



Evaluation of RNA extraction procedure for drought gene expression in basmati rice

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Basmati rice (*Oryza sativa* L.) is the most economically significant grain. Basmati rice occupies a prime position in the Indian subcontinent and more than 90% of rice is produced and consumed in Asia. Basmati rice plant development is adversely affected by water stress, which results in a substantial loss in yield. Studying the stress-induced changes in gene expression in Basmati rice of stress-tolerant and stress-susceptible cultivars are important to understand the molecular basis of stress adaptation in Basmati rice. Extraction of high quality RNA is imperative for many biological studies, viz. cDNA libraries, isolating genes by RT-PCR and investigating gene expression profiles by cDNA-AFLP. Isolation of good quality RNA in adequate amount is a bit difficult from especially the plant tissues and leaves owing to the presence of high polysaccharides and polyphenolic compounds. These products bind to RNAs during extraction, resulting in the formation of a viscous, insoluble material that interferes with the isolation steps. Qualitative and quantitative differences in composition of plant phenolics and polysaccharides in different plant tissues significantly affect the efficiency of RNA isolation. Various protocols for RNA isolation from different plant tissues exist though with several limitations. Very few protocols related to RNA isolation have been published in Basmati rice and those that have been published are not giving satisfactory results. Hence, to overcome all the drawbacks of earlier published protocols, an attempt has been made to re-examine the existing RNA isolation protocols with various modifications in order to develop an efficient RNA isolation protocol from leaves of basmati rice.

The experiments were conducted at SVP University of Agriculture & Technology, Meerut. The seeds of Nagina-22 and Annada (drought tolerant cultivars) and Pusa Basmati-1

and Basmati-370 (drought susceptible cultivars) were germinated on 3 mm whatman paper for 3-4 days and then transferred in a pot soil in controlled green house at 25°C with 16 hours light a day and were allowed to grow for 40 days with 80% humidity. A time course drought stress experiment was carried out, viz. 0, 1, 3 and 5 days under water stressed condition where, 0 represent the time when pots were lastly filled with 100 ml of water. Leaves were collected at each time point from every plant of each genotype and frozen in liquid nitrogen till the RNA isolation was carried out.

The inimitable and trustworthy extraction method was attempted to extract sufficient quantities of high-quality RNA from a leaf tissue of diverse Basmati rice cultivars. All the glass wares, plastic wares, pestle and mortar were treated in unautoclaved DEPC-treated water for at least 12 h at 37°C followed by autoclaving at 1.05 kg/cm². Glass wares were baked at 250°C for 4-12 h before use. Electrophoresis apparatus was cleaned with 0.5% SDS solution and washed with DEPC treated autoclaved water and finally treated with 3% H₂O₂ for 1 h. The plant material (1 g leaf tissue of Basmati rice) was crushed in liquid nitrogen until it was light green. A 2 ml eppendorf tube was filled up to one third with the frozen powder. 1 ml of RNA extraction solution was added and vortexed vigorously. Then 200 µl of chloroform was added and vortexed again. Suspension was kept on ice during transfer period followed by incubation on a shaker for 10 min at room temperature and centrifuged at 13500-14000 rpm for 10 minutes at 4°C. Supernatant was transferred in another fresh eppendorf tube (avoided contamination with interphase) and 850 µl chloroform was added and vortexed for 15 seconds. Eppendorf tube was then centrifuged at 13500 rpm for 15 min at 4°C. Supernatant was taken in a new eppendorf tube with 1 ml isopropanol and vortexed. After 2 hr of incubation at -20°C the eppendorfs tube was centrifuged at 13,500 for 20 minutes. Supernatant was discarded and pellet was washed with 900 µl of 70% ethanol. Pellets were dried until the white pellets became clear. Then, 53 µl H₂O was added and pellet was dissolved by frequent vigorous vortexing.

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Centrifugation was done at 13,500 rpm for 10 min. 50 μ l of the RNA solution supernatant was transferred to a new 1.5 ml reaction tube leaving behind polysaccharide slime. This method can also be used for leaf tissue above 1 gm by adjusting the volumes of buffers accordingly. Before using the RNA as a template for RT-PCR, a *DNase I* treatment was performed in order to eliminate the presence of eventual DNA contaminations in the samples. Reaction mixture of 20 μ l total volume for purification of extracted RNA comprised of total RNA 15 μ l, *DNase I* (1U/ μ l) 2 μ l, DEPC water 3 μ l. The reaction mixture was incubated at 37°C for 1 hour followed by inactivation of enzyme was done at 95°C for 10 min. The RNA was stored at 4°C for immediate use in H₂O DEPC and -80°C for long term in double volume of absolute ethanol.

