

# Development of an Efficient Protocol of RNA Isolation from Recalcitrant Tree Tissues

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**Abstract** Isolation of RNA from recalcitrant tree tissues has been problematic due to large amounts of secondary metabolites and interfering compounds in their cells. We have developed an efficient RNA extraction method, which yielded high-quality RNA preparations from tissues of the lychee tree. The method reported here utilized EDTA, LSS, and CTAB to successfully inhibit RNase activities. It was found that a high ionic strength brought about by 2 M NaCl was necessary. In addition, secondary metabolites and other interfering compounds were effectively removed using sodium borate and PVPP under a deoxidized condition. The quality of purified RNA was tested by both RACE and Northern blotting analysis, ensuring that the RNA could be used for subsequent gene expression analysis. This method has been successfully applied to purify RNA from 15 other plant species. In conclusion, the protocol reported here is expected to have excellent applications for RNA isolation from recalcitrant plant tissues.

**Keywords** Lychee · Recalcitrant plant tissues · RNA extraction method · Trees

## Abbreviations

CMO Choline monooxygenase  
CTAB Cetyltrimethylammonium bromide

DEPC Diethylpyrocarbonate  
EDTA Ethylenediaminetetraacetic acid  
LSS *N*-lauroyl sarcosine sodium  
PVPP Polyvinylpyrrolidone  
RACE Rapid amplification of cDNA ends

## Introduction

Isolation of high-quality RNA from plants is critical for subsequent molecular experiments such as reverse transcription polymerase chain reaction (RT-PCR), RACE, Northern hybridization, and microarray analysis [2, 9, 21, 25, 26]. RNA isolation from recalcitrant plant tissues has been problematic due to the presence of the rigid cell walls, large amounts of tannins [12], pigments [14], polysaccharides [15, 19], polyquinones [2, 19, 20], or other secondary metabolites [6, 11]. Such interfering compounds often coprecipitate with RNA, thereby affecting the yield and quality of RNA purification [2, 17, 27, 31]. Although common methods have been developed for total RNA extraction from plant tissues, they are often not applicable for a wide range of plant species, especially for woody plants [2, 27].

We wanted to extract high-quality RNA from Lychee (*Litchi chinensis* Sonn.), an important tropical and subtropical fruit tree. By modifying and combining the techniques that address the aforementioned problems simultaneously, we have developed a protocol to successfully isolate RNA from many tissues of lychee. The method has also been tested for RNA extraction from tissues of other 15 plant species, including several woody plants. Based on satisfactory results of RACE and Northern blotting, RNA extracts prepared by this method were concluded to be intact and suitable for the subsequent gene expression analysis.

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## Materials and Methods

### Plant Materials

Plant materials used in this study were listed in Table 1, including different organs and tissues from 16 plant species in 12 families of nine herbaceous species and seven woody plants. Young leaf, leaf, and old leaf were defined as the first half-expanded trifoliate leaf from the apical meristem, the matured one, and the fifth fully expanded leaf from the tip, respectively. The herbaceous plants were grown in a greenhouse at a thermo period of  $25/20 \pm 2^\circ\text{C}$  of day/night temperatures, a photoperiod of 16 h, and a relative humidity of  $50 \pm 10\%$ . The temperate trees and the tropical trees were grown under natural condition in Beijing Botanical Garden and Hainan Tropical Botanical Garden, respectively. Seedlings (30 days old) of halophytic plant *Salicornia europaea* grown on vermiculite were irrigated weekly with half-strength Hoagland nutrient solutions containing 0 (control), 200 or 600 mM NaCl for 1 month, respectively. Then, the shoots were sacrificed for total RNA isolation. All the plant tissues were collected, frozen immediately, and subsequently ground into fine powders in liquid nitrogen using a mortar and a pestle. The resulting tissue powders were stored at  $-80^\circ\text{C}$  until use.

