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Isolation of High-Quality RNA from High-Phenolic Tissues of Eelgrass (*Zostera marina* L.) by Keeping Temperature Low

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Abstract. We describe a modified LiCl method for isolating good-quality RNA from leaves, rhizomes and roots of eelgrass (*Zostera marina* L.) which are rich in phenolic compounds. In ordinary protocols, RNA extraction was strongly inhibited by contamination with the large amounts of polyphenol compounds. Only by keeping the temperature low during extraction could RNA be successfully isolated. The resulting high-quality RNA was suitable for reverse transcription–polymerase chain reaction, reverse transcription followed by quantitative real-time PCR, cDNA library construction and Northern blot analysis. The total RNA extracted from leaves was much higher than that from rhizomes and roots. Using this method, 20-40 µg of total RNA was routinely obtained from 1 g of fresh tissue. This is the first report of low temperature RNA extraction from plant tissues rich in phenolic compounds.

Key words: eelgrass (*Zostera marina* L.), low temperature extraction, marine plant, phenolic compounds, high-quality RNA

Abbreviations: CI, chloroform-isoamyl alcohol; DEPC, diethyl pyrocarbonate; NM, new method; PCI, phenol-chloroform-isoamyl alcohol; RT-PCR, reverse transcription–polymerase chain reaction; RT-qPCR, reverse transcription followed by quantitative real-time PCR; SM, standard method.

Introduction

Several protocols for RNA extraction have been successfully applied to various plant species (Chirgwin et al., 1979; Logemann et al., 1987; Hughes and Galau, 1988). However, in some cases, the quality and quantity of RNA isolated by these methods are too low for further application because of contamination of polyphenols and other secondary metabolites. Although several reports have described different methods to eliminate phenolic compounds using polyvinylpyrrolidone

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(PVP) (Woodhead et al., 1997; Salzman et al., 1999) or polyethylene glycol (PEG) (Gehrig et al., 2000), they were still not efficient for isolating high-quality RNA from some plants such as eelgrass (*Z. marina*) (Alemzadeh et al., 2005, in preparation).

Eelgrass is a monocotyledonous angiosperm that can thrive in seawater with a salinity of 0.5-3.3% (Fukuhara et al., 1996; Ye and Zhao, 2003). Therefore, this plant is a good candidate for studying the molecular mechanisms involved in salt resistance. Northern blot analysis and reverse transcription followed by quantitative real-time PCR (RT-qPCR) using RNA isolated from different organs would allow us to study the expression of eelgrass genes possibly involved in salt resistance. In addition, high-quality RNA would allow construction of a cDNA library from which individual salt resistance genes could be isolated. For this reason, we need a procedure that gives high-quality RNA from each organ. Here, we describe a simple and efficient protocol based on the LiCl method for isolating high-quality RNA from different eelgrass organs. The proposed method is likely to be useful with other plants rich in phenolic compounds.

Materials and Methods

Plant materials

Leaves, rhizomes and roots of *Z. marina* were collected at Yasuura in Hiroshima Prefecture, Japan. The plants were washed immediately with tap water, frozen in liquid nitrogen and stored at -80°C.

Solutions and reagents

- Liquid nitrogen
- Cold extraction buffer: 100 mM Tris-HCl (adjusted to pH 8 with 1 M HCl), 10 mM EDTA (adjusted to pH 8 with 1 M NaOH), 1% SDS, 100 mM LiCl.
- 2 M LiCl solution: 2 M LiCl, 50 mM EDTA, pH 8.
- 10 M LiCl
- 3 M sodium acetate (adjusted to pH 5.2 with CH_3COOH)
- 0.1 M sodium acetate (adjusted to pH 5.2 with CH_3COOH)
- Cold water-saturated phenol
- Cold chloroform-isoamyl alcohol (49:1 v/v)
- Cold phenol-chloroform-isoamyl alcohol (50:49:1 v/v/v)
- Cold 80% ethanol
- Cold absolute ethanol
- 0.1% diethyl pyrocarbonate (DEPC) (Nacalai Tesque, Kyoto, Japan)-treated-autoclaved water

Note

1. Glassware and mortar and pestles were baked overnight at 160°C; pipette tips were autoclaved; gel-running apparatus was treated with 70% ethanol and dried; all solutions were made with 0.1% DEPC-treated-autoclaved water.
2. Keep temperature lower than 4°C at all stages.

