

High-quality total RNA extraction from *Magnolia sieboldii* K. Koch seeds: a comparative evaluation

Jun Wei¹ · Xiaolin Zhang¹ · Zhe Hou² · Xiujun Lu¹

Received: 23 November 2016 / Accepted: 19 February 2017 / Published online: 20 March 2018
© Northeast Forestry University and Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract *Magnolia sieboldii* K. Koch seed is characterized with having deep dormancy. The inner molecular regulation mechanism has not been investigated because of the absence of a protocol for total RNA (ribonucleic acid) extraction. The extraction of high-quality RNA is important and can be a limiting factor in plant molecular biology experiments. Sufficient high-quality total RNA is required to elucidate the molecular regulation mechanism of germination. However, *M. sieboldii* seeds with large amounts of secondary metabolites also contain recalcitrant tissues for RNA isolation. We found two simple and low-cost RNA extraction methods for *M. sieboldii* seeds by evaluating and selecting eight types of methods and further optimizing these methods. The two methods were not only suitable for extracting *M. sieboldii* seed RNA but also applicable to RNAs from several other tissues. Total RNA

extracted through these approaches was applicable for general molecular biology experiments such as qRT-PCR (quantitative real-time polymerase chain reactions). The protocols also meet the strict harsh requirements for transcriptome sequencing and small RNA sequencing. This study provides a powerful approach for future studies at the transcription level.

Keywords Seed dormancy · RNA isolation · qRT-PCR · Small RNA · *Magnolia sieboldii*

Introduction

Magnolia sieboldii K. Koch belongs to the Magnoliaceae family and is a deciduous tree geographically distributed in the most northern part of China. *M. sieboldii* is important in scientific studies with a high development prospect because of its ornamental, medicinal, and economic values. However, germination, considered the most vulnerable stage of plant development, is stimulated by long periods of warm and cold stratification (Lu et al. 2016). To date, germination of this species has been investigated using physiological studies and proteomics analysis (Lu et al. 2013, 2016; Zhang et al. 2014). Studies at the transcription level are limited because of the absence of an appropriate RNA extraction method.

Differential protein expression and biological functions of *M. sieboldii* were analyzed through proteomics. However, only a low percentage of differentially expressed proteins could be detected because of the lack of a reference *M. sieboldii* genome (Lu et al. 2016; Zhang et al. 2014). Transcriptomes, such as mRNA (messenger RNA) and small RNA (a type of non-coding RNA), are important in molecular biology research. Transcriptomes assist in

Project funding: This work was supported by the National Natural Science Foundation of China (No. 31570621).

The online version is available at <http://www.springerlink.com>

Corresponding editor: Tao Xu.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11676-018-0615-8>) contains supplementary material, which is available to authorized users.

✉ Xiujun Lu
lxjsyau@126.com

¹ Department of Forestry, Shenyang Agricultural University, No. 120, Donglin Road, Shenyang City 110866, Liaoning Province, People's Republic of China

² Research Institute of Forestry, Chinese Academy of Forestry, No. 1, Xiangshan Road, Haidian District, Beijing City 100091, People's Republic of China

increasing the rate of protein identification; they can also provide a database for subsequent research at the transcription level. Moreover, reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT-PCR), among others, based on high-quality RNA can also validate subsequent gene and protein expression and biological function. However, obtaining a large volume of high-quality RNA is a preliminary step for these investigations (Christou et al. 2014). Therefore, an efficient method for extracting RNA from *M. sieboldii* seeds should be explored.

In this study, we evaluated and optimized several methods for *M. sieboldii* seeds to determine a suitable RNA isolation method. The approach was to extract total RNA in high quality, substantial yield, and high purity suitable for transcriptome sequencing, small RNA sequencing, RT-PCR, qRT-PCR, and other downstream molecular applications. Among these processes, transcriptome sequencing provides a database for the subsequent validation of key differentially expressed proteins and biological functions. This database was used to explore key differentially expressed genes in seed germination, thereby providing a basis for further exploration. In addition, small RNAs, especially microRNA, which are important regulators of gene expression both within defined regulatory pathways and at the epigenetic scale (Klevebring et al. 2009; Gupta et al. 2014; Li et al. 2014), have gradually become a new research hotspot. Meanwhile, the application of RT-PCR and qRT-PCR, among others, can also validate subsequent gene and protein expression and biological functions.

Materials and methods

Plant materials

Mature seeds of *M. sieboldii* were harvested randomly from eight plants at the botanical garden of Shenyang Agricultural University (41°82'N, 123°56'E) in October 2015. Seeds were packaged in Eppendorf tubes after the shell was removed, frozen in liquid nitrogen, and stored at − 80 °C until required.