### RNA Isolation Protocol

One gram of plant tissues (lyophilized powders or fresh materials) plus 1% (w/w) quartz sand was ground sufficiently at room temperature in 5 ml extraction buffer containing 2 M NaCl, 25 mM EDTA, 200 mM Tris (pH 8.0), 20 mM sodium borate, 2% PVPP (w/v), 2% CTAB (w/v), 1% LSS (w/v), and 2%  $\beta$ -mercaptoethanol (v/v). Note that the buffer, stored at room temperature, was a viscid fluid and should be stirred sufficiently to dissolve precipitated ingredients prior to each use. The homogenate was then transferred into a sterile disposable polypropylene tube, vortexed vigorously, and incubated at  $65^\circ\text{C}$  in a water-bath shaker over 10 min with occasional shaking. Add 6 ml of chloroform/isoamyl alcohol (24:1, v/v) into the mixture, and then vortexed, followed by centrifuging at 15,000g for 15 min at  $4^\circ\text{C}$ . The resulting upper aqueous phase was transferred into another tube containing 10 ml of water-saturated phenol/chloroform/isoamyl alcohol (25:24:1, v/v, pH 4.5). Following vortexing, the sample was then centrifuged at 12,000g for 10 min at  $4^\circ\text{C}$ . The above step was repeated until a clean interface was observed.

The upper phase containing RNA extract was extracted with 8 ml chloroform/isoamyl alcohol (24:1, v/v) and centrifuged at 12,000g for 10 min at  $4^\circ\text{C}$ . After the upper phase was transferred into another centrifuge tube, a half volume

(2–3 ml) of 8 M LiCl (Sigma, St. Louis, MO, USA) was added into the tube. The mixture was incubated at  $-20^\circ\text{C}$  for at least 1 h or sometimes overnight, followed by centrifugation at 15,000g for 15 min at  $4^\circ\text{C}$ . Decant the supernatant immediately. The pellet was rinsed with 75% ethanol and collected by centrifugation at 12,000g for 5 min at  $4^\circ\text{C}$  for three times. The air-dried pellet was finally dissolved in 50  $\mu\text{l}$  of DEPC-treated water and stored at  $-80^\circ\text{C}$ .

Note when RNA was used for reverse transcription analysis, the final pellet was dissolved in 200  $\mu\text{l}$  of TE containing 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA, and precipitated again with 600  $\mu\text{l}$  absolute ethanol and 10  $\mu\text{l}$  3 M NaAc solution (pH 5.2) at  $-20^\circ\text{C}$  for at least 30 min, followed by centrifugation at 15,000g for 15 min at  $4^\circ\text{C}$ .

### RNA Quality Analysis

The quality and quantity of the RNA extracts were assessed spectrophotometrically at 260 and 280 nm as described previously [25]. In order to verify RNA integrity, 10  $\mu\text{g}$  of total RNA was subjected to gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light.

### Rapid Amplification of cDNA Ends for the Gene Encoded a 22 kDa Protein

The N-terminal amino acid residues of a 22 kDa protein in lychee were determined by Edman stepwise degradation using Applied Biosystem Procise-PROCISE491 (ABI, USA), as N-ASE PLY DIN GDE VIT GTE YY-C. The 3'RACE degenerate primers based on the above sequence were 5-TA (CT) AT (TCA) AA (CT) GGIG A (TC) GA (AG) GTI AT-3. The adapted primer was 5-AAG CAG TGG TAA CAA CGC AGA GTA CTT TTT TTT TTT TTT TTT TTT TTT T-3. Total RNA was extracted from phloem and xylem of lychee, and the 3'RACE and 5'RACE were carried out according to the manufacturer's specifications (RACE Kit, TaKaRa Biotechnology, Dalian, China). Finally, specific primers were designed according to the 3'RACE and 5'RACE sequences to clone the full-length cDNA.

