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Extraction of High-quality RNA from Metabolite and Pectin Rich Recalcitrant Calyx Tissue of *Hibiscus sabdariffa* L.

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ABSTRACT

Hibiscus sabdariffa L. is no stranger to the field of pharmacology, as its calyx extract is highly rich in beneficial compounds and has been demonstrated to possess antihyperglycemic, antihypertension, anticancer and antioxidant properties. Thus, it is labelled as a functional food with great health benefits and therapeutic potentials. The medicinal and nutritional components of the calyx are well reported. On the contrary, not much is known about the molecular machineries governing the biosynthesis of beneficial compounds in this plant. Obtaining good yields of high-quality RNA is crucial for the success of downstream research pertaining to molecular biology. However, the presence of high quantities of phenolic compounds, polysaccharides, mucilage and pectin in the fibrous calyx tissue poses major challenges for RNA extraction in *H. sabdariffa*. Here, we modified a CTAB-based method for efficient extraction of high-quality RNA from the calyx tissue. High quality RNA samples having RNA integrity number of more than eight were successfully extracted. The purities of RNA samples were also confirmed by the A260/280 and A260/230 values. Subsequent successful preparation of a sequencing library using one of the RNA samples extracted via the modified CTAB method further emphasized the efficiency of this extraction protocol and quality of the RNA samples. The results showed that the modified CTAB method was effective in extracting good quality RNA from the challenging calyx tissue of

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Hibiscus sabdariffa L. suitable for sensitive downstream application.

Keywords: CTAB, high viscosity, mucilage, polysaccharide, RNA integrity, roselle, transcriptome profiling

INTRODUCTION

Ribonucleic acid (RNA) of high quality is the key to success for any downstream molecular work associated with transcriptomics such as cDNA library preparation, RNA-sequencing and real time quantitative reverse transcription polymerase chain reaction (RT-qPCR). RNA quality and quantity which emphasize on the yield, purity and reliable integrity require fundamental attention. However, with the high content of polyphenols, polysaccharides, and other secondary metabolites in flavonoid rich plants such as *Hibiscus sabdariffa* L. (Olaleye, 2007), it is difficult or perhaps impossible to extract pure RNA without contaminations. A major issue is the oxidation of phenols abundantly present in such tissues that forms quinone, an aromatic compound that binds RNA and inevitably affects downstream applications (Loomis, 1974). These polyphenolic compounds together with polysaccharides are able to bind and potentially co-precipitate with the nucleic acids (Gasic et al., 2004) leading to RNA extract of poor yield and low purity.

Many efficient and rapid extraction kits are developed and introduced to ease the challenges faced in RNA isolation from difficult plant samples. One example of such commercial kits is the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) that incorporates chemicals such as guanidine thiocyanate and guanidine hydrochloride in its extraction buffers, which enhance disruptive ability during the lysis step. In addition, the washing steps in the protocol that include two different types of wash buffers, one of which (Buffer RW1) contains the chemical guanidine thiocyanate further aids in the purification of the RNA samples. The potent binding capacity of the silica membrane is exceptionally sensitive and does not bind to 5S/5.8S rRNA, tRNA and other low-molecular-weight (LMW) RNAs. This alleviates the chances of high-quality RNA being extracted from tissue samples with profuse phenolic and other interrupting compounds. However, the cost incurred for RNA extraction using commercial kits are normally more expensive than conventional methods.

Thus, efforts to develop or modify conventional method for RNA extractions are continuously being carried out. CTAB extraction methods have consistently been used in extracting RNA in previous studies done on an array of troublesome plants including those that are rich in polysaccharides and phenolic compounds (Chang et al., 1993; Gambino et al., 2008; White et al., 2008; Wong et al., 2014). The extraction buffer that is based on cetyl trimethylammonium bromide (CTAB), a cationic surfactant that acts as a robust detergent, aids in breaking cell walls and in separating nucleic acids

from polysaccharides (Jaakola et al., 2001). Like many other conventional extraction methods, the CTAB method too includes four basic steps with the initiation of a nucleic acid extraction step, phase segregation step utilizing chloroform, and a precipitation step to separate the RNA from DNA, followed by a final washing step. Nonetheless, customized modifications to the basic CTAB protocol are almost always required in order to be suitable for use in each of the designated plant species.

