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AN IMPROVED METHOD FOR TOTAL RNA ISOLATION FROM RECALCITRANT LOQUAT (*ERIOBOTRYA JAPONICA* LINDL.) BUDS

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Abstract

Loquat buds during floral differentiation which contain large amounts of polysaccharides, proteins and secondary metabolites were not amenable to conventional RNA isolation protocols. Here a concise and efficient RNA isolation protocol based on cetyl trimethyl ammonium bromide (CTAB) and lithium chloride (LiCl) named improved CTAB-LiCl protocol was developed. The RNA isolated by this improved protocol was not only of high purity and integrity ($A_{260/280}$ ratio was range from 1.85 to 2.0 and $A_{260/230}$ ratio was over 2.0), but also high yield as 400-600 μg of total RNA per gramme fresh tissue, which was 6 to 10 folds more than that by the improved CTAB I or II protocols assessed. These results depended on the following crucial improved steps: the addition of 2 ml (3M) potassium acetate to further deposit polysaccharides, three times efficient separation of total RNA from polysaccharide and DNA residues with 2 M lithium chloride (LiCl) which was never applied in the traditional CTAB-based protocols, the reduction of phenol-chloroform-isoamylalcohol extraction times (only once) to avoid the great loss of total RNA. Further, the quality of total RNA isolated was verified to be competent for the reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis with *EJTFL1-1* and *EJTFL1-2* genes related to floral bud differentiation. Moreover, this improved protocol is cost-saving and reduces the risk of chemical carcinogen to operator as the abandon of diethyl pyrocarbonate (DEPC).

Introduction

Isolation of total RNA free of polysaccharides, proteins, genomic DNA and secondary metabolite contamination is an essential step for research on gene expression pattern and function by the techniques such as reverse transcription polymerase chain reaction (RT-PCR)/Northern blot/real-time PCR, cDNA cloning and gene transfer. However, it was difficult to isolate high-quality and high-yield RNA from the leaf and floral buds during differentiation in loquat as a recalcitrant woody fruit tree, due to its high levels of polysaccharides, proteins and secondary metabolites such as polyphenols (Liu *et al.,* 2005). These interfering chemicals can cause the degradation and low yield of functional mRNA through such approaches as oxidation of polyphenols and co-precipitation with polysaccharides (Wang *et al*., 2000). Traditional methods including common and improved cetyl trimethyl ammonium bromide (CTAB) methods (Chang *et al*., 1993; Kiefer *et al*., 2000), improved sodium dodecyl sulfate (SDS) methods (Bugos *et al*., 1995; Xu *et al*., 2004) and the commercial Trizol kit have been practiced repeatedly to extract total RNA from loquat leaf and floral buds before morphological differentiation, but offered inferior RNA products. Thus, this study developed an improved protocol to meet the need of the RNA isolation with high-quality and high-yield in loquat buds rich in polysaccharides, proteins, polyphenols and secondary metabolites, so as to reveal the expression patterns and functions of the genes related to floral bud differentiation in loquat.

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Liu (unpublished Ph.D. thesis) established a CTAB-based RNA isolating protocol for loquat tissues in 2005. However, this protocol produced an interior quality of RNA from early leaf and floral buds before morphological differentiation in loquat (rich in polysaccharides, polyphenols and secondary metabolites). Moreover, low-yield, tedious and time-consuming procedures limit its spread application in molecular research of loquat. In this study, we developed a simple and efficient method named improved CTAB-LiCl protocol for isolating high-quality and high-yield RNA from leaf and floral buds at various differential periods in loquat.

Materials and Methods

Plant material: Fresh leaf and floral buds at various development stages of 'Zaozhong No. 6' loquat (*Eriobotrya japonica* Lindl.) were collected from an orchard at South China Agricultural University, and froze immediately by liquid nitrogen, then stored at -80ºC.

RNA extracting protocols

Pretreatment: Plastic wares were immersed in 0.1% (v/v) diethyl pyrocarbonate (DEPC) treated water at 37ºC overnight then autoclaved 1 hour (h) at 121ºC (Note: this step was abandoned in the improved CTAB-LiCl protocol). Glassware and the mortar were baked for 6 h at 200ºC. Frozen buds from loquat were ground into fine power in liquid nitrogen with a pre-cooled mortar. Total RNA from loquat buds was extracted through the following three protocols respectively.

