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High-quality RNA preparation for cDNA library construction of the Antarctic sea–ice alga *Chlamydomonas* sp. ICE-L

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Abstract To study the molecular mechanism of the Antarctic sea–ice alga in adaptation to polar sea–ice environments, the RNA was prepared for cDNA library construction of *Chlamydomonas* sp. ICE-L. Three different methods were tested to prepare total RNA from this psychrophilic, unicellular green alga rich in protein and polysaccharide. Lauryl sodium sulfate- based method allowed a most effective extraction of high-quality total RNA compared to the other methods. Total RNA extracted with this protocol was used for cDNA library construction. The recombination rate of constructed cDNA library was 98.60%, the primary titer was 7.15×10^6pfu, and an average sequence length was 1.2 kb. These results show that with a high-quality RNA preparation, a cDNA library can be constructed successfully for *Chlamydomonas* sp. ICE-L.

Keywords Sea–ice alga · *Chlamydomonas* sp. ICE-L · RNA extraction · Lauryl sodium sulfate (SDS) · cDNA library construction

Introduction

Antarctica constitutes a unique and extreme environment on earth. On this extraordinary continent, floating sea–ice with a temperature ranges from 0 to −35°C and is supposed to be one of the coldest habitats on earth for marine-living creatures (Mock and Thomas 2005). The survival of microalgae in floating sea–ice matrix requires a complex suite of physiological and metabolic mechanisms for acclimatization in this extreme living space.

A unicellular green alga *Chlamydomonas* sp. ICE-L was isolated from Antarctic floating ice and kept in clonal culture in our laboratory (Liu et al. 2006). It is a psychrophilic alga and demonstrates some physiological properties such as freezing and hypersaline adaptations that correlate to Antarctic ice habitats (Kan et al. 2006). In addition, ice green algae are less sensitive or, in other words, more resistant to UV-B radiation compared to mesophilic green algae (Miao et al. 2003) and have been shown to survive in a condition of four times seawater salinity (Kan et al. 2006), while the temperate algae *Thalassiasira* sp. could only maintain negative growth merely at a salinity below two times seawater (Zhu et al. 2003). All these extremophile features indicate intrinsic genetic mechanisms of ice algae and suggest the need for molecular genetic research.

Construction of cDNA library and analysis of expressed sequence tags (ESTs) are powerful tools to reveal the acclimatization mechanism of this sea–ice alga under stress such as freezing, hypersalinity, and UV irradiance. In recent years, several EST studies have been conducted on polar diatoms (Mock et al. 2005; Jung et al. 2007); however, there have been few studies on polar sea–ice green microalgae. Janech et al. (2006) identified an ice-binding protein gene by EST analysis of the sea–ice diatom *Fragilariopsis cylindrus* which plays an important role in its adaptation to the freezing environment. Microarray studies based on ESTs from the sea–ice diatom *Chaetoceros neogracile* have shown that the expression patterns of genes related to photosynthesis were

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different in response to thermal stress (Gyeongseo et al. 2007; Hwang et al. 2008). As for the other important inhabitants in Antarctic floating ice, to our knowledge, the molecular mechanisms of acclimation of green algae has not been thoroughly studied.

To conduct a molecular investigation, the integrity and purity of RNA is a critical determinant for cDNA library construction. There have been many RNA extraction studies on seaweeds, especially on red algae which are rich in polysaccharide (Vanessa et al. 2008; Yao et al. 2009). The frequently used methods in RNA extraction for plant and seaweeds focus on using polysaccharide and phenolic eliminating reagents such as cetyltrimethylammonium bromide (CTAB), polyvinylpyrrolidone (PVP) and lithium chloride (LiCl; Gasic et al. 2004; Zeng and Yang 2002), as well as lauryl sodium sulfate (SDS; Kim et al. 1997). A modified phenol-guanidine method was used for the isolation of high-quality RNA from cyanobacteria (Pinto et al. 2009). Trizol reagent (Invitrogen, USA), is a monophasic solution of phenol and guanidinium isothiocyanate based on the methodology developed by Chomczynski and Sacchi (1987) and has been successfully used for RNA isolation from many eukaryotes and prokaryotes such as bacteria, yeast, and animals. In addition to polysaccharide and phenolic substances, high levels of proteins and secondary metabolites are also the important components of unicellular green algae. There are only few reports on the isolation of RNA from green microalgae such as *Ankistrodesmus convolutes* where Thanh et al. (2009) used an improved CTAB-based method and acquired high-quality RNA. Because of the complex composition of unicellular microalgae, protocols for isolating RNA from them need further exploration.

