Journal of Root Crops, 2016, Vol. 42 No. 1, pp. 53-56
Indian Society for Root Crops
ISSN 0378-2409, ISSN 2454-9053 (online)

A Simple Protocol for Isolating Small RNA from Cassava (*Manihot esculenta* Crantz)

D.C. Deepthi and T. Makeshkumar

ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram, 695 017 Kerala, India
Corresponding Author: T. Makeshkumar; e-mail: makeshctcri@gmail.com
Received: 12 May 2016; Accepted: 20 June 2016

Abstract

Small RNAs of 20-30 nucleotide are involved in re gulatory processes at DNA or RNA levels in many eukaryotic plant systems. Good and efficient protocols to isolate small RNAs are needed for characterization. A simple method for isolating small RNAs from virus infected plant species has been developed. The method involves precipitation of low molecular weight RNA using NaCl, PEG 8000 and LiCl. The purity of isolated small RNA was confirmed by A260/280 and A260/230 ratio. The described protocol also resulted in isolation of sufficiently higher yield of small RNA from virus infected leaf samples of cassava. The current protocol can be completed in a day and is cost effective. With regard to cost, the per sample cost associated with commercially available kits were approximately 32 times and for Trizol it is 5 times higher than that of current protocol.

Key words: Small RNA isolation, LiCl, denaturing PAGE, cassava

Introduction

Eukaryotes have several types of tiny regulatory RNAs (Ruvkun, 2001; Waterhouse et al., 2001; Grosshans and Slack, 2002). Small temporal RNA (stRNA) and small interfering RNA (siRNA) are involved in the post-transcriptional control of gene expression. Small RNAs are 18-30 nt non-coding regulatory elements found in diverse organisms, which were initially identified as small double-stranded RNAs in *Caenorhabditis elegans*. With the development of new and improved technologies, small RNAs have also been identified and characterized in plant systems. Among them, micro RNAs (miRNAs) and small interfering RNAs (siRNAs) are found to be very important riboregulators in plants (Praveen Guleria et al., 2011).

The siRNAs are associated with RNA silencing (RNA interference [RNAi]), serving as guides for sequence-specific nucleolytic activity of the silencing-associated RNA Induced Silencing Complex (RISC) (Hamilton and Baulcombe D.C., 1999; Hammond et al., 2000, 2001; Zamore et al., 2000). During viral infection in plants, double stranded RNAs (dsRNAs) are produced by viral/cellular-encoded RNA Dependent RNA Polymerases (RDRs) and are processed to siRNAs by cellular DICER-LIKE enzymes (DCLs) (Hamilton et al., 2002; Macrae et al., 2006). siRNAs deriving from the negative strand of viral RNAs can guide cleavage of complementary positive-strand viral RNAs, resulting in a strong decrease in virus accumulation, a cellular immune response referred to as viral recovery (Mallory and Vaucheret., 2006). The relevance of small RNAs may be much broader than was suspected previously.

Various protocols are available to isolate small RNAs from plant species. Most of them are based on the commercially available TRIZOL reagent (Invitrogen) which is not efficient for cassava. The option of using commercially available kits is much expensive. So the aim of the present study is to develop a protocol for isolation of small RNA, which is cost effective and less time consuming.

Materials and Methods

Plant material

Leaves of five different cassava varieties viz., H226, H165, CMR 1, CMR 117 and MNga-1 were collected from the

field of ICAR-Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala, India.

Reagents and solutions

Extraction buffer consisting of 100mM Tris HCl (pH 9.0), 100mM LiCl, 1% SDS and 10mM EDTA was prepared. In addition phenol (pH 8.0), 3M sodium acetate, 5M NaCl, 40% PEG 8000, absolute ethanol, chloroform:isoamyl alcohol (24:1) and 0.1% DEPC treated water were also used. 10X TBE buffer was prepared by mixing 54 gm Tris Base, 27.5 gm Boric acid and 20 ml 0.5 M EDTA (pH8.0). 5X loading dye (87% glycerol, 1M Tris (pH 7.7), 0.5 M EDTA and a little bit bromophenol blue), poly acrylamide stock solution (40% acrylamide: bis acrylamide (37.5:1)),10x TBE buffer (pH 8.4) and 7M Urea) and denaturing poly acrylamide gel (15 ml poly acrylamide stock solution, 7.5μl TEMED and 30μl APS (25%)) was prepared and used for running Poly Acrylamide Gel Electrophoresis. 0.5X TBE with 0.5μg/ml ethidium bromide was used as staining solution.