Improved CTAB I protocol: The improved CTAB I protocol for loquat tissues was developed by Liu (unpublished data). Transfer 2 g sample powder to a 50 ml centrifuge tube with 20 ml extraction buffer [2% (w/v) CTAB, 1.4M NaCl, 200 mM EDTA, 100 mM Tris-HCl (pH 8.0), 2% (w/v) PVP K-40, 2% (w/v) β-mercaptoethanol and 80μg/ml proteinase K], mix well and incubate at 65^{o}C water for 1 h with occasional shaking; Extract RNA crude product with an equal volume of phenol-chloroform (1:1,v/v) twice, separate the phenol-chloroform (1:1,v/v) and aqueous phases by centrifuging at 4,000 g for 20 min at 10ºC. Transfer the supernatant to a new tube and add 10 M LiCl to a final concentration of 3 M, then store at 4ºC overnight. Recover the RNA by centrifugation at 9,000 g for 30 min at 4ºC, and dissolve the pellet with 2 ml DEPC-treated water. Extract RNA three times with water-saturated phenol, phenol-chloroform (1:1, v/v) and chloroform-isoamylalcohol (24:1,v/v) respectively; Add 1/30 volume of 3 M sodium acetate (pH 5.2) and 1/10 volume of anhydrous alcohol into the collected supernatant, mix well and keep on ice for 30 min; Centrifuge at 12,000 g for 20 min at 4ºC, add 1/10 volume 3 M sodium acetate (pH 5.2) and 2 volumes of anhydrous alcohol to the supernatant; Mix well and precipitate RNA at -80ºC overnight; After centrifuging at 12,000 g for 20 min at 4ºC, wash the RNA pellet twice with 70% (v/v) alcohol, air-dried, then dissolved in 200 μl RNase-free water and stored at -80ºC.

Improved CTAB II protocol: The improved CTAB II protocol was modified based on the previously improved CTAB extraction (Asif *et al*., 2000; Cao *et al*., 2003). Transfer 2 g sample powder to a 50 ml centrifuge tube containing 10 volume of preheated extraction buffer (65ºC) [3% (w/v) CTAB, 1.4M NaCl, 200 mM EDTA, 100 mM Tris-HCl (pH

8.0), 2% (w/v) PVPP, 2% (w/v) β-mercaptoethanol and 80μg.ml^{-1} proteinase K] and mix well; Incubate at 65ºC water for 30 min with occasional shaking; Extract RNA crude product with an equal amount of phenol-chloroform (1:1, v/v), mix well and centrifuge at 8,000 g for 8 min at 15ºC; Add an equal volume of chloroform-isoamylalcohol (24:1, v/v) to the aqueous phase, mix well and centrifuge at 10,000 g for 10 min at 15ºC; Collect supernatant and add 1/4 volume of 10 M LiCl, mix well and store at -20ºC overnight; Recover the RNA by centrifugation at 12,000 g for 20 min at 4ºC; After dissolving the pellet with 4 ml of 0.1M Tris·HCl-EDTA buffer, the aqueous phase was separated with equal amount of chloroform-isoamylalcohol (24:1, v/v), then mix well and centrifuge at 10,000 g for 10 min at 4ºC; Collect the supernatant, add 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volume of pre-cooled anhydrous alcohol, mix well and keep for 3 h at -80ºC; Collect total RNA by centrifugation at 12,000 g for 30 min at 4ºC; Then wash the pellet twice with 75% (v/v) alcohol, air-dried, dissolved in 100 μl RNase-free water and store at -80ºC.

