Short Communication

An efficient method for simultaneous extraction of high-quality RNA and DNA from various plant tissues

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ABSTRACT. Determination of gene expression is an important tool to study biological processes and relies on the quality of the extracted RNA. Changes in gene expression profiles may be directly related to mutations in regulatory DNA sequences or alterations in DNA cytosine methylation, which is an epigenetic mark. Correlation of gene expression with DNA sequence or epigenetic mark polymorphism is often desirable; for this, a robust protocol to isolate high-quality RNA and DNA simultaneously from the same sample is required. Although commercial kits and protocols are available, they are mainly optimized for animal tissues and, in general, restricted to RNA or DNA extraction, not both. In the present study, we describe an efficient and accessible method to extract both RNA and DNA simultaneously from the same sample of various plant tissues, using small amounts of starting material. The protocol was efficient in the extraction of high-quality nucleic acids from several *Arabidopsis thaliana* tissues (e.g., leaf, inflorescence stem, flower, fruit, cotyledon, seedlings, root, and embryo) and from other tissues of non-model plants, such as *Avicennia*

schaueriana (Acanthaceae), *Theobroma cacao* (Malvaceae), *Paspalum notatum* (Poaceae), and *Sorghum bicolor* (Poaceae). The obtained nucleic acids were used as templates for downstream analyses, such as mRNA sequencing, quantitative real time-polymerase chain reaction, bisulfite treatment, and others; the results were comparable to those obtained with commercial kits. We believe that this protocol could be applied to a broad range of plant species, help avoid technical and sampling biases, and facilitate several RNA- and DNA-dependent analyses.

Key words: RNA and DNA extraction; *Arabidopsis thaliana*; *Avicennia schaueriana*; *Theobroma cacao*; *Sorghum bicolor*

INTRODUCTION

Modulation of gene expression underpins all aspects of developmental processes and responses to environmental signals; thus, analyses of RNA profiles are fundamental to the study of the regulatory networks involved in biological processes. Many studies indicate that isolation of high-quality RNA is a critical step in achieving this (Fleige et al., 2006; Copois et al., 2007). Gene expression is primarily regulated by transcription factors that bind to specific cis-regulatory DNA sequences. Mutations affecting regulatory genes or cis-sequences can be responsible for adaptive key features and define natural variation among species (van Dijk et al., 2010). Moreover, epigenetic marks such as DNA cytosine methylation can be stably inherited and may also participate in establishing specific gene expression patterns (Henderson and Jacobsen, 2007; Silveira et al., 2013).

The need to obtain high quality RNA and DNA from the same tissue sample to correlate unbiased gene expression with DNA sequence or epigenetic changes, prompted us to develop a methodology fulfilling such a requirement. A protocol that enables isolation of nucleic acid from different plant tissues and from small amounts of starting material is also desirable in order to avoid technical bias (Sellin Jeffries et al., 2014). Several techniques and commercial kits are available (Berendzen et al., 2005; Triant and Whitehead, 2009; Peña-Llopis and Brugarolas, 2013); however, a majority of them are optimized for animal tissues and do not enable efficient extraction of both types of nucleic acids simultaneously. The protocol described in the present study is based on phenol-chloroform phase separation, followed by the sequential precipitation of RNA with lithium chloride (LiCl) and DNA with isopropanol (Sambrook et al., 1989). The method was determined to be applicable to different tissues of *Arabidopsis thaliana* (Brassicaceae), such as, leaf, inflorescence stem, flower, fruit, cotyledon, seedlings, root, and embryo; and to those of *Avicennia schaueriana* (a typical mangrove species from South America; Acanthaceae), such as, leaf, stem, and roots. Furthermore, it was also efficient for extraction of RNA from the leaves and flowers of *Paspalum notatum* (Poaceae) and the leaves of *Theobroma cacao* (Malvaceae) and *Sorghum bicolor* (Poaceae). Thus, we believe that this protocol could be applied to many other plant species and may facilitate several RNA- and DNA-dependent analyses, without any sampling bias.

