A modified protocol for isolation of high quality total RNA from ginger (*Zingiber officinale* Rosc.) rhizomes

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Abstract

Ginger rhizomes contain high amount of water, polysaccharides, polyphenols and secondary metabolites which interfere with isolation of total RNA. High quality RNA with sufficient quantity is crucial for conducting gene expression studies. An effective protocol for isolation of RNA from ginger rhizomes is essential. The present study is focused on isolation of high quality RNA from ginger rhizome which can be further utilized for downstream applications like cDNA library preparation and differential gene expression studies. The protocol reported by Kumar et al. (2007) was modified and was compared with the original protocol. The modified protocol was found effective in getting higher quality (A260/A280 – 1.95 to 2.05) and quantity (51-58.6 µg/g) of isolated total RNA from fresh and frozen rhizomes. In the original protocol the quality obtained was (A260/A280 - 1.47 to 1.54) and quantity was 24.0-42.0 µg/g.

Keywords: Acid phenol, Ginger rhizome, Modified protocol, Total RNA

Introduction

Ginger is one of the most important spice crops, valued for its medicinal properties. It has a very big genome of 23,618 Mbp distributed in 22 chromosomes, which is not much exploited (Chandrasekar et al., 2009). To explore the vast genomic information stored in this medicinal herb, post translational modifications and gene expression studies are to be carried out. High quality RNA with sufficient quantity is crucial for conducting gene expression studies. All the downstream applications viz., gene isolation, cDNA library construction, transcriptome sequencing, qPCR analysis, northern blot hybridization, microarray analysis and RNA interference rely on good quality and quantity of total RNA. Due to high instability of RNA, there are chances of degradation during downstream manipulations. Often single stranded, RNA contains ribose sugar that carries a 2'-OH group which is prone to hydrolysis, as compared to DNA which has a 2'-H (Sah et al., 2014).

Considering the above, total RNA isolation from plants becomes more challenging as plants contain cell wall, pigments, tannins, polysaccharides and polyphenols and other secondary metabolites (MacRae, 2007). Ginger rhizomes contain high amount of polysaccharides, polyphenols and other secondary metabolites. Polysaccharides tend to co-precipitate with RNA resulting in low yield of RNA (Deepa et al., 2014). The co-precipitated compounds hinder the downstream activity of enzymes viz., reverse transcriptase, DNA polymerase, and DNA restriction endonuclease, thereby affecting the purification as well as quantification of total RNA (Moser et al., 2004). Secondary metabolites include polyphenols that form covalently linked quinines on oxidation and bind to proteins and nucleic acids in an irreversible fashion (Loomis, 1974). Genomic DNA and protein also make complexes with RNA leading to contamination and difficulty in isolation, resulting in low yield of RNA. Further, biotic and abiotic stresses pose more difficulty in isolating

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good quality and quantity of total RNA as stressful conditions increase the production of secondary metabolites (Chang et al., 1993). Most plants also have high levels of RNase activity which degrade RNA. Ginger rhizomes also have high water content which hinders the recovery of good quality total RNA.

Many protocols for isolating total RNA from plants were reported but they vary among species and tissue types. Hence the existing protocols require modifications to get high yield and quality of total RNA from different tissues. An effective protocol for isolation of total RNA from ginger rhizomes is scarce, though Kumar et al. (2007) reported RNA isolation from rhizome/tubers of different crops like ginger, potato, radish, sweet potato and turnip. Deepa et al. (2014) reported RNA isolation from turmeric rhizomes. The present study is focused on isolation of high quality total RNA from ginger rhizome which can be further utilized for downstream applications like cDNA library preparation and differential gene expression studies.