Improved CTAB-LiCl extraction: The improved CTAB-LiCl extraction protocol was developed based on the above improved CTAB II protocol and the improved hot borate method (Wan and Wilkins, 1994; Yao *et al*., 2005). Transfer 2 g frozen powder to a 50 ml centrifuge tube containing 10 volume of CTAB extraction buffer, as described in the above improved CTAB II protocol. Incubate at 65ºC water for 25 min with occasional shaking; Extract total RNA crude solution with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v) after the addition of 2 ml potassium acetate (3M) on the ice for 20 min; Recover the RNA by centrifugation at 10,000 g for 10 min at 4ºC, add 1/4 volume of 10 M LiCl to the collected aqueous layer and incubate on ice overnight; After centrifugation at 10,000 g for 20 min at 4ºC, the RNA pellet was washed three times with 2 ml of 2 M LiCl; Collect pellet by centrifugation at 10,000 g for 20 min at 4ºC and suspend it with 2 ml of 10mM Tris-HCl (pH7.5), add 1/10 volume of 3M potassium acetate (pH 5.2) and keep on ice for 30 min, then separated by centrifuging at 10,000 g for 15 min at 4ºC; Collect the supernatant, add 2.5 volume of cold anhydrous alcohol and store for 2 h at -80ºC; After centrifugation at 10,000 g for 30 min at 4ºC, the RNA pellet was collected, washed twice with 75% (v/v) alcohol, vacuum-dried, dissolved in 150 μl RNase-free water and store at -80ºC.

RNA quantity and quality analysis: The purity and yield of total RNA isolated was assessed by measuring optical density (OD value) at 230 nm, 260 nm and 280 nm using a spectrophotometer (BIO-RAD, Germany). The integrity of total RNA was examined by electrophoresis on 1.2% formaldehyde denaturing agarose gels.

RT-PCR analysis: First-strand cDNA was synthesized from 1 μg of total RNA using cDNA synthesis kit (TaKaRa, Japan) according to the manufacturer's instructions. In order to assay the quality of RNA, RT-PCR and Northern blot were performed with primers from the coding region of *EJTFL1-1* and *EJTFL1-2* genes related to floral bud differentiation. The sequences of forward and reverse primers for *EJTFL1-1* were 5′-GCCTTGGAGCCTCTGGT-TGT-3′ and 5′-TGGATGGAGGAGTTCTGGGTAG-3′. The sequences of forward and reverse primers for *EJTFL1-2* were 5′-TGTTGGGAGAGTGA TAGGAGAT-3′ and 5′-TGGATGTAGGAGTTTTGGGTAG-3′. The PCR for *EJTFL1-1*

was performed as follows: 94ºC for 5 min, 35 cycles of 85ºC for 60 s, 53ºC for 60 s and 72ºC for 70 s, a final extension at 72ºC for 10 min. The amplifying program for *EJTFL1-2* was the same as *EJTFL1-1* with the exception of the annealing temperature at 50.8ºC. PCR products were electrophoresed on 1.5% agarose gels, visualized by ethidium bromide (EtBr) staining and photographed under ultraviolet light.

Northern blot analysis: 10 µg of total RNA was separated on 1.2% formaldehyde agarose gel and capillary blotted onto biodyne nylon membrane (PALL, Japan) according to the manufacturer's instructions (Roche Applied Science, Mannheim, Germany). The blotted membrane was dried at 80ºC for 2 h and cross-linked with ultraviolet for 10s. The digoxin-labeled probes with cDNA full length of *EJTFL1-1* and *EJTFL1-2* (about 500 bp) from loquat were performed using PCR DIG Probe Synthesis kit according to the manufacturer's instructions (Roche, Germany). The probe primers were the same as those in RT-PCR. The membranes were hybridized with digoxin-labeled probe for 16 h at 43ºC in high-SDS buffer [7% (w/v) SDS, 5×SSC, 50 mM sodium-phosphate (pH 7.0), 2% (w/v) blocking reagent and 0.1% (w/v) N-lauroylsarcosine] and 50% deionized formamide (v/v) (Roche, Germany). Blotted membranes were washed twice by 2×SSC and 0.1% SDS for 10 min at 37 ºC, followed by washing twice with 0.1×SSC and 0.1% SDS at 58ºC for 30 min. The membranes were then subjected to immunological detection according to the manufacturer's instructions.

