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Efficient isolation of high quality RNA from tropical palms for RNA-seq analysis

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Abstract

Currently, RNA-seq as a high throughput technology is being widely applied to various species to elucidate the complexity of the transcriptome and to discover large number of novel genes. However, the technology has had poor success in elucidating the transcriptome of tropical palms, as it is difficult to isolate high quality RNA from tropical palm tissues due to their high polysaccharide and polyphenol content. Here, we developed an RNA-isolation protocol for tropical palms, the MRIP method (Methods for RNA Isolation from Palms). The integrity of the RNA molecules extracted using this protocol was determined to be of high quality by means of gel electrophoresis and Agilent 2100 Bioanalyzer microfluidic electrophoresis chip examination with a RIN (RNA Integrity Number) value of more than 9, indicating that the mRNAs were of good integrity. Subsequently the isolated RNA was used for transcription analysis without further purification. With Illumina sequencing, we obtained 54.9 million short reads and then conducted *de novo* assembly to gain 57,304 unigenes with an average length of 752 base pairs. Moreover, the RNA isolated with this protocol was also successfully used for real-time RT-PCR. These results suggested that the RNA isolated was suitable for Illumina RNA sequencing and quantitative real-time RT-PCR. Furthermore, this method was also successful in isolating total RNA from the leaves of various Palmaceae species.

Keywords: RNA isolation, RNA-seq, *Cocos nucifera*, Palmaceace, rRNA.
Abbreviations: RIN, RNA Integrity Number**;** MRIP, Methods for RNA Isolation from Palms; CTAB, Cetyltrimethylammonium bromide; DEPC, Diethylpyrocarbonate; RT-PCR, Reverse Transcription Polymerase Chain Reaction.

Introduction

Coconut palm (*Cocos nucifera* L.), one of the tropical palm trees, is an economically important oil crop and is widely cultivated in tropical regions due to its extensive applications in the fields of agriculture and industry. Such applications include food, fibre, oil, soil fertilizers, spa ingredients, furniture, fashion accessories, garments, construction, building materials and biofuels. Despite its importance, few coconut genomic sequences are publically available, which limits the application of functional genomics to coconut and other related palm trees. Currently, some strategies are being developed to elucidate the functional complexity of transcriptomes (Hass and Zody, 2010; Cloonan et al., 2008). For example, next-generation transcriptome sequencing (RNA-seq) has been validated as an efficient methodology for production of genomic sequences by assembling short-read sequences (Xia et al., 2011). RNA-seq is particularly attractive for non-model organisms, like coconut, with genomic sequences that are yet to be determined. However, implementing these RNA-Seq strategies has still provided a challenge, as abundant and high quality RNA is required for this kind of analysis. This is especially true for coconut and related species (such as oil palm, date palm and areca palm) which grow in tropical regions, as these are usually rich in polysaccharides and polyphenolics, and have leaves with a waxy cuticle and high fibre content (Angeles et al., 2005). Polysaccharides are known to be easily co-precipitated with RNA during the extraction process. Moreover, polyphenolic components in these samples are readily oxidized and can bind to RNA. Subsequent to extraction, samples contaminated with polysaccharides and polyphenolics may cause RNA degradation and be problematic for RNA-Seq analysis. Some methodologies and commercial kits have been successfully used to extract RNA from different plant tissues. Invitrogen TRIZOL Reagents are commercially available kits applied widely for rapid RNA isolation in species such as Arabidopsis (Box et al., 2011), rapeseed (Pant et al., 2009) and rice (Shi et al., 2007). However, Invitrogen TRIZOL Reagents have proved unsuitable for extracting RNA from plant tissues rich in polysaccharides and polyphenolics (Tattersall et al. 2005). Tiangen RNAplant reagent kits (Tiangen Company) were developed to isolate high-quality ribonucleic acid from plant tissues rich in polyphenolics and

starch, such as potato tubers, banana, apple and pear (Zheng et al., 2011). Alemzadeh et al. (2005) also described a modified Lithium chloride method that kept the temperature low during the process of RNA isolation. This method was successfully used for isolating good-quality RNA from plant tissues rich in phenolic compounds. The CTAB (Cetyltrimethylammonium bromide) method provides a low cost protocol for RNA isolation from a wide range of plant tissues. However, this method is time-consuming, and RNA extracted with this method may be degraded during the long extraction process. Another problem for this method is that the presence of contaminating DNA often requires an additional step for DNA removal, which also increases the possibility of RNA degradation. Here, we describe a novel protocol (MRIP) for extracting high quality total RNA in 1-2 hours after sampling from coconut and related species. Total RNA isolated using this protocol was suitable for RNA-Seq analysis with high quality data output. The extracted RNA can also be used for quantitative real-time RT-PCR.