RNA extraction procedure

1. Add 10 ml cold extraction buffer to a new 50-ml polypropylene tube; then, add 10 ml cold phenol and keep at 4°C.
2. Grind leaves, rhizomes or roots with a mortar and pestle in liquid nitrogen as finely as possible. Avoid thawing the tissue to keep hydrolytic enzymes (ribonucleases) inactive.
3. Transfer 1 g of ground tissue to a tube containing cold extraction buffer and phenol at 4°C, invert immediately and shake vigorously for 15 min. At this stage, the suspension should appear milky white in color; if the temperature is higher than 4°C, the color will change to brown.
4. Centrifuge at 10,000 *g* for 30 min at 4°C.
5. Transfer the supernatant to a new 50-ml polypropylene tube and extract with an equal volume of phenol. Centrifuge at 10,000 *g* for 10 min at 4°C.
6. Transfer the supernatant to a new 50-ml polypropylene tube and re-extract with an equal volume of phenol-chloroform-isoamyl alcohol (PCI). Centrifuge as in step 5.
7. Transfer the supernatant to a new 50-ml polypropylene tube and re-extract with an equal volume of chloroform-isoamyl alcohol (CI). Centrifuge as in step 5.
8. Transfer the final supernatant to a new 15-ml polypropylene tube and add 0.3 volume of 10 M LiCl, mix well, and keep at 4°C overnight. After adding LiCl, the suspension color should immediately change to milky white.
9. Recover the nucleic acids by centrifugation at 8,000 *g* for 60 min at 4°C.
10. Remove the supernatant and add 1 ml of 2 M LiCl, vortex, and centrifuge at 8,000 *g* for 30 min at 4°C.
11. Repeat the last step twice more.
12. Add 400 µl of TE to the pellet, transfer the resulting solution to 1.5-ml microfuge tube and extract using phenol, PCI and CI as above.
13. Precipitate the nucleic acids with cold absolute ethanol, wash the pellet with 80% ethanol and dry for 10 min.
14. Add 400 µl 0.1 M sodium acetate and 40 µl cold absolute ethanol, mix well, and centrifuge at 10,000 *g* for 15 min at 4°C.
15. Transfer the supernatant to a new tube and precipitate RNA with cold absolute ethanol. Wash the pellet with 80% ethanol, dry pellet for 10 min, resuspend in 50 µl TE and store at -80°C.

RT-PCR

cDNAs were reverse transcribed from total RNA (1 µg) extracted from eelgrass leaves, rhizomes and roots using a ReverTra Ace kit (Toyobo, Osaka). The cDNAs were amplified by PCR with specific primers for *zha2*, a gene encoding a plasma membrane H^+-ATPase from eelgrass expressed in leaves, rhizomes and roots (Alemzadeh et al., 2005, in preparation). PCR reactions in a final volume of 25 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl 1.5 mM $MgCl_2$, 200 µM dNTP, 0.2 µM each primer (forward: 5′-GAG AAA GCT GAT GGA TTT GCT GG-3′; reverse: 5′-CCA GAA AAA GAT AAC TGT CAT AAT TGC C-3′), and 0.5 units of *ExTaq* DNA polymerase (Takara Shuzo, Kyoto) were

carried out under the following conditions: 4 min at 95°C, 30 cycles at 95°C for 30 s, 57°C for 1 min, and 72°C for 5 min, with a final extension for 7 min at 72°C.

