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Precise isolation of high-quality RNA from leaves and storage roots of cassava (*Manihot esculenta* Crantz) for gene expression studies

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Abstract

Background The isolation of Ribonucleic Acid (RNA) from leaves and storage roots of cassava (*Manihot esculenta* Crantz) is a challenging one, due to the presence of large amounts of polyphenolic compounds, polysaccharides, and tuber proteins. RNA with high quality and intact integrity is vital for gene expression studies. We hereby report a precise, reproducible, and less cumbersome technique for isolating high-quality RNA from leaves and storage roots of cassava with minimal contamination from polyphenols, polysaccharides, and other secondary metabolites, using affordable reagents. This protocol functions without guanidinium salts in the extraction buffer. The presence of guanidinium salts usually leads to the formation of agglomerates during the extraction of RNA from plant tissues with high starch contents.

Results The isolated RNA from leaves and storage roots of the ten cassava genotypes yielded between 1576.1 and 2861.9 µg/ml for RNA isolated from the leaf tissues and 2761.2–3873.5 µg/ml for RNA isolated from the storage roots. The A260:A280 ratios of the total RNA were more than 2.0 for both leaf and storage root samples, indicating minimal contamination from polysaccharides and polyphenols. The RNA samples recorded intact integrity, as demonstrated by clear 28 S and 18 S rRNA bands observed on agarose gel electrophoresis. The RNA integrity number (RIN) values ranged between 7.2 and 8.0. Also, the RNA samples were successfully used for transcriptome sequencing.

Conclusion The present method which yielded high-quality and transcriptionally competent RNA samples is suitable for use in gene expression studies and downstream applications in the molecular breeding of cassava and related root/tuber crops.

Keywords Cassava (*Manihot esculenta* Crantz), Leaf and storage root, RNA isolation, Polyphenols, Polysaccharides, RT-qPCR, RNA sequencing

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Background

Cassava leaf and storage roots contain large amounts of secondary metabolites. The storage root which is the main harvestable part of cassava accounts for starch content of about 19.7–36.8% of its fresh weight depending on the genotype (Sun et al. 2022). Isolation of RNA from tissues of root and tuber crops containing large amounts of secondary metabolites such as cassava leaves and storage roots is not a very easy process as these compounds strongly limit the extraction of high-quality RNA. RNA samples meant for transcriptome or gene expression studies should be of high quality and purity to obtain a complete representation of the genes being expressed in the analyses.

Secondary metabolites such as polysaccharides and polyphenols present in plant tissues containing high starch and fiber could greatly interfere, degrade or co-precipitate with RNA during the nucleotide purification process due to the similarity in physical and chemical properties between RNA and polysaccharides. The elimination of starch co-precipitation during RNA isolation results in obtaining enhanced yield and high-quality total RNA (Amaranatha et al. 2020; Impa et al. 2020).

There are several reported RNA isolation techniques developed for crops like Arabidopsis, rice, maize, wheat, millet and sunflower, which are not suitable for isolating total RNA from cassava leaves and storage roots (Guan et al., 2019).

The traditional protocols for RNA isolation usually require much labor and time which are not suitable when applied to plant tissues with a high concentration of phenolic compounds and polysaccharides. This study is aimed to develop an efficient and reproducible protocol for the isolation of total RNA from cassava leaf and storage roots meant for molecular cloning, construction of cDNA libraries, RNA sequencing, differential expression analyses of RNA-Sequence data and other gene expression studies.

In this study, we describe a precise and less cumbersome technique for isolating high quality and transcriptionally competent RNA from the leaf and storage root of cassava. The protocol avoided the use of guanidinium salts in the extraction buffer which was replaced with sodium dodecyl sulfate, sodium acetate and ethylenediamine tetraacetic acid. Before the standardization of the present protocol we are reporting, we explored the isolation of RNA from cassava tissues using two different commercial kits, which failed at the first stage of the isolation protocol.