The RNA dissolved in H₂O DEPC was taken for quantification by UV absorbance at 260 nm. To evaluate the concentration, Biophotometer (Eppendorf, -Germany) was used. Reference was set against H₂O DEPC and then after thorough rinsing of quartz cuvette, the absorbance of the sample was considered at ODs 260 nm and 280 nm for estimating purity of nucleic acid (Zhen *et al.* 2011). Pure preparation of RNA had the ratio between 1.95 and 2.11 and RNA integrity was verified by electrophoresis in 2% agarose gel (Maniatis *et al.* 1982). Reverse transcription–polymerase chain reaction (RT-PCR) amplification was performed to demonstrate that the RNA could also be used for cDNA library construction and gene expression analysis of plant tissue with drought stress content. cDNA were synthesized, using total RNA as a template, with gene specific primers. Oligo dT (18 mer) was used to construct cDNA. The three steps were used to synthesize cDNA through reverse transcription. In the 1st step a reaction mixture containing 5 μ l total RNA (1 μ g), 1 μ l Oligo dT (18) 10 μ M and 5 μ l DEPC water was incubated for 5 min. at 70°C to disrupt the secondary structure of RNA molecules. During the 2nd step in the reaction mixture 4 μ l Enzyme buffer (5X), 2 μ l dNTPs (2.5 mM each) and 2 μ l DEPC water was added and incubated at 37°C for 5 min. In the 3rd step 1 μ l MMuLV reverse transcriptase enzyme (5U/ μ l) was added to perform the reaction in 20 μ l of volume with high fidelity MMLV reverse transcriptase enzyme. The integrity of cDNA was checked by running it on 1.2% agarose gel stained with Ethidium bromide at 100 volts for 40 minutes. Following initial denaturation at 94°C for 4 min, the PCR reaction was carried out for 45 cycles under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The finishing extension was carried out at 72°C for 7 min. After the finishing extension (polymerization) step, the samples were transferred to 4°C or to -20°C if they were not used immediately.

Generally, high quality RNA could be obtained from plant tissues when the *RNase* activities are inhibited. Several commonly available methods reported for RNA isolation from plant tissues failed to give either high quality or high yield of RNA (Srivastava *et al.* 2012). Mostly small and large-scale RNA extraction protocols are facing the problems

of solidification of samples in extraction buffer and starch co-precipitation. To resolve the solidification of samples, guanidine isothiocyanate and ammonium thiocyanate containing buffers are added. In the existing procedure SDS or β -mercaptoethanol was not used (Delira *et al.* 2008). Phenol or chloroform was used to promote cell lysis and removal of proteins and was found much more effective than chloroform and β -mercaptoethanol. Another modification of protocol, high concentration of sodium acetate (3M) in the extraction buffer was used for the simple isolation of inhibitor-free genomic DNA from plant leaf samples.

Our refined procedure extracted adequate results in term of yields and $A_{260}/_{280}$ ratio in basmati rice. Typical yields of RNA for the four Basmati rice genotypes ranged from 35-50 μ g. The $A_{260}/_{280}$ ratio ranged from 1.95-2.11 for all the samples and indicated that the RNA is of high quality free from impurities like polyphenols and polysaccharides contaminations (Zhang *et al.* 2012). On the other hand, at $A_{260}/_{280}$ ratio we observed as the average basmati rice leave derived RNA extracted through the phenol-SDS procedure ranged from 1.64–1.70, which indicated that the samples contained protein contamination and were low in purity. The veracity of the RNA is apparent on 2% agarose gel, where 28S and 18S bands were clearly noticeable. No evident degradation of RNA was detected manifesting that RNA resulting from our procedure is comparatively free of *RNases*. Mature leaves contain higher amounts of polysaccharides, polyphenols and other interfering compounds inducing less RNA and more secondary metabolites. Therefore, it is essential to use pre-warmed extraction buffer and extend the extraction phase to recover ample RNA (Jhala *et al.* 2015). For tissues containing low to moderate levels of polyphenols (young leaves), the procedure can be shortened by precipitating the RNA for only 1½ hr in extraction step. Inclusion of an additional re-extraction step with chloroform helped in eliminating contaminants and recover high quantity of RNA. Inefficient removal of polysaccharides and polyphenolic compounds results in co-precipitation with RNA, which impacts the yield and quality (Soni *et al.* 2009). In the customized procedure RNA was directly precipitated using cold absolute ethanol/isopropanol in lieu of LiCl to avoid any water insoluble precipitation and loss of RNA. PCR amplification of Nagina-22 genotype with primer, for2/rev2 in semi-quantitative PCR at variable drought stressed samples obtained profiles, giving the differential expression cDNAs were successfully recognized, eluted and re-amplified. The quality of RNA was further verified through RT-PCR using degenerated synthetic oligonucleotide primer, which was designed, based on the conserved domain of actin gene (Mawlong *et al.* 2016). The RT-PCR yielded the expected cDNA fragment (Yadav *et al.* 2016) (350 bp) as a single band with actin gene, representing that the RNA is PCR amplifiable and is free of any inhibitors. Actin an abundant gene in the plant system was also used in RT-PCR reaction to establish the quality of the extracted total RNA. The expression of actin was found to be higher with RNA

extracted using our modified method compared to other methods consisting of 350 bp fragments, when a control RT-PCR was done with each of the above RNA samples, and the transcript level appeared to be equal in all the lanes, suggesting equal amount of total RNA used.

SUMMARY

Basmati rice contains large amounts of polysaccharides and polyphenols. Due to these compounds, isolation of superior quality RNA from rice tissue is complicated. Most of the published procedures failed to yield adequate quantity of high-quality RNA from various rice tissues appropriate for gene expression studies. We conducted experiments and observed as the average ratio at $A_{260/280}$ of basmati rice leave derived RNA extracted through the phenol-SDS procedure ranging from 1.64 to 1.70, which indicated that the samples contained protein contamination and were low in purity. On the other hand, in our modified protocol typical yields of RNA for the four Basmati rice genotypes ranged from 35–50 μg . The $A_{260/280}$ ratio ranged from 1.95–2.11 for all the samples and indicated that the RNA is of high quality free from impurities like polysaccharides and polyphenols contaminations. Thus, the quality RNA with high yield from rice leaves or may be other plant tissues having high concentration of impurities like polyphenols and polysaccharides may be extracted and purified by this modified protocol can be used for various downstream applications including RNA-seq, RT-PCR and qPCR.

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