Chlamydomonas sp. ICE-L has a tough cell wall and the cell protein content is more than 45% (Miao et al. 2002). Many intracellular starch granules can be distinguished by transmission electron microscopy (Liu et al. 2006). The yield and quality of RNA from it can be significantly reduced by contaminants such as polysaccharides and proteins. In order to determine the best methods for RNA extraction and subsequent cDNA library construction, CTAB- and SDS-based methods were chosen and modified and compared with Trizol, the most commonly used commercial RNA extraction reagent. In this study, a modified RNA preparation was developed and used for the construction of high-quality cDNA library for EST analysis of the sea–ice microalga *Chlamydomonas* sp. ICE-L

Materials and methods

Chlamydomonas sp. ICE-L, provided by the Polar Research Institute of Shanghai, was collected from the floating ice near the Zhongshan Research Station of Antarctica (69° S, 77° E). Fresh alga cells were cultivated in Provasoli seawater medium (Provasolil 1968) in a 16:8 light/dark cycle at 6±1°C. The alga samples were collected by centrifugation (4°C, 5 min, 3,000×*g*), immediately frozen in liquid nitrogen, and stored at −80°C before use.

Total RNA extraction

Plastic hardware was RNase-free materials. The glassware was treated with 0.1% diethypyrocarbonate (DEPC) water and autoclaved. The mortar and pestle were treated for 5 h at 200°C. Frozen samples were ground into powder in liquid nitrogen.

Method 1: Extraction of total RNA by modified CTAB-based method (Zeng and Yang 2002)

Frozen alga powders were transferred into a 1.5-mL tube containing 600 μL of CTAB extraction buffer [2% (*w*/*v*) CTAB (Sigma), 2% (*w*/*v*) PVP (Sigma), 100 mM Tris–HCl (pH8.0), 25 mM EDTA, 2.0 M NaCl], and 10% β-mercaptoethanol was added just before use, mixed thoroughly, and incubated at 65°C for 10 min with occasional shaking. Thereafter, an equal volume of chloroform/isoamyl (24:1, *v*/*v*) was added, mixed thoroughly, and centrifuged at 10,000×*g* for 10 min at 4°C. The upper aqueous phase was transferred into a fresh tube and another equal volume of chloroform/isoamyl (24:1, *v*/*v*) was added. The upper aqueous phase was transferred into a fresh tube and 1/3 volume of 8.0 M LiCl was added. The mixture was well mixed and stored at −80°C for 1 h. After centrifuging at 10,000×*g* for 30 min at 4°C, the pellet was collected, washed by 75% (*v*/*v*) ethanol and air-dried, then dissolved in 50 μL of DEPC-treated water and stored at −80°C.

Method 2: Extraction of total RNA by Trizol reagent RNA isolation kit (Invitrogen)

RNA was extracted following the manufacturer's protocol, and an additional isolation step was added according to the recommendation: for samples with high content of proteins and polysaccharides, following homogenization, insoluble materials were removed from the homogenate by centrifugation at 10,000×*g* for 10 min at 2°C to 8°C.