Methods

Plant materials

The following cassava varieties: YTP-1, YTP-2, Sree Jaya, S. Athulya, H-165, TME-419, UMUCAS-36, MVD-1, H-226 and white Thailand were grown at the organic orchard of Tamil Nadu Agricultural University Coimbatore, India. Freshly harvested 12 months old cassava storage root and leaf samples were used for the RNA isolation.

Reagents

All the reagents were prepared with 0.1% diethylpyrocarbonate (DEPC) treated water.

1. Extraction buffer.
2. DEPC-treated water.
3. Chloroform.
4. Isopropanol.
5. 70% Ethanol.

The extraction buffer contains the following: Phenol solution (Equilibrated with10mM Tris HCL pH 5.7 ± 0.2 with 1 mM EDTA) procured from HIMEDIA laboratories pvt LTD, India (#catalog number MB082), Sodium acetate [NaOAc; 0.32 M (w/v)], Sodium dodecyl sulfate [SDS; 0.1% (w/v)] and ethylenediamine tetra acetic acid (EDTA; 0.01 M pH 8.0). For the working concentration of the extraction buffer, in a 50 ml falcon tube, add 10 ml of phenol solution, 5 ml of 0.32 M NaOAc, 5 ml of 1% SDS and 5 ml 0.01 M EDTA. Before commencing the isolation process, all plastic materials, spatulas, mortars and pestles were autoclaved.

RNA isolation protocol

Before standardizing the present protocol, two different commercial RNA isolation kits were explored in the isolation of RNA from cassava storage roots, and agglomerate formation was observed after adding the extraction buffer to the extracts (Fig. 1), which made us discontinue using the kits and proceeded with the protocol as follows:

100 mg of cassava leaf or storage root was ground to a fine powder in liquid nitrogen using a mortar and pestle. 3 ml of extraction buffer was added and ground well. (The extraction buffer freezes the plant tissue when added, therefore to make a fine powder of the frozen material, grind further to mix well).

900 µL of DEPC-treated water was added and mixed well with the pestle. The content was transferred to a 15 ml falcon tube and kept at room temperature for 5 min. Then, 300 µL of chloroform was added to the extract and vortexed for 5–8 s, and kept at room temperature for 10 min. The extract was centrifuged at

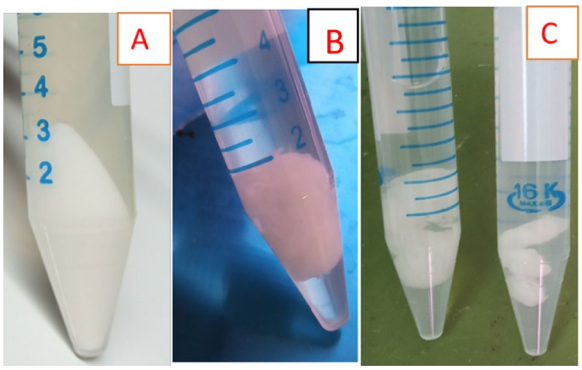


Fig. 1 Standardization of RNA isolation protocol. **a** The standardized protocol. **b** Spectrum RNA kit (Sigma) protocol. **c** RNeasy Plant Midi Kit (Qiagen) protocol. No agglomerate was formed after adding the extraction buffer to the cassava root extract while following the standardized protocol (**a**). Agglomerates were formed during vortexing after adding solution 1 (extraction buffer) to the cassava root extracts while using Spectrum (Sigma) and RNeasy Midi Kit (Qiagen) RNA isolation kits (**b, c**).

10,000 rpm for 10 min at 4 °C, and the upper phase was transferred to a new tube. 0.7 volume of ice-cold isopropanol was added and vortexed for 5–8 s and kept for 10 min at 4 °C. The tube was centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant was discarded. RNA pellet was washed with 70% ethanol, air dried and dissolved in 50–100 µL of DEPC-treated water (Sanjay et al. 2011).

Assessment of RNA quality

The concentration and purity of the RNA samples were assessed by determining the absorbance at 260 and 280 nm using a spectrophotometer (Genona Nano 69357, Cole-Parmer LTD, Uk).

