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Improved methods for basic molecular manipulation of the red alga *Porphyra umbilicalis* (Rhodophyta: Bangiales)

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Abstract The isolation of DNA and RNA from the leafy gametophyte (thallus) of red algal seaweeds such as *Porphyra* is still a challenge because of the high content of polysaccharides. Further molecular analysis, including restriction enzyme digestion, polymerase chain reaction (PCR), and reverse transcription (RT), could be interfered by the remaining trace amount of polysaccharides. Various protocols have been developed for molecular biological studies of *Porphyra* species to avoid polysaccharide contamination. Here, we compare different methods in DNA and RNA isolation from the thallus of *Porphyra umbilicalis*. The quality of the resulting DNA for restriction enzyme digestion and PCR and the quality of RNA for RT and RT-PCR were analyzed with *PuActin3*, a *P. umbilicalis* homolog of *Porphyra yezoensis Actin3* gene, which is a potential marker for monitoring thallus maturation. Our results show that the polysaccharides could be simply, but efficiently, removed from DNA by coprecipitation with potassium acetate, and RNA could be purified from polysaccharides by a single step of lithium chloride precipitation. We successfully utilized the RNA preparation for the rapid amplification of cDNA ends to amplify the unknown upstream flanking region, including the 5′-UTR, of *PuDnm1*, a *P. umbilicalis* homolog of the *Cyanidioschyzon merolae* mitochondrial division gene *Dnm1*, and locate the putative transcription start point at −162 bp from the translation initiation site (A of the ATG codon). Our work provides a simplified basic workflow for the future molecular cloning work on the gametophyte phase of *P. umbilicalis* and can be adopted easily for other polysaccharide-rich seaweeds.

Keywords *Porphyra umbilicalis* · Polysaccharides · Nucleic acid isolation · Rapid amplification of cDNA ends (RACE)

Introduction

Species of *Porphyra* have been utilized for food for more than a thousand years in Asian countries such as China, Japan, and Korea. However, artificial seeding was impossible until the life history of *Porphyra umbilicalis* was discovered by Drew (1949). Now it is clear that *Porphyra* has a heteromorphic alternation of generations, with a filamentous sporophyte phase (conchocelis) and a leafy gametophyte phase (thallus). The meiosis of conchospores produces four daughter cells, which grow together and thus make the leafy thallus genetically chimeric (Ohme and Miura 1988). This makes *Porphyra* thallus an ideal material for genetic studies (Xie et al. 2010).

The aquaculture of *Porphyra* species is worth US $1.3 billion a year (Blouin et al. 2011), and its molecular biological studies are receiving more and more attention worldwide. The plastid genome of *Porphyra purpurea* was among the earliest plant genomes completely sequenced (Reith and Munholland 1995). From the 1990s, different molecular approaches have been used for the discrimination of cultivated strains or ecological types of various *Porphyra* species. Both restriction fragment length polymorphism (RFLP) (Iitsuka et al. 2002; Niwa et al. 2004; Niwa and Aruga 2006) and random amplified polymorphic DNA (Mizukami et al. 1996; Kuang et al. 1998; Song et al.

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1998; Jia et al. 2000) strategies and the analysis of specific target sequences, such as microsatellite (Kong et al. 2009; Niwa and Sakamoto 2010), ribosomal DNA internal transcribed spacer (Mizukami et al. 1999; Niwa and Aruga 2003; Niwa et al. 2005a, b, 2008; Hu et al. 2010; Niwa and Sakamoto 2010), Rubisco spacer (Niwa and Sakamoto 2010; Teasdale et al. 2002; Niwa et al. 2005a, b, 2008) and genes coding Rubisco large subunit (*rbcL*) (Niwa et al. 2008; Xu et al. 2011), actin-related protein 4 (Niwa and Sakamoto 2010), and small subunit ribosomal RNA (Mizukami et al. 1998; Yamazaki et al. 2003), have been adopted. Because of the difficulties in the identification of some species in this polyphyletic genus (Oliveira et al. 1995; Müller et al. 1998; Broom et al. 1999; Oliveira and Bhattacharya 2000; Nelson et al. 2006), molecular information also provides powerful support for both identifying new species and resolving taxonomic and biogeographic puzzles (Dutcher and Kapraun 1994; Brodie et al. 1996, 2007; Broom et al. 2002; Klein et al. 2003; Milstein and Oliveira 2005; Bray et al. 2006, 2007). In addition to these, recent studies also focus on the unique tolerance of *Porphyra* species to stress conditions, such as high irradiance, high salinity, desiccation, etc. (Gantt et al. 2010), and the rhythmic growth (Lüning 1992, 2001; Lüning et al. 1997) and developmental control (Li et al. 2011; Lu and Yarish 2011).