MATERIAL AND METHODS

Plant material

For RNA and DNA extractions, different tissues from *A. thaliana* ecotype Columbia-0

cultivated at 22°C under long day-photoperiod (16-h light/8-h dark) were used. Rosette leaf, cauline leaf (bract), node, internode, flower, and fruit tissues were collected from plants cultivated in soil 60 days after germination (DAG), whereas cotyledons and rosette discs were obtained from plants 21 and 30 DAG, respectively. Seedlings and dissected roots were obtained 9 and 12 DAG, respectively, from plants cultivated in MS/2 medium (Murashige and Skoog, 1962) supplemented with 0.7% sucrose. Embryo tissue was obtained from macerated seeds as described by Raissig et al. (2013). For RNA and DNA extractions from *A. schaueriana* and *P. notatum*, the tissues were collected directly from naturally growing plants, and for *S. bicolor* and *T. cacao*, it was collected from plants grown in a green house. All plant material was directly collected and macerated in liquid nitrogen.

RNA and DNA quantification, DNAse treatment and PCR reactions

RNA samples were quantified by NanoVue Plus Spectrophotometer (GE Healthcare life Sciences, Little Chalfont, UK) and DNA samples by Qubit® dsDNA HS Assay kit (ThermoFisher Scientific, Carlsbad, CA, USA, cat. No. #Q32851). RNA samples were treated with Ambion® Turbo DNA-free DNAse (ThermoFisher Scientific, cat. No. #AM1907) and the polymerase chain reaction (PCR) was performed with Platinum® Taq DNA polymerase (ThermoFisher Scientific, cat. No. #10966) following the respective manufacturer instructions.

Quantitative real time-PCR (qRT-PCR) analyses

cDNA was synthesized by reverse transcription with the kit ImPromII (Promega, Madison, WI, USA, cat. No. #A3801), using oligo-dT as a primer. For qRT-PCR, 0.5 µL cDNA was used as a template together with forward and reverse oligonucleotide primers for housekeeping genes in 1X Platinum SYBR Green SuperMix-UDG (ThermoFisher Scientific, cat. No. #11733). Cycling conditions (7500 Fast Real-Time PCR Systems, ThermoFisher Scientific) were as follows: 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C, linked to a default dissociation stage program to detect non-specific amplification.

DNA sodium bisulfite analyses

DNA samples isolated from the rosette disc of plants with 30 DAG were treated with EZ DNA Methylation-Lightning™ kit (Zymo Research, Irvine, CA, USA, cat. No. #D5030) and PCR was performed using Platinum® Taq DNA polymerase (ThermoFisher Scientific, cat. No. #10966) following the manufacturer instructions. The primers used, Bis_13440_F2: 5'-GGATTATTTTAAAGTTGG TTAATTTATA-3' and Bis_13440_R2: 5'-AACCCATTTAAACAAAAATACTAC-3' for the At5g13440 negative control and Bis_FWA1_F: 5'-GGTTTTATATTAATATTAAAGAGTTATGGG-3' and Bis_FWA1_R: 5'-AAACCAAAATCATTCTCTAAACAAAATA-3' for *FWA* (At4g25530), were modified from those used by Fujimoto et al. (2008). The analyses were performed as described by Slotkin et al. (2009).

RESULTS

Protocol for simultaneous RNA and DNA extraction

We modified RNA extraction methods based on phenol:chloroform phase separation and LiCl precipitation (Suzuki et al., 2004; Oñate-Sánchez and Vicente-Carbajosa, 2008) and

isolated the DNA from the usually discarded supernatant of the same sample. The rationale for the approach described here relies on the fact that DNA is retained in the inorganic phase when basic phenol, pH 8.0, is used for RNA extraction and on the differential RNA precipitation by LiCl salt (Sambrook et al., 1989), thus allowing the separation of RNA from DNA. Therefore, the present protocol enabled the isolation of high-quality RNA and DNA from the same initial sample of different plant tissues.

The necessary amount of extraction buffer to be used (800 µL x number of samples) was prepared. One 2.0-mL and three 1.5-mL tubes were labeled for each sample. Extraction buffer: 0.4 M LiCl, 0.2 M Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 1% SDS, and 4.7% β-mercaptoethanol, always prepared using ultrapure and diethylpyrocarbonate (DEPC)-treated water (H_2O_{DEPC}).