The calyx tissue of *H. sabdariffa* is highly abundant in secondary metabolites and pectin. Pectin is a high-molecular-weight carbohydrate polymer, which poses additional challenges to RNA extraction from plant tissue. Hence, in this study, a CTAB-based protocol (Zeng & Yang, 2002) was improved with customized modifications (hereafter referred to as MCM) to suit our tissue sample. We had also extracted RNA from the same tissue type using Qiagen RNeasy Plant Mini Kit (hereafter referred to as RNeasy Kit), a guanidine-based protocol, for comparison. Comprehensive comparisons were made on the quantity and quality of the RNA samples extracted. One of the RNA samples extracted using the MCM was further tested for construction of RNA sequencing library. Based on the results obtained, we strongly recommend MCM protocol as a promising method to isolate high quality and quantity RNA from recalcitrant tissue such as the calyx of *Hibiscus sabdariffa* L.

MATERIALS AND METHODS

Materials

Calyx Tissue Sample. Calyx tissues were harvested at >25 days after blossoming, flash-frozen in liquid nitrogen, segregated into individually labeled bags and stored in a -80˚C freezer.

Chemicals, Reagents and Extraction Kit. All chemicals and reagents used were of molecular grade. Cetyltrimethyl ammonium bromide (Bio Basic Inc, Canada); disodium salt, dihydrate (Bio Basic Inc, Canada); chloroform (VWR Chemicals, USA); ethanol absolute (VWR Chemicals, USA); Tris hydrochloride (Amresco, USA); polyvinylpyrrolidone (Bio Basic Inc, USA); β-mercaptoethanol (Bio Basic, USA); isoamyl alcohol (Merck Schuchardt ohg, Germany); lithium chloride (Sigma-Aldrich, USA); sodium chloride (Sigma-Aldrich, USA); RQ1 RNase-free DNase (Promega, USA); diethyl pyrocarbonate (Bio Basic Inc, Canada); RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

DEPC-treated and RNase-free Apparatus. All the apparatus used for the RNA extraction were pre-treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) to remove any presence of RNase. All apparatus was immersed completely in DEPC-treatment solution overnight and autoclaved for 45 minutes at 121˚C. Subsequently, the apparatus was dried in an oven for 3 days and cooled down before use. The benches and working area were sprayed, wiped and

cleaned with 70% ethanol and RNaseOut™ solution (G-Biosciences, India), a chemical that acts as an inhibitor towards RNases.

Methods

Sample Grinding with Liquid Nitrogen. Preceding the addition of samples, the mortar and pestle were pre-chilled with liquid nitrogen. A total amount of 100mg calyx sample was pulverized into fine powder in liquid nitrogen using the pre-chilled mortar and pestle. The resulting talcum powder-like sample was then scraped quickly into a pre-chilled 1.5ml micro-centrifuge tube, sealed off with its cap and kept back into liquid nitrogen to maintain its frozen state before being extracted using the two methods discussed in the sections below. RNA extractions were conducted in triplicate for each method.

MCM Protocol.

Pre-preparation of Chemicals. The CTAB extraction buffer used had a composition of 2% (w/v) CTAB, 2% (w/v) NaCl, 100mM Tris-HCL, 25mM EDTA and 2% (v/v) dH_2O. Final addition of 2% (w/v) of polyvinylpyrrolidone (PVP) and 2% (v/v) β-mercaptoethanol were done separately and the buffer was warmed up in a water bath at 65˚C for 10 minutes prior to being used for extraction.