The integrity of the RNA samples was evaluated by the 28S, 18S and 5S rRNA bands observed from 2 µL total RNA in the 1.2% agarose gel electrophoresis. The gels were stained with ethidium bromide and visualized under UV light. The photographs were taken using Vilber Bio-print (Vilberlourmat, India) gel documentation system.

Reverse transcription-polymerase chain reaction (RT-PCR)/ real-time quantitative PCR (RT-qPCR) analyses.

The total RNA was treated with DNase 1-RNase-Free solution (Thermo Fischer Scientific, India), 4 µL of total RNA from the cassava tubers were reverse transcribed in a total of 20 µL reaction using Oligo dT primer (1 µM) and digested with RNase I following the instructions in PrimeScript™ 1st strand cDNA synthesis kit (Takara, India). Synthesis of the first strand cDNA of the treated total RNA was carried out, then the cDNA was used as a template for amplification in the RT-PCR. Separate reactions containing no-RT and no-template were added as controls. PCR was carried out with Taq DNA polymerase (Thermo Fischer Scientific, India) using gene-specific primers. For PCR, the condition was as follows: initially, denaturation at 94 °C for 2 min, followed by annealing for 30 cycles of 30 secs at 94 °C, 30 secs at 54 °C, and 30 secs at 72 °C. The final extension occurred at 72 °C for 5 min.

The quantitative Real-Time PCR analysis was carried out in a Mastercycler® nexus thermocycler (Eppendorf, India). The selected reference genes used were Ubiquitin conjugating enzyme E2-10 (U10), Glyceraldehyde-3-phosphate dehydrogenase (G3pdh) and Ribosomal 18S, with the following pairs of primer sequences: forward: 5′-AAGCCAGTCACTGTTTTCGG-3′, reverse: 5′-CGTACCTTCAAGTGAGCAGC-3′, forward: 5′-AGTCACTATTCATTTCCCGCCCGA-3′, reverse: 5′-TGTCCAGACAAATGCTTCCGTTGC-3′ and forward: 5′-ATTGGAGGGCAAGTCTGGTG-3′, reverse: 5′-CTTCAAAGTAACAGCGCCGG-3′ respectively (Table 1).

The reaction was carried out in a final volume of 20 µl using SYBR® Green Master Mix (India) following the manufacturer's instructions. The qPCR condition was set as follows: denaturation at 94 °C for 30 s; 45 cycles of 94 °C for 5 s, 60 °C for 15 s and 72 °C for 10 s, including melting and cooling. Finally, the PCR products were analyzed on 1.2% agarose gel electrophoresis and visualized with the Vilber Bio-print gel doc system.

Table 1 Primer sets of the selected reference genes used for RT-qPCR

S/N	Gene name	Forward 5′ → 3′	Tm	Length	Reverse 5′ → 3′	Tm	Length
1	Ubiquitin conjugating enzyme E2–10 (U10)	AGTCACTATTCATTTCCCGCCCGA	59.9	24	TGTCCAGACAAATGCTTCCGTTGC	59.9	24
2	Glyceraldehyde-3-phosphate dehydrogenase (G3pdh)	AAGCCAGTCACTGTTTTCGG	58.69	20	CGTACCTTCAAGTGAGCAGC	58.93	20
3	Ribosomal 18S	ATTGGAGGGCAAGTCTGGTG	59.96	20	CTTCAAAGTAACAGCGCCGG	59.83	20

Results

The quantity and quality of isolated total RNA samples were determined by Nano-spectrophotometer and gel electrophoresis without compromise of purity, as A_{260}/A_{230} ratios ranged between 1576.1 and 2861.9 µg/ml for total RNA isolated from the leaf samples (Table 2), and 2761.2–3873.5 for total RNA isolated from the storage root samples (Table 3), while the A_{260}/A_{280} ratios were > 2.0 in all the RNA samples isolated from both leaf and the storage roots.

The representative electropherograms (Fig. 4a–c for RNAs isolated from the leaf and Fig. 4d–f for RNAs isolated from storage roots) showed clear peaks of rRNAs.

