Gene 538 (2014) 155–163

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Cloning of ubiquitin-activating enzyme and ubiquitin-conjugating enzyme genes from *Gracilaria lemaneiformis* and their activity under heat shock

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ARTICLE INFO

Article history:
Accepted 11 December 2013
Available online 21 December 2013

Keywords:
Gene cloning
Gracilaria lemaneiformis
Heat shock
Transcription analysis
Ubiquitin-activating enzyme
Ubiquitin-conjugating enzyme

ABSTRACT

To study the response of *Gracilaria lemaneiformis* to heat stress, two key enzymes – ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) – of the Ubiquitin/26S proteasome pathway (UPP) were studied in three strains of *G. lemaneiformis*—wild type, heat-tolerant cultivar 981 and heat-tolerant cultivar 07-2. The full length DNA sequence of E1 contained only one exon. The open reading frame (ORF) sequence was 981 nucleotides encoding 326 amino acids, which contained conserved ATP binding sites (LYDRQIRLWGLE, ELAKNVLLAGV, LKEMN, VVCAI) and the ubiquitin-activating domains (VVCAI…LMTEAC, VFLDLGDEYSYQ, AIVGGMWGRE). The gene sequence of E2 contained four exons and three introns. The sum of the four exons gave an open reading frame sequence of 444 nucleotides encoding 147 amino acids, which contained a conserved ubiquitin-activating domain (GSICLDIL), ubiquitin-conjugating domains (RIYHPNIN, KVLLSICSLL, DDPLV) and ubiquitin-ligase (E3) recognition sites (KRI, YPF, WSP). Real-time-PCR analysis of transcription levels of E1 and E2 under heat shock conditions (28 °C and 32 °C) showed that in wild type, transcriptions of E1 and E2 were up-regulated at 28 °C, while at 32 °C, transcriptions of the two enzymes were below the normal level. In cultivar 981 and cultivar 07-2 of *G. lemaneiformis*, the transcription levels of the two enzymes were up-regulated at 32 °C, and transcription level of cultivar 07-2 was even higher than that of cultivar 981. These results suggest that the UPP plays an important role in high temperature resistance of *G. lemaneiformis* and the bioactivity of UPP is directly related to the heat-resistant ability of *G. lemaneiformis*.

1. Introduction

Gracilaria lemaneiformis (Bory de Saint-Vincent) Greville, as an agarophyte, is one of the most important economic marine algae in China. The temperature range for growth of wild populations is between 11–23 °C. As a consequence, in its normal habitat, the growing season is short and the biomass production is low. Thus, the wild population is unsuitable for the development of large-scale cultivation. The successful development and cultivation of two heat-tolerant strains, cultivar 981 and cultivar 07-2, prolongs the cultivation season and provides an important basis for large-scale cultivation and industrial application of *G. lemaneiformis* in the South China Sea (Gu et al., 2012; Lu et al., 2012). However, these two cultivars still cannot survive during the summer in the South China Sea, and thus new seedlings have to be transferred annually from the North China Sea. With the large-scale cultivation, it is urgent to breed a new strain that can tolerate still higher temperatures. To breed a temperature-tolerant new strain, it would be helpful to better understand the mechanism by which *G. lemaneiformis* responds to heat stress.

The ubiquitin/26S proteasome pathway (UPP) can degrade unwanted proteins in the cytoplasm and nucleus using ATP to provide energy. This pathway efficiently and specifically eliminates damaged and otherwise abnormal proteins, as well as short-lived functional proteins (Ciechanover et al., 1978, 1980, 1981; Wilkinson et al., 1980). The UPP is a cascade reaction involving ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). In this pathway, proteins are targeted for degradation by covalent ligation to ubiquitin (Brzovic et al., 2006; Hershko and Ciechanover, 1998; Shang and Taylor, 2011). In algae, there is accumulating evidence that the level of the ubiquitin–protein complex increases under heat shock. For example, heat treatment of *Chlamydomonas* cells (>40 °C) caused a rapid increase of high-molecular-weight ubiquitin–protein complex (>60 kDa), and concomitantly a coordinated redistribution or decrease of other ubiquitin–protein complexes and free ubiquitin (Shimogawara and Muto, 1989). The appearance of high-molecular-weight ubiquitin–protein complex was observed when *Skeletonema costatum* cells were subjected to heat treatment (Scoccianti et al., 1995). The total amount

Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; w, wild populations of *G. lemaneiformis*; 981, cultivar-981 of *G. lemaneiformis*; 07-2, cultivar-07-2 of *G. lemaneiformis*.