For molecular biology studies on *Porphyra* species, various protocols have been developed for the isolation of DNA and RNA and further analysis, using either the leafy gametophyte thallus (e.g., Hong et al. 1995; Kitade et al. 1996; Mizukami et al. 1998) or the filamentous sporophyte (e.g., Niwa and Aruga 2003, 2006; Wang et al. 2010). For both gametophytes and sporophytes, polysaccharides have been found to interfere not only with nucleic acid isolation but also to restriction enzyme digestion and the polymerase chain reaction (PCR) (Pandey et al. 1996), and protocols have been developed for easy and/or rapid isolation of DNA as a template for PCR identification (e.g., Wang et al. 2009, 2010; HwangBo et al. 2010). However, methods for further analysis, such as reverse transcription (RT) and beyond, have not been well documented.

The complete nuclear genome of *P. umbilicalis* and transcriptomes of both *P. umbilicalis* and *P. purpurea* under various conditions are currently being sequenced by the Joint Genome Institute, USA. A large amount of genome and transcriptome information is now available for analysis (Chan et al. 2012; Stiller et al. 2012). Based on the comparison and compilation of previous studies, we propose here the protocols we developed for DNA and RNA isolation, RT, and the rapid amplification of cDNA ends (RACE). With the current protocols, we achieved good restriction enzyme digestion of *P. umbilicalis* DNA and also successfully isolated the unknown 5′-end of *PuDnm1*, a *P. umbilicalis* homolog of the *Cyanidioschyzon merolae* mitochondrial division gene *Dnm1*, and located the transcription start point.

Materials and methods

The *Porphyra umbilicalis* strain P.um.1 was isolated by Dr Susan Brawley (University of Maine). The thallus was cultivated in Provasoli's enriched seawater (Provasoli 1966) at around 12 °C under 50 μmol photons m^{-2} s^{-1} irradiance with a light/dark regime of 12/12 h. Leafy thalli were rinsed briefly with distilled water, surface dried with paper towel, then ground into fine powder with pestle and mortar in liquid nitrogen, and stored at −80 °C before further analysis.

pMD-19 T vector, restriction enzymes, and ExTaq DNA polymerases, RNAiso Reagent, RNAiso for Polysaccharide-Rich Plant Tissue, and DL2,000 and DL15,000 DNA markers were purchased from Takara (Dalian, China). Moloney murine leukemia virus reverse transcriptase (MMLV-RT), SuperScript Reverse Transcriptase II (SSRT-II), and SuperScript Reverse Transcriptase III (SSRT-III) were from Invitrogen (Carlsbad, USA). Protease K was from Sigma (St. Louis, USA). All chemicals were analytical pure or of equivalent grade. Gel Extraction Kit and Plasmid Mini-Prep Kit were from Bio Basic Inc. (Canada). The SMART II A (AAGC AGTGGTATCAACGCAGAGTACGCGGG), UPML (CTAATACGACTCACTATAGGGCAAGCAGTGGTATC AACGCAGAGT), UPMS (CTAATACGACTCACTAT AGGGC), and NUP (AAGCAGTGGTATCAACGC AGAGT) oligonucleotides were designed according to the sequences from Clontech (Mountain View, USA). The synthesis of oligonucleotide primers and DNA sequencing were by Genscript (Nanjing, China).

