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EXTRACTION OF HIGH QUALITY RNA AND EXPRESSION ANALYSIS OF ALDEHYDE DEHYDROGENASE FROM *BIXA ORELLANA* L.

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ABSTRACT

Bixin is an important natural orange-red pigment derived from lycopene produced and stored in seeds of Bixa orellana L. Approximately 80% of the pigments present in annatto seeds correspond to bixin, widely used in the pharmaceutical, textile, dairy, food, beverage, paint, and cosmetics industries Though biochemical and molecular basis of bixin biosynthesis is known, most enzymes and genes regulating the biosynthesis of carotenoids and other natural products have not been characterized in annatto. RNA extraction is a prerequisite technique for gene expression studies, analyzing the etiology and disease progression, treatment effects, as well as designing the diagnostic methods. Isolating sufficient yields of high quality RNA from tissues of annatto has been particularly difficult due to the presence of large amounts of polyphenols and polysaccharides that coprecipitate with nucleic acids upon isolation. The present study aimed to isolate high quality RNA and to characterize aldehyde dehydrogenase gene (ALDH) through RT-PCR from Bixa leaves, mature and immature seeds which has a key regulating role in bixin biosysnthesis pathway.

KEY WORDS

Annatto, Bixin, RNA extraction, Lithium chloride, Aldehyde dehydrogenase, Gene expression

INTRODUCTION

Bixa orellana L. is a commercial crop originally from the southwest Amazon region which considered being the center of diversity for the species [1, 2]. Annatto is currently cultivated in Africa, Asia, and Latin America [3]. The production of annatto in Brazil is around 12,000-13,000 t/year [4]. Annatto has been used for centuries in many parts of the world for the prevention and treatment of a number of health disorders such as constipation, fevers, heartburn, asthma, scabies, ulcers, diarrhea, stomach upset, skin diseases, measles, anecdotal treatment of diabetes, allergy, leprosy, infectious diseases, burns, measles, gonorrhea, diarrhea, asthma, tumors, skin problems, and urinary infections [5]. Bixa is also an important medicinal species [6, 7, 8] having antigenotoxic and provitamin like activity, [9, 10] antimutagenic [11] and antimicrobial properties [12]. It is grown for its unique capability to produce bixin, a natural pigment, found predominantly in the seed aril and widely used in the food industry [13]. It is obtained from the outer coatings of the seeds of the Annatto tree (*B. orellana*) [14, 15]. The natural dye, known as bixin, is used in the pharmaceutical, textile, paint and cosmetics industries [16]. This makes annatto a very attractive and healthy pigment as a substitute for the many synthetic colorants and the demand for natural colorants of annatto has increased for its natural nutrients. The world demand for annatto is increasing together with the interest in natural food dyes. In spite of recent clarification of the biochemical and molecular basis of bixin biosynthesis [17], most enzymes and

genes regulating the biosynthesis of carotenoids and other natural products have not been identified in annatto.

Bixin, the first cis-carotenoid to be isolated from natural sources, is the only one found in *B. orellana*. This 24-carbon compound belongs to the apocarotenoid family; Apocarotenoids as bixin are derived from the oxidative cleavage of carotenoids, which might occur randomly through photo-oxidation or lipoxygenase co-oxidation**.** Bixin is derived from the enzymatic cleavage of lycopene [17, 18]. A biosynthetic pathway for bixin has been proposed and supported using a heterologous expression system [19]. Three *B. orellana* cDNAs encoding the enzymes required for bixin synthesis derived from the linear C_{40} lycopene have been identified: lyco-pene cleavage dioxygenase (BoLCD), bixin aldehyde dehydrogenase (BoALDH) and norbixin methyltransfer-ase (BonBMT) [17]. The second step in the bixin pathway is the oxidation of aldehyde groups in bixin aldehyde, into carboxylic acids by aldehyde. BoALDHs has the ability to oxidize aldehyde groups from apocarotenoides into carboxylic acids [20, 21]. Cardenas-Conejo et al. 2015 [22] identified 20 BoALDHs cDNAs from the ten families constituting the common core group. The best candidates for catalyzing the second step in bixin pathway to produce nor-Bixin occurs in plastid or cytosol by BoALDH3I1 and BoADLH3H1 and these enzymes can act on various substrates in plastids or cytosol.