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http://dx.doi.org/10.1016/j.gene.2013.12.017

of ubiquitin, particularly the high-molecular-weight ubiquitin conjugates, increased greatly upon a temperature shift from 18 to 30 °C (Penna et al., 1996). These results indicate that the UPP may play a role in the heat resistance of algae. To further study the mechanisms, researchers separated and cloned the genes of the key enzymes of this pathway. Schunn et al. (1999) used a biochemical approach to identify families of ubiquitin-activating and ubiquitin-conjugating enzymes in *Chlamydomonas reinhardtii*. Tonon et al. (2003) isolated two novel cDNAs, Plubc1 and Plubc2, encoding ubiquitin-conjugating enzyme from the cDNA library of *Pavlova lutheri*. Liu et al. cloned the gene of ubiquitin-conjugating enzyme from *Griffithsia japonica* (GenBank: AF517850.1). Nierman WC et al. cloned the gene of ubiquitin-conjugating enzyme from *Pyropia yezoensis* (GenBank: FJ232910.1). However, there is no report on the UPP in *G. lemaneiformis* at present.

In our previous work, we constructed a suppression subtractive hybridization (SSH) library between the cDNA libraries of *G. lemaneiformis* growing under either normal conditions or under heat stress (Zang et al., 2011). By sequencing and BLAST analysis, 56 differentially expressed genes responding to the heat stress were initially identified from the library, including the ESTs encoding ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2). This indicated that the UPP might also play a role in the heat resistance of *G. lemaneiformis*. On this basis, the full-length gene sequences of E1 and E2 in *G. lemaneiformis* were cloned by RACE and genome walking and then transcriptions of the mRNAs of E1 and E2 under high temperature were measured. This work lays the foundations for exploring the mechanism of *G. lemaneiformis* response to heat stress and may provide an indicator for heat-resistant strain breeding.

2. Materials and methods

2.1. Algae and cultivation conditions

The wild-type of *G. lemaneiformis* was collected from Zhanshan Bay in Qingdao, China (36. 0° N, 120.2° E), and cultivar 981 and cultivar 07-2 of *G. lemaneiformis* were collected from farms at Nan'ao Island in Shantou, China (23.4° N, 117.0° E).

After rinsing with sterilized seawater, *G. lemaneiformis* of about 10 g was cultured in 5 L flasks containing 2 L sterilized seawater with continuous aeration at 22 ± 1 °C for 1 month. Sterilized fresh seawater was changed every three days. The light intensity was 50 ± 5 μE · m^{-2}·s^{-1} for a 12:12 (light:dark) cycle.

2.2. Extraction of genomic DNA

Genomic DNAs of wild-type, cultivar 981 and cultivar 07-2 of *G. lemaneiformis* were extracted using a Universal Genomic DNA Extraction Kit Ver.3.0 (TaKaRa, Dalian, China). The DNA quality was determined by 1.0% agarose gel electrophoresis and the purity was assessed by determining the 260/280 nm absorbance value using a NANODROP 2000c spectrophotometer.

2.3. Total RNA extraction and cDNA synthesis

Total RNAs of wild-type, cultivar 981 and cultivar 07-2 of *G. lemaneiformis* were extracted using an RNAprep pure Plant RNA Purification Kit (TIANGEN, Beijing, China) and the quality was determined by 1.0% agarose gel electrophoresis.

