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POLYSACCHARIDE-FREE NUCLEIC ACIDS AND PROTEINS OF *Abelmoschus esculentus* FOR VERSATILE MOLECULAR STUDIES

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Abelmoschus esculentus **(okra) is one of the polysaccharide rich crop plants. The polysaccharides interfere with nucleic acids and protein isolation thereby affecting the downstream molecular analysis. So, to understand the molecular systematics of okra, high quality DNA, RNA and proteins are essential. In this study we present a method for extracting genomic DNA, RNA and proteins from polysaccharide rich okra tissues. The conventional extraction procedures were integrated with purification treatments with pectinase, RNase and proteinase K, which improved the quality and quantity of DNA as well. Using SDS, additional washes with CIA and NaCl precipitation improved the RNA isolation both quantitatively and qualitatively. Finally, ammonium acetate mediated protein precipitation and re-solubilization increased the quality of total protein extracts from the okra leaves. All of the methods above not only eliminated the impurities but also improved the quality and quantity of nucleic acids and proteins. Further, we subjected these samples to versatile downstream molecular analyses such as restriction endonuclease digestion, RAPD, Southern, reverse transcription-PCR and Western analysis and were proved to be successful.**

Keywords: ***Abelmoschus esculentus***, **nucleic acid isolation, total proteins, superior quality, molecular analysis.**

INTRODUCTION

High quality nucleic acids and protein samples are essential for most of molecular analysis techniques. Though large numbers of DNA and RNA isolation methods were developed in the recent years for a variety of plant species, they are indeed not always simple and moreover cannot be reproduced in other species [1]. In plants, several factors are known to limit the isolation of pure DNA or RNA due to co-extraction of impurities such as polysaccharides, terpenes, polyphenols, melicera colloidal hyalosome etc. Of these impurities polysaccharide contamination is the most common problem while isolating DNA and RNA of higher plants. Viscous or glue-like texture of polysaccharides makes the nucleic acids unmanagable during pipetting and unsuitable for PCR, since they inhibit the activity of Taq polymerase, ligases and restriction enzymes [15]. The samples contaminated with melicera colloidal hyalosome, are often more difficult to dissolve in water or the TE buffer [6]. Sometimes, phenolic compounds also interfere with extraction procedures, bringing down the purity of DNA and RNA, making them unfit for most of molecular analyses [7, 8]. Many perennials are rich with these contaminants which greatly affect the quality and quantity of the extracted nucleic acids [9–11], as a result, rehydrated ethanol-precipitated DNA will be a viscous slurry. The most common CTAB method [12] and its modifications [13, 14] are widely used in various laboratories, but they are not suitable for all the plant species. Extraction protocols using diatomaceous earth and spin filters for DNA extraction and benzyl chloride for RNA extraction, though capable of isolating good quality samples, are tedious and expensive [15–17] for small scale laboratories. The conventional DNA extraction protocols, which can remove some contaminants [18], require large amounts of tissue samples.

Similarly, protein extraction from plant tissues has been of a great deal for an array for proteomic analysis [19]. Plant tissues usually contain relatively low amounts of proteins and high quantities of secondary metabolites and polysaccharides which are indeed responsible for tissue disintegration and interfere with protein resolution on SDS-PAGE [20]. Hence, an effective protein extraction protocol is essential to eliminate or minimize the secondary metabolite variations across the

Abbreviations: PC – phenol (pH 4.5): chloroform; CIA – chloroform:isoamyl alcohol; PCIA – phenol:chloroform:isoamyl alcohol; CTAB – hexadecyltrimethylammonium bromide; DTT – dithiotreitol; PMSF– phenylmethanesulfonyl fluoride; PVPP – polyvinyl polypyrrolidone; DEPC – diethyl pyrocarbonate; RH – relative humidity; RT – room temperature.

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various tissues such as leaves, roots, fruit, seeds and stems, and also between different species [21].