CTAB Protocol (Zeng & Yang, 2002) with Modifications. A total of 100mg of ground powder-like calyx sample was used for RNA extraction. The sample was allowed to slightly thaw right before the addition of 800µl pre-warmed CTAB buffer. The sample and CTAB buffer were mixed using a vortex and incubated in a water bath for 10 minutes at 65˚C. An equal volume of chloroform: isoamyl alcohol solution was then added into the mixture and mixed vigorously using a vortex to form a homogenized mixture. The homogenized mixture was centrifuged at 10,000 × *g* for 15 minutes at 4˚C to separate the mixture into different phases according to density. The upper aqueous phase containing nucleic acid was transferred into a new sterile 1.5ml microcentrifuge tube. A second addition of an equal volume of chloroform: isoamyl alcohol solution was done but mixed thoroughly with gentle inversions of the tube. Once completely mixed, the mixture was then centrifuged again at 10,000 × *g* for 10 minutes at 4˚C. The upper aqueous phase was transferred into a new 1.5ml microcentrifuge tube, and 0.33 volumes of 8M LiCl was added and mixed thoroughly via gentle pipetting. The sample was precipitated for 24 hours at -20˚C. After overnight precipitation, the sample was centrifuged for 35 minutes at 10,000 × *g* at 4˚C. The supernatant was carefully removed without disturbing the RNA pellet. The washing steps were commenced with subsequent additions of 75% ethanol, 80% ethanol, and 95% ethanol with repeated centrifugation at 10,000 × *g* at 4˚C and removal of supernatant, after each addition of ethanol. The pellet was air dried. A final volume of 20µl of RNase-free water was added onto

the pellet and mixed via gentle pipetting. The RNA sample was kept in -80˚C freezer immediately.

RNeasy Kit Protocol. Similarly, a starting material of 100mg of ground calyx sample was used and the RNeasy Kit protocol (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) was conducted based on the manufacturer's handbook.

DNase-treatment. The DNase-treatment of RNA samples extracted using MCM and RNeasy Kit were performed post extraction and on-column respectively using RQ1 RNase-Free DNase (Promega Corporation, Madison, USA) according to the manufacturer's instructions.

Quality and Quantity Check (QC) of Samples. The isolated RNA samples were electrophoresed on a 1% agarose gel in 1× TAE buffer and stained with 4% (v/v) Midori Green staining dye (Genetics Nippon, Genetics Europe GmBH). A 2-Log DNA Ladder was used (BioLab, New England) and the gel was visualized using the ENDURO™ gel imaging system (Labnet International, Inc, Edison, NJ, USA). Sample purity and yield, and RNA integrity number (RIN) were further evaluated by means of a Qubit Fluorometer 2.0 (Life Technologies Corporation, Carlsbad, USA), NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) respectively.

Preparation of Sequencing Library using Sample 3(b). A sequencing library was prepared using sample 3(b) extracted via MCM. Terminator™ 5'-phosphate-dependant exonuclease (Epicentre, Madison, USA) and ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre, Madison, USA) were used for rRNA-depletion and library construction respectively following the manufacturer's protocol. The ScriptSeq cDNA libraries were quantified using Qubit® 2.0 Fluorometer (Life Technologies Corporation, Carlsbad, USA) and the size distribution was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) via a high sensitivity DNA chip. In order to achieve optimum sequencing of reads, at least more than 60% of the libraries should fall between the size fragment range of 200-1000 bp stipulated on the electropherogram profile. Sequencing was performed by Illumina NextSeq 500 Sequencer Platform (Illumina, Inc, California, USA). Raw sequencing reads were further subjected to quality trimming and filtering, in which good quality reads were acquired and quantified using the Bowtie 2 (Langmead et al., 2009; Langmead & Salzberg, 2012) and BBDuk (Bushnell, 2014).

RESULTS

The RNA samples extracted using the MCM denoted as 3(a), 3(b), and 3(c), and those extracted using the RNeasy Kit denoted as 3(i), 3(ii), and 3(iii) as seen in the gel image presented in Figure 1 clearly

showed two apparent double bands in each sample. For all the six samples, the upper band conspicuously appeared brighter and much thicker than their respective lower bands. Moreover, minimal background and smearing were seen for all samples indicating minor to no occurrence of RNA degradation. Table 1 documents the yield, absorbance ratios of A260/230 and A260/280, and RNA integrity numbers (RIN) of these samples. On average, RNA samples extracted via MCM recorded an average yield of 6803.33ng; whilst RNA samples extracted through RNeasy Kit recorded an average yield of 4721.67ng, from 100mg of starting material each. Thus, this indicated that the MCM had exceeded the RNeasy Kit in terms of yield.