The RNA samples were reverse transcribed and the target genes were amplified from cDNA using RT-PCR. The melting curve was at about 80 °C, 75 °C and 80 °C for G3pdh, R18S, and U10 genes, respectively (Fig. 5a–c), and the values for qPCR cycle thresholds (Ct) ranged between 24 and 30 cycles (Table 4).

The reverse-transcribed RNA samples and the selected target genes were amplified from cDNA via PCR and checked in 1% agarose gel electrophoresis for objective bands. For the R18S gene, objective bands were observed at approximately 169 bp, while for U10 and G3pdh genes, the objective bands were observed at approximately 110 bp and 180 bp, respectively (Fig. 6).

Discussion

Standardization of RNA isolation protocol

A standardized phenol-based and guanidinium salt-free protocol was developed for the isolation of total RNA from leaves and storage roots of ten different varieties of cassava. The reagents used were selected based on their specific properties: phenol solution as a strong denaturant of protein, SDS, and EDTA as RNase inhibitors. Thus, the extraction buffer provided a cocktail of RNase inhibitors and protein denaturants. Moreover, phenol is more suitable for high-throughput RNA extraction from a broad range of plants rich in polysaccharides and polyphenols and acidic phenol lowers the pH and decreases DNA contamination and polysaccharide contents (Liu et al. 2018). Also, the pH of the extraction buffer was adjusted to be in the acidic range to enable efficient partitioning of RNA in the aqueous phase and allow DNA to be in the phenolic phase. When using phenol for RNA isolation, the partitioning of nucleic acids is sensitive and the major determinant for nucleic acid partitioning is the equilibrated pH of the aqueous phase, which is acidic, because, at acidic pH, almost all genomic DNA will be partitioned into the phenol phase, and DNA-free total RNA will be retained in the aqueous phase (Xu et al. 2019).

For suitability of RNA isolation from the Euphorbiaceae plant family, an appropriate concentration of NaOAc was included in the extraction buffer to facilitate the precipitation of RNA in the presence of isopropanol, the addition of SDS in the extraction buffer served as a strong anionic detergent to solubilize lipid proteins and lipids, while EDTA was added to increase the RNA yields, and these are in agreement with previous studies (Babak et al. 2019).

Meanwhile, the addition of DEPC-treated autoclaved water into the extract before transferring it to falcon

Table 2 Nano-spectrophotometer result showing yield and purity of the total RNA isolated from the cassava leaf samples

S/N	Genotype	Yield µg/ml	A260/280	A260/230
1	YTP-1	1801.9	2.086	1.587
2	YTP-2	1980.2	2.054	1.921
3	S. Jaya	1826.8	2.701	1.621
4	S. Athulya	1961.8	2.077	1.546
5	H-226	1834.1	2.096	1.567
6	H-165	1871.2	2.601	1.825
7	TME-419	1576.1	2.107	1.783
8	UMUCAS-36	1578.4	2.104	1.534
9	MVD-1	2861.9	2.714	1.827
10	W. Thai	1827.1	2.133	1.561

Table 3 Nano-spectrophotometer result showing yield and purity of the total RNA isolated from the cassava storage root samples

S/N	Genotype	Yield (µg/ml)	A260/280	A260/230
1	YTP-1	2876.1	2.134	1.837
2	YTP-2	3482.9	2.196	1.641
3	S. Jaya	2871.1	2.159	1.566
4	S. Athulya	2901.3	2.714	1.624
5	H-226	2865.2	2.711	1.571
6	H-165	2767.2	2.154	1.864
7	TME-419	3376.1	2.179	1.842
8	UMUCAS-36	2761.2	2.764	1.573
9	MVD-1	3873.5	2.646	1.736
10	W. Thai	2957.9	2.701	1.568

Table 4 qPCR cycle thresholds (Ct) for the selected reference genes from both RNA isolated from the leaf and storage root of cassava

S/N	Leaf	Ct value	Storage root	Ct value
1	U10	30. 02	U10	30.04
2	G3pdh	28.54	G3pdh	29.52
3	R18S	24.50	R18S	24.58

tubes provided a sufficient aqueous environment for partitioning of RNA in the aqueous phase, preventing oxidation of the phenolics present in the extract, hence easing the RNA extraction and subsequent re-suspension in DEPC-treated water. The presence of chloroform promoted protein precipitation and also decreased polysaccharide contents in the extract (Sanchez et al. 2016).