Abelmoschus esculentus (L.) Moench, (okra) is a potential multiple-purpose crop [22] reported to have high polysaccharide content in leafs, buds, flowers, and pods, resulting in a highly viscous solution with a slimy appearance when okra is extracted with water or standard buffer solutions [23]. Isolation of nucleic acids (DNA and RNA) and proteins is much more difficult with this crop because of the impurities mentioned above. Such samples with high contamination levels have higher risks of interference during further molecular manipulations with the nucleic acids associated with enzymes and subsequent electrophoresis and with proteins during SDS-PAGE and Western analysis. In this article, we suggest isolation methods giving high quality nucleic acids and proteins from okra suitable for a variety of versatile molecular studies such as RAPD, Southern, Reverse Transcription-PCR and Western analyses.

EXPERIMENTAL

Plant material and sampling. Seeds of *A. esculentus cv Arka Anamika* were surface sterilized with 96% ethyl alcohol for 1 min followed by 10% sodium hypochlorite for 15 min. The seeds were washed 4 times for 5 min with sterile distilled water after treatment with each of the above and were soaked overnight in sterile distilled water with low agitation (30 rpm) on a shaker. They were germinated on sterile moistened filter paper in Petri dishes (10 cm diameter) for 2 days in the dark at 37°C. Healthy germinated seeds were transferred into sterile soil moistened with a nutrient solution in polypropylene pots. The pots were kept in a green house at 28 ± 1°C, ~55% RH and irrigated daily. Young, fully opened, healthy leaves from two week old plants were collected and frozen in liquid nitrogen and were stored at –80°C till use.

Nucleic acids isolation. *Genomic DNA:* About 0.4 g of tissue plus ~40 mg of polyvinyl pyrrolidone (PVP) were homogenized with a pestle in a pre-cooled sterile mortar containing liquid nitrogen until a fine powder was obtained, then the frozen powder was transferred to a 15 mL falcon tube. 10 folds (w/v) of sterilized C-TEN buffer (3% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0 and 1.4 M NaCl: preheated at 55°C) amended with 0.006% β-mercaptoethanol (add just prior to use) was added to the homogenized powder. The tubes were incubated for 30 min at 55°C and mixed at every 10 min interval. The samples were brought to RT and an equal volume of 24 : 1 CIA was added and mixed thoroughly. The tubes were centrifuged twice at 2500 g for 20 min at RT. $1/10^{th}$ volume of 5 M NaCl and 2.5 volumes of absolute ethanol were added to the supernatant and incubated at 4°C overnight. The DNA was pelleted at 2500 g for 20 min at RT and the pellet was washed with 70% ethanol. The pellet was dried for 15 min at RT and re-suspended with 300 μL of 0.1 × TE at 4°C. To purify the DNA, RNase (10 μg/mL) and pectinase (30–80 U) were added and incubated at 37°C for 45–60 min, followed by proteinase K (20 μg/mL) treatment for 15 min. Later an equal volume (v/v) 25 : 23 : 2 of PCIA was added, mixed thoroughly and centrifuged at maximum speed for 5 min. Then $1/10^{th}$ volume of 3 M $C_2H_3NaO_2$ and 2.5 volumes of chilled absolute ethanol were added to the supernatant and incubated at 4°C overnight. The DNA was pelleted at maximum speed for 10 min at RT and washed once with 70% ethanol. The pellet was dried for 30 min at RT and then dissolved in 100 μL of 0.1 × TE at 4°C.

Total RNA: (All of the reagents should be prepared with DEPC water and centrifuged at 14000 g at 4°C). Approximately 100–150 mg of leaf sample were ground with a pestle in a pre-cooled sterile mortar containing liquid nitrogen until a fine powder was obtained, then 500 μL of buffer-1 (38% phenol, 0.8 M ammonium thiocyanate, 0.4 M guanidium thiocyanate, 10 mM $C_2H_3NaO_2$, 5% glycerol) were added and homogenized (may take 10–15 min). The homogenate was transferred to a 1.5 mL sterile tube and half the volume of PC (1 : 1 v/v) was added, mixed by inverting and centrifuged. 500 μL of freshly prepared buffer-2 (1.5% SDS, 100 mM Tris pH 8, 50 mM EDTA, 150 mM LiCl, 1.5% β-mercaptoethanol) were added to the supernatant, the tubes were inverted for 1 min and incubated for 10 min at RT. $1/6^{th}$ volume of 24 : 1 CIA was added, mixed thoroughly and centrifuged for 15 min. The supernatant was transferred to a fresh tube and 300 μL isopropanol plus 250 μL NaCl (1.2 M) were added and mixed gently for 1 min. The tubes were incubated at –80°C for 20 min and centrifuged for 20 min. The samples were washed twice with 70% chilled ethanol, dried and finally re-suspended in 75–100 μL of sterile DEPC water at 4°C.

