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Comparative evaluation of total RNA extraction methods in *Theobroma cacao* using shoot apical meristems

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ABSTRACT. *Theobroma cacao* is a species of great economic importance with its beans used for chocolate production. The tree has been a target of various molecular studies. It contains many polyphenols, which complicate the extraction of nucleic acids with the extraction protocols requiring a large amount of plant material. These issues, therefore, necessitate the optimization of the protocols. The aim of the present study was to evaluate different methods for extraction of total RNA from shoot apical meristems of *T. cacao* 'CCN 51' and to assess the influence of storage conditions for the meristems on the extraction. The study also aimed to identify the most efficient protocol for RNA extraction using a small amount of plant

material. Four different protocols were evaluated for RNA extraction using one shoot apical meristem per sample. Among these protocols, one that was more efficient was then tested to extract RNA using four different numbers of shoot apical meristems, subjected to three different storage conditions. The best protocol was tested for cDNA amplification using reverse transcription-polymerase chain reaction; the cDNA quality was determined to be satisfactory for molecular analyses. The study revealed that with the best RNA extraction protocol, one shoot apical meristem was sufficient for extraction of high-quality total RNA. The results obtained might enable advances in genetic analyses and molecular studies using reduced amount of plant material.

Key words: Nucleic acid; cDNA; RT-qPCR; Endogenous gene actin; Plant RNA; Transcriptomics

INTRODUCTION

RNA extraction has always been a challenge in the progress of molecular research. Several studies require RNA samples of high quality and quantity for analyses. RNA extraction in some plants is difficult due to the presence of proteins, polysaccharides, and polyphenolic compounds. The presence of proteins causes errors, for example, incorrect quantification of sample concentration; the polysaccharides contaminate the RNA because they have similar physical and chemical characteristics and precipitate with the nucleic acid; the polyphenols oxidize and bind to nucleic acids (Mattheus et al., 2003; Sharma et al., 2003). In this context, it is necessary to adapt the existing protocols or to develop new protocols (Chang et al., 1993; Mitra and Kootstra, 1993; Salzman et al., 1999; Zeng and Yang, 2002; Gesteira et al., 2003; Gasic et al., 2004) for extraction of the desired quality and quantity of RNA.

Several RNA extraction protocols are based on the elimination of proteins, polyphenols, and polysaccharides (Hu et al., 2002; Vasanthaiah et al., 2008; Rai et al., 2010; Ghawana et al., 2011; Rubio-Pina and Zapata-Perez, 2011; Dash, 2013). The type of reagents and homogenization procedures used in the extraction process modifies the RNA extraction efficiency (Portillo et al., 2006). The extraction protocols generally utilize reagents such as acidic guanidinium thiocynate, cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), phenol, chloroform, lithium chloride, sodium acetate, among other reagents, in order to obtain pure RNA samples free from proteins, polysaccharides, and polyphenolic compounds (Gesteira et al., 2003; Verica et al., 2004).

Several protocols have been developed to efficiently extract RNA. Various companies now offer kits to optimize the extraction procedures; such kits reduce the time of extraction but are often costly. However, generally, a single kit or a protocol does not always results in satisfactory quality and quantity of RNA, extracted from all types of plant tissues, requiring tissue-specific modifications (Yu et al., 2012).

Many extraction protocols require a significant amount of plant material; however, depending on the conditions encountered in the field, plant material available for collection could be limiting. Therefore, studies that seek to optimize the extraction conditions using reduced amount of plant material are extremely important for the advancement of molecular analyses (Moser et al., 2004; Portillo et al., 2006; Christou et al., 2014).

In the present study, we evaluated four different methods for extracting total RNA from

shoot apical meristems of *Theobroma cacao* (cacao), a species with high levels of proteins, polyphenols, and polysaccharides. This tree is of great economic importance because of the production of cocoa liquor, cocoa butter and pulp, and derivatives as ice cream, jellies, cosmetics and chocolate, and it is a target of several genetic studies (Araújo et al., 2009; Silva et al., 2011, 2014). We also used three storage conditions and four different numbers of shoot apical meristems for identifying the most efficient protocol.

MATERIAL AND METHODS

Plant material

The shoot apical meristems used in this study were collected from approximately 6-year-old cacao "Colección Castro Naranjal" (CCN) 51 trees growing at the Mars Center for Cocoa Science, Barro Preto, Bahia, Brazil (14°42'45.021171''S and 39°22'13.008369''W). Healthy and active meristems, about 6 mm in size, were selected for use in the experiments (Figure 1).

Figure 1. Active and healthy shoot apical meristem of *Theobroma cacao*.

RNA extraction

Four different protocols for RNA extraction from the shoot apical meristems were tested: Protocol 01 (Verica et al., 2004); Protocol 02 (Gesteira et al., 2003); ZR Plant RNA mini Prep-Zymo kit (#R2024, Zymo Research, Irvine, CA, USA); and RNAqueous Total RNA Isolation kit (#AM1912, Life Technologies, Carlsbad, CA, USA). For each protocol, one shoot apical meristem was used. It was macerated using a mortar and pestle in liquid nitrogen; the macerate was transferred to 2-mL plastic tubes and processed as per the protocols or the recommendations of the manufacturers of the kits used.