It is remarkable to note that, while the two kits failed at the very first stage of RNA extraction, the reported protocol isolated high-quality RNA as shown in Tables 1 and 2 with clear bands on 1.2% agarose gel electrophoresis as shown in Figs. 2 and 3. Distinct 28 S and 18 S rRNA bands were observed on the gel electrophoresis before DNase treatment was given to the RNA samples, which indicated intact RNA.

It has been reported that Trizol-based methods of isolating RNA from plant tissues are not suitable for plants belonging to the Euphorbiaceae family such as cassava, which is due to the presence of some inhibitory compounds in them. Previous studies have also shown that polyphenolic compounds oxidize and covalently link with quinones, which in turn bind to RNA to form high molecular weight complexes that can interfere with RNA quantity and integrity. Polysaccharides co-precipitate with RNA in the presence of alcohols and stand as contaminants up to the final stage of RNA extraction which hamper its use in subsequent molecular applications as high-quality RNA is a pre-requisite for various techniques in molecular biology, including gene expression quantification and validation by RT-PCR, RT-qPCR, RNA-sequence analysis, northern blot analysis, and PCR amplification-based gene cloning. Therefore, for efficient isolation of RNA with high quality, it is consequential to remove polyphenols, polysaccharides, proteins, and other secondary metabolites (Amaranatha et al. 2020).

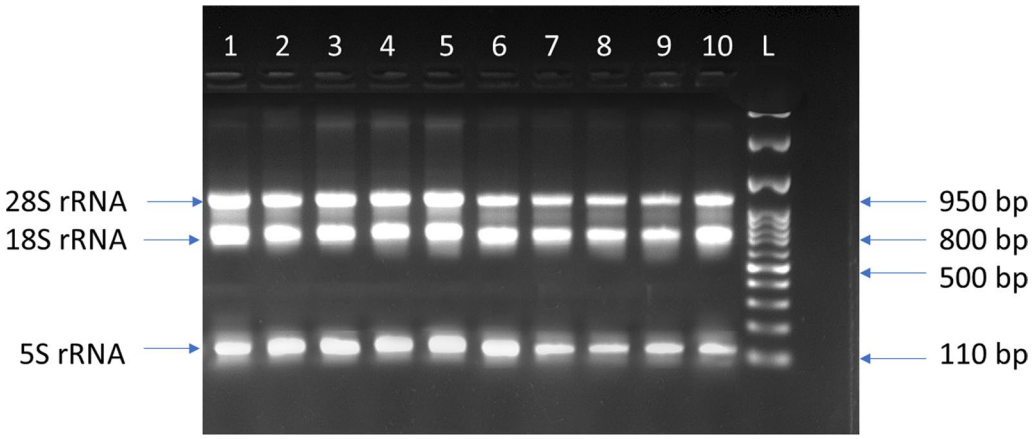


Fig. 2 Gel electrophoresis of the total RNA isolated from storage root samples of the 10 cassava varieties. (1) YTP-2, (2) YTP-1, (3) White Thailand, (4) Sree Jaya, (5) Sree Athulya, (6) UMUCAS-36, (7) TME-419, (8) MVD-1, (9) H-165, (10) H-226, (L) DNA Ladder

