aA}	2.21 ± 0.05^{aA}	147.10 ± 8.52^{aA}
C2	2.0	2.16 ± 0.02^{aA}	2.13 ± 0.03^{aA}	115.33 ± 8.79^{bB}
C3	3.0	1.92 ± 0.29^{bB}	2.22 ± 0.15^{aA}	132.27 ± 3.91^{aAB}

Results are means ± SE of three independent measurements, and the small letters as well as the capital letters denote the significances at p ≤ 0.05 and p ≤ 0.01, respectively; only those parts marked with different letters significantly differ.

Table 4. The absorbance ratios and yields of RNA isolated by S11 and C1-C3.

Treatment	Absorbance ratio		Contents (ng/µl)
	A260/A280	A260/A230	
A	2.14 ± 0.03^{aAB}	2.34 ± 0.02^{abAB}	110.65 ± 5.98^{dD}
B	2.12 ± 0.05^{abAB}	2.33 ± 0.03^{abAB}	117.33 ± 6.80^{dD}
C	2.12 ± 0.01^{abAB}	2.27 ± 0.04^{bAB}	101.02 ± 2.33^{dD}
D	2.16 ± 0.04^{aA}	2.28 ± 0.04^{bAB}	113.65 ± 3.92^{dD}
E	2.15 ± 0.01^{aAB}	2.36 ± 0.05^{abA}	198.12 ± 3.02^{aA}
F	2.13 ± 0.04^{aAB}	2.31 ± 0.04^{abAB}	136.90 ± 4.10^{cC}
G	2.13 ± 0.02^{aAB}	2.38 ± 0.04^{aA}	173.30 ± 6.50^{bB}
H	1.97 ± 0.01^{cB}	2.22 ± 0.02^{bB}	214.47 ± 1.08^{aA}
I	2.15 ± 0.01^{aAB}	2.29 ± 0.02^{bAB}	101.75 ± 2.06^{dD}
J	2.12 ± 0.02^{abAB}	2.27 ± 0.02^{bAB}	100.82 ± 3.88^{dD}
K	2.09 ± 0.02^{abAB}	2.23 ± 0.03^{bB}	110.62 ± 7.98^{dD}
L	2.06 ± 0.01^{bB}	2.10 ± 0.02^{cC}	148.07 ± 7.16^{cC}

A to L indicates the treatments illustrated in Figure 1.
Results are means ± SE of three independent measurements, and the small letters as well as the capital letters denote the significances at p ≤ 0.05 and p ≤ 0.01, respectively; only those parts marked with different letters significantly differ.

davidii var. *unicolor* by S11 and C1, selected based on the previous experiments showed that; both methods could extract high quality RNA. The yields of RNA in the middle scales were significantly higher than those in other scales (Table 4). There was no difference between S11 and C1 in isolating RNA from the middle and the interior scales, while CTAB with low NaCl concentration was superior to CTAB with higher NaCl concentration and SDS method from the outer scales (Figure 1d and Table 4). Overall, CTAB extraction C1 (1.4 M NaCl) is suitable to

Table 5. The absorbance ratios and yields of RNA isolated by improved Trizol method.

Tissue	Absorbance ratio		Contents (ng/μl)
	A260/A280	A260/A230	
Outer scales	1.82±0.04^{aA}	0.45±0.01^{bB}	77.21± 4.24^{aA}
Middle scales	1.84±0.01^{aA}	0.56±0.02^{abAB}	86.35± 7.48^{aA}
Inner scales	1.86±0.03^{aA}	0.88±0.01^{aA}	90.22±5.68^{aA}

Results are means ± SE of three independent measurements, and the small letters as well as the capital letters denote the significances at $p \leq 0.05$ and $p \leq 0.01$, respectively; only those parts marked with different letters significantly differ.