Method 3: Extraction of total RNA by SDS-based method (Zhang et al. 2004)

Frozen alga powders were transferred into a 1.5-mL tube containing 600 μL SDS extraction buffer [50 mM EDTA, 2% (*w*/*v*) PVP, 2% SDS (*w*/*v*), 100 mM Tris–HCl (pH8.0), 1.4 M NaCl], and 10% β-mercaptoethanol was added just

before use. The mixture was incubated at 65°C for 10 min with occasional shaking. An equal volume of chloroform/isoamyl (24:1, *v*/*v*) was added, mixed thoroughly, and centrifuged at 10,000×*g* for 10 min at 4°C. The aqueous phase was transferred into a new tube and extracted with chloroform–isoamyl (24:1, *v*/*v*) again. The upper aqueous phase was transferred into a fresh tube and 1/3 volume of 8.0 M LiCl was added. The mixture was well mixed and kept at −20°C for 3 h. After centrifugation at 10,000×*g* for 20 min at 4°C, the RNA pellet was washed twice with 70% ethanol, dried by air, and dissolved with DEPC-treated water.

The purity of prepared RNA was detected by the absorption at 260 nm and the ratio of absorption at 260:280 nm (A_{260}/A_{280}). Formaldehyde agarose gel (1.2%) electrophoresis of extracted RNA and their 1/10 and 1/100 concentration dilutions were performed as well in a volume of 5 μL, respectively.

Isolation of mRNA and construction of cDNA library

After RNA quantification, 500 μg of extracted RNA prepared by SDS-based method was used for mRNA isolation by using Oligotex Mini Kit (Qiagen, Germany) following the kit's user manual recommendation. The ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, USA) was used to construct cDNA library of *Chlamydomonas* sp. ICE-L.

cDNA library evaluation, amplification, and sequencing

After construction of cDNA library of *Chlamydomonas* sp. ICE-L, determination of titer and recombination efficiency of the primary cDNA library was performed following the manufacturer's protocol and using following formula:

$$\begin{aligned}\text{Titer}\left(\text{pfu mL}^{-1}\right) = [&\text{Number of plaques (pfu)}\\ &\times\text{dilution factor}/\ \text{Volume plated}\ (\mu\text{L})]\\ &\times 1,000\ \mu\text{L mL}^{-1}\end{aligned}$$

$$\text{Recombination efficiency}\ (\%) = (\text{white plaques}/\ \text{total plaques}) \times 100.$$

Primary cDNA library was amplified and converted into plasmid form by mass excision according to the manual described. To determine the average cDNA insert size and the cDNA length distribution profiles, 96 individual cDNA clones were randomly picked up from mass excision plate and incubated in 1.5 mL of Luria–Bertani with 100 μg mL^{-1} ampicillin at 37°C with 200 rpm shaking overnight. One microliter of bacterial suspensions was used as template directly for PCR reaction. The program for PCR was: 5 min denaturation at 94°C, followed by 32 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and finally extension at 72°C for 10 min. Afterwards, 10 μL of each product was separated by electrophoresis on a 1.2% agarose gel containing 1% gelview (Bioteke, China) and visualized by the BIO-RAD GelDoc 2000 (Bio-Rad, USA). For EST analysis, a total of 2,000 samples were sequenced by commercial DNA sequencing service provider (Genscript Corporation, China).

Results and discussion

The cDNA library construction of organism with high quantity of polysaccharides and protein was frequently troubled by the low yield of RNA extraction. To derive EST libraries from Grapevine, Iandolino et al. (2004) tried several standard protocols and commercially available kits to obtain high-quality RNA. They finally chose the CTAB-based method and derived a high-quality EST library with the average insert length of 1.1 kb. Lichen is also a plant rich in polysaccharides. Junttila et al. (2009) tried several methods to isolate RNA from the Reindeer lichen, *Cladonia rangiferina*, for cDNA library construction. They used the CTAB-based method plus a Rneasy Midi kit (Qiagen) and finally acquired a lichen cDNA library with average sequences between 200 and 800 bp. Their studies suggested that the construction of a cDNA library with high quality is possible from the polysaccharide-rich materials if a proper RNA extraction method is chosen.

