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ORIGINAL PAPER

Isolation and Purification of RNA from Tissues Rich in Polyphenols, Polysaccharides, and Pigments of Annatto (*Bixa orellana* L.)

Simone M. Rodrigues · Virgínia L. F. Soares · Tahise M. de Oliveira · Abelmon S. Gesteira · Wagner C. Otoni · Marcio G. C. Costa

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Abstract The tropical plant *Bixa orellana* L. (annatto) produces an array of natural products, including the pigment bixin used in the food and cosmetics industries. In order to understand the biochemical and molecular basis of the biosynthesis of these natural products, a reliable method for isolating high yields of high-quality RNA is required. Here we described a successful and reproducible method for isolation and purification of high-quantity and high-quality RNA from different tissues of annatto. This protocol overcomes the usual problems associated with large amounts of polyphenols, polysaccharides, pigments, and other secondary metabolites that are not easily removed by conventional extraction procedures. Furthermore, the proposed protocol can be easily carried out in any laboratory and it could also be extended to isolate RNA from other plant species showing similar abundance of compounds that interfere with RNA extractions. The yield and quality of the RNA were monitored by spectrophotometric analysis, separation on agarose gel, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and construction of a cDNA library.

Keywords RNA extraction · MATAB (mixed alkyl trimethyl ammonium bromide) · RT-PCR · cDNA library

S. M. Rodrigues · W. C. Otoni
Plant Biology Department, Federal University of Viçosa,
36570-000 Vicosa, Minas Gerais, Brazil

V. L. F. Soares · T. M. de Oliveira · A. S. Gesteira ·
M. G. C. Costa (✉)
Biological Sciences Department, State University of Santa Cruz,
45662-000 Ilheus, Bahia, Brazil
e-mail: mcosta@labbi.uesc.br

Introduction

Bixa orellana L. (annatto) is a perennial tree, native to tropical America, which accumulates several carotenoid derivatives (including bixin and norbixin), terpenoids, tocotrienols, arenes, and flavonoids in seeds and/or leaves [1–7]. This plant has raised a growing interest by the scientific community worldwide, since it is the sole source of the natural pigment bixin extracted from the pericarp of the seeds and used in food and cosmetics industries [8]. Due to the prohibition on the use of specific synthetic color additives in several countries, bixin is second only to caramel as the most used natural colorant in industry [9].

In spite of recent clarification of the biochemical and molecular basis of bixin biosynthesis [10], most enzymes and genes regulating the biosynthesis of carotenoids and other natural products have not been identified or characterized yet in annatto. A pre-requisite to conduct such research is obtaining intact nucleic acids, especially RNA. Unfortunately, isolating sufficient yields of high-quality RNA from tissues of annatto has been particularly difficult. Besides pigments, the major obstacles are large amounts of polyphenols and polysaccharides that co-precipitate with nucleic acids upon isolation [11]. Furthermore, most published protocols [e.g., guanidinium thiocyanate [12], hot phenol [13]] and commercial kits [e.g., RNeasy (QIAGEN), Trizol (Gibco-BRL Life Technologies)] available for RNA isolation were not designed for use with plant tissues rich in polyphenols and polysaccharides [14, 15].

As far as we are concerned, to date, only one study has examined the isolation of RNA from different tissues of annatto [16], resulting in a relatively low RNA yield. Here we presented a reproducible method for isolation and purification high-quality RNA in large amounts from different tissues of annatto based upon a modification of the

protocol described by Gesteira et al. [17]. The proposed protocol overcomes the problems associated with polyphenol, polysaccharide, and pigment contaminations and it can be easily carried out in any laboratory. The yield and quality of the RNA obtained were consistently high, as confirmed by spectrophotometric analysis, separation on agarose gel, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and construction of a cDNA library.

Materials

Plant Material

Samples of seeds and leaves at different developmental stages were collected from 14-years-old annatto plants (*Bixa orellana* L. var. 'Bico-de-Pato') growing in an experimental field plot at Federal University of Viçosa, Brazil. After harvesting, the samples were immediately submerged in RNA*later*™ RNA stabilization reagent (Qiagen, USA), incubated overnight at 4°C, and then maintained at –20°C until use.