Results

Quantity analysis of isolated RNA: The yield and purity of total RNA in loquat buds from different periods were apparently different adopting the three CTAB-based improved protocols (Table 1). The improved CTAB-LiCl extraction produced the highest RNA yield (400-600 ng RNA per gram fresh sample), which was 6-10 times more than that by the other two protocols. Compared with the improved CTAB I protocol, total RNA isolated using the improved CTAB-LiCl and CTAB II protocol exhibited much better purity for loquat buds at various periods. Their A_{260}/A_{280} and A_{260}/A_{230} ratios were 1.85-2.0 and 2.0-2.5 respectively (Table 1), suggesting that RNA samples were little contaminated by polysaccharides, proteins, DNA, phenol or salts, while the ratios of A_{260}/A_{280} and A_{260}/A_{230} were under 1.7 via the improved CTAB I protocol for the leaf and early floral buds of loquat.

Table 1. Quantity and yield analyses of total RNA from loquat buds.

Extracting methods	Bud stages	A_{260}	A_{280}	A_{230}	A_{260}/A_{280}	A_{260}/A_{230}	RNA yield ($\mu g \cdot g^{-1} FW^{-1}$)
Improved CTAB I protocol	Leaf buds	0.504	0.293	0.298	1.72	1.69	21.7
	Early floral buds	0.461	0.262	0.327	1.76	1.41	16.4
	Advanced floral buds	0.783	0.408	0.386	1.92	2.03	48.9
Improved CTAB II protocol	Leaf buds	0.625	0.332	0.305	1.88	2.05	29.5
	Early floral buds	0.919	0.469	0.429	1.96	2.14	58.2
	Advanced floral buds	1.047	0.528	0.463	1.98	2.26	86.7
Improved CTAB-LiCl extraction	Leaf buds	1.494	0.799	0.658	1.87	2.27	413.9
	Early floral buds	1.569	0.800	0.645	1.96	2.43	465.4
	Advanced floral buds	2.322	1.191	0.988	1.95	2.35	586.1

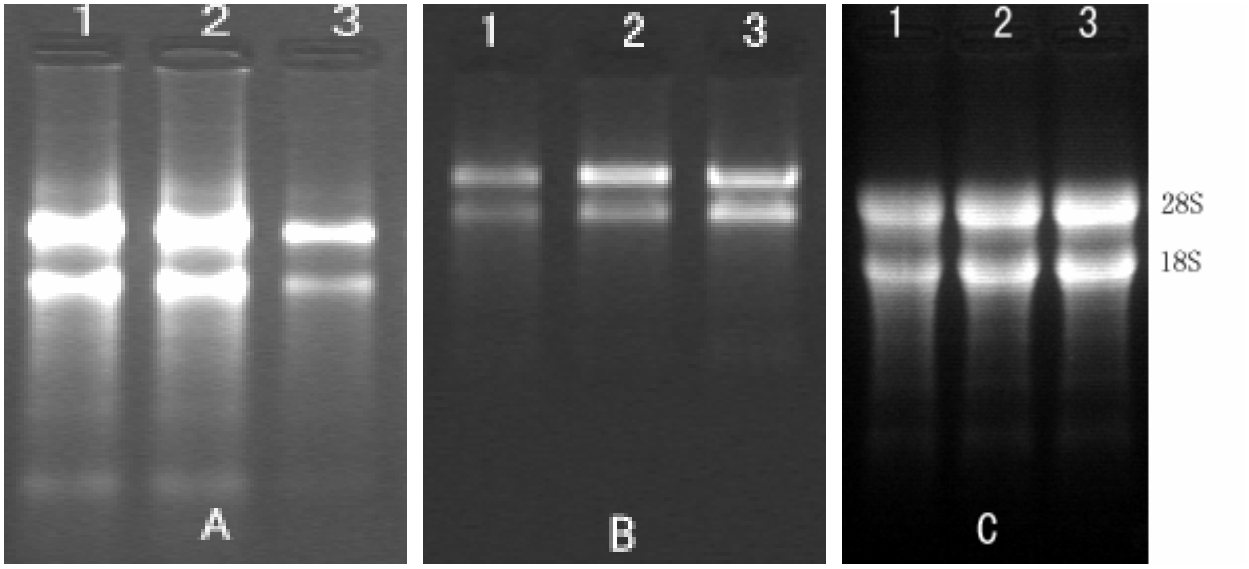

Fig. 1. Visualization of total RNA isolated from loquat buds at different development stages. The samples of total RNA were separated on 1.2% formaldehyde denaturing agarose gel containing EtBr (ethidium bromide) and photographed under ultraviolet light. A: improved CTAB I protocol; B: improved CTAB II protocol; C: improved CTAB-LiCl extraction. Lane 1: leaf buds; lane 2: the early floral buds before morphological differentiation; lane 3: the advanced floral buds in inflorescence.