DNA isolation, restriction enzyme digestion, and polymerase chain reaction

For extracting DNA from *P. umbilicalis* leafy thalli, three different lysis buffers were used respectively to break cells and release DNA, cetyltrimethylammonium bromide (CTAB) buffer (3 % CTAB, 1.4 mol L^{-1} NaCl, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris–HCl, pH 8.0, 0.2 % polyvinylpolypyrrolidone (PVPP), and 0.2 % β-mercaptoethanol), sodium dodecyl sulfate (SDS) buffer (2 % SDS, 500 mmol L^{-1} NaCl, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris–HCl, pH 8.0, 0.2 % PVPP, 0.2 % β-mercaptoethanol) and guanidinium-sarkosyl buffer (4.2 mol L^{-1} guanidinium thiocyanate, 0.05 % sarkosyl, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris–HCl, pH 8.0, 0.2 % PVPP, 0.2 % β-mercaptoethanol). Ground powder of thallus material (100 mg) was mixed with 0.5 mL of each lysis buffer, respectively, and 5 μL protease K (20 mg mL^{-1}). The mixture was incubated at 55 °C for 2 h with gentle shaking every 15 min. After incubation, the extract was centrifuged at 15,000×*g* for 15 min at room temperature and the supernatant was transferred into a new tube, gently mixed with 200 μL 5 mol L^{-1} potassium acetate (pH 8.0) (Hu et al. 2004), and then kept on ice for 30 min. After centrifugation at 15,000×*g* for

15 min under 4 °C, the supernatant was moved into a new tube and extracted by an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) by gentle shaking for 15 s and centrifugation at 12,000×*g* for 5 min at 4 °C. The supernatant was re-extracted once with chloroform by gentle shaking for 15 sec and then centrifugation at 12,000×*g* for 5 min. DNA in the supernatant was precipitated with equal volume of ice cold isopropanol by gently mixing in a new tube and then centrifuging at 12,000×*g* for 15 min. The pellet was washed with 0.5 mL 75 % ethanol, air dried, and dissolved in 50 μL sterilized water. To compare the quality of DNA samples extracted by different lysis buffers, 5 μL of genomic DNA was digested with *Bam*H I, *Eco*R I, and *Hin*d III, respectively, resolved on 1.0 % agarose in Tris–acetate–EDTA (TAE) buffer by electrophoresis, stained with ethidium bromide and photographed, following standard protocols (Sambrook et al. 1989).

Actin is a housekeeping gene and widely studied in both plants and animals. Recently, it has been reported that *Porphyra yezoensis* has four *Actin* homologs (*PyAct1-4*), different from other seaweeds such as *Fucus*, *Chondrus*, and *Ulva* with only one homolog (Kitade et al. 2008). Among these homologs, *PyAct3* was reported to expresses significantly high in mature gametophyte and can be used as a potential molecular marker for monitoring thallus maturation (GenBank No. AB126237, Kitade et al. 2002, 2008). In addition to its physiological importance, *PyAct3* also has the highest (G+C)% among the known seaweed *Actin* homologs (Kitade et al. 2008). By searching transcriptome sequencing information, counterparts of each *PyAct* member were found in *P. umbilicalis* transcriptome. To test the readiness of the DNA extracted for further PCR analysis, we amplified *PuAct3*, the *P. umbilicalis* homolog of *PyAct3*. Primer pairs PuAct-F (5′-CGAGCGTATGACGCAGATTATG-3′) and PuAct-R (5′-GCAATGCCCGCAAACATTGTC-3′) were used. ExTaq DNA polymerase was used for the amplification according to the manufacturer's instruction. In brief, each 50 μL reaction system contained 5 μL of 10× ExTaq buffer (without Mg^{2+}), 4 μL of 25 mmol L^{-1} $MgCl_2$, 4 μL of dNTPs mixture (2.5 mmol L^{-1} each), 2 μL of each of the primers at 10 mmol L^{-1}, 2 μL genomic DNA as template, 0.5 μL (2.5 units) of ExTaq. Dimethyl sulfoxide (DMSO) was added to the reaction system to a final concentration of 5 % (*v*/*v*) to overcome the high GC content of the templates (Kitade et al. 2003; Sun et al. 2010). The reaction was carried out at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, 62 °C for 30 sec, and 72 °C for 30 sec. A final extension step at 72 °C for 5 min was used to complete the amplification. PCR products were resolved on 1.5 % agarose gel in TAE, stained, and photographed.

