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BIOTECHNOLOGY

A Simple and efficient protocol for isolation of RNA from different tissues of chickpea and pea

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Abstract

A simple and efficient protocol is developed for isolation of high quality RNA from roots and leaves of chickpea and pea. The procedure is based on use of SDS, sodium acetate and EDTA in an extraction buffer in order to eliminate polysaccharides and prevent oxidation of phenolic compounds. The current method is modification of a method described for RNA isolation from pea leaves only, and yields excess amount of high-quality RNA suitable for cDNA based gene expression analysis. The protocol requires only three disposable micro centrifuge tubes during extraction, single phenol extraction step and a single precipitation step to yield high-quality RNA. RNA extracted with this method was free from protein and phenolic contaminants as evident from gel electrophoresis analysis. This method is applicable not only for leaves but also for roots and shoots and equally applicable to both chickpea and pea. cDNA is prepared and PCR amplification have been done with universal ubiqutin primer to check the integrity of RNA and absence of inhibitory compounds in RNA samples, which proves the suitability of samples towards qRTPCR.

Highlights

- An efficient and cost effective method for RNA isolation from different parts of chickpea and pea.
- Purity and quality of isolated RNA isolated was found sufficient for gene expression studies.

Keywords: RNA extraction; chickpea; pea; high yield; high quality

Utilization of molecular techniques for studies related to differential gene expression requires synthesis of cDNA from high quality RNA. A number of methods are available for extraction of RNA from plants but there are limitations as a single technique is not equally applicable to all plant species as well as different parts of a same species. It is due to differences in the metabolite content (phenolics and pigments) in different tissues such as root, shoot and leaf (Cardenas *et al*. 2011). There is no universal protocol available that suits isolation of RNA from all plant parts from a single plant without modifications. However, RNA isolation is a pre-requisite to the gene expression studies and has been considered increasingly important in physiological and genetic investigations of plant. Compared to genomic DNA, RNA is more delicate and prone to degradation. Plant tissues having starch, fibers and secondary compounds create hurdle in isolation of high quality RNA (Kiefer *et al*. 1984). Phenolic compounds bind proteins and nucleic acids to form high molecular weight complexes. The polysaccharides tend to co-precipitate with

RNA in the presence of alcohols, remaining as contaminants in one fourth of extract and interfering with subsequent applications (Salzman *et al*. 1999). Conventional methods for RNA isolation involve use of detergents such as SDS or CTAB, denaturing organic solvents (phenol and chloroform), reducing agents (β-mercaptoethanol and dithiothreitol), or denaturing agents (guanidinium isothiocyanate salts).

Chickpea (*Cicer aritunum*) and pea (*Pisum sativum*) are among the widely growing pulse crops in Asia. A large number of researchers are engaged with studying different aspects of plant physiological processes by taking these two plants as model organisms (Salzman *et al*. 1999; Jain *et al*. 2012; Yadav *et al*. 2013; Patel *et al*. 2016). Bioinformatical information was also available about leguminoseae family which can help for primer designing during RTPCR study (Patel and Panchal, 2014). The types and quantities of RNA present in an organism tell the story of genes expressed during a particular time and conditions. Most RNA extraction protocols developed so far are tailored for a particular crop plant or model organism making their utility limited for non-model plant species as well as tissue specificity. With the current protocol, we can successfully extract high-quantity and quality RNA from different tissues of chickpea and pea.

The fresh tissue (leaf and root, 200mg) of chickpea and pea was grinded with mortar and pestle in liquid nitrogen followed by 0.8ml extraction buffer [50ml containing sodium acetate 3M (3.3ml; pH 5.2), SDS 10% (5ml), EDTA 0.5 M (1ml, pH 8) and DEPC water]. Fresh proteinase-K (50-100µg/ml) was added before crushing and equal volume of water saturated phenol and 10µl of β-mercaptoethanol was added at the time of crushing. Crushed samples were thawed, transferred to eppendorf tubes and mixed by vortexing. Samples were then incubated at room temperature till the mixtures turned brown (nucleoprotein complexes breakdown). Tubes were centrifuged at 10,000 rpm for 5 min, clear supernatant was transferred to new eppendorf tubes and 400µl chloroform was added. Content of the tubes were mixed by inverting the eppendorf tubes gently and centrifuging at 10,000 rpm for 10 min. The upper aqueous layer containing RNA was carefully transferred to fresh eppendorf tubes without disturbing the lower layers. 1/3 rd volume of 10 M LiCl was added to the aqueous layer (for precipitation of RNA) and kept overnight at 4°C. Tubes were centrifuged at 10,000 rpm at 4°C for 20 min. At the end of the centrifugation step, precipitate was found at bottom or walls of the eppendorf tubes. The precipitate was washed with 2.5 M LiCl (by centrifugation) followed by washing with 70% ethanol. Tubes were left for drying at room temperature for complete evaporation of ethanol and care was taken not to over dry the RNA as it can cause problem in its dilution. Latter on the RNA was dissolved in 30µl of RNase free water [0.1% (v/v) DEPC water]. Eight replicates were used to increase reliability and precautions were taken to treat all glasswares and plastic wares with water containing DEPC (RNase inhibitor). After extraction of RNA, DNase enzyme treatment was given to remove residual DNA contamination. All the chemicals except 70 % ethanol were autoclaved before use. RNA purity and concentration were assessed by ratios of *A*260:*A*280 and *A*260:*A*230 by using Nanodrop 2000 (Thermo Scientific, Waltham, MA). RNA integrity was evaluated from the 28S and 18S rRNA bands on 1.2% agarose gel with ethidium bromide after electrophoresis and visualization in UV light (Figure 1).

