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Efficient oil palm total RNA extraction with a total RNA extraction kit

S.H. Habib[1,2], H.M. Saud[1] and H. Kausar[3]

[1]Department of Agriculture Technology, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[2]Oilseed Research Centre, Bangladesh Agricultural Research Institute, Joydebpur, Gazipur, Bangladesh
[3]Laboratory of Food Crops, Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Corresponding author: S.H. Habib
E-mail: hasna_brri@yahoo.com

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ABSTRACT. Oil palm tissues are rich in polyphenols, polysaccharides and secondary metabolites; these can co-precipitate with RNA, causing problems for downstream applications. We compared two different methods (one conventional and a kit-based method - Easy-Blue™ Total RNA Extraction Kit) to isolate total RNA from leaves, roots and shoot apical meristems of tissue culture derived truncated leaf syndrome somaclonal oil palm seedlings. The quality and quantity of total RNA were compared through spectrophotometry and formaldehyde gel electrophoresis. The specificity and applicability of the protocols were evaluated for downstream applications, including cDNA synthesis and RT-PCR analysis. We found that the conventional method gave higher yields of RNA but took longer, and it was contaminated with genomic DNA. This method required extra genomic DNA removal steps that further reduced the RNA yield. The kit-based method, on the other hand, produced good yields as well as well as good quality RNA, within a very short period of time from a small amount of

starting material. Moreover, the RNA from the kit-based method was more suitable for synthesizing cDNA and RT-PCR amplification than the conventional method. Therefore, we conclude that the Easy-Blue™ Total RNA Extraction Kit method is suitable and superior for isolation of total RNA from oil palm leaf, root and shoot apical meristem.

Key words: TLS; Total RNA extraction; Easy Blue™ Total RNA Extraction kit; RT-PCR

INTRODUCTION

Oil palm is one of the most important oil crops in the world, with yields tenfold higher than for soybean (Nair, 2010). Still, there is a huge gap in between demand and supply of palm oil. To fulfill the demand of an ever increasing population, an improvement both in yield and quality of palm oil is crucial (Sambanthamurthi et al., 2009). *In vitro* micro-propagation based on somatic embryogenesis is an appropriate solution for mass propagation of elite oil palm varieties with desired characteristics such as high oil yield, slow vertical growth and disease resistance (Low et al., 2008). However, large-scale clonal propagation is an obstacle due to the occurrence of truncated leaf syndrome (TLS) somaclonal abnormality (Tan et al., 1996). The detailed morphology of TLS seedlings compared to their normal counterparts has been reported by Habib et al. (2012a,b). They also suggested that TLS abnormality could be due to high production of cytokinin and brassinosteroid. However, the molecular mechanism of TLS occurrence has not yet been reported. To study the molecular mechanism behind the TLS occurrence, high-quality RNA needs to be extracted from TLS tissues.

Most of the plant material contains high levels of polyphenols and polysaccharides that decrease RNA yield and quality (Tai et al., 2004; Wang et al., 2008). The extraction of intact RNA is also difficult due to the enzymatic degradation of RNA by RNase present in the tissues (Rubio-Piña and Vázquez-Flota, 2008). Due to the presence of polyphenols or polysaccharides, many RNA isolation methods result in a very low yield of RNA or form complexes with these contaminants resulting in low quality poly (A+) RNA, which is unsuitable for first strand cDNA synthesis and RT-PCR analysis (Koonjul et al., 1999).

High-quality RNA is the primary requirement for molecular cloning and gene expression studies (Wanqian et al., 2005; Wang et al., 2008). There are various RNA isolation protocols for different species and for the same species grown under different environmental conditions (Salzman et al., 1999; Li et al., 2006; Chun et al., 2008; Thanh et al., 2009; Takahashi et al., 2010). Researchers utilize sodium dodecyl sulfate (SDS), soluble polyvinyl pyrrolidone (PVP) and ethanol steps to isolate RNA from plants rich in phenolics and polysaccharides (Dong and Dunstan, 1996). Acetone treatment is a representative protocol for freeze-dried and powdered plant materials (Schneiderbauer et al., 1991). Many researchers have modified the cetyl trimethyl ammonium bromide (CTAB) method to isolate RNA (Apt et al., 1995; Gareth et al., 2006; Wang et al., 2008). Several commercial kit reagents are available for isolating RNA from plants, such as Trizol (Gibco-BRL Life Technologies) and RNeasy plant kit (QIAGEN), but they are not effective for all plant tissues (Hou et al., 2011). So far, there are no standard methods for the isolation of high-quality RNA applicable for all plant species. Therefore, this study was undertaken to select a more sensitive total RNA extraction technique

