Conference Paper

Transcription Pattern of Catalase Gene from *Gynostemma pentaphyllum* (Thunb.) Makino during Various Abiotic Stresses

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Abstract

Catalase (CAT) is a group of enzymes that protect cells against oxidative damage generated by reactive oxygen species. A CAT cDNA was previously isolated and characterized from 3-month-old hydroponically cultured *Gynostemma pentaphyllum* (Thunb.) Makino plants. The ORF is 1 479 bp with a deduced amino acid sequence of 492 residues. CAT from *G. pentaphyllum* has a molecular mass of 56.97 kDa with an isoelectric point (pI) of 6.95. The temporal expression analysis of leaf samples demonstrated that *GpCAT* expression could be up-regulated by various environmental stresses such as jasmonic acid electro, oxidative, salt, heavy metal, chilling and heat stress in a certain time period. A three-dimensional structural model of *G. pentaphyllum* based on its *GpCAT* cDNA sequence. The temporal expression pattern suggests that the *GpCAT* could play a role in the molecular defense response of *G. pentaphyllum* to abiotic stresses.

Keywords: Abiotic stress; Catalase; Gene expression; *Gynostemma pentaphyllum* (Thunb.) Makino.

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1. Introduction

Reactive oxygen species (ROS) are free radical substances that contain one or more unpaired electrons, which include hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH^-), etc. [1]. Plants contain several types of antioxidant enzymes that are able to control ROS concentrations during fluctuating environmental conditions. A complex antioxidant defense system has been developed with several antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), peroxidase (POD) and glutathione-S-transferase (GST) [2]. CAT (oxidoreductase, EC 1.11.1.6) is a tetrameric heme-containing intracellular enzyme which can rapidly degrade two molecules of H_2O_2 to water and molecular oxygen [3]. CAT, the first antioxidant enzyme to be discovered and characterized in plants, is found in almost all aerobic organisms and serves to break down hydrogen peroxide rapidly [2]. In contrast to animals, CAT in plants was encoded by a

small gene family. Recently, interest in plant CATs has gained significant momentum as their important role in plant signal perception, plant defense, and plant metabolism. CATs are generally composed of a multi-gene family and have been biochemically and/or genetically purified, studied and characterized from many plants such as *Gossypium hirsutum* L. [4], *Zea mays* L. [5], and *Panax ginseng* C.A. Mey. [6]. Despite their importance as antioxidant enzymes, no prior report on CAT gene sequences in *Gynostemma pentaphyllum* (Thunb.) Makino exists. *G. pentaphyllum* is herbaceous vine plant of the family *Cucurbitaceae* (cucumber or gourd family) that is indigenous to and used widely throughout Asian countries, including China, Japan, and Korea as traditional medicines or tea with powerful biological effects that lead to longevity [7]. In this work, the cDNA clone encoding CAT was isolated from the cDNA library from 3-month-old hydroponically cultured *G. pentaphyllum* and we are interested in checking its transcription pattern in response to various abiotic stresses. This is the first report of the isolation of the CAT gene from *G. pentaphyllum* (*GpCAT*) and its transcription pattern in response to various abiotic stresses.

2. Materials and methods

2.1. Materials

In this study, we used *in vitro* cultured *G. pentaphyllum* plants which originated from Gyeryong-si, Chungcheongnam-do, Korea. *In vitro* conditions included a photoperiod of 16 h light and 8 h dark at 24 °C for 2 mo using ½ MS media.