Solutions and reagents

All reagents were molecular biology grade. The CTAB extraction buffer consisted of 2% CTAB (w/v), 2% PVP (w/v), 2 M NaCl, Tris-HCl (0.1 M, pH 8.0), EDTA (25 μM, pH 8.0), 3.44 μM spermidine, and 2% β-ME (w/v), added just before use. The SSTE buffer consisted of 1 M NaCl, 0.01 M Tris-HCl (pH 8.0), 1 μM EDTA, and 0.5% SDS (w/v). An SSTE optimal buffer consisted of 2 M NaCl, 0.05 M Tris-HCl (pH 8.0), 1 μM EDTA, and 1%

SDS (w/v) (Table S1). Other reagents were TRIzol (Invitrogen), 75% ethanol (v/v), chloroform: isoamyl alcohol (24:1; v/v), phenol: chloroform: isoamylalcohol (25:24:1; v/v/v), and isopropanol. First-stand cDNA synthesis kit (TOYOBO) and TaKaRa TaqTM Version 2.0 plus were also used.

RNA extraction protocol

Protocol 1: TRIzol method

This protocol was essentially as described in the manufacturer's instructions of TRIzol Reagent (Invitrogen) but slightly modified by using chloroform: isoamyl alcohol (24:1; v/v) rather than chloroform, and adding centrifugation in the final process because the RNA solution contained insoluble substances (Fig. S2). Turbid RNA solution was centrifuged at 6000 rpm for 5 min at 4 °C, and the supernatant was collected into a 1.5 ml Eppendorf tube.

Protocol 2: Modified TRIzol method

(1) Seeds (0.1 g) were frozen in liquid nitrogen, pulverized by grinding with a pre-chilled mortar and pestle to a fine powder, and added to a 1.5 mL Eppendorf tube containing 1 ml of TRIzol reagent and 20 μl of β-ME. The mixture was mixed by vortexing, incubated at room temperature for 10 min, and centrifuged at 12,000 rpm for 5 min at 4 °C. (2) The supernatant was transferred to a 1.5 ml Eppendorf tube. An equal volume of chloroform: isoamyl alcohol (24:1; v/v) was added and mixed by inverting the tube. The mixture was centrifuged at 12,000 rpm for 5 min at 4 °C. (3) The supernatant was transferred to a 1.5 ml Eppendorf tube and an equal amount of isopropanol was added and mixed by inverting the tube. The mixture was incubated at − 20 °C for 10 min and centrifuged at 12,000 rpm for 10 min at 4 °C. (4) The supernatant was removed and the pellet re-suspended in 500 μl of SSTE buffer (incubated at 65 °C for 20 min before being used). An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1; v/v/v) was added and mixed by inverting the tube, followed by centrifugation as described above. (5) Step (4) was repeated until the middle tier without any white precipitate was observed. (6) The supernatant was collected an equal amount of isopropanol added, and incubated at − 20 °C for 10 min to precipitate RNA. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. (7) The supernatant was discarded and the pellet washed with chilled 75% ethanol, the volume depending on the pellet amount. The pellet was air dried at room temperature and re-suspended in RNase-free water.

Protocol 3: CTAB method

This protocol was based on the description of Ma (2012).

Protocol 4: Modified CTAB method

This protocol is essentially as described by Protocol 2 but was modified by using CTAB extraction buffer (incubated at 65 °C for 30 min before use) rather than the TRIzol reagent.

Protocol 5: SDS method

This protocol is essentially as described by Ma et al. (2015) but modified by rejecting the use of the spin column in Step 7. Moreover, the mixture was transferred to a 1.5 ml Eppendorf tube and centrifuged at 4 °C for 10 min at 13,000×g. The supernatant was discarded and the pellet washed with chilled 75% ethanol, the volume depending on the pellet amount. The pellet was air dried at room temperature and re-suspended in RNase-free water.

Protocol 6: RNAPrep pure plant kit extraction buffer method (HL and RL)

The RNAPrep pure plant kit was obtained from TIANGEN Biotech Co. Ltd. (Beijing, China), and contained two types of cracking buffer, namely HL and RL. The protocol is similar to Protocol 1 except the TRIzol reagent was replaced with HL or RL.

Protocol 7: CTAB combined with the TRIzol method

This protocol was essentially as described by Peng et al. (2014) but modified by adding an equal amount of isopropanol to precipitate total RNA at – 20 °C for 10 min in Step 3. The solution was centrifuged at 12,000 rpm for 10 min at 4 °C and the pellet collected as in Step 4.

Protocol 8: TRIzol combined with the CTAB method

(1) Seeds (0.1 g) were frozen in liquid nitrogen, pulverized by grinding with a pre-chilled mortar and pestle to a fine powder, added to a 1.5 ml Eppendorf tube containing 1 ml of TRIzol reagent and 20 µl of β-ME, and then vortex mixed. The mixture was incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 5 min at 4 °C. (2) The supernatant was transferred to a 1.5 ml Eppendorf tube and an equal volume of CTAB extraction buffer (incubated at 65 °C for 30 min) and 100 µl of chloroform: isoamylalcohol (24:1; v/v) added. The mixture was mixed by inverting the tube and incubating at room temperature for 5 min. It was then centrifuged at 12,000 rpm for

10 min at 4 °C. (3) The supernatant was transferred to a 1.5 ml Eppendorf tube and an equal amount of phenol: chloroform: isoamylalcohol added. The mixture was mixed by inverting the tube and centrifuged at 12,000 rpm for 5 min at 4 °C. (4) The supernatant was transferred to a 1.5 ml Eppendorf tube and an equal amount of isopropanol added to precipitate total RNA at – 20 °C for 10 min. (5) The supernatant was discarded and the pellet washed with chilled 75% ethanol, the volume depending on the amount of pellets. The pellets were air dried at room temperature and re-suspended in RNase-free water.