by comparing the conventional method of Rochester et al. (1986) and a kit-based method by spectrophotometry and agarose gel electrophoresis as well as downstream application of cDNA synthesis and RT-PCR analysis.

MATERIAL AND METHODS

Plant materials

Tissue culture-derived six-month-old (TLS) somaclonal oil palm seedlings were collected from Felda Agricultural Services Sdn Bhd, Malaysia. After collection, the plants were uprooted, washed thoroughly with water, and leaves, roots and shoot apical meristems (SAM) were separated with a scalpel. The leaves, roots and SAMs were immediately frozen in liquid nitrogen and stored separately at -80°C until the RNA was extracted.

Total RNA extraction from SAM, leaf, and root using the Rochester et al. (1986) method with modification

Approximately 0.1 g oil palm SAM, leaf, or root was ground in liquid nitrogen to a very fine powder with a pestle in a pre-chilled mortar. The ground tissues were then transferred to a 1.5-mL microcentrifuge tube containing 500 µL lysis buffer [1 % (w/v) sodium-N-lauroyl sarcosinate, 6 % (w/v) 4-aminosalicylic acid sodium salt, 100 mM Tris-HCl, pH 7.6, 50 mM ethylene glycol tetraacetic acid (EGTA), pH 7.5, 100 mM NaCl, 1% (w/v) SDS and 50 mM 2-β-mercaptoethanol]. An equal volume of phenol:chloroform:isoamylalcohol [PCI = 25:24:1(v/v/v)] was added to the tube. The buffer and tissues in PCI were mixed vigorously. The mixture was then centrifuged at 10,000 *g* for 10 min at room temperature to separate the mixture into two phases. The top aqueous phase was transferred to a new tube. An equal volume of PCI was added to the tube and the mixture was mixed for approximately 5 min before centrifuging under the same conditions to separate the phases. The extraction was repeated with an equal volume of PCI until a white protein interface was observed. A final extraction was performed to remove traces of phenol with an equal volume of chloroform:isoamylalcohol [CI = 24:1(v/v)].

A one-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% (v/v) ethanol were added to the recovered aqueous phase to precipitate nucleic acid. The mixture was then stored at -80°C until frozen. The nucleic acid was then pelleted by centrifugation at 10,000 *g* for 25 min at 4°C. The pellet was rinsed with 70% ethanol and air-dried. The pellet was then dissolved in DEPC-treated water, with one-half of the original lysis buffer volume. An equal volume of cold 4 M LiCl was added, and the suspension was mixed vigorously and stored at 4°C for 2 h. The RNA was then pelleted by centrifuging at 10,000 *g* for 25 min at 4°C. The supernatant was discarded and the pellet was rinsed with 70% ethanol. The pellet was dissolved in DEPC-treated water, with one-tenth original volume of lysis buffer. An equal volume of cold 4 M LiCl was added and the suspension mixed vigorously. The tube was stored at 4°C for 1 h, and the RNA was then pelleted by centrifuging at 10,000 *g* for 25 min at 4°C. The supernatant was discarded and the pellet was rinsed with 70% ethanol. The RNA pellet was dissolved in 100 µL DEPC-treated water and recovered by precipitation in the presence of a 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol. The mixture was then stored at -80°C until frozen. The RNA was then pelleted by centrifuging at 10,000 *g*

for 25 min at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol and air-dried. The nucleic acid was dissolved in 30 µL DEPC-treated water and stored at -80 °C for further applications.