2.2. Methods

2.2.1. Plant Materials and application of abiotic stresses

In vitro cultured *G. pentaphyllum* plants, 3 mo old were used for the treatments and nucleic acid extractions, as previously described. For chemical stress, the plantlets were placed for various periods in ½ Murashige and Skoog (MS) medium containing the indicated concentrations of chemicals; 500 μM $CuSO_4$ and 100 μM jasmonic acid (JA). For saline stress treatment, 100 mM NaCl were used in the ½ MS media. For the heat and chilling stress treatment, the plantlets were exposed to temperatures at 37 °C and 4 °C. For osmotic and oxidative stresses, mannitol (11 %) and H_2O_2 (10 mM) were given to the ½ MS media, respectively. In all cases, stress treatments were carried out in ½ MS media and ten planlets were treated with each stress for 1 h , 4 h, 8 h, 24 h, 48 h, and 72 h. Control plants were maintained in a growth room at 25 °C under a 16 h photoperiod. The stressed plant materials from all completed treatments were

immediately frozen in liquid nitrogen and stored at −70 °C until it required for the next analysis. The expression pattern of CAT genes against several environmental stresses was investigated by quantitative real time-PCR.

2.2.2. Sequence analysis

The search of Catalase ESTs was performed using *G. pentaphyllum* EST database (Kyung Hee Database) and the BLAST program at the National Center for Biotechnology Information server. By comparing the *G. pentaphyllum* ESTs, we identified and selected CAT gene based on their open reading frames encoding the specific protein via BlastX program (NCBI BlastX program). Nucleotide and amino acid sequence analyzes were performed using the DNASIS program (Hitachi, Japan). ClustalX with default gap penalties was used to perform multiple alignments of CAT isolated from *G. pentaphyllum* and previously registered in other species. The protein properties were estimated using ProtParam [8] and the hydropathy value was estimated by the method described by Kyte and Doolittle [9]. Identification of conserved motifs within CAT was accomplished with MEME [10]. Another database also used to analyze the full-length *GpCAT* gene, such as MotifScan, HMMTOP, SOPMA and PSORT. A three-dimensional model was prepared using CAT as a template on an SWISS-MODEL WORKSPACE in automated mode [11]. The generated 3-D structure was visualized using the UCSF Chimera package.

2.2.3. Real-time quantitative PCR

Total RNA was extracted from plants using RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacture's instruction. The quality and concentration of RNA were measured using a spectrophotometer (GE Nanovalue, USA). To obtain the first strand of cDNA, 5 μg of total RNA were reverse transcriptase using RT-&GO^{TM} Mastermix (MP Biomedicals, LLC., USA) following the instruction given by the manufacturer. qRT-PCR was performed by RT rotary analyzer (Rotor- Gene 6000, Corbett Life Science, Sydney, Australia) using 10 ng of cDNA in a 10 μL reaction volume using SYBR®. Green Sensimix Plus Master Mix (Quantace, Watford, England) using the specific primer *GpCAT* (5'-GTAACCAAGACCTGGCCTGA-3' and 5'-GGTGCATTAGCTGGGA GTTG-3'). The gene encoding actin protein (5'-ACATACCGGTGTCATGGTTGGT-3' and 5'-CTTCAGGAGCAA CACGAAGCT-3') was used as a housekeeping gene in the experiment. PCR conditions for each 40 cycles are 95 °C for 10 s, 58 °C for 10 s, and 72 °C 20 s. The fluorescent product was detected at the last step of each cycle. Amplification, detection, and data analysis were carried out with a Rotor-Gene 6 000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia). The threshold cycle (Ct) represents the number of cycles at which the fluorescence intensity was significantly higher than

the background fluorescence at the initial exponential phase of PCR amplification. To determine the relative fold differences in template abundance for each sample, the Ct value for *GpCAT* was normalized to the Ct value for β-actin and calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Three independent experiments were performed in triplicate.