Estimation of RNA purity, yield and integrity

Total RNA (2 µl) was loaded on a standard 1.3% agarose gel, stained with GoldView and visualized using Bio-Rad ChemiDoc MP to verify the total RNA integrity. The quantity and quality of the isolated RNA were assessed by observing the absorbance at 260 and 280 nm. The A260/A280 ratio was calculated to determine the purity of the RNA sample. RIN values were determined on an Agilent 2100 bioanalyzer.

RT-PCR

Given that the nucleotide sequences of actin genes in *M. sieboldii* have not been reported, the sequence homology among the genes of interest from *Annona cherimola* Mill. (JN786945), *Betula luminifera* H. Winkl. (FJ410442), *Betula platyphylla* Suk. (EU588981), *Elaeis guineensis* Jacq. (XM_010907399), and *Gossypium hirsutum* Linn. (AY305737), available in the GenBank of the National Center for Biotechnology Information (NCBI), was used. The conserved regions were identified to design the degenerate primers by using the Primer Premier 5.0 software to obtain the sequence of the actin gene in *M. sieboldii* (unpublished). Meanwhile, we designed a pair of specific primers on the basis of the sequences. The sequences were 5'-ATGGCCGAAGAGGATATTCAAC-3' for the forward primer and 5'-CATTTCGGTGCACAA TAGATG-3' for the reverse primer.

The reaction followed the manual of Premix Taq™ (TaKaRa Taq™ Version 2.0 plus dye). After the reaction, 2.5 µl of the assay mixture was used for agarose electrophoresis using 0.5 × TBE buffer following the usual procedure.

Results

Analysis of total RNA isolated with different protocols

The quality of isolated total RNA described in this study was confirmed using several approaches. Agarose gel (1.3%) electrophoresis showed that total RNA was obtained through the protocols described in this article (Fig. 1). However, total RNA extracted with Protocol 3, 4 and 6-HL had visible gDNA contamination (Fig. 1). The total RNA isolated with Protocol 5 and Protocol 7 had dispersive and rayless bands (Fig. 1), although their A260/280 ranged from 1.8 to 2.1 (Table 1), indicating that the total RNA was degraded. Moreover, RNA extracted with Protocol 6-RL also had dispersive and rayless bands. The bands of total RNA extracted with Protocol 1 were intact and bright, indicating that the total RNA was not degraded (Fig. 1) but its A260/A280 was only approximately 1.65 (Table 1). In addition, Nanodrop 2000 (thermo scientific) showed that the figure of RNA extracted with Protocol 1 had abnormal peaks (Fig. S1).

Synthesizing the luminance of ribosomal bands, purity, and yield shows that the total RNA isolated with Protocol 2 and Protocol 8 indicates intact and bright bands (Fig. 1), with a A260/A280 ratio of more than 1.7. The total RNA isolated with Protocol 8 was better and could reach approximately 1.9, and had highest yield (Table 1). In addition, results from the Agilent 2100 Bioanalyzer showed that RINs can exceed 8.0 (Table 1 and Fig. 2). This finding indicates that high-quality RNA may be obtained through Protocol 8 and that total RNA extracted with Protocol 2 was also of high quality but still need to be improved.

Optimization of Protocol 2

Although the RIN of the total RNA extracted with Protocol 2 could reach more than 8.0, its A260/A280 was less than 1.80 (Table 1). The ratio was low, which suggests that the

purity of RNA extracted with the method was poor. Therefore, we optimized the design for Protocol 2.

SSTE buffer S1 to S10 and S1 to S9 were tested using the L_9 (3^4) orthogonal test (Table 2) applied according to different concentrations of SDS, Tris-HCl, EDTA, and NaCl in the extraction solutions. S10 was the optimal SSTE buffer (Table 2). Each test was repeated three times. The SPSS17.0 software was used for ANOVA.

Distinct 28S, 18S, and 5S ribosomal bands were observed for S1–S9 when the total RNA was separated on a 1.3% agarose gel stained with Goldview. However, each test showed relative protein or polysaccharide contamination (Fig. 3). Moreover, the use of different components of SSTE buffer resulted in remarkable differences in A260/A280 but not for yield (Table 2). Intuitive analysis (Table S1) shows that the most optimal SSTE buffer consisted of 2 M NaCl, 0.05 M Tris-HCl (pH 8.0), 1 μ M EDTA, and 1% SDS. High-quality RNA was obtained using the optimal SSTE buffer in Protocol 2. The RNA samples showed well-resolved 28S, 18S, and 5S rRNA bands with no visible signs of degradation (Figs. 3 and S10). A260/A280 reached 1.8 (Table 2 and S10) and RIN was more than 8.0 (Table 2 and Fig. 3), which were suitable for transcriptome and small RNA sequencing, RT-PCR, qRT-PCR, and other downstream molecular applications.

