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Cloning, characterization and expression analysis of a microsomal glutathione S-transferase gene from the seagrass *Zostera marina*

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Abstract

The response of glutathione S-transferase (GST) in *Zostera marina* to temperature variation was analyzed at molecular level by cloning the microsomal GST gene and texting the microsomal GST expression regularity under different temperature. Specific speaking, express *Zm*GST in *Escherichia coli*, then purify the recombinant protein and make the thermal stability analysis. Therefore, the experiments were carried out to provide a theoretical basis for the further elaboration to the population degradation mechanisms of *Z. marina*. In conclusion, the thermostability and the response of *Zm*GST gene to temperature changes can determine its temperature tolerance range, and affect its resilience in turn.

Key words: *Zostera marina*, antioxidant enzyme, glutathione S-transferase (GST), temperature, enzyme activity

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1 Introduction

Seagrasses, a polyphyletic assemblage of basal monocots (Larkum et al., 2006; Les et al., 1997), have adapted to exist fully saline environments and functioned as ecological engineers (Wright and Jones, 2006), significantly influenced the physical, chemical and biological environments in coastal ecosystems, and provided foundation or ecological services or highly productive ecosystems functioning as tropical rain forests and coral reefs in ecosystem services (Costanza et al., 1997; Fourqurean, 2012), such as altering water flow, stabilizing sediments, driving nutrient cycling and changing food web structure (Hemminga and Duarte, 2000).

Zostera marina, an important species of seagrass, widely distributes in coastal marine ecosystem of the northern hemisphere (Den, 1970; Green and Short, 2003) and has attracted more attention because of its rapid population recession.

Temperature can affect the process of plant physiology and biochemistry and determine the growth, propagation, metabolism and distribution (Logue et al., 1995). The rising of sea surface temperature caused by the global warming has shown increasingly adverse effects on marine plants. The main characteristic of plant suffering from heat-stress is breaking the balance between the production and elimination of reactive oxygen species (ROS), such as O_2^-, ·HO, and H_2O_2 (Zhang and Lu, 2011), causing the increasing of lipid permeability in intramembrane, which is one of the natures of high temperature damage (Martin et al., 1978).

Under such heat-stress, *Z. marina* is not passively standing with the oxidative damage, but taking the initiative to regulate and adapt. It is a continuous process of adaptation, so that the organisms form a perfect and complex enzymes antioxidant system to scavenge ROS in the long-term evolution, such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione S-transferase (GST) (Yan et al., 2008). Among these, GST, a supergene family of dimeric multifunctional protein, distributes in animals (Wilce and Parker, 1994), mainly in cytosol of plants (Dixon et al., 2002), and microorganisms such as bacteria, fungus (Sheehan et al., 2001); and plays an important role in the toxicology, including catalyzes the combination of glutathione with exogenous toxic and oxidized compounds, and catalyze the combination of glutathione in organisms with exogenous toxic and oxidized compounds in metabolic reaction II so as to protect organisms from oxidative damage (Edwards et al., 2000; Mannervik et al., 1988; Rushmore and Pickett, 1993).

Although the genome of *Z. marina* has been sequenced and the results have been published in *Nature* (Olsen et al., 2016) and

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adequate information of the structure and regulation mechanisms of GST genes and proteins has been generated from plants, little research is available on the response mechanism of the *Zm*GST to the temperature.

The current research analyses the response of *Zm*GST to temperature variation at the molecular level in order to provide evidence to the molecular mechanism of temperature stress on *Z. marina*.

2 Materials and methods

2.1 *Species and laboratory culture*

Entire *Z. marina* used in the experiments was collected in November 2014 from Huiquan Cove, a subtidal zone in Qingdao, Shandong Province, China; and were transported to the laboratory within half an hour. After being kept in filtered seawater at 15°C for 7 d, healthy shoot of *Z. marina* was subsequently used in the following experiments.

