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Characterization and expression analysis of *hsp70* gene from *Ulva prolifera* J. Agardh (Chlorophycophyta, Chlorophyceae)

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ABSTRACT

In the Yellow Sea of China, large-scale green tides have broken out consecutively from 2007 to 2011. *Ulva prolifera*, the causative species of green tide, showed great ability to acclimate to adverse circumstance. To explore the mechanisms of rapid growth and stress resistance during the bloom, we characterized and analyzed *hsp70* from *U. prolifera*. The results showed that *hsp70* gene had 6 exons and 5 introns. The promoter-like region contained multiple *cis*-acting elements. The transcription of *hsp70* was up-regulated by UV irradiation, heat treatment and salinities induction, but less influenced by desiccation. In vitro expression of HSP70 protein and western blot was also conducted, and the recombinant protein will be used in detecting the interaction between HSP70 and related functional proteins in the future. The study suggested that *hsp70* could be used in prediction of stress tolerance in algae and monitoring environmental changes.

1. Introduction

Ulva (Enteromorpha) prolifera is widely distributed in the coastal area of China. Recently with the global ecosystem variations and climatic influences, especially the coastal eutrophication (Paerl, 1997), green algae blooms occurred consecutively in the Yellow Sea areas in summer from 2007 to 2011 (Liu et al., 2009a, 2010a), and resulted in side effects to coastal environments (Liu et al., 2009b). Exposed to strong ultraviolet irradiation and other harsh stresses in summer, the intertidal seaweed exhibits a remarkable physiological potential. Some specific genes and related enzymes are involved in the acclimation to environmental challenges (Gutteridge and Gatenby, 1995; Houtz and Portis, 2003; Jensen, 2000), such as heat shock proteins (Preczewski et al., 2000).

Heat shock proteins are a family of highly conserved cellular proteins (Gupta and Golding, 1993). Generally, they could be divided into HSP90, HSP70, HSP60, and small HSPs (Schlesinger, 1990). Among them, HSP70 is the most extensively studied (Feder and Hofmann, 1999; Georgopoulos and Welch, 1993; Morimoto, 1998). HSP70 plays an essential role in protecting cells, folding and translocation of proteins (Gething and Sambrook, 1992; Morimoto et al., 1997; Boorstein et al., 1994), and response to heat shock, oxidant injury (Lindquist and Craig, 1988) and ultraviolet radiation (Zhou et al., 1998). Schroda et al. (1999) believed that HSP70 are important to the plant normal photosynthesis.

In algae, *hsp70* can facilitate *Chlamydomonas* sp. to acclimate the polar environment (Liu et al., 2010b), and help adjust asymmetric divisions in *Volvox carteri* (Cheng et al., 2006). Yokthongwattana et al. (2001) studied *hsp70* activities in the photosystem II damage and repairing in *Dunaliella salina*. In addition, Fu et al. (2011) found that there was a correlation between transcriptions and stress induction in *Ulva pertusa* and held that *hsp70* played an important role in the stresses.

U. prolifera showed a great ability to acclimate to an adverse circumstance, *hsp70* was believed to play an important role in its stress tolerance. In this study, through characterization of *hsp70* from *U. prolifera*, we conducted transcription and in vitro expression analysis of *hsp70*, the objective of our work is to verify the function of *hsp70* and screen the potential bio-indicator to monitor the stresses in seawater environments in the future.

2. Materials and methods

2.1. Materials and preculture

The sporophytes of *U. prolifera* were collected from the green tide in Zhanqiao, Qingdao, China (36° 03′ 35.03″N; 120° 18′ 54.20″E) on 25th July 2009, after rinsed with autoclaved seawater for 3–5 times to eliminate the epiphytes, the algae were subsequently cultured at 25 °C, 50 μmol m^{-2} s^{-1}, 12:12 h (L/D). To each test, about 5 g fresh algal samples were used.

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2.2. Stress treatments

For heat treatments, the algal samples were cultured under different temperatures (5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C and 40 °C) for 1 h. For salinities treatments, the algal samples were cultured in the artificial seawater (ASW; General Sea Salt Factory) with various concentrations (0‰, 8‰, 16‰, 24‰, 32‰, 40‰, 48‰ and 56‰) for 2 h. In desiccation treatments group, the algal samples were kept in the incubator at 25 °C at 60% humidity for 0, 1, 2, 3, 4, 5, 6 and 7 h, respectively. While for the UV irradiation tests, the algal samples were kept at 60 cm distance from the fluorescent ultraviolet lamp (40 W) for 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h, respectively. All the treated algal samples were collected respectively for the following RNA extraction.

2.3. RNA extraction and cDNA synthesis

Generally, about 0.3–0.5 g of algal sample was grinded to powder in liquid nitrogen, transferred into a 10 mL tube, added 3 mL extraction buffer (100 mM Tris (pH 7.5), 50 mM EDTA, 2 M NaCl and 55 mM CTAB) and incubated at 60 °C for 10 min. After mixed with 330 μL KAc (3 M) and 750 μL ethanol (100%), the solution was added 2 mL of phenol/chloroform/isoamyl alcohol (25:24:1, V/V/V) and mixed thoroughly, finally centrifuged at 10,200 *g* for 25 min at 4 °C. Supernatant was transferred to a new 10 mL tube. The extraction was repeated 2 times for the purification of RNA. Finally about 1 mL supernatant was transferred into a new 1.5 mL tube containing 0.25 mL of 12 M LiCl solution, mixed thoroughly and stored at −80 °C for 30 min. RNA precipitation was collected by centrifugation at 13,600 *g* for 30 min at 4 °C. The pellet was washed twice with 1 mL of 75% ethanol, dried in vacuum and redissolve in 30 μL RNase-free water, finally DNase I (Fermentas) was added into the solution for the removal of DNA. Total RNA concentrations were determined at 260 nm with ultraviolet (UV) absorbance under the BioPhotometer Plus (Eppendorf), and the purity of RNA was determined by measuring the A260/A280 ratio, RNA integrity was checked on agarose gel (1%) electrophoresis.