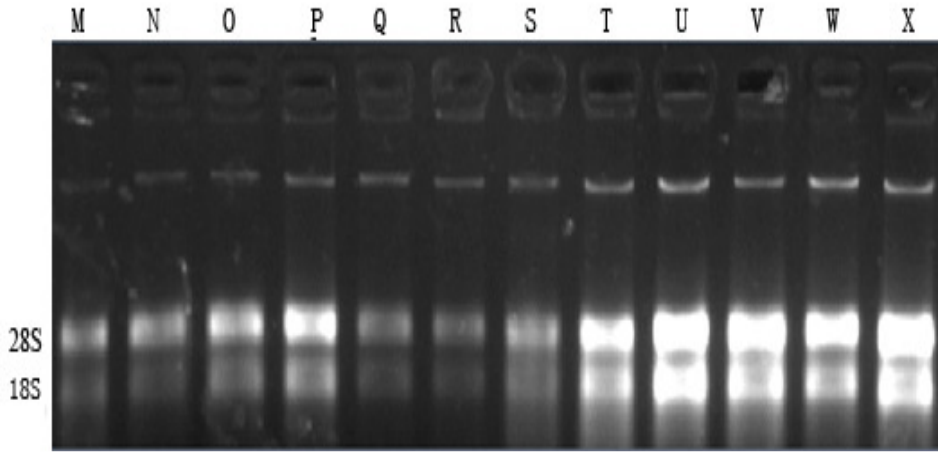

Figure 2. Agarose gel electrophoretic analysis of RNA extracted from different parts of scales in 'Elite' and 'Sorbonne' (M to R--RNA isolated from 'Sorbonne': M-RNA isolated from the outer scales by S11; N- RNA isolated from the outer scales by C1; O- RNA isolated from the middle scales by S11; P- RNA isolated from the middle scales by C1; Q- RNA isolated from the inner scales by S11; R- RNA isolated from the inner scales by C1; S to X RNA isolated from 'Elite': S-RNA isolated from the outer scales by S11; T- RNA isolated from the outer scales by C1; U-RNA isolated from the middle scales by S11; V- RNA isolated from the middle scales by C1; W-RNA isolated from the inner scales by S11; X- RNA isolated from the inner scales by C1).

extract RNA from all scales.

RNA isolation from different parts of scales by improved Trizol method

For all RNA samples tested, distinct 28S and 18S rRNA bands without apparent DNA were observed (Figure1e). But the A260/A230 absorbance ratios were very low indicating that, the heavy contamination of polysaccharides (Table 5). Unlike the improved CTAB and SDS method, the quality of RNA isolated from the interior scales by the improved Trizol method was superior to the exterior and the middle scales.

RNA extraction from different part of scales in 'Elite' and 'Sorbonne'

Total RNA was isolated from different scales in 'Elite' and 'Sorbonne' by S11 and C1 according to the results of *L. davidii* var. *unicolor*. The results show that both S11 and C1 could also obtain high quality from different scales of the two lilies (Figure 2 and Table 6). However, C1 was better than S11. A comparison of yields based on the spectrophotometric determination of the RNA samples showed that, the middle scales gave the highest yield similar to 'Elite'.

RT-PCR amplification

RT-PCR is an important process to gene cloning, transgenic plants identification and other important molecular experiments. It requires high-quality, intact RNA, free from any polysaccharides, proteins and other inhibitors, because nucleic acids can form tight complexes with polysaccharides and form a gelatinous pellet containing embedded RNA. RNA prepared with modified CTAB

Table 6. The absorbance ratios and yields of RNA isolated from different scales in ‘Elite’ and ‘Sorbonne’.

Treatment	Absorbance ratio		Contents (ng/µl)
	A260/A280	A260/A230	
M	2.08±0.05	3.02±0.04	48.41±0.74
N	2.12±0.03	2.83±0.05	67.85±0.87
O	2.01±0.02	2.67±0.02	74.22±3.53
P	2.04±0.02	3.15±0.10	90.55±3.77
Q	2.21±0.03	3.55±0.03	30.70±3.26
R	2.21±0.04	2.69±0.06	51.05±0.96
S	2.17±0.01	1.96±0.03	79.92±1.63
T	2.10±0.01	2.72±0.05	150.05±0.15
U	2.06±0.05	2.60±0.05	195.45±3.96
V	2.17±0.03	2.56±0.04	215.09±0.64
W	2.08±0.04	2.46±0.11	124.65±5.10
X	2.19±0.01	2.74±0.10	248.75±6.14

Results are means ± SE of three independent measurements. M to X indicates the treatments illustrated in Figure 2.