In the context of sample purity, the MCM isolated RNA samples have average A260/230 and A260/280 absorbance ratios of 1.70 and 1.78, respectively; whereas the RNeasy Kit had isolated RNA samples with an average absorbance value of 0.81 for A260/230 and 1.22 for A260/280. These absorbance values clearly showed that the purity of RNA samples obtained using MCM are notably closer to the ideal range 1.8-2.0 of standard values accepted for both A260/230 and A260/280 compared to those extracted using RNeasy Kit. Figures 2 and 3 show the bioanalyzer graph results recording the RIN values measured based on the development of two prominent peaks representing the 18S and 25S bands for all RNA samples alongside a digital gel image for samples 3(a), 3(b), and 3(c), and samples 3(i), 3(ii), and 3(iii) respectively. RNA samples obtained using MCM documented RIN values ranging from 8.00 to 8.20; whereas samples extracted using RNeasy Kit documented RIN values ranging from 7.60 to 8.50.

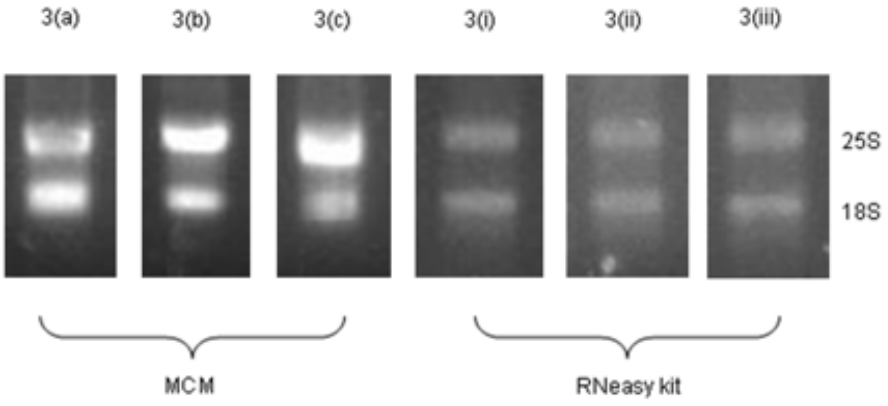

Figure 1. RNA samples of *Hibiscus sabdariffa,* samples 3(a), 3(b), and 3(c) extracted using MCM, and samples 3(i), 3(ii) and 3(iii) extracted using the RNeasy Kit electrophoresed on 1% agarose in 1× TAE buffer. The double bands represent an upper 25S and a lower 18S rRNA bands

Table 1

Yields, absorbance ratios and RNA integrity numbers (RIN) of RNA samples extracted from the calyx tissue of Hibiscus sabdariffa

Extraction Method	Sample	Yield (nanogram)	A260/230 ratio	A260/280 ratio	RIN
CTAB	3(a)	7150	1.58	1.63	8.10
	3(b)	4180	1.74	1.84	8.20
	3(c)	9080	1.80	1.88	8.00
RNeasy Plant Mini Kit	3(i)	4660	0.66	1.24	8.50
	3(ii)	4690	0.92	1.25	7.60
	3(iii)	4815	0.84	1.18	7.80