Equipment and Reagents

1. Mortar, pestles, and spatulas.
2. Diethylpyrocarbonate (Sigma).
3. 2-ml sterile microcentrifuge tube (Scientific Specialties).
4. Homogenization buffer: 0.2 M boric acid, 10 mM EDTA, pH adjusted to 7.6 with Tris, 0.5% sodium dodecyl sulfate (SDS), and 0.286 M β-mercaptoethanol.
5. Phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v).
6. Mixed alkyl trimethyl ammonium bromide (MATAB) solution (2% MATAB, 3 M NaCl).
7. Chloroform/isoamyl alcohol (24:1; v/v).
8. Cold isopropyl alcohol.
9. 3 M sodium acetate pH 4.5.
10. Ethyl alcohol.
11. DEPC-treated water.
12. DNase I (Invitrogen).
13. GeneQuant pro spectrophotometer (Biochrom).
14. 10 mM Tris–HCl, pH 7.5.
15. Taq DNA polymerase (Promega).
16. PTC-200 thermocycler (MJ Research).

Methods

RNA Extraction

All solutions were treated with 0.1% (v/v) diethylpyrocarbonate (DEPC). Mortars, pestles, and spatulas were soaked overnight in 0.1% (v/v) DEPC and then autoclaved prior to using.

1. About 100 mg of each tissue sample were ground to a fine powder using liquid nitrogen and then transferred into a 2-ml sterile microcentrifuge tube.
2. About 1 ml of homogenization buffer was added. The mixture was shaken for 5 min and then incubated at room temperature, for the same time just before centrifugation at 15,000*g* for 10 min, at room temperature.
3. The supernatant containing nucleic acids and a large amount of polysaccharides and pigments was carefully removed into a new microcentrifuge tube and treated with equal volume of phenol/chloroform/isoamyl alcohol, shaken for 5 min, and then centrifuged at 15,000*g* for 10 min, at room temperature.
4. About 500 µl of the upper aqueous phase was carefully removed into a new microcentrifuge tube. In order to eliminate residual polyphenols and polysaccharides, purification of the upper aqueous phase was carried out by adding one volume of MATAB solution and 1-ml of phenol/chloroform/isoamyl alcohol, followed by incubation at 65°C for 10 min.
5. About 900 µl of the upper aqueous phase was carefully removed into a new microcentrifuge tube and an equal volume of chloroform/isoamyl alcohol was added. Samples were shaken for 5 min prior to centrifugation at 15,000*g* for 10 min, at 4°C.
6. The upper aqueous phase was collected and the nucleic acids were precipitated by adding one volume of cold isopropyl alcohol and one-tenth volume of 3 M sodium acetate pH 4.5. The tubes were kept on ice for 15 min and then centrifuged at 15,000*g* for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 70% (v/v) ethyl alcohol and resuspended in 60 µl of DEPC-treated water.
7. The samples were treated with RNase-free DNase I (Invitrogen, USA), according to the manufacturer's instructions, and stored at –80°C for later use.
8. An aliquot of the RNA was electrophoresed on 1% DEPC-treated agarose gel and stained with ethidium bromide (0.5 µg/ml) to confirm its integrity.

RNA Analysis

Spectrophotometric Analysis

The purity and concentration of the isolated RNA were assayed spectrophotometrically at 260 and 280 nm. For accurate measure, samples were diluted (1:50) in 10 mM Tris–HCl, pH 7.5, since the A_{260}/A_{280} ratio is influenced considerably by pH.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The first-strand cDNA was synthesized from 1 μg total RNA of developing seeds using the First Strand cDNA Synthesis kit (Fermentas, USA) and the provided random hexamer primer, following the instructions of the manufacturer. The cDNA was used for PCR amplification of the annatto 18S rRNA sequence under the following conditions: 1 μl of first-strand cDNA, 0.2 μM of 18S forward and reverse primers, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, and 1 U of Taq DNA polymerase (Promega Co., USA). The following primers were used: 18SF (5′-CAAGCGA TCTTTTCGTAGGC-3′) and 18SR (5′-CGAAGATAAAA TCCGAGCTTGT-3′). PCR was done in a PTC-200 thermocycler using the following program: 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, and a final step of 72°C for 7 min.