RNA isolation

The standard methods using Takara RNAiso Reagent and RNAiso for Polysaccharide-Rich Plant Tissue were used to isolate intact RNA from *P. umbilicalis* thalli according to the manufacturer's instructions. And for comparison, we added the step of LiCl precipitation for RNA purification. In brief, the fine powder of *P. umbilicalis* leafy thalli (100 mg) was mixed with 1 mL of either RNAiso Reagent or RNAiso for Polysaccharide-Rich Plant Tissue, shaken on a vortex for 20 sec and kept at room temperature for 5 min. The mixture was extracted with 200 μL chloroform by shaking on a vortex for 20 s and left at room temperature for another 5 min. After centrifugation at 15,000×*g* for 15 min at 4 °C, the supernatant was moved to a new tube, mixed with 1/3 volume of 8 mol L^{-1} LiCl and kept at 4 °C for 2 h. The mixture was centrifuged at 15,000×*g* for 10 min at 4 °C and the supernatant was discarded. The pelleted RNA was re-suspended with 800 μL 2 mol L^{-1} LiCl by gentle pipetting and then centrifuged at 15,000×*g* for 5 min at 4 °C. The pellet was thoroughly dissolved in 200 μL DEPC-treated water, mixed with 20 μL 3 mol L^{-1} sodium acetate (pH 5.0) and 550 μL ethanol. RNA was precipitated by leaving at −20 °C overnight and being centrifuged at 15,000×*g* for 10 min at 4 °C. The pellet was washed by 75 % ethanol and finally dissolved in 20 μL DEPC-treated water; 3 μL of the total RNA was mixed with 18 μL deionized formamide, 3 μL 6× loading buffer (30 mmol L^{-1} EDTA, pH 8.0, 40 % glycerol, and 0.05 % bromophenol blue), and 1.5 μL ethidium bromide (1 mg mL^{-1}), heated at 65 °C for 10 min, immediately cooled on the ice for 2 min and then resolved on 1 % agarose gel with TAE buffer by electrophoresis and photographed, following standard protocols (Sambrook et al. 1989). The rest RNA was stored at −80 °C for further use.

Reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends

Three different reverse transcriptases from Invitrogen, MMLV-RT, SSRT-II, and SSRT-III were used in this study. The reverse transcription reactions were conducted according to the manufacturer's protocols, using 3 μg of total RNA for each reaction and Oligo $(dT)_{18}$ as the primer. The reactions was carried out at different temperatures according to the enzyme used, respectively, i.e., 37 °C for MMLV-RT, 42 °C for SSRT-II, and 50 °C for SSRT-III, for 60 min and finally terminated by heating at 70 °C for 15 min. The generated first strand cDNA samples were store at −20 °C until further use. For detecting the cDNA size distribution, the first strand cDNA products generated by different reverse transcriptases were resolved on a 1.2 % agarose gel in TAE by electrophoresis, stained, and photographed as before. For testing the readiness of the cDNAs for further PCR analysis, such as semiquantitative comparison, we also amplified *PuAct3* with the same primer pair PuAct-F and PuAct-R and the same PCR conditions as described above, except that 2 μL of first strand cDNA was used instead of 2 μL of genomic DNA. PCR

products also resolved on 1.5 % agarose gel in TAE, stained, and photographed as before.