Evaluation of RT-PCR for Protocol 8 and optimized Protocol 2

Total RNA extracted with Protocol 8 and optimized Protocol 2 was further assessed using RT-PCR. RNA samples were successfully reverse-transcribed and the target gene amplified from cDNA by PCR. The PCR products were checked for the presence of the objective band on 1.2% agarose gel (Fig. 4). PCR products for detecting the actin gene exhibited the target band with the expected size of approximately 1124 bp (Fig. 4).

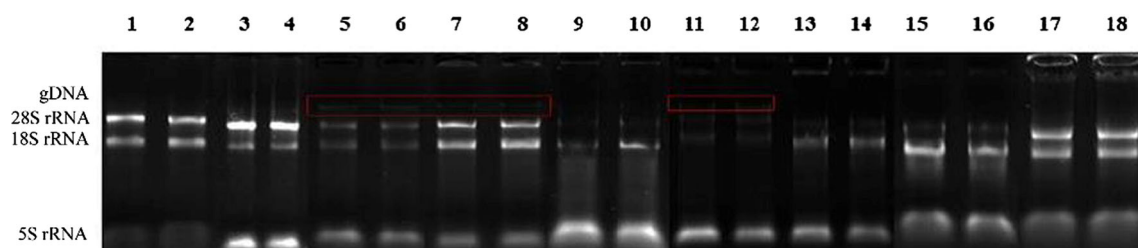


Fig. 1 Total RNA isolated using different protocols, separated on an agarose gel (1.2%). Lanes 1 and 2: Protocol 1; lanes 3 and 4: Protocol 2; lanes 5 and 6: Protocol 3; lanes 7 and 8: Protocol 4; lanes 9 and 10:

Protocol 5; lanes 11 and 12: Protocol 6-HL; lanes 13 and 14: Protocol 6-RL; lanes 15 and 16: Protocol 7; lanes 17 and 18: Protocol 8

Table 1 Test result of total RNA extracted under different protocols

Lanes	Protocol	A260/A280	Highest RIN	Yield ($\mu\text{g/g}$)
1, 2	1. TRIzol method	1.65 ± 0.02 Dd	–	37 ± 0.8 CDd
3, 4	2. Modified TRIzol method	1.68 ± 0.03 Ccd	8.4	110 ± 20.9 Bb
5, 6	3. CTAB method	1.90 ± 0.05 Bb	6.9	156 ± 20.9 Ccd
7, 8	4. Modified CTAB method	1.85 ± 0.09 BCbc	6.7	83 ± 8.9 Dd
9, 10	5. SDS method	1.88 ± 0.04 Bbc	–	114 ± 1.3 Bb
11, 12	6. HL	–	–	–
13, 14	6. RL	–	–	–
15, 16	7. CTAB combined with the TRIzol method	2.05 ± 0.03 Aa	–	162 ± 10.8 Aa
17, 18	8. TRIzol combined with the CTAB method	1.89 ± 0.01 Bb	8.6	214 ± 13.4 Bbc

Results are expressed as the averages of three samples \pm standard errors. Different lowercase and capital letters in the same column indicate significant differences at $p \leq 0.01$ and $p \leq 0.05$, respectively. “–” means the RNA sample was of poor quality as shown by agarose electrophoresis and was not tested

