Extraction of Total RNA from Leaves of *Eucalyptus* and Other Woody and Herbaceous Plants Using Sodium Isoascorbate

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ABSTRACT

Rapid extraction of total RNA from *Eucalyptus* leaves is difficult due to the high content of polyphenolics and polysaccharides. A rapid and simple method was developed by using an extraction buffer containing sodium isoascorbate at a concentration of 500 mM. This method consisted of one or two chloroform extractions, one acid guanidium-phenol-chloroform extraction, and isopropanol precipitation alone. The yields of the RNA fractions were 246–1750 µg/g fresh weight when leaves of *Eucalyptus*, five other woody plants, and four herbaceous plants were used as samples. The contamination of the RNA fractions by proteins and polysaccharides was very limited as judged spectrophotometrically. When the RNA fractions were subjected to agarose gel electrophoresis, intact rRNA bands were detected. The RNA fractions could be used for RT-PCR. These results indicate that our new method achieves a simple and rapid preparation of high-quality RNA from leaves of *Eucalyptus* and other plant species.

INTRODUCTION

Eucalyptus is one of the attractive resources for the production of biomass and industrial materials because it has good properties, such as a fast growth rate, the ability to adapt to various environments, and aptitudes as lumber, pulp, or firewood. Further improvements in their properties would be related to the breeding of transgenic *Eucalyptus* (1). To characterize such transformants, it is necessary to analyze changes in the patterns of transgene expression.

However, RNA extraction from leaves of *Eucalyptus* is troublesome in many cases, possibly due to the high

content of polyphenolics and polysaccharides. To avoid negative effects on RNA extraction by such compounds, one must use time-consuming and labor-intensive methods including LiCl precipitation or ultracentrifugation (2,3).

A solution for eliminating the contamination of the total RNA fraction by polyphenolics is to prevent their oxidation using a strong reductant [e.g., NaBH₄ (4)]. However, this reagent should be handled very carefully due to the high reactivity. Sodium isoascorbate is used as an antioxidant for foods such as meat and fish products, fruit, beer, and wine (5). It has also been used as a reagent in the isolation of chloroplasts to prevent the oxidation of molecules such as proteins and lipids (6), but its effect on the extraction of nucleic acids has been unknown. Here we report that the addition of sodium isoascorbate to the extraction buffer enables a rapid and simple extraction of RNA from the leaves of *Eucalyptus* and other woody and herbaceous plants.

MATERIALS AND METHODS

Plant Materials

Mature leaves were collected from the following plants: seedlings of *Eucalyptus camaldulensis* about two months after germination; mature trees of the Japanese apricot (*Prunus mume*); tea (*Thea sinensis*); big scentless mockorange (*Philadelphus grandiflorus*); southern magnolia (*Magnolia grandiflora*); and fragrant olive (*Osmanthus fragrans* var. *aurantiacus*); soybean (*Glycine max*); rice (*Oryza sativa*); buckwheat (*Fagopyrum esculentum*); and bitter melon (*Momordica charantia*) plants after anthesis.

Chemicals

Sodium isoascorbate and formaldehyde were obtained from Wako Pure Chemicals Industry Ltd. (Tokyo, Japan), isopropanol was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan), and SeaKem® GTG® agarose was obtained from CAMBREX (Rockland, ME, USA). All the other reagents used were obtained from Nacalai Tesque (Kyoto, Japan).

RNA Extraction

Our new method consists of the use of an extraction buffer containing sodium isoascorbate, chloroform extraction, and acid guanidium-phenol-chloroform (AGPC) extraction adapted from the method of Chomczynski and Sacchi (7). Thus, we designated our method the sodium isoascorbate-acid (SIA) guanidium extraction. Frozen leaves (>0.2 g) were ground to a fine powder in liquid nitrogen and immediately suspended with 5 volumes of extraction buffer [500 mM sodium isoascorbate, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 5% (v/v) 2-mercaptoethanol, and 2% (w/v) SDS]. After transfer to a disposable 1.5-mL tube, the suspension was extracted with an equal volume of chloroform-isoamyl alcohol (24:1). When *Eucalyptus* was used as a sample, one chloroform-isoamyl alcohol extraction had a sufficient effect. When other plants were used, the chloroform-isoamyl alcohol extraction was repeated once to minimize the contamination of RNA by other compounds. The resulting upper phase was transferred to a new 1.5-mL tube, and DNA and proteins were removed from the RNA by the AGPC treatment (7), with the following slight modifications. The upper phase was mixed with a half volume of 66.7% (w/v) guanidium thiocyanate, an equal volume of water-saturated phenol, and a 15% volume of 3 M sodium acetate, pH 5.2, and incubated for 3 min at room temperature. After the addition of chloroform-isoamyl alcohol (1/5 volume of the added phenol), the sample was shaken vigorously by hand, incubated on ice for 15 min, and centrifuged. We routinely centrifuged at 20 000× g for 15 min at 4°C, but the speed could be reduced to 12 000× g with a standard tabletop centrifuge. The resulting upper phase was transferred to a new 1.5-mL tube. Polysaccharides were removed from the RNA using a previously published method (8), with the following slight modifications. A 1/3 volume of 1.2 M NaCl-0.8 M sodium citrate and a 2/3 volume of isopropanol were added to the upper phase. The mixture was incubated for 10 min at room temperature. The RNA in the mixture was precipitated by centrifugation, washed with 75% (v/v) ethanol twice, air-dried, and dissolved in Tris-EDTA