Isolating sufficient yields of high quality RNA from tissues of annatto has been particularly difficult due to the presence of large amounts of polyphenols and polysaccharides that coprecipitate with nucleic acids upon isolation [23]. Isolation of high quality RNA is the first step for a variety of gene expression analysis. Extraction of high quality RNA and integrity is essential for gene expression studies and all downstream RNA-based techniques. Several RNA isolation protocols have been reported using various compounds in the extraction buffer, such as guanidinium thiocyanate [24], hot phenol [25] and commercial kits RNeasy (QIA-GEN), Trizol (Sigma Aldrich) available for RNA isolation were not designed for use with plant tissues rich in polyphenols and polysaccharides [26, 27], However, none of the above methods produced *B. orellana* RNA of sufficient quality or quantity. Successful and reproducible method for isolation and purification of high-quality RNA from different tissues of annatto was done previously by [28 and 29]. In the present study, an attempt was made to develop an alternate protocol to get high quality RNA from Bixa leaves and developing seeds according to our laboratory conditions. We used the basic protocol described by Sajeevan et al. [30] with slight modifications for tissues rich in polyphenols and polysaccharides to develop an efficient and rapid RNA isolation method from *B. orellana* L. plant tissues for studying gene expression related to BoALDH, which plays a key regulatory role in bixin biosynthetic pathway.

MATERIALS AND METHODS

Plant Material:

Samples were collected from different tissues (leaves, immature and mature seeds) of 12-year-old annatto plants (*B. orellana* L.) growing in Botanical Park at Sri Venkateswara University, Tirupati, Andhra Pradesh, India (Fig. 1). After harvesting, the samples were immediately frozen in liquid nitrogen before transportation to the laboratory and stored at −80˚C until use.

Reagents and Solutions

1. RNase free sterile water (0.1% DEPC—treated autoclaved water)
2. 5% PVPP (Polyvinylpoly pyrollidone)
3. Homogenization buffer: 0.1 M Tris (pH adjusted to 7), 0.01M EDTA (pH adjusted to 8), 0.5M NaCl (w/v), 3% (w/v) Sodium dodecyl sulfate (SDS), and 5% (v/v) β -mercaptoethanol (freshly added before use).
4. 1M Tris pH 7
5. 0.05M EDTA pH 8
6. 2M NaCl
7. 5% SDS
8. Phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v).
9. Chloroform/isoamyl alcohol (24:1; v/v).
10. Lithium chloride - 8M and 2M
11. Cold isopropyl alcohol
12. Ice cold 70% ethanol (v/v)
13. β-mercapto ethanol
14. DNase I (Invitrogen).
15. Reverse transcriptase (Thermo scientific)
16. Oligo DT_{18} (Thermo scientific)
17. Ribolock (Thermo scientific)
18. Taq DNA polymerase (Thermo scientific)

Note: 0.1 percent DEPC (Diethyl pyrocarbonate) treated and autoclaved glass and plastic ware (eppendorfs, PCR tubes, tips) and mortar and pestles have been used for the experiment.

RNA Extraction Protocol:

Plant tissue (100mg) was ground to fine powder in a mortar and pestle using liquid nitrogen along with PVPP (5%) and homogenized in 1ml of extraction buffer containing 50µl of *β*-mercaptoethanol. 1 ml of phenol: chloroform: isoamyl alcohol mixture was added and mixed thoroughly. The mixture was transferred to 2ml eppendorf tube and centrifuged at 15,000 rpm for 15 min. The upper colourless aqueous phase containing nucleic acids was then transferred to a fresh eppendorf tube and equal volume of chloroform: isoamyl alcohol mixture was added and mixed thoroughly by inverting the tube for 15 to 20 times. The mixture was then centrifuged at 15,000 rpm for 10 min at 4˚C, and the aqueous phase was transferred to a fresh eppendorf tube. The above step was repeated twice and 8M LiCl was added to a final concentration of 3M to the transferred upper aqueous phase and incubated in −80˚C for 5 h or −20˚C for 12 h. The solution was then centrifuged at 15,000 rpm for 15 min at 4˚C to collect RNA as precipitate. The precipitate was resuspended in 2M LiCl and centrifuged at 15000 rpm for 20 min at 4˚C. The supernatant was discarded, and the RNA pellet was washed with 70% ice cold ethanol. The pellet was then air dried and alcohol-free pellet was dissolved in 50 to 100 µl of DEPC treated sterile water. 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.