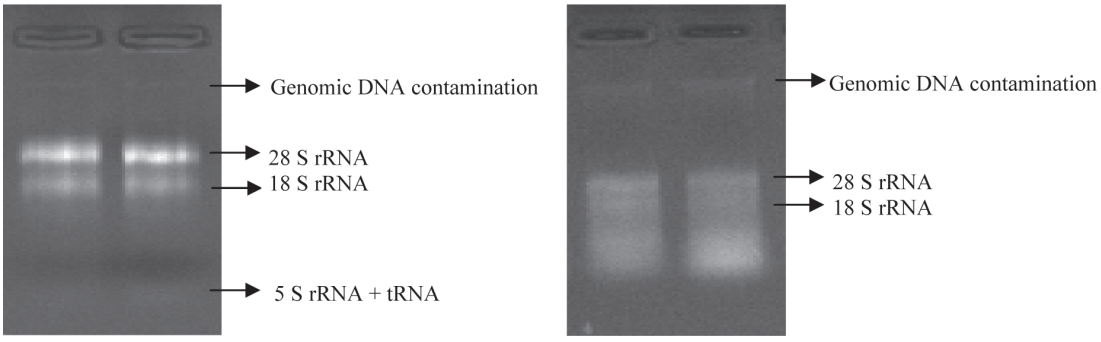
Materials and methods

The rhizome samples for the present work were collected from the ongoing DBT-BIRAC project at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur. The plants were maintained at field condition and rhizomes harvested at the seventh month of planting were used. The sample was harvested, cleaned, weighed and stored at -80°C for later use as frozen rhizomes. Also fresh rhizomes were transferred to liquid nitrogen for immediate isolation purpose.

The protocol reported by Kumar et al. (2007) was modified by using acid phenol (pH 4.2) instead of Tris-saturated phenol (pH 7.9), introducing PVP (2%) in liquid form, using SDS (2%) instead of SDS (10%), 100mM borate-Tris buffer instead of 200mM borate-Tris buffer, 25mM EDTA instead of 10mM EDTA, introducing 3M sodium acetate, using chilled isopropanol and chilled ethanol to isolate total RNA from fresh and frozen (stored at -80°C) ginger rhizomes. Chilled acetone was used in the original protocol to remove polyphenols and pigments from the lyophilized tissues but the modified protocol introduced chilled acetone (5 ml/g rhizome) wash for both fresh and frozen rhizome samples for effective RNA isolation without polyphenol impurities. Also the modified method included pre-warmed extraction buffer and incubation of sample in freezer for one hour for precipitation.

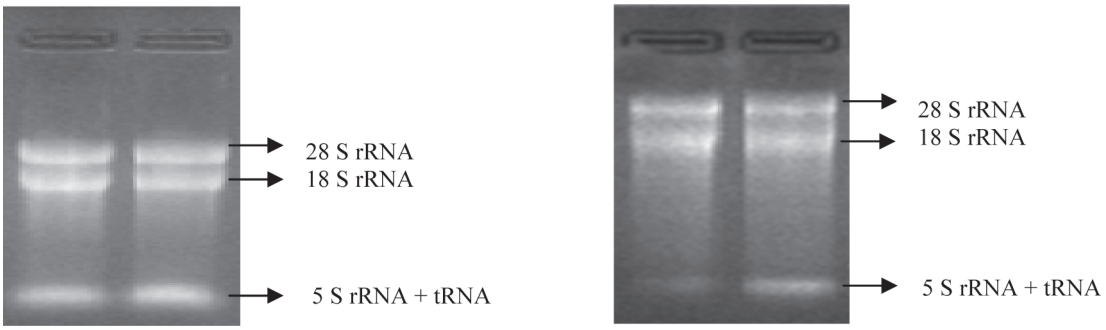
Results and discussion

Total RNA isolation from rhizome tissues of ginger are less reported and it poses more difficulty when compared to RNA isolation from leaves because rhizomes are rich in polyphenols and polysaccharides along with a good amount of water which often complicates the whole procedure of



a) Fresh rhizome b) Frozen rhizome

Plate 1. Total RNA isolated from a) fresh and b) frozen rhizomes using the original protocol by Kumar et al. (2007)



a) Fresh rhizome b) Frozen rhizome

Plate 2. Total RNA isolated from fresh and frozen rhizomes using the modified protocol

good quality RNA isolation. Trizol is a common reagent utilized for RNA extraction from different tissues but gives a low yield and purity for rhizome samples (Deepa et al., 2014).

In the present study the original protocol reported by Kumar et al. (2007) was compared with the modified protocol with respect to quality and quantity of total RNA isolated from fresh and frozen rhizomes of ginger. The total RNA quality and quantity for both fresh and frozen rhizomes were analyzed by taking the optical density values using the Nanodrop Spectrophotometer. Also the quality analysis was done by electrophoresis of the samples in 1 per cent denaturing agarose gel (Masek et al., 2005) using formaldehyde (37%) as a denaturant for removing the RNA secondary structures.