Total RNA extraction from SAM, leaf, and root using the Easy-Blue™ Total RNA Extraction kit

Total RNA from SAM, leaf, and root of TLS seedlings were extracted using the Easy-Blue™ Total RNA Extraction kit (iNtRON Biotechnology, Inc. Korea). Approximately, 0.1 g frozen tissue was ground in liquid nitrogen to a very fine powder with a pestle in a pre-chilled mortar. The ground material was transferred to a 1.5-mL microcentrifuge tube containing 1 mL buffer (provided in the kit). The tube was then mixed vigorously for 10 s, and subsequently, 200 µL chloroform were added. After vigorously mixing the components, the tube was then centrifuged at 4°C for 10 min at 11,000 *g*. A volume of 400 µL upper aqueous phase was transferred to a new tube and 250 µL P-buffer (0.8 M sodium citrate and 1.2 M NaCl) and 250 µL isopropanol (2-propanol) were added to the tube. The mixture was mixed well by inverting the tube 2-3 times followed by incubation for 10 min at room temperature. The tube was then centrifuged for 5 min at 4°C at 11,000 *g*. After discarding the supernatant, the pellet was washed twice with 75% ethanol and air-dried. The nucleic acid was dissolved in 30 µL DEPC-treated water and stored at -80°C for further applications.

Quantity and quality of total RNA

The quality and quantity of RNA were determined by taking spectrophotometer (Implen Gmbh, Germany) readings at 230, 260, and 280 nm. One absorbance unit at 260 nm corresponds to 40 µg/mL RNA. According to Sambrook et al. (1989) pure RNA has an A_{260}/A_{280} ratio of between 1.8 and 2.0. To determine the quality, the RNA was also separated on a denaturing formaldehyde agarose gel.

Denaturing formaldehyde agarose gel electrophoresis

The total RNA was electrophoresed in 1.5% (w/v) formaldehyde denaturing gels according to the method of Sambrook et al. (1989). A 1.5% formaldehyde gel of 25 mL was prepared by mixing 0.375 g agarose with 18.45 mL sterile DEPC-treated distilled water, and 1X MOPS buffer [10X MOPS (0.2 M MOPS), 20 mM sodium acetate, 10 mM EDTA, pH 8.0]. The mixture was heated until the agarose melted and was cooled to 55°C. Next, 37% (v/v) formaldehyde was added to the melted agarose to a final concentration of 6 % (v/v). The gel was then poured into a gel cast and allowed to harden. The running buffer used was 1X MOPS buffer with 6% formaldehyde.

The total RNA loaded into each well was approximately 2 µg (calculated according to spectrophotometer readings). RNA samples were prepared by adding RNA to 1X MOPS buffer, 6% formaldehyde, 50% (v/v) formamide and the necessary amount of DEPC-treated distilled water to a final volume of 15 µL. The mixture was then heated at 65°C for 15 min and immediately chilled on ice to denature the RNA sample. One microliter of loading dye (50% (v/v) glycerol, 10 mM EDTA, pH 8.0, 0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol) and 1 µL (1 mg/mL) ethidium bromide were added to the sample, which was then loaded

into the wells. The gel was run at 70 V for 1.5 h or until the blue dye had migrated about three-fourth of the gel. The gel was then viewed with an ultraviolet (UV) transilluminator (Bio-Rad, USA) to assess the quality of the RNA after electrophoresis.

cDNA synthesis and RT-PCR

The first-strand cDNA was synthesized from 1 µg total RNA using the QuantiTect® Reverse Transcription kit (Qiagen, USA) according to manufacturer instructions. A total of 50 ng first-strand cDNA was used for PCR to amplify the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene. GAPDH-specific primers were used: F (5'-GGTGACAGCAGGTCCAGCAT-3') and R (5'-ATCAAAGCCAGGCAAGCATC3'). The PCR mixture consisted of 50 ng first-strand cDNA, 0.125 µM forward primer, 0.125 µM reverse primer, 0.2 mM dNTP mix, 2.5 U Taq polymerase (MPOB) and 1X PCR reaction buffer (MPOB) in a 50-µL total volume. The reaction mixtures were subjected to PCR amplification with the following conditions: 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by an additional 5 min at 72°C. The PCR products were separated on a 1.5 % (w/v) agarose-ethidium bromide gel and viewed under UV light.