### RNA Gel Blotting Analysis

Northern blotting was carried out according to Malnoy and co-workers [18]. Approximately 15  $\mu\text{g}$  of total RNA was separated in 1.5% agarose gel containing 1.8% formaldehyde, and transferred to a nylon membrane. The blot was hybridized at  $65^\circ\text{C}$  overnight, and then the hybridized membrane was washed twice with  $1\times$  SSC, 0.1% SDS for

**Table 1** Purity and yield of total RNA extracted from different plant species and tissues

Families	Genera	Plant species	Tissues	Purity ( $A_{260/280}$ )	Yield ( $\mu\text{g/g}$ )
Cruciferae	<i>Arabidopsis</i>	<i>Arabidopsis thaliana</i> L.	Leaf	2.15 $\pm$ 0.13	387 $\pm$ 121
			Leaf, callus	2.09 $\pm$ 0.08	323 $\pm$ 87
			Root, callus	2.13 $\pm$ 0.06	288 $\pm$ 65
Solanaceae	<i>Nicotiana</i>	<i>Nicotiana tabacum</i> L.	Leaf	2.10 $\pm$ 0.22	298 $\pm$ 69
			<i>Lycopersicon</i>	<i>Lycopersicon esculentum</i> M.	Leaf
Graminae	<i>Oryza</i>	<i>Oryza sativa</i> L.	Leaf	1.89 $\pm$ 0.17	158 $\pm$ 37
			<i>Triticum</i>	<i>Triticum aestivum</i> L.	Leaf
Malvaceae	<i>Gossypium</i>	<i>Gossypium hirsutum</i> L.	Leaf	1.78 $\pm$ 0.15	176 $\pm$ 67
Compositae	<i>Taraxacum</i>	<i>Taraxacum officinale</i> Weber	Leaf	1.94 $\pm$ 0.22	313 $\pm$ 85
Chenopodiaceae	<i>Salicornia</i>	<i>Salicornia europaea</i> L.	Stem	2.13 $\pm$ 0.15	325 $\pm$ 93
			Root	2.07 $\pm$ 0.28	128 $\pm$ 55
Pinaceae	<i>Pinus</i>	<i>Pinus bungeana</i> Zucc.	Needle	2.01 $\pm$ 0.22	138 $\pm$ 46
Musaceae	<i>Musa</i>	<i>Musa nana</i> Lour.	Leaf	2.10 $\pm$ 0.10	156 $\pm$ 33
Salicaceae	<i>Populus</i>	<i>Populus canadensis</i> Moench	Leaf	2.06 $\pm$ 0.10	333 $\pm$ 58
			Phloem	1.82 $\pm$ 0.09	258 $\pm$ 49
			Xylem	1.98 $\pm$ 0.13	289 $\pm$ 75
Euphorbiaceae	<i>Hevea</i>	<i>Hevea brasiliensis</i> Mull.	Leaf	2.14 $\pm$ 0.09	312 $\pm$ 58
			Phloem	2.05 $\pm$ 0.11	282 $\pm$ 96
			Xylem	2.12 $\pm$ 0.24	235 $\pm$ 86
Meliaceae	<i>Swietenia</i>	<i>Swietenia macrophylla</i> King	Leaf	2.11 $\pm$ 0.25	313 $\pm$ 106
			Phloem	1.82 $\pm$ 0.18	254 $\pm$ 88
			Xylem	1.98 $\pm$ 0.21	285 $\pm$ 65
Sapindaceae	<i>Sapindus</i>	<i>Sapindus mukorossi</i> Gaertn	Leaf	2.01 $\pm$ 0.07	284 $\pm$ 104
			Phloem	2.05 $\pm$ 0.09	198 $\pm$ 69
			Xylem	2.11 $\pm$ 0.11	195 $\pm$ 25
Sapindaceae	<i>Dimocarpus</i>	<i>Dimocarpus congan</i> L.	Leaf	1.98 $\pm$ 0.31	184 $\pm$ 67
			Phloem	1.75 $\pm$ 0.29	154 $\pm$ 68
			Xylem	1.80 $\pm$ 0.25	179 $\pm$ 48
Sapindaceae	<i>Litchi</i>	<i>Litchi chinensis</i> Sonn.	Leaf	2.08 $\pm$ 0.25	285 $\pm$ 123
			Flower	1.65 $\pm$ 0.21	58 $\pm$ 32
			Pericarp	1.74 $\pm$ 0.32	83 $\pm$ 43
			Phloem	1.85 $\pm$ 0.30	108 $\pm$ 55
			Xylem	1.86 $\pm$ 0.16	145 $\pm$ 48

Note: The RNA yield was calculated based on the following formula: yield =  $A_{260} \times 40 \times 1,000 \times \text{volume} (\mu\text{l}) / \text{material weight} (\text{g})$ . Each value indicated was the mean of 3 ( $n = 3$ ) replicates recorded  $\pm$ SE, and the statistic analysis was performed by the software SPSS 10.0

15 min, and twice with 0.1 $\times$  SSC, 0.1% SDS for 20 min at 65°C. BASTation (Fuji Film) was used for visualizing the blot.