During experiment, the low A_{260}/A_{280} ratio (1.38) of the RNA prepared by TRIzol reagent suggested the remainder of polysaccharides and/or protein in the sample, while the A_{260}/A_{280} ratio of RNA isolated by methods 1 and 3 was 2.01 and 1.98, respectively. Afterwards, it was further checked by denature agarose gel electrophoresis, and the RNA isolated by three methods are shown in Fig. 1. The RNA isolated by methods 1 and 3 had sharp 18- and 28-s strips on the electrophoresis gel, indicating its high yield. The result also revealed that little degradation of RNA occurred and that contamination from protein and polysaccharide had been eliminated. The RNA isolated by method 2 had a dull strip, indicating a low yield and contamination of polysaccharides which co-precipitated with RNA and obstructed the loading wells.

The CTAB-based method has been successfully used to extract RNA from the green microalga *Ankistrodesmus convolutus* (Thanh et al. 2009), and the SDS-based method has been shown to be effective in the isolation of RNA from *Chlamydomonas reinhardtii* (Zhang et al. 2004). In the present study, these two methods were trialed and modified for RNA isolation from the sea–ice green microalgae

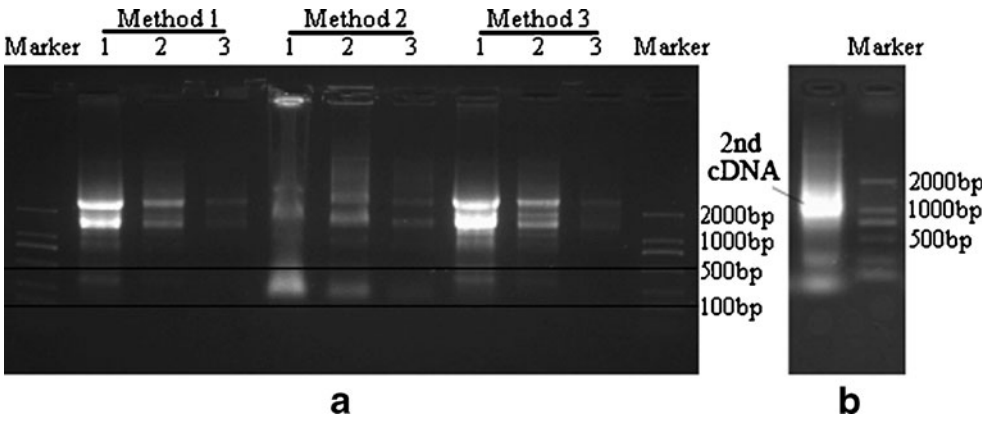

Fig. 1 RNA integrity checking. **a** Electrophoresis profile of *Chlamydomonas* sp. ICE-L RNA isolated by three different extraction methods. Total RNA preparations were analyzed on a 1.5% formaldehyde denature agarose gel with 0.1‰ gelview (Bioteke) at a voltage of 200 V for 10 min. Each three lines of three methods are: *1* stock solution, *2* ten-fold dilution, and *3* 100-fold dilution, respectively, from the left side to the right side. **b** Electrophoresis profile of Reversed cDNA, 1.2% agarose gel electrophoresis analysis

Chlamydomonas sp. ICE-L. Compared with Zeng's (Zeng and Yang 2002) CTAB-based method, RNA precipitation was modified from 4°C overnight to −80°C for 1 h. The results demonstrated that the yield of RNA was almost the same while the extraction time was sharply reduced. Also, spermidine trihydrochloride was omitted in our CTAB-based protocol, and this did not affect the efficiency of extraction. In the SDS-based method, PVP was added to Zhang's (Zhang et al. 2004) RNA extraction buffer which has been used in RNA isolation from woody plant conifer tissues to bind and remove phenolic compounds (Dong and Dunstan 1996). There are also some reports introducing polyvinylpolypyrrolidone (PPVP) to the RNA extraction lysis buffer to bind phenolic compounds (Geuna et al. 1998), but the smaller size and solubility characteristics of PVP suggested that it may be superior to PPVP with respect to binding phenolic compounds (Bekesiova et al. 1999). In our experiments, polysaccharides were extracted from the aqueous phase by chloroform/isoamyl (24:1, *v*/*v*) in lysis solution under high Na^+ density. RNA was especially precipitated by LiCl and avoided the contamination of polysaccharides and proteins (Su and Gibor 1988). The two extractions with chloroform/isoamyl (24:1, *v*/*v*) were also essential for the efficient elimination of protein. The use of SDS could denature proteins into insoluble forms and inhibit harmful enzyme activities. The purification procedures were simplified by omitting ethanol precipitation before using LiCl (Zhang et al. 2004). In this way, both the yield and efficiency were improved. Due to its high efficiency and the high quality of RNA obtained, the SDS method was finally chosen and proven to be efficient in RNA extraction and successful for cDNA library construction from *Chlamydomonas* sp. ICE-L in the present study.