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Table 1. Yield and Quality of RNA Extracted from *E. camaldulensis* Leaves Using Different Conditions

Method	Concentration of Sodium Isoascorbate	Relative RNA Yield (%)	A ₂₆₀ /A ₂₈₀	A ₂₆₀ /A ₂₃₀
CTAB	—	100 ^a	2.07 ± 0.02	2.06 ± 0.12
SIA	0 mM	11 ± 4	1.23 ± 0.07	0.60 ± 0.08
	100 mM	17 ± 7	1.29 ± 0.14	0.81 ± 0.24
	250 mM	44 ± 14	1.75 ± 0.17	1.45 ± 0.42
	500 mM	143 ± 8 ^b	2.03 ± 0.02	2.37 ± 0.04
	saturated ^c	137 ± 6	2.02 ± 0.02	2.40 ± 0.05

Data are means ± SD (*n* = 3–5).
^{a,b}The absolute values of the yields were 879 ± 88 and 1260 ± 70 µg/g fresh weight, respectively.
^cSodium isoascorbate becomes saturated in water at a concentration of 740 mM (15).

buffer or diethyl pyrocarbonate-treated water. The concentration of the extracted RNA was determined by the absorbance at 260 nm (A₂₆₀). The ratios of A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ were used as the indexes of contamination by proteins and polysaccharides, respectively. When leaves of *Eucalyptus* were used as samples, extraction buffers with different concentrations of sodium isoascorbate

(0, 100, and 250 mM and the saturated concentration) were also used.

RNA was also extracted with the cetyltrimethylammonium bromide (CTAB) method (2) with slight modifications. Polyvinylpyrrolidone and spermidine were removed from the extraction buffer, and 2-mercaptoethanol was added to the buffer at a concentration of 5% (v/v).

Gel Electrophoresis of Total RNA and RT-PCR

A portion of the total RNA (5 µg) was subjected to formaldehyde-agarose gel electrophoresis according to the method of Suzuki et al. (9). RT-PCR was performed with the extracted RNA (1 µg) and degenerated primers for a coding region of NADP-ICDH according to the method of Koyama et al. (10). The size of the amplified product was expected to be about 1.1 kb. An aliquot (10 µL) of the RT-PCR products was separated in a 1% agarose gel and stained with ethidium bromide.

RESULTS AND DISCUSSION

In preliminary experiments, RNA was extracted from *Eucalyptus* leaves with a series of conventional methods. When the AGPC method (7) and the method of Shirzadegan et al. (11) were used, the pellet obtained was unusually big, translucent, and hard to dissolve in water. Therefore, the pellet was judged to contain considerable contaminants to the RNA. Because the latter method includes the steps for the removal of poly-

saccharides, it has been suggested that the difficulty of RNA extraction from *Eucalyptus* leaves is not only attributed to the polysaccharides. On the other hand, a total RNA fraction with very limited contamination as judged from the ratios at A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ (Table 1) was obtained with the CTAB method, which is suitable for RNA extraction from plant tissues with high contents of polyphenolics and polysaccharides (12) and was previously applied to *Eucalyptus* (2). These results imply that the critical problem for RNA extraction from *Eucalyptus* leaves is the high content of polyphenolics rather than that of polysaccharides.

Therefore, we examined whether the use of sodium isoascorbate was effective for the RNA extraction because of its action as an antioxidant to prevent the oxidation of polyphenolics. Buffers containing sodium isoascorbate at different concentrations were used for the RNA extraction in our new method, the SIA method. Without the addition of sodium isoascorbate, the RNA yield was only 11% of that obtained with the CTAB method (Table 1). Such a reduction in the yield was probably due to a negative effect of the polyphenolics, as it has been pointed out that polyphenolics bind to the nucleic acids during their extraction (13) and possibly degrade them (14). Although there was no change in the RNA yield at a concentration of 100 mM sodium isoascorbate, improvements were found with increases in the concentration greater than 250 mM. At a concentration of 500 mM, the yield became maximal (the absolute value of 1260 ± 70 µg/g

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fresh weight) and was 1.4-fold higher than that obtained with the CTAB method. When the concentration of sodium isoascorbate was saturated, the RNA yield was almost the same as that obtained in the presence of 500 mM. These results suggest that the negative effects of polyphenolics on the RNA yield are eliminated by the addition of sodium isoascorbate to the extraction buffer at a concentration greater than 250 mM.