The quality and quantity of total RNA isolated from ginger rhizomes (fresh and frozen) using the original and modified protocol is presented in Table 1. The quality (A260/A280) of RNA isolated from fresh rhizome using the original protocol was 1.47 while the quality was 1.92 in the modified protocol. From frozen (stored for 2 months in -80°C) rhizomes the quality observed in original protocol was 1.54 and in the modified protocol the quality was 2.05. There was 30.61 per cent improvement in quality of RNA from fresh rhizome over the original protocol. The concentration of total RNA showed 39.52 per cent increment for fresh rhizome isolated using the modified protocol. Similarly in the quality of RNA from frozen rhizome, there was an improvement of 33.12 per cent over the original protocol and the concentration also increased to 112.5 per cent for frozen rhizomes when the modified method was adopted.

The pH of the phenol is an important criterion determining the partitioning of DNA and RNA between the organic phase and the aqueous phase (Brawerman et al., 1972; Perry and Kelley, 1972). The modified protocol utilized phenol at acidic pH (4.2). At alkaline or neutral pH (pH 7-8), both DNA and RNA partition into the aqueous phase as the phosphate diesters in nucleic acids are negatively charged at alkaline/ neutral pH. At acidic pH bigger DNA fragments and protein remain at the interphase and small DNA fractionate into the organic phase (Brawerman et al., 1972; Chomczynski and Sacchi,

Table 1. Quality and quantity of total RNA isolated from ginger rhizomes (fresh and frozen)

Optical density (OD)	Original protocol		Concentration (µg/g)	
	Fresh	Frozen	Fresh	Frozen
A260/A280	1.47	1.54	42.0	24.0
A260/A230	1.73	1.75		
	Modified protocol		Concentration (µg/g)	
	Fresh	Frozen	Fresh	Frozen
A260/A280	1.92	2.05	58.6	51.0
A260/A230	2.00	2.13		

1987; Puissant and Houdebine, 1990) as the phosphate group in DNA gets neutralized more easily at acidic pH than RNA. Hence RNA remains in the aqueous phase (Bradley et al., 2001; Revest and Longstaff, 1998). The RNase activity also gets reduced at acidic pH (Brown, 1967).

The protocol by Kumar et al. (2007) used only Tris-saturated phenol for phase separation but in the modified protocol the combination of acid phenol: chloroform (5:1) was used to reduce the partitioning of the poly(A) + mRNA into the organic phase. It further reduced the formation of insoluble RNA-protein complexes at the interphase (Perry et al., 1972). The original protocol also required additional borate-Tris buffer for phase separation which further diluted the RNA sample reducing the final concentration of RNA after dissolving the pellet. The combination of acid phenol: chloroform in modified method removed the difficulty of phase separation and improved the final concentration of total RNA. Further addition of chloroform prevented the retention of the aqueous phase by phenol, thus increasing the total RNA yield (Palmiter, 1974). The 5:1 ratio of phenol: chloroform at the acidic pH resulted in absence of DNA from the upper aqueous phase (Kedzierzki and Porter, 1991). The addition of isoamyl alcohol (chloroform 25: isoamyl alcohol 1) prevented foaming.

The use of SDS (2%), PVP (2%) in liquid form, 100 mM Tris buffer concentration, 25 mM EDTA and 5M NaOAc + phenol: chloroform concentration was adopted as the given concentration was found optimum for high quality RNA isolation based on the standardizations done by Deepa et al. (2014). Sodium dodecyl sulfate detergent was used to disrupt the cells (Hou et al., 2011) and PVP removed oxidation preventing the browning of the tissues. The chelating agent EDTA acted on Mg^{2+} ions required for DNases which if not removed could later on degrade cDNAs formed from mRNA. The combination of 5M NaOAc (pH 5.2) and phenol: chloroform enhanced the recovery of total RNA. Sodium acetate with isopropanol reduced the precipitation time for RNA and increased the yield (Greco et al., 2014). The combination of sodium chloride, sodium sulphite and SDS during the initial phase of extraction was reported to increase the RNA recovery. Also sodium sulphite was found effective against polyphenol oxidase activity (Kumar et al., 2007). The use of 1% β-mercaptoethanol as mentioned in original protocol was helpful in removing the RNase activity by reducing the disulphide bonds in the enzyme. The use of chilled isopropanol increased the rate of precipitation and chilled ethanol removed the salt content in final pellet maintaining the RNA integrity. Pre-warmed extraction buffer and sample incubation in freezer for one hour also resulted in better recovery of RNA.

The modified protocol was found effective in getting higher quality and quantity of isolated total RNA from fresh and frozen rhizomes.

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