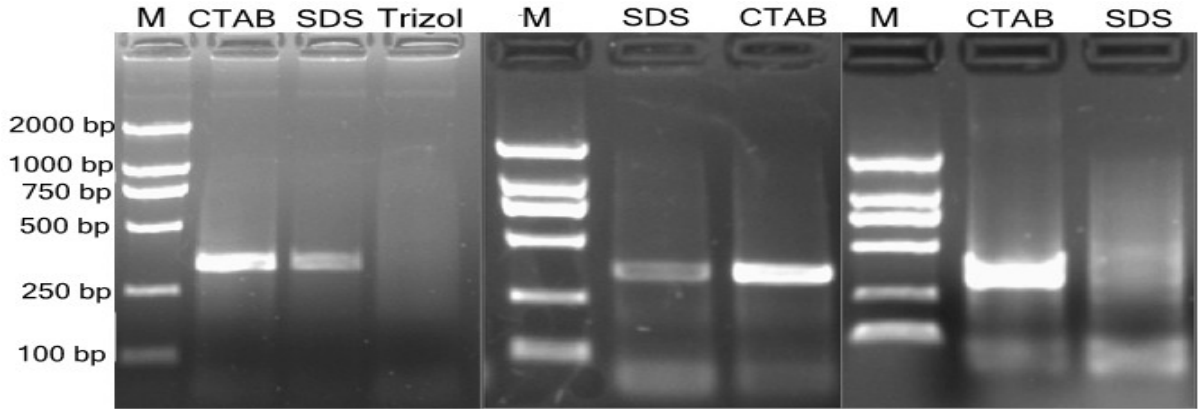

Figure 3. Agarose gel electrophoresis of RT-PCR products. M is molecular weight marker; lanes from left to right are PCR product of RNA isolated from *L. davidii* var. *Unicolor* by improved CTAB, improved SDS and improved Trizol method, amplified product of RNA isolated from ‘Sorbonne’ by improved SDS and improved CTAB method, and PCR product of RNA isolated from ‘Elite’ by improved CTAB and improved SDS method.

method and modified SDS procedure served as a robust template for reverse transcription which resulted in the expected amplification of a 320 bp amplicon, whereas no RT–PCR product was amplified with total RNA samples isolated by Trizol reagent (Figure 3). The brightness of CTAB was higher than that of SDS, which indicated that only improved CTAB method can extract biologically active RNA from lily bulbs.

DISCUSSION

The presence of RNase, which can be classified into endogenous and exogenous, is the major cause for the failure of RNA extraction. In order to remove exogenous RNase, the plastics and solutions used in this experiment were directly sterilized for long time without DEPC treatment. This can not only guarantee RNA quality, but prevent the threat to experimenters' health by DEPC. As for the inhibition of endogenous RNase, the samples should be ground in continuous liquid nitrogen. Moreover, the excessive possession of LiCl precipitation should be avoided. In this research, the precipitation time was controlled in the range of 8 to 12 h to prevent RNA yields decrease and RNA degradation. Besides, a ratio of the sample to extraction at 1:10 was found to be the optimum for RNA extraction. In this study, PVP and β-ME were added into all methods together to inhibit the effects of polyphenols for the reason that β-ME can provide reductive conditions in which polyphenols can integrate with the water-soluble PVP by forming a chelate which can be removed through the next steps [30]. H_2O-

saturated phenol was not used in this experiment because it can irreversibly combine with PVP [31].

Trizol method is the favorite reagent to extract RNA from different plant materials for the advantage of being simple, relatively short and easy to operate. By this method, Wang et al (2008) isolated RNA from the young flower buds of *Lilium longiflorum* 'Snow Queen' for *LiLFY1* gene cloning and expression [24]; Xin et al (2010) obtained RNA that can be used to *hsf* gene cloning and expression analysis from the culture lily [32]. Total RNA was extracted from lily bulbs using Trizol method in this experiment, the results showed that RNA quality of the inner scales was better than the outer and the middle, UV detection revealed a lot of impurities despite the gel electrophoresis bands were clear, high brightness and no DNA contamination and the RT-PCR results showed that the RNA can not be used in molecular biology. This might be related to the high quantity of starch in lily bulbs, which can form insoluble jelly with denaturing agent such as the isopropanol [19] which can induce coprecipitation of DNA and polysaccharides with RNA [33]. As reported [31], LiCl precipitation can effectively remove the substances whose absorption peak is 230 nm, so we make modification to Trizol method by taking LiCl to remove polysaccharides, but failed. Therefore, how can polysaccharides be removed efficiently is the most important issue to extract RNA from lily bulbs.

SDS, a protein denaturation was used for plant RNA extraction widely. Wu et al (2006) found that modified SDS method could isolate high quality RNA without DNA contamination from *Lilium longiflorum* leaves, stems, petals and stamens after compared SDS method with CTAB and Trizol method [25], and cloned *LLGLO1* [34]. It was discovered in this study that LiCl precipitation would appear white turbidity at low NaCl concentration and resume clear by increasing the concentration of NaCl. It might be the reason that there are a large number of polysaccharides dissolved in extraction buffer and chloroform can remove part of the polysaccharides under high salt [35]. In this experiment, modified SDS method can extract intact and high purity RNA from three different parts of lily scales, but RT-PCR results showed a weak band, possibly due to SDS remnant.