The fist-strand of cDNA was synthesized in a total 20 μL reaction solution containing 2 mM dNTP, 1× reaction Buffer, 0.25 U MMLV reverse transcriptase (Takara), 1 U inhibitor, 1 μL total template RNA (2 μg), and 25 nM oligo dT-adaptor primer. The reaction was carried out in the PCR Thermal Cycler (Takara) at 42 °C for 1 h, and was terminated at 70 °C for 15 min. Finally, the reaction tube was taken out and put on ice, subsequently stored at −80 °C.

2.4. DNA extraction

Genomic DNA of *U. prolifera* was obtained using Plant Genomic DNA kit (Tiangen), and the concentration, purity and integrity of DNA were checked using the same methods as RNA protocols described above.

2.5. Genomic cloning of hsp70

A pair of specific primers, Dhsp70-S (5′-ATCAAGGGAACCGCACTA-3′) and Dhsp70-A (5′-CGCCTCTATCATCCCAA A-3′) was designed according to the *hsp70* mRNA sequence of *U. prolifera* (GenBank ID: GQ285222). The PCR profile was performed at 94 °C for 10 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 3 min, and a final extension at 72 °C for 10 min. The PCR product was electrophoresis on the agarose gel (1%), and the objective bands was selected and purified by DNA fragment recovery kit (Sangon), then was used for sequencing (Sangon).

2.6. Cloning of upstream region of hsp70

Two designed specific primers Phsp70-1 (5′-GTAGGTTGTGCCAAGGTCA-3′) and Phsp70-2 (5′-CGATGGCAGGAGCGGACTTT-3′) were applied for the nest PCR amplification of the upstream region of *hsp70* with LA PCR in vitro Cloning kit (Takara). The PCR reactions were carried out as follows, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 4 min, and a final extension at 72 °C for 10 min. The amplified product was electrophoresis on agarose gel (1%), and the objective bands were excised, purified by DNA fragment recovery kit (Sangon), and subsequently cloned into PMD-19T vector (Takara) for the following sequence analysis (Sangon).

2.7. Real-time PCR detection of hsp70 transcription

Two specific primers of hsp70-S (5′-CATCAGTAACGATCAAGGGAA-3′) and hsp70-A (5′-CACAGTGTTGGTCGGGTTC-3′) were designed for amplification of a 118 bp fragment. A pair of *β-actin* primers, actin-S (5′-AGGATGCATACGTTGGTGAA-3′) and actin-A (5′-TTGTGGTGCCAAATCTTCTC-3′) based on sequence of actin gene (GenBank ID: FJ775527) was used to amplify a *β-actin* gene fragment of 140 bp as internal control. The reaction was conducted in a total volume of 25 μL containing 1× SYBR Premix Ex *Taq*™, 5 μL of diluted cDNA, 0.2 μM each forward and reverse primers. The PCR reaction was performed on the Takara TP800 Thermal Cycler Dice™ (Takara) as protocols: 95 °C for 1 min, followed by 45 cycles of 95 °C for 5 s and 55 °C for 1 min. Three independent experiments (biological replicates) were carried out, and expression level was calculated by $2^{-\Delta\Delta}C_T$, and then subjected to one-way analysis of variance and Tukey honestly significant difference test. When necessary, Data were transformed to meet assumptions of ANOVA by log transformation. Differences were considered statistically significant at $P<0.05$. Statistical analysis was performed using the statistical software SPSS 18.0 for Windows.

2.8. In vitro expression of HSP70

One pair of specific primer Hsp70-FS (5′-ATGGCAAAGTCCGCTC-3′) and Hsp70-FA (5′-GTCAACCTCCTCGATGG-3′) was designed to amplify the open reading frame (ORF) of *hsp70* (1980 bp). The PCR protocol included an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min and a final extension at 72 °C for 10 min. The amplified product was separated on agarose gel electrophoresis (1%), and the objective band was selected and purified according to the method stated above. The purified product was ligated into *pEASY*-E2 vector (TransGen Biotech), and was transformed into competent *E. coli* (BL21). After the verification of positive clones with PCR detection, enzyme digestion and sequences analysis, the selected positive clones was re-inoculated in LB solution over night. OD detection was conducted with Eppendorf BioPhotometer (Eppendorf). When the OD value reached 0.6, IPTG (5 mM final concentration) was added into the medium. The crude incubation solution was centrifuged at 5000 rpm for 5 min, and the sediment parts were chosen for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), after boiled for 5 min in the 2× loading buffer (Tris 25 mM, DTT 100 mM, SDS 2%, Glycerol 20%, Bromophenol Blue 0.016%). SDS-PAGE was performed with the compact PAGE electrophoresis instrument (ATTO). After the electrophoresis (40 mA) for 30 min, the gels were stained with Coomassie Brilliant Blue R-250 (Sangon) and discolored in the destaining solution (methanol/glacial acetic acid/ddH_2O, 3:1:6, V/V/V).

2.9. Western blot analysis

After the electrophoresis, the gel was transferred onto the nitrocellulose membrane in semi-dry blotting system AE-6675 (ATTO), at constant current of 2 mA/cm^2 for 1 h at room temperature. After