For RACE, 3 μg of total RNA was used as well. Different from the general reverse transcription protocol using SSRT-II and SSRT-III, 1 μL of water in each reaction in the beginning was substituted with 1 μL of SMART II A adaptor oligonucleotide at 10 mmol L^{-1}. The other steps and constituents were not changed. After reverse transcription, we did nested PCR to amplify the 5′-end of *PuDnm1*, a *P. umbilicalis* homolog of the *Dnm1* gene from *C. merolae* (GenBank No. AY162473.1), which is known to be involved in mitochondrial division (Nishida et al. 2003) and might have significant importance in cell cycle. A partial sequence of *PuDnm1* was found from the transcriptome sequencing and we did not find any other homolog of this gene family in *P. umbilicalis*. For the first round of PCR, we used the primer pair of gene-specific Dnm1-ER1 (GGGAGAAGACGCGCATGATGAC) and UPM (a mixture of UPML at 2 mmol L^{-1} and UPMS at 10 mmol L^{-1}). The 50-μL reaction system contains 5 μL of 10× ExTaq buffer (without Mg^{2+}), 4 μL of 25 mmol L^{-1} $MgCl_2$, 4 μL of dNTPs mixture (2.5 mmol L^{-1} each), 2 μL of Dnm1-ER1 primer at 10 mmol L^{-1} and 2 μL of UPM, 2 μL cDNA as template, 5 % (*v*/*v*) DMSO, and 0.5 μL (2.5 units) of ExTaq. The reaction was carried out at 94 °C for 5 min, followed by 39 cycles of 94 °C for 30 sec, 58 °C at 30 sec, and 72 °C at 90 sec. A final extension step at 72 °C for 5 min was used to complete the amplification. For the second round of PCR, new primer pair of gene-specific Dnm1-ER2 (GCAATCTCTGTCCGCACCACG) and NUP, both at 10 mmol L^{-1}, was used to instead of Dnm1-ER1 and UPM, with the annealing temperature increased to 63 °C for each PCR cycle. After two rounds of amplification, the products were resolved on 1.0 % agarose in TAE, stained, and photographed as before. The bands of the PCR products were purified from the gel and subcloned into pMD-19T vector following standard protocols and sequenced.

Results

DNA extraction, restriction enzyme digestion, and polymerase chain reaction

From our results, the extraction of genomic DNA from *P. umbilicalis* thallus with either CTAB or SDS buffer worked fine, and we did not get good DNA preparations with the guanidinium-sarkosyl buffer (Fig. 1). Although the major fractions of the SDS buffer-extracted DNA samples looked larger, their electrophoretic pattern was not as clear as the CTAB extracted ones. Further restriction enzyme treatment also showed an incomplete digestion (Fig. 2), which might result from the binding of contaminated polysaccharides to DNA molecules. Lanes 5–12 had some fast migrating fractions, which might be RNA and/or other small size nucleic acids. During the preparation of genomic DNA samples, the precipitation of polysaccharides with high concentration of potassium acetate on ice is a critical step for further restriction enzyme digestion and polymerase chain reaction.

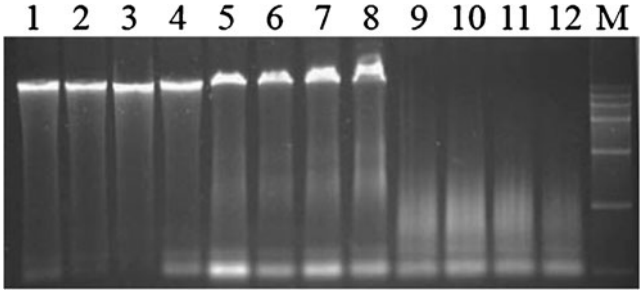

Fig. 1 DNA extracted from gametophyte (leafy thallus) of *P. umbilicalis*. DNA samples were extracted by CTAB buffer (*lanes 1–4*), SDS buffer (*lanes 5–8*), and guanidinium-sarkosyl buffer (*lanes 9–12*). A DL 15,000 DNA marker (*M*) was loaded for indicating the size

With the DNA samples from both CTAB and SDS extraction methods, we analyzed their quality by restriction enzyme digestion. Our results showed that the genomic DNA isolated with CTAB buffer was digested well by *Eco*R I, *Bam*H I, and *Hin*d III (Fig. 2), whereas the SDS buffer-extracted DNA had some problems in the digestion.

To test if the genomic DNA sample isolated with CTAB or SDS buffer can be used for PCR, we amplified *PuAct3* with primers designed according to the transcriptome sequencing result. Our result showed that the DNA prepared by either buffer was ready for further PCR amplification (Fig. 3), although the restriction enzyme digestion was partially interfered with the SDS buffer-extracted samples (Fig. 2).