Estimation of Quantity and Quality of RNA:

Determination of the RNA concentrations in the samples was done based on values given by measurement on a NanoDrop 300 N.A. The A260/A280 ratio gives the purity of the RNA (ratio should be between 1.8 -2.1). The concentrations were confirmed by comparison to the fragment intensities in electrophoresis of the individual samples on a 1% agarose gel. For checking the quality, 2 µl of the total RNA was loaded on 0.1 % DEPC-treated agarose gel stained with ethidium bromide (10mg/ml) and visualized under UV transilluminator.

Synthesis of cDNA and PCR:

About 5µg of total RNA was heat denatured at 65˚C for 5 min and reverse transcribed by MMLV reverse transcriptase (100 U, Thermo scientific, USA) in 20 µl reaction mixture containing oligo dT primer (10µM), 1X reaction buffer with 10mM dNTPS mix. Reverse transcription is performed at 42˚C for one hour and the enzyme was heat inactivated at 65˚C for 15 min and the cDNA synthesized was either used immediately or stored at 4˚C until further use.

Polymerase chain reaction (PCR) was performed using the cDNA as template. The PCR was carried out to amplify housekeeping gene of annatto 18s rRNA sequence using specific primers and 1 µl of reverse transcribed product in 20 µl reaction mixture. The reaction mixture contained 1 X *Taq* buffer, 1.5 mM dNTPs, and 1 U *Taq* polymerase (Thermo Scientific, USA). The reaction conditions were 94˚C for 7 min followed by 30 cycles of 94˚C for 30 sec, 55 - 60˚C for 30 sec, and 72˚C for 45 sec and a single final elongation step of 72 ˚C for 10 min. Similarly, bixin aldehyde dehydrogenase genes [22] were amplified using the cDNA as template with the primers listed in Table. 2 under standardized PCR conditions and analyzed by separating the PCR product on agarose gel.

RESULTS AND DISCUSSION

The modified protocol overcomes the problems associated with polyphenols and polysaccharides, and it can be easily carried out in any laboratory. We modified the protocol described by Sajeevan et al. [30] with slight modifications in concentrations of Sodium dedecyl sulpahte (SDS), Polyvinylpolypyrrolidone (PVPP) and β-mercaptoethanol as mentioned in Table.1. The yield and quality of the RNA obtained were consistently high, as confirmed by nanodrop analysis, separation on agarose gel and Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Sajeevan et al. 2014	Modified protocol
Tris-HCl-0.1 M (pH 7.0)	Tris-HCl-0.1 M (pH 7.0)
Sodium Chloride (NaCl)-0.5 M	Sodium Chloride (NaCl)-0.5 M
Ethylene diamine tetra aceticacid (EDTA)-10 mM (pH 8)	Ethylene diamine tetra aceticacid (EDTA)-10 mM (pH 8)
Sodium Dodecyl Sulphate (SDS)-2% (w/v)	Sodium Dodecyl Sulphate (SDS)-3% (w/v)
β-mercaptoenathol-4% (v/v)	β-mercaptoenathol-5% (v/v)
Polyvinylpolypyrrolidone (PVPP)-4% (w/v)	Polyvinylpolypyrrolidone (PVPP)-5% (w/v)

Table 1. Comparison of components of extraction buffers between two RNA isolation protocols.