Totally 30 plants were divided into six groups and were exposed to six different temperatures (5, 10, 15, 20, 25 and 30°C) for 96 h. Each group had five shoots in glass aquaria, which were aerated and lighted under same conditions of light intensity (150 μmol/(m^2·s)), and same light: dark ratio of 12:12. After all these treatments, leaf samples were collected for RNA preparation.

2.2 *RNA extraction and cDNA synthesis*

Approximately 50 mg of *Z. marina* leaf tissue was used for total RNA isolation with the EASYspin Plus Plant RNA Kit (Aidlab, Beijing, China) according to the manufacture's protocol. The crude RNA was treated with 0.1 U/μL RNase-free DNase I RQ1 (Promega, Madison, WI, USA) at 37°C for a total of 30 min. Oligo-dT-resistant receptor primers (5′-GGATCCGAATTCCCCGGG (T)24-3′) and Superscript™ III Reverse Transcriptase were used (Lifetechnologies, Carlsbad, CA, USA) to reverse transcribed the total purified RNA after phenol:chloroform:isoamyl alcohol (50:49:1) extraction and ethanol precipitation. The reaction was performed at 55°C for 1 h, terminated by heating at 70°C for 5 min, and store at –80°C subsequently. The obtained cDNA sample was used to amplify the cDNA sequence of *Zm*GST.

2.3 *Gene cloning from the synthesized cDNA*

The EST sequence of *Z. marina* used to amplify the full-length cDNA sequence in NCBI dbEST was analyzed by Blastx. The EST sequence (AM772768) homologous to previously identified GST sequence were selected for further cloning the *Zm*GST sequence by rapid-amplification of cDNA ends (RACE) technique. The full-length cDNA sequence of *Zm*GST was deposited in GenBank under the accession No. KJ766308. In order to amplify the 3′ end of the *Zm*GST cDNA, two gene specific primers, *Zm*GST_Race_F_1 (5′-GGCTTGCTTATGACAAAGAGTTGAAGA-3′) and *Zm*GST_Race_F_2 (5′-TGGAAATACGCCAGCCAGGTCTAC-3′), were designed by 3′-rapid identification of cDNA ends (RACE) technique, while the 3′ end has been completed in this EST. The T-A cloning vector pMD19-T simple (Takara, Otsu, Shiga, Japan) ligated the PCR products which then be tansformed into *E. coli* DH5α competent cells (TransGen, Beijing, China). Recombinants were determined by blue-white selection on ampicillin-containing Luria-Bertani (LB) plates, and using PCR to screen white colonies with BcaBEST sequencing primers M13-47 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and RV-M (5′-GAGCGGATAACAATTTCACACAGG-3′) (Takara). Positive clones were sequenced with the ABI Prism™ 3730 automated DNA sequencer (Thermoscientific, Carlsbad, CA, USA) to testify the full-length cDNA sequence.

2.4 *Sequence analysis and phylogenetic tree construction*

The search for protein sequence similarity was conducted with Blast algorithm. The protein sequences of *Zm*GST were analyzed with the EditSeq module of Lasergene suite 12.3.1. SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) was employed to predict the presence and location of signal peptide. The protein domain and motif features of *Zm*GST were predicted by Simple Modular Architecture Research Tool (SMART) 7.0 (http://smart.embl-heidelberg.de). Multiple sequence alignment was performed with ClustalW multiple alignment program 2.1 (http://www.clustal.org). A phylogenetic tree was constructed by Neighbor-Joining (NJ) method in MEGA 6.06.

2.5 *mRNA expression by real time quantitative reverse transcription PCR*

Total raw RNA was isolated from leaf tissue of *Z. marina*. The cDNA was prepared as described in Section 2.2 and diluted to 20 ng/μL. The mRNA expression of *Zm*GST at different temperatures was detected by fluorescent real-time PCR (qRT-PCR) using the real-time PCR Master Mix (Toyobo). The qRT-PCR assay was performed on a Bio-Rad iQ5™ Multicolor Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) and carried out in a total volume of 25.0 μL, containing 1×SYBR real-time PCR Master Mix, 4.0 μL of the diluted cDNA mix, 0.4 mmol/L of each primer and 7.5 μL of DEPC-treated RNA-free water. The comparative Ct method (2-ΔΔCt method) was used to analyze the relative mRNA expression levels of *Zm*GST. All data were given as means±SD (n=5) and subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison (S-N-K) via IBM SPSS Statistics 23.0.0.0. The p values less than 0.05 were considered statistically significant.