cDNA was prepared by following the protocol (Sambrook and Russell 2001) with help of oligo (dT) primers and reverse transcriptase enzyme. Semi-quantitative RTPCR has performed Fallowing PCR conditions according to (Marone *et al*. 2001) by using ubiquitin primers (forward: CCCCCAGACCAGCAAAGGTTGA, reverse: TGTGTCTGAGCTCTCCACCTCCA).

Using the current protocol 50-60 µg of RNA was yielded from 1 g roots and 190-200µg from 1 g leaves of pea while 130-140 µg from 1 g roots and 250-260 µg from 1 g leaves of chickpea (Table1-1). Disposable eppendorf tubes were used throughout the process instead of larger (50-mL) tubes required at the beginning. The process resulted in reliable and reproducible RNA yield. Washing pellet with 70% ethanol after the LiCl precipitation was an essential step for the purity of RNA. Due to the LiCl precipitation and use of acid phenol (pH ~4.3) in phenol-chloroform extraction, which selectively removes DNA, the resulting RNA is free from DNA contamination. After this step, if DNase treatment is further required, RNA pellet is resuspended in

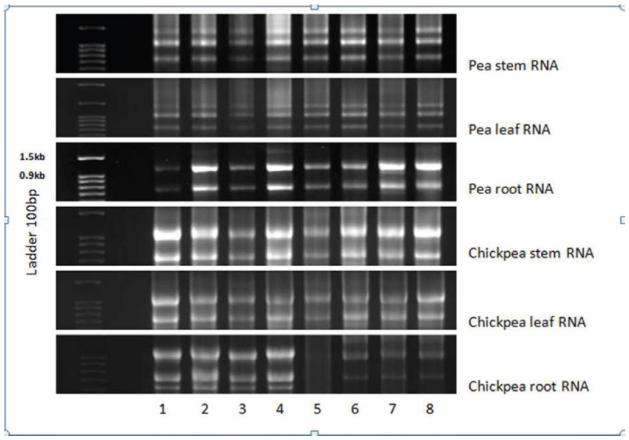

Fig.1: Gel electrophoresis of extracted RNA from different parts of chickpea and pea.

Table 1: Quality and quantity of RNA extracted from different parts of chickpea and pea

Plant sample	260/280 ratio	260/230 ratio	Concentration µg/g of fresh weight
Chickpea leaf	1.987	2.05	250-260 µg/g
Chickpea root	1.9675	2.172	130-140 µg/g
Chickpea stem	1.982	2.102	140-160 µg/g
Pea leaf	2.056	2.28	190-200µg/g
Pea root	2.095	1.808	50-60 µg/g
Pea Stem	2.022	1.914	110-120 µg/g

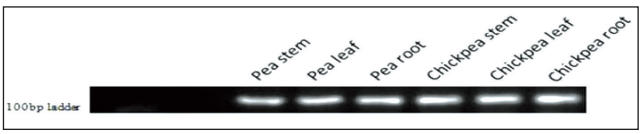

Fig. 2: Gel electrophoresis of PCR Products with ubiqutin primer and cDNA samples prepared from different RNA samples

Flow chart for Isolation of RNA from Plant Material

Grind the 200mg of Plant material in liquid N_2

↓

Immeditely add 800µl extraction buffer and water satureted Phenol

↓

Mix properly in Mortar and pestle and keep for some time for thawing

↓

Transfer the material in 2ml disposable centrifuse tube

↓

Incubate at room temperature till browning of mixture

↓

centrifuge the matter at 10000 rpm for 5 minutes in cooled centrifuge at 4°C

↓

Take the upper layer in fresh 2ml disposable centrifuge tube

↓

Add 400µl of chloroform and mix by inverting

↓

centrifuge the matter at 10000 rpm for 10 minutes in cooled centrifuge at 4°C

↓

Take the upper aqueous layer very carefully without disturbing lover layer in the fresh centrifuge tube