S.No	Oligonucleotide	Sequence
1	18S rRNA-F	CGGCTACCACATCCAAGGAA
	18S rRNA-R	GCTGGAATTACCGCGGCT
2	BoALDH-1F	GTAGCGACCTCAAGAGTGTATG
	BoALDH-1R	GAGCTCATCCTGGGCTATATTC
3	BoALDH-2F	CCACGAATGTGAGGCTATGAA
	BoALDH-2R	TTCTCTAGCCGCCTCTACTATC
4	BoALDH-3F	ACCATCGCAGAAGCAGATAAG
	BoALDH-3R	GGACCCATGTTGGCTTTAGT
5	BoALDH-4F	ATGGAAGAAACCTTGGCAG
	BoALDH-4R	TCAGTTGGGTGCTTTCCT
6	BoALDH-5F	TGGTAGATACTTTCAGAGAGG
	BoALDH-5R	GTTGGAGCAATCTTCAGC
7	BoALDH-6F	CATTCCACTTCAGACGTC
	BoALDH-6R	TTCTTGCATGATCTGTGC

Table 2. List of primers used for RT-PCR amplification.

Protocol used	Concentration (ng/ µl)	Absorbance Ratio	
		260/280 ratio	260/230 ratio
Sajeevan et al. (2014)			
Leaf	200	1.56	1.70
Immature seed	150	1.71	1.40
Mature seed	120	1.68	1.54
Modified Protocol			
Leaf	600	2.09	1.86
Immature seed	535	2.05	2.0
Mature seed	727	2.11	1.93

Table 3. Nanodrop quantification of total RNA extracted using Sajeevan et al. (2014) and modified protocols from leaf, immature and mature seed tissues of *B. orellana* L.

RNA Quantification and Agarose Gel Electrophoresis: Determination of the RNA concentrations in the samples was done based on values given by measurement on a NanoDrop 300 N.A (Table. 3). Denaturing agarose gel stained with ethidium bromide indicated intact RNA as there was clear distinct bands of 28 and 18S rRNA (Fig. 2).

Fig. 1 *Bixa orellana* tissues used as sources for isolation of RNA. a) Leaf, b) Immature seed, and c) Mature seed

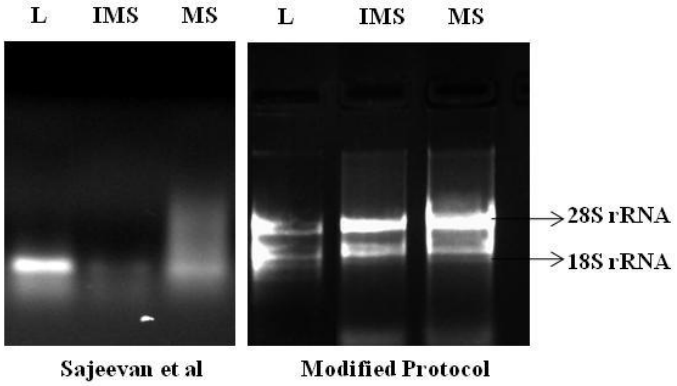

Fig. 2 Denatured agarose gel electrophoresis of Bixa RNA extracted samples.
L- Leaf, IMS-Immature seed, MS- Mature seed

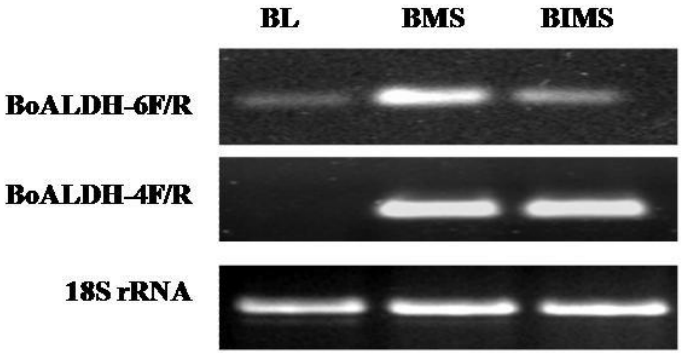

Fig. 3 RT-PCR analysis of Bixa samples using ALDH gene specific primers. L- Bixa Leaf, MS-Mature seed, IMS- Bixa Immature seed.