Results and Discussion

Comparison of different methods for RNA isolation from the young leaves of coconut

RNA extraction is essential for molecular investigations, such as gene expression profiling, construction of cDNA libraries and more recently RNA-Seq. Numerous protocols have been developed for isolation of RNA from plant tissue. To validate the suitability of commonly used RNA isolation protocols for coconut leaves, we compared CTAB extraction, Tiangen RNAplant Reagent and Invitrogen TRIZOL protocols with MRIP for total RNA isolation from young coconut leaves. The integrity of RNA extracted by different RNA extraction methods was examined by gel-electrophoresis on a 1.5% agarose gel (Fig.1). From the electrophoresis diagram, in the RNA sample extracted with MRIP protocol, the 28S, 18S and 5S rRNA bands were clearly visible without the presence of smearing. In addition, the 28S rRNA band was significantly more intense than the 18S rRNA band, which is an indicator of good RNA quality. As shown in Figure 1, the other three RNA extraction methods (CTAB extraction, Tiangen RNAplant Reagent and Invitrogen TRIZOL Reagent) are not suitable for RNA isolation from young tissues of coconut due to RNA degradation and DNA contamination. A clear smear was found with contaminating DNA in RNA samples isolated with the CTAB and RNAplant protocols, suggesting RNA extracted with these two methods has been degraded. In the electrophoresis diagram for RNA isolated with Invitrogen TRIZOL Reagent, 28S and 18S rRNA bands were absent but an intensive low-molecular weight band was visible, indicating the RNA isolated using Invitrogen TRIZOL Reagent had been completely degraded into low-weight RNA molecule fragments which have the same size as 5S rRNA. Commercial kits that have showed some success in isolating high quality RNA from the Palmaceae species were also validated in our study. Using the TRIZOL method, a massive coloured pellet was precipitated with isopropanol and was difficult to dissolve in nuclease-free water. Obviously, the TRIZOL method cannot efficiently separate RNA from proteins and other organic compounds in coconut tissues with high levels of polysaccharide and polyphenol. Subsequently, these high levels of impurities may lead to the complete degradation of RNA molecules. Although Tiangen RNAplant reagent kits (Tiangen Company) were developed to isolate high-quality ribonucleic acid from plant tissues rich in polyphenolics and starch, this method did not work well with

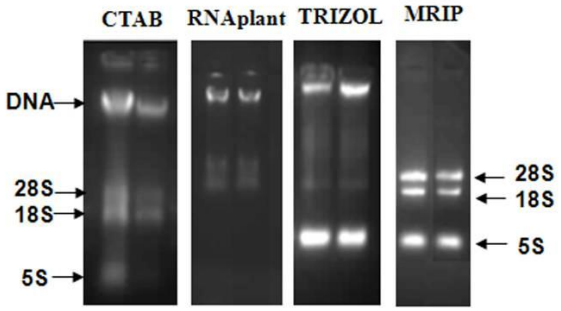

Fig 1. Electrophoresis diagram of RNA isolated from young leaves of coconut with different protocols: CTAB, Tiangen RNAplatn, Invitrogen TRIZOL and a MRIP extraction protocol.