RESULTS

Quantity and quality of total RNA

Two methods were used to isolate total RNA. The methods used were a conventional Rochester et al. (1986) method with modification and the other was a kit-based method (Easy-Blue™ Total RNA Extraction kit). The quantity and purity of extracted RNA were determined using a spectrophotometer (Implen Gmbh, Germany). The absorbance ratios $A_{260/280}$ and $A_{260/230}$ for the tissues using two different methods ranged 1.79 - 2.11 and 1.90 - 2.15, respectively. The absorbance reading obtained for RNA extracted using both methods in all tissues indicated high purity (Table 1). The Rochester et al. (1986) method gave a higher yield compared to the kit-based method. About 2 µg total RNA were electrophoresed on a 1.5% (w/v) formaldehyde agarose-ethidium bromide gel to examine the quality of the extracted RNA. The extracted total RNA using the kit-based method showed two distinct bright bands of 28S rRNA and 18S rRNA with a ratio of approximately 1.5 to 2:1, indicating intact RNA (Figure 1). Though distinct bands of 28S rRNA and 18S rRNA were also obtained in the RNA extracted using the Rochester et al. (1986) method, the total RNA was contaminated by genomic DNA (Figure 2), which further reduced the yield of total RNA.

cDNA synthesis and RT-PCR analysis

cDNA was synthesized with reverse transcriptase using the same amount of total RNA isolated by both methods. Subsequently, the transcribed RNA was characterized with RT-PCR using gene-specific primer of GAPDH. Using the kit-based method, RT-PCR amplification yielded a bright band of approximately 190 bp for the GAPDH fragment, on a 1.5% agarose-ethidium bromide gel for all tissue types. However, using the Rochester et al. (1986) method, RT-PCR yielded only a faint band for the root tissue (Figure 3).

Table 1. Absorbance and yield of total RNA from different tissues using the two methods.

Method	Tissue	Purity		Yield (µg/g of fresh weight)
		A_{260}/A_{280}	A_{260}/A_{230}	
Rochester et al. (1986) method	Leaf - TLS	1.88	1.99	339
	Root - TLS	1.79	2.05	110
	SAM - TLS	1.88	1.99	311
Kit method	Leaf - TLS	1.87	1.98	251
	Root - TLS	1.82	2.07	63
	SAM - TLS	2.09	1.91	211

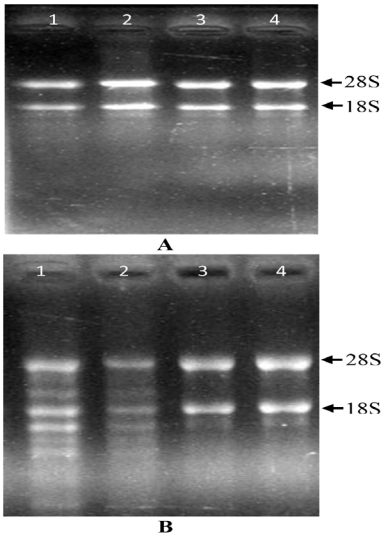

Figure 1. Total RNA isolated from shoot apical meristems (SAM), leaves and roots of TLS somaclonal oil palm seedlings using the Easy-Blue™ Total RNA Extraction Kit. **A.** *Lanes 1-4* = TLS shoot apical meristem. **B.** *lanes 1-2* = TLS leaf; *lanes 3-4* = TLS root.

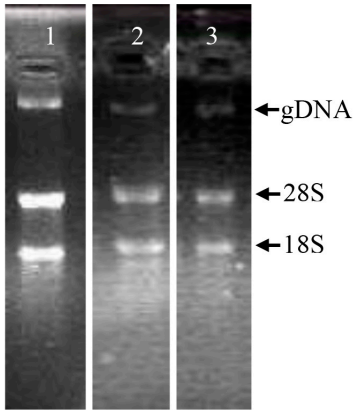

Figure 2. Total RNA isolated from shoot apical meristems (SAM), leaves and roots of TLS somaclonal oil palm seedlings using the Rochester et al. (1986) method. *Lane 1* = TLS shoot apical meristem; *lane 2* = TLS leaf; *lane 3* = TLS root. gDNA = oil palm genomic DNA.