3. Results and discussion

3.1. Amino acid sequence analysis of GpCAT

From our expressed sequence tags (EST) analysis of a cDNA library, which was prepared with the hydroponic culture of *G. pentaphyllum* we identified a cDNA clone encoding the CAT gene. We named this gene *GpCAT* (*Gynostemma pentaphyllum* (Thunb). Makino catalase). The full-length clone contained a *GpCAT* cDNA 1,909 bp long and had an ORF 1.479 bp long. The GC content of the coding region was 45.5 %. The *GpCAT* gene encoded a putative protein of 492 amino acid (aa) residues. CAT from *G. pentaphyllum* has a molecular mass of 56.97 kDa with an isoelectric point (pI) of 6.95, as determined by ProtParam program. *GpCAT* catalytic active site and heme binding motifs were detected by MotifScan software (PROSITE patterns [pat]). Seventeen amino acids at the position of Phe 54 to Ser 70 reported being involved in catalytic activity while the direct association with the heme group was detected on the nine amino acids at the position of Arg 344 to Glu 352. At the carboxyl terminus of *GpCAT*, the putative peroxisomal targeting sequence was present as a tri-peptide Pro-Ser-Ile. The presence of Pro-Ser/Thr/Asn-Met/Ile and context of adjacent residues are essential for directing proteins to the peroxisome [12]. Regarding PSORT prediction, this protein has a tendency to be located at peroxisome. These results clearly indicate that this cDNA encodes peroxisomal catalase. In addition, transmembrane helix prediction (HMMTOP) showed that there are no transmembrane helices in the deduced protein, implying that the protein does not function on the membranes in the cytosol or nucleus. One putative polyadenylation sites, AATAAA [13], can be found 178 nucleotides downstream of the stop codon (solid underline). Conserved GTGTT sequences also can be found 19 nucleotides downstream of the stop codon (dotted lines). It has been proposed that the GTGTT motif is required for efficient polyadenylation [14]. The full-length cDNA sequence has been assigned to GenBank under the accession numbers of KJ562361.

3.2. Homology analysis

A GenBank BlastX search revealed that the deduced amino acid sequences of *GpCAT* showed higher degrees of identity with the catalase of *G. hirsutum* (P17598) and *Prunus*

persica (L.) Batsch (CAD42908) with 93 % identity and 97 % similarity, followed by catalase of *Cucurbita pepo* L. (92 % identity, 98 % similarity). Alignment analysis was made to compare *GpCAT* with catalase from *C. pepo*, *Arabidopsis thaliana* (L.) Heynh., and *Raphanus sativus* (L.) Domin. Multiple sequence alignment revealed that all the sequences have high similarity between the CAT genes which have 492 amino acids length (Fig. 1). In addition, the heme group that reported being involved in the catalytic activity is conserved in Motif 2. Totally, there are three motifs as representative of conserved residues, as analyzed by MEME (Fig. 1). Heme group in *GpCAT* probably bind on the nine amino acids at the position of Arg 344 to Glu 352 while seventeen amino acids at the position of Phe 54 to Ser 70 reported being involved in the catalytic activity. Among those three representatives of conserved residue motifs, as analyzed by MEME, the heme group that reported being involved in the catalytic activity is conserved in Motif 2 (Fig. 1).

The hydrophilicity profile of the estimated CAT protein is shown in Fig. 2a. Peptides around the heme group are very hydrophobic in GpCAT and similar CATs, and the N and C-terminal peptides are very alike to each other. Secondary structure analysis and molecular modeling for *GpCAT* were conducted by SOMPA (Fig. 2b). The secondary structure analysis revealed that *GpCAT* consists of 132 α-helices, 27 β-turns joined by 71 extended strands, and 262 random coils. This result is highly similar to the secondary structure of *GhCAT1* from *G. hirsutum* 132 α-helices, 25 β-turns joined by 76 extended strands and 251 random coils; to *PpCAT1* from *P. persica* 132 α-helices, 27 β-turns joined by 70 extended strands and 263 random coils; and to *CpCAT* from *C. pepo* consist of 140 α-helices, 25 β-turns joined by 76 extended strands and 251 random coils. The PDB Sum results prediction of the 3-D structures of *GpCAT* by UCSF Chimera package was shown in Fig. 2c.