The total DNA or RNA was spectrophotometrically quantified by using NanoDrop1000. The samples were resolved on appropriate gels according to Sambrook and Russell [24].

Southern and RAPD analysis. In order to analyze the digestibility of the total genomic DNA and to ensure its quality for Southern analysis, 10 μg of genomic DNA were digested using 3U/μg of DNA for 16 h with EcoRI ("Fermentas®", USA), which does not cut the target gene sequence of the elongation factor (*elf*). The digested DNA samples were electrophoresed on a 0.8% agarose gel. The separated fragments were transferred onto a positively charged nylon membrane. The membrane was hybridized with [α-^{32}P]-radioactive probe which was made from the purified *elf* PCR product (amplified using forward, 5'-ACTGCTCT-

TCTTGAAATGATG-3'; reverse, 5'-GATTCTC-GAGACCGTCTCACG -3') labeled by random prime labeling ("Fermentas"). Hybridization was carried out at 65°C in Church buffer [25] for 18 h. Membranes were washed for 30 min in 2 × SSC, 0.1% SDS; 0.1 × SSC, 0.1% SDS at 65°C [24]. Later the membrane was wrapped, placed overnight on FUJI Image Plate (IP) and the IP was read using a phosphor imager (FUJI FILM FLA-5100, "Fuji Photo Film Co. Ltd.", Japan).

About 30 ng of freshly isolated genomic DNA was used for RAPD analysis. The RAPD profiling was done according to [26] with minor modifications as described in our earlier work [27]. In the present study two randomly selected decamer RAPD primers, OPC-6 and OPH-12 were used as representatives for RAPD analysis. The products were resolved on a 1% agarose gel and visualized under ultraviolet light [24].

Reverse transcription-PCR analysis. cDNA was synthesized from total RNA using a reverse transcriptase ("Invitrogen") and oligo dT primers ("Invitrogen"). Using the freshly prepared cDNA as the template, a housekeeping actin gene was PCR-amplified with gene specific primers (forward, 5'-CTAGTG-GATGTGCCTGAAGCATC-3'; reverse, 5'-CATGA-GTCTTTAGAGAACCCAAAG-3'). PCR was performed to amplify a 223 bp actin gene fragment from wild type okra plants using thermo cycler condition which were initiated by a hot start at 94°C for 7 min followed by 30 cycles of 94°C/1 min, 60°C/1 min and 72°C/1.5 min with a 72°C/10 min final extension. The reaction mixture without cDNA served as a water blank (negative control). The products were resolved on a 1% agarose gel and visualized under ultraviolet light [24].

Protein extraction. Total proteins were extracted from okra leaves using the phenol extraction method [28] with the following changes. The leaf sample (~300 mg) was homogenized with a pestle in a precooled sterile mortar containing liquid nitrogen until a fine powder was obtained and 5 mL each of Tris saturated phenol (pH 7.8) and freshly prepared extraction buffer (50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 3 mM DTT, 6 mM PMSF, and 30 mg insoluble PVPP) were added and homogenized for additional 2 min. The homogenate was transferred into 15 mL falcon tubes, vortexed for 15–20 min at 4°C and centrifuged at 18000 g for 15 min at 4°C. The phenol layer was transferred into a fresh tube and proteins were precipitated with five volumes of chilled ammonium acetate (0.1 M) plus methanol, mixed by inverting for 1 min and incubate at –20°C for 16 h. The proteins were pelleted by centrifugation at 18000 g for 30 min at 4°C and washed with chilled acetone (80%) followed by acetone (80%) containing 10 mM DTT. In each washing step, the pellet was completely dissolved at –20°C before centrifugation. The pellet was re-suspended in extraction buffer amended with 0.5% SDS. The protein quantity was determined using a spectrophotometer [29]. The crude protein (25 μg) samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [24].