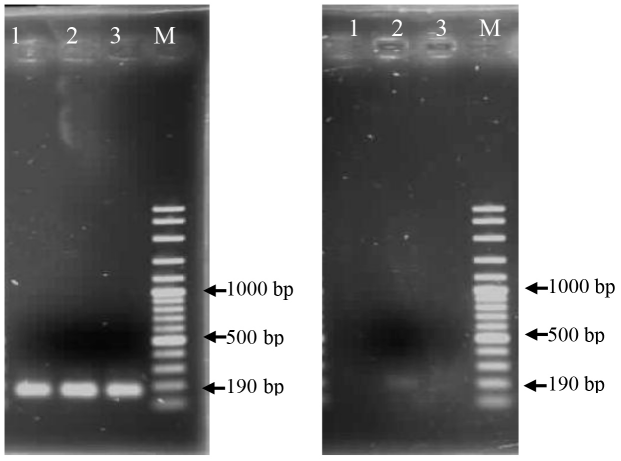

Figure 3. Agarose gel electrophoresis of the RT-PCR analysis. RT-PCR amplification of GAPDH fragment using total RNA isolated by kit method (**A**); total RNA isolated by Rochester et al. (1986) method (**B**). *Lane 1* = leaf; *lane 2* = root; *lane 3* = SAM; *lane M* = 100-bp DNA ladder.

DISCUSSION

As starting material, good quality RNA is a prerequisite for any downstream application. To obtain good quality RNA for gene expression studies, RNA isolation is a critical step. RNA isolation is difficult particularly in plants containing high levels of polysaccharides and phenolic compounds, which is the case in oil palm (Singh and Cheah, 2000). In the present study, two methods were employed to extract good quality and high-yield RNA from oil palm tissues. The methods were the Rochester et al. (1986) method and a kit-based method (Easy-Blue™ Total RNA Extraction kit). The kit-based method was found to be more suitable for isolating RNA from oil palm tissues, since it provided a sufficient amount of RNA with high purity and integrity within a very short period of time from a small amount of tissue, compared to the other method. Clear 28S and 18S ribosomal RNA bands were visualized on a denaturing formaldehyde agarose-ethidium bromide gel without any degradation of total RNA using either method, indicating the integrity of total RNA.

Generally, the Rochester et al. (1986) method was employed in the isolation of total RNA from oil palm tissues and required a large amount of starting material for RNA extraction. Singh and Cheah (2000) used the two methods described by Rochester et al. (1986) and Chang et al. (1993) and extracted good-quality RNA from young etiolated oil palm seedlings, leaves and inflorescences. However, they also mentioned that these methods are applicable when large amounts (approximately 2 g) of tissues are available. Therefore, when the sample amount is scarce, especially in the case of tissue culture, these methods would not be feasible. However, the present study demonstrated that this method can be scaled down to as little as 100 mg of any oil palm tissue to isolate total RNA. Thus, this method may be extensively used to extract total RNA from different oil palm tissues. When the total RNA extracted by this method was separated on gel electrophoresis, a band of genomic DNA was observed suggesting genomic DNA contamination in all tissues tested. The high absorbance reading might

have been due to the genomic DNA contamination that could not be removed in the extraction process. Another demerit of this method was that it took three days to complete the RNA isolation. The kit-based method on the other hand was simple, took only two hours to complete the isolation, and required only 100 mg starting material.

Reverse transcription is very sensitive to impurities. Therefore, we verified the RNA quality by reverse transcription and RT-PCR analysis. The PCR product separated on a 1.5% agarose-ethidium bromide gel using the cDNA synthesized from total RNA isolated by the kit method produced a single bright band for all tissue types without any smearing, indicating that the RNA was intact and could be used in downstream application. Though a faint band was observed only for the root tissue, no PCR products for the leaf and SAM tissue were observed on the gel using the cDNA synthesized from total RNA isolated by the Rochester et al. (1986) method. This was due to the contamination of total RNA with genomic DNA, and after DNaseI treatment, total RNA might have been drastically reduced, making it unsuitable for use as a template for cDNA synthesis or for RT-PCR amplification. The kit-based method was found to be more suitable for isolating RNA from oil palm tissues, since it yielded a sufficient amount of RNA with high purity and integrity within a very short period of time from a small amount of tissue, which could be used in downstream application, compared to the other method.

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