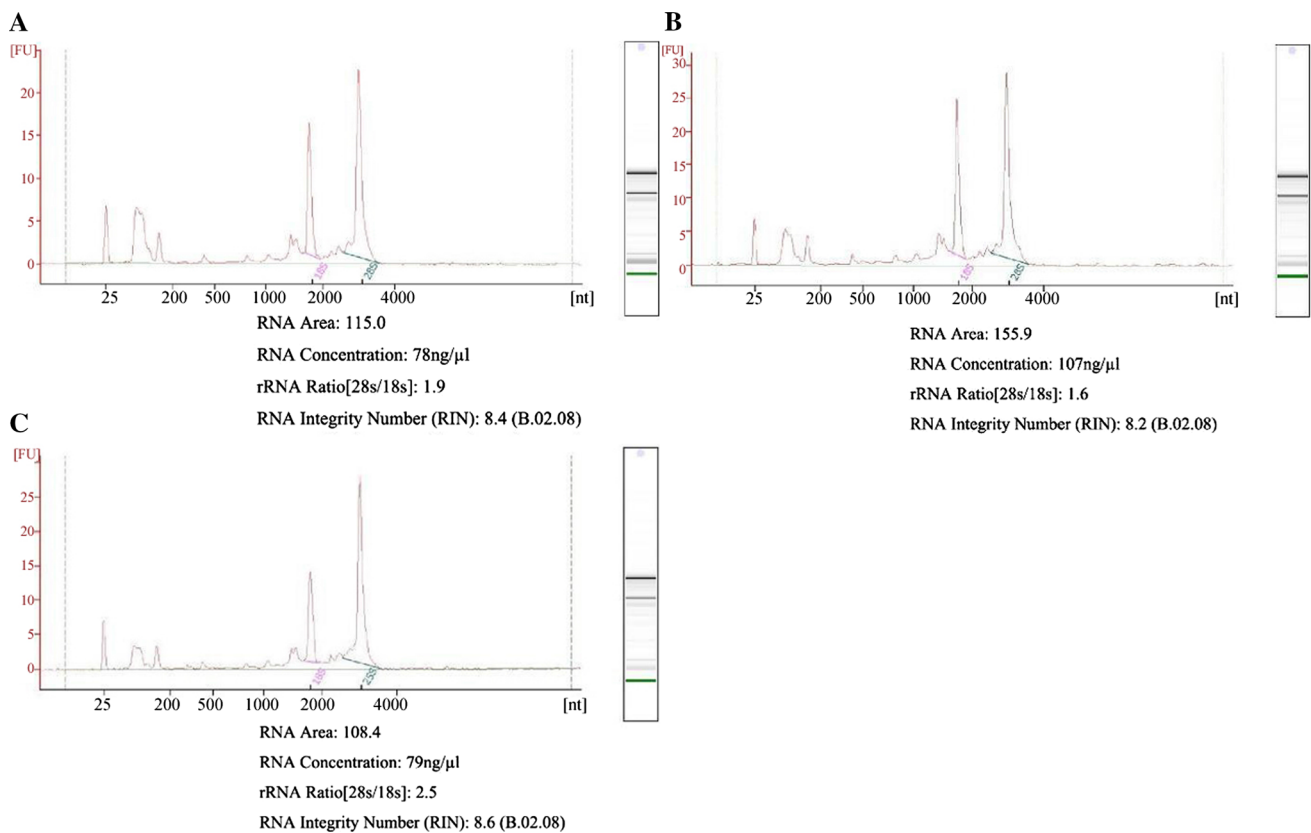


Fig. 2 Analyzed total RNA extracted with different protocols in the Agilent 2100 Bioanalyzer. **a** Protocol 2 (Test results after 5 times dilution); **b** Optimized Protocol 2 (Test results after 5 times dilution); **c** Protocol 8 (Test results after 7 times dilution)

Extraction of RNA from other tissues of *M. sieboldii* using Protocol 8 and Optimized Protocol 2

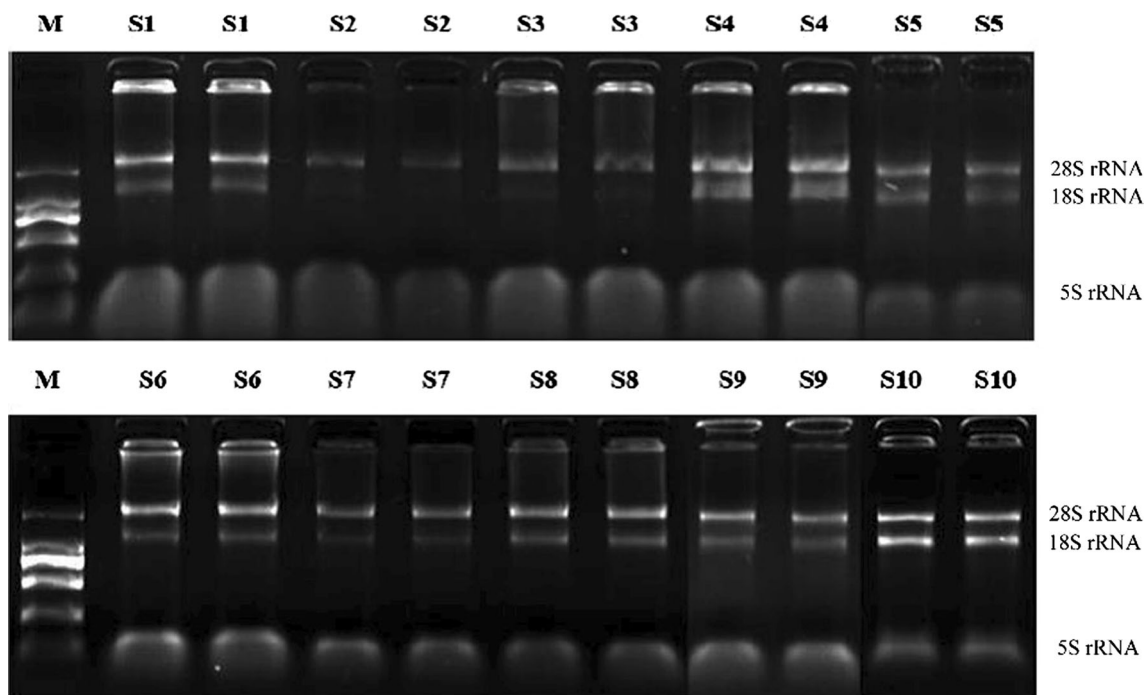
The RNA extracted from other tissues of *M. sieboldii* (Fig. S3) was assessed by the sharpness of ribosomal RNA (rRNA) bands visualized on a 1.0% agarose gel. RNA extracted with Protocol 8 had visible signs of degradation

(Fig. 5) whereas well-resolved 28S and 18S rRNA bands were observed with no visible signs of degradation for RNA extracted with the Optimized Protocol 2 from young roots, stems, cotyledons, and terminal buds. This finding shows that the Optimized Protocol 2 has a better applicability for other tissues from *M. sieboldii* than Protocol 8.