coconut palm leaf samples. Using the RNAplant method, large quantities of DNA molecules were precipitated, but the RNA yield was very low. The RNA solution obtained using the RNAplant method was also still sticky, indicating that polysaccharides were not removed during the process of RNA extraction. In oil palm (an important oil crop in the Palmaceae family), the CTAB protocol has been used to extract total RNA from suspension cells and from embryogenic callus for subsequent RT-PCR and real-time RT-PCR (Tranbarger et al., 2012; Low et al., 2008). However, this method is not suitable for isolating RNA from the leaves of coconut palm as shown on Fig. 1. Similar to the TRIZOL method, the coloured RNA pellet obtained by the CTAB method was difficult to dissolve. Moreover, the CTAB method is time consuming and very laborious. In comparison to these conventional RNA-isolation methods, the MRIP protocol is more robust and effective for tropical palm species. The combined use of ammonium thiocyanate, guanidine thiocyanate and acidic phenol in the lysis buffer has been validated to be able to remove more organic compounds and improve $A_{260/A280}$ ratios (Bilgin et al., 2009). In our study, the $A_{260/280}$ ratios obtained using the MRIP method varied from 1.86 to 2.08, yet the ratio for other conventional methods ranged from 1.1 to 1.42. The combined use of ammonium thicyanate, guanidine thiocyanate and acidic phenol in the lysis buffer also inactivates nucleases on contact and prevents RNA degradation by endogenous ribonucleases, thereby increasing the RIN (RNA integrity number) (Schroeder et al., 2006). Moreover, to simplify the RNA isolation procedure, only a single step extraction using chloroform and a single step precipitation using isopropanol were used in the MRIP protocol, such that total RNA could be obtained in 1-2 h after sampling. The quality of RNA isolated with the CTAB and the MRIP protocols was also assessed by an Agilent 2100 Bioanalyzer microfluidic electrophoresis chip, and the results were consistent with the agarose gel electrophoresis analysis of the RNA (Fig.2). RNA isolated with the MRIP protocol exhibited three peaks, with the highest peak corresponding to the 28S rRNA, an rRNA 28s/18s ratio of 2.3 and a RIN (RNA integrity number) (Schroeder et al., 2006) value of 9, indicating that the mRNAs were of good integrity. However, more than four peaks were found in RNA isolated with the CTAB protocol: the additional peak between the 18S and 5S bands may come from the degradation of 28S or 18S rRNA. The rRNA 28s/18s ratio was 0.7 and the RIN value was 5.5, which suggests that the mRNAs had been partially degraded. The gel electrophoresis and the microfluidic electrophoresis correspondingly indicated that the MRIP protocol is suitable for isolating high quality RNA from coconut leaves.

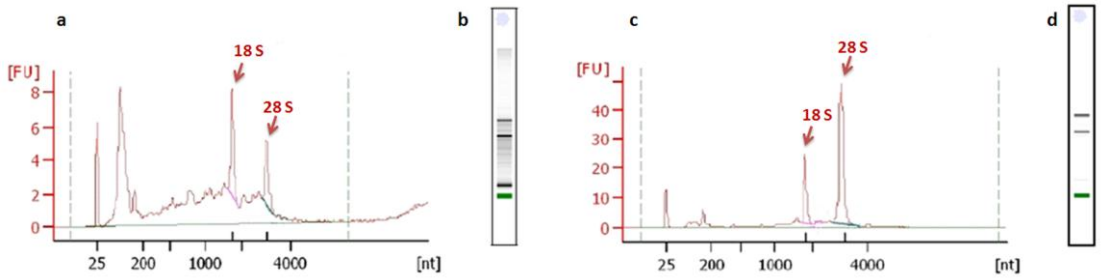

Fig 2. The quality of the RNA isolated using CTAB and MRIP methods measured with an Agilent 2100 Bioanalyzer microfluidic electrophoresis chip. (a) The electropherogram of the RNA samples extracted with CTAB method helps to determine the intensity of each band on the gel shown on (b). (c) The electropherogram of the RNA samples extracted with MRIP method helps to determine the intensity of each band on the gel shown on (d). The arrows indicate 28S and 18S ribosomal bands. (FU = Fluorescence Units, nt = nucleotide)