The influence of the number of apical meristems used (one, three, five, or ten) and their storage conditions (lyophilized, stored in freezer at -80°C, and fresh apical meristems) on the performance of the most efficient protocol was also tested by assessment of RNA concentrations obtained from the extracted samples. The extracted total RNA samples were purified with RNeasy Mini Kit (#74106, Qiagen, Valencia, CA, USA), treated with RQ1 RNase-Free DNase (#M6101, Promega, Madison, WI, USA), quantified with a spectrophotometer GeneQuant™ pro RNA/DNA Calculator (Amersham Biosciences, England, UK), and stored in a freezer at -80°C.

Statistical analysis

All tests were performed on five biological replicates. BioEstat 5.3 (Ayres, 2011) was used for determining the average RNA extracted with each protocol, analysis of variance ($P < 0.01$ by ANOVA), and for performing the Tukey test ($P < 0.05$).

cDNA synthesis

The extracted RNA was used for cDNA synthesis. cDNA was synthesized with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and stored in a freezer at -20°C. A 20-µL reaction mixture consisted of the following reagents: 4 µL 5X Vilo Reaction Mix, 2 µL 10X SuperScript Enzyme Mix, 4 µL total RNA, and 10 µL nuclease-free water. The reactions were incubated in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) at 25°C for 10 min, 42°C for 120 min, and 85°C for 5 min. The synthesized cDNA was quantified using the spectrophotometer, GeneQuant™ pro RNA/DNA Calculator and stored in a freezer at -20°C.

Relative standard curve

The cDNA was diluted in a 1:2 proportion with nuclease-free water, amplified on an Applied Biosystems® 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and quantified using the Relative Standard Curve method. The endogenous control gene, actin (Table 1), specific for *T. cacao*, was used to ensure the suitability of the samples for molecular analyses.

Table 1. Primers used for amplification of the endogenous actin gene (Pinheiro et al., 2011), showing the amplification efficiency, and R^2 obtained from RT-qPCR.

Endogenous gene	Sequence	Fragment size (bp)	Efficiency	R^2
Actin	5'-TCCTCTTCCAGCCATCTCTC-3' (F)	171	1.91	0.99
	5'-TCTCCTTGCTCATTCGGTCT-3' (R)			

R^2 is the correlation coefficient.

Sybr Green Master Mix (Applied Biosystems) was used for quantification. The concentration of different reagents in each 25 µL reaction was: 2X SYBR Green Master Mix, 0.05 mM primers (forward and reverse), and one of the five different cDNA concentrations (400, 200, 100, 50, and 25 ng/µL). All the samples, including the negative control (contained nuclease-free water in place of cDNA), were analyzed in triplicate. The amplification conditions were as follows: 50°C for 2 min, 95°C for 10 min (holding stages), followed by 40 cycles at 95°C for 1 s, 60°C for 1 min (cycling stage), followed by 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s (melt curve stage).

RESULTS

Protocol efficiency, number of meristems and storage conditions

Total RNA was extracted from a single-active shoot apical meristem. The average concentration of the total RNA extracted with each protocol is shown in Table 2. Protocol 01 was determined to be the most efficient with high concentration and quality of the extracted RNA.

Table 2. Concentration and absorbance of total RNA samples extracted from a single-active shoot apical meristem of *Theobroma cacao*.

Protocol	Concentration (ng/μL)	Absorbance (260/280)	Absorbance (260/230)
01 (Verica et al., 2004)	242.1	1.9	1.8
02 (Gesteira et al., 2003)	24.5	1.7	0.7
ZR Plant RNA mini Prep-Zymo (Zymo Research)	31.5	1.7	0.7
RNAqueous (Life Technologies)	56.6	2.0	0.7

Subsequent tests indicated that the extraction of total RNA using only one shoot apical meristem was more efficient than using three, five, or ten shoot apical meristems. The protocol 01 was tested using different numbers of apical meristems and concentration of total RNA extracted was 242.1, 175.2, 150.0, and 112.8 ng/μL using one, three, five, and ten apical meristems, respectively.

The efficiency of protocol 01 for total RNA extraction from a single-apical meristem subjected to different storage conditions was also assessed. The RNA concentration obtained from the lyophilized, ultra-frozen (at -80°C), and freshly collected samples was 177.5, 242.1, and 250.2 ng/μL, respectively. In addition, the mean concentration of cDNA, reverse transcribed from the RNA extracted from a single-active shoot apical meristem using the protocol 01, was 1384.5 ng/μL.

Statistical differences among protocols

ANOVA (F values, 1% probability) performed on the obtained data revealed statistically significant differences among the different protocols (Table 3). However, no significant differences were observed among the biological replicates used in each protocol.

Table 3. Analysis of variance for the different RNA extraction protocols and for the biological replicates used in each protocol. ($P < 0.01$).

Origin of variations	Sum of squares	Average square	F calculated	F critical value
Different protocols	159,777.3	53,259.1	1,460.6	5.292
Biological replicates per protocol	144.9	36.2	0.99	5.292

In addition, after ANOVA results indicated a significant difference between the extraction protocols, we assessed the magnitude of these differences, using the Tukey test at 5% probability (Table 4).