## Results

### Purity and Yield of RNA from Different Plant Species and Tissues

The yield and purity of RNA isolated from different tissues of 16 plant species by our method were listed in Table 1.

The average  $A_{260/280}$  ratio and yield of the RNA isolated from leaves of these species were around 2.02 and 259  $\mu\text{g/g}$ , respectively. For the six tree species, the average purity and yield values of the phloem were 1.89 and 209  $\mu\text{g/g}$ , respectively. However, higher values from the xylem (1.97 and 221  $\mu\text{g/g}$ , respectively) were obtained than from the phloem (Table 1).

In general, our protocol resulted in purer and higher yield of RNA from the herbaceous plants than the woody plants (Table 1). High-purity RNA was also isolated from some particular organs and tissues, such as the callus of *Arabidopsis thaliana* L., roots and shoots of *Salicornia*

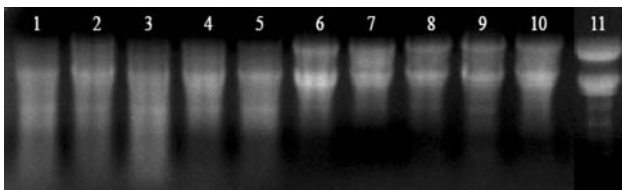
*europaea* L., needles of *Pinus bungeana* Zucc, the flowers and pericarps of *L. chinensis*. Fine quality RNA had also been obtained from several subtropical and tropical trees, such as *H. brasiliensis*, *Swietenia macrophylla* King, *Swietenia mukorossi* Gaertn, *Dimocarpus congan* L., *L. chinensis*, indicating the suitability of this method for recalcitrant tree tissues. Among them, the flower and fruit tissues of lychee were the most problematic materials (Table 1).

In some cases, the  $A_{260/280}$  ratio reached about 2.1 (Table 1), indicating low concentration of proteins in the RNA extracts. In addition, the  $A_{230}$  value was also determined, and the  $A_{260/230}$  ratio was ranged from 2.0 to 2.8 (data not shown), indicating little contamination of salt ions and polysaccharides.

#### Comparison of Total RNA from Barks of Lychee by Different Methods

Using our method, the RNA isolated from barks of the terminal branches in *L. chinensis* was compared to those extracted by other 10 standard methods (Fig. 1, lanes 1–10), including three commercial kits (Fig. 1, lanes 1, 2, and 9). The electrophoresis results demonstrated that the Trizol reagent (Fig. 1, lane 1) and RNAout kit (Fig. 1, lane 2) could produce partially degraded RNA.

Although the RNA extracts generated by Rneasy Plant Mini Kit (Fig. 1, lane 9) and the improved CTAB methods (Fig. 1, lanes 7, 8, and 10) resulted in superior results than others, the commonly used approaches (Fig. 1, lanes 3–8 and 10) could hardly render high-quality RNA from this species. On the contrary, our protocol was successful in recovering intact RNA from barks of *L. chinensis* as the 28S and 18S ribosomal RNA (rRNA) bands were clearly visible without apparent degradation (Fig. 1, lane 11). The aforementioned comparative results indicated that our



**Fig. 1** Comparison of RNA extracted by different methods from barks of lychee. Lanes 1–11, total RNA isolated by 11 different methods or commercial kits from the bark of 2-year-old branches in 20-year-old *L. chinensis*. Lane 1, Trizol kit from Invitrogen, USA; lane 2, RNAout kit from Huass, China; lane 3, guanidinium isothiocyanate method [5]; lane 4, acid phenol extraction method [8]; lane 5, improved hot borate method [28]; lane 6, SDS-phenol method [18]; lane 7, CTAB precipitation method [10]; lane 8, SDS and CTAB method [6]; lane 9, Rneasy Plant Mini Kit from Qiagen, USA; lane 10, CTAB-DNase method [13]; and lane 11, our method

method was the preferred protocol for isolating RNA from recalcitrant tree tissues.