The ZAP-cDNA Gigapack III Gold Cloning Kit from Stratagene was chosen to construct the cDNA library. The use of this cDNA construction kit requires a great quantity of high-quality mRNA as templates. In order to meet the requirements, three methods were compared and the SDS-based method was finally chosen. The Oligotex mRNA Kits (Qiagen) can avoid degradation of the RNA effectively

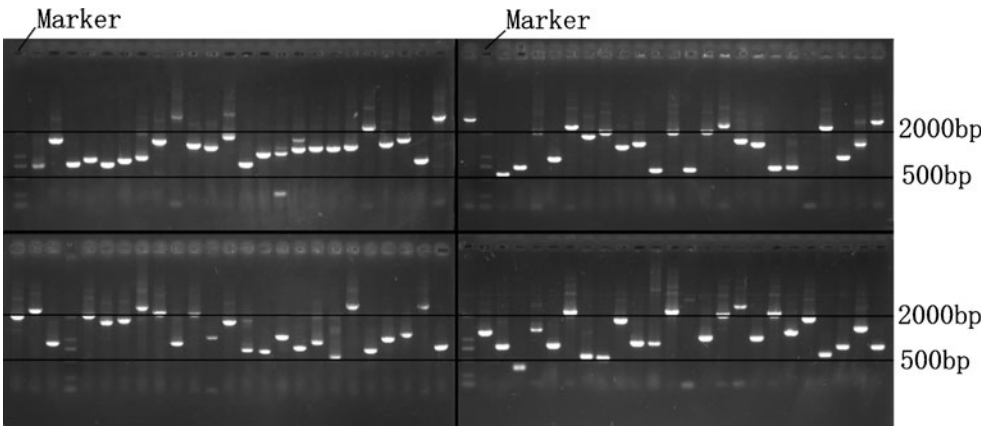

Fig. 2 Inserted sequence length of cDNA library detection. Inserted cDNA sizes of the 96 samples were detected after PCR amplification. The electrophoresis was conducted on 1.2% agarose gel

due to its easy manipulation and short handling time and was used in the isolation of mRNA from total RNA in this study. The second-strand cDNA electrophoresis result indicated that the integrity and quantity of extracted mRNA was satisfactory (Fig. 1b).

The cDNA library was constructed and recombination efficiency and titer were determined. The results demonstrated that there were 71.5 plaques on one plate on average after spreading 1 μL of 100-fold diluted primary library on plate to take count of titer. This indicated that the titer of the cDNA library was 7.15×10^{6}pfu mL^{-1}. The recombination efficiency was 4/282 (98.60%). The average size of inserted cDNA sequence was determined (Fig. 2). It demonstrated that most of the size ranged from 0.5 to 2 kb, and an average length was about 1.2 kb. There were few sequences shorter than 0.5 kb. All these characters showed that a typical cDNA library with high quality was constructed.

In conclusion, the SDS based one-step method can consistently yield large amounts of high-quality total RNA from the psychrotrophic unicellular green alga *Chlamydomonas* sp. ICE-L. Total RNA extracted by this method has been successfully used for cDNA library construction by ZAP-cDNA Gigapack III Gold Cloning Kit. The library was shown to be of high quality by the titer, the recombination efficiency, and the insert sequence length determination.

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