1) Tissue was macerated in liquid nitrogen (using either a mortar and pestle or a small pestle to macerate small samples directly in the tube).

2) Material (50-100 mg) was transferred to a 2.0 mL pre-cooled Eppendorf tube, 800 µL extraction buffer was added, and tubes were vortexed. Note 1: The quantity of material mentioned corresponded approximately to the lowest-volume marking of the Eppendorf tube and was required for optimal extraction condition, although lower amounts could also be used (see Discussion). Note 2: Until the end of the extraction procedure, the tubes should be maintained in ice to avoid RNA degradation and all the centrifugations should be performed at full speed (≥ 13.000 rpm or 18,000 *g*).

3) Pre-cooled chloroform:isoamyl alcohol (24:1; 800 µL) was added to the tubes and they were then vortexed for 20 s. Centrifuged for 10 min at 4°C and the supernatant or upper phase (~ 600 µL) was transferred to a new 1.5-mL tube. Basic phenol (500 µL), pH 8.0, was added and tubes were vortexed for 10 s. Pre-cooled chloroform:isoamyl alcohol (24:1; 200 µL) was added and tubes were vortexed.

4) Tubes were centrifuged for 5 min at 4°C and the supernatant (~ 500 µL) was transferred to a new 1.5-mL tube. Next, 1/3 volume (~ 170 µL) 8 M LiCl was added and mixed by inversion. Tubes were maintained at -20°C for at least 1 h or up to a maximum of 16 h overnight (O/N).

5) Tubes were centrifuged for 30 min at 4°C and the supernatant (~ 600 µL) was transferred to a new 1.5-mL tube without disturbing the pellet. The tube containing the pellet was used for RNA precipitation (RNA tube), whereas the tube containing the supernatant was used for DNA precipitation (DNA tube).

RNA tube

1) H_2O_{DEPC} (500 µL) was added and the pellet was dissolved by vortexing (~ 5 s).

2) Next, 3 M sodium acetate (50 µL), pH 5.2, and 800 µL 100% ethanol (EtOH) were added and mixed by inversion. Tubes were maintained at -20°C for 1 h or O/N. Note: This point permits transportation at room temperature (RT).

3) Tubes were centrifuged for 30 min at 4°C and the supernatant was discarded.

4) The pellet was washed with 500 µL 70% EtOH (used H_2O_{DEPC} for diluting). Tubes were centrifuged for 2 min at 4°C and the supernatant was discarded.

5) The pellet was washed with 500 µL 100% EtOH. Tubes were centrifuged for 2 min at 4°C and the supernatant was discarded.

6) The samples were dried for 10 min in a speed vacuum or, alternatively, for 30 min at RT. H_2O_{DEPC} (20 µL) was added, and tubes were vortexed and stored at -80°C.

DNA tube

1) In the tube containing the supernatant from step 5, 2/3 volume (~400 µL) pre-cooled isopropanol was added, mixed by inversion and maintained at -20°C for at least 1 h or O/N.

2) Tubes were centrifuged for 10 min and the supernatant was discarded.

3) The pellet was washed with 500 µL 70% EtOH. Tubes were centrifuged for 5 min and the supernatant was discarded.

4) The pellet was washed with 500 µL 95% EtOH. Tubes were centrifuged for 5 min and the supernatant was discarded.

5) The samples were dried for 10 min in speed vacuum or, alternatively, for 30 min at RT. Next, 20-50 µL 0.1X TE, pH 8.0 (10 mM Tris and 1 mM EDTA) or water was added, and the solution was mixed gently.

6) Tubes were maintained at 4°C for one day and stored at -20°C. If RNAse treatment was necessary, RNAse was added to the TE or water (final concentration 50 µg/mL) and the samples were incubated for 30 min in a water bath at 37°C before storing.