RNA-seq analysis of RNA isolated with the MRIP protocol

The total RNA extracted with the MRIP protocol was used for RNA-seq analysis to see if the quality was suitable for that purpose. The RNA samples isolated from young leaves, fruit flesh and spear leaves were mixed for subsequent mRNA preparation, fragment and cDNA synthesis. Two independent Illumina sequencing processes produced 54,931,406 reads of short sequences, containing a total of 4,943,826,540 nucleotides (nt), with an average sequence length of 90 bp for each short read, similar to that described in previous works (Xia et al., 2011; Gibbons et al., 2009). These short reads were then combined into 127,952 contigs with an average sequence length of 344 bp using Trinity, a *de novo* assembler for RNA-seq, following the protocol proposed by Grabherr et al. (2011). Sequence lengths of assembled contigs varied from 100 to more than 3000 bp. As sequence lengths of contigs increased, the number of contigs decreased. Subsequently, using pair-end joining and gap-filling, the short reads were re-aligned onto contigs, and contigs from the same transcript and the distance between these contigs was determined. Trinity software suite (Grabherr et al., 2011) was then used to join these contigs into longer sequences until the sequence could not be extended at either end. These assembled sequences were termed unigenes (unique genes). We obtained 57,304 unigenes. The sequence length distribution of the unigenes is shown in Figure 4. Sequence length for 17,889 unigenes was between 300 and 400 bp. With increases in sequence length, the number of unigenes decreased. There were 1104 unigenes with sequence lengths of more than 3000 bp (Fig. 3). The other unigenes varied from 400 to 3000 bp in sequence length. The average size of these unigenes was 752 bp. This value is higher than or equal to the average sizes reported by recent related researches (Xia et al., 2011; Gibbons et al., 2009; Guo et al., 2011; Gahlan et al., 2012; Zhang et al., 2012; Feng et al., 2012; Wei et al., 2011). These results suggest that using the MRIP protocol to isolate RNA from young tissues of coconut is compatible with RNA-Seq analysis.

Quantitative real-time RT-PCR

To further confirm the quality of total RNA extracted by this protocol, serial dilutions (1:10, 1:100, 1:1000 and 1:10000) of single strand cDNA prepared from the total RNA isolated with this protocol was used for quantitative real-time RT-PCR. A dilution series of the cDNA samples showed normal amplification curves, and amplification efficiency was close to one (Fig. 4). Both genes (28S rRNA and Actin genes) used in RT-PCR showed the similar result and the quality of the extracted RNA met the demand of such basic experiment' needs.

Isolating RNA from young leaves of 15 Palmaceae species

Palmaceae species are ubiquitous in tropical regions, with approximately 2800 species distributed among 202 genera. Some of these species are of economic importance in tropical regions and have a long history of cultivation, such as coconut, oil palm and areca palm. To see if the MRIP protocol is also suitable for total RNA isolation from wider range of Palmaceae species, we took samples of young leaves of 15 Palmaceae species in liquid nitrogen for subsequent RNA isolation. High quality total RNA could be isolated in 12 out of 15 Palmaceae species using the MRIP protocol: *Serenoa repens*, *Trachycarpus fortunel*, *Areca catechu*, *Borassus flabellifera*, *Bactris gasipaes*, *Elaeis guineensis*, *Pritchardia gaudichaudii*, *Cocos nucifera*, *Chamaedorea elegans*, *Areca triandra*, *Ravenea rivularis* and *Pritchardia pacifica* (Fig. 5). On the electrophoresis diagram in lane 2 with the sample from *Wodyetia bifurcate*. only a low-molecular weight band was visible, which corresponded to 5S rRNA. In lanes 9 and 12 with RNA samples isolated from the other two Palmaceae species (*Hyophorbe lagenicaulis*, *Carvota ochlandra*), 28S, 18S and 5S rRNA bands were not detected after electrophoresis, indicating low or no yield of RNA. These results suggest that the protocol did not work equally well on all Palmaceae species tested. This is not surprising since the RNA isolation is affected by the nature of the cell wall and central vacuole, which vary by species in dissolved organic and inorganic compounds. Hence, the same cell disruption methods will have different efficacies between species, therefore affecting the final quality and yield of the RNA isolated. Another confounding effect may come from rich secondary metabolites contained in the central vacuole, which may react with RNA during the extraction to the detriment of the extraction. Therefore, further optimization may be required for some of the palm trees, although the MRIP protocol could be used for RNA isolation from most of the Palmaceae species tested.