Isolation of small RNA

One hundred mg of leaf tissue was ground with liquid nitrogen using motor and pestle. The ground sample was homogenized with 500 μl extraction buffer and 500 μl phenol (pH 8.0). The sample was vortexed for one minute, incubated for 5 minutes at 65ºC and then centrifuged at 20000 rpm for 20 minutes at 4ºC. Upper phase was transferred to a new tube and equal volume of chloroform: iso amyl alcohol (24:1) was added. It was incubated at 65 ºC for 15 minutes and centrifuged at 15,000 rpm for 10 minutes at 4 ºC. Upper phase was transferred to new tube, 50 μl of 5M NaCl and 63 μl of 40% PEG 8000 was added and vortexed for one minute. This was followed by incubation on ice for 30 minutes. After centrifugation for 10 minutes at 20000 rpm at 4ºC, the supernatant was transferred to new tube and 50 μl of 3M sodium acetate (pH 5.2) and 1 ml of absolute ethanol were added. After incubating at -20 ºC for 30 minutes, the sample was centrifuged at 20000 rpm for 10 minutes at 4ºC. Supernatant was discarded and the pellet was air dried. The pellet was dissolved in 20μl DEPC treated water.

Analysis of small RNA in denaturing Poly Acrylamide Gel

Small gel plates, combs, spacers and gel tank were washed with 0.4N NaOH. 17% poly acrylamide gel was prepared and heated at 42ºC for complete dissolution followed by addition of TEMED and APS. The gel was poured in to the gap between the glass plates and was allowed to polymerise for 1-2 hours. The gels were pre run for 30-90 minutes with 0.5(X) TBE buffer.

Sample preparation

RNA sample (5-15 μg) was used at lowest volume possible. All samples were adjusted to equal volume with autoclaved water. Two volumes of formamide were added to one volume of 5X loading dye. One and a half volumes of resulting dye were added to each sample. Samples were boiled at 95ºC for 4 minutes and were immediately placed on ice. Before loading, each well was washed with 0.5(X) TBE to remove the urea crystals that were formed. Each sample was carefully loaded in wells while the empty wells were loaded with same volume of loading buffer. The gel was run on 180V at 4ºC until blue dye reached at the bottom of the gel. The gel was taken out of running chamber and placed in staining solution (0.5X TBE with ethidium bromide) for about 5 minutes. The gel was pictured with a UV transilluminator (AlphaImager, AlphaInnotech).

Results and Discussion

Extraction of high-quality small RNAs is essential and also a limiting factor in some experiments such as Northern blotting assay and the investigation of specific miRNA or siRNA expression profiles using end-point RT-PCR amplification or microarray. Consistent and reproducible methods for isolation and detection of small RNA currently pose a major problem in molecular biology experiments. We report a standardized method for isolating small RNA from cassava plants which reduces time and cost among different protocols. Most of the currently available isolation methods for small RNA are based on commercially available TRIZOL reagent or with commercially available kits. The present protocol based on LiCl precipitation was compared with other published protocols using TRIZOL and CTAB (Carra et al., 2007; Peng et al., 2014). The methods using TRIZOL and CTAB did not yield small RNAs in cassava while the present method with LiCl yielded good quality small RNA (Fig. 1).

The success of the RNA isolation protocol can be judged by the quality, quantity and integrity of small RNAs recovered (Peng et al., 2014). The high quality of small RNAs described here was confirmed with absorbance ratios and electrophoretic analysis. The A260/280 absorbance ratio ranged from 2-2.5 and the A260/230 ratio

