AN EFFECTIVE PROTOCOL FOR ISOLATION OF HIGH-QUALITY RNA FROM *GARCINIA PRAINIANA* (CERAPU) FRUIT FLESH

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ABSTRACT

Plant tissues containing large amounts of polysaccharides and compounds of polyphenol have been found to be difficult to isolate RNA. Commercial kits in the market to date have been developed for isolation of high-quality RNA but not specific from tissues with this characteristic especially for fruit. Most published protocols are tedious and give poor yields of RNA when applied to recalcitrant plant tissues. An efficient RNA isolation protocol has been developed to extract high-quality RNA from Cerapu fruit tissue. This protocol has been modified from RNA isolation protocol that was developed specifically to extract RNA from loquat fruit. The protocol is based on cetyltrimethylammonium bromide (CTAB) and 10M lithium chloride (LiCl) precipitations steps. RNA with high purity and integrity (A260/A280 ratios between 1.89 to 1.91 and above 2 for A260/A230) was successfully isolated by this improved protocol and total yield of RNA per gram tissue was above 3ug. The protocol was tested on two different stages of cerapu fruit which are ripe (Index 6) and unripe (index 2). High quality of RNA obtained suits the criteria for transcriptome sequencing and also efficient to be applied in research on gene characterization, expression and function.

Key words: RNA extraction, Improved protocol, Cerapu, Garcinia prainiana

INTRODUCTION

Because of its nutritional benefits, cerapu has been subjected to many studied, including those involving molecular biology. Several molecular studies have been carried out in the genomics era of woody plants (Jbir et al., 2008; Zarei et al., 2009; Zamani et al., 2010; Sarkhosh et al., 2011) but transcriptomics studies, which is an important growing field in molecular biology, is scarce on this plant species. Thus, isolation of high-quality RNA from plants is critical for such study and as well as for reverse transcription polymerase chain reaction (RT–PCR), Northern hybridization, and microarray analysis (Birtic & Kranner, 2006; Shi & Bressan, 2006). To obtain high-quality RNA from recalcitrant plant tissues particularly from fruit has been found to be problematic due to the presence of abundant compounds of polysaccharides, polyphenolic compounds, proteins, and genomic DNA contamination that interact with nucleic acids to form insoluble complexes. Due to similarity in physical and chemical properties between RNA and polysaccharides, it can coprecipitate with RNA during nucleotide purification process (Shu et al., 2014). Moreover, polysaccharides, polyphenols and other secondary metabolites interfere with or degrade RNA (Birtic & Kranner, 2006). These interactions can cause degradation and low yield of functional messenger RNA (mRNA) through mechanisms such as oxidation of polyphenols and coprecipitation with polysaccharides (Wang & Hunter, 2000). Therefore, removing polysaccharide effectively is the key to extract high quality RNA from woody plants.

The difficulty of isolating RNA from these tissues often requires modifications of existing protocols for developing tissue-specific procedures. Few methods have been developed to isolate total RNA from plant tissues but most of them are not applicable to extract high-quality of RNA from wide range of plant species, especially of woody plants (Birtic & Kranner, 2006; Su & Gibor, 1988). Some protocols to isolate RNA from plant tissues rich in polysaccharides and polyphenolic compounds have included the use of soluble polyvinylpyrrolidone (PVP) and precipitation with ethanol (Salzman et al., 1999). Modifications to the guanidinium–phenol–chloroform method (Vareli & Frangou-Lazaridis, 1996), hot borate method (Wan & Wilkins, 1994) and pre-treatment of

lyophilized plant material with acetone to remove polyphenolics from plant tissues (Schneiderbauer et al., 1991) have also been reported too. Furthermore, cetyltrimethylammonium bromide (CTAB) based methods also has been developed for tissues containing high levels of polysaccharides and phenols (Chang et al., 1993; Hu et al., 2002; Zeng & Yang, 2002). Unfortunately, this method is time consuming, technically complex and in some cases the RNA obtained is low yield. Furthermore, available commercial plant isolation kits, such as the RNeasy plant mini kit (Qiagen) and TRIzol reagent have not been designed for extraction of high-quality RNA from plant tissues that have an elevated amount of secondary metabolites (Tattersall et al., 2005). Two commercials reagent-TRIsure (Bioline, USA) and TRIzol (Thermo Fisher Scientific, USA) together with wash reagent Fruit-mate solution (Takara, Japan) were not suitable as alternative method to extract RNA from cerapu fruit flesh even with modification of published methods by the kits.