RT-qPCR

Real-time PCR was performed using a Line Gene Fluorescence quantitative detection system (BioFlux, Tokyo) with cDNAs prepared from plant leaves, rhizomes and roots. A PCR mixture containing SYBR-green (SYBR premix *ExTaq*, Takara Shuzo, Kyoto) was used. PCR reactions in a final volume of 10 µl reaction mixture containing 5 µl PCR mixture, 1 µl diluted cDNA and 0.5 µM each primer (forward *zha2*: 5′-TAC ATT CTC AGT GGC AAA GC-3′; reverse *zha2*: 5′-CGT TCT TCC CTA CCA TAA TCC-3′; forward *actin*: 5′-AGG TTC TCT TCC AGC CTT C-3′; reverse *actin*: 5′-CCT TGC TCA TCC TAT CTG C-3′) were carried out under the following conditions: 1 min at 95°C and 45 cycles at 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s. At the end of the program, the specificity of the primer set was confirmed by melting curve analysis (65-95°C with a heating rate of 0.5°C/min). The copy numbers of *zha2* and *actin* mRNA were estimated by comparing the results of real-time PCR with several dilutions (10^1, 10^2, 10^3, 10^4, 10^5, 10^6 copies/µl) of *zha2*- and *actin*-containing plasmids as the template. The mRNA level of *actin* was used to normalize the expression ratio of each gene.

Northern blot hybridization

Total RNA isolated from leaves was separated by 1.4% agarose gel electrophoresis and transferred to a Biodyne A nylon membrane (Pall). A 522 bp fragment close to the 3′-end of *zha2* was labelled with a Gene Images labelling kit (Amersham Biosciences, New Jersey, USA) and used as a probe for hybridization. Hybridization was performed at 65°C for 18 h and then the hybridized membrane was washed with 0.1 x SSC containing 0.1% SDS at 65°C twice for 15 min. The signal was detected with a Gene Images Detection Kit (Amersham Biosciences) by exposing onto X-ray film (RX-U Fuji Film).

Construction of a cDNA library

Using total RNA-based cDNAs prepared from leaves (10 µg) a cDNA library was constructed with a SMART cDNA library construction kit (Clontech, California, USA). The library contains 5.5×10^5 independent clones.

Results and Discussion

Standard methods (SM) for RNA extraction typically can not be used for some plant tissues rich in phenolic compounds because polyphenolic compounds are readily oxidized to quinines (Loomis, 1974; McMurry, 1992) and bind to nucleic acids (Salzma et al., 1999). The general approach in isolating nucleic acids from these plants is thus to prevent the formation of these complexes and remove phenolic compounds as quickly as possible. Oxidization of polyphenolic compounds and the subsequent binding to nucleic acids involves chemical reactions. Because lowering the temperature is thought to decrease the rate of chemical reactions

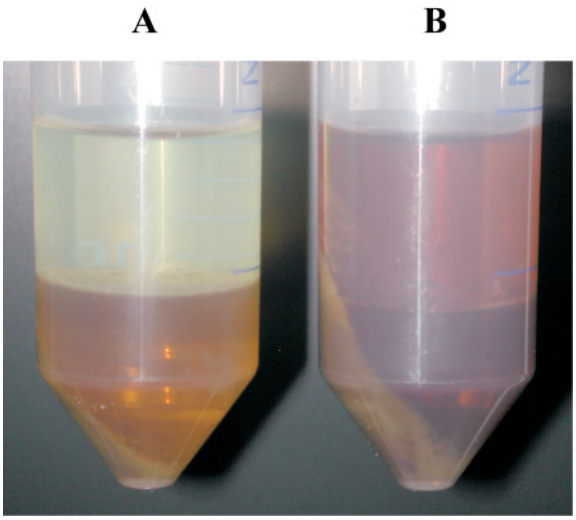

Figure 1. Removal of phenolic compounds from the aquatic phase using the new method (NM). When the temperature was kept below 4°C during RNA extraction, phenolic compounds did not react with nucleic acids and were precipitated in the first centrifugation step (A). In contrast, when RNA was extracted using a standard method (SM), phenolic compounds remained in the aquatic phase with nucleic acids (B).