3.3. Temporal expression of GpCAT genes in response to abiotic stresses

The expression patterns of *GpCAT* at different time points after treatments were analyzed using real-time PCR. *GpCAT* expression response against abiotic stress (Fig. 3) demonstrated different results. The expression of the *GpCAT* gene was enhanced in response to various abiotic stresses. Oxidative stress by H_2O_2 caused a strong induction of *GpCAT* at 1 h (Fig. 3a). *GpCAT* expression quickly increased until 4-fold greater than control, and then gradually became similar to control. Oxidative stress by applying exogenous 10 mM H_2O_2 induced rapid expression of CAT on *Z. mays* and *P. ginseng* [5, 6]. Initial increasing of CAT expression by 10 mM H_2O_2 treatment also happened in cassava catalase—*MecCAT1* [15]. Using the same treatment (exogenous application of 10 mM H_2O_2), the *GpCAT* transcript was also induced compared to the control (Fig. 3a).

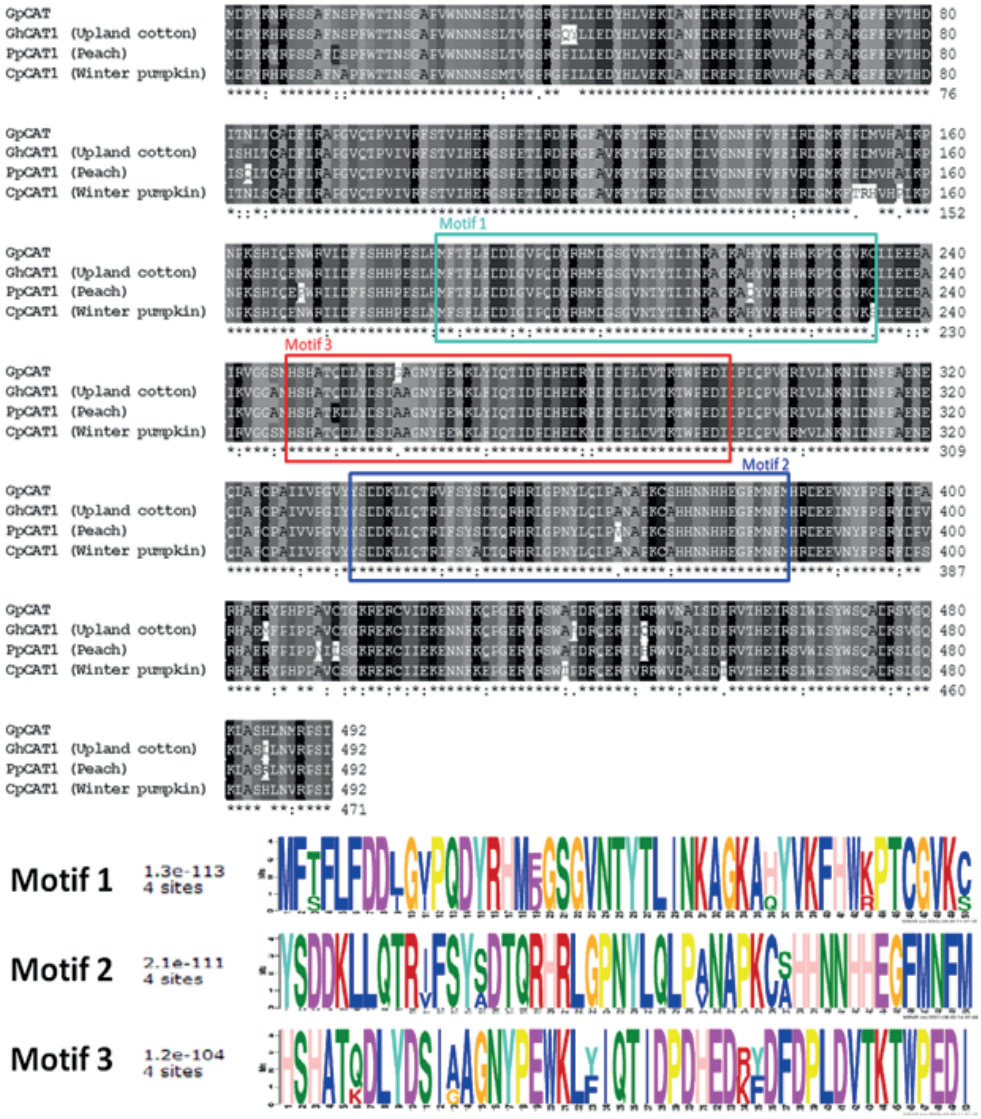

Figure 1: Putative amino acid sequence comparison of GpCAT with those of CAT genes from other plants: *C. pepo* (P48350), *R. sativus* (AAB86582) and *A. thaliana 2* (AEE86842). The hyphen was inserted within the amino acid sequence to denote gaps. Shadow box indicates well-conserved residues, * represents conserved amino acid, while: represents a very similar amino acid. Three conserved motifs obtained by MEME analysis contain 50 conserved amino acid residues. Motif 1, 2, and 3 are designated by the cyan, blue, and red boxes, respectively.

