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Isolation of High-Quality mRNA from Tannin-Rich Persimmon Fruit

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고 Tannin 함유 감과실로 부터 mRNA의 분리

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Abstract

In our studies on the role of β-galactosidase in fruit softening, significant difficulty, was encountered in our attempts to extract RNA from persimmon(***Diospyros kaki*** L. cv. Fuyu) fruit due to astringency and tannin content. Initial, unsuccessful RNA extractions involved methods using guanidinium isothiocyanate/CsCl with and without polyvinylpyrrolidone(PVP), phenol/sodium lauryl sulfate(SDS), guanidinium hydrochloride, as well as polysomal RNA purification method that used 0.2 M Tris-HCl (pH 9.0) containing KCl, Mg-acetate, EDTA, β-mercaptoethanol, and sucrose. A method was devised which employed treatment of fruit with CO_2 gas to diminish astringency prior to RNA extraction, followed by extraction of tissue powders with Proteinase K extraction buffer containing PVP and ascorbate at an alkaline pH. This procedure resulted in the removal of tannins and other polyphenolics and extraction of relatively large amount of high-quality RNA suitable for cDNA library construction and polymerase chain reaction(PCR). Futhermore, the procedure does not use the toxic and corrosive chemical guanidinium isothiocyanate or require ultracentrifugation.

Key words : persimmon(***Diospyros kaki*** L. cv. Fuyu), ascorbate, polyphenolics, PVP, RNA extraction, tannin

Introduction

Use of molecular biology techniques is becoming increasingly important in the area of postharvest biology. Genic manipulation of key genes in an effort to increase shelf life and quality

of fruits and vegetables is currently underway in a number of laboratories worldwide. Therefore, the ability to isolate relatively pure, intact RNA from a wide range of horticultural crops is critical for use in cDNA synthesis, RNA blots, PCR, and *in vitro* translations. However, this is difficult in tissues which are high in tannins and/or other polyphenols, such as persimmon[1, 2], apple[3], peach[4], *Cornus officinalis*[5], banana[6], pecan[7], and many other fruit types [8]. The problem has been attributed to the high content of phenolic terpenoids and tannins [9] which become associated with RNA upon tissue homogenization. These are difficult to remove using conventional extraction procedures and render the RNA unsuitable for *in vitro* translation or cDNA synthesis.

Our objective was to devise a method to extract high-quailty RNA from persimmon fruit. We report here on a simple and effective method for extraction of RNA from persimmon fruit tissue using buffer containing Proteinase K, PVP and ascorbate at pH 9.0.

Materials and Methods

Materials

Persimmon fruits(*Diospyros kaki* L. cv. Fuyu) were picked at the ripe stage in the Republic of Korea and stored in air at 4℃ for 3 months. Fruits were shipped by express air to Baltimore, MD, U. S. A. Upon arrival, fruits were placed in a 5% Co_2 at 20℃ for 1 day[10]. Pericarp tissue was excised directly into liquid nitrogen and stored at -80℃. For RNA extraction, tissue was then ground for 1 min. in an equal amount(w/w) of dry ice and stored at -80℃ for at least 72 hours prior to the extraction procedure.