irrespective of whether the reaction is exothermic or endothermic (Zumdahl, 1997), we attempted to keep temperature low during RNA extraction from eelgrass. When the temperature was kept lower than 4°C (new method, NM), the phenolic compounds did not react with nucleic acids and were precipitated with other debris after the first centrifugation. As a result, the supernatant appeared completely clear (Figure 1). In contrast, samples extracted by SM appeared brown, indicating that the phenolic compounds reacted with the nucleic acids and remained in the supernatant (Figure 1). The quality of RNA was assessed by electrophoresis on 1% non-denaturing agarose gel. With samples extracted by NM, bands corresponding to 18S and 28S rRNA were distinctly visible in all lanes, indicating high quality and non-degraded RNA (Figure 2). However, the band corresponding to 28S rRNA was significantly weaker than that of 18S rRNA in RNA extracted from roots by SM, indicating partly degraded RNA in this preparation (Figure 2). The A260/A280 ratios of RNA isolated by NM were 1.7 to 2 with a peak at 260 nm, indicating low amounts of contaminating proteins, polysaccharides and polyphenol compounds. In the case of SM, the yield and quality of RNA extracted from various organs, particularly the rhizome, were consistently less than those extracted at low temperature (Figure 2). The A260/A280 ratios of RNA isolated using SM were 1.0 to 1.2.

RT-PCR is an amplification procedure sensitive to the presence of inhibitors in the extract or to degradation of RNA. Therefore, reverse transcription followed by standard PCR or quantitative real-time PCR can be used to test RNA quality. After extraction of RNA from leaves, rhizomes and roots by NM and SM, cDNA was prepared from them and used as a template in standard PCR with specific primers (described in Materials and Methods) to amplify *zha2*, a plasma membrane

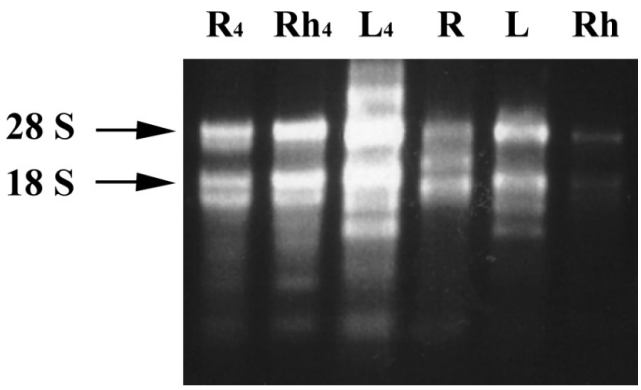

Figure 2. Total RNA isolated from leaves (L_4), rhizomes (Rh_4) and roots (R_4) using the new method (NM) or from leaves (L), rhizomes (Rh) and roots (R) using the standard method (SM) were separated by 1% non-denaturing agarose gel electrophoresis and stained with ethidium bromide. In all NW lanes, the bands corresponding to 18S and 28S rRNA were more distinctly visible than in the SM lanes, indicating that low temperature during RNA extraction can be effective in isolating high-quality non-degraded RNA from phenolic tissues of eelgrass.

H^+-ATPase gene. *zha2* cDNA was successfully amplified from all RNA preparations (from leaves, rhizomes and roots) by NM, but in the case of samples prepared by SM there was no band amplified in the rhizome lane and bands that appeared in leaf- and root- lanes were considerably weaker than those prepared with NM (Figure 3). In addition, when cDNA of different organs was used as templates for real-time PCR to quantify *zha2* and *actin* expression levels, the sample prepared by NM always gave reproducible levels of gene expression, while there were no detectable PCR products from rhizome-RNA extracted by SM (Figure 4).

To study the mechanisms of salt resistance in eelgrass, high-quality RNA is necessary for construction of a full length-enriched cDNA library. Using total RNA-based cDNA from eelgrass leaves, we were able to construct a cDNA library with 550,000 independent clones. The cDNA library was rich in full-length cDNA and we were able to isolate a full-length transcript (1464 bp encoding 488 aa) of *Zmvha-b1*, a vacuolar H^+-ATPase (subunit B) gene, through a cDNA library screening approach.

When total RNA extracted from leaves by NM was subjected to Northern blot hybridization with a 522-bp fragment of *zha2* as a probe, an approximately 3.0 kbp distinct and strong band with no smear appeared, indicating intact and non-degraded RNA (Alemzadeh et al., in preparation).