It had been reported that plant CATs play diverse roles in oxidative stress resistance, and possibly in the mediation of signal transduction which involving H_2O_2 as a second messenger. It also has been promoted that H_2O_2 could act as a cellular signal for the catalase gene induction in maize [16]. Thus, the increasing of CAT expression level may be included as one of the plant efforts to maintain the H_2O_2 homeostasis.

Similar to *GpCAT* response against oxidative stress, JA elicitor treatment to *G. pentaphyllum* make the rapid response of *GpCAT* upregulated expression (Fig. 3b). In the different pattern, *PgCAT1* transcription level was also upregulated but slowly, by giving 0.2 mM of JA [6]. CAT of *G. pentaphyllum* showed strong expression against salinity

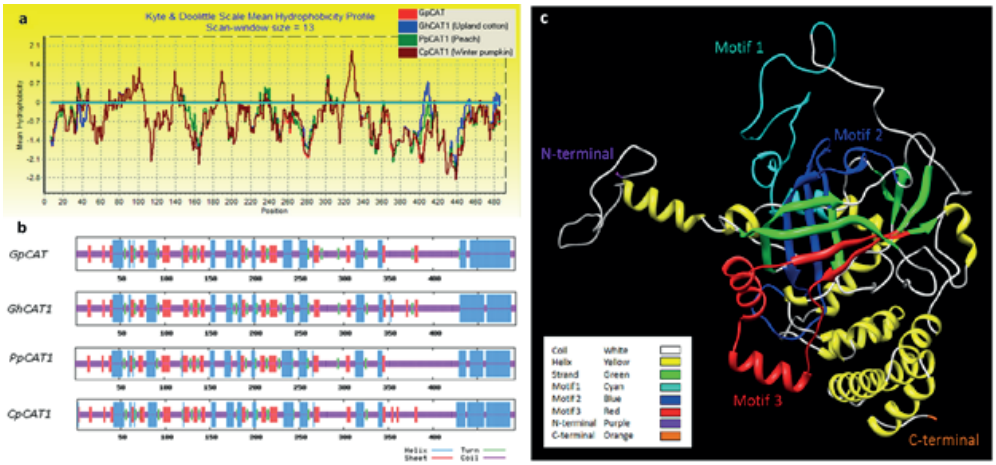

Figure 2: Superimposed hydrophilicity profiles and secondary structure predictions for *GpCAT* and homologous plant CATs; (a) Hydrophobic domains are indicated by positive numbers; hydrophilic domains are above the line and hydrophilic domains are below the line. The arrow indicates the conserved three cysteine residues. (b) Comparison of GPX secondary structures by SOMPA. The helix, sheet, turn, and coil are indicated in order from the longest to the shortest. (c) The predicted 3-D structures of *GpCAT*. Comparative representation was performed by UCSF Chimera package. Motif 1, 2, and 3 are depicted as blue, red, and cyan, respectively.