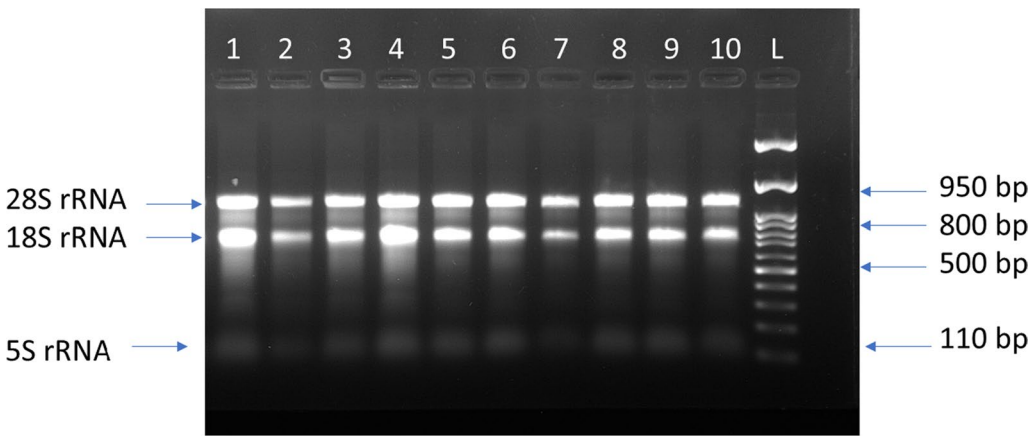
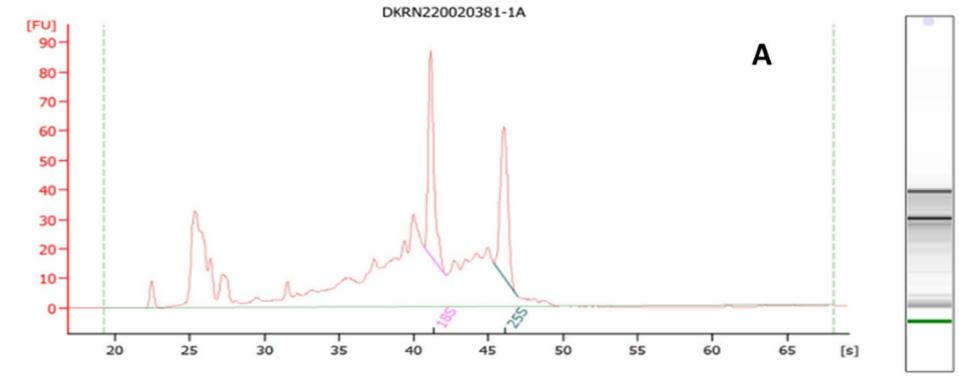


Fig. 3 Gel electrophoresis of the total RNA isolated from leaf samples of the 10 cassava varieties. (1) YTP-2, (2) YTP-1, (3) White Thailand, (4) Sree Jaya, (5) Sree Athulya, (6) UMUCAS-36, (7) TME-419, (8) MVD-1, (9) H-165, (10) H-226, (L) DNA Ladder



B

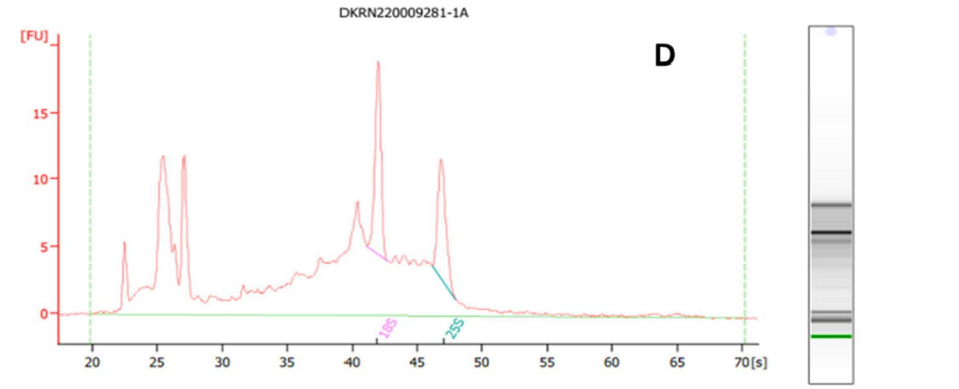
Overall Results for sample 3: **DKRN220020381-A**

RNA Area:	843.6	RNA Integrity Number (RIN):	8 (B.02.10)
RNA Concentration:	1,068 ng/µl	**Result Flagging Color:**	
rRNA Ra110 (25s I 18s):	1.5	Result Flagging Label:	RIN: 8