In this study, we modified the phenol-free CTAB-based procedure that improved earlier by Morante-Carriel (2014). The method was specifically adapted to extract RNA from fruit and tissues (with different extents of water content) of recalcitrant woody plant rich in polysaccharides, polyphenolics and other interfering substances. Washing step was the critical part in the procedure and a few modifications were introduced to RNA isolation procedure in order to increase RNA yield and minimize contamination with polysaccharides and polyphenol compounds. We demonstrated that high quality and quantity of RNA can be obtained systematically from cerapu fruit flesh by introducing modification of the procedure developed by Morante-Carriel (2014).

MATERIAL AND METHODS

Plant materials

Ripe and unripe fruits of cerapu were collected from Putrajaya Botanical garden at Putrajaya. Upon arriving, the fruits skin were peeled off and immediately the flesh tissues were sliced out, wrapped into small pouch and freeze in liquid nitrogen. The frozen samples were kept at -80°C until needed.

Protocol tested

As a first step, we selected a protocol from currently existing RNA isolation methods developed for fruit of recalcitrant woody plants. We decided to proceed with the protocol developed by Morante-Carriel and coworkers (Morante-Carriel et al., 2014) with slightly modification. The protocol developed to isolate the RNA from tissues with different extents of water content, rich in polysaccharides, polyphenolics and other interfering substances.

Equipments, reagents and extraction buffers

All chemicals and reagents used in this study were products of SIGMA-ALDRICH, USA. DEPC (diethyl pyrocarbonate) treated water (1st Base Bioreagent, Malaysia) was used for all solutions. All glasswares, mortar and pestle were treated with 0.1% DEPC-treated water overnight and baked for at least 3 hours at 200°C. All centrifuge tubes, micro tubes and tips were bought from the supplier that purposely for RNA work.

Washing buffer: Modification was made by using PEG 400 instead of PEG 6000 used by Morante-Carriel and coworkers. The buffer consisted of 100mM Tris-HCL (pH8.0), 0.35M Sorbitol, 10% (w/v) PEG (polyethylene glycol) 400 and 2% (v/v) β-mercaptoethanol (added just before use). All the stock solutions except for β-mercaptoethanol were autoclaved and used to prepare fresh solution.

Extraction buffer: The formulation was essentially as described in Morante-Carriel and coworkers but was modified by changing the chemical used to prepare the buffers. Consisted of 300mM Tris-HCl (pH8.0), 25mM EDTA (ethylenediaminetetraacetic acid), 2M NaCl, 2% (w/v) CTAB, 2% (w/v) polyvinyl pyrrolidone (PVP K40), 0.05% (w/v) spermidine trihydrochloride and 2% (v/v) β-mercaptoethanol (added just before use). The buffer every time was freshly prepared using autoclaved stock solutions. Thus, no precipitation occurred due to long storage at room temperature.

Other solution: Chloroform: isoamyl alcohol (Chl/Iaa, 24:1); 3M Sodium Acetate (NaOAc, pH5.2); 10M Lithium Chloride; Isopropanol; Absolute ethanol; 70% ethanol (prepared from absolute ethanol with DEPC-treated and autoclaved water); distilled, DEPC-treated and autoclaved water (DTW).

Procedure

Grinding step

One gram of fruit tissue (freshly frozen material) was placed in a precooled mortar. The samples were ground to obtain a fine powder in liquid nitrogen and quickly transferred into sterile disposable 50ml centrifuge tube while frozen and further kept in liquid nitrogen until all samples were prepared.

Washing step

This step was introduced as cerapu fruit tissue is highly hydrated tissue (higher water content) as suggested in Hu and coworkers (Hu *et al.*, 2002) and the step was performed on ground tissues prior to isolation step. Washing buffer was added in a proportion

of 10 ml per gram tissue powder, vortexed for 1 min and centrifuged at 3500g for 15 min at 4°C. The suspension was decanted and the supernatant and floating cell debris was discarded.