Total RNA extraction methods

Total RNA extraction using proteinase K with PVP and ascorbate

The following protocol is a modification of the RNA extraction methods of Callahan *et al.*[4] and Morgens *et al.*[11]. One hundred g of powdered tissue was ground in volumns(w/v) of 0.1 M Tris -HCl(pH 9.0) containing 0.1 M NaCl, 1% SDS(w/v, sigma L-4390), 1% PVP(w/v, Sigma PVP-40), 0.1% Proteinase K(w/v, Sigma P-2308), 10 mM ascorbate and 1% β-mercaptoethanol(v/v) at 65℃ using a mortar and pestle. Proteinase K, β-mercaptoethanol, and ascorbic acid were added to the buffer just prior to use. The mixture was centrifuged at 14,500×g for 10 min. at 20℃ and the supernatant poured into another centrifuge tube containing 0.5 volume of 65℃ Tris-saturated phenol(10mM, pH 6.7); the sample was mixed and centrifuged at 14,500×g for 10 min. at 20℃. The supernatant was transferred to another tube containing 0.5 volume of phenol : chloroform : isoamylalcohol(25 : 24 : 1, v/v/v), mixed well, and centerfuged at 14,500×g for 10 min. at 20 ℃. If the interphase was relatively large or the aqueous layer highly colored, the aqueous phase was reextracted. This was repeated until the aqueous phase was clear. The supernatant was then poured into another centrifuge tube containing 0.5 volume of chloroform : isoamylalcohol (24 : 1, v/v), mixed, and centrifuged at 14,500 ×g for 10 min. at 20℃. The supernatant was transferred to another centrifuge tube, kept on ice, and brought to a concentration of 0.3 M Na-acetate using a 3 M stock solution and adjusted to pH 5.0 using acetic acid. The supernatant was then brought to a concentration of 0.1% SDS using a 10% SDS stock solution and 0.5 M NaCl using a 5 M NaCl stock solution, incubated for 2 hours on ice and centrifuged at 16,000×g for 20 min. at 4℃. The supernatant was transferred to a new tube, brought to a concentration of 2 M LiCl using a 10 M LiCl stock solution, in-

cubated at 4℃ for at least 12 hours and centrifuged at 16,000×g for 30 min. at 4℃. The supernatant was discarded and the pellet resuspended in 3 ml of H_2O. Sixty $\mu\ell$ of 5 M NaCl and 2.5 volumes of ice cold 100% EtOH were then added, and the sample was mixed and incubated at -20℃ overnight. The mixtire was then centrifuged at 16,000×g for 30 min. at 4 ℃ and the supernatant discarded. The pellet was dried in vavuo until the EtOH was completely removed, resuspended in a minimal amount of ddH_2O, and made to 0.5 ml of 0.1 M NaCl. Then, 2.5 volumes of ice cold 100% EtOH were added. The sample was mixed and incubated at -20℃ for at least 12 hours. The mixture was centrifuged at 16,000×g for 30 min. at 4℃, the supernatant discarded, and the pellet dried *in vacuo* until the EtOH was completely removed. The RNA was resuspended in ddH_2O, frozen in liquid nitrogen and stored at -80℃ until used.

Other methods of total RNA extraction

For the 4 M guanidinium isothiocyanate/CsCl method, total RNA was extracted using the basic procedure of Chirgwin *et al.*[12] as modified by Starrett and Laties[13]. Briefly, the ground tissue powder(100g) was homogenized in 4 M guanidinium isothiocyanate extraction buffer in a mortar and pestle, filtered, and the homogenate centrifuged 10 min at 12,000×g. The supernatant was layered over a 10 ml pad of 5.7 M CsCl and centrifuged 6.5 hours at 200,000×g. The pellet was resuspended in ddH_2O and precipitated with 0.1 volume of 3 M Na-acetate(pH 5.2) and 2.5 volumes of 100% EtOH at -20℃. For the 5 M guanidinium isothiocyante/CsCl with PVP method, total RNA was isolated using 5 M guanidinium isothiocyanate/CsCl as described by John[14] as modified from Chirgwin *et al.*[12]. This procedure is similar to the 4 M guanidinium isothiocyanate/CsCl described above, but with addition of PVP to the extraction buffer.

The 8 M guanidinium hydrochloride method involved isolating total RNA using 8 M guanidinium hydrochloride(GIBCO-BRL # 15502-016) as described by Chirgwin *et al.*[12]. The ground tissue powder(100g) was homogenized in the guanidine/HCl extraction buffer with a mortar and pestle, filtered, and the homogenate centrifuged 10 min at 14,500×g. The supernatant was mixed with 0.2 volume of 1 M acetic acid and 0.5 volume of 100% EtOH. The extract was held at -70℃ for 1 hours and then centrifuged at 14,500×g for 30 min at 4℃. The residue was dissolved in extraction buffer by vortexing at ambient temperature. This precipitation procedure was repeated four times. The resulting pellet was washed once with 70% EtOH. The extract was centrifuged at 14,500×g for 30 min at 4℃ and the pellet dissolved in a minimal amount of ddH_2O.