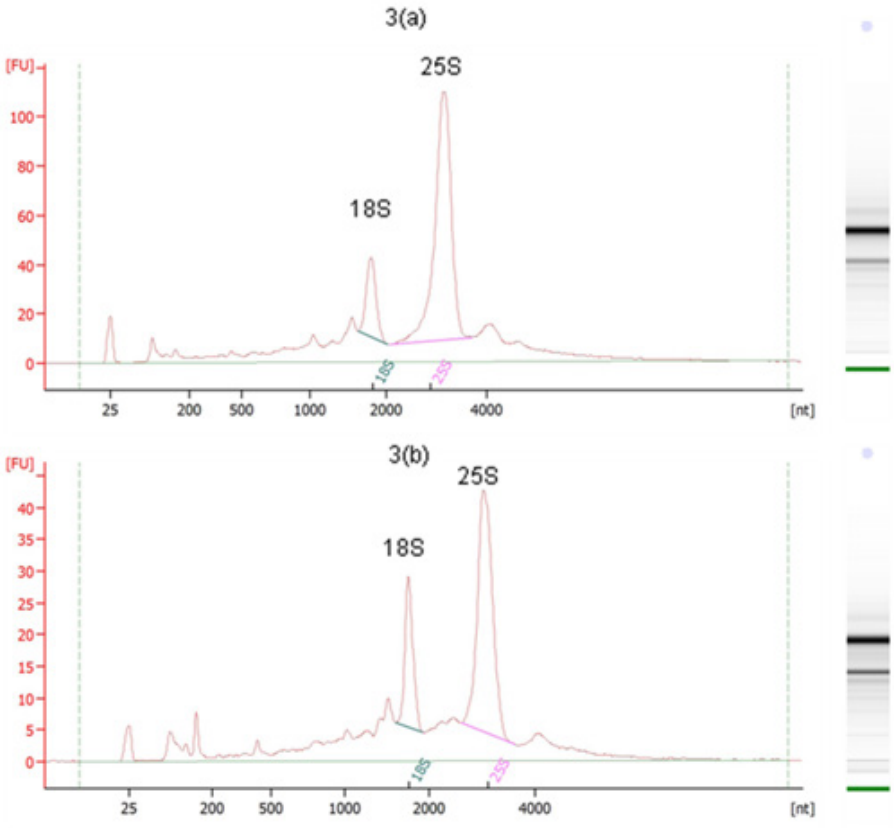

Figure 2. Digital image representations of the RNA bands and graphic representation of RNA integrity based on the two rRNA peaks (18S and 25S) for samples 3(a), 3(b), and 3(c) extracted from *Hibiscus sabdariffa* using the modified CTAB method (MCM)

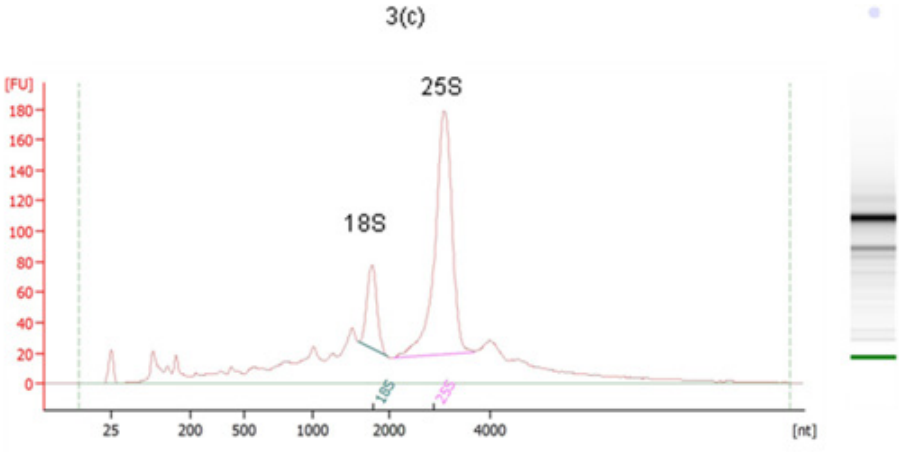

Figure 2. (Continued)

