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Original Articles

An efficient method for the isolation of RNA from blueberry leaves

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Summary : The isolation of high-quality RNA from blueberry leaves is difficult due to the presence of many organic acids and polyphenols in the leaves. We established an efficient method for isolating RNA from rabbit-eye blueberry (*Vaccinium virgatum* Aiton) leaves using a modified cetyl trimethyl ammonium bromide (CTAB) method. More than 200 μg of RNA from 1 g of blueberry leaves was obtained with a HEPES or a MOPS buffer changed from a Tris-HCl buffer. The quality and quantity of the total RNA was sufficient for the cDNA synthesis. We succeeded in the amplification of cDNAs using polymerase chain reaction (PCR).

Key words : CTAB, pH, RT-PCR.

Blueberry leaves are rich in organic acids (Kramer and Schrader 1945) and polyphenols mainly composed of proanthocyanidins (Matsuo *et al.* 2010). Proanthocyanidins extracted from blueberry leaves inhibited hepatitis C virus replication (Takeshita *et al.* 2009). These polyphenols have been expected to be useful as components of functional foods. Functional analyses of polyphenol synthesis-related genes are very important to elucidate the polyphenol synthesis system in blueberry leaves. It is necessary to isolate a sufficient amount of high-quality RNA from blueberry leaves to isolate and analyze polyphenol synthesis-related genes. However, it is difficult to isolate RNA from blueberry leaves because the leaves include large amounts of organic acids and polyphenols. Cetyl trimethyl ammonium bromide (CTAB) methods are easy to perform and inexpensive, and have been used to extract nucleic acids in plants generally (Chang *et al.* 1993). We report the efficient isolation of RNA from blueberry leaves using a modified CTAB method.

Mature leaves from rabbit-eye blueberry, northern highbush blueberry (*Vaccinium corymbosum*), tea (*Camellia sinensis*), loquat (*Eriobotrya japonica*) and persimmon (*Diospyros kaki*) were collected from field-grown plants (the University of Miyazaki, Japan). The leaves were immediately frozen in liquid nitrogen and kept at -80 °C until use.

We isolated RNA from rabbit-eye blueberry leaves as described by Asif *et al.* (2000) with modifications. One gram of rabbit-eye blueberry leaves was first ground to a fine powder in liquid nitrogen and then transferred to a 50 ml polypropylene tube containing 10 ml of an extraction buffer (0.2 mM buffer, 20 mM EGTA, 1.4 M NaCl, 2 % [w/v] CTAB, and 10 mM DTT) preheated at 65 °C. We used eight different buffers : MES (pH 6.7), PIPES (pH 7.5), MOPS (pH 7.9), HEPES (pH 8.2), TES (pH 8.2), Tricine (pH 8.8), Tris-HCl (pH 9.0) and borate (pH 10.0). The extraction solution was mixed by vortexing for 2 min and incubated for 10 min at 65 °C. The mixture was added to an equal volume of chloroform and mixed by vortexing for 2 min. The mixture was centrifuged at 2,000 x g for 10 min at 4 °C. The upper aqueous phase (UAP) was transferred to a new 50 ml polypropylene tube. The pH of the UAP was measured by a HORIBA pH METER F-52. After a 1/4 volume of 10 M LiCl was added to the UAP, the tube was kept overnight on ice to precipitate the RNA. The pellet was collected by centrifugation at 12,000 x g for 10

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min at 4 °C and washed with 70% ethanol. The pellet was dissolved in 200 μl of diethyl pyrocarbonate (DEPC)-treated water, and was transferred to a new 1.5-ml microcentrifuge tube. The solution was added to 20 μl of 3M sodium acetate (pH5.2) and mixed well. The mixture was kept on ice for 30 min and centrifuged at 12,000 x g for 10 min at 4 °C. The supernatant was transferred to a new 1.5 ml microcentrifuge tube and an equal volume of phenol/chloroform (1:1) was added and mixed by vortexing for 5 min. The mixture was centrifuged at 12,000 x g for 10 min at 4 °C, and the upper layer was transferred to a new microcentrifuge tube, and an equal volume of chloroform was added and mixed by vortexing for 5 min. The mixture was centrifuged at 12,000 x g for 10 min at 4 °C. The upper layer was transferred to a new 1.5 ml microcentrifuge tube, a 1/4 volume of 10 M LiCl was added, and the mixture was kept on ice overnight. The pellet was collected by centrifugation at 12,000 x g for 10 min at 4 °C, and washed with 70% ethanol twice. The pellet was dissolved in a suitable amount of DEPC-treated water.

The absorbancies of the samples were measured at 260 nm and 280 nm for analysis of the yield and purity of the RNAs (Table 1). A total RNA content of more than 200 μg was extracted from 1 g of blueberry leaves with the HEPES or the MOPS buffer, and less than 100 μg was extracted with the Tris-HCl or the MES buffer (Table 1). The decreases of the RNA yield were probably caused by the low pH during extraction because RNA was slightly soluble in acidic solutions and the reducing power of DTT decreased at pH lower than 7 (Han and Han 1994). The A_{260}/A_{280} ratios were about 1.8 with the HEPES or the MOPS buffer and less than 1.6 with the Tricine or the borate buffer (Table 1). The decreases of the A_{260}/A_{280} ratios seemed to have been caused by the high pH during extraction because polyphenols were easily dissolved in alkaline solution. The RNAs in blueberry leaves can be isolated efficiently with the HEPES or MOPS buffer that maintains the pH value at around neutral during extraction. We succeeded in isolating RNA from the leaves of tea, loquat and persimmon that included many polyphenols (Wei *et al.* 2011 ; Tanaka *et al.* 2010 ; Tsurunaga *et al.* 2008), by the modified CTAB method (Table1). We believe that the modified CTAB method is useful for isolating RNAs from polyphenol-rich plants.