RT-PCR analysis of bixin pathway key gene – Aldehyde dehydrogenase:

The relative mRNA levels were normalized according to housekeeping gene (18SrRNA) and expressed relative to the corresponding intensities of leaves, mature and immature seeds. Among the various Bixin Aldehyde dehydrogenase specific primers screened, BoALDH-6F/R and BoALDH-4F/R showed amplification with consistent results. When BoALDH-4F/R specific primer used, expression was not observed in Bixa leaves and the expression is same with respect to mature and immature seeds and with BoALDH-6F/R, relative expression of aldehyde dehydrogenase is high in mature seeds than immature seeds (Fig. 3).

B. orellana contain high amount of polysaccharides, polyphenolics and other secondary metabolites that rapidly oxidize during tissue grinding. Although there are many protocols standardized for isolating RNA from various plant species and tissues with varying amount of cellular composition, the methods were not suitable for all types of tissues and plant species. The existing methods could not yield high quality RNA in sufficient quantities in bixa probably because of high amount of interfering compounds such as polyphenols and polysaccharides. The difficulty in RNA extraction from

bixa grown under different environments have been earlier reported by many researchers. Increasing the concentrations of PVPP, SDS and β-mercaptoethanol in homogenization buffer compared to protocol of Sajeevan et al. [30] was shown to be very effective for preventing tissue browning and enhanced the dissolution of cell wall and denaturation of protein yielding quality RNA. The phenol/chloroform step was repeated twice to eliminate the denatured proteins and pigments and also to aid in the precipitation of large amounts of polysaccharides.

By using the method proposed in this work, high yields of good quality RNA were recovered. The modified protocol yielded a white, water soluble RNA precipitate from different tissues of bixa. Clear distinct bands of 28S and 18S rRNA were observed when denaturing agarose gel stained with ethidium bromide indicating intact RNA. The isolated RNA conserved its integrity as shown in Fig. 1. Many protocols failed to yield quality RNA in sufficient quantities, However, using this standardized protocol, we could isolate quality RNA in sufficient quantities from different tissues of bixa plant. The high yield (~700ng/µl), and quality of the RNA obtained with this methodology are appropriate for expression studies.

Recently several methodologies for molecular biology studies of *B. orellana* [31, 22], have been developed. Some of the genes involved in bixin biosynthesis have been characterized for future use as molecular tools in improvement of annatto production. In a study using standard molecular techniques, the genes involved in the biosynthesis of bixin were cloned and functionally characterized by Bouvier et al. [17]. The pathway determined by the gene isolation has been reviewed in the literature [32]. Bixin is an orange-red apocarotenoid that accumulates in high quantities in seeds which accounts for 80 % of the total carotenoids. Concentration of bixin increases continuously during development of immature seeds until they reach maximum size [18]. mRNAs encoding different BoCCDs, BoALDH and BoMTs enzymes have been identified and believed that these are involved in bixin synthesis [22]. The levels of expression and regulation of bixin biosynthetic genes are still not known and further confirmation is warranted through molecular techniques. The RNA recovered using this method has been used with good results in RT-PCR analysis. The expression was high in mature seeds of *B.orellana* when screened with BoALDH-4F/R and BoALDH-6F/R and in all cases the lowest expression levels were displayed in leaves.

CONCLUSIONS

The proposed protocol can be easily carried out in any laboratory to get high quality RNA and it is suitable for use in further molecular biology experiments. The protocol can also be used to isolate RNA from different plant species rich in polysaccharides, polyphenols and secondary metabolites. Among the various enzymes in Bixin biosynthetic pathway, aldehyde dehydrogenase plays a major role and it is a key regulatory enzyme in the biosynthesis of Bixin. Results suggested that mature seeds play a major role in the biosynthesis of Bixin when compared to leaves and immature seeds. Thus, the protocol facilitates future investigations on expression analysis at different reproductive stages through transgenics to evaluate the importance of BoALDH gene in the biosynthesis of Bixin.

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