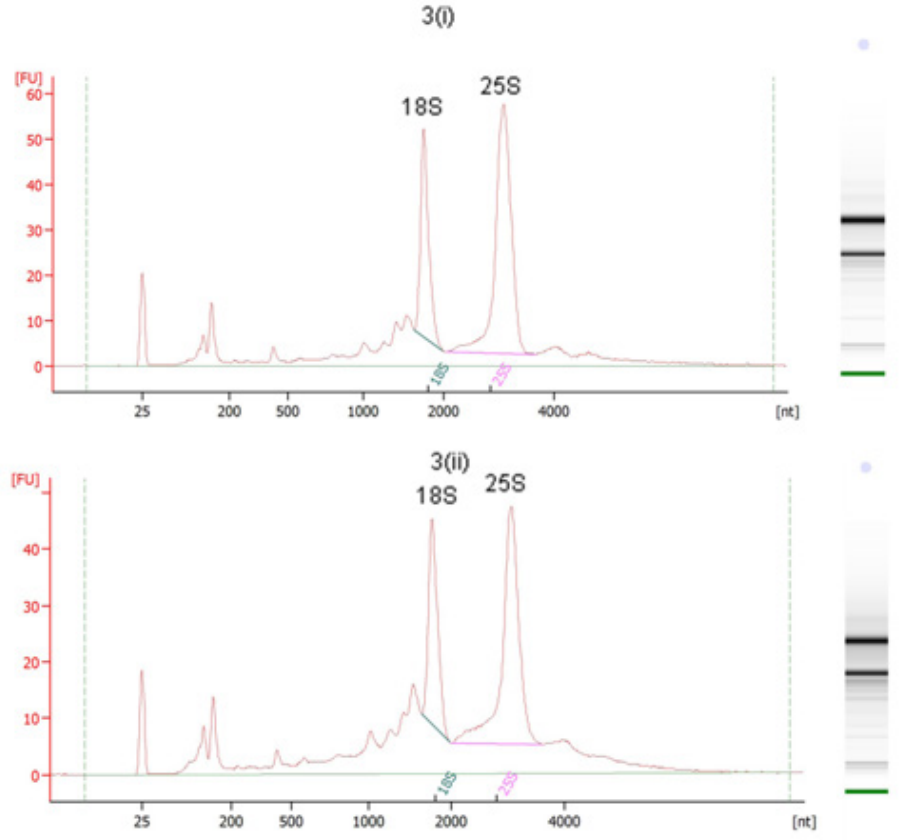

Figure 3. Digital image representations of the RNA bands and graphic representation of RNA integrity based on the two rRNA peaks (18S and 25S) for sample 3(i), 3(ii), and 3(iii) extracted from *Hibiscus sabdariffa* using RNeasy Kit

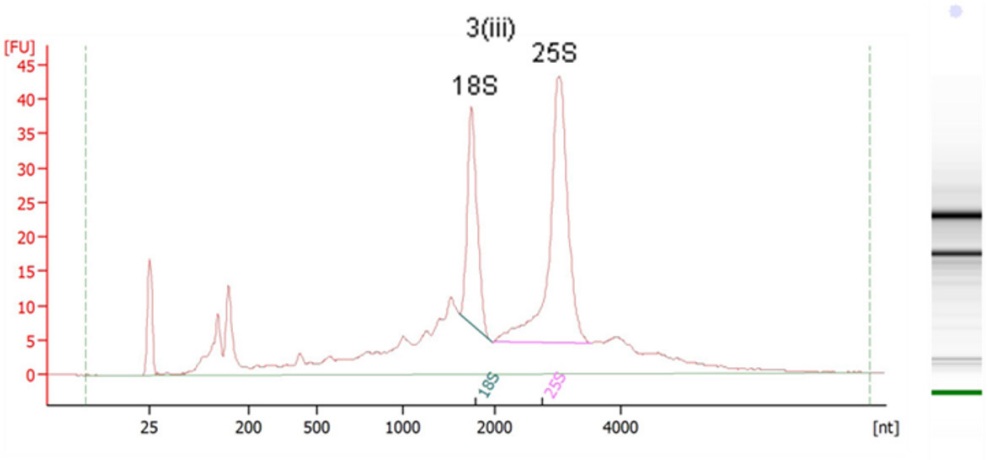

Figure 3. (Continued)

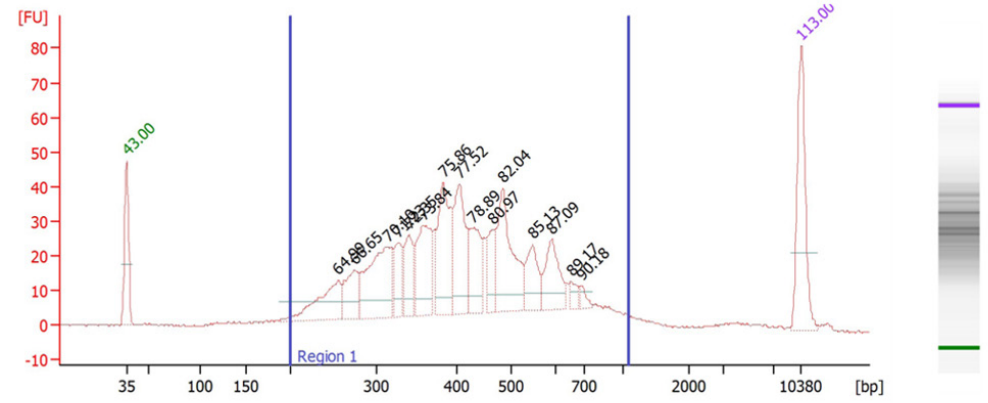

Figure 4. Electropherogram of the sequencing library prepared from sample 3(b)