3 Results

3.1 *Gene cloning and molecular characteristics of the cDNA sequence of ZmGST*

The full-length cDNA of *Zm*GST (Fig. 1) was 720 bp, includ-

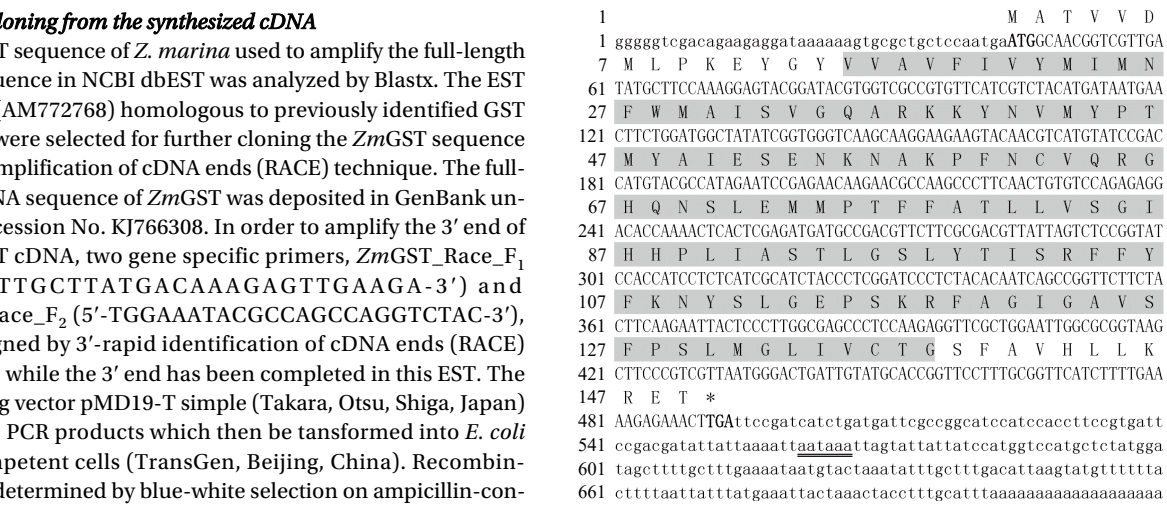

Fig. 1. The full-length cDNA of *Zm*GST. Nucleotide and deduced amino acid sequences of *Zm*GST (MAPEG domain (V15 to G138)) is shaded. The single typical polyadenylation signal was double underlined. The asterisk indicated the stop codon.)

ing the 450 bp open reading frame (ORF), 43 bp 5′UTR and 227 bp 3′UTR. 3′ untranslated regions (UTR) contained an 18 bp poly (A) tail and a signal sequence of polyadenylic acid. The GC content of the coding region was 49.44%. The cDNA encoded a polypeptide consisting 149 amino acid residues with a theoretical molecular weight mass of 16.8 kDa and isoelectric point of 9.42. The completed nucleotide sequence and the deduced amino acid are shown in Fig. 1. The amino acid sequence structural domain of *Zm*GST predicted by SMART contained a MAPEG domain (V15 to G138) and three transmembrane domains: Y12 to G34, F77 to Y99 and F119 to A141.

The deduced amino acid sequence of *Zm*GST by Blastp showed 64% identity with GST from *Oryza sativa*, 58% identity with GSTs from *Zea mays*, *Populus trichocarpa* and *Ricinus communis*, 57% identity with GST from *Gossypium arboretum*, and 54% identity with GST from *Medicago truncatula*, respectively (Fig. 2). The sequences of known GSTs members from different taxa were aligned. Using MEGA 6.06, a phylogenetic tree was constructed by Neighbor-Joining (NJ) method (Fig. 3). *Zm*GST with other GSTs from angiosperms was combined in the branch of higher plants, but GSTs from mammals and algaes were respectively into different branches. *Zm*GST was positioned within angiospermous cluster (Fig. 3), showing the close affinity with gramineous species, and declared it was one of homologues of GSTs from higher plants and typical genes in antioxidant enzymes.