stress (Fig. 3c). Salinity stress enhanced *GpCAT* expression at 8 h to 72 h. This result confirms previous transcription level enhancement of *Avicennia marina* (Forrsk.) Vierh. CAT1 by giving 500 mM NaCl stress [17] and sugarcane CAT by giving 250 mM NaCl [18]. Study in rice revealed that salt stress triggered a defense mechanism against oxidative stress. Moreover, antioxidant response patterns operating in different plant species are well conserved. Hence, antioxidant enzymes transcripts like CAT are being up-regulated in response to salinity defenses to ensure proper protection against ROS generated after salt treatment [19].

Under $CuSO_4$ stress (Fig. 3d), *GpCAT* expression was rapidly induced by 1 h (9-fold). In contrast with previous *PgCAT1* transcript result, there is no significant change of *GpCAT* expression during the osmotic stress (11 % mannitol) compared to control (Fig. 3e). It seems that *GpCAT* is not responsive to mannitol stress. Chilling stress caused an up-regulation of *GpCAT* (Fig. 3f), which expressed rapidly and then decreased transiently. These results confirm expression pattern of maize CAT3 under cold stress [16]. Similar but not same, *PgCAT1* [6] was induced and reached a maximum after 24 h of cold treatment. The previous study by Saruyama and Tanida [20] has also correlated chilling tolerance with an induced antioxidant mechanism and suggested that it will be responsible for cold injury in plants. These outcomes were consistent with GpCAT expression (Fig. 3g), which gradually increased during 3 d treatment with the highest expression after 2 d. However, CAT2 of *A. thaliana* showed a quick but temporary decrease response against heat treatment [21]. The previous report showed that ROS production and ROS-scavenging enzymes were known to be a part of the heat-stress response. Higher expression of CAT in high-temperature treated plants can reduce