Western blot analysis. Accumulation of native rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) protein in the wild type okra plants was analyzed by Western blot analysis. About 50 μg of total protein samples were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane ("Millipore (India) Pvt. Ltd.") using a semidry transfer system. The blot was subsequently probed with an antibody against rubisco ("Agrisera-AB Ltd.", Sweden) at a 1 : 10000 dilution. Membranes were incubated with the goat anti-rabbit IgG-ALP conjugate as a secondary antibody and the reaction was detected according to the manufacturer's instructions using BCIP/NBT as a substrate ("GeNei", India).

RESULTS

In the present work we demonstrated successfully, the use of pectinase, RNase and proteinase K to purify the okra genomic DNA isolated using the C-TEN (a modified C-TAB) buffer. This method improved the genomic DNA quality, without RNA contamination and degradation of DNA (fig. 1*a*). The quantity of DNA isolated using this method was in the range of 1.82.3 μg/μl, further spectrophotometer based observations using the A_{260}/A_{280} and A_{260}/A_{230} ratios of the DNA samples showed that DNA was not contaminated with proteins (table). Later, the DNA was subjected to molecular analyses such as Southern and RAPD analysis in order to verify its suitability for molecular manipulations. In the present study, a single restriction enzyme, EcoRI absolutely digested 10 μg of genomic DNA within 16 h (fig. 1*a*) and the EcoRI digested genomic DNA when probed with the labelled *elf* gene fragment on Southern blot showed a single copy gene pattern (fig. 1*c*). On the other hand, we used OPH12 and OPC6 RAPD decamer random amplified polymorphic DNA (RAPD) primers in order to verify the quality of genomic DNA by PCR analysis. With the above selected primers, the PCR fragment profile ranging from ~500 to 2000 bp obtained with very small or no smears (fig. 1*d*,*e*). The DNA quality and reproducibility were ensured by repeating the amplification several times.

Good quality of the total RNA is mandatory for understanding the regulation of gene expression in plants. A modified conventional RNA extraction method [30] was used in this study to isolate good quality RNA from high polysaccharide containing tissue, okra. Use of 1.5% SDS [30], an additional wash with CIA [31] and NaCl precipitation improved the

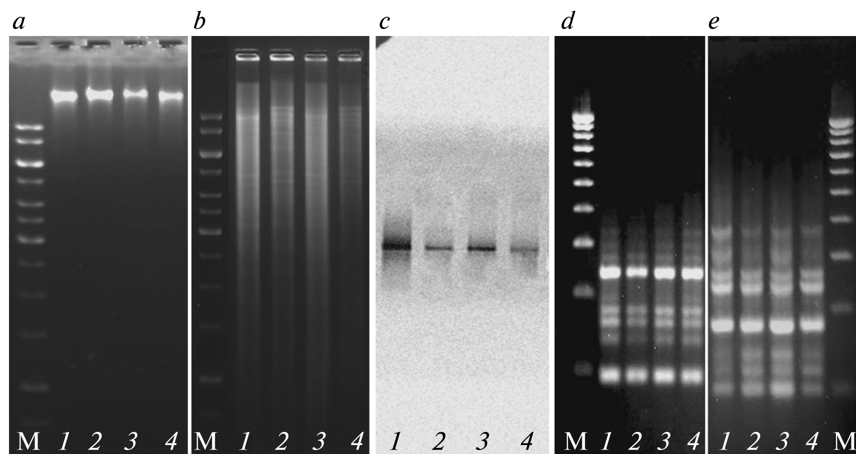

Fig. 1. Genomic DNA based molecular analysis. *a* – Qualitative analysis of *Abelmoschus esculentus* genimic DNA (2 μL) electrophoresed on a 0.8% agarose gel. *b* – About 10 μg of genomic DNA was digested for 16 h with EcoRI and the digested samples were electrophoresed on a 0.8% agarose gel at 25 V. *c* – Southern analysis: the digested samples were transferred onto a positively charged nylon membrane and the membrane was probed with a 726 bp radio labelled *elf* gene fragment. *d* – RAPD analysis: amplification of *A. esculentus* DNA using a decamer primer OPH-12. *e* – RAPD analysis: amplification of *A. esculentus* DNA using a decamer primer OPC-6. M – 1 Kb marker; *1–4* – wild type *A. esculentus* plant samples.