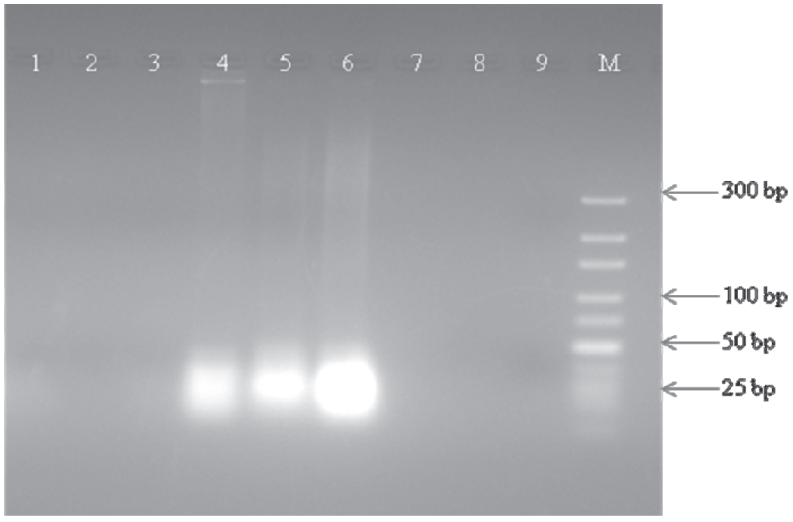

Fig.1. Small RNA isolated in 4% agarose gel
Lane 1 to 3: Trizol method
Lane 4 to 6: LiCl method
Lane 7 to 9: CTAB method
Lane M : Ultra low range ladder

was in a range of 2.0–3.0 for LiCl method, indicating that the small RNAs were relatively free from proteins and other impurities. But in CTAB method, the A260/280 absorbance ratio was below 1.8 and A260/230 ratio was very low, indicated protein or phenol contamination. The yield and absorbance ratio of small RNA from 100 mg of plant tissue was quantified using Nanodrop is tabulated in Table. 1. 17% PAGE electrophoresis showed clear, discrete small RNA bands (Fig. 2).

LiCl based extraction and precipitation has been successfully used for the isolation of total RNA from different plant species (Fu et al., 2004; Rekha et al., 2008). Rosas-Cardenas et al., 2011 reported LiCl based extraction method for plant tissues with high polysaccharide content, which is time consuming. This efficient method is optimised in the present study for small RNA extraction from cassava plants which can be completed within three hours. LiCl precipitation offers major advantages over other RNA precipitation methods as it does not efficiently precipitate DNA, proteins or carbohydrates (Barlow et al., 1963). Tris HCl helps to prevent degradation of RNA. High concentration of EDTA was used for inhibiting the oxidization of polyphenol to polyquinones and the activities of degrading enzymes as well (Chang et al., 1993; Kiefer et al., 2000). Moreover, the use of NaCl in higher concentrations effectively separates the contaminating compounds in plant tissues (Bekesiova et al., 1999).

Table 1. Yield and spectrophotometric absorbance ratio of small RNA isolated with different extraction methods

Extraction methods	Tissue	Concentration (ng/μl)	Average 260/280	Average 260/230	Yield (μg)
LiCl	H226	1224.67	2.1	2.7	24.49
	H165	841.55	2.03	2.5	16.83
	CMR 1	1305.64	2.16	2.25	26.11
	CMR 117	1007.86	2.21	2.9	20.16
	MNga-1	565.86	2.02	2.24	11.32
CTAB	H226	325.52	1.28	1.02	6.51
	H165	368.95	1.87	1.22	7.38
	CMR 1	610.19	1.76	1.09	12.2
	CMR 117	435.5	1.88	0.73	8.71
	MNga-1	110.52	1.27	0.68	2.21
TRIZOL	H226	——	——	——	No yield
	H165	——	——	——	No yield
	CMR 1	——	——	——	No yield
	CMR 117	——	——	——	No yield
	MNga	——	——	——	No yield

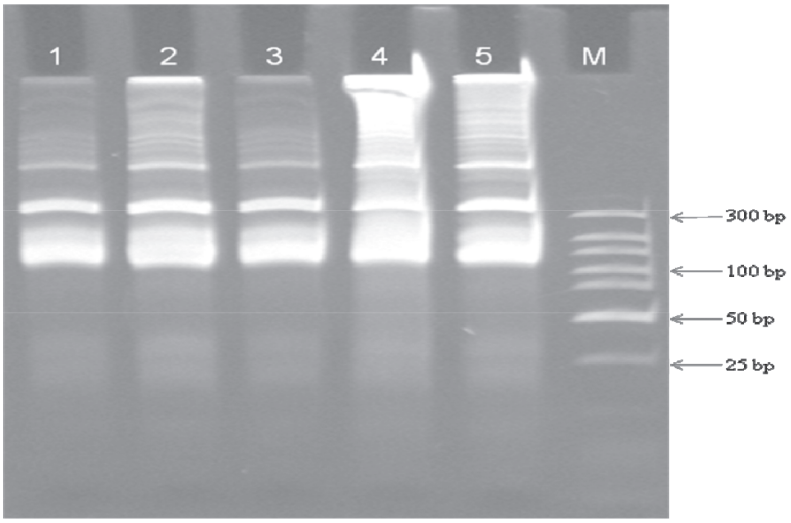

Fig.2. Small RNA isolated in 17% PAGE gel
Lane 1 to 5: Cassava samples
Lane M : Ultra low range ladder

Conclusion

Our results emphasize the importance of comparing different extraction methods in order to standardise small RNA isolation procedures. A simple, effective and rapid method for extraction of small RNA from cassava leaf samples is established. The current method gives good yield and quality of small RNA and can be completed within three hours. The method can be utilised for good quality small RNA extraction from other plant species. The high quality low molecular weight small RNAs obtained in this method can be used for various downstream applications.