3.2 *mRNA expression of ZmGST at different temperatures*

The solubility curve with a single peak near 83°C showed that the primer with fair specificity was right and can be used in relative quantitative analysis.

The influence of different temperatures on mRNA expression of *Zm*GST illustrated the relative mRNA expression level was significantly up-regulated from 10°C to 20°C; the minimum expression level was observed at 30°C, which reflected heat-stress significantly suppressed *Zm*GST expression; and the maximum expression level was observed at 10°C, which was twice higher than 5°C and 25°C, nine folds higher than 30°C (Fig. 4).

3.3 *Effect of temperature on the activity of recombinant ZmGST protein—the stability analysis of rZmGST*

As a heterologous expression system, *E. coli* was utilized to express *Zm*GST protein and demonstrate its antioxidant activity so as to obtain highly purified proteins. The r*Zm*GST protein was analyzed on SDS-PAGE and appeared as a distinct band at a molecular size of ~17 kDa (Fig. 5), which is close to the size of calculated molecular mass of *Zm*GST (16.8 kDa).

The enzymatic activity of r*Zm*GST dealing with different temperatures firstly increased and then decreased from 0°C to 40°C. The maximum was (181.35±13.3) mU/mg at 25°C for 96 h. More than 90% of the relative enzymatic activity was retained in the temperature range of 15°C to 25°C for 1 h; about 45% of the relative enzymatic activity was kept at 5°C and 30°C for 96 h; however, only approximately 7% of the relative enzymatic activity was surplus at 40°C (Fig. 6).

4 Discussion

The response between relative mRNA expression and temperature rising, the relative mRNA expression level changes reflect heat-stress suppressed *Zm*GST expression. The results of r*Zm*GST protein illustrate the recombined protein had a stronger correlation cooperativity of temperature, and *Z. marina* can enhance oxidation resistance by boosting antioxidant enzyme activities as temperatures go up.

According to previous researches, the optimum growth temperature for *Z. marina* was (15.3±1.6)°C, however, in the text, the mRNA expression of *Zm*GST got to the second-highest level, which declared *Zm*GST actively transcribed at the optimum tem-