Polysomal RNA was extracted using buffer containing 0.2 M Tris-HCl(pH 9.0), 0.4 M KCl, 0.06 M Mg-acetate, 0.05 M EDTA, 0.05% β-mercaptoethanol and 250 mM sucrose and pelleting through a 1.5 M sucrose pad as described by Boyd[15] except that the pellet(polysomal RNA) was resuspended in ddH_2O and precipitated with 0.1 volume of 3 M Na-acetate (pH 5.2) and 2.5 volumes of 100% EtOH at -20℃.

Purification of Poly A^+ mRNA

Poly A^+ mRNA was oligo-purified according to the protocol of Starrett and Laties[20]. Essentially, total RNA was chromatographed through a cellulose pre-column and an oligo dT-cellulose purification column. A^+ RNA was eluted, fractions were pooled and the A^+ RNA was precipitated in EtOH. The poly A^+ mRNA was then resuspended in ddH_2O, quantified spectrophotometrically and used for the indicated procedures.

RNAse precautions

Precautions were taken to eliminate RNAse activity in all extraction procedures. Solution were made to 0.05% diethylpyrocarbonate (DEPC) and autoclaved. Non-autoclavable solutiona were filter sterilized. All glassware was rinsed in 0.05% DEPC and baked at 180℃ overnight. Small utensils and plastics were pre-sterilized or autoclaved. Next boxes of pipet tips, microcentrifuge tube, etc., were opened each time RNA manipulations were performed.

PCR amplification of persimmon fruit β-galactosidase

In order to amplify persimmon β-galactosidase cDNA, two degenerate primers were synthesized. One primer(BG) was designed based on a consense peptide sequence of apple, asparagus, and carnation β-galactosidase and second primer(PB) was based on the N-terminal amino acid sequence of persimmon 44 kD β-galactosidase[16]. First strand cDNA synthesis and PCR amplification were performed using the SuperScript™ preamplification system[17] with ***Taq*** polymerase from Perkin-Elmer. For this, 160 ng Poly A^+ mRNA was reverse transcribed and target cDNA was amplified with 35 cycles at 94℃ for 1 min., 57℃ for 30 sec., and 72℃ for 2 min. The PCR products amplified using these two primers were colned and sequenced[18]. Based on sequenced data, two internal primers specific to persimmon β-galactosidase cDNA were synthesized(T3S and T7S).

Results and Discussion

A number of different RNA extraction protocols had been unsuccesful at yielding sufficient quantities of high-quality RNA for PCR or library constuction. However, addtion of ascorbate and PVP to the extraction protocol allowed extraction of high-quality RNA. Extraction of total RNA using the Proteinase K method with PVP and ascorbate resulted in an average yield of RNA from persimmon fruit of 29.6㎍ per gram fresh weight of tissue(Table 1). However, the yield of RNA when using the same Proteinase K method without PVP and ascorbic acid was 0.6㎍/g·fw. A significant reduction in extractable RNA was also observed when using the phenol/SDS method. Methods that used guanidinium isothiocyanate yielded no detectable RNA(Table 1). The polysomal extraction method was used in an effort to precipipate polysomes away from polyphenols. However, this method yielded poor-quality RNA in low quantity(Table 1). The RNA isolated using the Proteinase K method with PVP and ascorbate was used to prepare a cDNA library, whereas RNA isolated with the other extraction methods repeatedly resulted in low yields and low quality RNA which was not sufficiently pure for library construction by the Stratagene(data not shown). RNA from the Proteinase K method with PVP and ascorbate was also of sufficient quality for PCR(Fig. 1) and allowed the cloning of β-galactosidase from persimmon fruit[8]. The original amplification of β-galactosidase was carried out using the primers BG and PB. The primers T3S and T7S were designed as internal sequencing primers for the β-galactosidase clone and were also used as PCR primers for verification of high-quality RNA(Fig. 1). In the case of PCR of RNA extracted using the proteinase K method with PVP and ascorbate, the BG and PB primers yielded fragments of approximately 850 bp (Fig. 1, lane 3) while T3S T7S yielded fragments roughly 450 bp in length(Fig. 1, lane 6),

Table 1. Comparison of the amount of total RNA extracted from persimmon fruit using various methods