Quality analysis of isolated RNA: Compared with the improved CTAB I protocol, higher purity and better integrity with distinct bands of 28S, 18S ribosomal RNA (rRNA) were observed using the improved CTAB-LiCl and CTAB II protocols (Fig. 1B, C), particularly for loquat buds before morphological differentiation. The brightness of 28S rRNA was approximately twice than that of 18S. In terms of the improved CTAB-LiCl protocol, the clear and neat rRNA was obtained without genomic DNA, polysaccharides and other contaminant (Fig. 1C), indicating a good quality of RNA isolated. Besides, the improved CTAB-LiCl protocol is high-yield, cost-saving and reduces the risk of chemical carcinogen to operator owing to the abandon of diethyl pyrocarbonate (DEPC) and less use for phenol/chloroform, compared with the improved CTAB I and II protocols. Therefore, the improved CTAB-LiCl protocol was proposed, which is competent for recalcitrant leaf and floral buds during differentiation in loquat.

RT-PCR analysis: The functional intactness of isolated message RNA (mRNA) can be measured by reverse transcription-polymerase chain reaction (RT-PCR) analysis since reverse transcriptase is highly sensitive to impurities (Li *et al*., 2006; Ghangal *et al*., 2009). As shown in Fig. 2, employing the improved CTAB II and CTAB-LiCl protocols with the primers based on the coding regions of *EJTFL1-1* and *EJTFL1-2* genes related to floral bud differentiation, two distinct and regular bands without any smearing by RT-PCR analysis were represented in RNA samples isolated from the advanced leaf buds before physiological differentiation and from the floral buds between physiological and morphological differentiation phases in loquat. The results indicated that total RNA samples isolated by the two improved protocols were of high integrity, purity and suitable for downstream molecular analysis.

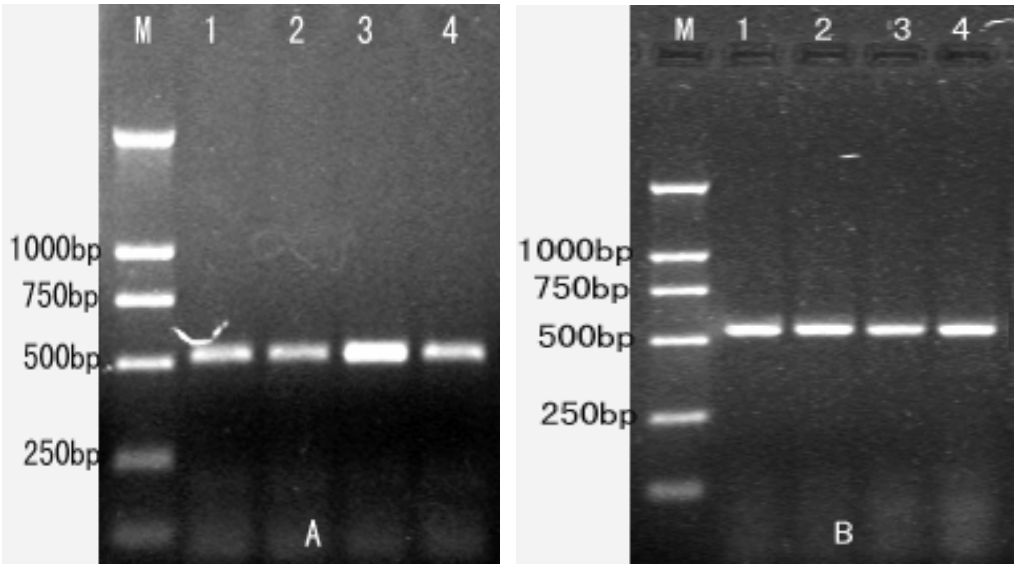

Fig.2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis for total RNA isolated from loquat buds at different development stages using the improved CTAB II protocol (lane 1,2) and the improved CTAB-LiCl protocol (lane 3,4) with *EJTFL1-1* (A) and *EJTFL1-2* (B) genes related to floral bud differentiation. Lane 1, 2: the advanced leaf buds; Lane 3,4: the floral buds before morphological differentiation in loquat.