By using the extracted RNA as a template and using 3′-CDS and 5′-Oligo (Table 1) as primers, Ploy-A mRNA was reverse transcribed using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The cDNA was stored at −20 °C.

Table 1
Sequences of primers used in this study.

cDNA primer	Sequence (5′ → 3′)
A1	GATTCGGACTTTTGGTAGC
A2	TCCGTCATCAACAGTTCTTCCTTTC
A3	TACTTGTCATCGTATTCCACCAG
S1	CGGTGTGTGCCATTGTAGGT
3′-CDS	AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTVN
5′-Oligo	AAGCAGTGGTATCAACGCAGAGTACGCGGG
DNA primer	**Sequence (5′ → 3′)**
P1	AGTGGGCCTTGTGCCGTT
3d	TTATCCAGGCGGTCCAACG
U1	GTTGCTTCATTTACTACCAAAAGACGGA
D1	ATGGCTTTGAAGCGAATCAAGAAGGAG
E2-SP1	CTCACACACTAGACCACTTG
E2-SP2	CGGACGTAGTGTGTTACAAGG
E2-SP3	CATCGTTTCGACGTGATGGTC
Real time-PCR primer	**Sequence (5′ → 3′)**
E1-RT-U2	AAGCAAACGGACAAATACCA
E1-RT-D2	TCTTCCCCACATCCCACCTA
E2-RT-U1	CCGTACTCGGGAGGTGTCTA
E2-RT-D1	TGATGTTGGGGTGGTAGATG
R2A	TTGGCCCGTTCAGTGTAGC
R2S	TGGTGGAGTGATCTGTCTGGTT

2.4. Cloning of ubiquitin-activating enzyme (E1) gene

Cloning primers S1, A1, A2 and A3 (Table 1) were designed according to the EST of E1 screened from the differential expression library of *G. lemaneiformis*. Additionally, primer S1 and Oligo-dT adapter primer 3′-CDS were used for 3′RACE to amplify the fragment of the E1 gene by using cDNA as template. Primers A1, A2, A3 and the primer 5′-Oligo were used for nested PCR to amplify the 5′ end fragments of the E1 gene by using cDNA as template. The full-length cDNA sequences were obtained by overlapping the EST fragments and the sequences of 3′ and 5′RACE.

Cloning primer-P1, corresponding to the 5′ end fragment, and primer-3d, corresponding to the 3′ end cDNA fragment, were used for cloning the full-length ubiquitin-activating enzyme gene sequences from wild type, cultivar 981 and cultivar 07-2 of *G. lemaneiformis*. Finally, the primers A1, A2, and A3 were used to amplify the 5′ end fragments of the E1 gene from genomic DNA using a Genome Walking Kit (TaKaRa, Dalian, China).

For the obtained sequence of the E1 gene, the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and the GenBank nucleic acid database were used for homology analysis (Wheeler et al., 2006). The cDNA and DNA sequences of E1 of wild-type, cultivar 981 and cultivar 07-2 of *G. lemaneiformis* were aligned by ClustalX1.81 (Thompson et al., 1997). The encoded amino acid sequences were deduced from cDNA sequences using DNAStar software. The homology of full-length amino acid sequences were analyzed with Blastp. The promoter region of the obtained 5′ upstream sequence was analyzed with PlantCARE (Lescot et al., 2002). The final phylogenetic trees were constructed by neighbor-joining algorithms of MEGA5.1 (Tamura et al., 2011). Bootstrapping was performed 1000 times to obtain support values for each branch.

2.5. Cloning of ubiquitin-conjugating enzyme (E2) gene

The cDNA sequence of E2, obtained from a differential expression library of *G. lemaneiformis* under heat shock, was analyzed with BLAST, and was found to be the full-length ORF of the E2 gene. Based on the cDNA sequence, primers U1 and D1 were designed for cloning the full-length DNA sequences of the E2 gene from wild type, cultivar 981 and cultivar 07-2 of *G. lemaneiformis*. Finally, the primers E2-SP1, E2-SP2 and E2-SP3, corresponding to the DNA sequence of E2, were used