References

Barlow, J. J., Mathias, A. P., Williamson, R. and Gammack, D. B. 1963. A simple method for the quantitative isolation of undegraded high molecular weight ribonucleic acid. *Biochem. Biophys. Res. Commun*., **13**: 61-66.

Bekesiova, I., Nap, J. P. and Mlynarova, L. 1999. Isolation of high quality DNA and RNA from leaves of the carnivorous plant *Drosera rotundifolia*. *Plant Mol. Biol. Rep.,* **17**: 269–277.

Carra, A., Gambino, G. and Schubert, A. 2007. A cetyltrimethylammonium bromide based method to extract low-molecular-weight RNA from polysaccharide-rich plant tissues. *Anal. Biochem*., **360**: 318–320.

Chang, S., Puryear, J. and Cairney, J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep*., **11**: 113–116.

Fu, X., Deng, S., Su, G., Zeng, Q. and Shi, S. 2004. Isolating high-quality RNA from mangroves without liquid nitrogen. *Plant Mol. Biol. Rep*., **22**(2): 197a-198e.

Grosshans, H. and Slack, F. J. 2002. Micro-RNAs: small is plentiful. *J. Cell Biol.*, **156**(1): 17-21.

Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. 2002. Two classes of short interfering RNA in RNA silencing. *EMBO J.,* **21**(17): 4671-9.

Hamilton, A. J. and Baulcomb, D. C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, **286**(5441): 950-2.

Hammond, S. M., Bernstein, E., Beach, D. and Hannon, G. J. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*., **404**: 293–296.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. and Hannon, G. J. 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science*., **293:** 1146–1150.

Kiefer, E., Heller, W. and Ernst, D. 2000. A simple and efficient protocol for isolation of functional RNA from plant tissues rich in secondary metabolites. *Plant Mo.l Biol. Rep*., **18**: 33–39.

Macrae, I. J., Zhou, K., Li, F., Repic, A., Brooks, A. N., Cande, W. Z., Adams, P. D. and Doudna, J. A. 2006. Structural basis for double-stranded RNA processing by Dicer. *Science*., **311**(5758): 195-8.

Mallory, A. C. and Vaucheret, H. 2006. Functions of microRNAs and related small RNAs in plants. *Nature Genetics*., June 38 (Suppl): S31-S36.

Peng, J., Xia, Z., Chen, L., Shi, M. and Pu, J. 2014. Rapid and efficient isolation of high-quality small RNAs from recalcitrant plant species rich in polyphenols and polysaccharides. *PLoS ONE.,* **9**(5): e95687.

Praveen Guleria., Monica Mahajan., Jyothi Bharadwaj. and Sudesh Kumar Yadav. 2011. Plant small RNAs: biogenesis, mode of action and their roles in abiotic stresses. *Genomics Proteomics and Bioinformatics*., **9**(6): 183-199.

Rekha Kansal., Kalika Kuhar., Isha Verma., Ram Niwas Gupta., Vijay Kumar Gupta. and Kirpa Ram Koundal. 2008. Improved and convenient method of RNA isolation from polyphenols and polysaccharide rich plant tissues. *Ind. J. Exp. Biol.*, **47**: 842-845.

Rosas-Cárdenas Flor de Fátima, Durán-Figueroa Noé, Vielle-Calzada1 Jean-Philippe, Cruz-Hernández Andrés, Marsch-Martínez Nayelli, Stefan de Folter.2011. A simple and efficient method for isolating small RNAs from different plant species. *Plant Methods*., **7**: 4.

Ruvkun, G. 2001. Molecular biology. Glimpses of a tiny RNA world. *Science*., **294**: 797–799.

Waterhouse, P. M., Wang, M. B. and Lough, T. 2001. Gene silencing as an adaptive defence against viruses. *Nature*., **411**: 834–842.

Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. 2000. RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell.*, **101**: 25–33.