Complementary DNAs were synthesized from distilled water, total RNA, total RNA treated with RNaseA (TypeII-A, Sigma) and total RNA treated with RNase-free DNaseI (TaKaRa), respectively. RNase treatment or DNase treatment was performed according to the instructions provided by the manufacturer. Complementary DNAs were synthesized as follows : One microgram of the total RNA was reverse-transcribed with Superscript™ III Reverse Transcriptase (Invitorogen) in the presence of oligo $(dT)_{20}$ primers according to the instructions provided by the manufacturer. The cDNA was amplified with *Takara Ex Taq* (Takara) in the thermal cycler (ASTEC PC707) using the following program : 1 min at 94 °C, followed by 40 cycles of 30 s denaturing at 94 °C, 30 s annealing at 64 °C, 1 min elongation at 72 °C, and then storage at 4°C. The primers (ACT-F2, 5'- GATTCTGGTGATGGTGTGAG-3' ; and ACT-

Table 1. The yield and purity of RNA isolated from rabbit-eye blueberry, tea, loquat and persimmon leaves

Plant materials	Buffer	pH	Total RNAcontent (g/g FW)	A_{260}/A_{280}	pH of UAP[2]
rabbit-eye blueberry	MES	6.7	81±15[1, d]	1.70±0.05[1]	6.10±0.01[1]
	PIPES	7.5	101±22[cd]	1.60±0.03	6.84±0.05
	MOPS	7.9	287±126[ab]	1.80±0.04	6.98±0.02
	HEPES	8.2	240±26[abcd]	1.78±0.01	7.35±0.05
	TES	8.2	177±40[bcd]	1.64±0.04	7.43±0.05
	Tricine	8.8	116±63[cd]	1.56±0.07	8.07±0.01
	Tris-HCl	9.0	84±33[d]	1.69±0.04	5.43±0.36
	Borate	10.0	180±45[bcd]	1.52±0.07	9.13±0.10
tea	HEPES	8.2	376±41[a]	1.78±0.04	7.95±0.03
loquat	HEPES	8.2	217±61[abcd]	1.77±0.03	7.90±0.02
persimmon	HEPES	8.2	254±60[abc]	1.78±0.02	7.86±0.02

[1] Data represent the mean±SE of three replicates.
[2] UAP : an upper aqueous phase
Means in columns followed by a common letter are not significantly different (P>0.05, as calculated by Tukey's method).

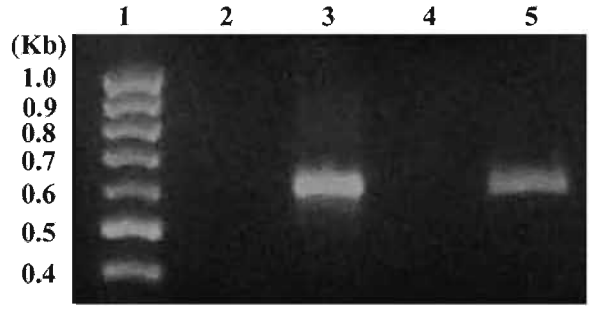

Fig. 1. Agarose gel electrophoresis analysis of blueberry actin cDNA amplified by PCR
Lane 1, DNA size marker ; lane 2, negative control ; lane 3, PCR amplification from total RNA ; lane 4, PCR amplification from total RNA treated with RNaseA and lane 5, PCR amplification from total RNA treated with DNaseI.

R2, 5'- TCCACATCTGTTGGAAGGTG-3') were used to amplify the blueberry actin gene (GenBank accession number AB694898, amplified DNA size, 610bp). The reaction products were electrophoretically separated on a 1.5% agarose gel. After being stained with ethidium bromide, the gel was visualized and photographed under UV light.

The cDNA was amplified in the non-treated RNA and the DNase-treated RNA, and not in the distilled water or the RNase-treated RNA (Fig. 1). The results suggested that cDNA syntheses and RT-PCR analyses could be used with isolated RNAs.

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ブルーベリー葉からRNAを効率的に抽出する方法

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要　約

ブルーベリー葉には有機酸やポリフェノールが大量に含まれるため，高品質なRNAを抽出することが難しい．我々は，改良した臭化セチルトリメチルアンモニウム法を用いることにより，ラビットアイブルーベリー（*Vaccinium virgatum* Aiton）の葉から，RNAを効率的に抽出する手法を確立した．トリス緩衝液をHEPES緩衝液若しくはMOPS緩衝液に変えることで，1 gのブルーベリー葉から200 μg以上の全RNAを得ることができた．cDNA合成を行うのに十分な質と量であった．また，我々は，RT-PCRによるcDNAの増幅にも成功した．

キーワード：臭化セチルトリメチルアンモニウム，pH，RT-PCR．