quantity (fig. 2*a*) and quality of RNA (table), resulting in samples with no protein contaminants. The feasibility of RNA for expression studies was successfully confirmed by cDNA synthesis and its subsequent amplification with a house keeping gene actin. As expected, a 223 bp fragment was obtained in all of the samples and no such fragment was registered in the water blank (fig. 2*b*).

During the okra protein extraction, an additional step such as ammonium acetate-protein precipitation and re-solubilization was included. The appreciable protein quality obtained from high polysaccharide containing okra leaves samples was confirmed by resolving on a 10% 1D-SDS PAGE (fig. 2*c*) and the quantity was estimated by Bradford [29] (fig. 2*e*). A sharp band of 52 kDa was obtained in western analysis when, a low amount of the total protein (25 μg) samples was probed with a rubisco antibody at a high dilution (1 : 10000) confirming the suitability of okra proteins for western analysis.

DISCUSSION

A. esculentus is the most commonly used vegetable in the Southern world, reported to have medicinal properties and also reported to be polysaccharide rich in all of its tissues [23]. To understand the molecular systematics of okra, obtaining high quality DNA, RNA and protein samples is essential. There are several handy protocols available for isolating DNA, RNA [32] and proteins from plant tissues [19]. However, high polysaccharide containing crops, such as *A. esculentus*, require modified or special protocols for nucleic acid and protein isolation. One of such modified and integrated methods is described in this work. A modified CTAB extraction buffer [13] integrated with different purification treatment agents such as pectinase [33], RNase and proteinase K, drastically improved the quality of genomic DNA samples, without evidence of RNA contamination and DNA degradation. The when the CTAB method was used alone to isolate DNA from the same tissue, without these modifications, it showed very poor results, both in quality and quantity (data not shown). Further, application of pectinase treatment successfully eliminated the polysaccharides by digesting them into smaller mole-

Quantity and quality of DNA and RNA extracts from *Abelmoschus esculentus*

Sample	Quantity, μg/μL*	Purity, A_{260}/A_{280}	Purity, A_{260}/A_{230}
DNA			
AeD-1	2.3	1.80	1.84
AeD-2	2.2	1.81	1.85
AeD-3	1.8	1.79	1.91
AeD-4	2.0	1.80	1.88
RNA			
AeR-1	2.8	1.88	1.79
AeR-2	3.5	1.90	1.89
AeR-3	3.5	1.97	1.95
AeR-4	2.9	1.95	1.90

* The mean value of three replications.

Note: The DNA pellet was dissolved in 100 μL of 0.1× TE buffer, whereas the RNA pellet was dissolved in 75 μl of DEPC water and the quantities were determined by the UV absorbance ratios measured using a NanoDrop ND-1000 spectrophotometer.