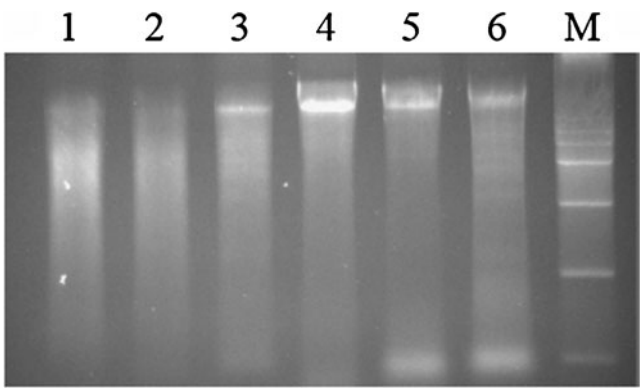

Fig. 2 Restriction enzyme digestion of DNA extracted from *P. umbilicalis*. CTAB-extracted DNA was digested by *Eco*R I (*lane 1*), *Bam*H I (*2*), and *Hin*d III (*3*), and SDS-extracted DNA was digested by *Eco*R I (*4*), *Bam*H I (*5*), and *Hin*d III (*6*), respectively. A DL 15,000 DNA marker (*M*) was loaded for indicating the size

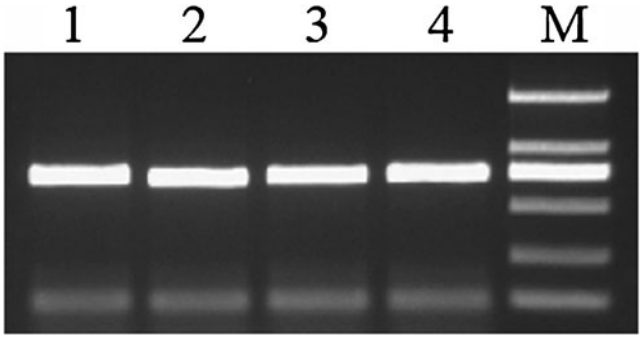

Fig. 3 PCR amplification of *PuAct*, the *P. umbilicalis* homolog of the *Actin* gene, from CTAB-extracted DNA (*lanes 1, 2*) and SDS-extracted DNA (*3, 4*), respectively. A DL 2,000 DNA marker (*M*) was loaded for indicating the size

RNA extraction

Both RNAiso Reagent and RNAiso for Polysaccharide-Rich Plant Tissue gave jelly pellets after ethanol precipitation, which were difficult to completely dissolve in DEPC-treated water because of the coprecipitated polysaccharides. Although such a pellet containing polysaccharides can be better dissolved in deionized formamide and used for Northern blot, the formamide solvent made it incompatible with reverse transcription (data not shown). The RNAiso for Polysaccharide-Rich Plant Tissue did not help with the leafy thallus of *Porphyra* (Fig. 4). Thus we incorporated the LiCl precipitation step (Sambrook et al. 1989), which has been used for RNA purification from higher plant materials. The coprecipitation of RNA with LiCl resulted in a smaller pellet, which was much easier to dissolve in DEPC-treated water. A further ethanol precipitation removed LiCl from the RNA preparations. This LiCl precipitation step increased the reproducibility of the RNA isolation methods, and the elimination of polysaccharide contamination also promoted the solubility of the pelleted RNA into DEPC-treated water (Fig. 4).

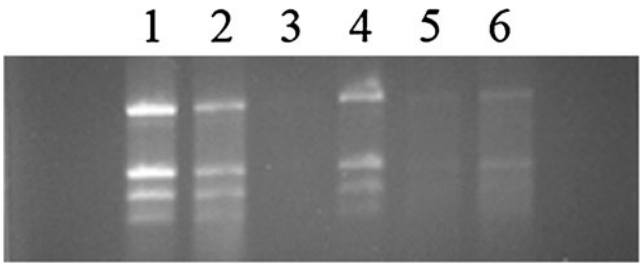

Fig. 4 Gel electrophoresis of RNA samples isolated from *P. umbilicalis* leafy thallus by RNAiso isolation and LiCl purification (*lanes 1, 2*), RNAiso only (*3, 4*), and RNAiso for Polysaccharide-Rich Plant Tissue (*5, 6*)