#### RNA Extracted from Several Subtropical and Tropical Trees

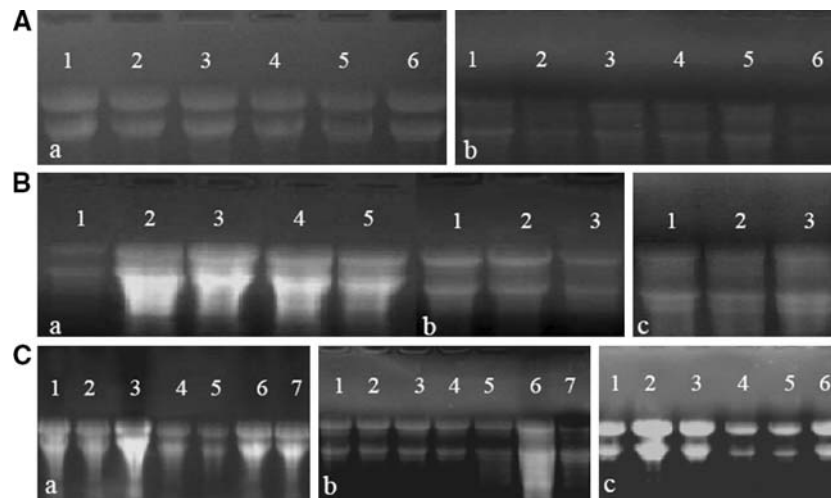
We have also attempted to isolate total RNA from different tissues of several subtropical and tropical trees as shown by the electrophoresis profiles (Fig. 2A–C). For lychee tree, the quality of different RNA samples prepared from the same tissues in different months demonstrated no significant difference (Fig. 2A, lanes 1–6 of gels a, and b). But the RNA sample obtained from the phloem of terminal branches (Fig. 2A, lanes 1–6 of gel a) were more intact than that from the xylem (Fig. 2A, lanes 1–6 of gel b). As seen in Fig. 2, the RNA extracts of the xylem was degraded to some extent (Fig. 2A, lanes 2–6 of gel b).

Furthermore, the electrophoresis results also confirmed that this method was suitable for many tropical trees (Fig. 2B, gels a, b, and c). The RNA extracts obtained from the phloem (Fig. 2B, lane 1 of gel b) and xylem (Fig. 2B, lane 2 of gel b) of *S. macrophylla* had high quality. However, the RNA samples obtained from some older tissues of *L. chinensis* (Fig. 2B, lanes 4 and 5 of gel a; Fig. 2A, lanes 4–6 of gel b), especially pericarps (Fig. 2B, lane 1 of gel a), were somewhat degraded.

Among these tropical trees, *H. brasiliensis* rendered the highest quality RNA. As shown in Table 1, both the purity and yield of RNA from this species were higher than those obtained from other subtropical and tropical trees (Table 1). The electrophoresis results also support the same conclusion, as the rRNA bands were distinct and the gel's background was clear. Additionally, the gels around the loading wells were very clean, indicating minimal contamination from DNA in the RNA extracts (Fig. 2C, gels a, b, and c). With the development of new twigs, RNA isolation from the xylem (Fig. 2C, lanes 1–7 of gel b) became more challenging, whereas there was no obvious difference for the phloem (Fig. 2C, lanes 1–7 of gel a) and leaf tissues (Fig. 2C, lanes 1–6 of gel c).