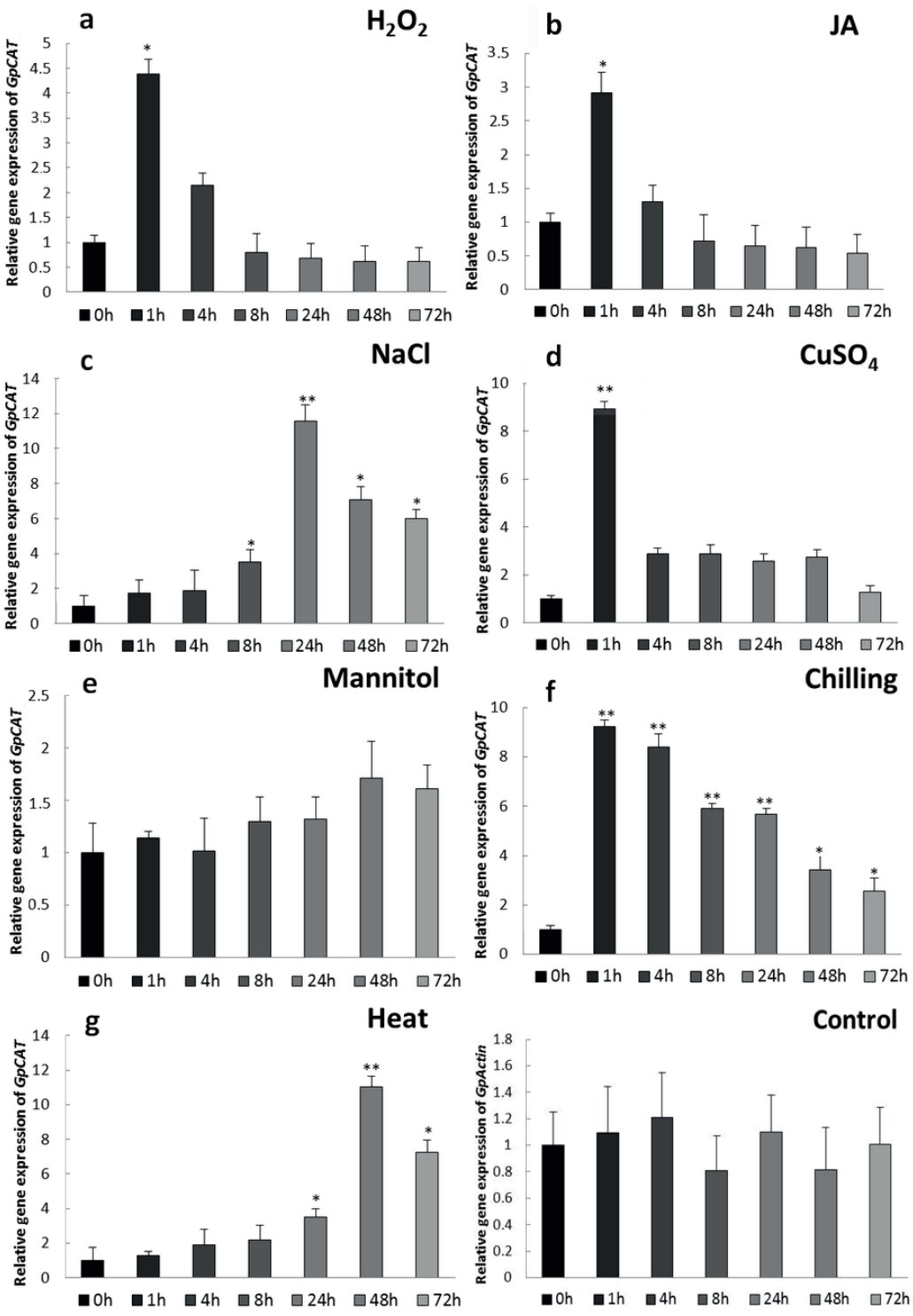

Figure 3: Relative quantities of *GpCAT* mRNA at various time points (hour) post-treatment with various stresses: 10 mM H_2O_2 (a), 100 μM Jasmonic Acid (b), 100 mM NaCl (c), 500 μM $CuSO_4$ (d), 11 % Mannitol (e), chilling (f), and heat (g) treatment. *GpActin* gene expression as control during the time course also provided. The error bars represent the standard error of the means of three independent replicates. "**" means the gene expression level was very significant compared to the control (0 h) and "*" means the gene expression level was significant compared to the control (0 h). Statistical analysis was performed by using an unpaired t - test with 95 % confidence interval.

the accumulation of H_2O_2 and prevent cell membrane damage. The plant capacity to enhance the expression and synthesis of CAT is limiting the plant's adaptation to heat stress [21].

4. Conclusion

A three-dimensional structural model of *G. pentaphyllum* CAT based on its *GpCAT* cDNA sequence. This study described the first characterization and formulation of an important enzyme, GpCAT, from G. *pentaphyllum*. Our results show that *GpCAT* was sensitively induced in 3 mo old *in vitro* culture of *G. pentaphyllum* by various kinds of stimuli, including oxidative stress (H_2O_2), plant hormones (JA), salinity stress, heavy metal stress (copper), chilling and heat stress. However, *GpCAT* was not responsive to osmotic stress made by mannitol treatment. Some similar reports about CAT expression in plants have been already reported and our results confirm that GpCAT is also affiliated with environmental stresses. Putative peroxisomal targeting sequence (Pro-Ser-Ile) was found at the C-terminal indicating that its location must be at peroxisome. Further studies on *GpCAT* are needed for specific localization within the cell, better substrate accessibility, and different regulation results under stress. Information on CAT variants in plants is still few, so this study might provide support for further characterization of CATs.

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