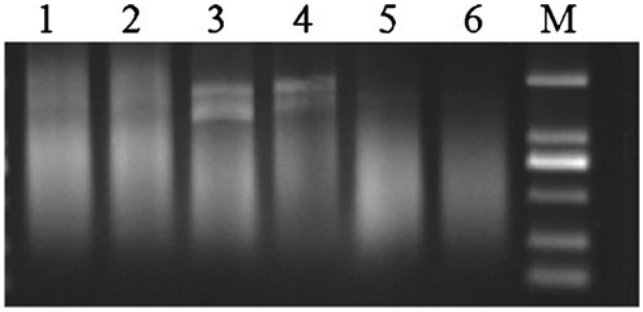

Fig. 5 Gel electrophoresis of the first strand cDNA reverse transcribed by MMLV Reverse Transcriptase (*lanes 1, 2*), SuperScript Reverse Transcriptase II (*3, 4*), and SuperScript Reverse Transcriptase III (*5, 6*) from 3 μg of total RNA isolated by RNAiso and purified by LiCl. A DL 2,000 DNA marker (*M*) was loaded to indicate the size

Reverse transcription-polymerase chain reaction and rapid amplification of cDNA ends

Because of the high GC content in *Porphyra* genome (Kitade et al. 2003), we compared the efficiency of different commercial enzymes for RT, which is a key step for gene expression analysis. Although MMLV-RT is commonly used for RT, both SSRT-II and SSRT-III could be used at higher reaction temperatures, which might be helpful in overcoming the high GC content of the template. In addition to the reaction temperature, the terminal transferase activity of reverse transcriptase was our other consideration. This activity enables us to amplify the unknown 5′-end of a certain gene as long as a part of the cDNA sequence is known. Here we compared the performance of MMLV-RT, SSRT-II, and SSRT-III in RT and also studied the feasibility of utilizing SS-II for RACE, using the RNA samples isolated by RNAiso and purified by LiCl precipitation.

Figure 5 shows that all three enzymes worked in RT with purified RNA and produced a bouquet of cDNAs in various sizes, with the majority ranging from 0.5 to 1.5 kb. Both MMLV-RT and SSRT-II worked more efficiently than SSRT-

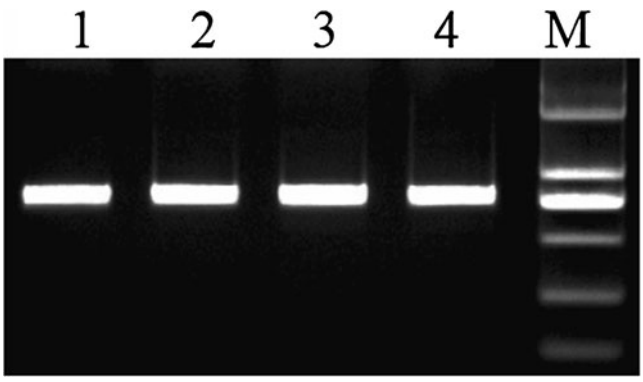

Fig. 6 RT-PCR amplification of *PuAct*, the *P. umbilicalis* homolog of the *Actin* gene, from cDNA reverse transcribed by MMLV-RT (*lanes 1, 2*) and SSRT-II (*3, 4*), respectively. A DL 2,000 DNA marker (*M*) was loaded for indicating the size

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-200  CGACGATTAA GCAGTGGTAT CAACGCAGAG TACGCGGGGG CCGCTCCCAG
-150  CCCGACTCGT TGGTGGTCGA TGTGTGCCAC TGCCGGCGTT ATACCCGCTG
-100  ATCTAGCGCC GAGAGCCAGG CCGCCGCGCC AACCTCATTC CCCTTTGCCT
-50   CCCCCCCAAA GCTGTATGCC TTGGTGCCGT CTGGCGCCAC CCCTTCCACC
      ATGGAGCGCC TCATCCCCGT CATCAATCGG CTCCATGAGA CGTTTGCCAA
      GTGCGGCCTC AAGGCCGCCC CCGTCGACCT CCCCCAAATC ACCGTCGTCG
      GCTCCCAGTC GTCGGGCAAG TCGTCGGTCC TCGAGGCGCT CGTCGGCTTT
      GACTTTCTCC CCCGCGGCTC GGGCATCGTG ACGCGGTGCC CCATCGTCAT
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Fig. 7 Sequencing of the 5′-end of the *P. umbilicalis Dnm1* gene homolog. The sequence of the SMART II A adaptor nucleotide for the RACE is *underlined*, and the translation starting codon ATG is in *boldface*

III. Because of this result and that SSRT-III has been engineered to eliminate the terminal transferase activity, we did not work forward to study SSRT-III for *P. umbilicalis* leafy thallus material. The higher reaction temperature of SSRT-II (42 °C) resulted in a different electrophoretic pattern of reverse-transcribed cDNA, comparing with MMLV-RT (Fig. 5).