Evaluation of the efficiency of the extraction procedure and integrity of RNA and DNA

The integrity of nucleic acids was assessed on an agarose gel and the purity calculated by the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}), with values ≥ 1.8 indicating highly pure samples (Sambrook et al., 1989; Fleige et al., 2006). The method described in the present study was found to be very efficient for nucleic acid extraction from different *A. thaliana* tissues; on average, approximately 20 µg total RNA and 1.6 µg DNA was obtained per sample, with A_{260}/A_{280} being approximately 2.0 (**Table S1**), with no apparent signs of degradation (Figures 1A and B, **Figures S1-S4**). The tissues tested included leaf, inflorescence stem, fruit, seedling, flower, root, cotyledon, and embryo; the nucleic acid yield varied for the last four mentioned tissues in relation to the total RNA average, being 2-3 folds more for flower and 2-4 folds less for embryo, cotyledon and root samples (**Table S1**). Similar results were obtained for leaf, stem, and root samples from *A. schaueriana* (Figure 2A and B, **Table S1**) and also for RNA extractions from *T. cacao*, *S. bicolor*, and *P. notatum* (Figure 2C, **Table S1**), which demonstrated the efficiency of the protocol across different tissues and species. However, RNA extractions from the roots of plants cultivated in soil may present impurities and the samples should be further purified by commercial columns to allow efficient cDNA synthesis.

The total RNA and DNA yield (per unit fresh weight of macerated tissue) was determined from the rosette discs of *A. thaliana* (Figure 3). On average, 2.5 µg total RNA was obtained from 10 mg fresh tissue with no loss of yield up to 50 mg material (Figure 3A and B). Although higher amounts of total RNA were obtained from 100 mg samples, the yield was lower in comparison to that obtained using other initial amounts (Figure 3B) defining a threshold for the maximum achievable RNA yield. Nevertheless, the amount of total RNA and yield varied with the tissue (**Table S1**). The DNA extraction step was as efficient as the CTAB protocol (Doyle and Doyle, 1990) with similar yield and quality standards obtained from the same material (**Figure S2**) and it also worked for different *A. thaliana* and *A. schaueriana* tissues (Figures 1B and 2B). Yields and quantities increased with the amount of the initial material in a non-linear correlation (Figure 3C and D), with the optimum results obtained from 100-mg samples.

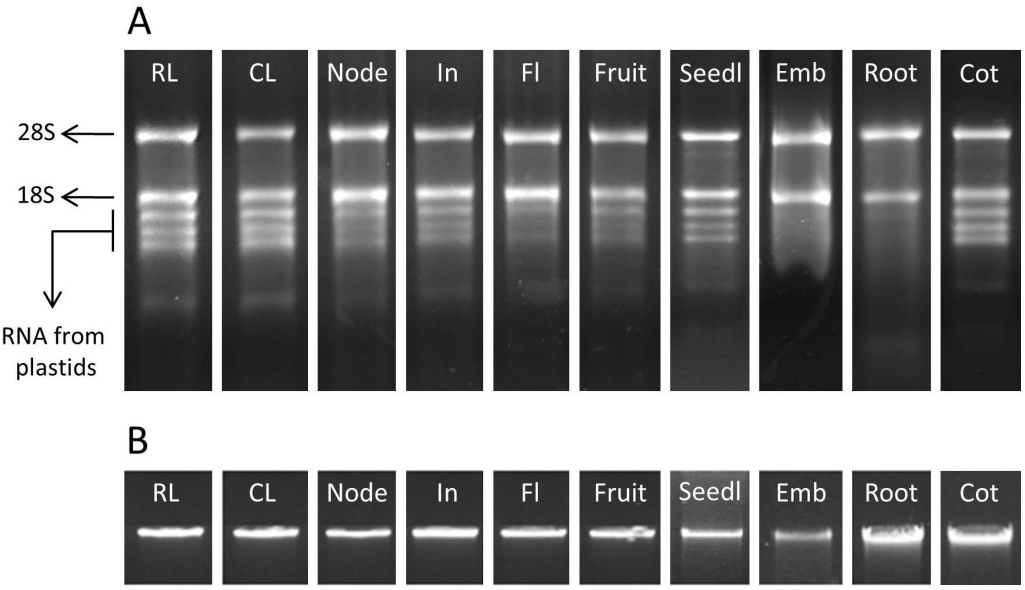

Figure 1. Integrity of RNA and DNA extracted from various *Arabidopsis thaliana* tissues. **A.** Total RNA (1 µg) extracted from various *Arabidopsis thaliana* tissues was fractioned on a denaturing 1% agarose gel. **B.** Fractionation of genomic DNA obtained from the same tissues as those shown in A on a 1% agarose gel. No apparent degradation (smear) is observed on either gel. RL = Rosette leaf; CL = Cauline leaf (bracts); In = Internode; Fl = Flower; Seedl = Seedlings nine days after germination, Emb = Embryo, and Cot = Cotyledon.