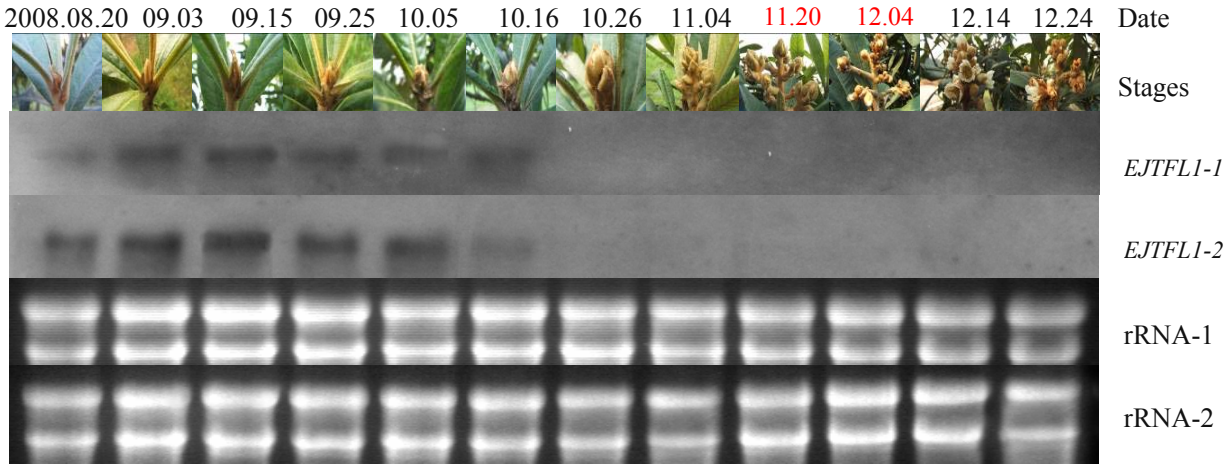

Fig. 3. Northern blot analysis for total RNA isolated from the leaf and floral buds at various development stages in loquat using the improved CTAB-LiCl protocol. 10 µg of total RNA sample from developmental buds was loaded in each lane of 1.2% formaldehyde denaturing agarose gel, and hybridized by *EJTFL1-1* and *EJTFL1-2* cDNA probes labeled by digoxin. *EJTFL1-1* and *EJTFL1-2* genes play the important parts in switching vegetative development and floral formation.

Northern blot analysis: The functional intactness of total RNA isolated from the leaf and floral buds at various phases in loquat was further assessed by Northern blot analysis. As shown in Fig. 3, the distinct hybridization signals were obtained using digoxin-labeled probes of *EJTFL1-1* and *EJTFL1-2* genes related to floral bud formation, inferring the high similarity in expression pattern between *EJTFL1-1* and *EJTFL1-2* genes, although *EJTFL1-2* was disappeared slightly earlier than *EJTFL1-1* in expression phase. Moreover, there was a span of intense expression from the secondary-advanced leaf buds to the expanding stage of single floral bud in morphological differentiation. These results demonstrated the functional integrity of isolated mRNA and the competence of the improved CTAB-LiCl protocol for loquat buds at various phases.

Discussion and Conclusion

Although there are many methods for RNA isolation presently, it is necessary to develop new optimized protocols of RNA isolation for different plant species or organs, even the identical tissues at different development stages, attributing to the considerable variability of chemical compositions and their content levels (Sharma *et al*., 2003; Wang *et al*., 2007). Loquat is a recalcitrant woody fruit tree, its leaf and floral buds during differentiation contain more abundant and intricacy polysaccharides, proteins and secondary metabolites such as polyphenols than those in herbaceous plants (Liu *et al*., 2005), causing great difficulty to isolate total RNA with high quality and high yield.