To test whether the quality of the cDNAs reverse transcribed by either MMLV-RT or SSRT-II was sufficient for RT-PCR or semiquantitative PCR, a fragment for *PuAct3* was amplified again. The result showed that both of the cDNAs reverse transcribed by MMLV-RT and SSRT-II were suitable for PCR amplification (Fig. 6).

We then tried to amplify the 5′-end of the *PuDnm1* transcript. A partial cDNA sequence from the 3′-end was found from the sequencing of different transcriptomes of *P. umbilicalis*, and two reverse primers, Dnm1-ER1 and Dnm1-ER2, were designed within this region to amplify the upstream flanking fragment with two adaptor primers UPM and NUP, respectively, after RT with SMART II A adaptor primer. After two rounds of nested PCR, a clear band was revealed in the agarose gel (data not shown). TA cloning and subsequent sequencing results showed a successful amplification of the 5′-end of this gene. There was a 162-bp sequence from the transcription starting point (immediately after the SMART II A adaptor sequence) to the translation starting codon (ATG) (Fig. 7).

Discussion

Different strategies have been adopted to overcome the polysaccharide problem for further molecular analyses of seaweeds. For examples, for cell disruption, grinding in liquid nitrogen has been widely used, whereas softening tissues in lithium chloride (Hong et al. 1995) and other methods to help release the DNA also have been tried. For DNA isolation, different CTAB methods, with or without further purification by cesium chloride gradient centrifugation (Kitade et al. 1996), diatomaceous earth (Kim et al. 2006) or resin (Kitade et al. 2008) absorption, were used, and for RNA, Trizol or other chemicals with similar property were generally used (e.g., Uji et al. 2012). Some of these methods are time consuming and/or there is the need to take special care with the complicated procedures. Commercially available kits such as DNeasy (Qiagen) and ISOPLANT II (Nippon Gene) for DNA isolation and RNeasy Plant Mini Kit (Qiagen) for RNA isolation have also been used (e.g., Nakajima et al. 2000; Niwa and Aruga 2006). Although commercial kits using membrane technology are easy to use and might provide good purity of extracted nucleic acids, their recovery efficiencies are lower compared with direct precipitation, which is also easy to scale up. Some kits such as ISOPLANT II are not easy to purchase worldwide. In this study, we tried to simplify the isolation strategies with common chemicals and benchtop instruments, so that it can be carried out in most laboratories.

With the improved method of using high concentration potassium acetate to precipitate polysaccharides from DNA at 4 °C, our experiment with CTAB lysis buffer produced genomic DNA with high quality, which was easily digested by restriction enzymes and can be adopted for further work, such as RFLP, bacterial artificial chromosome library construction, Southern blotting, etc. For RNA isolation, we added the LiCl precipitation step to purify RNA from the RNAiso-extracted samples, and this improvement eliminated the problems of polysaccharide contamination. The pellet after precipitation contained a relatively low amount of polysaccharides, and this made the rehydration of pelleted RNA into DEPC-treated water much easier and also helped the RT afterwards. For RT, because the information of the 5′-UTR of a cDNA is of great value for further analysis, such as to figure out where the transcription starts from in vivo, we not only compared the efficiency of different reverse transcriptases but also used one of these enzymes for RACE. Our results suggest that the SuperScript Reverse Transcriptase II can be used for *Porphyra*, benefiting from its high optimum temperature and catalytic efficiency to overcome the high GC content in *Porphyra* RNA templates.

Plant polysaccharides have presented a significant problem for various molecular biological manipulations (Pandey et al. 1996; Do and Adams 1991). This becomes a critical issue for studies of red algal seaweeds, most of which are rich in polysaccharides. Although different methods can be utilized to break algal cells and to release DNA and RNA, the strategies of using potassium acetate to remove polysaccharides from the lysate and of using LiCl to coprecipitate RNA from the lysate should be helpful in generating high-quality starting samples for further analysis. Limited additional optimization might be needed to adopt the protocols we propose here for either scaling up or handling other red seaweeds, especially with their leafy thallus.

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