The purity of these total RNA fractions was judged by the ratios of A_{260}/A_{280} and A_{260}/A_{230} . There was severe contamination by proteins and polysaccharides at the concentrations of 0 and 100 mM. The purity of the RNA was improved with increased concentration greater than 250 mM sodium isoascorbate. At a concentration of 500 mM and the saturated concentration, the ratios of A_{260}/A_{280} and A_{260}/A_{230} were about 2.0 and 2.4, respectively. These results show that the use of an extraction buffer containing sodium isoascorbate at a concentration of 500 mM or more yields high-quality RNA that is comparable to that obtained with the CTAB method.

The major advantage of SIA method is the reduction in the number of steps. In particular, the LiCl precipitation, which is necessary for the CTAB

Table 2. Yields and Qualities of RNA Extracted with the SIA Method from Five Woody Plants and Four Herbaceous Plants

Plant Species	RNA Yield ($\mu\text{g/g}$ fresh weight)	A_{260}/A_{280}	A_{260}/A_{230}
<i>P. mume</i>	601 $\pm$ 100	1.94 $\pm$ 0.06	2.18 $\pm$ 0.17
<i>T. sinensis</i>	1010 $\pm$ 200	1.99 $\pm$ 0.03	2.45 $\pm$ 0.07
<i>M. grandiflora</i>	246 $\pm$ 106	2.00 $\pm$ 0.02	2.39 $\pm$ 0.01
<i>O. fragrans</i> var. aurantiacus	585 $\pm$ 137	1.95 $\pm$ 0.06	2.30 $\pm$ 0.19
<i>P. grandiflorus</i>	869 $\pm$ 130	1.98 $\pm$ 0.05	2.39 $\pm$ 0.09
<i>G. max</i>	1750 $\pm$ 290	2.00 $\pm$ 0.09	2.29 $\pm$ 0.16
<i>O. sativa</i>	645 $\pm$ 159	2.03 $\pm$ 0.05	2.19 $\pm$ 0.21
<i>F. esculentum</i>	1200 $\pm$ 300	1.95 $\pm$ 0.05	2.07 $\pm$ 0.17
<i>M. charantia</i>	667 $\pm$ 124	1.99 $\pm$ 0.03	2.36 $\pm$ 0.12

Data are means $\pm$ SD ($n = 2-5$).

method and requires a long time (from several hours to overnight) (2,11), is unnecessary. Thus, the time required for RNA extraction became much less than that for the CTAB method. At the same time, the fewer steps in the RNA extraction probably led to the increase in the RNA yield by reducing its loss during the extraction. Thus, the SIA method enables the simple and rapid extraction of high-quality RNA from *Eucalyptus* leaves.

We also tried the SIA method for RNA extraction from the leaves of five woody plants and four herbaceous plants in the presence of 500 mM sodi-

um isoascorbate (Table 2). The RNA yields were 246–1750 $\mu\text{g/g}$ fresh weight. The ratios of A_{260}/A_{280} and A_{260}/A_{230} were 1.94–2.03 and 2.07–2.45, respectively, which indicated little contamination of these RNA fractions by protein and polysaccharides. These results show that the SIA method can be applied to other woody and herbaceous plant species.

The integrity of the RNA was examined by electrophoresis using a formaldehyde agarose gel (Figure 1). There was no difference between the properties of the RNA extracted from *Eucalyptus* with the SIA method and

Figure 1. Electrophoresis of RNA extracted from the leaves of *E. camaldulensis*, five woody plants, and four herbaceous plants with the SIA method in the presence of sodium isoascorbate at a concentration of 500 mM. When *Eucalyptus* was used as a sample, RNA was also extracted with the CTAB method as a control.

Figure 2. Products of RT-PCR amplification of RNA extracted from leaves of *E. camaldulensis*, five woody plants, and four herbaceous plants with degenerated primers for a coding region of NADP-ICDH.

that extracted with the CTAB method, and intact rRNA bands were detected in all the RNA fractions from different plants. This result indicates that the RNA extracted with the SIA method can be directly applied for various analyses (e.g., Northern blot analysis, RT-PCR, etc.). To examine this possibility, we performed RT-PCR using degenerated primers derived from the coding region of NADP-ICDH. According to our previous work, the expected size of the amplified band is approximately 1.1 kb (10). As expected, an amplified band at approximately 1.1 kb was observed in all the plant species (Figure 2). We could thus infer that the RNA extracted with the SIA method can be used for further enzymatic reactions.

Overall, the SIA method achieves the rapid and simple extraction of RNA from leaves of woody and herbaceous plants by using sodium isoascorbate as an additive to the extraction buffer. This method would be very useful for the development in the molecular biology of these plants.

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