Table 2 Test result of total RNA with different SSTE buffers in the modified TRIzol method

SSTE buffer	Composition and concentration				Ratio of A 260/A280	Yield ($\mu\text{g/g}$)	Highest RIN
	SDS (W/V, %)	Tris-HCl ($\mu\text{mol/L}$)	EDTA ($\mu\text{mol/L}$)	NaCl (mol/L)			
S1	0.5	10	1	1	1.58 ± 0.03 AaBbC	109 ± 23.7 Aa	8.4
S2	0.5	30	3	1.5	1.38 ± 0.06 Cb	101 ± 7.8 Aa	7.0
S3	0.5	50	5	2	1.42 ± 0.01 BaCa	148 ± 28.6 Aa	6.2
S4	1	10	3	2	1.62 ± 0.03 AaBb	109 ± 39.2 Aa	8.0
S5	1	30	5	1	1.46 ± 0.04 BbCa	168 ± 88.1 Aa	7.5
S6	1	50	1	1.5	1.52 ± 0.01 AaBb	132 ± 3.0 Aa	7.8
S7	1.5	10	5	1.5	1.47 ± 0.04 BbCa	99.8 ± 11.0 Aa	8.1
S8	1.5	30	1	2	1.62 ± 0.02 AaBb	72 ± 11.2 Aa	7.6
S9	1.5	50	3	1	1.58 ± 0.01 AaBbC	133 ± 4.8 Aa	7.9
S10	1	50	1	2	1.78 ± 0.04 Aa	155 ± 27.5 Aa	8.2

Results are expressed as the averages of three samples \pm standard errors. Different lowercase and capital letters in the same column indicate significant differences at $p \leq 0.01$ and $p \leq 0.05$, respectively

**Fig. 3** Total RNA extracted with different SSTE buffers in Protocol 2, separated on an agarose gel (1.2%)

Discussion and conclusions

The method for high-quality RNA extraction is difficult to validate in molecular biological research on woody plants. *M. sieboldii* seeds are characterized by deep dormancy and contain complex metabolites. Extraction of its RNA is challenging. We employed eight types of protocols which cover CTAB, SDS, TRIzol and two types of pyrolysis buffer of the RNeasy pure plant kit, to extract RNA from *M. sieboldii* seeds. We found that the individual application

of each method failed to obtain high-quality RNA which confirmed the difficulty in extracting RNA from this seed.

RNA extraction from plant tissues has been extensively investigated. However, many of these methods are sample-dependent because of the presence of a diverse array of metabolites among different plant tissues. By contrast, the TRIzol method is frequently used to extract total RNA from plant tissues (An et al. 2015; Liu et al. 2014; Hackenberg et al. 2015). Nevertheless, in this experiment, we utilized TRIzol reagent to crack tissue cells, extract

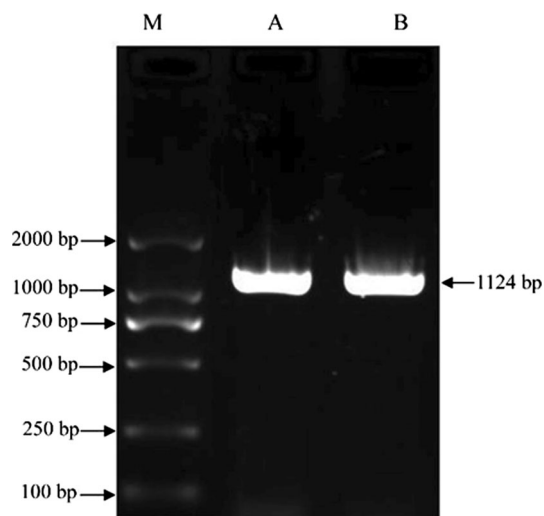
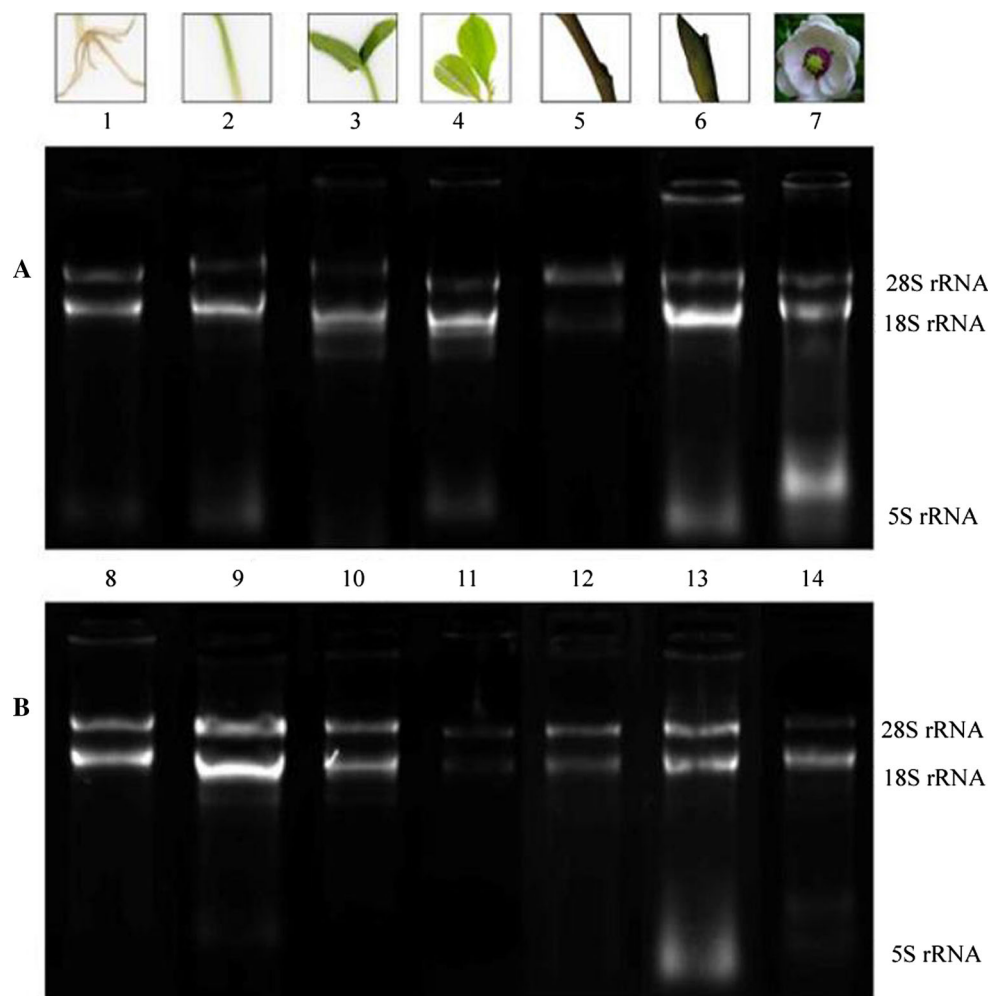