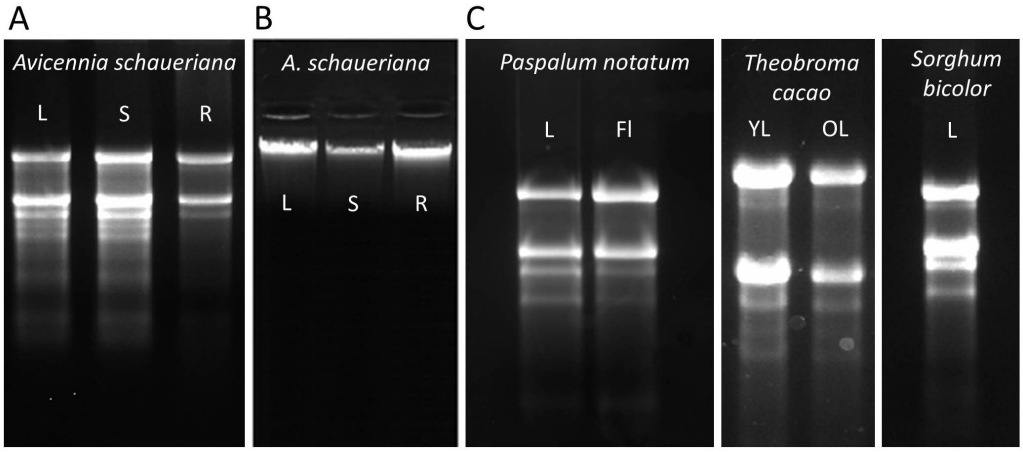

Figure 2. Integrity of RNA and DNA extracted from tissues of different plant species. **A.** Total RNA (1 µg) extracted from different *Avicennia schaueriana* tissues was fractioned on a denaturing 1% agarose gel. **B.** Fractionation of genomic DNA obtained from the same tissues as those shown in A on a 1% agarose gel. **C.** Total RNA (1 µg) extracted from other plant species was fractioned on a denaturing 1% agarose gel. No apparent degradation (smear) is observed on the gels. L = leaf; S = stem; R = root; YL = young leaf; OL = old leaf, and Fl = flower.