In this proposed procedure, unlike in pre-existing protocols for phenolic plants (Woodhead et al., 1997; Salzman et al., 1999; Gehrig et al., 2000), no special chemicals such as PVP or PEG are necessary. This protocol is very simple and inexpensive to perform, allowing highly efficient extraction of high-quality RNA from eelgrass, a plant rich in polyphenols. Keeping the temperature lower than 4°C can also be considered a critical point for extracting intact RNA from other plants containing high levels of phenolic compounds.

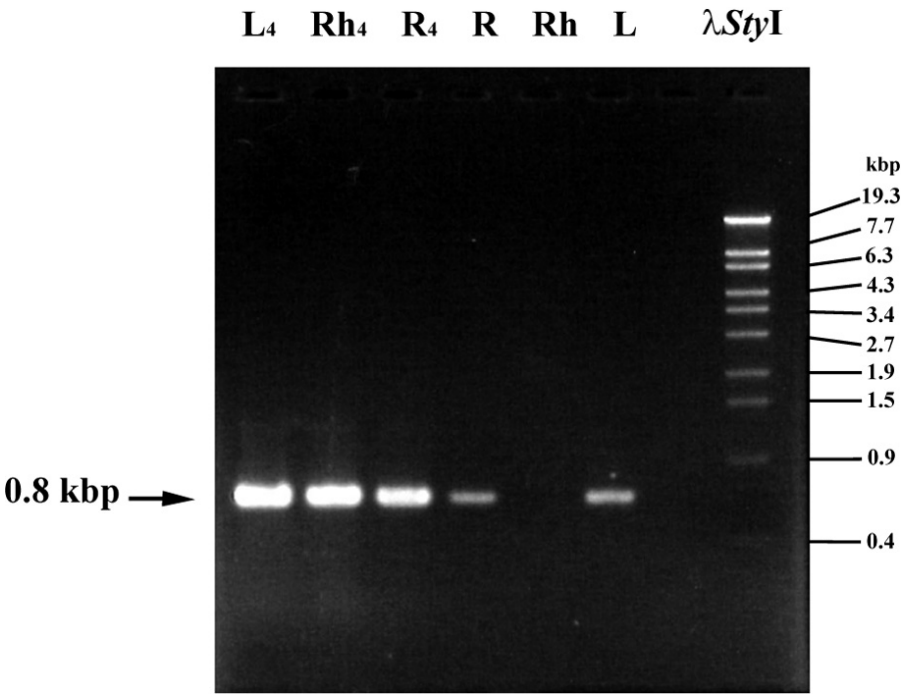

Figure 3. Agarose gel electrophoretic analysis of RT-PCR–amplified cDNA of a plasma membrane H^+-ATPase gene (*zha2*). Using the new method (NM), *zha2* was always amplified well from leaves (L_4), rhizomes (Rh_4) and roots (R_4), but using the standard method (SM), *zha2* was not amplified from the rhizomes (Rh), and was amplified very weakly from the leaves (L) and roots (R).

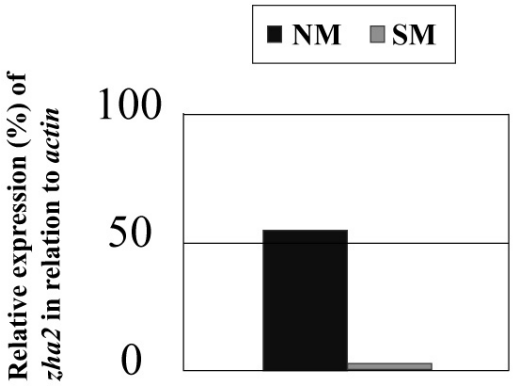

Figure 4. Quantitative real-time PCR analysis. cDNAs prepared from rhizome-RNA extracted using the new method (NM; Rh_4) and standard method (SM; Rh) was used as templates for real-time PCR. The relative expression level of *zha2* (normalized by the level of actin, a housekeeping gene) is shown. There was no expression in RNA extracted by SM.

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