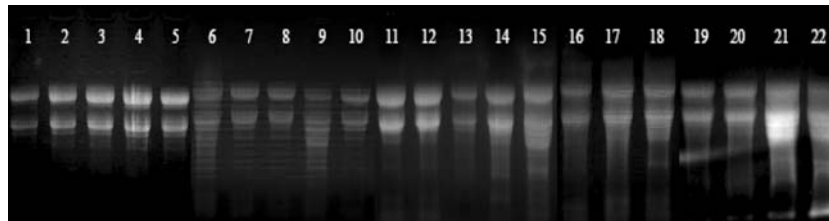
#### RNA Isolated from Some Herbaceous Plants and Temperate Trees Including Several Model Plants

RNA extracted from 12 plant species demonstrated distinct rRNA bands as shown in Fig. 3 (Fig. 3, lanes 1–22). Furthermore, the 28s rRNA band was equal to or more abundant than the 18s rRNA band (Fig. 3, lanes 1–22), indicating the isolated RNA was likely intact. Several main bands, as well as the 28S and 18S rRNA bands, were clearly visible without smearing in most samples (Fig. 3,



**Fig. 2** RNA isolated from several subtropical and tropical trees by our method. **A**, RNA extracts of the phloem (a, lanes 1–6) and xylem (b, lanes 1–6) in 2-year-old terminal branches from an adult lychee tree (20 years old). The tissues were collected during the first week in February (lane 1), April (lane 2), June (lane 3), August (lane 4), October (lane 5), and December (lane 6). **B**, RNA isolated from *L. chinensis* (a, lanes 1–5), *S. macrophylla* (b, lanes 1 and 2), *D. congan* (b, lane 3; c, lane 1), and *S. mukorossi* (c, lanes 2 and 3). In gel a, RNA samples were from the mature pericarp (lane 1), the xylem of young branch (lane 2), 2–4 years old branch (lane 3), stem (lane 4),

and main root tissues (lane 5) of the adult lychee tree. Gels b and c, RNA extracts of the phloem (lane 1 in gel b, lane 3 in gel b, lane 3 in gel c) and xylem (lane 2 in gel b, lane 1 in gel c, lane 2 in gel c) for *S. macrophylla*, *D. congan*, and *S. mukorossi*, respectively. **C**, RNA isolated from the phloem (a, lanes 1–7), xylem (b, lanes 1–7) and leaves (c, lanes 1–6) of the spring-flush branches in 30 years old *H. brasiliensis*. Plant materials were collected during the first weeks in March (lane 1), April (lane 2), June (lane 3), August (lane 4), October (lane 5), December (lane 6) and February (lane 7, without leaf in February), respectively



**Fig. 3** RNA isolated from herbaceous plants and temperate trees by our method. 1, *A. thaliana*, leaf; 2, *A. thaliana*, leaf, callus; 3, *A. thaliana*, root, callus; 4, *N. tabacum*, young leaf; 5, *N. tabacum*, old leaf; 6, *L. esculentum*, old leaf; 7, *L. esculentum*, young leaf; 8, *T. aestivum*, leaf; 9, *P. bungeana*, old needle; 10, *P. bungeana*, young needle; 11, *S. europaea*, young shoot; 12, *S. europaea*, old shoot; 13,

*S. europaea*, root; 14, *S. europaea*, young shoot treated with 200 mM NaCl; 15, *S. europaea*, young shoot treated with 600 mM NaCl; 16, *T. mongolicum*, leaf; 17, *G. hirsutum*, leaf; 18, *O. sativa*, leaf; 19, *M. nana* Lour., leaf; 20, *P. canadensis*, leaf; 21, *P. canadensis*, phloem; 22, *P. canadensis*, xylem

lanes 1–10 and 18–22). In addition, there were no visible bands around the loading wells (Fig. 3), indicating little genomic DNA pollutants. In contrast to the woody species (Fig. 3, lanes 9, 10, and 20–22), the herbaceous plants rendered more qualified RNA extracts (Fig. 3, lanes 1–8 and 11–19).

In particular, the quality of RNA isolated from the poplar *P. canadensis* was not as good as that from other plants as strong background was apparent on the lanes (Fig. 3, lanes 20–22). This might be attributed to the abundance of secondary metabolites in the tissues of poplar. The quality of RNA isolated from the untreated young shoot of *S. europaea* (Fig. 3, lane 11) was better than that from the 600 mM NaCl-treated plant (Fig. 3, lane 15). It

was probably due to the accumulation of considerable amount of osmoticum, such as the sodium ion, in the latter. It was worthy noting that the high-quality RNA from the needles of pine tree, which had been considered as a difficult material for RNA isolation, was also isolated successfully employing the method reported here (Table 1; Fig. 3, lanes 9 and 10).