C

Fragment table for sample 3: **DKRN220020384-1A**

Name	Start Time (s)	End Time [s]	Area	Start size [nt]	End size [nt]	% of total Area
185	40.71	42.04	57.9	1,569	1,986	18.2
255	45.35	46.76	77.2	2,563	3,641	25.5



E

Overall Results for sample 6: **DKRN220009281-A**

RNA Area:	291.5	RNA Integrity Number (RIN):	7.5 (B.02.10)
RNA Concentration:	989 ng/µl	**Result Flagging Color:**	
rRNA Ra110 (25s I 18s):	1.2	Result Flagging Label:	RIN: 7.50

F

Fragment table for sample 6: **DKRN220009281-1A**

Name	Start Time (s)	End Time [s]	Area	Start size [nt]	End size [nt]	% of total Area
185	41.13	42.76	39.8	1,462	1,732	16.8
255	46.17	48.00	67.4	2,371	3,131	24.0

Fig. 4 Bioanalyzer results of total RNA isolated from lead and storage root of cassava. Electropherograms of the total RNA isolated from the leaf and storage root of cassava showed the 18S and 25S rRNA regions with RNA concentrations, RIN values, and results of rRNA analysis. The RIN values ranged between 7.1 and 8, which is optimal for obtaining good results in cDNA library construction and next-generation sequencing analysis

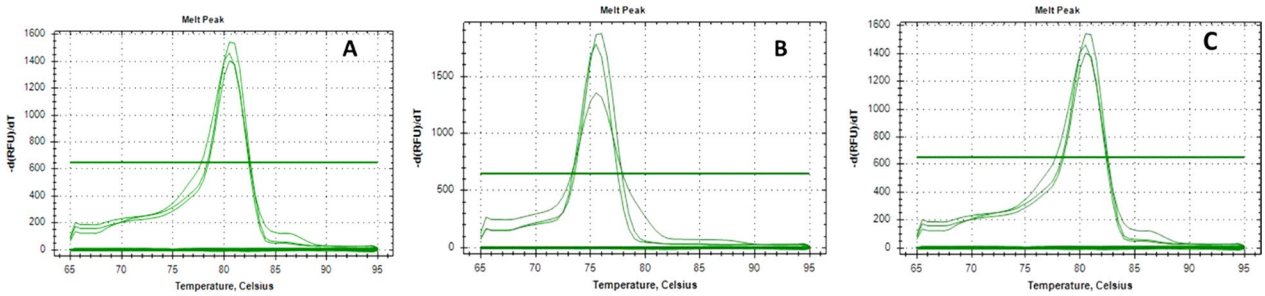


Fig. 5 RT-qPCR analysis of three selected reference genes in the leaf and storage root of cassava, showing the melting peaks of the PCR products for the amplified genes

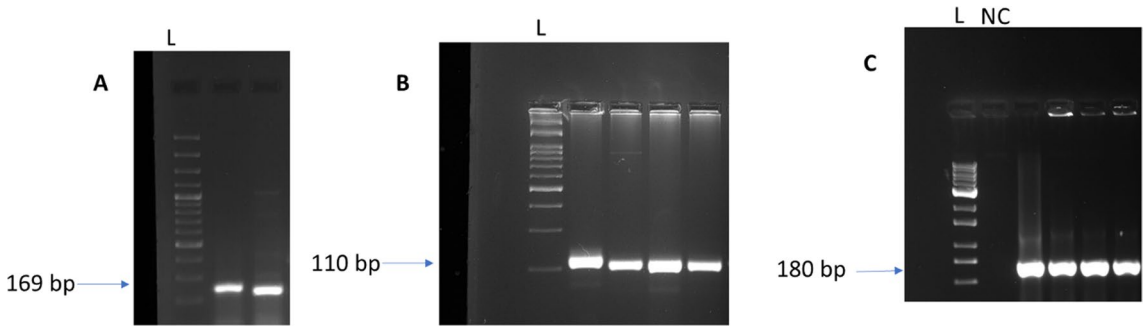


Fig. 6 Analysis of amplified cDNAs in 1% agarose gel electrophoresis. L: 500 DNA marker, NC: Negative control, A: Ribosomal 18S, B: Ubiquitin conjugating enzyme E2-10, C: Glyceraldehyde-3-phosphate dehydrogenase