One of the RNA samples 3(b) extracted using MCM was further tested for the preparation of RNA sequencing library. Quality of the RNA sample is a critical and fundamental factor in determining the quality of sequencing library and output data. The quality of the library was assessed using high sensitivity DNA assay on 2100 Bioanalyzer (Agilent Technologies, Germany). Figure 4 shows the electropherogram result of

sequencing library prepared from sample 3(b). The library had a concentration of 1295.95pg/µl and average fragment size of 433bp. Ninety-five percent of the fragment were distributed between 200-1079bp and size distribution (co-efficient variation) was 32.6%. Sixty percent of the sequencing output attained PHRED score quality ≥30 (reads with probability of error = 0.001), generating a total of 71,210,684 high-quality reads (unpublished data). Thus, the number of high-quality reads demonstrate a good and successful sequencing library preparation, which meets the requirements for subsequent *de novo* assembly alongside further downstream analysis.

DISCUSSION

One of the main problems faced in studying plant genetics is the difficulties in isolating high-quality nucleic acid (i.e. DNA and RNA) from plant tissue. RNA is important when gene expression profiling and functional genomics are the subjects of the study. The presence of phenolic compounds, polysaccharides, and various secondary metabolites in the fibrous calyx tissue of *Hibiscus sabdariffa* has made RNA isolation very challenging. The presences of mucilage and pectin in particular, makes complete homogenization of the calyx tissues impossible during isolation of RNA.

RNA extract with high yield or concentration is always desirable for downstream application such as preparation of sequencing library. In the context of RNA concentration, both methods yielded good amounts of RNA. However, on average, a difference of more than 2,000ng in yield between samples extracted via MCM and RNeasy Kit were recorded, which indicated the greater capacity of the MCM in extracting higher yields of RNA. Eukaryotic RNA is theoretically characterized as having double rRNA bands that are made up of an upper 25S band and a lower 18S band in plants. The presence of these two bands with the 25S band showing higher intensity is used as an indicator to reflect RNA intactness. In this study, the RNA samples extracted using MCM (Figure 1) clearly showed a thicker and more intense 25S band in relative to the lower 18S band, similar to those were extracted using the RNeasy Kit.

The RNA samples extracted from the calyx tissue using the two methods were intact in structure reflected by the reasonably superior RNA integrity numbers ranging from 7.60 to 8.50 as shown in Table 1, Figure 2, and Figure 3. The RIN values ranged from 1 to 10 with 10 representing the most intact RNA (Schroeder et al., 2006). RIN values were determined through the 25S and 18S peaks formed on the bioanalyzer graphs with the integration of algorithms. These peaks symbolize the quantity of rRNA molecules present in the samples which are mostly composed of cytosolic, chloroplastic and mitochondrial rRNAs (Kim & Haj-Ahmad, 2014). An ideal graph would show two prominent peaks with the 18S peak being lower in height in comparison to the 25S peak as demonstrated by samples extracted in this study. A noticeable difference in peak heights acts

as a strong indicator of intact RNA having minor/no degradation that consequently affects the RIN value (Schroeder et al., 2006). It had been shown in studies that RIN values can affect gene expression profile leading to misinterpretation of the result particularly in qPCR and RNA sequencing (Fleige & Pfaffl, 2006; Wang et al., 2016). Recommended RIN values of more than 5 were considered good; whereas RIN values of more than 8 were considered excellent in regards to total RNA most efficient for downstream work (Fleige & Pfaffl, 2006).

RNA purity as indicated by the A260/230 and the A260/280 ratios showed comparative deviations for samples extracted using both methods, from the ideal range of 1.8 to 2.0 ratio value that is generally considered as "pure" RNA sample (Skrypina et al., 2003). The three replicates of RNA extracts isolated using MCM recorded absorbance values ranged from 1.58 to 1.88, with an average value of 1.70 for A260/230 and an average value of 1.78 for A260/280. In contrast, for the RNA samples extracted using RNeasy Kit, the absorbance values deviated further from the range of acceptable purity with A260/230 and A260/280 ratios obtaining average values of 0.81 and 1.22, respectively. Deviation of the A260/230 and A260/280 absorbance ratios from the acceptable range of 1.8-2.0 may indicate either the contamination of phenol, guanidine or other organic salts, and the contamination of proteins in the DNA samples, respectively (Wang & Stegemann, 2010). Based on these values, it is worth noting that the MCM produced RNA extracts with less contaminants comparatively.