Isolation step

Prewarmed isolation buffer (10 ml/gm sample placed in water bath at 65°C) was added to 1 gm of washed ground tissue. Homogenized by vortexing and incubated at 65°C for 10 min with shaking every 2 min. The mixture was extracted with an equal volume of Chl/Iaa (24:1) and centrifuged at 5000g (10 min at 4°C). The aqueous layer was transferred to a new tube, mixed with an equal volume of Chl/Iaa, (24:1) and centrifuged at 10,000g (10 min at 4°C). Aqueous supernatant was transferred to a new tube and added with 0.1 vol of 3M NaOAc, pH5.2 and 0.6 vol of isopropanol. Mixture was mixed prior to store at -80°C for 30 min or alternatively at -20°C for 1 h. Precipitated material, including nucleic acids and remaining carbohydrates was recovered by centrifugation at 8,000g for 20 min, 4°C.

Purification step

Resulting pellets were dissolved in 1 ml of DTW and transferred to a microcentrifuge tube. The RNA was selectively precipitated by the addition of 0.3 vol of 10M LiCl, mixed and incubated on ice for 90 min, followed by centrifugation at 8,000g for 30min, 4°C. This step was repeated twice. Alternatively, a single 10M LiCl precipitation may be sufficient to obtain high-quality RNA by precipitate overnight at 4°C. However, for the case of cerapu flesh sample, purification step must be repeated twice in order to obtain sufficient amount of high-quality RNA. The RNA pellet was resuspended in 0.1 ml of DTW, 0.1 vol of 3M NaOAc, pH5.2 and 2 vol of cold absolute ethanol. The mixture was immediately centrifuged at 8,000g for 20 min at 4°C. Formed pellet was washed with ice-cold 70% (v/v) ethanol, let to dry and dissolved in volume of 100µl DTW prior to proceed with DNase treatment step.

Total RNA cleanup with DNase digestion.

This step was using RNeasy RNA isolation kit and RNase-Free DNase set which both products were from QIAGEN, USA. The sample (100µl) was mixed with 350µl of RLT buffer (10µl/ml of β-ME was added into RLT buffer just before use), mixed thoroughly and subsequently added 250µl absolute ethanol to the lysate. Mixture was mixed well by pipetting 2 to 3 times and immediately applied 700µl of the sample mixture into RNeasy mini spin column that sitting in a 2 ml collection tube and centrifuged at full speed for 15 sec. RNeasy column was transferred into a new 2 ml collection tube, pipetting 350µl buffer RW1 onto column, incubated 1 min. and span for 25 sec at maximum speed. The flow-through was discarded. Before proceed for the next step, DNase incubation mix was prepared by mixing 10µl DNase 1 with 70µl buffer RDD. About 80µl DNase 1 incubation mix was directly pipetting onto column and incubated for 15 min at room temperature. The column then added with 350µl buffer RW1 and span at maximum speed for 15 sec. The flow through was discarded. Pipette 500µl buffer RPE onto RNeasy column and incubate for 5 min. Centrifuge for 15 sec at maximum speed to wash the column. The washing step was repeated one more time. Discard flow through and reuse the collection tube

Estimation of RNA purity, yield and integrity

The purity and concentration of the isolated RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was evaluated from the 28S and 18S ribosomal RNA (rRNA) bands on 1.2% agarose gel after electrophoresis (90Vol, 90 min), staining with ethidium bromide and visualizing under UV (312 nm) light.

RESULTS AND DISCUSSION

Due to composition and content level of phytochemicals in different species of plants, it is necessary to develop new RNA extraction method even though many different RNA extraction methods are actively developed. This is because each method is given variability in yield and quality of RNA (Sharma et al., 2002; Wang et al., 2007). Polysaccharides and polyphenols are the main compounds that released out together with RNA once the cells are ground. These compounds are the most contaminant substances during RNA extraction due to the ability of polyphenols to be oxidised and physicochemical properties similarity between polysaccharides and RNA. This can lead to coprecipitation with RNA at the RNA precipitation steps. Cerapu is recalcitrant woody fruit tree contains abundant intricate polysaccharides, proteins, secondary metabolites and high-water content (Fruit tissue) (Liu et al., 2005) that contribute to difficulty of isolation high quality and high yield of RNA from the fruit tissue.