The method for extracting total RNA using CTAB-containing buffer has been frequently applied in polysaccharides-rich tissues and abnormal organs since only CTAB cannot remove polysaccharides from nucleic acids under high NaCl concentration effectively but inhibit RNase activity interacting with β-ME. High-quality RNA can be obtained by CTAB method from Lily leaves [24], petals [25] and other organizations. For instance, Zheng (2009) succeeded in extracting RNA from OT and Oriental lily bulbs for lily virus detection [20]. However, the traditional CTAB method failed in RNA extraction from all three hybrids lily bulbs in this study, only succeed when increased NaCl concentration. The improved CTAB protocol established in this study can extract high purity, intact and high yield RNA from three different parts of scales in different types of lilies and RNA yield in the middle scale was the highest. The extracted RNA checked by agarose gel electrophoresis show distinct 28S and 18S ribosomal RNA bands and the brightness of 28S is probably twice as much as that of 18S RNA; the ratio of A260/A280 is ranged from 1.8 to 2.2 and A260/A230 is greater than 2.0 and can be used directly for molecular biological experiments after RT-PCR text. In conclusion, the method reported here allowed for the isolation of RNA from lily bulbs rich in polysaccharides, for which other methods failed to deliver RNA suitable for reverse transcription. RNA obtained by this protocol was of good quality as indicated by spectrophotometry and amenable to downstream molecular applications of lily bulbs. Besides, the new protocol is likely to find application in RNA isolation from other medicinal plant species or tissues which are extraordinarily rich in secondary metabolites especially polysaccharides.

ACKNOWLEDGEMENTS

This project was supported by the National Natural Science Foundation of China (30972023), China Post-doctoral Science Foundation (20090451280) and Science Foundation for the Key Laboratory in Liaoning Province of China (LS2010148), respectively.

REFERENCES

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

Curatti L, Giarrocco LE, Cumino AC, Salerno GL (2008). Sucrose synthase is involved in the conversion of sucrose to polysaccharides in filamentous nitrogen-fixing cyanobacteria. Planta. 28: 617-625.

Garbarino JE, Rockhold DR, Belknap WR (1992). Expression of stress-responsive ubiquitin genes in potato tubers. Plant Mol. Biol. 20: 235-244.

Ghangal R, Raghuvanshi S, Sharma PC (2009). Isolation of good quality RNA from a medicinal plant seabuckthorn, rich in secondary metabolites. Plant Physiol. 47: 1113-1115.

Hamby RK, Sims L, Issel L, Zimmer E (1988). Direct ribosomal RNA sequencing optimization of extrantion and sequencing methods for work with higher plants. Plant Mol. Biol. Rep. 6(3): 175-192.

Hao FL, Liu YL, Wang YJ (2005). Extracting methods of the total RNA in the petals of *Lilium* flowers. Xibei Zhiwu Xuebao. 25: 1143-1147.

Hu CG, Honda C, Kita M, Zhang Z, Tsuda T, Moriguchi T (2002). A simple protocol for RNA isolation from fruit trees containing high levels of polysaccharides and polyphenol compounds. Plant Mol. Biol. Rep. 20: 69-69.

Hu XL, Jin GQ, Chen P, Ye BP, Li P (2007). Total RNA extraction from the bulbs of *Fritillary thunbergii* bulbs. Pharm. Biotechnol. 14: 361-363.

Kim SS, Shin DI, Park HS (2007). Transient β-glucuronidase expression in lily (*Lilium longflorum* L.) pollen via wounding-assisted Agrobacterium-mediated transformation. Biotechnol. Lett. 29: 965-969.

Klotz KL, Haagenson DM (2008). Wounding, anoxia and cold induce sugarbeetsucrose synthase transcriptional changes that are unrelated to protein expression and activity. J. Plant Physiol. 165: 423-434.