Extraction Method	Total RNA (μg/g·fw)
Proteinase K :	
with PVP and ascorbate	29.6
without PVP and ascorbate	0.6
Phenol/SDS	11.3
4M Guanidinium isothiocyanate/CsCl	ND[1]
5M Guanidinium isothiocyanate/CsCl with PVP	10.7
8M Guanidinium isothiocyanate/CsCl	ND
Polysomal RNA extraction	1.5

[1]ND, non detected

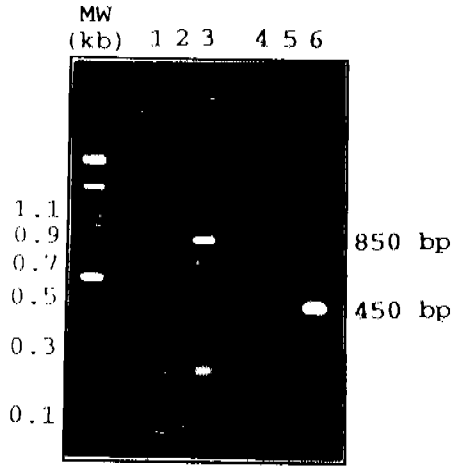

Fig. 1. PCR amplification of β-galactosidase cDNA using BG, PB, T3S and T7S primers with RNA extracted from persimmon fruit using various methods. 8μℓ of the 50μℓ PCR reaction were loaded per lane. Lane 1 and 4, total RNA extracted using phenol/SDS; lanes 2 and 5, total RnA extracted with Proteinase K without PVP and ascorbate; lane 3 and 6, total RNA extracted using Proteinase K with PVP and ascorbate; lane 1, 2 and 3 were amplified by PCR using BG and PB primers. Lane 4, 5 and 6 were amplified by PCR using T3S and T7S primers. Molecular weight marker(MW) were a 100 bp ladder from GIBCO-BRL.

all of which were clearly evident, this was not the case when PVP and ascorbate were omitted from the extraction buffer, or when the phenol/SDS method was used for extraction(Fig. 1, lane 1, 2, 4 and 5).

Methods which did not include both PVP and ascorbate yielded lower quantities of RNA. Further, the RNA that was extracted was low quality. cDNA synthesis was unsuccesful or yielded very low qualtities of cDNA at best. Ascorbate, which serves as an antioxidant, and PVP which binds via hydrogen bonding to the tannins[19, 20], both served to counter the effect of the tannins on RNA quality by binding to the tannins and other polyphenols, thereby removing them and limiting their binding to RNA during extraction.

In summary, the Proteinase K method we describe was effective for isolating RNA suitable for cDNA synthesis and PCR from persimmon fruit and potentially other fruits or tissues that are recalcitrant to other RNA extraction methods due to the presence of tannins and other polyphenolics.

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요 약

과실연화에 관여하는 β-galactosidase의 역할을 구명하는 연구를수행함에 있어서, 감 과실내에는 떫은 맛을 지닌 탄닌의 함량이 매우 높기 때문에 良質의 RNA를 추출하는데 매우 어려운 점이 있었다. 처음에 여러가지 RNA추출방법, 즉, PVP 함유 및 미함유 guanidinium

isothiocyanate/CsCl, phenol/SDS, guanidinium hydrochloride방법들과 KCl, Mg-acetate, EDTA, β-mercaptoethanol 그리고 sucrose가 함유된 0.2M Tris-HCl(pH 9.0)을 사용한 polysomal RNA정제방법을 사용하였으나, 良質의 RNA를 추출하는데는 성공하지 못하였다. 그래서 본 연구에서는 RNA를 추출하기 전에 과실의 떫은 맛을 줄이기 위해서 CO_2 gas를 감 과실에 처리한 후 PVP와 ascorbate를 함유한 Proteinase K 추출 buffer(pH 9.0)를 사용하여 감 조직으로부터 RNA를 추출하는 방법을 고안하였다. 이 방법은 감 과실내의 탄닌과 다른 polyphenolic compound를 제거하고 cDNA library 제작과 polymerase chain reaction(PCR)에 적합한 良質의 RNA를 다량으로 추출하는데 우수한 방법임을 알 수 있었다. 더우기 이 방법은 다른 방법들에서 사용되는 초원심분리기나 독성과 부식성이 강한 guanidinium isothiocyanate와 같은 화학약품이 사용되지 않는 장점이 있다.

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