Most published CTAB protocols are involved cell disruption in standard lysis buffer, phenol-chloroform separation to remove cellular compounds and RNA precipitation by lithium chloride together with anhydrous alcohol (Chang et al., 1993; Kiefer et al., 2000). Unfortunately, this standard CTAB protocol produces low quality and quantity of RNA for plants rich in polysaccharides, proteins and secondary metabolites as phenol-chloroform precipitation steps is inefficient to remove polysaccharides and proteins (Sambrook & Russell, 2006) and large losses of RNA (20-25%) occur during supernatant discarding each time as needed by the protocols. Thus, the improved protocol in this paper is combination between the use of optimal agents in a CTAB-based extraction buffer and some critical improved steps.

PVPP in this study was replaced by PVP due to its hard to be dissolved during buffer preparation. The function of PVP is quite similar with PVPP which to react strongly with polyphenols to form complexes by hydrogen bonding (Hu et al., 2002; Kolosova et al., 2004), thereby avoiding RNA losses. This is supported by adding ß-mercaptoethanol in CTAB buffer which the chemical shows function as a strong reducing agent prevented polyphenols from oxidizing and also made RNases denature irreversibly

(Wang & Stegemann, 2010). Washing step (Hu et al., 2002) we found is a critical step to be included especially for the cerapu and other underutilized fruits due to their tissues are highly hydrated. Moreover, to make sure we isolate high quality of RNA from the fruit tissue, two total RNA separation steps by using 10M LiCl was included to separate the RNA from polysaccharide and DNA residues. 10M LiCl is function as a strong dehydrating agent that promotes specific RNA precipitation (Sambrok & Russell, 2006; Chen et al., 1997). We found that this step is important step otherwise we obtain low quality of RNA or RNA will be lost if this step is skipped. The protocol that we improved is easy/straight forward protocol and need the whole one working day to complete. The protocol is using common equipments of a molecular biology laboratory and we routinely handle in parallel four samples each time to prevent any mistakes during the experiment and also to make sure we isolate good quality of RNA. All steps involve phenol was eliminated in this protocol event we know common RNA extraction protocol is using phenol (Baker et al., 1990; Shellie et al., 1997). This to lead cost saving and less chemical toxicity. In addition, we also carried out the centrifugation steps at medium speed (5000 – 8000 rpm) using high-speed (not ultra-) centrifuge.

High-quality RNA was obtained through our modified protocol by analyse the RNA integrity by the sharpness of ribosomal RNA (rRNA) bands upon visualized on 1.2% agarose gel. RNA samples well resolved 28S and 18S rRNA bands with no visible signs of degradation (Fig 1), and sharp 5S RNA peaks were also detected with the Bioanalyzer (Fig 2). The yields of total RNA from unripe and ripe cerapu fruit flesh are 1242.5 ng/µl and 216.9ng/µl respectively (Table 1). The A260/A230 ratio for both samples were over than 2.0 indicating high RNA purity had been isolated and the RNA is free from the contamination of polyphenolic and polysaccharide compounds. The A260/A280 ratios ranged from 1.80 to 1.90 indicating low or no protein contamination (Table 1), and the RIN (RNA integrity number) values shows by the samples were above 8 (Fig. 2). DNA contamination may appear as high molecular weight (MW) bands in agarose gels, but as observed in Fig. 1, high MW bands of DNA contamination are not visible in our gels.

CONCLUSION

Overall, the quality (purity and integrity) and yield of total RNA isolated from ripe and unripe fruit flesh of cerapu by our improved method are very high and reproducible. The essence this developed protocol lies in the fact that polysaccharides can be efficiently removed, which is very hard for other conventional method to tackle. The result almost similar compared to other improved protocol developed previously based on CTAB, phenol, and LiCl in term of isolation of high-quality RNA (A260/A280 and A260/A230). Our improved protocol leads to isolate significantly higher quality of RNA from cerapu fruit tissue and will be exclusively protocol to isolate good quality RNA from other Malaysian underutilized fruits. Therefore, a major strength of our improved method is to isolate robustness of RNA from watery, savoury and higher with secondary metabolite which all of these are common characters of Malaysian underutilized fruits and also no limitation to extract RNA from fruits which less phenolic compounds. As in this article, we have improved the existing protocol to be suitable for extracting RNA from cerapu fruit flesh as well as other fruit samples which are rich in polysaccharides, proteins, and secondary metabolites. The produced RNA was competent for RNA sequencing to study the pathway involved in producing the useful metabolite.