cDNA Library Construction

The cDNA library was constructed using the Creator™ SMART™ cDNA library construction kit (Clontech, USA), according to manufacturer's instructions, except for cDNA fragments that were cloned into pGEM-T Easy vector (Promega Co., USA) and subsequently introduced into ElectroMAX DH10B competent cells (Invitrogen, USA). In order to validate the library, PCR was performed from randomly selected colonies. Each PCR reaction was carried out in 25 μl volume containing 0.2 mM of each dNTP, 0.25 μM of each M13 forward and M13 reverse oligonucleotide primers, 0.25 U *Taq* DNA polymerase and 100 ng of plasmidial DNA. The reactions were amplified in a PTC-200 thermocycler with an initial denaturing at 94°C for 2 min, followed by 30 cycles of 1 min of denaturing at 94°C, 30 s of annealing at 55°C, and 2 min elongation at 72°C for 2 min, followed by a final cycle of 10 min at 72°C. Amplified DNA fragments were monitored in an 1% agarose gel.

Notes

1. A few protocols have previously reported the isolation of RNA from recalcitrant plant tissues [14–22], mainly because the high content of polyphenols, polysaccharides, pigments, and other unidentified compounds that preclude the isolation of feasible amounts of high-quality nucleic acids. We have chosen to improve the method of RNA isolation developed for recalcitrant tissues of cacao [17], a tropical tree showing similar problems of high content of polyphenol, polysaccharide, and pigments in its organs, which makes difficult to remove them by conventional extraction procedures. Annatto RNA extraction was successfully carried out using the modified method described in this report, which requires few reagents and can be easily carried out in any laboratory.
2. The homogenization buffer containing boric acid, associated with SDS and β-mercaptoethanol, facilitated the recovery of RNA by enhancing the dissolution of the cell wall and denaturation of proteins. Moreover, the use of the homogenization buffer avoided the oxidation of phenolic compounds and, therefore, the binding of the phenolics to the RNA [17]. Also, the polyphenol oxidase activity was reduced due to the alkaline pH of the homogenization buffer.
3. The phenol/chloroform/isoamyl alcohol step contributed not only to eliminate the denatured proteins and pigments, but also to aid in the precipitation of large amounts of polysaccharides, as observed in the bottom of the tubes after the centrifugation step.
4. In order to better purify nucleic acids from polyphenols and polysaccharides, the tertiary-butanol step [17] was further replaced by an efficient purification step using 2% of the anionic detergent MATAB associated to 3 M of NaCl. As confirmed by our results, this method was very effective in avoiding co-precipitation of polysaccharides, polyphenols and nucleic acids, resulting in the precipitation of nucleic acids free from these contaminants (see below).
5. The procedures for RNA isolation must be evaluated by monitoring the quantity, quality, and integrity of the RNA obtained [20]. The integrity of the RNA samples from different tissues of annatto was confirmed by visualizing intact bands of 28S and 18S rRNAs in an 1% agarose gel (Fig. 1). The RNA integrity was not affected by the subsequent DNase treatment step for removal of genomic DNA.
6. Spectrophotometric quantification at 260 nm, after DNase treatment (step 7 of RNA extraction protocol) of the samples, demonstrated that our method in fact yielded large amounts of RNA (138.9–745.2 μg/g of fresh weight; Table 1). In contrast, only 36.3–90 μg of RNA per gram of fresh weight was produced by the modified hot-phenol method previously reported for annatto [16]. The absorbance at 260/280 nm ranged from 1.70 to 1.80 (Table 1), suggesting that the RNA could be applied for further analysis.
7. In order to determine whether the isolated RNA could in fact be used for further analysis, it was used as a template for RT-PCR. By using the first-strand cDNA from developing seeds, RT-PCR was performed with 18S primers and the products were separated in 1% agarose gel electrophoresis. A major PCR product of