#### The RNA Integrity Determined by RACE and Northern Blotting

The suitability of the isolated RNA for the downstream studies was demonstrated by RACE and Northern

hybridization. At first, the total RNA isolated by our method from lychee tissues was used to clone a full-length cDNA for a 22 kDa vegetative storage proteins (VSP), an economically important protein whose sequence had not been determined. In this research, we cloned this gene via RACE technology. As shown in Fig. 4, the 3'-end sequence with a 700 bp length in phloem (Fig. 4A, lane 1 in gel a) and xylem (Fig. 4A, lane 2 in gel a) was obtained by 3'RACE. Then, the 5'-end sequences about 300 bp length were also amplified by 5'RACE from phloem (Fig. 4A, lane 1 in gel b) and xylem (Fig. 4A, lane 2 in gel b). Finally, an 855 bp length cDNA fragment, named *LcVSP1*, was obtained from both the phloem and xylem of lychee by designing specific primers according to the above RACE sequences (GenBank accession number: QD659678).

Furthermore, Northern blotting was carried out to determine the *CMO* gene expression in response to high salinity stress in a succulent euhalophyte *S. europaea*, which has been known as one of the most salt tolerant plant species in the world [7]. In order to cope with salt stress, *S. europaea* accumulated high concentrations of inorganic ions and large amounts of secondary metabolic compounds in its shoots. When exposed to NaCl over a

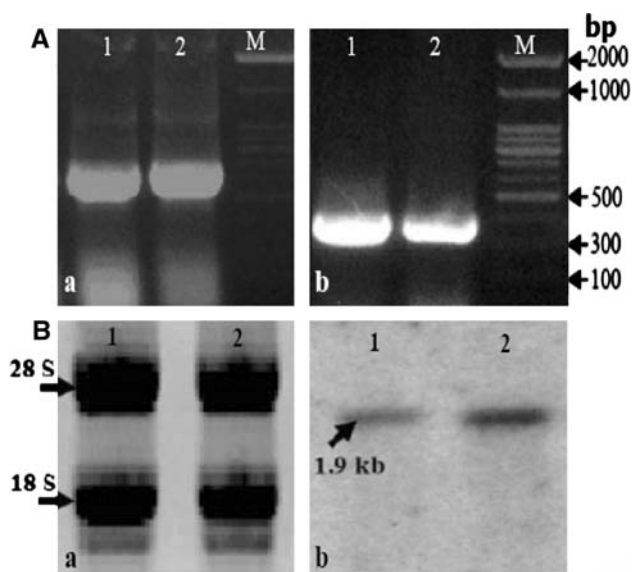
long period of time, its shoots will be lignified to some extent [7, 22]. These characteristics make the shoot tissues recalcitrant to common RNA isolation methods. However, high-quality RNA can be routinely obtained from this plant by our method. As indicated by the arrows, the clear 28S and 18S rRNA bands revealed that the RNA was undegraded (Fig. 3, lanes 11–15; Fig. 4B, lanes 1 and 2 in gel a).

In order to determine the integrity of mRNA from *S. europaea*, Northern blotting was performed using a probe of the *CMO* gene, encoding the limiting enzyme in the biosynthesis of glycine betaine in many plants in response to abiotic stress [23]. As seen in Fig. 4, the *CMO* gene expression was increased dramatically upon salt treatment (Fig. 4B, lane 2 in gel b). Distinct and sharp signals, as well as clear background, were obtained when the *CMO* gene probe was hybridized with total RNA extracted from both the control and the salt treated plant (Fig. 4B, lanes 1 and 2 in gel b). These results demonstrated that the mRNA was intact.

## Discussion

In this report, our results demonstrated that our modified protocol was efficient for isolating intact RNA from recalcitrant plant tissues, and the RNA extracts were suitable for the subsequent gene expression experiments, such as RT-PCR, RACE, and Northern blotting analysis.