Polysaccharides and polyphenols are the most difficult removal substances at RNA isolation process, as the oxidation of polyphenols and the similarity of physicochemical properties between polysaccharides and RNA, which lead to the co-precipitation with RNA at the RNA precipitation step (Wang *et al*., 2000). Traditional CTAB-based protocols including the improved CTAB I and II described in this study almost involve an initial digestion of tissues in standard CTAB lysis buffer, the separation of RNA aqueous phase from proteins, genomic DNA and polysaccharide residues with phenol-chloroform over twice, and subsequent RNA precipitation with lithium chloride and anhydrous alcohol. However, the conventional protocols produce the inferior quality, low yield RNA and poor RT-PCR amplification for the leaf and floral buds of loquat rich in polysaccharides, proteins and secondary metabolites. This is mainly because that repeated extraction with phenol-chloroform not only make an inefficient removal to polysaccharides other than proteins (Sambrook & Russell, 2001), but also cause the massive loss of total RNA as the discard of one fourth or fifth supernatant each time. Besides, polyvinyl pyrrolidone (PVP) can combine irreversibly with the long Poly (A) tail of message RNA (Sambrook and Russell, 2001) and the phenol, resulting co-precipitation loss (Hu *et al*., 2002; Kolosova *et al*., 2004).

When studying expression profiling of the genes related to floral formation in loquat using quantitative RT-PCR and Northern blot techniques, the acquisition of high quality and sufficient quantity RNA at each differential stage is required so as not to miss the rare expression genes in limited floral buds. The improved CTAB-LiCl protocol assessed in this study offered not only high yield of total RNA (400-600 μg of total RNA per gramme fresh tissue) which fulfilled the quantity demand of Northern hybridization through once cost-saving extraction, but also high purity and integrity of RNA as assayed by spectrophotometer reading, formaldehyde denaturing gel electrophoresis, RT-PCR and Northern hybridization. In CTAB buffer modified, proteinase K was supplied to digest proteins efficiently, β-mercaptoethanol as a strong reducing agent prevented polyphenols from oxidizing and also made RNase denature irreversibly (Wang and Stegemann, 2010), polyvinyl-polypyrrolidon (PVPP) as a insoluble cross-linked agent instead of PVP was also combined exclusively with polyphenols to form complex through hydrogen bonds (Hu *et al*., 2002; Kolosova *et al*., 2004).

However, the crucial improved steps include: the addition of 2 ml (3M) potassium acetate to the collected supernatant to further deposit polysaccharides; three times efficient separation of total RNA from polysaccharide and DNA residues with 2 M lithium chloride (LiCl) which is a strong dehydrating agent to make RNA not DNA precipitation specially (Chen *et al*., 1997; Sambrook and Russell, 2001), this procedure is never applied in conventional CTAB-based protocols; the reduction of phenol-chloroform-isoamylalcohol extraction times (only once) to avoid the great loss of total

RNA. Therefore, the improved CTAB-LiCl protocol proved to be competent for the leaf and floral buds at any developmental phase in loquat and other fruit crops including apple, banana, litchi (data not shown), while the improved CTAB I and II protocols failed for the leaf and floral buds before morphological differentiation in loquat.

The quality and yield of total RNA isolated by the improved CTAB-LiCl protocol fulfilled well the demand of RT-PCR and Northern hybridization. In addition, the procedure of improved CTAB-LiCl protocol is easy to manipulate and reduces the risk of chemical carcinogen to operators due to the abandon of DEPC treatment.

In conclusion, experimental results demonstrated that total RNA isolated by the improved CTAB-LiCl protocol was quite good in purity and integrity, especially high yield, in addition to cost-saving, less chemical toxicity. It proved to be completely suitable for the leaf and floral buds rich in polysaccharides, proteins and secondary metabolites in loquat at various development periods and competent for molecular downstream applications such as RT-PCR and Northern blot analysis.

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