Fig. 4 Analysis of agarose gel electrophoresis of the RT-PCR product. M: marker (2000 bp); lane A: RT-PCR product of total RNA isolated with Protocol 8; lane B: RT-PCR product of total RNA isolated with Optimized Protocol 2

chloroform: isoamyl alcohol, and precipitate isopropanol precipitation (Protocol 1). We found that a considerable amount of white insoluble substance precipitated together with RNA (Fig. S2). However, agarose gel electrophoresis using the turbid RNA sample also showed that the bands were intact and bright (Fig. S2) with a low A260/A280 ratio of approximately 1.6. This demonstrates that the integrity of the RNA sample extracted with the TRIzol reagent was satisfactory but its purity was poor. This may be related to *M. sieboldii* seeds as the leaves and flowers contain more than 20 types of aromatic oils and numerous terpenoids (Wu et al. 2010; Lim et al. 2002). Moreover, seeds often contain a highly complex chemical composition and the chemical properties of some components were similar to RNA, rendering the isolation of high-quality RNA difficult. Li et al. (2011) also obtained similar findings when they extracted total RNA from lily bulbs using the TRIzol method and suspected that the white insoluble substance may be polysaccharides. The A260/A280 ratio of the inferior RNA sample greatly improved after re-sus-

Fig. 5 Total RNA from different tissues of *M. sieboldii*, separated on an agarose gel (1.2%). **a** Gel electrophoresis of total RNAs extracted with Protocol 8. **b** Gel electrophoresis of total RNAs extracted with the Optimized Protocol 2. Lanes 1 and 8: young root; lanes 2 and 9: young stem; lanes 3 and 10: cotyledon; lanes 4 and 11: young leaf; lanes 5 and 12: annual branch; lanes 6 and 13: terminal bud; lanes 7 and 14: petal



pension in SSTE buffer and subsequent extraction (Protocol 2 and Optimized Protocol 2). We found that a high concentration of NaCl is beneficial to obtain high-quality RNA in SSTE buffer (Table S1).

Commercial kits have also been used in addition to the TRIzol method and are convenient but display poor applicability for tissues rich in secondary metabolites. In this experiment, no high-quality total RNA was obtained using the extraction buffer HL or RL (Protocol 6) of the RNAPrep pure plant kit from TIANGEN Biotech Co. Ltd., and visible gDNA contamination was even observed using HL extraction buffer (Fig. 1). The use of SDS extraction buffer to crack cells and high-concentration NaCl (Protocol 5) also did not result in the procurement of high-quality RNA. This indicates that SDS is not suitable for the RNA extraction of *M. sieboldii* seeds.

The test results show that the use of CTAB (Protocol 3, Protocol 4, Protocol 7 and Protocol 8) consistently led to a high ratio of A260/A280 (Table 1). Thus, CTAB can remove polysaccharides, polyphenols, grease, and other metabolic substances of *M. sieboldii* seeds. However, visible gDNA contamination was often observed (Fig. 1; Protocol 3 and Protocol 4). Small RNA will be lost together with gDNA if LiCl is used to specifically precipitate RNA (Peng et al. 2014). Meanwhile, the cost will increase and RNA yields may be reduced if DNase is used to digest gDNA. Nevertheless, we attempted to find a low-cost method to acquire high-quality total RNA and analyze mRNA and small RNAs, including microRNA. This method can also be used in other downstream molecular applications. Thus, we used isopropanol rather than LiCl to precipitate total RNA.