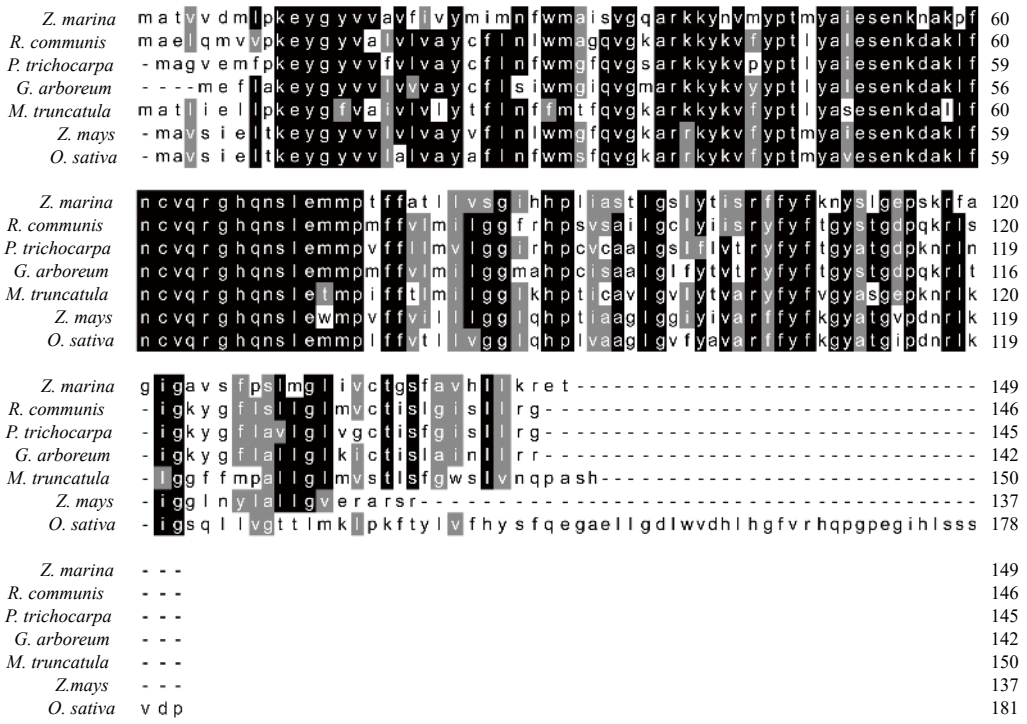

Fig. 2. Alignment of deduced amino acid sequences of GST from *Z. marina* and other plant species. The identical amino acids are indicated by black and similar amino acids are indicated by gray. Dashed lines are gaps introduced to maximize alignments.

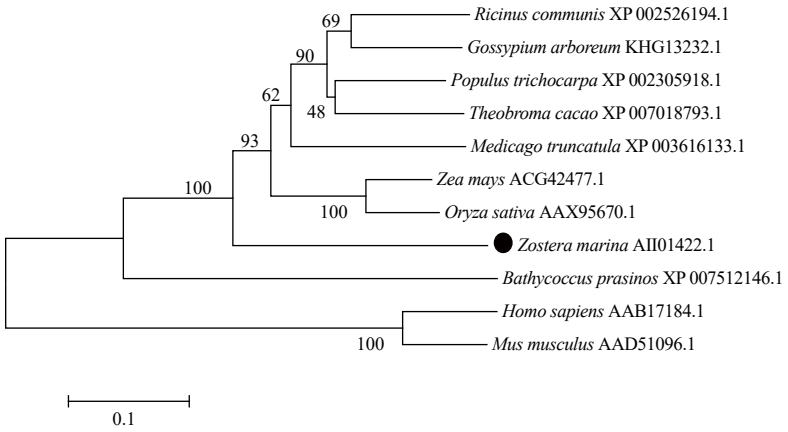

Fig. 3. Consensus neighbour-joining tree based on amino acid sequences of *Zm*GST and other known GSTs. Numbers at tree nodes refer to percent bootstrap values after 1 000 replicates. GenBank accession numbers are shown next to each sequence.

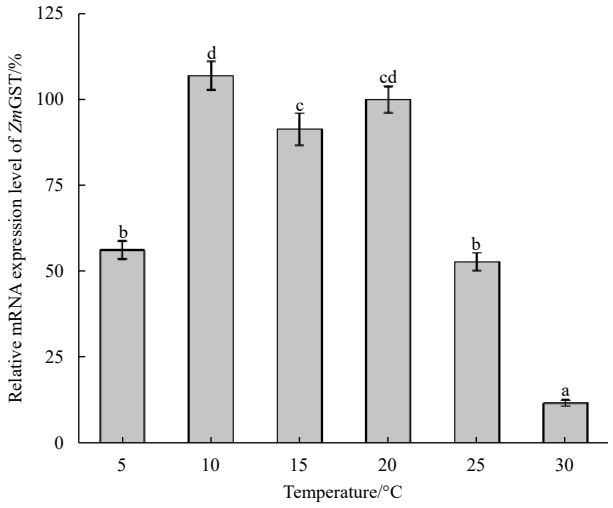

Fig. 4. The effect of temperature on the relative mRNA expression levels of *Zm*GST. The eIF4A gene was used as an internal control to calibrate the cDNA template for all the samples. The mRNA expression level at 20°C was set to 100%. Each values was shown as mean±SE (n=5), and bars with different characters were significantly different, p<0.05.