Materials and Methods

Plant materials

The young leaves, fruit flesh samples from a Chinese coconut variety (Hainan Tall) were collected and frozen with liquid nitrogen for RNA isolation and subsequent RNA-Seq. Young leaves from another 15 Palmaceae species were also used to examine if the MRIP protocol could be used for RNA

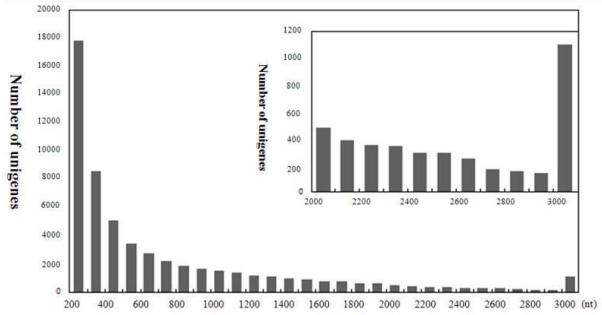

Fig 3. The length distribution of the assembled unigenes from next-generation transcriptome sequencing of coconuts. The inset gives an enhanced view of distribution of long unigenes over 2000 bp.

extraction across a range of Palmacease species: *Serenoa repens*, *Wodyetia bifurcate*, *Trachycarpus fortunei*, *Areca catechu*, *Borassus flabellifera*, *Bactris gasipaes*, *Elaeis guineensis*, *Pyitchardia gaudichaudii*, *Hyophorbe lagenicaulis*, *Cocos nucifera*, *Chamaedorea elegans*, *Carvota ochlandra*, *Areca triandra*, *Ravenea rivularis* and *Pritchardia pacifica*.

Reagents

- ✓ Liquid nitrogen
- ✓ The MRIP extraction buffer (modified from Bilgin et al. (2009)): to make 100 ml of buffer add 3.05 g ammonium thiocyanate, 9.44 g guanidine thiocyanate and 3.33 ml 3M sodium acetate (pH 5.2), add 38 ml phenol and adjust pH to 5.0, then make up the volume to 100 ml with DEPC (Diethylpyrocarbonate) ddH_2O. The buffer should be handled in a fume hood as it contains phenol.
- ✓ Invitrogen TRIZOL Reagent
- ✓ Tiangen RNAplant Kit
- ✓ CTAB extraction buffer based on Tang et al. (2010)
- ✓ Chloroform
- ✓ Chloroform and isoamyl alcohol solution (24:1, V/V)
- ✓ Phenol, chloroform and isoamyl alcohol solution (25:24:1, V/V/V)
- ✓ Isopropanol
- ✓ 70% ethanol

Isolation of RNA using different methods and commercial kits

✓ The extraction procedure for the MRIP protocol:

1. Grind about 0.08g frozen tissue into fine powder with a mortar and pestle which have been pre-cooled with liquid nitrogen.
2. Immediately transfer pulverized tissue to a 1.5 ml tube with 1 ml MRIP extraction buffer, vortex, and then incubate at room temperature for 5 minutes.
3. Add 1/5 volume of chloroform and vortex thoroughly for 15 seconds, then incubate at room temperature for 5 min, centrifuge for 10 minutes at 12,000g at 4°C.
4. Carefully transfer the upper aqueous phase of the solution to a fresh, RNase-free tube, then add an equal volume of isopropanol, mix the solution by inverting the tubes and incubate for 10 minutes on ice.
5. Centrifuge for 10 min at 12,000g at 4°C then discard the supernatant. The RNA pellet is generally in the bottom of tube but occasionally will be present on the wall of the tube.
6. Add 1 ml of 70% EtOH, centrifuge for 5 minutes at 8,000g and discard supernatant.
7. Repeat step 6.
8. Remove all residual ethanol, dry pellet, and dissolve RNA with 20-30 µl of RNase-free water by pipetting gently for 1-2 minutes.

✓ The RNA isolation procedure for the Invitrogen TRIZOL Reagent and Tiangen RNAplant reagent was carried out according to the corresponding instruction manual.

✓ The CTAB extraction protocol was performed according to Tang et al. (2010).

RNA analysis

The quality of RNA extracted with different methods was examined by running 3 µl of each RNA sample on a 1.5% non-denaturing agarose gel electrophesis (Sambrook et al. 2000) and an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) microfluidic electrophoresis chip. RNA quality was also evaluated with an RNA integrity number (RIN) (Schroeder et al. 2006).