Even though some commercial RNA extraction kits, like Spectrum RNA kit (Sigma), RNaesy Plant Mini Kit (Qiagen) and Pure link RNA Reagent (Thermo Fisher) to mention but a few, are available for RNA extraction from various plant tissues, however, these kits are not suitable for analyzing plant tissues containing high concentrations of starch and polyphenols like cassava roots and leaves and still obtain high-quality RNA. Also, the use of these kits while extracting RNA requires additional purification steps for such samples which, however, reduces the quality and quantity of extracted RNA. The cost of research is a critical part of any research project and most RNA extraction kits are expensive as cost keeps increasing with an increased number of samples to be analyzed, therefore, using the present method is cost-effective as well as time-saving and can be applied in other root and tuber crops.

Use of the RNA isolates in downstream applications

DNA contamination is usually an issue in most rapid RNA isolation protocols; hence the RNA samples were treated with RNase-free DNase solution to eliminate DNA contamination before being used for further molecular analyses since DNase digestion is a common step in protocols involving the use of RNA for downstream applications (Khairul-Anuar et al. 2019; Tang et al. 2019). High purity and concentration of RNA samples are required for transcriptomics analyses, therefore for next-generation sequencing, the isolated total RNA was sent to Oneomics laboratories, Tiruchirappalli, Tamil Nadu, India, and all the samples passed the quality control analysis as shown in the bioanalyzer results in Fig. 4 and were successful in the cDNA library construction which sowed their amenability to downstream applications, and finally used for RNA sequencing, in line with low-cost protocol and data analysis pipeline for RNA-sequencing (Huang et al. 2023).

The functional intactness was confirmed via RT-qPCR analysis as recorded in Fig. 5. The RT-qPCR method has been used widely in the analysis of gene expression due to its specificity, sensitivity rapidity, and reproducibility (Nong et al. 2019). In the RT-PCR analysis carried out U10, G3pdh, and R18S primer sequences were successfully amplified from the cDNA. The obtained melting curve values (Table 4) showed that the primers used were able to amplify a single major peak. Also, the single PCR product of the anticipated size observed on the gel electrophoresis image in Fig. 6 indicated that the PCR product was specific, showing that the present protocol is suitable for the isolation of RNA samples

free of inhibitors and contaminants, from cassava tissues and other related root and tuber crops, as it is well-reported that reverse transcriptase is highly sensitive to impurities as well as confirmed the suitability of the isolated RNA samples for molecular applications including RNA sequencing, which is in line with previous reports (Luo et al. 2018).

Conclusions

We standardized a rapid and precise protocol for isolating high-quality RNA from cassava tissues such as leaf and storage roots. The total RNAs isolated from both the leaf and storage roots of the ten cassava genotypes were found to be suitable for the construction of cDNA libraries, RNA sequencing, gene expression studies and other downstream molecular applications, not only for cassava research but for other related root and tuber crops. The present protocol is cost-effective and can be carried out using basic reagents and laboratory consumables easily accessible by cassava researchers in continents like Africa and Asia who may not have access to sufficient research funding.

Abbreviations

RNA	Ribonucleic Acid
RIN	RNA integrity number
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
EDTA	Ethylene diamine tetra acetic-acid
NaOAc	Sodium acetate
SDS	Sodium dodecyl sulfate
rRNA	Ribosomal Ribonucleic Acid
PCR	Polymerase Chain Reaction
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

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Author contributions

Conceptualization of the research was given by RM, the methodology was provided by UD, Planting and provision of plant samples was done by KPS, sample preparation and laboratory analysis were carried out by JIR-K, preparation of original draft was done by JIR-K, supervision of the research work was done by KE, UD, BV, RM, and KPS. All the authors read and approved the final manuscript.

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Availability of data and materials

The data generated and analyzed during this study have been included in this article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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