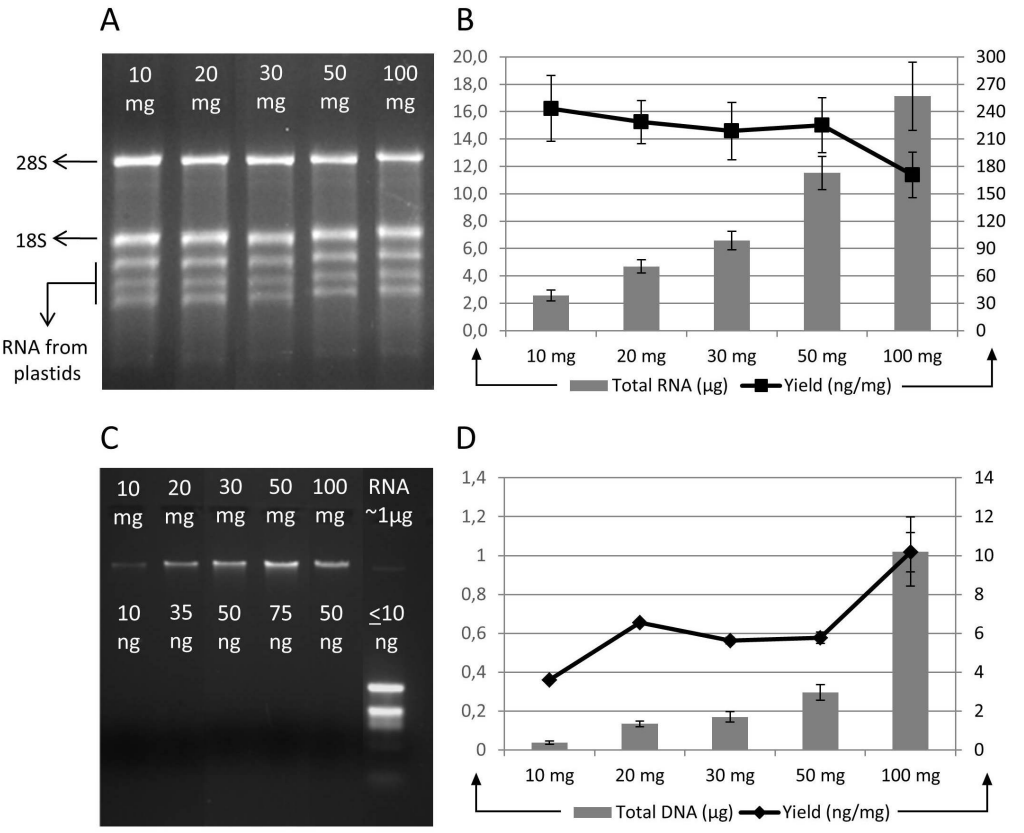

Figure 3. Yield of RNA and DNA from *Arabidopsis thaliana* rosette discs. **A.** Total RNA (0.5 µg) extracted from different amounts of *A. thaliana* rosette discs was fractionated on a denaturing 1% agarose gel. **B.** Efficiency and yield of RNA obtained per milligram of the macerated tissue. The data are the averages of three biological replicates (see **Figure S1**). **C.** Fractionation of genomic DNA obtained from the same samples as those shown in A on a 1% agarose gel. After quantification, 10, 35, 50, 75, and 50 ng of DNA samples corresponding to 10-100 mg samples, respectively, were loaded and verified on an agarose gel. Furthermore, RNA samples not treated with DNAse presented ≤10 ng DNA regardless of the starting quantity of material, as shown in the last well and in **Figure S3**. **D.** Efficiency and yield of DNA obtained per milligram of the macerated tissue. Three replicates were used (see **Figure S3**); a non-linear correlation was observed between the total DNA extracted and the starting material quantity.

RNA samples usually possessed some (less than 10 ng) DNA contamination (last lane in Figure 3C and **Figure S3**), which precipitated together with the RNA by LiCl, regardless of the quantity of the starting material. Thus, for procedures that require DNA-free RNA samples, treatment with commercial DNAse kits would be necessary; we determined Ambion® Turbo DNA-free DNAse (**Figure S4A** and **S4B**) to be effective. To check for the efficiency of DNA elimination while maintaining the integrity of RNAs, we performed PCR using RNA samples treated with Improm II DNAse as templates. No genomic DNA amplification was observed even after 40 cycles of amplification (**Figure S4C**). As an alternate to DNAse treatment, we suggest the designing of primers encompassing exon-exon regions to save time and reagents, whenever possible (Sambrook et al., 1989).

To evaluate the integrity of the RNA obtained with our protocol, qRT-PCR was performed with RNA samples from *A. thaliana* tissues. RNA levels of reference genes, such as, *ACTIN2* (*Act2*, At3g18780) and *PDF2* (*PP2A*, At1g13320) published by Czechowski et al. (2005) and *UBIQUITIN E2* (*Ubq*, At2g36060; Bourbousse et al., 2012), were evaluated by qRT-PCR. As shown in Figure 4, the Ct for these genes did not vary among the samples from different tissues and was equal to that obtained with high quality RNA purified with commercial columns. These results demonstrate the high integrity of RNA obtained with this protocol and the efficiency of cDNA synthesis. To evaluate DNA integrity and its usefulness in downstream applications, we treated DNA samples with sodium bisulfite, which converts methylated cytosines to thymines and, thus can be distinguished from non-methylated cytosines after sequencing of specific genomic loci (Slotkin et al., 2009). Figure 5 shows that 99% non-methylated cytosines in the At5g13440 loci, used as a non-methylated control, were converted after the sodium bisulfite treatment and that around 30% methylation was found in *FWA* from the DNA of vegetative tissues, which was in agreement with previously reported data (Fujimoto et al., 2008). This result proved that the DNA extracted using the present protocol was suitable for routine and sensitive analyses. Moreover, the RNA samples obtained from *A. schaueriana*, *S. bicolor*, and *P. notatum* were used to perform mRNA sequencing (RNA-seq) on the Illumina platform, and more than 95% of the reads had a Phred quality score of 30 (Q30) for all the species.