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Table 1: Yields and quality of RNA isolated from different ripening stage of cerapu fruit with high levels of polysaccharide and polyphenolic compounds using spectrophotometric determinations.

Plant	Sample	Tissue analyzed	Absorbency ratio		Yield ng/µl
			OD 260/280	OD 260/230	
Garcinia prainiana (Cerapu)	Fruit	Unripe fruit flesh	1.91	2.10	1242.5
		Ripe fruit flesh	1.89	2.46	216.9

Figure 1: Total RNA isolated from fruit flesh tissues at different ripening stage, ripe and unripe. Lane 1: DNA ladder; Lane 2: Ripe fruit flesh tissue; Lane 3: Unripe fruit flesh tissue.

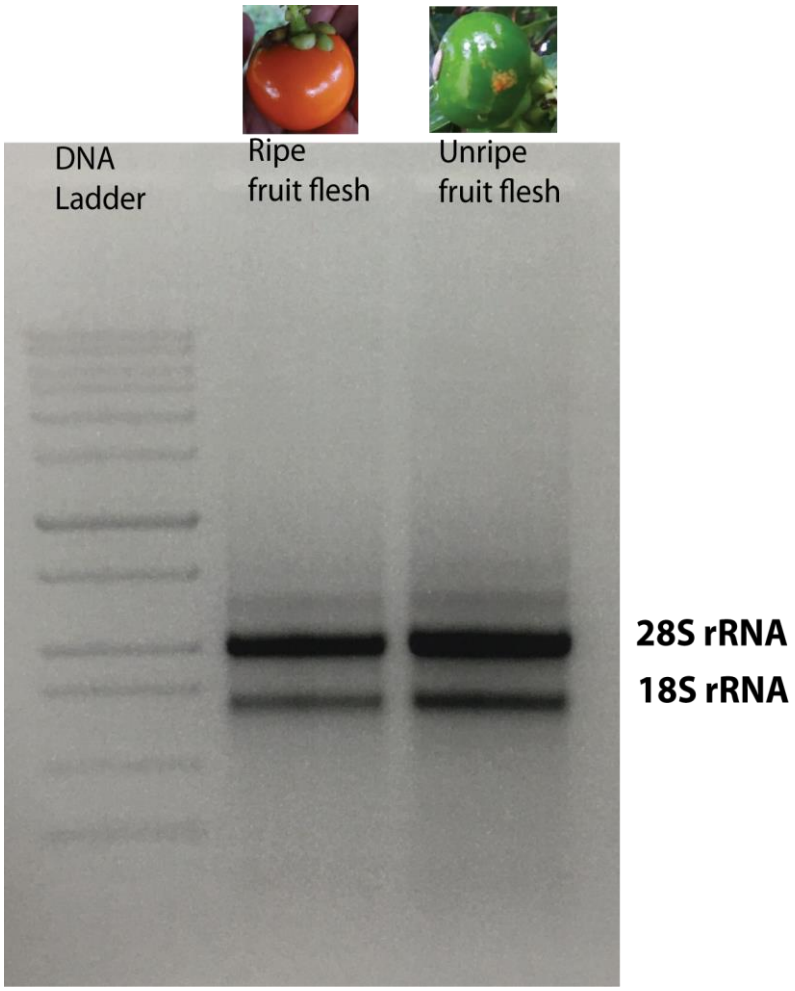

Figure 2: Total RNA samples analysed in the Agilent TapeStation with Nanodrop. M: Ladder/marker; Lane 1: Ripe fruit flesh; Lane 2: Unripe fruit flesh. The quality of RNAs are indicated by the RIN value at each lane.