Considering the advantages and disadvantages of TRIzol and CTAB, we attempted to combine both methods to overcome the drawbacks of both protocols. High-quality total RNA was not obtained using CTAB with the TRIzol method (Protocol 7) but by using TRIzol combined with the CTAB method (Protocol 8), in which the order of the two reagents was reversed. This phenomenon is probably due to the fact that some metabolites may have remained in the supernatant after centrifugation using CTAB extraction buffer to crack tissues which participate in and hinder the subsequent separation steps. However, these influential metabolites could have been removed using TRIzol reagents to crack tissues first, laying a foundation for subsequent separation. This mechanism needs further validation.

In conclusion, we found two simple, low-cost RNA extraction methods (Protocol 8 and Optimized Protocol 2) for *M. sieboldii* seeds. The methods efficiently yielded high-quality RNA. TRIzol combined with the CTAB method (Protocol 8) was more convenient, simple, and stable than the optimized modified TRIzol method

(Optimized Protocol 2). However, the latter is also a good choice and has better applicability for other tissues than the former. High-quality total RNA with high purity and good integrity was obtained through the two protocols. Its A260/A280 and RIN could exceed 1.8 and 8.0 which are suitable for transcriptome sequencing, small RNA sequencing, RT-PCR, qRT-PCR, and other downstream molecular applications. Thus these protocols may be used by future transcriptome studies.

Acknowledgements We thank Beijing Biomarker Technologies Co. Ltd. for technology support.

References

- An WY, Gong WF, He SP, Pan ZE, Sun JL, Du XM (2015) MicroRNA and mRNA expression profiling analysis revealed the regulation of plant height in *Gossypium hirsutum*. *BMC Genom* 16:886
- Christou A, Georgiadou EC, Filippou P, Manganaris GA, Fotopoulos V (2014) Establishment of a rapid, inexpensive protocol for extraction of high quality RNA from small amounts of strawberry plant tissues and other recalcitrant fruit crops. *Gene* 537:169–173
- Gupta OP, Sharma P, Gupta RK, Sharma I (2014) Current status on role of miRNAs during plant–fungus interaction. *Physiol Mol Plant Pathol* 85:1–7
- Hackenberg M, Gustafson P, Langridge P, Shi BJ (2015) Differential expression of microRNAs and other small RNAs in barley between water and drought conditions. *Plant Biotechnol J* 13:2–13
- Klevebring D, Street NR, Fahlgren N, Kasschau KD, Carrington JC, Lundeberg J, Jansson S (2009) Genome-wide profiling of populus small RNAs. *BMC Genom* 10:620
- Li XY, Wang CX, Sun HM, Li TL (2011) Establishment of the total RNA extraction system for lily bulbs with abundant polysaccharides. *Afr J Biotech* 10(78):17907–17915
- Li JY, Reichel M, Li YJ, Millar AA (2014) The functional scope of plant microRNA-mediated silencing. *Trends Plant Sci* 19(12):750–756
- Lim SS, Shin KH, Ban HS, Kim YP, Jung SH, Kim YJ, Ohuchi K (2002) Effect of the essential oil from the flowers of *Magnolia sieboldii* on the lipopolysaccharide-induced production of nitric oxide and prostaglandin E2 by rat peritoneal macrophages. *Planta Med* 68(5):459–462
- Liu HJ, Qin C, Chen Z, Zuo T, Yang XR, Zhou HK, Xu M, Cao S, Shen YO, Lin HJ, He XJ, Zhang YC, Li LJ, Ding HP, Lübberstedt T, Zhang ZM, Pan GT (2014) Identification of miRNAs and their target genes in developing maize ears by combined small RNA and degradome sequencing. *BMC Genom* 15:25
- Lu XJ, Zhang XL, Liu GL, Li TL (2013) Polyethylene glycol fraction analysis of low-abundant proteins in *Magnolia sieboldii* seeds. *Sci Silvae Sin (China)* 49(11):189–193
- Lu XJ, Zhang XL, Mei M, Liu GL, Ma BB (2016) Proteomic analysis of *Magnolia sieboldii* K. Koch seed germination. *J Proteom* 133:76–85
- Ma WL (2012) Nucleic acid extraction and purification experiment guide. Chemical Industry Publishing, China

- Ma ZH, Huang BL, Xu SS, Chen Y, Li SB, Lin SZ (2015) Isolation of high-quality total RNA from Chinese Fir (*Cunninghamia lanceolata* (Lamb.) Hook). PLoS ONE 10(6):e0130234
- Peng J, Xia ZH, Chen L, Shi MJ, Pu JJ, Guo JR, Fan ZF (2014) Rapid and efficient isolation of high-quality small RNAs from recalcitrant plant species rich in polyphenols and polysaccharides. PLoS ONE 9(5):e95687
- Wu D, Wang RP, Song SJ, Wu LJ, Gao HY (2010) A new isoquinoline derivative from the leaves of *Magnolia sieboldii* K. Koch. Chin Chem Lett 21(12):1446–1448
- Zhang XL, Liu GL, Li TL, Qi MF, Mei M, Lu XJ (2014) Differential proteome analysis of mature and germinated seeds of *Magnolia sieboldii* K. Koch. Trees 28(3):859–870