↓

Add 10M LiCl 1/3rd volme of aquous layer and mix by inverting the tube and incubate the solution at 4°C in the freeze over night

↓

Centrifuge the matter for 20 minutes on 10000rpm at 4°C

↓

Decant the supernatant and add 150µl of 2.5M LiCl and again centrifuge at 13000rpm for 10minutes

↓

Decant the supernatant and wash the pellets with 150µl 70% ethanol

↓

Decant the ethanol and keep the tube on ice until evaporation of ethanol

↓

Add30µl of RNase free water and dissolve the RNA peelets by gentle shaking

↓

Preseve the RNA in the deep freeze for further use

↓

DEPC-treated water and treat with DNAse using a standard protocol. Quality check revealed that A260/A230 ratio was higher than 2.0 for all samples (average = 2.1) which indicates high RNA purity and the absence of contamination with polyphenolic and polysaccharide compounds. The A260/A280 ratio ranged from 1.91 to 2.14 (average = 1.94) indicating low or no protein contamination (Table 1). Overall, these data demonstrated that the extraction protocol described here was efficient in yielding good quality RNA with high integrity and quantity. Good PCR amplification (Fig.1) with Ubiqutin specific primers from the cDNA (Prepared by using RNA samples), confirms absence of any inhibitory factor in RNA samples.

Although a number of protocols are available currently for RNA isolation from plant tissues, it is necessary to develop new standardized protocols for different plant species and organs. There is also variability in yield and quality of RNA from the same tissue at different developmental stages due to change in level of metabolites and phytochemicals (Sharma *et al*. 2003; Wang *et al*. 2007). Removal of polysaccharides and polyphenols are difficult during RNA isolation because of their similarity with RNA and they tend to co-precipitated with RNA at precipitation step (Wang *et al*. 2007). Traditional CTAB methods includes an initial disruption of tissues in standard CTAB lysis buffer and two times separation by phenol–chloroform of the RNA aqueous phase from other mixed cellular compounds (proteins, genomic DNA, and polysaccharide residues), and the subsequent precipitation of RNA with lithium chloride and anhydrous alcohol. However, these protocols are specific for certain plant tissues and produce an inferior quality, low-yield of RNA that creates difficulty in cDNA preparation compared to the current protocol required for gene expression analysis by RT–PCR. The main reason for poor yield is repeated extraction with phenol–chloroform and it is inefficient at removing polysaccharides and proteins (Sambrook and Russell 2001). However, there are also huge losses of total RNA when we need to discard approximately 20 to 25% of the supernatant at each step of aqueous RNA phage separation. A report on chickpea by (Singh *et al.* 2002) used the buffer containing 1% SDS, included use of 100 mM LiCl in buffer and one of step includes 90°C heat treatment which may cause degradation of RNA. However, in the present protocol we have used 10% SDS for better breakage of cell; buffer not includes LiCl so no chance of early precipitation of RNA and there is no heat treatment required. In the previous protocol precipitation was done with 2M LiCl in contrast present protocol 10m LiCl used for better precipitation. In the previous protocol Phenol was used in buffer, however in the present protocol it is used in the subsequent step, results better aqueous phase separation. Another study on pea by (Lapopin *et al*. 1999) showed use of 2M LiCl for the precipitation and twice used 80% ethanol for washing, in contrast present protocol uses of 10M LiCl and single washing with 70% ethanol gives better result.

The present protocol gives high quality (purity and integrity) and yield of total RNA isolated and the results are reproducible irrespective of tissue and physiological state of plant. Compared to an improved protocol based on CTAB, phenol and $LiCl_2$ for extraction of high-quality RNA from pea leaves (Chandler *et al*. 1983; Macherel *et al*. 1990), we obtained improved results in quality (A260/A280 and A260/A230) and quantity of total RNA. In comparable samples of young tissues (e.g., leaf, root) the protocol also shows better quality and quantity of RNA from both chickpea and pea. Therefore, a major strength of our improved method is its robustness irrespective of the type, age, and physiological state of the tissues. In addition, it avoids using the extra step of phenol, leading to lower cost and chemical toxicity. Although commercial reagents and kits for RNA extraction may provide high sample throughput but the cost per sample is very high. However, the present method results high quality and yield at very low cost. Our improved protocol proved to be completely suitable for extracting RNA from roots and leaf tissues of chickpea and chickpea and the RNA was pure enough for using molecular downstream applications such as cDNA synthesis, RT–PCR and Northern blot analysis.

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Note: Add 10 µl of BME per sample in the buffer before use and Fresh proteinase-K (50-100µg/ml).