Table 4. Tukey test for comparison between average concentrations of RNA extracted using the different protocols.

Protocols	Student range (Q)
01 (Verica et al., 2004) and 02 (Gesteira et al., 2003)	80.6
01 and ZR Plant RNA mini Prep-Zymo	78.0
01 and RNAqueous	68.7
02 and ZR Plant RNA mini Prep-Zymo	2.6
02 and RNAqueous	11.9
ZR Plant RNA mini Prep-Zymo and RNAqueous	9.3

Tukey = 3.65 (critical value), $P < 0.05$.

Extracted RNA suitable for reverse amplification

We showed that the cDNA amplifications with the actin marker by real-time-quantitative polymerase chain reaction (RT-qPCR) were satisfactory (amplification efficiency of 98.387%; Figure 2). The correlation coefficient ($R^2 = 0.99$) confirms the efficiency of these amplifications using the samples extracted with the protocol 01.

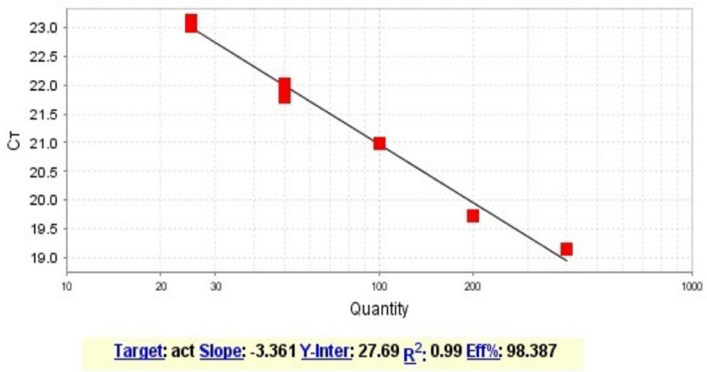

Figure 2. Standard curve of the actin marker amplified with cDNA of 'CCN 51' used at five different concentrations (25, 50, 100, 200, and 400 ng/µL). Ct = cycle threshold; R^2 = correlation coefficient; Eff = efficiency.

DISCUSSION

The extraction of nucleic acids from cacao trees is a laborious process because of the presence of several polyphenolic compounds. The four protocols for RNA extraction evaluated in the present study differed in the reagents used. Protocol 01 used CTAB, phenol, chloroform, lithium chloride, and sodium acetate; it allowed the isolation of total RNA, its dissociation from the proteins and precipitation, and the removal of polysaccharides. Protocol 02 used SDS, boric acid, and tert-butanol; it allowed the denaturation of the proteins, prevented the oxidation of phenolic compounds, and prevented the binding of phenolic compounds to the RNA. The RNAqueous kit had a high concentration of guanidine salts, which enabled cell lysis and inactivation of RNases. The details of the reagents present in the ZR Plant RNA mini Prep-Zymo were not available but the protocol ensured efficient extraction of RNA in 15 min.

We have demonstrated the superiority of the protocol 01 concerning to average concentration and quality of the RNA extracted (Table 2). The ratio of absorbance at 260 and 280 nm ($A_{260/280}$) is used to assess the contamination of RNA samples with proteins whereas $A_{260/230}$ indicates contamination with polyphenols and polysaccharides (Gasic et al., 2004). The RNA extracted using Protocol 01 showed purity of the samples from polyphenols and polysaccharides while that obtained using all the other protocols was contaminated with these compounds.

The different values of concentrations of total RNA (using one, three, five, or ten shoot apical meristems) were most likely due to the fact that the increased amount of the macerated material in the 2-mL tubes encumbered the extraction process. Also, increasing the amount of material means a greater time required for macerating and greater amount of contaminants and RNAses. This condition favors faster oxidation of macerated material, and degradation of RNA. In addition, although the extraction was effectively achieved from the samples stored under different conditions, the use of fresh samples for RNA extraction could be infeasible for some experiments because nucleic acids degrade very quickly if the samples are not handled or properly stored. The lyophilized samples worked well, but it is advisable to keep the samples in vacuum bags to maintain their integrity for longer period. Finally, storing the samples in an ultra-freezer at -80°C was effective and could be chosen as a fast, safe, and practical option.

ANOVA revealed that the results obtained using the protocol 01 were statistically different from those obtained using all the other protocols. However, the results obtained using the protocol 02

were not statistically different from those obtained using the ZR Plant RNA Mini Prep-Zymo protocol.

The amplification of the actin gene by RT-qPCR was efficient, which was another indication of the good quality of the samples. In summary, total RNA extracted from a single-shoot apical meristem with the protocol 01 was consistently of satisfactory quality and concentration and yielded good-quality cDNA after reverse transcription. The results indicate that this protocol could be very promising, especially when limited amounts of plant materials are available. This protocol for RNA extraction could, therefore, contribute to the success of molecular studies and the genetic improvement of plants, like the cacao tree, in future.

Conflicts of interest

The authors declare no conflict of interest.

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