RNA sequencing

The RNA extracted with the MRIP protocol was fragmented using divalent cations under increasing temperature. The short RNA fragments were taken as a template to synthesize the first-strand cDNA with random hexamer primers and Super-script™ III (Invitrogen™, Carsbad, CA, USA). A mixture containing buffer, dNTPs, RNaseH and DNA polymerase I was used to synthesize second-strand cDNA. Short fragments were purified using a QiaQuick PCR extraction kit (Qiagen) and resolved with EB buffer for end reparation and poly (A) addition. Sequence adaptors were used to link to these short fragments. Gel electrophoresis was carried out to screen for suitable fragments to use as templates for PCR amplification. Finally the library consisting of 200 bp inserts was sequenced using an Illumina Hiseq™ 2000 (Illumina Inc., San Diego, CA, USA). The library was established according to the Paired-End Sample Preparation Kit protocol (Illumina). The library was linked to a paired-end flow cell containing complementary adapters, and then inverted sequences were amplified to create "clusters". The adaptors were designed for selective cleavage of the forward DNA strand after synthesizing the reverse strand during sequencing, and then the reverse strands were used for sequencing from the opposite end of these short fragments. The short sequences were read after removing adaptor sequences, empty reads and low quality sequences.

Transcriptome* de novo *assembly

Transcriptome *de novo* assembly was carried out using the short read assembling program Trinity following previously described methods (Grabherr et al. 2011). Inchworm in Trinity software was firstly used to combine short reads into contigs with certain overlap lengths conference to default values in trinity manual. Using pair-end joining and gap-filling, the short reads were re-aligned onto contigs. A minimum of three read pairs was used as the criteria to define the order and distance between two contigs to prevent the small fraction of chimeric reads from mis-assembling. With paired-end reads, contigs from the same transcript could be detected. Assembled contigs were then combined to obtain longer sequences which could not be extended on either end

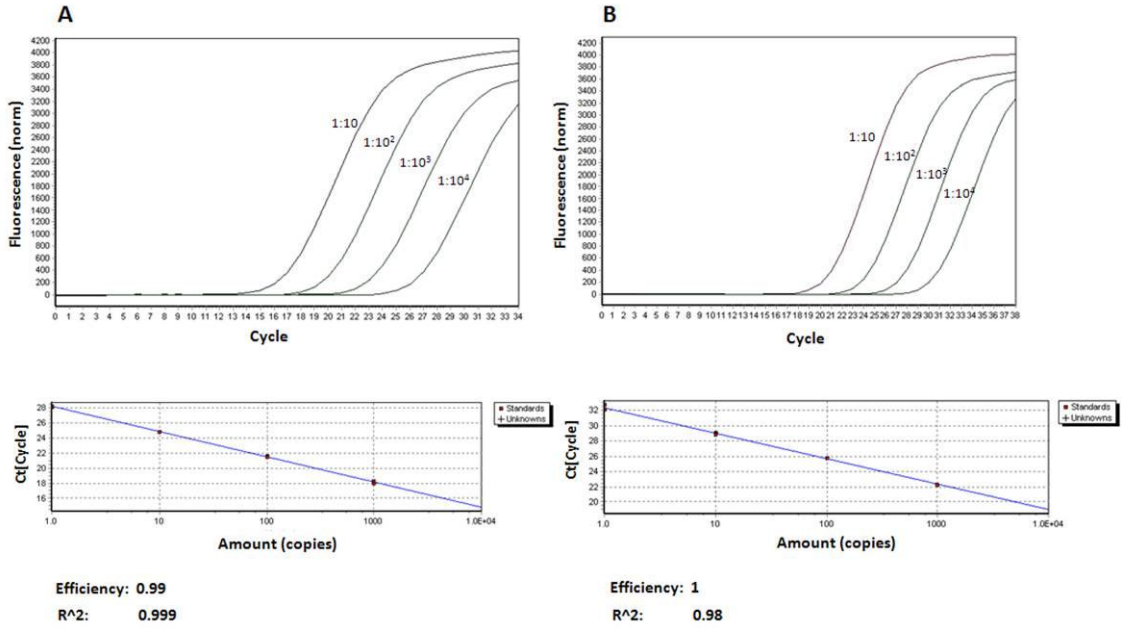

Fig 4. Amplification plot showing the expression level of housekeeping genes, 28S rRNA (A) and Actin genes (B), measured by Sybergreen qPCR. The cDNA was prepared using the total RNA isolated with the protocol described. The reactions were set up with serial dilutions of the cDNA, 1:10, 1:100, 1:1000 and 1:10000.