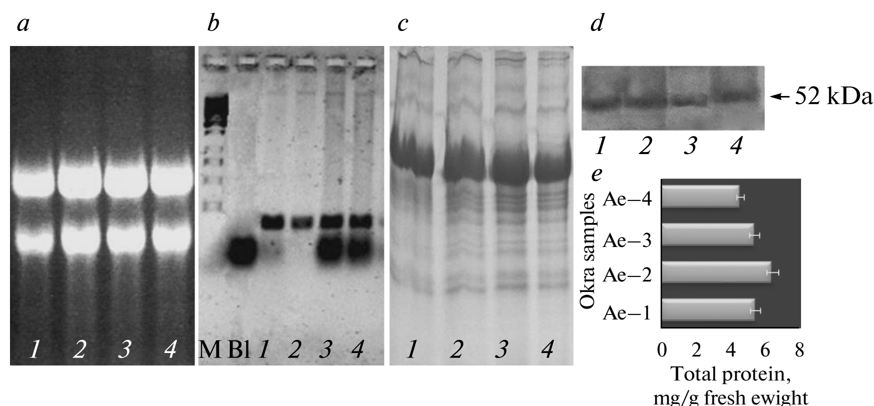

Fig. 2. *Abelmoschus esculentus* total RNA and protein based analysis. *a* – Qualitative analysis of *A. esculentus* total RNA resolved on a 1% formaldehyde agarose gel. *b* – Semi-quantitative Reverse Transcription-PCR analysis of actin transcripts in four wild type plants using gene specific primers. *c* – Total proteins isolated from *A. esculentus* by the phenol extraction method separated on a 10% 1D-SDS PAGE followed by Coomassie Brilliant Blue staining. *d* – Western analysis: about 25 μg of total protein samples were separated on SDS-PAGE and blotted onto a nitrocellulose membrane by a semidry transfer system. The blot was subsequently probed with an antibody against rubisco at a 1: 10000 dilution. Membranes were incubated with a goat anti-rabbit IgG-ALP conjugate as a secondary antibody and the reaction was detected according to the manufacturer's instructions using BCIP/NBT as a substrate ("GeNei", India). *e* – Quantity of total proteins (mean value) estimated using the Bradford method [29]. Error bars represent ± SE. M – 1 Kb marker; BI – blank (reaction mixture without cDNA); *1–4* – wild type *A. esculentus* plant samples; Ae *A. esculentus*.

cules in order to avoid their co-precipitation with the DNA [33]. Genomic DNA isolated using this method further proved to be suitable for molecular manipulations such as Southern blot and RAPD analysis. A restriction enzyme EcoRI, digested the genomic DNA completely overnight (16 h) indicating the digestibility and suitability of the obtained DNA samples for any restriction endonuclease digestion-based studies [34]. Further, a single copy gene pattern was shown, when the digested DNA was probed with the labelled *elf* gene demonstrating successful genomic Southern analysis. The elongation factor gene was reported as a single copy gene in tomato [35] using Simple Sequence Repeats markers.

The random amplified polymorphic DNA technique based on polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques for developing DNA markers [36]. Primarily, good DNA quality is essential in order for the RAPD analysis to amplify a variety of fragment sizes related to the polymorphism [36]. In this study, we demonstrated the quality of the DNA isolated from polysaccharide rich tissue samples using OPH12 and OPC6 RAPD decamer primers. The PCR fragment profile shows an array of sharp bands with very small or no smears representing the quality of the DNA. Therefore, good quality DNA can ease the detection of molecular DNA makers and help in further development of DNA-marker assisted breeding in recalcitrant plant species [17] such as okra.

In the gene expression analysis of plants, it is important to record the levels of total RNA or mRNA of a particular gene per unit of leaf area. Therefore, the good quality of total RNA is a platform for understanding the regulation of gene expression. For okra, a polysaccharide rich crop, we used a modified conventional RNA extraction method [30] to isolate the total RNA. The use of 1.5% SDS [37], an additional wash with CIA [31] and NaCl precipitation improved the quantity and quality of RNA, resulting in samples with no protein contaminants. RNA isolated using this method has been shown to be sufficient for versatile molecular studies, such as RT-PCR, by a successful amplification of a housekeeping gene actin. Thus, the improved RNA isolation method will allow to analyse gene expression in complex tissues such as okra.

With the growing research interests in plant proteomics, a verity of protein extraction and purification methods are being developed routinely [38, 39]. The conventional, single-step protein isolation method [40], not only results in a low protein yield, but also in low sample quality due to high amounts of different contaminants in the crude extract. Hence, in our method an additional step such as ammonium acetate-protein precipitation and re-solubilization was included [38]. This not only improved the protein quality but also the resolvability on SDS PAGE. Western analysis using a rubisco antibody further confirmed the suitability of these protein samples for further molecular analysis.

The described above extraction procedures for the DNA, RNA and proteins from polysaccharide rich tissues of okra are highly reproducible and can be easily adopted to any laboratory conditions. The presented

augmented methods for nucleic acid and protein isolation can also be extended to various plant tissues, such as leaves, roots, seeds, fruits or flowers of okra; and other crops and tree species with little modifications.

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