A large number of plant RNA isolation methods have been previously published, indicating many difficulties especially for RNA isolation. Generally, high-quality RNA could be obtained from plant tissues when the RNase activities were successfully inhibited [19, 21]. Guanidinium thiocyanate was the most widely used reagent for inhibiting RNase activity [5]. Subsequently, the Trizol reagent was devised by mixing guanidinium isothiocyanate, phenol, sodium acetate, LSS, and other strong denaturants together [4, 15, 16, 21]. On the other hand, some improved methods could produce better results [12, 16, 19]. Several patents have also been applied based on the fact that RNA could bind selectively on solidified membranes, and accordingly, some RNA isolation kits are commercially available for routine RNA isolation from plant tissues [8, 11, 21, 30]. Often, these methods have to be modified when dealing with the different plant species or even with different tissues of the same plant species [18, 19, 27]. However, they were still proven to be insufficient in obtaining fine quality or high yield of RNA in many recalcitrant tissues, especially those with abundant polysaccharides or secondary metabolites [2, 9, 14–16, 19, 20], such as trees [13, 18, 24, 28].



**Fig. 4** RACE and Northern blotting results demonstrated the integrity of RNA isolated by our method. **A**, Cloning of a 22 kDa VSP cDNA ends from *L. chinensis* by RACE technology. Lanes 1 and 2, the amplification results of 3'RACE (gel a) and 5'RACE (gel b) from the phloem and xylem of 2-year-old branches in 20 years old *L. chinensis*, respectively. M, DNA ladder. **B**, RNA gel-blotting analysis of the *CMO* gene expression in shoots of *S. europaea*. Total RNA was isolated by our method from shoots of *S. europaea* treated with 0 and 200 mM NaCl (from left to right) for 30 days, respectively. Gel a, reference RNA stained with EB. The arrows indicate the 28S and 18S rRNA bands. Gel b, *CMO* gene expression determined by Northern blotting analysis. The arrow indicates the detected *CMO* gene transcripts

Here, we developed a novel protocol for plant RNA extraction by modifying several reported methods [3, 24, 31] to make it suitable for recalcitrant plant tissues. Our method differs from the existing ones mainly at the composition of the extraction buffer. At first, 2 M NaCl was included in the extraction buffer to result in a high ionic strength, which has been proven to be sufficient to separate the contaminating compounds in plant tissues [3, 10, 20]. Then, EDTA and LSS were added into the buffer to inhibit the RNase activity [12, 13]. As a strong ionic denaturing detergent, CTAB was widely used in both DNA and RNA isolation from plant [10, 14, 20, 29]. CTAB was also included in the buffer with a final concentration of 2% (w/v). By grinding *plus* quartz sands at room temperature and incubating the mixture at 65°C, the plant tissues were disrupted efficiently and the cells were lysed in the presence of the extraction buffer [1]. As a strong H-receptor, PVPP can absorb polyphenols efficiently. It had also been used for the removal of secondary metabolites from nucleic acid in plant RNA isolation [3, 8, 18]. Almost all macromolecular substances, such as polysaccharides, polyquinones, and phenolic compounds, can be co-deposited with PVPP [8, 18] as well as borate [28]. Therefore, in order to remove the interfering compounds, we added sodium borate and PVPP to our extraction buffer. Additionally, the oxidation of polyphenol to polyquinones and the activity of many enzymes were inhibited efficiently under the de-oxidized condition generated by  $\beta$ -mercaptoethanol, EDTA, and PVPP [3]. Proteins and pigments could be removed by the following organic solvents extraction steps [14]. Finally, the RNA pellet was precipitated selectively by lithium chloride, whereas the DNA fragments remained in the supernatant [1, 6].

In conclusion, this article presented an RNA isolation method with a wide range of applications for plant, especially recalcitrant plant tissues such as the xylem and phloem of tropical trees. The described method was comparatively simple and convenient, which allowed for extraction of high-quality RNA in a much shorter time than other previously reported methods. It was expected to be used as an alternative method or a testing one to design a new RNA extraction kit for recalcitrant plant tissues.

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