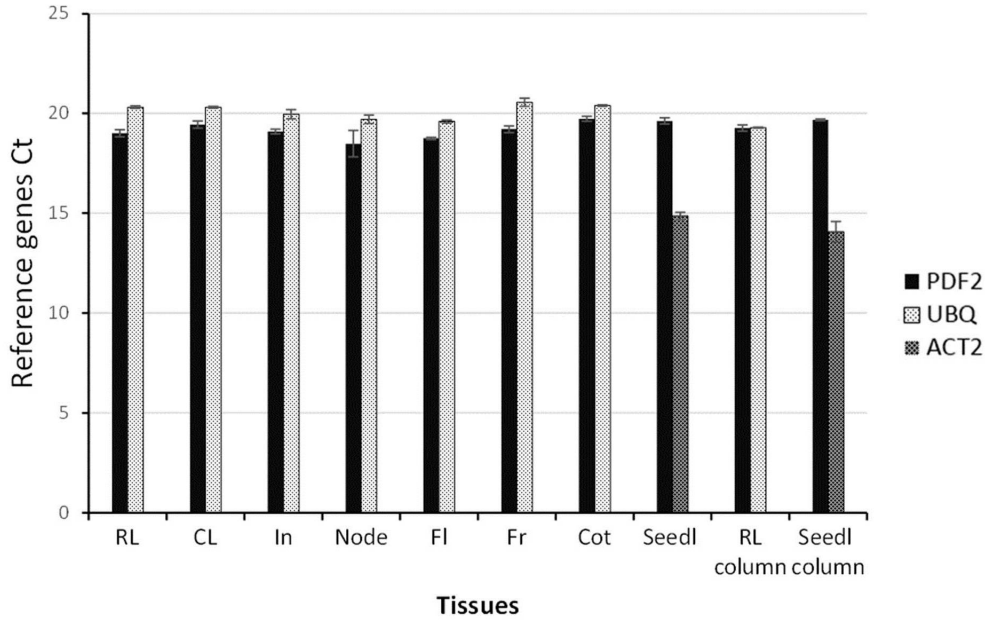

Figure 4. qRT-PCR analysis of reference genes. The Ct of the reference genes did not vary between samples from different tissues, indicating homogeneous RNA quality and cDNA synthesis efficiency. The statistical significance between the Cts of each reference gene in the different tissues was calculated by the Student's *t*-test ($P < 0.05$). *ACTIN2* (*Act2*, At3g18780) and *PDF2* (or *PP2A*, At1g13320) have been published as reference genes by Czechowski et al. (2005) and *UBIQUITIN E2* (*Ubq*, At2g36060) by Bourbousse et al. (2012). RL = Rosette leaf; CL = Cauline leaf (bracts); In = Internode; Fl = Flower; Fr = Fruit, Cot = Cotyledon and Seedl = Seedlings nine days after germination.