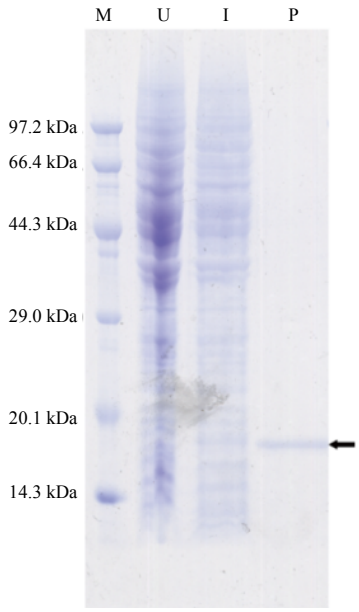

Fig. 5. SDS-PAGE analysis of recombinant *Zm*GST protein expressed in *E. coli* BL21 (DE3). M represents low molecular weight protein standards (Takara), U un-induced expression of pEASY-E1/*Zm*GST in total BL21 (DE3) cellular extract, I induced expression of pEASY-E1/*Zm*GST in BL21 (DE3) driven by IPTG, and P purified recombinant protein (r*Zm*GST).

perature, and the r*Zm*GST was more sensitive to the heat thermotherapy, which may be associated with its growing environment. *Zostera marina* grows in subtidal zone and submerged in sea water for a long time. In order to adapt to the lower and constant temperature, GST was formed to endure temperature variation. In particular, with temperature increasing, metabolism rate of *Zm*GST enlarged and ROS increased in plant; thereby the expression of *Zm*GST was induced; consequently, the mRNA expression of *Zm*GST rapidely augmented.

In a word, the seawater temperature rises can directly inhibit the activity of GST in the organisms, thus can weaken the ability of antioxidant system to scavenge ROS. As a result, excessive ROS were accumulated in plants, influenced the normal physiological process and caused oxidative damage. Consequently, antioxidant enzymes, such as GST, are sensitive to temperature, which was one of the important reasons that seawater surface temperature rising led to the population of *Z. marina* degradation.

5 Conclusions

The study investigated the expression pattern of *Zm*GST under different temperatures and found the temperature had a significant impact on the expression of *Zm*GST. It is a certain correlation between the *Zm*GST expression and temperature variation. The enzymatic activity will be increased as well as the gene transcription to keep its antioxidant effect with moderate temperature changes, and the antioxidant ability will be weakened by extreme temperature stress. *Zm*GST might play a pivotal role in reducing oxidative damage under heat-stress and then affect its adaptability to global warming. The results declared that high temperature can inhibit the expression of *Zm*GST and influence the ability of eliminating ROS, which led to changes of the redox equilibrium in organisms and affected several physiological func-

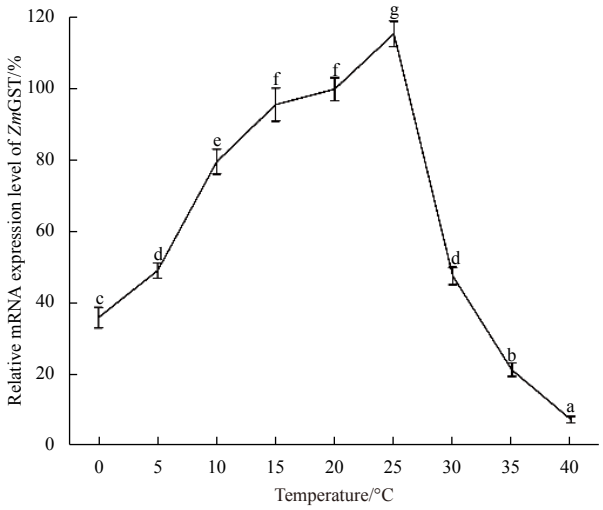

Fig. 6. The effect of temperature on the enzymatic activity of the recombinant *Zm*GST protein. The enzymatic activity at 20°C was set to 100%. Each value is shown as mean±SD (n=5), and bars with different characters were significantly different (p<0.05).

tions and survival of the *Z. marina*.

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