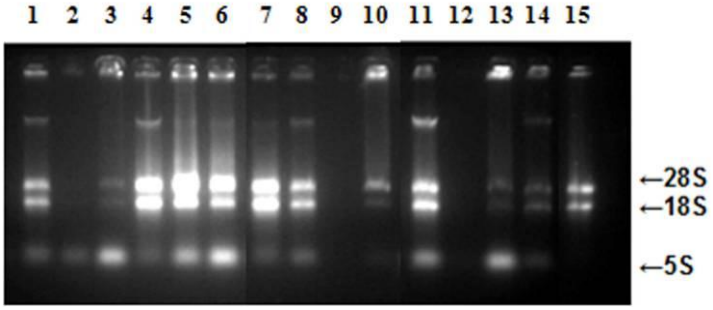

Fig 5. The electrophoresis diagram of RNA isolated from young leaves of 15 Palmaceae species using a MRIP protocol. Arabic numbers 1 to 15 represent different Palmaceae species: 1: *Serenoa repens*, 2: *Wodyetia bifurcate*, 3: *Trachycarpus fortunei*, 4: *Areca catechu*, 5: *Borassus flabellifera*, 6: *Bactris gasipaes*, 7: *Elaeis guineensis*, 8: *Pyitchardia gaudichaudii*, 9: *Hyophorbe lagenicaulis*, 10: *Cocos nucifera*, 11: *Chamaedorea elegans*, 12: *Carvota ochlandra*, 13: *Areca triandra*, 14: *Ravenea rivularis*, and 15: *Pritchardia Pacifica.*

using the Trinity software. Such sequences were defined as unigenes.

cDNA synthesis and real-time RT-PCR

To test the quality of the RNA obtained, first strand cDNA were synthesized according to manufacturer's instruction (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific). Briefly, RT reaction were conducted as follows: 1 µg total RNA and 1 µl oligo(dT)$_{18}$ primer (100 µM) were mixed and incubated at 65°C for 5 minutes, and then chilled on ice. The remaining reagents (5 × reaction buffer, 10 mM dNTP mix, Ribolock RNase inhibitor, RevertAid M-MuLV reverse transcriptase) were added and the mixture was incubated at 42°C for 60 minutes. The reaction mixture was heated to 70°C for 5 minutes to terminate the reaction. Real-time PCR was performed following a standard SYBR Premix Ex Taq^{TM} kit (TaKaRa) protocol in 96-well optical plates (Genuine AXYGEN Quality) using a final volume of 10 µl. The reactions were incubated in 0.1 ml tubes of Mastercyler ep realplex^4 (Eppendorf) machine as follows: 95°C for 30 s, then 40 cycles of the program (95°C for 5 s, 55°C for 15 s and 68°C for 20 s). The procedure ended by a melt-curve ramping from 60 to 95°C for 15 minutes.

Conclusion

Obtaining high quality RNA from plant tissue is an important step for the success of RNA-Seq analysis. However, isolating RNA from tropical palm species is made difficult by high concentrations of polyphenolics, polysaccharides, waxes and high fibre content. In this study, we describe a MRIP protocol for extracting high quality total RNA in 1-2 hours after sampling from coconuts. In isolating good-quality RNA molecules from tropical palm species, it is crucial to effectively remove organic compounds and prevent the degradation of RNA molecules in the extraction procedure. To separate RNA from DNA, proteins and high levels of polysaccharides and polyphenols, the combined use of ammonium thiocyanate, guanidine thiocyanate and acidic

phenol can facilitate the removal of organic compounds during the extraction procedure. To prevent the degradation of RNA molecules, two strategies were applied in our study. Firstly, simultaneous application of guanidine thiocyanate and acidic phenol was used to effectively inhibit the activity of numerous endogenous ribonucleases existing in the tissues of tropical palm species. Secondly, as a long RNA extraction procedure will increase the possibility of RNA degradation, only a single step extraction using chloroform and a single step precipitation using isopropanol were used in the MRIP protocol, so that total RNA could be obtained in 1-2 h after sampling. Total RNA isolated using this protocol was suitable for RNA-Seq analysis with high quality output. Therefore, this protocol provides an efficient and cheap total RNA isolation solution for coconut palm and most tropical palm species tested, although further optimization may be required on a case-by-case basis for some species and will have to be determined empirically.

Acknowledgements

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