The customized modifications made to the CTAB protocol that was used initially in an oil palm study conducted by Zeng and Yang (2002) had led to effective results in enhancing RNA quality and quantity. In our initial attempt, adoption of the Zeng and Yang (2002) CTAB method with no modification had resulted in consistent failures with the persistent formation of either a brown pellet, absence of pellet or the inability of the pellet to dissolve completely during the elution step. Hence, either non-intact RNA was continually being produced or no RNA was extracted. Other conventional method that integrated sodium chloride precipitation and isopropanol precipitation in order to extract RNA from samples having high starch levels (Li & Trick, 2005) was also tested but without any success. Under the modified conditions, the starting material used was decreased to 100mg from the 500-700mg suggested by Zeng and Yang (2002). Nevertheless, the rest of the procedure was not scaled down despite the reduced amount of plant starting material but was maintained the same as that used for 500-700mg of oil palm material. This was due to the consistency of the calyx tissue sample that turned thick and viscous upon homogenization with the extraction buffer because of the presence of pectin and mucilage. Initial trials using the minimum amount of 500mg suggested in the CTAB protocol by Zeng and Yang (2002) consistently led to incomplete breakage of cell walls and separation of nucleic acid due

to the challenging nature of the calyx tissue. Moreover, the tough and fibrous nature of the calyx tissue lengthened the time required to completely mix the sample in the extraction buffer after grinding, to the extent of allowing oxidation to occur rapidly. This unavoidably had led to the degradation of the nucleic acid. Thus, a reasonable amount of 100mg of sample was found to work well with the protocol due to the nature of the tissue sample used after several attempts. The extraction buffer was able to work well at homogenizing the sample entirely with the reduced sample input amount.

The ingredients used for the extraction buffer were like those of the Zeng and Yang's (2002) method, with only two changes made. Firstly, the addition of PVP was done separately just before warming up the CTAB buffer. PVP helps to prevent the oxidation of polyphenols in cell walls (Loomis & Battaile, 1966) and the addition of PVP into the CTAB buffer just prior to extraction prevented the formation of colloids and ensured no alteration was done towards its main function. Secondly, the inclusion of 0.05% spermidine trihydrochloride in Zeng and Yang's (2002) protocol was omitted since it binds and precipitates DNA rather than RNA. In our protocol, the separation between these two nucleic acids (i.e. RNA and DNA) was done solely using LiCl precipitation which had aided in reduction of steps. Precipitation was optimally effective at a molarity of 2.67M rather than 2.5M used in the original procedure of Zeng and Yang (2002). The increase in molarity had resulted in a better precipitation of RNA.

As for the protocol, the RNA samples were precipitated at -20˚C during the 24 hours incubation period rather than at 4˚C as was done by Zeng and Yang (2002). Lastly, the washing steps were also improved to suit the calyx RNA sample whereby three consecutive washing steps were performed using three different concentrations of ethanol in order to gradually wash off any unwanted residual compounds meticulously. As such, 75% and 80% ethanol were used to separate and dissolve residual compounds such as salts present in the sample and 95% ethanol was used to bind any leftover water clinging to the RNA pellet to allow swift drying of the pellet before elution. The combination of all these modifications had yielded high quality RNA from recalcitrant calyx tissue of the *Hibiscus sabdariffa* L. reproducibly.

CONCLUSION

The overall results of the RNA quality for the samples extracted using the CTAB method adapted from Zeng and Yang (2002) with modifications (MCM) and the RNeasy Plant Mini Kit (RNeasy Kit) produced comparatively similar qualities in terms of the RIN values. Both extraction protocols produced credible RNA qualities with RIN values exceeding 7.0. However, the absorbance values and yields of the RNA obtained via these two methods differed substantially with MCM scoring slightly higher points above the RNeasy Kit. Hence, in this study it is evident that MCM is efficient to extract RNA of excellent quality and quantity from the calyx tissue of roselle,

which allows downstream molecular studies on this important crop. We are optimistic that the modified CTAB method (MCM) developed in this study will be useful for RNA extraction from other difficult plant tissues of similar nature.

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