Li H, Wang XL (1991). The difficulties in the isolation of RNA from plant tissues and their resolving strategies. Shengwu Jishu Tongbao. 1: 36-39.
Li XR, Wang GF, Xi ML, Shi SJ (2008). Cloning and expression analysis of the *LsCCR1* Gene from *Lilium* Oriental Hybrid. Mol. Plant Breed. 6: 141-147.
Logemann J, Schell J, Willmitzer L (1987). Improved method for isolation of RNA from plant tissues. Anal. Biochem. 163: 16-20.
Manning K (1987). Isolation of nucleic acids from plants by differential solvent precipitation. Anal. Biochem. 195: 45-50.
Moser C, Gatto P, Moser M, Pindo M, Velasco R (2004). Isolation of functional RNA from small amounts of different grape and apple tissues. Mol. Biotechnol. 26(2): 95-99.
Nakatsuka A, Yamagishi M, Nakano M, Tasaki K, Kobayashi N (2009). Light-induced expression of basic helix-loop-helix genes involved in anthocyanin biosynthesis in flowers and leaves of Asiatic hybrid lily. Sci. Hortic. 121: 84-91.
Pirttila AM, Halonen M, Hohtola A (2001). Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. Mol. Biotechnol. 19(2): 201-203.
Salzman RA, Fujita T, Salzman KZ, Hasegawa PM (1999). An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. Plant Mol. Biol. Rep. 17: 11-17.
Salzman RA, Fujita T, Salzman KZ, 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.
Scottjr DL, Clark CW, Deahl KL, Prakash CS (1998). Isolation of functional RNA from periderm tissue of potato tubers and sweet potato storage roots. Plant Mol. Bio. Rep. 16: 3-8.
Sharma AD, Gill PK, Singh P (2002). DNA isolation from dry and fresh samples of polysaccharide-rich plants. Plant Mol. Bio. Rep. 20: 415-417.
Stiekema WJ, Heidekamp F, Diekse WG, Beckum J, Hann P, Rosch C, Louwerse JD (1988). Molecular cloning and analysis of four potato tuber mRNAs. Plant Mol. Biol. 11: 255-269.
Su X, Gibor A (1988). A method for RNA isolation from marine macroalgae. Anal. Biochem. 174: 650-657.
Sun HM, Li TL, Li YF (2004). Starch metabolism and sprouting of bulb in *Lilium davidii* var. *unicolor* stored at different cold temperatures. Yuan Yi Xue Bao. 31: 337-342.
Tzeng JD, Hsu SW, Chung MC, Yeh FL, Yang CY, Liu MC, Hsu YF, Wang CS (2009). Expression and regulation of two novel anther-specific genes in *Lilium longiflorum*. J. Plant Physiol. 166: 417-427.
Wang AJ, Tang JF, Li DY, Chen CY, Zhao XY, Zhu LH (2009). Isolation and functional analysis of *LiYAB1*, a *YABBY* family gene, from lily (*Lilium longiflorum*). J. Plant Physiol. 166: 988-995.
Wu XP, Xi ML, Miao HY, Shi JS (2006). Comparison and analysis of extracting methods of the total RNA in lily. Mol. Plant Breed. 4: 871-876.
Wu XP, Xi ML, Shi JS (2006). Cloning and expression of AMADS Box Gene *LLGLO1* from *Lilium longiflorum* Thunb. Zhi Wu Sheng Li Xue Tong Xun. 42: 871-876.
Xin HB, Chen L, Li XY, He XL, Yi MF (2010). Cloning and expression analysis of *hsf* from *Lilium longiforum*. Yuan Yi Xue Bao. 37: 435-440.
Yan YS, Lin XD, Zhang YS, Wang L, Wu KQ, Huang SZ (2005). Isolation of peanut genes encoding arachins and conglutins by expressed sequence tags. Plant Sci. 169: 439-445.
Yin H, Chen L, Li XY, Chen QM, Yi MF (2008). Analysis and improvement of high-quality RNA extraction in leaves of lily. Zhongguo Nong Ye Da Xue Xue Bao. 13: 41-45.
Zeng Y, Tang T (2002). RNA Isolation from highly viscous samples rich in polyphenols and polysaccharides. Plant Mol. Bio. Rep. 20: 417-417.
Zhang JJ, Wang YJ, Wang XP, Yang QK, Yang JX (2003). An improved method for rapidly extraction total RNA from vitis. Guoshu xuebao. 20: 178-181.
Zheng LN (2009). Studies on the virus detection and elimination techniques in lily bulb. M.A. Dissertation. Beijing Forestry University.
Zhou JH, Pesacreta TC, Brown RC (1999). RNA isolation without gel formation from oligosaccharide-rich onion epidermis. Plant Mol. Biol. Rep. 17: 397-407.