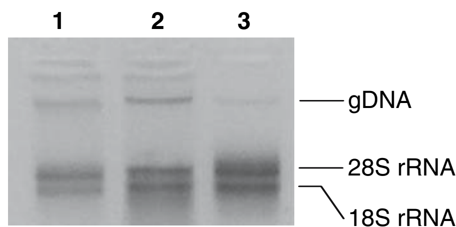

Fig. 1 RNA visualization in agarose gel electrophoresis after the polyphenol and polysaccharide elimination step using MATAB associated to NaCl. RNA was extracted from the following tissues of annatto: developing seeds (lane 1), young leaves (lane 2), and mature leaves (lane 3). gDNA, genomic DNA; rRNA, ribosomal RNA

Table 1 Spectrophotometric quantification of total RNA extracted from different tissues of annatto

Tissues	Total RNA (μg/g FW)	$A_{260/280}$ ratio
Developing seeds	745.2 ± 35.4	1.80 ± 0.05
Young leaves	138.9 ± 3.6	1.76 ± 0.03
Mature leaves	320.4 ± 15.0	1.70 ± 0.02

±Standard deviation, $n = 4$

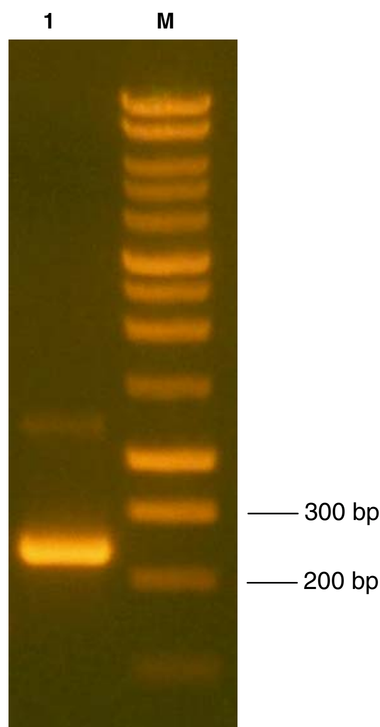

Fig. 2 Agarose gel electrophoresis of PCR-amplified 250-bp 18S fragment from developing seed cDNA. M, 100-bp DNA ladder; lane 1, 18S cDNA

250-bp, corresponding to the amplification of 18S cDNA sequence, was successfully obtained (Fig. 2). This result confirms the high-quality of the RNA obtained by our protocol.

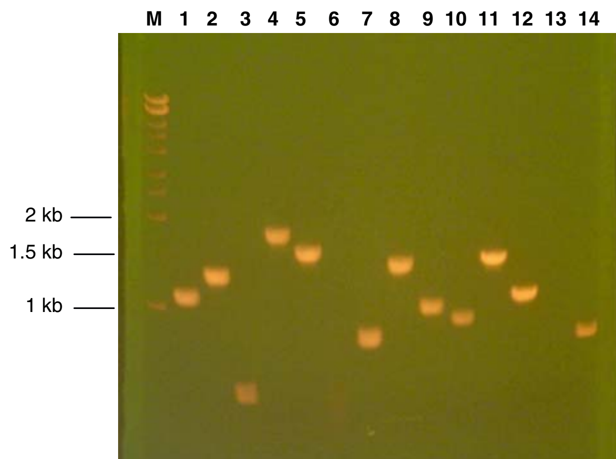

Fig. 3 Agarose gel electrophoresis of PCR-amplified cDNA fragments from selected clones of the library. Primers M13 forward and reverse were used in the PCR reactions. M, 1-kb DNA ladder; lanes 1–14, *E. coli* clones. Lanes 6 and 13 indicate false-positive clones

8. Confirmation whether the isolated RNA was suitable for use in further experiments also came from construction of a cDNA library and evaluation of randomly selected colonies by PCR. PCR products from 14 clones demonstrated that most of them had a cDNA size ranging from 1 kb to 2 kb (Fig. 3).

Taken together, these results confirmed that the isolated RNA is suitable for use in further molecular biology experiments, without any inhibition of enzymatic reactions. Thus, the protocol presented here facilitates future investigations of biochemical and molecular basis of natural product biosynthesis in annatto. It could also be useful for other plant species containing high levels of polyphenols, polysaccharides, and pigments, as we have observed for *Musa* spp. (banana) and *Genipa americana* (jenipapo) (data not shown).

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