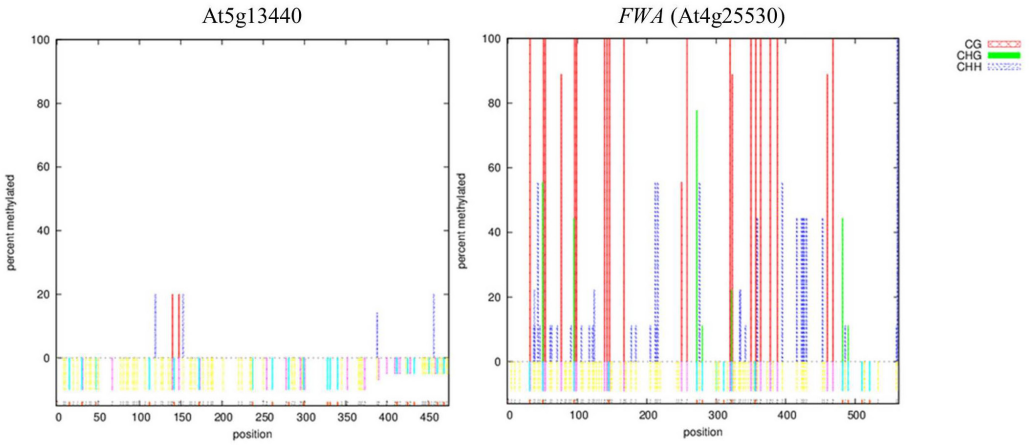

Figure 5. DNA methylation profile of At5g13440 and *FWA*. DNA sodium bisulfite treatment was performed and analyzed as described by Slotkin et al. (2009). At5g13440 did not show any cytosine methylation whereas *FWA* (At4g25530) was methylated at repeated sequences found in its promoter. The figure shows that around 99% of the cytosines were converted in thymine at the At5g13440 loci, from which high efficiency of DNA bisulfite conversion can be inferred. On the other hand, *FWA* showed approximately 30% DNA cytosine methylation which is in accordance with previous data (Fujimoto et al., 2008) and proved the specificity of DNA obtained by the present method.

DISCUSSION

To the best of our knowledge, most commercial kits and the available protocols do not provide an efficient and low cost method to extract both nucleic acids from different plant tissues suitable for quantitative expression analyses (Berendzen et al., 2005; Triant and Whitehead, 2009; Peña-Llopis and Brugarolas, 2013). To facilitate studies involving correlated RNA and DNA analyses, we developed a protocol to isolate both the nucleic acids from the same sample of several plant tissues and tested *A. thaliana* and the mangrove species, *A. schaueriana*. The technique was based on protocols that use phenol-chloroform (Suzuki et al., 2004; Oñate-Sánchez and Vicente-Carbajosa, 2008). We took advantage of the fact that both RNA and DNA are retained in the inorganic phase when basic phenol is used and that LiCl preferentially precipitates RNA (Sambrook et al., 1989).

The main features of our protocol are that the DNA is retained in the aqueous phase after denaturation by the basic phenol and that the RNA is precipitated with LiCl, which does not efficiently precipitate DNA, proteins or carbohydrates (Sambrook et al., 1989). Hence, the DNA is maintained in the supernatant and only precipitated with isopropanol after its separation from RNA, without affecting RNA yield or quality. LiCl precipitation is the method of choice to remove translation or cDNA synthesis inhibitors from RNA preparations (Sambrook et al., 1989). The expression analyses in the present study demonstrate that its use does not cause any detectable differences compared to the conventional protocols (Figure 4). Moreover, LiCl does not appear to preferentially precipitate higher-molecular-weight RNA over smaller RNA (Technical Bulletin #160, Life Technologies), which reinforces the appropriateness of its use for gene expression analyses.

The present analyses indicated that RNA extraction was optimal with 50 mg macerated tissues whereas 100 mg was required for the extraction of both RNA and DNA. However, minor

amounts of starting material could also be used efficiently (Figure 3) and the nucleic acid yields varied with the tissue (**Table S1**). Additionally, acid phenol can be used if DNA extraction is not desired. The protocol was also efficient for RNA isolation from tissues of other plant species besides the model plant, *A. thaliana*. Thus, we believe that the protocol could be used for a wide range of plants and for the extraction of their DNA, as well. The method has proved to be very useful since it allows for the nucleic acid extraction from the same sample of different plant tissues, even in small amounts, avoiding technical and sampling bias (Peña-Llopis and Brugarolas, 2013; Sellin Jeffries et al., 2014). Routine and sensitive analyses using RNA and DNA as templates, such as RNA-seq, PCR, qRT-PCR, Sanger sequencing, bisulfite treatment, and enzyme digestions (including the McrBC enzyme) have been demonstrated to work normally and did not present differences from the conventional protocols.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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Supplementary material

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